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Investigating the developmental and behavioral consequences of maternal immune activation on affected offspring

Thesis presented by

Megan E. Straley

in fulfilment of the requirements for the degree of

Doctor of Philosophy

Under the supervision of

Dr. Gerard O'Keeffe

July 2015

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Declaration

This thesis comprises original work carried out by the author (unless otherwise stated) and has not been submitted, in part or in whole, to this or any other university for any degree.

Author Contribution

All of the work described herein was performed independently by the author, with the following exceptions:

Chapter 2:

Katie Togher assisted with the immunohistochemistry experiments and conducted the placental cell culture experiments. Dr. Aoife Nolan conducted the SCG cell culture experiments.

Chapter 4:

Dr. Sean Crampton conducted the cell culture experiment in which VM neurospheres were treated with cytokines. Dr. Shane Hegarty conducted the experiment in which primary cultures of VM DA neurons were treated with cytokines in culture. Dr. Sinead Walsh assisted with embryonic immunohistochemistry experiments. Sarah Theze assisted with the analysis of postnatal immunohistochemistry.

Signed,

_____

Megan E. Straley

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Abstract

Maternal infection during pregnancy increases the risk of several neuropsychiatric disorders later in life, many of which have a component of dopaminergic (DA) dysfunction, including schizophrenia, autism spectrum disorders (ASD), and attention deficit hyperactivity disorder (ADHD). The majority of DA neurons are found in the adult midbrain; as such the midbrain is a key region of interest regarding these disorders. The literature is conflicting regarding the behavioral alterations following maternal immune activation (MIA) exposure, and the cellular and molecular consequences of MIA on the developing midbrain remain to be fully elucidated. Thus, this thesis aimed to establish the consequences of acute and mild MIA on offspring dopamine-related behaviors, as well as the associated cellular and molecular disturbances of MIA on offspring midbrains.

We utilized a rat model of MIA using low dose (50μg/kg, I.P.) of LPS administered at different gestational ages. Our first study indicated that MIA at later gestational ages significantly increased pro-inflammatory IL-1β expression, and reduced HSD11B2 expression in the placenta, which is an important regulator of fetal development. *In utero* LPS exposure at later gestational ages also impaired the growth of neurons from affected offspring. This study identified key gestational stages during which MIA resulted in differential effects. We utilized these time points in subsequent studies, the next of which investigated neurobehavioral outcomes following MIA. Our results from that study showed that motor differences occurred in juvenile offspring following MIA at E16 only, and these differences were compensated for in adolescence. Then, there was a decline in motor behavior capabilities in adulthood, again only for animals exposed to MIA on E16 (and not E12). Furthermore, our results also demonstrated adolescent and adult offspring that were exposed to MIA at E12 had diminished responses to amphetamine in reward seeking behaviors. In our final study, we aimed to investigate the molecular and cellular changes following MIA which might explain these behavioral alterations. This final study showed a differential inflammatory response in fetal midbrains depending on gestational age of exposure as well as differential developmental alterations. For example, LPS exposure at E16 resulted in decreased VM neurosphere size after 7DIV and this was associated with an increased susceptibility to neurotoxic effects of pro-inflammatory cytokines for VM neurospheres and VM DA neurons treated in culture. *In utero* LPS exposure at E16 also reduced DA

neuron count of fetal VM, measured by TH staining. However, there were no differences in DA neuron number in juvenile, adolescent, or adult offspring. Similarly, LPS exposure did not alter cell number or morphology of glial cells in the midbrains of affected offspring.

In conclusion, this thesis indicated later rat pregnancy (E16) as vulnerable time for MIA to affect the development of the nigrostriatal pathway and subsequent behavioral outcomes, possibly implicating a role for MIA in increased risk for disorders associated with motor behavior, like PD. These effects may be mediated through alterations in the placenta and altered inflammatory mediators in the offspring brain. This thesis has also shown that MIA in earlier rat pregnancy (E12) results in altered mesocorticolimbic function, and in particular MIA on E12 resulted in a differential response to amphetamine in affected offspring, which may implicate a role for MIA in increasing the risk for disorders associated with this pathway, including drug tolerance and addiction.

Chapter 1:

General Introduction

1.0 Introduction

During development the nervous system is assembled and sculpted by an orchestrated series of neurodevelopmental events that ultimately generate and refine the neural circuitry that govern all facets of human behavior. Fetal programming describes how alterations in the fetal environment during specific periods of vulnerability during pregnancy may cause perturbations in this normal developmental program, thereby altering the structure and function of organs including the nervous system (Schlotz & Phillips, 2009). This phenomenon has the potential for physiological changes, leading to susceptibility for disease later in life (known as the Barker Hypothesis) (Bateson *et al.*, 2004; de Boo & Harding, 2006; Schlotz & Phillips, 2009). Indeed, much evidence suggests that perinatal factors, including nutrition deficiency, maternal exposure to drugs or toxins, maternal stress, fetal hypoxia, low birth weight, and prenatal maternal infection have been implicated in adverse outcomes, most notably adult disease (Del Giudice, 2012; Faa *et al.*, 2014; Seremak-Mrozikiewicz *et al.*, 2014).

Many clinical and epidemiological studies have now shown that prenatal maternal infection and subsequent maternal immune activation (MIA) can increase the risk in affected offspring for a number of neurodevelopmental and neuropsychiatric disorders later in life, including schizophrenia (Brown *et al.*, 2004a; Brown *et al.*, 2005; Brown & Derkits, 2010), autism spectrum disorders (ASD) (Zerbo *et al.*, 2013a; Zerbo *et al.*, 2013b) and cerebral palsy (CP) (Pakula *et al.*, 2009; Shatrov *et al.*, 2010). Many of the epidemiological findings have been corroborated using pre-clinical models of MIA, including administration of lipopolysaccharide (LPS) to pregnant rats which has been shown to result in cognitive impairments, brain lesions, behavioral abnormalities, and neurological disease (Bell & Hallenbeck, 2002; Golan *et al.*, 2005; Bilbo & Schwarz, 2009; Boksa, 2010; Patterson, 2011b). Furthermore, research from animal models, which is supported by epidemiological evidence (Brown *et al.*, 2000b; Atladóttir *et al.*, 2010), has suggested that timing and nature of immunogen exposure, combined with environmental factors and genetic predisposition, are critical determinants of offspring outcome (Meyer *et al.*, 2007; Boksa, 2010). While MIA is indeed likely to affect many brain regions, these findings have significant implications for the development of the midbrain and the associated dopaminergic neurons therein,

which have been the focus of intensive investigation given their degeneration in Parkinson's disease (PD) (Toulouse & Sullivan, 2008; Lees *et al.*, 2009). Indeed, the increase risk in many of the neuro-related diseases post MIA are dependent on dopaminergic neurons and/or proper functioning of the dopaminergic system, including schizophrenia (Brown, 2006; Brown & Derkits, 2010; Anderson & Maes, 2012; Seeman, 2013). Thus, it is imperative to fully elucidate the consequences and the mechanisms of how MIA influences the midbrain and dopaminergic systems in order to better understand the etiology and progression of many of these diseases and disorders.

The aim of this review chapter is to provide a detailed account of the literature: I begin by reviewing the epidemiological and clinical evidence linking MIA and with adverse neuro-related outcomes, and then describe the relationship of neurodevelopmental processes to timing of infection. Next, I detail the animal models used to investigate these associations further and review the data showing the developmental and behavioral abnormalities in affected offspring as a result of MIA. I conclude with an overview of midbrain development and the implications of MIA for midbrain development and subsequent dopaminergic function in affected offspring. Finally, I describe the aims of this PhD thesis.

1.1 Epidemiological and Clinical Evidence

Epidemiological evidence has shown that MIA leads to increased risk of neurodevelopmental and neuropsychiatric disorders including CP, ASD, ADHD, and schizophrenia in affected offspring. While the comprehensive etiologies of these disorders are not yet fully elucidated, prenatal MIA has been implicated as a risk factor in epidemiological and clinical studies, the findings from which are reviewed below.

1.1.1 Cerebral Palsy

"Cerebral palsy" is an umbrella term used to describe a group of syndromes with varying levels of severity affecting gross motor function and posture, which arise following lesions or developmental abnormalities in the immature/developing fetal or infant brain (Mutch *et al.*, 1992; Rosenbaum *et al.*, 2002; Sankar & Mundkur, 2005; Rosenbaum *et al.*, 2007). The brain lesion/abnormalities associated with CP are not progressive, and motor impairments are permanent (Mutch *et al.*, 1992; Cans, 2000; Smithers-Sheedy *et al.*, 2014). Epidemiological evidence including retrospective studies, population based studies, as well as meta-analyses have provided evidence for a link between prenatal or intrauterine infection and brain lesions or CP (Himmelmann *et al.*, 2011; McIntyre *et al.*, 2013). In fact, Odding et al. reports that intrauterine infections are one of "the most important risk factors for CP", amongst low birth weight and multiple gestation (i.e. twin or triplet pregnancies) (Odding *et al.*, 2006). Indeed, one or more indicators of intrauterine maternal infection, including maternal fever exceeding 38°C or chorioamnionitis (infection of the fetal amnion and chorion membranes), were present in only 2.9% of control children, versus 22% of children with CP in a population-based case-control study (Grether & Nelson, 1997).

Prenatal infection, including urinary tract infection, clinical sepsis, and chorioamnionitis, which is possibly the most comprehensively researched form of MIA in terms of CP, have been associated with increased risk of CP development and white matter damage in affected offspring (Dammann & Leviton, 1998; Himmelmann *et al.*, 2011; McIntyre *et al.*, 2013; Bangash *et al.*, 2014). Periventricular leukomalacia (PVL) is a form of white matter brain injury, consisting of brain lesions near the lateral ventricles which are the most important identifiable

risk factors for developing CP (Yoon *et al.*, 2003). The lesions have been reported in 7-26% of low birth weight babies (under 1500g), and 62-80% of these children developed CP (Yoon *et al.*, 1997). Maternal diagnosis of chorioamnionitis significantly increased the risk for PVL in low birth weight infants (500-1700g) (Verma *et al.*, 1997). Similarly, the risk of CP increased from 12 to 39 per 1000 births in low birthweight babies from mothers diagnosed with chorioamnionitis (Nelson & Ellenberg, 1978). Furthermore, chorioamnionitis significantly increased the risk for PVL in preterm infants (Perlman *et al.*, 1996; Shevell *et al.*, 2013), and approximately 28% of instances of CP in preterm infants is attributable to this infection as well (Nelson & Willoughby, 2000). Indeed, chorioamnionitis increases the risk of CP from 3 to 17% (Murphy *et al.*, 1995). Population based studies as well as meta-analysis reviews have also found significant relationships between chorioamnionitis and increased risk of CP in normal weight and full term babies (O'Shea *et al.*, 1998; Wu *et al.*, 2000; Wu *et al.*, 2003). Indeed, infection-related factors including bacterial growth in urine during pregnancy, any infection during pregnancy, severe infection during pregnancy, and antibiotic therapy during pregnancy constituted strong independent risk factors for spastic hemiplegia subgroup of CP in a case-controlled, population-based study of full term children with CP (Ahlin *et al.*, 2013). However another recent study showed no association between antibiotic use during pregnancy and risk of CP (Meeraus *et al.*, 2015). Indeed, self-reported vaginal infections which received no antibiotic treatment were associated with an increased risk of spastic CP, more particularly than antibiotic treated vaginal infections (Streja *et al.*, 2013). These data suggest that adverse effects of MIA in relation to increase CP risk are not associated with the treatment of maternal infection but perhaps the illness itself and/or related processes.

An important question is whether the timing of infection is significant with relation to increased risk of offspring CP development, as MIA at different stages of pregnancy is likely to impact on the neurodevelopmental events occurring at that time. Interestingly, chorioamnionitis and maternal genitourinary infections that occur in the first two trimesters have been associated with increased risk of CP in preterm or low birthweight infants (Mann *et al.*, 2009). In agreement with this, another study found that maternal lower urinary tract infections treated with antibacterial prescriptions in the first trimester were associated with increased risk of CP in all births, however they also showed that genito-urinary tract infections in the

third trimester were associated with CP as well (Miller *et al.*, 2013). Finally, a population based control study demonstrated an increased risk of CP in both preterm and full term infants of mothers who had any infection during their hospitalization for delivery, however this study neglected to examine maternal infection during any other stage of pregnancy so it is not known if there was another infection earlier acting as a confounding factor (Neufeld *et al.*, 2005). These data seem to suggest that infection at any point during pregnancy is a risk factor of CP, but as the studies were not well controlled for this, more precise investigations must be conducted to validate a critical window or the lack thereof.

Results from clinical studies have also supported the epidemiological data. For example histological examination of the placenta post maternal infection, including chorioamnionitis, has confirmed infection-induced alterations in placental structure as a risk factor for CP (Grether & Nelson, 1997; Shevell *et al.*, 2013). Examination of tissue for levels of cytokines has also proved valuable as umbilical cord plasma concentration of interleukin (IL)-6 was significantly higher with the co-occurrence of PVL (Yoon *et al.*, 1996), and Martinez et al. showed higher median amniotic fluid levels of IL-6 as well as more positive amniotic fluid cultures (for aerobic and anaerobic bacteria) in cases where the child developed PVL and intraventricular hemorrhage (Martinez *et al.*, 1998). Thus, concentrations of IL-6 and IL-1β in the amniotic fluid have been shown to identify infants who are at risk for developing white matter lesions (Yoon *et al.*, 1997). This suggests a potential role for cytokine levels to be used as potential predictors of CP risk in future.

1.1.2 Autism Spectrum Disorders

Autism spectrum disorders (ASDs) are developmental disorders characterized by communication and social interaction deficits, repetitive behaviors, and restricted interests which present in early childhood and limit/impair everyday functioning for affected people throughout life (Nightingale, 2012; Lauritsen, 2013). Individuals with copy number variants as well as a history of maternal infection during pregnancy were associated with increased occurrence of the social communicative impairments and repetitive/restrictive behavioral symptoms of ASD (Mazina *et al.*, 2015), thus prenatal or early life infection is widely hypothesized as a possible step in the etiology for ASD along with genetic contributions (Libbey *et al.*, 2005; Brown, 2012; Samsam *et al.*, 2014). Indeed, viral infection has been referred to as

the "principal non-genetic cause of autism" (Ciaranello & Ciaranello, 1995) and inpatient diagnosis of infection during pregnancy has been attributed to a 30% increased risk in ASD, according to a study using the Swedish nationwide register based birth cohort encompassing 2,371,403 people of which 24,414 have ASD (Lee *et al.*, 2015).

Women with infections (namely bacterial) diagnosed during an admission to the hospital had increased risk of delivering an infant with ASD (Zerbo *et al.*, 2013b), and multiple infections during pregnancy was a further risk factor (Zerbo *et al.*, 2013b). Specifically, numerous case studies are available detailing an association of cytomegalovirus (CMV) infection and the development of ASD in affected offspring (Stubbs, 1978; Markowitz, 1983; Ivarsson *et al.*, 1990; Yamashita *et al.*, 2003; Sweeten *et al.*, 2004). Furthermore, one early study looked at 243 children exposed to maternal congenital rubella and 7% had autism in preschool (Chess *et al.*, 1978), demonstrating an increased risk of ASD following maternal rubella during pregnancy; more recent work is in agreement with these findings (Landrigan, 2010). Also, a study looking at 143 children diagnosed with ASD and 200 control children demonstrated that maternal respiratory infection/asthma as well as maternal vaginal infection are possible risk factors for ASD (George *et al.*, 2014). Finally, placental abnormalities have also been implicated in ASD as Anderson found a significantly greater occurrence of trophoblast inclusions in placenta from mothers of children with ASD (Anderson *et al.*, 2007).

As developmental processes during different stages of pregnancy might be differentially affected by maternal infection, a number of studies have also examined whether the timing of infection affected the increased risk of ASD. The study based on the Swedish nationwide register showed that timing of infection did not influence risk, and an elevated risk was associated with MIA in all trimesters (Lee *et al.*, 2015), however they did not distinguish between types of infection. Maternal rubella in early pregnancy is associated with increased risk of ASD (Landrigan, 2010). In a population study of Denmark, looking at children born between 1980 and 2005, maternal viral infection in the first trimester or bacterial infection in the second trimester were shown to be associated with a diagnosis of ASD in the offspring (Atladóttir *et al.*, 2010). The findings of case studies suggest that timing of CMV in the third trimester increases risk for offspring to develop ASD (Yamashita *et al.*,

2003). These data may suggest that the timing and type of infection have differential associations to the risk of ASD in affected offspring.

Clinical findings have demonstrated immune dysregulation and/or inflammation in some people with ASD, including changes in cytokine profiles (Gupta *et al.*, 2010; Gesundheit *et al.*, 2013; Goines & Ashwood, 2013; Rossignol & Frye, 2014). Indeed, a number of studies have investigated cytokine and chemokine levels, including tumor necrosis factor (TNF), interferon (IFN)-γ, IL-6, IL-8, and C-C motif chemokine 2 (MCP-1), and have demonstrated increased levels of these immunological biomarkers in the cerebrospinal fluid (CSF) of people with ASD (Pardo *et al.*, 2005; Vargas *et al.*, 2005; Chez *et al.*, 2007). Autoimmune markers have even been implicated in disease severity as positive associations with ASD severity level have been shown from serum levels of anti-nuclear antibodies (Mostafa & Kitchener, 2009), anti-ganglioside MI antibodies (Mostafa & Al-Ayadhi, 2011), and antineuronal antibodies (Mostafa & Al-Ayadhi, 2012) in children with ASD. Furthermore, using a high-sensitivity c-reactive protein (hs-CRP) test to evaluate the inflammatory response in individuals with different ASD severity diagnosis demonstrated a significantly higher hs-CRP level in severe ASD compared to the mild-to-moderate group (Khakzad *et al.*, 2012). The altered immune profile associated with ASD may be related to an initial insult of MIA, as mid-gestational maternal serum levels of IFN-γ, IL-4, and IL-5 were significantly associated with a 50% increased risk of ASD (Goines *et al.*, 2011), and furthermore amniotic fluid with increased TNF-α and TNF-β levels (Abdallah *et al.*, 2013) as well as altered pathology of the placenta, including trophoblast inclusions (Anderson *et al.*, 2007), are associated with increased risk of ASD. Indeed, preclinical research has shown that the placenta provides the fetus with hematopoietic stem cells (Gekas *et al.*, 2005), allowing for the possibility that maternal infection could permanently alter the peripheral immune system of the offspring (Patterson, 2009), later resulting in the immune profiles observed in these clinical studies.

1.1.3 Attention Deficit Hyperactivity Disorder

Attention-Deficit Hyperactivity Disorder (ADHD) is a disorder associated with significant problems in attention, increased impulsiveness, and excessive activity symptoms which impact daily social and occupational activities (American

Psychiatric Association [APA], 2013; Barkley, 2014). ADHD is commonly referred to as a 'childhood disorder' as the age of onset is in early childhood, most symptoms usually presenting by age 12 (Willcutt *et al.*, 2012; APA, 2013), however though symptoms decline with age, it is generally accepted as a life-long disorder (McGough & Barkley, 2004; Faraone *et al.*, 2006; Willcutt *et al.*, 2012; Barkley, 2014). For ADHD comparatively less is known about the impact of maternal infection as a risk factor for its development. However, perinatal exposure to infection has been shown to alter the behavior in offspring, including increasing hyperactivity (Hornig *et al.*, 1999; Silva *et al.*, 2014). In a cohort study of children with ADHD, children with undifferentiated attention –deficit disorder (UADD), and a control group, researchers found that the greater number of medical conditions prior or during their first pregnancy increased the risk of delivering a child with an ADHD (McIntosh *et al.*, 1995). Furthermore, maternal illness/infection has been associated with ADHD and impaired cognitive functioning in progeny (Milberger *et al.*, 1997). In a retrospective study, using data from 84,721 children who were born from 1996 to 2002, showed that there is an increased risk of developing ADHD following maternal genitourinary infection (GU) (Mann & McDermott, 2011). Interestingly specific diagnosis mattered as chlamydia, trichomoniasis, urinary tract infection (UTI), and candidiasis have been associated with the increased risk of ADHD, while gonorrhea was not (Mann & McDermott, 2011). In agreement with this, another study correlated maternal UTI with increased risk of offspring ADHD diagnosis (Silva *et al.*, 2014). Finally, mothers who had both a GU infection as well as pre-eclampsia (a disorder of the placenta which leads to systemic inflammation) were 53% more likely to have a child with ADHD (Mann & McDermott, 2011).

1.1.4 Schizophrenia

Schizophrenia is a psychiatric disorder characterized positive symptoms, including hallucinations and/or delusions; negative symptoms, including flat affect and inability to experience pleasure; and cognitive symptoms, including impaired attention and memory (APA, 2013). Dopamine plays a key role in the pathophysiology this disorder (Lau *et al.*, 2013), evidence which stems from the 'dopamine hypothesis' which proposes that the positive symptoms of schizophrenia are due to hyperactive mesolimbic dopamine projections and subsequent hyperstimulation of striatal D2 receptors and the negative symptoms and cognitive

impairments result from reduced mesocortical dopamine projections and associated hypostimulation of prefrontal cortex D1 receptors (Davis *et al.*, 1991; Howes & Kapur, 2009).

It has been proposed that if infections could be prevented during pregnancy, more than 30% of schizophrenia cases could be eliminated (Brown & Derkits, 2010). Indeed, the Danish national register allowed for a population based study in which they investigated the risk of schizophrenia associated with numerous obstetric events, and found a modest association between maternal infection and the risk of offspring developing schizophrenia (Byrne *et al.*, 2007). Furthermore, evidence has shown increased risk of schizophrenia in individuals that were exposed to the influenza virus during pregnancy (Mednick *et al.*, 1988; Mednick *et al.*, 1994), rubella (Patterson, 2009), measles (Brown & Patterson, 2011), mumps (O'Callaghan *et al.*, 1994), varicella zoster (O'Callaghan *et al.*, 1994), diphtheria (Watson *et al.*, 1984), toxoplasmosis (Patterson, 2009), and genital or reproductive infections, including herpes simplex virus type 2 (HSV-2) (Buka *et al.*, 2008; Patterson, 2009). There is increasing evidence showing heightened risk of offspring developing schizophrenia from mothers who contracted infections during pregnancy, and a few reviews have been published on this topic (Brown & Derkits, 2010; Brown & Patterson, 2011). While this body of evidence is enormous, this next section attempts to succinctly review the most important literature on this topic.

At first, epidemiological studies used ecologic designs, whereby data used at a population level compared to epidemics were used to look at disease exposure. Mendick pioneered investigations associating influenza epidemics to neurological adversity in affected offspring, importantly showing an association between second trimester influenza exposure with an increase in schizophrenia incidence in offspring (Mednick *et al.*, 1988; Mednick *et al.*, 1994). Following Mendick's work, over 26 other studies have looked at the incidence of schizophrenia following flu epidemics. These include studies from a Denmark cohort with cases from 1911 to 1950 (Barr *et al.*, 1990), a UK cohort and any effect of the 1957 A2 influenza epidemic (O'Callaghan *et al.*, 1991; Cooper, 1992; Fahy *et al.*, 1992; Sham *et al.*, 1992), and a Japanese cohort examining the 1957 influenza epidemic (Kunugi *et al.*, 1992; Izumoto *et al.*, 1999), among numerous others, including (Wright *et al.*, 1995), which have found significant increases in schizophrenia risk among offspring exposed to maternal influenza in the second trimester. However, studies with similar

design, including one with a much larger case sample from the US, have reported no correlation between second trimester maternal flu and schizophrenia (Torrey *et al.*, 1991; Selten & Slaets, 1994; Susser *et al.*, 1994; Westergaard *et al.*, 1999; Sørensen *et al.*, 2009). While important as the initial reports in this field, these studies had key mechanistic faults, including the fact that cases of schizophrenia were not directly related to a maternal diagnosis of infection, but instead only correlated to pregnancies occurring at the same time as a flu epidemic, which could lead to overestimations and misclassification of offspring exposure to maternal flu.

As the previous studies use ecologic study designs, resulting in inconsistent and nonspecific data, there was a motivation for further research to utilize well characterized birth cohorts, which provide many mechanistic improvements compared to the ecologic design including the use of clinical diagnosis of infection and longitudinal assessment of offspring. Two major studies using only documented perinatal infections, found no correlation with influenza and schizophrenia within a small sample size (Crow *et al.*, 1991; Cannon *et al.*, 1996). When a larger sample size was used, medical records of over 12,000 pregnant women showed a clear association between maternal respiratory infection and 2-fold increased risk of offspring developing schizophrenia (Penner & Brown, 2007). Similarly, specific diagnostic criteria as well as maternal interviews regarding maternal respiratory infection during the second trimester were used along with follow up of offspring status to find an increased risk of schizophrenia and schizophrenia spectrum disorders (SSD) in offspring adulthood (Brown *et al.*, 2000b). Influenza was not the only illness investigated in this way. Indeed, a Copenhagen Perinatal Cohort, which was made up of 9,125 offspring delivered by 8,949 women from 1959 to 1961, was used to find an increased risk of schizophrenia correlated to bacterial infection in the first trimester (Sørensen *et al.*, 2009). Furthermore, maternal genital/reproductive infections within the first few weeks of pregnancy is associated with a significantly increased risk of offspring developing schizophrenia (Babulas *et al.*, 2006).

Studies further improved as the use of biomarker measurement during individual pregnancy and subsequent follow up of offspring became common practice. One study used influenza antibody levels from the mother at different time points during gestation in order to directly associate maternal flu with incidence of schizophrenia, and demonstrated that the risk of developing schizophrenia increased by a factor of 7 after flu exposure in the first trimester, as well as a 3-fold increased

risk associated with early-mid gestation influenza exposure (Brown *et al.*, 2004a). Serological confirmation has also been used to establish a correlation between mothers with clinical rubella in early pregnancy with a 5 fold increased risk of non-affective psychosis in young adulthood (Brown *et al.*, 2000a), as well as a 20% increased risk for schizophrenia or SSD in middle-aged adults (Brown *et al.*, 2001). Furthermore, maternal serum from birth cohort studies from the US and Denmark has shown that increased levels of maternal IgG antibody to HSV-2 is a risk factor for schizophrenia in offspring (Buka *et al.*, 2001a; Buka *et al.*, 2008; Mortensen *et al.*, 2010). These maternal HSV-2 findings were not replicated in a smaller cohort (Brown *et al.*, 2006), though it has been suggested that the null result is due to lack of power and small sample size and other mechanistic differences (60 schizophrenia cases) (Brown & Derkits, 2010). Elevated levels of maternal IgG antibodies to *Toxoplasma gondii*, a parasite which causes toxoplasmosis, were also associated with increased risk of schizophrenia in the US Prenatal Determinants of Schizophrenia (PDS) cohort (Brown *et al.*, 2005) as well as the Danish birth cohort (Mortensen *et al.*, 2007). Immune biomarkers including TNF-α, (Buka *et al.*, 2001b), IL-8 (Brown *et al.*, 2004b), IL-5 and IL-6 (Abdallah *et al.*, 2013), and C-reactive protein levels (Canetta *et al.*, 2014) are associated with increased risk of psychosis and/or schizophrenia in offspring. Some believe that the relationship of maternal infection and schizophrenia initiates continued altered immune functioning in affected offspring because medication naïve patients with schizophrenia have demonstrated elevated blood levels of IL-1β, IL-6, TNF, and IL-2 (Upthegrove *et al.*, 2014) and, following prenatal vaginal infection, a susceptibility to illness in infancy was associated with increased risk of later psychotic experience (Betts *et al.*, 2014).

As has been touched on above, many studies have tried to assess specific infection type as well as relationship to timing of infection and the relationship to schizophrenia risk. To briefly summarize, peri-conceptual genital/reproductive infection (Babulas *et al.*, 2006), influenza in the first or second trimester (Brown *et al.*, 2004a), bacterial infection in the first trimester (Sørensen *et al.*, 2009), respiratory infection in the second trimester (Brown *et al.*, 2000b), and HSV-2 in the third trimester/at delivery (Buka *et al.*, 2001a; Buka *et al.*, 2008) are associated with increased risk of schizophrenia in affected offspring. While most studies seem to lack the power to make firm statistical conclusions, according to the systematic

review by Khandaker et al, it seems that there is a trend for increased risk of schizophrenia following exposure to maternal infection in earlier stages of pregnancy (Khandaker *et al.*, 2013), and as such this should certainly be investigated and validated further.

1.1.5 Parkinson's Disease

A final disease of note is Parkinson's disease (PD), which is a degenerative disorder most notably affecting the motor system. The hallmark clinical symptoms associated with PD include resting tremor, bradykinesia (slow movement, often associated with impaired ability to adjust body position), rigidity, or postural imbalance (Litvan *et al.*, 2003). These motor symptoms arise from a slow and progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) (Gibb & Lees, 1991; Damier *et al.*, 1999; Toulouse & Sullivan, 2008). Epidemiological databases are not yet old enough to examine the association of maternal infection and later risk of PD in affected offspring and as such, a clear association (or the lack there of) between MIA and PD remains to be determined. However, initial investigations took place in the 80s using influenza epidemic data and a cohort of people with PD. The initial study identified an increased risk of developing PD if an individual was born in specific years which were close to influenza pandemics of 1890-1930 (Mattock *et al.*, 1988). Hence, they evaluated if the risk of developing PD was associated with the influenza mortality rate for the year of the individual's birth (ranging from 1900-1930), and the investigations resulted in a significant correlation between influenza mortality rates and the year of birth of people who later developed PD by some (Mattock *et al.*, 1988), but were not replicated by others (Ebmeier *et al.*, 1989). As mentioned previously, this type of study has numerous mechanistic concerns and that may account for the discrepancy in results as well as the reason that no further crude epidemiological investigations were subsequently conducted regarding the relationship of MIA to PD. Regardless of the lack of epidemiological evidence, the idea that MIA might have a role in PD etiology remains an intriguing focus of on-going research. A common postmortem finding in the SN of Parkinson's patients is increased microglial activation and cytokine levels: TNF-α, IL-1ß, IL-6, IL-8, IL-10 and IL-12, implicating immune/ inflammatory involvement the pathology of PD (McGeer *et al.*, 1988; Banati *et al.*, 1998; Imamura *et al.*, 2003; Koziorowski *et al.*, 2012; Perry, 2012). Also, many of the genes posing

genetic risk factor to PD, including LRRK2 (Wallings *et al.*, 2015), NR4A2 (Han & Cao, 2012), and PARK7 (Waak *et al.*, 2009; Voros *et al.*, 2013), have immune-related functions. Furthermore, a hypothesis has emerged that MIA may prime microglia to maintain inflammatory processes throughout the lifespan, leading to altered responses to neuronal death and protein aggregation, which in turn results in subsequent progression of the α-synuclein aggregates into Lewy bodies that are the pathological hallmark of PD (reviewed in Glass *et al.*, 2010; Tomé *et al.*, 2013; Knuesel *et al.*, 2014). Thus, there seems to be a relationship between immune mechanisms and PD pathology which must be investigated further to be fully understood, and equally it will be imperative to determine if an association exists between MIA and PD once the epidemiological data becomes available.

1.2 Critical Windows

The development of the human brain is a highly complex and dynamic sequence of overlapping events. These events are highly orchestrated and controlled through genetic direction, epigenetic regulation, and environmental influence (Tau & Peterson, 2010). For these reasons, elucidating how disruptions in this neurodevelopmental program may lead to neurodevelopmental and neuropsychiatric diseases and disorders remains a challenge for neuroscientists, particularly as many of the most prevalent disorders are thought to originate following combined alterations in genes and environment (Fishbein, 2000; Tsuang, 2000; Warner & Schapira, 2003; Le *et al.*, 2008; Berardi *et al.*, 2015). For example, there is a genetic component in schizophrenia, as the heritability index for schizophrenia is 75% (Andersen, 2003), but twin studies have demonstrated that concordance is never 100% for monozygotic (identical) twins, suggesting that environmental factors have an important impact in disease development (Tsuang, 2000). Similar evidence has also been reported for PD, which has numerous genetic risk factors but they only account for about 30% of the familial and 3-5% of the sporadic cases (Houlden & Singleton, 2012; Klein & Westenberger, 2012). This is because environmental factors are able to mediate and modulate gene functions which are subsequently expressed in the final phenotype (Fishbein, 2000). Thus, the importance of environmental factors on the final outcome and disease progression is obvious. Pregnancy is the first instance that developing brains are exposed to different environments, and we know from an insurmountable body of evidence that what happens during pregnancy has lifelong impact on the developing embryo (Barker, 2002; de Boo & Harding, 2006; Schlotz & Phillips, 2009; Faa *et al.*, 2014; Seremak-Mrozikiewicz *et al.*, 2014) because disruptions in developmental processes can lead to profound structural and functional alterations in brain development (Thompson *et al.*, 2009a; Ben-Ari, 2013). MIA is one such disruption, and it may be the initiating insult which alters the developmental trajectory toward increased vulnerability to later insults and subsequent development of future disease (Schlotz & Phillips, 2009; Faa *et al.*, 2014; Knuesel *et al.*, 2014). Importantly, insults such as MIA may have a greater effect on neural development if the event occurs during certain developmental windows more than others, giving rise to critical windows for sensitivity (Andersen, 2003). Therefore, it is important to investigate the timing of

neurodevelopmental processes in order to understand the potential consequences of infection at different developmental stages.

1.2.1 Neurogenesis

Beginning around the 5th gestational (GA) week, neuronal progenitor cells (NPCs) are born and by about GA week 8 they differentiate into neuronal cell types or glia (Tau & Peterson, 2010). Then cells are subsequently laid down in an evolutionarily conserved manner, establishing older regions first (Jacobson & Rao, 1991; Andersen, 2003). Hence, different brain regions have specific developmental schedules. For example, hippocampal neurogenesis peaks around GA week 9 (Rakic & Nowakowski, 1981) but may continue into the postnatal period (Borre *et al.*, 2014), and cortical neurogenesis began around the same time, but continued up to 2.5 years of age (Workman *et al.*, 2013). This suggests that a disruption may alter process of neurogenesis more in one brain region than the other, depending on the timing the insult occurred.

In order for the developmental program to progress correctly, gene expression is tightly controlled. Epigenetic mechanisms play a large role in this control, and in fact regulate neurogenesis (Shen *et al.*, 2005). These mechanisms, including DNA methylation and histone modification, are processes that result in functional changes to the genome, without altering the DNA sequence itself (Bird, 2007). Importantly, these changes can be maintained through cell divisions for the duration of the existence of the cell and into future cell generations, and as such are essential for development (Jaenisch & Bird, 2003; Bird, 2007; Saitou *et al.*, 2012), but also have the potential for negative consequences, including cancer (Muñoz *et al.*, 2012). Hence, non-genetic factors have the power to alter how genes are expressed, and disruptions in this process have cascading effects. For example, DNA methylation that occurs during the differentiation of germ cells has the potential to alter the function of all cells stemming from the affected progenitors (Reik *et al.*, 2001; Saitou *et al.*, 2012), which may have implications for neuronal function later in development. Importantly, it has been proposed that increased cytokine production, such as that which occurs during MIA, may alter the epigenetic regulation of genes (Jasoni *et al.*, 2014). Furthermore, evidence has supported the association of copy number variants (CNVs; variations in the number of copies of one or more sections of DNA in a cell) and maternal infection during pregnancy in

increased severity of ASD symptomology (Mazina *et al.*, 2015), providing a link between epigenetics, MIA, and more severe ASD symptoms. Thus, MIA during the period of neurogenesis has potential to lead to adverse consequences, possibly stemming from epigenetic modifications.

1.2.2 Neuronal Migration

Immature neurons must migrate from the germinal zones within which they are generated to their final place of residence (Sidman & Rakic, 1973). Migration peaks at GA weeks 12-20 and the process is largely completed by GA weeks 26-29 (de Graaf-Peters & Hadders-Algra, 2006). The migrating neurons require the guidance of glial fibers along which the neurons move (Hatten, 2002; Chao *et al.*, 2009), and neurotrophic factors, including glial cell line-derived neurotrophic factor (GDNF) (Pahnke *et al.*, 2004), to accomplish this.

Errors in neuronal migration following genetic mutations can lead to improper gyri and culi patterning, resulting in the smooth brain disorder called lissencephaly, which is associated with significant developmental delays as well as decreased life expectancy (Olson & Walsh, 2002). Epidemiological evidence from patients with neuronal migration disorders has suggested a role for prenatal events including infections in the pathogenesis of these disorders (Palmini *et al.*, 1994). Furthermore, schizophrenia, which has increased risk following MIA (Patterson, 2007; 2009; Brown & Patterson, 2011), has been proposed to be linked to disturbance in neuronal migration by some (Weinberger, 1999), but this was not seen by others (Heinsen *et al.*, 1996). In schizophrenia postmortem studies, however, abnormal expression of *reelin* (a gene involved in neuronal migration) has been observed (Fatemi *et al.*, 2000b) and subsequently others have indicated the need for further research to substantially identify the role of cell migration in schizophrenia pathology (Lewis & Levitt, 2002). Poor neuronal migration can also cause ectopic cortical tissue in white matter which may lead to seizure disorders (Tau & Peterson, 2010). White matter injury is a hallmark of CP (Yoon *et al.*, 1997; Dammann & Leviton, 1998), the increased risk of which is also associated with MIA (Ahlin *et al.*, 2013; Miller *et al.*, 2013). Thus, there is cause for further investigation into the possible effects of MIA on neuronal migration.

1.2.3 Axonal and Dendritic Growth

By about the 16th week of gestation, neurons that have completed migration begin to branch outward, extending axons and arborizing dendrites (Jacobson & Rao, 1991). Both of these actions are promoted by neurotrophins and growth factors, including nerve growth factor (NGF), basic fibroblast growth factor (FGFb), brain derived neurotrophic factor (BDNF), and neurotrophin (NT)-3 (Cohen-Cory & Fraser, 1995; McAllister, 2000; Pérez-Domper *et al.*, 2013). As axons extend they begin to form connections, and these connections along with growth factors BDNF, NGF, GDNF, as well as neuronal and glial cross talk (Lemke, 2001) assist in the final placement of neurons (Jacobson & Rao, 1991; Andersen, 2003).

Loss of dendrites and spines has been implicated in schizophrenia (Glantz & Lewis, 2000; Broadbelt *et al.*, 2002). Furthermore, cytokines are able to cross the placenta following MIA (Zaretsky *et al.*, 2004), and cytokines have been shown to inhibit dendritic growth and complexity of cortical neurons (Gilmore *et al.*, 2004), suggesting possible implications for MIA in this developmental window as well. Indeed, this is in complete agreement with research from our lab group which showed that this developmental window (late gestation in the rat) is sensitive to the effects of MIA as we found increased IL-1β levels in the placenta as well as altered neurite length in superior cervical ganglion (SCG) neurons cultured from offspring that had been exposed to LPS on E18 (Straley *et al.*, 2014).

1.2.4 Programmed Cell Death

The developmental program initially produces more cells than are actually needed (Yamaguchi & Miura, 2015), and so it must eliminate the excess. Programmed cell death (PCD) is initiated in the second trimester, peaking in GA weeks 19-23 to get rid of post-mitotic cells (Tau & Peterson, 2010). Approximately 50% of neurons are destroyed during this phase in which PCD is the default mechanism and only those neurons which are able to access enough growth factors can avoid apoptosis (Pérez-Domper *et al.*, 2013). This neuronal refinement results in morphological rearrangement as the system strives to match the numbers of neurons to the requirements of the target field they innervate (Andersen, 2003).

PCD is highly conserved evolutionarily, suggesting critical importance in developmental processes (Yamaguchi & Miura, 2015), and there is a general absence of PCD in the adult brain as there is no need for a global decline in neuronal numbers (Tower, 2014). Yet, aberrant PCD in adulthood is associated with progressive degenerative diseases such as PD (Vila & Przedborski, 2003; Perier *et al.*, 2012; Franco-Iborra *et al.*, 2015) and Alzheimer's disease (AD) (Glass *et al.*, 2010; Tower, 2014). Some have suggested that immune mediators, including TNF-α and IL-1β, might directly initiate neuronal apoptosis in degenerative diseases because the neuropathology is associated with neuroinflammation (increased astrogliosis and microgliosis) (Glass *et al.*, 2010), but also apoptosis can result from deficits in its own regulation (Vaux, 1993). As such, it is possible that disturbances during the development phase might prime this process to remain unusually active throughout life, possibly driven by increased inflammatory mediators, resulting in increased cell death and associated neurological disease.

1.2.5 Synaptogenesis

While PCD is occurring, during the second trimester, neurons that do not become fated for apoptosis continue migrating toward their targets, and subsequently dendritic branching and axon extension increase with the assistance of growth factors (Pérez-Domper *et al.*, 2013) and glial cells signaling (Haydon, 2001; Eroglu & Barres, 2010). These processes, along with the help of scaffolding cells (Lopez-Bendito *et al.*, 2006) help orchestrate synaptogenesis. Synaptogenesis is the process of forming functional synapses between neurons, and it involves precisely aligning a neurotransmitter release site in the presynaptic neuron's axon terminal with receptors on the dendrite of the postsynaptic target neuron (Jacobson & Rao, 1991). Synaptic density continues to increase in a near logarithmic and region dependent manner from the late second trimester, following birth and throughout infancy, resulting in overproduction of synapses and receptors in early youth (Andersen, 2003; Levitt, 2003; Tau & Peterson, 2010). For example, the timing of synaptogenesis differs across specific cortical layers, beginning during the second trimester in the deeper layers first (Huttenlocher, 1990; Huttenlocher & Dabholkar, 1997; Zecevic, 1998), and synaptic density peaks at around 6 months of age in the primary visual cortex (Huttenlocher & De Courten, 1986) after which levels begin to decline, and the

prefrontal cortex reaches maximum levels around 8 months, at about 50% higher than adult levels, continuing through 2-4 years of age (Huttenlocher, 1979; Kostović *et al.*, 1995; Huttenlocher & Dabholkar, 1997; Lenroot & Giedd, 2006). The regionally dependent timeline for cortex development was further validated by a subsequent longitudinal MRI study that showed the primary visual cortex and somatic sensory cortex reach peak thickness first, followed by higher order areas like the prefrontal cortex (Shaw *et al.*, 2008)

The axonal glycoprotein neural cell adhesion molecule L1 (mediator of cytoskeleton and extracellular matrix interaction) is impaired by fetal alcohol exposure (Ramanathan *et al.*, 1996). Thus, it has been suggested that the occurrence of fetal alcohol exposure during the cell migration/synaptogenesis phase of development, contributes to characteristics of fetal alcohol syndrome (Tau & Peterson, 2010). Indeed, events that occur during this developmental stage, such as maternal separation or neglect, are implicated in adverse outcomes for the affected infant brain, including decreased synaptogenesis (Perry, 2002). This has been confirmed in animal studies which showed improved hippocampal synaptogenesis, and associated increases in N-methyl-D-aspartate (NMDA) receptor subunit, (which is important in synaptic plasticity and memory function (Li & Tsien, 2009)) and BDNF following improved maternal care in rats (Liu *et al.*, 2000).

1.2.6 Myelination

Myelination begins in the last trimester, but the vast majority occurs after birth (Ulfig *et al.*, 1998; Back *et al.*, 2001; Jakovcevski *et al.*, 2007), following maturation of oligodendrocytes, the myelin forming glial cells (Lappe-Siefke *et al.*, 2003; Funfschilling *et al.*, 2012). This process of encasing myelin around neuron axons is essential for normal brain function as it allows for rapid and efficient nervous system communication (Deoni *et al.*, 2011). Myelination is a feature of the dramatic brain growth seen in newborns and toddlers (Dekaban, 1978; Tau & Peterson, 2010). Following this most productive phase in infancy, myelination begins to slow after about 1 year of life, though it continues steadily afterward into adulthood (Barnea-Goraly *et al.*, 2005; Gao *et al.*, 2009).

Disturbance of myelination is hallmark characteristic of PVL (Iida *et al.*, 1995) and as such is associated with CP, which has increased risk following a number of prenatal events including maternal infection (Wu *et al.*, 2000; Sankar &

Mundkur, 2005; Pakula *et al.*, 2009; Ahlin *et al.*, 2013). Importantly, it may be the pre-myelinating oligodendrocytes which are sensitive to environmental insults as a study examining myelin production and oligodendrocyte progression demonstrated that the vulnerable window for PVL occurs during late oligodendrocyte progenitor phase at 23-32 gestational weeks, before the process of myelination is highly active (Back *et al.*, 2001). This indicates that while a certain developmental process may be altered in disease pathology, the important critical window could actually be a preceding phase. For example, if MIA occurs during GA week 23 and alters oligodendrocyte maturation, this could lead to poor myelination and later development of PVL and/or CP.

1.2.7 Synaptic Pruning/ Brain Re-wiring

Concurrently with myelination, during infancy, synaptic density reaches peak levels and subsequently the brain undergoes a competitive elimination of synapses, allowing for correction of superfluous cells and synapses (Cowan *et al.*, 1984). Indeed, it has been proposed that pruning of synapses occurs in order to work toward the desired synaptic density for the brain regions to have the best synaptic transmission efficiency (Andersen, 2003; Eroglu & Barres, 2010), as synaptic pruning helps with the 'rewiring' of the brain toward more adult-typical, more energy efficient connections (Spear, 2013). The time course for this developmental phase depends on brain region: sensory and motor regions begin after birth, association cortices and corpus callosum comes next, finally followed by regions which support higher cognitive functions (Levitt, 2003). The brain does not finish pruning synapses until much later, reaching average adult levels by adolescence (Huttenlocher, 1979; Glantz *et al.*, 2007) or young adulthood (Petanjek *et al.*, 2011). Neurochemical and molecular modifications also occur during adolescence, along with the notable structural changes associated with the synaptic pruning and subsequent neuronal rewiring of the adolescent brain (Paus *et al.*, 2008). All of these changes occur in response to genetic cues as well as environmental stimuli, and are extremely important in determining the final appearance of the mature brain (Paus *et al.*, 2008; Berardi *et al.*, 2015). Indeed, environmental information from before and during adolescence influences the structural and functional alterations (Berardi *et al.*, 2015), and in fact the information is incorporated permanently into the mature brain (Andersen, 2003). This suggests that the rearrangements that occur in the adolescent

developmental phase set the final stage for how the brain functions from that point forward.

Abnormalities in synaptic pruning and plasticity processes have been associated with ASD (Onore *et al.*, 2012) because of the increased size in infant ASD brains observed with MRI (Dager *et al.*, 2008). Though there are other possible explanations for the enlarged brain size, including reduced apoptosis or abnormal brain patterning (Belmonte *et al.*, 2004; Tau & Peterson, 2010), implicating other developmental phases in ASD as well. During adolescence, the remarkable developmental changes observed coincide with altered pharmacological sensitivity, with reports of enhanced sensitivity to the positive pleasure rewards initiated by drugs and decreased sensitivity to the negative aspects of a stimuli (Doremus-Fitzwater *et al.*, 2010), as well as dramatic changes in the hypothalamic-pituitary-adrenal (HPA) axis in response to stress (Eiland & Romeo, 2013). Developmental changes are especially responsive to this altered pharmacological sensitivity and stress response changes (Andersen, 2003), and as such the highly plastic adolescent brain is exceptionally vulnerable to pathological insult during this time. Therefore, if a predisposition due to genetic risk and *in utero* adversity like MIA previously occurred, and the adolescent rewiring would incorporate this information, another insult during adolescence has the potential to continue the brain down a developmental trajectory toward neurodevelopmental and neuropsychological disorders, such as schizophrenia and mood disorders which happen to have a peak age of onset in adolescence (Paus *et al.*, 2008; Berardi *et al.*, 2015).

1.2.8 Conclusion

Brain development is a highly orchestrated temporally specific, and yet dynamic process, involving genetic input as well as adaptation to environmental factors (Berardi *et al.*, 2015). MIA is one type of prenatal insult which has the ability to affect different aspects of fetal brain development depending on the time during pregnancy in which the infection occurs (Andersen, 2003; Knuesel *et al.*, 2014), and therein lies a potential for certain developmental periods to be more vulnerable to MIA and result in subsequent alterations in developmental trajectory. This suggests that the key to understanding disease development is dependent on our understanding of the long lasting effects of initial insults like MIA as the first step in

a potential for disease development. Therefore, having insight into critical windows of vulnerability is imperative with regards to disease prevention. There is abundant evidence suggesting potential vulnerable windows for MIA to increase the risk of disease (see first section "Epidemiological and Clinical Evidence"); however it will be important for future research to establish links in causality, and which critical windows and developmental processes are associated with which specific diseases. And of extreme importance, we need to identify the initial vulnerable period within which altered environment might instigate adverse developmental outcomes. For example, dendrite loss is a characteristic of schizophrenia (Glantz & Lewis, 2000; Broadbelt *et al.*, 2002) but it may not be the dendrite growth developmental phase which is most vulnerable, but instead a previous developmental window, which when altered then leads to poor dendrite growth downstream. Only within that framework of knowledge, can we identify where MIA is most detrimental in terms of fetal outcome, and attempt to identify appropriate preventions. Therefore, continued study of normal neurodevelopmental processes, as well as identifying the systems which are most vulnerable to deviations in their developmental trajectory will help us to identify where prevention needs to occur to protect from MIA and its adverse effects on neurodevelopmental outcomes in affected offspring.

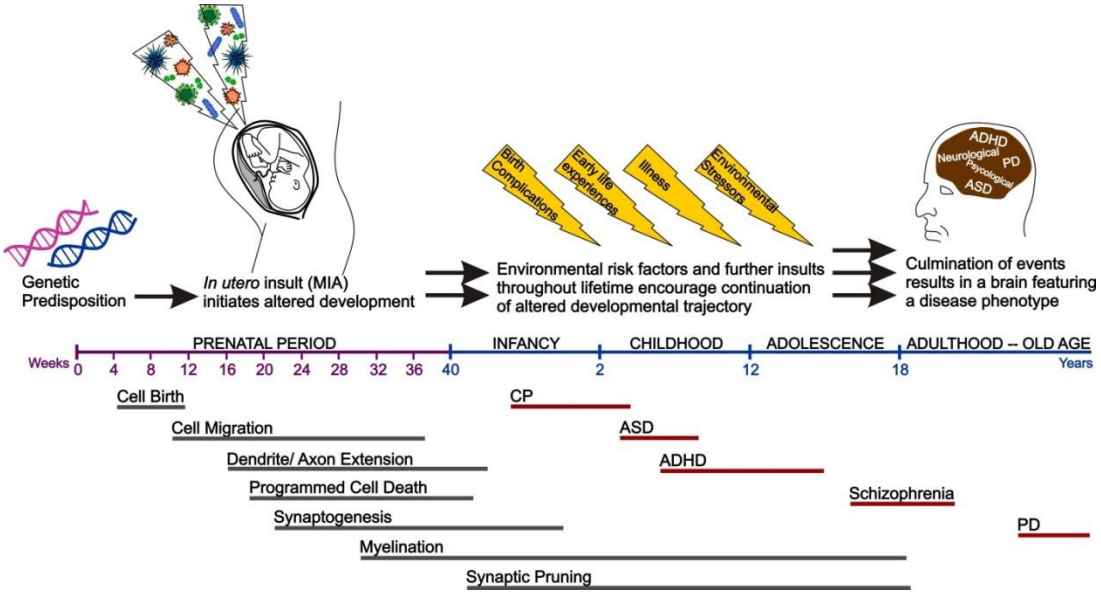


Figure 1: Hypothesized sequence of events contextualized within the temporal profile of neurodevelopment and neuropsychological disorder onset.
Neurodevelopmental phases are temporally organized and tightly regulated, providing the possibility for specific critical windows of vulnerability in which there is an increased risk of aversive outcomes following an insult such as MIA. If MIA occurs during a critical window, it may alter the developmental program in such a way as to leave the offspring more susceptible to further insults over time and to different neuropsychological disorders throughout life. Numerous diseases, with unique etiologic and pathologic characteristics, are associated with MIA, although the exact mechanistic role(s) of MIA in disease progression is still part of ongoing investigations. In order to fully elucidate the effects of MIA, and subsequently discern how and when to prevent negative outcomes, continued investigations into the critical windows of vulnerability is crucial.

1.3 Animal Models

While correlations between maternal infection and increased risk of developing numerous diseases later in life has been shown (see section "Epidemiological and Clinical Evidence"), causation has yet to be fully elucidated. To this end, animal models are widely used, most commonly with murines. Animal models allow researchers to ask specific questions within the wide area of MIA, especially in relation to timing of infection (both gestational time point and duration of infection) and dose (i.e. modeling mild or severe infections). The main animal models used are described in detail below, and then the effects of these models on offspring will be discussed in subsequent sections.

1.3.1 Influenza

The first animal models in MIA utilized infecting pregnant rodents with an intranasal (IN) droplet inoculation of human influenza (Fatemi *et al.*, 1999; Fatemi *et al.*, 2002a; Fatemi *et al.*, 2002b). Influenza is a virus, consisting of an increasing number of subtypes, which commonly induces antiviral as well as proinflammatory innate immune responses (Wu *et al.*, 2011). Pattern-recognition receptors (PRRs) recognize viral RNA and hence are necessary for detecting influenza infection within the cell. PRRs consist of TLR-7, retinoic acid inducible gene-I (RIG-1), and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) (Pang & Iwasaki, 2011). Single stranded viral RNA binds to TLR7 and double-stranded viral RNA binds to TLR3 and RIG-1 which causes a signal cascade leading to increased production of proinflammatory cytokines and type 1 interferons (Alexopoulou *et al.*, 2001; Heil *et al.*, 2004). Interferons such as IFN-α and IFN-β inhibit protein synthesis and thus limit the ability of the virus to replicate (Kreijtz *et al.*, 2011). Type 1 interferons also stimulate dendritic cells, which help to initiate innate immune response (Banchereau & Steinman, 1998; Steinman, 1998). As many rodent models of influenza resulted in death from the infection (Van Reeth, 2000), studies now generally uses a lower dose than estimated human disease viral load, such as $10^{-4.5}$ of 6.5 log 10 ($CCID_{50}$), which induces a moderate but non-lethal viral infection (Fatemi *et al.*, 2008). Experimental influenza is usually administered intranasally.

The time course for this infection is pretty well known for humans and rodents. In humans, nasal lavage fluid IL-6 and IFN-α levels and IL-6 plasma levels peak at day 2 along with the most significant symptoms, including temperature and mucus production (Hayden *et al.*, 1998). TNF-α peaks after 3 days in plasma and 4 days in nasal fluid, concurrent with viral shedding. IL-8 peaks at 4-6 days, which is the same time most symptoms in the lower respiratory tract are observed. Importantly, similar results have been reported from patients with naturally occurring acute influenza (Kaiser *et al.*, 2001), suggesting that the viral time course is consistent irrespective of how it was caught. In order to translate rodent influenza studies to human, the time course was determined using a normal rodent, i.e. animals that were not pregnant. These studies provided numerous reports of early or immediate rises in numerous cytokine levels, including IFN-α, TNF-α, IL-1 α, IL-1β and IL-6, in bronchoalveolar lavage (BAL) fluids along with the normal rodent clinical symptoms which includes anorexia, lethargy, and decreased body temperature (Vacheron *et al.*, 1990; Hennet *et al.*, 1992; Peper & Van Campen, 1995; Kurokawa *et al.*, 1996). Specifically, increases in BAL IL-6 is observed first within 1 day of infection, and with a slight decrease remains significantly above basal levels until day 6 which is when the virus is fatal for most mice strains (Hennet *et al.*, 1992). TNF-α BAL levels increase significantly the first day, peak by 36h and then decrease while IFN-γ BAL levels are first detected at 36h, peak at 2 days and decline until death (Hennet *et al.*, 1992). Rats show a similar time course in that when administered influenza, IL-1α, IFN-α, IL-6, TNF-α, GRO-α, and MIP1β are reported at increased levels in lung tissue shortly following inoculation, and IFN-γ, IL4, IL10 and IL12-p40 are seen at increased levels slightly later, during viral replication (3 d), and then again during pulmonary inflammation (up to 3 weeks) (Ottolini *et al.*, 2005). This model has decreased in popularity over the past decade, largely due to the long time course and the development of more controllable models. Indeed, influenza has a very different time course to its subsequent models (described below) in that the immune responses to influenza takes a day or two to appear and certain symptoms take weeks to resolve, whereas the other models are much more acute making them easier to monitor and control.

1.3.2 LPS

LPS is a large molecule that constitutes a major component of the cell wall in Gram negative bacteria (Raetz & Dowhan, 1990). LPS consists of three main constituents: a lipid domain, a core oligosaccharide, and a polysaccharide (Raetz & Whitfield, 2002). The hydrophobic area of the molecule (Lipid A) is an endotoxin and induces a strong immune response in animals, and as such is a well-characterized model for bacterial infection (Galanos *et al.*, 1985; Raetz & Whitfield, 2002). The immunogen binds to toll like receptor (TLR)-4, which activates an immune response (Faure *et al.*, 2000; Lin *et al.*, 2000). TLR-4 recruits numerous proteins, resulting in the activation of mitogen-activated protein kinases (MAPKs) as well as nuclear translocation of nuclear Factor-kappaβ (NF-kβ) and the activation for transcription factors, which initiate expression of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 (Wang *et al.*, 2006; Girard *et al.*, 2010).

LPS has been injected intraperitoneally (I.P.), intravenously (I.V.), intranigrally (I.N.), or subcutaneously (S.C.) in doses ranging from 50μg/kg up to 700μg/kg [for comprehensive review (Boksa, 2010)]. Following administration, LPS has shown to increase blood levels of IL-1β, IL-6, and TNF-α, as well as produce fever in rodents (Gayle *et al.*, 2004; Ashdown *et al.*, 2006). Furthermore, an increase in pro-inflammatory cytokines IL-1β, IL-6, and TNF-α has been observed in the placenta and increased TNF-α levels have been demonstrated in amniotic fluid following maternal exposure to LPS (200μg/kg (Girard *et al.*, 2010); 500μg/kg or 2.5mg/kg (Urakubo *et al.*, 2001)). In particular, IL-1β significantly increases following maternal LPS exposure, with levels 20-fold higher than IL-6 and 50-fold higher than TNF-α in the placenta (Girard *et al.*, 2010) suggesting that IL-1β may play a greater role in fetal programming compared to the other cytokines. In fact, when LPS is co-administered with IL-1Ra, LPS induced cytokine expression in the placenta was reduced (Girard *et al.*, 2010), implicating a potential preventative therapy in protecting against MIA and its adverse outcomes.

The time course for this endotoxin is pretty well understood. In humans, LPS injection induces an early increase of pro inflammatory cytokines TNF-α and IFN-γ, quickly followed by a dramatic increase in IL-6, which has been shown to reach peak levels 3-4 hours post injection (Kemna *et al.*, 2005). Anti-inflammatory cytokine IL-10 peaks 2-3 hours post injection, and at 4 hours there was no change in

IL-1β in humans (Kemna *et al.*, 2005). In pigs, TNF-α is increased 10 fold 2 h post I.P. injection and is still elevated at 4 h, but is returned to normal by 8 h; IL-6 is increased at 2 h and peaks at 4 h with levels 200 fold greater than baseline and has returned to control levels by 12 h (Webel *et al.*, 1997). In the rat, plasma cytokine levels have also been analyzed following I.V. and I.P injections: IL-1β increases shortly following LPS, peaking at 1.5 h (6.5 fold from basal level) and returns to basal levels by 3.5 h post; IL-6 begins to increase 1 h after LPS and peaks at 1.5 h (15.1 fold increase) and returns to basal levels after 5 h; TNF-α increases as early as 30 min post LPS and peaks at 1 h post injection (11.6 fold increase) and returns to normal at 3.5 h post injection (Kakizaki *et al.*, 1999). In another study, radiolabeled LPS was present in maternal organs and amniotic sac 1h after injection and in the placenta 2-8h post injection, and importantly this study demonstrated that LPS does not cross the placental barrier as no radiolabeled LPS was observed in the fetus (Ashdown *et al.*, 2006).

It has been known for some time that there is a wide variability in the endotoxin, as it is produced from bacteria. Indeed, different batches or lots result in different pyrogenic and cytokinogenic activity (Ray *et al.*, 1991). For example, as Boksa first reported, there are numerous factors that have been shown to alter the effect of LPS on animal temperature: higher room temperature, I.V. administration, and higher doses of LPS all increase the likelihood of the animal being hypothermic rather than hyperthermic (Derijk *et al.*, 1994; Fofie & Fewell, 2003; Boksa, 2010). Because of this information, researchers using LPS must be aware of the different results possible, and in order to compare studies in the literature effectively the specifics of the batch/lot and other experimental factors need to be explicit.

1.3.3 Poly I:C

Polyinosinic:polycytidylic acid (Poly(I:C)) is a synthetic double-stranded RNA (ds-RNA) analog which mimics a viral infection (Field *et al.*, 1967; Fortier *et al.*, 2004). It consists of a pair of strands of polyinsoinic and polyctidylic acids. Poly(I:C) works through TLR-3, which is expressed in the endosomal compartment where it recognizes microbial nucleic acids like dsRNA (Alexopoulou *et al.*, 2001; Savva & Roger, 2013). After Poly(I:C) binds to TLR3, signals are sent via Toll-IL-1 (TIR) domain-containing adaptor molecule-1 (TICAM-1) to activate the transcription factors interferon regulatory factor 3 (IRF-3), NF-kB, and AP-1, leading to the

induction of type I IFN, cytokine/chemokine production and dendritic cell (DC) maturation (Alexopoulou *et al.*, 2001; Oshiumi *et al.*, 2003; Yamamoto *et al.*, 2003). Initiation of the immune response depends heavily on DCs as they stimulate B and T lymphocytes (Banchereau & Steinman, 1998; Steinman, 1998). Poly I:C has been shown to induce pro-inflammatory cytokine production from dendritic cells (Cella *et al.*, 1999; Verdijk *et al.*, 1999) while decreasing anti-inflammatory cytokine IL-10 (Gilmore *et al.*, 2005; Meyer *et al.*, 2006b). In addition, Poly(I:C) also induces type 1 interferons IFN-α and IFN-β (Alexopoulou *et al.*, 2001).

Poly(I:C), like LPS, is injected intraperitoneally, intravenously or subcutaneously. In rodents, Poly(I:C) has been shown to increase sickness behaviors as early as 30min post treatment, including anhedonia, lethargy, hunched posture, hind limb stiffness, decrease locomotion, decrease body weight up to 6 h (Cunningham *et al.*, 2007; Gandhi *et al.*, 2007; Smith *et al.*, 2007). There is also an observed bi-phasic temperature response whereby hyperthermia occurs from 4-8h post treatment and hypothermia is observed 12-24 h post treatment (Smith *et al.*, 2007). Poly I:C (750μg/kg) has been shown to increase rat plasma levels of IL-1β, IL-6, and TNF-α (Fortier *et al.*, 2004). Furthermore, maternal administration of Poly(I:C) (10 or 20 mg/kg) resulted in increased TNF-α levels, and decreased BDNF and NGF levels in the rat placenta (Gilmore *et al.*, 2005).

The time course of cytokine induction following Poly(I:C) has been investigated in rodents. Firstly, in mice, systemic Poly(I:C) induced IFN-β beginning 1 h and peaking to 5000-fold increase on control levels at 3 h with a decrease toward control levels after 24h; systemic Poly(I:C) also increases levels of IL-6 which peaks at 3 h with 10,000-fold over control levels and continues to be elevated even at 24 h; IL-1β induction increases 100-fold at 3 h and subsequently decreases rapidly back to basal levels; finally, TNF-α is clearly induced by 1 h, peaks at 1000-fold above control level at 3 h, and persists through 12 h (Cunningham *et al.*, 2007). Following maternal administration of Poly(I:C) (4.5mg/kg on E16.5) in the mouse, there were two waves of cytokine expression in placenta, one at 2h post injection characterized by increased IL-6 and another at 4h demonstrating increased levels of IL-1β, TNF-α, and IFN-γ (Koga *et al.*, 2009). In rats, peripheral Poly(I:C) increased plasma IL-6 concentration 5-fold 2 h post injection and this returned to baseline by 4 h; plasma TNF-α increased 4-fold at 2 h; plasma

concentration of IL-1β was unaffected by Poly(I:C) but was only measured up to 8 h (Fortier *et al.*, 2004).

1.3.4 Cytokine Administration

The final, and least common, animal model is direct administration of cytokines themselves. IL-6 and IL-1β are most commonly used (Samuelsson *et al.*, 2006; Smith *et al.*, 2007; Meyer *et al.*, 2008d). At pathophysiological concentrations, IL-1β (0.5, 1.0, 2.0 and 8.0 ng/24 h), and less effectively TNF-α (20, 100, or 300 ng/24 h), IL-6 (100 ng/24 h), and IL-8 (20 ng/24 h), induced dose dependent anorexia in rats (Plata-Salamán *et al.*, 1996). Furthermore, an increase in cortisol (CORT) and adrenocorticotropic hormone (ACTH) levels 4 h post IL-6 injection (9 μg/kg) has also been observed (Samuelsson *et al.*, 2004). However, IL-6 (9 μg/kg) has also been reported to not produce any obvious sickness behavioral as measured by food intake, weight change, body temperature, or maternal care (Samuelsson *et al.*, 2006). Some studies investigating the mechanisms behind MIA use direct cytokine administration alongside one of the aforementioned models. They have found that the addition of IL-6 increases the severity of sickness in the animals. For example, when IL-6 was administered alongside influenza, animals were more severely ill and either died or suffered miscarriage, compared to when they were subjected to influenza alone (Smith *et al.*, 2007).

1.3.5 Importance of Immunogen, Dosage, and Timing

While all of these animal models have expanded knowledge of numerous diseases, they are not without caveats. Firstly, different diseases are associated with increased risk following different types of maternal infections (see section "Epidemiological Evidence"). Animal models are in agreement with this, showing different, though sometimes overlapping, immune responses following administration of different immunogens (Meyer *et al.*, 2007; Meyer *et al.*, 2009; Boksa, 2010; Harvey & Boksa, 2012). Each immunogen affects the immune response differently, and are not directly comparable to the live infection which they model. For example, LPS administration does not have the exact same physiological response as sepsis, and Poly(I:C) has a very different time course to influenza virus (Meyer *et al.*, 2009).

However, they do initiate the immune response with the key feature being the cytokine induction mechanisms of innate immunity (Meyer *et al.*, 2009).

Secondly, the dosage of the immunogen used in animal models also has a range of effects. For example, a particularly high dose can lead to fetal mortality and small litter sizes (Entrican, 2002; French *et al.*, 2013). Furthermore, immunogen dose can also play a role in the cytokine response. As cytokines are thought to contribute to the MIA effects on fetal brain development (Smith *et al.*, 2007), it is important to understand how different dosages of immunogen alter cytokine profiles of the mother and fetus. For example, injections with different doses of Poly(I:C) resulted in dose dependent IL-10 expression in mouse serum at 1h and 6h post injection (Meyer *et al.*, 2005). Furthermore, in studies that utilize a 'chronic infection' model in which the animal receives multiple injections throughout pregnancy, the additional stress the dam experiences due to the injections may confound results (Meyer *et al.*, 2009). This is because maternal stress itself has been shown to lead to epigenetic programming and adverse behavioral outcomes in offspring (Talge *et al.*, 2007; Zucchi *et al.*, 2013), so it would be challenging to discern if any adverse effects were due to the MIA or the stress caused by multiple injections.

Finally, the timing during pregnancy which an infection occurs is an intrinsic variable to fetal developmental outcome (see section "Critical Windows"). Indeed, many preclinical studies have observed different developmental and behavioral consequences from MIA depending on gestational time points (reviewed in Meyer *et al.*, 2007; Boksa, 2010), and these effects will be described in detail in the next section. Phenotypical differences that result depending on timing of MIA may help researchers to distinguish disease pathologies. For instance, most MIA studies administer the immunogen in mid to late gestational period of the rat, roughly correlating to the central nervous system (CNS) development occurring in the second trimester in the human (Clancy *et al.*, 2007), which is also the time point most associated with infection leading to increased risk of schizophrenia and ASD (see "epidemiological and clinical evidence" section). Alternatively, in CP models, MIA has been administered during the developmental window associated with myelination (approx. E18-P9 in the rat) (Burd *et al.*, 2012). Hence, the fact remains that the gestational time point must be included as a controlled variable in MIA animal models.

1.3.6 Translational Validity

Developmental progression, in terms of major events and general temporal arrangement, seems to be widely evolutionarily conserved (Finlay & Darlington, 1995; Andersen, 2003; Katz, 2007) and as such, preclinical research helps to hypothesize theories and validate knowledge for human situations. The most common preclinical models are from rodents, and the rat model in particular has been validated as a good model for most human developmental processes (Watson *et al.*, 2006). In fact, the location of regulatory genes required for much of development has been conserved for both the human and rat (Akashi, 2001). Furthermore, both humans and rats are altricial species (they are relatively immobile and require mother for nourishment after birth), so they have relatively low CNS development at birth and this similarity allows the rat to be a well suited model for human CNS development particularly in the postnatal developmental period (Watson *et al.*, 2006). The rat undergoes key developmental stages on average 2 days later than the mouse, and therefore results in difference of rodent gestation length (about 22 days for rat and 19 for mouse) (Mason, 1993; Clancy *et al.*, 2007). It is usually accepted that the gestational period of rats and mice roughly correlates to the developmental biology of the first and second trimester of human pregnancy, and the first week after birth in rodents would correspond to the final trimester of human pregnancy (Clancy *et al.*, 2007) (see Figure 2). This 'rule of thumb' is presumed to stem from comparative neuroanatomy studies which demonstrated that neural tube formation occurs during weeks 3-4 in human gestation and on E10.5-11 in the rat (Semple *et al.*, 2013), and that the brain growth spurt that occurs around birth in humans, peaks at P7 in the rat (Dobbing & Sands, 1979; Semple *et al.*, 2013). Furthermore, the weight of the cortex plateaus at around 2-3 years of age in the human, and the corresponding age in the rat is about P20 (Dobbing & Sands, 1979; Semple *et al.*, 2013).

While the rodent models are common, they are far from perfect. Comparisons cannot be made in a direct linear manner between any mammals because developmental processes progress at different rates depending on brain region, and the temporal profile of these differs between species (Jakovcevski *et al.*, 2009). For example, studies of human brain development show that neocortical neurogenesis begins at the same relative time as mouse (GA week 5/E10), but new

neurons are still being born in the human brains long after they have relatively ceased in the mouse (Mo *et al.*, 2007; Mo & Zecevic, 2008), and similar findings of differential temporal length with regards to oligodendrogenesis between humans and mice has also been shown (Jakovcevski *et al.*, 2009). Furthermore, changes in grey matter take much longer in the human compared to the rat, for example, human frontal lobe grey matter reaches peak volume at around puberty (11-12 years) (Bansal *et al.*, 2008) and the temporal lobe grey matter is maxed in adolescence (16-17 years) (Semple *et al.*, 2013) but these volumetric plateaus are observed in most brain structures at P20 in the mouse (Chuang *et al.*, 2011). Another concern in species translation is that of precocial (more mobile and independent infants) and altricial differences, as precocial offspring like guinea pigs have relatively more developed CNS at birth compared to altricial animals like humans (Watson *et al.*, 2006). It is difficult to translate developmental information across species if one species undergoes more extensive development postnatally and is therefore affected by a greater range of environmental factors, versus a species which has a more developed brain at birth. However, even animals with the same developmental pattern show differences. For example, human dentate gyrus neurogenesis is only about 80% complete in the human at birth, whereas only about 20% in the rat, even though both animals are considered altricial (Watson *et al.*, 2006). In fact, the peak of this hippocampal neurogenesis in the human occurs in the first trimester (Clancy *et al.*, 2007), but from E14-E17 in the rat (Rice & Barone Jr, 2000), which does not fit into the normal translational timeframe used by many preclinical models. Along similar lines, behavioral developmental outcomes are extremely difficult to parallel across animal models, as different species have evolved with different behavioral needs and responses due to circumstantial adaptive pressures (Watson *et al.*, 2006).

While important to discuss, these differences may be of minimal consequence as researchers have demonstrated that developmental event order is highly evolutionarily conserved, and even developmental programs which could be separated throughout the course of evolution are so well preserved as to be easily predicted with one model for all neurodevelopmental events in all species (Workman *et al.*, 2013). Also, researchers have identified many of the evolutionary and developmental differences which may influence the translational validity, thus making it more legitimate to make claims in instances that appear consistent (Watson *et al.*, 2006; Katz, 2007). For example, though the time scales differ, the process that

humans and rodents follow for cell proliferation are almost parallel (Bayer *et al.*, 1993). Furthermore, the primary interneurons of the cerebral cortex arise in slightly different ways, with the human neurons originating from two different locations (dorsal forebrain or ventral forebrain) (Letinic *et al.*, 2002), and the rodent interneurons arising mainly from the ventral region (Powell *et al.*, 2001); even though the development procedures involve slightly different process, the necessity and mechanism for interneurons to migrate long distances to reach the correct area of the cortex appears to be conserved in both species (Levitt, 2003). Furthermore, cerebral cortex development in the human begins at about E56 and lasts through P400, whereas in the rat the same developmental processes goes from E12-P16, so even though the development processes take different lengths of time, the mechanisms and key stages are conserved enough that they are easily compared (Levitt, 2003; Semple *et al.*, 2013). Importantly, the major developmental phases seem to line up with major developmental stages as is evident from synaptic pruning and remodeling, processes that are most prolific during adolescence in both species (Semple *et al.*, 2013). Following on from those observations, researchers have used neuroscience, evolutionary science, and statistic modeling to establish models that extrapolate timing of brain development from animal to human, and these are improving all the time (Clancy *et al.*, 2001; Clancy *et al.*, 2007; Workman *et al.*, 2013). These models are based on a comprehensive sampling of developmental literature, as well as allometric growth, which is the idea that increases in size of different organs occurs at different rates, and that is used because a correlation was shown between developmental duration and adult brain volume across mammals (Passingham, 1985; Workman *et al.*, 2013). While these methods are relatively dependable, we must strive for continued improvement in translation strategies as well as verification of the same. To help with this, work with nonhuman primate studies (Liu *et al.*, 2008), and human imaging studies, such as ultrafast MRI which allow for capture and quantification of brain images of progeny during pregnancy (Clouchoux & Limperopoulos, 2012) and after birth (Gousias *et al.*, 2013) are becoming more popular.

In summary, animal models provide insight into MIA events and are valuable for neuroscience research, and most neural events fall into a predictable pattern allowing for generalized theoretical translation of preclinical studies. However, it is an oversimplification to directly extrapolate from animal models to human

pathologies, and depending on the developmental process and brain region a translation may not fit into our generalized "rule of thumb" timeline comparison. Thus, when researchers describe temporal features of developmental processes, it is imperative to remember that the developmental timeline must be in the context of the specific animal tested and the brain maturation processes of that species. To then draw conclusions or extrapolate about other species, namely human, researchers should properly address the inexact nature of translating and make relative comparisons as opposed to exact connections while we continue to increase validity in translational modeling.

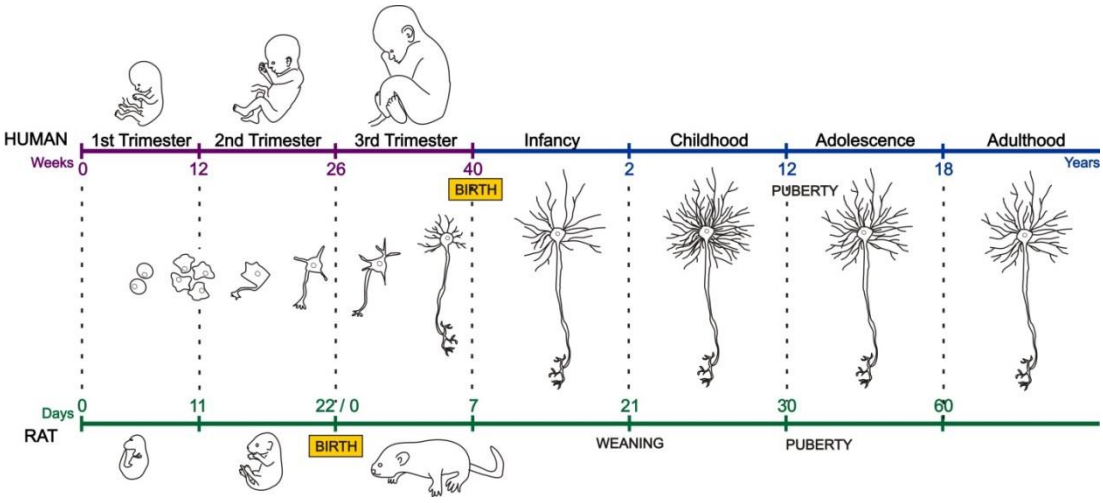


Figure 2: Temporal relativity of rat and human neuron development
This timeline depicts neuronal development within generalized temporal profiles and key developmental milestones illustrated for rats relative to humans. These species have structured developmental patterns which are mostly evolutionarily conserved, and the developmental timelines are somewhat understood for each. Thus, researchers utilize the rat as a good translational model for many, but not all, human neuronal processes.

1.4 Effects of MIA on Offspring Behavior

One of the most common ways animal models are used to understand the effects of MIA is through behavior studies. These models of MIA have shown that exposure to LPS, Poly(I:C), and influenza result in behavioral outcomes consistent with symptoms of neuropsychiatric disorders such as schizophrenia and ASD, including deficits in attention, memory, social behavior, and pre-pulse inhibition (Patterson, 2011a; Harvey & Boksa, 2012; Meyer, 2013). These and other behavioral characteristics that arise in animals following MIA are reviewed.

1.4.1 Pre-pulse Inhibition

Pre-pulse inhibition (PPI) is a neurological phenomenon present across species in which a weaker stimulus presented before a stronger stimulus reduces the amplitude of the final response elicited by the stronger stimulus (Braff *et al.*, 2001). PPI is a measure of sensorimotor gaiting, as such deficits in PPI are associated with an inability to filter out extraneous environmental stimuli, which leads to sensory overload and cognitive fragmentation (Braff & Geyer, 1990; Swerdlow *et al.*, 1994). People with schizophrenia and ASD exhibit sensorimotor gating deficits (including PPI) with characteristic inability to regulate sensory, motor, and/or attentional processes (Braff *et al.*, 2001; Wynn *et al.*, 2005; Perry *et al.*, 2007; Braff *et al.*, 2014), thus PPI is widely studied in animal models of neurological disease (Geyer *et al.*, 2001; Nestler & Hyman, 2010; Powell *et al.*, 2012; Young *et al.*, 2014) and is one of the most prolific behavioral assays used in MIA models (Meyer *et al.*, 2007; Boksa, 2010).

Deficits in PPI have been observed in adult rat offspring following chronic exposure to LPS (1mg/kg, every other day of gestation (Borrell *et al.*, 2002); 2mg/kg, every day (Romero *et al.*, 2010)) or more acute LPS exposure (50 or 100 μg/kg, E15 and E16 or E18 and E19) (Fortier *et al.*, 2007)). In agreement with this, acute Poly(I:C) (4mg/kg on E15) exposure resulted in PPI impairments in juvenile (P34-35) and adult (P56-57 (Wolff & Bilkey, 2008; Howland *et al.*, 2012) or >P90 (Dickerson *et al.*, 2010; Mattei *et al.*, 2014)) rat offspring. Interestingly, Wolff and Bilkey demonstrated that adverse behavioral effects following MIA were not due to maternal weight loss (Wolff & Bilkey, 2010) which is a common symptom of illness in rodents (Sim *et al.*, 2009; Hill *et al.*, 2015). However, others did not replicate this

finding, and in fact demonstrated that offspring who were exposed to Poly(I:C) (4mg/kg on E15) and whose mothers lost weight in response to the immunogen had more behavioral deficits than the offspring exposed to Poly(I:C) whose mothers gained weight following the injection (Missault *et al.*, 2014), suggesting that maternal response to infection is a factor in fetal outcome. Another group demonstrated that the duration of MIA may contribute to the difference in effects as a one-time administration of Poly(I:C) (8mg/kg) on E14 had no association to maternal weight change and PPI of offspring, but after chronic administration (E14-E18) offspring from mothers who lost weight after the Poly(I:C) exposure demonstrated significantly altered PPI (Vorhees *et al.*, 2012; 2015).

Deficits in PPI following MIA have also been shown in mice. For example, one of the earliest studies investigating how MIA affects this behavior, by Shi, Fatemi and others, showed a deficit in PPI in the acoustic startle response of offspring exposed to influenza virus on E9.5 (Shi *et al.*, 2003). Consistently, chronic Poly(I:C) (5mg/kg) exposure from E12-E17 (Ozawa *et al.*, 2006) or on E10.5, 12.5 and 14.5 (Malkova *et al.*, 2014) resulted in inhibited PPI. Interestingly, others have demonstrated a greater effect when MIA occurs earlier in mouse pregnancy compared to later. Indeed, Poly(I:C) (5 mg/kg) on E9, but not on E17, resulted in inhibited PPI in adult offspring (Meyer *et al.*, 2008b; 2008c; 2008d; Vuillermot *et al.*, 2010; 2011; 2012). This was confirmed by others who found PPI deficits in adolescent and young adult offspring (P28-56; P63) following prenatal exposure to Poly(I:C) on E9.5 (20mg/kg (Shi *et al.*, 2003); 60mg/kg (Makinodan *et al.*, 2008)). Importantly, lower doses of Poly(I:C) did not lead to deficits in PPI: a 2.5mg/kg dose administered to pregnant mice on E9 resulted in no differences in PPI of adult progeny, however the 5mg/kg dose did result in deficits (Lipina *et al.*, 2013). Taken together, these suggest that PPI deficits are age and dose dependent, and specifically that the middle of gestation in the mouse is a particularly vulnerable time for higher doses of Poly(I:C) to affect PPI of affected offspring later in life.

Interestingly, the mouse Poly(I:C) model has demonstrated that the adverse behavioral outcomes resulting from MIA are mediated, at least in part, by IL-6 (Smith *et al.*, 2007; Lipina *et al.*, 2013). Indeed, maternal injection of IL-6 on its own, administered on E12.5, resulted in PPI and other behavioral deficits in adult offspring, while MIA (PolyI:C, 20mg/kg) in IL-6 knockout mice did not result in behavioral changes (Smith *et al.*, 2007). Furthermore, co-administration of anti-IL-6

antibody with Poly(I:C) (20mg/kg) prevented the behavioral deficits caused by Poly(I:C) MIA (Smith *et al.*, 2007). This is consistent with a subsequent study utilizing a mutant mouse model combined with MIA: Disrupted-In-Schizophrenia-1 (*Disc1)*-L100P$^{+/-}$ mice were administered Poly(I:C) (5mg/kg) on E9 and the offspring demonstrated more profound behavioral deficits with the combination of genetic and environmental factors, compared to just the mutation alone, and co-administration of anti-IL-6 antibody and Poly(I:C) prevented the schizophrenic behaviors in the offspring (Lipina *et al.*, 2013).

1.4.2 Latent Inhibition

Latent inhibition (LI) is another attentional phenomenon found across species in which acquisition of a conditioned response is slower after pre-exposure to the conditioned stimulus. Hence, LI demonstrates an organism's ability to ignore irrelevant stimuli, and as such is a good measure of attentional processes or disruption of the same, and organisms with impaired attentional shifting express the LI effect in conditions where control animals do not (Weiner, 2003). Attentional processes, including LI deficits are symptomatic in schizophrenia (Braff, 1993; Braunstein-Bercovitz *et al.*, 2002). Therefore, LI protocols are used in many rodent models of schizophrenia (Weiner & Arad, 2009), even though the translational validity has been scrutinized recently (Schmidt-Hansen & Le Pelley, 2012). In MIA studies, a few different paradigms have been used to examine LI, including a drinking suppression after tone-shock pairing (Zuckerman *et al.*, 2003; Zuckerman & Weiner, 2005; Piontkewitz *et al.*, 2011a; Piontkewitz *et al.*, 2011b) or else initiating a freezing conditioned response after tone-shock pairing (Meyer *et al.*, 2006a; Meyer *et al.*, 2006c). MIA has resulted in adult LI deficits following *in utero* Poly(I:C) exposure in the rat (4mg/kg) on E15 or E17 (Zuckerman & Weiner, 2005). This has also been demonstrated in the mouse, with LI deficits resulting from Poly(I:C) exposure on E9 (5 mg/kg) (Meyer *et al.*, 2006c; 2008d; Lipina *et al.*, 2010; 2011; 2012), E12.5 (20mg/kg) (Smith *et al.*, 2007), or E15 (4mg/kg) (Piontkewitz *et al.*, 2011a; 2011b). Interestingly, LI deficits were not found in adolescent or young adult rats (P35, P45, P56), but they were seen at later ages in males (at P70 and P90) and females (at P90) following Poly(I:C) (4mg/kg) on E15, suggesting that effects of MIA on LI are sex and age dependent (Piontkewitz *et al.*, 2011a).

1.4.3 Maze Testing

The Morris water maze (MWM) is another of the more common behavioral assays in MIA studies. It is a navigation task in which the animal is placed in a pool and must locate a platform, thus this behavioral assay provides a robust readout of spatial learning and memory (Mulder & Pritchett, 2003). This test has been used to demonstrate a spatial learning deficit in adolescent rats (P28) that had been exposed to LPS (500 μg/kg) on E19 (Lanté *et al.*, 2008). This is in agreement with another study that found rat offspring had diminished platform acquisition in MWM following Poly(I:C) (8mg/kg) on E14-E18 (Vorhees *et al.*, 2015). Similarly, multiple exposure to prenatal cytokines (IL-6, 9 μg/kg) in middle (E8, 10 and 12) or late (E16, 18 and 20) pregnancy also decreased adult offspring (P140) spatial learning in MWM (Samuelsson *et al.*, 2004; Samuelsson *et al.*, 2006). Interestingly, the spatial learning differences commonly found in the MWM are exacerbated by age, as one study looked at young adult (3 month), adult (10 month) and aged (20 month-old) rats after they had been exposed to prenatal LPS (0.79mg/kg) on E8, 10, and 12; the spatial learning and memory deficits observed in MIA offspring were more significant with age, implicating MIA in increased risk of memory deterioration in older age (Hao *et al.*, 2010). The MWM is also used to assess reversal learning by using a paradigm where the animal learns an initial discrimination task, and then they must learn to reverse this choice. MIA has resulted in conflicting effects on reversal learning in rodents because maternal exposure to Poly(I:C) (4mg/kg) on E15 or E17 resulted in enhanced reversal learning in two different MWM tasks for rats (Zuckerman & Weiner, 2005). However, others showed impaired reversal learning in the MWM following Poly(I:C) in mice (5mg/kg, on E17 (Meyer *et al.*, 2006b) and in rats (8mg/kg, E14-E18 (Vorhees *et al.*, 2015)). As the timing of MIA was similar for all of these studies, one possible explanation for the differences is the dose of Poly(I:C) used and taken together these data might suggest that lower dose of Poly(I:C) leads to reversal learning benefits while a higher dose leads to detrimental effects. Another use of the MWM can be to assess associative learning whereby a cue, such as a platform with a ball or flag mounted above the water, is used to help the animal find the platform. In this scenario, researchers found no difference in cued learning for rats exposed to *in utero* Poly(I:C) (8mg/kg) from E14-E18 (Vorhees *et al.*, 2015). Certain cells of the entorhinal cortex and hippocampus, which work to map space outside of the

organism, increase in firing when rats approach a hidden platform MWM trials but not a cued platform (Vorhees & Williams, 2014), and perhaps this difference explains why rats exposed to MIA suffer in the hidden platform MWM task, but not in the cued protocol. However, another group did find a decrease in associative learning in the cued water maze for adult mice that were exposed to LPS *in utero* (120 μg/kg) on E17 (Golan *et al.*, 2005). Rats and mice have demonstrated different abilities in water mazes previously (Whishaw & Tomie, 1996), with mice showing an inferior performance compared to rats as well as displaying off task behaviors that rats do not (Vorhees & Williams, 2014), and perhaps this predisposition to substandard performance in the behavioral assay is exacerbated by MIA in the mouse, accounting for the difference in results.

Dry mazes are also often used to assess memory of MIA offspring. One such example, that is often used to assess spatial recognition memory, is the elevated y-maze. It is based on the principle that animals would rather explore a novel arm of the maze versus revisiting one they had previously encountered (Conrad *et al.*, 2003). Mice exposed to Poly(I:C) (5mg/kg) in late gestation (E17) developed severe spatial learning and memory deficits as demonstrated by increased time exploring the familiar arm versus the novel one and compared to control animal times; these memory deficits are similar to those associated with Alzheimer's disease (Krstic *et al.*, 2012). This is in agreement with others who have demonstrated decreased spatial and working memory performance in adult mice following exposure to Poly(I:C) (5 mg/kg) on E17 (Meyer *et al.*, 2008d) and chronic LPS (8μg/kg) exposure from E8-E15 in the radial arm maze (Wang *et al.*, 2010), which is similar to the y-maze except for having more arms to utilize in experimental design. Finally, the elevated plus maze is a different test which provides key insight into the anxiety and/or depression levels of an animal. It is based on the rodent characteristic aversion to open spaces, and animals with increased anxiety will avoid the open arms (Hogg, 1996; Costa *et al.*, 2014). MIA increased this anxiety behavior as MIA offspring that were expose to LPS (25μg/kg) on E9 had reduced time spent in the open arms of the elevated plus maze compared to control offspring (Depino, 2015).

1.4.4 Novel Object Recognition

The novel object (NO) task is another memory assay in which the animal is presented with a familiar object and a novel one. Control animals generally remember the familiar object and therefore spend more time investigating the new object, thus an animal that spends similar amounts of time with the two objects, or more time with the familiar object, is considered to have alterations memory function (Antunes & Biala, 2012). MIA seems to affect this process in rats as those exposed to gestational LPS (0.5mg/kg, administered every other day E14-E20) demonstrated impaired NO recognition (Graciarena *et al.*, 2010). In agreement with this, rats exposed to Poly(I:C) (4mg/kg) on E15 had reduced NO preference compared to control animals (Wolff *et al.*, 2011). Importantly, this group demonstrated that the behavioral decline was not due to decreased preference for novelty, providing evidence that it is strictly a memory impairment (Wolff *et al.*, 2011). Research with mice is in complete agreement with these findings, as decreased NO recognition has been demonstrated following *in utero* exposure to Poly(I:C) (5mg/kg) from E12-E17 in the affected adult mouse offspring (Ozawa *et al.*, 2006). Interestingly, LPS exposure in mice at E9 (300 μg/kg) resulted in increased exploration of the familiar object (Coyle *et al.*, 2009), indicative of a memory deficit, while LPS administered at E17 (120 μg/kg) showed increased novel object recognition (Golan *et al.*, 2005), suggesting that LPS exposure in middle pregnancy of mice leads to more detrimental memory outcomes in offspring compared to a later exposure.

1.4.5 Social Interaction/ Communication

Because social behavior deficits are common in ASD and schizophrenia, a social interaction test is commonly used in animal models of these disorders (Tordjman *et al.*, 2007; Harvey & Boksa, 2012). Many researchers have reported deficiencies in social interaction behaviors of offspring that were exposed to MIA in mid-pregnancy. For example, when two novel mice from the same experiment group were placed together for social interaction, mice born to mothers infected with influenza on E9.5 engaged in social interaction 2.7-fold less frequently than mice born to saline injected mothers (Shi *et al.*, 2003). Aside from this basic experimental paradigm, most other social interaction assessments utilize the three chamber apparatus to compare the time a rodent spends investigating each area, and these

compartments will hold a similar or novel animal, a novel object, or else remain empty (Silverman *et al.*, 2010). These different experimental paradigms provide evidence for different aspects of social behavior. For example, preference for a novel or familiar mouse assesses social memory and ability for the animal to discriminate familiar or novel social stimuli (O'Tuathaigh *et al.*, 2007). Interestingly, this paradigm showed no differences between mice exposed to MIA (Poly(I:C), 5mg/kg) or saline on E9 (Lipina *et al.*, 2013), suggesting that MIA does not affect social memory. In another paradigm, preference for another animal over an empty chamber indicates social motivation (Brodkin *et al.*, 2004). Utilizing this protocol, mice exposed to Poly(I:C) on E9 (5mg/kg)(Lipina *et al.*, 2013), on E9.5 (20mg/kg) (Soumiya *et al.*, 2011a), or on E12.5 (Smith *et al.*, 2007) showed a decline in motivation for social interaction as they opted to spend more time in a chamber with only an empty cylinder rather than a interacting with a novel same-sex mouse. The final experimental paradigm, which compares the time a rodent spends with a novel object versus an unfamiliar animal, is one of the most common experimental procedures because this paradigm does not require memory functioning (Malkova *et al.*, 2012) and because it is thought to mirror human ASD symptoms in that a socially impaired animal will spend more time investigating the novel object compared to the other animal (Silverman *et al.*, 2010). Indeed, one group saw this social interaction deficit in adult MIA offspring that had been exposed to Poly(I:C) (5mg/kg) on E10.5, 12.5, and 14.5 (Malkova *et al.*, 2012).

This group went a step further by investigating communication behaviors, including acoustic and chemical signals, as these communication techniques are important to rodent social behavior (Malkova *et al.*, 2012) and deficits in communication are also characteristic of ASD (APA 2013). In this study, MIA male and female pups born to mothers who were administered Poly(I:C) (5mg/kg, on E10.5, 12.5, and 14.5) demonstrated deficits in the total number and syllable repertoire of ultrasonic vocalization (USV)s, and adult males had reduced USV response to social stimuli (Malkova *et al.*, 2012). This is entirely consistent with another group that has found communication deficits in male rat pups and social interaction impairments in adult offspring following *in utero* LPS exposure (100μg/kg) on E9.5 (Kirsten *et al.*, 2010b; Kirsten *et al.*, 2012; Kirsten *et al.*, 2015). Furthermore, as scent marks (urinary traces) are used to identify mice, mark

territory, and attract mates, olfactory communication is also important for mice (Hurst, 2009). MIA males showed diminished olfactory communication as they deposited half scent marks compared to controls when stimulated with female urine (Malkova *et al.*, 2012). Taken together, these impairments in social interactions and associated communication behaviors may be indicative of numerous symptoms observed in people with ASD, further validating these preclinical models for disorders associated with MIA.

1.4.6 Open Field and Pharmaceutical Treatments

Locomotion and exploratory behaviors have also been assessed following MIA because these behaviors can provide information on motor functioning and anxiety in test subjects (Eilam, 2003; Prut & Belzung, 2003). Locomotor activity in an arena, termed the "open field" (OF) test, was increased at 3 months, but decreased at 17 months in adult rats following prenatal exposure to LPS (1mg/kg) on E10.5, indicating a greater age-related decline in motor functioning in animals exposed to LPS *in utero* compared to control animals (Ling *et al.*, 2009). Furthermore, influenza exposure on E9.5 (Shi *et al.*, 2003; Moreno *et al.*, 2011) and Poly(I:C) exposure on E12.5 (20mg/kg) (Smith *et al.*, 2007) resulted in decreased OF exploration in adult mice. Depending on the measurement, decrease exploration can also be indicative of anxiety-like behavior as a more anxious animal will tend to avoid the center of the arena (Prut & Belzung, 2003). Hence, some researchers have reported increased anxiety in offspring exposed to MIA in mid pregnancy using the OF test, including mice exposed to LPS (25μg/kg on E9) (Depino, 2015) or Poly(I:C) (20mg/kg on E9.5) (Soumiya *et al.*, 2011a). Inconsistent findings are prevalent with the OF test, however, as another group reported increased locomotor activity in adult mice following Poly(I:C) on E17 (2mg/kg) (Vuillermot *et al.*, 2012).

OF testing is not always a simple case of placing an animal in an arena, and indeed pharmaceuticals are often used in OF behavior assessments as they can exacerbate subtle differences in motor functioning (Bernardi *et al.*, 1986; Jackson *et al.*, 1994; Rodríguez *et al.*, 2013) and because excessively increased amphetamine (AMPH)-induced activity is considered to mimic the exacerbation of psychotic symptoms following AMPH exposure in people with schizophrenia (Laruelle *et al.*, 1996). One group showed an increase in adult rat AMPH-induced locomotion

following an LPS model of MIA (50 or 100 μg/kg) on E18 and E19 (Fortier *et al.*, 2007). This was confirmed by multiple groups using Poly(I:C) MIA models which demonstrated increased AMPH, methamphetamine (MAP) and/or MK-801 (*N*-methyl-D-aspartate (NMDA) agonist) induced locomotion following *in utero* MIA exposure in rats (4 mg/kg, on E15 or E17) (Zuckerman *et al.*, 2003; Zuckerman & Weiner, 2005; Piontkewitz *et al.*, 2011a; Piontkewitz *et al.*, 2011b) and mice (2 or 5 mg/kg, on E9 or E17 (Meyer *et al.*, 2007; Meyer *et al.*, 2008d); 5mg/kg, E12-17 (Ozawa *et al.*, 2006)). However, there is some inconsistency in the literature as some have reported that MIA resulted in decreased response to pharmaceuticals. For example, reduced AMPH-induced locomotion was observed for adult rats exposed to Poly(I:C) (8mg/kg) on E14 (Richtand *et al.*, 2011), and locomotion response to MK-801 was reduced in pre-pubescent rat offspring following *in utero* Poly(I:C) (4mg/kg) exposure on E15 (Howland *et al.*, 2012). Interestingly, maternal starting weight predicted the weight change in response to Poly(I:C) administration (8mg/kg on E14), which was associated with differential progeny responses to dopaminergic and glutamatergic drug challenges (Bronson *et al.*, 2011; Vorhees *et al.*, 2012). Specifically, one study showed that maternal weight loss resulted in decreased locomotion response to AMPH and MK-801, whereas absence of maternal weight loss following Poly(I:C) injection resulted in increased locomotion response to MK-801 and similar performance to controls following AMPH administration (Bronson *et al.*, 2011). However, another study demonstrated that the Poly(I:C)/maternal weight loss group exhibited exaggerated hyperactivity response to AMPH, and an attenuated hyperactivity response to MK-801 (Vorhees *et al.*, 2012). It is of note that these studies had opposite results with regards to AMPH responding even though the MIA and offspring behavior protocols were identical (same dose, timing, rout), suggesting there are more environmental or genetic factors which need to be elucidated in order to have replicable models across laboratories. Even though the direction of response is unconfirmed for many of these pharmaceutical treatments, the evidence still clearly shows that MIA affects the responsiveness to drugs in affected offspring, and that in and of itself is of huge importance with regards to drug addiction and schizophrenia where altered response to drugs may play a role (Hambrecht & Häfner, 1996; Chambers *et al.*, 2001).

Along similar lines, serotonin and glutamate systems are hypothesized to be involved in schizophrenia etiology (Aghajanian & Marek, 2000; Rao *et al.*, 2012;

Selvaraj *et al.*, 2014), and are also required for anti-psychotic drug function (Jakab & Goldman-Rakic, 1998; Aghajanian & Marek, 1999; De Bartolomeis *et al.*, 2013). While not applied in an OF paradigm, hallucinogens have also been administered to MIA offspring to investigate motor drug-response consequences of MIA. In response to hallucinogens (which require the serotonin 5-HT(2A) receptor), mice exposed to the influenza virus on E9.5 had increased head-twitch responses, and this was associated with upregulated 5-HT(2A) receptor (Moreno *et al.*, 2011). This study also showed a diminished antipsychotic effect of metabotropic glutamate receptor (mGlu(2)) agonist with an associated down regulated mGlu(2) receptor in the same offspring (Moreno *et al.*, 2011). These data further suggest that MIA may affect the ability of the offspring to respond normally to pharmaceuticals due, at least in part, to differential receptor phenotypes. The difference in these OF and pharmaceutical administration studies results emphasizes yet again the incongruent findings observed from different animal models. The need for more in depth studies that closely monitor and compare effects of experimental differences as well as studies that will help to elucidate which environmental factors exacerbate MIA effects on fetal outcome is becoming ever more important.

Reference	**Animal Model**	**Immunogen, Dose, Rout**	**Timing**	**Key Findings**
Smith, 2007	Mouse Cytokine	IL-6, 5 µg in 200 µl 0.9% saline I.P.; or Poly IC, 20 mg/kg, IP	E12.5	IL-6: ↓ PPI, LI (adult offspring); Poly IC: ↓ PPI, LI, OF exploration, social interaction (adult age); Co-administration of anti-IL-6 antibody with poly (I:C) prevents the deficits caused by poly I:C (adult)
Moreno, 2011	Mouse Influenza	Influenza (H1N1), $5*10^3$ pfu IN	E9.5	↓ locomotor activity, ↑ head-twitch after hallucinogen, ↓ antipsychotic effect of glutamate agonist (10-12 weeks)
Shi, 2003	Mouse Influenza	Influenza, IN; or Poly IC, 20 mg/kg, IP	E9.5	Flu: ↓ PPI, OF and NO exploration, social behavior (P28-56); Poly IC: ↓ PPI (P42-P56)
Coyle, 2009	Mouse LPS	LPS, 300 µg/kg SC	E9	↑ exploration of familiar vs novel object (P85)
Depino, 2015	Mouse LPS	LPS, 25µg/kg, SC	E9	↑ anxiety/depressive behaviors: avoided open arms in EPM, center of OF, and the light side of light/dark box; ↑ immobility in forced swim test and tail suspension (8-10 wks old at start)
Golan, 2005, 2006a,b	Mouse LPS	LPS, 120 µg/kg, IP	E17	↓ learning in cued water maze, ↑ novel object recognition, ↑ anxiety, ↓ passive avoidance (P240), ↑ OP exploration (P240, P600)
Wang, 2010	Mouse LPS	LPS, 8µg/kg, IP	E8-E15, Daily	↓ learning and retention radial arm maze, altered OF and burrowing/hoarding, impaired beam walking (P70, P200, P400, P600)
Bitanihirwe, 2010	Mouse Poly IC	Poly IC, 5 mg/kg, IV	E17	↓ Social interaction, ↑ anhedonia in sucrose preference, ↑ LI, alterations in locomotor and stereotyped behaviors following APO treatment (8 + wks)
Lipina, 2010, 2011, 2012, 2013	Mouse Poly IC	Poly IC, 2.5mg/kg or 5mg/kg, IV	E9	↓ cognitive and social behavior; ↓ PPI (14-16wks); *Disc1*-L100P+/- mutants > sensitivity to MIA vs WT or Disc1-Q31L+/- (7-15 wks)
Makinodan, 2008	Mouse Poly IC	Poly IC, 60mg/kg, IP	E9.5	↓ PPI (P63)
Malkova, 2012, 2014	Mouse Poly IC	Poly IC, 5mg/kg, IP	E10.5, E12.5, and E14.5	↓ USVs (P9 and adult), ↓ socializing and communicating behaviors, ↑ marble burying, ↑ self-grooming, ↓ PPI of startle response, ↑ sensitivity to hallucinogenic drug DOI = similar to schizophrenia (8-10 weeks old)
Meyer, 2006a,b, 2008b,c,d	Mouse Poly IC	Poly IC, 2 or 5 mg/kg, IV	E9 or E17	E9: ↓ PPI, ↑ startle response, ↓LI, OF exploration, ↑ AMPH and/or MK801 induced locomotion (P35, P90-P120); E17: ↓ reversal learning of L-R discrimination; ↓ working memory in water maze; ↑ AMPH and MK801 induced locomotion (P98-P112)
Ozawa, 2006	Mouse Poly IC	Poly IC, 5 mg/kg, IP	E12-E17	↓ PPI, thigmotaxix, NO recognition; ↑ MAP-induced locomotion (P63-P70)
Soumiya 2011	Mouse Poly IC	Poly IC, 20mg/kg, IP	E9.5	↑ anxiety in OF, ↓ Social interaction (P56-63)
Vuillermot 2010, 2011, 2012	Mouse Poly IC	Poly IC, 5mg/kg or 2mg/kg, IV	E9 or E17	↑ OF locomotion, ↑ AMPH-induced locomotion, altered apomorphine-induced locomotion, ↓ PPI (adult), ↑ LI, visual discrimination task, ↓ working

				memory in dry maze, (P30 and P70)
Wolf, Susanne, 2011	Mouse Poly IC	Poly IC, 5mg/kg, IP	E15	Voluntary exercise (P50) rescues behavioral deficits in PPI, OF (P90)
Samuelsson, 2004, 2005, 2006	Rat Cytokine	IL-6, 9 µg/kg IP	E8, E10, &E12 OR E16, E18, &E20	abnormal/extended stress responses to restraint and novel environment and CRF/ACTH stimulation in adulthood; ↓ special learning in Morris water maze (P140)
Borrell, 2002	Rat LPS	LPS, 1mg/kg, SC	Alternate days whole pregnancy	↓ PPI (P70, P100, P300)
Fortier, 2004, 2007	Rat LPS	LPS, 50 or 100 µg/kg, IP	E15 and E16 or E18 and E19	↓ PPI; just E18/E19: ↑ AMPH induced locomotion (P70)
Girard, 2009, 2010	Rat LPS	LPS, 200 µg/kg, IP	2X/day from E17 to birth	↓ latency to fall off rotarod (P30, P35, P40)
Graciarena, 2010	Rat LPS	LPS, 0.5mg/kg, SC	Every other day E14-E20	↓ NO recognition (>P60)
Hao, 2010	Rat LPS	LPS, 0.79mg/kg IP	E8, 10 and 12	↓ spatial learning and memory (3, 10 and 20 month old)
Kirsten, 2010, 2012, 2015	Rat LPS	LPS, 100 µg/kg, IP	E9.5	↓ general activity in OF aft immune challenge; ↓ Social Interaction, T-maze learning and memory (adults); ↓ communication (P11); Treatment of zinc post LPS prevented USV impairment; no difference: plus maze; normal OF, apomorphine stereotypy, nor haloperidol induced catalepsy
Lanté, 2007, 2008	Rat LPS	LPS, 500 µg/kg, IP	E19	↓ Spatial learning in water maze (P28)
Ling, 2009	Rat LPS	LPS, 1mg/kg, IP	E10.5-E11	↑ locomotor activity (P90), ↓ locomotor activity (P480)
Liu, 2004	Rat LPS	LPS, 1mg/kg, SC	Alternate days whole pregnancy	↑ ethanol intake and preference, ↓ rearing (P100-P130)
Romero, 2007, 2010	Rat LPS	LPS, 2 mg/kg, SC	Daily whole pregnancy	↓ PPI (P35, P70, P170, P180, P400)
Wijkstra, 1991	Rat LPS	LPS, 0.2 or 2.0 µg/ pregnant dam, IV	E18	↑ latency to initiate sexual behavior; ↓ intromission by males (P91)
Bronson, 2011	Rat Poly IC	Poly IC, 8mg/kg, IP	E14	Maternal weight loss = ↓ MK-801 and AMPH locomotion; no maternal weight loss = ↑ MK-801 locomotion, AMPH locomotion similar to control
Dickerson, 2010	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	↓ PPI (3months)
Howland, 2011; Zhang, 2012	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	↓PPI, ↓ MK-801 induced locomotor activity (P35-36 and P56-57); ↓ object-in-place memory (>P60); Impaired cognitive flexibility (males only) in operant paradigm (P53)

Mattei, 2014	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	↓ PPI and startle response (P90-98)
Missault, 2014	Rat Poly IC	Poly IC, 4mg/kg, SC	E15	Mothers lost weight = deficits in: PPI; locomotor activity w/ AMPH, y w/out AMPH, w/ MK-801, w/out MK-801; ↓ sucrose preference; Mothers gained weight = no clear behavioral deficits (P76-104)
Piontkewitz, 2011a, 2011b	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	↓ LI, ↑ AMPH induced activity (P35, 46, 56, 70, 90)
Richtand, 2011	Rat Poly IC	Poly IC, 8mg/kg, IP	E14	↓ response to AMPH in locomotion (P90)
Van den Eynde, 2014	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	No difference in PPI; ↓ basal startle response and spontaneous locomotion (P56, 90, and 180)
Vorhees, 2012, 2015	Rat Poly IC	Poly IC, 8mg/kg, IP	E14 or E14-E18	Acute exposure: no effect on elevated zero maze, OF, object burying, light-dark test, swimming, MWM, cued fear, LI; females of MIA+ mat weight gain group had ↓ cued water maze; MIA+ mat weight loss had ↑ hyperactivity to AMPH, ↓ hyperactivity to MK-801 (P65-70). Chronic exposure: NS outcomes w/= elevated zero maze, OF locomotion, marble burying, straight swimming channel, Cincinnati water maze, MWM, conditioned fear; ↑ PPI, ↓ time on dark side of light-dark test, ↓ Morris water maze acquisition, ↑ OF AMPH, ↓ OF MK-801 (P65-92)
Wolff, Amy 2008, 2010, 2011	Rat Poly IC	Poly IC, 4 mg/kg, IV	E15	↓ PPI, ↓NO memory (P56)
Zuckerman, 2003; Zuckerman and Weiner, 2003, 2005	Rat Poly IC	Poly IC, 4 mg/kg IV	E15 or E17	↓ LI, ↑ reversal learning of L-R discrimination, ↑ AMPH and MK801 induced locomotion (P90)

Table 1: Behavioral changes observed in offspring following MIA

Table summarizing a wide sample of literature that has investigated the effects of MIA on behavioral characteristics of offspring. In the "key findings" column the age at which offspring were tested is listed in parenthesis.

1.5 Effects of MIA on Brain Development

In order to understand how or why the aforementioned behavioral alterations occur, researchers utilize animal models to better understand the morphological, molecular, and neurochemical changes resulting from *in utero* exposure to MIA. Indeed, these investigations provide some of the most important evidence for determining the role of MIA in fetal programming and the neuroanatomical basis of the etiology of neurological impairments.

1.5.1 Cytokine Profiles

The first focus of many MIA studies includes analyzing the immune response in offspring by examining the expression of immune biomarkers in the brain and/or serum samples of the offspring. This is because cytokines are important in cell signaling, they are produced by immune cells, and they have been implicated in neurodegenerative disease (Capuron & Miller, 2011; Smith *et al.*, 2012). Some researchers have reported acute (within 24h) increases in mRNA for IL-1β and TNF-α in the fetal rat brain following MIA on E18 induced by LPS (4mg/kg (Cai *et al.*, 2000); 0.7 or 1mg/kg (Paintlia *et al.*, 2004)). However, inconsistent results have also demonstrated a decrease in TNF-α in whole fetal rat brain following LPS (2.5mg/kg) (Urakubo *et al.*, 2001) or Poly(I:C), (10 or 20 mg/kg) (Gilmore *et al.*, 2005) on E16. Furthermore, lower doses of LPS (100μg/kg (Gayle *et al.*, 2004) or 50μg/kg (Ashdown *et al.*, 2006)) on E18 resulted in no change in cytokine profiles in fetal rat brain for IL-1β, TNF-α, IL-6, and IL-10. This has also been investigated in mouse models of MIA. In this model, LPS (120 μg/kg (Golan *et al.*, 2005); 500 μg/kg (Ning *et al.*, 2008)) on E17 resulted in increased TNF-α and IL-6 in the fetal mouse brain. Another study analyzed fetal brains 6h post administration of Poly(I:C) (20mg/kg) to pregnant mice on E16 and found increased levels of cytokines: IL-1β, IL-7, and IL-13; and chemokines: monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α, interferon gamma-induced protein (IP)-10, and monokine induced by IFN-γ (MIG) (Arrode-Brusés & Brusés, 2012). Also, Poly(I:C) administration (2 or 5 mg/kg) to pregnant mice on E9 increased fetal brain IL-1β, IL-6, and TNF-α levels, while it decreased IL-10 levels, and administration on E17 increased IL-1β and IL-6 (but not TNF-α) and also increased IL-10 (Meyer *et al.*, 2006b; Meyer *et al.*, 2008d), further implicating that the timing of MIA exposure

is critical to fetal outcome. Therefore, even though the cytokine profiles of fetal brains can be quite different depending on dose, timing of immunogen exposure, as well as timing of cytokine measurement, it is clear that MIA alters this complex system.

Importantly, cytokine alterations are not only apparent in fetal brains, but also characteristic of MIA offspring all the way through to adulthood. For example, IL-2 and IL-6 serum levels were increased in adult rat offspring exposed to prenatal LPS (1mg/kg, alternate days whole pregnancy) (Borrell *et al.*, 2002), and IL-1β and TNF-α were upregulated in adult rat brains that were exposed to maternal administration of Poly(I:C) (4mg/kg) on E15 (Mattei *et al.*, 2014). Furthermore, transforming growth factor beta (TGF-β) plays a role in proliferation and cellular differentiation and this cytokine was down regulated in the adult brains of rats exposed to *in utero* LPS (0.5mg/kg; every other day from E14-E20), suggesting a potential mechanistic role for TFG-β in the altered proliferation observed in MIA models (Graciarena *et al.*, 2010). Another, and more comprehensive study, looked at the effects of Poly(I:C) (20mg/kg) administration on E12.5 on mouse offspring levels of 23 different cytokines in the frontal cortex, cingulate cortex, hippocampus, and serum across the progeny lifespan (P0, P7, P14, P30, and P60), and found that most of the cytokines analyzed were altered in a region- and age-specific manner (Garay *et al.*, 2013). Specifically, several pro- and anti-inflammatory cytokines were elevated compared to controls in frontal and cingulate cortices at birth, decreased during adolescence, but then a few cytokines increased again in adulthood (Garay *et al.*, 2013). Interestingly, the hippocampus had distinct cytokine profile differences for MIA offspring, including both increased and decreased levels of different cytokines at every age except adult (Garay *et al.*, 2013). These data correspond with the knowledge that cytokines are part of intricate feedback loops which strive to maintain cytokine levels within a homeostatic range (Careaga *et al.*, 2010). As it is such a complex and dynamic system it makes sense that there will be increases in some cytokines while others are decreased, but again the key finding of importance is that MIA seems to substantially alter the cytokine profiles of its offspring during development and throughout the course of life.

1.5.2 Structural Alterations

Work from Fatemi et al pioneered the investigation into structural consequences of *in utero* exposure to MIA. They noted decreased neocortical and hippocampal thickness following human influenza infection on E9 in mice (Fatemi *et al.*, 1999), and later confirmed this idea when the same group showed significant brain atrophy (~4%) in numerous regions of P35 mice that had been exposed to MIA on E18 (Fatemi *et al.*, 2008). Others have similarly demonstrated alterations in brain region size post MIA; for instance, Poly(I:C) (4mg/kg) on E15 in the rat resulted in progeny with decreased volume of the hippocampus, striatum, and prefrontal cortex, as well as an increased ventricular volume (Piontkewitz *et al.*, 2011a; Piontkewitz *et al.*, 2011b). In agreement with this, MRI scans of humans demonstrated decreased cerebral volume and increased ventricular volume in schizophrenia patients (Wright *et al.*, 2000), suggesting that altered volume of brain regions may be a physiological characteristic for MIA associated disorders.

Aside from the volume analysis, other generalized morphology studies have been conducted in MIA models. For example, animal studies have investigated MIA-induced lesions and altered white matter development following MIA as neonatal brain lesions are associated with CP (Dammann & Leviton, 1998; Bangash *et al.*, 2014). MIA (LPS, 300μg/kg, E19 and E20) lead to significant white matter injury in young rat pups (P1 or P7), consistent with the proposed pathophysiology of CP in humans (Rousset *et al.*, 2006). Interestingly, MIA has also been associated with cerebral cortical lesions, characterized by shrunken neuronal cell bodies and nuclei, described in rat pups (P3, P8) following maternal administration of LPS (200 μg/kg) twice per day E17 through to birth (Larouche *et al.*, 2005). PVL was shown to be followed by impaired cerebral cortical development (Inder *et al.*, 1999) and gray matter lesions occurred in a third or more of PVL cases in premature infants, suggesting that white matter lesions may not occur in isolation, implicating more adverse outcomes of MIA as well as a more complex pathophysiology for CP (Pierson *et al.*, 2007). Because white matter is largely made up of myelinated axons, this has also been studied with regards to MIA. For example, delayed postnatal myelination has been observed in the whole brains of adult mouse offspring (P84) exposed to Poly(I:C) (5mg/kg) on E9 or E17 (Li *et al.*, 2010), and also decreased myelin basic protein staining and decreased myelin thickness was observed in the

hippocampus of juvenile mouse offspring (P14) exposed to Poly(I:C) (60mg/kg) on E9.5 (Makinodan *et al.*, 2008). MIA appears to promote a decrease in brain complexity as seen by this myelin data, and also because prenatal LPS exposure (0.79mg/kg) promoted synaptic loss, shown by decreased synaptophysin (integral regulator of synaptic vesicle function and neurotransmitter release) staining in the hippocampus of 3-,10-, or 20-month old rat offspring (Hao *et al.*, 2010). Furthermore, MIA resulted in decreased hippocampal neurogenesis in rats following LPS (50 or 100 μg/kg, E18 and E19, (Cui *et al.*, 2009)) or Poly(I:C) exposure (4mg/kg on E15 (Mattei *et al.*, 2014)). In agreement with these findings, diminished hippocampal neurogenesis was found in adult mice exposed to Poly(I:C) (5mg/kg) on E9, E17(Meyer *et al.*, 2006b), or E15 (Wolf *et al.*, 2011). However, others did not find differences in adult hippocampal neurogenesis of MIA exposed mice (LPS, 25μg/kg, on E9) (Depino, 2015), though the dose of LPS in this later study may have been too low to produce the same results as the larger doses of LPS or Poly(I:C). Taken together, these studies provide evidence that hippocampal alterations are apparent across the lifespan of affected offspring. Furthermore, it is evident that MIA can result in large structural and developmental alterations in the developing brain.

1.5.3 Cellular Discrepancies

The structural differences discussed presumably stem from developmental disturbances affecting specific cell types. Indeed, a decrease in total proliferating cells and numbers of immature neurons has been observed in the dentate gyrus following MIA in later pregnancy (LPS; 0.5mg/kg; every other day E14-E20) (Graciarena *et al.*, 2010). More specifically, hippocampal γ-Aminobutyric (GABA) neurons seem to be affected by MIA. Indeed, numbers of glutamic acid decarboxylase 67 (GAD67)- and reelin- (markers for GABA neurons) immunoreactive neurons were counted in the hippocampus from pre-adolescent brains following MIA exposure on E15 and E16 (LPS, 100 μg/kg), and they found decreased numbers of GAD67 and reelin immunoreactive cells in the dentate gyrus on P14, and decreased GAD67 positive cells in the dentate gyrus and CA1 on P28 (Nouel *et al.*, 2012). This is consistent with findings in mice exposed to MIA on E9, as those offspring demonstrated reduced reelin-positive cells in the hippocampus at

P24 (Poly-I:C, 5mg/kg, (Meyer *et al.*, 2006b)) as well as specifically in the dentate gyrus in adult offspring (LPS, 25μg/kg, (Depino, 2015)). The hippocampus is not the only brain region with these neurons, though, and indeed MIA leads to alterations in other areas. For example, alterations in Purkinje neuron (a GABA neuron) number, size, and density have been demonstrated in the cerebellums of offspring exposed to Poly-I:C (20mg/kg) on E12.5 (Shi *et al.*, 2009) as well as prenatal influenza in early (E9) or later (E16) pregnancy (Fatemi *et al.*, 1999; 2002c; 2009).

Along similar lines, one group investigated the morphological consequences of MIA on cortical neurons, as inhibitory neurons of the cerebral cortex commonly signal via GABA (Ascoli *et al.*, 2008), and in this study, pregnant mice received an injection of Poly(I:C) (20mg/kg) on E9.5 and offspring brains were analyzed with BrdU labeling to determine the fraction of proliferating cells within the entire cell population as well as information on the cell cycle one week post MIA (Soumiya *et al.*, 2011b). The later stages of corticogenesis were affected following MIA, in that cell-cycle parameters and gene expression of daughter neurons were altered (Soumiya *et al.*, 2011b). They hypothesized that this altered corticogenesis lead to the altered synaptic development of upper-layer neurons (but not deeper layer) found in the cerebral cortex following MIA they found in a previous paper (Soumiya *et al.*, 2011a).

Along with GABA neurons, DA neurons are also a specific neuron type investigated in MIA studies. Cultures of rat mesencephalic neurons from rat offspring at E14.5 and P21 that were exposed to maternal LPS (1mg/kg) on E12.5 demonstrated decreased numbers of tyrosine hydroxylase (TH; a marker for DA neurons) immunoreactive cells in the E14.5 cultures and in P21 SN and ventral tegmental area (VTA) (Ling *et al.*, 2002), findings which were confirmed in numerous follow up studies by the same group (Ling *et al.*, 2004; Ling *et al.*, 2006; Ling *et al.*, 2009). In agreement with this, cultures from rat offspring exposed to LPS on E10, E14 or E18 demonstrated an accelerated loss of DA cells in the cultures over time compared to the control cultures, and the E10 exposure also resulted in impaired dopaminergic innervation into the striatum (Snyder-Keller & Stark, 2008). *In vivo* assessments further demonstrated the toxic effects of LPS (1mg/kg, on E10.5) because SN slices examined from numerous ages showed a persistent loss in

DA neurons across the lifespan (P21, up to 16 months), as measured by stereological cell counts of TH immune-positive cells (Carvey *et al.*, 2003), and this was replicated in other papers from the same lab (Ling *et al.*, 2006; Ling *et al.*, 2009), Importantly, this study also showed that there was not an accelerated rate of DA neuron loss in the LPS animals, as researchers hypothesized, suggesting that MIA does not alter the normal rate of DA neuron loss with age (Ling *et al.*, 2009). However, there were Lewy body like inclusions in the older adult animals along which may suggest a role for MIA in the morphological characteristics associated with PD symptomology. Other groups have found conflicting results regarding the effects of MIA on DA neurons, however. For example, some have demonstrated an increase in DA cell numbers, measured by TH and dopamine transporter (DAT) positive neurons in the mouse fetal mesencephalic flexure at E11, E13, and E17 as well as increased TH immunoreactivity in adult striatum and nucleus accumbens (DA innervated regions), following Poly(I:C) (5mg/kg) administration to the mothers on E9 (Meyer *et al.*, 2008a; Winter *et al.*, 2008). Furthermore, an increase in TH immunoreactivity in the shell of the nucleus accumbens and in the bed nucleus of the stria terminalis was observed from rats exposed to prenatal LPS (1mg/kg) every other day throughout the entire pregnancy (Borrell *et al.*, 2002). Importantly, these groups were interested in different regions brain and this may explain the discrepancy in results. Indeed, the effects of MIA on DA neurons may be locally specific, with MIA exposure resulting in decreased DA neuron numbers in the SN, and increased numbers in the nucleus accumbens.

MIA does not only affect the neuronal population within the brain, but also glial cell types. For example, studies that investigated more specific disturbances in white matter development resulting from MIA, found decreased numbers of oligodendrocyte precursor cells, immature oligodendrocytes, as well as increased cell death and decreased myelin basic protein staining following administration of LPS to mothers in late pregnancy (E18-20) in the rat (700 μg/kg or 1mg/kg (Paintlia *et al.*, 2004; Paintlia *et al.*, 2008); 300 or 400 μg/kg (Rousset *et al.*, 2008). Hence, the previously mentioned disturbances in myelination are a possible consequence of the disrupted oligodendrocyte development as these are the main myelin producing cells. Subsequent studies have also investigated astrocytes and microglia. In one such example, immunohistochemistry of P8 hippocampus and cortex from rat pups following exposure to MIA (500μg/kg LPS) on E18 and E19 demonstrated increased

glial fibrillary acidic protein (GFAP) positive astrocytes, decreased OX-42 (type 3 complement receptor) positive microglia, however there was no change in tomato lectin staining of amoeboid and ramified microglia in compared to control group, providing somewhat inconclusive glial morphological information (Cai *et al.*, 2000). Following this, other groups have conclusively determined increased microglial activity and astrogliosis following MIA. For example, one group found increases in hippocampal microglia at birth in mouse offspring exposed to prenatal LPS (120 μg/kg on E15) (Roumier *et al.*, 2008). Another group showed astrogliosis, associated with elevated IL-1β, postnatal cell death, and hypomyelination in 1 week old pups following LPS (300 μg/kg) on E19 and E20 (Rousset *et al.*, 2006). A subsequent study later related these findings to CP symptoms in that rats exposed to LPS *in utero* displayed exacerbated responses to intracerebroventricular injections of ibotenate (a CP model) with a noted increase in microglial markers, astrogliosis, and reduced white matter myelination (Rousset *et al.*, 2008).

Others have demonstrated similar findings across the lifespan. For example, Ling and colleagues administered LPS (1mg/kg) on E11 and analyzed the morphology of rat offspring whole brains 4, 14, and 17 months after birth; their results showed an increase in total microglia numbers as well as greater than normal amounts of these cells in their active or amoeboid form (OX-6ir positive) (Ling *et al.*, 2009). Subsequent studies have looked into specific brain regions and results from these studies suggest that the changes following MIA are age and region dependent. For example, one study stained sections of frontal cortex, cingulate cortex, and hippocampus, from mouse offspring exposed to Poly(I:C) (20mg/kg) on E12.5, at P0, P7, P14, P30, and P60 and found no differences in microglial morphology compared to controls in any region or at any age (Garay *et al.*, 2013). Then, at around 4 months of age (~P120), microglial density in rat brains does not differ from that of controls in the hippocampus, medial prefrontal cortex, ventral striatum, cingulate cortex, and cerebellum, but the microglial density was increased in the nucleus accumbens at this age, following *in utero* exposure to Poly(I:C) (4mg/kg) on E15 (Mattei *et al.*, 2014). Finally, in aged rat offspring (>P180) that were exposed to Poly(I:C) (4mg/kg) on E15, microglia density was increased in the hippocampus, corpus callosum, pons and thalamus (Van den Eynde *et al.*, 2014). Persistent microglial activation in the adult hippocampus of affected offspring has

been reported by others, including rats exposed to LPS (0.5mg/kg) every other day from E14-E20 (Graciarena *et al.*, 2010). Furthermore, Hao et al subjected pregnant rats to LPS (0.79mg/kg) on E8, 10, and 12 and demonstrated an increase in GFAP expression in the CA1 region of hippocampus in the affected offspring compared to controls and this difference increased with age (measured at 3, 10, and 20 months) (Hao *et al.*, 2010). Taken together, these data suggest that glial abnormalities, and in particular microglial density differences become more pronounced at different ages, depending on brain region. There are also other possible explanations for the difference in results, though, as some studies used rats versus mice, and furthermore, as noted by Mattei et al, different studies use different markers for microglia including Iba1 (Garay *et al.*, 2013; Mattei *et al.*, 2014), OX42 and ED-1 (Van den Eynde *et al.*, 2014) which may also account for the difference in results.

1.5.4 Neurochemical and Electrophysiological Changes

As alterations in neurotransmitter systems are implicated in numerous diseases associated with MIA (Howes & Kapur, 2009; Volkow *et al.*, 2009; Brunelin *et al.*, 2013), studies have also used animal models to investigate the specific consequences of MIA on neurotransmitter systems and electrophysiological function. First of all, MIA (LPS, 1mg/kg, on E 10.5) has been shown to decrease serotonin in multiple rat brain regions as measured by tryptophan hydroxylase- (enzyme involved in serotonin synthesis) and 5-HT (serotonin receptor) levels (Wang *et al.*, 2009). In agreement with this, mouse models of MIA also demonstrated deficits in serotonin and/or 5-hydroxyindoleacetic acid (5-HIAA, the main metabolite of serotonin) in multiple brain regions, including the hippocampus and nucleus accumbens, following LPS (25μg/kg) (Depino, 2015) or Poly(I:C) (5mg/kg) on E9 (Winter *et al.*, 2009), or influenza on E16 or E18 (Fatemi *et al.*, 2008; Winter *et al.*, 2008).

Secondly, prenatal exposure to LPS (1mg/kg) on E10.5 also resulted in decreased striatal dopamine in adult (4, 14, and 17 months) offspring (Ling *et al.*, 2009). This result was replicated with a lower dose of LPS (100μg/kg) which was administered to pregnant rats on E9.5 and subsequently offspring demonstrated decreased striatal DA and DA metabolite levels at P60 (Kirsten *et al.*, 2010a). Numerous others have also demonstrated a decrease in DA along with decreased innervation in nucleus accumbens or striatum of offspring exposed to MIA via LPS

in the rat (100 μg/kg, E10, E14, or E18, (Snyder-Keller & Stark, 2008); 1mg/kg, E10.5, (Ling *et al.*, 2006; Ling *et al.*, 2009); 20-80 μg/kg increasing daily, E15-E19, (Bakos *et al.*, 2004)). This has also been confirmed in the mouse Poly(I:C) model (5mg/kg, on E17) in that DA levels were reduced in the medial prefrontal cortex and hippocampus from adult (P140) offspring (Bitanihirwe *et al.*, 2010). However, results are inconsistent with regards to MIA effects on offspring DA because some have observed increased DA levels in adult brains following maternal administration of Poly(I:C) (5mg/kg) to pregnant mice on E9 (Meyer *et al.*, 2008c; 2008d). More specifically, chronic MIA exposure (Poly(I:C), 5mg/kg, once daily, E12-E17) increased striatal DA with reduced binding of D2 receptors in young adult mice (Ozawa *et al.*, 2006). Furthermore, an increase in DA activity in the rat fetal midbrain was shown following *in utero* LPS exposure (1mg/kg, on E12.5), however this was hypothesized to be a compensatory response to the decrease in DA neurons also observed in this study (Ling *et al.*, 2002). The discrepancy in DA results is most certainly due to differences in animals used, the immunogen, as well as timing of administration as all of these factors produce differing results (Meyer *et al.*, 2007; 2009; Boksa, 2010; Harvey & Boksa, 2012; Meyer, 2014), but they may also depend on other factors as well. For example, results depend on the age at which offspring brains are analyzed as one group showed decreased DA levels in the nucleus accumbens in adolescence (P39), while they found an increase in DA in the same region at later ages (P170, P120, P210, P420, P510) for rats exposed to LPS (2mg/kg) daily for the duration of pregnancy (Romero *et al.*, 2007; Romero *et al.*, 2010). Thus, the effects of MIA on DA activity are multifactorial, and further research is needed to fully understand the specific alterations resulting from the numerous variables.

Finally, glutamate and GABA were not affected by MIA in one mouse Poly(I:C) model (5mg/kg, on E9) (Winter *et al.*, 2009). However, basal levels of extracellular glutamate were elevated in the prefrontal cortex of post-pubertal (~P55) rats exposed to Poly(I:C) (8mg/kg) on E14 (Roenker *et al.*, 2011). Furthermore, MK-801-induced glutamate increases were blunted in the MIA exposed offspring (Roenker *et al.*, 2011), suggesting decreased NMDA receptor function. Similarly, mice that were exposed to Poly(I:C) (5mg/kg) on P2-P6 demonstrated elevated basil hippocampal extracellular glutamate levels when tested at 10-12 weeks of age (Ibi *et al.*, 2009). Importantly, NMDA receptor-dependent synaptic current and plasticity

are decreased following LPS (500 μg/kg) on E19 (Lanté *et al.*, 2007). These data are consistent with the NMDA glutamate receptor hypofunction theory of schizophrenia, which postulates that decreased activity of NMDA receptors, leading to decreased stimulation of GABAergic inhibitory neurons, resulting in increased glutamate release from glutamatergic efferent neurons (especially in the prefrontal cortex) accounts for many symptoms of schizophrenia (Olney *et al.*, 1999; Carlsson *et al.*, 2000; Moghaddam & Javitt, 2012).

While looking at specific neurotransmitters provides some insight into which pathways MIA affects, researchers need other means to investigate functionality within these pathways. To this end, electrophysiological studies have provided evidence for altered electrical properties of brain cells following MIA. For example, one group investigated whether MIA affected the synchrony of neural firing from the medial prefrontal cortex and the hippocampus and found that Poly(I:C) (4mg/kg) exposure *in utero* (E15) resulted in reductions in the EEG coherence of those regions, suggesting mechanistic disturbances in long range neuronal communication in brains of affected offspring (Dickerson *et al.*, 2010). This is similar to the abnormal synchronization of neural avidity thought to be at the core of schizophrenic symptomology (Spencer *et al.*, 2009; Uhlhaas & Singer, 2010). Furthermore, when the two main glutamate receptors were investigated, Lante *et al* found that MIA (LPS,500 μg/kg, on E19) exposure resulted in increased ratio of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) / NMDA currents in the hippocampi of affected offspring (Lanté *et al.*, 2008). In agreement with these studies, CA1 pyramidal cells from juvenile offspring (P28) exposed to *in utero* LPS (100 μg/kg) on E15 and E16 showed decreased presynaptic transmission associated with a compensatory increase in postsynaptic excitability (Lowe *et al.*, 2008). Taken together, these studies indicated disturbances in overall synaptic function in MIA exposed offspring. In agreement with this, Lowe demonstrated reduction in short term plasticity but no difference in long term potentiation (LTP) (Lowe *et al.*, 2008). However another study did find a decrease in LTP in the hippocampus of juvenile offspring following LPS (500 μg/kg) on E19 (Lanté *et al.*, 2008), though the difference in dosage and timing presumably accounts for the discrepancy in results. As the mechanisms responsible for synaptic plasticity include changes in neurotransmitter quality, rate of neurotransmitter release, as well as effectiveness of cell response to those neurotransmitters (Gaiarsa *et al.*, 2002; Citri & Malenka,

2007), these MIA-induced alterations in synaptic plasticity may result from the aforementioned neurotransmitter alterations, possibly in combination with the numerous cellular differences seen in affected offspring exposed to MIA.

1.5.5 Molecular Consequences

Aside from the cytokine alterations, other molecular differences have been observed in MIA offspring and of key importance are the observations of MIA genetic structure and function. Interest in genetic modifications resulting from MIA has increased because it has been demonstrated that morphological and behavioral consequences of MIA are exacerbated by genetic alterations. One example of this is the heterozygous deletion of nuclear receptor related 1 protein (Nurr1) in mice (Vuillermot *et al.*, 2012). This genetic alteration in combination with MIA (Poly(I:C), 2mkg/kg, E17) resulted in additive effects of behavioral consequences as well as triggering improper development of prefrontal cortical and ventral striatal DA systems (Vuillermot *et al.*, 2012). In agreement with this, another group investigated effects of MIA on mice with two different point mutations (L100P and Q31L) in the *Disc1* gene, which regulates progenitor cell proliferation, migration and differentiation, thus acting as an important susceptibility gene for schizophrenia (Chubb *et al.*, 2008; Jaaro-Peled *et al.*, 2009; Brandon & Sawa, 2011; Lipina *et al.*, 2013). The *Disc1*- $L100P^{-/-}$ mutant mice demonstrated schizophrenia related behaviors previously (Lipina *et al.*, 2010; Lipina *et al.*, 2011; Lipina *et al.*, 2012). Then, *Disc1*- $L100P^{+/-}$ mice were found to be more sensitive to MIA (Poly(I:C) , 5mg/kg at E9), which exacerbated schizophrenia phenotypic behaviors in offspring (Lipina *et al.*, 2013). These studies exemplify the theory that MIA is a key risk factor for, and plays a role in, the progression of mental disorders. Hence, these findings have huge implications for the study of neuropsychiatric diseases, and in particular schizophrenia, which has a complex etiology combining numerous gene and environmental interactions (Cannon *et al.*, 2003; Meyer *et al.*, 2005; Meyer, 2013).

One group in particular was interested in the effects of MIA on expression of genes that have been associated with schizophrenia and ASD, and in this study, pregnant mice were inoculated with human influenza virus (H1N1) at E18 (Fatemi *et al.*, 2008). They found elevation of numerous genes in the brains of MIA offspring: Arhgef9 in the frontal cortex; Efnb2, Erbb4, Vldlr, and Sema3a in the hippocampus;

and Trfr2 and Sox2 in the cerebellum at P0; and Dby was elevated seven fold in frontal cortex at P56 (Fatemi *et al.*, 2008). Most of these genes that were increased in the MIA offspring have developmental roles. Sox2, for example, is required for neural stem cell maintenance, having the potential to act as the "master regulator" in programming neural precursor cells (Maucksch *et al.*, 2013), and deletion of this gene in mice resulted extreme developmental disturbances including hypoplasia of the dentate gyrus (Favaro *et al.*, 2009). Furthermore, Erbb4 is involved in proliferation, differentiation, and migration of neurons and glia cells (Jaaro-Peled *et al.*, 2009), and Sema3a plays a role in axonal extension, synaptogenesis, and synaptic pruning (Tillo *et al.*, 2012). Efnb2 is a member of the ephrin (EPH) family and as such this gene is involved in dendritic development, cell adhesion, and migration (Irie & Yamaguchi, 2004; Senturk *et al.*, 2011), and it is also an essential member of the Reelin receptor/signaling pathway that controls neuronal migration and positioning (Senturk *et al.*, 2011). Playing similar roles in the developmental process, Vldlr is a Reelin receptor and a regulator of PPI in mice (Barr *et al.*, 2007), which is deficient in MIA models (Boksa, 2010) as well as in schizophrenia patients (Braff & Geyer, 1990; Wynn *et al.*, 2005). Indeed, many of these genes are associated with schizophrenia and/or ASD and are shown to be elevated in these patients, including Sema3a which is known to be increased in cerebella of schizophrenia patients (Eastwood *et al.*, 2003), Tfr2 levels are elevated in plasma of schizophrenia (Maes *et al.*, 1995), and Vldlr which is upregulated in ASD (Fatemi *et al.*, 2005). Importantly, the Fatemi group was able to conclude that MIA in later pregnancy (E18) altered more genes than in mid pregnancy (E9) suggesting that effects of influenza infection are more pronounced later in gestation (Fatemi *et al.*, 2008). While influenza may have exacerbated effects in later pregnancy, others have shown that Poly(I:C) in earlier pregnancy leads to genetic alterations in offspring. Indeed, maternal administration of Poly(I:C) (5 mg/kg on E9) resulted changes in fetal brains 2, 4, or 8 days post MIA (E11, E13, or E17) consisting of altered transcription factors Nurr1 and Pitx3 and inductive signals Sonic hedgehog (Shh) and fibroblast growth factor (FGF8) (Meyer *et al.*, 2008a), all of which are required for the development of dopaminergic neurons (Hegarty *et al.*, 2013). A subsequent longitudinal study showed increased expression of Nurr1 in ventral midbrain areas found in adult mice from the same MIA animal model (Poly(I:C, 5mg/kg, on E9) (Vuillermot *et al.*, 2010). Nurr1 plays an important role in differentiation as well as

survival of midbrain dopaminergic precursor neurons (Saucedo-Cardenas *et al.*, 1998) and it helps to regulate important proteins, including TH in mature midbrain DA neurons (Iwawaki *et al.*, 2000; Kim *et al.*, 2003). Taken together, these studies emphasize the association of MIA to genetic abnormalities, and the implicated subsequent developmental alterations related to the specific genes, which may result in disease later in life.

MIA effects on genes are not all detrimental, however and some evidence of compensatory genetic alterations exists, for example one study demonstrated three different MIA treatments, influenza, Poly(I:C) (20mg/kg), and IL-6, all upregulated α, β, and γ crystalline family genes in the embryonic brain (Garbett *et al.*, 2012). Crystallin proteins are proposed to serve a neuroprotective role, as pretreatment with α-crystallin protected against experimentally induced neuroinflammation (Masilamoni *et al.*, 2006) and in agreement with this, αβ-crystallin down regulated TNF-α and nitric oxide synthase in activated microglia (Wu *et al.*, 2009). As altered levels of αβ-crystallin protein levels were detected in ASD frontal cortices (Pickett, 2001), these data may suggest that the up-regulation of crystallins observed by Garbett may be a mechanism for the brain to protect itself from the neuroinflammation pathology characteristic of ASD (Vargas *et al.*, 2005; Prandota, 2010).

There is evidence to show that MIA affects the physical makeup of DNA itself, for example, following MIA on E15 (Poly(I:C), 5mg/kg) mice offspring had decreased activity of telomerase (an enzyme with important roles in DNA sequencing which adds the sequence repeats to the 3' end of DNA strands) and associated shortened telomeres in hippocampal NPCs (Wolf *et al.*, 2011). Furthermore, as telomere shortening has been shown to disrupt neuronal differentiation (Ferrón *et al.*, 2009), this effect resulting from MIA presumably leads to impairment in proper neuronal development by limiting proliferation and differentiation of NPCs in offspring. It has been proposed that epigenetic mechanisms regulate telomere length (Blasco, 2007), implicating a possible mechanism through which MIA exerts its effects on the developing brain. Indeed, abnormalities in epigenetic modifications such as DNA methylation have been linked to complex neurodevelopmental disorders including schizophrenia and ASD (Dempster *et al.*, 2011; Wong *et al.*, 2014). For example, one study demonstrated

that prenatal exposure to Poly(I:C) (5mg/kg) on E9 resulted in significant global DNA hypomethylation in the hypothalamus and a similar trend in the striatum of adolescent offspring (Basil *et al.*, 2014). In particular, the Methyl CpG-binding protein2 (*Mecp2*), which plays a key role in activation and repression of gene expression, was significantly hypomethylated in the hypothalamus (Basil *et al.*, 2014). This evidence should be further investigated as gene modifications have the potential to be modified by therapeutic targets, and as Basil *et al* point out, *Mecp2* could be utilized as a potential biomarker for alterations associated with prenatal immune exposure (Basil *et al.*, 2014).

Reference	Animal Model	Immunogen, Dose, Rout	Timing	Key Findings
Fatemi, 1999, 2002, 2008, 2009	Mouse Influenza	Influenza IN	E9, E16, or E18	E9: ↑ pyramidal cell density in hippocampus (P0, P98); ↓ thickness of cerebral cortex, ↓ total area of brain hemisphere (P0), ↑ brain weight and size, ↓ VBR (P98). E16: ↓ ventricular area (P0), ↓ total brain area and cerebellar area (P14), ↓hippocampal area (P35); altered white matter anisotropy by DTI (P0, P14, P56). E18: ↓ anisotropy in corpus callosum; ↓ brain by DTI volume by MRI (P35)
Depino, 2015	Mouse LPS	LPS, 25µg/kg, SC	E9	↓ reelin pos cells in dentate gyrus; adult neurogenesis not affected (>P60)
Golan, 2005	Mouse LPS	LPS, 120 µg/kg, IP	E17	↓hippocampal CA1 width (P7), ↑ hippocampal CA1 width and ↓ dentate length (P14); ↑ dentate width (P240); ↑ cell density in hippocampal CA1 (P240)
Roumier, 2008	Mouse LPS	LPS, 120 µg/kg, IP	E15	↑ microglial density in hippocampus (P0)
Garay, 2013	Mouse Poly IC	Poly IC, 20mg/kg, IP	E12.5	no change in microglia density of frontal cortex, cingulate cortex, and hippocampus (P0, P7, P14, P30, and P60)
Li, 2009, 2010	Mouse Poly IC	Poly IC, 5 mg/kg, IV	E9 or E17	Delayed postnatal myelination; ↓ purkinje cell density; ↑ lateral and 4th ventricle volumes by MRI (P84)
Makinodan, 2008	Mouse Poly IC	Poly IC, 60 mg/kg, IV	E9.5	↓ axonal size and myelin thickness in hippocampal CA1; ↓ MBP staining in hippocampal CA1 and CA3 (P14)
Meyer, 2006a,b, 2008	Mouse Poly IC	Poly IC, 5 mg/kg, IV	E9 or E17	↓ neurogenesis in hippocampus (P24). E9: ↓ reelin-ir cells in hippocampus (P24) and in mPFC (P180); ↓ paravalbumin-ir cells in mPFC (P180). E17: ↓ reelin-ir cells in mPFC, ↓ paravalbumin-ir cells in mPFC and ventral hippocampus (P180)
Shi, 2009	Mouse Poly IC	Poly IC, 20 mg/kg, IP	E12.5	↓ cerebellar Purkinje cells (P120)
Soumiya 2011a, 2011b	Mouse Poly IC	Poly IC, 20mg/kg, IP	E9.5	Abnormalities in cortical progenitor cells; ↓ synaptic development of upper-layer neurons; ↓ synaptophysin, ↓ glutamic acid decarboxylase-67 positive puncta surrounding neural cell bodies; ↑ dendritic spine density (P56)
Wolf, Susanne, 2011	Mouse Poly IC	Poly IC, 5mg/kg, IP	E15	↓ adult hippocampal neurogenesis
Samuelsson, 2006	Rat Cytokine	IL-6, 9µg/kg, IP	E8, E10 and E12; or E16, E18, and E20	↓ neurons and ↑ astrocytes in hippocampus (P168)
Baharnoori, 2009	Rat LPS	LPS, 100 µg/kg, IP	E15 and E16	↓ dendritic arborization in mPFC and hippocampal CA1 (P10, P35, P60); ↓ dendritic length in mPFC (P10, P35) and CA1 (P60); ↓ spine density in mPFC (P60)
Cai, 2000	Rat LPS	LPS, 500 µg/kg IP	E18	↑ GFAP-astrocytes, ↓ OX-42-microglia, no change in tomato lectin-microglia in hippocampus and cortex (P8)
Cui, 2009	Rat LPS	LPS, 50 or 100 µg/kg, IP	E15 and E16, or E18 and E19	↓ neurogenesis in hippocampal dentate gyrus (E18, P14)
Graciarena, 2010	Rat LPS	LPS, 0.5mg/kg, SC	Every other day E14-E20	↓ proliferating cells and immature neurons of dentate gyrus; persistent microglial activation (>P60)
Hao, 2010	Rat LPS	LPS, 0.79mg/kg IP	E8, 10 and 12	↓ neuron, ↓ SYP expression, ↑ GFAP in hippocampal CA1 (3, 10, 20 month-old)
Larouche, 2005 and Girard, 2010	Rat LPS	LPS, 200 µg/kg, IP	2X day from E17 to birth	↑ cerebral cortical lesions in form of shrunken neuronal cell bodies and nuclei (P3, P8); ↑ proliferating and total microglia in forebrain white matter (P9)

Ling, 2002, 2006, 2009	Rat LPS	LPS, 1 mg/kg, IP	E14.5 and P21; E10.5-11	Morph: ↓ DA neurons in SN and VTA + inc in DA activity; ↑ microglia cells that were also larger cell body and thicker processes (i.e. activated microglia), ↓ DA cell counts, LPS animals exhibited Lewy body like inclusions after 14months (4, 14, 17 months)
Nouel, 2011	Rat LPS	LPS, 100 μg/kg, IP	E15 and E16	↓ GAD67 cell count in dentate gyrus (P14); ↓ GAD67 cell count in dentate gyrus and CA1 (P28); ↓ reelin cells in dentate gyrus (P14)
Paintlia, 2004, 2008	Rat LPS	LPS, 700 μg/kg or 1mg/kg, IP	E18	↓ OPCs and immature oligodendrocytes in corpus callosum and lateral ventricles with ↓ MBP and PLP mRNA in brain; ↓ MBP immunostaining in corpus callosum and cingulum (P9, P16, P23, P30)
Rousset, 2006, 2008	Rat LPS	LPS, 300 or 400 μg/kg, IP	E19 and E20	↑ apoptosis in striatum, PWM and germinative ventricular zone (P1, P7); ↓ MBP immunostaining in external and internal capsule (P7); ↑ ibotenate-induced cortical lesions and PWM cysts (P9); ↑ ibotenate-induced microglial activation and astrogliosis in various regions (P9)
Snyder-Keller, 2008	Rat LPS	LPS, 100 μg/kg IP	E10, E14, or E18	Accelerated loss of DA neurons in culture
Mattei, 2014	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	↓ net-neurogenesis; minocycline for 10 weeks rescued neurogenesis (adult); no change in microglia density in hippocampus, medial prefrontal cortex, ventral striatum, cingulate cortex, nor cerebellum; ↑ in microglia in nucleus accumbens (4months)
Piontkewitz, 2011a, 2011b	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	↓ volume of hippocampus, striatum, and prefrontal cortex; ↑ ventricular volume (P35, 46, 56, 70, 90)
Van den Eynde, 2014	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	↑ microglia density in hippocampus, corpus callosum, pons and thalamus (P180)

Table 2: Morphological changes observed in offspring following MIA

Table summarizing a wide literature sample of the effects of MIA on brain morphology from affected offspring. In the "key findings" column the age at which offspring samples were tested is listed in parenthesis.

Reference	Animal Model	Immunogen, Dose, Rout	Timing	Key Findings
Asp 2005	Mouse Influenza	Influenza IN	E14	↑ mRNA neuroleukin, FGF5, ring finger protein 1B, Akv MuLV in whole brain (P90 and/or P280); ↑ neuroleukin in whole brain (P90)
Fatemi, 1999, 2002, 2005, 2008, 2009	Mouse Influenza	Influenza IN	E9, E16, or E18	E9: 7 genes altered in whole brain (P0); ↓ reelin-ir cells in hippocampus, cortex, (P0); altered SNAP-25 immunoreactivity in hippocampus (P0); ↑ GFAP immunostaining in cortex (P14) and hippocampus (P35); altered nucleolin, microcephalin, connexin 43 and aquaporin 4 protein in various regions (P35, P65). E16: Microarray/PCR- 13 genes altered (P0), 4 genes altered (P14), 3 genes altered (P56) in hippocampus ; ↓MBP, ↓ MA, ↑ splice variant of PLP1 in cerebellum (P14, P35, P56). E18: microarray/PCR: 12 genes altered (P0), 2 genes in various brain regions (P56); ↑ Foxp2 in cerebellum (P0, P35)
Moreno, 2011	Mouse Influenza	H1N1 (influenza), IN	E9.5	↑ 5-HT(2A) receptor, ↓ mGlu2 receptor in frontal cortex (10-12 weeks)
Golan, 2005	Mouse LPS	LPS, 120 µg/kg, IP	E17	↑ IL-6 (3h), ↑ BDNF (9 h) in brain; ↓ BDNF in cortex, hippocampus (P21); ↑ BDNF in thalamus (P270); ↓ NGF in hippocampus (P21); ↑ NGF in thalamus (P7, P270)
Liverman, 2006	Mouse LPS	LPS, 50µg, IP	E18	↑ mRNA for IL-1β, IL-6, mCP-1, needin, VEGF, YB-1 (1-12 h); ↓ mRNA for groucho, semaphoring 5b (6h)
Ning, 2008	Mouse LPS	LPS, 500 µg/kg, IP	E17	↑ TNF in brain, liver an amniotic fluid (1.5h)
Garbett, 2012	Mouse Multiple	Influenza, IN; Poly IC, 20mg/kg, IP; IL-6, 5µg/150µl saline, IP	E9.5 or E12.5	Upregulation of crystallin gene family (3h)
Basil, 2014	Mouse Poly IC	Poly IC, 5mg/kg, IV	E9	global DNA hypomethylation in hypothalamus (6wk old)
Bruses, 2012	Mouse Poly IC	Poly IC, 20mg/kg, IP	E16	↑ IL-1β, IL-7, IL-13, MCP-1, MIP-1α, IP-10, MIG (6h and 24h post injection)
Garay, 2013	Mouse Poly IC	Poly IC, 20mg/kg, IP	E12.5	23 cytokine measurements from frontal cortex, cingulate cortex, hippocampus, and serum (P0, P7, P14, P30, and P60)
Lipina, 2012	Mouse Poly IC	Poly IC, 2.5mg/kg or 5mg/kg, IV	E9	*Disc1*-L100P+/- mutants > sensitivity to MIA vs WT or Disc1-Q31L+/-; Anti-IL6 co-admin w/ Poly IC reversed adverse effects
Makinodan, 2008	Mouse Poly IC	Poly IC, 60 mg/kg, IV	E9.5	↓ MBP, ↓ mRNA for MBP, ↓ pAkt in hippocampus (P14)
Malkova, 2014	Mouse Poly IC	Poly IC, 5mg/kg, IP	E10.5, E12.5, and E14.5	↑ DOI-induced egr-1, COX-2 and BDNF mRNA in PFC; ↑ 5-HT2AR, phospholipase C β1, and regulator of G protein signaling 4 mRNA in PFC (adult)
Meyer, 2006a,b, 2008	Mouse Poly IC	Poly IC, 5 mg/kg, IV	E9 or E17	↑ TH pos cells in region ventral to fetal mesencephalic flexure in E13 and VM for E17; DAT expression ↓ at E13 but ↑ at E17. E9: altered mRNA for genes involved in DA development: Shh, Fgf8, Nurr1, Pitx3 (E11, E17); ↓ IL-1β, ↑ IL-6m, ↓ IL10 in brain (3h); ↑IL-1β, IL-6, TNF in brain (5 or 6h). E17: ↑ IL-1β, IL10 (3h); ↑ IL-6 (6h)
Vuillermot 2010, 2011, 2012	Mouse Poly IC	Poly IC, 5mg/kg or 2mg/kg, IV	E9 or E17	↑ Nurr1 positive cells in SN at E19 & P70, no difference at P35; ↑ VTA Nurr-1 positive cells at P70, no difference at E19 or P35
Wolf, Susanne, 2011	Mouse Poly IC	Poly IC, 5mg/kg, IP	E15	↓ telomerase activity in NPCs, telomere shortening (P1, P3, P10, P60)
Samuelsson, 2006	Rat Cytokine	IL-6, 9µg/kg, IP	E16, E18, and E20	↑ procaspase-3, active caspase-3; ↑ mRNA for GFAP, IL-6, and caspase-3 in hippocampus (P28, P168)
Ashdown, 2006	Rat LPS	LPS, 50 µg/kg, IP	E18	↑ IL-1β, IL-6, TNF-α in placenta (2-8h); no change in fetal brain IL-1β, IL-6, TNF

Cai, 2000	Rat LPS	LPS, 500 µg/kg IP	E18	↑ IL-1β (1h), TNF-α (4-24h); ↑ GFAP in various brain regions (P8)
Gayle, 2004	Rat LPS	LPS, 100 µg/kg, IP	E18	↑ CRH mRNA (6, 12h), no change in IL-1β, TNF-α, IL-6, and IL-10
Gilmore, 2005	Rat LPS	LPS, 100 µg/kg, IP	E14, E15, and E16	↓ TNF-α in brain (24h) and in FC (P7)
Kumral, 2007 and Yesilirmak 2007	Rat LPS	LPS, 500 µg/kg, IP	E18 and E19	↑ IL-1β, IL-6, TNF-α mRNA in whole brain (P7)
Lanté, 2007, 2008	Rat LPS	LPS, 500 µg/kg, IP	E19	↑ protein carbonylation (1-4 h), ↓reduced/oxidized glutathione (16 h), ↓ α-tocopherol in hippocampus (4h)
Ling, 2002, 2004a,b, 2006; Zhu 2007	Rat LPS	LPS, 1 mg/kg, IP	E10.5-11	↑TNF-α in striatum (P21, P120, P210, P510) and SN (P210); ↓ reduced/ oxidized GSH (P120, P210, P510) ↓ enzyme activity for GSH synthesis, ↑ GSH redox recycling enzyme activities, ↑ lipid peroxidation (P120, P510) and protein oxidation (P480) in many brain regions
Nilsson, 2001	Rat LPS	LPS, 790 µg/kg, IP	E8, E10, and E12	↑ GR in hippocampus after euglycemic hyperinsulinemia (P84)
Paintlia, 2004, 2008	Rat LPS	LPS, 700 µg/kg or 1 mg/kg, IP	E18	↓ peroxisome markers : DHAP-AT, PMP70, PPAR-α mRNA and protein; ↑ GFAP immunostaining in corpus callosum and/or cingulum (P9, P16, P23, P30); ↑ IL-1β mRNA (1-48h), TNF (1-24h), iNOS (24-48); ↑apoptosis in subventricular zone, ↓ OPCs and ↑ microglial activation (48h); ↑ oxidative stress, ↓ glutathione, ↓ peroxisomal function (24h, 48h)
Romero, 2007, 2010	Rat LPS	LPS, 2 mg/kg, SC	Daily whole pregnancy	↓ synaptophysin in FC (P21, P400), ↑ synaptophysin in FC, hippocampus (P170, P180), ↓ DARPP-32 in FC (P180), ↓ GSK-3β (P400)
Rousset, 2006	Rat LPS	LPS, 300 µg/kg, IP	E19 and E20	↑ IL-1β mRNA (P1), ↓ TNF-α whole brain (P7)
Urakubo, 2001	Rat LPS	LPS, 2.5 mg/kg IP	E16	↓ TNF fetal brain (2h)
Mattei, 2014	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	↑ microglia IL-1β and TNF-α in hippocampus, not cerebellum (P128)

Table 3: Molecular and genetic changes observed in offspring following MIA
Table summarizing research investigating effects of MIA on molecular and genetic characteristics of offspring brains. In the "key findings" column the timing at which offspring samples were tested is listed in parenthesis.

Reference	Animal Model	Immunogen, Dose, Rout	Timing	Key Findings
Fatemi, 2008 and Winter 2008	Mouse Influenza	Influenza IN	E16, or E18	↓ 5-HT, ↓ 5-HIAA in cerebellum (P14); ↓ 5-HT, ↓ taurine in cerebellum (P35)
Depino, 2015	Mouse LPS	LPS, 25µg/kg, SC	E9	↓ serotonin and noradrenaline in hippocampus (>P60)
Roumier, 2008	Mouse LPS	LPS, 120 µg/kg, IP	E15	↑ ratio of AMPAR/NMDAR currents in hippocampus (P28)
Bitanihirwe, 2010	Mouse Poly IC	Poly IC, 5 mg/kg, IV	E17	Changes in mult systems/depends on brain region, but main results: ↓ DA and glutamate in PFC and hippocampus, ↑ serotonin in amygdala females only, ↑ 5-HIAA in amygdala (~P140)
Meyer, 2008a,c,e and Winter 2008	Mouse Poly IC	Poly IC, 5 mg/kg, IV	E9 or E17	E9: ↑THir cell (E13, E17), ↑ DATir cells (E17) in mesencephalon; ↑ TH immunoreactivity in striatum and NAc, ↓ immunoreactivity for Glu-R1 subunit of AMPA receptor in NAc (P120); ↑ DA, DOPAC in PFC and globus pallidus, ↓ serotonin and metabolite in hippocampus, nucleus accumbens and lateral globus pallidus, ↓ taurine in hippocampus, no effect on glutamate nor GABA (P84); ↓ D1 receptor immunoreactivity in medial PFC (P180). E17: ↓ NR1 receptor subunit immunoreactivity in dorsal hippocampus (P180)
Nyffeler, 2006	Mouse Poly IC	Poly IC, 5 mg/kg IV	E9	↑ $GABA_{A\alpha5}$ receptor subunit immunoreactivity in ventral dentate and basolateral amygdala (P180)
Ozawa, 2006	Mouse Poly IC	Poly IC, 5 mg/kg, IP	E12-E17	↑DOPAC, ↑HVA, ↓ D2 receptor binding in striatum (P63-P70)
Samuelsson, 2006	Rat Cytokine	IL-6, 9µg/kg, IP	E16, E18, and E20	↑ mRNA for NR1 and $GABA_{A\alpha5}$ receptor subunits in hippocampus (P28, P168)
Bakos, 2004	Rat LPS	LPS, 20-80 µg/kg increasing daily, SC	E15-19	↓ DA in NAc (P83)
Borrell, 2002	Rat LPS	LPS, 1 mg/kg, SC	Alternate Days whole pregnancy	↑ TH immunoreactivity in NAc and bed nucleus of striaterminals (P100, P300)
Cambonie, 2004	Rat LPS	LPS, 300 µg/kg, IP	E19	↑Glu-induced hydroxyl radical release (mGlu I mediated) in striatum (P14)
Kirsten, 2010a,b, 2012, 2015	Rat LPS	LPS, 100 µg/kg, IP	E9.5	↓ striatal DA and metabolite levels (P60)
Lanté, 2007, 2008	Rat LPS	LPS, 500 µg/kg, IP	E19	↑ ratio of AMPAR/NMDAR currents in hippocampus, ↓ LTP in hippocampus (P28)
Ling, 2002, 2004a,b, 2006, 2009, and Wang, 2009	Rat LPS	LPS, 1 mg/kg, IP	10.5	↓ THir cells in SN (P21, P120, P210, P420, P510); ↑↑ DA neuron loss in SN after postnatal rotenone, 6-OHDA or intranigral LPS; ↓ DA in SN, ↑ HVA/DA in striatum (P120, P510) and other brain regions (P120)
Lowe, 2008	Rat LPS	LPS, 100 µg/kg, IP	E15 and E16	↑ hippocampal CA1 synaptic transmission and ↑ CA1 pyramidal cell excitability; ↓ presynaptic excitability in CA1 (P20-P25)
Romero, 2007, 2010	Rat LPS	LPS, 2 mg/kg, SC	Daily for whole pregnancy	↓ DA in NAc (P39), ↑ DA in NAc (P170, P120, P210, P420, P510), ↑ DOPAC in striatum (P180)
Snyder-Keller, 2008	Rat LPS	LPS, 100 µg/kg, IP	E10, E14, or E18	↓ THir cells in SN/VTA and ↓ DA innervation of striatum in organotypic cultures
Wang, 2009	Rat LPS	LPS, 1 mg/kg, IP	E10.5	↓ TPHir cells in dorsal raphe, ↓ 5-HT and ↑ 5-HIAA/5-HT in various brain regions (P120)
Dickerson, 2010	Rat Poly IC	Poly IC, 4mg/kg, IV	E15	Electrophysiological recordings = ↓ in mPFC-HPC EEG coherence (5months of age)
Roenker, 2011	Rat Poly IC	Poly IC, 8mg/kg, IP	E14	↑ PFC glutamate; blunted MK-801 induced increase in glutamate (P55-58)
Zuckerman, 2003	Rat Poly IC	Poly IC, 4 mg/kg, IV	E15	↑KCL-induced DA release from striatal slices (P100)

Table 4: Neurochemical and electrophysiological alterations in offspring following MIA

Table with summarized literature detailing the neurochemical characteristics and electrophysiological functioning in brains of offspring exposed to MIA. In the "key findings" column the timing at which offspring samples were tested is listed in parenthesis.

1.6 Mechanisms

Even though infections during pregnancy are fairly common, (Petersen, 1992; Longman & Johnson, 2007; Parveen *et al.,* 2012), most offspring exposed to MIA do not develop disorders (Selten & Slaets, 1994). This suggests that there are multiple factors at play, including genetic vulnerability and environmental insults that work with the effects of MIA to result in disease onset for a subset of affected individuals. Therefore, elucidating the mechanisms through which MIA evokes developmental changes leading to disease susceptibility is of huge importance. As mentioned previously, numerous infections are associated with increased risk of offspring development of neurological disease, such as CP, or neuropsychological disorders, like schizophrenia, ADHD or ASD (see "Epidemiological and Clinical Evidence" section), and there is increasing evidence indicating that many of these disorders share numerous risk factors (including MIA) and brain dysfunctions (Cheung *et al.*, 2010; Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Moreno-De-Luca *et al.*, 2013; Meyer, 2014). Furthermore, most of our information comes from animal models, and as none of these could ever presume to encompass one human disease in its entirety, the data are often applicable to numerous disease pathologies. As such, the mechanistic factors are usually associated with MIA only, as opposed to a specific disease etiology. Hence, researchers have begun to elucidate possible mechanistic factors that potentially apply to the development of many diseases and to determine how MIA may result in future neurobiological and behavioral alterations in affected offspring. The current factors that are hypothesized to play a mechanistic role in the effects of MIA in offspring neuropathology are herein reviewed.

1.6.1 Cytokines

One of the first reactions of the innate immune response during an infection, tissue damage, stress or other physiological insults, is to induce inflammation (Gallin *et al.*, 1999). Inflammation is characterized by the secretion of numerous inflammatory factors, including prostaglandins, chemokines, and cytokines. Immunological factors, and specifically cytokines are the most widely hypothesized mediator of the aversive outcomes associated with MIA because MIA in the absence of an infectious agent is sufficient to induce neurodevelopmental alterations and behavioral

impairments in affected offspring (Kneeland & Fatemi, 2013; Meyer, 2013; Knuesel *et al.*, 2014). Indeed cytokines are implicated in all of the other mechanistic players in MIA, which is evident throughout this section.

Cytokines are primarily produced by microglia, but astrocytes and neurons are also sources (Breder *et al.*, 1994; Smith *et al.*, 2012; Sofroniew, 2013). They function in many processes within the immune system, including immune cell differentiation, maintenance, and homeostasis (Huang & August, 2015) as well as inducing cell death (Curfs *et al.*, 1997). More importantly for our purposes, many cytokines and their receptors are expressed in fetal brain development (Mehler & Kessler, 1997; Mousa *et al.*, 1999), and therefore also affect neurodevelopmental processes (Meyer, 2013). Indeed, it has been noted that cytokines serve numerous essential functions in normal neurodevelopment, including neurogenesis, migration, and survival (Bauer *et al.*, 2007; Deverman & Patterson, 2009; Meyer, 2013), and cells (including neurons and microglia) use cytokines for paracrine and autocrine signaling within the developing CNS (Deverman & Patterson, 2009). Cytokines have also been shown to influence synthesis and reuptake of neurotransmitters, including dopamine and serotonin (Capuron & Miller, 2011); altered functioning of both of these neurotransmitters are implicated in neuropsychological diseases, most notably schizophrenia (Winter *et al.*, 2008; Howes & Kapur, 2009; Lau *et al.*, 2013; Selvaraj *et al.*, 2014). Clearly, cytokines have important roles to play in normal brain functioning, and as such, discrepancies in the proper protein levels could lead to devastating developmental alterations.

Animal models have demonstrated significant increases in protein and mRNA levels of IL-1β, IL-6, IL-10, and TNF-α in the fetal brain following MIA, suggesting that these may be of particular interest in MIA mechanisms (Meyer *et al.*, 2005; Patterson, 2009). Our group has demonstrated that high amounts of IL-1β limited proliferation and altered differentiation of neural progenitor cells and altered fetal neuron development *in vitro* (Nolan *et al.*, 2011; Crampton *et al.*, 2012). Furthermore, IL-1β, IL-6 and TNF-α were toxic to fetal midbrain DA neuron in some *in vitro* studies (Jarskog *et al.*, 1997; McGuire *et al.*, 2001), however they promoted cell survival when at higher concentrations in another (Akaneya *et al.*, 1995). Furthermore, TNF-α had neurotrophic effects on DA neurons cultured from mice in early fetal development (E12.5), but neurotoxic effects in later

developmental phases (E14 or E16) (Doherty, 2007). This suggests the ultimate effect of MIA induced cytokine alterations on DA neuron development is highly dependent upon developmental stage.

Importantly, *in vivo* studies confirm the potential mechanistic role of cytokines. The main cytokine implicated in MIA animal model studies seems to be IL-6, with IL-1 and TNF-α closely behind. Indeed, studies investigating the potential roles of IL-6 and IL-1 have demonstrated that blocking the cytokine action following MIA eliminated aversive outcomes, including behavioral deficits as well as brain and placental phenotypical alterations, which previously resulted from immunogen induced MIA or cytokine administration (Smith *et al.*, 2007; Girard *et al.*, 2010).

1.6.2 Microglia Function

Microglia are the major immune cells and resident macrophages of the CNS, regulating induction and inhibition of inflammatory processes and helping maintain homeostasis through crosstalk with neurons, astrocytes, oligodendrocytes, and circulating immune cells (Ransohoff & Perry, 2009; Ransohoff & Cardona, 2010; Aguzzi *et al.*, 2013). These cells accomplish their immune regulatory functions by up- or down- regulating cytokines and associated receptors, as well as other immune-related cell surface receptors (Meyer, 2013), and interestingly, cytokines also regulate microglia, as do chemokines, neurotrophic factors, complement factors, and neurotransmitters (Glass *et al.*, 2010; Hagberg *et al.*, 2012). Aside from the immune roles, microglia cells also have key roles in developmental processes: they help astrocytes regulate neuronal differentiation and maturation and they play key roles in synaptic pruning and neural circuit formation (Boulanger & Shatz, 2004; Glass *et al.*, 2010; Schafer *et al.*, 2012; Ueno & Yamashita, 2014). Therefore, while microglia cells do play necessary roles in neuroprotection and development, they are also potentially problematic if their functionality in these roles becomes disrupted as result of MIA. For instance, chronic microglial activation leads to heightened release of pro-inflammatory factors and this process is linked to neurodegenerative processes (Block *et al.*, 2007; Knuesel *et al.*, 2014). Indeed, chronic microglia activation is a characteristic of PD brains, first noted in the SN (McGeer *et al.*, 1988) and then subsequently noted in the hippocampus, putamen, and cortex of patients

with PD (Imamura *et al.*, 2003). Furthermore, chronic microglial activation has been implicated as a potential mechanism in neuropsychiatric disorders, including those with increased risk following MIA (Smith *et al.*, 2012; Knuesel *et al.*, 2014). Also numerous MIA associated diseases are characterized by chronic immune abnormalities, including ASD (Pardo *et al.*, 2005; Careaga *et al.*, 2010; Gesundheit *et al.*, 2013) and schizophrenia (Strous & Shoenfeld, 2006; Müller & Schwarz, 2010), further implicating a role for chronic changes in the immune system of affected individuals.

Microglia can be primed following an initial inflammatory stimulus to respond more strongly to subsequent stimuli (Cunningham *et al.*, 2009; Meyer, 2013; Knuesel *et al.*, 2014; Perry & Holmes, 2014). This microglial priming state is associated with alterations in microglial morphology, increased microglia cell number, as well as elevated cytokine / immune mediator levels (Knuesel *et al.*, 2014; Perry & Holmes, 2014). MIA models have shown that exposure to LPS or Poly(I:C) *in utero* resulted in increased microglial activation. For example, increased levels of microglial IL-1β and TNF-α were observed in rats exposed to Poly(I:C) (4 mg/kg) on E15(Mattei *et al.*, 2014). This increase in microglia cytokine output was associated with decreased neurogenesis and PPI because when microglial cytokine production was normalized through minocycline treatment, neurogenesis and the behavioral deficit were rescued (Mattei *et al.*, 2014). Interestingly, though, when a second hit of Poly(I:C) was administered in adulthood to MIA exposed offspring, the inflammatory response was significantly heightened with increased numbers of active microglia and cytokine expression (Krstic *et al.*, 2012). Similar indications of microglial priming were found following a secondary insult of stress in puberty (as opposed to another immune specific insult) in MIA exposed mice (Giovanoli *et al.*, 2013). These findings demonstrate the ability of MIA to prime microglia for heightened response later in life. It has been proposed that this priming action is what mediates the improper neuromodulatory and immune functions of microglia in MIA offspring, resulting in susceptibility to disease later in life (Knuesel *et al.*, 2014).

1.6.3 Placental Roles

The placenta is a key regulator of maternal-fetal interactions, regulating fetal growth and development (Huppertz, 2008), and as such a proper intrauterine environment is imperative for normal placental functioning in protecting fetal development (Hsiao & Patterson, 2012). The LPS and Poly(I:C) MIA models have been shown to increase pro-inflammatory cytokine levels in all of the maternal-fetal environments: the placenta, amniotic fluid and the fetus itself (Cai *et al.*, 2000; Urakubo *et al.*, 2001; Paintlia *et al.*, 2004; Ashdown *et al.*, 2006; Meyer *et al.*, 2006b; Boksa, 2010). In the placenta, signaling of cytokines along with growth factors and hormones play important roles in maternal and fetal cell cross talk (Hsiao & Patterson, 2012). Hence, placental alterations may lead to important deviations in developmental processes, and indeed placental health may even predict neurodevelopmental outcome (O'Keeffe & Kenny, 2014). Therefore, it is likely that the placenta plays a role in the mechanisms underlying MIA induced changes in fetal development.

Initially, researchers investigated if maternal immune factors or the immunogens themselves are able to cross the placenta and directly affect fetal development. Interestingly, neither influenza (Shi *et al.*, 2005) nor LPS were shown to cross the placenta in animal models (Ashdown *et al.*, 2006). This is consistent with findings that LPS does not cross chorioamniotic membranes *in vitro* in humans (Romero *et al.*, 1987), possibly due to antimicrobial proteins found on fetal membrane epithelium (Kim *et al.*, 2002). However, cytokines, and in particular IL-6, have been shown to cross the placental barriers. Indeed, radioactive IL-6 was observed in the fetus 30 min after its administration to the pregnant rat (Dahlgren *et al.*, 2006). Importantly, this group also demonstrated a significant difference in the fetal levels of IL-6 depending on gestational age, with mid gestation (E11-E13) allowing for higher concentration of cytokine transfer compared to later gestation (E17-E19) (Dahlgren *et al.*, 2006), suggesting that placental permeability changes throughout the course of pregnancy allowing for different levels of potentially harmful agents to reach the fetus depending on timing.

Animal models have also demonstrated structural and molecular abnormalities in the placenta and subsequent adverse fetal outcomes following MIA (Koga & Mor, 2010; Kneeland & Fatemi, 2013). For example, after pregnant mice were infected with influenza on E7, placenta that were harvested on E16 displayed cytoarchitectural disorganization and an increased number of immune cells and there

was major genetic variation with upregulation of 77 genes and downregulation of 93 others (Fatemi *et al.*, 2012). Furthermore, our group demonstrated that infection-induced placental changes occurred, followed by altered development of neurons isolated from affected offspring, following late pregnancy MIA exposure in the rat (Straley *et al.*, 2014). The placental changes included increased levels of IL-1β, reduced placental weight, and down regulation of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11β2) (Straley *et al.*, 2014), which is an enzyme that plays an important role protecting the fetus from excessive maternal cortisol exposure (Togher *et al.*, 2014). Other placental differences, including massive necrosis and edema, and subsequent neurodevelopmental and behavioral deficits in affected offspring have also been reported (Girard *et al.*, 2010; Carpentier *et al.*, 2011). Importantly, the negative effects of these studies were attributed to cytokine signaling because targeting TNF-α (Carpentier *et al.*, 2011) or IL-1 (Girard *et al.*, 2010) with receptor agonists prevented the placental pathology and alterations in progeny. Furthermore, increases in IL-6 mRNA and protein levels in placenta were observed 3, 6, or 24h following Poly(I:C) (20mg/kg) exposure on E12.5, and IL-6 mediated numerous endocrine changes in these placenta samples, including increased infiltration of uterine natural killer (uNK) cells and macrophages (Hsiao & Patterson, 2012). We know from previous findings that IL-6 is a key mediator of MIA-induced behavioral deficits observed in affected offspring (Smith *et al.*, 2007). Furthermore, evidence has shown that insult to the placenta itself (as opposed to a maternal systemic infection, etc.) is sufficient to induce many of the same neuropathological changes observed in MIA studies (Hutton *et al.*, 2008; Bassan *et al.*, 2010), suggesting that the placental consequences of MIA may mediate the unfavorable effects on the progeny. Taken together, these data may implicate IL-6 at the heart of an indirect mechanism through which MIA alters fetal development.

1.6.4 Oxidative Stress

Oxidative stress is the imbalance that occurs when the body is not able to eliminate excessive levels of reactive oxygen species (ROS) in the body, and so the amount of free radicals in the body remains above a tolerable level (Di Carlo *et al.*, 2012). When levels are maintained well, ROS are important in immune processes and are secreted by innate immune cells to help rid the body of invading pathogens (Nathan

& Shiloh, 2000), but when oxidative stress occurs it can lead to devastating outcomes. Indeed, oxidative stress can lead to major cell damage (Sies, 2013), including neurodegeneration, apoptosis, and reduced neuroprotection (Sayre *et al.*, 2007; Niizuma *et al.*, 2009) as well as mitochondrial dysfunction (Yan *et al.*, 2012). Hence, oxidative stress has been implicated in neurodegenerative diseases (Sayre *et al.*, 2007), specifically including PD (Jenner, 2003) and Alzheimer's disease (AD) (Wang *et al.*, 2014).

In terms of MIA, the offspring from mice infected with influenza on E9 demonstrated altered levels of nitric oxide (a common ROS) with increases in adolescence (P35) and decreases in early adulthood (P56) compared to control offspring (Fatemi *et al.*, 1998; Fatemi *et al.*, 2000a). Similarly, maternal administration of LPS (700μg/kg or 1mg/kg) on E18 resulted in immediate (several hours) increase in oxidative stress markers in fetal rat brains (Paintlia *et al.*, 2004; Paintlia *et al.*, 2008). This increase in oxidative stress in offspring brains following exposure to LPS *in utero* was shown consistently across the rat lifespan, from P9 through P510 by multiple groups (1mg/kg on E10.5-E11, (Ling *et al.*, 2004; Ling *et al.*, 2006) and (700μg/kg on E18, (Paintlia *et al.*, 2008). Even more compelling is the finding that maternal treatment with the anti-oxidant *N*-acetylcysteine prevented fetal brain oxidative stress as well as some of the spatial learning deficits observed in adolescent offspring that had been exposed to LPS (500μg/kg) on E19 (Lanté *et al.*, 2008). Therefore, oxidative stress may play a key mechanistic role in MIA associated disturbances, accounting for some of the detrimental outcomes that occur because of MIA (Meyer, 2013).

Interestingly, it has been shown that IFN-γ, a type 11 interferon cytokine, mediates neuronal oxidative stress and apoptosis (Vartanian *et al.*, 1995; Kneeland & Fatemi, 2013), and it is released during a bacterial infection (Kim *et al.*, 2011). Furthermore, IFN-γ is found at higher levels in normal pregnancy because it plays a key role in placental development, modifying decidual arteries (Croy *et al.*, 2002). Indeed, mice lacking uNK cells (the main lymphocytes found at human and murine implantation sites) had inadequate levels of IFN-γ which resulted in improper modification of decidual arteries, and uNK cell maturation (Ashkar *et al.*, 2000). Interestingly, nitric oxide is released by microglia (Nakajima & Kohsaka, 2001; Smith *et al.*, 2012) in response to high levels of IFN-γ (Meda *et al.*, 1995). Taken

together, these reports suggest that there may be a connection between microglia, cytokines, oxidative stress, and malfunctioning placenta, all of which are thought to play mechanistic roles in MIA.

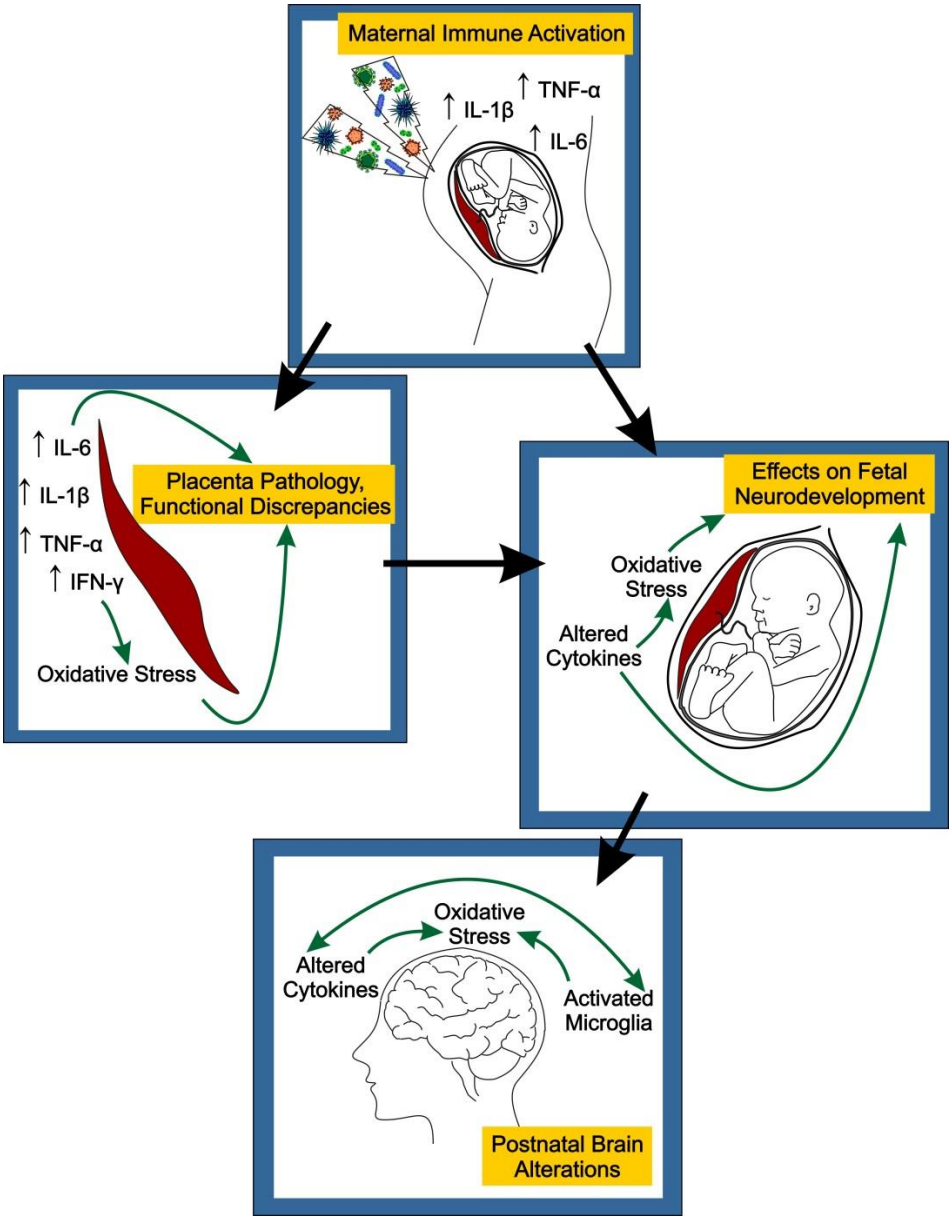


Figure 3: Key MIA Mechanisms

The current mechanisms though to mediate the adverse effects of MIA on offspring neurodevelopmental outcome. Altered cytokines (and in particular IL-6) in the maternal system, placenta and fetal brain, are thought to be the most potent factors in terms of MIA as they are able to alter the placenta as well as act directly on neurodevelopmental processes. Oxidative stress is implicated in similar roles, and can be promoted by cytokines or active microglia. Incidentally, altered functionality of microglia is another key mechanistic factor to MIA as microglia can alter any number of immunological factors within the CNS. Finally, the placenta, which itself can be adversely affected by MIA as well as associated alterations in systemic cytokines and oxidative stress, is a contributor to detrimental outcomes following MIA exposure due to its roles in fetal protection and developmental regulation.

1.7 Relevance to Midbrain/ Dopaminergic System Development

As described earlier, epidemiological and preclinical data have associated MIA with increased risk for a range of neurodevelopmental and neuropsychiatric disorders in affected offspring. Many of these MIA-associated diseases are related to malfunctions in dopaminergic systems, including PD (Toulouse & Sullivan, 2008; Lees *et al.*, 2009), schizophrenia (Brown *et al.*, 2004a; Brown, 2006; Howes & Kapur, 2009; Brown & Patterson, 2011; Canetta & Brown, 2012), and ADHD (Pineda *et al.*, 2007; Gold *et al.*, 2014; Silva *et al.*, 2014). While the causative molecular basis of these conditions is an area of on-going investigation, it has been hypothesized that "fetal programming" as a result of MIA may alter the development of fetal dopaminergic neurons and associated dopamine (DA) pathways, and those alterations in combination with other genetic and environmental risk factors may converge into a final disease phenotype later in life (Howes & Kapur, 2009). Thus, investigations into the effects of MIA on midbrain specific development and DA related behaviors are imperative for elucidating the role of MIA in DA-related disorders.

1.7.1 Normal Development/ Anatomical Description

Development of ventral mesencephalon (VM) DA neurons begins early in mammalian development in the floor plate of the mesencephalon. Indeed, VM DA neurogenesis begins at E10 in the rat, induced by signaling factors FGF8, Shh, and Wnt1 (Hegarty *et al.*, 2013). These factors induce expression of FoxA2, Lmx1a/b, and Msx1, indicating the presence of VM DA NPCs (Ono *et al.*, 2007). The peak of proliferation of these DA NPCs occurs at E12 in the rat (Gates *et al.*, 2006). From about E12 to E16, the NPCs differentiate toward DA phenotype which is promoted by Nurr1 and Pitx3 expression (Smidt & Burbach, 2007; Deng, 2010). Indeed, Nurr1 levels peak from E13-E15 in the rat, which is indecently when most VM DA neurons undergo differentiation (Volpicelli *et al.*, 2004). Then, the cells begin to migrate and extend axons at E13 in the rat, and their projections begin to reach the striatum by E14 and the cortex by E16 (Hegarty *et al.*, 2013). These projections ultimately result in the main dopaminergic pathways, which continue to be refined throughout postnatal development (Levitt, 2003; Tau & Peterson, 2010).

In the adult mammal, three quarters of all dopaminergic neurons are located in the midbrain, specifically in the SN and VTA (German *et al.*, 1983; Pakkenberg *et al.*, 1991; Blum, 1998; Hegarty *et al.*, 2013). The A9 DA cells of the SN primarily project to the dorsal striatum, forming the nigrostriatal pathway (Hegarty *et al.*, 2013). Nigrostriatal dopaminergic function is crucial for voluntary motor control, and dopaminergic neuronal loss results in PD (Toulouse & Sullivan, 2008; Lees *et al.*, 2009). A10 and A8 DA neuron clusters from the VTA and retrorubal field (RRF), respectively, innervate the ventral striatum and prefrontal cortex (PFC), forming the mesocorticolimbic DA system (Björklund & Dunnett, 2007; Van den Heuvel & Pasterkamp, 2008). Mesocorticolimbic dopaminergic function is crucial for the regulation of emotion and reward (Tzschentke & Schmidt, 2000), and alterations in this system have been implicated in schizophrenia (Meyer-Lindenberg *et al.*, 2002; Howes & Kapur, 2009), drug addiction (Robinson & Berridge, 1993; White, 1996), depression (Martin-Soelch, 2009), and ADHD (Volkow *et al.*, 2009). Importantly, the mesocorticolimbic system consists of two distinct subset pathways, the mesolimbic pathway which consists of A10 DA neurons that project from the VTA to the nucleus accumbens (NAc) (Joel & Weiner, 2000; Björklund & Dunnett, 2007), and the mesocortical pathway in which the VTA DA neurons project to the PFC (Van den Heuvel & Pasterkamp, 2008). The mesolimbic pathway functions are primarily concerned with reward processes, including emotional and motivational aspects of reward seeking along with goal-directed behavior (Van den Heuvel & Pasterkamp, 2008; Salamone & Correa, 2012); whereas the mesocortical pathway deals more with cognitive control and behavioral inhibition related to reward, emotion, and motivation (Tzschentke, 2001), and as such, these two pathways function synergistically for proper regulation of those processes.

1.7.2 Developmental Effects of MIA

VM DA neuron development is regulated by various well characterized genetic pathways and subsequent molecular pathways (Smidt & Burbach, 2007; Hegarty *et al.*, 2013). Interestingly, MIA (Poly(I:C), 5mg/kg, E9), altered gene expression of genes that have critical roles in DA neuron development including Shh and FGF8 as well as transcription factors Pitx3 and Nurr1 (Meyer *et al.*, 2008a). Indeed, Nurr1has been subjected to numerous investigations recently because of its important roles in the final differentiation, migration, and survival of DA neurons

(Hegarty *et al.*, 2013) as well as regulation of TH and DAT in maturing and adult DA neurons (Saucedo-Cardenas *et al.*, 1998; Kadkhodaei *et al.*, 2009). Furthermore, Nurr1 abnormalities have been observed in relation to PD (Jankovic *et al.*, 2005), schizophrenia (Buervenich *et al.*, 2000), and ADHD (Smith *et al.*, 2005) implicating a role for this nuclear receptor transcription factor in MIA-induced alterations. Indeed, Nurr1$^{+/-}$ mice demonstrated more of behavioral consequences along with improper development of prefrontal cortical and ventral striatal DA systems following Poly(I:C) (2mkg/kg) on E17 (Vuillermot *et al.*, 2012).

These genetic alterations are associated with structural and functional changes. Indeed, Poly(I:C) (5mg/kg) administered on E9 to pregnant mice lead to a significant increase in TH-positive cells of affected offspring compared to controls in the SN at E19 and P70 and VTA at E70, and this increase was associated with an increase in Nurr1 protein expression (Vuillermot *et al.*, 2010). These results suggest that MIA has the ability to affect DA neuron development, possibly through alterations in Nurr1. In contrast, however, other studies have found loss of DA neurons in the midbrain in rats following intracerebral injection of LPS at P5 followed by analysis of DA neuron numbers in the midbrain P21 (Fan *et al.*, 2005) as well as following *in utero* (E10.5) maternal LPS injection and offspring rat brains analyzed at P21, P120, P210, P420, and P510 (Ling *et al.*, 2002; Ling *et al.*, 2004; Ling *et al.*, 2006; Ling *et al.*, 2009). While the difference in immunogen and species may make these studies less than ideal candidates for comparison, the recognition that MIA is able to alter DA neuron development is not trivial.

Importantly, the ability of MIA to affect DA neuron development expands to include the regions of dopaminergic projection. For instance, exposure to *in utero* Poly(I:C) (5mg/kg) on E9 resulted in a decrease in TH density in the nucleus accumbens of mouse adolescent offspring (P35), followed by an increase in TH density adulthood (P70) (Vuillermot *et al.*, 2010). These cellular differences are in parallel with neurochemical findings of decreased striatal DA levels in adolescence (P39), but increased in adulthood following LPS exposure (2mg/kg) every day throughout pregnancy (Romero *et al.*, 2007). Consistently, Poly(I:C) exposure (5mg/kg) daily from E12-E17 resulted in hyperfunction of striatal DA in adulthood for mice (Ozawa *et al.*, 2006). Cortical projections are also affected by MIA, as decreased expression of dopamine receptor (DR) 1 and DR2 (Meyer *et al.*, 2008c; Meyer *et al.*, 2008d), along with significantly increase basal DA levels (Winter *et al.*,

2008) were observed in PFC of adult offspring following exposure to Poly(I:C) (5mg/kg) *in utero* (E9).

1.7.3 Behavioral Effects of MIA

Given that MIA can lead to altered neurodevelopment and subsequent neurochemical alterations, and because DA is required for proper functioning of motor control (Volkow *et al.*, 1998; Jenkinson & Brown, 2011) and regulation of emotion and reward (Tzschentke & Schmidt, 2000), subsequent studies have investigated the behavioral consequences in affected offspring behavior following MIA in animal models.

A number of studies have focused on the nigrostriatal dopaminergic pathway by measuring motor behavior (Girault & Greengard, 2004). Some have shown delayed achievement of certain developmental milestones (Toso *et al.*, 2005; Xu *et al.*, 2013), while other studies report no difference following MIA (Golan *et al.*, 2005; Kirsten *et al.*, 2010a). Rotarod and open field have been used to test motor coordination following MIA, but results are again conflicting with some studies reporting differences (Roberson *et al.*, 2006; Girard *et al.*, 2009), and others none in affected offspring (Zager *et al.*, 2012). One of the most common ways of studying the motor system has been with the use of amphetamine (AMPH) challenges (Bernardi *et al.*, 1986; Robinson & Becker, 1986; Jackson *et al.*, 1994; Rodríguez *et al.*, 2013) because of the ability of the drug to induce locomotion through promotion of increased DA release (Pijnenburg *et al.*, 1976; Ikemoto, 2002; Zager *et al.*, 2012; Shim *et al.*, 2014). Furthermore, enhanced susceptibility to AMPH-induced DA stimulation has been observed in disorders affecting the DA system, including schizophrenia (Laruelle *et al.*, 1996; Laruelle *et al.*, 1999). This is consistent in some animal models of MIA. For example, the locomotion response to AMPH was enhanced for animals exposed to prenatal Poly(I:C) (2 or 5 mg/kg, on E9 or E17) (Meyer *et al.*, 2007). However, inconsistent findings have also been reported as reduced response to AMPH in locomotion behavior was observed for adult rat offspring that had been exposed to Poly(I:C) *in utero* (on E14) (Richtand *et al.*, 2011).

Due to the contribution of DA imbalance in schizophrenia (Laruelle *et al.*, 1999; Howes & Kapur, 2009; Brunelin *et al.*, 2013; Lau *et al.*, 2013) and ADHD (Levy, 1991; Swanson *et al.*, 2000; Volkow *et al.*, 2009; Volkow *et al.*, 2011)

pathology, other studies have utilized motivation or goal directed behavior assays in order to investigate functionality of the mesocorticolimbic DA system following MIA. Adult rats that had been exposed to Poly(I:C) (4mg/kg) on E15 did not differ in discrimination learning, motivation, nor gross locomotor behavior compared to the saline exposed controls in a basic operant conditioning task with food restriction (Zhang *et al.*, 2012). However, another study administered betamethasone, an anti-inflammatory and immunosuppressive glucocorticoid, to pregnant baboons and subsequently tested juvenile offspring in progressive ratio task, among other learning and motivation behaviors, in which they found a decrease in motivation for affected juveniles, suggesting that alterations to the fetal environment have the ability to alter later motivation and reward behaviors (Rodriguez *et al.*, 2011). Furthermore, maternal malnutrition resulted in restricted learning and motivation of offspring as demonstrated by operant conditioning scenarios in rats (Reyes-Castro *et al.*, 2011; Reyes-Castro *et al.*, 2012; Rodriguez *et al.*, 2012b) and baboons (Rodriguez *et al.*, 2012a; Keenan *et al.*, 2013). Poor maternal nutrition has resulted in a number of detrimental effects on offspring neurodevelopment including poor neuronal circuit formation, down regulation of growth factors, increased cell death, and inability for normal developmental maturation and organization of certain cell types (for reviews see (Morgane *et al.*, 1993; Monk *et al.*, 2013)), and is associated with fetal origins of adult diseases similarly to MIA (Barker & Osmond, 1986; Barker, 2002; Armitage *et al.*, 2004).

As pointed out by Meyer, these findings have not provided us with a direct causal link from MIA to the DA-associated structural and functional alterations observed throughout the life span of affected offspring, because MIA may result in any number of neurodevelopmental sequelae, but they have demonstrated that DA abnormalities begin *in utero* following MIA and they are developmentally regulated (Meyer, 2013). As such, MIA-induced atypical fetal midbrain development is the potential starting point on a trajectory which, in combination with genetic factors and other environmental insults, could lead to malfunctioning nigrostriatal or mesocorticolimbic pathways and the eventuality of related disorders. However, before we can put all of the pieces of this multifaceted story together, we must understand each stage in its entirety. In order to do this, more comprehensive analysis of MIA effects using standardized animal models will need to occur. This is because the current literature is varied and conflicting due to differences in

experimental designs and animal models, which makes it hard to draw conclusions between lab groups or even between studies.

1.8 Aims and Objectives of the Thesis

This thesis aims to comprehensively investigate the effects of MIA on offspring midbrain development and function, and in particular to gain a better understanding of how and why the developing brain is more susceptible to the effects of MIA at different gestational ages. There is currently a great deal of conflicting information regarding the behavioral effects of MIA exposure, and the cellular and molecular consequences of MIA on the developing midbrain remain to be fully elucidated. Thus, the overall objective of my project is to establish the consequences of acute and mild MIA on offspring dopamine-related behaviors, as well as the associated cellular and molecular disturbances of MIA on the midbrain of the offspring.

1.8.1 Establish an animal model of acute and mild maternal infection

While there are currently a wide variety of animal models of MIA, they are paralleled with inconsistent findings in developmental and behavioral outcomes (Boksa, 2010; Harvey & Boksa, 2012). Thus we initially sought to validate our own model of acute and mild MIA with which we could reproduce and compare across studies. Our low dose of LPS aimed to represent an animal model of chorioamnionitis, a bacterial infection of the fetal membranes which is often subclinical and associated with negative fetal outcomes (De Felice *et al.*, 2005; Miyazaki *et al.*, 2007).

1.8.2 Determine the effects of MIA on placenta

As the placenta is a key regulator of fetal development (Huppertz, 2008; Togher *et al.*, 2014), and placental health is a great indicator of neurodevelopmental outcome (O'Keeffe & Kenny, 2014), determining the effects of our model of MIA on the placenta was extremely important. In particular, investigating changes in placental cytokine levels as well as expression of HSD11B2 would help to understand the MIA-induced, placenta-mediated alterations in neurodevelopment because cytokines are known to alter neuron development (Deverman & Patterson, 2009; Stolp, 2013) and HSD11B2 is a critical regulator of fetal neurodevelopment (Togher *et al.*, 2014).

1.8.3 Investigate any changes in offspring dopamine-related behaviors in the MIA model

There is a large body of literature relating to behavioral consequences of MIA (Meyer *et al.*, 2007; Meyer *et al.*, 2008d; Meyer *et al.*, 2009; Boksa, 2010; Harvey & Boksa, 2012; Meyer, 2014), however inconsistency in results plagues the research field. Differences in experimental methods are widely attributed as the reason for this. Therefore, there was a need for a comprehensive study that would elucidate the lifelong behavioral consequences following MIA exposure at different time points during pregnancy, using one validated animal model. Furthermore, we are most interested in midbrain development and associated functioning as it relates to numerous disease etiologies, including PD (Toulouse & Sullivan, 2008), schizophrenia (Meyer-Lindenberg *et al.*, 2002; Howes & Kapur, 2009), drug addiction (Robinson & Berridge, 1993; White, 1996), depression (Martin-Soelch, 2009) and ADHD (Volkow *et al.*, 2009). Therefore, we utilized a wide array of behavioral assays which would provide information into the functionality of dopaminergic systems across the lifespan of affected offspring.

1.8.4 Establish molecular and cellular effects of MIA on midbrains of affected offspring

The final aim of the thesis was to investigate possible factors that could lead to behavioral deficits. As MIA results in increased levels of pro-inflammatory cytokines, including IL-1β and TNF-α, in the fetal brain (Urakubo *et al.*, 2001), and we have demonstrated that IL-1β impairs proliferation and alter differentiation of VM NPCs *in vitro* (Crampton *et al.*, 2012), it was important to determine the cytokine profiles of offspring subjected to our MIA model. Following from this, we then needed to establish if MIA resulted in any alteration to VM NPC proliferation, as well as determine how inflammatory mediators affected differentiation in culture. Due to the different age of onset for MIA associated disorders, it was also important to identify the cytoarchitecture of the midbrain across the lifespan. Importantly, as timing of MIA determines fetal outcome due to the different developmental processes that occur at different gestational stages (Andersen, 2003), we sought to determine these characteristics following MIA at different time points to establish critical windows for our model.

Chapter 2:

LPS alters placental inflammatory and endocrine mediators and inhibits fetal neurite growth in affected offspring during late gestation

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Abstract

Introduction

During pregnancy, maternal infection at different stages of gestation increases the risk of developing several psychiatric and neurological disorders later in life for affected offspring. As placental health is intrinsically linked to neurodevelopmental outcome, maternal infection may adversely affect the placenta at or before the gestational stages it affects fetal neurodevelopment. Here we examined this premise.

Methods

Pregnant-Sprague Dawley rats were administered saline or lipopolysaccharide (LPS) by intraperitoneal injection on embryonic days 12 – 18. We then examined a number of key placental inflammatory and endocrine mediators, along with fetal, birth and neuronal characteristics at different stages of development.

Results

Maternal exposure to LPS at later gestational ages significantly increased pro-inflammatory IL-1β expression and reduced placental HSD11β2 expression. This was accompanied by a reduction in placental weight and embryo number without an effect on embryo weight or crown rump length. *In utero* LPS exposure at later gestational ages also impaired the growth of neurons from affected offspring.

Discussion

These data show that maternal infection at later gestational ages modifies placental inflammatory and endocrine mediators that may adversely affect the growth of developing neurons in affected offspring.

Key words: Maternal infection, placenta, pregnancy, LPS, neuron.

2.1 Introduction

Fetal programming describes how *in utero* adversity influences developmental trajectories and disease risk in affected offspring (Schlotz & Phillips, 2009). Many epidemiological studies have now provided evidence that maternal infection during pregnancy, increases the risk for neurodevelopmental and neuropsychiatric disorders in affected offspring that include schizophrenia (Brown *et al.*, 2004a; Brown *et al.*, 2005), autism spectrum disorders (Zerbo *et al.*, 2013a) and cerebral palsy (Shatrov *et al.*, 2010) (for recent excellent reviews see (Boksa, 2010; Canetta & Brown, 2012)). Critical to these outcomes, is the role of the placenta at the maternal-fetal interface in regulating fetal growth and development (Huppertz, 2008). There is increasing evidence that placental health plays a key role in determining fetal neurodevelopmental outcome (for review see (Baker & Sibley, 2006; von Beckerath *et al.*, 2013; O'Keeffe & Kenny, 2014)). This was directly assessed in a recent retrospective cohort study which showed that neurodevelopmental impairment was significantly more frequent in infants with distinct signs of placenta dysfunction compared to those with normal placental function (von Beckerath *et al.*, 2013). These data show that placental health during pregnancy is likely to be intrinsically linked to neurodevelopmental outcome, thus infection-induced changes in the placenta may contribute to the molecular sequale of how these affect the developing fetal nervous system.

Important to the protective functions of the placenta is the enzyme 11β-hydroxysteroid dehydrogenase type-2 (HSD11β2) which protects the fetus from the much higher levels of maternal glucocorticoids seen in pregnancy (Demey-Ponsart *et al.*, 1982; D'Anna-Hernandez *et al.*, 2011; Jung *et al.*, 2011), by converting active cortisol into inactive cortisone (Sun *et al.*, 1997; Cuffe *et al.*, 2012). This enzyme is critical in protecting the developing fetus from potential adverse fetal programming due to heightened cortisol exposure (Holmes *et al.*, 2006; Cottrell *et al.*, 2014; Togher *et al.*, 2014). Interestingly, the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β have been shown reduce HSD11β2 expression *in vitro* (Chisaka *et al.*, 2005; Suzuki *et al.*, 2005; Kossintseva *et al.*, 2006). Despite this, whether maternal infection reduces placental HSD11β2 expression *in vivo* is unknown.

The increase in risk for adverse neurodevelopmental outcome for affected offspring following maternal infection has been shown to be dependent on the stage at which the infection occurs during pregnancy. For example, bacterial infection in the second, but not the first trimester of human pregnancy was found to increase the risk for autism spectrum disorders and schizophrenia in the offspring (Sørensen *et al.*, 2009; Atladóttir *et al.*, 2010; Patterson, 2011b). This has been confirmed by a large body of work in pre-clinical models that have confirmed the presence of "critical developmental periods" during which the developing nervous system is susceptible to the effects of maternal immune activation (Boksa, 2010; Harvey & Boksa, 2012). In agreement with this, we have recently shown, using the well-characterized superior cervical ganglion (SCG) *in vitro* model (Glebova & Ginty, 2005), that the growth of fetal neurons can be inhibited by the pro-inflammatory cytokine, interleukin (IL)-1β, at late embryonic and early postnatal stages of development, but not before or after this developmental window of sensitivity (Nolan *et al.*, 2011). This suggests that maternal infection during this window of sensitivity may impair fetal neurite growth and/or affect the placenta. Here we use the lipopolysaccharide (LPS) rat model to examine the effects of maternal infection on placental expression of proinflammatory cytokines and HSD11β2 which plays a critical role in protecting the fetus against excessive exposure to maternal cortisol (Holmes *et al.*, 2006; Cottrell *et al.*, 2014; Togher *et al.*, 2014), and determine its effects on the developing fetus and the growth of fetal neurons at different stages of gestation.

2.2 Materials and Methods

2.2.1 LPS injections and sample collection

The study carried out under license with full ethical approval from the Ethics Committee of UCC. Time mated Sprague-Dawley rats (Biological Services Unit, UCC) were maintained in a controlled environment on a 12 hour light/dark cycle with *ad libitum* access to food and water. Pregnant dams (n=4/group/time point) received an intraperitoneal (I.P.) injection of LPS (Sigma: 50µg/kg) or normal saline on embryonic day (E)12, E14, E16, or E18. 48 h post injection, dams were anesthetized with inhaled Isoflurane (Abbeville Veterinary hospital, Cork) and culled by decapitation. Embryonic sacs were removed via laparotomy, and washed in ice-cold Hank's Balanced Salt Solution (HBSS). Embryos were removed from the yolk sac and transferred to fresh HBSS where then they were weighed and crown-to-rump length (CRL) was measured. Placentae were cleaned of all membranes, quickly weighed, and either snap frozen in liquid nitrogen or fixed in 4% PFA for 24h, then cryoprotected for 48h. SCG samples were dissected as previously described (Zareen & Greene, 2009). Briefly, the head was faced ventral side up in order to identify the trachea. Then forceps were used to clear away skin and fat around the trachea in order to expose parallel muscles that run alongside the trachea. The muscle was removed from one side first to expose the carotid artery, which runs along the trachea as well before it branches into a "Y" shape. The carotid artery was severed at the most caudal end and then lifted up to locate the almond-shaped mass of glossy tissue that is the SCG just at the point of the "Y". The SCG was gently detached, and the same method was used to obtain the SCG tissue from the other side of the trachea.

2.2.2 Real-time PCR

A motorized pestle and mortar were used to disrupt the placenta and total RNA was extracted using an RNeasy tissue mini kit (Qiagen). RNA concentration was determined using a Spectrophotometer (NanoDrop Technologies). 500ng of RNA was reverse transcribed using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 20µl reaction mixture consisting of 2.0µl 10X RT Buffer, 0.8µl 25X dNTP mix (100mM), 2.0µl 10X RT Random Primers, 1.0µl Reverse Transcriptase, and 4.2µl Nuclease-free H_2O, using the following parameters: 25°C

for 10 min; 37°C for 120 min; 85°C for 5 min; 4°C for at least 10 minutes. The cDNA was stored at -80°C prior to use. For real time PCR, all samples were run in triplicate, with the reaction mix for a single reaction consisting of 1.0 µl 20X TaqMan® Gene Expression Assays (Applied Biosystems), 10 µl 2X TaqMan® Gene Expression Master Mix (Applied Biosystems), and 8.0 µl RNase-free H2O (Applied Biosystems). 19 µl of the mix was added to each well of a 96 well PCR reaction plate (Applied Biosystems) as well as 1.0 µl of the relevant cDNA. The plate was centrifuged briefly and then loaded into the qRT-PCR system (StepOne Real-Time PCRY System, Applied Biosystems) and run using the following cycling parameters; 50°C for 2 minutes; 95°C for 10 minutes; and 50 repetitions of 95°C for 15 seconds and 60°C for 1 min. Analysis was carried out using the 2-δCT method (Livak & Schmittgen, 2001).

2.2.3 Immunohistochemistry

Post-mortem immunohistochemistry for HSD11β2 was performed on 20µm cryosections of the fixed placentae. To block endogenous peroxidase, sections were treated with 10% H_2O_2 for 5 min, washed in 10mM PBS and blocked for 1 h in 10% normal goat serum in 10mM PBS with 0.4% Triton-X. Sections were then incubated in primary antibody to HSD11β2 (1:250; Sigma) in 1% normal goat serum in 10mM PBS with 0.4% Triton-X overnight at 4°C. Following a 3 x 10 min wash in 10mM PBS, sections were incubated with a biotinylated secondary antibody (1:200, goat anti rabbit, Vector Labs) for 2 h at room temperature. Following another 3 x 10 min wash in 10mM PBS, sections were incubated in ABC solution (1:200; Vector Labs) for 45 minutes at room temperature followed by immersion in diaminobenzidine substrate/chromogen reagent for 2-3 min at room temperature. Sections were dehydrated, cleared, mounted and imaged using an Olympus AX70 Provis upright microscope. Negative controls received the same treatment except PBS was substituted for the primary antibody.

2.2.4 Corticosterone ELISA

Pregnant dams were culled by rapid decapitation between 10:00am and 12:00pm and trunk blood was collected, centrifuged for 15 min at 4°C to isolate serum, which was stored at -80°C. Serum corticosterone concentration was determined using a

commercial ELISA assay kit (Enzo Life Sciences, NY, USA) according to the manufacturer's instructions. The corticosterone concentration was calculated via reference to the standard curve.

2.2.5 Cell culture and quantification

Study 1: JEG-3 cells treated with IL-1β

JEG-3 human choriocarcinoma cells were grown and maintained in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Sigma), supplemented with 10% fetal calf serum (Sigma), 100 nM L-Glutamine (Sigma), 100 U/ml Penicillin (Sigma) and 10 µg/ml Streptomycin (Sigma), in a humidified atmosphere containing 5% CO_2 at 37 °C. Cultures were treated with 10ng/mL IL-1β for 24h.

Cultures were fixed in ice-cold methanol for 5 min at room temperature, blocked with 5% bovine serum albumin (BSA; Sigma) in PBS before incubation with rabbit anti-11HSDB2 (1:250; Santa Cruz Biotech) in 1% BSA in 10mM PBS at 4°C overnight. Cultures were washed in 10mM PBS and incubated for 2h at room temperature with an appropriate secondary antibody (Alexa Fluor; Invitrogen; 1:500) in 1% BSA in PBS in the dark. The fluorescence intensity of HSD11β2 expressing JEG-3 cells was measured using the Image J analysis software. The relative fluorescence intensity was calculated per individual cell after subtraction of the background.

Study 2: SCG investigation

Following *in utero* LPS exposure on E16 or E18, dissociated neurons from the superior cervical ganglia (SCG) of embryonic (E18) and postnatal (P1) Sprague-Dawley rats were plated at a low density on poly-ornithine/laminin-coated 4 well 35mm tissue culture dishes (Sigma; Greiner Bio-One) and cultured in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h as previously described (Nolan *et al.*, 2011).

All cultures were fixed in ice-cold methanol for 5 min at room temperature, blocked with 5% bovine serum albumin (BSA; Sigma) in PBS before incubation with mouse anti-β-III tubulin (to visualize neurons and quantify neuronal survival) (1:250; Promega) in 1% BSA in 10mM PBS at 4°C overnight. Cultures were washed in 10mM PBS and incubated for 2h at room temperature with an appropriate

secondary antibody (Alexa Fluor; Invitrogen; 1:500) in 1% BSA in PBS in the dark. Neuronal survival and the total neurite length and number of branch points of neurons was measured using Sholl analysis as previously described (Gutierrez & Davies, 2007; Nolan *et al.*, 2011).

2.2.6 Data analysis

In this study we were interested in how the LPS exposed group compared to the corresponding control for each variable investigated, therefore all data were analyzed using student's T-tests.

2.3 Results

2.3.1 Effect of maternal LPS exposure on placental, fetal and birth characteristics

Firstly, we carried out a morphometric analysis of the placenta and fetal birth characteristics to determine if LPS induced any overt change in these parameters. I.P. administration of LPS to the pregnant dams on E16 resulted in a significant decrease in placental weight by E18 (Fig. 1a), but we found no significant change in placental weight when LPS was administered on E12 or E14 (Fig. 1a). I.P. administration of LPS on E12 did not result in any significant difference in the number of embryos per dam on E14, however LPS administration on E14 and E16, resulted in a significant reduction in the number of embryos per dam on E16 and E18 respectively (Fig. 1b). We next examined the crown-rump length (CRL) and fetal weight at these time points and found that LPS exposure did not significantly change these parameters irrespective of when LPS was administered (Fig. 1c-e). I.P. administration of LPS on E16, which reduces placental weight at E18 (Fig. 1a), had no effect on birth weight, however repeated exposure to LPS on E12, E14, E16 significantly reduced birth weight (data not shown). These data suggest that the duration of LPS exposure may dictate its effects on birth weight, despite that shorter term LPS exposures can affect the placenta.

2.3.2 The effects of maternal LPS on placental cytokine and HSD11B2 expression vary by developmental age.

We next sought to determine the mRNA expression profile of TNFα and IL-1β in the placenta following maternal exposure to LPS at different stages of gestation. I.P. administration of LPS to the pregnant dams on E14 and E16 resulted in a significant increase in placental IL-1β mRNA expression on E16 and E18 respectively (Fig. 2a), but we found no significant change in TNF-α expression at these time points (Fig. 2b). The greatest increase in IL-1β mRNA expression was observed at E18 (Fig. 2a).

To determine if maternal exposure to LPS altered the expression of placental HSD11B2, which is a critical regulator of fetal development (Togher *et al.*, 2014), we next examined the expression of placental HSD11β2 using real-time PCR and immunocytochemistry at each of these time points. HSD11β2 protein (Fig. 2c) and mRNA (Fig. 2d) were expressed in the placenta at all gestational ages studied. Using

quantitative real-time PCR, we found that levels of HSD11β2 mRNA significantly increased from E14 to E16 and from E16 to E18 (Fig. 2d). I.P. administration of LPS to pregnant dams on E12 induced a significant up-regulation of HSD11β2 mRNA expression in the placenta (Fig. 2e). In contrast, I.P. administration of LPS on E14 or E16 resulted in a significant decrease in HSD11β2 expression at E16 and E18 respectively (Fig. 2e).

As cortisol/corticosterone levels are known to regulate HSD11β2 expression (Togher *et al.*, 2014), we next examined maternal corticosterone levels at E18 in pregnant dams that received I.P LPS or saline on E16. We found that maternal corticosterone levels were significantly lower in the LPS group compared to saline treated controls using an ELISA on maternal plasma samples (Fig. 2f), suggesting that the decrease in HSD11β2 expression is not due to an increase in maternal cortisol levels. Given that we found a significant up-regulation of placental IL-1β at E18, we next examined if IL-1β could reduce the expression of HSD11β2 in the placental JEG3 cell line. Treatment of JEG3 cells with 10ng/ml IL-1β for 24h resulted in a significant down regulation in HSD11β2 expression (Fig. 2g, h) suggesting that LPS-induced increase in placental IL-1β at E18 may mediate the observed decrease in placental HSD11β2 expression.

2.3.3 Effect of maternal LPS exposure on fetal neurite growth

Maternal infection is a known risk factor for a neurological dysfunction later in life (Boksa, 2010). We have previously shown that IL-1β exerts a growth inhibitory effect on developing fetal neurons that becomes apparent on E18, but not at developmental stages preceding this (Nolan *et al.*, 2011). Given that maternal exposure to LPS increases placental IL-1β mRNA in late but not early stages of pregnancy (Fig. 2), we next examined if developing neurons in LPS exposed offspring were affected at specific stages of pregnancy. To explore this further, we cultured neurons isolated from the SCG of rat pups or embryos that were exposed to maternal inflammation *in utero*. The SCG model system is a well characterized and experimentally tractable population of neurons extensively used to analyze the molecular basis of neuronal survival and axonal growth (Nolan *et al.*, 2011). Neuron cultures from P1 pups of pregnant dams that were exposed to LPS at E18 displayed a significant reduction in neurite length (Fig. 3a-c), whereas those neurons from pups

of dams exposed to LPS on gestational day 16 and grown at E18 or P1 grew normally (Fig. 3a). There was no effect on neuronal survival at any stage (data not shown). To confirm these findings, we used Sholl analysis, which provides a graphic illustration of neurite length and branching with distance from the cell body. Neurons from pups exposed *in utero* to LPS at E18 displayed a significant reduction in their Sholl profile (Fig. 3b) indicating a decrease in neuronal arbor elongation and complexity, whereas neurons from pups exposed *in utero* to LPS at E16 grew normally (Fig. 3b). This affect was not due to a direct effect of LPS, as neurons cultured in the presence of LPS were not adversely impacted in terms of axonal length (Fig. 3d), Sholl profile (Fig. 3e) and survival (Fig. 3f).

2.4 Figures and Figure Legends:

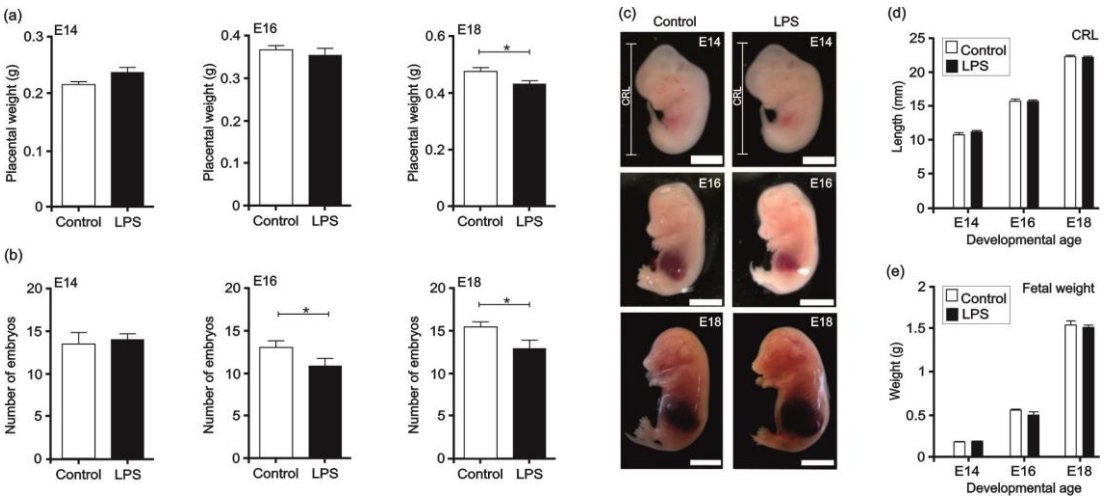


Figure 1: Maternal LPS exposure in late stages of pregnancy reduces placenta weight and fetal number.
(a) Graphical representation of placental weight, and (b) number of embryos, in pregnant dams at E14, E16 and E18 that received an I.P. injection of saline (control) or 50µg/kg LPS at E12, E14, or E16 respectively. (c) Representative examples of embryos (d) crown rump length (CRL) and (e) average fetal weight at each age from pregnant dams that received an I.P. injection of saline (control) or 50µg/kg LPS at E12, E14 or E16 with the embryos assessed following removal by laparotomy 48 h later. All data are expressed as mean ±SEM. (* $P < 0.05$).

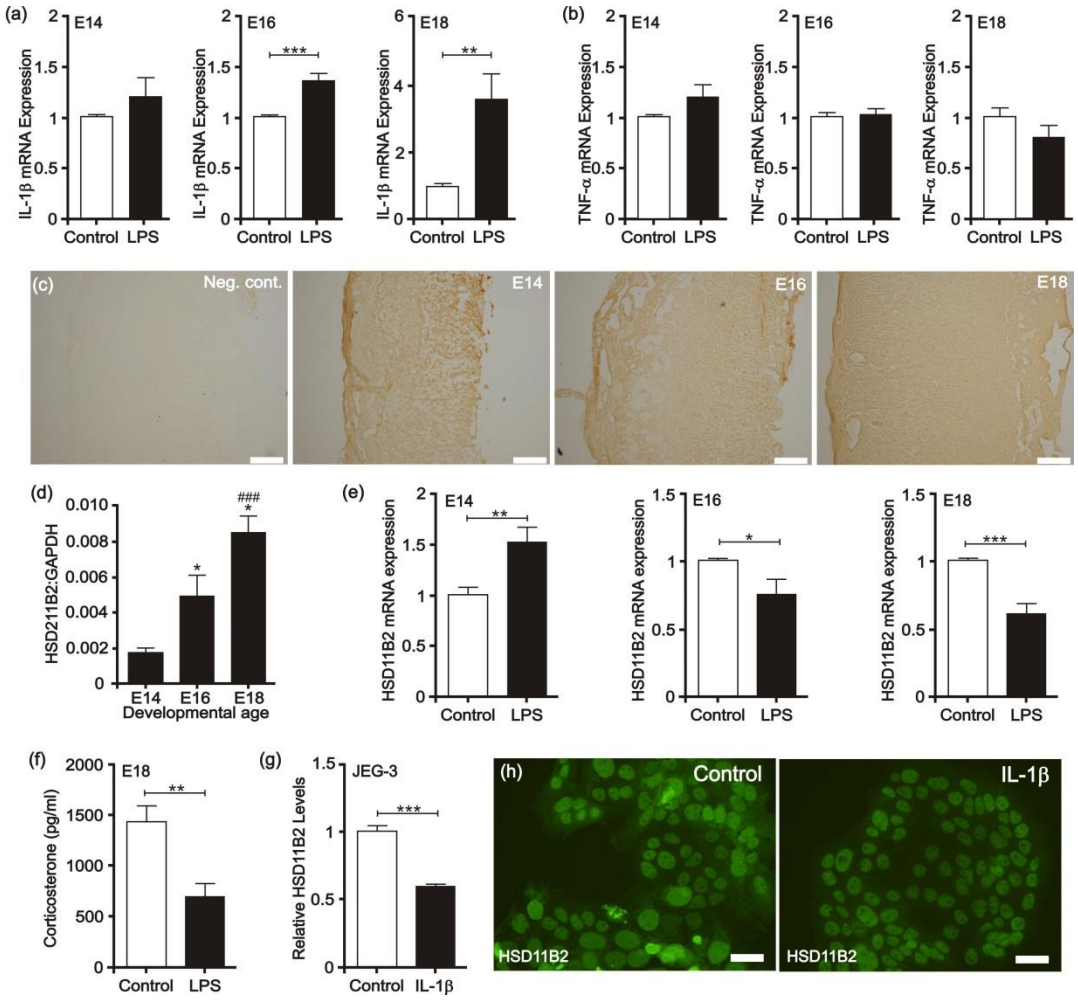


Figure 2: The effects of maternal LPS exposure on placental cytokine and HSD11β2 expression vary by developmental age.

Graphical representations of (a) IL-1β and (b) TNF-α mRNA expression in the placentae of pregnant dams that received an I.P. injection of saline (control) or 50µg/kg LPS at E12, E14, or E16. (c) Immunohistochemistry and (d) real-time PCR data showing HSD11β2 expression in the placenta at each gestation studied. (e) Graphical representation of real-time PCR data showing HSD11β2 mRNA expression in control and LPS exposed dams at E14, E16, and E18. (f) Graphical representation of maternal corticosterone concentration in pg/ml at E18, 48h post LPS or saline exposure. (g) Relative levels of HSD11β2 expression, and (h) representative photomicrographs of HSD11β2 expression in JEG3 cells treated with 10ng/ml IL-1β for 24h. All data are expressed as mean ± SEM. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ### $P < 0.001$).

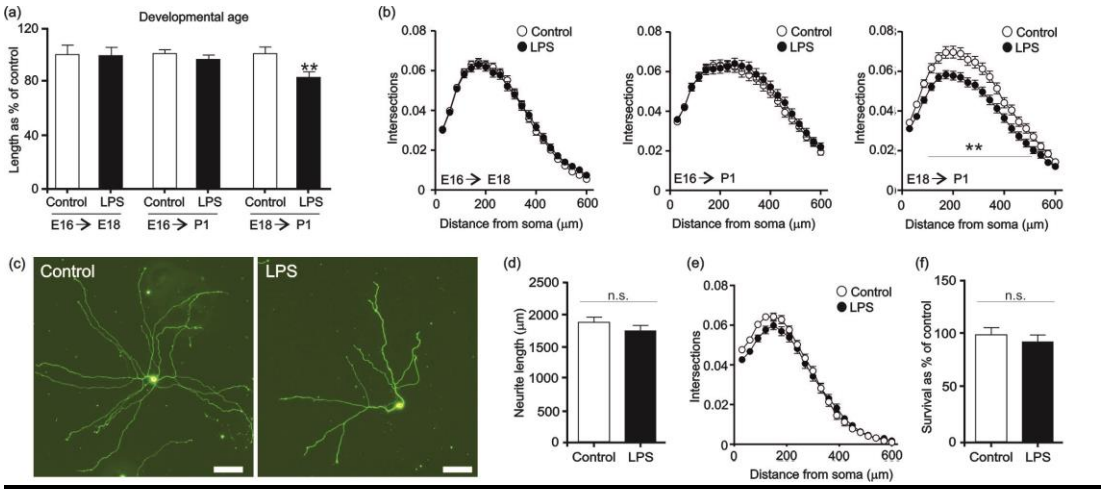


Figure 3: Maternal LPS exposure in late stages of pregnancy inhibits neurite growth.

(a) Graphical representation of normalized total neurite length as percentage of control, and (b) Sholl profiles of SCG neurons from embryos or pups from dams that received an I.P. injection of saline (control) or 50µg/kg LPS at E16 or E18 with neurons cultured for 24h at E18 or P1 as indicated (day of I.P. injection → day of culture). (c) Representative photomicrographs of P1 SCG neurons after 24h *in vitro* from P1 pups that were exposed to either saline or 50µg/kg LPS on E18 *in utero.* (d) Total neurite length, (e) Sholl profile and (f) survival of E18 SCG neurons directly treated with 50ng/ml LPS for 24h. All data are expressed as mean ±SEM. (** $P < 0.01$).

2.5 Discussion

During this study, we found that placental HSD11β2 expression increased from E14 to E18. This progressive increase in HSD11β2 expression with advanced pregnancy has been demonstrated in the human (Benediktsson *et al.*, 1993; Shams *et al.*, 1998; Togher *et al.*, 2014) and baboon (Pepe *et al.*, 1996) placenta previously. Conversely, HSD11B2 mRNA is abundantly expressed in the labyrinth zone and extra embryonic membranes of the mouse placenta until E15.5 but then halts at E16.5 (Brown *et al.*, 1996). Contradictory trends have been previously observed in the rat with increasing enzymatic activity of rat placental HSD11β2 with advancing pregnancy (E16 to E22) has been demonstrated by some (Burton & Waddell, 1994) but not others (Staud *et al.*, 2006). However there may be zone specific effects, as HSD11β2 mRNA expression in the labyrinth zone and decidua decreases, while it increases in the basal zone with advancing gestation (Burton *et al.*, 1996; Waddell *et al.*, 1998). Interestingly we found that maternal exposure to LPS has a bi-phasic effect on placental HSD11β2 expression in that it increased placental HSD11β2 expression at mid-gestation (E14), but decreased it at later stages (E18). These findings are supported by a study by Cottrell et al. that showed that a maternal low protein diet increased placental HSD11β2 expression at mid-gestation (E13.5), but reduced it at late gestation (E17.5) in the mouse (Cottrell *et al.*, 2012).

As we found that maternal exposure to LPS induced a down-regulation of HSD11β2 expression at later stages of pregnancy we next examined its effect on maternal corticosterone and found that at the same developmental stage, LPS induced a down-regulation of maternal corticosterone levels (Fig. 2). These finding are supported by previous studies showing that cortisol infusion into pregnant mice between embryonic day E12 and E15 up-regulated HSD11β2 expression in the placenta two days later (Cuffe *et al.*, 2012), indicating that LPS-induced decrease in cortisol levels post LPS, may lead to the observed decrease in HSD11β2 expression. However, we also observed an increase in placental IL-1β expression and found that IL-1β treatment of the JEG3 placental cell line down-regulated HSD11β2 expression after 24h. This is in agreement with previous studies showing that TNF-α reduced HSD11β2 expression in LLC-PK cells (Heiniger *et al.*, 2001). Similarly, TNF-α, and IL-1β have been shown to reduce HSD11β2 in term human placental villi explants (Kossintseva *et al.*, 2006). However IL-1β (but not TNF-α) decreased

HSD211β2 expression in primary cultures of term trophoblasts, indicating a predominant role for IL-1β in regulating placental HSD11β2 expression (Chisaka *et al.*, 2005). In agreement with this, we found that LPS induced placental IL-1β but not TNF-α expression. Collectively these data suggest that the down-regulation in placental HSD11β2 expression post LPS, may be mediated by a drop in maternal cortisol levels and an increase in placental IL-1β expression.

Interestingly we also found that maternal LPS reduced placental weight without affecting fetal weight only at E18. This is supported by a study showing that in the HSD11β2-/- mice at E15 (~E17 rat) there was a reduction in placental weight while fetal weight was maintained suggesting that compensatory changes occur in the placenta to maintain function (Wyrwoll *et al.*, 2009). What these changes mean for fetal exposure to cortisol remains to be determined. However as demonstrated by Cottrell et al. placental HSD11β2 expression *in vivo* protect the fetus from the effects of maternal cortisol overexposure, and a reduction in placental HSD11β2, increased fetal concentration of corticosterone (Cottrell *et al.*, 2012). This suggests that in our study the significant decrease in placental HSD11β2 expression post LPS at late gestation may result in elevated fetal exposure to maternal corticosterone. However, we also observed a significant reduction in maternal corticosterone levels at this stage, but as corticosterone levels are many times higher in the maternal circulation, this may still result in elevated fetal exposure to maternal corticosterone as a result of decreased placental HSD11β2 expression, the exact scale of which will depend potentially on the change in the ratio between both parameters. The neurodevelopmental ramifications of this will likely depend on the degree of change in fetal cortisol levels (for excellent review see (Cottrell *et al.*, 2014)), and may also be influenced and even exacerbated by corresponding changes pro-inflammatory cytokine levels, which have been shown to have many adverse effects on the developing brain (Boksa, 2010).

In agreement with this, we found that *in utero* LPS exposure, during the stage at which the placenta becomes sensitive to LPS (E18), inhibits the growth of neurons isolated from affected offspring. This is entirely consistent with previous work showing that IL-1β exerts a growth inhibitory effect on these neurons during this developmental window from E18 to P1 (Nolan *et al.*, 2011), and that IL-1β was the causative agent of behavioral change induced by maternal infection (Girard *et al.*,

2012). Though translating developmental dates between species in a linear manner is an over simplification, the first and second halves of rat gestation are often equated to the first and second trimesters of human pregnancy (Clancy *et al.*, 2007). Thus these findings are consistent with the notion that second trimester maternal infection can increase the risk an adverse neurodevelopmental outcome (Sørensen *et al.*, 2009; Atladóttir *et al.*, 2010; Patterson, 2011b). As maternal infection during specific periods may induce long lasting placental molecular change (O'Keeffe & Kenny, 2014), it is possible that these placental signatures may be useful in predicting subsequent risk for averse neurodevelopment.

Chapter 3:

LPS-induced maternal immune activation results in differential neurobehavioral outcomes depending on gestational age of exposure.

Straley ME, Theze SA, Hegarty SV, Crampton SJ, Cyran JF, O'Mahony SM, O'Keeffe GW. LPS-induced maternal immune activation results in differential developmental and neurobehavioral outcomes depending on gestational age of exposure. ***In preparation*** *for submission to the Journal of Neuroscience.*

Abstract

Introduction

The majority of DA neurons are found in the adult midbrain and they project outwards to form two of the major dopaminergic pathways: the nigrostriatal and mesocorticolimbic pathways. Nigrostriatal dopaminergic function is needed for proper motor control and degeneration of these DA neurons leads to Parkinson's disease. Regulation of emotion and reward depends on the mesocorticolimbic pathway and dysfunction of this pathway has been implicated in disorders such as schizophrenia, drug addiction, depression, and ADHD. While the full etiology of these diseases has yet to be fully elucidated, epidemiological and preclinical data suggest that fetal programming as a result of maternal immune activation (MIA) may alter neurodevelopment trajectories and leave affected offspring more susceptible to these diseases later in life. Here we aim to assess the differential effects of MIA at multiple gestational ages on two major DA pathways and associated behaviors.

Methods

To do this, pregnant rats were administered a 50μg/kg dose of LPS or saline via I.P. injection on E12 or E16. To determine if the timing of MIA altered neurobehavioral outcome, we performed an array of behavioral tests which target utilization of the dopaminergic system, including motor and reward behavior assays, across the lifespan of affected offspring.

Results

Our results show that motor differences can be seen in juvenile offspring following maternal infection at E16 only. These differences are compensated for in adolescence, but subsequently there is a decline in motor function in adulthood. Furthermore, our results also demonstrate differential responses to amphetamine in reward seeking behaviors of adolescent and adult offspring that were exposed to maternal infection at E12.

Conclusions

Our data show that timing of MIA is important, and infection earlier in gestation (E12) may have greater consequences for the mesocorticolimbic pathway whereas infection in later pregnancy (E16) may have greater consequences for the function of the nigrostriatal pathway.

3.1 Introduction

Three quarters of all dopaminergic neurons found in the adult brain are located in the midbrain (German *et al.*, 1983; Pakkenberg *et al.*, 1991; Blum, 1998; Hegarty *et al.*, 2013). These neurons form two major output projections as the nigrostriatal pathway and the mesocorticolimbic pathway. Nigrostriatal dopaminergic function is crucial for voluntary motor control, and dopaminergic neuronal loss results in Parkinson's disease (PD), which is a neurodegenerative disorder characterized by impaired motor function (Toulouse & Sullivan, 2008; Lees *et al.*, 2009). Mesocorticolimbic dopaminergic function is crucial for the regulation of emotion and reward (Tzschentke & Schmidt, 2000), and alterations in this system have been implicated in schizophrenia (Meyer-Lindenberg *et al.*, 2002; Howes & Kapur, 2009), drug addiction (Robinson & Berridge, 1993; White, 1996), depression (Martin-Soelch, 2009) and ADHD (Volkow *et al.*, 2009).

While the causative basis of these disorders is an area of intensive investigation, it has been suggested that "*fetal programming*" as a result of maternal immune activation (MIA) may alter the development of the fetal dopaminergic circuitry leaving the offspring more susceptible to these disorders later in life (Howes & Kapur, 2009; Knuesel *et al.*, 2014). In agreement with this, epidemiological and pre-clinical data show *in utero* exposure to an inflammatory stimulus increases the risk for a range of neurodevelopmental and neuropsychiatric disorders including schizophrenia (Brown *et al.*, 2004a; Brown *et al.*, 2005), autism spectrum disorders (ASD) (Zerbo *et al.*, 2013a; Zerbo *et al.*, 2013b; Lee *et al.*, 2015) and cerebral palsy (Shatrov *et al.*, 2010) in affected offspring.

One of the most widely used pre-clinical models of MIA is intraperitoneal administration of the bacterial endotoxin lipopolysaccharide (LPS) to pregnant dams (Boksa, 2010; Knuesel *et al.*, 2014). Observations of a causal link between LPS-exposure and loss of dopamine (DA) neurons in the midbrain have been reported in rats following intracerebral injection of LPS at P5 followed by analysis of DA neuron numbers in the midbrain P21 (Fan *et al.*, 2005) as well as following *in utero* (E10.5) maternal LPS injection and offspring rat brains analyzed at P21, P120, P210, P420, and P510 (Ling *et al.*, 2002; Ling *et al.*, 2004; Ling *et al.*, 2006; Ling *et al.*, 2009). Subsequently, as relative function of DA neurons can be measured using behavior analysis (Girault & Greengard, 2004; Goldberg *et al.*, 2011), a number of

studies have focused on the impact of prenatal LPS exposure on the development and subsequent functioning of the nigrostriatal system utilizing motor behavior assays. Some studies have shown delayed achievement of certain developmental milestones (Toso *et al.*, 2005; Xu *et al.*, 2013), while other studies report no difference following maternal infection (Golan *et al.*, 2005; Kirsten *et al.*, 2010a). Similarly, other studies have examined motor coordination following MIA, but results are conflicting with some studies reporting differences in juvenile or adult motor activity (Girard *et al.*, 2009), and others finding none in affected offspring (Zager *et al.*, 2012).

To the best of our knowledge, there has only been one study examining the effects of MIA on basic motivation based behaviors in affected offspring. These adult rats, which were exposed to Poly(I:C) (4mg/kg) on E15 did not show marked differences in discrimination learning, motivation, nor gross locomotor behavior compared to the saline exposed controls in a basic operant conditioning task with food restriction (Zhang *et al.*, 2012). In contrast, maternal malnutrition resulted in restricted learning and motivation of affected offspring as demonstrated by operant conditioning scenarios in rats (Reyes-Castro *et al.*, 2011; Reyes-Castro *et al.*, 2012; Rodriguez *et al.*, 2012b), which suggests a potential for *in utero* exposure to LPS to alter motivation and reward behaviors in affected offspring later in life (Rodriguez *et al.*, 2011). Interestingly, Poly-I:C exposure at E17 resulted in a significant reduction in the sucrose preference test for adult mice, indicating that late prenatal MIA may lead to anhedonia in adulthood (Bitanihirwe *et al.*, 2010), a symptom which is commonly associated with reward deficits as well as anxiety and depressive behaviors (Der-Avakian & Markou, 2012). Many studies have also analyzed anxiety-like behaviors as another indicator of the functionality of the mesocorticolimbic dopaminergic pathway; behavior tests measuring anxiety include ultrasonic vocalization, open field, and elevated plus maze (EPM), among others (Kalinichev *et al.*, 2002; Schmidt & Müller, 2006; Baharnoori *et al.*, 2012; Solati *et al.*, 2015). Prenatal influenza exposure in mice during mid-gestation (Shi *et al.*, 2003) or prenatal exposure to LPS in mid or late gestation increased anxiety levels and depression in male rat (Enayati *et al.*, 2012; Lin *et al.*, 2012) and mouse offspring (Hava *et al.*, 2006; Babri *et al.*, 2014). However, in contrast, adult male rats that were exposed to LPS at E15 and E16, demonstrated no difference in anxiety behavior (Wischhof *et al.*, 2015).

Here, we use a LPS, Sprague-Dawley rat model of MIA (Straley *et al.*, 2014), to examine the impact of *in utero* exposure to MIA on both nigrostriatal and mesocorticolimbic-mediated behaviors in affected offspring in the neonatal period, adolescence and adulthood. Throughout the study we utilize amphetamine (AMPH) administration in the behavioral experiments due to its role in increasing extracellular DA which can result in heightened locomotor behavior (Jackson *et al.*, 1994; Pontieri *et al.*, 1995; Underhill *et al.*, 2014) as well as increased reward responding in operant conditioning (Fletcher *et al.*, 1998; Schmidt *et al.*, 2013). As rat midbrain dopaminergic neurons extend axons at E12/E13 to establish functional circuitry and these projections begin to reach the striatum by E14, and the cortex by E16 (Hegarty *et al.*, 2013), we administered LPS on E12 or E16 to determine the impact of the timing of exposure to MIA on dopaminergic-mediated behavior in affected offspring.

3.2 Materials and Methods

3.2.1 Animals/LPS injections

All experimental animal work was carried out under license with ethical approval from the Ethics Committee of UCC. Sprague-Dawley rats (Biological Services Unit, UCC) were maintained in a controlled environment on a 12 hour light/dark cycle with *ad libitum* access to food and water. As the timing of infection during pregnancy is often a critical determinant of fetal outcome, we administered LPS at different time points (E12 or E16). For analysis of behavior, pregnant dams (n=6 or 7/group/time point) received an intraperitoneal (I.P.) injection of LPS (Sigma: 50μg/kg) or normal saline on E12 or E16. Pregnancies continued without disturbance and post-partum the offspring were randomly assigned for future behavior testing at P9, P30 or $P60^{+}$. Once the animals reached weaning age (21days), female offspring were culled and male offspring received ear punches and tail marks for identification and were randomly assigned to groups with which they were housed. This was done to ensure that no animal was disrupted until it was time to perform behavior testing and so that treatment groups remained caged together. Every experimental group consisted of male offspring from multiple litters with no more than 3 rats from the same litter. In all cases, experimenters were blinded to the cage codes for the duration of the study in order to prevent experimental bias. Upon completion of assigned behaviors, animals were culled by rapid decapitation (see Figure 1 for a timeline of the experiment).

3.2.2 Maternal care behavior

To determine if LPS administration adversely affected maternal care behavior in the post-partum period, which may confound the behavioral results, maternal behavior in all the saline and LPS groups was scored daily for the first 4 days postpartum, at the same time every day (beginning at 8am), and each trial lasted for 30 minutes. The animals habituated to the testing room for 10 minutes prior to scoring. During this time, nests were observed and scored as follows: 1 = nesting material was untouched or nest had no form; 2 = nesting material was in one section, flat, but nest could be distinguished; 3 = nesting material was loosely gathered into a definite shape of moderate height; 4 = nest was symmetrical, tightly packed and high walls or in a spherical shape (Capone *et al.*, 2005). Then the camera was started and scoring

began. For each session, 4 dams were under inspection at a time, and the observer examined dam #1 for 20 sec, #2 for 20 sec, #3 for 20 sec, #4 for 20 sec, and so on until the 30 minutes finished. With this method each dam was observed 22 times over the course of a trial. During each observation, the dam was scored for care behaviors, including: gathering pups, licking/grooming any pup, nursing pups as well as other behaviors such as exploring or remaining stationary (Francis *et al.*, 1999; Liu *et al.*, 2000; Franks *et al.*, 2011). Nursing behaviors were broken down into sub-categories as follows: blanket posture was defined as the dam lying flat over the pups, passive posture was defined as the dam allowing feeding while either laying on her back or side, and arched-back nursing was defined as a tense posture where the dam remained upright with the back strained (Liu *et al.*, 2000). The frequency of each behavior was calculated.

3.2.3 Reflex behaviors (P9 rat pups)

Three natural reflex behaviors were observed, with methods described by Heyser (2004). To score the righting reflex, the P9 rat pup was placed in a supine position on a flat surface and latency for the pup to turn itself to its belly was recorded. This test was repeated three times per animal and average time was calculated. The cliff test was next performed whereby the pup was placed on the edge of a platform with its nose and forefeet hung over the edge. The aim was to determine if the pup moved away from the cliff by backing up or turning sideways which was scored as a "pass" or "fail". Finally, the grasp reflex was examined by using a wooden toothpick to lightly stroke the paw of each individual pup. The reflex was confirmed as present if the animal grasped at the probe.

3.2.4 Ultrasonic vocalization (P9 only)

Neonatal rat pups produce alarm calls that can be measured at 40Hz by a bat detector (Walker *et al.*, 2003). P9 pups were subjected to USV at the beginning (pre test) and the end (post test) of the juvenile test battery (which included reflex testing, bar hold, and open field). The equipment was tested before each trial to ensure the detector was not picking up extraneous noise within an empty cage to ensure it would only register noise made by pups. Pups were placed individually into the Plexiglas isolator box which was cleaned with 70% EtOH before each animal and vocalizations were recorded using a mimi-3 bat detector for 3 minutes.

3.2.5 Bar hold (all ages)

The animal was lifted by the trunk and brought within reaching distance of a thin metal bar. The animal was allowed to grab hold with its front paws, and once it had grasped the bar it was released, the duration the animal held on was recorded, with a maximum cut off of 10s for pups and 180s for adolescent/adult animals (Heyser, 2004).

3.2.6 Rotarod (adolescent and adult offspring)

Adolescent (P30) and adult (>P60) animals were tested on a rotarod every day for 3 days, with the same 4 trial pattern as follows. Animals were firstly trained for 5 min at a fixed rate (4RPM) (trial 1). Then rats completed 3 trials on a rod that accelerated smoothly from 4 to 40 RPM over 5 min. There was at least 15 min break between each of the trials. The length of time that each animal was able to stay on the rod was recorded as the latency to fall, registered automatically by a trip switch under the floor of each rotating drum.

3.2.7 Open field (all ages)

An open field was conducted at each age: juvenile (P9), adolescent (P30) and adult (>P60). The apparatus was cleaned with 70% EtOH before each individual trial as to prevent any location bias due to smell. Rats were placed in the middle of the arena, always facing the same direction, and then the experimenter left the room while locomotion was recorded. P9 pups underwent open field for 5 min in a small arena (40cm x 32 cm rectangular arena), and the P30/P60 animals had trials which lasted 10 minutes in a larger arena (90cm diameter circular arena). Ethovision software was used to analyze locomotion behaviors (Noldus Information Technology, The Netherlands). For open field tests, locomotion was assessed using distance traveled and velocity measurements. Immobile frequency was an additional variable investigated to understand the number of times the animal froze during the test and was identified using controlled parameters with the Ethovision software.

3.2.8 Open field amphetamine challenge

Animals were habituated to the testing room and the open field chamber as described previously for 10 min (see ‘open field’ protocol). Following this, they were

habituated to injections by receiving I.P. injections of saline (10ul/kg). Two days following the habituating injections, they were subjected to an AMPH challenge in order to flood the system with extracellular DA which could exacerbate functional differences in the MIA offspring. Firstly, normal saline (10ul/kg) was injected I.P. in the left quadrant and the animal was immediately placed in the center of the open field apparatus for 15 minutes as previously described (Eisenstein *et al.*, 2009). After this point, the animal received an I.P. injection of amphetamine in the right quadrant (1mg/kg) and was placed back into the chamber undisturbed for 20 minutes. Locomotion behaviors were again recorded and subsequently analyzed using Ethovision software (Noldus Information Technology, The Netherlands).

3.2.9 Food reward operant conditioning (adolescent and adult)

Food reward operant conditioning test was performed to examine the effects of MIA at different gestational ages (E12 or E16) on the reward seeking behavior of food restricted animals. Furthermore, as we were interested in the possible adverse outcomes following maternal infection upon the dopaminergic system we utilized the reward-potentiating effect of d-amphetamine to fully elucidate any potential differences in the mesocorticolimbic dopaminergic pathways of our saline or LPS exposed groups. In a progressive ratio (PR) schedule, the number of lever presses required for the animal to obtain 1 sweetened pellet was arithmetically increased over a 1hr long test session. Reinforcer efficacy was expressed as the "break point" and is herein defined as the highest ratio completed before responding ceased or the session ended.

A food reward protocol was adapted from (Richardson & Roberts, 1996; Mobini *et al.*, 2000; Levin *et al.*, 2003; Hanlon *et al.*, 2010). Every day, starting 1 week before conditioning, rats were habituated to the sweet pellets (45mg, Sandown Scientific, UK). 48 hours before training, 4 weeks old rats were food restricted to 5g food / rat /day, and 24 hours before training rats received no food in order to create optimal reward seeking behavior. During the experiments, animals were kept food restricted at 12g food/ rat/day in order to maintain 90% of free-feeding body weight. Rats were placed in dual lever test chambers (Med Associates, USA) which were equipped with a house light and cue light above each lever. During food reward sessions, only one lever was active and the cue light over said active lever would be

illuminated indicating the active one. MED-PC software was used to program experiments and control data collection.

Rats were put initially on a fixed ratio (FR) training schedule FR1 to FR2 to FR5, receiving one training session of 1 h duration per day and progressed forward only once they were able to receive 30 rewards (food pellets) in 30 minutes, or 60 in an hour. Following training, rats were put on a PR schedule for 3 consecutive days in order to get the average number of rewards obtained from PR in 1 h (the average of the three days is reported as "baseline"). The next day, animals received an injection of saline (10µl/kg) and underwent PR test in order to establish a vehicle control for each animal. Finally on the last day, animals received an injection of 0.25 mg/kg d-amphetamine sulfate (Sigma, UK), which has been previously reported as the breaking point dose of amphetamine to use on PR (Richardson & Roberts, 1996), 10 minutes prior to being put in the operant chamber for a final session of PR (Mobini *et al.*, 2000). Animals were then returned to *ad libitum* food and water and went undisturbed from this point until they were grown into adults (week 10). Then they were again food restricted for 2 days (5g food / rat /day the first day and 0g the second) and maintained at 90% of free-feeding body weight during the experiment. On the first day of adult food reward they had one session of FR training confirm they all remembered the rules. All 47 animals progressed from FR1 to FR5 within the 1 h training session. Following this, the animals went through 3 consecutive days of PR again to reach a valid average (baseline) for each animal. As we were not under time constraints for this stage of the study (as the animals were full grown) we were able to perform a full dose response using 0.10, 0.25, and 2.5 mg/kg doses of d-amphetamine sulfate (Sigma, UK) and normal saline as vehicle control. Using a within-subject study design, animals were randomly assigned to a drug schedule, with two days between sessions, as two days has been shown previously to allow for amphetamine wash out (Gentry *et al.*, 2004; Milesi-Hallé *et al.*, 2005).

Helpful definitions for operant conditioning with food reward experiments

Fixed Ratio (FR)	When the number of lever presses required for 1 food reward remains the same for the entire session (e.g. 1 lever press for 1 pellet = FR1; 5 lever presses for 1 pellet = FR5)
Progressive Ratio (PR)	When the number of lever presses required to obtain 1 reward exponentially increases throughout the session. For example, to get the first pellet, the animal must press the lever 5 times. Then, to receive the second food reward they must press it 11 times, and so on.
Baseline	The average of 3 PR trials, indicating the baseline reward responding.
Break Point	The highest level of PR the animal was able to reach before they stopped responding or the session timed out.
Rewards	Sweet food pellets
Active Lever	The lever the animal needs to press to obtain a reward
Inactive Lever	The lever which does not connect to the food pellet
Time Out	There is an interval following a successful production of a food reward where a light turns on in the operant box indicating to the animal that any lever presses during the "time out" will not result in a reward.
Time out active lever presses	The number of times the animal presses the active lever when the house light indicating a 'time out' was on.

3.2.10 Statistics

For multiple variable situations, statistics were calculated using multi-way ANOVA in *statistica* software program. Following the ANOVA, Fisher's Least Significant difference (LSD) *post hoc* test was conducted where appropriate. As we were only interested in how E12 control compared to E12 LPS, and how E16 control compared to E16 LPS and we were not interested in how E12 compared to E16 overall, the cohorts were kept separate for ease of statistical calculations. In many instances, we were specifically interested to see if the saline control group had a better behavioral performance compared to the relative LPS group at a single time point, and in these cases, *graph pad prism* statistical software was used to conduct one-tailed student t-tests.

3.3 Results

3.3.1 In utero exposure to LPS does not affect maternal care behavior in the postnatal period

Firstly, we sought to investigate maternal care behavior towards the offspring post LPS, as it has been shown previously that alterations in maternal care can have a significant effect on offspring behavior (Weaver *et al.*, 2006). We investigated a multitude of maternal behaviors, including nesting abilities, nursing, maternal activity, grooming behavior, and pup location (grouped or scattered) over the first 4 days post-partum. Overall, there were no significant differences in the maternal behaviors, save for a decrease in frequency of blanket nursing in the E12 LPS group compared to the E12 control group on the first 3 days only. However, when all nursing behavior was analyzed together there were no significant differences (Table 1). Collectively these data show that all the pups received maternal care that was overall similar in nature, meaning any differences in offspring behavior are not due to differences in maternal care.

3.3.2 Effect of in utero LPS exposure on primitive reflexes in early life

Primitive reflexes are highly stereotypical automatic responses elicited in very early life, and weak reflexes are indicative of poor neurodevelopment (Zafeiriou, 2004). We first examined primitive reflexes at P9, as this is the age when many primitive reflexes are prominent in the rodent (Heyser, 2004). Firstly, to assess vestibular and proprioceptive skills, we examined the righting reflex (Roberts & Roberts, 1967), followed by the grasping reflex, which is often used for early detection of neurodevelopmental abnormalities including diagnosis of CP (Zafeiriou, 2004; Udo *et al.*, 2014), and can demonstrate insufficient peripheral or spinal cord involvement (Futagi *et al.*, 2012). Finally, the cliff avoidance reflex was examined to assess the sensory and motor function of the pups (Xi *et al.*, 2009). Our results demonstrate no differences in these reflexes (Table 2), suggesting that there were no extensive developmental abnormalities following *in utero* exposure to LPS at the two gestational stages we examined.

3.3.3 In utero exposure to LPS on day 16 of gestation significantly reduces locomotion and alters juvenile adaptation to anxiety

We next measured ultrasonic vocalization (USV) of rat pups (De Vry *et al.*, 1993; Hofer *et al.*, 2001; Wöhr & Schwarting, 2008) and examined open field behavior to look for characteristics widely associated with anxiety like behaviors (Prut & Belzung, 2003). The USV pretest showed no significant differences between any of the groups suggesting that their anxiety levels were similar (Figure 2B,C). However, in the post test differences were observed in the E16 cohort. The E16 LPS group had a significantly shorter duration of calls compared to controls, while there were no differences in the overall number of calls (Figure 2B,C). This suggests that the E16 LPS group adapted differently than the E16 control group during the battery of tests. We also measured the time spent in center (Figure 2F) and time spent on edges (data not shown) of the open field to assess anxiety, and found no significant differences in any of the groups, suggesting no differences in anxiety levels during the open field test.

We next investigated voluntary motor function at P9. LPS exposure significantly reduced the bar holding time compared to the relative control group in both cohorts (Figure 2A). Furthermore, there were significant differences in the open field as a result of *in utero* LPS exposure on E16 but not E12 (Figure 2D-G). Specifically, the E16 LPS group displayed decreased locomotor activity compared to E16 controls as there was a significant decrease in distance traveled (Figure 2D) and velocity (Figure 2E). Taken together, these results suggest an impairment in motor skills at P9 following *in utero* LPS exposure later (E16) but not earlier (E12) in gestation.

3.3.4 The detrimental effects of late gestation in utero exposure to LPS on motricity is resolved by adolescence

To determine the life-long consequences of *in utero* LPS exposure on neurobehavioral function in affected offspring, we also carried out behavioral testing in adolescence (P30) and adulthood (>P60) and male rats were subjected to a battery of motor tests at each time point. By P30, we observed no significant differences between any of the groups in the bar hold test (Figure 3A), rotarod (Figure 3B), or in the open field in the numerous motor variables measured (Figure 3C-I). These data

suggest that the behavioral disturbances that we observed at P9 (Figure 2), have been eliminated or compensated for by the time these animals reach adolescence.

Interestingly, when the open field experiment was used to analyze anxiety behaviors, unlike the P9 result, there was a significant increase in time spent in the center in the E16 LPS group compared to E16 controls while still no difference between the E12 groups (Figure 3H). These data suggest that while the E12 groups were similar in anxiety level, the E16 groups differ in their anxiety response. However, the fecal pellet output, which is another indicator of anxiety levels used in open field assessments, was not significantly different between groups (Figure 3I).

3.3.5 Age-related decline in motor function as a result of in utero exposure to LPS on E16

We next conducted the same battery of tests once animals reached adulthood (over P60) including bar hold, rotarod, and open field. Similarly to the adolescent behavior assay, the group that was exposed to LPS *in utero* on E12 had no statistically significant differences on any test (Figure 4). In contrast, although the E16 cohort displayed no significant difference in the bar hold test (Figure 4A), there was a group difference observed with the rotarod test. Over the 3 days, the E16 LPS group remained on the apparatus for a shorter duration than the E16 control group, with statistically significant differences observed on days 1 and 3 specifically (Figure 4B). In the open field, there were initial differences in locomotion behavior of the E16 animals as demonstrated by distance moved (Figure 4D) and velocity (Figure 4F); however after 5 minutes the animals were covering similar distances at similar speeds. Furthermore, the number of times E16 LPS animals were reported as immobile, or the number of times the animals froze, was significantly higher compared to the E16 controls (Figure 4G).

We next examined anxiety behavior again using the open field test. There were no differences shown between the relative control vs LPS groups regarding time spend in the center (Figure 4H) or time spent on the edges (data not shown). While the E12 groups were not significantly different, the E16 LPS group had a higher fecal output compared to E16 control (Figure 4I). While exploratory behavior demonstrates similar anxiety levels between groups, the fecal pellet output may suggest a subtle level of heightened anxiety in the E16 LPS group compared to E16 controls.

3.3.6 MIA differentially affects amphetamine response in open field task depending on gestational age of exposure

Drug challenges are extensively used to elucidate abnormal dopaminergic function, and one of the most common ways of studying the dopaminergic motor system has been with the use of amphetamine (AMPH) challenge in an open field test (Bernardi *et al.*, 1986; Robinson & Becker, 1986; Jackson *et al.*, 1994; Rodríguez *et al.*, 2013). Amphetamine is an indirect DA receptor agonist, and as such has been known to induce locomotion in a control animal by promoting significant increases in DA release (Pijnenburg *et al.*, 1976; Carboni *et al.*, 1989; Pontieri *et al.*, 1995; Ikemoto, 2002; Zager *et al.*, 2012; Shim *et al.*, 2014; Underhill *et al.*, 2014). Furthermore, enhanced susceptibility to the dopaminergic stimulation caused by amphetamine has been observed as a symptom of dopaminergic system disorders including schizophrenia (Laruelle *et al.*, 1996; Laruelle *et al.*, 1999). This appears to hold true for certain models of maternal infection. For example, in the Poly I:C mouse MIA model, the locomotion response to amphetamine was enhanced for the animals exposed to prenatal Poly I:C (Meyer *et al.*, 2007). Therefore, we sought to determine if prenatal exposure to LPS altered the locomotion response to amphetamine in affected offspring (Robinson & Becker, 1986; Laruelle *et al.*, 1999).

Interestingly, amphetamine affected motor behaviors in a similar manner between in the E16 groups, altering distance covered, time moved and velocity by statistically similar amounts (Figure 5D-F). That is, the amphetamine response was the same for the E16 control and E16 LPS groups. Surprisingly, there were significant differences observed within the E12 cohort. The E12 LPS group reacted to the amphetamine differently than the E12 control as seen by the difference in locomotion distance (Figure 5A) and velocity (Figure 5B).

We also used fecal pellet output during this paradigm to investigate anxiety related behaviors again, this time investigating how amphetamine might alter anxiety response. Importantly, there was a significant increase in the control groups' amphetamine trial fecal pellet output per minute compared to saline trials (i.e. E12 control saline trial compared to E12 control AMPH trial), suggesting that the amphetamine increased anxiety in the control groups (Figure 5C,F). However this difference was not seen with the LPS groups; there was not a statistically different fecal pellet output from the saline trial to the AMPH (Figure 5C,F). Interestingly,

the LPS groups both had significantly higher fecal pellet output than their respective control during the saline trial, suggesting a heightened anxiety response following saline injection for the LPS animals (Figure 5C,D). Finally, only the E16 cohort showed significant differences in the AMPH trial, with the E16 LPS group having a significantly lower fecal pellet output compared to E16 control (Figure 5F).

3.3.7 Adolescent and adult offspring demonstrate differential amphetamine response in food reward paradigm following maternal LPS exposure at E12

We next investigated motivation behaviors in affected offspring using food reward operant conditioning. Using baseline active lever press and inactive lever press during progressive ratio (PR) to rate the ability of animals to understand the test, E12 LPS animals performed similarly to E12 control and E16 LPS also compared comparably to E16 control (Figure 6C,F,H,J). These data suggest that all groups of animals were able to understand that the active lever would result in a food reward while the inactive lever would not. During the baseline trial where animals were not exposed to amphetamine, as well as saline vehicle control trials there was no difference seen between any of the groups, suggesting that the animals have similar functionality of reward behavior in normal circumstances. This was consistent across both adolescent and adult testing time points.

At the adolescent age (P30), we administered a moderate dose (0.25mg/kg) (Richardson & Roberts, 1996) of d-amphetamine sulfate via I.P. injection in order to investigate how this indirect dopamine agonist would affect reward seeking behavior in adolescent animals. The goal directed behavior of the E12 LPS group was not altered by amphetamine exposure, unlike the other groups which saw a significant increase in reward responding as measured by break point (Figure 6A,D) and active lever presses (Figure 6C,F) following the amphetamine injection. While there were difference between E12 control and E12 LPS groups, there were no difference between E16 control and E16 LPS, suggesting that the E12 cohort responded differently to the drug.

Similarly, the groups did not differ in the baseline PR task nor following a saline injection in goal directed behaviors in adulthood (Figure 7A-F). Using a within subject study design, we administered 3 different doses of d-amphetamine sulfate in order to determine a dose response. Interestingly, for all animals reward seeking (as measured by break point, rewards obtained, and active lever presses) was

most heightened with the lowest dose (0.1mg/kg), whereas as expected, the highest dose of 2.5mg/kg resulted in a complete lack of reward seeking behavior (Figure 7A-F). The E12 LPS exposed group differed from the E12 control group in numerous instances; rewards obtained (Figure 7B) and active lever presses (Figure 7C) with the 0.1mg/kg dose; and break point (Figure 7A), rewards obtained (Figure 7B), and active lever presses (Figure 7C) with 0.25mg/kg dose. There were no significant differences seen between the E16 groups in any measurement. These data taken together suggest that MIA did not leave the dopaminergic system vulnerable to motivation or goal directed behavior dysfunction under normal food deprivation. However, the animals exposed to MIA at E12 responded differently to a dopamine specific pharmacological intervention compared to their saline controls suggesting that MIA at E12, but not E16, might reduce sensitization to amphetamine in reward behaviors.

3.4 Figures and Figure Legends

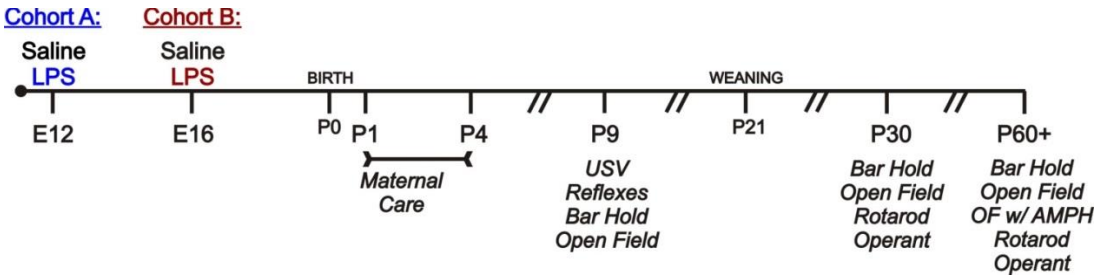


Figure 1: Schema of experimental design.
Schematic of the experimental design showing two cohorts of animals divided by gestational age of exposure: E12 or E16. On the designated day (E12 or E16) these cohorts were then further divided into saline control or LPS group and mother was administered the randomly assigned vehicle/LPS via I.P. injection (n=6 or 7 mothers per group). The four groups (E12 cont, E12 LPS, E16 cont, and E16 LPS) received no other differences throughout the study and experimenters were blind to the groups. Maternal care behaviors were observed on days 1-4 post-natal. Following weaning, offspring were caged according to treatment group and assigned behavior (i.e. E12 LPS offspring that were tested at P30 were grouped together). Juvenile behaviors were assessed on P9. Adolescent behaviors were assessed at P30. Adult behaviors were assessed beginning when animals reached the age of P60 and testing took approx. 1 month for completion.

Category	Behavior	E12	E16
Nesting	Nest Score	Control: 3.0 ± 0.4 LPS: 3.4 ± 0.3	Control: 3.2 ± 0.2 LPS: 2.6 ± 0.4
Nursing	Passive Nursing	Control: 24.7 ± 12.2 LPS: 9.7 ± 4.9	Control: 9.7 ± 4.5 LPS: 10.58 ± 5.462
	Blanket Nursing	Control: 3.2 ± 1.3 LPS: 9.7 ± 4.1	Control: 18.8 ± 6.7 LPS: 18.18 ± 6.6
	Arched Back Nursing	Control: 33.8 ± 11.4 LPS: 49.4 ± 7.3	Control: 33.8 ± 7.1 LPS: 50.8 ± 11.6
	Any Nursing Behavior	Control: 61.7 ± 13.5 LPS: 68.8 ± 9.0	Control: 62.3 ± 7.7 LPS: 79.6 ± 7.9
Moving Pups	Grouping	Control: 3.9 ± 1.5 LPS: 1.9 ± 1.9	Control: 2.0 ± 0.9 LPS: 1.5 ± 1.0
	Scattering	Control: 0 ± 0 LPS: 1.3 ± 1.3	Control: 1.4 ± 0.9 LPS: 0 ± 0
Maternal Activity	Stationary	Control: 85.7 ± 3.0 LPS: 92.2 ± 2.6	Control: 89.0 ± 1.9 LPS: 93.94 ± 3.3
	Active	Control: 21.4 ± 3.7 LPS: 16.2 ± 4.2	Control: 16.2 ± 4.9 LPS: 5.3 ± 1.4
	Exploring	Control: 11.7 ± 1.7 LPS: 11.7 ± 2.6	Control: 6.5 ± 1.7 LPS: 5.3 ± 1.8
Grooming	Groom Pup	Control: 24.7 ± 3.6 LPS: 18.8 ± 6.1	Control: 17.5 ± 3.5 LPS: 17.4 ± 3.4
	Groom Self	Control: 11.0 ± 4.2 LPS: 5.2 ± 1.8	Control: 5.2 ± 1.8 LPS: 2.3 ± 1.0
Pup Location	Grouped	Control: 90.3 ± 8.4 LPS: 99.4 ± 1.5	Control: 78.6 ± 15.3 LPS: 103.8 ± 1.4
	Scattered	Control: 9.7 ± 9.0 LPS: 0.7 ± 0.7	Control: 23.4 ± 15.2 LPS: 0.0 ± 0.0

Table 1: Exposure to LPS at the two gestational ages studied does not affect maternal care behavior in the postnatal period.

Table showing results of maternal care behaviors on Day 4. Nest score was marked on a scale of 1-4. All other variables are reported as frequency (the number of times the behavior was observed per the 22 viewings of that mother in this 30 minute session). Results compare E12 control group to E12 LPS, and E16 control group to E16 LPS. Analyzed using 2way ANOVA, data expressed as mean ±SEM (n=6-7 mothers per group).

Reflex	E12	E16
Righting Reflex	Control: 1.3 ± 0.08 LPS: 1.1 ± 0.08	Control: 1.2 ± 0.04 LPS: 1.4 ± 0.2
Cliff Test # Failures	Control: 2 / 13 LPS: 2 / 8	Control: 1 / 10 LPS: 0 / 8
Grasping Reflex # Failures	Control: 0 / 13 LPS: 0 / 8	Control: 1 / 10 LPS: 0 / 8

Table 2: Prenatal injection of LPS does not affect normal reflexes in pups.
Table showing results of reflex behavior testing on P9 rat pups. Righting reflex mean and SEM scores. Cliff test and Grasping are reported as pass/fail. Student one-tailed t-test was used to analyze data (n=8-13).

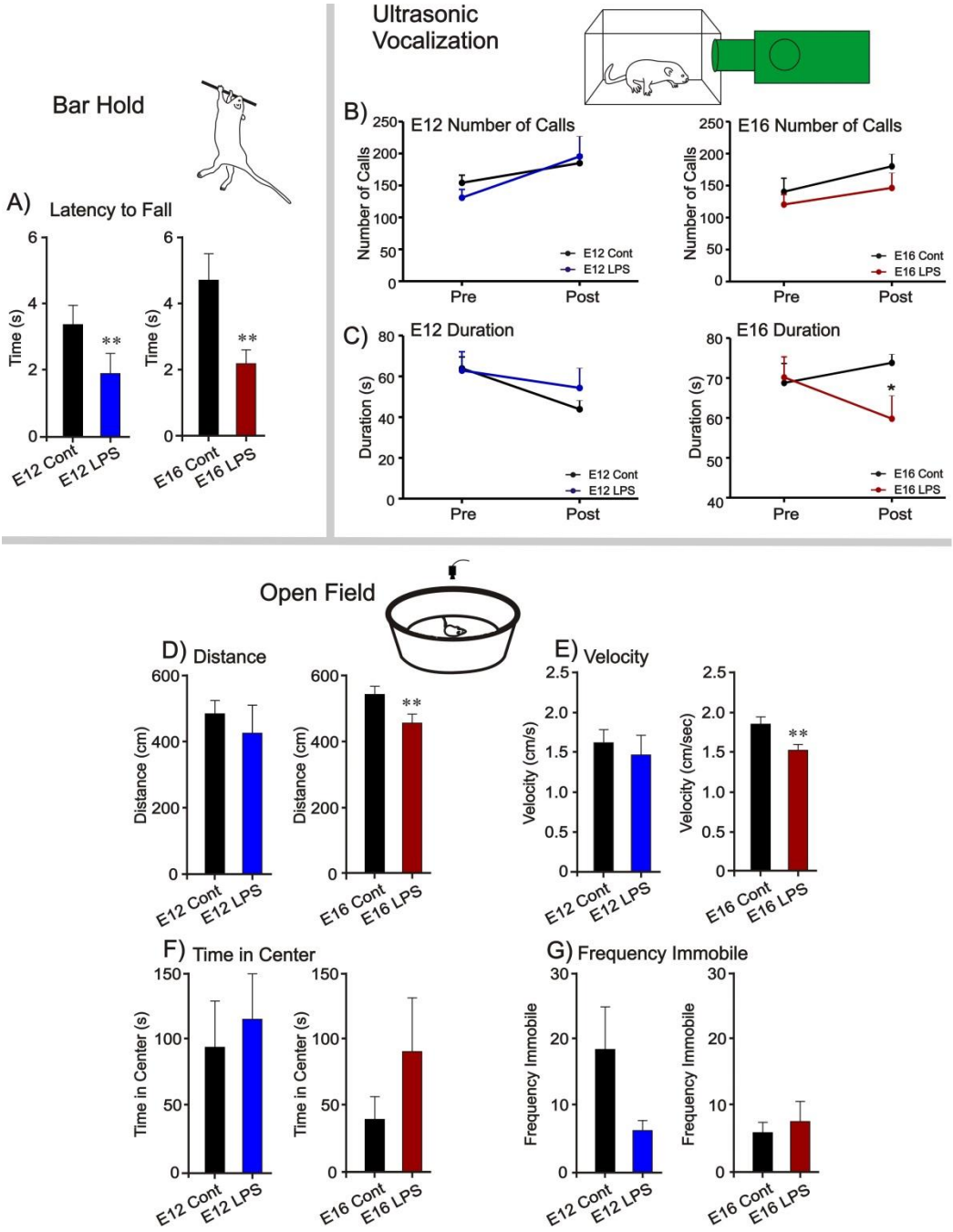


Figure 2: Exposure to *in utero* LPS on day 16 of gestation significantly reduces locomotion and alters juvenile adaptation to anxiety.

(A) Bar hold test, (B-C) ultrasonic vocalization (USV), and (D-G) open field behavioral assessment was performed at P9 for offspring that were exposed *in utero* to saline (Cont) or LPS at E12 (blue bars) or E16 (red bars) as indicated. (B-C) Ultrasonic vocalization was measured pre and post behavioral test battery conducted in P9 rat pups. (B) Number of calls and (C) duration of calls during each 3 minute session are reported. In Open Field assessment, (D) total distance moved, (E) velocity, (F) the total amount of time spent in the "center" and (G) frequency animal was immobile are shown. Data expressed as mean +SEM (n=7-12 per group; * = $P < 0.05$, ** = $P < 0.01$; compared to corresponding saline treated control).

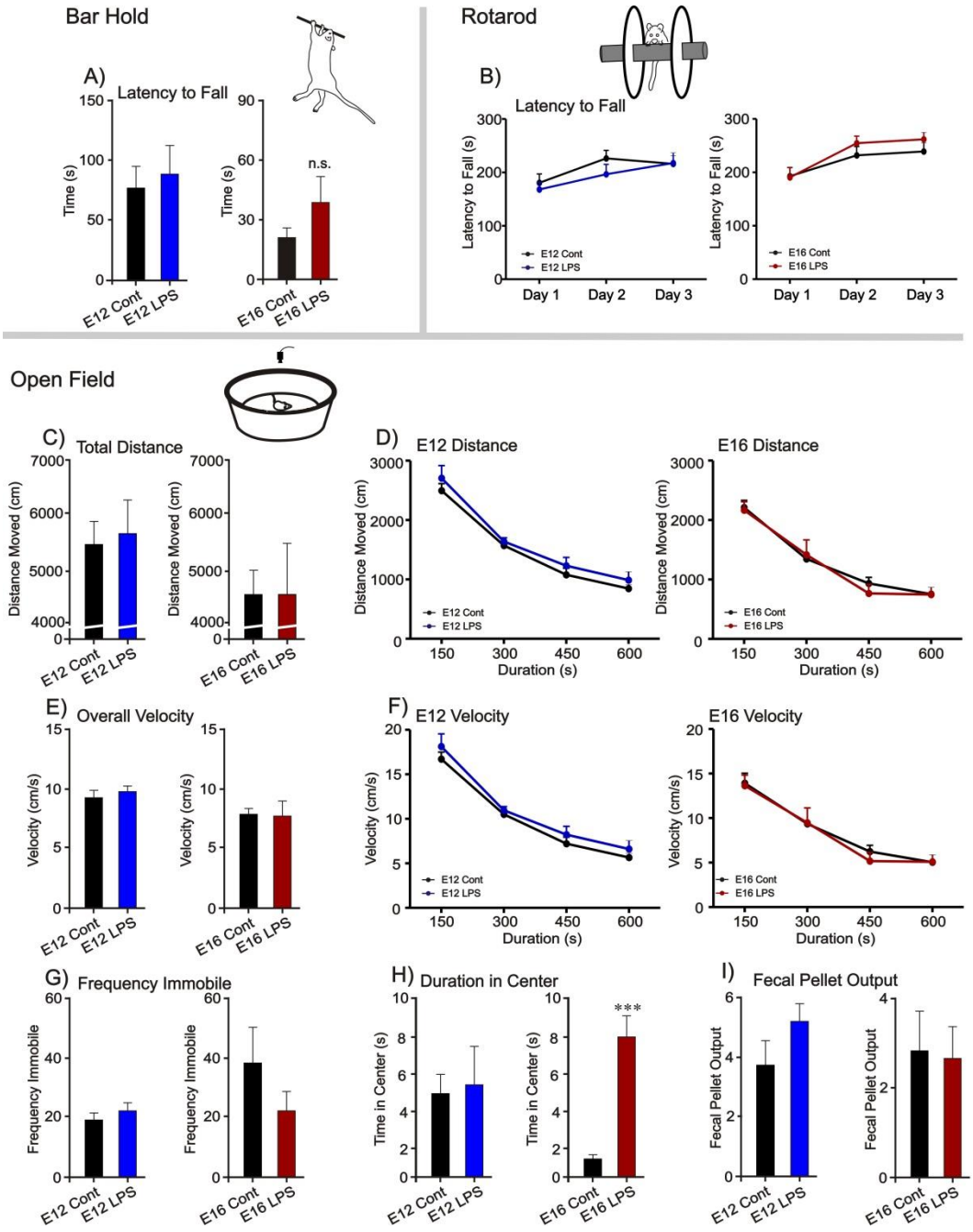


Figure 3: The detrimental effects of late gestation in utero LPS exposure on motricity is resolved by adolescence.

(A) Bar hold test and (B) Rotarod test and (C-I) open field test was performed at P30 for offspring that were exposed *in utero* to saline (Cont) or LPS at E12 (blue bars) or E16 (red bars) as indicated. (C) Total distance moved, (D) breakdown of distance covered over the duration of the open field test, (E) overall velocity, (F) breakdown of velocity over the duration of the open field test, (G) immobile frequency, (H) the total amount of time spent in the center of the arena, and (I) number of fecal pellets produced. Data expressed as mean +SEM (n = 8-14 rats per group; *** = $P < 0.001$ *in utero* exposed LPS group compared to relative saline control group).

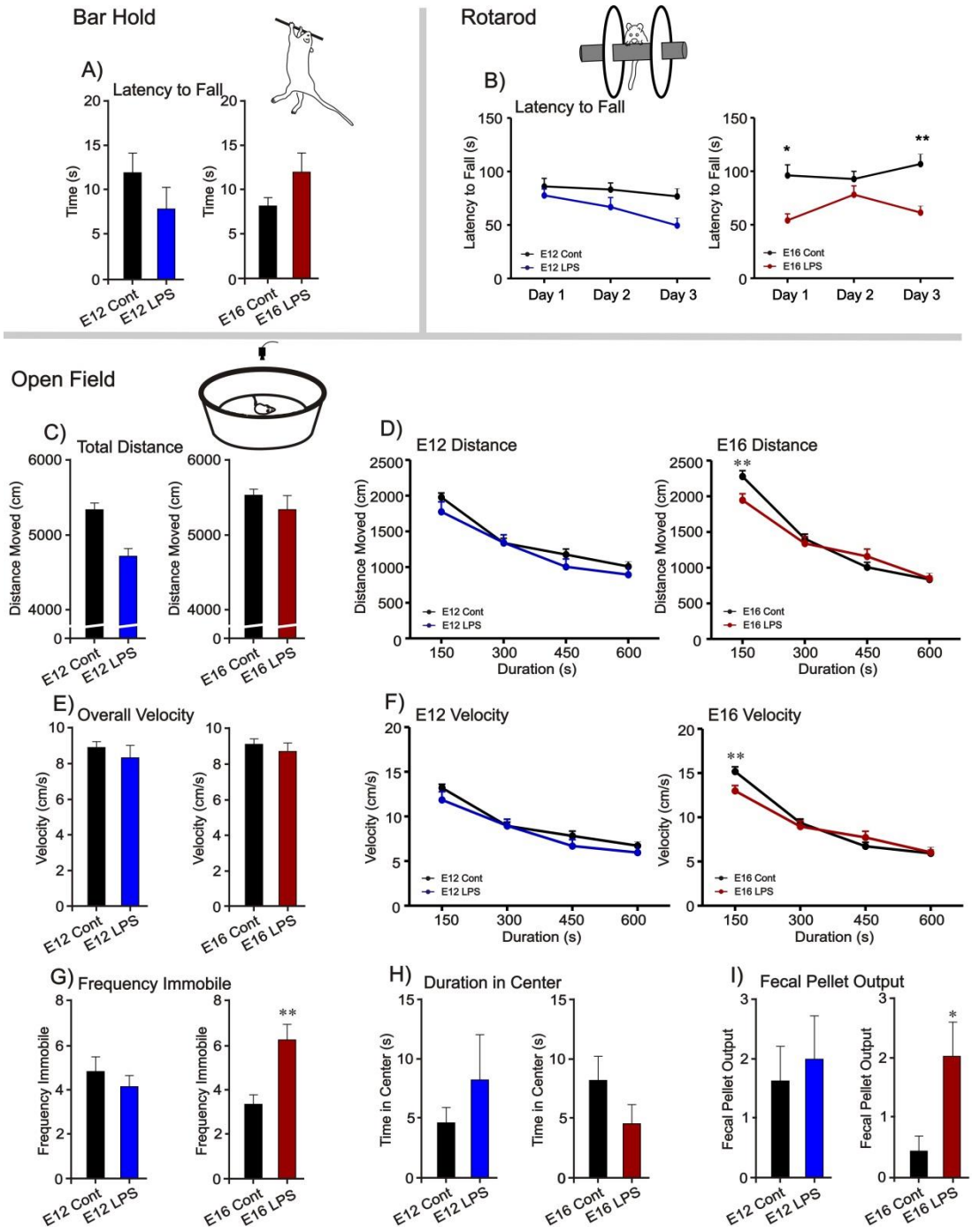


Figure 4: Age-related decline in motor function as a result of late gestation *in utero* LPS exposure.

(A) Bar hold test and (B) latency to fall from an accelerating rotarod that were performed after P60 for offspring that were exposed *in utero* to saline (Cont) or LPS at E12 (blue bars) or E16 (red bars) as indicated. (C-I) Open field behavioral assessment was performed after P60 as indicated. (C) The total distance moved, (D) distance moved at four 150s intervals, (E) velocity, (F) velocity moved at four 150s intervals, (G) Frequency Immobile, (H) total time spent in the center of the arena, and (I) fecal pellet output during the open field. Data expressed as mean +SEM (n = 9-11 rats per group; * = P <0.05; ** = P <0.01; *in utero* exposed LPS group compared to relative saline control group).

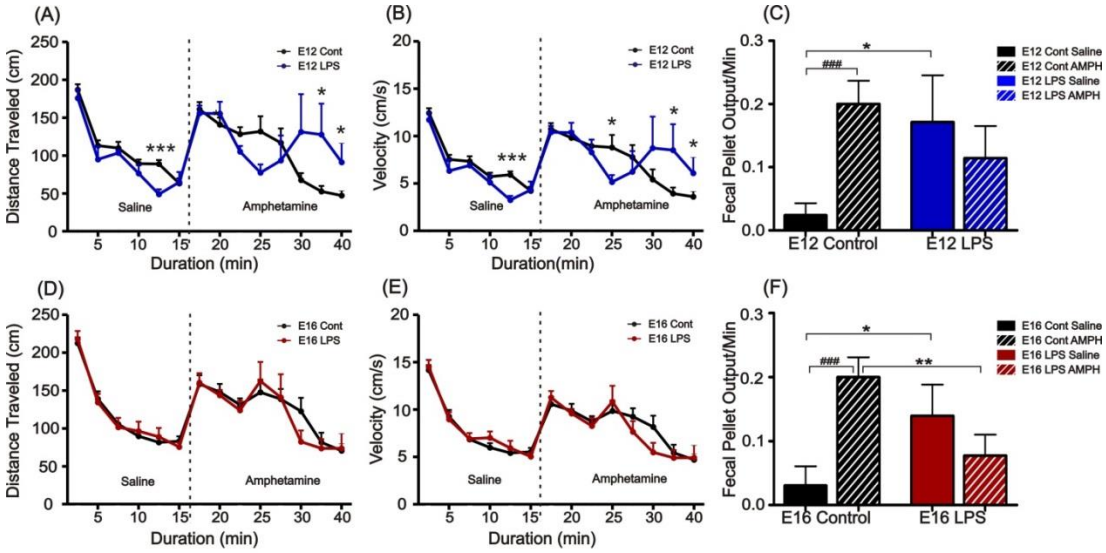


Figure 5: MIA at E12 results in differential response to AMPH in open field test of adult offspring

Open field behavioral assessment with amphetamine challenge was performed on adult offspring that were exposed *in utero* to saline (Cont) or LPS at (A-C) E12 (blue bars) or (E-F) E16 (red bars). The total distance moved (A,D), velocity (B,E), and fecal pellet output (C,F) are indicated. Data expressed as mean +SEM (n = 6-10 rats per group; *P < 0.05, **P < 0.01 *in utero* exposed LPS group compared to relative saline control group; ### = P < 0.001 OF saline trial compared to OF AMPH trial within same group)

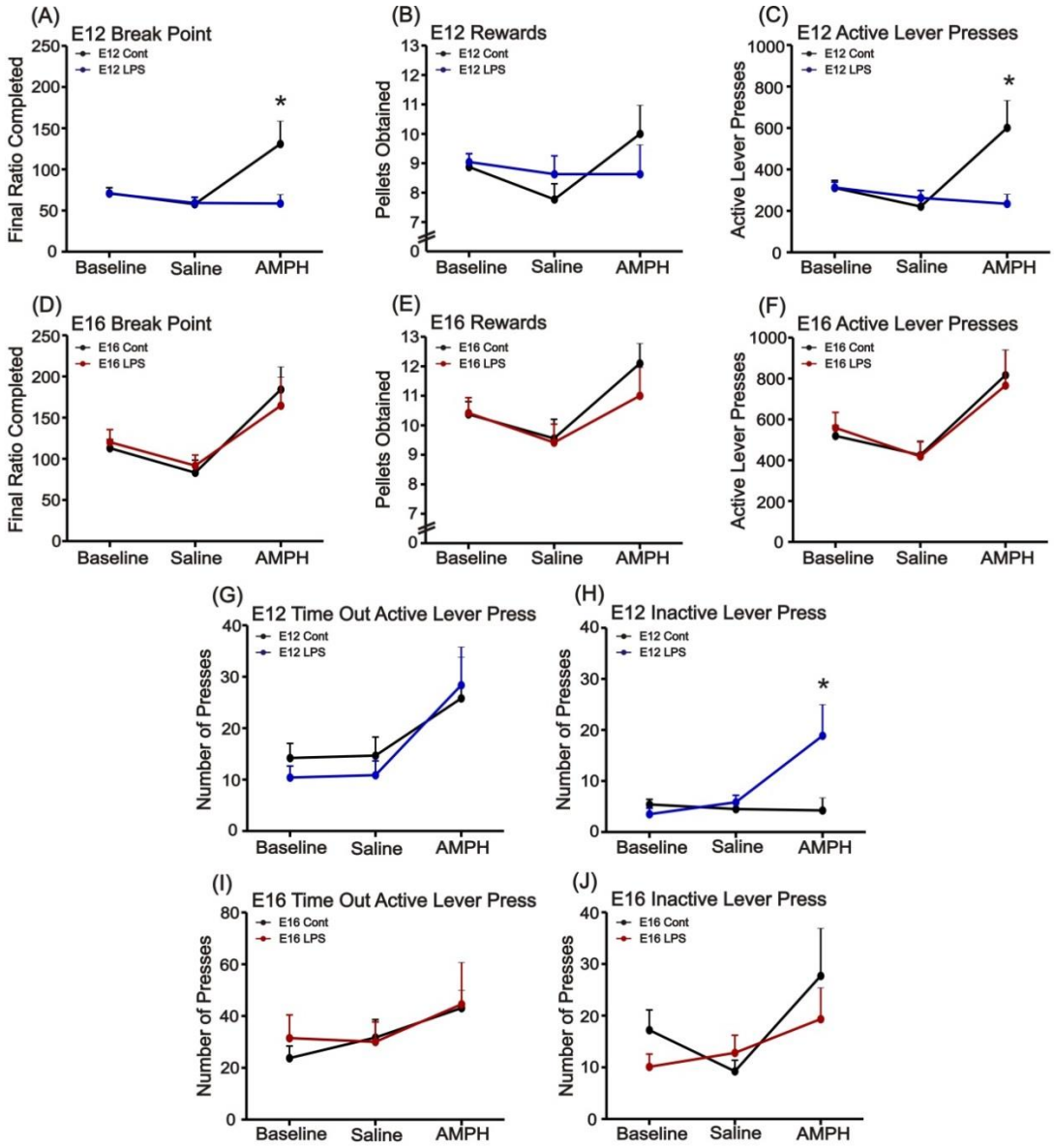


Figure 6: Altered amphetamine response in goal directed and impulsivity behaviors of adolescent offspring following maternal LPS at E12

Motivation behavior was assessed using an operant conditioning task with food restricted animals in adolescence offspring following exposure to MIA or saline *in utero* at either E12 (Blue Bars) or E16 (Red Bars). Goal directed behavior for animals exposed at (A-C) E12 or (D-F) E16 as measured by (A,D) Break point, (B,E) Rewards Obtained, and (C,F) Active Lever presses. Impulsive behavior for offspring exposed at (G-H) E12 or (I-J) E16 as measured by (G,I) Time Out Active Lever Presses and (H,J) Inactive Lever Presses. Amphetamine dose = 0.25 mg/kg. Data expressed as mean +SEM (n=7-13 per group; * = $P < 0.05$).

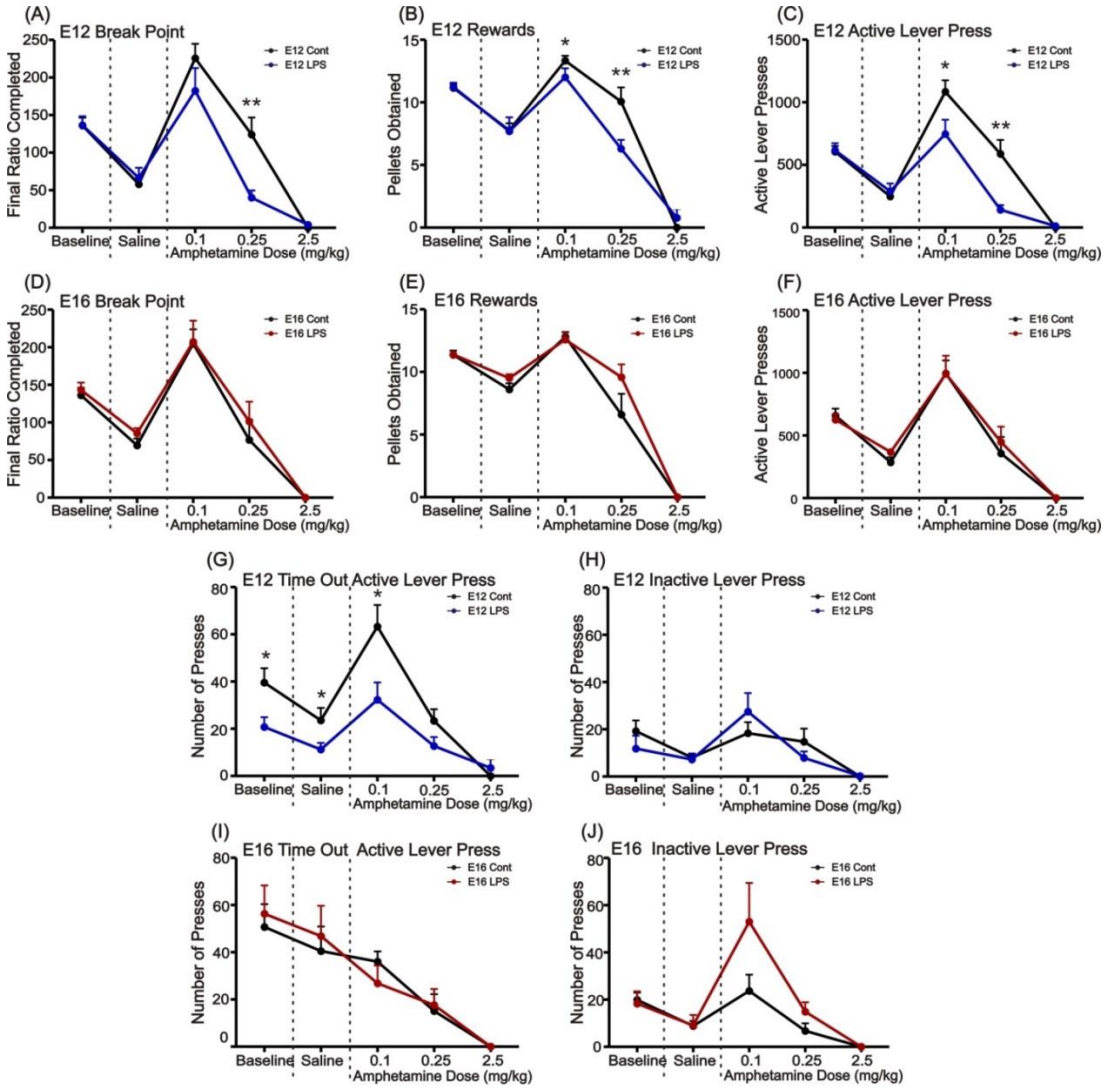


Figure 7: Maternal LPS at E12 results in reduced responsiveness to amphetamine in food reward paradigm, altering reward seeking behavior of adult offspring injected with AMPH

An operant conditioning paradigm utilizing food reward with systemic amphetamine challenges was used to assess motivation behavior in adult offspring who had exposure to systemic maternal LPS or saline at either E12 (Blue Bars) or E16 (Red Bars). Goal directed behavior for animals exposed at (A-C) E12 or (D-F) E16 as measured by (A,D) Break point, (B,E) Rewards Obtained, and (C,F) Active Lever presses. Impulsive behavior for offspring exposed at (G-H) E12 or (I-J) E16 as measured by (G,I) Time Out Active Lever Presses and (H,J) Inactive Lever Presses. Data expressed as mean +SEM (n=7-13 per group; * = $P < 0.05$, ** = $P < 0.01$).

3.5 Discussion

The two major dopaminergic pathways begin to develop at E12 in the rat as midbrain dopaminergic neurons extend projections that reach the striatum to form the nigrostriatal pathway, and the prefrontal cortex and nucleus accumbens to form the mesocorticolimbic pathway (Tzschentke & Schmidt, 2000; Janhunen & Ahtee, 2007; Hegarty *et al.*, 2013). This work investigated the impact of MIA as a result of *in utero* exposure to LPS at E12 (earlier) or at E16 (later) on relevant behavior mediated by these circuits in affected offspring during three stages of postnatal life. We used a number of behavioral tests associated with motor and motivation/reward behavior when the rats reached different ages including P9, P30, and P60+ in order to determine the effects of MIA on the dopaminergic system of the offspring across the lifespan. The overall results of this study demonstrate that MIA at different time points during pregnancy differentially affects behavioral outcome, with exposure at E12 altering the response to amphetamine in motivation/reward behavior of adolescent or adult offspring, and LPS exposure at E16 affecting motor behaviors in juvenile and adult offspring.

In agreement with a previous study, which administered a 100μg/kg dose of LPS at E11 we found no differences in maternal care as a result of *in utero* LPS exposure (Connors *et al.*, 2014). Therefore it is important to note that any behavioral changes seen in offspring are not due to differences in maternal care, which is a known determinant of the neurobehavioral outcome of neonates (Weaver *et al.*, 2006).

Due to the fact that weak primitive reflexes are indicative of poor neuro-development (Zafeiriou, 2004), and an MIA study reported delays in several neurological reflexes following an *in utero* exposure to a high dose of LPS (300μg/kg) at E19-20 in the rat (Rousset *et al.*, 2013), we next examined primitive reflexes including righting reflex, cliff test, and grasp reflex at P9 in our experimental paradigm. We found no differences in these reflexes, which is consistent with previous findings that have also shown no effect on pup reflex development following *in utero* exposure to LPS (120μg/kg) at E17 in mice (Golan *et al.*, 2005), at E9.5 in rats (100μg/kg) (Kirsten *et al.*, 2010a), and also in rats exposed to LPS (200μg/kg) at E10-15 and P5-10 (Xu *et al.*, 2013). These data

suggest that MIA, in numerous animal models, does not adversely affect overall CNS integrity in affected offspring.

We next found impaired performance in the bar hold test as a result of *in utero* LPS exposure at both gestational ages, in affected offspring at P9. In agreement with this, *in utero* exposure to 300μg/kg LPS on E19 and 20 in rats was found to decrease motor coordination in affected juvenile offspring (Rousset *et al.*, 2013). Consistent with this, offspring exposure to maternal LPS at E16 had impaired locomotion behavior in the open field test, in contrast to those exposed at E12, although these differences were no longer apparent by P30. In agreement with this, others have reported no difference in juvenile rat offspring motor behavior (measured at P21), following MIA (100μg/kg LPS) in mid-gestation (E9.5) (Kirsten *et al.*, 2010a). This is consistent with our data from the E12 cohort, suggesting that this period is not a vulnerable window during which MIA affects the nigrostriatal pathway. Also consistent with our findings, is a previous study showing a decrease in rat juvenile offspring motor performance from birth up to 3 weeks following exposure to 300μg/kg of LPS administered to pregnant dams at later pregnancy (E19 and 20) (Rousset *et al.*, 2013). Furthermore, dams administered 50μg/kg LPS on E15 and E16 had offspring with no differences in rotarod performance at P35 (Harvey & Boksa, 2014). Taken together, these data indicate a critical window of later pregnancy during which MIA affects the motor behavior of young offspring, an impairment which is subsequently compensated for by adolescence.

By the time offspring reached adulthood, we observed an age-related decline in complex motor functioning in the rotarod test in the group that was exposed to LPS at E16. Along similar lines, our data demonstrated an initial decrease in locomotor activity in the open field for the E16 LPS group, which attenuated after 5 minutes. Previous work has shown a greater decline in motor skills including diminished rotarod performance and a more robust decline in spontaneous locomotor activity in offspring following a much higher dose of LPS (200ug/kg) administered every 12 h from E17 to birth (Stigger *et al.*, 2013). These results suggest a potential dose response associated with *in utero* LPS exposure whereby the degree of MIA can be related to extent of the neurobehavioral impairment in affected offspring later in life. Our data also demonstrated an increase in frequency immobile, or frequency freezing in the group exposed to *in utero* LPS at E16 only. Previous findings have

demonstrated increased immobility following MIA via LPS (500µg/kg on E17) in mouse offspring (Babri *et al.*, 2014) as well as in rat offspring (66µg/kg on E10.5) (Lin & Wang, 2014) subjected to the forced swim test, however immobility frequency following MIA has not been reported in the open field test until now. As immobility is a hallmark symptom of PD, a disease associated with a degeneration of dopaminergic neurons and subsequent functional decline of the nigrostriatal system (Xia & Mao, 2012; Winner & Winkler, 2015), these results taken together indicate that MIA at E16 may lead to adverse outcomes in offspring motor behavior later in life, which I discuss further in the final discussion.

In the current study, we also conducted an amphetamine challenge in the open field to assess overall dopaminergic functioning, as amphetamine leads to increases in extracellular dopamine in numerous brain regions and pathways (Di Chiara & Imperato, 1988; Heuer *et al.*, 2012; Bardo, 2013). Our results demonstrate that MIA alters amphetamine response with regards to locomotion as seen by altered distance and velocity in the E12 LPS group, compared to the E12 control group. We saw an initial decrease in locomotion following AMPH administration, consistent with some literature which has demonstrated decreased sensitivity to the locomotor enhancing effects of amphetamine in adult rat offspring following MIA (8mg/kg Poly-I:C at E14) (Bronson *et al.*, 2011; Richtand *et al.*, 2011). Conversely, in the second half of the AMPH trial we saw a significant increase in locomotion behavior, which is similar to previous studies that have shown an exaggerated hyperactivity response to amphetamine following exposure to acute Poly-I:C (8mg/kg on E14 (Vorhees *et al.*, 2012) or 4.0mg/kg on E15 (Zuckerman & Weiner, 2005; Piontkewitz *et al.*, 2011b)) or chronic Poly-I:C (E14-18, 8mg/kg/day) (Vorhees *et al.*, 2015) in rats. Importantly, many of these studies assessed the locomotion response to amphetamine for a longer duration than the present study, suggesting that a long testing period may provide more comprehensive insight into the effects of MIA on AMPH-induced locomotion and future studies should take this into consideration. However, it is still clear from our data that the animals exposed to MIA at E12 responded to AMPH differently than the relevant control while the E16 cohort demonstrated no differences, indicating a possible critical window for differential responding to this indirect dopamine agonist.

We next analyzed anxiety behavior to investigate functionality of the mesocorticolimbic system in our MIA model, as previous work (using $Grin1^{\Delta/loxP}$;$Slc6a3^{crel+}$ knockout mice to alter dopamine neuron activation) has indicated a role for proper mesolimbic dopamine functioning in normal anxiety behavior (Zweifel *et al.*, 2011). We found a decrease in the duration of USVs in the post test following MIA at E16, but not E12. This is consistent with previous data which demonstrated reduced number and duration of USVs from P3 and P5 rat pups following *in utero* exposure to LPS (100µg/kg LPS at E15 and E16) (Baharnoori *et al.*, 2012). Impairment in the generation of USVs in isolation is an indicator of aversive affective states in young rodents (Hofer *et al.*, 2001). This suggests our E16 LPS group experienced increased feelings of anxiety, depression and/or anhedonia by the end of the test battery in comparison to the relative saline control. The USV tests in young rodents can predict anxiety response in adult age (Hofer *et al.*, 2001); this finding suggests that the E16 LPS group may have heightened anxiety later in life and therefore we assessed anxiety behaviors again at P30 and after P60.

Adolescent offspring exposed to MIA at E16 spent significantly more time in the center of the open field arena compared to E16 controls, and again there were no differences in the E12 cohort. Greater duration in the center of the open field arena is usually indicative of anxiolysis as the open field test exploits the natural aversion rats have to exposed areas (Prut & Belzung, 2003), which would suggest that the E16 LPS group demonstrated decreased anxiety behaviors compared to E16 control. In agreement with this, exposure to LPS in early life (10µg/kg every 24h P5-P8) has been shown to decrease anxiety in adolescence (Fan *et al.*, 2005), while administration of LPS to pregnant mice (50µg/kg on E15, E16 and E17) resulted in no significant differences in adolescent (P40) anxiety behavior (Enayati *et al.*, 2012). However, high LPS exposure *in utero* (300 and 500µg/kg on E16; 500µg/kg on E17) resulted in heightened levels of adolescent anxiety in the EMP (Enayati *et al.*, 2012). This suggests that the greater the MIA, the greater the resultant disturbances in adolescent anxiety behavior.

Previous literature that has shown that animals with low anxiety behavior have higher local dopamine release in the nucleus accumbens and this is thought to be associated with increased activation of the reward system (Beiderbeck *et al.*, 2012). To the best of our knowledge this is the first study that has examined the

effects of LPS specifically on offspring motivation and reward behaviors; however, it has been reported that maternal administration of Poly-I:C (4mg/kg, on E15) impaired cognitive flexibility, but did not affect motivational behaviors, in adult male offspring subjected to an operant conditioning task (Zhang *et al.*, 2012). Furthermore, maternal exposure to 3,4-methylenedioxy-N-methamphetamine (MDMA, 15mg/kg, twice daily from E14-E20) (which releases DA) did not affect food reward nor cocaine self-administration in adult offspring (Thompson *et al.*, 2009b) and maternal exposure to Poly-I:C (5mg/kg) on E17 resulted in anhedonic behavior as demonstrated by the sucrose preference test (Bitanihirwe *et al.*, 2010). In the present study, there were no major differences in baseline trials (in which animals did not receive amphetamine or saline injections), indicating that MIA does not affect normal baseline reward function in adolescent and adult offspring. However, amphetamine has been shown to affect reward processing in humans (O'Daly *et al.*, 2014) and rats (Wyvell & Berridge, 2000; Schmidt *et al.*, 2013), and previous findings demonstrate the ability of amphetamine to potentiate responding for a conditioned reward in a normal animal (Fletcher *et al.*, 1998). Interestingly, animals exposed to *in utero* LPS at E12 appear to escape the characteristic AMPH-induced increase in reward responding. Following AMPH injection in adolescence, when all other groups increased active lever presses, rewards obtained, and break point, the E12 LPS group remained at baseline levels. Furthermore, E12 LPS had increased number of inactive lever presses compared to the relative control group during the AMPH trial. Inactive lever presses are associated with non-reward seeking behavior (Robinson *et al.*, 2007), indicating the E12 LPS group showed increased impulsivity following AMPH injection. In adulthood, we observed the same decreased AMPH response in goal directed behavior with these animals (E12 LPS), which is consistent with previous studies which have showed that adult rat offspring exposed to high fat diet the last week of pregnancy displayed reduced sensitization to amphetamine and alterations to mesocorticolimbic system functionality (Naef *et al.*, 2008). Taken together, these data suggest that MIA has differential effects on amphetamine intervention in a reward paradigm depending on gestational age at which MIA occurs, with E12 as a potential critical age allowing for decreased AMPH sensitivity in adolescence and adulthood. An important implication of this finding concerns drug addiction. The DSM-V defines tolerance, or a diminished response to a drug that is repeatedly used, as a hallmark symptom of

addiction (APA, 2013), and therefore our findings of reduced sensitivity to AMPH following MIA at E12 may indicate a potential susceptibility for drug tolerance and subsequent abuse later in life, which is discussed fully in the final discussion.

In conclusion, our data show that timing of MIA is important determinant of neurobehavioral outcome in affected offspring whereby an infection earlier in gestation (E12) may have greater consequences for the mesocorticolimbic pathway whereas infection in later pregnancy (E16) may have greater consequences for the function of the nigrostriatal pathway.

Chapter 4:

Molecular and cellular effects of MIA on offspring midbrain development depend on gestational age of insult

Straley ME, Theze SA, Hegarty SV, Crampton SJ, Cyran JF, O'Mahony SM, O'Keeffe GW. LPS-induced maternal immune activation results in differential developmental and neurobehavioral outcomes depending on gestational age of exposure. ***In preparation*** *for submission to the Journal of Neuroscience.*

Abstract

Introduction

Maternal infection during pregnancy increases the risk several neurological and psychiatric disorders later in life, many of which have a component of dopaminergic (DA) dysfunction. In Chapter 3, we showed that behavioral deficits that emerge after MIA are dependent on when the insult occurs. In this study we sought to examine the molecular and cellular changes that occur in the midbrain of affected offspring that may lead to these behavioral deficits.

Methods

Pregnant-Sprague Dawley rats were administered saline or lipopolysaccharide (LPS) by intraperitoneal injection on embryonic day 12 or 16. We then examined a number of key inflammatory mediators, along with neurodevelopmental and cytoarchitectural characteristics at different stages of offspring development. In vitro studies of VM neurospheres and DA neuron growth were used to supplement in vivo findings.

Results

Our results show a differential inflammatory response in fetal midbrains depending on gestational age of exposure. LPS exposure at E16 resulted in decreased VM neurosphere size after 7DIV. This was associated with an increased susceptibility to neurotoxic effects of pro-inflammatory cytokines for both VM neurospheres and VM DA neurons treated in culture. *In utero* LPS exposure at later gestational ages also altered the cellular architecture of fetal VM stained for TH, a DA neuron marker. However, there were no differences in DA neuron number in juvenile, adolescent, or adult offspring. Similarly, LPS exposure did not alter cell number or morphology of glial cells in the midbrains of affected offspring.

Conclusions

Exposure to MIA in later pregnancy results in developmental alterations in the fetal VM, which may result from increased levels of pro-inflammatory cytokines at this developmental stage.

4.1 Introduction

Fetal programming is the idea that events during pregnancy influence developmental trajectories (Schlotz & Phillips, 2009). For example, epidemiological and preclinical evidence has associated prenatal inflammatory events with offspring susceptibility to neurological disorders later in life (Bell & Hallenbeck, 2002; Stolp & Dziegielewska, 2009). Parkinson's disease (PD) is an adult-onset neurological disorder which is highly associated with neuroinflammation because microglial activation (McGeer *et al.*, 1988; Imamura *et al.*, 2003) and elevated levels of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and IL-6 (Mogi *et al.*, 1994a; Mogi *et al.*, 1994b; Koziorowski *et al.*, 2012) are common characteristics of PD brains. Furthermore, it has been proposed that maternal immune activation (MIA) may prime microglia to maintain heightened inflammatory processes throughout life, which in turn may alter microglial response to cellular processes like protein aggregation or neuron death, subsequently resulting in progression of the α-synuclein aggregates into Lewy bodies that are the pathological hallmark of PD (reviewed in Glass *et al.*, 2010; Tomé *et al.*, 2013; Knuesel *et al.*, 2014). Hence, fetal programming as a result of MIA has the potential to lead to an increased susceptibility to PD later in life. PD results from the degeneration of dopaminergic (DA) neurons of the substantia nigra (SN) in the midbrain, cells which differentiate from neural progenitor cells (NPCs) in the ventral mesencephalon (VM) (Toulouse & Sullivan, 2008; Hegarty *et al.*, 2013). Furthermore, other disorders associated with an increased risk following MIA have characteristic components of dopaminergic (DA) dysfunction, including schizophrenia (Howes & Kapur, 2009; Howes *et al.*, 2012; Seeman, 2013) and ADHD (Levy, 1991; Swanson *et al.*, 2000; Volkow *et al.*, 2011). Indeed, MIA has been shown to induce numerous genetic, structural, and functional alterations to dopaminergic development in affected offspring (Meyer & Feldon, 2009; Meyer, 2013). As such, understanding the development of these cells within the context of MIA will help elucidate contributory factors of MIA processes to disease susceptibility in affected offspring.

To this end, inflammatory mediators have been investigated with results suggesting that they may exert a range of effects in the developing brain. They are known to have productive roles, and in fact IL-1β was sufficient to lead VM NPCs

toward DA specificity differentiation (Ling *et al.*, 1998; Potter *et al.*, 1999). Furthermore, depending on the concentration, IL-1β, IL-6, and TNF-α acted as either neurotoxic (at low-medium concentrations) or neurotrophic (at higher concentrations) toward fetal VM DA neurons (Meyer, 2013). Temporal profiles of neurodevelopmental stages also determine the potential effect of cytokines on the developing VM. For instance, in earlier development, TNF-α has been shown to enhance cultures of DA neurons (E12.5, mouse), whereas TNF-α inhibited DA neuron cultures isolated from E14 or E16 mouse VM (Doherty, 2007). Along these lines, it has been shown that offspring outcome is dependent upon the timing during pregnancy at which MIA occurs, and indeed animal models have identified "critical developmental windows" during which fetal neurodevelopment is more vulnerable to adverse effects of MIA (Andersen, 2003; Marco *et al.*, 2011). Importantly, these critical windows also depend on animal model, including immunogen and dose used (Boksa, 2010; Harvey & Boksa, 2012). In our MIA model, which consists of a single exposure to low dose of LPS in rats, superior cervical ganglion (SCG) neuron growth was inhibited by LPS exposure only in later stages of rat pregnancy (Straley *et al.*, 2014). This was associated with late gestational modifications to placental inflammatory factors and HSD11β2 (Straley *et al.*, 2014), which is a critical regulator for fetal development (Cottrell *et al.*, 2014; Togher *et al.*, 2014), implicating a role for placental function in neurodevelopment. Furthermore, in the previous chapter we demonstrated that negative behavioral outcomes in MIA exposed offspring depend on the timing during pregnancy at which the insult occurred. For instance, motor behavior deficits in juvenile offspring were observed following MIA in later pregnancy (E16), but not earlier (E12). Taken together, these findings suggest that the potential for MIA to adversely affect fetal VM and/or DA neuron development may be greatest during a later developmental window in our model.

In the current study, we use an LPS rat model of MIA (Straley *et al.*, 2014), to examine the impact of *in utero* exposure to MIA on fetal expression of pro-inflammatory cytokines as well as VM development and subsequent cellular architecture of offspring midbrains in the fetal stages, neonatal period, adolescence and adulthood. Rat VM DA NPCs peak in proliferation at E12 (Gates *et al.*, 2006), and differentiate toward the DA lineage which peaks from E13-E15 (Volpicelli *et*

al., 2004). Furthermore, the DA neurons begin to extend axons at E12/E13 to establish functional circuitry, with projections reaching the striatum by E14, and the cortex by E16 (Hegarty *et al.*, 2013). Therefore, we administered LPS on E12 or E16 to determine the impact of the timing of exposure to MIA on midbrain development in affected offspring.

4.2 Materials and Methods

4.2.1 LPS injections and sample collection

All experimental animal work was carried out under license with ethical approval from the Ethics Committee of UCC. Sprague-Dawley rats (Biological Services Unit, UCC) were maintained in a controlled environment on a 12-hour light/dark cycle with *ad libitum* access to food and water. As the timing of infection during pregnancy is often a critical determinant of fetal outcome, pregnant dams received an I.P. injection of LPS (50µg/kg) or normal saline on E12 or E16 for most experiments. The first experiment examined chronic vs acute exposure on cytokine profiles at P1 and so for these, pregnant rats were injected on E12, E14, E16, and E18 (chronic) or just on E18 (acute).

For embryonic developmental experiments, 48 h post injection, dams were anesthetized with inhaled Isoflurane (Abbeyville Veterinary hospital, Cork) and culled by decapitation. Embryos were removed via laparotomy, and washed in ice-cold Hank's Balanced Salt Solution (HBSS). Embryos were removed from the yolk sac and transferred to fresh HBSS. The litter was divided so that some embryos were fixed in 4% PFA for 24 h, then cryoprotected for 48h, and other embryos were used for obtaining VM samples via micro-dissection as previously described (Long-Smith *et al.*, 2010). Briefly, embryos were kept in HBSS on ice until transferred to another petri dish with HBSS for VM micro-dissection. The mesencephalic region was dissected out of the fetal brain and then dissection needles were used to cut along the dorsal midline to open out the tissue. Then the lateral edges were trimmed to remove the dorsal mesencephalon. Finally, the tissue was cleaned of the meninges. Half of the VM samples were immediately used for cell culture experiments and the other half were snap frozen for PCR.

For postnatal studies, offspring were divided and culled at different ages in order to analyze brains of juveniles (P11), adolescents (P32), and adults (>P60). The whole brains of the offspring were immersed fixed in 4% paraformaldehyde for 24h at 4˚C. They were subsequently transferred to 30% sucrose for cryoprotection and incubated at 4˚C. After a minimum of 48 hours in this 30% sucrose, the brains were snap-frozen in isopentane that had been pre-cooled in liquid nitrogen, and stored at -80˚C to await cyrosectioning.

Sample embryos/ brains were derived from four different mothers in each treatment condition (LPS or vehicle) and sampling interval (E12 or E16). This served to minimize potential confounds resulting from litter effects (Zorrilla, 1997), and to rule out the possibility that putative differences between LPS and vehicle-exposed fetuses may stem from individual differences in prenatal development.

4.2.2 Real-time PCR

A motorized pestle and mortar were used to disrupt the VM samples and total RNA was extracted using an RNeasy tissue mini kit (Qiagen). RNA concentration was determined using a Spectrophotometer (NanoDrop Technologies). 500ng of RNA was reverse transcribed using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 20µl reaction mixture consisting of 2.0µl 10X RT Buffer, 0.8µl 25X dNTP mix (100mM), 2.0µl 10X RT Random Primers, 1.0µl Reverse Transcriptase, and 4.2µl Nuclease-free H_2O, using the following parameters: 25°C for 10 min; 37°C for 120 min; 85°C for 5 min; 4°C for at least 10 minutes. The cDNA was stored at -80°C prior to use. For real time PCR, all samples were run in triplicate, with the reaction mix for a single reaction consisting of 1.0 µl 20X TaqMan® Gene Expression Assays (Applied Biosystems), 10 µl 2X TaqMan® Gene Expression Master Mix (Applied Biosystems), and 8.0 µl RNase-free H2O (Applied Biosystems). 19 µl of the mix was added to each well of a 96 well PCR reaction plate (Applied Biosystems) as well as 1.0 µl of the relevant cDNA. The plate was centrifuged briefly and then loaded into the qRT-PCR system (StepOne Real-Time PCRY System, Applied Biosystems) and run using the following cycling parameters; 50°C for 2 minutes; 95°C for 10 minutes; and 50 repetitions of 95°C for 15 seconds and 60°C for 1 min. Analysis was carried out using the 2-δCT method (Livak & Schmittgen, 2001).

4.2.3 Cell culture

For the preparation of rat VM cultures, embryos were obtained by laparotomy from date-mated female Sprague-Dawley rats following decapitation under terminal anesthesia induced by the inhalation of isoflurane. The first experiment consisted of neurosphere assays using cells isolated from VM of embryos 2 days post exposure to LPS (exposure occurred on E12 or E16). The second investigation utilized VM cells

from E12 or E16 embryos of naive animals which were treated with pro-inflammatory cytokines as outlined below. Cultures of VM NPCs were prepared as previously described (O'Keeffe and Sullivan 2005). The cells were seeded in T-25 culture flasks (Sarstedt) at a density of $2x10^6$ cells per flask in 10 ml of DMEM/F12, 1% L-Glutamine (Sigma) supplemented with 2% B27 (Invitrogen), 20 ng/ml epidermal growth factor (EGF; SIGMA) and 20ng/ml of basic fibroblast growth factor (bFGF; Sigma). Neurospheres were allowed to proliferate for 2, 4 or 7 DIV in a humidified atmosphere containing 5% CO_2 at 37˚C, and half of the media was replaced every second day. In the second experiment, neurospheres grown from the E12 and E16 VM of naïve animals were treated with IL-1β, TNF-α and IL-6 (Promokine) every 2DIV for 6days. Cytokine treatments were all at 10ng/ml, which was previously shown to affect NPCs in culture (Crampton *et al.*, 2012).

For primary cultures of VM DA neuron cultures, dissected VM tissue was centrifuged at 1100rpm for 5min at room temperature. The tissue pellet was incubated in a 0.1% trypsin-Hank's Balanced Salt solution for 5min, at 37˚C with 5% CO_2. Fetal calf serum was then added to the tissue, which was then carefully triturated using a sterile plugged flame-polished Pasteur pipette, followed by a 25-gauge needle and syringe. The VM cells were centrifuged at 1100 rpm for 5min. The resulting cell pellet was re-suspended in 1ml of differentiation media (Dulbecco's modified Eagle's medium/F12, 33mM D-glucose, 1% L-glutamine, 1% FCS, supplemented with 2% B27) using a P1000 Gilson pipette, and cell density was then estimated using a haemocytometer. Cells were plated on poly-D-lysine (Sigma)-coated 24-well tissue culture plates at a density of 5×10^4 cells per well in 500µl of differentiation media at 37˚C with 5% CO_2. E14 and E18 VM cell cultures were treated with 100ng/ml of IL-1β or 10ng/ml of TNF-α daily for 4DIV, and these doses were chosen as they were shown to affect neurite growth in a previous publication (Nolan *et al.*, 2011).

4.2.4 Analysis of neuronal complexity

The neurite length and branching of individual E14 and E18 VM DA neurons was measured at 4DIV using Sholl analysis as previously described (Gutierrez & Davies, 2007; Nolan *et al.*, 2011; Straley *et al.*, 2014). Briefly, neurite length (NL) was calculated with the following formula; $NL = \alpha \times T \times (\pi/2)$, where α is the number of

times the neurite intersects the grid lines, and T is the distance between the gridlines on the magnified image (taking into account the magnification factor). VM DA neurons with intact processes were analyzed from 20 random fields per condition, where a process that was at least 1.5 times the length of the neuron soma was defined as an intact process (which precludes analysis of apoptotic neurons (Hegarty *et al.*, 2014)). This is based on previous papers demonstrating that the average size of DA neuron soma is 10-20μm and the average length of a primary neurite is 85±10μm (Rayport *et al.*, 1992; O'Keeffe *et al.*, 2004), suggesting that our parameters are able to account for somatic shape and neurite length diversity as well as exclude cells that have initiated the axonal retraction process associated with apoptosis (Collins *et al.*, 2013).

4.2.5 Immunohistochemistry (embryonic samples)

Following LPS exposure at either E12 or E16, rat embryo brains were dissected at E14 and E18 respectively. The whole brains were placed in 4% paraformaldehyde for overnight post-fixation. They were subsequently transferred to 30% sucrose for cryoprotection and incubated at 4˚C. After a minimum of 48 hours equilibration in this 30% sucrose, the brains were snap-frozen in isopentane that had been pre-cooled in liquid nitrogen, and stored at -80˚C to await cyrosectioning. Coronal sections (20μm) of fixed embryonic brains were cut using a cryostat (Leica M1900) and collected as a 1:6 series and subsequently used for immunohistochemistry. To block endogenous peroxidase, sections were treated with 10% H_2O_2 for 5 min, washed in 10mM PBS and blocked for 1 h in 10% normal goat serum in 10mM PBS with 0.4% Triton-X. Sections were then incubated in primary antibody to TH (1:1000; mouse monoclonal; Millipore) in 1% normal goat serum in 10mM PBS with 0.4% Triton-X overnight at 4°C. Following a 3 x 10 min wash in 10mM PBS, sections were incubated with a biotinylated secondary antibody (1:200, goat anti rabbit, Vector Labs) for 2 h at room temperature. Following another 3 x 10 min wash in 10mM PBS, sections were incubated in ABC solution (1:200; Vector Labs) for 45 minutes at room temperature followed by immersion in diaminobenzidine substrate/chromogen reagent for 2-3 min at room temperature. Sections were dehydrated, cleared, coverslipped, and allowed to air dry overnight, and then they were imaged using an Olympus AX70 Provis upright microscope. Negative controls

received the same treatment except PBS was substituted for the primary antibody solution. To estimate the cellular architecture of VM regarding DA neurons, the relative number of TH-positive VM cells were expressed as the total number of the cells counted per section, and three sections per embryonic brain were analyzed, similarly to previous work (Nakao & Brundin, 1996).

4.2.6 Immunohistochemistry (postnatal samples)

For postnatal immunohistochemistry, a slightly different protocol was used. Coronal sections of fixed brains were cut using a cryostat (Leica M1900) at 20μm thickness in 1:6 series, dried at room temperature and stored at −80 °C until immunohistochemistry was performed, which has been used previously when assessing the numbers of DA neurons in the postnatal midbrain (Sullivan *et al.*, 1997; O'Sullivan *et al.*, 2010; Costello *et al.*, 2012). For immunostaining, the slices were thawed in incubator for 1hr and rehydrated in 10mM Phosphate Buffered Saline (PBS) for 5 min. They were then blocked for 40 mins in 10% normal serum (normal goat serum for TH staining and normal donkey serum for GFAP/Iba1 staining) in 10mM PBS with 0.4% Triton-X. Sections were then incubated with primary antibody (TH, 1:1500, Millipore; or co-staining with both: GFAP, 1:1000, Dako; and Iba1, 1:1000, Abcam) in 2.5% normal serum in 10 mM PBS with 0.4% Triton-X overnight at -4°C. Following a 3 x 5 min wash in 10 mM PBS, sections were incubated with secondary antibody (TH = Alexa Fluor 488 anti-mouse, 1:500, Invitrogen; or co-staining with both: GFAP = Alexa Fluor 594 anti-rabbit, 1:500, Invitrogen; Iba1 = Alexa Fluor 488 anti-goat, 1:250, Invitrogen) in 2.5% normal goat serum in 10mM PBS with 0.4% Triton-X for 2 h at room temperature. Following another 3 x 5 min wash in 10 mM PBS, sections were incubated with bisbenzimide (1:3000) for 4 min at room temperature. Following 1 more 5 min wash, slides were cover-slipped and allowed to dry for at least 24 hours. Once dry, sections were imaged using an Olympus AX70 Provis upright microscope. Negative controls received the same treatment except PBS was substituted for the primary antibody. For the postnatal immunohistochemistry, 1 out of every 6 sections was stained for TH or GFAP and Iba1. Then, we chose different levels throughout the midbrain relative to bregma (AP-5.2, -5.6, -6.0, -6.4) to examine, similarly to previous studies (Nakao & Brundin, 1996; O'Sullivan *et al.*, 2010). Representative images were

analyzed using Image J software to compare relative numbers of TH, GFAP, and Iba1-positive cells, reported as count/ area (μm^2). As we had determined differential behavioral outcomes associated with either the nigrostriatal pathway or the mesocorticolimbic pathway in the previous chapter, for the P60 group we also sub-divided the analysis by specific midbrain region, either SN or VTA using anatomical landmarks as previously described (Nakao & Brundin, 1996).

4.3 Results

4.3.1 Effect of maternal LPS exposure on postnatal cytokine profiles

MIA is known to alter the expression of numerous cytokines in the fetal brain (Urakubo *et al.*, 2001; Meyer *et al.*, 2005; Arrode-Brusés & Brusés, 2012). Hence, we initially investigated whether LPS exposure *in utero* would induce changes in mRNA expression of pro-inflammatory cytokines in the midbrain of affected offspring that are commonly associated with MIA. Chronic LPS exposure (LPS on E12, E14, E16, and E18), acting as a positive control, resulted in significant decreases of IL-6, IL-1β, and TNF-α mRNA levels at P1 (Fig 1A, B, C). I.P. administration of LPS to the pregnant dams only on E18 resulted in less robust effects, with decreases in IL-1β and TNF-α mRNA expression (Fig 1B, C), but no difference in IL-6 mRNA levels (Fig 1 A) in the P1 midbrain.

4.3.2 Differential effect of MIA on developing VM depending on gestational age

Given that MIA induced different levels of cytokine mRNA in postnatal midbrain samples from affected offspring (Fig. 1), we next examined if the inflammatory modulators affected VM development, as we have previously shown that inflammation impairs the proliferation of rat NPCs from the E14 rat VM (Crampton *et al.*, 2012). Firstly, we cultured VM NPC neurospheres that were isolated from embryos that were exposed to *in utero* saline (control) or LPS on either E12 or E16; VM samples were isolated 48h post MIA. Neurosphere area of the E12 (Fig 2A) and E16 (Fig 2B) LPS exposed groups were statistically comparable to the relative saline control group at 2DIV. By 7DIV, however, the area of VM NPC neurospheres isolated from the E16 LPS group (Fig 2B) were significantly reduced compared to the relative saline controls, whereas the E12 groups (Fig 2A) remained similar in area.

To determine if the differential alterations in neurosphere size over time in vitro were related to exposure to inflammatory cytokines, we next investigated the mRNA expression of cytokines in the midbrains from embryos 48h after LPS exposure at E12 or at E16. I.P. administration of LPS to pregnant dams on E12 resulted in decreased levels of IL-6 (Fig 2D) and increased levels of IL-1β (Fig 2E), whereas there were no difference in TNF-α (Fig 2F) mRNA expression for the MIA exposed group compared to control. LPS administered to pregnant rats on E16

resulted in increased mRNA expression of IL-1β (Fig 2E) and TNF-α (Fig 2F) as well as a trend toward an increase in IL-6 mRNA expression (Fig 2D).

As there were different cytokine profiles depending on gestational age of LPS exposure (Fig 2D-F), and this was in line with the difference in neurosphere growth following *in utero* LPS exposure at different gestational ages (Fig 2A, B), we next wanted to determine which specific cytokines alter the development of VM NPC neurospheres. Neurospheres grown from E12 VM were reduced only when grown in the presence of TNF-α, but the diameter of these spheres was not affected by either IL-1β or IL-6 (Fig 2G). In contrast, the diameter of neurospheres established from E16 VM was significantly reduced when grown in the presence of all three cytokines: IL-1β, TNF-α, and IL-6 (Fig 2H).

4.3.3 Differential sensitivity of VM DA neurons to inflammation

Given that we have previously shown SCG neuron complexity is affected by *in utero* LPS exposure at E16, but not E12 (Straley *et al.*, 2014), and that maternal administration of LPS on E16 decreased VM neurosphere area at 7DIV (Fig 2), we next sought to determine the effects of cytokines specifically on VM DA neurons. To this end, we applied Sholl analysis, which illustrates neurite length and branching with distance from the cell body, to differentiated VM DA neurons which had been treated in culture with pro-inflammatory cytokines. When E18 VM DA neurons were grown in the presence of TNF-α, we observed a decrease in DA neuron branching (Fig 3E) and neurite length (Fig 3F), whereas IL-1β treatment did not result in significant alterations to neuronal complexity (Fig 3E,F). The growth and complexity of E14 VM DA neurons was not altered following 4 day cytokine treatments (Fig 3 A-C).

4.3.4 Effects of MIA on DA neuron cytoarchitecture

Because cytokine mRNA levels were increased in the midbrains of offspring exposed to LPS in later pregnancy (Fig 2), and because TNF-α treatment inhibited the growth of differentiated DA neurons cultured from the same age (Fig 3), we next investigated the number of TH-positive cells in midbrain sections of offspring exposed to saline (control) or LPS on E12 of E16. LPS exposure on E16 reduced the total number of TH-positive DA neurons counted in the fetal midbrain 2 days later

(Fig 4C), whereas LPS at the earlier time point (E12) did not result in differences in TH cell count in embryonic midbrain sections (Fig 4A). Subsequently, midbrain sections were stained for TH at three other time points throughout the offspring lifetime, providing cytoarchitecture data for juvenile (Fig 4E-H), adolescent (Fig 4I-L), and adult (Fig 4M-P) offspring following MIA exposure *in utero*. There were no differences in TH count/ area at any of these ages, comparing the LPS exposed group to relevant saline, age-matched control. Importantly, because of the differential behavior characteristics observed in adulthood, we also analyzed TH cell count specifically in the SN and VTA. When we compared the LPS exposed groups to their relative saline age-matched controls, there were no significant differences in TH positive cell count/area for either the SN or the VTA (data not shown).

4.3.5 Effects of MIA on glial cytoarchitecture

Maternal infection increases the risk of a number of neuropsychological disorders which include symptoms of altered immune functioning including ASD (Onore *et al.*, 2012) and schizophrenia (Strous & Shoenfeld, 2006; Richard & Brahm, 2012). Furthermore, MIA has been shown to alter the numbers and morphological characteristics of glial cells in the brains of affected offspring (Boksa, 2010; Harvey & Boksa, 2012; Meyer, 2014). Similarly, *in vitro* studies have shown that IL-1β, which is increased in the brains of MIA offspring (Urakubo *et al.*, 2001; Zaretsky *et al.*, 2004), alters the differentiation of VM NPCs toward that of glial cell lineage, as opposed to neuronal (Crampton *et al.*, 2012). Therefore, we next examined the impact of MIA on glial development in the midbrains of juvenile (Fig 5 A, B) and adolescent (Fig 5 C, D) offspring exposed to saline (control) or LPS *in utero* at different gestational ages. Numbers of astrocytes, as indicated through GFAP staining, were not altered by LPS exposure at E12, nor E16 when analyzed at juvenile (Fig 5A) or adolescent (Fig 5C) ages. Similarly, numbers of microglia, identified using Iba1 as a marker, in midbrains of control and LPS groups were statistically similar at both ages, for both E12 and E16 cohorts (Fig 5B, D).

4.3.6 Effects of MIA on microglial phenotype

Changes in microglia morphology have been observed following MIA, including increased ratio of active to ramified microglia in affected offspring (Knuesel *et al.*,

2014; Perry & Holmes, 2014). Therefore, even though we did not see a change in overall microglial cell counts following MIA (Fig 5), it was important to determine if exposure to LPS *in utero* altered the morphology of microglial cells in affected offspring midbrains. We again analyzed the Iba1 stained midbrain sections from offspring at juvenile (Fig 6 A, D) and adolescent ages (Fig 6 B, E) following maternal administration of saline (control) or LPS on E12 or E16. Offspring that had been exposed to LPS at the later age (E16) demonstrated a trend toward a decrease in the number of ramified microglial cells (Fig 6A), but had similar numbers to that of the control group in amoeboid numbers (Fig 6D) at the juvenile age. Inversely, the same group demonstrated a trend toward a decrease in amoeboid numbers (Fig 6E), and no difference in ramified counts (Fig 6B) when analyzed at the adolescent age. Exposure to LPS at E12 resulted in no differences in numbers of ramified or amoeboid cells at either offspring age (Fig 6).

4.4 Figures and Figure Legends

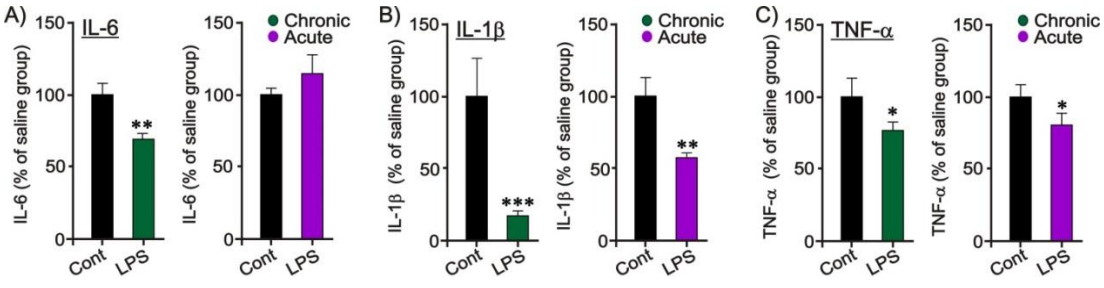


Figure 1: Postnatal cytokine profiles comparing chronic versus acute LPS exposure *in vivo.*

(A-C) Quantitative real-time PCR examining the expression of (A) IL-6, (B) IL-1β, and (C) TNF-α mRNA in the postnatal (P1) VM, following *in utero* exposure to saline (Cont) or LPS chronically (i.e. on E12, E14, E16, and E18; green bars) or acutely (on E18 only; purple bars) as indicated. Data are expressed as mean ±SEM (N=4/group/time point). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired Student's t-test.

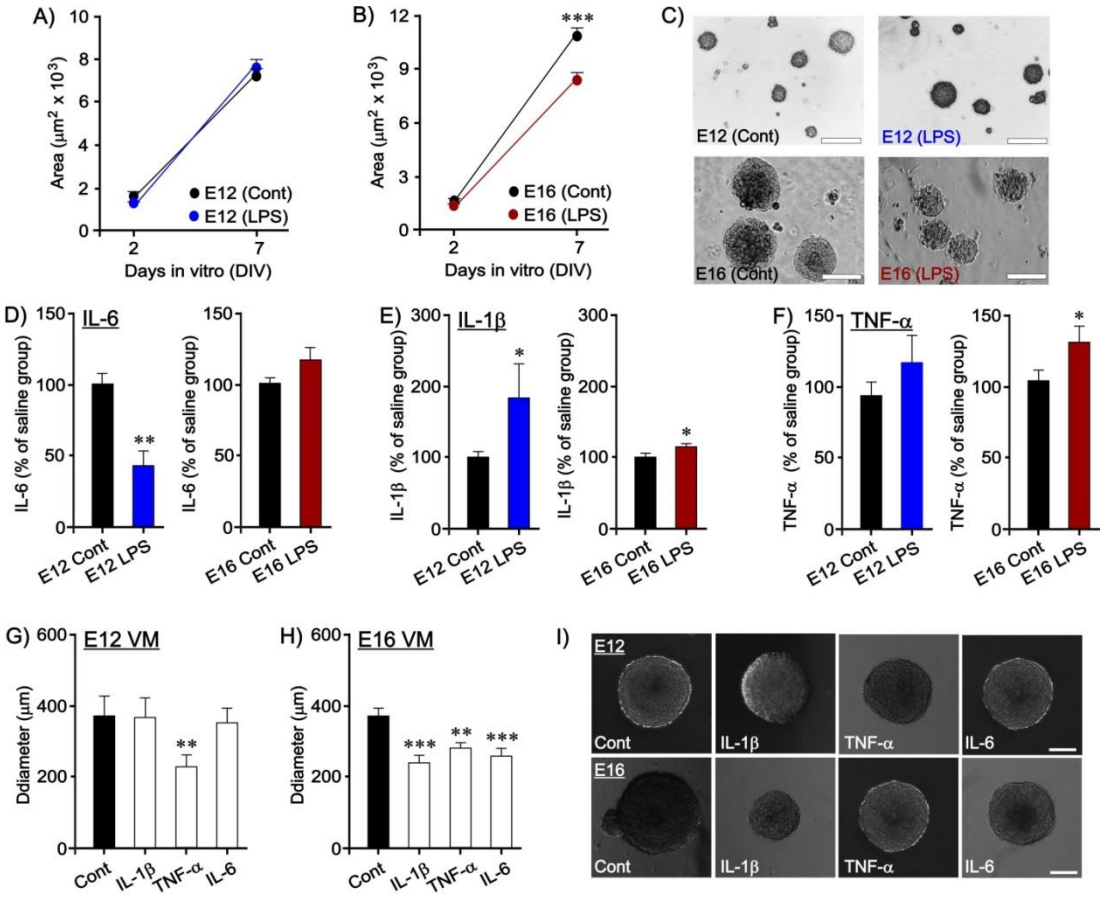


Figure 2: Differential sensitivity of the developing ventral midbrain to inflammatory cytokines.

(A-C) A neurosphere assay was carried out using cells isolated from the ventral midbrain (VM) of embryos 48h after being exposed *in utero* to saline (Cont) or LPS at E12 (blue bars) or E16 (red bars) as indicated; (A, B) neurosphere area at 2 and 7 DIV, and (C) representative photomicrographs of neurospheres at 7DIV. (D-F) Quantitative real-time PCR examining the expression of (D) IL-6, (E) IL-1β, and (F) TNF-α mRNA in the VM, 48h after *in utero* exposure to saline (Cont) or LPS at E12 (blue bars) or E16 (red bars) as indicated (LPS group was compared to saline treated control group). (G-I) A neurosphere assay examining diameter of neurospheres established from cells isolated from the (G) E12 or (H) E16 VM and grown for 4DIV in the presence of IL-1β, TNF-α and IL-6 (all at 10ng/ml). (I) Representative photomicrographs of the experiments shown in (G, H); scale bar = 50μm. Data are expressed as mean ±SEM (N=3-4/group/time point). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired Student's t-test.

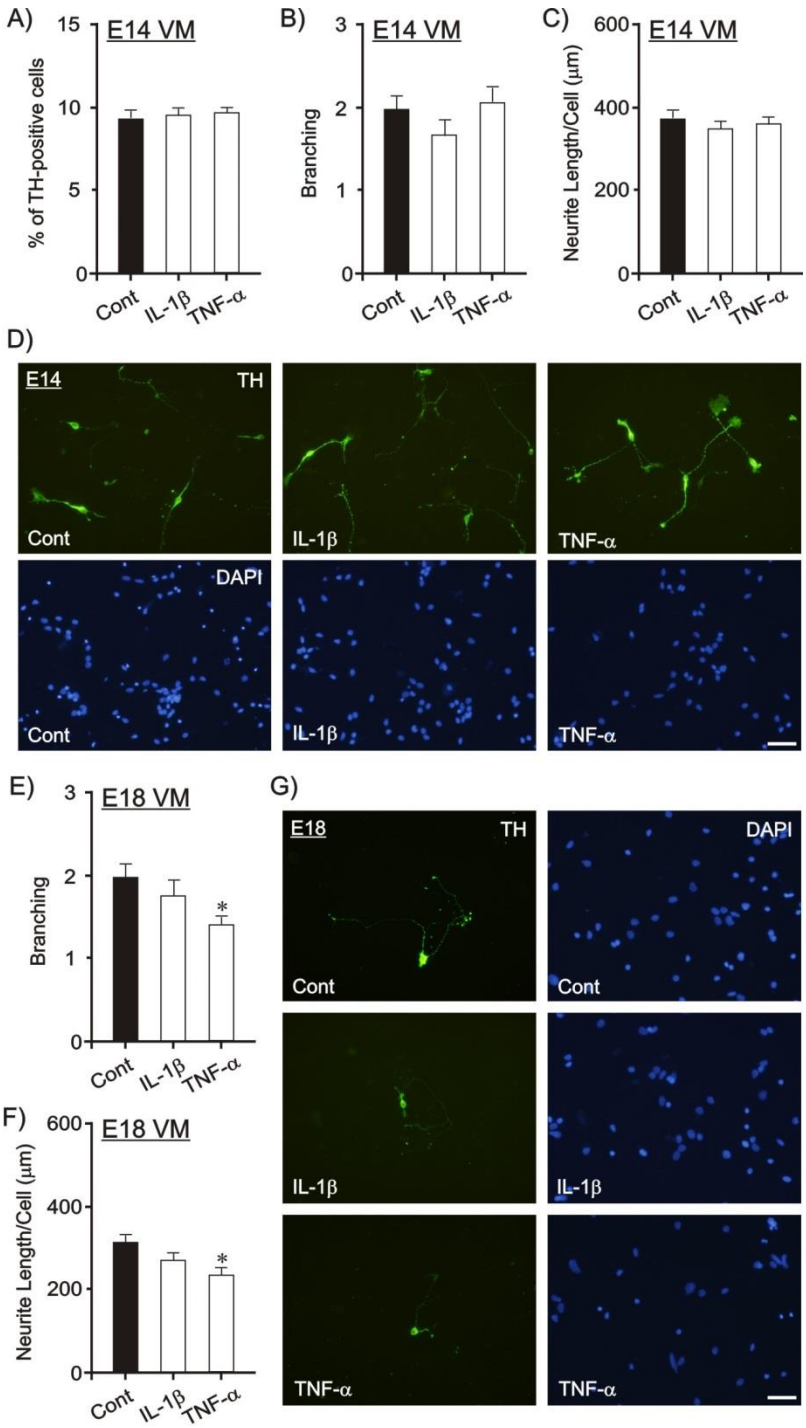


Figure 3: Differential sensitivity of cultured VM DA neurons to inflammatory cytokines

(A) Percentage of total cells, (B) branching and (C) neurite length/cell of TH-positive DA neurons at 4DIV following daily treatments with IL-1β (100ng/ml) or TNF-α (10ng/ml) in cultures of E14 rat VM. (D) Representative photomicrographs of the experiments shown in (A-C). (E) Branching and (F) neurite length/cell of TH-positive DA neurons at 4DIV following daily treatments with IL-1β (100ng/ml) or TNF-α (10ng/ml) in cultures of E18 rat VM (*P < 0.05 compared to controls). (G) Representative photomicrographs of the experiments shown in (E, F). Scale bar = 50μm. Data are expressed as mean ± SEM.

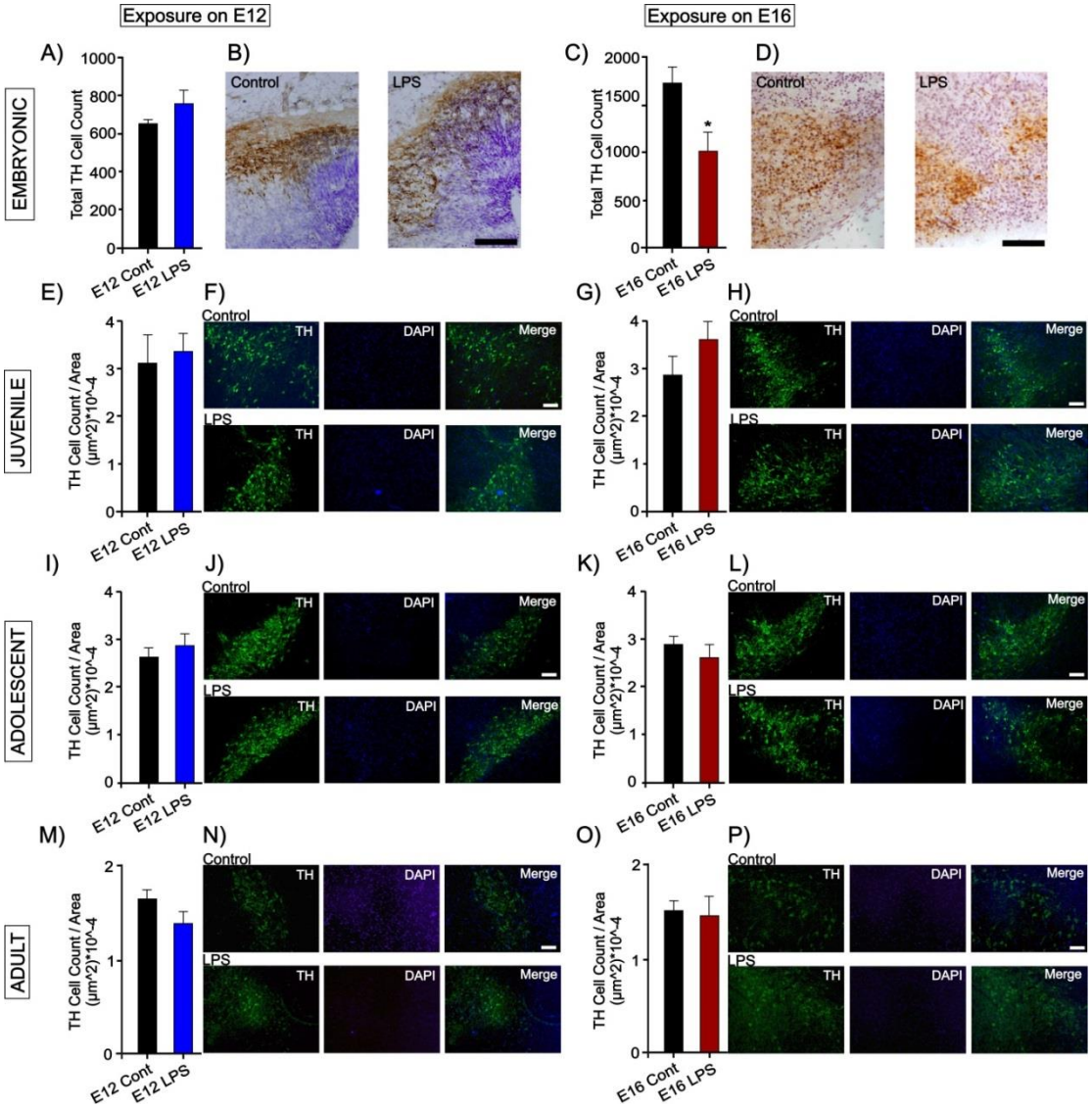


Figure 4: MIA alters fetal DA neuron count in later pregnancy, which is corrected by P9.

(A-D) TH positive total cell count of ventral midbrain (VM) from embryos 48h following *in utero* exposure to saline (Cont) or LPS at (A) E12 (blue bars) or (C) E16 (red bars). Representative photomicrographs of (B) E14 VM and (D) E18 VM from embryonic immunohistochemistry experiments; scale bar = 100µm. Data expressed as mean ±SEM (N=5-6/group/time point); * $P < 0.05$. (E-P) TH positive cell counts per area from stained midbrain sections of brains from (E-H) juvenile, (I-J) adolescent, or (M-P) adult offspring that were exposed to saline (Cont) or LPS at (E, I, M) E12 (blue bars) or (G, K, O) E16 (red bars). Representative photomicrographs of postnatal immunohistochemistry experiments, including (F) juvenile, (J) adolescent, and (N) adult offspring exposed to *in utero* insult on E12 (blue bars), as well as (H) juvenile, (L) adolescent, and (P) adult offspring exposed to saline or LPS on E16 (red bars). Data are expressed as mean ±SEM (N=3-4/group/time point), unpaired Student's t-test. Scale bars = 100µm.

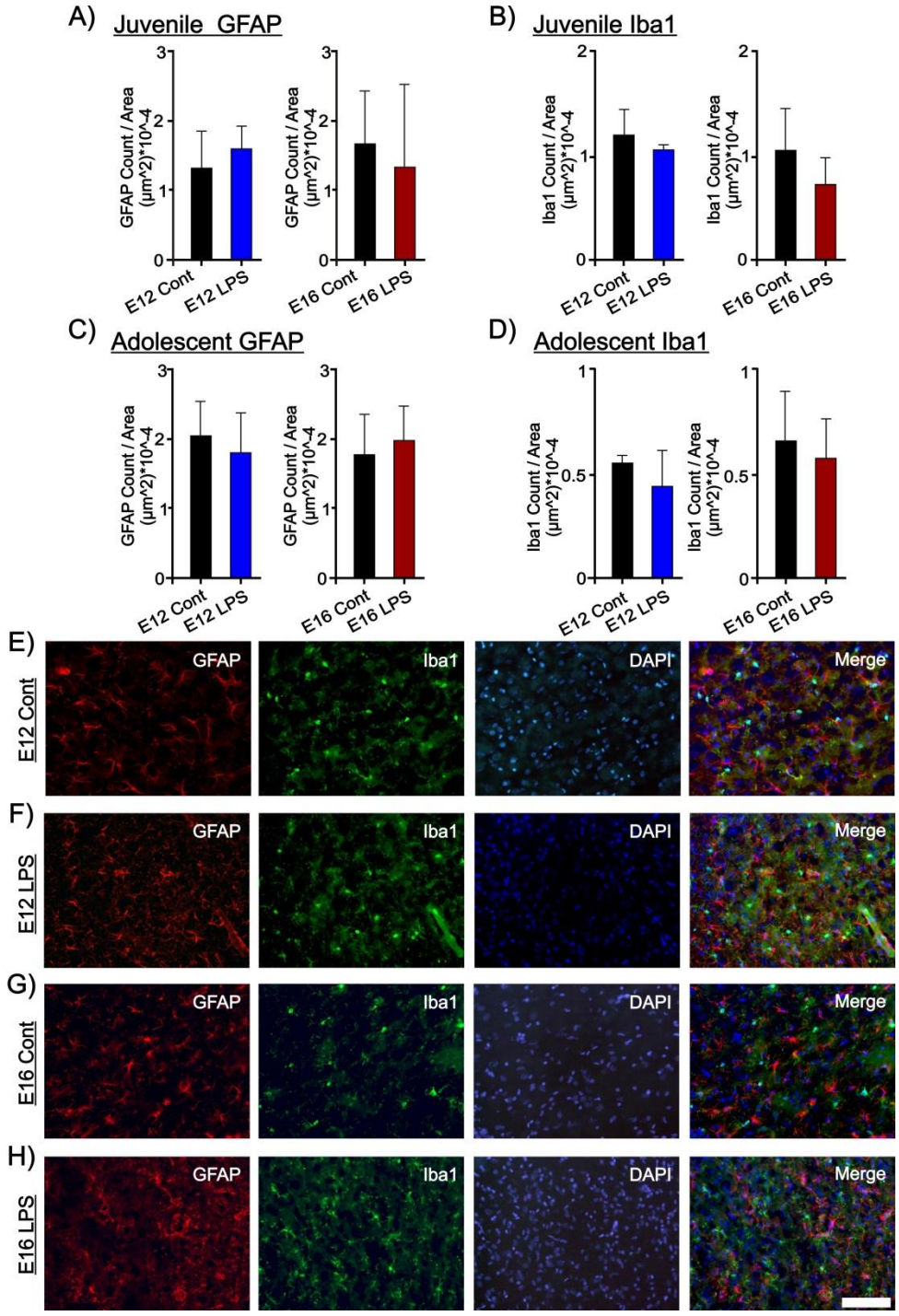


Figure 5: No difference in offspring midbrain glial cell number following MIA
(A-D) Graphical representations of cell count / area of (A-B) juvenile and (C-D) adolescent offspring midbrains co-stained for (A, C) GFAP and (B, D) Iba1 following maternal administration of saline (control) or LPS on either E12 (blue bars) or E16 (red bars). Data are expressed as mean ±SEM (N=4/group/time point), unpaired Student's t-test. (E-H) Representative photomicrographs of adolescent midbrains immunofluorescently labelled for astrocytes (GFAP) or microglia (Iba1) following *in utero* exposure to (E) saline (cont) or (F) LPS on E12, or else (G) saline (cont) or (H) LPS on E16. Scale bar = 100µm.

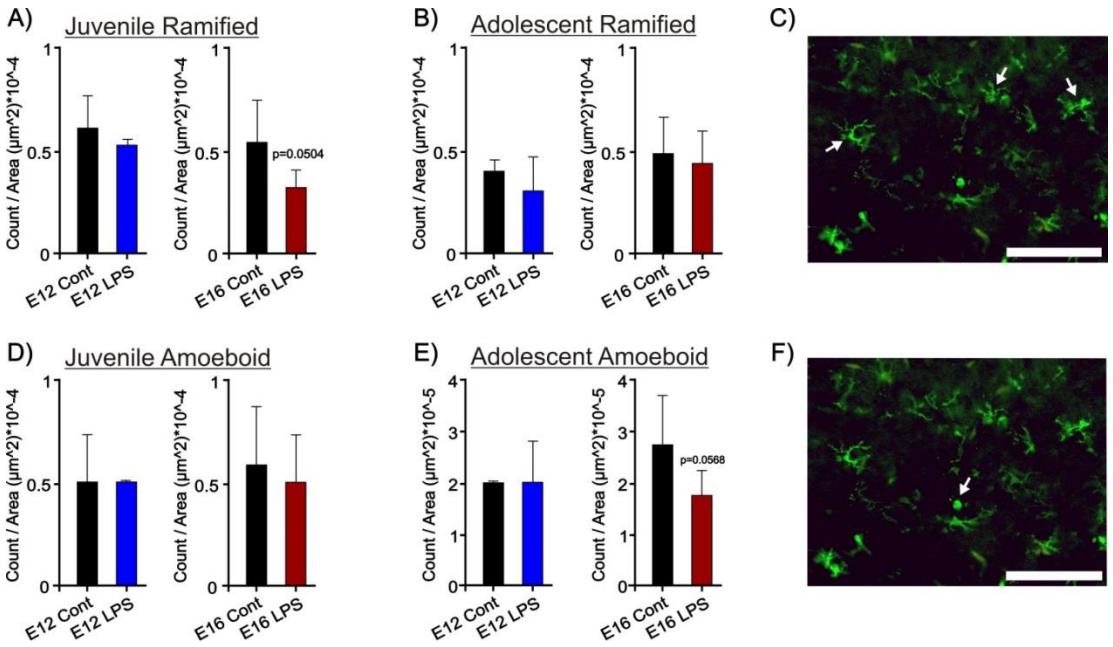


Figure 6: MIA does not significantly alter microglial phenotype in offspring midbrain

Graphical representation of (A-B) ramified or (D-E) amoeboid microglia cell count/ area in midbrain sections of (A, D) juvenile and (B, E) adolescent offspring exposed to saline (cont) or LPS on E12 (blue bars) or E16 (red bars) as indicated. Representative photomicrographs of juvenile midbrain section stained for Iba1 identifying (C) ramified and (F) amoeboid microglial morphology. Data are expressed as mean ±SEM (N=4/group/time point), unpaired Student's t-test. Scale bars = 100µm.

4.5 Discussion

This study investigated the molecular and cellular aspects of MIA-induced alterations of fetal midbrain development. We discovered intriguing developmental alterations in the offspring VM following later pregnancy (E16) LPS exposure, but not earlier exposure (E12), and inhibited proliferation may be mediated by increased levels of pro-inflammatory cytokines. Interestingly, we saw no postnatal effects of MIA on the cellular architecture of offspring midbrains, suggesting that the developmental alterations were compensated for. Taken all together, our findings suggest that the cytokine response and subsequent neurodevelopmental ramifications observed in the offspring VM are dependent upon the temporal responsiveness of developmental stages.

Once we established that our model of MIA, a low dose of LPS, would be sufficient to induce an inflammatory response in our region of interest (assessed though cytokine profiles of P1 VM samples), we investigated the effects of MIA on VM neurodevelopment. We noted that while LPS exposure on E12 resulted in no differences in E14 neurosphere area, LPS exposure on E16 resulted in inhibited E18 VM neurosphere growth after 7DIV. This is entirely consistent with our recent findings that later developmental stages is the critical window of vulnerability to MIA in our LPS model (Straley *et al.*, 2014). Furthermore, the neurosphere findings mirror previous work from our group which demonstrated that IL-1β limits neurosphere growth, progressively worsening over the 7DIV (Crampton *et al.*, 2012). Therefore, we next investigated whether difference in cytokine levels might explain the difference in neurosphere growth.

The inhibition on neurosphere growth of this study was indeed associated with differential cytokine mRNA expression levels depending on age of LPS exposure. Specifically, the E16 exposure group had midbrains with significantly higher IL-1β and TNF-α mRNA levels, whereas the E12 exposure group demonstrated significantly decreased IL-6 but increase IL-1β compared to the relative saline control group. These differential cytokine profiles implicate the importance of timing of MIA on resulting inflammatory response and downstream effects of that. For example, IL-1β regulates proliferation and is critical for gliogenesis and neurogenesis in the normal brain and in fact the cytokine peaks during this stage of development (Deverman & Patterson, 2009; Bilbo & Schwarz,

2012). This suggests the possibility that increases in IL-1β in earlier developmental windows may produce beneficial effects. In agreement with this, some reports indicate neuroprotective effects, including up-regulation of NGF and BDNF, resulting from mild systemic infection in the CNS (Petcu *et al.*, 2008; Song *et al.*, 2013). Furthermore, as IL-6 can have devastating effects on fetal development and offspring behavioral outcome (Smith *et al.*, 2007), the decreased IL-6 mRNA levels in the E12 LPS group suggests a protective mechanism preventing the developing fetus from adverse outcomes associated with MIA-induced IL-6 elevation. However, in the E16 exposure group, IL-1β and TNF-α demonstrated significantly elevated expression and IL-6 levels were not decreased. These elevated cytokine levels were accompanied by diminished VM neurosphere size at this age. This is in agreement with a plethora of literature suggesting that increased levels of pro-inflammatory cytokines in offspring brains mediated neurodevelopmental ramifications of MIA, and the differential effects of MIA depending on timing of exposure (Boksa, 2010; Knuesel *et al.*, 2014).

In order to confirm our suspicions of differential effects of cytokines on VM NPCs depending on developmental age, we then conducted a neurosphere assay with VM cells from naive embryos from earlier (E12) or later (E16) developmental time points. E16 neurospheres in this experiment had reduced diameter after 4DIV when treated with IL-1β, TNF-α, and IL-6 in culture. This is consistent with previous studies which demonstrated the power of IL-1β to inhibit proliferation of E16 rat forebrain NPCs (Wang *et al.*, 2007) and TNF-α to reduce the growth of neurospheres made from spinal cord of E13.5 mouse embryos (~E15.5 in the rat) (Deleyrolle *et al.*, 2006). The E12 VM neurospheres of the current study were only diminished following TNF-α treatment. Incidentally, TNF-α mRNA levels were not altered in our E12 LPS group providing a possible explanation why we saw no differences in neurosphere growth for this group. Taken together, these data suggest that a neurodevelopmental environment characterized by increased levels of pro-inflammatory cytokines is detrimental only to development of later stage VM neurospheres, and not earlier.

Because IL-1β and TNF-α mRNA expression significantly increased following *in utero* LPS exposure, we next conducted an *in vitro* study to assess the effects of those cytokines on VM DA neuron development in culture. Only the E18

VM cultures (and not E14 cultures) were affected by the treatments, with TNF-α significantly reducing neuronal complexity. This is in agreement with another study that demonstrated the ability of low dose TNF-α (as well as higher concentrations of IL-6, IL-1β, and TNF-α) to disrupt dendrite development of cortical neurons (Gilmore *et al.*, 2004). Also, another study found that TNF-α acted in a neurotrophic manner to VM DA cultured from E12.5 mice, but the cytokine was neurotoxic at later ages (E14 or E16) (Doherty, 2007). Consistently, we have previously shown that IL-1β exerted inhibitory effects on SCG neurons during similar developmental window (E18-P1) (Nolan *et al.*, 2011), and that exposure to LPS *in utero* on E16 also resulted in reduced neuronal growth in SCG neurons isolated from affected offspring (Straley *et al.*, 2014), but there were no effects in earlier developmental stages. Similarly, other studies have indicated roles for TNF-α and IL-1β in altered differentiation of NPCs in multiple brain regions (Wang *et al.*, 2007; Cacci *et al.*, 2008; Zhang *et al.*, 2013). These findings suggest that MIA-induced upregulation of cytokines in the fetal brain may affect the cellular architecture of the developing VM. Indeed, when we analyzed the numbers of TH-positive DA neurons in the developing VM, we found significantly decreased numbers following MIA in later pregnancy. This is consistent with previous studies that have found a loss of DA neurons grown in culture from LPS (0.1mg/kg on E10, E14 or E18) exposed embryos (Snyder-Keller & Stark, 2008). There are also reports of decreased cell counts of TH-immuno positive cells in midbrain sections from offspring exposed to LPS (1mg/kg, on E10.5) across the lifespan (Carvey *et al.*, 2003; Ling *et al.*, 2009). We were unable to replicate these findings, as our examination of TH cell counts across the lifespan revealed no differences between either LPS group compared to the relative, age-matched control. This is presumably due to the extreme difference in LPS dosage, as dose-dependent LPS-induced alterations to offspring neurodevelopmental outcome have been reported in many animal models (Boksa, 2010), including guinea pigs (Harnett *et al.*, 2007) and rats (Urakubo *et al.*, 2001).

Finally, we wanted to examine glial cytoarchitecture in the midbrains from offspring at different postnatal ages because it has been shown previously that cytokines are sufficient to induce gliogenesis and prohibit neurogenesis (Cacci *et al.*, 2008; Peng *et al.*, 2008; Crampton *et al.*, 2012) and because MIA has been proposed to prime microglia to respond more strongly to subsequent stimuli (Krstic *et al.*,

2012; Giovanoli *et al.*, 2013; Knuesel *et al.*, 2014). Interestingly, there were no significant differences in astrocyte or microglia cell count in either the E12 or the E16 cohort, in agreement with our TH findings. Taken together these data suggest that our low dose of LPS is not sufficient to induce long lasting changes in the cellular architecture of midbrains of offspring across the lifespan. Although, there was a trend toward age-dependent differences in the cell counts of the microglial morphological sub-types only in our E16 cohort, which raises intriguing questions for future research concerning microglial priming and the implications for multiple-hits in the etiologies of MIA-associated disorders.

Chapter 5:

Final Discussion

5.1 Summary of Thesis

The present thesis focused on characterizing the behavioral, molecular, and cellular consequences of maternal immune activation (MIA) on the midbrain of affected offspring. Though previous studies into the differential effects of MIA on offspring neurodevelopmental outcome exist (Boksa, 2010; Harvey & Boksa, 2012), conflicting findings resulting from inconsistent animal models and experimental methods created a need for a midbrain specific comprehensive investigation. It is essential to understand the neurodevelopmental and behavioral consequences of MIA if we ever hope to prevent the adverse effects that lead to increased susceptibility for offspring to develop numerous dopamine-associated neurological and neuropsychiatric disorders.

In an attempt to address this, we initially set out to establish a rat model of MIA (Chapter 2), and in particular of subclinical chorioamnionitis, which is an acute infection often associated with negative neurodevelopmental outcomes including CP (Wu *et al.*, 2000; De Felice *et al.*, 2005; Miyazaki *et al.*, 2007). Importantly, as fetal outcome may be dependent on the timing of MIA due to different developmental processes which occur at different gestational time points (Andersen, 2003), we sought to determine outcomes following MIA at different time points during pregnancy in order to establish possible critical windows for our model. In terms of our model, an overwhelming amount of evidence suggested that LPS exposure at a later time point (E16) had more robust consequences on the various factors we investigated, compared to the earlier gestational age (E12).

In this study (Chapter 2) we then wanted to determine the effects of our animal model on the placenta as the placenta is a key regulator of fetal development (Huppertz, 2008; Togher *et al.*, 2014), and placental health is a great indicator of neurodevelopmental outcome (O'Keeffe & Kenny, 2014). In particular, investigating placental cytokine response as well as HSD11B2 expression was of key interest because cytokines affect neuron development (Deverman & Patterson, 2009; Stolp, 2013) and HSD11B2 plays a critical role in protecting the fetus from heightened maternal cortisol levels which can have deleterious effects on fetal neurodevelopment (Togher *et al.*, 2014). Interestingly, this initial study demonstrated that LPS exposure has a bi-phasic effect on HSD11B2 mRNA expression in the placenta in that it increased following MIA on E12, but decreased

following MIA on E16. The decrease in the mRNA expression of this enzyme was associated with a decrease in maternal cortisol following LPS exposure at the same gestational time point. Also, LPS exposure at E16 (but not at E12) resulted in elevated IL-1β mRNA levels in the rat placenta. Our *in vitro* treatment of JEG3 placental cells with this cytokine resulted in a similar down regulation of HSD11B2 expression, suggesting a role for IL-1β in the regulation of HSD11B2. Overall, the data from these experiments suggested that LPS-induced IL-1β elevation and maternal cortisol depletion may mediate the observed down regulation of HSD11B2 following LPS at E16. We also demonstrated reduced placental weight, and no alterations to fetal weight or CRL following LPS administration on E16, and no placental weight changes were observed following exposure to LPS on E12. Even though these larger developmental characteristics were not altered post LPS, there were detrimental outcomes regarding neuron growth and complexity following LPS exposure at E16 (but not at E12).

In the next part of the thesis (Chapter 3), we utilized our MIA animal model and key critical time points which we established in the Chapter 2 (Straley *et al.*, 2014) to investigate behavioral consequences of MIA across the lifespan of offspring. We specifically utilized behavioral assays which would provide insight into the functioning of dopamine (DA) pathways due to its role in numerous disease etiologies, including PD (Toulouse & Sullivan, 2008), schizophrenia (Meyer-Lindenberg *et al.*, 2002; Howes & Kapur, 2009), drug addiction (Robinson & Berridge, 1993; White, 1996), depression (Martin-Soelch, 2009) and ADHD (Volkow *et al.*, 2009). The overall results of this study demonstrated that behavioral outcome was determined by gestational age of MIA occurrence, with exposure at E16 effecting motor behaviors in juvenile and adult offspring, and LPS exposure at E12 altering the response of adolescent or adult offspring to amphetamine (AMPH).

Specifically, offspring exposed to LPS on E16 demonstrated motor impairments as assessed by the bar hold test and open field locomotion at P9, but these deficits were later compensated for in adolescence (P30). Interestingly, though, these animals (E16 LPS exposed) later displayed an age-related decline in motor function during the rotarod test, as well as the open field test as demonstrated by decreased initial locomotion and increased frequency of freezing. Furthermore, the E16 group also demonstrated differences in anxiety behaviors, whereas the group

exposed to LPS on E12 did not. At P9, the animals that had been exposed to LPS on E16 demonstrated altered anxiety response to brief maternal separation, measured by USV, and at P30 these same animals remained in the center of the open field for a greater duration compared to E16 controls. With regards to the group of animals exposed to LPS *in utero* on E12, they demonstrated differential responding to amphetamine as compared to the relative age-matched controls, which were subjected to saline on E12. Previous findings established the ability of amphetamine to potentiate responding for a conditioned reward in a normal animal (Fletcher *et al.*, 1998), yet our animals that had been exposed to LPS at E12 avoided the characteristic AMPH-induced increase in reward responding at both adolescent and adult ages. Importantly, offspring exposed to LPS on E16 responded to AMPH similarly as their relative age-matched controls, thus indicating a possible critical window for differential responding to AMPH in affected offspring.

In the final stage of this thesis, in Chapter 4, we aimed to determine the molecular and cellular consequences that occur in the midbrain of affected offspring which might lead to the behavioral deficits we observed in Chapter 3. In order to accomplish this, we first sought to determine the cytokine profiles of offspring subjected to our MIA model. This was based on the knowledge that other MIA models have demonstrated increased levels of pro-inflammatory cytokines, including IL-1β and TNF-α, in the fetal brain (Urakubo *et al.*, 2001). Interestingly, results demonstrated differential midbrain cytokine profiles which were dependent upon the gestational time point at which animals were administered LPS. Following this, based on our previous finding that IL-1β impaired proliferation and altered differentiation of VM NPCs *in vitro* (Crampton *et al.*, 2012), we then investigated if MIA and associated inflammatory mediators alter proliferation or development of fetal VM neurospheres and primary DA neuron cultures. LPS exposure at E16 resulted in decreased VM neurosphere size after 7DIV. In order to then supplement our hypothesis that developmental effects were related to the differential cytokine profiles, we then conducted *in vitro* experiments. These results indicated that VM samples (both neurospheres and primary DA neuron cultures) from later gestational ages (≥E16) are more susceptible to neurotoxic effects of pro-inflammatory cytokines than cultures isolated from earlier gestational ages. Finally, due to the different onset ages for MIA associated disorders, we wanted to map the

cytoarchitecture of offspring midbrains across the lifespan following *in utero* exposure to saline or LPS on E12 or E16. *In utero* LPS exposure at E16 decreased the number of VM neurons stained for TH, a DA neuron marker. However, there were no differences in DA neuron number in juvenile, adolescent, nor adult offspring suggesting that the developmental alterations following MIA exposure are compensated for early in life. Similarly, LPS exposure did not alter cell number or morphology of glial cells in the midbrains of affected offspring.

5.2 Implications of this Work

This thesis has shown that LPS exposure can induce placental and fetal alterations during development, with behavioral consequences for the offspring dependent upon the gestational time point when the MIA insult occurs. The findings of this thesis have brought up numerous interesting implications as well as areas for future research to consider.

The initial wider implications from the first study (Chapter 2) is that our model of MIA agrees with previous epidemiological evidence associating second trimester maternal infection with an increased risk for adverse neurodevelopmental outcomes (Sørensen *et al.*, 2009; Atladóttir *et al.*, 2010; Patterson, 2011b). Obviously translating developmental dates between species comes with numerous caveats, yet there is a mounting body of evidence to suggest that the second week of rodent pregnancy correlates with key neurodevelopmental processes in the second trimester of human pregnancy (see Chapter 1) (Clancy *et al.*, 2001; Clancy *et al.*, 2007; Katz, 2007; Semple *et al.*, 2013; Workman *et al.*, 2013). Therefore, our finding that the placental and neuronal alterations post LPS occurred only following exposure at E16, suggest that second trimester is a more vulnerable developmental window for MIA to alter these parameters. Importantly, because of the temporal specificity and longevity of maternal infection- induced placental changes it is possible that placental characteristics may be useful in predicting subsequent risk for adverse neurodevelopment and subsequent outcomes in terms of neuropsychiatric disease (O'Keeffe & Kenny, 2014). Therefore, it would be interesting for future research to investigate further the precise predictive capabilities of placental phenotypes in determining the likelihood of neurodevelopmental disorders in affected offspring.

It would also be interesting for future research to determine what LPS-induced placenta changes mean for fetal exposure to cortisol because increased fetal exposure to maternal glucocorticoids is associated with a number of adverse outcomes including preterm birth and increased risk of neuropsychiatric disorders (Entringer *et al.*, 2012; Reynolds, 2012; Togher *et al.*, 2014). Reduction in placental HSD11β2 was shown by others to increase fetal levels of corticosterone (Cottrell *et al.*, 2012), and we found a reduction in HSD11β2 post LPS on E16. As this enzyme converts cortisol to its inactive form cortisone, thereby protecting the fetus from

excessive maternal cortisol exposure (Togher *et al.*, 2014), it is interesting to speculate about the neurodevelopmental ramifications of these observations. Of note, our other observation of reduced maternal cortisol levels following LPS should also be taken into consideration for this discussion as the drop in HSD11β2 expression may be of a sufficient level to protect the fetus from the altered post LPS cortisol levels, though maternal cortisol levels are greatly increased during pregnancy so it could be that the fetus is still subjected to higher than normal levels of cortisol regardless of this drop. Thus, future research should investigate these parameters further. In order to accomplish this, it would be interesting to compare cortisol levels from naive animals with those exposed to our MIA model, at different gestational ages, in numerous regions, including maternal circulation, placental and fetal compartments, as well as in the fetal brain.

As we also showed that IL-1β affected HSD11β2 expression in cultured placental cells, and LPS exposure in later pregnancy increased IL-1β mRNA expression in the placenta of our *in vivo* study, cortisol is not the only factor worth paying attention to post LPS. As we proposed in the paper (Straley *et al.*, 2014), the neurodevelopmental outcome resulting from LPS exposure may also depend on changes in pro-inflammatory cytokines, which have been shown to adversely affect the developing brain (Boksa, 2010). It would be interesting for future research to investigate differential cytokine expression following LPS exposure because neurodevelopmental consequences may involve cytokines coming from the fetus and/or the mother. Elucidating the location associated with the most detrimental up-regulation in cytokines will help to determine future preventative measures for protecting fetal neurodevelopment from MIA-induced, cytokine-mediated alterations. An interesting approach to investigate this would be to utilize recombinant cytokines labelled with fluorescent dye to trace the movement of maternal cytokines in live pregnancy.

Following on from this, the thesis also demonstrated an age-related decline in motor function for animals exposed to LPS on E16, and these findings have important implications for Parkinson's disease (PD). The initial decline in locomotion our animals demonstrated in the open field could represent a difficulty initiating movement, and they also exhibited a greater frequency freezing during the testing period compared to controls. These are both common clinical features of

motor symptoms of PD (Jankovic, 2008), suggesting a possible relationship between PD and MIA. Interestingly, these same animals (that were exposed to *in utero* LPS on E16) also demonstrated increased fecal pellet output in the open field, which is a measure of increased anxiety in animal studies. Common non-motor PD symptoms include problems with gastrointestinal motility as well as anxiety (Braak *et al.*, 2003; Langston, 2006; Taylor *et al.*, 2009), further implicating a possible PD phenotype following exposure to *in utero* LPS in later pregnancy.

Interestingly, we did not find a loss in DA neurons in the substantia nigra, which is the true hallmark of PD (Damier *et al.*, 1999; Sulzer, 2007; Venderova & Park, 2012), nor the ventral tegmental area of affected offspring, suggesting that motor function was not altered due to loss of DA neurons in the midbrain. Thus, the question arises what cause the deficits in motor behavior? Future investigations into DA production using neurochemical analyses (such as high-pressure liquid chromatography, HPLC) could provide insight into this, as MIA has been shown to alter concentrations of DA and its metabolites (Ozawa *et al.*, 2006; Ling *et al.*, 2009; Kirsten *et al.*, 2010a). Furthermore, it is possible that DA transmission between the substantia nigra and striatum reaches a ceiling effect whereby additional stimulation to the DA system (by AMPH) is blunted. Utilizing HPLC experiments examining DA levels in target regions, following AMPH administration in affected animals would help to determine this. Also to this end, it would be interesting to conduct an electrophysiological study. Electrophysiological analysis can provide important insights into the function of regionally specific DA neurons, and comparisons between DA neuron discharge properties and biochemical measures of neurotransmitter regulation can elucidate information about DA regulation in a pathway of interest. Alternatively, the observed motor deficits could be an issue with DA receptors, as several neuropsychiatric disorders are associated with abnormal dopamine receptor signaling (Girault & Greengard, 2004), and as such a simple analysis of DA receptor expression, through immunohistochemistry and/or PCR would be useful. Alternatively, other neurotransmitters such as glutamate, which are also involved in motor function and interacts with DA to produce PD symptoms, may also be involved in these processes (Chase & Oh, 2000; Rylander *et al.*, 2009).

Even more compelling in this regard, perhaps, is molecular and cellular data from Chapter 4. As MIA can prime microglia for chronic activation, altering

microglial response to cellular processes like protein aggregation or neuron death, it has been proposed that chronic microglial activation may subsequently result in progression of the α-synuclein aggregates into Lewy bodies that are the pathological hallmark of PD (reviewed in Glass *et al.*, 2010; Tomé *et al.*, 2013; Knuesel *et al.*, 2014). This suggests microglial priming as a result of MIA has the potential to lead to an increased susceptibility for PD later in life. In support of this, PD is highly associated with neuroinflammation because microglial activation (McGeer *et al.*, 1988; Imamura *et al.*, 2003) and elevated levels IL-1β and TNF-α, and IL-6 (Mogi *et al.*, 1994a; Mogi *et al.*, 1994b; Koziorowski *et al.*, 2012) are common characteristics of postmortem PD brains. In the current thesis, we demonstrated increased cytokine expression in fetal midbrains, and it would be of interest for future research to determine similar cytokine profiles across the lifespan in order to ascertain if chronic inflammation is characteristic in our MIA model.

Furthermore, the idea of microglial priming raises intriguing questions for future research in the context of multiple-hit scenarios. Indeed, it has been proposed in recent years that PD is the culmination of numerous insults or "hits" which may begin with MIA (Carvey *et al.*, 2006; Knuesel *et al.*, 2014) and include microglial priming and associated chronic inflammation (Sulzer, 2007; Long-Smith *et al.*, 2009; Collins *et al.*, 2012). Thus, MIA may be the initial step along the path which leads to predisposition of disease development later in life. In complete agreement with this, our data demonstrated altered VM neurosphere/ DA neuron development along with subsequent deficits in nigrostriatal-mediated behaviors in affected offspring, following exposure to LPS at E16. Furthermore, our subsequent investigation of microglial morphology indicated that LPS exposure at E16 resulted in a trend toward a difference in the numbers of ramified microglia at the juvenile age and in the amoeboid morphology at the adolescent age in the midbrains of affected offspring, however these results were not significant. Due to this, it is interesting to speculate what would happen if a subsequent hit occurred. Others have demonstrated that offspring exposed to MIA (Poly (I:C), 5mg/kg, E17) display chronic cytokine up-regulation along with impairments in working memory in old age, and if the MIA is followed by a second hit of Poly (I:C) in adulthood, these outcomes are strongly exacerbated along with a surge in microglia activation, mimicking the pathology of neurodegenerative progression of AD (Krstic *et al.*, 2012) which incidentally has

commonalities with PD pathology (Witt, 2012; Winner & Winkler, 2015). Similarly, when MIA (LPS 200μg/kg, every 12h, from E17 to the end of gestation) was combined with a secondary insult of hypoxia-ischemia, lesions to the substantia nigra as well as motor deficits were greatly increased, compared to either insult alone, for affected offspring (Girard *et al.*, 2009). Along similar lines, findings from both clinical and animal studies have suggested that there are lasting changes in the brains of cerebral palsy patients (CP) following perinatal insult which manifest as mechanisms that prevent regeneration and/or exacerbate brain damage, the processes and downstream effects are termed "tertiary brain damage" (Fleiss & Gressens, 2012). Persistent inflammation, associated with aberrant gliosis, and epigenetic changes are thought to be the major tertiary mechanisms, as they can alter numerous neurodevelopmental processes, and importantly the authors implore that many instances of tertiary brain damage may only become apparent following a secondary insult (Fleiss & Gressens, 2012). Therefore, if I were to investigate this, it would be interesting to conduct the same experiments as I have already done, including the behavioral assays as well as investigations into cellular architecture following MIA, but with an additional LPS administration in adolescence. I would choose this because of the dynamic neurodevelopmental and re-organizational processes that occur during adolescence, and because numerous diseases are associated with insults that occur during this developmental stage (schizophrenia, for example (Howes *et al.*, 2004)). Furthermore, if the microglia had been "primed" by *in utero* LPS exposure, the subsequent response following a second hit of the same immunogen could potentially result in more robust morphological and cytoarchitectural alterations.

The previous discussion has centered around the consequences of LPS on E16, as the majority of alterations were found following insult at that time point. However, we did see some behavioral alterations following LPS exposure in the earlier age (E12). Indeed, our other key findings in Chapter 3 demonstrated that MIA has differential effects on amphetamine (AMPH) responding depending on gestational age at which MIA occurs, and this has interesting implications for drug addiction. The DSM-V defines tolerance, or a diminished response to a drug that is repeatedly used, as a hallmark symptom of addiction (APA, 2013), and therefore our findings of reduced sensitivity to AMPH following MIA exposure at E12 in adult

animals may indicate a potential susceptibility for drug tolerance and subsequent abuse later in life. Current research poses the theory that drug addiction results from a series of events, beginning with an initial drug use voluntarily taken, followed by many subsequent exposures along with interactions between conditioned and instrumental learning processes, ultimately resulting in compulsive drug seeking behavior (Everitt & Robbins, 2005; Everitt *et al.*, 2008). Regardless of behavioral outcome, repeated exposure to drugs of abuse induces adaptations within the brain that compensate for the drug exposure (Chao & Nestler, 2004). Furthermore, studies in animal models demonstrates that impulsivity behavior is associated with an increased predisposition for drug seeking behavior/ drug abuse , as well as an increased probability of relapse (Everitt *et al.*, 2008); human studies have also implicated personality differences, including impulsivity or sensation seeking, in vulnerability to drug use and abuse (Hawkins *et al.*, 1992; Kelly *et al.*, 2006; White *et al.*, 2006). Taken together, these findings suggest that an individual with an initial blunted response to a drug, and who is prone to impulsive behavior may experiment again with the substance, resulting in the eventuality of habituation and biological dependency on the substance. Therefore, it would be of particular interest for future research to establish the effect of MIA more clearly on impulsive behavior of offspring as well as the propensity to engage in drug seeking behavior. In order to elucidate such information, there are numerous ways of measuring impulsivity in animals, most of which utilize similar food reward paradigms that I used for my investigations into motivation and reward. If I were to do that, it would be interesting to administer a range of food reward paradigms (some of which are described here: Everitt *et al.*, 2008), following LPS exposure, that would target impulsive behaviors of the animals. For example, monitoring the anticipatory responses made before a food reward, or else a delay-of-reward task paradigm, in which the animal learns that it can choose a small immediate reward or wait for a larger but delayed reward, are both interesting experimental possibilities. In order to examine if MIA increases drug seeking behavior in affected offspring, it would be interesting to use a drug self-administration paradigm following *in utero* LPS exposure. In this type of scenario, animals would be exposed to chronic administration (>40 days) of a drug, such as cocaine or AMPH, after which a certain percentage of control animals begin to demonstrate addiction-like behaviors (Deroche-Gamonet *et al.*, 2004; Everitt *et al.*, 2008). This test alone could

demonstrate if MIA has the ability to increase likelihood of drug seeking in affected offspring. Though, it would be even more compelling if the animals were subjected to a subsequent punishment paradigm, in which a punishment, such as a foot shock (Pelloux *et al.*, 2007), is administered at the same time as the drug reward because maintaining drug use despite aversive consequences is characteristic of addictive-like behaviors (Deroche-Gamonet *et al.*, 2004).

Within this context, it is interesting to speculate the reasons for different AMPH responding we observed in our study. AMPH-induced effects on locomotor behavior has been attributed primarily to the ability of the drug to bind to dopamine transporter (DAT) in the nucleus accumbens and increase DA release (Naef *et al.*, 2008). DAT is the main membrane protein responsible for clearing dopamine from the synapse and therefore DAT is critical for terminating DA signaling (Zahniser & Sorkin, 2004). Furthermore, DAT is implicated in numerous DA-related disorders associated with nucleus accumbens and the mesolimbic pathway including ADHD (Spencer *et al.*, 2007; Volkow *et al.*, 2007) and addiction, including alcohol (Repo *et al.*, 1999; Samochowiec *et al.*, 2006) and drugs of abuse (Zhu & Reith, 2008). Indeed, studies with knockout mice have demonstrated the contribution of DAT to the reinforcing, motivational, and rewarding effects of psychomotor stimulants such as cocaine or amphetamine (Rocha, 2003). Thus, DAT contributes to the potential of psychomotor simulants to be abused (Zahniser & Sorkin, 2004). DAT is regulated by acute AMPH exposure, which causes rapid up-regulation and slower down-regulation of DAT (Zahniser & Sorkin, 2009). Furthermore, uptake of amphetamine inhibits DAT transport of dopamine, resulting in the characteristic increase in extracellular DA associated with AMPH administration (Zahniser & Sorkin, 2004). Thus, altered AMPH responding in LPS exposed offspring may implicate a MIA-induced problem with DAT. Therefore, it would be of interest for future studies to determine the effects of MIA on DAT expression/ function in affected offspring.

As mentioned previously, basal levels of DA could be altered following *in utero* LPS exposure, and therefore an initial inquiry should be made into the transmission of DA from the VTA to the nucleus accumbens in drug naive animals as well as following AMPH treatment, in order to determine if MIA results in blunted AMPH-induced stimulation of this DA pathway. Another important aspect of this would be to examine postsynaptic receptors, such as D1 and D2, because these would need to be functioning properly in order to stimulate behaviors.

Furthermore, impulsivity (which predicted the intake escalation of cocaine), was associated with low D2 and D3 DA receptor availability in the ventral striatum of rats (Dalley *et al.*, 2007). Also, reduced D2 receptors in the dorsal striatum have been observed in recovering alcoholics and abstinent cocaine, heroin and methamphetamine addicts (Volkow & Wise, 2005; Volkow *et al.*, 2014). Thus, it is interesting to speculate whether the postsynaptic receptors of affected offspring were desensitized and/or decreased in number, resulting in the delayed AMPH response we observed in the open field test, and whether this might contribute to drug abuse vulnerability.

To conclude, this thesis has indicated later rat pregnancy (E16) as vulnerable time for MIA to affect the development of the nigrostriatal pathway and subsequent behavioral outcomes, possibly implicating a role for MIA in increased risk for disorders associated with motor behavior, like PD. These effects are likely mediated through alterations in the placenta as well as altered inflammatory mediators. This thesis has also shown that MIA in earlier rat pregnancy (E12) results in a differential response to amphetamine in affected offspring, implicating a role for MIA in mesocorticolimbic functional alterations, possibly leaving vulnerability for disorders associated with this pathway. Epidemiological evidence has correlated first trimester (often equated to first half of rat pregnancy (Clancy *et al.*, 2007)) human bacterial infection to increased risk of schizophrenia (Sørensen *et al.*, 2009), a disease associated with malfunctioning mesocorticolimbic system, differential AMPH responding with characteristic excessive dopamine release in response to the drug (Breier *et al.*, 1997), as well as increased risk of substance addiction (Chambers *et al.*, 2001). Taken together, this may implicate a role for MIA in increased risk for disorders associated with the mesocorticolimbic pathway, including drug abuse or schizophrenia. While results of preclinical studies cannot be directly translated to humans, the current thesis suggests that timing of MIA could be useful in speculating subsequent adverse neurobehavioral outcome and associated risk factor of neurodevelopmental disorders associated with dopaminergic pathways. Using this information we may then be able to more appropriately target preventative therapies that could protect the developing fetus from adverse neurodevelopmental outcomes.

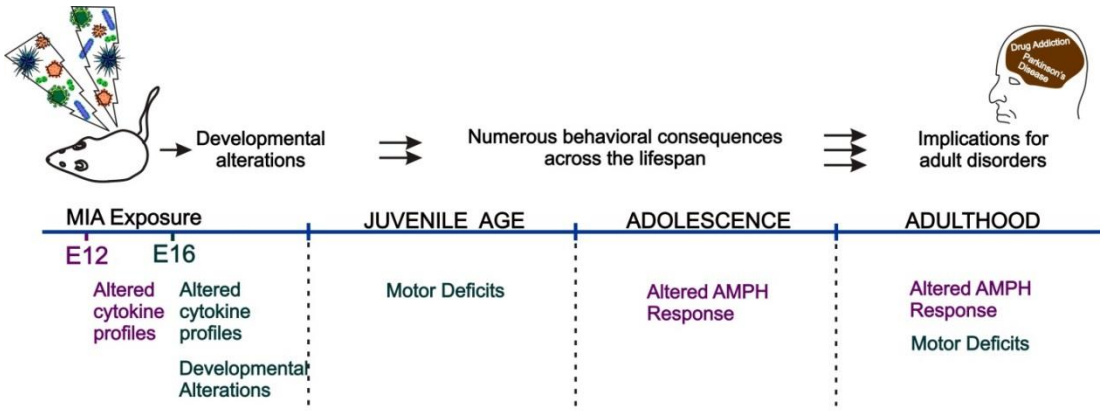


Figure 5.1: Summary figure outlining the overarching findings of this thesis

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Appendix A: List of Abbreviations

5-HIAA: 5-hydroxyindoleacetic acid

ACTH: Adrenocorticotropic hormone

AD: Alzheimer's disease

ADHD: Attention Deficit Hyperactivity Disorder

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPH: Amphetamine

ASD: Autism spectrum disorders

BAL: Bronchoalveolar

BDNF: Brain derived neurotrophic factor

CP: Cerebral Palsy

CMV: Cytomegalovirus

CNS: Central Nervous System

CNV: Copy number variants

CORT: Cortisol

CSF: Cerebrospinal fluid

DA: Dopamine/Dopaminergic

DAT: Dopamine (active) transporter

DC: Dendritic cell

DISC: Disrupted in schizophrenia

DR: Dopamine receptor

ds-RNA: Synthetic double-stranded RNA

E: embryonic day

EPH: Ephrin

FGF8: Fibroblast growth factor

FGFb: Basic fibroblast growth factor

GA: Gestational

GABA: γ-amionobutyric acid

GAD67: Glutamic acid decarboxylase 67

GDNF: Glial cell line-derived neurotrophic factor

GFAP: Glial fibrillary acidic protein

GU: Genitourinary

HPA: Hypothalamic pituitary adrenal

hs-CRP: High-sensitivity c-reactive protein

H1N1: Human influenza virus

HSD11B2: 11β-hydroxysteroid dehydrogenase type 2

IFN: Interferon

IL: Interleukin

I.N.: Intranigral

I.P.: Intraperitoneal

IRF: Interferon regulatory factor

I.V.: Intravenously

LI: Latent inhibition

LPS: lipopolysaccharide

LTP: Long term potentiation

NF-kβ: Nuclear translocation of nuclear factor-kappaβ

NPC: neuronal precursor cell

MAPK: Mitogen-activated protein kinase

MCP: monocyte chemoattractant protein 1

MIA: maternal immune activation

MIG: monokine induced by IFN-γ

MIP: macrophage inflammatory protein

MWM: Morris water maze

NAc: Nucleus accumbens

NMDA: N-methyl-D-aspartate

NO: Novel object

NPC: Neuronal progenitor cell

Nurr1: Nuclear receptor related 1 protein

NGF: Nerve growth factor

NLR: NOD-like receptors

NOD: Nucleotide binding oligomerization domain

NT: Neurotrophin

OF: Open field

PCD: Programmed cell death

PD: Parkinson's disease

PDS: Prenatal determinants of schizophrenia

Poly(I:C): Polyinosinic:polycytidylic acid

PFC: Prefrontal cortex

PPI: Pre-pulse inhibition

PRR: Pattern-recognition receptor

PVL: Periventricular leukomalacia

ROS: Reactive oxygen species

SCG: Superior cervical ganglion

Shh: Sonic hedghog

SNpc: Substantia nigra pars compacta

SSD: Schizophrenia spectrum disorders

TH: Tyrosine hydroxylase

TIR: Toll-IL-1

TNF: Tumor necrosis factor

TLR: Toll like receptor

UADD: Undifferentiated attention deficit disorder

uNK : Uterine natural killer cells

USV: ultrasonic vocalization

UTI: Urinary tract infection

VM: Ventral mesencephalon

VTA: Ventral tegmental area

Appendix B: List of Publications and Abstracts

Straley ME, Togher KL, Nolan AM, Kenny LC, O'Keeffe GW. (2014) LPS alters placental inflammatory and endocrine mediators and inhibits fetal neurite growth in affected offspring during late gestation. *Placenta*. 2014 Aug;35(8):533-8. doi: 10.1016/j.placenta.2014.06.001. Epub 2014 Jun 12.

Straley ME, Crampton SJ, Cryan JF, O'Mahony SM*, O'Keeffe GW* (2014) "The differential effects of prenatal LPS challenge on neurobehavioural outcomes in affected offspring depend on gestational age". *SFN meeting 2014*.

Straley ME, O'Keeffe GW (*in preparation*). "Current developments in maternal infection and offspring outcome: a review of neurodevelopment, behavior and disease". *To be submitted to Journal of Neuroscience.*

Straley ME, Theze SA, Hegarty SV, Crampton SJ, Cyran JF, O'Mahony SM, O'Keeffe GW (*in preparation*). LPS-induced maternal immune activation results in differential developmental and neurobehavioral outcomes depending on gestational age of exposure. *In preparation for submission to the Journal of Neuroscience*.