The Role of Early Versus Late Gestational Maternal Immune Activation in the Aetiology of Schizophrenia: Establishing a Rat Model with a Focus on Cognitive Symptomology and Neuroinflammation.

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Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository**, subject to the provisions of the Copyright Act 1968.

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Statement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included as a part of this thesis the below statement clearly outlining the extent of collaboration, with whom, and under what auspices.

Chapter 1 consists of a literature review written by myself and edited by Dr Lauren Harms, Emeritus Professor Patricia Michie, and Professor Deborah Hodgson. I was responsible for the data collection and analysis of data presented in Chapter 2. Statistical assistance for the weight data analysis in Chapter 2 was provided by statistician Kim Colyvas. Chapter 2 was written by myself and edited by Dr Lauren Harms, Emeritus Professor Patricia Michie, and Professor Deborah Hodgson. Chapter 3A is a published manuscript that I am a co-author of. Full details of the contributions to Chapter 3A are detailed in the *'Statement of Authorship'* found on page ix. I and Dr Lauren Harms are responsible for conducting the experimental the work presented in all figures in Chapter 3B. Dr Ross Fulham and Emeritus Professor Patricia Michie contributed to data processing and data analysis. Chapter 3B was written by myself and edited by Dr Lauren Harms, Emeritus Professor Patricia Michie, and Professor Deborah Hodgson. The introduction to Chapter 3 was written by myself and edited by Dr Lauren Harms, Emeritus Professor Patricia Michie, and Professor Deborah Hodgson. Chapter 4 is a published manuscript of which I am first author, in conjunction with Dr Lauren Harms. Full details of the contributions to Chapter 4 are detailed in the *''Statement of Authorship'* found on page ix. The work in Chapter 5 is a manuscript of which I am joint first author, in conjunction with Mr Ryan Duchatel. This manuscript is a final draft that was submitted to the journal *Progress in Neuropsychopharmaoclogy and Biological Psychiatry* for peer review without success. A revised version of the manuscript presented in this thesis was submitted to the journal *Psychiatry Research* for peer review on 17 November 2017, revisions were requested by the journal on 22 January 2018, and a further revised version was re-submitted to *Psychiatry Research* on 20 March 2018. Full details of the contributions to Chapter 5 are detailed in the *''Statement of Authorship'* found on page ix. Chapter 6 was written by myself and

edited by Dr Lauren Harms, Emeritus Professor Patricia Michie, and Professor Deborah Hodgson.

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Professor Deborah Hodgson

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Dr Lauren Harms

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Emeritus Professor Patricia Michie

Statement of Authorship

I hereby certify that the work embodied in this thesis contains published papers/scholarly work of which I am a joint author. I have included as part of the thesis, the below statement, endorsed by my supervisor, attesting to my contribution to the joint publications/scholarly work.

Chapter 3A is a published manuscript of which I am a co-author. I contributed to performing the experiments in conjunction with Dr Lauren Harms. I also contributed to the preparation of the manuscript for publication in conjunction with Dr Lauren Harm, Dr W Ross Fullham, Dr Juanita Todd, Dr Timothy W Budd, Conjoint Associate Professor Mick Hunter, Adjunct Professor Markku Penttonen, Professor Ulrich Schall, Dr Katerina Zavitsanou, Professor Deborah Hodgson, and Emeritus Professor Patricia Michie. Emeritus Professor Patricia Michie, Professor Deborah Hodgson, Dr Lauren Harm, Dr W Ross Fullham, Dr Juanita Todd, Dr Timothy W Budd, Conjoint Associate Professor Mick Hunter, Adjunct Professor Markku Penttonen, Professor Ulrich Schall, Dr Katerina Zavitsanou contributed to the conception and design of the experiments. While, Dr Lauren Harm, Dr W Ross Fullham, and Adjunct Professor Markku Penttonen were responsible for analysis of the data.

Chapter 4 is a published manuscript which myself and Dr Lauren Harms are joint first authors. I was responsible for performing the experiments detailed in the publication, with assistance from Dr Lauren Harms and Jade Frost. The qPCR was conducted by Rafael Barreto, on tissue samples prepared by myself. I and Dr. Lauren Harms were responsible for data analysis. The manuscript was written by myself and Dr Lauren Harms, and edited by Jade Frost, Dr Rafael Barreto, Dr Juanita Todd, Professor Ulrich Schall, Professor Cynthia Shannon Weickert, Dr Katerina Zavitsanou, Emeritus Professor Patricia Michie, and Professor Deborah Hodgson. I along with Dr Lauren Harms, Dr Juanita Todd, Professor Ulrich Schall, Professor Cynthia Shannon Weickert, Dr Katerina Zavitsanou, Emeritus Professor Patricia Michie, and Professor Deborah Hodgson contributed to the conception and design of the experiments.

Chapter 5 is a manuscript of which I am joint first author with Mr Ryan Duchatel. The manuscript wasill be submitted to the journal Progress in Neuropsychopharmaoclogy and Biological Psychiatry without success. A revised

version of the manuscript presented in this thesis was submitted to the journal Psychiatry Research for peer review on 17 November 2017, revisions were requested by the journal on 22 January 2018, and a further revised version was re-submitted to Psychiatry Research on 20 March 2018in the near future.. The experimental work presented in all figures of Chapter 5 was conducted by myself in collaboration with Mr. Ryan Duchatel from the School of Biomedical Sciences, The University of Newcastle. Assistance with immunohistochemistry data processing and use of laboratory equipment was provided by Associate Professor Rohan Walker. Assistance with qPCR analysis was provided by Mr Mark Bigland and Associate Professor Douglas Smith. Data analysis was undertaken by myself and Mr Ryan Duchatel. Chapter 5 was written by myself and Mr Ryan Dutchatel, and edited by Associate Professor Paul Tooney, Dr Lauren Harms, Emeritus Professor Patricia Michie, Associate Professor Rohan Walker, Mr Mark Bigland, Associate Professor Douglas Smith, Dr Phillip Jobling, and Professor Deborah Hodgson. I along with Mr. Ryan Dutchatel, Dr Lauren Harms, Associate Professor Paul Tooney, Emeritus Professor Patricia Michie, and Professor Deborah Hodgson contributed to the conception and design of the experiment.

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Emeritus Professor Patricia Michie

Published Works Incorporated in this Thesis

1. **Harms, L.,** Fulham, W. R., Todd, J., Budd, T. W., Hunter, M., Meehan, C., Penttonen, M., Schall, U., Zavitsanou, K., Hodgson, D. M., Michie, P. T. (2014). Mismatch negativity (MMN) in freely-moving rats with several experimental controls. *PLoS One, 9*(10), e110892.

2. **Meehan, C., Harms, L.,** Frost, J. D., Barreto, R., Todd, J., Schall, U., Shannon Weickert, C., Zavitsanou, K., Michie, P. T., Hodgson, D. M. (2017). Effects of immune activation during early or late gestation on schizophrenia-related behaviour in adult rat offspring. *Brain Behaviour and Immunity, 63*, 8-20.

3. **Duchatel, R. J., Meehan C. L.,** Harms L., Michie P. T., Bigland M. J., Smith D. W., Walker F. R., Jobling, P., Hodgson D. M., Tooney, P. A. Maternal immune activation increases microglia levels in the white matter of the corpus callosum of adult rat offspring. [Submitted to *Progress in Neuropsychopharmacology and Biological Psychiatry* without success. A revised version of the manuscript presented in this thesis was submitted to the journal *Psychiatry Research* for peer review on 17 November 2017, revisions were requested by the journal on 22 January 2018, and a further revised version was re-submitted to *Psychiatry Research* on 20 March 2018]

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Abstract

Schizophrenia is a debilitating disorder of neurodevelopmental origins that likely stems from the cumulative action of a range of genetic and environmental factors. Epidemiological evidence has identified maternal infection during gestation as one significant environmental risk factor for the development of the disorder. Evidence from animal models has further validated the link between maternal immune activation (MIA) in the absence of an active infection and the later life development of schizophrenia-like pathology in the offspring. In particular, work in mouse models has suggested that the gestational time at which MIA occurs can alter the behavioural and neurobiological phenotype displayed. Specifically, that MIA in late gestation is involved in schizophrenia-relevant cognitive dysfunction and altered NMDA receptor expression, whereas MIA in early gestation is more closely associated with behavioural deficits reminiscent of positive symptomology and dopaminergic neurotransmission. The aim of the current thesis was to extend the mouse findings to another species, the rat, and further explore the effects of MIA. In addition to producing a reliable rat model of schizophrenia where distinct behavioural and neurological phenotypes associated with schizophrenia are produced following MIA at either early or late gestational time-points (gestational day 10 or 19, respectively), the current thesis extends on previous work by examining the schizophrenia biomarker of mismatch negativity and assessing the neuroinflammatory state of offspring.

Behavioural assessments revealed that MIA in either early or late gestation produced transient impairments in working memory and reductions in PPI. In these behavioural studies, there was no clear distinction between a dopamine and glutamate-related behavioural phenotype based on the gestational timing of exposure. However,

early but not late gestation MIA did produce alterations in the dopaminergic system of males, as indicated by increased dopamine 1 receptor mRNA in the nucleus accumbens. EEG experiments demonstrated that although the male rat brain is able to generate human-like (adaptation-independent) mismatch responses (MMRs), and although MIA (regardless of gestational timing) does alter MMRs, it does not do so in a manner comparable with schizophrenia. Immunohistochemical techniques revealed that MIA does result in subtle neuro-immune changes in adult offspring, with an increase in microglial immunoreactivity identified in the frontal white matter of late, but not early, gestation MIA animals. Furthermore, a strong trend towards increased astrocyte immunoreactivity that approached significance was identified in the prefrontal cortex of late, but not early MIA offspring.

The combined results have demonstrated that MIA during the chosen gestational time-points are sufficient to disrupt neurodevelopmental processes producing long-term alterations in behavioural and neuropathological measures relevant to schizophrenia. However, the phenotype characterised here deviates slightly from previous findings from mouse models indicating potential differences in the critical periods of neurodevelopmental susceptibility to MIA exposure between the rat and mouse. Importantly this research has provided insights into the underlying neuro-immune changes which may contribute to the behavioural abnormalities seen in adult MIA offspring and has provided evidence that MIA in rats can alter the prominent schizophrenia relevant electrophysiological biomarker of adaptation-independent MMRs, providing a basis to further investigate these measures and their underlying mechanisms.

Abbreviations

Abbreviation	Description
µg	micrograms
µl	microliter
µm	micrometre
µV	microvolts
♀	female
♂	male
11β-HSD	11β-hydroxysteroid-dehydrogenase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPH	amphetamine
ANOVA	Analysis of Variance
BMI	body mass index
cDNA	complimentary DNA
cm	centermeter
CNS	central nervious system
CON	control
CORT	corticosterone
CPu	caudate putamen
D1r	dopamine 1 receptor
D2r	dopamine 2 receptor
D3r	dopamine 3 receptor
D4r	dopamine 4 receptor
D5r	dopamine 5 receptor
DA	dopamine
DAr	dopamine receptor
DAT	dopamine transporter
dBL	decibels
DEV	deviant
DISC1	disrupted-in-schizophrenia-1
EDTA	Ethylenediaminetetraacetic acid
EEG	electroencephalogram

ELISA	enzyme-linked immunosorbent assay
ERP	event related potential
g	grams
GABA	gamma-aminobutyric-acid
GD	gestational day
h	hours
HPA	hypothalamic-pituitary-adrenal
Hz	hertz
i.p.	intraperitoneal
ITI	intertrial interval
kg	killogram
kHz	kilohertz
LAC	left auditory cortex
L-DOPA	dihydroxyphenylalanine
LFC	left frontal cortex
LML	left of midline
LMM	linear mixed model
LPS	lipopolysaccharide
m	meter
M	mole
mg	milligram
MIA	maternal immune activation
min	minute
MK-801	Dizocilpine
mL	milliliter
MLR	mid latency response
MMN	mismatch negativity
MMR	mismatch response
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
ms	milliseconds
MSC	Many-standards control
mV	millivolts

NAc	nucleus accumbens
ng	nanograms
NMDA	N-methyl-D-aspartate
NMDAr	N-methyl-D-aspartate receptor
PBS	phosphate buffered saline
PCP	phencyclidine
PET	positoron emission tomography
PFC	prefrontal cortex
pg	picograms
PND	postnatal day
Poly (I:C)	polyriboinosinic-polyribocytidilic acid
PPI	prepulse inhibition
qPCR	real-time quantitative polymerase chain reaction
RAC	right auditory cortex
RFC	right frontal cortex
RNA	ribonucleic acid
RNase	Ribonuclease
s.c.	subcutanious
SEM	standard error of the mean
SN	substantia nigra
SOA	stimulus onset asynchrony
SPSS	statistical package for the social sciences
STD	standard
TH	tyrosine hydroxilaise
TLR3	toll like receptor 3
TLR4	toll like receptor 4
VHL	ventral hippocampal lesion
VTA	ventral tegmental area

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1. Chapter 1: General Introduction

Schizophrenia is a chronic neuropsychiatric disorder associated with disrupted mental functioning and abnormal behaviour which results in significant and persistent disability in all aspects of an affected patient's life (Lewis & Lieberman, 2000; Wilson & Terry, 2010). The disorder affects approximately 1% of the population with onset and diagnosis typically occurring between late adolescence and early adulthood (Lewis & Moghaddam, 2006). Patients with schizophrenia can present with a wide range of symptoms which fall into three main categories; positive symptoms, negative symptoms and cognitive impairments. Positive symptoms, which produce psychosis, include disordered thought, agitation, delusions and hallucinations. Negative symptoms include flat affect, anhedonia (inability to experience pleasure), lack of motivation, and social withdrawal. Cognitive impairments are characterised by deficits in executive function, verbal learning and memory, attention, working memory, processing speed, and social cognition (Gray & Roth, 2007; Lewis & Lieberman, 2000; Wilson & Terry, 2010; Young, Powell, Risbrough, Marston, & Geyer, 2009). The presentation profile of each patient can vary significantly as individual patients will differ with regard to which symptoms they present with and also the severity of those symptoms. Currently, there is no universal symptom or biomarker which can be used to aid in diagnosis of the disorder.

There are a number of genetic variations which have been shown to be involved in schizophrenia; however, the heritability rate and concordance rate among monozygotic twins suggests that there must also be other factors which contribute to the aetiology of the disorder (Olgiati et al., 2009; Shih, Belmonte, & Zandi, 2004; Stefansson et al., 2008; Sullivan, 2005). In fact, despite the onset of symptoms usually

occurring in adulthood, there is a strong empirical basis for the disorder having a neurodevelopmental origin as far back as the prenatal stages of development (Lewis & Levitt, 2002; Meyer, 2011; Meyer & Feldon, 2010; Weinberger, 1987). There are a wide range of environmental factors which have been associated with an increased risk of schizophrenia including obstetric complications (Cannon, Jones, & Murray, 2002b; Hultman, Sparén, Takei, Murray, & Cnattingius, 1999), maternal nutritional deficiency (Brown & Susser, 2008; Brown et al., 1996; Hoek, Brown, & Susser, 1998; Susser et al., 1996; Susser & Lin, 1992), maternal stress (Khashan et al., 2008; Malaspina et al., 2008; van Os & Selten, 1998), migration (Selten, Cantor-Graae, & Kahn, 2007), and maternal infection (Babulas, Factor-Litvak, Goetz, Schaefer, & Brown, 2006; Brown et al., 2004a; Brown & Derkits, 2010; Brown et al., 2004b; Brown et al., 2005; Sorensen, Mortensen, Reinisch, & Mednick, 2009; Torrey, Rawlings, & Waldman, 1988). Maternal immune activation (MIA) through exposure to infectious agents during the prenatal period is one environmental factor which has received increased attention in recent years. There is considerable evidence from epidemiological studies which indicate that infections during the prenatal period can result in up to a 7 fold increase in the risk of developing schizophrenia (Brown et al., 2004a; Brown & Derkits, 2010). In addition to this epidemiological data a number of animal models have also now demonstrated that maternal infection during pregnancy can lead to schizophrenia-related behavioural and neurobiological changes in the adult offspring (Howland, Cazakoff, & Zhang, 2012; Li et al., 2009; Meyer et al., 2008a; Meyer et al., 2008b; Wolff & Bilkey, 2008; Wolff, Cheyne, & Bilkey, 2011; Zuckerman, Rehavi, Nachman, & Weiner, 2003; Zuckerman & Weiner, 2005). Further developing our understanding of how such environmental factors can impact on the developing prenatal brain would not only extend our knowledge on the aetiology and mechanisms involved in the disorder, but

may lead to possible preventative measures or new treatment targets. To date, alterations in both the dopaminergic and glutamatergic neurotransmitter systems have been strongly linked to schizophrenia in both the human population and also in animal models of the disorder (Eyles, Feldon, & Meyer, 2012; Howes & Kapur, 2009; Konradi & Heckers, 2003; Stone, Morrison, & Pilowsky, 2007). There is evidence to suggest that positive symptomology is more strongly related to altered dopamine (DA) neurotransmission (Howes & Kapur, 2009), whereas alterations in glutamatergic function have been more closely associated with the cognitive symptoms of the disorder (Lewis & Moghaddam, 2006; Moghaddam & Javitt, 2012). However, a complete understanding of how dysfunction in these systems is related to the disorder and its specific symptoms, or how environmental factors such as MIA influence these systems has not yet been obtained. Evidence from a mouse model of MIA has suggested that the specific prenatal timing at which exposure to an immune stimulating agent occurs may be one factor that could influence the final symptom profile displayed in the offspring (Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008c; Meyer, Yee, & Feldon, 2007). It was found that MIA occurring in the earlier stages of gestation was more likely to result in mice displaying altered dopaminergic function and a behavioural phenotype more reminiscent of positive symptoms (Li et al., 2009; Meyer et al., 2008a; Meyer et al., 2008b; Meyer et al., 2008c). Whereas MIA in the later stages of gestation was found to produce alterations in the glutamatergic system and more cognitive symptom-like behavioural changes (Meyer et al., 2006; Meyer et al., 2008c).

The use of animal models provides a way for the scientific community to investigate how exposure to environmental risk factors for a disorder may alter the brain, its functioning, and any subsequent behavioural disturbances. Rodents, both rats and mice, are widely used in psychological and neuroscience research due to the

thorough understanding of their brain structure and function, cost effectiveness, and ease of use in research settings. Although mice are the more commonly used animal, especially for investigating genetic risk factors, rats may actually be a better choice. The more complex and wider behavioural repertoire of rats allows for a more comprehensive range of behavioural and electrophysical assessments, and may be better suited when aiming to model aspects of a highly complex human disorder such as schizophrenia.

1.1. Psychosis and Dopamine in Schizophrenia

1.1.1. Positive Symptomology in Schizophrenia

The positive symptoms of schizophrenia are the most recognisable of the three symptom categories and are historically considered to be the core deficits of the disorder. Positive symptoms are episodic in nature with the duration of psychotic episodes being variable (Addington & Addington, 1991). The presentation or relapse of psychotic symptoms often results in hospitalisation of the patients and results in increased health care costs (Gilmer et al., 2004). The duration of untreated psychosis has also been associated with poorer functional outcome measures in schizophrenia patients (Perkins, Gu, Boteva, & Lieberman, 2005). A number of studies have reported that the longer the period of psychosis before antipsychotic treatment is commenced, the poorer patients perform on a number of functional outcome measures including the Heinrich-Carpenter Quality of Life measure (Harrigan, McGorry, & Krstev, 2003), the Global Assessment of Functioning Scale (Melle et al., 2004), and measures of social functioning and disability (Addington, Van Mastrigt, & Addington, 2004; Wiersma et al., 2000). These findings highlight that although psychosis in schizophrenia is episodic in nature, there are long term consequences to these psychotic episodes which persist after symptom amelioration.

Current antipsychotic medication, both first generation (typical) and second generation (atypical) antipsychotics, are largely effective in treating the positive symptomology of schizophrenia (Carpenter & Koenig, 2008). The majority of patients experience considerable to full remission of positive symptoms following their initial psychotic episode when treated promptly (Lieberman et al., 1993). However, response to treatment in subsequent psychotic episodes can be reduced, and may not result in complete remission of symptoms (Lieberman et al., 1996). Indeed, relapse of psychotic episodes often occurs in patients as a result of self-discontinuation of medication, and compliance with medication is reduced with increased severity of positive symptomology (Fenton, Blyler, & Heinssen, 1997). Typical antipsychotics are high affinity antagonists of the dopamine 2 receptor (D2r) and are known to produce severe neurological side effects. The mode of action of atypical antipsychotics is also antagonism of the D2r but at a much lower affinity than of the typical antipsychotics (Carpenter & Koenig, 2008). In addition, atypical antipsychotics also have a strong affinity for several other receptor types, most notably, the serotonin receptors (Lewis & Moghaddam, 2006; Meltzer & McGurk, 1999; Wilson & Terry, 2010). Atypical antipsychotics tend to produce less severe neurological side effects than typical antipsychotics, however, they are known to produce serious metabolic side effects (Carpenter & Koenig, 2008). The often severe side effects of these antipsychotic agents is known to contribute to medication non-compliance and subsequent relapse of psychotic symptoms (Fenton et al., 1997). A number of studies have reported an association between discontinuation of medication and physician ratings of side effect severity (Buchanan, 1992; Nelson, Gold, Hutchinson, & Benezra, 1975; Pan & Tantam, 1989), while patient self-reports also highlight antipsychotic side-effects as a significant

factor contributing to medication non-compliance (del Campo, Carr, & Correa, 1983; Hogan, Awad, & Eastwood, 1983; Kelly, Mamon, & Scott, 1987).

The common mode of action for all antipsychotic medications, in addition to the correlation between their effectiveness in treating positive symptomology and affinity for the D2r (Creese, Burt, & Snyder, 1976; Seeman & Lee, 1975) provides evidence for the role of hyperactive subcortical DA function in active psychosis. These observations contributed to the establishment of the DA hypothesis of schizophrenia (Howes & Kapur, 2009).

1.1.2. Dopamine Hypothesis of Schizophrenia

DA is one of the major catecholamine neurotransmitters in the central nervous system (CNS) (Meisenzahl, Schmitt, Scheuerecker, & Moller, 2007). DA synthesis involves the hydroxylation of tyrosine to dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH), which is the rate limiting step of the DA synthesis process. L-DOPA is then converted to DA by Aromatic-amino-acid-decarboxylase (Meisenzahl et al., 2007). There are 5 DA receptors (DAr) which can be divided into two subtypes, the D1-like receptors (D1r & D5r) and the D2-like receptors (D2r, D3r, & D4r). Each subtype is differentially located within the brain with D1r most prominent in cortical regions including the prefrontal cortex (PFC), D2r are mostly concentrated in the striatum, D3r is predominantly located in the ventral striatum, D4r is largely found in the PFC and hippocampus, while D5r is prominent in the hippocampus (Meador-Woodruff et al., 1996; Meisenzahl et al., 2007). Dysregulation of the dopaminergic system has long been implicated in the generation of psychotic symptoms. The DA hypothesis of schizophrenia originally suggested the symptoms of the disorder, particularly the positive symptoms, are due to an over-activation of subcortical DA

neurotransmission at the D2r (Carlsson & Lindqvist, 1963; Carpenter & Koenig, 2008; Eyles et al., 2012). Later iterations of the hypothesis have expanded to include new evidence which implicated reduced D1r neurotransmission in the PFC, postulating that this may contribute to the negative and cognitive symptoms of the disorder (Davis, Kahn, Ko, & Davidson, 1991; Laruelle, Kegeles, & Abi-Dargham, 2003).

Many converging lines of evidence including pharmacological, neuroimaging, post-mortem and animal model studies support the involvement of altered dopaminergic neurotransmission in the pathophysiology of schizophrenia, specifically psychotic symptomology (Meisenzahl et al., 2007). In addition to the now well established finding that antagonists of the D2r are effective in reducing psychotic symptoms (Carpenter & Koenig, 2008), direct and indirect DA-stimulating drugs, such as amphetamine (AMPH) and Phencyclidine (PCP), are capable of inducing or exacerbating psychotic symptoms in both schizophrenia patients and health controls (Abi-Dargham et al., 1998; Curran, Byrappa, & Mcbride, 2004; Laruelle et al., 1996; Lieberman, Kane, & Alvir, 1987; Srisurapanont et al., 2011). Patients with schizophrenia are also known to have more reactive dopaminergic systems than healthy individuals and are more sensitive to the effects of such psychostimulant drugs as a result (Janowsky, el-Yousel, Davis, & Sekerke, 1973; Lieberman et al., 1987).

A number of studies using positron emission tomography (PET) to assess the dopaminergic neurotransmission in the brains of living patients have reported increased density of D2r, increased AMPH-induced DA neurotransmission, and increased Aromatic-amino-acid-decarboxylase activity (reviewed in Laruelle (1998)). Although, antipsychotic drug treatment is a potential confounding factor in such studies, research using drug-naïve patients indicates that altered DA neurotransmission is related to the disorder itself, rather than solely a consequence of antipsychotic treatments (Abi-

Dargham et al., 2000; Wong et al., 1986). A number of neuroimaging studies have now convincingly demonstrated that, in comparison to controls, patients with schizophrenia have increased striatal synaptic DA levels in response to psychomimetic drug administration (Abi-Dargham et al., 1998; Breier et al., 1997). In addition, neuroimaging studies have also identified elevations in pre-synaptic DA synthesis in the striatum of schizophrenia patients (McGowan, Lawrence, Sales, Quested, & Grasby, 2004; Meyer-Lindenberg et al., 2002). Although increased striatal post-synaptic D2r density has been reported in schizophrenia patients using PET (Abi-Dargham et al., 2000; Wong et al., 1986), there are inconsistencies in the literature (Farde, Wiesel, Stone-Elander, & et al., 1990; Nordstrom, Farde, Eriksson, & Halldin, 1995), indicating that such changes are not a well-established phenomenon in the disorder (Howes et al., 2009a). It has also been reported that patients in the prodromal stage of the disorder have altered DA functioning, with a PET study revealing increased DA uptake in the striatum of patients pre-diagnosis, further indicating that dysfunction in DA signalling may contribute to symptom onset (Howes et al., 2009b).

Given that current antipsychotic treatments, all acting through antagonism of D2r, are largely ineffective in treating the cognitive and negative symptoms of schizophrenia (Carpenter & Koenig, 2008), it has become widely accepted that models of dopaminergic dysfunction alone are not able to completely account for the full range of symptomology seen in schizophrenia, but rather are more aptly suited to explaining psychosis. Indeed, dysfunction of other neurochemical systems which are modulated by DA, specifically the glutamatergic system, have now been closely associated with the negative and cognitive symptoms of the disorder (Konradi & Heckers, 2003), and D2 agonist based antipsychotics are known to also influence the glutamate system (Leveque et al., 2000).

1.2. Cognitive Dysfunction, Mismatch Negativity (MMN), and Glutamate in Schizophrenia

1.2.1. Cognitive Symptomology in Schizophrenia

Although the positive symptoms of schizophrenia are the most 'typical' or obvious symptoms of schizophrenia, it has been shown that cognitive deficits are a core feature of the disorder with estimates that up to 85% of patients suffer from cognitive dysfunction (Gray & Roth, 2007). Indeed, cognitive symptoms usually manifest long before the onset of positive symptoms and not only persist after the successful remediation of psychotic symptoms but remain stable throughout the course of the disease (Cornblatt, Obuchowski, Roberts, Pollack, & Erlenmeyer-Kimling, 1999; Gold, 2004; Heaton et al., 2001). And although reduced performance motivation, fear about the outcome of cognitive tests, and distracting symptoms have been identified as factors which contribute to the poor performance of patients with schizophrenia on cognitive assessments, when accounted for such factors were not able to completely explain the difference in cognitive performance between controls and patients (Moritz et al., 2017). In addition, the severity of cognitive dysfunction has been shown to be more closely related to a range of long-term functional outcome measures (Green, 1996, 2006), increased risk of relapse (Chen et al., 2005) and reduced medication compliance than is the severity of positive symptoms (Gray & Roth, 2007).

A meta-analysis conducted by (Green, 1996) highlighted that the degree of cognitive deficits, in particular deficits in verbal memory and executive function, were good predictors of a range of functional outcome measures which included level of social and occupational functioning, skills acquisition, and social problem solving. There was, however, a consistent lack of association found between positive symptoms and functional outcomes across all of the studies examined. A later study by McGurk,

Mueser, Walling, Harvey, and Meltzer (2004) has also shown that patients with more severe executive function deficits required more outpatient services than those with milder deficits and that the severity of positive and negative symptoms was not a good predictor of the amount of outpatient services required. The severity of cognitive dysfunction has also been identified as a main factor which contributes to reduced medication compliance, which in turn can lead to poorer functioning and increased risk of relapse (Kazadi, Moosa, & Jeenah, 2008). A study by Jeste et al. (2003) found that the severity of cognitive impairment was more directly related to a patient's ability to comply with a medication treatment plan than severity of positive symptoms. Taken together these studies highlight the significant impact which cognitive dysfunction can have on patients rehabilitation and their ability to function on a daily basis. The majority of schizophrenia patients remain reliant on family as well as clinical, community, and financial support services to assist with basic living skills as many are unable to independently maintain treatment regimes, full-time employment, or interpersonal relationships (Bowie & Harvey, 2006).

Although the need for effective management of cognitive dysfunction is clear, there has been, until recently, limited emphasis placed on the development of new treatments which target cognitive symptoms (Lyon, Saksida, & Bussey, 2012; Wilson & Terry, 2010). In fact, no major developments in the psychopharmacology of schizophrenia have been made since the introduction of the current treatment options in the 1950's, the first generation (typical) and second generation (atypical) anti-psychotics (Carpenter & Koenig, 2008). Both classes of drugs are effective in treating positive symptoms, but have only been shown to produce small and inconsistent improvements in some areas of cognition, however, overall cognition is still impaired (Gold, 2004; Meltzer & McGurk; Mishara & Goldberg, 2004).

Research into new pharmacological approaches which target the range of cognitive dysfunctions present in schizophrenia is needed. The fact that current DA-based treatments are predominantly ineffective in reducing cognitive dysfunction, along with the fact that cognitive symptoms present before positive symptoms and are stable throughout the course of the disease, despite fluctuations in the positive symptoms, suggests that the cognitive and positive symptoms of schizophrenia may be the result of dysfunction in separate neural systems (Gold, 2004). Indeed, involvement of the neurotransmitter glutamate has been implicated particularly in cognitive and negative symptoms leading to the identification of the glutamate synapse as a major target for future drug development (Lewis & Moghaddam, 2006; Moghaddam & Javitt, 2012).

1.2.2. Glutamate Hypothesis of Schizophrenia

Glutamate is the principal excitatory neurotransmitter released from the majority of neurons, including all pyramidal neurons, within the mammalian brain. It has three different receptor types, the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and Kainate receptors (Gray & Roth, 2007). Glutamatergic neurotransmission plays a major role in synaptic formation, development and maintenance throughout all stages of brain development and senescence. Glutamate is also vital to long term potentiation and synaptic plasticity, the process by which the strength of synaptic connections is changed, which has been identified as one of the mechanisms which underlies important cognitive functions such as memory and learning. Perturbation of the glutamate system can disrupt all of these neuronal functions resulting in atypical neuronal development, plasticity, function, neurotoxicity and neuronal death (Konradi & Heckers, 2003). Dysfunction in the glutamatergic system has been strongly associated with schizophrenia and is now widely believed to

be involved in the aetiology of the disorder (Lewis & Lieberman, 2000; Lyon et al., 2012; Moghaddam & Javitt, 2012). In the normally functioning brain, activation of glutamate receptors typically causes depolarisation of the post-synaptic neuron resulting in its subsequent activation. The firing of pyramidal neurons is under the regulation of inhibitory gamma-aminobutyric-acid (GABA)-ergic interneurons which are also activated by the release of glutamate. These GABA interneurons regulate pyramidal neuron activation via feed-forward inhibition; the simultaneous activation of these inhibitory GABAergic interneurons dampens or inhibits the activation of the post-synaptic pyramidal neuron and prevents an uncontrolled runaway excitation effect. It is now understood that hypo-function of NMDA receptors (NMDAr) on these inhibitory GABA interneurons decreases their firing which in turn disinhibits pyramidal neurons resulting in their over-activation (Moghaddam & Javitt). The consequence of this is increased release of glutamate and excessive activation of other glutamate receptor types which causes even further overstimulation of downstream neurons resulting in a large amount of unregulated activity or noise (Farber, 2003).

The first line of evidence which suggested the involvement of NMDAr hypo-function in schizophrenia came from the study of the NMDAr antagonists, Ketamine, and Dizocilpine (MK-801) which non-competitively block the channel of the NMDAr preventing it from being activated (Moghaddam & Javitt, 2012). It was discovered that when NMDAr antagonists are given to healthy subjects at sub-anaesthetic doses, they produce a range of positive, negative and cognitive symptoms analogous to those seen in schizophrenia (Konradi & Heckers, 2003). In addition to producing psychotic states in healthy subjects, blockade of the NMDAr by its antagonists also significantly exacerbates symptoms in patients with schizophrenia (Lahti, Koffel, LaPorte, & Tamminga, 1995). NMDAr antagonists have been shown to induce thought disorder,

altered perception, blunted emotions, and decreased motivation in healthy individuals, but most notably produce deficits in multiple cognitive domains including working memory and learning which are unable to be distinguished from those of patients with schizophrenia (Krystal et al., 1994; Krystal et al., 2005; Rowland et al., 2005). In fact, one study found no differences in a direct comparison of deficits seen in patients with schizophrenia and those seen in healthy subjects administered with low doses of Ketamine, lending weight to the notion that a similar mechanism may underlie deficits in both groups (Adler et al., 1999). The ability of NMDAr antagonists to produce schizophrenia-like behaviour in animals such as rodents and monkeys has also been well established, with this technique often used to model the cognitive symptoms of the disorder (Konradi & Heckers, 2003).

Further evidence which lends support to NMDAr hypo-function in the aetiology of schizophrenia, particularly the cognitive symptoms, has come from the post-mortem study of brains from patients with schizophrenia (Catts, Lai, Weickert, Weickert, & Catts, 2016; Konradi & Heckers, 2003). It has been reported that patients with schizophrenia have reduced mRNA and protein expression of the NMDAr NR1 subunit and reduced mRNA expression of the NMDAr NR2C subunit in the PFC (Beneyto & Meador-Woodruff, 2008; Catts et al., 2016; Shannon Weickert et al., 2013; Sokolov, 1998). In addition, it has been shown that in brains from schizophrenia patients who showed severe cognitive deficits, transcription of the NR1 subunit is decreased in the frontal and temporal cortices. However, no such reduction was found in the brains of schizophrenic patients who did not show marked cognitive deficits. This finding highlights the involvement of NMDAr dysregulation in the cognitive aspects of the disorder (Humphries, Mortimer, Hirsch, & de Belleroche, 1996). In addition to NMDAr irregularities, a number of studies have also reported a reduction in the expression of

AMPA and kainite receptor subunits and reduced receptor binding in the hippocampus and parahippocampal regions (Eastwood, Kerwin, & Harrison, 1997; Eastwood et al., 1995; Kerwin, Patel, & Meldrum, 1990; Porter, Eastwood, & Harrison, 1997). This down-regulation of AMPA and kainite receptors may be a consequence of the increased glutamate levels caused by the disinhibition of pyramidal neurons which is in line with the NMDAr hypo-function hypothesis (Konradi & Heckers).

The NMDAr hypo-function model has also been assessed using genetic rodent models in which a gene or genes that code for the NMDAr subunits have been removed or 'knocked out' leaving the animals without fully functioning NMDAr (Konradi & Heckers, 2003). Mice with knockout of NMDAr subunits in the hippocampus and frontal regions, two areas strongly associated with schizophrenia and cognition, have been shown to display many behavioural symptoms, particularly cognitive deficits, analogous to those seen in schizophrenic patients and in NMDAr antagonist models of the disorder. It has been found that mice with knockout of the NR2B subunit in the forebrain region show deficits in spatial working memory, reference memory, recognition memory and contextual memory tasks while knockout in the hippocampus produces impairments in spatial working memory (von Engelhardt et al., 2008; Zhao et al., 2005). NR1 knockout in the dentate gyrus hippocampal region of mice has also been shown to result in spatial working memory deficits, but not in reference memory deficit on the radial arm maze task. In addition, long term potentiation in these NR1 knockout mice was also found to be impaired (Niewoehner et al., 2007). In addition to deficits in memory which indicate cognitive dysfunction, NR1 knockout mice have also shown deficits in social interactions and sexual behaviour which is analogues to negative symptomology, as well as showing positive symptoms of hyper-locomotion, stereotypy,

and sensorimotor gating deficits (Duncan et al., 2004; Halene et al., 2009; Mohn, Gainetdinov, Caron, & Koller, 1999).

In addition to inducing a range of schizophrenia-like behaviours, NR2A knockout mice have been found to have reduced GABA release (Miyamoto et al., 2001). This observation lends support to the notion that reduced NMDAr function causes a decrease in GABA release which in turn disinhibits pyramidal neurons (Konradi & Heckers, 2003). More evidence which supports NMDAr hypo-function in GABAergic dysfunction came from a study by Belforte et al. (2010) in which they produced a mouse strain with selective NR1 deletion only on cortical and hippocampal GABAergic interneurons. These mice displayed reduced GABA function in the NR1 deleted cortical and hippocampal interneurons as indicated by reduced glutamic-acid-decarboxylase-67, the GABA synthesising enzyme. In addition to reduced GABA synthesis, in vivo recordings showed increased activation of pyramidal neurons and disruptions to neural synchrony in the primary somatosensory cortical region of NR1 deficient mice.

Taken together, these lines of evidence confirm the involvement of NMDAr hypo-function in the aetiology of schizophrenia and although NMDAr hypo-function is known to result in altered perceptions and other positive symptomology, it has been most prominently implicated in the cognitive and negative symptoms of the disorder. This is not surprising given that glutamate and the NMDAr are known to play a vital role in neuronal plasticity and long term potentiation which is critical for normal cognitive functioning (Gray & Roth, 2007; Rezvani, 2006). Altered NMDAr functioning has also been linked to the abnormal electroencephalography (EEG) components found in schizophrenia, specifically reductions in the mismatch negativity

(MMN), which has been identified as the most promising biomarker for the disorder to date.

1.2.3. MMN in Schizophrenia

The MMN is a component of the event-related potential (ERP), typically measured by EEG, that occurs when an unexpected stimulus (a *deviant*, DEV) is presented at random intervals in an established pattern of repetitive stimuli (*standards*, STD). This EEG component is most commonly measured using auditory stimuli with a DEV that differs in some physical attribute (pitch, intensity or duration) from the STDs (Harms, Michie, & Naatanen, 2016).

Reductions in the size of the MMN (the difference between the DEV response and the STD response) in patients with schizophrenia is a robust and well-established finding. A large number of studies have consistently reported MMN reductions in patients with schizophrenia (Bodatsch, Brockhaus-Dumke, Klosterkotter, & Ruhrmann, 2015; Umbricht & Krljes, 2005) since it was first reported by Shelley et al. (1991) 25 years ago. Reductions in MMN are not only common in schizophrenia but also appear to be reasonably specific to the disorder (Umbricht et al., 2003), with patients diagnosed with other psychopathologies including depression (Umbricht et al., 2003), bipolar disorder (Catts et al., 1995; Umbricht et al., 2003), and obsessive-compulsive disorder (Oades, Dittmann-Balcar, Zerbin, & Grzella, 1997; Oades, Zerbin, Dittmann-Balcar, & Eggers, 1996; Towey et al., 1994) failing to show any alterations in the MMN. Reductions in MMN also appear to be largely stable in patients with schizophrenia. MMN deficits have been shown to persist well beyond the acute phase of the disorder (Shinozaki et al., 2002) and persist in patients following treatment with a range of antipsychotic medications (Korostenskaja et al., 2005; Schall, Catts, Karayanidis, &

Ward, 1999; Umbricht et al., 1998). First degree relatives of patients with schizophrenia have also been reported to display reductions in MMN (Michie, Innes-Brown, Todd, & Jablensky, 2002) in addition to individuals identified as being at high-risk of developing the disorder (Perez et al., 2014). Reductions in MMN have been shown to be predictive of progression to symptom onset and diagnosis in these high-risk individuals (Bodatsch et al., 2011; Perez et al., 2014). Reduced MMN has also been linked to poorer functional outcome measures and level of independence in patients with schizophrenia. It has been demonstrated that patients with larger MMN reductions are rated more poorly than those with smaller MMN reductions on the Global Assessment of Functioning Scale and are more likely to live in highly structured rather than independent environments (Kawakubo & Kasai, 2006; Light & Braff, 2005a, 2005b).

Glutamatergic functioning, specifically NMDAr function, is known to play a role in MMN. There is now ample evidence from NMDAr antagonist models indicating that hypo-function of the NMDAr may contribute to the MMN reductions seen in the schizophrenic population. It has been demonstrated that healthy controls exposed to NMDAr antagonists such as Ketamine exhibit reductions in MMN similar to those seen in patients (Heekeren et al., 2008; Umbricht, Koller, Vollenweider, & Schmid, 2002; Umbricht et al., 2000) in addition to producing psychosis-like symptoms (Gunduz-Bruce et al., 2012). A number of animal models have also found similar results, with administration of a range of NMDAr antagonists shown to produce reduced MMN-like responses (MMR) in primates (Gil-da-Costa, Stoner, Fung, & Albright, 2013; Javitt, Steinschneider, Schroeder, & Arezzo, 1996), rats (Sivarao et al., 2014; Tikhonravov et al., 2008), and mice (Chen, Helmchen, & Lutcke, 2015; Ehrlichman, Maxwell, Majumdar, & Siegel, 2008). In addition to producing MMRs, animals from NMDAr

antagonist models also exhibit behavioural deficits relevant to schizophrenia (Andine et al., 1999; Becker et al., 2003; Sams-Dodd, 1999).

The relationship between MMN reductions and functional outcome measure, in addition to the predictive ability, specificity, stability, and robust nature of MMN reductions in schizophrenia has led to reduced MMN being identified as a potential biomarker for the disorder (Light & Swerdlow, 2015). MMN being an automatic pre-attentive sensory process allows it to be easily measured in a range of species using the same techniques and testing procedures as in humans, making it more directly translatable for use in animal models of the disorder than other measures involving more higher-order symptomology (Javitt & Sweet, 2015).

1.3. Neurodevelopmental Hypothesis of Schizophrenia

It is widely accepted that schizophrenia has a neurodevelopmental origin in which adverse events experienced during critical periods of prenatal brain development produce permanent neurological alterations which then remain largely dormant until later life. It is believed that these brain insults alter neurodevelopmental trajectories and can lead to the disruption of normal brain maturational processes which occur during adolescence to early adulthood, leading to the manifestation of clinical symptoms at this time (Lewis & Lieberman, 2000; Lewis & Moghaddam, 2006; Lyon et al., 2012).

Many lines of evidence now support this neurodevelopmental view with some of the earliest evidence coming from the observation that during early childhood abnormal psychomotor, cognitive, and social development is often observed in those at high risk of developing schizophrenia (Lewis & Levitt, 2002; Rapoport, Addington, Frangou, & Psych, 2005). Many studies have also assessed the development of children who later

went on to develop schizophrenia. These studies have shown that children who later develop schizophrenia, when under 2 years of age, display delays in neuromotor development, such as age at which walking occurred, and other motor skill abnormalities when compared to both healthy controls and controls that later developed affective disorders (Jones, Rodgers, Murray, & Marmot, 1994; Walker, Savoie, & Davis, 1994). Problems with language, social isolation, and social anxiety have also been found to be increased in these children between the ages of 2 to 15 years (Jones et al.). Intellectual and cognitive deficits have also been observed children who later develop schizophrenia. Cannon et al. (2002a) found that pre-schizophrenic children who later develop schizophrenia between the ages of 3 and 11 years scored lower on standard IQ tests than healthy controls and controls that later developed affective disorders. Reduced educational achievement at age 14 has also been reported in children who are later diagnosed with schizophrenia (Isohanni et al., 1998). The presence of these various deficits in children who later developed schizophrenia suggests that disruptions to brain development and function have already occurred by early childhood, long before the clinical manifestation of the disorder (Meyer & Feldon, 2010).

The results of post-mortem studies have also been interpreted as supporting the involvement of early life neurological perturbation in the aetiology of schizophrenia (Akbarian et al., 1993; Arnold, Hyman, Van Hoesen, & Damasio, 1991; Jakob & Beckmann, 1986). Although these studies suffer from small sample size and their results have not always been replicated, they have none the less identified disruptions to the cortical layers of the brains of patients with schizophrenia. These disruptions are suggestive of interruptions to normal cell death and neuronal migration which occurs during prenatal brain development (Rapoport et al., 2005). In addition to this, little to no

signs of gliosis have been found in the post-mortem study of schizophrenia brains which eliminates the possibility of a neurodegenerative process in the disorder (Lewis & Levitt, 2002).

The neurodevelopmental hypothesis has also found support in genetics. Many of the genes which are believed to be involved in schizophrenia are also known to play a part in neurodevelopment (Wilson & Terry, 2010). The neuregulin gene is involved in synaptic development and plasticity through regulation of the NMDAr while the glutamic-acid-decarboxylase-1 gene controls the synthesis of GABA and is also involved in synaptic plasticity (Akbarian & Huang, 2006; Stefansson, Steinthorsdottir, Thorgeirsson, Gulcher, & Stefansson, 2004). The disrupted-in-schizophrenia-1 (DISC1) gene is also believed to be involved in the neurodevelopmental processes of cell migration and signalling. Although the presence of such genes may make an individual more susceptible to developing schizophrenia, they are not the only factor involved, nor does their presence guarantee that the disorder will develop. It has been shown that the prevalence of schizophrenia among monozygotic twins ranges from 33% to 50% and among first degree relatives is as low as 6%, suggesting that environmental factors also play a critical role (Hilker et al., 2018; Wilson & Terry).

Epidemiological studies have identified a number of environmental factors which increase the risk of schizophrenia, with the pre- to perinatal period being identified as a critical time-point of exposure (Meyer & Feldon, 2010; Rapoport et al., 2005). Obstetric complications are one such environmental factor which has long been associated with the disorder. Many studies have found that both complications of pregnancy such as preeclampsia, bleeding and retarded foetal growth as well as complications of delivery including hypoxia and emergency caesarean section are increased in the schizophrenia population (Cannon et al., 2002b; Lewis & Levitt, 2002).

Several experimental studies conducted using animal models have further confirmed these epidemiological findings with caesarean section, hypoxia (Boksa & El-Khodor, 2003; Juarez, Silva-Gomez, Peralta, & Flores, 2003; Vaillancourt & Boksa, 1998), and insufficient foetal blood flow (Rehn et al., 2004) all being shown to result in brain and behavioural abnormalities associated with schizophrenia (Boksa, 2004; Meyer & Feldon, 2010).

Severe maternal stress during the prenatal period is another factor which has been associated with many psychological disorders, including schizophrenia (Meyer & Feldon, 2010). Epidemiological studies have shown that psychological stressors including the loss or serious injury of a close relative, war, and natural disaster increase the risk of schizophrenia, particularly when exposure occurs in the first trimester (Khashan et al., 2008; Malaspina et al., 2008; Selten, van der Graaf, van Duursen, Gispen-de Wied, & Kahn, 1999). This has also been confirmed by rodent models in which exposure of pregnant dams to psychological stressors such as restraint or overcrowded housing result in behavioural and neurobiological deficits relevant to schizophrenia in the offspring (Koenig et al., 2005; Shalev & Weiner, 2001; Son et al., 2006). Moreover, many of these deficits have also been shown to only develop after the onset of puberty, following the developmental time course of schizophrenia (Koenig et al.). In addition to psychological stressors the administration of stress hormones to pregnant rodents has also been shown to induce behavioural changes relevant to schizophrenia (Shalev & Weiner).

1.4. Prenatal Infection in Schizophrenia and the Poly (I:C) Rodent Model

Maternal infection during the prenatal period has also been identified as a factor which may contribute considerably to the development of schizophrenia. Epidemiological investigations have revealed that the presence of a bacterial infection during the first trimester of pregnancy, but not the second or third, significantly increases the risk of schizophrenia in the offspring (Babulas et al., 2006; Sorensen et al., 2009). Support for the involvement of bacterial infection in schizophrenia has also been reinforced by experimental studies using animal models. Administration of the bacterial endotoxin Lipopolysaccharide (LPS) to pregnant rats has been shown to produce multiple behavioural and cognitive deficits relevant to schizophrenia (Lante et al., 2008; Lante et al., 2007; Romero, Guaza, Castellano, & Borrell, 2010; Wijkstra, Valkhof, Koolhaas, & Schuiling, 1991). The offspring of LPS treated rats have also been found to show abnormalities in the development of pyramidal neurons in the PFC and hippocampus, two areas largely implicated in schizophrenia (Baharnoori, Brake, & Srivastava, 2009; Cui, Ashdown, Luheshi, & Boksa, 2009). Although substantial support has been shown for the involvement of bacterial infection in the aetiology of schizophrenia, the implication of maternal infection in the disorder first came from the study of viral rather than bacterial infections. In fact, considerably more evidence has been found to support the role of viral infection in the development of the disorder.

A large number of epidemiological studies have found an association between maternal viral infection and an increased risk of schizophrenia in offspring. Maternal influenza infection was first linked to increase risk of schizophrenia by Mednick, Machon, Huttunen, and Bonett (1988) when he found that the offspring of women who were in their second trimester of pregnancy during the Helsinki 1957 influenza epidemic were at an increased risk of developing schizophrenia. Since that time, numerous other studies have shown a link between schizophrenia risk and maternal

viral exposure in the first to second trimesters of pregnancy (for review see Brown and Derkits (2010)). In addition, some epidemiological studies have been able to establish the same association using serological evidence in which maternal infection was confirmed by the presence of influenza antibodies in maternal blood samples (Brown et al., 2004a). A study by Brown et al. (2009) showed that schizophrenia patients who were exposed to influenza or toxoplasmosis during the first half of gestation exhibited more severe executive function deficits, as measured on a number of different cognitive tasks, in comparison to schizophrenic patients not exposed to infection, indicating that cognitive set-shifting was particularly vulnerable in this subgroup. The association between maternal viral infection and schizophrenia is not restricted to the influenza virus; an array of other viral pathogens including rubella (Brown et al., 2001), Polio (Suvisaari, Haukka, Tanskanen, Hovi, & Lonnqvist, 1999), herpes simplex (Buka et al., 2001), and measles (Torrey et al., 1988) have also been identified as increasing schizophrenia risk through birth cohort studies. The wide number of differing viral and bacterial pathogens so far implicated in the aetiology of schizophrenia suggests that the critical factor involved may be an aspect of the human body's immune response which is common to these pathogens (Meyer & Feldon, 2010).

The association between maternal viral infection and schizophrenia shown in these human epidemiological studies has also been confirmed using experimental animal models. It has been shown that mice exposed prenatally to the human influenza virus exhibit an array of behavioural and cognitive changes associated with schizophrenia (Shi, Fatemi, Sidwell, & Patterson, 2003). In addition to behavioural abnormalities, the same group has also found morphological changes and alterations in gene expression relevant to schizophrenia in the brain of prenatally exposed offspring (Fatemi et al., 1999; Fatemi et al., 2008). Prenatal administration of the viral mimetic

Polyriboinosinic-polyribocytidilic acid (Poly (I:C)), a double strand RNA commonly used to mimic viral infection, has also been shown to produce numerous schizophrenia-like behavioural abnormalities including deficits in working memory, spatial learning, latent inhibition, social interactions, novel object recognition, pre-pulse inhibition of the acoustic startle response (PPI), and altered reversal learning in both rats (Howland et al., 2012; Wolff & Bilkey, 2010; Wolff et al., 2011; Zuckerman & Weiner, 2005) and mice (Bitanihirwe, Peleg-Raibstein, Mouttet, Feldon, & Meyer, 2010; Ibi et al., 2009; Ozawa et al., 2006). Enhanced sensitivity to the locomotor stimulating effects of the DA agonist AMPH and the NMDAr antagonist MK-801 have also been observed in prenatally Poly (I:C) treated rodents suggesting that both of these neurotransmitter systems may be disturbed, which is in line with the human literature on schizophrenia (Meyer & Feldon, 2010; Meyer et al., 2008b; Zuckerman et al., 2003; Zuckerman & Weiner, 2005). In addition, a number of these studies have found aberrations in object recognition memory, latent inhibition, PPI, and AMPH and MK-801-induced locomotion to be present in adult but not in pre-pubertal offspring, which is consistent with the post-pubertal onset of schizophrenia symptoms (Meyer et al., 2008b; Ozawa et al., 2006; Wolff & Bilkey, 2008; Zuckerman et al., 2003; Zuckerman & Weiner, 2003).

In addition to the alterations in behaviour, prenatal administration of Poly (I:C) to rodents has also been shown to produce disturbances of the DA and glutamate systems, as suggested by the increased sensitivity to AMPH and MK-801. Dysfunction in the glutamate system was observed by Roenker et al. (2011), where prenatal Poly (I:C) exposure on gestational day (GD) 14 was shown to result in elevated basal levels of extracellular glutamate in the PFC of rats in adulthood. In addition, these Poly (I:C) exposed animals also displayed a blunted MK-801-induced increase in glutamate in comparison to controls, which, is indicative of NMDAr hypo-function. Hypo-

functionality of the AMPA receptor has also been implicated with reductions in the GluR1 subunit of the AMPA receptor found in the nucleus accumbens (NAc) of mice administered with Poly (I:C) on GD9 (Meyer et al., 2008b). Furthermore, alterations in the GABA system have been identified with adult mice exposed to Poly (I:C) on GD9 exhibiting increased levels of the GABA receptor subunit α2 in the hippocampus and amygdala (Nyffeler, Meyer, Yee, Feldon, & Knuesel, 2006). Schizophrenia-related alterations in the DA system have also been associated with prenatal Poly (I:C) exposure. In mice exposed to Poly (I:C) on GD9, reduced D1r and D2r levels have been found in the medial prefrontal cortex (mPFC) (Meyer et al., 2008b). Elevated release of DA from the striatum of adult rats parentally treated with Poly (I:C) on GD15 has also been reported (Zuckerman et al., 2003). A number of neuroanatomical changes have also been observed in the brains of rodents following prenatal Poly (I:C) exposure including disruptions to neurogenesis and increased apoptosis cell loss in the dentate gyrus, as well as cell loss in the CA1 region of the hippocampus (Meyer et al., 2006; Zuckerman et al., 2003).

The precise timing of exposure to an immune activating pathogen such as Poly (I:C) has been identified as an important factor which can influence the behavioural and neurochemical phenotype eventually displayed by the offspring (Boksa, 2010; Meyer & Feldon, 2012). Specifically, differences have been identified between exposure occurring in early/middle (GD9) and late gestation (GD17) in the mouse. These early/middle and late exposure periods in the mouse are analogous to the end of the first trimester and middle to end of the second trimester in human pregnancy, respectively (Macedo et al., 2012). Poly (I:C) exposure during early/middle gestation (GD9) in the mouse results in offspring exhibiting deficits in PPI and latent inhibition. In contrast, the offspring of dams administered with Poly (I:C) during late gestation (GD17) display

abnormally perseverative behaviour as indicated by impaired reversal learning, deficits in working memory and enhanced MK-801-induced locomotion. In addition, mice exposed to Poly (I:C) on GD9 displayed decreased D1r expression in the PFC and enlarged lateral ventricles but exposure on GD17 primarily produces aberrations in the glutamate system as indicated by reduced expression of the NMDAr NR1 subunit in the hippocampus. Increased apoptotic cell loss in the dentate gyrus was also observed only following exposure on GD17. Conversely, it was found that enhanced AMPH-induced locomotion and reduced Reelin and Parvalbumin, both markers of GABAergic neurons, were independent of the time of exposure (Li et al., 2009; Meyer et al., 2006; Meyer et al., 2008c). These findings propose an interaction between the maternal immune response to the pathogen and the stage of brain development at the time of exposure with late exposure producing a phenotype reminiscent of the cognitive and negative symptoms of schizophrenia while early exposure is more synonymous with the positive symptomology (Meyer & Feldon, 2012). Further investigation into the distinction of different behavioural and neurochemical phenotypes following immune challenge at varying gestational time-points may provide a new understanding of the neuroimmunological processes which underlie schizophrenia (Meyer et al., 2008c).

1.5. Maternal Immune Activation: Neuroinflammation as a Mechanism of Action

Due to the wide range of pathogens and other environmental insults which have been implicated in the aetiology of schizophrenia it has been suggested that it is not actually the pathogen itself but rather an aspect of the body's immune response, inflammation, which is common to all pathogens that is involved (Meyer & Feldon, 2009). Cytokines and microglia are the main aspect of the body's immune-inflammation

response and both have been identified as potential mediators between prenatal infection and schizophrenia-relevant brain and behavioural changes (Meyer, Feldon, & Yee, 2009). Microglia are immune surveillance cells found in the CNS and are responsible for regulating both pro and anti-inflammatory responses through the synthesis of cytokines and reactive oxidative species, and by altering the expression of cell surface receptors including cytokine receptors and pathogen recognition receptors. Microglial cells also act as macrophages within the CNS, devouring damaged cells and infectious agents and are also involved in cellular growth and repair through the synthesis of cytokines and other signalling molecules (Deverman & Patterson, 2009; Meyer, 2011). When activated by an immune challenge microglia release small proteins known as cytokines which serve a dual role within the CNS; they are involved heavily in directing firstly the pro-inflammatory and then anti-inflammatory response to pathogens, cell damage and stress. The second role of cytokines is in cell-cell signalling which governs many aspects of neurodevelopment including neurogenesis, cell migration, differentiation, axon pathfinding, and apoptosis within the developing brain (Deverman & Patterson). This dual role of microglial cells and the cytokines they release means that induction of cytokines in response to prenatal inflammatory inducing insults has the potential to interfere with the normal neurodevelopmental processes occurring during this time, resulting in permanent abnormalities which may later contribute to the development of schizophrenia (Meyer).

Support for the involvement of cytokines in the development of schizophrenia following prenatal infection has come from a number of sources. Human epidemiological studies have found an association between elevated levels of pro-inflammatory cytokines in maternal blood during pregnancy and an increased risk of schizophrenia in the offspring (Brown et al., 2004b), while post-mortem studies have

shown increased cytokine mRNA expression in the mPFC of patients with schizophrenia (Fillman et al., 2012). Further evidence has come from animal models in which administration of pro-inflammatory cytokines to pregnant rats produces schizophrenia-relevant behavioural and cognitive deficits in the offspring (Samuelsson, Jennische, Hansson, & Holmang, 2006). Further studies in mice have also shown that when an antibody which inhibits the effects of the pro-inflammatory cytokine IL-6 is administered concurrently with Poly (I:C), it prevents the behavioural deficits seen in the offspring of Poly (I:C) only treated mice. In addition, it has also been found that transgenic mice unable to express IL-6 do not show many of the behavioural deficits seen in normal mice following prenatal administration of Poly (I:C) (Smith, Li, Garbett, Mirnics, & Patterson, 2007). Another study by Meyer et al. (2008a) has suggested that the moderating factor involved may be an imbalance between pro- and anti-inflammatory cytokines rather than just an increase of pro-inflammatory cytokines in response to the pathogen. Their work showed that mice overexpressing the anti-inflammatory cytokine IL-10 were protected from the deficits induced by prenatal Poly (I:C) exposure. However, when the IL-10 overexpressing mice were not exposed to Poly (I:C) prenatally they also displayed schizophrenia-relevant behavioural and cognitive deficits. This indicates that an increase in either pro- or anti-inflammatory cytokines can disrupt the balance required for normal neurodevelopment, resulting in a schizophrenia-like phenotype in the offspring.

It has been speculated that prenatal infection and the associated release of cytokines may disrupt neurodevelopment by two means, either by directly altering neuronal cells at the time of exposure or indirectly by altering microglial cell function (Meyer et al., 2007). It is possible that prenatal inflammation and the subsequent release of cytokines can alter ongoing microglial function resulting in overactive

microglia and long-term neuroinflammatory abnormalities, thus enhancing an organism's response to future environmental challenges such as stress or infection (Meyer, 2011; Perry & Holmes, 2014). Overactivity of microglia is known to result in the excessive release of pro-inflammatory cytokines that can potentially disrupt brain maturational process occurring throughout childhood and adolescence (Monji, Kato, & Kanba, 2009).

Evidence to support long-term alterations to microglial function in schizophrenia has been found with increased markers of microglial activation being identified in the brains of patients with schizophrenia using both PET and post-mortem techniques (Doorduin et al., 2009; Fillman et al., 2012; van Berckel et al., 2008). Excessive microglia activation also fits with studies which have identified elevated levels of circulating cytokines in adult patients with schizophrenia, suggesting an over-activation of the immune system which may stem from altered microglial function (Miller, Buckley, Seabolt, Mellor, & Kirkpatrick, 2011; Potvin et al., 2008; van Kammen, McAllister-Sistilli, Kelley, Gurklis, & Yao, 1999). It has also been observed that the prenatal administration of Poly (I:C) to pregnant mice not only alters schizophrenia-relevant behavioural outcomes in the offspring but also disrupts pro- and anti-inflammatory cytokine levels in the brains of offspring as soon as 3 h post-administration and up until 60 days following birth, indicative of permanent alterations to the function of microglial cells (Garay, Hsiao, Patterson, & McAllister, 2012; Meyer et al., 2006).

1.6. Aims and Thesis Outline

The main aim of this project was to establish a reliable rodent MIA model of schizophrenia, and determine whether prenatal immune activation at two separate

gestational time-points (early verse late gestation) differentially influences the behavioural and neurobiological phenotypes associated with schizophrenia. Specifically, the aims were to determine whether prenatal exposure to the viral mimetic Poly (I:C) during late gestation (GD19) causes long-term alteration in the functioning of the NMDAr system more so than early gestation (GD10) exposure, resulting in a predisposition to develop NMDA-sensitive behavioural and cognitive deficits in adulthood. In addition, it aimed to determine if the dopaminergic system is more sensitive to Poly (I:C) administration at early gestation (GD10) rather than late gestation (GD19), resulting in a predisposition to develop more DA-sensitive behavioural deficits and DA-related mRNA changes within the brain. Behaviours assessed included prepulse inhibition of the acoustic startle response, working memory, AMPH-induced locomotion, MK-801-induced locomotion, and spontaneous locomotion. The data from these assessments are detailed in the peer-reviewed publication found in Chapter 4.

Another aim was to establish this MIA model with minimal confounds, or at least to be aware of confounds if they existed. Specifically, to produce a model where: (1) exposure to Poly (I:C) at either early (GD10) or late (GD19) gestation produced an appropriate immune-stress response in the dams, (2) the reproductive fitness of the dams was not compromised, (3) the growth and weight of the offspring was not adversely impacted. Assessment of IL-6 and corticosterone (CORT) responses were conducted, in addition to assessment of dam weigh following Poly (I:C) exposure, litter size, male-to-female ratio of offspring in litters, and offspring weigh. These data are presented in Chapter 2.

Moreover, this thesis aimed to assess three different methods often used to elicit MMN (the flip flop control, many-standards control, and cascade control sequences) in an attempt to establish the most appropriate method for use in rats using a new multi-

channel recording system. These data are presented in the peer-reviewed publication detailed in Chapter 3A. This methodology was then used to further examine the effects of prenatal Poly (I:C) exposure at either early (GD10) or late (GD19) gestation on MMRs in the rat. These data are presented in Chapter 3B.

Finally, this thesis aimed to explore the potential long-term effects of Poly (I:C) exposure at either early (GD10) or late (GD19) gestation on neuroinflammation. Examination of markers for microglia, astrocyte, and cytokine function within the PFC were conducted using real-time quantitative polymerase chain reaction (qPCR) and immunohistochemistry techniques. These data are presented in Chapter 5, in a manuscript that will be submitted for peer-review in the near future.

1.7. References

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2. Chapter 2: Establishing the Early Versus Late Gestation MIA Model

2.1. Introduction

The early verses late gestational MIA model used throughout this thesis was established using outbred Wistar rats. Rats were the preferred species of rodent, rather than mice, due to their ability to successfully and reliably perform more complex forms of behavioural and cognitive assessment that are suited to the assessment of schizophrenia-related constructs, such as working memory. In addition, rats were also chosen as proposed biomarkers for schizophrenia measured using EEG, such as MMN, are more readily performed in rats. Mice are more commonly used for investigating the genetic contribution to schizophrenia due to the wide range of transgenics available. Further, the effects of early versus late gestational MIA have previously been described in a series of studies using a mouse model developed by Meyer and colleagues (Li et al., 2009; Meyer et al., 2006; Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008). Therefore, extension of the effects described in this mouse model to the rat will provide evidence to support the cross-species translatability of the early verse late gestational MIA effects. The Wistar is a strain of outbred rat commonly used in neuroscience research and its use in prenatal and neonatal immune activation models of a variety of psychopathologies is well established (Dalton, Verdurand, Walker, Hodgson, & Zavitsanou, 2012; Walker, Hawkins, Sominsky, & Hodgson, 2012; Walker et al., 2009; Zavitsanou et al., 2013; Zuckerman & Weiner, 2005). This, in conjunction with our laboratory's extensive previous experience working with the Wistar strain guided the choice of the Wistar for this model.

Epidemiological evidence indicates that prenatal infection with either viral (Brown et al., 2004; Brown et al., 2001; Brown et al., 2009; Buka et al., 2001; Suvisaari, Haukka, Tanskanen, Hovi, & Lonnqvist, 1999; Torrey, Rawlings, & Waldman, 1988) or bacterial (Babulas, Factor-Litvak, Goetz, Schaefer, & Brown, 2006; Sorensen, Mortensen, Reinisch, & Mednick, 2009) agents are associated with an increased risk of the offspring developing schizophrenia in adulthood. However, there is considerably more evidence to support the role of viral (specifically influenza virus), rather than bacterial induced immune activation in the aetiology of schizophrenia (Brown & Derkits, 2010). This is an important distinction as viruses initiate an immunological response via the unique pathogen recognition receptor, toll-like receptor 3 (TLR3), which is distinct from the toll-like receptor 4 (TLR4) activated by bacterial infection. Therefore, the most appropriate agent for the current research would be a TLR3 activating agent that more closely models the majority of the epidemiology findings indicating an association between influenza and schizophrenia. Poly (I:C) is a double stranded RNA commonly used to mimic viral infection that initiates an immunological response via the TLR3 receptor. However, as Poly (I:C) is not a live virus it cannot replicate inside the host animal, this means that the dose of Poly (I:C) and subsequent intensity and length of the immune response can be controlled. Previous investigations have shown that the acute phase immunological response to Poly (I:C) exposure is time limited to between 24 – 48 h depending on the dose administered (Cunningham, Campion, Teeling, Felton, & Perry, 2007; Meyer, Feldon, Schedlowski, & Yee, 2005). With a live virus such as influenza the length and intensity of the infection cannot be controlled as easily, potentially leading to large variability between subjects and the introduction of confounding factors such as secondary bacterial infections. With a viral mimetic such as Poly (I:C), the dose can be controlled and the

short time frame of the immunological response makes it possible to target a narrow time-point of neurodevelopment. The use of a viral mimetic such as Poly (I:C) also eliminates the need for strict biosecurity and contamination procedures inherent in the use of live infectious agents (Meyer & Feldon, 2012; Reisinger et al., 2015). These factors in combination with the well-established nature of the use of Poly (I:C) in MIA models of schizophrenia, (for review see Meyer and Feldon (2012)) resulted in it being the method of choice for the research detailed in this thesis.

It was important to ensure that exposure to the Poly (I:C) produced an immunological response in the pregnant dams. As such, we examined the circulating levels of the pro-inflammatory cytokine IL-6 in dams 2 h following Poly (I:C) exposure. IL-6 is one of a range of pro-inflammatory cytokines that is released following the Poly (I:C)-induced activation of the TLR3 receptor. Previous studies have confirmed that exposure to Poly (I:C) produces a significant increase in the circulating levels of pro-inflammatory cytokines IL-6, TNF, IL-17a, and IL-1β and type 1 interferons INF-α and INF-β (Cunningham et al., 2007; Fortier et al., 2004b; Suh, Brosnan, & Lee, 2009). Exposure to immune activating agents is also known to activate not just the immune system, but also activates the hypothalamic-pituitary-adrenal (HPA) axis. In general, most forms of stress, whether they be immunological in nature (infection), or a psychological stressor (such as restraint or a traumatic experience), are found to activate the HPA axis, leading to increased levels of the stress hormone cortisol (or corticosterone, CORT, in rats) (Reul et al., 1994). Exposure to increased levels of CORT during prenatal development has previously been linked to schizophrenia relevant behavioural and neurological changes in adult rodents (Diaz, Ogren, Blum, & Fuxe, 1995), and could potentially be a confounding factor. For this reason CORT

levels were also measured in pregnant dams to ensure that levels of stress induced were consistent across our two MIA groups (GD10 MIA and GD19 MIA).

Another factor which has previously been identified as potentially playing a role in the successful development of MIA models of schizophrenia is whether or not the dam loses or gains weight following exposure to Poly(I:C). One group found that while there was an overall reduction in body weight of pregnant rats in the 24 h following Poly (I:C) exposure there was also a great deal of variability, with only 51% of Poly (I:C) treated dams losing weight. Furthermore, the presence or absence of maternal weight loss in the days following Poly (I:C) exposure was found to be related to distinct behavioural effects in the offspring, with offspring from dams that lost weight or gained minimal amounts of weight more affected than those from dams which gained the most weight and controls (Bronson, Ahlbrand, Horn, Kern, & Richtand, 2011; Vorhees et al., 2012, 2015). Although this phenomenon has only been shown by the one group and in a select range of behavioural assessments, while others have failed to find any change in offspring behavioural response based on maternal weight change following Poly (I:C) administration (Wolff & Bilkey, 2010), we also measured the weight of the dams for two days following their exposure to Poly (I:C) or saline, to rule it out as a confounding factor in our model.

In addition to dam weight, we also investigated the weight of the offspring throughout their development. This was done to ensure the offspring did not suffer any severe or chronic metabolic alterations as a result of prenatal Poly (I:C) treatment. Perinatal immune activation via exposure to LPS on PND3 and 5 in Wistar rats has previously been shown to produce changes in the weight and growth rate of exposed animals throughout the adolescent to adult period, indicating potential metabolic changes (Sominsky et al., 2012). Although weight gain and metabolic changes in

patients with schizophrenia are common, they are predominantly due to the adverse side effects of antipsychotic medications (Deng, 2013; Gebhardt et al., 2009). No difference in body weight between drug-naïve first episode patients and healthy controls has been previously reported (Wyatt, Henter, Mojtabai, & Bartko, 2003; Zhang, Yao, Liu, Fang, & Reynolds, 2004), however, drug-naïve first episode patients have been reported to have increased levels of intra-abdominal fat stores in comparison to controls (Ryan, Flanagan, Kinsella, Keeling, & Thakore, 2004) in addition to some metabolic changes (Guest et al., 2010; Harris et al., 2013). As differences in weight are not overtly evident in drug-naïve first episode schizophrenia patients, the presence of such changes in our model may suggest that the developmental impact of our MIA is farther reaching than the schizophrenia related brain systems we are aiming to disrupt. Significant differences in weight and rate of development between our MIA and control groups has the potential to be a significant confounding factor and was therefore measured and assessed.

A final factor which was assessed in the establishment of this model was that the experimental treatment did not significantly modify the reproductive capabilities of the dams by altering the size of the litters produced or the ratio of male-to-female pups within the litters. Variability in litter size is a factor that could influence behavioural assessments and offspring health. It has been previously documented in both mice and rats that offspring from larger litters, which have to share limited milk production among more offspring, display reduced weight, growth rate, and altered body composition (Rogowitz & McClure, 1995; Romero, Villamayor, Grau, Sacristan, & Ortiz, 1992; Wurtman & Miller, 1976), altered endocrine function and pubertal maturation (Bourguignon, Gerard, Alvarez Gonzalez, Fawe, & Franchimont, 1992), and in some instances altered behavioural outcomes (Bechard, Nicholson, & Mason, 2012;

LaBarba, White, Stewart, & Buckley, 1973; Priestnall, 1973) in comparison to those of smaller litters. In some instances these alterations persist into adulthood. As with offspring weight, litter size and male-to-female ratio are potential confounding factors which may indirectly influence the behavioural and neurobiological alterations that are the focus of the current model, and as a result need to be considered when interpreting the behavioural and neurobiological changes identified in this MIA model.

2.1.1. Aims

The aims of the experiments reported in this chapter were to establish (1) that dams produced an immunological and stress response to the administration on Poly(I:C), but not to the administration of phosphate-buffered saline (PBS); (2) that the dams reproductive process (dam weight during gestation, litter size, male-to-female pup ratio) was not adversely influenced by exposure to Poly (I:C) ; (3) that offspring growth and weight was not severely impacted by prenatal exposure to Poly (I:C). Fulfilling these objectives will ensure the successful establishment of a MIA model and reduce confounding factors in the behavioural and neurobiological experiments mentioned in following chapters.

2.2. Methods

2.2.1. Animals

Outbred Wistar breeding rats were obtained from the University of Newcastle Central Animal House at the age of 8-10 weeks. All offspring used in this thesis were bred from this pool of rats in the University of Newcastle Behavioural Sciences vivarium. Breeding males were single-housed while female breeders were pair housed with their litter mates until conception, after which point they were also single-housed.

Breeders and their offspring were contained in a single colony room until offspring were weaned at PND21, after which, offspring were separated into pairs and held in a separate colony room. Offspring were pair-housed with same-sex litter mates allocated to the same experiment although in some instances offspring were housed three to a cage due to uneven numbers. All colony rooms were maintained at a constant temperature (21±1 °C) and humidity (34±2%) under a normal 12 h light/dark cycle (lights on at 0730 h). Animals were housed in plastic wire top cages (41.5cm x 28cm x 22cm cages; Mascot Wire Works, Australia) containing recycled compressed paper bedding and had ad libitum access to food (Rat and Mouse Pellets, Specialty Feeds, Western Australia) and water, unless otherwise specified in experimental procedures mentioned below. All animals were handled twice per week for weighing and cage/bedding changes. Each experiment contained in this thesis used animals from a number of separate litters and a number of separate breeding cycles/waves in an effort to account for variations caused by seasonal, genetic or environmental variations. Sample sizes for the dam assessments included in this chapter are shown in Table 2.1.

Table 2.1. *Sample size for dam IL-6, dam CORT, dam litter size, male-to-female ratio of pups per littler, and dam weight analysis.*

	GD10		GD19	
	Control	MIA	Control	MIA
Dam IL-6	24	25	18	23
Dam CORT	13	20	12	17
Dam Weight Day of Injection	23	26	18	22
Dam Weight 24 h post-injection	17	17	12	15
Dam Weight 48 h post-injection	16	17	10	12
Dam litter size	27	30	22	28
Proportion of male pups per litter	27	30	22	28

Abbreviations: IL-6 (interleukin 6), CORT (corticosterone).

2.2.2. Breeding

Breeding rats were given 1-2 weeks to acclimate to the University of Newcastle Behavioural Sciences vivarium prior to commencement of breeding. After the acclimation period, female breeders underwent daily monitoring of oestrous cycle using vaginal impedance probe (Muromachi Kikai, Tokyo, Osaka). Impedance was assessed between the hours of 1500 h and 1800 h daily. On the evening of proestrous (impedance > 3) females were housed with a male breeder overnight to allow mating to occur. Successful copulation was determined by the presence of sperm via vaginal smear between 0700 h and 0900 h the following morning. Vaginal smears were obtained by gently restraining the animals in a cotton restraint sock and while in an inverse position using a sterile glass pipette to administer a small amount of sterile 0.9% sodium chloride (saline) solution into the vaginal canal. The saline was then drawn back into the pipette before removing it from the vagina and placing the sample on a glass microscope slide and assessed for the presence of sperm cells using a light microscope with 10x magnification. The presence of sperm confirmed copulation and the day of sperm detection was identified as GD0. Pregnant dams were then randomly allocated to one of four treatment group (GD10 Control, GD10 MIA, GD19 Control, GD19 MIA).

2.2.3. Preparation of Poly (I:C)

Powdered Poly (I:C) was dissolved in RNase-free distilled water to make 10 mg/mL Poly (I:C) solution which was divided into aliquots and stored at -20°C until use. At the time of injection aliquots were thawed at room temperature and further diluted with 0.1M of PBS to make a 4 mg/mL Poly (I:C) solution which was used for injection. Once diluted to 4 mg/mL, the Poly (I:C) solution was only refrozen and thawed once more before being discarded. Poly (I:C) was made to the two mentioned

concentrations under RNase-free conditions to ensure its integrity. To ensure RNase free conditions all Poly (I:C) was prepared in the same PC2 laboratory space and class II biosafety cabinet as PCRs are performed. Prior to the preparation of Poly (I:C) all benchtops, pipettors, tube racks, the outside of all tubes, and gloved hands were cleaned with a commercial RNase decontamination solution (RNase Zap, ThermoFisher Scientific) and autoclaved pipette tips were used.

2.2.4. Maternal Immune Activation & Stress Response

On the appropriate gestational day (10 or 19), dams were anaesthetised with isoflurane via face mask (induction 5%, maintenance 2.5-3%) and administered with either 4.0 mg/kg of Poly (I:C) (P9582; Sigma-Aldrich, Buchs, St. Gallen, Switzerland; "MIA" group) or 0.1M PBS ("Control" group) via injection to the lateral tail vein (at 1 mL/kg body weight). During anaesthesia, dams were placed on an electric heat mat and had their eyes covered with a small towel to protect them from direct light. Following the injection and removal from anaesthetic, pregnant dams were immediately returned to their home cage for recovery and were monitored until fully conscious and able to walk normally.

In order to confirm activation of the maternal immune response following Poly (I:C) administration, circulating IL-6 levels were assessed in the pregnant dams. Blood samples were obtained via a saphenous vein puncture of the hind limb from dams 2 h following treatment with either Poly (I:C) or PBS. Samples were collected in EDTA-coated tubes before being centrifuged at 1000xG for 15 min at 4°C, plasma was separated from the remaining sample and stored at -20 °C until assayed. Circulating levels of IL-6 in the maternal serum were measured using a commercially available rat IL-6 immunoassay kit (R&D Systems, Australia) following the manufacturer's

instructions. Absorbance was detected using an ELISA plate reader (Thermotrace, Australia) and the kit detection limit was 10 pg/ml for IL-6. The intra-assay coefficient of variability ranges between 4.5% and 8.8% and the inter assay variability ranges from 7% to 10%, as reported by the manufacturer. In addition to IL-6 levels, the plasma obtained from dams 2 h following injection was also assessed for levels of CORT using a rat corticosterone ^{125}I radioimmunoassay kit (MP Biomedicals, USA), following manufacturer instructions. The intra and inter-assay variability ranges of the CORT kit are 4.4% to 10.3% and 6.5% to 7.3%, respectively. Sufficient samples were obtained from 90 dams for IL-6 analysis and from 85 dams for CORT analysis, out of the 97 dams used throughout this thesis.

Dams were weighed at the time of injection in order to calculate the appropriate volume of Poly (I:C) or PBS to be given. Dams were also weighed once daily for 2 days following injections to monitor for any loss of body weight due the injections.

2.2.5. Offspring Weights

At the time of weaning, all offspring were weighed by placing them inside a container set on top of a set of scales. Offspring were handled and weighed in this same manner once weekly until such time as they were euthanased. At the time of weighing offspring also had their identification numbers redrawn on their tails using a permanent marker, in some instances animals were restrained in restraint sock for this process. All rats were weighed on one day per week, resulting on offspring from different litters being weighed at different PNDs. The analysis of offspring weight presented in this chapter was conducted on a subset of the offspring used throughout this thesis, predominantly those that underwent the behavioural experiments presented in chapter 4.

2.2.6. Statistical Analysis

Litter size, male-to-female ratio of pups, and plasma concentrations of IL-6 and CORT in dams were analysed using Univariate Analysis of Variance (ANOVA), with treatment and GD as between-subjects factors. A reflect and square root transformation was used for litter size data, a square root transformation was used for CORT data, and for IL-6 data a logarithmic transformation with the addition of a constant of 10 (as IL-6 data contained zero values) was used, to correct for violations of normality and homogeneity of variance. All figures show raw untransformed data.

Analysis of dam weight following injection of Poly (I:C) or saline was conducted using independent samples t-tests with dam weight on the day of injection (day 0), weight 24 h following injection (day 1), and weight 48 h following injection (day 2) as dependent variables and treatment as the between-subjects factor. Due to the obvious weight differences between dams in the middle verses late stages of pregnancy analysis was conducted on each GD separately. Not all dams were able to be weighed each day following injections. Therefore, a repeated measures analysis over time could not be implemented because of missing data.

Offspring growth rate was assessed using growth Linear Mixed Models (LMM) because not all weight data was collected for each animal at the same age, leading to many animals missing values at any particular age. The nature of this LMM analysis resulted in a very large sample size, as weight data for each animal at each separate age of measurement was treated as a separate sample. This resulted in a total *n* of 4960 male and 4903 female weight observations (the sample size), which were taken from a total of 279 male and 273 female offspring at varying ages (Table 2.2). The LMM included fixed predictors of age-centred (with polynomials up to power 5 to account for curvature in the relationship), prenatal treatment, GD, and the interaction of treatment

with GD. Also, terms were added to test whether there were growth related interactions with treatment, GD, and the interaction of GD and treatment, these interaction terms with age-centred being with polynomials up to a power of 5. Random effects terms were added to the model to account for differences between rats in their mean weights, using a random intercept term. To account for possible differences between rats in growth rates, random terms were included for the linear slope and for polynomials to power 2 and 3. We fitted different models by firstly removing non-significant random effects, followed by the removal of fixed predictor interaction terms, also based on significance. A combination of significance tests on individual terms and Akaike's Information Criterion was used to select the most appropriate model. Residuals plots were used to assess the quality of fit of the model, especially the effectiveness of the polynomial terms in fitting the curvature in the model. Separate models were used for male and female data to reduce the complexity of the model due to the large differences in weight and weight variability between the two sexes. Statistical analyses were performed using SPSS V21.

Table 2.2. *The number of weight observations* (*sample size), and the total number of offspring contributing to the offspring weight LMM analysis.*

	Male				Female			
	GD10		GD19		GD10		GD19	
	Control	MIA	Control	MIA	Control	MIA	Control	MIA
Sample Size (Observations)	1194	1282	1190	1294	1138	1292	1141	1332
Number of Offspring	66	72	69	72	63	74	64	72

2.3. Results

2.3.1. Maternal Immune & Stress Response

MIA dams exhibited a significant increase in both plasma IL-6 ($F_{(1, 86)} = 420.09$, $p < 0.001$) and CORT ($F_{(1, 58)} = 62.15$, $p < 0.001$) concentrations in comparison to control dams 2 h following exposure to Poly(I:C). There was no significant effect of GD ($F_{(1, 86)} = 0.00$, $p = 0.98$; $F_{(1, 58)} = 0.02$, $p = 0.88$) nor any treatment by GD interaction ($F_{(1, 86)} = 0.65$, $p = 0.42$; $F_{(1, 58)} = 1.05$, $p = 0.31$) observed on either IL-6 or CORT levels, respectively (Figure 2.1).

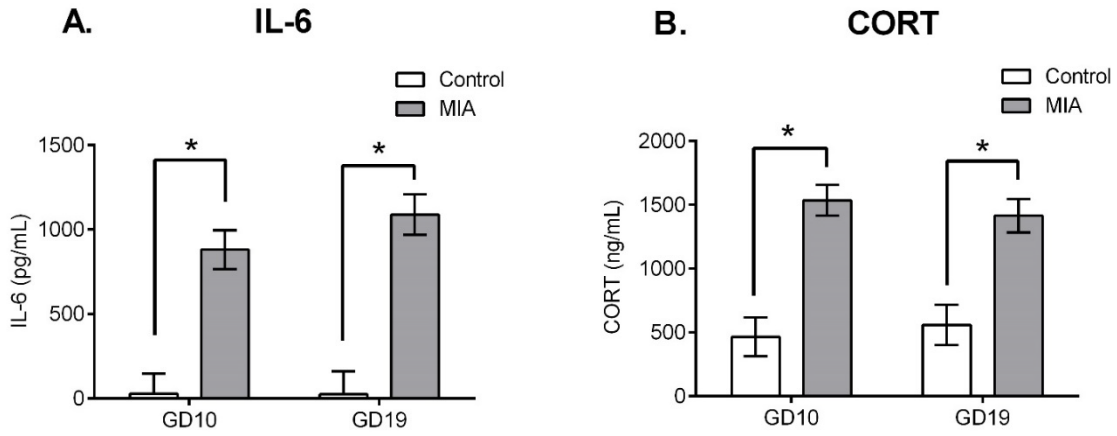

Figure 2.1. Effects of MIA and GD on mean plasma IL-6 (A), and CORT (B) in dams 2 h post Poly (I:C) and saline injection indicating significantly increased levels of IL-6 (A), and CORT (B) in dams exposed to Poly (I:C) in comparison to controls at both GD 10 and 19. Data is expressed as Mean ± SEM. * = $p \leq 0.05$. Abbreviations: IL-6 (interleukin 6), CORT (corticosterone).

2.3.2. Breeding Performance

There was no significant effect of MIA ($F_{(1, 103)} = 1.73$, $p = 0.21$), GD ($F_{(1, 103)} = 1.73$, $p = 0.19$) or interaction of GD and MIA on the number of offspring born in each litter ($F_{(1, 103)} = 1.88$, $p = 0.17$, See Figure 2.2A). The proportion of male-to-female pups

born within each litter was also found to be unaffected by MIA ($F_{(1, 103)} = 2.16$, $p = 0.15$), GD ($F_{(1, 103)} = 0.14$, $p = 0.71$), or their interaction ($F_{(1, 103)} = 0.97$, $p = 0.33$, See Figure 2.2B).

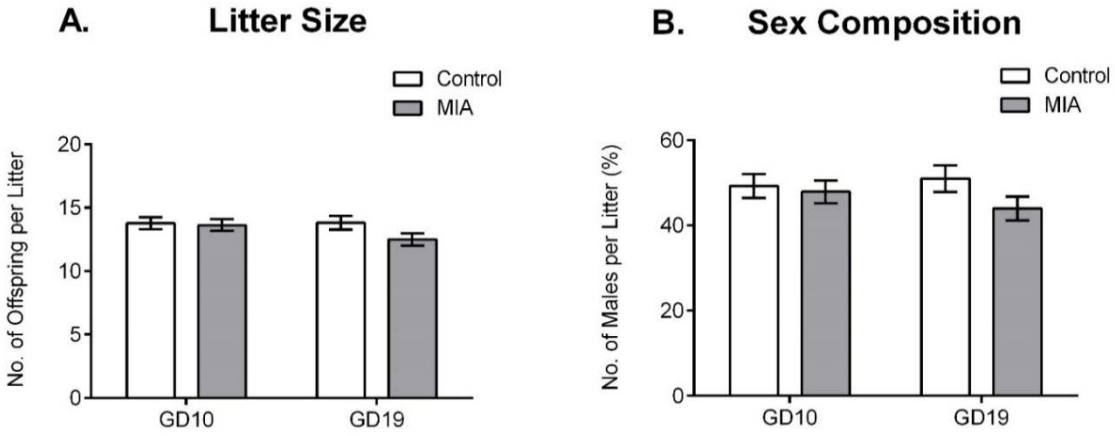

Figure 2.2. Effects of MIA and GD on the number of offspring born within each litter (A), and the ratio of male to female offspring within each litter (B). The number of offspring born in a litter and the proportion of male to female offspring within litters was not significantly influenced by prenatal treatment (MIA v Control) or the GD of exposure (GD10 v GD19). Data is expressed as Mean ± SEM.

Dam weights prior to and following injection were analysed separately for dams allocated to the GD10 and 19 groups, due to the obvious differences in weight between rats in the early/middle stage of pregnancy compared to those in the last days of gestation. No significant effect of treatment, on the day of injection, was found in weights of GD10 dams ($t_{(47)} = 0.67$, $p = 0.50$), indicating that dams allocated to the GD10 Control vs. MIA groups did not differ in terms of body weight prior to prenatal treatment. No significant effect of treatment was found on GD10 dam weight 24 h following injection ($t_{(32)} = -1.44$, $p = 0.16$), or 48 h following injection ($t_{(31)} = -0.28$, $p =$

0.78), indicating that Poly (I:C) exposure did not significantly reduce dam weight (Table 2.3). Analysis of GD19 dam weights revealed no significant effect of treatment on the day of injection ($t_{(38)} = 1.07$, $p = 0.29$), nor 24 h ($t_{(25)} = 1.66$, $p = 0.11$) or 48 h following injection ($t_{(20)} = 0.92$, $p = 0.37$), indicating that GD19 Control and MIA dams did not differ in body weight prior to, or following Poly (I:C) exposure (Table 2.3).

Table 2.3. *Effects of prenatal treatment with either Poly (I:C) or saline on dam body weight (g) at time of injection, 24 h, and 48 h post-injection.*

	GD10		GD19	
	Control	MIA	Control	MIA
Day of injection	321.76 ± 5.52	316.56 ± 5.41	418.84 ± 8.18	408.26 ± 5.94
24 h post-injection	318.52 ± 5.76	330.52 ± 6.04	432.46 ± 10.72	412.32 ± 6.71
48 h post-injection	320.84 ± 6.50	323.67 ± 7.46	454.26 ± 11.60	440.38 ± 9.89

Data presented as Mean ± SEM.

2.3.3. Offspring Weights

LMM of male weight data up to PND 86 indicated that treatment had a significant effect on the rate of growth as indicated by the interaction between age to the power of 2 and treatment ($F_{(1, 1162.07)} = 16.23$, $p \leq 0.001$), and age to the power of 4 and treatment ($F_{(1, 1708.88)} = 12.37$, $p \leq 0.001$), while age to the power 1 and 3 was not significant. Neither the interaction of GD with age nor the 3-way interaction of treatment, GD and age were significant, indicating that neither GD nor the combination of GD and treatment had any age related effect on weight. However, the interaction of GD with treatment was found to be significant ($F_{(1, 292.68)} = 14.73$, $p \leq 0.001$), indicating that there were mean differences between the combinations of the two factors, but that these differences were not related to age, simply differences between the mean weights

for the groups. To further investigate the above mentioned interaction effects, pairwise comparisons were performed at the ages of PND35, 50 and 70. At PND70 no significant differences in weight between control and MIA animals at either GD were identified. While no difference were found between GD10 MIA and control animals at earlier ages, GD19 MIA animals were found to weigh significantly more than GD19 controls at both PND50 ($p = 0.008$) and PND35 ($p = 0.004$). However, these differences between treatment conditions at GD19 were approximately a 3% and 4.5% increase in body weight relative to controls, respectively, and although they are statistically significant increases they are not of a size substantial enough to be considered clinically relevant (Table 2.4). The fixed age effect polynomials up to power 4 were significant, as were the interaction terms for age by treatment to the power 4. For the random slope terms polynomials to power 2 were required. Examination of residuals showed systematic differences with age indicating that the polynomial terms did not fully explain the curvature for predicted weights < 300 (above this the fit was unbiased) however the mean departures from the fitted line did not exceed ±2 g which was considered to be acceptable variation and not a biologically significant difference.

LMM of female weight data up to PND 86 showed that treatment significantly influenced the rate of growth in the female data, as indicated by a significant treatment by age interaction ($F_{(1, 262.03)} = 7.99$, $p = 0.005$). As with the males, neither the GD by age interaction nor the 3-way treatment by GD by age interaction were significant, indicating that neither GD nor the combination of GD and treatment had any age related effect. The same significant (non-growth related) treatment by GD interaction as displayed in the males was also seen in the females ($F_{(1, 277.65)} = 6.02$, $p = 0.015$), again indicating that there were mean differences between the combinations of the two factors. Pairwise comparisons were performed at the ages of PND35, 50 and 70 to

further investigate the effects of treatment and GD on weight, revealing that MIA animals weighed significantly more than controls for both GDs at PND70 (GD10 p = 0.015; GD19 $p \leq 0.001$) and PND50 (GD10 p = 0.048; GD19 $p \leq 0.001$), with increases in body weight relative to controls of 3%, 5.3%, 2.2%, and 5.1% respectively. The same pattern of heavier weight in MIA animals was seen at PND35 in GD19 animals only ($p \leq 0.001$) with an increases in body weight relative to controls of 5.9%. Again, these differences in weight between treatment conditions are statistically significant but are not large enough in size to be considered clinically relevant (Table 2.4). The fixed effect age polynomial terms were significant up to power 4, but the interaction terms for age by treatment were only significant to power 1. Polynomials to power 2 were required for the random slope terms. Inspection of residuals exposed differences with age indicating that the polynomial terms did not fully explain the curvature for predicted weights < 180 (above this the fit was unbiased). However, as with the male model, this was considered to be acceptable variation as the mean departures from the fitted line did not exceed ±3 g.

Table 2.4. *Effects of MIA and GD of treatment on offspring body weight (g) at PND35, 50 and 70 for both males and females.*

	GD10		GD19	
	Control	MIA	Control	MIA
Male				
PND 35	151.77 ± 1.63	150.14 ± 1.57	**147.59 ± 1.62**	**154.18 ± 1.57***
PND 50	280.18 ± 2.24	280.22 ± 2.16	**276.00 ± 2.22**	**284.26 ± 2.16***
PND 70	407.68 ± 3.44	402.45 ± 3.33	403.50 ± 3.43	406.49 ± 3.33
Female				
PND 35	129.98 ± 1.34	131.99 ± 1.25	**127.55 ± 1.33**	**135.05 ± 1.26***
PND 50	**194.07 ± 1.68**	**198.35 ± 1.57***	**191.64 ± 1.67**	**201.40 ± 1.58***
PND 70	**244.34 ± 2.23**	**251.63 ± 2.08***	**241.91 ± 2.22**	**254.69 ± 2.08***

Data presented as Mean ± Standard Error from the LMM. * = $p \leq .05$, MIA compared to control within GD.

2.4. Discussion

The data presented in this chapter indicate that the three objectives identified for the successful establishment of the MIA model and reduction of potential confounds were met. Specifically, that increased circulating levels of IL-6 and CORT were produced in pregnant dams injected with Poly (I:C) but not those injected with PBS indicating that the MIA model was successfully established, with no difference in the immune or stress response seen between the two gestational time-point of exposure. The second objective that reproductive outcomes of the dams were not significantly compromised by exposure to Poly (I:C) at either gestational time-point was also met, with no difference in the number of offspring produced in each litter or the male-to-female ratio of offspring within litters being present between the four treatment groups, eliminating these as potential confounds in the model. And finally, the third objective that offspring weight is not severely impacted by prenatal exposure to Poly (I:C) or the gestational timing of the exposure was partially achieved, with only small changes in offspring weight found, indicating that offspring weight change is unlikely to be a significant confounding factor in the model.

As expected, assessment of maternal plasma collected 2 h post-treatment showed that injection with Poly (I:C) effectively induced a pro-inflammatory immune response in pregnant dams at both gestational time-points. This is in line with previous studies which have shown that intravenous injection with Poly (I:C) in both rats (Dalton et al., 2012; Missault et al., 2014) and mice (Connor et al., 2012; Meyer et al., 2006; O'Leary et al., 2014) produces a sharp increase in a range of cytokines **(**IL-6, TNF, IL-10, IL-8, IL-1β, IFN-γ,) that is indicative of an acute phase immune response associated with viral infection.

As expected, injection with Poly (I:C) did produce a significant rise in CORT levels 2 h post-injection in pregnant dams. The increase in CORT 2 h post-injections is in line with other studies which have reported similar increases following Poly (I:C) exposure (Dalton et al., 2012; Dunn & Vickers, 1994; Ellis, Mouihate, & Pittman, 2006) and indicates that Poly (I:C) is an effective immunological stressor capable of activating the HPA axis. Although CORT levels were not different between dams in the MIA groups at the early and late gestational time-points, there is a caveat in terms of interpreting this result that needs to be noted. Comparable CORT levels does not necessarily translate to the same levels of exposure in the foetus of the MIA early and late groups. CORT is able to cross the placenta to the foetus directly, however, the permeability of the placenta to CORT is known to change throughout pregnancy. The placental enzyme 11β-hydroxysteroid-dehydrogenase (11β-HSD)-2, which coverts CORT into the inactive metabolite 11-dehydrocorticosterone, is known to decrease between the 16^{th} and 22^{nd} day of gestation in the rat (Burton, Smith, Krozowski, & Waddell, 1996; Mark, Augustus, Lewis, Hewitt, & Waddell, 2009; Waddell, Benediktsson, Brown, & Seckl, 1998). Concurrently, the levels of placental 11β-HSD1, the enzyme which converts 11-dehydrocorticosterone into active CORT is known to increase at this time (Burton et al., 1996; Mark et al., 2009; Waddell et al., 1998). This change in 11β-HSD1 and 2 during late gestation allows for in increased trans-placental movement of glucocorticoids from the mother to the foetus, and coincides with the late gestational time-point of exposure (GD19) used in this model. In future, it would be advantageous to account for this potential confound by sacrificing a subset of dams and assessing the level of CORT in foetal brain tissue at the two separate time-points. This would ensure that the CORT concentrations experienced by the foetus does not differ between the two gestational time-points due to placental permeability differences as a

result of gestational timing of exposure. If there are significant GD effects seen in the behavioural and neurobiological assessments presented throughout the remainder of this thesis, they must be considered in light of this potential CORT difference.

A large proportion of the dams used throughout this thesis underwent IL-6 and CORT assessment, with only a small proportion not being assessed. However, experimenters monitored all dams for signs of successful injection in an effort to identify any injections that may potentially have been unsuccessful (and therefore would of failed to produce the desired immunological and stress response). It was noted at the time of injection by the experimenter if the injection was not successful (i.e. there was no easy flow of the injection into the vein without resistance and no blood flash back present with removal of the needle from the vein). Any dam which was observed to have an unsuccessful injection was removed from the IL-6 and CORT analysis and its offspring were excluded from all testing. Poly (I:C) is well established as immune activating agent and therefor systematic confirmation of a cytokine response in all dams following injection with Poly (I:C) is not standard practice within the MIA literature, in fact, many studies do not confirm successful MIA via cytokine assessment at all (Bitanihirwe, Peleg-Raibstein, Mouttet, Feldon, & Meyer, 2010; Deslauriers, Larouche, Sarret, & Grignon, 2013; Hadar et al.; Ito, Smith, Hsiao, & Patterson, 2010; Li et al., 2009; Ozawa et al., 2006; Richetto, Calabrese, Meyer, & Riva, 2013; Wolff & Bilkey, 2015; Zhang & van Praag, 2015; Zuckerman, Rehavi, Nachman, & Weiner, 2003). The criteria implemented for determining successful administration of the Poly (I:C) in this thesis, in addition to the confirmed IL-6 and CORT response in the majority of dams is sufficient to conclude that MIA was successful established in this model. However, future studies may benefit from the assessment of additional cytokines known to be altered in the acute phase viral immunological response (such as TNFα, IL-1β, IL-10,

IL-8) to further elucidate the mechanism which mediate the effects of viral infection on the prenatal development of schizophrenia-related brain systems, and if any of these other potential mediators differ between the two gestation time-points used in this model.

Assessment of dam weight data revealed that there was no difference in the weight of MIA and control dams at either gestational time-point immediately before injection with Poly (I:C), nor in the 48 h following injection. This result is similar to that of Wolff and Bilkey (2010) and Dalton et al. (2012) who also failed to find and significant difference in dam weight in the four days following intravenous Poly (I:C) administration on GD15. However, it is contrary to the work of one group (Vorhees group) who identified a subset of dams that lost weight or gained only small amounts of weight following exposure to Poly (I:C) (low gain group), and found that the offspring from this subset of 'low gain' dams showed discernible behavioural outcomes in comparison to the offspring of dams that gained more weight following Poly (I:C) exposure (high gain group) (Bronson et al., 2011; Vorhees et al., 2012). There is however some inconsistency between the findings of this group with one study reporting a significant difference between 'low gain' and 'high gain' groups 24 h post-injection (Bronson et al., 2011), whereas in a later study the same group only found a difference from the third to sixth day post-injection (Vorhees et al., 2012). It is possible the time-frame of 48 h post-injection may not have been long enough to elucidate any potential weight difference and a longer period such as the three to six day period used by Vorhees et al. (2012) may have allowed for differences to be detected. However, the majority of dams injected on GD19 gave birth within three days of injection (GD22-23) therefore not allowing for a longer period of monitoring. Differences between the current model and that of the Vorhees group are however the most likely reason for the

differences in dam weigh seen between the models. The Vorhees group use an 8mg/kg dose of Poly (I:C) via an intraperitoneal (i.p.) route at GD14, which differs from our model with a dose of 4mg/kg via an intravenous route at an early (GD10) or late (GD19) gestational time-point. It is possible that the differing weight change in response to Poly (I:C) in pregnant dams is specific to the i.p. route or higher dose of Poly(I:C), and therefore not seen in our model.

No difference in the size of litters or the male-to-female ratio of pups within litters was observed in the current MIA model. This was in line with the previously mentioned Poly (I:C) MIA model in rats of the Vorhees group (Vorhees et al., 2012, 2015) and indicates that the reproductive capabilities of MIA dams was not adversely effected by Poly (I:C) exposure. As a result, litter size and male-to-female ratio of pups are unlikely to be confounding factors in our model, and were not included as covariates in the behavioural and neurobiological assessments mentioned throughout the remainder of this thesis.

Analysis of offspring weight data demonstrated that MIA at either gestational time-point resulted in statistically significant but small and non-substantial long term changes in the weight of the offspring. Although we did find statistically significant increases in the weight of MIA animals across the postnatal period, particularly in females, these differences were of a small magnitude not considered to be clinically relevant (2.2 – 5.9% increase in body weight relative to controls), and mostly normalised by adulthood. It is important to note that the LMM analysis used to assess offspring weight was highly powered due to the very large sample size (n = 4960 Male; 4903 Female) and therefore was capable of detecting very small differences, that may not necessarily be clinically significant differences, as being statistically significant. This seems to be the case in this instance, where only very small changes in offspring

body weight were seen, yet these small changes presented as being statistically significant in the analysis. Previous research from Vorhees et al. (2012) also assessed offspring post weaning body weight in a rat MIA model. In contrast to our findings of *increased* weight in MIA offspring, they identified small *reductions* in weight in male and female rats exposed prenatally to Poly (I:C) at mid-gestation (GD15) in comparison to controls at PND84 and PND91. On the other hand, other MIA models have failed to find any differences in offspring weight (Fortier, Joober, Luheshi, & Boksa, 2004a; Ratnayake, Quinn, LaRosa, Dickinson, & Walker, 2014; Romero, Guaza, Castellano, & Borrell, 2010). In the current study, we found that rats exposed to MIA at GD19 had increased weight during the juvenile and peri-adolescent periods, which had normalised to control levels by adulthood in males, but not females. It is unclear how such changes reflect what occurs in persons with schizophrenia. While significant weight gain in persons with schizophrenia is common, it is primarily attributable to antipsychotics such as olanzapine (Deng, 2013), with reports of first episode drug-naïve patients having comparable body weight, waist circumference and body mass index (BMI) to controls (Venkatasubramanian et al., 2007; Wyatt et al., 2003; Zhang et al., 2004). However, there is also evidence of insulin resistance and metabolic alterations in drug-naïve first-episode schizophrenia patients (Guest et al., 2010; Harris et al., 2013), including at least one incidence where no difference in BMI and waist circumference were found (Venkatasubramanian et al., 2007). Similar changes may be present in rats exposed to MIA, promoting a subtle shift in body weight during growth. However, because the weight changes seen in our model are relatively small (2.2 – 5.9% increase in body weight relative to controls), they are unlikely to play a significant role or be significantly associated with any behavioural and neurobiological changes observed in MIA offspring. Future experiments may benefit from assessing the metabolic status of

offspring throughout development to fully elucidate if this system is affected by prenatal exposure to Poly (I:C).

The data presented here confirm that the administration of Poly (I:C) at the two time-points of GD10 and GD19 both successfully induced an immunological stress response in pregnant dams, and that the magnitude of the immunological response was not significantly different between the two gestational time-points. Poly (I:C) administration did not have any effect of dam weight or breeding performance, indicating that MIA did not detrimentally effect the dams fertility or growth. In addition, prenatal Poly (I:C) treatment did not severely disturb offspring growth, with only minor changes in this measure detected. This confirms that MIA was successfully produced in Poly (I:C) treated dams with minimal disturbances to other physiological systems in the dams and their offspring, indicating that these offspring are suitable for use in investigating the behavioural and neurodevelopmental consequences of MIA.

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3. Chapter 3: Assessing Mismatch Negativity (MMN) in Wistar Rats and the MIA Model

3.1. Introduction

MMN is a component of the ERP which occurs in response a deviation in an established pattern of auditory inputs, often called an auditory oddball paradigm. An oddball sequence capable of eliciting MMN consists of a train of repetitive and expected auditory stimuli (the standards) interrupted at random intervals by an uncommon and unexpected auditory stimulus (the deviant) that differs in some physical attribute (usually in pitch, intensity or duration) from the standards (Harms, Michie, & Naatanen, 2016). MMN is extracted by subtracting the ERPs recorded in response to the standard from those elicited following exposure to the deviant, which produces a large negative potential usually between 100 to 200 ms after the deviant is presented. MMN is believed to reflect deviance detection, which is the result of a memory-dependent comparison process that detects violations of a predictive model (Näätänen, Jacobsen, & Winkler, 2005). There is also, however, a known adaptation component which can contribute to MMN responses. Adaptation is the process by which neurons become less activated or responsive to a particular stimuli the more that stimulus is repeated, but those same neurons will be normally activated by a different stimuli (Ulanovsky, Las, Farkas, & Nelken, 2004; Ulanovsky, Las, & Nelken, 2003). This reduced activation to the repetitive stimuli (the standard in an oddball paradigm) makes the difference between the reduced standard response and the non-adapted response to a deviating stimuli larger, when in fact it is the neuronal response to the standard which has been reduced. The distinction between these two components, adaptation and true or

adaptation-independent deviance detection, can be made by using specific control sequences such as the *many-standards* or *cascade* control sequences (described in detail in Ch3A). A number of studies have now demonstrated, using these controls, that MMN in humans is partly the result of true deviance detection and involves higher order functioning (Jacobsen, Schröger, Horenkamp, & Winkler, 2003; Todd, Harms, Schall, & Michie, 2013).

Reduction in MMN is a well-established phenomenon within the schizophrenia population, with a multitude of studies and meta-analyses over the last 25 years reporting reductions (Bodatsch, Brockhaus-Dumke, Klosterkotter, & Ruhrmann, 2015; Erickson, Ruffle, & Gold, 2016; Shelley et al., 1991; Umbricht & Krljes, 2005). In addition, those identified as being at high-risk of developing schizophrenia also display MMN deficits, with progression to symptom onset and schizophrenia diagnosis predicted by MMN reductions (Bodatsch et al., 2011; Perez et al., 2014). In addition, first degree relatives of schizophrenia patients also display deficits in MMN (Michie, Innes-Brown, Todd, & Jablensky, 2002). MMN reduction has also been linked to functional outcome measures in patients with schizophrenia. It has been demonstrated that poor functional status as measured by the Global Assessment of Functioning Scale and the probability of patients living independently are significantly associated with MMN deficits (Kawakubo & Kasai, 2006; Light & Braff, 2005a, 2005b). Moreover, the finding of reduced MMN appears to be relatively specific to schizophrenia and schizoaffective disorder (Umbricht et al., 2003), as reductions in MMN are not found in other psychopathologies including depression (Umbricht et al., 2003), bipolar disorder (Catts et al., 1995; Umbricht et al., 2003), or obsessive-compulsive disorder (Oades, Dittmann-Balcar, Zerbin, & Grzella, 1997; Oades, Zerbin, Dittmann-Balcar, & Eggers, 1996; Towey et al., 1994). Reduced MMN also appears to be very stable, with the

deficit persisting in patients following treatment with a range of antipsychotic medications and long after the acute phase of the disorder has passed (Korostenskaja et al., 2005; Schall, Catts, Karayanidis, & Ward, 1999; Shinozaki et al., 2002; Umbricht et al., 1998, 1999). The robust nature of MMN deficits in schizophrenia, specificity to the disorder, stability of the deficit, and relation to functional outcome measures has led to reduced MMN being identified as a potential biomarker for the disorder (Light & Swerdlow, 2015). Furthermore, the lower-order sensory nature of MMN makes it more translatable to other species than other higher-order symptoms of the disorder such as hallucinations or executive function deficits, and therefore ideal for use in animal model (Javitt & Sweet, 2015). In fact, there is now considerable evidence that non-human mammals are capable of producing mismatch responses (MMR) analogous to the MMN seen in humans (Bickel & Javitt, 2009).

MMN has been linked to functioning of the glutamatergic NMDAr, with evidence suggesting that NMDAr hypo-function may underlie the reduced MMN amplitude seen in patients with schizophrenia. The administration of NMDAr antagonist Ketamine to healthy controls has been shown to result in reduced MMN responses to both pitch and duration deviants (Heekeren et al., 2008; Umbricht, Koller, Vollenweider, & Schmid, 2002; Umbricht et al., 2000), and concurrently produce psychotic-like symptomology similar to that seen in schizophrenia (Gunduz-Bruce et al., 2012). Furthermore, a range of NMDAr antagonist animal models of schizophrenia also report reduced MMR amplitude following administration of Ketamine (Gil-da-Costa, Stoner, Fung, & Albright, 2013) and PCP in non-human primates (Javitt, Steinschneider, Schroeder, & Arezzo, 1996), MK-801 (Tikhonravov et al., 2008) and CP-101606 in rats (Sivarao et al., 2014), and Ketamine in mice (Ehrlichman, Maxwell, Majumdar, & Siegel, 2008). Not only do NMDAr antagonist-treated animals display

reductions in MMRs but they also demonstrate behavioural deficits relevant to schizophrenia (Andine et al., 1999; Becker et al., 2003; Sams-Dodd, 1999). The involvement of NMDAr functioning in MMN, specifically reduced NMDAr function, is pertinent as NMDAr dysfunction is now well established in schizophrenia. NMDAr antagonists are known to produce schizophrenia-like symptoms in healthy controls in addition to exaggerating symptoms in patients with schizophrenia (Adler et al., 1999; Krystal et al., 1994; Krystal et al., 2005; Rowland et al., 2005). Post-mortem studies have also identified reduced mRNA expression for the NR2C subunit, and reduced protein and mRNA expression of the NR1 subunit of the NMDAr in the PFC of patients with schizophrenia (Beneyto & Meador-Woodruff, 2008; Catts, Lai, Weickert, Weickert, & Catts, 2016; Shannon Weickert et al., 2013; Sokolov, 1998)

The occurrence of MMN in schizophrenia is also confirmed by neurodevelopmental animal models of the disorder, such as the VHL and social isolation models (Harms, 2016). Rats which have undergone VHL during the early neonatal period of development have been shown to develop a range of behavioural deficits analogous to those seen in schizophrenia (Tseng, Chambers, & Lipska, 2009). In addition to modelling aspects of the behavioural symptomology of schizophrenia, the VHL rats have also been shown to display reductions in MMRs (Cabungcal et al., 2014). Similar findings come from the social isolation model, where weaning-age rats are removed from their mothers and litter mates and raised in total isolation. When tested in adulthood, socially-isolated animals display reduced MMRs in comparison to controls (Witten et al., 2014), in addition to a range of schizophrenia-like behavioural abnormalities (Cilia, Reavill, Hagan, & Jones, 2001; Fabricius et al., 2010; Fone & Porkess, 2008). This evidence not only supports MMRs as a potential biomarker for

schizophrenia-like pathology that is translatable to animal models, but also links MMRs to a number of the known neurodevelopmental risk factors for the disorder.

While these studies mentioned above have investigated the impact of neurodevelopmental risk factors for schizophrenia on MMRs, no studies to date have assessed MMRs in the MIA model. Rodent MIA models of schizophrenia have previously been found to exhibit altered NMDA function, such as hyper-sensitivity to NMDAr antagonists (Zuckerman & Weiner, 2005), increased extracellular glutamate and blunted glutamate release in response in MK-801 (Roenker et al., 2011), and reduced NMDAr NR1 expression (Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008). Therefore, it is possible that this NMDAr dysfunction could contribute to a schizophrenia-like reduction in MMRs in the MIA model. In particular, the early (GD9) versus late (GD17) gestation MIA model in mice indicated a distinction in the neurochemical and behavioural dysfunction of offspring based on the gestational timing of MIA (Meyer et al., 2006), with early gestation MIA producing alterations in positive-like behavioural measures associated with disturbances in dopaminergic system, whereas exposure to MIA in late gestation resulted in NMDAr alterations and cognitive deficits (Meyer et al., 2008).

Section 3.2 (*Chapter 3A: Mismatch Negativity (MMN) in Freely-Moving Rats with Several Experimental Controls*) of this chapter is a peer-reviewed publication which outlines a number of different control methods often used to elicit MMN (the flip flop control, many-standards control, and cascade control) in an attempt to establish the most appropriate method for use in rats, that can then be used to assess MMRs in the MIA model. These methods are then used to assess adaptation and adaptation-independent deviance detection separately giving a more thorough picture of MMRs produced in early (GD10) verses late (GD19) gestational MIA rats in section 3.3

(*Chapter 3B: Deviance Detection and Mismatch Negativity (MMN) Responses in Awake, Freely-moving Rats Following MIA Exposure*).

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3.2. Chapter 3A: Mismatch Negativity (MMN) in Freely-Moving Rats with Several Experimental Controls

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3.2.1. Abstract

Mismatch negativity (MMN) is a scalp-recorded electrical potential that occurs in humans in response to an auditory stimulus that defies previously established patterns of regularity. MMN amplitude is reduced in people with schizophrenia. In this study, we aimed to develop a robust and replicable rat model of MMN, as a platform for a more thorough understanding of the neurobiology underlying MMN. One of the major concerns for animal models of MMN is whether the rodent brain is capable of producing a human-like MMN, which is not a consequence of neural adaptation to repetitive stimuli. We therefore tested several methods that have been used to control for adaptation and differential exogenous responses to stimuli within the oddball paradigm. Epidural electroencephalographic electrodes were surgically implanted over different cortical locations in adult rats. Encephalographic data were recorded using wireless telemetry while the freely-moving rats were presented with auditory oddball stimuli to assess mismatch responses. Three control sequences were utilized: the *flip-flop* control was used to control for differential responses to the physical characteristics of standards and deviants; the *many-standards* control was used to control for differential adaptation, as was the *cascade* control. Both adaptation and adaptation-independent deviance detection were observed for high-frequency (pitch), but not low-frequency deviants. In addition, the *many-standards* control method was found to be the optimal method for observing both adaptation effects and adaptation-independent mismatch responses in rats. Inconclusive results arose from the *cascade* control design as it is not yet clear whether rats can encode the complex pattern present in the control sequence. These data contribute to a growing body of evidence supporting the hypothesis that the rat brain is indeed capable of exhibiting human-like MMN, and that

the rat model is a viable platform for the further investigation of the MMN and its associated neurobiology.

3.2.2. Introduction

One of the most commonly reported and replicable electrophysiological abnormalities observed in people with schizophrenia is the reduction in the amplitude of the mismatch negativity (MMN) in response to deviations in the acoustic environment (Michie, 2001; Todd, Harms, Schall, & Michie, 2013; Umbricht & Krljes, 2005). In adult humans, MMN is evident as a negative shift in the auditory event-related potential (ERP) elicited by a rare, unexpected stimulus (the *deviant*) when it interrupts a train of common, expected stimuli (the *standards*), and typically occurs 100-200 ms after stimulus onset (Näätänen, 1992; Picton, Alain, Otten, Ritter, & Achim, 2000). A meta-analysis reported that persons with schizophrenia exhibit reductions in the size of the MMN with an overall effect size of 0.99 (Umbricht & Krljes, 2005). MMN responses can be observed in different states of consciousness and in the absence of attention to the stimuli, leading to its characterisation as an automatic, pre-attentive process (Näätänen, 1992). MMN is primarily generated in the auditory cortex, with some contribution from the frontal cortex and other areas (Alho, 1995; Molholm, Martinez, Ritter, Javitt, & Foxe, 2005). It has been observed in neural activity measured using electroencephalography (EEG) (Näätänen & Escera, 2000), magnetoencephalography (Hari et al., 1984), and optical imaging (Rinne et al., 1999). MMN is typically measured using *oddball* sequences of auditory stimuli, in which a repeated train of standards is unexpectedly interrupted by a low-probability deviant. MMN is commonly elicited by presenting deviants that differ from the standards in some simple characteristic feature, such as frequency or duration (Näätänen & Escera, 2000).

In recent years, the MMN research community has begun to focus on developing animal models of MMN, in order to investigate the neurobiological mechanisms underlying the MMN, such as the role of specific neurotransmitter systems, contributions from different cortical layers or brain regions to the surface potential, and relationship to upstream effects that appear to be related to MMN such as stimulus specific adaptation (SSA) (Nelken & Ulanovsky, 2007; Ulanovsky, Las, & Nelken, 2003). Several models in rats, mice and non-human primates have been studied with varied results (for detailed review see Todd et al. (2013)). There are two important factors that need to be examined and controlled when identifying an animal homologue of the human MMN: first is the possibility of differential responses to the physical characteristics of the stimuli used as the standards and deviants. This is addressed in *flip-flop* sequences, where two oddball sequences are presented with the identity of the standard and deviant reversed (e.g. a particular tone is the deviant in one sequence and the standard in the other). This permits the response to a stimulus when it is a deviant to be compared to the same stimulus when it is a standard (Figure 3.1A). The second factor is the role of *adaptation* versus *'true' deviance detection*, which, within some theoretical frameworks, is considered to be a memory-based or a predictive coding error signal (Fishman, 2014; Friston, 2005; Winkler, Karmos, & Näätänen, 1996). Several studies in both humans and animals have shown that with repeated exposure to a stimulus, the neural populations responding to that stimulus undergo *adaptation*[1], in which their responses are dampened with higher probabilities of stimulation (Eriksson & Villa, 2005; Farley, Quirk, Doherty, & Christian, 2010; Fishman & Steinschneider,

[1] While the term *adaptation* is used here, other terms are often used to describe similar, but not exactly synonymous phenomena (the reduction of a response to a stimulus with repeated exposure), such as habituation, refractoriness, and stimulus-specific adaptation.

2012; Maess, Jacobsen, Schroger, & Friederici, 2007; Opitz, Schroger, & von Cramon, 2005; Taaseh, Yaron, & Nelken, 2011; Ulanovsky, Las, Farkas, & Nelken, 2004; Ulanovsky et al., 2003; von der Behrens, Bauerle, Kossl, & Gaese, 2009). This means that a larger response to a deviant stimulus may simply be due to lower levels of adaptation of neural populations responding to a rare stimulus (the deviant) compared to a frequent stimulus (the standard). This is addressed in several studies by using a *many-standards control* sequence (Figure 3.1B). In this sequence, the deviant tone from the oddball sequence is presented with the same probability as it is presented within the oddball sequence, but it is nested within many other equally-probable tones. The tones are presented pseudo-randomly (without repetition) so that no pattern of regularity is established. This lack of regularity ensures that no specific 'prediction' is set that can be violated. Comparing the response to the same physical stimulus when it is a deviant within the oddball sequence, to when it is the control stimulus within the many-standards sequence, provides a measure of the adaptation-independent comparison process contribution that is thought to underlie MMN.

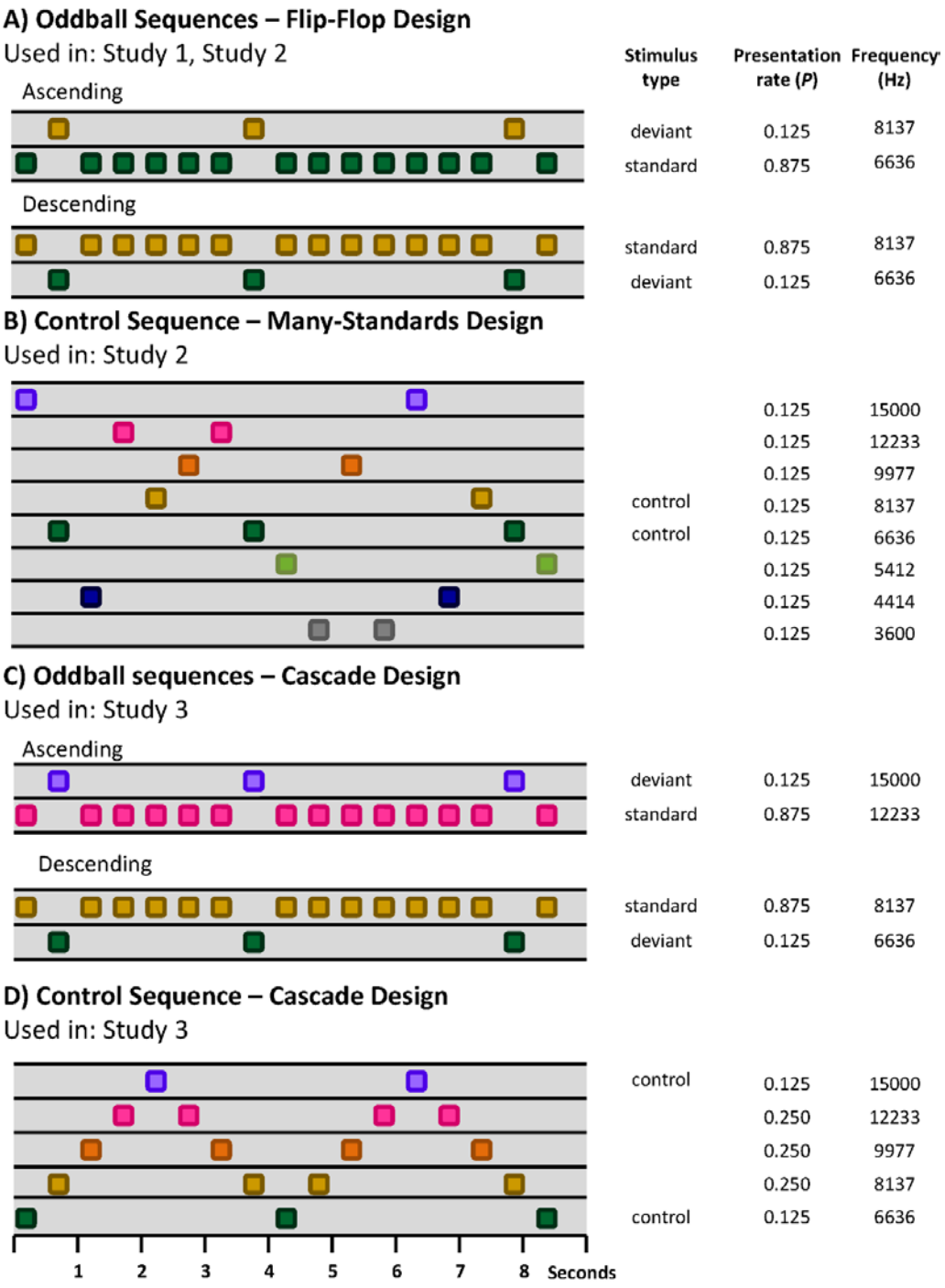


Figure 3.1. Control sequence designs used in the current investigation. (A) Both Study 1 and Study 2 used a flip-flop design for oddball sequences. This design allows for the comparison of the response to a stimulus when it is a rare, unexpected deviant to the same tone when it is a common, expected standard, controlling for differences in responses to the physical characteristics of the stimuli, but not for differential adaptation. (B) The many-standards control sequence was used in Study 2 to test responses to stimuli that would have prompted the same level of adaptation as the

deviant stimulus. Comparing the responses to the deviant used in the oddball sequence, where it defies established stimulus regularity predictions, to the control in the many-standards sequence, which does not defy regularity predictions (there is no established regularity) yields a measure of adaptation-independent deviance detection. Comparing the responses to the control (presented rarely) in the many-standards sequence to the standard (presented often) in the oddball sequence yields a measure of adaptation to a rare stimulus vs. a common stimulus. (C) The cascade sequence designs were used for Study 3. The oddball sequences are similar to those used in Study 1 and Study 2, except that a flip-flop design was not used, and the stimuli presented in the ascending and descending sequences are respectively on the upper and lower end of the range of sequences used in the control sequence. (D) The control sequence (like the many-standards sequence) presents the stimuli used as deviants in the oddball sequences at the same probability as they are when deviants in a context where they do not defy established patterns in regularity, thus enabling the control of differential adaptation.

Using these approaches, MMN in humans has been found to comprise two 'elements' after controlling for the physical characteristics of the stimulus using the flip-flop control method: an adaptation element ('sensorial' element, represented by the difference between the rare control stimulus and the common standard stimulus), and a prediction error-like element ('cognitive' element, represented by the difference between the rare control stimulus and the rare deviant stimulus) (Jacobsen & Schroger, 2001; Jacobsen & Schröger, 2003; Jacobsen, Schröger, Horenkamp, & Winkler, 2003; Maess et al., 2007; Opitz et al., 2005). While this feature of MMN has several designations: memory-based MMN (Astikainen, Ruusuvirta, Wikgren, & Penttonen, 2006; Astikainen et al., 2011), prediction error (Jung et al., 2013), cognitive (versus sensory) MMN (Maess et al., 2007; Opitz et al., 2005), and deviance detection (Grimm & Escera, 2012; Imada, Morris, & Wiest, 2012; Shiramatsu, Kanzaki, & Takahashi, 2013; Taaseh et al., 2011) to name a few, for the purposes of clarity in the remainder of

the paper, *adaptation-independence* and/or *deviance detection* will be used to describe the element of MMN that remains when adaptation is controlled for.

While the two elements comprising MMN (adaptation and adaptation-independent deviance detection) are not commonly disentangled in human studies (for exceptions, (Maess et al., 2007; Opitz et al., 2005), it is important for animal models to test for both. This is because a) it is unknown whether a given species is capable of generating an adaptation-independent response as humans do, and b) these two elements likely have diverging neural mechanisms and signatures. If the aim of establishing an animal model of MMN is to investigate the underlying neural mechanisms of MMN, then the MMN elements being investigated should be identified.

The many-standards control sequence, as mentioned, controls for the effects of stimulus presentation probability so that differential adaptation can be observed in the absence of the established models of regularities. However, this control has been subject to two criticisms (Ruhnau, Herrmann, & Schroger, 2012). First, it may be overly conservative, because the variety (usually frequency range) of stimuli presented in the many-standards sequence is substantially larger than that in the oddball paradigm. It has been demonstrated using local field potential and multiunit activity recordings that many-standards sequences using a broad range of frequencies produce larger responses than those from narrow sequences, regardless of presentation rate (Taaseh et al., 2011). This would indicate that potentially, the control response in the many-standards sequence is not affected as much by adaptation and therefore is increased in amplitude. However, the deviant response from the oddball sequence (containing a narrow range of stimuli) would presumably undergo *more* adaptation than the control response, and consequently be reduced in size. This possible imbalance in the amount of adaption undergone by the control and deviant responses could result in an underestimation of

the difference between the control and deviant responses (deviance detection). Second, in the oddball paradigm, deviants are presented within a repetitive, predictable sequence, but no such repetition is established within the many-standards control sequence. To avoid these issues, a cascade control sequence has been proposed (Ruhnau et al., 2012). In this method, firstly, there are a small number, nominally five, stimuli that vary from low- to high-frequency, with the highest frequency stimulus corresponding to the deviant, and the second highest frequency stimulus corresponding to the standard within an ascending oddball sequence (Figure 3.1C, D). Secondly, the stimuli are presented in a regular pattern from low- to high-frequency, then back down to low-frequency, repetitively (Figure 3.1D). The high-frequency stimulus at the upper extreme of the stimulus range is used as a control for high-frequency deviants in the ascending oddball task. An equivalent sequence can be adapted for low-frequency deviants in a descending oddball sequence. Within the cascade sequence, the variety of stimuli presented is more comparable to that in the oddball task, and the control stimulus is always preceded by a stimulus that is physically identical to the standard within the oddball task. This can improve the estimation of adaptation effects. In addition, the cascade control incorporates a background regularity, albeit a more complex one than oddball sequences, but where the occurrence of the high- (and low-) frequency tones at the extremes of the cascade sequence are predictable, in contrast to the equivalent high (and low) frequencies of oddball sequences. Therefore, the cascade control provides the opportunity to observe adaptation-independent deviance detection in the context of unpredictable deviants versus predictable deviants, assuming of course that the rat brain is able to model the regularity of the cascade sequence.

In a recent review of animal models of MMN (Todd et al., 2013), several important trends were identified. First, mismatch responses (MMR) in animals typically

occur earlier than in humans, likely due to the smaller brain size. Second, the difference between the deviant and the standard can be either negative or positive in polarity (positive shifts are far more common in recordings in anaesthetised animals, particularly when anaesthetised with urethane). Finally, deviance detection that is independent of the effects of differential adaptation is rarely observable in small-field recordings (e.g. local field potentials, multiunit activity) from primary auditory cortex (Farley et al., 2010; Fishman & Steinschneider, 2012; Taaseh et al., 2011), but are more often observed with epidural electrodes (Ahmed et al., 2011; Astikainen et al., 2011; Jung et al., 2013; Nakamura et al., 2011; Ruusuvirta, Penttonen, & Korhonen, 1998; Tikhonravov et al., 2008, 2010). This is in agreement with emerging studies suggesting that non-primary areas of the auditory cortex are involved in adaptation-independent MMN (Opitz et al., 2005). Indeed a recent study has demonstrated that while non-MMN components of the auditory ERP are localised to primary (core) auditory cortical areas and can be tonotopically mapped, adaptation-independent MMN is highly distributed over the auditory cortex, including secondary auditory 'belt' regions and are not tonotopically localised (Shiramatsu et al., 2013). All of the studies that instituted a control method for adaptation effects used either a deviant-alone control (another method for controlling for adaptation) or the many-standards control sequence. To our knowledge, the cascade control method has not been tested in an animal model thus far.

Our laboratory has previously published one of the aforementioned animal model studies (Nakamura et al., 2011), in which adaptation-independent deviance detection was observed in awake rats to deviants in stimulus frequency. These were only observed for high-frequency deviants (3600 Hz), not low-frequency deviants (2500 Hz), in agreement with similar findings in anaesthetised rats (Astikainen et al., 2011), indicating a possible enhanced salience for increments in frequency, compared to

decrements. Another possible reason for why rats in our previous study exhibited deviance detection to high but not low-frequency stimuli could be the frequency of the tones that were tested (2500 and 3600 Hz), which were at the lower end of the rats' frequency sensitivity (Kelly & Masterton, 1977; Mazurek et al., 2010). Examining whether deviance detection can be elicited to higher-frequency tones that are closer to the peak of rats' frequency sensitivity (e.g. ~16000 Hz) will determine if the preference for high-frequency deviants was an artefact resulting from the use of low-frequency sounds.

Many previous studies in animal models have only investigated MMRs in single locations, typically over auditory cortex. However, it is possible that MMRs in rats are more readily observed at other locations depending upon the orientation and location of generators. In humans for example, the major generators of MMN are located in auditory cortex, yet the largest response is seen over frontal areas even though there may be only a small contribution from frontal generators (Alho, 1995; Alho, Woods, Algazi, Knight, & Naatanen, 1994). Therefore, an examination of the effect of recording location on the amplitude of MMRs is warranted.

In the current investigation, we aimed to replicate our laboratory's previous evidence of adaptation-independent deviance detection in the rat, with the overall aim of determining which conditions produce the most robust adaptation responses and adaptation-independent deviance detection responses. In Study 1, we used the same recording system previously used in our laboratory (Nakamura et al., 2011), to determine if MMRs are produced to tones of higher frequencies (closer to the rats' peak frequency sensitivity). In Study 2, we used a new system allowing multichannel recordings, to characterize the morphology at different locations of adaptation and adaptation-independent deviance detection using the many-standards control. Finally, in

Study 3, we investigated the utility of the cascade control method for recording MMRs in rats.

3.2.3. Methods

3.2.3.1. Ethics Statement

All experiments were performed under strict adherence to the National Health and Medical Research Council's Australian code of practice for the care and use of animals for scientific purposes and were approved by the University of Newcastle's Animal Care and Ethics Committee (Approval number A-2009-108). Surgical procedures were performed under well-maintained anaesthesia and all efforts were made to reduce the number of animals used and alleviate pain and discomfort following surgery through use of analgesics.

3.2.3.2. Animals and Surgery

3.2.3.2.1. Study 1

Nine male Wistar rats (sourced from the University of Newcastle's Central Animal House) were used for Study 1. All rats were on a 12 h light/dark cycle with lights on at 0630 h. The surgery was performed when the animals were on average 96 days old (89-111 days old). The average weight of the animals was 456.9 g (381-513 g) on the day of surgery. Animals were anaesthetised with fentanyl (300 μg/kg i.p.) and medetomidine (300 μg/kg i.p.), and/or isoflurane and the rat was fixed onto a stereotaxic frame (Stoelting, IL, USA) and placed on a heating pad during surgery. A battery operated biotelemetric radiotransmitter (model # TA11CA-F40, Data Sciences International, St. Paul, MN, USA) was implanted in the peritoneal cavity. Insulated biopotential leads from the transmitter were passed subcutaneously to the base of the

skull. The skin over the skull was exposed and 2 small burr holes were drilled in the skull, one hole for the active electrode over the right auditory cortex (4.5 mm posterior to Bregma and 3.5 mm lateral to the midline) and the other for the reference electrode in the left cerebellum (2 mm posterior to the lambda and 2 mm lateral to the midline). These locations are based on previous research demonstrating MMN-like epidural responses in the rat (Tikhonravov et al., 2008). The leads were fixed with dental acrylic. Carprofen (5 mg/kg s.c.) and buprenorphine (0.05 mg/kg s.c.) were administered pre-operatively as analgesics. The animals were allowed to recover for at least 6 days after surgery before the first ERP recordings.

Testing occurred within an experimental chamber covered with grounded copper mesh acting as a Faraday cage. The rat was placed in a partition (internal dimensions: length 23.5 cm, width 12.0 cm, height 24.0 cm) within the experimental chamber. EEGs were recorded using custom acquisition software written in LabVIEW (version 8.2.1). Two channels of data were continuously digitised (1000 Hz): a single EEG channel, and an analogue trigger pulse generated by the PC sound card in parallel with the auditory stimulus. Stimulus event codes were logged with the EEG data. The bandwidth of the data acquisition system was 0.2-150 Hz and the input voltage range was ±10 mV.

3.2.3.2.2. Studies 2 and 3

Eighteen male Wistar rats were used for Study 2, 15 of which were also used for Study 3. These rats were on a 12 h light/dark cycle with lights on at 0000 h (midnight) and were used as controls for another study investigating the role of developmental exposure to immune activation on electrophysiological measures. Seven female Wistar rats (sourced from the University of Newcastle's Central Animal House) were time-mated with three male Wistar breeders. The day of positive sperm detection was

designated as gestational day (GD) 0. Four pregnant females were injected with saline on GD10 and three were injected with saline on GD19, resulting in nine male offspring exposed to prenatal saline injection at GD10 and nine at GD19, all of which were used for Study 2. For Study 3, only eight rats exposed to GD10 injection and seven exposed at GD19 were used. Pregnant females were anesthetised with isoflurane, and given an intravenous administration (via the lateral tail vein) of 0.1 M phosphate buffered saline (at 1 mL/kg body weight).

The surgery to implant electrodes was performed on the male offspring of these pregnant animals when they were, on average, 108 days old (76-137 days) and weighed on average 481.76 g (370-593 g). Rats were anaesthetised with isoflurane, placed on a heating pad, and fixed to a stereotaxic frame (Stoelting, IL, USA). The dorsal surface of the skull from +4.00 mm to -12.00 mm relative to Bregma and 4-5 mm lateral from the midline was exposed and the periosteum was removed. A custom-made electrode connector was implanted onto the rat's skull. The connector consisted of a 10-pin male-female socket (BD075-10-A-1-L-D from Global Connector Technology, Lawrence, MA, USA), with the pins soldered to magnet wire (8057 from Belden, St Louis, MO, USA) and embedded in epoxy resin (RS 1991402, RS Components, Sydney, Australia). Seven wires from the connector were soldered to stainless steel screws (B002SG89S4, Amazon Supply, USA). Seven 0.9 mm burr holes were made into the skull of the rat, penetrating all the way through the skull, but not through the dura. The screw electrodes were implanted into these holes until they were fixed in place. Five screws were used as recording electrodes and were placed above the left and right auditory cortices (LAC and RAC, 5.00 mm posterior to Bregma and 4.00 mm lateral to the midline), the left and right frontal cortices (LFC and RFC, 2.00 mm anterior to Bregma and 2.00 mm lateral to the midline), and a location to the left of the midline (LML, 3.50 mm posterior

to Bregma and 1.00 mm left of the midline). The ground screw was placed over the right posterior cortex (2.0 0 mm anterior to Lambda and 2.50 mm right of the midline), and the reference screw over the cerebellum (1.00 mm posterior to Lambda and 1.00 mm to the right of the midline). The wire connecting the screw electrodes to the connector was wound around their respective screws and the wires, screws and socket were fixed to the animal's head using dental cement (Dentsply, Mount Waverly, VIC, Australia). Carprofen (5 mg/kg s.c.) and buprenorphine (0.05 mg/kg s.c.) were administered pre-operatively as analgesics. The animals were allowed to recover for at least 4 days after surgery before the first ERP recordings.

Immediately prior to testing, a wireless telemetric 8-channel headstage from Multi Channel Systems (Reutlingen, Germany) was connected to a battery using reusable adhesive, and then attached to the electrode connector previously implanted on the rat's head. Testing occurred within an expanded PVC sound-attenuating chamber (ENV-018V, Med Associates, St. Albans, VT USA) with the interior covered with sound-absorbing foam. The awake rat was placed in a 32 cm diameter plastic bucket, containing pressed-paper bedding, where it was free to roam. EEG data were recorded using Multi Channel Systems MCRack software. Each channel of EEG data was digitised at 2000Hz (high pass filter 0.1 Hz; low pass filter 5000 Hz; voltage range ±12.4 mV)[2]. Event code markers and a trigger pulse generated by the sound card in

[2] The multichannel system used for these recordings implements a 5KHz low pass filter on the head-stage as an analog filter prior to the digitisation stage. The low pass cut-off frequency cannot be changed. Normally this would mean that any sampling frequency below 10kHz might be affected by aliasing of high frequency noise signals (in this case between 1kHz – 5kHz). However, the Multi-channel system uses over-sampling techniques, meaning the head stage samples at 20kHz and then down-samples to the requested 2kHz output rate. It does this by digitally averaging every 10 samples (taken at 20KHz) to produce one output sample (at 2 KHz). This in effect acts as a digital 'comb' filter with notch frequencies at 2KHz and all

parallel with the auditory stimuli were recorded as digital signals at the same sampling rate.

3.2.3.3. Sound Generation

3.2.3.3.1. Study 1

Auditory stimuli were generated with a custom program written in Presentation (version 14.1, Neurobehavioral Systems, Inc.), amplified and delivered through a speaker (50 Hz-19000 Hz frequency response) mounted at an approximate height of 1 m above the floor of the experimental chamber. Sound intensity was calibrated with a sound meter (Brüel & Kjær Model 2260) using a linear weighting to an average of 78 dB_L SPL across locations within the chamber for the sounds in the 6636 and 8137 Hz range used in this study.

3.2.3.3.2. Studies 2 and 3

Auditory stimuli were generated with a custom program written in Presentation (version 14.1, Neurobehavioral Systems, Inc.), amplified and delivered through a speaker (1 kHz – 30 kHz frequency response) mounted at an approximate height of 50 cm above the floor of the experimental chamber. Sound intensity was calibrated with a sound meter (Brüel & Kjær Model 2260) using a linear weighting to an average of 70 dB_L SPL across locations within the chamber for the sounds in the 3600 and 15000 Hz range used in these studies.

3.2.3.4. Experiment Design and Stimuli

harmonic frequencies above that (i.e. 4, 8, 12, ... kHz). This comb filter has a 3dB low-pass cu-toff somewhere below 2 kHz, which will 'almost' act as an anti-aliasing filter.

3.2.3.4.1. Study 1 – Flip-flop Control

Rats were tested for one half-hour session each day, for three days. The awake rat was placed in the experimental chamber for 15 min before each session to acclimatise. Each session consisted of an ascending and a descending oddball sequence separated by a 3 min break. The order of the two sequences alternated for each rat across test sessions, and was balanced across rats.

Two sequences were presented in Study 1. These were oddball sequences where the roles of the deviant and standard were reversed (flip-flop condition) resulting in either an ascending deviant sequence (low-frequency standard and high-frequency deviant) or a descending deviant sequence (high-frequency standard and low-frequency deviant) (Figure 3.1A). In the ascending and descending oddball sequences, 87.5% of the tones were standards and 12.5% deviants. Previous findings have demonstrated that certain components of the deviant response were sensitive to the recent stimulus history of standards, in that it increased in amplitude as the number of preceding standard increased from 1 to 5 or more (Nakamura et al., 2011). In order to maximise MMRs, the oddball sequences of Studies 1-3 reported here were designed to have at least 3 standards prior to each deviant. For all sequences, tones had a 10 ms rise and fall time and a stimulus onset asynchrony (SOA) of 500 ms. Two tones of 100 ms duration were used: a low-frequency tone of 6636 Hz and a high-frequency tone of 8137 Hz, equivalent to a 0.29 octave difference or normalised frequency difference (or Δf) of $(f_2 - f_1)/(f_2 \times f_1)^{1/2} = 0.20$ where f_1 = 6636 Hz and f_2 = 8137 Hz (Ulanovsky et al., 2003) . Each of the sequences consisted of 1600 tones and ran for 13.33 min.

3.2.3.4.2. Study 2 – Many-Standards Control

Rats were tested for MMRs on one 62 min session a day for three days, and were exposed to three different testing orders for each of the three days. The rat was placed in the experimental chamber with bedding for 5 min before each session to acclimatise. The rat did not have access to food or water during the session but was free to explore the testing chamber during the recordings. On each of the three testing days, the rat was also tested in separate sessions on two other auditory paradigms that are not reported here.

Each session in Study 2 consisted of four types of sequences each presented twice, resulting in eight sequences per session. Two of the four types of sequence were the ascending and descending oddball sequences described for Study 1 (Figure 3.1A). The other sequences were many-standards control sequences in which tones equivalent to the deviants in the ascending and descending oddball sequences were presented at the same probability as in the oddball sequences (12.5%) but randomly interspersed with six other tones (also presented at 12.5%), ensuring that a pattern of regularity in the auditory stimuli was not established (Jacobsen & Schroger, 2001; Nelken & Ulanovsky, 2007) (Figure 3.1B).

The two many-standards control sequences were subtly different in order to accommodate the pseudo-random stimulus orders within the ascending and descending oddball sequences. For all sequences, tones had a 10 ms rise and fall time, a duration of 100 ms and a SOA of 500 ms. Eight frequencies (each of 100 ms duration) differing on a logarithmic scale were presented: 3600 Hz, 4414 Hz, 5412 Hz, 6636 Hz (equivalent to oddball low-frequency deviant), 8137 Hz (equivalent to high-frequency oddball deviant), 9977 Hz, 12233 Hz and 15000 Hz. In the first of the control sequences, the 8137 Hz stimulus was presented in exactly the same temporal location (relative to the beginning of the sequence) as in the ascending oddball deviant sequence. In the second

of the control sequences, the 6636 Hz stimulus was presented in the same temporal location as in the descending oddball deviant sequence, but neither of the sequences controlled for the tone preceding the deviant. The remaining tones were presented in pseudorandom order except that no tone was ever repeated. In order to avoid the possibility of an MMN being elicited by tones at the extremes of a range for either the frequency control conditions, known as the extreme substandard effect (Jacobsen & Schroger, 2001; Jacobsen et al., 2003; Winkler et al., 1990), the standard and deviant used in the ascending and descending sequences were the fourth and the fifth highest frequencies (Figure 3.1B).

Within each session, sequences were presented in one of four orders, and repeated in that same order. Blocks of sequences began with one oddball sequence, followed by the two control sequences (with two order combinations), and ending with the other oddball sequence. Within a block, sequences were separated by 1 min silent breaks and a 3 min silent break separated the two blocks. Each sequence contained 800 stimuli and ran for 6.67 minutes, and each session ran for 62 min.

3.2.3.4.3. Study 3 – Cascade Control

The same animals used for Study 2 were also used in Study 3. Rats were tested for one recording session using the cascade control sequences. The rat was placed in the experimental chamber for 5 min before the commencement of the session and was free to explore during recordings. Three types of sequence were presented, and similar to Study 2, each sequence consisted of 800 tones, each played with 100 ms duration, 10 ms rise and fall time and SOA of 500 ms. Similar to Studies 1 and 2, Sequences 1 and 2 were ascending and descending oddball sequences (Figure 3.1C). The ascending sequence consisted of a low-frequency standard (12233 Hz, 87.5%) and a high-

frequency deviant (15000 Hz, 12.5%), and the descending sequence consisted of a high-frequency standard (8137 Hz, 87.5%) and a low-frequency deviant (6636 Hz, 12.5%). Sequence 3 was a cascade control sequence (Figure 3.1D). Five tones were presented in this sequence: 6636 Hz, 8137 Hz, 9977 Hz, 12233 Hz and 15000 Hz, played in order from lowest frequency to highest frequency, back to lowest frequency in a 'cascading' order, similar to Ruhnau et al. (2012). In this sequence, the two tones used as deviants in ascending and descending oddball sequences are presented with the same probability as in the oddball sequences (12.5%), whereas the other tones are presented at a probability of 25%. Within a session, sequences were presented in one of two orders: either 1) Ascending, Control, Descending; or 2) Descending, Control, Ascending. Each of these blocks was presented twice within a session with a 3 min silent break between the two blocks and 1 min silent breaks between each sequence within a block. The total session time was approximately 49 min.

3.2.3.5. Data Extraction

Data processing was performed off-line with EEGDisplay 6.3.12 (Fulham, 2012). Intervals of gross artefacts in the continuous EEG record were excluded using an automated algorithm that rejected signals exceeding ±1400 µV. Epochs were extracted from the continuous EEG consisting of a 100 ms pre-stimulus baseline and a 400 ms post-stimulus interval. The first 25 tones at the start of each tone sequence were excluded from analysis to allow for transitory effects associated with switching between different types of sequences or the beginning of the session. Within oddball sequences, the first standard following each deviant was excluded from the analysis to allow for recovery of a stable response to standards. Following these pre-processing steps, epochs were averaged off-line for each animal and session separately and ERPs extracted for

each of the stimulus types, including the responses to deviants and standards, as well as their respective controls for each of the studies. ERPs were baseline corrected over a 50 ms pre-stimulus interval for Study 1 and a 100 ms pre-stimulus interval for Studies 2 and 3.

The ERPs recorded in these studies exhibited distinct components over the first 200 ms, although the amplitudes of these components differed according to the type and frequency of the stimulus. For Study 1, they were characterised by a negative component peaking at approximately 22 ms (denoted as N22), followed by a positive peak at 37 ms (P37) and a second broad negative component with a peak latency of approximately 60-100 ms (N80). For Studies 2 and 3 (using different EEG recording and acoustic delivery), the components were identified to occur slightly earlier with an additional early positive component being identified, and a clear separation of the broad late negative shift into two distinct peaks. The ERP was characterised by an initial positive peak at 13 ms (P13), a negative peak at 18 ms (N18), followed by a positive peak at 30 ms (P30) and a broad negative component with two discernible peaks from approximately 45-65 ms (N55) and 65-105 ms (N85).

For Study 1, three mean amplitude measures were extracted over latency windows corresponding to the ERP peaks: a 15 ms window from 15-30 ms for N22[3], an 8 ms window from 35-43 ms for P37 and a 40 ms window from 60-100 ms for N80. For Studies 2 and 3, five mean amplitude measures were extracted over the following latency windows: a 4 ms window from 11-15 ms (P13), a 7 ms window from 15-22 ms

[3] A wide window was used to assess N22 because there were relatively large individual differences in the latency of the peak, which was far more variable across animals than P37 (Figure 3.3).

for N18, a 21 ms window from 22-43 ms for P30, a 23 ms window from 43.5-65.5 ms for N55 and a 40 ms window from 65.5-105.5 ms for N85.

3.2.3.6. Statistical Analysis

All analyses were performed controlling for stimulus identity. That is, only responses to stimuli of the same frequency were compared. For example, although the 8 kHz deviant in Study 2 was presented in an ascending oddball sequence with a 6 kHz standard, all analyses performed on the 8 kHz deviant involved comparisons with the 8 kHz standard (used in the descending oddball sequence) and the 8 kHz control (used in the many-standards control sequence). Therefore, for this study, when referring to a Deviant vs. Standard comparison, we do not refer to the Deviant and Standard tones within an individual sequence, rather we refer to the deviant tone of a certain frequency and its respective flip-flop controlled standard tone of the same frequency.

Mean amplitudes of the ERP components were analysed using Analysis of Variance (ANOVA) with one or more repeated measures factors depending upon the study. Within-subjects factors were *Stimulus Type* (Study 1: Deviant and Standard, Study 2: Deviant, Control and Standard, Study 3: Deviant and Control), *Stimulus Frequency* (Studies 1 and 2: 6636 Hz, 8137 Hz; Study 3: 6636 and 15000 Hz) and *electrode location* (in the case of Studies 2 and 3, left and right auditory cortices, LAC and RAC; left and right frontal cortices, LFC and RFC; and left of the midline, LML). Each ERP component was analysed separately. Gestational age of maternal treatment (for Studies 2 and 3 only) with saline was also used as a between-subjects factor to ensure that the different gestational day of treatment did not impact findings in this group of animals. In instances where sphericity was violated, Huynh-Feldt adjusted degrees of freedom were used to determine significance levels.

Given the large number of regions and components analysed in Study 2 and 3, significance levels for the first-pass, omnibus ANOVA were set at $p < 0.01$, to reduce the likelihood of Type 1 errors. Once an effect was identified in this first ANOVA, follow-up ANOVAs and *post-hoc* comparisons used a significance level of $p < 0.05$. *Post hoc* pairwise comparisons were made using Bonferroni correction and *p* values will be expressed as the Bonferroni-corrected value, p_b. These pairwise comparisons were used to determine whether *oddball effects*, *deviance detection* or *adaptation* were present to a statistically significant degree. *Oddball effects* occur when the amplitude of the response to the deviant is significantly larger than that to the standard stimulus (i.e. more positive for positive components and more negative for negative components) and these were assessed in Studies 1 and 2 (designs in which deviants and standards of the same frequency were presented). This measure of MMN, while controlling for different stimulus frequencies, does not comprise a control for different levels of adaptation to the standard and deviant stimuli. Therefore, two other comparisons were made. In Studies 2 and 3, the amplitude response to the deviant was compared to the control to determine the level of *deviance detection*, and in Study 2, the amplitude response to the control was compared to the standard to determine the level of *adaptation.* The magnitude of these oddball, adaptation and deviance detection effects were expressed as effect sizes measured by Cohen's *d*.

3.2.3.7. Incomplete Data

In Studies 2 and 3, some animals did not have a complete dataset. This was caused by poor-quality, noisy EEG traces from particular electrodes that caused certain regions to be removed from analysis. For Study 2, only two rats had incomplete data

(both were missing data for three regions). These rats were removed from the analysis, resulting in a final sample size of 16 for Study 2.

For Study 3, on the other hand, there were a large number of animals with incomplete data: no regions produced usable data in all animals. A total of seven rats had incomplete data, but none were missing data for more than three regions. The data for Study 3 were initially analysed without the seven animals with incomplete data. However, removing so many from the analysis could result in a dramatic loss of useful information and reduced power. For instance, only one animal had missing data from each of the frontal cortex sites. Therefore, in order to still utilize the full dataset from Study 3, automatic imputation was used to impute the missing data for the incomplete samples. Five data imputations were made in SPSS for variables with missing data points. Data were imputed using linear regression separately for each component, after which, analyses were performed as described above for the original (incomplete) dataset and the five datasets containing imputations. Effects will be reported as significant if they are present in the majority of datasets (>4 of 6), and statistics (*F* and *p* values) will be reported for the most conservative change (lowest *F* value).

3.2.4. Results

3.2.4.1. Study 1 – Flip-flop Control

Raw mean amplitude data for Study 1 are available in Supplementary data S1. In Study 1, the early components, N22 and P37 were larger in response to deviant stimuli (Stimulus Type: N22 $F_{(1, 8)} = 11.22$, $p = 0.010$; P37, $F_{(1, 8)} = 13.11$, $p = 0.007$; Figure 3.2). The later component, N80, in addition to main effects of Stimulus Type ($F_{(1, 8)} = 37.05$, $p < 0.001$) and frequency ($F_{(1, 8)} = 8.08$, $p = 0.022$), also exhibited a Stimulus Type x Frequency interaction ($F_{(1, 8)} = 11.06$, $p = 0.010$) due to the deviant producing a

larger N80 than the standard only for high-frequency stimuli (p_b = 0.001; d = 1.74). No other significant changes in the response to the deviant were identified although a similar trend-level effect was observed for N22 to the high-frequency deviant (p_b = 0.087; d = 0.65).

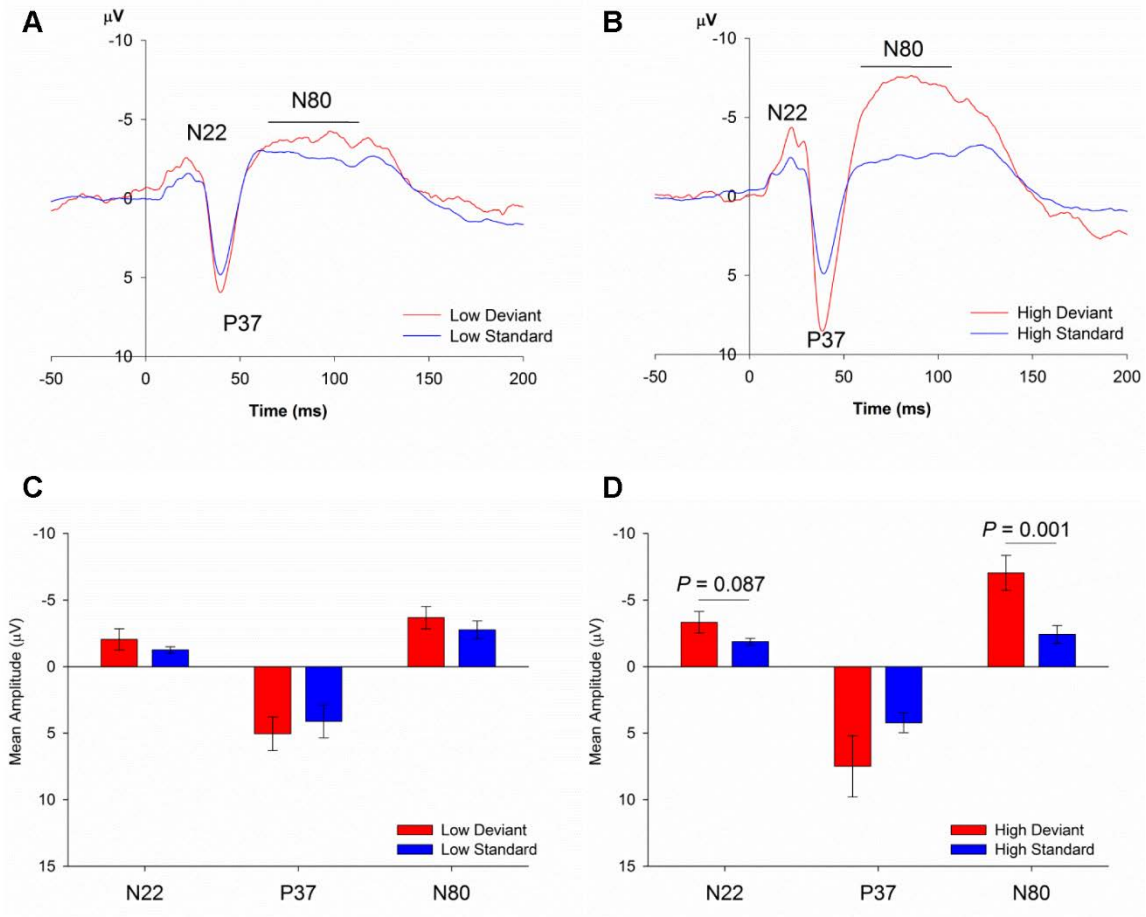


Figure 3.2. Rat ERPs in Study 1. (A, B) ERPs to the oddball deviant (red) and the standard (blue) for the low (A) and high (B) frequency stimuli. All stimuli show a similar pattern with the same components (N22, P37, N80): responses to deviants are larger in amplitude in comparison to standards. (C, D) Mean amplitudes (± standard error, SE) of N22, P37 and N80 generated by oddball deviants (red) and standards (blue), showing that responses to the deviant compared to the standard were larger for the N80 component (p = 0.001), and the N22 (although not significantly, p = 0.087).

3.2.4.2. Study 2 – Many-standards Control

Raw mean amplitude data for Study 2 are available in Supplementary data S2.

3.2.4.2.1. Effects of GD of Saline Treatment

In order to ensure that the pool of animals used in this study was relatively homogenous and not differentially affected by the developmental intervention at different GDs, GD was included as a between subjects factor in all statistical analyses. The only effect of GD was seen for the P30 component to high frequencies. For the P30 component, a main effect of GD ($F_{(1, 14)} = 5.01$, $p = 0.042$) and a Stimulus Type x GD interaction ($F_{(2, 28)} = 3.53$, $p = 0.043$) were observed. There was a significant effect of Stimulus Type for GD19 rats ($F_{(2, 16)} = 5.88$, $p = 0.012$), but not for GD10 rats ($F_{(2, 12)} = 2.51$, $p = 0.122$). Although not significant, the mean values for the deviant, control and standard P30 responses for the GD10s followed the expected pattern, with largest values for the response to the deviant and smallest values for the response the standard (Deviant = 7.60, Control = 6.22, Standard = 5.83). However, GD19 rats had a larger control response than both deviant and standard (Deviant = 3.72, Control = 5.02, Standard = 2.98). However, with the exception of the P30 component, overall, GD had little effect on component amplitudes and conditions. Half of the rats in Study 2 (the GD19 rats) having unexpectedly large control P30 responses relative to deviant responses may result in an overestimation of adaptation effects (Control vs. Standard) and an underestimation or reversal of deviance detection (Deviant vs. Control). This will be considered when interpreting and discussing results for P30.

3.2.4.2.2. Overall Effects of Region

Figure 3.3 shows the ERPs generated for each of the different frequencies in the many-standards control condition over each of the different sites, as well as for all of the

sites averaged together (Figure 3.3F). Analysis of the effects of region were only performed on deviant and standard stimuli from the oddball sequences and the 6636Hz and 8137Hz (low- and high-frequency) stimuli from the many-standards control sequence. There were significant region effects for all five components. For the earliest component, P13, amplitudes were largest at the midline and auditory cortex sites and smallest at the frontal sites ($F_{(3.25, 21.47)} = 9.47$, $p < 0.001$). N18 amplitudes, on the other hand, were largest at auditory sites compared to frontal and midline sites ($F_{(2.85, 39.94)} = 5.33$, $p = 0.004$). The later components (P30, N55 and N85) were largest at frontal cortex sites (P30: $F_{(4, 56)} = 25.73$, $p < 0.001$; N55: $F_{(1.90, 26.63)} = 7.50$, $p = 0.003$; N85: $F_{(2.05, 28.75)} = 7.92$, $p = 0.002$). P30 was smallest at the midline site, but both N55 and N85 exhibited equal amplitudes over auditory cortex and midline sites.

3.2.4.2.3. Effects of Stimulus Type and Frequency

Figures 3.4A and 3.4B illustrate the ERPs to deviant, control and standard stimuli for low-, and high-frequency stimuli, respectively. The components P13, N18 and N55 had similar Type and Frequency effects regardless of the region recorded from. That is, while there were main effects of Region on component amplitudes, there were no interactions between Region and Frequency or Type. Therefore, the effect of Type and Frequency on these component amplitudes pooled over Regions was analysed. These effects are represented in Figures 3.4C and 3.4D. A Type x Frequency effect was present for the P13 component ($F_{(1.31, 18.37)} = 7.16$, $p = 0.010$). The effect of type was limited to high-frequencies $F_{(1.33, 18.56)} = 10.36$, $p = 0.003$. The P13 amplitude to deviant stimuli was larger than control stimuli (deviance detection, $p_b = 0.028$; $d = 0.73$; Figure 3.4B, D) and standard stimuli (oddball effect, $p_b = 0.014$; $d = 0.85$; Figure 3.4B, D) for high-frequency stimuli, but not low-frequency stimuli. In addition, P13 amplitude was

larger to high-frequency control stimuli than to high-frequency standards (adaptation, p_b = 0.035; d = 0.76; Figure 3.4B, D).

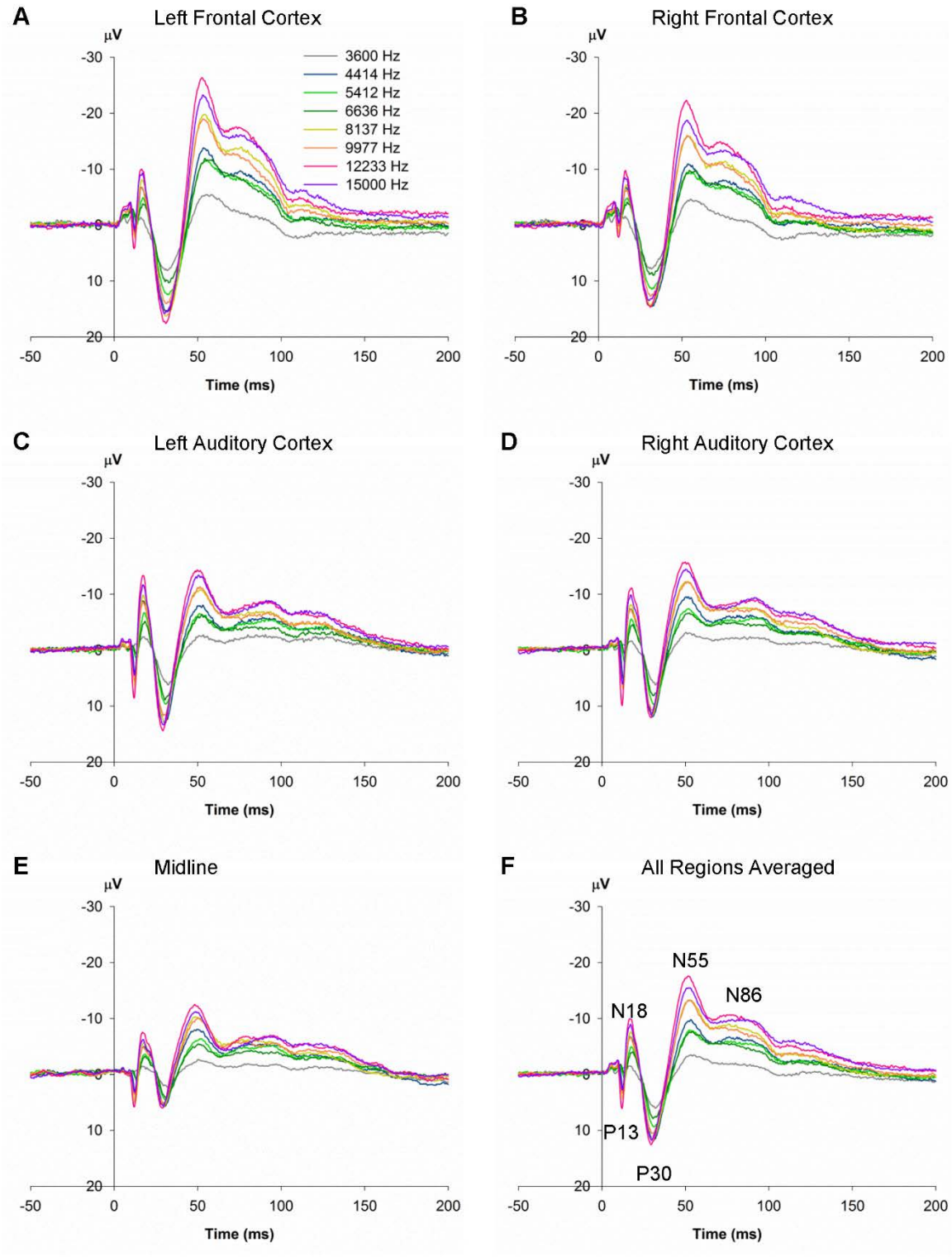


Figure 3.3. Rat ERPs in the many-standards control sequence (Study 2). ERPs recorded from electrodes implanted into the skull above the left frontal cortex (A), right frontal

cortex (B), left auditory cortex (C), right auditory cortex (D), midline (E), and averaged over all of the regions (F).

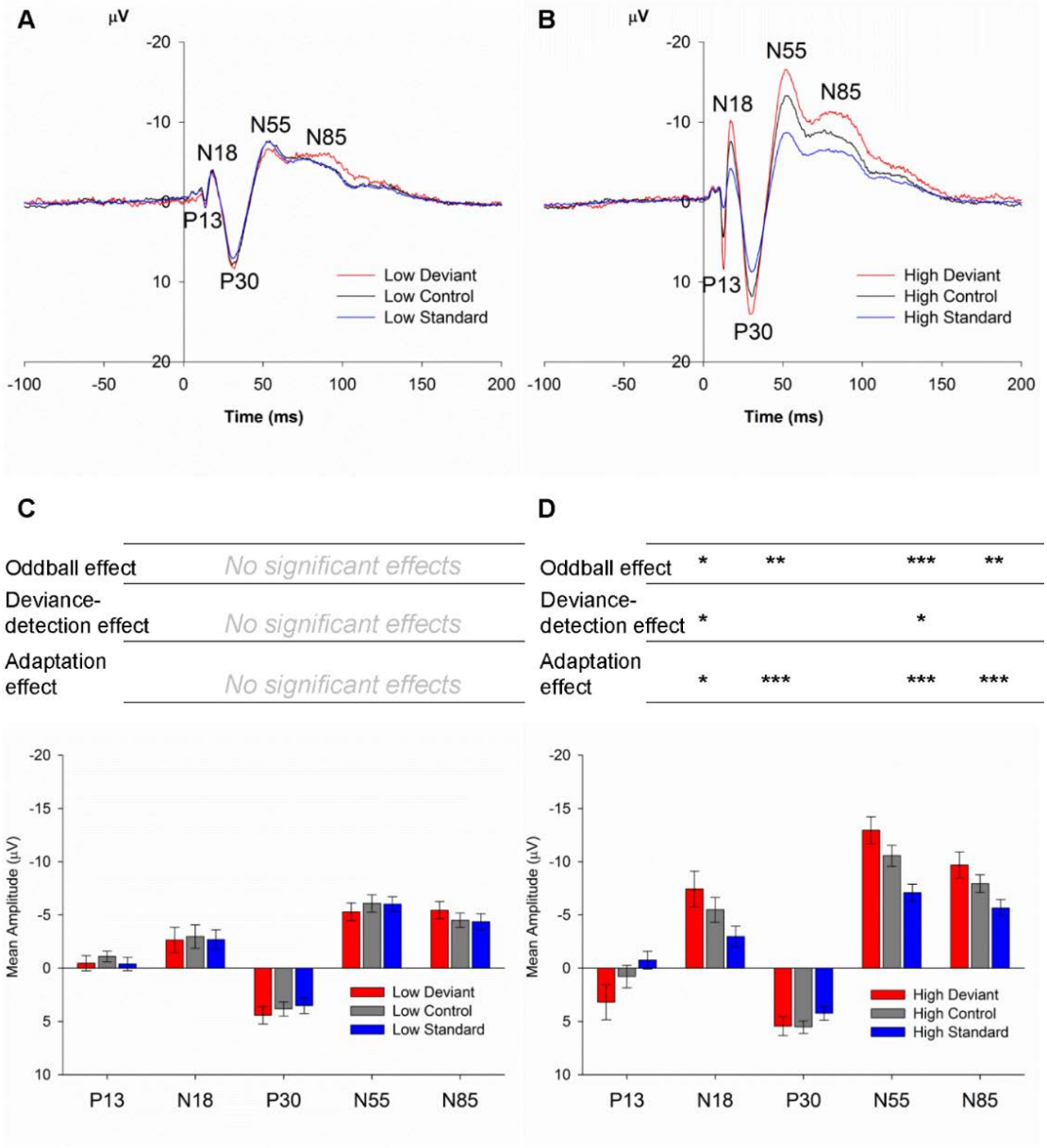


Figure 3.4. Rat ERPs in Study 2, averaged over all regions. (A, B) ERPs (averaged over all five regions) to the oddball deviant (red), the many-standards control (black) and the standard (blue) for the low- (A) and high- (B) frequency stimuli. All stimuli show a similar pattern with the same components (P13, N18, P30, N55 and N85): responses to deviants are larger in amplitude in comparison to the controls for high-frequency, but not low-frequency stimuli for P13 (p_b = 0.023, d = 0.73) and N55 (p_b = 0.010, d = 0.87). (C, D) Mean amplitudes (± standard error, SE) of P13, N18, P30, N55 and N85 generated by oddball deviants (red), many-standards controls (grey) and standards

(blue), averaged over all five regions. Significance levels for statistical comparisons between stimulus types for each component are shown above the bars for their respective components. Asterisks indicate statistical significance under 0.05, with * $0.050 < p > 0.010$, ** $0.010 < p > 0.001$, *** $p < 0.001$.

A similar Type x Frequency effect was observed for N18 amplitudes ($F_{(2, 28)} = 20.90$, $p < 0.001$), and again, the effect of Type was limited to the high-frequency stimuli ($F_{(1.59, 22.23)} = 17.30$, $p < 0.001$), for which N18 amplitudes to both control and deviant stimuli were larger compared to those to standard stimuli (adaptation and oddball effects, respectively; Adaptation $p_b < 0.001$; $d = 1.33$; Oddball $p_b = 0.001$; $d = 1.24$; Figure 3.4B, D). Unlike the P13 component, deviance detection to the high-frequency stimuli did not reach significance for the N18 component ($p_b = 0.072$; $d = 0.66$; Figure 3.4B, D).

The strongest effects of Stimulus Type were observed to high-frequency stimuli for the N55 component (Type x Frequency $F_{(1.68, 23.53)} = 52.12$, $p < 0.001$), where statistically significant oddball ($p_b < 0.001$; $d = 2.04$; Figure 3.5B, D), deviance detection ($p_b = 0.010$; $d = 0.87$; Figure 3.5B, D) and adaptation effects ($p_b < 0.001$; $d = 1.99$; Figure 3.5B, D) were observed. Such effects on the N55 component were not seen for low-frequency stimuli (Figure 3.4A, C)

For the remaining ERP components in Study 2 (P30 and N85), the recording site played a significant role in the expression of MMN-like responses (indicated by Type x Frequency x Region interactions). These effects are represented in Table 3.1 for all components (even though in the omnibus analysis, region interactions were not identified for the other components, P13, N18 and N55, these are included in Table 3.1 for consistency).

Table 3.1. *Oddball effects, deviance detection and adaptation for each frequencies and component combination in Study 2.*

		Low-Frequency					**High-Frequency**				
		Component									
		P13	**N18**	**P30**	**N55**	**N85**	**P13**	**N18**	**P30**	**N55**	**N85**
Oddball effect (deviant > standard)	LFC			*				*	*	**	*
	RFC									**	*
	LAC							*		**	*
	RAC									**	*
	LML									**	*
	Pooled							*		**	*
Deviance detection (deviant > control)	LFC										*
	RFC										*
	LAC										
	RAC								†		
	LML										
	Pooled										
Adaptation (control > standard)	LFC							*		**	*
	RFC									**	
	LAC							*		*	
	RAC								*	**	**
	LML							*		**	**
	Pooled							**		**	**

Levels of significance for the statistical difference between responses to deviant and standard stimuli (Oddball effect), deviant and control stimuli (Deviance detection) and control and standard stimuli (Adaptation) are shown for individual components and frequency conditions. Significant levels of oddball effects, deviance detection and adaptation were rarely observed for the low-frequency stimuli. By contrast, high-frequency stimuli often elicited both deviance detection and adaptation responses.

Significance levels are indicated as: * or $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p < 0.001$. In the large majority of cases, the changes were in the expected direction for MMN-like effects (i.e. deviant > control > standard), indicated by unformatted asterisks. Dagger symbols (†) indicate that the change was in the opposite direction to expected (i.e. standard > control > deviant).

A Type x Frequency x Region effect was identified for P30 ($F_{(3.76, 52.63)} = 5.96$, $p = 0.001$). MMN-like effects were present over several regions for both high- and low-frequency stimuli. As illustrated in Table 3.1, the majority of MMRs for the P30 component occur over the left and right frontal sites (Deviance Detection in the RFC $p_b = 0.043$, $d = 0.72$; Adaptation in the LFC $p_b = 0.001$, $d = 1.22$; Oddball effects in the LFC $p_b = 0.004$, $d = 0.98$, and RFC $p_b = 0.033$, $d = 0.42$). No significant MMRs were observed over the midline site. At auditory sites, an adaptation effect (P30 amplitude to control > standard, $p_b = 0.002$, $d = 1.01$) was observed for the RAC, but this was accompanied by a *reversed* deviance detection effect (i.e. amplitude to control > deviant, $p_b = 0.009$, $d = 0.85$), and no oddball effect. These puzzling results may be driven by the interaction with GD noted in section 3.2.4.2.1 (*Effects of GD of Saline Treatment)*, where it was found that half of the rats (the rats exposed to saline at GD19), had unusually high P30 amplitudes to control stimuli. Similar to P30, N85 amplitudes were also affected by a Type x Frequency x Region interaction ($F_{(5.49, 76.90)} = 5.60$, $p < 0.001$). Oddball and adaptation effects were observed in most regions for high-frequency stimuli (Table 3.1), but deviance detection was only observed to a statistically significant degree over the left ($p_b = 0.004$, $d = 1.01$) and right frontal sites ($p_b = 0.008$, $d = 0.90$) and the left auditory site ($p_b = 0.039$, $d = 0.61$).

3.2.4.3. Study 3 – Cascade Control

Raw mean amplitude data for Study 3 are available in Supplementary data S3. Contrary to Study 2, no effects of Stimulus Type (deviant vs. control) or Stimulus Type interactions were identified for Study 3 (Figure 3.5). GD of saline treatment did not affect responses for any of the components extracted in Study 3 (no significant main effects or interactions with GD in ANOVAs). The most prominent effects observed in Study 3 were that of Region, with main effects of Region being present for all components. The regional effects for each component from Study 2 were replicated in Study 3. As in Study 2, the P13 component in Study 3 was largest at the midline site and smallest at frontal cortex sites (Effect of Region $F_{(4, 24)} = 5.12$, $p = 0.004$, observed in original data and 5/5 imputations of missing data). Similar trends were observed for the other components, all of which showed the same regional distribution of responses as in Study 2 (N18: $F_{(4, 52)} = 3.78$, $p = 0.009$ for original data and 4/5 imputations; P30: $F_{(4, 24)} = 5.88$, $p = 0.002$ for original data and 5/5 imputations; N55: $F_{(4, 24)} = 13.008$, $p < 0.001$ for original data and 5/5 imputations; N85 $F_{(4, 52)} = 6.68$, $p < 0.001$ for original data and 5/5 imputations).

When the imputed data were analysed for the later components (P30, N55 and N85), significant effects of Frequency were revealed for all three components. The P30 component was larger for low-frequency compared to high-frequency stimuli ($F_{(1, 13)} = 16.81$, $p < 0.001$ for 5/5 imputations) and the N55 and N85 components were larger for the high-frequency stimuli (N55: $F_{(1, 13)} = 25.79$, $p < 0.001$; N85: $F_{(1, 13)} = 21.34$, $p < 0.001$; both for 5/5 imputations).

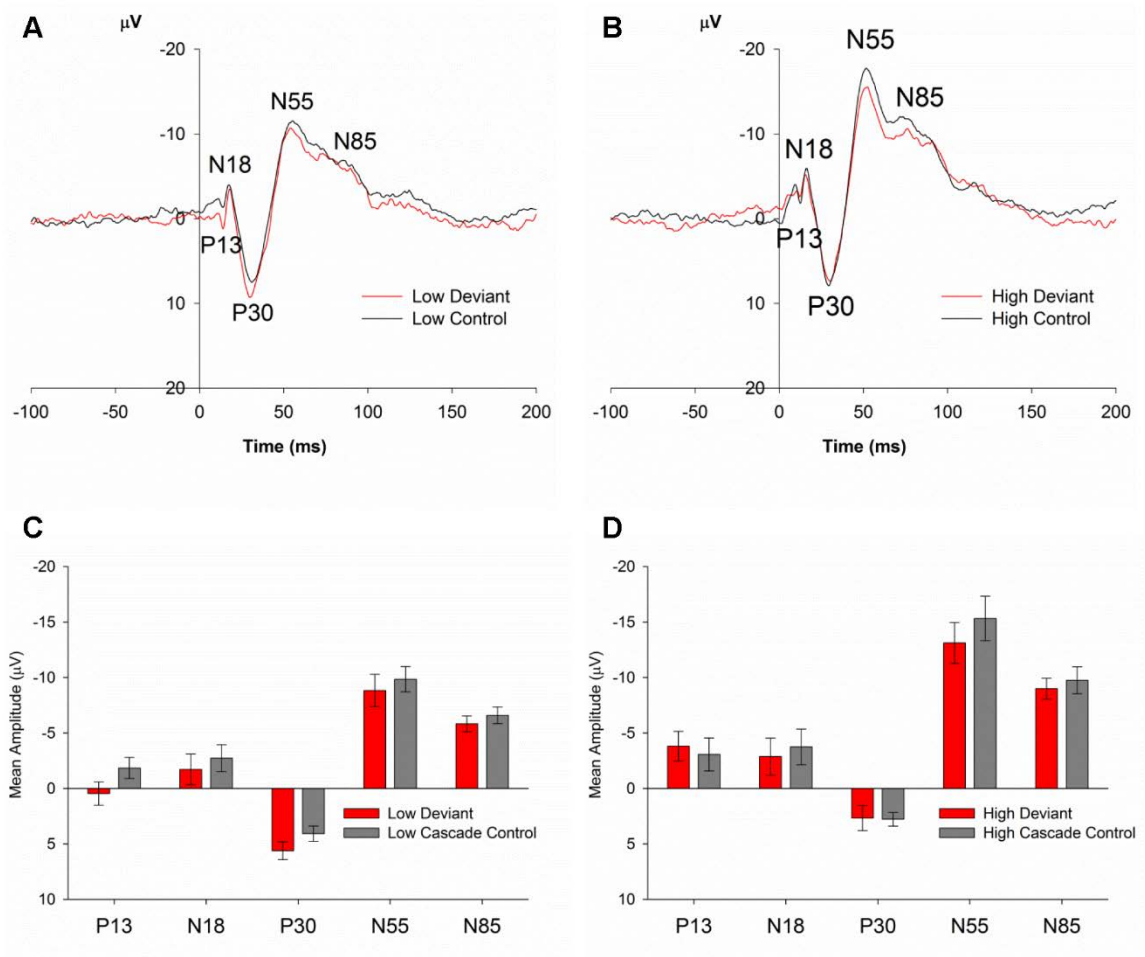


Figure 3.5. Rat ERPs in Study 3, averaged over all regions. (A, B) ERPs (averaged over all five regions) to the oddball deviant (red) and the cascade control (black) for the low- (A) and high- (B) frequency stimuli. As in Study 2, all stimuli show a similar pattern with the same components (P13, N18, P30, N55 and N85), however in this case, responses to deviants were not larger in amplitude in comparison to the controls and the standards. (C, D) Mean amplitudes (± standard error, SE) of P13, N18, P30, N55 and N85 generated by oddball deviants (red) and the cascade controls (grey), averaged over all five regions.

3.2.5. Discussion

The overall aim of the current investigation was to determine optimal conditions with which to observe robust human-like MMRs in rats, with a particular focus on the

type of sequence used to control for potential contributions to the size of MMR in rats. The flip-flop sequences controlled only for differences in the physical characteristics of deviant and standard stimuli but not differential adaptation. The many-standards control sequence (i) controlled for the probability of presentation and hence adaptation; (ii) it precluded the development of a predictive model and therefore (iii) no stimulus including the control deviant violated predictions. The cascade control sequence (i) controlled for the probability of presentation and adaptation; (ii) it provided the basis for a predictive model but (iii) the control deviant did not violate that predictive model. Significant MMRs were observed for high-frequency deviants with the flip-flop design of Study 1, which as noted, did not include a control for differential adaptation. The many-standards control for adaptation, used in Study 2, replicated findings from Study 1, suggesting that the MMRs observed were due to both deviance detection and adaptation, but use of a cascade control design in Study 3, did not replicate these effects, although this control did not allow extraction of adaptation effects.

3.2.5.1. Oddball Effects, Deviance Detection and Adaptation

Both Study 1 and Study 2 confirmed the presence of oddball effects, that is, significant increases in ERP amplitudes in response to deviant stimuli compared to standard stimuli. While in Study 1, oddball effects were only observed to a statistically significant degree for the later negative component (N80), Study 2 using a different sound generation and recording system revealed that these oddball effects can also be observed at earlier components (P13, N18, P30), in addition to the later negative components N55 and N85. In Study 2, a many-standards control sequence was used to separate oddball effects into separate elements: an *adaptation* index, a measure of the degree of reduction in peak amplitudes to frequent standard stimuli versus control

stimuli, and adaptation-independent *deviance detection* index, a measure of the degree of enhancement of peak amplitudes to deviant stimuli versus control stimuli. We therefore can separate two processes that contribute to the oddball effect: (i) deviance detection, the difference between stimuli that conform to patterns of regularity and those that defy predicted patterns and (ii) adaptation, the difference between frequently- and rarely-presented stimuli. In Study 2, by far the strongest oddball effect on peak amplitude to deviant stimuli was observed for the late N55 component, but significant effects were also observed for the earlier P13 and N18 components as well as the later N85 component. Significant levels of deviance detection and adaptation were identified for the N55 component (Figure 3.4D), indicating that the oddball effect on this component was driven by both adaptation-independent and –dependent processes. Similar effects were found for P13 and N85, where both deviance detection and adaptation contributed to the oddball effect, but not for N18, where only adaptation effects were observed. These findings indicate that the rat brain is capable of generating human-like MMN, and that like human MMN, these effects are in-part, independent of adaptation and driven by memory-based or prediction error signalling processes.

3.2.5.2. The Role of Frequency for MMR in Rats

Studies 1 and 2 replicated the results of previous investigations in our lab (Nakamura et al., 2011), where deviance detection was observed for high-, but not low-frequency deviants when all tone frequencies were selected from a relatively low-frequency range (2500-3600 Hz). In Study 1, while control for differential adaptation was not employed in this flip-flop only design, we observed similar increases in the response to the deviant, compared to the standard when tone frequencies were selected from higher frequency range (6636-8137 Hz), and closer to the optimum auditory

sensitivity range of rats. The morphology of ERPs was similar to those described previously in Nakamura et al. (2011), with a negative peak at approximately 20-30 ms, and a positive peak at approximately 30-40 ms (Figure 3.2A). Two additional negative components were identified in Nakamura et al. (2011): a negative peak at 42 ms, and a late negative difference between the deviant and the control stimuli from 50-70 ms after stimulus onset. In the current Study 1, however, these two components were replaced by a broad, and much larger negative peak from 60-100 ms. It was hypothesised that by increasing the frequency range used in Study 1 compared to Nakamura et al. (2011), MMRs would also be observed for low-frequency deviants. However, as in Nakamura et al. (2011) increased responses to low-frequency deviants were not observed (Figure 3.3A and C), indicating that the lack of observable MMRs for low-frequency deviants is not due to the relative sensitivity of the rat's auditory system to low-frequencies, but perhaps associated with a lower salience for unexpected decreases in frequency compared to frequency increases (Brudzynski, 2013). A similar effect was also evident in Study 2. When differential adaptation was controlled for, both adaptation and deviance detection were observed in several ERP components from several sites for high-frequency deviants, but rarely occurred for low-frequency deviants. Table 3.1 illustrates this dramatic difference between high and low frequencies in terms of capacity to elicit oddball, deviance detection and adaptation effects. Other researchers using anesthetised rats have observed similar effects, namely evidence of MMRs to high-frequency, but not low-frequency deviants (Astikainen et al., 2011). The same effect has also been observed previously for human MMN (Peter, McArthur, & Thompson, 2010), as well as for changes in evoked potentials to alterations in tone frequency (increases in frequency were associated with larger ERP changes) (Pratt et al., 2009). These findings indicate an overall trend towards a higher sensitivity of the rat

brain to increments in frequency rather than decrements. A possible explanation for such a trend may be that the ultrasonic vocalizations that rats use to communicate with each other are of a much higher frequency and range from 22 kHz (alarm/distress call) to 50 kHz (reward, appetitive call) (Brudzynski, 2013). The auditory system of the rat may therefore be somewhat 'primed' to perceive high-frequency noises. Future studies could examine this by measuring MMRs to low-frequency alarm (22 kHz) calls and high-frequency appetitive (50 kHz) calls in a flip-flop condition. It should be noted that adaptation to low-frequency changes was not observed in Study 2. While adaptation has been shown to occur for low-frequency changes in other rat models (Duque, Perez-Gonzalez, Ayala, Palmer, & Malmierca, 2012; Farley et al., 2010; Taaseh et al., 2011), it has been found that neural populations in the rat inferior colliculus exhibit less adaptation to low-frequency tones compared to high-frequency tones (Duque et al., 2012).

Effects of stimulus frequency were seen for ERPs in the many-standards condition (Figure 3.3), with increments in frequency producing larger responses (except for one frequency, 4414 Hz, which produced a larger response compared to the 5412 and 6636 Hz stimuli). As all stimuli were presented at the same intensity (70 dB_L), the altered responses to the different frequencies could possibly be due to the sensitivity of the rat's auditory system to different frequencies. Rats exhibit low-sensitivity to tones at low-frequencies (<1kHz), but this increases with increasing frequency until peak sensitivity is reached at 8 (Kelly & Masterton, 1977) to 16 kHz (Mazurek et al., 2010). The current data indicate a similar effect, with responses to the 3600 Hz stimulus being relatively low-amplitude, but with responses increasing in magnitude with increasing frequency, and a peak response seen to the stimuli presented at 12233 Hz, indicating that this may be the peak sensitivity range for the rats in our study. This dramatic effect

of frequency on the response to different stimuli highlights the importance of controlling for stimulus identity and only comparing deviant stimuli to their respective standards and controls of the same frequency.

3.2.5.3. Effects of the Cascade Control Method

Deviance detection was not observed in Study 3, which used the same recording system, electrode array and animals as Study 2, but instituted a different control method, the cascade control. Such results would indicate that contrary to Study 2, Study 3 did not find evidence of 'true' adaptation-independent MMN in rats. There could be a number of reasons for this lack of replication of MMRs using this method.

Firstly, the cascade control necessitated the use of a higher-frequency deviant for the ascending oddball condition, in comparison to Studies 1 and 2 (15000 Hz vs. 8137 Hz). At face value, the findings from Study 3 do not conform to the suggestion proposed above, that rats are most sensitive to increments in frequency – if this is the case, one would expect to observe evidence of deviance detection for frequency increments of 12233 to 15000 Hz. It is unlikely that the lack of deviance detection to 15000 Hz stimuli is due to a lack of auditory sensitivity to the tone, because the frequency is well within the rat's frequency sensitivity range, if not at the peak of the auditory sensitivity for the rat (Mazurek et al., 2010). Indeed, this peak sensitivity may be the reason why deviance detection is not observed to tones of 15000 Hz. Study 2 results revealed that in the many-standards control condition (where tones from 3600 – 15000 Hz were presented), by far the largest ERP amplitudes (for every recording site) were observed for stimuli presented at 12233 and 15000 Hz (Figure 3.3), the two frequencies used as standards and deviants respectively for the ascending oddball sequence of Study 3. Therefore, perhaps the lack of deviance detection observed for

deviants of 15000 Hz can be explained by a ceiling effect: the exogenous evoked potentials to both the standard and deviant tones are so large that any deviance-associated increase in the ERP simply cannot be observed. By contrast, ERP amplitudes to frequencies used as deviants and standards in Studies 1 and 2 (6636 and 8137 Hz) sit closer to the middle of the amplitude response range to differing frequencies (Figure 3.3), such that any amplitude changes related to deviance or adaptation are more readily observable. If indeed a ceiling effect is occurring that 'masks' possible effects of deviance in the cascade control condition, such effects could be minimized by shifting the range of frequencies used for the cascade control condition down (e.g. 3000 Hz – 9000 Hz) so that the tones used as standards and deviants are not at the peak level of auditory sensitivity for the rat. In addition, the sound intensity of stimuli could be reduced.

Secondly, while it was suggested that the wide range of stimuli used in the many-standards condition may result in an overestimation of adaptation effects (Ruhnau et al., 2012), it has also been suggested that stimuli at the extreme ends of a range in control sequences (as used for the control deviants in the cascade control sequence) may result in control stimuli at the outer ends being perceived as deviants and again, an overestimation of adaptation in the oddball sequences (Jacobsen & Schroger, 2001; Winkler et al., 1990). Therefore, the use of the cascade control sequence, with the stimuli used as deviants sitting at the outer extremes of the range of stimuli, may result in an underestimation of deviance detection. This issue would be trivial in human studies, as the pattern of regularity used in the control sequence would negate this. The third explanation for why deviance detection may not have been observed using the cascade control method is therefore that it is unknown whether the pattern of regularity established by the cascade sequence can be modelled by the rat brain. If not, higher

order expectations based on these more complex statistical regularities within the environment cannot be generated and therefore, the frequencies at the extremes of the cascade sequence are as unexpected as any other of the cascade frequencies. In fact, given that the extreme frequencies occur with a lower probability than other cascade frequencies (12.5% vs. 25%), in the absence of a rule that governs the cascade sequence regularity, they will appear to be aberrant (rare) and therefore generate deviance detection in their own right. In addition, without data from a flip-flop control standard being measured, it is not known whether the animals in Study 3 are even exhibiting any oddball response (a larger response to the deviant vs. the standard). The results from Study 3 therefore remain somewhat inconclusive. Future studies should include four oddball sequences for the cascade control method, the two used in the current study, as well as flip-flop controls for each of them. In addition, further examination of the cascade control, how it compares to the many-standards control, and the ability of the rat brain to model such complex regularities is warranted.

3.2.5.4. Relationship of These Data to MMRs in Humans

The results from Study 2 revealed that MMRs (both adaptation and deviance detection) can be observed in the late, negative components, N55 and N85, which most closely resemble human MMN in their polarity (negative) and their relative latency (it is expected for ERP components to occur with a reduced latency in the rat brain (Bickel & Javitt, 2009). However, oddball effects were also observed on earlier components such as P13 and N18 (and P30 to a lesser degree). This may seem at variance with the human MMN literature that has focussed on late effects. However, recent research has shown evidence of adaptation-independent deviance detection on human middle latency responses (MLRs). In human investigations of MMN, a bandpass filter (e.g. 0.1-35 Hz)

is typically applied, which filters out early high-frequency mid-latency ERP components, but allows the slower MMN component to be observed. However, when a suitable bandpass filter is applied so that early ERPs can be detected (e.g. 15-200 Hz), additional MLR components can be observed (Grimm, Escera, Slabu, & Costa-Faidella, 2011). These include positive peaks at approximately 12 and 30 ms (P0 and Pa) and negative peaks at approximately 22 and 40 ms (Na and Nb) (Grimm et al., 2011). Several human studies have now confirmed evidence of deviance detection in MLRs, notably Na peaking at 20 ms (Grimm, Recasens, Althen, & Escera, 2012), Pa at 30 ms (Slabu, Escera, Grimm, & Costa-Faidella, 2010), and Nb peaking at 40 ms (Grimm et al., 2011). The bandpass filters associated with our data acquisition system permitted the detection of a series of early responses that exhibited adaptation and/or deviance detection. These early components might be homologues for the human MLR components that show deviance detection, although further research is required to support this view.

This study also highlights the importance for including controls for differential adaptation in human studies, which rarely occurs (for review, see Todd et al. (2013)). While it is known that MMN in healthy subjects includes an adaptation-independent deviance detection component, we thus far do not know if observed reductions in MMN (for example, in patients with schizophrenia, or in subjects given NMDAr antagonists (Todd et al., 2013)), are due to reductions in adaptation or deviance detection, a very important question for future research into the functional importance of MMN in disease states (Todd et al., 2013).

3.2.5.5. The Role of Recording Site in Component Amplitudes and the Expression of Adaptation and Deviance Detection

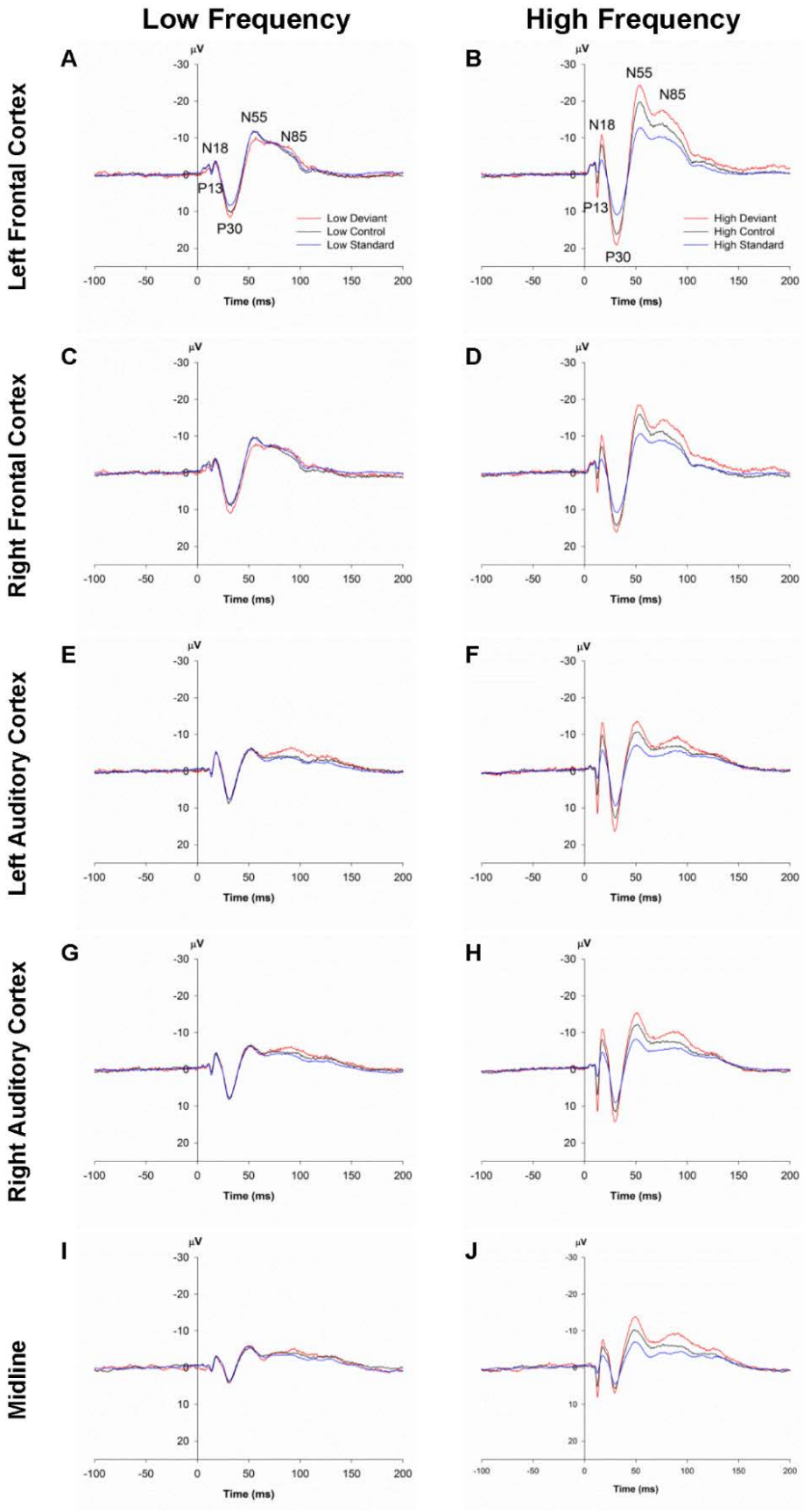
Studies 2 and 3 recorded ERPs from five separate sites over the rat cortex: over left and right auditory cortices (LAC and RAC), left and right frontal cortices (LFC and RFC) and another at the midline, similar to the vertex in human recordings (due to the bone suture at the exact midline the electrode was placed just left of the midline, LML). Marked effects of site were found for the amplitudes of all ERP components in Studies 2 and 3 (Supplementary Figure 3.1). P13, the earliest component, was largest at the midline and auditory sites and smallest at frontal sites. This pattern of scalp topography contrasts with the later components, which were smallest at the midline site. N18 was largest at the auditory sites and the remaining later components (P30, N55 and N85) were maximal at frontal sites.

The site of the recording electrode also had a significant impact on the expression of rat MMRs for P30 and N85, but not P13, N18 and N55. MMRs were rarely observed for P30, but tended to be over the frontal cortex and auditory cortex sites, not midline sites (where P30 was smallest). Similarly, deviance detection at N85 was observed only for the sites where N85 was largest (both frontal sites and LAC site). These findings do not necessarily indicate that MMRs can only be observed at particular locations, but rather suggest that the capability of detecting statistically significant changes at a particular recording site or region is reliant on that site producing a strong signal. Since deviance detection was strongest for the N55 and N85 components, and these components are largest at frontal cortex sites, recording from frontal sites is most likely the best choice for observing human-like deviance detection in the rat.

3.2.5.6. Conclusions

This study presents a careful characterisation of different control paradigms, stimulus frequencies and recordings sites and how readily they detect MMN-like responses in the rat brain. The data presented in this study contribute to a growing body of evidence (Ahmed et al., 2011; Astikainen et al., 2011; Jung et al., 2013; Nakamura et al., 2011; Ruusuvirta et al., 1998; Shiramatsu et al., 2013; Tikhonravov et al., 2008, 2010) supporting the conclusion that the rat brain is quite capable of producing MMRs that are similar to the human MMN and are not entirely dependent on neural adaptation but rather are in part, contributed to by a more complex deviance detection process. This model can now be used to investigate the neurobiology of both adaptation and adaptation-independent deviance detection, using different pharmacological, developmental and neurobiological manipulations.

3.2.6. Supplementary Figures



Supplementary Figure 3.1. ERPs in rats to deviant, control and standard stimuli for low- and high-frequency conditions in Study 2 for each region. ERPs for each of the five

regions recorded from to the oddball deviant (red), the many-standards control (black) and the standard (blue) for the low- (left) and high- (right) frequency stimuli. All stimuli show a similar pattern with the same components (P13, N18, P30, N55 and N85).

3.2.7. Supplementary Data

3.2.7.1. Data S1 Mean Amplitude Data from Study 1

Data values are mean amplitudes (in mV) of the components N22, P37 and N80, measured in Study 1. 'High' or 'Low' in the variable name refers to whether the ERP response was to the high-frequency or low-frequency stimulus. 'Dev' or 'Std' refers to whether the ERP response was to a deviant or standard stimulus, respectively. Supplementary Data S1 spreadsheet files can be viewed at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0110892#s5

3.2.7.2. Data S2 Mean amplitude data from Study 2

Data values are mean amplitudes (in mV) of the components P13, N18, P30, N55 and N85, measured in Study 2. 'High' or 'Low' in the variable name refers to whether the ERP response was to the high-frequency or low-frequency stimulus. 'Con', 'Dev' or 'Std' refers to whether the ERP response was to a control, deviant or standard stimulus, respectively. 'GD' is the gestational day of saline exposure. Recording sites are identified in the variables by the following abbreviations: AvReg – ''Averaged region'', mean amplitude averaged over all regions; LAC, Left auditory cortex; LFC, Left frontal cortex; LML, midline (slightly to the left of); RAC, Right auditory cortex; RFC, Right frontal cortex. Supplementary Data S2 spreadsheet files can be viewed at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0110892#s5

3.2.7.3. Data S3 Mean amplitude data from Study 3

The data for the mean amplitudes (in mV) of each component of the ERPs from Study 3 (P13, N18, P30, N55, N85) are represented on separate sheets of the spreadsheet file. Incomplete, original data are labelled as imputation =0, the imputed data are labelled as imputations 1– 5. 'High' or 'Low' in the variable name refers to whether the ERP response was to the high-frequency or low-frequency stimulus. 'Con', 'Dev' or 'Std' refers to whether the ERP response was to a control, deviant or standard stimulus, respectively. 'GD' is the gestational day of saline exposure. Recording sites are identified in the variables by the following abbreviations: AvReg – ''Averaged region'', mean amplitude averaged over all regions; LAC, Left auditory cortex; LFC, Left frontal cortex; LML, midline (slightly to the left of); RAC, Right auditory cortex; RFC, Right frontal cortex. Supplementary Data S3 spreadsheet files can be viewed at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0110892#s5

3.2.8. Acknowledgements

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3.2.9. References

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3.3. Chapter 3B: Deviance Detection and Mismatch Negativity (MMN) Responses in Awake, Freely-moving Rats Following MIA Exposure

3.3.1. Introduction

In the current experiment, MIA was implemented at an early (GD10) or late (GD19) stage of gestation. MMRs were assessed in awake freely-moving offspring using the many-standards control method which was identified in the publication detailed in section 3.2 (Harms et al., 2014) as being the most suitable for distinguishing adaptation and true deviance detection MMRs in rats. In light of the evidence strongly implicating NMDAr function in MMN (Heekeren et al., 2008; Umbricht, Koller, Vollenweider, & Schmid, 2002; Umbricht et al., 2000), and the evidence that late gestation MIA in the mouse model produces alterations of the NMDAr system (Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008), it was expected that MIA during late gestation (GD19), but not early gestation (GD10) or controls, would produce reductions in deviance detection MMRs analogous to those seen in schizophrenia.

3.3.2. Methods

3.3.2.1. Animals and Generation of MIA

Adult female (n = 31) and male (n = 12) Wistar rats were obtained from the University of Newcastle's Central Animal House at 8 weeks of age. Rats were acclimated for 2 weeks prior to daily monitoring of oestrous cycling using an impedance probe. Female rats were mated overnight in the males' home cage during the proestrus phase of the cycle. The morning following mating vaginal smears were performed to confirm the presence of sperm, with the day of detection being identified

as GD0. Pregnant dams were randomly allocated to a treatment group. On GD10 or GD19, pregnant dams were anaesthetised with isoflurane (induction 5%, maintenance 2.5-3%) and administered with either 4.0 mg/kg of Poly (I:C) (Sigma-Aldrich, St. Louis, MO) or 0.1M PBS via injection to the lateral tail vein (at 1 mL/kg body weight).

Animals were weaned and pair housed at PND21, after which they were handled and weighed weekly until adulthood (PND70) when testing commenced. A total of 31 male offspring were used in the experiment (GD10 Control n = 8, GD10 MIA n = 7, GD19 Control n = 9, GD19 MIA n = 7). All animals were pair housed with same-sex litter-mates and maintained at a temperature of 21°C under a 12 h light/dark cycle.

All experiments were performed under strict adherence to the National Health and Medical Research Council's Australian code of practice for the care and use of animals for scientific purposes and were approved by the University of Newcastle's Animal Care and Ethics Committee (Approval number A-2009-108). Every effort was made to reduce the number of animals used. All surgical procedures were performed under well-maintained anaesthesia and the appropriate use of analgesics was implemented to alleviate pain and discomfort during and following surgery.

3.3.2.2. Surgery

Surgery was performed as described previously in section 3.2 of this thesis. Briefly, rats were anaesthetised with isoflurane before being placed in a stereotaxic frame. The skin and periosteum were removed to expose the dorsal surface of the skull and a custom-made electrode connector consisting of seven stainless steel screws (B002SG89S4, Amazon Supply, USA) attached to a male-female socket (BD075-10-A-1-L-D from Global Connector Technology, Lawrence, MA, USA), which was left exposed, were implanted into the skull and covered with dental cement (Dentsply,

Mount Waverly, VIC, Australia). Five screw electrodes were implanted in the skull as recording electrodes, but only the two placed above the left and right frontal cortices (2.00 mm anterior to Bregma and 2.00 mm lateral to the midline) were used for the current study. The reference electrode was placed over the cerebellum (1.00 mm posterior to Lambda and 1.00 mm to the right of the midline). Five mg/kg of Carprofen and 0.05 mg/kg of buprenorphine were administered pre-operatively via the subcutaneous route as analgesics. All animals were allowed to recover for at least five days after surgery before the first EEG recordings.

3.3.2.3. Sound Generation and Stimuli Presentation

A custom program written in Presentation (version 14.1, Neurobehavioral Systems, Inc.) was used to generate auditory stimuli. The auditory stimuli were amplified and delivered through a speaker (1 kHz – 30 kHz frequency response) mounted at approximately 50 cm above the floor of the experimental chamber. Sound intensity was calibrated with a sound meter (Brüel & Kjær Model 2260) using a linear weighting to an average of 70 dBL SPL across locations within the chamber for the sounds in the 3600 and 15000 Hz range used in this study. In order to check if the speakers were producing distortions in the ultrasonic frequency range the sound generated was assessed by recording and digitising at a very high rate from within the chamber and examining the signal. There was no obvious distortion of the sound, with the expected ramp at the start and at the end of the sound as expected and a regular sinusoidal wave at the expected frequency during the plateau.

MMR testing sequences were the same as those detailed in Study 2 of section 3.2 of this thesis, and consisted of ascending (high-frequency DEV) and descending (low-frequency DEV) oddball sequences and two many-standards control sequences.

STDs were presented at a rate of 87.5% and DEVs at a rate of 12.5% in the oddball sequences. For the many-standards control sequences all stimuli were presented at a rate of 12.5%.

3.3.2.3.1. Procedure

In order to assess MMRs, rats underwent one 62 min session a day for three days, with the same procedure to that reported in Section 3.2 of this thesis. EEG data were recorded using Multi Channel Systems MCRack software and digitized as described in Section 3.2 of this thesis.

3.3.2.3.2. Data Extraction

Data processing was performed as reported for Section 3.2 of this thesis. However, an additional component (NLD) was extracted in the current study that was not observed in Section 3.2 due to the lower sample size. The ERP was characterised by an initial positive peak at 13 ms (P13), a negative peak at 18 ms (N18), followed by a positive peak at 30 ms (P30), a large negative component with two discernible peaks from approximately 45-65 ms (N55) and 65-105 ms (N85), and a low-amplitude, broad late negative component from 106-206 ms (Negative Late Difference, NLD). Six mean amplitude measures were extracted over the following latency windows: a 4 ms window from 11-15 ms (P13), a 7 ms window from 15-22 ms for N18, a 21 ms window from 22-43 ms for P30, a 23 ms window from 43.5-65.5 ms for N55, a 40 ms window from 65.5-105.5 ms for N85, and a 100 ms window from 106-206 ms for NLD. Mean amplitudes were extracted for responses to the DEV, STD and CON stimuli, which were then used to calculate measures of deviance detection (DEV – CON), adaptation (CON – STD) and 'classic' MMN (DEV – STD). Mean amplitude measures were

averaged over the left and right frontal cortex electrode locations. Individual responses were averaged over the three sessions and for the two frequency conditions (low, high).

3.3.2.4. Statistical Analysis

Mixed Analyses of Variance (ANOVA) were performed for the six components using the within-subjects factor of *MMR Type* (deviance detection, adaptation, MMN) or *Stimulus Type* (DEV, CON, STD), and the between-subjects factors of *GD* (10, 19) and *Treatment* (Control, MIA). Significant main or interaction effects of Treatment on the dependent variables were followed up using pairwise Bonferroni-corrected comparisons between the control and MIA conditions. Bonferroni-corrected post-hoc *p* values are shown as p_b. Only data from the high-frequency condition (8137 Hz) was used. Greenhouse-Geisser or Hundt-Felt corrections were used where sphericity assumptions were violated.

All EEG analyses were performed controlling for stimulus identity. That is, only responses to stimuli of the same frequency were compared. For example, although the 8137 Hz DEV was presented in an ascending oddball sequence with a 6636 Hz STD, all analyses performed on the 8137 Hz DEV involved comparisons with the 8137 Hz STD (used in the descending oddball sequence) and the 8137 Hz CON (used in the many-standards control sequence). ERP mean responses to DEV, STD and CON stimuli for the six ERP components were analysed, as were deviance detection (DEV – CON), adaptation (CON – STD) and 'classic' MMN (DEV – STD) difference measures.

3.3.3. Results

3.3.3.1. Effects of MIA

The impact of early (GD10) and late (GD19) MIA on ERPs and MMRs elicited in rats (Figure 3.6) was conducted on the data from the high-frequency (8137 Hz) stimuli. Analysis of MMR data demonstrated no significant interaction between Treatment and MMR Type (deviance detection, adaptation, MMN) or a Treatment by GD by MMR Type interaction. Main effects of Treatment were observed for the P30 ($F_{(1, 27)}$ = 6.40, p = 0.018) and N85 ($F_{(1, 27)}$ = 8.19, p = 0.008) components, with MMRs observed to be increased in MIA-exposed rats compared to controls (Table 3.2). No other main effects of Treatment (P13: $F_{(1, 27)}$ = 0.80, p = 0.378; N18: $F_{(1, 27)}$ = 3.97, p = 0.057; N55: $F_{(1, 27)}$ = 0.38, p = 0.543; NLD: $F_{(1, 27)}$ = 1.23, p = 0.278) or interactions with GD or MMR Type were identified for any other components of the ERP.

Stimulus Type x Treatment effects were identified for N18 ($F_{(1.42, 38.31)}$ = 3.81, p = 0.044), P30 ($F_{(2, 54)}$ = 4.52, p = 0.015), and N85 ($F_{(1.94, 52.33)}$ = 5.92, p = 0.005) on DEV, CON and STD mean amplitude responses. Responses for the N18 component were increased in MIA-exposed rats, but no pair-wise comparisons with control rats were significant (Table 3.2). For the P30 component, DEV responses were increased in MIA compared to control animals (p_b = 0.009, Table 3.2). In addition MIA rats exhibited larger P30 responses in general compared to controls (Treatment effect: $F_{(1, 27)}$ = 6.08, p = 0.020), which was particularly pronounced for GD19 MIA rats (p_b = 0.003) compared to GD10 rats (p_b = 0.846 ;Treatment x GD effect: $F_{(1, 27)}$ = 4.77, p = 0.038). For the N85 component, DEV responses were increased in MIA compared to control animals (p_b = 0.042, Table 3.2). No other main effects of Treatment (P13: $F_{(1, 27)}$ = 1.63, p = 0.212; N55: $F_{(1, 27)}$ = 0.40, p = 0.533; NLD: $F_{(1, 27)}$ = 0.39, p = 0.539) or interactions with GD or Stimulus Type were identified for any other component (Table 3.2).

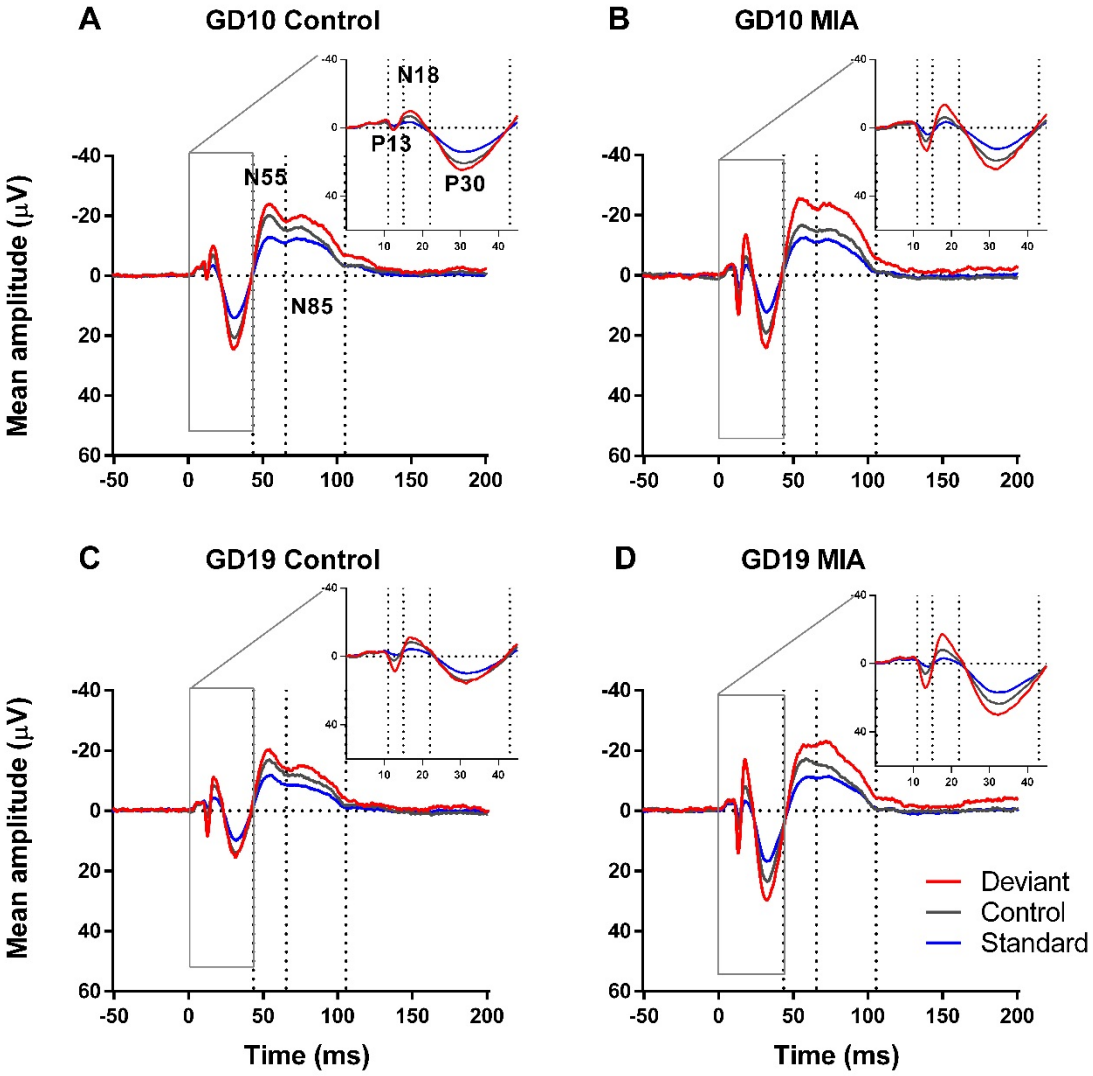

Figure 3.6. Effect of maternal immune activation (MIA) exposure at two different gestational days (GDs) on responses to deviant (red), control (grey) standard STD (blue) stimuli. Graphs show grand-averaged event related potentials (ERPs) from 100 ms prior to stimulus onset to 300 ms after stimulus onset for GD10 Control (A, *n* = 8), GD10 MIA (B, *n* = 7), GD19 Control (C, *n* = 9), and GD19 MIA (D, *n* = 7) rats.

Table 3.2. *Effect of maternal immune activation (MIA) on individual responses to stimuli and mismatch responses.*

		Treatment Group				Effect
Component	MMR Effect	GD10 Control	GD10 MIA	GD19 Control	GD19 MIA	
P13	Dev detection	-0.24 ± 0.68	2.17 ± 2.55	3.85 ± 1.46	5.04 ± 2.23	
	Adaptation	0.33 ± 0.97	1.22 ± 1.62	1.49 ± 0.68	1.97 ± 1.51	
	MMN	0.1 ± 1.41	3.39 ± 4.15	5.34 ± 2.05	7.01 ± 3.27	
N18	Dev detection	-1.82 ± 1.46	-5.37 ± 1.38	-1.54 ± 1.58	-4.94 ± 1.6	
	Adaptation	-2 ± 0.6	-2.46 ± 0.7	-3.08 ± 1.19	-3.11 ± 1.19	
	MMN	-3.82 ± 1.57	-7.83 ± 1.49	-4.63 ± 2.39	-8.05 ± 1.38	
P30	Dev detection	1.99 ± 1.03	2.21 ± 0.75*	-0.04 ± 1.04	4.71 ± 1.45*	Treatment p = 0.018
	Adaptation	2.56 ± 0.96	4.24 ± 1.86*	2.49 ± 0.53	3.8 ± 1.75*	
	MMN	4.55 ± 1.11	6.45 ± 2.26*	2.46 ± 1.32	8.51 ± 1.57*	
N55	Dev detection	-3.35 ± 0.8	-6.49 ± 2.08	-2.6 ± 1.56	-3.88 ± 1.69	
	Adaptation	-5.31 ± 1.32	-2.29 ± 1.26	-3.88 ± 0.45	-4.58 ± 1.52	
	MMN	-8.66 ± 1.51	-8.78 ± 1.55	-6.48 ± 1.5	-8.46 ± 2.25	
N85	Dev detection	-3.69 ± 1.36	-6.14 ± 2.13**	-2.68 ± 0.7	-6.7 ± 1.28**	Treatment p = 0.008
	Adaptation	-1.41 ± 0.85	-4.27 ± 1.67**	-2.55 ± 0.57	-2.04 ± 0.78**	
	MMN	-5.09 ± 1.89	-10.41 ± 1.55**	-5.23 ± 1.19	-8.74 ± 1.47**	
NLD	Dev detection	-1.54 ± 0.94	-2.85 ± 1.23	-1.33 ± 0.68	-2.55 ± 0.67	
	Adaptation	-0.55 ± 0.74	0.06 ± 0.99	0.17 ± 0.43	-0.14 ± 0.51	
	MMN	-2.08 ± 1.08	-2.79 ± 1.37	-1.16 ± 0.67	-2.69 ± 0.9	
Component	ERP Response	GD10 Control	GD10 MIA	GD19 Control	GD19 MIA	
P13	DEV	-1.57 ± 3.13	4.31 ± 5.53	3.52 ± 2.59	6.96 ± 4.38	
	CON	-1.33 ± 2.79	2.14 ± 3.1	-0.32 ± 1.34	1.93 ± 2.61	
	STD	-1.66 ± 1.92	0.92 ± 1.67	-1.82 ± 0.78	-0.04 ± 1.31	
N18	DEV	-4.68 ± 2.37	-9.55 ± 1.98	-8.02 ± 4.05	-8.85 ± 1.66	Treatment x Stimulus p = 0.044
	CON	-2.86 ± 1.22	-4.18 ± 1.43	-6.48 ± 2.8	-3.91 ± 1.22	
	STD	-0.87 ± 0.87	-1.72 ± 1.04	-3.4 ± 1.77	-0.8 ± 0.78	
P30	DEV	12.93 ± 1.72	14.08 ± 3.47**	7.81 ± 1.76	18.91 ± 1.37**	Treatment x Stimulus p = 015
	CON	10.93 ± 1.58	11.88 ± 3.03	7.84 ± 0.92	14.2 ± 1.55	
	STD	8.38 ± 1.63	7.64 ± 1.72	5.35 ± 0.68	10.4 ± 0.65	
N55	DEV	-17.66 ± 2.64	-17.42 ± 2.98	-15.63 ± 2.59	-15.23 ± 3.51	
	CON	-14.31 ± 2.98	-10.93 ± 2.59	-13.04 ± 1.32	-11.35 ± 2.68	
	STD	-9 ± 2.05	-8.64 ± 1.88	-9.16 ± 1.2	-6.77 ± 1.61	
N85	DEV	-13.92 ± 1.99	-18.49 ± 2.77*	-11.44 ± 2.02	-16.34 ± 2.07*	Treatment x Stimulus p = 0.005
	CON	-10.24 ± 1.65	-12.35 ± 2.76	-8.76 ± 1.4	-9.64 ± 1.87	
	STD	-8.83 ± 1.36	-8.08 ± 1.47	-6.21 ± 1	-7.6 ± 1.17	
NLD	DEV	-3.29 ± 1.45	-2.47 ± 1.63	-1.25 ± 0.66	-2.64 ± 0.98	
	CON	-1.76 ± 1.4	0.38 ± 1.14	0.08 ± 0.33	-0.1 ± 0.79	
	STD	-1.21 ± 0.78	0.32 ± 0.77	-0.09 ± 0.23	0.05 ± 0.5	

Data shown are mean ± SEM. * 0.010 < p_b 0.050, ** 0.001 < p_b 0.010, *** p_b < 0.001 for Bonferroni-corrected pairwise comparisons. *Abbreviations: n*= 31, ERP – Event related potential, DEV – Deviant, CON – Many-standards control, STD – Standard, MMR – Mismatch response, Dev detection – Deviance detection (difference in response

to DEV – CON), Adaptation (difference in response to CON – STD), MMN – 'classic' computation of mismatch negativity (difference in response to DEV – STD), MIA – maternal immune activation.

3.3.4. Discussion

This study reports the impact of MIA on MMRs in rats. Specifically, it was demonstrated that MIA male rats in the current study exhibited significantly larger MMRs (adaptation, deviance detection, and MMN responses) to high-frequency deviants than controls when assessing the P30 and N85 components, with no difference found for any other ERP components or between the two GD groups. This increase to MMRs was driven by significantly increased responses to the high-frequency DEV stimuli in MIA animals compared controls for the P30 and N85 components of the ERP. This is contrary to the hypothesis that responses to the high-frequency DEV stimuli and subsequent MMRs in MIA animals would be reduced, specifically in late gestation MIA animals. These results indicate that MIA at GD19 and GD10 does not produce the changes to deviance detection mechanisms that are commonly seen in schizophrenia patients (Bodatsch, Brockhaus-Dumke, Klosterkotter, & Ruhrmann, 2015; Erickson, Ruffle, & Gold, 2016; Umbricht & Krljes, 2005).

One possible explanation for the increased, rather than decreased, MMRs found in the current study could be altered NMDAr functioning, specifically *increased* function or expression rather than the *reduced* NMDAr function that is implicated in schizophrenia (Rubio, Drummond, & Meador-Woodruff, 2012). As mentioned previously, reductions in the expression of NMDAr subunits (NR1, NR2C) have been recorded in patients with schizophrenia (Beneyto & Meador-Woodruff, 2008; Shannon Weickert et al., 2013; Sokolov, 1998). In addition, reduced NMDAr functioning,

induced via administration of NMDAr antagonists, has also been linked to reduced MMN amplitude in both humans (Heekeren et al., 2008; Umbricht et al., 2002; Umbricht et al., 2000) and animals (Ehrlichman, Maxwell, Majumdar, & Siegel, 2008; Gil-da-Costa, Stoner, Fung, & Albright, 2013; Javitt, Steinschneider, Schroeder, & Arezzo, 1996; Sivarao et al., 2014; Tikhonravov et al., 2008). In a separate study conducted using the same early versus late MIA model used in the current study, it was discovered that adult MIA rats had *increased* auto-radiographic binding to the NMDAr NR2A subunit ([^{3}H]CGP39653) in the cortex, hippocampus and striatum, with this difference specific to male animals (Rahman et al., 2017). Furthermore, NMDA channel binding ([^{3}H]MK-801) was found to be increased in the cortex of MIA animals, and an increase was also identified in the striatum of male but not female MIA offspring (Rahman et al., 2017). NR2A mRNA expression as assessed via in situ hybridisation was also found to be significantly increased in the cortex and hippocampus of male MIA offspring (Rahman et al., 2017). These results suggest potential increased functioning of the glutamatergic NMDAr system in this MIA model. Given that NMDA hypo-function has been associated with MMN reductions, it is possible that the increased NR2A expression observed in MIA rats is associated with the increased MMR identified in the current study. However, it is not yet clear what the functional implications of increased NR2A expression in MIA-exposed rats are: it is possible that NR2A over-expression in favour of the NR2B subunit may alter the NMDA channel kinetics such that calcium influx through these channels is reduced, thus reducing function (Cui et al., 2013). Further investigations assessing the NMDAr system in the same animals which undergo EEG testing will need to be conducted to confirm this association.

As mentioned, reduced MMN in schizophrenia is associated with poor functioning in the disorder and its association with NMDAr hypo-function suggests that MMN may be an 'index' of NMDAr dysfunction in schizophrenia. However, it is unknown what the functional correlates of *increased* MMRs at the P30 and N85 components are. Using the control rats from the current experiments, another study has examined the impact of escalating doses of the NMDAr antagonist MK-801 on MMRs in rats (Harms et al., 2017). Mk-801 was found to *reduce* MMRs at the N55 component (as hypothesised) but *increased* MMRs at the earliest P13 component. These results indicate that for the current experiments, the component of the ERP that is most 'MMN-like', at least in regard to sensitivity to NMDAr antagonism, is the N55 component. Neither P30 nor N85 MMRs were affected by MK-801, indicating that these components may reflect auditory information processing that is NMDA-independent.

This is the first study to examine the influence of MIA at any gestational time-point on MMRs in the rat. The early (GD10) and late (GD19) gestational time-points chosen for this study were translated from the C57 mouse model (reviewed in Meyer (2014)), which revealed distinct phenotypic differences in the offspring based on the gestational timing of MIA exposure. However, MMRs have not to date been examined in this mouse model and it is currently unknown if our finding of increased, rather than decreased MMRs is common to MIA exposure in general, or the specific gestational time-points used in the current study. In fact, the majority of the studies which examine the effects of MIA in rats use a mid-gestational time-point of exposure (GD14 or 15) (Howland, Cazakoff, & Zhang, 2012; Wolff & Bilkey, 2008, 2010; Wolff, Cheyne, & Bilkey, 2011; Zuckerman & Weiner, 2003, 2005). Although none of these studies have investigated MMRs, they have revealed strong alterations in schizophrenia-like behavioural measures (social interaction deficits, working and recognition memory

deficits, PPI deficits, and pharmacologically induced hyper-locomotion) similar to those seen in the mouse model. It is perhaps possible that the neurodevelopmental processes occurring at the gestational time-points of MIA exposure used in the current study are not as 'sensitive' to the effects of MIA as the more well established (at least in terms of behavioural findings) mid-gestational time-point. However, due to the lack of MMR studies in both the early verse late C57 mouse model and mid-gestational rat model it is currently not possible to discern if the gestational timing or MIA alone play a role in the current finding of increased MMR in MIA animals.

3.3.4.1. Conclusion

MMN deficits are one of the most consistent and replicable biomarkers for schizophrenia. In addition, MMN has the ability to be directly measured in animal models making it an excellent tool to help validate animal models of the disorder. Contrary to our expectations, MIA was observed to *increase* MMRs in the rat. This data confirms that MIA, regardless of gestational timing, does not alter MMRs in a schizophrenia-like fashion. However, further work is required to fully elucidate how the underlying neurobiological system is altered and its relation to the alterations in MMRs reported here.

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4. Chapter 4: Effects of Immune Activation during Early or Late Gestation on Schizophrenia-related Behaviour in Adult Rat Offspring

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4.1. Abstract

Maternal exposure to infectious agents during gestation has been identified as a significant risk factor for schizophrenia. Using a mouse model, past work has demonstrated that the gestational timing of the immune-activating event can impact the behavioural phenotype and expression of dopaminergic and glutamatergic neurotransmission markers in the offspring. In order to determine the inter-species generality of this effect to rats, another commonly used model species, the current study investigated the impact of a viral mimetic Poly (I:C) at either an early (gestational day 10) or late (gestational day 19) time-point on schizophrenia-related behaviour and neurotransmitter receptor expression in rat offspring. Exposure to Poly (I:C) in late, but not early, gestation resulted in transient impairments in working memory. In addition, male rats exposed to maternal immune activation (MIA) in either early or late gestation exhibited sensorimotor gating deficits. Conversely, neither early nor late MIA exposure altered locomotor responses to MK-801 or AMPH. In addition, increased dopamine 1 receptor mRNA levels were found in the nucleus accumbens of male rats exposed to early gestational MIA. The findings from this study diverge somewhat from previous findings in mice with MIA exposure, which were often found to exhibit a more comprehensive spectrum of schizophrenia-like phenotypes in both males and females, indicating potential differences in the neurodevelopmental vulnerability to MIA exposure in the rat with regards to schizophrenia related changes.

4.2. Introduction

It is widely accepted that risk for schizophrenia has a neurodevelopmental origin in which adverse events experienced during critical perinatal periods of brain development, as well as genetic predispositions, produce lasting but often behaviourally

dormant neurodevelopmental changes, which manifest later in life (Lewis & Lieberman, 2000; Lewis & Moghaddam, 2006; Lyon, Saksida, & Bussey, 2012; Owen, Craddock, & O'Donovan, 2005; Wilson & Terry, 2010). Maternal immune activation (MIA) as a result of infection during the critical prenatal period considerably increases the risk of developing schizophrenia. Epidemiological studies have revealed that maternal exposure to a range of bacterial (Babulas, Factor-Litvak, Goetz, Schaefer, & Brown, 2006; Sorensen, Mortensen, Reinisch, & Mednick, 2009) and viral agents (Brown et al., 2001; Buka et al., 2001; Suvisaari, Haukka, Tanskanen, Hovi, & Lonnqvist, 1999; Torrey, Rawlings, & Waldman, 1988) during pregnancy significantly increases the risk of schizophrenia developing in the offspring. In addition, an association between maternal infection and increased risk of schizophrenia in offspring has been identified using serological evidence in which infection (and cytokine up-regulation) during pregnancy was confirmed in blood samples from mothers of those who later developed the disorder (Brown et al., 2004a; Brown et al., 2004b; Brown et al., 2005; Buka et al., 2001). Studies using animals have provided additional support for the link between MIA and adult behavioural and neurobiological changes related to schizophrenia. Rats and mice exposed prenatally to various immune activating agents such as the influenza virus (Fatemi et al., 1999; Fatemi et al., 2008; Shi, Fatemi, Sidwell, & Patterson, 2003), the viral mimetic Polyriboinosinic-polyribocytidilic acid (Poly(I:C)) (Howland, Cazakoff, & Zhang, 2012; Ozawa et al., 2006; Wolff & Bilkey, 2010; Wolff, Cheyne, & Bilkey, 2011; Zuckerman & Weiner, 2005), the bacterial endotoxin lipopolysaccharide (LPS) (Fortier, Joober, Luheshi, & Boksa, 2004; Lante et al., 2008; Lante et al., 2007; Romero, Guaza, Castellano, & Borrell, 2010) and pro-inflammatory cytokines (Samuelsson, Jennische, Hansson, & Holmang, 2006) develop behavioural and neurological ‘schizophrenia-like’ phenotypes.

The timing of prenatal exposure to an immune activating agent such as Poly (I:C) has been identified as an important factor which can influence the behavioural and neurochemical phenotype displayed by adult mouse offspring (Boksa, 2010; Meyer & Feldon, 2012). Specifically, differences between exposure in early/middle (gestational day; GD9) and late gestation (GD17) have been identified in a mouse model of MIA, approximately translating to the mid-first and early-second trimester in humans, respectively (Workman et al., 2013, http://www.translatingtime.net). The majority of evidence from retrospective epidemiological studies implicates the second trimester as a particularly sensitive period with regard to the association between maternal infection and schizophrenia risk (for review see, Brown and Derkits (2010)). However, a birth cohort study found that influenza infection (determined by presence of antibody in maternal serum samples) during the first trimester was associated with a 7-fold increase in schizophrenia risk, which fell to 3-fold when the entire first half of pregnancy was considered (Brown et al., 2004a). Poly (I:C) exposure at GD9 in mice was associated with dopamine (DA)-related behavioural and neurobiological alterations including deficits in prepulse inhibition (PPI) and latent inhibition, reduced expression of dopamine 1 and 2 receptors (D1r, D2r) in the prefrontal cortex (PFC) and D2r in the striatum, as well as increased striatal tyrosine hydroxylase (TH) (Li et al., 2009; Meyer et al., 2008a; Meyer et al., 2008b; Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008c). In contrast, the offspring of dams administered with Poly (I:C) during late (GD17), but not early, gestation displayed phenotypes more consistent with negative/cognitive symptoms of schizophrenia and N-methyl-D-aspartate (NMDA)-receptor alterations including impaired reversal learning, working memory deficits, increased locomotor sensitivity to the NMDA receptor (NMDAr) antagonist dizocilpine (MK-801) and reduced hippocampal expression of the obligate NMDAr NR1 subunit in the

hippocampus (Meyer et al., 2006; Meyer et al., 2008c). Taken together, these findings show that early gestational MIA may be more informative as a model for investigating the rodent equivalents of the positive, DA-related symptoms of schizophrenia in humans, including how they could potentially be treated. Accordingly, late gestational exposure in rodent models could be used to do the same for negative/cognitive symptoms (Meyer & Feldon, 2012).

While the observation of differential early/late phenotypes has been demonstrated in the C57 strain of mouse (Meyer, 2014), it is thus far unknown if such findings translate to other species of rodent, such as the rat. Many studies in rats have used Poly (I:C) to show that prenatal exposure to MIA produces a range of schizophrenia-like phenotypes such as increased sensitivity to AMPH and MK-801 (Vorhees et al., 2012, 2015; Zuckerman & Weiner, 2005), reduced PPI (Howland et al., 2012; Wolff & Bilkey, 2008, 2010), disrupted latent inhibition (Zuckerman & Weiner, 2003, 2005), and impaired cognition (Howland et al., 2012; Wolff et al., 2011). However, these studies have only demonstrated the effects of MIA during mid-gestation (GD14-17), and to our knowledge, no study in a species other than the mouse has replicated the divergent effects of MIA in early versus late gestation on schizophrenia-like behaviour. Extension of these effects to rats will provide evidence of inter-species generality of the early versus late gestation effects, increasing the likelihood of these results also being relevant to human neurodevelopment. Additionally, it will facilitate further investigations that are more readily performed in rats (such as proposed biomarkers for schizophrenia that are measured using EEG) (Dickerson, Wolff, & Bilkey, 2010; Harms, 2015; Harms et al., 2014; Light & Swerdlow, 2015).

In the current study, we investigated the effect of MIA at GD10 and 19 in the rat, which are approximately equivalent to GD9 and 17 in the mouse, respectively

(Workman, Charvet, Clancy, Darlington, & Finlay, 2013). We examined a range of behaviours that were altered in both early and late MIA-exposed mice (locomotor sensitivity to AMPH), and those that were selectively altered by early (PPI) or late (working memory, locomotor sensitivity to MK-801) gestational MIA. For all behavioural assessments, we used both male and female offspring and had sufficient power to detect Sex x Maternal Treatment effects. It was predicted that if there were sex differences in the sensitivity to behavioural perturbation by MIA, effects would be most pronounced in males. Males tend to develop schizophrenia at a higher incidence than females (McGrath et al., 2004), and have been demonstrated to have more prominent negative symptoms (Andreasen, Flaum, Swayze, Tyrrell, & Arndt, 1990; Chang et al., 2011; Fenton & McGlashan, 1991; Kay, Opler, & Fiszbein, 1986; Morgan, Castle, & Jablensky, 2008; Ring et al., 1991; Schultz et al., 1997). With regard to cognitive deficits, however, there is mixed evidence for sex differences, with some cognitive deficits more pronounced in males and some deficits more pronounced in females, potentially depending on the neural network involved (for review see, Mendrek and Mancini-Marie (2015)). In addition, there is evidence that estrogen may serve a somewhat 'protective' role with respect to schizophrenia (Kulkarni, Gavrilidis, Worsley, & Hayes, 2012). To follow up MIA-associated changes in DA-related behaviour found in male rats only, we examined the impact of MIA exposure on midbrain and striatal mRNA expression of TH, the D1r, D2r, and the dopamine transporter (DAT).

4.3. Methods

4.3.1. Animals and Prenatal Treatment

Male and female Wistar rats (*n* = 16 and 94 respectively) were obtained from the University of Newcastle's Central Animal House at 8 weeks of age. Animals were acclimated for two weeks before daily monitoring of oestrous cycle using an impedance probe began. On the day of proestrus females were mated with male rats overnight. The following morning, the presence of sperm via vaginal smear was used to identify day of conception, with the day of detection being identified as GD0. Pregnant dams were then randomly allocated to a treatment group (GD10 Control *n* = 24, GD10 MIA *n* = 26, GD19 Control *n* = 20, GD19 MIA *n* = 24). Control and MIA allocations within each GD were paired and time-matched (e.g. if one pregnant rat was allocated to the GD19 MIA group the next one would be allocated to the GD19 Control group), ensuring that the resulting offspring from Control and MIA dams were of similar ages. On the appropriate gestational day (10 or 19) dams were anaesthetised with isoflurane (induction 5%, maintenance 2.5-3%) and administered with either 4.0 mg/kg of Poly (I:C) (MIA group; Sigma-Aldrich, St. Louis, MO) or 0.1M phosphate-buffered saline (Control group) via injection to the lateral tail vein at 1 mL/kg body weight. Following injection, dams were weighed once daily for 4 days, except if birth occurred during this time. All experiments were performed under strict adherence to the National Health and Medical Research Council Australian Code of Practice for the care and use of animals for scientific purposes and were approved by the University of Newcastle's Animal Care and Ethics Committee (Approval numbers A-2009-108 and A-2013-319).

Two hours following prenatal treatment, blood samples were collected from the saphenous vein of pregnant dams and placed in EDTA-coated tubes before being centrifuged at 1000xG for 15 min at 4°C. Plasma was stored at -20°C until assayed.

Circulating levels of the pro-inflammatory cytokine IL-6 were assessed using a rat cytokine assay kit (R&D) following manufacturer instructions. Sufficient levels of plasma were collected to assay from 73 of the 94 dams. Circulating levels of corticosterone (CORT) were also measured in 57 of the dams using a rat corticosterone ^{125}I radioimmunoassay kit (MP Biomedicals, USA), following manufacturer instructions. The intra assay coefficient of variability ranges between 4.5% and 8.8% and the inter assay variability ranges from 7% to 10%, as reported by the manufacturer.

Litters were left undisturbed until weaning at postnatal day (PND) 21, at which time no more than 3 animals per sex from each litter were allocated to any of the behavioural assessments. All efforts were made to allocate as few animals per litter to each experiment. A summary of how many litters contributed 1, 2 or 3 offspring to each experiment is shown in Table 4.1. A total of 556 offspring were used for these experiments. Sample sizes for the behavioural and qPCR assessments are shown in Table 4.2. Animals were pair housed with same-sex litter-mates and maintained at a temperature of 21° C under a 12 h light/dark cycle (lights on at 0730 h). Following weaning, all offspring were weighed and handled once weekly until 10 weeks of age, at which time brain collection or behavioural testing commenced. Weight measurements that were collected close to PND70 (PND68-72; Control n = 172 and MIA n = 180) were used to assess adult body weight in males and females exposed to early or late MIA.

Table 4.1. *Summary of distribution of offspring allocation to each experiment.*

Experiment	Total offspring	Number of offspring (and litters) from litters contributing:			Number of offspring (and litters) from litters contributing:		
		1 male	2 males	3 males	1 female	2 females	3 females
PPI	169	36 (36)	46 (23)	6 (2)	29 (29)	52 (26)	3 (1)
DNMTP	88	15 (15)	28(14)	0	13 (13)	32 (16)	0
Amphetamine (low dose)	104	8 (8)	40 (20)	0	8 (8)	42 (21)	6 (2)
Amphetamine (high dose)	117	3 (3)	22 (11)	20 (10)	0	26 (13)	36 (12)
MK-801	116	8 (8)	50 (25)	0	8 (8)	50 (25)	0
Dopamine qPCR	24	24 (24)	0	0	0	0	0

Abbreviations: Prepulse inhibition (PPI); Delayed-non-match-to-position (DNMTP); real time quantitative polymerase chain reaction (qPCR).

Table 4.2. *Sample sizes for each experiment.*

		Males		Females		Exp Total	Total
		GD 10	GD 19	GD 10	GD 19		
PPI	Control	10	14	10	14	102	551
(exclusively)	MIA	14	15	12	13		
DNMTP	Control	3	4	3	4	26	
(exclusively)	MIA	4	2	4	2		
Amphetamine	Control	12	11	13	10	94	
(low dose, exclusively)	MIA	11	9	16	12		
PPI and DNMTP	Control	8	7	8	8	62	
	MIA	8	7	8	8		
PPI and Amphetamine	Control	1	0	1	1	10	
(low dose)	MIA	3	1	2	1		
Amphetamine	Control	13	14	15	16	117	
(high dose, total)	MIA	14	14	16	15		
MK-801	Control	14	14	13	13	116	
(total)	MIA	17	13	20	12		
Dopamine qPCR	Control	6	6	0	0	24	
(total)	MIA	6	6	0	0		
PPI	Control	19	21	19	23	172	
(total)	MIA	25	23	22	20		
Amphetamine	Control	13	11	14	11	104	
(low dose, total)	MIA	14	10	18	13		
DNMTP	Control	11	11	11	12	88	
(total)	MIA	12	9	12	10		
DNMTP	Control	10	11	11	12	82	
(1s delay condition)	MIA	9	8	12	9		
DNMTP	Control	9	8	11	9	69	
(variable short delays)	MIA	6	6	12	8		
DNMTP	Control	7	7	9	8	60	
(variable long delays)	MIA	7	6	8	8		

Abbreviations: Prepulse inhibition (PPI); Delayed-non-match-to-position (DNMTP); quantitative polymerase chain reaction (qPCR); Experiment (Exp).

4.3.2. Behavioural Phenotyping

All behavioural assessments commenced at between PND70 to 84. Table 4.2 shows the sample sizes for each experiment. Most animals in the study were only allocated to one experiment, except for 62 rats (*n* = 7-8 per Sex/GD/Treatment, Table 4.2) that underwent PPI testing then went on to complete delayed non-match to position (DNMTP) testing for working memory after a 6-day rest period and 10 rats (*n* = 1-3 per Sex/GD/Treatment, Table 4.2) that underwent testing for locomotor responses to low-dose AMPH 7 days after PPI testing.

4.3.2.1. Prepulse Inhibition (PPI) of the Acoustic Startle Response

PPI testing was conducted using two identical SR-LAB system startle chambers (San Diego Instruments, USA). Startle chambers consisted of sound attenuated chambers containing a plexiglass cylinder (9 cm diameter x 20 cm length) mounted on a plexiglass board attached to a piezoelectric accelerometer. A speaker was situated at the top of the chamber and 70 dB background noise was maintained throughout testing.

The PPI protocol used in the current study was based on that used previously to assess sensorimotor gating in other rodent models of risk factors for schizophrenia (Foldi, Eyles, McGrath, & Burne, 2010; Harms, Eyles, McGrath, Mackay-Sim, & Burne, 2008; Karl, Burne, Van den Buuse, & Chesworth, 2011; Kesby et al., 2012). Animals were first habituated to the apparatus with the 70dB background noise for 5 min prior to testing. Rats were then presented with a mix of different pulse-alone and prepulse trials, presented in a pseudorandom order. There were 25 pulse-alone trials consisting of 5 trials each at 80, 90, 100, 110, and 120 dB intensities to assess acoustic startle response. Ninety PPI trials were presented, consisting of a low-intensity prepulse (either 4, 8, or 16 dB above the background), followed by a 120 dB startle pulse

presented at varying intervals (either 8, 16, 32, 64, 128 or 256 ms). The combination of prepulse intensities and intervals resulted in 18 different types of PPI trials and each was presented five times. Each startle pulse was 40 ms in duration and each prepulse was 20 ms in duration. The inter-trial interval (ITI) varied between 10-20 s. The movement of the rats was recorded by the accelerometer and mean startle responses were averaged from 0 to 100 ms after the onset of the startling pulse. PPI was calculated as a percentage of startle inhibition using the formula 100 x [(120 dB pulse-alone trials – prepulse trials) / 120 dB pulse-alone trials]. The dependent variables used for analyses were %PPI over 3 different prepulse intensities (4, 8, 16 dB above background) and 6 different prepulse to pulse intervals (8, 16, 32, 64, 128, 256 ms).

4.3.2.2. Delayed Non-match to Position (DNMTP)

DNMTP testing was conducted using two operant chambers with retractable levers residing within sound and light-attenuating chambers (Med Associates, USA). Each chamber was equipped with a house light, two retractable levers, and a reward receptacle where a strawberry milk (“Breaka”, Parmalat, Brisbane, Australia) reward was presented from an automatic dipper. Prior to training rats were placed on a food-restricted diet in which body weight was maintained at 85-90% of ad libitum weight plus an additional 10-15 g increase per week to allow for normal growth. Training for the task began six days following the introduction of food restriction at PND70. All training and testing occurred during the light phase with animals being tested once per day, five days a week. The majority of rats learned to collect reward from the reward receptacle following lever press to instigate reward delivery through spontaneous acquisition. However, the few rats that failed to spontaneously acquire this skill were trained to lever press by successive approximations. Following successful acquisition of

lever pressing, rats were then trained on the non-match to position rule. In the non-match to position trials, rats were presented with one of the two levers (selected randomly). Upon pressing the lever, the reward receptacle was lit and a reward was delivered on 20% of trials. Once the rat made a head entry to the reward receptacle to check for the reward, both levers were presented. To receive a reward, the rat needed to press the lever that *was not* presented previously (correct response). An incorrect response was recorded if the rat pressed the wrong lever and an omission response was recorded if the rat failed to lever press or make a head entry after 20 s. The house light remained on throughout the trials, but was turned off for a 20 s 'time out' period in response to both incorrect and omission responses at which time no stimuli or rewards were presented. The ITI was 5 s and each session ended after 100 trials or 30 min, whichever was reached first.

Rats progressed to the *delayed* non-match to position testing sessions once the non-match rule had been learned, i.e. performing ≥ 80 trials per session with ≥ 80% correct responses [100x (no. correct responses/ no. of trials)] for three out of four consecutive days. Rats were then tested for working memory performance using the same procedure, except that a 1 s delay was introduced prior to the two levers being presented in the choice phase of the procedure. After reaching criterion in the 1 s delay phase (≥ 80% correct responses for 3 consecutive days) animals then underwent 5 days of testing with a varied short delay. Delays of 1, 2, 3, 4, 5, and 6 s were presented in a random order throughout the testing session. Regardless of performance in the varied short delay phase, animals then moved on to five days of testing with a varied long delay. Delays were of 1, 2, 4, 8, 16, and 24 s. Each session lasted for a maximum of 30 minutes or 100 trials (whichever occurred first), and the ITI, time-out and limited hold for the study and choice phases were the same as previous training levels. Performance

was assessed by calculating the percentage of correct responses out of total responses (correct and incorrect): 100 x n correct responses / (n correct responses + n incorrect responses). Rats that exhibited high levels (>50%) of omission responses for the 1 s delay, varied short delay, or varied long delay test sessions were removed from the analysis for that session. Therefore, the DNMTP analyses were performed on slightly different sample sizes (1 s delay: *n* = 9-12; Short delays: *n* = 6-12; Long delays: *n* = 6-9 /Sex/GD/Treatment). The dependent variables used for analysis were days to criterion on the 3 stages of training, %correct responses for the last day of training on the no delay condition (as a baseline) and %correct responses over the days of testing and variable delays (1-6 s or 1-24 s).

4.3.2.3. Psychomimetic-induced Locomotion

Both spontaneous and drug-induced locomotor activity was assessed using four identical square open field apparatus, which were 48cm^3 in dimension and made of grey Perspex with an open top. An infrared camera and infrared light array, mounted above the apparatus, were used to simultaneously record from all four open fields in a dark room and CAT-TRACK tracking software was used to record the distance travelled within the open field arena, which was used as the dependent variable for assessment of baseline and drug-induced locomotion. Animals were allocated to one of three drug groups: low-dose AMPH (Low-AMPH; 1mg/kg), high-dose AMPH (High-AMPH; 2.5mg/kg) or MK-801 (0.1mg/kg for ♀ or 0.3mg/kg for ♂) and were tested once only. The choice of drug doses was based on results from pilot experiments using smaller samples of control and MIA and a range of MK-801 and AMPH doses. Doses that produced a significant elevation of drug-induced locomotion in MIA rats, compared to controls in the pilot experiments were chosen for the final experiment.

For the testing procedure, animals were first placed in the centre of the open field and recorded for a 30 min habituation period before being removed from the arena and administered with an intraperitoneal (i.p.) injection of 0.9% saline (Livingstone International, Australia). Immediately following saline injection the animal was returned to the arena and the distance travelled was recorded for a further 30 min baseline phase. Following the baseline phase the animal was again removed from the arena and administered via i.p. injection with either AMPH (D-amphetamine, National Measurement Institute, Australia), or MK-801 (dizocilpine, (+)-MK-801 hydrogen maleate, Sigma-Aldrich, St. Louis, MO), depending on group allocation, and returned to the open field for a further 120 min locomotor assessment. All substances were administered at 1mL/kg body weight.

4.3.3. Tissue Collection & Dopaminergic Real-time Quantitative Polymerase Chain Reaction (qPCR)

Expression of D1r and D2r, DAT and TH mRNA levels was determined by qPCR in the nucleus accumbens (NAc), caudate putamen (CPu), substantia nigra (SN) and ventral tegmental area (VTA) for the dopamine receptors (DAr) and just the SN and VTA for DAT and TH. A total of 24 behaviourally naive ♂ animals were included with $n = 6$ from each of the four experimental groups. Between PND70 and 84 rats were sacrificed via i.p. injection of Lethabarb (2 mL/kg i.p.; Virbac, Pty. Ltd, Milperra, Australia) and whole brains were removed. Brains were rinsed in diethyl pyrocarbonate (DEPC, Sigma-Aldrich, St. Louis, MO) treated phosphate-buffered-saline (PBS) for 1 min before being snap frozen in isopentane at -40 °C. Brains were stored at -80°C. A series of 500 μm-thick coronal cryosections were obtained through the rostro-caudal extent of the NAc (Bregma +2.28 to +1.20 mm), CPu (Bregma +2.28 to +1.20 mm), SN

(Bregma -4.44 mm to -6.24mm), and VTA (Bregma -4.44 mm to -6.24mm) (Paxinos & Watson, 2005). Sections were then placed onto chilled glass microscope slides and the regions of interest bilaterally excised using a tissue punch. The excised tissue was homogenized with TissueLyzer® (Qiagen, Netherlands; 4 min at 20 Hz) in an RNAse-free microtube containing 1 mL of QIAzol® Lysis Reagent (Qiagen, Netherlands) and a 5 mm diameter stainless steel bead (Qiagen, Netherlands). Homogenized samples were stored at −80°C until needed.

Total RNA was then extracted using RNeasy® Mini Kit and DNase I reagents (Qiagen, Netherlands) and RNA concentration, integrity and purity were assessed using the Agilent 2100 Bioanalyser (Agilent Technologies, CA, USA). All samples showed RIN over 8.5, indicating that all RNA obtained were of high quality. cDNA was generated using SuperScript® VILO™ cDNA synthesis kit (Invitrogen, CA, USA). qPCR reactions were carried out using 2X SYBR® Select Master Mix (Applied Biosystems, MA, USA) and gene-specific primer pairs listed in Table 4.3 (Sigma-Aldrich, St. Louis, MO). PCR efficiency for each pair of primers was screened and only primer pairs that presented efficiency between 90% and 110% were used. Reactions were carried out on an AB 7500 fast Real-Time PCR System (Applied Biosystems, CA, USA) and analyzed using the 7500 Software (version 2.0.6). The data were normalized to the endogenous control, β-actin, using the equation $2^{-\Delta\Delta C(t)}$ where C(t) is the cycle at which fluorescence was first detected above background and presented as a fold increase relative to the samples from control rats. The choice of an appropriate reference gene as an endogenous control is a critical step for normalization of PCR results. qPCR were performed with the reference gene β-actin in all samples. For the NAc region, where significant effects of MIA were detected, another potential reference gene (tubulin) was screened for all samples. RefFinder, a web-based tool that integrates

major computational programs (geNorm, Normfinder, BestKeeper, and the $\Delta\Delta$Ct method) for evaluating and screening reference genes (Xie, Xiao, Chen, Xu, & Zhang, 2012) was used to assess the stability of β-actin, tubulin and their geometric average, and it was determined that β-actin was most stable. In addition, expression levels (as a % change from control average) of reference genes were compared between Control and MIA rats to ensure that Treatment did not impact reference gene levels.

Table 4.3. *Gene specific primer pairs used for qPCR.*

ACTβ	TCTGTGTGGATTGGTGGCTCTA	GACGAACGACTAGGTGTAGAC	92%
D1r	TGTCCCCAGCCTTATCGGTCATATTGG	TCCACTGTGTGTGACAGGTTGGATCTT	98%
D2r	TGAAGACACCACTCAAGGGCAA	TCCATTCTCCGCCTGTTCACT	100%
TH	TGTGTCCGAGAGCTTCAATG	GCTGGATACGAGAGGCATAGTTC	101%
DAT	ACGCAGGAGTCAGTCGAAGA	TTGGTCCCACGGAGCATTTG	98%

Abbreviations: ACTβ (Beta actin), D1r (Dopamine 1 receptor), D2r (Dopamine 2 receptor), TH (tyrosine hydroxylase), DAT (dopamine transporter, Solute carrier family 6, member 3)

4.3.4. Statistical Analysis

Statistical analyses were performed using SPSS V21. Dam IL-6 and CORT levels were analysed using an Analysis of Variance (ANOVA), with Treatment and GD as between-subjects factors. A square root transformation was conducted on CORT data and a log transformation was used on IL-6 data to correct for violations of normality. *T*-tests comparing Control and MIA-exposed dams were performed on the weight of dams at injection and for two days following injections.

Offspring data (including adult weight and behavioural data) were analysed using ANOVAs, with Maternal Treatment (Control or MIA), Gestational Day of treatment (GD10 or GD19) and Sex (when both males and females used) as between-subjects factors. Repeated measures analyses were also used where appropriate (e.g. prepulse intensity and interval for PPI experiments and time for behavioural pharmacology experiments). Statistical significance was set at $p < 0.05$. Post-hoc pairwise comparisons were used to explore significant main effects and interactions and SPSS Bonferroni adjusted *p*-values are reported (the *p* values are adjusted for the number of comparisons but using a cut-off of $p < 0.05$ to determine significance).

Analyses of MK-801 data were split by sex due to the differing dose administered to males and females. Additional analyses of behavioural pharmacology data were performed using 2-way ANOVAs (with Treatment and GD as factors) separately for each sex and drug/dose. The dependent variable in these analyses was the arithmetic difference between the distance travelled in the 10 min bin with the highest drug response and the average distance travelled per 10 min in the saline phase of the experiment. For gene expression data, *t*-tests were conducted on the normalised fold-change values for each gene and for each brain region to assess changes between MIA versus control groups within each gestational time-point. In addition, *t*-tests were used to compare reference gene expression levels between control and MIA rats. As with pairwise comparisons from the ANOVAs (above), Bonferroni adjusted *p*-values are reported. Mann Whitney non-parametric test was used to assess DAT in the VTA due to normality violation. For all ANOVAs involving repeated measures, Greenhouse-Geisser corrections were used in cases of violations of sphericity. All data presented in graphs are the raw, untransformed data.

4.4. Results

4.4.1. Dam IL-6 and Corticosterone Concentrations

MIA dams exhibited a significant increase in plasma IL-6 ($F_{(1, 69)} = 284.21$, $p < 0.001$, Table 4.4) and CORT ($F_{(1, 53)} = 52.75$, $p < 0.001$, Table 4.4) concentrations in comparison to control dams 2 h following administration of prenatal treatment. No significant effect of GD or any Treatment by GD interaction was observed. There was no significant effect of Treatment on the body weight of dams at GD10 and GD19 injection, and 24 h and 48 h after injection.

Table 4.4. *Effect of MIA on IL-6, CORT and body weight in pregnant dams.*

	GD10		GD19		
	Control	MIA	Control	MIA	
IL-6 (pg/mL)	26.32 ± 5.79	800.15 ± 119.76	23.82 ± 5.90	1036.39 ± 209.40	Treatment $p <$ 0.001
CORT (ng/mL)	458.80 ± 65.89	1511.42 ± 142.92	557.98 ± 88.16	1327.98 ± 186.56	Treatment $p <$ 0.001
Body weight (g):					
At injection	321.76 ± 5.52	316.56 ± 5.41	418.84 ± 8.18	408.26 ± 5.94	No effect
24h post-injection	318.52 ± 5.76	330.52 ± 6.04	432.46 ± 10.72	412.32 ± 6.71	No effect
48h post-injection	320.84 ± 6.50	323.67 ± 7.46	454.26 ± 11.60	440.38 ± 9.89	No effect

Abbreviations: IL-6 (interleukin 6), CORT (corticosterone)

4.4.2. Offspring Weights in Adulthood

Male rats were found to be predictably heavier than female rats (Males: 400.39 ± 2.64 g; Females: 244.37 ± 2.79g; Sex effect: $F_{(1, 343)} = 1648.28$, $p < 0.001$). There was

no effect of Treatment on offspring weight in adulthood (PND70, $p = 0.966$), nor was there a GD ($p = 0.280$), or GD x Treatment ($p = 0.127$) effect.

4.4.3. Prepulse Inhibition of the Acoustic Startle Response

Analysis of acoustic startle responses to 80-120 dB pulses revealed a significant effect of Pulse Intensity on acoustic startle response ($F_{(1.31, 214.46)} = 344.58$, $p < 0.001$, increasing from 80-120 dB, Figure 4.1). A significant main effect of Sex ($F_{(1, 164)} = 35.54$, $p < 0.001$) and a significant Pulse Intensity x Sex ($F_{(1.31, 214.46)} = 34.71$, $p < 0.001$) interaction were also observed, with startle responses found to be predicably larger for males, compared to females, an effect driven by the increased weight of the males. No significant effects of Treatment ($F_{(1, 164)} = 2.16$, $p = 0.144$) or interactions between Treatment, GD, and/or Sex were observed.

Analysis of PPI data revealed a significant main effect of Sex ($F_{(1, 164)} = 7.23$, $p = 0.008$, increased PPI in males). No significant main effect of immune activation (Treatment) was observed ($F_{(1, 164)} = 0.96$, $p = 0.330$). A significant Treatment x Sex interaction was observed ($F_{(1, 164)} = 6.50$, $p = 0.012$). Post-hoc investigation of this interaction revealed that %PPI was reduced in MIA males relative to control males ($p = 0.013$, Figure 4.2A and B), an effect that was not observed for female rats ($p = 0.273$, Figure 4.2C and D). An apparent increase in %PPI can be seen in GD10 female MIA-exposed rats compared to controls (Figure 4.2C). However, the Treatment x GD x Sex interaction failed to reach statistical significance ($F_{(1, 164)} = 2.83$, $p = 0.094$). There was a main effect of prepulse intensity on %PPI ($F_{(1.64, 269.51)} = 364.92$, $p < 0.001$), with increasing prepulse intensity from 4-16 dBL above background increasing %PPI (Figure 4.2). In addition, a main effect of prepulse-to-pulse interval was found ($F_{(4.36, 714.24)} = 97.10$, $p < 0.001$), with %PPI increasing over increasing prepulse-to-pulse

intervals from 8 – 64 ms (%PPI at 8ms: 13.31 ± 1.84; 16ms: 22.90 ± 1.58; 32ms: 27.34 ± 1.45; 64ms: 40.19 ± 1.31), and decreasing from 128 – 256 ms (%PPI at 128ms: 29.45 ± 1.53; 256ms: 25.10 ± 1.62). There was no effect of GD on PPI ($F_{(1,164)} = 0.96$, $p = 0.328$)

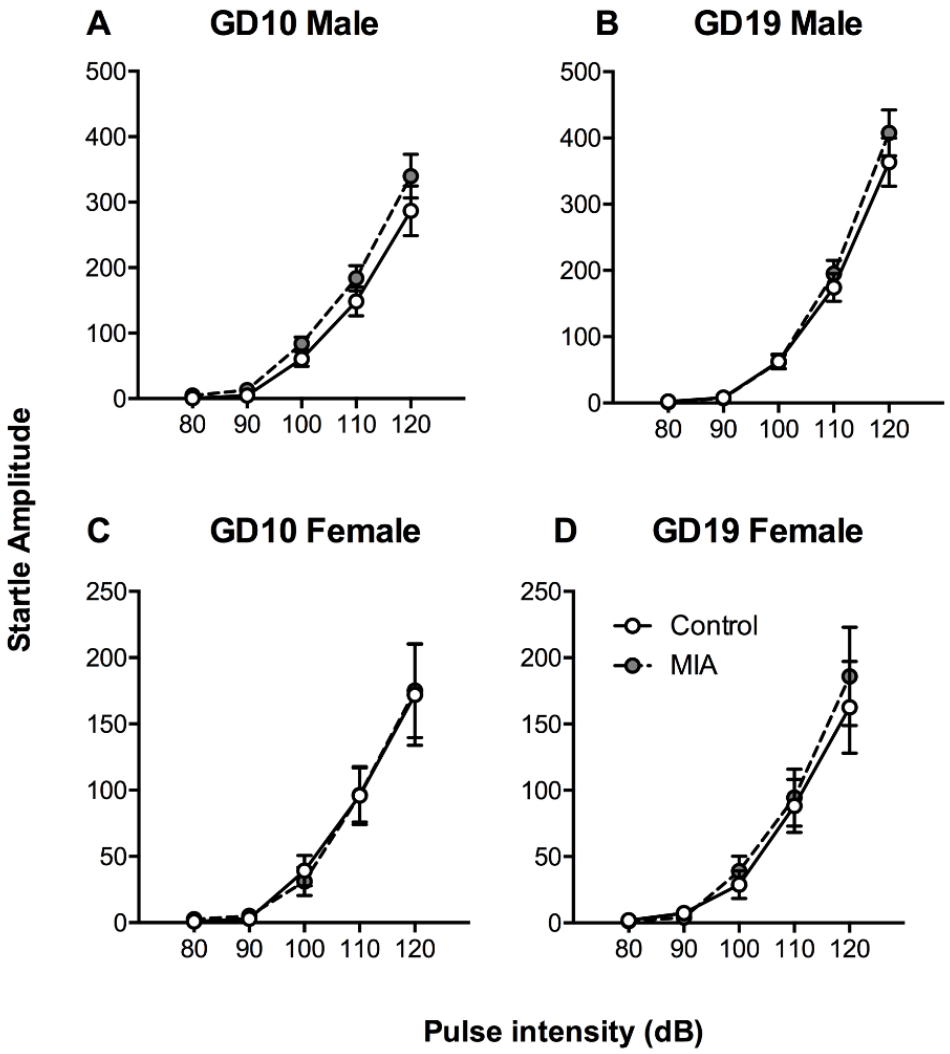


Figure 4.1. Effects of MIA on acoustic startle response over different pulse intensities. Startle response (arbitrary units; Mean ± SEM) in GD10 male (A), GD19 male (B), GD10 female (C), and GD19 female (D) Control (white circles) and MIA (grey circles) animals (n = 19-25). *$p \leq .05$ difference between control and MIA.

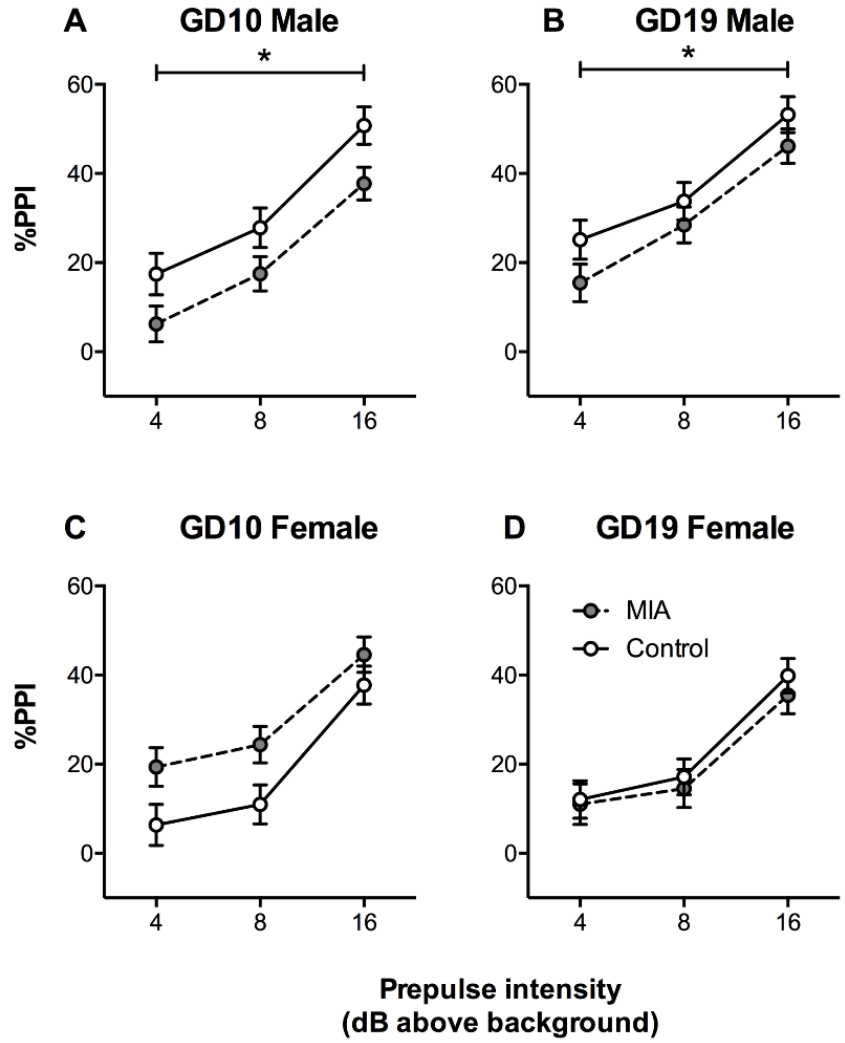


Figure 4.2. Effects of MIA on sensorimotor gating over different prepulse intensities. Prepulse inhibition (%; Mean ± SEM) in GD10 male (A), GD19 male (B), GD10 female (C), and GD19 female (D) Control (white circles) and MIA (grey circles) animals indicating a significant overall reduction of PPI in male MIA animals in comparison to male controls and no effect of MIA in female animals (n = 19-25). Values are presented as raw %PPI values, prior to transformation and are averaged over all six prepulse to pulse intervals. *$p \leq .05$ difference between control and MIA.

4.4.4. Working Memory

No significant effects were observed on days to reach criterion for the first 3 training stages (learning to collect reward, learning to lever press, learning the non-

match to sample rule) indicating that learning was not influenced by treatment or timing of treatment.

Working memory performance was assessed with a 1 s retention period (delay) over three consecutive days of testing. A significant Treatment by GD interaction ($F_{(1, 74)} = 4.87$, $p = 0.03$) was evident. Subsequent post-hoc tests indicated that GD19 MIA animals had significantly reduced performance in comparison to GD19 control animals ($p = 0.048$). Performance at the 1 s delay significantly improved over the three days of testing for all treatment groups ($F_{(1.83, 135.16)} = 36.74$, $p \leq 0.001$). However, a significant 3-way interaction between Treatment, GD and Day of testing was also observed ($F_{(1.83, 135.16)} = 3.89$, $p = 0.026$, Figure 4.3A). Post hoc analysis revealed that GD19 MIA animals showed reduced performance in comparison to GD19 controls on each day of testing. However, this difference was significant on the first day of testing only ($p = 0.041$). There were no significant differences in performance identified for GD10 controls compared to GD10 MIAs ($F_{(1, 74)} = 1.96$, $p = 0.278$). In addition, there were no significant effects of Sex ($F_{(1, 74)} = 0.32$, $p = 0.576$) or GD ($F_{(1, 74)} = 0.01$, $p = 0.932$).

Data from the varied short delay (1, 2, 3, 4, 5, 6 s) and varied long delay (1, 2, 4, 8, 16, 24 s) phase of testing was averaged over the 5 days of testing for each delay. Working memory performance decreased with increased delay for both the varied short ($F_{(3.92, 239.04)} = 199.73$, $p \leq 0.001$, Figure 4.3B), and varied long delay phase ($F_{(3.36, 174.95)} = 214.25$, $p \leq 0.001$, Figure 4.3C). No significant effects of Sex (Short delay: $F_{(1, 61)}= 0.04$, $p = 0.843$; Long delay: $F_{(1, 52)}= 0.001$, $p = 0.979$), Treatment (Short delay: $F_{(1, 61)}= 0.72$, $p = 0.400$; Long delay: $F_{(1, 52)}= 1.81$, $p = 0.184$), GD (Short delay: $F_{(1, 61)}= 52$, $p = 0.475$; Long delay: $F_{(1, 52)}= 0.001$, $p = 0.976$) or interaction with any other variable was identified for either the varied long (Figure 4.3B) or varied short delays (Figure 4.3C).

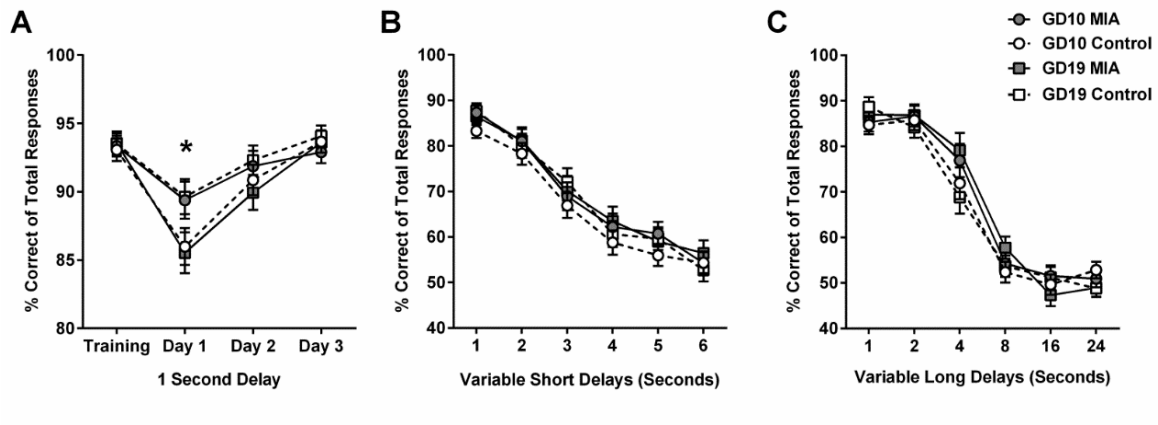


Figure 4.3. Effect of MIA on working memory. The percentage correct of total responses (Mean ± SEM) in the DNMTP task, shown for rats exposed to MIA (grey) or control (white) rats on GD10 (circles) and GD19 (squares). A: % correct responses on the last day of training and the three days of DNMTP testing at a 1 s delay, showing a significant reduction in performance of GD19 MIA animals in comparison to GD19 controls on the first day of testing at the 1 s delay. n = 17-21. B: % correct responses on the varied short delay sessions. n = 14-21. C: % correct responses of the varied long delay sessions. n = 14-16. *$p \leq .05$ difference between control and MIA.

4.4.5. Amphetamine-induced locomotion

Spontaneous locomotor activity was not influenced by Treatment, GD or by the interaction of the two. Throughout the baseline phase of testing the distance travelled significantly decreased with Time interval ($F_{(1.71, 364.51)} = 2428.75$, $p \leq 0.001$) and females travelled significantly more than males ($F_{(1, 213)} = 115.51$, $p \leq 0.001$; Figure 4.4). Locomotor response after the injection with saline was also unaltered by MIA, GD or an interaction effect. A significant Time x Sex interaction that was further modified by Treatment was also observed ($F_{(1.79, 382.06)} = 19.75$, $p \leq 0.001$; $F_{(1.79, 382.06)} = 159.79$, $p = 0.049$, respectively; Figure 4.4). Post-hoc analyses revealed that there were no

significant differences between Control and MIA rats on saline-induced locomotion in either sex.

The administration of AMPH resulted in a marked increase in locomotor activity, with a significant Time x Dose ($F_{(3.98, 815.91)} = 68.28$, $p \leq 0.001$, Figure 4.4) effect identified. Locomotor activity increased in the 30 min after drug administration, an effect that was more pronounced in rats given the high-dose (2.5 mg/kg), compared to those given low-dose (1 mg/kg). A significant main effect of Sex was also confirmed with females travelling further than males during the drug stage of testing ($F_{(1, 205)} = 324.93$, $p \leq 0.001$). Neither MIA, GD, nor an interaction influenced locomotion in response to i.p. AMPH administration at either dose as shown in Figure 4.4.

Additional analyses examined the impact of Treatment and GD on the difference between the peak distance travelled in a 10 min bin following drug administration and the average distance travelled per 10 min in the saline phase of the experiment. For the low AMPH condition, peak distance travelled in males occurred at the 20 min time bin (10-20 min after injection). No effects of Treatment ($F_{(1, 44)} = 0.08$, $p = 0.774$) or Treatment x GD ($F_{(1, 44)} = 0.002$, $p = 0.962$) interactions were identified (Supplemental Figure 4.1A). For females, the peak distance travelled occurred in the 30 min time bin, and no effects of Treatment ($F_{(1, 52)} = 0.28$, $p = 0.598$) or Treatment x GD ($F_{(1, 52)} = 0.18$, $p = 0.670$) interactions were observed (Supplemental Figure 4.1A). For the high AMPH condition, peak distance travelled in males occurred at the 40 min time bin (30-40 min after injection). No effects of Treatment ($F_{(1, 51)} = 1.48$, $p = 0.230$) or Treatment x GD ($F_{(1, 51)} = 0.001$, $p = 0.974$) interactions were found (Supplemental Figure 4.1B). For females, the peak distance travelled occurred in the 30 min time bin, and no effects of Treatment ($F_{(1, 58)} = 0.17$, $p = 0.679$) or Treatment x GD ($F_{(1, 58)} = 0.53$, $p = 0.468$) interactions were observed (Supplemental Figure 4.1B).

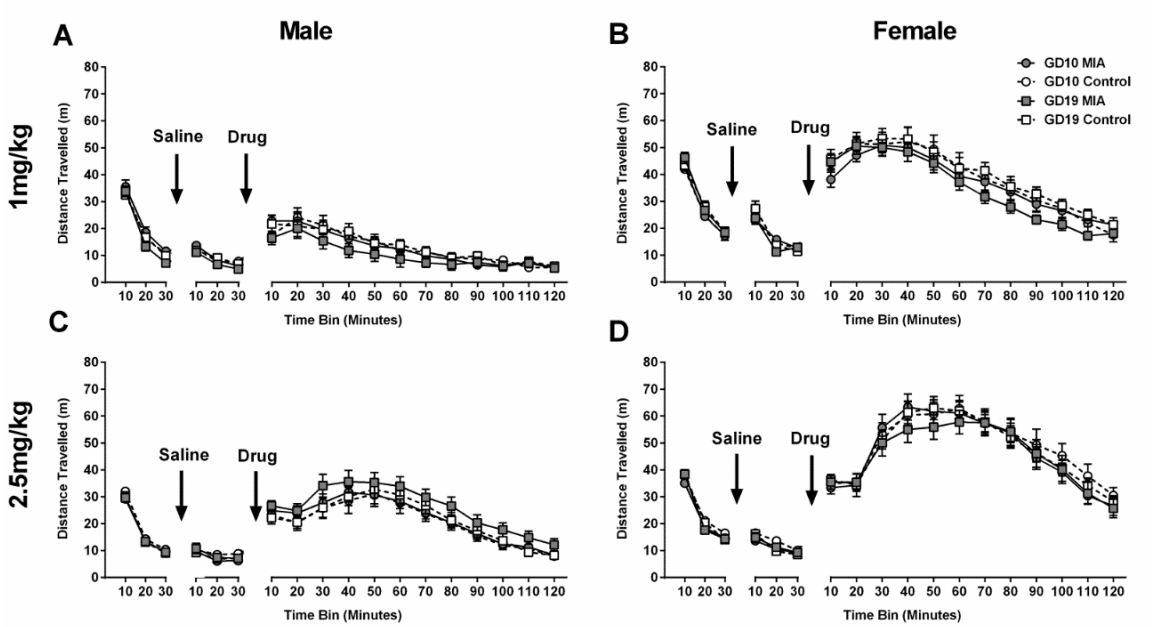


Figure 4.4. Effect of MIA on locomotor sensitivity to two doses of amphetamine (AMPH). Graphs show total distance travelled (m; mean ± SEM) for spontaneous, saline, and AMPH-induced locomotion for rats exposed to MIA (grey) or control (white) rats on GD10 (circles) and GD19 (squares), showing no effect of MIA, GD or their interaction. A: Locomotor activity for male rats given 1 mg/kg AMPH (*n* = 10-14). B: Locomotor activity for female rats given 1 mg/kg AMPH (*n* = 8-14). C: Locomotor activity for male rats given 2.5 mg/kg AMPH (*n* = 9-11). D: Locomotor activity for female rats given 2.5 mg/kg AMPH (*n* = 11-12).

4.4.6. MK-801 Induced Locomotion

As female rats have increased sensitivity to MK-801, males and females were administered with different doses and their locomotor responses were analysed separately. Spontaneous locomotion in the initial exposure to the open field was found to differ as a result of Treatment in male animals ($F_{(1, 54)} = 6.13$, $p = 0.016$; Figure 4.5A)

with MIA rats travelling significantly less than controls. Pairwise comparisons revealed that this difference was only significant in GD19 ($p = 0.014$) and not GD10 rats ($p = 0.364$). There was a similar effect of Treatment on locomotor response after i.p. saline administration in males ($F_{(1, 54)} = 5.55$, $p = 0.022$; Figure 4.5A) with pairwise comparisons indicating this difference was not significant at either GD. The administration of MK-801 to male rats resulted in a minor increase in locomotor activity peaking at 70 min post-injection, leading to a significant main effect of Time ($F_{(2.77, 149.36)} = 35.32$, $p \leq 0.001$; Figure 4.5A). However, there was no effect of Treatment, GD or an interaction between these factors (Figure 4.5A).

Female rats also showed changes in spontaneous locomotor activity however this was in the opposite direction to that displayed in the males with control animals travelling significantly less than MIA rats ($F_{(1, 54)} = 5.62$, $p = 0.021$; Figure 4.5B). Pairwise comparisons showed this treatment effect was not significant at either GD. No main effect of GD or Treatment was found in locomotion response to i.p. saline administration in females. The administration of MK-801 (0.1 mg/kg) to female rats gave rise to a significant increase in locomotor activity, peaking at 80 min as indicated by a significant main effect of Time ($F_{(2.73, 147.49)} = 84.84$, $p \leq 0.001$; Figure 4.5B). However, this increase was consistent for all groups with no differences resulting from Treatment or GD.

Peak distance travelled in males occurred at the 70 min time bin (60-70 min after injection). No effects of Treatment ($F_{(1, 54)} = 0.97$, $p = 0.328$) or Treatment x GD ($F_{(1, 54)} = 0.002$, $p = 0.968$) interactions were found (Supplemental Figure 4.1C). For females, the peak distance travelled occurred in the 80 min time bin, and no effects of Treatment ($F_{(1, 54)} = 0.002$, $p = 0.963$) or Treatment x GD ($F_{(1, 54)} = 0.04$, $p = 0.836$) interactions were observed (Supplemental Figure 4.1C).

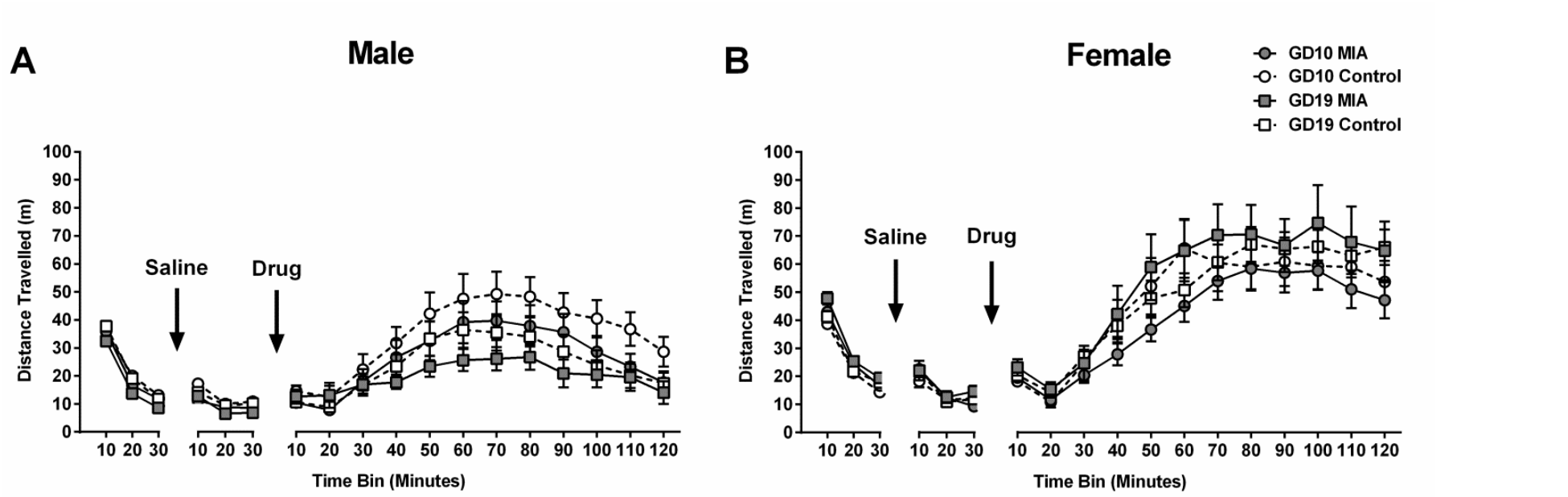


Figure 4.5. Effect of MIA on locomotor sensitivity to MK-801. Graphs show total distance travelled (m; Mean ± SEM) for spontaneous, saline, and MK-801-induced locomotion for rats exposed to MIA (grey) or control (white) rats on GD10 (circles) and GD19 (squares), showing no effect of MIA, GD or their interaction. A: Locomotor activity for male rats given 0.3 mg/kg MK-801 (*n* = 6-9). B: Locomotor activity for female rats given 0.1 mg/kg MK-801 (*n* = 9-11).

4.4.7. Dopamine Receptor mRNA Expression

Analysis of gene expression for brain tissue samples in male animals indicated subtle changes in dopaminergic markers as a result of MIA and gestational timing (Table 4.5). A significant increase of 20% in D1r mRNA levels were observed in the NAc of GD10 MIA animals in comparison to GD10 controls ($t_{(10)} = 2.55$, $p = 0.05$) but no significant differences were found for GD19 animals ($t_{(10)} = 0.19$, $p = 0.832$). No significant differences in D1 mRNA levels for either the CPu or SN were observed for either GD10 or GD19 animals. In addition, no significant differences in gene expression were observed for remaining gene targets (D2r in the CPu, SN, VTA; DAT in the SN, VTA; TH in the SN, VTA). Expression of the utilized reference gene, β-actin, as well as the alternative reference genes, tubulin, and their geometric mean were assessed for effects of MIA using NAc tissue. Expression of these genes (as measured by the % of control expression) was not altered by MIA in GD10 rats (where significant effects of MIA on target gene expression were observed): β-actin (Control: 100.00% ± 3.96; MIA: 105.48 ± 5.47; $t_{(1, 10)} = 0.81$, $p = 0.436$), tubulin (Control: 100.00% ± 9.57; MIA: 96.88 ± 8.67; $t_{(1, 10)} = 0.24$, $p = 0.814$), geometric mean (Control: 100.00% ± 5.44; MIA: 101.51 ± 5.94; $t_{(1, 10)} = 0.19$, $p = 0.855$). MIA at GD19 also was not found to alter reference gene expression: β-actin (Control: 100.00% ± 4.39; MIA: 108.15 ± 4.40; $t_{(1, 10)} = 1.31$, $p = 0.219$), tubulin (Control: 100.00% ± 3.78; MIA: 113.71 ± 9.57; $t_{(1, 10)} = 1.33$, $p = 0.212$), geometric mean (Control: 100.00% ± 3.69; MIA: 110.34 ± 4.94; $t_{(1, 10)} = 1.68$, $p = 0.124$). The observation of increased D1r mRNA in GD10 MIA-exposed males was also replicated when the data were normalised against the tubulin reference gene (Control: 1.00 ± 0.08; MIA: 1.41 ± 0.10; $t_{(1, 10)} = 2.67$, $p = 0.023$), as well as the geometric mean of β-actin and tubulin (Control: 1.00 ± 0.08; MIA: 1.30 ± 0.08; $t_{(1, 10)} = 2.72$, $p = 0.022$).

Table 4.5. *Gene expression of dopaminergic markers in the Nucleus accumbens (NAc), Caudate putamen (CPu), Substantia nigra (SN), and the Ventral tegmental area (VTA).*

	GD10		GD19	
Target Gene	Control	MIA	Control	MIA
Dopamine 1 Receptor				
NAc	**1.00 ± 0.05**	**1.20 ± 0.06***	1.00 ± 0.05	1.02 ± 0.10
CPu	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.06	0.99 ± 0.03
SN	1.00 ± 0.08	1.00 ±0.07	1.00 ± 0.09	0.99 ± 0.06
Dopamine 2 Receptor				
NAc	1.00 ± 0.08	1.09 ± 0.08	1.00 ± 0.06	1.09 ± 0.12
CPu	1.00 ± 0.09	0.99 ± 0.08	1.00 ± 0.08	1.00 ± 0.07
SN	1.00 ± 0.10	0.98 ± 0.05	1.00 ± 0.15	0.95 ± 0.05
VTA	1.00 ± 0.18	1.00 ± 0.25	1.00 ± 0.15	1.02 ± 0.16
Dopamine Transporter				
SN	1.00 ± 0.16	0.95 ± 0.05	1.00 ± 0.17	0.63 ± 0.22
VTA	1.00 ± 0.20	0.98 ± 0.21	1.00 ± 0.18	0.98 ± 0.13
Tyrosine Hydroxylase				
SN	1.00 ± 0.16	0.91 ± 0.06	1.00 ± 0.20	1.29 ± 0.08
VTA	1.00 ± 0.19	0.98 ± 0.21	1.00 ± 0.12	1.00 ± 0.12

Data presented as a fold change relative to saline control (Mean ± SEM). *$p \leq .05$ MIA compared to control.

4.5. Discussion

Our findings in the current study indicate that prenatal exposure to MIA disrupts neurodevelopment in rats, producing behavioural and neurobiological changes in adulthood. These changes following MIA exposure, some of which are similar to those observed in schizophrenia emerge in a time-specific manner. The results here partially replicate previous findings reported by Meyer et al. (2008c), where a dissociation was identified between glutamatergic and dopaminergic-related behavioural phenotypes in mice exposed to MIA in early versus late gestation, respectively.

PPI was used to assess sensorimotor gating, a commonly used test of the ability to filter sensory information at a pre-attentional level. Sensorimotor gating impairments have been found in patients with schizophrenia (Braff, Geyer, & Swerdlow, 2001). In particular, such impairments are believed to reflect a state of subcortical DA-hyper-function in animal models of schizophrenia, as direct DAr agonists, such as apomorphine, and indirect DAr agonists such as AMPH, have been shown to disrupt PPI in a manner similar to that observed in MIA-exposed rats in the current study (Geyer, Krebs-Thomson, Braff, & Swerdlow, 2001). As such, PPI deficits are believed to be somewhat analogous, at least on the neurotransmitter level, to the positive symptoms of the disorder (Braff, Swerdlow, & Geyer, 1999). Here, we found that MIA exposure was associated with deficits in sensorimotor gating in male, but not female, rats, regardless of the time of gestational exposure. These findings suggest that MIA is associated with schizophrenia-like changes in subcortical DA-function. This notion is further supported here by an increase in D1r mRNA levels in the NAc of male animals exposed to MIA at GD10 (female rats were not tested). While a wealth of evidence indicates that D2r activity appears to be more closely associated with PPI than D1r activity, there is some supporting evidence for D1r involvement, with D1r antagonists reported to restore PPI when disrupted by cocaine, apomorphine or ovariectomy (Doherty et al., 2008; Gogos, Kwek, Chavez, & van den Buuse, 2010). It is thus far not known if such mRNA changes seen in MIA rats directly contribute to PPI disruption, nor whether they reflect a change in synaptic D1r protein. However, there is some evidence from a mouse model of MIA demonstrating that such D1r changes may contribute to PPI deficits. Mice exposed to MIA during early gestation (GD9) were found to exhibit similar deficits in PPI (33%, compared to 29% in current study) and increased D1r expression in the NAcc (15% compared to 20% in the current study)

(Vuillermot, Weber, Feldon, & Meyer, 2010). In addition, it was found that administration of the D1r agonist SKF38393 to control mice (i.e. not exposed to any prenatal manipulation) produced deficits in PPI similar to the MIA-exposed mice, and that administration of the D1r antagonist SCH2339 to MIA mice restored PPI to the same level as recorded in control mice (Vuillermot et al., 2010).

The changes in PPI and DAr mRNA observed in the current investigation of MIA-exposed rats were not accompanied by a change in sensitivity to the locomotor-stimulating effects of the indirect DA-agonist AMPH. AMPH sensitivity is often observed in animal models of schizophrenia-like hyper-dopaminergia (El-Khodor & Boksa, 1998; Flores, Wood, Liang, Quirion, & Srivastava, 1996), including other models of MIA (Fortier et al., 2004; Meyer et al., 2008c; Ozawa et al., 2006; Richetto, Calabrese, Meyer, & Riva, 2013; Vuillermot et al., 2010; Zuckerman, Rehavi, Nachman, & Weiner, 2003). However, altered locomotor activity after exposure to AMPH is not always found in animal models of MIA (Missault et al., 2014). Like PPI deficits, increased sensitivity to AMPH is believed to reflect an endogenous sensitization of the DA-system which has also been found in persons with schizophrenia (Howes & Kapur, 2009; Lieberman, Kane, & Alvir, 1987). It is unclear why both AMPH sensitivity and PPI deficits were not observed in MIA rats in the current study. The doses used in the current study are commonly used to demonstrate locomotor sensitivity in other schizophrenia risk factor models (Kesby et al., 2010; Zuckerman et al., 2003). However, it is possible that very *subtle* alterations may only be demonstrated by low doses of AMPH, such as 0.5 mg/kg as demonstrated in another rat MIA model using the bacterial mimic lipopolysaccharide (Fortier et al., 2004).

In the current study, we observed sex-specific effects, with deficits in PPI present in male but not female offspring. In line with our PPI findings, previous

research in a mouse model using early gestational exposure to Poly (I:C) found deficits in PPI predominantly in male offspring (O'Leary et al., 2014). Prenatal LPS exposure in Wistar rats also produced a more pronounced PPI deficit in male offspring (Romero et al., 2010). This suggests that male rats are more prone than females to the effects of MIA on sensorimotor gating. This is in agreement with studies suggesting that estrogen or agents that increase brain estrogen activity potentially have a protective effect on PPI disruption (Gogos & van den Buuse, 2015) or even act as an adjunct therapeutic for schizophrenia (Weickert et al., 2015). We did not control for oestrous cycle in female rats, so it is possible that potential influences of estrogen on MIA-induced behavioural alterations were somewhat 'masked' by the noise inherent in assessing a group of female rats presumably at different stages of their cycles. It would be informative to investigate whether ovariectomised female rats exposed to MIA exhibit similar PPI deficits as males, whether estrogen treatment in MIA-exposed males reverses or normalises PPI deficits, or whether the behavioural effects of MIA in females differ as a function of oestrous cycle.

Visuo-spatial and verbal working memory deficits are considered core cognitive symptoms of schizophrenia (Lee & Park, 2005). Working memory deficits in patients are also not delay-dependent and do not deteriorate any further with delays longer than 1 s (Lee & Park, 2005). Rather, people with schizophrenia exhibit impairments in goal maintenance (the act of activating working memory, as measured in the continuous performance AX task) and interference control (the act of guarding working memory contents from interference, as measured in *n*-back tasks) (Barch & Smith, 2008). The current study used the DNMTP task to assess working memory, which is considered an appropriate test for schizophrenia-like impairments in animal models, particularly in *goal maintenance* (Dudchenko, Talpos, Young, & Baxter, 2013). In the current study,

we found working memory deficits specific to MIA in late but not early gestation, independent of sex. Specifically, we found that all rats performed at a similar level by the end of training on the non-match to position task (without the delay that would necessitate the recruitment of working memory). However, when a 1 s delay was introduced, rats exposed to MIA in late gestation performed worse than control animals indicating impaired working memory, which improved to control levels after subsequent days of testing. Impairments in working memory are found in the mouse model of late gestational MIA (Bitanihirwe, Weber, Feldon, & Meyer, 2010), and are believed to be associated with the range of NMDA-specific abnormalities also present in mice exposed to MIA at GD17 including increased locomotor sensitivity to the NMDAr antagonist, MK-801, and reduced expression of the obligate subunit of the NMDAr, NR1, in the hippocampus (Meyer et al., 2008c). Our results demonstrate that sensitivity to the locomotor stimulating effects of the NMDAr antagonist MK-801 are not altered by MIA at either prenatal time-point. These findings indicate that while late gestational MIA in rats can recapitulate some features found after late gestational MIA in mice (namely, working memory impairments), MIA at GD19 was not sufficient to produce other alterations reminiscent of schizophrenia in humans.

We have observed some subtle and/or transient alterations in behaviour and DAr expression in rats exposed to MIA. To our knowledge, this is the first study to investigate the impact of early versus late MIA on behaviour in a species other than the mouse. While the evidence for some phenotypic divergence is strong for early versus late MIA in C57 mice (as reviewed in Meyer (2014)), the findings from the current study suggest this is not the case for the rat. This may be due to inaccurate translation of gestational exposures. For instance, perhaps the development of the DA-system is not at the same stage at GD10 in the rat as it is at GD9 in the mouse and therefore, MIA

exposure at GD10 in the rat is less impactful. For the current study, we used the best available evidence for 'translating' the developmental ages from rats to mice (Workman et al., 2013). However, it is possible that the different windows of sensitivity that lead to the phenotypic disassociation of changes to DA- versus glutamate-related behaviours (or positive symptom versus negative/cognitive symptom-related behaviours) in early versus late MIA-exposed mice, respectively, is unique to mice. Indeed, many of the studies that investigate the impact of MIA in rats administer Poly (I:C) at GD14-15 (Howland et al., 2012; Piontkewitz, Arad, & Weiner; Wolff & Bilkey, 2008; Wolff et al., 2011; Zuckerman & Weiner, 2005), and typically find common a range of behavioural alteration in MIA-exposed rats, including the DA-related behaviours believed to model positive symptomology (increased AMPH-induced motor hyperactivity, PPI deficits) and behaviours that are more related to negative symptoms and cognitive impairments (social interaction, working memory deficits). The findings from this study indicate that along with GD14-15, GD10 and 19 in the rat also coincide with neurodevelopmental stages that are sensitive to Poly (I:C), but perhaps are not as sensitive as GD14-15 as, we did not observe the entire 'spectrum' of schizophrenia-related brain and behavioural changes often reported in other models of MIA via Poly (I:C) (Meyer, 2014).

Despite the absence of dramatic phenotypic divergence between early and late MIA that has been previously reported in mice (Li et al., 2009; Meyer et al., 2008a), one effect, that of transient working memory impairments, was only identified in rats exposed to MIA at GD19, not GD10. Differences in the effects of MIA at different ages can be due to innumerable factors of three main types: maternal factors (shifts in the maternal immune response throughout pregnancy), placental factors (shifts in placental permeability throughout pregnancy) and foetal factors (changes in periods of the

vulnerability of the foetal immune and nervous systems throughout prenatal development). With regard to maternal factors, in the current study we found that IL-6 and CORT were elevated in MIA-exposed dams regardless of GD, potentially indicating that there was no difference in the maternal response to the Poly (I:C). However, CORT and IL-6 are not the only possible mediators of maternal immune response, so it is entirely possible that MIA affects other cytokines or hormones to different degrees over the course of pregnancy. With regard to placental factors, there is evidence that the permeability of the placenta to circulating maternal cytokines differs throughout pregnancy (Bowen, Chamley, Keelan, & Mitchell, 2002; Zourbas, Dubanchet, Martal, & Chaouat, 2001). In particular, permeability to IL-6 is substantially increased during mid-gestation in rats compared to late gestation (Dahlgren, Samuelsson, Jansson, & Holmang, 2006). However, in the current study, we find more alterations in rats exposed to MIA during late (GD19) gestation. Possibly the most prominent factor is that of the stage of neurodevelopment that MIA affects. Throughout prenatal (and postnatal) brain development, the proliferation, differentiation, migration, synaptogenesis and signalling of a variety of different types of neuron and glia cell would result in some systems having different levels of vulnerability throughout development. For example, peak neurogenesis of midbrain dopaminergic neurons occurs at approximately GD11 in the mouse (Bayer, Wills, Triarhou, & Ghetti, 1995) and GD12 in the rat (Gates, Torres, White, Fricker-Gates, & Dunnett, 2006). These processes may be particularly vulnerable to MIA at GD9 in the mouse, leading to substantial shifts in DA-ontogeny in MIA-exposed mice. While we do not replicate the range of DA-related behavioural alterations, we do find that rats exposed to MIA on GD10 exhibited changes to D1r expression, which were not found in GD19-exposed rats. In a similar fashion, it is possible that the developmental

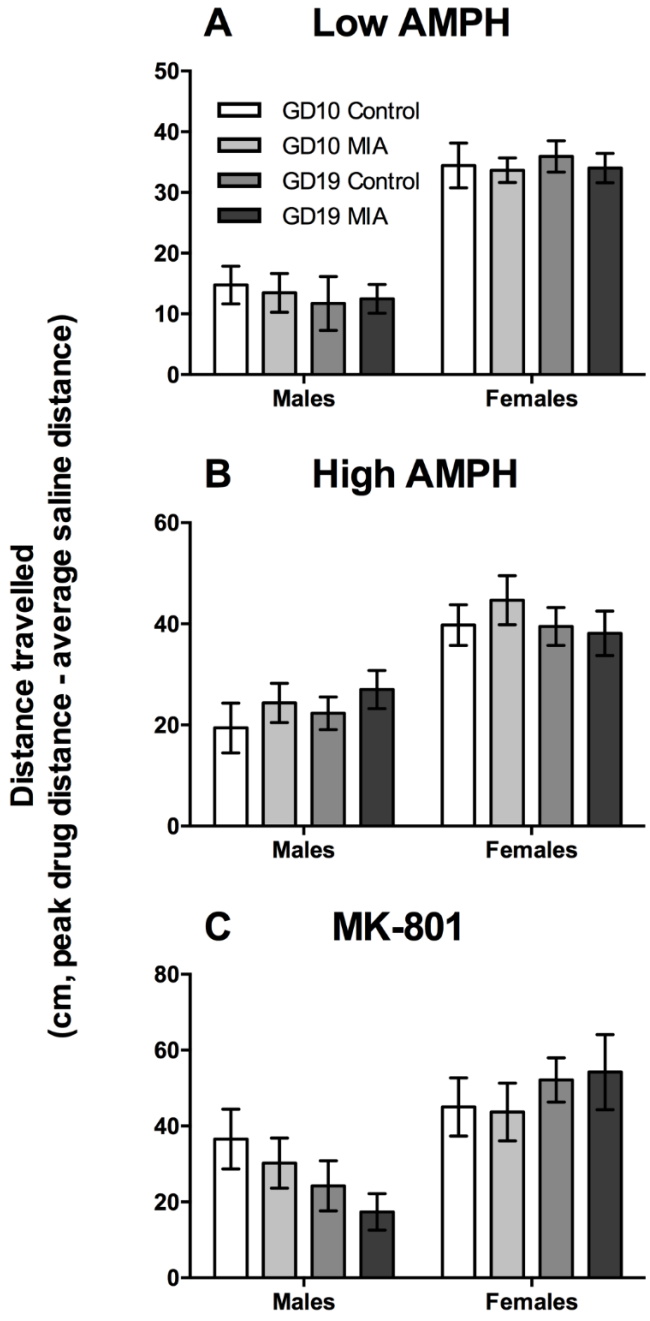
processes taking place at GD19 in rats were altered specifically by GD19 MIA to promote the specific effects of late MIA on behaviour. Both the neocortex and hippocampus undergo peak neurogenesis during late-gestation in the rat (Bayer & Altman, 2004), so it is therefore possible that MIA disrupts this process, shifting the developmental trajectory and altering cortical and/or hippocampal function, with the result of altering spatial working memory. Furthermore, direct NMDA-dependent alterations are likely only possible during late gestation, as mRNA for the obligate NMDAr subunit, NR1, is not expressed in the embryonic rat brain until late-gestation (GD17) (Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994).

It should be noted that one key methodological difference between the current study and similar ones in mice, apart from the species of rodent, is that the rats in the current study were anesthetised for the intravenous injection. To our knowledge, all studies that administer Poly (I:C) intravenously to pregnant mice do this without anaesthesia. However, similar to many other rat MIA studies that administer the Poly (I:C) intravenously (Dickerson et al., 2010; Howland et al., 2012; Piontkewitz, Arad, & Weiner, 2011b; Savanthrapadian et al., 2013; Wolff & Bilkey, 2008; Yee, Schwarting, Fuchs, & Wohr, 2012; Zhang, Cazakoff, Thai, & Howland, 2012; Zuckerman & Weiner, 2005), we used an inhalation anaesthetic (isoflurane), which has the advantage of having a very short half-life. For a short procedure, such as the injection in the current study, the animal is only anesthetised lightly for approximately 5 min, and returns to an active and alert state within 5 min of being removed from anaesthesia.

We have employed a novel model of MIA to investigate the impact of early versus late gestational immune challenge on DA- and glutamate-related behaviour and neurobiology in the rat. We identified two changes in schizophrenia-related behaviour including PPI deficits, which were observed in rats exposed to MIA in both early and

late gestation, and a transient working memory impairment found only in rats exposed to MIA in late gestation (GD19). Alterations in the DA-system found here, namely an increase in D1r mRNA expression in male MIA rats, could potentially underlie PPI deficits in these rats. The present findings provide continued support for the use of MIA rodent models in schizophrenia research but indicate that GD10 and 19 in the rat may not be the most sensitive periods of rat gestation for the observation of schizophrenia-like behavioural and neurobiological effects.

4.6. Supplemental Figures



Supplemental Figure 4.1. Behavioural pharmacology peak activity data. Graphs show the Mean ± SEM difference between locomotor activity (distance travelled in cm) during the 10 min peak response period and the average distance travelled (cm) in the saline phase. Data shown are for male and female rats exposed to saline (control) or Poly (I:C) (MIA) at GD10 or GD19 after treatment with (A) low-dose amphetamine (AMPH), for which the peak drug response time was 10-20 min for males and 20-30 min for females; (B) high-dose AMPH, for which the peak drug response time was 30-40 min for males and 20-30 min for females; and (C) MK-801, for which the peak drug response time was 60-70 min for males and 70-80 min for females.

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5. Chapter 5: Maternal Immune Activation Increases Microglia Levels in the White Matter of the Corpus Callosum of Adult Rat Offspring

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5.1. Abstract

Animal models of maternal immune activation (MIA) are used to study the effects of infection on brain development in the offspring that may increase their risk of schizophrenia. Microglia activation and cytokine upregulation have been proposed to play key roles in the neuropathology of schizophrenia. We hypothesised that MIA induces changes in microglia and cytokines in the brains of the adult offspring. To test this, experimental MIA was produced by injecting Polyriboinosinic-polyribocytidilic acid (Poly (I:C)) or saline into pregnant rats on gestational day (GD) 10 or GD19, with brain tissue from the offspring collected for analysis at adulthood (12 weeks of age). There was no change in Iba1, Gfap, IL1-β and TNF-α mRNA expression in the medial prefrontal cortex (mPFC) in adult offspring of GD10 (n = 12) and GD19 Poly (I:C) dams (*n* = 12) compared to GD10 (*n* = 12) and GD19 controls (*n* = 12) respectively. In a separate cohort of animals, microglial IBA1-positive (IBA1+) and astrocytic GFAP-positive (GFAP+) immunoreactivity was determined using a pixel intensity thresholding method in the PFC and white matter of the corpus callosum in 12 week old offspring of GD10 (*n* = 10) or GD19 Poly (I:C) dams (*n* = 18) compared to controls (*n* = 20). ANOVA showed that MIA had a significant main effect on IBA1+ immunoreactivity in the corpus callosum, with post-hoc analyses identifying a significant increase in IBA1+ immunoreactivity at GD19, but not GD10. No change in IBA1+ immunoreactivity was observed in the PFC. In contrast, ANOVA showed that MIA had a significant main effect on GFAP+ immunoreactivity in the PFC, with post-hoc analyses identifying a strong trend towards increased GFAP+ immunoreactivity that approached significance in the offspring of GD19 Poly(I:C), but not GD10 Poly (I:C) dams. No change in GFAP+ immunoreactivity was observed in the corpus callosum. These findings suggest that late gestation MIA is capable of causing subtle alterations to the trajectory of

microglial and astrocyte development. How these findings relate to the risk of developing schizophrenia requires further investigation to determine the developmental time frame for these changes, whether other brain regions are also affected and if later life insults relevant to schizophrenia, exacerbate the neuropathology.

5.2. Introduction

Schizophrenia is a chronic psychiatric disorder characterised by a wide array of symptoms which ultimately result in substantial disability in the affected person's life (Lewis & Lieberman, 2000). Schizophrenia is understood to have complex neurodevelopmental origins that are underpinned by both genetic and environmental factors (Owen, Craddock, & O'Donovan, 2005; Wilson & Terry, 2010). Many of the environmental risk factors implicated in schizophrenia occur in the prenatal stages of development, with alterations in the maternal-foetal environment having the potential to produce long-lasting and significant influence on normal neurodevelopmental processes (Lewis & Levitt, 2002). Maternal immune activation (MIA) through exposure to infectious agents during the prenatal stages of development is one of the most robust environmental risk factors identified to date for schizophrenia. Considerable epidemiological evidence has shown that exposure to bacterial (Babulas, Factor-Litvak, Goetz, Schaefer, & Brown, 2006; Sorensen, Mortensen, Reinisch, & Mednick, 2009) or viral infection (reviewed in Brown and Derkits (2010)) during pregnancy increases the risk of schizophrenia developing in the offspring by up to 7-fold (Brown et al., 2004a).

Rodent models of MIA have provided evidence to support the link between prenatal infection and the later-life development of schizophrenia. Exposure to a number of immune activating agents including the influenza virus, the viral mimetic Poly(I:C), and the bacterial endotoxin lipopolysaccharide (LPS) during gestation have

all been shown to produce schizophrenia-like behavioural and neurobiological abnormalities in the adult offspring of both rats and mice (reviewed in Meyer (2014)). Due to the array of pathogens which have now been linked to schizophrenia, it is believed that a common feature of the maternal immune response, rather than direct actions of the individual pathogens are responsible for the altered neurodevelopment and long-term neurobiological abnormalities seen in the MIA offspring (Meyer & Feldon, 2009). The exact mechanisms by which MIA disrupts neurodevelopmental processes to produce schizophrenia pathology are still not fully elucidated, but what is recognised is the important role the body's immune-inflammation response plays in this process.

Microglia and astrocytes are the primary immune cells of the central nervous system (CNS) with cytokines and chemokines being the main immune-signalling molecules released from these cells in response to an immunological challenge. Cytokines play a dual role in the CNS, where they are crucial to pro- and anti-inflammatory immune responses to infection, stress, and cell damage (Deverman & Patterson, 2009). In addition, cytokines play a vital role in neurodevelopmental processes such as neurogenesis, cell migration, differentiation, axon pathfinding, and apoptosis (Deverman & Patterson, 2009). Microglia are immune surveillance cells and are heavily involved in the CNS innate immune-inflammatory response (Perry, Andersson, & Gordon, 1993). When activated by an immunological challenge, microglia are known to release a range of pro- and anti-inflammatory cytokines and chemokines in addition to acting as macrophages that become phagocytic in the presence of pathogens and cell damage (Deverman & Patterson, 2009). Further to their role in the immune response, microglia are also involved in neurodevelopmental processes of synaptogenesis and synaptic pruning (Dong & Benveniste, 2001). Whilst

astrocytes are traditionally believed to be involved in maintaining CNS homeostasis and neuronal support processes (Dong & Benveniste, 2001), they have now been implicated in CNS immune regulation. Much like microglia, activated astrocytes release a range of cytokines and chemokines whilst also having modulatory effects on microglia (Aloisi, 2001; Bianchi, Kastrisianaki, Giambanco, & Donato, 2011; Zhang et al., 2011).

Numerous findings indicate that cytokines, and by association microglia are potential mediators between MIA and the development of behavioural and brain disturbances relevant to schizophrenia (Meyer, Feldon, & Yee, 2009). For example, the mothers of patients with schizophrenia where found to have elevated serum levels of the cytokines IL-8 and TNF-α during gestation in comparison to the mothers of healthy controls (Brown et al., 2004b; Buka et al., 2001), whilst elevated maternal IL-8 has been associated with neuroanatomical changes associated with schizophrenia (Ellman et al., 2010). In addition, a MIA model in mice found that injection of the cytokine IL-6 during gestation produces similar effects (altered sensorimotor gating and latent inhibition) in the offspring as those administered with Poly(I:C), and that when administered in conjunction with an IL-6 antibody, the development of these changes is attenuated indicating that IL-6 plays a role in mediating the effects of prenatal infection on brain and behavioural alterations (Smith, Li, Garbett, Mirnics, & Patterson, 2007). Another MIA mouse model also showed that the brain and behavioural alterations in response to prenatal Poly (I:C) was attenuated in mice overexpressing the anti-inflammatory cytokine IL-10 (Meyer et al., 2008a).

The multiple roles of microglia, astrocytes, cytokines and chemokines in neurodevelopment, homeostasis and immune function opens the possibility that immune activation occurring during prenatal development could directly and permanently disrupt the neurodevelopmental process (Meyer, 2011). It is also possible that MIA and

the subsequent immune response in the foetus can indirectly disturb neurodevelopment by permanently altering or 'priming' microglial and astrocyte functioning to produce long-term neuroinflammatory abnormalities. This in turn may alter responses to future inflammation-inducing environmental factors such as stress or infection and disturb the normal brain maturational process that occurs throughout childhood and adolescence (Bland et al., 2010; Meyer, 2011; Monji, Kato, & Kanba, 2009; Upthegrove & Barnes, 2014).

Support for the involvement of long-term immuno-inflammatory abnormalities in schizophrenia has come from studies in both the periphery and CNS of affected patients. Increased levels of a range of circulating cytokines (IL-1β, IL-6, IL-12, TNFα, IFN-γ, sIL-2R, IL-1RA) have been identified in up to 1/3 of schizophrenia cases, some of which normalise following antipsychotic treatment (Miller, Buckley, Seabolt, Mellor, & Kirkpatrick, 2011; Potvin et al., 2008; van Kammen, McAllister-Sistilli, Kelley, Gurklis, & Yao, 1999). This suggests the involvement of inflammatory processes in active psychotic states. Indeed, numerous antipsychotics have now been shown to have anti-inflammatory effects by reducing circulating levels of inflammatory cytokines in schizophrenia patients, and it is believed that these anti-inflammatory effects may contribute to the effectiveness of these drugs in psychotic symptom reduction (Lu et al., 2004; Tourjman et al., 2013).

Human post-mortem studies have also shown elevated mRNA expression for cytokines IL-1β, IL-6 and IL-8 in the dorsolateral prefrontal cortex (PFC) of schizophrenia cases (Fillman et al., 2012). The increased levels of cytokines is suggestive of an over-active immune state which may be related to altered microglial and astrocyte function. Indeed, increased microglial density has been identified in the PFC, hippocampus, temporal gyrus, anterior cingulate cortex, and dorsal thalamus

(Bayer, Buslei, Havas, & Falkai, 1999; Fillman et al., 2012; Radewicz, Garey, Gentleman, & Reynolds, 2000; Steiner et al., 2008b). Positron Emission Topography (PET) studies have also identified increases in microglial activation in grey matter and the hippocampus of schizophrenia patients within the first five years of symptom onset (Doorduin et al., 2009; van Berckel et al., 2008) as well as in those at ultra-high risk of developing schizophrenia (Bloomfield et al., 2016). Long-term changes to astrocytes have also been demonstrated in schizophrenia cases (Rothermundt, Ahn, & Jorgens, 2009). Increased astrocyte activation and altered astrocyte morphology have both been identified in many cortical regions (cingulate, prefrontal, orbitofrontal, and temporal) in the post mortem brains of schizophrenia cases (Steiner et al., 2008a). Increased glial fibrillary acidic protein (GFAP) mRNA expression was detected in the post mortem PFC of schizophrenia cases who were also identified as having increased inflammatory markers (Catts, Wong, Fillman, Fung, & Shannon Weickert, 2014). In addition, increased levels of the astrocyte marker SB-1000 in the serum and CSF of patients with schizophrenia have been reported (Rothermundt, Ponath, Glaser, Hetzel, & Arolt, 2004; Steiner, Bielau, Bernstein, Bogerts, & Wunderlich, 2006).

This begs the question: Are these long-term changes also seen in rodent models of MIA? Indeed, elevations in circulating levels of cytokines IL-2, IL-6, and TNFα have been observed in the prepubescent and adult offspring of MIA rats, and occur in conjunction with schizophrenia-like behavioural and neurobiological deficits (Romero, Guaza, Castellano, & Borrell, 2010; Samuelsson, Jennische, Hansson, & Holmang, 2006). MIA in mice alters mRNA and protein expression of pro and anti-inflammatory cytokine markers (IL-6, IL-1α, IL-9, IL-10, IFN-γ) in a variety of schizophrenia relevant brain regions (including the hippocampus, frontal cortex, and cingulate cortex) in offspring from the foetal stage through to young adulthood (Garay, Hsiao, Patterson,

& McAllister, 2012; Mattei et al., 2014; Samuelsson et al., 2006). Furthermore, increased microglial density and activated morphology have been reported in the brains of MIA offspring in neonatal (Girard, Tremblay, Lepage, & Sebire, 2010) and adult rat offspring (Van den Eynde et al., 2014) and neonatal (Ratnayake, Quinn, Castillo-Melendez, Dickinson, & Walker, 2012; Roumier et al., 2008) and adolescent mouse offspring (Juckel et al., 2011). In addition, MIA has also been shown to result in increased astrocyte density and activation, as well as elevated GFAP mRNA expression in adolescent to adult rat offspring (de Souza et al., 2015; Samuelsson et al., 2006). However, some studies have failed to find any changes to microglial and astrocyte density or activation status in adult rat and mice offspring following MIA (Garay et al., 2012; Missault et al., 2014; Nyffeler, Meyer, Yee, Feldon, & Knuesel, 2006).The majority of MIA models that investigated postnatal neuroinflammatory markers focused primarily on the foetal to early postnatal stages of development, with few studies examining them in adulthood. Furthermore, most models used a mid-gestational time-point for MIA.

Previous work in the mouse has demonstrated that the gestational timing at which MIA occurs can differentially alter the neurochemical and behavioural phenotype of offspring (Meyer et al., 2006; Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008b). Early gestational day (GD9) versus late gestation day (GD17) MIA by exposure to Poly (I:C) identified a potential interaction between the maternal immune response and the stage of brain development at the time of exposure (Meyer et al., 2008b). Indeed, late MIA produced a phenotype suggestive of the cognitive/negative symptomology whereas early MIA resulted in a phenotype more identical with the positive symptomology of schizophrenia (Meyer & Feldon, 2012; Meyer et al., 2006; Meyer et al., 2008b). Until recently, the differential effects of MIA at early versus late gestational

time-points had only been demonstrated in the C57 strain of mouse (Meyer, 2014). More recently, we established an early (GD10) versus late (GD19) MIA model in the rat that demonstrates some of these differences in the behavioural and neurobiological phenotypes of offspring based on the gestational timing of Poly (I:C) exposure (Duchatel et al., 2016; Meehan et al., 2017).

Currently, there is no evidence to suggest if the differences in behaviour and neurobiology observed in the early versus late MIA models are associated with differences in neuro-immune status. Further understanding of the neuroinflammatory status resulting from MIA at differential gestational time-points may help in elucidating the potential mechanisms involved in mediating the effects of MIA at early (GD10) versus late (GD19) gestation on neurodevelopment. This study aimed to further validate the early versus late MIA model of schizophrenia-like changes in the rat, by exploring the potential long-term neuroinflammatory effects of Poly (I:C) exposure at either GD10 or GD19 on microglial, astrocyte, and cytokine levels in the adult PFC, a brain region strongly implicated in schizophrenia.

5.3. Methods and Materials

5.3.1. Maternal Immune Activation using Poly (I:C) in Rats

The use and monitoring of animals in this project was performed in accordance with the National Health and Medical Research Council's Australian code of practice for the care and use of animals for scientific purpose, with approval from the University of Newcastle Animal Care and Ethics Committee, Newcastle, Australia (Approval numbers A-2009-108 and A-2013-319).

Wistar rats were obtained from the University of Newcastle's Central Animal House at 8 weeks of age and acclimated for 2 weeks before daily monitoring of oestrous

cycle using an impedance probe. Food and water was available *ad libitum*. On the day of proestrous, females were mated overnight with male rats. Day of conception was identified via the presence of sperm in vaginal smears taken the following morning, the day of positive sperm detection was identified as GD0. Pregnant dams were randomly allocated to a treatment group (Poly (I:C) or saline) and on the appropriate gestational day (10 or 19) dams were anaesthetised with isoflurane (induction 5%, maintenance 2.5-3% - Abbott Australasia Pty Ltd – Botany - Australia) and administered with either 4.0 mg/kg of Poly (I:C) (Sigma-Aldrich – Sydney – AUS) or phosphate-buffered-saline (PBS) via lateral tail vein injection (at 1 mL/kg body weight).

5.3.2. Confirmation of Immune Activation

To confirm successful immune activation, pregnant dams underwent saphenous vein puncture 2 h following prenatal treatment with blood samples collected in EDTA-coated tubes (Thermo Fischer Scientific – Richlands - AUS). Blood was centrifuged at 1000 xG for 15 min at 4°C with resulting plasma being stored at -20°C until assayed. An ELISA kit (Rat IL-6 Quantikine ELISA, R&D Systems – Minneapolis - USA) was used to measure circulating levels of the pro-inflammatory cytokine IL-6 to confirm MIA according to the manufacturer's instructions. Circulating IL-6 was significantly increased in all dams receiving Poly (I:C) but not dams that received PBS.

5.3.3. Animals and Tissue Collection for Gene Expression Analysis

Expression of Iba1, Gfap, TNF-α and IL-1β, mRNA levels in the mPFC were determined by real-time quantitative polymerase chain reaction (qPCR). A total of 24 male and 24 female rats were included with $n = 6$ per sex from each of the four experimental groups; GD10 Saline, GD10 Poly (I:C), GD19 Saline, GD19 Poly (I:C).

Between PND70 and PND84, rats were anesthetised via intraperitoneal injection of Lethabarb (2 mL/kg i.p.; Virbac, Pty. Ltd, Milperra, Australia) and transcardially perfused with 300ml of PBS (Sigma Aldrich) prior to decapitation and removal of the brain. mPFC samples were obtained using the methods previously described by Ong et al. (2014). Once removed brains were placed immediately into ice cold buffer for 5 min. Brains were then placed ventral side up in a brain matrix and a series of 5 coronal sections were made, from which subsequent dissection of the mPFC (including the infralimbic, prelimbic, and cingulate cortex; bregma +2.5 – +3.5) was made using a scalpel blade. Brain samples from each hemisphere were collected in 1.5mL microtubes kept on dry ice before being stored at -80^{o}C. Only samples from one hemisphere were used for qPCR analysis, left and right hemispheres were counterbalanced across the 4 treatment groups and sex. Tissue samples were thawed and homogenized with TissueLyzer® (Qiagen – Chadstone, Australia; 4 min at 20 Hz) in a RNAse-free microtube containing 1 ml of QIAzol® Lysis Reagent (Qiagen) and a 5mm diameter stainless steel bead (Qiagen). Total RNA was then extracted using the RNeasy® Mini Kit (Qiagen) and DNase I treated (Invitrogen – California – USA) per manufacturer's instructions. RNA was quantified using the NanoDrop Pearl (Implen – California – USA) and stored at -80^{o}C.

5.3.4. Quantitative Real Time Polymerase Chain Reaction Analysis

qPCR was used to examine MIA related gene expression changes. RNA was reverse transcribed using a Superscript III Reverse Transcription kit (Life Technologies – California – USA), as per manufacturer's instructions. A reverse transcriptase enzyme – negative control reaction was also performed for each sample. The cDNA was diluted in nuclease free water and stored at -20^{o}C. qPCR primers were designed using Primer

Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and are listed in Table 5.1. For each animal, qPCR reactions were performed in triplicate for each gene using 1x SYBR Green Select Master Mix (Thermo Fisher Scientific), 200 nM of each forward and reverse primer, and 5 µl of cDNA sample, in a total volume of 12 µl per reaction. Amplification reactions for Gfap and Iba1 were performed at 0.2 ng/µl cDNA concentration, and due to low expression of cytokines within the brain, 20 ng/µl for TNF-α and IL-1β. qPCR amplification was performed using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems – California – USA), with an initial denaturation and activation step at 95ºC for 10 min, followed by 40 cycles of 95ºC for 10 s and 60ºC for 30 s. Melt curves were generated to confirm amplification of a single gene product. The average threshold cycle (Ct) value was calculated using the 7500 SDS software v2.0.6 (Applied Biosystems) for each gene. The relative expression of each gene was determined using the delta-delta Ct method (Schmittgen & Livak, 2008) with the geometric mean of *18s-rRNA*, Actb, Gapdh and Tubb3 used as the internal reference for Iba1 and Gfap. Due to the very high concentration of cDNA required to examine TNF-α and IL-1β, only Actb and Gapdh were used as the internal reference for these genes; 18srRNA was no longer acceptable due to a product arising too early in the non-linear phase.

Table 5.1. *Primer sequences for qPCR*

Gene	Forward Primer	Reverse Primer
Actb	CCTAGCACCATGAAGATCAAGA	GCCAGGATAGAGCCACCAATC
18s-rRNA	CCCGAAGCGTTTACTTTGAA	CCCTCTTAATCATGGCCTCA
Gapdh	GGCTGGCATTCGTCTCAA	GAGGTCCACCACCCTGTTG
Tubb	CTTCCGACTCCTCGTCGTCA	GAGGCCGAGAGCAACATGAA
Iba1	CTAAGGCCACCAGCGTCTGA	AGCTTTTCCTCCCTGCAAATCC
Gfap	GCGGGATGGCGAGGTCATTA	TGGGCACACCTCACATCACA
TNF-α	GGCCCAGACCCTCACACTCA	CCGCTTGGTGGTTTGCTACGA
IL-1β	TGAAAGACGGCACACCCACCC	TTGTTTGGGATCCACACTCTCCAGC

5.3.5. Immunohistochemical Analysis of Microglia and Astrocytes

Brain sections used for immunohistochemistry are from the same cohort of rats previously used to investigate white matter neuron density following MIA (Duchatel et al., 2016), and separate to the cohort used in the above mentioned qPCR analysis. Methods for perfusion, brain collection, sectioning and immunohistochemical labelling are essentially as described in Duchatel et al. (2016) and briefly stated here. At 12 weeks of age (PND84), male and female offspring from MIA induced dams were deeply anaesthetised and then transcardially perfused with 0.1M PBS followed by 4% paraformaldehyde (Sigma-Aldrich) in 0.1M PBS. Brains were collected and stored in PBS + 0.1% sodium azide (Sigma-Aldrich).

For diaminobenzidine immunohistochemistry, 30 µm rat brain sections were pre-treated as described (Duchatel et al., 2016) then incubated with primary antibodies either rabbit anti-Ionized calcium binding adaptor molecule 1 (IBA1) (1:1000, Wako Chemicals, Virginia - USA; Cat. #019-19741) for microglia or rabbit anti-GFAP (1:500, Sapphire Bioscience, Redfern, AUS; Cat. LS-C7112#) for astrocytes, for 48 h at 4^{o}C. Sections were washed then incubated in donkey anti-rabbit IgG biotinylated secondary antibody (1:1000, Jackson ImmunoResearch, Cat. #711-065-152) for 1 h at RT. After

washing, sections were incubated at RT in the avidin–biotin–peroxidase complex (Vectastain ABC kit; Vector Laboratories, USA) for 1 h, and treated with 3,3'–diaminobenzidine (Sigma-Aldrich; 12 mmol/L final concentration in PBS with 0.03% H2O2) for 5–7 min on ice. Sections were mounted on gelatin-subbed microscope slides, dried overnight, defatted in ascending alcohols, counterstained with cresyl violet (VWR, Pennsylvania, USA), differentiated in ascending alcohols, cleared using Xylene and cover-slipped using Ultramount (Thermo Fisher Scientific). Sections from control and experimental animals were equally distributed across plates and processed at the same time to control for any variability in the immunohistochemistry process.

5.3.6. Quantification of Microglia and Astrocytes

Investigators were blind to the treatment status (i.e. MIA or vehicle) throughout experimental, analysis and quantification steps. Brain sections containing both the forebrain cortex and the white matter of the corpus callosum were pooled into two regions, region 1; 3.2 mm – 2.5 mm from Bregma and region 2; 2.3 mm – 0.7 mm from Bregma. This delineation is based on the anatomical difference in the white matter of the corpus callosum between these regions, with region 2 starting where the genu of the corpus callosum becomes visible and joins both hemispheres of the cortex. Four sections sampled 180 µm apart in region 1 and region 2 were used to measure the DAB labelling for IBA1+ microglia and GFAP+ astrocytes within both the forebrain cortex and the white matter. Images of sections (eight images per animal over four sections) immunolabelled for IBA1+ microglia and GFAP+ astrocytes using diamoinobenzidine immunohistochemistry were captured at 20x magnification using an Olympus DP72 microscope (Olympus – Notting Hill – AUS). The rat forebrain cortex and white matter brain regions were identified by comparing the Nissl staining pattern to sections at an

equivalent level in the Rat Brain in Stereotaxic Co-ordinates Atlas (Paxinos & Watson, 2006). The level of IBA1+ microglia and GFAP+ astrocyte DAB labelling was determined by cumulative threshold analysis as previously described by Johnson and Walker (2015), Jones et al. (2015), Ong et al. (2016), and Patience et al. (2015). Matlab software R2015a was used to analyse the cumulative pixel threshold of each image, which calculates the number of pixels occurring at a given pixel intensity (0-255), as a percentage of the overall number of pixels present within a given image. Group comparisons were then determined using ImageJ software to visualise thresholding of regions at individual pixel intensities (PI) related to the immunohistochemical labelling patterns of IBA1 and GFAP. Data for group comparisons was obtained by selecting a pixel intensity, based on the control group, which included all immunolabelling but no background. Supplementary Figures 5.1 and 5.2 show the cumulative threshold spectrum, which reflects the cumulative number of pixels that occur at or below each of the 256 pixel intensities. The cumulative threshold spectra operates in a manner very similar to a standard single point threshold analysis except it provides information on all potential points at which an image could be thresholded and therefore provides a much more comprehensive view of between group differences. The PI that detected genuine IBA1+ immunoreactive material were: PFC region 1 = PI120; PFC region 2 = PI90; white matter region 1 = PI120; white matter region 2 = PI125. The PI that detected genuine GFAP+ immunoreactive material were: PFC region 1 = PI120; PFC region 2 = PI120; white matter region 1 = PI120; white matter region 2 = PI1120. The number of pixels that were captured at and below each pixel intensity were expressed as a percentage of the total number of pixels in each image and these data were used to investigate between group differences.

5.3.7. Statistical Analysis

Changes in gene expression (qPCR) between MIA and control groups at GD10 and GD19 were assessed using two-tailed students t-tests on the normalised delta-Ct values for each gene of interest. All graphs are presented as increase or decrease in fold change with the standard error of the mean (SEM). Initial analyses showed there was no significant effect of sex on gene expression at GD10 or GD19 so all data was pooled for further analyses.

Immunohistochemistry data is expressed as a percentage change relative to the average of the control group ± standard deviation (SD), and were analysed using GraphPad Prism 6 software (GraphPad, California, USA). Based on previous studies of this animal cohort (Duchatel et al., 2016), showing no difference between GD10 and GD19 controls in interstitial white matter neuron density, these groups were pooled to form a single control group. One-way Analysis of Variance (ANOVA) with 3 levels of a group factor (GD10, GD19 and controls) and Bonferroni multiple comparisons were used to determine whether there was any significant difference between controls and offspring from MIA affected dams at either GD10 or GD19. Initial analyses showed there was no significant effect of sex on the levels of IBA1+ or GFAP+ immunoreactive cells after MIA and since the numbers per sex were low in some groups, data from males and females were pooled for further analyses.

5.4. Results

5.4.1. Effects of MIA on Gene Expression.

We examined Iba1 and Gfap gene expression in the mPFC in a cohort of animals from offspring of dams exposed to Poly (I:C) at either GD10 (n = 12) or GD19 (n = 12) hereafter referred to as Poly (I:C) rats and controls (n = 12 GD10 controls, n =

12 GD19 controls) (Figure 5.1A). No changes in Iba1 or Gfap gene expression were observed in either GD10 (Iba1: $t_{(22)} = 0.0936$, $p = 0.92$ or Gfap: $t_{(22)} = 0.99$, $p = 0.33$) or GD19 Poly (I:C) rats (Iba1: $t_{(22)} = 0.225$, $p = 0.82$ or Gfap: $t_{(22)} = 1.6$, $p = 0.12$) compared to their GD-specific control groups (Figure 5.1A).

Studies of schizophrenia have identified changes in genes relating to the immune response in human post-mortem brain (Fillman et al., 2012; Fillman, Sinclair, Fung, Webster, & Shannon Weickert, 2014). In the absence of a change in Iba1 and Gfap gene expression in the mPFC, we examined whether prenatal exposure to Poly (I:C) affected the gene expression of cytokines in the mPFC. We observed no difference in the expression of TNF-α or IL-1β in either GD10 (TNF-α: $t_{(22)} = 0.458$, $p = 0.65$ or IL-1β: $t_{(22)} = 0.659$, $p = 0.656$) or GD19 Poly (I:C) rats (TNF-α: $t_{(22)} = 0.679$, $p = 0.504$ or IL-1β: $t_{(22)} = 0.6725$, $p = 0.508$) compared to their GD-specific control groups (Figure 5.1B).

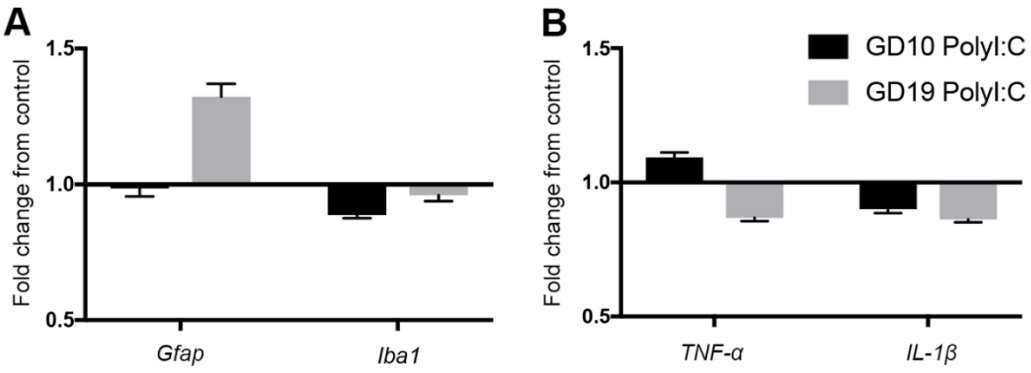


Figure 5.1. Effects of maternal immune activation on immune related gene expression. Relative gene expression for Gfap, Iba1 (A), TNF-α and IL-1β (B) in the medial prefrontal cortex (mPFC) of offspring from dams exposed to Poly (I:C) at either GD10 (black bars) or GD19 (grey bars). No significant changes were observed in the expression of Gfap, Iba1, TNF-α or IL-1β in Poly (I:C) rats compared to controls. Bars represent mean fold change from control ±SEM.

5.4.2. Effects of MIA on IBA1+ Immunoreactive Microglia in Offspring.

Since there was no change in gene expression, we next investigated the effects of MIA on the density of microglia in offspring similar to what has been observed previously (Van den Eynde et al., 2014). IBA1+ immunoreactive microglia were identified in abundance in the mPFC (Figure 5.2A) with fewer in the white matter of the corpus callosum (Figure 5.2B) in offspring of controls and dams exposed to Poly (I:C) at GD10 and GD19. Using one-way ANOVA we observed no alterations in the level of IBA1+ immunoreactive material in the mPFC in either region 1 [$F_{(2, 32)} = 2.269$, $p = 0.119$ – Figure 5.3A, B and E], or region 2 [$F_{(2, 39)} = 1.09$, $p = 0.345$ – Figure 5.3C, D and F]. However, in the white matter of the corpus callosum, one-way ANOVA showed a significant group difference in the level of IBA1+ immunoreactive material in region 1 [$F_{(2, 40)} = 4.619$, $p = 0.016$ – Figure 5.4A, B and E]. Bonferroni multiple comparisons showed the level of IBA1+ immunoreactive material in the region 1 white matter (Figure 5.4E) was significantly increased by ~46% in GD19 Poly (I:C) rats (2.065±1.26% of IBA1+ immunoreactive material, $p = 0.017$), but not GD10 Poly (I:C) rats (1.22±1.02% of IBA1+ immunoreactive material, $p = 0.99$) compared to control rats (1.099±0.584% of IBA1+ immunoreactive material). We did not observe any group differences in IBA1+ immunoreactive material in region 2 white matter [$F_{(2, 43)} = 0.8448$, $p = 0.4366$ – Figure 5.4C, D and F].

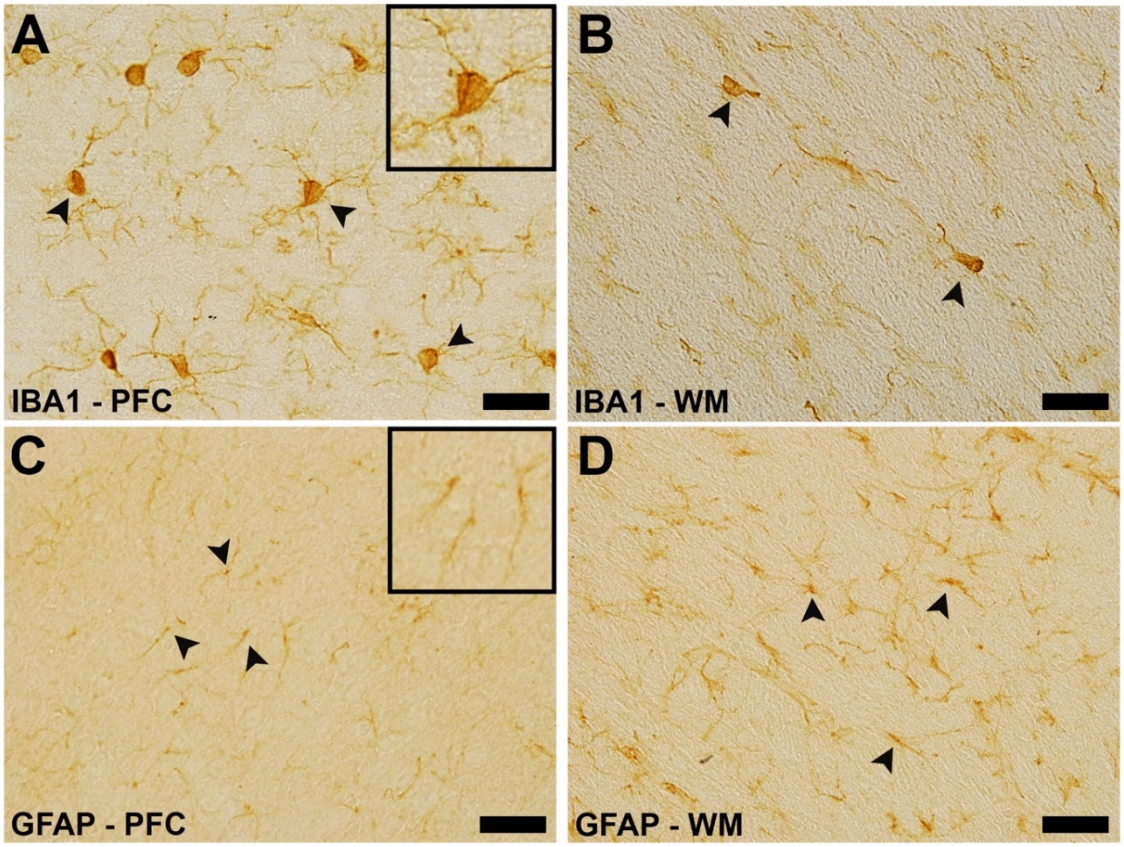


Figure 5.2. Representative images of IBA1+ immunoreactive microglia and GFAP+ immunoreactive astrocytes in the rat brain. IBA1+ immunoreactive microglia in the prefrontal cortex (PFC - A) and white matter (WM) of the corpus callosum (B) of control rats. GFAP+ immunoreactive astrocytes in the PFC (C) and white matter of the corpus callosum (D) in the rat brain of control rats. Scale bar: 25 µm

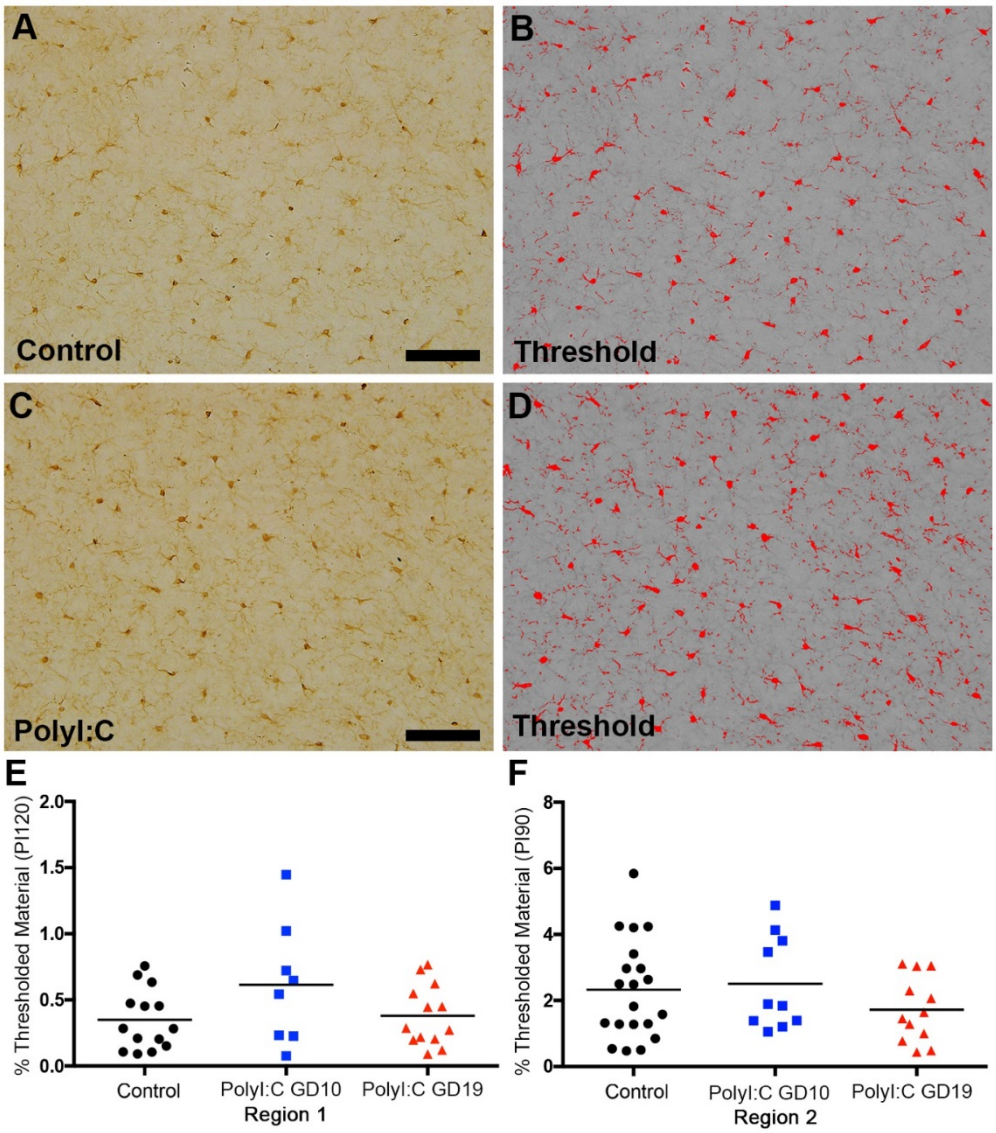


Figure 5.3. Effect of maternal immune activation on IBA1+ immunoreactive microglia in the medial prefrontal cortex (mPFC). Representative images of IBA1+ immunoreactive microglia in the PFC from region 1 of the rat brain in either controls (A) or offspring from animals exposed to Poly (I:C) at GD19 (C), with the corresponding images showing IBA1+ thresholded material at pixel intensity 120 (B,D). (E-F) The percentage of IBA1+ immunoreactive material is shown at a given pixel intensity for offspring exposed to Poly (I:C) at GD10 (blue squares) or GD19 (red triangles), as well as controls (black circles). Each data point represents the mean % of IBA1+ immunoreactive material for one animal. Comparisons are presented for two rostrocaudally adjacent areas, region 1 (E) and region 2 (F). The percentage of IBA1+ immunoreactive material was not significantly different between control and Poly (I:C) rats in the PFC of region 1 and region 2. Scale bar: 100 µm

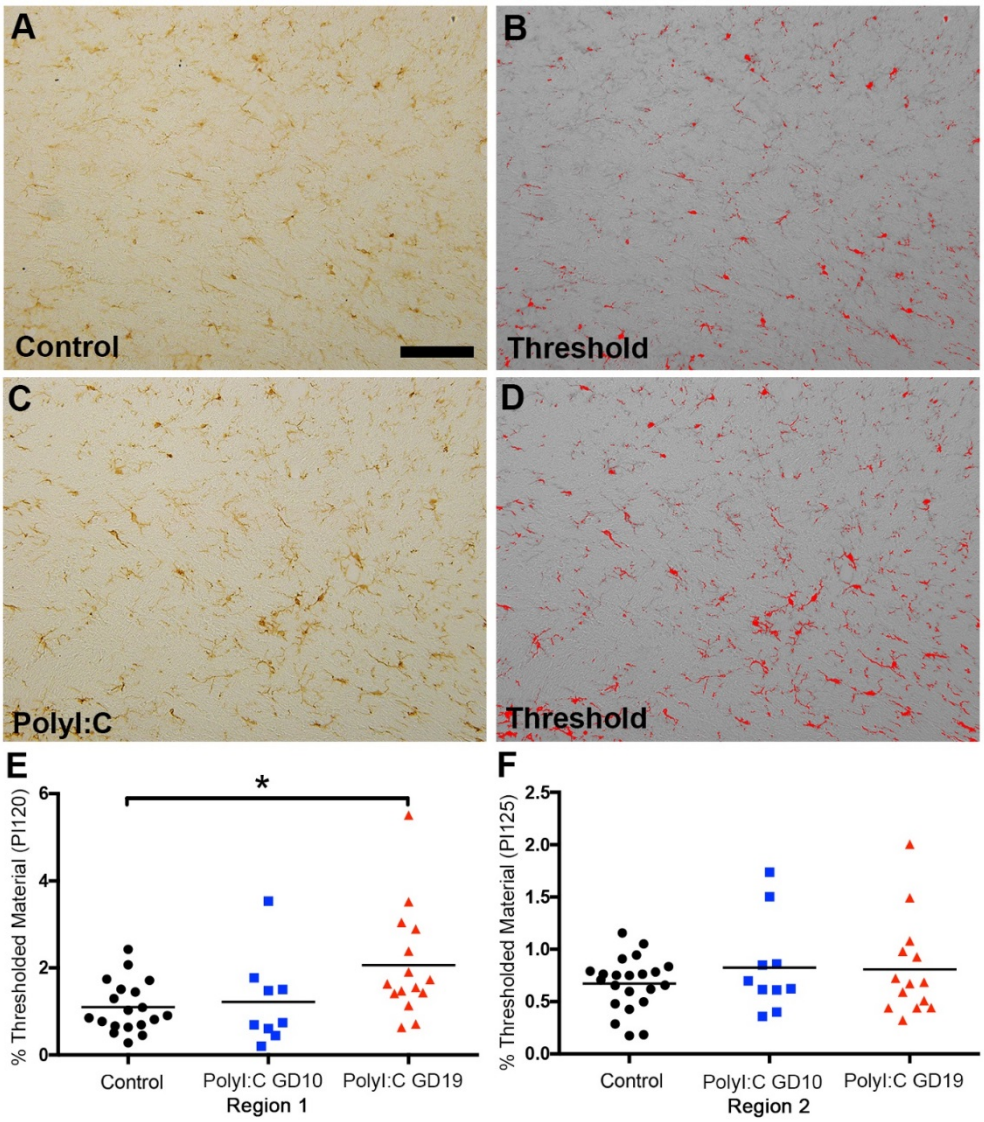


Figure 5.4. Effect of maternal immune activation on IBA1+ immunoreactive microglia in the white matter of the corpus callosum. Representative images of IBA1+ immunoreactive microglia in the white matter of the corpus callosum in region 1 of the rat brain in either controls (A) or offspring from animals exposed to Poly (I:C) at GD19 (C), with the corresponding images showing IBA1+ thresholded material at pixel intensity 120 (B,D). The percentage of IBA1+ immunoreactive material is shown at a given pixel intensity in two rostrocaudally adjacent areas region 1 (E) and region 2 (F) for offspring exposed to Poly (I:C) at GD10 (blue squares) or GD19 (red triangles), as well as controls (black circles). Each data point represents the mean % of IBA1+ immunoreactive material for one animal. The percentage of IBA1+ immunoreactive material in the white matter was significantly increased after MIA at GD19 in region 1 (p = 0.016). No difference was observed after MIA at GD10 nor in region 2 in both groups. Scale bar: 100 µm

5.4.3. Effects of MIA on GFAP+ Astrocytes in Offspring.

GFAP+ immunoreactive astrocytes were identified in the mPFC (Figure 5.2C) but were more abundant in the white matter of the corpus callosum (Figure 5.2D) in GD10 and GD19 Poly (I:C) rats as well as control rats. Interestingly, one-way ANOVA identified a significant group effect of MIA on GFAP+ immunoreactive material in region 1 of the mPFC ($F_{(2, 40)} = 2.08$, $p = 0.045$ – Figure 5.5A, B and E, F). Whilst Bonferroni multiple comparisons showed there was no change in GFAP+ immunoreactivity in GD10 Poly (I:C) rats (0.17±0.16% of GFAP+ immunoreactive material, $p = 0.99$), there was a strong trend towards increased GFAP+ immunoreactivity in GD19 Poly (I:C) rats (0.35±0.33% of GFAP+ immunoreactive material, $p = 0.054$) compared to controls. No effect of MIA on GFAP+ immunoreactivity was observed in the mPFC region 2 [$F_{(2, 43)} = 0.43$, $p = 0.6531$ – Figure 5.5C, D and F]. In contrast to the microglia data, overall we did not observe any alterations in the level of GFAP+ immunoreactive material in the white matter of the corpus callosum as a result of MIA [region 1: $F_{(2, 43)} = 1.739$, $p = 0.1879$ – Figure 5.6E; region 2: $F_{(2, 43)} = 1.183$, $p = 0.3161$ – Figure 5.6F].

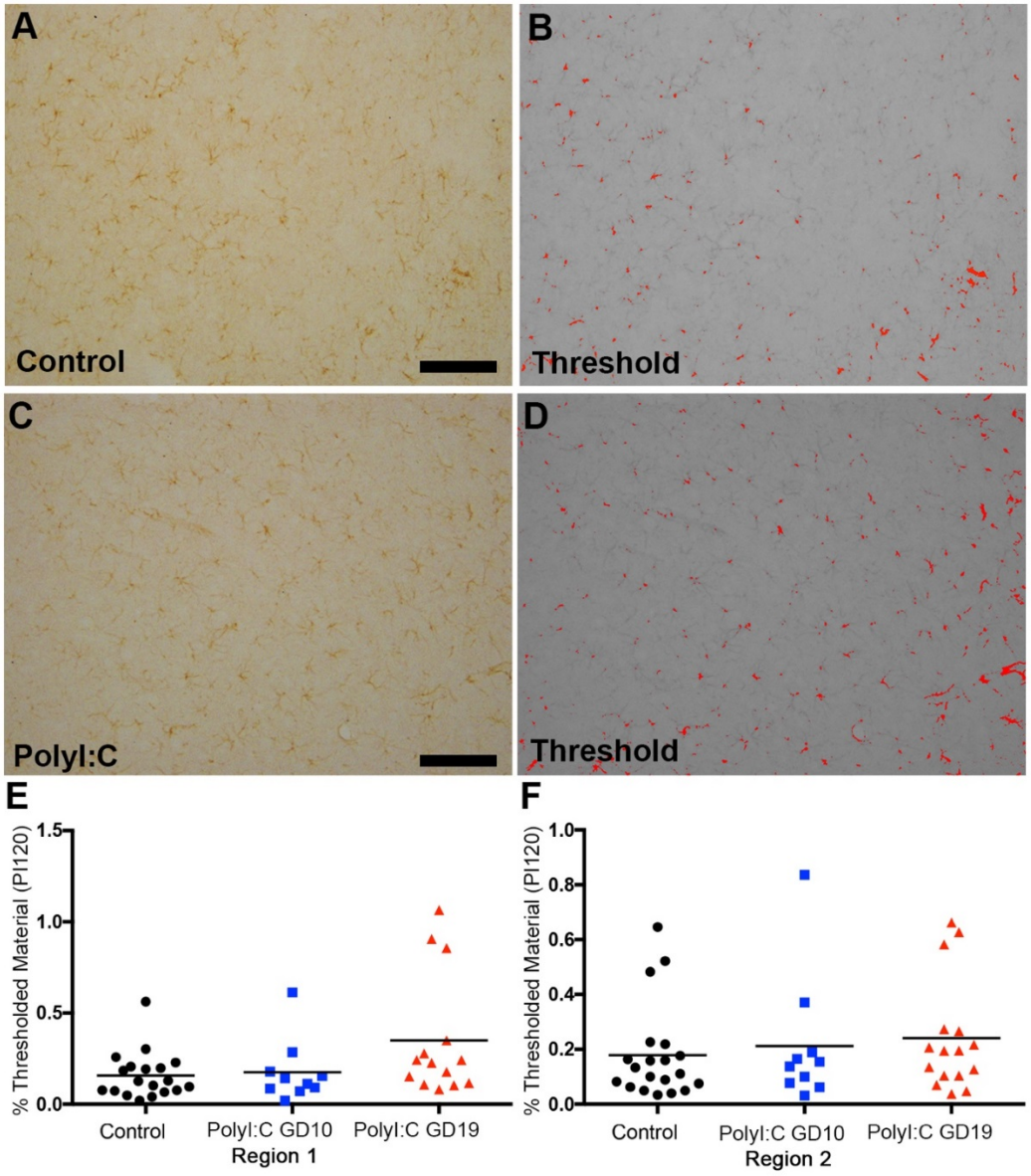


Figure 5.5. Effect of maternal immune activation on GFAP+ immunoreactive astrocytes in the medial prefrontal cortex (mPFC). Representative images of GFAP+ immunoreactive astrocytes in the rat brain mPFC in region 1 of either controls (A) or offspring from animals exposed to Poly (I:C) at GD19 (C), with the corresponding images showing GFAP+ thresholded material at pixel intensity 120 (B,D). (E-F) The percentage of GFAP+ immunoreactive material is shown at a given pixel intensity for offspring exposed to Poly (I:C) at GD10 (blue squares) or GD19 (red triangles, as well as controls (black circles). Each data point represents the mean % of GFAP+ immunoreactive material for one animal. Comparisons are presented for two rostrocaudally adjacent areas, region 1 (E) and region 2 (F). The percentage of GFAP+ immunoreactive material was not significantly different between control and Poly (I:C) rats in either region. Scale bar: 100µm

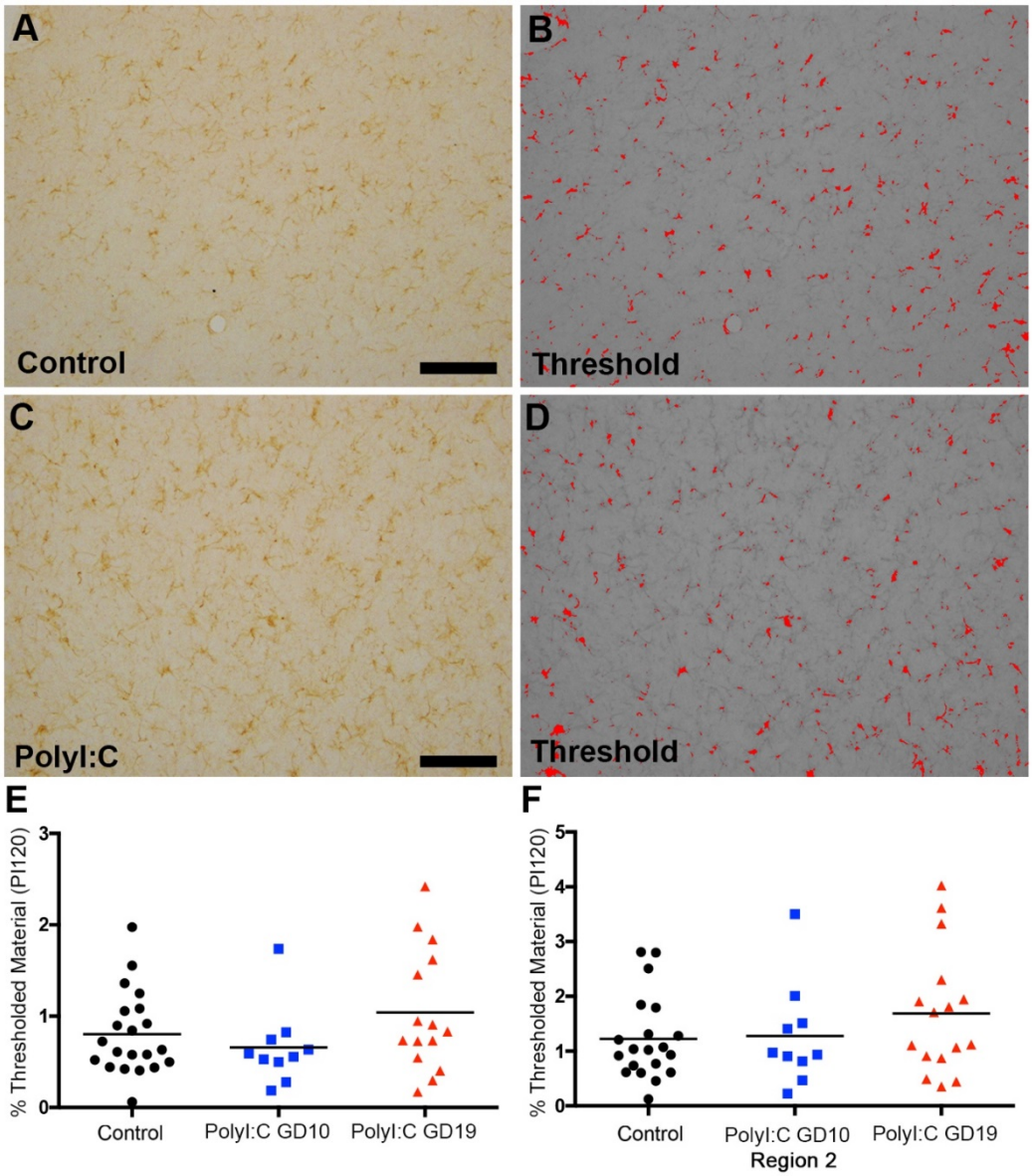


Figure 5.6. Effect of maternal immune activation on GFAP+ immunoreactive astrocytes in the white matter of the corpus callosum. Representative images of GFAP+ immunoreactive microglia in the white matter of the corpus callosum in region 1 of the rat brain in either controls (A) or offspring from animals exposed to Poly (I:C) (C), with the corresponding images showing GFAP+ thresholded material at pixel intensity 120 (B,D). (E-F) The percentage of GFAP+ immunoreactive material at a given pixel intensity for offspring exposed to Poly (I:C) at GD10 (blue squares) or GD19 (red triangles), as well as controls (black circles). Each data point represents the mean % of GFAP+ immunoreactive material for one animal. Comparisons are presented for two rostrocaudally adjacent areas, region 1 (E) and region 2 (F). The percentage of GFAP+ immunoreactive material was similar between control and Poly (I:C) rats in both regions. Scale bar: 100µm

5.4.4. Correlation of IBA1+ Microglia and White Matter Neuron Density.

We previously reported an increase in the density of interstitial white matter neurons (IWMNs) expressing neuronal nuclear antigen (NeuN) and somatostatin (SST) after MIA at GD10 but more prominently in GD19 within the same cohort of animals (Duchatel et al., 2016) used to examine IBA1+ microglia in this current study. Therefore, we examined whether the percentage of IBA1+ immunoreactive material in the white matter of region 1, correlated with the SST+ or NeuN+ IWMN density (Figure 5.7) as previously determined in our publication (Duchatel et al., 2016). We observed a statistically significant negative correlation between the percentage of IBA1+ immunoreactive material (PI120) and SST+ IWMNs (Figure 5.7A) in GD19 Poly (I:C) rats (r = -0.532, p = 0.041). No correlation was observed in GD10 Poly (I:C) rats (r = -0.296, p = 0.439), or control rats (r = -0.1216, p = 0.62). There was no correlation between the amount of IBA1+ immunoreactive material and NeuN+ IWMN density (Figure 5.7B) in either GD19 Poly (I:C) rats (r = -0.3246, p = 0.239), GD10 Poly (I:C) rats (r = 0.2, p = 0.606), or control rats (r = 0.362, p = 0.127).

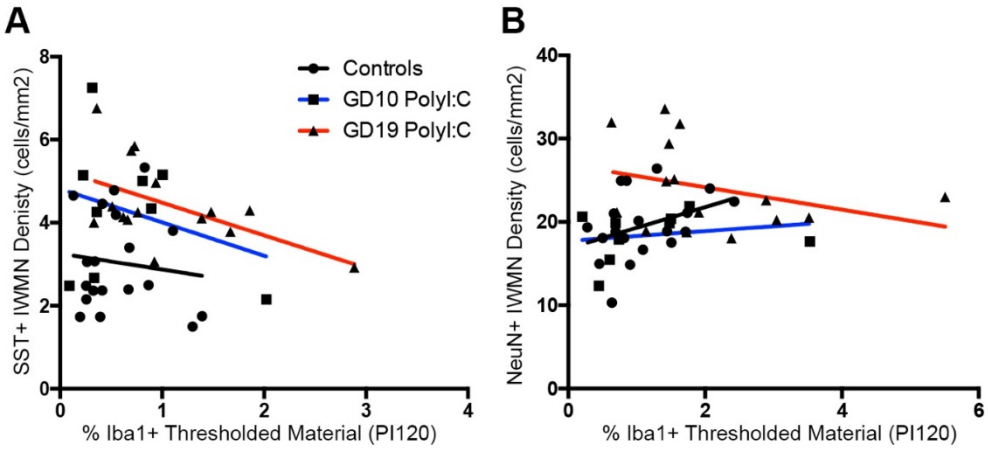


Figure 5.7. Correlation between IBA1+ immunoreactive microglia and neuronal density in the white matter. The percentage of IBA1+ immunoreactive material negatively

correlated with SST+ IWMN density (A) in offspring from rats exposed to MIA at GD19 (Triangles – Red line – r = -0.532, p = 0.0412), but not GD10 MIA animals (Squares – Blue line) or controls (Circles – Black line). No correlation was observed between the percentage of IBA1+ immunoreactive material and NeuN+ IWMNs (B) in either GD10 (Squares – Blue line) or GD19 (Triangles – Red line) Poly (I:C) rats or control rats (Circles – Black line). SST+ and NeuN+ IWMN density data was previously reported in Duchatel et al. (2016).

5.5. Discussion

In the current study, we examined the effects of MIA, a risk factor for schizophrenia, on the expression of immune related cytokine genes and the density of microglia and astrocytes. The findings indicate that prenatal exposure to MIA results in subtle neuro-immune changes in adulthood, specifically in microglial density. Previous work from our group has demonstrated that rats exposed to Poly (I:C) on either GD10 or GD19 exhibit behavioural changes in adulthood, namely sensorimotor gating deficits (in male but not female rats) and working memory impairments (in GD19 but not GD10 MIA rats), in addition to altered dopamine 1 receptor expression in GD10 MIA males (Meehan et al., 2017). Furthermore, previous work from our group has demonstrated that MIA-exposed adult rats have increased density of interstitial white matter neurons, particularly those that express SST (Duchatel et al., 2016). This effect is similar to the increase in interstitial white matter interneuron density observed in post-mortem brains from schizophrenia cases (for review see, Duchatel et al., 2016). The current findings add to the previous work from our group and further validates the early (GD10) verses late (GD19) rat model of MIA in producing schizophrenia-like phenotypes, albeit, subtle ones.

5.5.1. Cytokine Gene Expression is unaltered by MIA.

Under typical conditions, cytokines are produced by a number of different cell types in response to infection, including peripheral immunocompetent cells, glial cells and neurons (Woodroofe, 1995). A number of studies have investigated changes in the expression of cytokines in patients with schizophrenia both in the brain and periphery. These studies report increased levels of IL-6, TNF-α and IL-1β (Fillman et al., 2012; Fillman et al., 2014; Kim & Maes, 2003). However, while studies in patients with schizophrenia have provided strong evidence linking cytokine alterations to schizophrenia, it has been difficult to replicate these findings within animal models of schizophrenia risk factors. Indeed, in the current study we examined the expression of IL-1β and TNF-α in the mPFC from both GD10 and GD19 MIA offspring, but did not find any significant changes in mRNA levels.

These results are in line with those of Willi, Harmeier, Giovanoli, and Meyer (2013) who demonstrated that early gestation (GD9) MIA in mice was not sufficient to produce alterations in IL-1β, TNF-α or IL-6 in the mPFC of adult animals (PND85 – 100). Garay et al. (2012) did detect alterations in a range of cytokines at the protein level in the frontal cortex (FC) and cingulate cortex (CC) of mice exposed to mid-gestational (GD 12.5) Poly(I:C), these changes were dependent on the age of the offspring at assessment. With regards to IL-1β and TNF-α, Garay et al. (2012) found that in the frontal regions (FC and CC) of MIA animals, IL-1β protein levels were elevated at birth (FC only), normalised at PND7 (FC and CC), reduced at PND14 and 30 (FC and CC), and normalised again at PND60 (FC and CC). No significant alterations in TNF-α were identified, although some trends were apparent (Garay et al., 2012). Although the results of Garay et al. (2012) demonstrate many cytokine changes in the frontal brain regions of young adult offspring (IL-1α, IL-6, IL-9, IL-10, IFNγ),

the two cytokines examined in the current study were found to be unchanged at this stage of development. Based on these observations it seems the expression of cytokines is highly age dependent and it is entirely possible that the adult stage of development may not be the most sensitive period for detecting changes in these particular cytokines. It should be noted that cytokine and chemokine expression may also be region dependent. As the current investigation assessed only one of many schizophrenia relevant brain regions, it is possible that cytokine alterations may be present in other regions not examined here. Indeed, previous work from a mid-gestational (GD15) rat MIA model has reported increased microglial derived IL-1β, but not TNF-α, in the hippocampal region of adult offspring, with these changes being reversed by the tetracycline antibiotic minocycline (Missault et al., 2014). In addition, increased *hippocampal IL-6* levels were also reported in older rats (PND168) exposed to IL-6 on multiple days in late gestation (GD16, 18 & 20) but not early gestation (GD8, 10 & 12) (Samuelsson et al., 2006). Future studies using the current model should aim to assess a wider range of pro and anti-inflammatory chemokines and cytokines, at multiple postnatal time-points, and in multiple schizophrenia-relevant brain regions in an effort to further establish the developmental nature of any central cytokine alterations resulting from MIA.

5.5.2. GFAP Immunoreactivity but not mRNA is Altered by MIA.

As long-term neuroinflammatory alterations are associated with schizophrenia, and have been identified as a potential mediating mechanisms between MIA and schizophrenia-related neurodevelopmental disruption (Na, Jung, & Kim, 2014), the current study assessed astrocytes and microglia, the two major immune cells of the CNS involved in neuroinflammatory processes. Immunohistochemistry techniques were used

to assess the density of astrocytes within the mPFC and frontal corpus callosum, areas of the brain strongly implicated in schizophrenia (Lewis & Levitt, 2002; Pomarol-Clotet et al., 2010; Salgado-Pineda et al., 2007; Wible et al., 2001). We observed subtle effects of MIA on GFAP+ immunoreactivity, but not mRNA, in the mPFC specific to region 1, with pairwise comparisons showing an increase in GFAP+ immunoreactivity which approached significance in GD19 but not GD10 MIA animals. No alterations in GFAP+ immunoreactivity or mRNA expression were observed in the frontal white matter at either gestational time-point or region.

These results are somewhat contradictory to those of de Souza et al. (2015) who reported that LPS exposure on consecutive days in late gestation (GD18 & 19) resulted in increased GFAP levels in the PFC in juvenile (PND30) but not young adult (PND60) rats. However, this study also revealed increased levels of the astrocyte marker S100B in the PFC and hippocampus of both juvenile (PND30) and young adult (PND60) rats (de Souza et al., 2015), indicating that GFAP levels, but not S100B, may normalise throughout adolescence. This reported change in GFAP markers throughout postnatal development suggests the adult time-point (PND70 - 84) assessed in the current study may not be ideal for identifying such alterations, and could explain why the increase in GFAP density only approached significance in our GD19 group ($p = 0.054$). Perhaps assessment of GFAP markers at an earlier postnatal stage, as previously mentioned in regards to the cytokine mRNA data, may demonstrate a stronger effect. However, at least one other MIA model has demonstrated GFAP alterations in adult animals. A study by Samuelsson et al. (2006) showed that prenatal exposure to the pro-inflammatory cytokine IL-6 at either early (GD8, 10 & 12) or late gestation (GD16, 18 & 20) increased GFAP mRNA expression in adult rats (PND168). It is possible that the extended period of MIA over a 5 day period used in that study produced more

pronounced deficits than the single day of exposure used in the current study. This could account for why Samuelsson and colleagues found increased GFAP mRNA expression in adult animals and also found a difference in their earlier gestational MIA group. This notion is in line with the results of Nyffeler et al. (2006) who failed to find any GFAP changes in adult (PND180>) mice following a single dose of Poly (I:C) in early gestation (GD9).

5.5.3. IBA1 Immunoreactivity but not mRNA is Altered by MIA.

In addition to astrocytes, the current study also examined the effects of MIA at early or late gestation on the other major immune cells of the CNS, microglia, within the mPFC and frontal white matter regions. We observed that MIA in late gestation (GD19), but not early gestation (GD10), was associated with increased microglial density, but not mRNA expression, in frontal white matter as assessed by quantification of IBA1. In contrast, no change in Iba1 mRNA expression nor IBA1+ immunoreactivity was observed in the cortex. These findings suggest that late gestation MIA is associated with subtle long-term neuroinflammatory alterations to the frontal white matter.

Similar increases in microglial density have previously been reported in the corpus callosum of adult rats following Poly (I:C) exposure at mid-gestation (GD15) (Van den Eynde et al., 2014), and in frontal white matter of neonatal rats (PND9) following late gestation (GD17 through to birth) LPS exposure (Girard et al., 2010). It has previously been reported that Poly (I:C) exposure at early gestation (GD9) in the mouse did not produce any microglial changes in the PFC of juvenile (PND30) offspring (Juckel et al., 2011), nor in the PFC and striatum of adult offspring (Giovanoli et al., 2016; Willi et al., 2013). Thus, the lack of any microglial changes in our early gestation group or in the frontal grey matter is consistent with these findings, and taken

together is suggestive of mid to late gestation being a more sensitive period than early gestation with regards to microglial changes. However, drawing conclusions on the effects of gestational timing across multiple studies is difficult due to the varying inconsistencies in methodologies used between studies (species, age of assessment, and microglial marker). Indeed, the varying methods used may account for the inconsistency in the MIA rodent literature, with a number of studies also failing to find any microglial alterations in the PFC following mid-gestational MIA. Mid-gestational (GD12.5) Poly (I:C) in mice did not produce any changes in microglial density in the PFC, cingulate, or hippocampus at any postnatal time-point examined (PND0, 7, 14, 30, and 60) (Garay et al., 2012). Furthermore, mid-gestational (GD15) Poly (I:C) exposure in rats also failed to yield microglial alterations in the mPFC of adult offspring (PND126 – 133) (Hadar et al., 2017).

Interestingly, a number of these studies that did not observe altered microglial markers in the PFC reported significant changes in other schizophrenia relevant brain regions, including the hippocampus and striatum, suggesting that the mPFC may not be the most suitable brain region in which to investigate microglial changes in adult animals. Although the above mentioned study by Juckel et al. (2011) did not find microglial changes in the PFC following (GD9) MIA, they did observe increased microglial density and activation in the hippocampus and striatum of juvenile MIA mice (PND30). Furthermore, the previously mentioned study by Hadar et al. (2017) reported increased microglial density in the hippocampus and NAc, in the absence of PFC changes, in adult MIA rats exposed to Poly (I:C) on GD15. In addition, the study by Van den Eynde et al. (2014) found that MIA at GD15 in rats not only produced microglial changes in the corpus callosum but also resulted in increased activated microglia in the hippocampus and increased density in the pons. Hippocampal

microglial immunoreactivity was not measured in the current study, however, based on these findings highlighting the hippocampus as an area vulnerable to microglial alterations future studies using the current model would benefit from investigating this region.

Previously, our group demonstrated that MIA exposure increased the density of SST+ neurons in the white matter using tissue from the same animals in the current study (Duchatel et al., 2016) and this effect was most prominent after MIA at GD19 and in the most rostral region of the corpus callosum (i.e. region 1) where we now show an increase in IBA1+ immunoreactivity. As these two findings were observed in the same tissue, we hypothesised that the higher the IAB1+ immunoreactivity levels reflects a stronger immune response in the brain, leading to more pronounced brain pathology and a larger increase in density of SST+ neurons in the white matter. Contrary to our hypothesis, the correlation analysis showed that SST+ immunoreactivity from Duchatel et al. (2016) was negatively correlated with IBA1+ immunoreactivity in the current study. This correlation does not give any indication to the direction of causation of these effects and thus requires further investigation.

5.5.4. Priming of the Neuro-immune System.

It is conceivable that the subtle neuro-immune changes demonstrated in the current investigation may be enhanced following exposure to further inflammatory-inducing insults in the postnatal period. There is evidence to suggest that the neuro-immune system, in particular microglia, are “primed” by an initial inflammatory-inducing challenge occurring at vulnerable stages of prenatal development, and that this priming makes microglia more vulnerable to subsequent inflammatory-inducing insults resulting in exaggerated responses (Czeh, Gressens, & Kaindl, 2011; Monji et al., 2013;

Perry & Holmes, 2014). Indeed, a number of so called “two-hit” models where offspring are exposed to an initial immunological insult prenatally which is then followed by a second insult or “hit” (infection, stress, drug treatment etc.) in postnatal development, have demonstrated that prenatal and postnatal environmental risk factors can work synergistically to cause neuro-immune system disruption. An over-reactive neuro-immune system was observed in a study by Krstic et al. (2012) where the hippocampal cytokine and microglial changes induced by late gestation (GD17) Poly (I:C) exposure in mice were exacerbated by the addition of a second Poly (I:C) challenge in adulthood. A series of studies using early gestation (GD9) Poly (I:C) in mice also found that increased microglial activation and cytokine levels (IL-1β & TNF-α) were only present in the hippocampus and PFC of MIA animals that were also exposed to peri-pubertal stress (Giovanoli et al., 2016; Giovanoli et al., 2013). Furthermore these alterations were only present shortly after the second hit in adolescence (PND41) and not in adulthood (PND70) (Giovanoli et al., 2016; Giovanoli et al., 2013). It is clear from this evidence that a potential priming effect may explain why we only see subtle neuro-immune changes in the present investigation. Further investigations in this model should include the addition of a postnatal physiological or immunological stressor to investigate if this may exaggerate the subtle alterations observed in this study, or help to elucidate other potentially latent neuro-immune disruptions.

5.5.5. Methodological Considerations of Experimental Data

Some methodological considerations are important in the interpretation of these results. This study had two main parts, an investigation of MIA effects on the gene expression of Iba1, Gfap, TNF-α and IL-1β, and an investigation of microglia and

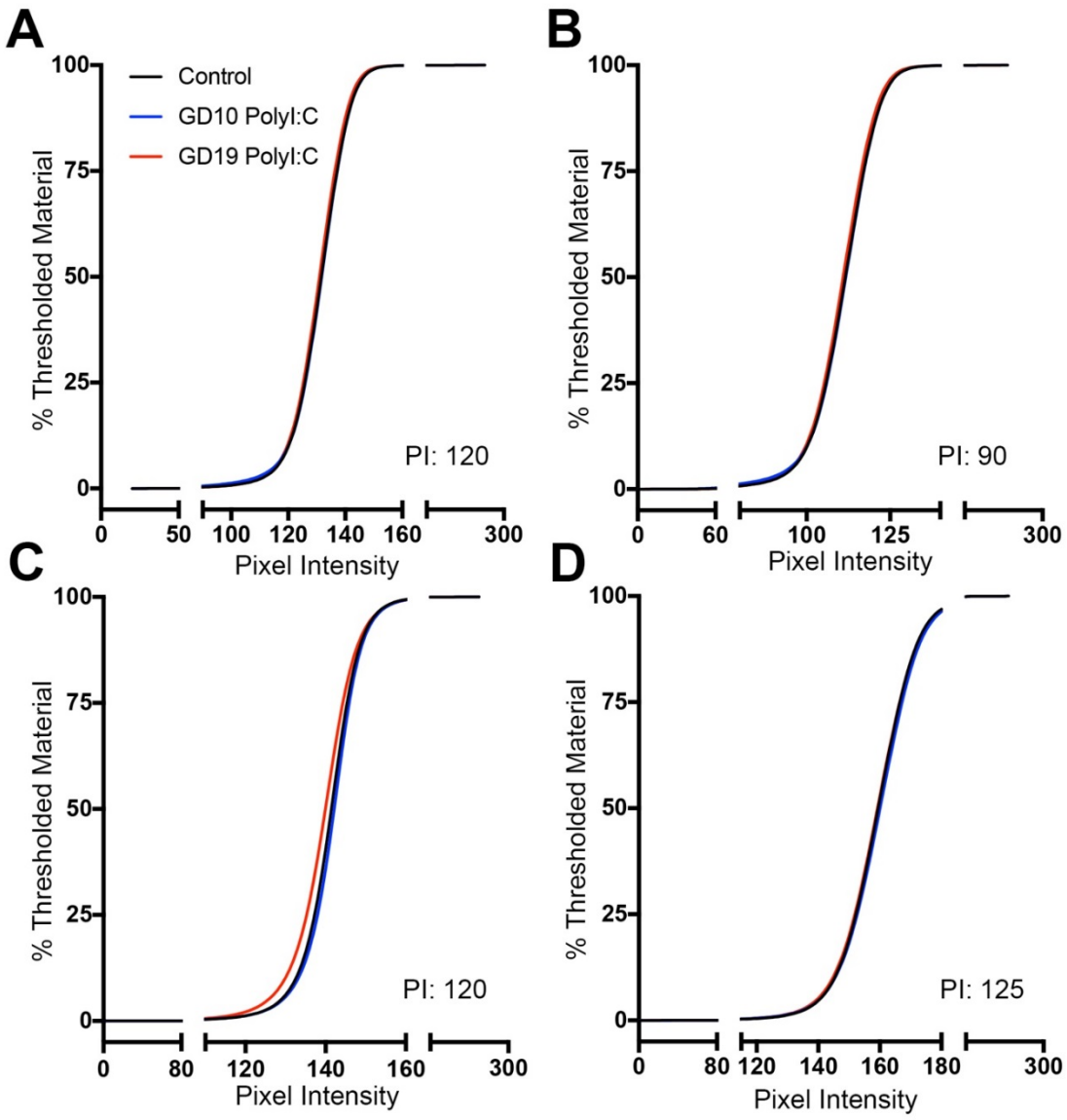
astrocytes density using immunohistochemistry measures. Due to these methodological differences where the brain tissue used for gene expression was fresh frozen and the tissue for immunohistochemistry was perfuse-fixed, we thus utilised two separate animal cohorts and were unable to examine the correlation between gene expression and microglia or astrocytic immunoreactivity. Within the microglia/astrocyte immunohistochemistry cohort, we examined the level of microglia/astrocyte immunoreactivity in both the mPFC and the white matter of the corpus callosum. However, within the gene expression cohort, due to methodological limitations in isolation of white matter devoid of cortical tissue from the blocks of rat brains, we examined only the mPFC. Stereological counting methodologies have been used to determine microglia density. In this study, we used a pixel intensity thresholding method to examine IBA1+ immunoreactivity that has been shown to be a powerful method for examining alterations to microglia (Jones et al., 2015; Ong et al., 2016; Patience et al., 2015). The IBA1 protein is selective to microglial cells and is found throughout the processes and cytoplasm of the cells making it not only an excellent marker for assessing cell density as performed here, but also ideal for assessing structural changes which are indicative of activation status (Korzhevskii & Kirik, 2016). Such assessment of the activation status of microglial cells identified in this study is an important future direction which may provide further insight into the neuroimmune changes identified in the current study.

5.5.6. Conclusion

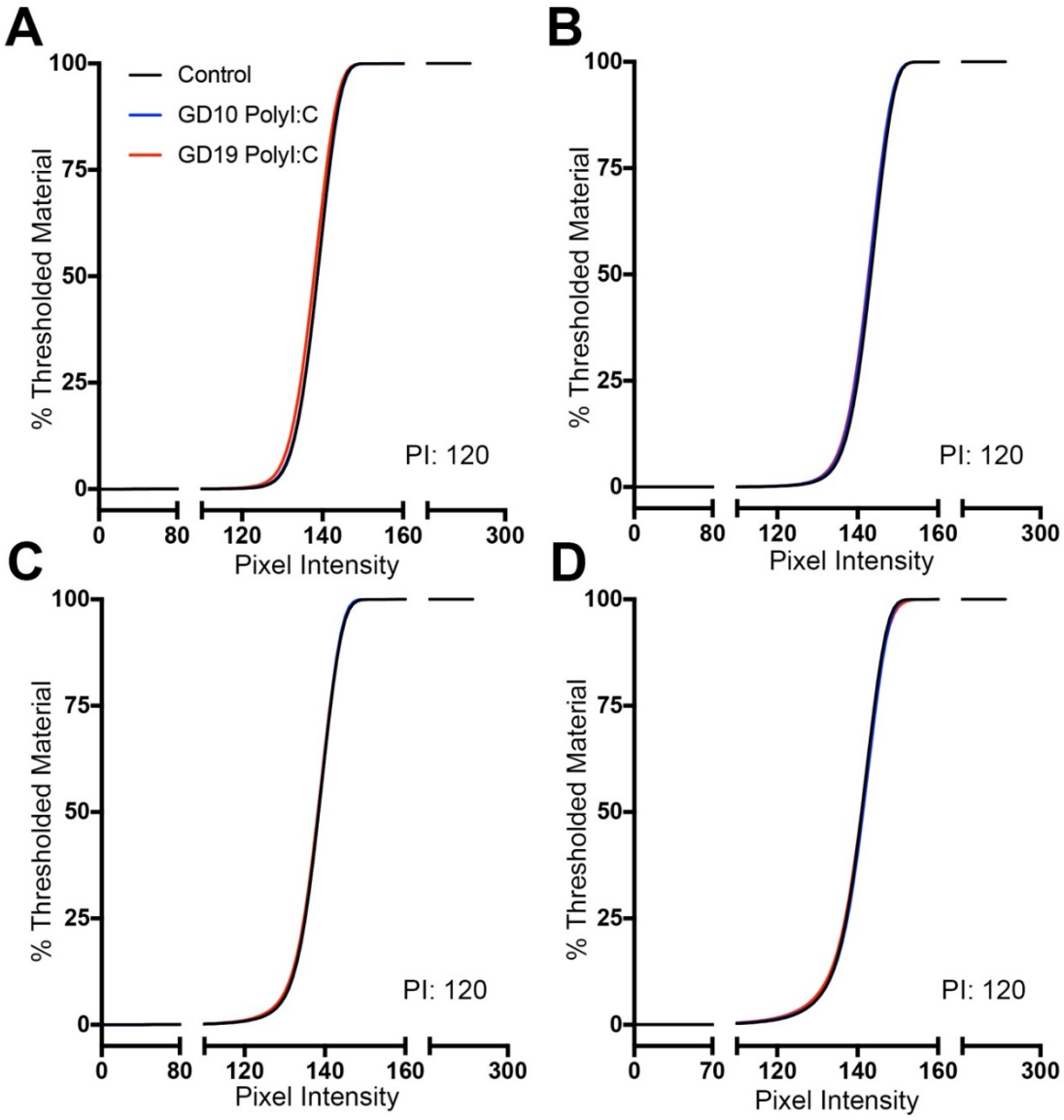
Although MIA models of schizophrenia-like pathologies are becoming better established, little is still known about how MIA alters foetal brain development to result in the wide ranging behavioural and neurobiological alterations commonly reported.

Altered neuro-immune function has been identified as a potential mediating mechanism, and the current study provides further evidence supporting the involvement of long-term neuro-immune alterations in a MIA model which has previously demonstrated schizophrenia-relevant behavioural and brain dysfunction (Duchatel et al., 2016; Meehan et al., 2017). Further research should aim to establish a developmental time course for the neuro-immune changes identified here, if alterations are present in other schizophrenia-relevant brain regions, and whether these changes are indicative of a "primed" system that produces overactive inflammatory responses to later-life insults that impact on the brain and/or immune system.

5.6. Supplementary Figures



Supplementary Figure 5.1. Cumulative thresholding spectra of IBA1+ immunohistochemistry. Cumulative thresholding analysis was conducted in the medial prefrontal cortex (Region 1 – A and Region 2 – B) and the white matter (Region 1 – C and Region 2 – D). The PI that detected genuine IBA1+ immunoreactive material that was used for the analysis is shown in the bottom right corner of each graph.



Supplementary Figure 5.2. Cumulative thresholding spectra of GFAP+ immunohistochemistry. Cumulative thresholding analysis was conducted in the medial prefrontal cortex (Region 1 – A and Region 2 – B) and the white matter (Region 1 – C and Region 2 – D). The PI that detected genuine GFAP1+ immunoreactive material that was used for the analysis is shown in the bottom left corner of each graph.

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6. Chapter 6: General Discussion

6.1. Summary of Main Findings

The central premise of this research was that activation of the maternal immune response during gestation would differentially disrupt trajectories of foetal neurodevelopment resulting in long term neurological and subsequent behavioural deficits. The primary aim was to establish a reliable rat MIA model of schizophrenia, and determine whether prenatal immune activation at two separate gestational time-points (early versus late gestation) differentially influences the behavioural and neurochemical phenotypes associated with schizophrenia; with early exposure more severely disrupting dopaminergic function and DA-related behaviours, and late exposure more severely perturbing glutamatergic function and NMDA-associated behaviours.

In order to assess this aim, a series of behavioural assessments were performed to investigate the effect of MIA via Poly (I:C) exposure at the two differential gestational time-points in adult offspring (Chapter 4). To do this we focused on behavioural measures that are implicated in schizophrenia in addition to being well-established for use in rodent. The measures used included tests for sensorimotor gating as evaluated via the PPI paradigm, working memory measured using an operant chamber based DNMTP paradigm, and pharmacologically induced (DA-agonist and NMDA-antagonist) locomotion evaluated by means of exposure to AMPH and MK-801 respectively. These assessments revealed that MIA in either early or late gestation produced subtle alterations in schizophrenia-related behaviours, however there was no clear distinction between a DA- and glutamate-related behavioural phenotype based on the gestational timing of the exposure. Male rats exposed to MIA in either early or late

gestation both exhibit sensorimotor gating deficits. In addition, no difference in AMPH-induced or MK-801-induced locomotion was observed following MIA at either gestational time-point. On the other hand, MIA only in late gestation generated transient working memory impairments. Secondly, we conducted post-mortem investigations using qPCR techniques which assessed mRNA expression of dopaminergic markers in the PFC of adult MIA offspring (Chapter 4). These assessments demonstrated that MIA in early, but not late, gestation produces subtle alterations in the development of the dopaminergic system in males. Increased Dr1 mRNA expression in the NAc was found in this group, however no alterations were found in any other dopaminergic markers assessed, highlighting the subtle nature of the effect. Together, these findings deviate somewhat from previous findings in mouse model studies, where early and late MIA produce a comprehensive spectrum of schizophrenia-like behavioural and neurochemical phenotypes with a distinct dichotomy between early and late gestation MIA.

A second aim of this research was to establish the most appropriate method for eliciting human-like MMRs in rats using a multi-channel recording system new to our lab, and once the optimal methodology was identified to then examine the effects of MIA at the two gestational time-points on MMRs in the rat. To achieve this we firstly assessed a number of different methodologies often used to elicit MMN (flip flop control, many-standards control (MSC), and cascade control) using untreated control animals (Chapter 3A). This investigation demonstrated that the MSC was the most appropriate method for use in rats to assess human-like MMRs, with the more complex cascade control proving to be unsuitable. The MSC data showed that the male rat brain is able to generate human-like MMRs, in that the MMRs produced were in-part adaptation-independent and therefore in-part determined by memory-based error

signalling processes. The MSC method was then used to assess MMRs in male rats from the MIA model (Chapter 3B). This assessment revealed increased, not decreased, MMRs in MIA rat regardless of gestational timing, indicating that although MIA does alter MMRs it does not do so in schizophrenia-like manner.

Interpretation of this finding is not straight forward. It has been assumed in the schizophrenia clinical literature that reduced amplitude of MMN reflects a decrease in the activity of the neural tissue generating the response. The brain activity recorded via a surface electrode on the scalp is an aggregate measure of post-synaptic potentials of pyramidal cells oriented perpendicular to the cortical surface (Nunez, Nunez, Srinivasan, Srinivasan, & Press, 2006). This activity is conducted through the brain, skull bone and skin virtually simultaneously – such that surface recorded voltages reflect neural activity happening exactly at that point in time (Kappenman & Luck, 2016). However, post-synaptic potentials last tens to hundreds of milliseconds and occur in response to a stimulus or event at many different areas of the brain at the same time. Further, because the potentials originating in a given brain region spread widely across the scalp, the voltages recorded at a given site on the scalp could reflect activity from a wide area of brain (Kappenman & Luck, 2016). This aspect of volume conduction makes it very difficult to localise the source of activity in specific regions of the brain with confidence. A further complication for interpretation of changes in the amplitude of MMN is that there are at least two potential contributors to the appearance of the signal recorded via a surface electrode in response to an auditory event – there could be new phase-locked neuronal activity triggered by the stimulus event or there could be a phase re-set of ongoing oscillations triggered by the stimulus. In either case, averaging over many such events results in an evoked response such as the MMN. It turns out that it is quite difficult to distinguish between these two possibilities although

there is evidence for both occurring (Michie, Malmierca, Harms, & Todd, 2016; Telenczuk, Nikulin, & Curio, 2010). What is uncontested is that a key property affecting the strength of the electric field (the amplitude of the evoked response) recorded at the scalp is the amount of synchronisation between neurons (Musall, von Pfostl, Rauch, Logothetis, & Whittingstall, 2014; Telenczuk et al., 2010). That is, changes in the degree of spatial synchronisation will lead to changes in the amplitude of the recorded field. Therefore, an increase in MMRs in the MIA rat most likely reflects an increase in synchrony of post-synaptic activity generated within pyramidal cells over a region of the rat cortex. Further research is required to understand the mechanisms whereby MIA affects synchrony of activity across neural networks.

With the aim of exploring the potential mechanisms involved in mediating the effects of MIA on altered schizophrenia-like behaviour and neurodevelopment, the neuroinflammatory phenotype of adult offspring was investigated in rats exposed to MIA at either gestational time-point. qPCR and immunohistochemistry techniques were used to assess a range of neuro-immune markers including microglia, astrocytes, and cytokines within the mPFC and frontal white matter. This examination showed that MIA does result in subtle neuro-immune changes in adult offspring, with an increase in microglial immunoreactivity identified in the frontal white matter of late, but not early, gestation MIA animals. In addition, a strong trend towards increased astrocyte immunoreactivity that approached significance was identified in the mPFC of late, but not early MIA offspring. However, no alterations in gene markers for microglia, astrocytes or cytokines was seen in frontal white matter nor in the mPFC. This finding again highlights the subtle nature of MIA on neurodevelopment at the two gestational time-points used in this research.

6.2. The Differential Influence of Sex Hormones in Psychopathologies

Sex hormones are known to influence neurodevelopment in the foetus in addition to being involved in maturational processes which occur during puberty (Barth, Villringer, & Sacher, 2015; de Vries & Sodersten, 2009; Forger, 2006; Gore, Martien, Gagnidze, & Pfaff, 2014). The neuro-modulatory nature of sex hormones allows them to have an impact on schizophrenia and other psychopathologies of a neurodevelopmental origin. Indeed, sex hormones are known to influence the incidence, symptomology, and course of schizophrenia (reviewed in, Abel, Drake, and Goldstein (2010)). A number of studies and meta-analyses have observed the incidence of schizophrenia to be higher among males than females with a ratio approximate of 1.4:1 (Aleman, Kahn, & Selten, 2003; McGrath, Saha, Chant, & Welham, 2008; McGrath et al., 2004). Symptom presentation and course have also been shown to differ as a function of sex. Symptom onset occurs and peaks earlier in males than females (Hafner, 2003). Males are also more likely than females to display prominent negative and cognitive symptoms, and less likely to display affective symptoms that often accompany the disorder, at both first presentation and throughout the course of the disorder (Andreasen, Flaum, Swayze, Tyrrell, & Arndt, 1990; Castle, Wessely, & Murray, 1993; Chang et al., 2011; Fenton & McGlashan, 1991; Goldstein & Link, 1988; Kay, Opler, & Fiszbein, 1986; Morgan, Castle, & Jablensky, 2008; Ring et al., 1991; Schultz et al., 1997). Brain structural changes in schizophrenia have also been shown to vary as a result of sex, with males displaying more prominent abnormalities than females (Gur et al., 2004; Gur et al., 2000; Walder et al., 2007). Such differences in brain structure have been attributed to the effects of sex hormones and genes throughout development (Goldstein et al., 2002).

In the current MIA model, we identified subtle sex dependent effects, specifically in the behavioural assessment of sensorimotor gating, with reductions in PPI evident in male but not female MIA animals. In addition, we also found altered D1r in the NAc of male animals. Given that sensorimotor gating is related to enhanced striatal dopaminergic activity (Meyer & Feldon, 2009; Swerdlow, Caine, Braff, & Geyer, 1992), together this evidence suggests that the developing DA-system is more prone to the effects of MIA in males. However, it must be noted that DA mRNA markers were not assessed in females in the current study, therefore it is currently unknown if measurable dopaminergic neurotransmitter measures are unaffected in females. Similar findings indicating more prominent PPI deficits in males compared to females have previously been reported in MIA models using both mice (O'Leary et al., 2014) and rats (Romero, Guaza, Castellano, & Borrell, 2010). In addition, alterations in D1r in male but not female MIA mice have also been reported, although these changes were found to present in the PFC and not the NAc, and were also in the opposite direction to our findings (decrease rather than increase) (Meyer et al., 2008b). It is possible that sex hormones specific to females have a protective or shielding effect to some extent on symptom development resulting in more pronounced deficits in males. Indeed, the suggestion that estrogen has a protective effect in schizophrenia is supported by a number of lines of evidence (reviewed in Kulkarni, Gavrilidis, Worsley, and Hayes (2012)). Fluctuations in symptoms across the menstrual cycle, during menopause, and during pregnancy have been noted in female patients with schizophrenia (da Silva & Ravindran, 2015; Hafner, 2003). A number of studies have reported an inverse relationship between these factors, indicating that decreases in estrogen are associated with exacerbation in symptoms (Markham, 2012; Mendrek & Stip, 2011; Riecher-Rössler & Häfner, 2000). Furthermore, the combination of oestradiol with traditional

antipsychotic medications has also shown some promise for increased efficacy in the treatment of positive symptomology (Kulkarni et al., 2002). Moreover, raloxifene, a drug known to stimulate estrogen-like activity in the brain, has been shown to improve cognitive function in schizophrenia patients of both sexes (Weickert et al., 2015) and administration of estrogen and raloxifene to rats has been revealed to have a protective effect on PPI disruption induced by D1r/D2r agonists (Gogos & van den Buuse, 2015).

The sex effects seen in our model are subtle and specific to sensorimotor gating, as all other behavioural or neuro-immune assessment conducted in our model were found to be independent of sex. Such sex independent effects are not uncommon in the MIA literature with other models of prenatal Poly (I:C) exposure identifying a range of brain and behavioural changes which are mutual to both sexes (Bitanihirwe, Peleg-Raibstein, Mouttet, Feldon, & Meyer, 2010). Why our model identified sex effects in PPI and no other behavioural measures is not clear, and further assessment of oestrous cyclicity, sex hormones, sex dimorphic brain differences, or the addition of an ovariectomised female group for comparison is needed to further investigate this result.

6.3. Gestational Timing of MIA: Critical Periods of Exposure

Differences between the findings from the current study and the early versus late gestation mouse model, introduced by Meyer and colleagues group, are clearly apparent. The mouse studies demonstrated a distinct differentiation in the behavioural and neurobiological phenotypes between the early and late gestation MIA offspring (Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008c). It was found that MIA via Poly (I:C) in early gestation (GD9 in the mouse) resulted in deficits in PPI, impaired LI, and reductions in the expression of D1r and D2r in the PFC and increased TH in the striatum (CPu and NAc shell) (Li et al., 2009; Meyer et al., 2008a; Meyer et al., 2008b;

Meyer et al., 2008c). In contrast, late gestation (GD17 in the mouse) exposure to Poly (I:C) resulted in impaired working memory, deficits in reversal learning, and reduced expression of the NR1 subunit of the NMDAr in the hippocampus (Meyer et al., 2006; Meyer et al., 2008c). Locomotor response to MK-801 in the mouse model was found to be enhanced by MIA at late gestation only (Meyer et al., 2008c), however further studies found a similar response following early MIA (Meyer et al., 2008a; Meyer et al., 2008b). Enhanced locomotion in response to AMPH treatment was also found following both early (Meyer et al., 2008a; Meyer et al., 2008b; Meyer et al., 2008c) and late gestation exposure (Meyer et al., 2008c).

In the current studies, it was observed that the same exposure in rats does not result in the same clear differentiation between MIA at early versus late gestation which would be suggested by the mouse findings. MIA in rats during early gestation altered D1r expression in the PFC, whereas MIA in late gestation produced transient deficits in working memory and subtle alterations in microglial and astrocyte density specific to frontal white and grey matter, respectively. However, the current study also showed that MIA results in PPI deficits and altered MMRs, regardless of gestational timing. In addition, in our current model significant changes in AMPH and MK-801 induced locomotion, or alterations in central cytokine expression were not observed. These results indicate that GD10 and 19 in the rat may not be the most sensitive periods of neurodevelopment for the production of a schizophrenia-like phenotype, and do not follow the same differentiation between dopaminergic and glutamatergic neurotransmitter system disruption as seen in the mouse.

Although the stages of development are comparable between the mouse and rat model (Workman, Charvet, Clancy, Darlington, & Finlay, 2013), and the sequence of neurodevelopmental milestones are largely conserved across species (Finlay &

Darlington, 1995), it is still possible that minor variations in the neurodevelopmental processes between these two species at these gestational time-points (GD9 in the mouse versus GD10 in the rat, and GD17 in the mouse versus GD19 in the rat) may account for the lack of consistency across species. In the mouse, neurogenesis of dopaminergic midbrain neurons begins in early gestation and is at its highest at GD11, and further development of the dopaminergic system continues throughout prenatal development (Bayer, Wills, Triarhou, & Ghetti, 1995). To date, results from the mouse have confirmed that GD9 is definitely a vulnerable period for MIA with relation to disrupted development of the dopaminergic system. The dopaminergic system in the rat also begins development prenatally and continues to maturate well into adulthood (Andersen, Rutstein, Benzo, Hostetter, & Teicher, 1997; Gates, Torres, White, Fricker-Gates, & Dunnett, 2006), resulting in extended periods of development which are critical to normal functioning of the dopaminergic system and therefore vulnerable to disruption. The first critical period of dopaminergic development in the rat occurs prenatally, beginning with DA neuron differentiation at GD10 which peaks at GD12 (Gates et al., 2006; Lauder & Bloom, 1974), continues with innervation of the developing basal ganglia between GD14 – 17 (Voorn, Kalsbeek, Jorritsma-Byham, & Groenewegen, 1988) and closes with a period of neuron cell death in the SN occurring between GD20 and PND14 which establishes the final number of adult DA neurons (Burke, 2004; Oo & Burke, 1997). A second period critical to development of the dopaminergic system in the rat occurs postnatally between PND20 – 80 when DAr numbers are being stabilised, an initial increase and then reduction of DAr occurs during this time (Andersen et al., 1997). The subtle and minimal disruption to the DA-system and associated behavioural measures following early MIA in the current study when combined with the above information about the timing of development of DAr in

the rat indicate that the GD10 time-point may be too early to cause significant disruption to the developing dopaminergic system and perhaps a later gestational time-point may be more effective.

In agreement with this notion, the only other investigation examining early gestation MIA in the rat to our knowledge, failed to find any changes in the dopaminergic based behavioural measure of PPI following either Poly (I:C) or LPS exposure at GD10-11 (Fortier, Luheshi, & Boksa, 2007). However, PPI deficits were found as a result of LPS exposure at later time-points (GD15-16 and GD18-19), but not following Poly (I:C) at the later time-point (Fortier et al., 2007). The very low-dose of Poly (I:C) used in the Fortier et al. (2007) study (1000 ug/kg in comparison to the more common 4-5 mg/kg) may account for this. Indeed, the majority of MIA models in rats use a mid-gestational time-point at GD14 or 15 which has been shown to produce more prominent dopaminergic dysfunction than the current study. Disruption to the dopaminergic based behavioural measures of PPI, (Dickerson, Wolff, & Bilkey, 2010; Howland, Cazakoff, & Zhang, 2012; Wolff & Bilkey, 2008, 2010) latent inhibition (Piontkewitz, Arad, & Weiner, 2011; Zuckerman, Rehavi, Nachman, & Weiner, 2003; Zuckerman & Weiner, 2003) and altered sensitivity to the locomotor stimulating effects of AMPH (Piontkewitz et al., 2011; Zuckerman et al., 2003) have all been well documented in the GD15 Poly (I:C) rat model. Increased striatal DA release (Zuckerman et al., 2003) and reduced striatal volumes (Piontkewitz et al., 2011) have also been reported. This mid-gestational time-point overlaps with dopaminergic differentiation and innervation in the developing foetal brain (Gates et al., 2006; Voorn et al., 1988), and appears to be a period of dopaminergic development that is more sensitive to the effects of MIA than the early GD10 time-point.

The mid-gestational time-point (GD15) which is often used in rat MIA models also produces significant alterations in glutamatergic functioning and associated cognitive behavioural deficits. Poly (I:C) exposure at GD15 in the rat has been shown to produce deficits in recognition memory and alterations in reversal learning (Wolff, Cheyne, & Bilkey, 2011) in addition to deficits in object-in-place recognition memory (Howland et al., 2012) and altered sensitivity to the locomotor stimulating effects of MK-801 (Zuckerman & Weiner, 2005). Furthermore, MIA at GD15 has been associated with altered neurogenesis and loss of interneurons in the hippocampus (Piontkewitz et al., 2012), and with volumetric changes in the hippocampus and PFC (Piontkewitz et al., 2011), both areas involved in cognitive function. Disruption to cognitive function and associated brain regions is not surprising given that the neurogenesis in the neocortex occurs throughout mid (GD14) to late (GD20) gestation in the rat, peaking between GD14 – 18 depending on the cortical layer (Bayer & Altman, 2004), making this region particularly vulnerable to the disrupting effects of MIA at GD15. The hippocampus, excluding the dentate gyrus, also undergoes neurogenesis during mid (GD16) to late (GD20) gestation in the rat, peaking around GD17-19 (Bayer & Altman, 2004). In addition, the NMDAr NR1 subunit is not expressed in the rat until later in gestation, becoming detectable around GD17 (Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994). The fact that a large part of hippocampal and neocortical development, including NMDAr, occurs prior to the GD19 time-point used in the current study may account for our subtle finding in relation to cognitive function and MMRs.

The effect that timing of prenatal infection has on long term neuroinflammatory markers is less clear. The majority of studies which investigate neuro-immune markers in MIA offspring do so in the foetal or early postnatal stages of development, with few assessing the long-term consequences in adult offspring (Girard, Tremblay, Lepage, &

Sebire, 2010; Ratnayake, Quinn, Castillo-Melendez, Dickinson, & Walker, 2012; Roumier et al., 2008). Of those that have assessed markers in the post-pubertal stages of development, the gestational timing, immune agent, brain region, methodology and species have all varied, producing mixed results. MIA in early gestation has predominantly been done in mice, with alterations in microglial markers found in a range of brain regions (Juckel et al., 2011), however early gestational MIA in the mouse has also failed to find, or identified only subtle neuro-immune changes in some cases (Giovanoli, Weber-Stadlbauer, Schedlowski, Meyer, & Engler, 2016b; Nyffeler, Meyer, Yee, Feldon, & Knuesel, 2006; Willi, Harmeier, Giovanoli, & Meyer, 2013). MIA at GD15 in the rats has been shown to produce increased microglial immunoreactivity in the hippocampus, thalamus, and corpus callosum, but not the mPFC which is in line with our findings (Hadar et al., 2017; Van den Eynde et al., 2014). In addition, mid gestation exposure in the mouse (GD12.5) has also produced changes in central cytokine profiles in post pubertal offspring (Garay, Hsiao, Patterson, & McAllister, 2012). However, mid gestational MIA in the rat has also failed to produce significant neuro-immune changes in at least one study (Missault et al., 2014). One rat study that used injection of the cytokine IL-6 across 3 consecutive days at either early (GD8, 10 & 12) or mid-late (GD16, 18 & 20) gestation in the rat also found alterations in astrocyte markers at both gestational points (Samuelsson, Jennische, Hansson, & Holmang, 2006). In addition, exposure to LPS on two consecutive days in late gestation (GD18 & 19) produced alterations in astrocyte markers in the frontal and hippocampal regions of adult rats (de Souza et al., 2015). The variations and inconsistent methodologies between studies make it difficult to accurately compare and draw conclusions on the influence that gestational timing alone may have on neuroinflammatory processes. However, these findings in conjunction with our result of altered microglial markers in

late but not early gestation, indicate that mid to late gestation (in addition to extended periods of exposure over consecutive gestational days) may be a more susceptible time of development with regards to long term neuro-immune changes in that rat.

In addition, it is also possible that differences in the phenotypes between our model and that presented in the mouse could potentially stem from difference in survival rates between the two models. The Poly (I:C) administered at GD10 and GD19 in the current model produced an immunological response sufficient to alter brain and behavioural measures, but not strong enough to induce spontaneous abortion and reabsorption of the foetus as evidenced by normal litter sizes within all groups (see section 2.3.2). It has been noted by Meyer, Feldon, Schedlowski, and Yee (2005) that exposure to Poly (I:C) on GD 9 in mice at doses of 5 mg/kg (similar to our 4 mg/kg) and 10 mg/kg was associated with increased spontaneous abortion. The increased abortion rate reported in mice, as suggested by Meyer et al. (2005), could be indicative of potential genetic or other difference between the surviving and aborted offspring, and therefore differences in the phenotype displayed by these surviving mice and models such as ours where abortion was not increased.

6.4. Contribution of Postnatal Mechanisms

Although in the current study we have focused on the effects of one specific risk factor for schizophrenia, prenatal infection, it is unlikely that exposure to a single risk factor alone produces such a multifaceted and complex disorder as schizophrenia in all individuals. Indeed, schizophrenia is most likely the cumulative effect of multiple risk factors combining to disrupt normal neurodevelopmental and maturational processes occurring at multiple time-points throughout the lifespan (Knuesel et al., 2014; Meyer, 2014). While not directly assessed in the current investigation, the peri-pubertal stage of

postnatal development has been identified as a critical period involved in the aetiology of a number of psychopathologies (Holder & Blaustein, 2014). In particular, environmental risk factors which occur at this stage of development, such as psychological stress and trauma, postnatal infection, or drug abuse are known to be associated with schizophrenia (Brown, 2011; Corcoran et al., 2003; Henquet, Di Forti, Morrison, Kuepper, & Murray, 2008), and are believed to act synergistically with other risk factors such as insults that occur in prenatal development or genetic susceptibilities (Ayhan, Sawa, Ross, & Pletnikov, 2009; Clarke, Tanskanen, Huttunen, Whittaker, & Cannon, 2009; Knuesel et al., 2014). This "two-hit" hypothesis proposes that an insult experienced during prenatal brain development results in a dormant neuropathology making the brain more susceptible to the effects of a second, or even multiple, environmental "hit" experienced in later development (Bayer, Falkai, & Maier, 1999; Maynard, Sikich, Lieberman, & LaMantia, 2001). The validity of the two hit model has been well established in the animal literature with a number of studies using both mice (Deslauriers, Larouche, Sarret, & Grignon, 2013; Deslauriers, Racine, Sarret, & Grignon, 2014), and rats (Choy & van den Buuse, 2008; Choy, de Visser, & van den Buuse, 2009; Hill et al., 2014) showing that an early life insult followed by a second stressor during postnatal development, results in a range of schizophrenia-like behavioural and neurological abnormalities.

It is possible that the subtle brain and behavioural changes demonstrated in the current model may be enhanced or further alterations revealed by the addition of a second risk factor or "hit" in the peri-pubertal stage, such as a psychological stressor. Indeed, a number of rodent MIA models have found that the addition of a second hit in postnatal development (Burt, Tse, Boksa, & Wong, 2013; Dalton, Verdurand, Walker, Hodgson, & Zavitsanou, 2012; Giovanoli et al., 2016a; Giovanoli et al., 2013), or the

presence of a genetic susceptibility (Abazyan et al., 2010; Granholm et al., 2011; Vuillermot et al., 2012), can help to illuminate latent neurological changes induced by prenatal infection.

The neuro-immune system has also been demonstrated to be more susceptible to the effects of postnatal insults if it has previously been exposed to an initial immunological insult in prenatal development. This so called "priming" effect has been reported in microglia, whereby prenatal infection renders microglia more reactive to environmental insults in later life (Monji et al., 2013; Perry & Holmes, 2014). Mixed results have been identified with regards to long-term neuro-immune changes following MIA, with some studies identifying alterations in microglial markers in adolescent – adult offspring (Juckel et al., 2011; Van den Eynde et al., 2014), while others have not (Garay et al., 2012; Giovanoli et al., 2016b; Missault et al., 2014). However, several studies have found that an exaggerated immune response is induced by a second hit in "primed" MIA offspring. Early gestation MIA in mice was found to work synergistically with peri-pubertal stress to increase markers of cytokine and microglial activation, but not astrocytes, in the PFC and hippocampus (Giovanoli et al., 2016a; Giovanoli et al., 2013), and when peri-pubertal stress was concurrently administered with the tetracycline antibiotic minocycline, the exacerbated microglial activation and cytokine markers were not seen (Giovanoli et al., 2016a). Moreover, late gestation MIA in mice altered microglial activation and cytokine expression in the hippocampus, which was then further exacerbated following adult immune challenge, indicating an over-activated neuro-immune state which is hypersensitive to subsequent exposures (Krstic et al., 2012). This evidence suggests that the subtle neuro-immune changes identified in the current model may be further exacerbated, and more substantial neuro-

immune disruptions identified, in MIA offspring following the presence of second environmental insult.

6.5. Relevance of Animals in Modelling Schizophrenia

Attempting to use a rodent, or any other mammal, to replicate the full spectrum of symptomology and neuropathology of a multi-faceted and complex psychiatric disorder is not possible (Knuesel et al., 2014; Meyer, 2014). This especially so in a disorder where so many of the primary behavioural disturbances, such as hallucinations, delusions, and paranoid thought, are not able to be observed and/or evaluated in non-human species (Reisinger et al., 2015), and when the model uses but one of the many recognized risk factors known to contribute to the aetiology of the disorder. However, this does not make MIA animal models, such as the one established in this thesis, unimportant. The MIA model presented here, like many other animal models of complex psychopathologies, not only aimed to but was successful in reproducing a limited set of behavioural and neurobiological disturbances associated with a number of neurodevelopmental psychopathologies, including schizophrenia. Such models provides us with a platform to further investigate the complex neurobiological mechanisms which underlie the behavioural disturbances displayed, and the processes by which the specific insult used alters these neurobiological mechanisms (Knuesel et al., 2014; Reisinger et al., 2015). This in turn allows for the identification of novel therapeutic targets and provides a platform for early development and testing of new therapies.

In order to be useful in enhancing our understanding of the underlying mechanisms of a disorder and identifying new therapeutic targets, some degree of validity must be established for individual models. Face validity of an animal model refers to extent to which the model is able to reproduce behavioural and neurobiological

disturbances in the animal of choice which are analogous to those seen in human patients (Nestler & Hyman, 2010; Reisinger et al., 2015). The MIA model in rodents has obtained a high level of face validity over the years as it has now been linked at a wide range of behavioural, pharmacological, neurochemical, and neuroanatomical measures which are strongly implicated in schizophrenia, and a number of studies have also shown that symptom onset follows the post-pubertal time course seen in humans (Canetta & Brown, 2012; Meyer & Feldon, 2009). Offspring in the current study were also found to display alterations in a range of measures associated with schizophrenia, including working memory, PPI, D1r expression, and microglial density, indicating that our early verses late gestation MIA model in the rat also meets the criteria of face validity. In order for animal models to provide useful insights into the human disorders they model it is also important for them to have a high level of construct validity, which is described as replicating the disorders aetiological process, such as grounding the model on the environmental and/or genetic risk factors which are known to be involved in the development of the disorder or aspects of it pathological process (Nestler & Hyman, 2010). The current model is successful in achieving this by inducing one of the more well known risk factors for schizophrenia, prenatal exposure to infection, as its basis. However, the current model could further add to its construct validity by the addition of a second well established risk factor such as peri-pubertal stress. The model would then more closely represent the complex interaction of multiple risk factors occurring throughout development which is believed to underpin schizophrenia. The predictive validity of an animal model is also important, and refers to the ability of pharmacological agents used in the treatment of humans (such as antipsychotics) to be efficacious in treating the analogous behavioural disturbances produced in the model (Nestler & Hyman, 2010; Reisinger et al., 2015). Although predictive validity has been

established in other MIA models of schizophrenia, where the use of antipsychotic treatments have been shown to reverse or prevent MIA-induced deficits in LI, object-recognition memory, spatial working memory, PPI, and extracellular glutamate levels (Meyer, Knuesel, Nyffeler, & Feldon, 2010; Meyer, Spoerri, Yee, Schwarz, & Feldon, 2010; Ozawa et al., 2006; Roenker et al., 2011; Zuckerman et al., 2003), it has not yet been assessed in the current model. This however, is not necessarily a limitation of the current model. It has been suggested that in relation to psychopathologies where the current pharmacological interventions are limited in their ability to effectively ameliorate the full range of symptomology in patients, such as in schizophrenia, that assessment of how behavioural deficits in animals models respond to these treatments is not particularly informative (Reisinger et al., 2015). Especially when the aim of many of these animal models is to identify novel therapeutic targets aimed at treating the symptoms which are not addressed by the current pharmacological treatment.

In the current model we chose to focus on behavioural measures which were highly comparable between human and rodents, such as PPI, working memory, and MMRs. The methodologies which were selected to measure MMRs, PPI, and working memory are the most current and comprehensive available for use in rats. The DNMTP task is a well-established test for assessing working memory in rodents (Dudchenko, 2004), and has been acknowledged by the Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia (CNTRICS) group as a valid and useful measure of working memory in rodents (Dudchenko, Talpos, Young, & Baxter, 2012). The DNMTP task removes the spatial memory component which is needed to complete the Morris water maze, another commonly used test of working/spatial memory in the MIA literature. In addition, the techniques and equipment used to assess MMRs in the electrophysical experiments are state-of-the-art, allowing for recordings from multiple

sites using untethered and fully conscious animals without the need for invasive abdominal surgeries. In addition, the methodology implemented enabled for a distinction to be made between adaptation and true MMN-like (adaptation independent) responses. Thus far, MMRs have been assessed in very few animal models of schizophrenia. The methodologies and equipment used to assess PPI in the current model are standard across the animal literature. Deficits in sensorimotor gating, as assessed by PPI of the acoustic startle reflex, are one of the most influential behavioural correlates used in schizophrenia research having repeatedly been reported in patients and widely established in translational animal models (Braff, Geyer, & Swerdlow, 2001; Van den Buuse, Garner, & Koch, 2003). Overall, the methodologies and techniques used to assess behavioural outcomes in the current model are strong, and in some instances more comprehensive than those used in other studies. Although the behavioural measures used here are not directly translatable to humans, they are some of the most comparable measures across species which are currently available. The subtle nature of the behavioural changes displayed in the current model can therefore be attributed to limiting effect of our MIA, and not to methodological insensitivities in the chosen behavioural assessments.

6.6. Limitations and Future Directions

Although the current model has been successful in producing behavioural and neurobiological disturbances with relevance to schizophrenia, it is not without its limitations. Although many of the measures we investigated in the current model were assessed in both male and female offspring there were two exceptions to this, with male only offspring being assessed in the electrophysiology studies and in the dopaminergic mRNA expression study. Future investigations using the model established here need to

be extended to include female offspring in order to assess for any potential sex specific effects in dopaminergic and electrophysiological neuropathology. This is particularly relevant to the dopaminergic mRNA expression study as we did find sex differences in PPI, a dopaminergic based behaviour, and were not able to confirm if the sex differences seen in PPI were also associated with sex differences in D1r expression. Furthermore, for female offspring the stage of the oestrous cycle at the time of behavioural testing should be accounted for, or an ovariectomised female groups should be added to the experiments in order to identify any potential protective effects which sex hormones such as estrogen may have.

In addition, this model could be further validated by future investigations examining the time course of behavioural and neurobiological deficit presentation. By assessing the brain and behavioural measures looked at in the current study at multiple time-points throughout development, in particular assessing if behavioural abnormalities present after but not prior to puberty onset, would further strengthen the face validity of our model. Indeed, a number of MIA models have shown that at deficits in LI, PPI, and recognition memory follow a post-pubertal onset similar to that seen in schizophrenia (Ozawa et al., 2006; Zuckerman et al., 2003; Zuckerman & Weiner, 2003). However, this phenomenon has not yet been assessed in the early or late gestational time-point in the rat: rat models have so far only assessed the time course of behavioural symptom onset in the GD15 MIA model (Zuckerman et al., 2003; Zuckerman & Weiner, 2003). The rat model presented here would be strengthened by such an investigation.

Furthermore, future investigations using the model established here should aim to assess pathological changes, particularly neuroinflammatory changes, in other schizophrenia-relevant brain areas. Specifically, investigations should firstly focus on

regions associated with behavioural and electrophysiological deficits identified in the current thesis. Increased MMRs may implicate alterations in the auditory systems such as the auditory cortex, while the PPI deficits identified implicate subcortical DA regions including the CPu, NAc, SN, and VTA. In addition, areas such as the hippocampus which are involved in cognitive function and associated with schizophrenia should also be assessed.

Finally, given the subtle nature of the phenotype displayed in the current model, in addition to the strong evidence indicating that multiple risk factors work synergistically together in the aetiology of schizophrenia, future investigations using this model could benefit from the introduction of a second hit (peri-pubertal stress). As discussed above, the addition of an aetiologically relevant risk factor during the peri-pubertal stage following MIA has been shown to elucidate latent behavioural, neuro-immune, and neuropathological markers associated with schizophrenia (Dalton et al., 2012; Giovanoli et al., 2013; Krstic et al., 2012). By adding a second hit to the current model, a more robust and extensive phenotype may be expressed in the adult offspring, which could potentially allow for the revelation of hidden mediating mechanisms.

6.7. Conclusions

The data presented in this thesis provides evidence that transient activation of the maternal immune-inflammatory system during varying stages of gestation is sufficient to disrupt neurodevelopmental processes producing long-term alterations in behavioural and neuropathological measures relevant to schizophrenia. The findings from this thesis deviate slightly from previous findings coming from early verses late gestation MIA in mice, which were found to display a more comprehensive range of behavioural and neurobiological alterations relevant to schizophrenia. The clear

distinction between the dopaminergic and glutamatergic phenotypes resulting from MIA at early verses late gestational time-points in the mouse was not replicated in the current model, and overall a more subtle phenotype was demonstrated in our current investigation, indicating differences in the critical periods of neurodevelopmental susceptibility to MIA exposure between the two species. The subtle phenotype displayed in the current model may also be taken as an indication that the addition of further environmental risk factors/insults may be needed to trigger a more robust phenotypic display. The findings presented here extend the current body of MIA literature by furthering our understanding of the neuropathological and behavioural outcomes occurring in the rat following MIA at the specific and novel gestational windows of exposure used. In addition, the current research has extended the already established MIA models by examining the prominent schizophrenia relevant electrophysiological biomarker of MMRs, which has not previously been assessed in any MIA rodent models. Our findings indicated that MMRs are indeed altered by MIA, but not in a manner consistent with schizophrenia. Furthermore, the present investigation has also provided insights into the underlying neuro-immune changes which may contribute to the behavioural abnormalities seen in adult MIA offspring, although further investigation is needed to fully elucidate these changes and their causative mechanisms.

With the limits of current treatments and the significant impact cognitive symptoms have on the quality of life of patients with schizophrenia now well established, recognition of the need to develop new treatments targeting these largely untreated cognitive symptoms has increased. The development of valid animal models that display cognitive impairment analogous to those seen in schizophrenia has been noted as vital for the identification of new treatment targets and testing treatment-related

hypothesis (Wilson & Terry, 2010). In order to identify potential treatment targets it is important to uncover the mechanisms which underlie prenatal infections influence on neurochemical, neuroanatomical, neuro-immune and behavioural dysfunction, a task in which reliable aetiological based animal models are crucial. In the future, additional investigation extending on the MIA model established in this thesis may be able to illuminate the mechanisms by which MIA alters neurodevelopmental processes. By further developing our understanding of the disturbances that early life viral infection has on cognitive dysfunction, the glutamatergic system and MMRs, and microglial activity, animal models such as the one established in this thesis can provide a platform for identifying novel targets which may be suitable for the developing new treatments, or potentially preventative therapies.

6.8. References

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