Effects of maternal immune activation on adult brain neurobiology

Ryan J Duchatel

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Thesis

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Abstract

Prenatal immune challenge is an environmental risk factor for the development of psychiatric illnesses including schizophrenia. Modelling this epidemiological link in animals shows that maternal immune activation (MIA) is capable of inducing long-lasting changes in brain structure, function and behaviour in the offspring; which is very promising for elucidating the underlying mechanisms for schizophrenia. Indeed, one interesting anatomical finding within schizophrenia research is an increased density of neurons residing in the white matter under grey matter cortical regions. These interstitial white matter neurons (IWMNs) have been hypothesised to have a number of neural origins, but the role they play in the underlying schizophrenia aetiology is unknown. Chapter 1 introduces schizophrenia and presents an in-depth literature review on the changes to IWMNs in schizophrenia. Chapter 1 then presents the evidence for MIA and its relationship to schizophrenia. However, to fully elucidate their role in disease pathogenesis an animal model is necessary to study IWMNs in an environment other than human post-mortem brain.

In Chapter 2, the aim was to characterise IWMNs subjacent to the frontal cortex of the adult rodent brain, including markers and location, and then examine if MIA affected the density of IWMNs in this model. MIA was induced by early or late gestation exposure of pregnant rats to polyriboinosinic-polyribocytidylic acid (PolyI:C) with IWMN density assessed in the adult rat offspring. While NeuN+ IWMNs trended to be increased by this model, both early (gestational day 10; GD10) and late (GD19) gestation MIA induced a significant increase in somatostatin positive (SST+) IWMN density in the white matter of the corpus

callosum. Interestingly the increase in SST+ IWMN density was regionally more widespread in those rats exposed to MIA at GD19. These changes are similar to that observed in post-mortem brain studies of schizophrenia. This established an animal model of increased white matter neuron density induced by a known risk factor for schizophrenia. Then in Chapter 3 the aim was to determine if other IWMN subtypes were altered by MIA. The density of both NPY+ IWMNs and GAD+ IWMNs was examined but neither were affected by MIA – suggesting that SST+ IWMNs are particularly susceptible to MIA. Chapter 3 also provided a gene expression analysis to determine if MIA affected cortical GABAergic gene expression.

The role of an abnormal immune system in schizophrenia has recently come to light. Alterations in immune related genes within the cortex of people with schizophrenia has provided an underlying “immune signature” in the disease. Furthermore, studies of post-mortem brains in schizophrenia have identified changes to glia, the brain’s immune cells. I hypothesised that the MIA-induced increase in IWMNs reported in Chapter 2 may be driven by inflammation in the cortex reflected by alterations in glial cells and inflammatory gene expression. In Chapter 4 I tested this hypothesis by examining inflammatory gene expression and immunohistochemistry for microglia and astrocytes in the brains of rats exposed to MIA induced by PolyI:C. Whilst there were no changes in cortical inflammatory gene expression, a significant increase in microglia (Iba1+) immunoreactivity was observed in the white matter of the corpus callosum, but not the cingulate cortex, suggesting that disrupted microglia were specific to the white matter. Furthermore, no alterations in astrocyte (GFAP+) immunoreactivity

was identified in rats exposed to MIA, which is congruent with current literature on their role in schizophrenia.

Further links to immune dysfunction in schizophrenia came from a ground-breaking discovery by Sekar et al. (2016), who identified a significant association of the complement component 4 (*C4*) gene with schizophrenia and that people with schizophrenia have a significant increase in the expression of the complement component 4 (*C4*) gene. In Chapter 5 I showed that this alteration in cortical *C4* gene expression was also present in the cingulate cortex of rats exposed to MIA at late gestation.

In summary the data in this thesis provides a link between white matter pathology, including increased white matter neurons, and increased microglia reactivity, with cortical innate immune system gene expression changes. Finally, this thesis provides a discussion, summarising the work presented within, linking MIA with increased IWMN density, increased microglial reactivity in the white matter and cortical changes in *C4* gene expression.

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List of Abbreviations

BA	Brodmann's area
ChR	channelrhodopsin
CC	cingulate cortex
Cr	calretinin
CWM	cingulate white matter
DA	dopamine
DCX	doublecortin
DLPFC	dorsal lateral prefrontal cortex
DSM-V	diagnostic and statistical manual, fifth version
DTI	diffusor tensor imaging
eGFP	enhanced green fluorescent protein
EPS	extrapyramidal syndrome
GABA	γ-aminobutyric Acid
GAD	glutamic acid decarboxylase
GAT-1	GABA transporter
GD	gestational day
GSEA	gene set enrichment analysis
GWAS	genome wide association study
ICD10	tenth revision of the international classification of disease
IPC	inferior parietal cortex
IWMN	interstitial white matter neuron
MAP2	microtubule associated protein 2
MIA	maternal immune activation
mPFC	medial prefrontal cortex
MRI	magnetic resonance imaging
NADPH	nicotinamide–adenine dinucleotide phosphate-diaphorase
NeuN	neuronal nuclei
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NR	not reported
NPY	neuropeptide Y
OFC	orbitofrontal cortex

PET	positron emission topography
PHG	para hippocampal gyrus
PND	postnatal day
PolyI:C	polyriboinosinic-polyribocytidylic acid
Pv	parvalbumin
RelN	reelin
SD	standard deviation
SST	somatostatin
vGAT	vesicular GABA transporter
vGLUT	vesicular glutamate transporter

CHAPTER ONE

Introduction

1.1 Introduction

Historically the term “Schizophrenia”, was derived from two Greek words; schizo (split or divide) and phrenos (mind), but today comes to represent one of the most debilitating mental illnesses known to society. Schizophrenia affects approximately 7 in every 1000 persons (McGrath et al., 2008) and places a high social and economic burden on communities with an estimated $1.44 billion cost to the Australian health care system in 2003 (Carr et al., 2003).

Schizophrenia is a complex disorder, with diagnosis relying solely on the presentation of a diverse array of clinical symptoms including positive and negative symptoms, as well as cognitive impairments (Makinen et al., 2010). Positive symptoms are displayed as a gain of function, represented by a loss of contact with reality and may include hallucinations and delusions. Negative symptoms are a loss or decline in function such as weakening or lack of normal thoughts, social interaction, emotions or behaviour in schizophrenia patients. Cognitive deficits observed in schizophrenia include decreases in attention to tasks, learning ability and problem solving (Bowie and Harvey, 2006; Lett et al., 2014). The onset of schizophrenia is generally during adolescence or early adulthood, persisting for the lifetime of the affected individual. Approximately 30% of all sufferers will attempt suicide (Jablensky, 2000), thus schizophrenia comes at a great cost to the individual, families and the health care system.

Sex differences have been widely reported in schizophrenia, and may have important implications for both future research on schizophrenia pathophysiology as well as clinical practice (Javed, 2000). These differences include, but are not

limited to: sex differences in prevalence estimates, incidence and its distribution of onset over the life-cycle; differences in the determinants of premorbid functioning and consequences of the gender difference in age at onset; gender-specific course and outcome; sex differences in brain development and functioning, specifically as they relate to sex differences in brain in schizophrenia and sex differences in treatment response (Mendrek and Mancini-Marie, 2015). In particular, women have a later onset of schizophrenia and a better course of illness than men and that these two phenomena are related to one another, i.e. a worse illness subtype occurs earlier and therefore accounts for the worse prognosis in men; or, later illness onset in women represents a less aggressive illness and allows for better outcome (Rapoport et al., 2005).

Despite decades of research, only incremental advances have been made in the understanding of the condition, no conclusive aetiology of the disorder exists and there has been only modest gains in diagnosis, treatment and epidemiology of schizophrenia.

1.2 Grey and white matter pathology in schizophrenia

Schizophrenia neuropathology was initially prominently focused on functional regions of the human grey matter, where wide spread alterations have been reported including structural changes such as decreased volumes of cortical grey matter in the dorsolateral prefrontal cortex (DLPFC), cingulate gyrus, medial temporal lobe and superior temporal gyrus (Reviewed together in: Andreasen et al. (1994); Gaser et al. (2004); Lawrie and Abukmeil (1998); Wright et al. (2000)). At a cellular level, changes to interneurons containing γ-aminobutyric acid

(GABA) termed GABAergic interneurons, have been a widely reported neuropathological finding in schizophrenia (Reviewed by Hoftman et al. (2015)). Interneurons are vital for maintaining a balance of excitatory signalling within the brain. Indeed, one of the most robust findings reported in the schizophrenia literature is a decrease in the expression of glutamic acid decarboxylase-67 (GAD67), the enzyme that synthesises GABA (Hashimoto et al., 2008a). The literature consistently reports decreased GAD67 mRNA (Gonzalez-Burgos et al., 2011; Gonzalez-Burgos et al., 2010; Lewis, 2011) and protein (Curley et al., 2011; Guidotti et al., 2000) in 25-35% of interneurons (Akbarian et al., 1995; Volk et al., 2000) in the DLPFC, as well as the anterior cingulate cortex, superior temporal cortices, striatum and thalamus (Thompson et al., 2009), suggesting that this is a phenomena observed in a variety of brain regions in schizophrenia. Decreased GAD67 protein and mRNA has been shown to occur in a variety of interneuron subsets, particularly chandelier and basket neurons that express parvalbumin (PV), a calcium binding protein (Lewis et al., 2012). These changes in interneurons are postulated to affect GABA-mediated inhibition, pyramidal neuron excitation and the generation of gamma oscillations in the cortex which is thought to be dependent on fast spiking PV+ interneurons (Sohal et al., 2009). This has been postulated to contribute to the development of cognitive deficits in schizophrenia implicating a role for interneuron pathology in the disorder (Uhlhaas and Singer, 2010).

Whilst most of the early studies investigated the grey matter, white matter pathology in schizophrenia has been increasing in focus particularly as neuroimaging techniques developed. White matter in the brain consists of the

axonal projections to other neurons and brain areas and forms the basis for connectivity in the brain. Structural integrity of white matter can be examined indirectly with a diffusion-weighted imaging technique called diffusion tensor imaging (DTI). DTI is a magnetic resonance imaging-based technique that allows the investigation of location, orientation, and anisotropy of the brains' white matter tracts and more recently has grown in popularity amongst schizophrenia researchers (reviewed in Wheeler and Voineskos (2014). DTI revealed abnormal changes in morphology and decreased integrity of white matter tracts in the brain in people with schizophrenia (Adler et al., 2006; Szeszko et al., 2005). Furthermore, these changes in white matter tracts detected by DTI were present in first-episode schizophrenia, as well as individuals at high risk prior to onset of disease symptoms (Hoptman et al., 2008). A progressive reduction of frontal lobe white matter has also been identified using DTI in schizophrenia patients post diagnosis, and this continued with antipsychotic treatment (Ho et al., 2003). During development, there are long lasting structural changes to the white matter that are necessary for the generation of cognitive functions (Nagy et al., 2004), in particular working memory and reading ability. This is most pronounced during the transition from adolescence to early adulthood, which overlaps a key time in the development of schizophrenia, namely the onset of the disorder. Therefore, any alteration to white matter development or damage to white matter structures at this time point may contribute greatly to the development of schizophrenia. The building evidence for white matter pathology within schizophrenia suggest the time has come to reassess white matter neuropathology at a cellular level in a bid to determine if functional changes predominantly attributed to cortical abnormalities in schizophrenia may also relate to white matter alterations.

1.3 White matter neurons – neurobiology and neurochemistry

In traditional neuroscience, the grey matter of the cortex was thought to contain all the neuronal cell bodies in the brain with the underlying white matter thought to consist of myelinated fibres of neuronal axons. However, a small number of neurons have been found to reside within the white matter spaces of the human brain and whilst not as comprehensively studied, should not be discounted when considering the pathophysiology of schizophrenia. These interstitial white matter neurons (IWMNs) were first described by Meynert in 1867 when "solitary" neurons were observed between myelinated nerve fibres of the subcortical white matter (Meynert, 1867). A similar population of neurons was identified by Cajal, who observed neurons between axon bundles of the trigeminal nerve and the white matter of the cerebellum (Cajal, 1895). In addition, many neurons were observed with ascending projections towards the overlying cortex and Cajal was the first to postulate that these neurons may be cells displaced from the grey matter (Cajal, 1901a).

Initial studies of IWMNs focused on their location within the brain and morphological characteristics. IWMNs are a morphologically and neurochemically heterogeneous population of cells. From the earliest drawings of Meynert and Cajal of Golgi stained neurons, it was clear that IWMNs had different morphologies and orientations in the human brain (Garcia-Lopez et al., 2010; Judas et al., 2010). Since then, immunohistochemistry utilising nicotinamide-adenine dinucleotide phosphate-diaphorase (NADPH) histochemistry and antibodies to neuronal markers has become the most utilised method for examining IWMN subtypes and their distribution. In adults IWMNs are

more abundant in the superficial white matter close to the grey matter border, with numbers declining towards the deep white matter (García-Marín et al., 2010). The frontal cortices contain the highest IWMN density in the adult human brain, followed by the cingulate cortex, then the visual and temporal cortices (García-Marín et al., 2010; Meyer et al., 1992). Studies of IWMNs in fetal and adult human brains observed that NADPH-positive (NADPH+) IWMNs have predominantly a fusiform shape with some having pyramidal-like, bipolar or multipolar shapes within the superficial white matter under the temporal and frontal cortices (García-Marín et al., 2010; Judaš et al., 2010; Kostovic et al., 2011b). Axons from some NADPH+ IWMNs in the adult brain project into the overlying cortical layers (Judaš et al., 2010). In the visual cortex from early postnatal rats, biocytin filled IWMNs were shown to have pyramidal-like, fusiform, bipolar and multipolar somata with extensive dendrites within the white matter some of which projected into layer VI of the cortex (Clancy et al., 2001). Furthermore, axons from some superficial IWMNs projected through layer VI to layer V whereas deep IWMNs projected axons only to Layer VI (Clancy et al., 2001). This indicates there is variability in the morphological characteristics of IWMNs, as well as their dendrites and axonal projections that in turn may be dependent on their location within the white matter. Excitingly, it suggests that IWMNs may be communicating with neurons within the grey matter.

Studies in humans and animals have investigated the neurochemistry of IWMNs using antibodies directed at proteins expressed in neurons. Antibodies to neuronal nuclei antigen (NeuN), which is used as a marker of mature neurons, appears to identify the overall population of mature IWMNs (Connor et al., 2009;

Eastwood and Harrison, 2003a, 2005a; García-Marín et al., 2010; Joshi et al., 2012; Yang et al., 2011a). Other markers appear to detect only a subpopulation of IWMNs. For example, NADPH histochemistry labels a proportion of IWMNs, as do antibodies to microtubule associated protein-2 (MAP-2) (Judaš et al., 2010; Meyer et al., 1992). Some IWMNs contain neuronal nitric oxide synthase (nNOS) suggesting they release nitric oxide as a transmitter. IWMNs are immunoreactive for glutamate and type II Ca^{2+}/calmodulin-dependent protein kinase-α indicating some are likely excitatory (Tighilet et al., 1998). Some suggest that IWMNs are remnants of the developmental subplate that gives rise to cortical neurons and Clancy et al. (2009) suggests that 15-25% of these persistent subplate neurons in rodents are GABAergic. Indeed, IWMNs are immunoreactive for glutamic acid decarboxylase (GAD) (Joshi et al., 2012), the calcium binding proteins parvalbumin (PV) and calretinin (CR) (Meyer et al., 1992), and the neuropeptides neuropeptide Y (NPY), somatostatin (SST) (Duchatel et al., 2016; Hogan and Berman, 1993; Yang et al., 2011a), substance P and cholecystokinin (Friedlander and Torres-Reveron, 2009). These studies suggest IWMNs are morphologically and neurochemically diverse, but could IWMNs be truly integrated into cortical circuitry?

Cajal's drawings of Golgi labelled sections of early postnatal human and rodent brain provided the first evidence that not only do IWMNs project axons to the cortex, but cortical neurons project dendrites into the superficial white matter. Just under a century later, Kostovic and Rakic (1980) described both symmetrical (putative GABAergic) and asymmetrical (putative glutamatergic) synapses on the dendrites and soma of IWMNs situated below the monkey and human visual,

somatosensory and motor cortices. García-Marín et al. (2010) studied the synaptic innervation of the white matter from adult human frontal, striate and visual cortex using immunolabelling for GAT-1 (GABA transporter-1) and vGAT (vesicular GABA transporter) to identify GABAergic terminals and vGlut-1 (vesicular glutamate transporter) to identify glutamatergic terminals. The cortical layers had abundant labelling for GABAergic and glutamatergic terminals and whilst they were also evident in the white matter, their density was significantly less (García-Marín et al., 2010). This anatomical data shows that IWMNs receive synaptic inputs from either other IWMNs, neurons in the overlying cortex or possibly from subcortical structures, suggesting they are integrated into local circuits. However, the functional consequences of these putative synapses on IWMNs in frontal brain regions remain a mystery.

Indeed, very few studies have investigated the electrophysiology of IWMNs due to their sparse distribution amongst dense fibre tracts of the white matter making it difficult to locate and record from IWMNs. Clancy et al. (2001) utilised post recording analysis of biocytin filled neurons in the white matter underneath the rat visual cortex at postnatal day (P)4-35. Most IWMNs had short-duration action potentials with fast after-hyperpolarisations and virtually no spike-frequency adaptations (Clancy et al., 2001). In the visual cortex of rats at P10-20, IWMNs and subplate neurons had similar intrinsic properties and threshold for spike initiation, as well as action potential kinetics (Torres-Reveron and Friedlander, 2007). IWMNs were, however, more depolarized at rest than subplate cells (Torres-Reveron and Friedlander, 2007). Both IWMNs and subplate neurons received mostly AMPA/kainate receptor synaptic inputs, with some also receiving

NMDA receptor and GABAergic inputs; these inputs also underwent long-term plasticity (Torres-Reveron and Friedlander, 2007). Friedlander and Torres-Reveron (2009) in a follow-up study used paired whole cell patch clamp recordings from a IWMN and a neuron in cortical layer VI of the rat visual cortex at P20 and suggested that they received both excitatory and inhibitory synaptic inputs, potentially participating in local synaptic networks. One of the difficulties faced by these earlier studies was the identification of IWMNs to record from using normal light microscopy, plus then knowing what subpopulation of neuronal type the recordings were made from. To solve this problem, Engelhardt et al. (2011) used transgenic mice (P10-P30) where the serotonin $5HT_3$ receptor gene was tagged with enhanced green fluorescent protein (eGFP) in the only electrophysiology study of a subset of GABAergic IWMNs. $5HT_3^+$ eGFP IWMNs underneath the motor cortex at the level of the hippocampus had firing patterns typical of interneurons, receiving excitatory and inhibitory synaptic inputs from cortical and subcortical structures (Engelhardt et al., 2011). In only four paired recordings they showed that $5HT_3^+$ IWMNs inhibited cortical neurons (Engelhardt et al., 2011). These studies provide tantalising evidence that IWMNs are indeed functionally integrated into cortical circuitry. Could this impact on normal brain function or disease states?

1.4 White matter neurons in schizophrenia

One of the most robust white matter pathologies in people with schizophrenia, is an increased density of IWMNs directly underneath particular cortical regions implicated in the disorder. To date thirteen studies have examined the density of IWMNs in the post-mortem brains of schizophrenia subjects. Eight out of the thirteen studies have shown an increase in the density of neurons in the superficial white matter (Akbarian et al., 1996; Anderson et al., 1996; Connor et al., 2009; Eastwood and Harrison, 2003a, 2005a; Joshi et al., 2012; Kirkpatrick et al., 2003a; Yang et al., 2011b) below the cortex, with three studies showing increases in the deep white matter (Akbarian et al., 1993; Ikeda et al., 2004; Rioux et al., 2003). In contrast, two studies could not show any change in the density of IWMNs in either superficial or deep white matter (Beasley et al., 2002; Molnar et al., 2003). A summary of these studies is provided in Table 1. The studies of altered IWMN density in schizophrenia have focused on three key markers, NADPH, MAP2 and NeuN, the latter because it detects all mature neurons.

Akbarian et al. (1993) published the first study showing that subjects with schizophrenia had a significant reduction in the NADPH+ neurons in the superficial white matter adjacent to the cortex in the DLPFC, but in contrast a significant increase in the density of IWMNs in the deep white matter region (Akbarian et al., 1993). Molnar et al. (2003) in the only other study using NADPH as a IWMN marker observed no significant changes in the density of IWMNs in either the superficial or deep white matter. This confounded the use of NADPH as an IWMN marker in studies of their density in schizophrenia. Other early studies examined the use of MAP2 as a marker for IWMNs. In a second study,

Akbarian et al. (1996) reported that the density of MAP2-positive (MAP2+) IWMNs was increased in the superficial white matter subjacent to the DLPFC of subjects with schizophrenia. In contrast to their earlier study using NADPH, Akbarian and colleagues did not observe a significant difference in the density of MAP2 IWMNs in the deep white matter compared to controls (Akbarian et al., 1996). Increased MAP2+ neuron density in the superficial white matter was then replicated by Anderson et al. (1996) subjacent to the DLPFC and unchanged in the deep white matter (Akbarian et al., 1996). Interestingly, Kirkpatrick et al. (2003a) showed that schizophrenia subjects with cognitive impairments had a significantly higher density of MAP2+ IWMNs in the superficial white matter subjacent to the inferior parietal cortex (BA39) than either patients with little or no cognitive deficits or healthy controls(Kirkpatrick et al., 2003b).

Table 1. Summary of the studies investigating the density of IWMNs underneath the cortex in subjects with schizophrenia.

Neuronal Marker[c]	Brain Region[a]	Brodmann Area[g]	Sample Size[b]	Reference	Density of IWMNs	
					Superficial White Matter	Deep White Matter
NAPDH	DLPFC	NR	5:5	Akbarian et al. (1993)	Decreased	Increased
NADPH	DLPFC	NR	18:18	Molnar et al. (2003)	Unchanged	Unchanged
MAP2	DLPFC	NR	20:20	Akbarian et al. (1996)	Increased	Unchanged
MAP2	DLPFC	BA46	5:5	Anderson et al. (1996)	Increased	Unchanged
MAP2	DLPFC	BA9/10	15:15	Beasley et al. (2002)	Unchanged	Unchanged
MAP2	IPC	BA39	5:7[d]	Kirkpatrick et al. (2003a)	Increased[e]	Not examined
MAP2	PHG	NR	41:15	Rioux et al. (2003)	Unchanged	Increased
NPY	DLPFC	BA9	14:6	Ikeda et al. (2004)	Unchanged	Increased
NeuN and Reelin	DLPFC	BA22	12:14	Eastwood and Harrison (2003a)	Increased	Unchanged
NeuN	DLPFC	BA22	11:12	Eastwood and Harrison (2005a)	Increased	Unchanged
NeuN	CWM	BA33	22:45:(15)[f]	Connor et al. (2009)	Increased[f]	Unchanged
NeuN and SST	DLPFC	BA46	29:37	Yang et al. (2011b)	Increased	Unchanged
GAD NeuN	OFC	BA11	38:38	Joshi et al. (2012)	Increased	Unchanged

Adapted from Eastwood and Harrison et al. (2003).

[a] DLPFC: dorsolateral prefrontal cortex. IPC: Inferior Parietal Cortex. PHG: Parahippocampal Gyrus. CWM: Cingulate White Matter. OFC: Orbitofrontal cortex

[b] Number of subjects with schizophrenia: to the number of control subjects.

[c] NeuN: neuronal nuclear antigen; MAP2: anti-microtubule associated protein 2; NADPH: nicotinamide–adenine dinucleotide phosphate-diaphorase; Reelin; SST: Somatostatin; NPY: Neuropeptide Y.

[d] Subjects with schizophrenia split into deficit and nondeficit subgroups.

[e] Significant in the deficit subgroup only.

[f] Number of cases with bipolar disorder where the increase of IWMN was also observed is shown in brackets.

[g] NR: Not reported.

The consistency in IWMN alterations was then confounded when Beasley et al. (2002) published a conflicting result to Akbarian et al. (1996) and Anderson et al. (1996), and could not show a significant change in MAP2+ IWMN density or spatial distribution between cases with schizophrenia and matched controls, in either the superficial or deep white matter subjacent to the DLPFC. Rioux et al. (2003) examined the distribution of MAP2+ IWMNs in the white matter of a different brain region, the parahippocampal gyrus, in subjects with schizophrenia and also showed no change in density of superficial IWMNs when compared to controls. Indeed, MAP2 is only expressed in a small subset of IWMNs and may not provide a true indication of the density of these neurons in the white matter.

The breakthrough in the consistency of identifying altered IWMN pathology in schizophrenia came when a series of studies investigating the density of IWMNs was published using antibodies directed against NeuN. Eastwood and Harrison (2003b) provided the first study examining altered IWMN density using immunohistochemical labelling for NeuN and showed that their density was increased in the superficial white matter of the DLPFC. They also showed that some IWMNs express less reelin protein and mRNA (Eastwood and Harrison, 2003b), and thus reelin expression may identify a subset of IWMNs. In a second study, Eastwood and Harrison (2005b) replicated their results showing an increased density of NeuN+ IWMNs in the DLPFC of a separate cohort of subjects with schizophrenia. Using NeuN as a marker, Connor et al. (2009) then showed that the density of neurons was increased in the superficial white matter subjacent to the cingulate cortex. This was further supported by a larger study from Yang et al. (2011c), who had access to the DLPFC from 29 schizophrenia

subjects and 37 controls and confirmed an increased density of NeuN+ IWMNs in the superficial white matter in schizophrenia subjects. Yang et al. (2011c) also showed that there was a subset of NeuN+ IWMNs that expressed the neuropeptide SST and that these SST+ IWMNs were also increased in density in the white matter of the DLPFC in subjects with schizophrenia. Interestingly, a proportion of these SST^+ neurons also co-expressed NPY and an earlier study by Ikeda et al. (2004) showed that the density of NPY+ IWMNs was increased in the deep white matter of the DLPFC. These studies further suggest that specific subsets of IWMNs might be altered in the brain in people with schizophrenia. Interestingly, Morris et al. (2009) demonstrated that *NPY* mRNA, but not *SST* mRNA was significantly reduced in the superficial white matter in those with schizoaffective disorder compared to controls. In the most recent and largest study of the density of IWMNs conducted, Joshi et al. (2012) showed that in a cohort of 38 schizophrenia subjects and matched controls there was an increase in GAD+ and NeuN+ IWMNs in the superficial white matter of the orbitofrontal cortex.

These studies that have accessed some of the largest brain tissue cohorts available at the time, provide powerful evidence for a significant increase in IWMN density subjacent to the cortex in several brain regions implicated in schizophrenia including the frontal, temporal and parietal lobe white matter. These studies also show that NeuN is the most reliable immunohistochemical marker for detecting these differences. Collectively, these studies reported a generalised increase in the density of neurons in these selected compartments of white matter space (Akbarian et al., 1993; Anderson et al., 1996; Eastwood

and Harrison, 2003a, 2005a) or a redistribution towards the deeper white matter (Akbarian et al., 1996; Rioux et al., 2003). No studies on IWMNs examined whether sex effects were present, though all studies examined cohorts of mixed gender. From this we could conclude that IWMN alterations may be exhibited across both genders.

1.5 The neurodevelopmental hypothesis of schizophrenia

It has long been suggested that schizophrenia has a neurodevelopmental origin. A key aspect of this hypothesis is that exposure to an environmental factor(s) early in development triggers a pathophysiological process that alters brain development, before the onset of symptoms of schizophrenia. Indeed, maternal infection during pregnancy is one of the known environmental factors that can significantly increase the risk of schizophrenia in the offspring (Brown, 2006). Another established risk factor for the development of schizophrenia is birth in winter or spring months when respiratory infections are more prevalent (Brown and Derkits, 2010b). Furthermore, epidemiological studies provide compelling evidence that prenatal exposure to infection significantly increases the risk of schizophrenia, with a large number of infectious agents shown to be involved including viral pathogens such as influenza (Brown et al., 2004; Mednick et al., 1988), rubella (Brown et al., 2001b), measles (Torrey et al., 1988), polio (Suvisaari et al., 1999), herpes simplex virus (Buka et al., 2001), as well as infections with bacterial pathogens (Sorensen et al., 2009), the protozoan toxoplasma gondii (Brown et al., 2005; Mortensen et al., 2007) and reproductive tract infections (Babulas et al., 2006). The large number of pathogens that have been implicated in the maternal infection hypothesis suggests that factors common in the immune response to these pathogens, rather than the pathogens

themselves, may be critical in schizophrenia development through prenatal infection.

1.6 Modelling maternal immune activation using PolyI:C

The maternal immune activation (MIA) model builds on the association between maternal exposure to infection during pregnancy and the increased risk of schizophrenia. Maternal infection paradigms used mice exposed to human influenza virus during prenatal stages of development and observed a wide array of behavioural and cognitive responses, such as decreased pre-pulse inhibition, associated with schizophrenia pathologies (Shi et al., 2003). In addition to this, morphological changes such as lateral ventricle enlargement and GABAergic gene expression changes relevant to schizophrenia were observed in the same animal model (Fatemi et al., 1999; Fatemi et al., 2008).

Maternal immune activation differs from maternal infection, as non-infectious substances are used to activate the immune system, representative of a specific infection, such as Influenza. MIA using the viral mimic PolyI:C has been shown to induce schizophrenia-like phenotypes in rodent models, but is a safer alternative for experimental use than live human influenza virus (Wilson and Terry, 2010). MIA using PolyI:C exhibits numerous neurochemical and brain morphological abnormalities similar to people with schizophrenia (Meyer and Feldon, 2010b; Meyer et al., 2009a; Piontkewitz et al., 2011), and importantly with the same maturation delay (Ozawa et al., 2006b; Piontkewitz et al., 2011). Behaviourally, prenatal exposure to PolyI:C has been shown to alter working memory, spatial learning, latent inhibition, social interactions, pre-pulse inhibition

in both rat models (Howland et al., 2012; Wolff and Bilkey, 2010; Wolff et al., 2011; Zuckerman and Weiner, 2005), as well as mouse models (Bitanihirwe et al., 2010; Ibi et al., 2009; Ozawa et al., 2006a). In addition to this, alterations in neurotransmitter systems that have been implicated in people with schizophrenia, have been observed after prenatal exposure to PolyI:C, with both enhanced sensitivity to locomotor stimulating effects of amphetamine and NMDA receptor antagonist MK-801, reportedly altered (Meyer and Feldon, 2010a; Meyer et al., 2008b; Zuckerman et al., 2003; Zuckerman and Weiner, 2005). Most importantly, these MIA models follow the developmental time course associated with schizophrenia where these schizophrenia-associated behavioural changes only occur in the adult, but not pre-pubertal offspring (Meyer et al., 2008b; Ozawa et al., 2006a; Wolff and Bilkey, 2008; Zuckerman et al., 2003; Zuckerman and Weiner, 2003).

The timing of gestational exposure to maternal immune activating pathogens such as PolyI:C has been shown to be a critical factor in the development of behavioural and cellular changes displayed by the offspring of mothers exposed to maternal infection paradigms (Boksa, 2010; Meyer and Feldon, 2012). MIA paradigms have primarily focused on early/middle vs. late gestation insults. Specific differences have been identified between early/middle (GD9) and late (GD17) gestation exposur periods. These periods are homologous to end of the first trimester and middle to end of the second trimester in humans (Macedo et al., 2012b). Specific differences identified in mice include deficits in PPI and latent inhibition at GD9 (Meyer et al., 2005), but impaired reversal learning, deficits in working memory and enhanced MK-801 locomotion at GD17 (Meyer et al.,

2008c) . In addition to these behavioural changes, specific changes in brain structure at both the anatomical and cellular level are also present at different MIA time points. Decreased dopamine D1 receptor expression in the prefrontal cortex and enlarged lateral ventricles after GD9 MIA (Meyer et al., 2008a), whereas offspring from GD17 MIA animals showed decreases in NMDA receptor NR1 subunit expression suggesting glutamatergic system dysfunction (Luchicchi et al., 2016). Some changes are also shared between time points for example, enhanced amphetamine induced locomotion and reduced reelin and parvalbumin mRNA (GABAergic markers) were observed independent of the time of exposure (Li et al., 2009; Meyer et al., 2006; Meyer et al., 2008c). This suggests that there is an interaction between the maternal immune response and stage of brain development at the time of exposure with early exposure producing a phenotype reminiscent of the positive symptoms of schizophrenia while late gestational exposure is more representative of the negative symptoms of schizophrenia (Meyer and Feldon, 2012). The gestational timing utilised in this model, mimics the extensive GD9 and GD17 data in mice – GD10 and GD19 are analogous time points when you consider the gestational lengths of both rats and mice. Indeed, specific behavioural findings from this model have been published by Meehan et al. (2017), where they identified 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.

Understanding specific differences between viral and bacterial infections are important when attempting to determine the most appropriate MIA model to use

to recapitulate schizophrenia pathologies. 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 (Vercammen et al., 2008). Therefore, the use of a TLR3 activating agent would be more appropriate for attempting to model the majority of the epidemiology findings indicating an association between influenza and schizophrenia (Mednick et al., 1988). In this regard, PolyI:C is a double stranded RNA commonly used to mimic viral infection that initiates an immunological response via the TLR3 receptor (Zhou et al., 2013). Importantly, as PolyI:C is not a live virus it cannot replicate inside the host animal, this means that the dose of PolyI:C and subsequent intensity and length of the immune response can be controlled (Zuckerman et al., 2003). Previous investigations have shown that the acute phase immunological response to PolyI:C exposure is time limited to between 24 – 48 h depending on the dose administered (Cunningham et al., 2007; Meyer et al., 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. The use of a viral mimetic such as PolyI:C also eliminates the need for strict biosecurity and contamination procedures inherent in the use of live infectious agents (Meyer and Feldon, 2012). These factors in combination with the well-established nature of the use of PolyI: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.

1.7 Microglia, neuroinflammation, and maternal immune activation

There have been several mechanisms proposed to explain why prenatal exposure to infection can cause abnormalities in structural brain and behavioural development. 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, namely inflammation, which is common to all pathogens that is involved (Meyer et al., 2009b).

Changes in the expression of genes involved in the immune system and inflammation have been identified in the brains of people with schizophrenia (Fillman et al., 2013; Gardiner et al., 2013; Kumarasinghe et al., 2013). In particular, genes encoding inflammatory markers, such as IL-6, IL-8 and IL-1β have been reported to be altered in people with schizophrenia. In relation to the model presented here, abnormal foetal brain development has been shown to be associated with altered expression of pro-inflammatory cytokines and other mediators of inflammation in the maternal host, such as IL-10 (Ashdown et al., 2006; Gilmore and Jarskog, 1997; Meyer et al., 2009b), suggesting this may be a shared pathology. In addition to this, a recent study by Sekar et al. (2016) observed increased *C4* gene expression in the post mortem brain of schizophrenia. *C4* is part of the complement innate immune system and plays a crucial role in the recognition of pathways instigated by antibody – antigen complexes to other effector proteins of the innate immune response {Vercammen, 2008 #4264}. Its implication in schizophrenia provides further evidence of immune activation in the brains of people with schizophrenia.

Indeed, cytokines and microglia are the main aspect of the body's innate immune-inflammation response and potential mediators between prenatal infection and schizophrenia-relevant brain and behavioural changes (Meyer and Feldon, 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 (Deverman and Patterson, 2009). 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. Cytokines also play a key role in cell-cell signalling which influence numerous areas of neurodevelopment including neurogenesis, cell migration, differentiation, axon pathfinding, and apoptosis within the developing brain (Deverman and Patterson, 2009). This dual cooperating role of microglial cells and the cytokines they release means that induction of cytokines in response to prenatal immune responses has the potential to interfere with the normal neurodevelopmental processes occurring during this time, potentially resulting in permanent abnormalities which may later contribute to the development of schizophrenia.

Relating to IWMNs, evidence suggests that schizophrenia subjects that have this cortical gene expression profile which is representative of increased neuro-inflammation, also have a larger increase in NeuN+ and GAD65/67+ IWMN density in the OFC compared to those schizophrenia subjects with low

expression of these genes (Fung et al., 2014b). As previously examined, GABAergic interneurons form the main inhibitory network in the brain and it has been hypothesised that maternal infection may affect the development of this network by affecting expression of GABAergic markers leading to abnormal synaptic inputs by these interneurons (Behrens and Sejnowski, 2009). In the context of the developing brain, alterations in GABAergic markers could disrupt the normal development of inhibitory circuitry that is seen in schizophrenia. Indeed, GABAergic interneurons are a subgroup of IWMNs and thus the possibility arises that in the case of maternal infection, these neurons may be increased in density in the white matter of the adult brain in this model and contribute to the development of schizophrenia-like changes to cortical functions by making abnormal synaptic contacts in the white matter and the overlying cortex.

1.8 Rationale

The brain is made up of billions of neurons linked together in a complex network of connections. Most neurons in the brain are located in the cortex and subcortical nuclei. But, nearly 150 years ago, early neuroscientists observed neurons in the white matter parts of the brain which is made up of mostly nerve fibres running from one brain area to the next. IWMNs are less well characterised and consist of excitatory and GABAergic inhibitory neurons. Schizophrenia is underpinned by deficits in cortical GABAergic interneurons. Interestingly post-mortem studies have identified increased IWMN density underneath the cortex in schizophrenia however, we still do not fully understand the role of these neurons and if they contribute to schizophrenia pathogenesis. This is a concern because, there is a building body of evidence that suggests exposure of mothers to viruses during pregnancy increases the risk of their children developing schizophrenia. In addition to this, recent data suggests that people with schizophrenia have an increased number of neurons in the white matter of their brains. These IWMNs are poorly characterised and their role in normal or pathological brain functions are unknown. One hypothesis that integrates this knowledge is that MIA may trigger the build-up of neurons in the white matter that may in turn have some role in the development of schizophrenia. My project used an animal model of maternal infection to determine if MIA during pregnancy increases the density of IWMNs. The results of these studies will help determine what these white matter neurons actually do and whether they may have some role in the disorderly transmission of brain messages in schizophrenia. In addition, this work will help to show how infections during pregnancy influence brain development and provide new insights into the aetiology of schizophrenia.

1.9 Aims and hypotheses

The main aim of this project was to establish a reliable rodent MIA model of increased IWMN density and GABAergic interneuron alterations. In addition this thesis aimed to determine whether prenatal immune activation at two separate gestational time-points (early verse late gestation) differentially influences the neurobiological phenotypes associated with schizophrenia. In particular, this thesis focuses on the role MIA has on interstitial white matter neurons (IWMNs), microglia (as part of the brain immune response to this insult), and neuro-immune system interactions.

A rat model was utilised to examine MIA induced by PolyI:C in dams at early GD10 versus late gestation and the effects on the brains of the offspring, particularly the presence of schizophrenia-like neuropathology. 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 (Castner et al., 2004). Whilst understanding the behavioural consequences of the MIA model are important, the specific behavioural consequences of this MIA model were not examined in this thesis (a summary of behavioural findings in this particular model was published by Meehan et al. (2017)).

The first aim of this thesis was to identify and characterise IWMNs in the brain of adolescent/early adult rats, including markers and location, subjacent to the frontal cortex of the adult rodent brain. As the schizophrenia literature suggests that IWMNs are altered in post-mortem brain tissue, and there is a strong link

between maternal immune activation and schizophrenia, we also sought to establish whether the density of IWMNs was altered in offspring from dams exposed to a maternal immune activation (MIA) protocol during gestation. To do this, brains were obtained from offspring of dams exposed to MIA, at both GD10 (early) and GD19 (late), and the density of IWMNs determined by immunohistochemistry for known IWMN subtypes. In addition, we aimed to determine if MIA caused changes in cortical expression of genes expressed in GABAergic interneurons.

Following this, as an immune response is central to the MIA model, our second aim was to determine if MIA at either GD10 or GD19 was capable of inducing long term effects on glia, the brain's immune cells, which may be indicative of an altered inflammatory state. Here examined the expression of immune related genes by qPCR, as well as the density of microglia and astrocytes by immunohistochemistry.

Finally, based on recent findings by Sekar et al. (2016) we aimed to determine if the gene expression of *C4* was altered in the prefrontal cortex of offspring from dams exposed to the MIA protocol by qPCR. As *C4* is an important part of the innate immune system, we hypothesised that any long lasting effects of prenatal MIA may involve alterations in *C4*.

1.10 List of publications

I warrant that I have obtained, where necessary, permission from the copyright owners to use any third-party copyright material reproduced in this thesis, or to use any of my own published work in which the copyright is held by another party, such as a publisher or co-author.

Ryan J. Duchatel, Philip Jobling, Brett A Graham, Lauren R. Harms, Patricia T. Michie, Deborah M. Hodgson, Paul A. Tooney. **Increased white matter neuron density in a rat model of maternal immune activation – Implications for schizophrenia.** Progress in Neuro-Psychopharmacology & Biological Psychiatry 2016. **65:**118-126.

Ryan J. Duchatel, Crystal L. Meehan, Lauren R. Harms, Patricia T. Michie, Mark J. Bigland, Doug W. Smith, Frederik R. Walker, Phillip Jobling, Deborah M. Hodgson, Paul A. Tooney. **Late gestation immune activation increases IBA1-positive immunoreactivity levels in the corpus callosum of adult rat offspring.** Psychiatry Research 2018.

Ryan J. Duchatel, Crystal L. Meehan, Lauren R. Harms, Patricia P. Michie, Mark J. Bigland, Douglas W. Smith, Phillip Jobling, Deborah M. Hodgson, Paul A. Tooney. **Increased complement component 4 (C4) gene expression in the cingulate cortex of rats exposed to late gestation immune activation.** Schizophrenia Research 2018.

CHAPTER TWO

Effects of maternal immune activation on somatostatin positive white matter neuron density

Statement of Contribution

Ryan J. Duchatel, Philip Jobling, Brett A Graham, Lauren R. Harms, Patricia T. Michie, Deborah M. Hodgson, Paul A. Tooney. **Increased white matter neuron density in a rat model of maternal immune activation – Implications for schizophrenia.** Progress in Neuro-Psychopharmacology & Biological Psychiatry 2016. **65:**118-126.

*"I attest that Research Higher Degree candidate **Ryan Duchatel**, was the primary contributor to the development of this publication. This extensive contribution included: contributing to the initial design of the study; contributing to the intellectual development of this study; developing, optimising, executing, analysing and interpreting all experiments contained within this publication; and writing the manuscript in full".*

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Ryan Duchatel

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Philip Jobling

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Brett Graham

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Lauren Harms

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Patricia Michie

_____
Deborah Hodgson

_____
Paul Tooney
(Primary PhD Supervisor)

_____
Robert Callister
(Deputy Head of Faculty)

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Increased white matter neuron density in a rat model of maternal immune activation — Implications for schizophrenia

Ryan J. Duchatel [a,c,d,e], Phillip Jobling [a,c,d], Brett A. Graham [a,c,d], Lauren R. Harms [b,e], Patricia T. Michie [b,e], Deborah M. Hodgson [b,e], Paul A. Tooney [a,c,d,e,*]

[a] Preclinical Neurobiology Research Group, School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, Callaghan, NSW 2308, Australia
[b] School of Psychology, Faculty of Science and IT, University of Newcastle, Callaghan, NSW 2308, Australia
[c] Centre for Translational Neuroscience and Mental Health, The University of Newcastle, Callaghan, NSW 2308, Australia
[d] Hunter Medical Research Institute, New Lambton Heights, NSW 2305, Australia
[e] Schizophrenia Research Institute, Sydney, NSW, Australia

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ABSTRACT

Interstitial neurons are located among white matter tracts of the human and rodent brain. Post-mortem studies have identified increased interstitial white matter neuron (IWMN) density in the fibre tracts below the cortex in people with schizophrenia. The current study assesses IWMN pathology in a model of maternal immune activation (MIA); a risk factor for schizophrenia. Experimental MIA was produced by an injection of polyinosinic:polycytidylic acid (Poly I:C) into pregnant rats on gestational day (GD) 10 or GD19. A separate control group received saline injections. The density of neuronal nuclear antigen ($NeuN^+$) and somatostatin (SST^+) IWMNs was determined in the white matter of the corpus callosum in two rostrocaudally adjacent areas in the 12 week old offspring of GD10 ($n = 10$) or GD19 poly I:C dams ($n = 18$) compared to controls ($n = 20$). $NeuN^+$ IWMN density trended to be higher in offspring from dams exposed to poly I:C at GD19, but not GD10. A subpopulation of these $NeuN^+$ IWMNs was shown to express SST. Poly I:C treatment of dams induced a significant increase in the density of SST^+ IWMNs in the offspring when delivered at both gestational stages with more regionally widespread effects observed at GD19. A positive correlation was observed between $NeuN^+$ and SST^+ IWMN density in animals exposed to poly I:C at GD19, but not controls. This is the first study to show that MIA increases IWMN density in adult offspring in a similar manner to that seen in the brain in schizophrenia. This suggests the MIA model will be useful in future studies aimed at probing the relationship between IWMNs and schizophrenia.

1. Introduction

Schizophrenia is thought to have neurodevelopmental origins where genes and environmental factors alter brain development leading to symptoms that emerge in late adolescence/early adulthood (Brown, 2006). The search for an underlying mechanism has identified cortical pathology including alterations to gamma-aminobutyric acid (GABA)ergic interneurons in the brains of people with schizophrenia (Palaniyappan et al., 2012). For example a number of studies in post-mortem tissue observed decreased levels of mRNA and protein for 67 kDa isoform of glutamic acid decarboxylase (GAD67) — a key synthetic enzyme for GABA production (Akbarian et al., 1995; Duncan et al., 2010; Guidotti et al., 2000; Hashimoto et al., 2008a; Thompson et al., 2009). Decreased levels of mRNA for other GABAergic markers including parvalbumin (Fung et al., 2014a; Hashimoto et al., 2003, 2008b), somatostatin (SST) (Fung et al., 2014a; Hashimoto et al., 2008a, 2008b; Morris et al., 2008), and neuropeptide Y (Fung et al., 2014a; Hashimoto et al., 2008a), within prefrontal cortex areas, suggest an inhibitory interneuron pathology in schizophrenia.

Meynert (reviewed in Judas et al. (2010)) first described a population of 'interstitial cells' among the fibre tracts of the adult white matter below the cortex. Dramatic changes in the density of these interstitial white matter neurons (IWMN) have been observed in schizophrenia. Of the 13 studies reported, eight show an increase in IWMN density in the superficial white matter (Akbarian et al., 1996; Anderson et al., 1996; Connor et al., 2009; Eastwood and Harrison, 2003, 2005; Joshi et al., 2012; Kirkpatrick et al., 2003; Yang et al., 2011), three described changes in the deep white matter (Akbarian et al., 1993; Ikeda et al., 2004; Rioux et al., 2003), and two reported no changes (Beasley et al., 2002; Molnar et al., 2003) in IWMN density below the cortex in

Abbreviations: GD, gestational day; GAD, glutamic acid decarboxylase; IL, interleukin; IWMN, interstitial white matter neuron; MIA, maternal immune activation; Poly I:C, polyinosinic:polycytidylic acid; NeuN, neuronal nuclei protein; SST, somatostatin.

* Corresponding author at: School of Biomedical Sciences and Pharmacy, University of Newcastle, University Drive, Callaghan, NSW 2308, Australia.
E-mail addresses: ryan.duchatel@uon.edu.au (R.J. Duchatel), Phillip.Jobling@newcastle.edu.au (P. Jobling), Brett.Graham@newcastle.edu.au (B.A. Graham), Lauren.Harms@newcastle.edu.au (L.R. Harms), Pat.Michie@newcastle.edu.au (P.T. Michie), Deborah.Hodgson@newcastle.edu.au (D.M. Hodgson), paul.tooney@newcastle.edu.au (P.A. Tooney).

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postmortem brains in schizophrenia. This work has focussed largely on IWMN density in frontal brain regions implicated in schizophrenia, including the dorsolateral prefrontal cortex (Akbarian et al., 1993, 1996; Anderson et al., 1996; Eastwood and Harrison, 2003, 2005; Yang et al., 2011), orbitofrontal cortex (Joshi et al., 2012) and anterior cingulate white matter (Connor et al., 2009).

All five studies utilising neuronal nuclei protein (NeuN) to identify IWMNs reported increased density in the brains of schizophrenia subjects (Connor et al., 2009; Eastwood and Harrison, 2003, 2005; Joshi et al., 2012; Yang et al., 2011). Furthermore, approximately 25% of IWMNs are believed to be GABAergic and increased density of GAD65/67 positive ($GAD65/67^+$) (Joshi et al., 2012) and SST^+ (Yang et al., 2011) IWMNs were observed in the superficial white matter of the orbitofrontal cortex and dorsolateral prefrontal cortex respectively in schizophrenia. Recent evidence also suggests that schizophrenia subjects with a high inflammatory profile categorised by the overexpression of interleukin (IL; e.g. IL-1β, IL-6 and IL-8) mRNAs have a more pronounced increase in $NeuN^+$ and $GAD65/67^+$ IWMN density in the orbitofrontal cortex compared to those schizophrenia subjects with a low inflammatory gene expression profile (Fung et al., 2014b).

This raises the possibility that neuroinflammation could have a role in the increased density of IWMN observed in schizophrenia. Indeed, there is a growing body of evidence supporting changes in the expression of genes involved in the immune system and inflammation in the blood and brains of people with schizophrenia (Fillman et al., 2013; Gardiner et al., 2013; Kumarasinghe et al., 2013). In addition, epidemiological studies suggest prenatal exposure to infectious agents including viral pathogens (e.g. influenza (Brown et al., 2004; Mednick et al., 1988), rubella (Brown et al., 2001) and measles (Torrey et al., 1988)), bacterial pathogens (Sorensen et al., 2009), the protozoan *Toxoplasma gondii* (Brown et al., 2005; Mortensen et al., 2007) and reproductive tract infections (Babulas et al., 2006) significantly increases the offspring's risk of developing schizophrenia. Furthermore, animal models that mimic these environmental insults replicate behavioural, structural and cellular brain changes, with strong face and construct validity to schizophrenia (reviewed in Meyer (2014)).

One of these models, maternal immune activation (MIA), involves the administration of the viral mimic polyriboinosinic–polyribocytidylic acid (polyI:C) to pregnant dams. Offspring of dams exposed to polyI:C exhibit numerous neurochemical and brain morphological abnormalities similar to people with schizophrenia (Meyer and Feldon, 2010; Meyer et al., 2009a; Piontkewitz et al., 2011), and with the same maturational delay (Ozawa et al., 2006; Piontkewitz et al., 2011). Rodent models of MIA display alterations in sensorimotor gating, social behaviour, and working memory, as well as increased sensitivity to psychotomimetic drugs (Meyer and Feldon, 2010, 2012; Meyer et al., 2009a). Animals exposed to MIA also exhibit structural brain abnormalities such as increased lateral ventricle volume (Piontkewitz et al., 2011) and decreased cortical brain volumes (Piontkewitz et al., 2009). The gestational timing of maternal immune activation has been shown to be a critical factor in the development of behavioural and cellular changes displayed by the offspring (Boksa, 2010; Meyer and Feldon, 2012). MIA paradigms have primarily focused on early/middle vs. late gestation insults (Meyer and Feldon, 2012). In mouse models, early/middle (gestational day — GD9) polyI:C exposure leads to impaired sensory motor gating and reduced dopamine D1 receptor levels in the prefrontal cortex, whilst late gestation (GD17) exposure potentiates NMDA receptor antagonist locomotor effects and reduced hippocampal NMDA NR1 subunit expression (Meyer et al., 2008). These gestational stages are homologous to the end of the first trimester and middle to end of the second trimester in humans and are thought to model positive and negative/cognitive symptoms of schizophrenia, respectively (Macedo et al., 2012). In contrast, reduction in neurons expressing reelin and parvalbumin, and enhancement of amphetamine-induced locomotion was observed in mice exposed to prenatal polyI:C at both gestational stages (Meyer et al., 2008).

In light of these findings we hypothesised that maternal infection may trigger a change in density of IWMNs in the adult offspring, similar to reports in schizophrenia. It was our expectation that MIA at both gestational stages would alter the density of IWMNs in adult offspring compared to control offspring. To test this, we used polyI:C to induce MIA in pregnant rats, at two different gestational days and studied the effects on IWMNs density in the corpus callosum from the brains of the adult offspring.

2. Methods and materials

2.1. Animals and maternal immune activation using polyI:C

The use and monitoring of animals 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 purposes, and with approval from the University of Newcastle Animal Care and Ethics Committee, Newcastle, Australia (Numbers A-2009-108 and A-2013-319).

Wistar rats were housed individually under a 12-h light/dark cycle, with food and water available ad libitum. Female Wistar rats in the proestrous phase (determined by vaginal impedance reading >3 Ω) were time-mated and the morning of successful mating (determined by presence of sperm using a vaginal smear) designated as GD0. Pregnant females were allocated to one of four groups and received either polyI:C (Sigma-Aldrich, Sydney, AUS) or Phosphate-buffered saline (PBS, Sigma-Aldrich) on GD10 or GD19. Pregnant rats were anaesthetised with isofluorane (Abbott Animal Health, Illinois, USA) and injected with 4 mg/kg body weight polyI:C or an equivalent volume of PBS into the tail vein. MIA was confirmed by measuring IL-6 levels from serum extracted from whole blood taken from the saphenous vein of the dam 2 h after injection of polyI:C using the IL-6 enzyme-linked immunosorbent assay (ELISA) standard protocol (Biorad; Gladesville, AUS). Circulating IL-6 was significantly increased in all dams receiving polyI:C but not dams that received PBS (Supplementary Fig. 1). Offspring were kept in full litters until postnatal day 21 when they were removed from their mothers and kept in same-sex littermate pairs until 12 weeks of age.

2.2. Preparation of brain tissue

At 12 weeks of age, rats were deeply anaesthetised by intraperitoneal injection of 160 mg/kg sodium pentobarbitone (Virbac Animal Health, Milperra, AUS), then transcardially perfused with saline followed by 4% paraformaldehyde (Sigma-Aldrich) in 0.1% Phosphate Buffer. Brains were extracted, post-fixed in 4% paraformaldehyde for 24 h before being stored in Phosphate Buffered Saline (PBS) containing 0.01% sodium azide (Sigma-Aldrich) at 4 °C. Brains were then submerged in PBS with 12.5% sucrose (ChemSupply, Port Adelaide, AUS) overnight at 4 °C, mounted in TissueTek Optimal Temperature Compound (ProSciTech, Kirwan, AUS), frozen at -20 °C and sectioned at 30 μm using a Leica SM2000R microtome (Leica Biosystems, North Ryde, AUS).

2.3. Immunohistochemistry

For diaminobenzidine immunohistochemistry, rat brain sections were processed essentially as described previously (Tooney and Chahl, 2004). Briefly, rat brain sections were treated with 50% ethanol containing 0.9% H_2O_2 for 20 min at room temperature (RT). Sections were washed in triton diluent (0.1% Triton X-100 + 1% normal donkey serum) for 15 min, blocked using 10% normal donkey serum in PBS, and incubated with primary antibodies either mouse anti-NeuN (1:1000, Millipore, California, USA; Cat. #MAB377) or rabbit anti-SST (1:500, Santa Cruz Biotechnology, California, USA; Cat. #sc-139,099) in Triton diluent for 48 h at 4 °C. Sections were next washed in PBS (3 × 15 min) and incubated in donkey anti-mouse (for NeuN — 1:1000,

Jackson ImmunoResearch, Pennsylvania, USA; Cat. #715–065–150) or anti-rabbit (for SST — 1.1000, Jackson ImmunoResearch, Cat. #711–065–152) IgG biotinylated secondary antibody for 1 h at RT. Sections were then washed in PBS (3 × 15 min), incubated at RT (1 h) in the avidin–biotin–peroxidase complex (Vectastain ABC kit; Vector Laboratories, USA), and treated with 3,3′– diaminobenzidine (Sigma-Aldrich; 12 mmol/L final concentration in PBS with 0.03% H_2O_2) for 5–7 min on ice. The tissue was mounted on gelatin-subbed microscope slides and dried overnight before defatting in ascending alcohols (70%, 95% and 100%) for 1 min, counterstained using 0.1% cresyl violet (VWR, Pennsylvania, USA) for 30 s, and differentiated using a series of incubations in ascending alcohols (dH_20, 70%, 95% and 100%) for 3 min. Finally, sections were cleared using Xylene (4 min) and cover-slipped using Ultramount (Thermo Fisher Scientific, Richlands, AUS).

2.4. Immunofluorescence

For immunofluorescence, sections were blocked using 10% normal donkey serum in PBS (30 min at RT), and then incubated in a combination of primary antibodies (mouse anti-NeuN, 1:1000, and rabbit anti-SST, 1:1000) overnight at 4 °C. Sections were next washed with PBS (3 × 15 min) and incubated for 1 h at RT with species-specific secondary antibody (donkey anti-mouse FITC, 1:1000, Jackson ImmunoResearch, Cat. #715–095–151 or donkey anti-rabbit Cy3, 1:1000, Jackson ImmunoResearch, Cat. #711–095–152). Finally, sections were washed with 4′,6-diamindino-2-phenylindole (DAPI — 1.1000, Life Technologies, Mulgrave, AUS) in 0.1% NaCl for 5 min at RT to identify nuclear lipofuscin fluorescence, mounted on gelatin-subbed microscope slides and then cover-slipped.

2.5. Neuronal counting

Images of sections immunolabelled for $NeuN^+$ and SST^+ using diaminobenzidine immunohistochemistry were captured using the Aperio™ Digital Pathology System (Leica Biosystems) at 20X magnification. The grey/white matter boundary of the rat corpus callosum was identified from the cresyl violet counterstain, as well as the distribution of $NeuN^+$ stained neurons which decreased abruptly at the transition from grey to white matter (Supplementary Fig. 2). The white matter of the corpus callosum was delineated by manual tracing using the Aperio ImageScope™ software (Version 11.1.2.760 — Leica Biosystems) and the area of white matter calculated in each section. Sections were pooled into two regions: region 1; 3.2 mm-2.5 mm from Bregma representing the most rostral region of the rat brain, where the forceps minor of the corpus callosum is visible; and region 2; 2.3 mm-0.70 mm from Bregma, where the main body of the corpus callosum is present. Four sections sampled 180 μm apart in region 1 and 2 were used to count $NeuN^+$ or SST^+ IWMNs in the entire corpus callosum (Supplementary Fig. 3). Those immunopositive IWMNs touching the grey/white matter boundary were not counted. The final $NeuN^+$ and SST^+ IWMN density was calculated as the mean of the sections counted for each region in each animal. Investigators were blind to the treatment status (i.e. MIA or vehicle) throughout experimental, analysis and quantification steps.

2.6. Statistical analysis

GraphPad Prism 6 software (GraphPad, California, USA) was used to analyse data and produce descriptive statistics, including the mean and standard deviation (presented as mean ± SD). All other statistics are reported as the true p-value, where $p < 0.05$ was considered statistically significant. Grouped data sets were tested for normality. One-way ANOVA with Bonferroni multiple comparisons were used to compare IWMN density between control and polyI:C GD10 and GD19 rats for each region separately. Two-way ANOVA with Bonferroni multiple comparisons were used to examine the impact of sex on IWMN density in the GD19 polyI:C cohort compared to controls. Pearson correlation was used to determine the relationship between $NeuN^+$ and SST^+ IWMN density and a two-tailed Z-test was used to determine if there was a significant effect of treatment status on the correlation. As the IWMN densities between saline treated rats at GD10 and GD19 were not significantly different ($p = 0.80$), they were pooled into a single control group. This pooling resulted in a total of 23 rats (13 M, 10 F) in the control group, 8 in the GD10 group (3 M, 5 F) and 19 in the GD19 group (7 M, 12 F). These sample sizes were sufficient to detect a large effect size (≈0.45) equivalent to approximately a 20% change in NeuN density (assuming SD of 4.5) and approximately 35% change in SST^+ IWMN density (SD of 1.2) in the MIA groups relative to the control group with power of 80%.

3. Results

3.1. Effects of maternal immune activation on $NeuN^+$ IWMN density in offspring

$NeuN^+$ IWMNs were identified within the white matter of the corpus callosum in offspring of controls ($n = 20$) and dams exposed to polyI:C (hereafter referred to as polyI:C rats) at GD10 ($n = 10$) and GD19 ($n = 18$) (Fig. 1A–B).

Overall, one-way ANOVA showed a significant group difference in $NeuN^+$ IWMN density [$F(2,43) = 3.96$, $p = 0.026$] in region 1, but not in region 2 [$F(2,46) = 0.72$, $p = 0.49$]. The mean density of $NeuN^+$ IWMNs was not changed in GD10 polyI:C rats, in either region 1 (18.26 ± 3.14 cells/mm^2 vs. 19.40 ± 3.69 cells/mm^2, $p = 0.99$, Fig. 1C) or region 2 (16.27 ± 3.39 cells/mm^2 vs. 17.45 ± 3.27 cells/mm^2, $p = 0.99$, Fig. 1D) compared to controls. In GD19 polyI:C rats, $NeuN^+$ IWMN density in region 1 was ~17% higher compared to controls (22.80 ± 5.63 cells/mm^2 vs. 19.40 ± 3.69 cells/mm^2, respectively), which approached statistical significance ($p = 0.07$; Fig. 1C). There was no difference in $NeuN^+$ IWMN density in region 2 in GD19 polyI:C rats (18.06 ± 3.89 cells/mm^2 vs. 17.45 ± 3.27 cells/mm^2, $p = 0.99$; Fig. 1D). Increased $NeuN^+$ IWMN density approached significance in GD19 polyI:C rats compared to GD10 polyI:C rats in region 1 (22.80 ± 5.63 cells/mm^2 vs. 18.26 ± 3.14 cells/mm^2, $p = 0.06$, Fig. 1C), but not region 2 (18.06 ± 3.89 cells/mm^2 vs. 16.27 ± 3.39 cells/mm^2, $p = 0.71$, Fig. 1D).

3.2. Effects of maternal immune activation on SST^+ IWMN density in offspring

Given Yang et al. (2011) reported SST^+ IWMNs were increased in schizophrenia, we examined the effect of MIA on SST^+ IWMN density. Using dual-label immunofluorescence in six control animals (Fig. 2), we determined that ~15% of rat $NeuN^+$ IWMNs also expressed SST (113 of 743 cells).

Overall, one-way ANOVA showed a significant group difference in SST^+ IWMNs in region 1 [$F(2,47) = 7.28$, $p = 0.002$], and region 2 [$F(2,46) = 3.32$, $p = 0.045$]. The mean density of SST^+ IWMNs was significantly increased by 49% (4.66 ± 1.39 cells/mm^2 vs. 3.13 ± 1.14 cells/mm^2; $p = 0.008$; Fig. 3) in region 1 of GD10 polyI:C rats ($n = 8$), compared to controls ($n = 23$). No change was observed in SST^+ IWMNs density in region 2 of GD10 polyI:C rats, compared to controls (3.71 ± 1.09 cells/mm^2 vs. 3.16 ± 0.99 cells/mm^2; $p = 0.57$; Fig. 3). However, in GD19 polyI:C rats ($n = 19$), the mean density of SST^+ IWMNs was significantly higher when compared to controls in region 1 (~35%; 4.24 ± 1.10 cells/mm^2 vs. 3.13 ± 1.14 cells/mm^2; $p = 0.011$; Fig. 3C), and region 2 (~25%; 3.96 ± 0.97 cells/mm^2 vs. 3.16 ± 0.99 cells/mm^2; $p = 0.045$; Fig. 3D). There was no difference between GD19 polyI:C rats compared to GD10 polyI:C rats in either region 1 (4.24 ± 1.10 cells/mm^2 vs. 4.66 ± 1.39 cells/mm^2; $p = 0.99$) or region 2 (3.96 ± 0.97 cells/mm^2 vs. 3.71 ± 1.09 cells/mm^2; $p = 0.99$).

The mean density of SST^+ IWMNs from immunohistochemistry experiments was significantly less than $NeuN^+$ IWMNs ($p = 0.001$).

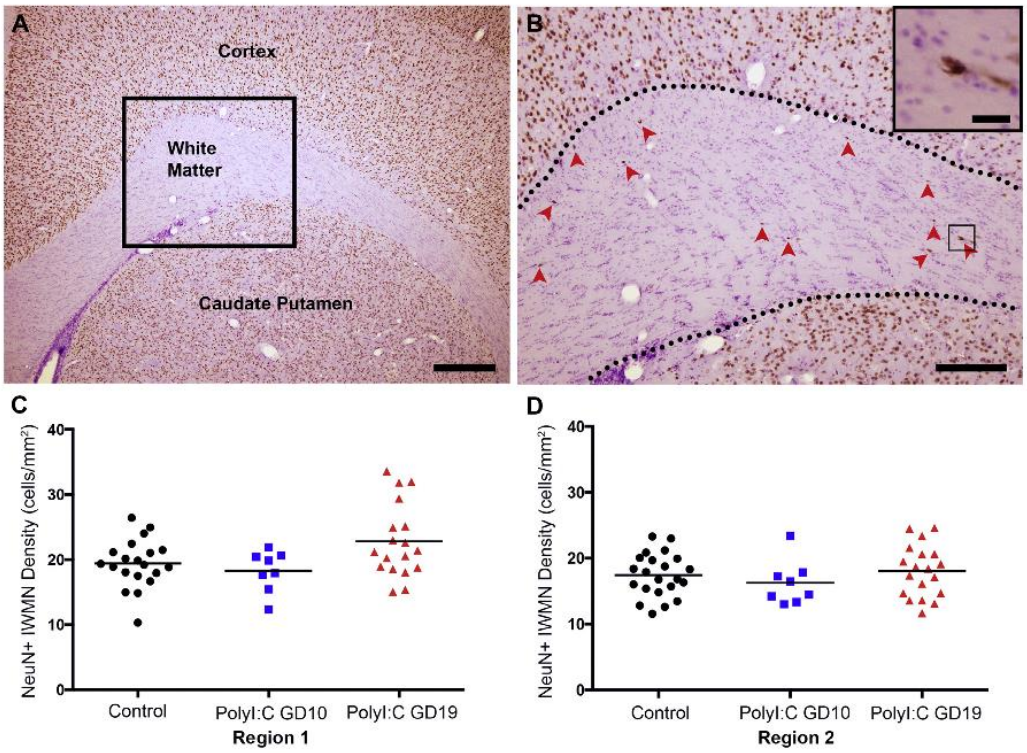

Fig. 1. Affects of maternal immune activation (MIA) on neuronal nuclear antigen immunopositive ($NeuN^+$) interstitial white matter neurons (IWMNs) in the rat corpus callosum. Representative images of $NeuN^+$ neurons in the cortex and white matter of the corpus callosum in control rats (A–B). Boxed area in A is shown at higher magnification in B. Red arrowheads indicate representative $NeuN^+$ IWMNs that were quantified. (C–D) Density of $NeuN^+$ IWMNs assessed in the corpus callosum from offspring of animals exposed to MIA via polyinosinic:polycytidylic acid (polyI:C) treatment at either gestational day 10 (GD10) (blue) or GD19 (red), and controls (black). Each data point represents the mean density (cells/mm^2) from one animal. Comparisons are presented for two rostrocaudally adjacent areas, (C) region 1 and (D) region 2. The density of $NeuN^+$ IWMNs was similar in control and MIA rats. Scale bar: A = 200 μm, B = 100 μm, inset = 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The average mean ratio of SST^+ IWMN density to $NeuN^+$ IWMN density for each animal was calculated to determine the percentage of SST^+ cells/IWMN in control (~16%), GD10 polyI:C rats (~26%) and GD19 polyI:C rats (~20%).

3.3. *Impact of sex on IWMN density after maternal immune activation*

Since males and females with schizophrenia can have different courses in their illness (Zhang et al., 2012), we examined the impact of sex on IWMN density after MIA. Due to the small numbers of rats in the GD10 group and the more widespread changes in IWMN density in GD19 polyI:C rats, we examined sex differences within the GD19 polyI:C cohort (Fig. 4). We used a two-way ANOVA where IWMN density was the dependent variable with between subject factors of polyI:C treatment and sex. For $NeuN^+$ IWMN density in region 1, this analysis identified a significant overall effect of treatment [$F(1,38) = 4.48$; $p = 0.042$] confirming the strong trend observed in Fig. 1, but no main sex effect [$F(1,38) = 1.621$; $p = 0.212$] and no interaction of treatment with sex [$F(1,38) = 0.205$; $p = 0.584$] (Fig. 4). In region 2, there was no main effect of treatment [$F(1,41) = 0.122$; $p = 0.729$] or sex [$F(1,41) = 3.04$; $p = 0.089$] on $NeuN^+$ IWMN density, nor any interaction [$F(1,41) = 0.279$; $p = 0.6$] (Fig. 4).

For SST^+ IWMN density, this analysis confirmed the significant effect of treatment [$F(1,42) = 8.323$; $p = 0.006$] observed in Fig. 3

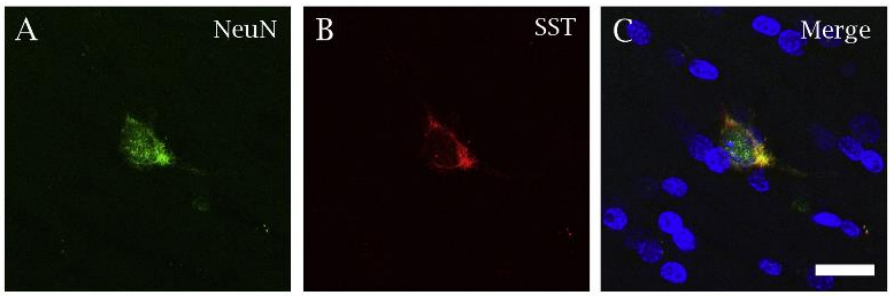

Fig. 2. Somatostatin immunopositive (SST^+) interstitial white matter neurons (IWMNs) are a subset of mature neuronal nuclear antigen immunopositive ($NeuN^+$) IWMNs. Images show immunofluorescent labelling for NeuN (A; green), SST (B; red), and merge (C; yellow) with 4′,6-diamindino-2-phenylindole nuclear counterstaining (blue) in a representative IWMN from region 1 of control animals. Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

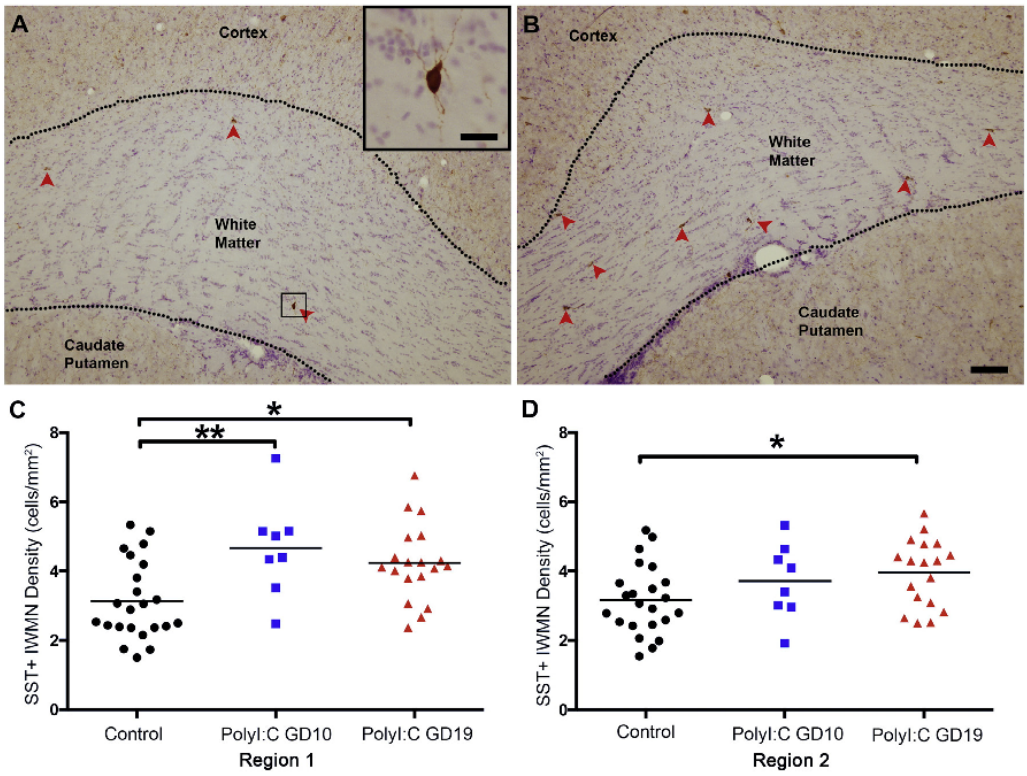

Fig. 3. Effects of maternal immune activation (MIA) on somatostatin immunopositive (SST^+) interstitial white matter neurons (IWMNs) in the rat corpus callosum. Representative SST^+ neurons in the white matter of control rats (A) and from offspring of animals exposed to polyinosinic:polycytidylic acid (polyI:C) at gestation day 19 (GD19) (B). IWMN in boxed area in A is shown at higher magnification in the inset. Red arrows indicate the SST^+ interstitial white matter neurons that were quantified. Density of SST^+ IWMNs (cells/mm^2) in the corpus callosum in the offspring of animals exposed to polyI:C at either GD10 (blue) or GD19 (red) compared to controls (black). Each data point represents the mean density (cells/mm^2) from one animal. Comparisons are presented for two rostrocaudally adjacent areas in region 1 (C) and region 2 (D). SST^+ IWMNs were increased in density after MIA at GD10 in region 1 and GD19 in both region 1 and region 2 compared to vehicle controls ($^*p < .05$, $^{**}p < 0.01$). Scale bar: A–B = 100 μm, inset = 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

plus a strong trend effect of sex [$F(1,42) = 3.751$; $p = 0.06$], but no interaction [($F(1,42) = 0.686$); $p = 0.413$] (Fig. 4). In region 2, a significant effect of treatment [$F(1,41) = 5.679$; $p = 0.022$] and sex [$F(1,41) = 4.188$; $p = 0.048$] on SST^+ IWMN density was observed, but no interaction [$F(1,41) = 1.875$; $p = 0.179$] (Fig. 4). This suggests that female rats have higher density of SST^+ IWMNs than male rats. The impact of sex on IWMN density after MIA at GD10 was not analysed due to the smaller size of the GD10 polyI:C cohort.

3.4. Correlation of $NeuN^+$ and SST^+ IWMN density

We then examined the relationship between $NeuN^+$ and SST^+ IWMNs across the whole dataset. Whilst there was no relationship between $NeuN^+$ and SST^+ IWMN density in the control group alone ($r = -0.05$, $p = 0.828$; Fig. 5), a strong positive correlation between $NeuN^+$ and SST^+ IWMN density was evident in the GD19 polyI:C data alone ($r = 0.53$, $p = 0.0009$; Fig. 5). The correlation in the GD19 polyI:C group was significantly higher than the correlation in the control group ($z = 2.61$, $p = 0.0091$). Correlations were not examined in the GD10 group due to the smaller sample size.

4. Discussion

This study was provoked by the increased density of IWMNs reported in the brains of people with schizophrenia (Akbarian et al., 1996; Anderson et al., 1996; Connor et al., 2009; Eastwood and Harrison, 2003, 2005; Joshi et al., 2012; Kirkpatrick et al., 2003; Yang et al., 2011). Epidemiological evidence also suggests that infection during pregnancy increases the risk of offspring developing schizophrenia (Fillman et al., 2013; Gardiner et al., 2013; Kumarasinghe et al., 2013). Here we show that mimicking immune activation caused by a viral infection with a single injection of PolyI:C during late pregnancy in rats increased the density of IWMNs particularly those that contain SST, in the corpus callosum of adult offspring.

Before discussing the implications of our findings on IWMNs in the MIA model, some methodological considerations concerning the measurement of cell density are important. Previous studies of IWMNs in humans distinguished between superficial and deep white matter. Since the rodent white matter is significantly smaller in volume compared to humans and the corpus callosum is directly bounded by cortical regions and subcortical structures, we did not distinguish between superficial and deep white matter in this study. Furthermore, IWMNs were non-uniformly distributed and at much lower density in our rodent brains compared to humans. Consequently, we used a two-dimensional protocol for measurement of cell density on non-serial sections to avoid double counting of neurons. This is consistent with some previous studies measuring IWMN density in human brains (Beasley et al., 2002; Connor et al., 2009; Eastwood and Harrison, 2003, 2005).

$NeuN^+$ IWMNs are consistently reported as increased in subjects with schizophrenia. Our power was insufficient to detect significant changes in our data. Nonetheless, we identified a strong trend towards increased $NeuN^+$ IWMN density in the most rostral region (region 1–17%) of the brain in the polyI:C GD19 rats that is similar to the averaged increase (~22%) from all studies using NeuN in schizophrenia (Connor

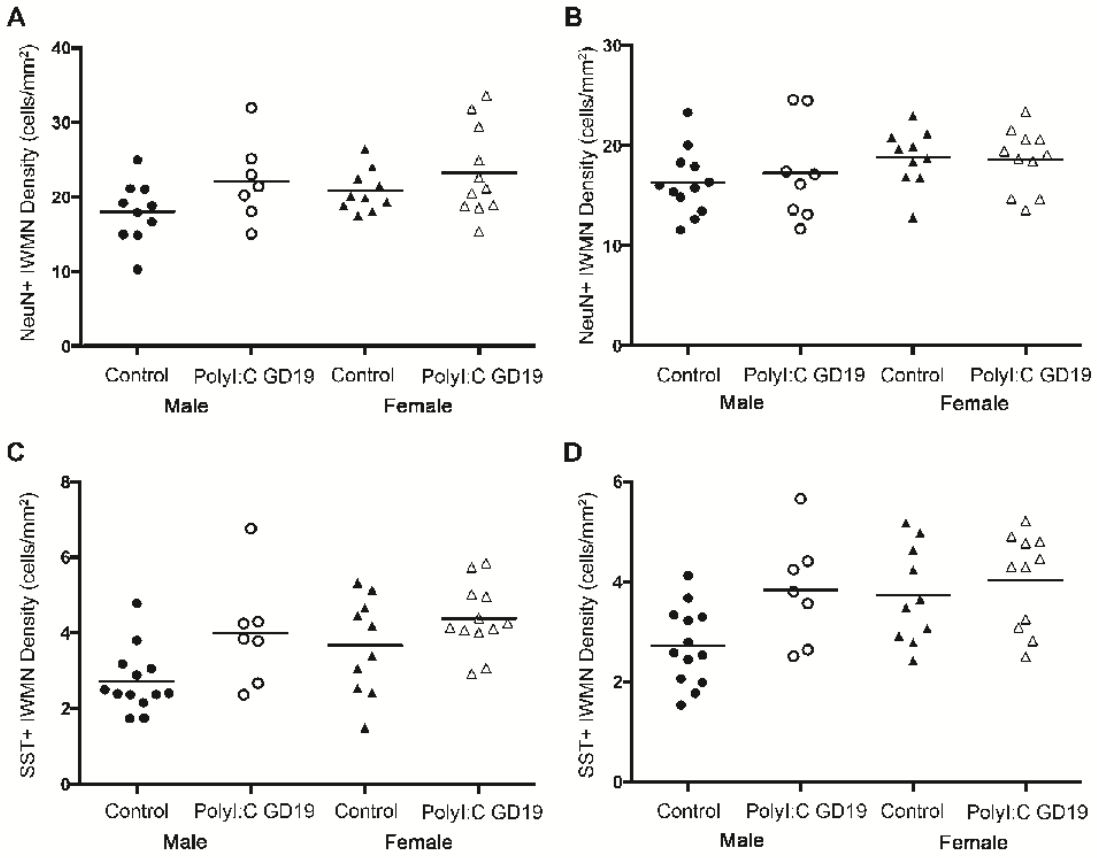

Fig. 4. Effects of sex on interstitial white matter neuron density (IWMN) after maternal immune activation (MIA). Plots compare IWMN density for each sex, assessed by neuronal nuclei protein (NeuN) immunolabelling (A–B), and somatostatin (SST) immunolabelling (C–D). Each data point represents the mean density (cells/mm^2) from one animal in the corpus callosum from male (circles) and female (triangles) offspring of animals exposed to polyinosinic:polycytidylic acid (Polyl:C) at gestation day 19 (GD19). Sex did not affect the density of NeuN$^+$ IWMNs after Polyl:C treatment in either region 1 (A; $p = 0.212$) or 2 (B; $p = 0.729$). Female rats appear to have a higher density of SST$^+$ IWMNs that was a strong trend in region 1 (C; $p = 0.06$) and a significant effect in region 2 (D; $p = 0.048$).

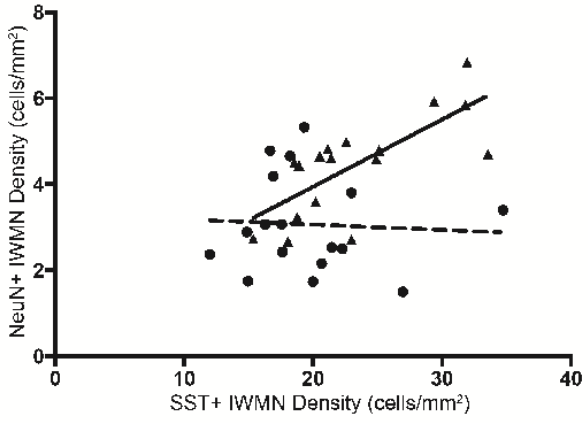

Fig. 5. Correlation of neuronal nuclei protein immunopositive (NeuN$^+$) and somatostatin immunopositive (SST$^+$) interstitial white matter neuron (IWMN) density. NeuN$^+$ IWMN density (cells/mm^2) positively correlates with SST$^+$ IWMN density in offspring from rats exposed to polyinosinic:polycytidylic acid (polyI:C) at gestation day 19 (GD19) (triangles, solid line $r = 0.53$, $p = 0.0009$), but not in the control cohort (dashed line $-r = \quad 0.05$, $p = 0.828$). The correlation within the GD19 polyI:C group was significantly higher than the correlation between the control group ($z = 2.61$, $p = 0.0091$).

et al., 2009; Eastwood and Harrison, 2003, 2005; Joshi et al., 2012; Yang et al., 2011). Indeed, Joshi et al. (2012) used the largest sample size to date ($n = 38$) and reported a 24% increase in NeuN$^+$ IWMN density in the orbitofrontal cortex in schizophrenia. Joshi et al. (2012) also reported a significant increase in GAD65/67$^+$ IWMN density in the orbitofrontal cortex in schizophrenia. We used two antibodies to investigate GAD65/67$^+$ IWMNs in our rat model, however, whilst GAD65/67$^+$ dendrites and boutons were observed in the corpus callosum, detection of GAD65/67$^+$ IWMN cell soma was not reliable enough to determine the effect of MIA on the density of GAD65/67$^+$ IWMN in our rat model.

In contrast, the effect of MIA on the density of SST$^+$ IWMNs was striking suggesting this subpopulation is highly susceptible to prenatal immune challenge. Increased density of SST$^+$ IWMNs was observed in polyI:C GD10 rats in region 1 (49%) with more widespread changes in polyI:C GD19 rats where increased density was recorded in both region 1 (35%) and region 2 (25%). Given this elevated SST$^+$ IWMN density one might have expected a related increase in NeuN$^+$ IWMN density, however, the relative density of SST$^+$ IWMNs was low compared with NeuN$^+$ IWMNs. Interestingly, Yang et al. (2011) observed a 54% increase in SST mRNA$^+$ IWMNs in subjects with schizophrenia. Thus while MIA does not produce a dramatic change in the overall number of IWMNs, this developmental perturbation may activate SST mRNA transcription leading to the de novo expression of SST protein in NeuN$^+$ IWMNs representing an expansion of the SST$^+$ population.

Indeed, Fung et al. (2010) reported decreased SST^+ mRNA in the cortex in subjects with schizophrenia. In this study we did not measure the levels of SST^+ mRNA nor SST^+ neurons in the cortex in our rats, thus whether MIA affects cortical neurons remains to be determined. Furthermore, as mentioned above, to date only the SST^+ (Yang et al., 2011), $GAD65/67^+$ (Joshi et al., 2012) and NPY^+ (Ikeda et al., 2004) GABAergic IWMN subpopulations have been examined in work on human brain, with increases in $GAD65/67^+$ (Joshi et al., 2012) and SST^+ (Yang et al., 2011) IWMN density reported in schizophrenia. Therefore, the human data suggests not all subsets of IWMNs are affected in the same way in the brains of people with schizophrenia. It is currently unknown if SST^+ IWMNs in the rat brain are specifically susceptible to prenatal immune challenge or whether other subsets of GABAergic IWMNs are also affected.

Considered together, our data suggests that whilst rat SST^+ IWMNs are increased in density after MIA at GD10 and GD19, the effects of prenatal immune challenge appear to be more widespread in the GD19 animals where the increase was observed in both region 1 and 2. Related to this, SST^+ interneurons are born from the medial ganglionic eminence (MGE) during development (Sussel et al., 1999; Xu et al., 2008). Studies suggest these interneurons begin migrating from the MGE towards the cortex at embryonic day (E) 14, with a larger cohort migrating from E15–17 in rats (Metin et al., 2006). Therefore, immune activation triggered by PolyI:C at GD10 comes four days before the smaller cohort of neurons are born and emerge from the MGE possibly accounting for the more regionally restricted effects on IWMN density at this developmental stage. In contrast, MIA at GD19 may have more widespread effects on IWMN density since it occurred after the larger cohort of MGE-derived neurons were born and whilst they were migrating. Additional experiments investigating the origin of IWMNs in the brain, the timing of the MIA and changes in WMN density are required to shed further light on the importance of these temporal relationships.

In consideration of their origin, all IWMNs that expressed SST in our study also expressed $NeuN^+$, suggesting they have a mature neuronal phenotype. In contrast, Yang et al. (2011), observed a small number of SST^+ IWMNs in human brains that did not express NeuN and suggested that these IWMNs were developmentally immature. This is critical when trying to determine the origin of these neurons in different species as some assume IWMNs are remnants of the subplate that gives rise to cortical neuronal populations during development. Specifically, most subplate neurons undergo programmed cell death after the dissolution of the subplate, with a small population of these neurons surviving as IWMNs into adulthood (Kostovic et al., 2011). This is somewhat at odds with our data, which suggests all SST^+ IWMNs in the rat brain have a mature phenotype.

An alternative to this 'developmental vestige' theory suggests IWMNs may represent neurons actively migrating towards the cortex in response to cortical neuronal damage (Yang et al., 2011). From rodent models, it is evident that certain forms of brain injury upregulate neurogenesis in the subependymal zone, with new neurons migrating through the subcortical white matter to the cortex (Gu et al., 2000; Li et al., 2010; Magavi et al., 2000; Ohira, 2011). Thus a neurogenic and potentially restorative response is possible within the human brain. In addition, a study by Meechan et al. (2009), showed that the 22q11.2 deletion, a genetic lesion associated with schizophrenia, disrupted early brain development by altering the normal distribution and migration of neurons, suggesting altered neurogenesis may be a feature of schizophrenia. Future studies that manipulate neuronal migration in the MIA model will be required to determine if the increased density of IWMNs represents a neuroprotective process.

The timing of maternal immune activation in animal models suggest that both early and mid/late gestational insults result in changes to the brain and behaviour of offspring that may relate to schizophrenia (Meyer et al., 2009b). We observed a highly significant increase in SST^+ IWMN density after polyI:C exposure at GD10. This is interesting given that Brown et al. (2004) reported a seven fold increase in the risk of developing schizophrenia in the offspring of mothers exposed to influenza infection during their first trimester. In contrast, other studies suggest the more regionally widespread changes observed after MIA at GD19 in this study are also consistent with human epidemiological evidence regarding the timing of infection. Indeed, maternal influenza infection was first linked to increased risk of schizophrenia by Mednick et al. (1988), who observed that the offspring of women in their second trimester of pregnancy during the Helsinki 1957 influenza epidemic, had increased risk of developing schizophrenia. This has been supported by several other studies (reviewed in Brown and Derkits (2010)). We observed significant changes in SST^+ IWMN density, and a strong trend in $NeuN^+$ IWMN at GD19 (i.e. late gestation). Interestingly, insults later in gestation may be more representative of a negative/cognitive deficit symptomology (Macedo et al., 2012), thus increased IWMN density may be a more pronounced feature in people with negative/cognitive deficit symptoms of schizophrenia. This has support from the human IWMN literature where increased $MAP2^+$ IWMN density was observed in a small number of schizophrenia cases with negative symptoms compared to those with positive symptoms (Kirkpatrick et al., 2003), however this has not been validated in a larger data set.

Several studies have shown people with schizophrenia have an underlying immune signature (Gardiner et al., 2013; Kumarasinghe et al., 2013), with observations of increased expression of pro-inflammatory genes such as IL-6 in the prefrontal cortex (Fillman et al., 2013). In addition, higher cortical IL-6 expression (i.e. indicative of a 'high cortical inflammatory state') positively correlated with IWMN density in the orbitofrontal cortex in schizophrenia (Fung et al., 2014b). This could suggest the level of cortical inflammation may reflect the numbers of IWMNs recruited in response to cortical damage. We observed an increase in IL-6 levels in serum from pregnant dams 2 h after polyI:C injection, but no discernable grouping of high versus low IL-6 levels, and no significant correlation between dam IL-6 and IWMN density (data not shown). We measured IL-6 protein in serum to confirm MIA in dams; we have not examined IL-6 gene expression in the brains of MIA affected offspring. Thus, we cannot rule out the possibility of longer-term changes in cytokine mRNA expression following MIA.

Whilst there was no overall effect of sex on IWMN density because our sample size when split on gender lacked statistical power, female rats generally had a higher density of IWMNs, particularly SST^+ IWMNs, than males. As a result, the increase in SST^+ IWMN density observed in our study was mostly due to the larger difference observed in males after MIA at GD19 (Fig. 4). Furthermore, MIA at GD19 increased the IWMN density in males bringing it closer to levels that were observed in females, suggesting that MIA attenuated any effects of sex on SST^+ IWMNs. Joshi et al. (2012) also noted a gender-related trend towards higher density of $NeuN^+$ IWMNs in females versus males with schizophrenia. Whilst males are more likely to have an earlier onset of schizophrenia, and a more chronic disorder than females (Zhang et al., 2012), whether this is related to changes in IWMN density remains to be determined.

This study provides evidence for the first time that infection during pregnancy is one factor that contributes to increased IWMN density in the brains of the adult offspring, particularly affecting the subpopulation that expresses SST. This MIA model provides a platform to study the mechanisms by which maternal immune activation affects the white matter and its cellular components in the brains of offspring, and the consequences of these changes to normal brain function. The implications and mechanisms underlying IWMNs accumulation in the subcortical white matter of people with schizophrenia remain to be determined, as does the issue of whether this phenomenon is causal or a consequence of schizophrenia. Our data suggest that future studies in the MIA model will greatly enhance our ability to address these questions.

Conflicts of interest

None.

Contributors

PAT, RJD, PJ, BAG designed the study. RJD conducted all experiments. LRH, PTM and DMH developed the model of maternal immune activation and provided animals from this model. RJD developed the figures and wrote the first draft of the manuscript. All authors contributed to writing the manuscript and approved the final version of the manuscript.

Role of the funding sources

The funding sources listed in the acknowledgements had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pnpbp.2015.09.006.

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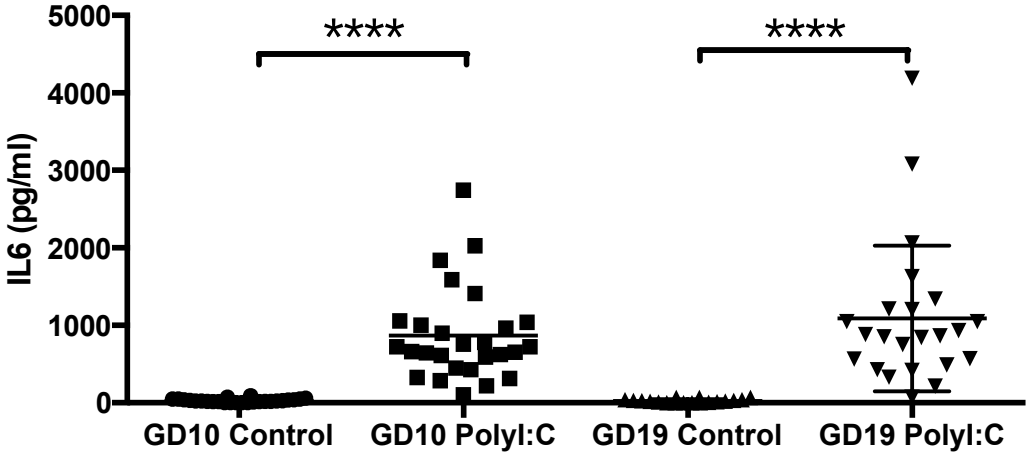
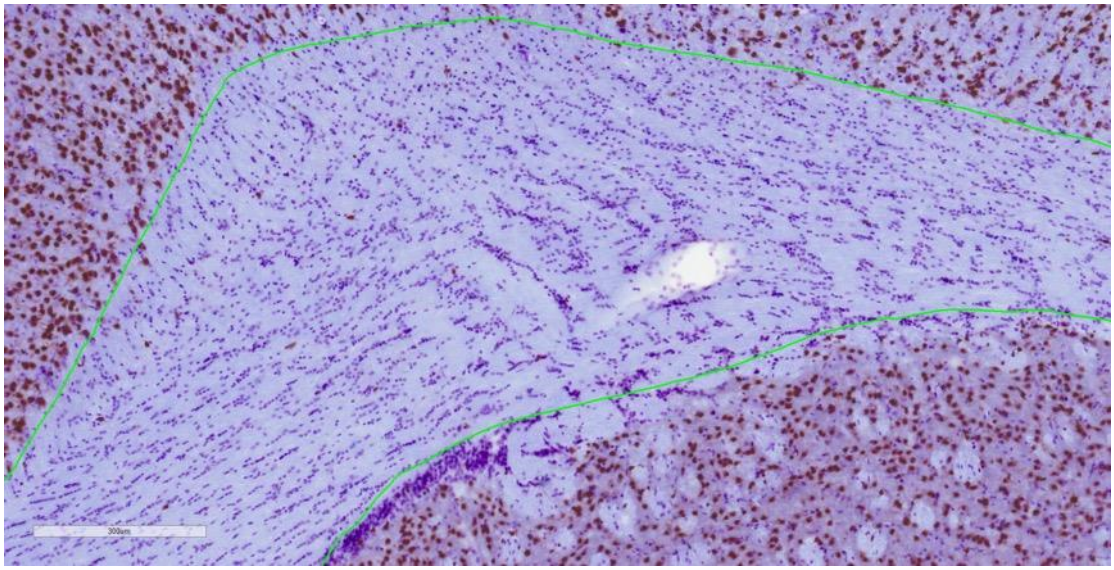
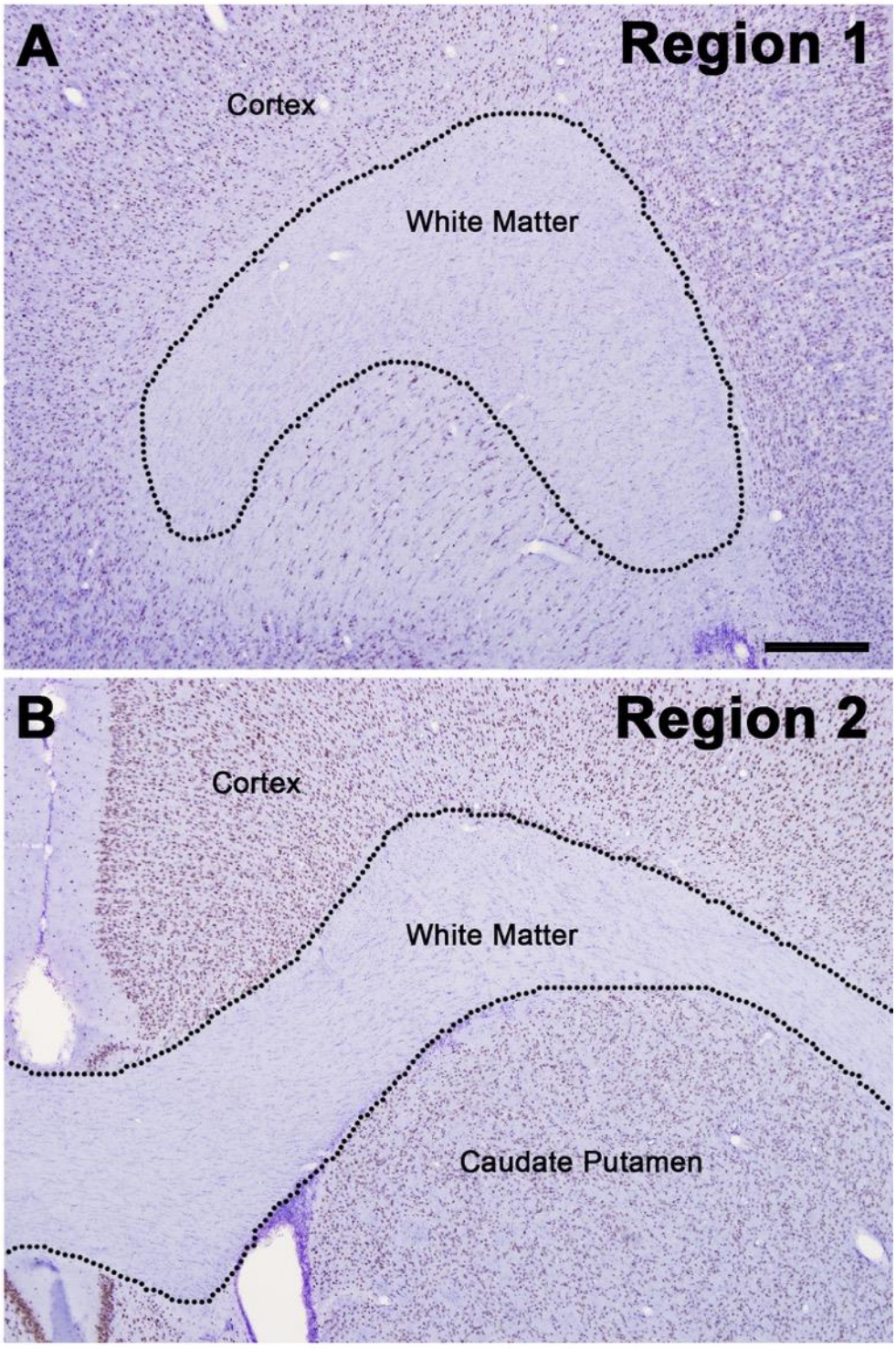
Supplementary Figures



Supplementary Fig. 1 IL-6 levels in dams 2 h after injection of PolyI:C determined by ELISA. IL-6 was significantly increased in dams exposed to PolyI:C at both GD10 and GD19. There was no significant difference in IL-6 levels between the dams exposed to PolyI:C at GD10 or GD19 (p = 0.48). **** p = < 0.0001.



Supplementary Fig. 2 Aperio^{TM} annotated region of white matter in the rat brain. Using the Aperio^{TM} ImageScope analysis software, the region of interest was "annotated" or selected (Green outline) in each section that was counted for each animal. The boarder was drawn based on the cresyl violet counterstain, where the white matter stays more white than the purple cortex, as well as a clear distinction of NeuN^{+} cells in the cortex and caudate putamen.



Supplementary Fig. 3 Regions of white matter in the rodent brain where IWMN density was determined. Sections of rat brain were immunolabelled with antibodies to NeuN using DAB IHC and counterstained with cresyl violet. **(A)** shows region 1 between 3.20 - 1.70mm from Bregma, and **(B)** shows region 2 between 1.60 and 0.20mm from Bregma. Scale bar: 400µm

CHAPTER THREE

Effects of MIA on the density of additional white matter neuron subtypes

3.1 Introduction

In Chapter 2, I reported that SST+ IWMN density was increased after GD10 and GD19 PolyI:C MIA. Indeed, it has been shown that IWMNs express a number of markers congruent with cortical GABAergic interneurons (reviewed in Chapter 1). This chapter aims to elucidate whether other IWMN subtypes are altered by MIA.

GABAergic interneurons are the main inhibitory neuron in the brain. These interneurons can be identified by the expression of various markers such as glutamic acid decarboxylase (GAD; the enzyme that synthesises GABA), calcium binding proteins; parvalbumin (Pv) and calretinin (Cr) and neuropeptides such as neuropeptide Y (NPY) and SST (Lewis et al., 2012). Indeed, alterations to GABAergic interneuron markers is the longest standing and most central pathology implicated in schizophrenia. These include a reduction of GAD mRNA and protein (Akbarian et al., 1995; Duncan et al., 2010; Guidotti et al., 2000; Hashimoto et al., 2008a; Thompson et al., 2009), Pv mRNA (Fung et al., 2014a; Hashimoto et al., 2008b; Hashimoto et al., 2003), SST+ mRNA (Fung et al., 2014a; Hashimoto et al., 2008a; Hashimoto et al., 2008b; Morris et al., 2008), and NPY mRNA (Fung et al., 2014a; Hashimoto et al., 2008a), suggesting that an inhibitory interneuron pathology in schizophrenia may be more expansive that previously thought.

One of the major functions of GABA-mediated transmission in the cortical grey matter is in producing synchronised network oscillations. These oscillations are thought to be essential for normal cognitive function in humans (Gonzalez-Burgos et al., 2011; Lewis et al., 2012), and driven by Pv-positive interneurons (Lewis et

al., 2012). In particular, Pv+ interneurons exert inhibitory synaptic effects on firing and synchronisation of pyramidal neurons, which in turn effects the development of cognitive functions associated with the prefrontal cortex (Bartos et al., 2007; Behrens and Sejnowski, 2009; Salinas and Sejnowski, 2001). Further evidence was provided in rats treated with methylazoxymethanol acetate (an alternative immune model used to establish schizophrenia like neuropathology in rodents – (for a review see Lodge and Grace (2009)) that were shown to have a decreased density of Pv+ interneurons in the medial prefrontal cortex, which coincided with reduced GABA-mediated inhibition and diminished gamma oscillations (Lodge et al., 2009).

Post-mortem studies have been conducted that have identified changes at a cellular level to GABAergic interneurons in the cortex in schizophrenia. Indeed, a consistently reduced level of GAD67 protein and mRNA in interneurons from the cortex in schizophrenia is the most robust finding reported in post-mortem studies (Gonzalez-Burgos et al., 2011; Lewis, 2011). Decreased GAD67 protein (Curley et al., 2011; Guidotti et al., 2000) and mRNA (Gonzalez-Burgos et al., 2010) has been shown in the DLFPC where levels are markedly lower in 25-35% of interneurons (Akbarian et al., 1995; Volk et al., 2000), with similar deficits shown in other cortical regions such as the sensory and motor cortices as well as limbic regions (Thompson et al., 2009). Decreased GAD67 protein and mRNA has also been shown in a variety of subsets of interneurons, including those that express Pv (Lewis et al., 2012). Indeed one study showed that GAD67 mRNA was not expressed in half the interneurons that are immunoreactive for Pv (Hashimoto et al., 2003).

Whilst, most IWMN studies have focused on general neuronal markers (MAP2/NADPH/NeuN – Reviewed in Chapter 1), there have only been three studies to date examining the densities of known GABAergic markers in IWMNs. These studies include SST (Yang et al., 2011b), GAD (Joshi et al., 2012) and NPY (Ikeda et al., 2004). In particular, SST+ and GAD+ IWMNs were shown to be altered in people with schizophrenia, with NPY remaining unchanged. Having previously identified that SST+ IWMNs were altered by MIA, in this chapter I aimed to determine if other GABAergic IWMN markers may be altered by MIA, and what effects MIA had on cortical GABAergic gene expression, with a view to determine if MIA specifically affects the SST+ IWMN subpopulation.

3.2 Methods

3.2.1 Animals

Animals utilised in this study are outlined in Chapter 2 and Chapter 4. Briefly, Pregnant Wistar rats were randomly allocated to a treatment group (PolyI:C or saline) and on the appropriate gestational day (GD10 or 19) dams were anaesthetised with isoflurane (induction 5%, maintenance 2.5-3% - Abbott Australasia Pty Ltd - Australia) and administered with either 4.0 mg/kg of PolyI:C (Sigma-Aldrich – AUS) or phosphate-buffered saline (PBS) via lateral tail vein injection (at 1 mL/kg body weight). Litters were weaned at postnatal day (PND) 21, at which no more than 3 animals per sex from each litter were allocated for qPCR and IHC/IF analyses. Confirmation of MIA was verified using IL-6 ELISA (Chapter 2 – Supplementary Figure 1).

3.2.2 qPCR

Expression of GABAergic mRNA markers in the cingulate cortex (CC) was determined by quantitative RT-PCR (qPCR). A total of 24 male and 24 female rats at PND70-84 were included with n = 6 per sex from each of the four experimental groups; GD10 Saline, GD10 PolyI:C, GD19 Saline, GD19 PolyI:C. CC samples were obtained using the methods previously described by Ong et al. (2014), from which subsequent dissection of the CC (including parts of the infralimbic, prelimbic cortices; bregma +2.5 – +3.5) was made. Brain samples from each hemisphere were 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 –

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 – USA) as per manufacturer's instructions. RNA was quantified using the NanoDrop Pearl (Implen – USA) and stored at -80ºC. RNA was reverse transcribed using a Superscript III Reverse Transcription kit (Life Technologies – 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 -20ºC. qPCR primers (Table 1) were designed using Primer Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For each animal, qPCR reactions were performed in triplicate for each gene using 1x SYBR Green Select Master Mix (Thermo Fisher Scientific), 200nM of each forward and reverse primer, and 5µl of cDNA sample, in a total volume of 12µl per reaction. Amplification reactions were performed at 0.2ng/µl cDNA concentration. qPCR amplification was performed using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems – USA), with an initial denaturation and activation step at 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 10 seconds and 60ºC for 30 seconds. Melt curves were generated to confirm amplification of a single gene product. The average cycle threshold (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 and Livak, 2008) with the geometric mean of, *Actb*, *Gapdh* and *Tubb3* used as the internal reference genes. 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.

Table 1. Primer sequences for GABAergic qPCR

Gene	Protein	Forward Primer	Reverse Primer
Actb	Actin	CCTAGCACCATGAAGATCAAGA	GCCAGGATAGAGCCACCAATC
Gapdh	GAPDH*	GGCTGGCATTCGTCTCAA	GAGGTCCACCACCCTGTTG
Tubb3	Tubulin	CTTCCGACTCCTCGTCGTCA	GAGGCCGAGAGCAACATGAA
Gad1	Glutamate Decarboxylase 1	AAAGCCAAGCGGGGGACGTT	GAACGGGCTCGATTCAGCCAC
Gad2	Glutamate Decarboxylase 2	AGAACCCGGGAACAGCGAGA	TTTCCGATGCCGCCCGTGAA
Sst	Somatostatin	TGCCACCGGGAAACAGGAAC	TGGGCTCGGACAGCAGTTCT
Npy	Neuropeptide Y	GCCCGCCATGATGCTAGGTAAC	AGCGAGGGTCAGTCCACACA
Pvalb	Parvalbumin	GAGCTGGGGTCCATTCTGAAG	TTAGCTTTCGGCCACCAGAG
Calb1	Calbindin	AGGCTGGATTGGAGCTATCACCTG	TGGCATCGAAAGAGCAGCAGG
Calb2	Calretinin	TATGGAGGCTTGGCGGAAGT	AGGGACGGTTGGCCTTCTTCA
Nos1	Neuronal Nitric Oxide Synthase	TTCGACGTGCTGCCTCTCCT	CAGTCGAACTTGGGGTGCCTG
Reln	Reelin	GCAGCCACGACACAACGGAA	TTGTTTGCGAGTGAGGACGAC

*: Glyceraldehyde-3-phosphate dehydrogenase

3.2.3 Immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry experiments are as described in Chapter 2. Brain sections used for immunohistochemistry are from the same cohort of rats used to investigate IWMN density in our previous study of SST+ IWMN density by Duchatel et al. (2016) – Chapter 2. This is a separate cohort to those used in the above-mentioned qPCR analysis. Briefly, 12 week old (PND 84), male and female offspring from maternal immune activation induced dams were perfused, brains collected, sectioned at 30µm then processed for DAB immunohistochemistry as described in Duchatel et al. (2016). This current study used primary antibodies directed against GABAergic neuron markers as per Table 2.

Table 2. Antibodies used for GABAergic Immunofluorescence and Immunohistochemistry

Antibody	Species	Supplier	Concentration
GAD	Rabbit	Merck-Millipore	1:1000
NPY	Rabbit	Santa-Cruz Biotechnology	1:3000
Pv	Rabbit	Sigma-Aldrich	1:1000
Pv	Mouse	Santa-Cruz Biotechnology	1:1000
Cr	Rabbit	Santa-Cruz Biotechnology	1:1000
nNOS	Rabbit	Abcam	1:3000

3.2.4 Neuronal counting, quantification and statistical analysis

Neuronal counting and quantification is as described in Chapter 2. Images of sections immunolabelled for GAD^+ and SST^+ using DAB immunohistochemistry were captured using the Aperio™ Digital Pathology System (Leica Biosystems) at 20X magnification and then used to calculate the area of white matter sampled in each section. Four sections sampled 180µm apart from region 1 and 2 were used to count GAD+ or NPY+ IWMNs in the entire corpus callosum, contained in previously annotated white matter area (Chapter 2- Supplementary Figure 3). All immunopositive IWMNs within this area were counted, except those touching the grey/white matter boundary. The final GAD+ and NPY+ IWMN density was calculated as the mean of the sections counted for each region in each animal. Investigators were blind to the treatment status (i.e. MIA or vehicle) throughout experimental, analysis and quantification steps. GraphPad Prism 6 software (GraphPad, California, USA) was used to analyse data and produce descriptive statistics, including the mean and standard deviation (presented as mean $^+$/- SD). All other statistics are reported as the true *p*-value, where $p<0.05$ was considered statistically significant. Grouped data sets were tested for normality. One-way ANOVA with Bonferroni multiple comparisons were used to compare IWMN density between control and PolyI:C GD10 and GD19 rats for each region separately. Two-way ANOVA with Bonferroni multiple comparisons were used to examine the impact of sex on IWMN density in the GD19 PolyI:C cohort compared to controls. As the IWMN densities between saline treated rats at GD10 and GD19 were not significantly different ($p = 0.80$), they were pooled into a single control group.

3.3 Results

3.3.1 Expression of GABAergic interneuron markers in the white matter of the corpus callosum

Chapter 2 reported that rat IWMNs expressed NeuN and SST and that SST+ IWMNs were significantly increased in density after MIA. It is known that SST is a marker of GABAergic interneurons in the cortex. Immunofluorescence co-localisation experiments were used to determine what other GABAergic interneuron markers were expressed by NeuN+ IWMNs. Indeed, NeuN+ IWMNs also expressed GABAergic markers including GAD (Fig. 3.1A-C), NPY (Fig. 3.1D-F), Pv (Fig. 3.1G-I), Cr (Fig. 3.1J-L) and nNOS (Fig. 3.1M-O) in the corpus callosum of the white matter of control rats. All GAD+, NPY+, Pv+, Cr+ and nNOS+ IIWMNs within the white matter also expressed NeuN, suggesting that these were all populations of mature neurons. Interestingly, GABAergic markers were not co-expressed between all IWMN subtypes. Figure 3.2 shows that neither SST+ IWMNs (Fig. 3.2A-F) nor NPY+ IWMNs (Fig. 3.2G-L) co-express Pv suggesting that whilst these may be mature neurons (Fig. 3.1), they may be distinct individual populations.

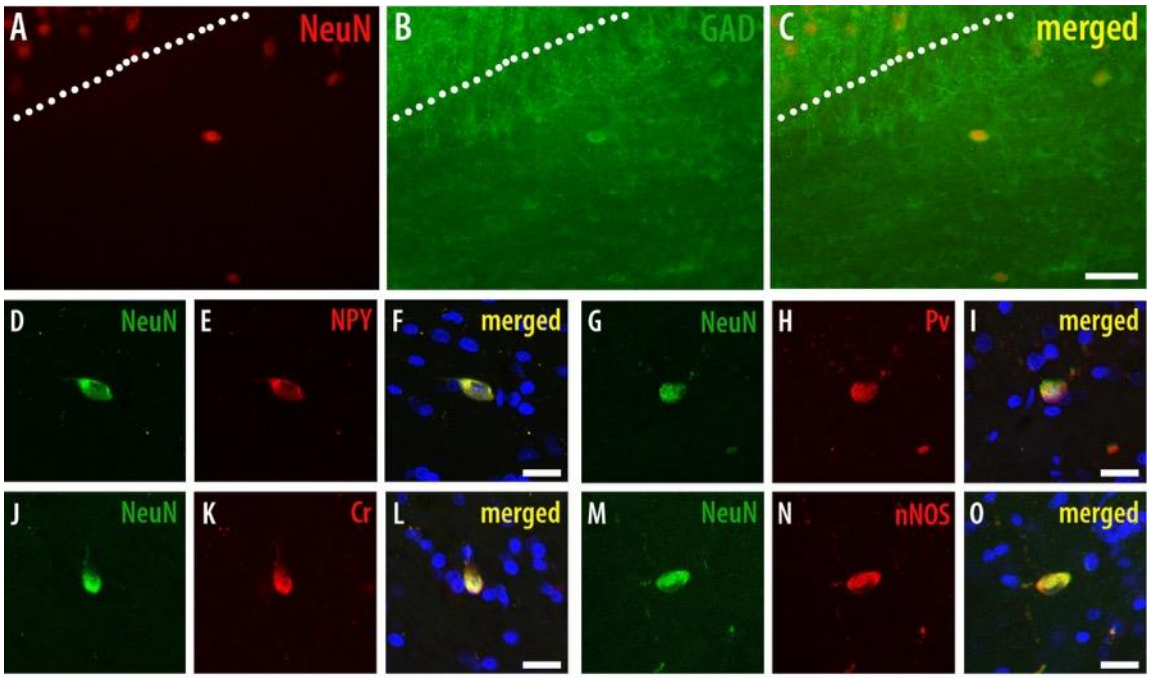


Figure 3.1: NeuN+ IWMNs co-express GABAergic interneuron markers. IWMNs identified by immunofluorescence in the corpus callosum of the adult rat brain by the expression of NeuN (A, D, G, J, M), were shown to co-express the GABAergic interneuron markers glutamic acid decarboxylase (GAD65/67; A-C), neuropeptide Y (NPY; D-F), parvalbumin (Pv; G-I), calretinin (Cr; J-L) and neuronal nitric oxide synthase (nNOS; M-O). (F,I,L,O) Merged overlays of NeuN, and GABAergic interneuron subset (Yellow), with DAPI (blue). Scale bar 10µm.

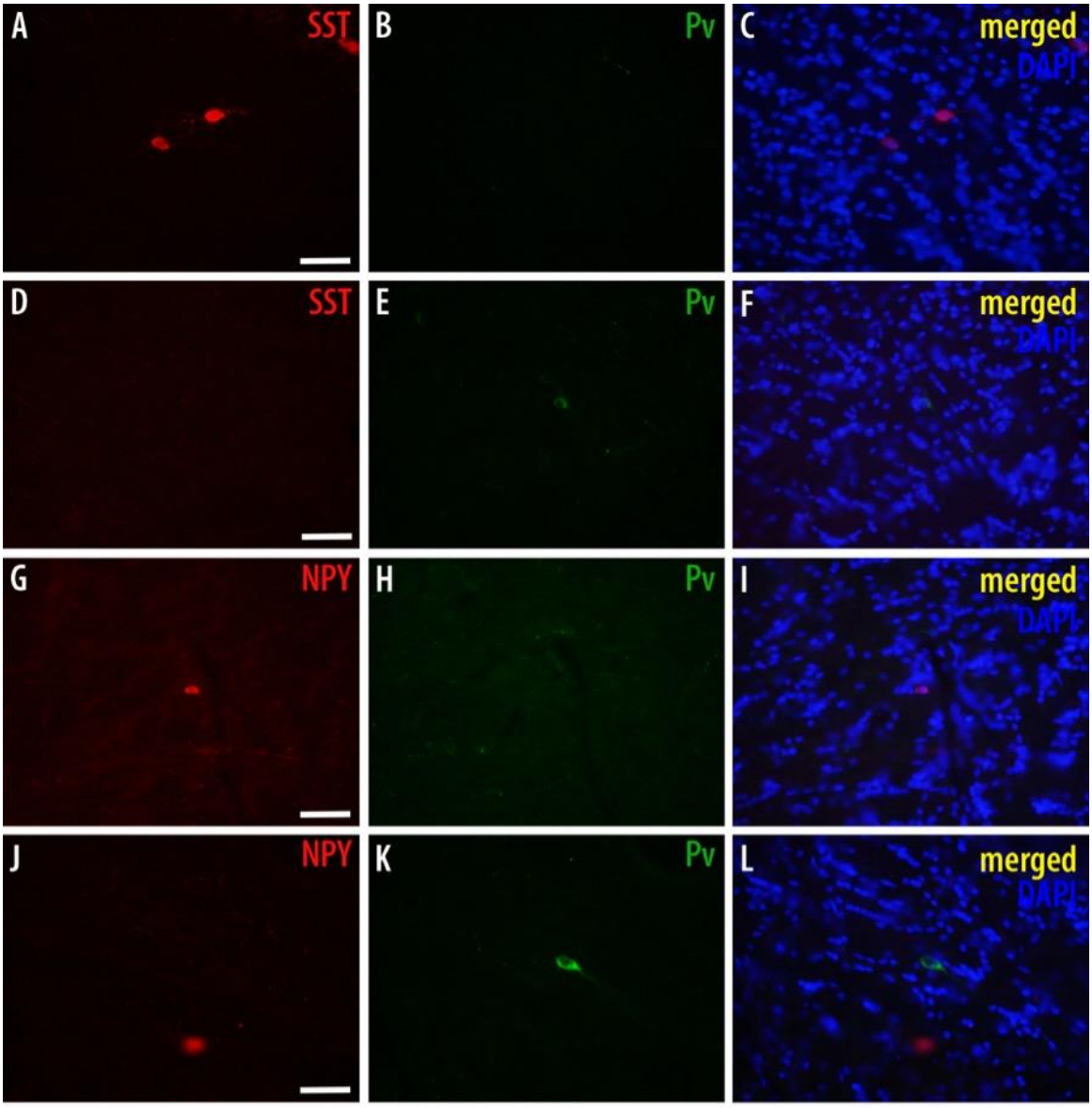


Figure 3.2: Somatostatin+ and Neuropeptide Y+ IWMNs do not co-express Parvalbumin. IWMNs identified using immunofluorescence co-localisation in the corpus callosum of the adult rat brain by the expression of somatostatin (SST: A), or neuropeptide Y (NPY: G), did not co-express GABAergic interneuron marker parvalbumin (Pv – E/K). (C, F, I, L) Merged overlays (Yellow), with DAPI (blue). Scale bar 10µm.

3.3.2 GAD+ IWMN density in a Maternal Immune Activation Model

To determine if MIA selectively increased the density of SST+ IWMNs, I examined if other subsets of GABAergic interneurons may also be altered by MIA. GAD+ IWMNs had previously been reported to be altered in people with schizophrenia by Joshi et al. (2012). Indeed, GAD+ IWMNs were identified within the white matter of the corpus callosum in control and PolyI:C rats (Fig. 3.3A-E). The mean density of GAD+ IWMNs in the white matter was not changed in GD10 PolyI:C rats, in either region 1 (0.29±0.18 cells/mm^2 vs. 0.32±0.24 cells/mm^2, p = 0.99, Figure 3.3F), or region 2 (0.16±0.06 cells/mm^2 vs. 0.17±0.14 cells/mm^2, p = 0.99, Figure 3.3G) compared to controls. In addition, the mean density of GAD+ IWMNs in the white matter was not changed in GD19 PolyI:C rats, in either region 1 (0.29±0.18 cells/mm^2 vs. 0.24±0.24 cells/mm^2, p = 0.99, Figure 3.3F), or region 2 (0.16±0.06 cells/mm^2 vs. 0.24±0.24 cells/mm^2, p = 0.99, Figure 3.3G) compared to controls.

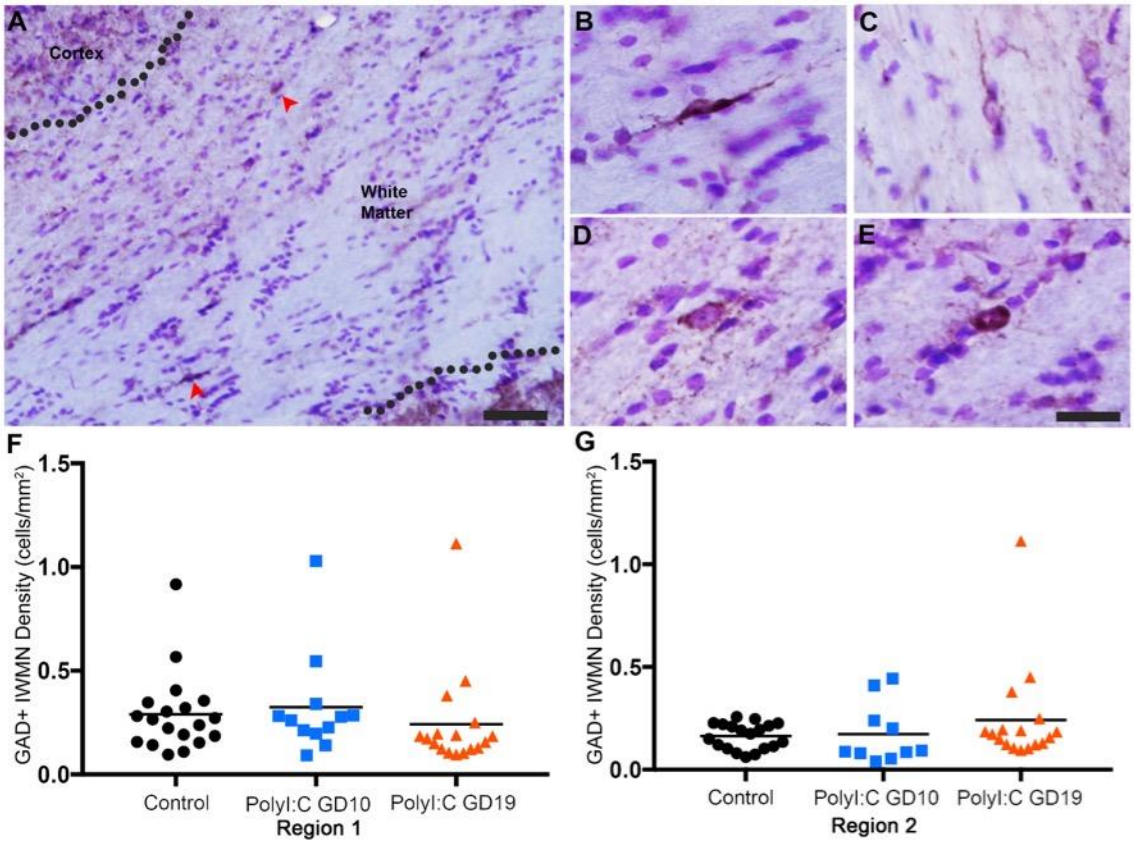


Figure 3.3: Effects of maternal immune activation (MIA) on glutamic acid decarboxylase positive (GAD+) interstitial white matter neurons (IWMNs) in the rat corpus callosum. Representative images of GAD+ neurons in the cortex and white matter of the corpus callosum in control rats (A). Higher magnification in (B-E). Red arrowheads indicate representative GAD^+ IWMNs that were quantified. Density of GAD+ IWMNs assessed in the corpus callosum from offspring of animals exposed to MIA via Polyinosinic:polycytidylic acid (PolyI:C) treatment at either gestational day 10 (GD10, n = 12, blue) or GD19 (n = 18, red), and controls (n = 22, black). Each data point represents the mean density (cells/mm^2) from one animal. Comparisons are presented for two rostrocaudally adjacent areas, region 1 (F) and region 2 (G). There was no difference in the density of GAD^+ IWMNs between control and MIA rats at either gestational time point. Scale bar: A – 30µm, B-E – 10µm.

3.3.3 NPY+ IWMN density in a Maternal Immune Activation Model

NPY has previously been investigated in people with schizophrenia where Ikeda et al. (2004), found no change in NPY+ IWMN density. Other than SST and GAD, NPY is the only other GABAergic IWMN marker examined in people with schizophrenia to date. NPY+ IWMNs were identified within the white matter of the corpus callosum in control (*n* = 20) animals and the offspring of mothers exposed to PolyI:C at GD10 (*n* = 7) and GD19 (*n* = 17) (Fig. 3.4A-E). The mean density of NPY+ IWMNs was not changed in GD10 PolyI:C rats, in either region 1 (3.547±1.70 cells/mm^2 vs. 5.17±1.88 cells/mm^2, *p* = 0.08, Fig. 3.4F), or region 2 (3.25±1.53 cells/mm^2 vs. 4.09±1.12 cells/mm^2, *p* = 0.99, Fig. 3.4G) compared to controls. In addition, the mean density of NPY+ IWMNs in the white matter was not changed in GD19 PolyI:C rats, in either region 1 (3.547±1.70 cells/mm^2 vs. 3.54±1.62 cells/mm^2, *p* = 0.99, Fig. 3.4F), or region 2 (3.25±1.53 cells/mm^2 vs. 2.99±1.24 cells/mm^2, *p* = 0.99, Fig. 3.4G) compared to controls.

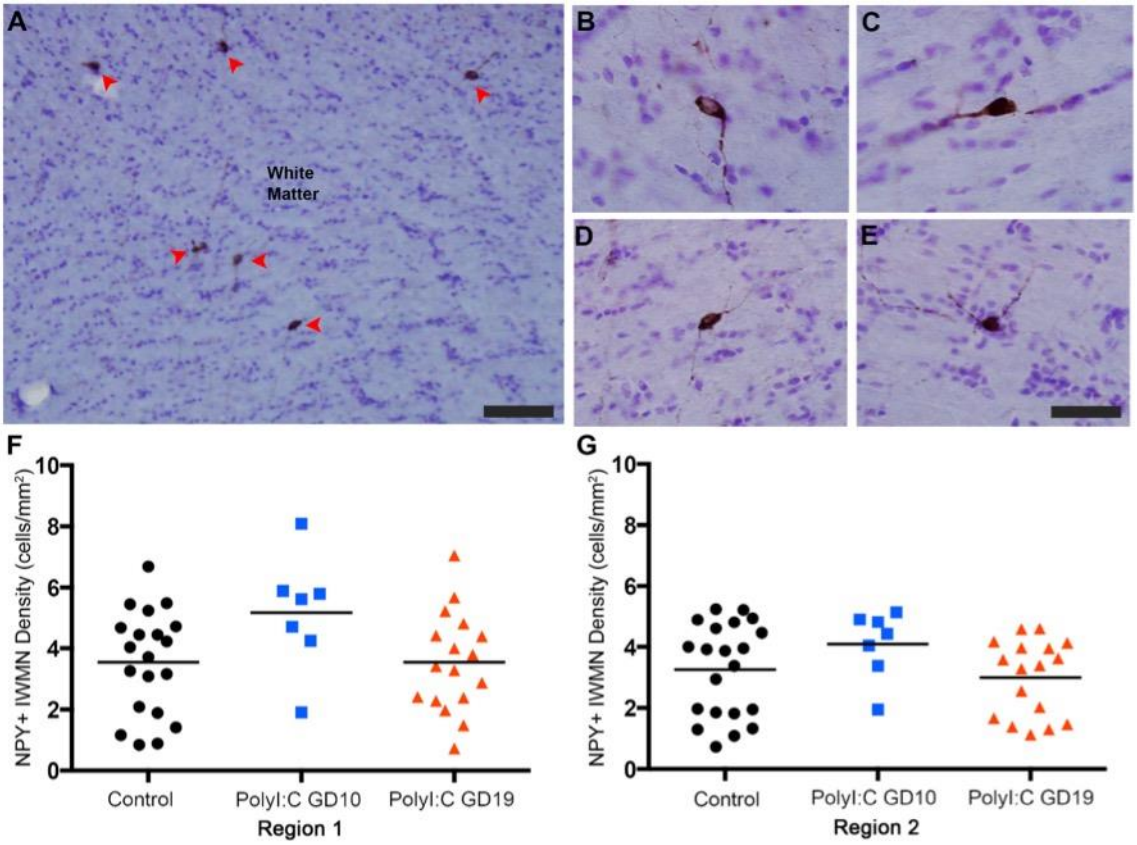


Figure 3.4: Effects of maternal immune activation (MIA) on neuropeptide Y positive (NPY+) interstitial white matter neurons (IWMNs) in the rat corpus callosum. Representative images of NPY+ neurons in the cortex and white matter of the corpus callosum in control rats (A). Higher magnification in (B-E). Red arrowheads indicate representative NPY^+ IWMNs that were quantified. (F-G) Density of NPY+ IWMNs assessed in the corpus callosum from offspring of animals exposed to MIA via Polyinosinic:polycytidylic acid (PolyI:C) treatment at either gestational day 10 (GD10, n = 7, blue) or GD19 (n = 17, red), and controls (n = 20, black). Each data point represents the mean density (cells/mm^2) from one animal. Comparisons are presented for two rostrocaudally adjacent areas, region 1 (F) and region 2 (G). The density of NPY+ IWMNs was similar in control and MIA rats. Scale Bar: A – 30µm, B-E – 10µm.

3.3.4 Cortical GABAergic gene expression is not altered by MIA.

Since cortical GABAergic interneuron pathology is implicated in the abnormal brain function in people with schizophrenia, the effect of MIA on the expression of a number of GABAergic interneuron related genes was examined in the cingulate cortex in the offspring of dams exposed to PolyI:C at either GD10 (n = 12) or GD19 (n = 12) hereafter referred to as PolyI:C rats and controls (n = 12 GD10 controls, n = 12 GD19 controls) (Fig 3.5). We chose *Gad1*, *Gad2*, *Sst*, *Pvalb and Reln* since these genes have altered cortical expression in schizophrenia (as described above) including in the cingulate cortex. *Npy*, *Calb1*, *Calb2*, *Nos1* were chosen since they are not changed in schizophrenia and thus served to rule out global effects on gene expression. No changes in *Gad1*, *Gad2*, *Sst*, *Npy*, *Pvalb*, *Calb1*, *Calb2*, *Nos1*, or *Reln* gene expression were observed in either GD10 or GD19 compared to their GD-specific control groups (Fig. 3.5, Table 3). No changes were observed between sexes (data not shown).

Table 3: Statistical analysis of qPCR for GABAergic gene expression

Gene	GD10	GD19
Gad1	$t = 0.09$, $df = 22$, $p = 0.92$	$t = 1.48$, $df = 22$, $p = 0.14$
Gad2	$t = 0.33$, $df = 22$, $p = 0.74$	$t = 0.25$, $df = 22$, $p = 0.80$;
Sst	$t = 0.45$, $df = 22$, $p = 0.65$	$t = 0.48$, $df = 22$, $p = 0.63$
Npy	$t = 0.35$, $df = 22$, $p = 0.73$	$t = 0.02$, $df = 22$, $p = 0.97$
Pvalb	$t = 0.92$, $df = 22$, $p = 0.36$	$t = 1.07$, $df = 22$, $p = 0.29$
Calb1	$t = 0.21$, $df = 22$, $p = 0.83$	$t = 0.30$, $df = 22$, $p = 0.76$
Calb2	$t = 0.23$, $df = 22$, $p = 0.81$	$t = 1.15$, $df = 22$, $p = 0.26$
Nos1	$t = 0.93$, $df = 22$, $p = 0.36$	$t = 1.10$, $df = 22$, $p = 0.28$
Reln	$t = 0.74$, $df = 22$, $p = 0.46$	$t = 1.37$, $df = 22$, $p = 0.18$

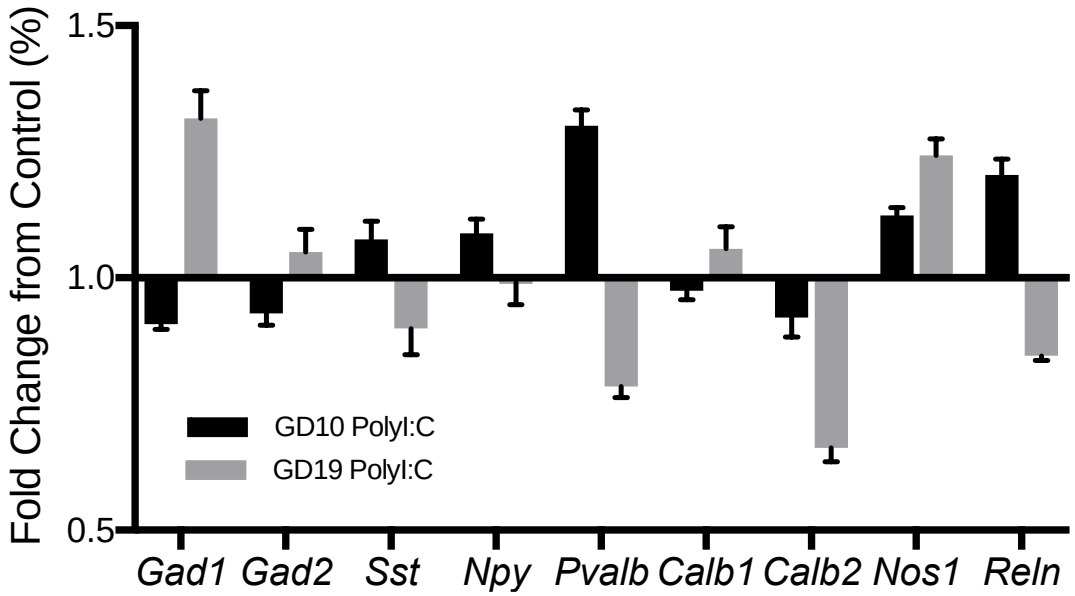


Figure 3.5: Effects of maternal immune activation on GABAergic related gene expression. Relative gene expression for Glutamic Acid Decarboxylase 1 (*Gad1*), *Gad2*, somatostatin (*Sst*), neuropeptide Y (*Npy*), Parvalbumin (*Pvalb*), Calbindin (*Calb1*), Calretinin (*Calb2*), neuronal nitrox oxide synthase (*Nos1*) and reelin (*Reln*) in the cingulate cortex of offspring from dams exposed to PolyI:C at either GD10 (black bars) or GD19 (grey bars). No significant changes were observed in the expression of *Gad1*, *Gad2*, *Sst*, *Npy*, *Pvalb*, *Calb1*, *Calb2*, *Nos1* or *Reln*, in PolyI:C rats compared to controls. Bars represent mean fold change from control +/- SEM.

3.4 Discussion

This chapter examined both cortical GABAergic gene expression as well as IWMNs that express GABAergic interneuron markers and how these were affected by MIA. No alterations in GABAergic gene expression were observed in the cingulate cortex of MIA rats. Alterations to GABAergic gene signatures are well established in people with schizophrenia and when developing schizophrenia-like models in rodents, researchers attempt to recapitulate these GABAergic interneuron pathologies as validation of the model. A number of IWMNs were identified within the white matter of the corpus callosum that also expressed GABAergic interneuron markers. All IWMNs that were positive for GAD65/67, NPY and Pv were also NeuN+, which suggests these are all subsets of mature neurons. Interestingly, IWMNs did not seem to co-express a number of these markers, with SST+ and NPY+ IWMNs not expressing Pv. Though, it must be noted that we did not examine the co-expression in IWMNs of every GABAergic marker we previously identified. Indeed, Chapter 2 identified that SST+ IWMNs were particularly susceptible to MIA – interestingly MIA did not induce a corresponding change to *SST* gene expression in the cortex. Though I did not identify any cortical GABAergic gene expression changes, we examined if MIA effected the density of IWMNs expressing GABAergic interneuron markers GAD and NPY. We did not observe any effect of MIA at either GD10 or GD19 on GAD+ or NPY+ IWMNs.

Only one study to date has examined the density of NPY+ IWMNs in post mortem brains from people with schizophrenia. Ikeda et al (2004) showed that the density of NPY+ IWMNs was increased (~8%) in the deep white matter (compared to

superficial white matter) in the brains of subjects with schizophrenia compared to controls (Ikeda et al., 2004). This is an interesting observation, as all studies that examined NeuN+ IWMNs observed only increases in the superficial white matter and not the deep white matter. However, the rodent white matter is significantly smaller in volume and the corpus callosum is directly bounded by cortical regions and subcortical structures unlike the cortex investigated in the human studies of IWMNs. Since IWMNs are a non-uniformly distributed and at much lower density in rodent brain, we did not distinguish between superficial and deep white matter in this study, which may confound identifying this change in rodents.

GAD65/67 has only been investigated as a marker of IWMNs in post-mortem human brain by Joshi et al (2012). They showed that the density of GAD65/67+ IWMNs in the OFC was increased by 42% in subjects with schizophrenia compared to controls. This current study also attempted to determine if GAD65/67 was responsible for the changes in the trend towards increased density of NeuN+ IWMNs in response to MIA. I was unable to replicate the increased GAD+ IWMN found in people with schizophrenia, as no change in GAD+ IWMN density was observed after MIA. Whilst GAD+ neurons could be identified mostly in the cortex but also the white matter of the corpus callosum in the rat brain best with immunofluorescence, the DAB immunohistochemical labelling was not consistent between brain sections or animals, despite multiple optimisation attempts with four different anti-GAD antibodies. Diffuse immunolabelling for GAD predominantly in the cortex was consistently observed with all antibodies in DAB immunohistochemistry, likely representative of terminal bouton labelling. However consistent labelling of GAD+ neuronal cell bodies was

difficult. This often-confounded identifying GAD+ IWMNs in the white matter – and was confirmed when comparing the density of GAD+ IWMNs to NPY+ IWMNs, where the density of GAD+ IWMNs was significantly less. Indeed, we utilised four differing primary antibodies to GAD and all provided the same results. Since Fig. 3.1 and Fig. 3.2 showed that there are multiple GABAergic populations in the rat corpus callosum, it was expected that there would be a high density of GAD+ IWMNs, though we observed the opposite. One interpretation of the low density of GAD+ IWMNs in the rat corpus callosum, is that this may be representative that GAD is not highly expressed in rat IWMNs until they reach the cortex. Indeed, studies in the human brain have suggested that not all IWMNs are mature (NeuN+). Yang Yang et al 2011, observed a number of SST+ IWMNs that did express NeuN, but expressed doublecortin (Dcx), suggesting that these IWMNs may be immature neurons. This is an important finding when trying to determine the origin of these neurons, especially considering we found a stark lack of GAD+ immunolabelling in the white matter. Perhaps an explanation for this is that GAD is not actively transcribed until neurons reach the cortex. Future methodological approaches may consider utilising in-situ hybridisation methodologies to examine GAD+ IWMNs in the white matter of the rat corpus callosum to determine the effects of MIA.

Two studies have reported the effects of MIA on GAD gene expression. Richetto et al. (2014) used PolyI:C at late gestation (GD17) and showed a reduction in *GAD* mRNA and protein expression in the offspring at adulthood (PND100). This study was conducted in mice in the medial prefrontal cortex (mPFC) which overlaps with the region used in this current study. It is possible that the mouse

brain is more susceptible to the effects of maternal immune activation. However, Cassella et al. (2016) used PolyI:C injected at GD14 in rats and showed a reduction in *GAD* mRNA in the offspring at P60. Whilst it appears a similar region of the cortex was sampled to our study here, there were a number of differences that may account for the lack of concordance between the two studies. We induced MIA at early and late gestation in Wistar rats and used qPCR on brains collected between PND70-84, whereas Cassella et al. (2016) used a mid-gestation time point in Sprague Dawley rats and used *in situ* hybridisation. Several studies have also investigated the density of Pv+ neurons in the mPFC in MIA models. Meyer et al. (2008c) showed a reduction in Pv+ neurons and reelin+ neurons in the mPFC of six month old mice exposed to PolyI:C at GD9 and GD17. Wischhof et al. (2015) reported a reduction in Pv+ neurons in the mPFC of Wistar rats at PND90 after exposure to lipopolysaccharide at GD15 and 16, whereas Boksa (2010) using a similar rat model showed a significant increase in density of Pv+ cortical neurons at PND14. Whether these differences in MIA agent, timing, techniques or rat species can account for the lack of concordance of our study with the published literature requires further investigation.

In conclusion, we showed that early and late gestation MIA did not induce a change in GAD+ IWMNs like that observed in schizophrenia. Furthermore, MIA did not affect the gene expression of markers for GABAergic interneurons in the cingulate cortex in adult rat offspring. This contrasts with the literature on prenatal immune activation models and the post mortem literature for schizophrenia. Even though we confirmed MIA was induced by PolyI:C in the dams in our model via IL6 ELISA, it appears that this alone was not sufficient to recapitulate these

schizophrenia-like changes observed in other studies. Schizophrenia is known to be multifactorial likely involving several environmental insults and genetic changes. It is possible that a second environmental hit in conjunction with MIA might be needed to induce these changes in our model.

CHAPTER FOUR

Effects of maternal immune activation on Glial cells

Statement of Contribution

Ryan J. Duchatel, Crystal L. Meehan, Lauren R. Harms, Patricia T. Michie, Mark J. Bigland, Doug W. Smith, Frederik R. Walker, Phillip Jobling, Deborah M. Hodgson, Paul A. Tooney. **Late gestation immune activation increases IBA1-positive immunoreactivity levels in the corpus callosum of adult rat offspring.** Psychiatry Research.

*"I attest that Research Higher Degree candidate **Ryan Duchatel**, is a co-primary author of this publication and contributed extensively to the development of this publication including input into the initial design and intellectual development of the study; extensive contributions to optimising, executing, analysing and interpreting all experiments contained within this publication; and writing the manuscript".*

_____	_____
Ryan Duchatel	***Crystal Meehan***
_____	_____
Mark Bigland	***Douglas Smith***
_____	_____
Lauren Harms	***Patricia Michie***
_____	_____
Frederick Walker	***Phillip Jobling***

_____ _____

Deborah Hodgson ***Paul Tooney***
(Primary PhD Supervisor)

_____

Robert Callister
(Deputy Head of Faculty)

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Late gestation immune activation increases IBA1-positive immunoreactivity levels in the corpus callosum of adult rat offspring

Ryan J. Duchatel[a,c,d,1], Crystal L. Meehan[b,c,d,1], Lauren R. Harms[b,c,d], Patricia T. Michie[b,c,d], Mark J. Bigland[a,c,d], Doug W. Smith[a,c,d], Frederick R. Walker[a,d], Phillip Jobling[a,c,d], Deborah M. Hodgson[b,c,d], Paul A. Tooney[a,c,d,*]

[a] School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, Callaghan, NSW 2308, Australia
[b] School of Psychology, Faculty of Science, University of Newcastle, Callaghan, NSW 2308, Australia
[c] Priority Centre for Brain and Mental Health Research, University of Newcastle, Callaghan, NSW 2308, Australia
[d] Hunter Medical Research Institute, New Lambton Heights, NSW 2305, Australia

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ABSTRACT

Animal models of maternal immune activation study the effects of infection, an environmental risk factor for schizophrenia, on brain development. Microglia activation and cytokine upregulation may have key roles in schizophrenia neuropathology. We hypothesised that maternal immune activation induces changes in microglia and cytokines in the brains of the adult offspring. Maternal immune activation was induced by injecting polyriboinosinic:polyribocytidylic acid into pregnant rats on gestational day (GD) 10 or GD19, with brain tissue collected from the offspring at adulthood. We observed no change in *Iba1, Gfap, IL1-β* and *TNF-α* mRNA levels in the cingulate cortex (CC) in adult offspring exposed to maternal immune activation. Prenatal exposure to immune activation had a significant main effect on microglial IBA1-positive immunoreactive material (IBA1 + IRM) in the corpus callosum; post-hoc analyses identified a significant increase in GD19 offspring, but not GD10. No change in was observed in the CC. In contrast, maternal immune activation had a significant main effect on GFAP + IRM in the CC at GD19 (not GD10); post-hoc analyses only identified a strong trend towards increased GFAP + IRM in the GD19 offspring, with no white matter changes. This suggests late gestation maternal immune activation causes subtle alterations to microglia and astrocytes in the adult offspring.

1. Introduction

Schizophrenia has complex neurodevelopmental origins underpinned by genetic and environmental factors (Owen et al., 2005; Wilson and Terry, 2010). Many of these environmental risk factors impact on 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 and Levitt, 2002). Considerable epidemiological evidence has shown that exposure to bacterial (Babulas et al., 2006; Sorensen et al., 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). Indeed, maternal immune activation through exposure to infectious agents during the prenatal stages of development is one of the most robust environmental risk factors for schizophrenia (Brown et al., 2004a).

Rodent models of maternal immune activation have provided evidence to support the link between prenatal infection and the later-life development of schizophrenia. Exposure to immune activating agents including the influenza virus, the viral mimetic polyriboinosinic:polyribocytidylic acid (PolyI:C), and the bacterial endotoxin lipopolysaccharide during gestation produce schizophrenia-like behavioural and neurobiological abnormalities in the adult offspring of rats and mice (reviewed in Meyer, 2014). It is believed that the common features 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 offspring exposed to maternal immune activation (Meyer and Feldon, 2009). The exact mechanisms by which maternal immune activation disrupts neurodevelopmental processes to produce

* Corresponding author at: School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, University Drive, Callaghan, NSW 2308 Australia.
E-mail addresses: Ryan.Duchatel@uon.edu.au (R.J. Duchatel), Crystal.Meehan@utas.edu.au (C.L. Meehan), Lauren.Harms@newcastle.edu.au (L.R. Harms), Pat.Michie@newcastle.edu.au (P.T. Michie), Mark.Bigland@uon.edu.au (M.J. Bigland), Douglas.Smith@newcastle.edu.au (D.W. Smith), Rohan.Walker@newcastle.edu.au (F.R. Walker), Phillip.Jobling@newcastle.edu.au (P. Jobling), Deborah.Hodgson@newcastle.edu.au (D.M. Hodgson), Paul.Tooney@newcastle.edu.au (P.A. Tooney).
[1] These authors contributed equally to this work.

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0165-1781/

schizophrenia-like pathology are not fully elucidated, but what is recognised is the important role the body's immune-inflammation response plays in this process.

Cytokines are potential mediators between maternal immune activation and the development of behavioural and brain disturbances relevant to schizophrenia (Meyer et al., 2009). For example, the mothers of patients with schizophrenia have elevated serum levels of the cytokines TNF-α and IL-8 during gestation in comparison to the mothers of healthy controls (Brown et al., 2004b; Buka et al., 2001) and elevated maternal IL-8 has been associated with neuroanatomical changes associated with schizophrenia (Ellman et al., 2010). Furthermore, increased levels of circulating cytokines including TNF-α, IL-1β, IL-6, have been identified in up to 1/3 of schizophrenia cases (Miller et al., 2011; Potvin et al., 2008; van Kammen et al., 1999) and post-mortem studies have shown elevated *IL-1β, IL-6* and *IL-8* mRNA expression in the dorsolateral prefrontal cortex (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. Increased microglial density has been identified in the prefrontal cortex (PFC), hippocampus, temporal gyrus, cingulate cortex (CC), and dorsal thalamus (Bayer et al., 1999; Fillman et al., 2012; Radewicz et al., 2000; Steiner et al., 2008b). Positron Emission Topography identified microglial activation in the grey matter and hippocampus of schizophrenia cases within the first five years of symptom onset (Doorduin et al., 2009; van Berckel et al., 2008) and in those at ultra-high risk of developing schizophrenia (Bloomfield et al., 2016). However, more recently a number of other PET studies have failed to identify microglial activation in a range of patients, from those at ultra-high risk of schizophrenia to those chronically affected (reviewed in De Picker et al., 2017). Many would argue that the best assessment of microglia activation comes from using specific markers in post-mortem brain tissue to assess morphological changes. A recent meta-analyses of studies investigating microglia activation in post-mortem brains in schizophrenia showed that microglia activation was reported in 11 of 22 studies, with three studies reporting a decrease and a further 8 studies showing no change in microglia activation (Trepanier et al., 2016).

Postmortem studies have also investigated other cell types such as astrocytes in schizophrenia. Indeed increased astrocyte activation and altered astrocyte morphology have been identified in the CC, prefrontal, orbitofrontal, and temporal cortices in post mortem brains of schizophrenia cases (Steiner et al., 2008a). Also, 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 et al., 2014). However, in their meta-analyses Trepanier et al. (2016) have shown that a large variability exists in post-mortem studies of GFAP expression in schizophrenia. The bulk of the studies (21 of 33) do not show any association of GFAP expression or immunoreactivity with schizophrenia, with the remaining 12 studies split equally amongst those showing increased or decreased GFAP levels (Trepanier et al., 2016). Whilst there is variability in the findings from these studies, it would appear that in those patients with schizophrenia that have an inflammatory phenotype, likely involves activation of microglia, not astrocytes.

This begs the question: Are these long-term changes also seen in rodent models of maternal immune activation? Elevated circulating levels of TNF-α, IL-6, and IL-2, in conjunction with schizophrenia-like behavioural and neurobiological deficits have been observed in the prepubescent and adult rat offspring exposed to maternal immune activation (Romero et al., 2010; Samuelsson et al., 2006). Maternal immune activation in mice altered mRNA and protein expression of pro and anti-inflammatory cytokine markers (IL-6, IL-1α, IL-9, IL-10, IFN-γ) in the hippocampus, frontal cortex, and CC in offspring from the foetal stage through to young adulthood (Garay et al., 2012; Mattei et al., 2014; Samuelsson et al., 2006). Furthermore, increased microglial density and activation occurs in the brains of offspring in neonatal (Girard et al., 2010) and adult rat offspring (Van den Eynde et al., 2014) and neonatal and adolescent mouse offspring (Juckel et al., 2011) exposed to maternal immune activation. In addition, maternal immune activation resulted 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 maternal immune activation (Garay et al., 2012; Missault et al., 2014; Nyffeler et al., 2006).

One brain region that could be vulnerable to maternal immune activation is the white matter (WM) tracts of the corpus callosum, a region known to be altered in schizophrenia. Indeed, diffusion tensor imaging has been extensively used to study the WM tracts to investigate potential connectivity deficits in schizophrenia. Most studies show reduced fractional anisotropy in the corpus callosum, but many have not been able to replicate these findings (reviewed in Wheeler and Voineskos, 2014). However, it has been known for some time that interhemispheric transfer of information across the corpus callosum is impaired in schizophrenia (reviewed in Innocenti et al., 2003). These findings suggest that abnormalities in the structure/function of the corpus callosum might be indicative of abnormal connectivity argued to be an aspect of the pathophysiology of schizophrenia (Innocenti et al., 2003). In addition, early investigations of the corpus callosum at an electron microscopic level identified that during fetal development in primates (and other mammalian species) there is a significant increase in callosal axons during late gestational stages, three-quarters of which are pruned back during the first six months of life (LaMantia and Rakic, 1990). Given that evidence suggests prenatal exposure to infectious agents during mid-late gestation increases the risk of the offspring developing schizophrenia (Mednick et al., 1988), it is possible that the resulting immune activation could affect connectivity by disrupting the normal fetal development of the corpus callosum.

Indeed, with respect to the timing of infection, previous work in the mouse has demonstrated that the gestation stage when maternal immune activation actually occurs can differentially alter the neurochemical and behavioural phenotype of offspring (Meyer et al., 2006,2008). Early gestational day (GD9) versus late GD17 exposure of mice to PolyI:C identified that early maternal immune activation produced a more positive symptom schizophrenia-like phenotype with late maternal immune activation a more cognitive/negative symptom phenotype (Meyer and Feldon, 2012; Meyer et al., 2006,2008). Until recently, the differential effects of early versus late maternal immune activation had only been demonstrated in the C57 strain of mouse (Meyer, 2014). More recently, we established an early (GD10) versus late (GD19) maternal immune activation model in the rat that demonstrates some differences in the behavioural and neurobiological phenotypes of offspring based on the gestational timing of PolyI:C exposure (Duchatel et al., 2016; Meehan et al., 2016).

Currently, there is no evidence on whether the differences in behaviour and neurobiology observed in the early versus late maternal immune activation models are associated with differences in neuroimmune status. Further understanding of the neuro-inflammatory status resulting from maternal immune activation at differential gestational time-points may help in elucidating the potential mechanisms involved in mediating the effects of maternal immune activation at early (GD10) versus late (GD19) gestation on neurodevelopment. This study explored the potential long-term neuro-inflammatory effects of PolyI:C exposure at either GD10 or GD19 on microglial, astrocyte, and cytokine levels in the adult cingulate cortex and underlying WM, both implicated in schizophrenia.

Table 1
Primer sequences for qPCR.

n	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

2. Methods

2.1. Maternal immune activation using PolyI:C in rats

The use and monitoring of animals 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, 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 (PolyI:C or saline) and on the appropriate gestational day (GD10 or 19) dams were anaesthetised with isoflurane (induction 5%, maintenance 2.5–3% - Abbott Australasia Pty Ltd - Australia) and administered with either 4.0 mg/kg of PolyI:C (Sigma-Aldrich – AUS) or phosphate-buffered saline (PBS) via lateral tail vein injection (at 1 mL/kg body weight). Litters were weaned at postnatal day (PND) 21, at which no more than 3 animals per sex from each litter were allocated for qPCR and IHC analyses.

2.2. Confirmation of immune activation

Immune activation was confirmed by measuring IL-6 levels using ELISA kit (Rat IL-6 Quantikine ELISA, R&D Systems – USA) in saphenous vein blood samples drawn from pregnant dams two hours following prenatal treatment as described previously (50). Circulating IL-6 was significantly increased in all dams receiving PolyI:C but not controls (previously reported in Duchatel et al., 2016).

2.3. Quantitative real time PCR analysis

Expression of *Iba1, Gfap, TNF-α* and *IL-1β* mRNA levels in the CC were determined by quantitative RT-PCR (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 PolyI:C, GD19 Saline, GD19 PolyI:C. Between PND 70 and PND 84, rats were anesthetised via intraperitoneal injection of Lethabarb (2 ml/kg i.p.; Virbac, Pty. Ltd, Australia) and transcardially perfused with 300 ml of PBS (Sigma Aldrich) prior to decapitation and removal of the brain. CC 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 CC (including parts of the infralimbic, prelimbic corticies; bregma +2.5 to +3.5) was made. Brain samples from each hemisphere were kept on dry ice before being stored at −80 °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 – Australia; 4 min at 20 Hz) in a RNAse-free microtube containing 1 ml of QIAzol® Lysis Reagent (Qiagen) and a 5 mm diameter stainless steel bead (Qiagen). Total RNA was then extracted using the RNeasy® Mini Kit (Qiagen) and DNase I treated (Invitrogen – USA) as per manufacturer's instructions. RNA was quantified using the NanoDrop Pearl (Implen – USA) and stored at −80 °C.

RNA was reverse transcribed using a Superscript III Reverse Transcription kit (Life Technologies – 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 °C. qPCR primers (Table 1) were designed using Primer Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). 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 cDNA for *TNF-α* and *IL-1β*. qPCR amplification was performed using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems – USA), with an initial denaturation and activation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C 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 and 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.

2.4. Immunohistochemical analysis of microglia and astrocytes

Brain sections used for immunohistochemistry are from the same cohort of rats used to investigate WM neuron density in our previous study of maternal immune activation (Duchatel et al., 2016), and separate to the cohort used in the above mentioned qPCR analysis. Briefly, 12 week old (PND 84), male and female offspring from maternal immune activation induced dams were perfused, brains collected, sectioned at 30 µm then processed for diaminobenzidine immunohistochemistry as described in Duchatel et al., (2016). This current study used primary antibodies to either rabbit anti-Ionized calcium binding adaptor molecule 1 (IBA1) (1:1000, Wako Chemicals - USA; Cat. #019-19741) for microglia or rabbit anti-GFAP (1:500, Sapphire Bioscience, AUS; Cat. LS-C7112#) for astrocytes with a donkey anti-rabbit IgG biotinylated secondary antibody (1:1000, Jackson ImmunoResearch, Cat. #711-065-152).

2.5. Quantification of microglia and astrocytes

Investigators were blind to the treatment status (i.e. PolyI:C or vehicle) throughout experimental, analysis and quantification steps. Brain sections containing the CC and the WM of the corpus callosum were divided into two regions, region 1; 3.2–2.5 mm from Bregma and region 2; 2.3–0.7 mm from Bregma (Supplementary Fig. S1). This delineation is based on the anatomical difference in the WM tracts 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 from each region sampled 180 µm apart were used to measure the DAB labelling for IBA1+ microglia immunoreactive material (IBA1+IRM) and GFAP+ astrocyte immunoreactive material (GFAP+IRM) within both the CC and WM. Images of IBA1+IRM and GFAP+IRM on sections of CC or WM (eight images per animal over four sections) were captured at 20x magnification using an Olympus DP72 microscope (Olympus – AUS). Images were taken in the deep cortical layers of the CC and in the forceps minor of the corpus callosum below the CC. Images for each region were taken in one session, with standardised microscope lighting intensity, exposure and sensitivity settings. The rat CC and WM brain regions were identified by comparing the Nissl staining pattern of adjacent sections to sections at an equivalent level in the Rat Brain in Stereotaxic Coordinates Atlas (Paxinos and Watson, 2006). The level of IBA1+IRM and GFAP+IRM was determined by cumulative threshold analysis as previously described by Johnson and Walker (2015) and Ong et al. (2016). 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 Figs. S1 and S2 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+IRM were: PFC region 1 = PI120; PFC region 2 = PI90; WM region 1 = PI120; WM region 2 = PI125. The PI that detected genuine GFAP+IRM were: PFC region 1 = PI120; PFC region 2 = PI120; WM region 1 = PI120; WM 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. Each region was analysed separately since they were thresholded separately based on the optimal pixel intensity to detect genuine immunoreactive material in that particular region.

2.6. Statistical analysis

Changes in gene expression (qPCR) between maternal immune activation at GD10 and GD19 and control groups 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 are expressed as a percentage change relative to the average of the control group ± SEM, and were analysed using GraphPad Prism 6 software (GraphPad - USA). Previous studies of this animal cohort (Duchatel et al., 2016) showed no difference between GD10 and GD19 controls, so 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 maternal immune activation affected dams at either GD10 or GD19. Nonparametric Kruskal–Wallis tests were also performed, and revealed the same results indicating that the ANOVA outcomes were robust against violations of normality of the data and sensitivity to outliers. Initial analyses showed there was no significant effect of sex on the levels of IBA1+ or GFAP+ immunoreactive cells after maternal immune activation. Therefore, the data from males and females were pooled for further analyses. However, we cannot rule out the possibility that the study is underpowered to detect sex effects since the numbers per sex were low in some groups (e.g. GD10 there was only $n = 4$ per sex).

3. Results

3.1. Effects of maternal immune activation on gene expression in the brains of the offspring

We examined *Iba1* and *Gfap* gene expression in the CC in the offspring of dams exposed to PolyI:C at either GD10 ($n = 12$) or GD19 ($n = 12$) hereafter referred to as PolyI:C rats and controls ($n = 12$ GD10 controls, $n = 12$ GD19 controls) (Fig. 1(A)). No changes in *Iba1* or *Gfap* gene expression were observed in either GD10 (*Iba1:* $t = 0.0936$, $df = 22$, $p = 0.92$ or *Gfap:* $t = 0.99$, $df = 22$, $p = 0.33$) or GD19 PolyI:C rats (*Iba1:* $t = 0.225$, $df = 22$, $p = 0.82$ or *Gfap:* $t = 1.6$, $df = 22$, $p = 0.12$) compared to their GD-specific control groups (Fig. 1(A)). With respect to cytokines previously implicated in schizophrenia, we observed no difference in the expression of *TNF-α* or *IL-1β* in either GD10 (*TNF-α:* $t = 0.458$, $df = 22$, $p = 0.65$ or *IL-1β:* $t = 0.659$, $df = 22$, $p = 0.656$) or GD19 PolyI:C rats (*TNF-α:* $t = 0.679$, $df = 22$, $p = 0.504$ or *IL-1β:* $t = 0.6725$, $df = 22$, $p = 0.508$) compared to their GD-specific control groups (Fig. 1(B)).

3.2. Effects of maternal immune activation on IBA1+IRM in brains of the offspring

Since there was no change in gene expression, we next investigated

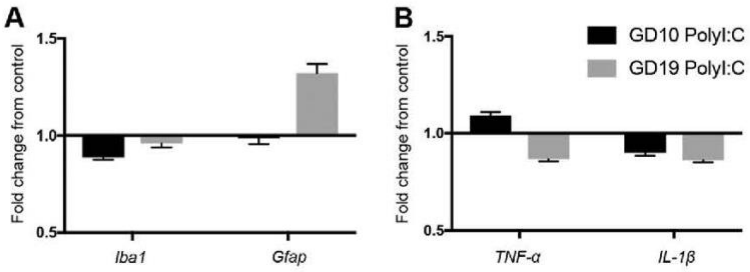

Fig. 1. Effects of maternal immune activation on immune related gene expression. Relative gene expression for *Iba1, Gfap* (A), *TNF-α* and *IL-1β* (B) in the CC of offspring from dams exposed to PolyI:C at either GD10 (black bars) or GD19 (grey bars). No significant changes were observed in the expression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

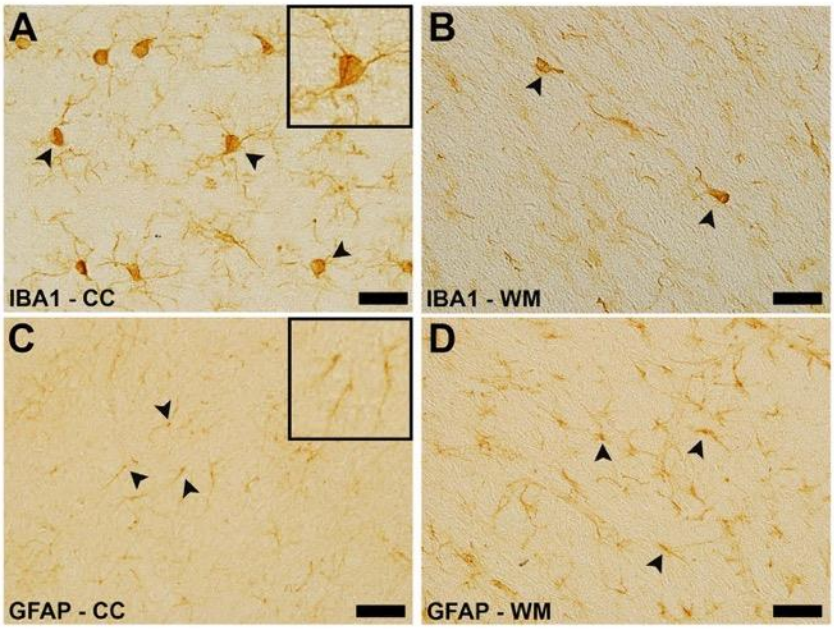

Fig. 2. Representative images of IBA1+ immunoreactive microglia and GFAP+ immunoreactive astrocytes in the rat brain. IBA1+ immunoreactive microglia (black arrowheads) in the cingulate cortex (CC; A) and white matter (WM) of the corpus callosum (B) of control rats. GFAP+ immunoreactive astrocytes (black arrowheads) in the CC (C) and WM of the corpus callosum (D) in the rat brain of control rats. Scale bar: 25 μm.

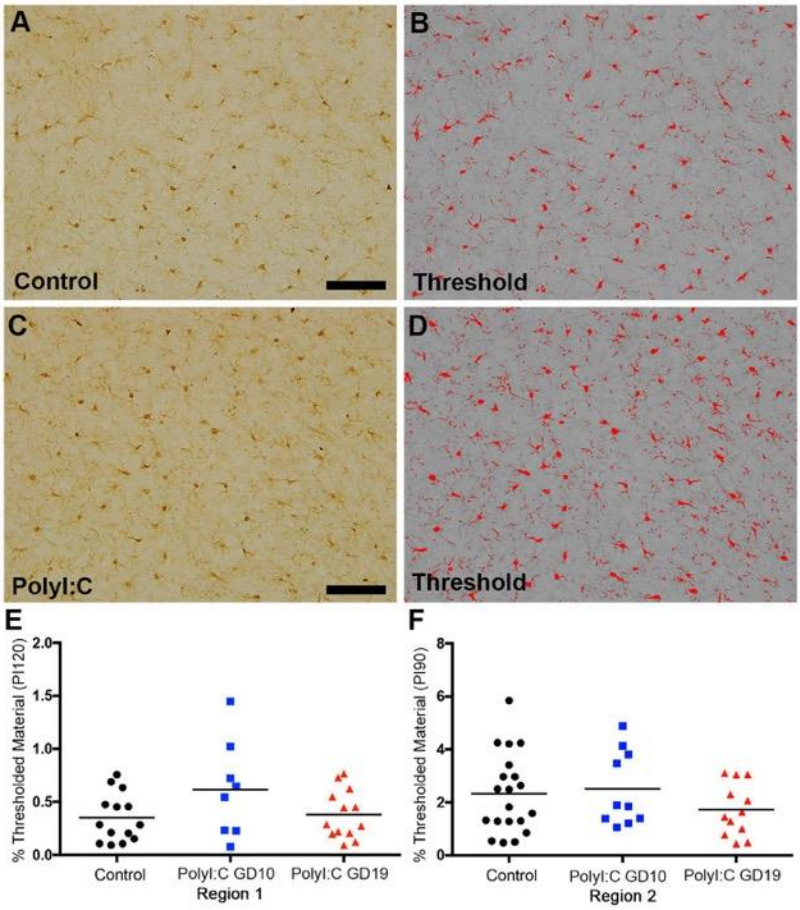

Fig. 3. Effect of maternal immune activation on IBA1+ immunoreactive material (IBA1+IRM) in the cingulate cortex (CC). Representative images of IBA1+ immunoreactive microglia in the CC from region 1 of the rat brain in either controls (A) or offspring from animals exposed to PolyI:C at GD19 (C), with the corresponding images showing IBA1+ thresholded material at pixel intensity 120 (B) and (D). (E) and (F) The percentage of IBA1+IRM is shown at a given pixel intensity for offspring exposed to PolyI:C at GD10 (blue squares) or GD19 (red triangles), as well as controls (black circles). Each data point represents the mean % of IBA1+IRM for one animal. Comparisons are presented for two rostrocaudally adjacent areas, region 1 (E) and region 2 (F). The percentage of IBA1+IRM was not significantly different between control and PolyI:C rats in the CC of region 1 and region 2. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

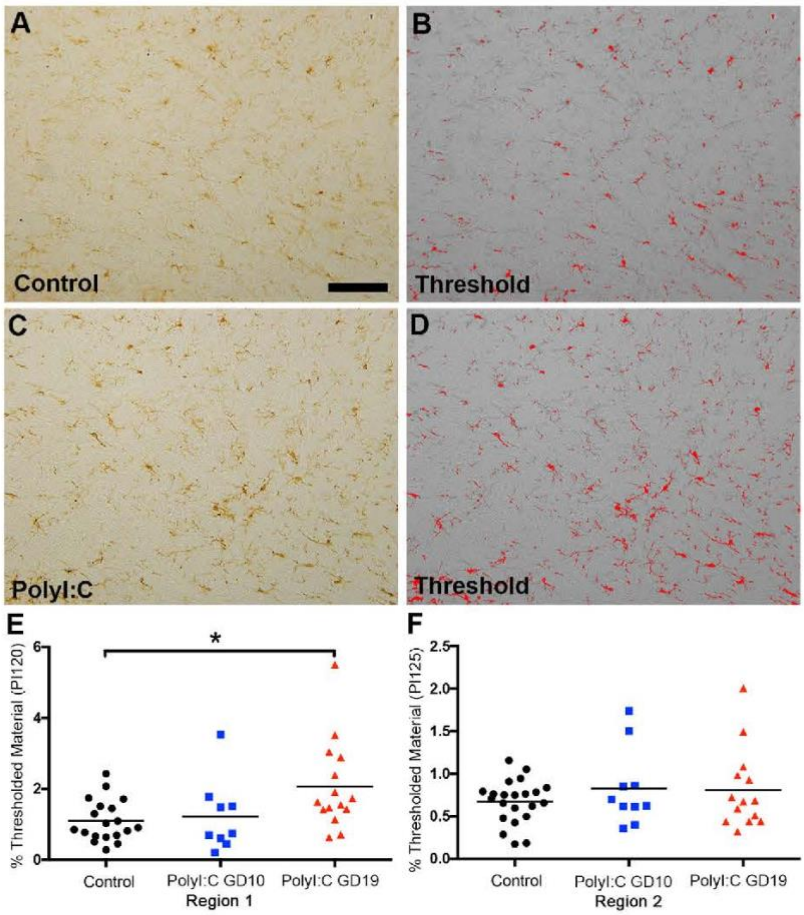

Fig. 4. Effect of maternal immune activation on IBA1+ immunoreactive material (IBA1+IRM) in the white matter (WM) of the corpus callosum. Representative images of IBA1+ immunoreactive microglia in the WM of the corpus callosum in region 1 of the rat brain in either controls (A) or offspring from animals exposed to PolyI:C at GD19 (C), with the corresponding images showing IBA1+ thresholded material at pixel intensity 120 (B,D). The percentage of IBA1+IRM is shown at a given pixel intensity in two rostrocaudally adjacent areas region 1 (E) and region 2 (F) for offspring exposed to PolyI:C at GD10 (blue squares) or GD19 (red triangles), as well as controls (black circles). Each data point represents the mean % of IBA1+IRM for one animal. The percentage of IBA1+IRM in the WM was significantly increased after maternal immune activation at GD19 in region 1 ($p = 0.016$). No difference was observed after maternal immune activation at GD10 nor in region 2 in both groups. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the effects of maternal immune activation on the density of microglia in the offspring. IBA1+ immunoreactive microglia were identified in abundance in the CC (Fig. 2(A)) with fewer in the WM of the corpus callosum (Fig. 2(B)) in offspring of controls and dams exposed to maternal immune activation. Using one-way ANOVA, we observed no alterations in the level of IBA1+IRM in the CC in either region 1 [$F(2,32) = 2.269$, $p = 0.119$ – Fig. 3(A), (B) and (E)], or region 2 [$F(2,39) = 1.09$, $p = 0.345$ – Fig. 3(C), (D) and (F)]. However, in the WM of the corpus callosum, one-way ANOVA showed a significant group difference in the level of IBA1+IRM in region 1 [$F(2,40) = 4.619$, $p = 0.016$ – Fig. 4(A), (B) and (E)]. Bonferroni multiple comparisons showed the level of IBA1+IRM in the region 1 WM (Fig. 4(E)) was significantly increased by ~46% in GD19 PolyI:C rats (2.065 ± 0.32% of IBA1+IRM, $p = 0.017$), but not GD10 PolyI:C rats (1.22 ± 0.34% of IBA1+IRM, $p = 0.99$) compared to control rats (1.099 ± 0.13% of IBA1+IRM). We did not observe any group differences in IBA1+IRM in region 2 WM [$F(2,43) = 0.8448$, $p = 0.4366$ – Fig. 4(C), (D) and (F)].

3.3. Effects of maternal immune activation on GFAP+IRM in the brains of the offspring

GFAP+ immunoreactive astrocytes were identified in the CC (Fig. 2(C)) but were more abundant in the WM of the corpus callosum (Fig. 2(D)) in the offspring of controls and dams exposed to maternal immune activation. Interestingly, one-way ANOVA identified a significant group effect of maternal immune activation on GFAP+IRM in region 1 of the CC ($F(2,40) = 2.08$, $p = 0.045$ – Fig. 5(A), (B), (E) and (F)). Whilst Bonferroni multiple comparisons showed there was no change in GFAP+IRM in GD10 PolyI:C rats (0.17 ± 0.16% of GFAP+IRM, $p = 0.99$), there was a strong trend towards increased GFAP+IRM in GD19 PolyI:C rats (0.35 ± 0.33% of GFAP+IRM, $p = 0.054$) compared to controls. No effect of maternal immune activation on GFAP+IRM was observed in the CC region 2 [$F(2,43) = 0.43$, $p = 0.6531$ – Fig. 5(C),(D) and (F)]. In contrast to the IBA1 data, Fig. 6(A)–(F) shows that overall we did not observe any alterations in the level of GFAP+IRM in the WM of the corpus callosum in rats exposed to maternal immune activation [region 1: $F(2,43) = 1.739$, $p = 0.1879$ – Fig. 6(E); region 2: $F(2,43) = 1.183$, $p = 0.3161$ – Fig. 6(F)].

3.4. Correlation of IBA1+IRM and GFAP+IRM with white matter (WM) 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 maternal immune activation at GD10 but more prominently in GD19 within the same cohort of animals (Duchatel et al., 2016) used in this current study. Therefore, we examined whether the percentage of IBA1 + IRM (Fig. 7) or GFAP + IRM (Fig. 8) in the WM of region 1, correlated with the SST positive (SST+) or NeuN positive (NeuN+) IWMN density (Duchatel et al., 2016). We observed a statistically significant negative correlation between the percentage of IBA1+IRM (PI120) and NeuN+ IWMNs (Fig. 7(C)) and SST+ IWMNs (Fig. 7(D)) in GD19 PolyI:C rats (NeuN: $r = -0.624$,

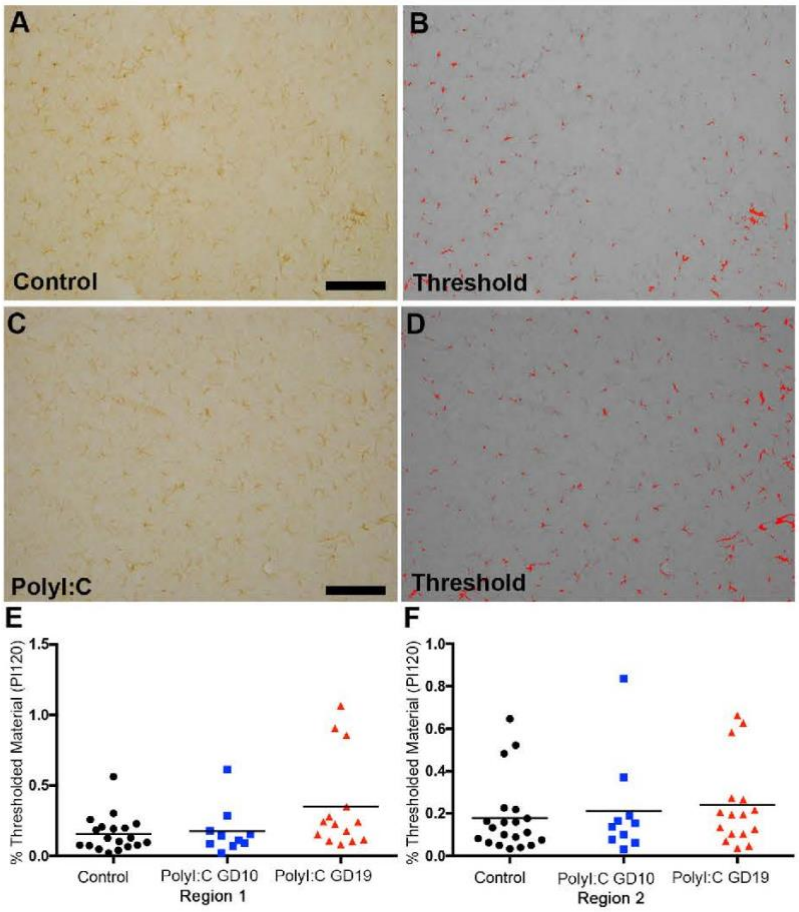

Fig. 5. Effect of maternal immune activation on GFAP+ immunoreactive material (GFAP+IRM) in the cingulate cortex (CC). Representative images of GFAP+ immunoreactive astrocytes in the CC in region 1 of either controls (A) or offspring from animals exposed to PolyI:C at GD19 (C), with the corresponding images showing GFAP+ thresholded material at pixel intensity 120 (B,D). (E) and (F) The percentage of GFAP+IRM is shown at a given pixel intensity for offspring exposed to PolyI:C at GD10 (blue squares) or GD19 (red triangles, as well as controls (black circles). Each data point represents the mean % of GFAP+IRM for one animal. Comparisons are presented for two rostrocaudally adjacent areas, region 1 (E) and region 2 (F). The percentage of GFAP+IRM was not significantly different between control and PolyI:C rats in either region. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$p = 0.017$ and SST: $r = -0.604$, $p = 0.022$) but not control rats (Fig. 7(A) and (C)) NeuN: $r = -0.417$, $p = 0.122$ and SST: $r = 0.011$, $p = 0.968$), nor GD10 PolyI:C rats (Fig. 7(B) and (E)) NeuN: $r = -0.280$, $p = 0.544$ and SST: $r = -0.555$, $p = 0.196$). There was no correlation between GFAP+IRM and NeuN+ IWMN density (Fig. 8(A)–(C)) or SST+ IWMN density (Fig. 8(D)–(F)) in either control rats (NeuN: $r = 0.054$, $p = 0.849$ and SST: $r = 0.225$, $p = 0.421$), GD10 PolyI:C rats (NeuN: $r = -0.214$, $p = 0.611$ and SST: $r = -0.082$, $p = 0.846$) or GD19 PolyI:C rats (NeuN: $r = 0.142$, $p = 0.628$ and SST: $r = 0.395$, $p = 0.162$).

4. Discussion

The current study examined the effects of maternal immune activation on immune-related cytokine gene expression and the density of microglia and astrocytes. The findings indicate that prenatal exposure to maternal immune activation results in subtle neuro-immune changes in adulthood, specifically in IBA1+ microglia immunoreactivity. Previous work from our group has demonstrated that rats exposed to PolyI: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 PolyI:C rats), in addition to altered dopamine 1 receptor expression in GD10 PolyI:C male rats (Meehan et al., 2016). Furthermore, previous work from our group has demonstrated that adult rats exposed to maternal immune activation have a schizophrenia-like increase in IWMN density, particularly those that express SST (Duchatel et al., 2016). Thus the current findings further suggest this early (GD10) verses late (GD19) rat model of maternal immune activation produces subtle schizophrenia-like phenotypes.

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 *TNF-α*, *IL-1β*, and *IL-6* (Fillman et al., 2012; Fillman et al., 2014; Kim and Maes, 2003). In the current study we examined the expression of *IL-1β* and *TNF-α* in the CC from offspring exposed to maternal immune activation at both GD10 and GD19, but did not find any significant changes in mRNA levels. These results are in line with those of Willi et al. (2013) who demonstrated that early gestation (GD9) maternal immune activation 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) detected alterations to cytokine protein levels in the frontal cortex (FC) and CC of mice exposed to mid-gestational (GD 12.5) PolyI:C, however these changes were dependent on the age of the offspring at assessment. Garay et al. (2012) observed that in animals exposed to maternal immune activation, 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 (Garay et al., 2012) thus the two cytokines examined in the current study were found to be unchanged at the same stage of development. Based on these observations it seems

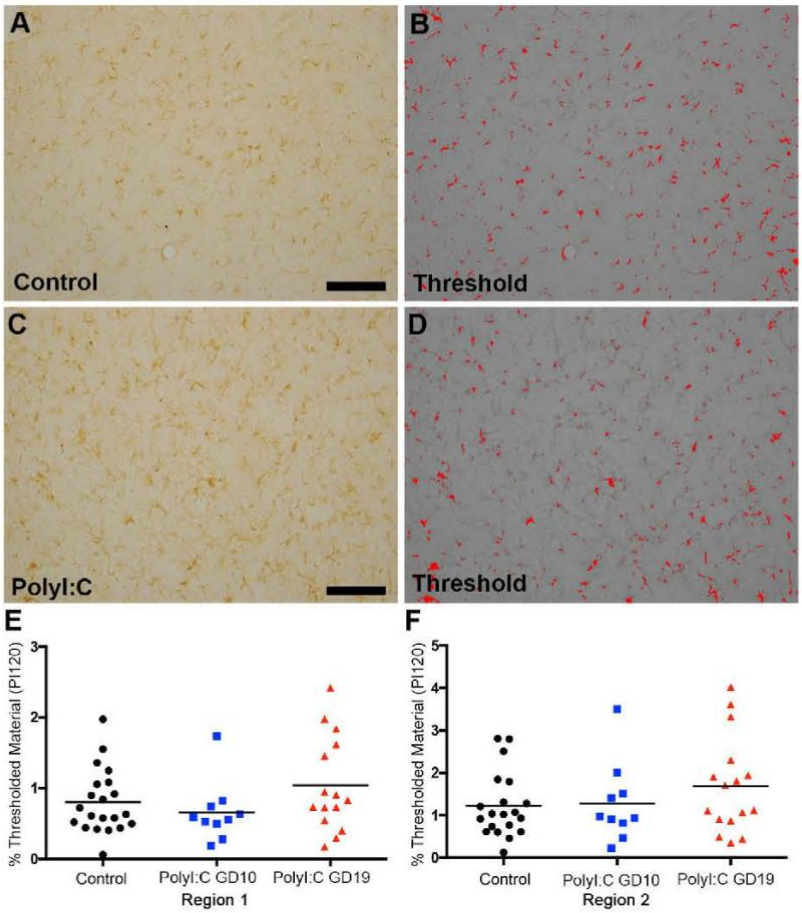

Fig. 6. Effect of maternal immune activation on GFAP+ immunoreactive material (GFAP+IRM) in the white matter (WM) of the corpus callosum. Representative images of GFAP+ immunoreactive microglia in the WM of the corpus callosum in region 1 of the rat brain in either controls (A) or offspring from animals exposed to PolyI:C (C), with the corresponding images showing GFAP+ thresholded material at pixel intensity 120 (B,D). (E) and (F) The percentage of GFAP+IRM at a given pixel intensity for offspring exposed to PolyI:C at GD10 (blue squares) or GD19 (red triangles), as well as controls (black circles). Each data point represents the mean % of GFAP + IRM material for one animal. Comparisons are presented for two rostrocaudally adjacent areas, region 1 (E) and region 2 (F). The percentage of GFAP+IRM was similar between control and PolyI:C rats in both regions. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the expression of cytokines is highly age dependent and it appears the adult stage of development may not be the most sensitive period for detecting changes in these particular cytokines.

Cytokine and chemokine expression may also be region dependent and so it is possible that cytokine alterations may be present in other regions not examined here. Previous work from a mid-gestational (GD15) maternal immune activation rat model reported increased microglial derived *IL-1β*, but not *TNF-α*, in the hippocampal region of adult offspring (Missault et al., 2014). 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 should aim to assess a range of pro and anti-inflammatory chemokines and cytokines, at multiple postnatal time-points in multiple schizophrenia-relevant brain regions to further establish the developmental nature of any central cytokine alterations resulting from maternal immune activation.

As long-term neuro-inflammatory alterations are associated with schizophrenia, and have been identified as a potential mediating mechanisms between maternal immune activation and schizophrenia-related neurodevelopmental disruption (Na et al., 2014), the current study assessed astrocytes and microglia using immunohistochemistry within the CC and frontal WM, areas of the brain strongly implicated in schizophrenia (Lewis and Levitt, 2002; Pomarol-Clotet et al., 2010; Salgado-Pineda et al., 2007; Wible et al., 2001). We observed subtle effects of maternal immune activation on GFAP+ immunoreactivity, but not mRNA, only in the CC (not WM) with pairwise comparisons showing an increase in GFAP+IRM which approached significance in GD19 but not GD10 PolyI:C rats. These results are in line with the results of Nyffeler et al. (2006) who also failed to find any GFAP changes in adult (PND 180>) mice following a single dose of PolyI:C in early gestation (GD9). Interestingly, de Souza et al. (2015) reported that lipopolysaccharide 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 indicating that GFAP levels may normalise throughout adolescence. In direct opposition to this suggestion, Paylor et al. (2016) who induced maternal immune activation with PolyI:C at GD15 did not observe any change in GFAP optical density in the medial prelimbic cortex at four postnatal stages (PND7, 21, 35, 90). Thus whilst the increase in GFAP+IRM approached significance in our GD19 group ($p = 0.054$) further assessment of GFAP markers at a range of postnatal stages and brain regions needs to be conducted to confirm that GFAP+ astrocytes are not affected by maternal immune activation in our model.

The current study also examined the effects of maternal immune activation at early or late gestation on microglia within the CC and frontal WM. We observed that maternal immune activation in late gestation (GD19), but not early gestation (GD10), was associated with increased IBA1+IRM, in the frontal WM of the corpus callosum. Increases in IBA1+ microglial density have previously been reported in the corpus callosum of adult rats following PolyI:C exposure at mid-gestation (GD15) (Van den Eynde et al., 2014), and in frontal WM of neonatal rats (PND 9) following late-gestation (GD17 through to birth) lipopolysaccharide exposure (Girard et al., 2010). These findings suggest that activation of the mother's immune system during mid-late

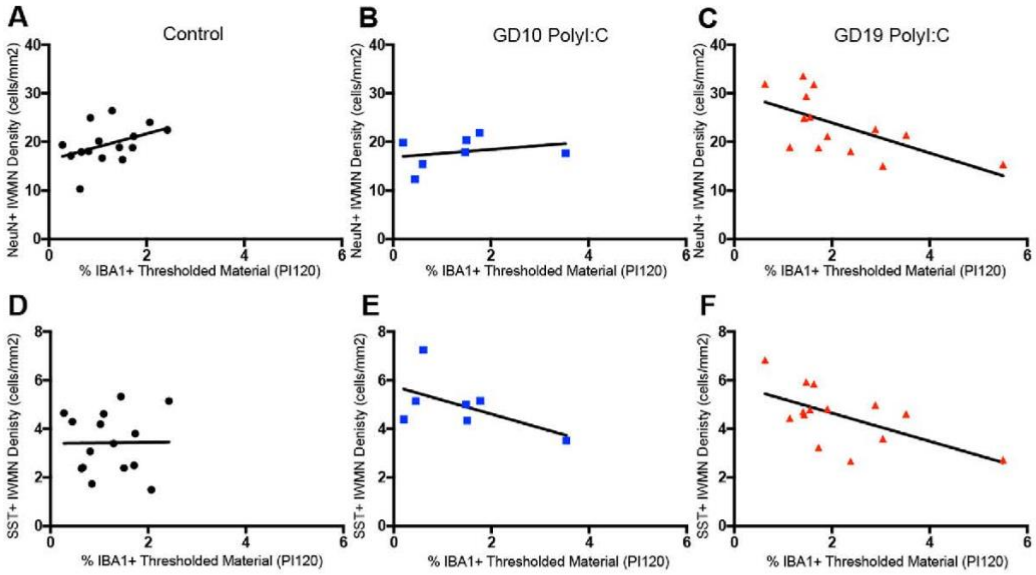

Fig. 7. Correlation between IBA1+ immunoreactive material (IBA1+IRM) with neuronal density in the white matter (WM) of the corpus callosum. Correlations of IBA1+IRM with Neun (A)–(C) and SST (D)–(F) IWMN density. The percentage of IBA1+IRM negatively correlated with both NeuN+ IWMN density (C) and SST+ IWMN density (F) in offspring from rats exposed to maternal immune activation at GD19 (Red triangles – NeuN: $r = -0.624$, $p = 0.017$ and SST: $r = -0.604$, $p = 0.022$). There was no correlation between IBA1+IRM and NeuN+ or SST+ IWMN density in either control rats (A) and (D) or GD10 maternal immune activation rats (B) and (E). SST+ and NeuN+ IWMN density data was previously reported in Duchatel et al. (2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gestation is associated with subtle long-term neuro-inflammatory alterations to microglia in the frontal WM of the offspring.

With respect to the cortex, no change in *Iba1* mRNA expression nor IBA1+IRM was observed in the CC which is consistent with the literature. For example, PolyI: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). Furthermore, two studies investigated mid-gestational (GD15) PolyI:C exposure in rats but also failed to observe microglial alterations in the medial prelimbic cortex of the offspring at a range of postnatal stages up to adulthood (Hadar et al., 2017; Paylor et al., 2016), consistent with our study. However, a number of these studies reported significant changes in microglia in other schizophrenia relevant brain regions, including the hippocampus and striatum, suggesting that the microglia in the frontal cortices of adult rodents are not as susceptible or affected by the

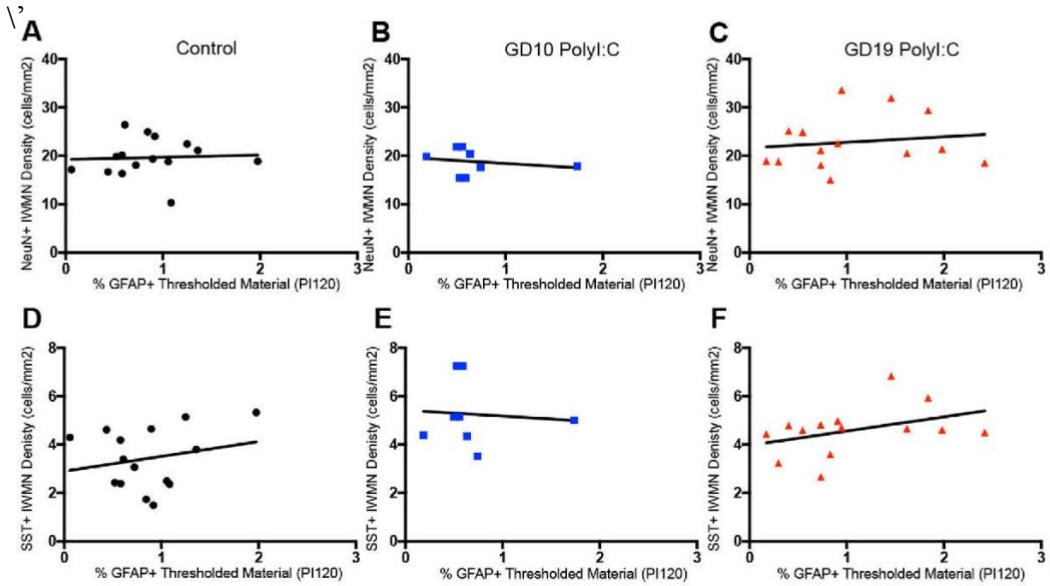

Fig. 8. Correlation between GFAP+ immunoreactive material (GFAP+IRM) with neuronal density in the white matter (WM) of the corpus callosum. No correlation was observed between the percentage of GFAP+IRM and NeuN+ IWMNs or SST+ IWMNs in either control rats (A) and (D), or those exposed to maternal immune activation at either GD10 (D) and (E) or GD19 (C) and (F). SST+ and NeuN+ IWMN density data was previously reported in Duchatel et al. (2016).

current maternal immune activation protocols. Future studies using the current model would benefit from investigating IBA1 + IRM in the hippocampus and other brain regions or determine if increasing the maternal immune activation exposure over several days induced a response in microglia located in frontal cortices.

Previously, our group demonstrated that exposure to maternal immune activation increased the density of IWMNs particularly those expressing SST using tissue from the same animals in the current study (Duchatel et al., 2016); an effect most prominent after maternal immune activation at GD19 and in the most rostral region of the corpus callosum (i.e. region 1) where we now show an increase in IBA1 + IRM. As these two findings were observed in the same tissue, we hypothesised that the higher the IBA1+ 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 WM. Contrary to our hypothesis, the correlation analysis showed that SST+ immunoreactivity 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. Since the GFAP+IRM was not significantly different in GD19 PolyI:C rats, we did not expect and consequently did not observe a correlation with GFAP+IRM and IWMN density.

Some methodological considerations are important in the interpretation of these results. This study had two main parts, an investigation of the effects of maternal immune activation on the gene expression of *Iba1, Gfap, TNF-α* and *IL-1β*, and an investigation of IBA1+IRM and GFAP+IRM using IHC. Due to these methodological differences where the brain tissue used for gene expression was fresh frozen and the tissue for IHC 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 IHC cohort, we examined the level of IBA1+IRM/GFAP +IRM in both the CC and the WM of the corpus callosum. However, within the gene expression cohort, due to methodological limitations in isolation of WM devoid of cortical tissue from the blocks of rat brains, we examined only the CC. In addition, whilst GFAP is regarded as a sensitive and reliable marker that labels the majority of reactive astrocytes in central nervous system injury models, it is not an absolute marker of all non-reactive astrocytes and in some cases can be immunohistochemically undetectable in astrocytes in healthy central nervous system tissue (Sofroniew and Vinters, 2010). Stereological counting methodologies have been used previously to determine microglia density. In this study, we used cumulative threshold spectra analysis to examine IBA1+ and GFAP+ immunoreactivity, a method shown to be powerful for detecting subtle alterations to IBA1+ immunoreactive microglia and GFAP+ immunoreactive astrocytes (Jones et al., 2015; Ong et al., 2016; Patience et al., 2015). This thresholding methodology is able to sensitively detect differences in immunoreactive material (see Supplementary Figs. S2 and S3), across the entire pixel intensity spectrum. However, this method is unable to detect morphological differences such as those between activated or non-activated microglia. Thus these findings can be extended in the future by subtyping the microglia into the M1 and M2 phenotypes and associated morphological analyses, to determine whether maternal immune activation affects microglia activation in the offspring.

Although maternal immune activation models of schizophrenia-like pathologies are becoming better established, little is still known about how maternal immune activation 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 maternal immune activation model which has previously demonstrated schizophrenia-relevant behavioural and brain dysfunction (Duchatel et al., 2016; Meehan et al., 2016). 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.

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Competing interests

The authors have no competing interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2018.05.063.

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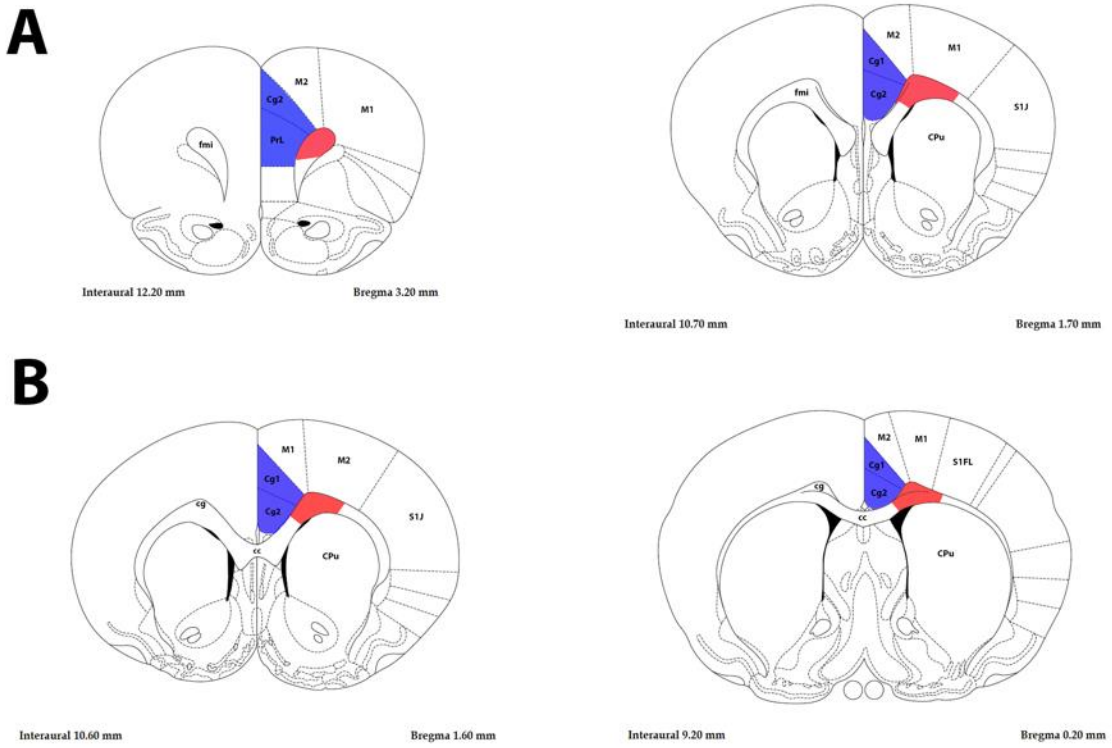
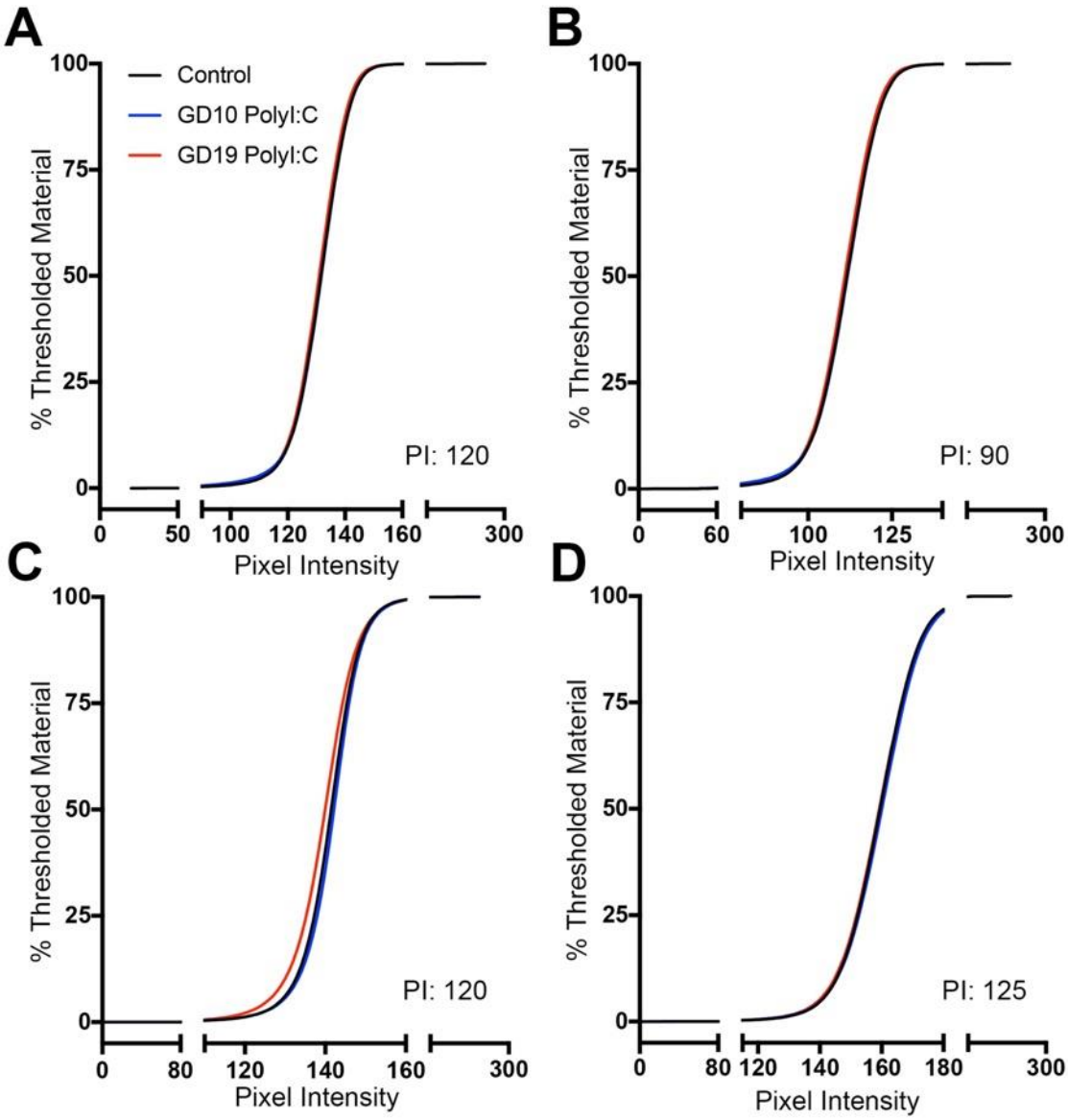
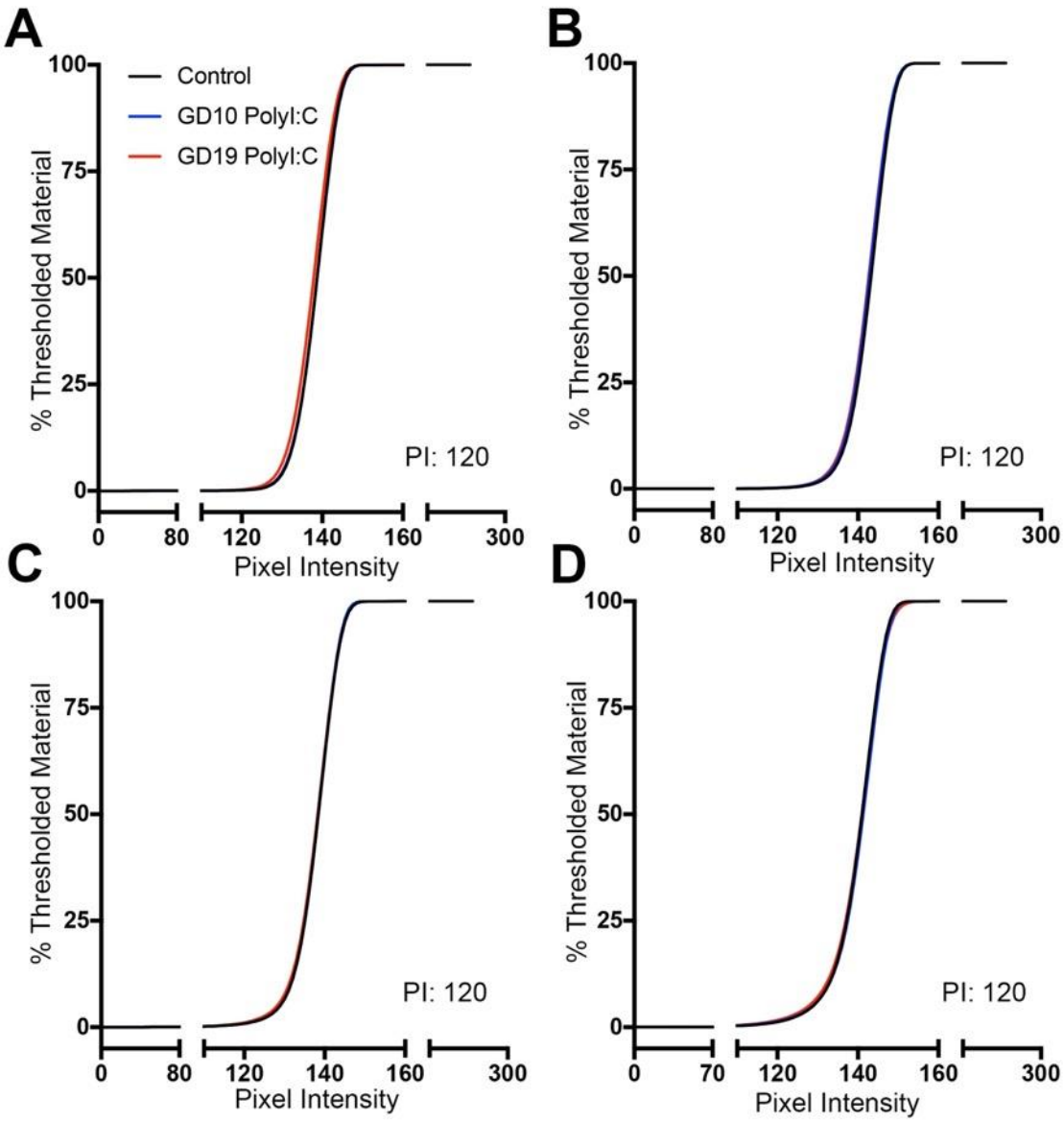
Supplementary Figures



Supplementary Figure S1: Anatomical co-ordinates used for immunohistochemical analyses. The rat brain was binned into two regions based on the different proximity to Bregma and brain structure. Region 1 was binned from 3.20mm to 1.70mm from Bregma (A). Region 2 was binned from 1.60mm to 0.2mm from Bregma (B). The areas sampled for experimentation are shaded in blue for the cingulate cortex and red for the white matter. M2: Secondary Motor Cortex, M1: Primary Motor Cortex, Cg1: Cingulate Cortex Area 1, Cg2: Cingulate Cortex Area 2, PrL: Pre-Limbic Cortex, fmi: Forceps Minor of the Corpus Callosum, S1J: Primary Somatosensory Cortex, CPu: Caudate Putamen, cc: Corpus Callosum, cg: Cingulum, S1FL: Primary Somatosensory Cortex Forelimb Area. Adapted from Watson and Paxinos (2005).



Supplementary Figure S2: Cumulative thresholding spectra of IBA1+IRM. Cumulative thresholding analysis was conducted in the Prefrontal Cortex (Region 1 – A and Region 2 – B) and the WM (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 left corner of each graph.



Supplementary Figure S3: Cumulative thresholding spectra of GFAP+IRM. Cumulative thresholding analysis was conducted in the Prefrontal Cortex (Region 1 – A and Region 2 – B) and the WM (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.

CHAPTER FIVE

Complement Component 4: Linking Maternal Immune Activation and Schizophrenia

Statement of Contribution

Ryan J. Duchatel, Crystal L. Meehan, Lauren R. Harms, Patricia P. Michie, Mark J. Bigland, Douglas W. Smith, Phillip Jobling, Deborah M. Hodgson, Paul A. Tooney. **Increased complement component 4 (C4) gene expression in the cingulate cortex of rats exposed to late gestation immune activation.** Schizophrenia Research.

*"I attest that Research Higher Degree candidate **Ryan Duchatel**, was the primary contributor to the development of this publication. This extensive contribution included: contributing to the initial design of the study; contributing to the intellectual development of this study; developing, optimising, executing, analysing and interpreting all experiments contained within this publication; and writing the manuscript in full".*

_____	_____
Ryan Duchatel	***Crystal Meehan***
_____	_____
Lauren Harms	***Patricia Michie***
_____	_____
Mark Bigland	***Douglas Smith***
_____	_____
Phillip Jobling	***Deborah Hodgson***

_____	_____
Paul Tooney	***Robert Callister***
(Primary PhD Supervisor)	(Deputy Head of Faculty)

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Letter to the editor

Increased complement component 4 (*C4*) gene expression in the cingulate cortex of rats exposed to late gestation immune activation

Keywords:
Schizophrenia
Complement component C4
Maternal immune activation
Cingulate cortex
Late gestation
Rat

The largest genetic study of schizophrenia confirmed the strong genetic association with the major histocompatibility complex (MHC) locus (Ripke et al., 2011). Whilst the link between schizophrenia and the MHC locus is not yet fully elucidated, there is significant interest in immune dysfunction as an underlying pathology in schizophrenia. Recently Sekar et al. (2016) presented strong evidence for a role of complement component 4 (C4) in schizophrenia by showing that some of the variation in the MHC locus in schizophrenia is linked to the *C4* gene. Whilst complement dysfunction in schizophrenia has been known for some time (reviewed in Nimgaonkar et al. (2017)), Sekar et al. (2016) provided the first pathophysiological link between increased *C4A* expression in schizophrenia and reduced synaptic pruning in $C4^{-/-}$ knockout mice. Thus, the increased *C4A* expression in the brain in schizophrenia could be related to the reduced synaptic density observed in post-mortem studies of schizophrenia affecting several cortical regions (for review see Glausier and Lewis (2013)) including the anterior cingulate cortex (CC), a region known to be structurally and functionally altered in individuals with the disorder (Roberts et al., 2015).

Infections during pregnancy increase the risk of schizophrenia and this may be exacerbated by complement system dysfunction (Nimgaonkar et al., 2017). We and others have reported that rodent models of maternal immune activation (MIA), induce behavioural changes and neuropathology in the offspring like those observed in schizophrenia (Duchatel et al., 2016; Meehan et al., 2017). Relevant to this current study, Short et al. (2010) reported reduced volume of several cortical regions including the CC in rhesus monkeys exposed prenatally to influenza infection during mid-late gestation. Furthermore, late gestation exposure of mice to PolyI:C decreased expression of the synaptic marker synaptophysin in the hippocampus (Giovanoli et al., 2015). It is tantalising to suggest that the reduction in cortical volume induced by MIA could be due to changes in synaptic pruning brought about by alteration to the brain's complement component system during development. As a step towards this, we evaluated whether maternal immune activation induced by polyriboinosinic-polyribocytidylic acid (polyI:C) in rats in early or late gestation altered complement component *C4* gene expression in the cingulate cortex (CC) of the offspring.

Pregnant Wistar rats were injected with polyI:C at gestational day (GD) 10 or GD19 as described previously (Duchatel et al., 2016; Meehan et al., 2017) and offspring euthanized at postnatal day 72–84 (Animal Research Ethics Committee University of Newcastle approval A-2013-319). RNA was extracted from fresh frozen CC tissue, with *C4* gene expression measured by qPCR using cycle conditions described by Parkinson et al. (2016). Since rodents do not have the *C4A* and *C4B* isotypes present in humans, one set of *C4* primers was designed for qPCR (Supplementary Table 1). The relative expression of *C4* was determined by the delta-delta Ct method (Schmittgen and Livak, 2008) utilizing the geometric mean of β-actin (*Actb*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and β-tubulin (*Tubb*) as internal reference genes. Groups were analyzed by ordinary one-way ANOVA with post-hoc correction for multiple comparisons using the Holm-Bonferroni correction method.

Here we report for the first time a significant increase in *C4* gene expression in the CC of offspring exposed to prenatal polyI:C treatment at GD19 ($p = 0.02$) but not GD10 ($p = 0.99$) (Fig. 1A). This supports our previous work demonstrating that schizophrenia-related pathologies including working memory impairments (Meehan et al., 2017) and increased white matter neuron density (Duchatel et al., 2016) are more prominent in rat offspring exposed to MIA during late gestation. Furthermore, a study by Han et al. (2017) observed increased complement component C1q protein expression in the prefrontal cortex of offspring exposed to polyI:C during mid-late gestation in mice. In contrast, Mattei et al. (2014) did not detect a change in C4 protein expression in animals exposed to MIA, however they investigated the hippocampus in mouse offspring after mid gestation MIA (GD15). Clearly further studies need to investigate the complement component system in both rodent MIA models with early/mid/late gestational exposures and in different brain regions.

When we divided by sex, the significant increase in *C4* gene expression after MIA at GD19 was only detected in male ($p = 0.02$) but not female ($p = 0.99$) offspring (Fig. 1B). This gender effect on *C4* gene expression after late gestation MIA is consistent with our previous findings showing more schizophrenia-like behavioural deficits in male rats in our model (Meehan et al., 2017). Why this sex effect occurs and whether this relates to evidence showing that males with schizophrenia generally have a more severe and chronic course of the illness, remain to be determined.

In conclusion, recent findings by Sekar et al. (2016) implicated complement component *C4* in schizophrenia pathology, providing strong evidence for links between increased expression of *C4* in the brain in schizophrenia with increased synaptic pruning. Here we provide evidence that maternal immune activation during pregnancy, a known environmental risk factor for schizophrenia, is indeed itself capable of inducing long-term increases in *C4* gene expression in the rat cingulate cortex of their offspring later in life. Thus, it is possible that if infection occurs late in pregnancy and long lasting changes to complement components are induced in the offspring's brain, increased synaptic pruning

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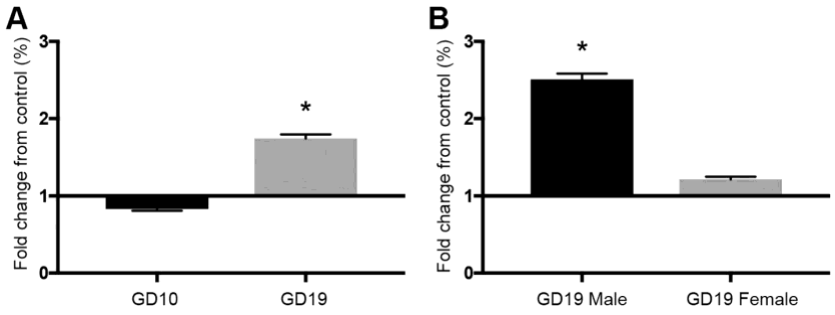

Fig. 1. Effects of maternal immune activation on the gene expression of Complement Component 4 (*C4*). Relative gene expression for *C4* in the cingulate cortex (CC) of offspring from dams exposed to polyI:C at either GD10 (n = 12) or GD19 (n = 12) (controls n = 12 per GD; 6 females and 6 males per group). (A) A significant increase in *C4* gene expression was observed in offspring from GD19 polyI:C rats (Fold change: 1.74, $p = 0.02$) but not GD10 PolyI:C rats. (B) Separation on gender showed that *C4* gene expression was significantly increased in male GD19 polyI:C rats (Fold Change: 2.51, $p = 0.02$) but not female GD19 polyI:C rats. Bars represent mean fold change +/— SEM.

could be triggered leading to development of behavioural disturbances and schizophrenia later in life.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.schres.2018.03.035.

Author disclosure

Funding body agreements and policies

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Contributors

PAT, RJD, DMH designed the study. RJD conducted all experiments. PTM, DMH, LRH and CLM developed the MIA model. CLM did all the animal husbandry associated with the MIA model which provided animals and tissues for this study. MJB and DWS assisted with the gene expression analyses. RJD developed the figures. RJD conducted all statistical analyses. All authors contributed to writing and editing the manuscript and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Ryan J. Duchatel
School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, Callaghan, New South Wales 2308, Australia
Priority Centre for Brain and Mental Health Research, University of Newcastle, Callaghan, New South Wales 2308, Australia
Hunter Medical Research Institute, New Lambton Heights, New South Wales 2305, Australia
E-mail address: Ryan.Duchatel@newcastle.edu.au.

Crystal L. Meehan
Lauren R. Harms
Patricia T. Michie
School of Psychology, Faculty of Science, University of Newcastle, Callaghan, New South Wales 2308, Australia
Priority Centre for Brain and Mental Health Research, University of Newcastle, Callaghan, New South Wales 2308, Australia
Hunter Medical Research Institute, New Lambton Heights, New South Wales 2305, Australia
E-mail addresses: Crystal.Meehan@uon.edu.au (C.L. Meehan), Lauren.Harms@newcastle.edu.au (L.R. Harms), Pat.Michie@newcastle.edu.au (P.T. Michie).

Mark J. Bigland
Doug W. Smith
Phillip Jobling
School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, Callaghan, New South Wales 2308, Australia
Priority Centre for Brain and Mental Health Research, University of Newcastle, Callaghan, New South Wales 2308, Australia
Hunter Medical Research Institute, New Lambton Heights, New South Wales 2305, Australia
E-mail addresses: Mark.Bigland@uon.edu.au (M.J. Bigland), Douglas.Smith@newcastle.edu.au (D.W. Smith), Phillip.Jobling@newcastle.edu.au (P. Jobling).

Deborah M. Hodgson
School of Psychology, Faculty of Science, University of Newcastle, Callaghan, New South Wales 2308, Australia
Priority Centre for Brain and Mental Health Research, University of Newcastle, Callaghan, New South Wales 2308, Australia
Hunter Medical Research Institute, New Lambton Heights, New South Wales 2305, Australia
E-mail address: Deborah.Hodgson@newcastle.edu.au.

Paul A. Tooney*
School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, Callaghan, New South Wales 2308, Australia
Priority Centre for Brain and Mental Health Research, University of Newcastle, Callaghan, New South Wales 2308, Australia
Hunter Medical Research Institute, New Lambton Heights, New South Wales 2305, Australia
*Corresponding author at: School of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan, New South Wales 2308, Australia.
E-mail address: Paul.Tooney@newcastle.edu.au.

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Supplementary Figures

Table 1: Primer sequences for qPCR

Gene	Forward Primer	Reverse Primer
Actb	CCTAGCACCATGAAGATCAAGA	GCCAGGATAGAGCCACCAATC
Gapdh	GGCTGGCATTCGTCTCAA	GAGGTCCACCACCCTGTTG
Tubb	CTTCCGACTCCTCGTCGTCA	GAGGCCGAGAGCAACATGAA
C4	CTAAGGCCACCAGCGTCTGA	AGCTTTTCCTCCCTGCAAATCC

CHAPTER SIX

Thesis discussion and remarks

6.0 Discussion

This study utilised a rat model of MIA, at two gestational time points, GD10 (early) and GD19 (late), to attempt to identify 1) if MIA induced an increase in IWMN density, 2) if glia were altered after MIA suggesting immune dysfunction, and 3) if *C4* gene expression was altered in the cortex, providing evidence of neuroinflammation.

In line with these aims, this thesis identified the following significant differences after maternal immune activation:

- SST+ IWMN density is increased in the white matter of the corpus callosum after MIA, after both GD10 and GD19 PolyI:C
- Increased Iba1+ immunoreactivity was observed in the white matter of the corpus callosum, but not the prefrontal cortex after late gestation (GD19) MIA
- *C4* gene expression was increased in the prefrontal cortex after late gestation (GD19) MIA

In addition, a number of important hallmarks remained unchanged including:

- Except for an increase in SST+ IWMN density at GD10, there was a lack of significant alterations after early MIA (GD10)
- No change in GABAergic gene expression in the prefrontal cortex
- No change in gene expression of cytokine markers in the cortex
- No change in microglia density in the cortex

6.1 White matter neurons in schizophrenia

A number of hypotheses have been put forward to explain the pathogenesis of schizophrenia, but the cause still remains undetermined. Evidence suggests perturbations during *in utero* brain development could contribute to the pathogenesis of schizophrenia (Brown, 2006). Specifically, this thesis focussed on the effects of maternal immune activation on the density of IWMNs. IWMNs are a poorly characterised neuronal subpopulation which exist within the white matter regions of the brain and have been shown to be increased in post mortem brains of people with schizophrenia. Unfortunately studying the development of neural circuits or measuring the function of discrete populations of neurons is impossible in human patient populations with presently available technology. The best resolution imaging technologies cannot resolve down to the level of individual neurons in humans. Therefore, a major aim of this PhD project was to determine if an animal model of maternal immune activation could induce an increase in the density of IWMN, like what is observed in schizophrenia. This would provide a model system to shed light on this mysterious neuronal population.

Chapter 1 provided a detailed review into white matter neurons, their biology and role in the development of schizophrenia. Even though, the origins of IWMNs and their specific role in the disease pathogenesis of schizophrenia remain to be determined. Indeed, Chapter 2 and Chapter 3 provided a series of published and unpublished works examining specific subsets of IWMNs after PolyI:C exposure. In Chapter 2, I examined the density of NeuN+ IWMNs, which trended to be increased after GD19 PolyI:C but did not reach statistical significance.

Interestingly though, the SST+ subpopulation of IWMNs was significantly increased after MIA, especially after later gestation immune activation, supporting the difference reported by Yang et al. (2011b). Yang et al. (2011b), who observed a 22% increase in SST mRNA+ cells in the white matter of the DLPFC – suggesting that SST+ IWMNs are particularly susceptible to MIA.

Chapter 3 continued the examination of IWMNs in the MIA model and identified that rat IWMNs also expressed the GABAergic interneuron markers GAD and NPY as previously determined for the human brain by Joshi et al. (2012) and Ikeda et al. (2004). However, when their density was examined no differences were observed in GAD+ or NPY+ IWMNs after MIA. I then sought to examine if our MIA model mimicked the cortical gene expression changes seen in people with schizophrenia. No difference was observed in the expression of GABAergic genes (e.g. GAD, Pv) that are so robustly reported in people with schizophrenia (Hoftman et al., 2015). Although we did not observe cortical gene expression changes seen in people with schizophrenia, prenatal PolyI:C exposure was shown to provide a model for increasing SST+ IWMN density that could provide a foundation to further examine the role of IWMNs in a schizophrenia related context.

The changes observed in this study are consistent with human epidemiological evidence regarding timing of infection. Maternal influenza infection was first linked to increased risk of schizophrenia by Mednick et al. (1988), who observed that the offspring of women in their second trimester of pregnancy during the Helsinki 1957 influenza epidemic, had increased risk of developing

schizophrenia. This has been supported by several studies (reviewed in Brown and Derkits (2010a)). We observed significant changes in SST^+ IWMN density, and a strong trend in $NeuN^+$ IWMN at GD19 (i.e. late gestation). Interestingly, insults later in gestation may be more representative of a negative/cognitive deficit symptomology (Macedo et al., 2012a), thus increased IWMN density may be a more pronounced feature in people with negative/cognitive deficit symptoms of schizophrenia. This has support from the human IWMN literature where increased MAP2+ IWMN density was observed in a small number of schizophrenia cases with negative symptoms compared to those with positive symptoms (Kirkpatrick et al., 2003a), however this has not been validated in a larger data set.

Female rats in this MIA model generally had a higher density of IWMNs, particularly SST^+ IWMNs, than males. Joshi *et al.* (Joshi et al., 2012) also noted a gender-related trend towards increased density of IWMNs in females versus males with schizophrenia. In general, males are more likely to have an earlier onset of schizophrenia, and a more chronic disorder than females (Zhang et al., 2012). These may be initiated during foetal and early postnatal development, but are also likely to be influenced by gender differences in psychosocial experience and exposures that occur throughout life. Such differences are likely to be non-specific to schizophrenia and may pertain to other mental illness. Indeed, white matter is involved in interhemispheric connectivity and cell packing density (or number of neurons per unit volume) in the planum temporale was also greater in women than men (Witelson et al., 1995). Some have argued that men have more neurons across the entire cortex, and women more neuropil (Jacobs et al., 1993)

However, it is likely that sex differences in neuronal characteristics depend on the brain region and/or cortical layer assessed (Harasty et al., 1997). Given the relatively small number of studies in healthy brain, further investigations in sex-specific developmental brain trajectories are critical.

6.2 Underlying mechanisms for increased white matter neuron density.

The origin of IWMNs has caused some debate and is a key point in understanding the biological significance of increased IWMN density in schizophrenia. Early reports about IWMNs mainly focused on their role in development. This hypothesis focused on the cortical subplate a transient structure beneath the cortical plate that gives rise to cortical neurons in the prenatal and early postnatal brain (Ayoub and Kostovic, 2009). Though most subplate neurons undergo programmed cell death, some believe IWMNs may represent foetal subplate neurons that have escaped apoptosis and subsequently reside in the subcortical white matter of the mature adult brain (Fig. 6.1A) (Kanold and Shatz, 2006). In support of this notion, is the fact that we observed many SST+ and NPY+ IWMNs which had leading processes perpendicular with the pial/grey matter surface. This suggests that they moving the white matter towards the grey matter. In contrast, {Joshi, 2012 #2588@@author-year}, observed a number of GAD+ IWMNs with leading processes that had were pointing towards the grey matter and may be representative of migrating neurons. Indeed, supportive of the notion suggesting that IWMNs may represent neurons migrating in response to cortical damage, for example by neuroinflammation or physical damage (Fig. 6.1C). Hansen et al. (2010) demonstrated that the prenatal human cerebral cortex is a site of significant neurogenesis, in particular the areas outside the proliferative

neuroepithelium lining the ventricular walls. Indeed, rodent brain injury models suggest that upregulation of subependymal zone neurogenesis and recruitment of new neurons that migrate through the subcortical white matter to the injury site is a physiological response to cortical damage (Gu et al., 2000; Li et al., 2010; Magavi et al., 2000; Ohira, 2011). Neurogenesis in the subependymal zone has also been observed in neurodegenerative diseases of the human brain such as Huntington's disease, where death of striatal neurons is thought to induce proliferation of neuronal precursors (Curtis et al., 2003; Curtis et al., 2005). This demonstrates that a neurogenic and potentially restorative response is possible within the human brain (Fig. 6.1B). Importantly in a schizophrenia context, *GAD*, *SST*, *NPY*, *Calb1*, and *VIP* mRNA have been shown to be decreased in the DLPFC, with *SST* mRNA also decreased in the OFC, suggesting cortical damage is active in people with schizophrenia (Fung et al., 2014a; Hashimoto et al., 2008a; Morris et al., 2008). We did not observe any of these reported mRNA alterations in the MIA model (*VIP* not assessed), and correspondingly, this may have contributed a failure to identify other IWMN alterations, such as GAD+ IWMNs. Indeed, future experiments will seek to identify if there is upregulated neurogenesis in the MIA model.

Indeed, these and many other reports have identified many changes to the cortex in schizophrenia that might be detected as cortical damage. Thus the brain is capable of generating new neurons and it is possible that IWMNs may be neurons migrating postnatally in response to cortical damage. However, what happens if their ability to migrate is disrupted?

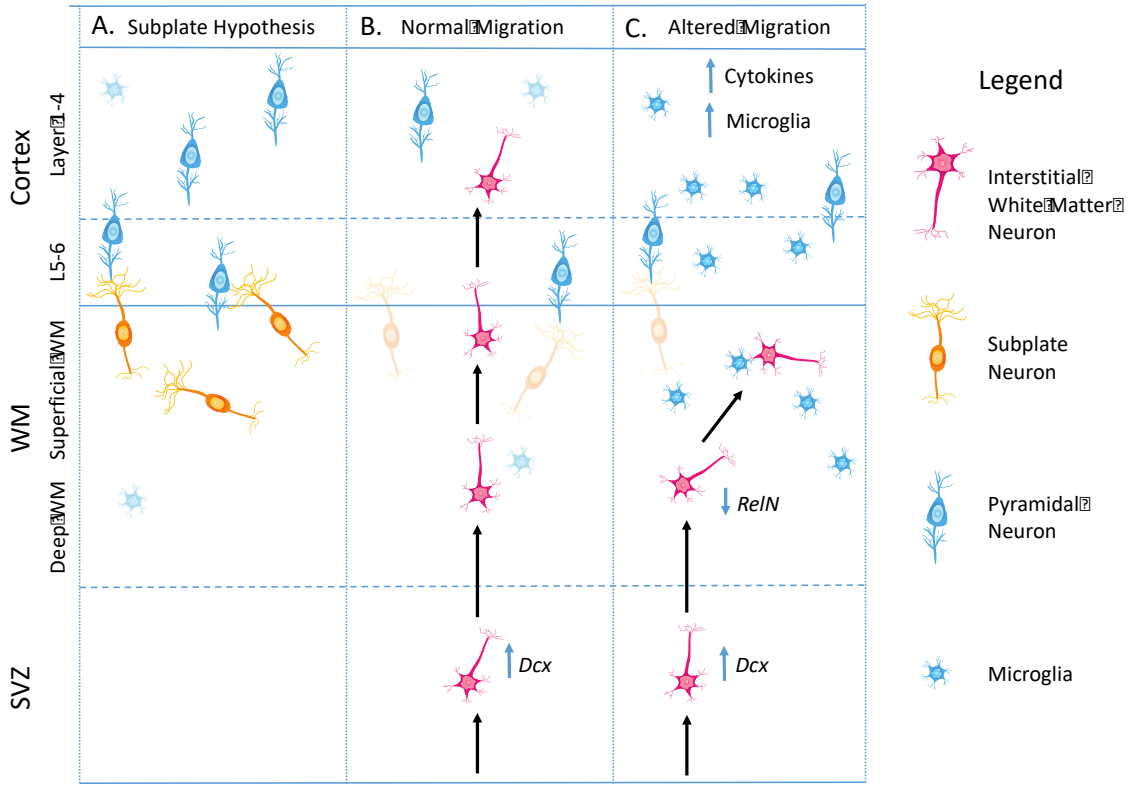

Figure 6.1. Hypothetical mechanisms for the role of interstitial white matter neurons (IWMNs) in schizophrenia. A) that IWMNs represent neurons residing in the subplate zone, B) that they are part of normal restorative brain mechanism or C) that in schizophrenia, IWMNs fail to migrate radially.

There is evidence for disrupted neuronal migration in schizophrenia. Studies show that in adulthood not all IWMNs are NeuN+. Fung et al. (2010) reported that IWMNs expressed doublecortin mRNA (a marker of immature neurons) and this negatively correlated with IWMN density in schizophrenia patients. Meanwhile, Yang et al. (2011b), observed a number of SST+ IWMNs that did not express NeuN and suggested that these IWMNs may be immature neurons. This accumulation of neurons in the white matter of schizophrenia would be congruent

with a negative correlation between doublecortin mRNA and support a migration deficit hypothesis in schizophrenia (Fung et al., 2011) (Fig 6.1C). Furthermore, reduced expression of reelin has been observed in the DLPFC in schizophrenia (Fatemi et al., 2000; Guidotti et al., 2000; Impagnatiello et al., 1998). Reelin is secreted during development and is known to guide neuronal migration, positioning and proliferation during corticogenesis (D'Arcangelo et al., 1995; Meyer and Goffinet, 1998; Meyer et al., 2000). In addition, reelin has been shown to have a role in cortical lamination, columnarity and synaptic connectivity (Borrell et al., 1999; Ogawa et al., 1995) and is reduced in expression in schizophrenia (Harvey and Boksa, 2012). To model decreased reelin expression, Tueting et al. (1999) developed a heterozygote *reeler* mouse (rl +/-), which showed a variety of morphological, behavioural, and neurochemical changes compared to wild-type mice, that were similar to those observed in people with schizophrenia (Tueting et al., 1999). Of importance to the work presented here, Tueting et al. (1999) also observed an increased number of IWMNs under the frontal cortex in these mice. However if migration is incorrectly regulated these new neurons may fail to migrate radially or fail to survive, becoming trapped in the white matter thereby failing to correct the cortical interneuron deficit (Fig. 6.1C). In consideration of the origin of IWMNs, all IWMNs that expressed SST in our study also expressed NeuN suggesting they have a mature neuronal phenotype. In contrast, Yang et al. (2011b), observed a small number of SST^{+} IWMNs in human brains that did not express NeuN and suggested that these IWMNs were developmentally immature. This is critical when trying to determine the origin of these neurons as some assume IWMNs are remnants of the subplate that gives rise to cortical neuronal populations during development. Specifically, most subplate neurons

undergo programmed cell death after the dissolution of the subplate, with a small population of these neurons surviving as IWMNs into adulthood (Kostovic et al., 2011a). This is somewhat at odds with our data, which suggests all SST+ IWMNs have a mature phenotype.

Considered together, our NeuN and SST data suggests that rat IWMNs may be more susceptible to MIA at GD19 than GD10. Related to this, SST+ interneurons are born from the medial ganglionic eminence (MGE) during development (Sussel et al., 1999; Xu et al., 2008). Studies suggest these interneurons begin migrating from the MGE towards the cortex at embryonic day (E) 14, with a larger cohort migrating from E15-17 in rats (Metin et al., 2006). Therefore, immune activation triggered by PolyI:C at GD10 comes four days before the smaller cohort of neurons are born and emerge from the MGE possibly accounting for the more tempered effects on IWMN density at this developmental stage. In contrast, MIA at GD19 may have more significant effects on IWMN density since it occurred after the larger cohort of MGE-derived neurons were born, whilst they were migrating. Additional experiments investigating the origin of IWMNs in the brain, the timing of the MIA and changes in IWMN density are required to fully elucidate their role in schizophrenia neuropathology.

6.3 Neuroinflammation in the MIA model

As inflammation is at the core of the MIA model, in Chapter 4, I sought to determine if glial cells, were playing a part in the IWMN pathology seen in the MIA model. In the normal brain, microglia produce and respond to a variety of cytokines (Prokop et al., 2013). Post-mortem, serological and imaging studies

have further elucidated the role for increased microglia activity in people with schizophrenia (Steiner et al., 2008; Steiner et al., 2006; van Berckel et al., 2008; Wierzba-Bobrowicz et al., 2005). Indeed, a number of relevant cellular processes, including neurogenesis, apoptosis, and white matter development are affected by altered microglia (Reviewed by Monji et al. (2013)). In this regard, there is also increasing evidence that prenatal and early life infections can lead to "priming" of microglia that are more easily activated later in life (Bland et al., 2010; Juckel et al., 2011) – putting greater emphasis on the contributions of MIA presented in this thesis. As glial cells are the main immune cells within the brain, Iba1+ (representative of microglia) and GFAP+ immunoreactivity (representative of astrocytes) were examined in both the cortex and white matter of the PolyI:C rats. Interestingly, we did not observe any alterations in Iba1+ or GFAP+ immunoreactivity in the cortex, though a significant increase in Iba1+ immunoreactivity was observed in the white matter of the corpus callosum at GD19, which was the same white matter brain region which I observed an increase in SST+ IWMN density. This suggests that perhaps the increase in SST+ IWMN density could be related to increased inflammation assessed as increased Iba1+-IR within the white matter of the corpus callosum. We did not observe a change in microglia density in the cortex, this goes against the hypothesis that schizophrenia has an underlying immune signature, however it is not unreasonable to suggest that prenatal PolyI:C exposure in this model, may not have been sufficient enough to cause long term inflammatory changes in the brain.

We can potentially integrate inflammation and microglia in the MIA model, with synaptic pruning. In Chapter 5, I presented novel data showing that the gene expression for complement component 4 (*C4*), was increased in prefrontal cortex in offspring after GD19 PolyI:C exposure. This was a timely study built upon an important publication by Sekar et al. (2016) showing that *C4* was increased in people with schizophrenia, and in animal models, was responsible for aberrant synaptic pruning. Pruning is the process by which synapses are culled in activity dependent manner, most notably in late adolescence (Hua and Smith, 2004; Schafer et al., 2012). This process preserves highly loaded synaptic connections and removes low use/potentially extraneous connections (Chung and Barres, 2012; Paolicelli et al., 2011). In this regard, in addition to reduced grey and white matter in people with schizophrenia (Chan et al., 2009; van Haren et al., 2008), there is a disproportionate decrease in the density of dendritic spines that begins near adolescence (Glantz and Lewis, 2000; Glausier and Lewis, 2013) – which may be representative of aberrant synaptic pruning. The overlap of critical periods of synaptic pruning and first onset of schizophrenia symptoms implicate pruning as a potentially altered process in schizophrenia.

Indeed, synaptic plasticity and synaptic pruning during development is also known to be linked to glial signalling through microglia (Paolicelli et al., 2011; Tremblay et al., 2011), which are both also altered by inflammatory processes (Schafer and Stevens, 2010). It is therefore feasible to suggest that increased IBA1 immunoreactivity in the white matter and increased cortical *C4* gene expression are linked. A potential mechanism to explain this, is that within the PolyI:C MIA model, fewer synapses subsequently results from the over activation

of synaptic pruning via *C4* mediated processes, possibly by means of increased microglial activity and cortical brain inflammation – however this has not been examined in this mode, which will require further experiments. This provides further evidence that MIA has subtle effects on the immune system and further implicates the immune system dysfunction in schizophrenia.

6.4 Limitations of modelling human pathologies in rodents

Limitations of specific studies outlined in this thesis, are presented in the discussion of each chapter. Though there are some overarching limitations regarding modelling schizophrenia pathologies in animals that should be noted. Indeed, the absence of a granular zone in the rat prefrontal cortex still confounds what areas truly are homologous to the human DLPFC (Preuss, 1995). Despite this it is generally considered that, the medical prefrontal cortex (inclusive of the cingulate cortex – examined here) of the rat is most anatomically similar to the DLPFC in the human (Ongur and Price, 2000). The medial prefrontal cortex has been shown to be important for several cognitive and executive processes such as attention, decision-making and working memory (Fuster, 2000, 2001; Goldman-Rakic, 1995; Miller, 2000; Ragozzino, 2007). When it is considered that the density of IWMNs is highest in this area of the rat brain (Kostovic et al., 2011a), it could suggest cyto-architectural alterations in this area may have similar implications for rat behaviour to that of dysfunction in the DLPFC in humans with schizophrenia.

6.5 Future directions

This group of studies exclusively utilised a model of MIA where pregnant dams were given an injection of PolyI:C during gestation and then examined the offspring at late adolescence/early adult hood. Whilst only changes to SST+ IMWNs, Iba1+ immunoreactivity in the white matter and cortical *C4* gene expression were observed in this model of MIA, it has failed to replicate other robust changes seen in people with schizophrenia (e.g. reduced cortical GAD expression CH3). In this regard, it is important to appreciate the notion that schizophrenia likely results from multiple factors that contribute to changes in neurodevelopment including adolescent/early-adulthood stressors that may assist in progressing these underpinning neurodevelopmental abnormalities to full blown psychosis. Traditionally, schizophrenia is typically considered an adult disease, though there are incidences in children (Gogtay et al., 2004). In this regard, it is known that neural development is highly active during the transition from childhood to adolescence, with some white and grey matter changes not fully developed until well into adulthood (Fields, 2008). Indeed, there is evidence that stressful childhood events are associated with psychosis in later life (Sideli et al., 2012). Following on from this – other events that have been linked to the future development of schizophrenia include cannabis use – indicating that neurochemical alterations can predispose individuals to schizophrenia (Brown et al., 2001a).

Therefore, researchers have begun to integrate multiple risk factors in animals in order to tease out their links to schizophrenia development and to produce a more robust phenotype. For example, “two hit” models, have included an in-utero

environmental insult coupled with a secondary hit when the offspring are approaching late adolescence/early adulthood. The second hit in some two hit models have focused on adolescence cannabis exposure. Cannabis is one of the most commonly used recreational drugs among teens/early adults (Hilt, 2014), and has been associated with a more than two fold increased risk of developing psychosis in adulthood (Arseneault et al., 2004; Jacobus and Tapert, 2014) and increases more with chronic use (Valmaggia et al., 2014). This is not surprising as the endocannabinoid system is known to play a vital role in neurodevelopment including maturation processes such as synaptic pruning, and myelination (Lubman et al., 2015). Indeed, the main receptor subtypes of the endocannabinoid system, the Cannabinoid receptors (CB1 and CB2) are known to regulate the psychoactive effects of cannabis (Mackie, 2005; Mackie and Stella, 2006) – mostly regulated by CB1 receptors which are expressed in the brain, and control neurotransmitter release (Gerdeman and Lovinger, 2001; Shen et al., 1996). Therefore cannabis exposure during adolescence can alter the normal physiological functions of the endocannabinoid system, increasing the likelihood for the development of schizophrenia (Galve-Roperh et al., 2009).

Animal models utilising adolescent cannabinoid exposure (ACE) have been able to reproduce behavioural and cognitive deficits similar to those observed in people with schizophrenia, including impaired object recognition memory, reduced social interaction (O'Shea et al., 2006; Quinn et al., 2008) and spatial working memory deficits (Rubino et al., 2009a; Rubino et al., 2009b). HU210 is a synthetic cannabinoid that resembles THC as a potent partial CB1 agonist with high affinity and long-lasting pharmacological effects *in vivo* (Howlett et al., 2002),

has also been used in ACE animal models. HU210 induced ACE has shown to impair working memory, hippocampal synaptic plasticity (Hill et al., 2004), spatial memory and reduced hippocampal firing in rats (Robinson et al., 2007).

In studies that have utilised MIA and ACE as a two-hit model of schizophrenia, combining the effects of mid-gestation MIA at GD15 and ACE, was shown to down regulate GABA gene expression in the entorhinal cortex, a region implicated in learning and memory processes (Hollins et al., 2016), as well as exhibiting synergistic effects of MIA and ACE on serotonin receptor $5HT_{1A}R$ (Dalton et al., 2012). It would be useful to replicate the studies on IWMNs conducted in this thesis utilising a two-hit model of MIA and ACE. It is reasonable to suggest that the combination of MIA and ACE may produce more robust changes to IWMN subsets in addition those expressing SST, more brain inflammation and changes in cortical gene expression when compared to that observed in this thesis where MIA was modelled alone. This is an important step in continuing to strive for a model that reliably replicates schizophrenia pathologies. However, it is important to acknowledge that other potential risk factors for schizophrenia, such as adolescent stress, may also provide the basis for valid two-hit models of MIA and schizophrenia {Mizrahi, 2014 #3705;Debnath, 2015 #2234}.

The overarching objective of this PhD thesis was to understand how MIA effects IWMN density and to uncover potential mechanisms in which IWMNs may contribute to the pathogenesis of schizophrenia. Data suggests that IWMNs are electrically active, and the anatomical and electrophysiological data suggests

they are integrated into cortical circuitry (Engelhardt et al., 2011). An intriguing thought is that these cells may be able to influence cortical processing, though the jury remains undetermined whether there are enough neurons to do this. Characterizing what neurotransmitters are released from IWMNs will be crucial in examining this further. Indeed, complete phenotyping of the IWMN subgroups will be necessary to fully elucidate their biological roles. Nonetheless, IWMNs remain an intriguing cell population, certainly worthy of future research and investigation. At the present time, information on IWMNs has predominantly only been gathered from human post mortem tissue, with a small number of studies investigating IWMNs in animals. To be able to understand how IWMNs contribute to functional brain circuitry, and thus to be able to gain inferences on how this could be altered in schizophrenia, IWMNs must be investigated in live brain slices; information that is unattainable from human post mortem tissue. By using transgenic animals, where enhanced Green Fluorescent Protein (eGFP) is tagged to the promoters of markers of IWMNs, such as SST, GAD65/67, NPY and Pv, this may provide a method of identifying IMWNs in live slices of brain. Leading on from this, whole cell patch clamping techniques can then be employed to functionally record from these IWMNs and investigate the intrinsic cellular properties of specific subsets of WMNs. In addition to this, new methods of selectively activating neuron populations by utilising light sensitive channelrhodopsins (ChRs) (Zhao et al., 2011) have been developed in an emerging field called “optogenetics”. By expressing the light-responsive ChR in neurons using cell type selective promoters or other methods such as viral vectors (Zhao et al., 2011), specific neurons can be depolarised by light in a temporally precise manner with millisecond resolution, and even to sustain

depolarisation over a long period of time, which cannot be achieved by traditional methods. By using these electrophysiological techniques, the functional properties of IWMNs could be investigated and pave the way for studies of the consequences of the increased density of IWMNs induced by infections during pregnancy, that may shed light on our understanding of how this may manifest in relation to schizophrenia.

6.6 Conclusion

To uncover the secrets of the pathology of schizophrenia, animal models will be vital in elucidating the mechanisms and pathways controlled by the disorder. Whilst this thesis provided evidence that MIA plays a role in inducing schizophrenia like neuropathology's, the specific lack of schizophrenia associated pathologies such as GABAergic interneuron alterations in this model is concerning. By further elucidating specific timing, doses, and second hits, this model may be able to recapitulate schizophrenia related neuropathology more closely.

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"Either you decide to stay in the shallow end of the pool or you go out in the ocean"

Christopher Reeve