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The impact of maternal inflammation and maternal stress in the regulation of neurodevelopment and physiological function

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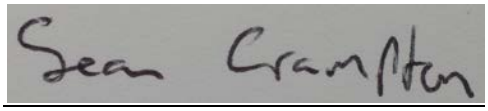
Preface

All work presented in this thesis is original and entirely my own. The work was carried out under the supervision of Dr. Gerard O'Keeffe between July 2011 and October 2014 in the Department of Anatomy and Neuroscience, University College Cork, Ireland. This dissertation has not been submitted in whole or in part for any other degree, diploma or qualification at any other University.



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1. Abstract

The mechanisms governing fetal development follow a tightly regulated pattern of progression such that interference at any one particular stage is likely to have consequences for all other stages of development in the physiological system that has been affected thereafter. These disturbances can take the form of many different events but two of the most common and widely implicated in causing detrimental effects to the developing fetus are maternal immune activation (MIA) and maternal stress. The first studies to link prenatal or early life events with the development of disorders later in life were conducted in the 1980s by Barker and colleagues following observations that low birth weight was associated with the development of cardiovascular disease in later life (Barker and Osmond, 1986; Barker et al., 1989). Since this early discovery multiple epidemiological studies have investigated the associations between prenatal infections and stress with an increased risk of incidence of numerous disorders.

MIA has been shown to cause an increase in circulating proinflammatory cytokines in both the maternal and fetal circulation as well as resulting in a febrile response in some particular types of infection. This increase in proinflammatory mediators in the fetus is thought to occur by fetal production rather than through exchange between the maternal-fetal interface. In the case of maternal stress it is increased levels of stress related hormones such as cortisol/corticosterone which is thought to elicit the detrimental effects on fetal development. There are placental barrier mechanisms which under normal conditions serve to control the quantities of these glucocorticoids that pass through to the fetus but these can become downregulated or saturated under conditions of maternal stress. In the case of both maternal infection and stress the timing and nature of the insult generally dictates the severity and type of effects seen in affected offspring.

We first investigated the effect of a proinflammatory environment on neural precursor cells (NPC). Exposure resulted in a significant decrease in the normal rate of proliferation of NPCs in culture but did not have any effect on cell survival, rather interleukin-1 beta (IL-1β) caused these NPCs to exit the proliferative cycle and differentiate towards a glial phenotype. This gliogenic effect of IL-1β subsequently resulted in a decrease in neurogenesis. Antagonism of the functional IL-1 receptor, IL-1R1 prevented any of these adverse effects that were previously observed. IL-1β was also found to elicit its effects through p38-MAPK kinase signalling and ablation of this signalling also negated any effects of IL-1β.

Secondly, we examined whether these NPCs were particularly susceptible to the effects of proinflammatory cytokines at particular developmental ages. NPCs isolated from E12 embryos were completely resistant to the effects of any cytokines that were used in treatments whereas E16 NPCs were affected in a similar manner to that of E14 NPCs from out first study. Using an *in vivo* lipopolysaccharide (LPS) model we tested whether a single insult was sufficient to alter NPC development. Cultured NPCs from E12 and E16 LPS injected animals were cultured in the absence of any treatment yet E16 NPCs displayed a decline in proliferation as was observed in our *in vitro* studies, E12 NPCs as before were unaffected. This demonstrates that an infection during pregnancy is capable of inducing lasting changes in the fetus and also that certain periods during development are more sensitive to these effects than others.

Lastly using a restraint stress model we investigated the effects of prenatal stress (PNS) on the development of a number of different physiological systems in the same cohort of animals. PNS animals exhibited a number of aberrant changes in cardiovascular function with altered responses to stress and hypertension, modifications in respiratory responses to hypercapnic and hypoxic challenges and discrepancies in gastrointestinal innervation. In addition to this we also observed behavioural abnormalities and modified corticosterone (CORT) release in response to restraint stress.

Taken together these findings suggest that both maternal infection and maternal stress are detrimental to the normal development of the fetus and may increase the risk of affected offspring developing a range of developmental disorders as well as disorders that may not manifest until later life.

2. Abbreviations

11β-HSD2 - 11 β-hydroxysteroid dehydrogenase type 2

5-HT – Serotonin

ADHD – Attention Deficit Hyperactivity Disorder

AMPA – α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA – Analysis of Variance

APdias – Diastolic Arterial Pressure

ApoE – Apolipoprotein E

APsys – Systolic Arterial Pressure

ARS – Acute Restraint Stress

BCH – Bethanechol

bFGF – Basic Fibroblast Growth Factor

BPD – Bronchopulmonary Dysplasia

BSA – Bovine Serum Albumin

BWIS – Baltimore-Washington Infant Study

ChAT – Choline Acetyltransferase

CHD – Congenital Heart Defect

CHDS – The Child Health and Development Study

CORT – Corticosterone

CP – Cerebral Palsy

CPP – Collaborative Perinatal Cohort

CRD – Colorectal Distension

CRH – Corticotropin-Releasing Hormone

CSF – Cerebrospinal Fluid

DIV – Days *in vitro*

DMEM – Dulbecco's Modified Eagle's Medium

DNMT – DNA Methyltransferase

E – Embryonic Day

EAAT – Excitatory amino-acid transporter

EGF – Epidermal Growth Factor

Egr2 – Early Growth Response 2 Transcription Factor

ENS – Enteric Nervous System

EPM – Elevated Plus Maze

f – Breathing Frequency (Breaths per minute)

FCS – Fetal Calf Serum

GABA – γ-Aminobutyric Acid

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GFAP – Glial Acidic Fibrillary Protein

HBSS – Hanks Balanced Salt Solution

HPA – Hypothalamic-Pituitary-Adrenal Axis

HR – Heart Rate

IL-1R1 – Interleukin-1 Receptor 1

IL-1β – Interleukin-1 β

IL-6 – Interleukin-6

IL-6R – Interleukin-6 Receptor

I_{SC} – Short Circuit Current

LDH – Lactate Dehydrogenase

LPS – Lipopolysaccharide

MBP – Myelin Basic Protein

MIA – Maternal Immune Activation

NE – Norepinephrine

NMDAR – N-methyl-D-aspartate Receptor

NOR – Novel Object Recognition

NPC – Neural Precursor Cell

OF – Open Field

P – Postnatal Day

PARP – poly (ADP-ribose) polymerase

PBS – Phosphate Buffered Saline

PD – Parkinson's Disease

PNS – Prenatal Stress

Poly(I:C) – Polyriboinosinic-Polyribocytidilic Acid

RT – Room Temperature

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

RT-QPCR – Real-Time Quantitative PCR

SD – Sprague-Dawley

SDHA – Succinate Dehydrogenase Complex, Subunit A

SEM – Standard Error of the Mean

SI – Social Interaction Test

SN – Substantia Nigra

TER – Transepithelial Resistance

TH – Tyrosine Hydroxylase

TLR3 – Toll-Like Receptor 3

TLR4 – Toll-like Receptor 4

TNF – Tumour Necrosis Factor

TNFR1 – Tumour Necrosis Factor Receptor 1

UBQC – Ubiquitin C

USV – Ultrasonic Vocalisations

V_E – Minute Ventilation

VM – Ventral Mesencephalon

VSD – Ventricular Septal Defect

V_T – Tidal Volume

WBP – Whole Body Plethysmography

WHO – World Health Organisation

3.0 Introduction

3.1 'Fetal Programming'

One of the most compelling challenges that face the field of medicine is trying to understand and elucidate the underlying molecular basis driving the development of complex disorders such as obesity, diabetes and schizophrenia. Current medical practice aims to treat the individual rather than treating the disease as the aetiology for an individual disease may stem from a number of potential events from perinatal or postnatal periods of life or in some cases both. It has only been in the past 30 years that research has examined what effects the 'state' of the intrauterine environment may have on the developing fetus. The first discovery that abnormalities *in utero* may affect the normal developmental trajectory and thus have detrimental effects in later life was made by Barker and colleagues in the late 1980's. Through epidemiological studies an association was made between infants that were born with low birth weight or other signs of growth restriction and an increased risk of developing coronary heart disease in later life (Barker and Osmond, 1986; Barker et al., 1989; Barker et al., 1993). In 1995, the British Medical Journal coined these observations the "Barker Hypothesis" which is now widely accepted across the scientific and medical communities.

An additional factor to take into account when examining the long-term consequences of *in utero* adversity is that during organogenesis and the development of complex functional systems within the body that there is particular periods during which an organ or system may be especially vulnerable to changes in the surrounding environment. These are what may be referred to as 'critical periods' during development and vary from organ to organ and tissue to tissue (Widdowson and McCance, 1975). For example maternal infection during the first or early second trimester is associated with a threefold increase in the risk of developing schizophrenia (Brown et al., 2004). Some biological mediators that play a role in normal healthy development such as cortisol and cytokines (Ohrlander et al., 1976; Mousa and Bakhiet, 2013; Stolp, 2013) can also cause detrimental effects in normal development if the delicate balance is disturbed. Instances in which this may occur may be during in periods of maternal stress where activation of the maternal hypothalamic-pituitary-adrenal axis (HPA) leads to elevated cortisol levels or in cases of maternal immune activation (MIA) where levels of circulating cytokines are elevated. Elevated levels of cortisol in response to increased stress levels in the mother have been linked to the development of several disorders including autism (Beversdorf et al., 2005; Kinney et al., 2008a; Kinney et al., 2008b; Li et al.,

2009c; Fine et al., 2014; Walder et al., 2014), obesity (Tamashiro et al., 2009; Tamashiro and Moran, 2010; Ingstrup et al., 2012; Wright et al., 2013; Hohwü et al., 2014) and asthma (Pincus-Knackstedt et al., 2006; Kumar, 2008; Khashan et al., 2012). MIA, similarly has been implicated in elevating the risk of developing a number of disorders such as schizophrenia (Ashdown et al., 2005; Meyer et al., 2006b; Meyer et al., 2009c; Brown and Derkits, 2010; Anderson and Maes, 2013; Khandaker et al., 2013; Kneeland and Fatemi, 2013), diabetes (D'Mello and Liu, 2006; Niklasson et al., 2006; Lindehammer et al., 2011; Oikarinen et al., 2011; Tauriainen et al., 2011; Viskari et al., 2012; Stene and Gale, 2013) and cardiovascular malformations (Tikkanen and Heinonen, 1991; Hornberger et al., 2000; Loffredo, 2000; Ács et al., 2005). In this next section I will review the effects of both PNS and maternal infection on the development of offspring looking at both epidemiological studies and animal models providing supporting evidence for these clinical observations.

3.2 Maternal Infection

Cytokines encompass a broad spectrum of small proteins generally in the 5-20 kDa size range which are responsible for eliciting most of the biological effects of the immune system. Although there are numerous cytokines they can be functionally divided into two groups: proinflammatory and anti-inflammatory (Elenkov and Chrousos, 2002). Within the immune system T lymphocytes are a major source of cytokine production, $CD4^+$ T lymphocytes known as helper T cells are considered the most prolific cytokine producers and can be further divided into two subgroups, namely Th1 and Th2. Respectively, they produce Th1- and Th2-type cytokines, Th1 cytokines such as tumour necrosis factor (TNF) and IFN-γ generally cause a proinflammatory response whilst Th2 such as IL-4 and IL-10 predominantly elicit an anti-inflammatory response (Landgraf et al., 2012).

During development of the fetus the balance between Th1 and Th2 cytokines is tightly regulated to prevent an proinflammatory environment being established. However, in cases of prenatal infection leading to MIA, this balance can be altered leading to increased production of Th1 cytokines (Boksa, 2010). Although MIA is the most common cause for elevated levels of proinflammatory cytokines in the maternal circulation during pregnancy, maternal stress has also been implicated in causing this effect (Maes et al., 1998; Laviola et al., 2004; Diz-Chaves et al., 2012). Interestingly the converse is also possible by which elevated levels of proinflammatory cytokines in the periphery have been shown to activate the HPA axis causing an increase in CORT levels (Dunn, 2000; Hashimoto et al., 2001). A point of note in regards to fetal cytokine levels is that the increase in proinflammatory cytokine production

occurs within the fetus itself rather than being, at least fully, derived from maternal sources via maternal-placental transfer (Zaretsky et al., 2004; Aaltonen et al., 2005a; Boksa, 2010).

MIA through prenatal infection has been implicated in increasing the risk of developing multiple disorders and diseases in later life such as neurodevelopmental and neuropsychiatric disorders including autism spectrum disorders (ASD) and schizophrenia (Sørensen et al., 2009; Atladóttir et al., 2010), congenital heart defects (Botto et al., 2014), chronic lung disorders (Prendergast et al., 2011) and metabolic disorders (Stene and Gale, 2013). A growing body of evidence in the form of both epidemiological and animal models is providing further support for these theories previously put forward by individual groups.

3.3 Epidemiological Studies

3.3.1 Neurodevelopmental and Neuropsychiatric Disorders

The initial association between the increased risk of developing schizophrenia and maternal infection arose from the early studies that suggested winter/spring births were associated with a higher incidence of schizophrenia in later life (Brown and Derkits, 2010). This led to epidemiological investigations which examined specific types of infection as presumptive schizophrenia risk factors (Mednick et al., 1988). Earlier research failed to produce reproducible results between studies due to shortcomings in study design but nonetheless they provoked further more refined approaches, birth cohort studies being of particular importance. Birth cohorts are a collection off offspring that are born within a particular geographical area and during a particular time span (Kraemer, 2009). The main advantages of these types of studies are that exposures occurring during term can be documented for prospective reviews in addition to the ability to be able to follow up offspring for outcomes of interest. Samples that are collected during the period of the pregnancy may also be examined for specific biomarkers therefore not relying on self-reporting which is often highly subjective. The Child Health and Development Study (CHDS) contained subjects born from 1959 to 1967 in Almeda County, California. Study participants were followed up by databases and received structured interviews at the Kaiser Permante Division of Research. In this nested case-control study conducted by Brown and colleagues exposure to influenza during early to mid-pregnancy was shown to lead to a threefold increase in the risk of developing schizophrenia among offspring during adulthood (Brown et al., 2004). Influenza infection was determined based on antibodies in maternal serum. In a separate study using the same birth cohort Brown and colleagues displayed evidence that an increased IgG antibody titre (≥1:128) to *toxoplasma gondii* was indicative of a greater than twofold increase in the

risk for schizophrenia (Brown et al., 2005). This study did not differentiate between the developmental stage at which the infection occurred but rather by the *T.gondii* IgG titre divided into negative, moderate and high groupings. An additional study using a Danish cohort from which blood spots were collected on filter paper within the first week of life also showed that elevated IgG antibodies to *T.gondii* were associated with an increased prevalence of schizophrenia in offspring thus supporting Brown's earlier study (Mortensen et al., 2007). Another study performed by Brown and colleagues used data from the Rubella Birth Defects Evaluation Project, a birth cohort containing patients from the rubella pandemic of 1964. 20% of offspring from mothers who were confirmed with infection during pregnancy were diagnosed with schizophrenia or other schizophrenia spectrum disorders equating to a 10- to 15-fold increase in risk (Brown et al., 2001). The Collaborative Perinatal Cohort (CPP) used archived maternal sera from mothers from the Providence, Rhode Island. Buka and colleagues investigated the association between increased maternal Herpes simplex virus-2 (HSV-2) IgG levels and seropositivity to this infection displaying evidence that it was related to schizophrenia in offspring (Buka et al., 2001; Buka et al., 2008).

Using data from the CHDS cohort Babulas and colleagues demonstrated a fivefold increase in the risk of developing schizophrenia was associated with periconceptional urogenital infections (Babulas et al., 2006). Maternal exposure to a respiratory infection during the second trimester of pregnancy has also been demonstrated to cause a twofold increase in the risk of schizophrenia (Brown et al., 2000). In a cohort from Helsinki, Finland women who contracted pyelonephritis, a urinary tract infection, and who had a family history of schizophrenia faced a fivefold increase in the chance of offspring developing schizophrenia when they compared the exposed and unexposed without genetic vulnerability (Clarke et al., 2009). Further studies into the CHDS birth cohort found that prenatal infection was associated with structural abnormalities, namely enlargement of the cavum septum pellucidum, a neuromorphologic marker of schizophrenia amongst patients indicating disruption in brain formation. Also noted were deficits in executive function (Brown et al., 2009a; Brown et al., 2009b). Another study that examined brain structural abnormalities in cases where there were elevated levels of the cytokine IL-8 in subjects from the Developmental Insult and Brain Anomaly in Schizophrenia cohort. It had previously been shown that there were structural neuroanatomic alterations linked to schizophrenia (Wright et al., 2000) but Ellman and colleagues provided the first link between fetal exposure to increases in maternal cytokines and neuroanatomic disruptions associated with schizophrenia (Ellman et al., 2010). In this study they demonstrated increases in ventricular cerebrospinal

fluid (CSF) and decreases in the left entorhinal cortex and right posterior cingulate volumes (Wright et al., 2000; Sigmundsson et al., 2001; Gur et al., 2007). Collectively, these studies provide evidence for a role of prenatal infections in the development of schizophrenia and also in contributing to brain structural abnormalities. Although some particular types of infection cause a higher risk than others, it is more probable that it is the nature of the immune response to the infection rather than the actual infectious agent that causes these differences.

In comparison to studies examining the role of MIA on the risk of schizophrenia, relatively few have investigated the effects of maternal infection on the incidence of autism. One of the largest studies to date was conducted using data from the Danish Medical Birth Register encompassing children born from 1980 to 2005 totalling 1,612,342 newborns. In this study a link was established between maternal hospitalisation for a viral infection in the first trimester relating to a nearly threefold increase in the risk of offspring developing ASD (Atladóttir et al., 2010). However, hospitalisation during the second trimester for a bacterial related infection although causing a significant increase in the risk of developing ASD did not represent the same risk as that of viral infections (Atladóttir et al., 2010). There were several limitations of this study such as the inclusion limitation to infections that only warranted hospital admission therefore neglecting a large amount of infection data from other infections. Infections were also very broadly grouped making it impossible to analyse specific pathogens and their relative associated risk. Grether and colleagues used the California Department of Developmental Services to identify children with ASD born in six counties during 1994 and examined newborn IgG levels in blood specimens obtained from the California Genetic Disease Screening Program (Grether et al., 2010). In this study they demonstrated an association between lower neonatal IgG levels and an increased incidence of ASD.

Another study which acquired data from the Early Markers for Autism study used a multiplex bead assay kit to assess the levels of 17 cytokines in maternal serum samples (Goines et al., 2011). It was found that elevated levels of IL-4, IL-5 (Th2) and interferon-γ (Th1) were significantly elevated in mothers whose children were born with ASD. An interesting conclusion by the authors in this study was that this cytokine profile was consistent with that found in an allergic asthma phenotype. The potential relationship between MIA and the immune response in relation to asthma will be discussed later on. In an additional study from the Danish Historic Birth Cohort amniotic fluid, which is considered to better reflect fetal rather than maternal cytokine profiles, was analysed to establish cytokine profiles (Abdallah

et al., 2013). Very similar findings to that of Goines and colleagues were made in this study with results showing that elevated levels of TNF-α, TNF-β (Th1), IL-4 and IL-5 were associated with an increased risk of ASD in affected offspring.

Changes in the cytokine balance leading to a proinflammatory state have been documented in post-mortem brains of autistic patients as well as evidence of activated microglia and astrocytes, these changes were evident in patients spanning a wide age range (4-44 years of age) which suggests that these changes are established early and persist throughout adulthood (Vargas et al., 2005). CSF samples from living autistic children aged from 3 to 10 have also presented evidence for abnormal cytokine levels and additional findings from microarray studies have shown perturbations in the expression and regulation of immune-related genes in autistic brains (Chez et al., 2007; Morgan et al., 2010; Lintas et al., 2012). Results from a study of a large cohort of ASD children drew a correlation between increases in several cytokines and chemokines within the plasma of children aged 2 to 5 and the level of impairment in communication and aberrant behaviours (Ashwood et al., 2011b; Ashwood et al., 2011a; Lintas et al., 2012).

Maternal infection has also been implicated in causing an increased risk of cerebral palsy (CP) and attention-deficit hyperactivity disorder (ADHD) in affected offspring. Epidemiological studies have shown that maternal infection especially those causing a febrile illness can cause over a sevenfold increase in the incidence of CP when compared with mothers who experienced no infection (Eastman and DeLeon, 1955; Grether and Nelson, 1997). White matter lesions in proximity to the lateral ventricles are generally indicative as a risk factor for developing CP and periventricular leukomalacia is characterised by this type of pathology. These injuries are characterised by elevated levels of proinflammatory cytokines which may be the causative agents in developing these white matter injuries (Yoon et al., 1997; Martinez et al., 1998; Yoon et al., 2003). In studies examining maternal infection as a risk factor for ADHD the relative risk association is related to the nature of the infection, with urogential infections posing the most significant risk (Mann and McDermott, 2011; Silva et al., 2014).

In summary there is evidence that prenatal infection with resulting MIA may be a causative factor in the development of a range of neurodevelopmental disorders in affected offspring. MIA may also cause aberrant programming of the fetal immune system which could contribute to the development of these disorders with cytokine and chemokine profiles possibly serving as an indicator of the severity of the condition.

3.3.2 Cardiovascular Disorders

Febrile illnesses are defined as any illness such as an infection which results in the sudden onset of fever, internal body temperature 38˚C or greater. The occurrence of these types of illnesses are relatively common with an estimated incidence of 5-10% during early pregnancy but their incidence varies depending on several factors such as season or immunisation status (Tikkanen and Heinonen, 1991; Botto et al., 2002; Oster et al., 2011). Congenital heart defects (CHD) occur with a relatively high incidence of roughly 1 in 110 births and therefore even moderate teratogenic risks may cause an significant increase in the burden of disease (Hoffman et al., 2004). The National Birth Defects Prevention Study is a large ongoing case-control study encompassing 30 major structural malformations within the United States. In a study by Botto and colleagues this data was used to investigate the potential relationship between heart defects, source of fever and multivitamin supplement use (Botto et al., 2014). Urogenital infections displayed the highest risk factor for developing CHD and this was followed by respiratory infection. These risk estimates were limited to specific heart defects though such as obstructive defects and heterotaxy. In the Hungarian medical system it is mandatory for physicians to report all cases of congenital abnormalities to the Hungarian Congenital Abnormality Registry (HCAR) which has provided a valuable data set for epidemiological studies (Czeizel, 1997). In a study by Csáky-Szunyogh and colleagues data from this cohort was examined to investigate whether there was a relationship between maternal infection and ventricular septal defects (VSD), the CHD which occurs with the highest prevalence in Hungary (Mészáros and Czeizel, 1977). The findings of this study provided supporting evidence for several previous studies which reported that influenza or common cold can lead to an increased incidence of CHD with high fever being a probable mediator in addition to establishing a potential link with cytomegalovirus infection and a higher risk of VSD (Csáky-Szunyogh et al., 2013).

The Atlanta Birth Defects Case-Control Study was conducted between 1982 and 1983 and case infants for the study by Botto and colleagues were selected using the Metropolitan Atlanta Congenital Defects program (Botto et al., 2001). This population-based study found evidence showing that contraction of a febrile illness in early pregnancy or around the period of conception was associated with over a twofold increase in the risk of developing major heart defects such as tricuspid atresia or aortic stenosis. Two earlier studies conducted using data from Finland (Tikkanen and Heinonen, 1991) and China (Zhang and Cai, 1993) have also demonstrated evidence for a similar increase in the risk of offspring developing heart defects associated with maternal febrile illness. In the Finnish cohort the left sided obstructive

defect, hypoplastic left heart, was associated with febrile illness and outflow tract defects were positively associated with upper respiratory tract infection both with or without fever (Tikkanen and Heinonen, 1991). In the Baltimore-Washington Infant Study (BWIS) maternal fever was strongly associated with tricuspid atresia whilst influenza was shown to be associated with aortic stenosis, hypoplastic left heart and transposition of the great arteries (Ferencz et al., 1997). Another study using the BWIS displayed evidence that antipyretic agents tended to attenuate the associations between contracting fever or influenza in the periconceptional period of pregnancy and CHD (Oster et al., 2011). A study examining the effect of HIV-infected mothers on the development of CHD also found significant differences between normal fetuses and those of HIV-infected mothers in cardiovascular structure and function (Hornberger et al., 2000). Collectively these data suggest that contraction of an infection during pregnancy, particularly one that elicits a febrile response, may cause an increase in the incidence of CHDs in affected offspring (Botto et al., 2001; Oster et al., 2011; Botto et al., 2014).

3.3.3 Respiratory Disorders

Maternal infection has also been studied in relation to the development of respiratory-related disorders with a particular focus on asthma and bronchopulmonary dysplasia (BPD). Chorioamnionitis is caused by inflammation at the maternal-fetal interface and complicates roughly 8% of all pregnancies, potential mechanisms of the condition include ascending bacterial infections at the maternal-fetal interface and inflammation of the genital tract (Krohn et al., 1995; Offenbacher et al., 2001). In cases of chorioamnionitis the fetal lung is in constant contact with amniotic fluid therefore exposing the developing airways to inflammatory mediators, predominantly proinflammatory, in addition to microorganisms which may be detrimental to development (Hsu et al., 1998). A retrospective cohort study of data acquired by the Kaiser Permanente Southern California examined the association between chorioamnionitis and asthma. In this study fetal exposure to chorioamnionitis in conjunction with preterm delivery was associated with an increased risk of asthma at 8 years or younger, there was also an inverse correlation between gestational age at delivery and asthma risk (Getahun et al., 2010). A second study consisted of 805 consecutive mother-child pairs that were delivered by caesarean section Kuopio University Hospital, Finland at which time intrauterine bacterial cultures were recorded (Keski-Nisula et al., 2009). This study indicated that intrauterine growth of specific microbes at the time of delivery may increase the risk of the development of asthma in offspring through inflammatory mechanisms. A

potential confounder in this study may be the fact that all babies were delivered by caesarean section in which amniotic fluid may be not be dispelled as efficiently as by natural birth thus potentially having an effect on the development of asthma. The reason for which these mothers underwent caesarean was not stated in this study therefore it is unclear whether this method of delivery was related to the infection. A large cohort of singleton births to residents in New South Wales, Australia between 2001 and 2003 were analysed to assess the associative risk of *in utero* exposures and the development of asthma (Algert et al., 2011). A positive association between prenatal infections and an increased risk of asthma was established, the most significant increases in risk being related to maternal urinary tract infections and pre-term pre-labour rupture of membranes. As previously mentioned in an earlier section the risk associated with maternal infection does not seem to be microbe specific therefore implicating the nature of the immune response as the causative factor.

BPD is a serious chronic lung disorder that generally results in respiratory distress syndrome and is most common in premature infants. The aetiology is complex and not yet fully understood but is caused in part by the lack of adequate surfactant which prevents the lungs collapsing. It has been proposed that a potential causative factor may be exposure to intrauterine inflammation and it has been reported that neonates who develop the condition had higher concentrations of proinflammatory cytokines in the amniotic fluid (Watterberg et al., 1996; Yoon et al., 1999). A study using data from 203 preterm singleton neonates delivered at Seoul National University Hospital examined umbilical cord plasma and amniotic fluid for IL-6 levels (Yoon et al., 1999). Their findings displayed that IL-6 concentrations were significantly increased in preterm neonates who developed BPD when compared to those who did not and it was proposed that a possible mechanism for BPD predisposition may be due to damage of the pulmonary microcirculation by the inflammatory response.

3.3.4 Metabolic Disorders

With a decreasing age of onset for diabetes risk factors in the gestational period in addition to early childhood such as infections are being investigated (Menser et al., 1967; Dahlquist et al., 1995; Hyöty et al., 1995). Several birth cohort studies have investigated the potential of maternal infections to predispose offspring to the development of diabetes. One such study conducted using data from the Diabetes Prediction in Skane cohort examined cord blood and serum samples from mothers for markers of Type I diabetes and autoimmune exposure (Lindehammer et al., 2011). Samples were also assayed for cytokine concentrations in

addition to completing a questionnaire on potential infection during pregnancy. They revealed that elevated cytokines (IFN-γ, IL-1β and IL-2) during early pregnancy predicted development of at least 2 islet autoantibodies which showed elevations by 5 years after birth. Of these 48 children, 34 had developed type I diabetes before 7 years of age. This study postulated that elevated levels of IFN-γ, IL-1β and IL-2 where indicative of maternal infection. Most of the other studies conducted have focused on enterovirus infections as a risk factor for the development of diabetes. The largest of these to date was performed using children from the Type 1 Diabetes Prevention and Prediction Study participants of which came from three university hospitals in Finland (Viskari et al., 2012). From this study they demonstrated that a slightly increased frequency of enterovirus infections was observed in mother of children who went on to develop type I diabetes. Taken together with other studies conducted that examined enterovirus RNA, IgM antibodies and blood virus load it is reasonable to conclude that maternal enterovirus infections that occur during pregnancy may contribute to the development type I diabetes (Dahlquist et al., 1995; Hyöty et al., 1995; Dahlquist et al., 1999; Sadeharju et al., 2003; Dahlquist et al., 2004; Oikarinen et al., 2011; Tauriainen et al., 2011).

3.4 Animal Models

3.4.1 Neurodevelopmental and Neuropsychiatric Disorders

Although epidemiological studies in which specific inflammatory markers or pathogens may be quantified in maternal and neonatal samples offer a robust approach to the establishment of links between maternal illness and developmental disorders they face ethical and technical limitations. This is where the importance of animal models which allow the investigation of molecular mechanisms that may underlie the development of these disorders. There are currently several different approaches to mimicking the effects of a maternal infection in animal models which vary in the pathways by which they elicit an immune response in regards to cells that respond and cytokine profiles in addition to whether they cause a systemic or localised immune response (Bsibsi et al., 2006; Reimer et al., 2008; Figueiredo et al., 2009).

The main agents currently used to model maternal infection are viral or bacterial components which can be recognised by the immune system to provoke an immune response. However, some groups have focused on developing models of infection using live viruses such as Fatemi et al who pioneered a mouse model of maternal infection by the influenza virus based on studies that have drawn an association between prenatal influenza infection and

schizophrenia (Mednick et al., 1988; Brown et al., 2004; Brown et al., 2009a; Kneeland and Fatemi, 2013). This model uses a sublethal dosage of a mouse-adapted influenza virus delivered via intranasal infusion at gestational day 9 in pregnant dams, the effects of this infection are then examined in offspring in which behaviour and long-term changes within the brain are assessed relative to mock infected or control animals. Utilising this approach pregnant dams were exposed at specific gestational ages to investigate the effect of timing of infection on the outcome in affected offspring and thus explore critical periods during brain development (Kneeland and Fatemi, 2013). One of the main advantages of using live viruses is also its major limitation, as the virus is live it follows a naturalistic time course of infection and nature of immune response in the host but it also proves a challenge in the fact that it is more difficult to control the dosing and window of exposure. Although influenza is the most commonly used other viruses such as cytomegalovirus and Borna virus have been used to investigate the effects of congenital and neonatal infection on neural development (Hornig and Lipkin, 2001; Barry et al., 2006).

The pathology from maternal influenza infection is dependent on the timing of exposure and produces a range of abnormalities including reduced corticogenesis and hippocampal volumes, impaired corpus callosum development and decreased reelin expression (Fatemi et al., 1999; Fatemi et al., 2008; Moreno et al., 2011; Kneeland and Fatemi, 2013). Long term deficiencies in serotonin (5-HT) are also observed in mouse models of prenatal influenza infection (Winter et al., 2008) which may contribute to the set of induced behavioural deficits (Shi et al., 2003; Meyer and Feldon, 2012) many of which can be normalised through the use of typical or atypical antipsychotic drugs (Shi et al., 2003; Moreno et al., 2011). Studies in non-human primates using prenatal maternal influenza infection models have also shown patterns of reduced white and grey matter in specific parieto-cortical and cortical brain regions (Short et al., 2010). The use of non-human primates that possess more complex prenatal corticogenesis in comparison to rodents assists in relating findings in animal models to human conditions.

The other classes of animal models of MIA use agents that result in a cytokine-associated immune response without the use of live viral or bacterial pathogens (Meyer et al., 2009b; Meyer and Feldon, 2010). These models were initially designed to explore the theory that altered cytokine profiles in either the mother or fetus may be the main causative agent in mediating the effects of maternal infection during gestation and the subsequent aberrant brain development in offspring (Gilmore and Fredrik Jarskog, 1997; Meyer et al., 2009a). The most favoured and widely used of these immune activating agents are polyriboinosinic-

polyribocytidilic acid (poly(I:C)) and LPS. Poly(I:C) consists of a synthetic analogue of double-stranded RNA which is commercially produced and replicates the double-stranded RNA produced during viral replication as an intermediate for single-stranded RNA or as a by-product of symmetrical transcription in DNA viruses (Akira and Takeda, 2004). Poly(I:C) acts by activating the transmembrane receptor toll-like receptor 3 (TLR3) which serve as pathogen recognition receptors for the immune system, upon activation of TLR3 a signalling cascade induces the expression of multiple innate immune response genes and proteins (Akira and Takeda, 2004). This response stimulates the release and production of a range of proinflammatory cytokines including IL-1β and TNF in addition to being a potent stimulator of type I interferons (Kimura et al., 1994; Cunningham et al., 2007). Treatment with Poly(I:C) is therefore capable of closely replicating an acute phase response to a viral infection and efficiently causes an inflammatory response in the fetal brain when administered systemically to pregnant dams (Meyer et al., 2006d; Abazyan et al., 2010).

Maternal poly(I:C) administration in mouse models of prenatal infection have shown that this model causes a number of behavioural alterations in affected offspring which include deficits in prepulse inhibition which tests for abnormalities in sensorimotor gating a trait observed in patients with schizophrenia. Additional changes observed are decreased social behaviour, ultrasonic vocalisation deficits, repetitive behaviours and increased markers of anxiety all of which are traits displayed by people with autism (Smith et al., 2007b; Malkova et al., 2012). In both of these studies the viral immunogen was administered during early to midgestation which correlates with findings from epidemiological studies on autism. Poly(I:C) treatment at gestational day 9 in mice also results in deficits in 5-HT and its main metabolite in multiple brain regions which may contribute to the aetiology of autism and schizophrenia (Winter et al., 2008). Latent inhibition is a subtle measure of the processing of information and attention, in poly(I:C) or IL-6 prenatally treated rats and mice offspring demonstrate deficits in latent inhibition but this deficit is not evident in animals treated with LPS (Zuckerman et al., 2003; Zuckerman and Weiner, 2003; Meyer et al., 2006d; Smith et al., 2007a).

Dysfunction of the dopaminergic system has been implicated in the pathogenesis of schizophrenia and is considered a hallmark of its neurochemistry, alterations in tyrosine hydroxylase (TH), dopamine metabolites and amphetamine-induced locomotor activity have been used as markers of activity of the dopaminergic system. Poly(I:C) administration in pregnant mice and rats has been reported to cause extensive alterations to the dopaminergic system, in particular a significant increase in amphetamine-induced rotations (Zuckerman et al., 2003). Poly(I:C) treatment of pregnant dams has also been shown to cause some

structural alterations within the brains of affected offspring namely changes to cerebellar and hippocampal volume, organisation and receptor function (Samuelsson et al., 2006; Shi et al., 2009).

LPS is a component of the bacterial cell wall of gram-negative bacteria which is recognised mainly by the transmembrane receptor toll-like receptor 4 (TLR4) and elicits an innate acute phase response to a bacterial infection (Akira and Takeda, 2004). Following TLR4 activation, LPS stimulates the expression of a range of proinflammatory cytokines (Akira and Takeda, 2004). Even though LPS and poly(I:C) share similarities in their respective immune responses they can produce completely converse effects in animal models. For instance early prenatal exposure to LPS produces a reduction in midbrain dopaminergic cells (Carvey et al., 2003) whilst poly(I:C) causes an increase in total number (Vuillermot et al., 2010). Another example comes from studies in rhesus monkeys where treatment with LPS results in a significant increase in overall white matter volume (Willette et al., 2011) whereas viral infection causes the opposite pattern (Short et al., 2010).

LPS exposure displays some similar behavioural abnormalities that are seen using the poly(I:C) model. Prenatal treatment of rats with 100 μg/kg of LPS via an intraperitoneal route on gestational day 9 causes a reduction in social behaviour in male offspring in both infancy and adulthood (Kirsten et al., 2010). Prenatal exposure to LPS also results in impaired communication and deficits in learning and memory in male adults as well as a reduction in striatal TH expression in combination with decreased striatal dopamine and metabolite levels which may be indicative of impairments in the striatal dopaminergic system of LPS treated animals (Kirsten et al., 2012). Another study by Bahanoori et al also demonstrated behavioural deficits in the offspring of LPS treated animals which exhibited a reduced number and duration of isolation-induced ultrasonic vocalisations (USV), decrease nest seeking response and impaired odour-stroke associative learning (Baharnoori et al., 2009). Impairment of USVs is recognised as an indicator of an aversive state in neonate rodents (Hofer et al., 2001; Shair, 2007). The same study found significant decreases in 5HT1A and 5HT2B gene expression in the frontal cortex of LPS treated animals.

Maternal exposure to LPS has been demonstrated by several studies to induce multiple alterations in brain morphology as well as affecting the developmental trajectory of some brain regions and their specific cell types. Administration of LPS at gestational day 18 causes the loss of developing oligodendrocytes in the fetal brain resulting in a decreased number of oligodendrocytes and reduced expression of myelin-associated proteins in the postnatal brain (Paintlia et al., 2008). Exposure to LPS around midgestation in the rat also causes significant

changes in dendritic arbours and spine structure in the forebrain. Interestingly not all of these changes persist through postnatal ages even though they were evident at all neonatal ages perhaps indicating a compensatory or repair mechanism at later ages (Baharnoori et al., 2009). LPS administration in mid-late gestation also significantly perturbs brain development through disturbing signalling pathways involved in neuronal patterning. Evidence of microglial activation, reactive astroglia and increased proinflammatory cytokine expression may implicate immune mediators in interfering with signalling pathways (Ghiani et al., 2011b). In this study LPS exposed animals display an enlarged cortical plate and abnormal expression of immature neuronal markers within the neocortex two days after maternal LPS injection. A number of important regulatory molecules including reelin, GLAST and Arc were also decreased in foetal brains, these results suggest inflammation causes improper cleavage of reelin which plays a crucial role in cortical layer formation by regulating neuronal migration (Fatemi, 2004; Förster et al., 2010; Ghiani et al., 2011b). The cerebral cortex of LPS-exposed animals was also significantly larger at P1 than age matched controls with cells appearing compacted which may affect their ability to extend processes (Ghiani et al., 2011b).

There is also evidence from several studies linking LPS exposure to fetal brain injury. In a preterm model of maternal infection sheep received intravenous LPS injections over a period of 5 days during late gestation after which there was evidence of diffuse subcortical damage and periventricular leukomalacia (Duncan et al., 2002). Another preterm model looked at the effects of intrauterine exposure in mice which resulted in significant fetal brain injury and similar results were observed in a ‘to term’ model (Ernst et al., 2009; Burd et al., 2010; Elovitz et al., 2011). In an intrauterine model of maternal infection pregnant rats received a single dose of LPS at gestational day 15 and by 3 weeks of age offspring showed signs of cortical cell death and dysmyelination resembling periventricular leukomalacia lesions (Bell and Hallenbeck, 2002). Administration of LPS towards later periods in gestation has been previously shown to increase markers of oxidative stress in the fetal brain (Paintlia et al., 2008). In the brains of CP patients periventricular leukomalacia lesions are caused through a loss of oligodendrocytes resulting in hypomyelination. This damage is partly mediated by oxidative stress and circulating cytokines which demonstrates a potential role of prenatal infection in the aetiology of CP (Kinney and Back, 1998).

Animal models utilising the administration of a single cytokine were developed in an attempt to delineate the potential roles of cytokine mediated mechanisms in the effects of maternal infection. Data thus far has identified the proinflammatory cytokine IL-6 as a potentially

critical mediator in the link between prenatal infection and aberrant brain development. Treatment with exogenous IL-6 in pregnant dams causes very similar functional and structural deficits in adult offspring that are observed in other models of MIA such as poly(I:C) (Samuelsson et al., 2006; Smith et al., 2007b). Furthermore, elimination of IL-6 using either antibodies against this cytokine or through the generation of IL-6 mutant animal models prevents the behavioural abnormalities usually elicited by immune activating agents such as poly(I:C) (Smith et al., 2007b). The use of other proinflammatory cytokines (IL-1β, IFN-γ and TNF) in models of maternal infection are also capable of causing behavioural changes in adult animals although blocking their action in poly(I:C) models does not prevent its effect as is seen with IL-6 blockade (Smith et al., 2007a). Although there is little supporting evidence for the theory it is possible that IL-6 may be such a key mediator in the effects of maternal infection and abnormal brain development as it is readily capable of crossing the placental barrier unlike other proinflammatory cytokines (Zaretsky et al., 2004). To date there have not been any significant studies or findings using these approaches in non-human primates although there are several which are currently being undertaken.

3.4.2 Cardiovascular, Respiratory and Metabolic Disorders

In comparison to studies on the neurodevelopmental effects of prenatal infection there are relatively few studies examining the above disorders in animal models. Nonetheless there are several reports which provide support for epidemiological studies. One such study looked at the effects of maternal viral infection on the development of diabetes in affected offspring using a novel virus, Ljungan virus (Niklasson et al., 2006). They found that exposure during pregnancy altered glucose tolerance and led to significantly increased bodyweight in adult male offspring. The gestational period in which exposure occurred also influenced the diabetic outcome with an earlier exposure time leading to a more severe diabetic state. Another interesting finding from this study was that behavioural stress applied during adulthood was found to be essential for the induction of a diabetic state bearing similarities to the 'two-hit' hypothesis of schizophrenia (Maynard et al., 2001; Niklasson et al., 2006).

Multiple epidemiological studies have established a link between chorioamnionitis and respiratory disorders or fetal lung damage. Using a lamb model to examine fetal lung responses to chorioamnionitis two separate groups have shown the lung first recruits proinflammatory cells that produce hydrogen peroxide (Kallapur et al., 2001; Kramer et al., 2001). This is followed by an upregulation of proinflammatory cytokine mRNAs with a subsequent maturational effect within the lungs within 7 days following onset of infection

(Jobe et al., 2000; Bachurski et al., 2001; Kallapur et al., 2001). However, this maturational effect is accompanied by an arrest in alveolarisation which may lead to gas exchange deficiencies if an insufficient number of alveoles have been formed (Willet et al., 2000). In addition the initial immune cells that were recruited to the lungs and elevated cytokine mRNA levels persist for a prolonged period of time demonstrating impaired or ineffective clearing of the inflammatory process (Kallapur et al., 2001; Kramer et al., 2001).

Most forms of maternal infection result in a febrile state of the mother which would therefore also result in a hyperthermic state in the developing fetus. Studies conducted in chick embryos, which allow easy study of the developing vasculature due to the semi-translucent shell, have shown that hyperthermia causes a range of developmental defects in the vasculature. Embryos that were exposed to hyperthermic conditions displayed pathologic leakage of plasma through injured endothelium as well as inter-endothelial gaps which allow for leakage of macromolecules and blood cells (Grotte, 1956; Nilsen, 1984a). In a separate study by the same group heart deformities such as perivascular bulging were observed in addition to defects in some of the major vessels of the heart (Nilsen, 1984b). In both of these studies exposure to hyperthermic conditions only occurred for 3 days in total yet caused persisting abnormalities in the cardiovascular system supporting the theory than an acute exposure to infection during fetal development is sufficient to cause lasting changes.

3.5 Prenatal Stress

Maternal stressors during pregnancy can take on multiple forms and may not be confined to a single event. During recent years investigations into the effects of PNS on the development of offspring have grown significantly as it has been linked to increasing the risk of development of a number of disorders. A systematic review that investigated the prevalence of depression amongst women during pregnancy reported that during the second and third trimester that 12.8% and 12% respectively experienced some form of depression with only 7.4% of women in their first trimester reporting depression (Bennett et al., 2004). Another study within the US examined the incidence of reported psychosocial stress amongst pregnant women with 6% of women reporting high levels, 78% reporting low to moderate levels and only 16% reporting no stress at all during the last two trimesters (Woods et al., 2010).

Chronic exposure to maternal stress has been shown to cause both an increase in the levels of proinflammatory cytokines in addition to elevating levels of the stress hormone cortisol (Elenkov and Chrousos, 2002; Coussons-Read et al., 2005; Davis et al., 2011). One potential mechanism by which PNS may affect fetal outcome is overexposure of the fetus to maternal

glucocorticoids known as the 'glucocorticoid hypothesis' (Reynolds, 2013) (Fig 1). The reasoning behind this hypothesis comes from the fact that PNS causes abnormal increases in cortisol levels from both maternal and fetal sources which subsequently disrupt the developmental processes that are extremely sensitive to changes especially within their 'critical periods' and therefore may predispose offspring to an increased risk of disease in later life.

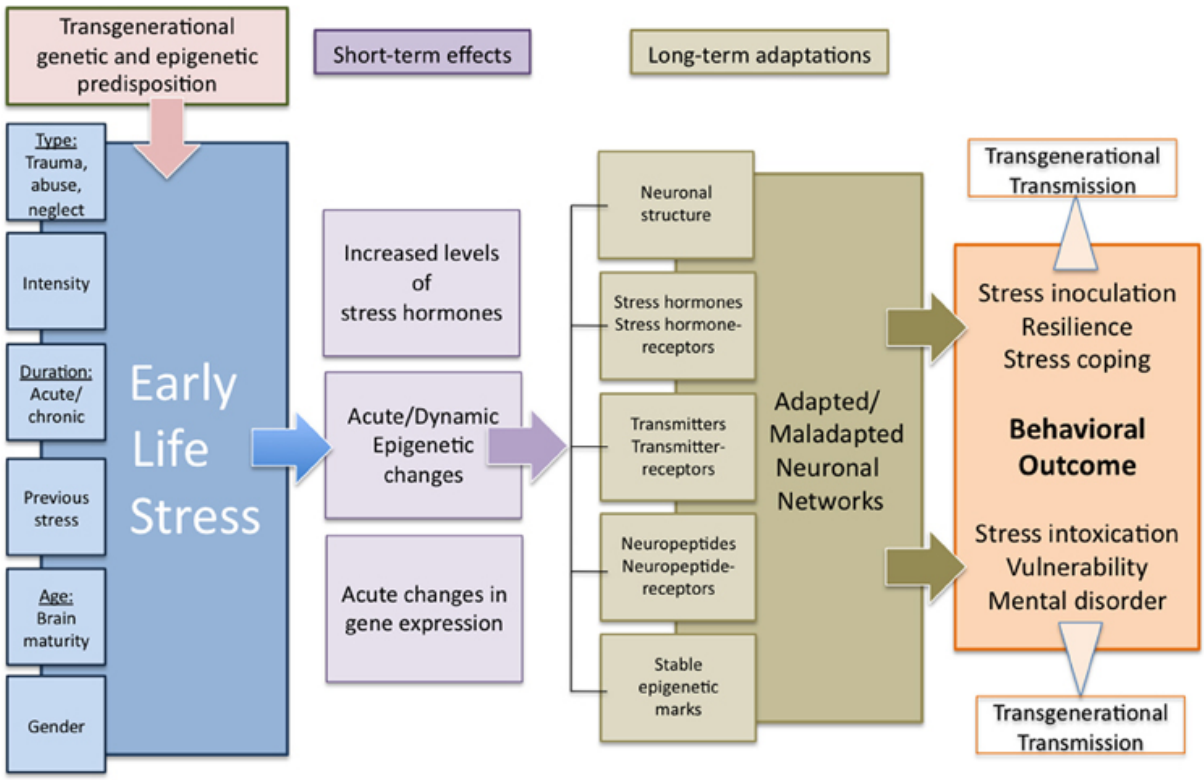


Figure 1 Glucocorticoid Hypothesis

During the course of pregnancy the function of the maternal HPA axis is altered to result in an increased production of cortisol which steadily increases in the circulation throughout pregnancy. It is thought that this observed increase plays a normal in the normal development and maturation of organs and tissues within the fetus (Smith and Shearman, 1974). As both a protective and regulatory function there is usually a parallel upregulation of the placental enzyme 11 β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) which serves to convert cortisol to its inactive form, cortisone (Murphy and Clifton, 2003; Sandman et al., 2006). However, this enzyme only serves as a partial barrier allowing a certain amount of cortisol to cross the placental barrier (Gitau et al., 1998; Gitau et al., 2001), therefore if there are unusually high levels of cortisol excess quantities will pass through the placental-fetal interface. In addition to this it has also been shown that stress may cause a reduction in the

expression of 11β-HSD2 thus causing a further increase in the levels of circulating fetal cortisol (Glover et al., 2009; O'Donnell et al., 2009).

Under normal conditions the production of cortisol by the adrenal glands is self-regulated by a negative feedback loop whereby a rise in cortisol levels causes the inhibition of further production of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (Myers et al., 2012). However, during pregnancy the placenta becomes an additional source of CRH and unlike hypothalamic CRH an increase in cortisol results in further release of CRH from the placenta in a feed forward manner which in turn increases cortisol production (Demey-Ponsart et al., 1982; D'Anna-Hernandez et al., 2011; Jung et al., 2011). The purpose of placental CRH is thought to be as a 'placental clock' which regulates events leading to parturition (Smith et al., 2002). As mentioned previously it has been demonstrated that pregnant women who report increased stress levels also display alterations in the levels of circulating cytokines such as the proinflammatory cytokines IL-6 and TNF both of which have been associated with premature labour, preeclampsia and an increased risk of offspring developing schizophrenia and autism in later life (Coussons-Read et al., 2005; Coussons-Read et al., 2007; Merlot et al., 2008; Bale, 2009). It has also been previously demonstrated that increases in the levels of peripheral cytokines may cause elevated levels of cortisol through activation of the HPA axis (Dunn, 2000; Hashimoto et al., 2001). From these observations the data suggests that there may possibly be a certain level of crossover between the pathologic mechanisms of MIA and PNS as both can result in elevated cytokine levels and increased amounts of circulating stress hormones. PNS as evidenced from the studies discussed above may increase the risk of incidence of a number of developmental disorders in addition to others that may not manifest until later life. In this section I will review and discuss the data available from epidemiological and animal studies pertaining to the effects of PNS on fetal development.

3.6 Epidemiological Studies

3.6.1 Neurodevelopmental and Neuropsychiatric Disorders

There is a growing body of evidence supporting the theory that PNS increases the risk of developing a range of neurodevelopmental and neuropsychiatric disorders in affected offspring. Attention Deficit Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder which is characterised by impulsivity, hyperactivity and deficits in attention span (Childress and Berry, 2012). A prospective cohort study from a Danish population presented

evidence that maternal bereavement during pregnancy was a risk factor for ADHD but that this effect was sex specific effecting only males. This increase in risk was greatest when bereavement occurred during the third trimester and was unexpected to the mother (Li et al., 2010a). Another study which also demonstrated an increased risk of ADHD following maternal bereavement was conducted in a Swedish population in which they found that this occurrence during the third trimester resulted in a 30% increase in the risk of developing ADHD (Class et al., 2014). From these studies it has been proposed that the third trimester is a window of sensitivity in which PNS is particularly likely to induce ADHD in affected offspring.

Schizophrenia is a neuropsychiatric disorder that affects roughly 24 million people worldwide according to the World Health Organisation. A number of studies have implicated PNS as a potential risk factor in the development of the disorder. In a large cohort study of a Danish population there was an established link between maternal bereavement and an increased risk for the development of schizophrenia, this association was most prevalent in those women who experienced the death of a close relative within the first trimester of pregnancy (Khashan et al., 2008). An additional study that provides support for these findings is from a cohort of mothers who were pregnant during the Arab-Israeli war, children from mothers in their third month of pregnancy were found to have a higher incidence of schizophrenia than others (Malaspina et al., 2008). Together these studies indicate that the first trimester may be a 'critical period' for the development of schizophrenia in offspring through fetal programming. However, there is conflicting data from two separate cohorts; one cohort from a Swedish population and the second from an Israeli population. Both of these studies found that maternal stress during the preconceptional, prenatal or postnatal periods did not have any significant effect in increasing the relative risk factor for the development of schizophrenia in affected offspring (Selten et al., 2003; Class et al., 2014).

Several studies have also investigated the affect of maternal stress on the incidence of epilepsy. A large cohort study from a Danish population initially found that there was no strong association between PNS and the aetiology of epilepsy, however when this data was stratified into individual trimesters they found that bereavement within the first trimester led to a twofold increase in the risk of epilepsy in offspring. (Li et al., 2008). The authors do identify a possible weakness in this conclusion due to the low number of cases in the exposed group. Another study using a Danish cohort found that death of a child either during pregnancy or in the 12 months preconception was associated with an increased risk of

developing CP in later life (Li et al., 2009b). The most significant effect was observed when the bereavement occurred during the perinatal period, this data was not stratified into individual trimesters however. The greatest risk was evident in mothers who experienced the bereavement within 7-12 months prior to pregnancy with those in the 0-6 months window were less at risk (Li et al., 2009b). Li et al conducted an additional study using a Danish cohort in which they established that there was no increase in the risk of febrile seizures in offspring in which mothers experienced a form of bereavement in a 12 month period before pregnancy or during the gestational period (Li et al., 2009a).

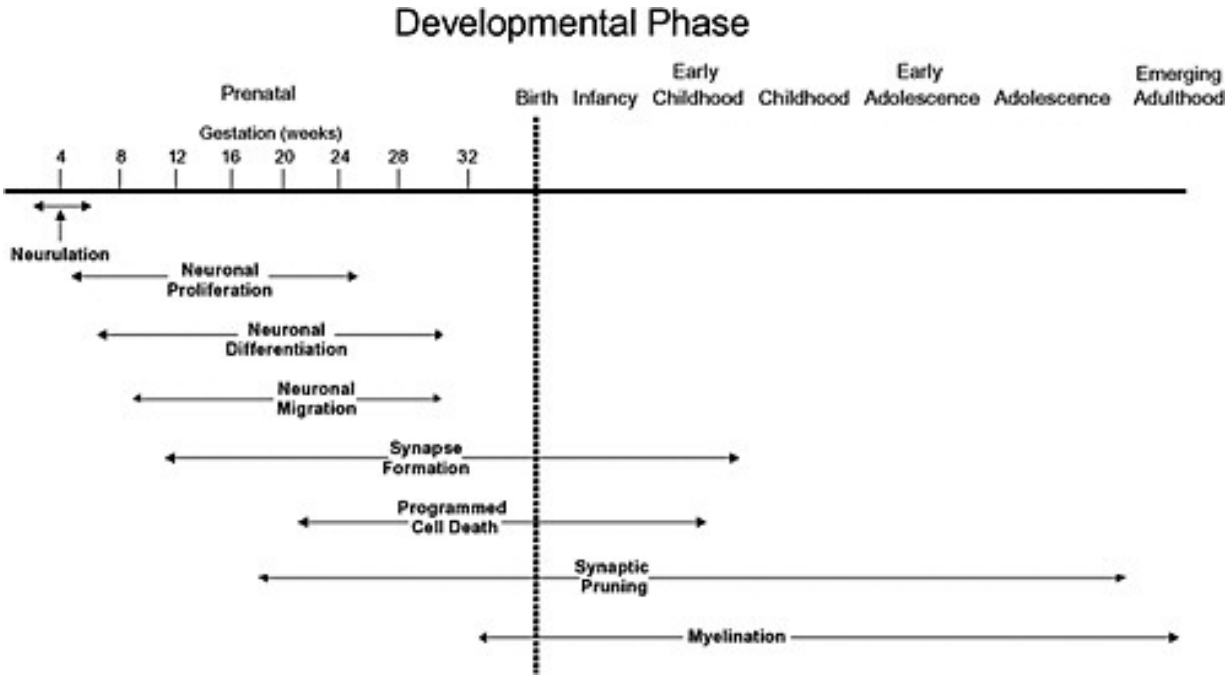


Figure 2 Neural Development Schematic

Autism is a neurodevelopmental disorder in which there are deficits in the processing of sensory information and is estimated to effect 1 in every 68 persons (Baio, 2014). A number of epidemiological reports have suggested a potential role for PNS in the aetiology of autism. One such study performed by Beversdorf et al showed that mothers whose children developed autism reported higher stress levels due to adverse life events during pregnancy in comparison to healthy children. Mothers who experienced these stressful events between 21-32 weeks of pregnancy had the highest prevalence of children with autism (Beversdorf et al., 2005). There is also evidence for environmental stressors increasing the risk of autism; women pregnant during the Louisiana storms between 1980 and 1995 had an increased frequency of births of children with autism - the rate of incidence increasing in line with the severity of the storm (Kinney et al., 2008a). There was a drastic increase in autism prevalence

in mothers who experienced stressful circumstances during the 5th, 6th, 9th or 10th months of gestation with a nearly fourfold increase when compared to other months (3.72 per 10,000 births) (Kinney et al., 2008a). Stressful life events were shown to be a predictor of autistic like traits in male offspring at 2 years of age in a small population of women in Australia (Ronald et al., 2010). Another supporting study that implicates PNS in the aetiology of autism was conducted in a Swedish cohort and found a 58% increase in the risk of developing the disorder related to maternal exposure to a bereavement during the third trimester (Class et al., 2014). There is one study in disagreement with these previous findings, a Danish cohort study which examined the association between maternal bereavement during pregnancy and an increased risk of autism found that there was no trend towards in increased risk (Li et al., 2009c). In a morphological investigation, elevated maternal cortisol levels at 15 weeks of gestation was positively correlated with an increase in right amygdala volume along with increased affective symptoms in girls at 7 years of age (Buss et al., 2012). Enlarged amygdala volumes are a characteristic feature in the brains of autistic children which suggests that fetal programming by elevated cortisol levels as a result of PNS may contribute to the aetiology of autism (Schumann et al., 2004).

The development of other mood disorders in affected offspring has also been attributed to PNS. A study cohort involving women who were in their first trimester of pregnancy during the Arab-Israeli war had up to a threefold increase in the likelihood of offspring developing mood disorders in later life. A particularly strong effect was noted in mothers who were exposed during their third month of pregnancy with a greater than fivefold increase in risk (Kleinhaus et al., 2013). Another study in support of these findings was conducted in a Danish cohort in which they demonstrated a link between maternal bereavement in the second trimester and an increased risk of affective disorders in male, but not female offspring (Khashan et al., 2011). A report by Class et al found contradicting results to these other two studies in that they found no association between maternal bereavement and bipolar disorder. Exposure during any period of gestation including periconceptual and postnatal did not have any effect on the incidence of affective disorders, however, this study failed to stratify its data based on gender (Class et al., 2014).

3.6.2 Cardiovascular Disorders

Congenital heart defects are reported to affect over 1.35 million live births worldwide each year (van der Linde et al., 2011). Psychosocial stress has been shown to be related to an increased incidence of CHD including cotruncal defects, transposition of the great arteries

and Teratology of Fallot when exposure occurs during the periconceptional period (Carmichael and Shaw, 2000). A study in a Danish cohort has provided evidence that maternal bereavement during pregnancy or up to 6 months prior to conception is an associated risk factor for the development of CHD in offspring. Bereavement occurring during the first trimester representing an increased risk of 23% whilst exposure during the second trimester representing an increased risk of 26% (Zhu et al., 2013). Psychosocial stress has also been shown to result in increased systolic and diastolic blood pressure as well as an increase in the mean arterial pressure which manifests in children as young as 5 years of age (Van Dijk et al., 2012). Potential mechanisms by which these aberrant changes in the cardiovascular system may occur could be due to fetal programming by elevated cortisol levels due to maternal stress. Studies by Rondo and colleagues determined that there was a positive association between elevated maternal cortisol levels in late gestation with systemic vascular resistance (Rondó et al., 2010) and low arterial elasticity (Rondo et al., 2011) both of which may contribute to hypertension. There are no studies to date that look at affected offspring beyond the age of 7 years so as of yet the true consequences of PNS in humans during adulthood has not been examined.

3.6.3 Respiratory Disorders

Asthma like symptoms including wheezing illnesses and breathlessness are a major cause of morbidity in children between the ages of 3 and 5 (Bisgaard and Szefler, 2007). In a Boston cohort of English speaking or Hispanic women, mothers who reported experiencing a high number of adverse life events during late pregnancy were more than three times as likely to have offspring that displayed a form of wheezing illness when compared to those of mothers who experienced low levels of maternal stress (Mathilda Chiu et al., 2012). The relationship between maternal bereavement was investigated in a large cohort of Swedish women in which a link was established between exposure to maternal stress during the first and third trimester and an increased incidence (48%) of hospital admissions for asthma (Khashan et al., 2012). Elevated maternal cortisol levels within the third trimester have also been shown to cause a twofold increase in wheezing illnesses in children at 2 years of age (Wright et al., 2013).

3.6.4 Metabolic and Immune Disorders

Over the period of the last 30 years there has been a substantial increase in the number of cases of obesity with numbers doubling since the 1980s according to data from the World Health Organisation. It has been postulated that there may be a potential role for fetal

programming due to PNS in contributing to this observed increase over recent years and several studies have investigated this line of thought. A study of a large cohort of Danish mothers who experienced a form of maternal bereavement within a 6 month period prior to conception or during pregnancy were at an increased risk of their child being overweight at ages 7-13. Those at the highest risk of their child being overweight, with a threefold increase, experienced bereavement up to 6 months before pregnancy (Li et al., 2010b). In a similar study amongst a Danish cohort elevated maternal stress levels, as assessed by means of a telephone interview, during mid to late gestational periods was not associated with an increased incidence of obesity in affected children at 7 years of age. Though when this data was stratified based on gender a minor increase in risk was observed in males but not females (Ingstrup et al., 2012). A more recent study examined a cohort of only men between the ages of 17 and 31 in which maternal bereavement in the 6 months before their conception was related to an increased risk for being overweight or obese. If the maternal stressor took place during the first trimester this posed the most significant risk with those men having a 25% increased risk of obesity and 14% increase in the risk of being overweight (Hohwü et al., 2014).

Metabolic disorders such as diabetes may also have some basis of origin relating to maternal stress during development. In a study investigating the potential relationship between psychosocial stress during pregnancy and the development of diabetes-like symptoms their findings showed that women whose mothers reported elevated levels of stress during their gestational period had an elevated insulin response to a glucose tolerance test which can be an early indicator for the development of insulin resistance (Entringer et al., 2008). Similar results were observed in a separate study whereby women who were pregnant during the 1988 Quebec ice storm who reported high levels of stress as a result were found to have children with elevated insulin secretion (Dancause et al., 2013). A recent study in which the association between glucose metabolism at 5 years of age and maternal stress, depression and anxiety was examined did not find any relationship between the two. However, this study specifically selected mothers who experienced stress at 16 weeks of pregnancy so this limited time window may not reflect the window of sensitivity for maternal stress to affect glucose metabolism in the offspring. One final study that supports PNS causing an increased risk of developing diabetes comes from a Danish cohort in which maternal bereavement during the second trimester was associated with a 15 % increase in the risk of developing type 2 diabetes in affected offspring (Li et al., 2012).

Exposure to maternal stressors during pregnancy can also cause programming of the immune system and its related components. In a study where psychosocial stress in mothers during pregnancy was assessed through use of the modified Chinese version of Short Form 36 Health Survey whereby mothers who self-reported elevated stress levels were found to have increased cord serum levels of IgE (Lin et al., 2004). Elevated levels of IgE can serve as an early indicator for the development of atopic diseases in childhood and adulthood (Pesonen et al., 2009). In a small case control study, with a relatively small sample size of only 34 young women, those whose mothers reported experiencing negative life events during pregnancy displayed elevated production of proinflammatory cytokines in comparison to mothers who reported no stress when stimulated with phytohaemagglutinin (Entringer et al., 2008).

3.7 Animal Models

3.7.1 Neurodevelopmental and Neuropsychiatric Disorders

As discussed previously epidemiological studies are a valuable research method which allow direct correlations between observations in a population relating to a specific set of criteria and patient outcomes. Although it is possible to examine obtainable samples from these populations in some cases in order to assess changes in sets of biological markers in bloods or other fluids such as CSF it is not possible to perform many of the manipulations to systems that are possible in animal models. In this section I will present findings from some of the more recent studies from *in vivo* and *in vitro* studies and the potential mechanisms which are thought to contribute to the pathogenesis of neurodevelopmental and neuropsychiatric disorders discussed in the previous section.

It is important to note that maternal psychosocial stress may affect normal fetal development through a number of mediators some of which directly relate to the stress response system whilst others such as proinflammatory cytokines are elevated through secondary effects of stress (Laviola et al., 2004; Coussons-Read et al., 2007; Dunkel Schetter, 2011; Diz-Chaves et al., 2012). Elevations in the levels of circulating proinflammatory cytokines have also in turn been shown to activate the HPA axis thus further stimulating the production of stress related hormones therefore potentially establishing a cyclical series of events (Dunn, 2000). The development of the central nervous system involves a series of events that are initiated in sequence and that are therefore dependent on each other in various ways. Disturbances in the neuroendocrine system or other events that may result in epigenetic changes that occur at one particular stage therefore may influence those at later stages of development.

Alterations to glutamate signalling, which comprises the main excitatory pathways within the brain, are thought to be involved in the aetiology of schizophrenia and other mood disorders. Specifically the mGlu2 receptors are thought to play a key role in the pathophysiology of anxiety related disorders and schizophrenia and have been investigated as potetential therapeutic targets (Moghaddam and Javitt, 2012). Studies have shown that maternal stress during pregnancy alters the expression of mGlu2 receptors in addition to the 5-HT receptor 5HT2A within the mouse frontal cortex which results in a schizophrenia-like phenotype in adult offspring. Specifically, PNS results in the downregulation of mGlu2 receptor expression and an increase in expression of the 5-HT2A receptor in the brain (Holloway et al., 2013). 5-HT and glutamate systems have been implicated in the pathophysiology of schizophrenia in addition to the mechanism of action of antipsychotics (González-Maeso and Sealfon, 2009; Moreno et al., 2009). The frontal cortex plays a significant role in cognition and perception and therefore dysfunction in this region has been proposed to play a role in schizophrenia and other psychotic disorders (González-Maeso et al., 2007; Gonzalez-Maeso et al., 2008; González-Maeso and Sealfon, 2009). Changes in receptor expression as reported by Holloway and colleagues have been found in post-mortem analysis of the prefrontal cortex of schizophrenic subjects (Gonzalez-Maeso et al., 2008). Therefore the findings in this animal model as a result of PNS demonstrate that maternal stress causes brain alterations that may lead to the development of schizophrenia in affected offspring.

Rab proteins assist in the regulation of membrane trafficking among subcellular compartments and are members of the Ras superfamily of monomeric GTP-binding proteins. Of the Rab proteins the Rab3A isoform is the most prominent and widely distributed within the brain where it has key functions in the regulation of glutamate release and synaptic plasticity by governing late synaptic vesicle fusion (Südhof, 2004; Coleman and Bykhovskaia, 2010). In a study performed by Orlando et al they hypothesised from their results that defective glutamate release in the hippocampus caused by a reduction in the activity of Rab3A may possibly contribute to the schizophrenic phenotype of PNS offspring (Orlando et al., 2014). Pregnant dams exposed to restraint stress as a form of psychosocial stress give birth to offspring that exhibit similar behavioural traits to those of animal models of schizophrenia. Namely they display locomotor hyperactivity and alterations in fear conditioning, pre-pulse inhibition and social interaction (SI) (Matrisciano et al., 2012; Holloway et al., 2013; Matrisciano et al., 2013). These data suggest that PNS may cause a schizophrenic phenotype in these animals. In agreement with the theory of ‘fetal

programming' these mice have a down regulation of glutamate decarboxylase-67 and reelin in the frontal cortex (Matrisciano et al., 2013). Glutamate decarboxylase plays a crucial role in catalysing glutamate to γ-aminobutyric acid (GABA) which regulates neuronal excitability (Watanabe et al., 2002). These same alterations are also observed in post-mortem analysis of brains from schizophrenic patients (Grayson and Guidotti, 2013). It is likely that these alterations occur through epigenetic mechanisms, an increased expression of methyltransferases would lead to promoters of genes, including those governing glutamatergic and GABAergic function, to become hypermethylated resulting in their downregulation (Weaver, 2007; Sweatt, 2009; Zhang et al., 2010). This reasoning was confirmed by Matrisciano et al who demonstrated a sustained upregulation of the DNA methyltransferases (DNMT) 1 and 3a in hippocampal and cortical GABAergic neurons during development in addition to sustained binding of DNMTs to GABAergic promoters (Matrisciano et al., 2012). These changes result in deficits in GABAergic signalling and are likely to manifest as the behavioural and cognitive abnormalities in addition to the observed deficits in sensory-motor gating which are typical of psychotic patients (Guidotti et al., 2005; Lewis et al., 2005). Studies have also shown that the use clozapine and sulpiride which are both atypical antipsychotics, alone or in conjunction with the histone deactylase inhibitor valproate at clinically relevant doses down-regulates reelin and GAD67 promoter methylation in the frontal cortex and striatum (Dong et al., 2008).

Multiple animal studies have shown that offspring from maternally stressed mothers exhibit increases in affective-related behaviours (Abe et al., 2007; Maccari and Morley-Fletcher, 2007; Weinstock, 2008). Animals born from mothers who were subjected to a prenatal stressors such as restraint stress display prolonged periods of immobility in the forced swim test which is used to assess a depressive like state in rodents (Drago et al., 1999; Szymańska et al., 2009). Both male and female rats that are exposed to PNS in the mid to late periods of gestation exhibit maladaptive behavioural responses to the tail-suspension test including prolonged immobility periods and diminished immobility latency (Zhang et al., 2013). The glutamatergic system has also been speculated to have a potential role in causing these depressive like states in PNS animals. A study by Jia et al demonstrated that PNS caused elevated glutamate levels in the hippocampus with a concordant decrease in the NR1 subunit of the N-methyl-D-aspartate receptor (NMDAR) in offspring (Jia et al., 2010). These findings initiated further studies investigating the potential role of glutamate and its receptors in causing depressive-like behaviours induced by PNS. Excitatory amino-acid transporters

(EAATs) predominantly regulate the reuptake of glutamate within the brain thereby maintaining a proper concentration of glutamate in the synaptic cleft (Shigeri et al., 2004). PNS has been shown to significantly reduce the mRNA levels of EAAT2 in the hippocampus, striatum and frontal cortex of both male and female rats and EAAT3 in the hippocampus only. This decrease in the level of EAATs can subsequently result in the decrease of glutamate reuptake thus causing an accumulation of glutamate in the synaptic cleft (Zhang et al., 2013). In addition to this, although PNS has been shown to have no effect on the α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subunit GluR1 in the frontal cortex or hippocampus of affected offspring it is thought that there may be a resultant decrease in Ser-845 phosphorylation of this subunit. This may result in defective trafficking of AMPAR to the postsynpatic membrane thus effecting the binding of glutmate (Zhang et al., 2013).

Based on reports from both clinical and preclinical studies it has been proposed that 5-HT signalling is involved in the aetiology of anxiety, impulsiveness and depression (Murphy and Pigott, 1990; Nordquist and Oreland, 2010). Several animal studies have established links between PNS and the aberrant development of central serotonergic neurons. One study discovered that maternal stress results in increased 5-HT synthesis in rats as well as displaying region-specific changes in brain 5-HT, 5-hydroxyindoleacetic acid and NE levels in infancy (Peters, 1982). In one report it was shown that PNS induces synaptic loss which is associated with disruption of 5-HT neurotransmission and resultant developmental disabilities in offspring (Hayashi et al., 1998). Excessive PNS has also been demonstrated to induce long-term emotional vulnerability to stress associated with functional abnormalities in central 5-HT neurons of affected offspring. In these animals there is a decreased expression of tryptophan hydroxylase and decreased expression of the transcription factor Lmx1b, which is known to be crucial for both the maintenance of normal function and differentiation of central 5-HT neurons in the adult raphe and embryonic hindbrain of PNS animals (Miyagawa et al., 2014). The Five-Choice Serial Reaction-Time Task was developed as a useful test for evaluating sustained attention and inhibitory control to investigate impulsive or compulsive-like behaviours. Using this test it was found that exposure to PNS results in impairments of sustained attention and inhibitory response control which are intensified by NMDA antagonism possibly indicating an ADHD-like phenotype arising from PNS exposure (Wilson et al., 2012).

Offspring from maternally stressed animals have also be shown to exhibit autistic-like behavioural traits with significantly increased fear of strange or unfamiliar environments as indicated by decreased exploration in the open field (OF) test in addition to a more defensive withdrawal from threatening environments when compared to controls (Weinstock, 2002). A separate study by Son et al found that adult male mice from maternally stressed mothers had an impaired motor response habituation to a novel stimulus.

PNS is known to cause an increase in proinflammatory cytokines leading to a proinflammatory status (Vanbesien-Mailliot et al., 2007). In ovariectomised female mice, which prevents the anti-inflammatory effects of ovarian hormones (Tapia-Gonzalez et al., 2008), PNS has been reported to cause an increase in the mRNA levels of IL-1β and also significantly increase the number of Iba1 immunoreactive cells which mark microglia (Diz-Chaves et al., 2012). Increased expression of IL-1β in the hippocampi of PNS animals is thought to contribute to anhedonic and depressive-like behaviours (Maccari et al., 2003; Kubera et al., 2011). It had previously been described that an elevation in IL-1β in the hippocampus produces a marked reduction in neurogenesis, as IL-1β expression is induced by stress it is probable that this cytokine may mediate the antineurogenic effect of stress (Ben Menachem-Zidon et al., 2007; Goshen et al., 2007; Koo and Duman, 2008; Yirmiya and Goshen, 2011). PNS has also been shown to inhibit neurogenesis in the dentate gyrus and hippocampus in monkeys as well as rats (Lemaire et al., 2000; Coe et al., 2003).

3.7.2 Cardiovascular Disorders

The early epidemiological studies which formed the basis for the 'fetal origins of disease' theory initiated further studies into cardiovascular disease arising from adverse events during the development of the fetus. A number of studies in animal models have now formed a body of evidence in support of this theory. It has been demonstrated that inducing a chronic stress response during pregnancy significantly increases systolic arterial pressure (APsys) in adult male offspring with no effect on diastolic pressure (Holst et al., 2002). A follow up study by a separate group in relation to this previous study investigated the effect of PNS on cardiovascular responses. PNS animals exhibited a greater peak in APsys along with a greater duration of APsys responses both during acute stress and also during the recovery period. These animals also displayed a greater variability in blood pressure increase in response to stress when compared to their control counterparts (Igosheva et al., 2004). These reports demonstrate that PNS induces lasting effects on offspring leading to maladaptation to stressful experiences demonstrated by enhanced blood pressure responses with longer

recovery periods to return to baseline levels. Ultimately this may lead to offspring exposed to PNS being at an increased risk for developing hypertension in addition to other stress-related cardiovascular disorders.

The comorbidity of major depressive disorders and cardiovascular disease has been shown to have a population prevalence of almost 20% suggesting some overlapping aetiology between the two (Scherrer et al., 2003). Depressive disorders have been characterised by some researchers as aberrant stress-induced neuroplastic alterations in the medial prefrontal cortico-amygdalo/hippocampo-hypothalamo-brainstem circuitry (Krishnan and Nestler, 2008; Murray et al., 2011). Vagal dyscontrol of the motor and solitary nucleus in the brainstem, both of which are innervated by preautonomic neurons of the paraventricular nucleus may affect cardiovascular function (Swaab et al., 2003). Thus PNS may induce changes in innervation or molecular mechanisms that regulate cardiovascular function. Ruijtenbeek et al have provided evidence that a suboptimal fetal environment can promote hyperinnervation of the sympathetic branch of the peripheral arterial system (Ruijtenbeek et al., 2000). There are multiple mechanisms that regulate sympathetic control of cardiovascular function including stimulation of various receptor subtypes and changes in expression of these receptors may cause deviations in normal function. Intrauterine stress has been demonstrated to cause regionally selective alterations in arterial adrenergic function in affected offspring, although femoral and saphenous ateries were not affected, renal arteries displayed pharmacological alternations namely changes in the density of β-adrenoreceptors and possibly their coupling to adenylyl cyclise (Sanders et al., 2004).

Apolipoprotein E (ApoE) is an essential apolipoprotein which is crucial for the catabolism of lipid lipoprotein constituents such as cholesterol, mice lacking this gene display cholesterol levels nearly five times that of normal animals (Andersson et al., 2009). One study has shown that PNS perpetuates the development of atherosclerosis and inflammation in these ApoE knockout mice. PNS was shown to cause accelerated atherosclerotic lesion formation in which elevated systemic inflammation was deemed to be a contributing factor (Ho et al., 2013). PNS animals also demonstrated formation of larger plaques at the level of the aortic root a finding that was most pronounced in female animals. It has been shown that programmed effects may not be limited to the F1 generations but may be passed down through subsequent generations (Drake and Liu, 2010). Using a dexamethasone model of PNS animals prenatally exposed have elevated cholesterol levels which is a known risk factor in the development of cardiovascular disease. The defective changes in lipid metabolism

from the F1 generation were passed through to both the F2 and F3 generations which had higher levels of cholesterol but lower levels of triglycerides than control animals (Buchwald et al., 2012). Of particular note was the significant increase in LDL cholesterol, a major contributor to atherosclerosis (Lusis et al., 2004), in F2 and F3 generations of the dexamethasone group (Buchwald et al., 2012).

3.7.3 Respiratory Disorders

In comparison to other areas of investigation there have been relatively few studies that have examined the role of PNS in contributing to the development of respiratory disorders. From the research that has been carried in this area two neuronal systems, the GABAergic and serotonergic, have been demonstrated to be effected by PNS with subsequent aberrant changes in respiratory function. The GABAergic system is involved in the regulation and generation of breathing with the GABAA receptor being located in the respiratory nuclei of the brainstem, activation of these receptors serves to inhibit respiratory activity (Darnall et al., 2006; Ren and Greer, 2006). The maturation process of respiratory system control can be affected by a number of environmental factors during development (Verkuyl et al., 2005). Disturbances in GABAergic transmission have been implicated in the pathophysiology of a number of respiratory disorders in newborn offspring (Darnall et al., 2006; Abu-Shaweesh and Martin, 2008; Zhao et al., 2011). Delhaes et al found that PNS significantly reduces respiratory depression resulting from the administration of the GABAA agonist muscimol demonstrating alterations in the GABAergic system. This effect was also sex specific with females showing a marked difference in the minute ventilation (V_E) when compared to male animals of the same group (Delhaes et al., 2014).

Another study which examined the effect of PNS on apneas and the 5-HT system discovered that PNS alone is sufficient to cause disruption to respiratory regulation. They found that the hypoxic ventilatory response of PNS animals was lower than control which poses significant physiological consequences as this mechanism is an essential defence against hypoxia (Fournier et al., 2013). The proportion of apneas with 0_2 desaturations is also more frequent in PNS animals which may indicate that cardio-respiratory coupling is also affected. PNS also results in a transient deficiency in medullary 5-HT and NE although this does not persist past birth. However, this temporary deficiency during gestation may be sufficient to cause alterations in respiratory control. The use of 5-HT agents produced greater effects both *in vivo* and *in vitro* in PNS animals indicating that deficits in medullary 5-HT contributes to respiratory disorders (Fournier et al., 2013). Finally through non-invasive methods evaluation

of 0_2 saturation and heart rate (HR) displayed evidence that the apneas are physiologically relevant and could compromise brain development (Talge et al., 2007; Zhao et al., 2011).

3.7.4 Metabolic and Immune Disorders

As discussed previously with an increase in the number of cases of diabetes and obesity related disorders combined with a younger age of onset, adverse prenatal events are being investigated as potential causative agents. Animal models of PNS aim to attempt to elucidate the potential mechanisms by which these disorders may occur. Pregnant dams exposed to stressors during their third week of pregnancy produced offspring that were hyperinsulinemic and hyperglycaemic during adulthood (Lesage et al., 2004; Tamashiro et al., 2009). Under high-fat diets PNS animals display increased insulin and glucose responses to an oral glucose tolerance test, interestingly these effects are more pronounced in rats with a passive stress-coping manner. In addition to this, passive stress-coping animals gained significantly more weight on a high fat diet than control or active coping animals PNS animals (Boersma et al., 2014). This phenotype closely resembles that which would be observed in human subjects of type II diabetes. Another observation from this study was that PNS animals had elevated leptin levels which may suggest these animals have a degree of leptin resistance in addition to insulin resistance (Boersma et al., 2014). In a separate study it was demonstrated that PNS exposure results in significantly increased glucose responses to ARS in males only whilst following an oral glucose load female animals were hyperinsulinaemic but males did not display this response (Brunton et al., 2013). This group also reported that genes necessary for glucocorticoid and lipid metabolism were influenced by PNS, these changes were also sex specific with males displaying alterations predominantly in skeletal muscle and the liver whereas in females this occurred in the subcutaneous fat. Additionally, in males PNS resulted in a decrease in hepatic peroxisome proliferator-activated receptor α mRNA which is a key modulator of systemic and intrahepatic lipid homeostasis (Leone et al., 1999; Akiyama et al., 2001). A finding consistent with this result was an increase in plasma triglyceride concentrations in PNS males.

Maternal social stress during gestation has been shown to effect the nature of immune reaction to social stress in adult offspring with males exhibiting lower numbers of T and NK cells, neutrophils and monocytes accompanied by a lower level of lymphocyte proliferation in whole blood cultures (Götz et al., 2007). PNS can also modify T and B lymphocytes responses to specific antigens, this effect being dependent on the timing and nature of the stressor as well as the age of offspring (Merlot et al., 2008). Immune system components like

cells from other systems express receptors for hormones and neurotransmitters thus making them sensitive to changes in circulating stress hormones therefore activation of these receptors results in modulation of immune activity (Elenkov and Chrousos, 2002). In a study by Pascuan et al animals that were exposed to PNS did not display any changes in IgG antibody production in response to a T-cell dependent antigen, but did show a decreased humoral response after acute stress exposure (Pascuan et al., 2014). It has also been shown that transient changes that occur during development may cause altered immune-reactivity in later life towards infections (Vanbesien-Mailliot et al., 2007).

In this study by Vanbesien and colleagues they demonstrated that PNS results in a proinflammatory state during adulthood evidenced by an increased percentage of $CD8^+$ and NK cells within the blood along with an elevated proliferative response to phytohaemagglutinin *in vitro*. This response was also associated with an increased level of secretion of IFN-γ (Vanbesien-Mailliot et al., 2007). An interesting finding in this study was that cellular alterations that were observed in adulthood could not be identified in younger animals despite there being a proinflammatory profile observed at the mRNA level. Another study conducted by Ho et al showed that PNS caused affected offspring to have a proinflammatory immune state with a decrease in regulatory T cells and perturbations to the innate immune response. These animals were susceptible to inflammation resulting in increased concentrations of TNF and IFN-γ.

3.8 Aims of Study

- Create an *in vitro* model of a proinflammatory environment during development to investigate the effects of the proinflammatory cytokine IL-1β on NPCs determining effects on cell survival, proliferation and cell fate specification.
- Use this same *in vitro* model to examine the theory of 'critical periods' during embryonic development examining several different developmental time-points and using several cytokines to assess the 'critical periods' of this model.
- Utilise an *in vivo* animal model of PNS, using a well established protocol of restraint stress, to examine the effects of elevated stress levels on multiple physiological systems from the same cohort of animals.

4. Material and Methods

4.1 Preparation of E14 Rat Ventral Mesencephalon (VM) NPCs

E12, E14 and E16 rat embryos were obtained by laparotomy following decapitation under terminal anaesthesia induced by Isoflurane (Abbeyville Veterinary; 2% minimum alveolar concentration.). Each VM was dissected out in ice-cold Hank's Balanced Salt Solution (HBSS) (Sigma), as previously described (Clayton and Sullivan, 2007). The tissue pieces were incubated in 2 ml of 0.1% trypsin (Sigma) at 37˚C for 5 min to enzymatically dissociate the cells. 500 µl fetal calf serum (FCS) (Sigma) was added as a trypsin inhibitor and the suspension was triturated through a 25 gauge needle and syringe. After centrifugation at 1100 rpm for 5 min, the HBSS was removed and the cell pellet was resuspended in 1 ml of 'basic medium' (Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), penicillin 100 U/ml (Sigma), streptomycin 10 µg/ml (Sigma), L-glutamine 100 mM (Sigma) and 33mM D-glucose (Sigma)). Cells isolated from E12, E14 or E16 rat VM were seeded in a Costar^{TM} Ultra Low Cluster Plate 24-well plate (Corning) and grown in 1 ml of 'basic medium' supplemented with 20 ng/ml epidermal growth factor (EGF; Sigma) and 20ng/ml of basic fibroblast growth factor (bFGF; Sigma). Cytokines (IL-1β, TNF, IL-6 all at 10ng/ml (Promokine)) were added on 0 DIV and subsequently every second day with fresh media change where indicated. The IL-1R1 antagonist, IL1ra (R&D Systems) (20 ng/ml) or the p38 MAP kinase pathway inhibitor, SB203580 (Calbiochem) (10 µM) were also added where indicated. After proliferation for 2, 4 or 7 DIV in a humidified atmosphere containing 5% CO_2 at 37˚C the neurospheres were enzymatically dissociated to a single cell suspension as described previously (O'Keeffe and Sullivan 2005). A portion of the cell suspension was stored at -80˚C for RNA extraction or lysed for protein analysis. The remaining cells were seeded at a density of $2x10^4$ in poly-D-lysine-coated 96-well tissue culture plates (Sarstedt) and allowed to adhere for immunocytochemical staining.

4.2 LPS Injection Protocol

This study was performed under license with full ethical approval from the Animal Experimentation Ethics Committee of University College Cork, Ireland. Time mated Sprague-Dawley (SD) rats (provided by University College Cork Biological Services Unit) were maintained in controlled facilities where temperature (37°C), humidity (40-60%) and light (12 hour light/dark cycle) were regulated. Animals had access to standard rat chow and

water ad libitum. Pregnant dams received inraperitoneal injections of 50μg/kg bodyweight LPS (Sigma; Serotype 055:B5) or saline at E12 or E16.

4.3 Immunocytochemistry

Cultures were fixed in ice-cold methanol for 10 min. Following 3 x 5 min washes in 10mM phosphate buffered saline (PBS) containing 0.02% Triton X-100 (PBS-T) all cultures were incubated in blocking solution (5% bovine serum albumin (BSA) (Sigma), 0.2% Triton X-100 in 10 mM PBS) overnight at 4˚C. Cultures were incubated in the following antibodies; TH (1:300; goat polyclonal; Millipore), β-III tubulin (1:300; mouse monoclonal; Promega), nestin (1:300; mouse monoclonal; Millipore), IL-1R1 (1:300; rabbit polyclonal; Santa Cruz), Myelin Basic Protein (MBP) (1:300; mouse monoclonal), Glial Acidic Fibrillary Protein (GFAP) (1:300; mouse monoclonal; Sigma), phospho-p38 1:300; Cell Signalling), IL-6R (1:300; rabbit polyclonal; Santa Cruz), TNFR1 (1:300; rabbit polyclonal; Santa Cruz) or DCX (1:300; goat polyclonal; Santa Cruz) diluted in 1% BSA in 10mM PBS at 4˚C overnight. Following washes in PBS-T, cells were incubated in the appropriate secondary antibodies for 2 h at room temperature (RT) (22-25 °C); Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 594 donkey anti-mouse IgG or Alexa Fluor 594 goat anti-rabbit IgG (all 1:500; Invitrogen). Cultures were counterstained with bisbenzimide (1:1000; SIGMA). Cells were imaged under an Olympus IX70 inverted microscope fitted with an Olympus DP70 camera and AnalysisD^{TM} software. The total number of cells, assessed as bisbenzimide positive, and the numbers of each cell type were counted in each individual image. For each treatment, cells from four independent wells were stained and analysed, each experiment was repeated three times. For calculation of neurosphere volume the formula $4/3 \times \pi \times r^3$ was used. To get a true presentation of culture compositions at the time of plating, cells were plated for 2-3 hours before being fixed and stained.

4.4 Western Blotting

Western blots were performed based on methods originally described by Towbin et al (Towbin et al., 1979). Whole cell lysates were standardized to 20 μg/ml of protein per lane and electrophoresed on 5% stacking and 10% resolving polyacrylamide gels. Proteins were transferred to a PVDF membrane in transfer buffer at 100 V for 1 h. Membranes were blocked in TBS (pH 7.4) with 0.05% Tween 20 (Sigma) (TBS-T) and 5% BSA at RT for 1 h. Primary antibodies against either β-Actin (1:5000; Sigma), Caspase-3 (1:1000; Cell Signalling) or poly (ADP-ribose) polymerase (PARP) (1:1000; Millipore) were exposed to

the antigen blots at overnight at 4˚C. Membranes were rinsed three times for 10 min with TBS-T. Membranes were then incubated with horseradish peroxidase labelled anti-rabbit or anti-mouse IgG (1:2000; Promega). The membranes were rinsed again three times for 10 min with TBS-T and developed with ECL Plus from Amersham.

4.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis

Total cellular RNA was extracted from VM NPCs using an RNeasy kit (Qiagen). cDNA synthesis was performed on RNA (1 μg) using oligo(dT)s, random primers and reverse transcriptase (Promega) at 37ºC for 1 h. RNA was incubated with DNAse (Qiagen) for 30 minutes to exclude DNA contamination. Amplification of IL-1β, IL-1R1, IL-1RII, Nurr1, Pitx3, Lmx1b, TH and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA by PCR was carried out using the primers in table 1.

Table 1 PCR Primers

Target	Primer Sequence
IL-1β	*GAC CTG TTC TTT GAG GCT GAC (+), TTC ATC TCG AAG CCT GCA GTG (-)*
IL-1RI	*AGA TTG AAG GAC CTA TGA TG (+), TGC AGC ATC TGA CGA CAG GA (-)*
IL-1RII	*GGC AAG GAA TAC AAC ATC AC (+), TGG TTG TCA GTC GGT AGC TT (-)*
Nurr1	*CTG TCT CCC GCC TTT CAC TCT TCT (+), ATT TCG GCG GCG CTT ATC CA (-)*
Pitx3	*GCA GTA ATT CAC AGC CTC TCT GG (+), GTC CCT GTT CCT GGC CTT AGT (-)*
Lmx1b	*CGT GAG CCC GGA TGA GTC TGA (+), AGG GGT CGC TGC TTC CGT AGG (-)*
TH	*TGT CAC GTC CCC AAG GTT CAT (+), GGG CAG GCC GGG TCT CTA AGT (-)*
GAPDH	*TGG CAC AGT CAA GGC TGA GA (+), CTT CTG AGT GGC AGT GAT GG (-)*

PCR was performed with the following temperatures; an initial denaturation for 2 min at 95˚C, amplification for 26 cycles (GAPDH: 95˚C for 40 s, 55˚C for 30 s and 72˚C for 60 s), amplification for 30 cycles (IL-1R1: 95˚C for 40 s, 54˚C for 40 s and 72˚C for 30 s; IL-1R2: 95˚C for 30 s, 55˚C for 30 s and 72˚C for 60 s), amplification for 32 cycles (IL-1β: 95˚C for 30 s, 55˚C for 30 s and 72˚C for 60 s) or amplification for 34 cycles (Nurr1: 95˚C for 30 s, 60.9˚C for 30 s and 72˚C for 60 s; Pitx3: 95˚C for 30 s, 58.8˚C for 30 s and 72˚C for 60 s; Lmx1b: 95˚C for 30 s, 63.2˚C for 30 s and 72˚C for 60 s; TH: 95˚C for 30 s, 60˚C for 30 s and 72˚C for 60 s). The final extension was at 72ºC for 5 min. Products (10μl) were run on 1.5% agarose gels containing SafeView (NBS Biologicals) and visualised on an ultraviolet transilluminator. Gels were photographed and semi-quantified using densitometry in Adobe Photoshop CS5. Each experiment was independently carried out 3 times.

4.6 Quantitative Real-Time PCR (RT-QPCR)

Midbrain and striatum samples were homogenised in 1 ml of QIAzol Lysis Reagent (Qiagen). Following the addition of 200 µl chloroform, homogenates were separated into aqueous and organic phases by centrifugation at 13,000 rpm for 15 min. The upper aqueous phase was mixed with an equal volume of 70% ethanol to precipitate the RNA which was then transferred to an RNeasy Mini spin column within a 2 ml collection tube. Total RNA was purified using the Qiagen RNeasy Lipid Tissue Mini extraction kit and RNase-free DNase set, according to the manufacturer's instructions. After purification, total RNA was reverse transcribed using Stratascript reverse transcriptase (Agilent Technologies), for 1 h at 45 ˚C, in a 30 µl reaction according to the manufacturer's instructions. In order to amplify cDNAs encoding the normalising reference genes, GAPDH, succinate dehydrogenase complex, subunit A (SDHA) and ubiquitin C (UBQC), 2.5 µl of cDNA was amplified in a 25 µl PCR mix containing 1X FastStart Universal SYBR Green Master Mix (Rox) (Roche) and 150 nM of the forward and reverse primers. In the case of amplifying cDNAs encoding TH 2 µl of cDNA was amplified in a 20 µl PCR containing 1X of Brilliant III Ultra-Fast RT-QPCR Master Mix (Agilent Technologies), 150 nM of each forward and reverse primers and 300 nM cDNA-specific FAM/BHQ1 dual-labelled hybridization probe (Eurofins), and 3 nM ROX reference dye. Quantitative real-time PCR amplification was performed using the Stratagene MX3000P thermal cycler. GAPDH, SDHA and UBQC quantitative real-time PCR amplification products were verified using melting curve analyses (melting temperatures 83.5, 80 and 85 ˚C, respectively) of the completed PCR. The initial quantities of each cDNA in each PCR were determined by comparison to a standard curve incorporated into the PCR run and constructed from serial dilutions of cDNA reverse transcribed from RNA extracted from P11 striatum and midbrain samples. Values for each gene of interest were normalised to the geometric mean of the three reference genes. Cycling parameters for GAPDH, SDHA and UBQC were 10 min at 95 ˚C followed by 40 cycles of 95 ˚C for 30 s; 55 ˚C for 1 min; 72 ˚C for 1 min. Cycling parameters for IL-1R1 were 3 min at 95 ˚C followed by 45 cycles of 95 ˚C for 13 s and 60 ˚C for 30 s.

4.7 MTT Assay

Neurospheres expanded in 'proliferation medium' for 7 DIV with or without IL-1β treatment (as previously described) were dissociated and seeded at a density of $5x10^4$ cells per well in poly-D-lysine coated 96-well tissue culture plates. Cells were incubated in 200 µl of the MTT solution (0.5 mg/ml) for 4 h. Formazan product was dissolved in 100 µl dimethyl sulfoxide

(DMSO) and absorbance of the wells was measured at 570 nm and 630 nm (reference wavelength) with a microplate spectrophotometer (Tecan Sunrise).

4.8 Statistical Analysis of *In Vitro* Experiments

Unpaired Student's t-test or ANOVA with a post hoc Dunnett's multiple comparisons test were performed as appropriate to determine significantly different treatment groups. Results were expressed as means with SEM and deemed significant when $p < 0.05$.

4.9 Animals for Prenatal Stress Study

Animal experimentation protocols were approved by the Animal Experimentation Ethics Committee of University College Cork and performed in accordance with EU Directive 86/609/EEC. Adult female and male Sprague-Dawley rats (Harlan, UK), aged 10-12 weeks, where housed in same-sex groups prior to breeding. For mating, females were individually housed with a male rat; males were then removed from the cages. Following birth, pups were raised with their mothers before weaning at 21 days of age. Following weaning, offspring were group housed unless otherwise stated (2-6 animals per cage). Animals were housed on a 12h light/dark cycle; standard rodent chow and water were given ad libitum.

4.10 Prenatal Stress Protocol

Pregnant females were randomly assigned to prenatally stressed (n=6) or control (n=5) groups. Prenatal psychological stress was performed during the last week of pregnancy, from E14 to E21. To induce stress, pregnant dams were placed into transparent flat bottom plastic restraint devices (8.6 cm in diameter x 21.6 cm in length, LABEX of MA, MA, US) under a bright light every day, 3 times a day for 45 min at any one time: at 10:00 am, 14:00 pm and 18:00 pm. Control dams were left undisturbed in their home cages. Upon delivery, pup numbers, sex and weight were recorded. Average litter size was similar in both control and PNS groups: 15±1 and 14±1 pups, respectively. Neither litter weights (5.9±0.2 g in Control and 6.3±0.1 g in PNS litters), nor male-to-female ratio (1.3±0.1 in Control and 1.2±0.1 in PNS group) differed between groups.

4.11 Experimental Layout for *In Vivo* Study

Male offspring from the five control and six PNS litters were grown until the age of 2 months (Fig. 7.3.1) after which two animals from each litter were randomly selected for behavioural testing in the OF, elevated plus maze (EPM), novel object recognition (NOR) and SI tests (n=10 for both groups). A minimum three days were allowed between tests. At 4 months of age rats were split across three cohorts so that each cohort had 1–2 animals from each litter.

In cohort one, eight control and ten PNS animals were sacrificed for sampling and distal colon tissue samples were harvested for ex vivo transepithelial ion transport measurements (Ussing chambers study) and enteric nervous system (ENS) staining. In cohort two, eight control and nine PNS rats were used to assess hypothalamic-pituitary-adrenal (HPA) axis reactivity to acute stress. Following one week of a recovery period, these animals underwent a hot plate test to assess somatic pain sensitivity and also respiratory recordings using a whole body Plethysmography (WBP) protocol. In cohort three, eight control and nine PNS animals were analysed for blood pressure and HR responses to intestinal nociceptive stimulus (colorectal distension) and acute stress exposure (restraint stress). Female rats were excluded from the study to avoid potential effects of the oestrous cycle on functional outcomes.

4.12 Behavioural Testing

4.12.1 Open field Test

Animals were placed in the centre of open-topped circular arena (90 cm in diameter) with white walls, and behaviour was videotaped for 10 min. The following parameters were automatically scored in Ethovision XT 8.5 software (Noldus): total distance travelled (cm); average velocity of locomotion (cm/s), time spent in the centre of the arena (the inner 50cm wide circle); time spent on the periphery (the outer 20cm wide circle) and the fecal output of each animal.

4.12.2 Elevated Plus Maze

The EPM consisted of a cross-shaped platform raised 55 cm above the floor, with two open arms (51×10 cm) and two closed arms (51×10×41 cm) extending from a central platform (10×10 cm). Animals were placed in the centre of the maze facing an open arm, and behaviour was videotaped for 5 min in dimmed red light (80 lux). The following parameters were manually scored by a blinded researcher: number of open arm entries (all four paws were in an open arm); number of closed arm entries (all four paws in a closed arm); head dips (directed explorative behaviour) and stretches (directed explorative behaviour).

4.12.3 Novel Object Recognition Test

This test assesses the animal's ability to discriminate between familiar and novel objects. Prior to the test, animals were habituated to the open-topped, rectangular arena (42×62×36 cm) for 10 min daily for two consecutive days. In the acquisition phase of the test rats were exposed to two identical objects placed in the corners of the arena 10 cm from the nearest walls for 10 min. In the retention phase, delayed for 3 h, one object was substituted with a

novel object and animals were allowed to explore the objects for 10 min. To analyse the effect of stress on memory consolidation half of the animals in each group were randomly assigned to ARS exposure for 1 h immediately after the acquisition phase. Between acquisition and retention phases rats remained in their home cages. Test and habituation trials were performed under dimmed red light (80 lux at the level of arena). Objects and arena were cleaned with 70% ethanol after each trial to eliminate olfactory cues. The position of novel object was counter-balanced within and between both groups. Animal behaviour was recorded and object exploration was analysed manually by a blinded researcher. Exploratory behaviour was defined as orienting the nose towards the object at a distance <2cm, or direct contact with the object. Discrimination ratio was calculated as time spent exploring novel object compared to familiar object relative to the total time spent exploring all objects according to the formula: (t [novel] − t [familiar]) / (t [novel] + t [familiar]).

4.12.4 Social Interaction (SI) Test

To estimate SI activity each rat was tested with an unknown male partner from the opposite group for 10 min in an open-topped, rectangular arena (42×62×36 cm). Testing arena was unfamiliar for both animals. Animal behaviour was videotaped and active interaction between animals was manually scored by a blinded researcher. Only one animal from each pair was scored. The following behaviours were scored: time spent performing general sniffing; anogenital sniffing; grooming and rearing (in sec). Passive contact (sitting or lying with bodies in contact) was not included in this SI score.

4.12.5 Hot Plate Test

To estimate somatic pain sensitivity, animals were placed with all four paws on a hot plate heated to 52°C (Plantar Test Analgesia Meter, Stoelting, IL, US); and the time latency to first hind paw lick or jump was recorded. The cut-off time was set at 30 s to avoid tissue damage.

4.13 Plasma Corticosterone Response to ARS

Plasma CORT levels were analysed in control (n=8) and PNS (n=9) adult males from cohort two during acute stress exposure. Animals were placed in transparent restraint devices under bright light for 60 min. Blood samples (400 µl) were taken from the tail vein within first 2 min of stress exposure to determine baseline levels, and again at 30 and 60 min after the onset of restraint stress. Protocol was carried out between 9:00 AM and 12:00PM. Total plasma CORT levels were measured using an ELISA kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol.

4.14 Blood Pressure and Heart Rate Responses to Colorectal Distension (CRD) and Acute Restraint Stress (ARS)

Blood pressure and HR responses to CRD were analysed in control (n=8) and PNS (n=9) adult males from Cohort 3. On day 1, animals were anaesthetised with an i.p. injection of a ketamine (90 mg/kg)/ xylazine (10 mg/kg) cocktail; and polyethylene cannula (PE10 glue-sealed with PE50) was implanted into the abdominal aorta through the femoral artery for blood pressure recordings. The cannula was tunnelled subcutaneously with emergence at the level of the withers. Animals were given a single pre-operative analgesic treatment with carprofen (1 mg per animal s.c.) and post-operative injection of 0.9% NaCl (2 ml per animals s.c.) to prevent dehydration. Following the surgery animals were allowed to recover for 3 days in individually housed cages. Cannulas were flushed daily with sterile heparinized 0.9% NaCl (500 U/ml). Body weight was monitored daily and animals were visually examined for the presence of pain or distress (piloerection, hunched appearance, reduced mobility, ptosed eyes). Weight loss was negligible and never exceeded 2%; no signs of deterioration in general wellbeing was observed in post-surgery animals.

On day 5 animals were subjected to CRD protocol. Animals were transferred to the testing room 30 min prior to procedure to allow habituation, anaesthetised with 4% isoflurane and a latex 8cm length balloon was carefully inserted into the colorectal cavity. The balloon was connected to a Distender Series IIR™ Barostat (G&J Electronics Inc.) and catheter was connected to a miniature pressure transducer (1.6 F, Cath-SCI-1200, WPI, Sarasota, FL); the pressure signal was collected at a 1000 Hz sampling rate using Lab-Trax-4 data acquisition hardware and analysed in LabScribe software (WPI, Sarasota, FL). To achieve the stability of pulse wave throughout the experiment, a heparin solution in sterile 0.9% NaCl (50 U/ml) was infused in a contra-flow at a constant rate of 5 μl/min. Animals were allowed to recover for 15 min to allow stabilisation of haemodynamic parameters before beginning procedure. CRD was performed as described in (O'Mahony et al., 2012a). An ascending phasic distension paradigm from 0 mmHg to 80 mmHg over 8 minutes or until it was deemed to exceed acceptable pain levels. Blood pressure was continuously recorded for 15 min prior to CRD, 8 min of the CRD session and for a further 15 min during the recovery period.

Apart from cardiovascular response, noxious colonic distension evokes a visceromotor reflex consisting of both phasic and tonic contractions of abdominal musculature (Ness and Gebhart, 1988b). To further estimate visceral pain sensitivity, we analysed the frequency of phasic abdominal contractions, which is a validated measure of colonic sensitivity in rats

(O'Mahony et al., 2012b), by scoring the blood pressure spikes originating from each abdominal contraction/relaxation cycle. The average amplitude of pressure spikes was 49.4 ± 2.3 mmHg in control and 53.0 ± 1.2 mmHg in PNS group. Spikes with amplitude <20 mmHg were excluded from the analysis as they were most likely artefacts of laboured breathing and were separated out as a single cluster in hierarchical cluster analysis. The parameters of interest were (1) the total number of pressure spikes over the 8 min of CRD procedure and (2) the threshold pressure (mmHg) that evoked the first pressure spike.

On day 6, blood pressure and HR responses to ARS exposure were analysed in control (n=5) and PNS (n=7) rats. In three animals from control and two animals from the PNS group catheters were blocked (probably as a result of stress-induced activation of blood coagulation); and these rats were excluded from the study. Animals were transferred to the testing room 30 min prior to procedure to allow habituation. Blood recording was set up as described above. For ARS animals were placed in transparent restraint devices under bright light. Blood pressure was continuously recorded for 15 min prior to, during the 30 min of ARS and also during the 30 min recovery period in their home cage.

APsys, diastolic arterial pressure (APdias) and HR were derived from blood pressure recording on cycle-to-cycle basis. Pre-stressor periods were averaged as a whole; haemodynamic responses during CRD, ARS and recovery sessions were averaged per minute. Haemodynamic parameters were presented in absolute values, as well as changes from the pre-stressor values (Δ, delta).

4.15 Whole Body Plethysmography

Baseline breathing during normoxia and ventilatory responses to acute hypoxia (10% O_2) and hypercapnia (5% CO_2 in O_2) were assessed in control (n = 8) and PNS (n=9) adult males from Cohort 2. Respiratory recordings were obtained in unrestrained and freely-behaving rats using WBP (Buxco Research Systems, Wilmington, USA). Briefly, animals were placed into the experimental chambers, supplied with air or gas mixture at a constant flow rate of 2 L/min. The net pressure changes, as well as oscillations in air temperature and humidity, occurring due to the inspiration/expiration-induced air flows, were continuously recorded and analysed using specialised software (BioSystem, XA for windows, Buxco Electronics). Throughout the protocol animal behaviour was continuously monitored; only stable periods of quiet rest were included in the data analysis.

Rats were allowed to habituate to the experimental chambers for approximately 30-60 min prior to recording. When ventilatory parameters stabilized, baseline breathing in room air flow-through (normoxia) was recorded for 30 min. The gas supply to the chambers was then switched to 10% O_2 for 20 min (hypoxia). Following a recovery period under normoxia a second normoxic period lasting 10 min was recorded. Finally, a 10 min hypercapnic (5% CO_2 in O_2) challenge was administered.

The values for minute ventilation (V_E, ml/min), tidal volume (V_T, ml) and breathing frequency (f, breaths per minute) were derived from the plethysmograph recordings. Volume based parameters, i.e. V_E and V_T were normalized per 100g body weight. Body weight values were similar in both groups being 425±10 g in control and 429±9 g in PNS rats. Each normoxic period was averaged as a whole; breathing parameters during the gas challenges were averaged initially per minute (to estimate the time course of the response) and then per challenge, i.e. over the entire 20 minutes of hypoxia or 10 minutes of hypercapnia. Ventilatory responses were presented in absolute values, as well as changes from the preceding normoxic values (Δ, delta).

4.16 Transepithelial Ion Transport (Short-Circuit Current) Measurements in Ussing Chambers

Distal colon (a distal 1.5 cm segment adjacent to rectum) was dissected and placed in chilled Krebs solution prior to seromuscular stripping. Tissue samples were taken from control (n = 6) and PNS (n = 6) adult males from Cohort 1. Seromuscular stripping was carried out by blunt dissection and both the longitudinal and circular muscle layers as well as the myenteric plexus were removed. The resulting mucosal-submucosal segments (two from each animal) were mounted in Ussing chambers with an exposed tissue area of 0.12 cm^2. Tissue samples were bathed with 5 ml of Krebs solution on each side of the specimen, infused with 95% O_2 and 5% CO_2, and maintained at 37°C. Krebs buffer was prepared as follows, 1.2mM NaH_2PO_4, 117mM NaCl, 4.8mM KCl, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, 11mM $CaCl_2$ and 10mM glucose, pH 7.4. Tissues were voltage clamped to zero using an automatic voltage clamp (DVC-1000/EVC-4000, WPI, Sarasota, US). The short-circuit current (I_{sc}) measurements were continuously recorded with a 20 Hz sampling rate on Lab-Trax-4 data acquisition hardware and analysed in LabScribe software (WPI, Sarasota, FL). After an equilibration period of 60 min, tissue samples were treated with either norepinephrine (NE) or bethanechol (BCH) added to the serosal reservoir of the Ussing chamber in increasing concentration every 15 minutes. Serial dilutions of drugs (both from Sigma-Aldrich, Ireland)

were prepared in dH_2O; NE was tested in the 0.005-50 μM range and Bch in the 0.05-10 μM range. Transepithelial resistance (TER) was assessed by discharging a 2mV pulse across the tissue and measuring the peak change in I_{sc}.

The baseline I_{sc} value (in $\mu A/cm^2$) in each sample was averaged over the 5 min interval in the end of the equilibration period, prior to drug application. I_{sc} response to each dose was averaged per 30 s interval (to estimate the time course of the response) and presented as changes from the baseline value expressed in % (Δ%, delta %). The maximal change of I_{sc} was taken as a peak response value. TER was calculated using Ohm's law and expressed in $\Omega \cdot cm^2$.

4.17 Distal colon innervation in whole mount muscular preparations

Distal colon (1.5 cm length segment) was dissected and placed in pre-chilled Krebs buffer. Tissue samples were taken in control (n = 6) and PNS (n = 6) adult males from Cohort 1. Intestinal segments were opened along the mesenteric border, washed with Krebs and pinned to the bottom of Sylgard-coated dishes with the mucosal side down. The seromuscular layer consisting of longitudinal muscle layers, circular muscle layers and myenteric plexus between them, was gently peeled away, fixed in 4% paraformaldehyde in PBS (pH= 7.4) for 1 h at RT, washed with PBS, cut into 2 squares and processed for immunofluorescent staining. Tissue preparations were incubated in 50 mM NH_4Cl quenching solution for 1 h, followed by blocking and permeabilisation in 5% horse serum in PBS containing 0.3% Triton X-100 2 h at RT. Tissues were then incubated with primary antibodies overnight at +4°C and with secondary antibodies for 3 h at RT. Antibodies were diluted in 0.1% TBST with 1% horse serum. Nuclei were stained with 3 μM DAPI for 1 h at RT. Primary antibodies were: mouse anti-β-III tubulin (1:300) (Millipore, MAB1637); rabbit anti-TH (1:300) (Abcam, ab41528); goat anti-choline acetyltransferase (ChAT) (1:100) (Millipore, AB144P). Secondary antibodies were goat anti-mouse IgG conjugated with Alexa 594, goat anti-rabbit and donkey anti-goat IgG conjugated with Alexa 488 (all from Invitrogen, 1:500 dilution). For each animal one tissue preparation was double stained for β-III tubulin and TH, the other was stained only for ChAT.

Fluorescent imaging of muscular preparations was performed using an Olympus FV1000 confocal laser scanning microscope. On each tissue specimen six visual fields (1270x1270 μM) were randomly chosen and acquired using a 10x objective. For ChAT staining all confocal planes were combined in a single z-stack image using FV1000 Viewer software

(Olympus). Imaging of β-III tubulin- and TH-expressing fibres revealed an impressively dense neuronal network in circular muscle layer masking the rest of the terminals. For this reason confocal planes were z-stacked separately for circular muscle layer and myenteric plexus with underlying longitudinal muscles. The resulting images were analysed using Adobe Photoshop CS5.

The density of innervation in confocal images was analysed using a counting grid to assess the total number of intersections through the grid. This was performed using a superimposed grid measuring 200 μm x 200 μm with 40 μm intervals between dividing lines. The grid was randomly assigned to four different fields within each image, and the total numbers of intersections of nerve fibres through the grid were counted excluding the uppermost and far right border lines. All images were analysed at a predetermined magnification setting. The terminal length (in mm per 1 mm^2 of specimen), as a readout of innervation density, was calculated using the formula N x T x $\pi/2$ where: N is the total number of points of intersection per image and T being the distance between each line in the grid. For ChAT staining the terminal length values were averaged per visual field. For β-III tubulin and TH staining the terminal length was analysed separately in both the circular muscle layer and in the myenteric plexus; the values were summated and averaged per visual field. The density of neural ganglia in the myenteric plexus was quantified as the number of ganglia per visual field.

4.18 Statistical analysis of Prenatal Stress Study

Data are presented as mean ± SEM. Pre-stressor values of plasma corticosterone, APsys, APdias, HR, V_E, V_T, f, as well as behavioural scorings, visceral pain scoring, TER, I_{sc} peak responses, number of ganglia and nerve terminal length were compared between groups with independent Student's t-test. Significance of stress-induced changes in CORT levels within each group were analysed with paired samples t-test. Time courses of blood pressure, HR, V_E, V_T and f changes in response to stress challenges and during recovery periods, as well as I_{sc} changes to NE stimulation, were analysed in factorial ANOVA with PNS and time as two independent predictor factors; further between-group comparisons for each time point were done with independent Student's t-test. Averaged ventilatory responses to hypoxic and hypercapnic challenges were analysed in mixed design ANOVA with PNS as a between-group factor and gas mixture as a repeated-measured within-group factor. A p value < 0.05 was deemed significant in all cases.

5. Exposure of foetal NPCs to IL-1β impairs their proliferation and alters their differentiation – a role for maternal inflammation?

5.0 Abstract

During pregnancy, activation of the maternal immune system results in inflammation in the foetal nervous system. The causative agents are proinflammatory cytokines such as interleukin-1β (IL-1β), produced by the foetus. Here we examined the effect of IL-1β on the proliferation and differentiation of NPCs to better understand its potential effects on the developing brain.

We found that the IL-1β receptor, IL-1R1, is expressed in the ventral mesencephalon (VM) of the developing brain. Furthermore, IL-1R1 is expressed on nestin-positive NPCs. IL-1β treatment reduced the numbers of proliferating neural precursor cells (NPCs), an effect prevented by the IL-1R1 receptor antagonist. LDH and MTT assays, and western blot analysis for cleaved Caspase-3 and PARP, confirmed that this was not due to an increase in cell death but rather an induction of differentiation. To further study the effects of IL-1β on cell fate determination, we differentiated NPCs in the presence and absence of IL-1β. Il-1β promoted gliogenesis and inhibited neurogenesis, an effect that required p38-MAPK kinase signalling.

In summary, these data show that exposure of NPCs to IL-1β affects their development. This necessitates an examination of the consequences that maternal immune system activation during pregnancy has on the cellular architecture of the developing brain.

5.1 Introduction

During pregnancy, it has been proposed that prenatal inflammatory events can adversely contribute to an individual's susceptibility to an adult onset neurological disease (Nelson and Willoughby, 2000; Bell and Hallenbeck, 2002; Huleihel et al., 2004; Stolp and Dziegielewska, 2009). One such disorder is Parkinson's disease (PD) which results from the degeneration of dopaminergic (DA) neurons of the substantia nigra (SN) in the midbrain. During development, these cells differentiate from progenitor cells in the VM, which becomes the midbrain in the adult. Inflammation is a major contributing factor in the pathogenesis of PD, and proinflammatory cytokines such as IL-1β and TNF have been shown to be elevated in the brains of PD patients (Mogi et al., 1994b; Mogi et al., 1994a). Furthermore, microglial activation is a common post mortem finding in the SN of patients with PD (McGeer et al. 1988). As such the possibility that a prenatal inflammatory event can contribute to a life-long susceptibility to inflammation thus rendering an individual more susceptible to PD is being explored (Ling et al., 2004).

As the effects of maternal intrauterine infection results in the production of IL-1β production by the fetal brain (Zaretsky et al 2004; Aaltonen et al 2005), it is crucial to understand the direct effects of these cytokines individually on the developing VM. Furthermore, as maternal intrauterine infection can affect the expression of genes involved in the induction of midbrain DA neurons (Meyer et al. 2008), it is also possible that it can affect a much more fundamental aspect of neuronal differentiation and regulate cell fate choice from progenitor cells and thus alter the cellular architecture. To begin to address these questions, we examined the direct effects of IL-1β on NPCs isolated from the developing rat VM. These data show that IL-1β inhibits NPC proliferation by promoting their differentiation towards a glial lineage at the expense of neuronal differentiation. These effects are mediated by IL-1R1 via p38 MAPK kinase signalling. This suggests that maternal intrauterine infection leading to increased levels of IL-1β in the foetal VM, has the potential to alter the cellular architecture of the developing nigrostriatal system. This lends further support to the hypothesis that MIA during *in utero* development has the potential to affect the developing brain and for the first time shows the molecular basis of this effect in NPCs isolated from the developing VM.

5.2 Results

5.2.1 Culture Composition of Rat E14 VM

We set out to establish whether IL-1β and its receptors were expressed in the developing rat VM. Firstly, we characterised the type of cells present in cultures of freshly dissociated E14 rat VM tissue to determine the cell type most likely to express IL-1β and/or its receptors. To do this, E14 rat VM cells were plated as single cells and fixed and stained for a variety of precursor, neuronal and glial cell markers after 2 h *in vitro* (Fig. 5.3.1A-F). We found that 94.2% and 83.5% of cells present in the VM at this stage of development expressed the neuronal markers DCX and β-III tubulin respectively (Fig. 5.3.1A, B). Of these cells, 3.7% were DA neurons as assessed by the expression of TH, the enzyme involved in the rate limiting step in DA biosynthesis (Fig. 5.3.1D). GFAP-positive cells comprised less than 1% of the total number of cells in these cultures, while approximately 35% of cells expressed the neural precursor marker, nestin (Fig. 5.3.1E). RT-PCR for a variety of DA genes was used to confirm the accuracy of the dissections.

We next carried out RT-PCR for IL-1β, IL-1R1 and IL-1R2 using RNA extracted from freshly dissected E14 rat VM tissue. We found clear expression of IL-1R1 but not IL-1R2 in the E14 rat VM (Fig. 5.3.2B). Western blotting was also performed on whole tissue lysates of freshly dissected E14 VM to show protein expression of IL-1R1 *in vivo* (Fig. 5.3.2C). To determine which cells expressed the IL-1R1 receptor, we carried out immunocytochemistry using IL-1R1 specific antibodies. IL-1R1 was strongly expressed in cells that expressed nestin in these cultures (Fig. 5.3.2A), suggesting that IL-1β, may exert an effect on the proliferation and/or differentiation of these cells.

5.2.2 Interleukin 1 Receptor Expression

To begin to examine the potential effects of IL-1β on nestin-positive cells, we grew cultures of NPCs from the E14 rat VM as neurospheres. When proliferated in this manner for 7 DIV, there was a significant increase in the percentage of nestin-positive cells from approximately 30% to 90% of the total cell number ($p < 0.001$; student's t-test) (Fig. 5.3.3A, B). As IL-1R1 was predominantly expressed on nestin-positive cells in freshly dissociated cultures from the E14 rat VM, we examined its expression level using RT-PCR in neurospheres at 2, 4 and 7 DIV. There was a dramatic increase in IL-1R1 expression which reached a peak at 4 DIV (Fig. 5.3.5A). There was no induction of IL-1R2 at any of the time points studied. Interestingly there was a transient induction of IL-1β at 2 DIV, but its expression was lost

following this stage (Fig. 5.3.5A). To confirm that the increase in transcripts for IL-1R1, leads to an increase in the expression of the protein, we used immunocytochemistry with specific anti-IL-1R1 antibodies to compare the presence of IL-1RI in nestin-positive cells at 0 DIV and 3 DIV. These cells were plated at the same time and subsequently imaged in the same manner. The relative expression of IL-1R1 was compared between both groups using densitometry which showed that there was approximately a two-fold increase in IL-1R1 protein expression levels on nestin-positive cells between 0 DIV and 3 DIV (Fig. 5.3.4A, B).

5.2.3 Effects of IL-1β on NPC Proliferation

The strong expression of IL-1R1 on nestin-positive cells, suggested that IL-1β may influence the proliferation, survival and/or differentiation of proliferating NPCs. To begin to determine if IL-1β affected the proliferation of these cells, we measured the neurosphere diameter and volume of both control neurospheres and of those that had been exposed to IL-1β (10ng/ml) at four separate time points; 4 h after seeding, 2 DIV, 4 DIV and 7 DIV (Fig. 5.3.6 A to C). At 4 DIV there was a significant decrease in both neurosphere diameter (Fig. 5.3.6D) and volume (Fig. 5.3.6E) of IL-1β treated cultures when compared to controls ($p<0.05$; student's t-test). This observed effect became more pronounced by 7 DIV ($p<0.001$; student's t-test) (Fig. 5.3.6A to E).

5.2.4 IL-1β Induces Differentiation Not Cell Death of Proliferating NPCs

The reduction in neurosphere volume could be as a result of IL-1β inducing cell death, halting cell proliferation, and/or inducing differentiation. To investigate these possibilities, we firstly performed western blot analysis for the pro-apoptotic factors, cleaved caspase-3 and PARP using protein extracts from control and IL-1β treated neurospheres (Fig. 5.3.8A-C). Interestingly, there was no increase in the levels of these proteins as a result of IL-1β treatment, which indicates that the reduction in IL-1β treated neurosphere volume is not as a result of increased cell death. To confirm this we carried out LDH assays on medium taken from control and IL-1β treated neurospheres at 2, 4 and 7 DIV. In agreement with the western blot findings, there were no significant differences in the levels of lactate dehydrogenase (LDH) in the culture medium between control and IL-1β treated cultures at any time points (Fig. 5.3.8D). An MTT assay was performed to assess the rate of cellular respiration between control and IL-1β treated neurosphere cultures. IL-1β treatment induced a significant decrease in the MTT absorbance ($p<0.05$; student's t-test) (Fig. 5.3.8E). As a reduction in MTT absorbance could indicate a reduction in the metabolic rate of IL-1β treated cells, it is possible that IL-1β induced differentiation of the proliferating nestin positive cells. In

agreement with this hypothesis, we found a significant reduction in the percentage of nestin-positive cells ($p<0.001$; student's t-test) in IL-1β treated cultures when compared to control at 7 DIV (Fig. 5.3.7C). Furthermore, nestin-positive cells plated from IL-1β treated neurospheres appeared to be more differentiated than those from control neurospheres (Fig. 5.3.7A). When we quantified these morphological changes, we found that nestin-positive cells at 7 DIV from IL-1β treated neurospheres had a significant increase in the complexity of their somal architecture, as assessed by measuring the distance from the nucleus to the end of the longest somal process ($p<0.001$; student's t-test) (Fig. 5.3.7B).

5.2.5 IL-1β Inhibits Proliferation by Activating p38 MAP Kinase Signalling in NPCs

We next identified the molecular basis of the effects of IL-1β on NPCs. IL-1β is known to activate a variety of intracellular signalling pathways, including, p38 and NF-κB. To determine the effect of IL-1β on intracellular signalling in NPCs cells that had been proliferated for 7 DIV were dissociated and plated down as a single cell layer prior to stimulation with IL-1β. Whole cell lysates were then analysed by western blot. We found that IL-1β induced the phosphorylation of p38 at 5 min that peaked at 5 minutes and declined thereafter (Fig. 5.3.11A). Subsequently we performed immunohistochemistry for phospho-p38 on nestin-positive NPCs that had been treated for either 5 or 60 min to confirm our findings by immunohistochemistry (Fig 5.3.11B, C). We found there to be no activation of the NF-κB pathway by IL-1β in these NPCs (data not shown).

The compound SB203580 is a selective inhibitor of p38 MAPK (Cuenda et al., 1995). Treatment of NPC cultures with SB203580 fully prevented the IL-1β induced reduction in neurosphere diameter (Fig. 5.3.11D, E), indicating that p38 activation mediates the effects of IL-1β on NPCs from this region of the developing brain.

5.2.6 Effects of IL-1β on Cell Fate Specification

As IL-1β treatment of nestin-positive cells promoted their differentiation (Fig. 5.3.6), we next assessed the fate specification of these cells. Cells from the E14 rat VM were proliferated as neurospheres for 7 DIV with or without IL-1β (10ng/ml). These cells were then plated and allowed to differentiate for another 7 DIV in medium with or without IL-1β, before they were fixed and immunocytochemically stained for neuronal (β-III tubulin and TH), oligodendroglial (MBP) and astrocytic (GFAP) markers (Fig. 5.3.9). There was a significant increase in the percentage of GFAP-positive cells ($p<0.001$; student's t-test) (Fig. 5.3.9A) and a significant decrease in the percentage of nestin-positive cells ($p<0.01$; student's t-test)

(data not shown) in cells that had been treated with IL-1β during proliferation. There was no effect on the percentage of MBP or β-III tubulin-positive cells (Fig. 5.3.9B, C).

IL-1β treatment during differentiation significantly increased the percentage of GFAP-positive cells (p<0.001; student's t-test) (Fig. 5.3.9J) and significantly decreased the percentage of β-III tubulin-positive cells (p<0.05; student's t-test) (Fig. 5.3.9K) but had no effect on the percentage of MBP (Fig. 5.3.9L) or nestin-positive cells (data not shown) at 7DIV, an effect that was not seen at 2DIV (data not shown). Co-treatment of IL-1β treated cultures with IL1ra during differentiation prevented the significant increase in GFAP-positive cells observed in cultures treated with IL-1β only (Fig. 5.3.10D, E).

To confirm that the observed reduction in neurosphere size was a specific effect of IL-1β, we used IL1ra, the endogenous IL1R1 receptor antagonist, to examine whether this blocked the previously observed effects. We found that IL1ra (20 ng/ml) when added in conjunction with IL-1β prevented the IL-1β induced reduction in neurosphere diameter and volume (Fig. 5.4.10 A-C).

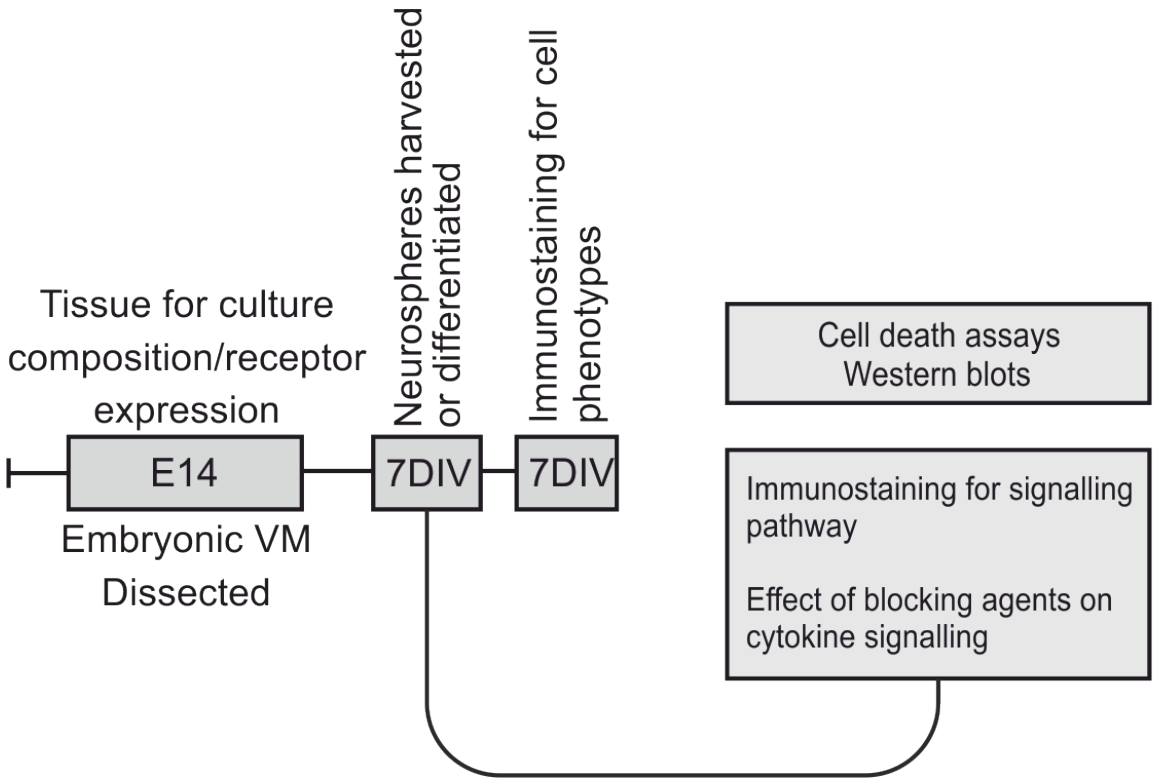


Figure 3 Methods Schematic

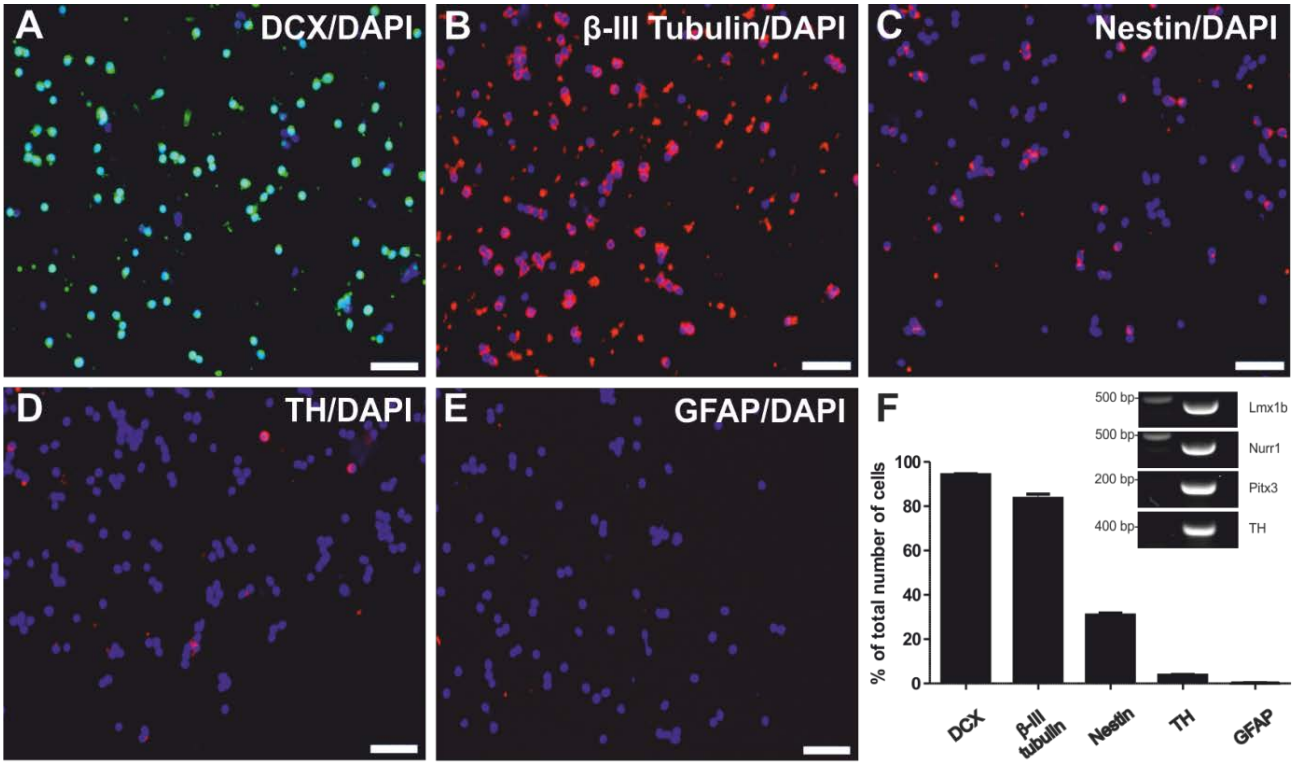


Figure 5.3.1: Culture composition of E14 VM tissue at 0DIV.

(A-E) Immunocytochemical staining of dissociated fresh tissue isolated from E14 VM for the neuronal markers DCX and β-III tubulin, the NPC marker nestin, the glial marker GFAP and the dopaminergic marker TH. (F) Graphical representation of the percentage of each cell marker taken as a percentage of total cells. Scale bar = 50 μm.

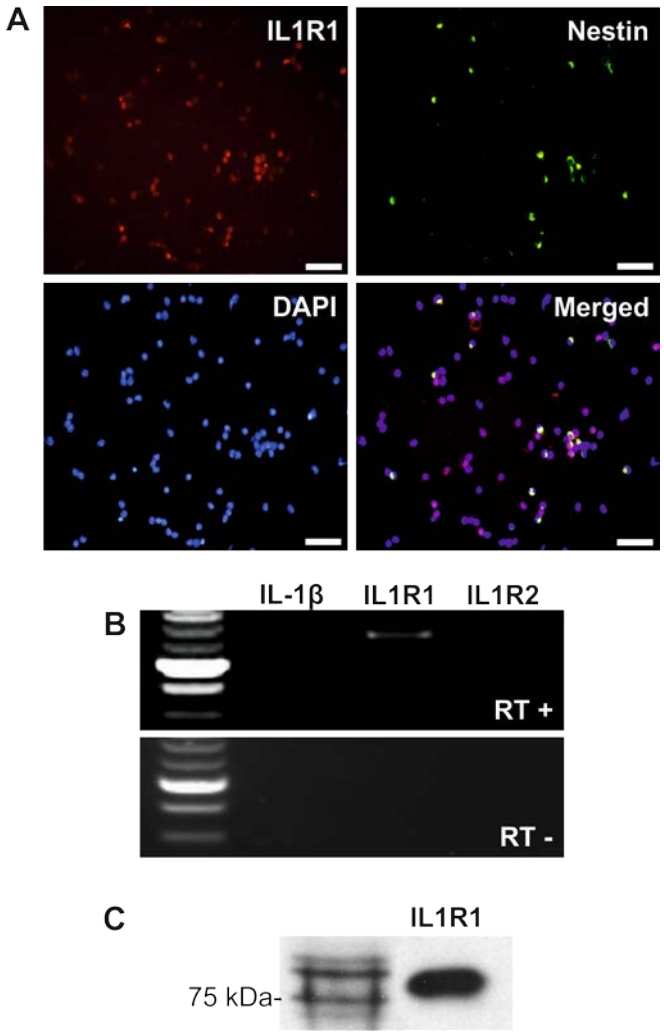


Figure 5.3.2: E14 VM NPCs express the receptor IL1R1 at both protein and mRNA levels.

(A) Immunocytochemical staining of E14 VM NPCs demonstrating coexpression of IL1R1 on nestin positive cells. (B) RT-PCR of E14 VM NPCs showing expression of IL1R1 but no expression of IL1R2. (C) Western blot of E14 VM tissue showing the presence of IL1R1. Scale bar = 50 μm

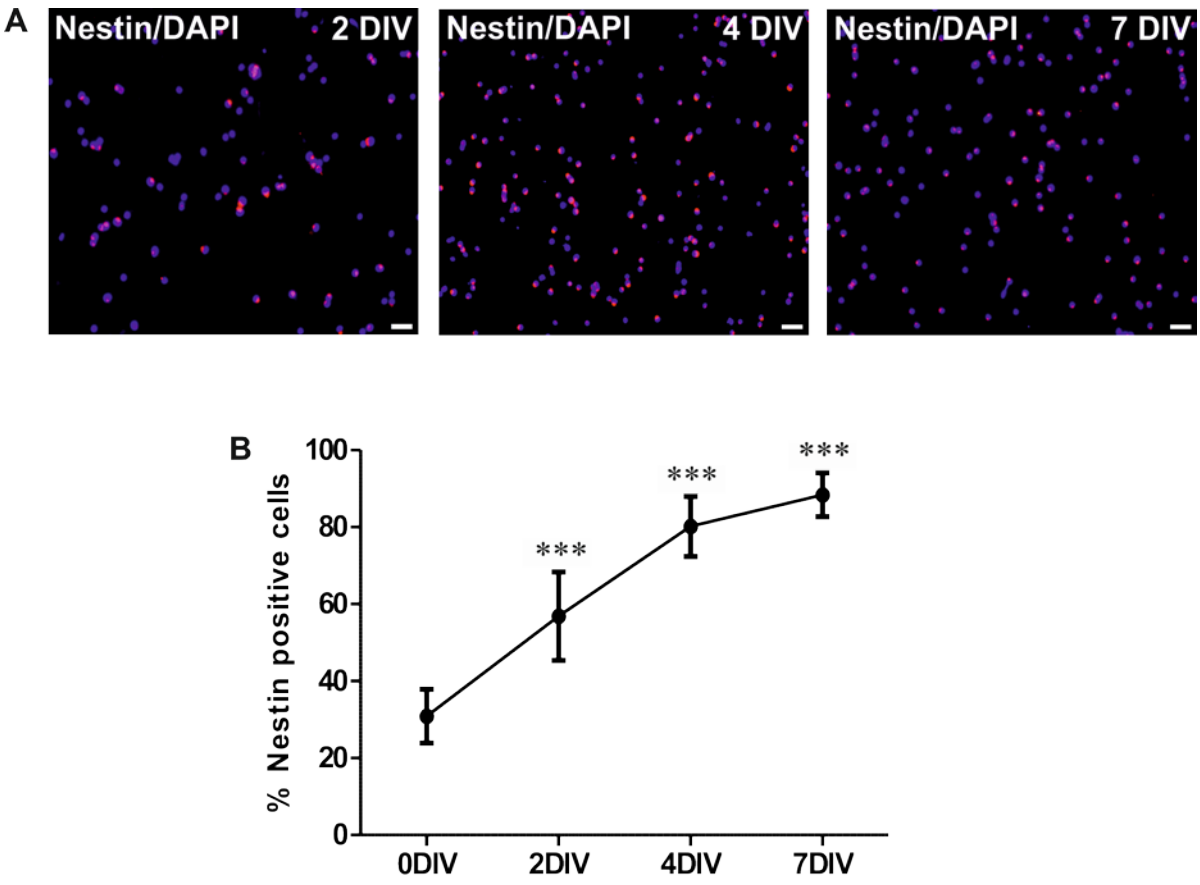


Figure 5.3.3: The percentage of nestin positive NPCs in culture increases with number of DIV.

(A) Immunocytochemical staining of dissociated neurospheres at 2, 4 and 7DIV for the NPC marker nestin. (B) Graphical representation showing an increase in the percentage of nestin positive cells in culture with increasing time. ***$p < 0.001$ compared to 0DIV; $n = 3$ (mean ± SEM). Scale bar = 50 μm

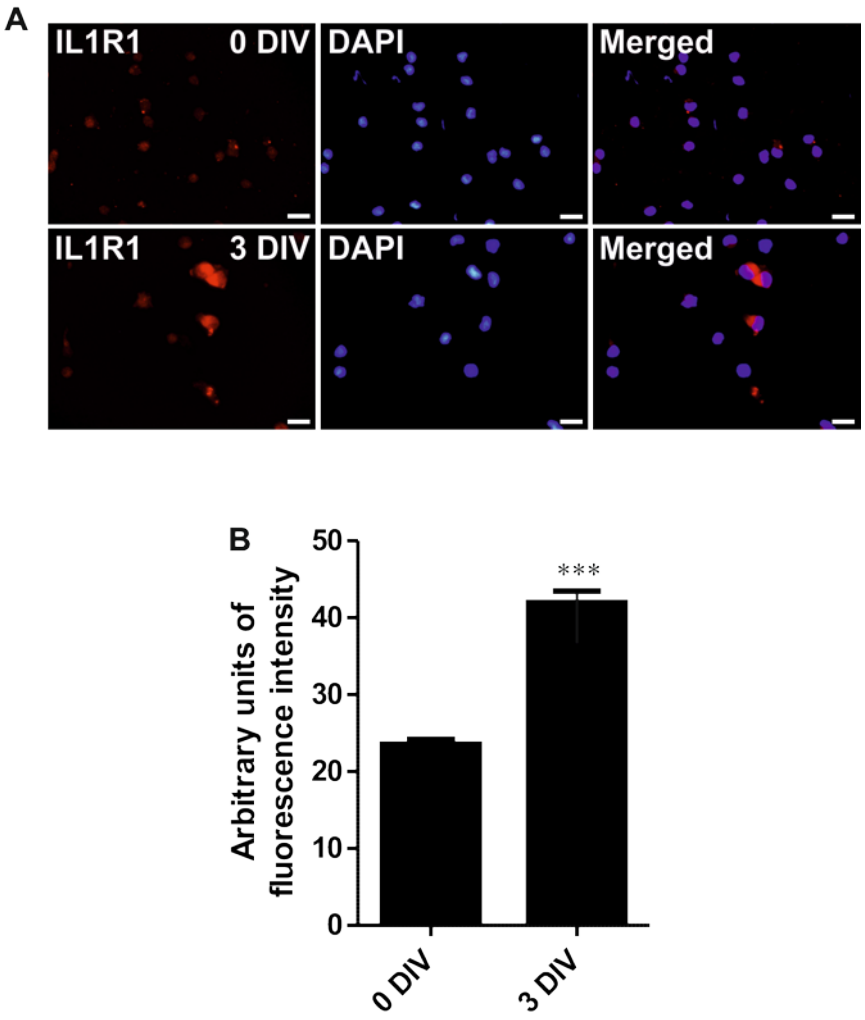


Figure 5.3.4: Expression of IL1R1 is upregulated in NPC cultures in the absence of cytokine treatment.

(A) NPCs at 0 and 3 DIV stained for IL-1R1. (B) Quantification by densitometry of protein levels of IL-1R1 at 0 and 3 DIV in expanded NPCs. ***$p < 0.001$ compared to 0DIV; $n = 3$ (mean ± SEM). Scale bar = 50 µm

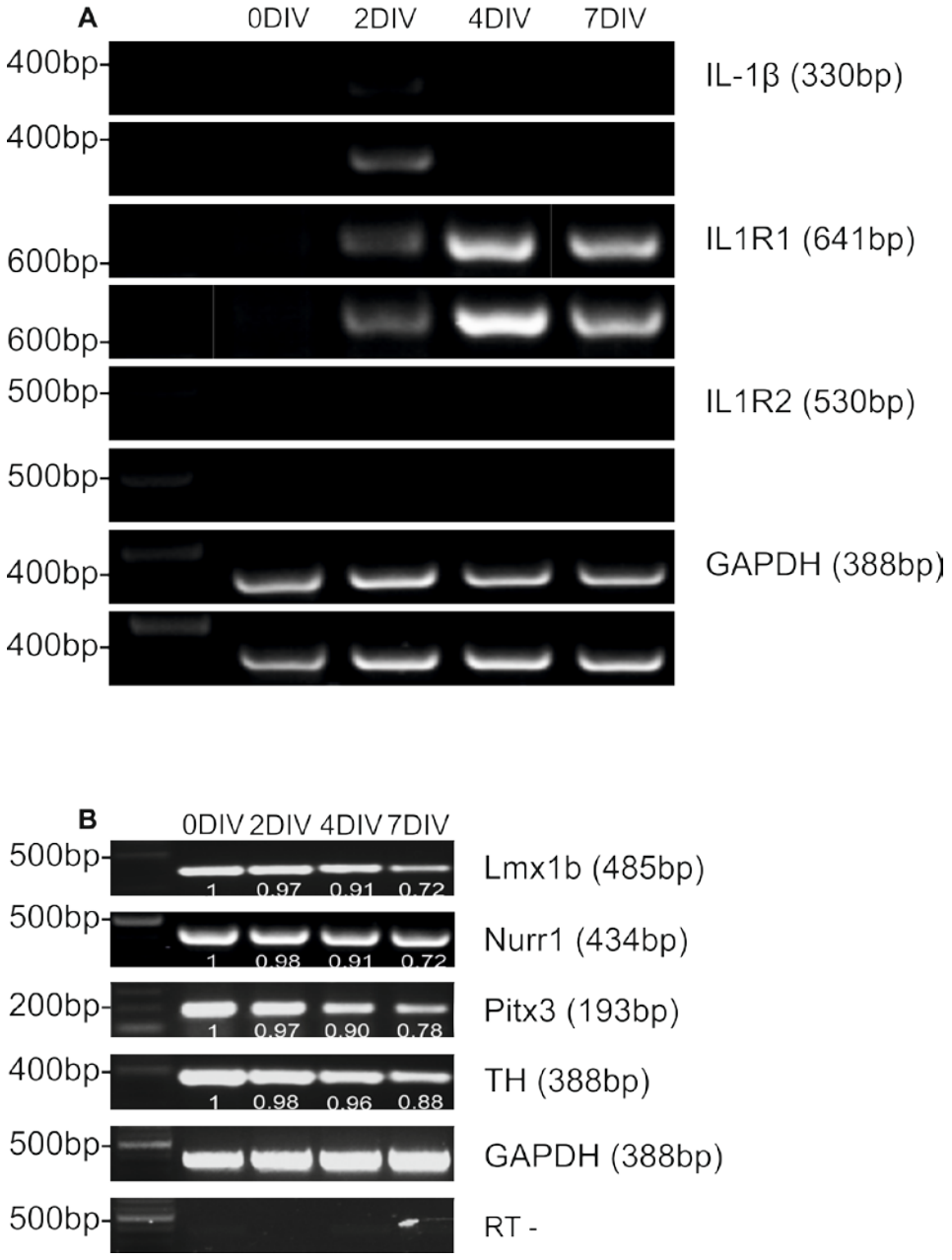


Figure 5.3.5: IL-1β treatment of NPCs induces a transitory increase in the expression of both IL-1β and IL1R1.

(A) RT-PCR for IL-1β, IL1R1 and IL1R2 using control and treated NPC samples at 0, 2, 4 and 7DIV. IL1R2 was not found to be expressed at any stage in either control or treated groups with IL1R1 expression peaking at 4DIV. An upregulation of IL-1β was seen in treated samples at 2DIV. (B) RT-PCR for the DAergic genes Lmx1b, Nurr1, Pitx3 and TH from fresh tissue and expanded NPCs demonstrating that expression of these genes decreases with increasing time spent in culture.

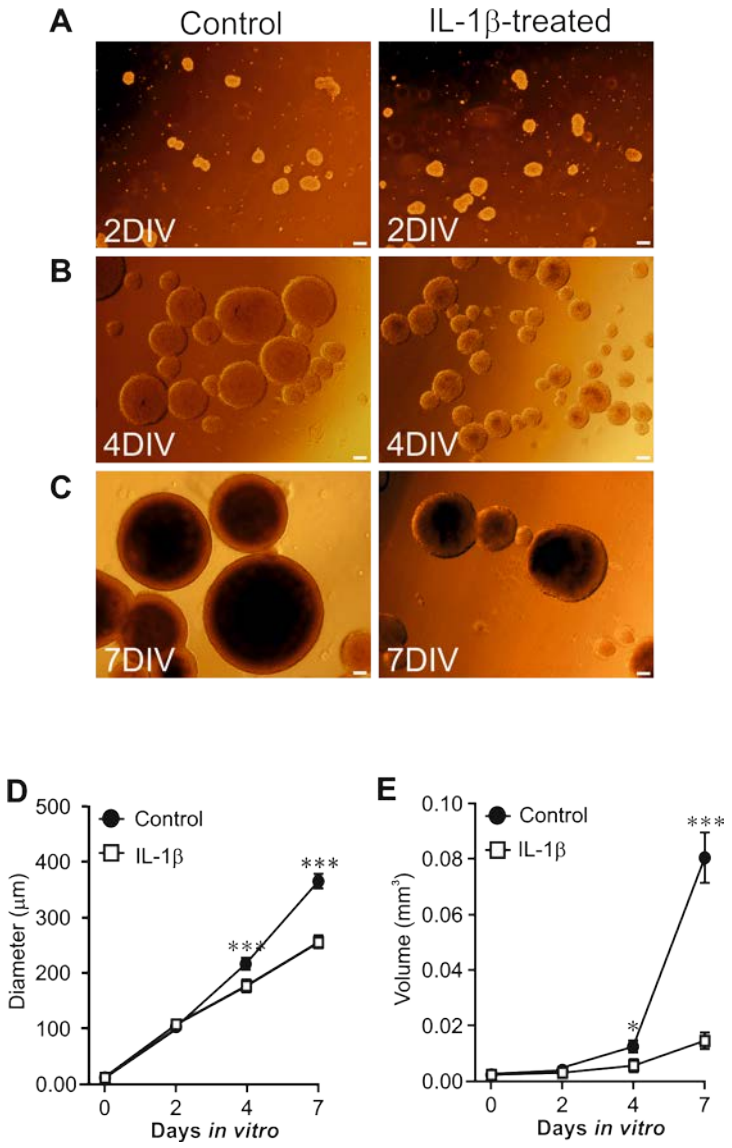


Figure 5.3.6: IL-1β treatment impedes proliferation of E14 VM NPCs.

(A-C) NPC spheres at 2, 4 and 7DIV showed a decrease in size of IL-1β treated spheres in relation to control. (D, E) Quantitative data showing the significant decrease in sphere diameter and volume at 4 and 7 DIV between control and IL-1β treated NPCs. *$p < 0.05$; ***$p < 0.001$ compared to 0DIV; n = 3 (mean ± SEM). Scale bar = 100 µm.

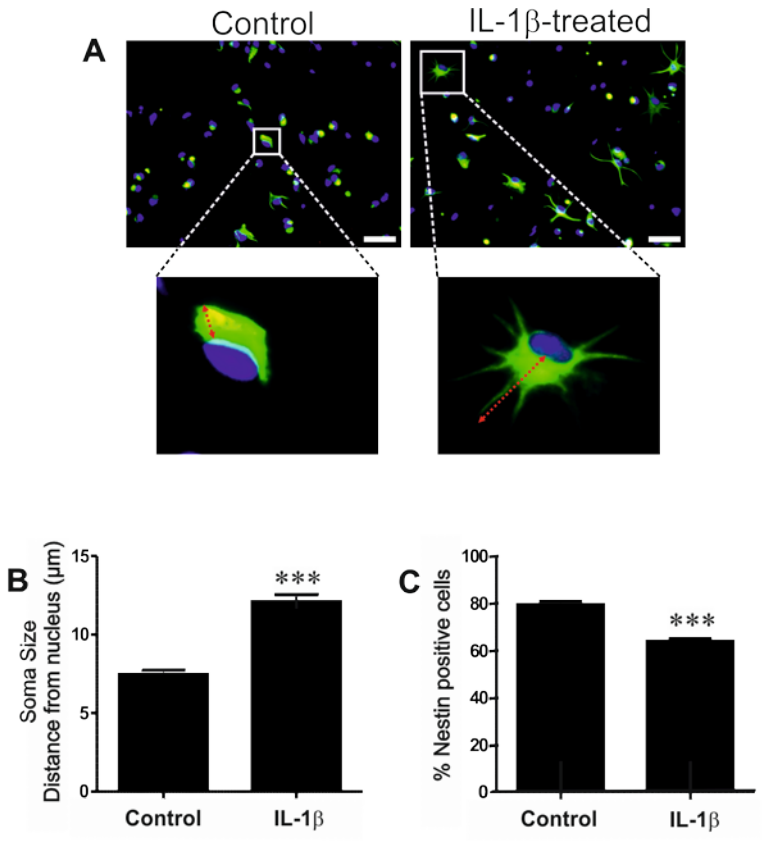


Figure 5.3.7: IL-1β treatment causes a reduction in the percentage of nestin positive cells at 7DIV and induces glial-like morphology in NPCs.

(A) Control and treated NPCs at 7DIV stained for nestin showed a decrease in the percentage of nestin$^+$ cells in IL-1β treated NPCs and also a change in morphology. (B) Quantitative data showing a significant increase in the mean somal outgrowth from the nucleus in IL-1β treated nestin$^+$ NPCs. ***$p < 0.001$; $n = 3$ (mean ± SEM). (C) Quantitative data showing a significant decrease in the percentage of nestin positive cells in IL-1β treated NPCs compared with control. ***$p < 0.001$; $n = 3$ (mean ± SEM). Scale bar = 50 μm.

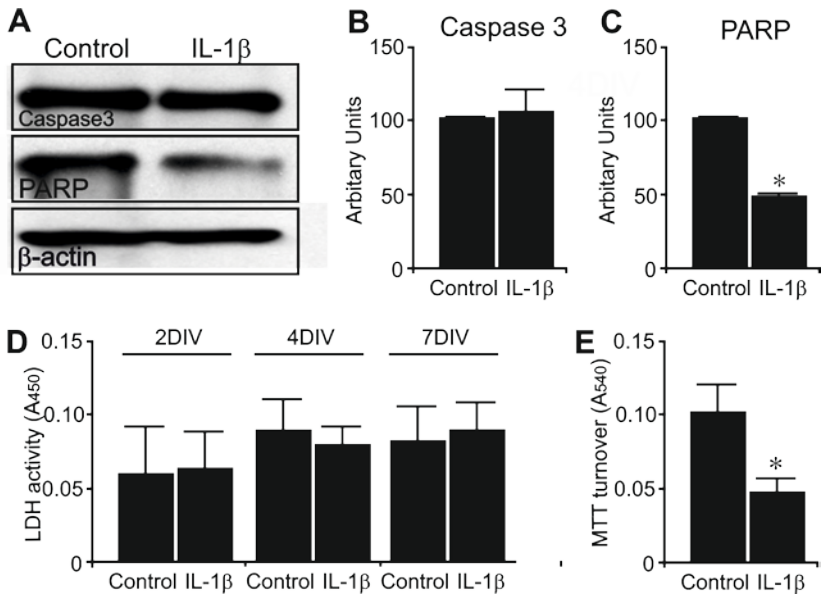


Figure 5.3.8: IL-1β does not induce cell death in proliferating E14 VM NPCs.

(A) Western blot analysis showed that treatment of NPCs with IL-1β did not induce apoptosis in these cells as levels of cleaved caspase 3 or PARP were not elevated compared with control cultures, β-actin was used as the internal control. (B, C) Semi-quantitative analysis of western blots for caspase 3 and PARP. *$p < 0.05$; $n = 3$ (mean ± SEM). (D) Graphical representation of results from LDH assay at 2, 4 and 6 DIV from proliferating NPCs. (E) MTT assay on NPCs at 7 DIV showed a decreased rate of respiration in IL-1β treated cells compared to control cells. *$p < 0.05$; $n = 3$ (mean ± SEM).

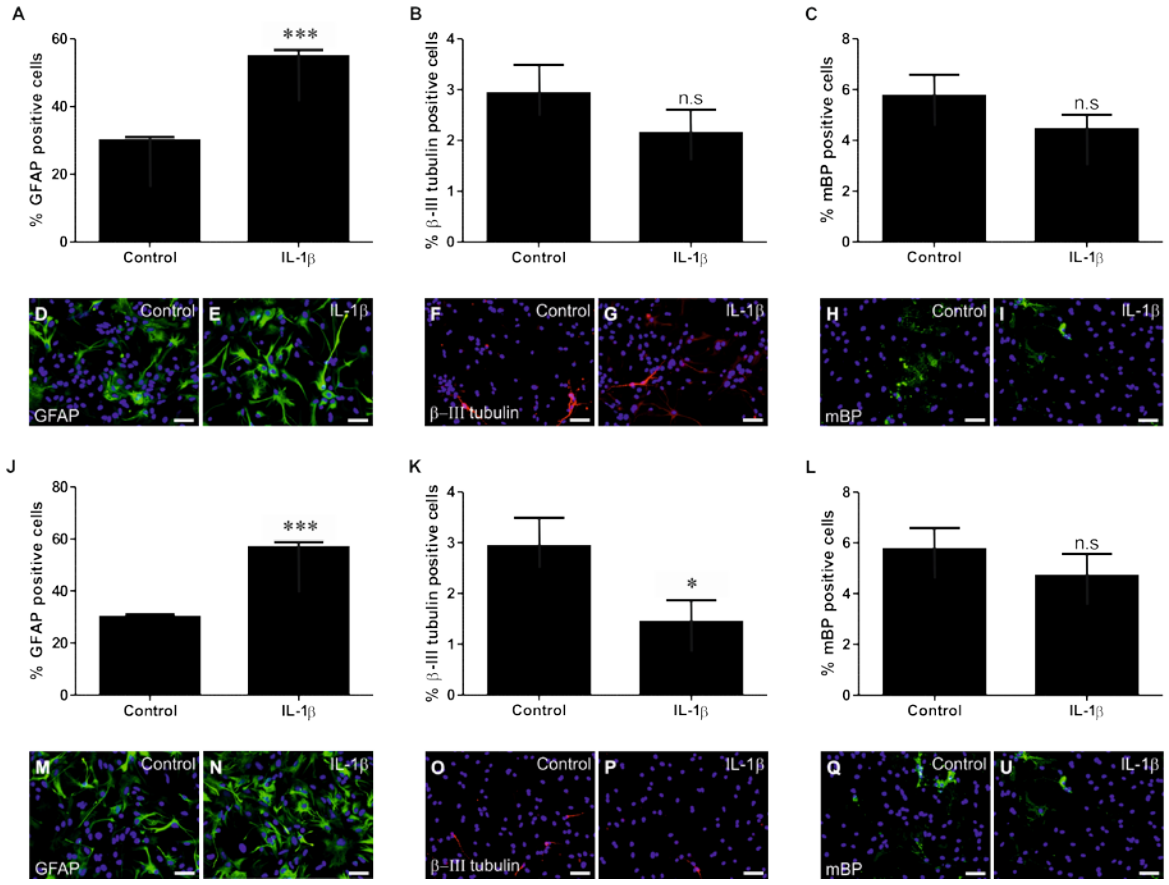


Figure 5.3. 9: Effects of IL-1b on the lineage fate of E14 rat VM NPCs.

(A-C) Quantitative data showing a significant increase in the percentage of $GFAP^+$ cells (A) but no difference in the percentage of β-III $tubulin^+$ (B) or MBP^+ (C) cells when treated with 10 ng/ml IL-1β for 7 DIV during proliferation. n.s $p>0.05$; *** $p < 0.001$; n=3 (mean ± SEM). (D-I) Representative photomicrographs of cells stained for GFAP (D, E), β-III tubulin (F, G) and MBP (H, I). Scale bar, 50 µm. (J-L) Quantitative data showing a significant increase in the percentage of $GFAP^+$ cells (J) but a significant decrease in β-III $tubulin^+$ cells (K) and no difference in the percentage of MBP^+ (L) cells when treated with 10 ng/ml IL-1β for 7 DIV during differentiation. n.s $p > 0.05$; *** $p < 0.001$; n=3 (mean ± SEM). (M-U) Representative photomicrographs of cells stained for GFAP (M, N), β-III tubulin (O, P) and MBP (Q, U). Scale bar, 50 µm.

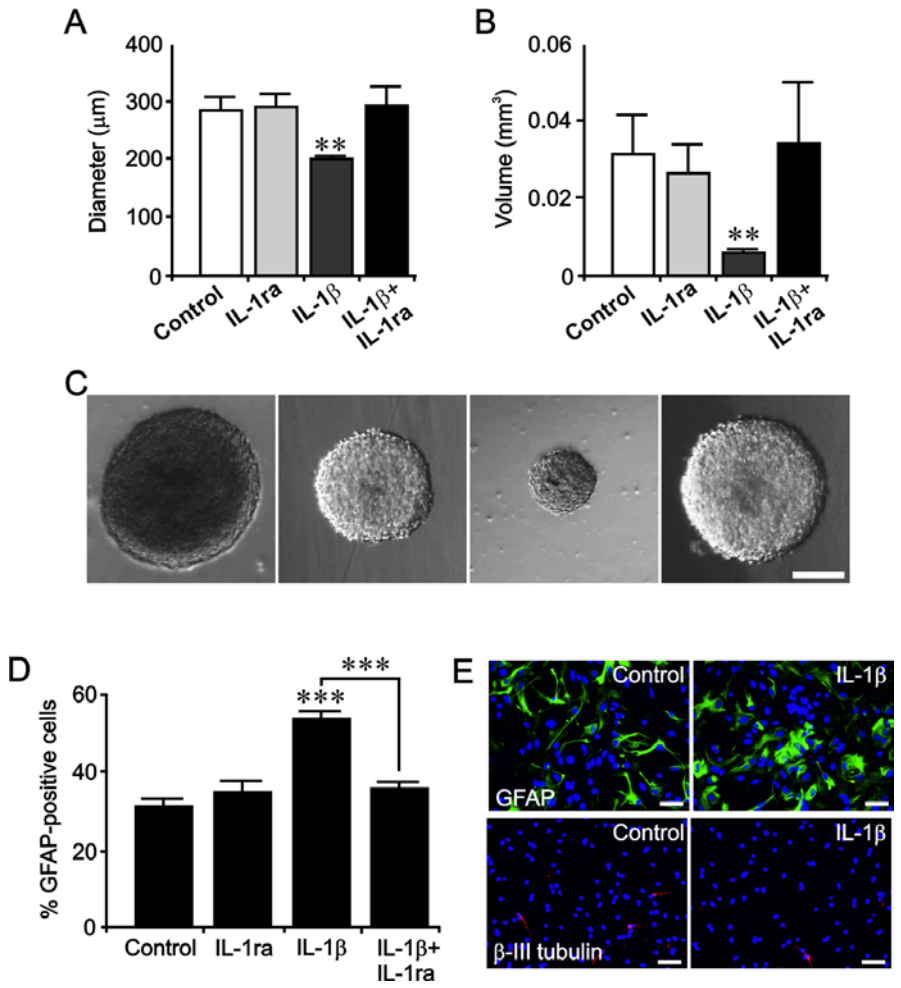


Figure 5.3.10: IL1ra rescues NPCs from the detrimental effects of IL-1β treatment *in vitro.*

(A, B) Quantitative data showing that the IL1R1 antagonist, IL1ra, prevents the IL-1β mediated reduction in size of proliferating neurospheres. **p < 0.01; n = 3 (mean ± SEM). (C) Representative photomicrographs of control and treated neurospheres. (D) Quantitative data showing that the IL1R1 antagonist, IL1ra, prevents the IL-1β mediated induction of gliogenesis in NPCs. *** $p<0.001$; n=3 (mean ± SEM). (E) Representative images of GFAP immunostaining in control and treated cultures. Scale bar = 50 μm

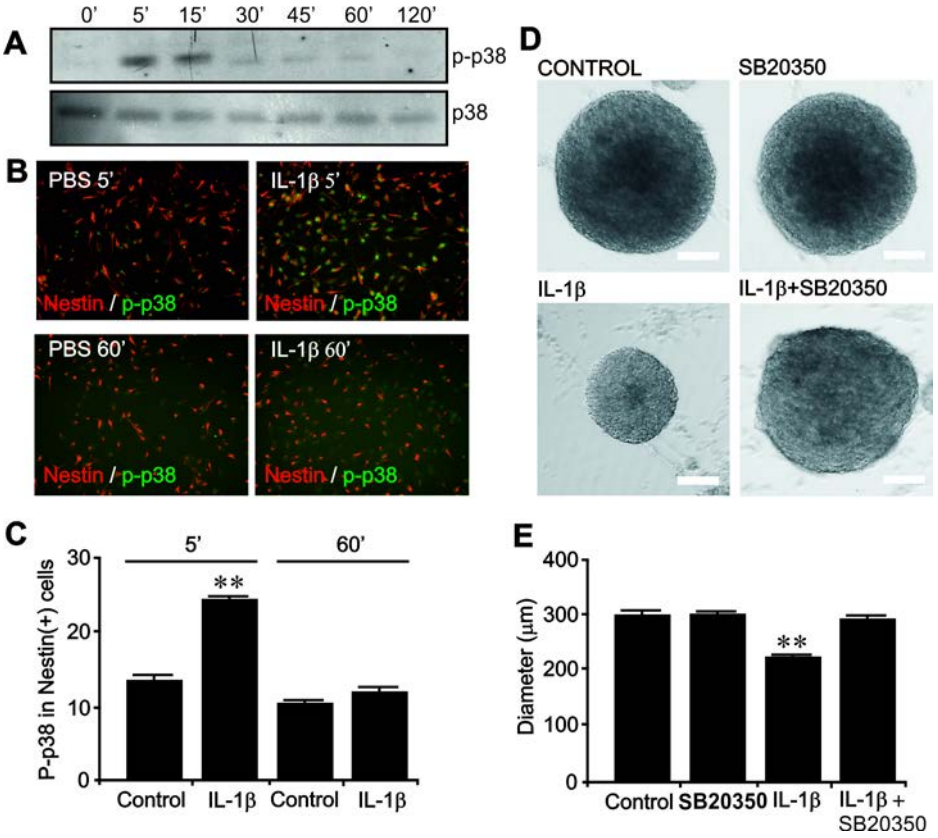


Figure 5.3.11: IL-1β activates the p38 MAP kinase pathway in E14 VM NPCs.

(A) Western blot showing that phospho-p38 was significantly increased at 5 min post-IL-1b stimulation and then declined returning to baseline at 2 h. (B) Immunofluorescent staining for nestin and phospho-p38 in proliferated NPCs. Scale bar 50 lm. (C) Semi-quantitative analysis of phospho-p38 levels in IL-1β treated, nestin-positive NPCs at 5 and 60 min. **p < 0.01; n = 3 (mean ±SEM). (D) Representative images of proliferating neurospheres demonstrating that blockade of the p38 MAPK pathway with the selective p38 MAPK inhibitor, SB203580, inhibits the effects of IL-1β on NPC proliferation. Scale bar, 100 μm. (E) Quantitative data from NPCs treated with p38 MAPK inhibitor, SB203580. **p < 0.01; n = 3 (mean ± SEM).

5.4 Discussion

In this study we investigated whether the effects of the proinflammatory cytokine IL-1β on the proliferation, survival and differentiation of cultured rat E14 VM. We have demonstrated that IL-1β acts directly on NPCs isolated from embryonic rat VM that express the IL-1R1 receptor. The widespread expression of IL-1R1 in the developing brain has been previously reported (Vitkovic et al., 2000; Friedman, 2001; Sabolek et al., 2009; Long-Smith et al., 2010), but this work extends these findings by showing that IL-1R1 is expressed on nestin-positive cells in the developing VM. We have also shown that mRNA expression and protein levels of IL-1R1 increase with time *in vitro* in agreement with previous findings (Friedman, 2001). This increase in receptor density of IL-1R1, with no detected presence of IL-1R2, suggests that NPCs to become more sensitive to the effects of IL-1β treatment in culture.

IL-1β-treated neurospheres were significantly smaller compared to control neurospheres, an effect that was mediated by IL-1R1. This is the first description of this in the developing VM, and is in agreement with other studies on other cell types (Vela et al., 2002; Wang et al., 2007). As nestin expression is restricted to immature NPCs and immunoreactivity is lost upon their terminal differentiation at a cellular level, it is interesting to speculate that the decrease in nestin-positive cells in IL-1β-treated cultures compared to untreated cultures may be partly due to IL-1β inducing the differentiation of NPCs if the cells are exposed to it whilst they are proliferating. An important question arising from these results is whether IL-1β encourages directed differentiation of NPCs along a specific lineage or whether it causes an inhibition of NPC proliferation, allowing the cell to continue along its default lineage pathway. As the protein levels of cleaved caspase-3 and PARP, both of which are involved in programmed cell death, were not increased in IL-1β treated NPCs, and as there were no differences in LDH levels between control and IL-1β treated cultures, the decrease in neurosphere size by IL-1β-treated cultures is not due to cell death, but is rather more likely to be due to IL-1β-mediated differentiation. IL-1β has, however, also been shown to increase proliferation in cultured human NPCs whilst inhibiting neuronal differentiation (Whitney et al., 2009).

Collectively data suggests that IL-1β induces differentiation of NPCs when they are proliferating as there was a reduction in the number of nestin-positive cells in IL-1β treated cultures. This is supported by the finding that many NPCs treated with IL-1β displayed a distinct glial morphology with a significant increase in the maximum outgrowth of somal processes when compared to control. Members of the interleukin family have previously been

shown to be pro-gliogenic when added to cultures at differentiation phase, but this effect has not been shown before at proliferative phases (Nakanishi et al., 2007; Ideguchi et al., 2008; Ajmone Cat et al., 2010).

The effects of IL-1β have been shown to be mediated through several intracellular signalling pathways, including p38, NF-κB and JNK (Wagner and Nebreda, 2009; Gabay et al., 2010). In NPCs grown from the E16 forebrain, IL-1β has been shown to signal through the SAPK/JNK and not the p38 pathway (Wang et al, 2007). Interestingly, our study shows that the effects of IL-1β are fully mediated by p38 in NPCs from the VM. Rather than these data being conflicting, this highlights an extremely important point regarding the effects of IL-1β on the developing nervous system. That is that the effects of IL-1β and the molecular mechanisms underlying them will depend very much on the development age and cell-type in question. This is highlighted by studies showing that IL-1β exerts its effects in sympathetic neurons by activating NF-κB (Nolan et al. 2011), forebrain NPCs by activating JNK (Wang et al. 2007) and VM NPCs by activating p38. These studies show that it will be important to fully characterise the effects of IL-1β on distinct cell populations as inferring generalities in mechanism of action from cell type to another may be misleading.

To assess the effect of IL-1β on the lineage fate of NPCs, cells were differentiated for 7 DIV in the presence or absence of IL-1β. IL-1β treatment during either proliferation or differentiation caused a marked increase in GFAP-positive cells suggesting that IL-1β is a strong inducer of gliogenesis. These findings are in agreement with previous studies using IL-1β and other proinflammatory cytokines which show that they promoted cells to follow a glial lineage fate (Giulian et al., 1988; Ajmone Cat et al., 2010; Keohane et al., 2010). With the resultant increase in the numbers of GFAP-positive cells, there was a reduction in the extent of neurogenesis as assessed by β-III tubulin staining of cells in these cultures. This is in agreement with other studies showing that IL-1β has a detrimental effect on neurogenesis when administered during the differentiation phase of adult and embryonic NPCs (Koo and Duman, 2008; Kuzumaki et al., 2010). When IL1ra was added in addition to IL-1β during differentiation the significant increase in gliogenesis, as seen with IL-1β only treated NPCs, was prevented. This demonstrates that IL-1β binding to the IL1R1 receptor is required to elicit the gliogenic effects of IL-1β in NPCs from the developing VM. This has implications also for the fate and/or proliferation of transplanted stem cells into the adult CNS (Lie et al., 2002; Jiao and Chen, 2008). Transplanted NPCs into the diseased or inflamed brain will be subjected to an inflammatory response which may be exacerbated due to the presence of

'primed' microglia (Depino et al., 2003; Godoy et al., 2008). In allografts of rat embryonic tissue into the adult rat brain expression of the proinflammatory cytokines IL-1β and TNF-α where shown to be increased around the graft demonstrating that these inflammatory mediators could affect the local microenvironment of transplanted cells (Mirza et al., 2004). Furthermore, our data shows that the proliferation and differentiation of an endogenous population of actively dividing progenitor cells in the adult rodent SN which are capable of differentiating to neurons (Lie et al., 2002; Zhao et al., 2003) could be altered by increases in IL-1β.

From a neurodevelopmental standpoint, in rodent models of maternal intrauterine infection there are significantly increased levels of inflammatory cytokines such as IL-1β and TNF in the foetal brain following maternal administration of common immune activators such as LPS, poly(I:C) or turpentine (Boksa 2010; Cai et al 2000; Urakubo et al 2001). The primary source of inflammatory cytokine production following a maternal immune challenge is from the fetus itself (Aaltonen et al 2005; Zaretsky et al 2004). In terms of the development of the VM, our study shows that progenitor cells in this region will be potentially affected by increases in IL-1β as a result of maternal inflammation *in utero.* Although this was reported to not affect the mean number of DA neurons in organotypic cultures of the E14 rat midbrain, at longer durations a reduction in DA neurons was observed (Snyder-Keller and Stark 2008). Whether this is due to some specific effect on progenitor cells or due to a potential change in the cellular architecture of the region remains to be determined. This raises intriguing questions for future research concerning what consequences maternal immune system activation, leading to an in circulating IL-1β levels in the embryo, has for the formation of the developing VM and whether this can in some way predispose to the development of neurological disease later in life is an intriguing question for future research.

6. Gestational age of NPCs dictates their susceptibility to the detrimental effects of an elevated proinflammatory environment.

6.0 Abstract

Previous studies have shown that maternal immune activation (MIA) during pregnancy can have a profound impact on the developmental trajectory of the fetal brain leading to increased risk of developing neurological disorders such as autism and schizophrenia in later life. There are thought to be particular windows of sensitivity during periods of the developmental process in which the fetal brain is especially susceptible to changes in the balance of cytokines. In this study we investigated how embryonic age influences the effects of an increased proinflammatory environment. Neural precursor cells (NPCs) isolated from embryonic day (E)12 and E16 SD rats were exposed *in vitro* to IL-1β, IL-6 or TNF and the effects on proliferation and cell death were assessed. In addition to this pregnant Sprague Dawley (SD) rats were administered a single dose lipopolysaccharide (LPS) injection (50μg/kg) at E12 or E16 and NPCs from the embryos were cultured to assess proliferation. In both experiments there were no significant differences in proliferation of treated groups at E12 when compared to controls, however, at E16 there was a significant reduction in neurosphere volume and area in treatment groups when compared to control. Similarly there were no differences seen in the percentage of nestin positive cells at 7 days *in vitro* (DIV) in any of the E12 groups when compared to controls yet at E16 there was a significant reduction in nestin positive cells in all treatment groups when compared to controls. We found no evidence for an increase in cell death across treatment groups at either age. These data demonstrate that the detrimental effects of proinflammatory cytokines on NPCs are limited to certain periods of development and that at earlier ages these NPCs seem relatively impervious to their effects.

6.1 Introduction

During pregnancy different organs and tissues go through what are known as 'critical periods' of development in which they may be particularly sensitive to disturbances in their environment (Widdowson and McCance, 1975). Alterations to the balance of pro- and anti-inflammatory cytokines within the developing embryo are thought to be detrimental to the normal course of embryonic brain development, (Meyer et al., 2009a; Watanabe et al., 2010; Meyer et al., 2011) however, the effects of these imbalances may not manifest until later periods in life. These changes in cytokine expression profiles may be caused through activation of the maternal immune system or PNS, both of which have been shown to increase the expression of a number of proinflammatory cytokines including IL-1β, IL-6 and TNF in the fetal brain resulting in fetal neuroinflammation (Maes et al., 1998; Rozlog et al., 1999; Boksa, 2010). This increase in proinflammatory cytokine production occurs in the fetal brain rather than being (at least fully) derived from maternal-placental transfer (Zaretsky et al., 2004; Aaltonen et al., 2005b; Boksa, 2010). It is also thought that maternal psychosocial stress may have an effect on fetal development through neurochemicals that are involved in the stress response system which may alter inflammatory markers (Coussons-Read et al., 2007; Dunkel Schetter, 2011). In conjunction to this point peripheral cytokines have been demonstrated to activate the HPA axis causing an elevation in plasma CORT levels (Dunn, 2000; Hashimoto et al., 2001). PNS has been shown by several studies to cause elevated levels of proinflammatory cytokines in both the periphery and embryonic brain (Laviola et al., 2004; Diz-Chaves et al., 2012).

MIA has been shown to increase the risk for neurodevelopmental and neuropsychiatric disorders such as ASDs and schizophrenia in affected offspring later in life (Machón et al., 2002; Sørensen et al., 2009; Atladóttir et al., 2010; Patterson, 2011). This increased risk has been shown to be dependent on the developmental age at which MIA occurs (Meyer et al., 2006c); for example, maternal bacterial infection in the second trimester, but not the first of human pregnancy was found to increase the risk of developing ASDs and schizophrenia in offspring (Sørensen et al., 2009; Atladóttir et al., 2010; Patterson, 2011). It has previously been shown that the levels of both circulating and fetal brain cytokine levels are also subject to the timing of the infection, with increased quantities of IL-10 and TNF-α present during early to mid gestation compared to late gestation (Meyer et al., 2006a). How MIA increases this risk, and why this risk is restricted to specific developmental stages is unclear. However, a variety of studies have described the structural changes that occur in the fetal brain as a

result of MIA suggesting that neuroanatomical alterations may be (at least in part) involved. Administration of LPS to pregnant dams on gestational day 15/16, resulted in a significant increase in the size of the cortical plate and hippocampi in the fetal brain, coupled with a reduction in nestin expression (a marker of NPCs) (Ghiani et al., 2011a). Similarly, Girard et al showed that maternal LPS administration from gestational day 15 onwards in mice resulted in a reduction in the number of newly born neurons in the hippocampi of affected offspring (Girard et al., 2012). Interestingly, in this study these effects of MIA were IL-1β–dependant (Girard et al., 2012). Similarly, systemic administration of LPS to pregnant mice on gestational day 13.5 resulted in reduced cellular proliferation at the ventricular surface in the fetal brain which resulted in altered cortical organisation in the postnatal period (Stolp et al., 2011). Some of these neuroanatomical alterations are present in the ASD brain and the presence of an inflammatory-like state in postmortem autistic brains suggests that elevations in pro-inflammatory cytokines may be a causative factor (Vargas et al., 2005; Lintas et al., 2012). However, why maternal infection only increases risk for ASDs (and schizophrenia) at specific stages of pregnancy is unknown.

We have previously shown that NPCs from a region of the fetal rat brain known as the VM, the presumptive midbrain, express the IL-1R1 at gestational day 14 (Crampton et al., 2012). As these cells proliferate in culture as neurospheres, there is an increase in expression of IL-1R1 (Crampton et al., 2012). IL-1β treatment of these proliferating E14 NPCs impairs their proliferation and promotes their differentiation as evidenced by loss of nestin expression, through a p38-MAPK-dependant pathway (Crampton et al., 2012). IL-1β also inhibited neuronal differentiation while promoting glial differentiation from these cells without affecting cell death, as they underwent differentiation (Crampton et al., 2012). Given that MIA in mid-pregnancy, rather than early pregnancy increases ASD and schizophrenia risk, and given that the structural alterations in the fetal brain following MIA are IL-1β-dependant (Sørensen et al., 2009; Atladóttir et al., 2010; Girard et al., 2012), it raises the possibility that the cellular basis of both may depend on differing sensitivity of NPCs to IL-1β at different stages of fetal development. To test this hypothesis we examined the effects of IL-1β, and related cytokines, TNF and IL-6, which have also been implicated in mediating alterations in the fetal brain following MIA (Smith et al., 2007b), on the lineage potential of NPCs from the E12 rat VM and we directly compared the effects of IL-1β on E12 and E14 NPCs from the rat VM.

6.2 Results

6.2.1 Cytokine Receptor Expression in vitro

Firstly, we aimed to assess whether the necessary cellular signalling machinery to elicit the effects of our cytokines of interest were present in both E12 and E16 NPCs. To ascertain this E12 and E16 tissue that had been freshly dissected and dissociated were plated as single cells then fixed and immunocytochemically stained for the signal transducing receptors for IL-1β (IL-1R1), IL-6 (IL-6R) or TNF (TNFR1) following 2 h *in vitro*. We found that the functional receptors for all cytokines of interest were present on both E12 and E16 cells and that at E12 IL-1R1 seemed to be more highly expressed than the others whereas at E16 IL-6R appeared to be the most highly expressed (Fig 6.3.1). All cells were double-stained with the NPC marker, nestin, to validate that the observed cytokine receptor expression was present on the NPC population of cells within these cultures. These results demonstrate that NPCs from both E12 and E16 ages possess the required receptors to generate a response to cytokine exposure.

6.2.2 Differential Effects of Cytokine Exposure on VM NPC Proliferation is Dependent on Embryonic Age

Following confirmation that NPCs of both E12 and E16 rat VM expressed functional receptors for IL-1β, IL-6 and TNF we next directly compared the effects of these cytokines on E12 and E16 VM NPC proliferation through the measurement of neurosphere volume and diameter. NPCs of both ages were treated *in vitro* from 0DIV with IL-1β, IL-6 or TNF for a total of 7DIV; neurospheres were imaged at 2, 4 and 7DIV for volume and diameter analysis. We found that treatment with IL-1β, IL-6 or TNF did not result in significant reduction in E12 neurosphere volume at any of the time points examined (Fig 6.3.2A and C). Exposure of E16 NPCs to TNF caused a significant reduction in neurosphere diameter at 2DIV when compared to control although this difference was not evident at 4 or 7DIV (Fig. 6.3.2B and C). Treatment of E16 NPCs with IL-1β, IL-6 or TNF caused a significant decrease in neurosphere volume at 2DIV when compared to control. This reduction in volume persisted only in the IL-1β group of E16 NPCs at 7DIV with both other groups returning to that of control (Fig. 6.3.3A and C). Significant differences in neurosphere diameter were visible across all treatment groups of E16 NPCs at 2 and 4DIV although this difference was only observed in the IL-1β group at 7DIV (Fig. 6.3.3B and C).

6.2.3 Cytokine Treatment Reduces the Number of Nestin Positive Cells in E16 but not E12 VM NPC Cultures

Having shown that the effects of IL-1β, IL-6 and TNF on the proliferation of VM NPCs are dependent on the embryonic age of the tissue we then aimed to assess the effects of these cytokines on the percentage of nestin positive cells in culture. To do this neurospheres that had been treated in culture for 7DIV were dissociated and plated for 2 h as single cells then stained for the NPC marker nestin. None of the treatment groups at E12 displayed any difference in the total number of nestin positive cells in culture at 7DIV when compared to controls (Fig. 6.3.4A and B). Administration of IL-1β, IL-6 or TNF to cultures of E16 VM cultures all caused a significant decrease in the total number of nestin positive cells at 7DIV in comparison to control group with TNF resulting in the greatest reduction (Fig. 6.3.5A and B).

6.2.4 IL-1R1 Expression in vivo During Development

We previously demonstrated that IL-1R1 was expressed in dissociated tissue under *in vitro* conditions, to follow on from this we examined IL-1R1 expression in the striatum and midbrain at developmental ages from E11 to postnatal day (P) 90 using both RT-PCR and RT-QPCR. Expression of IL-1R1 within the developing striatum steadily increases from E14 until it reaches a peak at around P60 and then declines (Fig. 6.3.6A). Within the midbrain IL-1R1 expression also increases gradually from E14 but the peak of expression occurs at an earlier age, around P31 (Fig. 6.3.6B).

6.2.5 Maternal Immune Activation in vivo Results in Impaired Proliferation of VM NPCs in vitro at E16 but not E12

Exposure of cultured NPCs, isolated from the developing VM, to proinflammatory cytokines can result in reduced proliferation depending on the embryonic age of the tissue. We have displayed that E12 tissue is not susceptible to these effects but that at E16 a pronounced decrease in the neurosphere volume and diameter is caused through cytokine addition cultures (Fig. 6.3.2A and B, 6.3.3A and B). Subsequent to these findings we investigated whether *in vivo* MIA at E12 or E16 via a systemic LPS insult results in persistent alterations to NPCs within the developing VM. VM NPCs obtained from control and LPS animals injected at either E10 or E14 where cultured under proliferation conditions without the addition of any cytokines. Similar to our previous observations there was no difference between the control and LPS groups at E12 (Fig. 6.3.7A and C) yet at E16 there was a

significant decrease in neurosphere volume of the LPS group when compared to the control at 7DIV (Fig. 6.3.7B and C).

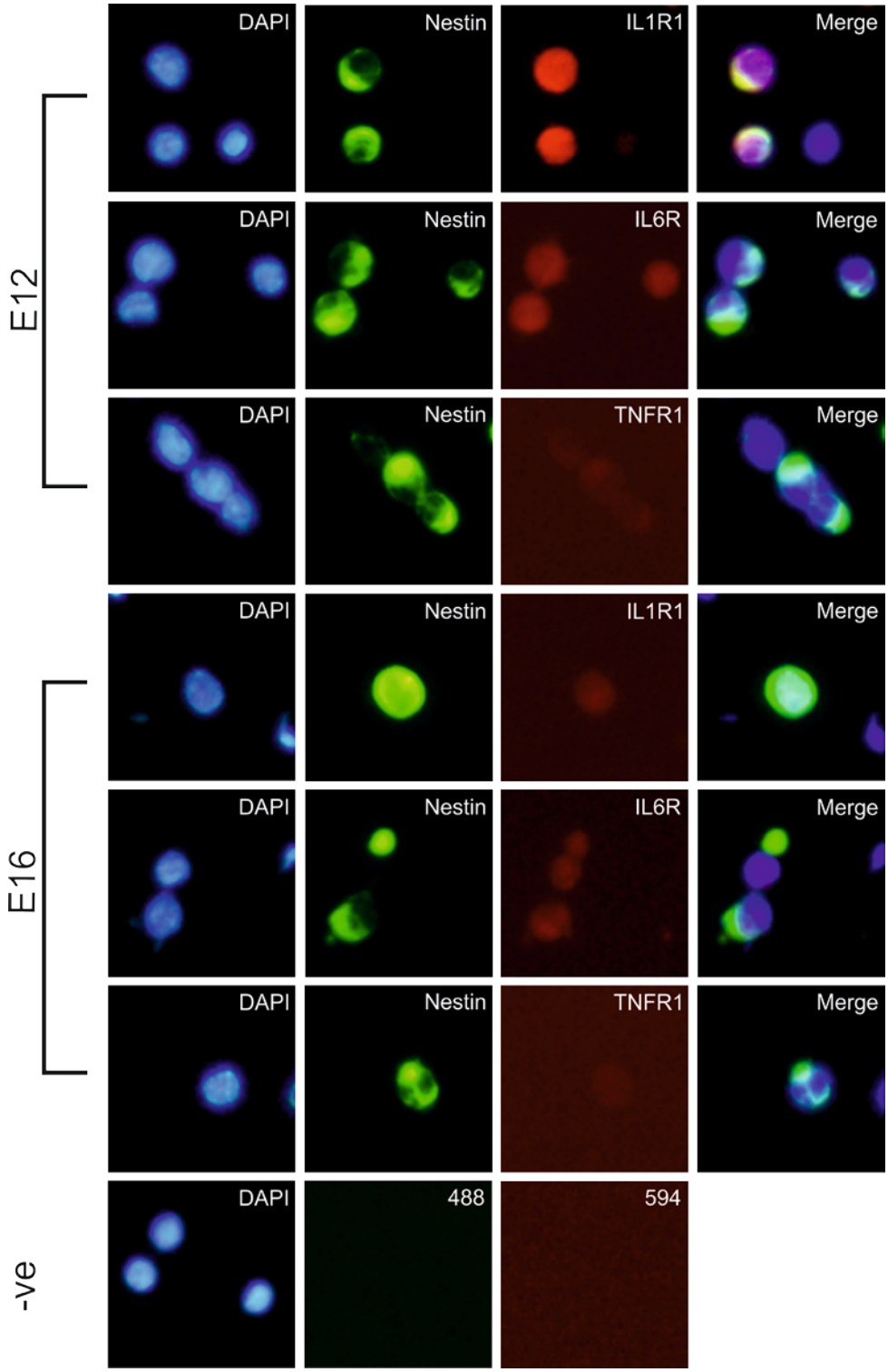


Figure 6.3.1: Cytokine receptor expression on E12 and E16 VM NPCs.

Photomicrographs of immunofluorescent staining illustrating the presence of the functional proinflammatory cytokine receptors IL-1R1, IL-6R and TNFR1 (594; red) and their co-expression with the NPC marker nestin (488; green).

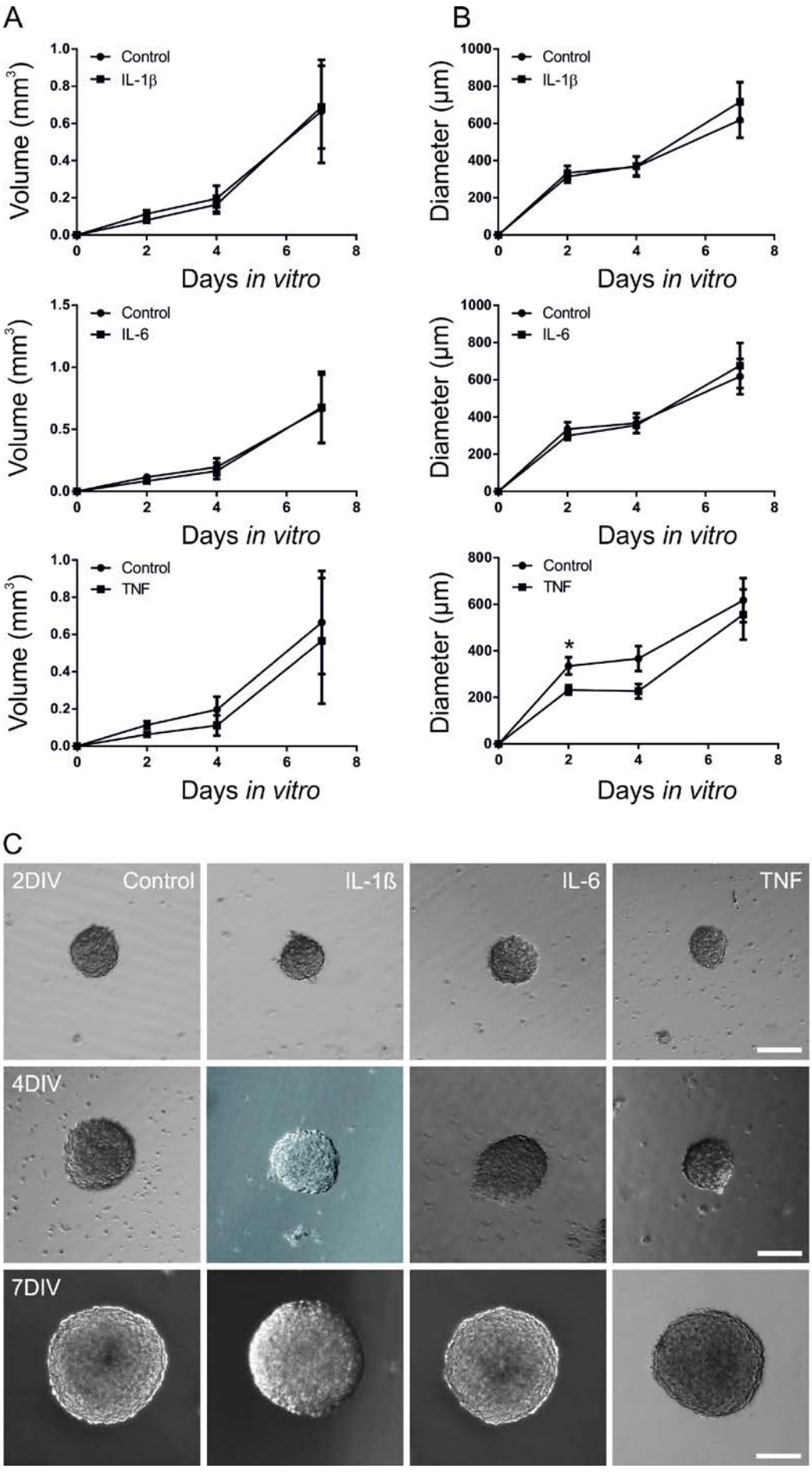

A
B
Control
IL-1β
IL-6
TNF
Volume (mm3)
Diameter (µm)
Days in vitro
*
C
2DIV
Control
IL-1ß
IL-6
TNF
4DIV
7DIV

Figure 6.3.2: Cytokine exposure has no effect on the proliferation of E12 VM NPCs.

(A) Graphical representation of the effects of IL-1β, IL-6 and TNF treatment on neurosphere volume at 2, 4 and 7DIV. (B) Graphical representation of the effects of IL-1β, IL-6 and TNF treatment on neurosphere diameter at 2, 4 and 7DIV. *$p < 0.05$; n=3 (mean ± SEM). (C) Representative photomicrographs of control and treated neurospheres at 2, 4 and 7DIV. Scale bar, 100μm.

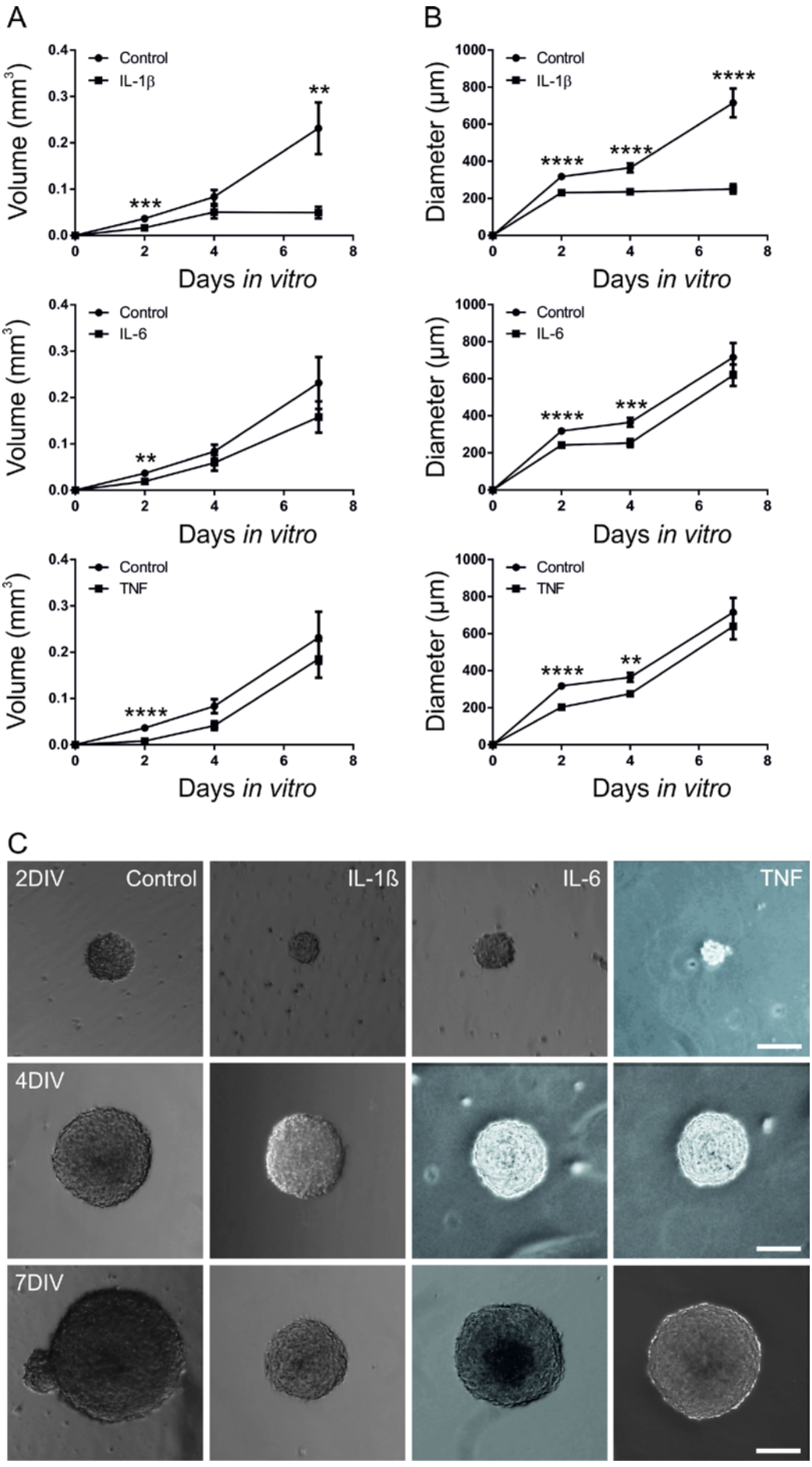

A
B
Control
IL-1β
IL-6
TNF
Volume (mm3)
Diameter (µm)
Days in vitro
***
**
****
C
2DIV
4DIV
7DIV
Control
IL-1ß
IL-6
TNF

Figure 6.3.3: Cytokine exposure results in a decrease in proliferation of E16 VM NPCs.

(A) Graphical representation of the effects of IL-1β, IL-6 and TNF treatment on neurosphere volume at 2, 4 and 7DIV. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; n=3 (mean ± SEM). (B) Graphical representation of the effects of IL-1β, IL-6 and TNF treatment on neurosphere diameter at 2, 4 and 7DIV. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; n=3 (mean ± SEM). (C) Representative photomicrographs of control and treated neurospheres at 2, 4 and 7DIV. Scale bar, 100μm.

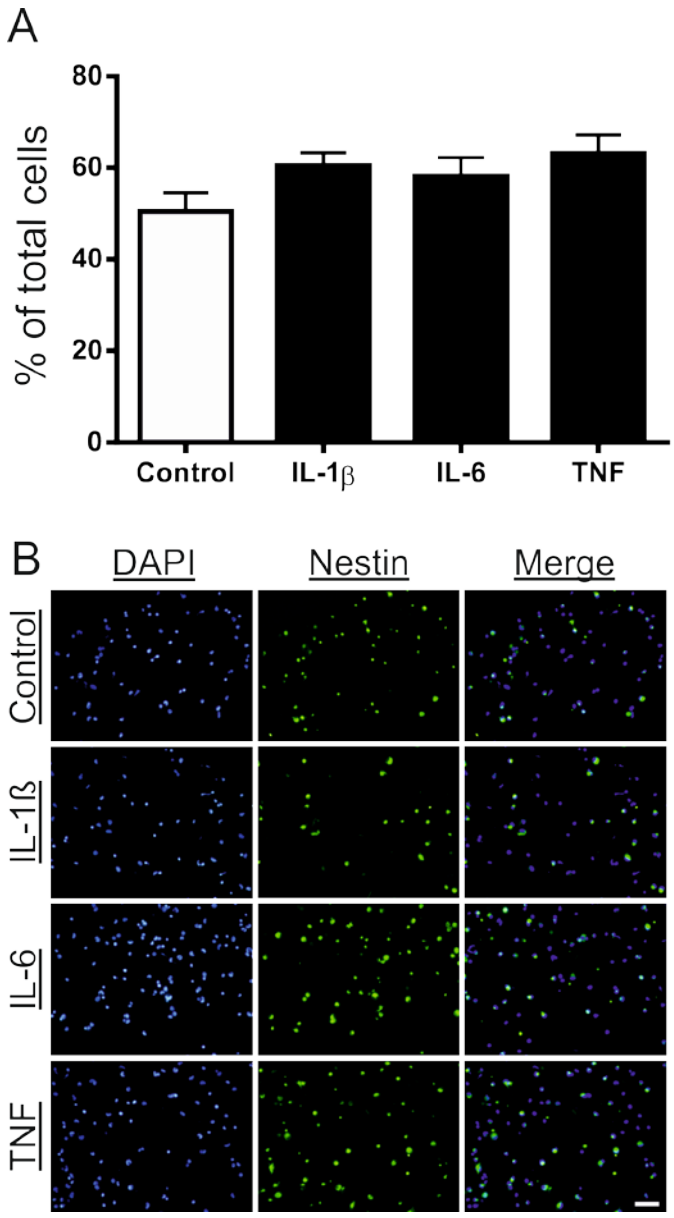


Figure 6.3.4: Cytokine treatment has no effect on the percentage of nestin positive cells at 7DIV in E12 VM NPCs.

(A) Quantitative data showing no significant differences in the percentage nestin positive cells between treatment groups and control in E12 NPCs. (B) Representative photomicrographs of control and treated E12 NPCs at 7DIV. Scale bar, 50µm. (A) Quantitative data showing a significant decrease in the percentage nestin positive cells in all treatment groups when compared to controls in E16 NPC cultures. *$p < 0.05$, **$p < 0.01$; n=3 (mean ± SEM). (B) Representative photomicrographs of control and treated E16 NPCs at 7DIV. Scale bar, 50µm.

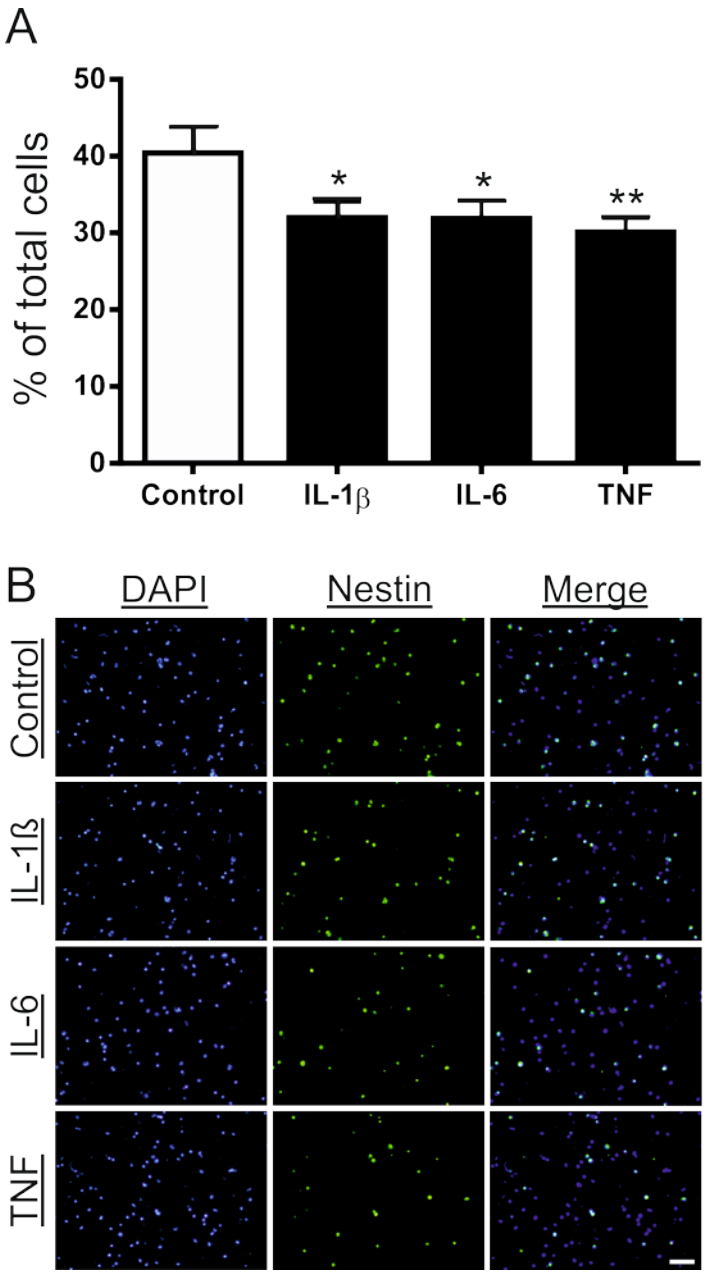


Figure 6.3.5: Cytokine treatment causes a significant decrease in the percentage of nestin positive cells at 7DIV in E16 NPCs.

(A) Quantitative data showing a significant decrease in the percentage nestin positive cells in all treatment groups when compared to controls in E16 NPC cultures. *$p < 0.05$, **$p < 0.01$; n=3 (mean ± SEM). (B) Representative photomicrographs of control and treated E16 NPCs at 7DIV. Scale bar, 50μm.

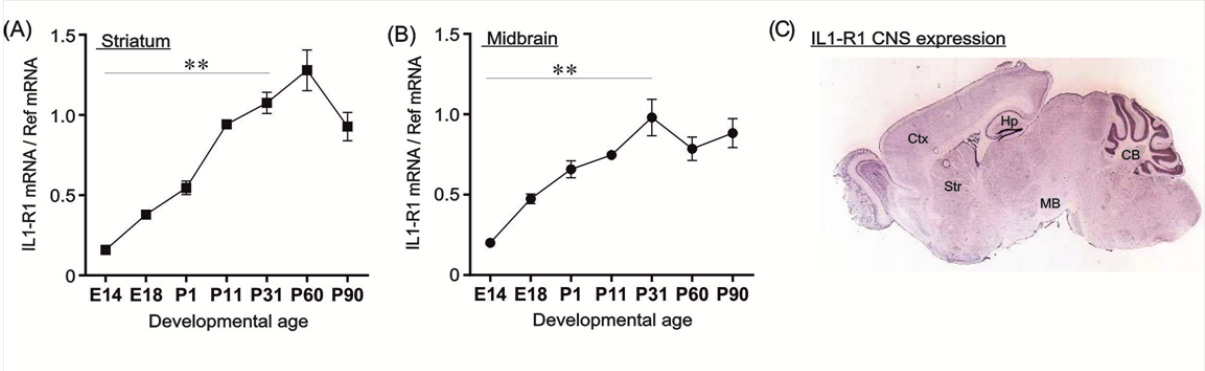


Figure 6.3.6: IL-1R1 is expressed in the striatum and midbrain during embryonic and postnatal development.

(A-B) Quantitative RT-QPCR data showing the levels of IL-1R1 mRNA in the developing midbrain and striatum, from E14 to P90, relative to the levels of the reference mRNAs GAPDH, SDHA and UBQC. Each data point represents pooled data from four samples from three separate litters/animals, and all data are presented as the mean ± SEM. (C) In situ hybridization images taken from the Allen Developing Brain Atlas (© (Allen) Developing Mouse Brain Atlas, 2012) showing IL-1R1 expression (purple colour) in sagittal sections of adult rat brain.

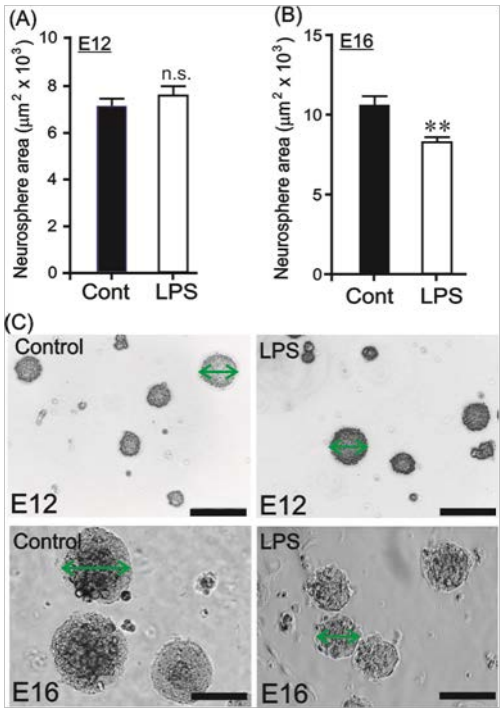


Figure 6.3.7: The effects of maternal immune activation on the proliferation of embryonic NPCs is dependent on developmental age.

(A) Graphical representation of the effects of maternal infection, by means of a single LPS injection, on the proliferative capabilities of E12 VM NPCs. (B) Quantitative data showing a significant decrease in neurosphere area of neurospheres from E16 LPS treated animals when compared to control group. $^{**}p < 0.01$; n=3 (mean ± SEM). (C) Representative photomicrographs of neurospheres from E12 and E16 control and LPS treated animals. Scale bar = 100 μm

6.4 Discussion

In this study we have provided further evidence which supports the theory that during fetal development there are 'critical periods' in which tissues and organs may be particularly sensitive to changes in their environment (Widdowson and McCance, 1975). We specifically focused on the effect of elevated levels of proinflammatory cytokines and the consequences that this altered environment had on NPCs *in vitro*. There are multiple causative agents that have the potential to induce increased levels of proinflammatory cytokines within the mother and developing fetus including stressful experiences (Maes et al., 1998; Dunkel Schetter, 2011) or induction of the maternal immune system through infection (Meyer et al., 2009a; Watanabe et al., 2010). However, it has been shown that cytokines may variably regulate the development of subsets of neurons within the brain as cytokines and their receptors are expressed in the normal developing brain (Deverman and Patterson, 2009). One such example of this is the ability of TNF to have opposing effects on VM neuron survival dependent on the developmental time-point (Doherty, 2007).

We have shown that treatment with the cytokines IL-1β, IL-6 and TNF does not elicit any effects on the neurosphere volume of E12 NPCs but at E16 all cytokines cause a significant reduction in neurosphere size. In our previous study we have shown that the window of sensitivity to the effects of IL-1β extends to tissue isolated from E14 rats (Crampton et al., 2012). This observed reduction is not thought to be due to an increase in cell death in treated groups but rather is caused by a decrease in NPC proliferation as evidenced by no significant differences in LDH levels between treatment groups and controls. LDH being released from the cell into the surrounding environment in response to cell damage. In addition to proliferation being differentially affected by cytokine exposure dependent on the developmental age of the tissue we also found that this extended to the influence of proinflammatory mediators on the nestin percentage in culture. Cultures from E12 tissue displayed no decrease in the percentage of cells expressing the NPC marker nestin following treatment for 7 days whereas a significant reduction was observed in all treatment groups of E16 cultures at 7 days. This difference in susceptibility between gestational ages demonstrates that during certain periods of fetal development tissues may be particularly sensitive to changes in basal cytokine levels. It has been previously shown that depending on the timing of a prenatal immune challenge that different behavioural outcomes are observed. If this challenge occurs during early to middle gestation the offspring suffers impaired selective associative learning and spatial exploration (Meyer et al., 2006a; Meyer et al.,

2006c) whilst if this same challenge occurs in late gestation it results in the emergence of perseverative behaviour (Meyer et al., 2006c). Given the ages that we examined E12 correlates roughly to the end of the first trimester and E16 to the middle-late second trimester of human pregnancy with respect to the percentage of gestation from rodent to human (Clancy et al., 2001). Meyer et al have in addition demonstrated that the timing of a maternal immune challenge also dictates the expression profiles of proinflammatory cytokines both in maternal circulation and also within the fetal brain (Meyer et al., 2006a).

We also investigated whether an individual maternal immune challenge in the form of a single dose LPS injection at either E12 or E16 would cause similar effects to those seen when cultured NPCs from tissue of these ages were treated *in vivo*. NPCs isolated from the embryos of dams which received either control or LPS injections at E12 or E16 were cultured without any treatments for 7DIV to assess proliferation. Similar results to our earlier experiments were observed in that no differences where present between control and LPS groups at E12 but there a significant reduction in neurosphere area at E16 in the LPS group. This data shows that the effects of MIA are capable of persisting outside of the proinflammatory environment and thus the initial insult may cause lasting molecular changes within NPCs having detrimental consequences to development. Events such as this may contribute to 'fetal programming' which predisposes affected offspring in later life to neurological disorders such as schizophrenia (Machón et al., 2002; Meyer et al., 2011). Peripheral cytokines are also known to activate the HPA axis (Dunn, 2000) and therefore if the balance of cytokines within affected offspring is altered permanently this may lead to changes in a normal stress response.

Several studies have investigated the potential of antagonising proinflammatory cytokine receptors, in particular the IL-1 receptor, in an attempt to ameliorate the effects of elevated proinflammatory cytokines. This approach has demonstrated relative efficacy in preventing some of the detrimental effects that are seen in animal models of systemic inflammation such as fetal cortical injury and decreased motor performance in offspring (Girard et al., 2010; Leitner et al., 2014). Utilising anti-inflammatory therapies in cases of maternal infection therefore may offer a possibility of minimising damage. Furthering our knowledge of periods of sensitivity within the developing fetal brain and the potential outcomes of disturbances within these periods will also assist in predicting potential complications in later life of affected offspring.

7. Chronic Prenatal Restraint Stress Causes Lasting Molecular and Physiological Alterations in Adult Rat Offspring.

7.0 Abstract

The theory of 'fetal programming' has gained a great deal of support in recent years with numerous epidemiological and preclinical studies providing evidence to support initial findings by Barker et al (Barker and Osmond, 1986; Barker et al., 1989). Maternal stress during pregnancy results the both the elevation of stress related hormones such as cortisol and also increases the levels of circulating proinflammatory cytokines (Gitau et al., 1998; Coussons-Read et al., 2005; Coussons-Read et al., 2007; Glover et al., 2009). Prenatal stress (PNS) has now been implicated in the development of a number of neuropsychiatric (Markham and Koenig, 2011), cardiovascular (Loffredo, 2000), respiratory (Khashan et al., 2012) and metabolic (Hohwü et al., 2014) disorders. In this study we used a maternal restraint stress protocol whereby pregnant dams were subjected to 3 x 45 minute sessions of restraint stress for the last 7 days of gestation, embryonic day (E)14 to E21. We then performed behavioural analysis, cardiovascular and respiratory functional analysis in addition to examining gastrointestinal tract innervation and functional responses to specific agonists. Stress responses to two different types of stressors were also analysed.

PNS animals showed a number of behavioural alterations including deficits in the novel object recognition (NOR) test and signs of hyperactivity in the open field (OF) test. In cardiovascular function experiments PNS animals were hypertensive at baseline recording and under the influence of psychosocial or noxious stimuli showed an enhanced cardiovascular response which persisted into the recovery period. Alterations in respiratory responses to both hypercapnic and hypoxic challenges were also observed in PNS animals when compared to controls. Measurements of corticosterone release in response to restraint stress also provided evidence of aberrant changes in the stress response of PNS animals that showed a prolonged and elevated release of corticosterone following the stressor. PNS also caused several changes within the gastrointestinal tract resulting in decreased overall innervation of the colon in conjunction with an exaggerated response to noradrenaline (NE). From our results we can conclude that PNS affects a number of different physiological systems and that these changes persist into adulthood potentially increasing the risk of developing numerous disorders in later life.

7.1 Introduction

The prenatal period is a sensitive window of development in which the developing fetus can be particularly susceptible to alterations in the surrounding environment. As organ systems undergo massive structural changes and begin to mature towards a fully functional state they are especially receptive to both organising and disorganising cues. However, each system has critical periods during development where they may be notably affected whilst other systems may remain unaffected by the same imbalances. These influences are thought to contribute to 'fetal programming' in which an insult or stimulus during a critical period of development may cause long-lasting or permanent changes which may not manifest until later periods of life (Widdowson and McCance, 1975; Davis and Sandman, 2010).

These insults can arise as a result of multiple events such as chronic stress or maternal infection which both cause a multitude of changes in the maternal and subsequently the fetal environment. Chronic prenatal maternal stress has been shown to cause an increase in both the levels of circulating proinflammatory cytokines as well as increasing the levels of the stress related steroid hormone cortisol in humans (Elenkov and Chrousos, 2002; Coussons-Read et al., 2005; Coussons-Read et al., 2007; Davis et al., 2011). Although maternal cortisol levels increase over the course of normal gestation, as it plays a role in the maturation of some tissues, there is usually a parallel upregulation of the placental enzyme 11β-HSD2 which converts cortisol to its inactive variant cortisone (Murphy and Clifton, 2003; Sandman et al., 2006). However, 11β-HSD2 is only a partial barrier and a certain amount of cortisol is capable of crossing the placental barrier (Gitau et al., 1998; Gitau et al., 2001) therefore elevations of cortisol within maternal circulation leads to increased levels in the fetus. In addition to this stress may reduce the expression of 11β-HSD2 causing a further increase in maternal to fetal cortisol transfer leading to even higher fetal levels (Glover et al., 2009; O'Donnell et al., 2009).

Pregnant women who report experiencing elevated stress levels display alterations in the levels of circulating cytokines such as IL-6 and TNF which are associated with the development of preeclampsia and premature labour as well as schizophrenia and autism in later life (Coussons-Read et al., 2005; Merlot et al., 2008; Bale, 2009). Increased levels of peripheral cytokines may also serve to perpetuate elevated levels of cortisol through activation of the HPA axis as has been shown previously (Dunn, 2000; Hashimoto et al., 2001). It has been established that PNS not only increases levels of cytokines within the periphery but also within the brain which may affect neurogenesis and cell fate specification

potentially contributing to the development of psychiatric disorders (Laviola et al., 2004; Diz-Chaves et al., 2012; Diz-Chaves et al., 2013). Prenatally stressed animals also display altered behavioural responses to an immune challenge, such as an LPS challenge, showing an enhanced or exaggerated immune response including increased concentrations of IL-1β in both the spleen and frontal cortex (Laviola et al., 2004; Merlot et al., 2008).

Other biological systems that have been less extensively studied but yet may still be affected by PNS include the respiratory and cardiovascular systems. In a study by Igosheva et al in which pregnant dams were subjected to a regimen of heat, light and restraint stress over the last trimester of pregnancy, their offspring displayed several alterations in cardiovascular function. It was demonstrated that PNS causes long-term effects on affected animal's ability to effectively cope with stressful situations in adulthood, this was evident through enhanced blood pressure and slow adaptation of stress induced tachycardia and hypertension in the post-stressor period (Igosheva et al., 2004). Findings such as this indicate that PNS may increase the risk of developing stress-related cardiovascular disorders in later life. In regards to the effects of PNS on the respiratory system there is only a small volume of data available, one study in a cohort of urban children did, however, draw a relationship between chronic stress in the perinatal period and reduced lung function at a young age (Suglia et al., 2008).

In this study we aimed to examine the effects of PNS across a broad range of biological systems using chronic restraint stress for the last trimester of pregnancy as our model of stress.

7.2 Results

7.2.1 PNS Animals Display Traits of Hyperactivity and Impairments in Recognition Memory

A number of behavioural tests were performed to assess whether PNS resulted in behavioural or learning and memory deficits in these animals (Fig 7.3.1). The OF test was used to measure whether PNS showed increased levels of anxiety and also to determine levels of locomotor activity. PNS animals showed no increase in anxiety as measured by time spent in the periphery but did display significantly increased locomotor activity with greater distance travelled and an increased velocity when compared to control animals (Fig 7.3.2A). We also carried out an additional test as a means of assessing anxiety levels, the EPM, in which we found no differences between control and PNS animals in the number of closed or open arm entries (Fig 7.3.2B). However, we did note a decrease in the number of head dips in PNS animals which can be interpreted as a measure of explorative behaviour (Fig 7.3.2B). The

NOR test was used to investigate the effects of PNS on recognition memory. Results from these tests demonstrated that rats from maternally stressed litters performed significantly worse than their control counterparts with a decreased discrimination ratio of the novel object (Fig 7.3.2C). As a measure of social behaviour we performed a SI test in order to examine impairments in SI which may indicate an autistic-like phenotype. In this test we found no significant differences between control and PNS in any of the parameters that were assessed (Fig 7.3.2D).

7.2.2 PNS Offspring Exhibit Elevated Systolic Arterial Pressure and an Enhanced Corticosterone Response to Acute Stress in Adulthood

To assess if PNS affected the HPA axis response to stress in adulthood, plasma corticosterone release was analysed under ARS conditions (Fig 7.3.3A). Control and PNS offspring had similar baseline CORT levels, averaging 8 – 10 ng/ml. In both groups, stress exposure resulted in more than a 20-fold elevation of plasma CORT within the first 30 min of stress. However, in control animals the CORT levels remained unaltered during the second 30 min of stress session whereas PNS offspring had a further elevation of CORT levels by 12%, thus showing a more sustained HPA axis activation.

We further analysed blood pressure and HR responses to stress exposure to address the activity of the sympathoadrenal axis (Fig 7.3.3B-E). At rest, prior to restraint stress, PNS offspring had higher APsys levels in comparison with control animals, with no significant changes in APdias and HR values (Fig 7.3.3B). Following exposure to restraint stress, all animals immediately responded with a 20-25% increase in APsys, APdias and HR (Fig 7.3.3C-E). The response reached its maximum within the first three minutes of stress onset, then started to decay slowly during the following 30 min, indicating a habituation effect (significant effect of restraint stress, $p<0.0001$). Return to the home cages for the 30 min recovery phase caused an initial rapid spike of blood pressure and HR due to animal arousal (explorative activity, rearing, grooming), which was followed by a gradual decline of assessed parameters to pre-stressor values (significant effect of recovery, $p<0.0001$).

PNS animals exhibited enhanced values of APsys both under stress exposure and during the recovery period (significant effect of group, $F_{(1,243)} = 33.2$ for stress and $F_{(1,223)} = 33.6$ for recovery, $p < 0.0001$). However, no interaction effect between group and stress was observed, indicating that stress evoked similar overall changes in APsys in control and PNS offspring. Similarly to APsys, stress induced an equal spike of HR in PNS and control groups. Interestingly, unlike APsys response, PNS rats displayed a faster decline of HR

throughout the stress session (significant effect of group, $F_{(1,272)} = 5.4$, $p = 0.02$, Fig 7.3.3E); the effect being even more prominent after correction for differences in pre-stressor values (significant effect of group, $F_{(1,272)} = 15.9$, $p = 0.0001$, Fig 7.3.3F). APdias values were unaffected by PNS.

7.2.3 PNS Results in Dysregulation of Ventilatory Response to Hypoxic and Hypercapnic Challenges

Baseline ventilatory parameters, measured under normoxic conditions at rest, are listed in Table 2. PNS animals had similar values of V_T, f, V_E and apnoea index as control group; however, they showed higher variability in f($p=0.032$, independent Student's t-test).

An acute hypoxic challenge (10% O_2 for 20 min) caused a significant increase in f by 56% ($F_{(1,11)} = 135.16$, $p = 0.0001$), V_T by 25% ($F_{(1,11)} = 45.67$, $p = 0.0001$) and V_E by 84% ($F_{(1,11)} = 292.51$, $p = 0.0001$) (Fig 7.3.4A-C). There were no differences in the averaged-per-challenge magnitude of hypoxic response between control and PNS groups ($F_{(1,11)} < 1$ both for prenatal condition and PNS x hypoxia interaction). However, time course analysis of ventilatory response to hypoxia revealed that PNS animals developed a decreased increment of V_E during the first 12 min of hypoxic onset ($F_{(1,119)} = 8.33$, $p = 0.005$) (Fig 7.3.4F). This effect was determined by the blunted response of fin PNS offspring ($F_{(1,111)} = 10.95$, $p = 0.001$, Fig 7.3.4D), since the V_T changes were comparable to those observed in control rats ($F_{(1,119)} < 1$, Fig 7.3.4E). Between-group comparisons in each time point confirmed a significant decrease in both f and V_E responses in PNS animals on the 7th and 8th min of hypoxic challenge. During the last 8 min of hypoxic exposure (from 13th to 20th min), the ventilation was stable and similar in both groups (Fig 7.3.4D-F).

Following a recovery period under normoxia, allowing for stabilisation of the ventilatory parameters back to basal levels (see Table 1 and Fig 7.3.5A-C, Normoxia), animals were subjected to the acute hypercapnic challenge. Exposure to 5% CO_2-enriched gas mixture for 10 min induced an increase in f on average by 53% ($F_{(1,15)} = 89.98$, $p = 0.0001$), V_T by 28% ($F_{(1,15)} = 188.93$, $p =0.0001$) and V_E by 98% ($F_{(1,15)} = 154.21$, $p = 0.0001$) both in control and PNS animals. Similar to hypoxia, no significant between-group differences in averaged per challenge response were observed ($F_{(1,15)} < 1$ both for prenatal condition and PNS x hypercapnia interaction (Fig 7.3.5A-C). Further analysis of the time course of ventilatory response to hypercapnia showed that PNS rats developed an enhanced increment of f over time, particularly during the second half of exposure ($F_{(1,136)} = 18.67$, $p = 0.0001$) (Fig 7.3.5D). The V_T elevation was, on the contrary, slightly reduced in PNS animals; the effect

being prominent within the first half of hypercapnic challenge ($F_{(1,68)}$ = 9.04, p = 0.004) (Fig 7.3.4E). Thus, the f and V_T responses were affected by PNS in the opposing manner during the first 5 min of hypercapnic exposure. In agreement with this, the V_E response in PNS group was similar during the first half of hypercapnic challenge ($F_{(1,69)}$ < 1 for prenatal condition), while being significantly increased during the second half ($F_{(1,69)}$ = 8.19, p = 0.006) (Fig 7.3.5F).

7.2.4 Colorectal Distension Does Not Indicate Visceral Hypersensitivity in PNS Animals

To assess if prenatally stressed animals have different sensitivity to noxious visceral stimuli, we exposed animals to CRD which evokes pain-related cardiovascular and visceromotor responses. Following exposure to CRD all animals demonstrated a blood pressure response and tachycardia; APsys, APdias and HR were significantly elevated vs pre-CRD values starting from 2-3 min of CRD protocol, which corresponds to 20-30 mmHg of distension pressure (two-way ANOVA, analysis of contrasts); which is in agreement with previous studies (Ness and Gebhart, 1988a). With increasing intensities of colonic distension, both blood pressure and HR gradually increased indicating intensification of nociceptive stimuli. Peak APsys response was on average 33 mmHg in Control and 32 mmHg in PNS group; APdias – 33 and 30 mmHg; HR – 47 and 31 bpm, respectively. Cessation of colonic distension resulted in an immediate restoration of APsys and APdias to the pre-CRD values; although HR recovery to baseline was less rapid.

Time course analysis of haemodynamic response showed that PNS animals had significantly higher values of APsys over the CRD session and the recovery period (significant effect of PNS, $F_{(1,216)}$ = 65.37 for CRD and $F_{(1,212)}$ = 48.68 for recovery, p < 0.0001) (Fig 7.3.6A). However, no interaction effect between group and CRD/recovery was observed indicating that PNS animals developed a similar pressure response to both CRD and recovery as controls. Similar patterns of change were shown for APdias (Fig 7.3.6B). PNS animals exhibited significantly higher values of APdias over the CRD session and the recovery period (significant effect of PNS, $F_{(1,216)}$ = 14.92 for CRD and $F_{(1,212)}$ = 10.04 for recovery, p < 0.002). A non-significant interaction between PNS and CRD/recovery indicated an equal magnitude of APdias response in both groups. Unlike pressure responses, HR values were significantly higher in PNS animals over the recovery period only (significant effect of PNS, $F_{(1,212)}$ = 22.98, p < 0.0001) (Fig 7.3.6C). Interestingly, time course analysis of Delta HR changes (subtracting pre-CRD values from response) showed that PNS offspring had a

significantly blunted HR response to CRD compared to control controls (significant effect of PNS, $F_{(1,216)} = 13.5$, $p < 0.0001$) (Fig 7.3.6D).

Apart from cardiovascular response, noxious colonic distension evokes visceromotor reflex consisting of both phasic and tonic contractions of abdominal musculature. To address the sensitivity of visceromotor reflex in PNS animals, we further analysed the frequency of blood pressure spikes originating from phasic abdominal contractions (pain scoring); and the distension pressure that evoked the first pressure spike (pain threshold). Neither pain scoring, nor pain threshold differed between groups (Fig 7.3.6E, F).

7.2.5 PNS Offspring Have Elevated Transepithelial Ion Transport Response to Adrenergic Stimulation of Distal Colon

After an equilibration time of 60 min, the baseline I_{sc} values were similar in control and PNS distal colon samples, amounting to 66.0±8.2 and 52.1±7.9 $\mu A/cm^2$, respectively. TER measures showed no difference between groups as well: 57.9±5.0 $\Omega \cdot cm^2$ in control and 54.3±6.8 $\Omega \cdot cm^2$ in PNS tissues (Fig 7.3.7A-B).

Application of NE, a non-selective agonist of adrenergic receptors, on the serosal side of distal colon mucosal preparations induced a dose-dependent decrease in I_{sc} (Fig 7.3.7A). The I_{sc} response typically had a rapid onset, reaching its peak value within the first 1.5 – 3 min after NE application, followed by a slow decline. A decrease in I_{sc} induced by adrenoceptors stimulation was shown to be caused by a sustained increase in K^+ secretion in the colonic epithelium (Horger et al., 1998) and mediated by both α- and β-receptor subtypes (Schultheiss and Diener, 2000). We did not observe a transient increase in I_{sc} preceding a long lasting decrease of current in response to NE or epinephrine application, as previously seen (Horger et al., 1998; Schultheiss and Diener, 2000) in both proximal and distal rat colon. However, in the studies cited above the NE response was not constant and displayed a biphasic pattern only in 48% of distal colon specimens tested. Tissue samples collected from PNS animals exhibited a considerably higher I_{sc} response to NE stimulation at the highest dose applied (50 µM), increasing on average 48% decrease in I_{sc} in comparison to a 28% decrease in control specimens ($p = 0.02$, independent Student's t-test) (Fig 7.3.7A). At a submaximal concentration of 5 µM NE induced an overall prolonged response in PNS colonic tissue (time x PNS interaction effect, $F_{(10,80)} = 2.54$, $p = 0.01$), although the maximal I_{sc} response was not significantly changed ($P > 0.05$, independent Student's t-test) (Fig 7.3.7A).

BCH, an agonist of M_3 muscarinic receptors, induced a dose-dependent increase of I_{sc} in 1 – 10 μM concentrations (Fig 7.3.7B). This I_{sc} response is known to be caused by activation of Cl^- secretion across the epithelial layer and is mediated predominantly by the M_3 receptor subtype (O'Malley et al., 1995). PNS had no effect on colonic ionic transport response to cholinomimetic stimulation (Fig 7.3.7B).

7.2.6 PNS Affects the Innervation Density Pattern in Distal Colon Muscular Layers

Staining of distal colon muscular layers against pan-neuronal microtubule protein β-III tubulin revealed an impressively dense and fine neuronal network in circular muscle layers (Fig 7.3.8A, right panel) with a less densely innervated in the longitudinal muscles, and a clear pattern of myenteric plexus between them (Fig 7.3.8A, left panel). The total density of innervation, summed in both muscular layers, was found to be 12% lower in PNS samples than in the control group (Fig 7.3.8B). To understand what type of innervation was affected by PNS, we further stained TH-expressing neurons which belong to sympathetic nerve system. In agreement with other studies (Park et al., 1995), sympathetic terminals were present in both longitudinal and circular muscle layers, as well as innervating neuronal ganglia (Fig 7.3.8C, left and right panels). Quantitative analysis showed a strong trend towards decreased sympathetic innervation in the PNS group ($p=0.068$, independent Student's t-test, Fig 7.3.8D). Anti-ChAT staining, revealing parasympathetic terminals and cholinergic neurons originating from ENS, showed no differences between control and PNS group (Fig 7.3.8E-F). The density of myenteric plexus ganglia were also similar in both groups (Fig 7.3.8G). Further investigation is required to determine if neurogenesis of these neurons could be affected by PNS.

Table 2 Baseline breathing parameters in normoxia

Parameter	Control (n=8)	PNS (n=9)	
Tidal volume, ml	0.52 ± 0.02	0.54 ± 0.01	ns
Breathing frequency, breaths/min	71.1 ± 2.9	74.4 ± 1.9	ns
Minute ventilation, ml/min	35.3 ± 1.2	38.2 ± 1.3	ns
CV of breathing frequency, %	13.1 ± 1.7	**22.0 ± 3.2**	p = 0.032*
Apnoea index	8.3 ± 0.3	9.9 ± 0.9	ns

Baseline breathing during normoxia (room air-flow) was recorded for 30 min in 8 Control and 9 PNS adult males from Cohort 2. Data are presented as Mean ± SEM. * $p<0.05$ - significant difference from control group (independent Student's t-test).

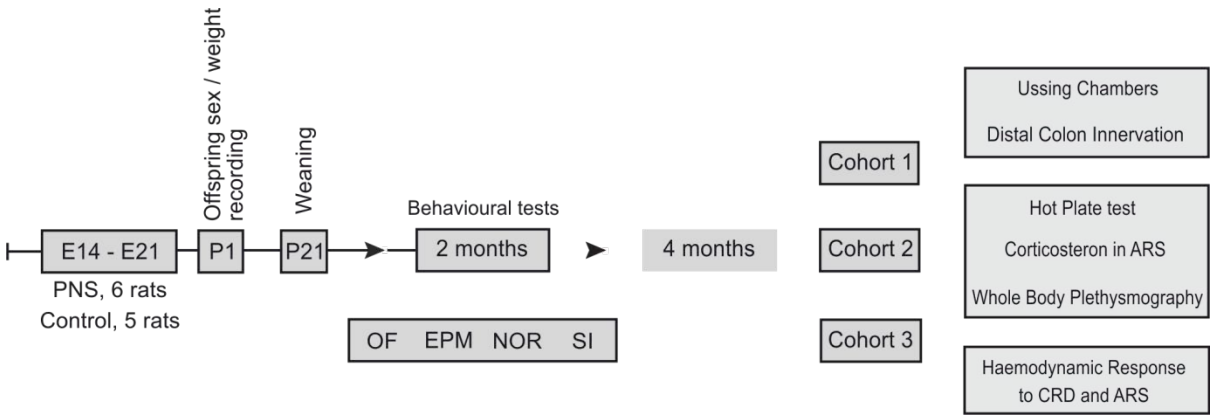
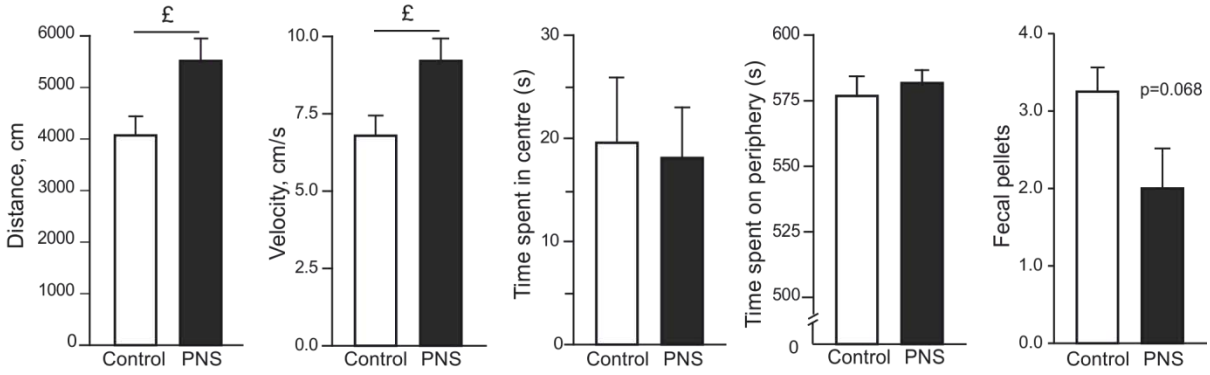
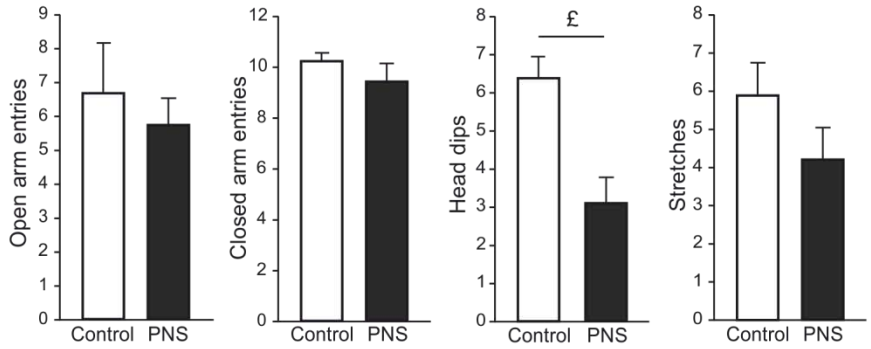


Figure 7.3.1: Outline of experimental procedures performed in chronological order. E14-E21 – embryonic days 14 – 21; P1 and P21 – postnatal days 1 and 21; PNS – prenatal stress; OF - open field; EPM – elevated plus maze; NOR – novel object recognition; SI – social interaction; ARS - acute restraint stress; CRD – colorectal distension. For procedure details, see text.

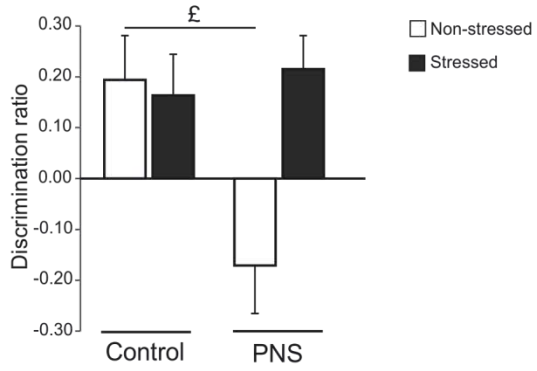
A. Open Field test



B. Elevated Plus Maze test



C. Novel Object Recognition test



D. Social Interaction test

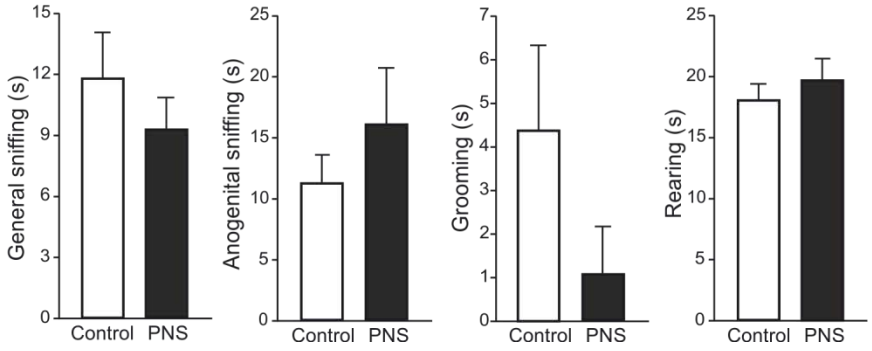


Figure 7.3.2: Effect of prenatal stress on anxiety, cognition and social interaction. Control (n=10) and PNS (n=10) rats at 2 months of age were subjected to the battery of behavioural tests. (A) OF test revealed hyperactivity in PNS offspring; distance travelled and average velocity were significantly higher in PNS group. (B) EPM did not show significant elevation of anxiety like behaviour in PNS animals; only numbers of head dips were reduced. (D) SI activity was unaffected by PNS. £ $p < 0.05$ with respect to control; independent Student's t-test for distance travelled and velocity, Mann-Whitney U-test for fecal pellets and head dips.

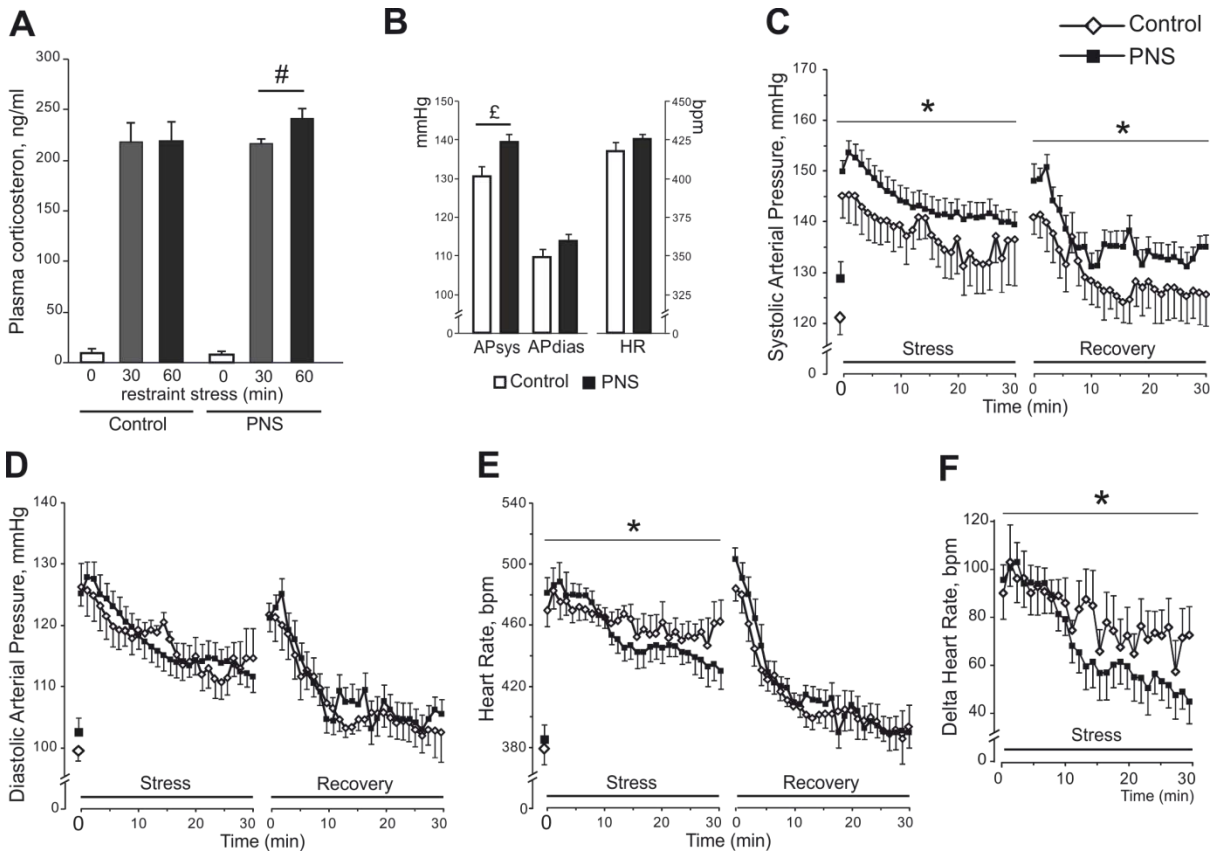


Figure 7.3.3: Effect of prenatal stress on corticosterone release and haemodynamic response to acute stress exposure.

(A) Control (n=8) and PNS (n=9) rats from cohort 2 were exposed to 60 min restraint stress. Plasma CORT was analysed immediately after (time point 0, baseline), and in 30 and 60 min after the onset of stress session. PNS offspring had unchanged baseline CORT levels, but more sustained CORT release in response to stress. (B–E) Control (n=5) and PNS (n=7) rats from cohort 3 were exposed to 30 min restraint stress followed by 30 min of recovery. Blood pressure was recorded prior to (time point 0), during the stress session and the recovery period. PNS offspring had elevated levels of APsys at a quiet rest (B), during the stress exposure and the recovery period (C). APdias response remained unchanged (D). The HR peak response was similar in magnitude in PNS and Control animals; however, PNS rats showed faster decline of HR throughout the stress session (E, F). Data are presented as Mean ± SEM. # - significant CORT increment from 30 min ($p = 0.02$, paired samples t-test); £ - significant difference from control group ($p = 0.02$, independent Student's t-test); * - significant main effect of PNS on APsys ($p < 0.0001$), HR ($p = 0.02$) and delta HR ($p < 0.0001$, two-way ANOVA).

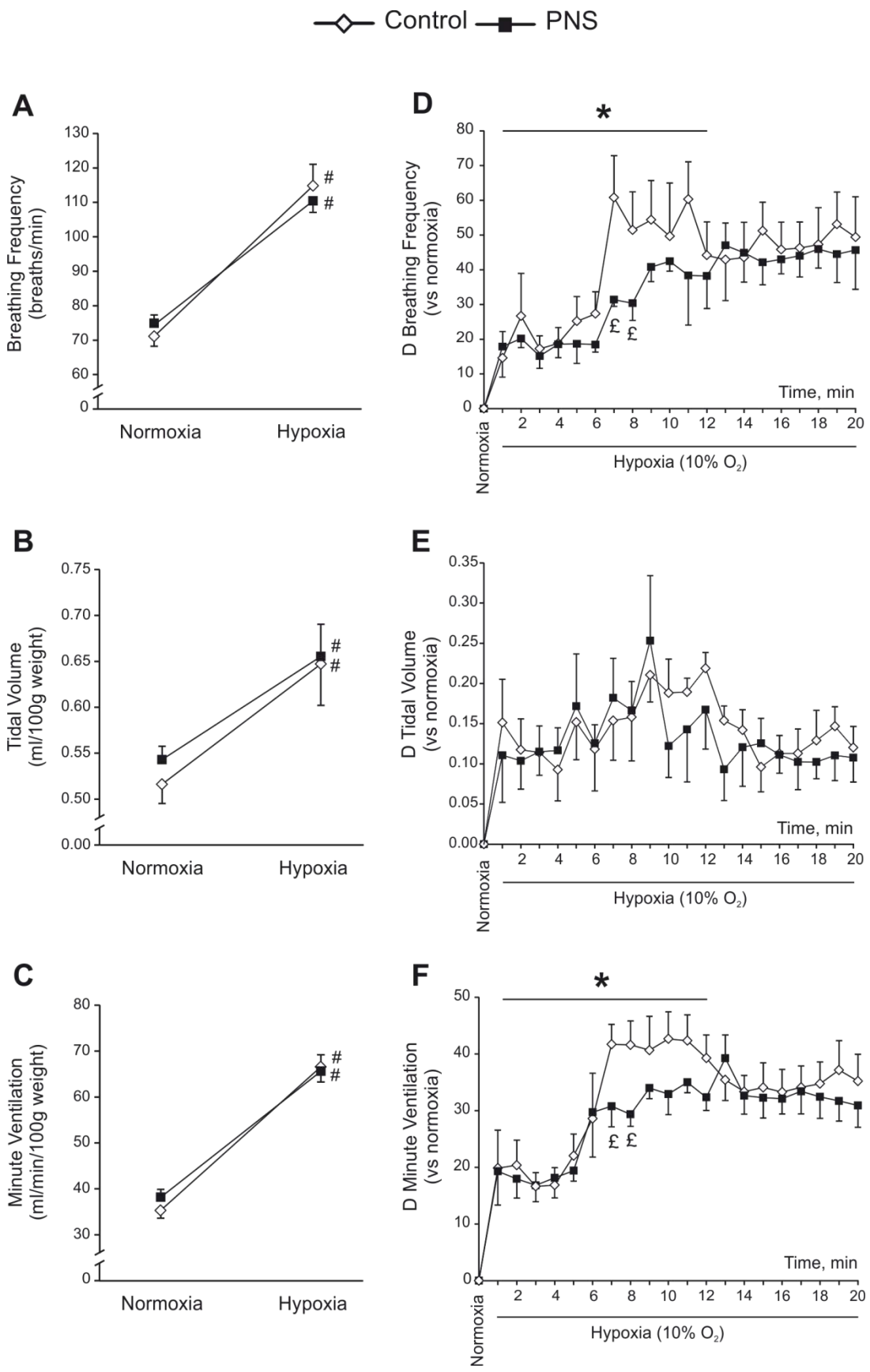

Control
PNS
A
Breathing Frequency (breaths/min)
Normoxia
Hypoxia
B
Tidal Volume (ml/100g weight)
C
Minute Ventilation (ml/min/100g weight)
D
D Breathing Frequency (vs normoxia)
E
D Tidal Volume (vs normoxia)
F
D Minute Ventilation (vs normoxia)
Time, min
Hypoxia (10% O2)

Figure 7.3.4: Effect of prenatal stress on ventilatory response to hypoxia.

Key ventilatory parameters (f, V_T and V_E) were assessed in freely-behaving rats using WBP. Baseline respiratory recordings were obtained in Control (n=8) and PNS (n=9) rats from cohort 2 in normoxic (room air flow) conditions (Normoxia). Following this animals were exposed to acute hypoxic (10% O_2 for 20 min) challenge. (A-C) Hypoxic exposure induced a similar increase in ventilatory parameters, averaged per challenge, in both groups. (D-F) Time course analysis of the ventilatory response indicated a decreased increment of f and V_E in PNS animals within the first 12 min of hypoxic exposure; V_T response was not changed. Data are presented as Mean ± SEM. # - significant change from baseline values in normoxia ($p < 0.001$ for hypoxia effect, mixed design ANOVA); * - significant effect of PNS on V_E and f ($p < 0.005$, two-way ANOVA); £ - significant difference from corresponding time point in Control group ($p < 0.05$, independent Student's t-test).

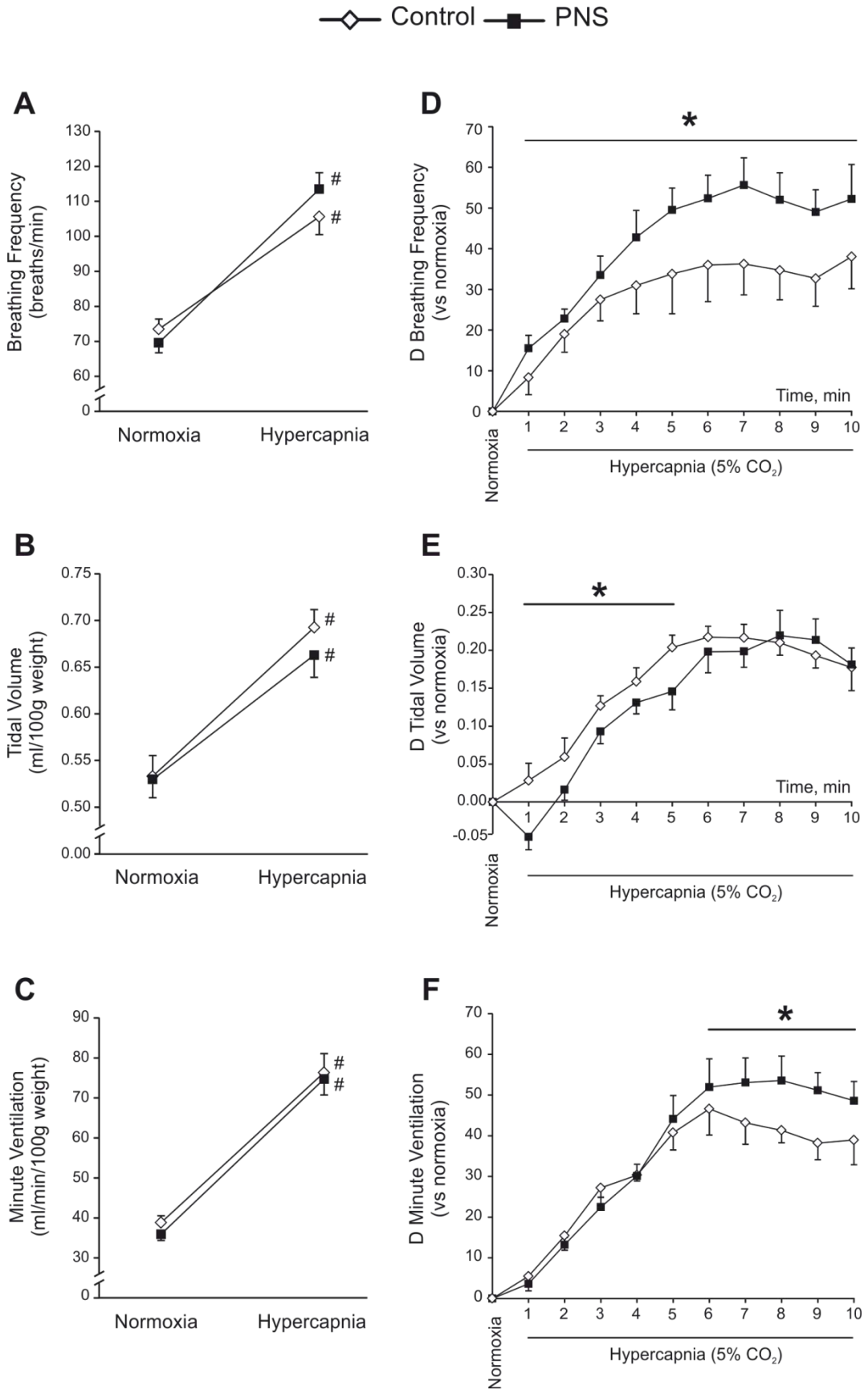

Control
PNS
A
Breathing Frequency (breaths/min)
Normoxia
Hypercapnia
#
#
D
D Breathing Frequency (vs normoxia)
*
Time, min
Normoxia
Hypercapnia (5% CO2)
B
Tidal Volume (ml/100g weight)
Normoxia
Hypercapnia
#
#
E
D Tidal Volume (vs normoxia)
*
Time, min
Normoxia
Hypercapnia (5% CO2)
C
Minute Ventilation (ml/min/100g weight)
Normoxia
Hypercapnia
#
#
F
D Minute Ventilation (vs normoxia)
*
Normoxia
Hypercapnia (5% CO2)

Figure 7.3.5: Effect of prenatal stress on ventilatory response to hypercapnia.

Key ventilatory parameters (f, V_T and V_E) were assessed in freely-behaving rats using WBP. Respiratory recordings were obtained in control (n=8) and PNS (n=9) rats from cohort 2 in normoxia and in response to acute hypercapnic (5% CO_2 in 95% O_2, 10 min) challenge. (A-C) Exposure to hypercapnia induced an increase in f, V_T and V_E values in Control and PNS animals, without significant between-group differences in averaged per challenge response. (D-F) Time course analysis of the ventilation showed an overall increase in f response in PNS group, accompanied by a decrease in tidal volume response during the first 5 min of hypercapnia exposure. V_E response was accordingly enhanced within the last 5 min of hypercapnic challenge. Data are presented as Mean ± SEM. # - significant change from normoxic values ($p < 0.001$ for gas mixture effect, mixed design ANOVA); * - significant effect of PNS on f, tidal volume and V_E ($p < 0.006$, two-way ANOVA).

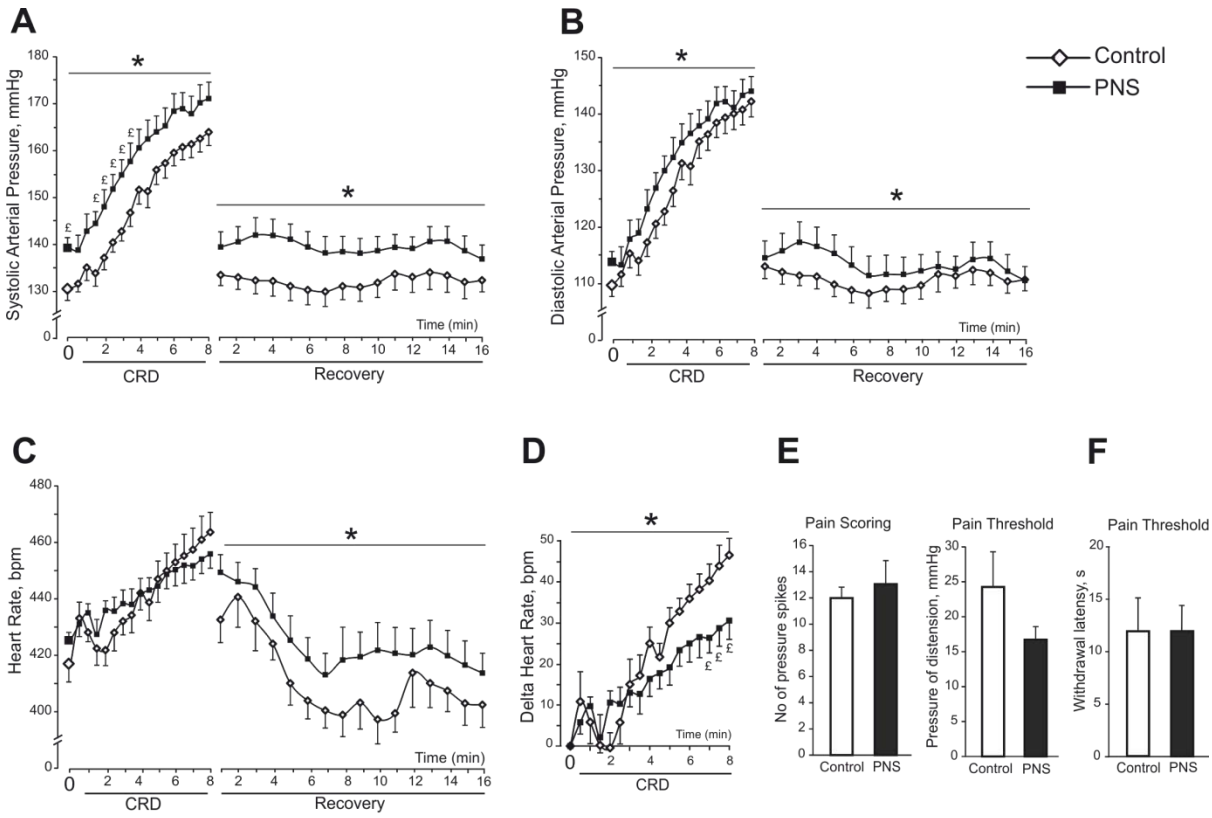


Figure 7.3.6: Effect of PNS on haemodynamic response to noxious CRD.

Control (n=8) and PNS (n=9) rats from cohort 3 were exposed to CRD procedure (8 min, 0-80mmHg ascending paradigm), followed by 15 min of recovery. Blood pressure was recorded prior to (time point 0), during the CRD session and the recovery period. (A, B) Time course analysis of haemodynamic response to colonic distension showed that PNS animals had elevated values of APsys and APdias during the CRD and the recovery period. (C) HR values were elevated in PNS group during the recovery period only. (D) Delta HR analysis (eliminating differences in pre-CRD values) showed that PNS animals had a decreased HR response to CRD. (E) The total number of blood pressure spikes (left panel), generated by pain-evoked abdominal contractions, as well as the threshold distension pressure (right panel) did not differ between PNS and Control groups, indicating similar visceral sensitivity to CRD. (F) Somatic pain sensitivity, estimated by hind paw withdrawal latency in hot plate test, was not affected by PNS. * - significant effect of PNS on APsys, APdias and HR ($p < 0.001$, two-way ANOVA); £ - significant difference from corresponding time point in Control group ($p < 0.05$, independent Student's t-test).

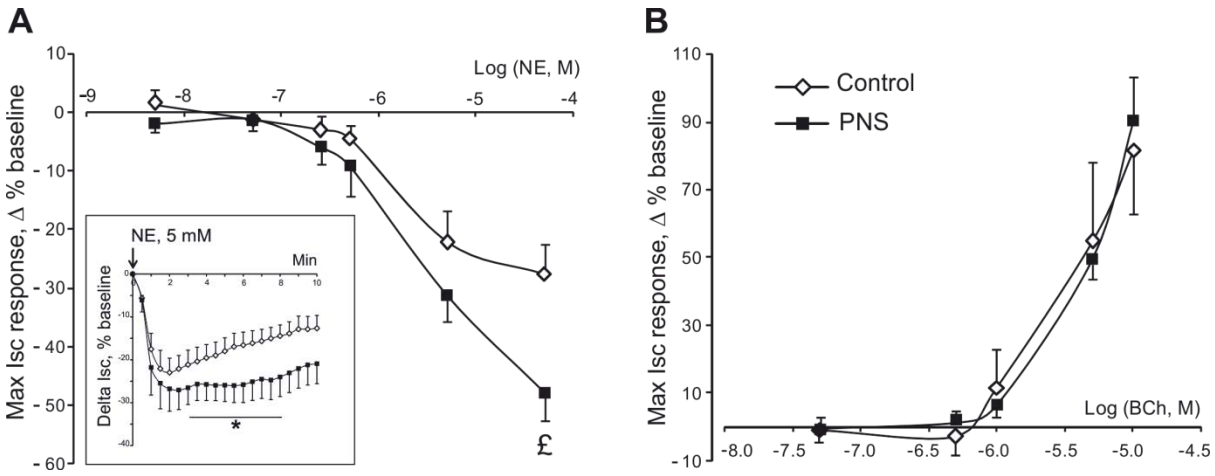


Figure 7.3.7: Effect of prenatal stress on short-circuit current (I_{sc}) response to adrenergic and muscarinic receptors stimulation in distal colon.

I_{sc} measurements were taken in distal colon mucosal/submucosal preparations obtained from control (n=6) and PNS (n=6) rats from cohort 1. (A) Dose-dependent effect of NE on I_{sc}; NE was tested in $5 \cdot 10^{-9}$, $5 \cdot 10^{-8}$, $2.5 \cdot 10^{-7}$, $5 \cdot 10^{-7}$, $5 \cdot 10^{-6}$ and $5 \cdot 10^{-5}$ M concentrations. Tissue specimens from PNS group had an enhanced peak I_{sc} response to the maximal dose of NE (50 µM). The inset depicts the dynamics of I_{sc} changes induced by submaximal dose of NE (5 µM). Time course analysis indicated a more prolonged I_{sc} response to NE in PNS colon. (B) Dose-dependent effect of BCH on I_{sc}; BCH was tested in $5 \cdot 10^{-8}$, $5 \cdot 10^{-7}$, $1 \cdot 10^{-6}$, $5 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ M concentrations. An increase in I_{sc} induced by muscarinic receptor activation remained unaltered in PNS animals. Data are presented as Mean ± SEM. £ - significant difference from corresponding dose in control group ($p = 0.02$, independent Student's t-test); * - significant effect of PNS on I_{sc} response ($p = 0.01$, two-way ANOVA).

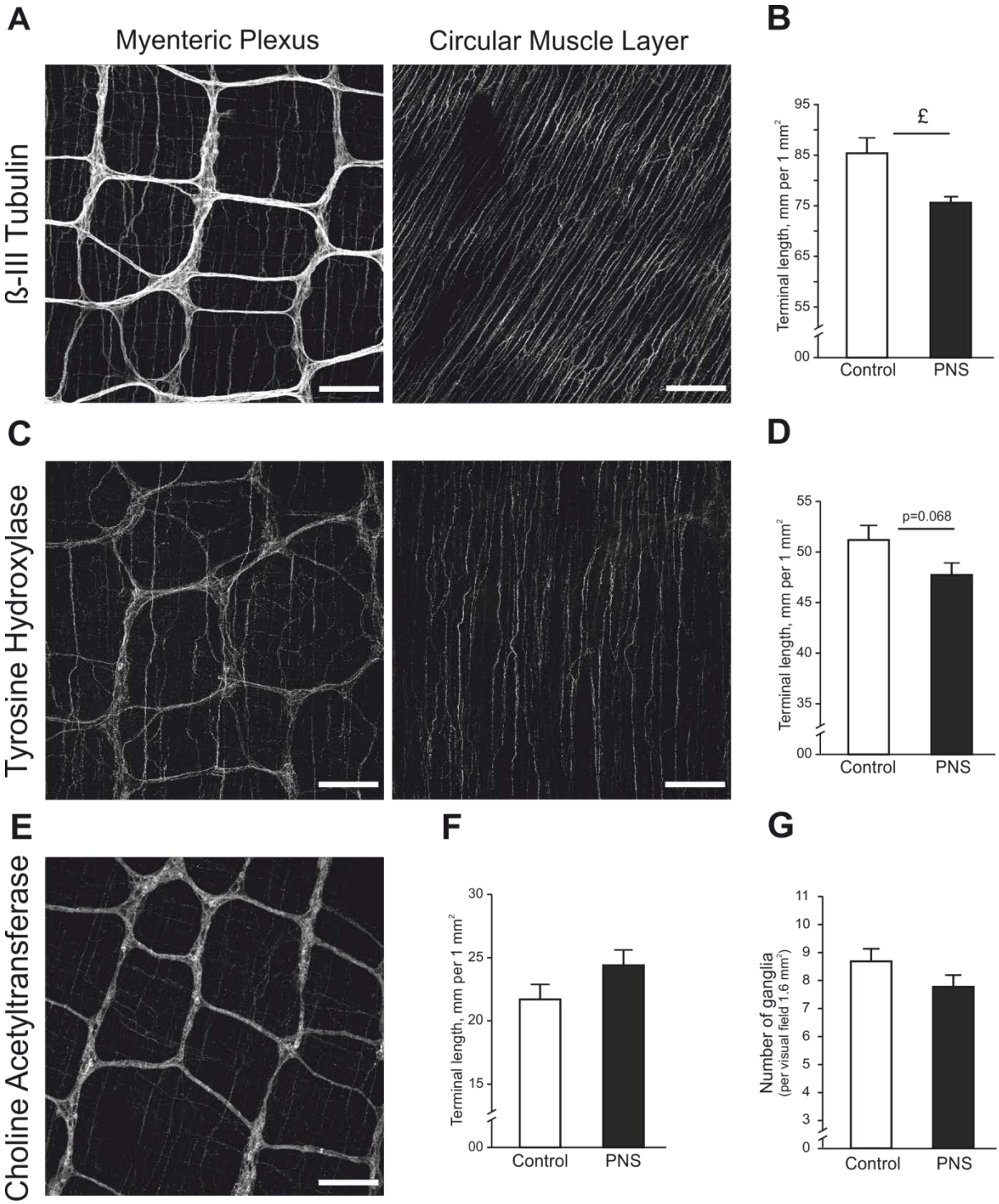

A
Myenteric Plexus
Circular Muscle Layer
ß-III Tubulin
B
£
Terminal length, mm per 1 mm²
95
85
75
65
55
00
Control
PNS
C
Tyrosine Hydroxylase
D
p=0.068
Terminal length, mm per 1 mm²
55
50
45
40
35
00
Control
PNS
E
Choline Acetyltransferase
F
Terminal length, mm per 1 mm²
30
25
20
15
00
Control
PNS
G
Number of ganglia
(per visual field 1.6 mm²)
11
10
9
8
7
6
5
4
3
0
Control
PNS

Figure 7.3.8: Effect of prenatal stress on innervation density in distal colon muscular preparations.

Whole mount muscular preparations of distal colon, taken in control (n=6) and PNS (n=6) rats from cohort 1, were stained against β III tubulin (total innervation), TH (sympathetic innervation) and ChAT (ENS and parasympathetic fibres). Density of innervation was expressed in terminal length (mm) per 1 mm^2 of specimen. (A, B) Beta III tubulin staining of terminal network in longitudinal muscle layer with overlying myenteric plexus (left panel) and in circular muscle layer (right panel). The total innervation density, summed in both layers, was decreased in PNS group (graph). (C, D) TH staining in longitudinal muscle layer with overlying myenteric plexus (left panel) and in circular muscle layer (right panel). The density of sympathetic fibres, summed in both layers, showed a strong trend to be decreased in PNS animals (graph). (E, F) ChAT staining in two muscular layers with myenteric plexus in between. Density of ChAT-expressing fibres was similar in both Control and PNS groups. (G) Density of neural ganglia in myenteric plexus was unaffected by PNS. £ - significant difference from Control group ($p = 0.002$, independent Student's t-test).

7.4 Discussion

Our results demonstrate several novel findings on the effects of PNS on the development of offspring in addition to supporting previous studies findings. Behavioural analysis was conducted at 2 months of age in male offspring only to eliminate the effects of the oestrous cycle potentially obscuring data. Animals in the PNS group displayed traits of hyperactivity illustrated by increases in distance travelled and velocity in the OF test. Similar traits of hyperactivity in offspring as a result of PNS have been reported in a study by Bronson and Bale in which PNS animals displayed increased locomotor activity in a light-dark exploration test (Bronson and Bale, 2014). However, this effect was only observed in males and was not evident in females. A potential cause of the hyperactive traits in PNS animals may possibly be due to changes in the mesocorticolimbic dopaminergic circuitry which is involved in spontaneous and novelty-stimulated locomotion (Hooks and Kalivas, 1995; Canales and Iversen, 2000; Son et al., 2007). Bronson and Bale found alterations in both D1 (increased) and D2 (decreased) receptor expression in the prefrontal cortex and nucleus accumbens respectively (Bronson and Bale, 2014). These changes in receptor expression may lead to the observed hyperactivity in PNS animals and also support the theory that PNS may increase the likelihood of affected offspring developing neuropsychiatric disorders.

PNS animals also exhibit changes in their stress reactivity, as measured by CORT release, in response to restraint stress with offspring from stressed mothers demonstrating an altered pattern of CORT release. Concentrations of CORT would be expected to reach maximal values at roughly 30 minutes post onset of the stressor, yet in PNS animals there is a significant increase in CORT levels from 30 miuntes to 60 minutes when compared to control animals. A human study which examined cortisol release in 7 month old children whose mothers had experienced clinical levels of maternal anxiety (according to DSM-IV criteria) during mid/late gestation found that these children had higher cortisol concentrations, or a lower decline in cortisol levels, 40 minutes after a psychological stressor (Grant et al., 2009). Abnormal concentrations of CORT/cortisol in the circulation can lead to a number of complications including hypertension of which we found evidence for in our study of cardiac function.

The first study to reveal that PNS causes a hypertensive like condition was conducted by Holst and colleagues in which they demonstrated a sex specific increase in APsys in only male rats (Holst et al., 2002). However, in this study they failed to investigate cardiovascular

response to stress. A more in depth follow up to this original study was carried out by a separate group in which they examined specific cardiovascular responses to stress in prenatally stressed animals (Igosheva et al., 2004). This study utilised a similar restraint stress protocol during the last trimester of pregnancy as our own study and found that PNS animals had an increased peak of APsys with an extended duration of APsys responses both during ARS and also during recovery. In our study we show that PNS animals have an elevated APsys under baseline conditions and also display increased APsys under restraint stress when compared to controls. In addition to examining cardiovascular responses to restraint stress we also investigated the effects of a noxious stimulus, in this case CRD, on cardiovascular responses. Similar to our findings under restraint stress conditions PNS animals displayed elevated APsys; however, there was also an increase APdias both during CRD and also during recovery. An interesting observation in both restraint stress and CRD tests was that even though there was increased APsys and APdias in PNS animals controls had a greater rise in HR in response to either stressor. A potential explanation for this could be due to adrenergic receptor downregulation or decreased sensitivity to NE in the sinoatrial node. As PNS animals demonstrate a heightened stress response this may occur as a compensatory mechanism to avoid ischaemic damage to cardiac tissue through regular periods of elevated HR. Overall our results display evidence that PNS may increase the risk of developing hypertension in addition to other stress-related cardiovascular disorders.

A novel section of this study was the investigation of the effects of PNS on respiratory control and function under basal conditions in addition to both hypercapnic and hypoxic challenges. Under normoxic conditions there were no observed differences between control and PNS animals in any of the parameters measured. Under hypoxic conditions although there was the expected elevation in f and V_E in both groups, PNS animals showed a slower incline in both parameters when compared to controls. The respiratory response to hypercapnia showed an opposite trend in PNS animals when compared to controls with f and V_T being elevated to a greater extent in PNS group. These differences in response to either hypercapnic or hypoxic challenges could indicate changes in the function of the respiratory control centres in the brainstem as a result of PNS. Within the central nervous system respiratory output is dictated by complex interactions between both central and peripheral chemosensory elements (Day and Wilson, 2009; Blain et al., 2010; Smith et al., 2010). NMDARs play a role in mediating peripheral chemoreceptor afferent input, specifically being involved in responses to hypoxia whilst not appearing to be essential for the hypercapnic

ventilatory response (Ohtake et al., 2000; Waters and Machaalani, 2005). The hypercapnic respiratory response in adults is controlled by a population of early growth response 2 transcription factor (Egr2) positive neurons within the brainstem, namely the pons and medulla (Ray et al., 2013). It has been shown that perturbation of these Egr2 cells causes a significant decrease in respiratory response to a hypercapnic challenge with no change in baseline breathing under normoxia (Ray et al., 2013). The observed differences in our study between control and PNS animals may be due to several possible reasons. Firstly, PNS may induce changes in central or peripheral chemosensory elements causing them to be hypersensitive to changes on CO_2 or O_2 levels therefore eliciting an exaggerated respiratory response. An alternative theory is that PNS causes alterations in the receptors or pathways that control the respiratory response to a hypercapnic or hypoxic challenge resulting in an aberrant ventilatory response. Taken together our results demonstrate that under normoxic conditions there are no differences between control and PNS animals but that if faced with respiratory challenge PNS animals display a defective response.

A physiological system that to the best of our knowledge had not been examined with regard to the effects of PNS was the lower gastrointestinal tract. There have been several studies conducted using the maternal separation model of early life stress which provided strong evidence for this model predisposing offspring to visceral hypersensitivity and irritable bowel syndrome in later life (Malley, 2011; O'Mahony et al., 2011; Moloney et al., 2012). In our study we found no evidence for visceral hypersensitivity in the CRD test with no differences in pain scores between control and PNS animals. However, using an ussing chamber as a method of assessing cholinergic and adrenergic receptor activation and net ion transport across the mucosa, in response to their respective agonists BCH and NE, we discovered that PNS animals had a heightened response to NE. This observed response may be an indication of decreased gut motility in PNS animals, potential support of this concept may be taken from the observation that there were less fecal pellets present in the OF arena following testing of PNS animals even though this result was just outside significance. It has been previously been shown in mice that BCH induced changes in I_{sc} can be independent of M3 receptors, compensatory mechanisms exist in the cholinergic system which may account for the absence of change in stressed mice in response to BCH and further immunohistochemical or more specific pharmacological studies are required to fully determine the impact of PNS on the colonic cholinergic system (Hirota and McKay, 2006).

Further to this we examined distal colonic innervation in the circular muscle and myenteric plexus layers using confocal microscopy. Here we investigated total innervation through β-III tubulin staining, sympathetic innervation by staining for TH and ChAT staining which identifies parasympathetic neurons as well as a subpopulation of sympathetic neurons both of which play roles in dictating gut motility. We found no differences between control and PNS animals with TH or ChAT immunostaining but there was a significant overall deficit in innervation (β-III tubulin) in the PNS group. This observed reduction in innervation of the distal colon may lead to disturbances in normal gastrointestinal function in addition to what we have already shown and warrants further investigation.

Taken together these results illustrate that there are multiple subtle changes to a number of physiological systems caused by prenatal exposure to maternal stress. Some of the systems effected such as the cardiovascular and respiratory systems may pose a greater risk than others for affected offspring developing more serious complications in later life. We provide evidence for alternations in the gastrointestinal and respiratory systems that had previously not been demonstrated which require further in depth studies to elucidate possible mechanisms that lead to our observed changes. Overall maternal exposure to stressful circumstances over prolonged periods of time proves detrimental to the normal development of offspring and mothers exposed to these conditions require careful monitoring and interventional therapies where possible during pregnancy.

8. Final Discussion and Future Studies

In this thesis I set out to investigate the effects of MIA and PNS on the developmental process. Although these models are often dealt with separately there are several factors which overlap between the two and thus essentially link them through common traits and effects. Maternal stress has previously been shown to cause an increase in the levels of the stress hormone cortisol but also results in elevated levels of cytokines (Elenkov and Chrousos, 2002; Coussons-Read et al., 2005; Coussons-Read et al., 2007). MIA has been demonstrated to increase the concentrations of a number of proinflammatory cytokines (Cai et al., 2000; Boksa, 2010) which in turn are capable of activating the HPA axis resulting in the release of cortisol (Dunn, 2000). From these observations we can establish that these two separate models even though primarily activating two separate systems, stress and immune, cause similar effects namely an increase in circulating stress hormones and proinflammatory cytokines.

IL-1β has been shown by several studies to be increased in the developing fetal brain following maternal infection (Zaretsky et al., 2004; Aaltonen et al., 2005b). In the first chapter I set out to investigate the effects of elevated levels of IL-1β, which mimics an *in vivo* proinflammatory environment, on NPCs examining several different aspects which would be relevant from a developmental standpoint. A number of neurodevelopmental and neuropsychiatric disorders are thought to arise from improper migration of NPCs or alterations in the makeup of cellular phenotypes (Meyer et al., 2009b; Short et al., 2010; Vuillermot et al., 2010). We provided evidence that a proinflammatory environment results in several detrimental consequences for developing NPCs in relation to the points above. IL-1β causes a reduction in the proliferation of NPCs which we have shown not to be caused by an increase in cell death through analysis of specific protein makers. Rather this halt in proliferation seems to be caused by an induction of differentiation by IL-1β which promotes a glial fate with a subsequent decrease in neurogenesis which in an *in vivo* setting would result in an imbalance between neurons and glia potentially resulting in an insufficient number of neurons. This reduction in the neuronal population may lead to improper connections being established within and between brain regions, in cases where these NPCs could have been in the process of migration it would ultimately produce perturbed formation of organisational layers that would affect function in these brain regions.

In this section we also elucidated the signalling pathway through which IL-1β mediates its effects on NPCs as understanding these molecular events aids in creating effective interventional therapies. IL-1β has also been shown to signal through alternative pathways such as NF-κB and SAPK/JNK depending on the cell type and also age (Wang et al., 2007). We have also shown that antagonism of the functional IL-1 receptor, IL-1R1, is sufficient to prevent the negative effects of exposure during development. The main issue with blocking IL-1β signalling during developmental stages is that even though it produces detrimental effects in a specific region IL-1β signalling may be necessary for normal development in other cells types (Vitkovic et al., 2000; Deverman and Patterson, 2009; Stolp, 2013). Approaches that would allow site specific delivery of antagonists have two main disadvantages; firstly they involve invasive techniques and secondly these techniques would be especially complicated in an *in utero* setting. Systemic administration of antagonists would risk interference in too many other cell types other than the ones specifically being targeted therefore the risk to benefit ratio outweighs the potential therapeutic value of this approach.

Both MIA and PNS produce an inflammatory phenotype in affected offspring through fetal programming (Vanbesien-Mailliot et al., 2007; Nicholas et al., 2013) which potentially contributes to the developmental of neuropsychiatric disorders in later life. A number of affective disorders have been associated with deficits in adult neurogenesis which has been linked to elevated levels of proinflammatory cytokines which could be caused by fetal programming of the immune system towards a proinflammatory phenotype (Lemaire et al., 2000; Goshen et al., 2007; Kuzumaki et al., 2010; Kubera et al., 2011; Zunszain et al., 2012; Wu et al., 2013) or by altered stress responses to stressful life events (Hunter et al., 2011). Such instances of 'fetal programming' often depend on the timing and nature of the insult, a prenatal event that leads to either transient or lasting aberrant changes may have no effect at a different developmental time point. These windows of sensitivity were coined 'critical periods' (Widdowson and McCance, 1975) and this is what the second section of this thesis examined.

Identification of the various 'critical periods' for different functional systems, tissues and cells types are crucial for developing strategies to alleviate damage that may be caused during these times and also for predicting potential negative outcomes for offspring. In the previous section we provided evidence that E14 NPCs isolated from the developing midbrain were susceptible to the detrimental effects of a proinflammatory environment. We next looked at

two other embryonic ages, one younger and one older and investigated whether exposure of NPCs to the cytokines TNF, IL-6 or IL-1β exhibited similar effects to those previously observed. Interestingly we discovered that NPCs isolated from E12 embryos were unaffected whilst those from E16 embryos displayed the same vulnerability as E14 NPCs. E12 NPCs were displayed no changes in cell fate specification or alterations in proliferation. It has previously been shown that the timing of a maternal immune challenge dictates the nature of behavioural abnormalities observed in offspring as well as influencing the nature of the immune response and expression profiles of cytokines (Meyer et al., 2006a; Meyer et al., 2006c). Results from this study have provided significant evidence for the presence of 'critical periods' and have demonstrated how drastically they can alter the susceptibility of specific cell types to an insult. At the age which cytokine exposure had no negative effects it is important to mention that we did not any observe any trophic effect of these cytokines either.

For the *in vivo* study of this section pregnant dams received a single dose of LPS at either E12 or E16 before embryos were harvested and NPCs cultured without the presence of any cytokines. Here we demonstrated how a single short-term exposure to an immune antigen resulting in MIA is capable to producing 'fetal programming' effects which persist even when the affected tissues have been removed from the proinflammatory environment. NPCs from control and LPS animals of both ages were cultured under proliferative conditions with E12 NPCs not displaying any reduction of proliferation whereas E16 NPCs displayed a significant reduction in neurosphere size. These findings indicate that MIA causes epigenetic changes that can influence receptor expression and alter signalling pathways. Epigenetic changes such as these have the potential to persist for the entirety of the life-span and even be passed onto future generations leading not only to the development of disorders in the affected generations but possibly even their offspring (Drake and Liu, 2010; Meyer et al., 2011; Buchwald et al., 2012).

In the final chapter of this thesis we used an *in vivo* model of PNS utilising the well established restraint stress protocol in which pregnant dams were subjected to 3 sessions of restraint stress per day for a total of 7 days from E14 to E21 (Clancy et al., 2001). Results from behavioural testing indicated that PNS animals displayed signs of locomotor hyperactivity which may be indicative of a hyperactivity disorder although this behavioural trait has also been described as a sign of a schizophrenic-like state in other studies (Holloway et al., 2013; Matrisciano et al., 2013). This observed behavioural alteration could be as result

of epigenetic changes in either the GABAergic, DAergic or NMDA-related systems of which some evidence has been provided before (Wilson et al., 2012; Matrisciano et al., 2013; Bronson and Bale, 2014). We did not find any depressive-like symptoms as have been reported in several studies before (Szymańska et al., 2009; Zhang et al., 2013) which may be due to the nature, duration or timing of restraint stress in our model. Maternal stressors during certain periods of development is thought to cause an altered stress response in adult offspring, here we provide evidence for this line of thought. CORT levels did not differ between groups when baseline recordings were taken demonstrating no change in basal activity, however, when subjected to restraint stress PNS animals differed in their response to control animals. CORT release generally peaks within 30 miuntes after a stressful stimulus is applied and this is what we observed in the control group, the PNS group though displayed a further increase in CORT levels from 30 to 60 minutes. This prolonged CORT release leads to sustained levels of this stress hormone in the circulation which may increase the risk of affected animals developing hypertensive and metabolic disorders such as type II diabetes. Similar observations have been made in human studies which support our findings and demonstrate a clinical relevance of these findings (Grant et al., 2009; Hunter et al., 2011).

There have been relatively few animal studies investigating the potential role for PNS as a risk factor for developing cardiovascular disorders in later life. The first study examined only baseline recordings of arterial pressure in offspring following maternal stress but found that mean APsys was increased in PNS animals (Holst et al., 2002). A second study built on these original findings and subsequently looked at cardiovascular responses to a psychological stressor. Their results showed that PNS animals exhibited an elevated APsys response to the stressor with a prolonged duration of this response during restraint stress and also during recovery (Igosheva et al., 2004). In our study we provided supporting evidence for these previous studies finding similar results but we also examined an additional cardiovascular response. Along with a psychological stressor we also measured cardiovascular responses to a noxious stimulus, CRD, in which we could also monitor pain behaviours between groups and relate them to responses. PNS animals displayed an increased APsys when compared to controls both during the testing period and this also persisted into the recovery period. A noxious stimulus also elicited this response in APdias which had not been shown previously in the other studies. Another novel finding from this experiment was that although APsys and APdias were elevated in PNS they showed a decreased HR in comparison to controls. We hypothesise that this may due to epigenetic changes in receptor expression or innervation of

the cardiac pacemakers or the cardiac control centre in the brainstem. Supporting evidence for this theory comes from findings that PNS is capable of altering the density of β-adrenoreceptors and possibly their coupling to adenylyl cyclase in arteries. Additionally it has been shown that changes in the fetal environment can cause hyperinnervation of the sympathetic branch of the peripheral arterial system meaning that this effect or indeed hypoinnervation may be possible in other regions of the cardiovascular system (Ruijtenbeek et al., 2000).

The maturation of the respiratory system and its control mechanisms can be affected by a number of factors during development (Verkuyl et al., 2005). Modifications to the GABAergic system have been shown to contribute to the pathophysiology of several respiratory disorders in newborns (Darnall et al., 2006; Abu-Shaweesh and Martin, 2008). Disturbances in the serotonergic system such as deficits in medullary 5-HT have also been implicated in contributing to respiratory disorders (Fournier et al., 2013). In our plethysmography study we looked firstly at baseline measurements of f, V_E and V_T between control and PNS groups. We then examined respiratory responses to hypercapnic and hypoxic challenges; under both conditions PNS and control animals displayed the expected increase in f and V_E. However, PNS did show differences to control animals in both challenges, under hypoxic conditions these animals showed a slower incline in reaching the same level of response as controls. Under hypercapnic conditions the PNS group displayed a converse trend having a greater elevation in both parameters in relation to the control group. These discrepancies in PNS animals lead us to conclude that there are alterations in either the chemosensory elements of the respiratory system or the respiratory control centres located within the brainstem. Previous studies have shown that PNS is sufficient to cause changes in multiple systems of neurotransmission controlling a number of aspects of respiratory control, therefore it is reasonable to speculate that aberrant alterations to innervation or expression of chemosensory elements is possible. Basal control of breathing patterns in PNS animals are not affected by PNS as evidenced by no significant differences in any parameters measured at baseline.

A stressful environment during the neonatal period has been implicated in the development of dysfunction in the lower gastrointestinal tract. Multiple studies using a maternal separation model have provided numerous lines of evidence in support of this observation (O'Malley et al., 2011; O'Mahony et al., 2012a). To the best of our knowledge we have investigated for the first time the effects of PNS on the development of the lower gastrointestinal tract and its

function. We used a CRD test to assess whether there was any evidence of visceral hypersensitivity in PNS animals as this effect has been previously demonstrated in the maternal separation model (Moloney et al., 2012). We found no differences between control and PNS animals. We then looked at innervation of the colon examining total innervation through β-III tubulin staining and also sympathetic and parasympathetic neurons only through TH and ChAT staining respectively. There was a significant decrease in overall innervation in the PNS group but this deficit was not observed when TH and ChAT subtypes were analysed on their own. Ussing chamber evaluation of cholinergic and adrenergic receptor activation and the respective net ion transport across the mucosa was achieved though treatment with their respective agonist BCH and NE. Here we found that PNS animals exhibited a heightened response to NE treatment which could be related to a decrease in gut motility. An earlier observation of decreased fecal output in PNS animals during behavioural testing lends to the possibility of this effect.

Overall this thesis has provided significant evidence in support of the theory that MIA or maternal stress during pregnancy can cause numerous detrimental effects on the developing fetus. Further investigations into several findings are necessary to increase our understanding of how these changes occur and what their lasting effects may be. From our study which looked at 'critical periods' it would be beneficial to carry out molecular studies to examine the changes in signalling pathways that lead to the increased sensitivity to cytokine exposure at particular ages and immunity at other points in development. Elucidation of these mechanisms would allow additional research into how elements of these signalling pathways could be manipulated in order to prevent the negative effects that are observed during periods of susceptibility.

In our study of cardiovascular function further pharmacological studies in combination with immunohistological investigation of the cardiac control centres within the brain and the pacemaker nodes would allow identification of whether specific neurotransmitter systems were altered by PNS resulting on the observed effects. The peripheral vasculature also warrants examination as changes to this network may contribute to the basal elevations in APsys. Similar studies are necessary in regards to the respiratory control centres and their branches in an attempt to ascertain the causative changes resulting in the alterations in respiratory function that were observed. As the gastrointestinal system had not been examined in any detail before there is much work yet to be done in relation to the effects of PNS as we only looked at the colonic portion. Pharmacological studies are required to gain a

more in depth understanding of how the observations we made may affect gut function and whether than changes in innervation contribute to pathologies that were not evident from the aspects we studied. It would also be interesting to explore the makeup of the gut microbiome and see if PNS alters it constituents as this has been shown to have multiple effects on both brain and gastrointestinal system. Additionally it would be worth extending the time points at which the functions of physiological systems were investigated to see whether the observed alterations in these systems worsened or improved as the age of the animal progressed.

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11. Publications

Papers

Crampton SJ, O'Keeffe GW (2013) NF-κB: Emerging roles in hippocampal development and function. The International Journal of Biochemistry & Cell Biology 45:1821-1824.

Crampton SJ, Collins LM, Toulouse A, Nolan YM, O'Keeffe GW (2012) Exposure of foetal neural progenitor cells to IL-1β impairs their proliferation and alters their differentiation – a role for maternal inflammation? Journal of neurochemistry 120:964-973.

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Crampton SJ, Walsh S, Straley M, Gavin A, O'Keeffe GW. The gestational age of neural progenitor cells dictates their susceptibility to the detrimental effects of an elevated proinflammatory environment. *In preparation*

Crampton SJ, Carmody RJ, Perkins ND, O'Keeffe GW. Different p65 phosphorylation sites play non-redundant roles in mediating the growth inhibitory effects of NF-κB in developing neurons. *In preparation*

Crampton SJ[*], Golubeva AV[*], Desbonnet L, Edge D, Lomasney KW, O'Halloran, Cryan JF, O'Keeffe GW. Chronic prenatal restraint stress causes lasting molecular and physiological alterations in the adult rat. *In preparation*

Abstracts

Crampton S.J. & O'Keeffe, G.W. 2011. Effect of Interleukin-1β on proliferating embryonic rat ventral mesencephalic precursor cells. *Ir J Med Sci.* 5th Annual Neuroscience Ireland Conference, National University of Maynooth, September 2011.

Crampton S.J. & O'Keeffe, G.W. 2012. The phosphorylation status of p65 (RelA) determines the growth regulating effects by NF-κB signalling in the peripheral nervous system. *Ir J Med Sci.* 6th Annual Neuroscience Ireland Conference, Royal College of Surgeons, Dublin, September 2012.

Crampton SJ[*], Golubeva AV[*], Desbonnet L, Edge D, Lomasney KW, O'Halloran, Cryan JF, O'Keeffe GW. Neuro-immune and behavioural consequences of chronic prenatal stress in affected offspring. 43rd Annual Society for Neuroscience Conference, San Diego, November 2013.

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