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Rodent models of immunologically induced depression and anxiety related behaviour and associated biomarkers

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A thesis submitted to Trinity College Dublin for the degree of Doctor of Philosophy

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2014

I Declaration

I declare that this thesis is submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College and has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, with the exception of the following:

- In chapter 3, high performance liquid chromatography in Wistar and Wistar Kyoto rats was carried out by Mr. Eoin Sherwin.
- In chapter 4, assistance was received from Mr. Barry Moran from the School of Biochemistry and Immunology TCD with fluorescence associated cell sorting. Microarray analysis was carried out by Dr. Wouter Beumer from Erasmus MC and assistance with analysis of the microarray data was provided by Dr. Sinéad Gibney in the Trinity College Institute of Neuroscience.

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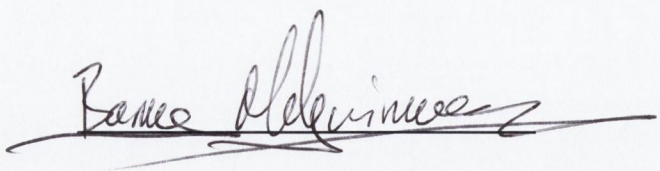

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III Abstract

A series of rodent animal models of immune activation were assessed to develop an understanding of interactions between the immune system and brain leading to changes in behaviour and the determination of biomarkers of relevance to depression symptoms and its underlying pathophysiology.

The effect of challenge with the TLR3 agonist, polyinosinic:polycytidylic acid (poly I:C) on the development of depressive and anxiety related behaviour was assessed in the first instance. In response to systemic poly I:C, Sprague-Dawley rats showed anhedonic and anxious-like behaviours which persisted in the absence of sickness behaviour. These were accompanied by an increase in the expression of pro-inflammatory cytokines and the microglial activation marker CD11b which were accompanied by decreased expression of the neurotrophic marker BDNF and its receptor TrkB in the frontal cortex and hippocampus. A poly I:C induced increase in the expression of the tryptophan degrading enzyme indoleamine 2,3 dioxygenase (IDO) was also found in the frontal cortex and hippocampus which was associated with increased concentrations of the tryptophan metabolite, kynurenine. These findings suggest that the depressive-like behaviour elicited by poly I:C administration in Sprague-Dawley rats may be associated with alterations in cytokines and induction of the kynurenine pathway.

The Wistar Kyoto (WKY) rat is an inbred rat model of genetic predisposition to anxiety and depression related traits. Here WKY rats displayed depressive-like phenotypes at steady state and did not show an enhanced response following poly I:C challenge relative to the Wistar control strain. WKY rats however showed reduced activation of microglia, matched by low expression of IDO with no change in the kynurenine concentrations, while expression of BDNF was reduced in the frontal cortex, following poly I:C administration. While this model does not match with the response observed in Sprague-Dawley rats, further investigations of the WKY rat model may provide insights into immune related mechanisms associated with the behavioural phenotype of this rat strain.

Further investigation of the role that immunity has in the pathogenesis of depressive and anxiety related behaviours focussed on the non-obese diabetic (NOD) mouse, a murine model of autoimmune related thyroiditis and diabetes. NOD mice possess an anxious and depressive-like phenotype relative to the

CD1 mouse at steady state and following challenge with the TLR4 agonist, lipopolysaccharide (LPS). Characterisation of peripheral monocytic and central microglial cells of the NOD mouse revealed an aberrant expression profile at steady state, which was also impaired following LPS administration. An altered steady state and inflammatory response in the NOD mouse was also observed when inflammatory markers, growth factors and kynurenine pathway genes were measured in the hypothalamus. Exposure to a psychopysiological stressor, a two hour period of immobilisation, revealed that NOD mice exhibit a sustained elevation of circulating corticosterone and a blunted glucose response in response to stress. These changes were accompanied by increased expression of GR inducible genes in NOD mice that was not present in the CD1 strain. The findings from these studies add support to the view that the NOD mouse may be a suitable model in which to study the relationship between an autoimmune phenotype, dysregulation of the HPA axis in response to stress and a related predisposition to mood disorders.

Meta analyses have reported IL-6 to be consistently elevated in clinical studies of depressed patients, however the influence of raised circulating concentrations of IL-6 on CNS function is unclear. In this thesis, IL-6 was shown to be elevated in response to a period of immobilisation stress in the mouse which led to IL-6 related signalling in peripheral and central compartments. Following systemic administration of IL-6 to CD1 mice, systemic IL-6 produced an IL-6 response in the periphery and brain including IL-6 receptor activation and stimulation of the expression of IL-6 regulated genes associated with neuronal activation within the CNS.

Investigations of the effects of immunological challenge, genetic predisposition related to autoimmune disease and/or anxiety and depression related behaviour coupled to assessment of the effects of individual cytokines such as IL-6, in animal models add to accumulating evidence for a role for immune and inflammatory response system activation in the pathophysiology of depression and anxiety. Insights gained from such investigations may aid in the discovery of biomarkers associated with depression and anxiety disorders. Furthermore a better understating of immunological mechanisms underlying psychiatric disorders will pave the way for future drug development and the possibility of an immune based therapeutic for mood and/or anxiety related disorders.

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Accepted by 21st annual meeting of Psychoneuroimmunology Research Society, May 2014 244

Accepted by 8th annual meeting of Neuroscience Ireland, September 2013 244

Accepted by 22nd annual meeting of International Behavioural Neuroscience Society, June 2013 244

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Accepted by 7th annual meeting of Neuroscience Ireland, September 2012 245

Accepted by 6th annual meeting of Neuroscience Ireland, September 2011 245

Accepted by EURON Workshop School – Drugs and the brain: an update in psychopharmacology from experimental to clinic, April 2012 245

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VII List of abbreviations

°C	Degree Celcius
1-MT	1-methyl tryptophan
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine / Serotonin
Ab	Antibody
ACTH	Adrenocorticotropic hormone
Adr	Adrenaline
ANOVA	Analysis of variance
AR	Adrenoceptor
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BCG	Bacille Calmette-Guerin
BDNF	Brain derive neurotrophic factor
BrdU	5'-brom-2'deoxyuridine
BSA	Bovine serum albumin
C/EBP β	CCAAT/enhancer binding protein beta
CC	C-C motif
CD	Cluster of diferentiation
cDNA	Complimentary deoxyribonucleic acid
CMS	Chronic mild stress
CNS	Central nervous system

COX	Cyclooxygenase
CRH	Corticotrophic releasing hormone
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CXC	C-X-C motif
CXXXC	CXXXC-motif
DA	Dopamine
DC	Dendritic cell
dH_2O	Deionised water
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders version IV
dsRNA	double stranded RNA
EAE	Experimental autoimmune encephalitis
ELISA	Enzyme-linked immunosorbant assay
EPM	Elevated plus maze
EtOH	Ethanol
FACS	Fluorescence associated cell sorting
FAM	6-carboxyfluorescein
FKBP	FK506 binding protein
FRET	Fluorescence resonace energy transfer
FST	Forced swim test
GC	Glucocorticoid
GFAP	Glial fibrillary acid protein

GILZ	Glucocorticoid inducible leucine zipper
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive DNA element
H_2O	Water
HCA	Home cage activity
HCl	Hydrochloric acid
HPA	Hypothalamic pituitary adrenal
HPLC	High performance (pressure) lipid chromatography
HRP	Horse radish peroxidase
i.c.v.	intracerebroventricularly
I.P.	Intraperitonial
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K	Potassium
KAT	Kynurenine amino transferase
kDa	Kilo Dalton
kg	Kilogram
KMO	kynurenine hydroxylase
KO	Knockout

Kyn	Kynurenine
Kyna	kynurenic acid
LBP	LPS-binding protein
LPS	Lipopolysaccharide
mA	mili Amp
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
MeOH	Methanol
MHC	Major Histocompatability complex
MIA	Maternal immune activation
mm	Milimeter
mM	Milimolar
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NA	Noradrenaline
NaCl	Sodium chloride
NET	Noradrenergic transporter
NFκB	Nuclear factor κB
ng	Nanogram
NICE	National Institute of Health and Clinical Excellence
NK	Natural killer
NMDA	N-methyl-D-aspartate

NO	Nitric oxide
NOD	Non obese diabetic
NOS	Nitric oxide synthase
OFT	Open field test
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission topography
PNS	Peripheral nervous system
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pathogen recognition receptor
pSTAT	Phosphorylated-signal transducer and activator of transcription
QPCR	Quantitative PCR
QUIN	Quinolinic acid
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RS	Restraint stress
SAM	Sympathetic adrenal medullary
SD	Standard deviation
SDS	Sodium dodecyl sulphate

SEM	Standard error of the mean
SERT	Serotonin reuptake transporter
SGK	Serum- and glucocorticoid induced protein kinase
SOCS	Supression of cytokine signalling
SPT	Saccharin preference test
SSRI	Selective Serotonin re-uptake inhibitor
STAT	Signal transducer and activator of the transcription
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline – tween
TDO	Tryptophan 2,3 dioxygenase
TEMED	N,N,N,N'-Tetramethylethylene-diamine
TGF	Transforming growth factor
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
Trp	Tryptophan
TST	Tail suspension test
V	Volt
WB	Western blot
µg	Microgram
µL	Microlitre
µm	Micrometer
µM	Micromolar

1. Introduction

1.1 Preface: Immune system as a contributor to major depressive illness; insights from animal models.

Bi-directional communication between the brain and immune system is a research area gaining in prominence in the search for biological factors associated with the development of a number of psychiatric disorders. In this regard, animal models make substantial contributions to our understanding of these interactions. For instance, long-term adaptive changes to the immune system, in response to stress, have been documented and include stress-induced desensitisation of immune cell glucocorticoid receptors (GR) and β_2-adrenoceptors (AR) which in turn can impact on the ability of these factors to elicit their natural anti-inflammatory actions. It is of interest that psychological stress has been reported to induce microglial activation and pro-inflammatory cytokine expression in the central nervous system (CNS) in animal studies, and whilst there is little evidence of microglial activation or an increase in CNS cytokines in depressed humans to date, animal studies are providing important insights into the potential role of central inflammatory events in the pathophysiology of stress related psychiatric disorders. Stress can be linked to the development of a pro-inflammatory state and it is of interest to consider the reciprocal impact of peripheral inflammatory mediators on the brain. In this regard, there is accumulating evidence that inflammation plays a role in the pathogenesis of major depressive illness. To further explore the behavioural effects associated with immune system activation, a number of animal models are currently in use and include a range of agents used to initiate immune activation including bacterial endotoxin lipopolysaccharide (LPS), the inflammatory cytokine interferon (IFN) α, inoculation with Bacille Calmette-Guerin (BCG), experimental autoimmune encephalitis (EAE) and administration of synthetic double stranded ribonucleic acid (dsRNA) polyinosinic:polycytidylic acid (poly-IC), a viral mimetic. Immune activation in these models provokes signs of depression which may be ameliorated following treatment with antidepressant drugs. Despite these findings the question as to how an immunological basis for a disorder can be reconciled with more traditional hypotheses including impairment of monoamine transmission and the functional impairment of neurotrophins, remains to be addressed. In this regard, the activation of indoleamine 2,3 dioxygenase (IDO) and activation of the kynurenine pathway or the ability of cytokines to influence monoamine transport, receptor activation or neurotrophic factor expression are possibilities. The impact of inflammation on

such systems is providing new avenues to develop a better understanding of the role of stress and related immune changes in the pathophysiology of depression and to find new and improved therapies to address current unmet clinical needs.

1.2 Introduction to the immune system

In order to deal with invading pathogens that would otherwise be detrimental and possibly lethal to the body, a specific set of organs, tissues, cells and molecules exist, which monitor and eliminate pathogens and insults interpreted as a danger to the body, referred to collectively as the immune system. The organs of the immune system are divided into primary (thymus and bone marrow) and secondary lymphoid organs (spleen and lymph nodes) and allow for maturation, differentiation and proliferation of lymphocytes. The immune system can be broadly separated into two different systems, mediating innate (short-term) and adaptive (acquired and long-term) responses.

The innate immune response is comprised of a series of non-antigen-specific mechanisms through which the body can protect itself. It is rapidly activated in response to a noxious infectious or inflammatory stimulus. It is made up of three types of defensive barriers including anatomic, physiologic and inflammatory. The anatomic barrier is the first line of defence against an invading pathogen and consists of physical barriers that prevent entry of the pathogen into an organism, which includes the skin and mucosal membranes. The physiological barrier that contributes to the overall innate immunity includes temperature and pH, induction of a fever can inhibit the growth of pathogens. The inflammatory barrier involves the release of molecules and proteins that reduce the hospitability of the environment for the invading pathogen and act as signals to innate and adaptive immune cells.

Cells of the innate immune system include macrophages, dendritic cells (DC), neutrophils and natural killer (NK) cells (Figure 1.1). These cells lack antigen specificity but can act very quickly to eliminate an invading pathogen or if the infectious agents are too large or too progressed, they can delay proliferation. It is a non-specific response which triggers an inflammatory reaction owing to the recognition of the highly evolutionarily conserved structures on pathogens termed pathogen-associated molecular patterns (PAMPs), to pathogen recognition receptors (PRRs) on cells of the immune system. Upon PAMP recognition the

PRRs trigger a number of intracellular signalling cascades to evoke an inflammatory response, the cardinal signs of which are heat, pain, swelling, redness and loss of function. Rather than expressing antigen specific receptors, innate immune cells express toll-like receptors (TLR), which can bind to and detect generalised pathogen markers. For example TLR4 binds to LPS located on the surface of gram negative bacterial cells while TLR3 binds to dsRNA found in viruses.

Innate immune cells including macrophages and DCs subsequently present antigens to the adaptive arm of the immune system, activating it and enabling a stronger more specific response. Macrophages circulate in an inactive state until they encounter an antigen at which point they engulf, internalise and destroy it. This process also activates the macrophage to increase the amount of surface major histocompatability complex (MHC) class II molecules, which serve to present antigenic elements on the cell surface thus performing the role of an antigen presenting cell (APC). Macrophages also produce soluble protein mediators of immune function including cytokines, complement components and diffusible factors such as oxygen metabolites and nitric oxide (NO). Activated macrophages can operate in different modes with characteristic expression and functional profiles including "M1" and "M2". The M1 profile is induced by toll like receptor ligands and is pro-inflammatory in nature, up-regulating pro-inflammatory cytokines including IL-1β, IL-6, whereas the M2 profile is stimulated by IL-4 and exhibits an anti-inflammatory phenotype, focussing on repair, regulation and suppression of the M1 response (Murray and Wynn, 2011, Gordon, 2003).

DCs serve as antigen presenting cells. Immature DCs are located in the periphery where they are exposed to pathogens. They express higher levels of MHC II than macrophages and are more potent APCs when stimulated. Upon stimulation, DCs migrate to secondary lymphoid organs and up-regulate a series of co-stimulatory molecules including cluster of differentiation (CD) 40, CD80 and CD86, where they facilitate the activation of naive T-cell lymphocytes and the adaptive arm of the immune system (Mellman et al., 1998, Janeway and Medzhitov, 2002).

Mast cells can be found resident in a wide variety of tissues including skin, connective tissues, mucosal epithelial tissues, genitourinary and digestive tracts (Kindt et al., 2007). They are one of the primary cell responders to an immune

stimulus. Upon stimulation, mast cells release histamine and prostaglandins resulting in vasodilation causing redness and an increase in temperature and extravasation which results in swelling (Nathan, 2002).

Neutrophils are granulocytes that make up 50-70% of the circulating white blood cells and are recruited to the site of infection (Witko-Sarsat et al., 2000). Like with macrophages these cells also phagocytose invading pathogens, however using the granules within their cytoplasm, they are able to destroy the pathogen via lytic enzymes and bactericidal substances. They are also able to generate reactive oxygen species which are toxic to the engulfed pathogen. Other granulocytes include eosinophils and basophils, which are much less numerous than neutrophils but modulate the immune response in a similar manner. Basophils are responsive to IgE antibodies and have been reported to have a role in the allergic response (Schroeder, 2009). Eosinophils are not phagocytic, relying on the cytotoxic function of the granules contained in their cytoplasm and have been reported to have a role in protection from parasites (Parkin and Cohen, 2001). They are also responsive to IgE antibodies and have been reported as effector cells in allergic inflammation (Hogan et al., 2008).

NK cells are cytotoxic lymphocytes, which do not bear an antigen specific or CD3 complex (Trinchieri, 1989), indicating that they are part of the innate immune response, whose primary role includes killing off virally- and cancer-infected cells. This is carried out using receptors to identify abnormalities in cells, including reduced surface MHC class I molecules and antiviral or antitumor antibodies bound to the cell surface on the target cell (Moretta et al., 1996). NK cells become cytotoxic and lyse target cells once identified via the formation of a cytotoxic synapse. The secretion of perforins create holes in the plasma membrane of the target cell at which point granzymes are injected through the pore inducing apoptosis (Leong and Fehniger, 2011). Once activated NK cells produce pro-inflammatory cytokines such as IFNγ and tumour necrosis factor (TNF) α, which signal to other immune cells (Lauwerys et al., 2000).

Whereas the innate immune response relies on speed of action to exert its effects, the adaptive immune system response is more targeted to specific invading pathogens. The acquired immune response is mediated by T-lymphocytes (T-cells) and requires the presentation of antigens via APCs usually in lymphoid organs, which activate the T-cells. Owing to its ability to identify specific antigenic elements, the adaptive immune response must be able to

distinguish self from non-self antigens. This prevents activation of the immune system by self proteins in the absence of an immune challenge. Failure of this safeguard leads to the development of auto-immunity, a process involved in diseases such as rheumatoid arthritis, lupus erythematosus, Grave's disease and myasthenia gravis (Eggert et al., 2010).

T-cells originate from the bone marrow and migrate to the thymus gland where they mature. T-cells express a receptor on their surface unique to an individual antigen. The two main subgroups of T-cells are the cytotoxic T-cell, which display CD8 on their cell surface and only respond to antigens presented by MHC I, and the T-helper (TH) cell, which have CD4 on their cell surface and respond to antigens presented by MHC II (Kindt et al., 2007). $CD8^+$ T-cells elicit their cytotoxic effects on cells presenting the complimentary antigen and carry out this function via perforins and granzyme granules, similar to natural killer cells. $CD4^+$ TH-cells respond to MHC II antigen presenting cells. There are two subsets of TH cells Th1, which releases cytokines including IL-2 that activate $CD8^+$ T-cells, inducing a cell mediated response and Th2 cells which releases cytokines like IL-4, IL-5 and IL-10 which lead to antibody production (Parkin and Cohen, 2001).

B lymphocytes (B-cells) are also involved in the adaptive immune response (Figure 1.1). These cells display antigen binding cell surface receptors and mediate the specificity, diversity, memory and self non-self recognition associated with acquired immunity. B-cells arise from the bone marrow, mature there and evoke the humoral antibody response to an invading pathogen. B-cells express surface antibodies unique for a specific antigen. When a naive B-cell binds to its complimentary antigen, it differentiates into plasma B-cells with a short life span that produces soluble antibodies. Memory B-cells do not produce soluble antibodies, but have a longer life span and serve to produce a faster response upon re-exposure to an invading pathogen and antigenic determinant. Antibodies secreted by activated B-cells aid the work of the innate immune response by activating complement, opsonising parasites for phagocytosis and inducing antibody dependent cytotoxic shock on tumour and cells infected by pathogens (Parkin and Cohen, 2001).

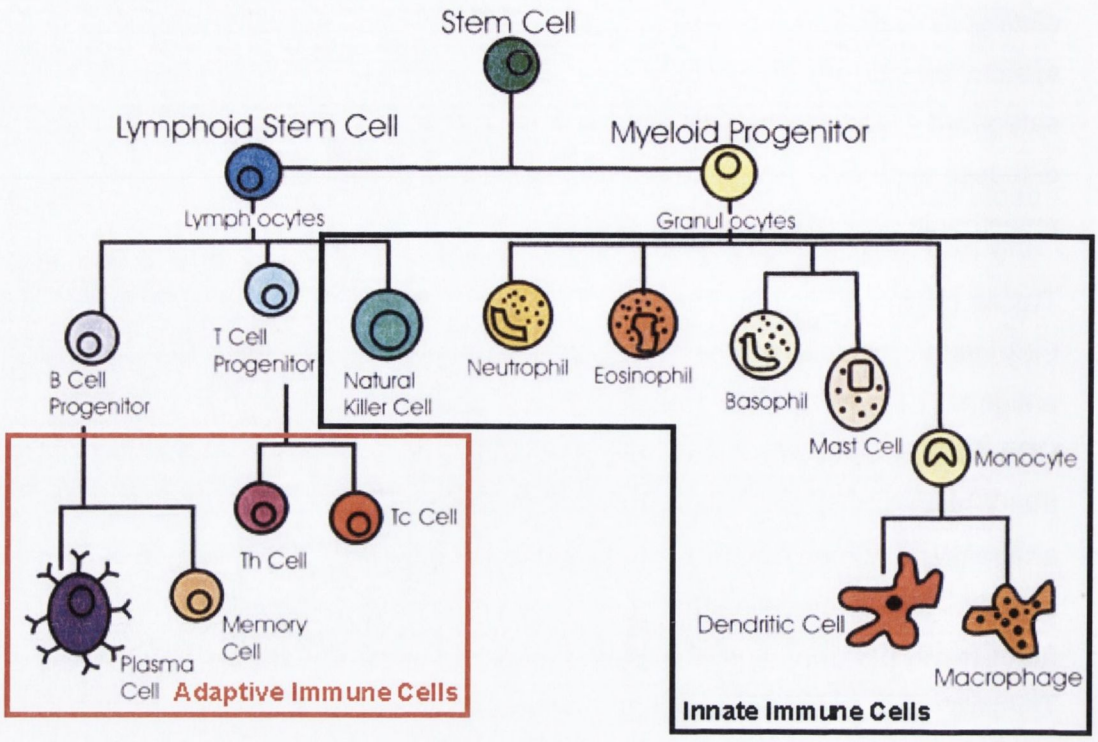

Figure 1.1 – Cells of the Immune system

Most innate immune cells, and microglia in the CNS (not shown), are derived from myeloid progenitor cells. Adaptive immune cells and innate natural killer cells originate from lymphoid stem cells. Figure adapted from (Todar, 2009).

Cells of both the innate and adaptive immune systems communicate with each other via the release of inflammatory mediators known as cytokines and chemokines.

Cytokines are a set of structurally diverse soluble proteins (8-80 kilo Dalton (kDa)) that are produced by a wide variety of cells and tissues including macrophages, neutrophils, microglia, T-cells, epithelial cells and endothelial cells. They exert their effect by binding to specific receptors both soluble and membrane bound that in turn lead to signalling within the cell evoking changes in gene expression, protein activation and cell migration. Distinct groups of cytokines have been described including interleukins (IL), IFNs, TNF and transforming growth factors (TGF). Two main categories of cytokine exist, pro-inflammatory, which promote inflammation, recruit and activate immune cells, and

anti-inflammatory cytokines, which play a regulatory role, reducing the expression of pro-inflammatory cytokines and suppressing immune activation.

Chemokines (*Chemo*tactic cyto*kines*) are a family of structurally related small cytokines (8-10kDa) which can induce activation and migration of immune cells via chemotaxis (Bajetto et al., 2002). There are 4 main families of chemokines, defined by the location of their first two cysteine residues, CC, CXC (where X is another amino acid), C and CXXXC.

1.3 Immunosurveillance in the brain and the role of glial cells

Within the CNS 90% of the cells are glial cells comprising of microglia, astrocytes and oligodendrocytes. Their role within the CNS is to support and sustain neuronal function and to elicit a CNS specific immune response in times of challenge.

Microglia, which comprise 5% of the total glial population (Pelvig et al., 2008), are the immunocompetent cells of the CNS derived from myeloid progenitors that enter the CNS during embryonic development and differentiate into microglia (Ginhoux et al., 2010). Microglia respond to insult and injury and have a role in tissue repair (Harry and Kraft, 2012), but are not limited solely to immune related functions. They have also been shown to have a role in neuronal support and synaptic plasticity (Tremblay and Majewska, 2011). Distribution and density of microglia have been shown to vary in different brain regions, for example the substantia nigra has been shown to comprise of ~12% microglia while the corpus callosum comprises ~5% of microglia (Tremblay et al., 2011a). Inactive, "resting" microglia express low levels of surface antigens and reduced cytokine synthesis and cannot phagocytose (Garden and Moller, 2006), but are quite motile and always testing and monitoring the surrounding environment, continuously extending and retracting processes from their cell body (Davalos et al., 2005, Nimmerjahn et al., 2005). Microglia detect changes in the local environment via receptors which bind and are activated by complement, cell adhesion molecules, cytokines, chemokines, immunoglobulins and prostaglandins (Streit, 2002). Activation of microglia involves detection of factors perceived to be noxious including bacterial and viral mimetics such as LPS and poly I:C respectively. This activation leads to a morphological change in microglia, which retract their

processes and change from a highly ramified state to a more amoeboid shape (Kreutzberg, 1996). Expression of immune markers like MHC II, complement receptors and adhesion molecules are increased, as is the release of reactive oxygen species and the phagocytic capacity of the cells (Aloisi et al., 2000, Galea et al., 2007). Coupled with this, there is also an increase in the production of pro-inflammatory mediators including TNFα, IL-1β, IL-6 and IFNγ, which promote recruitment of other microglia and infiltration of peripheral immune cells leading to an inflammatory response which can be detrimental to neuronal survival (Liu and Hong, 2003, Garden and Moller, 2006). Normal microglial function involves the detection of a danger signal, activation and removal of this perceived threat, followed by a return to a "resting" monitoring state again. Dysregulation of these functions however, either through hyper- or hypo-activation of microglia, may contribute to the development of diseases or syndromes harmful to the brain. Microglia have been reported to have a role in chronic neurodegenerative diseases including Alzheimer's and Parkinson's disease (Liu and Hong, 2003) and neuropsychiatric disorders including bipolar disorder, depression and schizophrenia (Beumer et al., 2012).

Astrocytes make up 20% of the total glial population (Pelvig et al., 2008). While originally thought to serve only as nutrient providers and buffers for extracellular neurotransmitters and ions, astrocytes are responsible for processes including water transport, blood brain barrier (BBB) modulation, the conversion of glucose to lactate as a fuel source for neurons, clearance of free radicals, and provision of trophic support for the development and sustainability of neurons (Kimelberg and Nedergaard, 2010, Jou, 2008). Astrocytic expression of neurotransmitter receptors including receptors for noradrenaline (NA), acetylcholine, serotonin (5-HT), dopamine and glutamate (Kimelberg, 1995) and their ability to release glutamate in a calcium (Ca^{2+}) dependent manner (Parpura et al., 1994) have indicated a signal transmission role for astrocytes. Evidence also exists for an immunological function of astrocytes within the CNS, owing to the expression of molecules involved in the immune response including TLRs, mannose receptor, scavenger receptors and components of the compliment system (Farina et al., 2007). Upon immune stimulation, astrocytes release key mediators of the innate inflammatory reaction including complement, IL-1β, IL-6 and chemokines (Ransohoff and Brown, 2012) and have also been shown to have phagocytic properties, expressing proteins present in phagocytic cells (Nguyen et al., 2011).

They have been shown to engulf colloidal carbon particles following their injection into the brain (al-Ali and al-Hussain, 1996).

Oligodendrocytes, which account for 75% of total glia in the CNS (Pelvig et al., 2008), are the myelinating cells of the CNS, providing insulating myelin sheaths which cover axons and enables efficient propagation of action potentials. The myelin is formed via wrapping of a cell process around the axon of a neuron followed by the withdrawal of the cytoplasm, leaving only the insulating lipid bilayer membrane (Bear et al., 2007). Each oligodendrocyte can insulate multiple neurons in the CNS, while the peripheral based insulating Schwann cells only insulate one neuron per cell. Oligodendrocytes form during embryonic development (Bradl and Lassmann, 2010) but can also form in the adult CNS from neural stem cells in the sub ventricular zone (Nait-Oumesmar et al., 1999).

1.4 Immune system activation is associated with anxiety and depression related behaviours

Activation of the immune system in vivo occurs when a foreign or non-self antigen is detected by cells of the immune system. Various pathogen mimics exist which can induce a range of immune responses based on the different receptors that recognise these mimics including numerous TLRs, depending on the nature of the pathogen.

LPS is a component of the cell wall of gram negative bacteria e.g. E.coli and is recognised by the innate immune cells as a foreign antigen via its PAMP (Figure 1.3). The first process in LPS recognition by the immune system is the binding of LPS to soluble LPS-binding protein (LBP). Once bound by LBP, LPS then associates with CD14, a protein necessary for TLR activation by LPS. This complex binds to TLR4 receptors on innate immune cells leading to downstream intracellular signalling. LPS challenge is routinely used as an experimental tool to act as an immunostimulus and mimic the early stages of a bacterial infection. TLR4 receptors are also present on the cell surface of astrocytes and microglia. The physiological changes following administration of LPS have been well characterised in animal models including peripheral and central immune activation (Olson and Miller, 2004), elevations in circulating and central pro-inflammatory cytokines (Connor et al., 2005, Chung and Benveniste, 1990),

behavioural changes (Frenois et al., 2007) and impairments in synaptic plasticity including reduced neurogenesis (Guan and Fang, 2006).

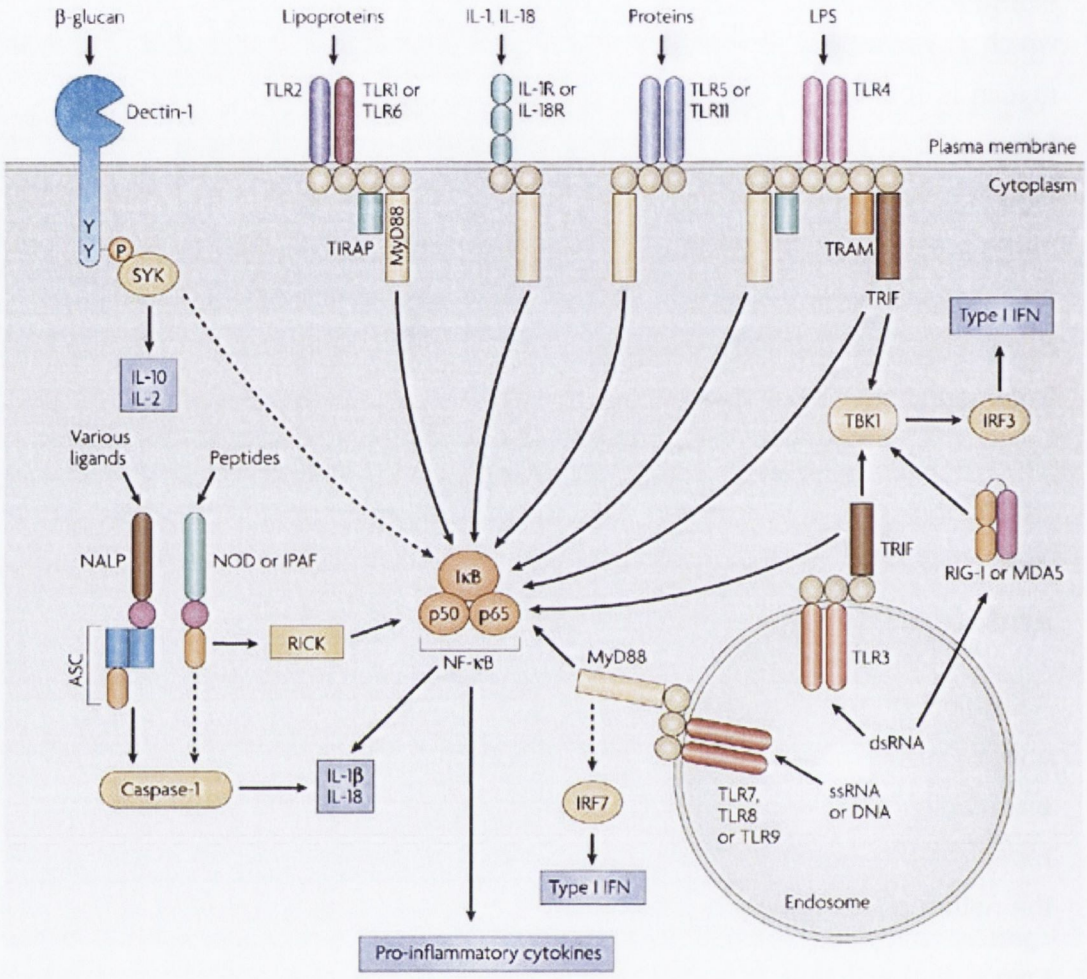

Figure 1.2 – Pathogen Recognition Receptors and their ligands

PRRs are present on both the extracellular membrane and intracellularly in endosomes. Upon binding of their ligand, such as LPS (TLR4) and poly I:C (TLR3), this leads to intracellular signalling that culminates in the activation of a pro-inflammatory response by the cell. All TLR responses except for TLR3 (dsRNA) are dependent on MyD88 for signal transduction. ASC, apoptosis-associated speck-like protein containing a CARD (caspase-recruitment domain); ds, double-stranded; IFN, interferon; IκB, inhibitor of NF-κB; IL, interleukin; IPAF, ICE-protease-activating factor; IRF, IFN-regulatory factor; LPS, lipopolysaccharide; MDA5, melanoma-differentiation-associated gene 5; MyD88, myeloid differentiation primary-response gene 88; NALP, NACHT-, LRR- and pyrin-domain-containing protein; NFκB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NOD, nucleotide-binding oligomerization domain; RICK, receptor-interacting

serine/threonine kinase; RIG-I, retinoic-acid-inducible gene I; ss, single-stranded; TBK1, TANK-binding kinase 1; TIRAP, Toll/IL-1R (TIR)-domain-containing adaptor protein; TLR, Toll-like receptor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor protein inducing IFNβ; SYK, spleen tyrosine kinase. Figure adapted from (Trinchieri and Sher, 2007).

Poly I:C is a viral mimetic that can be used to activate the immune system (Figure 1.3). Whereas LPS activates TLR4 receptors on innate immune cells, poly I:C binds to TLR3 receptors. Poly I:C comprises of dsRNA, similar to that produced during the viral life cycle and is detected by TLR3 in intracellular endosomes. Like LPS it is widely used as an experimental immune stimulus and has been shown to reliably induce sickness behaviour in animals, inducing fever and an inflammatory response characterised by an increase in the expression and release of pro-inflammatory cytokines both in the periphery and in the brain.

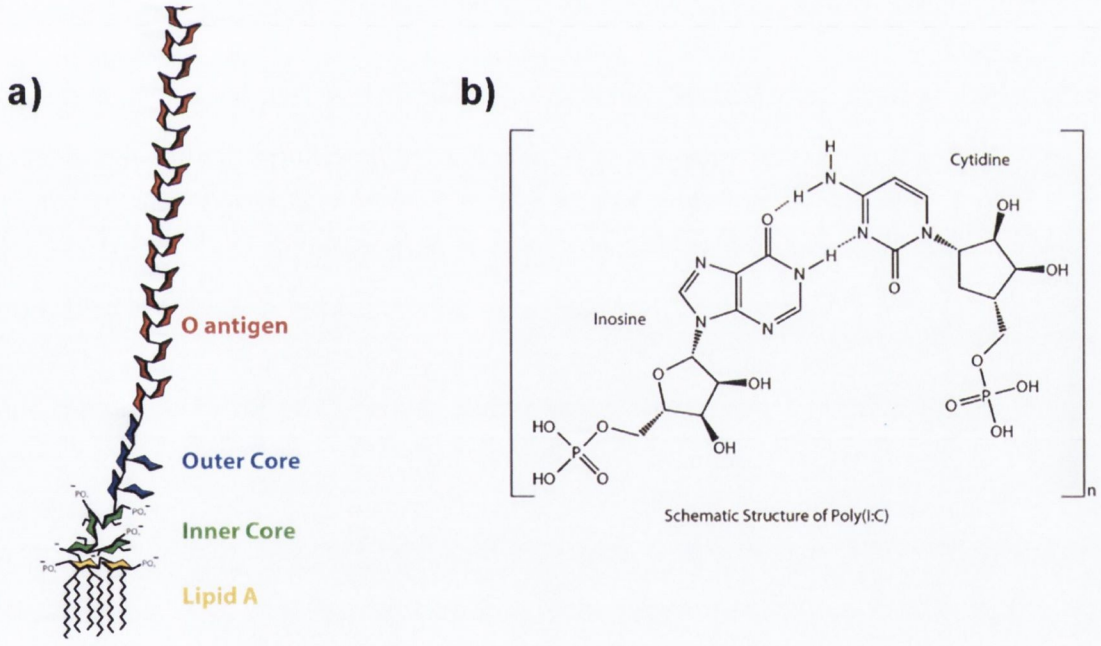

Figure 1.3 – Structure LPS and poly I:C

The bacterial mimetic LPS (a) comprises of three main sections, a lipid A base, a core and O antigen (O polysaccharide). The hydrophobic Lipid A connects to the outer membrane of the bacteria and much of the immune responsiveness originates from this part of the LPS. The O antigen is a repetitive glycan polymer and can vary in size, e.g. E.coli has >160 antigen sizes between different strains. Poly I:C (b) comprises of a strand

of poly(I) annealed to a strand of poly(C) which can come in varying sizes (1.5-8kilo bases)

BCG is an attenuated strain of mycobacterium bovis, which is commonly used to immunise against mycobacterium tuberculosis. Administration of this vaccine to mice has been shown to induce a sustained pro-inflammatory response accompanied by sickness behaviour (O'Connor et al., 2009a). The demonstration of depression-like behaviours in these models however, is frequently confounded by the overlap of cytokine-induced sickness behaviours such as fever, hypoactivity, weight loss and the suppression of feeding. Reports that antidepressant responsive depression-like behaviours in immune stimulated animals can develop independently of sickness related performance impairments, however, have enhanced the validity of this model. For instance, peripheral administration of LPS to laboratory mice or rats induces sickness behaviour, including hyperpyrexia, hypoactivity, suppression of feeding and bodyweight loss, which peaks 2-6 hours (hr) later and gradually subsides. Depression-like behaviours as measured by increased immobility, regarded as behavioural despair in the forced swim test or tail suspension test, reduced voluntary wheel running and decreased preference for a sweet solution, indicative of anhedonic behaviour, emerge later. Such behaviours are believed to require the activation of pro-inflammatory cytokine signalling in the brain (Dantzer et al., 2008, Dunn et al., 2005). In this regard, IL-1β and TNFα are considered the main cytokines as there are numerous reports that systemic or central administration of these cytokines to laboratory rats and mice induce sickness behaviour in a dose and time dependent manner. Systemic administration of IL-1β to mice has been reported to mediate endotoxin induced sickness behaviour (Bluthe et al., 2000) and to increase immobility (behavioural despair) in the forced swimming and tail suspension tests (Dunn and Swiergiel, 2005). Skelly and co–workers have reported that systemic administration of TNFα and IL-1β to mice provoked a reduction in activity in the home-cage (Skelly et al., 2013). It has been shown that IL-1β can instigate anhedonia in laboratory rodents independent of effects on anorexia (Larson et al., 2002, Merali et al., 2003). Other cytokines too are considered to play a role including IL-6 which contributes to the expression of brain IL-1β and TNFα in response to immune stimuli and may play

a role in LPS induced hippocampal mediated cognitive impairments (Sparkman et al., 2006). A recent study by Karson and co-workers reported that treatment of rats with the TNFα inhibitor infliximab reduced depressive-like behaviours induced by a chronic mild stress exposure (Karson et al., 2013). In other reports to date, IL-2, but not IL-1β or IL-6, has been reported to provoke long-lasting anhedonic effects in mice and rats (Anisman et al., 2002, Dunn et al., 2005). A systemic injection of IFNα was reported to induce long-term behavioural effects including reduced locomotor activity and sickness behaviour in rats (Kentner et al., 2006). Central administration of IL-6 in mice induced depressive behaviours, such as increased immobility in the forced swim and tail suspension test (TST) which was sustained for 48hr in the TST and reduced interactions in a social paradigm which were blocked via co-administration with an anti-IL-6 antibody (Sukoff Rizzo et al., 2012).

Cytokine immunotherapy for viral hepatitis or various forms of cancer provokes severe psychological disturbances including depression. Numerous studies have reported that previously psychiatrically healthy individuals, treated with high doses of exogenous cytokine IL-2 or IFNα, develop depressive-like symptoms such as depressed mood, increased somatic symptoms and stress reactions and cognitive impairment (Capuron et al., 2000, Capuron et al., 2003b, Capuron et al., 2004, Valentine and Meyers, 2005). It is not entirely clear why only a proportion of individuals, typically less that 50%, that receive cytokine immunotherapy become depressed. It has been suggested that psychological stress may sensitise to the neurochemical and behavioural actions of cytokines and therefore individuals with an anxious/stress-prone phenotype may be more susceptible to developing psychiatric sequelae in response to cytokine administration (Anisman et al., 2002). In support of this idea, it was reported that patients with a higher cortisol response (stress response) to the initial injection of IFNα, displayed a greater propensity to develop depression following treatment with IFNα (Capuron et al., 2003b). Further support for this notion stems from a recent pre-clinical study demonstrating that mice exposed to psychosocial stress showed exaggerated central monoamine changes, HPA axis reactivity and sickness behaviours to IFNα treatment (Anisman et al., 2007).

When one is considering the literature on cytokines as a trigger for depressive illness it is indisputable that treatment with IL-2 or IFNα can induce depression. However one must remember that the doses of cytokines administered to patients in these studies far exceeds physiological concentrations. This is a factor

that should be considered when implicating elevated endogenous cytokine secretion as a causal factor in the development of depressive symptoms. Thus one cannot simply regard the psychiatric and biological sequelae that occur following exogenous administration of cytokines such as IFNα or IL-2 as being akin to the biology of idiopathic depression where no obvious exogenous agent is driving changes in the psychiatric state.

A connection between inflammation and depression may be supported also given the high levels of co-morbidity of depression reported in patients also suffering with inflammatory disorders. Rheumatoid arthritis patients are twice as likely to suffer from depression than the general population (Isik et al., 2007, Katon and Sullivan, 1990) and a meta analysis by Anderson and co workers reported that people suffering with diabetes were also twice as likely to develop depressive disorders than non-diabetic controls (Anderson et al., 2001). Prevalence of depression is also greater in patients with multiple sclerosis (MS) than in healthy controls (Minden and Schiffer, 1990). Sadovnick reported that MS patients had a lifetime prevalence for depression of 50% (Sadovnick et al., 1996). Indeed, even people with a history of allergies were more likely to be diagnosed with major depression (Hurwitz and Morgenstern, 1999). The prevalence of depression is 3 times higher in IBD patients when compared to the general population (Graff et al., 2009), the incidence of depression and anxiety in patients with Crohn's disease reported to be five-fold higher when compared to healthy controls (Walker et al., 2011).

1.5 Biological mechanisms implicated in mediating the depressive effects of cytokines

Currently, elucidation of mechanisms mediating the ability of immune activation to induce depression-like behaviours is an area of considerable interest and includes cytokine effects on neurotransmitter metabolism, neuroendocrine function and neuronal plasticity. Such mechanisms have been reviewed extensively elsewhere (Anisman et al., 2008, Anisman, 2009, Miller et al., 2009).

Of these mechanisms, considerable attention has recently been focused on the ability of pro-inflammatory cytokines such as IL-1β, TNFα and IFNγ to enhance the activity of IDO. IDO breaks down tryptophan, the primary amino acid precursor of serotonin, into kynurenine. The breakdown of tryptophan is believed

to contribute to reduced serotonin availability and increased kynurenine. In mice, IDO is induced following an immune challenge in both the periphery and brain in a time dependent fashion and peaks at 24hr following LPS administration (Lestage et al., 2002). In an alternative model to LPS administration in mice, chronic stimulation of the immune system by inoculation with BCG induces a sustained elevation in circulating levels of IFNγ and a chronic activation of IDO (O'Connor et al., 2009a). Activation of IDO results in decreased circulating tryptophan concentrations and increased production of kynurenine and other tryptophan derived metabolites (Figure 1.4).

Activation of IDO is also associated with late onset depression-like behaviours following BCG inoculation that may be blocked in IFNγ knockout and IDO knockout mice (O'Connor et al., 2009a). Pre-treatment with the second generation tetracycline minocycline which has potent anti-inflammatory effects both peripherally and in the brain, blocks both LPS-induced sickness and depression-like behaviours. By contrast, treatment with the competitive IDO inhibitor 1-methyl tryptophan (1-MT), without influencing IL-1β or IFNγ expression or LPS-induced sickness behaviour, attenuates late onset depression like behaviours in tandem with a reduction in the plasma kynurenine/tryptophan ratio. Similar mechanisms were reported in the BCG inoculation model in mice (Dantzer et al., 2008, O'Connor et al., 2009b). Although the activation of IDO by cytokines provokes a reduction in circulating levels of tryptophan, this may not translate into alterations in the synthesis of serotonin as the attenuation of LPS induced depression-like behaviours in mice by the inhibition of IDO are not associated with central changes in serotonin turnover. Rather degradation of tryptophan into kynurenine and related metabolites such as quinolinic (QUIN) and kynurenic acid (KYNA) with effects at the N-methyl-D-aspartate (NMDA) receptor could account for changes in glutamatergic transmission which promote the development of depression-related behaviours (Muller and Schwarz, 2007). In support of this administration of kynurenine to mice dose dependently induces depression-like behaviour (O'Connor et al., 2009c). Such findings support a role for the kynurenine pathway in mediating late onset depression-like behaviours induced following immune challenge.

Studies by Capuron et al. Have shown that patients undergoing treatment for cancer with IL-2 and/or IFNα develop depressive symptoms associated with decreased circulating concentrations of tryptophan (Capuron et al., 2000, Capuron et al., 2001, Capuron et al., 2004, Capuron et al., 2002b). Similarly

patients receiving chronic cytokine treatment for hepatitis C also exhibited depressive phenotypes (Alavi et al., 2012a, Raison et al., 2005a, Raison et al., 2009). Investigations into this IFNα immunotherapy in patients have reported an increase in the circulating and CSF concentrations of kynurenine, a key metabolite of the kynurenine pathway (Raison et al., 2010).

Alternative mechanisms to kynurenine pathway activation have also been proposed in the development of depression associated with inflammation including the ability of cytokines to influence tryptophan uptake in the brain (Dunn et al., 2005), serotonin uptake in nerve terminals (Morikawa et al., 1998, Tsao et al., 2008, Zhu et al., 2010, Zhu et al., 2006) and the ability of cytokines to influence the expression of 5-HT1A receptors in cells in culture (Cai et al., 2005) which when taken together are suggestive that cytokines modulate serotonergic neurotransmission by mechanisms other than an IDO related decrease in tryptophan and the generation of kynurenine related metabolites. Increased serotonin transporter expression has been associated with elevated IFNα (Morikawa et al., 1998) and following immune stimulus (Zhu et al., 2010). This could mediate the depressive effects of cytokines by removing 5-HT from the synapse and reducing signal duration

It is also of interest that auto-antibodies, antibodies produced by the body which respond to resident "self" protein signals, implicated in autoimmune diseases such as rheumatoid arthritis, lupus erythematosus and Grave's disease (Eggert et al., 2010), have been found against 5-HT (Maes et al., 2012, Schott et al., 2003) and the 5-HT_{1A} receptor (Tanaka et al., 2003) in circulating serum of depressed patients.

Figure 1.4 – Outline of the kynurenine metabolic pathway

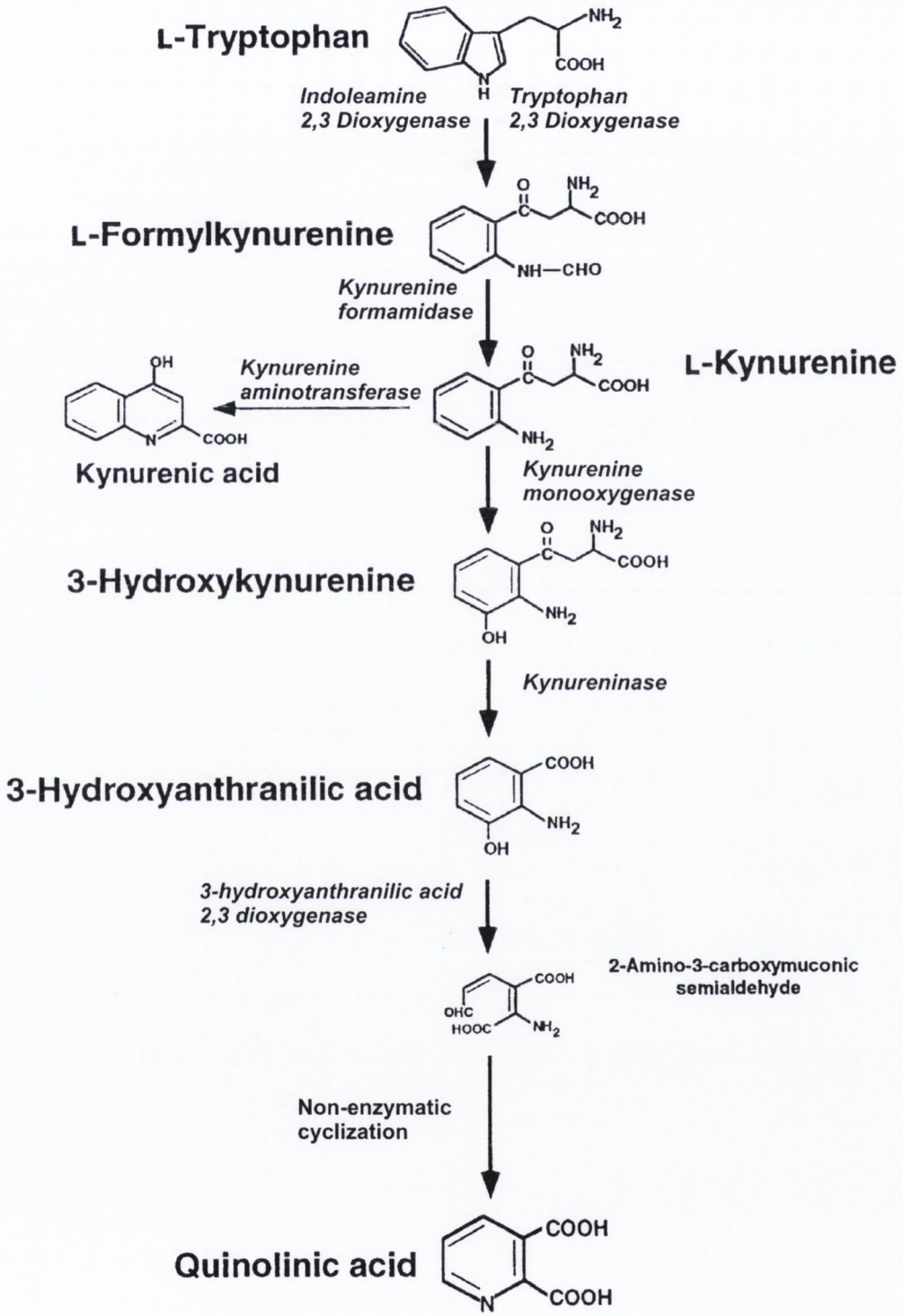

Figure 1.4 – Outline of the kynurenine metabolic pathway

Tryptophan, an essential dietary amino acid, can be degraded along two pathways, the serotonergic pathway and the kynurenine pathway, through which ~99% of tryptophan is metabolised (Guillemin et al., 2007, Russo et al., 2009). The rate limiting enzymes of this pathway are tryptophan 2,3 dioxygenase (TDO) and IDO. TDO is found mainly in the liver and its activity is controlled by tryptophan itself (Myint, 2012) and glucocorticoids (Maes et al., 2011). IDO is found peripherally and centrally and its expression is increased by pro-inflammatory cytokines (Carlin et al., 1989, Fu et al., 2010, Zunszain et al., 2012). Tryptophan is metabolised to form kynurenine, at which point it can progress down two further separate pathways. In the first pathway kynurenine is metabolised by kynurenine amino transferase (KAT) to yield kynurenic acid (KYNA), which is an NMDA glutamate receptor antagonist (Wichers et al., 2005). In the other pathway, kynurenine monooxygenase (KMO) catabolises kynurenine to create 3-Hydroxykynurenine (3-HK), which is a free radical generator and an NMDA receptor agonist (Guidetti and Schwarcz, 1999, Leonard, 2007). 3-HK is subsequently acted upon by kynureninase which yields 3-hydroxyanthranilic acid (3-HAA) which is metabolised by 3-hydroxyanthranilic acid 2,3 dioxygenase (HAAO) forming QUIN, also an NMDA receptor agonist (Wichers and Maes, 2004). Within the CNS there is compartmentalisation of specific enzymes of the kynurenine pathway; astrocytes preferentially express KAT and the production of KYNA which was reported to have a neuroprotective role (Guillemin et al., 2001), whereas microglia have been shown to express KMO, producing 3-HK and QUIN which can be neurotoxic (Heyes et al., 1996). Figure adapted from (Heyes et al., 1997).

Non serotonergic mechanisms such as hyperactivity of the hypothalamic pituitary adrenal (HPA) axis may also be significant in mediating depression associated with inflammation on account of the ability of cytokines to activate the HPA-axis (Pace et al., 2007). In addition, pro-inflammatory cytokines promote the expression of the β isoform of the GR that can bind to response elements in the nucleus but is inactive, while pro-inflammatory cytokines have been reported to decrease expression of the active α isoform (Pace and Miller, 2009). A recent study has shown that depressed patients have lower expression of GRα which is strongly correlated with increases in pro-inflammatory cytokines (Carvalho et al., 2014). Such mechanisms promote resistance to glucocorticoids and may account for cytokine induced dysregulation of the HPA axis on account of the reduced ability of glucocorticoids to feedback to the hypothalamus. Depressed patients show elevated levels of corticotrophin releasing hormone (CRH) a hypothalamic neuropeptide under the regulation of glucocorticoids that drives HPA axis activation and plays an important central role in behavioural, endocrine and immune responses to stress (Irwin, 2008, Pace et al., 2007). It has therefore been proposed that at the level of peripheral and central immune cells, the normally inhibitory effects of glucocorticoids on cytokine production would no longer be operative facilitating an unrepressed increase in the production of pro-inflammatory cytokines which in turn drives activation of the HPA axis. Such a mechanism consolidates a stress driven inflammatory loop in tandem with an over production of CRH (Dantzer et al., 2008).

Central administration of CRH induces a robust decline in innate and central immune responses by acting within the CNS to co-ordinate changes in peripheral immunity e.g. T-cell response to antigen and reduced natural killer cell activity (Irwin and Miller, 2007). Such central mediated actions of CRH are supported by the fact that the immune effects of CRH can be antagonised by benzodiazepines which alter the perception of stress and that CRH mediated changes in immune responsivity are linked to CRH receptors in the amygdala rather than the pituitary/adrenal axis. Repeated administration of CRH leads to a down-regulation of CRH receptors in the amygdala which is associated with reduced CRH mediated immune responses. With regard to the role of stress-induced activation of CRH in the brain, central but not peripheral immunoneutralization of CRH antagonised the immunosuppressive effects e.g. decline in natural killer cell activity following exposure to footshock stress demonstrating that CRH in the

CNS is involved in the regulation of immune function. Such reports support a role for CRH in the brain rather than HPA axis activation in mediating stress-induced immune suppression. By contrast the non selective β-blocker propanolol and the β_2-AR antagonist butoxamine blocked CRH induced immune suppression highlighting the functional significance of noradrenergic innervation of the spleen in mediating CRH effects on immunity. A model of CNS stress circuitry activation involving CRH activation and ultimately sympathetic nervous system outflow are proposed to link the brain to immune responses to stress in this instance. Further consideration of sympathetic effector mechanisms in stress and depression and the critical role of sympathetic output in mediating immune response, competence and inflammation is provided elsewhere (Irwin, 2008).

Additional research is required to establish the physiological role of individual cytokines in the brain and the mechanisms by which cytokines mediate changes in behaviour of relevance to depression. There are a limited number of reports to date which have helped to bridge this gap in our understanding. Increased expression of IL-1β and TNFα observed in the circumventricular organs and BBB is associated with the initial wave of LPS-induced sickness. The secondary wave of depression-like behaviours, which occurs 12hr to 24hr later, may be associated with increased expression of cytokines within parenchymal regions. Mapping of neuronal activation in response to LPS administration has been carried out and the peak of sickness behaviour is associated with increased expression of the cellular activation marker and immediate early gene product c-fos in the paraventricular nucleus and the bed nucleus of the stria terminalis (BNST), areas involved in endocrine and autonomic components of LPS-induced sickness. By contrast, immunostaining for FosB and its truncated splice variant ΔfosB, both of which have a longer half life than c-fos and accumulate during repeated or long lasting stimulation, were increased in several hypothalamic nuclei and had extended to the amygdala and hippocampus. Such activation patterns point towards the involvement of these structures in cytokine-induced depression (Dantzer et al., 2008, Frenois et al., 2007).

It is also of significance that immunological stimulation with agents such as LPS provoke a reduction in the expression of brain derived neurotrophic factor (BDNF) and neurogenesis in the hippocampus (Fujioka and Akema, 2010, Ormerod et al., 2013). In rodent models IFNα treatment was reported to reduce the expression of BDNF within the hippocampus (Kaneko et al., 2006). BDNF expression is also reduced following immune challenge with LPS (Tanaka et al.,

2006) which is antagonised by inhibition of IL-1β signalling following intrahipocampal administration of IL-1 receptor antagonist (IL-1ra). Moreover IL-1ra prevents stress related reductions in BDNF expression suggesting a role for that pro-inflammatory cytokine in stress related mechanisms (Barrientos et al., 2003). Such reports of reduced hippocampal growth factor expression and neurogenesis may contribute to reduction in hippocampal volume as indicated in numerous neuroimaging studies on depressed patients (Chen et al., 2001, Sheline et al., 2003). It is proposed that reduced hippocampal volume may reflect neuronal atrophy in the region associated with chronic stress or inflammatory coupled mechanisms.

1.6 Effects of stress on the immune system

The role of stress-induced changes in the immune system have been more routinely considered in disease vulnerability, wound healing and peripheral or central inflammatory conditions (Glaser and Kiecolt-Glaser, 2005) e.g. inflammatory bowel disease (Mawdsley and Rampton, 2005) or multiple sclerosis (Gold et al., 2005). In this regard, stressful events and the distress that they evoke can impact on infectious disease risks, impair wound healing and exacerbate the course of inflammatory disease. Stress related immune dysregulation may be an important mechanism underlying the development of cancer, cardiovascular disease, osteoporosis, arthritis, type 2 diabetes and age related frailty and functional decline (Glaser and Kiecolt-Glaser, 2005, Godbout and Glaser, 2006, Irwin, 2008, Kemeny and Schedlowski, 2007, Moynihan and Ader, 1996). As this research topic has been the subject of a number of published research reviews to date, it will not be covered in detail here. Accumulating data indicate that stress related effects on the immune system and inflammation may also play a role in neuropsychiatric disorders including major depression (Miller et al., 2009) and analyses of stressor and immune activation effects in animal models have been useful in defining some of the alterations that may subserve pathophysiology.

Interactions between the brain and immune system are bidirectional involving both the ability of cells and products of the immune system to alter brain function and behaviour and also the brain to influence immune function. The most widely applied manipulation to study brain immune interactions has been the application

of stressors of varying type, duration and intensity. Upon exposure to stress the brain sends signals to the periphery to stimulate the release of glucocorticoid hormone via the HPA-axis and catecholamine transmitters via the sympathoadrenal medullary (SAM) axis in addition to a neuronal component that regulates peripheral activity by direct sympathetic innervations. These peripheral stress signals in turn have direct effects on various components of the immune system influencing immunity, host resistance and disease outcomes. In addition the immune system also communicates with and influences the brain. Although usually considered to be immune privileged, receptors for numerous cytokines, soluble protein mediators of immune cells, are expressed throughout the brain. Moreover there are mechanisms by which cytokines communicate with the brain via the circumventricular organs, via active transport or the activation of cells lining the cerebrovasculature. Cytokines are also capable of activating peripheral nerves such as the vagus nerve which transmit signals to the brain (Miller et al., 2009, Quan and Banks, 2007). Researchers working in the field have used both human and animal models to contribute to our understanding of these interactions and their impact on behaviour. This field has also been the subject of a number of reviews to date (Connor and Leonard, 1998, Dantzer et al., 2008, Dunn et al., 2005)

CRH can directly alter mood independently, functioning as a neuroregulator and acting via its own receptors CRHr1 and CRHr2 (Gallagher et al., 2008). CRH pathways have been shown to interact with 5-HT and NA circuits (Nemeroff and Owens, 2002) and signalling via CRHr1 has been associated with anxious behaviour, anhedonia and reduced exploratory behaviour in rodent models (Refojo and Holsboer, 2009). Indeed mice genetically altered to lack the CRHr1 gene have been shown to have decreased anxiety behaviour and be more resistant to stress (Smith et al., 1998). Altered CRH signalling has been implicated in depression, with victims of suicide shown to have lower levels of CRH receptors in their frontal cortex (Pace et al., 2007), and it has been suggested as a possible treatment target in anxiety and depression (Reul and Holsboer, 2002). Rectification of this HPA-axis imbalance is the most reliable indicator of a patient's response to antidepressant treatment and whether the patient is likely to suffer a relapse. Patients who exhibit sustained dysregulation of the HPA-axis at point of discharge from an institution have a 4-6 fold higher risk of relapse than those who's HPA-axis is functioning normally (Hennings et al., 2009, Zobel et al., 1999).

Figure 1.5 – Outline of the bi-directional communication between the CNS and the periphery

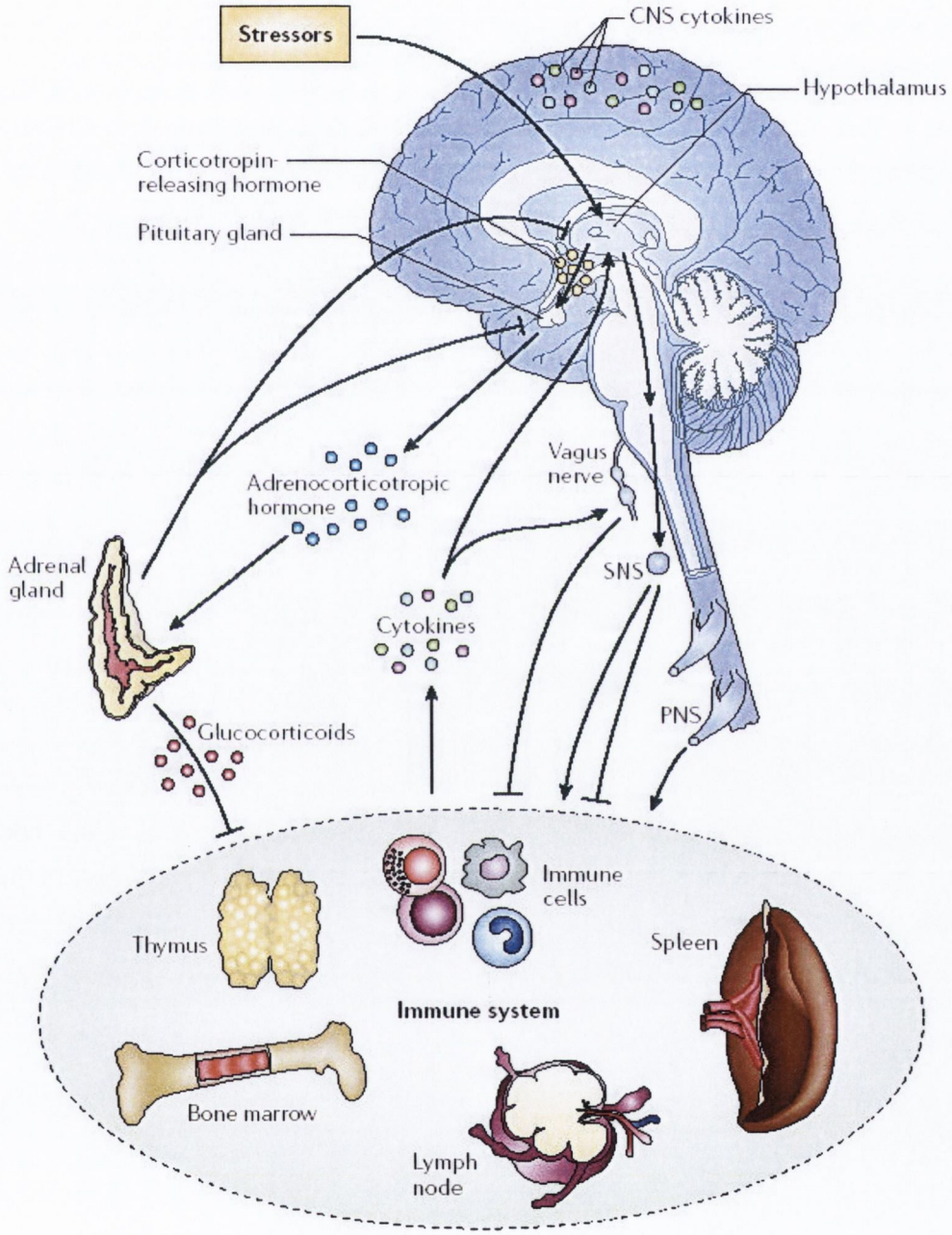

Figure 1.5 – Outline of the bi-directional communication between the CNS and the periphery

The HPA-axis is activated in response to stresses within the body, including immunological and psychological. Upon activation, the parvocellular neurosecretory neurons of the paraventricular nucleus of the hypothalamus release CRH that bind to receptors at the anterior lobe of the pituitary, which subsequently releases adrenocorticotropic hormone (ACTH). ACTH binds to receptors in the adrenal cortex, causing the release of glucocorticoids into the general circulation which bind to GR. There exists two subtypes of GR, a genetically active subtype (GRα) that induces GR related signalling and a genetically inactive subtype (GRβ) which does not bind to glucocorticoids but can bind to glucocorticoid response elements in the nucleus and is transcriptionally inactive (Bamberger et al., 1995). Glucocorticoids mediate a self-regulation on the HPA-axis itself via signalling onto the hypothalamus, pituitary and hippocampus, avoiding prolonged activation (Bear et al., 2007). The SAM-axis also has an effect on the immune response, with direct innervation of lymphoid organs including lymph nodes and the spleen resulting in local release of adrenaline (Adr) and NA and release into general circulation. Signalling for NA and Adr is via two receptor types, α and β, of which there are two (α_1, α_2) and three (β_1, β_2 and β_3) further subtypes, all of which are G-protein coupled receptors (Rang and Dale, 2007). It has been widely reported that most lymphoid cells express β-AR and that the effect that the SAM-axis has on immune function is mediated mainly via the β_2-AR (Sanders and Straub, 2002). Immune products such as cytokines can also signal in the CNS via vagus nerve stimulation, access the brain directly across the BBB or through "leaky" circumventricular organs. Dysregulated HPA- and SAM- axes have been associated with depressive symptoms. One of the most consistent states observed in people presenting with depression is a hyperactivity of the HPA-axis marked by chronic elevations in CRH and glucocorticoids (McKay and Zakzanis, 2010). Figure by (Sternberg, 2006).

Investigations of the immunomodulating effects of stress are commonly carried out in animal models as it is difficult to experimentally induce stress in a quantifiable way in humans. In this regard animal models have made a good contribution to our current understanding, enabling an invasive study of the influence of stress on brain-immune interactions not possible in humans for ethical reasons. Numerous manipulations are used to simulate stressful experiences in animals which vary in construct, presentation, intensity and duration including restraint or immobilisation, foot shock, conditioned fear, water avoidance, forced swimming, social disruption/separation, altered housing conditions, perinatal stress and maternal separation. In general, stressors are characterised as being either physical (e.g. forced swimming,) or psychological (e.g. social disruption) or elements of both (e.g. immobilisation). Those of a psychological, or psychological combined with physical, nature are considered to more closely mimic psychological stress in humans and to be of greater relevance. Such interventions are associated with reduced host resistance to infectious agents, impaired wound healing, increased metastatic potential of cancer cells and an altered course of autoimmunity and inflammation. The manner in which stress translates into physiological changes in the immune system is also characterised in human and animal studies and includes altered cellular trafficking, suppressed T-cell proliferation, modified antibody production, altered neutrophil phagocytosis, reduced natural killer cell activity and dysregulation of the cytokine network (Padgett and Glaser, 2003). Currently, elucidation of mechanisms mediating the ability of stress to influence such changes is an area of considerable interest.

CNS and immune response to stress and resultant disease vulnerability can vary substantially depending on a variety of interacting factors including genetic background, sex, age, coping style, social support, previous exposures and experience, the nature of stressor employed and type of immune response generated (Avitsur et al., 2006, Bonneau et al., 2007, Kavelaars and Heijnen, 2006, Moynihan and Ader, 1996, Koolhaas, 2008). The most common approach taken in developing animal models of stress-immune interactions has been to determine responses to stress in various laboratory strains of mice or rats. In view of the hundreds of strains available, identifying differentiating characteristics of interest between strains is no small task although there are some pre-existing examples of dissociation identified in some commonly used mouse or rat strains. For example, sex and/or strain can influence the activation of cell mediated or

Th1 and humoral or Th2 responses. The popular mouse strain C57Bl/6 exhibits a strong Th1 response to intracellular pathogens whereas BALB/c mice produce a humoral response that is not very effective in clearing the pathogen. Another potential variable is the neuroendocrine state of the animal, where glucocorticoids affect the balance between Th1 and Th2 cells. High levels of glucocorticoids can shift the balance between Th1 and Th2 towards a Th2 response (Calcagni and Elenkov, 2006). For example, Lewis rats show reduced HPA-axis response to stress on account of lower hypothalamic CRH concentrations. Consequently these rats are more susceptible to inflammatory disorders including arthritis and colitis. A more comprehensive review of the effects of stressors on the course of a variety of infectious and/or inflammatory and immunologically mediated disease processes in various strains of rats and mice may be found elsewhere (Bonneau et al., 2007, Moynihan and Ader, 1996). Although much research remains to be carried out particularly in the areas of genetic predisposition, gene environment interaction and the impact of development in early life amongst other vulnerability factors, it is evident to date that animal models, which enable a study of the role of stress in immunologically mediated disease processes, are critical as they identify interactions between stress, immune system and disease and determine whether or not the effects of stress on the immune system may be particularly detrimental in a disease setting (Bonneau et al., 2007, Moynihan and Ader, 1996).

Data generated in our laboratory using a mouse model of bacterial endotoxin (LPS) induced immune activation, shows that restraint stress alters the response to this immune challenge. Mice, restrained in tubes with breathing and cooling holes such that they can move forwards or backwards but cannot move around, for 2hr were subsequently challenged with LPS and trunk blood/spleen were collected and analysed for cytokine responses. The spleen is a model organ for testing brain-immune interactions as it is innervated by sympathetic nerve fibres that are distributed in close proximity to immune cells (Meltzer et al., 1997, Nance and Burns, 1989). Moreover the observed time course for splenic cytokines in rodents follows a similar pattern for serum cytokines (Meltzer et al., 2003). Restraint stress suppressed the LPS induced increase in splenic IFNγ expression and circulating IFNγ concentrations. This suppression of IFNγ, a key cytokine in priming antigen presenting cells and in anti-viral mediated immunity, was accompanied by a reduction in IFNγ associated phosphorylation of the transcription factor STAT1 and a reduction in the transcription of a number of

STAT1 regulated genes including the chemokine IP-10, the co-stimulatory molecule CD-40 and the inducible form of nitric oxide synthase (NOS) (Curtin et al., 2009a).

IFNγ is not produced directly following LPS challenge rather its induction is regulated by IFNγ inducing cytokines of which IL-12 is the main driver. IL-12 is produced by macrophages and dendritic cells and in turn stimulates the production of IFNγ in natural killer cells, the major source of IFNγ in the spleen. In this regard restraint stress suppresses the expression of the active subunit of IL-12, IL-12p40, following LPS challenge. To confirm that IL-12 was driving IFNγ production in this model it was possible to rescue the stress-induced suppression of IFNγ by the administration of exogenous IL-12 to cultured splenocytes (Curtin et al., 2009a).

Activation of the HPA and SAM axes play an important role in mediating the effects of stress on the immune system (Besedovsky and del Rey, 2000, Malarkey and Mills, 2007). To address the mechanisms mediating the stress-induced suppression of IFNγ, it was shown that restraint stress provoked an increase in circulating and splenic corticosterone concentrations. Furthermore, the suppressive effect of stress on IFNγ and IL-12 could be mimicked by the addition of exogenous corticosterone to spleen cells *in vitro*. Thus corticosterone was a likely candidate to account for the suppressive effect of stress on IL-12 and IFNγ production. In support of this, prior administration of the GR-antagonist mifepristone prevented the stress-induced suppression of IL-12 and IFNγ production (Curtin et al., 2009a). Another candidate of interest to account for the immunosuppressive effects of stress was the anti-inflammatory cytokine IL-10 which is known to be a key regulator of IL-12 production and to possess the ability to suppress the production of a number of pro-inflammatory cytokines. Stress was determined to increase circulating IL-10 concentrations following LPS challenge. However, using IL-10 knockout mice, it was reported that IL-10 does not mediate the suppressive effect of restraint stress on innate IFNγ production as this response remained intact in the knockout animals and thus increased IL-10 does not account for the stress-induced suppression of IL-12 and IFNγ (Curtin et al., 2009a). Further work to determine the mechanism involved showed that the β-AR antagonist nadolol prevents the stress-induced increase in circulating IL-10 concentrations and splenic IL-10 expression confirming that the stress-induced enhancement of IL-10 is mediated through β-Ars subsequent to the activation of the SAM-axis or innervating sympathetic nerves (Curtin et al.,

2009b). These and related studies (Glaser and Kiecolt-Glaser, 2005, Konstantinos and Sheridan, 2001) illustrate that the HPA and SAM axes are the main immunoregulatory pathways that influence the immune response to stressor exposure.

In a further series of experiments Curtin and co-workers determined the effect of the anxiolytic drug chlordiazepoxide, a drug commonly prescribed to relieve stress by altering perception, in the restraint stress model. Prior treatment with chlordiazepoxide can prevent the stress-induced suppression of LPS-induced IFNγ and IL-12 and the stress related increase in LPS-induced IL-10 (Curtin et al., 2009b, Curtin et al., 2009a). These results support a mechanism where central perception of the stressor is critical to mediating the immunosuppressive effects of stress.

Similar effects to those observed following acute stressor exposure were also obtained following repeated stress consisting of 2hr restraint stress over 7 consecutive days. However it was noted that although LPS-induced IFNγ and IL-12p40 expression was suppressed in splenocytes immediately after stress, an increase in splenic IFNγ and IL-12 concentrations was obtained 24hr later. In tandem with raised cytokine concentrations, a reduction in the expression of glucocorticoid and β_2-Ars in splenocytes 24hr after stressor termination was accompanied by a reduction in the sensitivity of LPS-induced IFNγ and IL-12 to corticosterone and the β_2-AR agonist salbutamol (Curtin, 2008). Thus prolonged exposure to stress can precipitate a low grade inflammatory response associated with a down-regulation and reduced sensitivity of the glucocorticoid and β_2-Ars on splenocytes.

Stress has long been associated with changes in immune status and there are many reports of both immunoenhancement and immunosuppression where the impact of stressor exposure on the immune response depends on a variety of factors. The duration of stressor exposure seems to be important in determining the immune response. In a recent meta-analysis of human studies carried out by (Segerstrom and Miller, 2004), they showed that a range of altered immune profiles could be induced depending on the time frame of stressor exposure. Acute time limited stressors were associated with an up-regulation of some innate immune responses and suppression of specific immunity. Brief naturalistic stressors or real life short term challenges were associated with a shift in the profile of some cytokines whereas chronic stress was linked to global

immunosuppression. The nature of the stressor is also influential; where there is a smaller behavioural and endocrine response upon repeated exposure to a stressor of a physical nature, the intensity of a psychological stressor increases with repeated exposures (Blanchard et al., 1998, Sato et al., 1996). For example, studies have shown that exposure to chronic social disruption stress results in altered splenocyte function including increased splenic monocytes and neutrophils and decreased lymphocytes, with implications for the healing of bite wounds that are often associated with social stress in rodents (Avitsur et al., 2002a, Avitsur et al., 2002b). However in the studies of social stress, and in other studies that show an immunoenhancing effect of chronic stress exposure, the temporal proximity between the stressor termination and the immune challenge is delayed. Such studies have reported that an immunoenhanced state becomes evident within 24hr of stressor termination (Avitsur et al., 2005, Zalcman et al., 1988, Zalcman et al., 1989, Zalcman and Anisman, 1993). Therefore an immune challenge administered immediately following stress and 24hr after stressor termination gives alternative immune responses suggestive of cellular alterations occurring during the recovery period following stress.

It has long been demonstrated that stress induces adaptations in receptor expression and sensitivity during exposure to chronic stress (Quan et al., 2003). Alterations in immune response due to changes in receptor expression and sensitivity are commonly referred to as receptor desensitisation which involves alterations in receptor expression that remain for a period following stressor termination. As GR mediates tonic anti-inflammatory effects, down-regulation of these receptors in response to stress could promote the development of an inflammatory state. Exposure to chronic stress which chronically activates the HPA-axis has been shown to result in substantial increases in systematic glucocorticoid levels by both enhancing the release of glucocorticoid hormones and by decreasing the plasma binding capacity of corticosterone binding globulin (Stefanski, 2000, Spencer et al., 1996). This has been demonstrated to lead to adaptive changes in immune cells with reduced nuclear translocation of GR and impaired immunosuppressive action of the receptor in monocytes. This stress-induced reduction of GR expression and function may be considered an adaptive response to chronic stress to alleviate immunosuppressive effects associated with sustained levels of glucocorticoids. When exposure to stress is terminated, the concentrations of glucocorticoids return to pre-stress basal levels. However the cellular adaptations persist for some time and during this interim an

immunoenhanced/pro-inflammatory state may prevail as a result of GR desensitisation to normal physiological levels of glucocorticoids.

Reduced GR expression in mouse spleen 18hr following exposure to chronic social stress has been reported by (Quan et al., 2001) who also reported that GR expression was reduced in the hippocampus, an important neural structure for the negative feedback action of glucocorticoids (De Kloet et al., 1998). Chronic stress-induced down regulation of GR mRNA and protein in mouse hippocampus has also been demonstrated by other groups (Sapolsky et al., 1984, Sapolsky and Meaney, 1986, Herman et al., 1995, Kitraki et al., 1999). The implications of stress-induced downregulation of GR in the hippocampus for the activity, regulation and proliferation of resident microglia is a subject of considerable current interest in this field and will be discussed later.

Catecholamines, acting through α- and β-Ars, have been shown to have pro- and anti-inflammatory properties and play a role in the maintenance of an immunoregulated environment (Johnson et al., 2005, Nance and Sanders, 2007, Feinstein et al., 2002). It has long been shown that following stimulation of β_2-Ars on immune cells subsequent production of cAMP promotes the production of cytokines that can suppress cell mediated immune responses (Suberville et al., 1996, Platzer et al., 2000). For instance, activated monocytes and dendritic cells exposed to NA have reduced IL-12 concentrations, in parallel with increased production of the anti-inflammatory cytokine IL-10 (Elenkov et al., 1996) Such an alteration in the cytokine network could potentially diminish the cell based immune response. In a similar fashion to persistent HPA-axis activation with chronic stress, chronic activation of the SAM-axis results in substantial increases in circulating catecholamine concentrations and this can lead to adaptive changes in the expression of β_2-AR. For example, it has been demonstrated that continuous exposure to catecholamines leads to a reduced receptor response by a process of receptor phosphorylation, internalisation and down-regulation (Bawa-Khalfe et al., 2007). In addition, it has been reported that forced exercise which can be considered to provoke robust activation of the SAM-axis and to be a stressor for laboratory animals, provokes a reduction in β_2-AR expression in peritoneal macrophages associated with an increase in IL-12 production 24hr following termination of the regime (Itoh et al., 2004). Therefore, in a similar fashion to GR, stress-induced reduction of β_2-AR could be involved in a diminished negative feedback response to catecholamines on immune cells. Conversely others have reported an increase in catecholamine reactivity in mice

subjected to chronic mild stress (CMS) (Edgar et al., 2002, Edgar et al., 2003). Normally catecholamines exert a β_2-AR mediated inhibitory effect on mitogen induced T-cell proliferation and a stimulatory effect on B-cell proliferation in response to selective B-lymphocyte mitogens. Lymphocytes from mice subjected to CMS have an increased response to catecholamine mediated inhibition or enhancement of proliferation in T and B-cells coupled with an increase in β_2-AR density and responsivity. In this model, chronic stress is associated with an increased sympathetic influence on the immune response suggestive of an alternative mechanism through which stress can alter immunity.

In addition, the parasympathetic nervous system (PNS) may also play a role in the regulation of inflammation. Activation of efferent vagus nerve fibres by acetylcholine release and subsequent α_7-nicotinic receptor activation on immune cells can inhibit cytokine responses to endotoxin in laboratory animals (Pavlov and Tracey, 2005) suggesting that the sympathetic and parasympathetic nervous systems may have opposing regulatory influences over innate immunity and inflammation during stress (Miller et al., 2009). Increased muscarinic receptor expression has been reported in T and B-cells isolated from mice exposed to chronic stress (Edgar et al., 2002). Thus long term adaptive responses are also evident in the PNS in response to stress which may impact on immune function.

It is well established that reactivity to psychological stress is a contributing factor to depressive disorders and relapse and that severely depressed patients display hypercortisolaemia and glucocorticoid resistance related at least in part to impaired GR function (Pariante and Miller, 2001, Pace et al., 2006, Maier and Watkins, 1998). In this regard, it is known that inflammatory cytokines can promote glucocorticoid resistance (Pace et al., 2007). Thus the possibility exists that stress, which promotes glucocorticoid resistance, may precipitate a state of increased innate immune cytokines which in turn further drives glucocorticoid resistance in stress-related disorders like major depression. In that regard, chronic stress/depression has been consistently linked with the increased peripheral production of pro-inflammatory cytokines, most notably IL-6. It is well known from human studies that stressful experiences including depression, bereavement, marital separation, care-giving and examination stress can induce immune dysregulation and alter rates of clinical illness and influence recovery. In this regard high serum levels of IL-6 have been linked to risks for several conditions, such as cardiovascular disease, type 2 diabetes, mental health complications, and some cancers (Godbout and Glaser, 2006).

Taken together, reports to date suggest that increased activity through the SAM-axis coupled with reduced sensitivity to the inhibitory effects of corticosterone may, during chronic stress, contribute to activation of the inflammatory response system. Given the role of glucocorticoids and catecholamines in regulating innate immune responses and the capacity of cytokines to influence behaviour, it is not unreasonable to suggest that the inter-relationship between SAM-axis activation, HPA-axis dysregulation and cytokines contributes to the pathophysiology of depression, at least in some patients where inflammation is evident (Miller et al., 2009, Pace and Miller, 2009).

Preclinical studies have shown that exposure to a chronic stress can reduce the reactivity and amount of GR protein in immune cells (Quan et al., 2001, Curtin, 2008) and human tests have shown that people who had long term stressful exposure displayed glucocorticoid resistance which was accompanied by an increased pro-inflammatory profile following infection with a rhinovirus (Cohen et al., 2012).

Quan et al. (2001) reported that GR expression in the spleen and hippocampus was reduced following chronic stress exposure, while elevated levels of CRH and ACTH were also observed (Quan et al., 2001), Subsequent exposure to an LPS stimulus yielded higher sepsis mortality in chronically stressed mice than control equivalents. GR signalling also induces the expression of a GR co-chaperone, FK506 binding protein (FKBP5), which reduces the binding capability of GR to glucocorticoids (Jääskeläinen et al., 2011, Stechschulte and Sanchez, 2011),and has also been shown to increase the nuclear translocation of the non-active GRβ isoform, further reducing GR responsiveness (Zhang et al., 2008).

Pro-inflammatory cytokines have also been shown to affect GR signalling within the cell. Activation of p38 MAPK, JNK1/2/3, ERK1/2, STAT and NFκB signalling pathways have all been reported to reduce GR functioning within fibroblast cells. IL-1a treatment has been shown to reduce dexamethasone induced GR translocation to the nucleus and GR mediated transcription *in vitro*, via induction of p38 MAPK (Pariante et al., 1999, Wang et al., 2004). These reductions were reversed following co treatment with IL-1ra or antisense oligonucleotides targeting p38. IL-2 has been reported to reduce translocation of GR and the formation of heterodimers with STAT5 (Goleva et al., 2002). Tliba and co-workers have also reported that IFN regulatory pathways can reduce GR gene transcription following treatment with TNFα and IFNγ (Tliba et al., 2008).

NFκB has been shown to interact with GR in the nucleus in a mutually inhibitory protein-protein interaction (McKay and Cidlowski, 1999) as well via competition for the co-activators CREB-binding protein and steroid receptor co-activator 1 (Sheppard et al., 1998). Thus activation of pro-inflammatory cytokine signalling pathways can interfere with GR signalling via disruption to binding in the nucleus that prevents transcription and by limiting the availability of common co-activators that would otherwise be available to activate GR signalling. *In vitro* experiments have shown greater increases in GRβ expression following treatment with TNFα and IL-1 than GRα (Webster et al., 2001).

Chronic activation of the SAM-axis has been shown to reduce β_2-AR thereby limiting the effectiveness of its influence on the immune response to stimulation. Trials involving a forced exercise regime revealed downregulation of β_2-AR expression in peritoneal macrophages 24hr following exposure (Itoh et al., 2004). Subsequent LPS stimulation of macrophages harvested from these stressed mice revealed that they produced elevated levels of the pro-inflammatory cytokine IL-12 when compared to non stressed controls.

1.7 Evidence for activation of immune system in depressed patients

Patients with depression have been shown to have elevated levels of pro-inflammatory cytokines, chemokines, adhesion molecules and prostaglandins in the blood and cerebrospinal fluid (CSF) (Leonard, 2007, Miller et al., 2009). Meta analyses have reported that depressed patients have elevated levels of pro-inflammatory cytokines including IL-1β, IL-6 and TNFα circulating peripherally (Dowlati et al., 2010, Howren et al., 2009, Liu et al., 2012). IL-6 and IFNγ were found to be elevated in treatment resistant depressed patients when compared to controls, however no difference in TNFα concentrations were observed (Hughes et al., 2012). IL-6 has been the most consistently elevated cytokine in depressed patients and the presence of elevated IL-6 and C-reactive protein (CRP) can predict a subsequent development of depression over the course of a decade even in individuals with no background of depression when samples were taken (Gimeno et al., 2009, Pasco et al., 2010). Interestingly, while Simon et al. (2008) reported increased circulating concentrations of pro-inflammatory cytokines in depressed patients, they also reported increased concentrations of IL-10 and IL-4

(Simon et al., 2008). Maes and co-workers also reported that the anti-inflammatory IL-1ra was elevated in conjunction with increased pro-inflammatory cytokines (Maes et al., 1997) suggesting a complex profile of activation within the innate immune system.

People with autoimmune disorders have been shown to have a higher incidence of depressive disorders, including MS and RA, suggestive of an association between activation of the adaptive immune system and depression (Gibney and Drexhage, 2013). Depressed patients have been reported to have fewer resting $CD3^{+}/CD25^{-}$ T_{reg}-cells, increased T-cells expressing activation markers, accompanied by increased expression of the soluble IL-2 receptor (sIL-2R), induction of IDO, higher levels of circulating tryptophan metabolites and lower levels of tryptophan (Maes, 2011).

Investigations that have examined functional responses in peripheral blood mononuclear cells (PBMC) have reported elevated levels of IFNγ and sIL-2R (Seidel et al., 1995) and IL-1β and IL-6 (Maes et al., 1995), however other studies have reported the opposite effect, reporting reduced IL-1β, IL-2 and IL-3, in stimulated PBMCs from depressed patients (Weizman et al., 1994). This inhibited response observed by Weizman and co-workers could be as a result of hypo-responsivity in PBMCs caused by raised circulating cytokines signalling through T-cells, suppressing the ability of cells to respond to stimulation (Haroon et al., 2012, Maes et al., 2012).

CSF is produced by the choroid plexus within the brain and circulates throughout the CNS in the subarachnoid space and the ventricular system. Examination of human CNS tissue while the person is still alive is very difficult so CSF can be used to monitor the levels of pro-inflammatory cytokines, neurotransmitter metabolites and other molecules to gain insight into immune responses within the CNS. CSF of depressed patients has been found to contain elevated levels of pro-inflammatory cytokines and their soluble receptors (Müller et al., 1997, Raison et al., 2006). Increased concentrations of IL-1β have been reported in patients suffering from depression and a positive correlation found between concentration of IL-1β concentration and symptom severity (Levine et al., 1999). Elevated levels of IL-6 and TNFα have also been reported in patients who had attempted suicide (Lindqvist et al., 2009) while in a separate study IL-1, IL-6 and TNFα were also correlated with depression severity in patients (Martinez et al., 2012). In relation to cytokine therapy as mentioned previously, patients with

hepatitis C treated with IFNα who subsequently developed depression related symptoms, were reported to have elevated CSF levels of IFNα and IL-6 (Raison et al., 2009).

Post mortem, the expression of IFNα and its receptor IFNα/βR1 were reported to be elevated in dorsolateral prefrontal cortex of depressed patients when compared to non-depressed controls (Kang et al., 2007). Higher expression of the tryptophan/ kynurenine metabolite, QUIN was reported to be elevated in activated microglia in the subgenual anterior cingulated cortex and anterior midcingulate cortex of severely depressed patients (Steiner et al., 2011). These limited studies would suggest a role for microglial activation within the CNS in the progression of depressive disorders. Patients with depression have been shown to have a lower density of astrocytes when compared to healthy controls. A reduction in the astrocytic activation marker glial fibrillary acidic protein (GFAP) has been reported in post mortem tissue from depressed when compared to non-depressed controls (Miguel-Hidalgo et al., 2010, Muller and Schwarz, 2007, Ongür et al., 1998). As mentioned above previously, astrocytes preferentially express KAT II which leads to the production of an NMDA receptor antagonist KYNA (Guillemin et al., 2001). Astrocytes also express the highest density of glutamate transporters and play an integral role in removal of glutamate from the synapse controlling the extent of excitation of glutamate receptors (Huang and Bergles, 2004, Thomas et al., 2011). Reduction of astrocytes could lead to reduced production of the KYNA and decreased removal of glutamate from the synapse creating an excitotoxic extracellular environment.

1.8 Development of animal models of depression based on immunological activation and autoimmunity

Animal models are most useful if they are based on the underlying cause and can elucidate mechanisms underlying the pathophysiology of depression and predict useful treatment strategies. A number of proposed mechanisms have emerged from animal studies bridging immune dysregulation to the pathophysiology of depression in the literature in recent times and these are summarised as follows:

1. Decompensation or dysregulation of the mechanisms that regulate sickness behaviours has been proposed to be of consequence for depression (Dantzer et

al., 2008). Normally infectious episodes are reversible as the immune system combats the infectious pathogen and recovery mechanisms that oppose inflammation take effect. In depression however such mechanisms may be dysfunctional where the inflammatory response is more intense due to an imbalance between pro and anti-inflammatory cytokines or glucocorticoid resistance. Depression may also be associated with a greater sensitivity to immune related changes due to pre-existing vulnerability such as changes in genotype e.g. serotonergic receptors, transporters or metabolism or disturbances in HPA-axis or sleep quality. Overall Dantzer and colleagues (2008) suggest that depression may represent a maladaptive version of cytokine-induced sickness which could occur when activation of the innate immune response is exacerbated in intensity or duration.

2. Changes in brain plasticity associated with immune activation may also account for mechanisms underlying cytokine-induced depression. Cytokines are considered important for providing trophic support to neurons and enhancing neurogenesis while contributing to cognitive functions such as memory in laboratory animals. However with excessive or prolonged activation, cytokines such as IL-1β and TNFα can promote abnormalities that are thought to be relevant to the pathophysiology of depression, including cognitive impairment, diminished neurotrophic support, enhanced oxidative and apoptotic mechanisms and decreased neurogenesis in the hippocampus favouring impaired neuroplastic processes (Anisman, 2009, Goshen et al., 2008, Miller et al., 2009). In support of a causative role for cytokines, blockade of cytokine activity through administration of IL-1ra, transplantation of IL-1ra secreting neural precursor cells into the hippocampus or through the use of IL-1 knockout mice, can prevent such changes (Barrientos et al., 2003, Koo and Duman, 2008).

3. Stress and cytokines act synergistically and promote several common neurotransmitter, endocrine and behavioural measures and cross sensitisation of the effects of stressors on inflammatory immune system activation or vice versa may predispose to major depressive disorder. In this regard Anisman (2008) proposes that the CNS may perceive immune activation as a stressor and by virtue of stressor like effects may contribute to mood disorder vulnerability. For example it has been reported that when an immune challenge (e.g. IL-1β, LPS or poly I:C) is administered against a backdrop of psychosocial stress, the central transmitter and endocrine changes ordinarily elicited by immune challenge are greatly enhanced coupled with a marked enhancement of pro-inflammatory

cytokine expression in hypothalamic and extra-hypothalamic regions (Anisman et al., 2007, Gandhi et al., 2007, Gibb et al., 2008). Further work is required to clarify if sensitisation may also occur to behavioural changes upon exposure to a cytokine following a period of stress or vice versa.

4. Conditions of chronic inflammation are associated with priming of the CNS e.g. age, diabetes or prion disease exacerbate signs of sickness and neurovegetative, somatic and psychological symptoms of depression that occur in response to acute peripheral inflammation. Such an exacerbation is associated with an exaggerated inflammatory response in the brain in response to peripheral immune activation and is proposed to reveal a primed state of the microglial compartment. Priming of macrophage in the periphery has been characterised e.g. increased TLR4 signalling and NFκB activation in response to LPS following an initial exposure to IFNγ. Whilst the priming of microglia is also thought to occur, it is less well characterised and is considered to be associated with increased IL-1β, reduced IL-10 and IL-1 type II receptor and reduced IL-1ra, increased leucocytes and microglia in the brain coupled with mast cell migration to the perivascular space (Dantzer, 2009) .

Microglia have been reported to show an increased expression of IDO following treatment with TNFα and IFNγ (O'Connor et al., 2009a). Pre-treatment of mice with the microglial inhibitor minocycline was shown to attenuate LPS-induced increases of pro-inflammatory cytokines in the CNS, sickness behaviour and anhedonia (Henry et al., 2008). As mentioned above (figure 1.4) microglia preferentially express KMO which produces the NMDA receptor agonist QUIN, facilitating prolonged signalling of this receptor in the brain (Heyes et al., 1996). Recent studies involving positron emission topography (PET) imaging have been able to show microglial activation states in living brain tissue. Mice injected with LPS were shown to have activated microglia on day 2 and day 3 following LPS administration, which was accompanied by depressive-like behaviour on Day3 and an increased kynurenine/tryptophan ratio in the serum (Dobos et al., 2012).

Although a number of animal models support a role for inflammatory mediators in the induction of depression-like behaviours it is also of interest to consider the possibility that cytokine-induced depression may occur in the absence of a direct immune stimulus. In this regard there is evidence from animal studies that stress promotes cytokine expression and microglial activation in the CNS through the induction of the pro-inflammatory cytokine IL-1β and the microglial activation

marker CD11b, respectively, and inhibition of IL-1β receptors using IL-1ra blocks the ability of stress to cause cognitive dysfunction (Pugh et al., 1999). In a separate report, exposure of mice to chronic mild stress (CMS) increases central IL-6 expression without the use of an immune stimulus to induce the cytokine response (Mormède et al., 2003). However the functional significance of such changes for the pathophysiology of stress or depression remains to be determined. IL-1ra can inhibit stress-induced increases in hypothalamic NA, dopamine and 5-HT concentrations and stress-induced HPA-axis activation in rats (Shintani et al., 1995). These data suggest that IL-1β can mediate behavioural, neurochemical and endocrine effects of stress. Yirmiya and co-workers have shown that brain IL-1β mediates chronic stress-induced depression in mice via adrenocortical activation and the suppression of hippocampal neurogenesis (Goshen et al., 2008). Mice subjected to CMS displayed increased depression related behaviours, increased circulating levels of corticosterone coupled with an increase in IL-1β concentrations in the hippocampus. Mice with a deletion of the IL-1 receptor type 1 (IL-1rKO) or mice with transgenic, brain restricted over-expression of IL-1ra did not display CMS-induced behavioural, neuroendocrine or neurogenesis changes. Removal of endogenous glucocorticoids by adrenalectomy abolished the depressive-like behaviours induced by CMS suggestive of a causal role of the reduced corticosterone response observed in IL-1rKO mice in their resistance to CMS-induced depression.

Given the ability of stress to activate IL-1β in the brain, it is conceivable that cytokines like IL-1β may be of relevance to the neurotrophic hypothesis of depression which proposes that stress-induced depression is mediated by a suppression of hippocampal BDNF that is counteracted following chronic antidepressant treatment (Duman et al., 1997). Such changes in BDNF have been proposed to underlie reductions in hippocampal volume and neuronal atrophy associated with depression. As pro-inflammatory cytokines increase glucocorticoid concentrations which in turn suppress the expression of growth factors in the brain including BDNF it is not unreasonable to suggest that stress-induced activation of IL-1β may be related to reductions in hippocampal BDNF. In support of this mechanism it has previously been reported that systemic LPS administration to rats reduces BDNF expression in cortex and hippocampus (Guan and Fang, 2006). Repeated administration of LPS into the hippocampus reduces mRNA expression of BDNF and its receptor TrkB (Tanaka et al., 2006).

Moreover reductions in BDNF expression produced by social isolation in rats is blocked by intrahippocampal administration of IL-1ra (Barrientos et al., 2003). It is also of note that BDNF has also been reported to be reduced in the hippocampus of rats following central administration of IFNα which was mediated by IL-1β (Kaneko et al., 2006) indicating that other cytokines may converge on IL-1β to mediate changes in hippocampal BDNF.

C57BL/6J mice subjected to a chronic prolonged restraint stress, displayed a decrease in IL-10 expression centrally accompanied by increased total immobility and decreased time to first immobility in the forced swim test paradigm. These depressive-like behavioural deficits were reversed via administration of recombinant IL-10 (Voorhees et al., 2013).

The role of microglia in mediating inflammation in the brain following exposure to psychological stress has been substantiated by Blandino and co-workers (2006) who demonstrated that pre-treatment with the microglial activation inhibitor minocycline substantially attenuated the increase in IL-1β found following inescapable shock (Blandino et al., 2006). Chronic stress has been reported to increase the expression of microglial activation markers and reduce the expression of glucocorticoid inducible genes in mice indicating glucocorticoid insensitivity. These changes, induced by repeated social defeat, were reversed by administration of propanolol suggesting a link between the SAM-axis, a GR signalling impairment and immune activation within the CNS (Wohleb et al., 2011b). Increased microglial activation was also reported in a study by Couch and co-workers, in stress sensitive C57BL/6J mice who displayed anhedonic and depressive-like behaviours following exposure to a chronic stress paradigm that included exposure to predation and restraint stress. When compared to stress resilient mice, increased TNFα, IDO and SERT expression were reported in the frontal cortex of stress susceptible mice (Couch et al., 2013). In rats, elevated TNFα concentrations have been reported in the CNS of rats subjected to a prolonged restraint stress (Madrigal et al., 2002), while an inescapable foot shock stress leads to an increase in the expression of the microglial activation markers CD11b and MHC II in the CNS and a down-regulation of CD200, which helps to keep microglia in a quiescent state (Frank et al., 2007). These changes were also accompanied by an increased response of hippocampal microglia to LPS. In addition immobilisation stress promotes changes to the number of microglia (Nair and Bonneau, 2006) and the morphology of microglia from a ramified resting state to activated state that occurs in a number of stress sensitive regions of the

brain (Sugama et al., 2007, Tynan et al., 2010). Stress-induced activation of cytokine responses in the CNS appear to be largely dependent on activation of microglia (Frank et al., 2007).

However despite these interesting leads from animal experiments indicating that stress can promote a pro-inflammatory phenotype and microglial activation in the CNS in the absence of an overt immune stimulus, to date there is no solid evidence of microglial activation or increased CNS cytokines in depressed humans. There is increasing recognition that psychosocial stress can activate the inflammatory response system in the periphery albeit with limited evidence for activation of central inflammatory pathways within clinical populations of relevance for depression. NFκB and IL-6 responses to psychosocial stress have been shown to be exaggerated in patients with depression (Glaser et al., 2003, Pace et al., 2006). In addition there are numerous reports that chronic stress in humans is associated with increases in the acute phase protein, CRP as well as IL-6 and other inflammatory markers (Kiecolt-Glaser et al., 2005, McDade et al., 2006, Miller, 2008). There is evidence for the loss of glial elements including oligodendrocytes and astrocytes in multiple mood-relevant brain regions including the subgenual prefrontal cortex and amygdala which has emerged as a morphological abnormality in major depression (Hamidi et al., 2004, Ongür et al., 1998). Moreover recent post mortem data suggests that suicide is associated with increased microglial numbers in the prefrontal cortex (Steiner et al., 2008).

The mechanisms responsible for the activation of microglia in psychiatric disease remain elusive. A direct microbe-driven activation of microglia is possible (Kristensson, 2011). But apart from direct microbial/gDNA activation of microglia, inborn errors in the growth and differentiation of mononuclear phagocyte system (MPS) cells can make the cells vulnerable for hyper-stimulation. A good model of this mechanism is the non-obese diabetic (NOD) mouse, widely used for studying autoimmune thyroiditis and diabetes, which are also clinically associated with psychiatric disorders. This mouse has been shown to express organ specific autoantibodies, autoreactive T-cells and aberrant expression profiles of MHC genes (Leiter et al., 1987). It typically develops diabetes spontaneously at 12-14 weeks old in an specific pathogen free environment, with the first mononuclear infiltrates occurring at 3-4 weeks old (Anderson and Bluestone, 2005a). The diabetic phenotype found in NOD mice is mediated by CD4+ and CD8+ T-cell infiltration of the pancreas and destruction of the β-cells of the islets of Langerhans (Boitard et al., 1989). NOD mice have been shown to have a series

of defects in their leukocytes, including low levels of natural killer cells and defective macrophage maturation and function which have been shown to produce higher levels of pro-inflammatory cytokines when stimulated (Anderson and Bluestone, 2005a, Stoffels et al., 2004). Previous investigations of NOD mice have reported that their inflammatory state may be associated with anxiety-like behaviours (Amrani et al., 1994a, Bluth et al., 1999a). The NOD model thus opens new avenues to study gene-environment (immune stimulation) interactions of relevance to psychiatric disorders.

The development of models which combine genetic susceptibility to depressive-like behaviours with environmental manipulations may lead to models with better face and construct validity. One such model of susceptibility is the Wistar-Kyoto (WKY) rat strain, which was originally bred as the normotensive control of the spontaneously hypertensive rat (SHR). It has subsequently been shown to exhibit altered behaviour, as well as changes in neurotransmitter and endocrine systems that are similar to those exhibited in human depressed patients. The model has been shown to possess good face validity across a number of paradigms and its behaviour has been shown to be sensitive to treatment with a number of standard and novel antidepressant agents (Durand et al., 1999, Hurley et al., 2013, Tizabi et al., 2012). In the current project we have adopted the WKY rat to determine if microglial activation may be associated with the anxiety and depressive-like behaviours exhibited by the strain.

The maternal immune activation (MIA) is a model in which a pregnant female is administered an immune stimulus, such as LPS or poly I:C, which subsequently leads to raised circulating pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6, in the pregnant dam and offspring *in utero* (Boksa, 2010, Patterson, 2009, Wang et al., 2006). Changes in the levels of BDNF and other growth factors have been subsequently observed in the neonatal brain (Gilmore et al., 2003). The offspring have also been reported to have increased neuronal cell death, reduced neurogenesis and reduced brain size (Boksa, 2010, Wang et al., 2006). Administration of pro-inflammatory cytokines individually is sufficient to induce MIA. IL-6 has been reported to cause deficits in offspring, including impaired spatial learning, neuronal loss, astrogliosis and changes in neurotransmitter expression (Samuelsson et al., 2006). Treatment with anti-IL-6 antibodies protected against these developmental and behavioural abnormalities and IL-6KO subjected to an immune stimulus were also protected against abnormal behavioural developments (Patterson, 2009, Smith et al., 2007). Treatment with

the anti-inflammatory cytokine IL-10 has been reported to protect against white matter damage observed in MIA (Pang et al., 2005). Similarly administration of anti-TNFα antibodies to the pregnant dam following LPS challenge reduced foetal death (Silver et al., 1994), and use of the drug pentoxifylline, to block TNFα synthesis, reduced foetal mortality and reversed previously observed growth and skeletal development restrictions of the foetus (Xu et al., 2006). The brain abnormalities and the associated behaviours, such as increased anxiety, impaired locomotor activity, altered social behaviour and deficits in learning and memory, associated with MIA appear to best mimic symptoms associated with schizophrenia (Meyer and Feldon, 2009). In agreement with this, MIA leads to hyper-functionality of the dopaminergic system in the offspring, including increased tyrosine hydroxylase and dopamine transporter expression in neuronal cells (Bakos et al., 2004, Meyer and Feldon, 2009). A recent study by Khan and co-workers reported an effect of poly I:C MIA on depressive-like behaviours in adult mouse offspring, accompanied by deficits in cognition and hippocampal long-term potentiation (Khan et al., 2014). Thus this provides a suitable model for assessing the long term behavioural effects of an inflammatory stimulus during development with long term phenotypic consequences.

1.9 Thesis aims and objectives

The overall aim of the this project was to engage with preclinical paradigms of immune stimulation and autoimmunity with a view towards their development as putative animal models of anxiety and/or depression-related behaviours coupled to microglial activation and alterations in neuronal systems including HPA and GC regulation, kynurenine pathway activation and deficits in neurotrophins.

In the first instance the effects of the immune stimulus poly I:C were assessed in the Sprague Dawley rat and genetically pre-disposed WKY rat. Second, the project uses the NOD mouse as a novel animal model of psychiatric disorders based on immune abnormalities. The model allows for the possibility of manipulation of gene-environment (immune challenge) interaction leading to changes in immune set points, enabling in-depth studies on the molecular mechanism of immune mediated behavioural abnormalities. Behaviour of the NOD mouse was assessed under basal conditions and following LPS challenge coupled to a genomics wide screen of peripheral and central markers of monocytic and microglial activation, GR related and regulated genes, kynurenine pathway induction and alterations in growth factor expression.

Finally the effects of psychological stress were assessed on the circulating and central expression of IL-6 and IL-6-related and regulated genes. Following this, a study of the effects of systemic IL-6 administration was undertaken to determine what effects IL-6 may have on IL-6 related and regulated genes centrally.

2. Materials and Methods

2.1 Materials

2.1.1 Animals

Female CD1 mice	Harlan, U.K.
Female Non-Obese Diabetic mice	Charles River, Italy
Male Sprague-dawley rats	Harlan, U.K.
Male Wistar Kyoto rats	Harlan, U.K.
Male Wistar rats	Bio-resources, TCD
Rodent feed	Bio-resources, TCD

2.1.2 Immune stimuli

Lipopolysaccharide	Sigma-Aldrich, Ireland
Polyinosinic:polycytidylic acid	Amersham Biosciences, UK

2.1.3 ELISA Kits

Corticosterone EIA Kit	Enzo Life Sciences
Glucose Assay Kit	Cayman Chemical Company
IL-6 ELISA kit	BioLegend
IL-6 soluble receptor ELISA kit	R&D Systems

2.1.4 mRNA related products

Nucleospin RNA II	Macherey-Nagel
AB 1900 PCR plate	Thermo Fisher Scientific, USA
Rnase Zap	Ambion, U.K.

TaqMan® gene expression assays	Applied BioSystems, U.K.
TaqMan® universal PCR master mix	Applied BioSystems, U.K.

2.1.5 Microarray related products

PicoPure RNA isolation kit	Applied BioSystems, UK
Ovation Pico WTA v2	NuGEN, The Netherlands
Encore Biotin Module	NuGEN, The Netherlands
Mouse Genome 430 2.0 Array	Affymetrix, UK

2.1.6 General Laboratory Chemicals

2-Propanol	Sigma-Aldrich, Ireland
Acrylamide	Sigma-Aldrich, Ireland
Ammonium persulphate (APS)	Sigma-Aldrich, Ireland
Bicinchoninic acid (BCA) protein kit	Pierce
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Ireland
Bromophenol blue	Sigma-Aldrich, Ireland
Ethanol (EtOH)	Sigma-Aldrich, Ireland
Glycerol	Sigma-Aldrich, Ireland
Glycine	Sigma-Aldrich, Ireland
Hydrochloric acid (HCl)	BDH
Methanol (MeOH)	Sigma-Aldrich, Ireland
N' N' Bis Acrylamide	Sigma-Aldrich, Ireland
N,N,N',N'-Tetramethylethylende-diamine (TEMED)	Sigma-Aldrich, Ireland
Potassium Chloride (KCl)	Merck

Sodium Chloride (NaCl)	BDH
Sodium dodecyl sulfate (SDS) 99%	Sigma-Aldrich, Ireland
Sodium Hydroxide (NaOH)	Sigma-Aldrich, Ireland
Sodium Hydroxide (NaOH)	Sigma-Aldrich, Ireland
Triton X	Sigma-Aldrich, Ireland
Triton X-100	Sigma-Aldrich, Ireland
Trizma-base	Sigma-Aldrich, Ireland
Tween-20	Sigma-Aldrich, Ireland
β- Mercaptoethanol	Sigma-Aldrich, Ireland

2.1.7 General Laboratory Consumables

15ml Universal collecting tubes	Sarstedt, Ireland
50ml Universal collecting tubes	Sarstedt, Ireland
50ml Yellow capped containers	Sarstedt, Ireland
Blood collection tubes (5ml)	Sarstedt, Ireland
Eppendorfs (1.5/2ml)	Sarstedt, Ireland
F96 Maxisorp immunoplates for ELISA	Sarstedt, Ireland
Laboratory rolls	Fisher Scientific
Microtest 96-well flat bottomed plates	Sarstedt, Ireland
Parafilm laboratory rolls	Sarstedt, Ireland
Pasteur pipettes (1ml)	Sarstedt, Ireland
Pipette tips (Filter and non-filter)	Sarstedt, Ireland

2.1.8 Western blotting reagents and antibodies

Anti-mouse IgG	Sigma-Aldrich, Ireland
Anti-rabbit IgG	Amersham, U.K.
β-actin antibody	Santa-Cruz Inc., USA
C/EBPβ antibody	Cell Signalling, USA
Dual colour molecular weight marker	Biorad, USA
ECL Western Blotting Substrate	Pierce, USA
Phosphatase inhibitor cocktail I and II	Sigma-Aldrich, Ireland
Phospho-C/EBPβ antibody	Cell Signalling, USA
Phospho-STAT3 antibody	Cell Signalling, USA
Polyvinylidene	Millipore, Ireland
Protease inhibitor cocktail	Sigma-Aldrich, Ireland
Re-blot plus	Millipore, Ireland
STAT3 antibody	Cell Signalling, USA

2.1.9 High Performance Liquid Chromatography

5-hydroxyindole-3-acetic acid	Sigma-Aldrich, Ireland
Citric Acid	BDH Chemicals, Poole, U.K.
Ethylenediaminetetra-acetic acid (EDTA)	BDH Chemicals, Poole, U.K.
HPLC grade water	Fisher Chemical, U.K.
Methanol, 100%	Lab-Scan, Ireland
N-methyl-5-HT	Sigma Chemical Co., U.K.
Octane-1-sulfonic acid	Sigma-Aldrich, Ireland
Serotonin (5-HT)	Sigma Chemical Co., U.K.

Sodium phosphate monobasic monohydrate	Sigma-Aldrich, Ireland

2.1.10 Instruments and software used

AM1051 data logger	Benwick Electronics
Ethovision 3.0 software	Noldus, The Netherlands
ABI Prism 7300 Instrument	Applied BioSystems, Germany
ABI StepOne 7500 Instrument	Applied BioSystems, Germany
Applied BioSystems RQ software	Applied BioSystems, UK
Applied Biosystems StepOne software	Applied BioSystems, UK
Peltier Thermal Cycler- 200	BioRad, USA
ND-1000 Spectrophotometer	Thermo Fisher Scientific, USA
Elx800 Microtitre plate reader	Biotek, Germany
Fastblot B43	Biometra, Germany
PowerPac^{TM} HC	BioRad, USA
Fujifilm Luminescent Image Analyzer LAS-3000	Fujifilm Life Science, Japan
Image J software	NIH Image, USA
Ingenuity Pathway Analysis software	Qiagen, USA
GB-STAT software	Dynamic Microsystems, USA
CLASS-VP software	Shimadzu, Japan
RF-20A XS prominence fluorescence detector	Shimadzu, Japan
Reverse phase analytical column	Phenomenex, USA
Digital electrochemical amperometric detector	Mason Technology, Ireland

2.2 Methods

2.2.1 Animal Husbandry

Female NOD/ShiLtJ mice were obtained from Charles River (Italy), while male Sprague-Dawley rats and Wistar Kyoto rats and female CD-1 mice were obtained from and Harlan (UK). Male Wistar rats were obtained from the Bio-resources unit of Trinity College Dublin. All animals were group housed unless otherwise stated and kept on a 12 hour (hr) light: 12hr dark cycle (lights on at 08:00hr) in a temperature controlled room (22±2 degrees Celsius (°C)). Food and water were available *ad libitum.* The experimental protocols were in compliance with the European Communities Council directive (86/609/EEC).

2.2.2 Behavioural Profiling

In order to determine whether an animal could be used as a model for depression, a series of behavioural tests were carried out both under basal conditions and in response to a treatment.

2.2.2.1 Open Field Test

The Open Field Test (OFT) is a behavioural test used to assess anxiety levels in rodents. At the beginning of each trial, animals were placed in the centre of a dimly lit open field arena, measuring 90 centimetre (cm) in diameter, and observed for 10 minutes (mins) (Figure 2.1). The trials were conducted between 10am and 2pm. The arena was cleaned with 70% ethanol between each trial. The behaviour of the animals was recorded by an overhanging camera that was attached to a personal computer. Ethovision 3.0 (Noldus, The Netherlands) was used to track the movement of the animal. For the purposes of this test the arena was divided into an inner and outer zone. The total distance moved in the arena, distance moved in the inner zone of the arena and number of transitions between the inner and outer zones were recorded. Mice with lower levels of anxiety will spend more time in, move more inside and make more entries into, the inner zone.

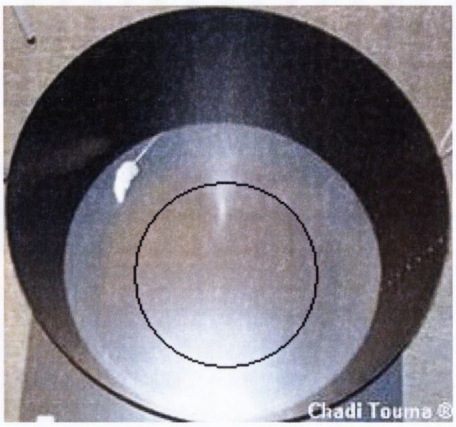

Figure 2.1 – Image of an open field, superimposed circle indicates the division between inner and outer zones.

2.2.2.2 Elevated Plus Maze

The Elevated Plus Maze (EPM) is a behavioural test used to judge anxiety levels in rodents. The maze (Figure 2.2) consists of a raised cross-shaped platform, with four arms originating from a common centre point. Two of the arms, facing each other, contain raised walls, providing the mouse with a dark enclosed environment, whilst the two other arms contain no walls and are exposed. At the start of the test, the mouse is placed in the centre square. The mouse's movements are monitored using a digital video camera connected to the computer program Ethovision, with time spent in the open arms compared to the time spent in the closed arms. The number of entries into the open and closed areas was also monitored. Mice that venture into, and spend more time in the open arms than the closed arms are judged to display less anxious behaviour than those that do not.

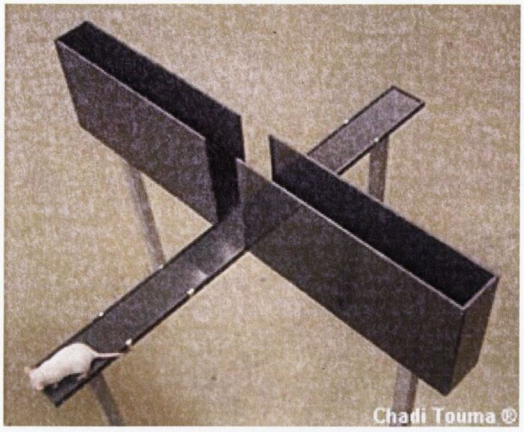

Figure 2.2 – Image of an elevated plus maze

2.2.2.3 Tail Suspension Test

The Tail Suspension Test (TST) is an inescapable stress that measures the presence of despair behaviour in the mice subjected to the test. Mice were attached by their tails to wire affixed to a horizontal bar of a retort stand (Figure 2.3) so that they are suspended 25cm above the base of the stand. The mice are left in suspension upside down for a period of 6mins, of which the last 5mins are scored for immobility. Behaviour was scored by a trained blind observer.

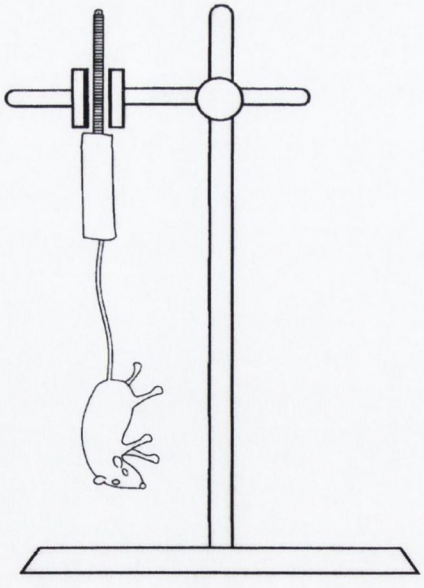

Figure 2.3 – Image depicting the apparatus used in the tail suspension test

2.2.2.4 Home Cage Activity

Animals were individually placed in activity cages measuring 32cm x 20cm x 18cm (length x width x height). These cages were connected to an AM1051 data logger (Benwick Electronics). Each cage contained 2 sets of horizontal infrared beams, one set at 3cm and one at 6cm from the bottom of the cage. Each set consisted of a 12 beam by 7 beam matrix which formed a grid of 66 x 2.54cm^2 cells in each cage. Together the beams and data logger recorded activity a rearing. Activity was determined by the number of times a beam was broken and was measured from when the beam was first broken until no beam had been broken for 5 seconds. Rearing was measured as the number of times the higher set of beams were broken. The trials were conducted between 10am and 2pm. After 30mins of behavioural recording the animals were returned to the home cage. This protocol was carried out both before the treatment, in order to determine the baseline levels of locomotor and rearing activity, and following treatment.

2.2.2.5 Saccharin Preference Test

The saccharin preference test, used as a measure of anhedonia, was carried out as previously described (Harkin et al., 2002). Animals were singly housed and exposed to a saccharin solution (0.1%) in a standard drinking bottle and also a bottle containing tap water in their home cage. The position of the bottles was varied and counter balanced across the left or right side of the feeding compartment. Bottles were weighed over a number of days before the commencement of the study. This was carried out to ensure that animals were subdivided into groups with comparable baseline preference. Bottles were subsequently weighed daily. Fluid consumption was calculated by calculating the daily difference in bottle weight. Preference scores were calculated by dividing the amount of saccharin consumed by the total amount of fluid consumed (water and saccharin) during the 24hr period.

2.2.3 Restraint stress paradigm

Restraint stress reduces an animal's movement and access to food and water. The restraint stress procedure used in the presented studies was based on previous studies carried out in this lab (Curtin et al., 2009a). For the acute stress protocol, mice were immobilised by placing in a well ventilated restrainer (modified 50ml collecting tube, Figure 2.4) for one single exposure. The mice were inserted into the tube and the lid closed tightly. The tubes were then placed parallel to the table top in a brightly lit room with an ambient temperature of 20-24°C. The mice were restrained for 2hr and then either euthanised immediately or returned to the home cage for 2hr before euthanisation. Control animals remained in their home cage and were removed only for euthanisation.

Figure 2.4 – Image of a modified 50ml collecting tube used to restrain mice

2.2.4 Immune stimulants

2.2.4.1 LPS administration

Lipopolysaccharide (LPS) was prepared from Echerichia coli (Sigma) for injection by resuspending in sterile saline and was stored at -20°C until use. Animals received an i.p. injection of LPS (100µg/kg) or an i.p. injection of saline.

2.2.4.2 Poly I:C administration

Poly I:C (Little Chalfont, Buckinghamshire, UK) was prepared for injection by resuspending in sterile saline, heating to 50°C at a concentration of 2mg/ml to ensure complete solubilisation and then allowing to cool at room temperature to ensure proper annealing of the double-stranded RNA, at which point it was stored at -20°C until use. Animals were injected i.p. with either Poly I:C (6mg/kg) or a saline control.

2.2.5 Post mortem blood and organ harvesting from animals

2.2.5.1 Blood sampling and serum preparation

Mice were decapitated using a sharp scissors. Trunk blood was collected and centrifuged at 2000 revolutions per minute (rpm) for 15mins at 4°C. The serum was carefully removed and stored at -80°C until corticosterone analysis was carried out.

2.2.5.2 Harvesting of brain tissue

The skin around the skull and the skull itself were removed using a scissors. The brain was then carefully removed from the head and placed on the petri dish. The areas of interest isolated in this study included, the frontal cortex, hippocampus, hypothalamus, striatum and amygdala. Each isolated tissue sample was then placed in an individual 1.5ml eppendorf and snap frozen in liquid nitrogen to prevent any degradation of nucleic acids or proteins.

2.2.6 Enzyme Linked Immunosorbant assay

The sandwich enzyme-linked immunosorbant assay (ELISA) method was used to determine corticosteroid and cytokine concentrations in circulating serum.

2.2.6.1 Corticosterone Enzyme Linked Immunosorbant assay

The concentrations of corticosterone in serum were determined using a commercially available corticosterone assay kit (Enzo Life Sciences). Unlike typical ELISA kits, this enzyme immunoassay kit works via competitive binding of an anti-corticosterone polyclonal antibody (sheep origin) to either the corticosterone present in the serum and standards, or to an alkaline phosphatase molecule covalently bound to corticosterone. The standards were prepared for loading on the plate by preparing the stock of corticosterone, using a pre-reconstituted corticosterone standard (200,000pg/ml). The concentrated stock solution was diluted to a working concentration of 20,000pg/ml, which was used as the top standard, after which 1in5 dilutions were carried out using assay buffer (provided in kit). 100µl of each standard and sample was loaded onto a mcirotitre 96-well plate pre-coated with donkey anti-sheep IgG in duplicate. 50µl of the sheep anti-cort antibody (provided in kit) and 50µl of a conjugate containing the alkaline phosphatase bound to cort (provided in kit) were also added to each well; the plate was sealed and incubated for 2hr at room temperature (RT) with shaking at 500rpm. Higher concentrations of corticosterone present resulted in less alkaline phosphatase bound corticosterone being able to bind to the polyclonal antibody. Following incubation, the wells were washed with 400µl wash buffer (provided in kit) 3 times and blotted on tissue paper thoroughly after each wash. At this point, 200µl of p-nitrophenyl phosphate substrate (provided in kit) was added to each well and incubated for 1hr at RT. 50µl of stop solution (trisodium phosphate in water (provided in kit)) was then added and the absorbance of each well was measured using a microtitre plate reader (Elx800; Biotek, Germany) set to 405nm. A standard curve was constructed by plotting standards against the absorbance and results obtained expressed as pg/ml of serum or % control.

2.2.6.2 Glucose Assay

The concentration of glucose in serum was determined using a commercially available glucose assay kit (Cayman Chemical Company). Standards were prepared using glucose provided in the kit, delivered at a concentrated stock solution of 1,000mg/dl. The stock solution was diluted using assay buffer (provided in kit) to a working concentration of 250mg/dl, which was used as the top standard. Remaining standards of 200mg/dl, 150mg/dl, 100mg/dl, 50mg/dl,

25mg/dl and 12.5mg/dl were created by diluting the stock top standard with assay buffer. Glucose enzyme mixture was prepared by reconstituting the provided lyophilized enzyme mixture with 500µl with dH_20, and adding 12ml of assay buffer to the reconstituted solution. 5µl of each standard and sample was added to labelled 1.5ml eppendorfs and 500µl of enzyme mixture was added forcefully down the side of each tube. The tubes were mixed thoroughly and incubated at 37°C for 10mins. 150µl of each tube was then added in duplicate to a 96-well plate and absorbance in each well was measured using a microtitre plate reader (Elx800; Biotek, Germany) set at 514nm. A standard curve was constructed by plotting standards against the absorbance and results obtained expressed as pg/ml of serum or % control.

2.2.6.3 Mouse IL-6 ELISA

The concentration of IL-6 in serum was determined using a commercially available mouse IL-6 ELISA kit (BioLegend). 100µl of the capture antibody (CA) solution (1:200 dilution of pre-titrated CA in coating buffer (provided in kit)) was added to a flat-bottomed 96 well plate (NUNC, F96 MAXISORP-immuno plate), which was sealed and incubated overnight at 4°C. These wells were washed 4 times with 300µl of wash buffer (PBS with 0.05% Tween-20) and excess wash buffer removed by blotting the plate on a paper towel. 200µl of blocking buffer (provided in kit) was added to the wells, the plate sealed and incubated for 1hr at RT on a plate shaker at 200rpm. The wells were washed again four times with 300µl of wash buffer and blotted on a paper towel. The standards were prepared for loading on the plate by preparing the stock of IL-6 (recombinant mouse IL-6). The recombinant IL-6 was reconstituted in 200µl of reagent diluent (provided in kit) and the concentrated stock solution, diluted to a working concentration of 500pg/ml, which was used as the top standard, after which 1in2 serial dilutions were carried out using assay diluent, as low as 7.8pg/ml. 100µl of each standard and sample was loaded onto the plate in duplicate, the plate sealed and incubated for 2hr at RT with shaking at 200rpm. Following incubation, the plates were washed as before and 100µl of biotinylated detection antibody (1:200 dilution, in assay diluent) was added to the plates, which were then sealed and incubated for 1hr with shaking at 200RPM at RT. Plates were washed as before and 100µl of the working solution streptavidin-horseradish peroxidise (HRP) conjugate (1:1000 dilution in assay diluent) was added to each well, the plate

was sealed and incubated for 30mins at RT with shaking at 200RPM avoiding direct light. Plates were washed as before and 100µl of tetramethylbenzidine (TMB) substrate solution was added to each well. This was incubated for 20mins in the dark, at which point 100µl of stop solution (2N H2SO4) was then added to each well and the absorbance measured at 450nm using a microtitre plate reader (Elx800; Biotek, Germany). A standard curve was constructed by plotting standards against the absorbance and results obtained expressed as pg/ml of serum or % control.

2.2.6.4 Mouse Soluble IL-6 receptor ELISA

The concentration of the IL-6 soluble receptor (IL-6sR) in serum was determined using a commercially available mouse IL-6sR ELISA kit (R&D systems). CA was reconstituted in 1ml of PBS to a working concentration of 144µg/ml. 100µl of CA solution (1:180 dilution of reconstituted CA in PBS) was added to a flat bottomed 96-well plate (NUNC, F96 MAXISORP-immuno plate), which was sealed and incubated overnight at RT. These wells were washed 3 times with 400µl of wash buffer (PBS with 0.05% Tween-20) and excess wash buffer removed blotting the plate on a paper towel. 300µl of blocking buffer (reagent diluent; 1% BSA in PBS) was added to the wells, the plate sealed and incubated for 1hr at RT. The wells were washed again 3 times with 400µl of wash buffer and blotted on a paper towel. The standards were prepared for loading on the plate by preparing the stock of IL-6sR (recombinant mouse IL-6sR). The stock was reconstituted in 500µl of reagent diluent to give a receonstituted standard of 130ng/ml. The concentrated stock solution was diluted to a working concentration of 3000pg/ml, which was used as the top standard, after which 1in2 serial dilution using assay diluent. 100µl of each standard and sample was loaded onto the plate in duplicate, the plate sealed and incubated for 2hr at RT. Following incubation, the plates were washed as before and 100µl of detection antibody (1:180 dilution, in reagent diluent) was added to the plates, which were then sealed and incubated for 2hr at RT. Plates were washed as before and 100µl of the working solution streptavidin-HRP conjugate (1:1000 dilution in assay diluent) was added to each well, the plate was sealed and incubated for 20mins at RT while avoiding direct light. Plates were washed as before and 100µl of TMB substrate solution was added to each well. This was incubated for 20mins in the dark, at which point 50µl of stop solution (2N H2SO4) was then added to each well and the

absorbance measured at 450nm using a microtitre plate reader (Elx800; Biotek, Germany). A standard curve was constructed by plotting standards against the absorbance and results obtained expressed as pg/ml of serum or % control.

2.2.7 High Performance Liquid Chromatography

Measurements of tryptophan, kynurenine, 5-HT and 5HIAA were made in the brain and serum via high performance liquid chromatography (HPLC) coupled to a range of detection systems.

2.2.7.1 Monoamine HPLC

In experiments involving analysis of biogenic amine content in the brain, animals were euthanised at the appropriate time point and the required brain regions dissected out. These tissue samples were weighed and then added to 500µl ice-cold homogenisation buffer. Homogenisation buffer consisted of mobile phase buffer [100mM citric acid, 100mM NaH_2PO_4, 1.4mM octane-1-sulfonic acid, 100mM EDTA, 10% (v/v) methanol in double-distilled, refiltered NANOpure H_2O, adjusted to pH 2.8] spiked with 5ng/20µl N-methyl-5-HT as an internal standard. Samples were homogenised by sonication (MSE Sonicator), centrifuged for 20mins at 20,000rpm at 4°C and the supernatant removed to a fresh collection tube. Standards of 5-HT, 5-HT and N-methyl-5-HT were prepared as standard calibration points and to assess retention times on the HPLC column. 10µl of the sample supernatant was injected onto a reverse phase column (KinetexTM Core Shell Technology column, specific area 100mm x 4.6mm, particle size 2.6µm, Phenomenex) at a flow rate of 0.8ml/min for separation of the neurotransmitters. 5-HT and 5-HIAA concentrations were quantified by electrochemical detection (Antec Decade) and chromatograms generated (Class VP software integration). Final results were expressed as ng neurotransmitter/ g wet tissue weight.

2.2.7.2 Kynurenine HPLC

HPLC coupled to UV and fluorescence detection was used to determine concentrations of tryptophan (Tryp) and kynurenine (Kyn) within the brain. Animals were euthanised at the appropriate time point and the required regions

dissected out. Samples were weighed and then added to 1ml of mobile phase [50 nM glacial acetic acid, 100 mM zinc acetate and 3% acetonitrile dissolved in double-distilled NANO-pure HPLC grade water at pH 4.9] containing 7% perchloric acid and 50ng/ 20µl N-methyl-5-HT. Samples were then homogenised by sonication (MSE Sonicator), centrifuged for 20,000rpm at 4°C and the supernatant removed to a fresh collection tube using a syringe fitted with a 0.45µm filter (Phenomenex). Standards of N-methyl-5-HT, L-Kynurenine, L-Tryptophan, KYNA and 3-HAA were prepared as standard calibration points and to assess retention times of the HPLC column. 20µl of the filtered sample supernatant was injected onto a reverese phase column (KinetexTM Core Shell Technology column, specific area 100mm x 4.6mm, particle size 2.6µm, Phenomenex) at a flow rate of 0.8ml/min for separation of neurotransmitters. Tryp and Kyn concentrations were quantified via a PDA-UV detector (Shimadzu SPD-M10A VP) and a fluorescent detector (Shimadzu RF-20A XS prominence fluorescence detector) and chromatograms generated (Class VP software integration). Final results were expressed as ng neurotransmitter/ g wet tissue weight.

2.2.8 Preparation of tissue for and procedure of real-time polymerase chain reaction

2.2.8.1 Total RNA extraction

Isolation of total RNA from dissected tissue was carried out using NucleoSpin® RNA II kits (Macherey-Nagel) as instructed in the manufacturer's protocol. Prior to this all surfaces and equipment were wiped down with RNAse away wipes, which degrades any RNAse present that might reduced the yield of total RNA being extracted. In brief, up to 30mg of tissue, that had been snap frozen in liquid nitrogen and stored at -80°C, was weighed out and placed into a lysis buffer mixture of 350µl RA1 buffer and 3.5µl β-mercaptoethanol. This tissue was then disrupted using a polytron, until there was a uniform mixture present. Following homogenisation, the lysate was placed in a Nucleospin Filter unit and filtered by centrifuging for 1min at 13,000rpm. 350µl of 70% ethanol (etOH) was then added to the lysate and pipetted up and down several times until dissolution occurred. This lysate-ethanol mixture was loaded into a Nucleospin II column and centrifuged for 30sec at 13,000rpm. The column containing nucleic acid was retained and desalted by adding 350µl of a membrane desalting buffer and

centrifuging for 1min at 13,000rpm. At this point Dnase was added to the column and incubated for 15mins at room temperature to remove any DNA present in the column. This was then deactivated by passing 200µl of the "RA2" solution through the column by centrifuging the column for 30sec at 13,000rpm. The column was then washed on two occasions with the "RA3" buffer, 600µl centrifuged at 13,000rpm for 30sec and 200µl centrifuged for 2mins at 13,000rpm. The column was then placed in a nuclease free collecting tube and the total RNA present was eluted with 60µl Rnase free water via centrifugation at 13,000rpm for 1min. The total RNA samples were then either stored at -80°C until ready for the next step or equalised immediately post extraction.

2.2.8.2 RNA equalisation

Total RNA samples, previously extracted and stored -80°C, were removed from the freezer, allowed to defrost slowly on ice and were kept on ice for the duration of this procedure. A laptop computer connected to a spectrophotometer (Nanodrop ND-1000) was used to measure total RNA concentrations. The spectrophotometer was blanked with nuclease free H_2O. 1µl of each sample was placed on the pedestal and read, with results expressed as ng/µl. The purity of the sample was judged using the $A_{260/280}$. Once all of the concentrations were calculated, the samples were all equalised to the sample with the lowest concentration detected, using nuclease free H_2O.

2.2.8.3 Complimentary DNA Synthesis

In brief, a master mix solution was made up using reverse transcription buffer, dNTPs, random primers, multiscribe reverse transcriptase and Rnase free H_2O (Table 2.2.7.3.1). 10µl of RNA from each sample was added to 10µl of the master mix in micro tubes. These tubes were then placed in a thermocycler (Peltier Thermal Cycler- 200) and set to the following program: 25°C for 10mins, 37°C for 120mins and 85°C for 5mins. When the cDNA synthesis was completed, each sample was diluted 1-in-5 times with nuclease free H_2O (20µl cDNA + 80µl of H_2O), and samples were stored at -20°C until needed.

Table 2.1 – Volumes of the components of a cDNA synthesis master mix

Master Mix	Volume per sample (µl)
10x RT buffer	2
25x dNTPs	0.8
10x Random Primers	2
Multiscribe RT (50u/µl)	1
Nuclease free H_2O	4.2
Total Volume	10

2.2.8.4 Real-time polymerase chain reaction (PCR)

Target gene expression was assessed using a Taqman® gene expression assay (Table 2.2.7.4.1), which contain specific target primers and FAM-labelled MGB target probes (Applied Biosystems). Abl-1 gene expression was used to as an endogenous control between samples and was quantified using specific primers and a VIC-labelled MGB probe.

Table 2.2 – List of mouse target probes used in QPCR

Target	Taqman® Assay ID	Target	Taqman® Assay ID
β-actin	4352341E	**IDO2**	Mm00524206_m1
Abl1	Mm00802029_m1	**IFNγ**	Mm00801778_m1
ARG-1	Mm00475988_m1	**IL-10**	Mm00439616_m1
BDNF	Mm04230607_m1	**IL-1β**	Mm01336189_m1
C/EBP β	Mm00843434_m1	**IL-6**	Mm00446190_m1
CD11b (ITGAM)	Mm00434455_m1	**IL-6RA**	Mm00439653_m1
CD40	Mm00441891_m1	**IL-6ST**	Mm00439665_m1
CD68	Mm03047340_m1	**KMO**	Mm00505511_m1
CD80	Mm00711660_m1	**Kynu**	Mm00551012_m1
CD86	Mm00444543_m1	**MHC II γ chain**	Mm00658576_m1
cFos	Mm00487425_m1	**NGF**	Mm00443039_m1
DUSP1	Mm00457274_m1	**NOS 2 (inducible)**	Mm00440502_m1
EGR-1	Mm00656724_m1	**P23**	Mm00727367_s1
FGF2	Mm00433287_m1	**PDGF**	Mm00440677_m1
FKBP4	Mm00487391_m1	**PP5**	Mm00803198_m1
FKBP5	Mm00487401_m1	**SGK-1**	Mm00441380_m1
GFAP	Mm00546086_m1	**SOCS3**	Mm00545913_s1
GILZ (TSC22 d3)	Mm00726417_s1	**STAT3**	Mm01219775_m1
GR (NR3C1)	Mm00433832_m1	**TNFα**	Mm00443258_m1
HSP90 aa	Mm00658568_gH	**TREM 1**	Mm01278455_m1
HSP90 ab	Mm00833431_g1	**TREM 2**	Mm04209424_m1
IDO	Mm00492586_m1	**VEGF**	Mm00437310_m1

Table 2.3 – List of rat primers used in QPCR

Target	Taqman® Assay ID
β-actin	Rn00667869_m1
BDNF	Rn02531967_s1
CD11b (ITGAM)	Rn00709342_m1
IDO	Rn00576778_m1
IFNγ	Rn00594078_m1
IL-1β	Rn00580432_m1
IL-6	Rn00561420_m1
TNFα	Rn93699017_m1
TrkB	Rn01441749_m1

In brief, diluted cDNA samples which had been previously synthesised and stored at -20°C were removed from the freezer and allowed to defrost on ice. Once defrosted each sample was added to an individual well on a fast PCR plate (Thermo Scientific). A master mix solution was made up with Taqman® fast Advanced Master Mix, the endogenous control primer, Abl1, and the target primer of interest (Table 2.2.7.4.2). This master mix was then added to each well and the plate sealed with an optically clear plastic cover, before being pulse centrifuged for a few seconds to ensure all of the sample cDNA and master mix was at the bottom of the wells.

Table 2.4 – Volumes of the components in each well of a PCR plate

PCR mix (per well)	Volumes (µl)
cDNA (diluted 1 in 5)	4
Fast Taq	5
Endogenous control	0.5
Target gene	0.5
Total Volume	10

The sealed plate was inserted into Step One Plus real time PCR system (Applied Biosystems) and run on the following settings: 50°C for a period of 2mins, followed by 95°C for 20seconds.This constituted the first step. The second step was repeated 40 times and consisted of a denaturation temperature of 95°C for 1sec, followed by a temperature of 60°C for 20secs, the annealing and extension phase, during which the fluorescence was read by the machine. During this second step, the double stranded DNA is denatured at 95°C for 1sec, as the temperature falls to 60°C, the target probe binds to the single stranded coda first, owing to its higher melting point in comparison to the target primers. This probe contains the FAM/VIC dye and a proprietary no-fluorescent quencher (NFQ) dye. This prevents the dye from emitting a fluorescence signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems). When the temperature reaches 60°C the target primers anneal and the strand is extended at the 5' end by the nuclease activity of Taq polymerase. This displaces the FAM/VIC labelled probe which in turn causes the FRET between the dye and quencher to be broken, leading to the generation of a fluorescent signal being generated. Due to the specificity of the probe and the primers of the cDNA sequence, one fluorescent signal is generated for each new cDNA copy.

2.2.8.5 Real-time PCR analysis

Assessment of gene expression of all the real-time PCR was carried out using the ΔΔCT method (Applied Biosystems RQ software). This method is used to assess relative gene expression by comparing the gene expression of treatment samples to that of a control sample, rather than quantifying the exact copy number of the target gene. This allows for fold difference to be assessed between samples of different treatments. The fold difference is calculated using the cycle number (CT) difference between samples. To measure this difference, the CT of the endogenous control (β-actin or Abl1) is subtracted from the CT of the target gene for each sample, which accounts for any variations in cDNA quantity (termed ΔCT). While β-actin is a commonly used endogenous control and utilised in studies in this thesis, previous work in our lab (unpublished) has suggested that NOD mice have variable levels of this gene. Thus for studies involving NOD mice, Abl1 was used as the endogenous control. The ΔCT of a control sample is subtracted from itself and all of the other samples giving the

ΔΔCT value (cycle difference corrected for endogenous control). This can then be converted into a fold difference from which the related expression change can be calculated.

2.2.9 Genome-wide analysis

2.2.9.1 Monocyte isolation

Whole blood was obtained by cardiac puncture and was directly stored on ice in tubes containing buffered sodium citrate. Erythrocytes were lysed in NH_4Cl (0.15M in DPBS) for 15mins at room temperature. Cells were washed twice in DPBS containing 0.1% BSA. The cells were re-suspended in DPBS containing 0.1% BSA and labelled with CD11b-APC and Ly6G-PE (Becton Dickinson) and Ly6C-AF488 (SeroTec) for 30mins on ice. Cells were washed and the $SSC^{low}CD11b^{pos}Ly6G^{neg}$: immature ($Ly6C^{high}$) and mature ($Ly6C^{low}$) monocytes were sorted in DPBS+0.1%BSA on a MoFlo FACS sorter (Dako), according to the gating strategy presented in Figure 2.5. Re-analysis of the sorted cells indicated a purity >99%. Finally cells were washed in DPBS and lysed in extraction buffer from the Picopure kit (Arcturus) and stored at -80°C until RNA isolation.

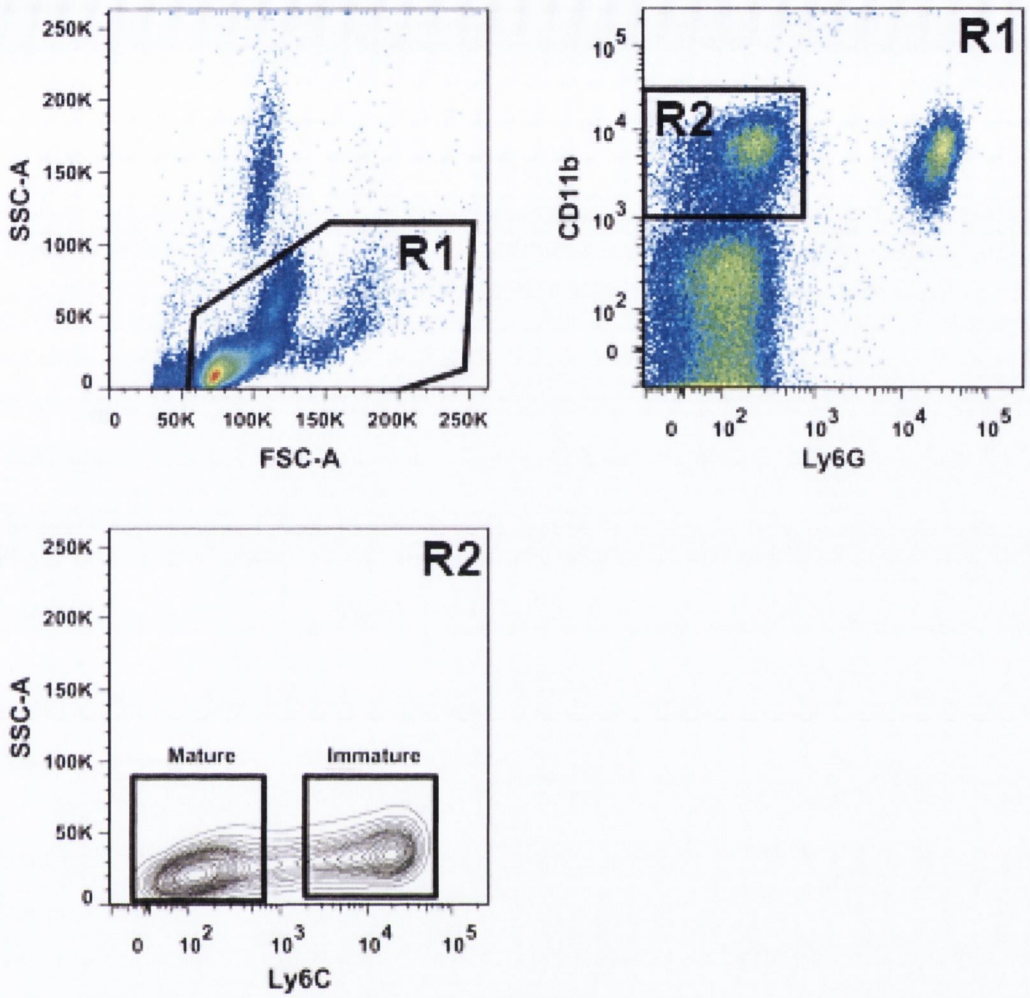

Figure 2.5 – Gating strategy for sorting blood monocyte subsets.

SSC^{low} were gated to exclude granulocytes and dead cells and debris in gate R1. Within gate R1, the $CD11b^{pos}$ and $Ly6G^{neg}$ cells (monocytes) were gated in gate R2. The two major monocyte populations in R2 were gated on their expression of Ly6C: $Ly6C^{high}$ for immature monocytes and $Ly6C^{low}$ for mature monocytes.

2.2.9.2 Microglial isolation

Microglia were isolated according to a modified version of the method described previously (de Haas et al., 2008). In short, the mice were perfused with PBS after blood sampling by cardiac puncture. Subsequently, the small-brains were removed and the cortex was stored in ice-cold 1x Hanks' balanced salt solution (HBSS, Gibco), containing 15 mM HEPES (Gibco) and 0.5% glucose (Sigma). Brains were cut in small pieces, dissociated in a glass tissue homogenizer (VWR, Amsterdam, The Netherlands) and titruated by fire-polished glass Pasteur pipets (VWR). The single-cell suspension was first filtered on a 70-µm cell strainer (Becton Dickinson) and pelleted for 10 min at 200·g, 4°C. A 100% Percoll stock (GE Healthcare) was made using 9 volumes of Percoll and 1 volume of HBSS 10x (Gibco). The 100% Percoll stock was diluted by adding 1x Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco) in order to obtain 75% and 25% Percoll stocks. The cell pellet was taken up in 10ml 75% Percoll stock and respectively overlaid with 10ml 25% Percoll stock and 6ml DPBS (0% Percoll). The density gradient was centrifuged in a swinging bucket rotor at 800g (slow acceleration without brake) for 30 min at 800·g, 4°C. After centrifugation, a thick myelin-containing layer at the 0/25% Percoll interface was discarded and the cells between the 25/75% interfaces were collected and washed in 30ml ice-cold DPBS. The cells were re-suspended in DPBS containing 0.1% BSA and labelled with CD11b-APC (Becton Dickinson) and CD45-PB (Biolegend) antibodies. Cells were washed and $SSC^{low}CD11b^{+}CD45^{low}$ microglia were sorted in DPBS+0.1%BSA on a MoFlo FACS sorter (Dako) according to the gating strategy presented in Figure 2.6. Re-analysis of the sorted cells indicated a purity >99%. Finally cells were washed and lysed in extraction buffer and stored at -80°C until RNA isolation.

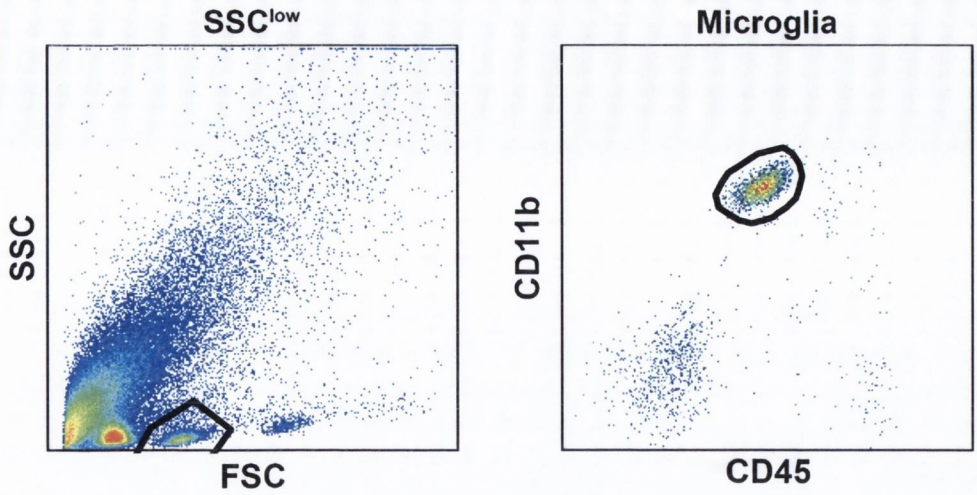

Figure 2.6 – Gating strategy for microglia isolation

SSC^{low} cells were selected in order to get rid of dead cells and debris (left panel). Microglia were gated on the expression of CD11b and low/medium expression of CD45 (right panel).

2.2.9.3 RNA isolation and amplification, Affymetrix microarrays

RNA was isolated with the PicoPure kit (Arcturus, Applied Biosystems) according to the manufacturers protocol including a Dnase I treatment (Qiagen, Venlo, The Netherlands) to remove genomic DNA contamination. The RNA was reverse transcribed, amplified, biotinylated and fragmented with the Ovation Pico WTA v2 and Encore Biotin Module (NuGEN Technologies, Leek, The Netherlands) and subsequently hybridized on Mouse Genome 430 2.0 Arrays (Affymetrix, High Wycombe, UK) according to the manufacturers protocols.

2.2.9.4 Microarray analysis

Quality analysis of the CEL data was assessed by running a standardized workflow developed at the BiGCaT department of Maastricht University – The Netherlands (http://www.arrayanalysis.org/). The expression data containing .CEL files were imported and processed further with BRB-Arraytools (R. Simon, http://linus.nci.nih.gov/BRB-ArrayTools.html). Gene expression data was normalized using RMA (Robust Multichip Average) (Bolstad et al., 2003). A list of differentially expressed genes (DEGs) among the two classes was identified by using a multivariate permutation test using the class comparison tool in BRB-arraytools. Ingenuity pathway analysis (Ingenuity® Systems, www.ingenuity.com) was used for annotation and to sort data relevant to fold change. Gene expression was considered statistically significantly different when $P<0.05$. Only genes where expression was 1.5 fold different from CD1 are reported in the present study.

2.2.10 Preparation of tissue for and procedure of Western Immunoblotting (WB)

2.2.10.1 Tissue preparation for Western Blotting

Tissue samples stored at -80°C were removed and adjusted to ~30mg. This tissue was then added to 300µl of lysis buffer (50nM Tris HCL, 150nM NaCL, 1% Triton-X-100) that contained 1% phosphatase and 1% protease inhibitors. The samples were mechanically homogenised with a polytron in a 2ml round bottomed microtube. These homogenised samples were centrifuged at 13,000

RPM at 4°C for 5mins and the supernatant removed. Protein concentration of this supernatant was determined using a bicinchoninic (BCA) assay and samples were equalised with lysis buffer. The equalised samples were then added to Laemmli sample buffer (1:1, 1M Tris-HCL [pH 6.8], 25% w/v (SDS), 50% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 2% (w/v) bromphenol blue) and boiled at 60°C for 5mins proper to loading on gels for SDS-PAGE.

2.2.10.2 Protein quantification – BCA protein assay

The BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. Protein content of samples used for western blot was quantified using this assay. A working solution of 2000µg/ml of bovine serum albumin (BSA) was prepared using lysis buffer stock as the diluent. A set of dilutions were prepared to give final protein concentrations of: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0µg/ml BSA. Next, 25µl of the dilutions and of the individual samples were pipetted in duplicate into the wells of a new 96-well plate. A BCA working solution was prepared by adding 1 part of the BCA working reagent B to 50 parts of reagent A. 200µl of this working solution was added to each sample/standard. The 96-well plate was then covered and incubated at 37°C for 30mins. The plate was subsequently cooled to room temperature and the absorbance read at 560nm using a microtitre plate reader (Elx800; Biotek, Germany). A standard curve was constructed by plotting the standards against the absorbance recorded. From this, the concentration of the samples were obtained and expressed as µg/ml of protein. The samples were equalised by adding the required volume of lysis buffer as a diluent.

2.2.10.3 SDS-PAGE

Sodium dodecylsulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was used on the equalised protein samples to separate them for subsequent analysis via western immunoblotting. Previously equalised samples were added

The polyacrylamide gel the samples were run on was divided in to two gels of distinct concentrations, a 10% separating gel (2.425ml d.H_2O, 1.25ml

Bis/acrylamide, 1.25ml Tris-HCL [pH8.8], 50µl SDS, 25µl APS, 3µl TEMED) and a 4% stacking gel (1.525ml d.H_2O, 325µl Bis/acrylamide, 625µl Tris-HCL [pH6.8], 25µl SDS, 12.5µl APS, 4µl TEMED). Two glass plates were cleaned with 70% ethanol (EtOH) and arranged 1mm apart using a spacer. The plates were placed on top of a rubber seal and held securely in place with a casting frame. The 10% separating gel was prepared and, after mixing, quickly pipetted between the two glass plates. A temporary layer of isopropanol was placed on top of this while it set to avoid dehydration of the gel. The gel was allowed to set for 30mins. Once the gel had solidified, the isopropanol layer was aspirated off and the gel washed with d.H_2O. The 4% stacking gel was then pipetted on top of the separating gel. A 10-well comb was carefully inserted into the stacking gel and allowed to set for 30mins. Once set the gels were arranged in the gel rig. The upper and lower reservoirs were filled with 1x running buffer (125mM Tris Base, 960mM glycine, 5% SDS (w/v)). The well combs were carefully removed and 10µl of equalised sample was added to each well. 5µl of a molecular marker (BioRad dual colour) was added to each gel. At least one representative of each treatment group was added to each gel. The proteins were separated by applying a constant current of 33mA per gel for 60mins or until the blue dye from the sample buffer had run to the bottom of the gel.

2.2.10.4 Semi-dry transfer

Following electrophoresis, separated proteins were transferred from the polyacrylamide gel onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry blotter at a constant current of 100mA per gel for a period of 70mins. A sandwich consisting of six pieces of filter paper, a piece of PVDF membrane and the gel was created on the transfer rig. Two pieces of filter paper soaked in Anode buffer I (0.3M Tris HCl, pH10.4, 10% methanol) were placed on the anode (+) plate of the semi-dry blotter and onto this a piece of filter paper soaked in anode buffer II (25mM Tris-HCl, pH 10.4, 10% Methanol) was placed. The PVDF membrane was first activated by submerging in methanol for 30sec followed by 2mins in dH_2O, before being added to the sandwich. A completed SDS-PAGE Gel was removed from the glass plates and placed on top of the PVDF membrane at this point and then lastly three pieces of filter paper soaked in cathode buffer (25mM Tris-HCl, pH 9.4, 40mM Glycine, 10% Methanol) were placed on to the gel to complete the sandwich. Any bubbles that may have been

present were removed by gently rolling a Pasteur pipette over the top of the sandwich. The cathode (-) plate was then placed on top of the sandwich and the current applied. In this set-up, the proteins will migrate from the gel towards the anode plate and hence into the PVDF membrane.

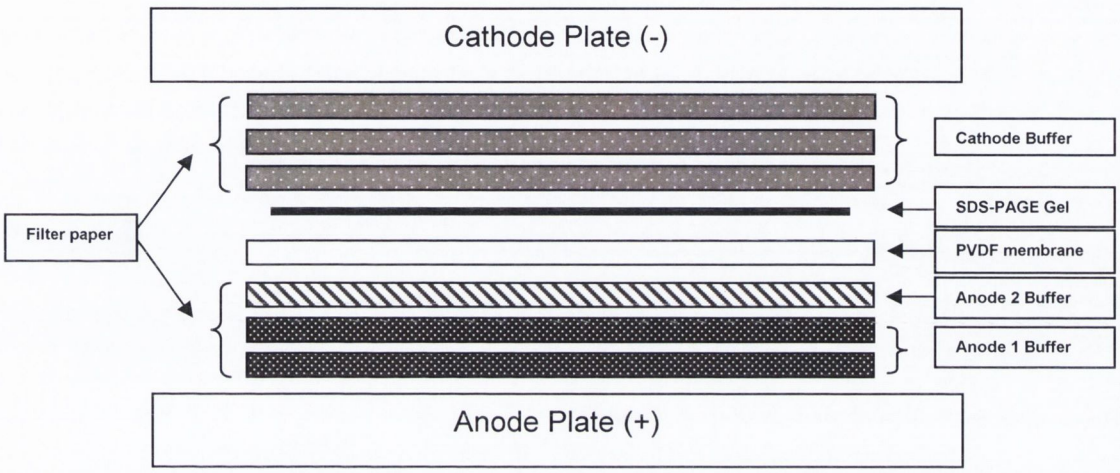

Figure 2.7 – Diagram of sandwich preparation for semi-dry transfer of protein from SDS gel to PVDF membrane

2.2.10.5 Western Immunoblotting

Following transfer of the proteins from the gel onto the PVDF membrane, the membrane was immediately blocked in 5% milk in TBS-Tween20 (TBS-T) for 1hr on a rocker at room temperature. The membrane was then washed for 3 x 10mins in TBS-T on a rocker at room temperature before being transferred to the primary antibody at the appropriate dilution (see Table below) and incubated overnight at 4°C on a rocker. The membrane was washed 3 x 10mins in TBS-T and then the secondary antibody at the appropriate dilution (see Table below) was applied to the membrane for a period of 1hr at room temperature on a rocker. Following this, the membrane was washed 3 x 10 min in TBS-T. The membrane was then exposed to chemiluminescent solution (Milipore) and developed on the Fujifilm Luminescent Image Analyzer LAS-3000. Following this step, the membrane was washed 3 x 5mins in TBS-t and stripped in a striping solution for 15mins and then washed again 3 x 5mins. At this point the blot was

ready to be re-blocked and re-probed with another antibody. Protein bands were quantified using ImageJ software.

Table 2.5 – Protein targets for mouse Western Immunoblotting

Target	Mol Weight (kDa)	Primary Antibody Conc	Secondary Antibody Conc
B-actin	43	1 in 1000	1 in 5000
STAT 3	79 / 86	1 in 1000	1 in 5000
pSTAT 3	79 / 86	1 in 1000	1 in 5000
C/EBP β	35-38	1 in 1000	1 in 5000
pC/EBP β	36 / 38	1 in 1000	1 in 5000

2.2.11 Statistical analysis

All statistical analyses were carried out using the GB-STAT statistical software package (Dynamic Microsystems). Data were deemed to be outliers if they were two standard deviations away from the mean. Initial statistical comparisons used were a student's t-test, a one-way or two-way analysis of variance (ANOVA). If significant changes were observed following ANOVA, the data were further analysed with a student Newman-Keuls *post hoc* test. Data was determined to be significant when $P<0.05$.

3. Results Chapter 1 – Poly I:C as a potential inducer of depressive symptoms in rat models

3.1 Introduction

Whilst the pathophysiological mechanisms that underlie depression have not been fully elucidated, increasing evidence suggests that inflammation may play a role in the development of this disorder (Anisman, 2011, Dantzer et al., 2008, Leonard and Maes, 2012, Miller et al., 2009). Specifically, it has been reported that depression is associated with increased circulating concentrations of inflammatory cytokines, soluble cytokine receptors, chemokines and acute phase proteins (Cizza et al., 2008, Diniz et al., 2010, Grassi-Oliveira et al., 2009, Lanquillon et al., 2000, Maes et al., 1995, Simon et al., 2008). Also, cytokine immunotherapy with IFNα or IL-2 can induce depressive symptoms in otherwise psychiatrically normal individuals (Capuron et al., 2003a, Capuron et al., 2000, Capuron et al., 2001, Alavi et al., 2012a, Raison et al., 2005a, Loftis and Hauser, 2004, Udina et al., 2012). Further evidence supporting an inflammatory basis to depression comes from studies reporting an association of inflammatory and infectious diseases with depressive symptoms (Gao et al., 2009, Owen et al., 2001, Pollak and Yirmiya, 2002, Sakic et al., 1996, Loftis et al., 2008, Lehman and Cheung, 2002), and the induction of depressive-like behaviours in animals upon the administration of inflammatory cytokines (Dunn et al., 2005, Raison et al., 2006, Dantzer et al., 2008, Miller et al., 2009, Hayley et al., 2012).

Animal models are useful tools that allow the investigator to probe biological mechanisms that would not be possible in humans. Polyinosinic:polycytidylic acid (poly I:C) is a toll-like receptor-3 (TLR3) agonist that has been shown to initiate inflammatory responses comparable to that of a systemic viral infection. Systemic injection of poly I:C has been reported to induce an inflammatory response coupled with several symptoms of sickness behaviour (Katafuchi et al., 2003, Cunningham et al., 2007, Gandhi et al., 2007, Gibb et al., 2011, McLinden et al., 2012). Moreover, poly I:C has also been shown to affect memory consolidation in adults, anxiety-like behaviours in neonates and behavioural alterations in the offspring of dams challenged during pregnancy (Kent et al., 2007, Konat et al., 2011, Kranjac et al., 2012, Meyer and Feldon, 2012, Meyer et al., 2008, Meyer et al., 2006, Yee et al., 2011, Yee et al., 2012). However, to date depressive or anxiety-like behaviours in adults have not been comprehensively examined following poly I:C administration, and we suggest that poly I:C administration may represent a useful model of post-viral depression.

A specific mechanistic link between inflammation and depression remains elusive, however evidence suggests that alterations in tryptophan metabolism may play a role. Whilst a functional deficit in the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) is well-established as a contributor to the pathogenesis of depression (Coppen and Doogan, 1988, Cryan and Leonard, 2000), it is only in recent years that theories have emerged to suggest a correlation between inflammation and low serotonin (Leonard and Maes, 2012, Maes et al., 2011). The synthesis of serotonin is dependent on tryptophan availability (Russo et al., 2009). In this regard, the kynurenine pathway (KP) is the major metabolic pathway for tryptophan in the body resulting in the production of kynurenine and several downstream metabolites (Stone and Darlington, 2002), and induction of the rate limiting enzyme in the KP, indoleamine 2,3-dioxygenase (IDO) is driven by inflammatory cytokines (Carlin et al., 1989, Fujigaki et al., 2006, Zunszain et al., 2012). Consequently, IDO induction has been proposed as a mechanism by which inflammation can precipitate depression via tryptophan depletion (Christmas et al., 2011, Maes et al., 2011, Raison et al., 2009). As well as potentially reducing the availability of tryptophan for serotonin synthesis, activation of the KP can also lead to the formation of neuroactive metabolites which have been shown to be neuroprotective or neurotoxic, and in the central nervous system are primarily generated by glial cells (Schwarcz and Pellicciari, 2002). It is proposed that a shift in balance towards the neurotoxic arm of this pathway can lead to neurodegeneration and reduced neurogenesis in the brain which may be a contributing factor to depressive behaviour that occurs secondary to inflammation (O'Connor et al., 2009a, O'Connor et al., 2009c, Raison et al., 2010, Muller and Schwarz, 2007). In fact a recent study demonstrated that inhibition of the neurotoxic arm of the kynurenine pathway prevented the ability of the inflammatory cytokine IL-1β to inhibit neurogenesis *in vitro* (Zunszain et al., 2012). Also a systemic inflammatory challenge with bacterial LPS reduces expression of the neurotrophic factor brain-derived neurotrophic factor (BDNF) in rat brain (Guan and Fang, 2006) and intrahippocampal LPS administration has been shown to inhibit expression of BDNF and also its receptor TrkB (Tanaka et al., 2006). These are important findings considering the role that BDNF plays in driving neurogenesis (Henry et al., 2007, Lee et al., 2002); a process implicated in the pathogenesis of depression and in therapeutic response to antidepressants (Nibuya et al., 1995, Saarelainen et al., 2003).

The Wistar Kyoto (WKY) rat was selectively bred as a normotensive strain control for the spontaneously hypertensive rat (SHR), however it has also been proposed as a model for depressive disorders because it exhibits behavioural, endocrine and neurotransmitter changes also found in people suffering with depression (Kurtz and Morris, 1987, Redei et al., 2001). WKY rats have been shown to display increased anxious behaviours in the open field test (OFT) and elevated plus maze paradigms which can be reversed via treatment with antidepressants (Durand et al., 1999, Paré, 1994). WKY rats have also been shown to display increased immobility in the forced swim test (FST) and lower levels of preference for a saccharin solution, which are indicative of a depressive-like phenotype (Rittenhouse et al., 2002, Malkesman et al., 2005). While conflicting reports do exist as to their response to antidepressants, administration of the tricyclic antidepressant desipramine (López-Rubalcava and Lucki, 2000) or the NMDA receptor antagonist ketamine (Tizabi et al., 2012) produce a dose-dependent reduction in immobility in the FST. Chronic treatment with the serotonin re-uptake inhibitor escitalopram (Yamada et al., 2013) also produces an antidepressant response in the FST in WKY rats providing pharmacological validation of the antidepressant response in the WKY rat as a rodent model of depression.

The WKY rat has also been reported to have higher circulating concentrations of corticosterone which is analogous to high circulating cortisol in patients suffering from depression (Rittenhouse et al., 2002, Pardon et al., 2003, De La Garza and Mahoney, 2004, Solberg et al., 2001). Solberg et al. (2001) reported that WKY rats had higher levels of corticosterone and that peak diurnal levels remained elevated up to 5hr post peak when compared to WIS controls. Studies by De La Garza and Mahoney (2004) and Rittenhouse et al. (2002) have reported that WKY rats display a delayed return to basal concentrations following exposure to a swim stress when compared to WIS and Sprague-Dawley rats respectively. Similarly, Pardon et al (2003) reported a delayed return to basal concentrations of corticosterone when WKY rats were subjected to a chronic cold environment and a restraint stress. Higher circulating concentrations of corticosterone might indicate reduced glucocorticoid feedback to the hypothalamus mediated by reduced glucocorticoid receptor (GR) sensitivity and/or signalling. To date however there have been no studies to assess GR function in the WKY strain.

Dysregulation of the hypothalamic pituitary adrenal (HPA) axis may subsequently contribute to impaired immune function and development of an inflammatory

phenotype related to glucocorticoid insensitivity (Kiecolt-Glaser et al., 2003). Pro-inflammatory cytokines act synergistically to activate the HPA-axis leading to increased concentrations of glucocorticoids and prolonged activation of the axis (Salas et al., 1990, Ray and Prefontaine, 1994). There is however a paucity of data available on the inflammatory status within the WKY rat although there are a few reports of the development of autoimmune glomerulonephritis and diabetes associated with aged WKY rats (Reynolds et al., 2012, Solberg Woods et al., 2009).For these reasons it is of interest to assess the WKY rat for activation of the inflammatory response system and any potential association of immune system activation with the behavioural phenotype associated with the strain. In the present investigation, basal inflammatory state and temporal effects of the viral mimetic and immune stimulus poly I:C in the WKY rat were determined. The immune phenotype and response of the outbred Wistar-Ham strain to varying immunological stimuli have been characterised in previous studies (Bluthé et al., 1992, Katafuchi et al., 2006).

Previous studies have reported that the WKY rats show lower concentrations of 5-HT basally and following a psychological stress the ratio of 5HIAA, the metabolite of 5-HT, to 5-HT is greater in WKY rats when compared to a control strain (De La Garza and Mahoney, 2004, Scholl et al., 2010). The monoamine hypothesis of depression suggests that deficits in the bioavailability of monoamine neurotransmitters can lead to altered mood and rectification of this chemical imbalance can improve mood. Literature exists outlining that people suffering with depression have lower levels of 5-HT and the effect that inflammatory stimuli and cytokines can have on mood (Maes et al., 2011, Raison et al., 2009), coupled with the known anxious- and depressive-like phenotype WKY rats exhibit, this might suggest that the WKY rat presents as a viable model of depression. As such, changes in concentrations of tryptophan and serotonin metabolites in addition to the possibility of kynurenine pathway activation at baseline or following immune challenge are also of interest in the WKY rat strain.

The objective of this study was to characterise the ability of the viral mimetic poly I:C to induce symptoms of depression and anxiety in Sprague-Dawley rats secondary to its inflammatory actions. The ability of poly I:C to induce the tryptophan degrading enzyme IDO and impact upon central 5-HT and kynurenine concentrations, and also to impact upon expression of the neurotrophin BDNF and its receptor TrkB were examined as potential mechanisms to link inflammatory markers to depression and anxiety related behaviours. Furthermore

it was of interest to investigate the effects of poly I:C in the WKY rat, a genetically predisposed strain to depressive- and anxious-like behaviours and determine if immune challenge might induce an enhanced sickness or depression/anxiety related behavioural response when compared to the background Wistar-Ham (WIS) outbred control strain. Parallel experiments were performed with WKY and WIS rats and comparisons made following poly I:C administration on selected behavioural, inflammatory and metabolic markers as previously described with the Sprague-Dawley strain.

3.2 Materials and Methods

3.2.1 Animals

Male Wistar rats (WIS, 300-400g) were obtained from Bioresources, TCD and Sprague–Dawley (250–350g) and Wistar Kyoto (WIS, 300-400g) rats were obtained from Harlan, UK. Rats were maintained on a 12hr light:12hr dark cycle (lights on at 08:00 h) in a temperature controlled room (22 ± 2°C) and food and water were available *ad libitum*. All animals were singly housed for one week prior and for the total duration of the study to facilitate accurate measurements of food, water and saccharin intake. The experimental protocols were in compliance with the European Communities Council directive (86/609/EEC).

3.2.2 Systemic poly I:C challenge

Poly I:C was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). It was prepared for injection by re-suspending in sterile saline, heating to 50 °C at a concentration of 2 mg/ml to ensure complete solubilisation and then allowed to cool naturally to room temperature to ensure proper annealing of double-stranded RNA. Poly I:C was stored at −20 °C until use. Animals received either an intraperitoneal (i.p.) injection of poly I:C (6 mg/kg) or sterile saline and were analysed for behaviour or euthanised at time intervals post injection (6hr, 24hr, 48hr, 72hr). Body weight and food intake was measured daily.

3.2.3 Behavioural testing

3.2.3.1 Home Cage Activity Test (HCA)

Animals were individually placed in activity monitor cages measuring 32cm x 20cm x 18cm (length x width x height). These cages were connected to an AM1051 data logger (Benwick Electronics). Each cage contained 2 sets of horizontal infrared beams, one set at 3cm and one at 15cm from the bottom of the cage. Each set consisted of a 12 beam by 7 beam matrix, which formed a grid of 66 x 2.54cm^2 cells in each cage. Together the beams and data logger recorded activity and rearing. Activity was determined by the number of times a beam was broken and was measured from when the beam was first broken until

no beam had been broken for 5 seconds. Data was analysed using the total number of beam breaks over the 15 minute trial. The trials were conducted between 10am and 2pm. Activity was subsequently recorded at different time intervals post injection as described above. After 15 minutes of behavioural recording the animals were immediately sacrificed.

3.2.3.2 Saccharin preference

The saccharin preference test, used as a measure of anhedonia, was carried out as previously described (Harkin et al., 2002). Briefly, animals that were also used for measuring home cage activity were exposed to the saccharin solution (0.1%) in a standard drinking bottle and also a bottle containing tap water in their home cage. The position of the bottles was varied and counter balanced across the left or right side of the feeding compartment. Bottles were weighed for 4 days before the commencement of the study. This was carried out to ensure that animals were subdivided into groups with comparable baseline preference. Bottles were subsequently weighed every day post injection. Fluid consumption was calculated by calculating the daily difference in bottle weight. Preference scores were calculated by dividing the amount of saccharin consumed by the total amount of fluid consumed (water + saccharin) during the 24 hr period.

3.2.3.3 Open field test

A separate set of animals were used for behavioural analysis in the open field and these animals were tested 24 hr post poly I:C/vehicle injection. At the beginning of each trial, animals were placed in the centre of a dimly lit open field arena, measuring 1.2m in diameter, and observed for 15 minutes. The trials were conducted between 10am and 2pm. The arena was cleaned with 70% ethanol between each trial. The behaviour of the animals was recorded by an overhanging camera that was attached to a personal computer. Ethovision 3.0 (Noldus, The Netherlands) was used to track the movement of the animal. The total distance moved in the arena, distance moved in the inner zone of the arena and number of transitions between the inner and outer zones were recorded.

3.2.4 Analysis of inflammatory gene expression by real-time PCR

RNA was extracted from brain tissue using the NucleoSpin® RNA II total RNA isolation kit (Macherey-Nagel, Germany). Genomic DNA contamination was removed by the addition of Dnase to the samples. RNA was reverse transcribed into cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Darmstadt, Germany). Real-time PCR was performed using an ABI Prism 7300 instrument (Applied Biosystems) as previously described (Boyle and Connor, 2007). Taqman® Gene Expression Assays (Applied Biosystems) containing forward and reverse primers and a FAM-labelled MGB Taqman® probe were used to quantify each gene of interest. PCR was performed using Taqman® Universal PCR Master Mix and samples were run in duplicate. The cycling conditions consisted of 90 °C for 10 min and 40 cycles of 90 °C for 15 s followed by 60 °C for 1 min. B-actin was used as an endogenous control to normalize gene expression data. Relative gene expression was calculated using the ΔΔCT method with Applied BioSystems RQ software (Applied BioSystems, UK).

Table 3.1 – List of Taqman® Gene Expression Assays used and their gene codes

Target	Taqman® Assay ID
IL-1β	Rn00580432_m1
IL-6	Rn00561420_m1
TNFα	Rn93699017_m1
IFNγ	Rn00594078_m1
CD11b	Rn00709342_m1
BDNF	Rn02531967_s1
TrkB	Rn01441749_m1
IDO	Rn00576778_m1

3.2.5 Analysis of serotonin concentrations by HPLC

Serotonin (5-HT) and its metabolite 5-HIAA were measured by high performance liquid chromatography (HPLC) as previously described (Harkin et al., 2003).

Brain tissue was sonicated in 1 ml of mobile phase spiked with 5 ng/20 µL of N-methyl 5-HT (Sigma) as internal standard. The mobile phase contained 0.1 M citric acid (Sigma), 0.1 M sodium dihydrogen phosphate (Sigma), 0.1 mM EDTA (Sigma), 1.4 mM octane-1-sulphonic acid (BDH) in dd$H_2O$and was adjusted to pH 2.8 using 4 N sodium hydroxide. Homogenates were centrifuged at 20,000 rpm for 20 min and the supernatants were placed into new eppendorf tubes, using a syringe fitted with a 0.45µm filter (phenomenex). 10µl of the filtered supernatant was injected into a reverse phase analytical column (Kinetex™ Core Shell Technology C18 column with specific area of 100mm x 4.6mm and particle size of 2.6u, Phenomenex), for separation of the neurotransmitters. Neurotransmitter concentrations were quantified by electrochemical detection (Digital Electrochemical Amperometric Detector, Mason Technology Ltd) and chromatographs were generated by CLASS-VP software (Shimadzu). Results are expressed as ng of neurotransmitter per g fresh weight of tissue.

3.2.6 Analysis of tryptophan and kynurenine concentrations by HPLC

Tryptophan and kynurenine were measured in brain samples using HPLC. The mobile phase contained 50nM glacial acetic acid, 100mM zinc acetate (Sigma) and 3% acetonitrile dissolved in double-distilled NANOpure water HPLC grade H_2O (Sigma). The pH was adjusted to 4.9, using 5 M NaOH. Brain tissue was sonicated in 1 ml of mobile phase containing 7% perchloric acid spiked with 50 ng/20 µL of N-methyl 5-HT (Sigma) as internal standard. Brain homogenates were centrifuged at 20,000 rpm for 20 min and the supernatants were placed into new eppendorf tubes, using a syringe fitted with a 0.45µm filter (phenomenex). 20µl of the filtered supernatant was injected using a Waters autosampler and a Reverse Phase analytical column (Kinetex™ Core Shell Technology column with specific area of 100mm x 4.6mm and particle size of 2.6µm, Phenomenex) was used for separation of metabolites. A PDA-UV detector (Shimadzu SPD-M10A VP), calibrated to integrate at 230 nm and 250nm, as well as a fluorescent detector (Shimadzu RF-20A XS prominence fluorescence detector), set to excitation wavelength 254nm; emission wavelength 404nm, were used to detect the metabolites. Chromatographs were generated by CLASS-VP software (Shimadzu). Results are expressed as ng of neurotransmitter per g fresh weight of tissue.

3.2.7 Statistical analysis of data

All values are expressed as mean ± standard error of the mean (S.E.M.). Data was analysed using either a repeated measures two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test, a randomised two-way ANOVA followed by Newman-Keuls post-hoc test, a one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test or an unpaired student-t-test (GB stat). A value of $P<0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Poly I:C administration induces sickness behaviour followed by depressive and anxiety like behaviours in Sprague-Dawley rats

3.3.1.1 Poly I:C induces sickness behaviour in Sprague-Dawley rats characterised by reduced bodyweight gain and reduced locomotor activity

Poly I:C induced a significant decrease in body weight gain at 24hr post injection when compared to vehicle treated animals ($p<0.01$) (Interaction, $F_{(4, 36)} = 32.04$, $p<0.0001$). By 48 hr the change in body weight gain had returned to control levels (A). When poly I:C and vehicle treated animals were compared in the home-cage a significant decrease in locomotor activity was observed 6hr post injection ($p<0.01$) (Interaction, $F_{(4, 36)} = 46.86$, $p<0.0001$) which had returned to baseline levels by 24hr (B). [Figure 3.1, n=6-8]

3.3.1.2 Poly I:C induces anhedonia in Sprague-Dawley rats in the saccharin preference test

When animals were exposed to a 2-bottle saccharin preference test, those animals that were treated with poly I:C showed anhedonic behaviour as indicated by their reduced preference for the saccharin solution. At baseline levels both groups had a saccharin preference of approximately 95%. A significant effect of treatment was observed at 24hr with those animals exposed to poly I:C showing a reduced preference for saccharin, whereas preference for saccharin in the vehicle treated groups was not affected ($p<0.01$). This anhedonic behaviour was maintained at 48hr and 72hr post injection with poly I:C treated animals showing a significant reduction in saccharin preference compared to vehicle treated groups ($p<0.01$, $p<0.05$ respectively) (Interaction, $F_{(4, 36)} = 19.16$, $p<0.0001$, C). The reduced preference for saccharin observed was not related to a change in total fluid intake as no change in this parameter was observed when vehicle and poly I:C treated animals were compared (D). [Figure 3.1, n=6-8]

3.3.1.3 Poly I:C induces anxiety-like behaviour in Sprague-Dawley rats in the open field test

When exposed to a novel open field arena, poly I:C treated animals showed a significant reduction in distance moved in the inner zone when compared to vehicle treated controls ($p<0.01$, E), which is indicative of an anxiety-like behaviour. Data are presented as distance moved in the inner zone as a % of total distance moved in the open field arena. Furthermore, when the number of transitions made between the inner and outer zones were compared, those animals that had been treated with poly I:C showed a significant reduction in the number of transitions made ($p<0.01$, F), which again is indicative of anxiety-like behaviour. [Figure 3.1, n=6-8]

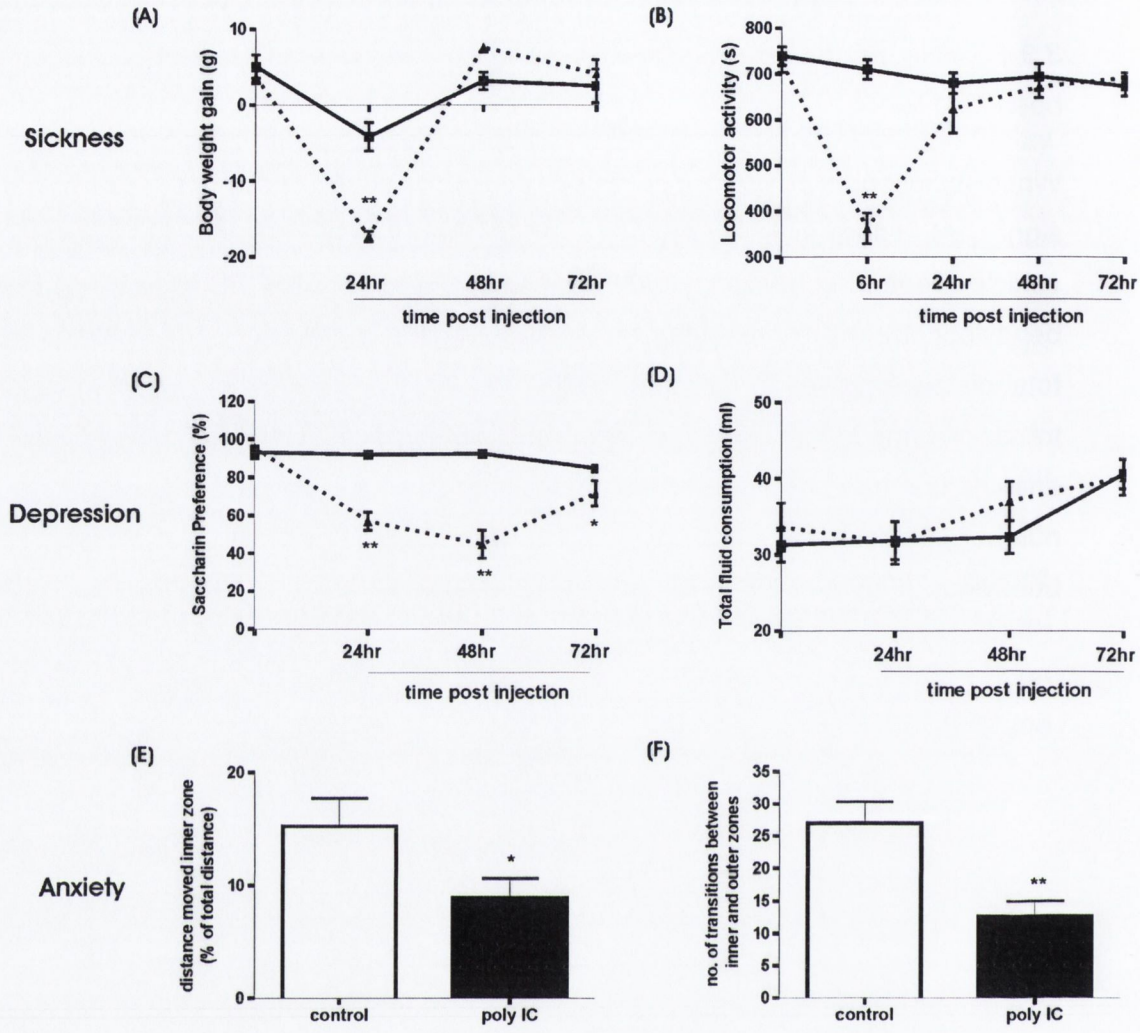

Figure 3.1 – Poly I:C administration induces sickness behaviour followed by depressive and anxiety like behaviours in Sprague-Dawley rats

Poly I:C induces sickness behaviour characterised by a reduction in body weight (A) and in home cage locomotor activity (B). Poly I:C administration induces depressive-like behaviour (anhedonia) characterised by reduced saccharin preference in the saccharin preference test (C) without affecting total fluid intake (D). Poly I:C induces anxiety-like behaviours in the open field test 24hr post administration as indicated by decreased movement in the inner zone (E) and decreased transitions between the inner and outer zones (E) of the open field arena. Dotted and solid lines correspond to poly I:C administration and vehicle administration respectively. Data expressed as mean ± SEM, n=6-8 per group (Body weight gain; Locomotor activity; Saccharin preference test: Two-way RM ANOVA with Bonferonni test; Open field test: unpaired t-test).

3.3.2 Poly I:C induces a neuroinflammatory response characterised by expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα and the microglial activation marker CD11b in frontal cortex and hippocampus in Sprague-Dawley rats

In response to poly I:C administration, a significant increase in IL-1β expression was found in the frontal cortex at the 6hr time point when compared to vehicle treated controls ($p<0.001$) ($F_{(4, 21)} = 4.17$, $p<0.01$, A). A 4-fold increase in IL-1β expression was also found in the hippocampus 6hr post poly I:C administration ($p<0.001$) ($F_{(4, 25)} = 5.25$, $p<0.003$, B). In both brain regions, IL-1β had returned to control levels by 24 post administration. [Figure 3.2, n=4-6]

When compared to vehicle treated controls, animals exposed to poly I:C showed a significant 10-fold increase in the expression of IL-6 in the frontal cortex at 6hr post administration ($p<0.001$) which had returned to control levels by 24hr ($F_{(4, 21)} = 7.86$, $p<0.0005$, C). A similar pattern of expression was found in the hippocampus with a significant 6-fold increase found in this brain region in poly I:C treated animals at 6hr post administration ($p<0.001$). Moreover, increased IL-6 expression was maintained at a significantly elevated level at 24hr ($p<0.05$) returning to control levels at 48hr ($F_{(4, 25)} = 7.49$, $p<0.0004$; D). [Figure 3.2, n=4-6]

At 6hr post administration, a significant increase in TNFα was also found in the frontal cortex and hippocampus ($p<0.001$) with increased expression remaining in both brain regions until 24hr ($p<0.05$) ($F_{(4, 21)} = 7.54$, $p<0.0006$, E) ($F_{(4, 25)} = 4.66$, $p<0.006$, F). [Figure 3.2, n=4-6]

Expression of IFNγ was very low and variable compared to the other inflammatory cytokines. Whilst poly I:C did not induce IFNγ expression in the frontal cortex at any time-point analysed, a transient increase in IFNγ expression was observed in the hippocampus 6hr post poly I:C administration (Data not shown).

Approximately a 2.5-fold significant increase in the expression of the microglial activation marker CD11b was found in the frontal cortex of the poly I:C treated animals at 24hr post administration ($p<0.001$). The expression of this marker in the frontal cortex remained significantly elevated at 48hr ($p<0.01$), returning to control levels by 72hr ($F_{(4, 21)} = 24.23$, $p<0.0001$, G). Similarly, a significant increase in CD11b expression was also found in the hippocampus at 24hr and

48hr post administration ($p<0.001$ and $p<0.01$ respectively), again returning to control levels by 72hr ($F_{(4, 25)} = 26.21$, $p<0.0001$, H). [Figure 3.2, n=4-6]

Figure 3.2 – Poly I:C induces a neuroinflammatory response characterised by expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα and the microglial activation marker CD11b in frontal cortex and hippocampus of Sprague-Dawley rats

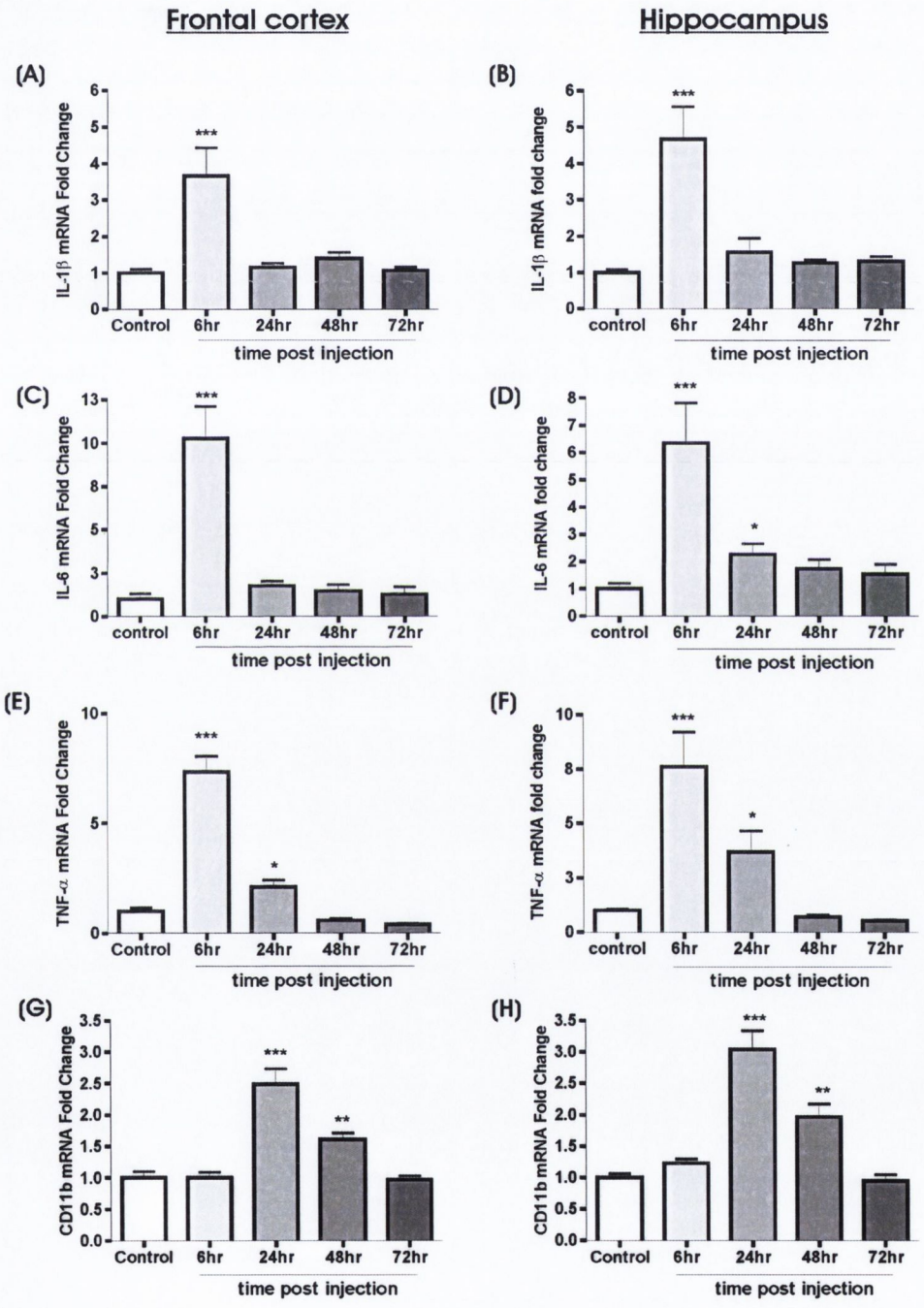

Figure 3.2 – Poly I:C induces a neuroinflammatory response characterised by expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα and the microglial activation marker CD11b in frontal cortex and hippocampus of Sprague-Dawley rats

Increased mRNA expression of the pro-inflammatory cytokines IL-1β (A-B), IL-6 (C-D), and TNFα (E-F) in the frontal cortex (A,C,E) and hippocampus (B,D,F) 6-24h following poly I:C administration. Increased mRNA expression of the microglial activation marker CD11b (G,H) in the frontal cortex (G) and hippocampus (H) 24-48h following poly I:C administration. Data expressed as mean ± SEM, n=4-6 per group.**P<0.01; ***P<0.001 poly I:C vs. Control (One-way ANOVA, Newman-Keuls post-hoc test), *P<0.05 poly I:C vs. Control (unpaired t-test).

3.3.3 Poly I:C decreases the expression of BDNF and its receptor TrkB in the frontal cortex and hippocampus of Sprague-Dawley rats

In response to poly I:C treatment, BDNF expression was significantly reduced in the frontal cortex at all time points analysed ($p<0.05$) ($F_{(4, 24)} = 6.06$, $p<0.001$, A). A similar pattern of expression was found in the hippocampus with significantly decreased BDNF expression found at 6hr, 24hr and 48hr post administration ($p<0.01$) however, by 72hr the level of expression had returned to control levels ($F_{(4, 24)} = 9.23$, $p<0.0001$, B). Furthermore, expression of the BDNF receptor TrkB was also found to be decreased at 24hr and 48hr post poly I:C administration in the frontal cortex ($p<0.05$) ($F_{(4, 22)} = 9.22$, $p<0.0002$, C) and hippocampus ($p<0.05$) ($F_{(4, 23)} = 2.42$, $p<0.03$, D), with expression in both brain regions returning to control levels by 72hr. [Figure 3.3, n=4-6]

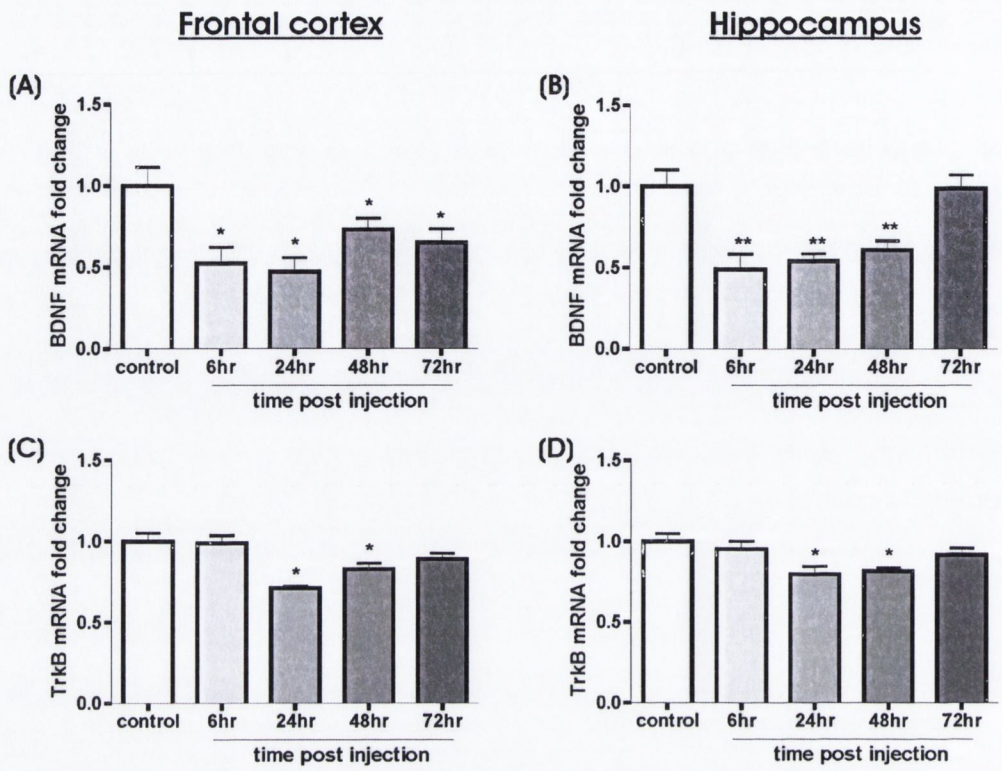

Figure 3.3 – Poly I:C reduces expression of the neurotrophic factor BDNF and its receptor TrkB in frontal cortex and hippocampus of Sprague-Dawley rats

Decreased mRNA expression of BDNF (A,B) and its receptor TrkB (C,D) in the frontal cortex (A,C) and hippocampus (B,D) 6-72h (BDNF) and 24-48h (TrkB) following poly I:C administration. Data expressed as mean ± SEM, n=4-6 per group. **P<0.01, *P<0.05 poly I:C vs. Control (One-way ANOVA, Newman-Keuls post-hoc test).

3.3.4 Poly I:C induces IDO expression coupled with kynurenine production in the frontal cortex and hippocampus of Sprague-Dawley rats

3.3.4.1 IDO expression

As mentioned previously, increased expression of the IDO-inducing cytokines IL-6, TNFα and IL-1β were found in the brain in response to poly I:C administration. Accordingly, a significant 70-fold increase in IDO expression was found in the frontal cortex of poly I:C treated animals at 6hr post administration when compared to control animals ($p<0.001$) ($F_{(4, 21)} = 9.39$, $p<0.0007$, A). Whilst the 3.3-fold change in IDO at 6hr post poly I:C administration in the hippocampus was not as great as observed in the frontal cortex it was still significantly elevated in comparison to the vehicle treated control group ($p<0.001$) ($F_{(4, 21)} = 6.68$, $p<0.0001$, B). [Figure 3.4, n=5-6]

3.3.4.2 Tryptophan and kynurenine concentrations

Increased concentrations of tryptophan were observed in frontal cortex at 24hr and 48hr post poly I:C administration ($p<0.05$), returning to control levels by 72hr ($F_{(4, 25)} = 2.77$, $p<0.05$, C). In the hippocampus, increased tryptophan was found at 6hr, 24hr and 48hr post administration ($p<0.05$), again returning to control levels by 72hr ($F_{(4, 25)} = 3.47$, $p<0.02$, D). Kynurenine could not be detected in the frontal cortex of vehicle treated controls or in those animals that were assessed 6hr after poly I:C administration, however, kynurenine was detected at 24hr, 48hr and 72 hr post-administration (E). This translated to an increased kynurenine/tryptophan ratio at 24hr, 48hr and 72hr post-poly I:C administration (G). Kynurenine was detected in the hippocampus of control animals and those at the 6hr time point post-administration, however the level of kynurenine produced was significantly higher at both 24hr and 48hr post poly I:C administration ($p<0.05$), returning to control levels by 72hr ($F_{(4, 23)} = 3.84$, $p<0.02$, F). Once again this increase in kynurenine translated to an increase in the kynurenine/tryptophan ratio at 24hr, 48hr ($p<0.05$), returning to control levels by 72hr ($F_{(4, 23)} = 5.34$, $p<0.01$, H). [Figure 3.4, n=5-6]

Figure 3.4 – Poly I:C induces IDO expression coupled with kynurenine production in the frontal cortex and hippocampus of Sprague-Dawley rats

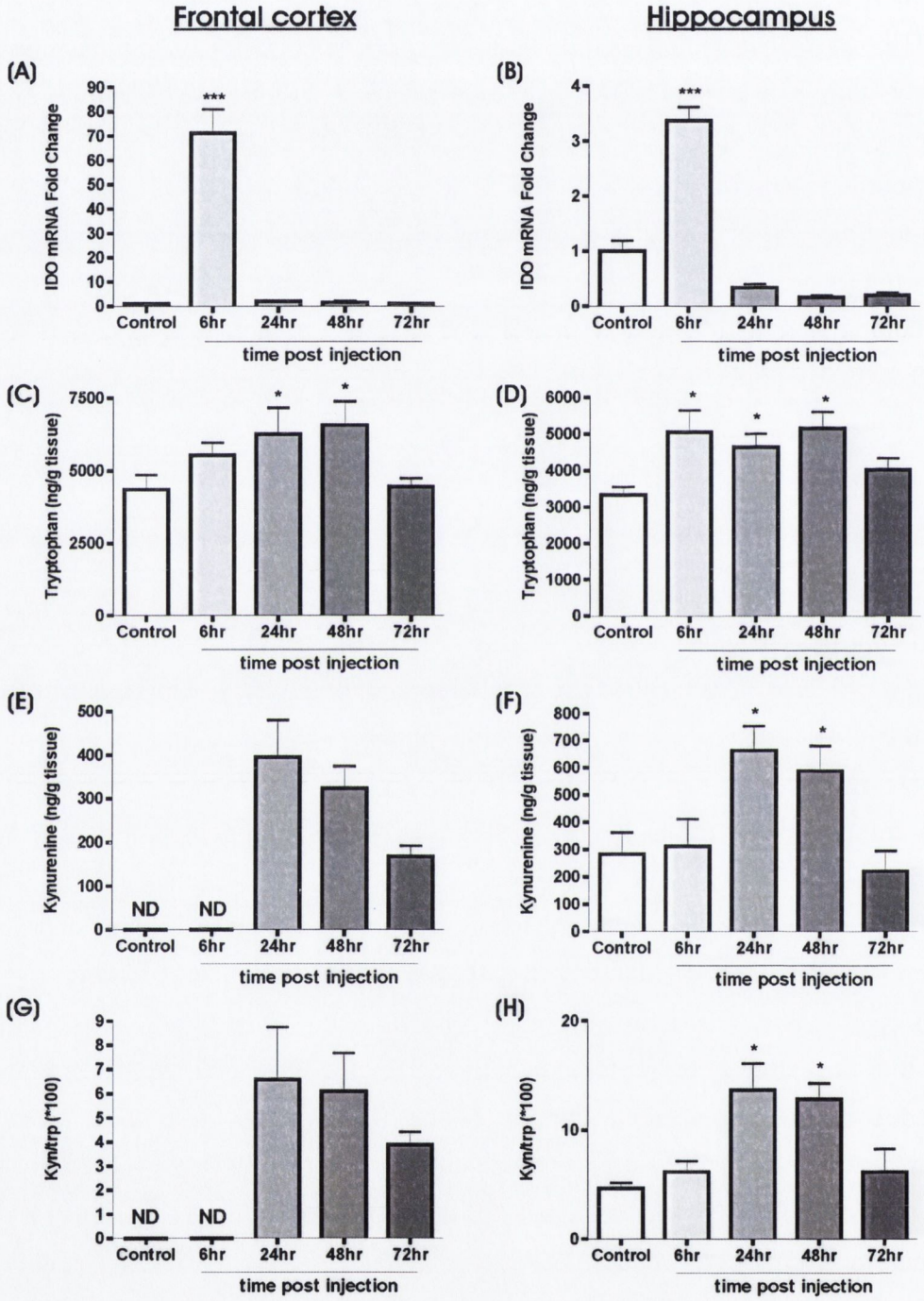

Figure 3.4 – Poly I:C induces IDO expression coupled with kynurenine production in the frontal cortex and hippocampus of Sprague-Dawley rats

Increased expression of IDO was detected in the frontal cortex (A) and hippocampus (B) at 6h following poly I:C administration. Increased tryptophan (C, D) and kynurenine (E, F) concentrations were detected in the frontal cortex (C, E) and hippocampus (D,F) in response to poly I:C and this was accompanied by an increase in the kynurenine/tryptophan (G, H) ratio indicative of increased metabolism of tryptophan to kynurenine. Data expressed as mean ± SEM, n=5-6 per group. ***P<0.001, *P<0.05 poly I:C vs. Control (One-way ANOVA, Newman-Keuls post-hoc test).

3.3.5 Poly I:C fails to alter 5-HT concentrations in frontal cortex and hippocampus of Sprague-Dawley rats

In response to poly I:C, no change in 5-HT concentration was detected in the frontal cortex, at any time point analysed ($F_{(4, 23)}$ = 0.64, p = 0.64, A). An increased concentration of the 5-HT metabolite 5-HIAA was however found in response to poly I:C at 24hr post administration ($p<0.05$), returning to control levels by 48hr ($F_{(4, 23)}$ = 3.22, $p<0.05$, C), indicative of increased 5-HT metabolism. In the hippocampus a similar pattern was observed where no poly I:C induced change in 5-HT concentration was detected ($F_{(4, 23)}$ = 0.68, p = 0.61, B). As with the frontal cortex, an increase in 5HIAA was detected in response to poly I:C but in this brain region was detected at 6hr post administration ($p<0.05$), returning to control levels by 24hr ($F_{(4, 23)}$ = 3.95, $p<0.01$, D), again indicative of increased 5-HT metabolism. [Figure 3.5, n=5-6]

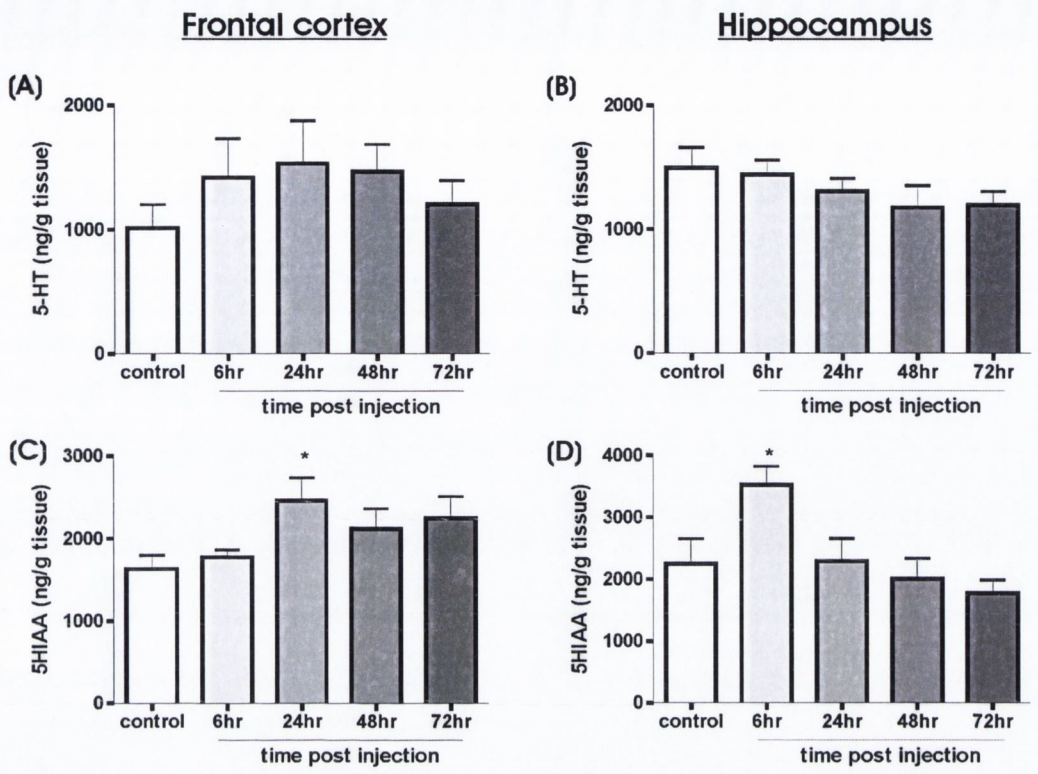

Figure 3.5 – Poly I:C fails to alter 5-HT concentrations but induces a transient increase in 5-HIAA concentrations in frontal cortex and hippocampus of Sprague-Dawley rats

5-HT concentrations in the frontal cortex (A) or hippocampus (B) were not altered following poly I:C administration. Increased 5-HIAA concentrations were observed in the frontal cortex (C) 24h post poly I:C administration, and in the hippocampus (D) 6h post poly I:C administration (D). Data expressed as mean ± SEM, n=5-6 per group. ***P<0.001, **P<0.01 poly I:C vs. Control (One-way ANOVA, Newman-Keuls post-hoc test). *P<0.05 poly I:C vs. Control (unpaired t-test).

3.3.6 The behavioural response of WIS and WKY rats to systemic poly I:C administration

Considering previous results which demonstrated transient sickness behaviour followed by anxious and depressive like behaviours in Sprague-Dawley rats following poly I:C administration, the current experiment aimed to assess the effect that poly I:C would have in two other rat strains. WIS and WKY rats were systemically administered poly I:C and home cage activity monitoring carried out at 6hrs, 24hrs and 48hrs following injection. Body weight, food consumption, saccharin preference, water, saccharin and total fluid consumption were recorded 24hrs and 48hrs following poly I:C administration.

3.3.6.1 Systemic administration of poly I:C causes a time-dependent reduction in body weight and food consumption of WIS and WKY rats

Two-way ANOVA showed an effect of poly I:C administration on the weight of WIS (a) ($F_{(1, 18)}$=31.31, P<0.0001) and WKY (c) ($F_{(1, 16)}$=9.78, P<0.01) rats. Post-hoc comparisons revealed a reduction in weight 24hrs (P<0.01) and 48hrs (P<0.05) in WIS rats following poly I:C administration when compared to vehicle treated controls. WKY rats showed a significant reduction in body weight 24hrs but not 48hrs following poly I:C administration when compared to vehicle treated controls. [Figure 3.6, n=5-6]

Two-way ANOVA showed an effect of poly I:C administration on the food consumption of WIS (b) ($F_{(1, 18)}$=42.05, P<0.001) and WKY (d) ($F_{(1, 18)}$=10.22, P<0.01) rats. Post-hoc comparisons revealed a decreased consumption of food in WIS rats 24hrs (P<0.01) and 48hrs (P<0.01) following poly I:C administration when compared to vehicle treated controls. Food consumption in the WKY rat was only reduced 24hrs following poly I:C administration when compared to vehicle treated controls. [Figure 3.6, n=5-6)

3.3.6.2 Systemic administration of poly I:C causes a reduction in the saccharin preference of WIS rats

Two-way ANOVA showed an effect of poly I:C administration on the preference towards a 0.1% saccharin solution in WIS rats (a) ($F_{(1, 18)}$=33.98, P<0.0001). Post-hoc comparison revealed a significant reduction in preference 24hrs

(P<0.01) and 48hrs (P<0.01) following poly I:C administration when compared to vehicle treated controls. ANOVA showed no effect of poly I:C on the preference towards a saccharin solution in WKY rats (b). [Figure 3.6, n=4-6]

Two-way ANOVA showed an effect of poly I:C administration on saccharin consumption in WIS rats (c) ($F_{(1, 18)}$=16.12, P<0.001). Post-hoc comparison revealed a significantly lower consumption of saccharin 48hr following poly I:C administration in WIS rats when compared to vehicle treated controls (P<0.05). ANOVA showed no effect of poly I:C on saccharin consumption in WKY rats (d). [Figure 3.7, n=4-6]

Two-way ANOVA showed an effect of poly I:C administration on total fluid consumption in WIS (e) ($F_{(1, 18)}$=7.46, P<0.05) and WKY (f) ($F_{(1, 16)}$=6.78, P<0.05) rats. Post-hoc comparisons revealed a significant reduction in total fluid consumption 48hrs following poly I:C administration in both WIS (P<0.05) and WKY (P<0.05) rats when compared to vehicle treated controls. [Figure 3.7, n=4-6]

Two-way ANOVA showed an effect of poly I:C administration on water consumption in WIS (g) ($F_{(1, 18)}$=16.69, P<0.001) rats. Post-hoc comparison revealed a significant increase in water consumption 48hrs following poly I:C administration in WIS rats (P<0.05) when compared to vehicle treated controls. ANOVA showed no effect of poly I:C on water consumption in the WKY rat (h). [Figure 3.7, n=4-6]

3.3.6.3 Systemic administration of poly I:C causes a time-dependent reduction in locomotor activity and investigative rearing of WIS and WKY rats

Two-way ANOVA showed an effect of poly I:C administration on the rearing of WIS (a) ($F_{(1, 23)}$=5.23, P<0.05) and WKY (c) ($F_{(1, 19)}$=18.16, P<0.001) rats. Post-hoc comparisons revealed significantly reduced exploratory rearing 6hrs following poly I:C administration in WKY rats (P<0.05) but not in WIS rats when compared to vehicle treated controls. [Figure 3.6, n=4-6]

Two-way ANOVA showed an effect of poly I:C administration on the locomotor activity of WIS (b) ($F_{(1, 23)}$=10.19, P<0.01) and WKY (d) ($F_{(1, 19)}$=8.93, P<0.01) rats. Post-hoc comparisons revealed significantly reduced activity 6hrs following poly

I:C administration in WIS rats ($P<0.01$) but not in WKY rats when compared to vehicle treated controls. [Figure 3.6, n=4-6]

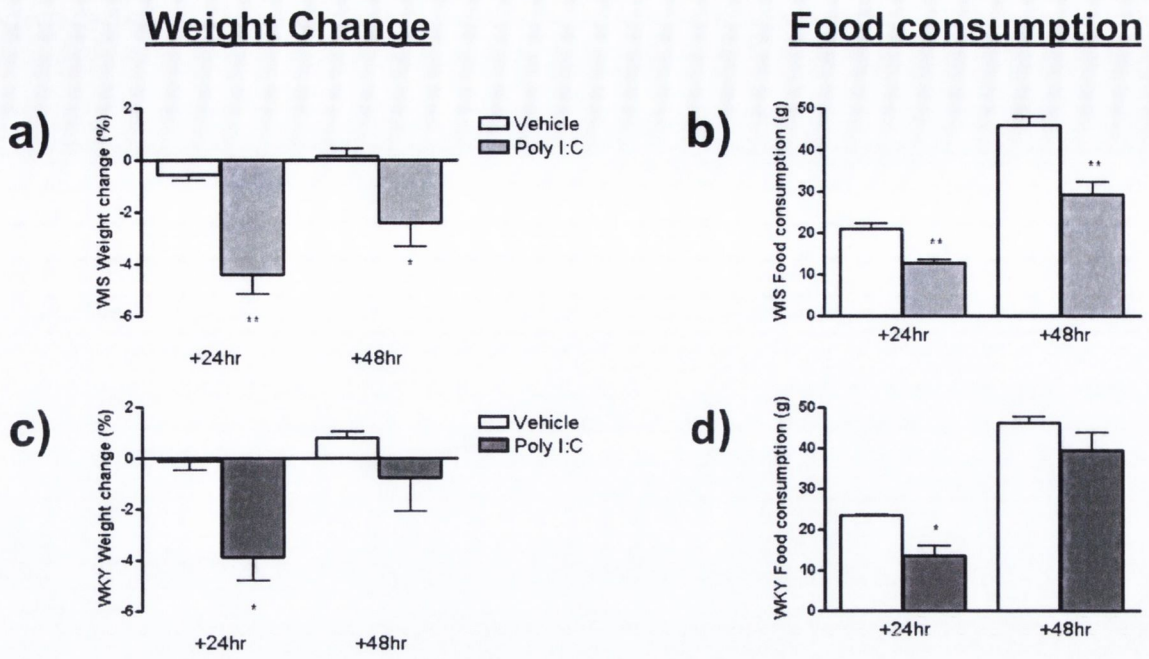

Figure 3.6 – Systemic poly I:C administration causes a decrease in body weight and food intake in WIS and WKY rats

Poly I:C (6mg/kg) reduced the body weight (a, c) and food consumption (b, d) of both WKY and WIS rats 24hrs post injection. Body weight and food consumption was decreased only in WIS rats 48hrs following poly I:C administration. Data expressed as mean ± SEM (n=4-6). ** $P<0.01$, * $P<0.05$ vs. Vehicle treated controls.

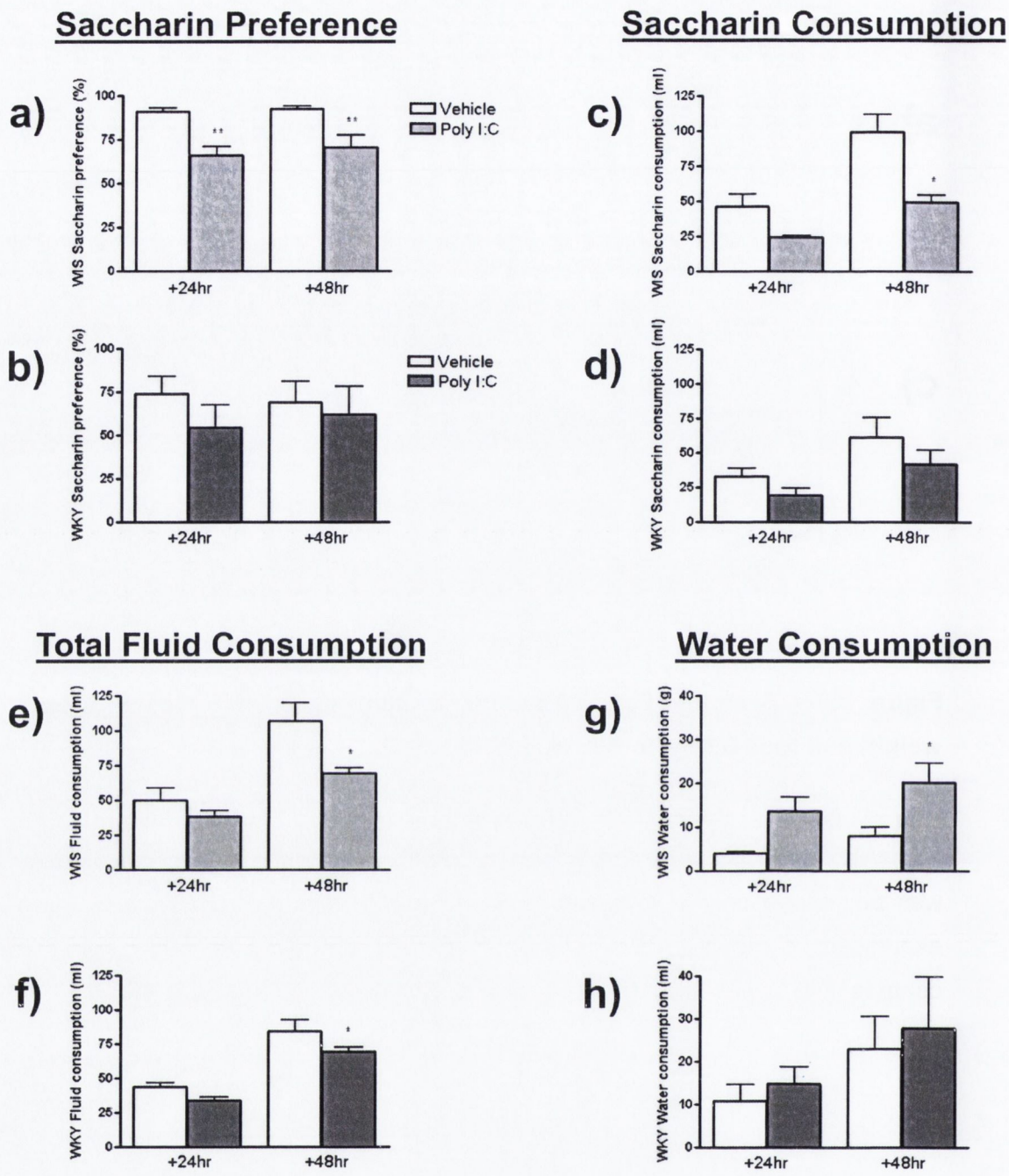

Figure 3.7 – Systemic poly I:C administration reduces saccharin preference and consumption in WIS but not WKY rats

Poly I:C (6mg/kg) reduced the saccharin preference in WIS (a) but not WKY (b) rats. While total fluid consumption was reduced in both WIS (e) and WKY (f) rats 48hrs post injection, saccharin consumption was decreased 48hrs post injection in WIS rats (c). Similarly consumption of water was increased only in WIS (g) and not WKY (h) rats following injection. Data expressed as mean ± SEM (n=4-6). ** $P<0.01$, * $P<0.05$ vs. Vehicle treated controls.

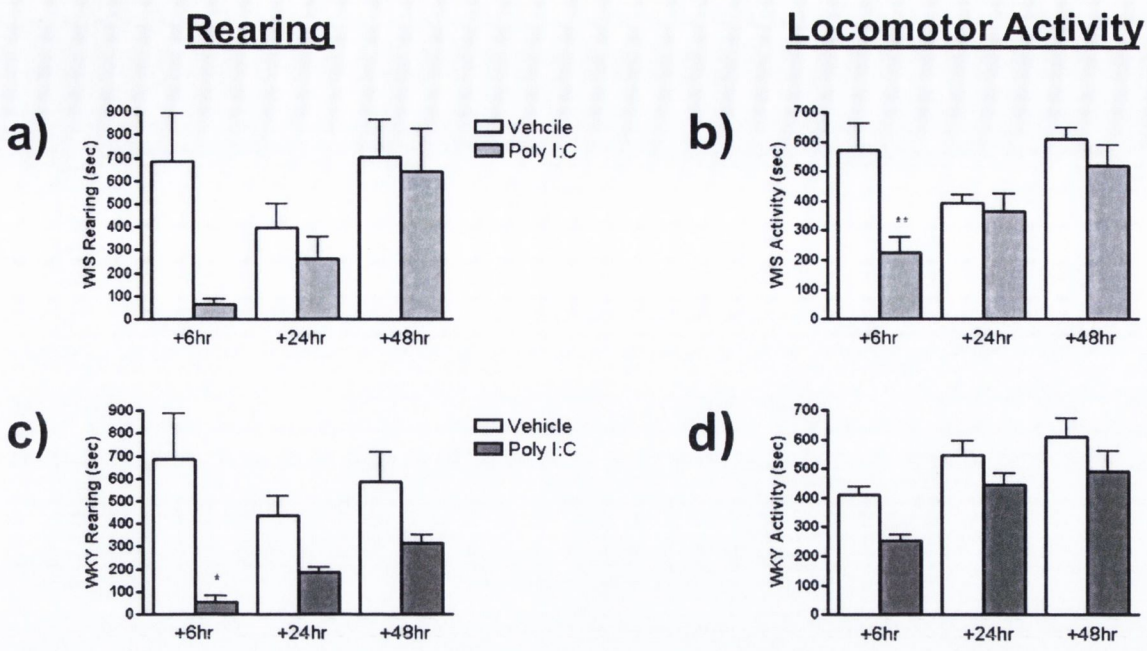

Figure 3.8 – Systemic poly I:C administration affects locomotor activity and rearing in the home cage in the HCA in WIS and WKY rats

Poly I:C (6mg/kg) decreased the locomotor activity of WIS rats (b) and the investigative rearing of WKY rats (c) 6hrs following injection. Non significant reductions in WIS investigative rearing (a) and WKY locomotor activity (d) were observed 6hrs post injection. Data expressed as mean ± SEM (n=4-6). ** $P<0.01$, * $P<0.05$ vs. Vehicle treated controls.

3.3.7 The temporal effect of poly I:C on the expression of neuroinflammatory cytokines and microglial activation marker CD11b in the brain of WIS and WKY rats

Considering previous results which demonstrated an increase in pro-inflammatory cytokine expression 6hrs following poly I:C administration followed by an increase in the microglial activation marker CD11b 24hrs post injection in the frontal cortex and hippocampus of Sprague-Dawley rats, the current experiment aimed to assess the effect that poly I:C would have in two other rat strains. WIS and WKY rats were systemically administered poly I:C and euthanised 6hrs, 24hrs and 48hrs following injection. The mRNA response of IL-1β, IL-6, TNFα and CD11b were examined in the frontal cortex and hippocampus.

3.3.7.1 Systemic administration of poly I:C causes a time-dependent increase in the mRNA expression of IL-1β in the frontal cortex and hippocampus of WIS and WKY rats.

Two-way ANOVA showed an effect of poly I:C administration on mRNA expression of IL-1β in the frontal cortex (a) ($F_{(3, 32)}$= 22.38, P<0.0001) and hippocampus (b) ($F_{(3, 30)}$= 13.50, P<0.0001) of WIS and WKY rats. Post-hoc comparison revealed elevated levels of IL-1β mRNA 6hrs following poly I:C administration in both WIS (P<0.01) and WKY (P<0.01) rats in the frontal cortex. In the hippocampus, post-hoc comparison revealed elevated levels of IL-1β mRNA 6hrs following poly I:C administration in both WIS (P<0.05) and WKY (P<0.01) rats, when compared to vehicle treated controls. [Figure 3.9, n=3-6]

3.3.7.2 Systemic administration of poly I:C causes a time-dependent increase in the mRNA expression of IL-6 in the frontal cortex and hippocampus of WIS and WKY rats.

Two-way ANOVA showed an effect of strain ($F_{(1, 32)}$=8.90, P<0.01) and poly I:C administration ($F_{(3, 32)}$=46.65, P<0.0001) on mRNA expression of IL-6 in the frontal cortex (c) of WIS and WKY rats. Post-hoc comparison revealed elevated levels of IL-6 mRNA 6hrs following poly I:C administration in the both WIS (P<0.01) and WKY (P<0.01) rats, when compared to vehicle treated controls.

Two-way ANOVA showed an effect of interaction between strain and poly I:C administration ($F_{(3, 30)}$= 3.72, P<0.05) on the mRNA expression of IL-6 in the hippocampus (d) of WIS and WKY rats. Post-hoc comparison revealed elevated levels of IL-6 mRNA 6hrs following poly I:C administration in both WIS (P<0.01) and WKY (P<0.01) rats when compared to vehicle treated controls, however IL-6 expression was significantly greater in WKY rats at this time point (P<0.01). [Figure 3.9, n=3-6]

3.3.7.3 Systemic administration of poly I:C causes a time-dependent increase in the mRNA expression of TNFα in the frontal cortex and hippocampus of WIS and WKY rats.

Two-way ANOVA showed an effect of interaction between strain and poly I:C administration ($F_{(3, 32)}$=3.86, P<0.05) on mRNA expression of TNFα in the frontal cortex (e) of WIS and WKY rats. Post-hoc comparison revealed elevated levels of TNFα mRNA 6hrs following poly I:C administration in both WIS (P<0.01) and WKY (P<0.01) rats, when compared to vehicle treated controls. This increase in mRNA expression was significantly higher in WIS rats when compared to WKY rats (P<0.01).

Two-way ANOVA showed an effect of poly I:C administration ($F_{(3, 30)}$=51.69, P<0.0001) on mRNA expression of TNFα in the hippocampus (f) of WIS and WKY rats. Post-hoc comparison revealed elevated levels of TNFα mRNA 6hrs following poly I:C administration in both WIS (P<0.01) and WKY (P<0.01) rats when compared to vehicle treated controls. [Figure 3.9, n=3-6]

3.3.7.4 Systemic administration of poly I:C causes a time-dependent increase in the mRNA expression of CD11b in the frontal cortex and hippocampus of WIS and WKY rats.

Two-way ANOVA showed an effect of interaction between strain and poly I:C administration ($F_{(3, 32)}$=5.87, P<0.01) on mRNA expression of CD11b in the frontal cortex (g) of WIS and WKY rats. Post-hoc comparison revealed elevated levels of CD11b mRNA 24hrs following administration of poly I:C in WIS (P<0.01) but not in WKY rats when compared to vehicle treated controls. This remained elevated 48hrs following poly I:C administration in the WIS rats (P<0.01).

Two-way ANOVA showed an effect of strain ($F_{(1,31)}$= 7.20, P<0.05) and poly I:C administration ($F_{(3, 31)}$=15.45, P<0.0001) on mRNA expression of CD11b in the hippocampus (h) of WIS and WKY rats. Post-hoc comparison revealed a significant increase in the mRNA expression of CD11b 24hrs following poly I:C administration in both WIS (P<0.01) and WKY (P<0.05) rats when compared to vehicle treated controls. [Figure 3.9, n=3-6]

Figure 3.9 – Differential expression of pro-inflammatory cytokines and microglial activation markers in WKY and WIS rats following poly I:C administration

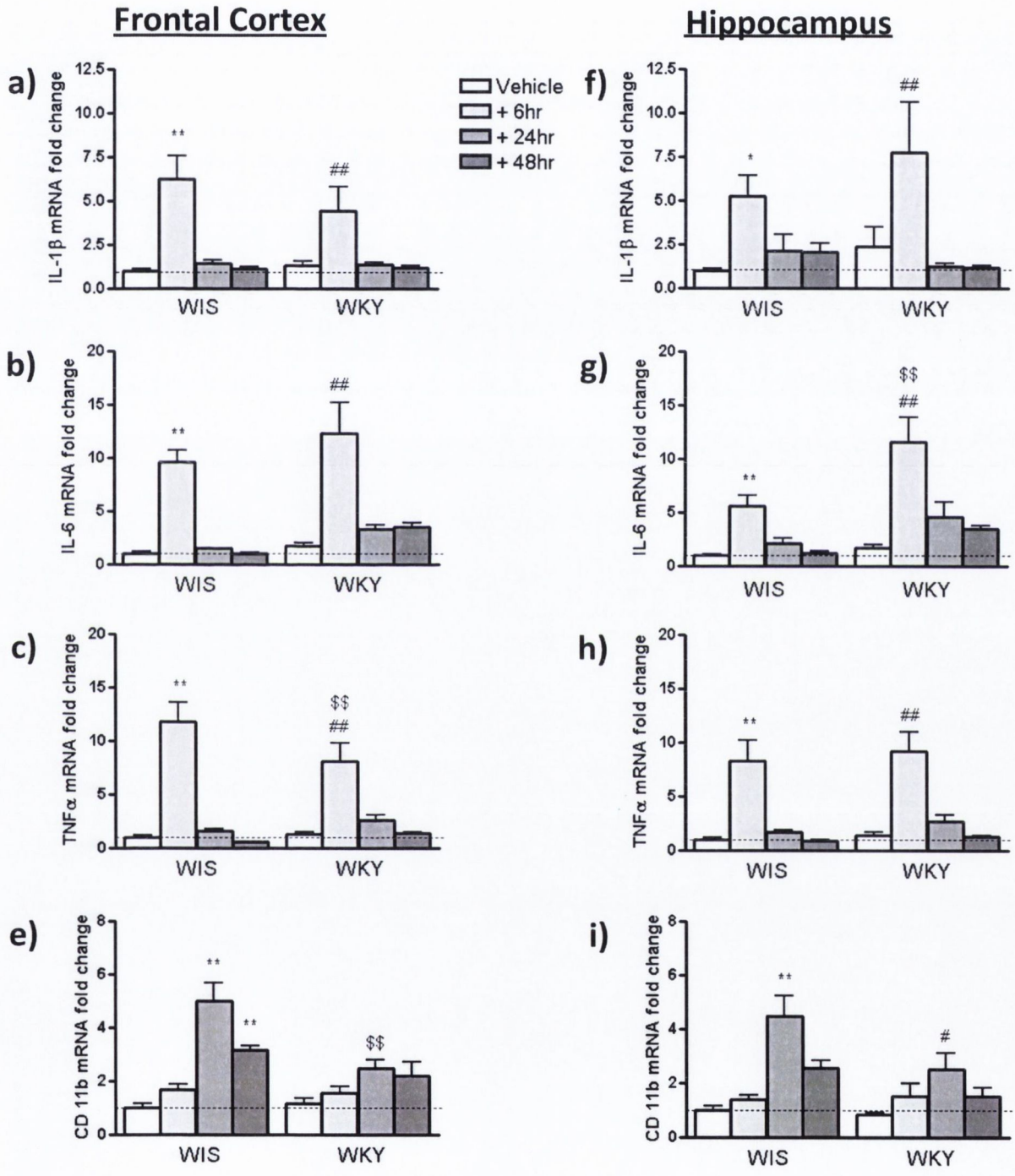

Figure 3.9 – Differential expression of pro-inflammatory cytokines and microglial activation markers in WKY and WIS rats following poly I:C administration

Poly I:C (6mg/kg) increased the expression of pro-inflammatory cytokines of WIS and WKY rats 6hrs post injection in the frontal cortex and hippocampus. Expression of IL-6 in the hippocampus of WKY rats was greater than that of WIS rats (d), while the expression of TNFα was lower in the frontal cortex of WKY rats. Similarly expression of microglial marker CD11b was lower in the frontal cortex of WKY rats 24hrs following injection. Data expressed as mean ± SEM (n=3-6). ** $P<0.01$, * $P<0.05$ vs. Vehicle treated WIS, ## $P<0.01$, # $P<0.05$ vs. Vehicle treated WKY, $$ $P<0.01$ vs. WIS time point equivalent.

3.3.8 The temporal effect of systemic poly I:C on the expression of BDNF and its receptor TrkB in the brains of WIS and WKY rats

Considering previous results, which demonstrated a prolonged reduction in the mRNA expression of BDNF and its cognate receptor in the frontal cortex and hippocampus of Sprague-Dawley rats, the current experiment aimed to assess the temporal response to systemic poly I:C in two other rat strains. WIS and WKY rats were systemically administered poly I:C (6mg/kg) and euthanised 6hrs, 24hrs or 48hrs following injection. The mRNA expression of BDNF and TrkB was measured in the frontal cortex and hippocampus.

3.3.8.1 Systemic poly I:C administration leads to a time-dependent decrease in the mRNA expression of BDNF in the frontal cortex and hippocampus of WIS and WKY rats

Two-way ANOVA showed an effect of poly I:C administration ($F_{(3, 32)}$=10.88, P<0.0001) on mRNA expression of BDNF in the frontal cortex (a) of WIS and WKY rats. Post-hoc comparison revealed reduced levels of BDNF mRNA 6hrs following poly I:C administration in WKY (P<0.05) but not WIS rats when compared to vehicle treated controls.

Two-way ANOVA showed an effect of interaction between strain and poly I:C administration ($F_{(3, 31)}$= 4.24, P<0.05) on mRNA expression of BDNF in the hippocampus (b) of WIS and WKY rats. Post-hoc comparison revealed reduced levels of BDNF mRNA 6 hrs following poly I:C administration in WKY (P<0.01) but not WIS rats when compared to vehicle treated controls. 24hrs following poly I:C administration mRNA levels in WKY were not significantly reduced, but mRNA in WIS rats were reduced (P<0.05). [Figure 3.10, n=3-6]

3.3.8.2 Systemic poly I:C administration leads to a time-dependent decrease in the mRNA expression of TrkB in the frontal cortex of WIS rats.

Two-way ANOVA showed an effect of strain ($F_{(1,32)}$= 9.10, P<0.01) and poly I:C administration ($F_{(3, 32)}$=7.89, P<0.001) on mRNA expression of TRKb in the frontal cortex (c) of WIS and WKY rats. Post-hoc comparison revealed reduced levels of TRKb mRNA 24hrs following poly I:C administration in WIS (P<0.01) but not WKY rats when compared to vehicle treated controls.

Two-way ANOVA showed no effect of strain or poly I:C administration on the mRNA expression of TRKb in the hippocampus (d) of WIS and WKY rats. [Figure 3.10, n=3-6]

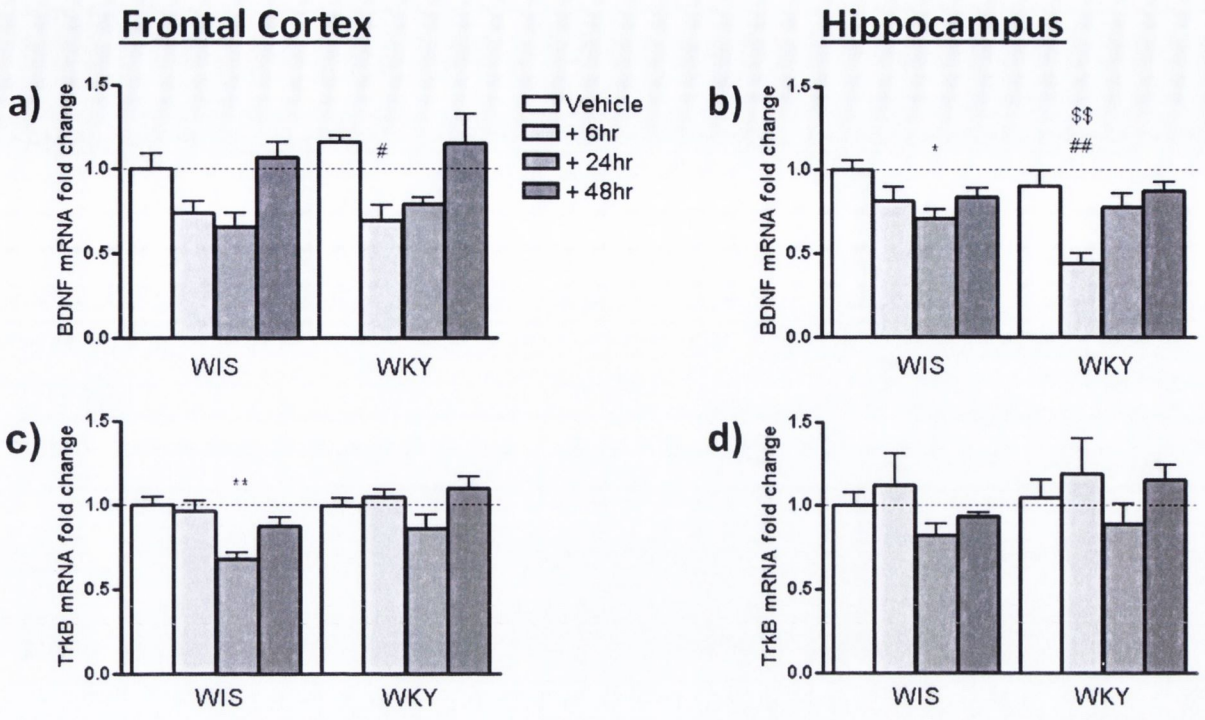

Figure 3.10 – Systemic poly I:C administration reduced BDNF and TrkB expression in the brain of WIS and WKY rats

Poly I:C (6mg/kg) reduced expression of BDNF in both WIS and WKY rats in the hippocampus (b) and only WKY in the frontal cortex (a). Expression of TrkB was only reduced in the frontal cortex of WIS rats 24hrs following injection (c). Data expressed as mean ± SEM (n=3-6). ** P<0.01, * P<0.05 vs. Vehicle treated WIS, ## P<0.01, # P<0.05 vs. Vehicle treated WKY, $$ P<0.01 vs. WIS time point equivalent.

3.3.9 The temporal effect of systemic poly I:C on the expression of IDO and the production of kynurenine in the brain of WIS and WKY rats

Considering previous results which reported a significant increase in the mRNA expression of IDO coupled with increases in kynurenine and tryptophan in the frontal cortex and hippocampus of Sprague-Dawley rats, the current experiment aimed to assess the temporal response to systemic poly I:C in two other rat strains. WIS and WKY rats were administered poly I:C (6mg/kg) i.p. and euthanised 6hrs, 24hrs or 48hrs following injection. The mRNA expression of IDO and concentrations of Kyn, Tryp and the Kyn:Tryp ratio were measureded in the frontal cortex and hippocampus.

3.3.9.1 Systemic administration of poly I:C causes an increase in the mRNA expression of IDO in the frontal cortex of WIS but not WKY rats.

Two-way ANOVA showed an effect of interaction between strain and poly I:C administration ($F_{(3, 32)}$=5.38, P<0.01) in the frontal cortex and an effect of poly I:C administration ($F_{(3, 31)}$=3.11, P<0.05) in the hippocampus, on mRNA expression of IDO in WIS and WKY rats. Post-hoc comparison revealed elevated levels of IDO mRNA 6hrs following poly I:C administration in WIS (P<0.01) but not WKY rats when compared to vehicle treated controls. The difference between strains 6hrs following injection was significant (P<0.01). In the hippocampus, post-hoc comparison showed no changes in the levels of IDO mRNA in either strain of rat following poly I:C administration when compared to vehicle treated controls. [Figure 3.11, n=3-6]

3.3.9.2 Systemic administration of poly I:C causes a time-dependent increase in the concentration of tryptophan in frontal cortex only, in WIS and WKY rats

Two-way ANOVA showed an effect of interaction between strain and poly I:C administration ($F_{(3, 32)}$=4.15, P<0.05) on the concentration of tryptophan in the fontal cortex of WIS or WKY rats (c). Post-hoc comparison revealed an increase in tryptophan in the frontal cortex of WIS rats 6hrs following poly I:C administration (P<0.05) when compared to vehicle treated controls. WKY rats displayed an increase in tryptophan 48hrs following poly I:C administration (P<0.05) when compared to vehicle treated controls. The increased

concentrations of tryptophan were also greater than those of WIS rats at the same time point ($P<0.05$). ANOVA showed no effect of poly I:C on the concentration of tryptophan in the hippocampus of WIS and WKY rats (d). [Figure 3.11, n=4-6]

3.3.9.3 Systemic administration of poly I:C does not cause a change in the concentration of kynurenine in the frontal cortex or hippocampus of WIS or WKY rats

Two-way ANOVA showed no effect of poly I:C administration on the kynurenine concentration in the frontal cortex (e) or hippocampus (f) of WIS and WKY rats. [Figure 3.11, n= 4-6]

3.3.9.4 Systemic administration of poly I:C does not cause a change in the kynurenine:tryptophan ratio in the frontal cortex or hippocampus of WIS or WKY rats

Two-way ANOVA showed no effect of poly I:C administration on the ratio of kynurenine to tryptophan in the frontal cortex and hippocampus of WIS and WKY rats. [Figure 3.11, n= 4-6]

Figure 3.11 – Systemic poly I:C administration induces IDO expression but does not influence the kynurenine:tryptophan ratio in the frontal cortex and hippocampus

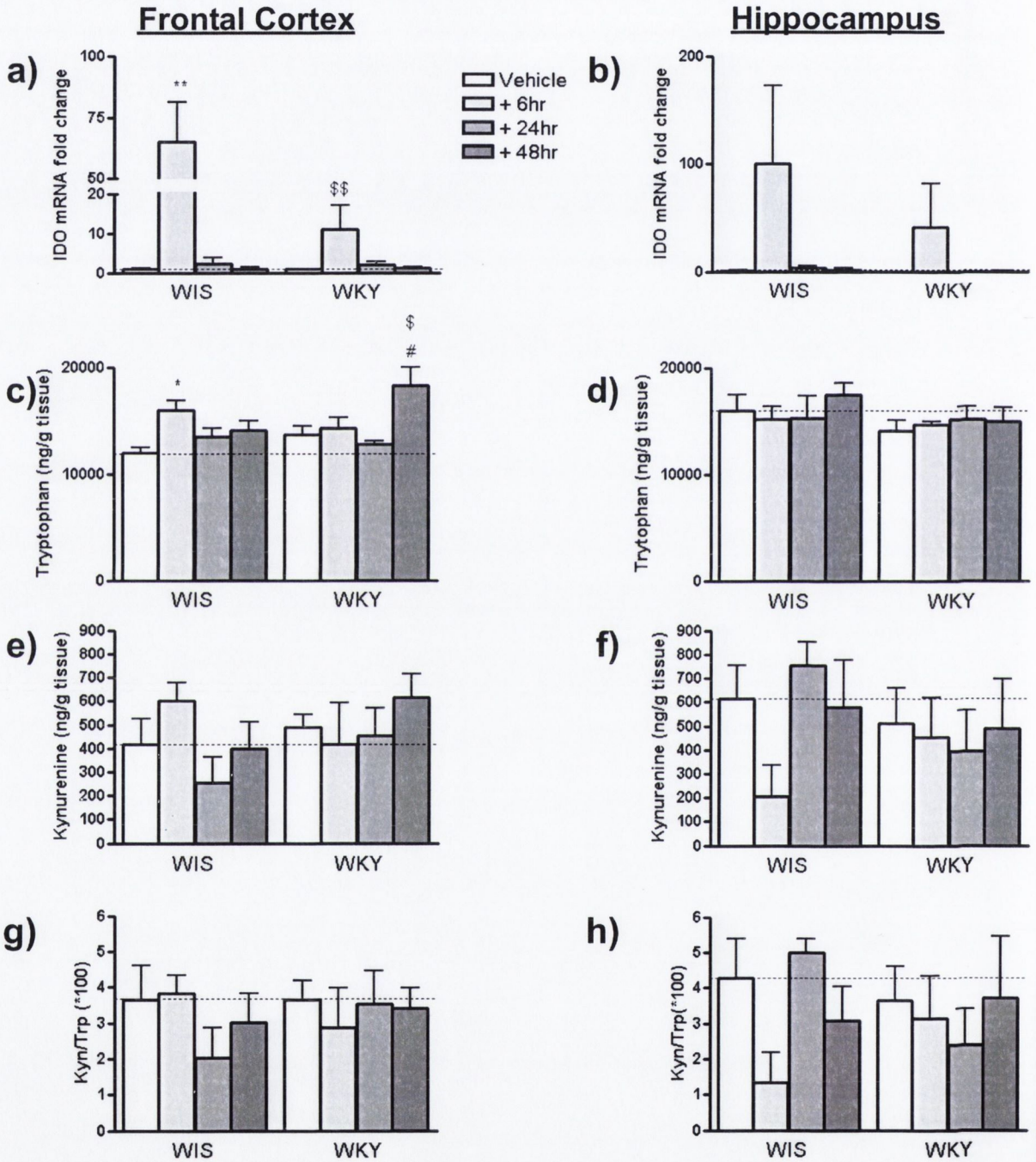

Figure 3.11 – Systemic poly I:C administration induces IDO expression but does not influence the kynurenine:tryptophan ratio in the frontal cortex and hippocampus

Poly I:C (6mg/kg) increased the expression of IDO in the frontal cortex in WIS but not WKY rats 6hrs following administration (a). Significant increases in the amount of tryptophan in the frontal cortex were obtained 6hrs post injection in WIS and 48hrs in WKY (c), however this did not lead to any change in the Kyn:Tryp ratio (g). No significant changes were obtained in the hippocampus in either strain (b, d, f, h). Data expressed as mean ± SEM (n=4-6). ** $P<0.01$, * $P<0.05$ vs. Vehicle treated WIS, # $P<0.05$ vs. Vehicle treated WKY, $$ $P<0.01$, $ $P<0.05$ vs. WIS time point equivalent.

3.3.10 The temporal effect of poly I:C on the concentrations of 5-HT and 5HIAA in the frontal cortex and hippocampus of WIS and WKY rats

Considering previous results which reported an increase in the 5-HT metabolite 5HIAA in the frontal cortex and hippocampus of Sprague-Dawley rats, the current experiment aimed to assess the temporal response to systemic poly I:C in two other rat strains. WIS and WKY rats were administered poly I:C (6mg/kg) i.p. and euthanised 6hrs, 24hrs or 48hrs following injection. Concentrations of 5-HT and 5HIAA were quantified in the frontal cortex and hippocampus.

3.3.10.1 Systemic administration of poly I:C provokes an increase in 5-HT concentrations in WKY rats in the hippocampus

Two-way ANOVA showed no effect of poly I:C administration or strain on 5-HT concentration in the frontal cortex (a) or hippocampus of WIS and WKY rats. [Figure 3.12, n= 4-6]

3.3.10.2 Systemic administration of poly I:C does not affect 5HIAA concentrations in the frontal cortex of WIS and WKY rats

Two-way ANOVA showed no effect of poly I:C or strain on the concentration of 5HIAA in the frontal cortex (c) or hippocampus (d) of WIS and WKY rats. [Figure 3.12, n= 4-6]

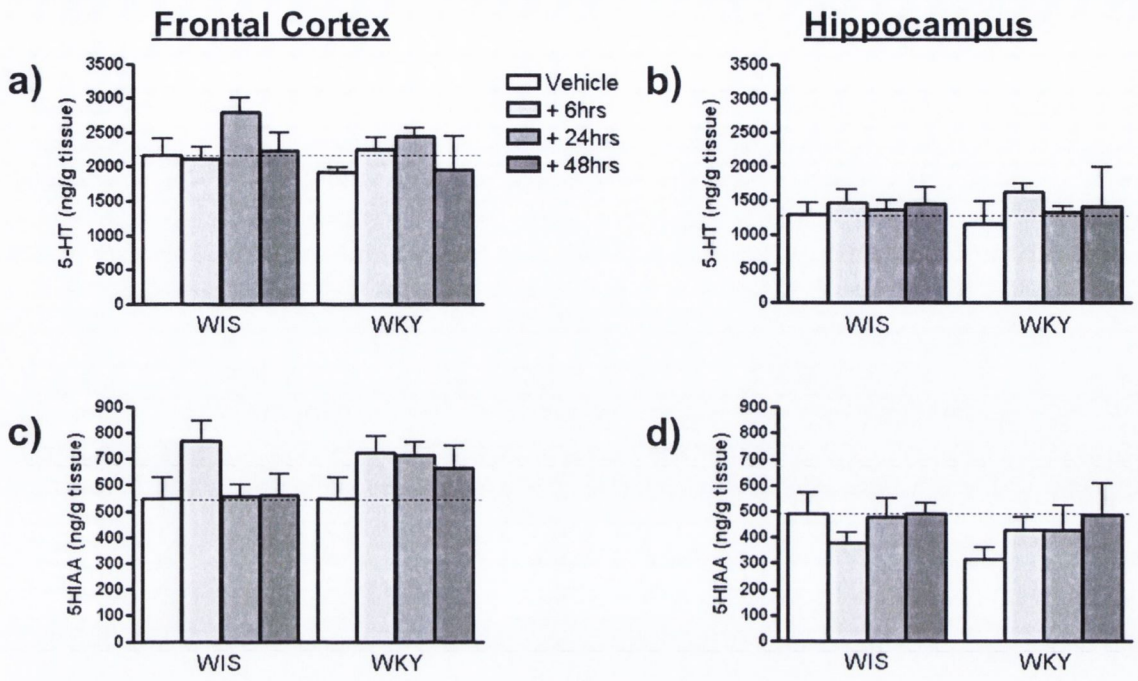

Figure 3.12 – Systemic poly I:C administration does not affect concentrations of 5-HT or 5HIAA in the frontal cortex or hippocampus of WIS and WKY rats

No changes were observed in the concentrations of 5-HT or 5HIAA in the frontal cortex or hippocampus of WIS and WKY rats following poly I:C (6mg/kg) adminsitration. Data expressed as mean ± SEM (n=4-6).

3.4 Discussion

In the present study poly I:C was used to study inflammation-induced depression and anxiety behaviours in rats, and determine the impact of this inflammatory challenge on expression of the hippocampal BDNF neurotrophin system and on the kynurenine/serotonin axis; two systems implicated in the pathogenesis of depression (Christmas et al., 2011, Maes et al., 2011, Muller and Schwarz, 2007, Nibuya et al., 1995, Raison et al., 2009, Saarelainen et al., 2003).

3.4.1 Poly I:C-induced depressive and anxiety related-behaviours in Sprague-Dawley rats persist beyond acute sickness behaviour

We have shown that the initial acute sickness behaviour that develops following poly I:C administration had resolved within 24hr of administration, by contrast, a depressive-like anhedonic response persisted for up to 72hr. These findings illustrate that in response to poly I:C, animals develop behavioural deficits characteristic of a depressive phenotype. These data tally with a recent study where LPS-induced sickness behaviour and depression-related behaviours in mice could be separated in a temporal fashion. Specifically, depression-related behaviour in the mouse TST and FST induced by LPS was evident following the resolution of sickness behaviour (O'Connor et al., 2009c).

Similarly, anxiety-like behaviour in the open-field was observed 24hr following poly I:C administration, a time-point at which sickness behaviour was no longer evident. The open field represents a novel environment which was subdivided into 2 zones, a small centre or inner zone and a larger outer zone along the wall of the arena. Reduced movement and exploration in the inner zone of the open field, is indicative of anxiety-like behaviour in rodents (Stanford, 2007). Those animals that had been exposed to poly I:C showed reduced movement in the inner zone of the open field when compared to vehicle treated controls. Moreover, the number of transitions made between the inner and outer zone was also significantly reduced in response to poly I:C administration. The open field activity was measured at 24hr post poly I:C, a time at which locomotor activity in the home cage had returned to normal, thereby dissociating sickness behaviour from anxiety behaviour.

3.4.2 Poly I:C induces a neuroinflammatory response in Sprague-Dawley rats

Poly I:C-induced inflammatory responses were assessed in the frontal cortex and the hippocampus, two brain regions that constitute part of the cortical-limbic neurocircuitry that had been implicated in depression (Piser, 2010). Increased expression of the inflammatory cytokines IL-1β, IL-6 and TNFα were detected following poly I:C administration, with maximal expression found at 6hr, a time at which sickness behaviour was also most apparent. These cytokines have previously been shown to be involved in the development of LPS-induced sickness behaviour (Bluthe et al., 2000, Harden et al., 2006), and have also been detected centrally in response to poly I:C administration (Cunningham et al., 2007, Konat et al., 2009). A role for these cytokines in the development of anxiety-like behaviours in rodents has been extensively characterised. For example, when IL-1β is administered either intraperitoneally or intracerebroventricularly, it induces anxiogenic-like effects similar to those found in the present study (Connor et al., 1998, Dunn and Swiergiel, 2005, Swiergiel and Dunn, 2006). IL-6 has also been shown to be involved in the development of anxiety related behaviours (Armario et al., 1998a, Butterweck et al., 2003), with IL-6 (-/-) mice showing attenuated stress-induced depressive behaviours (Chourbaji et al., 2006). A role for TNFα in the development of depressive-like symptoms has also been proposed using TNFR (-/-) mice (Simen et al., 2006). In their study, Simen et al., demonstrated that deletion of this receptor induces antidepressant-like effects in the forced swim test and the sucrose preference test, even in the absence of malaise. Overall we suggest that the early up-regulation of inflammatory cytokines observed in the present study is likely to mediate the behavioural effects of poly I:C administration.

Microglia are one of the major regulators of the CNS inflammatory response, possessing the ability to produce and secrete a range of inflammatory mediators including inflammatory cytokines, chemokines and reactive oxygen and nitrogen species (Block et al., 2007). Consequently we measured expression of the microglial activation marker CD11b in order to track microglial activation in this animal model, and observed a robust up-regulation of CD11b in both the hippocampus and cortex following poly I:C administration. Considering the established role of microglia as a major producer of inflammatory cytokines in the

CNS (Hanisch, 2002, Rock et al., 2004, Rock and Peterson, 2006, Smith et al., 2012, Garden and Moller, 2006), the ability of poly I:C to induce microglial activation was to be expected. However, the time-line of CD11b up-regulation relative to the time-line of inflammatory cytokine expression observed was somewhat surprising. Specifically, expression of the microglial activation marker CD11b was not observed until 24-48hr following poly I:C administration significantly lagging behind inflammatory cytokine expression that peaked 6h post-poly I:C administration. Thus in response to poly I:C, inflammatory cytokine production represents an earlier marker of microglial activation than the routinely used phenotypic microglial activation marker CD11b. This data is consistent with recent findings from our laboratory where CD11b expression in the hypothalamus was preceded by inflammatory cytokine expression following an *in vivo* challenge with LPS (Chapter 5). Based on these findings it is likely that inflammatory cytokines produced by poly I:C or LPS-stimulated microglia are responsible for the subsequent induction of CD11b expression. It also must be noted that CD11b is not exclusively expressed by microglia and is also found on peripheral immune cells (Kettenmann et al., 2011). As previous studies have demonstrated that these peripheral immune cells can infiltrate the brain in response to an inflammatory challenge (Wohleb et al., 2012, Wohleb et al., 2011a), one cannot rule out that at least some of the cells expressing CD11b in the current study may constitute another cell type. In any case, considering previous studies demonstrating that treatment with the microglial inhibitor minocycline attenuates sickness behaviour and depressive-like behaviours induced by LPS (Henry et al., 2008, O'Connor et al., 2009c, Tynan et al., 2010, Hinwood et al., 2011), it is likely that activated microglia play a role in the depressive and anxiety–like behavioural effects of poly I:C. Furthermore one cannot rule out a possible role for astrocytes in the observed changes in cytokines and/or kynurenine pathway metabolites within the brain prior to microglial activation. Investigation of astrocytic markers and kynurenine pathway enzymes known to be localised to astrocytes, e.g. KAT, could allow for further confirmation of the role of microglia in the observed results.

3.4.3 Poly I:C decreases the expression of BDNF and its receptor TrkB in Sprague-Dawley rats

BDNF is a member of the neurotrophin family that is integral to neurogenesis, neuronal growth, maintenance and synaptic plasticity, with most of these effects

mediated by its tyrosine kinase receptor, TrkB (Tapia-Arancibia et al., 2004). Here we observed that poly I:C treatment inhibited expression of both BDNF and TrkB in hippocampus and cortex. These findings are consistent with previous studies indicating a role for inflammation in suppressing BDNF and TrkB expression. For instance, studies have demonstrated that bacterial LPS inhibited expression of BDNF and TrkB in rat brain (Guan and Fang, 2006, Tanaka et al., 2006). IL-1β is thought to be one of the main cytokines to compromise the BDNF pathway. Administration of IL-1β has been shown to reduce hippocampal BDNF mRNA expression (Lapchak et al., 1993), and the IL-1 receptor antagonist has been shown to reverse stress-related reductions in BDNF (Barrientos et al., 2003). Alterations in BDNF expression and function are central to the 'neurotrophin hypothesis of depression' which suggests that reduced BDNF can lead to reduced neuronal protection and neurogenesis, and ultimately to the development of depressive symptoms (Martinowich et al., 2007). Thus the decreased expression of BDNF that occurs in response to poly I:C may therefore be related to the depressive and anxiety-like behaviours observed in Sprague-Dawley rats.

3.4.4 Poly I:C induces central IDO expression and kynurenine production without depleting tryptophan or serotonin in Sprague-Dawley rats

Tryptophan is an essential amino acid required for serotonin synthesis, however tryptophan is also degraded through the kynurenine pathway and it has been suggested that inflammation-related induction of IDO and kynurenine pathway activation may lead to depressive symptomatology via actions on the serotonergic and glutamatergic neurotransmitter systems (Muller and Schwarz, 2007). Here we demonstrate that poly I:C increased IDO expression in the hippocampus and frontal cortex which was followed by increased concentrations of both tryptophan, and its metabolite kynurenine. The increase in brain tryptophan concentrations observed in tandem with increased kynurenine concentrations following poly I:C administration is somewhat counterintuitive, and certainly argues against the hypothesis that IDO activation depletes tryptophan availability for serotonin synthesis. This suggests that reduced availability of tryptophan for serotonin synthesis is not necessarily required for the development of behavioural changes in the present study. In addition to the ability of central IDO expression to induce the observed increase in kynurenine concentrations in

the brain the possibility exists that uptake of kynurenine from the circulation also contributed to the poly I:C-induced increase in central kynurenine concentrations. In this regard it is known that kynurenine can pass through the blood brain barrier (Fukui et al., 1991), and we have observed that poly I:C induces a significant increase in plasma kynurenine concentrations 24-72hr post-administration (Unpublished data).

Our finding that brain tryptophan concentrations are increased by poly I:C is supported by previous studies where administration of inflammatory cytokines or LPS also induced an increase in brain tryptophan (Heyes et al., 1989, Dunn and Welch, 1991, Wang and Dunn, 1998, Ando and Dunn, 1999). Whilst brain serotonin concentrations were not altered by poly I:C administration a transient increase in the serotonin metabolite 5-HIAA was observed in both cortex and hippocampus following poly I:C. This finding is consistent with an acute increase in serotonin metabolism, and is consistent with a number of previous studies where inflammatory cytokines and LPS provoke a transient increase in central 5-HIAA concentrations (Heyes et al., 1989, Dunn and Welch, 1991). In fact, increased cerebral tryptophan concentrations and serotonin metabolism have previously been reported in response to poly I:C in mice (Dunn and Vickers, 1994). Despite these findings suggesting a lack of a role for IDO-induced serotonergic dysfunction in inflammation-related depressive symptoms, a role for IDO and kynurenine pathway activation in the development of depressive symptoms in animal models has previously been reported. Specifically, it has been demonstrated that pharmacological blockade of IDO can reverse the depressive phenotype observed in the mouse FST and TST following exposure to an acute LPS challenge, or in response to Bacille Calmette-Guérin (BCG) inoculation (O'Connor et al., 2009a), and that IDO deficient mice were resistant to BCG-induced depressive-like behaviour (O'Connor et al., 2009b). In addition, when kynurenine is administered to naive mice it has been shown to directly induce a depressive phenotype (O'Connor et al., 2009c). Thus the possibility exists that kynurenine or one of its downstream metabolites could be mediating poly I:C-induced depressive or anxiety related behaviours potentially via an action on the glutamatergic system (Muller and Schwarz, 2007).

3.4.5 Poly I:C induces behavioural and expression changes in neuroinflammatory and transmitter markers in WIS and WKY rats

Poly I:C induced depressive and anxiety related-behaviours are strain dependent, with both WIS and WKY rats displaying reduced performance in the HCA paradigm but only WIS rats showing a reduction in hedonic behaviour in the saccharin preference test. WIS rats also displayed a prolonged reduction in body weight when compared to WKY rats. Investigations into the expression of neuroinflammatory markers showed that, as with the Sprague-Dawley rats, WIS and WKY rats had increases in IL-1β, IL-6 and TNFα 6hrs following poly I:C administration. Investigation into the expression of the microglial marker CD11b revealed that WKY rats showed a lower level of induction 24hrs following poly I:C administration. These neuroinflammatory changes were accompanied by region specific reductions in the expression of BDNF in both WIS and WKY but a reduction in the expression of its cognate receptor, TrkB, was evident in the frontal cortex of WIS rats only. Investigation of a role for the kynurenine pathway revealed reduced expression of IDO in WKY rats when compared to WIS rats. There was however no changes in the amount of kyn or the kyn:tryp ratio in either strain suggesting that the kynurenine pathway is not activated following poly I:C administration in WIS or WKY rats. Monoamine analysis revealed no changes in 5-HT or 5HIAA in the frontal cortex or hippocampus of WKY or WIS rats.

3.4.6 Strain dependent changes in sickness and depressive-like behaviour following poly I:C administration to WIS and WKY rats

Poly I:C has been reported to induce sickness behaviours (Cunningham et al., 2007) and similarly we have shown that both WIS and WKY rats displayed sickness behaviours following poly I:C administration. Home cage activity and rearing was suppressed 6hrs following poly I:C administration and food consumption and body weight decreased 24hrs following poly I:C administration when compared to vehicle treated controls. Interestingly, WIS rats displayed a prolonged suppression of body weight and food consumption, whereas the WKY rats did not, suggesting that WIS rats may exhibit an increased sensitivity to poly I:C induced sickness behaviour when compared to WKY or Sprague-Dawley rats. The age of the rats at the time of poly I:C administration could potentially affect the duration of sickness behaviour, with both WIS and WKY rats older than

Sprague-Dawley rats at the time of injection. In a recent study, McLinden and co-workers reported that aged mice treated with poly I:C displayed an exacerbated and prolonged sickness behaviour when compared to younger mice up to 48hrs following poly I:C administration (McLinden et al., 2012). This could possibly account for the prolonged sickness behaviour observed in WIS rats when compared to the duration of sickness behaviour in the younger Sprague-Dawley rats, but not when compared to WKY rats of the same age.

This prolonged sickness behaviour was matched by the prolonged suppression of preference for a 0.1% saccharin solution in WIS but not in WKY rats, indicative of anhedonic behaviour. WIS rats displayed a significant reduction in preference for the saccharin solution 24hrs and 48hrs following poly I:C administration, however because of the prolonged sickness behaviour, the observed anhedonic behaviour cannot be attributed solely to a depressive phenotype. By contrast, WKY rats did not display a reduction in their preference for saccharin at either time point yet their preference to saccharin solution was lower under control conditions. WKY rats preferentially drank from a bottle on one side of the cage rather than seek out the saccharin solution, in line with what has been described by others in mice (Bachmanov et al., 2002). The lack of hedonic response of WKY rats following poly I:C administration may be interpreted as as a "flooring effect", where innate anhedonic behaviour cannot reduce any further, diminishing any additional effect of poly I:C administration (Yamada et al., 2013, Malkesman et al., 2005). Conversely the WIS control strain possessed a high preference for a saccharin solution and displayed no preference for the side of the cage on which the bottles were placed under control conditions. These findings coupled with previous reports of anxious and depressive phenotypes under basal conditions (Durand et al., 1999, Rittenhouse et al., 2002, Paré, 1994) suggest that WKY rats are not ideal for generation of depressive-like behaviours following immune challenge on account of the pre-existing phenotype.

3.4.7 WKY rats show increased hippocampal IL-6 expression and reduced expression of TNFα and CD11b following poly I:C administration compared to the WIS strain

Previous studies have reported WKY rats to respond to immunological challenge although evidence for differential responses to related strains is sparse. Abe et al. 2001 showed that peripheral administration of LPS (1mg/kg) induced IL-1β

expression in the heart of WKY rats when compared to spontaneous hypertensive rats (Abe et al., 2001). Similarly in this study, IL-1β was increased in response to poly I:C in frontal cortex and hippocampus yet no strain differences were observed.

There was a reduction in the expression of CD11b mRNA in response to poly I:C in the frontal cortex and hippocampus of WKY rats 24hr following poly I:C administration when compared to the WIS strain. These results indicate a lower level of microglial activation in the brain following poly I:C administration to WKY rats when compared to WIS controls. A reduced microglial response to poly I:C in WKY rats might indicate a greater degree of suppression of inflammatory responses on foot of elevated circulating glucocorticoids. In agreement with this, recent unpublished findings from the laboratory indicate that WKY rats display enhanced glucocorticoid sensitivity as measured by the activation of GR inducible genes including glucocorticoid-induced leucine zipper (GILZ) and serine/threonine-protein kinase (SGK-1) following exposure to psychological stress when compared to the WIS control strain. It is understood that glucocorticoids control the activation of innate immune cells and reduce their pro-inflammatory profile via induction of anti-inflammatory genes and inhibition of pro-inflammatory signalling (Curtin et al., 2009a, Baschant and Tuckermann, 2010). It is feasible that glucocorticoid hypersensitivity observed in the WKY rats could also influence the activation of microglia within the brain. A potential scenario may consist of activation of the inflammatory response system by poly I:C within both WIS and WKY rats, which in turn stimulates the HPA-axis, increasing circulating levels of corticosterone. GR signalling within the WKY rats is more sensitive to circulating steroids whereby an anti-inflammatory phenotype emerges when compared to WIS rats reflected by reduced microglial activation. In support, poly I:C related increases in mRNA expression of TNFα in the frontal cortex of WKY rats was lower when compared to WIS controls albeit similar levels of expression between both strains were obtained in the hippocampus. TNFα contributes to microglial activation (Kuno et al., 2005) and a deficiency of TNF receptors have been reported to suppress microglial activation (Sriram et al., 2006). Thus the reduction in cortical TNFα expression may be a plausible mechanism whereby CD11b expression is reduced in WKY rats. In the hippocampus, where TNFα expression following poly I:C administration was observed to be similar to that of WIS control rats, alternative mechanisms may

account for the reduction in CD11b expression and microglial activation obtained in this region.

Lower expression of CD-11b and reduced microglial activation following poly I:C administration in WKY rats might suggest that microglia are in a pre-existing activated state and as such are less responsive to additional stimulation. Given that there are no differences in central CD-11b expression between WKY and WIS strains at baseline and WKY rats show a reduced response following poly I:C administration, it seems unlikely that WKY rats exhibit an increase in microglial activation. At the expression level, the results indicate that microglial activation is in fact reduced in response to immunological challenge in the WKY rat. Further analysis of the activation state of microglia in the WKY rat is warranted and in particular measurement of protein to verify changes observed at the gene expression level.

By contrast to the aforementioned attenuated increase in TNFα and CD11b expression in WKY compared to WIS rats following poly I:C administration, an increase in IL-6 expression was observed in the hippocampus in WKY relative to the changes observed in WIS rats. The effect was regionally specific to the hippocampus as no such change was observed in the cortex. IL-6 may be produced by both microglia and astrocytes in response to immune stimuli and other inflammatory cytokines (Van Wagoner et al., 1999, Sawada et al., 1992, Aloisi et al., 1992) although astrocytes are the predominant producer of IL-6 within the brain (Van Wagoner and Benveniste, 1999). Thus in the presence of reduced activation of microglia, increased astrocytic function may account for the increased expression of IL-6 observed although additional work is necessary to verify this. Of interest is that sustained production of IL-6 by astrocytes in transgenic mice has been associated with reduced hippocampal neurogenesis in mice (Vallières et al., 2002) linking the production of this cytokine to synaptic plasticity deficits within the hippocampus which are characteristic of those changes observed following stress and in depression. A role for IL-6 in the depression or anxiety related behaviours or in cytoarchitectural changes within the cortex or hippocampus of the WKY rat strain has not been explored to date.

3.4.8 Differential changes in the expression of BDNF and TrkB in WKY and WIS rats following poly I:C administration

Under unstimulated conditions the expression of BDNF in the frontal cortex and hippocampus was comparable in both WIS and WKY strains in agreement with previous observations reported between WKY and the spontaneous hypertensive rat (SHR) (Lee et al., 2004). However lower BDNF protein levels have been reported in the hippocampus and frontal cortex of WKY compared to WIS rats and serum BDNF concentrations have been reported to be reduced following exposure to chronic stress when compared to the Sprague-Dawley strain (O'Mahony et al., 2011, Hauser et al., 2011, Vinod et al., 2012). In the current study, exposure to poly I:C provoked a reduction in BDNF expression in WKY rats in the frontal cortex and hippocampus and in the hippocampus only of WIS rats. Expression of BNDF was reduced further in WKY relative to the changes observed in WIS rats possibly relating to the greater increase in hippocampal IL-6 expression described earlier. It is of interest that changes in hippocampal BDNF expression in WKY rats may be reversed by antidepressant treatments. WKY rats treated with curcumin, the principal ingredient found in turmeric which possesses antidepressant-like effects, had increased hippocampal BDNF protein coupled with an antidepressant-related reduction in immobility behaviour in the FST when compared to vehicle administered controls (Hurley et al., 2013).

A previous investigation of the BDNF receptor, TrkB, reported reduced expression of the receptor in lung homogenate of WKY relative to SHR rats (Ricci et al., 2004), however investigations into TrkB protein levels in the brain are lacking. We found no change in the expression of TrkB mRNA in WKY rats following poly I:C administration. By contrast expression of TrkB in the frontal cortex of WIS rats was reduced 24hrs following poly I:C similar to the changes previously observed in Sprague-Dawley rats. Reduced expression of BDNF and/or TrkB following poly I:C treatment could possibly account for persistent changes in behaviour in the WIS relative to the WKY strain. Similar relative expression under non-stimulated conditions however indicate that the hippocampal expression of these markers does not readily associate with the depressive and anxiety-like behaviours in the WKY strain.

3.4.9 Poly I:C does not lead to a functional activation of the kynurenine pathway or changes in 5-HT metabolism in the brain of WKY and WIS rats

Poly I:C administration strongly induced expression of the tryptophan degrading enzyme IDO in the frontal cortex with a similar non–significant trend in the hippocampus of WIS rats. A similar increase was not apparent in WKY in line with reduced expression of CD11b and microglial activation relative to changes observed in WIS rats. There were however no differences in kynurenine concentrations or Kyn:Tryp ratio obtained in either strain despite an increase in tryptophan following poly I:C administration indicating a lack of functional activation of kynurenine pathway in the brain in either strain. This lack of kynurenine pathway activation in the brains of WIS and WKY rats differed from that observed in Sprague-Dawley rats following poly I:C administration.

Deficits in 5-HT transmission have been associated with symptoms in depressed patients. Reports of lower basal levels of 5-HT in the frontal cortex and hippocampus of WKY compared to WIS rats have implied that the WKY rat strain may be a useful analogue of reduced 5-HT function which may underlie some depression related symptoms (Scholl et al., 2010, Getachew et al., 2010, Maes et al., 2011). While not significant, our current data in the WKY rat shows a similar trend towards lower basal levels in the hippocampus. WKY rats have been previously reported to have lower levels of 5-HT accompanied by higher levels of 5HIAA following stress in the frontal cortex when compared to WIS rats (De La Garza and Mahoney, 2004). In this study no changes in 5-HT or 5-HIAA were observed in the frontal cortex or hippocampus of either WIS or WKY rats. Moreover previous research with WKY rats has reported no effect of chronic stress exposure on the concentrations of 5-HT and 5HIAA in the frontal cortex or hippocampus of WKY rats (O'Mahony et al., 2011). The changes obtained in Sprague-Dawley rats were however different whereby challenge with poly I:C provoked an increase in 5-HIAA and 5-HT metabolism indicating an increased sensitivity of Sprague-Dawley rats to the immune stimulus.

3.4.10 Conclusion

In this study we demonstrated that administration of the viral mimetic poly I:C to rats induced symptoms of depression and anxiety in Sprague Dawley rats that could be distinguished temporally from acute sickness behaviour. The initial

sickness behaviours seem to correlate with a robust increase in poly I:C-induced cytokine expression, whereas the subsequent anxiety and depressive-like behaviours observed may be related to cytokine-induced changes in tryptophan metabolism and/or effects on the neurotrophin BDNF. Whilst poly I:C induced an inflammatory response and robust activation of the kynurenine pathway in the hippocampus and cortex, it failed to deplete tryptophan or serotonin concentrations, largely ruling out a role for inflammation-induced serotonergic dysfunction in the behavioural effects observed. Poly I:C could have other effects on 5-HT transmission, the 5-HT transporter has been shown to be increased in the frontal cortex of WIS rats following poly I:C treatment (Katafuchi et al., 2006). However, considering the increase in central kynurenine concentrations observed following poly I:C administration a role for kynurenine or one of its metabolites in mediating the depressive and anxiety behaviours observed cannot be ruled out. In addition, as poly I:C reduced expression of the neurotrophin BDNF and its receptor TrkB in hippocampus and cortex, the possibility exists that neurotrophic deficit could contribute to the depressive and anxiety behavioural deficits observed.

WKY rats do not appear to be amenable to investigate the emergence of depression related behaviours following immune challenge given that they exhibit depressive like behaviours under control conditions and do not respond to immune challenge to the same extent as Sprague-Dawley and WIS rats. Reduced activation of microglia in the frontal cortex is matched by low induction of IDO and no change in tryp, kyn, 5-HT and 5HIAA in the frontal cortex and hippocampus of WKY rats. Increased IL-6 expression matched by lower BDNF expression provides a possible mechanism whereby deficits in hippocampal plasticity in the strain emerge yet this requires further investigation. Future studies with WKY rats, would involve investigating the reduction in microglial activation in greater detail via immunostaining and characterisation of the microglial phenotypes both basally and following immune challenge. It would be of interest to determine if the reduction in microglial activation is related to enhanced GR sensitivity and if GR receptor antagonists may have efficacy as antidepressants in the WKY strain.

3.4.11 Future work and directions

Development of these studies further would include measurement of protein levels in all strains to determine whether the changes observed in expression translated into protein changes also. Use of the IDO inhibitor 1-methyl-tryptophan has been shown to prevent depressive-like behaviours in a rodent model following an LPS immune challenge (Dobos et al., 2012), it would be interesting to test whether use of such an IDO inhibitor and prevention of kynurenine pathway activation in Sprague-Dawley rats could eliminate the depressive-like behaviours observed in this strain following poly I:C administration. Future studies involving only WKY/WIS rats should focus on the observed failure of induction of activation of the microglia activation marker CD11b. Immunostaining of microglia could allow for elucidation of the number and activation state of microglia in discreet brain regions.

4. Results Chapter 2 – The Non Obese Diabetic mouse

4.1 Introduction

There is an increased co-morbidity of major depressive disorder and endocrine autoimmune diseases such as autoimmune thyroiditis and type-1 diabetes. Evidence suggests that people with such endocrine autoimmune diseases are up to three times more likely to present with depressive symptoms than the general population and vice versa (Eaton et al., 2006, Kupka et al., 2002, Hillegers et al., 2007, Vonk et al., 2007, Bergink et al., 2011), and when these disorders are co-morbid, prognosis is worse for either disease than when they occur separately (Anderson et al., 2001, Lloyd, 2010, Roy and Lloyd, 2012).

While there is abundant evidence that a dysregulated immune system forms one of the cornerstones in the immune pathogenesis of endocrine autoimmune diseases, a clear understanding of the immune pathophysiological mechanisms that underlie major depressive disorder have not been fully elucidated. However, increasing evidence suggests that inflammation may play a critical role in the development of this disorder. Increasing support for this theory comes from 2 large recent meta-analysis studies which demonstrated that inflammatory markers such as interleukin (IL-) 6, IL-1, tumour necrosis factor (TNF) α and C-reactive protein (CRP) positively associated with depressive symptoms in patients (Howren et al., 2009, Dowlati et al., 2010). In addition a link between an aberrant monocyte phenotype and mood disorders has been shown, at least in a subset of patients (Drexhage et al., 2011a, Beumer et al., 2012, Gibney and Drexhage, 2013, Bergink et al., 2013).

Microglia, the resident macrophages of the CNS, are central to the neuro-immune response. They are involved in a variety of normal physiological processes including synaptic pruning, synaptogenesis and neuronal apoptosis in the developing and adult brain (Paolicelli and Gross, 2011, Wake et al., 2011). Microglia also express receptors for many neurotransmitters and neuromodulators such as glutamate, GABA, dopamine, glucocorticoids and neurotrophins and therefore play an important role in neural plasticity in both health and disease (Kettenmann et al., 2011). Once activated, microglia are involved in a number of inflammatory processes including activation of the astrocytic inflammatory response, oxidative stress, antigen presentation and phagocytosis, and reactivation of infiltrating T cells (Garden and Moller, 2006). They are also the main producers of inflammatory cytokines and other

inflammatory mediators in the CNS (Garden and Moller, 2006, Hanisch, 2002, Olson and Miller, 2004).

There is increasing evidence to suggest that microglia may play a role in the aetiology of psychiatric disorders. Several post-mortem and positron emission tomography (PET) studies have reported increased activated microglia in the brains of psychotic patients (Bayer et al., 1999, Busse et al., 2012, Doorduin et al., 2009, Fillman et al., 2013, Radewicz et al., 2000, Steiner et al., 2008, Steiner et al., 2006). Clinical evidence for microglial dysregulation in depression is as yet limited to only a few post-mortem studies (Bayer et al., 1999, Steiner et al., 2008, Vidal et al., 2012). However, as recently reviewed (Beumer et al., 2012), there is strong evidence from pre-clinical models of depression to suggest that they may be integral to the aetiology of this disorder, at least in a subset of patients.

The Non-Obese Diabetic (NOD) mouse derived, from the CD1 mouse, displays spontaneous development of autoimmune thyroiditis and type 1 diabetes similar to what is observed in humans (Chaparro and Dilorenzo, 2010). The autoimmune phenotype found in NOD mice is mediated by cluster of differentiation (CD) 4^+ and $CD8^+$ T cell infiltration of the endocrine glands and destruction of the endocrine cells, and is more prominent in females (Homo-Delarche, 2004). Mononuclear phagocytes such as macrophages and dendritic cells and their precursors in the endocrine glands and bone marrow have been shown to play crucial roles in the early stages of the onset of endocrine autoimmune diseases (Nikolic et al., 2004, Strid et al., 2001, Charre et al., 2002, Homo-Delarche and Drexhage, 2004, Diana et al., 2013, Welzen-Coppens et al., 2012, Anderson and Bluestone, 2005b). In addition, upon stimulation, these macrophages show an exaggerated inflammatory response characterised by increased secretion of cytokines (Stoffels et al., 2004). Furthermore, the NOD mouse has been shown to have several brain alterations including altered expression of hypothalamic neuropeptides, impaired neurogenesis, reduced neuronal survival, astrogliosis and disturbances in the hypothalamic-pituitary-axis (Beauquis et al., 2008a, Beauquis et al., 2008b, Saravia et al., 2001, Saravia et al., 2002).

Whilst there is substantial evidence that the NOD mouse is characterised by an aberrant peripheral immune system, there is only limited evidence suggesting that the inflammatory state of the NOD mouse may be associated with behavioural changes (Amrani et al., 1994b, Bluth et al., 1999b), and consequently data is still lacking.

Literature exists outlining that the hypothalamic pituitary adrenal (HPA) axis and the immune system can interact, reciprocally affecting each other's function, and that dysregulation of these communication pathways exist in forms of depression (Leonard, 2005, Besedovsky and del Rey, 2000). For example, pro-inflammatory cytokines have been shown to activate the HPA-axis (Dunn et al., 2000, Silverman et al., 2003) and patients undergoing chronic cytokine treatment for cancer (Capuron et al., 2001, Pace and Miller, 2009) and hepatitis C (Majer et al., 2008) have been shown to develop depressive symptoms which can be reversed via antidepressant treatment (Musselman et al., 2001, Capuron et al., 2002a). The NOD mouse is commonly used as a model of autoimmune disease as a result of a dysregulated immune system (Anderson and Bluestone, 2005b) and its macrophages have been shown to produce high levels of pro-inflammatory cytokines upon encountering apoptotic and necrotic cells (Stoffels et al., 2004). A higher turnover of serotonin (5-HT) metabolism has also been reported in depressed patients and is reduced following antidepressant treatment (Barton et al., 2008, Mitani et al., 2006) and pro-inflammatory cytokines have been shown to increase metabolism of 5-HT in the brain (Dunn, 2006). It is therefore of interest to investigate whether the dysregulated immune system present in the NOD mouse could lead to an impairment of the stress response, an alteration in monoamine neurotransmitter levels, and cause a change in the glucocorticoid related genes and pro-inflammatory genes.

The aims of the present study were therefore to 1) More extensively investigate the behavioural profile of the NOD mouse compared to a control strain (CD1) to determine any altered steady state depressive-like behaviour, and any altered behavioural response to an acute immunological challenge (lipopolysaccharide, LPS), 2) Compare the genotype of NOD monocytes and microglia with CD1 at steady state and in response LPS, 3) To determine whether there is an altered inflammatory or growth factor response and/or kynurenine pathway activation in the hypothalamus of the NOD mouse at steady state and in response to LPS and 4) to compare the peripheral steroid and glucose response, the central 5-HT concentrations and the central genetic response of the NOD mouse to the CD1 mouse following exposure to an acute psychological stressor.

4.2 Methods

4.2.1 Animals

Female NOD/ShiLtJ mice (20-25g) and CD1 mice (25-30g) were obtained from Charles River (Italy) and Harlan (UK) respectively. Mice were housed 6 per cage and were maintained on a 12hr light: 12hr dark cycle (lights on at 08:00hr) in a temperature controlled room (22±2°). Food and water were available *ad libitum.* The experimental protocols were in compliance with the European Communities Council directive (86/609/EEC). Experiments were carried out on the NOD mice in a pre-diabetic state, aged 8 weeks old.

4.2.2 Behavioural testing

4.2.2.1 Open Field Test

At the beginning of each trial, animals were placed in the centre of a dimly lit open field arena, measuring 90cm in diameter, and observed for 10mins. The trials were conducted between 10am and 2pm. The arena was cleaned with 70% ethanol between each trial. The behaviour of the animals was recorded by an overhanging camera that was attached to a personal computer. Ethovision 3.0 (Noldus, The Netherlands) was used to track the movement of the animal. The total distance moved in the arena, distance moved in the inner zone of the arena and number of transitions between the inner and outer zones were recorded.

4.2.2.2 Elevated Plus Maze

The maze consists of a raised cross-shaped platform, with four arms originating from a common centre point. Two of the arms, facing each other, contain raised walls, providing the mouse with a dark enclosed environment, whilst the two other arms contain no walls and are exposed. At the start of the test, the mouse is placed in the centre square. The behaviour of the animals was recorded by an overhanging camera that was attached to a personal computer. Ethovision 3.0 (Noldus, The Netherlands) was used to track the movement of the animal. Time

spent in the open and closed arms, and number of entries into the open and closed arms was recorded.

4.2.2.3 Tail Suspension Test

The Tail Suspension Test (TST) is an inescapable stress that measures the presence of despair behaviour in the mice subjected to the test (Cryan et al. 2005). Mice were attached by their tails to wire affixed to a horizontal bar of a retort stand so that they are suspended 25cm above the base of the stand. The mice are left in suspension upside down for a period of 6mins, of which the last 5mins are scored for time spent immobile. Behaviour was scored by two trained observers, blind to mouse identity.

4.2.2.4 Home Cage Activity

In order to monitor the behavioural response of the mice to a physical restraint stress, the activity in a novel home cage was monitored following stress exposure in a separate set of animals. Animals were individually placed in activity monitor cages measuring 32cm x 20cm x 18cm (length x width x height). These cages were connected to an AM1051 data logger (Benwick Electronics). Each cage contained 2 sets of horizontal infrared beams, one set at 3cm and one at 6cm from the bottom of the cage. Each set consisted of a 12 beam by 7 beam matrix, which formed a grid of 66 x 2.54cm^2 cells in each cage. Together the beams and data logger recorded activity and rearing. Activity was determined by the number of times a beam was broken and was measured from when the beam was first broken until no beam had been broken for 5 seconds. Data was analysed using the total number of beam breaks over the 30mins trial. The trials were conducted between 10am and 2pm. This protocol was carried out both before the immunological challenge (LPS) in order to determine the baseline levels of locomotor and rearing activity, and 4hr and 24hr post LPS injection.

4.2.3 LPS treatment

To evaluate the effect of lipolysaccharide (LPS), animals received an i.p. injection of LPS(100µg/kg) prepared from Echerichia coli (Sigma) or an i.p. injection of saline 4hr or 24hr before euthanisation.

4.2.4 Acute Restraint stress

Mice were immobilised by placing them in a well ventilated restrainer (modified 50ml collecting tube, Fig: 2.4) for a single exposure. The tubes were placed parallel to the table top in a brightly lit room with an ambient temperature of 20-24°C. The mice were restrained for 2 hours (hr), and then either euthanised immediately or returned to the home cage for 2hr after which time they were euthanised. Control animals remained in their home cage and were euthanised when removed from the cage.

4.2.5 Monocyte isolation

Whole blood was obtained by cardiac puncture and was directly stored on ice in tubes containing buffered sodium citrate. Erythrocytes were lysed in NH_4Cl (0.15M in DPBS) for 15mins at room temperature. Cells were washed twice in DPBS containing 0.1% BSA. The cells were re-suspended in DPBS containing 0.1% BSA and labelled with CD11b-APC and Ly6G-PE (Becton Dickinson) and Ly6C-AF488 (SeroTec) for 30mins on ice. Cells were washed and the $SSC^{low}CD11b^{pos}Ly6G^{neg}$: immature ($Ly6C^{high}$) and mature ($Ly6C^{low}$) monocytes were sorted in DPBS+0.1%BSA on a MoFlo FACS sorter (Dako). Re-analysis of the sorted cells indicated a purity >99%. Finally cells were washed in DPBS and lysed in extraction buffer from the Picopure kit (Arcturus) and stored at -80°C until RNA isolation.

4.2.6 Microglial isolation

Microglia were isolated according to a modified version of the method described previously (de Haas et al., 2008). In short, the mice were perfused with PBS after blood sampling by cardiac puncture. Subsequently, the small-brains were

removed and the cortex was stored in ice-cold 1x Hanks' balanced salt solution (HBSS, Gibco), containing 15 mM HEPES (Gibco) and 0.5% glucose (Sigma). Brains were cut in small pieces, dissociated in a glass tissue homogenizer (VWR, Amsterdam, The Netherlands) and titruated by fire-polished glass Pasteur pipets (VWR). The single-cell suspension was first filtered on a 70-µm cell strainer (Becton Dickinson) and pelleted for 10 min at 200·g, 4°C. A 100% Percoll stock (GE Healthcare) was made using 9 volumes of Percoll and 1 volume of HBSS 10x (Gibco). The 100% Percoll stock was diluted by adding 1x Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco) in order to obtain 75% and 25% Percoll stocks. The cell pellet was taken up in 10ml 75% Percoll stock and respectively overlayed with 10ml 25% Percoll stock and 6ml DPBS (0% Percoll). The density gradient was centrifuged in a swinging bucket rotor at 800g (slow acceleration without brake) for 30 min at 800·g, 4°C. After centrifugation, a thick myelin-containing layer at the 0/25% Percoll interface was discarded and the cells between the 25/75% interfaces were collected and washed in 30ml ice-cold DPBS. The cells were re-suspended in DPBS containing 0.1% BSA and 141eurotro with CD11b-APC (Becton Dickinson) and CD45-PB (Biolegend) antibodies. Cells were washed and $SSC^{low}CD11b^{+}CD45^{low}$ microglia were sorted in DPBS+0.1%BSA on a MoFlo FACS sorter (Dako). Re-analysis of the sorted cells indicated a purity >99%. Finally cells were washed and lysed in extraction buffer and stored at -80°C until RNA isolation.

4.2.7 RNA isolation and amplification, Affymetrix microarrays

RNA was isolated with the PicoPure kit (Arcturus, Applied Biosystems) according to the manufacturers protocol including a Dnase I treatment (Qiagen, Venlo, The Netherlands) to remove genomic DNA contamination. The RNA was reverse transcribed, amplified, biotinylated and fragmented with the Ovation Pico WTA v2 and Encore Biotin Module (NuGEN Technologies, Leek, The Netherlands) and subsequently hybridized on Mouse Genome 430 2.0 Arrays (Affymetrix, High Wycombe, UK) according to the manufacturers protocols.

4.2.8 Microarray analysis

Quality analysis of the CEL data was assessed by running a standardized workflow developed at the BiGCaT department of Maastricht University – The

Netherlands (http://www.arrayanalysis.org/). The expression data containing .CEL files were imported and processed further with BRB-Arraytools (R. Simon, http://linus.nci.nih.gov/BRB-ArrayTools.html). Gene expression data was normalized using RMA (Robust Multichip Average) (Bolstad et al., 2003). A list of differentially expressed genes (DEGs) among the two classes was identified by using a multivariate permutation test using the class comparison tool in BRB-arraytools. Ingenuity pathway analysis (Ingenuity® Systems, www.ingenuity.com) was used for annotation and to sort data relevant to fold change. Gene expression was considered statistically significantly different when $P<0.05$. Only genes where expression was 1.5 fold different from CD1 are reported in the present study.

4.2.9 Enzyme linked immunosorbent assay (ELISA)

4.2.9.1 Corticosterone Assay

Circulating serum concentrations of corticosterone were quantified using a commercially available corticosterone assay kit (Enzo Life Sciences). The assay was performed as per the manufacturer's guidelines and as previously described in chapter 2. Absorbance was measured at 405nm using a microtitre plate reader (Elx800; Biotek, Germany).

4.2.9.2 Glucose Assay

Circulating serum concentrations of glucose were quantified using a commercially available glucose assay kit (Cayman Chemical Company). The assay was performed as per the manufacturer's guidelines and as previously described in chapter 2. Absorbance was measured at 514nm using a microtitre plate reader (Elx800; Biotek, Germany).

4.2.10 Analysis of serotonin concentrations by HPLC

Serotonin (5-HT) and its metabolite 5-HIAA were measured by high performance liquid chromatography (HPLC) as previously described (Harkin et al., 2003). Brain tissue was sonicated in 1 ml of mobile phase spiked with 5 ng/20 µL of N-

methyl 5-HT (Sigma) as internal standard. The mobile phase contained 0.1 M citric acid (Sigma), 0.1 M sodium dihydrogen phosphate (Sigma), 0.1 mM EDTA (Sigma), 1.4 mM octane-1-sulphonic acid (BDH) in $ddH_2O$and was adjusted to pH 2.8 using 4 N sodium hydroxide. Homogenates were centrifuged at 20,000 rpm for 20 min and the supernatants were placed into new eppendorf tubes, using a syringe fitted with a 0.45μm filter (phenomenex). 10μl of the filtered supernatant was injected into a reverse phase analytical column (Kinetex™ Core Shell Technology C18 column with specific area of 100mm x 4.6mm and particle size of 2.6u, Phenomenex), for separation of the neurotransmitters. Neurotransmitter concentrations were quantified by electrochemical detection (Digital Electrochemical Amperometric Detector, Mason Technology Ltd) and chromatographs were generated by CLASS-VP software (Shimadzu). Results are expressed as ng of neurotransmitter per g fresh weight of tissue.

4.2.11 Analysis of hypothalamic gene expression by real-time PCR

RNA was extracted from hypothalamic tissue using the NucleoSpin® RNA II total RNA isolation kit (Macherey-Nagel, Germany). Genomic DNA contamination was removed by the addition of Dnase to the samples. RNA was reverse transcribed into cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Darmstadt, Germany). Real-time PCR was performed using an ABI Prism 7300 instrument (Applied Biosystems) as previously described (Boyle and Connor, 2007). Taqman® Gene Expression Assays (Applied Biosystems) containing forward and reverse primers and a FAM-labelled MGB Taqman probe were used to quantify each gene of interest. PCR was performed using Taqman® Universal PCR Master Mix and samples were run in duplicate. The cycling conditions consisted of 90 °C for 10 min and 40 cycles of 90 °C for 15 s followed by 60 °C for 1 min. Abl1 was used as an endogenous control to normalize gene expression data. Relative gene expression was calculated using the ΔΔCT method with Applied BioSystems RQ software (Applied BioSystems, UK).

Table 4.1 – List of Taqman® gene assays and codes used in this study

Target	Taqman® Assay ID	Target	Taqman® Assay ID
Abl1	Mm00802029_m1	**TREM2**	Mm04209424_m1
CD86	Mm00444543_m1	**IL-1β**	Mm01336189_m1
CD40	Mm00441891_m1	**IL-6**	Mm00446190_m1
CD68	Mm03047340_m1	**TNFα**	Mm00443258_m1
CD11b	Mm00434455_m1	**IFNγ**	Mm00801778_m1
TREM1	Mm01278455_m1	**NOS2**	Mm00440502_m1
ARG1	Mm00475988_m1	**IDO**	Mm00492586_m1
KMO	Mm00505511_m1	**KAT-II**	Mm00496169_m1
PDGF	Mm00440677_m1	**BDNF**	Mm04230607_m1
FGF2	Mm00433287_m1	**VEGF**	Mm00437310_m1

4.2.12 Statistical analysis of data

All values are expressed as mean ± standard error of the mean (S.E.M.). Data were analysed using either students t-test or a two-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test (GB stat). A value of $P<0.05$ was considered to be statistically significant.

4.3 Results

4.3.1 NOD mice display an anxious and depressive-like behavioural phenotype in the steady state when compared to the CD1 control strain.

In the elevated plus maze paradigm, NOD mice were found to spend less time in the open arms (a) ($P<0.001$) when compared to the CD1 control strain, which has been described before as indicative of an anxious phenotype. NOD mice were also found to enter the open arms of the EPM significantly less than the CD1 mice (b) ($P<0.01$).

When exposed to a novel open field environment, NOD mice showed a significant reduction in movement in the inner zone when compared to the CD1 mice (c) ($P<0.05$), which is indicative of anxiety-related behaviour. Data are presented as distance moved in the inner zone as a % of total distance moved in the open field arena.

In the tail suspension test, the NOD mice were found to be significantly more immobile than the CD1 control strain. The response to this inescapable stress is indicative of behavioural despair (d) ($P<0.001$). [Figure 4.1, n= 12]

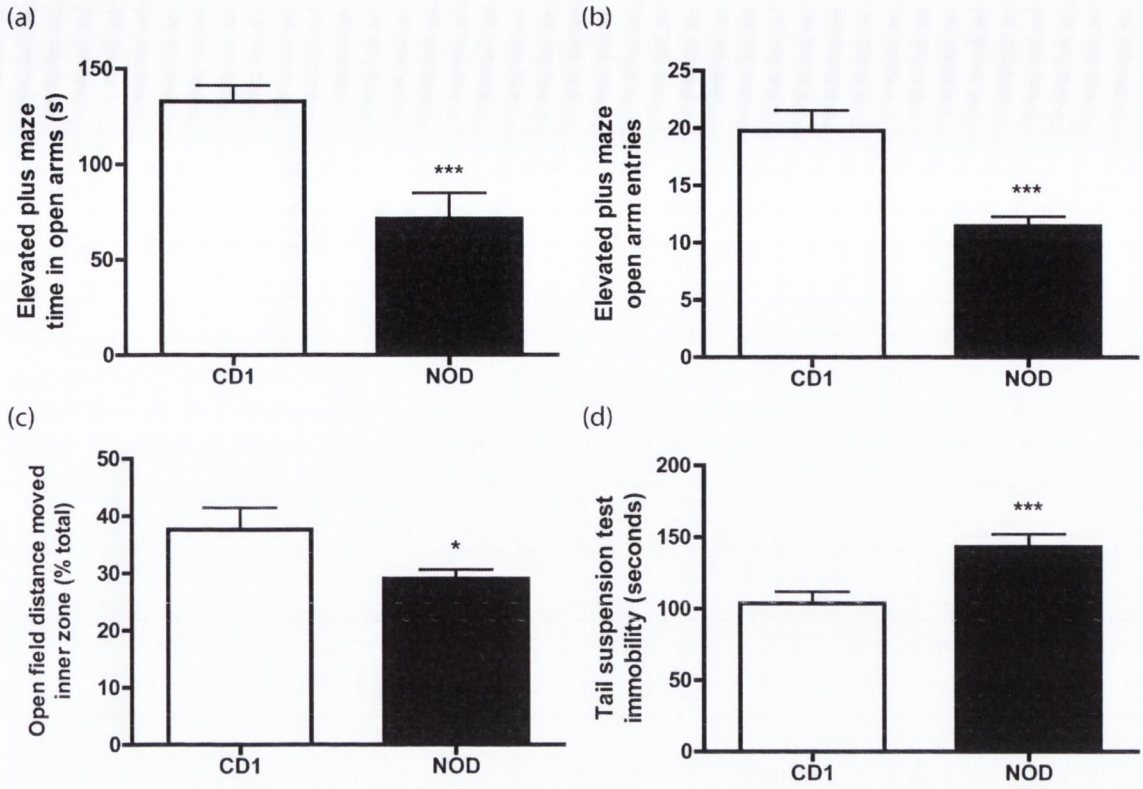

Figure 4.1 – Altered steady state behaviour in the NOD mouse (vs. CD1).

NOD mice at steady state show increased anxiety- and depressive-like behaviour as indicated by decreased time spent in (a) and decreased entries into the open arms of the elevated plus maze (b); decreased movement in the inner zone of the open field (c) and increased immobility in the tail suspension test (d). Data expressed as mean ± SEM, n=12 per group. ***P<0.001, **P<0.01, NOD vs. CD1.

4.3.2 NOD mice show increased weight loss 24hr post LPS and also display an exaggerated and prolonged LPS-induced sickness behaviour in the home cage

When body weight was recorded at baseline and then subsequently 24hr post injection, an effect of both strain and LPS was found (Interaction; $F_{(1, 12)}=25.42$, $P<0.001$). Post-hoc analysis revealed that while no difference in body weight was observed in the CD1 strain in response to LPS exposure, a significant increase in weight loss was found in NOD mice 24hr post-LPS when compared to saline treated controls ($P<0.01$) (a). This was independent of strain differences in food intake post LPS, where both CD1 and NOD mice showed similar reductions in food intake 24hr post-LPS (b).

To determine any differential behavioural response to LPS, activity and rearing were monitored in the home cage activity paradigm. An effect of both strain and LPS was found (Interaction; $F_{(1, 27)}=4.1$, $P<0.05$) when the activity of the mice was measured. Both CD1 and NOD showed comparable home cage activity at steady state. While they both showed reduced activity at 4hr post-LPS when compared to their respective controls ($P<0.05$ and $P<0.01$ respectively), this altered behaviour was more pronounced in the NOD mouse with activity significantly reduced at 4hr post-LPS when compared to CD1 mice at the same time point ($P<0.01$) (c).

Similarly, an effect of strain ($F_{(1, 27)}=6.8$, $P<0.05$) and LPS ($F_{(1, 27)}=15.7$, $P<0.001$) on rearing behaviour in this paradigm was also apparent. Once again no difference was found in the behaviour of the 2 strains at steady state. Furthermore, both strains showed comparable reductions in rearing 4hr post-LPS when compared to their relative controls ($P<0.01$). However, at 24hr, while rearing in the CD1 mouse had returned to control levels, it was still significantly reduced in the NOD mouse ($P<0.05$) suggesting a prolonged behavioural response to LPS (d). [Figure 4.2, n=5-6]

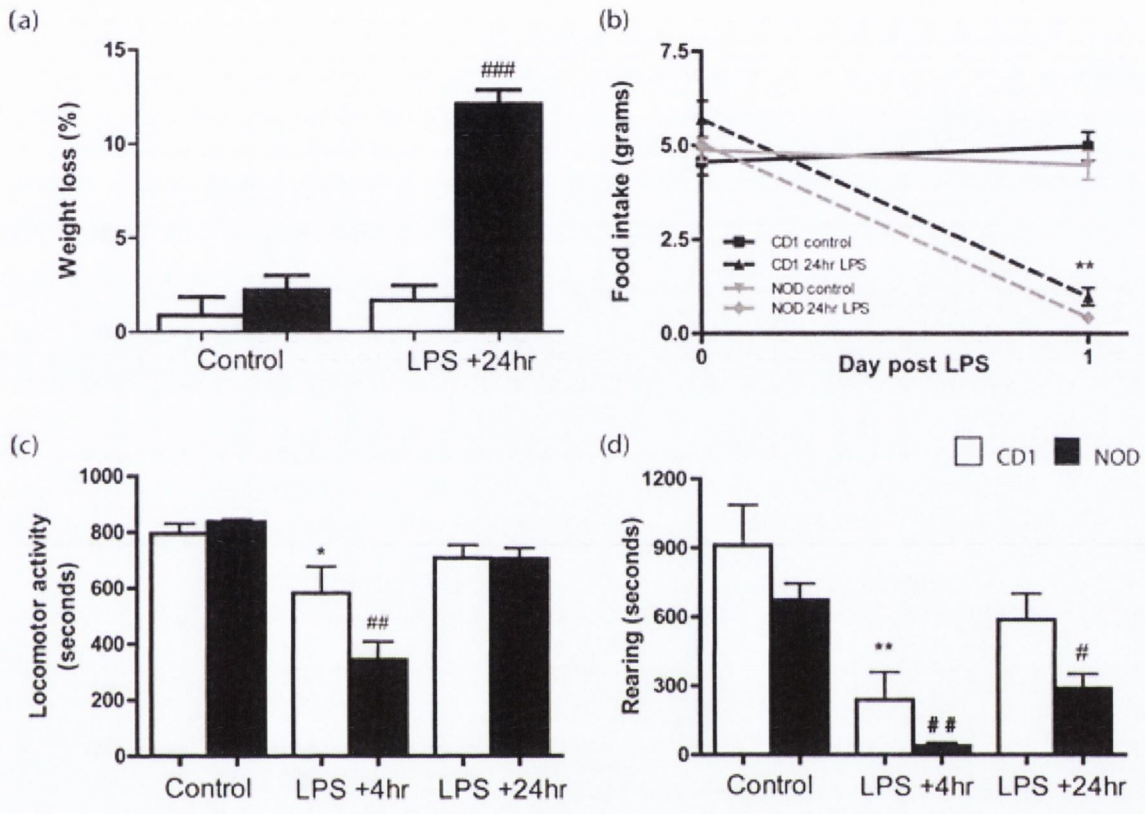

Figure 4.2 – LPS-induced behavioural alterations in NOD mouse (vs. CD1).

In response to LPS NOD mice show an exaggerated and prolonged behavioural response to LPS as indicated by a reduction in body weight 24hr post LPS (a) which was independent of food intake (b); a more pronounced reduction in home cage activity 4hr post LPS (c) and a reduction in rearing that was maintained at 24hr post LPS (d). Data expressed as mean ± SEM, n=5-6 per group. **P<0.01, *P<0.05, LPS versus control. $^{###}$P<0.001, $^{##}$P<0.01, $^{#}$P<0.05, NOD vs. CD1.

4.3.3 NOD monocytes at steady state show an altered gene expression suggestive of impaired development and inflammatory genotype

Using Ingeunity analysis, it was found that impaired development, differentiation and maturation were among the top physiological functions affected in the monocytes isolated from the NOD mouse at steady state (compared to CD1). In accordance, when the top 10 down-regulated genes were examined in these cells, 70% of them we found to be involved in these physiological processes. A biased analysis of the monocyte gene expression was carried out to determine if the inflammatory gene expression of these cells was altered in the NOD mouse at steady state. This analysis included chemokines, interleukins, interferon (IFN) and IFN-induced genes, TNFα and identifying markers of various M1 and M2 activation states as outlined previously (Gordon & Taylor, 2005). It was found that the profile of the mature monocytes did not differ greatly between strains with only marginal decreases in chemokines, MHC genes and TNFα found in the NOD mouse (Table 4.2). Conversely, steady state immature monocytes from the NOD mouse showed a large down-regulation of some inflammatory genes involved in IL-1 signaling, when compared to CD1 (Table 4.2).

4.3.4 NOD monocytes are hyper-responsive to LPS and display characteristics of a classic and type-1 IFN inflammatory profile

4hr post LPS. When the inflammatory profile of the NOD mature monocyte genes 4hr post LPS was compared with CD1 monocytes from the same time point, the profile did not differ greatly between strains with only marginal up- and down-regulation of some inflammatory genes. However, when genes unique to the NOD mouse were examined, it was found that the NOD monocytes were characterised by a strong type-1 IFN profile at this time point. In fact 6 of the top 10 genes uniquely up-regulated in these monocytes at 4hr post-LPS are involved in type-1 IFN signaling (Table 4.2).

As was seen at steady state, the inflammatory profile of the immature monocytes 4hr post-LPS was still impaired in the NOD mouse when compared to CD1, with expression of many inflammatory genes reduced in the NOD at this time point, although to a lesser degree (Table 4.2). Interestingly, while IFNγ was one of the top 10 up-regulated genes in the NOD mature monocyte at this time point (↑461 fold), its expression was reduced in the NOD immature monocytes when

compared to CD1. As with the mature monocytes, when genes unique to the NOD immature monocytes were examined at this time point, a strong up-regulation of genes involved in type-1 IFN signaling was found but also a classic inflammatory profile with up-regulation of IL-1R1 and 2 and TNF (Table 4.2).

24hr post LPS. At 24hr post LPS, when the inflammatory profile of NOD monocytes was compared to CD1, a strong classical inflammatory phenotype was apparent in the NOD monocytes. This included increases in the expression of IL-1β, IL-6R, IFNγ, several genes involved in the TNF pathway, MHC genes and chemokine genes, amongst others. As well as this classic pro-inflammatory phenotype, the NOD monocytes at this time point were also characterised by an overt type-1 IFN genotype when compared to their CD1 counterparts (Table 4.2). Conversely, the inflammatory profile of the NOD immature monocytes at this time point (when compared to CD1) was not as robust. Although many of the inflammatory genes found down-regulated at steady state and at 4hr post-LPS in these cells were now up-regulated in the NOD monocytes. While no overt type-1 IFN phenotype was evident when genes unique to the NOD were examined at this time point, they were characterised by an up-regulation of several chemokine genes and TNF (Table 4.2).

4.3.5 NOD microglia at steady state display an altered expression of genes involved in neuronal function and inflammatory processes

When the top 20 altered genes in the steady state NOD microglia were analysed (compared to CD1) it was found that a large percentage (65% of the top-20) of these altered genes were involved in neuronal functioning including neurite growth, axon guidance and neurogenesis. Also genes involved in antigen presentation, phagocytosis and inflammation were also differentially expressed in the top dysregulated genes.

As stated above for steady state monocytes, a biased analysis of the microglial gene expression was also carried out to determine if the inflammatory profile of these cells was altered in the NOD mouse. While differential expression of inflammatory genes between NOD and CD1 at steady state was limited, all of the genes that were differentially expressed were down regulated in the NOD mouse, suggesting that the NOD microglia have an impaired inflammatory profile at steady state (Table 4.2).

4.3.6 NOD microglia are less responsive to LPS stimulation and do not show a 'classic' inflammatory genotype

When the total number of genes altered in response to LPS were analysed (vs. The steady state situation) it was found that a greater number of genes were changed in the CD1 microglia than is observed in the NOD microglia at both 4hr and 24hr post stimulation (Number of genes altered 4hr LPS (vs. Steady state): CD1, 1664; NOD, 484. 24hr LPS (vs. Steady state): CD1, 1274; NOD, 506). This suggests that the NOD microglia are actually hypo-responsive to this stimulus. To determine if the inflammatory profile of the NOD and CD1 microglia also differed, the biased approach of inflammatory gene expression analysis as outlined in the previous section was used.

4hr post LPS. When NOD and CD1 were compared it was found that only a limited number of inflammatory genes were differentially expressed between these two strains 4hr post LPS. While expression of IL-6 or its receptor did not differ in either strain, IL-1β was induced in both NOD and CD1 microglia at this time point. Although the expression of IL-1β did not significantly differ when NOD and CD1 were compared, there was a trend for a higher increase in the expression of this gene in the CD1 microglia (488 fold increase in CD1 (vs. Steady state); 372 fold increase in the NOD (vs. Steady state)). Interestingly, induction of TNFα and IL-12 were unique to the CD1 microglia and not observed in NOD microglia.

In addition to these, other inflammatory genes were also uniquely induced in the CD1 microglia including chemokines, the alternative activation markers chitinase 3 and mannose receptor, as well as IFN gamma inducible genes. While the number of inflammatory genes uniquely altered in the NOD microglia was much less, they were predominantly made up of genes involved in IFN type I signalling, highlighting the differential inflammatory response of these microglia to LPS stimulation. It is also important to note, that the gene with the highest up-regulated expression when NOD and CD1 microglia were compared at this time point was the pro-inflammatory chemokine receptor CCR6, supporting the view that the NOD microglia are in fact responsive to this stimulus, but in an alternative way (Table 4.2).

24hr post LPS. When NOD and CD1 microglia were compared, again the differential expression of inflammatory genes was limited. However, it was found that while chemokine genes were up-regulated in the CD1 microglia at 4hr post

LPS, in the NOD microglia they were up-regulated at this later time point suggesting that these cells may have a delayed migratory response to this stimulus. However, it must also be noted that other markers of microglial activity such FCGR1A and the MHC I gene HLA-C were down-regulated in the NOD microglia compared to CD1 at this time point, suggesting that these cells may also have impaired function. While IL-1β expression was no longer increased in the NOD microglia at 24hr post LPS, it was still elevated in the CD1 microglia and was unique to these cells at this time point. Other inflammatory genes unique to CD1 microglia were predominantly reduced suggesting that these cells may be returning to a de-activated state. As observed at the earlier time point, inflammatory genes unique to NOD microglia at 24hr post LPS were again characterised by an type-1 IFN response (Table 4.2).

Table 4.2 – Characteristics of monocytes and microglia of NOD mice (vs. CD1)

Immature monocytes:

Steady state

- Impaired development (e.g. AAK1 ↓37, COG1 ↓162, NSMF ↓218)
- Reduced 'classic' inflammatory phenotype (e.g. IL-18R ↓259, IL-18RAP ↓168, IL-2R ↓324)

LPS 4hr

- Altered 'classic' inflammatory phenotype (e.g. IFNγ ↓72, IL-2R ↓86, IL-1R ↑48, TNF ↑11)
- Strong type-1 IFN phenotype unique to NOD (e.g. IFIT2 ↑184, OASL ↑342, IFIT3 ↑106)

LPS 24hr

- Mild 'classic' inflammatory response (e.g. IL-18 ↑20, TNF ↑5)

Mature monocytes:

Steady state

- Impaired development (e.g. LY6D ↓113, ATXN1L ↓66, JAKMIP ↓256)
- Mildly reduced inflammatory phenotype (e.g. CCR6 ↓18.7, HLA-B ↓2.3)

LPS 4hr

- Hyper-responsive
- Strong type-1 IFN phenotype unique to NOD (e.g. CXCL10 ↑1751, OASL ↑1015, IFIT3 ↑378)

LPS 24hr

- Hyper-responsive
- Strong 'classic' inflammatory response (e.g. IL-1β ↑310, IL-6R ↑391, IFNγ ↑72)
- Strong type-1 IFN phenotype (e.g. IRF8 ↑149, IFNAR2 ↑275, IFITM6 ↑487)

Microglia:

Steady state

- Impaired neuronal support (e.g. ADNP↓20, PHGDH ↓24, NEO1 ↑34, STX1A ↑5)
- Mildly reduced 'classic' inflammatory phenotype (e.g. HLA-C ↓6.8, CCL2 ↓5.6, IL-6R ↓2.7)
- Mild type-1 IFN phenotype (e.g. IRF3 ↑6, SP110 ↑8)

LPS 4hr

- Hypo-responsive
- No clear 'classic' inflammatory response
- Mild type-1 IFN phenotype unique to NOD (e.g. IFIH1 ↑6, STAT2 ↑9, IRF3 ↑5)

LPS 24hr

- Hypo-responsive
- No clear 'classic' inflammatory response
- Type-1 IFN phenotype (e.g. SP110 ↑38, LY6E ↑6, TGTP1 ↑8)

Table 4.2 – Characteristics of monocytes and microglia of NOD mice (vs. CD1)

In NOD mice, immature monocytes display impaired development and a reduced classic inflammatory phenotype under basal conditions. This altered inflammatory phenotype persists following immune stimulus coupled with a strong type-1 IFN phenotype. Mature monocytes also display impaired development and a reduced inflammatory phenotype under basal conditions. Following immune stimulus, they display hyper responsiveness accompanied by a strong type-1 IFN phenotype and a classic inflammatory response. NOD microglia display impaired neuronal support and elevated classic and type-1 IFN inflammatory phenotypes under basal conditions. Following immune stimulus, they display a hypo responsive phenotype accompanied with a mild type-1 IFN phenotype only. Abbreviations: AAK – Adaptor-associated protein kinase, ADNP – Activity dependent neuroprotector, ATXN1L – Ataxin-1 like, CCL – Chemokine (C-C motif) Ligand, CCR – Chemokine (C-C motif) receptor, COG – Conserved oligomeric Golgi complex subunit, CXCL – Chemokine (C-X-C motif) ligand, GADD – Growth Arrest and DNA-damage inducible, HLA – Human Luekocyte Antigen, IFIH – Interferon Induced With Helicase C Domain, IFIT – Interferon-induced protein with tetratricopeptide repeats, IFITM – Interfenon Induced Transmembrane Protein, IFN – Interferon, IFNAR – Interferon-α/β receptor, IL – Interleukin, IL-*X* R – Interleukin *X* Receptor, IL-*X*RAP – Interleukin *X* Receptor Accessory Protein, IRF – Inteferon Regulatory Factor, JAKMIP – Janus kinase and microtubule interacting protein, Ly6*X* – Lymphocyte Antigen 6 Complex Locus *X*, NEO – Neogenin, NSMF – NMDA receptor synaptonuclear signalling an neuronal migration factor, OASL – Oligoadenylate synthase-like, PHGDH – Phosphoglycerate dehydrogenase, SP – Speckled, STAT – Signal Transducer And Activator of Transcription, STX – Syntaxin, TGTP – T-cell Specific GTPase, TIA – T-cell-restricted Intracellular Antigen, TNF – Tumour Necrosis Factor,

4.3.7 LPS induces differential expression of microglial markers in the hypothalamus of NOD mice when compared to the CD1 strain

To further examine the differential genotype of the NOD mouse compared to CD1, several markers of microglial activity were analysed in the hypothalamus including CD40, CD11b, CD86, CD68 and TREM1 and the alternative activation markers ARG1 and TREM2.

While all of these markers were induced by LPS in both NOD and CD1 mice (Table 4.3), there were strain dependent differences in the level of induction of some of these genes. For example, an effect of strain and LPS was found ($F_{(2, 29)}$=13.19, P<0.001) on the mRNA expression of CD40. While a significant increase in the mRNA expression of CD40 at 4hr post-LPS was found in both NOD (P<0.001) and CD1 (P<0.001) mice when compared to their respective controls, this increase was significantly greater in the NOD mouse when compared to LPS treated CD1 mice at the same time point (P<0.01). By 24hr, mRNA expression of CD40 had returned to control levels in both strains (a).

A significant effect of strain and LPS was also found ($F_{(2, 29)}$=9.95, P<0.001) on the mRNA expression of CD86. As with CD40, while a significant increase in the mRNA expression of CD86 4hr post-LPS was found in both NOD (P<0.01) and CD1 (P<0.01) mice, this increase was significantly greater in the NOD mouse when compared to LPS treated CD1 mice at the same time point (P<0.01). By 24hr, mRNA expression of CD86 had returned to control levels in both strains (b).

When expression level of the M2 marker ARG1 was measured in the hypothalamus, an effect of both strain and LPS were found ($F_{(2, 29)}$=6.47, P<0.05). Expression of ARG1 was greater in the NOD mouse at steady state when compared to CD1 (P<0.05). While no change in expression was found in the CD1 4hr post LPS, a significant decrease in expression was found in the NOD mouse at this time point when compared to control (P<0.05). While expression in the NOD mouse returned to control levels at 24hr post LPS, a significant increase was found in the CD1 mouse at this later time point when compared to its relative control (P<0.01), which interestingly was comparable to the expression level observed in the NOD mouse at steady state (c).

Similarly, when expression level of TREM2 was measured in the hypothalamus an effect of strain ($F_{(2, 29)}$=22.34, P<0.0001) and LPS ($F_{(2, 29)}$=9.13, P<0.01) was found. As with ARG1, a significant increase in TREM2 expression was found in

the NOD mouse at steady state when compared to CD1 ($P<0.05$). While expression was significantly reduced in both strains at 4hr post-LPS compared to their relative controls ($P<0.05$), the level of expression was still significantly higher in the NOD mouse compared to CD1 at this time point ($P<0.05$). By 24hr post-LPS, levels in both strains had returned to control levels (d). [Figure 4.3, n=5-6]

4.3.8 LPS induces differential expression of pro-inflammatory markers in the hypothalamus of NOD mice when compared to the CD1 strain

To further examine the immune profile of NOD and CD1, expression levels of several pro-inflammatory cytokines were also measured in the hypothalamus including TNFα, IL-1β, IL-6, IFNγ and NOS2. As mentioned above, while all of these genes were induced by LPS (Table 4.3), only some of them displayed strain dependent differences in their expression levels. For example, an effect of LPS was shown ($F_{(2, 29)}=15.57$, $P<0.01$) in response to treatment and an increase in IL-1β was found in the NOD mouse 4hr post-injection, returning to control levels by 24hr. While LPS did induce an increase in IL-1β in the CD1 mice this time point, post-hoc analysis revealed that this increase was not significant, potentially due to the high variability of expression levels. When expression of IFNγ was measured, an effect of strain and LPS was found ($F_{(2, 28)}=6.41$, $P<0.05$). While no effect of LPS was found in the CD1, a 14-fold increase in IFNγ mRNA was found in the NOD mouse at 4hr post-LPS when compared to its relative control ($P<0.05$), returning to basal level by 24hr (f). [Figure 4.3, n=5-6]

4.3.9 LPS induces differential expression of kynurenine pathway and growth factor genes in the hypothalamus of NOD mice when compared to the CD1 strain

When mRNA expression of the kynurenine pathway enzyme IDO was measured in the hypothalamus, expression in the NOD mouse at steady state was below the limits of detection. Furthermore, expression of this gene was only found in 50% of CD1 mice at steady state. However, a significant effect of strain ($F_{(1, 28)}=8.77$, $P<0.01$) and LPS ($F_{(2, 28)}=3.38$, $P<0.05$) on IDO expression was found after LPS injection (g). While no effect of LPS was found in the CD1 mice, a significant increase in mRNA expression of IDO was found 4hr post-LPS in NOD

mice ($P<0.01$), with this expression remaining significantly elevated at 24hr post-LPS ($P<0.01$). [Figure 4.3, n=5-6]

While no effect of strain or LPS was found on KAT-II mRNA expression (Table 4.3), an interaction between strain and LPS was found ($F_{(2, 28)}=4.98$, $P<0.05$) on the mRNA expression of KMO. At steady state, a significantly higher expression of KMO was found in the NOD mice compared to CD1 ($P<0.05$). While no effect of LPS was found on KMO mRNA in the NOD mouse, significantly increased expression of this gene was found in the CD1 mice at both 4hr and 24hr post-LPS when compared to its relative control ($P<0.01$), with these levels comparable to what was found in the NOD mouse at steady state (h). [Figure 4.3, n=5-6]

Several growth factor genes were also analysed in the hypothalamus. While no change in the expression level of VEGF or BDNF was found in any of the groups analysed, increased FGF expression was found in both NOD and CD1 mice 4hr post-LPS (Table 4.3). Moreover, a significant effect of both strain and LPS were found when expression level of PDGF was measured in the hypothalamus. While no effect of LPS was found in the CD1, a significant decrease in mRNA expression was found in the NOD mouse at 4hr post-LPS when compared to its relative control ($P<0.05$), and to CD1 at the same time point ($P<0.05$). By 24hr, expression had returned to basal levels (Table 4.3).

Figure 4.3 – Strain and LPS dependent differences in hypothalamic target gene mRNA expression.

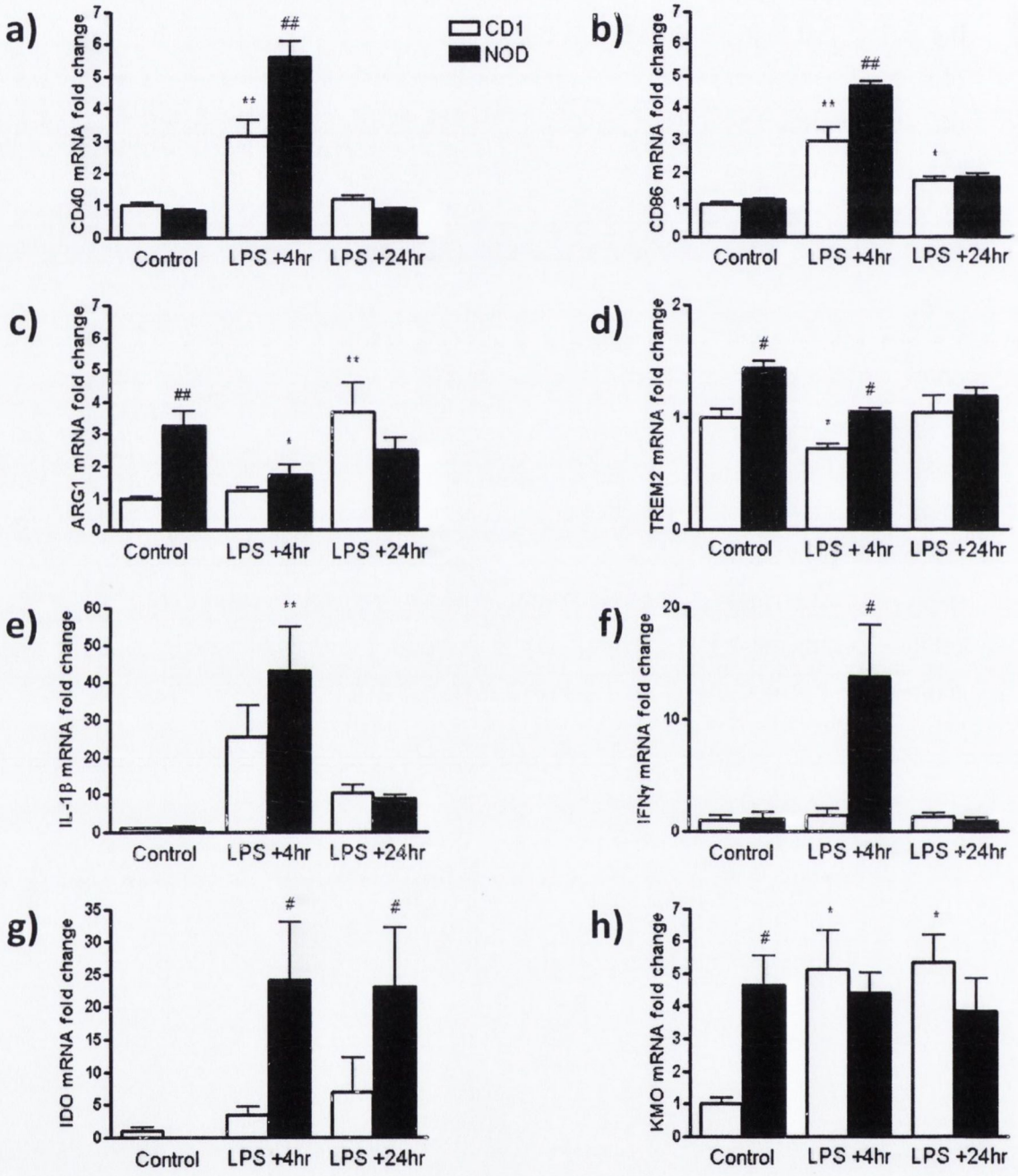

Figure 4.3 – Strain and LPS dependent differences in hypothalamic target gene mRNA expression.

LPS-induced a greater induction of CD40 and CD86 in the NOD mouse at 4hr post-LPS (a, b). Differential effects of both strain and LPS were seen in the expression levels of the alternative activation markers ARG1 and TREM2 (c, d). Increased expression of the pro-inflammatory markers IL-1β and IFNγ were found in the NOD mouse 4hr post-LPS (e, f) and increases in LPS-induced expression of IDO were only observed in the NOD mouse (g). Increased expression of KMO was seen in the NOD mouse at steady state, however LPS-induced effects on KMO expression were only seen in the CD1 mouse (h). Data expressed as mean ± SEM, n=5-6 per group. **P<0.01, *P<0.05, LPS versus control. $^{\#\#}$P<0.01, $^{\#}$P<0.05, NOD vs. CD1.

	Control		LPS + 4hours		LPS + 24 hours	
	CD1	NOD	CD1	NOD	CD1	NOD
CD40	1 ± 0.06	0.8 ± 0.04	**3.1 ± 0.5** *	***5.8 ± 0.5*** *	1.2 ± 0.1	0.86 ± 0.03
CD86	1 ± 0.07	1.1 ± 0.04	**2.9 ± 0.43** *	***4.7 ± 0.13*** *	1.7 ± 0.11	1.8 ± 0.1
CD68	1 ± 0.03	1.0 ± 0.09	1.1 ± 0.15	0.9 ± 0.04	**2.1 ± 0.11** *	**1.9 ± 0.05** *
CD11b	1 ± 0.06	0.9 ± 0.07	1.0 ± 0.09	0.94 ± 0.05	**2.4 ± 0.13** *	**2.2 ± 0.08** *
TREM1	1 ± 0.2	1.0 ± 0.2	**31.8 ± 8.9** *	**26.5 ± 2.8** *	3.1 ± 0.7	2.4 ± 0.35
ARG1	1 ± 0.07	*3.3 ± 0.5* *	1.2 ± 0.12	**1.7 ± 0.3** *	**3.7 ± 0.9** *	2.5 ± 0.4
TREM2	1 ± 0.06	*1.4 ± 0.06* *	**0.7 ± 0.4** *	***1.1 ± 0.04*** *	1.0 ± 0.15	1.2 ± 0.06
TNFα	1 ± 0.2	0.9 ± 0.3	**23.1 ± 9.4** *	**28.4 ± 6.9** *	12.9 ± 2	10.6 ± 1.1
IL-1β	1 ± 0.15	1.2 ± 0.19	25.5 ± 8.3	***43.1 ± 11.8*** *	10.5 ± 2.1	8.9 ± 0.9
IL-6	1 ± 0.16	0.8 ± 0.18	**25.1 ± 10.8** *	**39.9 ± 9.8** *	2.3 ± 0.7	1.3 ± 0.2
IFNγ	1 ± 0.4	1.0 ± 0.6	1.4 ± 0.6	***13.8 ± 4.6*** *	1.3 ± 0.3	0.8 ± 0.3
iNOS	1 ± 0.06	1.1 ± 0.06	3 ± 0.73	***5.2 ± 1.7*** *	1.2 ± 0.14	1.1 ± 0.06
IDO	1 ± 0.5	not detected	3.5 ± 1.2	*24.1 ± 8.9* *	6.9 ± 5.2	*22.9 ± 9.2* *
KMO	1 ± 0.18	*4.7 ± 0.9* *	**5.2 ± 1.2** *	4.4 ± 0.64	**5.4 ± 0.85** *	3.8 ± 0.9
KAT-II	1 ± 0.09	1.2 ± 0.04	1.1 ± 0.17	1.3 ± 0.08	1.2 ± 0.05	1.1 ± 0.08
VEGF	1 ± 0.08	1.2 ± 0.14	1 ± 0.19	1.4 ± 0.11	1.1 ± 0.11	1.1 ± 0.09
BDNF	1 ± 0.16	1.0 ± 0.13	0.8 ± 0.09	0.7 ± 0.08	0.9 ± 0.08	0.8 ± 0.08
FGF	1 ± 0.03	1.1 ± 0.04	**1.5 ± 0.13** *	**1.6 ± 0.08** *	1.0 ± 0.04	1.0 ± 0.06
PDGF	1 ± 0.05	1.1 ± 0.04	1 ± 0.11	***0.7 ± 0.08*** *	1.0 ± 0.06	0.9 ± 0.04

Table 4.3 – Hypothalamic mRNA expression of target genes in CD1 and NOD mice at steady state and in response to LPS

Data expressed as mean ± SEM. * indicates a significant difference. Bold indicates significantly different to respective controls. Italics indicate NOD significantly different to CD1. Bold italics indicate NOD significantly different to CD1 and its respective control. (normalised fold expression relative to CD1 control)

4.3.10 NOD mice display a persistent elevation in corticosterone but no change in glucose concentration in response to an acute restraint stress when compared to the CD1 strain

In this present experiment CD1 and NOD mice were subjected to an acute 2hr restraint stress and euthanised immediately or 2hr post stress. The effect of this psychological stressor on circulating concentrations of corticosterone and glucose were investigated.

4.3.10.1 NOD mice have a prolonged elevation of corticosterone in response to an acute restraint stress relative to CD1 mice

Two-way ANOVA revealed an effect of interaction between the strain of mouse and stress ($F_{(2, 30)}$=4.604,P=0.018) on circulating serum levels of corticosterone (a). Post-hoc comparison revealed an increase in the circulating levels of corticosterone immediately following stress in both NOD (P<0.01) and CD1 (P<0.01) mice when compared to non stressed controls. Corticosterone concentrations remained elevated in NOD mice when compared to CD1 controls of the same time point (P<0.05). [Figure 4.4, n=6]

4.3.10.2 NOD mice have an impaired glucose response to an acute restraint stress relative to CD1 mice

Two-way ANOVA revealed an effect of interaction between the strain of mouse and stress ($F_{(2, 29)}$=5.64, P<0.01) on circulating levels of glucose (b). Post-hoc comparison revealed an increase in circulating levels of glucose in CD1 mice immediately following stress (P<0.05) when compared to non stressed controls. NOD mice showed no increase in glucose concentration in response to stress. [Figure 4.4, n=6]

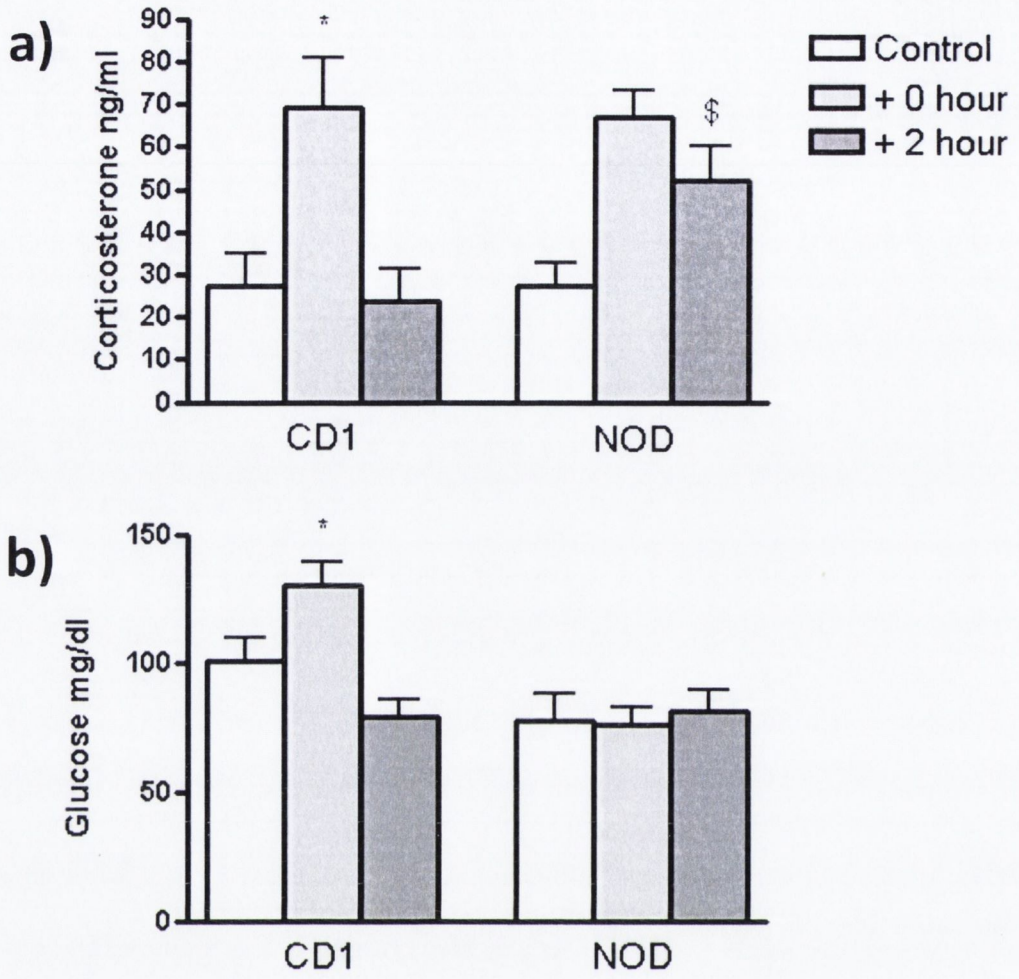

Figure 4.4 – NOD mice exhibit a delayed return to basal corticosterone levels and no glucose response following exposure to a psychological stressor

In response to an acute 2hr restraint stress a significant increase in (a) corticosterone was noted immediately post stress in the both CD1 and NOD mice. A delayed return to basal levels of corticosterone was observed in NOD mice compared to CD1 mice. (b) Glucose levels increased in the CD1 mice immediately post stress and returned to baseline 2hr later, however NOD glucose levels were unaffected by stress. Data expressed as mean ± SEM (n=6). * $P<0.05$ vs. CD1 non-stressed control; $\$<0.05$ vs. CD1 time point (Two-Way ANOVA, Newman Keuls post-hoc test).

4.3.11 Concentrations of 5-HT and 5HIAA vary in different brain regions in NOD and CD1 mice

Considering previous results, which identified an inhibited glucose response immediately post stress and a prolonged elevation of corticosterone 2hr following stress exposure, the current experiment aimed to assess the effect a psychological stressor on the concentrations of 5-HT and its metabolite 5HIAA (5-Hydroxyindoleacetic acid) in the brain.

4.3.11.1 Stress does not affect 5-HT metabolism in the amygdala

Two-way ANOVA showed an effect of strain ($F_{(1, 29)}$=6.0586, P<0.05) on the concentration of 5HIAA (b) and an effect of stress ($F_{(2, 29)}$=4.74, P<0.05) on the ratio of 5HIAA/5-HT (c) in the amygdala of NOD mice. Post-hoc comparisons could not determine differences between groups, however a trend increase in 5HIAA and the ratio of 5HIAA/5-HT was observed in NOD mice. Two-way ANOVA showed no significant effect of stress or strain on the concentration of 5-HT (a) in the amygdala of NOD and CD1 mice. [Figure 4.5, n=5-6]

4.3.11.2 Stress induces an increase in 5HIAA/5-HT ratio in the hippocampus

Two-way ANOVA showed an effect of strain ($F_{(1, 30)}$=31.44, P<0.001) and stress ($F_{(2,30)}$=7.63, P<0.01) on the concentration of 5HIAA (e) in the hippocampus of NOD and CD1 mice. Post-hoc comparison revealed an increase in 5HIAA immediately following a 2hr restraint stress when compared to non stressed controls (P<0.01). [Figure 4.5, n=6]

Two-way ANOVA showed an effect of strain ($F_{(1, 30)}$=25.11, P<0.001) and stress ($F_{(2,30)}$=3.83, P<0.05) on the concentration of 5-HT (d) in the hippocampus. Post-hoc comparison revealed a significantly higher concentration of 5-HT in the hippocampus of NOD mice when compared to CD1 mice under control conditions (P<0.01) and immediately following stress. 5-HT concentrations reduced 2hr following acute stress when compared to non stressed controls (P<0.05). [Figure 4.5, n=6]

Two-way ANOVA showed an effect of strain ($F_{(1, 30)}$=5.10, P<0.05) and stress ($F_{(2, 30)}$=10.14, P<0.001) on the ratio of 5HIAA/5-HT (f) in the hippocampus. Post-hoc

comparison revealed an increase in the ratio of 5HIAA/5-HT immediately following an acute restraint stress in the hippocampus of NOD mice only ($P<0.01$) when compared to non stressed controls. [Figure 4.5, n=6]

4.3.11.3 Stress does not affect 5-HT metabolism in the frontal cortex

Two-way ANOVA showed no effect of stress or strain on the concentration of 5-HT (g), 5HIAA (h) or the ratio of 5HIAA/5-HT (i) in the frontal cortex of NOD and CD1 mice. [Figure 4.5, n=6]

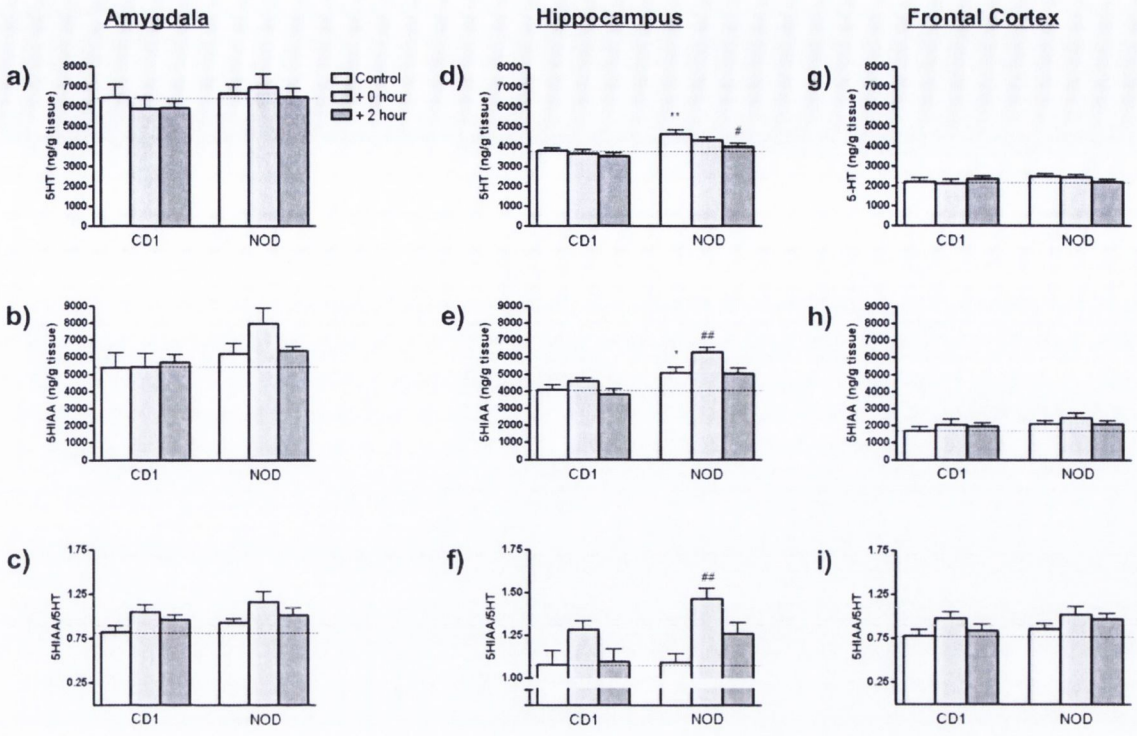

Figure 4.5 – NOD mice show enhanced 5-HT metabolism in the hippocampus in response to stress

Stress induced an increase in the concentrations of 5HIAA in the hippocampus of NOD mice which was accompanied by an increase in the ratio of 5HIAA/5-HT. Similar trends were also present in the amygdala of NOD mice also. NOD mice also showed a reduction in the concentration of 5-HT 2hr following stress exposure. No changes were recorded in the frontal cortex in either 5-HT or 5HIAA. Data expressed as mean ± SEM (n=5-6), ** $P<0.01$, vs. CD1 non-stressed controls, # $P<0.05$, ## $P<0.01$ vs. NOD control (Two-way ANOVA, Newman Keuls post-hoc test).

4.3.12 Stress provokes an increase in cortical IL-6 expression in CD1 and NOD mice

In this current experiment the mRNA expression of the pro-inflammatory cytokines interleukin (IL-) 1β and IL-6 and the microglial marker CD11b were examined to see whether there was a difference in the expression of these markers between strains in response to a 2hr restraint stress in the brain. Mice were euthanized immediately or 2hr following the stress, and mRNA extracted from the hypothalamus, frontal cortex and hippocampus.

4.3.12.1 Stress does not influence target gene expression in the hypothalamus

Two-way ANOVA showed no effect of stress or strain on the mRNA expression of IL-1β (a), IL-6 (b) and CD11b (c) in NOD and CD1 mice in the hypothalamus. [Figure 4.6, n=5-6]

4.3.12.2 Stress does not influence IL-1β or CD11b in the frontal cortex although raised IL-1β expression was evident in NOD compared to CD1 mice at steady state

Two-way ANOVA showed an effect of strain on the mRNA expression of IL-1β ($F_{(1, 29)}$=28.93, P<0.001) in the frontal cortex (d). Post-hoc comparison revealed a higher level of mRNA expression of IL-1β at a steady state in NOD mice (P<0.01) and this remained higher both immediately (P<0.05) and 2hrs (P<0.05) following 2hr restraint stress. Two-way ANOVA showed no significant effect of stress or strain on the mRNA expression of CD11b in NOD and CD1 mice in the frontal cortex (f). [Figure 4.6, n=5-6]

4.3.12.3 Stress provokes an increase in cortical IL-6 expression in CD1 and NOD mice

Two-way ANOVA showed an effect of stress on the mRNA expression of IL-6 ($F_{(2, 29)}$=8.01, P<0.01) in the frontal cortex (e). Post-hoc comparison revealed an increase in the mRNA expression of IL-6 immediately following 2hr restraint stress in both NOD (P<0.05) and CD1 (P<0.01) mice when compared to non

stressed controls. This mRNA expression remained elevated 2hr following restraint stress in both NOD ($P<0.05$) and CD1 ($P<0.05$) mice. [Figure 4.6, n=5-6]

4.3.12.4 IL-1β mRNA expression in the hippocampus was reduced in NOD mice in response to stress

Two-way ANOVA showed an effect of stress of the mRNA expression of IL-1β ($F_{(2, 29)}=6.23$, $P<0.01$) in the hippocampus (g). Post-hoc comparison revealed a reduction in the mRNA expression of IL-1β in NOD mice immediately following 2hr restraint stress ($P<0.01$) when compared to non stressed controls. Two-way ANOVA showed no significant effect of stress or strain on the mRNA expression of IL-6 (h) and CD11b (i) in NOD and CD1 mice in the hippocampus. [Figure 4.6, n=5-6]

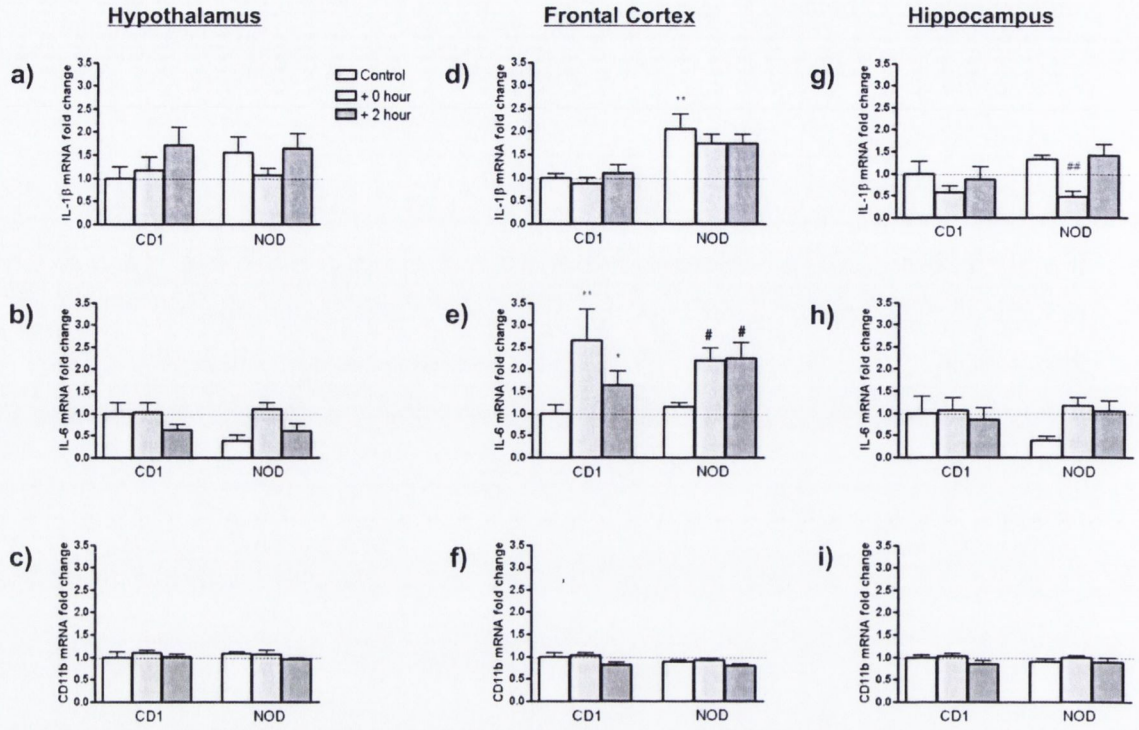

Figure 4.6 – Stress provokes an increase in cortical IL-6 expression in CD1 and NOD mice

In response to a restraint stress, expression of IL-6 was increased immediately following stress in both NOD and CD1 mice (e). Expression of IL-1β in the frontal cortex was greater in NOD mice at basal levels and remained unchanged following stress (d). Expression of IL-1β was reduced following stress in the hippocampus (g). Expression of CD11b was unchanged following stress in all regions in both strains (c, f, i). Data expressed as mean ± SEM (n=5-6), * P<0.05, ** P<0.01 vs. CD1 non-stressed controls, ##<0.01 vs. NOD non-stressed controls (Two-way ANOVA, Newman-Keuls post-hoc test).

4.3.13 Stress induces changes in expression of glucocorticoid related genes in the hypothalamus, hippocampus and liver

In this current experiment mRNA expression of the glucocorticoid receptor (GR) and the glucocorticoid related genes FK506 binding protein 5 (FKBP5), serum- and glucocorticoid-induced protein kinase 1 (SGK-1) and glucocorticoid-induced leucine zipper (GILZ) were examined to see whether there was a difference between NOD and CD1 mice in response to a 2hr restraint stress. Mice were euthanized immediately or 2hr post stress, and mRNA extracted from the hypothalamus, and hippocampus.

4.3.13.1 mRNA expression of the glucocorticoid receptor was reduced in the hippocampus in response to psychological stress

Two-way ANOVA showed no significant effect of stress or strain on the mRNA expression of GR in NOD and CD1 mice in the hypothalamus.

Two-way ANOVA showed an effect of stress on the mRNA expression of GR ($F_{(2, 29)}$=10.67, P<0.001) in the hippocampus. Post-hoc comparison revealed a decrease in the mRNA expression of GR immediately following a 2hr restraint stress in CD1 and NOD mice (P<0.05), which remained decreased 2hr post stress (P<0.05) when compared to non stressed controls.

Two-way ANOVA showed no significant effect of stress or strain on the mRNA expression of GR in NOD and CD1 mice in the liver. [Figure 4.7, n=5-6]

4.3.13.2 mRNA expression of FKBP5 is increased in response to psychological stress – comparison between NOD and CD1 strains

Two-way ANOVA showed an effect of strain ($F_{(1,30)}$=8.52, P<0.01) and stress ($F_{(2,30)}$=100.02, P<0.001) on the mRNA expression of FKBP5 in the hypothalamus. The interaction between stress and strain was not significant ($F_{(2,30)}$=3.2, P=0.0549). Post-hoc comparison revealed an increase in the mRNA expression of FKBP5 immediately following 2hr restraint stress in both NOD (P<0.01) and CD1 (P<0.01) mice when compared to non stressed controls. This mRNA expression remained elevated 2hr post stress in both NOD (P<0.01) and

CD1 ($P<0.01$) mice. Immediately following stress, the mRNA of FKPB5 in the hypothalamus of NOD mice was higher than that of CD1 mice ($P<0.01$).

Two-way ANOVA showed an effect of interaction between stress and strain ($F_{(2, 29)}=4.56$, $P<0.05$) on the mRNA expression of FKBP5 in the hippocampus. Post-hoc comparison revealed an increase in the mRNA expression of FKBP5 immediately following 2hr restraint stress in both NOD ($P<0.01$) and CD1 ($P<0.01$) mice when compared to non stressed controls. This mRNA expression remained elevated 2hr post stress in both NOD ($P<0.01$) and CD1 ($P<0.01$) mice. Immediately following stress, the mRNA of FKBP5 in the hippocampus of NOD mice was higher than that of CD1 mice ($P<0.05$).

Two-way ANOVA showed an effect of interaction between stress and strain ($F_{(2,30)}=6.02$, $P<0.01$) on the mRNA expression of FKBP5 in the liver. Post-hoc comparison revealed an increase in the mRNA expression of FKBP5 immediately following 2hr restraint stress in both NOD ($P<0.01$) and CD1 ($P<0.01$) mice when compared to non stressed controls. This mRNA expression remained elevated 2hr post stress in both NOD ($P<0.01$) and CD1 ($P<0.01$) mice. Immediately following stress, the mRNA of FKBP5 in the hippocampus of NOD mice was higher than that of CD1 mice ($P<0.01$). [Figure 4.7, n=5-6]

4.3.13.3 mRNA expression of SGK-1 is increased in response to psychological stress – comparison between NOD and CD1 strains

Two-way ANOVA showed an effect of stress ($F_{(2, 30)}=71.18$, $P<0.001$) on the mRNA expression of SGK-1 in the hypothalamus. Post-hoc comparison revealed an increase in the mRNA expression of SGK-1 immediately following 2hr restraint stress in both NOD ($P<0.01$) and CD1 ($P<0.01$) mice when compared to non stressed controls.

Two-way ANOVA showed an effect of stress ($F_{(2, 29)}=58.31$, $P<0.001$) on the mRNA expression of SGK-1 in the hippocampus. Post-hoc comparison revealed an increase in the mRNA expression of SGK-1 immediately following 2hr restraint stress in both NOD ($P<0.01$) and CD1 ($P<0.01$) mice. [Figure 4.7, n=5-6]

4.3.13.4 mRNA expression of GILZ is increased in response to psychological stress – comparison between NOD and CD1 strains

Two-way ANOVA showed an effect of stress ($F_{(2, 30)}$=27.51, P<0.001) on the mRNA expression of GILZ in the hypothalamus. Post-hoc comparison revealed an increase in the mRNA expression of GILZ immediately following 2hr restraint stress in both NOD (P<0.01) and CD1 (P<0.01) mice when compared to non stressed controls. GILZ mRNA expression was still elevated in the hypothalamus of the NOD mice 2hr following stress (P<0.05).

Two-way ANOVA showed an effect of stress ($F_{(2, 29)}$=28.03, P<0.001) on the mRNA expression of GILZ in the hippocampus. Post-hoc comparison revealed an increase in the mRNA expression of GILZ immediately following a 2hr restrain stress in both NOD (P<0.01) and CD1 (P<0.01) mice when compared to non stressed controls. This mRNA expression remained increased 2hr following stress in both NOD (P<0.01) and CD1 (P<0.01) mice. [Figure 4.7, n=5-6]

4.3.13.5 mRNA expression of TDO in the liver is increased in response to psychological stress – comparison between NOD and CD1 strains

Two-way ANOVA showed an effect of interaction between stress and strain ($F_{(2, 30)}$=3.88, P<0.05) on the mRNA expression of TDO in the liver. Post-hoc comparison revealed an increase in the mRNA expression of TDO immediately following 2hr restraint stress in both NOD (P<0.01) and CD1 (P<0.05) mice when compared to non stressed controls. This remained significantly elevated 2hr post stress in both NOD (P<0.01) and CD1 (P<0.01) mice. Immediately following stress, the mRNA of TDO in the liver of NOD mice was higher than that of CD1 mice (P<0.05). [Figure 4.7, n=5-6]

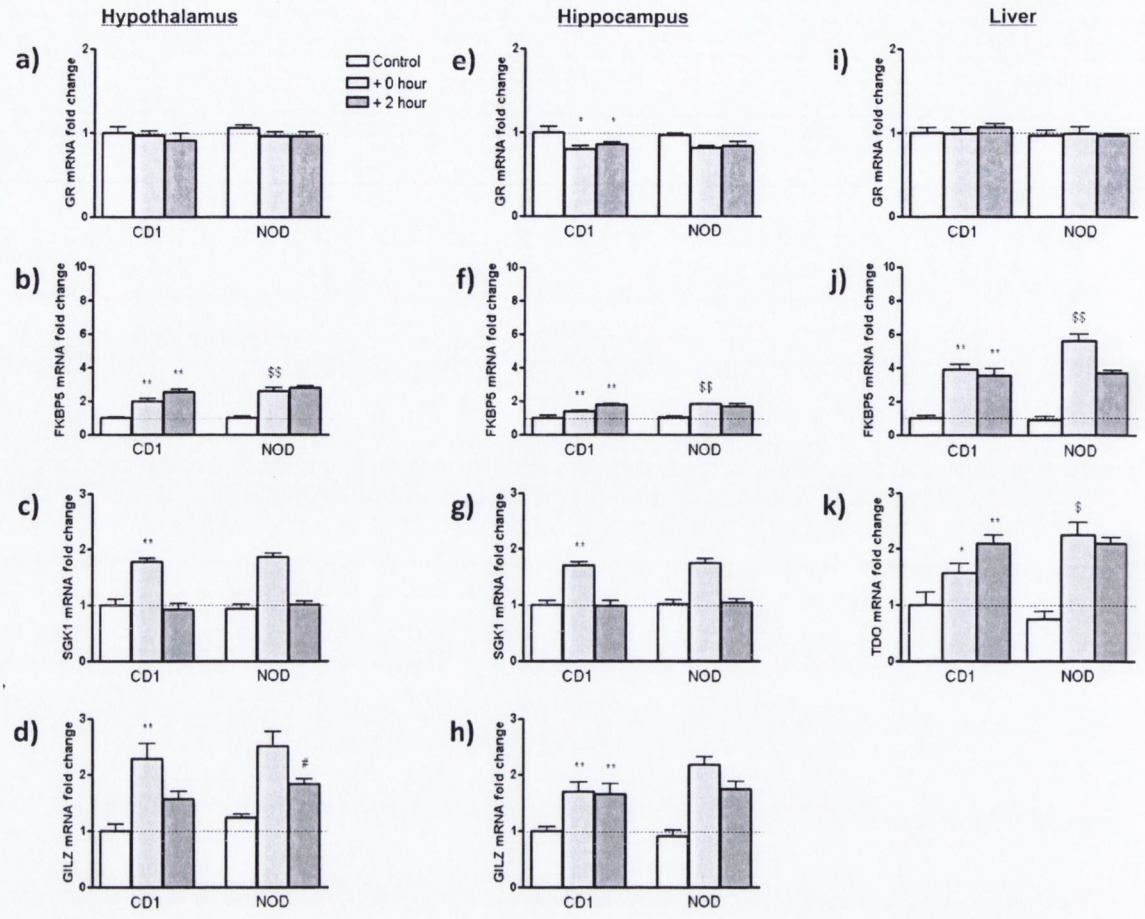

Figure 4.7 – Stress induces a change in mRNA expression in glucocorticoid related and inducible genes

In response to a restraint stress, the expression of GR did not change in the hypothalamus (a) or liver (i) but did reduce in the hippocampus (e). Expression of FKBP5 increased post stress in the hypothalamus (b), hippocampus (f) and liver (j). NOD mice had a greater mRNA increase immediately post stress than CD1 mice. Expression of SGK1 increased immediately post stress in both strains in the hypothalamus (c) and hippocampus (g). Expression of GILZ increased following stress and remained elevated in the NOD mouse 2hr post stress in the hypothalamus (d) and in both strains in the hippocampus (h). TDO expression was increased in both NOD and CD1 mice following stress (k), however expression immediately post stress was higher in NOD than CD1 mice. Data expressed as mean ± SEM (n=5-6), * $P<0.05$, ** $P<0.01$ vs. CD1 non-stressed controls, #<0.05 NOD vs. NOD control, $ $P<0.05$, $$ $P<0.01$ vs. CD1 time point (Two-way ANOVA, Newman-Keuls post-hoc test).

4.4 Discussion

4.4.1 NOD mice display altered behaviour at steady state and in response to LPS when compared to the CD1 strain

The elevated plus maze (EPM) and open field test (OFT) are behavioural tests routinely used to assess the anxious phenotype of rodents by exploiting their natural aversion to exposed environments (Stanford, 2007, Carola et al., 2002). Mice with higher levels of anxiety are prone to remaining in enclosed areas of the EPM, or the edges of open field arenas. In the present study, the NOD mice displayed a greater anxious phenotype in the EPM as indicated by the decreased entries and time spent in the open arms of the maze compared to the CD1 control strain. There was also a significant reduction in distance moved in the inner zone of the OFT by the NOD mice when compared to CD1 mice which again is indicative of an anxious phenotype. This was observed without a difference in the total distance travelled in the arena between strains suggesting that it was not specifically related to a reduction in overall movement.

Behavioural despair in animal models can be determined by exposing the test subject to an inescapable stress, and can be used to model depressive like phenotypes (Cryan et al., 2002). Animals with depressive-like symptoms will become immobile much quicker and for a longer duration than non-depressive or control mice (Cryan et al., 2005, Mason et al., 2009). Accordingly our results showed that the NOD mice spent more time immobile in the TST suggesting that this strain exhibits a more depressive-like phenotype in this paradigm.

The NOD mouse also showed exaggerated sickness behaviour when exposed to an acute immune stimulus (LPS), as indicated by an excessive weight loss and an exaggerated reduction in activity in the home cage. In addition, a behavioural effect of LPS was also seen in the home cage. Rearing is considered a measure of exploratory behaviour and reduced exploration can be indicative of anxiety in animals (Cryan and Holmes, 2005). Accordingly rearing was reduced in both strains at 4hr post-LPS but remained significantly reduced in the NOD mouse at 24hr. These findings are supported by previous studies where an altered anxiety-like behavioural phenotype was found when NOD mice were exposed to an immune stimulus (Amrani et al., 1994b, Bluth et al., 1999b).

4.4.2 NOD monocytes are impaired at steady state but are hyper-responsive to LPS when compared to the CD1 strain

As previously reviewed, a link exists between an aberrant monocyte phenotype and mood disorders in humans, at least in a subset of patients (Beumer et al., 2012, Drexhage et al., 2011b, Gibney and Drexhage, 2013). In addition, several previous studies have shown that NOD mice have an altered monocyte profile both at steady state and in response to stimulation in vitro (Nikolic et al., 2005). We therefore wanted to determine whether the behavioural alterations observed in the NOD mouse were paralleled by an altered monocyte genotype in vivo at steady state and in response to LPS. At steady state the predominant genotype of the NOD monocytes was impaired development. Interestingly, impaired development is a phenomenon that has previously been reported in bone marrow precursor cells and pancreatic dendritic cells in the NOD mouse (Welzen-Coppens et al., 2012, Nikolic et al., 2004). In addition, while minimal differences were found in the inflammatory profile of the mature monocytes when strains were compared, there was a large down regulation of some pro-inflammatory genes in the immature NOD population at steady state when compared to CD1, suggesting an impaired inflammatory genotype in this population.

It has been previously shown that NOD macrophages show an enhanced production of inflammatory cytokines upon stimulation (Stoffels et al., 2004). Accordingly, NOD monocytes in the present study were hyper-responsive to LPS, however there were some differences in the response of the immature and mature monocytes. At 4hr post-LPS, while neither population differed extensively when the NOD and CD1 monocytes were compared, both were characterised by a strong type-1 IFN genotype when genes unique to the NOD were examined, which was particularly evident in the mature monocytes. A type-1-IFN induced gene expression signature has been found before in NOD mice and treatment with a neutralizing antibody against the type-1 IFN receptor (IFNAR) delayed onset and reduced diabetes incidence (Li et al., 2008). At 24hr, while the increased inflammatory response of the immature NOD monocytes was limited to a few genes, the inflammatory response of the mature NOD monocytes was very robust. It was characterised by large increases in the expression of classic pro-inflammatory markers such as IL-1β, IL-6 and IFNγ but also by large increases in genes involved in type-1 IFN signalling. Interestingly, in 2 large recent meta-

analysis studies, IL-1 and IL-6 were two of the markers that positively associated with depressive symptoms in patients (Howren et al., 2009, Dowlati et al., 2010). Furthermore, a role for type-I IFN and especially IFNα in depression has gained much interest primarily due to the observation that IFNα immunotherapy can induce depressive symptoms in otherwise psychiatrically normal individuals (Alavi et al., 2012b, Capuron et al., 2001, Capuron et al., 2004, Majer et al., 2008, Raison et al., 2005a, Raison et al., 2005b, Schaefer et al., 2012, Udina et al., 2013). It is of interest that the most robust effect of LPS was seen in the mature monocytes as the NOD mouse has previously been shown to have an abnormally large population of these mature circulating monocytes compared to several control strains (Nikolic et al., 2005).

4.4.3 NOD microglia show impaired function at steady state and are hypo-responsive to LPS with a type-1 IFN skewed genotype when compared to the CD1 strain

As it has been repeatedly shown that microglia are involved in the development of many anxiety-like and depressive-like behaviours in rodents (Beumer et al., 2012), we also wanted to determine whether the genotype of microglia derived from NOD mice was divergent when compared with those derived from the control CD1 strain. It was observed that at steady state, microglia in the NOD mouse have an altered gene profile characterised by a differential expression of genes involved in neuronal support. When the top 20 genes altered in NOD microglia were compared to CD1 control, it was found that 65% of these genes were involved in neuronal function. For example, overexpression of GADD45A (the gene with the highest fold increase in expression in the NOD mouse compared to CD1), leads to impaired neurite outgrowth as well as increased neuronal apoptosis (Choi et al., 2011, Sarkisian and Siebzehnrubl, 2012). Several other dysregulated genes in the NOD mouse at steady state are also involved in regulating neurite growth and apoptosis including NEO1, STX1A, PA2G4, TIA1, ABCB1B and H60A (Hill et al., 2012, Kim et al., 2005, Kwon and Ahn, 2011, Lowin et al., 1996, Schumacher et al., 2012, Zhou et al., 2000). It has been well documented that microglia play a role in the normal physiological processes of synaptogenesis and neurogenesis both during development and in the adult brain (Tremblay et al., 2011b). If these normal physiological processes become dysregulated, as may occur in the NOD mouse due to the impaired

function of the genes mentioned above, the homeostasis can be disturbed and potentially lead to exaggerated synaptic stripping and neuro-degeneration and/or impaired neurogenesis. This is supported by the previous observation of reduced hippocampal neurogenesis in the NOD mouse, characterised by reduced cell proliferation and impaired neuronal survival (Beauquis et al., 2008a, Beauquis et al., 2008b, Tremblay et al., 2011b). However, a direct correlation of the genes identified in this study with these physiological processes remains to be elucidated.

Interestingly, two of the top up-regulated genes in the NOD microglia at steady state are involved in type-1 IFN signalling, IRF3 and SP110. IRF3 is a transcription factor that is integral to type-1 IFN signalling (Honda and Taniguchi, 2006, Taniguchi et al., 2001) and SP110 is a type-1-IFN inducible gene (Kadereit et al., 1993). It is known that IRF-3 is central to both TLR-3 and MYD88-independent TLR-4 signalling resulting in the induction of type-1-IFNs, and has been shown to be essential for LPS-induced IFNβ production (Sakaguchi et al., 2003). The up regulation of these genes in the NOD mouse compared to the CD1, in the absence of any exogenous inflammatory stimulus however, suggest that the type-1-IFN signalling pathway may be innately hyperactive in NOD mouse microglia. (Li et al., 2008).

NOD microglia also seem to be less responsive to LPS stimulation with a lower number of genes altered at both 4hr and 24hr post LPS than is observed in the CD1 mouse. Furthermore, CD1 microglia display a 'classic' pro-inflammatory response to LPS characterised by the up-regulation of several chemokine genes, interleukin genes, TNFα, and the TLR4 adaptor protein MYD88, whereas the NOD microglia were more characterised by differential expression of genes involved in type 1 IFN signalling. In addition, there was no detectable LPS-induced increase in MYD88 gene expression in NOD microglia which may explain their differential inflammatory response to LPS stimulation. LPS preferentially signals through MYD88, inducing the production of pro-inflammatory cytokines such as interleukins and TNFα (Takeda and Akira, 2004), as is observed in the CD1 microglia. However, as TLR4 can also signal independently of MYD88 through IRF3 (one of the genes that was highly up-regulated in the NOD at steady state) leading to the production of type-1 IFN (Sakaguchi et al., 2003, Takeda and Akira, 2004), it is plausible to suggest that this is the TLR4 signalling pathway active in NOD mice, at least in microglia.

Previous studies have shown that this MYD88-indpependent pathway results in a delayed induction of NFkB and thus a delayed downstream response (Kawai et al., 2001, Selvarajoo, 2006). It is not surprising therefore that at 24hr post LPS, there was a further induction of –type-1 IFN genes in NOD microglia at this later time point. In addition, they also display an increase in chemokine genes that were found to be up-regulated in the CD1 microglia at the 4hr time-point, again supporting the putative delayed kinetics of this alternative TLR-4 pathway. Once again the type-1 IFN response was not overt in CD1 microglia at 24hr post LPS, where expression of classic interleukins still characterised the inflammatory profile of these cells. Several studies have linked the neuro-modulatory effects of IFNα to kynurenine pathway activation and the subsequent production of the neurotoxic downstream metabolite quinolinic acid (QUIN) (Baranyi et al., 2013, Raison et al., 2010, Wichers et al., 2005), which in the brain occurs primarily in microglia (Guillemin et al., 2005), and may be one way in which the NOD microglia may be influencing the altered behaviour observed.

4.4.4 NOD mice show evidence of a pro-inflammatory phenotype, kynurenine pathway activation and impaired growth factor support in the hypothalamus

To determine whether the genotype observed in the microglia was paralleled in the brain in general we looked at mRNA expression of several inflammatory markers, growth factors and kynurenine pathway genes in the hypothalamus. When isolated LPS-stimulated microglia were examined for genes involved in growth factor support, it was found that several genes involved in growth factor signalling were impaired in NOD microglia compared to CD1 (data not shown). Therefore, expression of growth factor genes was also measured in the hypothalamus of these animals. While expression of BDNF and VEGF did not change in any group analysed, there was a clear induction of FGF in both strains in response to LPS. Moreover, there were strain dependent changes in the LPS-induced expression level of PDGF, which has been shown to have neurotrophic functionality (Smits et al., 1991), with decreased expression found in the NOD mouse in response to immune stimulation, lending further support to the hypothesis that NOD microglia have impaired neuronal support.

Interestingly, at steady state, the NOD mouse seems to be characterised by an 'M2' genotype with the up-regulation of ARG1 and TREM2 (Ydens et al., 2012,

Gordon and Taylor, 2005), compared to CD1 mice. As no strain dependent differences were observed in the mRNA expression of pro-inflammatory markers, it potentially suggests a skewed anti-inflammatory phenotype in the NOD mouse at steady state. The expression of these genes, or any other M2 markers, were not observed in the isolated microglia, however, microglial isolation was performed on the whole brain (excluding the hypothalamus), and may highlight discrete regional differences in the phenotype of these cells in the brain.

As expected, all of the pro-inflammatory markers measured were induced by LPS, however there were some strain and time-dependent changes in the expression of some of these markers. Expression of CD40 and CD86 for example were enhanced in the NOD compared to CD1 4hr post LPS exposure. CD40 is an important regulator of microglial activation and serves as an amplifier of pro-inflammatory responses in the CNS (Benveniste et al., 2004), and although it is found expressed on other cell types in the brain such as astrocytes, microglia are the most sensitive to the CD40 ligand (Chen et al., 2006). In addition, while LPS-induced expression of the pro-inflammatory cytokines TNFα and IL-6 did not differ between strains, expression of IL-1β was enhanced in the NOD mouse in response to immune stimulation. Once again these findings do not directly correlate with what was observed in the isolated microglia as LPS-induced TNFα expression was unique to the CD1 strain in these isolated cells. Furthermore, there was also a trend for higher expression of IL-1β in the CD1 microglia compared to NOD microglia. As previously mentioned, this may reflect regional differences in microglia but it also cannot be ruled out that these pro-inflammatory cytokines may be expressed in other cells types in the hypothalamus such as astrocytes (Chung and Benveniste, 1990, Didier et al., 2003), and highlights the sensitive interaction between various cell types in the brain. Astrocytes have been reported to exhibit immune functions, including cytokine production, phagocytosis and antigen presentation and astrogliosis has been reported in NOD mice (Saravia et al., 2002, Ransohoff and Brown, 2012, Nguyen et al., 2011). Investigation into the astrocytic profile and response to an LPS stimulus in NOD mice could allow for confirmation of the role the microglia play in the differences observed between isolated microglia and whole hypothalamic tissue

A very robust LPS-induced increase in IFNγ and iNOS expression was also found in the NOD hypothalamus when compared to CD1 further supporting a heightened inflammatory response of the NOD mouse to LPS-stimulation. Furthermore, IFNγ is one of the most potent inducers of the kynurenine pathway (KP) enzyme IDO (Leonard, 2007). Accordingly, a large increase in the expression of IDO was found in the NOD hypothalamus at 4hr post LPS which remained elevated at 24hr. It has been hypothesised that the delayed depressive-like behaviours observed in animal models that occur after the initial sickness behaviour induced by LPS may be due to IDO and kynurenine pathway activation (Dantzer et al., 2011). This is supported by the fact that LPS-induced depressive behaviours can be blocked by pharmacological inhibition of IDO (O'Connor et al., 2009c). Interestingly, expression of the KP enzyme KMO, which is expressed mainly by microglia in the brain, is significantly up-regulated in the NOD hypothalamus at steady state. This is one of the enzymes that facilitates metabolism of kynurenine to QUIN suggesting that NOD mice may be primed for preferential metabolism of kynurenine via the neurotoxic arm of this pathway. This is supported by the fact that expression of KAT-II, which is primarily expressed in astrocytes, and is involved in the metabolism of kynurenine via the neuroprotective arm of the pathway, does not change in the NOD mouse.

4.4.5 NOD mice exhibit impaired glucocorticoid concentration reduction and a muted glucose response following restraint stress

In the present study, NOD and CD1 mice had a similar maximal increase in corticosterone levels in response to restraint stress, as was shown in previous studies comparing NOD to C57BL/6 mice (Amrani et al., 1994b). However, while the corticosterone levels of CD1 mice had returned to basal levels 2hr post stress, the CORT levels of the NOD mice remained elevated. This delay in return to basal concentrations could be indicative of dysregulated HPA-axis negative feedback in the NOD mice following a psychological stress. In depressive disorders a common state observed in patients is the presence impaired GR feedback to the HPA-axis and glucocorticoid resistance (McKay and Zakzanis, 2010). Furthermore, an indicator for remission from depressive symptoms is the normalisation of glucocorticoid resistance and HPA-axis dysregulation (Hennings et al., 2009). Psychological stresses have also been shown to increase circulating blood glucose concentrations (Surwit et al., 1992), however NOD mice

showed no increase in glucose in response to restraint stress. This is supported by previous findings from a study by Amrani et al. (1994) which showed that NOD mice had a lower hyperglycaemic response at baseline and following exposure to immobilisation stress. This finding lends further support to a potentially impaired HPA-axis response in the NOD mouse.

4.4.6 Stress related changes in 5HT metabolism are increased in the hippocampus of the NOD mouse when compared to the CD1 strain

Mitani et al. (2006) have reported that depressed patients have a higher plasma concentration of 5HIAA, the metabolite of 5-HT, and that these elevated levels correlate with disease severity. In agreement Sullivan and co-workers reported elevated levels of 5HIAA in the cerebrospinal fluid of depressed patients (Sullivan et al., 2006). Preclinical studies have also shown that stress can induce increases in 5HIAA levels and that these can be reversed following antidepressant treatment (Dunn and Welch, 1991). Immunological stimulation via endotoxin or administration of pro-inflammatory cytokines can also increase 5-HT metabolism centrally (Wang and Dunn, 1999, Clement et al., 1997). In the present study, concentrations of 5HIAA and the ratio of 5HIAA/5-HT were elevated in NOD mice in response to stress in the hippocampus with a trend towards significance observed in the amygdala. 5-HT and 5HIAA concentrations were higher in the hippocampus of NOD mice compared to CD1 controls under basal conditions. No changes were observed in 5-HT or 5HIAA concentrations in the frontal cortex of either strain. These results indicate that there are regional and stress related differences in 5-HT turnover in NOD mice.

4.4.7 Changes in pro-inflammatory gene expression in response to stress

No change in IL-6 expression was observed in the hippocampus of either the NOD or CD1 mouse following restraint stress, however in the frontal cortex an increase in the mRNA expression of IL-6 was observed in both NOD and CD1 mice immediately following stress when compared to its own non-stressed control. Elevated levels of IL-6 have been reported following exposure to psychological stressors and IL-6 has been shown to elicit depressive-like behaviours when directly administered to the brains of mice (Girotti et al., 2013, Sukoff Rizzo et al., 2012). There was also no change in IL-1β expression in the

hypothalamus or frontal cortex in either strain of mouse, while a reduction was observed in hippocampal IL-1β mRNA expression in response to stress of the NOD mouse only. NOD mice did however have elevated levels of IL-1β expression under basal conditions. Interestingly, NOD mice have been shown to be sensitive to the behavioural effects of IL-1β, reducing social interaction and locomotion when compared to a control strain (Bluth et al., 1999b). An association between restraint stress, microglial activation and the development of a depressive phenotype has previously been reported (Beumer et al., 2012). However, neither the CD1 nor NOD mice displayed any changes in the expression of the microglial marker CD11b in the hippocampus or the hypothalamus in response to a 2hr restraint stress, suggesting that such an acute stress does not lead to microglial activation within the brain. This is supported by a previous study by Frank and co workers (2007) which showed that inescapable tail shock stress exposure does not alter the expression of CD11b in the hippocampus of Sprague Dawley rats (Frank et al., 2007).

4.4.8 Stress related changes in GR related and regulated genes

Feedback regulation of the HPA-axis is mediated by circulating glucocorticoids on the hypothalamus and pituitary (Bear et al., 2007). This signalling is mediated through the glucocorticoid receptor (GR) which binds to glucocorticoids in the cytosol of the cell and translocates to the nucleus where they alter the gene expression patterns of the cell. Exposure to a restraint stress decreased expression of GR in the hippocampus, but not the hypothalamus or liver, of NOD and CD1 mice immediately post stress. In the cytosol unbound GR associates with a series of chaperones and co-chaperones that help form the structure and influence the binding affinity of GR. Investigation into the expression of HSP90 A and B, DUSP, p23, pp5 and FKBP4 showed no changes following stressor exposure (data not shown). However, immediately following stress, while FKBP5 expression increased in both the NOD and CD1 mice, expression in the NOD was greater than that of the CD1 mice both centrally and peripherally. This GR co-chaperone has obstructive actions when bound to GR-chaperone complex, reducing the binding affinity of GR to glucocorticoids (Stechschulte and Sanchez, 2011), and is directly up-regulated by GR signalling and serves to limit the GR signalling in the cell (Jääskeläinen et al., 2011). Mice deficient in FKBP5 have

been shown to have decreased activity of the HPA-axis in response to stress when compared to wild type mice (Touma et al., 2011). FKBP5 has also been shown to increase the translocation of the non-expressive GRβ subtype to the nucleus where it can compete with GRα for the glucocorticoid response elements, further lowering the GR responsiveness (Zhang et al., 2008).

The link between FKBP5 and anxious and depressive-like symptoms in mice has previously been investigated. An FKBP5 knockout mouse was shown to be more resilient to behavioural despair in the TST than a control strain (O'Leary et al., 2011). Studies in humans have shown that FKBP5 is associated with a susceptibility to depressive episodes (Zimmermann et al., 2011, Binder et al., 2004). People found with a homozygous allele for increased expression of FKBP5 have been found to be more susceptible to depression (Binder et al., 2004). Elevated expression of FKBP5 observed immediately post stress in the hippocampus, hypothalamus and liver of the NOD relative to CD1, could lead to reduced GR signalling and as such induce glucocorticoid resistance. Investigation into protein levels by Western immunoblotting of both GR and FKBP5 yielded inconclusive results (data not shown) and further investigations into GR and FKBP5 protein levels and localisation within the cell are required. Expression of other GR inducible genes was also increased in response to restraint stress. SGK1 and GILZ expression was increased immediately following stress, however unlike FKBP5 no strain differences were observed. Expression of the GR inducible enzyme TDO was also increased in response to restraint stress, with a greater increase in expression evident in the NOD strain. Recent studies in this lab have shown that this tryptophan degrading enzyme can reduce circulating tryptophan concentrations and inhibition of this enzyme produces antidepressant-like activity in an animal model (Gibney et al., 2014). The greater increase in FKBP5 expression both centrally and peripherally and hepatic TDO expression indicates an increased sensitivity to glucocorticoid signalling in NOD mice relative to CD1.

4.4.9 Conclusion

In the present study it has been demonstrated that pre-diabetic NOD mice possess an anxious and depressive-like phenotype when compared to a control strain at steady state. While the exact cause of the altered steady-state behavioural phenotype in this model is still unclear it is possible that it may relate

in part to the putative impaired neuronal support of microglia at steady state, however further functional studies are required to determine if microglia do in fact play a causal role. In response to immune stimulation, an altered behavioural phenotype was also observed in the NOD mouse. This was paralleled by a robust increase in both a classic and type-1 IFN inflammatory response in the mature circulating monocytes. Several preclinical models of psychiatric disease exist which are induced by activation of the peripheral immune system (Bluthe et al., 2000, Fu et al., 2010, Gibney et al., 2012, O'Connor et al., 2009b, O'Connor et al., 2009c, Salazar et al., 2012, Yirmiya, 1996), and as such, the immune-stimulated behavioural alterations seen in the NOD mouse in the current paradigm may be related to an enhanced response to LPS and/or activation state of NOD microglia in response to this stimulus. Future functional studies are required to support this proposed association. Nevertheless, taken together the findings from this study add support to the view that the NOD mouse may be a suitable model in which to study the relationship between an augmented immune system and depression.

In response to an acute restraint stress, the NOD mouse showed a delayed return of circulating corticosterone concentrations to baseline and an impaired glucose response to stress. This was accompanied by enhanced expression of the GR co-chaperone FKBP5 which has been linked to depression susceptibility (Binder, 2009, Binder et al., 2004, Zimmermann et al., 2011). This suggests that NOD mice may have an increased sensitivity to GR activation whereby induction of this gene may subsequently allow for interference in GR signalling and delay negative feedback at the level of the HPA-axis. Further investigation is warranted to elucidate the exact mechanisms involved and to determine if the behaviour of these glucocorticoid hypersensitive mice could also be altered by GR modulation.

4.4.10 Future work and directions

It would be interesting to see if the type-1 IFN response observed in NOD mice may be induced to a greater extent by a viral mimetic, such as poly I:C. Monitoring of the behavioural response to this immune stimulus would be of interest, including tests for anhedonia, anxious- and depressive-like behaviours. Immunostaining of microglia in the brain under control conditions would allow for classification of resting microglia in the brain and for quantification of microglia in specific brain regions and determination of the activation state of the microglia

too. Further characterisation in response to an immune stimulus such as LPS or poly I:C would also be of interest considering the aberrant expression of immune biomarkers in the microglia of NOD mice.

5. Results Chapter 3 – Systemic and central IL-6 related changes in a mouse model following peripheral IL-6 administration

5.1 Introduction

The role of interleukin (IL-) 6 in immunity and inflammation is a complex one as it is associated with both pro- and anti-inflammatory effects (Petersen and Pedersen, 2006, Scheller et al., 2011, Xing et al., 1998). IL-6 is produced by innate immune cells, B cells and $CD4^+$ T-helper cells but can also be produced by non immune cells including endothelial cells, fibroblasts and epithelial cells (Hirano, 1998). It is largely responsible for the hepatic acute phase response to infections or systemic inflammation (Naugler et al., 2007). Elevated levels of IL-6 have been identified in patients suffering from rheumatoid arthritis (RA), osteoporosis, psoriasis and Crohn's disease, and higher levels have been correlated with disease severity (Rincon, 2012). Preclinical studies with IL-6 knockout mice and blockade of the IL-6 receptor have been shown to delay onset and reduce the severity of disease in a murine model of RA (Takagi et al., 1998, Sasai et al., 1999). Within the central nervous system (CNS), IL-6 can be produced by neurons and glial cells and its effects include activation of astrocytes, promotion of oligodendrocyte maturation and neuronal differentiation (Brunello et al., 2000, Perígolo-Vicente et al., 2013, Van Wagoner and Benveniste, 1999).

The main IL-6 signalling pathways have been elucidated (Rincon, 2012, Heinrich et al., 2003, Taga, 1997). Binding of IL-6 to its receptor (IL-6RA) leads to association with a signal transducer (IL-6ST), which in turn activates janus kinases (JAK) leading to phosphorylation of the transcription factor signal transduction and activator of transcription (STAT) 3. Following phosphorylation, STAT3 dimerises and translocates to the nucleus where it binds to specific DNA response elements and can induce transcription of IL-6 inducible genes. Another transcription factor activated by the ligand bound IL-6RA–IL-6ST complex is CCAAT/enhancer binding protein beta (C/EBPβ), which is phosphorylated by the GRB2-Ras-mitogen activated protein kinase (MAPK) pathway, and translocates to the nucleus. One of the genes induced by the STAT3 signalling method encodes for the suppressor of cytokine signalling (SOCS) 3 protein, which has been shown to act as part of a negative feedback mechanism that prevents phosphorylation of STAT3 by JAK (Croker et al., 2003). C/EBPβ has been shown to induce its own expression and that of haptoglobin (Hirano, 1998, Huntoon et al., 2013).

Membrane bound IL-6RA (mIL-6RA) is limited mainly to leukocytes and hepatocytes and has been identified *in vitro* on microglia, astrocytes and neurons in the CNS, but a soluble IL-6 receptor (sIL-6R) allows for IL-6 trans-signalling in cells lacking the mIL-6RA (Erta et al., 2012). This soluble receptor can be generated by both post –transcriptional and –translational cleavage and binds to membrane bound IL-6ST, which is expressed ubiquitously, initiating IL-6 signalling (Rose-John, 2012, Chalaris et al., 2011). The importance of this trans-signalling has been demonstrated with the use of a soluble IL-6ST (sIL-6ST) which blocks sIL-6R but not mIL-6RA based signalling (Jostock et al., 2001). Recent preclinical studies have shown that intracerebroventricularly (i.c.v.) administered or transgenically over expressed sIL-6ST reduced inflammation and sickness behaviour in response to systemic administration of lipopolysaccharide (LPS) (Rabe et al., 2008, Burton et al., 2011).

A role for pro-inflammatory cytokines has been proposed in the pathophysiology of psychiatric disorders including major depression in keeping with the macrophage/T lymphocyte theory of depression (Leonard, 2000). Circulating cytokines have been implicated as biomarkers and possible contributors to CNS conditions including dementia, anxiety and depression. For example, chronic cytokine immunotherapy treatment for certain cancers and hepatitis C, including interferon (IFN) α and IL- 2, have been shown to induce depressive symptoms in up to 40% of patients (Capuron et al., 2001, Udina et al., 2012) and these symptoms can be alleviated using antidepressants (Musselman et al., 2001). The specific contribution of IL-6 to behavioural symptoms are less well characterised when compared to IL-1β, tumour necrosis factor (TNF) α or the IFNs. For this reason it is of interest to consider IL-6 in isolation, both in clinical and pre-clinical studies, in order to elucidate a specific role for this cytokine in the aetiology of psychiatric disorders with a particular focus on major depression and its treatment. Meta analyses of clinical studies have shown IL-6 to be consistently elevated in depressed patients (Howren et al., 2009, Valkanova et al., 2013, Dowlati et al., 2010). Interestingly, selective serotonin reuptake inhibitor antidepressants have been shown to specifically reduce circulating levels of IL-6 and TNFα (Hannestad et al., 2011). As with depression, circulating IL-6 has been linked with schizophrenia and its expression has been associated with the duration of the disease (Ganguli et al., 1994). In addition increased levels of sIL-6R were reported in the serum and CSF of schizophrenic patients (Müller et al., 1997). IL-6 has also been associated with reduced hippocampal volume,

cognitive decline in elderly people and delirium (Weaver et al., 2002, Engelhart et al., 2004, van Munster et al., 2008).

Systemic inflammation has been reported to elicit effects on CNS function as demonstrated through administration of immunostimulatory agents like LPS and recombinant pro-inflammatory cytokines (Cunningham et al., 2007, Hayley et al., 2012). Studies using IFNα have shown that systemic administration of this pro-inflammatory cytokine can induce prolonged sickness behaviour (Kentner et al., 2006). Similarly administration of LPS or Bacillus Calmette-Guerin (BCG) reliably induce sickness behaviour in mice (Dantzer et al., 2011). LPS induced IL-6 itself does not have a direct role in inducing sickness behaviour (Anisman et al., 1998, Skelly et al., 2013), but may act synergistically with other pro-inflammatory cytokines in affecting CNS function (Harden et al., 2008). Evidence for this exists where blockade of sIL-6R trans-signalling reduces the inflammatory response to an immune stimulus and facilitates quicker recovery from sickness behaviour (Sukoff Rizzo et al., 2012, Burton et al., 2011, Rabe et al., 2008). Central administration of IL-6 induced depressive-like behaviours in mice were reversed by the co-administration of an antibody against IL-6 (Sukoff Rizzo et al., 2012).

Psychological stressors activating the hypothalamic pituitary adrenal (HPA) axis have been shown to have a stimulatory effect on the circulating levels of IL-6 in animal models (Girotti et al., 2013). IL-6 is co-localised in vasopressin neurons in the paraventricular nucleus and supraoptic nucleus of the hypothalamus and these magnocellular neurons are activated by psychological stress releasing IL-6 (Jankord et al., 2010). Studies investigating the neuroendocrine response to both acute (Nukina et al., 2001) and chronic (Voorhees et al., 2013) restraint stress have revealed elevations in circulating concentrations of IL-6. Studies using IL-6 knockout mice have demonstrated a role for the cytokine in the behavioural response to stress (Armario et al., 1998b). Butterweck et al. (2013) showed that IL-6 deficient mice had increased locomotor activity in the open field test and reduced time spent in the open arm of the elevated plus maze which is indicative of anxiety related behaviour (Butterweck et al., 2003). IL-6 knockout mice have been reported to be resistant to despair related behaviour in the tail suspension test and forced swim test and exhibit increased hedonic behaviour (Chourbaji et al., 2006). IL-6 has also been reported to induce activation of the HPA-axis and leads to elevations in circulating concentrations of adrenocorticotropic hormone (ACTH) (Dunn, 2000). IL-6 and ACTH also act synergistically to stimulate the release of corticosterone in rats (Salas et al., 1990). Similarly in humans,

exogenously administered IL-6 has been reported to lead to an increase in circulating cortisol levels (Mastorakos et al., 1993, Steensberg et al., 2003).

There are a number of possible mechanisms whereby raised concentrations of IL-6 in the periphery in response to immunological activation, inflammation or psychological stress can gain access and influence the CNS. An IL-6 specific saturable transport system has been previously described, which is unique from the IL-1 and TNFα transporters (Banks et al., 1994, Banks et al., 2002). Blood brain barrier (BBB) investigations showed that ~0.2% of intravenously administered radio-labelled IL-6 passed from the systemic circulation into the brain (Banks et al., 1994). IL-6 has been reported to impair the BBB, a transgenic mouse model where astrocytes over-expressed IL-6 possessed an impaired BBB (Brett et al., 1995). IL-6 secretion into the CNS by endotoxin stimulated endothelial cells has also been reported (Banks, 2008).

Cytokines including IL-6 produce behavioural symptoms reminiscent of those seen in depressed patients. Interpretation of these effects is complicated by the sickness induced following acute administration and it is unclear whether IL-6 acts directly on the brain to execute its CNS actions and involvement in the etiology of behavioural symptoms. Recent investigations into the effect of systemically administered IL-6 in the CNS have not reported a clear response following IL-6 administration. Skelly et al. (2013) recently explored the effect of two systemic doses of IL-6 (50 and 125μg/kg) on the induction of sickness behaviour and genes related to an inflammatory response in the mouse. Core body temperature was unchanged by IL-6 and only the high dose of 125μg/kg IL-6 reduced activity in the open field arena, yet even then the reduction was less than that observed by IL-1β and LPS administration, which suggests a lack of direct induction of sickness behaviour by IL-6 (Skelly et al., 2013).

There is to date no specific marker to demonstrate and confirm a direct CNS action for IL-6 following systemic peripheral administration. To define a possible direct CNS action by peripheral systemic IL-6, IL-6 stimulated genes were assessed as an indicator for a direct IL-6 action following peripheral IL-6 administration. Changes in expression of target genes in the brain following IL-6 administration at these doses were also minimal suggesting that they are below the threshold required for biological activity. Within the CNS, as mentioned above, IL-6 can signal on astrocytes, microglia and neurons. Release of serotonin (5-HT) in the CNS has been noted to increase with acute systemic

administration of IL-6 leading some investigators to propose that it might contribute to an antidepressant effect (Zhang et al 2001). This observed effect could possibly change following chronic exposure, as chronic administration of the pro-inflammatory cytokine TNFα reduced 5-HT availability (Hayley et al., 2003). IL-6 has also been shown to inhibit glutamate signalling within the CNS of rats upon application to brain slices *ex vivo* depressing the spread of evoked stimuli through the cortex (D'Arcangelo et al., 2000).

The objectives of the current study were to assess the effects of acute and chronic stress on circulating and central IL-6 expression and production in addition to the expression of IL-6 regulated genes. Following this, the dose and time course effects of systemic IL-6 administration were assessed. Doses of 1ng, 10ng, 100ng and 1μg/mouse were initially tested to evaluate the dose at which IL-6 related signalling within the CNS would be detectable. Based on the results obtained, which provided evidence for IL-6 related signalling within the brain 2 hours (hr) following administration to a limited extent, a high dose of 10μg/mouse IL-6 was evaluated in order to achieve a more robust response, and a time course implemented to monitor the temporal response.

The experiments assess the extent to which systemic IL-6 administration may trigger IL-6 related signalling and gene expression in the CNS which may underpin IL-6 mediated changes in CNS function.

5.2 Materials and Methods

5.2.1 Animal husbandry

Female CD-1 mice (25-30g) were obtained from Harlan (UK). Mice were housed 6 per cage and were maintained on a 12hr light: 12hr dark cycle (lights on at 08:00hr) in a temperature controlled room (22±2 degrees Celsius (°C)). Food and water were available *ad libitum*. The experimental protocols were in compliance with the European Communities Council directive (86/609/EEC).

5.2.2 Acute restraint stress procedure

Mice were immobilised by placing them in a well ventilated restrainer (modified 50ml collecting tube, Figure 2.2.3.1) for a single exposure. The tubes were placed parallel to the table top in a brightly lit room with an ambient temperature of 20-24°C. The mice were restrained for 2hr and then either euthanised immediately or returned to the home cage for 2hr after which time they were euthanised. Control animals remained in their home cage and were euthanised when removed from the cage.

5.2.3 Systemic IL-6 dose response

Mice were removed from the home cage, injected intraperitoneally (i.p.) with varying doses of IL-6, 1ng/mouse, 10ng/mouse, 100ng/mouse, 1µg/mouse and returned to a separate cage for 2hr at which point the mice were euthanised and tissue and serum harvested for post mortem analysis. Control mice were injected i.p. with saline, returned to a separate cage and euthanised 2hr post injection

5.2.4 Systemic IL-6 time course

Mice were removed from the home cage, injected i.p. with 10µg/mouse IL-6 and returned to a separate cage for varying durations, 15 minutes (mins), 30mins, 1hr, 2hr and 4hr. The mice were then euthanised and tissue and serum harvested for post mortem analysis. Control mice were injected with saline and euthanised 2hr post injection.

5.2.5 Enzyme linked immunosorbent assay (ELISA)

5.2.5.1 Corticosterone Assay

Circulating serum concentrations of corticosterone were quantified using a commercially available CORT assay kit (Enzo Life Sciences). The assay was performed as per the manufacturer's guidelines and as previously described in chapter 2. Absorbance was measured at 405nm using a microtitre plate reader (Elx800; Biotek, Germany).

5.2.5.2 IL-6 ELISA

Circulating serum concentrations of IL-6 were quantified using a commercially available IL-6 ELISA kit (BioLegend). The assay was performed as per the manufacturer's guidelines and as previously described in chapter 2. Absorbance was measured at 450nm using a microtitre plate reader (Elx800; Biotek, Germany).

5.2.5.3 Soluble IL-6 receptor ELISA

Circulating serum concentrations of soluble IL-6 receptor (sIL-6r) were quantified using a commercially available sIL-6r ELISA kit (R&D systems). The assay was performed as per the manufacturer's guidelines and as previously described in chapter 2. Absorbance was measured at 450nm using a microtitre plate reader (Elx800; Biotek, Germany).

5.2.6 Reverse transcriptase polymerase chain reaction (PCR)

PCR was carried out as previously described in chapter 2. In brief, RNA was extracted from tissue using the Nucleospin® RNA II total isolation kit (Macherey-Nagel) as instructed in the manufacturer's protocol. RNA was quantified using a spectrophotometer (Nanodrop ND-1000) and equalised to the lowest concentration of RNA using Rnase free H_2O, at which point it was reverse transcribed into cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was carried out using an ABI StepOne 7500 instrument. Taqman® Gene Expression Assays containing primers and a Taqman probe were used to quantify the mRNA expression of each gene of interest. A

total volume of 10µl for each sample reaction (4µl cDNA, 0.5µl primer of interest, 0.5µl endogenous control gene and 5µl Fast Taqman) was placed in a PCR plate and a 40 cycle PCR was carried out. B-actin was used as an endogenous control to normalise gene expression data, and an RQ value (2^{-DDCt}, where C_t is the threshold cycle) was calculated for each sample. These RQ values were then expressed as a fold change versus the control group, which was normalised to 1.

Table 5.1 – List of Taqman Gene Expression Assays used and their gene codes

Target	Taqman Assay ID
β-actin	4352341E
C/EBPβ	Mm00843434_m1
c-fos	Mm00487425_m1
EGR-1	Mm00656724_m1
IL-6	Mm00446190_m1
IL-6RA	Mm00439653_m1
IL-6ST	Mm00439665_m1
SOCS3	Mm00545913_s1
STAT3	Mm01219775_m1
HP	Mm00516884_m1
IL-1β	Mm01336189_m1
BDNF	Mm04230607_m1
TDO	Mm00451266_m1
IDO1	Mm00492586_m1
IDO2	Mm00524206_m1

5.2.7 Western Immunoblotting (WB)

WB was carried out as previously described in chapter 2. In brief, protein was extracted from tissue, via homogenisation of tissue in extraction buffer containing protease and phosphatase inhibitors. Protein concentration was quantified using a BCA colorometric method of protein determination (Pierce). Absorbances were measured using a microtitre plate reader (Elx800; Biotek, Germany) at 560nm.

Unknown protein concentrations were determined via a standard curve prepared using samples of a known concentration. Samples were equalised with lysis buffer to the sample of the lowest concentration and added to sample buffer 1in2. Samples were separated by size via SDS-PAGE and transferred to a PVDF membrane for probing with antibodies. After 1hr blocking in Marvel, the membrane was incubated with a protein specific primary antibody at 4°C overnight and then probed with a secondary antibody specific to the primary antibody (Cell Signalling, USA) for 1hr. Visualisation of the proteins was done using a chemiluminescent solution and a 195eurotrop (Fujifilm Luminescent Image Analyzer LAS-3000). It was possible to re-probe with a different primary antibody post visualisation by stripping the antibodies from the membrane with a stripping solution for 15mins and re-blocking in Marvel.

Table 5.2 – A list of protein specific antibodies used

Target	Mol Weight (kDa)	Primary Antibody Conc	Secondary Antibody Conc
β-actin	43	1 in 1000	1 in 5000
STAT 3	79 / 86	1 in 1000	1 in 5000
pSTAT 3	79 / 86	1 in 1000	1 in 5000
C/EBP β	35-38	1 in 1000	1 in 5000
pC/EBP β	36 / 38	1 in 1000	1 in 5000

5.2.8 Statistical Analysis

Data from all experiments were analysed using GB-STAT. One-way analysis of variance (ANOVA) was used for all experiments within this study. If significant changes were observed, the data was further analysed using a Newman-Keuls post-hoc comparison test. Data was deemed to be significant when $P<0.05$. Results are expressed as means ± standard error of the mean (SEM).

5.3 Results

5.3.1 The effect of an acute stress on IL-6 and the expression of IL-6 related and regulated genes

In this present experiment, mice were subjected to an acute 2hr restraint stress and euthanised immediately or 2hr post stress. The effect of this psychological stressor on circulating levels of corticosterone, IL-6, sIL-6R, and the peripheral and central mRNA expression of IL-6 related and regulated genes were investigated.

5.3.1.1 Exposure to an acute stress induces increases in circulating corticosterone, IL-6 and sIL-6r

ANOVA showed an effect of acute stress on the circulating serum levels of corticosterone (a) ($F_{(2, 15)}$= 7.31, P<0.01), IL-6 (b) ($F_{(2, 15)}$=7.87, P<0.01) and sIL-6r (c) ($F_{(2, 15)}$= 4.75, P<0.01). Post-hoc comparisons revealed an increase in circulating serum levels of corticosterone immediately following stress (P<0.01) when compared to non-stressed controls, but this had returned to basal levels 2hr post stress. Circulating serum levels of IL-6 were elevated immediately post stress (P<0.01) and remained elevated 2hr post stress (P<0.05). Circulating serum levels of sIL-6r were elevated 2hr post stress (P<0.05). [Figure 5.1, n=6]

5.3.1.2 Expression of IL-6 and IL-6 associated markers in the liver and spleen following acute stress

ANOVA showed an effect of acute stress on the mRNA expression of the IL-6RA ($F_{(2, 15)}$=16.15, P<0.001), IL-6ST ($F_{(2, 15)}$=18.71, P<0.001), SOCS3 ($F_{(2, 15)}$=7.79, P<0.01), STAT3 ($F_{(2, 15)}$=21.45, P<0.0001), C/EBPβ ($F_{(2, 15)}$=46.51, P<0.0001) and haptoglobin (HP) ($F_{(2, 15)}$=11.81, P<0.001), in the liver. Post-hoc comparisons revealed an increase in the mRNA expression of IL-6RA immediately following stress (P<0.01) and this remained elevated 2hr post stress (P<0.01) when compared to non-stressed controls. mRNA expression of IL-6ST (P<0.01), SOCS3 (P<0.01), STAT3 (P<0.01) and HP (P<0.01) were significantly increased 2hr post stress when compared to non-stressed controls. mRNA expression of C/EBPβ was significantly increased immediately following stress (P<0.01) and

remained significantly elevated 2hr post stress ($P<0.05$). ANOVA showed no effect of exposure to an acute restraint stress on the mRNA expression of IL-6. [Table 5.3, Liver]

ANOVA showed an effect of an acute stress on the mRNA expression of IL-6RA ($F_{(2, 15)}=12.69$, $P<0.001$), IL-6ST ($F_{(2, 15)}=5.53$, $P<0.05$) and STAT3 ($F_{(2, 15)}=22.39$, $P<0.0001$), in the spleen. Post-hoc comparisons revealed an increase in the mRNA expression of IL-6RA ($P<0.01$) and IL-6ST ($P<0.05$) 2hr post stress when compared to non-stressed controls. mRNA expression of STAT3 increased immediately post stress ($P<0.05$) and had increased further 2hr post stress ($P<0.01$). ANOVA showed no effect of exposure to an acute stress on the mRNA expression of IL-6, SOCS3, C/EBPβ and HP. [Table 5.3, Spleen]

5.3.1.3 Expression of IL-6 and IL-6 associated markers in the brain following acute stress

ANOVA showed an effect of exposure to an acute stress on the mRNA expression of IL-6 ($F_{(2, 14)}=3.79$, $P<0.05$) and C/EBPβ ($F_{(2, 14)}=3.82$, $P<0.05$), in the frontal cortex. Post-hoc comparisons revealed an increase in the mRNA expression of IL-6 ($P<0.05$) and C/EBPβ ($P<0.05$) immediately following stress, but this had returned to basal levels 2hr post stress when compared to non-stressed controls. ANOVA showed no effect of exposure to an acute stress on the mRNA expression of IL-6RA, IL-6ST, SOCS3, STAT3 and HP. [Table 5.3, Frontal Cortex]

ANOVA of the mRNA expression of C/EBPβ in the hippocampus showed an effect of exposure to an acute stress ($F_{(2, 15)}=7.58$, $P<0.01$). Post-hoc comparisons revealed a significant increase in the mRNA expression of C/EBPβ ($P<0.05$) immediately following stress, but this had returned to basal levels 2hr post stress when compared to non-stressed controls. ANOVA showed no effect of exposure to an acute stress on the mRNA expression of IL-6, IL-6RA, IL-6ST, SOCS3, STAT3 and HP. [Table 5.3, Hippocampus]

ANOVA showed an effect of exposure to an acute stress on the mRNA expression of SOCS3 ($F_{(2, 15)}$=6.22, P<0.05) and C/EBPβ ($F_{(2, 15)}$=12.98, P<0.001) in the hypothalamus. Post-hoc comparisons revealed a decrease in the mRNA expression levels of SOCS3 2hr post stress (P<0.05), when compared to non-stressed controls. mRNA expression of C/EBPβ increased immediately post stress (P<0.01), but this had returned to basal levels 2hr post stress. ANOVA showed no effect of exposure to an acute stress on the mRNA expression of IL-6, IL-6RA, IL-6ST, STAT3 and HP. [Table 5.3, Hypothalamus]

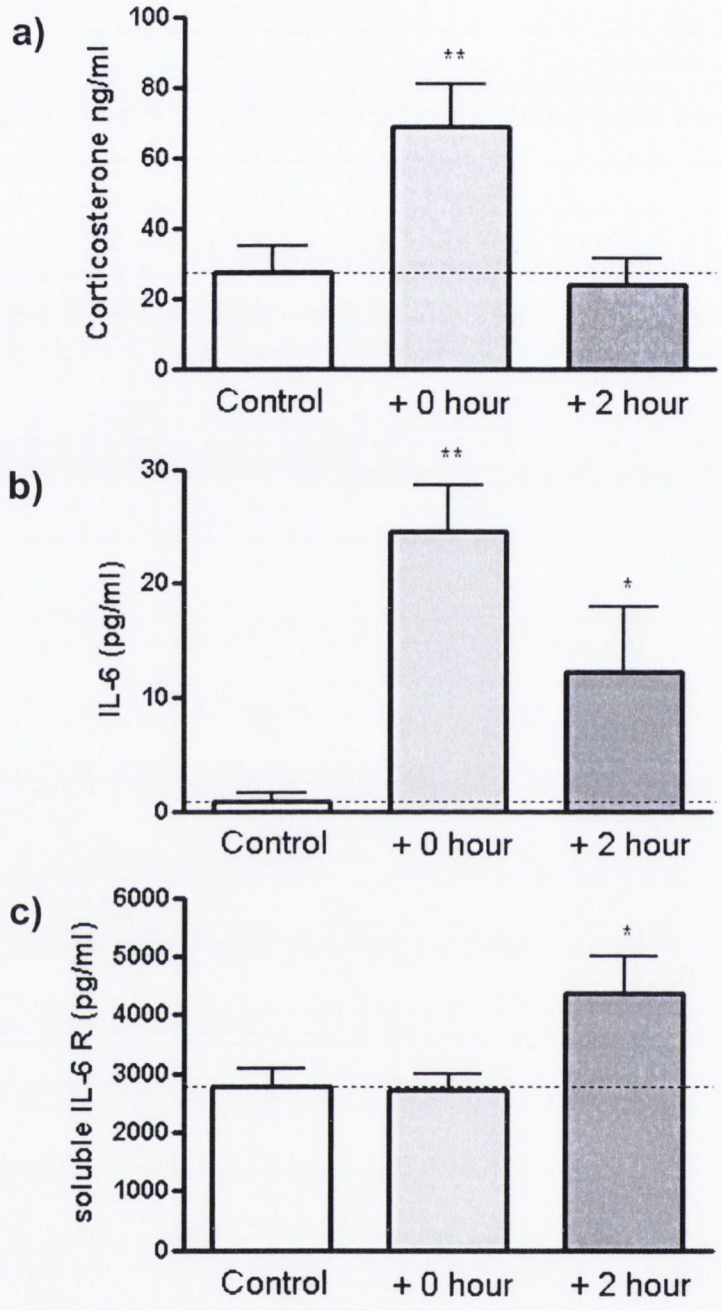

Figure 5.1 – Exposure to a psychological stressor leads to increases in serum concentrations of corticosterone, IL-6 and its soluble receptor sIL-6R

In response to an acute 2hr restraint stress an increase in circulating serum levels of corticosterone (a) was noted immediately post stress in CD1 mice, ($P<0.01$) which returned to basal levels 2hr post stress. Circulating levels of IL-6 (b) were also increased immediately following stress ($P<0.01$) and remained elevated 2hr later ($P<0.05$). Levels of the soluble IL-6 receptor (c) increased 2hr following stress ($P<0.05$). Data expressed as mean ± SEM (n=6). ** $P<0.01$, * $P<0.05$ vs. Non-stressed control.

	IL-6	IL-6 RA	IL-6 ST	SOCS 3	STAT 3	C/EBP β	HP
Liver							
Control	1.00 ± 0.11	1.00 ± 0.11	1.00 ± 0.03	1.00 ± 0.13	1.00 ± 0.07	1.00 ± 0.13	1.00 ± 0.09
+ 0 hr	1.95 ± 0.34	2.37 ± 0.22 (**)	0.84 ± 0.05	1.43 ± 0.20	1.39 ± 0.11	3.97 ± 0.30 (**)	1.42 ± 0.13
+ 2 hr	1.15 ± 0.33	1.74 ± 0.16 (**)	1.43 ± 0.11 (**)	3.02 ± 0.62 (**)	2.19 ± 0.18 (**)	1.78 ± 0.22 (*)	2.66 ± 0.40 (**)
Spleen							
Control	1.00 ± 0.11	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.25	1.00 ± 0.06	1.00 ± 0.38	1.00 ± 0.30
+ 0 hr	0.96 ± 0.11	1.09 ± 0.37	1.08 ± 0.06	1.74 ± 0.37	1.35 ± 0.08 (*)	2.22 ± 0.49	1.01 ± 0.21
+ 2 hr	1.01 ± 0.12	1.46 ± 0.09 (**)	1.28 ± 0.06 (*)	2.18 ± 0.52	1.93 ± 0.14 (**)	1.77 ± 0.40	1.42 ± 0.55
Frontal Cortex							
Control	1.00 ± 0.18	1.00 ± 0.10	1.00 ± 0.03	1.00 ± 0.12	1.00 ± 0.07	1.00 ± 0.10	1.00 ± 0.61
+ 0 hr	2.65 ± 0.70 (*)	0.95 ± 0.05	0.95 ± 0.05	0.98 ± 0.06	0.97 ± 0.05	1.35 ± 0.12 (*)	0.47 ± 0.28
+ 2 hr	1.65 ± 0.30	0.94 ± 0.06	0.97 ± 0.02	0.74 ± 0.02	1.01 ± 0.02	1.07 ± 0.06	0.21 ± 0.23
Hippocampus							
Control	1.00 ± 0.38	1.00 ± 0.12	1.00 ± 0.18	1.00 ± 0.19	1.00 ± 0.22	1.00 ± 0.14	1.00 ± 0.36
+ 0 hr	1.06 ± 0.29	0.89 ± 0.06	0.65 ± 0.03	0.54 ± 0.09	0.79 ± 0.04	1.43 ± 0.11 (*)	2.67 ± 1.11
+ 2 hr	0.85 ± 0.27	0.94 ± 0.09	0.74 ± 0.06	0.73 ± 0.16	0.83 ± 0.07	0.83 ± 0.09	9.71 ± 5.40
Hypothalamus							
Control	1.00 ± 0.24	1.00 ± 0.68	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.15	1.00 ± 0.23
+ 0 hr	1.02 ± 0.21	0.98 ± 0.06	0.96 ± 0.03	0.93 ± 0.06	1.03 ± 0.03	1.82 ± 0.11 (**)	1.15 ± 0.38
+ 2 hr	0.62 ± 0.12	1.15 ± 0.05	0.94 ± 0.09	0.74 ± 0.05 (*)	1.04 ± 0.02	0.99 ± 0.14	1.01 ± 0.45

Table 5.3 – Exposure to a psychological stressor leads to increases in mRNA expression of IL-6 and IL-6 related and regulated genes in the periphery but not in the brain except for increased C/EBPβ expression in the regions examined.

Summary of the changes in mRNA expression of IL-6 and IL-6 related genes following exposure to an acute 2hr restraint stress in the periphery (liver, spleen) and in the brain (frontal cortex, hippocampus, hypothalamus). Data expressed as mean ± SEM (n=6). ** P<0.01, * P<0.05 vs. non-stressed control.

5.3.2 The effect of varying doses of systemic IL-6 on IL-6 and the mRNA expression of IL-6 related and regulated genes

Given that a psychological stressor induces more than just IL-6, the current experiment aimed to explore the specific effects of systemic IL-6 in relation to the changes observed post restraint stress. Consequently, animals were administered varying doses of IL-6 (1ng/mouse, 10ng/mouse, 100ng/mouse and 1µg/mouse, i.p.) and euthanised immediately or 2hr following IL-6 administration.

5.3.2.1 Systemic IL-6 administration leads to a dose-dependent increase in circulating IL-6 but not sIL-6r

ANOVA showed an effect of varying doses of IL-6 administration on the circulating serum levels of IL-6 (a) ($F_{(4, 15)}$=3.48, P<0.05). Serum levels of IL-6 increased in a dose-dependent manner and post-hoc comparison revealed that the concentration obtained following administration of the highest 1µg/mouse dose was significantly greater than the control (P<0.05). ANOVA showed no effect of varying doses of exogenous IL-6 on the circulating serum levels of sIL-6r (b). [Figure 5.2, n=4]

5.3.2.2 Systemic IL-6 administration leads to a dose-dependent change in the mRNA expression of IL-6 related and regulated genes in the liver

ANOVA showed an effect of varying doses of IL-6 administration on the mRNA expression of IL-6RA (b) ($F_{(4, 15)}$=3.08, P<0.05) and STAT3 (e) ($F_{(4, 15)}$=3.81, P<0.05) in the liver. Post-hoc comparisons revealed an increase in the mRNA expression of STAT 3 in response to 1µg/mouse IL-6 (P<0.05), when compared to vehicle administered controls. However, post-hoc comparison of IL-6RA did not reveal any changes in mRNA expression at any dose of IL-6 administered. ANOVA showed no effect of systemic IL-6 on the mRNA expression of IL-6 (a), IL-6ST (c), SOCS3 (d) and C/EBPβ (f). [Figure 5.3, n=4]

5.3.2.3 Systemic IL-6 administration leads to a dose-dependent change in the mRNA expression of IL-6 related STAT3 in the frontal cortex

ANOVA showed an effect of varying doses of IL-6 administration on the mRNA expression of STAT3 (e) ($F_{(4, 15)}$=3.11, P<0.05) in the frontal cortex. Post-hoc comparison revealed a significant increase in the mRNA expression of STAT3 in response to 1µg/mouse IL-6 administration (P<0.05), when compared to vehicle administered controls. ANOVA showed no effect of exogenous IL-6 on the mRNA expression of IL-6 (a), IL-6RA (b), IL-6ST (c), SOCS3 (d) and C/EBPβ (f). [Figure 5.4, n=4]

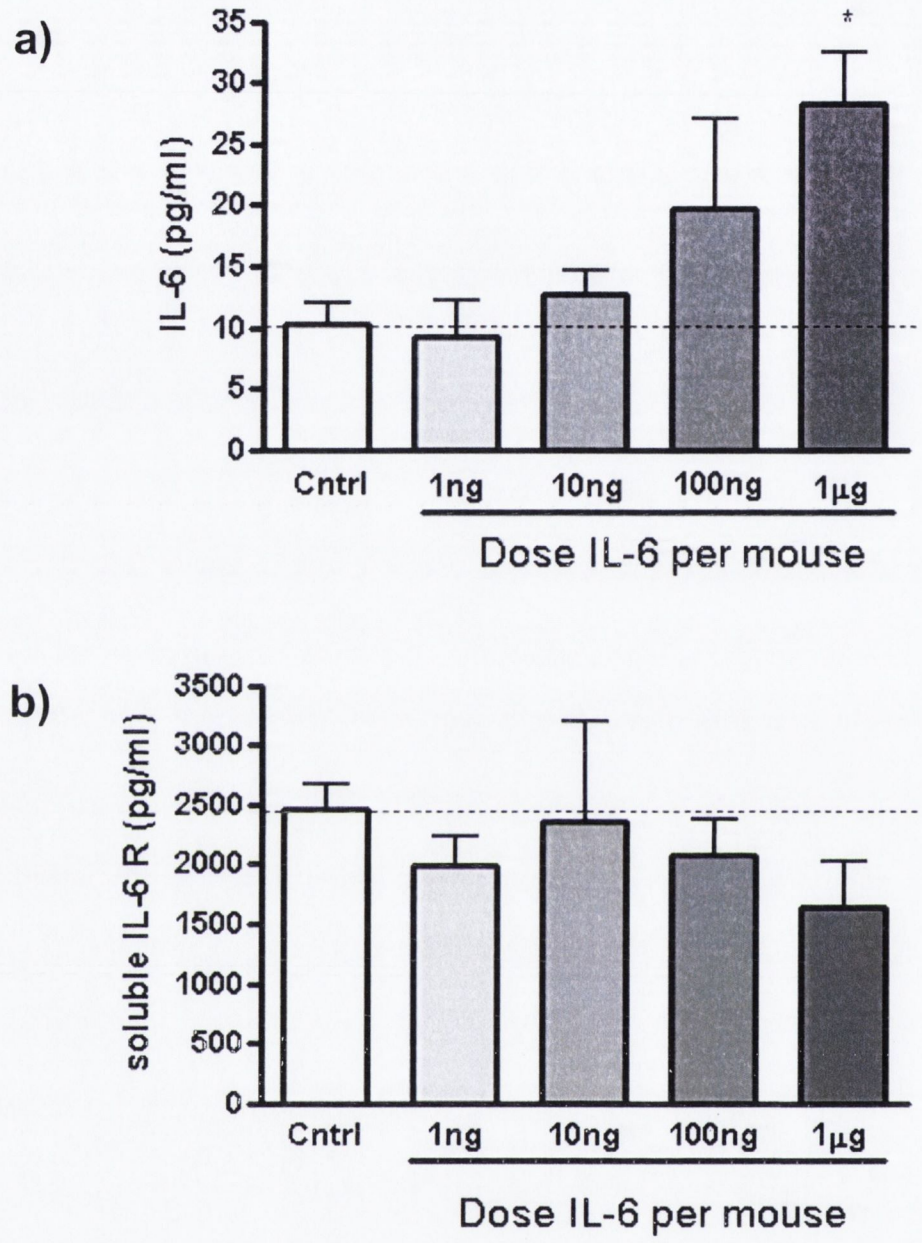

Figure 5.2 – IL-6 increased serum concentrations of IL-6, but not sIL-6R, in a dose-dependent manner

Systemic IL-6 administration leads to an increase in the circulating serum levels of IL-6 in a dose-dependent manner (a). Exogenous IL-6 did not have any effect on the circulating levels of the sIL-6R 2hr post injection. Data expressed as mean ± SEM (n=4). * $P<0.05$ vs. Vehicle treated controls.

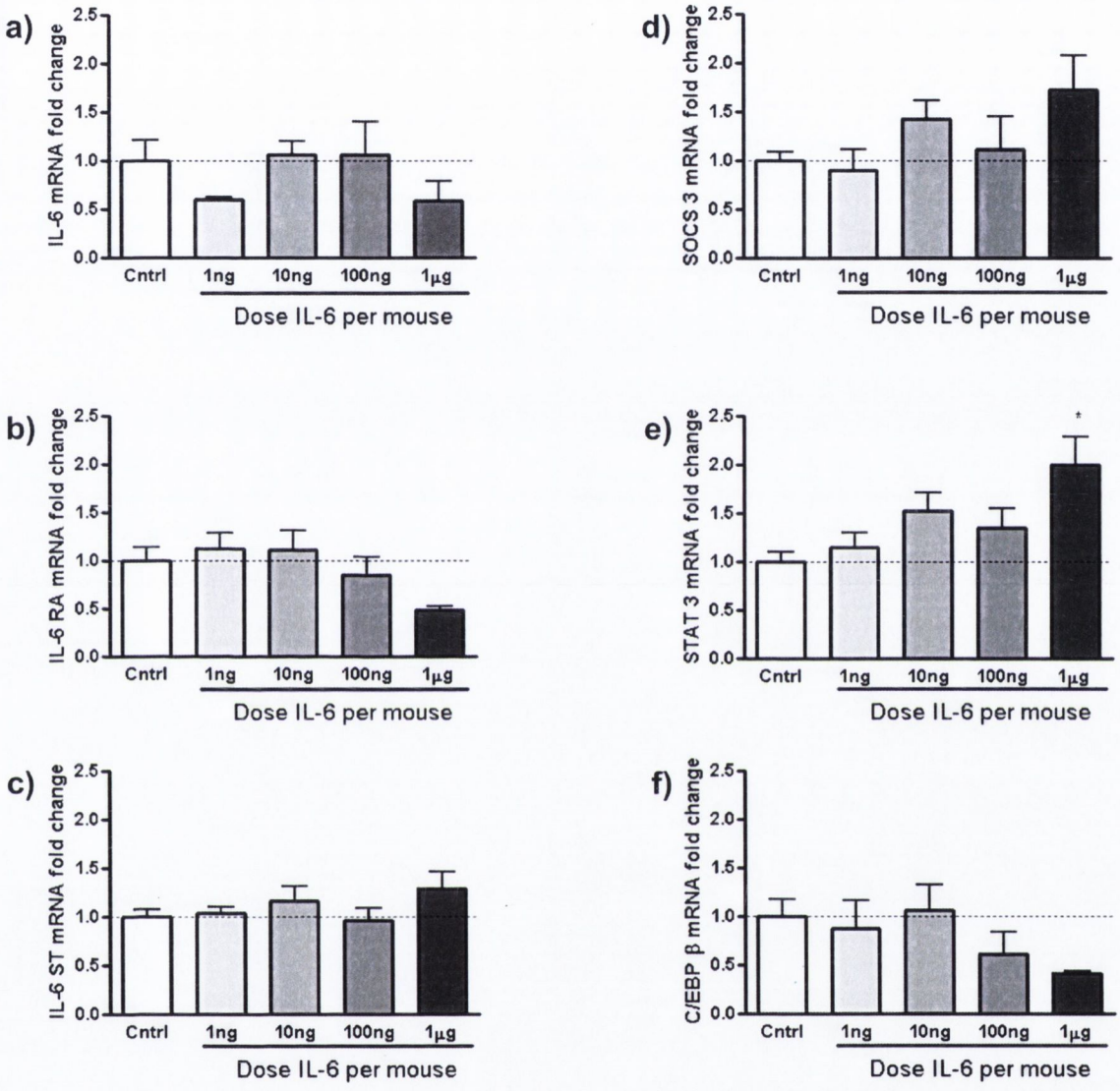

Figure 5.3 – IL-6 increased mRNA expression of STAT3 in a dose-dependent manner in the liver

Systemic IL-6 administration induces an increase in expression of STAT3 (e) in the liver 2hr post IL-6 injection and only at the highest dose of IL-6 administered (1µg/mouse). Data expressed as mean ± SEM (n=4). * $P<0.05$ vs. Vehicle treated controls.

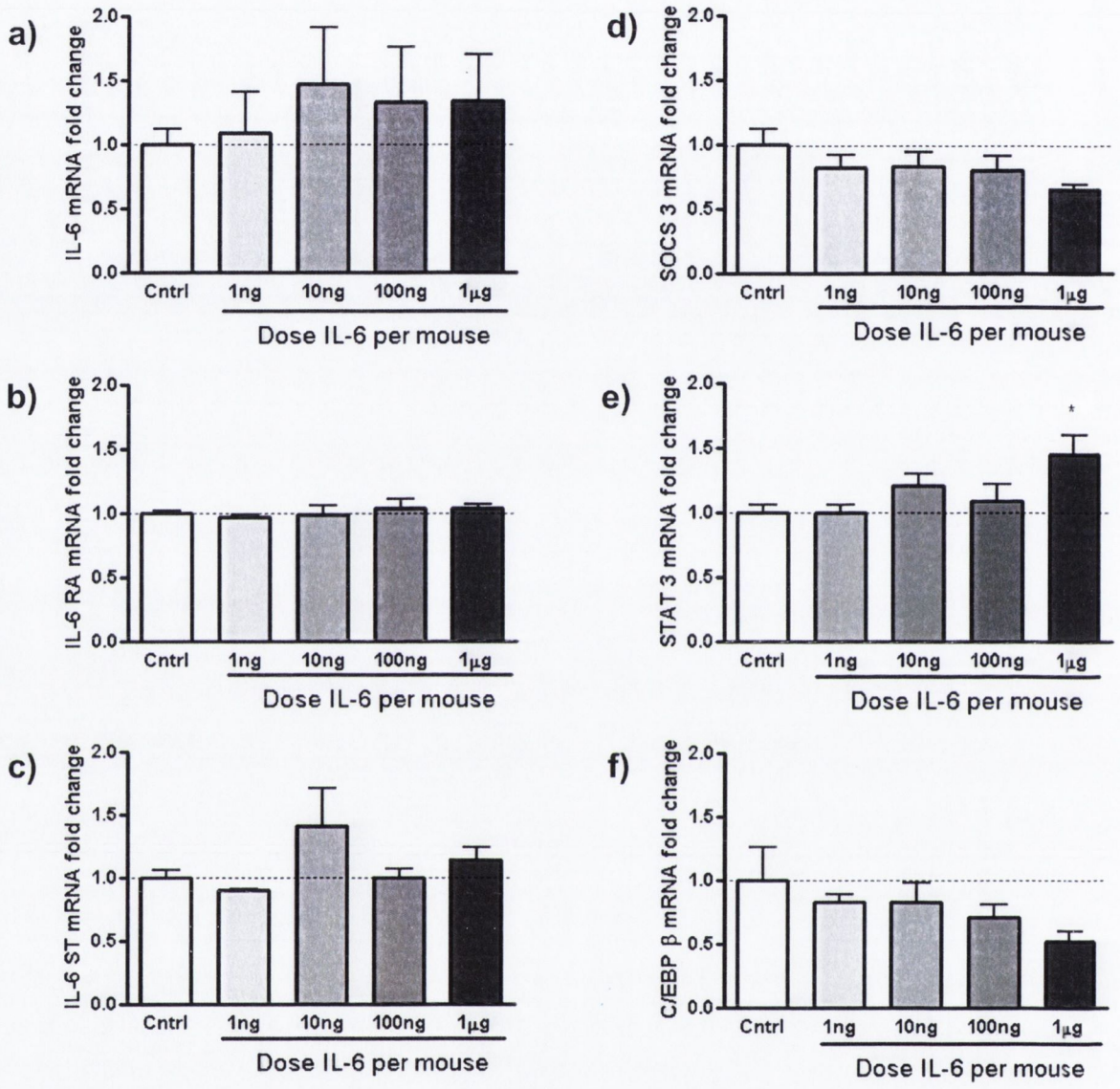

Figure 5.4 – IL-6 increased mRNA expression of STAT3 in a dose-dependent manner in the frontal cortex

Systemic administration of IL-6 induces an increase in the expression of STAT3 in the frontal cortex 2hr post IL-6 injection and only at the highest dose administered (1µg/mouse). Data expressed as mean ± SEM (n=4). * $P<0.05$ vs. Vehicle treated controls.

5.3.3 The temporal effect of systemic IL-6 (10µg/mouse) on IL-6 and IL-6 related and regulated genes in the periphery and the brain

Considering previous results, which demonstrated limited IL-6 signalling in the periphery and brain 2hr following administration of the highest dose of IL-6 (1µg/mouse), the current experiment aimed to assess the temporal response to a single high systemic dose of IL-6. Animals were injected 10µg/mouse IL-6, i.p. and the mice euthanised 15mins, 30mins, 1hr, 2hr and 4hr post injection.

5.3.3.1 Systemic administration of IL-6 (10µg/mouse) leads to a time-dependent change in the mRNA expression of IL-6 related and regulated genes in the liver

ANOVA showed an effect of a 10µg/mouse dose of IL-6 on the mRNA expression of IL-6 (a) ($F_{(5,28)}$=8.27, P<0.0001), IL-6RA (b) ($F_{(5,28)}$=4.61, P<0.01), SOCS3 (d) ($F_{(5,28)}$=22.89, P<0.0001), STAT3 (e) ($F_{(5,28)}$=17.83, P<0.0001), C/EBPβ (f) ($F_{(5,28)}$=6.05, P<0.001) and tryptophan 2,3 dioxygenase (TDO, g) ($F_{(5, 28)}$=2.55, P<0.05) in the liver.

Post-hoc comparisons revealed a decrease in mRNA expression of IL-6RA 30mins following IL-6 administration (P<0.05), when compared to vehicle administered controls. This decrease had returned to control levels by 2hr but had significantly reduced again 4hr post injection (P<0.05).

Post-hoc comparisons revealed elevated mRNA levels of SOCS3 15mins (P<0.05), 30mins (P<0.01), 1hr (P<0.01) and 2hr (P<0.01) following IL-6 administration, peaking 30mins post injection, when compared to vehicle administered controls.

Post-hoc comparisons revealed an increase in the mRNA expression of STAT3 2hr following IL-6 administration (P<0.01) which remained elevated 4hr post injection (P<0.01), when compared to vehicle administered controls.

Post-hoc comparisons of IL-6, C/EBPβ and TDO did not reveal any changes in mRNA expression at any time point following IL-6 administration. One-way ANOVA showed no effect of a 10µg/mouse dose of IL-6 on the mRNA expression of IL-6ST (c) in the liver or indoleamine 2,3 dioxygenase (IDO) in the spleen (data not shown). [Figure 5.5, n=5-6]

5.3.3.2 Systemic administration of IL-6 (10μg/mouse) causes a time-dependent increase in the activation of STAT3 protein in the liver

ANOVA of the ratio of 207eurotro-STAT3:total-STAT3 (pSTAT3:tSTAT3) showed an effect of IL-6 within the liver ($F_{(5,28)}$=4.29, P<0.01). Post-hoc comparison revealed a significant increase in phosphorylated STAT3 relative to total STAT3 15mins following IL-6 administration when compared to vehicle administered controls.

ANOVA showed no effect of a 10μg/mouse dose of IL-6 on the total protein levels of STAT3 or C/EBPβ or the phosphorylation state of C/EBPβ in the liver. [Figure 5.6, n=5-6]

5.3.3.3 Systemic administration of IL-6 (10μg/mouse) causes a time-dependent increase in IL-1β and IL-6 mRNA in the hypothalamus

ANOVA showed an effect of a 10μg/mouse dose of IL-6 on mRNA expression of IL-6 (c) ($F_{(5,28)}$=9.48, P<0.0001) and IL-1β (f) ($F_{(5,28)}$=4.07, P<0.01) in the hypothalamus. Post-hoc comparisons revealed an increase in mRNA expression of IL-6 (P<0.01) and IL-1β (P<0.01) 2hr following IL-6 administration in the hypothalamus, when compared to vehicle administered controls. ANOVA showed no effect of IL-6 on the mRNA expression of IL-6 or IL-1β in both the frontal cortex and the hippocampus. [Figure 5.7, n=5-6]

5.3.3.4 Systemic administration of IL-6 (10μg/mouse) causes a time-dependent increase in the mRNA expression of IL-6 related and regulated genes in the brain

ANOVA showed an effect of a 10μg/mouse dose of IL-6 on mRNA expression of SOCS3 in the frontal cortex (a) ($F_{(5,28)}$=34.83, P<0.0001), hippocampus (b) ($F_{(5,28)}$=10.86, P<0.0001) and hypothalamus (c) ($F_{(5,28)}$=12.6, P<0.0001). Post-hoc comparison revealed elevated levels of SOCS3 mRNA 15mins (P<0.01), 30mins (P<0.01), 1hr (P<0.01) and 2hr (P<0.05) following IL-6 administration, peaking 30mins post injection in the frontal cortex, when compared to vehicle administered controls. In the hippocampus, post-hoc comparisons revealed elevated levels of SOCS3 mRNA 30mins (P<0.01) and 1hr (P<0.01) following IL-6 administration, peaking at 1hr post injection. In the hypothalamus, post-hoc

comparisons revealed elevated SOCS3 mRNA 30mins ($P<0.01$) and 1hr ($P<0.01$) following IL-6 administration. [Figure 5.8 (a-c), n=5-6]

ANOVA showed an effect of IL-6 on mRNA expression of STAT3 in the frontal cortex (d) ($F_{(5,28)}=17.83$, $P<0.0001$), hippocampus (e) ($F_{(5,27)}=3.79$, $P<0.01$). Post-hoc comparisons did not reveal any significant changes in mRNA levels in either the frontal cortex or the hippocampus, when compared to vehicle administered controls. ANOVA showed no effect of a 10µg/mouse dose of IL-6 on the mRNA expression of STAT3 in the hypothalamus (f). [Figure 5.8 (d-f), n=5-6]

ANOVA showed an effect of IL-6 on mRNA expression of C/EBPβ in the hypothalamus (i) ($F_{(5,28)}=3.71$, $P<0.05$). Post-hoc comparisons revealed a significant increase in the mRNA of C/EBPβ in the hypothalamus 1hr ($P<0.05$) following IL-6 administration, when compared to vehicle administered controls. ANOVA showed no effect of a 10µg/mouse dose of IL-6 on the mRNA expression of C/EBPβ in the frontal cortex (g) or the hippocampus (h). [Figure 5.8 (g-i), n=5-6]

5.3.3.5 Systemic administration of IL-6 (10µg/mouse) causes a time-dependent increase in the mRNA expression of the neuronal activation markers c-fos and EGR1 in the brain

ANOVA showed an effect of IL-6 on mRNA expression of c-fos in the frontal cortex (a) ($F_{(5,28)}=17.17$, $P<0.0001$), hippocampus (b) ($F_{(5,28)}=25.14$, $P<0.0001$) and hypothalamus (c) ($F_{(5,28)}=22.12$, $P<0.0001$). Post-hoc comparisons revealed elevated c-fos mRNA in the frontal cortex 15mins ($P<0.01$), 30mins ($P<0.01$) and 1hr ($P<0.01$) following IL-6 administration, when compared to vehicle administered controls. In the hippocampus, post-hoc comparisons revealed increased c-fos mRNA 15mins ($P<0.05$) and 30mins ($P<0.01$) post injection. In the hypothalamus however, while post-hoc comparisons revealed an elevation in c-fos mRNA 15mins ($P<0.01$), 30mins ($P<0.01$) and 2hr ($P<0.01$) post injection, peaking at 30mins, it also revealed a subsequent reduction in c-fos mRNA 4hr following injection ($P<0.01$). [Figure 5.9 (a-c), n=5-6]

ANOVA showed an effect of IL-6 on mRNA expression of EGR1 in the frontal cortex (d) ($F_{(5,28)}=2.78$, $P<0.05$), hippocampus (e) ($F_{(5,27)}=12.38$, $P<0.0001$) and hypothalamus (f) ($F_{(5,28)}=19.07$, $P<0.0001$). Post-hoc comparisons revealed an increase in EGR1 mRNA 15mins ($P<0.01$), 30mins ($P<0.01$) and 1hr ($P<0.05$)

following IL-6 administration, in the hippocampus, when compared to vehicle administered controls. In the hypothalamus, post-hoc comparisons revealed an elevation in EGR1 mRNA 30mins ($P<0.01$) and 1hr ($P<0.01$) post injection, peaking at 30mins, however 4hr post injection a decrease was noted ($P<0.05$). Post-hoc comparison of EGR1 mRNA expression in the frontal cortex failed to show any changes following IL-6 administration. [Figure 5.9 (d-f), n=5-6]

5.3.3.6 Systemic administration of IL-6 (10µg/mouse) causes a time-dependent increase in the expression of IDO2 in the hypothalamus but no effect on BDNF or TDO

ANOVA showed an effect of IL-6 on mRNA expression of IDO2 in the hypothalamus (h) ($F_{(5,28)}=4.26$, $P<0.01$). Post-hoc comparisons revealed a significant increase in the mRNA expression of IDO2 15mins following IL-6 administration ($P<0.01$), when compared to vehicle administered controls. ANOVA showed no effect of IL-6 on mRNA expression of BDNF in the frontal cortex (a) or the hippocampus (b) and also failed to show any effect of IL-6 administration on the mRNA expression of TDO in the frontal cortex (c), hippocampus (d) or hypothalamus (e). ANOVA also showed no effect of IL-6 administration on IDO2 mRNA in the frontal cortex (f) and the hippocampus (g). Investigation of mRNA expression of IDO1 failed to determine a basal level of expression in any of the brain regions examined. [Figure 5.10, n=5-6]

Figure 5.5 – Systemic administration of IL-6 altered the expression of IL-6 related and regulated genes in the liver in a time-dependent manner

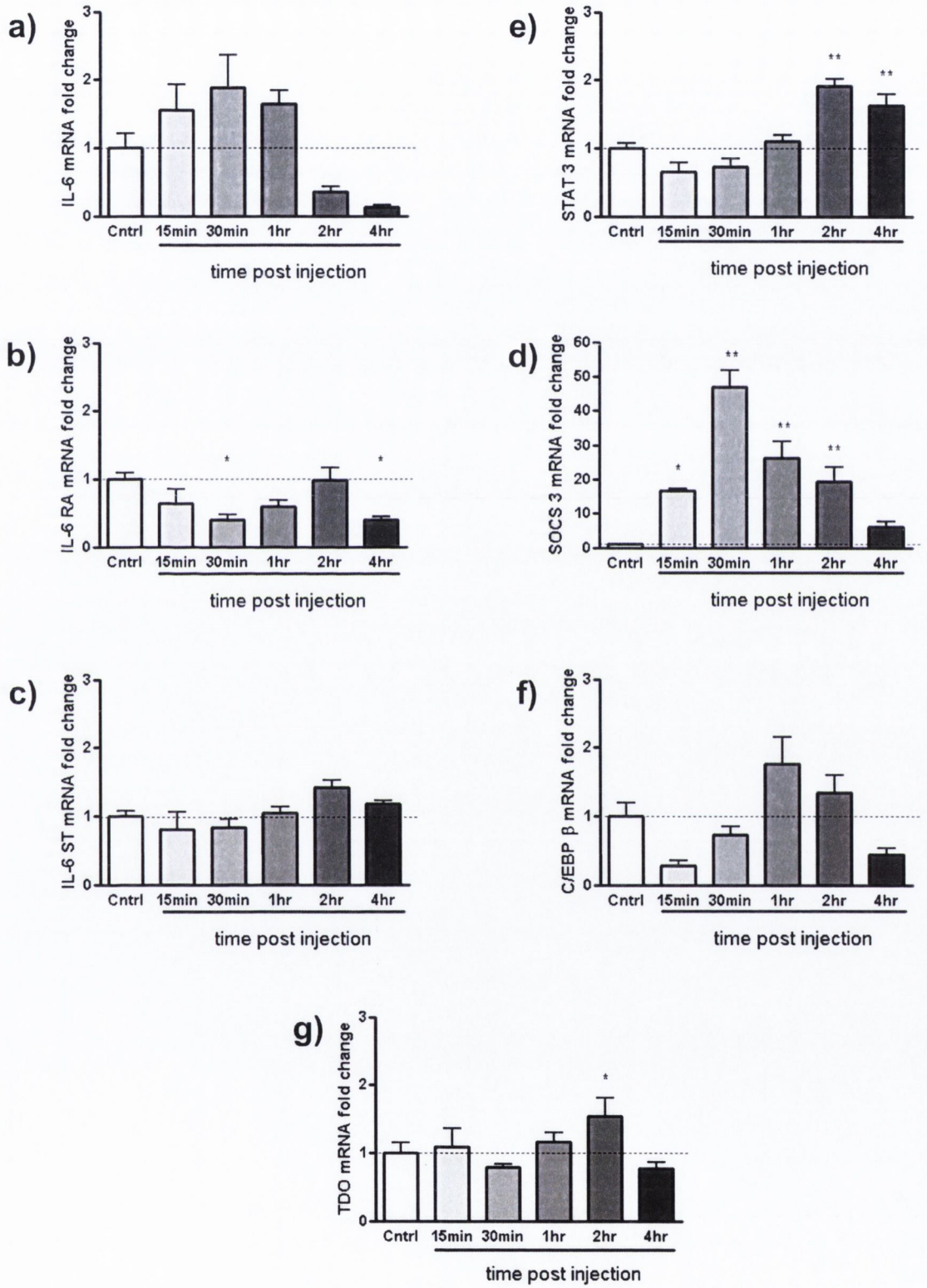

Figure 5.5 – Systemic administration of IL-6 altered the expression of IL-6 related and regulated genes in the liver in a time-dependent manner

IL-6 (10μg/mouse, i.p.) lead to a peak increase in the mRNA expression of SOCS3 (d, $P<0.01$) in the liver 30mins following injection. It also lead to a decrease in the mRNA expression of IL-6RA (b) 30mins and 4hr post injection ($P<0.05$). mRNA expression of STAT3 (e, $P<0.01$) increased 2hr following IL-6 administration. Data expressed as mean ± SEM (n=6). ** $P<0.01$; * $P<0.05$ vs. Vehicle treated controls.

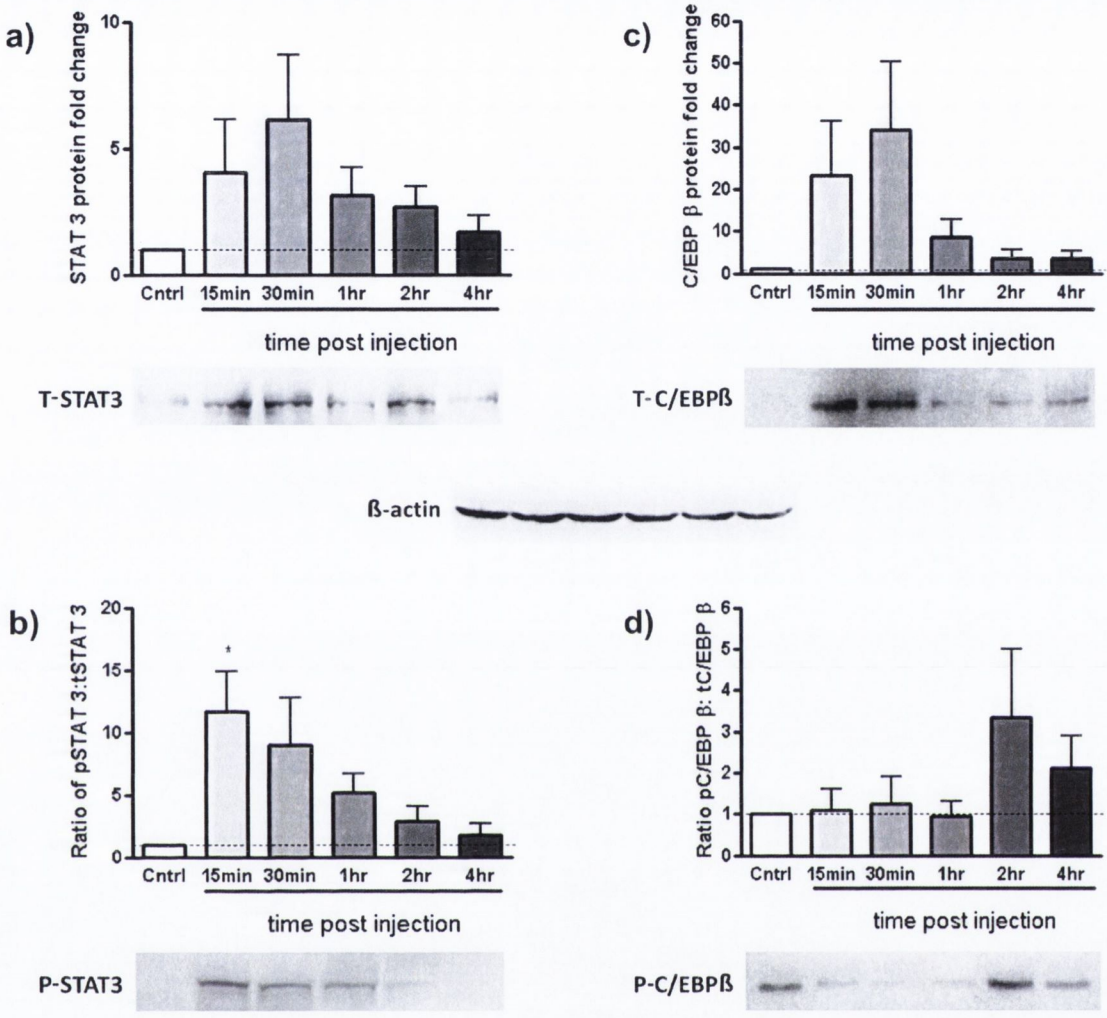

Figure 5.6 – Systemic administration of IL-6 altered the activation state of STAT3 in a time-dependent manner

IL-6 (10μg/mouse i.p.) increased the phosphorylation of the intracellular IL-6 signalling protein STAT3 (b) 15mins following injection ($P<0.05$) in the liver. A trend towards an increase was observed in the phosphorylation of the signalling protein C/EBPβ. Data expressed as mean ± SEM (n=6). * $P<0.05$ vs. Vehicle treated controls.

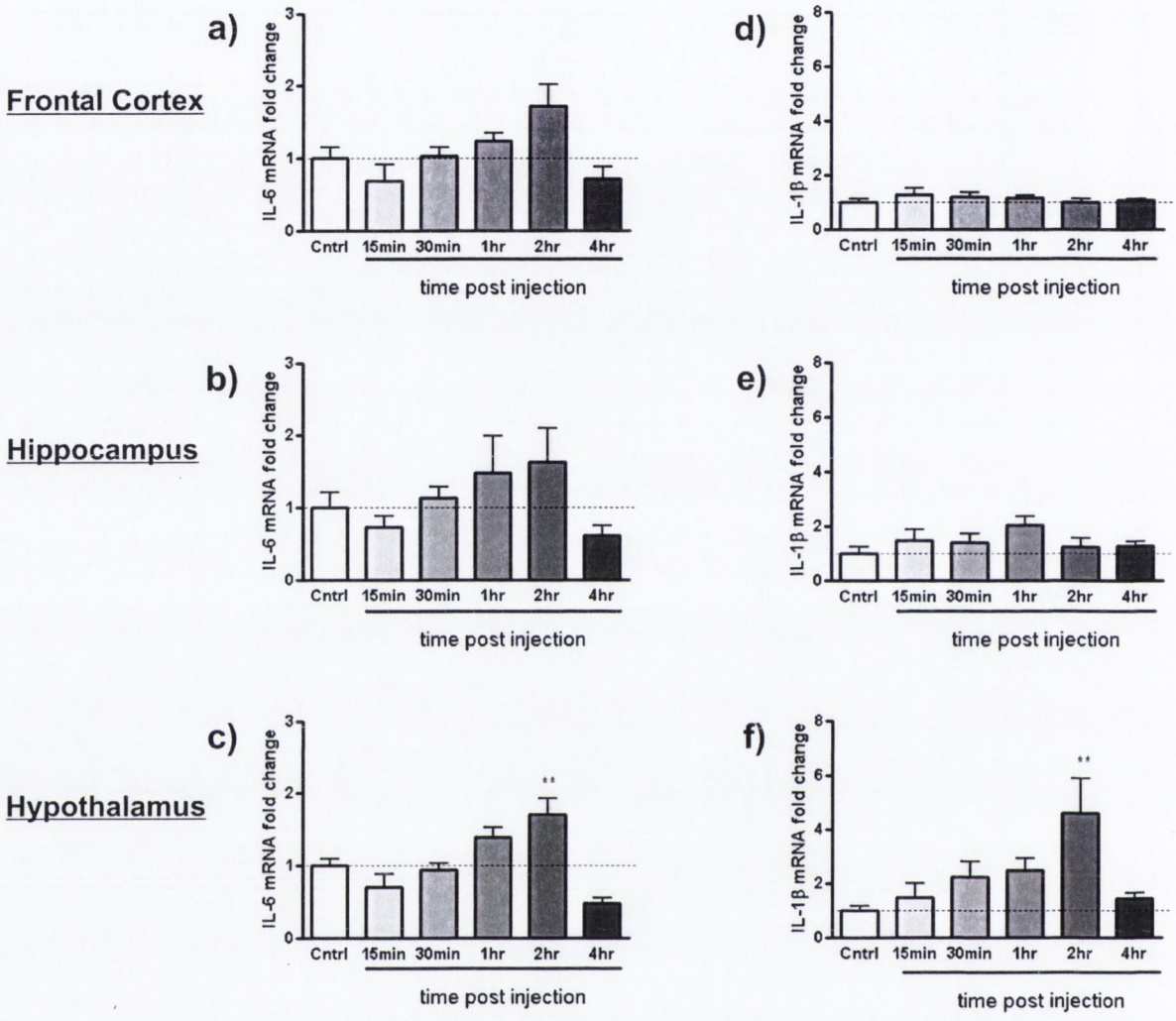

Figure 5.7 – Systemic administration of IL-6 increased the expression of IL-6 and IL-1β in a time-dependent manner in the hypothalamus

IL-6 (10ug/mouse, i.p.) increased the mRNA expression of IL-6 in the brain 2hr post injection in the hypothalamus (c, $P<0.01$). Similarly with IL-1β, mRNA expression was increased in the brain in the hypothalamus (f, $P<0.01$). No changes were obtained in the frontal cortex or hippocampus. Data expressed as mean ± SEM (n=6). ** $P<0.01$ vs. Vehicle treated controls.

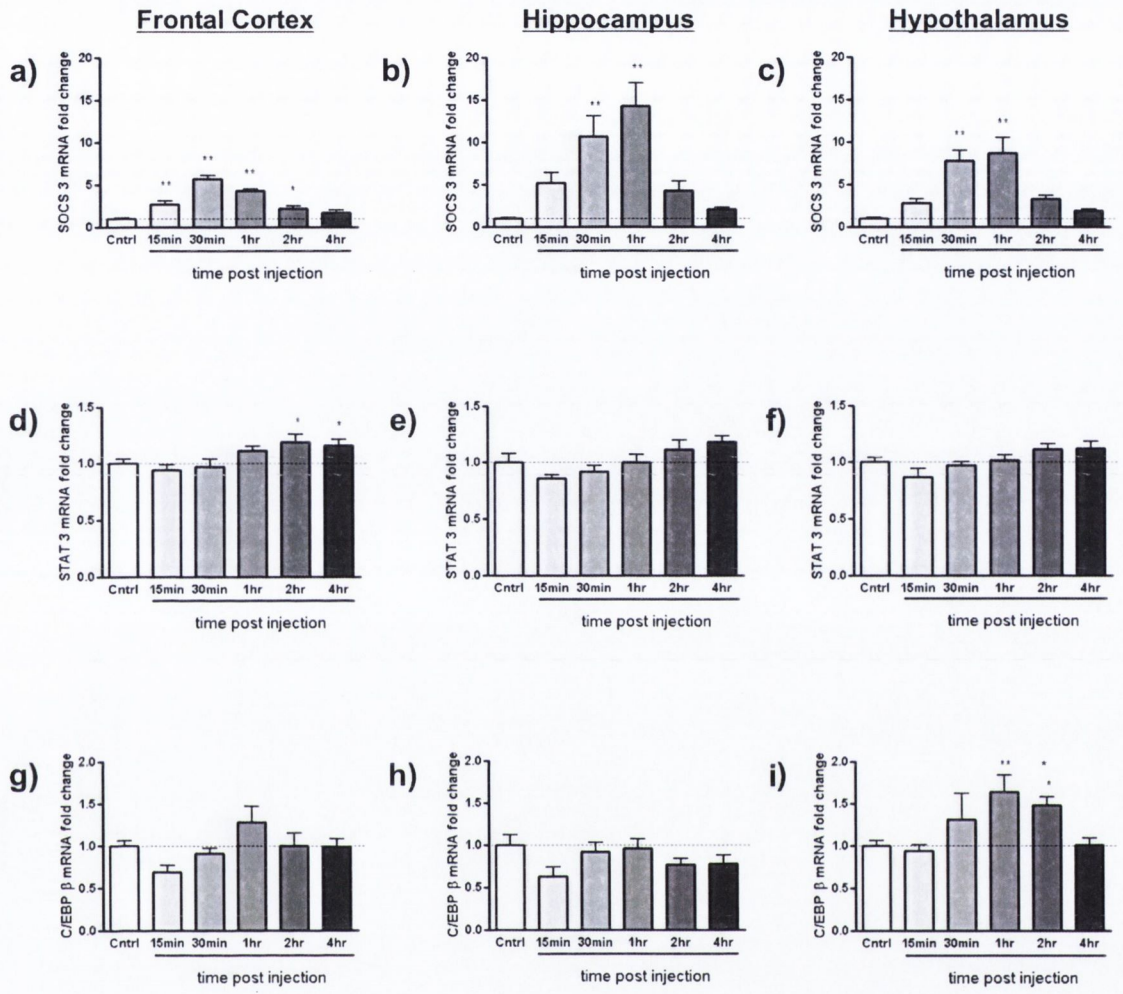

Figure 5.8 – Systemic administration of IL-6 increased the expression of IL-6 inducible genes in the brain

IL-6 (10ug/mouse, i.p.) increased the mRNA expression of the IL-6 inducible gene SOCS3 in frontal cortex (a, peak 30mins, $P<0.01$), hippocampus (b, peak 1hr, $P<0.01$) and hypothalamus (c, peak 1hr, $P<0.01$). mRNA expression of C/EBPβ was significantly increased 1hr ($P<0.05$) following injection in the hypothalamus (i). mRNA of STAT3 was unchanged following IL-6 administration. Data expressed as mean ± SEM (n=5-6). ** $P<0.01$; * $P<0.05$ vs. Vehicle treated controls.

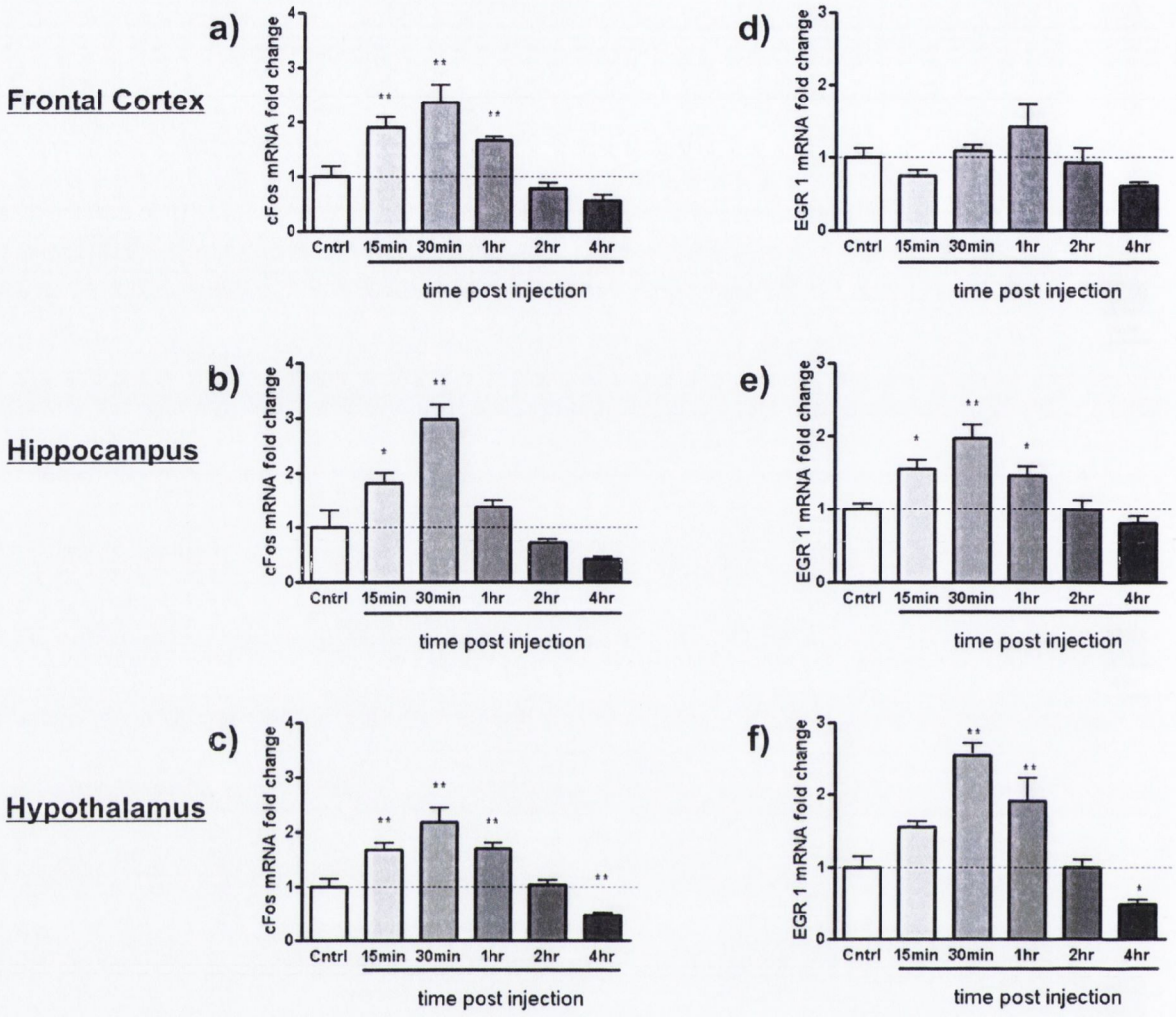

Figure 5.9 – Systemic administration of IL-6 increased the expression of neuronal activation markers c-fos and EGR1 in the brain

IL-6 (10ug/mouse, i.p.) increased the mRNA expression c-fos in the frontal cortex (a, peak – 30mins, $P<0.01$), hippocampus (b, peak – 30mins, $P<0.01$) and hypothalamus (c, peak – 30mins, $P<0.01$). In the hypothalamus, a reduction in mRNA expression was noted 4hr following injection ($P<0.01$). mRNA expression of EGR1 was increased in the hippocampus (e, peak – 30mins, $P<0.01$) and hypothalamus (f, peak – 30mins, $P<0.01$) following IL-6 administration. In the hypothalamus, a reduction in mRNA expression was noted 4hr following injection ($P<0.05$). Data expressed as mean ± SEM (n=6). ** $P<0.01$; * $P<0.05$ vs. Vehicle treated controls.

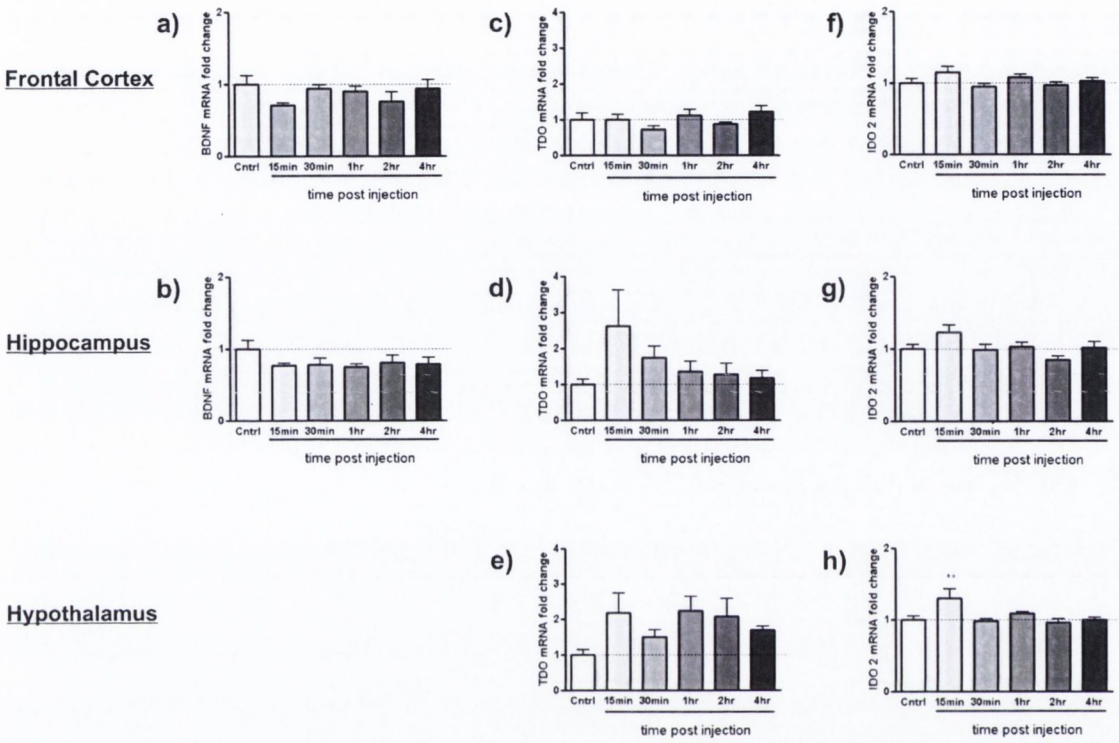

Figure 5.10 – Systemic administration of IL-6 increased hypothalamic IDO2 expression but did not affect BDNF or TDO expression in the brain

IL-6 did not have any effect the mRNA expression of BDNF in the frontal cortex (a) or hippocampus (b). Similarly mRNA expression of TDO did not significantly change following systemic IL-6 administration in any brain region investigated (c-e). IDO2 mRNA expression was found to be increased only in the hypothalamus (h) 15mins post injection ($P<0.01$). Data expressed as mean ± SEM (n=6). ** $P<0.01$; * $P<0.05$ vs. Vehicle treated controls.

5.4 Discussion

In the present study, we provide evidence that systemic IL-6 acts in the periphery and on the brain to activate IL-6 receptors and stimulate the expression of IL-6 regulated genes. The induction of IL-6 stimulated genes in the brain in response to stress or following i.p. administration of IL-6 indicates that circulating IL-6 can enter the CNS and act locally. This is supported by a similar temporal pattern of expression of IL-6 responsive markers in the brain and peripheral organs (liver and spleen) following IL-6 administration and suggests that cerebral parenchymal cells are sensitive to circulating IL-6.

Specifically the main findings of the current investigation are summarised as follows: Acute stress provoked an increase in circulating corticosterone accompanied by raised circulating concentrations of IL-6 and sIL-6R. Hepatic expression of IL-6RA, IL-6ST, STAT3, SOCS3, C/EBPβ and HP and expression of IL-6R, IL-6ST and STAT3 in the spleen were also raised following acute stress. Systemic administration of IL-6 (1μg/mouse) lead to raised circulating concentrations of the cytokine (25pg/ml) approximately equivalent to the circulating concentration observed following acute stress yet in the absence of a change in circulating concentrations of the sIL-6R. Moreover the expression of STAT3 only was increased in the liver and frontal cortex by comparison to the more extensive changes obtained following acute stress. Systemic administration of a high dose of IL-6 (10μg/mouse) provoked a time-dependent increase in the expression of IL-6, STAT3, SOCS3, C/EBPβ and TDO in the liver. Changes in the expression of STAT3 and C/EBPβ were accompanied by an increase in the ratio of pSTAT3:tSTAT3 and pC/EBPβ:tC/EBPβ indicative of enhanced IL-6 related signalling. Systemic IL-6 (10μg/mouse) provoked an increase in the expression of IL-6 and IL-1β, SOCS3 and C/EBPβ in the hypothalamus. SOCS3 expression was also raised in the frontal cortex and hippocampus whereas STAT3 expression was increased in the frontal cortex and hippocampus but not the hypothalamus. In all three brain regions assessed systemic IL-6 provoked an increase in the expression of the neuronal activation markers c-fos and EGR-1 in support of a direct action of systemic IL-6 on the CNS. Thus systemic IL-6 was found to induce a robust albeit more limited CNS response by comparison to the peripheral effects observed.

There are a limited number of reports to date on the central effects of systemically administered IL-6 in mice. A low dose of IL-6 (50μg/kg) did not affect

performance in the open field environment, however a higher dose (125μg/kg) reduced activity in the absence of a change in body temperature suggesting an anxious phenotype was generated in the absence of other classical sickness behaviour markers (Skelly et al., 2013). Behavioural changes were accompanied by an increase in the expression of the hepatic acute phase gene SAA2 indicating that IL-6 related signalling was occurring peripherally. By contrast to the increase in IL-1β and IL-6 expression in the hypothalamus observed 2hr following systemic administration of IL-6 (10μg/mouse) in the present investigation, Skelly and co-workers (2013) did not find changes in the expression of these pro-inflammatory cytokines 2hr following IL-6 administration. However the pattern of gene expression changes in the hippocampus as reported by Skelly et al. (2013) were more closely matched with those described in the present study. Differences between studies may be dose related, where the dose of IL-6 in the present investigation was considerably higher than that assessed by Skelly et al., (2013), and so it is not unreasonable to suggest that with a higher dose, they would have observed similar changes in hypothalamic mRNA.

A potential role for a CNS effect of systemic IL6 was demonstrated by Sakić et al., (2001) who observed that a spontaneous increase in serum IL-6 concentrations in lupus prone mice coincided in time with increased immobility in the forced swim test (FST). Also, intake of sucrose was decreased when IL-6 was over-expressed systemically in healthy mice suggesting that sustained elevation of plasma IL-6 might affect behaviour associated with motivation and reward (Sakić et al., 2001). High circulating concentrations of IL-6 in mice infected with Ad5MIL6 adenovirus were associated with reduced exploration of a novel object as well as food, water and sucrose intake resembling changes observed in sickness behaviour. Although elevations of IL-6 are commonly associated with sickness behaviour, whether or not IL-6 is responsible for the symptoms has not been established. It is of interest that systemic administration of IL-6 has not been shown to induce any sickness behaviours (Bluthe et al., 1998, Swiergiel and Dunn, 1999). Peripheral IL-6 also fails to induce endothelial NFκB or COX2 in the microvasculature of the CNS unlike other pro-inflammatory cytokines such as TNFα and IL-1β (Lacroix and Rivest, 1998, Laflamme and Rivest, 1999).

5.4.1 Stress related increase in circulating IL-6 and expression of IL-6 regulated genes

In the present investigation mice exposed to stress displayed an increase in the circulating levels of corticosterone immediately following stress, indicating a robust activation of the HPA-axis. Stress also provoked an increase in circulating levels of IL-6 and the sIL-6R in line with previous reports of increases in IL-6 in response to stress (Voorhees et al., 2013, Stark et al., 2002). In these investigations the nature of stress varied, Voorhees et al. (2013) reported elevated circulating concentrations of IL-6 following repeated restraint stress in mice, whereas Stark et al. (2012) reported an increase in circulating IL-6 following a period of social disruption stress. Within the brain, studies involving exposure to inescapable stress and dominant/aggressive cage mate paradigms have reported increased amounts of IL-6 present in cortical tissue (Sukoff Rizzo et al., 2012). An increase in the circulating concentration of the sIL-6R following acute stress as reported in the current investigation is significant as it potentially allows for trans-signalling of IL-6 on cells that do not have mIL-6RA (Chalaris et al., 2011, Rose-John, 2012). Changes in the circulating level of the sIL-6R occur following the immediate effects of stress on circulating IL-6 and so may enable the effects of IL-6 to persist beyond the 2hr time period over which the effects of stress were observed. IL-6 has been reported to act directly on adrenal cells to induce the release of corticosterone, but it also interacts synergistically with HPA-axis signalling messenger adrenocorticotropic hormone (ACTH) to produce increased amounts of corticosterone (Salas et al., 1990). The HPA-axis activating effect of IL-6 is shared by other cytokines including IL-1β and TNFα but is markedly less potent with limited physiological consequence. Treatment with antibodies to IL-6 and studies in IL-6 knockout mice indicate that IL-6 contributes only modestly to the elevation of plasma ACTH and corticosterone by LPS in mice (Wang and Dunn, 1999, Swiergiel and Dunn, 2006). It is nevertheless considered relevant in the context of infection and inflammation where IL-6 may contribute to the activation of the HPA axis (Silverman et al., 2003, Silverman et al., 2004, Dunn, 2004, Dunn et al., 2000).

Matsumoto et al. (2006) reported in rats that systemic IL-6 followed by exposure to a forced swim stress resulted in an increased hypothalamic response when compared to systemic IL-6 alone (Matsumoto et al., 2006). Similarly, a clinical study by Capuron et al. (2003) reported that patients who subsequently displayed depressive symptoms had a significantly higher ACTH and cortisol response to

IFNα treatment than patients who did not (Capuron et al., 2003b). IL-6 has also been shown to act synergistically with other stress responsive cytokines, like IL-1β, to induce behavioural changes (Harden et al., 2008, Bluthé et al., 2000). Harden et al. (2008) reported that in a study administering varying doses of IL-6, IL-1β or both i.c.v. to Sprague-Dawley rats, the rats administered both IL-6 and IL-1β exhibited the greatest decrease in food intake and body mass. Similarly, Bluthé et al. (2000) demonstrated that IL-6 knockout mice displayed greater social exploration, decreased levels of immobility and a decreased reduction in body weight following systemic administration with IL-1β or LPS when compared to IL-6 wild type mice. These studies have indicated a putative role for pro-inflammatory cytokines, including IL-6, to have synergistic effect on stress. The effect of a peripherally administered pro-inflammatory cytokine on animal behavioural phenotype could therefore be potentiated with the addition of a stress.

Exposure to stress also provoked an increase in the expression of a range of IL-6 related and regulated genes including IL-6RA, IL-6ST, STAT3, SOCS3, C/EBPβ, SOCS3 and HP in the liver. The changes obtained were largely not observed in the brain regions examined with the exception of an increase in the expression of C/EBPβ which was observed immediately following stress returning to basal levels 2hr later. In line with this, expression of the C/EBPβ inducible gene HP was significantly increased 2hr following exposure to stress in the liver, however while HP mRNA was detected in all of the brain regions examined, changes in expression were variable and not significant. The expression of IL-6 itself remained unaltered in the periphery changing only in the frontal cortex where an increase in expression was observed immediately following stress. IL-6 has been shown to be able to regulate its own expression via the transcription factor C/EBPβ, acting synergistically with NFκB to induce expression (Matsusaka et al., 1993), but IL-6 can be induced by other pro-inflammatory cytokines so we cannot be sure that the increase obtained in the frontal cortex is IL-6 mediated (Zhao et al., 2008). In addition no change in the expression of IL-6RA was observed in any of the brain regions assessed although there are conflicting reports of increases (Shizuya et al., 1998) and decreases (Miyahara et al., 2000) in the expression of IL-6RA in the hypothalamus and midbrain of rats in response to stress.

Changes in the expression of IL-6 and IL-6 related and regulated genes were also assessed in response to chronic stress. To test for any persistent effects of stress, the expression of all markers was measured 24hr following stressor

termination. No differences were obtained between the stressed and non-stressed control group indicating that repeated exposure to stress does not provoke persistent or sustained effects on the expression or IL-6 or related markers. Previously in our lab, it has been reported that chronic stressor exposure, using an identical paradigm to the one used in the present investigation, provoked an increase in the expression of IFNγ and IL-12 in splenocytes cultured from mice 24hr following exposure to chronic stress which was associated with a reduction in the expression of the glucocorticoid and β-adrenergic receptors (Curtin, 2008). It was proposed that the enhanced cytokine response observed is in keeping with receptor desensitisation whereby stress related adaptations in receptor expression remain for a period following stressor termination and consequently sensitivity to glucocorticoids and catecholamines is reduced leading to enhanced cytokine responses. This was supported where the suppressive effects of corticosterone and the β-adrenergic agonist salbutamol on IFNγ and IL-12 were impaired in splenocytes cultured from mice 24hr following exposure to chronic stress. Dysfunction in glucocorticoid and β-adrenergic signalling and the subsequent dysregulation of immune cells via these mechanisms has been proposed as a possible consequence of elevated pro-inflammatory cytokine expression and the mild inflammatory phenotype at times evident in psychiatric disorders (Raison and Miller, 2003). Such mechanisms however do not appear to be of relevance in the present investigation in relation to IL-6 as no changes in this cytokine were observed following chronic stressor exposure. Further work is required to clarify if chronic stress may influence the response to IL-6, specifically in the spleen, as reported with IFNγ and IL-12 in association with the stress-related downregulation of GR and catecholamine immnuoregulation.

Higher concentrations of circulating IL-6 and sIL-6R have also been reported in patients suffering from post-traumatic stress disorder (Maes et al., 1999) and major depressive disorder (Maes et al., 1995). Meta analyses have shown a clear association between circulating levels of IL-6 and depression. A recent meta-analysis of clinical studies involving 3,695 patients in all, revealed a significant positive association between increased IL-6 and depressive symptoms (Valkanova et al., 2013). Similarly, Howren's analysis of 62 investigations and Dowlati's analysis of 16 studies, revealed positive associations between depressive symptoms and circulating levels of IL-6 (Dowlati et al., 2010, Howren et al., 2009). In addition two meta analyses compared circulating levels of IL-6 in

depressed patients and non-depressed controls and the levels of IL-6 following treatment with antidepressants. Depressed patients displayed elevated levels of IL-6 when compared to non-depressed controls (Hiles et al., 2012a) while IL-6 levels were significantly reduced following antidepressant treatment (Hiles et al., 2012b),

A putative mechanism whereby peripheral IL-6 may increase in response to stress is via the sympathetic nervous system, which like the HPA-axis is activated by stress (Raison et al., 2006). Sympathetic nerve fibre afferents innervate lymphoid tissues including the spleen and lymph nodes allowing direct signalling on immune cells shown to possess adrenergic receptors (Felten and Felten, 1994). Interestingly, a link between noradrenergic release and circulating IL-6 has been demonstrated. In 2007, Tan et al. Demonstrated that macrophages treated with the β_2-adrenoceptor agonist salmeterol showed a robust increase in IL-6 mRNA and protein and this was blocked by the β_2-adrenoceptor antagonist ICI 118,551 (Tan et al., 2007). Similarly, non-specific blockade of the β-adrenoceptor suppressed stress-induced increases in circulating IL-6 (Soszynski et al., 1996). *In vitro* work on fibroblasts harvested from osteoarthritis patients has also shown an increase in the amount of IL-6 released by cells following treatment with noradrenaline (Raap et al., 2000).

5.4.2 Dose dependence of systemic and CNS inflammatory effects

Exposure to stress triggers changes in a wide array of endocrine and immunological makers including both pro- and anti-inflammatory cytokines (for review see (Black, 2002, Elenkov and Chrousos, 1999)). Specifically relating to IL-6 function, SOCS3 expression may also be induced by IL-4, which is influenced by glucocorticoids (Canfield et al., 2005, Bethin et al., 2000) and while C/EBPβ is induced by IL-6 it may also be induced by other pro-inflammatory cytokines (Baumann et al., 1992). In order to isolate the central response to raised circulating concentrations of IL-6, a study to investigate the dose dependence of systemic and CNS inflammatory effects was carried out following systemic IL-6 administration.

Systemic administration of IL-6 (1μg/mouse) lead to raised circulating concentrations of the cytokine matching those obtained following acute stressor exposure. This confirmed the bioavailability of IL-6 following IL-6 administration

i.p. and also the physiological relevance of the dose of IL-6 administered. Whereas in response to restraint stress circulating concentrations of sIL-6R were increased 2hr post cessation of stress, there was no induction of sIL-6R 2hr following systemic administration of IL-6 at any of the doses tested which would imply that changes to the sIL6-R in response to stress are not driven by IL-6 itself, but some other factor induced or activated in response to stress. Also noteworthy was the apparent lack of IL-6 related signalling in the periphery following systemic administration when compared to the response obtained following acute stress. An increase in the expression of hepatic STAT3 expression was obtained with the highest dose (1μg/mouse), however other IL-6 related or regulated genes were not affected following systemic IL-6 administration at any dose in the periphery. In line with the change observed in the liver, expression of STAT3 was also increased in the frontal cortex indicating that systemic IL-6 leads to a change in a signalling component relating to IL-6 function centrally. However no further changes were observed centrally in relation to IL-6 related or regulated genes including IL-6 itself, IL-6ST, IL-6RA, SOCS3 and C/EBPβ following systemic IL-6 administration. Reasons for the lack of response both peripherally and centrally to systemic IL-6 administration could relate to the doses tested whereby higher doses are required to initiate changes in IL-6 regulated genes such as SOCS3. In addition the markers were measured at a single time point 2hr following systemic IL-6 administration and so changes occurring either before or after this time may have been missed. In order to address these limitations a time course study following systemic administration of a single high dose of IL-6 (10μg/mouse) was undertaken where resultant peripheral and central changes were recorded at regular intervals over 4hr.

5.4.3 Temporal effects of a single high systemic dose of IL-6

Peak increases in the hepatic expression of IL-6, SOCS3 and C/EBPβ were obtained within 2hr returning to basal levels or below 4hr following systemic administration of IL-6. The time course was therefore appropriate to capture the full extent of changes associated with the IL-6 challenge. Raised expression of the IL-6 regulated genes indicates that IL-6 related signalling is activated in the liver in response to systemic administration confirmed also by increased phosphorylation of STAT3 and C/EBPβ.

IL-6 also lead to an induction of the expression of the glucocorticoid receptor activated tryptophan degrading enzyme, TDO in the liver 2hr following systemic IL-6 administration, suggestive of a possible activation of the kynurenine pathway. Activation of this pathway has been linked to the pathophysiology of major depression, whereby a shift in tryptophan metabolism away from serotonin biosynthesis in favour of kynurenine biosynthesis promotes neuronal transmission that favours a depressive phenotype. Previous work in our laboratory has reported stress related activation of TDO in rats which leads to tryptophan depletion, production of kynurenine and depression-related behaviour. Inhibition of TDO blocks these stress related changes and promotes antidepressant-like behaviour (Gibney et al., 2014). The mechanism whereby IL-6 leads to hepatic expression may be via activation of the HPA-axis, production of corticosterone and activation of hepatic glucocorticoid receptors, however such a mechanism remains to be confirmed. Moreover further experiments will need to be carried out to assess the ability of systemic IL-6 administration to lead to circulating changes in tryptophan and kynurenine pathway metabolites leading to depression related behaviours. A role for the closely related enzyme IDO is not likely as results to date indicate that the expression of IDO in the spleen is not altered by systemic IL-6 administration. In the present study, expression of the IDO2 subtype was increased in the hypothalamus in response to systemic IL-6 administration. IDO2 is structurally similar to the IDO1 isoform retaining the same catalytic sites and function as IDO1 (Ball et al., 2007) and its expression has been reported to change centrally in response to immune and stressful stimuli (Browne et al., 2012). Although primarily produced in the liver, TDO has also been reported in the rodent brain, warranting investigation centrally (Haber et al., 1993, Ohira et al., 2010). The relatively modest increase in expression of IDO2, the absence entirely of IDO1 and the lack of significant changes in TDO expression within the brain would suggest that the kynurenine pathway is not activated within the brain (Maes et al., 2011, Dantzer et al., 2011, Ball et al., 2009). In support of this, the observed increases in IL-1β and IL-6 expression in the hypothalamus, which could possibly account for changes in IDO2 expression, did not match up to the time course of increased expression of the cytokines making a cause and effect interaction unlikely. A role for IDO however has been reported in other immune stimulation models where there is a clear induction of IDO following immunological challenge which has contributed to depression related behaviour in rodent models (O'Connor et al., 2009b, O'Connor et al., 2009a).

In the CNS, systemic IL-6 administration leads to an increase in the expression of IL-6 and IL-1β in the hypothalamus. Coupled with an increase in SOCS3 and C/EBPβ expression, the hypothalamus is clearly sensitive to the systemic effect of IL-6 and may contribute to IL-6 mediated activation of the HPA-axis (Girotti et al., 2013). SOCS3 expression was also increased in the frontal cortex and hippocampus indicating that the effects of systemic IL-6 are regionally distributed beyond the hypothalamus. Of note is that the expression of C/EBPβ did not change in these regions suggestive that IL-6 regulated C/EBPβ expression is restricted to the hypothalamus. Further support for the central effects of systemic IL-6 administration is evident where neuronal activation, confirmed by raised expression of the neuronal activation markers c-fos and EGR-1, appears over a time course in all three brain regions assessed following IL-6 administration. Investigation of the growth factor response to a peripheral IL-6 injection, showed no change in the mRNA expression of BDNF in the hippocampus or the frontal cortex, suggesting that increased levels of IL-6 signalling in the brain does not negatively affect the expression of BDNF within the brain as has been reported with other pro-inflammatory cytokines including IL-1β and TNFα (Miller et al., 2009).

In conclusion, this present study demonstrates that systemic IL-6 administration leads to activation of IL-6 related signalling in the periphery coupled with the activation of IL-6 regulated genes and neuronal activation centrally.

5.4.4 Implications for the clinic

The only cytokine consistently reported to be elevated in the plasma of depressed patients is IL-6. Plasma concentrations of IL-6 are frequently elevated during infections, some forms of stress and other pathological conditions. Thus there is unlikely to be any specific relationship between plasma IL-6 and depression. Moreover in most studies in which IL-6 was administered to rats and mice, it fails to induce sickness or depression related behaviours. There are however numerous hypothetical mechanisms by which IL-6 could alter neurotransmitter systems and thus contribute to changes in behaviour of relevance to depression or anxiety related disorders. IL-6 may lead to activation of the kynurenine pathway as described previously to contribute to neural transmission changes which influence behaviour. Conversely IL-6 has been reported to activate brain serotonergic systems increasing brain tryptophan

concentrations and the metabolism of 5-HT as indicated by increases in the brain concentrations of its metabolite 5-HIAA (Zalcman et al., 1994, Clement et al., 1997, Dunn, 2006). Experiments administering 2μg IL-6 systemically to Sprague-Dawley rats revealed an increased release of 5-HT (measured via microdialysis) 3hr following IL-6 administration when compared to vehicle administered controls that persisted for the remaining 3hr of measurements (Zhang et al., 2001). The acute effects of IL-6 appear to increase the available tryptophan in the brain perhaps enhancing serotonergic activity which could contribute to an antidepressant effect. The relationships between these potential mechanisms and depression remain to be determined in depressed patients. It is possible that by activating the HPA-axis, central CRF and serotonergic mechanisms, cytokines like IL-6 may complement (or even synergize with) other factors that induce depression.

5.4.5 Future work and directions

Demonstration of the presence of mRNA only indicates the propensity for the synthesis of the proteins. Further work is necessary to determine if changes in the expression of target genes as reported in this investigation translate into protein with functional consequences. Assessing the behaviour exhibited following systemic administration of the high dose of IL-6 would also be of benefit. With relation to the specific behavioural impact of IL-6, Sukoff Rizzo et al. (2012) have recently demonstrated an important role of central IL-6 and IL-6 trans-signalling in the development of depression related behaviours in animal models. Within this study, Sprague-Dawley rats who expressed helplessness behaviour in an inescapable foot shock paradigm, had elevated central IL-6 protein levels when compared to sham exposed controls. Further investigation with 1μg IL-6 administered i.c.v. to Swiss Webster mice revealed a decrease in social interaction and an increase in immobility time in both the FST and tail suspension test (TST) in the absence of sickness behaviour, with immobility time in the TST remaining elevated up to 48hr following administration. Furthermore, mice genetically altered to overexpress IL-6 centrally also displayed increased immobility in the FST and TST. In both transgenic and centrally administered IL-6 models the increased immobility was reduced with pre-treatment with the antidepressant fluoxetine. In addition co-administration of centrally administered IL-6 with an anti-IL-6 antibody reduced immobility time. Similarly, co-

administration of IL-6 with sIL-6ST also reduced this immobility time via blockade of trans-signalling through sIL-6R. This study shows a direct role for central IL-6 in developing behavioural changes and inducing depressive-like phenotypes in the absence of sickness behaviour.

Our specific interest for the future would be whether peripherally administered IL-6 could induce the same increase in immobility in a TST exposure or alterations in hedonic behaviours, which would be indicative of a depressive phenotype. Inhibition of peripheral IL-6 signalling would determine whether the changes observed centrally are induced by peripheral IL-6 signalling. Systemic co-administration of IL-6 with a sIL-6ST could assess the role peripheral IL-6 trans-signalling in the CNS IL-6 response. Tocilizumab is a humanised monoclonal antibody for IL-6r that prevents binding of IL-6 to the receptor and is used in the treatment of RA (Emery et al., 2008). The very limited data available reports that Tocilizumab has low infiltration rates into the brain which suggests that the majority of its action occurs in the periphery (Salsano et al., 2013, Therapeutic-Goods-Aministration, 2011). An apparent half-life of 11 days for a 4mg/kg dose of Tocilizumab has been reported. Co-administration with this antibody would allow for removal of all peripheral IL-6 signalling meaning that any changes in central expression or behavioural responses would be caused by IL-6 that accesses the CNS alone.

6. Discussion

6.1 General Discussion

There is accumulating evidence in support of increased activation of immune cells in the brain in the development of major mental disorders and the identification and modulation of activated immune cells provides new avenues to improve diagnosis and treatment of major mental illnesses. In this thesis, a series of animal models of immune activation were assessed to develop an understanding of interactions between the immune system and brain leading to changes in behaviour and the determination of biomarkers of relevance to depression symptoms and its underlying pathophysiology. The main findings may be summarised as follows:

6.1.1 Poly I:C challenge: Poly I:C-induced activation of the innate immune response is accompanied by symptoms of depression and anxiety; a role for the kynurenine pathway

In response to poly I:C, Sprague-Dawley rats showed significant body weight loss, a reduction in saccharin preference (anhedonia), decreased locomotor activity in the home cage activity test and increased anxiety-related behaviours in the open field test. This altered behavioural phenotype was associated with poly I:C-induced temporal increases in the expression of the cytokines IL-1β, IL-6 and TNFα and the microglial activation marker CD11b in the frontal cortex and hippocampus of Sprague-Dawley. Decreased expression of the neurotrophic marker BDNF and its receptor TrkB, was also found in response to poly I:C administration and an increase in the expression of the tryptophan degrading enzyme IDO was also found in the brain which was associated with increased central concentrations of the tryptophan metabolite, kynurenine. These findings suggest that the depressive-like behaviour elicited by poly I:C administration in Sprague-Dawley rats may be associated with cytokine induced alterations in the kynurenine pathway and may be of importance for the development of viable therapeutic strategies for inflammation-related depression.

6.1.2 WKY rats show a characteristic response to immune challenge relative to other strains and species

In agreement with numerous reports from other groups and previous work in our laboratory, immune stimulants including LPS and poly I:C provoke depression and anxiety-related behaviours in rats (Sprague Dawley) and mice (CD-1 and NOD) that persist after sickness behaviours dissipate. WKY rats and NOD mice both displayed a depressive-like behavioural phenotype at basal level without stimulation, however WKY rats did not respond to immune challenge to the same degree as the other species and strains tested. Furthermore, whereas Sprague-Dawley and WIS rats and CD1 and NOD mice display an increase in microglial activation following immune challenge, WKY rats appear to exhibit lower levels of microglial activation indicating that this strain may be characteristically different to other rodent species and strains with regard to immunological response and related behavioural change. Poly I:C increased expression of IDO in the frontal cortex and hippocampus of Sprague-Dawley and WIS rats, however IDO expression was not increased in WKY rats. This failure to induce IDO was accompanied by the aforementioned impaired microglial activation and reduced expression of TNFα, a key inducer of IDO expression (O'Connor et al., 2009a), suggesting that impaired microglial activation could be responsible for the lack of IDO induction in the WKY strain.

These observations have recently been accompanied by data from the laboratory that indicates a degree of GR hypersensitivity in WKY rats in response to an immobilisation stress (unpublished data, Eoin Sherwin). WKY rats showed a greater expression of FKBP5 and TDO in the liver and measurement of hepatic TDO activity revealed WKY rats had a greater conversion of tryptophan to kynurenine, indicative of a putative link between glucocorticoid sensitivity and kynurenine pathway induction (Sherwin et al., 2014). Interaction between the stress-induced endocrine (neuro-) and immune axes may account for the depressive and anxiety-related phenotype characteristic of this strain. It is of interest that GR hypersensitivity has been reported in patients suffering from post traumatic stress disorder (Mello et al., 2011). Some forms of depression, particularly where depression and anxiety present together, may also exhibit signs of GR hypersensitivity although this remains largely uncharacterised to date.

6.1.3 NOD mouse: a new animal model for psychiatric disorder characterised by gene-environment interactive immune and neurotrophic dysregulation

Pre-diabetic NOD mice possess an anxious and depressive-like phenotype when compared to a CD1 control strain at steady state. Also at steady state both monocytes and microglia isolated from NOD mice show impaired development and function, and an impaired inflammatory phenotype. Furthermore, in response to immune stimulation, an altered behavioural phenotype was observed in the NOD mouse compared to CD1. This was paralleled by the observation that NOD monocytes were hyper-responsive and were characterised by a classic and type-1 interferon genotype, which was particularly evident in the mature $Ly6C^{low}$ monocytes. Conversely, NOD microglia were hypo-responsive to LPS and while they did not show a clear classic inflammatory response, they did however show a mild up-regulation of genes involved in type-1 interferon signalling. An altered steady state and inflammatory response in the NOD mouse was also observed when inflammatory markers, growth factors and kynurenine pathway genes were measured in the brain. Separate exposure to a psychological stressor revealed that NOD mice have a prolonged elevation of circulating corticosterone and a blunted glucose response. These changes were accompanied by increased expression of GR inducible genes in NOD mice that was not present in the CD1 strain, such as FKBP5 which has been reported to inhibit GR signalling and also been linked to depression (Binder, 2009, Jääskeläinen et al., 2011, Zimmermann et al., 2011).

6.1.4 Stress-induced IL-6 and systemic IL-6 administration

Immune stimuli including LPS and poly I:C increase circulating concentrations of pro-inflammatory cytokines including IL-6 both peripherally and centrally, which can induce depressive behaviour (Burton et al., 2011, Dunn et al., 2005, Fortier et al., 2004, Gibb et al., 2011, Konat et al., 2009). The role that individual cytokines play in the pathogenesis of depressive symptoms, and in particular IL-6, is not well characterised in general. IL-6 has also been shown to be a stress sensitive cytokine that can be induced following exposure to psychological stressors (Butterweck et al., 2003, Jankord et al., 2010, Maes et al., 1999, Voorhees et al., 2013). It is of particular interest as it has been the cytokine which has been reported to be most reliably increased in patients with depression

(Dowlati et al., 2010, Howren et al., 2009). Moreover direct central administration of IL-6 has been shown to induce depressive-like behaviours in mice (Sukoff Rizzo et al., 2012).

In this project stress related increases in the circulating concentrations of IL-6 were reported accompanied by changes in the expression of IL-6 inducible and related genes in both peripheral and central compartments. Administration of a high systemic dose of IL-6 also resulted in a central response of IL-6 inducible genes and induction of neuronal activation markers suggesting that peripheral IL-6 can lead to signalling within the brain. Monitoring of the activity of cytokines in depressed humans is mainly limited to observed changes in circulating concentrations of soluble factors in plasma. Information into the role that elevated circulating cytokine concentrations may have in the CNS is limited to changes in CSF concentrations of cytokines or downstream intracellular markers within blood cells. This limitation can be addressed in an animal model and we provide evidence in the present investigation that a rise in circulating IL-6 concentrations following systemic administration can provoke IL-6 related changes in CNS tissue. Limitations of this particular study stem around the lack of tests investigating a change in the behavioural phenotype of mice following IL-6 administration.

6.1.5 A potential role for astrocytes

In this thesis, the focus of the studies carried out has been on microglia, the primary immune cell of the brain. However within the CNS astrocytes significantly outnumber microglia (Pelvig et al., 2008). Initial descriptions of astrocytic function focussed on its role in neuronal support via buffering of ions, provision of nutrients and water, conversion of glucose to lactate, clearance of free radicals and its role in BBB integrity. As outlined briefly above (Chapter 1.3), astrocytes have also been reported to have a role in signal transmission as well as immunological functions (Kimelberg, 1995, Parpura et al., 1994). Astrocytes have been reported to express immune related cell surface molecules including MHCII, CD40 and CD80 and can respond to TLR agonists (Dong and Benveniste, 2001, Gurley et al., 2008). Phagocytic activities and production of inflammatory mediators following immune stimulation have also been reported in astrocytes, suggesting a broader immune role within the CNS (Ransohoff and Brown, 2012, Nguyen et al., 2011, Farina et al., 2007). Expansion of the topics covered in the

thesis to include astrocytic responses to immune stimuli and their profile in an inbred rodent model, could allow for a more accurate evaluation of the role that microglia specifically contribute to the observe results.

6.1.6 Future directions

Microglia, when activated, are involved in the regulation of neuroinflammation but in the steady state they have important functions assisting in neurodevelopment, including synapse formation and pruning (Tremblay et al., 2011a). There are now several lines of evidence showing that this physiological function in particular is perturbed in pre-psychiatric and psychiatric disease, resulting in an abnormal neurodevelopment and neuronal function due to an activated state of cells of the mononuclear phagocyte system (MPS), including the microglia in the brain. This concept is generally referred to as the "Macrophage-T cell or Immune Theory of Major Mental Disorders" first proposed in 1992 and supported by numerous reports of aberrant levels of inflammatory cytokines in the serum, plasma and CSF of patients with major mental illnesses (Beumer et al., 2012, Smith, 1992). Monocytic activation has been reported to precede the onset of symptoms indicating that immune parameters may serve as biomarkers for an at-risk state for major mental illness (Drexhage et al., 2010). Histopathological studies, though limited and carried out on post mortem material, also indicate an abnormal activation of microglia in patients with major psychiatric disorders (Steiner et al., 2011). Moreover it has been proposed that microglial activation may underlie white matter integrity disturbances which accompany illness episodes over the patient's lifetime (Benedetti et al., 2011).

The activation state may be useful in predicting the response of patients to drug treatment. Patients with an activated immune state are less likely to respond to regular therapy and it is hypothesised that treatment resistant patients will respond to conventional treatment in combination with immunosuppressive therapy expected to dampen the inflammatory state of non-responders. Monocyte and microglial activation may be reversed by administration of immunosuppressive drugs including non-steroidal anti-inflammatories (NSAIDs) (Müller, 2010, Sommer et al., 2012), N-acetylcysteine (NAC) (Dean et al., 2011) and minocycline (Levkovitz et al., 2010), although such treatments have not been systematically investigated in psychiatric disorders, with further potential for novel immunosuppressive interventions. The mechanisms responsible for the activation

of microglia in psychiatric disease remain elusive. A direct microbe-driven activation of microglia is possible (Kristensson, 2011), but apart from direct microbial/gDNA activation of microglia, inborn errors in the growth and differentiation of MPS cells can make the cells vulnerable for hyper-stimulation. For example, NOD mice display reduced expression of genes important for neuronal development, suggesting a defect in support function of NOD microglia for the development of neuronal circuitry. Utilisation of diffusion tensor magnetic resonance imaging (DT-MRI) would allow for characterisation of white matter tracts in NOD mice in a longitudinal fashion. Use of NSAIDs, NAC, minocycline, and novel immunomodulating drugs in all models could be examined for effectiveness in rectifying the behavioural changes observed in this thesis. Models will enable in-depth studies on the molecular mechanism of immune mediated behavioural abnormalities and their correction by drug treatment. Overall the animal models will enable us to study neuroimaging changes, blood parameters for immune and HPA-axis activation, central immune markers induced following immunological stimulation over the course of development and to correlate these to behavioural abnormalities and immune histopathological changes in the brain. Furthermore, use of animal models will help in developing a better understanding of the biological factors involved with the pathogenesis of depression.

6.1.8 General discussion on animal models

One of the main limitations of the studies outlined in this thesis as with any study involving animal models is that they do not completely model the human disease, exhibiting only a few select traits that are deliberately chosen either through breeding or the treatment regime. Furthermore, working with inbred strains of mice and rats that are genetically identical minimising population variability coupled to the use of controlled environments e.g. housing where temperature, light and diet exposure are kept constant minimising environmental influences, reduces variation between individuals and may therefore not be reflective of the human population. However, animal models present researchers with the opportunity to carry out invasive research to investigate target organs and the central nervous system directly which cannot be approached in studies on human beings. The validity of the animal models in use must be weighed up to include (i) construct i.e. based on the hypotheses about etiology of depression i.e. immune

stimulation, autoimmune activation and response to individual cytokine (ii) face, which seeks to address the similarities between immunologically induced symptoms and symptoms associated with depression and anxiety related disorders and (iii) predictive i.e. potential of a model in relation to predicting drugs or biological agents of potential therapeutic value in humans.

Understanding how stress and emotional distress modulates brain-immune-endocrine interactions has clear translational implications from laboratory data. Clearly there are numerous research opportunities in this area which would advance our current knowledge. The identification of genetic risk factors and the role that genetics might have in these complex relationships is largely unexplored territory to date. As the technology to manipulate the genome has come of age, such questions can be adopted from the clinic and readily explored in animal models. Strategies which attempt to address wide variation within animal studies by subdividing populations according to behavioural or physiological characteristics e.g. coping styles, will also help to unravel factors underlying susceptibility or vulnerability. Additional research employing stressors with a greater degree of ecological validity which challenge the natural defence and/or adaptive capacity of animals e.g. social stress, rather than pushing animals towards a physiological ceiling will serve to increase the face validity and relevance of existing models and practices (Koolhaas, 2008). Moreover an investigation of the influence of early life stress, and its long term impact on the developing endocrine and immune systems, is an important question that has not been well studied to date. The concept that stress may predispose to a premature ageing of the immune response has been proposed (Glaser and Kiecolt-Glaser, 2005) and to explore this further, a greater emphasis on longitudinal investigations is required by comparison to the predominance of cross sectional studies which have provided limited insights on this issue to date.

Leading on directly from developments with animal models, there is great potential for the development of biomarkers of inflammation to reflect pathophysiological mechanisms linking inflammation to sickness and depression. Such biomarkers would help to address the current inadequacy of psychological scales and over reliance on psychiatric diagnosis especially for the neurovegetative symptoms associated with depression. The availability of peripheral biomarkers that can both identify patients with specific pathophysiology and serve to objectively monitor therapeutic responses would represent a major advance in the diagnosis and treatment of depression. There

are new possibilities for treatments that target pathways by which the immune system influences the brain such as cytokines or growth factors and their downstream mediators or the activation of relevant CNS immune cell types (e.g. microglia) to emerge. Results from trials to determine the efficacy of anti-inflammatory drugs such as the use of anti-TNFα in patients with psoriasis (Kannan et al., 2013) with antidepressant potential or the adjunctive use of cyclooxygenase (COX) -2 inhibitors for treating depression (Müller, 2010) have been encouraging. The COX enzymes are responsible for the production of prostaglandins and their activity has been reported to increase with inflammation (Hawkey, 1999). Two isoforms exist, the constitutively expressed COX-1 and COX-2, which is expressed under inflammatory conditions (Fu et al., 1990, O'Banion, 1999). Muller et al. reported that using the COX-2 selective inhibitor celecoxib yielded therapeutic effects in a study of 40 patients suffering from major depression (Müller et al., 2006). However use of COX-2 inhibitors have been associated with increased risk of cardiovascular events in patients undergoing chronic treatment (Solomon, 2006). Solomon et al. reported an increase in cardiovascular events and blood pressure in patients receiving a dose of celecoxib which was lower than that which exhibited therapeutic effects in the study by Muller (Solomon et al., 2006). Subsequently further research is required to elucidate a dose of this drug that elicits a therapeutic response without the associated cardiovascular risks. Moreover treatments addressing the influence of stress and stress-induced activation of the SAM and HPA axes including behavioural interventions that address psychological and autonomic reactivity to stress such as psychotherapy, exercise and meditation may have efficacy regarding both treatment and prevention of depression.

Figure 6.1 Summary diagram

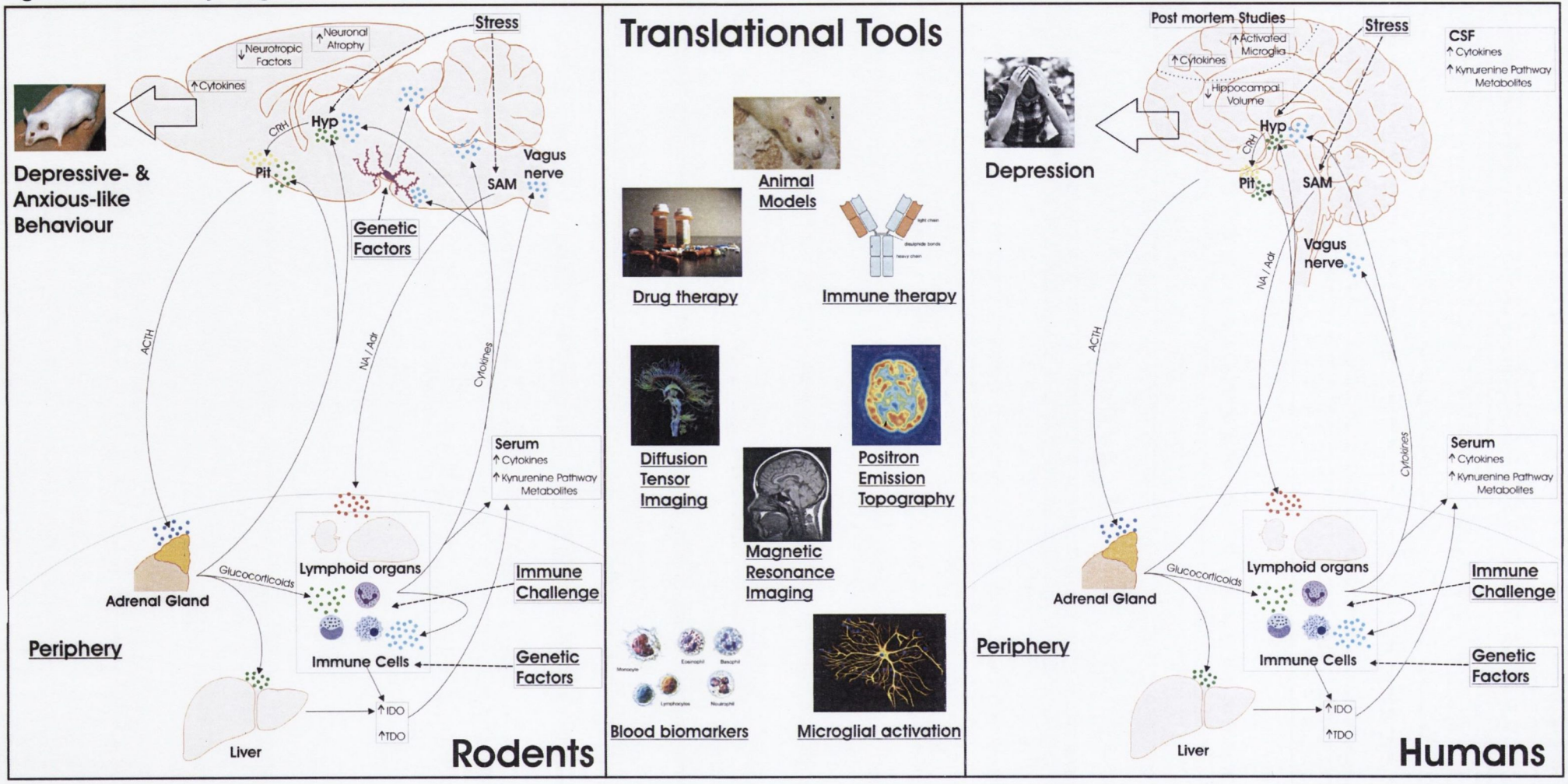

Figure 6.1 Summary Diagram

Animal models play an integral role in the development of an understanding of the effect of immune activation and inflammation on behavioural traits. Investigation of mechanisms linking immune-brain communication can be approached in a more invasive way in animal studies to elucidate changes in the brain in response to immune challenge. Such mechanisms include changes to the sensitivity of glucocorticoid/ catecholamine receptors and regulation of the hypothalamic pituitary adrenal (HPA) or sympathoadrenal medullary (SAM) axes. Furthermore animals may be selectively bred or genetically engineered to help assist in evaluating a role for immune related mechanisms underlying depression and anxiety related behaviours with associated neurobiological changes including the expression of neurotrophic factors or neurotransmitters such as serotonin or tryptophan metabolites related to kynurenine. Investigations of the CNS in humans are limited to CSF and post mortem tissue although MR and PET neuroimaging are providing investigators with a window into the brain and parallels may be drawn between outcomes in animal and human investigations. The possibility to translate from animal to human experiments in this way will pave the way for the development of biomarkers related to a role for the immune system in the pathophysiology of mood and anxiety disorders and future development of therapeutics which may target the immune system or brain microglial cells directly for a desirable clinical outcome.

Abbreviations: ACTH – adrenocorticotropic hormone, Adr – adrenaline, CRH – corticotropin releasing hormone, CSF – cerebrospinal fluid, Hyp – hypothalamus, IDO – indoleamine 2,3 dioxygenase, NA – noradrenaline, Pit – pituitary, SAM sympathoadrenal medullary axis, TDO – tryptophan 2,3 dioxygenase

7. Appendix

9.1 Journal publications

Published 2013, *Brain Behav Immun,* 28, 170-81.

Poly I:C-induced activation of the innate immune response is accompanied by symptoms of depression and anxiety; a role for the kynurenine pathway.

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Evidence suggests that depression is associated with activation of the innate immune system, and that infectious agents or inflammation can be a precipitating factor for depression. Poly I:C is a TLR3 agonist that initiates inflammatory responses comparable to viral administration. The objective of this study was to characterise the ability of poly I:C to induce an inflammatory response and induce symptoms of depression and anxiety in rats. Furthermore any poly I:C induced alterations in the kynurenine pathway were also examined. In response to poly I:C, rats showed significant body weight loss, a reduction in saccharin preference (anhedonia), decreased locomotor activity in the home cage activity test and increased anxiety-related behaviours in the open field test. These behavioural deficits were most prominent in the first 24 hr following administration but were still apparent up to 72 hr. This altered behavioural phenotype was associated with a poly I:C induced temporal increase in the expression of the cytokines IL-1β, IL-6 and TNF-α and the microglial activation marker CD11b in the frontal cortex and hippocampus. Decreased expression of the neurotrophic marker BDNF and its receptor TrkB, was also found in response to poly I:C administration. A poly I:C induced increase in expression of the tryptophan degrading enzyme indoleamine 2,3 dioxygenase (IDO) was also found in the brain which was associated with increased central concentrations of the tryptophan metabolite, kynurenine. These findings suggest that the depressive-like behaviour elicited by poly I:C administration may be associated with cytokine induced alterations in the kynurenine pathway and may be of importance for the development of viable therapeutic strategies for inflammation-related depression.

[Submitted] 2014, *Brain Behav Immun*

The NOD mouse. A new animal model for anxious and depressive-like behavior characterized by gene-environment interactive immune and neurotrophic deregulations.

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The Non-Obese Diabetic (NOD) mouse is an established inbred model for Type 1 diabetes and autoimmune thyroiditis, both co-morbidities of major depressive disorder. There is preliminary evidence that NOD mice display a depressive-like behaviour.

Aim: To investigate the behaviour of NOD mice versus its background strain CD1. Since there is increasing evidence that inflammatory processes and activated microglia play roles in the aetiology of major depression, we studied the microglia genotype and the expression of inflammatory markers in NOD versus CD1 mice.

Methods: The open field, tail suspension test and elevated plus maze were carried out, at steady state. Home cage activity was monitored before and after i.p. injection of LPS. Microglia were isolated, FACS sorted and Affymetrix microarray analysis was performed. Genome-wide expression data were analysed (BRB-Arraytools, Ingenuity, detailed literature search using PubMed for the top over- and under-expressed genes). Q-PCR was performed on hypothalamic tissue to measure the expression of growth, microglial and immune activation markers.

Results: At steady state pre-diabetic NOD mice possessed an anxious and depressive-like phenotype; after LPS injection NOD mice displayed an enhanced sickness behaviour. In both conditions NOD microglia did not show a classical M1-like inflammatory response, but an IFN type 1 skewed inflammatory machinery and a gene profile characteristic of compromised neuronal support; BDNF and PDGF expression was reduced in brain tissue after LPS. In brain tissue itself classical inflammatory genes (e.g. IL-1β, IFNγ, iNOS and IDO) were up regulated.

Conclusion: NOD mice represent a novel animal model to study gene-environment induced depressive-like behaviours characterized by microglial deficiencies for neuronal support and microglial and brain inflammatory responses.

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Rodent models of stress induced depression: the link between stress and immune system related changes

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Bi-directional communication between the brain and immune system is a research area gaining in prominence in the search for biological factors associated with the development of a number of psychiatric disorders. In this regard, animal models make substantial contributions to our understanding of these interactions. For instance, long term adaptive changes to the immune system in response to stress have been documented and include stress-induced desensitisation of immune cell glucocorticoid receptors (GR) and β_2-adrenoceptors (AR) which in turn can impact on the ability of these factors to elicit their natural anti-inflammatory actions. It is of interest that psychological stress has been reported to induce microglial activation and pro-inflammatory cytokine expression in the central nervous system (CNS) in animal studies, and whilst there is little evidence of microglial activation or an increase in CNS cytokines in depressed humans to date, animal studies are providing important insights into the potential role of central inflammatory events in the pathophysiology of stress related psychiatric disorders. Stress can be linked to the development of a pro-inflammatory state and it is of interest to consider the reciprocal impact of peripheral inflammatory mediators on the brain. In this regard, there is accumulating evidence that inflammation plays a role in the pathogenesis of major depressive illness. To further explore the behavioural effects associated with immune system activation, a number of animal models are currently in use and include a range of agents used to initiate immune activation including bacterial endotoxin lipopolysaccharide (LPS), the inflammatory cytokine interferon (IFN) α, inoculation with Bacille Calmette-Guerin (BCG), experimental autoimmune encephalitis (EAE) and administration of synthetic dsRNA polyinosinic:polycytidylic acid (poly-IC), a viral mimetic. Immune activation in these models provokes signs of depression which may be ameliorated following treatment with antidepressant drugs. Despite these findings

the question as to how an immunological basis for a disorder can be reconciled with more traditional hypotheses including impairment of monoamine transmission and the functional impairment of neurotrophins, remains to be addressed. In this regard the activation of indoleamine 2,3 dioxygenase (IDO) and activation of the kynurenine pathway or the ability of cytokines to influence monoamine transport, receptor activation or neurotrophic factor expression are possibilities. The impact of inflammation on such systems is providing new avenues to develop a better understanding of the role of stress and related immune changes in the pathophysiology of depression and to find new and improved therapies to address current unmet clinical need.

9.2 Poster abstracts

Accepted by 21st annual meeting of Psychoneuroimmunology Research Society, May 2014

Enhanced glucocorticoid sensitivity coupled with reduced microglial activation in a genetically predisposed animal model of depression.

E. Sherwin [1,3], S. Gormley [2,3], B. McGuinness[1,3], T. Connor [1,3], A. Harkin [2,3]

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Accepted by 8th annual meeting of Neuroscience Ireland, September 2013

Systemic administration of IL-6 induces time dependent IL-6 related signalling in the brain

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Accepted by 22nd annual meeting of International Behavioural Neuroscience Society, June 2013

Stressor-induced IL-6 production and signalling: Divergence in responses between the periphery and CNS

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Accepted by 22nd annual meeting of International Behavioural Neuroscience Society, June 2013

Exaggerated behavioural and HPA axis responses to stress in the non-obese diabetic (NOD) mouse

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Accepted by 7th annual meeting of Neuroscience Ireland, September 2012

The effect of stress on IL-6 production and signalling in a mouse model

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Accepted by 6th annual meeting of Neuroscience Ireland, September 2011

Accepted by EURON Workshop School – Drugs and the brain: an update in psychopharmacology from experimental to clinic, April 2012

Increased anxiety and stress sensitivity, and evidence of glucocorticoid resistance in the non-obese diabetic (NOD) mouse

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9.3 Awards

7th annual meeting of Neuroscience Ireland, September 2012 – Best poster

The effect of stress on IL-6 production and signalling in a mouse model

DRHEA Master Class: "Cytokine actions in the brain: The good, the bad and the ugly", February 2011 – Best poster

Poly I:C-induced activation of the innate immune response is accompanied by symptoms of depression and anxiety

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