The Role of IL-1 and Type I IFN in CNS Function.

by

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I Declaration

I declare that this thesis is entirely my own work, with the exception of one clearly identified results (figure 5.13d-i) and that it has not been previously submitted as an exercise for a degree at this or any other University. I give my permission to the library to lend or copy this thesis.

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II Summary

Cytokines including interleukins (ILs) and interferons (IFNs) are induced in response to tissue injury, infection or inflammation. Peripheral cytokines signal to the healthy Central Nervous System (CNS) to produce physiological, behavioural, affective, and cognitive changes collectively known as sickness behaviour. Sickness behaviour is an adaptive response that is beneficial to the organism, but when these inflammatory insults are superimposed upon neurodegeneration, they have deleterious effects such as delirium, an acute cognitive disorder prevalent in elderly and patients with dementia that has significant economic, medical and societal impacts.

It is unclear whether IL-1 is necessary or detrimental in learning and memory. The role of IL-1 in learning and memory was assessed in IL-1R1$^{-/-}$ mice. These mice were not impaired on an array of hippocampal-dependent tasks, including the T-maze or Y-maze, Morris water maze (MWM) but exhibited increased open field (OF) activity and decreased anxiety in the elevated plus maze. Furthermore, IL-1R1$^{-/-}$ mice showed shorter latencies in the MWM and mild deficits in first choice accuracy in a spatial discrimination beacon task. These results demonstrate that basal IL-1 does not contribute to learning and memory.

The effect of systemic inflammation in the ME7 prion model of neurodegeneration was then interrogated. The data here indicate that LPS has dissociable effects in two different hippocampal-dependent memory tasks; impairing contextual fear conditioning (CFC) but not T-maze performance in normal mice. In contrast, LPS impaired T-maze performance in mice with existing neurodegenerative disease (ME7) and the severity and duration of this deficit increased with progression of disease. IL-1RA protected against LPS-induced T-maze deficits but not CFC impairment without blocking brain IL-1 synthesis. Likewise, dexamethasone-21-phosphate failed to inhibit LPS-induced CNS cytokine transcription, did suppress systemic cytokines. and showed time-dependent protection against the LPS-induced working memory deficit, indicating that systemic cytokines contribute to LPS-induced cognitive impairment. However LPS also induced hypoglycaemia and glucose administration prevented LPS-induced changes in T-maze. The results demonstrate a role for hypoglycaemia in systemic inflammation during neurodegeneration.

Alongside IL-1, type-one IFN's (IFN-I) are induced systemically and centrally in response to viral infection and viral mimetics and can impact on CNS function. Patients treated with IFN-α- based drugs experience depression, sickness behaviour, anxiety and cognitive impairment. There remains limited research on how IFN-I affects brain function. Therefore, the roles of endogenous IFN-I in cognition and during poly I:C-induced sickness behaviour and aging were investigated. The data showed that IFN-I receptor 1 deficient mice (IFNAR1$^{-/-}$) are not impaired in hippocampal-dependent memory tasks and do not display differences in anxiety or locomotor activity. Upon

poly I:C challenge IFNAR1$^{-/-}$ mice displayed reduced sickness behaviour and attenuated poly I:C-induced hypothermia, hypoactivity and weight loss. This was associated with equivalent IL-1β and TNF-α but much reduced IL-6 levels in plasma, hypothalamus and hippocampus. IFNAR1$^{-/-}$ mice lack basal IFN-I activity, show significantly lower levels of STAT1 and several inflammatory transcripts. When poly I:C–treated IFNAR1$^{-/-}$ mice were also administered IL-6 full sickness behaviour was reconstituted and both inter- and independent roles for IFN-I and IL-6 in systemic inflammation-induced changes in brain function was demonstrated.

Aged IFNAR1$^{-/-}$mice, displayed impaired hippocampal function and microglial expression associated with neuronal maintenance was suppressed. Analysis of neuroinflammatory transcripts after poly I:C show that microglia in the aged brain are primed to produce exaggerated acute responses to these stimuli, but these exaggerated responses do not appear to be mediated via IFNAR1. These data indicate that in normal aging, basal IFN-I response facilitates maintenance of normal cognitive function.

Together, these data indicate basal IL-1 and IFN-I in the normal brain have no influence on cognitive function but may influence anxiety. In the induced state, IL-1 certainly contributes to acute cognitive dysfunction in the vulnerable brain, and IFN-I contribute to the sickness behaviour response to acute viral mimetics. However, the absence of IFN-I as the brain ages may contribute to age-related cognitive decline. These finding have significant implication for age-related cognitive function and the understanding of the contribution of neuroinflammation to those processes. Considerable further work is required to validate these findings in clinical populations.

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V List of Abbreviations

ABC	Avidin-biotin complex
2-DG	2-Deoxyglucose
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADA	American Diabetes association
ALS	Amyotrophic lateral sclerosis
ANLS	Astrocyte neuron lactate shuttle
ANOVA	Analysis of variance
APC	Antigen presenting cell
APP	Amyloid precursor protein
Aβ	Amyloid Beta
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CA1	Cornu ammonis 1
CA3	Cornu ammonis 3
CD 14	co-receptor cluster of differentiation molecule 14
CD11b	Cluster of differentiation molecule 11 b
cDNA	complementary Deoxyribonucleic acid
CFC	Contextual fear conditioning
CLP	Cecal ligation puncture
CNS	Central Nervous System
COX	Cyclooxygenase
CP	Choroid Plexus
cPGES	Cytosolic prostaglandin E-synthase
CRF	Corticotrophin-releasing factor
CSF	Cerebrospinal fluid
CT	Cycle threshold
CVO	Circumventricular organ
CVO	Circumventricular organs
DAB	Diaminobenzidine
DAMP	Damage-associated molecular pattern
DC	Dentrytics cell
d-GalN	d-galactosamine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPX	Di-N-Butyle Phthalate in xylene
E. Coli	Escherichia coli
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid

EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
FDG	2-18F-fluoro-2-deoxy-D-glucose
GAPDH	Glyceraldehyde-3-phosphate dehyrdrogenase
GC	Glucocorticoid
gDNA	genomic Deoxyribonucleic acid
GLUT	Glucose transporters
gp130	Glycoprotein 130
H_2O_2	Hydrogen peroxide
H_2S0_4	Sulphuric acid
HCB	Hepatitis B
HCl	Hydrochloric acid
HCV	Hepatitis C
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
i.c.	Intracerebral
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
IBA-1	Ionized calcium binding adaptor molecule-1
IDO	indoleamine 2,3-dioxygenase
IFN	Interferon
IFN-I-	Type one interferons
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IL-1	Interleukin-1
IL-1R1	Interleukin-1 receptor type 1
IL-1R1-/-	Interleukin-1 receptor type 1 genetic knock-out
IL-1RA	Interleukin-1 receptor antagonist
IL-1RAP	IL-1 receptor accessory protein
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-6-/-	Interleukin-6 genetic knockout
IL-6R	Interleukin-6 receptor
iNOS	inducible nitric oxide synthase
IP-10	Interferon-gamma-induced protein-10
IRAK-4	Interleukin-1 receptor-associated kinase-like 4
IRF	Interferon-regulatory factor
ISGF3	IFN-stimulated gene factor 3
IκB	Inhibitor of kappa B
JAK	Janus kinase

kDA	kiloDalton
LPB	lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAP	Mitogen-activated protein
MCI	Mild cognitive impairment
MCP-1	Monocyte chemoattractant protein-1
MHV	Mouse hepatitis virus
mRNA	messenger Ribonucleic acid
MS	Multiple sclerosis
mu-p75-sap	Murine p-75-saporin
MWM	Morris water maze
MyD88	Myeloid differentiation primary response gene (88)
Na2HPO4	Sodium phosphate dibasic
NaCl	Sodium Chloride
NaH2PO4	Sodium phosphate monobasic
NBH	Normal brain homogenate
NBH	Normal brain homogenate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
NLRP3	NOD-like receptor family, prying domain containing 3
NLRS	NOD like receptors
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
PAMP	Pathogen-associated molecular pattern
PAMP	Pathogen- associated molecular patterns
PBS	Phosphate-buffered saline
PCC	Posterior cingulate cortex
PCR	Polymerase chain reaction
PD	Parkinson's disease
PET	Positron emission tomography
PET	Positron emission tomography
PGE2	Prostaglandin E2
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
PRR	Pattern recognition receptor
PTX3	Pentraxin 3
Q-PCR	Quantitative-polymerase chain reaction
RA	Research assistant
RIPK1	Receptor-interacting serine/threonine-protein kinase 1(RIPK1)
RNA	Ribonucleic acid
RT	Reverse transcription
SAA2	Serum amyloid A protein
SEM	Standard error of the mean

SMI	Subjective memory impairment
SNC	Suprachiasmatic nucleus clock
SOCS3	Suppressor of cytokine signalling 3
SSRI	Selective serotonin reuptake inhibitors
STAT	Signal transducers and activators of transcription
TBK1	TANK-binding kinase 1
TIR	Toll-IL-1 receptor
TLR4	Toll-like receptor-4
TN	Trigeminal Neuralgia
TNF-α	Tumor necrosis factor-alpha
TNRF1	TNF receptor type 1
TNRF2	TNF receptor type 2
TRADD	TNF-receptor-associated death domain proteins
TRAF	Tumor necrosis factor-alpha receptor factor
TYK2	Tyrosine kinase 2
VSM	vesicular stomatitis virus
β-ME	Beta-mercaptoethanol

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Chapter 1

Introduction

1.1 The CNS and Brain and Immune Privilege

The Central Nervous system (CNS) consists of the spinal cord and the brain, which are protected by three layers of connective tissue called the meninges. The CNS primary function involves the integration of sensory input from the peripheral nervous system via the bundle of nerve fibres within the spinal cord and mediation of responses to this information from the brain itself. The brain can be subdivided into three regions. The forebrain which consists of the cerebrum, hypothalamus and hippocampus and is responsible for functions such as motor control, relaying sensory information and controlling autonomic functions. The second region is the midbrain which is part of the brainstem. The midbrain connects the forebrain to the hindbrain and it functions in auditory and visual responses as well as motor functions. The third region is the hindbrain also part of the brainstem, which contains the cerebellum and pons and the medulla oblongata. The functions of the hindbrain include controlling breathing, heart-rate, digestion, maintaining balance and movement coordination. The brainstem consisting of the midbrain and the hindbrain is pivotal in maintaining consciousness, sleep cycle, cardiac and respiratory functions. Cerebrospinal fluid surrounds cushions and protects the brain and spinal cord from trauma.

The brain was once thought of as an immune-privileged organ in the body, with structures such as the blood-brain barrier (BBB) and blood-cerebrospinal fluid (CSF) barrier protecting it from peripheral immune responses. In recent decades however, the concept of brain immune-privilege has been challenged and re-evaluated. Current research indicates that the brain has its own resident CNS macrophages known as microglia, and the brain and CNS is both immune competent and actively interactive with the peripheral immune system (Steinman, 2004). Systemic inflammation is capable of causing an impact on the brain. Systemic inflammation occurs in response to infection, surgery or trauma and results in the induction of innate immune responses. The immune cells travel to the site of infection or injury and release inflammatory cytokines and mediators (Cunningham et al., 2009).

The BBB is a highly selective semi-permeable membrane that separates circulating blood from the brain extracellular fluid in the CNS. It is composed of endothelial cells,

connected by tight junctions. Peripheral immune cells and immunomodulatory agents, can permeate through the BBB especially at circumventricular organs and at the choroid plexus as the BBB is weak or absent at these regions (Maier, 2003). Most of the evidence that the immune system and the nervous system can interact and influence each other comes from the earlier studies which looked at the fever and sickness behaviour response in rodents.

Sickness behaviour which will be discussed later in greater detail is an adaptive behaviour change as a result of systemic inflammation. The sickness behaviour studies in the literature, demonstrate that cytokines like IL-1β when injected in the periphery can cause the brain to elicit a fever response. Subsequently, blocking IL-1β action with an antagonist can inhibit the fever response (Kent et al., 1992). Fortier and colleagues demonstrated that when Poly I:C a synthetic dsRNA was injected into rats peripherally, the rats showed a fever response (Fortier et al., 2004). During the fever response the immune system signals the brain to respond to the virus, bacteria and pathogen and the CVO's such as organum vasculosum lateralis terminalis, subfornical organ, median eminence, area postrema allow transmission of soluble mediators released by the immune cells to sites located in the hypothalamus of the brain (Blatteis, 2000). Prostaglandins which can be induced by IL-1 are small lipophilic molecules that can diffuse into the brain parenchyma which leads to the activation of non-neuronal cells in the BBB that can spread to glial cells on the brain side of the vessels thereby initiating a cascade of neural communication events (Steinman, 2004, Louveau et al., 2015).

Additional research in neurodegenerative diseases has also shown the interaction of the immune system with the CNS. Elevated levels of IL-1 have been found in Alzheimer's disease (Cacabelos et al., 1994, Cacabelos et al., 1991). In Parkinson's disease chronic systemic expression of IL-1 has been shown to exacerbate neurodegeneration and microglial activation in the substantia nigra (Pott Godoy et al., 2008). Inflammation is regarded as playing a pivotal role in the disease process. Inflammation is the process where the body responds to injury, tissue ischemia, autoimmune response and infectious agents. There has been research focussing on the protective effects of long-term use of nonsteroidal anti-inflammatory drugs

(NSAIDS) in the treatment of Alzheimer's disease (Stewart et al., 1997). Follow up work from Holmes and colleagues have reported that there was a correlation between systemic circulating levels of TNF and progression of AD in Alzheimer's patient (Holmes et al., 2009). In Parkinsonian patients, TNF-α was elevated in the brain and cerebrospinal fluid (Mogi et al., 1994b). Baker and colleagues showed that anti-TNF antibody pre-treatment can inhibit the development of experimental autoimmune encephalomyelitis (EAE), an experimental model of Multiple Sclerosis (Baker et al., 1994). Taken together, these studies and many others demonstrate that systemic inflammation can impact on brain function which can lead to cognitive dysfunction. Cognitive dysfunction can be classified as the loss of thinking, memory, perception and problem solving and concentration. Cognitive dysfunction can be seen in a variety of diseases and disorders that include Dementias, Alzheimer's disease, Delirium, Schizophrenia, Depression, Multiple Sclerosis and chronic fatigue syndrome. Systemic inflammation involves the process of immune responses that results in the cascade of signalling pathways where the immune system plays a role.

1.2 The immune system

The immune system can be divided into innate or humoral immunity, and the adaptive or cell-mediated immunity. The innate branch is considered the first line of defence and therefore plays a crucial role in the early recognition and subsequent triggering of a pro-inflammatory response to invading pathogens (Medzhitov and Janeway, 2000). The innate immune system is relatively non-specific, in contrast to the adaptive system which is specific. The innate system comes into play immediately or within hours upon presentation of an antigen, whereas the adaptive system is more complex than the innate and provides an antigen-specific response. The innate immune system is based principally on physical barriers such as the skin and chemical barriers such as sweat, tears, saliva, mucus as well as different cell types recognizing invading pathogens and upon recognition of these pathogens, activating antimicrobial immune responses. These cell types are leukocytes which develop from the hematopoietic stem cells present in the bone marrow. These immune leukocytes include neutrophils, basophils, easinophils, natural killer cells (NK) , and antigen presenting cells (APC), such as macrophages and dendritic cells (Iwasaki and Medzhitov, 2004).

The adaptive immune response consists principally of 2 types of lymphocytes: T cells and B cells. B cells differentiate into plasma cells which circulate in blood and lymph, and their function is to produce antibodies which will bind specifically to foreign antigens. T-cells originate in the bone marrow and mature in the thymus. In the thymus T cells multiply and differentiate further into T-helper cells, T-regulatory cells, Cytotoxic T killer cells, and T-memory cells. The T-cells are then sent to peripheral tissues or circulate in the blood or lymphatic system. T-helper cells recognize foreign antigen on the surfaces of other cells, and stimulate B cells to produce antibodies by secreting chemical messengers called cytokines. T-regulatory cells act to control immune reactions, and cytotoxic T cells when activated by various cytokines, can bind to and kill infected cells and cancer cells. T-memory cells are T-cells which persist long-term after an infection has passed, and that have encountered a pathogen during a prior infection and can recall the strategy used to defeat the pathogen, thus allow faster antibody production for future infections. Suppressor T cells are another form of T-cells that can inactivate the B cells and killer T cells and return the immune system to normal. The adaptive immune response requires signals that are provided by the innate immune system that provide information about the origin of the antigen and type of response to be induced (Janeway and Medzhitov, 2002).

In the innate immune response, the NK cells, macrophages and dendritic cells all become activated during the inflammatory response and their main role is to clear the infection and pathogen. They do this by recognition of evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs) (Mogensen, 2009).PAMPS such as Lipopolysaccharide (LPS) an endotoxin found on the cell membrane of bacteria, and artificial microbial nucleic acids such as peptidoglycan and lipoteichoic acids found in Gram-positive cell wall bacteria and flagellin found in bacterial flagella are then recognised by pattern recognition receptors (PRR's). The principle function of these PRR's is activation of complement cascades, opsonisation, activation of pro-inflammatory signalling pathways and the induction of apoptosis (Janeway and Medzhitov, 2002). The PRR's can be divided into three types, humoral proteins circulating in the plasma, endocytic receptors that are expressed on the cell

surface and the signalling receptors that can be expressed on the cell surface or intracellularly (Fearon and Locksley, 1996, Medzhitov and Janeway, 1997). Upon recognition of the PAMP by the PRR's it ultimately results in the activation of gene expression and synthesis of a range of molecules that include cytokines, chemokine, cell adhesion molecules and immunoreceptors (Akira et al., 2006). Taken together these all orchestrate the early host response to infection and at the same time represent an important link to the adaptive immune response.

1.3 Microglia

Microglia are the largest population of tissue resident macrophages in the brain. They are part of the glial system of non-neuronal cells that support and protect neuronal functions. They are derived from primitive yolk sack myeloid progenitors that enter the brain parenchyma during embryonic development (Ginhoux et al., 2010). Microglia are broadly distributed throughout the brain and the spinal cord (Lawson et al., 1990) and they represent 10-15% of the total cell population within the brain parenchyma (Carson et al., 2006). In humans approximately 5% of brain cells are microglia (Pelvig et al., 2008). In order to maintain homeostasis of the CNS in both health and disease adequate and appropriate microglia function is crucial (Perry and Holmes, 2014). Microglia can be described as ramified in a resting or quiescent state (Glenn et al., 1992) however they are constantly surveying their environment in preparation for insult or injury (Nimmerjahn et al., 2005).

Upon activation they transform to a more condensed bushy morphology (Tambuyzer et al., 2009). Microglia have a high level of plasticity allowing them to change their shape and function in response to environmental cues (Saijo and Glass, 2011). Microglia have many roles in the CNS system. They monitor for tissue changes and maintain tissue homeostasis by clearing pathogens, dying cells and debris. In addition microglia are also capable of pruning synapses on neighbouring neurons during development by direct engulfment (Paolicelli et al., 2011). Microglia can be activated by LPS or IFN-γ and exhibit a M1 pro-inflammatory phenotype where they release pro-inflammatory mediators include Interleukin -1 and TNF-α and nitric oxide, eicasoanoids and quinolinic acid (Liu et al., 2002). If left activated the excess

production of pro-inflammatory cytokines by microglia and neurotoxic molecules can lead to detrimental effects and contribute to long-term neurodegeneration(Liu and Hong, 2003). IL-4 and IL-13 released from Th2 cells can drive the microglia to convert to a less active neuroprotective M2 state where anti-inflammatory cytokines are then released from microglia. The M2 state is driven by immune complexes, and TLR and IL-1β activation.

Microglia and other glial cells such as oligodendrocytes and astrocytes can impact on neuronal function and are significantly involved in the underlying pathophysiology of neurodegenerative diseases and psychiatric disorders. There have been numerous studies over the last number of years that have implicated microglia in neurodegenerative diseases, in particular, Alzheimer's and Parkinson's disease. Post-mortem studies in patients with Alzheimer's have shown that in the cortical regions of these brains, microglia were co- localising with neuritic plaques (Rogers et al., 1988). Rodent studies have also shown that there is an association of microglia and amyloid plaques in APP23 transgenic mice (Stalder et al., 1999). Microglia have also been found in the brains of multiple sclerosis patients (Benveniste, 1997). Taken together all of these studies indicate that microglia play a pivotal role in neurodegenerative diseases.

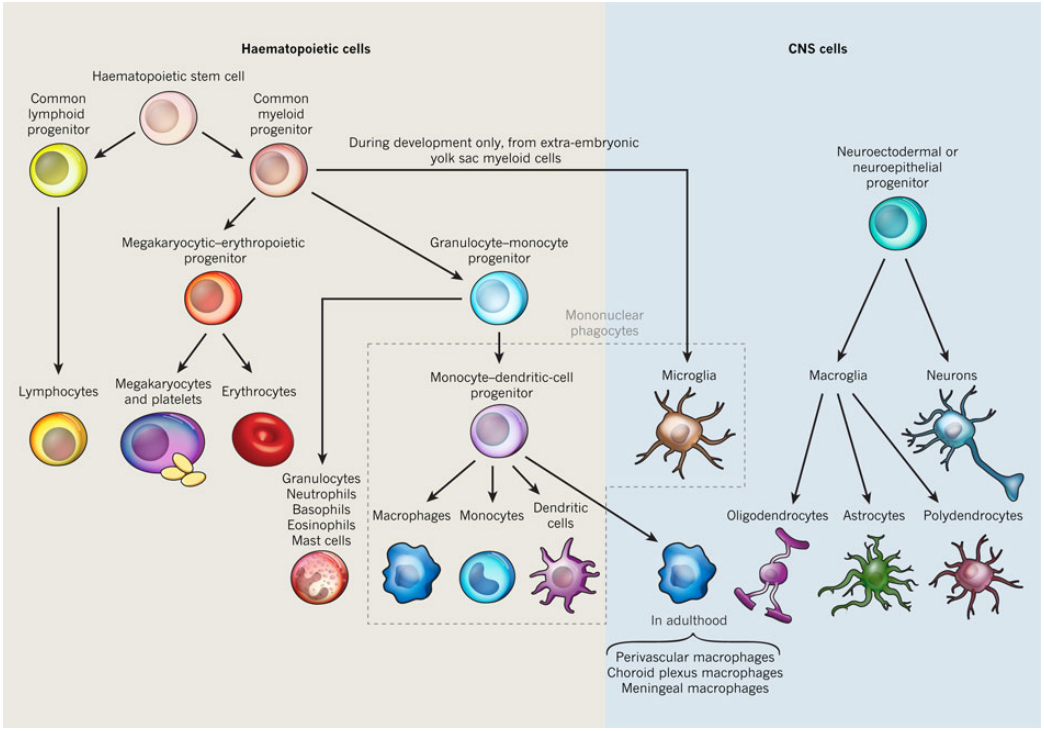


Figure 1 Immune Cell lineages

Cells of the haematopoietic system and CNS are depicted, with arrows indicating lineage relatedness. Microglia are the only haematopoietic cells found in the parenchyma of the CNS. Figure from (Ransohoff and Cardona, 2010).

1.4 Inflammatory ligands, receptors and pathways

1.4.1 Pattern recognition receptors

The pattern recognition receptors include, the Toll Like Receptors, the NOD like receptors (NLR's) and the RIG-I receptors and these can be expressed on the cell surface, in intracellular compartments or secreted into the bloodstream and tissue fluids (Medzhitov and Janeway, 1997). In 1996 Hoffmann *et al* while studying Drosophila fly identified TOLL the first receptor that was essential for the hosts defence against fungal infection. Following this discovery, in 1997 Medzhitov and Janeway identified TLR 4, a mammalian homolog to the TOLL receptor, and this engagement of this receptor was shown to induce expression of number of genes involved in inflammatory response (Medzhitov and Janeway, 1997). In 1998, Bruce Beutler and colleagues identified that lipopolysaccharide (LPS), which is an endotoxin found in the outer membrane of Gram negative bacteria, was the signalling molecule

for TLR4 (Poltorak et al., 1998b). TLRs are members of a larger superfamily of interleukin-1 receptors that contain a so-called TIR (toll-IL-1 receptor) domain. TLR structure consists of an extracellular ligand binding domain which contains leucine-rich repeat motif and a cytoplasmic signalling Toll/Interleukin (1L-1) receptor homology (TIR) domain (O'Neill and Bowie, 2007). To date there have been 13 TLR's identified in mammals and mice. These have been called TLR1-TLR13. Further research carried out by Akira and his team identified that the TLR's can be divided into subfamilies primarily recognizing related PAMPs; TLR1, TLR2, TLR4, and TLR6 recognize lipids, whereas TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids (Akira et al., 2006). The most important cell types expressing TLRs are macrophages, DCs, and B lymphocytes (Iwasaki and Medzhitov, 2004). Although there are still some of these receptors whose ligands have yet to be identified, many ligands have been identified. These include dsRNA for TLR3, ssRNA for TLR7 or TLR8, LPS for TLR4 and unmethylated CpG-DNA for TLR9 (Theofilopoulos et al., 2011). Ligand binding to TLRs through PAMP-TLR interaction induces expression of inflammatory cytokine and chemokines, antimicrobial peptides, MHC molecules and activation of nuclear factor kappa B (NFκB) which are necessary for the host cell to fight off invading pathogen (Janeway and Medzhitov, 2002). For the purpose of this thesis the two TLR's that are of interest to this work and will be discussed further are TLR4 which recognises LPS, a component present in many Gram-negative bacteria and TLR3 is the receptor that recognises dsRNA associated with viral infection and can induce the activation of IRF3 which in turn activates the production of interferons.

1.4.2 Toll like receptor 4 and lipopolysaccharide

TLR4 has been identified as the main and essential receptor for LPS. In 1998 Poltorak and colleagues showed that mice which are insensitive to LPS were shown to have a single point mutation in the TIR domain of TLR4 (Poltorak et al., 1998a). Further research in 1999 demonstrated that TLR4 deficient mice are hypo responsive to LPS (Hoshino et al., 1999). When TLR4 is activated, it leads to the activation of MYD88 dependent and independent pathways. In addition to recognizing LPS, TL4 has been shown to be involved in the recognition of endogenous ligands, such as heat shock

proteins (HSP60 and HSP70), HMGB1, the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen though these endogenous ligands need to be present at very high concentrations to activate TLR4 (Takeda and Akira, 2005).

LPS is an endotoxin found in the outer membrane of the cell wall of gram negative bacteria and is a powerful activator of the innate immune response. LPS can bind to the LPS receptor complex via it's hydrophobic Lipid A component. This complex consists of Toll like receptor 4, co-receptor cluster of differentiation molecule 14 (CD14) and MD2 receptor a cell surface protein that is required for surface expression and LPS-regulated activation of TLR4. The binding of LPS to this receptor complex promotes the activation of NF-κB and IRF3 and leads to the secretion of pro-inflammatory cytokines including Tumor Necrosis Factor-α (TNF-α) and Interleukin 1 (IL-1). As a result of this LPS is an agent that can be classically used as a pro-inflammatory stimulus in research, as it can activate systemic inflammation, mimic the symptoms of a bacterial infection, and induce sickness behaviour and induction of inflammatory mediators in addition to cytokines. LPS has also been used to induce sickness behaviour in rodents (Bluthe et al., 1992). It has been shown in multiple studies that LPS can impair memory in rodents (Shaw et al., 2001, Thomson and Sutherland, 2005, Pugh et al., 1998, Murray et al., 2010) as well as in humans (Reichenberg et al., 2001).

1.4.3 Toll like receptor 3 and dsRNA Poly I:C- Polyinosinic:polycytidylic acid

TLR3 is mostly expressed on the membrane of intracellular endosomal compartments but has been shown to be present on the surface of certain cells (Matsumoto and Seya, 2008). Research carried out by Alexopoulou in 2001 demonstrated that TLR-3 deficient mice are impaired in their response to dsRNA as they exhibited reduced responses to poly I:C resistance to the lethal effect of poly I:C when sensitized with d-galactosamine (d-GalN), and showed reduced production of inflammatory cytokines (Alexopoulou et al., 2001). This dsRNA is produced by almost all viruses during their replication and it induces the production of type one interferons interferon beta (IFN-

β) and interferon alpha (IFN-α). TLR-3 recognises dsRNA leading to activation of IRF-3 which results in the synthesis of IFN-β.

Poly I:C is a synthetic double-stranded RNA that is a ligand for the TLR3 receptor on endosomal membranes and for the cytoplasmic proteins RIG I and MDA5 (Alexopoulou et al., 2001, Yoneyama et al., 2004, Andrejeva et al., 2004, Kato et al., 2005). Poly I:C is a mismatched double-stranded RNA with one strand being a polymer of inosinic acid, and the other a polymer of cytidylic acid. It is used as an agent in research to mimic the acute phase of a viral infection. Poly I:C upon binding to its receptor is a potent stimulator of the type one interferons α and β and other inflammatory cytokines (Jacobs and Langland, 1996, Matsumoto and Seya, 2008). Poly I:C has been used in multiple rodent studies where it induces features of sickness behaviour, such as fever and/or hypothermia, malaise, anhedonia, sleep/wake cycle disruption and hypoactivity (Cunningham et al., 2007, Majde, 2000, Traynor et al., 2006, Fortier et al., 2004). Poly I:C has also been used as a model of chronic fatigue syndrome (Katafuchi et al., 2003) and has also been used in maternal immune activation studies during pregnancy in rodents, where the behavioural and neurological disorders in the offspring have been investigated (Meyer, 2014).

TLR 3 and TLR4 signal predominantly through 2 different pathways which are the MYD88–dependent pathway and the MYD88-independent pathway.

1.4.4 MYD88–dependent pathway

MyD88 is essential for the production of inflammatory cytokines in response to a variety of microbial components (Kawai et al., 1999). LPS (ligand) binds to CD14 a co-receptor in the presence of LBP (lipopolysaccharide binding protein). Upon binding of LPS to the TLR MYD88, an adaptor molecule, is recruited to the TLR receptor where it binds to the TIR domain. This binding leads to recruitment of Interleukin-1 receptor-associated kinase-like 4 (IRAK-4), which mediates the phosphorylation of IRAK 1 which then induces the interaction of TRAF 6 with TAK1, TAB1, and TAB2, which leads to two distinct signalling pathways. The activation of AP-1 transcription factor through

activation of MAP kinases and the activation of the TAK1/TAB complex which enhances the activity of IKK complex. TAK1-mediated activation of the IKK complex results in the phosphorylation of IKK-β, which then phosphorylates IκB causing its degradation and allowing NFκB to translocate to the cell nucleus and activate transcription and consequent induction of inflammatory cytokines (Takeuchi and Akira, 2010). The MyD88 pathway is used by all TLR's except TLR3 which utilizes the MYD88-independent pathway.

1.4.5 The MYD88-Independent pathway

TLR3 and TLR4 utilise this pathway to induce IFN beta (IFN-β). It does this by recruiting the adaptor protein TRIF. The recruitment of TRIF then leads to the activation of two enzymes known as TANK-binding kinase 1 (TBK1) and Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) The TRIF-TBK1 complex then phosphorylates a transcription factor known as interferon-regulatory factor 3 (IRF-3). This transcription factor then induces the interferon beta (IFN-β) gene and other genes such as IP-10. IFN-β, in turn then activates Stat1, a signal activator of transduction which then further leads to the induction of several IFN-inducible genes (Doyle et al., 2002, Toshchakov et al., 2002, Hoshino et al., 2002). The activation of RIPK-1 leads to the polyubiquitination and activation of TAK1 and NFκB transcription in the same manner as the MyD88-dependent pathway(Kawai and Akira, 2010).

1.5 Cytokines

Cytokines are a diverse group of polypeptides that contribute to nearly every aspect of inflammation including pro-inflammatory and anti-inflammatory processes, immune activation and cell differentiation. They include interleukins, interferons, tumor necrosis factors, growth factors and chemokines. They are rapidly induced in response to infections, tissue injury and inflammation. Cytokines are produced by many different cell types that include macrophages, endothelial cells, microglial cells and B and T lymphocytes. They generally act through specific receptors. The most studied cytokines are TNF-α, Interleukin 6, Interleukin 1, and Interferons which will be discussed in further detail below.

1.5.1 Tumor Necrosis Factor -TNF-α

Tumor necrosis factor is a pleiotropic cytokine of 26kDa that was originally recognized for its anti-tumor activity but now referred to as a pro-inflammatory cytokine. It was originally discovered in mouse serum during endotoxemia (Carswell et al., 1975), but in 1987 Frei and colleagues observed its role in CNS when microglia were found to produce it (Frei et al., 1987). It can induce the production of IL-1β and IL-6 (Fong et al., 1989). TNF-α can be induced by bacterial lipopolysaccharide, mitogens and viruses (Vassalli, 1992). TNF-α exists as both soluble and membrane bound versions. It can be produced by many other cells types such as CD4+ lymphocytes, natural killer cells, eosinophils and neurons. TNF-α signals through two receptors, TNF receptor type 1 (TNFR1) which is expressed in most tissues and TNF receptor type 2 (TNFR2) which is found in cells of the immune system (Tartaglia and Goeddel, 1992). Upon binding to its receptor it causes a conformational change to occur which recruits adaptor proteins, the TNF receptor factors (TRAF's) and TNF-receptor-associated death domain proteins (TRADD) which then leads to initiation of the NFκB pathway and the MAP kinase pathway. TNF-α-signalling can also induce apoptosis by recruitment of the cysteine protease caspase 8 (Gaur and Aggarwal, 2003).

1.5.2 Interleukin 6

Interleukin 6 can be described as a pro-inflammatory cytokine and an anti-inflammatory myokine. IL-6 can be produced by variety of cell types that include lymphocytes, endothelial cells, neurons, macrophages, astrocytes, fibroblasts and microglia (Sebire et al., 1993, Lafortune et al., 1996). Recent studies have also shown that IL-6 can be produced by skeletal muscle in response to exercise (Keller et al., 2001, Jonsdottir et al., 2000, Hiscock et al., 2004). The 26kDa cytokine can be described as a pleiotropic cytokine as it plays a central role in diverse host defence mechanisms such as the immune response, haematopoiesis, and acute-phase reactions (for reviews see (Van Snick, 1990, Kishimoto et al., 1995). IL-6 is mainly induced by pro-inflammatory cytokines IL-1 and TNF-α, LPS, and also Poly I:C. IL-6 mediates its signalling through the IL-6 receptor. This comprises of two membrane proteins, the ligand binding non-signalling α-subunit receptor (IL-6R) and the signal

transducing p subunit, glycoprotein gp130. A soluble IL-6R that lacks the transmembrane and intracytoplamic domains can bind IL-6 in association with cell-surface gp130 to transduce IL-6 signalling (Taga et al., 1989).

Upon binding of IL-6 to its receptor this initiates cellular events that include activation of Janus Kinases (JAK) which allows for the recruitment of signal transducer and activator of transcription STAT1 and STAT 3. Stat 1 and 3 then translocate into the nucleus, where they control the transcription of a variety of genes (Zhong et al., 1994, Hirano et al., 2000, Kamimura et al., 2003). The association of IL-6 with it's receptor can also lead to the activation of Ras-mediated signalling pathway and the mitogen-activated protein kinase (MAPK) pathway. IL-6 can also cross the blood brain barrier through a saturable transport system and is involved in the fever control response in the hypothalamus (Banks et al., 1994). IL-6 signalling is regulated by suppressor of cytokine signalling (SOCS) 1-3 through binding to JAK (Naka et al., 1997, Starr et al., 1997, Endo et al., 1997). Soluble gp130 in the circulation can also negatively regulate IL-6 signalling (Narazaki et al., 1993). IL-6 has been reported to induce IL-1RA and soluble TNF p55 (Tilg et al., 1994). IL-6 can activate COX-2 in a STAT-3 dependent manner which induces fever (Rummel et al., 2006).

IL-6 has been implicated in a wide array of diseases from Diabetes (Kristiansen and Mandrup-Poulsen, 2005), Arthritis (Srirangan and Choy, 2010), Atherosclerosis (Schieffer et al., 2004), Depression (Bob et al., 2010, Sukoff Rizzo et al., 2012), AIDS (Breen et al., 1990) multiple myeloma (Gado et al., 2000) and schizophrenia (Naudin et al., 1996). It has also been reported that in the brains of Alzheimer's disease patients, IL-6 expression is altered with more expression of IL-6 present and increased around amyloid plaques and in cerebrospinal fluid (Bauer et al., 1991, Hull et al., 1996, Strauss et al., 1992). In Parkinson's disease it has been reported that IL-6 immunoreactivity is markedly elevated in the nigrostriatal dopaminergic region of PD patients (Mogi et al., 1994a) and Parkinson patients show elevated levels of IL-6 in their CSF (Blum-Degen et al., 1995, Mogi et al., 1996). A more recent study by Barnes, has demonstrated that IL-6 plays a role in hyperglycaemia by amplifying glucagon secretion via the brain and also by direct action on islets (Barnes et al., 2014).

1.5.3 Interleukin 1

Interleukin 1 is a pro-inflammatory cytokine that is highly involved in the inflammatory and host defence response in the body. IL-1 is produced by macrophages and immune cells in the periphery as well as glia and neurons within the brain. It is elevated in both septic and aseptic inflammation (Chen and Nunez, 2010). It has many biological functions which include the upregulation of adhesion molecules on endothelial cells that are important for the recruitment of neutrophils and monocytes, and the transmigration of leukocytes to the sites of infection and the induction of additional pro-inflammatory mediators such as cytokines and chemokines and other inflammation associated molecules to form an amplified cascade to stimulate an immune response (Dinarello, 1996). There are 11 members of the IL-1 family but IL-1 α and IL-1β are the most widely studied. The IL-1 gene is NFκB-responsive and IL-1β is produced in response to surgery (Ozcinar et al., 2014), infection (Sahoo et al., 2011) and tissue damage (Sims and Smith, 2010). IL-1 signalling is triggered when IL-1 α and IL-1β bind to the interleukin type one receptor (IL-1R1). Another ligand called interleukin 1 receptor antagonist (IL-1RA) can inhibit IL-1 α and IL-β signalling by competing for the binding sites of the IL-1 receptor. Upon binding of IL-1α and IL-1β to this receptor, a co-receptor called IL-1 receptor accessory protein (IL1RAP) is recruited which is necessary for signal transduction and activation of NFκB and MAPKs. After the interaction of IL-1α or IL-1β with the IL-1R1 and IL1RAP, two adaptor proteins called MyD88 and TRAF-6 are recruited. IL-1R1 signals through a conserved intracellular region called Toll and IL-1R-like (TIR) domains. TRAF activates IKK which in turn then induces phosphorylation of IκB releasing NFκB's which then translocates to the nucleus. NFκB signalling occurs which induces inflammatory cytokines and COX-2. (Takeuchi and Akira, 2010). IL-1β is produced in a pro-IL-1 form and requires cleavage by caspase-1, an intercellular enzyme that forms mature IL-1β. Conversely pro-IL-1α is cleaved to its mature form by cell surface-bound calpain. IL-1β elicits most of its effects through the IL-1R1, however there is a second receptor IL-1R2, that is thought to be a decoy receptor as it does not seem to elicit any biologic effects upon binding with IL-1 (Dinarello, 2009, Colotta et al., 1993). IL-1β appears to be more rapidly

expressed and extracellularly active than the predominantly intracellular and locally acting IL-1α (Chen et al., 2007).

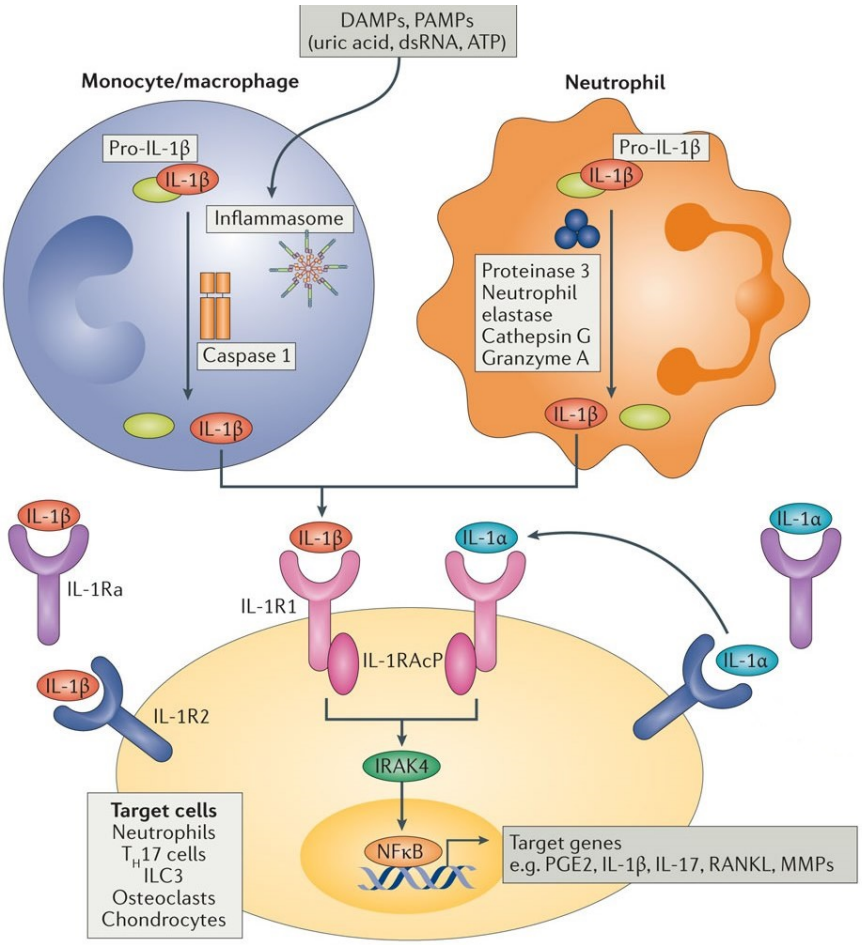


Figure 1.2 IL-1 signalling pathway 1

Figure 1.2 IL-1 signalling pathway

CNS injury results in the synthesis of IL-1. Caspase 1 activates the inflammasome which generates active IL-1α and IL-1β that are then released by the microglia. They bind to the IL-1 receptor type 1 and activate the mitogen activated kinase pathway and nuclear factor kappa B (NFκB) which leads to changes in gene expression, cell death and cellular repair. Adapted from (Schett et al., 2016).

1.5.4 Interferons

Interferons were first discovered in the 1950's by Isaacs and Lindenmann. Interferons have antiviral properties and the ability to interfere with virus replication (Isaacs and Lindenmann, 1987). Since their discovery, interferons, which are pleiotropic cytokines, are produced as a first-line response to viral infection by almost every cell type in the body. They are typically produced by macrophages (Solodova et al., 2011) and dendritic cells (Gary-Gouy et al., 2002) and play a key role in mediating immune responses. Interferons can be classified into 3 groups, Type 1 interferons (IFN-α, IFN-β, IFN-ε, IFN-K, IFN-ω, IFNd, and IFN-τ), Type 2 interferons (IFN-γ) and Type 3 interferons (IFN-λ1, IFN- λ2, and IFN- λ3). For the purpose of this thesis the focus will be on type 1 interferons (IFN-I) alpha and beta (α/β) which are the best defined and broadly expressed type one interferons. Type 1 IFN's in addition to being induced by viruses, can also be induced by bacteria, double stranded RNA and in response to TLR signalling by PAMPS (Hertzog et al., 2003).

As well as their antiviral properties, interferons can also be anti-proliferative and pro-apoptotic (Owens et al., 2014). After induction IFN-I signal through a heterodimeric transmembrane receptor IFNAR which is composed of IFNAR 1 and IFNAR2 subunits. The engagement of the IFNAR receptor leads to activation of the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which leads to the phosphorylation of cytosolic STAT1 and STAT2. The tyrosine-phosphorylated STAT1 and STAT2 dimerize and translocate to the nucleus, where they bind with IFN-regulatory 9 (IRF9) which then forms a trimolecular complex called IFN-stimulated gene factor 3 (ISGF3) which then induces transcription of IFN stimulated genes such as IRF-7 and PKR, via IFN-stimulated response elements (Taniguchi and Takaoka, 2001).

Interferons, which can be induced in the brain (Cunningham et al., 2007, Wang et al., 2008), play a considerable role in infectious and inflammatory disease, cancer and CNS function (Pestka et al., 1987). Their biological properties have led to their use as therapeutics in chronic hepatitis C (HCV), hepatitis B (HCB), virus infections and in

haematological cancers as well as Multiple Sclerosis. Recombinant IFN-β is a standard treatment for relapsing-remitting Multiple Sclerosis patients (Brandt et al., 1993). Targeting IFN-α in the autoimmune disease systemic lupus erythematosus has been shown to be therapeutic (Schmidt and Ouyang, 2004). IFN-β also been shown to induce interictal-like spontaneous activity in hippocampal neurons (Costello and Lynch, 2013). In contrast, IFN-α has been shown to induce depression, sickness behaviour, anxiety, cognitive impairment and delirium in patients being treated for cancer and hepatitis (Bonaccorso et al., 2001, Schaefer et al., 2002, Capuron and Miller, 2004, Raison et al., 2005, Zdilar et al., 2000). A recent study has demonstrated that chronic treatment with IFN-α decreases cell proliferation and neurogenesis in the dentate gyrus of mice (Zheng et al., 2014).

Interferons are produced by macrophages and dendritic cells in the brain (Gary-Gouy et al., 2002). IFN-alpha is produced by epithelial cells and IFN-beta is produced by fibroblast cells. Interferon receptors are found in macrophages, glia, and neurons within the brain. In response to a viral infection or systemic immune challenge plasmacytoid dendritic cells (PDs) secrete large quantities of type one interferons. Following induction interferons bind to the IFN receptor complex which initiates the activation of multiple signalling cascades including the JAK/STAT pathway leading to the induction of pro-inflammatory gene transcription leading to the secretion of cytokines including
TNF-α, IL-6 and IL-1β cellular recruitment and inflammatory progression (de Weerd and Nguyen, 2012, Ivashkiv and Donlin, 2014).

In the brain IL-1 can be expressed by endogenous brain cells, albeit at low levels. IL-1 is produced by glia and neurons. Following immune challenge, CNS injury or infection microglia appear to be the early primary source of IL-1. Astrocytes also produce IL-1, usually slightly later than microglia after an acute insult (Pearson et al., 1999, Davies et al., 1999). Expression of IL-1 has also been reported in oligodendroglia, neurones and cerebrovascular cells and circulating immune cells (Pearson et al., 1999, Blasi et al., 1999, Vitkovic et al., 2000). IL 1 receptors are found in typically neuron-rich sites of the brain such as granule cell layer of the dentate gyrus, the pyramidal cell layer of the

hippocampus, and the granule cell layer of the cerebellum as well as in the hypothalamus. (Farrar et al., 1987) IL-1 receptors are found in different regions of the CNS with the highest abdundance in the hippocampus. Following induction, IL-1 binds to IL-1R1 receptor leading to signal transduction and activation of NFκB and MAPKs leading to induction of cytokines and cox -2 (Takeuchi and Akira, 2010).

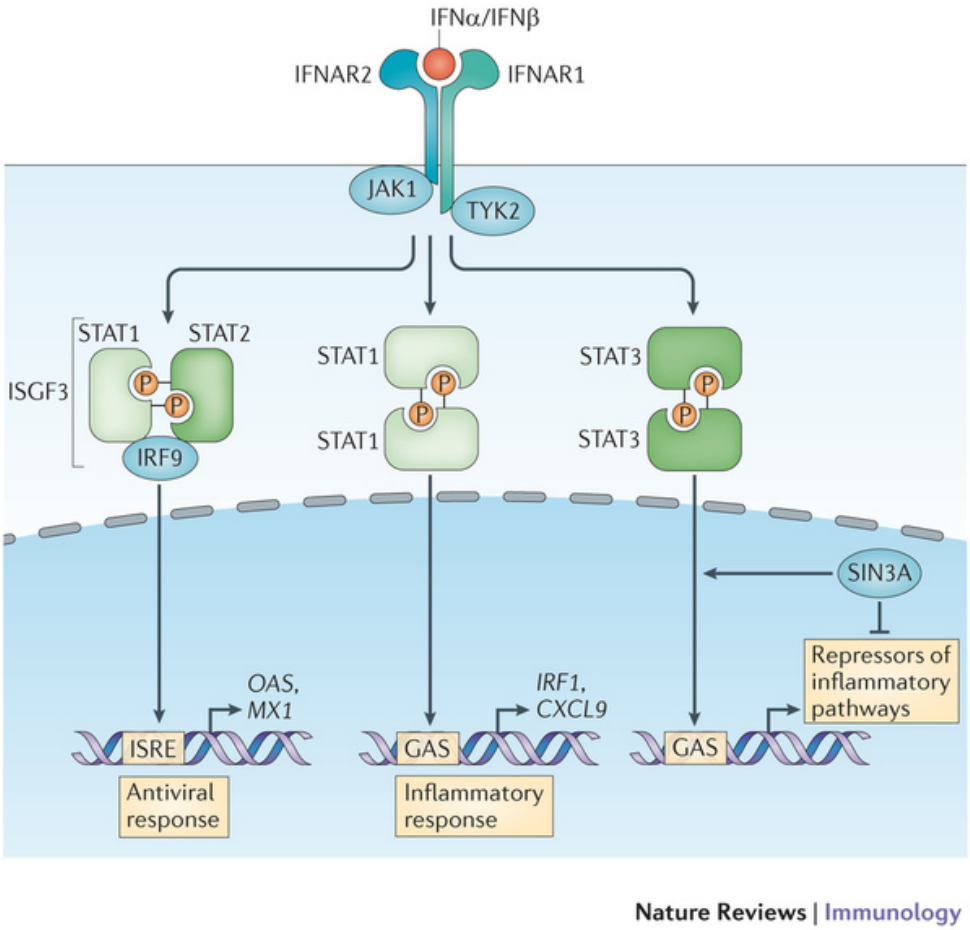


Figure 1.3 Interferon Signalling 1

Figure 1.3 Interferon Signalling

IFNα/β binds to interferon-α receptor IFNAR which has two subunits, IFNAR1 and IFNAR2. This leads to activation of kinases JAK1 and TYK2. Phophorylation of IFNAR by these kinases, leads to the recruitment of STAT proteins STAT1 and STAT2 and further phosphorylation. These STAT proteins then dimerize and translocate to the nucleus, where they bind with IFN-regulatory 9 (IRF9) which then forms a trimolecular complex called IFN-stimulated gene factor 3 (ISGF3). The ISGF3 complex then binds to IFN-stimulated response element (ISRE) sequences to activate classical antiviral genes, whereas STAT1 homodimers bind to gamma-activated sequences (GASs) to induce pro-inflammatory genes. Figure from (Ivashkiv and Donlin, 2014).

1.6 The Immune system affects the brain: Sickness behaviour

Sickness behaviour was first described by Hart in 1988 as an adaptive and highly organized strategy of an organism to fight off infection (Hart, 1988). This hypothesis was coherent with the already established role of fever in the host response to pathogens by Kluger (Kluger, 1979). Sickness behaviour is regarded as a motivational state that reorganizes the organisms priorities to cope with infectious pathogens (Aubert et al., 1997). The symptoms of sickness behaviour include malaise, depression, anxiety, lethargy, fever, anhedonia, weight loss, decreased social interaction and poor concentration (Kelley et al., 2003, Dantzer, 2004).The main pro-inflammatory cytokines involved in sickness behaviour are IL-1β and TNF-α. Studies have shown that animals in the laboratory injected acutely or chronically with these molecules, usually appear lethargic, show decreased motor activity, decreased food and water intake, social withdrawal and become anorexic (Kent et al., 1992, Plata-Salaman et al., 1988, Plata-Salaman, 1992).Cytokines, in particular IL-1β and TNF-α are key molecules for mediating systemic inflammation (Konsman et al., 2002, Dantzer, 2001, Larson and Dunn, 2001).

Cytokines play key roles in sickness behaviour, neurodegenerative and immune diseases, sleep, and cognition. Cytokines can signal to the brain via circumventricular sites lacking a blood brain barrier (BBB). Circumventricular organs (CVOs) are regions of the brain in which the capillary bed does not form a blood brain barrier (BBB), but instead the vessels are leaky. Blatteis was the first to show that cytokines can cross the circumventricular organs (Blatteis, 1992). Another route is the vagus nerve where Luheshi demonstrated that behavioural depression but not the pyrogenic effect of IL-1β was attenuated in vagotomised rats (Luheshi et al., 2000). The BBB is another route in which cytokines can signal to the brain as this has binding sites that alter intracellular function (receptors) or convey the cytokine across the BBB (transporters) (Banks and Erickson, 2010). Prostaglandin E2, is an inflammatory mediator which can be stimulated by blood-borne cytokines and this can diffuse into the brain parenchyma due it its small size and lipophilic properties (Davidson et al., 2001, Engblom et al., 2002).

LPS and Poly I:C have also been used to induce sickness behaviour in rodents. Bluthe et al demonstrated that LPS injected into rodents leads to reduced food intake, reduction in social exploration and reduction in body weight which are all symptoms of sickness behaviour (Bluthe et al., 2000a). Other authors have shown that LPS injected into rats can induce depressive-like symptoms represented by increased duration of immobility in both the forced–swim and tail suspension tests and in the open-field (O'Connor et al., 2009, Lawson et al., 2013, Yirmiya, 1996). In addition to LPS sickness behaviour studies, Poly I:C a synthetic dsRNA molecule has been also used to induce sickness behaviour. Poly I:C has been shown to induce fever in rats that is partially dependent on IL-1 (Fortier et al., 2004). Other research has shown that when Poly I:C is injected into mice, it can induce a biphasic temperature response consisting of an initial hyperthermia followed by mild hypothermia and reduced locomotor activity (Traynor et al., 2004). Cunningham *et al* showed that mice injected with Poly I:C exhibit decreased burrowing, locomotor activity and decreased body weight all symptoms characteristic of sickness behaviour. The authors also showed that poly I:C challenged mice showed hyperthermia 3-7h post challenge and an extended and marked hypothermia between 8-16h post challenge. The authors also reported elevated levels of IL-6, TNFα, IFN-β and IL-1 in the periphery and in the CNS of these mice (Cunningham et al., 2007).

1.6.1 Role of cytokines in sickness behaviour

IL-1 is one of the key cytokines that contributes to sickness behaviour. The symptoms of sickness behaviour include malaise, depression, anxiety, lethargy, fever, anhedonia, weight loss, decreased social interaction and poor concentration (Kelley et al., 2003, Dantzer, 2004)

Dunn et al showed that when IL-1 is administered i.p to mice it reduces the intake of sweetened milk (Dunn and Swiergiel, 2001, Swiergiel and Dunn, 2002). Another study from the same group showed that when IL-1β was administered in mice 30 or 60 mins prior to being put on the elevated plus maze, it resulted in less time spent in the open arms of the maze and entry to open arms which is considered to reflect anxiety like

behaviour. The same study showed that IL-1β injected mice had a reduction in locomotor activity in the open field (Swiergiel and Dunn, 2007).

Wang and colleagues demonstrated that when IL-1β is administered to mice it induces a profound hypothermia that lasted for more than 3h (Wang et al., 1997). Another study showed that when IL-ra was administered to mice it prevented the reduction in milk intake caused by IL-1β (Swiergiel et al., 1997). Other work by Bluthe and colleagues demonstrated that when IL-ra was administered i.p it blocked the LPS depressive effects on social exploration and on body weight (Bluthe et al., 1997). The same authors also showed that IL-1RI$^{-/-}$ mice were resistant to the sickness-inducing effects of IL-1β administered intraperitoneally (i.p) (2 μg/mouse) and intracerebroventricularly (i.c.v) (2 ng/mouse) but were fully responsive to LPS administered i.p or i.c.v. (Bluthe et al., 2000a). Another study showed that when IL-1β was given to rats, they had an increase in body temperature and a decrease in wheel running. The same study also demonstrated that rats given a higher dose of IL-1β showed significant decrease in food intake and body weight compared to vehicle controls (Harden et al., 2008). Thomson and colleagues also reported anorexia in rats after IL-1β was given (Thomson and Sutherland, 2006). Work from our laboratory has also shown that when IL-1β is given to mice it induces hypothermia and locomotor hypoactivity (Skelly et al., 2013). Cartmell and colleagues also demonstrated that IL-1β in areas of the brain such as the anterior hypothalamus and paraventricular nucleus has been shown to be a crucial event in the generation of sickness behaviour (Cartmell et al., 1999). Taken together these studies demonstrate that IL-1β is a key contributor to sickness behaviour.

There have also been studies that have looked at IL-6 and interferons as potential initiators of sickness behaviour but there is still some conflicting views on whether IL-6 alone can induce sickness behaviour. Lencczowski et al showed that when IL-6 was administered to rats, it resulted in a febrile response but failed to produce a reduction in social investigatory behaviour or locomotor activity alone but when combined with IL-1 the rats showed a reduction in locomotor activity and social investigatory behaviour (Lenczowski et al., 1999). Other studies have shown that peripheral

injections of IL-6 increased ambulatory exploration, rearing and increased grooming and locomotion in mice (Zalcman et al., 1998) however in another study i.c.v injection of IL-6 decreased locomotion and food intake in rats (Schobitz et al., 1995). In addition, Wang et al showed that core body temperature was not altered in mice after IL-6 challenges (Wang et al., 1997) and work by Bluthe et al demonstrated that deletion of the IL-6 gene in mice attenuates sensitivity to the depressing effects of IL-1 and LPS on body weight and social exploration (Bluthe et al., 2000b). More recent work has shown that IL-6 only modestly reduces baseline activity compared to saline-treated controls in the open field (Skelly et al., 2013) and also causes mild decreases in burrowing activity and weight loss (Murray et al., 2015). It has also been shown that when IL-6 trans-signalling is blocked, it reduces suppression of locomotor activity by LPS (Burton et al., 2013). Therefore, these experiments would suggest that IL-6 contributes to sickness behaviour induced by LPS, IL-1 and Poly I:C but has relatively limited effects alone.

Interferons have also been reported to play a role in sickness behaviour. Patients being treated with IFN-α drugs for the treatment of cancer, show signs of sickness behaviour, depression and malaise (Capuron and Miller, 2004). In addition Interferon α has also been shown to be involved in the regulation of temperature and sleep (Krueger et al., 1988) and circadian rhythm (Koyanagi and Ohdo, 2002) and feeding behaviour (Plata-Salaman, 1992).
Taken together, all of these experiments suggest that LPS and Poly I:C can induce sickness behaviour and cytokines all contribute to PAMP-induced sickness behaviour.

Sickness behaviour which is a set of adaptive behaviour changes can be accompanied by a fever response, which essentially helps the individual fight off the infection they are experiencing. Cytokine signalling to the brain during sickness behaviour is normal and useful in fighting off infections and allowing the individual to recover, however emerging evidence has suggested that when sickness behaviour is superimposed on neurodegeneration this can lead to deleterious effects.

1.7 Microglial activation and vulnerability to systemic inflammatory effects on the brain

It is clear that systemic inflammation can communicate with the brain and can impact on brain function via increased inflammatory responses. However during aging and neurodegenerative disease, the brain also shows chronic activation of microglia which potentially increases the impact of systemic inflammation. Neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Dementia, and ALS are a burden on society. As people are now living longer, the incidence of these diseases is increasing. Evidence for the role of chronic neuroinflammation in neurodegenerative conditions continues to grow. In Alzheimer's disease, histochemical studies have shown that there is an increasing presence of microglia and astrocytes, cytokines, complement proteins in AD brains in addition to reductions in brain glucose metabolism (Munch et al., 1998, Millington et al., 2014). Similarly activated microglia and increases in cytokines have been found in the substantia nigra and striatum of PD patients post-mortem (McGeer et al., 1988, Mogi et al., 1994a, Mogi et al., 1994b, Mogi et al., 1995, Hirsch and Hunot, 2009).

Our laboratory in particular is interested in the interaction between systemic inflammation and infection on existing chronic neurodegeneration. This can be studied using a variety of mice of existing brain pathology and superimposing acute systemic inflammation. ME7 is a model of prion disease which our laboratory uses as a tool to investigate behavioural, molecular and neuropathological changes in mice with neurodegenerative disease during episodes of systemic inflammation. The ME7 model will now be described in further detail.

1.8 ME7 model

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative diseases that target the CNS and affect both humans and animals. Prion diseases are caused by a conformational change in the ubiquitous cellular prion protein (PrPC) to the aberrant "scrapie" form (PrPSc) (Prusiner, 1982). PrPC is converted to PrPSc though a conformational misfolding event characterized by the transition of α-helixes into β-sheet structures. This structural transition is

accompanied by profound changes in the physicochemical properties of the PrP (Prusiner, 1998, Prusiner, 1982). This PrPsc isoform can cause disease to occur.

ME7 is a prion disease that originates from scrapie in sheep and that has been mouse adapted. It is used in murine animal models in laboratories as it has all the characteristics associated with neurodegenerative disease which include extracellular amyloidosis, microglial activation, synaptic loss , neuronal death and irreversible cognitive decline (Betmouni et al., 1999, Cunningham et al., 2003). Cell death is not seen until late in disease in the ME7 model, and is thought to occur by apoptosis (Lucassen et al., 1995, Williams et al., 1997).

1.8.1 Progression of ME7 disease

At 12-14 weeks in the progression of ME7 prion disease, hyperactivity is apparent as is reduced burrowing activity (Guenther et al., 2001, Deacon et al., 2001). There are also impairments in nesting and glucose consumption (Cunningham et al., 2003). The same authors also showed that 15 weeks post-inoculation with ME7, mice were performing well on the bar and screen tests, which are measures to access motor and neurological deficits (Cunningham et al., 2009). As ME7 disease progresses, neuropathology is also altered. At 13 weeks, there is a significant loss of synaptic terminals from the hippocampus representive of a reduction in synaptophysin staining in the stradiaum radiatum of the CA1 (Murray et al., 2012). At 19 weeks nearly 40% neuronal loss can be seen in the CA1 region of the hippocampus (Cunningham et al., 2003). Lack of weight, ataxia, huched posture, piloerection, incontinence and ruffled hair is evident at 23 weeks (Betmouni et al., 1996, Felton et al., 2005). The time at which ME7 prion-disease mice would succumb to the disease is generally about 23-25 weeks.

1.8.2 Microglial Priming

Microglial Priming can be defined as a change in the activation state of the microglial cell, which increases the microglial cell responsiveness to further challenge. Combrinck and colleagues demonstrated that ME7 mice after challenge with LPS had elevated levels of hippocampal and thalamic IL-1β in the brain. (Combrinck et al., 2002). Cunningham and colleagues were the first to demonstrate there was an altered

response to LPS peripheral challenges in ME7 inoculated mice. In this study they used LPS to mimic aspects of central or systemic infection and the ME7 as a model of chronic neurodegeneration and addressed whether microglial cells seen in chronic neurodegeneration were primed to produce a greater inflammatory response when challenged central and peripheral with LPS.

In this study the authors clearly showed that at 19 weeks post-inoculation, there were elevated levels of LPS-induced IL-1β and inducible nitric oxide synthase expression in the brain parenchyma of the ME7 mice compared to NBH controls, which was accompanied by exaggerated neutrophil infiltration after LPS challenges. It was also reported in this study that microglia in the ME7 mice showed no morphological change compared to NBH LPS controls but upon LPS stimulation changed to a more ramified activated morphology. This study demonstrated that primed microglia show an exaggerated IL-1 response to subsequent inflammatory challenge whether centrally or peripherally applied (Cunningham et al., 2005b). Follow up work from the same group demonstrated that at an earlier time point in ME7 disease, 12 weeks, ME7 mice show heightened IL-1, TNF-α and interferon beta transcription in the CNS (Cunningham et al., 2009). Walsh and colleagues, showed there was little expression of pro-inflammatory cytokine IL-1 and IL-6 in ME7 prion disease but COX-2 was highly expressed in the microglia of prion diseased mice and that as these mice progressed in disease, the number of COX-2 positive cells also increased (Walsh et al., 2000, Walsh et al., 2001). Low expression of these cytokines has been reported in animal models of AD and Parkinson's disease (Sly et al., 2001, Depino et al., 2003). Cunningham and colleagues showed that it was the anti-inflammatory cytokine transforming growth factor (TGF- β1) that was in fact elevated in ME7 inoculated mice (Cunningham et al., 2002). Importantly, the systemic inflammatory response to peripheral immune challenge is equivalent in diseased and normal animals (Murray et al., 2012).

1.8.3 Microglial priming in other models

Microglial Priming seen in the ME7 model is not unique to this model. Subsequent studies have demonstrated that an exaggerated CNS inflammatory response to peripheral inflammation is a relatively generic phenomenon also observed in aging

(Godbout et al., 2005, Barrientos et al., 2006), models of PD (Pott Godoy et al., 2008), and Alzheimer's disease (Sly et al., 2001, Sy et al., 2011), MS (Ramaglia et al., 2012) and Wallerian degeneration (Palin et al., 2008).

In the PD model, Godoy and colleagues showed using the 6-hydroxydopamine (6-OHDA) model, that chronic systemic IL-1β exacerbated the neurodegenerative effects of 6-OHDA with significantly less dopaminergic cells present following IL-1β treatment (Pott Godoy et al., 2008). In an aging study carried out by Godbout and colleagues, the authors showed that the levels of pro-inflammatory cytokines IL-1, IL-6 and TNF-α were highly expressed following LPS challenge in aged mice compared to the adult mice indicating that in the brains of these aged mice the microglia were primed. The same group showed in a further study that there were more IL-1 beta positive cells present in the dentate gyrus and hippocampal region in the aged mice than in the young adults following LPS challenge. These aged mice also then showed longer distances and latency in the radial arm maze which is a behaviour test for spatial memory compared to the young adult mice (Godbout et al., 2005, Chen et al., 2008). Similar studies showing cognitive impairment in aging animals following systemic challenge were also reported in rats. Aged rats challenged with *Escherichia coli* (*E. coli*) had increased levels of IL-1β in the hippocampus and displayed impaired memory in the Morris water maze and impaired contextual fear conditioning (Barrientos et al., 2006). In an AD model, 16-month-old Tg2576 (Tg) showed significant increases in IL-1 protein in the cortical and hippocampal region that was not present in the young Tg or non-transgenic mice. The authors also showed in the same study that there were increased levels of Aβ1-40 following LPS challenge in the aged Tg mice, which indicates that the microglia in these aged Tg mice appear to be primed by accumulating Aβ plaques (Sly et al., 2001). Other AD models using 3xTg mice have demonstrated that when mice were injected with mouse hepatitis virus (MHV) or LPS, resulted in a robust but transient neuroinflammation, exacerabated tau pathological characteristics that was accompanied with deficits in cognitive function in these mice (Sy et al., 2011). In an optic nerve crush model of Wallerian degeneration, microglial priming resulted in exaggerated levels of IL-1 and iNOS production following challenge with LPS peripherally (Palin et al., 2008). A more recent study by Holtman showed that microglia possess distinct gene expression signatures. In this study the authors looked

at the transcriptomes of microglia in aging, AD and ALS mouse models. They found that there was a highly consistent transcriptional profile of unregulated genes in the primed microglia in these models, which was distinctly different from the acute inflammatory gene network induced by LPS (Holtman et al., 2015).
Taken together all of the studies show that microglial priming is not unique to the ME7 model.

1.8.4 Why does microglial priming matter?

Microglial priming leads to increased expression of cytokines following a secondary insult such as infection, trauma or surgery. In the ME7 animal studies, the increase in IL-1 expression and pro-inflammatory transcripts in the ME7 mice resulted in acute behavioural deficits that were not observed in the NBH controls. The authors also reported that following LPS challenge, the ME7 mice displayed an exaggerated fever and sickness behaviour response, with a heightened hypothermic response and a reduction in locomotor activity (Combrinck et al., 2002). Cunningham and colleagues found that at 15 weeks ME7 mice were performing well, however when challenged with LPS, showed decreased performance on the bar and screen tests which are measures to access motor and neurological deficits, compared to their NBH controls. These data would suggest a single peripheral inflammatory event in a vulnerable animal can induce acute behavioural and cognitive deficits and contribute to the acceleration and progression of disease (Cunningham et al., 2009). Additional research from the same authors has also shown that when ME7 mice are repeatedly challenged with poly I:C, disease progression is accelerated compared to normal animals (Field et al., 2010).

Clinical evidence has shown that patients with systemic inflammation or suffered trauma or undergoing surgery are at risk of developing Dementia. Dementia can be defined as a chronic or persistent disorder of the mental processes caused by brain disease or injury which is marked by memory disorders, personality changes, and impaired reasoning. Dementias are more common with age and patients over the age of 65 undergoing surgery for hip fractures experience dementia the most common being Alzheimer's. Approximately 10% of people will develop AD at some point in their

life (Larson et al., 2013, Loy et al., 2014). Globally Dementia can affect 36 million people and the costs of caring for patients with dementia are increasing as the population is aging (Bleiler, 2014).

Hip fracture is one good example of how systemic inflammation and priming of microglia leading to increased cytokine circulation, can lead to deleterious effects. In patients undergoing hip fractures surgery, one of the most common post-operative complications is delirium with an incidence of up to 53.3% (Bruce et al., 2007). Patients undergoing hip fracture surgery are exposed to severe precipitating events for delirium such as major trauma, surgery and prolonged anaesthesia. Numerous studies have looked at the predisposing factors as well as the impact of delirium on mortality in patients after having hip fracture surgery (Lee et al., 2011, Lee et al., 2016, Gottschalk et al., 2015, Smith et al., 2017). Prevention and intervention strategies after hip fracture would be of great public health significance as there is a high increase in morbidity and mortality in these patients. Delirium is also seen in patients undergoing femoral neck surgery (Weed et al., 1995, Edlund et al., 1999).

Patients over the age of 65 undergoing hip and neck fractures surgeries are already vulnerable due to their age and fractures. Following trauma and surgery there is increased cytokine expression in the periphery that signals to the brain and impacts on CNS function. Patients undergoing surgery experience delirium. The priming models all share similar traits, that microglia are primed due to underlying pathology or when the brain is comprised/vulnerable during aging. The primed microglia then, following a secondary insult, such as surgery or trauma, induce a robust inflammatory reaction which leads to increased cytokine expression that then impacts on CNS function. These priming models are important in that they mimic key core features that are associated with patients experiencing dementia, delirium, PD, AD and aging. By having these models, it allows researchers to develop new and therapeutic approaches for these debilitating illnesses.

1.9 Delirium

Delirium, which is characterised by inattention and profound cognitive impairment, is an acute and transient disorder that is highly prevalent in elderly and demented patients (Burns et al., 2004). It is a very common medical condition and it is estimated that one in eight hospitalised patient will experience delirium (Cunningham and Maclullich, 2013). In surgical patients over the age of 65 years, 15-53% will have postoperative delirium which leads to multiple adverse outcomes that include increased length of stay, patient distress, increased morbidity and mortality, loss of independence and increased healthcare costs (Fong et al., 2009). There are large economic and social burdens associated with delirium. The care of older patients in hospital with delirium has accounted for more than 49% of all hospital days and $6.9 billion of hospital expenditures are attributable to delirium costs (Inouye, 2006).

Delirium can occur in three forms. These are hyperactive, hypoactive and mixed delirium. The most common form of delirium seen in the elderly population is hypoactive delirium and it is often not recognized (Inouye, 2006). Hypoactive delirious patients show signs of lethargy and low arousal and have slow responses to questions and very little spontaneous movement. In contrast hyperactive delirious patients are restless, agitated and often experience hallucinations and delusions (Fong et al., 2009). Up to 20% of patients with delirium are classed as 'hyperactive" and there is limited evidence that in drug-induced cases hyperactive delirium occurs more readily. Benzodiazepines, narcotics, and drugs with anticholinergic activity have a particular tendency to cause delirium (Meagher, 2001). In addition, the use of anti-cholinergic drugs can lead to an increase of delirium symptoms (Han et al., 2001), and there is now evidence that lack of acetylcholine, a neurotransmitter highly involved in memory function can cause delirium (Hshieh et al., 2008).

There are many risk factors associated with delirium. Delirium can be caused by factors in the patient such as age, dementia, alcohol abuse, medical illness as well as by pharmacological and environmental factors (Meagher, 2001). Dementia is the most prominent risk factor for delirium in the elderly and is present in two thirds of all cases of delirium (Cole, 2004). It is estimated that over the next 40 years the care for

patients with dementia will cost $1.2 trillion dollars (Bleiler, 2014). Changes in medication, head trauma, peripheral and systemic infections, surgery, stroke, psychological stress, hypoglycaemia, hyponatremia, acute hepatic encephalopathy are all risk factors that can cause delirium (Cunningham and Maclullich, 2013).

Delirium is poorly understood. With the incidence of delirium increasing, especially in the vulnerable aging population, and the burden of costs to care for patients with delirium also increasing, there is pressing need for further research into delirium. In order for this to be done, biologically relevant animal models in which to study the inflammatory pathways and neurodegeneration that occur during dementia and delirium need to be established. The lack of biologically relevant animal models in which to study the pathophysiology of delirium has limited basic research; however our laboratory is creating animals models in which key core features displayed in delirium can be investigated in these animal models. These models all share the same fundamental idea, that if animals with prior pathology are challenged systemically with an systemic inflammatory stimulus such as LPS, this will result in an acute and transient cognitive deficit in these animals, with impairment in attention, recall and short-term working memory which are all key core feature displayed in Delirium as defined by the DSM-IV (American Psychiatric Association, 1994) and ICD-10 (World Health Organization, 1993). Two animal models which have been published by our laboratory include the selective lesioning of the basal forebrain cholinergic system using the murine-p75-saporin (mu-p75-sap) immunotoxin, a ribosome-inactivating toxin saporin, bound to an antibody directed against the p75 neurotrophin receptor. Cholinergic insufficiency can lead to delirium (Trzepacz, 2000), and the selective lesioning in this model results in chronic cholinergic hypofunction in these mice. These p75 saporin-lesioned mice when challenged systemically with LPS show an acute and transient cognitive deficit (Field et al., 2012). The other model our laboratory has developed is the ME7 prion model which will be described in further detail below.

1.9.1 An animal model of Delirium

Using the ME7 prion disease, our laboratory has created a novel model in which to study the key core feature of delirium in mice. ME7 is used as it is a progressive

neurodegenerative disease with progressive cognitive and functional decline that is accompanied by synaptic loss, amyloidosis and microgliosis (Cunningham et al., 2005a). It is well known that systemic inflammation caused by injury, surgery or trauma can induce episodes of delirium in elderly and demented populations. Delirium can be defined as an acute onset and transient disorder. Previous studies showed that in the Y maze reference memory, systemic inflammation induced a learning deficit in ME7 mice that was not present in non-diseased mice also challenged with LPS (Cunningham et al., 2009) and microglia are primed by prior neurodegenerative disease to respond more robustly to systemic inflammation. The Y maze task fails to assess working memory in these mice; therefore the paddling alternation T-maze task was used in this novel model. This model aims to address whether systemic inflammation, induced in animals with early stage neurodegeneration (ME7 prion disease) would induce acute onset and transient working memory dysfunction that is not induced by similar challenges in normal animals. Mice were inoculated with ME7 or normal brain homogenate (NBH). The mice were then trained to alternate on the T-maze until performance of 80% correct alternation is obtained. The mice were then subsequently challenged with LPS. LPS induces an acute and transient working memory deficit but only in ME7 mice. The NBH mice challenged with the same dose of LPS do not have any working memory deficits. The ME7 mice show heightened and pro-longed transcription of inflammatory cytokines compared to the LPS-treated normal mice (Murray et al., 2012). This model demonstrates that systemic inflammation, induced in animals with early stage neurodegeneration (ME7 prion disease) induced an acute onset and transient working memory dysfunction that was not induced in the NBH mice. This model is appropriate to assess molecular mechanisms of systemic inflammation –induced working memory deficits with relevance to delirium.

In this thesis it is proposed to study impacts of systemic inflammation on cognitive function. In order to understand how learning and memory is assessed in mice it is important to describe the typical approaches used in the literature and in this thesis to assess cognition in mice.

1.10 Learning and memory and behavioural tasks in rodents

The two main behavioural tasks that are widely described in the literature to assess learning and memory in rodents include the Morris water maze (MWM) task and contextual fear conditioning task.

Morris water maze (MWM) - The MWM was conceived by Richard Morris in 1981 (Morris, 1981). It is a behavioural task that assesses spatial memory and learning. This task allows rodents to learn to locate a hidden platform that they cannot see, hear or smell provided the platform remains in a fixed spatial location relative to distal room cues. The testing area is a round pool filled with water and a hidden platform is submerged just below the water surface. Rodents learn to escape from the water by locating the hidden platform with the help of visual cues. Latency (time take to locate the hidden platform) and distance travelled and velocity can be measured to access learning of the rodents to this task. Anymaze or Ethovision are the most widely used tracking software used to measure the different parameters in this task. The shorter the latency and distance travelled suggest learning in this task. The platform can be placed in another quadrant, or removed during another phase of the experiment allowing memory retention and extinction to be investigated. Across repeated trials spatial learning is assessed and reference memory is determined by preference for the platform area when the platform is absent. This behavioural task is hippocampal dependent. The Morris water maze task is a key technique in the investigation of hippocampal circuitry as it has been linked to long-term potentiation (LTP) and NMDA receptor function (Morris et al., 1986, Jeffery and Morris, 1993, Moser et al., 1998). It has been shown that there is involvement of the entorhinal and perirhinal cortices, prefrontal cortex, the cingulate cortex, the neostriatum and the cerebellum in a more limited way (D'Hooge and De Deyn, 2001). When planning MWM experiments or analysing results, several characteristics of the experimental animals need to be considered. Some of these include age, body weight, gender and strain (Brandeis et al., 1989, Lipp and Wolfer, 1998, Gallagher and Nicolle, 1993, Geinisman et al., 1995).

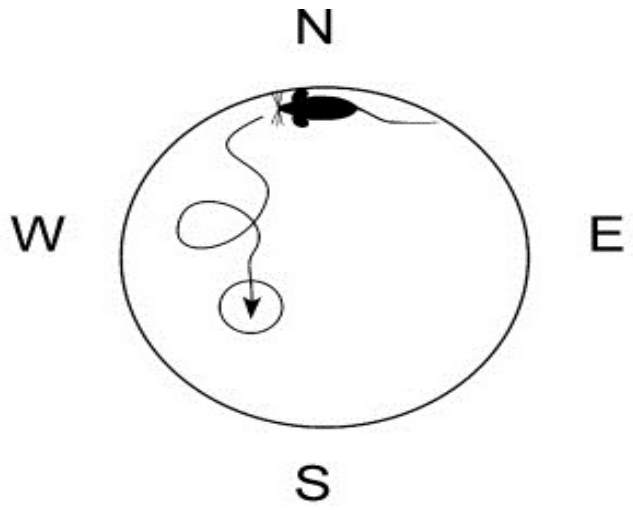


Figure 1.4 Schematic diagram of the Morris Water Maze

Contextual Fear Conditioning is a behavioural paradigm in which rodents learn to predict aversive events. In contextual fear conditioning a rodent is placed into an apparatus and receives pairings of a phasic auditory cue and electrical shock to its feet. When the rodent is tested in the original training context- it will display freezing like behaviour and becomes immobile (Blanchard and Blanchard, 1969). Freezing is regarded as the complete absence of movement, except those related to respiration as originally described (Fanselow, 2000). The rodent will also display the freezing response to the auditory cue when it is presented in a different context. Auditory cue is generally tested in a different context so that the freezing can be attributed to conditioning to the cue and not to the context in which shock occurred. The increase in time spent freezing in the context is an indication of learning in this task. Fear conditioning is thought to depend on the amygdala. Electrophysiological recording from the amydgala have demonstrated that cells in that region undergo LTP (Sah et al., 2008). Inhibition of neurons in the amygdala disrupts fear acquisition, while stimulation of those neurons can drive fear-related behaviours, such as freezing behaviour in rodents (Bocchio et al., 2017), thus indication that proper function of the

amygdala is both necessary for fear conditioning and sufficient to drive fear behaviours. The hippocampus is also involved in the contextual fear response (Anagnostaras et al., 2001). Glutamate is one of the major neurotransmitters involved in conditioned fear learning. NMDA receptors (NMDARs) in the amygdala are thought to be necessary for fear memory acquisition as disruption of NMDAR function disrupts development of fear responses in the rodent (Johansen et al., 2011).

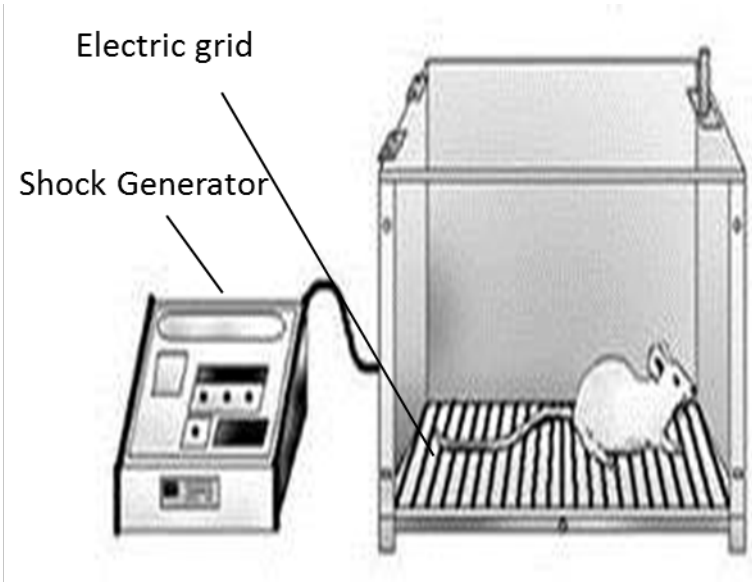


Figure 1.5 Schematic diagram of the contextual fear conditioning paradigm.

Diurnal Rhythm Monitoring- is the recording of daily rhythms, biological processes that cycle in 24-hour intervals and is synchronized with the day/night cycle. Diurnal rhythms differ from circadian rhythms as circadian rhythms are endogenously generated but are still susceptible to modulation by 24-hour environmental cycles. Diurnal rhythms should not be called circadian rhythms unless their endogenous nature is confirmed (Vitaterna et al., 2001). Light and temperature are environmental cues that can affect circadian rhythms. There are three criteria that must be met for a biological rhythm to be called circadian. These include that the 1) rhythm must continue under constant conditions with no environmental time cues, with a period close to 24 hours 2) Rhythm are entrainable, it can be reset by environmental cues

such as light and heat and 3) rhythms must exhibit temperature compensation they maintain circadian periodicity over a range of physiological temperatures. Circadian rhythms are controlled by the circadian clock which is located in the suprachiasmatic nuclei (SCN) in the hypothalamus. (Giebultowicz, 2004).

1.11 Role of interleukin 1 in learning and memory

IL-1 plays a role in sickness behaviour and in learning and memory. There are numerous studies that show that IL-1 can be detrimental in learning and memory but also may facilitate learning in some situations. Interleukin 1, produced in the brain by microglia and neurons and by macrophages in the periphery, is a pro-inflammatory cytokine that plays a considerable role in learning and memory. Many of the studies to date have looked at endogenous levels of IL-1 in learning and memory, and other studies have also investigated memory and learning performance after induction or administration of IL-1 either systemically or centrally or IL-1RA, which is the receptor antagonist for IL-1.

The first study that showed detrimental effects of IL-1β on learning and memory was conducted in 1993 in rats by Oitzl and colleagues using the MWM. Oitzl and colleagues showed that when IL-1β (100 ng) was administered i.c.v 1hr before the spatial water maze training, this resulted in a transient memory impairment in the first trial of the following day. They also showed in the same study that when IL-1β was injected immediately before training, there was no impairment in the water maze thus suggesting that IL-1β affects the retention of learning and not the acquisition of spatial memory (Oitzl et al., 1993). Subsequent studies have also demonstrated that when IL-1β (100ng i.p) was administered on the first and second day of spatial water maze training this also resulted in impaired spatial learning but no impairment of non-spatial memory (Gibertini et al., 1995). The same group also demonstrated that IL-1β impaired learning in a random start point version of water maze (hippocampus dependent spatial memory but not in fixed start point version of water maze (hippocampus-independent non-spatial memory) (Gibertini, 1996). A lower dose of IL-1β (15ng) i.c.v can also induce detrimental effects on spatial memory and IL-1β was

shown to impair the performance of rats in the hippocampus-dependent spatial version of water maze but not in the hippocampus-independent visually guided version of the water maze (Song and Horrobin, 2004).

In addition to the water maze behavioural tasks, there have also been studies using the contextual and auditory fear conditioning paradigms that also support the hypothesis that IL-1 can be detrimental on memory and learning. Contextual and auditory fear conditioning is a behaviour task that assesses the ability of rodents to learn and remember an association between environmental cues and aversive experiences. Intracerebral administration of IL-1β (10 or 20ng) injected immediately following conditioning, impaired contextual (hippocampus-dependent) but not auditory-cued (hippocampus-independent) fear conditioning (Pugh et al., 1999). Bilateral intra-hippocampal injection of IL-1β (10ng) impairs contextual fear conditioning (Barrientos et al., 2004).

Taken together, all of the above water maze and fear conditioning studies described all indicate that IL-1 given systemically or intracerebrally can be detrimental for learning and impair memory.

However, there are conflicting studies that have supported the idea that IL-1 has no effect on memory and is also necessary to facilitate learning. In the spatial memory task, Lacosta and colleagues administered IL-1β a week before training and during training and they showed no effect on spatial memory in mice (Lacosta et al., 1999). Additionally, mice that were trained in the water maze using a spaced-learning protocol displayed normal latency to reach the platform following IL-1 injection, despite the fact that the IL-1β-injected mice used a different strategy to find the platform (Gibertini, 1998). Similar studies by Cibelli and colleagues, demonstrated rats injected with IL-1β or IL-α had no impairment in the non-spatial version of the MWM (Cibelli et al., 2010). In a different version of the contextual fear conditioning paradigm in which rats were pre-exposed to the context before conditioning the authors report that the rats that received IL-1β(1ng) 48 hr after pre-exposure to the context showed less freezing than vehicle-treated rats (Barrientos et al., 2002). Rats treated with IL-1β

also showed improved memory in the passive avoidance response (Yirmiya et al., 2002). Together all of these studies would indicate that IL-1 action is beneficial for learning and memory.

In addition to these studies where IL-1 has been injected either systemically or centrally, there are multiple studies where IL-1RA has been administered. Similar to the IL-1 studies, the data from the IL-1RA studies are conflicting. Some studies have suggested that blocking IL-1 by IL-1RA can prove to be detrimental in memory and learning. Studies in rats have shown that when rats were given i.c.v injection of IL-1RA, it caused memory impairments in the Morris water maze (MWM) and also memory impairments in the passive avoidance task (i.e decreased latencies to cross into the goal area) (Yirmiya et al., 2002). Further studies from the same group showed that IL-1RATG mice, transgenic mice over expressing IL-1RA, have impaired hippocampal-dependent and intact hippocampal-independent memory in the water maze and fear-conditioning paradigms. They also show in the same study that when IL-1RA is continuously administered via osmotic minipumps during prenatal development it disrupts memory performance in adult mice (Goshen et al., 2007). . A study by Maier and colleagues showed that when IL-1RA is administered i.c.v, and given immediately before or after inescapable shock, it blocked the potentiation of fear conditioning and the interference with escape learning produced by inescapable shock (Maier and Watkins, 1995)

Conflicting studies have indicated that IL-1RA is necessary for learning and memory. Blocking IL-1 actions by administration of IL-1RA has also proven to be beneficial in protecting against memory deficits. Terrando and colleagues also showed that when IL-1RA was administered before LPS injection in mice, after exposure to the contextual fear conditioning paradigm it ameliorated the cognitive dysfunction of LPS on the hippocampal-dependent cognitive task (Terrando et al., 2010). Another study from the same group also demonstrated that surgery-induced impairments in contextual fear conditioning were significantly reduced by pre-emptive administration of IL-1RA i.p (Cibelli et al., 2010). This was similar in the studies by Pugh and colleagues where

they showed that the impairment in the contextual fear conditioning test after intracerebral administration of IL-1β could be blocked using IL-1RA (Pugh et al., 2001).

Perhaps a reason for these conflicting views could be seen in an important study from Goshen and colleagues where they administered different doses of IL-1 to rats i.c.v and examined the rats contextual fear conditioning response following administration. The authors showed in the contextual fear conditioning that when 1ng of IL-1 was administered to rats i.c.v, the rats, showed an increase in freezing response than saline-treated controls. However, when the dose of IL-1 was increased to 10ng, the rats showed decreased freezing that was also observed when IL-1RA was administered i.c v. This study was the first study that showed that very low "physiological" levels of IL-1β facilitate learning and blocking these levels can cause impairments and increasing the levels of IL-1β can also induce memory impairments (Goshen et al., 2007). The same authors in another studied demonstrated that IL-1 receptor type 1 knockout mice (IL-1rKO) display significantly longer latencies to reach the hidden platform compared to WT controls. They also show in the same study that the IL-1Rko mice have impairments in contextual memory compared to WT controls but exhibited no impairments in the auditory-cued memory (Avital et al., 2003).

Long-term potentiation (LTP) a persistent increase in synaptic efficacy following high frequency stimulation of that synapse is believed to be a major mechanism underlying learning and memory and IL-1β is thought to be implicated in this process. Research has shown that LTP in the hippocampus is accompanied by a long-lasting increase in IL-1β gene expression and that exposure to IL-1RA can impair the maintenance of LTP (Schneider et al., 1998). In addition, high levels of IL-1β can inhibit the expression of LTP in the CA3 region of the hippocampus (Katsuki et al., 1990) and similar results have also been seen in the CA1 region, and in the dentate gyrus (O'Connor and Coogan, 1999, Cunningham et al., 1996). The impairment of LTP is thought to be caused by IL-1β because IL-1RA can reduce the inhibitory effect of IL-1β on LTP expression. The IL1rKO mice used by Goshen *et al* above, showed no LTP either in vivo or in vitro, and showed severely impaired hippocampus-dependent but normal hippocampus-independent memory with only 35% of time spent freezing in the

context for the IL-1R1$^{-/-}$ mice compared to the 60% freezing of their controls (Avital et al., 2003). In addition, mice with knockout of the IL-1α and IL-β genes had no impairment on LTP (Ikegaya et al., 2003). These studies suggest that IL-1 can impair or assist learning dependent on the concentration of IL-1 and task being carried out. At low levels and concentrations, IL-1 may facilitate LTP, but if the levels of IL-1 increase this can lead to impairment of LTP.

1.12 Confounding factors in learning and memory

Each of the above studies all support the idea that IL-1 can play a detrimental as well as beneficial role in learning and memory, however there are confounding factors and methodological concerns that need to be taken into account when interpreting the data presented in these studies. Some of the confounding concerns which are highlighted in the review by (Cunningham and Sanderson, 2008) include lack of motivation, increased stress and anxiety responses and state-dependent effects. Sickness behaviour/malaise which can be induced by IL-1, can be a confounding factor during water maze testing. In order to interpret and separate between cognitive, emotional and locomotor impairments during MWM testing, when presenting data, in addition to presenting the latency and distance travelled, additional information such as route taken and a probe trial should also be included when presenting results. When animals are sick, latency to find the platform is increased with reduced locomotor speed in these sick animals. Some studies can just report distance travelled or route to the platform and show no difference in treated animals but not show the latency which would could indicate a difference in treated animals, therefore in order to interpret correctly what strategies sick animals are using to solve the MWM, it is essential that in addition to probe trial information, distance and route travelled be also reported. In the studies by Oitzl et al, the authors only showed latency to the platform and did not show distance travelled or route taken or results of a probe trial conducted during training. A probe trial should be included in all water maze testing as it measures the amount of time spent in the quadrant where the location of the hidden platform previously was. If mice spend more time in the same quadrant as the hidden platform was once located, this shows that they have learned this task using visuospatial cues. A probe trial can also tell you if mice are using thigmotaxic

strategies to avoid entering the center area of the maze (Treit and Fundytus, 1988, Simon et al., 1994). Thigmotaxis is regarded as the learning of a non-spatial strategy is where animals in the maze tend to cling or follow the wall around the outer perimeter of the tank (Vorhees and Williams, 2006). Increases in stress and anxiety responses in mice undergoing testing in the water maze can result in thigmotaxic strategies therefore, when water maze results are reported, authors should also provide the route taken by animals in order to distinguish whether there is a failure in animals to learn this tasks or whether they are using a stress-minimizing strategy. In addition, elevated plus maze and open field data should also be reported in the same mice undergoing testing in the maze which would provide further evidence of the anxiety levels in the mice being tested.

The temperature of the water in which testing and training is conducted should also be taken into account when measuring learning performance. A study by Gibertini demonstrates that when mice injected with IL-1β were tested in water temperature of 18°c there was no impairment seen, however when tested in water temperature of 23°c the IL-1β treated mice showed impairment on this task, suggesting that IL-1β may impair motivation to learn this task but not actually the ability to learn the task (Gibertini, 1998).

State-dependent effects are another confounding factor that should be considered in learning and memory tests. A state-dependent effect is where memory retrieval is most efficient when a person/animal is in the same state of consciousness as they were when the memory was formed. State dependent changes can be interrupted by administration of drugs during testing that were not present at the time of learning or retrieval of memory, or the context in which training occurred is not the same in where testing is carried out. It is well known that the context or state the human is in when learning occurs plays a pivotal role in subsequently retrieving the memories. Goodwin and colleagues demonstrated this when they assessed 4 memory tasks in men who were either sober or under the effects of alcohol when conducting the learning of this task. The retrieval of memory was poor when sober however when tested under the effects of alcohol was better (Goodwin et al., 1969). The timing of

drug before testing and training should also be taken into account. Oitzl and colleagues demonstrated that when IL-1β (100 ng) was administered i.c.v 1hr before the spatial water maze training, this resulted in a transient memory impairment in the first trial of the following day, however they also showed that there was no impairment in the water maze when IL-1β was injected immediately before training, which suggests that the timing of IL-1 before training can have an effect on any impairments observed during training.

1.13 Aims and Objectives

Cytokines which include interleukins, interferons, tumour necrosis factors, chemokines and growth factors are rapidly induced in response to tissue injury, infection or inflammation. We know that cytokines play a role in emotion and memory functions (Reichenberg et al., 2001) but the role these cytokines play in influencing brain function in health and disease is still being investigated. IL-1 and IFN-I are two key inflammatory mediators which are induced by bacterial and viral infections. They have documented effects on brain function with IL-1 involved in learning and memory, and IFN-I having a considerable role in infectious and inflammatory disease, cancer and CNS function. The extent of our understanding on the roles of basal endogenous IL-1 and IFN-I and the impacts of induced IL-1, IFN-I on the normal brain remains poorly understood. Therefore the overarching aim of this thesis is to examine the effects of endogenous and induced IL-1 and Type I IFN's on behaviour and cognition.

- A primary aim of this thesis will be to investigate the roles of endogenous IL-1 and IFN-I on learning and behaviour in IL-1R1$^{-/-}$ and IFNAR1$^{-/-}$ mice. In addition to assessing performance and learning of these mice on an array on memory tasks, anxiogenic behaviour, activity levels and diurnal rhythm will also be assessed. The expression of inflammatory markers in these mice through the use of immunohistochemistry and molecular techniques will also be investigated.

- The second aim will be to assess the impacts of systemically induced IL-1 on cognition and systemically induced IFN-I on sickness behaviour. IL-1R1$^{-/-}$ mice will be challenged with LPS and IFNAR$^{-/-}$ mice will be challenged with Poly I:C to look at the effects of induced IL-1 during systemic inflammation on cognition and induced IFN-I during poly I:C induced sickness behaviour. Molecular studies will try to determine what mediators are responsible for inducing sickness behaviour in IFNAR1$^{-/-}$ mice challenged with poly I:C.

- Finally, the role of induced IL-1 will be assessed in the ME7 neurodegenerative model in the CFC and T maze alternation task, after systemic challenge with LPS. The role of induced IFN-I during aging will also be investigated.

Chapter 2

Materials and Methods

2.1 Materials

Animals and *in vivo* Products

C57BL/6 mice	Harlan laboratories, UK
IL-1RI-/- / C57BL/6	Prof. Kingston Mills/Jackson Laboratory, USA
IFNAR1-/- C57BL/6	Prof Paul J Hertzog
Laboratory mouse diet	Red Mills, UK
T-maze	Custom designed in TCD
Y-maze	Custom designed in TCD
Open Field arena	Custom designed in TCD
Morris Water Maze	Custom designed in TCD
Beacon Water Maze	Custom designed in TCD
Elevated Plus Maze	Custom designed in TCD
Contextual Fear Conditioning	UGO Basile, Italy
Phenotyper Home Cage	Noldus, Nottingham, U.K
Thermocouple rectal probe	Thermalert , USA
Peristaltic pump	Gilson, France
Formalin solution, neutral buffered, 10%	Sigma-Aldrich, UK
Microsyringe	Hamilton, USA
Stereotaxic frame	David Kopf Instruments, USA
Glucose meter ACCU Chek Aviva	Roche, UK

Treatments

Dexamethasone-21-phospate	Sigma-Aldrich, UK
Mouse recombinant IL-1b	R & D Systems, UK
Mouse recombinant IL-6	Peprotech, UK

Human recombinant IL-1RA	Peprotech, UK
Interferon β Interferon Source	Stratech, UK
Interferon α PBL Interferon Source	Stratech, UK
Salmonella equine abortus Lipopolysaccharide, L5886	Sigma-Aldrich, UK
SC-560	Cayman Chemicals, USA
0.9% Sodium Chloride (Sterile)	Braun, Germany
Sodium pentobarbital	Merial Animal Health, UK
Glucose	Sigma-Aldrich, UK
Poly inosinic:poly cytidilic acid	Amersham Biosciences, UK
2-Deoxyglucose	Sigma-Aldrich, UK
Insulin Human, European Pharmacopoeia	Sigma-Aldrich, UK

ELISA

Mouse CXCL1 duo set	R & D Systems, UK
Mouse IFN-β	PBL Biomedical Laboratories, USA
Mouse IL-1β duo set	R & D Systems, UK
Mouse IL-1β Quantikine	R & D Systems, UK
Mouse IL-6	Biolegend, USA
Mouse TNF-α duo set	R & D Systems, UK
HPE Buffer	Sanquin, Netherlands
Streptavidin poly horseradish peroxidase	Sanquin, Netherlands
Substrate Solution	R & D Systems, UK
DC Protein Assay	Bio-Rad, UK
96 well maxisorb immunoplates	Nunc, UK

Immunohistochemistry Reagents and Products

Biotinylated horse-anti-mouse antibody	Vector Laboratories, UK
Bovine serum albumin	Sigma-Aldrich, UK
DPX mountant	Sigma-Aldrich, UK
Harris Haematoxylin solution	VWR, UK
Histoclear II	National Diagnostics, USA
Normal horse serum	Vector Laboratories, UK
Mouse monoclonal anti-Synaptophysin antibody [SY38]	Abcam, UK
PAP pen	Sigma-Aldrich, UK
Paraffin wax	Leica, Germany
SuperFrost Plus microscope slides	Menzel-Glaser, Germany
Vectastain ABC kit	Vector Laboratories, UK

Molecular Reagents and Products

Nucleospin RNA II kits	Macherey-Nagel , Germany
RNeasy Plus mini kits	Qiagen, UK
Qiashredder columns	Qiagen, UK
RNase-free DNase I enzyme	Qiagen, UK
SYBR green PCR master mix	Roche, UK
FastStart universal probe master mix	Roche, UK
High Capacity cDNA Reverse Transcriptase Kit	Applied Biosystems, UK
Molecular grade Absolute Ethanol	Sigma-Aldrich, UK
Molecular grade water	Sigma-Aldrich, UK
Optical adhesive covers	Applied Biosystems, UK

MicroAmp 96-well Reaction Plate	Applied Biosystems, UK

General Laboratory Chemicals

Ammonium nickel chloride	Sigma-Aldrich, UK
3,3' Diaminobenzidine	Sigma-Aldrich, UK
Dimethyl sulfoxide (DMSO)	Pierce, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, UK
Formic acid	Sigma-Aldrich, UK
Glycerol	Sigma-Aldrich, UK
Hydrochloric acid	VWR, USA
Heparin (bovine)	Leo Laboratories, UK
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich, UK
HPLC-grade Ethanol	Sigma-Aldrich, UK
Methanol	Fisher, UK
β-mercaptoethanol	Sigma-Aldrich, UK
Phosphate-buffered saline (PBS)	Thermo Fisher Scientific UK
Potassium bicarbonate	Sigma-Aldrich, UK
Sodium acetate	Sigma-Aldrich, UK
Sodium Chloride (NaCl)	Sigma-Aldrich, UK
Sodium Hydroxide	Sigma-Aldrich, UK
Sodium phosphate dibasic (Na2HPO4)	Merck, USA
Sodium phosphate monobasic (NaH2PO4)	Merck, USA
Sucrose	Sigma-Aldrich, UK
Sulphuric acid (H2SO4)	VWR, USA
2,2,2, tribromoethanol	Sigma-Aldrich, UK
Tris-base	Sigma-Aldrich, UK

Tris-HCl	Sigma-Aldrich, UK
Triton X-100	Sigma-Aldrich, UK
Tween 20	Sigma-Aldrich, UK
Xylene	VWR, USA

Western Blotting

Tris-HCl	Sigma-Aldrich, UK
Sodium Chloride (NaCl)	Sigma-Aldrich, UK
Protease Inhibitors	Roche, UK
Phosphatase Inhibitors	Roche, UK
30% Acryl/Bis-acrylamide	Sigma-Aldrich, UK
Tris Borate	Sigma-Aldrich, UK
Ammonium Persulphate	Sigma-Aldrich, UK
Sodium Chloride (NaCl)	Sigma-Aldrich, UK
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich, UK
STAT1 (SC346)	Santa Cruz, USA
Phosphorylated STAT1 (9167)	Cell signalling USA
Goat Anti-rabbit	Dako, Agilent Technologies, USA
Goat Anti-mouse	Jackson, USA
Beta Actin A5441	Sigma-Aldrich, UK
Super Signal West Dura ECL	Thermofisher, USA

General Laboratory Products

Nuclease-free PCR tube	Greiner, Germany
Needles 26G, 3/8	Braun, Ireland
Parafilm laboratory roll	Pechiney Plastic Packaging, USA

Plastic cassettes	Labonord, France
Plastic wax containers for cassettes	Labonord, France
Pipettes	Gilson, UK
Sterile Pipette tips (RNA)	Starlab, Germany
Non-sterile Pipette tips	Greiner, Germany
Standard grade No. 1 filter paper	GE Healthcare, UK
Sterile 7 ml Bijou and 30 ml tubes	Sterilin, UK
Sterile Falcon 15 ml and 50 ml tubes	Sarstedt, Ireland
Syringes (sterile 1ml)	Braun, Ireland
Tubes 200µl, 500µl, 1ml	Greiner, Germany

2.2 Animals

All mice were housed in cages of five at 21°C with a 12:12 hour light dark cycle (lights on from 0800 to 2000 with food and water ad libitum. All animal procedures were performed under licence from the Irish Department of Health & Children after a full ethical review by the TCD animal research ethics committee, in compliance with the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EEC. All efforts were made to minimise both the suffering and number of animals used.

2.2.1 Wild-types

Female and male C57BL/6 mice (Harlan, Bicester, UK) approximately 5-8 months in age were housed in groups of five, with food and water ad libitum. Prion surgery was performed in female mice that were approximately 8-10 weeks in age.

2.2.2 IL-1R1$^{-/-}$

5-8 month old female IL-1R1$^{-/-}$ mice, B6.129S7-Il1r1<tm1Imx>/J (Stock 003245), were obtained from the inbred colony of Prof. Kingston Mills in the Trinity College Bioresources Unit. In later experiments with male mice, both C57BL6 and IL-1RI$^{-/-}$ mice came from inbred colonies in the same Bioresources unit in TCD. The knockout mice, at the time of their initial arrival from JAX (Bar Harbor, US) had been backcrossed 7 times to a C57 background and thus C57BL/6 mice were an appropriate control strain

2.2.3 IFNAR1$^{-/-}$

IFNAR1$^{-/-}$ breeder pairs were obtained from Prof Paul J Hertzog of Monash Medical Centre, Monash University, Clayton, Victoria, Australia. Generation of mutant mice was as previously described (Hwang et al., 1995) :129Sv ES cells were transferred into the Balb/C background and offspring backcrossed onto a C57BL6/J background for >7 generations. These mice were then inbred in house to generate a homozygous colony. 5-8 month mice were used in experiments.

2.3 ME7 surgery -performed by Dr.Donal Skelly and Dr.Eadaoin Griffin

Mice were weighed and anesthetized with intraperitoneal Avertin (2,2,2-tribromoethanol; 0.1ml per 5g body weight) and positioned in a stereotaxic frame

(David Kopf Instruments, Tujunga, CA, USA). The incisor bar was set at -1 mm, to ensure an approximately level head. The head of the animal was shaved and an incision made on the scalp to expose the skull. A hole was drilled in the skull on each side of the midline for bilateral infusion of 1µl homogenate of a 10% w/v ME7-infected C57BL/6 brain homogenate, prepared in sterile PBS. Injections were made into the dorsal hippocampus (co-ordinates from bregma: anteroposterior, -2.0 mm; lateral, -1.6 mm; depth, -1.7 mm), using a microsyringe (Hamilton, Reno, NV, USA) with a 26 gauge needle. The needle was left in place for 2 minutes before being withdrawn slowly to minimize reflux. Mice were then placed in a heated recovery chamber (30°C) to recover and then returned to their home cage. Sucrose (5% w/v) and Carprofen (0.05% v/v; Rimadyl, Pfizer, Ireland) were added to drinking water for two days post-surgery for post-surgical analgesia. Control animals were injected with a 10% w/v normal brain homogenate (NBH) in PBS, derived from a naïve C57BL/6 mouse.

2.4 Systemic inflammatory intraperitoneal challenges:

2.4.1 Lipopolysaccharide

Animals were injected with 100 µg/kg of the bacterial endotoxin LPS (equine arbortus, Sigma, L5886, Poole, UK) prepared in non-pyrogenic 0.9% sterile saline (Sigma, Poole, UK). Control animals were injected with sterile saline (Sigma, Poole, UK) 200µl per 20 g body weight.

2.4.2 Poly I:C:

Poly I:C obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK) was prepared for injection by re-suspension in sterile saline, and was heated to 50 °C at a concentration of 2 mg/ml and was allowed to cool to room temperature to ensure proper annealing of double-stranded RNA. Poly I:C was stored at -20 °C until use. Experimental groups were challenged intraperitoneally (i.p.) with either poly I:C (12 mg/kg) or sterile saline, based on prior observations with this dose (Cunningham et al., 2007). Blood and hypothalamic and hippocampal tissues were collected, from a

separate group of animals, 1 and three hours post-poly I:C challenge for analysis of inflammatory markers.

2.4.3 IL-1 Receptor Antagonist

IL-1RA (10 mg/kg, i.p.; Kineret, Biovitrum, Sweden) was given immediately following LPS injection at a concentration of 10 mg/kg, 200µl per 20 g body weight. Control animals were injected with sterile saline (Braun).

2.4.4 IL-1β

Animals were injected with recombinant mouse IL-1β (R&D Systems, Minneapolis, MN, USA) at a dose of 25µg/kg prepared in 0.9% sterile saline. Control animals were injected with sterile saline (Sigma, Poole, UK) 200µl per 20 g body weight.

2.4.5 IL-6

Experimental groups were injected with recombinant mouse IL-6 i.p (Peprotech, Rocky Hill, NJ, USA) at a dose of 50 µg/kg prepared in 0.9 % sterile saline, judged to be a mild dose based on prior observations (Skelly et al., 2013). Control animals were injected with sterile saline (Sigma, Poole, UK) 10 ml/kg body weight.

2.4.6 IFN -β

Animals were injected with recombinant mouse IFN-β i.p (PBL Interferon source, NJ, USA) at a dose of 25,000 units or with vehicle solution, phosphate buffered saline containing 0.037 % Glycerol and 0.0094 % BSA, 10 ml/kg body weight.

2.4.7 IFN -α

Animals were injected with recombinant mouse IFN-α i.p (PBL Interferon source, NJ, USA) at a dose of 25,000 units or with vehicle solution, phosphate buffered saline containing 0.037% Glycerol and 0.0094 % BSA, 10 ml/kg body weight.

2.4.8 Dexamethasone

Dexamethasone-21-phosphate (2 mg/kg; Sigma, Poole, UK) was prepared in 0.9% sterile saline and administered 60 minutes before LPS. The solution was sonicated in 10 second on/off bursts for 5 minutes to ensure complete dissolution prior to

injection. Control animals were injected with sterile saline (Sigma, Poole, UK) 200µl per 20 g body weight.

2.4.9 2-Deoxyglucose

2-Deoxyglucose (2-DG) (2g/kg; D3179 Sigma, Poole, UK) was prepared in phosphate buffered saline and administered three hours prior to LPS administration 200µl per 20 g body weight.

2.4.10 Glucose

Glucose (2g/kg; D3179 Sigma, Poole, UK) was prepared in 0.9% sterile saline and administered thirty minutes prior to the start of T-maze or open field behaviour 200µl per 20 g body weight.

2.4.11 Insulin

Insulin (400µg/kg) was prepared in 0.9% sterile saline and administered 40 minutes prior to the start of the T-maze

2.4.12 SC-560

SC-560 COX-1-specific inhibitor SC-560 administered at a dose of 30 mg/kg, was prepared in 24% DMSO and administered i.p. 60 min before LPS injection.

2.5 Behaviour tests

Prior to all behavioural tests, mice were removed from their housing room and left in the behaviour room for 15 minutes to ensure an optimal state of arousal.

2.5.1 T-maze

To assess hippocampal-dependent working memory the paddling T-maze task was used. As described in (Murray et al., 2012), the T-maze was constructed of black Perspex with dimensions (cm): long axis 67, short axis 38, depth 20, and arm width 7. There was a single 40 mm diameter hole at the end of each choice arm, 2 cm from the floor. Black exit tubes were inserted into these holes (these may also be blocked to prevent exit). A “guillotine” door was inserted to prevent access to 1 or other choice arm. This maze was filled with water at 20 °C to a depth of 2 cm to motivate mice to

leave the maze by “paddling” or walking “on tip-toe” to an exit tube. Animals were taken with their cage mates to a holding cage. Each mouse was placed in the start arm of the maze with 1 arm blocked such that they were forced to make a left (or right) turn, selected in a pseudorandomised sequence (equal numbers of left and right turns, no more than 2 consecutive runs to the same arm). On taking the turn the mouse could escape from the shallow water and was held in a holding cage for 25 seconds (intra trial interval) during which time the guillotine door was removed and the exit tube was switched to the alternate arm. The mouse was then replaced in the start arm and could choose either arm. The mouse must alternate from its original arm to escape. Correct trials were recorded when the mouse alternated from its original turn and exited the maze. On choosing correctly mice escape from the maze and are returned to their home cage. On choosing incorrectly, the mice were allowed to self-correct to find the correct exit arm. Mice were trained for blocks of ten trials with a 20 minute inter-trial interval. Training was continued until all mice reached performance criterion. During training, a correction strategy was used if mice developed a side preference. Side preferences which develop early on in training can be broken by applying this strategy. During the correction strategy mice upon choosing the incorrect arm, were blocked from choosing the correct arm by closing the correct arm entrance with the guillotine door, thus preventing the mice from entering and making them instead return to the start arm where the trial would begin again. The correct arm entrance was only blocked when the mice chose the incorrect arm. When the mice returned to the start arm the guillotine door blocking the entrance to the correct arm was removed, and the mice were then allowed to choose an arm. The correction strategy was repeated until complete side preferences were broken. Once the side preference was broken the correction strategy was discontinued. The correction strategy was never used within 3 days of the mice being challenged. Mice reached alternation of 80% or above. Mice were only challenged if they performed at 70% for consecutive days but never less than 80% in the last block of 5 trials prior to challenge and with no side preference.

2.5.2 Y-maze

To investigate hippocampal dependent reference memory, the “paddling” Y-maze visuospatial task as described in (Cunningham et al., 2009) was used. A clear perspex Y-maze consisting of three arms with dimensions 30 x 8 x 13 cm was mounted on a white plastic base. The distal end of each arm contained a hole, 4 cm in diameter and two arms could be blocked by insertion of a closed black plastic tube thus preventing mice from exiting. The third hole had an open black tube where mice could exit the maze and enter a black burrowing tube to be returned to their homecage. Each of the three plastic tubes had a burrowing tube over them on the outside of the maze so that from the center of the maze all arms looked identical. The maze was filled with 2 cm of water at 20 to 22°C, sufficient to motivate mice to leave the maze by paddling to an exit tube at the distal end of one arm, 2 cm above the floor. Mice exits to a tube in which they are returned to their home cage. Mice were placed in one of two possible start arms in a pseudorandomised sequence for 10 trials and the groups were counter-balanced with respect to the location of the exit and start arm. For any individual mouse the exit arm was fixed. The task was conducted for 20 trials. In a subset of mice, the task was conducted for 12 trials. An arm entry was defined as entry of the whole body, excluding the tail. A correct trial was defined as entry to the exit arm without entering other arms.

2.5.3 Elevated plus maze

The elevated plus maze task was used to test for anxiety-like behaviour in mice(Walf and Frye, 2007). The maze consists of four arms (two open without walls denoted North/South and two enclosed by high walls denoted East/West) 35 cm long and 5 cm wide. The maze was elevated 45 cm above the surface it was placed on. The mouse was placed in a start arm which was a closed arm. The groups were counterbalanced with respect to start arms. The time spent in the open and closed arms, latency to first emerge from a closed arm and the number of open and closed arm entries were recorded for five minutes. The number of entries and the time spent in the junction were also observed. When at the junction the mouse was regarded as being neither in an open arm nor in a closed arm.

2.5.4 Burrowing

Black plastic cylinders, 20 cm long, 6.8 cm diameter, sealed at one end, were filled with 300 g of normal diet food pellets, and placed in individual mouse cages. The open end was raised by 3 cm above the floor preventing non-purposeful displacement of the contents. Mice were placed individually in the cages at appropriate times post-poly I:C, IFN-β or IL-6 challenges. The food remaining in the cylinders after 2h was weighed and the amount displaced (burrowed) was calculated. Baseline tests were run 24h before inflammatory challenges. Burrowing is typically performed during or just before the dark phase and is thus generally performed between 6-12 hours post-poly I:C or cytokine challenge. The burrowing tubes were also used in the T-maze and Y-maze tasks in which the mice were returned to their home cages upon exiting the mazes.

2.5.5 Open field

To investigate locomotor and rearing activity the open field task was used. Briefly, mice were placed in a box (58cm x 33 x 19cm). The box was divided into a grid of equal sized squares measuring 10cm x 10cm. Rearing is where a mouse stood on their hind legs in the open field. The number of times the mouse reared and crossed the squares in the box (distance travelled) was recorded for three minutes.

2.5.6 Temperature

Body temperature was measured using a rectal probe (RET-3- ISO Rectal Probe Physitemp, NJ) on three occasions in the week prior to poly I:C injections to habituate mice to this procedure. Mice were then assessed at the time of injection and at intervals afterwards. These intervals were 3, 6 and 9h post-poly I:C, based on prior experiments (Cunningham et al., 2007) and were not changed significantly (4, 9, and 24h) post-IFN-β challenges.

Body Weight: Body weight was recorded at the time of injection and at the following intervals afterwards; 12, 28 and 48h post-poly I:C and IL-6 challenges. For the IFN-β challenges body weight was measured at 7 and 24h post -challenge.

2.5.7 Morris water maze

The water maze consisted of a tank 120 cm in diameter, 60 cm in depth filled with water to a depth of 24 cm. The water was made opaque with the use of a white tempura paint powder (Crafty Devils, UK). A camera was fixed to the ceiling above the water maze and connected to the computer-based tracking programme Ethovision 3.1 (Noldus, Nottingham, UK). Mice were trained using extra maze visual cues to find the location of a hidden platform (15cm in diameter and 23 cm in height) submerged 1cm below the water surface and 13 cm from the edge of the pool wall. The pool was divided into four quadrants namely, north, south, east and west; using virtual bisectors using Ethovision. The platform was placed in one of these four different quadrants which were 13 cm from the edge of the pool. The groups were counterbalanced so that there were mice going to each of the four quadrants. The training protocol used was as described by (Goshen et al., 2007) consisting of three trials per day per mouse, for 3 days, with a 1 hour break between trials, followed by a probe trial the following day. Training was then continued for a further 6 days, followed by a further probe trial. Briefly, the mouse was placed in a holding cage and then gently lowered by the tail into the maze facing the pool wall at different start points. The order of these start points was randomised daily by means of a random sequence generator programme (random.org). The mouse was allowed one minute to find the platform. If, after one minute, the mouse did not locate the hidden platform, the experimenter would direct the mouse to the hidden platform where they would be left for 15 seconds. The mouse was then removed and placed into a heated holding cage and then returned to home cage for an hour (inter trial interval) until the next trial. The lighting conditions sound and distal visual cues on the walls were controlled and kept constant throughout the experiment. Latency to the hidden platform and distance travelled by the mouse were the parameters recorded. A probe trial was carried out every three days of training to assess memory for the platform location. This consisted of a single one-minute trial in which the hidden platform was removed from the water maze and the % time spent by the mouse in the quadrant where the hidden platform was previously located was recorded. On the last day of training, in order to assess visual impairment in the mice, a single flag trial was carried out. A flag

was placed onto the hidden platform, in its original position, and the time and distance taken by the mouse to swim to the platform was recorded.

2.5.8 Spatial discrimination beacon water maze

The spatial discrimination beacon water maze is an adapation of the water maze task where spatial memory and spatial-discrimination are assessed using two visually identical beacons (Bannerman et al., 2012). New mice, naïve to behavioural testing, were trained for 8 trials per day, for 3 days, to swim to a black plastic beacon (diameter 15cm; 24 cm high) sitting on the water surface which had, underneath, a hidden platform for escape from the water (24 trials across 3 days). In this training phase the black plastic beacon+platform (+ve beacon) was moved to different locations in the maze for each trial to ensure that the beacon, rather than other visuospatial cues, was used to locate the hidden platform. A second identical visible beacon, with no hidden platform underneath (-ve beacon), was then introduced to the maze and mice were trained to discriminate between the two identical beacons depending on their allocentric spatial locations. On top of each beacon was a circular (20cm diameter) piece of laminated white card. Both beacons and the escape platform now remained in the same spatial locations on every trial. The incorrect/decoy beacon was always located in the diametrically opposite quadrant to the correct beacon/platform location. The mice received 8 trials per day for 12 days. A block consisted of 3 days training, i.e 24 trials. First choice accuracy to choose the correct beacon was assessed and the percentage choice of the correct beacon when the start position was furthest from the correct beacon, closest to correct beacon and of equal distance from both beacons were plotted. Total errors were also recorded and analysed (i.e. number of times the mouse swam under the incorrect beacon, as defined by disappearance of the mouse video trace beneath the white circle of the incorrect beacon). A standard probe test was also conducted to assess learning about the spatial location of the platform after 72 trials. Both beacons and the platform were removed from the pool and the mouse allowed to swim freely for 60 sec and % time in the target quadrant plotted.

2.5.9 Contextual and Auditory Fear Conditioning

Contextual fear conditioning (CFC) was recorded using a box (40cm x 10cm x 16cm) with a transparent wall that had a floor containing metal rods which were wired to a shock generator (UGO Basile, Italy). The protocol was as described by Avital (Avital et al., 2003). The mice were placed into the box and allowed to explore for 2 minutes. A tone at 2.9 kHz for 20 seconds was presented, followed by a shock of 0.4 mA for two seconds. After 2 minutes the tone was repeated for 20 seconds and a second shock of 0.4 mA for two seconds was administered. After a further 30 seconds of exploration mice were removed to a holding cage. After 30 seconds in the holding cage, saline and LPS challenges (±saline or IL-1RA) were administered before returning to the home cage. Vehicles and treatments were administered in quick succession at discrete intraperitoneal injection sites. Fear conditioning was assessed in the same location 48h later for duration of 5 minutes. Freezing was regarded as the complete absence of movement, except those related to respiration as originally described (Fanselow, 2000). Auditory fear conditioning was also assessed 48h later, 3h post fear conditioning for 6 minutes by placing animals into an empty cage in a different context. Mice were allowed to explore for 3 minutes then the tone was presented for 20 seconds and the time spent freezing for final 3 minutes was recorded. Mice were then placed back into home cage after testing was completed.

2.5.10 Diurnal rhythm

Diurnal rhythm was recorded with the use of Phenotyper home cage (45cmx45cm) Noldus, Nottingham, U.K) and Ethovision 3.1 (Noldus, Nottingham, U.K). IL-1R1$^{-/-}$ mice and WT mice were left in the Phenotyper home cage for a period of 60 hours (3 nights and 2 days, starting at 8 pm) with free access to food and water. The recordings taken over the 60 hours were then binned into 4 hour periods and presented as 15 consecutive periods, showing night and day activities. Three Phenotyper home cages were used per strain, with five mice in each cage. The total activity for each strain was averaged across these three cages for night and day. Since the software did not allow viewing of separate traces for individual animals, statistically the groups are therefore presented as n=3, but this figure represents the activity of 15 animals of each strain.

2.6 RNA Extraction and quantitative PCR

Mice were terminally anaesthetised using Euthatal and transcardially perfused with heparinised saline 3h post-challenge with IL-1β (25µg/kg), poly I:C , IFN-β or saline i.p. The hypothalami and hippocampi were collected and stored at -80 °C. RNA was extracted from these samples using Qiagen RNeasy® Plus mini kits (Qiagen, Crawley, UK) and yields were determined by spectrophotometry at 260 nm using a nanodrop (Thermo Scientific, U.S) and as previously described (Murray et al., 2011). cDNA synthesis was performed according to manufacturer's instructions using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington). 400 ng of total RNA was reverse transcribed in a 20 µl reaction volume. One microliter of the RT reaction (equivalent to 20 ng of RNA) was used for PCR. A standard curve was prepared from total RNA from brains that were treated with 2.5µg LPS intra-cerebrally. This was used to allow relative quantification of transcript levels, followed by normalisation to GAPDH expression. As described in (Cunningham et al., 2005a), the standard curve was constructed using a higher concentration of RNA in the reverse transcription reaction than that in the samples for analysis to ensure that the cytokine transcription of all experimental animal groups would fall within the range of the standard curve constructed. The cDNA from the standard curve was then serially 1 in 4 diluted and run on the same plate as the samples under the standard Taqman PCR cycling conditions. A standard curve was plotted which consisted of the mean Ct value (the cycle number at which the florescence of the product of transcribed gene crosses the threshold of detection) versus the log concentration of an assigned arbitrary value since the absolute concentration of cytokine transcript is not known. Verification that this relationship is linear confirms the efficiency of the PCR reaction across the entire concentration range and the equation of this line can then be used to calculate the relative concentrations of the experimental samples. Since the top standard has been assigned an arbitrary value, it follows that the calculated concentrations of all experimental samples will also have arbitrary values, but these values will be directly related to the equation of the line. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured in each sample and all PCR data

were normalised to the expression of GAPDH. All Taqman primers and probes used in this thesis are listed in table 1.1

Table 1.1 Mouse Taqman primers and probe sequences

Target	Accession No	Oligonucleotide	Sequence	Amplicon Length
CXCL1	NM_008176	Forward Primer	5’-CACCCAAACCGAAGTCATAGC-3’	82
		Reverse Primer	5’-AATTTTCTGAACCAAGGGAGCTT-3’	
IL-6	NM_031168	Forward Primer	5'-TCCAGAAACCGCTATGAAGTTC-3'	72
		Reverse Primer	5'-CACCAGCATCAGTCCCAAGA-3'	
		Probe	5'-CTCTGCAAGAGACTTCCATCCAGTTGCC-3'	
IL-1β	M15131	Forward Primer	5’-GCACACCCACCCTGCA-3’	69
		Reverse Primer	5’-ACCGCTTTTCCATCTTCTTCTT-3’	
		Probe	5’-TGGAGAGTCTGGATCCCAAGCAATACCC-3’	
TNF-α	M11731	Forward Primer	5’-CTCCAGGCGGTGCCTATG-3’	149
		Reverse Primer	5'-GGGCCATAGAACTGATGAGAGG-3'	
		Probe	5'-TCAGCCTCTTCTCATTCCTGCTTGTGG-3'	
TGFβ-1	AJ009862	Forward Primer	5’-CGTGGAAATCAACGGGATCA-3’	84
		Reverse Primer	5’-GGCCATGAGGAGCAGGAA-3’	
		Probe	5’-ACCTGGGCACCATCCATGACATGA-3’	
IFN-β	X14455	Forward Primer	5’-CCATCATGAACAACAGGTGGAT-3’	67
		Reverse Primer	5’-GAGAGGGCTGTGGTGGAGAA-3’	
		Probe	5’-CTCCACGCTGCGTTCCTGCTGTG-3’	
STAT 1	NM_009283.4	Forward Primer	5'-AGGGGCCATCACATTCACAT-3'	173
		Reverse Primer	5'-AGATACTTCAGGGGATTCTC-3'	
IRF-7	NM_016850	Forward Primer	5'-CGAGGAACCCTATGCAGCAT-3'	108
		Reverse Primer	5'-TACATGATGGTCACATCCAGGAA-3'	
CCL2	NM_011333	Forward Primer	5'-GTTGGCTCAGCCAGATGCA-3'	81
		Reverse Primer	5'-AGCCTACTCATTGGGATCATCTTG-3'	
IDO	NM_008324.1	Forward Primer	5'-CGGGCAGCTTCGAGAAGA-3'	75
		Reverse Primer	5'-TGAGAGGACACAGGTTACAGCG-3'	
COX-2	NM_011198.4	Forward Primer	5’-TGGGTGTGAA GGGAAATAA GGA-3’	81
		Reverse Primer	5’-GAAGTGCTGG GCAAAGAATG-3’	
IL-10	M37897.1	Forward Primer	5'-GGTTGCCAAGCCTTATCGGA-3'	191
		Reverse Primer	5'-ACCTGCTCCACTGCCTTGCT-3'	
		Probe	5'-TGAGGCGCTGTCATCGATTTCTCCC-3'	
OAS1a	NM_145211)	Forward Primer	5'-CTTTGATGTCCTGGGTCATGT-3'	123
		Reverse Primer	5'-GCTCCGTGAAGCAGGTAGAG-3'	

Target	Accession No	Oligonucleotide	Sequence	Amplicon Length
CXCL10	M33266.1|	Forward Primer	GCCGTCATTTTCTGCCTCAT	127
		Reverse Primer	5'-GCTTCCCTATGGCCCTCATT-3'	
		Probe	5'-TCTCGCAAGGACGGTCCGCTG-3'	
TyroBP	NM_011662.2	Forward Primer	5'-CGTACAGGCCCAGAGTGAC-3'	91
		Reverse Primer	5'-CACCAAGTCACCCAGAACAA-3'	
Clec7a	NM_020008.3	Forward Primer	5'-CCCAACTCGTTTCAAGTCAG-3'	82
		Reverse Primer	5'-AGACCTCTGATCCATGAATCC-3'	
Cd68	NM_009853	Forward Primer	5'-CAAGGTCCAGGGAGGTTGTG-3'	75
		Reverse Primer	5'-CCAAAGGTAAGCTGTCCATAAGGA-3'	
		Probe	5'-CGGTACCCATCCCCACCTGTCTCTCTC-3'	
C1qa	NM_007572.2	Forward Primer	5'-GCCGAGCACCCAACGGGAAGG-3'	268
		Reverse Primer	5'-GGCCGGGGCTGGTCCCTGATA-3'	
C3	NM_009778.2	Forward Primer	5'-AAAGCCCAACACCAGCTACA-3'	115
		Reverse Primer	5;-GAATGCCCCAAGTTCTTCGC-3'	
Itgam	NM_001082960.1	Forward Primer	5'-TCATTCGCTACGTAATTGGG-3'	71
		Reverse Primer	5'-GATGGTGTCGAGCTCTCTGC-3'	
ctss	NM021281	Forward Primer	5'-GCCACTAAAGGGCCTGTCTCT-3'	80
		Reverse Primer	5'-TCGTCATAGACACCGCTTTTGT-3'	
ctsd	NM_009983.2	Forward Primer	5'-GGCGTCTTGCTGCTCATTCT-3'	90
		Reverse Primer	5'-CCGACGGATAGATGTGAACTTG-3'	
cybb	NM_007807	Forward Primer	5'-CAGGAACCTCACTTTCCATAAGATG-3'	113
		Reverse Primer	5'-TCCCGACTCTGGCATTCAC-3'	
		Probe	5'-CACACCGCCATCCACACAATTGC-3'	

2.7 Glucose measurements

Blood was taken from the mice injected with 100µg/kg LPS and placed into heparinised tubes and left for 1 hour at room temperature. Blood was then spun at 1.5g for 15 minutes at 4°C to remove cells and the remaining plasma was aliquoted and stored at -20 °C until use. Glucose was then read from the plasma on the YSI 2300

STAT Plus Glucose and Lactate Analyzer. For the 250μg/kg LPS study blood glucose was read directly from the atrium of the heart on the ACCU Chek Aviva glucometer.

2.8 ELISA

IL-1R1-/- and WT animals were terminally anaesthetised with Sodium pentobarbital (Merial Animal Health, UK) 3h post-IL-1β (25μg/kg), or saline challenges. IFNAR1-/- and WT animals were terminally anaesthetised 3h post-poly I:C (12 mg/kg i.p.), IFN-β (25,000 units, i.p.) or saline challenges. The thoracic cavity was opened and blood was collected into heparinised tubes directly from the right atrium of the heart. Blood was centrifuged at 1.5 x g for 15 minutes to remove cells and the remaining plasma was aliquoted and stored at -20 °C until use. Plasma levels of CXCL1 and IL-6 levels were analysed 3 hours post injection of IL-1β (25μg/kg i.p) or saline using R&D systems duo-set enzyme-linked immunosorbent assays (R&D systems UK) according to manufacturer's instructions. Samples from IFNAR1-/- and WT animals were analysed for plasma IL-6 (Biolegend) and TNF-α (R&D Systems U.K) and IFN-β (PBL Biomedical Laboratories, USA) using commercially available enzyme-linked immunosorbent assay kits, as per manufacturer guidelines. All ELISAs were performed according to the manufacturer guidelines.

2.9 Western blotting

Hippocampal punches, weighing 20-30 mg were homogenized in 100 μl of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton x10) with protease and phosphatase inhibitors. The homogenate was then centrifuged at 4 °C for 10 min at 10,000 rpm. Total protein content was quantified using a DC protein Assay (Biorad) and samples were boiled in loading buffer containing β-mercaptoethanol. 50 μg of each lysate was loaded onto a 10 % SDS-PAGE gel and the proteins were transferred to a PVDF membrane following separation by electrophoresis. Membranes were blocked in 5 % non-fat milk in TBS and 0.1 % Tween-20 for 1h at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies STAT1 (SC346, Santa Cruz), phosphorylated STAT1 (9167 cell signalling) at 1/1000 dilution and Beta Actin (A5441 Sigma) at 1/5000 dilution. Membranes were then washed in TBS-T and incubated with secondary antibodies: goat anti-rabbit (P0448 Dako, 1/1000 dilution)

for both STAT1 and Phosphorylated STAT1 and goat anti-mouse (Jackson, 1/10,000 dilution) for Beta Actin. Membranes were then developed using Supersignal West Dura Extended Duration ECL (Pierce) and quantified using Image J software (imagej.nih.gov/ij/).

2.10 Immunohistochemistry and quantification

Animals were terminally anaesthetised with sodium pentobarbital (~200µl i.p, Euthatal, Merial Animal Health, Essex, UK) and transcardially perfused with heparinised saline for approximately 3 minutes followed by 10 % formalin (Sigma, Poole, UK) for approximately 15 minutes. Brains were then removed post-fixed in 7ml sterilin flasks over 2 days in formalin and then placed in PBS until embedding.Formalin-fixed, paraffin-embedded sections were cut from the brains of WT (n=4) and IL-1R1$^{-/-}$ mice (n=6). Sections were dewaxed in Xylene and Histoclear then rehydrated through a series of decreasing alcohols ranging from 100-70% concentration and treated with 0.2 M boric acid, pH 9, 65 °C for 30 min and cooled to room temperature. Non-specific peroxidase activity was eliminated by incubating sections in 1% v/v H_2O_2 in ethanol (1ml H_2O_2/100ml) for 10 minutes. Sections were washed in PBS and blocked using 10% normal horse serum. Sections were incubated with SY38 Chemicon (CA, USA; 1:2000), at room temperature overnight. Sections were then washed in PBS before incubation with biotinylated horse anti-mouse secondary IgG (Vector U.K; 1:200). Sections were then washed in PBS and ABC was applied for 30 minutes followed by diaminobenzidine (DAB) reaction in the presence of ammonium nickel chloride (0.06% w/v) to intensify staining. The density of synaptophysin staining in IL-1R1$^{-/-}$ mice and WT mice was determined by mono-chrome pixel density analysis of digitally captured images using Cell A imaging software (Olympus). Areas of uniform staining within each of the strata of the hippocampal formation (avoiding unstained areas such as blood vessels) were quantified using pixel density analysis. One group of quantifications (comprising all strata) was performed on each section. Transmittance measurements were made at a light intensity within the linear transmittance range (0-255 Lux). The area of highest transmittance (the corpus callosum) was chosen as an internal standard and all other transmittances were subtracted from this layer. The adjusted values for each stratum were then used in ratio calculations to derive a ratio measure of synaptic density as

determined by the equation: Ratio=(T corpus callosum–T radiatum)/Tcorpus callosum–T lacunosum moleculare).

Ventricular volume was estimated Using Image J software (NIH, Bethesda, US), and 10 µm sections from WT and IL-R1-/- brains, the ventricles on each section were outlined and an area measurement in pixels obtained. Using a stage micrometer of 1mm in length the measurement in pixels was converted to mm2 (Image J area (pixels)/27.8=area (mm2)). Areas were calculated for sections from each animal at 2.0 and 2.3 mm posterior to Bregma and summed and multiplied by the summed section thickness (2x10µm: 0.02 mm).

2.11 Statistical Analysis.

All statistical analyses were performed using GraphPad Prism 5 for Windows with the exception of three-way ANOVA analyses, which were performed using GB-Stat Version 10.0. In all cases data are expressed as mean ± SEM. Different analyses used in each chapter are detailed below.

2.11.1 Chapter 3

Contextual fear conditioning, elevated plus maze, open field behavioural data and the flag and probe trials data from the Morris water maze were analysed by t-tests. Synaptic density in the hippocampus of the WT and IL-1R1$^{-/-}$ animals were also analysed using t-tests. Mann-Whitney tests were used to compare non-parametric ventricular volumes in these animals. All the Taqman qPCR and ELISA data were analysed using one-way ANOVA. For cognitive behaviour (Y-maze, T-maze Morris water maze, Beacon task and diurnal rhythm) repeated measures two-way ANOVA with strain as between subjects factor and trial block as within subjects factor were used for repeated measures analyses of performance across multiple test sessions.

2.11.2 Chapter 4

Bonferroni *post-hoc* tests were performed following a significant ANOVA. For cognitive testing and challenges with LPS, saline, IL-RA, or Dexamethasone two-way repeated measures ANOVA was used to compare treatments over a number of time-points.

Treatment was the between-subjects factor and time was the within-subjects factor. Bonferroni *post-hoc* tests were performed following a significant ANOVA. 1 way ANOVA was used to test for the effects of Dexamethasone, IL-1RA data and SC560 data on glucose levels.

2.11.3 Chapter 5

Elevated plus maze and open field behavioural data was analysed by t-tests. For cognitive behaviour (Y-maze and diurnal rhythm) repeated measures two-way ANOVA with strain as between subjects factor and trial block as within subjects factor were used for repeated measures analyses of performance across multiple test sessions. Bonferroni *post-hoc* tests were performed following a significant ANOVA.

Poly I:C challenges: An a priori prediction that poly I:C would produce robust suppression of rearing activity at 3h and hypothermia at 8h. Therefore a two-way ANOVA in WT and IFNAR1$^{-/-}$ animals with strain and treatment as factors for these analyses was performed. Body weight was analysed by three-way ANOVA with strain and treatment as between subjects factors and time as within subjects factor.

IFN- β challenges Open field and burrowing data were analysed by t-test

All ELISA, Taqman qPCR data and Western blots were analysed by two-way ANOVA or t-test.

Chapter 3

The role of endogenous IL-1 in learning and memory in WT mice

The data in this chapter have been published in Murray et al., 2013, *PLOS One*

3.1 Introduction

Interleukin-1 (IL-1) is a pleiotropic pro inflammatory cytokine that is produced by immune cells in the periphery as well as glia and some neuronal populations within the brain. There are 11 members of the IL-1 family but IL-1 α and IL-1β are the most widely studied. IL-1 signalling is triggered when IL-1 α and IL-1β bind to the interleukin type one receptor (IL-1R1). The signalling of IL-1 can be blocked using Interleukin 1 receptor antagonist (IL-1RA) that blocks the binding of IL-1 to the IL-1R receptor(Dinarello, 1996). Multiple studies have shown that IL-1 contributes to inflammation-induced sickness behaviour by inducing fever, decreased exploration, food and water reduction and depressed locomotor activity (Dunn and Swiergiel, 2001, Bluthe et al., 1992, Larson et al., 2002). In addition to playing a role in sickness behaviour, IL-1 is reportedly involved in learning, cognition and memory. Detrimental effects of IL-1β on learning and memory have been shown. Intracerebroventricular (i.c.v) or peripheral injection (i.p) of IL-1 can impair spatial memory in the water maze (Oitzl et al., 1993, Gibertini, 1996) and in the contextual fear conditioning (CFC) paradigm (Pugh et al., 1999, Barrientos et al., 2002). It has also been shown that IL-1RA can block the LPS or IL-1-induced impairment in the CFC (Pugh et al., 2001). Moore and colleagues showed that in transgenic mouse model over expressing IL-1β, the spatial memory of the mice was impaired in the Morris water maze but had no effect on non-spatial learning (Moore et al., 2009). The same group, also reported in the same mice, that contextual memory was impaired (Hein et al., 2010).

However, it is also suggested that constitutive IL-1 signalling is necessary in cognition as i.c.v infusion of IL-1RA impaired hippocampal-dependent performance in the water maze in rats (Yirmiya et al., 2002) and similarly impaired performance could also be seen in transgenic mice lacking expression of IL-ra (Avital et al., 2003). In another study, IL-1RA i.c.v impaired memory in the CFC (Goshen et al., 2007). Using knockout mice, the same group showed that IL-IrKO mice were impaired on both Morris water maze and contextual fear conditioning tasks with respect to wild type control mice (Avital et al., 2003) and impaired performance in rats in the passive avoidance test could be seen when injected with an adenovirus that overexpresses IL-1RA (Depino et

al., 2004). More recently, deletion of the P2X(7) receptor the receptor responsible for ATP-dependent IL-1 release (Ferrari et al., 2006), impaired memory in Y-maze spatial memory task (Labrousse et al., 2009) also perhaps suggesting a role for endogenous IL-1 in memory function. In addition, elevated levels of IL-1 are found in Alzheimer's disease patients particularly in the temporal lobe, frontal cortex, hippocampus and cerebrospinal fluid (Cacabelos et al., 1994, Cacabelos et al., 1991). It has also be shown that IL-1β is present in the regions surrounding amyloid plaques in Alzheimer's (Griffin et al., 1995). Low concentrations of IL-1 can support neuronal viability whereas higher concentrations can be neurotoxic and reduce neuronal survival (Araujo, 1992, Lawrence et al., 1998). It has also been reported that when IL-1 is administered systemically during ischemia, it increases ischemic damage similar to that seen with LPS and exacerbates brain edema in mice (McColl et al., 2007). Lawrence and colleagues also demonstrated that IL-1 can exacerbate cortical neuronal damage in the striatum in rats (Lawrence et al., 1998).

It is clear from the literature that LPS can impair memory in particular, perhaps most robustly shown in the CFC and IL-1 appears to be key in these effects. The data that endogenous IL-1 facilitates learning is significantly less clear. Moreover it is important to understand whether IL-1 contributes to dysfunction in other aspects of hippocampal function.

The aim of the experiments in this chapter was to assess the role of basal endogenous levels of IL-1 on behaviour. This will be assessed by comparing the behaviour phenotype of IL-1R1$^{-/-}$ mice to wildtype (WT) mice using a wide variety of spatial navigational hippocampal-dependent working memory (T-maze), reference memory (Y-maze, Morris Water Maze (MWM) and anxiety measures - Open field and Elevated Plus Maze (EPM). Diurnal rhythm will also be assessed in these mice. In addition to these behaviours, contextual and auditory fear conditioning will be investigated. Together, these behaviour measurements should give a fuller understanding of how endogenous levels of IL-1 influence cognitive and behavioural function.

3.2 Results

3.2.1 The role of IL-1 in WT and IL-1R1$^{-/-}$ deficient mice

The behavioural phenotype of female IL-1R1$^{-/-}$ mice and female wildtype (WT) mice was characterised using a variety of hippocampal-dependent learning and memory tasks, as well as tests of anxiety and general locomotor activity. Mice that were tested in the open field and elevated plus maze were tested in the Morris water maze and y maze. Mice that were tested in the CFC were tested in the beacon discrimination task and the diurnal rhythm monitoring and T-maze was performed in separate cohorts.

3.2.2. Y maze and T-maze

The performance of IL-1R1$^{-/-}$ mice (n=11) and WT (n=20) mice was assessed in a visuo-spatial reference memory Y-maze task (figure 3.1a) across four blocks of 5 trials. Mice had to learn and remember the location of the exit arm in a shallow water Y-maze. Two-way repeated measures ANOVA with strain as a between subjects factor and trial block as a within subjects factor indicated that there was no impairment on learning of the task in the IL-1R1$^{-/-}$ mice compared to their WT controls. Two-way ANOVA revealed no effect of strain ($F=0.10$, df 1,29, $p=0.75$), nor an interaction ($F=0.17$, df 3,87, $p=0.91$), but there was a significant effect of trial block ($F=18.33$, df 3,87, $p< 0.0001$) indicating that performance of both strains improved as training continued.

In the T-maze working memory task, a new cohort of animals (n=15 WT and 11 IL-1RI$^{-/-}$) were trained, for 12 days, to alternate their arm choices to escape from the shallow water maze. The performance of each mouse was assessed for 10 trials per day, for 12 days and these data were then divided into 3 blocks of 4 days (training block 1 (days 1-4) and block 2 (days 4-8) block 3 (days 9-12)). There was no impairment on this task in the IL-1R1$^{-/-}$ mice compared to controls (figure 3.1b). Two-way ANOVA with strain and trial block as factors revealed no significant effect of strain ($F=2.26$, df 1,24, $p=0.13$) but a significant effect of trial block ($F=13.24$, df 2,72, $p<0.0001$) and no significant interaction of these 2 factors ($F=1.71$, df 2,72, $p=0.28$).

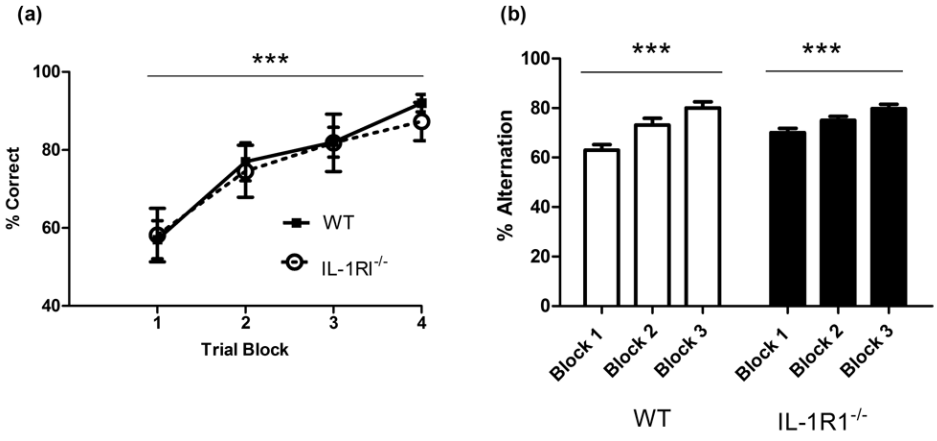


Figure 3.1 Performance of IL-1R1$^{-/-}$ mice versus WT on a visuo-spatial reference memory task and a working memory task. (a) Visuo-spatial reference memory was assessed using the Y-maze across 20 trials (5 trials per trial block) in IL-1R1$^{-/-}$ (n=11) compared to WT mice (n=20). (b) Working memory was assessed in IL-1R1$^{-/-}$ (n=11) and WT (n=15) by T-maze alternation, over 12 days, 10 trials per day with block 1 representing performance of mice on days 1-4, block 2 the performance of mice on days 5-8 and block 3 the performance of mice on days 9-12. Data are shown as mean±SEM and were analysed by two-way repeated-measures ANOVA with strain as between subjects factor and trial block as within subjects factor. Main effects of trial block for both mazes are depicted by ***p<0.001.

3.2.3 Contextual Fear Conditioning

In the contextual fear conditioning paradigm the time spent freezing 48 hours following exploration and foot shock at 0.4 mA for IL-1R1$^{-/-}$ mice (n=10) and WT mice (n=10 was recorded, for a period of 5 minutes. The data (figure 3.2) reveal that the IL-1R1$^{-/-}$ mice show equivalent contextual fear memory compared to relevant controls i.e they spend similar time freezing compared to the WT, indicating a similar recall of the memory of the context in which they received the foot shock. Data were analysed by two-way ANOVA with strain and session (pre-shock exploration, post-shock) as factors. There was no effect of strain ($F=1.07$, df 1,18 $p=0.32$) and no interaction of session and strain ($F=1.19$, df 1,18 $p=0.29$) but there was a significant effect of session ($F=220.03$, df 1,18 $p<0.0001$) indicating that all animals learned i.e they had a memory for the context in which shock was received. Bonferroni *post-hoc* tests showed that both strains significantly increased freezing between pre-shock and post-shock sessions ($p<0.001$).

3.2.4 Open field activity and a decrease in anxiety like behaviour in IL-1R1$^{-/-}$ mice

Distance travelled and number of rears in the open field for a period of 3 minutes was recorded. All data in figure 3.3 are expressed as mean±SEM and significant differences were analysed by unpaired t-tests. The results shown in figure 3.3a reveal that IL-1R1$^{-/-}$ mice were more active in the open field compared to controls, showing a significantly greater total distance travelled ($p=0.0002$). Their rearing activity in the open field was also significantly higher ($p<0.0001$, student's t-test) indicating that exploratory activity is also increased in IL-1RI$^{-/-}$ mice (figure 3.3b). In figure 3.3c, in which the time spent in open and closed arms of the elevated plus maze was investigated, the IL-1R1$^{-/-}$ mice spent more time in the open arms of the maze than the WT mice. Student's -test confirmed that IL-1R1$^{-/-}$ mice spend significantly more time in the open arms ($p<0.05$, and significantly less time in the closed arms (data not shown, $p<0.05$). These data suggest a less anxious phenotype in the IL-1R1$^{-/-}$ mice. However, latency to emerge from the initial closed arm was not significantly different between IL-1R1$^{-/-}$ and WT controls ($p=0.47$; figure 3.3d).

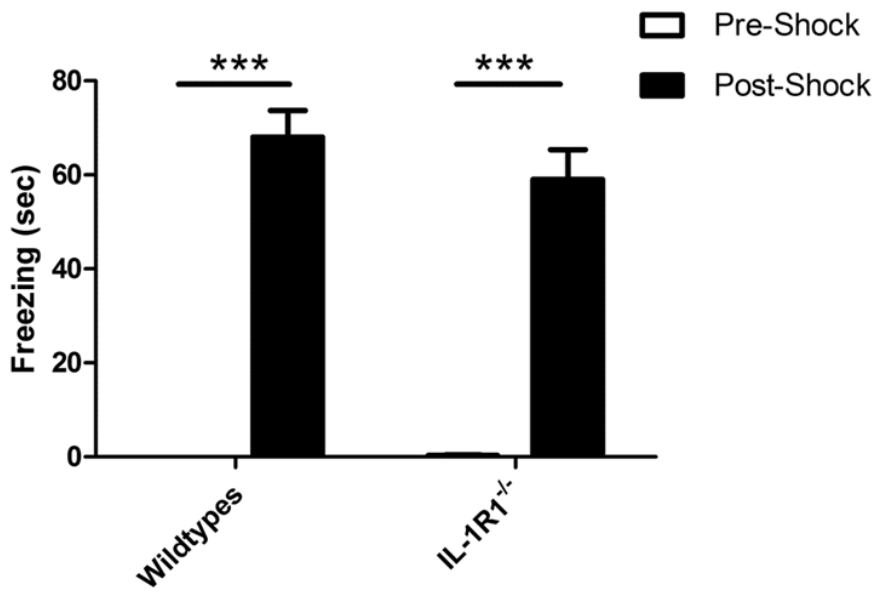


Figure 3.2 Performance of IL-1R1$^{-/-}$ mice versus WT in the contextual fear conditioning paradigm. The time spent freezing measured across 2 minutes (pre-shock) and 5 minutes, 48 hours later (post shock), following foot shock at 0.4 mA for 2 seconds for IL-1R1-/- mice (n=10) and WT mice (n=10) in the fear conditioning paradigm. Data are expressed as mean±SEM and were analysed by t-test, bars representing pre-shock freezing are insufficiently large to be clearly visible. Bonferroni post-hoc tests after a significant effect of treatment in ANOVA analysis showed that both strains showed significant differences from their pre-shock freezing (***$p<0.001$).

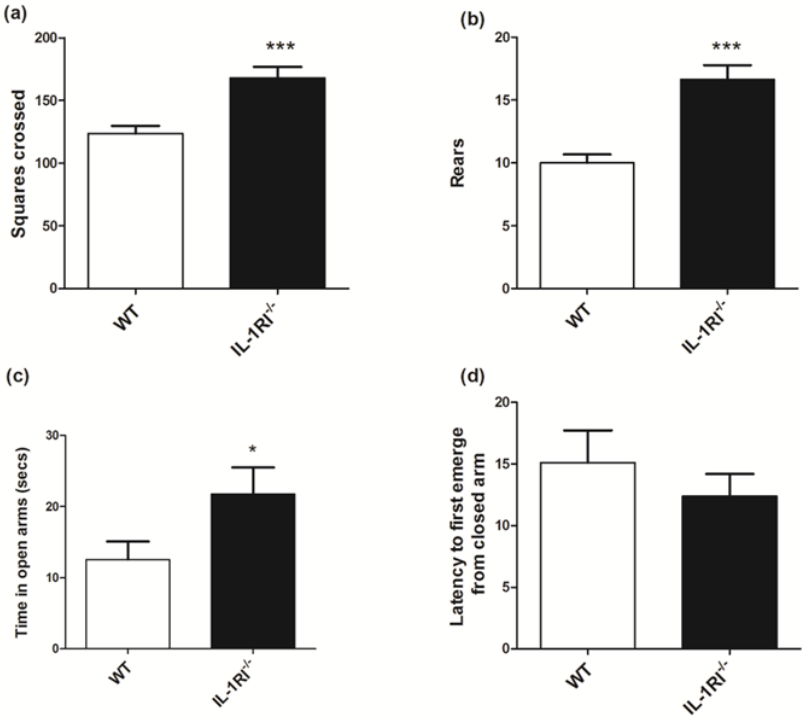


Figure 3.3 Open field and elevated plus maze activity in IL-1R1$^{-/-}$ and WT mice. (a) Distance travelled in open field in IL-1R1$^{-/-}$ mice (n=11) compared to WT (n=19) and (b) the number of rears recorded in open field, across 3 minutes. (c) The time spent in open arms of elevated plus maze and (d) the latency to first emerge from closed arm. Data are expressed as mean ± SEM and significant differences by t -test are denoted by ***p<0.001 and *p < 0.05

3.2.5 Learning and memory in the water maze

The ability of IL-1R1$^{-/-}$ (n=11) and WT mice (n=20) to locate the hidden platform in the Morris water maze spatial memory task during 3 days of 3 trials with an inter-trial interval of 1 hour (total of 9 trials) was assessed. The protocol used to test this was that of Avital and colleagues, in order that their original findings with the IL-1rKO mouse might be replicated (Avital et al., 2003). The latency (figure 3.4a) and path length (figure 3.4b) to reach the platform were recorded. Data are expressed as mean±SEM and were analysed using two-way repeated-measures ANOVA with strain as between subjects factor and trial number as within subjects factor. IL-1R1$^{-/-}$ mice displayed a similar rate of learning in the water maze compared to controls. The IL-1R1$^{-/-}$ mice on the first time entering the maze had a lower latency on the 1st trial but showed slightly, but statistically significantly, shorter latencies than the WT to get to the platform indicating that the IL-1R1$^{-/-}$ mice learned the task more rapidly then WT. Two-way ANOVA of the latency showed a significant effect of strain ($F=5.93$, df 1,29, $p=0.0213$) and a significant effect of trial number ($F=3.53$, df 8,232, $p=0.0007$) but no interaction ($F=0.68$, df 8,232, $p=0.71$) and there were no significant differences at any individual time point by Bonferroni *post-hoc* tests. There was also a slight, but non-significant, difference between the strains on distance swam (no effect of strain ($F=3.34$, df 1,29, $p=0.08$; no interaction between strain and trial ($F=0.74$, df 8,232, $p=0.66$). Both strains learned the task well (significant main effect of trial number ($F=5.15$, df 8,232, $p<0.0001$). Furthermore, no obvious differences could be observed in the types of routes taken by the 2 strains as assessed by mouse tracking in Ethovision. There was no difference in velocity in WT or IL-1R1-/- mice. Two-way ANOVA showed no significant effect of strain ($F=0.79$, df 1,232,$p=0.38$) or significant effect of time ($F=1.60$, df 8,232, $p=0.12$) or interaction of strain x time with ($F=1.25$, df 8,232, $P=0.27$). In a probe trial conducted after 3 days of training, during which the platform was removed from the pool (figure 3.4c), both strains spent an equivalent time in the target area where the platform was previously located. A further probe trial, conducted after a further 6 days training (figure 3.4c) showed that animals from both strains spend significantly more time in the target area than in any other quadrant (main effect of quadrant $F=29.66$, df 2,87, $p<0.001$). This performance was

equivalent in IL-1R1$^{-/-}$ and wild-type animals with p=0.9635 at 9 trials and p=0.6970 at 27 trials (unpaired t test of time in target quadrant). In the non-spatial flag trial in which a flag was added to the platform in its existing location, both strains reached the platform more quickly than in the hidden platform version of the maze but there was no significant difference between strains on this parameter (p=0.3593) (figure 3.4d). Collectively these data indicate that IL-1RI$^{-/-}$ perform no worse to WT mice in either the hidden and visible platform versions of the maze.

3.2.6 Learning and memory in the spatial-discrimination beacon water maze task

The next experiment used the spatial-discrimination beacon task to see whether response inhibition would be different in the IL-1R1$^{-/-}$ mice to controls. The ability of IL-1R1$^{-/-}$ mice and their controls to locate the hidden platform in a spatial discrimination beacon water maze task was assessed. New mice, naïve to any other behavioural experiment, were used for this task. Briefly mice were trained to discriminate between two identical beacons, one of which had a hidden platform beneath the surface of the water, depending on their allocentric spatial locations. Animals were started from various positions in the maze, some close to the correct beacon, some close to the incorrect beacon and some equidistant from both beacons. IL-1R1$^{-/-}$ mice performance in this task was equivalent to the wild type mice. They all learnt this task. Given (i) that previous studies have implicated IL-1 in spatial memory performance and (ii) that other aspects of watermaze performance may be more sensitive to disruption of hippocampal function (e.g. impaired synaptic plasticity: (Bannerman et al., 2012)) than the standard, open field version of the task, first choice accuracy for selecting between the beacons and the total errors made was analysed (figure 3.5a). First choice accuracy averaged across all sessions showed a small but significant difference between strains ($t(18)=1.96$; $p<0.05$, one-tailed). A similar, just significant group difference was also seen for total errors made across all test sessions ($t(18)=1.94$; $p<0.05$, one-tailed). ANOVA for first choice accuracy revealed a significant effect of trial block ($F=61.36$, df 2,36 $p<0.0001$), indicating that all animals showed learning across trial blocks (fig 3.2.5a) but there was no significant interaction between trial block and group ($F=0.94$, df 2,36, $p=0.40$) Likewise, for total errors, ANOVA demonstrated a significant effect of trial block ($F=68.38$, df 2,36 $p<0.0001$) but

no significant interaction between group and trial block (F=0.63, df 2,36, p=0.54). A three-way repeated measures ANOVA on these data was performed with strain as a between subjects factor and trial block and start position as within subjects factors. This analysis showed that there was a significant effect of trial block (F=3.96, df 2,36, p<0.0001), and of start position (F=9.35, df 2,36, p=0.005). However, there were no interactions of group and start position, or between group, trial block and start position which indicates that regardless of where the mice started there was no difference in strains or in trial block. A probe trial conducted after 72 trials (figure 3.5b) showed that mice spent more time in the target area than in any other quadrant. ANOVA revealed a main effect of quadrant (F=13.19, df 2,54 p<0.001). However, this performance was equivalent to wild-type animals with p=0.8690 (unpaired t-test). Thus, IL-1R1$^{-/-}$ and WT mice learned about the spatial location of the platform to the same extent. There was no difference in IL-1R1$^{-/-}$ mice compared to the wild-type mice in the performance of this task if they were started in positions close to the correct beacon (figure 3.5c), equidistant from correct beacon (figure 3.5d) or far from correct beacon (figure 3.5e). Taken together these results would suggest that IL-1R1$^{-/-}$ mice do not have any substantial spatial learning deficit compared to controls.

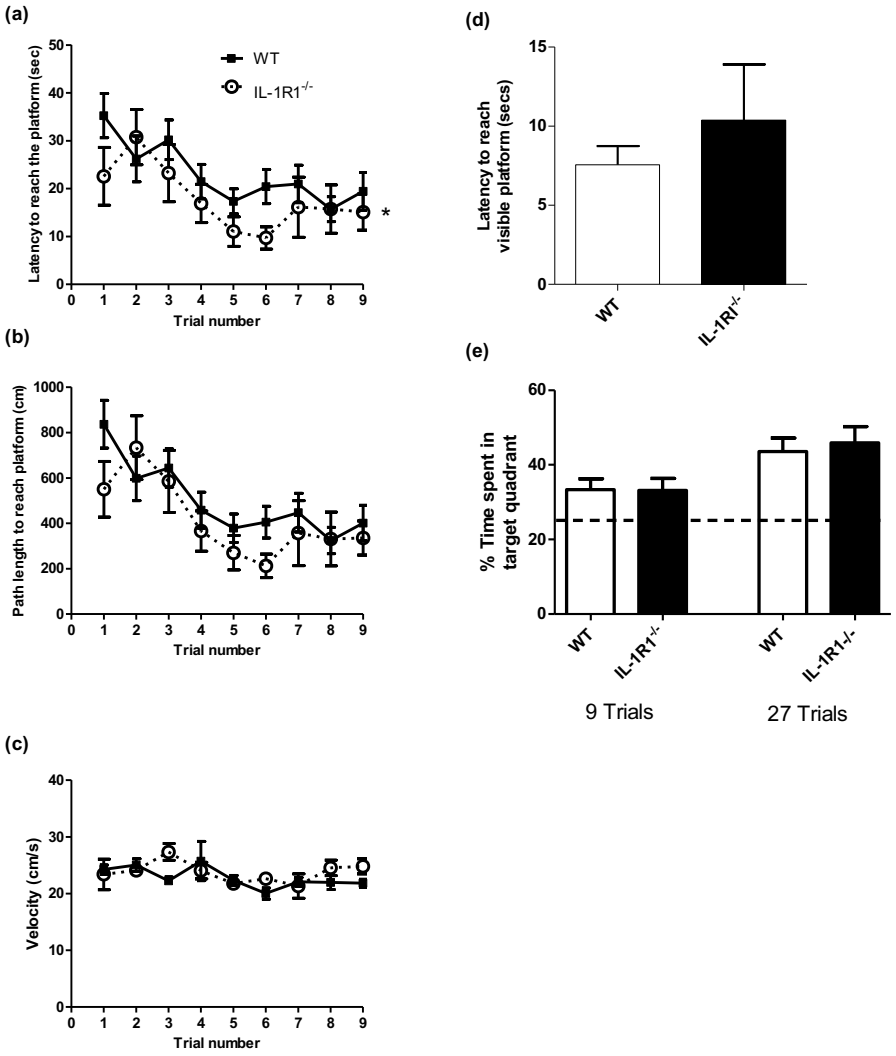


Figure 3.4. Spatial and non-spatial memory in the Morris water maze in IL-1R1$^{-/-}$ and WT mice. (a) The latency to reach the hidden platform, and (b) the path length and (c) the velocity were assessed in IL-1R1$^{-/-}$ mice (n=11) and WT mice (n=20) in the spatial memory water maze task across 9 trials with 1 hour inter trial interval. (d) Probe trials performed after 9 trials and 27 trials (dotted line shows 25%), and (e) non-spatial memory (flag trial) in IL-1R1$^{-/-}$ mice and WT. Data are expressed as mean±SEM and were analysed by two-way repeated-measures ANOVA with strain as between subjects factor and time as within subjects factor (a,b,c) or by t-test (d,e).* denotes main effect of strain(p=0.02)

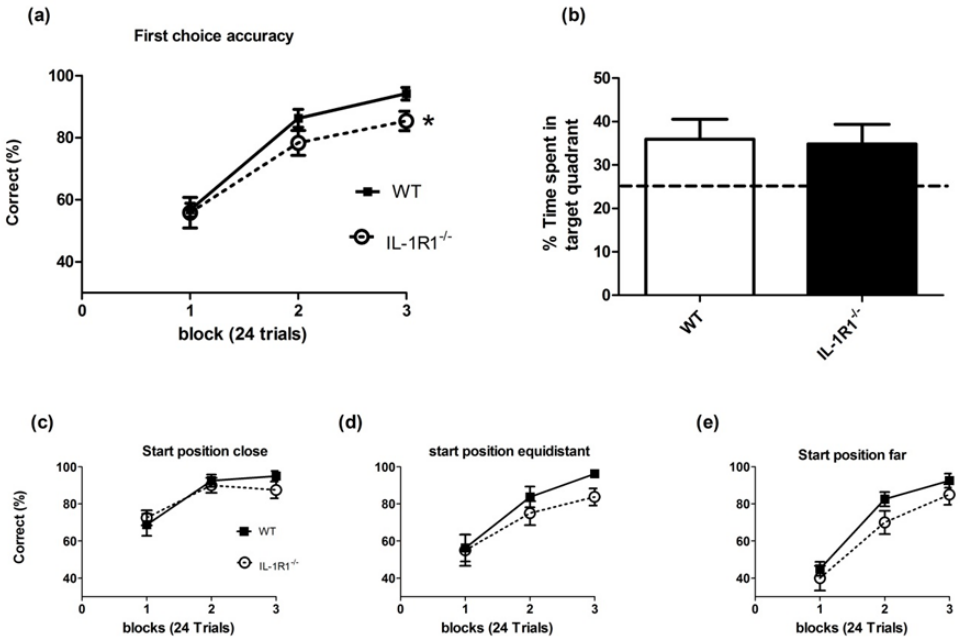


Figure 3.5 Spatial memory and choice performance on the spatial-discrimination beacon water maze task in IL-1R1$^{-/-}$ and WT mice. IL-1R1$^{-/-}$ and WT mice (n=10) were trained (8 trials per day for 12 days) to discriminate between two identical beacons (diameter 15cm; 24 cm high, sitting on the water surface) depending on their spatial locations, one of which had a hidden platform underneath. 1 block = 24 trials. a) First choice accuracy to choose the correct beacon was assessed. b) Probe test performed after 72 trials, with 25% depicted by dotted line. The percentage correct choices on choosing the correct beacon are shown when start position is c) close to correct beacon, d) equal distance from both beacons and e) furthest from correct beacon.* $p<0.05$ by one-tailed t-test comparison of first choice accuracy across all trials. Data are expressed as mean±SEM and full statistical analysis is described in the text.

3.2.7 Diurnal rhythm

In some previous published studies in which cognitive changes have been observed in IL-1RI$^{-/-}$ mice, animals were housed under a reversed light cycle such that housing rooms are dark during the daytime and cognitive experiments were therefore performed during the animals' 'subjective night' or more active period. The diurnal rhythm of IL-1R1$^{-/-}$ mice compared to WT controls was assessed. The total activity was measured for a cohort of 15 female IL-1R1$^{-/-}$ mice and 15 female WT in PhenoTyper home cage, 45cm x 45cm, (Noldus, UK) captured by Ethovision (for statistical purposes these constitute n=3 per group because each phenotyper cage monitored total activity for 5 animals each). As expected, over a period of three nights and two days plotted in 4 hour blocks, both the IL-1R1$^{-/-}$ and WT mice were clearly more active in the night phase compared to the day (figure 3.6) and there was a main effect of time ($F=57.09$, df 14,56, $p<0.0001$). Although distance travelled was not affected by strain ($F=0.27$, df 1,4, $p=0.63$) there was a significant interaction of time and strain ($F=3.55$, df 14,56, $p=0.0003$). This may reflect that IL-1RI$^{-/-}$ mice travelled shorter distances in the dark phase than did WT mice, although there were no significant differences of individual time points by Bonferroni *post-hoc* comparisons.

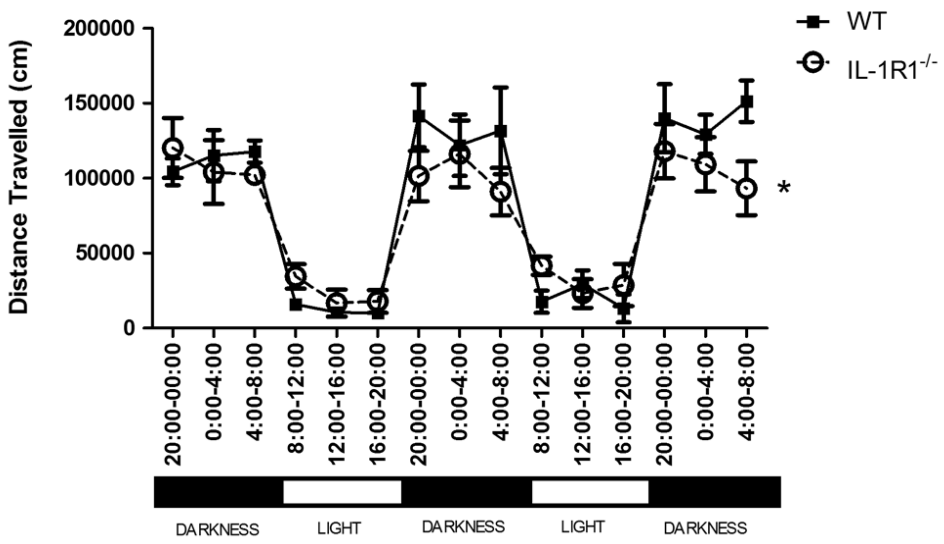


Figure 3.6 Diurnal rhythm of female WT and IL-1R1$^{-/-}$ mice. The activity of female WT (n = 15) and female IL-1R1$^{-/-}$ (n = 15) mice was recorded using the Phenotyper home cage over a period of three nights and two days. Data are plotted in 4 hour blocks and are expressed as mean±SEM and were analysed by two way repeated measures ANOVA with strain as between subjects factor and time as within subjects factor. There was no effect of strain but a significant time x strain interaction, which is denoted by *p<0.0003.

3.2.8 Systemic and hypothalamic cytokine responses in IL-1R1$^{-/-}$ mice and WT mice challenged with IL-1β

Given the failure to find any substantial spatial learning deficit in the IL-1RI$^{-/-}$ mice, acute IL-1β administration experiments were performed to confirm that the strain was indeed unresponsive to IL-1β. Systemic and CNS cytokine responses in IL-1R1$^{-/-}$ mice and WT mice challenged with IL-1β (25 μg/kg i.p) or saline i.p at 3 hours were assessed by ELISA and RT-PCR. As shown in Table 1.2, IL-6 and CXCL1 were detectable at very low levels in WT mice treated with saline or in IL-1R1$^{-/-}$ mice treated with IL-1β but these molecules were robustly expressed in WT mice treated with IL-1β. Plasma cytokine responses to IL-1β were equally limited in both male and female IL-1RI$^{-/-}$ mice. Similarly the CNS transcription of mRNA for these IL-1β-sensitive genes was assessed. IL-1β induced robust transcription of both cytokine transcripts in WT animals but did not produce any change from basal levels in either male or female IL-1RI$^{-/-}$ mice. The same patterns were true for hippocampal transcription. Thus, IL-1RI$^{-/-}$ mice, of both genders, are completely unresponsive to IL-1β stimulation. IL-1β protein (plasma) and mRNA (hypothalamus) was also analysed for these animals and found no evidence of elevated basal IL-1β in these animals.

	WT+Saline	WT + IL-1β	IL-1R1-/- + IL-1β	
			female	male
IL-6 Plasma	63 ± 8	2442 ± 240	3 ± 1	<1
CXCL1 Plasma	145 ±14	222,680 ± 13,080	196 ± 22	104 ± 31
IL-6 mRNA	<0.0015	0.113 ± 0.015	<0.0015	<0.0015
CXCL1 mRNA	<0.003	0.252 ± 0.0328	<0.003	<0.002

Table 1.2 Systemic and hypothalamic cytokine responses in IL-1R1$^{-/-}$ mice and WT mice challenged with IL-1β. Plasma was prepared from whole blood of WT and IL1R1$^{-/-}$ mice 3 hours post treatment with IL-1β (25 μg/kg i.p.) or saline and analysed by ELISA. CNS cytokine transcription was assessed by quantitative PCR on cDNA synthesised from total RNA isolated from hypothalamic tissue at the same time. All data are expressed as mean±SEM and were analysed by one way ANOVA where n=5 for WT/saline, n = 4 for WT/IL-1β and n=6 for female IL-1R1$^{-/-}$ mice and n=5 for male IL-1R1$^{-/-}$ mice challenged with IL-1β.

3.2.9 Effect of Gender

Since previous published studies on cognitive deficits in IL-1RI$^{-/-}$ mice were carried out in males, it was important to verify that the results (figure 3.1-3.3) with female mice generalised to males. The Y-maze, open field, fear conditioning and elevated plus maze were thus repeated in male IL-1R1$^{-/-}$ and male WT mice. The performance of male IL-1R1$^{-/-}$ mice (n=11) and male WT (n=9) mice on a visuo-spatial reference memory Y maze task (figure 3.7a) across four blocks of 5 trials was assessed. Two-way repeated measures ANOVA with strain as between subjects factor and trial block as within subjects factor showed that there was no impairment on learning of the task in male IL-1R1$^{-/-}$ mice compared to WT controls. There was no effect of strain ($F=0.16$, df 1,18, $p=0.69$) or any interaction ($F=0.37$, df 3,54, $p=0.77$) but a significant effect of trial block ($F=10.40$, df 3,54, $p<0.0001$) where performance of both strains improved as training continued.

The time spent freezing 48 hours following exploration and foot shock at 0.4 mA in the fear conditioning paradigm was recorded for 5 minutes. These data reveal that the male IL-1R1$^{-/-}$ mice show equivalent, unimpaired, contextual fear memory compared

to their controls i.e they spend similar time freezing compared to the WT (figure 3.7b). Data were analysed by two-way ANOVA with conditioning (pre vs. post-shock levels) and strain as factors. There was no effect of strain ($F= 0.26$, df 1,18 $p=0.61$) and no interaction between conditioning and strain ($F= 0.27$, df 1,35 $p=0.61$) but there was a significant effect of conditioning ($F= 47.99$, df 1,35 $p<0.0001$).

Distance travelled and the number of rears in the open field for a period of 3 minutes was recorded. Data are expressed as mean±SEM and significant differences were analysed by unpaired t-tests. Based upon an a priori prediction of increased activity in IL-1R1$^{-/-}$ mice, arising from the results in females, open field activity was compared (figure 3.7 c) in the male IL-1R1$^{-/-}$ versus WT mice by one-tailed t-test and demonstrated a significantly increased activity ($p=0.0362$). Similarly, as shown in figure 3.7d, there was a significant increase in rearing activity in the IL-1R1$^{-/-}$ mice ($p=0.0035$). Figure 3.7e shows that the IL-1R1$^{-/-}$ mice spend a significantly longer time in the open arms of the elevated plus maze compared to the WT mice ($p<0.05$). This increased time in the open arms suggests that male IL-1R1$^{-/-}$ mice are less anxious, as was observed in the female mice (figure 3.3c). Since increased time in open arms might be a product of more arm entries in a general sense (i.e since they show hyperactivity) this possibility was addressed by examining, in mice of both genders combined, the number of open arm entries as a ratio to the total arm entries (figure 3.7f). These data show that a greater proportion of the total arm entries of the IL-1R1$^{-/-}$ mice are into open arms than those of WT mice ($p=0.0160$ t-test), thus indicating a decrease in anxious behaviour in the IL-1RI$^{-/-}$ mice.

Thus across these parameters of hippocampal-dependent learning and memory, open-field activity and anxiety tests, male IL-1R1$^{-/-}$ mice showed the same characteristics as females.

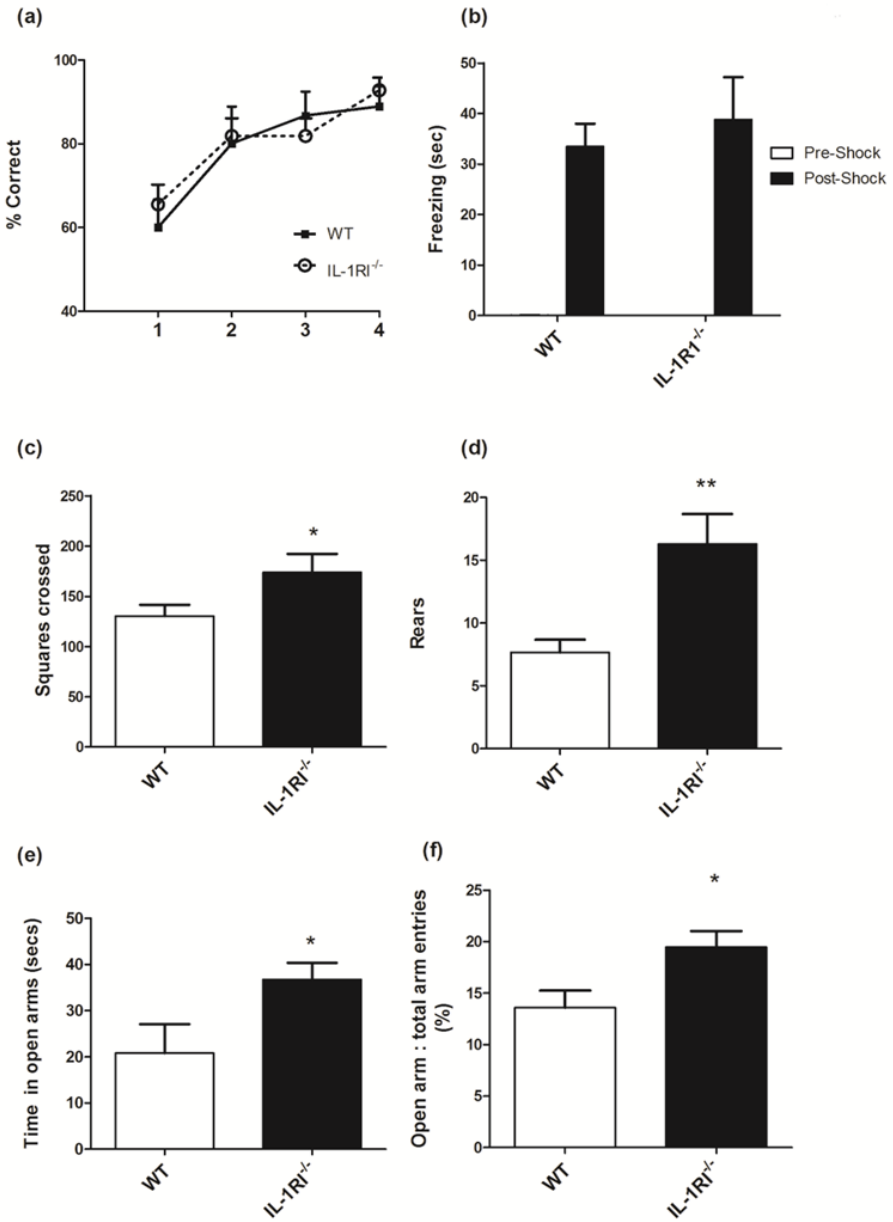


Figure 3.7 Performance of male IL-1R1$^{-/-}$ mice versus male WT on a battery of tasks. (a) Visuo-spatial reference memory was assessed using the Y-maze across 20 trials (n = 5 per trial block) in male IL-1R1$^{-/-}$ (n = 11) compared to male WT mice (n = 9). (b) The time spent freezing before (too small to be clearly visible), and 48 hours following, 0.4 mA foot shock for male IL-1R1$^{-/-}$ mice and WT mice in the fear conditioning paradigm. (c) The number of squares crossed in 3 minutes in the open field and (d) the number of rears recorded in open field, across 3 minutes (e) the time spent in open arms of the elevated plus maze and (f) entries into open arms as a proportion of total arm entries (for male and females combined). Data are expressed as mean ± SEM and figure (a,b) were analysed by two way repeated-measures ANOVA with strain as between subjects factor and time/block as within subjects factor and all other figures were analysed by one-tailed t-test and significant differences are denoted by ** $p<0.001$ and *$p < 0.05$.

3.2.10 Ventricular volume and synaptic density

The ventricular volume in IL-1R1$^{-/-}$ (n=6) and WT mice (n=4) was assessed with the use of ImageJ. Figure 3.8c, shows that there is no significant difference in the ventricular volume between WT or IL-1R1$^{-/-}$ mice (p=0.17) as assessed by Mann Whitney test following Kolmogorov–Smirnov test., It was also investigated whether there was a difference between the two strains in synaptic density in regions of the dorsal and ventral hippocampus. In figures 3.8f and 3.8i where mean values density ratios (layer of interest as ratio to reference layer) are presented, there were no differences in synaptic density in the stratum radiatum between the IL-1R1$^{-/-}$ mice and WT mice in either dorsal hippocampus (Bregma -2.0 mm AP, 1.2 mm DV) or in more ventral hippocampus (Bregma -3.1mm AP, -3mm DV from surface of brain). Unpaired t tests showed that there were no statistically significant differences ($p \geq 0.1324$), although there was generally more variability in values in the IL-1RI$^{-/-}$ animals.

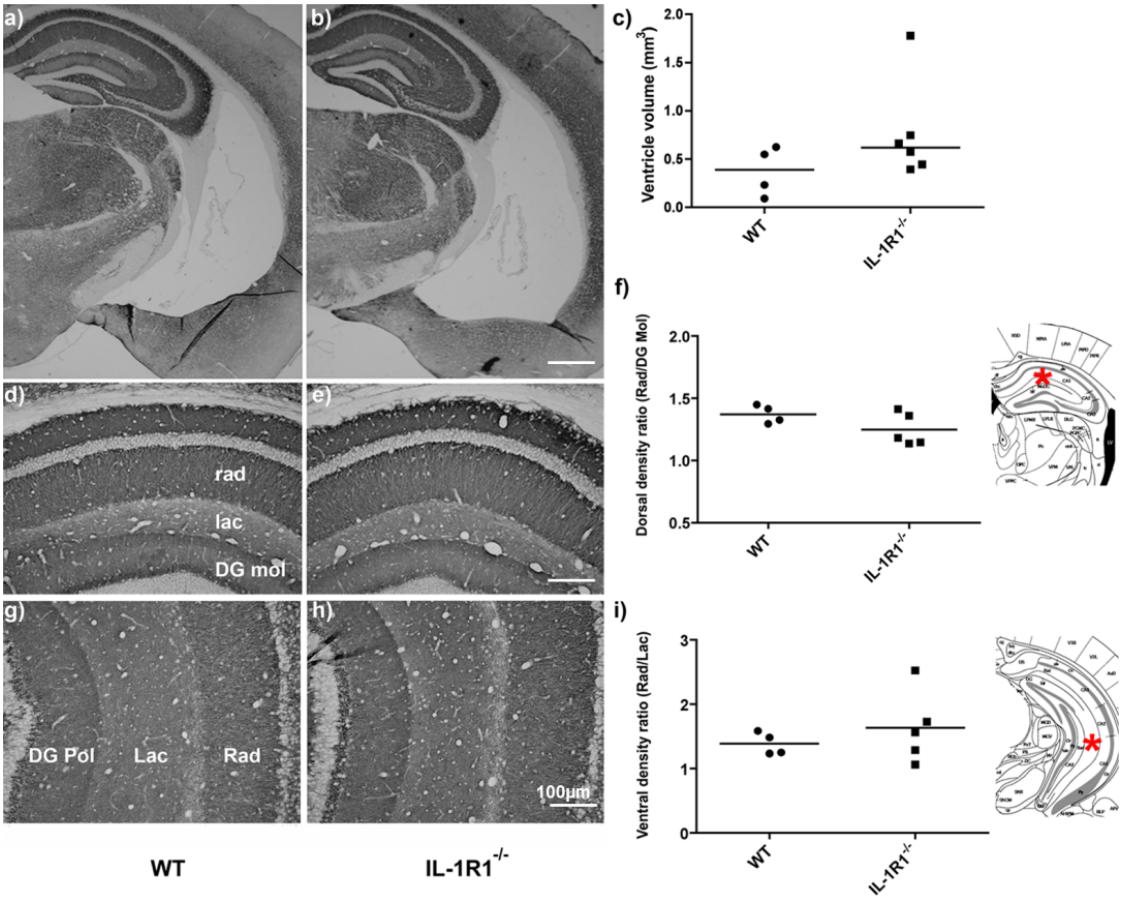


Figure 3.8 Ventricular volume and synaptic density in WT and IL-1R1$^{-/-}$ mice. (a-c) Representative images of the hippocampus and lateral ventricle of WT (a) and IL-1R1$^{-/-}$ (b) brain. Ventricular volume in WT (n=4) and IL-1R1$^{-/-}$ mice (n=6) was assessed using Image J Software (c). Areas were calculated for sections from each animal at 2.0 and 2.3 mm posterior to Bregma and summed and multiplied by the summed section thickness (2x10µm: 0.02 mm). Data are non-parametric and have been presented as dot plots with median value denoted by the bar. (d-i) Synaptophysin labelling in the dorsal (see inset *) and more ventral hippocampus (approximately -3.0 mm from surface of brain; see inset *) of WT (d,g) and IL-1R1$^{-/-}$ mice (e,h). Synaptic density analysis of the dorsal hippocampus: ratio of stratum radiatum to dentate gyrus molecular (f) and ventral hippocampus ratio of stratum radiatum to stratum lacunosum moleculare (i) for WT (n=4) and IL-1R1$^{-/-}$ (n=5).

3.3 Discussion

The results here presented in this chapter show that IL-1R1$^{-/-}$ mice are not impaired on an array of hippocampal dependent, spatial memory tasks, that include the T maze working memory task or the Y maze, water maze visuospatial tasks or the contextual fear conditioning paradigm. It appears that IL-1R1$^{-/-}$ mice from the open field data exhibit increased activity by displaying increase in squares crossed and increased rears. Elevated plus maze which measures anxiogenic behaviour showed that the IL-1R1$^{-/-}$ mice display decreased anxiety compared to WT control and these results were seen in both genders. IL-1R1$^{-/-}$ mice also showed a significantly shorter latency to reach the platform in the Morris, water maze but a slight deficit on first choice accuracy in the spatial discrimination beacon task.

3.3.1 IL-1 in anxiety

The data in section 3.2 suggest that IL-1R1$^{-/-}$ mice have increased activity compared to controls. It is of importance to note that when the overall activity of IL-1R1$^{-/-}$ mice was recorded in the phenotyper cages, it was not significantly different to the WT controls suggesting that this increase in open field activity is explained by activity in a novel environment. Rearing, may be a stereotype behaviour. Stereotypies can be defined as repetitive, unvarying and seemingly functionless behaviour patterns that can occur in a variety of species. Stereotypies can be induced by environmental conditions, drug administration or psychiatric disorders (Mason, 1993). Rearing which is seen as a measure of exploratory behaviour, was increased in IL-R1$^{-/-}$ mice suggesting they more readily explore their new environment. In addition to the data seen in the open field environment, the IL-1R1$^{-/-}$ mice tend to spend longer time in the open arms of the elevated plus maze and less time in the closed arms compared to WT (figure 3.3c). They also show a tendency, though not significant, to have a shorter latency to first emerge from the starting closed arm compared to controls (figure 3.3d). When the open arm entries and a % of total arm entries were calculated, IL-1R1$^{-/-}$ mice spend significantly more time in the open arms than do WT mice. All of these data suggest decreased anxiety and therefore support a role of IL-1 in anxiety. Reduction of anxiogenic behaviour in IL-1R1$^{-/-}$ mice was also seen in a similar study by Koo and Duman. In that study the authors demonstrated that mice with a null mutation for the

IL-1 type I receptor also displayed a reduction in anxiogenic behaviour by spending a significantly longer time in the open arms of the elevated plus maze compared to the WT controls (Koo and Duman, 2009). The authors also showed in the same study that the IL-1R1$^{-/-}$ mice had a longer latency to enter the dark chamber of the light dark box compared to the controls, supporting a reduction in anxiety like behaviour in this phenotype. A more recent study by Chiu and colleagues demonstrated that when IL-1 is increased in the brain by adenosine-dependent activation of caspase-1, it results in increased anxiety like behaviour in WT mice (Chiu et al., 2014).

In another study Connor and colleagues, observed an increase in anxiety like behaviour in rats in the elevated plus maze when injected with IL-1β. The IL-1β-treated rats spent significantly more time in the closed arms and less time in the open arms than the controls, despite showing a similar level of activity as reflected in their total arm entries (Connor et al., 1998). Swiergal and colleagues also report that mice injected with IL-1β spend less time in the open arms and less entries into open arm than vehicle controls all suggestive of anxiety like behaviour (Swiergiel and Dunn, 2007). These studies all provide evidence that IL-1β can induce anxiety like behaviour in rodents, and possibly in the data presented in section 3.2, may suggest that endogenous IL-1 can induce anxiety in normal WT mice and that by blocking this pathway, via IL-1R1-/- deletion anxiety like behaviour is reduced, as seen in the IL-1R1$^{-/-}$ mice in this study, All of these data support the hypothesis that IL-1 does play a role in anxiety like behaviour. It is possibly that the IL-1R1$^{-/-}$ mice have decreased anxiety as a result of decreased endogenous IL-1 action in brain anxiogenic centres. It is of course, impossible to pinpoint the locus of reduced anxiety in the brain of a global knockout animal. However, there is considerable evidence to suggest an important role for the hippocampus, and particularly the ventral subregion of the hippocampus, during ethological, unconditioned tests of anxiety like the elevated plus maze and the open field (Bannerman et al., 2002, Bannerman et al., 2003, Bannerman et al., 2004, McHugh et al., 2004, Barkus et al., 2012, Kjelstrup et al., 2002, Treit et al., 1993). It has been argued that during these unconditioned tests, anxiety is generated by the approach/ avoidance conflict experienced by the animals (Barkus et al., 2012, McNaughton and Gray, 2000). For example, on the elevated plus maze the mouse is

faced with the choice of either approaching and exploring the open arms, or avoiding them and staying safe in the enclosed sections of the maze. An important component of the anxiety response in normal animals is the behavioural inhibition of motor activity and, in particular, inhibition of the approach response towards the open arms (McNaughton and Gray, 2000) In the present study IL-1R1$^{-/-}$ mice were less able to inhibit the approach response during these approach/avoidance conflict tests. It is notable that the IL-1R1$^{-/-}$ mice were also less able to inhibit their approach responses to the decoy beacon during the spatial discrimination version of the MWM task. Although we should be cautious about over-interpreting what is only a mildly significant effect in the beacon watermaze task, it nevertheless may help to identify a key psychological process (behavioural inhibition of approach responses) which could be important, not only in interpreting certain tests of cognition but also during anxiety, and which could also be a key target for modulation in sickness behaviour.

Preliminary investigations into a neuropathological basis for these decreased anxiety effects in IL-1R1$^{-/-}$ mice were not definitive, with no significant effects on ventricular volume or hippocampal synaptic density in dorsal or more ventral regions, although changes in the lamination of the hippocampus in ventral regions make the analysis conducted here difficult in the most ventral regions. There is a recognised double dissociation between the dorsal hippocampus and ventral hippocampus with respect to spatial memory and anxiety (Bannerman et al., 2002) and it will be worth investigating, in IL-1R1$^{-/-}$ mice and littermate controls, the possibility that there are more pronounced structural or morphological differences in the anxiety-associated ventral hippocampus. It is also important to note that the described differences in open field activity and decreased anxiety also represent important potential confounding factors in studies of cognitive function. That is to say, in cognitive tasks that are aversively motivated, such as the MWM and contextual fear conditioning, an animal with decreased anxiety may show differences in these cognitive tasks because of its different response to the aversive stimulus rather than because of some cognitive impairment. Aspects of sickness behaviour can significantly confound cognitive testing (Cunningham and Sanderson, 2008) and given the divergent findings on endogenous IL-1 in cognitive function in this and previously published studies, altered levels of anxiety and activity may be a key consideration here.

3.3.2 Endogenous IL-1 in learning and memory

There is prior evidence that endogenous IL-1 can play an important role in hippocampal-dependent memory and learning tasks. Work by Avital and colleagues demonstrated that IL-1Rko mice are impaired on the spatial version water maze by displaying significantly longer latencies to reach the hidden platform compared to WT controls. In the same study the authors also show that the IL-1Rko mice spent less time freezing in the fear conditioning paradigm to the controls, but in the auditory-cued conditioning paradigm they showed no difference to the controls which suggests that endogenous IL-1 does play a role in hippocampal dependent memory but does not play a role in hippocampal independent memory. In another study from the same group, they showed that when IL-1RA was administered it also impaired memory in the water maze (Yirmiya et al., 2002). The same group also showed that transgenic mice with over-expression of IL-1RA displayed impaired hippocampal-dependent memory in the water maze indicating that the blocking of IL-1 signalling specifically disrupts hippocampal-dependent memory (Goshen et al., 2007).

However, the current data shows that there was no impairment in IL-1R1$^{-/-}$ mice compared to WT controls when tested on the spatial reference Y maze memory task, which uses visuospatial cues to escape shallow water. Mice were able to solve this task equally well to the WTs. To replicate previous studies that have already reported deficits in IL-1R1$^{-/-}$ mice in the Morris water maze, the exact protocol reported by Avital and colleagues was used to access the IL-1R1$^{-/-}$ mice in the water maze and these data also did not support a role for IL-1R1. This protocol consisted of 3 trials for 3 days with 1h inter-trial interval. The current study shows that IL-1R1$^{-/-}$ mice are not impaired on this task. They displayed a similar rate of learning in the water-maze compared to the WT controls. The IL-1R1$^{-/-}$ mice in the current study showed slightly but statistically significant shorter latencies to the platform than the WT controls. In their work, Avital and colleagues, showed rapid learning of the task by WT animals compared to a large literature of Morris water maze studies. In that study WT mice were showing quite stable performance by trial 4 whereas the IL-1R1$^{-/-}$ mice in their study were showing longer latencies to find the hidden platform and travelling longer

distances compared to the WT. Contrary to the Avital study, the data in figure 3.4 show that the WTs here did not learn this task as rapidly as the WT controls in the Avital study and a probe test that was performed after 9 trials, indicated only slight preference for the goal quadrant (33% in target quadrant). The rapid learning in the controls rather than the slow learning in the IL-1R1$^{-/-}$ mice in Avital's work may constitute a key difference between the data presented in 3.4 and Avital's research. This more rapid WT learning could conceivably be explained by strain differences (see discussion page 108). The overall distance travelled by the mice was also shorter than the distance travelled by the mice in Avital's study (figure 3.4b) and again the IL-1R1$^{-/-}$ mice did not show any difference in path length to the WT controls. As the protocol used was rather short, with only 3 days of training and after observing only 33% preference for the goal quadrant, training was continued for further trials. As can be seen in figure 3.4c, even after extending training to 27 trials, the IL-1R1$^{-/-}$ mice did not show impairment on this task. The % time spent in the target quadrant after 27 trials increased for both strains to above 40% and there was no difference in IL-1R1$^{-/-}$ mice to WT on the visible flag trials, all of this suggesting that even after this extended period of learning, IL-1R1$^{-/-}$ mice learn this task similarly well to the WT controls.

In a recent study by Bannerman and colleagues (Bannerman et al., 2012) it was shown that performance on a spatial discrimination version of the MWM task is more sensitive to disruption of hippocampal synaptic plasticity than performance on the standard open field version of the MWM. Mice lacking NMDARs in the dentate gyrus and CA1 subfields of the hippocampus were capable of learning the spatial location of the platform but were less accurate at discriminating between two visually identical beacons, one of which signified the position of the escape platform as defined by the allocentric, extramaze spatial cues. Given the deficits in hippocampal synaptic plasticity reported in the IL-1R1$^{-/-}$ mice (Avital et al., 2003) the IL-1R1$^{-/-}$ mice were assessed on the spatial discrimination/beacon version of the watermaze task. During standard probe tests in which both beacons and the escape platform were removed from the pool, both WT and IL-1R1$^{-/-}$ mice again showed an equivalent preference for the training quadrant, suggesting that both groups had learned about the spatial location of the platform to the same extent. In contrast, the IL-1R1$^{-/-}$ mice displayed a

subtle increase in the number of times they approached the wrong (decoy) beacon (both in terms of first choice accuracy and total errors). Bannerman et al., (Bannerman et al., 2012) argued that the impairment in spatial discrimination performance in mice lacking hippocampal NMDARs was not due to an impairment in associative spatial learning but instead reflected an inability to behaviourally inhibit the very strong conditioned response that the mice have to swim to the first beacon that they encounter. Given the normal acquisition of the standard, open field version of the MWM task in the IL-1R1$^{-/-}$ mice and their ability to learn about the spatial location of the platform during the beacon task, their deficit in choice behaviour may also reflect impaired behavioural inhibition rather than impaired associative spatial learning, as discussed above.

In addition to being tested on the water maze and spatial discrimination task, IL-1R1$^{-/-}$ mice showed no difference in diurnal rhythm compared to the WT controls. Both strains showed higher activity in the night phase compared to the day phase; however it is important to note that the phenotyper cage recorded the total activity per cage as opposed to activity per mouse therefore the data in graph represents the activity for 3 cages of 5 mice instead of the activity of all mice. Though there was no significant effect of strain, there was a significant effect of strain x time, suggesting that IL-1R1$^{-/-}$ mice travel shorter distances in the dark phase than controls.

As there have been reports to suggest that IL-1R1$^{-/-}$ mice are impaired on contextual memory (Avital et al., 2003) the IL-1R1$^{-/-}$ mice were also assessed on this paradigm. The results in figure 3.3 indicate that the IL-1R1$^{-/-}$ mice spend equivalent time freezing 48 hours post-conditioning and showed no impairment on this task compared to the WT controls. This data would suggest that the hippocampal-dependent fear conditioning task is not dependent on IL-1R1. Therefore, the work presented in this chapter provides a further understanding of the role of endogenous IL-1 in learning and memory and a more detailed behavioural assessment of IL-1R1$^{-/-}$ mice.

3.3.3 Divergent effects in IL-1R1$^{-/-}$ studies

The data presented in this chapter is different to the results found in the studies conducted by Avital and Yirmiya. In their studies, the authors report that that IL-1Rko mice are impaired in contextual fear conditioning paradigm and on the Morris water maze (Avital et al., 2003). The Avital studies were conducted in male mice, whereas the studies presented in this chapter were carried out in female mice. To assess whether gender influenced differences, additional experiments looking at the Y maze, EPM and contextual fear conditioning paradigms were carried out in male mice. As can be seen from figure 3.8 there was no impairment in the Y-maze or fear conditioning and similar results to that seen in the females were also observed in the open field and elevated plus maze. IL-1R1$^{-/-}$ male mice showed decreased anxiety and increased activity in the open field (figures 3.8 c-f) thus across these parameters of hippocampal-dependent learning and memory, open-field activity and anxiety tests, male IL-1R1$^{-/-}$ mice showed the same characteristics as females. These results would indicate that gender is not an important factor in either of the cognitive or anxiety tests used in the studies in this chapter.

It is important to note, that Goshen and colleagues demonstrated the effect of IL-1β pathways on behaviour using mice raised in reverse cycle and therefore conducted all of their behavioural experiments during the mice's more active period. In the data presented in this chapter, all of the behaviour was carried out in the mouse's less active period as they were not raised in reverse cycle. It has been reported that time of day influenced IL-1β and IL-1R1 expression and that IL-1β may contribute to the basal functioning of the suprachiasmatic nucleus (SCN) clock (Beynon and Coogan, 2010). The SNC which is found in the hypothalamus functions as the master circadian pacemaker. It could be suggested that IL-1β may play a role in influencing the SNC controlled oscillation of hippocampal dependent memory tasks. To address this, the IL-1R1$^{-/-}$ mice and WT controls diurnal rhythm were assessed in phenotyper cages. As can be seen in figure 3.6 there was no difference in overall distance travelled by both strains. It can also be seen that the IL-1$^{-/-}$ mice and WTs showed a peak in activity during the dark phase which was expected. However, the significant interaction of

time and strain may reflect that IL-1R1$^{-/-}$ mice are slightly less active in the dark phase and perhaps slightly more active in the light phase, so this could lead to a suggestion that that the strains may show other behaviour differences if tested during subjective night but does not offer an obvious explanation in the difference in cognitive function in Avitals research and the data presented in this chapter.

One possible explanation for the difference in results in the work presented here, and of that presented by Avital and Yirmiya, was the difference in strains used. In the studies presented here in this chapter, female or male Interleukin 1 receptor 1 knock-out mice (IL-1R12/2; B6.129S7- Il1r1tm1Imx/J; Stock number 003245) were obtained from an in-house colony, originally imported from Jackson Laboratories, USA. The initial generation of these mice (Glaccum et al., 1997) involved both 129Sv and C57 strains. Briefly, a null mutation in Il1r1 was generated by homologous recombination in 129/SvEv AB1 ES cells and targeted mutant mice were subsequently backcrossed 7 times to C57BL/6 background (that was not C57BL6/J). The IL-1RI$^{-/-}$ animals supplied by JAX have remained at generation N5+N2F2 (since October 2009). As such they were initially a C57/129Sv hybrid and were backcrossed 5 times onto a C57 background before importation by JAX and a further 2 times onto a C57BL6/J background after importation by JAX. They were subsequently maintained as an inbred colony and the current study has used C57BL6 as the control strain. Koo and Duman studies have also been performed with C57BL/6 mice. A recent (March 2011) 32 single nucleotide polymorphism panel analysis of the JAX mice, with 27 markers covering all 19 chromosomes and the X chromosome, and a further 5 markers distinguishing between C57BL/6J and C57BL/6N sub-strains, was performed on the re-derived living colony at The Jackson Laboratory Repository. 26 of 27 markers throughout the genome indicate a C57BL/6 genetic background (http://jaxmice.jax.org/strain/003245.html). Furthermore, 3 of 5 markers that distinguish C57BL/6J from C57BL/6N indicated that the mice originally sent to JAX were on a C57BL/6N genetic background or a mixed C57BL/6J;C57BL/6N genetic background. As such, it could be argued that C57BL/6 mice are an appropriate control strain. It is of interest that the studies of Koo and Duman were also performed with C57BL/6 controls, but not C57BL6/J originating from JAX. The data in this chapter, demonstrating decreased anxiety, increased activity and

a limited effect on cognitive performance are consistent with the Koo and Duman studies (Koo and Duman, 2009) and divergent from those of the Yirmiya group, which were performed using 129 x C57 crosses as controls. The Yirmiya group have used a different IL-1R1$^{-/-}$ animal: the B6;129S1-Il1r1tm1Roml/J (Stock Number:003018), which is maintained as a hybrid strain (129S1/Sv * C57BL/6 cross). Thus a 129/Sv X C57BL/6 cross was used as the control strain for their initial and subsequent studies (Avital et al., 2003, Goshen et al., 2009, Goshen et al., 2007, Yirmiya et al., 2002). The IL-1RI knockouts and the control animals in those studies are 50/50 129/C57 and thus while all three research groups have used appropriate control strains for their respective IL-1RI$^{-/-}$ mice, the effects of deletion of IL-1RI$^{-/-}$ may be different depending on the background strain of mouse. There has been evidence to suggest strain differences on performance of hippocampal-dependent tasks. Balogh and colleagues found 129 mice to be behaviourally and cognitively deficient compared to C57 mice (Balogh et al., 1999). Additional studies by Wolff and colleagues showed that C57 mice had better performance in some paradigms in the Morris water maze and worse performance than 129 mice in other paradigms in the same maze (Wolff et al., 2002). There is also some evidence that 129 x C57 hybrids learn the Morris water maze more rapidly than either 129 or C57 strains (Voikar et al., 2001) which is consistent with the rapid learning in the Morris water maze as seen in Avital's work (Avital et al., 2003).

With the detailed and large battery of cognitive tests performed in the IL-1R1$^{-/-}$ mice in this chapter, and in mice of both genders, and contrary to prior published studies, it can be suggested that the loss of IL-1R1 does not affect hippocampal-dependent learning and/or memory in these IL-1R1$^{-/-}$ mice. The IL-1R1$^{-/-}$ mice in this study however, did display a lower level of anxiety compared to WT controls, suggesting that IL-1 signalling is involved in normal anxiety responses. This is also found in the studies of Koo and Duman where the authors demonstrated that mice with a null mutation for the IL-1 type I receptor also displayed a reduction in anxiogenic behaviour by spending a significantly longer time in the open arms of the elevated plus maze compared to the WT controls. In the same studies the authors demonstrated that IL-1β infused rats had a reduction in number of entries to open arms and total arms in the elevated plus maze. The IL-1β infused rats also displayed a

tendancy to spend less time in the open arms of the maze, all suggesting IL-1 plays a role in anxiety like behaviour(Koo and Duman, 2009). Further analysis, both neuroanatomical and neurochemical in knock out animals and appropriate littermate controls should be carried out to assess this.

3.4 Conclusion

To date, there have been some studies that have assessed the role of IL-1R1 in learning and memory however, these studies failed to provide a detailed assessment of the role of IL-1R1 in anxiety and in working memory tasks. These studies also failed to show if deletion of IL-1R1 has an impact on diurnal rhythm. Contrary to prior published studies by Avital and Goshen the IL-1R1$^{-/-}$ mice in the current study are not impaired on hippocampal-dependent learning and memory tasks but do display a lower level of anxiety compared to WT mice. The results in this chapter have highlighted how endogenous levels of IL-1 do not contribute to learning and memory. The level of anxiety in mice lacking IL-1R1 was decreased to that of WT. This would suggest that endogenous IL-1 contributes to anxiety. This work offers some therapeutic value in the medical field, in that future drugs targeted to treat anxiety in patients could specifically target the IL-1 pathway.

Chapter 4

The role of systemically induced IL-1 on cognition in WT mice and those with existing neurodegenerative disease.

Elements of this chapter have been published in Murray et al., 2012, *Neurobiology of Aging* and Murray et al., 2011 *Journal of inflammation* and the majority of the work in this chapter has been accepted for publication at *Molecular Psychiatry.*

4.1. Introduction

Despite prior reports of behaviour impairments in IL-1R1$^{-/-}$ mice (Avital et al., 2003, Goshen et al., 2009), the results in the previous chapter showed very limited evidence of any cognitive deficits in IL-1R1$^{-/-}$ mice thus challenging the conception that endogenous IL-1 is necessary for learning and memory. However, LPS has been demonstrated to impair learning and memory (Pugh et al., 1998, Pugh et al., 1999, Avital et al., 2003, Thomson and Sutherland, 2005, Terrando et al., 2010) These studies typically show that rodents, after LPS administration, are impaired in consolidation of contextual memory suggesting that systemic inflammation impairs hippocampal function. IL-1 is believed to be key in these deficits. Studies have shown that intracerebral administration of IL-1β (10 or 20ng) injected immediately following conditioning in the contextual fear conditioning (CFC) task impaired contextual memory (Pugh et al., 1999 and bilateral intra-hippocampal injection of IL-1β (10ng) impairs CFC (Barrientos et al., 2004).

Peripheral infections are known to trigger episodes of acute cognitive impairment, including delirium, in older populations and in those with dementia (Elie et al., 1998). Delirium, which is characterised by inattention and profound cognitive impairment, is an acute and transient disorder that is highly prevalent in elderly and patients with dementia (Burns et al., 2004). It is a very common medical condition and it is estimated that one in eight hospitalised patients will experience delirium (Cunningham and Maclullich, 2013). In surgical patients over the age of 65 years, 15-53% will have postoperative delirium which leads to multiple adverse outcomes that include increased length of stay, patient distress, increased morbidity and mortality, loss of independence and increased healthcare costs (Fong et al., 2009).There are large economic and social burdens associated with delirium. The care of older patients in hospital with delirium has accounted for more than 49% of all hospital days and $6.9 billion of hospital expenditures are attributable to delirium costs (Inouye, 2006).

Cytokines are key mediators of septic and aseptic inflammation and, given its important role in coordinating CNS responses to systemic inflammation(Dantzer et al., 2008, Gosselin and Rivest, 2008), the pro-inflammatory cytokine IL-1β might

bepredicted equally to underlie infection-and sterile inflammation-induced cognitive dysfunction. Consistent with this idea, IL-1β levels have been associated with delirium in hip fracture patients (Cape et al., 2014). There is a high prevalence of delirium after surgery and infection which emphasises the deleterious consequences that systemic inflammation has for cognitive function especially in the older population (Davis et al., 2012). It is also clear that acute systemic inflammation and delirium can accelerate the course of existing dementia (Fong et al., 2009, Holmes et al., 2009). Despite the large economic and social burdens associated with delirium, the impact of systemic inflammation on the vulnerable brain still remains to be investigated.

This aim of the experiments in this chapter will therefore investigate whether LPS can induce other forms of hippocampal dysfunction in behavioural paradigms. Performance of WT mice in the alternation T-maze task and in the contextual and auditory fear conditioning paradigm after administration of LPS will be assessed to investigate the role of induced IL-1 in these tasks.

Another aim of the experiments in this chapter will address the impact of systemic inflammation on hippocampal function in the vulnerable brain. The ME7 prion disease model shows a robust chronic neurodegenerative disease centered on the hippocampus. ME7 prion disease has many of the characteristics all associated with progressing neurodegenerative disease such as synaptic loss, extracellular amyloidosis, neurodegeneration and progressive cognitive decline (Betmouni et al., 1999, Cunningham et al., 2003). Previous work from our laboratory has shown that LPS can induce IL-1 and other pro-inflammatory cytokines during ME7 disease (Cunningham et al., 2009, Murray et al., 2012). The effect of this systemically induced IL-1 in working memory and consolidation memory still remains to be investigated in WT mice and mice with ME7 disease.

Therefore, experiments in this chapter will aim to assess ME7 and NBH mice in hippocampal dependent memory tasks after administration of LPS. Further experiments using IL-1R1$^{-/-}$ mice and anti-inflammatory interventions will also be

carried out to unpick the inflammatory basis of these LPS-induced impairments in these mice.

4.2 Results

4.2.1 Dissociable effects of LPS on contextual fear conditioning and T-maze

Female WT mice were injected with saline or LPS (100µg/kg i.p) after exploration and a foot-shock of 0.4mA. The time spent freezing in the contextual fear conditioning paradigm was assessed 48 hours later. LPS induced a significant decrease in freezing compared to the saline-treated controls with $p < 0.0001$ (figure 4.1a). Auditory fear conditioning was also assessed 3 hours after contextual for the same mice. The time mice spent freezing before and after tone was played, was recorded for duration of 6 minutes. There was no impairment of freezing in any group in the auditory fear conditioning task (figure 4.1b). Data were analysed by t-test with $p=0.49$. In an independent cohort, WT mice (n=18) were trained on the escape from shallow water T-maze working memory task and then injected with saline or LPS (100µg/kg i.p) and their performance after treatment was assessed. LPS did not impair working memory in this task (figure 4.1c). The results in figure 4.1 (a-c) indicate that LPS can have differential effects on two hippocampal-dependent tasks.

4.2.2 Performance of NBH and ME7 mice post-inoculation at 12 weeks and 16 weeks after challenge with LPS in the working memory T-maze alternation task.

The LPS-induced deficits in hippocampal function seen in figure 4.1a were then repeated in mice inoculated with ME7 prion disease to assess whether underlying disease and prior hippocampal pathology could make the LPS-induced impairments more sensitive.

ME7 prion was used as it is a progressive neurodegenerative disease with progressive cognitive and functional decline that is accompanied by synaptic loss, amyloidosis and microgliosis (Cunningham et al., 2005a). The performance of mice inoculated with normal brain homogenate (NBH) or ME7 prion disease was assessed in the T-maze following systemic challenge with LPS or saline 12 weeks post-inoculation.

Briefly, ME7 and NBH animals were trained on a novel paddling alternation T-maze task for 10 blocks of 10 trials. By the end of training (24 hours before LPS or saline challenge) all animals had reached a steady baseline performance at or above 80%

correct choices. Student's t-test of all ME7 versus all NBH revealed that animals in the early stages of disease (12 weeks) are not impaired with respect to controls (p=0.70).

Systemic challenge with LPS (100µg/kg) did not induce memory impairments in NBH animals. However the same LPS challenge induced a marked transient working memory deficit in ME7 animals (figure 4.1d). The memory performance of NBH and ME7 groups of animals were compared across all time points post-injection with LPS or saline (i.e., 3–26 hours) using 3-way ANOVA with disease, treatment and time as factors. Three way ANOVA showed a significant interaction of disease (NBH versus ME7) x treatment (saline versus LPS) (F=11.098, df 1,43, p=0.001). Bonferroni *post-hoc* showed that at 3 hours post-treatment, there was a significant difference between the ME7+LPS group and NBH+LPS and ME7+saline group (p<.05). Together these results indicate that underlying prior hippocampal vulnerability make these mice more vulnerable to the systemic inflammatory insult such as LPS.

It was hypothesized that this acute transient deficit at 12 weeks would be greater at 16 weeks so the performance of NBH and ME7 mice at 16 weeks post-inoculation was assessed in the T-maze. NBH animals did not show a memory deficit nor did ME7 saline-treated mice. ME7 mice challenged with the same dose of LPS as the NBH however showed a marked transient working memory deficit 3 hours post-challenge but this deficit was further decreased by 5 hours. Data were analysed by 3-way ANOVA with disease, treatment and time as factors. Three way ANOVA showed a significant interaction of disease (NBH versus ME7) x treatment (saline versus LPS) (F=4.6439, df 1,32, p=0.03) and an interaction of treatment x time (F=2.448, df 6,216, p=0.02). Bonferroni *post-hoc* test demonstrated a significant difference in working memory performance between ME7+LPS and both ME7+saline and NBH+LPS at 5 hours (p<0.01).

Together the data in figure 4.1 (d-e) suggest that as underlying hippocampal pathology progresses, the severity and duration of systemic inflammation-induced cognitive dysfunction also increases. All future ME7 experiments therefore were conducted at 16 weeks

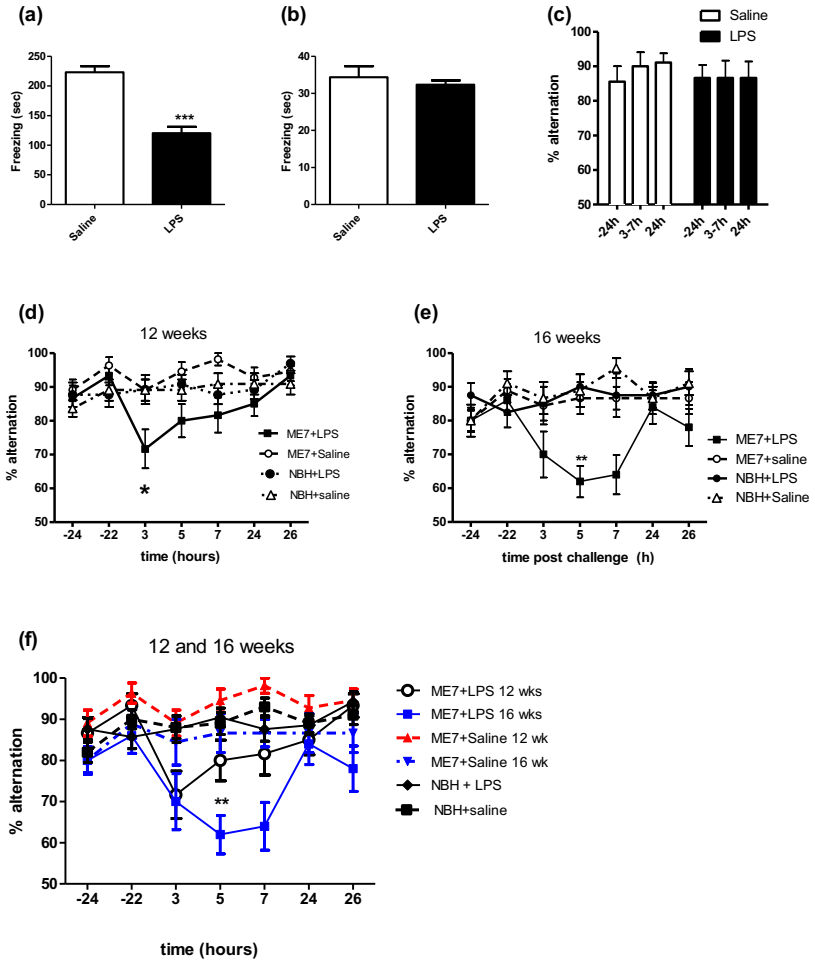


Figure 4.1 Dissociable effects of LPS on contextual fear conditioning and T-maze working memory task in WT mice and in NBH and ME7 mice post-inoculation at 12 and 16 weeks. Contextual (a) and auditory (b) fear conditioning performance (time spent freezing across 5 minutes) 48 hours post-challenge with saline (n=12) or LPS (100 µg/kg i.p.) (n=16). (c) Working memory performance by alternation T-maze in saline-treated and LPS-treated WT mice (n=9 for both groups) 24 hours before, 3-7 and 24 hours post-treatment. (d) Performance of ME7+LPS (n=12), ME7+saline (n=11), NBH+LPS (n = 13) and NBH+saline (n=11) animals 12 week post-inoculation, in the novel T-maze alternation task at baseline (−22, −24 hours), post-treatment with saline or LPS (3, 5, 7 hours) and upon recovery (24, 26 hours). (e) Working memory performance of ME7+LPS (n=10), ME7+saline (n=9), NBH+LPS (n=8) and NBH+saline (n=9) animals 16 weeks post-inoculation, in the novel T-maze alternation task at baseline (−22, −24 hours), post-treatment with saline or LPS (3, 5, 7 hours) and upon recovery (24, 26 hours). All data are expressed as mean±SEM and were analysed by t-test (*** $p<0.001$) for (a,b) and two-way ANOVA with time and treatment as the two factors for (c) and 3 way ANOVA with disease, treatment and time as factors (d,e). Significant Bonferroni post-hoc differences between ME7+LPS and both NBH+LPS and ME7+saline are denoted by ** ($p<0.01$) and * ($p<0.05$). (f) 12 and 16 week data combined.

4.2.3 Role of IL-1 in LPS-induced deficits in hippocampal-dependent tasks.

As shown in figure 4.1 LPS impairs multiple hippocampal-dependent processes in normal animals and those with underlying hippocampal pathology. It is known that IL-1 contributes to LPS-induced impairment in contextual fear conditioning experiments. Therefore it is important to assess the roles of IL-1 in these different LPS-impaired processes. The aim of the next experiments was to address whether the deficits in contextual fear conditioning in figure 4.1a and those seen in ME7 LPS-treated mice in figure 4.1 d,e were caused by IL-1. In order to address this question, a number of experiments were performed. IL-1R1$^{-/-}$ mice were utilized in order to examine endogenous/basal levels of IL-1 and IL-1RA, the receptor antagonist for IL-1 was utilized in order to examine IL-1 signalling and the role it plays in this behavioural task.

In the first experiment IL-1RA was administered to WT mice following LPS to assess if systemic IL-1 contributes to the LPS-induced deficit. In the second experiment IL-1R1$^{-/-}$ mice were assessed on contextual fear conditioning after LPS to address whether IL-1 signalling plays a role in this task.

Following exploration and foot-shock, the time spent freezing 48 hours later in the contextual fear conditioning box was recorded for 5 minutes for WT mice challenged intraperitoneally with saline (n=7), IL-1RA (10mg/kg i.p n=11), LPS 100µg/kg; n=11), or LPS+IL-1RA (n=11) (figure 4.2a). Data were analysed by two-way ANOVA with LPS treatment and IL-1RA treatment as the two factors. There was a significant effect of LPS treatment ($F=38.68$, df 1, 36, $p<0.0001$) but no significant effect of IL-1RA treatment ($F=0.03$, df 1,36, $p=0.88$) or significant interaction of these two factors ($F=2.13$, df 1,36, $p=0.15$), indicating that LPS significantly impaired time spent freezing in the context and that the addition of IL-1RA does not protect against this LPS-induced decrease in freezing, which suggests that systemically induced IL-1 does not play a role in the contextual fear memory paradigm. Mice showed no impairment in the auditory task following IL-1RA treatment. Two-way ANOVA analysis with LPS treatment and IL-1RA treatment as the two factors showed no significant effect of LPS treatment ($F=0.61$, df 1,36 $p=0.44$) or IL-1RA treatment ($F=0.09$, df 1,36 $p=0.76$) or significant interaction of these two factors ($F=0.01$,df 1,36 $p=0.94$).

WT mice treated intraperitoneally with saline (n=12) or LPS 100µg/kg; n=16) and IL-1R1$^{-/-}$ mice challenged intraperitoneally with saline (n=5) or LPS 100µg/kg; n=7) were assessed in the contextual fear paradigm. LPS impaired contextual memory in both WT and IL-1R1$^{-/-}$ mice (figure 4.2c). Two-way ANOVA analyses with treatment and strain as factors, show a significant effect of treatment ($F=49.08$, df 1,36 $p<0.0001$) but no significant effect of strain ($F=0.35$, df 1,36 $p=0.56$) or significant interaction ($F=0.01$,df 1,36 $p=0.91$), indicating that all mice had a memory for the context in which shock was received but that LPS impairs this memory equally in both strains. Therefore the results in figure 4.2c indicate that IL-1R1 is not essential for this deficit.

Together the experiments in figure 4.2 show that systemic IL-1 does not play a role in the LPS-induced deficit in the contextual fear conditioning and IL-1R1 activation is not essential for the LPS-induced deficit.

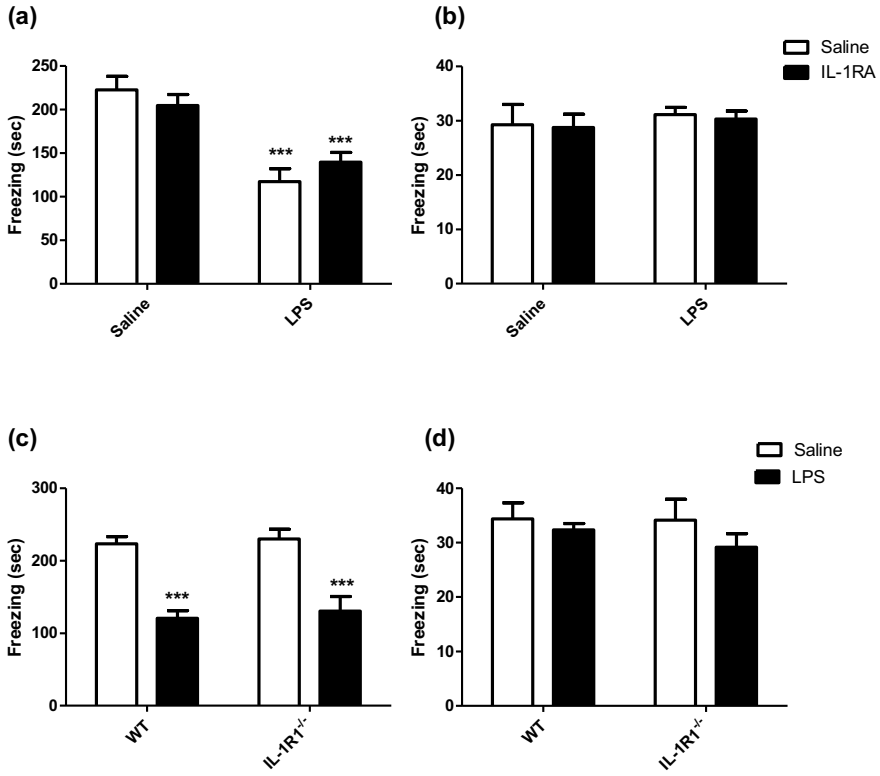


Figure 4.2 Performance in the contextual and auditory fear conditioning paradigm in WT mice challenged with saline, IL-1RA (10mg/kg i.p) or LPS (100µg/kg i.p) and in WT and IL-1R1$^{-/-}$ mice following systemic challenge with saline, or LPS (100µg/kg i.p). (a) The time spent freezing measured across 5 minutes, 48 hours following foot shock at 0.4 mA for WT+saline (n=7)**,** WT+IL-1RA, WT+LPS and WT+IL-1RA+LPS (n=11) in contextual (a) and auditory (b) fear conditioning. Data are expressed as mean±SEM and were analysed by two-way ANOVA with LPS treatment and IL-RA as factors. (c,d) Contextual and auditory fear conditioning for WT+saline (n=12)**,** WT+LPS (n=16) IL-1R1$^{-/-}$+saline (n=5) and IL-1R1$^{-/-}$+LPS (n=7). Data are expressed as mean±SEM and were analysed by two-way ANOVA. Significant Bonferroni *post-hoc* differences after significant ANOVA are denoted by *** p<.0.001 w.r.t saline-treated controls.

4.2.4 Contextual and Auditory Fear Conditioning in NBH and ME7 WT and IL-1R1$^{-/-}$ mice

Given that existence of underlying hippocampal synaptic pathology increased vulnerability to LPS-induced working memory deficits, it was hypothesized that vulnerability to contextual memory deficits might also be increased in ME7 animals with respect to NBH animals. Therefore the next set of experiments assessed the performance of ME7 mice in contextual fear conditioning.

There was a small decrease in time spent freezing in the ME7-treated WT compared to NBH-treated controls (figure 4.3a) however this was not significant when analysed by Student's t-test with p=0.1590. Auditory fear conditioning (figure 4.3b) showed that there was no difference in time spent freezing with p=0.96 thus animals with neurodegenerative disease are not impaired on this task. NBH and ME7 mice were then challenged with LPS or saline to see if systemic inflammation on neurodegenerative disease would induce a deficit. NBH and ME7 mice showed a decrease in time spent freezing in the contextual fear conditioning paradigm following challenge with LPS. Data analysed by 2-way ANOVA with disease and treatment as the 2 factors showed a significant effect of LPS treatment with ($F=34.75$, df 1,16 $p<0.0001$) and no significant effect of disease ($F=1.94$, df 1,16 $p=0.18$) or interaction ($F=0.82$, df 1,16 $p=0.38$). The LPS-induced deficit in the NBH was the same as that seen in the ME7 mice. Auditory fear conditioning showed no effect of LPS or disease. To see if IL-1R1 was essential for this LPS-induced deficit in contextual fear conditioning, IL-1R1$^{-/-}$ mice were challenged with LPS or saline. The data in figure 4.3c shows that all mice with neurodegenerative disease have impairment after LPS and show a reduction in freezing compared to controls. IL-1R1$^{-/-}$ mice do appear to have a higher level of freezing compared to the WT mice treated with saline. Two-way ANOVA comparing ME7 and IL-1R1$^{-/-}$ ME7 mice showed that there was a significant effect of LPS treatment ($F=16.71$, df 1,16 $p=0.00$) and strain ($F=6.31$, df 1,16 $p=0.02$) but no significant interaction of these two factors ($F=0.08$,df 1,16 $p=0.78$).

Neither IL-1R1$^{-/-}$ or IL-1RA had any significant impact on the LPS-induced impairments in the CFC in normal or ME7 mice whether this was also true for the hippocampal-dependent T-maze was next investigated.

4.2.5 T-maze performance following systemic challenge with IL-1RA, saline or LPS in WT and IL-1R1$^{-/-}$ ME7 mice.

It was hypothesised that systemic induced IL-1 could play a role in the LPS-induced deficit observed in the T-maze working memory task in figure 4.1. Therefore WT mice 16 weeks post-inoculation with ME7 prion disease were trained to alternate from their choice arms in the T-maze and then systemically challenged with LPS (100ug/kg i.p), IL-1RA (10mg/kg i.p), or LPS and IL-1RA. As previously seen, LPS had no effect in NBH mice (figure 4.1 d-e) therefore NBH animals were not included in this experiment. At 5 hours LPS-treated mice showed impairments in working memory T maze task (figure 4.4a). When IL-1RA was administered to mice treated with LPS these mice were protected against LPS-induced deficits at 5 hours however by 7 hours the effects of IL-1RA were diminished. Data were analysed by two-way repeated measure ANOVA with treatment and time as factors. There was a significant effect of time ($F=7.13$, df 6,273, $p<0.0001$) and treatment ($F=8.09$, df 2,39, $p=0.01$) and a significant interaction of these two factors ($F=3.25$, df 12,273, $p=0.00$). Further Bonferroni *post-hoc* tests showed a significant difference between ME7+LPS and ME7+LPS+IL-1RA at 5 hours ($p<0.001$) indicating that systemically induced IL-1 is causative in the LPS-induced deficit in the T-maze. It was then investigated if IL-1R1 was essential for this deficit. ME7 and IL-1R1$^{-/-}$ mice were assessed in the T-maze following challenge with LPS or saline. As previously seen in figure 4.1d,e, LPS did not induce a deficit in NBH mice therefore they were not included in the following experiment. IL-1R1$^{-/-}$ ME7 mice showed the same acute LPS-induced working memory deficits as WT ME7 mice in this task after LPS treatment (figure 4.4b). Two-way repeated measures ANOVA showed a significant effect of treatment ($F=16.61$, df 2,36, $p<0.0001$) and of time ($F=7.193$, df 6,216, $p<0.0001$) and a significant effect of these two factors ($F=2.123$, df 12,216, $p=0.02$). Further Bonferroni *post-hoc* tests demonstrated a significant difference between IL-1R1$^{-/-}$ ME7 + LPS animals and IL-1R1$^{-/-}$ ME7 + saline animals at 3 hours

(p<0.01) and 7 hours (p <0.05). There was no significant difference between IL-1R1$^{-/-}$ ME7 + LPS animals and WT ME7 + LPS mice, suggesting that IL-1R1 activation is not indispensable for LPS-induced working memory deficits in ME7 mice.

The results in figure 4.2 (a) and 4.3 (a) show that IL-1RA had no effect on the LPS-induced deficit in contextual fear conditioning; however the same dose of IL-1RA offered some protection against the LPS-induced deficit in the T-maze, therefore indicating that IL-1RA has dissociable effects with respect to its ability to protect against these two hippocampal-dependent behavioural tasks.

4.2.6 T-maze performance of IL-1R1$^{-/-}$ ME7 mice with pre-treatment of Dexamethasone-21-phophate and the impact of Dexamethasone on LPS-induced systemic and CNS cytokine synthesis.

Other pro-inflammatory cytokines are also reported to compensate for loss of IL-1 signalling in IL-1R1$^{-/-}$ mice (Bluthe et al., 2000a). Our laboratory has shown recently that both IL-1 (Griffin et al., 2013) and TNF-α challenge can induce deficits in the T-maze in ME7 mice (Hennessy et al., 2017). Dexamethasone-21-phosphate, an anti-inflammatory steroid has been shown to block systemic cytokine production in mice and prevent hypothermia in ME7 mice (Teeling et al., 2010). It was therefore hypothesised that blocking systemic cytokine induction with dexamethasone-21 phosphate would lead to an improvement in performance in the T-maze in WT ME7 and IL-1R1 ME7 mice.

T-maze performance in IL-1R1$^{-/-}$ animals, 16 weeks post–ME7 inoculation, in the presence or absence of LPS (100 µg/kg i.p.) and dexamethasone-21-phosphate treatment (2mg/kg) was assessed. Dexamethasone-21-phosphate alone did not have effect on performance of IL-1R1$^{-/-}$ ME7 mice in the T-maze. LPS induced a working memory deficit in these mice and mice pre-treated with dexamethasone-21-phosphate showed a reduction in this LPS- induced deficit (figure 4.5a). Two-way repeated measures ANOVA showed a significant main effect of treatment (F=8.47, df 2,29, p=0.00) and time (F=5.38, df 6,174, p=0.00) but no significant interaction

between these two factors (F=1.76, df 12,174, p=0.06). A two-way ANOVA comparing IL-1R$^{-/-}$ ME7 DEX mice to IL-1R1$^{-/-}$ ME7 LPS mice showed a significant main effect of dexamethasone-21 treatment (F=7.271, df 1,17, p<0.0153) which indicated that dexamethasone-21-phosphate can protect against these LPS deficits but that at 7 hours any protection from dexamethasone-21-phosphate had passed. These results suggest that systemic cytokines are contribute to LPS-induced working memory deficits in ME7 mice in the T-maze and that dexamethansone-21-phosphate can protect against these deficits in a time-dependent manner.

Plasma levels of IL-1β and IL-6 were assessed by ELISA assay at 4 hours post-challenge with LPS in the presence or absence of dexamethasone-21-phopshate. Levels of both cytokines were markedly increased after challenge with LPS and were very significantly diminished by pre-treatment with dexamethasone-21-phosphate. IL-1β was decreased by approximately 80% and this decrease was statistically significant as analysed by one-way ANOVA followed by Bonferroni *post-hoc* comparison of NBH+LPS versus NBH+LPS+DEX and of ME7+LPS versus ME7+LPS+DEX (both p<0.001; figure 4.5b). IL-6 production was diminished by approximately 90% and once again this decrease was statistically significant for both NBH+LPS+DEX and ME7+LPS+DEX groups when compared to their respective control LPS-treated groups (p<0.001; figure 4.5c). TNF-α levels were also assessed, but these were below reliable quantification limits, as would be predicted at 4 hours post-LPS (Murray et al., 2012).

Limited IL-1β was induced by LPS in NBH animals and indeed this increase did not reach statistical significance with respect to NBH+saline treated animals (p>0.05, Figure 4.5d). The increase was much more marked in the case of ME7 animals treated with LPS compared to ME7+sal (p<0.001) and to NBH+LPS (p<0.001). However neither in the case of NBH+LPS nor ME7+LPS did dexamethasone-21-phosphate inhibit this CNS transcription of IL-1β. That is to say, ME7+LPS animals were not significantly different from ME7+LPS+dex (p>0.05).

TNF-α transcription was higher in ME7 compared to NBH animals (figure 4.5e). Furthermore, there were significant increases in TNFα mRNA when either NBH

($p<0.001$) or ME7 ($p<0.001$) animals were challenged with LPS. This LPS-induced increase was greater in ME7 animals ($p<0.01$). However, dexamethasone-21-phosphate failed to inhibit this LPS-induced TNF-α mRNA increase in either NBH ($p>0.05$) or ME7 ($p>0.05$) animals.

IL-6 mRNA levels were extremely low in saline or dexamethasone-21-phosphate treated groups whether in NBH or ME7 animals. Levels of mRNA for this cytokine were dramatically induced after LPS treatment and these increases were greater in ME7+LPS than NBH+LPS ($p<0.001$, figure 4.5f). Dexamethasone-21-phosphate inhibited LPS-induced expression of IL-6 mRNA by approximately 50% in both NBH and ME7 animals but only in the latter case did this inhibition reach statistical significance (ME7+LPS > ME7+LPS+dex, $p<0.01$).

TGFβ1 mRNA was elevated in ME7 animals with respect to NBH animals. LPS did not produce substantial further increases in this mRNA species, although in the case of ME7+LPS this minor increase was statistically significant ($p<0.01$). Dexamethasone-21-phosphate did not have significant effects on CNS TGFβ1 transcription ($p>0.05$). These data are shown in figure 4.5g.

The results in figure 4.4 and figure 4.5 indicate that despite the failure of dexamethasone-21-phosphate to inhibit LPS-induced CNS cytokine transcription, it was successful in suppressing systemic cytokines IL-1β and IL-6 by approximately 80 and 90% and did offer some protection, though time-dependent, against the LPS-induced deficits suggesting that systemic cytokines are contributing to the LPS-induced deficits seen in the T-maze. This is consistent with the data in figure 4.4a showing that IL-1 contributes to T-maze deficit.

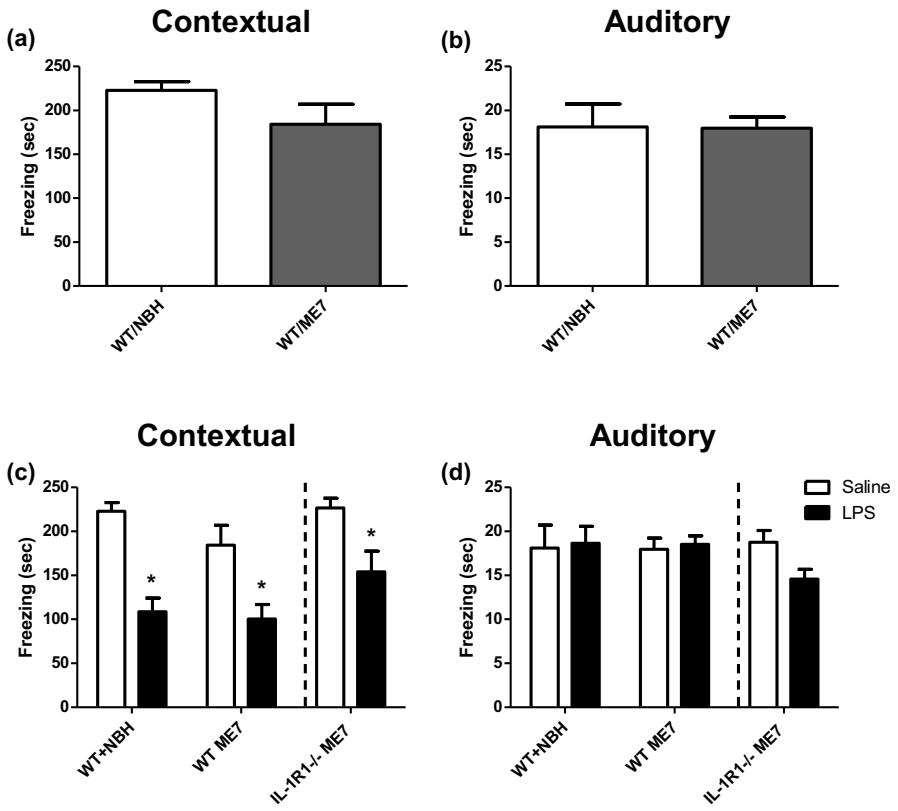


Figure 4.3 Performance of WT mice inoculated with NBH or ME7 on the contextual and auditory fear conditioning paradigm and following systemic challenge in WT NBH, WT ME7, and IL-1R1$^{-/-}$ ME7 mice with saline or LPS (100ug/kg i.p). Contextual (a) and auditory (b) memory was assessed in WT, 15 weeks post-inoculation with NBH or ME7 (n=5). The time spent freezing 48h following foot-shock at 0.4 mA for 2 seconds was recorded. Data are shown as mean ± SEM and were analysed by T-test. Performance of WT NBH, WT ME7 and IL-1R1$^{-/-}$ ME7 mice (n=5) in the contextual (c) and (d) auditory fear conditioning paradigm following systemic challenge with saline or LPS (100ug/kg). Data are expressed as mean±SEM. Data was analysed by 2-way ANOVA for WT NBH and WT ME7 and for WT ME7 and IL-1R1$^{-/-}$ ME7. Significant Bonferroni *post-hoc* after significant ANOVA are denoted by *p< 0.05 w.r.t saline controls.

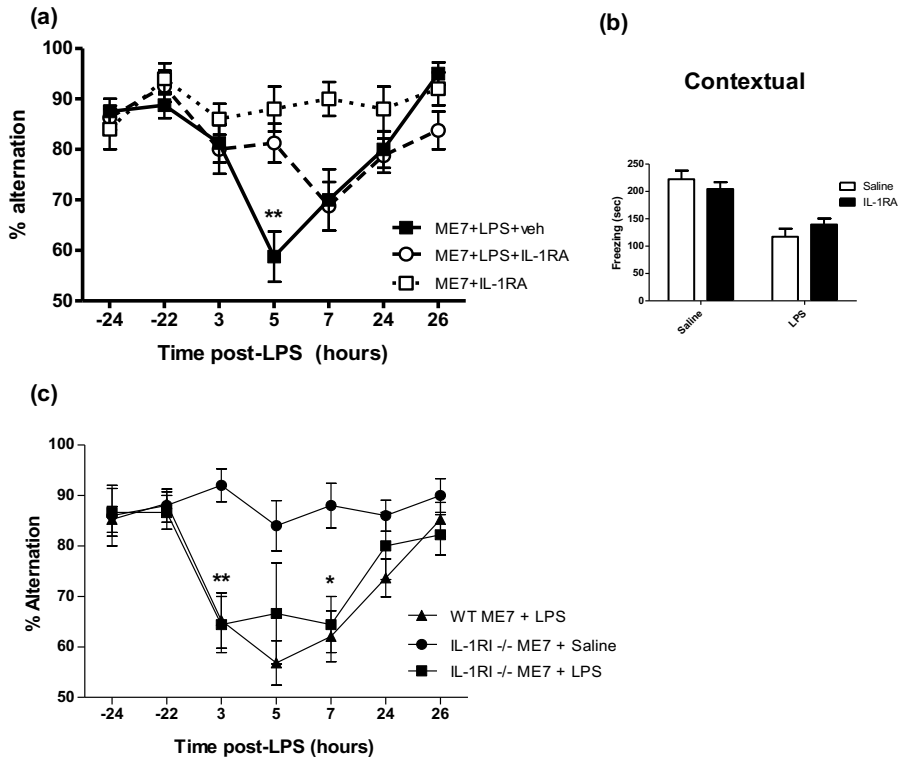


Figure 4.4 Performance of WT mice inoculated with ME7, challenged peripherally with LPS±vehicle or IL-1RA and WT and IL-1R1$^{-/-}$ ME7 mice challenged with LPS or saline on T-maze working memory task. (a)The performance of ME7 animals, 16 weeks post-inoculation with ME7 challenged with LPS (100µg/kg i.p.) was assessed by T-maze alternation in the presence or absence of IL-1 receptor antagonist (IL-1RA), administered at 10 mg/kg i.p. immediately following LPS. Data is shown as mean ± SEM and significant Bonferroni *post-hoc* difference between ME7+LPS and ME7+LPS+IL-1RA, after repeated measures ANOVA are denoted by **p < 0.01. (ME7+LPS n=16, ME7+LPS + IL-1RA n=16 and ME7 + IL-1RA n=10). (b) Consolidation memory for WT+saline (n=7), WT+IL-1RA, WT+LPS and WT+IL-1RA+LPS (n=11). Data are expressed as mean±SEM and were analysed by two-way ANOVA with LPS treatment and IL-RA as factors. (c) Working memory performance of IL-1RI$^{-/-}$ ME7 and WT ME7 mice in the T-maze alternation task. Mice were challenged with saline or LPS (100µg/kg i.p). Data are expressed as mean±SEM for WT ME7+LPS (n=19), IL-1R1$^{-/-}$ ME7 + LPS and IL-1R1$^{-/-}$ ME7 + saline (n=10). Data were analysed by 2-way repeated measures ANOVA with treatment and time as factors. Significant Bonferroni *post-hoc* differences between IL-1R1$^{-/-}$ ME7 + LPS and IL-1R1$^{-/-}$ ME7 + saline are denoted by *p<0.05 and **p<0.01. All mice were assessed for 10 trials 24 hours before acute challenge, 15 trials 3-9 hours post-challenge and 10 trials 24 hours after the challenge for figure a and c.

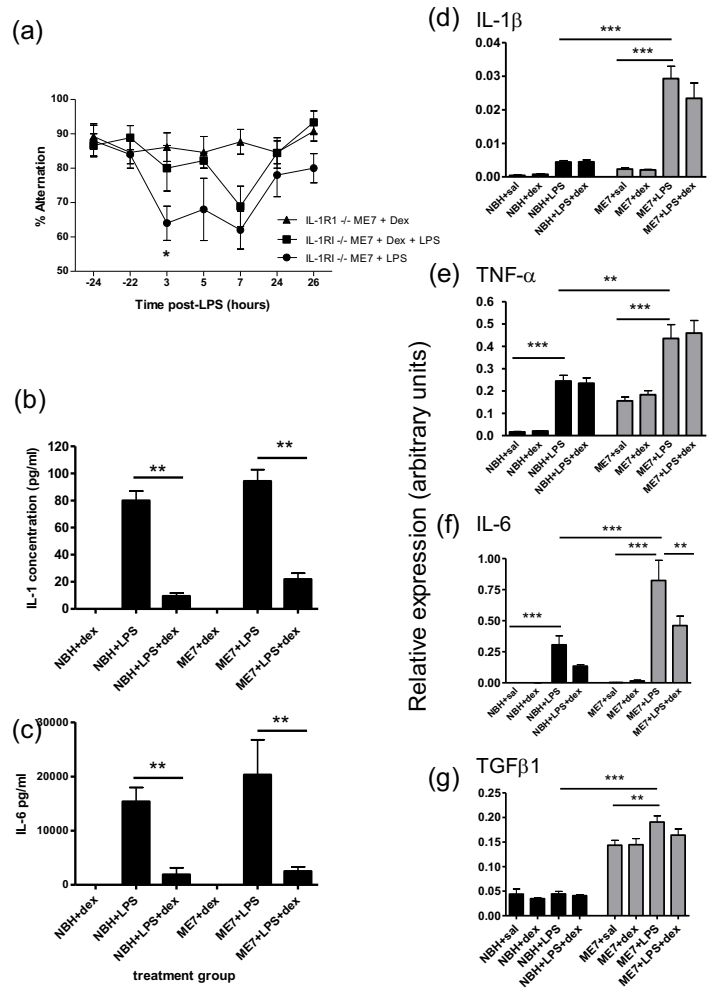


Figure 4.5 Impact of dexamethasone-21-phosphate on T-maze working memory task, LPS-induced systemic and CNS cytokine synthesis (a) Working memory performance of IL-1RI$^{-/-}$ and WT mice in the T-maze alternation task, 16 weeks post-ME7 inoculation. Mice were challenged with dexamethasone-21-phosphate (2mg/kg i.p.),LPS(100µg/kg i.p.) or LPS+dexamethasone. All mice were assessed for 10 trials 24 hours before acute challenge, 15 trials 3-9 hours post-challenge and 10 trials 24 hours after the challenge. Data are expressed as mean±SEM for IL-1R1$^{-/-}$ ME7 LPS (n=10), IL-1R1$^{-/-}$ ME7 DEX+ LPS (n=9) and IL-1R1$^{-/-}$ ME7+Dex (n=13).Data were analysed by 2-way repeated measures ANOVA with treatment and time as factors. Bonferroni *post-hoc* differences between ME7+LPS and ME7 LPS+ Dex are denoted by *p<0.05. (b) IL-1β and (c) IL-6 concentrations, as measured by ELISA, in NBH and ME7 animals (18-19 weeks) at 4 hours post-LPS (500 µg/kg) with or without pre-treatment with dexamethasone-21-phosphate (2 mg/kg) ** p<0.01 *** p <0.001 by Bonferroni *post-hoc* test after a significant main effect of treatment by one-way ANOVA; n=5 for NBH+LPS+dex and ME7+LPS+dex and n=4 for all other groups. TAQMAN quantitative PCR mRNA expression analysis of d) IL-1β, e) TNFα, f) IL-6 and g) TGFβ1. LPS (500µg/kg) induced marked expression of all cytokines except TGFβ1 but dexamethasone-21-phosphate did not inhibit this transcription in most cases (p > 0.05). Data were analysed by Bonferroni post-hoc test after a significant main effect of treatment by one-way ANOVA, ** p < 0.01, *** p < 0.001. n=9 for ME7+saline, n=4 for NBH+dex and NBH+LPS and n=5 for all other groups. All data have been presented as mean ± SEM.

4.2.7 Potential mechanism for systemic IL-1 effect on cognitive function

The results to date have demonstrated that IL-1RA can protect against the LPS-induced T-maze deficit. Background work in our laboratory has looked at the effects of IL-1RA blocking LPS and IL-1β action by looking at chemokine CXCL1 AND CXCL2 and cytokines IL-1β and TNF-α. Plasma was prepared 2 hours post-treatment with recombinant IL-1RA (10mg/kg, i.p.) and simultaneous injection of IL-1β, LPS or saline. IL-1 and LPS administration induced circulating levels of CXCL1 and CXCL2 in WT mice. IL-RA blocked the IL-1-induced increases on theses chemokines but had no effect on the LPS-induced increases. In addition, PTX-3 and IL-1β were increased in the hippocampus following LPS and IL-1 challenge, but only IL-1RA blocked the IL-1 induced increases in these transcripts. These data would suggest that that LPS signals to the brain by altering brain inflammatory profiles despite the absence of systemic IL-1 activity. When LPS is administered in normal and ME7 mice they had increased levels of chemokines CXCL1 and CXCL2 with IL-1RA not protecting against these increases. IL-1β and PTX-3 hippocampal expression was increased in both NBH and ME7 mice and IL-1RA did not impact on this increased expression. Indeed IL-1β mRNA was increased in ME7 LPS- treated mice after IL-1RA injection. These data would indicate that even though IL-1RA blocks circulating IL-1β actions, LPS still induces brain inflammatory activation including IL-1β (Skelly et al., 2018).

Furthermore, in this chapter Dexamethasone can block against the LPS-induced deficit without blocking central synthesis of TNF-α or IL-1β. Previous work from our laboratory has demonstrated that TNF-α and IL-1β when given systemically to mice can induce working memory deficit in the T-maze (Hennessy et al., 2017, Skelly et al., 2018). Together all of this would suggest that circulating IL-1 seems to be important for this deficit. How might this be? One possibility is via its effects on energy metabolism. TNF-α and IL-1β have been shown to induce hypoglycaemia in mice (Oguri et al., 2002, Endo, 1991, Del Rey et al., 2006) and LPS can induce hypoglycaemia in experimental animals. Hypoglycaemia is defined as blood glucose less than 8.2mg/100ml in mice (Rerup and Lundquist, 1966). IL-1 has been described as the major driver for LPS-induced hypoglycaemia (del Rey et al., 1998, Del Rey et al., 2006).

The next set of data addresses the question of whether this LPS-induced deficit in the T-maze observed in ME7 mice might be as a result of LPS-induced hypoglycaemia?

First, it was important to show that LPS can induce hypoglycaemia, therefore NBH and ME7 mice were challenged with vehicle or LPS (100μg/kg) and subsequent blood glucose levels were measured 5 hours post-challenge. There was no difference in basal levels of glucose in NBH and ME7 mice with p=0.6983 when analysed by student's T-test. LPS caused a decreased in glucose levels in the NBH and ME7 mice and disease per se did not influence the level of glucose after LPS challenge (figure 4.6a). Two-way ANOVA with treatment and disease as factors showed no significant effect of disease ($F=1.25$, df 1,23, $p=0.28$) but a significant effect of treatment ($F=121.74$, df 1,23, $p<0.0001$) and a significant interaction ($F=5.56$, df 1,23, $p=0.03$). Bonferroni *post-hoc* showed that there was a significant difference in glucose levels in NBH and ME7 mice after LPS challenge with $p<0.001$. The difference in glucose levels in ME7 mice to NBH mice is a modest increase with the levels of glucose in NBH LPS mice being 4.18mmol and ME7 mice are 3.54mmol.

In order to see what the effects of Insulin have on glucose concentration, NBH and ME7 mice were injected i.p with 400μg/kg of Insulin and the blood glucose concentrations at 90 minutes and 180 minutes were recorded. There was a significant decrease in glucose concentration after insulin treatment in NBH and ME7 mice (figure 4.6b). Two-way ANOVA with disease and treatment showed a significant effect of insulin treatment ($F=17.11$, df 2,20, $p=0<0001$) in NBH and ME7 mice and Bonferroni post-hoc analysis confirmed that this decrease in glucose levels was not statistically different in NBH mice compared to ME7 mice with $p>0.05$. It was then investigated whether this hypoglycaemia observed in NBH and ME7 insulin-treated mice would impact on their cognition in the T-maze. Briefly, NBH and ME7 mice were injected with insulin 40 minutes prior to starting the T-maze and their performance was assessed over a period of time. Figure 4.6c shows that only ME7 mice treated with insulin had a decrease on % alternation performance at 5 hours compared to the NBH insulin-treated mice. Data analysed with two-way repeated measure ANOVA with treatment and time as factors showed that there was a significant effect of treatment ($F=3.42$ df 3,135, $p=0.00$), time ($F=6.99$ df 3,135, $p=0.00$) and a significant interaction ($F=3.05$ df

9,135, p=0.00). Bonferroni post-hoc analysis showed that there was a significant difference in the ME7 insulin-treated mice to the NBH insulin-treated mice at 5 hours indicating that underlying pathology present in these mice leaves them more sensitive to cognitive disrupting effects of insulin-induced hypoglycaemia. Normal performance is restored at 24 hours. Together figure 4.6 b,c show that insulin provokes a profound hypoglycaemia in both NBH and ME7 mice but remarkably only ME7 insulin-treated animals show a decrease in working memory.

4.2.8 Uncoupling the roles of IL-1 and hypoglycaemia in LPS-induced sickness.

After establishing that hypoglycaemia induced working memory deficit in the T-maze, it was important to look at the effects of hypoglycaemia on IL-1 and glucose concentration and activity levels in mice. Therefore the next experiments investigated the effect of LPS-induced hypoglycaemia on IL-1 concentration, glucose concentration, and locomotor and rearing activity in the open-field.

The two doses of LPS administered during these sickness behaviour experiments were from two different batches of LPS. It was found in previous experiments in our laboratory that the higher dose of 250µg/kg of LPS in the new batch of LPS (#L5886) showed equivalent levels of CXCL1, TNFα production, and similar levels of decreased activity in the open field to the dose of 100µg/kg in the previous batch (#037K4086). All LPS was diluted in saline to the required dose. IL-1 levels were measured in the plasma using R&D Quantikine IL-1 ELISA according to the manufacturer's instructions.

7 month WT mice were injected with 2-deoxyglucose (2-DG; an inhibitor of glycolysis) 3 hours prior to LPS administration. LPS 100µg was administered 2 hours before the open field and glucose given 30 minutes prior to open field. The number of squares and rears in 3 minutes was recorded for all mice. Mice were then transcardially perfused 125 minutes post LPS-injection. Blood was collected in a heparinised tube and spun at 1.5g for 15 minutes. Glucose concentration in plasma was read on the YSI 2300 STAT Plus Glucose and Lactate Analyzer.

LPS treatment at 100µg/kg dose showed an increase in IL-1 production with glucose having no effect on this increase. However, when 2-DG was administered this resulted in a reduction in IL-1 being produced. Data was analysed by two-way ANOVA with LPS and glucose treatment as the two factors. There was a significant effect of LPS treatment with ($F=40.90$, df 1,15, $p<0.0001$) but no significant effect of glucose ($F= 0.2581$, df 1,15, $p=0.62$) and no significant interaction ($F=0.4504$, df 1,15 $p=0.51$).Bonferroni *post-hoc* showed a significant difference between the vehicle and the LPS treated group (***$p<0.001$). Furthermore, the effects of 2-DG on IL-1 production showed there was a significant difference between the LPS group to the 2DG+LPS group ($p=0.0296$) when analysed by t-test.

Glucose levels in the plasma were assessed in these mice. LPS resulted in decreased glucose levels in the plasma figure 4.8 (b). However, when glucose was administered this led to an attenuation of this decrease. When 2-DG, was co-administered with LPS, this resulted in significant increase in levels of glucose compared to LPS treated group due to the inability to utilise glucose in these mice. Data was analysed by two-way ANOVA with LPS and glucose treatment as the two factors. There was a significant effect of LPS treatment with ($F=27.76$, df 1,17, $p<0.0001$) but no significant effect of glucose($F= 2.608$, df 1,17, $p= 0.12$) and no significant interaction ($F=0.004517$, df 1,17 $p=0.95$). The administration of 2-DG, led to a significant increase in glucose levels compared to LPS-treated ($p=0.0031$) when analysed by t-test.

As glucose levels were decreased after LPS, it was then then important to assess whether sickness behaviour activity observed after treatment with LPS could be correlated with glucose levels? Could glucose be a predicator of activity?

To address this glucose concentration in the plasma was plotted against activity levels for vehicle, LPS and glucose treated mice. Linear regression analysis of this data shows that that there was a significant correlation for squares crossed (figure 4.7c) ($F=36.38$, df 1,21, $p<0.0001$) and for rearing (d) ($F=15.39$, df 1,21, $p=0.00$). Activity levels in the open field were assessed in these mice with co-administration of glucose and 2-DG. Macrophages go into a hyper glycolytic state in order to make IL-1β therefore it was predicated that inhibiting glycolysis with 2-DG would prevent the production of IL-1 as

it has been reported that LPS-induced expression of IL-1 is dependent on glycolysis (Tannahill et al., 2013).

LPS caused a decrease in squares crossed and rearing and glucose administration tended to attenuate this decrease. Despite the increase observed in glucose levels following 2-DG administration, there was a further significant decrease in activity in this group compared to the LPS vehicle group (figure 4.7 f). Data was analysed by two-way ANOVA with LPS and glucose treatment as the two factors. There was a significant effect of LPS treatment with ($F=37.02$, df 1,17, $p<0.0001$), but no significant effect of glucose ($F= 1.523$, df 1,17, $p=0.23$) and no significant interaction ($F=2.180$, df 1,17 $p=0.16$). Bonferroni *post-hoc* showed a significant difference between the vehicle and the LPS treated group ($p<0.001$). For Rears there was significant effect of LPS treatment with ($F=31.00$, df 1,17, $p<0.0001$), and a trend towards, though not no significant effect of glucose ($F= 3.687$, df 1,17, $p=0.07$) and interaction ($F=3.687$, df 1,17, $p=0.07$). Bonferroni *post-hoc* showed a significant difference between the vehicle and the LPS treated group ($p<0.001$). There was also a significant difference in the LPS and LPS plus glucose group ($p <0.05$).

The same experiments were carried out using 250µg /kg dose of LPS. In these experiments glucose concentration was measured in blood using a glucometer to rule out any confounding issues with decreased glucose concentration in vehicle treated mice because of the delay in reading glucose levels in these mice with the storing of plasma. Similar results to that seen in the 100 µg /kg dose could be seen in the mice challenged with 250 µg /kg dose. IL-1 levels were increased after LPS administration with glucose having no effect on this increase. 2-DG showed significant reduction in IL-1 production. Data were analysed by 2 way ANOVA and showed there a significant effect of LPS treatment with ($F=29.88$, df 1,25, $p<0.0001$) but no significant effect of glucose ($F=0.8106$, df 1,25, $p=0.38$) and no significant interaction ($F=0.2352$, df 1,25 $p=0.63$). Bonferroni *post-hoc* showed a significant difference between the vehicle and the LPS treated group (** $p<0.01$). Furthermore, the effects of 2-DG on IL-1 production showed there was a significant difference between the LPS group to the 2DG+LPS group ($p=0.0012$) when analysed by t-test.

Glucose concentration was decreased following LPS treatment and glucose protected against this LPS-decreased glucose. 2-way ANOVA analysis showed that there was a significant effect of LPS treatment with ($F=60.00$, df 1,27, $p<0.0001$) and a significant effect of glucose ($F=6.721$, df 1,27, $p=0.01$) but no significant interaction ($F=0.7495$, df 1,27 $p=0.39$). Bonferroni *post-hoc* showed a significant difference between the vehicle and the LPS treated group with ** $p<0.01$. The administration of 2-DG, led to a significant increase in glucose levels compared to LPS+vehicle-treated mice ($p=0.0142$) when analysed by t-test.

Linear regression analysis of activity levels against glucose concentration showed there were significant correlations for squares crossed (figure 4.8c; $r^2=0.659$; $F=15.89$, df 1,31, $p<0.0004$) and for rearing which was also significant figure 4.8d $r^2=0.4475$; $F=8.895$, df 1,31, $p=0.01$). Activity levels in the open field were assessed in these mice with co-administration of glucose and 2-DG. LPS caused a decrease in squares crossed and rearing and glucose administration tended to attenuate this decrease. Despite the increase observed in glucose levels following 2-DG administration, there was a further significant decrease in activity in this group compared to the LPS vehicle group (figure 4.8 f). Open field activity showed that there was a decrease in rearing and squares crossed after LPS treatment and glucose protected against this decrease. 2-DG co-administration resulted in further reduced activity in the open field compared to LPS treated mice. Data analysed by 2 way ANOVA showed a significant effect of LPS treatment for squares crossed with ($F=13.39$, df 1,27, $p=0.00$), and interaction between LPS and glucose ($F=10.48$, df 1,27 $p=0.00$). Bonferroni *post-hoc* analysis showed a significant difference between LPS and LPS+glucose ($p<0.01$). For rearing, there was significant effect of LPS treatment with ($F=4.53$, df 1,27, $p=0.04$), but no significant effect of glucose ($F= 1.12$, df 1,27, $p=0.30$) and no significant interaction ($F=0.49$, df 1,27, $p=0.49$). Bonferroni *post-hoc* analysis showed no significant difference between LPS and LPS+glucose.

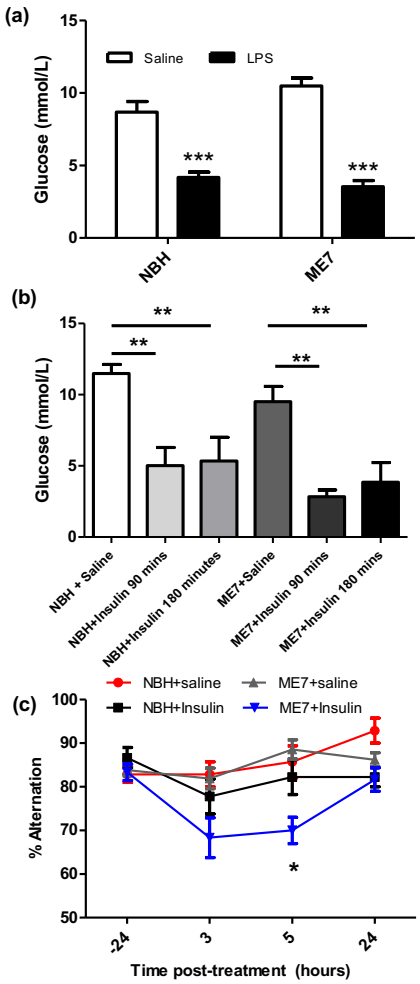


Figure 4.6 Glucose concentration in NBH and ME7 mice following systemic challenge with LPS, saline or insulin and cognition in the T-maze working memory task following challenge with Insulin or saline in NBH and ME7 mice (a) NBH and ME7 mice were injected with saline or LPS 250µg/kg i.p and the glucose levels in the blood were measured 5hrs post-injection. All data have been presented as mean ± SEM. Data was analysed by Bonferroni *post-hoc* test after significant main effect of LPS treatment by two-way ANOVA denoted by *** $p<0.001$; n=6 for saline+saline and n=7 for NBH+LPS, ME7+saline and ME7+LPS. (b)NBH and ME7 mice were injected with saline or insulin 400µg/kg i.p and the glucose levels in the blood were measured 90 minutes and 180 minutes post-injection. All data have been presented as mean ± SEM. Data were analysed by Bonferroni *post-hoc* test after a significant main effect of insulin treatment by two-way ANOVA, denoted by **$p<0.01$; n=4 for NBH+saline, n=5 for NBH+Insulin 90m, n=4 for NBH+Insulin 180m, n=4 for ME7+saline, n=4 for ME7+Insulin 90m, n=5 for ME7+Insulin 180m. No significant difference between NBH Insulin or ME7 insulin treated mice. (C) Cognition in the T-maze following insulin or saline administration in NBH (n=7 saline, n=9 insulin) and ME7 mice (n=21 saline, n=12 insulin). Data presented as mean ± SEM and were analysed by two-way repeated measures ANOVA with treatment and time as the two factors. Bonferroni *post-hoc* test after a significant main effect of insulin treatment in NBH v's ME7 at 5 hrs denoted by *$p<0.05$.

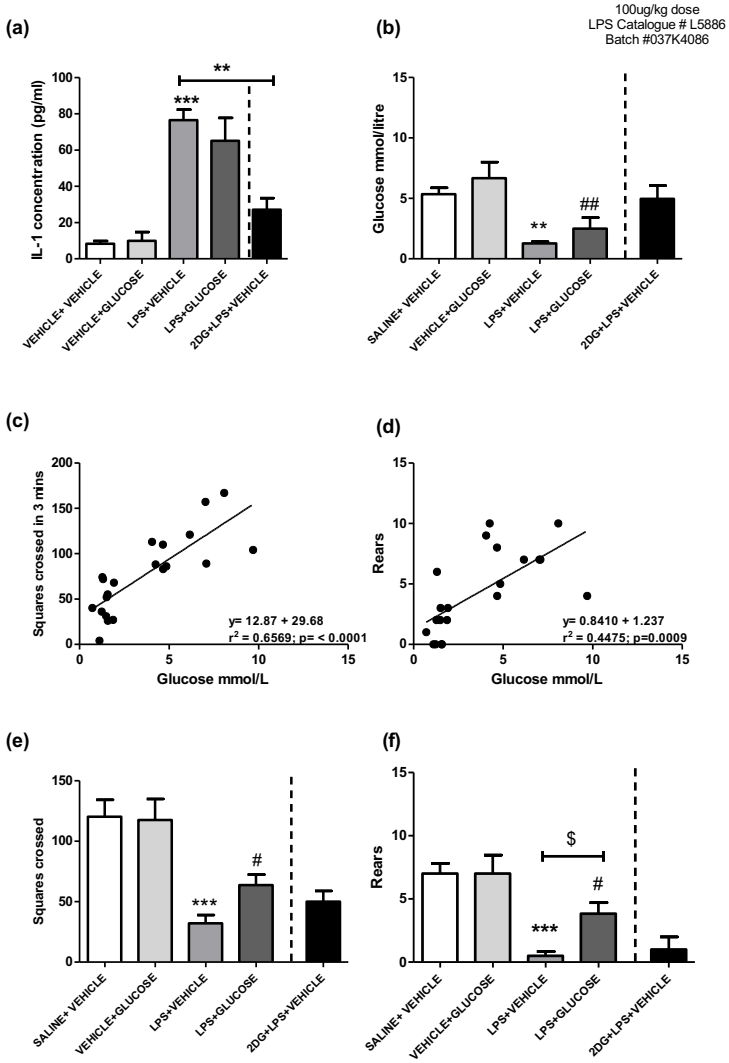


Figure 4.7 Systemic IL-1 levels and glucose concentration in plasma and activity levels following 100µg/kg LPS challenge with co-administration of glucose or 2-DG in WT mice. (a) Systemic levels of IL-1 and glucose (b)in the plasma following injection of Vehicle, LPS (100µg/kg) ±glucose(2g/kg) or ± 2-DG (2g/kg) in WT mice. All data have been presented as mean ± SEM for n=3 for vehicle+vehicle, n=4 for vehicle+glucose, n=6 for LPS+glucose ,LPS+Vehicle, n=3 for LPS+2-DG. Data were analysed by Bonferroni *post-hoc* test after a significant main effect of LPS treatment by two-way ANOVA, denoted by ***p<0.001 and **p<0.01. ## denotes significant difference between vehicle+glucose and LPS+glucose group with p<0.01 for 100µg/kg dose(c,d) Glucose concentration versus squares crossed (a) and rearing activity (b) in mice treated with vehicle, glucose and LPS 100ug/kg i.p. Activity and glucose levels were measured 2.5hr post-treatment. Data was analysed by linear regression analysis where n=5 for Vh+Vh and LPS+glucose, n=6 for LPS+glucose and n=4 for veh+glucose. (e) Squares crossed and (f) rearing activity measured in three minutes in the open field in WT mice following challenge with LPS or glucose. Data analysed by two- way ANOVA and significant Bonferroni *post-hoc* differences denoted by ***p<0.001, # p<0.05, and $ p<0.05.

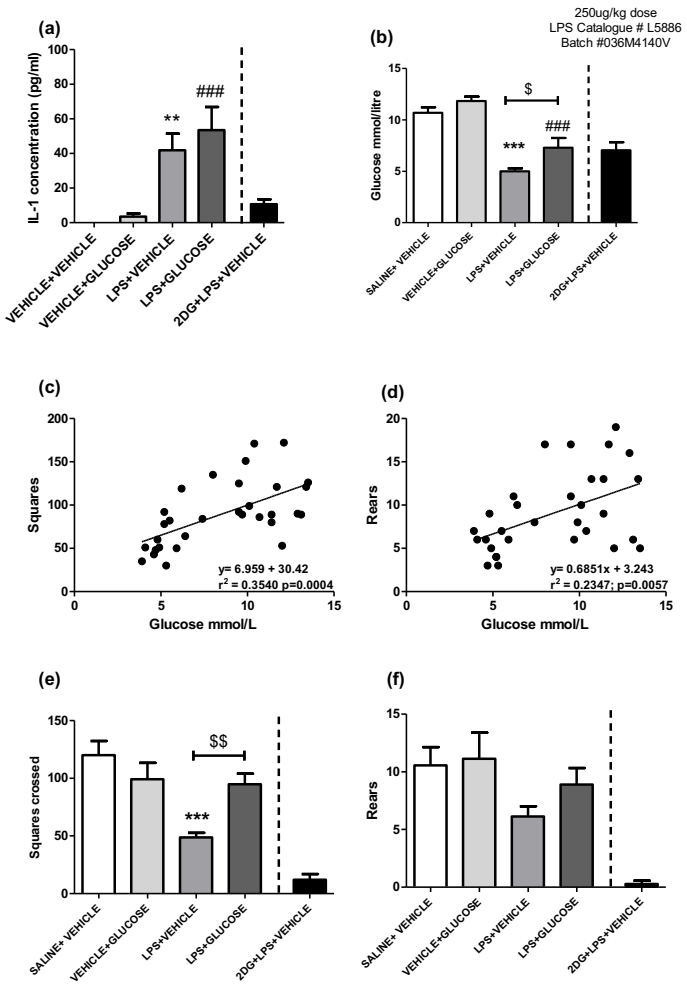


Figure 4.8 Systemic IL-1 levels and glucose concentration in blood and activity levels following 250µg/kg LPS challenge with co-administration of glucose or 2-DG in WT mice. (a) Systemic levels of IL-1 and glucose(b)in the blood following injection of Vehicle, LPS (100µg/kg) ±glucose(2g/kg) or ± 2-DG (2g/kg) in WT mice. All data have been presented as mean ± SEM for n=7 for vehicle+vehicle, vehicle+glucose, LPS+glucose and n=8 for LPS+Vehicle, n=5 for LPS+2-DG. Data were analysed by Bonferroni *post-hoc* test after a significant main effect of LPS treatment by two-way ANOVA, denoted by ***p<0.001 and **p<0.05. ### denotes significant difference between vehicle+glucose and LPS+glucose group with p<0.001 and $ denotes significant difference between LPS and LPS+glucose treated mice with p<0.05. (c,d) Glucose concentration versus squares crossed (a) and rearing activity (b) in mice treated with vehicle, glucose and LPS 250ug/kg i.p. Activity and glucose levels were measured 2.5hr post-treatment. Data was analysed by linear regression analysis where n=7 for Vh+Vh, Vh+Glucose, n=8 for Vh+LPS and n=9 for LPS+Glucose. (e) Squares crossed and (f) rearing activity measured in three minutes in the open field in WT mice following challenge with LPS or glucose. Data analysed by two- way ANOVA and significant Bonferroni *post-hoc* differences denoted by ***p<0.001, $$ denotes significant difference between LPS and LPS+ glucose treated mice with p<0.01.

4.2.9 The role of hypoglycaemia in LPS-induced deficit in the T-maze.

Figure 4.6 showed that hypoglycaemia can induce working memory deficit in the T-maze in ME7 mice and the data in figure 4.7-4.8 indicate that this hypoglycaemia can result in decreased activity in the open field. However this reduction in activity in the open field can be ameliorated by glucose administration. Therefore it was important to address whether restoring glucose levels to ME7 mice could lead to an improvement on cognitive function in the LPS-induced deficit observed in the T-maze in figure 4.1 c-d. To investigate this ME7 mice were administered with vehicle or LPS (100µg/kg i.p) 3 hours prior to starting the T-maze. Then mice were also given a vehicle or glucose (2g/kg) thirty minutes prior to the start of T-maze. Figure 4.9 showed that ME7 mice given LPS have a significant cognitive impairment at 5 hours and that glucose mitigates this deficit. Data were analysed by two-way ANOVA with treatment and time as factors and show that there was a significant effect of treatment ($F=13.57$, df 3,240, $p<0.0001$) and time ($F= 3.87$, df 4,240, $p=0.00$) and a significant interaction ($F=3.74$, df 12,240 $p<0.0001$). Bonferroni *post-hoc* analysis confirmed a significant difference between ME7+LPS mice and ME7+LPS+glucose mice (**$p<0.01$). This data would suggest that glucose is the driver of the LPS-induced deficit in the T-maze.

The data in figure 4.4 and 4.5 indicate that IL-1RA and Dexamethasone can protect against the LPS-induced deficit. Previous work in our laboratory has also shown that SC-560, COX-1 inhibitor, is successful at blocking the LPS-induced deficit in the T-maze (Griffin et al., 2013). It was therefore important to address if these inhibitors protect against the LPS-induced deficit by altering glucose levels? To investigate this question, WT mice were injected with LPS with co-administration of IL-1RA, Dexamethasone or SC-560 and levels of glucose in blood and activity levels in the open field were assessed in these mice.

LPS caused a decrease in glucose levels and IL-1RA, Dexamethasone or SC-560 offered no protection against this reduction. One way ANOVA analysis comparing all the LPS-treated groups showed a significant effect of treatment with ($F=5.737$, df 3,19 $p=0.01$)

Bonferroni *post-hoc* analysis showed no significant difference in the IL-1RA, Dexamethasone or SC-560-treated mice with $p>0.05$.

IL-1RA or SC-560 administration did not improve activity levels in the open-field compared to the dexamethasone which attenuated the LPS-induced deficits. There was a significant effect of treatment when analysed by one way ANOVA with ($F=9.506$, df 3,19 $p=0.00$) and Bonferroni *post-hoc* showed a significant effect of dexamethasone treatment against LPS treated mice with $p<0.05$. Finally IL-1 and TNF were increased in the plasma following LPS treatment and only dexamethasone showed an attenuation of this induction in IL-1 with Bonferroni *post hoc* test showed a significant difference between these two groups with $p<0.05$ after significant ANOVA ($F=4.763$, df 3,19 $p=0.01$).

Collectively this data would suggest that inhibiting COX1 and prostaglandin production, endogenous IL-1 and systemic cytokines does not affect glucose levels. This would suggest that the protection offered by these inhibitors against the LPS-induced deficit in the T-maze is acting via different pathways to that of glucose production, i.e LPS-induced hypoglycaemia is not the only factor driving memory impairment in the T-maze.

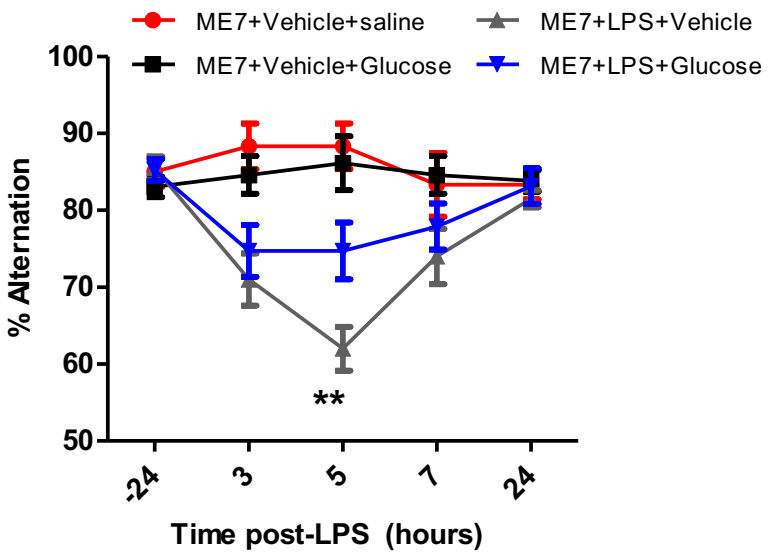


Figure 4.9 Cognition in the T-maze working memory task following challenge with glucose or vehicle in ME7 mice. Cognition in the working memory t-maze task for ME7 mice following i.p challenge with LPS or glucose (2g/kg). Data presented as mean ± SEM for n=12 ME7+Veh+Sal, n=13 ME7+Veh+glucose, n=20 ME7+LPS+Veh and n=19 ME7+LPS+glucose. Data were analysed by two-way repeated measures ANOVA with treatment and time as the two factors. Bonferroni *post-hoc* test after a significant main effect of glucose treatment in ME7 at 5 hrs denoted by **p< 0.01

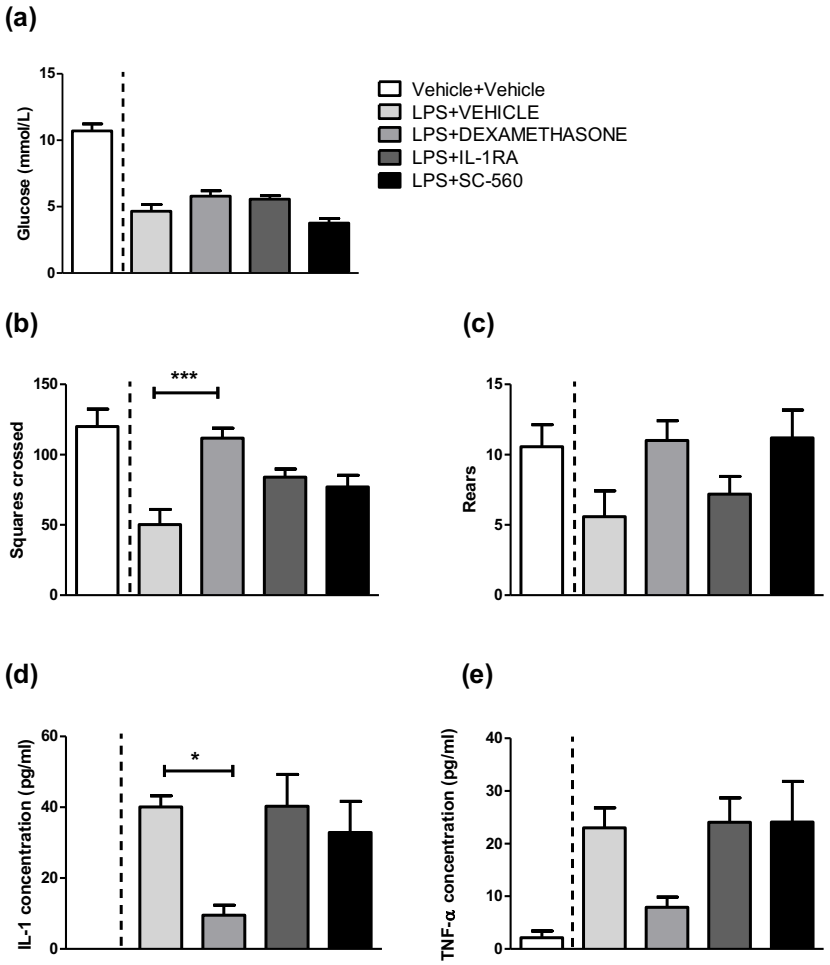


Figure 4.10 Glucose, open field activity, IL-1β and TNF-α concentration following co-administration of LPS with Dexamethasone, IL-1RA or SC-560 in WT mice. (a) Systemic levels of glucose in the plasma following i.p injection of LPS (250µg/kg) along with Dexamethasone (2mg/kg), IL-1RA (10mg/kg) or SC-560 (30mg/kg) in WT mice. Vehicle/vehicle data shown for comparative purposes.(b),(c) squares crossed and rearing activity measured in three minutes in the open field. Systemic levels of IL-1 (d) and TNF levels in the plasma (e); n=7 for vehicle+vehicle treatment, n=5 for all other treatments. All data have been presented as mean ± SEM. Data were analysed by Bonferroni post-hoc test after a significant main effect of treatment by one-way ANOVA. Significant Bonferroni post-hoc differences denoted by ***p<0.001 and * p<0.05.

4.3 Discussion

The data in this chapter indicate LPS has dissociable effects across two different hippocampal dependent memory tasks; LPS impairs in the CFC but not in the working memory T-maze task. In contrast, in the T-maze, LPS-induced deficit was observed in mice inoculated with ME7 prion disease, and the severity and duration of this LPS-induced deficit increased with progression of disease. IL-1RA can protect against this LPS-induced deficit in the T-maze but not in the CFC task without blocking brain IL-1 synthesis. Dexamethasone-21-phosphate failed to inhibit LPS-induced CNS cytokine transcription but was successful in suppressing systemic cytokines and showed some protection against the LPS-induced deficit though time-dependent, indicating that systemic cytokines contribute to the LPS-induced deficit in the T-maze. Further examination of possible peripheral explanations for this CNS dysfunction revealed that LPS-induced hypoglycaemia could be contributory. Inhibiting glycolysis and IL-1 with 2-DG, lead to decreased activity and increased sickness behaviour in the open field. Glucose administration could protect against this decreased activity and ameliorated the LPS-induced deficit in the T-maze, indicating that systemic inflammation mediated deficits are partially mediated by hypoglycaemia.

4.3.1. Systemic inflammation impacting on hippocampal function

The current study has demonstrated that LPS can induce a deficit in freezing in the contextual fear conditioning task thus impairing contextual fear memory consolidation, which is not observed in the hippocampal independent auditory cue fear conditioning task. In addition, the same dose of LPS did not induce a deficit in another hippocampal dependent working memory task, the T-maze alteration task; however it can impair working memory in mice inoculated with ME7 prion disease. This deficit in the T-maze in mice with prior hippocampal synaptic loss (Murray et al., 2012) could be partially protected by systemic IL-1RA but IL-1RA failed to protect against the LPS-induced deficit in the contextual fear conditioning paradigm. These data suggest that systemic inflammation induced by endotoxin LPS can induce cognitive dysfunctions in multiple domains and with the two hippocampal-dependent tasks presented in this chapter, these impairments are dependent on dissociable IL-1 processes.

Recent work from Czerniawski and colleagues demonstrated that when rats were injected with LPS, they had no impairment in context discrimination, however the same dose of LPS, resulted in impaired memory consolidation in the contextual fear conditioning task (Czerniawski and Guzowski, 2014). They also showed in a subsequent study that rats displayed impaired context-object discrimination, but no impairment in the Morris water maze task or novel recognition task (Czerniawski et al., 2015). These studies all suggest dissociations in hippocampal memory tasks following systemic challenge with LPS, consistent with the data presented in figure 4.9a-c.

From the literature, it has been well described that LPS can impair learning and memory (Pugh et al., 1998, Thomson and Sutherland, 2005, Terrando et al., 2010, Avital et al., 2003). Most of these studies have demonstrated that contextual memory, a hippocampal dependent memory, is impaired in rodents following surgery or challenge with LPS (Goshen et al., 2007, Cibelli et al., 2010). Further studies have shown that age can have a bigger effect in the CFC. Injection of *E-coli* into 24 month old rats resulted in decreased freezing in the CFC task and increased levels of IL-1β in the hippocampus (Barrientos et al., 2006). However the ME7 mice in this chapter at 15 weeks post-inoculation when challenged with LPS did not show exaggerated levels of decreased freezing compared to normal mice despite having exaggerated levels of IL-1β in the hippocampus. The ME7 mice after LPS also had exaggerated levels of TNF and IL-6 compared to normal mice. A possible explanation for difference in these results to that of Barrientos and colleagues is that they used 24 month old rats, and it is possible that if CFC was carried out at the later time point of 20 weeks of ME7 disease, where there is more vulnerability present , one would observe a difference in freezing levels. This would suggest that cognitive vulnerability can be mediated by age-related changes in the glial environment that can result in an exaggerated brain pro-inflammatory response to infection. It is important to point out that just because IL-1 impairs consolidation of contextual and impairs LTP this does not imply that all effects of IL-1 on cognitive function can be explained in this way.

Chen and colleagues showed an LPS-induced deficit in aged mice. In their study, the authors showed that when aged mice were challenged systemically with LPS, the hippocampal processing is more easily disrupted in these aged animals than in younger ones, represented by increased distance and latency to exit the radial arm maze (Chen et al., 2008). Similarly, Field and colleagues demonstrated that mice with selective lesioning of the basal forebrain cholinergic system after challenge with LPS systemically, show a deficit in the hippocampal-dependent working memory task (Field et al., 2012).

4.3.2 Systemically induced IL-1 on hippocampal-dependent memory tasks

In the current study, the CFC deficit was not dependent on IL-1R1 and while IL-1RA failed to protect against this LPS-induced deficit, it did offer partial protection in a time dependent manner in the alternation T-maze task.

IL-1RA data in the CFC presented in this chapter differ to the studies conducted by Pugh and colleagues (Pugh et al., 1998) and the studies by Terrando (Terrando et al., 2010). These authors showed that IL-RA can block LPS-induced deficits in the context. A possible explanation for the difference in results, is in the studies conducted by Pugh, the IL-1RA was injected i.c.v after conditioning, whereas in the experiments conducted by Terrando, the IL-RA was injected subcutaneously before LPS administration. In the study presented in this chapter, the IL-1RA was administered intraperitoneally. Peripherally administered IL-1RA (17 kDa) can cross the blood-brain barrier (BBB) to a limited extent (Gutierrez et al., 1994, Banks et al., 1994). In addition, centrally administered IL-1RA has been shown to offer protection from peripheral infection mediated-deficits in memory consolidation in aged animals (Frank et al., 2010, Barrientos et al., 2012). Cawthorne and colleagues showed using PET imaging that a very low level of IL-1RA can penetrate into the CNS when injected intravenously (Cawthorne et al., 2011). Other studies have shown that, a low percentage of IL-1RA reaches the CSF in rats and humans after being injected intravenously, but this is sufficient to be therapeutically effective in ischemia (Clark et al., 2008).

IL-1RA offered partial protection in the T-maze task that was time-dependent. Seven hours post-challenge, the protective effects of IL-1RA were diminished. A possible

explanation for this time-dependent effect is IL-1RA is reportedly cleared rapidly by the kidneys, (Cawthorne et al., 2011) and is also reported to have an initial half—life of 21 minutes and a terminal half-life of 108 minutes (Granowitz et al., 1992) so by the 7th hour in the T-maze the therapeutic efficacy would be predicted to be much lower with only a fraction of the original concentration being present. Further analysis in our laboratory has demonstrated that low levels of human IL-1RA are detectable in the hippocampus of NBH and ME7 mice and these concentrations of IL-1RA are equivalent in both. These levels (60-70 pg/ml/mg protein) are almost 7,500-fold lower than plasma levels (481,511 ± 95,460 pg/ml). This would indicate that a tiny fraction of IL-1RA penetrates the brain parenchyma and that this is not significantly higher in neurodegeneratively diseased (ME7) mice. Although IL-1RA is known to have a short half-life, falling to 20% 3 hours post-intraperitoneal injection (Barrientos et al., 2012), the blood levels at 3 hours post-treatment remained approximately 2000-fold higher than circulating IL-1β at what is a key stage in working memory dysfunction and contextual memory consolidation tasks.

The results in figure 5.12 show that in the T-maze IL-1R1 ME7 mice when treated with LPS show similar deficits to the WT, suggesting that IL-1R1 is not essential for the LPS-induced deficits in the however, IL-1 signalling obviously contributes since IL-1RA offered significant protection. It has been reported that other pro-inflammatory cytokines can compensate for loss of IL-1 signalling in IL-1R1$^{-/-}$ mice (Bluthe et al., 2000a) and previous work from our laboratory has shown that LPS can also induce IL-1 and other pro-inflammatory cytokines during ME7 disease(Cunningham et al., 2009, Murray et al., 2012). More recently, ME7 mice when injected with TNF-α display a deficit in the T-maze (Hennessy et al., 2017) and TNF-α has been shown to be expressed after systemic challenge with LPS. This would suggest that in the lack of IL-1 signalling, TNF-α can mimic LPS-induced deficits and also induce sickness behaviour (Bluthe et al., 2000a).

Dexamethasone-21-phosphate can block NFκB trans-activation and block the synthesis of multiple pro-inflammatory cytokines. In this chapter, systemic and central cytokine levels were measured after Dexamethasone-21-phosphate and figure 5.13 shows that

Dexamethasone did not block central cytokines, but was 90% effective in blocking systemic IL-1 and IL-6 secretion. The systemic blocking of IL-1 and IL-6 secretion in this study is consistent with the study by Teeling and colleagues, where the authors demonstrated that Dexamethasone-21 phosphate can inhibit the systemic secretion of IL-1β, TNF-α and IL-6 (Teeling et al., 2010). In addition, IL-1R1$^{-/-}$ ME7 mice pre-treated with Dexamethansone-21 -phosphate 60 minutes before LPS challenge, did not exhibit the LPS-induced deficit 3-7h post-challenge that were seen in IL-1R1$^{-/-}$ ME7 LPS treated mice, suggesting that systemic cytokines are contributing to LPS-induced deficits in diseased mice.

4.3.3 Hypoglycaemia contributes to LPS-induced deficit in the T-maze.

The data in this chapter showed that dexamethasone administration was successful in blocking systemic cytokines IL-1, IL-6 but central IL-1 was not blocked. The effect of this central IL-1 inducing hypoglycaemia was of interest. It has been reported in the literature that IL-1 can induce hypoglycaemia (Del Rey et al., 2006, del Rey et al., 1998).

The authors also showed in the same study that IL-1 induced hypoglycaemia lasts longer than insulin-induced hypoglycaemia. The mice injected with IL-1 were still below basal levels 24 hour later compared to the insulin-treated mice suggesting that IL-1 induced hypoglycaemia is prolonged. In this study the authors show that the IL-1 can reset glucose homeostasis at central levels by expression of IL-1 β in the hypothalamus of these IL-1 treated mice (Del Rey et al., 2006). Arising from these studies an interesting study to investigate would be injecting mice with LPS and administration IL-1RA icv to see if blocking IL-1 centrally would alleviate the LPS-induced deficit observed in the experiments in this chapter. This was also seen in the studies by Vogel and colleagues where they also showed that mice injected with a recombinant IL-1 receptor antagonist 3 days prior to LPS could reverse the LPS-induced hypoglycaemia (Vogel et al., 1991) Another study looking at IL-1β deficient mice showed a significant reduction in glucose levels after 5mg/kg but not 5μg/kg of LPS measured 6 hours post-challenge indicating that endogenous IL-1 does play a role in hypoglycaemia and that IL-1β is not essential (Fantuzzi et al., 1996). This is

contradictory to the studies of Del Rey and colleagues in which they showed that IL-1 induced hypoglycaemia. Oguri and colleagues demonstrated that mice that were deficient in IL-1 α or IL-1 β after challenge with LPS experienced hypoglycaemia but this hypoglycaemia was not present in IL-1 α/ β knock out mice. In the same studies, IL-1α and IL-β and TNF-α could also induce hypoglycaemia in IL-1 α/β knock out mice and WT control mice thus supporting the idea that IL-1 is a prerequisite for the hypoglycaemic effect of LPS (Oguri et al., 2002).

The data in this chapter also demonstrated that insulin-injected mice lead to cognitive impairment in the T-maze in ME7-insulin treated mice indicating that the ME7 mice are more sensitive to the hypoglycaemic effect of insulin. Coinciding with this exacerbated hypoglycaemia in ME7 mice, the mice exhibited an acute and transient deficit that was no longer apparent at 24hr. Similar results have shown that when insulin was injected into 12hr fasted mice, these insulin-injected mice had significant hypoglycaemia and near complete social withdrawal and this effect was absent 24 hr post-insulin injection. The hypoglycemia-associated withdrawal in this study was dependent on catecholamines as selective beta-2 adrenergic receptor antagonist butoxamine was successful at protecting against the insulin-induced social withdrawal (Park et al., 2008). Hypoglycemia can increase plasma levels of both epinephrine and norepinephrine (Hoffman, 2007). Follow up work by the same group also showed that insulin injected mice that were fasted 12hr prior to insulin administration displayed depressive-like behaviours with increased immobility in the forced swim test and reduced saccharin preference. The authors demonstrated in this study that these depressive behaviours induced by hypoglycaemia could be reversed when antiadrenergic agents were administered indicating that these depressive like behaviours are as a result of hypoglycaemia through adrenergic pathways (Park et al., 2012).

4.3.4 Glucose enhancement on cognitive function.

In this chapter, it was demonstrated that glucose co-treatment led to an attenuation of LPS-induced deficit in the T-maze. This would suggest that the addition of glucose lead to an enhancement of hippocampal dependent memories in these vulnerable

mice. This is supported by other studies in the literature where administration of glucose following LPS treatment led to improved performances in the WIN shift and win stay task in rodents, and improved working memory performance in the radial arm maze (Ragozzino et al., 1998). In the former study the authors showed that performance in rats, in the four arm cross maze for spontaneous alternation was increased following injection of glucose which was accompanied by an increase in hippocampal acetylcholine (ACh) while rats were performing the task. This increase in ACh indicates that glucose can enhance memory by increasing the release of ACh. Indeed the same group showed that intrahippocampal injection of glucose increased ACh in the hippocampus which lead to improved alternation scores suggesting a direct effect of glucose increasing ACh (Ragozzino et al., 1998)

Further studies supporting the role of glucose impacting on ACh and memory demonstrated that when Scopalamine, an muscarinic antagonist for muscarinic ACh receptor, was administered to mice, it lead to decreased performance in spontaneous alternation and these deficits were blocked when glucose was administered (Stone et al., 1991). The authors also showed in previous studies that glucose could attenuate the effect of cholinergic antagonists showing increased locomotor activity in mice following glucose administration (Stone et al., 1988, Stone et al., 1991). Messier and colleagues demonstrated that a post-training glucose injection in mice following the operant bar pressing task lead to an improved retention of the operant bar pressing response the next day in an appetitive- motivated task (Messier, 1998). Following work by the same group showed that glucose could significantly attenuate the amnesia produced by a post-training scopolamine injection in mice trained for an operant bar pressing task. In this study the authors showed that glucose did not interrupt with the high affinity choline uptake in resting mice, but did reduce the amplitude of the increase in high-affinity choline uptake in trained mice thus providing evidence of glucose on hippocampal cholinergic activity under conditions of high acetylcholine demand (Messier et al., 1990) More recently Kealy and colleagues demonstrated that when rats were pre-treated with glucose, that there was no enhancement of performance in the spontaneous alternation task, and no accompanying changes in hippocampal glucose. In their study, the authors showed by

using an oxygen sensor that there was an increase in hippocampal oxygen during plus maze exploration, indicating an increase in hippocampal activity despite a lack of any changes in hippocampal glucose (Kealy et al., 2017).

The attenuating effects of glucose on memory can also be seen in human studies. More recently a fluorodeoxyglucose PET scan showed that a reduction in medial temporal lobe glucose caused by an typhoid vaccination lead to reduced spatial memory performance but not medial temporal lobe independent memory in patients (Harrison et al., 2014). In addition Alzheimers patients with hyperinsulinaemia had improved memory compared to Alzheimers patients that were hyperglycemic indicating that glucose is necessary for memory improvement in these Alzheimer's patients (Craft, 2007).

Together all these studies would suggest that glucose can attenuate memory impairments in neurodegenerative and vulnerable rodents and patients which is consistent with what was seen in the data in this chapter. It would next be important to look at the metabolism of glucose to establish which glucose transporters are involved in glucose metabolism.

4.3.5 Delirium and Hypoglycaemia

Delirium, which is characterised by inattention and profound cognitive impairment, is an acute and transient disorder that is highly prevalent in elderly and demented patients (Burns et al., 2004). It is a very common medical condition and it is estimated that one in eight hospitalised patient will experience delirium (Cunningham and Maclullich, 2013). In surgical patients over the age of 65 years, 15-53% will have postoperative delirium which leads to multiple adverse outcomes that include increased length of stay, patient distress, increased morbidity and mortality, loss of independence and increased healthcare costs (Fong et al., 2009). There are large economic and social burdens associated with delirium. The care of older patients in hospital with delirium has accounted for more than 49% of all hospital days and $6.9 billion of hospital expenditures are attributable to delirium costs (Inouye, 2006). The American Diabetes association (ADA) defines hypoglycaemia as blood glucose less than 60mg/dl in humans. Hypoglycaemia can be found in hospitalized patients,

including those with Diabetes Mellitus. Hypoglycaemia is associated with several complications, including neurologic and ischemic events, longer hospital stay and increased mortality risk (Krinsley et al., 2011a, Krinsley et al., 2011b). It has been reported that patients with any blood glucose value of less than 70mg/dl have 7% higher odds of in hospital mortality compared with patients without hypoglycaemia (Curkendall et al., 2009). Furthermore, a recent study demonstrated that hospitalized patients with hypoglycaemia either by insulin use or spontaneous had increased short and long term mortality risk (Akirov et al., 2017). It has also been reported that patients with Diabetes mellitus experiencing hypoglycaemia have been shown to experience delirium (Fishbain and Rotundo, 1988).

The brain is highly dependent on glucose for its metabolism and is particularly vulnerable to hypoglycaemia especially in older people. Delirium has been thought to be brought on by acute metabolic changes in the brain. Derangement of cerebral metabolism is thought to be the driver for delirium (Engel and Romano, 1959, Engel and Romano, 2004) as well as reduced global and regional cerebral blood flow (Yokota et al., 2003, Caplan et al., 2014). 2-18F-fluoro-2-deoxy-D–glucose (FDG) positron emission tomography (PET) is a valuable and widely used functional neuroimaging technique that measures cerebral glucose metabolism. A recent paper showed that patients with delirium had cortical hypo metabolism that improved with delirium resolution. In the same study, the authors showed that glucose metabolism was higher post-delirium in the whole brain and bilateral posterior cingulate cortex (PCC) compared to during delirium (Haggstrom et al., 2017). The PCC-hypo metabolism was associated with inattention during delirium. This was an important study as it demonstrated that the metabolic impairments may be a common pathway in delirium pathophysiology and targeting the decreases in metabolism may be a therapeutic target in alleviating delirium in these patients.

4.3.6. Glucose transport to the brain

It is important to point out that despite equivalent levels of hypoglycaemia in NBH and ME7 mice seen in this chapter, the ME7 mice were more vulnerable to this hypoglycaemia. Why would this be? A possible explanation could be because of

impaired glucose transport in the brain of these mice. Glucose is supplied to the brain by the cerebral vasculature and can be taken up by the neuron or astrocytes and used as an energy substrate under basal conditions (Pellerin and Magistretti, 1994, Pellerin and Magistretti, 2012). However, neurons can also utilise lactate produced by lactate when needed and this is known as the astrocyte neuron lactate shuttle (ANLS) hypothesis. Briefly, during synaptic activity glutamate is released into the synapse and the glutamate reuptake system in astrocytes ensures removal of glutamate from the synaptic cleft. The entry of Na^+ cotransported with glutamate activates the Na^+/K^+ ATPase -pump. This activation of the Na^+/K^+ ATPase pump results in the stimulation of glucose uptake from the capillaries. Astrocytes convert glucose taken from capillaries to lactate. This lactate, when released from the astrocyte can be taken up by neurons and serve as an alternative energy substrate.

The passage of glucose across the cell membrane is facilitated by a family of integral membrane transporter proteins, the glucose transporters (GLUTs) GLUT 1 and GLUT3 are the main glucose transporters to be involved in glucose metabolism within the brain. GLUT 1 facilitates glucose transport across the blood brain barrier (BBB). GLUT3 is the most abundant glucose transporter in the brain having five times higher transport capacity than GLUT1 (Vannucci et al., 1997). GLUT 3 supplies glucose to neurons. In the studies presented in this chapter, when glucose level was carried out in the same animals in the CSF following LPS challenge, LPS caused a decrease in glucose in both NBH and ME7, the same results as to what was seen in the plasma of these animals. The effects of IL-1RA, SC-560, Dexamethasone, 2-DG on LPS decreases in the CSF were also the same as those seen in plasma. None of these interventions had any improvement on the decreased glucose levels induced by LPS.

The CSF data correlates to the plasma data and this has been found in another study by Boutelle and colleagues. In this study the authors showed in rats, that decreased glucose levels in the plasma following Insulin treatment correlated with decreased glucose in the striatum (Boutelle et al., 1986). In order to verify if there is any difference in glucose concentration in the brains of ME7 mice to normal mice is this study, further experiments using microdialysis or glucose biosensors implanted directly into the brain would have to be performed.

Previous work has shown that macrophages response to LPS are supported by a rapid and sustained glucose influx via GLUT1 so it would be important to see if this transporter is involved in the transport of glucose in the study presented in this chapter (Fukuzumi et al., 1996). A possible explanation for the vulnerability to ME7 mice to hypoglycaemia in this study would be a difference in GLUT transporters in these mice. ME7 mice at 8-12 weeks have been shown to have decreases in lactate and glucose (Broom et al., 2007). ME7 mice have also been found to have reduction in GLUT3 transporter while GLUT 1 remains unchanged (Yan et al., 2014). This decrease in GLUT3 would result in decreased or insufficient uptake of glucose into the neurons which can then have an impact on neuronal functioning and lead to cognitive processes being impaired in these mice as seen in the data presented in this chapter.

4.3.7 Alternative factors for inducing hypoglycaemia, cognitive impairment and sickness behaviour

While it has been reported that IL-1 can indeed induce hypoglycaemia it has also been shown that IL-6 and TNF-α can also induce hypoglycaemia in mice. Studies by Oguri demonstrated that TNF-α can induce hypoglycaemia in control mice and mice with IL-1α/β KO. Previous work from our laboratory has shown that when ME7 mice were challenged with TNF-α, lead to an acute cognitive deficit in these mice that was accompanied by increased levels of hippocampal and hypothalamic of IL-1β, TNF-α and CCL2. These mice also showed an exaggerated sickness behaviour response after TNF challenge which could be as a result of TNF-induced hypoglycaemia (Hennessy et al., 2017). However it is important to point out from the sickness behaviour data presented in this chapter, that the sickness behaviour response is only partially driven by glucose as glucose levels were not affected by Dexamethasone, IL-1RA or SC-560 despite all of these showing attenuating effects against the LPS-induced deficit in T-maze.

The IL-1RA showed a protective effect against the LPS-induced deficit in the T-maze however it is surprising that it did not have any effect on the glucose levels in these mice. Del Rey and colleagues showed in their studies that IL-1RA 300μg could block the LPS-induced decrease in glucose levels by 2 hours and continued to block this LPS induced decrease in glucose at 8 hours. In their study the dose of LPS used was much

lower (25µg/kg) than the dose of LPS (100ug/kg) used in the study presented in this chapter. As a result the LPS-induced hypoglycaemia presented here is greater than that seen in the Del Rey study. As a result, any protective effect of IL-1RA on glucose levels maybe confounded by this greater LPS induced hypoglycaemia in the study presented in this chapter. Another explanation for the difference in the protective effect of IL-1RA on glucose levels is the dose of IL-1RA in the del Rey was 15mg/kg whereas the dose used in the current study was 10mg/kg. Perhaps a higher dose of IL-1RA and lower dose of LPS might offer similar results to those seen by Del Rey. Another explanation for the difference in the protective effect of IL-1RA on glucose levels is the dose of IL-1RA. In the Del Rey study 15mg/kg of IL-1RA was used whereas the dose used in the current study was 10mg/kg dose. Perhaps a higher dose of IL-1RA and lower dose of LPS might offer similar results to those seen by Del Rey. Finally the timing of the IL-1RA is another possible explanation for the difference in results. In the current study the IL-1RA was given at the same time as the LPS, whereas in the Del Rey study the IL-1RA was given 30 minutes after LPS. IL-1RA is reported to have an initial half- life of 21 minutes (Granowitz et al., 1992) therefore by 30 minutes after LPS, the IL-1RA in the current study is 1.5 times less than what was injected by Del Rey 30 minutes after LPS. Perhaps a time course study looking at the effects of IL-1RA over a continued time period may help to address if IL-1RA would be affective in protecting against the LPS-induced hypoglycaemia in the current study.

IL-6 has also been shown to induce hypoglycaemia however to a lesser extent to that of IL-1 (Del Rey and Besedovsky, 1992). Fattori and colleagues showed that IL-6KO mice displayed decreased glucose than WT after challenge with LPS (Fattori et al., 1994). Another further study showed that the absence of IL-6 protected against LPS-induced hypoglycaemia in IL-6 KO mice; i.e IL-6 KO mice also exhibit profound hypoglycaemia 30 mins following LPS that is no longer apparent at 4 hours (Tweedell et al., 2011). In addition, our laboratory has shown that IL-6 can induce sickness behaviour in mice with reduced locomotor (Skelly et al., 2013). Together these data all indicate that IL-1 is not the only cytokine that can drive hypoglycaemia and sickness behaviour.

4.4 Conclusion

The data in this chapter have highlighted how LPS can have dissociable effects in 2 hippocampal dependent behaviours and when there is underlying neurodegenerative vulnerability present and a systemic inflammatory insult occurs, this resulted in a profound acute transient deficit that can be driven substantially by a decrease in glucose levels. This is important in the clinical field as it would suggest that patients with delirium or Dementia when they experience a bacterial infection can lead to impairment in memory that could be driven by hypoglycaemia. Therefore if we develop therapeutic targets to inhibit the decrease of glucose in these patients, this could reduce the incidence of cognitive dysfunction in these vulnerable patients. The question as to whether hypoglycaemia occurs in infection in the clinical field is one that needs to be taken into account. There are reports of hypoglycaemia in children with acute gastroenteritis (Qadori et al., 2018) and with classic congenital adrenal hyperplasia (Keil et al., 2010).

While there are reports of hypoglycemia in sepsis (Miller et al., 1980), hyperglycemia is more prevalent in septic patients than hypoglycaemia (Wernly et al., 2016, Hirasawa et al., 2009). Up to 90% of critical ill patients in intensive care unit can experience hyperglycaemia which has been reported to be associated with poor prognosis and morbidity and mortality (Zhu et al., 2015). Indeed in the study by Haggstrom and Caplan 2017, hypometabolism in the brain leading to hyperglycemia in these patients was associated with delirium (Haggstrom et al., 2017).

Chapter 5

Roles of IFN-I in the normal, inflamed and aged brain.

The majority of the data in this chapter have been published in Murray et al., 2015, *Brain, Behaviour and Immunity* and further data is in preparation for submission to *Brain Behaviour and Immunity.*

5.1 Introduction.

Interferons are pleiotropic cytokines produced by host cells in response to viral and bacterial pathogens (Colonna et al., 2002). For the purpose of this chapter, the focus will be on type 1 interferons alpha and beta (α/β). Type-one interferons are regulators of antiviral response, but are also anti-proliferative and pro-apoptotic (Owens et al., 2014). Type-one interferon signalling occurs through a heterodimeric receptor IFNAR that contains two subunits: IFNAR1 and IFNAR 2. Cross phosphorylation of, and activation of, TYK2 and JAK1 takes place, which leads to tyrosine phosphorylation resulting in activation of signal transducers and activators of transcription STAT1 and STAT2. These STATs associate with IRF9 to form ISGF3 which then translocates to the nucleus to induce transcription of IFN stimulated genes from IFN-stimulated response elements. (Taniguchi and Takaoka, 2001).

Interferons, which can be induced in the brain (Cunningham et al., 2007, Wang et al., 2008), can impact on CNS function. Recombinant IFN-β is a standard treatment for relapsing-remitting Multiple Sclerosis patients (Brandt et al., 1993) but has also been shown to induce interictal-like spontaneous activity in hippocampal neurons (Costello and Lynch, 2013). In contrast, IFN-α has been shown to induce depression, sickness behaviour, anxiety, cognitive impairment and to be associated with delirium in patients being treated for cancer and hepatitis (Bonaccorso et al., 2001, Schaefer et al., 2002, Capuron and Miller, 2004, Raison et al., 2005, Zdilar et al., 2000). Chronic treatment with IFN-α has also been reported to decrease cell proliferation and neurogenesis in the dentate gyrus of mice (Zheng et al., 2014). Interferon-α has also been implicated in regulating temperature and sleep (Krueger et al., 1988) circadian rhythm (Koyanagi and Ohdo, 2002) and feeding behaviour (Plata-Salaman, 1992). Baruch *et al* recently demonstrated that the choroid plexus, an interface between the brain and the circulation, shows a type I interferon (IFN-I)-dependent gene expression profile that was also found in aged human brains. The authors demonstrated that when IFN-I signalling was blocked in the CSF of aged animals, it resulted in improved cognitive function as well as hippocampal neurogenesis and diminished astrogliosis and microgliosis in these animals (Baruch et al., 2014). Taken together all of these studies demonstrate that interferons can impact on CNS function. As mentioned

above, interferons can induce sickness behaviour. Sickness Behaviour, described by Hart, (Hart, 1988) is a highly organised behavioural strategy of an organism to conserve energy to fight off viral or bacterial pathogens. Symptoms of sickness behaviour include malaise, lethargy, anhedonia, decreased social interaction and poor concentration (Dantzer, 2004). Numerous studies have shown that sickness behaviour can be induced by LPS, Poly I:C and to a lesser degree pro-inflammatory cytokines IL-1, TNF-α and IL-6. Poly I:C is a synthetic double stranded RNA commonly used as a viral mimetic as it can mimic the acute antiviral response and it can be detected by TLR3 receptors present on endosomal membranes and cytoplasmic proteins RIG I and MDA5 (Alexopoulou et al., 2001, Yoneyama et al., 2004, Andrejeva et al., 2004, Kato et al., 2005). Systemic administration of poly I:C in rodents can induce fever that is partially dependent on IL-1β (Fortier et al., 2004) while other poly I:C studies have replicated the symptoms of sickness behaviour including those of hypothermia, anhedonia, anorexia, hypoactivity and sleep wake cycle disruption (Cunningham et al., 2007, Traynor et al., 2006, Fortier et al., 2004, Majde, 2000). In addition, Poly I:C has also been shown to cause cognitive dysfunction in contextual fear conditioning (Weintraub et al., 2014, Kranjac et al., 2012, Dilger and Johnson, 2010). Despite all of these studies, whether and how type I interferons might contribute to sickness behaviour has been little studied.

IFNAR1$^{-/-}$ mice have been shown to have altered sleep patterns (Bohnet et al., 2004, Traynor et al., 2006) and to have altered expression of, and responses to, other inflammatory molecules such as IL-6 (Mitani et al., 2001). To date there have been no studies that have looked at the role of endogenous IFN-I on cognitive behaviour, activity, anxiety or diurnal rhythm.

This chapter will aim to characterise the phenotype of IFNAR1$^{-/-}$ mice (Hwang et al., 1995) using C57BL/6 mice as controls. Spatial reference memory will be assessed in these mice by Y-maze. Activity and anxiety will be assessed by open-field and elevated plus maze task and diurnal rhythm will also be assessed. After establishing what the normal function is in these IFNAR1$^{-/-}$ mice they will then be assessed after challenge with Poly I:C examine the role of induced type one interferons during dsRNA-induced sickness behaviour. Finally, as these IFNAR mice age, they will be assessed on

hippocampal dependent behaviour tasks and activity in the open field assessed to see if ageing has any impact on their locomotor activity, anxiety levels and cognition.

5.2 Results

5.2.1 Initial characterisation of the IFNAR1$^{-/-}$ mouse strain – Role of endogenous IFN-I on behaviour

Initially a subset of male wildtype (WT) and IFNAR1$^{-/-}$ (n=6) and female WT and IFNAR1$^{-/-}$ mice (n=6) were recorded on open field activity, elevated plus maze and Y-maze. There were no significant differences between genders on any of these three tasks, therefore apart from figure 5.1, all other data presented in this chapter were carried out with female mice. The behavioural phenotype of female IFNAR1$^{-/-}$ compared to female WT mice was characterised using a variety of hippocampal-dependent learning and memory tasks, as well as tests of anxiety and general locomotor activity.

5.2.2 Y maze, Open field and Elevated Plus maze mixed gender

The performance of IFNAR1$^{-/-}$ mice (n=6 female, n=6 male) and WT (n=6 female, n=6 male) mice in a visuo-spatial reference memory Y-maze task (figure 5.1a) across 2 blocks of 6 trials was recorded. There was no impairment in IFNAR1$^{-/-}$ mice to controls in this task. Both strains learned the task equally. Two-way repeated measures ANOVA with strain as between subjects factor and trial block as within subjects factor indicated that there was no impairment on learning of the task in the IFNAR1$^{-/-}$ mice compared to their WT controls. ANOVA revealed no effect of strain ($F=0.69$, df 1,22, $p=0.42$), nor an interaction ($F=0.89$, df 1,22, $p=0.36$), but there was a significant effect of trial block ($F=59.51$, df 1,22, $p<0.0001$) where performance of both strains improved as training continued.

Open-field activity was assessed in the same mice. There was no significant difference in activity between strains with ($p=0.9585$) figure 5.1b. IFNAR1$^{-/-}$ mice however displayed an increase in rearing activity compared to controls ($p=0.0597$). (figure 5.1c).

Anxiety levels were measured by elevated plus maze and figure 5.1d,e confirms that IFNAR1$^{-/-}$ mice spend equivalent time in the closed and open arms to the WTs with no significant difference in time spent in closed arms with ($p=0.64$) or open arms with ($p=0.89$).

5.2.3 Open field and Elevated Plus Maze in female WT and IFNAR1$^{-/-}$ mice

In order to establish whether endongenous type one interferons play a role in anxiety and whether the lack of type one interferon receptor would have an effect on anxiety, open field and elevated plus maze was recorded in WT (n=17) and IFNAR1$^{-/-}$ mice (n=18). All females were 4-6 months old. The behavioural experiments were powered based on prior experience with these behavioural assays. Since the intention was to age the mice and there was no clear indication of the likely hood of longevity- the power size was increased in order to ensure that there was sufficient amount of mice present throughout the entire study and to ensure sufficient amount of mice for processing of tissue for molecular and immunohistochemically analysis upon completion of the behavioural study.

Distance travelled and number of rears in the open field for a period of 3 minutes was recorded in 4-6 months old WT and IFNAR1$^{-/-}$ female mice (n=18). All data in figure 5.2 are expressed as mean±SEM and significant differences were analysed by unpaired t-tests. In figure 5.2a where squares crossed in 3 minutes in the open field were recorded, the results reveal that IFNAR1$^{-/-}$ mice were not more active compared to the WT mice with p=0.2193.

In figure 5.2b, although IFNAR1$^{-/-}$ mice seemed to show an increase in rearing activity from controls, this was not statistically different (p=0.10). In Figure 5.2 c,d where time spent in open and closed arms was recorded the IFNAR1$^{-/-}$ mice spend equivalent time in the closed and open arms of the elevated plus maze to the WT mice. There was no statistical difference in time spent in the closed arms (p= 0.44) and the same could also be seen in the open arms (p= 0.51).

Taken together these two experiments demonstrate that basal endogenous type one interferons do not play a role in anxiety-like behaviour.

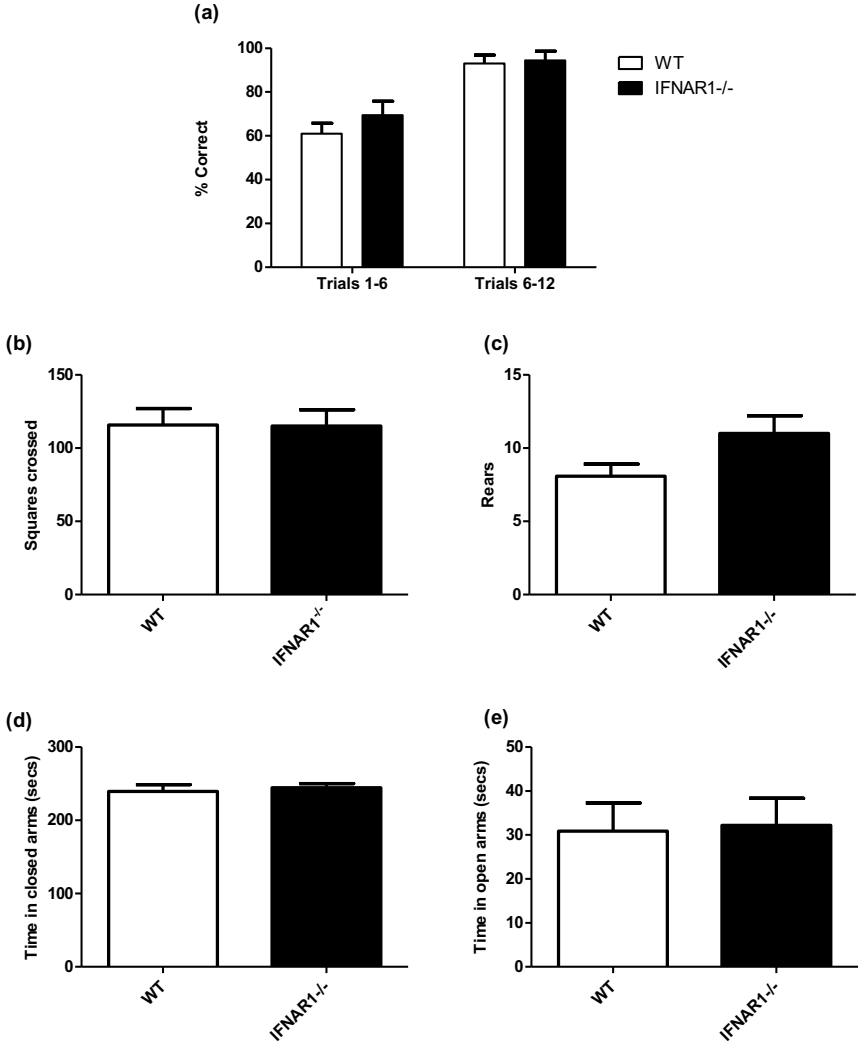


Figure 5.1 Performance of IFNAR1$^{-/-}$ and WT mice on a visuo-spatial reference memory task, open field and elevated plus maze mixed gender. (a) Visuo-spatial reference memory was assessed using the Y-maze across 12 trials (6 trials per trial block) in IFNAR1$^{-/-}$ (n=12 (6 female and 6 males)) compared to WT mice (n=12 (6 female and 6 males)), (b,c) open-field activity measurements,(d) time spent in closed and (e) open arms of the elevated plus maze. Data are shown as mean±SEM and were analysed by 2- way repeated-measures ANOVA with strain and time as factors for (a) and t-test for (b-e). No significant differences were detected in IFNAR1$^{-/-}$ or WT mice on these measures.

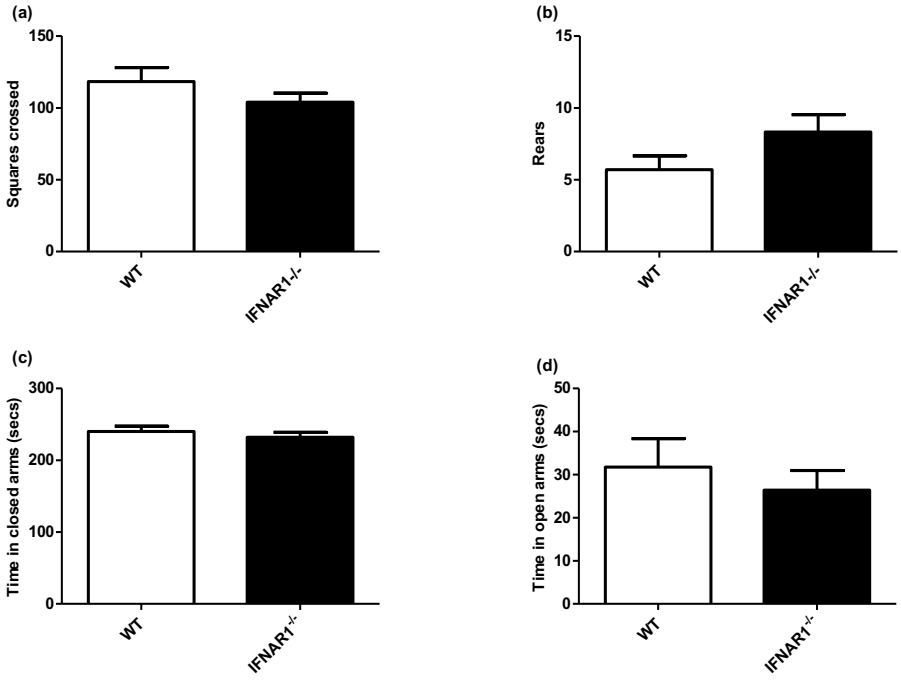


Figure 5.2 Open field and elevated plus maze activity in female IFNAR1$^{-/-}$ and WT mice. (a) Distance travelled in open field in IFNAR1$^{-/-}$ mice (n=18) compared to WT (n=17) and (b) the number of rears recorded in open field, across 3 minutes. (c) The time spent in closed arms of elevated plus maze in IFNAR1$^{-/-}$ mice and WT mice and (d) time spent in open arms of elevated plus maze. Data are expressed as mean ± SEM and all data were analysed by t-test. No significant differences were detected in IFNAR1$^{-/-}$ or WT mice on these measures.

5.2.4 Diurnal rhythm

In the current literature, it has been reported that IFNAR1$^{-/-}$ mice show altered sleep patterns with decreased time spent in spontaneous rapid eye movement (REMS) and non-rapid eye movement (NREMS) and a reduction in number of REMS episodes (Traynor et al., 2006, Bohnet et al., 2004). There have been no studies that have looked at circadian rhythm in IFNAR1$^{-/-}$ mice so to address this, the next experiment assessed the activity levels of IFNAR1$^{-/-}$ and WT mice during their diurnal rhythm.

The total activity was measured for a cohort of 18 female IFNAR$^{-/-}$ mice and 17 female WT mice, 6-8 months old using PhenoTyper home cage, 45cm x 45cm, (Noldus, UK) captured by Ethovision. Each PhenoTyper cage monitored total activity for 6 mice each, therefore for statistical purposes n=3 cages of mice per group. All mice were left for a period of 60 hours (3 nights and 2 days). The recordings taken in the 60 hours were then binned into four hour periods and presented as 15 consecutive periods, showing night and day activity.

As expected, over a period of three nights and two days plotted in 1 hour blocks, both the IFNAR1$^{-/-}$ and WT mice were clearly more active in the night phase compared to the day (figure 4.3) and there was a main effect of time ($F=5.98$, df 59,236, $p<0.0001$). Distance travelled was not affected by strain ($F=2.77$, df 1,236, $p=0.17$) and there was no significant interaction of time and strain ($F=1.12$, df 59,236, $p=0.28$) These data would suggest that endogenous IFN-I do not play a role in diurnal rhythm. However the graph in figure 5.3 indicates that IFNAR1$^{-/-}$ mice seem to have a higher activity to the WT during the dark phase. It is possible due to the reduced numbers of mice recorded in this study, it is difficult to obtain a significant effect therefore it would be important to repeat this experiment with more number of animals in order to see if there is an effect on diurnal rhythm in IFNAR1$^{-/-}$ mice.

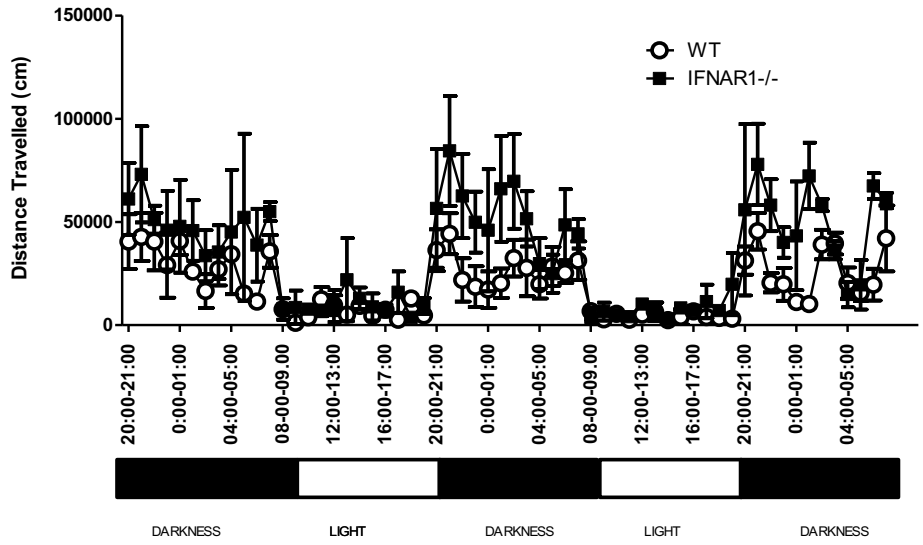


Figure 5.3 Diurnal rhythm of female WT and IFNAR1$^{-/-}$ mice. The activity of female WT (n=17) and female IFNAR1$^{-/-}$ (n=18) mice was recorded using the Phenotyper home cage over a period of three nights and two days. Each PhenoTyper cage monitored total activity for 6 mice each, therefore for statistical purposes n=3 cages of mice per group. All mice were left for a period of 60 hours (3 nights and 2 days). The recordings taken in the 60 hours were then binned into one hour periods and presented as 15 consecutive periods, showing night and day activity. Data are plotted in 1 hour blocks and are expressed as mean±SEM and were analysed by two-way repeated measures ANOVA with strain as between subjects factor and time as within subjects factor.

5.2.5 The role of IFN-I in sickness behaviour following challenge with poly I:C in WT and IFNAR1$^{-/-}$ mice.

The first experiments characterizing the IFNAR1$^{-/-}$ strain, have demonstrated that IFNAR1$^{-/-}$ mice have no impairment on visuo-spatial memory. In addition, although not significant, they displayed increased rearing activity in the open field and equivalent time spent in the open and closed arms of the elevated plus maze to their wild type controls suggesting that endogenous IFN-I do not play a role in anxiogenic behaviour or diurnal rhythm.

It has been well described in the literature that poly I:C a viral mimetic can induce acute inflammation and sickness behaviour in rodents (Traynor et al., 2006, Majde, 2000, Fortier et al., 2004). Previous work from our laboratory, has looked at the effects of different doses of poly I:C on a wide spectrum of sickness behaviour measures such as temperature, weight, burrowing and locomotor activity in mice. From these studies, it can be seen that following challenge of 12mg/kg poly I:C, these sickness behaviour measures were robustly decreased compared to when the mice were challenged with a lower dose of 2mg/kg poly I:C. The same studies also demonstrated that IFN-β was elevated 1 hr post-poly I:C challenge but at 3 hours was further increased to 5000 fold of control levels. This increase in IFN- β was also seen in the hypothalamic and hippocampal regions, where at 24 hours post-challenge the levels were still elevated (Cunningham et al., 2007).

Interferons in particular IFN alpha used for treatment of cancer and hepatitis can induce sickness behaviour and depression (Raison et al., 2005).Therefore the increase in IFN-β post-poly I:C and the decrease in sickness behaviour measurements, as reported in the Cunningham study merits investigation as to whether and how type one interferons might contribute to dsRNA-induced sickness behaviour. The previous data has shown that IFNAR1 deletion has no effect in normal mice. What is the role of IFNAR1 deletion during inflammatory induced sickness behaviour?

The next experiments therefore, investigate the role of type one interferons on poly I:C induced sickness behaviour in IFNAR1$^{-/-}$ and WT mice.

5.2.6 Poly I:C-induced type I Interferons and sickness behaviour

WT and IFNAR1$^{-/-}$ mice were injected with 12mg/kg of poly I:C or saline i.p and weights, temperature, burrowing and open field measurements were recorded. In addition, systemic and CNS responses in a different cohort of animals challenged with poly I:C were also analysed for pro-inflammatory cytokines. Poly I:C induced robust hypothalamic expression of IFN-β mRNA but not IFN-α mRNA (figure 5.4a) and induced some features of sickness in all treated animals. IFNAR1$^{-/-}$ mice appeared to show less hunched posture and piloerection than WT animals when challenged with poly I:C.

Temperature was measured, by rectal probe, at 3, 6, and 9h post-poly I:C challenge and early measures showed some variability and possible stress-related hyperthermia in most animals. Based on previous work (Cunningham et al., 2007), there was an *a priori* prediction that temperature would be affected in these mice at 9h. Figure 5.4b shows that WT poly I:C-treated mice showed significant hypothermia at 9h that was not seen in in poly I:C-treated IFNAR1$^{-/-}$ mice. Two-way ANOVA at 9h with treatment and strain as factors showed a significant effect of treatment ($F=13.91$, df 1,40, $p=0.00$), and a significant interaction between treatment and strain ($F=8.22$, df 1,40, $p=0.00$) indicating the impaired hypothermic response in IFNAR1$^{-/-}$ mice.

Rears in the open field were recorded for 3 minutes, 3h post-challenge with poly I:C or saline. There was a robust decrease in the number of rears in poly I:C-treated mice but this was less marked with IFNAR1$^{-/-}$ poly I:C-treated mice compared to WT poly I:C-treated (figure 5.4c). Two-way ANOVA revealed a significant effect of strain ($F=4.51$, df 1,40, $p=0.04$) and of treatment ($F=14.85$, df 1,40, $p=0.00$) but no significant interaction ($F=0.88$, df 1,40, $p=0.35$). Bonferroni *post-hoc* analysis indicated that suppression of rears by poly I:C was significantly more robust in WT animals than in IFNAR1$^{-/-}$ animals ($p<0.05$).

All poly I:C-treated mice showed a marked reduction in weight but recovery was significantly faster in IFNAR1$^{-/-}$ mice at 48h (figure 5.4d). Three-way ANOVA with strain, treatment and time as factors revealed a significant effect of treatment ($F=118.4$, df 1,43, $p<0.0001$) and of time ($F=102.95$, df 3,43, $p<0.0001$) and an

interaction of all three factors ($F=12.99$, df 1,43, $p<0.0001$). Therefore activity of IFN-I influences the time-course of recovery during poly I:C-induced sickness.

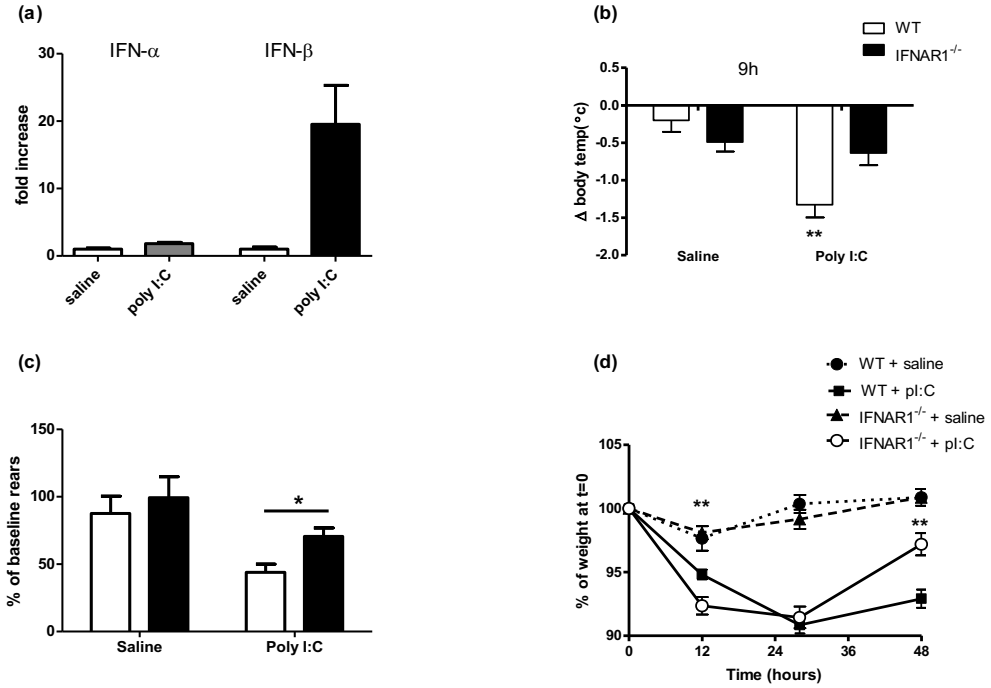


Figure 5.4 Poly I:C-induced type I interferons and sickness behavior in WT and IFNAR1$^{-/-}$ mice. (a) The hypothalamic transcription of IFN-α and IFN-β were assessed at 6 hours post-poly I:C (12 mg/kg i.p.). The sickness behaviour response of C57BL/6 and IFNAR1$^{-/-}$ mice to poly I:C was assessed: (b) temperature at 9h, (c) open field rearing activity and (d) % body weight loss. Differential effects of poly I:C on (b) temperature and (c) rearing were assessed using two-way ANOVA (with strain and treatment as between-subjects factors) and (d) body weight was assessed using 3 way ANOVA analysis with time, treatment and strain as factors in WT and IFNAR1$^{-/-}$ mice. Data are expressed as mean ± SEM, n=5 for WT+Saline, n=8 for WT+pI:C, n=13 for IFNAR1$^{-/-}$+Saline and n=14 for IFNAR1$^{-/-}$+pI:C. Full ANOVA analysis appears in the main text. Statistically significant differences by Bonferroni post-hoc are denoted by ** $p<0.01$ and * $p<0.05$ w.r.t WT +pI:C .

5.2.7 Systemic cytokine response in plasma

Plasma from WT and IFNAR1$^{-/-}$ mice 3h post-challenge with poly I:C or saline was analysed for systemic levels of pro-inflammatory cytokines (IL-6, TNF-α and IFN-β) by ELISA (figure 5.5). The plasma concentrations of IL-6, TNF-α and IFN-β in the saline-treated animals were below detection levels. There was almost complete ablation of the IL-6 response in poly I:C-treated IFNAR1$^{-/-}$ mice. Two-way ANOVA analysis of plasma IL-6 levels with treatment and strain as factors, revealed a significant effect of treatment ($F=28.18$, df 1,18, $p<0.0001$) and of strain ($F=21.04$, df 1,18, $p=0.00$) and an interaction between treatment and strain ($F=21.04$, df 1,18, $p=0.00$). Analysis of plasma TNF-α, IL-1β and IFN-β by two-way ANOVA demonstrated a significant effects of treatment ($F\geq42.03$, df 1,18, $p<0.0001$) but no effects of strain and no interaction, indicating that poly I:C-induced increases in plasma TNF-α, IL-1β and IFN-β are not affected by IFNAR1.Therefore there was a selective ablation of poly I:C-induced IL-6 in IFNAR1$^{-/-}$ mice.

5.2.8 Hypothalamic and hippocampal cytokine response

Hypothalamic and hippocampal brain regions were also extracted from WT and IFNAR1$^{-/-}$ mice 3h post-challenge with poly I:C or saline. IFN-β, IL-6, TNF-α and IL-1β expression was measured by RT-PCR. As in the plasma, there was a near-complete ablation of IL-6 in the IFNAR1$^{-/-}$ poly I:C-treated compared to the WT poly I:C-treated in the hypothalamus (figure 5.6a) and in the hippocampus (figure 5.6e). Two-way ANOVA of IL-6 mRNA revealed a significant effect of treatment ($F=32.08$, df 1,18, $p<0.0001$), of strain ($F=28.18$, df 1,18, $p<0.0001$) and an interaction ($F=27.39$, df 1,18, $p<0.0001$) of these two factors in the hypothalamus and this effect was replicated in the hippocampus: treatment ($F=26.75$, df 1,18, $p<0.0001$), strain ($F=22.46$, df 1,18, $p<0.0002$) and interaction of strain and treatment ($F=21.79$, df 1,18, $p<0.0002$).

Conversely, TNF-α was increased by poly I:C in the hypothalamus (figure 5.6b) and the hippocampus (figure 5.6f) in both WT and IFNAR1$^{-/-}$ mice. Two-way ANOVA analysis shows a significant effect of treatment ($F=428.72$, df 1,18, $p<0.0001$) but no effect of strain ($F=0.46$, df 1,18, $p=0.5073$) and no interaction of strain or treatment ($F=0.52$, df 1,18, $p=0.48$). Similarly in the hippocampus (Figure 5.6f), two-way ANOVA shows poly

I:C induced increases in TNF-α, with a significant effect of treatment ($F=53.39$, df 1,17, $p<0.0001$) but no effect of strain ($F=0.24$, df 1,17, $p=0.63$) and no interaction between strain and treatment ($F=0.64$, df 1,17, $p=0.44$), indicating that poly I:C-induced increases in the expression of TNF-α are not affected by IFNAR1.

IL-1β was also increased by poly I:C in the hypothalamus, but to a greater degree in IFNAR1$^{-/-}$ than WT mice (figure 5.6c). There was a significant effect of treatment ($F=57.17$, df 1,18, $p<0.0001$) and of strain ($F=10.83$, df 1,18, $p=0.00$) and an interaction between treatment and strain ($F=10.65$, df 1,18, $p=0.00$). In the hippocampus (figure 5.6g) poly I:C treatment once again had a significant effect on IL-1β expression ($F=12.78$, df 1,17, $p<0.0023$) but there was no effect of strain ($F=1.24$, df 1,17, $p=0.28$) and no interaction ($F=0.45$, df 1,17, $p=0.97$) indicating that the poly I:C induced increase in IL-1β mRNA is exacerbated in IFNAR1$^{-/-}$ mice, but only in the hypothalamus.

Poly I:C robustly induced IFN-β in the hypothalamus of both WT and IFNAR1$^{-/-}$ mice (figure 5.6d). There was a significant effect of treatment ($F=17.45$, df 1,17, $p<0.0006$) but no effect of strain ($F=0.46$, df 1,17, $p=0.51$) and no interaction ($F=0.45$, df 1,17, $p<0.5136$). In the hippocampus, poly I:C induced IFN-β (figure 5.6h) with a main effect of treatment ($F=16.89$, df 1,16, $p<0.00$), strain ($F=7.91$, df 1,16, $p<0.0125$) and an interaction of strain x treatment ($F=4.58$, df 1,16, $p<0.05$), indicating a lack of poly I:C-induced IFN-β response in the hippocampus but not the hypothalamus of IFNAR1$^{-/-}$ mice.

These results suggest that IFNAR1 expression appears to be necessary for robust plasma and CNS IL-6 responses to poly I:C and for the full expression of sickness behaviour, but not for IL-1β nor TNF-α responses. There is evidence for an exaggerated hypothalamic IL-1β response to poly I:C in IFNAR1$^{-/-}$ mice.

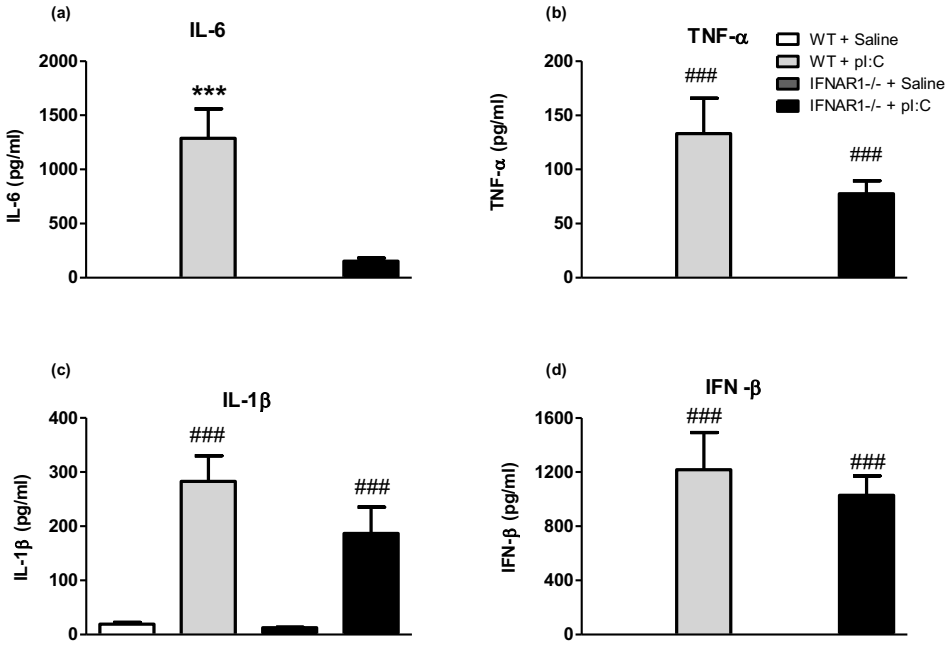


Figure 5.5 Poly I:C-induced systemic synthesis of inflammatory cytokines. ELISA analysis of plasma of IL-6, TNF-α, IL-1β and IFN-β (a-d) cytokine transcription 3h after intraperitoneal challenge with poly I:C (12 mg/kg). Data are expressed as mean ± SEM, n=5 for WT and n=6 for IFNAR1$^{-/-}$. Comparisons of pI:C treatment for WT and IFNAR1$^{-/-}$ mice were carried out by two-way ANOVA, with treatment and strain as factors. Main effects of treatment are denoted by ### $p<0.001$ and interactions between treatment and strain are denoted by ∗∗∗ $p<0.001$.

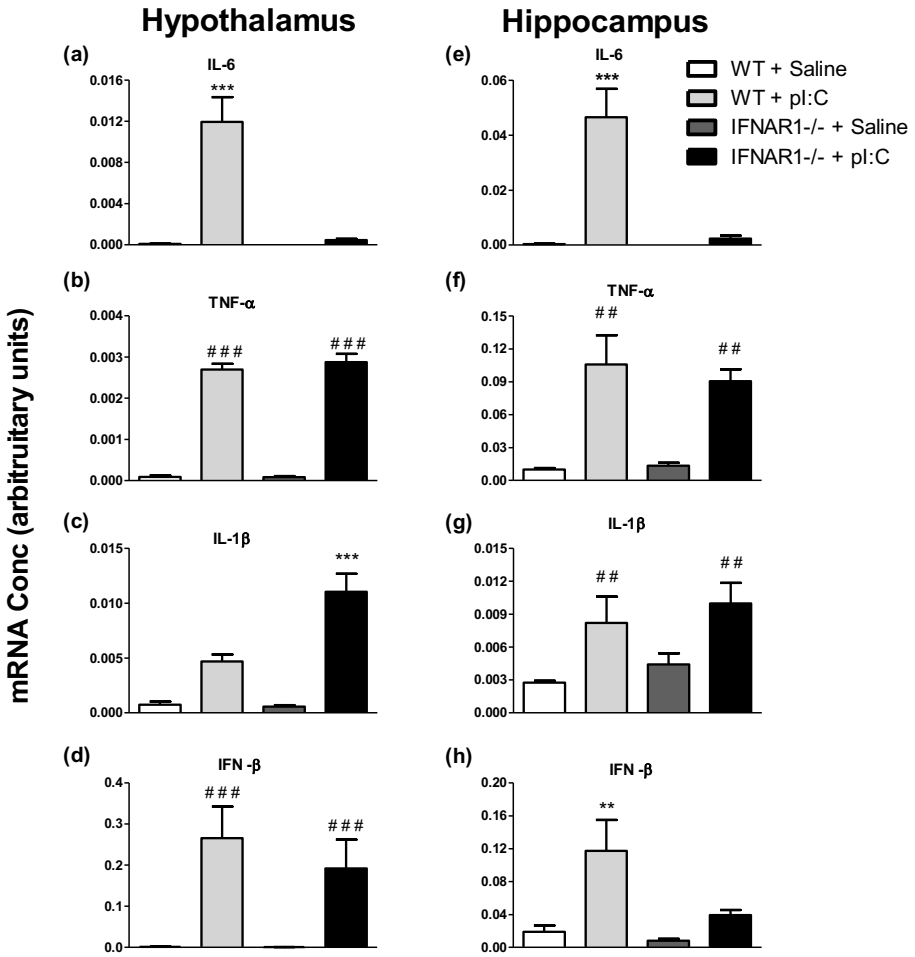


Figure 5.6 Poly I:C-induced hypothalamic and hippocampal synthesis of inflammatory cytokines. Hypothalamic and hippocampal cytokine transcription of IL-6, TNF-α, IL-1β and IFN-β (a-h) 3h after intraperitoneal challenge with poly I:C (12 mg/kg). Data are expressed as mean ± SEM, n=5 for WT and n=6 for IFNAR1$^{-/-}$. Comparisons of pI:C treatment for WT and IFNAR1$^{-/-}$ mice were carried out by two-way ANOVA, with treatment and strain as factors. Main effects of treatment are denoted by ### p<0.001, ## p<0.01 and interactions between treatment and strain are denoted by ∗∗∗ p<0.001 and ∗∗ p<0.001.

5.2.9 Systemic and CNS response of IL-6 after IFN-β challenge

The loss of poly I:C-induced IL-6 in IFNAR1$^{-/-}$ animals suggested a role for IFN-I in IL-6 expression. Therefore it was examined whether systemically administered IFN-β could induce IL-6 in wild-type C57BL6 mice and sickness behaviour. IFN-β increased plasma IL-6 (figure 5.7a; $p<0.05$ by unpaired Student's t test) and elevated expression of IL-6 mRNA in the hypothalamus compared to vehicle-injected controls (figure 5.7b; $p<0.05$ by unpaired Student's t test). However, this increase in IL-6 was not replicated in the hippocampus and levels of plasma IL-6 and brain IL-6 mRNA were trivial compared to those induced by poly I:C (fig 5.7 a,b).

5.2.10 Sickness behaviour measurements post IFN-β challenge

A number of measures of sickness behaviour were also assessed to address whether IFN-β alone could drive sickness behaviour. There were no effects of IFN-β (25,000 units) on body temperature or body weight (figure5.7). Open field activity was recorded for 3 minutes, 9h post-challenge with IFN-β or vehicle (figure 5.7 c,d) after pilot experiments indicated mild effects on activity only in the dark phase. There was a slight decrease in activity in the IFN-β-treated mice with respect to controls, $p<0.0011$ by unpaired Student's t test. Burrowing activity was measured at baseline and an a priori prediction of impairment was assessed at 7h post-challenge with IFN-β (25,000 units; figure 5.7b). Students t-test at 7h showed a small but statistically significant effect of treatment ($p=0.03$).

5.2.11 Expression of IL-6 and IFN-β in plasma and hippocampus post-poly I:C challenge

Having found that IFN-β could drive very limited IL-6 and sickness behaviour it was also necessary to establish the plausibility of the idea that acutely induced IFN-β was necessary for poly I:C-induced IL-6 (i.e. whether IFN-β was expressed significantly earlier than IL-6 after poly I:C stimulation *in vivo*). The sequence of expression of IL-6 and IFN-β in the plasma and hippocampus of WT and IFNAR1$^{-/-}$ mice 1 and 3h post-challenge with saline or poly I:C was examined. (figure 5.7g-j). There was no detectable IL-6 or IFN-β in the plasma of saline-treated mice. 1h post-poly I:C there was measureable plasma IL-6 and IFN-β and both these cytokines increased

substantially in wild-type mice at 3h. IL-6 was apparent at 1hr, it is not preceded by IFN-β and showed no further increase in the IFNAR1$^{-/-}$ mice while IFN-β continued to rise at 3h in both strains (figure 5.7.g-h). Two-way ANOVA of plasma IL-6 showed a significant effect of strain ($F=13.41$, df 1,25, $p=0.00$) and interaction between treatment and strain ($F=15.50$, df 2,25, $p=0.00$). Bonferroni *post-hoc* analysis showed a significant difference between poly I:C and saline at 3h in wild-type ($p<0.001$) but not in IFNAR1$^{-/-}$ mice. Two-way ANOVA for IFN-β showed equivalent responses to poly I:C in both strains (i.e. a significant effect of treatment ($F=44.39$, df 2,25, $p<0.0001$) but no significant effect of strain ($F=0.24$, df 1,25, $p=0.63$), or interaction between these two factors ($F=0.39$, df 2,25, $p=0.68$) indicating that IFNAR1 is necessary for the full poly I:C-induced plasma IL-6 response but the plasma IFN-β does not precede IL-6 and is unlikely to drive IL-6 production.

In the hippocampus IL-6 mRNA was not yet significantly induced in either strain at 1 hour and was elevated only in the WT mice at 3h post-poly I:C (figure 5.7c). Two-way ANOVA of IL-6 expression, with strain and treatment as factors, showed a significant effect of strain ($F=23.26$, df 1,21, $p<0.0001$) and treatment ($F=30.87$, df 2,21, $p=0.00$) and an interaction between treatment and strain ($F=15.50$, df 2,25, $p<0.0001$). Bonferroni *post-hoc* analysis showed a significant difference in WT poly I:C-treated at 3h when compared to controls ($p<0.001$). The expression of IFN-β in the hippocampus was different to that seen in the plasma (fig 5.7d). There was detectable IFN-β expression present at 1h in both strains, however IFN-β expression increased at 3h post-poly I:C in WT mice but not in IFNAR1$^{-/-}$ mice. There was a significant effect of strain ($F=5.10$, df 1,21, $p=0.03$), treatment ($F=4.84$, df 2,21, $p=0.01$), but no significant interaction ($F=2.25$, df 2,21, $p=0.13$).

Thus, similar to plasma IL-6, the poly I:C-induced hippocampal IL-6 response is facilitated by IFNAR1. In contrast to the plasma IFN-β response, poly I:C-induced hippocampal IFN-β is also mediated by IFNAR1 activity. Nonetheless, there was little evidence that acutely induced IFN-β might be responsible for the poly I:C-induced increase in IL-6 in WT mice. Rather the data suggest that IFNAR1 facilitates full IL-6 expression, perhaps through basal low level IFN-I activity.

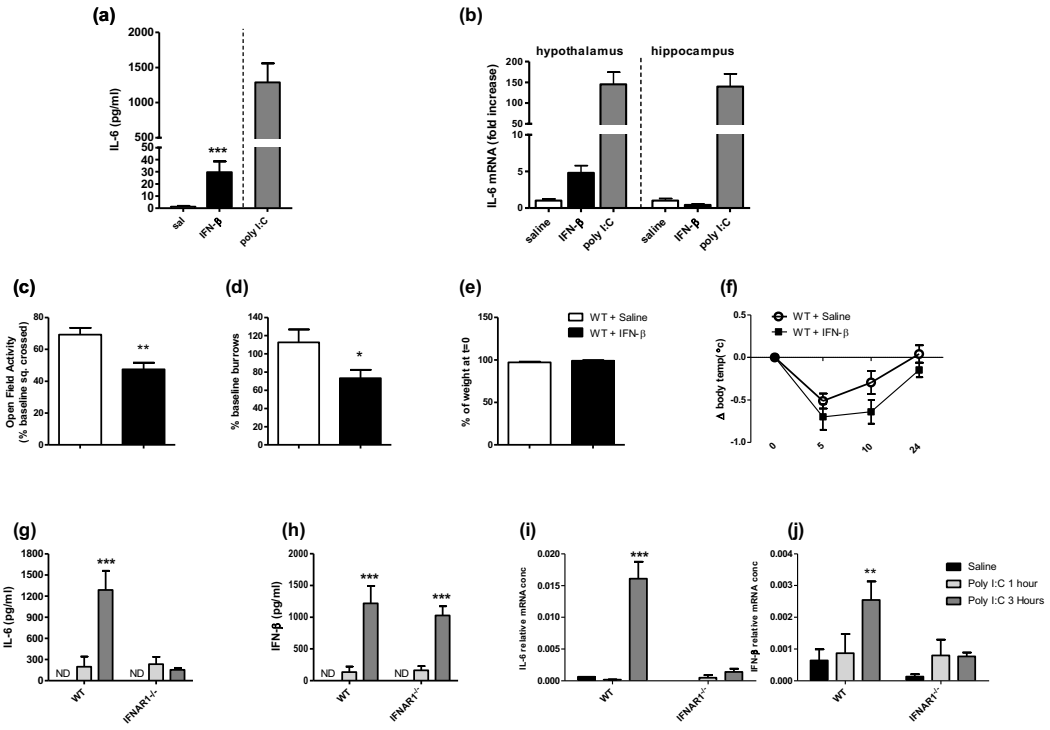


Figure 5.7 The role of acutely increased IFN-β in induction of IL-6 and sickness behaviour. Plasma IL-6 (a) and IL-6 expression in the hypothalamus and hippocampus (b) of C57BL/6 mice 3 h post-IFNβ challenge (25,000 units, i.p), compared to that induced by poly I:C (12 mg/kg). Data are expressed as mean ± SEM and were analysed by unpaired Student's t test, n = 4 for WT + vehicle and n = 5 for WT + IFN-β. The sickness behaviour response of C57BL/6 mice to IFN-β (25,000 units, i.p.) on (c) open field activity (expressed as % of baseline squares crossed at 9 h) (d-e) burrowing and weight at 7 h, and (f) temperature . Data are expressed as mean ± SEM and were analysed by Students t-test, n = 17 for WT + vehicle, n = 15 for WT + IFN-β. IL-6 and IFN-β levels in the plasma (g, h) and expression in the hippocampus (i,j) 1 and 3 h post-poly I:C challenge in WT and IFNAR1$^{-/-}$ mice. Data are expressed as mean ± SEM and were analysed by two-way ANOVA with strain and treatment as factors, n = 5 for WT + saline, n = 4 for WT + pI:C 1 h, n = 5 for WT + pI:C 3 h and n = 4 for IFNAR1$^{-/-}$ + saline, n = 4 IFNAR1$^{-/-}$ + pI:C 1 h and n = 5 for IFNAR1$^{-/-}$ + pI:C 3 h. Bonferroni post-hoc are denoted by *** p< 0.001 and ** p<0.001.

5.2.12 STAT1 expression

The previous data suggests that IFNAR1 facilitates IL-6 expression and it has been reported that STAT1 can contribute to the expression of IL-6 (Lee et al., 2006, Chaudhuri et al., 2008). Western blot analysis of STAT1 and phosphorylated STAT1 was performed in WT and IFNAR1$^{-/-}$ mice to assess whether lower basal levels of STAT1 in IFNAR1$^{-/-}$ mice might associate with the lack of IL-6 in the IFNAR1$^{-/-}$ mice.

Western blotting, as shown in figure 5.8a showed that basal total STAT1 is higher in WT than IFNAR1$^{-/-}$ mice and quantification by densitometry shows this difference to be significant (Student's t test, p=0.0015, fig 5.8c). Total and phospho-STAT1 were also assessed at 1 and 3 hours post-poly I:C in WT and IFNAR1$^{-/-}$ mice (figure 5.8b). Quantification (figure 5.8d) and analysis by two-way ANOVA with strain and treatment as factors showed a significant effect of strain ($F=16.31$, df 1,17, $p=0.00$) and a significant interaction ($F=7.61$, df 1,17, $p=0.01$) but there was no significant effect of treatment ($F=0.01$, df 1,17, $p=0.93$) indicating that poly I:C does not significantly alter total STAT1 levels within 3 hours. However, STAT1 became phosphorylated (i.e. activated) in all WT+poly I:C mice tested but was never detected in any other treatment/strain combination.

STAT1, which translocates to the nucleus upon phosphorylation(Gautron et al., 2003) has been shown to induce transcription of stat1 mRNA (Satoh and Tabunoki, 2013, Cheon and Stark, 2009) and therefore hippocampal stat1 mRNA was used as a readout of STAT1 activity was used, when levels of phosphoSTAT1 were too low to detect (figure 5.8d). There was a lower stat1 mRNA expression at baseline in IFNAR1$^{-/-}$ mice with respect to WT mice (i.e. in saline-treated mice) and an acute induction of stat1 mRNA by 3 hours which was visible only in WT poly I:C-treated animals. Two-way ANOVA showed a significant effect of strain ($F=149.93$, df 1,15, $p<0.0001$), and treatment ($F=84.10$, df 1,15, $p<0.0001$) and a significant interaction of these two factors ($F=49.98$, df 1,15, $p<0.0001$). Bonferroni *post-hoc* analysis revealed poly I:C treatment significantly increased STAT1 expression at 3h, only in WT mice ($p<0.001$ with respect to saline-treated controls), indicating that poly I:C-induced increases in hippocampal STAT1 expression are mediated by IFNAR1.

5.2.13 Genotype-dependent differences in basal and poly I:C-induced expression of inflammatory transcripts

Given the basal differences in STAT1 expression, it was predicted that there may be changes in basal levels of important STAT1-dependent inflammatory transcripts, therefore a number of inflammatory transcripts with reported STAT1-dependent regulation were examined in the hippocampus of WT and IFNAR1$^{-/-}$ mice (figure 5.9a). Interferon regulatory factor 7 (IRF7) is a transcription factor that is specifically induced by type I IFN action (via STAT1). CCL2-chemokine (C-C motif) ligand 2 (or monocyte chemoattractant protein-1) recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation. Indoleamine-2,3-dioxygenase (IDO) is an enzyme that converts tryptophan to kynurenine. Cyclo-oxygenase 2 (COX-2) is a key prostaglandin biosynthetic enzyme and interleukin 10 (IL-10) is a key anti-inflammatory cytokine. Basal IRF7 and CCL2 are lower in IFNAR1$^{-/-}$ than WT when expressed as a fraction of WT expression levels. Student's t tests demonstrate significantly lower basal IRF7 ($p<0.0001$) and CCL2 ($p=0.00$) in IFNAR1$^{-/-}$ and lower levels of IDO that did not reach statistical significance ($p=0.12$). Neither COX 2 ($p=0.14$) nor IL-10 ($p=0.53$) were significantly lower at basal levels.

Despite this lower basal level of CCL2, poly I:C still induces increased CCL2 expression in IFNAR1$^{-/-}$ mice, but it remains lower than that observed in WT mice (figure 5.9c). Two-way ANOVA analysis revealed a significant effect of strain ($F=26.65$, df 1,18, $p<0.0001$), and treatment ($F=47.56$, df 1,18, $p<0.0001$) and a significant interaction between strain and treatment ($F=25.28$, df 1,18, $p<0.0001$).

Transcription of the kynurenine biosynthetic enzyme indoleamine-2,3-dioxygenase (IDO), was analysed. Two-way ANOVA of IDO expression in the hippocampus (figure 5.9d) showed a significant effect of strain ($F=15.14$, df 1,15, $p=0.00$) but no effect of treatment ($F=0.01$, df 1,15, $p=0.93$) and no interaction ($F=0.02$, df 1,15, $p=0.90$), indicating a decrease in basal hippocampal IDO expression in IFNAR1$^{-/-}$ mice and no poly I:C-induced increase (at 3h).

COX-2 and IL-10 expression (figure 5.13 e,f) are similar in WT and IFNAR1$^{-/-}$ at baseline but are less robustly induced 3 hours post-poly I:C in IFNAR1$^{-/-}$. Two way ANOVA

analysis of COX-2 expression also showed a significant effect of strain ($F=7.95$, df 1,18, $p=0.01$), treatment ($F=29.70$, df 1,18, $p<0.0001$) and a significant interaction ($F=13.75$, df 1,18, $p=0.00$). Two-way ANOVA analysis of IL-10 expression showed a significant effect of treatment ($F=7.04$, df 1,15, $p=0.01$) but not of strain ($F=2.06$, df 1,15, $p=0.1718$) and no interaction ($F=3.40$, df 1,15, $p=0.09$) indicating that the poly I:C-induced increase in hippocampal COX-2 and IL-10 expression is attenuated in IFNAR1$^{-/-}$ mice.

Therefore, while the expression of IRF7, CCL2, IDO, COX-2 and IL-10 are all significantly different in poly I:C-treated IFNAR animals with respect to wild-types, lower basal expression of IRF7, CCL2 and IDO certainly contributes to this effect.

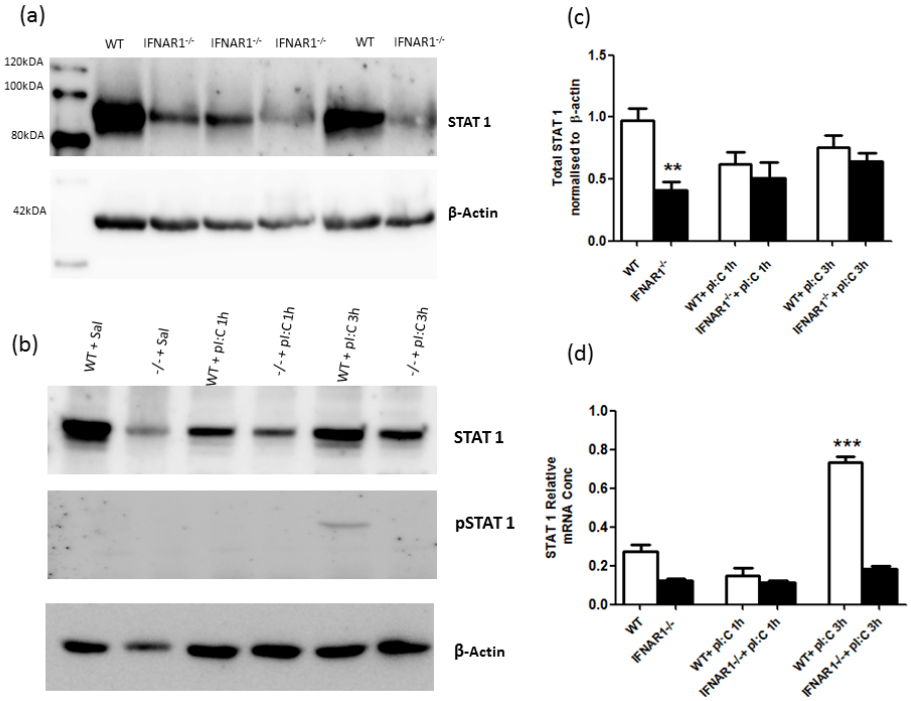


Figure 5.8 Expression of total and phosphorylated STAT1 in WT and IFNAR1$^{-/-}$ mice. Western blot of basal STAT1 in WT and IFNAR1$^{-/-}$ hippocampal homogenates (a). Western blot analysis of STAT1 and phosphorylated STAT1 in WT and IFNAR1$^{-/-}$ mice post-poly I:C challenge at 1 and 3h (b). Quantitative histogram of basal STAT1 in WT and IFNAR1$^{-/-}$ mice and levels of STAT1 at 1 and 3h post-poly I:C (c). Significant differences by Students t test are denoted by ** (p=0.0015). Expression of stat1 mRNA (d). Data are expressed as mean ± SEM and analyzed by two-way ANOVA with treatment and strain as factors; n=5 for WT+saline, n=4 for WT+pI:C 1h, n=5 for WT+pI:C 3h and n=5 for IFNAR1$^{-/-}$+saline, n=4 for IFNAR1$^{-/-}$+pI:C 1h, n=5 for IFNAR1$^{-/-}$ +pI:C 3h. Significant main effect of treatment by Bonferroni *post-hoc* is denoted by *** p<0.001 w.r.t. saline-treated controls.

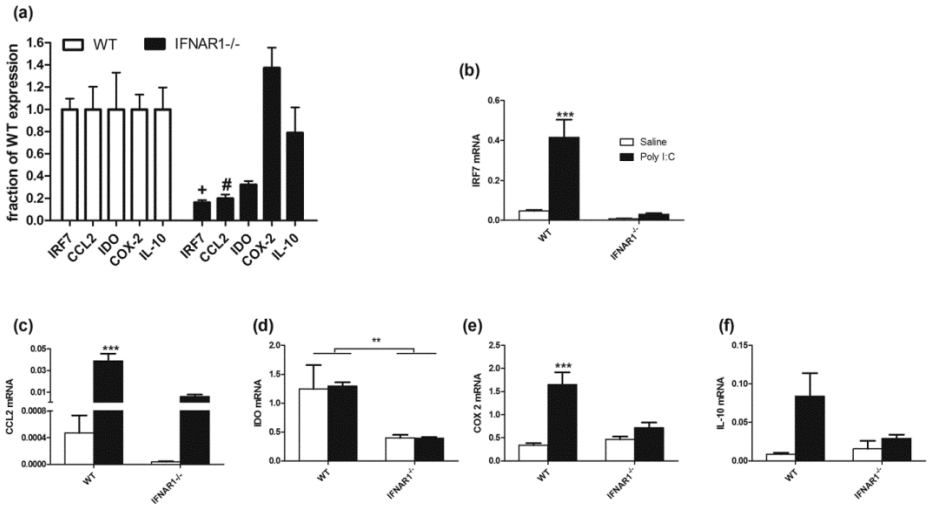


Figure 5.9 Differential basal and poly I:C-induced expression of inflammatory transcripts in WT and IFNAR1$^{-/-}$ mice. (a) Basal expression of multiple inflammatory transcripts in IFNAR1$^{-/-}$ mice are expressed as a fraction of WT expression levels and comparisons between WT and IFNAR1$^{-/-}$ were made by Student's t tests, with statistical significance denoted by + $p< 0.0001$ and # $p=0.0021$. (b-f) Expression of the same transcripts 3h post-challenge with saline or poly I:C (12 mg/kg i.p.): (b) IRF7, (c) CCL2, (d) IDO, (e) COX-2 and (f) IL-10. Data are expressed as mean ± SEM and analysed by two-way ANOVA with treatment and strain as factors, n=5 for WT and n=6 for IFNAR1$^{-/-}$. Significant interactions between strain and treatment are denoted by *** $p<0.001$ and a significant main effect of strain is denoted by * $p<0.05$ (d).

5.2.14 Poly I:C challenges ± IL-6

As can be seen in figure 5.5-5.6 the IL-6 responses to poly I:C challenge are significantly deficient in IFNAR1$^{-/-}$ animals and IL-6 is thought to be crucial for the full expression of sickness behaviour, therefore it was important to assess if the sickness behaviour response to poly I:C could be reconstituted by co-injecting exogenous IL-6 with poly I:C in IFNAR1$^{-/-}$ animals. However, it was important to first determine the effects of IL-6 injections alone in WT and IFNAR1$^{-/-}$ animals (figure 5.10a-c). As the effects of poly I:C were variable with rectal probe measurements, this was omitted and instead replaced with burrowing which is an extremely sensitive measure of suppression of species typical behaviours (Deacon, 2006).

Burrowing activity was measured at 9 and 30h, open field activity at 3h and weight loss at 12, 24 and 48h post-i.p. challenge with IL-6 (50 μg/kg) or saline. As seen in figure 5.10a-c, there were no statistically significant effects of IL-6 treatment or strain on any of these parameters using three way ANOVA ($F \leq 0.99$, df 1,31, $p \geq 0.3276$).

Poly I:C induced a decrease in burrowing in all groups but, as before, this decrease was attenuated in IFNAR1$^{-/-}$ mice. However, when IL-6 was co-administered with poly I:C, burrowing in IFNAR1$^{-/-}$ mice was decreased to levels equivalent to those in WT+poly I:C animals (figure 5.10d). That is, IL-6 could reconstitute, in IFNAR1$^{-/-}$ mice, the full deficit observed after poly I:C treatment in WT animals. An *a priori* prediction that burrowing would be maximally affected at 9h was assessed. Two-way ANOVA analysis of the four poly I:C-treated groups at this time showed a main effect of strain ($F=18.49$, df 1,31, $p=0.00$), of IL-6 treatment ($F=8.29$, df 1,31, $p=0.01$) and an interaction between these factors ($F=10.74$, df 1,31, $p=0.00$). Bonferroni *post-hoc* analysis of these data showed that when IL-6 was added simultaneously with poly I:C, burrowing in the IFNAR1$^{-/-}$ mice was no longer significantly different to poly I:C in the wild-type mice ($p>0.05$), while those challenged with poly I:C+vehicle burrowed significantly more ($p<0.001$ with respect to poly I:C+IL-6). Three way-ANOVA analysis with strain, treatment (saline) and co-treatment (IL-6) as factors showed a significant effect of strain ($p=0.04$) and treatment ($p=0<0.0001$) and there was a significant

interaction of all three factors (p=0.01). Thus, the poly I:C-induced decrease in burrowing, which is absent in the IFNAR1$^{-/-}$, is dependent on IL-6 levels.

Rearing when analysed by 3 way- ANOVA with strain, treatment (saline) and co-treatment (IL-6) as factors showed a significant effect of treatment (p=0.002) and treatment x strain (p=0.02) but no significant effect of strain or interaction of the three factors. However as was previously seen in figure 5.4c, poly I:C had less effect on rearing activity at 3h in IFNAR1$^{-/-}$ mice. Therefore, the ability of poly I:C+IL-6 to block rearing activity in IFNAR1$^{-/-}$ mice, compared to poly I:C in WT mice was examined (figure 5.10e). Exogenous IL-6 decreased rears equally in poly I:C-treated WT and IFNAR1$^{-/-}$ mice. Two way ANOVA at 3h with strain and co-administration of IL-6 as two factors showed a significant effect of IL-6 co-treatment ($F=4.43$, df 1,35, $p=0.04$) and of strain ($F=19.57$, df 1,35, $p<0.0001$) but no significant interaction ($F=0.05$, df 1,35, $p=0.82$).

Thus IL-6 exacerbates poly I:C-induced suppression of rears, but does so equally in WT and IFNAR1$^{-/-}$ animals. In other words, both IL-6 and IFNAR1 influence poly I:C-induced decrease in rears but these effects are independent of each other.

Poly I:C induced a marked weight loss in both strains as previously observed, but this recovered more quickly in IFNAR1$^{-/-}$ mice (figure 5.10d). The possibility that the addition of IL-6 with poly I:C might reconstitute the effect of poly I:C in WT animals was examined. The addition of IL-6 did not slow the recovery of weight in poly I:C-treated IFNAR1$^{-/-}$ mice (figure 5.14f). Three-way ANOVA of these data with strain and IL-6 as between subjects factors and time as the within subjects factor showed a main effect of strain ($F=21.71$, df 1,38, $p<0.0001$) but no effect of IL-6 ($F=1.18$, df 1,38, $p=0.28$) and no interaction of strain and IL-6 ($F=1.37$, df 1,38, $p=0.25$). Therefore the impact of type I interferon on weight loss during poly I:C-induced sickness behavior does not appear to be mediated or modulated by IL-6. Collectively these data show that IFNAR1 contributes to the sickness behaviour responses in both IL-6-dependent and independent mechanisms.

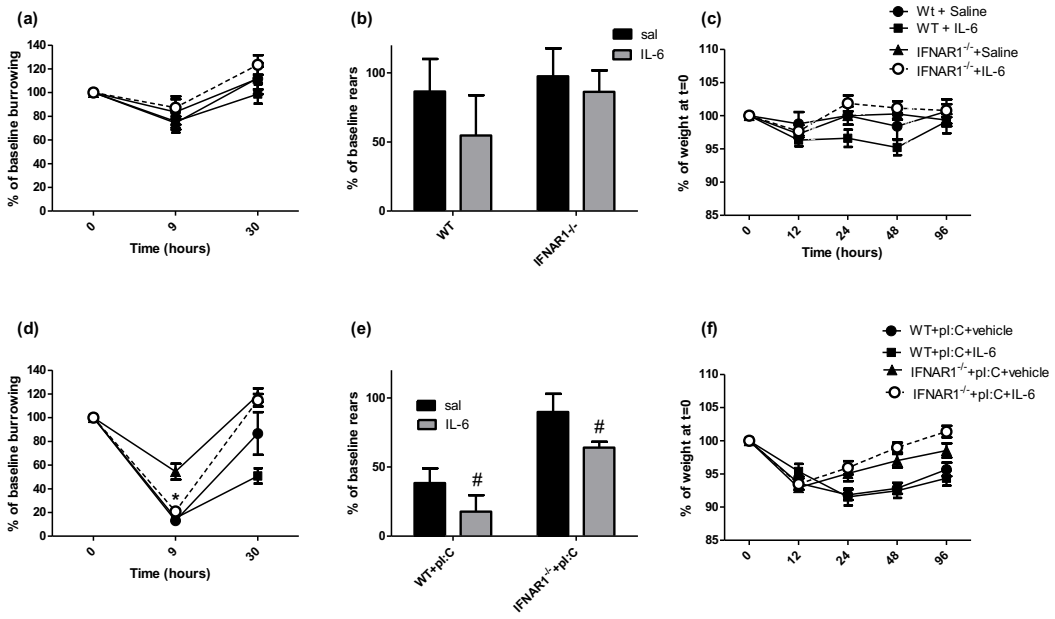


Figure 5.10 Sickness behavior post-IL-6 and post-poly I:C ± IL-6 challenges. (a-c) The sickness response of C57BL/6 and IFNAR1-/- mice to saline or interleukin-6 (50 µg/kg i.p.), as assessed by burrowing (a), rearing activity in the open field (b) and % body weight loss (c). Data are expressed as mean ± SEM, n=8 for WT+Saline, n=7 for WT+IL-6, n=10 for IFNAR+Saline, n=10 for IFNAR+IL-6. d-f) The sickness response of C57BL/6 and IFNAR1-/- mice to poly I:C+vehicle (12 mg/kg i.p.) versus poly I:C+IL-6 (50 µg/kg i.p.) challenges on burrowing (d), rearing (e) and body weight (f). Data are expressed as mean ± SEM, n=11 for WT+pI:C and IFNAR+pI:C, n=12 for IFNAR+pI:C+IL-6 and n=5 for WT+pI:C+IL-6. Rearing activity at 3h was analyzed by two-way ANOVA (b,e) with strain and treatment as factors. Burrowing at 9h (a, d) and body weight change (c, f) were analyzed by three-way ANOVA with strain, treatment and time as factors. * denotes significant difference between IFNAR+pI:C+vehicle and IFNAR+pI:C+ IL-6 at 9h after a significant two-way ANOVA at that time ($p<0.05$). denotes significant main effect of IL-6 treatment by two-way ANOVA (e).

5.2.15 Cognitive and behavioural changes in aged WT and IFNAR1$^{-/-}$ mice

It has recently emerged that IFN-I may have significant impacts on age-related neuroinflammation and cognitive decline. *In vivo* studies with $APP_{SWE}/PS1_{\Delta E9}$ IFNAR1$^{-/-}$ crosses revealed that the absence of IFNAR1 led to improved spatial and learning memory compared to positive controls as measured by Morris Water Maze and that microglial phenotype was altered with reduced *iNOS* and *CD11b* expression and increased *Arg1* and *Trem2* expression the knockout mice (Minter et al., 2016). Likewise, microarray analysis of the choroid plexus (CP) of the PDGFB-APP AD murine model revealed an IFN-I expression profile whilst, in the APP/PS1 murine AD model, a two-fold increase in IFN-α was noted in whole brain homogenates by ELISA (Mesquita et al., 2015). RT-PCR of human AD patient pre-frontal cortex also revealed a significant increase in IFN-I expression compared to non-AD patients (Mesquita et al., 2015, Taylor et al., 2014). IFN-I has also been implicated in α-synuclein toxicity and murine PD models were found to show reduced neuroinflammation and disease progression in IFNAR1$^{-/-}$ mice with respect to WT controls (Qin et al., 2016, Main et al., 2016).

With respect to ageing, it has been reported that the murine CP shows an elevated IFN-I response and aged mice injected (i.c.v) with an IFNAR1-neutralising antibody showed improved spatial memory and learning abilities and enhanced neurogenesis compared to IgG-treated controls (Baruch et al., 2014). Likewise, transcriptomic analysis of microglia has shown an age-associated, brain region-dependent, induction of the IFN-I response (Grabert et al., 2016). These data suggest that IFN-I signalling is detrimental in the ageing brain. The aim of the next experiments was to investigate whether an increased IFN-I response could be observed in the murine brain during ageing and whether the absence of IFNAR1 would mitigate effects of age on microglial phenotype and on age-associated cognitive impairments. The influence of IFNAR1 on the hippocampal response to systemic inflammatory stimuli was also investigated in order to assess whether age and IFN-I shape glial responses to acute inflammatory stimuli.

Therefore, the next set of behaviour tests aimed to assess the role of IFN-I in tests of hippocampal dependent memory which include the spontaneous alternation T-maze

task, the visuospatial reference memory Y maze and the contextual fear task. Open field activity was also assessed in IFNAR1$^{-/-}$ mice as they aged. 4-5 month mice were regarded as young adult mice, 16-17 months were regarded as middle aged mice and we sought to pursue behaviour tasks into older age however mortality in the timing curtailed these experiments and the last time point available was 18-19 months.

To assess working memory performance WT and IFNAR1$^{-/-}$ mice were assessed for 10 trials over two days, at 16-17 months old, in a spontaneous alternation T-maze task. The aged IFNAR1$^{-/-}$ mice showed significantly lower spontaneous alternation ($p<0.01$, Student's t-test) (figure 5.11a) suggesting impaired working memory.

Spatial reference memory was assessed using an escape from water Y-maze task at 17-18 months of age. All mice showed significant learning across trial blocks (significant effect of trial block, F = 25.08, $df_{2,48}$, p < 0.0001) but there was no significant effect of strain (F = 1.54, $df_{1,48}$, p = 0.24) or interaction between trial block and strain (F = 1.64, $df_{2,48}$, p = 0.20 (figure 5.11b).

Contextual memory consolidation was assessed using contextual fear conditioning. 48 hours after shock-context pairing, the time spent freezing in the original location of the foot-shock was assessed. IFNAR1$^{-/-}$ mice showed a significant impairment in memory consolidation with a significant decrease in time freezing post-shock compared to WT (figure 5.11c**,** p=0.008 by Student's t-test). Therefore IFNAR1$^{-/-}$ mice show impairments in hippocampal dependent memory.

Open-field activity was assessed as the mice progressed in age, starting at 4-5 months and continuing to 18-19 months in age, focusing on squares crossed (general locomotor activity) and rearing (exploratory) activity for WT and IFNAR1$^{-/-}$ mice (figure 5.11 d**-**e). Graphs shown represent all mice that were present at the time of behavioural assessment, however as the mice aged, some mice died or reached humane end-points resulting in the two way-repeated measures ANOVA being performed only on the mice that were present throughout the entire study. No significant difference was observed in squares crossed but there was a trend towards increased squares crossed with increasing age in IFNAR1$^{-/-}$ mice while age-matched WT mice remained relatively constant. Two-way repeated measures ANOVA including

all animals up to 16-17 months and excluding 18-19 months, to increase statistical power, suggested a strong trend towards an interaction between strain and age ($F=3.51$, $df_{1,29}$, $p=0.07$)

A significant difference was observed in the rearing activity with IFNAR1$^{-/-}$ mice showing a significantly higher level of rearing compared to the WT at 16-17 months and 18-19 months in age. Two-way repeated measures ANOVA showed no significant effect of age but a very significant effect of strain ($F = 9.84$, $df_{1,48}$, $p = 0.00$). Rearing is an exploratory behaviour and, when combined with the prior data in hippocampal memory function, the maintained rearing levels in IFNAR1$^{-/-}$ mice compared to the habituation to the familiar arena in WT mice is consistent with a visuospatial memory impairment. It is also important to note that these mice did not show differences in anxiety using the elevated plus maze as seen in figure 5.1 or as they continued to age. The data indicate that lack of IFN-I-mediated signalling during ageing actually has detrimental effects on hippocampal-dependent cognitive function.

The weight of the WT and IFNAR1$^{-/-}$ mice was also measured as they aged and it was apparent that, from approximately 14 months, IFNAR1$^{-/-}$ mice gained weight more readily than WT animals. Again, only mice that were present throughout the full study were analysed by two-way repeated measures ANOVA with strain and age as the two factors. There was a significant effect of age with all mice gaining weight as they aged ($F = 94.12$, $df_{15,405}$, $p < 0.0001$) and interaction between strain and age ($F = 9.29$, $df_{15,405}$, $p < 0.0001$ indicating a greater weight increase in IFNAR1$^{-/-}$ mice.

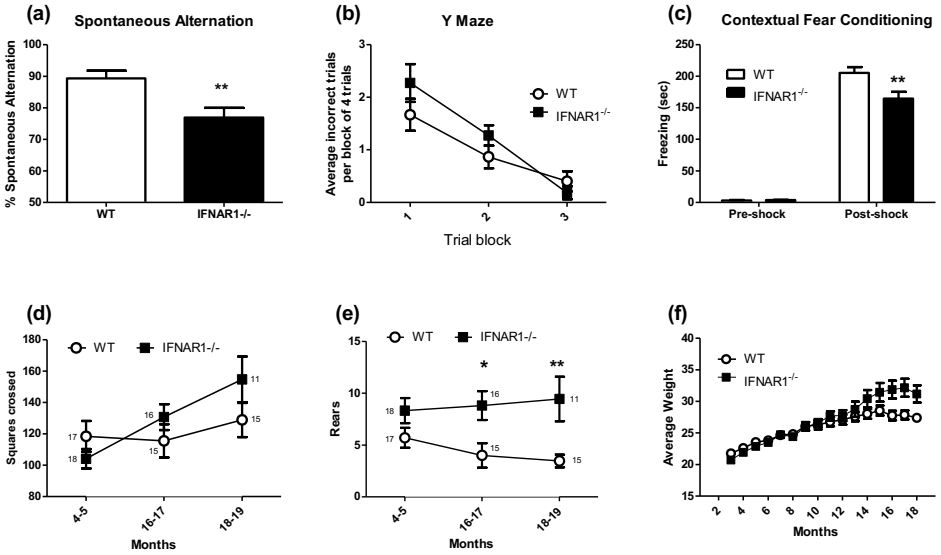


Figure 5.11 Working memory, spatial reference memory, memory consolidation and open-field activity in WT and IFNAR1$^{-/-}$ mice (a) Working memory was assessed using the spontaneous alternation T-maze task in WT and IFNAR1$^{-/-}$ mice (b) Visuo-spatial reference memory was assessed using the Y-maze across 12 trials (4 trials per trial block) in WT (n=15) and IFNAR (n=11) (c) contextual fear conditioning (d-e) open-field activity measurements and (f) average weight of WT and IFNAR1$^{-/-}$. Data are shown as mean ± SEM and were analysed by t-test for (a) and 2-way repeated-measures ANOVA with strain and trial block or shock or time as factors for (b-f). Significant difference denoted as ** $p<0.01$ and * $p<0.05$.

5.2.16 The effect of age on basal IFN-I responses in the murine hippocampus

In order to assess whether aging induced increased IFN-I activity in the murine hippocampus, RNA was extracted from the hippocampi of young and aged WT mice. Three IFN-1 dependent genes; *Irf7, Oas1a* and *Cxcl10* were examined in the cDNA of these mice. The young mice in this cohort were aged 3 months and the aged mice were between 19-21 months. The results in figure 5.12 (a-c) show that there is a significant increase in *Irf7, Oas1a* transcription in the aged mice compared to the young mice with $p< 0.05$ by student's t test. There was a trend towards significance for *Cxcl10* with p=0.06. In a second cohort of animals, these transcripts were also examined. In this cohort young animals were 7 months old and the aged animals ranged from 24-26 months. In this cohort of animals, there was surprisingly no age-associated increase in hippocampal IFN-I response, compared to that seen in figure a-e. A recent paper published by Grabert and colleagues in which they analysed IFN-I transcripts in the cerebellum showed a strong age-associated increase in inflammatory transcripts in the cerebellum (Grabert et al., 2016). With this in mind, the cerebellum of this second cohort of animal's was examined for *Irf7, Oas1a* and *Cxcl10*. In contrast to the results seen in figure 5.13d-f, there was elevated levels of each of these IFN-I dependent genes in the aged animals with *Irf7* ($p < 0.01$), *Oas1a* ($p < 0.001$) and *Cxcl10* ($p < 0.05$) when analysed by student's t test (Fig g-i) The data for figure 5.12 (d-i) was kindly provided by Dáire Healy. These results suggest that across 2 cohorts, that the age-associated hippocampal increase in IFN-I is relatively variable in the hippocampus. However, the observed age-associated increase in the cerebellar IFN-I response, despite being assessed in whole cerebellar homogenates does support the idea of relative reactivity of the cerebellum compared to other brain regions such as the hippocampus (Grabert et al., 2016).

Despite this somewhat variable IFN-I response in aged animals, it was important to demonstrate that these IFN-I dependent genes were suppressed in aged IFNAR1$^{-/-}$ mice. RT-PCR therefore was performed on cDNA derived from the hippocampi of aged WT and IFNAR1$^{-/-}$ mice, and the transcript levels of *Irf7*, *Oas1a* and *Cxcl10* were quantified. Figure 5.12 j-l shows that the absence of IFNAR1 during aging resulted in significantly decreased levels of *Irf7* ($p < 0.0001$), *Oas1a* ($p < 0.001$) and *Cxcl10* ($p <$

0.05) when analysed by student's t test, suggesting that the absence of IFNAR1 significantly reduces the IFN-I response in aged mice.

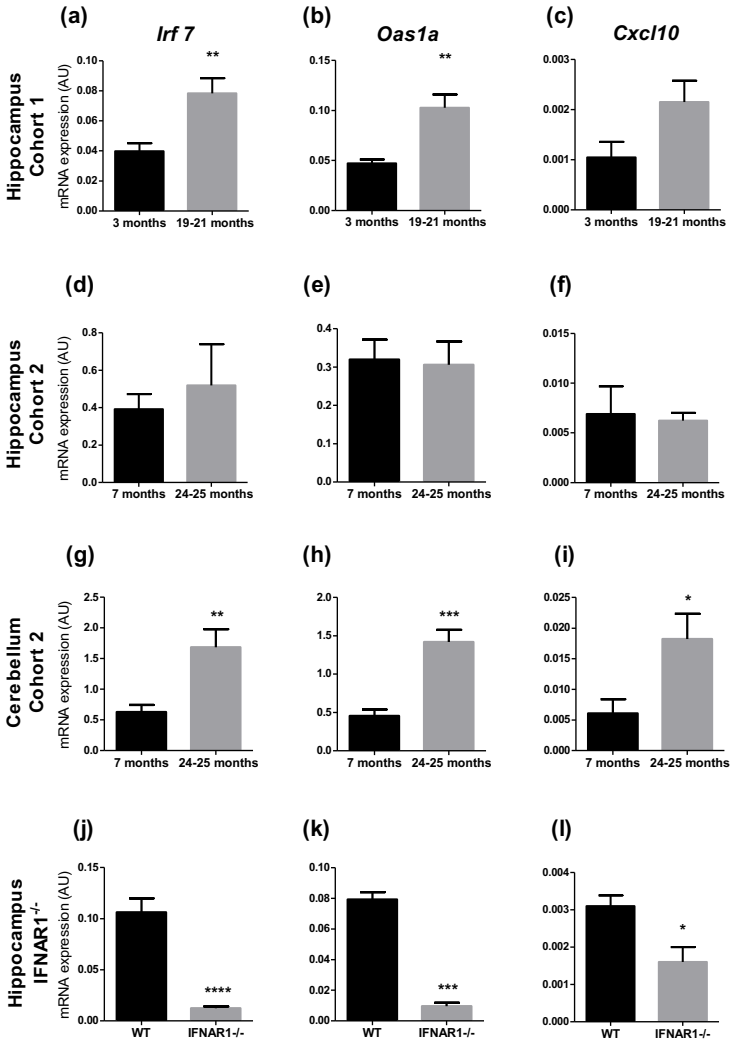


Figure 5.12 The impact of age on basal IFN-I responses in the hippocampus and cerebellum. All data were analysed by Student's *t-test* and expressed as mean ± SEM. Graphs in each row are in the order from left to right; *Irf7*, *Oas1a* and *Cxcl10*. (**a-c**) Hippocampal analysis of cohort 1. Young (3 months, *n* = 6), aged (19-21 months, *n* = 5) (**d-f**) Hippocampal analysis of cohort 2. Young (7 months, *n* = 6), aged (24-25 months, *n* = 6). (**g-i**)Cerebellar analysis of cohort 2. (7 months, *n* = 7), aged (24-25 months, *n* = 6). (J-l) Hippocampal analysis of aged WT and IFNAR1$^{-/-}$ mice. Statistical significance denoted by *** $p<0.001$, ** $p<0.01$ and * $p<0.05$.

5.2.17 Transcriptional analysis of aged WT and IFNAR1$^{-/-}$ mice

Although a definitive age-associated increase in the hippocampal IFN-I response was not observed in aged mice, IFNAR1$^{-/-}$ mice were more cognitively impaired and it remained important to assess the influence of IFN-I signalling on the microglia of the hippocampus in the aged mice. Therefore, RT-PCR analysis was performed on the hippocampi of aged WT and IFNAR1$^{-/-}$ mice to interrogate a neuroinflammatory transcriptional signature. The effect of chronic absence of IFN-I signalling on microglial number and activation state and on the complement system, and lysosomal/phagocytic activity were quantified and analysed by Student's t-test. *Tyrobp* has been reported to be strongly indicative of microglial number (Holtman et al., 2015). Here, transcription of *Tyrobp* was reduced in the IFNAR1$^{-/-}$ mice when compared to WT, ($p < 0.05$) (Fig. 5.13a). Analysis of *Clec7a*, an indicator of microglial priming, was also found to be significantly decreased in the aged IFNAR1$^{-/-}$ when compared to the aged WT ($p < 0.05$) (figure 5.13b) but no significant difference in transcription of the scavenger receptor *Cd68* was observed (figure 5.13c). Assessing the complement system, *C1qα, C3* and *Cd11b*/*ITGAM* transcription were all somewhat decreased in aged IFNAR1$^{-/-}$ compared to WT mice (33%, 28% and 26% respectively) but only the reduction in C1qa was statistically significant ($p<0.01$) (figure 5.13 d-f). To assess differences in lysosomal and oxidative activity indices of the lysosomal cathepsins and NADPH oxidase were assessed (transcription of *Ctsd*, *Ctss* and *Cybb;* figure 5.13 *g-i)*. Absence of IFNAR1 resulted in a significant decrease in *Ctsd* ($p < 0.05$), *Cybb* ($p < 0.0001$) and *Ctss* ($p < 0.01$). Collectively these data may indicate a modulation of microglial number and phenotype in aged IFNAR1$^{-/-}$ mice compared to WT mice, with perhaps a suppression of priming of this population. There was modest evidence of suppressed lysosomal activity but this cannot be definitely attributed to the microglia specifically.

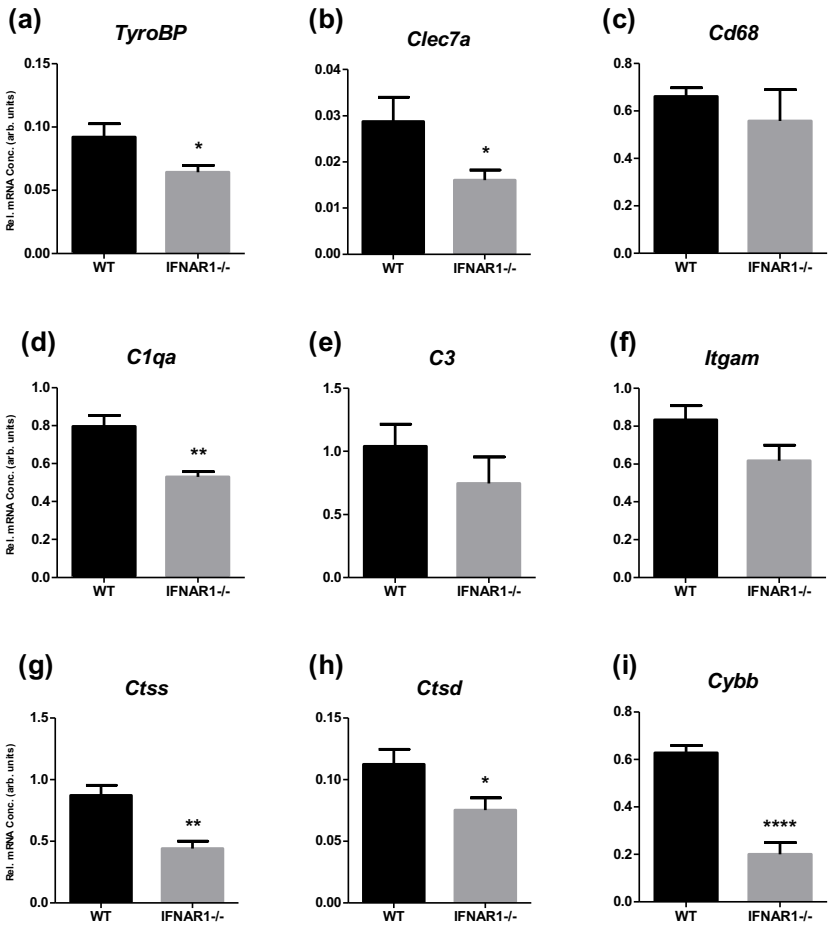


Figure 5.13 Transcriptional analysis of 19 month WT and IFNAR1$^{-/-}$ mice. Data were analysed by Student's *t-test.* Statistical significance was determined by Bonferroni *post hoc* tests and denoted by **** $p<0.0001$, ** $p<0.01$ and * $p<0.05$. Data are represented as mean ± SEM. WT (19 months old, $n = 6$), IFNAR1$^{-/-}$ (19 months old, $n = 6$).

5.2.18 Induction of IFN-I response and microglial activation by systemic inflammation

Since there were changes in microglial activation in IFNAR1$^{-/-}$ mice and previous work in our laboratory has observed exaggerated IFN-I response in animals with primed microglia (Field et al., 2010) it was important to examine the extent to which altered microglial phenotype in IFNAR1$^{-/-}$ might alter the CNS response to systemic inflammation. RNA was isolated from the hippocampi of WT and IFNAR1$^{-/-}$ mice post-challenge with saline or poly I:C and the mRNA levels quantified by RT-PCR. In the IFNAR1$^{-/-}$ post-poly I:C treated mice, IRF7 was completely ablated. There was a significant strain x treatment effect with F = 69.85, $df_{1,15}$, $p < 0.0001$). Expression of several microglial and phagocytic-related transcripts were examined in these mice. Absence of IFNAR1 reduced basal levels of transcripts for *C1qα*, *C3*, *Itgam*, *Cd68, Cybb* and *Ctss* (figure 5.14b-g). Poly I:C induced a relative increase in the expression of most of these transcripts (significant effect of treatment for 4 of 6 transcripts: $F \geq 5.523$, $df_{1,15}$, $p \leq 0.03$; Fig.5.14 g-i), while *cd68* and *Ctss* were unchanged by poly I:C. There was no interaction between strain and treatment for any of these transcripts. Collectively, the data in figure 5.14 suggests that systemic inflammation induced by poly I:C drives acute IFN-I responses in the hippocampus, but that IFNAR1 does not appear to contribute to acute systemic inflammation-induced changes in this small panel of microglial/phagocytic transcripts, although it does appear to influence their basal level of expression.

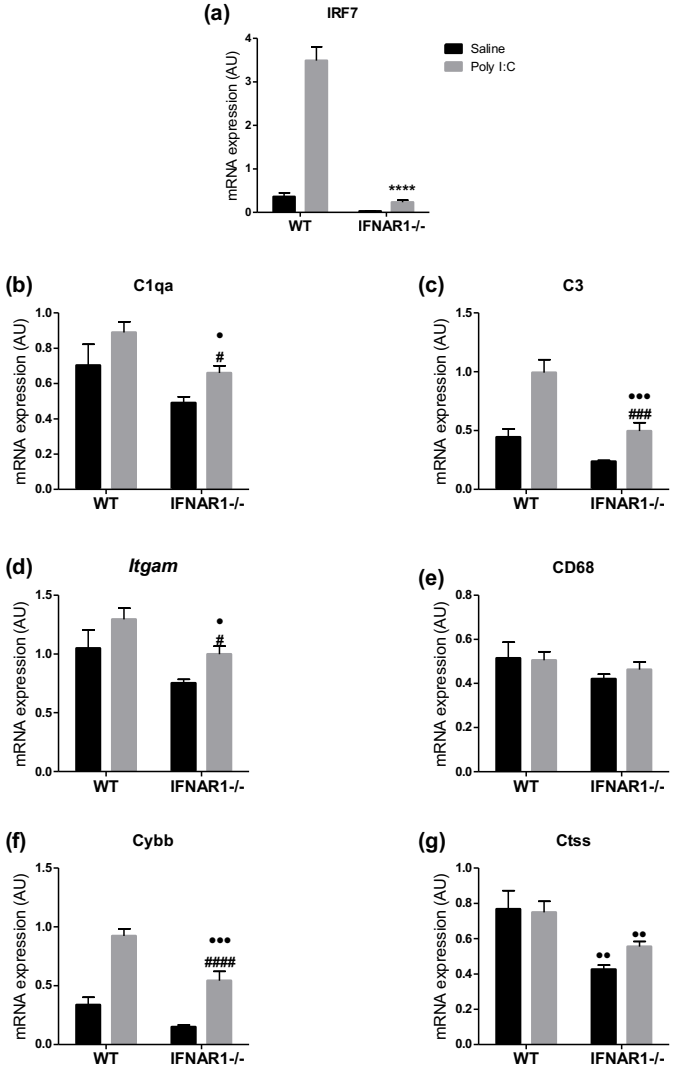


Figure 5.14 Induction of IFN-I response and microglial activation by systemic inflammation in WT and IFNAR1$^{-/-}$ mice. RT-PCR analysis of hippocampal mRNA of WT and IFNAR mice 3h post-i.p challenge with saline or poly I:C (12mg/kg poly I:C). Animal were aged 5-8 months; n=5 for WT+Saline, WT+ Poly I:C , IFNAR1$^{-/-}$ + saline and n=4 for IFNAR1$^{-/-}$ +Poly I:C. Data are shown as mean ± SEM and was analysed by two-way ANOVA. Statistical differences were determined by Bonferroni *post hoc* tests. Effects of treatment denoted by $^{\#\#\#}$ p<0.001, effects of age denoted by $^{\bullet\bullet\bullet}$ p<0.001, $^{\bullet\bullet}p$<0.01 and interaction between strain and treatment is denoted by $^{*}p$<0.05.

5.3 Discussion

5.3.1 Characterizing the IFNAR1$^{-/-}$ strain

The data presented in this chapter has shown that IFN-I receptor 1 deficient mice (IFNAR1$^{-/-)}$ mice are not impaired in hippocampal dependent memory task, they learn the Y maze task equally to controls. They also do not display increased anxiety or locomotor activity to controls and there is no difference in the diurnal rhythm of these mice to controls. IFNAR1$^{-/-}$ mice following challenge with poly I:C, displayed a reduction in sickness behaviour measurements compared to WT challenge with poly I:C. IFNAR1$^{-/-}$ mice displayed significantly attenuated poly I:C-induced hypothermia, hypoactivity and weight loss compared to WT C57BL/6 mice. This amelioration of sickness was associated with equivalent IL-1β and TNF-α responses but much reduced IL-6 responses in plasma, hypothalamus and hippocampus of IFNAR1$^{-/-}$ mice.

IFN-β injection induced trivial IL-6 production and limited behavioural change and the poly I:C-induced IFN-β response did not precede, and would not appear to mediate, IL-6 induction. Rather, IFNAR1$^{-/-}$ mice lack basal IFN-I activity, have lower STAT1 levels and show significantly lower levels of several inflammatory transcripts, including stat1. Basal IFN-I activity appears to play a faciliatory role in the full expression of the IL-6 response. The deficient IL-6 response in IFNAR1$^{-/-}$ mice partially explains the observed incomplete sickness behaviour response. Reconstitution of circulating IL-6 revealed that the role of IFNAR in burrowing activity is mediated via IL-6, while IFN-I and IL-6 have additive effects on hypoactivity, but the role of IFN-I in anorexia is independent of IL-6. Hence, this chapter demonstrated both interdependent and independent roles for IFN-I and IL-6 in systemic inflammation-induced changes in brain function. Aged IFNAR1$^{-/-}$ mice, displayed impaired hippocampal function compared to controls and had significant decreases in IFN- responsive genes compared to controls. Expression of microglial associated with neuronal maintenance was robustly suppressed in these mice. Analysis of neuroinflammatory transcripts after systemic inflammatory stimuli (poly I:C) indicated that microglia in the aged brain are primed to produce exaggerated acute responses to these stimuli, but these were not mediated by IFN-I. These data indicate that in normal aging, the IFN-I response contributes to normal neuronal homeostasis.

5.3.2 Role of endogenous IFN-I in cognitive behaviour, anxiety and locomotor activity and diurnal rhythm.

Cognitive behaviour was assessed in the Y-maze visuospatial reference memory task. IFNAR1$^{-/-}$ mice were not impaired on the learning of this task (fig 5.1a). They learned the exit from this behaviour task at the same rate as WT controls. This would suggest that endogenous IFN-I are not important in visuospatial reference memory tasks.

It is clear that when IFN-α is given to patients to treat cancer and hepatitis, that many patients show signs of anxiety as well as depression and sickness behaviour (Bonaccorso et al., 2001, Schaefer et al., 2002, Raison et al., 2005). Here, IFNAR1$^{-/-}$ and WT mice were tested in the open field and elevated plus maze which is used as a measure of anxiety (Walf and Frye, 2007). As can be seen in figure 5.1b,c, IFNAR1$^{-/-}$ mice did not show signs of hyperactivity in the open field. They showed similar levels of activity to their WT controls. In addition they did not exhibit any increase in rearing activity which is thought to be a measure of exploratory behaviour. In the elevated plus maze, the IFNAR1$^{-/-}$ mice showed equivalent amount of time spent in the open and closed arms as the WTs. These results would indicate that in the experiments in figure 5.1 type one interferons do not play a role in anxiety or locomotor behaviour. It is also important to note that when these experiments were repeated in male mice, there was no difference in behaviour to the females (figure 5.2) , therefore gender does not influence any hypothetical impact of IFN-I.

Traynor and colleagues reported that IFNAR1$^{-/-}$ mice show altered sleep patterns with decreased time spent in spontaneous rapid eye movement (REMS) and non-rapid eye movement (NREMS) and a reduction in number of REMS episodes (Traynor et al., 2006, Bohnet et al., 2004) however no reports have looked at diurnal rhythm in IFNAR1$^{-/-}$ mice. To address the role type one interferons may play in diurnal rhythm, the night and day activity of WT and IFNAR1$^{-/-}$ mice was recorded over a period of 3 nights and 2 days with the use of phenotyper home cages. The recording taken over 60 hours were binned into one hour periods and presented as 15 consecutive periods. As can be seen in figure 5.3, the WT and IFNAR1$^{-/-}$ mice were clearly more active in the night phase compared to the day and there was no difference in strains. However, it is important to note that there were 18 IFNAR1$^{-/-}$ and 17 WT mice used in this study, and

they were divided into groups of 6 and placed into each of the 3 phenotyper cages. Due to limitations of the phenotyper cage tracking, the total activity of a cage was recorded instead of the activity of each of the 6 mice. Therefore, the data presented in figure 5.3 represents the total activity of three cages of 6 mice instead of the activity of each individual mouse. Though the statistical analysis indicates that IFN-I do not play a role in diurnal rhythm, a more detailed analysis of individual activity per mouse would be better to assess instead of total cage activity as there could be an hyperactive mouse that is influencing the data being recorded per cage despite IFNAR1$^{-/-}$ mice showing no difference in activity levels to that of WT (figure 5.2).

5.3.3 Role of type one interferons in sickness behaviour

It can be seen that IFNAR1 deletion has limited effect of basal behaviour however there is evidence that acute IFN I induction can cause sickness behaviour. Interferon-α when given to cancer and hepatitis patients, can induce sickness behaviour symptoms (Raison et al., 2005) and poly I:C when given systemically can induce fever, hypothermia, decrease activity, weight, and disrupt sleep wake cycles (Fortier et al., 2004, Cunningham et al., 2007, Traynor et al., 2006).There has been very limited research into whether IFN-I-might contribute to the sickness behaviour response so this was addressed in this study.

Here, the levels of IFN-β but not IFN-α was highly expressed following poly I:C challenge. The sickness behaviour response is different in the IFNAR1$^{-/-}$ mice compared to their controls. During behavioural testing, a subjective observation indicated that the IFNAR1$^{-/-}$ mice 'appeared' less sick. They exhibited less ruffled hair and a less hunched posture than WT controls. Rearing activity which is a measure of exploratory behaviour, is less decreased in IFNAR1$^{-/-}$ mice and there was a less marked hypothermia in these mice compared to WT controls following poly I:C challenge. Traynor and colleagues showed that IFNAR1$^{-/-}$ mice showed protection against robust and progressive hypothermia a number of days after inoculation with influenza virus in wild type mice (Traynor et al., 2007) which is consistent with the data seen here. Other research has shown that IFN-α can induce hypothermia in rats (Sammut et al., 2001). It has also been reported that IFN-α can cause hyperthermia during the light

phase in mice and this has also been shown with IFN-β (Ohdo et al., 1997). The data though shown here would suggest that IFN-I are influencing temperature and activity levels during poly IC induced sickness behaviour.

Poly I:C is known to produce robust anorexia (Cunningham et al., 2007). When weight was measured in these mice, all poly I:C-treated mice showed a marked reduction but the IFNAR1$^{-/-}$ mice showed a significantly faster recovery of this weight reduction 48h post challenge. This data would suggest that IFN-I contribute to anorexia and that activity of IFN-I influences the time-course of recovery during poly I:C-induced sickness. Poly I:C-treated animals remained capable of rearing and climbing and, in previous studies, maintained muscle strength and motor coordination (Field et al., 2010) and it is therefore highly likely that weight loss reflected decreased drive to eat, rather than inability to obtain food. A recent paper demonstrated that influenza infected mice and mice challenged with poly I:C when glucose was administered to them had a higher survival rate, than mice that were injected with 2-DG an inhibitor of glycolysis. It was found in the same study that when IFNAR1$^{-/-}$ mice challenged with poly I:C were injected with 2-DG they had a higher survival rate, compared to WT, indicating that IFN-I is necessary for the detrimental effects of 2-DG (Wang et al., 2016).

5.3.4 Impact of IFNAR1$^{-/-}$ on cytokine profiles.

Examination of the pro-inflammatory cytokines in the plasma, hippocampal and hypothalamic brain region in the WT and IFNAR1$^{-/-}$ mice post poly IC challenge were assessed. There was only IL-6 production in WT animals after poly I: C challenge and there were elevated levels of IL-1β, TNF-α and IFN-β in both IFNAR1$^{-/-}$ and WT suggesting that IL-1β, TNF-α and IFN-β poly I:C inductions are not affected by IFNAR1. Similarly, TNF-α production after poly I:C is not affected by IFNAR1 in either the hippocampus or hypothalamus. IL-1β expression was induced in both strains post poly I:C challenge, though in the hypothalamus, IL-1β expression is exacerbated in IFNAR1$^{-/-}$ mice. IFN-β was expressed in both strains in the hypothalamus post-poly I:C challenge, but only in the WT in the hippocampus. IL-6 production was only seen in the WT post-poly I:C challenge. Together, the data suggest that IFNAR1 expression appears to be

necessary for robust plasma and CNS IL-6 responses to poly I:C and for the full expression of sickness behaviour, but not for IL-1β nor TNF-α responses. The data shown in the current study is consistent with data in a previous study by Cunningham and colleagues. The authors report that when WT mice are challenged with poly I:C there are elevated levels of IL-1β, IL-6, TNF-α and IFN-β in the periphery, hippocampus and hypothalamic brain region. The mice in the same study also showed decreased locomotor activity, burrowing and body weight and mild hyperthermia, all suggesting that increase in cytokines is contributing to the sickness behaviour response. Similar studies have also showed that poly I:C can induce fever and reduce food intake which is accompanied by increased levels of IL-6, TNF-α, IL-1β in the hypothalamus which is responsible for regulating temperature responses (Fortier et al., 2004).

5.3.6 Role for IFN-I in IL-6 expression

The milder sickness behaviour and loss of poly I:C-induced IL-6 in IFNAR1$^{-/-}$ mice suggested that IFN-I may play a role in IL-6 expression. To address this, IFN-β was injected into WT mice and IL-6 expression compared to poly I:C induced expression of IL-6 was measured in the plasma, hypothalamus and hippocampus of these mice. There was IL-6 expression in the plasma and hypothalamus (figure 5.7), however these levels were much less compared to those induced by poly I:C which is consistent with the limited evidence for IFN-α/β directly inducing IL-6. One study in patients with chronic hepatitis C showed that after injection of Interferon-α, there was a significant induction of IL-6 in the plasma 2 hours post-treatment that continued for 6 hours whereas TNF-α and IL-β levels were not affected. The induction of this IL-6 could be blocked by serine protease inhibitor gabexate mesilate in these patients. In the same study, it was also demonstrated that IL-6 transcripts were increased following IFN-α treatment. It was suggested that IFN- α may induce IL-6 by inducing or activating nuclear factor IL-6 and nuclear factor-$_K$B that positively regulate IL-6 production or inhibiting the degradation of IL-6 mRNA (Ito et al., 1996). Sickness behavioural measurements were taken in the mice post IFN-β challenge. IFN-β treatment had no effect on body temperature or body weight (figure 5.7), however open-field activity was decreased 9h post-challenge and at 7h post challenge, there was also a decrease in burrowing activity which is seen as a rewarding behaviour (Deacon, 2006).These

mild effects may be explained by the rapid clearance of injected cytokines (Abraham et al., 2010). This data would suggest that IFN- β can induce mild sickness behaviour, but not to the full spectrum seen in the poly I:C studies.

The diminished levels of IL-6 in IFNAR1$^{-/-}$ mice indicates that IL-6 expression upon poly I:C challenge is IFN-I dependent. One possible explanation of this effect could be that in normal mice, poly I:C induces IFN-β, which in turn induces IL-6. However this is not intuitive since NFκB is a key regulator of IL-6 and is induced rapidly after LPS, TNF-α or double stranded RNA (Libermann and Baltimore, 1990). Therefore, the levels of IL-6 and IFN-β were examined in the plasma and hippocampus of WT and IFNAR1$^{-/-}$ mice to establish whether IFN-β was expressed significantly earlier than IL-6 post poly I:C challenge, and whether the expression of IFN-β was essential for the poly I:C induced IL-6. IFN-β does not precede IL-6 expression as IL-6 was present at 1hr post-poly IC challenge. IL-6 continues to increase in WT mice at 3hr but this further increase in IL-6 is absent in IFNAR1 deficient mice. In contrast, IFN-β was induced 1 hr post-poly I:C in WT and IFNAR1$^{-/-}$ mice and continues to rise at 3hr in IFNAR1$^{-/-}$ mice whereas in the hippocampus, however IFN-β expression increased at 3h post-poly I:C in WT mice but not in IFNAR1$^{-/-}$ mice. These data would indicate that acute IFN-β expression is not the signal that triggers IL-6 expression. Rather the data suggest that IFNAR1 facilitates full IL-6 expression, perhaps through basal low level IFN-I activity.

5.3.7 STAT1 expression in WT and IFNAR1$^{-/-}$ mice

There is now ample evidence that low level actions of type I interferons are essential to enhance cellular responses to subsequent stimuli (Taniguchi and Takaoka, 2001) and these include optimisation of both type I interferon responses but also responses to other cytokines such as IFN-ɣand IL-6 (Muller et al., 1994, Mitani et al., 2001). It is also known that IFN-γ-induced factors can synergise with TLR agonists to produce optimal levels of TLR target genes and key among these factors is STAT1, which synergises with NFκB to maximise inflammatory mediator output (Schroder et al., 2006). STAT1 activity is also a major signalling mechanism in type I interferon actions and it has been reported that cells that are IFNAR1$^{-/-}$ provide less docking sites for

STAT1 and STAT3, thus impairing STAT1/ 3 and therefore also IL-6 and IFN-I signalling (Mitani et al., 2001).

IFNAR1$^{-/-}$ mice have suppressed levels of total STAT1 in comparison to WT controls (figure 5.8).Gough and colleagues have shown that this lower STAT1 results in lower expression of STAT1-dependent genes stat1, irf1 and junB (Gough et al., 2010), providing a simpler explanation of less efficient expression of STAT1-dependent genes in IFNAR1$^{-/-}$ mice. The data shown in figure 5.8 replicate, in the brain, this lower STAT1 expression level, decreased STAT1 phosphorylation post-poly I:C and the decreased transcription of the dependent gene stat1. This suggests that basal STAT1 has a role in facilitating poly I:C induced IL-6 in the CNS, probably in co-operation with NFκB which has been reported to influence IL-6 expression after double stranded RNA stimulation (Libermann and Baltimore, 1990). Lee and colleagues have shown that JAK-STAT1 signalling is sufficient for IL-6 expression in macrophages treated with phosphatidic acid (Lee et al., 2006) and IRF1 and NFκB have also been shown to co-operate, at low levels of expression, at the IL-6 promoter (Faggioli et al., 1997). It has also been reported in HIV infection of cultured cells, the STAT1 inhibitor fludarabine significantly decreased IL-6 expression (Chaudhuri et al., 2008) indicating the importance of STAT1 signalling for IL-6 expression during viral infection. Together these studies all indicate that STAT1 can regulate IL-6 production. In the current study, the IFNAR1$^{-/-}$ mice show lower basal levels of STAT1 which may explain diminished IL-6 levels in these mice.

5.3.8 Role of basal IFNAR1 signalling in inflammatory regulation in the brain

Given the basal differences in STAT1 expression, it was predicted that there may be changes in basal levels of important STAT1-dependent inflammatory transcripts. As can be seen in figure 5.9, basal levels of IRF7, CCL2 and IDO transcripts were all significantly lower in IFNAR1$^{-/-}$ mice than in WTs and these STAT-1-dependent inflammatory transcripts were significantly less induced in IFNAR1$^{-/-}$ mice. The data in figure 5.9 suggests that basal activity of IFNAR1 (likely activated by IFN-β, but we cannot rule out low levels of IFN-α) influences the basal expression of CCL2, IDO and perhaps many other inflammatory transcripts. These transcripts appear to remain responsive to poly I:C stimulation, but not to the same levels as in WT mice, and the

origins of this lower expression may lie in the facilitating effects of basal actions of IFN-I, not least via basal expression of STAT1. Like IL-6, CCL2 transcription can be regulated by NFκB, but there are studies showing that STAT1 can co-regulate its induction (Valente et al., 1998, Sikorski et al., 2014). The current study demonstrates that CCL2 transcripts are lower both at baseline and post-poly I:C, consistent with the total STAT1 levels. Du and colleagues reported that IDO expression can be induced by IFN-I in a STAT1 dependent manner (Du et al., 2000). IDO expression was also reduced in IFNAR1$^{-/-}$ mice. These data suggest that IFN-I is important in regulating the production of metabolites on this pathway. Since KYN can cross the blood brain barrier(Fukui et al., 1991) elevated plasma levels of KYN may also influence brain concentrations and indeed blocking the peripheral production of KYN, via inhibition of liver tryptophan-2,3-dioxygenase, produces antidepressant effects in a repeated restraint stress model of depression (Gibney et al., 2014). It is also possible, therefore, that basal IFN-I actions influence depressive states.

5.3.9 Role of IL-6 in poly I:C induced sickness behaviour

IFNAR1$^{-/-}$ mice displayed significantly attenuated poly I:C-induced hypothermia, hypoactivity and weight loss compared to WT (figure 5.4). This amelioration of sickness was associated with equivalent IL-1β and TNF-α responses but much reduced IL-6 responses in plasma, hypothalamus and hippocampus of IFNAR1$^{-/-}$ mice (figure 5.5-5.6). It has been reported in the literature that IL-6 contributes to sickness behaviour and thermoregulatory responses. Centrally injected IL-6 increased body temperature and suppressed locomotor activity and food intake in rats (Schobitz et al., 1995), although in other studies effects of centrally administered IL-6 on locomotor activity were not observed even at doses significantly higher than those producing febrile and hypothalamic pituitary adrenal axis responses (Lenczowski et al., 1999). In the same study when IL-6 was co-administered with IL-1β, IL-6 increases the severity of suppression of social interaction and immobility. Consistent with a contributory rather than direct causative role, deletion of the IL-6 gene in mice attenuates sensitivity to the depressing effects of LPS and IL-1 on social exploration and body weight (Bluthe et al., 2000b) and using soluble gp130 (sgp130) to block IL-6 trans-

signalling also reduces LPS-induced suppression of locomotor activity (Burton et al., 2013).

In the current study, IL-6 alone without poly I:C had no effect on burrowing in either strain, however IFNAR1$^{-/-}$ mice given poly I:C and IL-6 showed equivalent decreases in burrowing compared to WT mice given poly I:C, suggesting that the effects of poly I:C on burrowing are mediated by IL-6 which is consistent with reported anhedonic effects of IL-6 (figure 5.10). In addition the effects on open field rears are independent of IL-6, although IL-6 itself further decreases rearing. IDO has also been implicated in anhedonia emerging from sickness episodes. O' Connor and colleagues reports that blocking IDO activity can limit LPS-induced depressive-like behaviour (O'Connor et al., 2009) and this could have a role in the IFNAR1$^{-/-}$ and IL-6 dependent burrowing deficits seen in figure 5.10. Another possible mediator of the muted response of IFNAR1$^{-/-}$ mice to poly I:C might be COX-2, which is crucial in a number of aspects of the sickness behaviour responses including thermoregulatory responses (Saper et al., 2012). COX-2 is robustly induced by systemic poly I:C (Cunningham et al., 2007) and its mRNA induction was muted in IFNAR1$^{-/-}$ in the current study as can be seen in figure 5.9.

5.3.4 Role of endogenous IFN-I in the hippocampus and cerebellum during aging

In this chapter, an age-associated increase in IFN-I that was region-dependent and not consistent across the 2 cohorts examined was observed. Although the age-associated increase in IFN-dependent genes was somewhat uneven, it was found that the absence of IFNAR1$^{-/-}$ left the animals more vulnerable to age-associated cognitive decline. The IFN-I dependent genes from two aged cohorts examined in this thesis displayed contrasting results to each other. The first cohort showed an age-associated increase whereas the second cohort did not show this increase when quantified by RT-PCR. However, the same IFN-I dependent genes when quantified in the cerebellum of the second cohort revealed a significant increase in all of the three IFN-dependent genes. This would suggest that the age-associated IFN-I response is region-dependent. The inconsistent effects in the hippocampus may suggest that factors other than age may contribute to whether an animal displays hippocampal IFN-I responses with age.

There is much evidence to support the idea that there is significant heterogeneity in ageing even with inbred animals and co-morbid disease may be an influence. If this was the case, it would be most logical to accept the null hypothesis that the basal IFN-I response of the hippocampus does not increase with age per se, unlike other brain regions such as the cerebellum. As the hippocampus is very important in neurogenesis, learning and memory, it would be appropriate that the hippocampus would be more protected against increases in inflammation than other brain regions. Similar results have been reported in a recent microarray analysis of microglia derived from young (4-month old), adult (12-month old) and aged (22-month old) C57Bl/6 mice (Grabert et al., 2016). The study revealed that, in general, hippocampal microglia do not express the same extent of immune/inflammatory genes as microglia from other brain regions, such as the cerebellum (Grabert et al., 2016). A strong age-associated increase in inflammatory transcripts was reported in the cerebellum at age 12 months, whereas the gene expression profile of hippocampal microglia did not become significantly altered until 22 months. At this point, most of the genetic alterations were decreases in expression of genes relating to cell adhesion, migration, motility, membrane organisation and endocytosis, with only a relatively small number of inflammatory genes increasing with age, with other inflammatory genes also decreasing with age (Grabert et al., 2016). These results together with the results presented in this chapter, highlight the complexity of the murine hippocampus and would suggest that the hippocampus is more protected against age-related disturbances to inflammatory processes than other brain regions.

The inconsistent effects in the hippocampus of the two cohorts suggested that factors other than age might contribute to the presence or absence of hippocampal IFN-I responses with age. After the completion of this study, health screening showed that murine Norovirus (MNV-1) was present in the animal unit, as is now widespread in conventional and SPF units and even in commercial breeders (Ohsugi et al., 2013). Our analysis of archival plasma samples showed that the cohort that showed an elevated hippocampal IFN-I response was consistently positive for mouse norovirus, while in the young and aged groups with equivalent IFN-I responses just 2 of 6 mice tested positive. Given that this virus is relatively widespread in academic and commercial

colonies, the finding that norovirus infection was significantly associated with hippocampal IFN-I response has significant implications for studies of IFN-I expression and its effects in animal model studies.

One might predict that lack of IFNAR1$^{-/-}$, a key anti-viral gene, would increase susceptibility to this viral infection and indeed it has been shown that immune responses to murine Norovirus are STAT1-dependent (Karst et al., 2003). However, MNV-1 infection was not observed at a significantly higher rate in IFNAR1$^{-/-}$ compared to WT mice and indeed the successful mounting of an antibody response to MNV-1 is the basis of the diagnostic test and aged IFNAR1$^{-/-}$ mice mount this response successfully. In fact animals deficient in the IFN$\alpha\beta$R$^{-/-}$ have been shown not to be more susceptible to lethal MNV-1 infection and it has been demonstrated that IFNγ receptor signalling compensates for the lack of IFN$\alpha\beta$R, while IFN$\alpha\beta\gamma$R$^{-/-}$ mice were much more susceptible to infection than wild type mice (Karst et al., 2003). In the current study all mice gained weight normally during the many months of the experiment suggesting that there were not symptomatic infections (Mumphrey et al., 2007) and indeed IFNAR1$^{-/-}$ mice gained significantly more weight than WTs (Figure 5.11). Some mice lost weight after 15 months of age and some reached humane end-points but these mice were omitted from the behavioural analysis and none of those mice were used in tissue analysis. It is difficult to rule out the possibility that some aspect of MNV-1 infection could have negatively influenced the aging IFNAR1$^{-/-}$ brain but it is worth stressing that most microglial markers were suppressed in the IFNAR1$^{-/-}$ mice, an observation that neither supports hypotheses of additional neuropathological burden arising from viral infection nor provides an inflammatory explanation for why IFNAR1$^{-/-}$ mice might be worse affected by age than WT mice.

It has been found that the absence of IFNAR1 in mouse models of both AD and PD improved spatial learning and memory, reduced the expression of markers of neuroinflammation and limited disease progression (Minter et al., 2016, Qin et al., 2016, Main et al., 2016). This is most likely due reduced priming of the microglia by the surrounding neurodegeneration and by ageing itself. The data in this chapter also aimed to investigate the effects of systemic inflammatory stimuli on young WT and IFNAR1$^{-/-}$ mice and on young and aged WT mice. The absence of IFNAR1 reduced basal

levels of complement gene expression and genes relating to oxidative burst, with poly I:C treatment inducing similar increases in gene transcription in both genotypes. Poly I:C treatment in aged mice appeared to only induce exacerbated IFN-I responses, with complement gene expression only being affected by age and not treatment.

Transcriptional analysis showed interesting results, with a significant decrease in *Clec7a* expression but no significant change in *Cd68* expression. This may indicate that, at a molecular level, the microglia of the IFNAR1$^{-/-}$ mice are less primed by ageing than the WT mice. However, the mice in this experiment were not exposed to inflammatory stimuli nor did they have a neurodegenerative disease. Thus, the microglia of these mice had limited stimuli to respond to that could induce microglia reactivity. This may suggest that in the presence of neuropathology, the absence of IFNAR1 may be beneficial, thus explaining why experiments performed with IFNAR1$^{-/-}$ mouse models of AD or PD showed improved disease pathology whilst no difference is observed in the absence of pathology (Minter et al., 2016, Main et al., 2016). In fact, the present study has found that non-neurodegenerative aged IFNAR1$^{-/-}$ mice displayed impaired working memory, memory consolidation and open field activity when compared to WT. Similar results have been reported previously in the context of motor co-ordination and spatial/reference memory with significant impairments being noted from the age of 3 months in IFNβ$^{-/-}$ mice (Ejlerskov et al., 2015).

5.4. Conclusion

The current study demonstrates that basal endogenous type one interferons do not play a role in anxiogenic behaviour or in diurnal rhythm. In addition, the induction of type I interferons during peripheral innate immune activation, mimicking viral infection, has effects on CNS-mediated behavioural and metabolic changes by IL-6-dependent and independent mechanisms. Basal activity of the type I IFN receptor affects basal STAT1 levels which appear to alter basal expression of inflammatory transcripts in the CNS and facilitates the brain's inflammatory response to systemic challenge with double stranded RNA. However, the lack of IFN-I-mediated signalling during ageing has detrimental effects on hippocampal-dependent cognitive function with decreased cognitive performance observed in aged IFNAR1$^{-/-}$ mice. IFN-I

contributes to microglial activity in the hippocampus at both basal levels and during acute systemic inflammation.

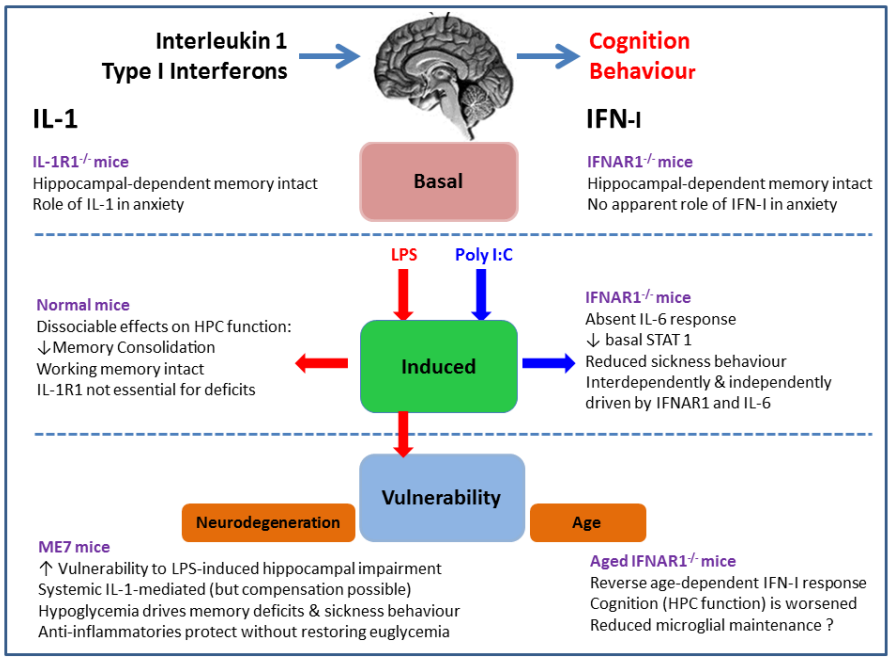
Chapter 6

Final Discussion

6.0 Discussion

This thesis set out to examine the impact of Interleukin 1 and IFN-I on the brain in cognition and behaviour. Their effects were interrogated at three discrete levels. 1) The effects of these cytokines were first examined at basal levels by using IL-1R1$^{-/-}$ mice and IFNAR1$^{-/-}$ mice to examine the behavioural and cognitive function of these knockouts. IL-1R1$^{-/-}$mice and IFNAR1$^{-/-}$ mice displayed intact hippocampal-dependent memory. The IL-1R1$^{-/-}$ mice had a reduction in anxiety as seen with increased time spent in the open arms of the EPM, while IFNAR1$^{-/-}$ mice displayed similar levels of anxiety to WT controls. 2) WT controls and knockout mice were then challenged systemically with LPS or Poly I:C to examine the effects of induction of these cytokines in cognition and behaviour. There were dissociable effects of LPS on two hippocampal-dependent memory tasks; working memory was intact while consolidation of memory was impaired in the CFC. IL-1R1 was not essential for these deficits and IL-1RA failed to protect against memory consolidation deficits. 3) The impact of these cytokines in aging and neurodegenerative disease were also examined. To examine the effect of IL-1 during neurodegeneration, WT and IL-1R1$^{-/-}$ mice were inoculated with NBH or ME7 disease. WT ME7 mice were vulnerable to the LPS-induced deficits in working memory and both dexamethasone and IL-1RA protected against the LPS-induced deficit in the T-maze, independently of CNS cytokine expression. LPS induced hypoglycaemia, and glucose administration to ME7 mice after LPS could ameliorate the LPS-induced deficits in the T-maze. When glycolysis and IL-1 synthesis were inhibited with 2-DG, this lead to decreased activity and increased sickness behaviour and glucose administration once again could ameliorate these effects, indicating that systemic inflammation deficits are partially mediated by hypoglycaemia.

IFNAR1$^{-/-}$ mice had reduced poly I:C induced sickness behaviour and absent IL-6 response in the periphery and brain and lower level of basal STAT1. The reduction in sickness behaviour responses in IFNAR1$^{-/-}$ mice were driven interdependently and independently by IFNAR1 and IL-6. Aged IFNAR1$^{-/-}$ had impaired hippocampal function and reduction in microglia which was not present in aged WT, indicating that IFN-I play a role in maintaining hippocampal function, perhaps via maintenance of optimal microglial numbers and phenotype.



Schematic diagram demonstrating how cytokines IL-1 and IFN-I impact on cognition and behaviour. The effects of these cytokines were examined at a basal level, induced level and in chronic vulnerable underlying states of neurodegenerative disease and aging. This diagram is a summary of the key findings of this thesis.

6.1 IL-1 influence on learning, memory and LTP

The question as to whether IL-1 is important in memory function is one that has been under investigation for many years. There are conflicting studies, some demonstrating that IL-1 is necessary for memory, while other studies have demonstrated that IL-1 is detrimental for memory. A number of these studies have looked at the endogenous levels of IL-1 and its role in learning and memory, while additional experiments have looked at memory and learning after induction or administration of IL-1 either injected systemically or centrally, or blocking IL-1 action with the IL-1 receptor antagonist IL-1RA. Chapter 3 and Chapter 4 demonstrated that memory is not impaired in IL-1R1$^{-/-}$ mice but IL-1 contributes to LPS induced deficits.

Studies have demonstrated that IL-1 is detrimental to learning and memory. These experiments have shown that when IL-1β was injected i.c.v or i.p, it impaired

hippocampal dependent memory in the spatial Morris water maze but not hippocampus independent non-spatial memory and IL-1β affected the retention of learning but not the acquisition of spatial memory (Gibertini, 1996, Gibertini et al., 1995, Song and Horrobin, 2004). IL-1β injected ic.v has also been shown to impair contextual (hippocampus dependent) but not auditory-cued (hippocampus-independent) fear conditioning (Pugh et al., 1999, Pugh et al., 2001, Barrientos et al., 2002, Barrientos et al., 2004, Goshen et al., 2007). Additional studies where IL-1RA has been used to block IL-1 whether induced by LPS, induced by surgery or injected has demonstrated that cognitive dysfunction in the CFC is reduced via blocking IL-1 (Terrando et al., 2010). Together all of these studies show that elevated levels of IL-1β in the hippocampus can impair cognitive performance.

Long term potentiation (LTP) is believed to be a major mechanism underlying learning and memory. IL-1 at physiological levels is believed to be necessary in the process of long term potentiation (LTP) however elevated levels of IL-1 has been shown to be detrimental to LTP. The first studies showed that IL-1β applied to rodent hippocampal slices reduced LTP in the CA1 (Bellinger et al., 1993). Further studies by Ross and colleagues showed that IL-1 can have opposing effects on LTP. In their study the authors demonstrated that mouse slices treated with IL-1β resulted in similar reduction in LTP in the CA1 area, and when a high dose of IL-1RA was administered in the absence of exogenous IL-1 this also resulted in reduction in LTP (Ross et al., 2003). This study indicated that there was a biphasic effect of IL-1 on LTP, it can facilitate LTP but elevated levels of IL-1 can be detrimental for LTP. LTP-induction protocols in the hippocampus are accompanied by a long-lasting increase in IL-1β gene expression but high levels of IL-1β can inhibit the expression of LTP in the CA3 region of the hippocampus (Katsuki et al., 1990). Similar results demonstrating that IL-1 can inhibit LTP have also been seen in the CA1 region, and in the dentate gyrus (O'Connor and Coogan, 1999, Cunningham et al., 1996). Elevated levels of IL-1β have been shown to inhibit LTP in several regions of the hippocampus in young mice (Lynch, 2010), and injection of IL-1β into the ventricles inhibited LTP in the dentate gyrus in vivo (Murray and Lynch, 1998, Kelly et al., 2003). IL-1RA has been shown to impair the maintenance of LTP demonstrating that IL-1 is necessary for LTP maintenance (Schneider et al.,

1998). The impairment of LTP is thought to be caused by IL-1β because IL-1RA can reduce the inhibitory effect of IL-1β on LTP expression (Katsuki et al., 1990, Coogan and O'Connor, 1997, Cunningham et al., 1996). There are studies that have supported the idea that IL-1 has no effect on memory. These studies have demonstrated IL-1β administered before and during training of the spatial memory task had no effect on spatial memory (Lacosta et al., 1999), while IL-1β or IL-α induced no impairment in the non-spatial version performance in adult mice (Goshen et al., 2007). Together these studies indicate that when the levels of IL-1 increase this leads to impairment of LTP however low concentrations of IL-1 may facilitate LTP.

There are also studies that have supported the idea that IL-1 is actually necessary to facilitate learning. Studies have demonstrated that IL-1RA when given immediately before or after inescapable shock i.c.v blocked the potentiation of fear conditioning and the interference with escape learning produced by inescapable shock (Maier and Watkins, 1995). Therefore, in that study IL-1RA is actually blocking memory consolidation indicating that IL-1 is important. While there are conflicting views as to whether IL-1 is necessary or detrimental to memory, one study demonstrated that low "physiological" levels of IL-1β (1ng) facilitated learning and blocking these levels with IL-1RA caused impairments and when the levels of IL-1β was increased it induced memory impairments in consolidation memory in the fear conditioning paradigm (Goshen et al., 2007), thus proposing the idea that at low levels IL-1 facilitates memory and is necessary but when levels increase it can be detrimental for memory.

However, the results in chapter 3 would indicate that IL-1 is not necessary for learning and memory. The data would support the statement that deletion of IL-1R1 does not affect hippocampal-dependent learning and/or memory in these mice but does reduce anxiety activity. IL-1R1$^{-/-}$ mice previously have been reported to be impaired in hippocampal-dependent memory Morris water maze task by displaying significantly longer latencies to reach the hidden platform and displaying impairments in contextual memory compared to controls (Avital et al., 2003). However, the results in chapter 3 of this thesis contradict these findings. The IL-1R1$^{-/-}$ mice were not impaired on the T maze or Y-maze or in the contextual fear conditioning paradigm. They

displayed reduced anxiety in the elevated plus maze and increased activity in the open field and this was true in both genders. In chapter 3 IL-1R1$^{-/-}$ mice, contrary to the studies of Avital, showed significantly shorter latency to reach the platform in the Morris water maze, even when the training was continued for a longer duration to that of Avital. They did display a very modest deficit on first choice accuracy in the spatial discrimination beacon task. While there are differences in the studies of Avital to the ones conducted in chapter 3, which include strain, time of day the experiment was conducted, rate at which control mice learnt the task, the experiments in chapter 3 used a larger battery of cognitive tasks than that of Avital or any other previous study with IL-1R1$^{-/-}$ mice and with mice of both sexes.

In addition to showing that IL-1R1 mice were impaired in memory tasks, Avital also demonstrated that IL-1RATG mice, (that had over expression of IL-1RA) or WT mice injected icv or i.p with IL-1RA had impaired performance in the MWM, CFC and passive avoidance test indicating that low levels of IL-1 were necessary to facilitate learning in the CFC, improving consolidation memory and improving memory in the passive avoidance task (Goshen et al., 2007, Yirmiya et al., 2002). Contrarily, in chapter 4, when IL-1RA alone was given to WT mice, it did not impair memory consolidation or working memory, indicating that IL-1RA is not detrimental to memory arguing against a crucial role for basal IL-1 in memory function. This finding is supported in other studies (Frank et al., 2010) where the authors showed that IL-1RA alone injected intra cisterna magna did not impair memory consolidation and IL-1RA injected subcutaneously did not impair memory consolidation in the CFC (Pugh et al., 2001) which is consistent to what was seen in Chapter 4 of this thesis. It can be seen from all of the studies mentioned that the role of IL-1 in memory is conflicting however from the data presented in Chapter 3, that included a battery of cognitive tests with a longer testing duration period and with mice of both sexes, compared to Avital, IL-1 is not important in hippocampal-dependent memory. Though Avital demonstrated with IL-1RA that memory was impaired, maybe that is not a robust effect based of the work of Frank and Pugh and the data presented in chapter 4.

6.2 IL-1: metabolic support or metabolic disruption?

Chapter 4 demonstrated that LPS can have dissociable effects on two hippocampal dependent memory tasks. WT mice injected with LPS had impaired consolidation memory but were intact in the working hippocampal dependent T-maze task. However, ME7 mice were more vulnerable to the LPS-induced deficits in the T-maze compared to normal mice. The data in chapter 4 have indicated that IL-1RA, systemically applied, protected against the LPS-induced deficit. Despite successfully blocking circulating IL-1 action with IL-1RA, robust brain activation of IL-1β CXCL1, CCL2 and other metrics of acute inflammatory stimulation still occurs. These data indicates that even though IL-1RA blocks circulating IL-1β actions, LPS still induces brain inflammatory activation including IL-1β (Skelly et al., 2018). Thus IL-1RA appears to act peripherally rather than centrally to block IL-1 action with respect to working memory in these mice. How IL-1, acting systemically, might bring about cognitive dysfunction was addressed in chapter 4 of this thesis.

Indeed both ME7 and normal mice were significantly hypoglycaemic following LPS administration and yet despite the equivalent blood glucose concentration, only the ME7 mice displayed deficits while LPS had no impact in controls. This LPS-induced deficit could be attenuated with glucose, dexamethasone and with IL-1RA. In parallel experiments, albeit with a different LPS preparation none of these anti-inflammatory drugs had any measurable effect on the hypoglycaemia in mice. When glucose was administered to these ME7 mice, it led to a protection against the LPS-induced deficit demonstrating that hypoglycaemia is a driver of this deficit. In chapter 4, WT NBH and ME7 mice when injected with insulin had decreased glucose levels, however only the ME7 insulin–treated mice displayed cognitive working memory impairment in the T-maze. Though LPS-induced hypoglycaemia was equivalent in ME7+ LPS and NBH+LPS only the former showed cognitive deficits. It is significant that when treated with insulin it was also only ME7+Insulin that showed cognitive deficits. Therefore, they are not more prone to become hypoglycaemic, they are more prone to show cognitive deficits when they become hypoglycaemic.

Hypoglycaemia has been shown to induce depressive like behaviour in mice characterised by increased immobility in the forced swim test (FST) and reduced saccharin preference (Park et al., 2012). In chapter 4, LPS induced sickness behaviour with decreases in squares crossed and rearing that was ameliorated with glucose administration. Despite this reported hypoglycaemia induced depression, and increased sickness behaviour it is remarkable that normal mice can maintain excellent function despite the levels of blood and CSF glucose falling below 50% of normal for several hours. ME7 mice are vulnerable compared to normal mice with increased synaptic loss in the hippocampus and increased microglial priming (Murray et al., 2012). It has been suggested by Del Rey and colleagues that IL-1 actually facilitates neuronal metabolism despite producing hypoglycaemia (Del Rey et al., 2016).

It is possible that, during the T-maze task and sickness behaviour responses the priorities under systemic inflammation are changed to reflect what is currently important for the organism and perhaps IL-1 can still facilitate neuronal activity in the normal mice by facilitating glucose uptake by these neurons. Indeed this explanation could be supported by studies from the literature in which IL-1 can reach the brain at the circumventricular organs that lack a patent BBB and endothelial cells all over the brain and interact with its receptor to induce NF_KB translocation (Konsman et al., 2008) and allowing cFOS to become activated by IL-1 of the neurons in the amygdala of the bed nucleus of the stria terminalis, the hypothalamus and the caudal brainstem (Nadjar et al., 2003). If IL-1 activates multiple neuronal groups to drive sickness perhaps it is prioritising activity in these key population that allow the animal to behave appropriately under inflammatory stress and the consequent hypoglycaemia. If these centres are the main drivers of sickness behaviour, it could be that IL-1 facilitates activity of those centres despite a generalised suppression of brain glucose metabolism. It is possible that in the ME7 mice where synaptic loss and a degenerating network is present that these mice have to work harder to achieve the same cognitive performance and engage compensatory networks but under 50% hypoglycaemia ME7 mice cannot do that and therefore display impairments.

Indeed a clinical study in humans, looking at Stroop performance after typhoid vaccination, demonstrated that Stroop performance was still good despite the

inflammation but a bigger network was engaged by f-MRI so perhaps a bigger network can compensate to maintain function, despite the inflammation and the hypoglycaemia, but when in neurodegeneration that ability to network to compensate is impaired then that is when you see dysfunction (Harrison et al., 2009). Recently a fluorodeoxyglucose PET scan showed that a reduction in medial temporal lobe glucose caused by an typhoid vaccination lead to reduced spatial memory performance but not medial temporal lobe independent memory in patients (Harrison et al., 2014).

In chapter 4, it was shown that LPS increases IL-1 and decreases glucose concentration, which caused a deficit in working memory. The LPS-induced deficit could be blocked with glucose and ongoing work from our laboratory has shown that IL-1RA can increase IL-1β mRNA in the brain (Skelly et al., 2018) in press. It is possible that this increase in IL-1β in light of the Del Rey study, be facilitating neuronal metabolism and IL-1RA may contribute to protection against cognitive deficits however this is highly speculative. However the glucose level in the CSF of these mice is halved by LPS and systemic glucose increases CSF glucose levels thus the most conservative account of the T-maze data in chapter 4 is that lack of available glucose impairs cognitive performance in vulnerable animals. Future experiments looking at glucose utilisation in these ME7 mice would need to be performed to determine how glucose is being metabolised in these mice. Interestingly it has been reported that in ME7 mice, GLUT 3 is significantly down-regulated (Yan et al., 2014). GLUT 3 is specifically expressed in neurons, found predominantly in axons and dendrites and also, but less prominently, in the cell body. Conversely GLUT 1 is expressed at the blood brain barrier in endothelial cells and in parenchymal glia (Vannucci et al., 1997). GLUT 3 transporter is regarded as the most abundant glucose transporter in the brain having five times higher transport capacity than GLUT1, thus glucose transport to neurons could be decreased in ME7 mice however in the same study where GLUT 3 was found to be significantly down-regulated in ME7 mice glucose levels were not examined in these ME7 mice (Yan et al., 2014). Ongoing experiments in our laboratory will be examining glucose metabolism in ME7 mice with the use of oxygen and glucose bio-sensors in the brain, to investigate glucose metabolism in real time in ME7 mice.

6.3 Reconciling IL-1-dependence and IL-1R1$^{-/-}$ - independence

In chapter 4, IL-1R1$^{-/-}$ ME7 mice when administered LPS displayed transient cognitive deficit to that seen in the WT ME7 LPS mice. This would suggest that deletion of the IL-1R1 receptor does not influence the LPS-induced deficit. However, Bluthe and colleagues have shown that there are compensatory mechanisms and cytokines such as TNF-α can replace IL-1 when IL-1 signalling is deficient. As a result of this compensatory mechanism IL-1R1 knock out mice remain fully susceptible to LPS-induced sickness behaviour (Bluthe et al., 2000a). In chapter 4 it has been shown that sickness behaviour is, at least part mediated by hypoglycaemia. Therefore an interesting experiment would be to address whether hypoglycaemia occurs despite IL-1R1 being absent. Injection of IL-1R1$^{-/-}$ and WT mice with LPS and IL-1β and assessment of sickness behaviour and blood glucose and CSF levels in these mice, would clarify whether hypoglycaemia occurs despite IL-1R deletion. This could explain why these mice show typical LPS-induced T-maze deficits. Furthermore it would clarify whether IL-1β alone induces sickness behaviour via induction of hypoglycaemia.

6.4 Delirium and glucose metabolism

Chapter 4 demonstrated that when vulnerable brains, i.e ME7 mice, are subjected to systemic inflammation (LPS) this leads to an impairment in cognitive function, thus supporting that systemic inflammation superimposed on neurodegeneration is detrimental to cognitive function. This is similar to effects seen in patients with delirium. Delirium, is characterised by inattention and profound cognitive impairment, and is an acute and transient disorder that is highly prevalent in elderly and demented patients (Burns et al., 2004). There are three forms of delirium hyperactive, hypoactive and mixed delirium. The most common form observed in the elderly population is hypoactive delirium and it is often not recognized. Patients with hypoactive delirium show signs of lethargy and low arousal and have slow responses to questions and very little spontaneous movement. The LPS-induced deficit in the ME7 mice was ameliorated with administration of glucose suggesting brain metabolism and hypoglycaemia as the driver for cognitive dysfunction in this model system. Indeed, in clinical studies, delirium has been proposed to be brought on by acute metabolic changes in the brain. In the 1950's Engel and Romano hypothesised that derangement

of cerebral metabolism lead to delirium (Engel and Romano, 1959), and they then performed several controlled experiments in humans demonstrating causative effect of hypoglycaemia (Engel and Romano, 2004). In chapter 4, the NBH LPS- treated mice despite having similar hypoglycaemia to ME7 mice did not show impairment in cognitive function so is there something about the diseased brain that handles or supplies glucose differently? Is impaired glucose metabolism the key risk factor and the acute hypoglycaemia just the last straw?

Studies have suggested hypometabolism as a vulnerability state. In a human study in patients with Alzheimer disease, with the use of FDG-PET imaging and MRI it was demonstrated that patients with subjective memory impairment (SMI) had hypometabolism and reduced glucose metabolism in the right precuneus of the parietal lobe (Scheef et al., 2012). Similarly in APP/PS1 mice, decreased blood brain-barrier-GLUT1 expression with reduced glucose uptake exacerbated cognitive deficits in these mice (Winkler et al., 2015).

A more recent study in 2017 showed that patients with delirium had cortical hypo metabolism that improved with delirium resolution. In the same study, the authors showed that glucose metabolism was higher post-delirium in the whole brain and bilateral posterior cingulate cortex (PCC) compared to during delirium (Haggstrom et al., 2017). The PCC hypo metabolism was associated with inattention during delirium. This was an important study as it demonstrated that the metabolic impairments may be a common pathway in delirium pathophysiology.

In the current data low glucose availability in ME7 mice lead to impairments in working memory and in the work above patients with delirium had reduced glucose metabolism. Perhaps targeting the decreases in metabolism may be a therapeutic target in alleviating delirium in these patients. Taken together all of these studies demonstrate that impairment of glucose metabolism in the brain may be a key driver of cognitive dysfunction in patients and in animals and these data emphasis the need to better understand the nature of brain hypometabolism and also imply possible clinical benefits of restoring normal brain energy metabolism.

6.5 LPS ≠ Infection

However, patients with infection do not typically become hypoglycaemic. It is important to point out that the bolus of LPS used in this model (100μg/kg), while low compared to many studies of sepsis leads to higher levels of endotoxin units and cytokine production compared to those seen in bacterial infection in patients with acute illness. In bacterial infection, it has been shown that the concentration of endotoxin units is far lower, then that induced by LPS in this study and as a result the level of inflammatory cytokines is less than what is produced with LPS (Barrientos et al., 2009a, Murray et al., 2012). Therefore the bolus LPS perhaps produces disruptions of metabolism greater than those seen with active infection. Therefore understanding the metabolic basis of delirium and whether metabolomics analysis may reveal key differences between ME7+LPS which show working memory deficits and NBH+LPS which do not needs to be examined.

The bolus of LPS used in the current study has higher levels of endotoxin units and cytokine production compared to those seen in bacterial infection. LPS produces hypoglycaemia which leads to cognitive dysfunction treating the hypoglycaemia with glucose protects against cognitive dysfunction, however an important study where mice were injected with *Listeria monocytogenes* infection and mice injected with LPS demonstrated that glucose administration lead to a higher mortality rate in these infected mice. Inhibiting glycolysis with 2-DG resulted in a protection against this mortality (Wang et al., 2016).

It is also important to comment that the relative sensitivity to LPS between rodents and humans is very different. Humans are more sensitive to LPS than rodents. Research by Copeland *et al* demonstrated that when healthy humans were injected intravenously with Escherichia coli type 0113 endotoxin, they exhibited a rapid physiological response (fever, tachycardia and slight hypertension) that was not exhibited in mice injected with the endotoxin (Wolff, 1973, Copeland et al., 2005)

It would be important to move towards models of real infection, closer to those experienced in the clinical field. One could models such as the *E.coli* model and cecal ligation puncture (CLP) sepsis model to understand the roles of cytokines during

behaviour and cognition in these models. Investigating blood glucose levels in these models would also be of importance.

6.6 Alternative mechanisms to explain LPS-induced deficits not explained by hypoglycaemia in ME7 mice?

Dexamethasone blocks the induction of systemic cytokines, IL-1RA blocks the systemic effect of LPS and SC-560 is a specific inhibitor of COX-1 production. Each of these anti-inflammatories have shown to be protective against the LPS-induced deficits in the T-maze (Griffin et al., 2013, Skelly et al., 2018). However, in chapter 4, these anti-inflammatories failed to inhibit the LPS-reduction in glucose levels which would suggest that it is not just glucose that is driving these LPS-induced deficits. The LPS-induced working memory deficit was blocked by dexamethasone 21 phosphate in chapter 4. Dexamethasone blocked synthesis of systemic cytokines in this study but not central cytokines. The blocking of induction systemic cytokines in our study can be also seen in another study in mice. Here the authors demonstrated that when mice were treated with the anti-inflammatory dexamethasone 21 phosphate, it prevented hypothermia in these mice after systemic inflammation with LPS i.p and blocked the induction of systemic cytokines in these mice (Teeling et al., 2010). Indeed, there is induction of TNF-α, IL-6 and iNOS in these mice and TNF-α has been shown to be sufficient when used alone to induce deficits in the ME7 mice alone (Hennessy et al., 2017, Skelly et al., 2018)and TNF-α has been shown to produce hypoglycaemia in control mice and IL-1α/β knock out mice (Oguri et al., 2002). In our laboratory it has been shown in other studies that TNF-α administration can decrease glucose levels in aged mice.

Although TNF-α has been shown to not induce hypoglycemia and to be necessary for glucose homeostasis (Satomi et al., 1985): One small clinical study showed that non-diabetic patients who received TNF-α inhibitors infliximab and adalimumab displayed low blood glucose supporting the important role of TNF-α in maintaining glucose equilibrium (Czajkowska et al., 2012). Rodent studies have shown that neutralizing peripheral TNF by etanercept (a p75 TNF receptor/Fc fusion protein) prior to the IL-1β microinjection inhibited certain IL-1β-mediated sickness behaviours, such as the depression of open-field activity and reduced glucose consumption (Jiang et al., 2008).

IL-6 has been reported to protect against LPS-induced hypoglycaemia (Fattori et al., 1994) and IL-6KO mice were protected against the LPS-induced hypoglycaemia (Tweedell et al., 2011). IL-6 has been shown to induce hypoglycaemia however to a lesser extent than IL-1 (Del Rey and Besedovsky, 1992). So clearly, there are conflicting reports on the effects of IL-6 in respect to hypoglycaemia. Studies in our laboratory have demonstrated that mice administered IL-6 display little or no sickness compared to IL-1 or TNF-α- treated mice (Skelly et al., 2013). Studies in aged mice injected with LPS and selective blocking of IL-6 with sgp130 showed amelioration against the LPS-induced sickness behaviour (Burton et al., 2013) and sgp130 can facilitate recovery from LPS-induced sickness (Burton et al., 2011). IL-6 deficient mice are also protected against disruption of working memory after LPS administration (Sparkman et al., 2006). In chapter 5 it was shown that poly I:C induced sickness behaviour could be driven by IL-6 in both dependent and independent mechanisms with burrowing being dependent on IL-6 levels, rearing activity influenced by IL-6 but weight loss not appearing to be mediated of modulated by IL-6. Future experiments therefore could look at blocking these cytokines using TNF-α inhibitors such as XPRO 1595, and an inhibitor of IL-6 production, Gabexate mesialte (Ito et al., 1996) or using anti-IL-6 receptor monoclonal antibody such as Tocilizumbab to see if blocking IL-6 could lead to a protection against hypoglycaemia and LPS-induced deficit in these mice.

Another putative mechanism previously published by our laboratory proposed that progressive microglial COX 1 expression and prostaglandins synthesis underpin the susceptibility to the LPS-induced deficits in ME7 mice (Griffin et al., 2013). In this study, the authors showed that disease induces PGE2 via COX-1. LPS impairs T-maze errors without significant further elevation of PGE2 but does increase IL-1 and when IL-1 or COX-1 was inhibited this was protective. This leads to the proposal that COX-1-induced PGE2 predisposes but deficits are not apparent until active elevation of IL-1. That idea then is that PGE2 predisposes and IL-1 is the trigger. The data in chapter 4 however do not support the idea that SC-560 might be protective against hypoglycaemia and this is consistent with the idea that PGE2 and IL-1 contribute to the deficits by distinct pathways and remains consistent with the hypothesis that PGE2 predisposes and IL-1 is the trigger.

CXCL10 and CCL3 are chemokines that are induced at brain surfaces (epithelial and endothelial) during systemic inflammation and these have been implicated in being contributors to cognitive deficits. Endothelial derived CXCL10 have been implicated as a direct contributor to acute cognitive deficits. In that study the authors demonstrated that mice, after vesicular stomatitis virus (VSV-M2) infection, had elevated levels of CXCL10 protein in the brain, spleen and serum. They showed that when recombinant CXCL10 was directly injected into the mouse brain, it elicited changes in synaptic plasticity and they suggested that CXCL10 could bind directly to CXCR3 expressed on neurons, which directly inhibits neuronal plasticity. In the same study the authors demonstrated that mice lacking CXCR3 or CXCL10 when treated with IFN-β were protected from depressive like behaviour and impairment of spatial learning and memory implicating this cytokine as being IFNAR-dependent and as the driver for these behaviours when challenged with virus or viral mimetics (Blank et al., 2016). Preliminarily data from our laboratory where IFNAR-dependence of poly I:C induced Y maze deficits were investigated have indicated that deficits post-poly i:C still occur in IFNAR1 knockout mice however they were significantly different to that in WT controls.

Finally, CCL3 has also been demonstrated to impair LTP and spatial memory. In this study, the authors showed that when CCL3 was injected i.c.v into mice, they had impaired LTP which resulted in significant impairment in spatial memory abilities in the Y-maze and long-term memory in the passive avoidance task. However, when Maraviroc a CCR5 specific antagonist, was administered, this lead to a protection against these impariments, thus suggesting that CCL3 is a hippocampal neuromodulator which is able to regulate synaptic plasticity involved in learning and memory functions (Marciniak et al., 2015). Taken together, one could suggest that LPS-induced cognitive impairments are likely being mediated partly by IL-1 and TNF-α induced hypoglycaemia, but also partly facilitated by other mechanisms, possibly inducing PGE2, CXCL10 and CCL3.

6.7 Role of IL-1 & IFN-I in anxiety, depression and the sickness behaviour response

Most of the experiments concerning IL-1 thus far have been focussed on cognitive function, but the only robust phenotype in IL-1R1$^{-/-}$ mice was decreased anxiety suggesting that IL-1 probably has a robust role in inducing anxiety. This has also been shown by other authors (Koo and Duman, 2009). Therefore both of the cytokines of focus in this thesis have demonstrable effects on emotional symptoms relevant to psychiatric conditions. In clinical terms IFN-I are better characterized as inducers of psychiatric symptoms. Indeed in the clinic, patients being treated with IFN-α for chronic hepatitis and cancer have been shown to have depressive-like behaviour, sickness behaviour and anxiety accompanied by cognitive impairment (Bonaccorso et al., 2001, Schaefer et al., 2002, Capuron and Miller, 2004, Raison et al., 2005, Zdilar et al., 2000). Interferon-α has also been implicated in regulating temperature and sleep (Krueger et al., 1988) circadian rhythm (Koyanagi and Ohdo, 2002) and feeding behaviour (Plata-Salaman, 1992).

In chapter 5, IFNAR1$^{-/-}$ mice showed no anxiety-like behaviour which was in contrast to IL-1R1$^{-/-}$ mice which showed reduced levels of anxiety compared to controls. This would suggest that IL-1 signalling contributes to anxiety but basal type-I interferons are not strongly involved in inducing or suppressing anxiety.

In chapter 5 IFNAR1$^{-/-}$ mice, when subjected to systemic inflammation with Poly I:C, displayed reduced sickness behaviour compared to WT controls. IFNAR1$^{-/-}$ mice displayed higher levels of burrowing activity compared to WT poly I:C-treated mice, and displayed higher levels of rearing activity. Poly I:C produced anhedonic-like behaviour and this was mitigated in IFNAR1$^{-/-}$ mice. It was also demonstrated that WT mice when injected directly with interferon β had reduced activity in the open field and reduction in burrowing activity compared to WT controls. IFNAR1$^{-/-}$ mice in the current study also had lower levels of indoleamine 2,3-dioxygenase (IDO) which is the enzyme that converts tryptophan to kynurenine. When this enzyme is activated in conditions of chronic inflammation, its degree of activation is correlated to the intensity of depressive symptoms observed in cancer patients chronically treated with IFN-α (Capuron et al., 2002). A study in mice also showed that the blocking of LPS-

induced IDO with 1-methyltryptophan, an IDO inhibitor, attenuated the development of depressive-like behaviour by decreasing the duration of immobility in both the forced swim test and tail suspension test (O'Connor et al., 2009). Based on the evidence of IDO being involved in the induction of depressive like behaviour, one could suggest that lower IDO levels in IFNAR1$^{-/-}$ mice in the current study, might mediate their less depressive phenotype.

Patients being treated with IFN-β therapy for multiple sclerosis display flu-like symptoms and it has been shown that these flu like symptoms can be ameliorated with low dose oral steroids (Martinez-Caceres et al., 1998). The authors in this showed a significant correlation between fever and increase in IL-6 producing cells that was not found with IFN-γ , TNF-α or IL-10. The low dose oral steroids ameliorated flu-like symptoms in these patients by decreasing the percentage of IL-6 producing cells. Indeed, in chapter 5, IFNAR1$^{-/-}$ mice had significantly decreased levels of IL-6 in the plasma, hypothalamus and hippocampus compared to WT controls and when IL-6 was co-administered with poly I:C in IFNAR1$^{-/-}$ mice this reconstituted the full sickness behaviour response seen in WT controls. Therefore from the studies mentioned here, IL-6 and IDO may mediate at least some of IFN-I effects of psychiatric symptoms.

6.8 Role of IFN-I in cognition in aging

In chapter 5 young mice lacking IFNAR1 receptor showed normal cognition in hippocampal dependent memory tasks. However IFN-I have been described to mediate age-dependent cognitive dysfunction. Baruch and colleagues demonstrated that aged mice had an elevated IFN-I response at the choroid plexus. When these aged mice were injected i.c.v with an IFNAR1-neutralising antibody, they showed improved spatial memory and learning abilities and enhanced neurogenesis compared to IgG-treated controls. This study suggested that blocking interferon action in the aged brain would lead to a protection against spatial memory in aged animals (Baruch et al., 2014).

However the results in Chapter 5 of this thesis demonstrated the opposite to that of Baruch and colleagues. Aged IFNAR1$^{-/-}$ mice in the current study had significantly impaired hippocampal function when measured on spontaneous alternation and CFC

compared to age-matched WT mice. IFNAR1$^{-/-}$ mice had decreased performance and decreased consolidation of memory with significant decrease in time spent freezing post-shock in the CFC. IFNAR1$^{-/-}$ mice also had significantly higher levels of rearing compared to WT controls at 16-17 months and progressing at 18-19 months indicating impaired habituation in a familiar open field arena as these mice aged. These data indicate that chronic deletion of the interferon pathway can be detrimental for memory in aged mice. It was confirmed that the IFNAR1$^{-/-}$ mice had significant decreases in the IFN-responsive genes, PKR, IRF7 and OAS. The microglial markers associated with neuronal maintenance were robustly suppressed in these IFNAR1$^{-/-}$ mice. Taken together these data would support a role of type one interferons in maintaining cognitive function during aging and chronic deletion of the interferon pathway can be detrimental for memory as highlighted in chapter 5.

There are procedural differences that need to be taken into account for the differences in results in the current study and that of Baruch and colleagues. In the current study mice with deletion of IFNAR1 receptor were used, whereas Baruch and colleagues used aged WT mice injected with a IFNAR1-neutralising antibody. In our IFNAR1$^{-/-}$ mice, there could be compensatory mechanisms for the lack of IFNAR1 receptor, whereas in Baruch studies it is an acute model of blocking the receptor.

The current study used the working memory spontaneous alternation task, assessed consolidation memory using the contextual fear conditioning and assessed spatial memory with the Y-maze, compared to the novel object recognition task used by Baruch and colleagues. Therefore a possible experiment to address whether interferons are important in driving cognitive function in an aging model, would be to create an inducible IFNAR receptor knockout mouse where the use of tamoxifen or doxycycline can be used to turn off the transcription of the IFNAR receptor gene during experiments and as these mice age behavioural assessments can be performed. Another approach would be to knock-down IFNAR1$^{-/-}$ for a more prolonged period using Antisense oligonucleotides and the laboratory is pursuing this.

The cognitive impairments in spatial memory observed in the aged IFNAR1$^{-/-}$ mice in the current study have been similarly observed in IFN-β knockout mice. In this study,

the authors demonstrated that from as early as 3 months of age, mice with a neuron-specific deletion of IFN-β had significant impairments in visuospatial and reference memory and learning in the Morris Water maze (Ejlerskov et al., 2015). Therefore age-dependent effects of cognitive function emerge in mice lacking neuronal expression of IFN-β. The authors also demonstrated these IFN-β knockout mice had accompanying α-synuclein-containing Lewy bodies in the brain and a reduction in dopaminergic neurons and defective dopamine signalling in the nigrostriatal region.

In contrast other studies have highlighted how deletion of IFNAR1$^{-/-}$ can lead to an improvement in memory. In one study APPSWE/PS1ΔE9 IFNAR1$^{-/-}$ mice were shown to have improved spatial and learning memory in the Morris Water maze compared to controls, which was accompanied by reduced iNOS and CD11b expression and increased Arg1 and Trem2 expression in the knockout mice (Minter et al., 2016). IFN-I has been suggested as having significant impact on age-related neuroinflammation. In an APP/PS1 murine model of AD, there was increased IFN-I expression at the choroid plexus when analysed by microarray. Indeed in patients with AD, a two fold increase in IFN-α was noted in whole brain homeogenates when they were analysed by ELISA (Mesquita et al., 2015) and in patients with AD there was a significant increase in IFN-I expression in the pre-frontal cortex compared to non-AD patients (Taylor et al., 2014, Mesquita et al., 2015). Reduction in neuro inflammation and disease progression in IFNAR1$^{-/-}$ mice has also been seen in α-synuclein toxicity and murine PD models (Qin et al., 2016, Main et al., 2016). Further studies in our laboratory have shown that in the ME7 model, IFNAR deletion helps, but in normal aging model IFNAR deletion is detrimental. Together these studies demonstrate that as you age you need some IFN-I but too much IFN- can be detrimental

6.9 Cytokines work in synergy

It is important to acknowledge that although the impacts of IL-1 and IFN-I have been studied in isolation in this thesis, during viral or bacterial infection, multiple cytokines are expressed. This has been seen in many studies where poly I:C or LPS (viral and bacterial mimetics) and with *E-Coli* and Influenza and viral models (Swiergiel et al., 1997, Majde, 2000, Bluthe et al., 2000a, Fortier et al., 2004, Cunningham et al., 2005a,

Cunningham et al., 2007, Field et al., 2010, Murray et al., 2012, Barrientos et al., 2009b). It has been demonstrated that IL-1 injected systemically can induce CNS TNF-α, while TNF-α has been shown to induce IL-1β in the brain and blood (Skelly et al., 2013).

There is evidence in the literature that type one interferon impacts on IL-1 production. IFN-α when given to healthy humans had a significant suppression of IL-1 β-induced IL-8 production 3 and 6 hours post-treatment compared to controls (Reznikov et al., 1998). Further studies showed IFN-I signalling via the transcription factor STAT1/2 repressed the activity of the NLRP1 and NLRP3 inflammasomes, thereby suppressing caspase-1-dependent IL-1β maturation (Guarda et al., 2011). The authors also showed in the same study that type I IFN induced IL-10 in a STAT1-dependent manner; autocrine IL-10 then signalled via STAT3 to reduce the abundance of pro-IL-1α and pro-IL-1β (Guarda et al., 2011). Data in chapter 5, demonstrated that poly I:C- induced increase in hippocampal IL-10 expression was attenuated in IFNAR1$^{-/-}$ mice. These studies support the data in chapter 5 demonstrating poly I:C increased IL-1 and IFNAR1$^{-/-}$ had no IL-10 and allowed further IL-1β expression all of which is broadly consistent with Guarda and colleagues.

In a previous study from our laboratory, it was demonstrated that ME7 mice injected with Poly I:C had amplified expression of both IFN-I and IL-1β (Field et al., 2010). If IFN-I tends to suppress IL-1 significantly, how might elevation of both, as observed in prior studies (Field et al., 2010) play out for cognitive function in the vulnerable brain? Further experiments looking at cognition in ME7/IFNAR1$^{-/}$ poly I:C- treated mice will be conducted in our laboratory and blocking of IFN-I and its impacts on IL-1 or blocking of IL-1 and its impacts on IFN-I will be assessed using this model.

6.10 Conclusion

The data in this thesis have shown that IL-1 seems to drive anxiety but is not important in hippocampal dependent memory in normal mice. However, during systemic inflammation in vulnerable mice with underlying neurodegenerative disease, IL-1 was shown to be detrimental to memory and this deficit was partially mediated by glucose. IL-1RA and dexamethasone was successful in blocking against the working

memory impairment. Indeed it has been seen in Alzheimer's patients that systemic inflammation can exacerbate and increase the progression of disease with increases in TNF-α in the serum of these patients being accompanied by decrease in cognitive decline over a 6 month period (Holmes et al., 2009).

Therefore one could suggest that in these vulnerable patients with cognitive dysfunction, using therapeutic agents such as IL-1RA and dexamethasone could improve the cognitive function in these vulnerable patients. In the clinic IL-1RA is used as a treatment for rheumatoid arthritis, however some of the side effects in patients talking IL-1RA (Anakinra) include infection at the injection site, pneumonia and gastroenteritis (Kullenberg et al., 2016). One study in rats demonstrated that IL-1RA was protective against brain damage by reducing blood-brain barrier breakdown in a model of sub arachnoid haemorrhage (Greenhalgh et al., 2012). From the data presented in chapter 4 perhaps IL-1RA could also be look at as a preventative treatment for patients with delirium and dementia.

It was also demonstrated in this thesis that interferons drive the poly I:C-induced sickness behaviour, by reduction of IL-6 levels. Therefore the use of interferon-α based drugs currently being used in patients with cancer, hepatitis and multiple sclerosis should be re-evaluated and potential inhibitors of IL-6 co-administered to alleviate the depressive sickness behaviour symptoms experienced by these patients.

Together, the data in this thesis indicate basal IL-1 and IFN-I in the normal brain have no influence on cognitive function but may influence anxiety. In the induced state, IL-1 certainly contributes to acute cognitive dysfunction in the vulnerable brain, and IFN-I contribute to the sickness behaviour response to acute viral mimetics. However, the absence of IFN-I as the brain ages may contribute to age-related cognitive decline. These finding have significant implication for age-related cognitive function and the understanding of the contribution of neuroinflammation to those processes. Considerable further work is required to validate these findings in clinical populations.

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