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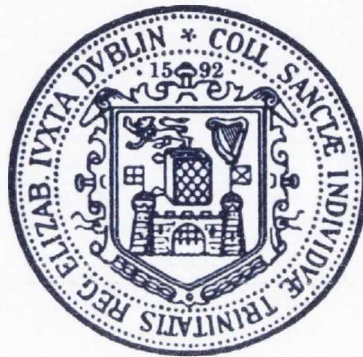
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Studies on the delirium-dementia continuum: CNS effects of systemic inflammation on hypocholinergia and chronic neurodegeneration



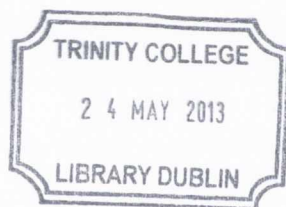
by

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Thesis submitted for the degree of Doctor of Philosophy at the
University of Dublin, Trinity College

January 2013

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Thesis 9912

I Declaration

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II Summary

Peripheral inflammation can signal to the normal, healthy CNS via multiple routes. During ageing and dementia, however, the brain is particularly susceptible to the deleterious effects of such insults. Delirium is an acute and severe disturbance in cognitive function with particular deficits in attention, memory, orientation and perception and a single episode of delirium is predictive of accelerated long-term decline. There are no licensed treatments for delirium and the pathophysiology of this distressing condition remains poorly understood. Dementia and prior cognitive impairment are the major risk factors for delirium, and systemic inflammation is a frequent trigger. The overarching aim of this thesis was to study the interaction between peripheral inflammation and neurodegeneration in order to gain a better understanding of how CNS function is affected by inflammatory insults, with particular relevance for the pathophysiology of delirium during dementia.

The cholinergic deficiency and neuroinflammatory hypotheses are two leading clinical hypotheses of delirium pathophysiology. Whether these are distinct pathways or are part of a continuum of risks and triggers remains unclear. The first study in this thesis used the murine-p75-saporin immunotoxin to produce, in C57BL/6 mice, a limited selective lesions of the basal forebrain cholinergic system in order to produce a lasting hypocholinergic state, mimicking early stages of dementia-associated cholinergic neuronal loss. Animals trained on our novel T-maze working memory paradigm were challenged systemically with bacterial LPS (100 µg/kg). Only those animal with a hypocholinergic predisposition exhibited acute and transient impairments in cognitive function, indicating that cholinergic loss confers increased susceptibility to the cognitive effects of systemic inflammation. These deficits occurred in the absence of exaggerated CNS inflammatory responses and treatment with the acetylcholinesterase inhibitor, donepezil, protected against the LPS-induced deficits, suggesting that reduction in cholinergic neuromodulation was responsible for the increased vulnerability. Nevertheless, more substantial lesions did lead to long-term priming of hippocampal microglia and it is predicted that both loss of tonic cholinergic inhibition of pro-inflammatory cytokine synthesis and loss of cholinergic neuromodulation would contribute to acute deficits in more severe hypocholinergia. These data demonstrate an important interaction between prior cholinergic pathology and subsequent systemic inflammatory insults, perhaps reconciling inflammatory and cholinergic hypotheses of delirium. Moreover, this paradigm offers a novel animal model with which to study aspects of the pathophysiology of delirium during dementia.

Previous work from our group demonstrated that microglia are primed by progressive neurodegenerative disease (ME7 prion disease) to produced exaggerated CNS inflammatory responses to systemic LPS, and that systemic LPS can accelerate disease progression. The second part of this thesis demonstrated that the degenerating brain is also primed to produce amplified responses to dsRNA. Systemic poly I:C (12 mg/kg) induces exaggerated IL-1 β and type I IFN responses only in animals with ongoing neuropathology. Furthermore, systemic poly I:C triggered acute exacerbations in neurological function along with longitudinal acceleration of disease. Removal of the type I IFN signal using IFNAR1^{-/-} mice actually increased survival in ME7 prion disease, indicating that type I IFNs are deleterious in disease per se. Investigation of the CNS inflammatory profile in IFNAR1^{-/-} ME7 animals challenged systemically with poly I:C revealed that despite the deleterious effects of type I IFN, these mediators have anti-inflammatory effects, promoting IL-10 expression and suppressing IL-1 β . Thus it seems apparent that type I IFNs are deleterious during prion disease despite anti-inflammatory effects.

The studies conducted in the current thesis have therefore begun to elucidate some of the mechanisms by which peripheral inflammation interacts with the brain during neurodegeneration, with particular relevance to delirium and progression of dementia in the geriatric population.

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V List of Abbreviations

ABC	Avidin-biotin complex
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
APP	Amyloid precursor protein
A β	Amyloid Beta
β -ME	Beta-mercaptoethanol
BBB	Blood-brain barrier
BFCN	Basal forebrain cholinergic neurons
BSA	Bovine serum albumin
CA1	Cornu ammonis 1
CD11b	Cluster of differentiation molecule 11 b
CD14	Cluster of differentiation molecule 14
CD45	Cluster of differentiation molecule 45
CD68	Cluster of differentiation molecule 68 (macrosialin)
CD206	Cluster of differentiation molecule 206 (mannose receptor)
cDNA	complementary Deoxyribonucleic acid
ChAT	Choline acetyltransferase
CNS	Central Nervous System
COX	Cyclo-oxygenase
CSF	Cerebrospinal fluid
CT	Cycle threshold
CVO	Circumventricular organ
DAB	Diaminobenzidine
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPX	Di-N-Butyle Phthalate in xylene
DSM-IV	Diagnostic and statistical manual of mental disorders -IV
dsRNA	Double-stranded RNA
E. coli	Escherichia coli
EAE	Experimental allergic encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
eIF2 α	Eukaryotic translation initiation factor- α
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	genomic Deoxyribonucleic acid
H ₂ O ₂	Hydrogen peroxide
H ₂ SO ₄	Sulphuric acid
HCl	Hydrochloric acid
HMGB1	High mobility group protein B1
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase

IBA-1	Ionized calcium binding adaptor molecule-1
ICD-10	International classification of diseases-10
ICU	Intensive care unit
IFN	Interferon
IFN- α	Interferon-alpha
IFN- β	Interferon-beta
IFN- γ	Interferon-gamma
IFNAR	Type I interferon receptor
IFNAR1	Type I interferon receptor subunit 1
IFNAR1 ^{-/-}	Type I interferon receptor subunit 1 genetic knockout
IFNAR2	Type I interferon receptor subunit 2
IL-1	Interleukin-1
IL-1RI	Interleukin-1 receptor type 1
IL-1RI ^{-/-}	Interleukin-1 receptor type 1 genetic knockout
IL-1RII	Interleukin-1 receptor type 2
IL-1 α	Interleukin-1 alpha
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-17	Interleukin-17
iNOS	inducible nitric oxide synthase
IP-10	Interferon-gamma-induced protein-10
IPS-1	Interferon-beta promoter stimulator-1
IRF	Interferon-regulatory factor
IRF3	Interferon-regulatory factor 3
IRF7	Interferon-regulatory factor 9
IRF9	Interferon-regulatory factor 9
I κ B	Inhibitor of kappa B
JAK	Janus kinase
kDa	kiloDalton
LPS	Lipopolysaccharide
LTP	Long-term potentiation
mAChR	Muscarinic acetylcholine receptor
MAP	Mitogen-activated protein
MCI	Mild cognitive impairment
MCP-1	Monocyte chemoattractant protein-1
mRNA	messenger Ribonucleic acid
MS	Multiple sclerosis
Mu-p75-sap	Murine-p75-saporin immunotoxin
MWM	Morris water maze
Mx1	Myxovirus (influenza virus) resistance protein 1
MyD88	Myeloid differentiation primary response gene (88)
nAChR	Nicotinic acetylcholine receptor
NaCl	Sodium Chloride
NBH	Normal brain homogenate
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor

NSAID	Non-steroidal anti-inflammatory drug
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PET	Positron emission tomography
PGE2	Prostaglandin E2
PKR	Double-stranded RNA-dependent protein kinase
Poly I:C	Poly inosinic:poly cytidylic acid
PRR	Pattern recognition receptor
PTX3	Pentraxin 3
PVM	Perivascular macrophage
qPCR	Quantitative-polymerase chain reaction
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor-interacting protein
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
SAA2	Serum amyloid A protein
SEM	Standard error of the mean
SOCS3	Suppressor of cytokine signalling 3
SRA2	Scavenger receptor A2
STAT	Signal transducers and activators of transcription
TACE	Tumour necrosis factor-alpha cleaving enzyme
TdT	Terminal deoxynucleotidyl transferase
TLR2	Toll-like receptor-2
TLR3	Toll-like receptor-3
TLR4	Toll-like receptor-4
TMB	Tetramethylbenzidine
TNF-R1	Tumor necrosis factor-alpha receptor 1
TNF-R2	Tumor necrosis factor-alpha receptor 2
TNF- α	Tumor necrosis factor-alpha
TNFAIP2	Tumor necrosis factor-alpha-induced protein 2
TRADD	Tumor necrosis factor-alpha-receptor-associated death domain protein
TRAF	Tumor necrosis factor-alpha receptor factor
TRIF	TIR-domain-containing adapter-inducing interferon- β
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling

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Chapter 1

Introduction

1. Introduction

1.1 Overview

Neurodegenerative disease is a deleterious state characterised by progressive neuronal death in the central nervous system (CNS). This leads to long-term progressive and irreversible neurological impairments in many facets of brain function including cognition, memory and movement, and in many cases ultimately results in death. This spectrum of diseases includes Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). Since ageing is the major risk factor for many neurodegenerative diseases, the ever-increasing elderly population will see a rise in the incidence of such disease. The global older population is predicted to double by the year 2030 (NIA, 2007) and here in Ireland, the number of people aged 80 and over (the oldest old) will increase by 250% to an estimated 460,000 between 2011 and 2041 (Barrett et al., 2011). This increase in the geriatric population will bring with it, increased incidence of neurodegenerative disease and complications that accompany them such as dementia and delirium, placing a massive economic and medical burden on society. It is clear that the problem of these currently intractable illnesses needs to be addressed from both a basic science and clinical perspective. The mechanisms that underlie neurodegenerative diseases and drive their progression remain poorly understood. Although it is now well accepted that inflammatory processes in the CNS are involved in the progression of AD and PD, how inflammation contributes to the disease process is still not clear.

There is good evidence that inflammation has a role to play in neurodegenerative disease. Numerous studies over the past 20 years have highlighted that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with significant protection against the subsequent development of both AD (in t' Veld et al., 2001) and PD (Chen et al., 2005). Further to this, there is a growing body of evidence suggesting that systemic inflammatory events can contribute to an exacerbation of the inflammatory conditions in the degenerating brain, causing acute brain dysfunction and accelerated progression of chronic neurodegenerative disease (Perry et al., 2007, Cunningham, 2012). The principal cellular mediators of the inflammatory response in the brain are microglia and much research has focussed on this population of cells since their numbers are significantly elevated in neurodegenerative disease and they have been shown to surround amyloid plaques that accumulate in the brains of AD patients (McGeer et al., 1987). Microglial

cells are part of the innate immune system, which provides non-specific responses to pathogens and does not confer long-lasting protection.

1.2 Innate inflammatory response

The ability to mount an immune response is vital to our survival. The innate immune system forms the “first line of defence” against invading pathogens and micro-organisms and confers protection in a non-specific manner and does not offer long-lasting immunity. Stimulation of the evolutionarily older innate immune system eliminates foreign entities and can direct activation of the more specialised adaptive immune response, which recognises specific antigens and offers long-lasting protection from future insults (Janeway and Medzhitov, 2002, Iwasaki and Medzhitov, 2010). That the adaptive response can also recruit and activate the same effector cells as the innate system highlights that these two systems are inextricably linked.

The cells of the innate immune system are leukocytes (white blood cells) and are of haematopoietic origin. These include NK cells, mast cells, basophils, eosinophils, along with phagocytic macrophages, dendritic cells and neutrophils. Following immune stimulation, these cells may be recruited to the site of injury or infection by resident innate immune cells. Small serum proteins known as the complement system are also part of the innate response and can activate phagocytic cells. Although the cells of the innate immune system do not recognise specific antigens, they are designed to detect and respond to conserved microbial signatures known as pathogen-associated molecular patterns (PAMPs), which include lipopolysaccharide (LPS) and double-stranded RNA. These PAMPs are recognised by specific pattern recognition receptors (PRRs) expressed on the resident innate immune cells. PRRs can be membrane-bound or cytoplasmic and include the toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). These PRRs can also recognise endogenous danger-associated molecular patterns (DAMPs) such as high-mobility group protein B1 (HMGB1), which are produced by innate immune cells in response to sterile tissue trauma or injury (Gordon, 2002). Activation of the innate response following stimulation of PRRs results in a co-ordinated set of events that leads to release of myriad inflammatory mediators (Nathan, 2002) including histamine, bradykinin, cytokines, chemokines and prostaglandins, all of which promote recruitment of neutrophils and phagocytosis which should culminate in the clearance of infection or injured tissue.

Although the dogma of an immune privileged brain persisted for many years and is still referred to, this has been superseded by the demonstration that there are in fact an elaborate system of communication between the immune system and the CNS (Steinman, 2004). Rather than lie outside the immune system, the brain is tightly controlled by its resident cells and by the blood-brain barrier (BBB) and does contain many features of the peripheral innate immune system. Much of the brain's innate response to infection or injury is directed by microglia, the resident macrophages of the brain (Kreutzberg, 1995).

1.3 Innate immune cells of the CNS – microglia

Microglia, the resident macrophages of the brain, are distributed throughout the mature CNS and account for about 15% of the total number of cells in the CNS. Comprising part of the innate immune system, microglia form the first line of defence against invading pathogenic organisms that may come in contact with the brain parenchyma (Aloisi, 2001). This provides defence in a non-specific manner and only recognises and responds to pathogens in a general manner and thus does not confer, upon the host, the long-lasting protection that the adaptive immune system provides. Unlike all other cell types in the CNS, microglia are of myeloid lineage and are members of the mononuclear phagocyte system. Their origins from haematopoietic stem cells were not always known, however, and there was much debate as to whether these cells were of neuroectodermal or myeloid origin. Conclusive evidence confirming their myeloid lineage arose from studies using PU.1-null mice. PU.1 is a transcription factor that is expressed exclusively in haematopoietic cells and studies have shown that mice with a homozygous disruption (McKercher et al., 1996) or knockout of this gene (Beers et al., 2006) exhibit a complete absence of microglia in the CNS. Further to this, a recent study by Ginhoux *et al.* has demonstrated that virtually all adult microglia arise from yolk sac macrophages formed before embryonic day 8 (Ginhoux et al., 2010), thus bringing to an end the long-running dispute about the origins of the microglial population.

The principal function of microglia in the healthy brain is to continually monitor and survey the CNS microenvironment in which they reside. In vivo two-photon imaging studies have demonstrated that these cells are highly motile and are sensitive to even the most subtle disturbances in the homeostasis of the brain parenchyma and will rapidly act to restore any detected imbalance (Nimmerjahn et al., 2005, Davalos et al., 2005). In the normal, healthy brain microglia are characterised by their highly branched, ramified

morphology and down-regulated phenotype, which distinguishes them from other macrophage populations. The historical binary classification of microglia was based largely on their morphology and the expression of cell-surface antigens (Kreutzberg, 1996, Streit et al., 1999). This classifies them into two states: “quiescent” and “activated”. The quiescent or resting state was so-called due to the low or undetectable expression of cell-surface antigens such as CD45 and MHC class I and II that are involved in macrophage function, a lack of evidence of cytokine synthesis, and also the observed ramified morphology. We now know that the term “quiescent” or “resting” is not accurate since Nimmerjahn and colleagues have reported microglia to be a highly alert and dynamic population of cells as already described (Nimmerjahn et al., 2005). Upon neurotoxic insult or disturbances in the homeostasis of the parenchyma, microglia undergo a rapid change in morphology whereby they appear much more condensed with shorter processes and take on a more amoeboid shape (Kreutzberg, 1996). These microglia are said to be “activated” although it now appears that this terminology is also much too simplistic. With this classical description of microglial activation comes an upregulation of myeloid cell markers and an increased secretory profile with the inflammatory mediator, interleukin-1 β (IL-1 β), typically released by these cells at significant levels. Cunningham *et al.* demonstrated that while microglia appear morphologically activated in neurodegenerative disease, it is only following subsequent central or systemic inflammatory stimulation with lipopolysaccharide (LPS) that they initiate production and secretion of inflammatory mediators into the brain parenchyma at significant concentrations and that this occurs without a further change in morphology (Cunningham et al., 2005b). This and other studies have contributed to the understanding that it is not possible to infer activation states from morphology alone and the binary classification of microglia has thus been re-evaluated. During neurodegenerative disease microglia appear activated by various aspects of the underlying pathology of neurodegeneration but it is now apparent that the activation profile of microglia is actually rather muted. Despite upregulation of macrophage cell-surface antigens there is very tightly regulated and, thus, limited production of inflammatory mediators.

Initially, much of what we knew about microglia in neurodegenerative disease came from neuropathological observations in post-mortem brain tissue. Increased numbers of morphologically activated microglia are apparent in the brains of AD (McGeer et al., 1987), PD (McGeer et al., 1988) and MS patients (Benveniste, 1997). More recently, animal models have provided a key platform with which to study microglia in disease. One

such model, the ME7 model of murine prion disease, has been used extensively by our laboratory to study the response of the local microglial population to progressive neurodegenerative disease. This represents a novel system with which to study neurodegenerative disease relevant to the human condition since the current animal models of AD (eg. APP/PS1 mice, Tg2576 mice) do not exhibit robust neuronal loss. These models present robust amyloidosis and modest memory impairments and even the originators of these first transgenic models view them as more representative of mild cognitive impairment (Ashe and Zahs, 2010). In terms of microglial activation, those models offer an excellent tool to study the microglial response to amyloid, but if one is interested in the microglial response to chronic neurodegeneration, robust loss of neurons across a protracted period is necessary. Microglia in the ME7 strain of prion disease, in which robust neurodegeneration occurs, appear morphologically activated but do not exhibit a “classically activated” phenotype. That is, they do not secrete the full profile of inflammatory cytokines typically associated with classical macrophage/microglia activation. Peripheral macrophages have been traditionally assigned two activation states based on the inflammatory profile of these cells. Firstly, the classical activation or M1 profile by which macrophages are activated by interferon- γ (IFN- γ) and LPS to produce IL-1 β , IL-12, tumour necrosis factor- α (TNF- α) and inducible nitric oxide synthase (iNOS) (Dalton et al., 1993). The second or alternate activation state (M2) may be split into three subcategories: M2a which responds to IL-4 and IL-13 giving an anti-inflammatory phenotype (Stein et al., 1992); M2b is driven by immune complexes and TLR and IL-1 β activation; M2c macrophages remain in a “deactivated” state and are influenced by glucocorticoids, transforming growth factor- β (TGF- β) and IL-10, and express low levels of MHC class II molecules (Gordon, 2003). Although microglia appear to exhibit aspects of these diverse phenotypes, their phenotype during chronic neurodegenerative disease remains rather different. Despite upregulation of cell surface markers and “morphological activation”, there is not detectable production of inflammatory cytokines (Walsh et al., 2001). In fact, it has been shown that these microglia are “primed” by the underlying disease pathology such that they are in a heightened state of reactivity to environmental disturbances such that subsequent inflammatory stimulation, central or systemic, will evoke a phenotypic switch to a more classical activated form, independent of a further morphological change (Cunningham et al., 2005b).

1.4 A model of progressive neurodegeneration – prion disease

Transmissible spongiform encephalopathy (TSE) or prion diseases are a group of progressive neurodegenerative diseases that are less common than the likes of AD and PD but have proved very useful in the study of neurodegenerative disease in animal models. This group of diseases were initially thought of as viral infections due the apparent replication of the infectious component. Consequently the early attempts to treat and slow the progression of disease was by stimulating production of type I interferons (Field et al., 1969), which had recently been identified as inhibitors of viral replication (Isaacs and Lindenmann, 1957), but this proved unsuccessful. In 1982, Prusiner demonstrated that prion diseases were actually caused by a conformational change in the ubiquitous cellular prion protein (PrP^C) to the aberrant “scrapie” form (PrP^{Sc}) (Prusiner, 1982). Although it is reasonably clear that neurodegenerative diseases are caused by this aberrant prion protein the mechanism of neuronal death remains poorly understood.

There are many different strains of prion disease currently used in mouse models, with some of the most common being the ME7 and RML strains that originated from scrapie in sheep in the UK. Our laboratory has used the ME7 murine strain of prion disease extensively. This, like other strains of prion disease is a terminal progressive neurodegenerative condition characterised by extracellular amyloidosis, synaptic loss, microglial activation, neuronal death and irreversible cognitive decline (Betmouni et al., 1999, Cunningham et al., 2003) and thus recapitulates *all* key aspects of progressive neurodegenerative disease. It is induced by intra-cerebral inoculation of 1 µl (bilaterally) of ME7-infected brain homogenate, with normal brain homogenate as control.

Prion diseased mice display a range of characteristic behavioural deficits. Early changes in behaviour that appear are reduced burrowing activity (Deacon et al., 2001, Guenther et al., 2001) that might reflect an affective state or a decreased motivational state of animals in the early stages of synaptic loss. Onset of hyperactivity in the open field arena typically follows the reduction in burrowing alongside progressive decline in the T-maze working memory task in which mice were trained early in disease (Felton et al., 2005). Later in prion disease, neurological functions begin to decline with loss of muscle strength and motor co-ordination along with overt ‘clinical’ signs including urinary incontinence, kyphosis and weight loss. The time at which ME7 prion-disease mice would succumb to disease is at about 25 weeks. In our laboratory we adopt a humane end point dictating that

euthanasia is performed upon 15% decrease from peak body weight in association with appearance of the 'clinical' signs described above.

Many of the current animal models of AD only replicate certain aspects of the human disease and do not exhibit progressive neuronal loss (Ashe and Zahs, 2010). Indeed although the APP/PS1 model displays amyloid plaques, there is often only mild cognitive impairment and limited microgliosis proximal to plaques. There is also limited evidence of neuronal death or neurofibrillary tangles, although new models with Tau tangles induced by the introduction of mutations in the Tau genes have been developed (Oddo et al., 2003). It is important to note that these mice rely on mutations that are not found in any AD patients. The principal strength of ME7 prion disease as a model is that it is a progressive neurodegenerative disease with widespread synaptic loss, amyloidosis and microgliosis concomitant with progressive cognitive and functional decline (Cunningham et al., 2005a). This facilitates the study of the host inflammatory response to progressive neurodegeneration in a way that is truly relevant to human disease. We do not suggest that this is an appropriate model for AD itself, but it is striking that predictions that systemic inflammation would exacerbate progression of AD patients, based on experiments in ME7 mice, have been borne out in the AD population (Cunningham et al., 2009; Holmes et al., 2009).

1.5 Systemic inflammation and the CNS: Sickness Behaviour

An organism must possess the ability to mount an adequate immune response to infection or injury so as to ensure its long-term survival. Those that lack this ability and are immune-compromised may have significantly reduced life expectancy. The first mechanism by which we deal with such infections is via our immune system's propensity to elicit an inflammatory response (Nathan, 2002). Under normal conditions, inflammation is a protective and beneficial response that initiates the body's healing process to repair damaged cells and tissues and as such, this inflammatory process must be tightly regulated and controlled. A dysfunctional or dysregulated immune system contributes to the pathophysiology of myriad diseases including rheumatoid arthritis, type I diabetes, Crohn's disease and MS. As has been mentioned, the dogma of a central nervous system that lies outside peripheral immune control has been supplanted by the demonstration that intricate mechanisms of immune modulation connect the periphery and the brain (Steinman, 2004).

Perhaps the most tangible way in which one can appreciate that systemic inflammation impacts upon the brain is through the phenomenon of sickness behaviour.

The immune response to infection by pathogenic micro-organisms or injury is initiated by activation of innate immune cells that recognise PAMPs or DAMPs via PRRs. Numerous types of PRR are known to be expressed on the immune cells of the brain. TLRs and RLRs are expressed on microglia (Town et al., 2006, Furr et al., 2008) and the mannose receptor (CD206) is expressed on perivascular macrophages (Galea et al., 2005). The TLRs form a key part of the host's innate immune response to invading pathogens and there are a number of different subtypes in this family that each respond to specific PAMPs. Each subtype of TLR recognises different ligands originating from a variety of pathogens. For example, TLR2 recognises components of gram-positive bacteria and fungi, TLR3 is responsible for detecting and responding to double-stranded RNA (dsRNA) from viruses or synthetic analogues such as poly inosinic:poly cytidilic acid (poly I:C), while TLR4 recognises LPS from the cell wall of gram-negative bacteria (Akira and Takeda, 2004). These TLRs all respond to their respective ligands and activate signalling pathways that in turn activate transcription factors, leading to translation of mRNA into proteins that form part of the inflammatory response.

This innate immune response is characterised by secretion of inflammatory mediators such as cytokines, chemokines and eicosanoids into the circulation by damaged cells, resident tissue macrophages and other local immune cells. Cytokines are small soluble secreted proteins that comprise some of the principal effector molecules of the innate inflammatory response (Feghali and Wright, 1997). Two of the best described of these mediators are IL-1 β and TNF- α and these molecules have been widely reported to be involved in the transduction of the inflammatory response from periphery to brain (Konsman et al., 2002)

IL-1 β

IL-1 β is a potent cytokine that is elevated in both septic and aseptic inflammation (Dinarello, 1996, Chen and Nuñez, 2010). Induction of the IL-1 β gene is NF κ B-dependent and IL-1 β is produced in response to local tissue damage (Ortiz et al., 2007), surgery (Haeflner-Cavaillon et al., 1989) and infection (Sansonetti et al., 2000). Upregulation of the IL-1 β gene results in transcription and translation of inactive pro-IL-1 β , which requires further processing to become the active IL-1 β protein. Following translation, immature and inactive pro-IL-1 β (31 kDa) is cleaved by a specific converting protease, caspase-1

(Thornberry et al., 1992), to active IL-1 β (17.5 kDa). In some instances of sterile inflammation this post-translational processing of IL-1 β is modulated by the NLRP3 inflammasome protein complex, which processes pro-caspase-1 into its active form that cleaves pro-IL-1 β (Rock et al., 2010). The NLRP3-caspase-1 inflammasome complex has been implicated in sterile inflammatory increases in mature IL-1 β in general (Chen and Nunez, 2010, Rock et al., 2010) and specifically in the CNS (Halle et al., 2008, Jha et al., 2010). Indeed, a recent study has demonstrated that NLRP3 is activated in Alzheimer's disease and is associated with increased neuropathology in APP/PS1 transgenic mice (Heneka et al., 2012). Two specific receptors for IL-1 have been characterised; IL-1 receptor type I (IL-1RI), and IL-1 receptor type II (IL-1RII) (Dinarello, 2009). Biological activity of IL-1 is mediated via signalling of the IL-1RI, whose expression has been demonstrated in the CNS. The IL-1RII however, does not seem to elicit any biologic effects upon binding with IL-1 and is therefore thought to be a decoy receptor. Signalling via IL-1RI regulates further gene transcription that is also largely dependent on NF κ B activation.

There is a clear body of work that demonstrates the potency of IL-1 β in neuro-immune interactions (Gosselin and Rivest, 2007, Gosselin and Rivest, 2008). In the CNS, its basal expression is low but seemingly physiologically active. IL-1 β expression is rapidly upregulated following ischemia, trauma, hypoxia and neurotoxic or inflammatory stimuli (Allan et al., 2005, Simi et al., 2007). There is also evidence for a role for IL-1 β in acute neuronal death, which is increased if IL-1 β is administered centrally or peripherally after acute brain injury (Lawrence et al., 1998, McColl et al., 2007). There have also been some reports of IL-1-positive microglia surrounding β -amyloid plaques in AD (Griffin et al., 1995). Despite some evidence of increased IL-1 β in the brains of AD patients, the inflammatory profile of degenerating brain and its microglia is actually rather muted compared to that after a direct brain insult (Cunningham et al., 2005b). Furthermore, a recent study from our laboratory has demonstrated that microglia/macrophages remain negative for IL-1 β during phagocytosis, suggesting that phagocytosis of degenerating cells does not produce a robust pro-inflammatory response (Hughes et al., 2010) and this is consistent with a well developed literature in the peripheral macrophages showing that phagocytosis of apoptotic cells is an anti-inflammatory process (Fadok et al., 1998). Introduction of exogenous systemic IL-1 β however, has recently been shown to increase central IL-1 β and proves detrimental for neurodegenerative disease (Skelly et al., in preparation).

TNF- α

TNF- α was first identified as a product of innate immune cells, which can cause lysis and necrosis in cell types, particularly tumour cells (Carswell et al., 1975). Chiefly produced by macrophages, TNF- α is a potent inflammatory mediator which can induce both IL-1 β and IL-6 (Fong et al., 1989) and has been identified as the key mediator in lethal septic shock (Tracey et al., 1986). This cytokine has also been implicated in rheumatoid arthritis and other autoimmune diseases (Feldmann and Maini, 2003). Following translation, TNF- α converting enzyme (TACE or ADAM-17) cleaves membrane-bound TNF- α to release the active inflammatory mediator (Black et al., 1997). TNF- α signalling is mediated via two receptors, TNF- receptor 1 (TNF-R1; p55) and TNF receptor 2 (TNF-R2; p75) (Tartaglia and Goeddel, 1992) which contribute to caspase-mediated apoptosis and NF κ B/MAP kinase activation respectively.

Similar to IL-1 β , TNF- α has been shown to be a mediator of neuro-immune interactions (Turrin and Rivest, 2004, Gosselin and Rivest, 2007). While TNF- α has physiological roles in the CNS, there is also evidence of dysregulation of TNF- α signalling in neurodegeneration. Increased CNS expression of TNF- α has been reported following ischemic stroke, PD, ALS and MS (McCoy and Tansey, 2008) and has also been observed surrounding amyloid plaques upon post-mortem examination of AD brains (Dickson et al., 1993). Like IL-1 β , TNF- α is suppressed in macrophages actively phagocytosing apoptotic cells (Fadok et al., 1998) but might well play a role in periphery-to-brain interactions during neurodegenerative disease.

The early local inflammatory reaction comprises a set of immune, physiological, metabolic, and behavioural processes known as the acute phase response (Moshage, 1997, Cray et al., 2009). Benjamin Hart first described the group of non-specific symptoms associated with this inflammatory response in 1988 and proposed to refer to them collectively as “sickness behaviour” (Hart, 1988). The classical symptoms of sickness include those subjective feelings that an organism experiences at times of illness and are characterised by general malaise, lethargy, hypersomnia, anhedonia, apathy, decreased social interaction and poor concentration. The body also commonly mounts a fever response in reaction to systemic inflammation and this is important for fighting invading pathogens and increased efficiency of the immune system. Sickness behaviour was historically viewed by clinicians as a group of uncomfortable but rather banal side-effects

of infection. However, it is now understood to be a highly evolutionarily conserved, co-ordinated set of adaptive behavioural changes that act to restore homeostatic imbalances in order to combat infection (Dantzer and Kelley, 2007). These changes in behaviour can be considered to be the expression of a motivational state (Aubert et al., 1997) that reshapes an organism's priorities in order to facilitate the immune system's fight against the invading pathogens and to enable its recovery from the resulting infection (Kelley et al., 2003). This co-ordinated response reduces activities that are metabolically expensive (exploration, foraging), and promotes those activities that favour heat production (shivering) and minimise heat loss (rest, piloerection). In doing this, the body creates sub-optimal conditions for microbial replication, and also reduces the organism's exposure to subsequent infections or other insults.

Much of what we know about the CNS consequences of systemic inflammation has come from the sickness behaviour field (Dantzer, 2001). It is well established that sickness behaviour is mediated by cytokines (Konsman et al., 2002) that are synthesised and secreted in the periphery but produce the range of behavioural changes by communicating to the CNS. That is, sickness behaviour is co-ordinated by the CNS, and peripheral mediators must signal to particular brain centres (such as the hypothalamus) to initiate this response. Despite the historical view that the brain was an "immune-privileged" organ, it is now clear that this privilege is not absolute and indeed it seems to refer, not to the absence of immunological components but, rather, their intricate regulation (Galea et al., 2007). Consequently, there has been much research to investigate the routes by which systemic inflammation and cytokines transduce their message to the brain.

1.6 Neuro-immune interactions – from periphery to brain

Several major communication pathways have been identified that transduce the peripheral inflammatory signal to the CNS, from fast neural to slower humoral routes, but all of which lead to synthesis of cytokines and inflammatory mediators in the brain parenchyma (Konsman et al., 2002, Dantzer et al., 2008). The routes of transduction during the adaptive sickness behavioural response are described below and in Figure 1.1.

1. Vagus

One such route of neural transmission from the periphery to the CNS is from the visceral organs via the sensory afferents fibres of the vagus nerve. Vagal nerve fibres express the

signalling IL-1 receptor type I (IL-1RI) and prostaglandin E2 receptors such that locally produced IL-1 β and PGE2 in injured or infected peripheral tissue stimulates vagal sensory activity (Ek et al., 1998). There is also evidence suggesting that LPS itself can directly activate the vagus nerve at the nodose ganglion (Hosoi et al., 2005). The role of the vagus in transduction of systemic inflammatory signals to produce aspects of the sickness behaviour response has been confirmed by studies using vagotomised animals whereby behavioural depression but not the pyrogenic effect of IL-1 β was attenuated (Luheshi et al., 2000). It also appears that efferent outflow through the vagus nerve from the CNS to the periphery can in turn control and modify systemic inflammatory events through acetylcholine secretion (Tracey, 2002). In fact, direct stimulation of the peripheral vagus nerve *in vivo* has been shown to attenuate the systemic inflammatory response to bacterial endotoxin (Borovikova et al., 2000). This has become known as the cholinergic anti-inflammatory pathway (Tracey, 2007).

2. Circumventricular organs

A slower humoral route of transmission from immune system to the brain involves pro-inflammatory cytokines and mediators entering the bloodstream at the site of infection in the periphery and circulating to the circumventricular organs (CVOs). The CVOs are specialised brain regions that lack a classical blood-brain barrier (BBB) and include the area postrema, median eminence, pineal body and choroid plexus (Dantzer et al., 2008). There is evidence that circulating cytokines such as TNF- α , IL-1 β and IL-6 and other inflammatory mediators can interact directly with the resident macrophages in the CVOs and propagate a signal into the brain parenchyma via the brain's microglia and astrocytes (Lacroix et al., 1998, Quan et al., 1998).

3. Cerebrovascular endothelium

It is also apparent that inflammatory molecules or PAMPs such as LPS or dsRNA present in the circulation can interact directly with the cerebrovascular endothelial cells of the BBB, since these cells have been shown to express TLR4 (Chakravarty and Herkenham, 2005) and TLR3 (Tissari et al., 2005). Thus these molecules can signal across the BBB via the endothelia and perivascular macrophages, leading to propagation of the initial inflammatory response into the brain. This occurs via induction of further cytokines or prostaglandins which are then free to act on regions of the brain parenchyma, leading to sickness behaviour. Indeed, our laboratory and many others have shown cerebrovascular endothelium activation after systemic inflammatory stimulation with poly I:C and LPS

(Cunningham et al., 2007, Murray et al., 2011, Zhou et al., 2009, Uchikado et al., 2004). There is also evidence that the synthetic glucocorticoid, dexamethasone, inhibits systemic but not central cytokine production (Murray et al., 2011), suggesting that LPS, even when present at relatively low doses, binds directly with the brain's endothelium to initiate the CNS inflammatory response, as is consistent with recent reports that identify the cerebrovascular endothelium as key modulators in the transduction of the inflammatory signal from the periphery to the brain (Chakravarty and Herkenham, 2005, Gosselin and Rivest, 2008).

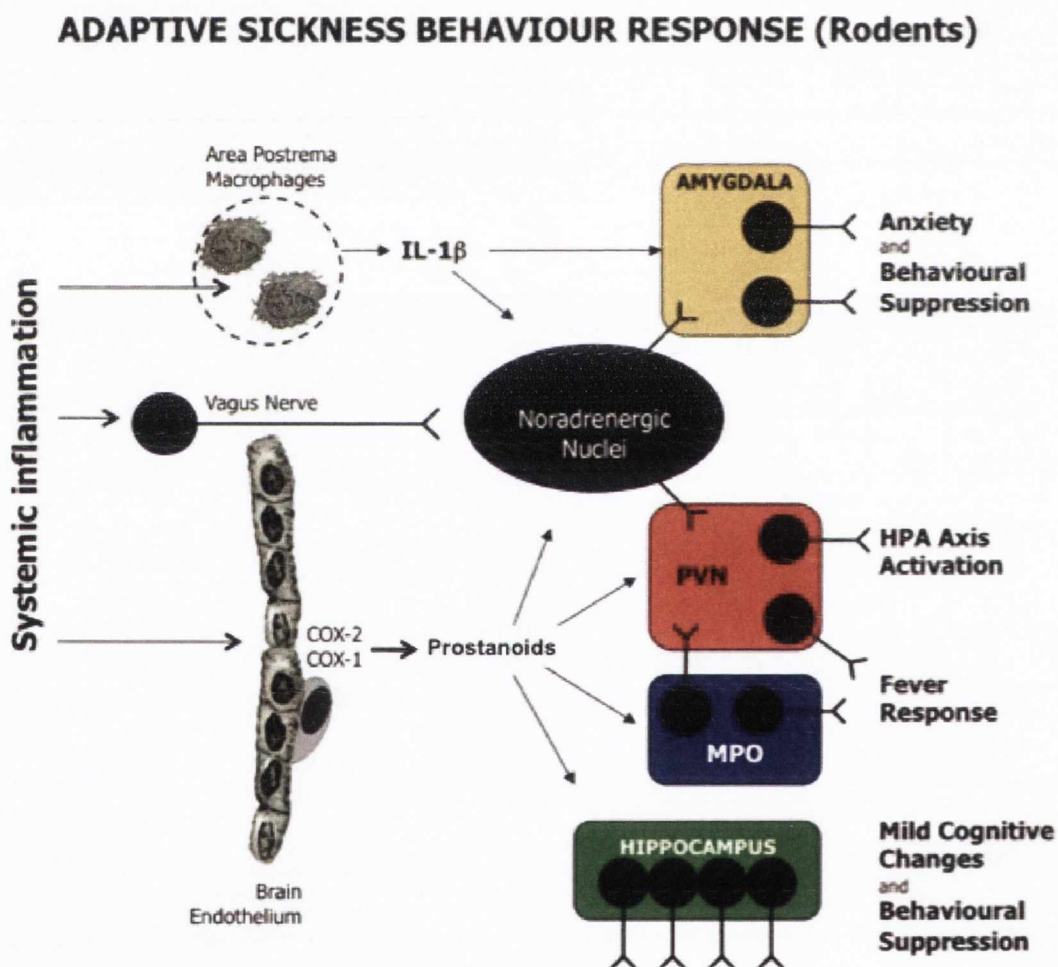


Figure 1.1 The adaptive sickness behaviour response that is present in normal healthy animals. Systemic inflammation communicates to the brain via the cerebrovascular endothelium, the vagus nerve and via the circumventricular organs such as the area postrema. This results in production of inflammatory mediators such as IL-1 β or prostaglandins in the brain and activation of various brain centres responsible for directing the sickness behaviour response. Adapted from Cunningham and Maclullich (2012).

4. Blood-brain barrier transport

There is also evidence that cytokines themselves can be directly transported across the BBB via saturable cytokine transporters (Banks, 2005, Banks and Erickson, 2010). These cytokines can then signal directly to microglia and astrocytes to induce further cytokine production or can act on specific brain centres responsible for the spectrum of sickness behavioural responses. Breakdown of the BBB is known to be a facet of normal ageing and a recent meta-analysis of BBB function showed that BBB permeability was further impaired with vascular or Alzheimer's dementia (Farrall and Wardlaw, 2009). Increased BBB permeability is likely to provide a further route of communication from the periphery to the CNS since circulating inflammatory mediators may leak into the brain parenchyma. The cells of the parenchyma may also be directly vulnerable to activation from LPS itself, or indeed circulating serum proteins such as fibrinogen and thrombin that are known to activate peripheral macrophages. There is also some evidence that LPS itself (Wispelwey et al., 1988) and poly I:C (Wang et al., 2004) can cause BBB breakdown such that the PAMPs themselves make the brain more vulnerable to other PAMPs or cytokines that are present in the blood.

1.7 Exaggerated sickness behaviour during disease

In a healthy individual, the inflammatory mediators that are expressed in the brain in response to systemic inflammation do not cause tissue damage or neurodegeneration, but act on the brain regions that are involved in fever and the co-ordination of sickness behaviour. One might predict that in the degenerating brain (AD) where there is increased permeability of the BBB and in which the tight regulation of molecular transport into the brain is diminished (Farrall and Wardlaw, 2009), and that there would be an altered central inflammatory response following systemic inflammation which could in turn effect the adaptive sickness behaviour response. Indeed in the ME7 model of murine prion disease, there is an amplified response to systemic inflammatory stimuli. Animals at the 'preclinical' stage of disease (19 weeks post-inoculation) challenged systemically with bacterial LPS (500 µg/kg) exhibit exaggerated sickness behaviour, characterised by decreased locomotor activity and a profound hypothermia by comparison to normal animals (Combrinck et al., 2002). This exaggeration of sickness behaviour, however, is not present 8 weeks into disease and only becomes apparent from 12 weeks onwards (Cunningham et al., 2009). Thus it appears that it is some facet of the progressive

underlying neuropathology that contributed the increased vulnerability to the effects of systemic inflammation.

The neurological changes that occur in the normal ageing process have also been shown to provide a background for this exaggerated sickness behavioural response to peripheral immune activation (Godbout et al., 2005), thus showing that this is not specific to the diseased or degenerating brain. The exaggerated sickness behaviour exhibited on a background of prior pathology may be viewed as a maladaptive response in the host organism, divergent from the adaptive nature of sickness behaviour in healthy individuals as described above. This maladaptive response could have deleterious consequences in individuals that are already more vulnerable due to underlying disease. Indeed, a recent patient study reported increased frequency of neuropsychiatric symptoms characteristic of sickness behaviour in AD patients that had an increased peripheral cytokine profile (Holmes et al., 2011). In elderly patients with dementia or AD, peripheral infection frequently causes episodes of delirium (Chang et al., 2008, Elie et al., 1998, George et al., 1997), which can perhaps be considered as a severe presentation of normal sickness behaviour, with negative consequences for the affected individual (Rockwood et al., 1999). Given the enormous economic and medical importance of delirium it is important to establish, in animal models, the mechanisms that underpin the exaggerated sickness response and to create models to interrogate the biological basis for delirium during aging and dementia.

1.8 Microglia in neurodegeneration – “priming”

The precise cause of the exaggerated sickness behaviour seen in neurodegenerative disease states after activation of the systemic immune response remains poorly understood. Recent work from our laboratory has aimed at investigating what role that the brain's microglia might play in the induction of the exaggerated response to peripheral inflammation in the degenerating brain. Microglial function in the healthy brain has already been discussed and we know that these cells exhibit a downregulated phenotype in this state. A key characteristic of neurodegenerative disease such as AD (McGeer et al., 1987), PD (McGeer et al., 1988) and prion disease (Miyazono et al., 1991) is the increased numbers of activated microglia present in the regions of pathology in patients with these conditions and this observation has been repeated in our ME7 model of murine prion disease (Betmouni et al., 1996, Cunningham et al., 2005a). Although these microglia appear

morphologically activated and show upregulation of macrophage antigens such as CD68, complement receptor 3 (CR3) and MHC class I and II (Ransohoff and Perry, 2009), it is important to note that morphology alone does not inform on the phenotype or function of these resident immune cells. In fact, it appears that microglia activation is quite muted during neurodegenerative disease (see figure 1.2), and that the inflammatory profile of these cells is dramatically lower than what has been shown with direct stimulation of microglia with endotoxin *in vivo* (Hughes et al., 2010). Indeed, intra-cerebral injection of LPS produces significant cytokine induction, even 24 hours after insult, and even 20-fold more microglia make manifold less IL-1 β in the prion and APP/PS1 models, highlighting this muted inflammatory profile.

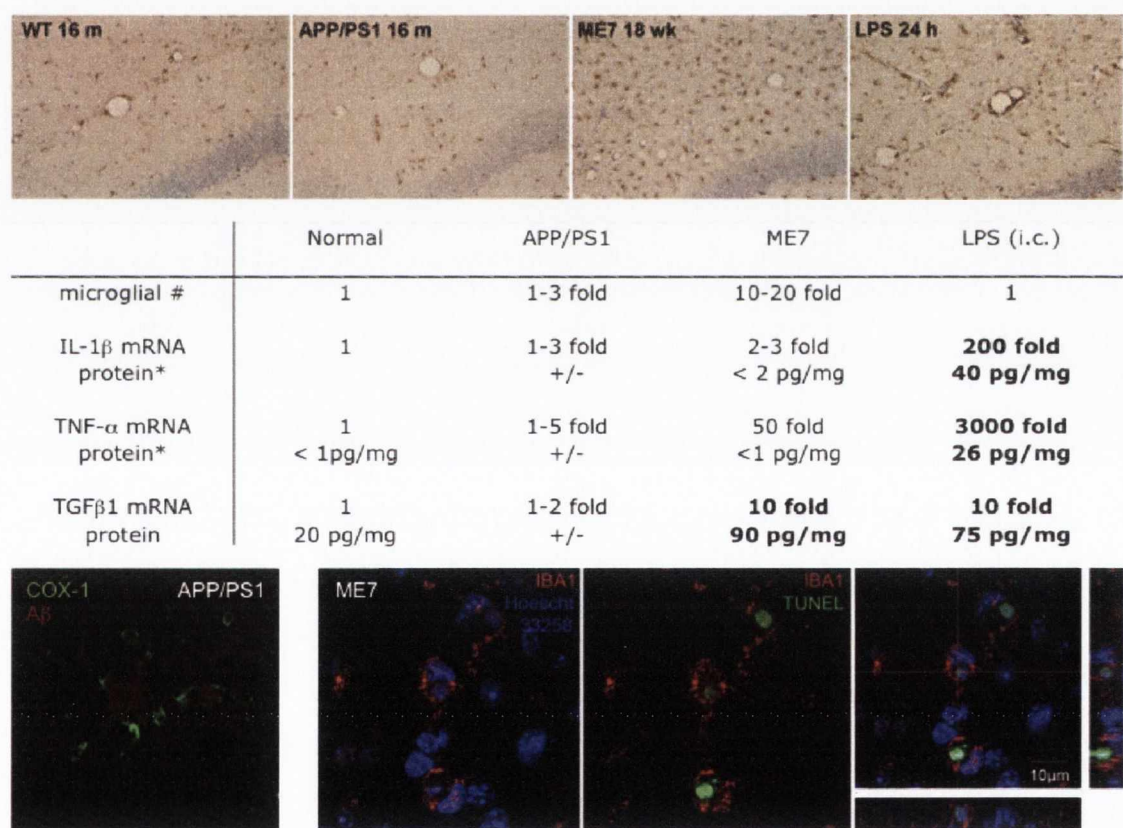


Figure 1.2 *Muted inflammatory profile and microglial activation in models of chronic neurodegenerative disease.* Comparison of microglial numbers, morphology, pro-inflammatory phenotype and phagocytic function in normal animals, 16-month old APP/PS1 double transgenic mice, ME7 prion-diseased animals at 18 weeks post-inoculation and normal animals challenged intrahippocampally with 2 μ g LPS. Top panel: only the ME7 model of prion disease displays widespread microgliosis in the region of pathology. APP/PS1 mice show small clusters of activation and LPS-injected animals show sparse microglia with activation of cerebrovascular endothelium. Table: microglial numbers and qPCR data are shown as fold change from normal. ELISA for cytokines shown in pg/mg protein are from our laboratory but where shown as +/- is from a summary of the literature. Bottom panel: COX-1-positive microglia surrounding an amyloid plaque in the APP/PS1 brain while IBA1-positive microglia in prion disease engulf TUNEL-positive apoptotic cells. Reproduced from Cunningham (2012).

It has therefore been proposed that these microglia exist in a partially activated or “primed” state which, upon subsequent insult, induce a robust inflammatory reaction. This priming phenomenon was first demonstrated in the ME7 model of murine prion disease (Cunningham et al., 2005b). In this study, the microglia are primed by some aspect of the ongoing neuropathology in the degenerating brain, such that following a subsequent central or systemic inflammatory insult, these cells respond more robustly to immune stimulation, giving rise to exaggerated expression of IL-1 β and iNOS in the brain, concomitant with neutrophil infiltration. Importantly, the peripheral inflammatory response to systemic immune challenge is equivalent in diseased and normal animals (Murray et al., 2012). These experiments highlighted the ability of microglia to undergo phenotypic switching independent of morphological change.

Classical “macrophage priming” described that prior exposure to IFN- γ resulted in more robust responses to subsequent LPS exposure, with only the combination of both resulting in the production of IL-1 β and iNOS (Johnson et al., 1983, Pace et al., 1983). Since these observations were repeated in the studies with microglia, the term “microglial priming” was adopted (Cunningham et al., 2005b). The process by which priming is initiated has been suggested to be caused by numerous diverse triggers, including increased amyloid load, neuronal loss, synaptic degeneration, dysregulation of complement system, loss of neurotransmitters such as acetylcholine and noradrenaline and many others. Although there has been some progress in research to further understand microglial priming, it remains ill-classified and the precise factors that underlie this phenomenon are still not clear. Recent evidence has demonstrated that microglial priming during neurodegenerative disease can occur as a result of uncontrolled activation of the alternative complement pathway via the C3 complement component (Ramaglia et al., 2012). Although this recently identified “priming factor” provides information on one mechanism underpinning priming, it is likely that this is not the only route to the primed state. Mechanisms of macrophage and microglial priming are shown in Figure 1.3.

A primed microglial cell, by the current definition, will produce an exaggerated CNS inflammatory response, with respect to a normal microglial cell, to a subsequent inflammatory stimulus. Further stimulation of primed microglia can be associated with exacerbation of neuropathology although exacerbation of CNS pathology can obviously occur following activation of the peripheral innate immune response independent of microglial priming. This priming phenomenon and the exacerbation of chronic CNS

pathology that follows it is not specific to prion disease, and is also not limited to TLR4-mediated, bacterial LPS-induced inflammation. Subsequent studies have demonstrated that an exaggerated CNS inflammatory response to peripheral inflammation is a relatively generic phenomenon also observed in ageing (Barrientos et al., 2006, Godbout et al., 2005) and in models of AD (Sly et al., 2001, Kyrkanides et al., 2011), PD (Pott Godoy et al., 2008), MS (Ramaglia et al., 2012) and Wallerian degeneration (Palin et al., 2008). Similarly, systemic inflammation can also impart deleterious long-term consequences and cause exacerbation of disease pathology as has been reported in models of AD (Kitazawa et al., 2005), amyotrophic lateral sclerosis (Nguyen et al., 2004) and stroke (McColl et al., 2007).

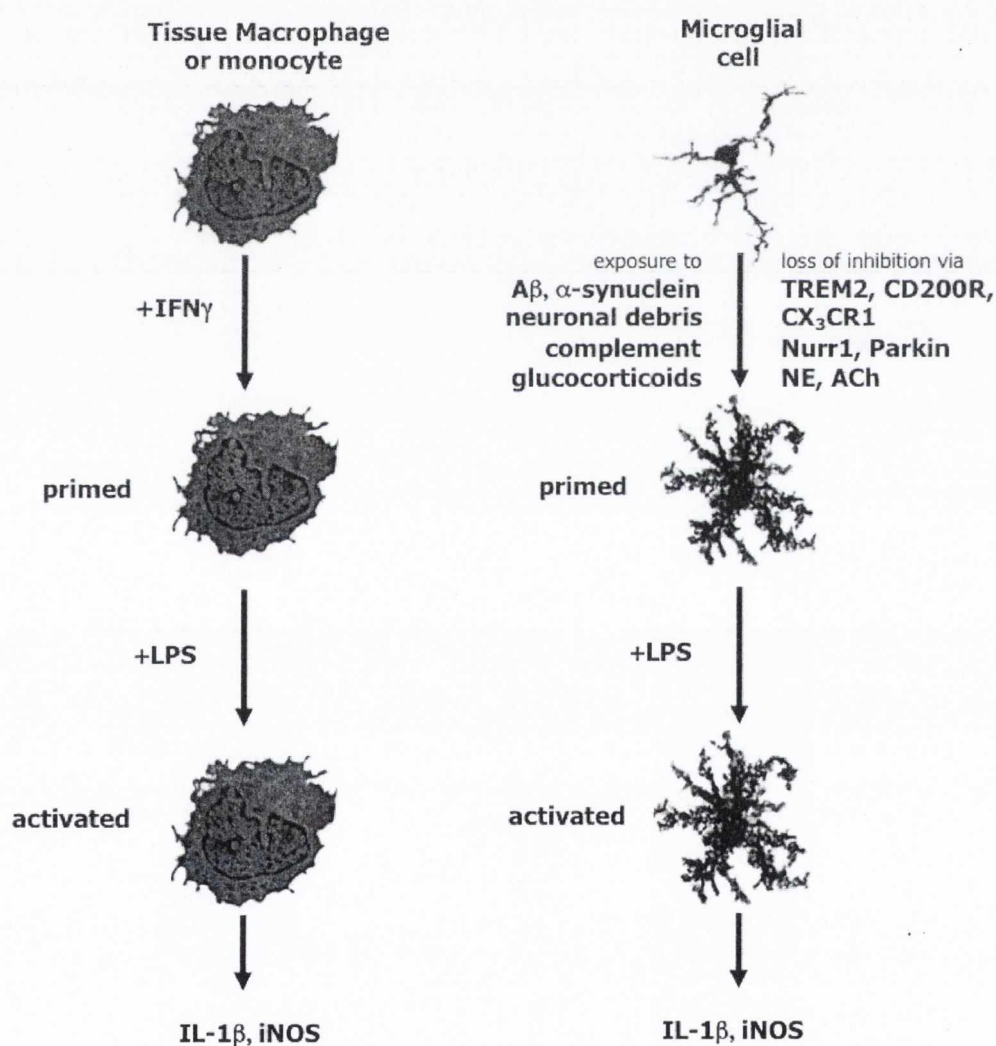


Figure 1.3 Macrophage and microglial priming. Classical macrophage priming was based on the observation that prior exposure to IFN- γ resulted in an exaggerated inflammatory response to subsequent LPS stimulation characterised by increased IL-1 β and iNOS. This priming phenomenon can also be applied to microglia since these cells are primed by some facet of neurodegenerative disease to elicit exaggerated responses to LPS. The priming factors have not yet been identified but several proposals have been made including increased amyloid burden, synaptic loss, complement activation and cholinergic depletion. Adapted from Cunningham (2012).

Importantly, the observations in different models of neurodegenerative disease that subsequent systemic inflammatory insults trigger exaggerated CNS inflammatory responses and accelerate disease progression have been “translated” to patient populations. A recent study in AD patients has reported acceleration of cognitive decline after a systemic inflammatory event with or without cognitive deficits (Holmes et al., 2009). In the same cohort, systemic inflammatory events induced exaggerated features of sickness behaviour and neuropsychological changes, consistent with the microglial priming hypothesis (Holmes et al., 2011). This further validates the use of the ME7 model of murine prion disease as a model of progressive neurodegenerative disease and dementia. Prion-diseased animals receiving a relatively mild single acute systemic challenge using bacterial LPS early in disease is sufficient to induce not only acute cognitive deficits (100 µg/kg LPS; Murray et al., 2012) but also cause an acceleration of disease progression (500 µg/kg LPS; Cunningham et al., 2009). Thus, systemic inflammation induced by LPS can exacerbate pathology in multiple chronic CNS disease models, and there is also no reason to believe that this should be an LPS-specific effect: it can be predicted that other inflammatory stimuli, for example viral infection, or the viral mimetic (poly I:C), might also elicit exaggerated CNS and sickness behaviour responses and degenerative consequences in susceptible populations. Given the high frequency of viral infections in the aged and demented population (Falsey et al., 2005, Estes et al., 2006), the effect of systemic viral infection on neurodegenerative disease has been relatively overlooked. It is important to assess whether systemic insults using a viral mimetic (poly I:C) can elicit a similar response in diseased animals. Systemic inflammation-induced acute cognitive deficits similar to those we have demonstrated have also been reported in patients with dementia or those suffering from neurodegenerative disease. There is now growing evidence that these inflammatory insults can trigger delirium and lead to exacerbation of dementia.

1.9 Viral infection and neurodegeneration

Much of the research that has sought to investigate the effect of inflammation on the brain during progressive neurodegenerative disease or in models of AD and PD has utilised the bacterial endotoxin, LPS. Though this has proved very useful and will continue to provide mechanistic information about the transduction of the peripheral immune signal into the brain, it should be noted that in geriatric populations that are most at risk of neurodegenerative disease, viral infections are much more common than bacterial

infections (Nicholson et al., 1997, Aiello et al., 2006). Thus it is somewhat surprising that the impact of viral infections during neurodegenerative disease and dementia has been relatively overlooked. There is however, some evidence that viral infections (herpes simplex virus and cytomegalovirus) are associated with more severe cognitive decline in the elderly (Strandberg et al., 2003). Given that the principal immune responders to viral infection are the pleiotropic type I interferon cytokines, it is possible that these mediators could underpin cognitive or functional impairment that manifests in the aged and demented population upon viral infection. A major goal of the current research is to examine the CNS consequences of a mimic of viral infection during progressive neurodegenerative disease (Chapter 4).

1.10 Type I Interferons

The type I IFNs were discovered by Isaacs and Lindenmann who described their ability to render cells resistant to viral infection (Isaacs and Lindenmann, 1957). These molecules were given the name “interferons” due to their ability to interfere with the process of viral replication and as such have attracted much attention since their discovery. Type I IFNs are some of the principal effector molecules in the innate immune response to viral infection and consequently form a key part of the host anti-viral defence mechanism. A further class, the type II IFNs of which there is only one member, IFN- γ , was later discovered and shares some anti-viral properties of type I IFN but is a markedly different cytokine (Wheelock, 1965). Two main subtypes of type I IFN exist, IFN- α and IFN- β (Havell et al., 1975), although several more subtypes (IFN- ω , - ϵ , - κ) have recently been discovered (Pestka et al., 2004). For the purpose of the current work, type I IFN refers to the best described subtypes, IFN- α and IFN- β . The IFN- α gene consists of 13 subfamily genes in humans and 14 in mice, whereas only a single IFN- β gene has been identified. Although initially described as anti-viral mediators, it has become clear that type I IFNs are a multifunctional pleiotropic family of cytokines, with effects ranging from T cell proliferation (Tough et al., 1996) to inhibition of tumour growth (Gresser et al., 1969), and used clinically to treat a wide range of conditions, from hepatitis, to cancer and MS. As part of the innate immune response, type I IFNs are produced in the early stages of viral infection and can be secreted by almost any cell type in the body but are typically produced by macrophages (Solodova et al., 2011) and dendritic cells (Gary-Gouy et al., 2002) following stimulation of cell surface or cytosolic PRRs.

Double-stranded RNA (dsRNA) is one of the most potent activators of the type I IFN response. Constituting the genome of one class of viruses, dsRNA is also produced as an intermediate during the viral replication process of many other viruses, particularly those with a positive sense single-stranded RNA genome, such as West Nile virus and Hepatitis C virus. The first identified PRR for dsRNA was the dsRNA-dependent protein kinase (PKR) (Clemens and Elia, 1997). Although PKR can detect dsRNA, its expression is also directly dependent on type I IFN production (Hovanessian, 1989, Meurs et al., 1990). Since the identification of PKR as a PRR for dsRNA, several more have been described and are effective stimulants of the anti-viral type I IFN response.

PRRs that are the most potent activators of type I IFNs are toll-like receptor 3 (TLR3), melanoma differentiation-associated gene 5 (MDA-5) and retinoic acid-inducible gene I (RIG-I). 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). For TLR3 to detect viral RNA, the RNA must first be taken into the endosome, a mechanism proposed to occur by endocytosis. In contrast to TLR3, MDA5 and RIG-I are present in the intracellular cytoplasmic space and are consequently much more readily exposed to viral dsRNA as it can bind them directly rather than requiring uptake into the endosome. Each of these PRRs were initially thought to be responsible for the sensing of viral or synthetic dsRNA (Yoneyama et al., 2004) but it has now become clear that *in vivo*, RIG-I is actually responsible for detecting 5'-triphosphate moieties of RNA (Hornung et al., 2006, Pichlmair et al., 2006, Myong et al., 2009) while TLR3 and MDA5 mediate the response to dsRNA (Alexopoulou et al., 2001, Kato et al., 2006).

1.10.1 Poly I:C

Polyinosinic:polycytidylic acid (poly I:C) is a synthetic dsRNA that has been widely used to mimic the acute phase of viral infection and is a potent stimulator of the IFN- α/β response (Jacobs and Langland, 1996, Matsumoto and Seya, 2008). An analogue of dsRNA, poly I:C is not composed of the traditional RNA nucleotide bases but instead consists of one strand of inosinic acid and one of cytidylic acid. Poly I:C has been shown to bind and transduce its inflammatory signal via TLR3 (Alexopoulou et al., 2001) and MDA5 (Kato et al., 2006). Since these PRRs are principally expressed in intracellular endosomes or in the cytosol, poly I:C must first be internalised into the cell. Since poly I:C is a synthetic molecule this process of internalisation cannot be facilitated by a viral particle and has been shown to be partly mediated by the LPS co-receptor CD14 (Lee et

al., 2006). It appears that extracellular poly I:C is incorporated in the endosomal compartment via clathrin-mediated endocytosis, from where it can stimulate TLR3 (Itoh et al., 2008).

Despite its extensive use in the field of immunology, the effects of poly I:C stimulation in the brain has received limited investigation, with small numbers of studies examining its effects on sleep-wake cycle patterns (Traynor et al., 2006) and immunologically-induced fatigue (Katafuchi et al., 2003). Poly I:C is being increasingly utilised to examine the effects of maternal infections on the lasting inflammatory and neuropathological profile of the brains of the offspring (Garay et al., 2012). Recent reports have shown AD-like neuropathology and cognitive decline in adult offspring following prenatal immune challenge during late gestation (Krstic et al., 2012).

Activation of TLR3 and MDA5 by viral dsRNA or poly I:C results in a signalling cascade that ultimately results in induction of type I IFN and other inflammatory mediators via activation and nuclear translocation of transcription factors interferon regulatory factor 3 (IRF3) and nuclear-factor kappa B (NF κ B) and is shown in Figure 1.4. TLR3 ligation stimulates the recruitment of the adaptor molecule TRIF (Toll/IL-1R-domain-containing adaptor protein inducing IFN- β), that functions in the MyD88-independent TLR signalling pathway (Yamamoto et al., 2003, Akira and Takeda, 2004, Kawai and Akira, 2010). TRIF can recruit TNF receptor associated factor-3 (TRAF3) and TRAF-family-member-associated NF κ B activator-binding kinase 1 (TBK1), which results in phosphorylation of IRF3, leading to its nuclear translocation and up-regulation of IFN- β gene transcription (Honda and Taniguchi, 2006). If TRIF recruits TRAF6, this leads to activation and nuclear translocation of NF κ B culminating in NF κ B-dependent gene transcription. Such NF κ B-dependent genes include the classical pro-inflammatory cytokines IL-6, TNF- α and IL-1 β . Binding of dsRNA to cytosolic MDA5 also results in activation of IRF3 and NF κ B but occurs through the use of a different adaptor molecule, IFN- β promoter stimulator-1 (IPS-1) (Kawai et al., 2005).

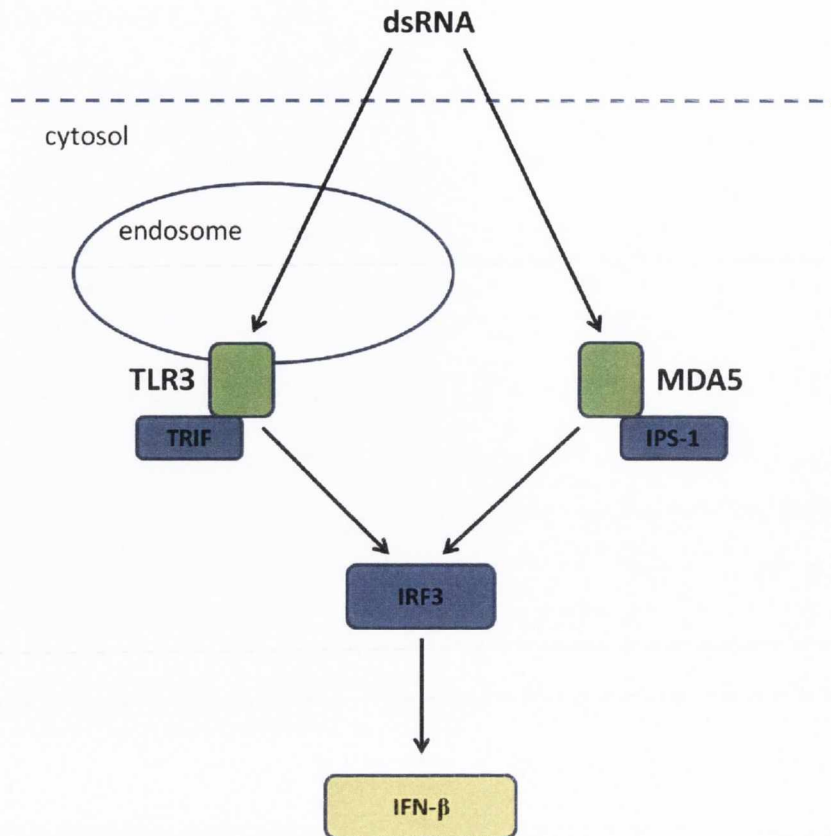


Figure 1.4 Double-stranded RNA-induced IFN- β induction. Poly I:C and viral dsRNA can activate PRRs: either endosomal TLR3 that uses TRIF as an adaptor, or cytosolic MDA-5 that uses IPS-1 as an adaptor. Stimulation of TLR3 and MDA5 results in activation and nuclear translocation of IRF3 and consequent production of IFN- β .

1.10.2 Type I IFN receptor

Stimulation of TLR3 and MDA5 by dsRNA leads to robust induction of type I IFNs which can in turn bind to their cognate receptor. The type I IFN receptor (IFNAR) binds to IFN- α/β and is a heteromeric cell surface receptor comprised of two subunits, IFNAR1 and IFNAR2. Host cells are known to upregulate expression of IFNAR proteins during viral infection (de Weerd et al., 2007), thus, allowing for a more robust type I IFN response to allow the immune system to combat infection. Genetic knockout studies have demonstrated that mice lacking a functional type I IFN receptor are more susceptible to and unable to effectively cope with viral infection (Muller et al., 1994, Hwang et al., 1995), indicating the importance of type I IFNs in these conditions.

The type I IFN receptor is constitutively associated with Janus kinase 1 (JAK1) and non-receptor tyrosine kinase 2 (TYK2) (Platanias, 2005) and binding of IFN- α/β to the receptor initiates activation of signal transducer and activator of transcription (STAT) family members. The biological response initiated by type I IFNs is dependent on which members

of the STAT family are phosphorylated and activated. Upregulation of anti-viral and IFN-stimulated genes is mediated by the formation of a heterotrimeric complex of STAT1, STAT2 and IRF9, known as IFN-stimulated gene factor 3 (ISGF3), which translocates to the nucleus to initiate mRNA transcription. This typically results in the production of IRF7 which stimulates the later phase of IFN- α/β production via a positive feedback loop mechanism, along with other IFN-stimulated genes such as PKR, 2'-5' oligoadenylate synthetase (OAS) and Mx proteins (Honda and Taniguchi, 2006). Thus type I IFNs when initially activated by viral infection or poly I:C can promote their own induction. Activation of the STAT3 homodimer following type I IFN binding to IFNAR results in upregulation of anti-inflammatory transcripts, including IL-10 (Gonzalez-Navajas et al., 2012), which might have beneficial influences by attenuation of the pro-inflammatory response during an infection or insult (Ito et al., 1999). Finally, type I IFN signalling can also promote activation of the STAT1 homodimer to induce a pro-inflammatory response that favours recruitment of immune cells to the site of inflammation. This is also the principal mechanism of type II IFN signalling by IFN- γ (Platanias, 2005). This activation of STAT1 has also been associated with upregulation of pro-apoptotic factors (Kim and Lee, 2007). Accordingly, removal of the type I IFN response via genetic knockout of either IFNAR1 or IFNAR2 subunits should elicit a dysregulation of many cellular processes and particularly interfere with those that are solely dependent on IFN- α/β .

Administration of type I IFNs has multiple effects on the human brain, including depression and in some cases delirium (Myers et al., 2002, Schaefer et al., 2002, Raison et al., 2005). Since the overarching aim of this thesis is to assess the ability of systemic inflammation to induce CNS dysfunction and pathology, the effect of poly I:C on the inflammatory profile in the prion-diseased brain will be examined in the absence of type I IFN signalling in IFNAR1^{-/-} mice (Chapter 5).

1.11 Delirium

Delirium is an acute and severe neuropsychiatric condition characterised by disturbance of thinking, memory, orientation, perception and emotion (Meagher et al., 2007, APA, 1994, WHO, 1992). There are a multitude of aetiologies that can precipitate an episode of delirium but the pathophysiology of this condition remains poorly understood (MacLullich and Hall, 2011). Although almost any individual can suffer from delirium, elderly populations are at much greater risk, probably due to multiple co-morbidities that are

associated with increasing age. Delirium in the elderly is associated with poor long-term outcomes and is predictive of functional deterioration, dementia and can shorten the time until death, even in older people without prior cognitive or functional impairment (George et al., 1997, Rockwood et al., 1999).

Delirium occurs in just 1-2% of community dwelling patients (Folstein et al., 1991, Fong et al., 2009b) but has massive prevalence in hospital populations and is one of the most common acute medical conditions. The overall prevalence of delirium in general medical inpatients is greater than 10% (Siddiqi et al., 2006). In older patients this rises to greater than 20% and in intensive care units and post-operative hip fracture patients the prevalence is greater than 50% (Holmes and House, 2000). At any one time, roughly 1 in 5 hospitalised patients are thought to be delirious though many of these cases go unreported. This appears to be due to a lack of basic knowledge of the diagnosis and management of delirium (Davis and MacLulich, 2009). As a result of the low detection rates, management of delirium remains inadequate in up to 80% of patients (Michaud et al., 2007). Even if delirium is correctly diagnosed, therapeutic options remain limited and there are currently no licensed treatments for this highly distressing syndrome (Skrobik, 2010).

There are large economic and social burdens associated with delirium. In hospitalised older patients, caring for individuals suffering from delirium accounts for 49% of all hospital days (Inouye, 2006). Delirium frequently complicates and lengthens hospital stays (Siddiqi et al., 2006) such that the cost of this syndrome is significant, with about \$6.9 billion of US hospital's annual expenditure attributable to delirium (Inouye, 2006). Furthermore, one study revealed that the direct 1-year healthcare costs of patients who have had a delirious episode is estimated to cost the US healthcare system up to \$152 billion annually (Leslie et al., 2008). Although making direct comparisons across conditions can be difficult due to differences in study methodology, the fact that diabetes mellitus and cardiovascular disease cost an estimated \$92 billion and \$258 million per year respectively highlight the prominence of delirium and the enormous economic burden that is associated this condition and its complications (Leslie and Inouye, 2011).

1.11.1 Risk factors

Although delirium pathophysiology remains poorly understood, there is good evidence of the existence of predisposing risk factors that increase the likelihood of an individual experiencing a delirious episode. The major predisposing factors in delirium pathogenesis

are ageing (> 65 years), prior cognitive impairment and dementia (Elie et al., 1998, Fick et al., 2002, Inouye, 2006). Whereas delirium is an acute and transient state of cognitive impairment, dementia is a chronic progressive and irreversible cognitive decline that usually occurs in those over the age of 65 years, with a detrimental impact on daily functioning. Although myriad CNS conditions can contribute to dementia, from traumatic brain injury to vascular dementia, the most common form of dementia is that which occurs with AD. These individuals typically present with multi-domain cognitive dysfunction and exhibit progressive memory loss (McKhann et al., 1984, Dubois et al., 2010). Diagnosis can only be definitively confirmed by post-mortem evidence of neurofibrillary tangles and extracellular amyloid deposits in excess of those found in the normal brain. Degeneration of basal forebrain cholinergic neurons is also a key characteristic of AD (Davies and Maloney, 1976, Whitehouse et al., 1982). Mild cognitive impairment (MCI) is a relatively new diagnosis that has its origins in memory clinics where patients were presenting with milder cases of cognitive loss that did not meet the criteria for diagnosis of dementia or AD (Mufson et al., 2012). MCI represents a transitional zone between normal cognitive ageing and dementia that does not compromise daily functions. It is often an amnesic disorder characterised by increased forgetfulness but non-amnesic manifestations do exist with subtle decline in functions not related to memory (Petersen, 2011). Importantly, MCI is frequently predictive of transition to dementia, with over 50% of cases progressing to dementia within 5 years (Gauthier et al., 2006). Thus, since it constitutes a risk factor for dementia, MCI also becomes a risk factor for delirium.

There is evidence to suggest that delirium when superimposed on dementia is altogether more detrimental to global brain function and is predictive of poor outcomes and indeed death (Fick et al., 2002). In healthy individuals, a severe stimulus is generally necessary to induce delirium. In the most vulnerable populations such as the aged and demented, however, it seems that much milder stimuli can trigger these episodes (Inouye and Charpentier, 1996). The concept of cognitive reserve has been proposed as an explanation for the increased susceptibility to delirium in demented patients (Fick et al., 2009). This can be defined as the manner in which the brain uses its available resources to build resilience to the cognitive effects of disease or injury. The cognitive reserve hypothesis posits that individual differences in the flexibility and adaptability of brain networks that underlie cognitive function may allow some people to cope better with brain changes than others (Steffener and Stern, 2012). Increased reserve typically describes the greater lifetime mental stimulation that individuals are exposed to, through the level of educational

attainment, occupational complexity and the mental complexity of leisure activities. Since this has been shown to be associated with reduced incidence of dementia (Valenzuela and Sachdev, 2006), interventions to enhance cognitive reserve might protect against the occurrence of delirium in vulnerable individuals.

Not only are individuals with prior cognitive impairment and dementia more susceptible to delirium, studies have demonstrated that delirium accelerates the trajectory of cognitive decline in AD (Fong et al., 2009a). When superimposed on dementia, delirium exhibits a slower rate of recovery and resolution compared to that experienced in a healthy cognitively normal individual (Boettger et al., 2011), an important point given that resolution within 2 weeks of onset is associated with better functional recovery (Kiely et al., 2006). Moreover, a recent meta-analysis has revealed that delirium in elderly patients is associated with poor outcomes including increased institutionalisation, dementia and death (Witlox et al., 2010). Even in cognitively normal patients, a single episode of delirium predicts a higher risk of dementia and cognitive decline in the years following the episode (MacLullich et al., 2009, Davis et al., 2012). Given that the global population is expected to double over the next 30 years (NIA, 2007) and that the oldest old in Ireland is predicted to increase by 250% in the same time, this increase in the elderly population will bring with it a dramatic rise in the incidence of age-related illnesses, particularly dementia. Thus this rise in individuals most susceptible to delirium will cause incidence of delirium itself to increase. As such there are both important medical and economic imperatives to gain a greater understating of the mechanisms that underpin this highly distressing syndrome.

1.11.3 Precipitating factors

There are myriad triggers that can precipitate an episode of delirium. These causes include but are not limited to peripheral and systemic infection, drug intoxication, surgery, pain, psychological stress, acute CNS trauma, metabolic disturbances such as hypoglycaemia, hypoxia, hyponatremia and acute hepatic encephalopathy. It has been suggested that the factors that trigger delirium can be conceptualised into two categories: (a) direct brain insults and (b) aberrant stress responses (MacLullich et al., 2008, Cunningham and MacLullich, 2012). Based on this classification, it may be possible to get a better understanding of how the multitude of aetiological factors can contribute to this deleterious syndrome. The category of direct brain insults is more straightforward and profoundly alters brain function through energy deprivation (hypoxia), metabolic derangement (hyponatremia), CNS trauma (stroke), primary CNS infection or drug-induced

neurotransmitter disturbances (cholinergic antagonists). The aberrant stress response is somewhat different and refers to how the brain becomes more susceptible to insults in vulnerable individuals with pre-existing brain pathology (ageing and dementia). These are typically adaptive responses in the healthy individual that can become dysregulated in vulnerable individuals. As discussed previously, the sickness behavioural response to systemic inflammation is adaptive in normal healthy individuals but becomes exaggerated and maladaptive during neurodegeneration (Combrinck et al., 2002) and dementia (Holmes et al., 2011), and indeed mild stimuli can be sufficient to trigger delirium (Inouye and Charpentier, 1996).

There have been numerous theories put forward suggesting possible pathophysiological mechanisms that underpin the events that precipitate delirium. One of the most widely propagated theories centres on the disruption of neurotransmitter systems, and suggests that acetylcholine deficiency and dopamine excess could mediate the onset of delirium (Trzepacz, 2000). The cholinergic deficiency hypothesis states that a reduction in CNS acetylcholine signalling leads to delirium (Tune and Egeli, 1999, Hsieh et al., 2008). This has its origins in the observation that anticholinergic medications can trigger delirium in surgical patients (Tune et al., 1981, Golinger et al., 1987, Han et al., 2001). Early animal models of delirium used the muscarinic cholinergic antagonist atropine to induce cognitive deficits and EEG slowing consistent with those observed in delirious patients (Trzepacz et al., 1992, Leavitt et al., 1994, Jacobson and Jerrier, 2000). Thus there has been an interest in modulation of cholinergic neurotransmission as a mechanism of resolving or preventing delirium, with one study suggesting that the cholinesterase inhibitor rivastigmine might be effective in treating delirium (Overshott et al., 2010). However, a recent clinical trial of rivastigmine in the treatment of delirium in intensive care patients was unsuccessful (van Eijk et al., 2010) and the trial was stopped due to increased mortality in the treatment group. It is one hypothesis of the current thesis that cholinergic disruption when combined with systemic inflammation may be sufficient to cause delirium, suggesting that cholinesterase treatment may be more suited to those individuals that display a pre-existing cholinergic deficit, as is apparent in dementia and AD (Davies and Maloney, 1976, Whitehouse et al., 1982). The interaction of cholinergic depletion and systemic inflammation will be studied in chapter 3.

Another avenue that has gained much attention in the drive to understand delirium pathophysiology is the neuroinflammatory hypothesis (Cerejeira et al., 2010, Cunningham,

2011). There is a growing body of evidence indicating that inflammatory processes are capable of eliciting delirium. Numerous clinical studies have demonstrated that delirium can be induced by sterile inflammation resulting from surgery, in particular hip replacement and cardiac procedures (Kat et al., 2008, Koster et al., 2008), burns (Perry and Blank, 1984) and hip fracture (van Munster et al., 2008), with further studies showing induction of delirious episodes by peripheral infections (George et al., 1997, Elie et al., 1998, Chang et al., 2008). All of these inflammatory events are mediated by PAMPs, DAMPs and circulating cytokines and prostaglandins all of which communicate to the brain via the various routes discussed earlier. Indeed pro-inflammatory cytokines have been associated with delirium (van Munster et al., 2010, MacLulich et al., 2011). It is clear that severe sepsis can induce delirium in almost any individual (Ebersoldt et al., 2007). In vulnerable individuals such as the elderly and demented, however, much milder inflammation is capable of inducing a delirious episode (Inouye and Charpentier, 1996) and this is associated with acceleration of dementia and death (Rockwood et al., 1999, Fong et al., 2009a). It is possible that the increased susceptibility that comes with advancing age and dementia is reflective of reduced brain reserve, as discussed above, thus minimizing the individual's ability to cope with significant disruption of homeostasis as would occur during infection, metabolic derangement or changes in medication. Decreased neuronal connectivity and synaptic density during normal ageing or neurodegenerative disease could thus decrease the threshold required for a mild stimulus to produce profound brain dysfunction. Similarly, increasing susceptibility may arise due to the priming of the microglial population, such that the brain now produces exaggerated inflammatory responses to equivalent systemic inflammation.

Despite the enormous medical and economic imperatives to gain a better understanding of delirium, the pathophysiology of this distressing condition remains poorly understood. This is certainly partly due to the dearth of biologically relevant animal models in which to interrogate the biological mechanisms that underpin this serious condition. Our laboratory has recently described the first animal model for inflammation-induced delirium during dementia (Murray et al., 2012). Using the ME7 model of murine prion disease as a model of dementia/neurodegeneration and superimposing systemic inflammation induced by LPS, this study demonstrated that LPS (100 µg/kg i.p.) elicits acute and transient impairment of working memory only in animals with prior pathology. The studies relied on a novel T-maze working memory task that allows testing of working memory even in animals experiencing acute sickness behaviour. It is clear that sickness behaviour, as described in

section 1.5 induces a number of changes in behaviour that include profoundly suppressed locomotor activity and appetite and this significantly decreases their motivation to engage with cognitive tasks that are based on exploratory or appetitive drives. These aspects of sickness have significantly confounded many prior studies of altered cognition in animals experiencing acute sickness behaviour (Cunningham and Sanderson, 2008) . Likewise swim water mazes such as the Morris Water Maze do not suffer these confounds but do induce high stress states and can also be confounded by decreased locomotor speed in sick animals. The novel T-maze task avoids all of these problems by training mice to escape from shallow water thus ensuring that motivation, under non-stressed conditions, is maintained during sickness. Since the alternation is a simple left or right turn choice, animals are scored simply as correct or incorrect, thus simplifying analysis.

The sudden onset and transient nature of the working memory deficits reported by Murray et al. (2012) recapitulate key features of clinical delirium as defined by the ICD-10 and DSM-IV (WHO, 1992, APA, 1994). The presence of robust synaptic loss and microglial priming in this model along with exaggerated production of CNS cytokines, suggests two possible mechanisms for the increased vulnerability to the cognitive effects of systemic inflammation during neurodegeneration and dementia. Recent observations in our laboratory using this model have shown that the observed deficits are mediated by prostaglandins, since COX-1 inhibitors offer protection against the deficits. Furthermore, systemic challenge with the pro-inflammatory cytokine IL-1 β has also been shown to be capable of inducing cognitive deficits in animals with prior pathology, with evidence of increased neuronal susceptibility to IL-1 β in hippocampal slices (Skelly et al., in preparation).

This first animal model of delirium during dementia has provided, and will continue to provide, mechanistic information about this heterogeneous condition. Nonetheless, there is a need to develop animal models away from the prion setting since, despite its predictive value for AD patients (Holmes et al., 2009), these diseases are less common in the human demented population and it may be perceived as somewhat ‘atypical’. There is a strong rationale to create a neuropathology that recapitulates aspects of the common neurodegenerative diseases such as AD and that is manifestly relevant to delirium as observed in the demented geriatric population.

1.12 A new animal model of delirium?

It is known that there are multiple disease processes occurring during dementia/neurodegeneration, and since prior cognitive impairment and established dementia are key susceptibility factors in delirium, it is necessary to assess the contribution of different facets of disease to the observed susceptibility to delirium. Prion disease, while a useful model of progressive neurodegenerative disease, is not capable of addressing all of these facets. Thus there is a need to explore other pathologies in order to create and model different aspects of the currently hypothesised pathophysiology of delirium. The two leading hypotheses of delirium pathophysiology are the cholinergic and neuroinflammatory hypotheses as described above, but whether these act in parallel or interact with one another remains unknown. A major goal of the current research is to attempt to reconcile these two lines of thinking in an animal model that is relevant to clinical delirium.

The cholinergic hypothesis of delirium is based on a deficiency in CNS cholinergic function (Hsieh et al., 2008). This theory gained attention from the observation that anticholinergic medications act as a trigger for delirious episodes (Tune et al., 1981, Golinger et al., 1987). Furthermore, there is significant and substantial degeneration of the basal forebrain cholinergic neurons in AD and dementia (Davies and Maloney, 1976, Whitehouse et al., 1981) and since AD/demented patients are more susceptible to delirium, the hypocholinergic state becomes an obvious point of focus. Also, perturbations in cholinergic function are consistent with some of the core features of delirium, such as inattention which has been thought to be caused by diminished acetylcholine reserves, as evidenced by increased cortical acetylcholine during a sustained attention task (Himmelheber et al., 2000). Thus, we hypothesised that cholinergic hypofunction would increase the susceptibility of mice to acute cognitive deficits induced by systemic inflammation.

1.12.1 Acetylcholine in the CNS

Acetylcholine is one of the principal neurotransmitters in both the peripheral and central nervous systems and can act as both an inhibitory and an excitatory neuromodulator. Acetylcholine is synthesised from its precursors, acetyl coenzyme A (a by-product of the citric acid cycle) and choline (from the diet), in the presynaptic terminal by the enzyme choline acetyltransferase (ChAT) (Oda, 1999). The newly formed neurotransmitter is then sequestered into vesicles via the vesicular acetylcholine transporter. Upon neuronal

stimulation and synaptic depolarisation, these vesicles fuse with the neuronal plasma membrane at the synaptic cleft and undergo exocytosis, releasing acetylcholine, which can interact with post-synaptic receptors to transmit its signal. Following cholinergic neurotransmission, acetylcholine is rapidly hydrolysed by the enzyme acetylcholinesterase (AChE) at the nerve terminal and the cholinergic signal is terminated.

The synaptic receptors for acetylcholine can be classified into two broad categories; the nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors. The nAChRs are ligand-gated ion channels and the principal CNS subtypes are the heteromeric $\alpha 4\beta 2$ and the homomeric $\alpha 7$ receptor. The $\alpha 7$ nAChR is also present in the periphery and is known to play a role in the regulation of peripheral inflammation via the vagus nerve (Wang et al., 2003). The mAChRs are metabotropic receptors and the main subclasses are M1 and M2. The M2 receptors are involved in maintenance of the normal sinus rhythm of the heart while the M1 receptor is thought to play a role in memory, since the M1 antagonist scopolamine has been shown to impair memory consolidation and acquisition (Klinkenberg and Blokland, 2010).

1.12.2 The basal forebrain cholinergic system

Cholinergic signalling pathways are widely distributed in the brain (Benarroch, 2010) (see figure 1.5) and are involved in a multitude of brain functions including learning and memory (Hasselmo, 2006), attention (Himmelheber et al., 2000, Klinkenberg et al., 2011) and the sleep-wake cycle (Jones, 2005). The entire cerebral cortex and hippocampus are innervated by cholinergic projections that originate from several cholinergic nuclei in the basal forebrain (Woolf, 1991). These discrete brain regions are known to play a role in cognitive function, particularly attentional processing and memory function (Baxter and Chiba, 1999). Thus given that these are two of the cognitive domains that are dysfunctional in delirium, it is, perhaps, not surprising that anticholinergic medications might contribute to this syndrome. However, it has not been clear whether hypocholinergia creates a susceptible state to some subsequent insults or whether hypocholinergia per se is the trigger for delirium. The basal forebrain shows significant degeneration in AD (Davies and Maloney, 1976, Whitehouse et al., 1981) and it has been suggested that this is the source of mnemonic impairment in the early stages of AD. Indeed AChE inhibitors such as donepezil and rivastigmine still remain the first line treatment for AD patients and do offer some stabilization of memory function in the mild to moderate phases of disease (Birks, 2006). Lesion studies in rodents have also demonstrated that the basal forebrain

cholinergic neurons are necessary for hippocampal-dependent working memory tasks (Berger-Sweeney et al., 2001). Given that AD patients are some of the most susceptible to delirium it seems plausible that a hypocholinergic predisposition in this patient group could underpin this vulnerability.

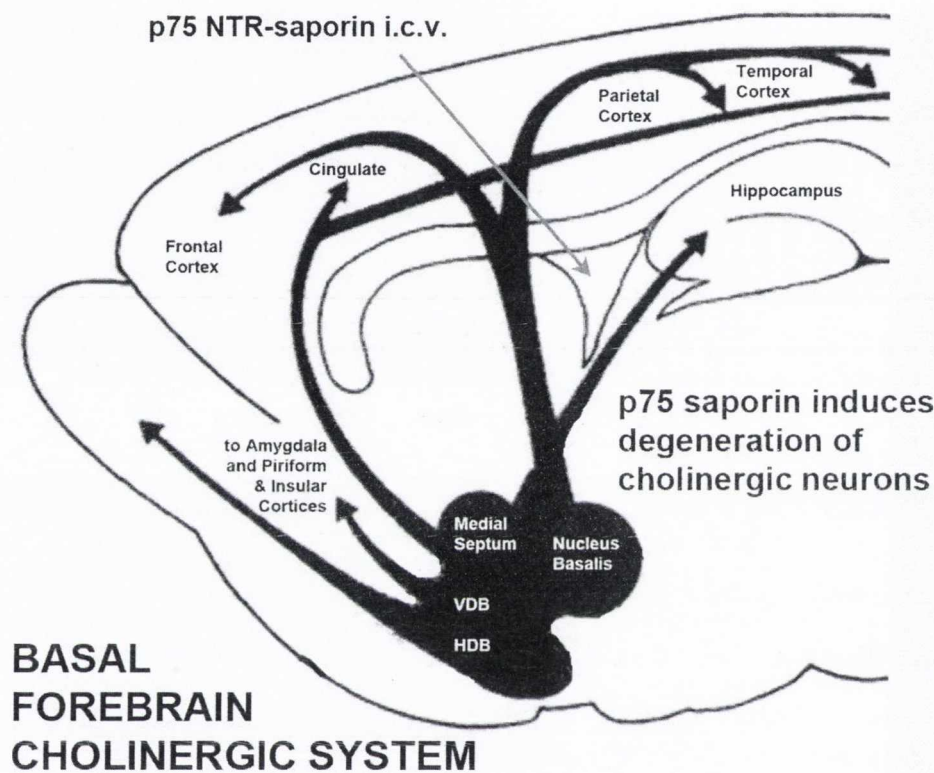


Figure 1.5 The basal forebrain cholinergic system and its projections in the mouse brain. The population of cholinergic neurons in the basal forebrain innervate myriad brain regions including the frontal cortex, amygdala and hippocampus (Woolf, 1991). The cholinergic neurotoxin mu-p75-saporin is injected into the lateral ventricle and diffuses to the medial septum/diagonal bands to selectively target cholinergic cells with the aim of creating a limited lesion analogous to dementia-associated cholinergic loss. Adapted from Cunningham and MacLullich (2012)

1.12.3 Towards a new model – cholinergic hypofunction

It was one key aim of this thesis project to create a novel animal model of delirium that can interrogate the relationship between cholinergic and neuroinflammatory hypotheses. Such a model would require novel combination of pathological ‘predisposition’ and typical systemic inflammatory ‘trigger.’ It was first necessary to create hypocholinergic pathology: We hypothesized that by placing pathology in the basal forebrain cholinergic system, it would be possible to create experimental conditions similar to the cholinergic state present in the early stages of dementia and AD. Thus a specific lesion of this population of neurons would allow for this investigation. The ribosome-inactivating toxin

saporin (192-IgG-saporin) has been used in several rodent studies to lesion the basal forebrain cholinergic system in order to assess the role of these neuronal populations in normal brain function (Baxter et al., 1995, Berger-Sweeney et al., 1994). A new form of this toxin, murine p75-saporin immunotoxin (mu-p75-sap), has been recently developed and consists of the saporin toxin conjugated to an antibody directed against the p75 neurotrophin receptor, which is enriched on cholinergic neurons of the basal forebrain (Berger-Sweeney et al., 2001, Moreau et al., 2008). This has made it possible to specifically lesion basal forebrain cholinergic neurons in the mouse, without damage to other populations of neurons (Hunter et al., 2004, Moreau et al., 2008). Such a lesion model would allow us to ask multiple questions about the interaction of systemic inflammation with prior CNS pathology, now with a specific focus on the loss of cholinergic function in the diseased brain. This model system facilitates the first fundamental scientific attempts to reconcile cholinergic and inflammatory hypotheses of delirium and is the most disease-relevant approach so far to assessing whether loss of cholinergic input affects microglial activation status.

1.13 Summary

The aged and demented population, as a cohort, are some of the most susceptible individuals to systemic inflammatory events such as infections and tissue trauma from falls or surgery. Increased CNS pathology is associated with progressive dementia and neurodegenerative disease that is characterised to varying degrees by cognitive and functional decline, neuronal loss, synaptic loss, amyloidosis and microglial activation. There is an emerging literature which has demonstrated that the degenerating brain is increasingly vulnerable to the effects of systemic inflammation and animal models from our laboratory have contributed to this literature. The adaptive sickness behavioural response is exaggerated in animals with underlying neurodegeneration (Combrinck et al., 2002) and there is now evidence from both animal studies (Cunningham et al., 2009) and clinical studies in AD (Holmes et al., 2009) that systemic inflammatory events can accelerate progression of disease and functional decline. Delirium during dementia is a distressing condition in its own right but it can also provide information on the cognitive effects of peripheral immune stimulation. By creating biologically relevant animal models of this syndrome we should be able to elucidate the CNS mechanisms that underpin the increased vulnerability in the geriatric population. Given the frequency of systemic inflammatory events in elderly individuals and the impact that this can have on quality of

life, exploring the mechanisms of how systemic inflammation impacts upon CNS pathology is imperative to both basic science and geriatric research.

1.14 Objectives

The overarching aim of this thesis is to gain a better understanding of how peripheral inflammation impacts upon CNS function, inflammation and pathology during neurodegeneration. The mu-p75-saporin model of permanent cholinergic hypofunction and the ME7 model of progressive neurodegenerative disease will be used and bacterial and viral mimetics will be superimposed in order to examine sickness behavioural, cognitive, inflammatory and pathological consequences of these insults on the vulnerable brain. The studies have important implications for the fields of CNS inflammation, psychoneuroimmunology, neurology and psychiatry as well as relevance for intensive care unit medicine. In particular, the work sits at the rather unclear transition zone between dementia and delirium, in which patients who suffer acute systemic inflammation suffer serious consequences, both acutely, and for their ongoing disease status.

The primary objectives of this thesis are:

- To investigate the interaction between systemic inflammation and prior cholinergic neurodegeneration (Chapter 3). This was approached by the development of a novel model of basal forebrain cholinergic hypofunction using mu-p75-saporin to selectively lesion this population of neurons. Specifically aimed at addressing the cholinergic hypothesis of delirium and reconciling this with the neuroinflammatory hypothesis, this study will investigate the effect of systemic LPS challenge on cognitive function when superimposed upon prior cholinergic hypofunction, with the specific hypothesis that hypocholinergia + systemic inflammation would induce a state of cognitive dysfunction resembling delirium. Furthermore, given the existence of cholinergic anti-inflammatory pathway in the periphery, it was a secondary hypothesis of this work that the loss of acetylcholine innervation of the hippocampus would lead to a priming of the hippocampal microglial population (due to a loss of cholinergic inhibition of microglia).
- To investigate the interaction of a systemically administered viral mimetic, double stranded RNA (poly I:C), with the brain during progressive neurodegenerative

disease (Chapter 4). In this case the ME7 model of murine disease is used as a model of progressive neurodegeneration. The first aim of this study was to investigate whether the degenerating brain and its microglial population are primed with respect to systemic poly I:C challenge. The second aim was to investigate whether there are deleterious consequences for disease progression following systemic poly I:C challenge and if so, to explore some possible mechanisms underpinning these exacerbations.

- The final study (Chapter 5) aimed to follow up on some of possibilities raised in chapter 4. Specifically, we hypothesised that the type I IFN response may have a role in disease progression in the ME7 model of prion disease. To this end, the progression of prion disease in the IFNAR1^{-/-} mouse, which lacks the ability to respond to IFN- α/β , was examined. The study also aimed to clarify the role of type I IFNs in microglial priming: are IFN- α/β the priming factors or factors limiting full activation? Furthermore, the dependence on IL-1 β for progression of prion disease and exaggerated sickness and pathological consequences of systemic poly I:C challenges was also assessed using IL-1RI^{-/-} mice, which do not respond to IL-1 α /IL-1 β .

Chapter 2

Materials and Methods

2.1 Materials

Animals and *in vivo* Products

C57BL/6 mice
C57BL/6 IL-1R1^{-/-}
C57BL/6 IFNAR1^{-/-} mice
Formalin solution, neutral buffered, 10%
Horizontal bar
Inverted screen
Lab mouse diet
Microsyringe
Open field arena
Peristaltic pump
Stereotaxic frame
Thermocouple rectal probe
T-maze

Harlan laboratories, UK
Prof. Kingston Mills
Prof. Paul Hertzog
Sigma-Aldrich, UK
Custom designed in TCD
Custom designed in TCD
Red Mills, UK
Hamilton, USA
Custom designed in TCD
Gilson, France
David Kopf Instruments, USA
Thermalert, USA
Custom designed in TCD

Treatments

Donepezil hydrochloride (Aricept™)
Lipopolysaccharide (*Salmonella equine abortus*)
Murine-p75-saporin
Poly inosinic:poly cytidilic acid
Scopolamine hydrobromide
Sodium chloride 0.9% (endotoxin-free)
Sodium pentobarbital

Pfizer, Ireland
Sigma-Aldrich, UK
Advanced Targeting Systems, USA
Amersham Biosciences, UK
Tocris Bioscience, USA
Sigma-Aldrich, UK
Merial Animal Health, UK

ELISA

Mouse CXCL1 duo set (DY453)
Mouse IFN- β (42400-1)
Mouse IL-1 β duo set (DY401)
Mouse IL-1 β Quantikine (MLB00C)
Mouse IL-6 duo set (DY406)
Mouse TNF- α duo set (DY453)
Mouse TNF- α Quantikine (MTA00)
HPE Buffer
Streptavidin poly horseradish peroxidase
Substrate Solution
DC Protein Assay
96 well maxisorb immunoplates
96 well minisorb immunoplates

R & D Systems, UK
PBL Biomedical Laboratories, USA
R & D Systems, UK
R & D Systems, UK
R & D Systems, UK
R & D Systems, UK
R & D Systems, UK
Sanquin, Netherlands
Sanquin, Netherlands
R & D Systems, UK
Bio-Rad, UK
Nunc, UK
Nunc, UK

Immunohistochemistry: reagents and products

Biotinylated anti-fluorescein antibody (BA0601)
Biotinylated goat-anti-rabbit antibody (BA1000)
Biotinylated horse-anti-goat antibody (BA9500)
Biotinylated horse-anti-mouse antibody (BA2001)
Biotinylated rabbit-anti-goat antibody (BA5000)
Bovine serum albumin

Vector Laboratories, UK
Vector Laboratories, UK
Vector Laboratories, UK
Vector Laboratories, UK
Vector Laboratories, UK
Sigma-Aldrich, UK

DeadEnd Fluorometric TUNEL System

DPX mountant

Goat polyclonal anti-ChAT antibody (AB144P)

Goat polyclonal anti-COX-2 antibody (sc-1747)

Goat polyclonal anti-IBA-1 antibody (ab5076)

Harris Haematoxylin solution

Histoclear II

Normal goat serum

Normal horse serum

Normal rabbit serum

Mouse monoclonal anti-NFκB antibody (sc-8008)

Mouse monoclonal anti-NeuN antibody (MAB377)

Mouse monoclonal anti-synaptophysin antibody (MAB5258)

Mouse monoclonal anti-PrP antibody (sc-58581)

PAP pen

Paraffin wax

Proteinase K from *Pichia pastoris* (yeast)

Rabbit polyclonal anti-caspase-3 antibody (AB3623)

Rabbit polyclonal anti-IL-1β antibody (500-P51)

Rabbit polyclonal anti-IRF3 antibody (51-3200)

Super premium microscope slides

SuperFrost Plus microscope slides

Vectastain ABC kit

Invitrogen, UK

Sigma-Aldrich, UK

Millipore, USA

Santa Cruz, USA

Abcam, UK

VWR, UK

National Diagnostics, USA

Vector Laboratories, UK

Vector Laboratories, UK

Vector Laboratories, UK

Santa Cruz, USA

Millipore, USA

Millipore, USA

Santa Cruz, USA

Sigma-Aldrich, UK

Leica, Germany

Sigma-Aldrich, UK

Millipore, USA

Peptotech, USA

Invitrogen, UK

Menzel-Glaser, Germany

Menzel-Glaser, Germany

Vector Laboratories, UK

Molecular Reagents and Products

FastStart universal probe master mix

High Capacity cDNA Reverse Transcriptase Kit

MicroAmp 96-well Reaction Plate

Molecular grade ethanol 200 proof (absolute)

Molecular grade water

Nucleospin RNA II kits

Optical adhesive covers

Qiashredder columns

RNase-free DNase I enzyme

RNeasy Plus mini kits

SYBR green PCR master mix

Roche, UK

Applied Biosystems, UK

Applied Biosystems, UK

Sigma-Aldrich, UK

Sigma-Aldrich, UK

Macherey-Nagel, Germany

Applied Biosystems, UK

Qiagen, UK

Qiagen, UK

Qiagen, UK

Roche, UK

General Laboratory Chemicals

S-Acetylthiocholine iodide

3-Aminopropyltriethoxysilane

Ammonium nickel sulfate

Boric Acid

Citric Acid

Copper sulfate

3,3' Diaminobenzidine

Ethopropazine hydrochloride

Ethylenediaminetetraacetic acid (EDTA)

Sigma-Aldrich, UK

Sigma-Aldrich, UK

Sigma-Aldrich, UK

Sigma-Aldrich, UK

Sigma-Aldrich, UK

Sigma-Aldrich, UK

Sigma-Aldrich, UK

Sigma-Aldrich, UK

Sigma-Aldrich, UK

Formic acid	Sigma-Aldrich, UK
Glycine	Sigma-Aldrich, UK
Hydrochloric acid	VWR, USA
Heparin sodium (bovine)	Leo Laboratories, UK
Hydrogen peroxide (H ₂ O ₂)	Sigma-Aldrich, UK
HPLC-grade ethanol	Sigma-Aldrich, UK
Isopentane	Sigma-Aldrich, UK
Magnesium sulfate	Sigma-Aldrich, UK
Methanol	Fisher, UK
2-Methyl-2-butanol (tert-amyl alcohol)	Sigma-Aldrich, UK
β-mercaptoethanol	Sigma-Aldrich, UK
Pepsin (from porcine gastric mucosa)	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
di-Sodium hydrogen phosphate (Na ₂ HPO ₄)	Merck, USA
Sodium di-hydrogen phosphate (NaH ₂ PO ₄)	Merck, USA
Sodium sulfide	Sigma-Aldrich, UK
Sucrose	Sigma-Aldrich, UK
Sulfuric acid (H ₂ SO ₄)	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

General Laboratory Products

Needles 26G, 3/8	Braun, Ireland
Non-sterile pipette tips	Greiner, Germany
Nuclease-free PCR tube	Greiner, Germany
Parafilm laboratory roll	Pechiney Plastic Packaging, USA
Pipettes	Gilson, UK
Plastic cassettes	Labonord, France
Plastic wax containers for cassettes	Labonord, France
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
Sterile Pipette tips (RNA)	Starlab, Germany
Syringes (sterile 1ml)	Braun, Ireland
Tubes 200µl, 500µl, 1ml	Greiner, Germany

2.2 Methods

All solutions were made using Millipore Direct-Q 3 UV Series purified water (resistivity 18.2 MΩ cm at 25°C and < 10 ppb Total Organic Carbon and conductivity 0.056 μS/cm) and pH calibrated using a Hanna pH 210 Bench Top pH Meter, complete with pH electrode, temperature probe and electrode holder. Chemicals were weighed on Mettler Toledo scales.

2.2.1 Animals

Female C57BL/6 (Harlan, Bicester, UK) were housed in groups of five and given access to food and water *ad libitum*. Females were used in order to avoid fighting and injury, which has significant effects on behaviour. Animals were kept in a temperature-controlled room (21°C) with a 12:12 h light-dark cycle (lights on at 0700 h). All animal experimentation was performed in accordance with Republic of Ireland Department of Health & Children licenses, with approval from the local ethical committee and in compliance with the Cruelty to Animals Act 1876 and the European Community Directive, 86/609/EEC, and all efforts were made to minimise both the suffering and number of animals used.

IL-1R1^{-/-} mice on a background of C57BL/6 (strain B6.129S7-*Il1r1 tm1Imx/J*) from an established colony, originally from The Jackson Laboratory (Bar Harbor, ME), were kindly provided by Professor Kingston Mills.

IFNAR1^{-/-} mice on a background of C57BL/6 from an established colony, originally from The Jackson Laboratory (Bar Harbor, ME), were kindly provided by Professor Paul Hertzog (Monash University, Clayton, Australia).

2.2.2 Surgery

2.2.2.1 ME7 surgery

Mice were weighed, anaesthetised intraperitoneally (i.p.) with 1.2% Avertin (2,2,2-tribromoethanol solution; 0.2 ml/10 g body weight) and positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The incisor bar was set at - 1 mm, to give an approximately level head. The scalp was incised and the skull exposed. Two small holes were drilled in the skull either side of the midline to allow for bilateral injection of 1 μl of a 10% w/v scrapie (ME7 strain)-infected C57BL/6 brain homogenate made 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 $10\ \mu\text{l}$ Hamilton microsyringe (Hamilton, Reno, NV, USA) with a 26 gauge needle. Control animals were injected with a 10% w/v normal brain homogenate (NBH) in PBS, derived from a naïve C57BL/6 mouse. Following intrahippocampal injection, 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 and finally re-housed. Sucrose (5% w/v) and carprofen (0.05% v/v; Rimadyl, Pfizer, Ireland) were added to drinking water for two days following surgery to provide post-surgical analgesia and to minimise weight loss.

2.2.2.2 mu-p75-saporin surgery

Basal forebrain cholinergic lesions were produced by intracerebroventricular (i.c.v.) injection of mu-p75-sap (Advanced Targeting Systems, San Diego, CA, USA). Mice were anaesthetised and prepared for surgery as for ME7 treatment. Two small holes were drilled in the skull either side of the midline. Two $1\ \mu\text{l}$ injections of mu-p75-sap at a concentration of either 0.04, 0.2 or $0.6\ \mu\text{g}/\mu\text{l}$ (total dosage 0.08, 0.4 or $1.2\ \mu\text{g}$ per mouse) were made into the lateral ventricles using a pulled glass micropipette (Sigma, Poole, UK) [co-ordinates from bregma: anteroposterior, $- 0.45$ mm; lateral, ± 1.0 mm; depth $- 1.9$ mm]. Control animals were injected with sterile PBS. Following injection, the needle remained in place for 5 minutes to ensure full circulation of the toxin in the ventricular system and to minimize reflux. Post-operatively, mice were treated as for ME7 animals with the exception that carprofen was not added to drinking water as this surgical procedure follows a more acute timecourse than that of prion disease and thus it was not desirable to interfere with any post-surgical inflammatory response. Animals recovered fully from surgical procedures (20 days) before starting cognitive experiments.

2.2.3 Intraperitoneal challenges

2.2.3.1 Lipopolysaccharide

For cognitive and microglial priming experiments, mu-p75-sap-lesioned animals were injected i.p. with $100\ \mu\text{g}/\text{kg}$ of the bacterial endotoxin LPS (*Salmonella equine abortus*; Sigma, L5886, Poole, UK) prepared in non-pyrogenic 0.9% sterile saline (Sigma, Poole, UK). This dose mimics a mild to moderate infection rather than severe sepsis, eliciting small changes (approximately 1°C) in core body temperature and previously shown to produce acute working memory deficits in ME7 animals (Murray et al., 2012). For cognitive experiments, LPS was administered 3 hours prior to behavioural testing. In all cases control animals were injected with sterile saline, $200\ \mu\text{l}$ per 20 g body weight.

2.2.3.2 poly I:C

Poly I:C was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). It was prepared for injection by resuspending in sterile saline, heating to 50°C at a concentration of 2 mg/ml to ensure complete solubilisation and then allowing to cool naturally to room temperature to ensure proper annealing of double-stranded RNA. Poly I:C was stored at -20°C until use. Experimental groups at 18 weeks post-inoculation with ME7 or NBH were injected i.p. with 12 mg/kg poly I:C delivered in sterile saline. A subset of naïve animals were challenged with one, two or three injections of poly I:C (12 mg/kg) to determine whether repeated systemic administration induces immunological tolerance or indeed primes the peripheral immune system to produce an exaggerated systemic response to subsequent stimulation. In this case, each injection of poly I:C was administered two weeks apart to ensure full recovery from the prior systemic inflammatory response. A further subset of ME7 animals received repeated systemic challenge with poly I:C (12 mg/kg i.p.) made at 14, 16 and 18 weeks post-inoculation in order to assess the effect of repeated “viral stimulation” on the progression of neurodegenerative disease. The two week interim period allowed for full recovery from each systemic inflammatory response before subsequent challenges. Control animals were injected with sterile saline, 200µl per 20 g body weight.

2.2.3.3 Scopolamine

To determine whether the T-maze task was dependent on normal cholinergic function, the muscarinic antagonist scopolamine hydrobromide (Tocris Bioscience, Bristol, UK) was injected i.p. at a dose of 1 mg/kg delivered in sterile saline. This was administered 1 hour prior to testing in a cohort of naïve C57BL/6 mice. Controls were administered sterile saline, 200µl per 20 g body weight.

2.2.3.4 Donepezil

The acetylcholinesterase inhibitor, donepezil hydrochloride (Aricept®; Pfizer, New York, NY, USA) was kindly donated by Professor Shane O'Mara, School of Psychology, Trinity College Dublin. 10 mg tablets were crushed and dissolved in 10 ml of endotoxin-free water to yield a concentration of 1 mg/ml. This solution was sonicated in 10 second on/off bursts for 5 minutes to ensure complete dissolution of tablets. The solution was then diluted 1:10 to give a 0.1 mg/ml stock solution for injection. For cognitive experiments, animals were injected i.p. with 1 mg/kg donepezil delivered in endotoxin-free water. Donepezil solution

was administered 1 hour after LPS and 2 hours prior to commencement of cognitive testing.

2.2.4 Intra-cerebral challenges

2.2.4.1 Poly I:C

Mice were weighed, anaesthetised and prepared as for ME7 stereotaxic surgery. 1 μ l injections of poly I:C (2 mg/ml – 2 μ g/mouse) were made into the right dorsal hippocampus (co-ordinates from bregma: anteroposterior, – 2.0 mm; lateral, – 1.6 mm; depth, – 1.7 mm) using a pulled glass micropipette (Sigma, Poole, UK). The needle was left in place for 2 minutes before being withdrawn slowly to minimize reflux. Mice were allowed to recover but were perfused for formalin fixation at 3 hours post-challenge.

2.2.5 Behavioural tests

For all behavioural experiments, mice were moved from their home-room and left in the test room for 15 minutes prior to beginning the task to ensure that they were in an optimal state of arousal.

2.2.5.1 Open field

The open field arena consisted of a plastic base (58 cm x 33cm) surrounded by walls of 19 cm. The floor of the box is divided into a grid of equal sized squares. Measurement was made of distance travelled (in terms of grid squares crossed) and total number of rears. Weekly measurements of open field activity were recorded for 3 minutes from when animals were placed facing any corner of the arena.

2.2.5.2 Burrowing

Black plastic burrowing tubes, 20 cm long, 6.8 cm diameter, sealed at one end were filled with 300 g of normal mouse diet food pellets, and placed in individual mouse cages. The open end was raised by 3 cm above the floor by a wooden support to prevent non-purposeful displacement of the contents. Mice were placed individually in the cages for 2 hours, at which point the food remaining in the cages was weighed and the amount displaced (burrowed) was calculated. Weekly measurements were taken until burrowing significantly decreased due to prion disease progression.

2.2.5.3 Y-maze reference memory

The “paddling” Y-maze visuo-spatial task was used to investigate hippocampal-dependent reference learning and memory following lesions of the basal forebrain cholinergic system. This Y-maze task combines elements of two hippocampal-dependent tasks: the paddling pool spatial cognition test and the appetitive Y-maze (Deacon and Rawlins, 2002, Reisel et al., 2002). The Y-maze was constructed of clear Perspex mounted on a white plastic base and consisted of three arms with dimensions 30 x 8 x 13 cm joined in the form of a Y (Figure 2.1a). The distal end of each arm contained a hole, 4 cm in diameter, set with its lower annulus 2 cm above the floor of the maze. Two of these holes were sealed off by insertion of a closed black plastic tube, making them impassable. The third hole, however, was filled with an open black tube thus allowing an escape route from the maze. Over each of the three tubes, on the outside of the maze, was placed a black burrowing tube which can be readily detached with a mouse inside to facilitate its return to the home cage. Burrowing tubes were also placed over the false exit tubes, so that from the centre, all three arms appeared identical. The Y-maze was filled with 2 cm of water at a temperature of 20 - 22°C. This depth of water is not overtly stressful but is sufficient to motivate mice to leave the maze by paddling to a black exit tube at the distal end of one arm, 2 cm above the floor. The maze was placed in the centre of a small room with by prominent visual cues on all walls. The location of each exit was fixed for each animal. Animals were taken with their cage mates to a holding cage. Mice were placed in one of the two possible start arms, facing away from the centre of the maze, in a pseudo-randomised sequence for 10 trials and all groups were counterbalanced with respect to the location of the exit. In a subset of animals the task was conducted for 15 trials. Animals were allowed 60 seconds to locate the correct arm. A correct trial was defined as entry to the exit arm without entering other arms. An arm entry was defined as entry of the whole body, excluding the tail, into the arm. If the mouse did not enter the correct arm within the time limit, it was gently pushed towards this arm using a clear plastic paddle. Upon choosing the correct arm, the mouse can escape from the water and enter the small exit tube followed by the transit tube, in which the animal is immediately returned to its home cage. An inter-trial interval of 20 minutes was adhered to during this task.

2.2.5.4 T-maze alternation: working memory

Hippocampal-dependent working memory was assessed by analysis of alternation behaviour using a novel “escape from shallow water” T-maze task. This task was designed to enable studies of cognitive function of animals during sickness behaviour, which

confounds analysis of cognitive performance in many other hippocampal-dependent tasks. The T-maze was constructed of black Perspex with dimensions: long axis 67 cm, short axis 38 cm, depth 20 cm, and internal arm width 7 cm (Figure 2.1b). There was a single 4 cm diameter escape aperture at the end of each choice arm, placed 2 cm from the floor of the maze. Black exit tubes were inserted into the escape holes, which may also be blocked to prevent exit. A “guillotine” door was inserted into the maze to prevent access to one or other of the choice arms. The maze was filled with water at a temperature of 20°C to a depth of 2 cm to motivate mice to leave the maze by “paddling” to an exit tube. Directly before testing, animals were taken with their cage-mates to a holding cage. Each mouse was placed in the start arm facing towards the junction, with one arm blocked such that mice were forced to make a left (or right) turn. Forced arm entry was varied in a pseudo-randomised sequence such that all animals were presented with equal numbers of left and right turns and that there were no more than two consecutive runs to the same arm, and also ensuring that the first two trials were not identical. On making this forced turn, the mouse could escape from the water by entering the small tube, and then a transit tube, in which it was carried to another holding cage. The mouse was held here 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. Alternation from its original turn constitutes a correct trial. On choosing correctly, mice escape to the transit tube as before and are returned to their home cage. On choosing incorrectly the mice are allowed to self-correct to find the correct arm. Animals were trained for blocks of ten trials (20 minute inter-trial interval) until reaching performance criterion.

If animals developed a side preference early in training, a correction strategy was employed. This side preference can be broken by not allowing mice to self-correct, such that instead of crossing the T from the incorrect arm to the correct arm, the entrance to the correct arm can be closed at the moment that the incorrect arm is chosen. Animals must then return to the start arm and once again have the option to choose the correct arm. This is repeated until the animal finally chooses the correct body turn when starting from the start arm. This correction strategy is only employed for animals displaying a complete side preference (always turning in one direction if that arm is open to them), is discontinued once their side preference is broken and is never employed within 2 days of administration of substances to examine effects on working memory. Most animals reached alternation of greater than 80% but no animals were challenged with LPS or saline unless they showed

consecutive days performing at 70% or above, never less than 80% in the last block of 5 trials prior to challenge and no evidence of a side preference.

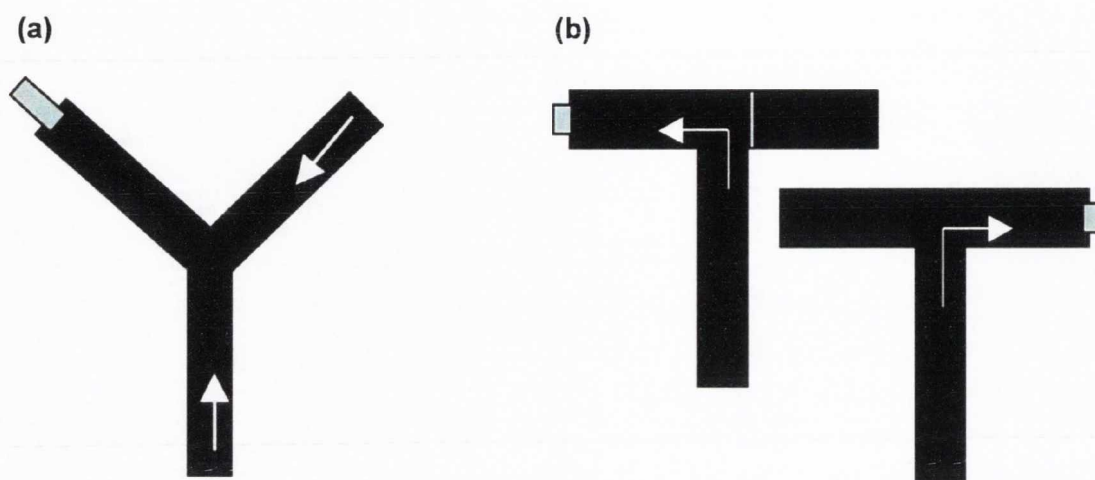


Figure 2.1 *Schematic representations of behavioural paradigms.* (a) Y-maze visuo-spatial test where a mouse learns to exit the maze from the same arm on each trial regardless of where it started. (b) T-maze working memory test where a mouse is firstly forced to the sample arm, then upon returning to the maze after 30 seconds, must alternate to the choice arm.

2.2.6 Motor co-ordination tests

2.2.6.1 Horizontal bar

The horizontal bar was designed to assess forelimb muscular strength and co-ordination. It consisted of a 26 cm long metal bar, 0.2 cm diameter, supported by a 19.5 cm high wooden column at each end. Each mouse was held by the tail and allowed to grip the central point of the bar with its front paws only. The tail was rapidly released and mice were scored based on whether they fell, held on for 60 seconds, or reached a platform on a supporting column, with the latter two results scoring the maximum of 60 seconds.

2.2.6.2 Inverted screen

The inverted screen (Kondziella, 1964) assessed muscular strength for all four limbs. It consisted of a wooden frame, 43 cm square, covered with wire mesh (12 mm squares of 1 mm diameter wire). The mouse was placed on the screen, which was then slowly (2 seconds) inverted. The time it took for the mouse to fall was measured, up to a criterion of

60 seconds. Padding was provided to cushion mice falling from both bar and screen apparatus.

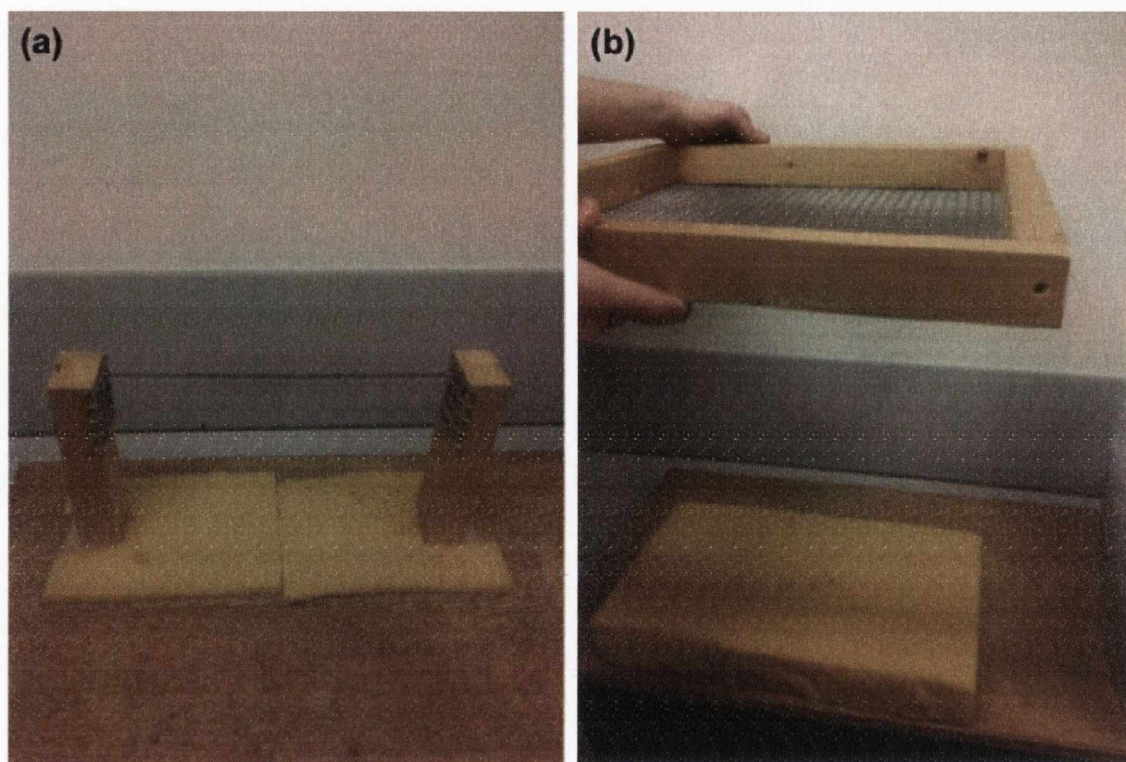


Figure 2.2 *Photographic representation of motor co-ordination tests. (a) Horizontal bar task for forelimb muscular strength and co-ordination. (b) Inverted screen task to assess muscular strength in all four limbs.*

2.2.7 Body temperature

Core body temperature was measured using a thermocouple rectal probe (Thermalert TH5, Physitemp, Clifton, NJ, USA). ME7 animals (wild-type C57BL/6 and IL-1R1^{-/-} mice) 18 weeks post-inoculation were habituated to measurements of rectal temperature on two separate occasions prior to the i.p. poly I:C challenge to minimise the effects of stress which could confound experimental results. Temperatures were recorded at baseline and then at 4, 9, and 13 hours following i.p. challenge with poly I:C.

2.2.8 ELISA for cytokines

2.2.8.1 Plasma ELISA

Animals were terminally anaesthetised with sodium pentobarbital (40 mg per mouse i.p., Euthatal, Merial Animal Health, Essex, UK). The skin overlying the sternum was incised

and the thoracic cavity opened to expose the heart. A butterfly needle connected to a Gilson Minipuls 3 peristaltic pump (Gilson, Villiers le Bel, France) was inserted into the left ventricle. Blood was collected in heparinised tubes directly from the right atrium of the heart. Whole blood was centrifuged for 15 minutes at 900 G to remove cells and the remaining plasma was aliquoted and stored at -20°C before assay. Samples from normal animals that received three peripheral poly I:C challenges (12 mg/kg) were analysed for IFN- β to determine the systemic inflammatory response following multiple systemic viral insults.

2.2.8.1.1 IFN- β ELISA

The IFN- β ELISA was carried out using a kit supplied by PBL Biomedical Laboratories (Piscataway, NJ, USA) and the procedure for the sandwich-type ELISA was carried out as per manufacturer's instructions. 100 μl plasma samples and standards diluted in sample diluent were added to a pre-coated plate at room temperature for 60 minutes before being washed 3 times with wash solution. Samples were serially diluted 1/3, 1/9 and 1/27, while standards were in a range of 15.6-1000 pg/ml. 100 μl of antibody solution (30 μl antibody per 12 ml sample diluent) was added to each well. After 60 minute incubation at room temperature, the plate was washed 3 times and horseradish peroxidase (HRP) solution (36 μl HRP per 12 ml concentrate diluent) was added to each well and incubated at room temperature for 60 minutes. The plate was washed 3 times and 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to each well. After a 15 minute incubation, stop solution was added to each well and mixed gently. Optical density was read at 450 nm with correction at 570 nm. Sample concentrations were determined by extrapolation from the standard curve.

2.2.8.1.2 IL-1 β , TNF- α , IL-6 and CXCL1

Mouse TNF- α , IL-6, and CXCL1 were quantified by sandwich-type ELISA, using R&D systems duo set kits (R&D systems, Minneapolis, MN, USA). A standard protocol was followed. The capture anti-TNF- α , IL-6 and CXCL1 antibodies were diluted 1/180 (approximately 1 $\mu\text{g}/\text{ml}$) in PBS, and used to coat a 96-well maxisorb microplate overnight (Nunc, Fisher Scientific, Leicestershire) with 100 μl per well. Plates were then washed with PBS + 0.05 % Tween and blocked with PBS + 1 % BSA before addition of 100 μl samples and standards for 2 hours at room temperature. Samples were serially diluted as follows: for TNF- α 1/2 and 1/4; for IL-6 1/120 and 1/240 for LPS challenges and 1/40, 1/80 and 1/160 for poly I:C challenges; for CXCL1 1/300 and 1/600. Standards were in the

range of 1000-4 pg/ml. Detection antibodies were used at a dilution of 1/180 and incubated on the plates for 1.5 hours. Streptavidin poly-horseradish peroxidase (HRP: Sanquin, Amsterdam, Netherlands) was diluted in 1:10,000 and incubated in the dark at room temperature for 20 minutes. Samples, standards, detection antibodies and streptavidin poly-horseradish peroxidase were diluted in high performance ELISA buffer (Sanquin; Amsterdam, Netherlands). TMB and H₂O₂ were used as substrate and the reaction was stopped with 1 M H₂SO₄ before optical density was read at 450 nm with correction at 570 nm. Sample concentrations were determined by extrapolation off the standard curve.

IL-1 β was analysed using a Quantikine mouse IL-1 β kit (R&D systems, Minneapolis, MN, USA). 50 μ l assay diluent and 50 μ l standard, control or undiluted sample was added to wells and the plate was incubated for 2 hours at room temperature. Following 5 washes with wash buffer, 100 μ l mouse IL-1 β conjugate was added to all wells and the plate was incubated for 2 hours at room temperature. Standards ranged 500-7.8 pg/ml and samples were diluted 1/2. The wash step was repeated and 100 μ l of substrate solution was added to each well and incubated for 30 minutes at room temperature. After the addition of 100 μ l stop solution, the optical density was read at 450 nm with correction at 570 nm.

2.2.8.2 ELISA for brain cytokines

Animals were perfused as for plasma ELISA. Brains were rapidly removed and the hippocampi dissected out, placed in eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C until further processing.

2.2.8.2.1 IL-1 β assay

Hippocampal tissue was weighed and homogenised 10% w/v in ice-cold buffer (150mM NaCl, 25mM Tris-HCL, 1% Triton-X; 10% w/v) containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche, Lewes, UK) and centrifuged at 21,000 G for 10 minutes. The IL-1 β ELISA was carried out using a Quantikine mouse IL-1 β kit (R&D systems, Minneapolis, MN, USA), following the manufacturer's instructions. 50 μ l of assay diluent was added to all wells of a pre-coated plate, followed by 50 μ l of sample supernatant, standards and control. Sample supernatants were undiluted and standards were in the range of 3.125 – 800 ng/ml. The plate was then covered and incubated for 2 hours at room temperature. All wells were washed 5 times with wash buffer before addition of mouse IL- β conjugate, 100 μ l per well. The plate was again covered and incubated for 2 hours at room temperature. All wells were again washed 5 times with wash buffer before

addition of substrate solution, 100 µl per well. The plate was incubated at room temperature in the dark for 30 minutes before addition of stop solution, 100 µl per well. Optical density was read at 450 nm with correction at 570 nm. Data was linearised by plotting the log of the IL-1 β concentration versus the log of the corrected optical density and sample concentrations were determined by extrapolation from this standard curve.

2.2.8.2.2 *TNF- α assay*

Hippocampal tissue was weighed and homogenised in a buffer (150mM NaCl, 25mM Tris-HCL, 1% Triton-X; 10% w/v) containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche, Lewes, UK) and centrifuged at 21,100 G for 10 minutes. The TNF- α ELISA was carried out using a Quantikine mouse TNF- α kit (R&D systems, Minneapolis, MN, USA), following the manufacturer's instructions. 50 µl of assay diluent was added to all wells of a pre-coated plate, followed by 50 µl of sample supernatant, standards and control. Sample supernatants were undiluted and standards were in the range of 375 – 5.9 pg/ml. The plate was then covered and incubated for 2 hours at room temperature. All wells were washed 5 times with wash buffer before addition of mouse TNF- α conjugate, 100 µl per well. The plate was again covered and incubated for 2 hours at room temperature. All wells were again washed 5 times with wash buffer before addition of substrate solution, 100 µl per well. The plate was incubated at room temperature in the dark for 30 minutes before addition of stop solution, 100 µl per well. Optical density was read at 450 nm with correction at 570 nm. Data was linearised by plotting the log of the TNF- α concentration versus the log of the corrected optical density and sample concentrations were determined by extrapolation from this standard curve.

2.2.8.2.3 *Protein assay*

Brain cytokine concentrations were normalised to protein concentration (quantified using DC Protein Assay [Bio-Rad, Hemel Hempstead, UK]). 5 µl hippocampal samples homogenised in homogenisation buffer were diluted in 1/10 in homogenisation buffer. Bovine serum albumin, diluted in homogenisation buffer in a range 2 – 0.0625 mg/ml, was used as a standard curve. 25 µl working reagent A, a 5 µl dilute hippocampus sample and 200 µl reagent B were added to each well and incubated on a minisorb assay plate for 15 minutes before optical density was read at 730 nm. Sample concentrations were determined by extrapolation off the standard curve. Sample cytokine levels were then divided by the corresponding protein concentrations to give a pg/mg of protein concentration for each hippocampal sample.

2.2.9 RNA extraction and Quantitative PCR

2.2.9.1 Tissue preparation for qPCR

Animals were terminally anaesthetised with sodium pentobarbital (40 mg per mouse i.p., Euthatal, Merial Animal Health, Essex, UK). The skin overlying the sternum was incised and the chest cavity opened to expose the heart. A butterfly needle connected to a peristaltic pump (Gilson, Villiers le Bel, France) was inserted into the left ventricle. The right atrium was cut and the animal was perfused transcardially with heparinised saline (0.1% v/v heparin [LEO Pharma, Princes Risborough, Buckinghamshire, UK] in 0.9% saline) for approximately 2 minutes to clear blood from all tissues. Brains were rapidly removed, the hippocampi, hypothalamus and medial septum (mu-p75-sap animals only) dissected out, placed in eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C until further processing.

2.2.9.2 RNA extraction

Total RNA was extracted from brain samples using Qiagen RNeasy[®] Plus mini kits (Qiagen, Crawley, UK) according to the manufacturer's instructions. Brain samples were weighed to ensure each lay in the range of 20 – 30 mg. Before use, β -mercaptoethanol (β -ME) was added to Buffer RLT Plus (10 μl per 1 ml β -ME). 600 μl Buffer RLT was added to each sample followed by homogenisation using a motorised pestle. The sample lysates were placed in Qiagen Qias shredder (Qiagen, Crawley, UK) columns and centrifuged at 21,100 G for 6 minutes to further homogenise the tissue. The flow-through was collected and transferred to the genomic DNA (gDNA) Eliminator spin column and centrifuged at 21,100 G for 30 seconds. The column was discarded and an equal volume of 70% ethanol was added to the flow-through and mixed until homogenous. Samples were placed in RNeasy mini spin columns in 2 ml collection tubes and centrifuged at 21,100 G for 15 seconds. The flow-through was discarded and the column was washed by addition 350 μl of Qiagen Buffer RW1 followed by centrifugation at 21,100 G for 15 seconds. The flow-through was discarded and an on-column DNase digestion was performed using the Qiagen RNase-free DNase I enzyme, as an extra precaution to ensure complete removal of contaminating gDNA. 10 μl DNase I stock solution was mixed with 70 μl Qiagen Buffer RDD and this DNase I incubation mix (80 μl) was added to each column and left for 15 minutes, after which the column was washed with 350 μl of Buffer RW1 and centrifuged at 21,100 G for 30 seconds. The flow-through was discarded and the column was inserted into a new 2 ml collection tube. 500 μl of Qiagen RPE buffer was added to the column and centrifuged at 21,100 G for 15 seconds. The flow-through was discarded, 500 μl of Qiagen

RPE buffer was added to the column and centrifuged at 21,100 G for 2 minutes to dry the silica membrane. To eliminate carryover of Buffer RPE, the spin column was transferred to a new 2 ml collection tube and centrifuged at 21,100 G for 1 minute. The RNeasy mini spin column was placed into a new 1.5 ml collection tube. 30 µl RNase-free water was added directly to onto the RNeasy silica-gel membrane and the followed by centrifugation for 1 minute at 21,100 G to elute the RNA. RNA yields were determined by spectrophotometry at 260 and 280 nm using the NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Dublin, Ireland). RNA was stored at -80°C until cDNA synthesis and PCR assay.

2.2.9.3 Reverse transcription cDNA synthesis

RNA was reversed transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). Four hundred nanograms of total RNA were reverse transcribed in a 20 µl reaction volume. 10 µl master mix (for each sample master mix contained: 2 µl 10X RT Buffer; 0.8 µl 25X dNTP mix, 100 mM; 2 µl 10X RT random primers; 1 µl MultiScribe™ Reverse Transcriptase; 4.2 µl RNase-free water) was added to 10 µl RNA for each sample in a nuclease-free PCR tube (Greiner Bio-One, Monroe, NC, USA). Two controls were prepared; one of which replaced RNA with RNase-free water, and the other which replaced reverse transcriptase with RNase-free water. PCR tubes were placed in a DNA Engine® Peltier Thermal Cycler PTC-200 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and samples were incubated at 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes (to inactivate reverse transcriptase). Samples were held at 4°C until collection and then stored at -20°C until assay.

2.2.9.4 Taqman quantitative PCR

Reagents were supplied by Applied Biosystems (Taqman® Universal PCR Master Mix; SYBR® Green PCR Master Mix) and Roche (FastStart Universal Probe Master [Rox]; FastStart Universal SYBR Green Master [Rox]; Lewes, UK). For all assays, primers were designed using the published mRNA sequences for the genes of interest, applied to Primer Express™ software. Where possible, probes were designed to cross an intron such that they were cDNA specific. In some cases the fluorescent DNA binding probe SYBR green has been used in place of a specific probe. Primer and probe sequences, along with accession numbers for mRNA sequence of interest may be found in table 1. Oligonucleotide primers were resuspended in 1X TE buffer (Tris Base 10 mM, EDTA 1 mM; pH 7.5-8.0) and diluted to 10 µM working aliquots. All primer pairs were checked for

specificity by standard reverse transcription (RT)-PCR followed by gel electrophoresis and each primer pair produced a discrete band of the expected amplicon size. Table 1 lists the sequences for primers and probes for each assay.

For Taqman PCR, 24 µl of PCR master mix containing 12.5 µl Taqman® Universal PCR Master Mix (or SYBR® Green PCR Master Mix), 0.5 µl of each of the forward primer, reverse primer and probe, and 10 µl of RNase-free water was added to individual wells of a MicroAmp™ Optical 96-well Reaction Plate (Applied Biosystems, Warrington, UK). Where SYBR green was used, RNase-free water was substituted in place of the probe. To this, 1 µl of cDNA (equivalent to 20 ng of RNA) was added to each well to give a final reaction volume of 25 µl. An optical adhesive cover was fixed to the plate and the samples were run in the 7300 Real-Time PCR System (Applied Biosystems, Warrington, UK). The PCR reaction was conducted over three steps as follows: step one, 50°C for 2 minutes; step two, 95°C for 10 minutes; step three, 95°C for 15 seconds and 60°C for 1 minute. Step three was repeated for 40 cycles, and data was collected during this amplification phase.

Since experimental work for thesis began, some reagents and consumables used for Taqman qPCR were changed for economical reasons. Thus, the above protocol was used for chapter 3 and Figures 5.13 and 5.14 in chapter 5. All other Taqman qPCR used the following master mixes and plates in place of their Applied Biosystems counterparts: FastStart Universal Probe Master [Rox] (Roche, Lewes, UK); FastStart Universal SYBR Green Master [Rox] (Roche, Lewes, UK); Framestar® 96-well PCR Plate (Anachem, Bedfordshire, UK).

2.2.9.5 Taqman PCR quantification

A standard curve was constructed from total RNA isolated from mouse brain tissue 24 hours after intra-cerebral challenge with 2.5 µg LPS, which is known to up-regulate most target transcripts of interest in this study. This standard curve was constructed using a higher concentration of RNA in the reverse transcriptase 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. Serial 1 in 4 dilutions of the cDNA synthesized from brains of LPS-injected mice were made and standards were run alongside samples under standard Taqman PCR cycling conditions. A curve was plotted of the C_t value (the cycle number at which the fluorescence of the product of transcribed gene crosses the threshold of detection) versus the log of the concentration (assigned an

arbitrary value since the absolute concentration of cytokine transcripts 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 using Applied Biosystems Rodent GAPDH Taqman Kit. All PCR data were normalised to the expression of GAPDH.

Table 1. Mouse Taqman primer and probe sequences

Target	Accession no.	Oligonucleotide	Sequence	Amplicon size (b
ADAM 8	NM_007403	Forward primer	CCGAAAGGCTCCGAGACAA-3'	126
		Reverse primer	5'-GAAGGGTCTGGAGGCCTGTT-3'	
		Probe	5'-AGGAGTGTGGCACCCAAGCCTATCTCG-3'	
ADAM 17	NM_009615	Forward primer	5'-AAGTGCAAGGCTGGGAAATG-3'	121
		Reverse primer	5'-CACACGGGCCAGAAAGTT-3'	
		Probe	5'-CCTGCGCATGCATTGACACTGACAAC-3'	
Bax- α	L22472	Forward primer	5'-GTTTCATCCAGGATCGAGCAG-3'	238
		Reverse primer	5'-CCCCAGTTGAAGTTGCCATC-3'	
		Probe	SYBR Green	
ChAT	NM_009891	Forward primer	5'-CAGCCAATTGGGTCTCTGAATAC-3'	107
		Reverse primer	5'-GAAGTGCTGCCGAGCAAAG-3'	
		Probe	SYBR Green	
COX-2	NW_000157	Forward primer	5'-GAGTGGTAGCCAGCAAAGCC-3'	81
		Reverse primer	5'- TTTAATTGGGAACCCCTTCTTTGTT-3'	
		Probe	5'-AGCAACAAAAGCGTTCTACAAAGGAACCTAACCA-3	
CXCL1	NM_008176	Forward primer	5'-CACCCAAACCGAAGTCATAGC-3'	82
		Reverse primer	5'-AATTTTCTGAACCAAGGGAGCTT-3'	
		Probe	5'-TCGCGAGGCTTGCCTTGACCC-3'	
Fas	M83649	Forward primer	5'-CTGCGATGAAGAGCATGGTTT-3'	208
		Reverse primer	5'-CCATAGGCGATTTCTGGGAC-3'	
		Probe	SYBR Green	
IFN β	X14455	Forward primer	5'-CCATCATGAACAACAGGTGGAT-3'	67
		Reverse primer	5'-GAGAGGGCTGTGGTGGAGAA	
		Probe	5'-CTCCACGCTGCGTTCCTGCTGTG-3'	
IFN- γ	X01992	Forward primer	5'-TCAAGTGGCATAGATGTGGAAGAA-3'	92
		Reverse primer	5'-TGGCTCTGCAGGATTTTCATG-3'	
		Probe	5'-TCACCATCCTTTTGCCAGTTCCTCCAG-3'	
IFNAR2	NM_010509	Forward primer	5'-GAGAGCAGAAAAACGGACTTAAGAG-3'	98
		Reverse primer	5'-TCGCAGACACCACAAGACACA-3'	
		Probe	5'-TGCACCGTCTCTGCCGTCGG-3'	

Target	Accession no.	Oligonucleotide	Sequence	Amplicon size (bp)
IL-1 β	M15131	Forward primer	5'-GCACACCCACCCTGCA-3'	69
		Reverse primer	5'-ACCGCTTTTCCATCTTCTTCT-3'	
		Probe	5'-TGGAGAGTCTGGATCCCAAGCAATACCC-3'	
IL-6	NM_031168	Forward primer	5'-TCCAGAAACCGCTATGAAGTTC-3'	72
		Reverse primer	5'-CACCAGCATCAGTCCCAAGA-3'	
		Probe	5'-CTCTGCAAGAGACTTCCATCCAGTTGCC-3'	
IL-10	M37897	Forward primer	5'-GGTTGCCAAGCCTTATCGGA-3'	191
	M84340	Reverse primer	5'-ACCTGCTCCACTGCCTTGCT-3'	
		Probe	5'-TGAGGCGCTGTCATCGATTCTCCC-3'	
IL-12p40	M86671	Forward primer	5'-GGAAGCACGGCAGCAGAATA-3'	180
		Reverse primer	5'-AACTTGAGGGAGAAGTAGGAATGG-3'	
		Probe	SYBR Green	
iNOS	U43428	Forward primer	5'-CAGCTGGGCTGTACAAACCTT-3'	95
		Reverse primer	5'-CATTGGAAGTGAAGCGTTTCG-3'	
		Probe	5'-CGGGCAGCCTGTGAGACCTTTGA-3'	
IP-10	NM_021274	Forward primer	GCCGTCATTTTCTGCCTCAT	127
		Reverse primer	GCTTCCCTATGGCCCTCATT	
		Probe	TCTCGCAAGGACGGTCCGCTG	
IRF7	NM_016850	Forward primer	CGAGGAACCCTATGCAGCAT	108
		Reverse primer	TACATGATGGTCACATCCAGGAA	
		Probe	CCAGCTCTCACCGAGCGCAGC	
MCP-1	BC154869	Forward primer	5'-GTTGGCTCAGCCAGATGCA-3'	81
		Reverse primer	5'-AGCCTACTCATTGGGATCATCTTG-3'	
		Probe	5'-TTAACGCCCCACTCACCTGCTGCTACT-3'	
MMP9	NM_013599	Forward primer	5'-CGAACTTCGACACTGACAAGAAGT-3'	114
		Reverse primer	5'-GCACGCTGGAATGATCTAAGC-3'	
		Probe	5'-TCTGTCCAGACCAAGGGTACAGCCTGTTC-3'	
Mx1	NM_010846	Forward primer	5'-GCCAGACGATGGATTCTGTGA-3'	149
		Reverse primer	5'-TGA ACTCTGGTCCCCAATGAC-3'	
		Probe	SYBR Green	

Target	Accession no.	Oligonucleotide	Sequence	Amplicon size (b
OAS1a	NM_145211	Forward primer	5'-CTTTGATGTCCTGGGTCATGT-3'	123
		Reverse primer	5'-GCTCCGTGAAGCAGGTAGAG-3'	
		Probe	SYBR Green	
PKR	NM_011163	Forward primer	5'-CCGAAACTGCCGGAACA-3'	97
		Reverse primer	5'-CTGACTGGGAAACACCATTACTTG-3'	
		Probe	SYBR Green	
PTX3	X83601	Forward primer	5'-ACAACGAAATAGACAATGGACTTCAT-3'	62
		Reverse primer	5'-CTGGCGGCAGTCGCA-3'	
		Probe	5'-CCACCGAGGACCCCACGCC-3'	
SRA2	L04274	Forward primer	5'-GTTCTGTGTCATGCCATGC-3'	76
		Reverse primer	5'-ATGTCAATGGAGGCCCCA-3'	
		Probe	SYBR Green	
TNF- α	M11731	Forward primer	5'-CTCCAGGCGGTGCCTATG-3'	149
		Reverse primer	5'-GGGCCATAGAACTGATGAGAGG-3'	
		Probe	5'-TCAGCCTCTTCTCATTCTGCTTGTGG-3'	
TNFAIP2	NM_009396	Forward primer	GCTGGCAAGGCACATCCT	74
		Reverse primer	AGGTTGCAGTGGAGCCATTCT	
		Probe	CAACGCGGATGCC	
TREM2	NM_031254	Forward primer	TGTGGTCAGAGGGCTGGACT	68
		Reverse primer	CTCCGGGTCCAGTGAGGA	
		Probe	CCAAGATGCTGGGCACCAACTTCAG	

2.2.10 Immunohistochemistry

2.2.10.1 Tissue preparation

All animals used for immunohistochemical analysis were terminally anaesthetized with sodium pentobarbital (40 mg/mouse i.p., Euthatal, Merial Animal Health, Essex, UK) and transcardially perfused with heparinised saline as for quantitative PCR. Following this, animals were perfused with 10% formalin (Sigma, Poole, UK) for 15 minutes to allow for fixation of all tissues. Brains were dissected out and post-fixed in small containers of 10% formalin for 2 days at which point they were transferred to PBS until embedding.

2.2.10.2 Paraffin wax embedding and sectioning of brains

Formalin-fixed brains were placed in plastic cassettes (Thermo Fisher Scientific, Dublin, Ireland) and transferred to a vessel of 70% ethanol for 20 minutes to begin the dehydration process. This was followed by 1.5 hourly changes through a graded series of ethanol, each 300 ml: 70% ethanol II, 80%, 95%, 100% I, 100% II. Cassettes were then placed in 100% ethanol III overnight after which they went through two five hour periods in the clearing agent, HistoClear II (National Diagnostics, Atlanta, GA, USA), followed by a final overnight period in HistoClear II. Brain cassettes were then placed through two changes of molten Paraplast paraffin embedding medium (McCormick Scientific, St Louis, MO, USA), heated to 60°C for two hours. Embedding moulds were filled with wax, placed on a cooling stage and brains were removed from their cassettes, one at a time, and placed in the mould with the anterior surface of the brain facing downwards. The cassette was placed on top of the mould and the wax was allowed to set and left on the cooling stage until completely hardened. The moulds were removed and brains were cut on a Leica RM2235 Rotary Microtome (Leica Microsystems, Wetzlar, Germany) to produce 10 µm coronal sections, which were placed on water heated to 50°C and floated onto SuperFrost®Plus electrostatically charged slides (Menzel-Gleser, Braunschweig, Germany) and dried at 37°C overnight.

2.2.10.3 General immunohistochemistry protocol

Immunohistochemistry was performed for IBA-1-positive microglia (Abcam, Cambridge, UK), COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-1 β (Peprotech, London, UK), TNF- α (R&D systems, Minneapolis, MN, USA), IRF3 (Invitrogen, Carlsbad, CA, USA), NF κ B (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cleaved caspase-3 (Millipore, Temecula, CA, USA), TUNEL (Promega, Southampton, UK), PrP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), NeuN (Millipore, Temecula, CA,

USA) and ChAT (Millipore, Temecula, CA, USA). Tissue sections were dewaxed in xylene for 15 minutes and passed to two separate vessels of HistoClear II for 10 minutes each to ensure further clearance of wax from the tissue. Sections were then rehydrated through a series of ethanols of decreasing concentration: 100% II, 100% I, 95%, 85%, 70%. Following rehydration, non-specific endogenous peroxidase activity was eliminated (quenched) by incubating sections in 1% v/v hydrogen peroxide in methanol (1 ml H₂O₂ per 100ml methanol). For some immunohistochemical reactions, antigen retrieval was performed by incubation in citrate buffer, pH 6, and microwaving twice for 5 minutes followed by 5 minutes of cooling after incubation (this ensures a temperature of >80°C for a period of 15 minutes). Slides were cooled to room temperature by gently bubbling through cold tap water. Sections were washed three times in PBS for 5 minutes, encircled with a ring of wax using a liquid blocking PAP pen (Sigma, Poole, UK), and the appropriate blocking serum applied (40 µl) and incubated at room temperature for 30 minutes. Serum was tapped off and sections were incubated in 30 µl of primary antibody. Primary antibodies were prepared in PBS unless otherwise stated. Following primary antibody incubation, sections were washed three times in PBS for 5 minutes and incubated with secondary antibody. All biotinylated secondary antibodies were supplied by Vector Laboratories (Peterborough, UK). Three 5 minute washes with PBS followed and staining was then visualized using the ABC method using peroxidase as enzyme, 0.05% v/v hydrogen peroxide as substrate, and diaminobenzidine (DAB) as chromogen. Sections were incubated in ABC solution (1 drop reagent A and 1 drop reagent B added to 5 ml PBS as per the manufacturers instruction; Vector Laboratories, Peterborough, UK) for 45 minutes followed by three washes in PBS, and finally developed in DAB solution (250 ml 0.1 M phosphate buffer, 500 µl DAB, 125 µl hydrogen peroxide). All sections (except those labelled for NFκB, PrP^{Sc}, NeuN and ChAT) were counterstained with haematoxylin for 20 – 60 seconds depending on intensity desired, acidified in 1% acid alcohol (1% v/v hydrochloric acid in 70% ethanol) for 5 seconds, and differentiated in Scott's tap water (500 ml distilled water, 15 g magnesium sulfate, 1 g potassium bicarbonate). Sections were dehydrated through a series of ethanols of increasing concentration for 5 minutes each (70%, 80%, 95%, 100% I, 100% II), followed by two changes of HistoClear II for 10 minutes each, and a final incubation in xylene for 15 minutes. Slides were finally mounted in DPX (Sigma, Poole, UK) and coverslips applied.

2.2.10.4 IBA-1

Sections for the microglial marker IBA-1 were quenched for 15 minutes, incubated in citrate buffer as above, blocked with 10% normal rabbit serum in PBS for 30 minutes before overnight incubation at 4°C with the goat antibody against murine IBA-1 at 1:200. Sections were washed and incubated in biotinylated rabbit-anti-goat secondary at 1:100 and continued as described above.

2.2.10.5 COX-2

Sections for cyclooxygenase-2 staining were quenched for 20 minutes, incubated in citrate buffer as above and blocked with 10% rabbit serum in PBS for 2 hours. The goat antibody against murine COX-2 was then diluted 1:1000 and incubated overnight at room temperature. Sections were washed and incubated in biotinylated goat-anti-rabbit secondary at 1:200 and continued as above.

2.2.10.6 IL-1 β

Sections for IL-1 β were quenched for 10 minutes, incubated in citrate buffer as above and blocked with 20% goat serum in PBS for 30 minutes. The primary antibody (rabbit anti-murine IL-1 β) was then applied at 1:50 and incubated overnight at 4°C. Sections were washed and incubated in biotinylated goat-anti-rabbit secondary at 1:200 and continued as above.

2.2.10.7 IRF-3

Sections labelled for interferon regulatory factor-3 were quenched for 10 minutes, incubated in citrate, blocked with 10% normal goat serum in PBS for 45 minutes, before overnight incubation at 4°C with the rabbit antibody against IRF3 at 1:200. Sections were washed and incubated in biotinylated goat-anti-rabbit secondary at 1:200 and continued as above. To ensure the nuclear localisation of IRF3 was not obscured, a weak counterstain was performed (10 seconds in haematoxylin).

2.2.10.8 TNF- α

Sections labelled for TNF- α were quenched for 10 minutes, incubated in citrate, blocked with 10% normal rabbit serum in PBS for 45 minutes, before overnight incubation at 4°C with the goat antibody against murine TNF- α (capture antibody from RnD systems ELISA duo set) at 1:1000. Sections were washed and incubated in biotinylated rabbit-anti-goat secondary at 1:200 and continued as above.

2.2.10.9 NFκB

Sections labelled for NFκB were quenched for 10 minutes, incubated in citrate buffer as above. Prior to blocking, sections were incubated in 1% Triton-X for 15 minutes to permeabilise the nuclear membrane. This was followed by a very brief wash in PBS (5 seconds), blocking with 10% horse serum in PBS for 45 minutes and overnight incubation at 4°C with the mouse antibody against human NFκB at 1:1000. Sections were washed and incubated in biotinylated horse-anti-mouse secondary at 1:100 and continued as above. No counterstain was performed so as not to obscure nuclear translocation.

2.2.10.10 Activated caspase-3

Sections were labelled for activated caspase-3 to identify cells undergoing the early stages of apoptosis. Sections were quenched for 10 minutes, incubated in citrate buffer, blocked with 10% normal goat serum for 30 minutes before overnight incubation at 4°C with the rabbit antibody against activated caspase-3 at 1:50. Sections were washed and incubated in biotinylated goat-anti-rabbit secondary at 1:100 and continued as above.

2.2.10.11 Choline acetyltransferase

Sections for ChAT were quenched for 20 minutes, incubated in citrate buffer and blocked with 10% normal rabbit serum for 30 minutes. The primary antibody (rabbit anti-human ChAT) was then applied at 1:500 and incubated overnight at 4°C. Sections were washed and incubated in biotinylated goat-anti-rabbit secondary at 1:200 and continued as above, with the exception that no counterstain was applied following this immunohistochemical reaction. For ChAT fibre labelling in the hippocampus, ammonium nickel sulfate (0.06% w/v) was added to DAB solution intensify immunolabelling.

2.2.10.12 PrP

Sections labelled for proteinase K-resistant prion protein (PrP^{Sc}) were autoclaved in distilled water for 20 minutes at 121°C. After PBS wash, sections were incubated in 90% formic acid for 5 minutes followed by quenching for 10 minutes as standard. No citrate antigen retrieval step was performed here. Following a PBS wash, sections were incubated with 10 µg/ml proteinase K solution for 30 minutes. Following a brief wash, Sections were blocked with 10% horse serum in PBS for 45 minutes and the primary antibody (mouse anti-PrP) was then applied at 1:100 and incubated overnight at room temperature. Sections were washed and incubated in biotinylated horse-anti-mouse secondary at 1:200 and

continued as above. No counterstain was applied following this immunohistochemical reaction.

2.2.10.13 NeuN

Sections labelled for NeuN were quenched for 10 minutes, , incubated in citrate buffer and blocked with 10% normal horse serum for 30 minutes. The primary antibody (mouse anti-NeuN) was then applied at 1:5000 and incubated overnight at 4°C. Sections were washed and incubated in biotinylated horse-anti-mouse secondary at 1:200 and continued as above. No counterstain was applied following this immunohistochemical reaction.

2.2.10.14 TUNEL

Staining for apoptotic cells was carried out using the DeadEnd™ Colorimetric TUNEL System (Promega, Peterborough, UK). Sections were quenched for 10 minutes followed by a single 5 minute wash in 0.85% w/v sodium chloride in distilled water and two 5 minute washes in PBS. Sections were incubated with 10 µg/ml proteinase K solution for 10 minutes at room temperature followed by three 5 minute washes in PBS. Equilibration of sections was carried out at 37°C for 10 minutes by applying 30 µl kit equilibration buffer. Sections were then incubated in 25 µl TUNEL buffer (24.5 µl equilibration buffer, 0.25 µl nucleotide mix, 0.375 µl rTdT enzyme) for 2 hours at 37°C. The reaction was stopped by incubation in 2X SSC solution for 15 minutes at room temperature (50 µl per section). Sections were washed three times in PBS and streptavidin HRP (1:500 in PBS) was applied and incubation carried out for 30 minutes at room temperature. Following a final three PBS washes, the standard DAB reaction was carried out as above followed by a brief counterstain.

2.2.11 Acetylcholinesterase histochemistry

2.2.11.1 Tissue preparation and embedding

Animals were terminally anaesthetized with sodium pentobarbital (40 mg mouse i.p., Euthatal, Merial Animal Health, Essex, UK) and transcardially perfused with heparinised saline as for quantitative PCR. Brains were dissected out and embedded in Tissue-Tek® O.C.T. (Sakura Finetek, Tokyo, Japan) in small foil containers. These were then frozen gently in isopentane suspended over liquid nitrogen and finally stored at – 20°C until ready for sectioning.

2.2.11.2 Slide coating for fresh frozen tissue

Super Premium microscope slides (Menzel-Gleser, Braunschweig, Germany) used for fresh frozen tissue were coated with 3-aminopropyltriethoxysilane (APS, Sigma, Poole, UK) to ensure proper adherence of sections to the slide. Slides were washed in 10% v/v Decon detergent in distilled water for 30 to 60 minutes, followed by a rinse under cold tap water for several minutes. Slides were rinsed twice in distilled water and then dried in an oven at 60°C overnight. Slides were coated with APS by immersion in a 2% v/v solution of APS in methanol for 10 to 15 seconds. Finally, slides were rinsed in methanol followed by distilled water and dried overnight at 37°C.

2.2.11.3 Sectioning of fresh frozen tissue

Coronal sections (20 µm) of fresh frozen tissue were cut on a Leica CM1850 Cryostat (Leica Microsystems, Wetzlar, Germany). Sections were collected onto APS-coated glass slides and allowed to air-dry overnight before being stored at – 20°C until histochemical processing.

2.2.11.4 Acetylcholinesterase histochemistry

Acetylcholinesterase (AChE) histochemistry was carried out at the level of the hippocampus using the classical thiocholine method for cholinesterases (Karnovsky and Roots, 1964, Paxinos and Watson, 1998). Sections were removed from – 20°C and allowed to dry at 37°C for 20 minutes. A stock solution (pH 5) containing 50 mM sodium acetate, 4 mM copper sulfate and 16 mM glycine was prepared. For every 100 ml of stock solution, 116 mg S-acetylthiocholine iodide and 3 mg ethopropazine were added to produce the incubation solution. Sections were incubated in this solution overnight at room temperature. Following incubation, sections were rinsed in distilled water and placed in 1% w/v sodium sulfide solution, pH 7.5, for 10 minutes to develop the histochemical reaction. Sections were rinsed in distilled water and then fixed overnight in 10% formalin (Sigma, Poole, UK). Slides were removed, allowed to dry, and then dehydrated in 100% ethanol for 5 minutes followed by xylene for 15 minutes. Slides were mounted in DPX (Sigma, Poole, UK) and coverslips applied.

2.2.12 Neuropathological quantification

Cell counting was performed for IL-1β, TUNEL, IBA-1 and ChAT, while density measurements were conducted for AChE histochemistry. In the study of viral mimetics during neurodegenerative disease, IL-1β- and TUNEL-positive cells were identified and

counted throughout the hippocampus and thalamus of all animal groups. For IBA-1, a 0.62 x 0.47 mm section of the centre of the dorsal hippocampus (containing the CA1 pyramidal layer and stratum oriens and radiatum, but not the dentate gyrus granule cell layer) was photographed and used for microglial counts in all animal groups. Positively labelled cells were identified and counted using the “analyse particles” function in Image J software (rsbweb.nih.gov). Colour photomicrographs were converted to black and white 8-bit images and then threshold and circularity parameters were set to ensure that all cells being counted were microglia. Counting was performed for two coronal sections per animal.

ChAT-positive cells were identified and counted using Image J, using the same function as for IBA-1. Cells were counted at three pre-defined areas moving rostral-caudally through the medial septum of all animal groups (anteroposterior: +1.10-1.05, 1.00-0.95, 0.90-0.85 mm from Bregma). AChE density measurements were also assessed in all animal groups using Image J software. Images were converted to 8-bit and the entire hippocampus area selected. Density was calculated using the “measure” function of Image J.

2.2.13 Statistics

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 $\pm$ SEM.

2.2.13.1 Chapter 3

For cognitive testing, 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. For histochemical quantification of ChAT cells, AChE density and NeuN-positive cell numbers, unpaired Student's *t*-test was used. Linear regression analysis used to identify if there was a correlation between cholinergic cell density and behavioural performance. Taqman qPCR data for the acute inflammatory response to cholinergic lesion were analysed by one-way ANOVA. Neurological scores were analysed by one-way ANOVA followed by Bonferroni post-hoc tests where significant difference was found. ELISA and Taqman qPCR data were analysed by two-way ANOVA using lesion (PBS vs mu-p75-sap) and treatment (saline vs LPS) as factors. Where a significant difference was found, Bonferroni post-hoc tests were performed.

2.2.13.2 Chapter 4

For ELISA and Taqman qPCR data, either one-way or two-way ANOVA analyses were performed. In the case of two way ANOVA analysis, disease state (NBH vs ME7) and treatment (saline vs poly I:C) were the factors examined. Bonferroni post-hoc tests were performed following a significant ANOVA. Immunohistochemical cell counting was analysed by one-way ANOVA, which if significant was followed by Bonferroni post-hoc tests. For core body temperature and neurological testing, two-way repeated measures ANOVA was used to analyse effects of disease and treatment over a number of time-points. Where significant differences were found, Bonferroni post-hoc analyses were performed.

2.2.13.3 Chapter 5

Longitudinal assessment of disease progression (food burrows, open field, horizontal bar, and inverted screen) was analysed by two-way repeated measures ANOVA with mouse strain (wild-type versus either IFNAR1^{-/-} or IL-1R1^{-/-}) as the between subjects factor and time (weeks post-inoculation) as the within subjects factor. Bonferroni post-hoc tests were performed following a significant ANOVA. Survival time (days) of animals post-inoculation with ME7 was assessed by Kaplan-Meier log rank survival analysis. Taqman qPCR for the type I IFN response in ME7 prion disease was analysed by Student's *t* test. Peripheral ELISA and Taqman qPCR data comparing the inflammatory response to poly I:C in wild-type versus IFNAR1^{-/-} mice in ME7 prion disease was analysed by three-way ANOVA. Factors used were mouse strain (wild-type versus IFNAR1^{-/-}), disease state (NBH versus ME7) and systemic challenge (saline versus poly I:C).

Chapter 3

Systemic inflammation and cholinergic hypofunction –
reconciling cholinergic and neuroinflammatory hypotheses
of delirium

3.1 Introduction

Delirium is a profound and highly prevalent neuropsychiatric syndrome of acute onset that is characterised by disturbances in attention, memory, orientation and perception (Meagher et al., 2007). The major risk factors for delirium are aging and dementia (Inouye, 2006) and it is now clear that episodes of delirium are predictive of poor outcomes such as long term cognitive decline (MacLulich et al., 2009), dementia (Fong et al., 2009a), institutionalization and death (Rockwood et al., 1999, Witlox et al., 2010). Despite this considerable medical and economic burden (Leslie and Inouye, 2011), there has been limited progress on development of therapeutic interventions for vulnerable populations (Skrobik, 2010), and the pathophysiology of delirium remains poorly understood (MacLulich and Hall, 2011). The lack of biologically relevant animal models with which to study delirium has certainly contributed to this, and has hampered progress in investigating the mechanisms that underlie this condition.

The neuroinflammatory hypothesis of delirium has been proposed, whereby peripheral inflammation induces an inflammatory response in the brain (Cerejeira et al., 2010). This has largely been based on observations that delirium may be triggered by systemic sterile inflammation, such as surgery or tissue trauma (Kat et al., 2008), or by peripheral infections (George et al., 1997, Elie et al., 1998). While it appears that severe sepsis can induce delirium in almost any individual, much milder inflammation can induce episodes in vulnerable populations such as the elderly and demented (Fong et al., 2009b). Our group has recently described the first animal model of delirium during dementia, superimposing LPS-induced systemic inflammation upon the ME7 model of murine prion disease (Murray et al., 2012). The ME7 model is characterised by robust synaptic and neuronal loss, extracellular amyloidosis and microglial activation, alongside characteristic progressive neurological and cognitive symptoms. This prior pathology increases the vulnerability of mice to show acute working memory deficits upon systemic challenge with bacterial endotoxin (lipopolysaccharide, LPS; Murray et al., 2012). Since the nature of these deficits are acute and transient, with impairments in attention, recall and short-term/working memory, this model displays key core features of delirium as defined by DSM-IV and ICD-10 classifications (WHO, 1992, APA, 1994) and will be useful in delineating mechanisms.

Additionally, there is clinical evidence that medications interfering with actions of the neurotransmitter acetylcholine can induce delirium (Tune et al., 1981, Han et al., 2001).

An early animal model used the muscarinic cholinergic antagonist atropine to induce cognitive deficits and EEG changes consistent with those observed in delirious patients (Trzepacz et al., 1992, Leavitt et al., 1994). Cholinergic hypofunction is also a cornerstone of the “final common pathway” hypothesis of delirium, which suggests that delirium resulting from a variety of aetiologies is caused by a common underlying mechanism (Trzepacz, 2000). Thus these factors, along with the fact that acetylcholine is known to play a key role in cognition and attention (Himmelheber et al., 2000), has led to the cholinergic deficiency hypothesis of delirium (Hsieh et al., 2008). The basal forebrain cholinergic system shows significant degeneration in AD and dementia (Davies and Maloney, 1976, Whitehouse et al., 1982) and these patient groups are among the most susceptible to delirium. It is therefore important to investigate whether the cognitive dysfunction apparent during delirium could be explained by decreased cholinergic function in an aetiological context other than simple interruption of cholinergic function using pharmacological antagonism. One possibility is that progression of dementia leads to an ongoing state of hypocholinergia, which leaves the brain vulnerable to subsequent insults such as systemic infection/inflammation.

The current study hypothesised that chronic basal forebrain cholinergic hypofunction would increase the susceptibility of mice to acute cognitive deficits induced by systemic inflammation. This study aimed to produce and characterise a limited selective lesion of the basal forebrain cholinergic system in mice, using the murine-p75-saporin (mu-p75-sap) immunotoxin. This consists of the ribosomal toxin saporin, bound to an antibody directed against the p75 neurotrophin receptor (Berger-Sweeney et al., 2001, Moreau et al., 2008) which is enriched on cholinergic neurons of the basal forebrain. Before this model system could be used to address the hypothesis above, characterisation of neuronal loss, acute inflammatory responses and cognitive consequences of the primary lesion (basal forebrain cholinergic neurodegeneration) had to be first examined. Thereafter, lesioned and control animals, were challenged systemically with LPS (100 µg/kg) and assessed for their differential susceptibility to acute working memory deficits. Furthermore, we hypothesized that the lesioned animals would show priming of the microglial population of the hippocampus due to the loss of cholinergic innervation, since microglia have been shown to express the $\alpha 7$ subunit of the nicotinic acetylcholine receptor (De Simone et al., 2005). This receptor is expressed on peripheral macrophages and exerts an anti-inflammatory role in sepsis (Wang et al., 2003) but direct evidence for muted microglial responses to systemic LPS has not been furnished.

Since acetylcholine can have both anti-inflammatory and neuromodulatory roles, we also assessed the cholinergic dependence of LPS-induced cognitive impairments by administration of the acetylcholinesterase inhibitor donepezil, 1 hour after systemic LPS challenge.

Thus, the current study addresses the interactions between systemic inflammation and prior hypocholinergia at multiple levels. The findings could potentially have important implications for our current understanding of delirium pathophysiology and also provide an important novel animal model with which to investigate the reconciliation of inflammatory and cholinergic hypotheses of delirium. The data also have implications for the recent clinical trial of the acetylcholinesterase inhibitor rivastigmine for treatment of delirium in the intensive care unit (van Eijk et al., 2010).

3.2 Results

3.2.1 Cognitive testing

The paddling Y-maze visuo-spatial task has been used to assess reference memory following lesion of the basal forebrain cholinergic system using the murine-p75-saporin immunotoxin (Figure 3.1). Since the initial aim of this study was to induce a cholinergic lesion that did not, of itself, impair cognition we assessed animals injected with 0.08 µg and 0.4 µg of mu-p75-sap on this task. PBS controls and mu-p75-sap-treated animals were tested in the Y-maze for two blocks of five trials. Two-way repeated-measures ANOVA revealed a main effect of treatment ($F = 3.147$, $df\ 2,76$; $p = 0.0486$) and Bonferroni post-hoc tests found that in the second block of five trials, the 0.4 µg mu-p75-sap group showed significantly fewer correct trials when compared to both PBS control animals and the lower dose 0.08 µg mu-p75-sap group. A subset of animals (PBS, $n = 14$; 0.08 µg, $n = 20$; 0.4 µg, $n = 10$) were tested for three blocks of 5 trials (15 trials in total) in the Y-maze. One-way-ANOVA analysis ($F = 0.5921$, $df\ 2,43$; $p = 0.5578$) revealed no difference for the final block of 5 trials, indicating that the higher dose mu-p75-sap-lesioned animals are able to learn the task but do so at a slower rate than the other groups.

Animals were also trained on the working memory T-maze alternation task to assess working memory performance in lesioned animals. Each experimental group was trained for 10 blocks of 10 trials (Figure 3.2). There was no effect of the cholinergic lesion on the ability of animals to learn this task, as determined by two-way repeated measures ANOVA ($F = 1.34$, $df\ 2,990$; $p = 0.2670$), although there was a trend towards a slower rate of learning in the higher dose (0.4 µg) mu-p75-sap animals.

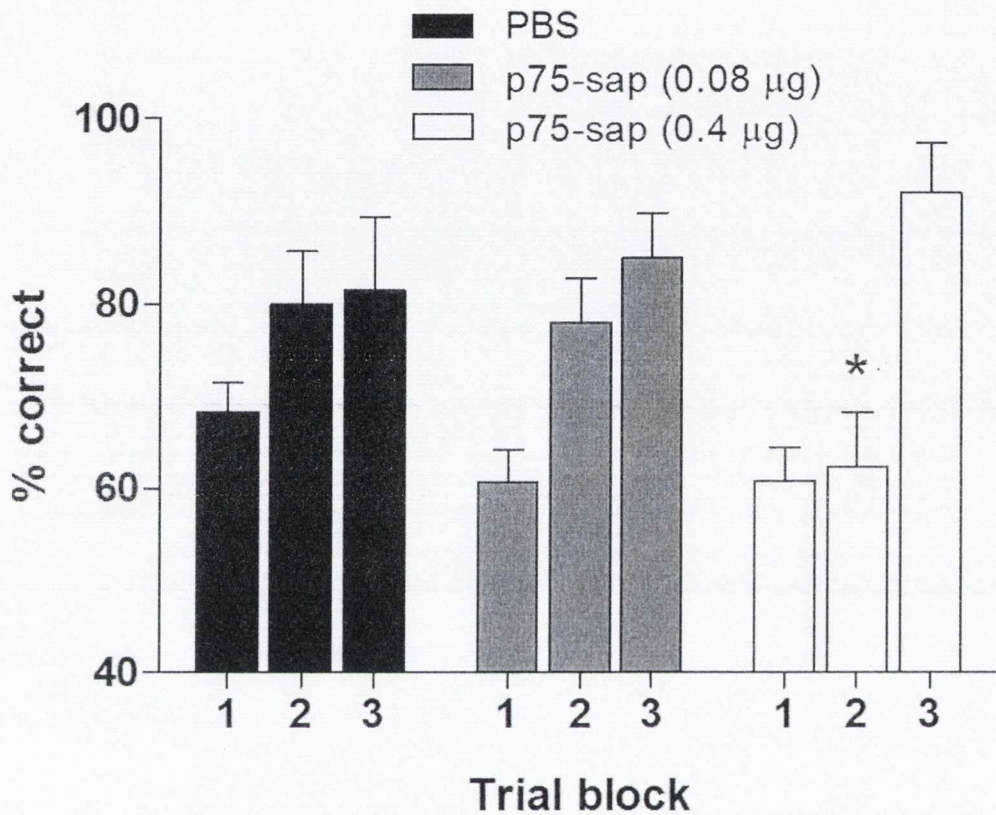


Figure 3.1 *Reference memory assessment following mu-p75-saporin lesions.* Visuo-spatial reference memory as assessed in the paddling Y-maze task (10 trials: PBS, n = 24; 0.08 µg, n = 30; 0.4 µg, n = 25; 15 trials: PBS, n = 14, 0.08 µg, n = 20; 0.4 µg, n = 10). Groups were compared by two-way repeated measures ANOVA with a main effect of treatment. Significant Bonferroni post-hoc differences between 0.4 µg mu-p75-sap and all other groups are denoted by * $p < 0.05$. Each trial block consists of 5 trials. Data are expressed as mean $\pm$ SEM.

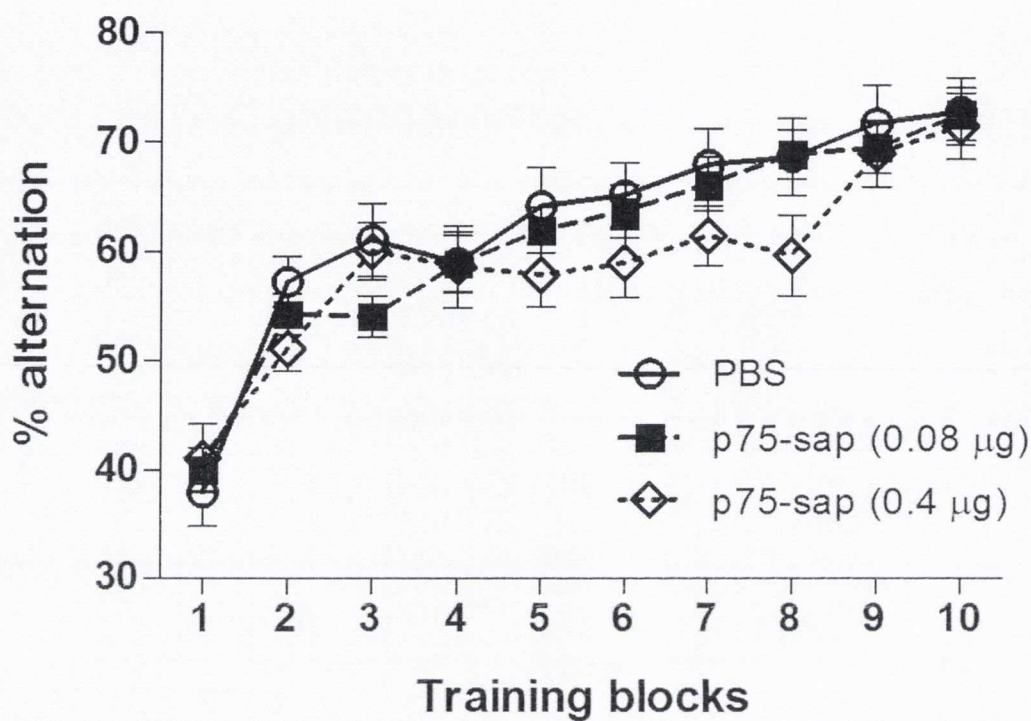


Figure 3.2 *Working memory performance following mu-p75-saporin lesion.* Working memory performance, assessed by the T-maze alternation task. Two-way repeated measures ANOVA revealed no significant differences between groups over 10 blocks of 10 trials. Data are expressed as mean $\pm$ SEM (PBS, $n = 29$; 0.08 μg , $n = 52$; 0.4 μg , $n = 32$).

3.2.2 Mu-p75-saporin lesions do not produce deficits in motor function

The horizontal bar and inverted screen tasks were utilised to assess motor co-ordination and muscular strength following a basal forebrain cholinergic lesion, since locomotor disturbances owing to cerebellar dysfunction have been reported with higher doses of the mu-p75-sap immunotoxin (Berger-Sweeney et al., 2001, Moreau et al., 2008). Mu-p75-sap, at either 0.08 μ g or 0.4 μ g, did not impair motor function on either the horizontal bar (Figure 3.3a) or inverted screen (Figure 3.3b), as analysed by one-way ANOVA (bar: $F = 0.7999$, $df\ 2,58$; $p = 0.4545$; screen: $F = 0.9741$, $df\ 2,58$; $p = 0.3838$).

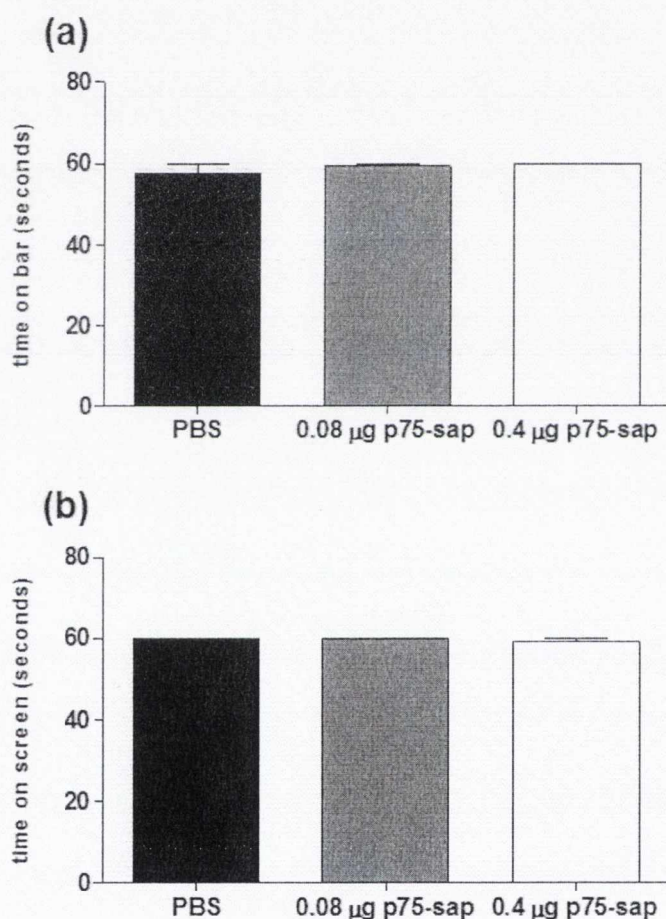


Figure 3.3 Motor co-ordination following mu-p75-sap lesion. PBS, 0.08 μ g and 0.4 μ g mu-p75-sap treated animals were assessed on the horizontal bar (a) and inverted screen (b) tasks for motor co-ordination and muscular strength 20 days following surgical lesion. One-way ANOVA revealed that neither dose of mu-p75-sap produced motor impairments in these tasks when compared to PBS controls. Data are expressed as mean $\pm$ SEM. (PBS, $n = 19$; 0.08 μ g, $n = 20$; 0.4 μ g, $n = 20$).

3.2.3 Neuropathology of cholinergic lesions

The survival of neurons positive for choline acetyltransferase (ChAT), the biosynthetic enzyme responsible for the final step in the synthesis of the neurotransmitter acetylcholine, was assessed. ChAT immunolabelling was conducted in the basal forebrain region encompassing the medial septum and ventral diagonal band in PBS, 0.08 μg and 0.4 μg mu-p75-sap-injected animals. Representative images are shown in Figure 3.4 (a) to (c). Depletion of ChAT-positive neurons in these regions rich in cholinergic neurons was evident in the two mu-p75-sap treatment groups, and this was clearly more severe in the 0.4 μg group.

The thiocholine method for the histochemical analysis of activity of the acetylcholine degradative enzyme acetylcholinesterase (AChE) was performed in the hippocampus, a major projection area from the basal forebrain cholinergic neurons (Mesulam et al., 1983). Representative images are shown in Figure 3.4 (d) to (f). Robust loss of AChE enzymatic activity is apparent in the hippocampus in each of the mu-p75-sap treatment groups, with more severe denervation at the 0.4 μg dose.

ChAT immunolabelling was also performed in the hippocampus of PBS and mu-p75-sap animals to highlight the degeneration of cholinergic terminal synaptic fields. Representative images are shown in Figure 3.5. A clear decrease in ChAT-positive synaptic terminals was observed in mu-p75-sap-lesioned animals compared to PBS-injected controls.

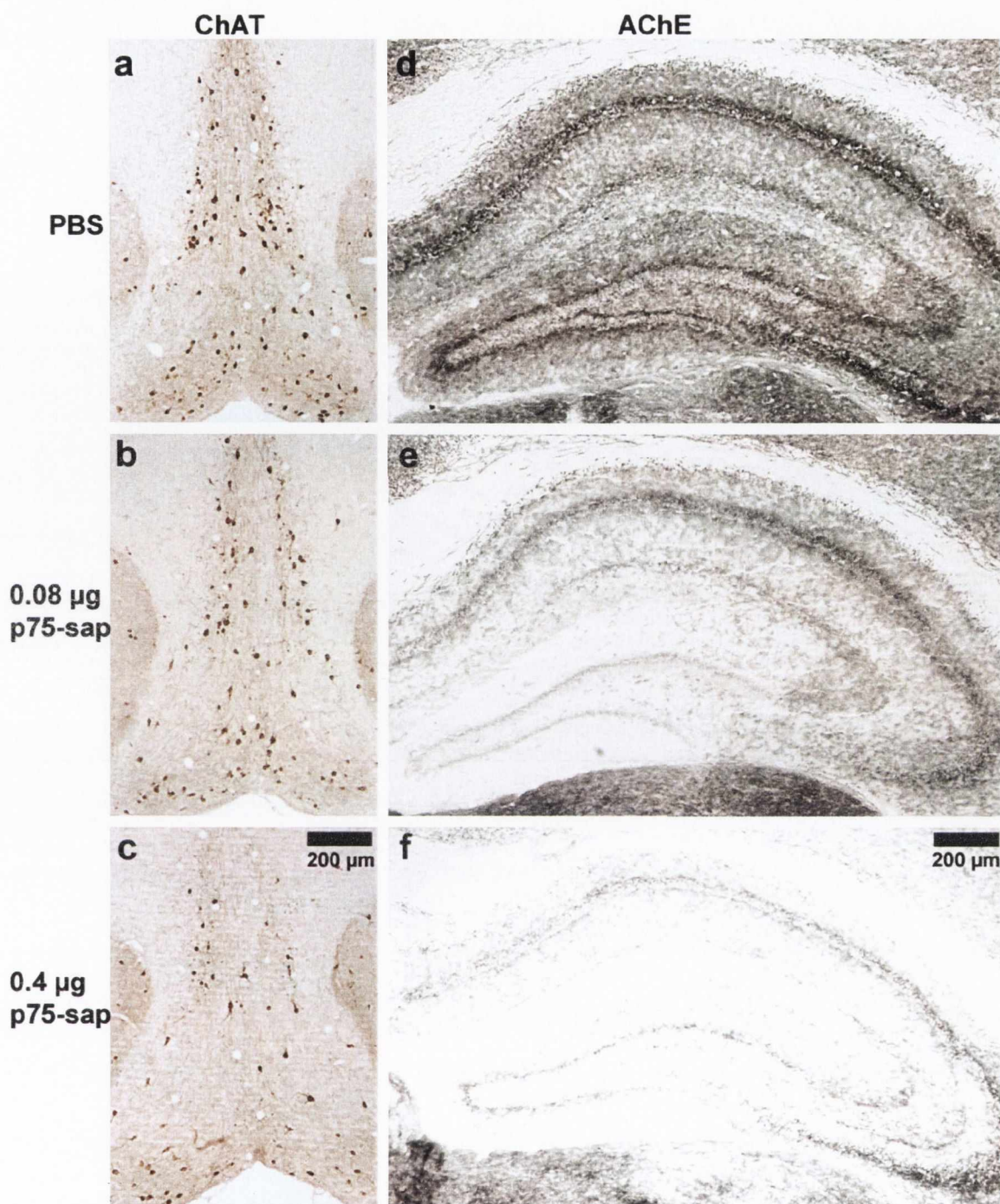


Figure 3.4 *Neuropathological consequences of saporin lesions.* (a-c) Representative photomicrographs of ChAT-positive neuronal depletion in the medial septum and ventral diagonal band regions of the basal forebrain in PBS (a), 0.08µg mu-p75-sap (b) and 0.4 µg mu-p75-sap (c). (d-h) Histochemical AChE enzyme activity using the thiocholine method shows clear reductions in the hippocampus of 0.08 µg mu-p75-sap-lesioned animals (e) and further reduction in 0.4 µg-treated animals (f) when compared to non-lesioned controls (d). Scale bar = 200 µm.

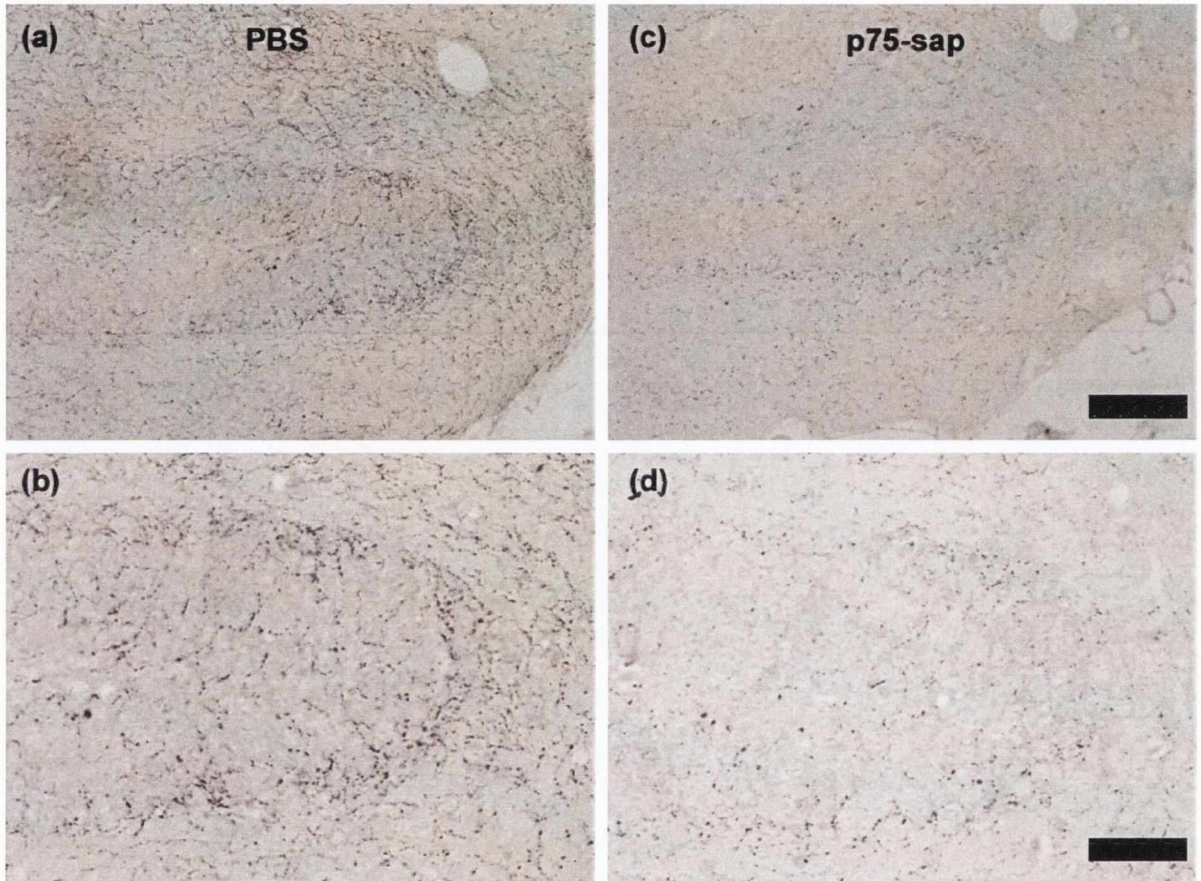


Figure 3.5 *Hippocampal cholinergic synaptic terminals in saporin lesions.* Representative photomicrographs of ChAT-positive synaptic terminal fields in the hippocampus of PBS and 0.4 μ g mu-p75-sap-lesioned animals. (a-b) ChAT fibres in PBS-injected animals at 20 and 40 X magnification. (c-d) Depletion of ChAT fibres in 0.4 μ g mu-p75-sap-lesioned animals compared to control animals at 20 and 40 X magnification. Scale bar = 100 μ m (a,c); 50 μ m (b,d).

3.2.4 Correlation of ChAT cell number with Y-maze performance

A subset of animals was assessed to investigate whether the number of neurons labelled positively for ChAT was correlated with the ability to perform the Y-maze visuo-spatial reference memory task. The animals assessed included PBS controls (n = 4) and mu-p75-sap animals at both doses (0.08 µg, n = 5; 0.4 µg, n = 9). ChAT counts were performed in the medial septum and ventral diagonal band, moving rostro-caudally towards Bregma from 0.85 mm to 1.1 mm. Linear regression analysis indicated a significant correlation between remaining cholinergic cell density and Y-maze performance during trial block two (Figure 3.6; $r^2 = 0.361$, $p = 0.0084$). Since our hypothesis was based upon limited cholinergic deficiency that, of itself, did not produce cognitive impairments, the lower dose of mu-p75-sap (0.08 µg) was used for all further cognitive experiments.

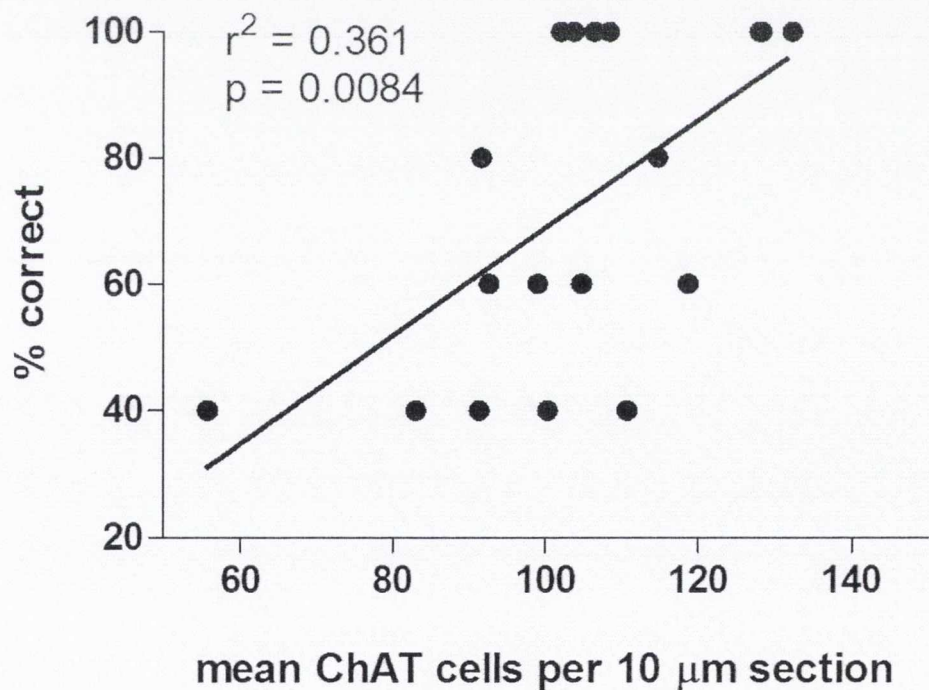


Figure 3.6 *ChAT-positive cell number positively correlates with Y-maze performance.* Linear regression analysis revealed a positive correlation between mean ChAT-positive cell numbers in the medial septum (0.85-1.1 mm from Bregma) and performance on the Y-maze reference memory task in trial block two ($r^2 = 0.361$, $p = 0.0084$).

3.2.5 Quantification of basal forebrain cholinergic degeneration

Three methods of quantification have been employed to quantify the basal forebrain cholinergic depletion following mu-p75-sap lesion. ChAT-positive neurons were quantified in three areas of basal forebrain so as to encompass the central regions of the medial septum/diagonal band, moving rostro-caudally towards Bregma (Figure 3.7a). Significant ChAT depletion was found in mu-p75-sap-lesioned mice compared to PBS controls. Repeated measures ANOVA revealed a main effect of lesion ($F = 7.61$, df 1, 34, $p = 0.0134$) and of rostro-caudal location ($F = 65.56$, df 2, 34, $p < 0.0001$), and Bonferroni post-hoc tests showed significant differences between PBS and mu-p75-sap at 1.0-0.95 and 0.9-0.85 mm from Bregma ($p < 0.05$).

Histochemical AChE enzyme activity was decreased in the hippocampus of mu-p75-sap-lesioned animals (0.08 μ g) (Figure 3.7b). Two-tailed t-test revealed significantly less AChE activity in lesioned animals compared to PBS-injected controls ($p < 0.01$). Four mu-p75-sap-injected animals showed limited AChE reduction and these were subsequently excluded from behavioural analysis.

ChAT mRNA expression was also assessed by quantitative PCR in the medial septum/diagonal band in each treatment group (Figure 3.7c). Two-tailed t-test revealed significantly less expression of ChAT mRNA in mu-p75-sap-lesioned animals compared to PBS controls ($p < 0.05$).

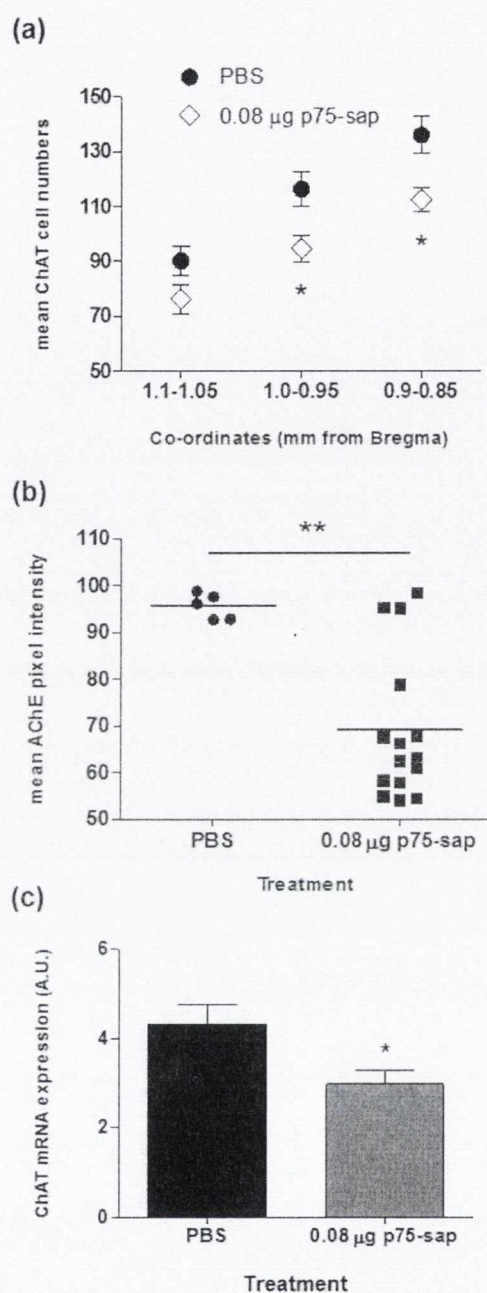


Figure 3.7 Cholinergic depletion following *mu*-p75-sap lesion. (a) Mean ChAT-positive cell numbers in three regions spanning the medial septum and diagonal band showed significant depletion in 0.08 µg *mu*-p75-sap ($n = 13$) animals relative to PBS controls ($n = 6$) 50 days post-lesion. Bonferroni post-hoc tests after significant main effects by two-way repeated measures ANOVA are denoted by $*p < 0.05$. Data are expressed as mean $\pm$ SEM. (b) Histochemical AChE enzyme activity in the hippocampus is significantly reduced in 0.08 µg *mu*-p75-sap ($n = 15$) animals relative to PBS controls ($n=5$). Significant difference by Student's *t*-test denoted by $**p < 0.01$. (c) ChAT mRNA expression in the medial septum is significantly reduced in 0.08 µg *mu*-p75-sap ($n = 10$) animals relative to PBS controls ($n=9$). Significant difference by Student's *t*-test denoted $*p < 0.05$. Data are expressed as mean $\pm$ SEM.

3.2.6 Do mu-p75-sap lesions also produce non-specific neuronal loss?

Other groups have reported some non-specific neuronal cell loss when using higher doses of the mu-p75-sap immunotoxin (Berger-Sweeney et al., 2001). A subset of animals was therefore assessed to determine if there was significant non-selective neuronal loss in the current study. Immunolabelling for the neuronal nuclei marker, NeuN, was conducted in PBS ($n = 3$) and 0.08 μg mu-p75-sap-injected ($n = 8$) animals. Representative photomicrographs are shown for PBS and mu-p75-sap animals in Figure 3.8 (a) and (b).

NeuN-positive cells were counted in the intermediate lateral septum, a region which is not populated by cholinergic neurons and is proximal to the lateral ventricles into which the immunotoxin is injected. Cells were counted in a standardised area of 0.48 mm^2 (Figure 3.8c). Student's *t*-test revealed that there was no difference in NeuN-positive cell numbers between PBS controls and 0.08 μg mu-p75-sap-injected animals.

Thus, there is no detectable non-selective neuronal loss following the 0.08 μg dose of mu-p75-sap immunotoxin used throughout the current study. This toxin is therefore specifically targeting p75 neurotrophin receptor-enriched cholinergic neurons.

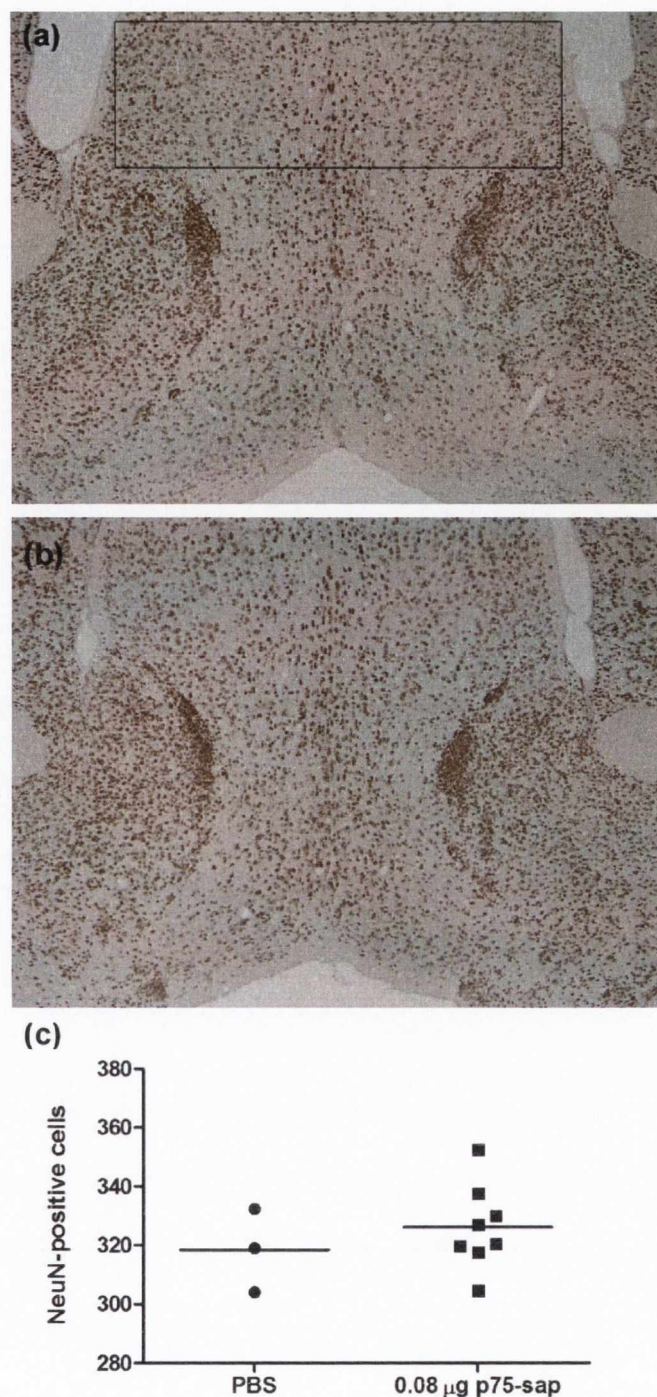


Figure 3.8 *NeuN-positive cell numbers following mu-p75-sap lesion.* Immunolabelling for the neuronal nuclei marker NeuN was performed to assess non-selective cell loss in an area proximal to the circulating cholinergic toxin – intermediate lateral septum. Representative photomicrographs of (a) PBS- and (b) 0.08 µg p75-sap-injected animals. The black rectangle represents an area of 0.48 mm² where cell counts were performed in each animal. (c) NeuN-positive neuronal cell numbers in PBS and p75-sap-injected animals. No differences in cell numbers were detected by Students *t*-test (PBS *n* = 3; p75-sap *n* = 8).

3.2.7 Acute inflammation following cholinergic lesion.

The acute inflammatory response to cholinergic neuronal death was assessed by qPCR analysis of inflammatory gene transcripts in the medial septum of animals injected with 0.4 µg mu-p75-sap. In order to identify whether acute inflammation was still present at the time that cognitive testing was conducted, a number of time-points following lesion (3, 7, 14, 28 and 35 days) were assessed to provide temporal information on the acute response and its eventual resolution. PBS animals were assessed at 3, 7 and 28 days but since inflammatory gene expression in each of these groups remained at basal levels, these three time-points have been combined for the analysis.

3.2.7.1 Classical pro-inflammatory cytokines

TNF- α expression increased acutely following cholinergic lesion, peaking at 7 days post-insult and showing resolution by 14 days (Figure 3.9a). One way ANOVA analysis ($F = 6.68$, $df\ 5,33$; $p = 0.0002$) followed by selected Bonferroni post-hoc comparisons revealed that TNF- α expression was significantly elevated at 7 days post-mu-p75-sap-injection compared to PBS controls ($p < 0.001$). Expression of TNF- α was not significantly different to PBS at 3, 14, 28 or 35 days post-lesion ($p > 0.05$).

Medial septal expression of IL-1 β was induced at 3 and 7 days following lesion with resolution evident at 14, 28 and 35 days (Figure 3.9b). One way ANOVA analysis ($F = 6.28$, $df\ 5,33$; $p = 0.0003$) followed by selected Bonferroni post-hoc comparisons revealed that IL-1 β expression was significantly elevated at 3 and 7 days post-mu-p75-sap-injection compared to PBS controls ($p < 0.01$). IL-1 β expression was not significantly different to PBS at 14, 28 or 35 days post-lesion ($p > 0.05$).

Mu-p75-sap-lesioned animals showed acute induction of IL-6 following lesion which peaked at 7 days and had resolved by 14 days (Figure 3.9c). One way ANOVA analysis ($F = 13.04$, $df\ 5,31$; $p < 0.0001$) followed by selected Bonferroni post-hoc comparisons revealed that IL-6 expression was significantly induced at 7 days post-mu-p75-sap-injection compared to PBS controls ($p < 0.001$). There was no significant difference in IL-6 expression in mu-p75-sap-injected animals compared to PBS at 3, 14, 28 or 35 days post-insult ($p > 0.05$).

CXCL1 expression was acutely induced by mu-75-sap lesion at 3 and 7 days followed with resolution by 14 days post-lesion (Figure 3.9d). One way ANOVA analysis ($F = 17.67$, $df = 5,33$; $p < 0.0001$) followed by selected Bonferroni post-hoc comparisons revealed that CXCL1 expression was significantly elevated at 3 and 7 days post-mu-p75-sap-injection compared to PBS controls ($p < 0.05$). Expression was not significantly different to PBS at 14, 28 and 35 days post-lesion ($p > 0.05$).

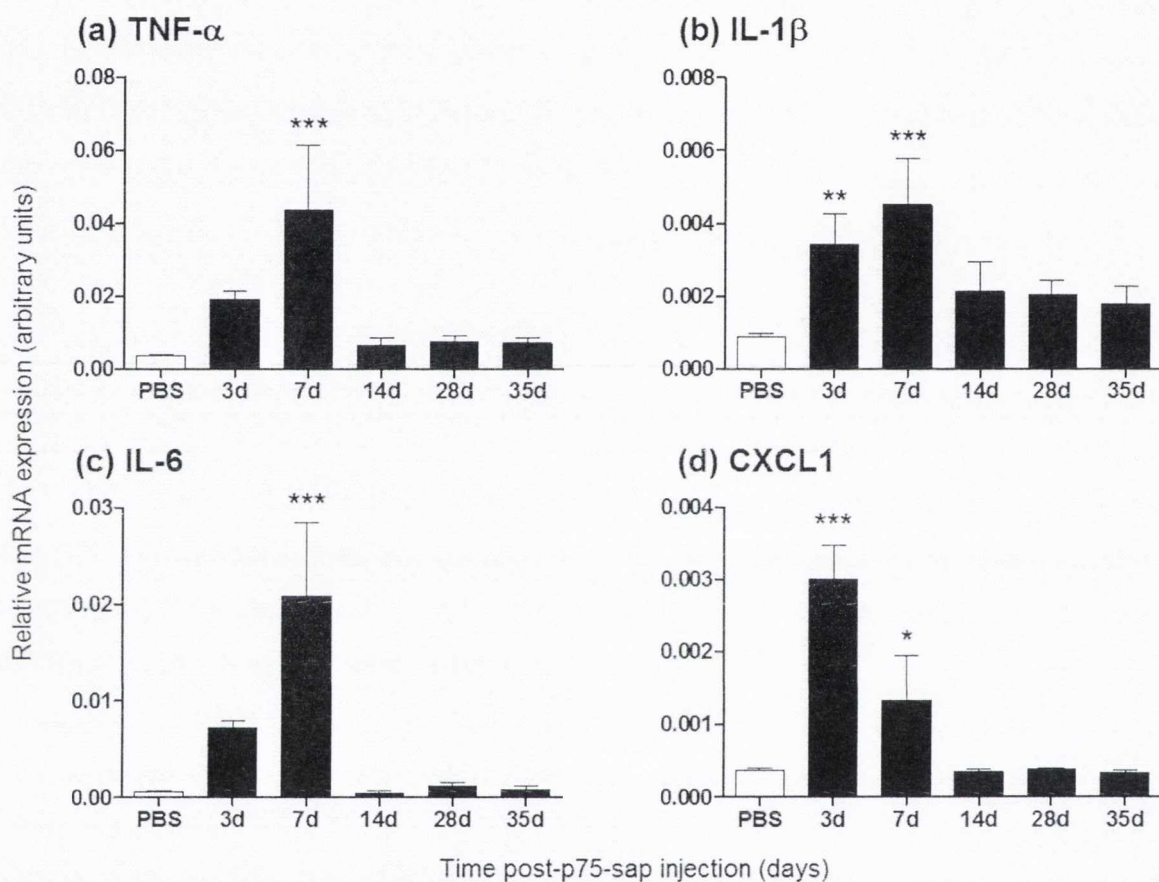


Figure 3.9 *Temporal inflammatory profile following mu-p75-sap lesion.* Medial septum mRNA expression of (a) TNF- α , (b) IL-1 β , (c) IL-6 and (d) CXCL1 was assessed by qPCR at a number of time-points following mu-p75-sap (0.4 μ g) injection. PBS temporal data was combined as all transcripts remained at basal levels throughout. Significant differences by Bonferroni post-hoc tests following one-way ANOVA analysis are denoted * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Data are expressed as mean $\pm$ SEM ($n = 15$ for PBS; $n \geq 4$ for p75-sap).

3.2.7.2 Type I IFN response

The type I IFN response, typically activated in response to viral infection, was also examined for acute activation in the medial septum following mu-p75-sap lesion. IFN- β expression increased acutely following cholinergic lesion, peaking at 7 days post-insult and showing resolution by 28 days (Figure 3.10a). One way ANOVA analysis ($F = 6.39$, df 5,32; $p = 0.0003$) followed by selected Bonferroni post-hoc comparisons revealed that IFN- β expression was significantly elevated at 3 and 7 days post-mu-p75-sap-injection compared to PBS controls ($p < 0.05$). Expression of IFN- β was not significantly different to PBS at 14, 28 or 35 days post-lesion ($p > 0.05$).

The transcription of IFN-dependent genes, IRF7, Mx1 and PKR was examined to determine whether IFN- β was being secreted and active in the brain acutely following cholinergic lesion. Expression of IRF7 was induced by mu-p75-sap injection and peaked at 7 days post-insult with resolution to baseline by 14 days (Figure 3.10b). One way ANOVA analysis ($F = 13.42$, df 5,33; $p < 0.0001$) followed by selected Bonferroni post-hoc comparisons revealed that IRF7 expression was significantly elevated at 7 days post-mu-p75-sap-injection compared to PBS controls ($p < 0.001$). Expression of IRF7 was not significantly different to PBS at 3, 14, 28 or 35 days post-lesion ($p > 0.05$).

Mx1 transcription in the medial septum was induced at 7 days post cholinergic lesion with resolution apparent by 14 days (Figure 3.10c). One way ANOVA analysis ($F = 8.59$, df 5,33; $p < 0.0001$) followed by selected Bonferroni post-hoc comparisons revealed that Mx1 was significantly induced by mu-p75-sap at 7 days post-injection compared to PBS controls ($p < 0.001$). Expression of Mx1 was not significantly different to PBS at 3, 14, 28 or 35 days post-lesion ($p > 0.05$).

Mu-p75-sap-lesioned animals showed acute induction of PKR in the medial septum at 7 days post-insult with resolution by 14 days (Figure 3.10d). One way ANOVA analysis ($F = 9.35$, df 5,33; $p < 0.0001$) followed by selected Bonferroni post-hoc comparisons revealed that PKR was significantly induced by mu-p75-sap at 7 days post-injection compared to PBS controls ($p < 0.001$). Expression of PKR was not significantly different to PBS at 3, 14, 28 or 35 days post-lesion ($p > 0.05$).

Thus, following intracerebroventricular injection of mu-p75-sap immunotoxin and consequent degeneration of basal forebrain cholinergic neurons, there is an acute inflammatory response in the medial septum. This is characterised by increased expression of classical pro-inflammatory mediators TNF- α , IL-1 β , IL-6 and CXCL1. Furthermore, there is also an acute type I IFN response to this sterile neurotoxic insult. Given that type I interferon expression is principally involved in mediation of the inflammatory response to viral infection, these are novel findings suggesting that the type I interferon response may be a key regulator of the CNS inflammatory response to acute neurodegeneration.

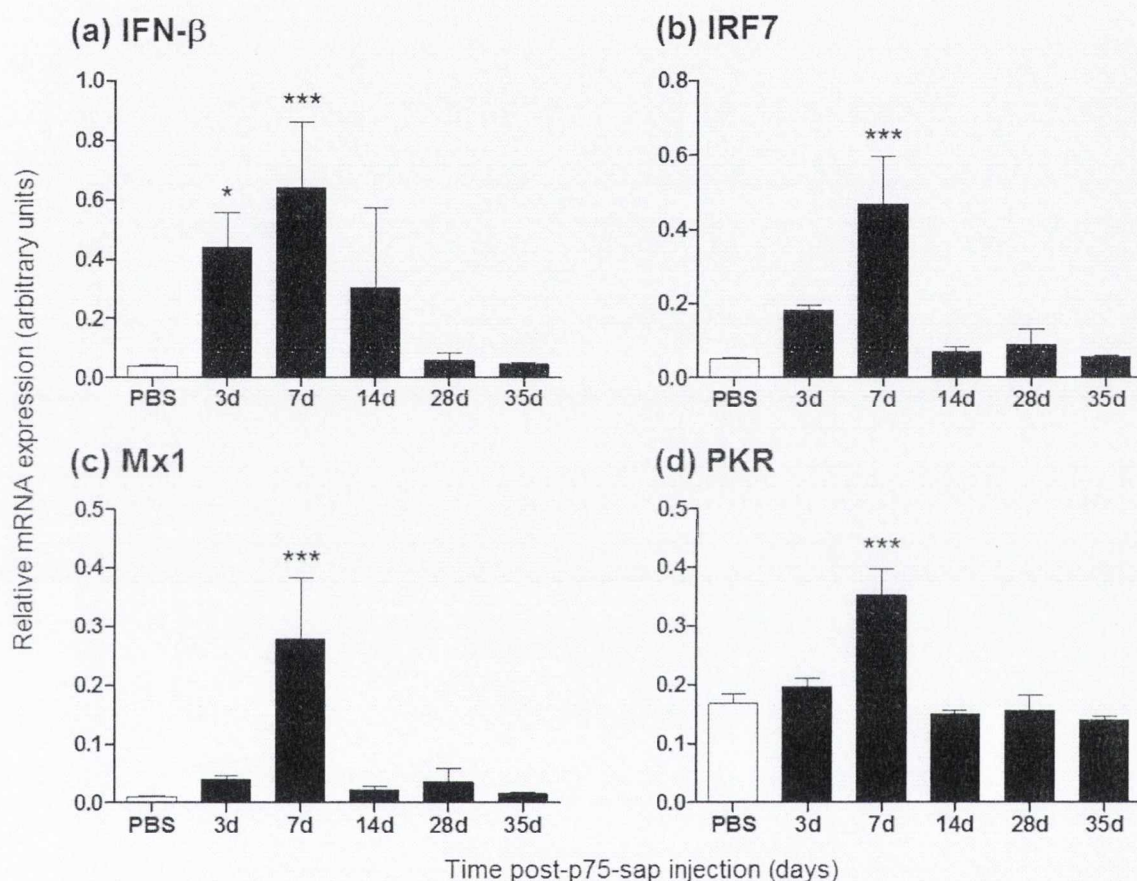


Figure 3.10 Temporal profile of the type I IFN response following mu-p75-sap lesion. Medial septum mRNA expression of (a) IFN-β, (b) IRF7, (c) Mx1 and (d) PKR was assessed by qPCR at a number of time-points following mu-p75-sap (0.4 μg) injection. PBS temporal data was combined as all transcripts remained at basal levels throughout. Significant differences by Bonferroni post-hoc tests following one-way ANOVA analysis are denoted * $p < 0.05$ and *** $p < 0.001$. Data are expressed as mean ± SEM ($n = 15$ for PBS; $n \geq 4$ for p75-sap).

3.2.8 Microglial response to cholinergic lesion.

The acute microglial response to mu-p75-sap-induced basal forebrain cholinergic neuronal degeneration was measured in the medial septum by quantitative PCR and immunolabelling. Expression of the macrophage scavenger receptor A2 (SRA2), showed induction at 3 and 7 days post-injection of mu-p75-sap, indicative of acute microglial activation (Figure 3.11a). One-way ANOVA analysis ($F = 22.30$, $df\ 5,33$; $p < 0.0001$) followed by selected Bonferroni post-hoc comparisons revealed that SRA2 mRNA expression was significantly increased in mu-p75-sap animals at 3 and 7 days post-injection compared to PBS controls ($p < 0.05$). SRA2 expression had returned to baseline levels at 14 days post-insult and was not significantly different to PBS ($p > 0.05$).

Expression of the myeloid restricted endosomal/lysosomal marker CD68 was upregulated 3 days post-insult followed by resolution (Figure 3.11b). One-way ANOVA analysis ($F = 3.03$, $df\ 5,33$; $p = 0.0233$) followed by selected Bonferroni post-hoc tests revealed a significant upregulation of CD68 expression in the medial septum 3 days following basal forebrain cholinergic lesion compared to PBS-injected control animals ($p < 0.01$). Acute activation of CD68 showed resolution from 7 days onwards and was no longer significantly different to PBS controls ($p > 0.05$).

Similarly, immunolabelling for the microglial marker IBA-1, showed clear evidence of activation in both the medial septum and in the dentate gyrus of the hippocampus at 6 days post-mu-p75-sap lesion (Figure 3.11e,f) but not in PBS-injected control animals (Figure 3.11c,d) or in mu-p75-sap animals at 40 days post-lesion (Figure 3.11g,h).

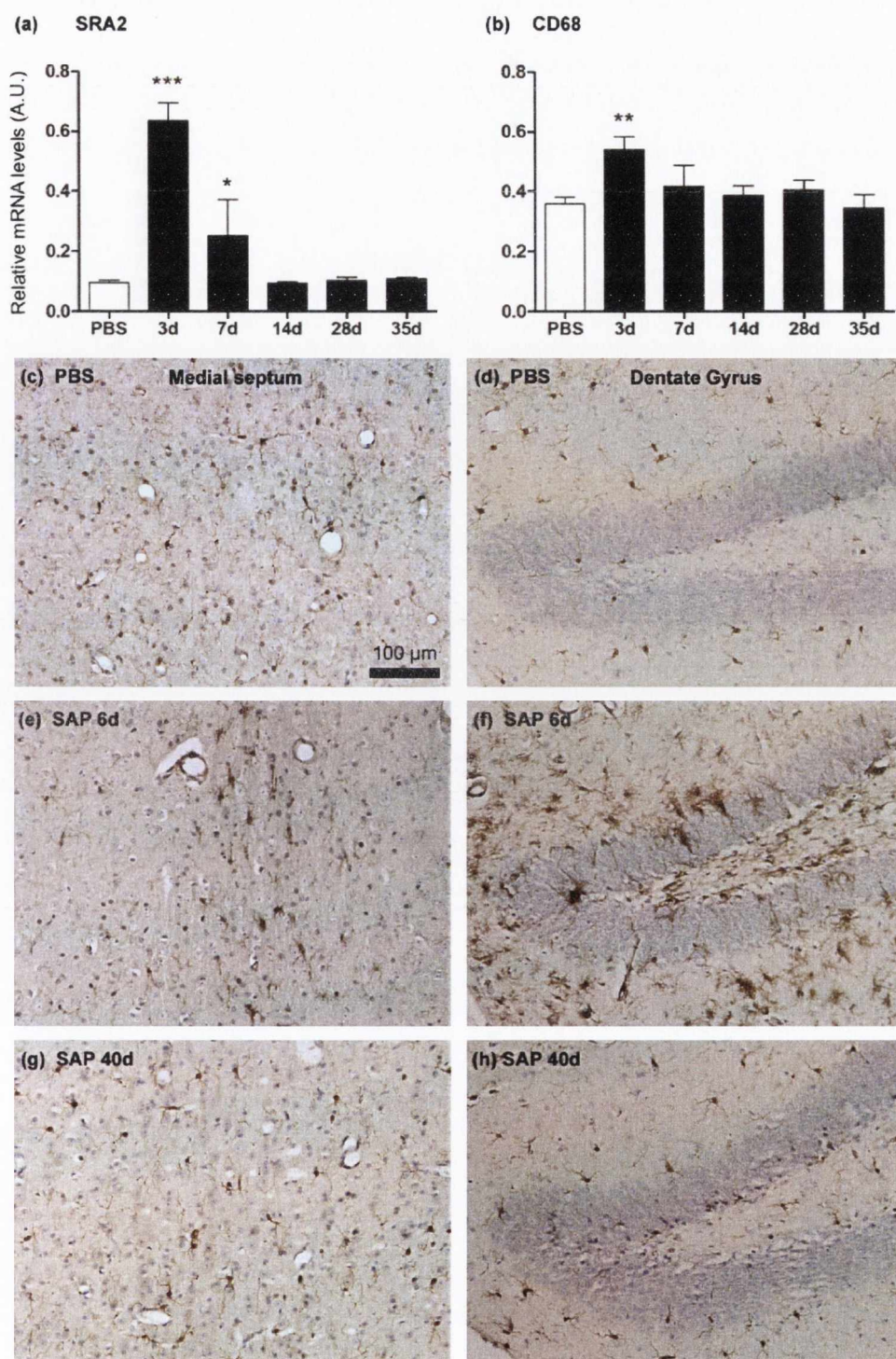


Figure 3.11 *Acute microglial response to basal forebrain cholinergic lesion.* Temporal qPCR data for (a) SRA2 and (b) CD68 following mu-p75-sap (0.4 μ g) injection. Bonferroni post-hoc tests compared to PBS control after significant one-way ANOVA are denoted * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Representative photomicrographs of microglial IBA-1 immunolabelling in the medial septum and dentate gyrus 6 days following PBS injection (c, d) and 6 days (e, f) and 40 days (g, h) after mu-p75-sap injection. Scale bar = 100 μ m. (n = 15 for PBS; n $\geq$ 4 for p75-sap).

3.2.9 Cholinergic depletion confers increased susceptibility to LPS-induced cognitive deficits

PBS-treated and mu-p75-sap-lesioned animals (0.08 µg) were trained on the T-maze working memory task as earlier described and subsequently challenged with either saline or LPS at 40 ± 2 days post-lesion. Cognitive testing was commenced 3 hours following peripheral challenge. This time-point was chosen based on previous studies by our laboratory that observed LPS-induced cognitive changes in the ME7 model of murine prion disease (Murray et al., 2012).

Systemic challenge with LPS (100 µg/kg i.p.) did not induce memory impairments in non-lesioned PBS animals. Saline-injected mu-p75-sap-lesioned (0.08 µg) animals also showed intact working memory performance. However, cholinergic-lesioned animals challenged systemically with LPS (100 µg/kg) showed marked working memory deficits (Figure 3.12), peaking 3 hours post-challenge, persisting to 7 hours and finally showing resolution 26 hours following challenge. Two-way repeated measures ANOVA analysis revealed a main effect of treatment ($F = 3.93$, df 3,240; $p = 0.015$) and an interaction between treatment and time ($F = 1.89$, df 18,240; $p = 0.00173$). Bonferroni post-hoc tests revealed that mu-p75-sap animals challenged with LPS were impaired with respect to other groups at 3 ($p < 0.001$) and 7 hours ($p < 0.05$) post-challenge. Thus, systemic LPS selectively induce cognitive deficits only in those animals with prior basal forebrain cholinergic neuropathology.

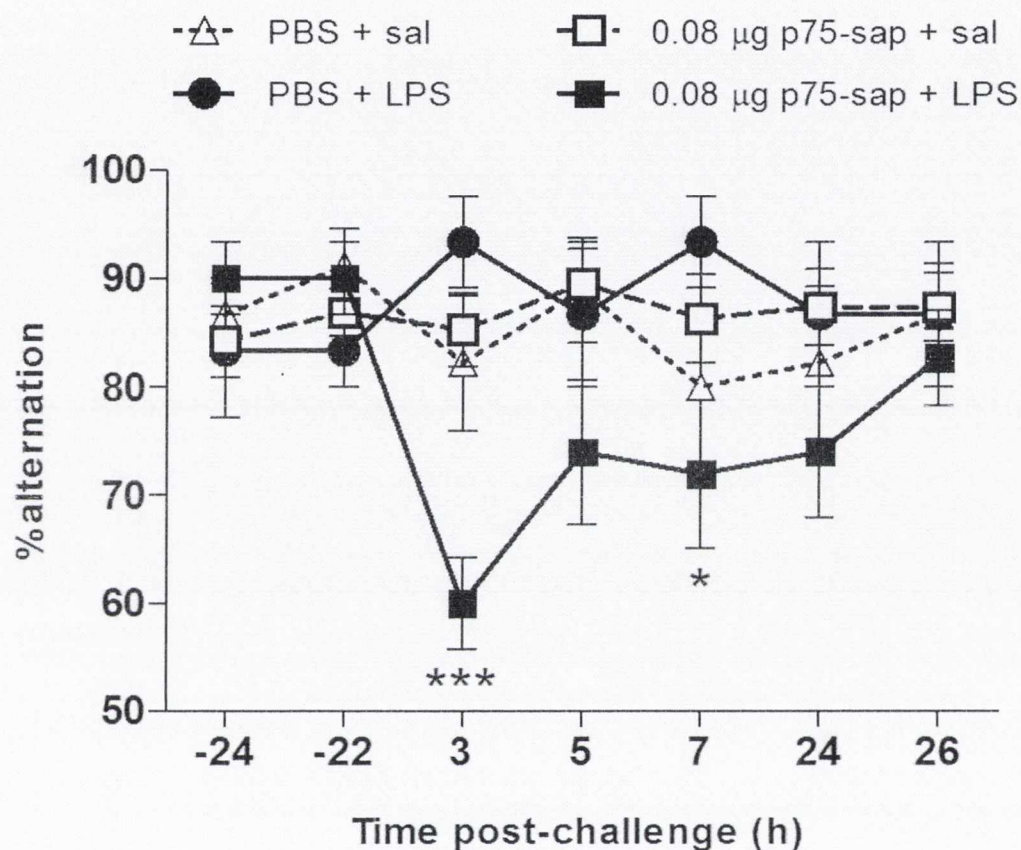


Figure 3.12 *Systemic LPS-induced working memory deficits in cholinergic-lesioned animals.* T-maze alternation of PBS animals challenged intraperitoneally with LPS (100 µg/kg) or sterile saline 40 ± 2 days post-surgery versus 0.08 µg p75-sap animals challenged with LPS or sterile saline. Bonferroni post-hoc tests after significant main effects and interactions by two-way repeated measures ANOVA are indicated by *** $p < 0.001$ and * $p < 0.05$. Data are expressed as mean ± SEM. (PBS + sal, $n = 9$; PBS + LPS, $n = 6$; p75-sap + sal, $n = 10$; p75-sap + LPS, $n = 19$)

3.2.10 Microglia are not primed in response to cholinergic neuronal degeneration

Previous work in our laboratory has shown that microglia are primed by neurodegenerative disease to produce exaggerated responses to subsequent inflammatory insults (Cunningham et al., 2005b). Chapter 4 of this thesis will further assess this with respect to stimulation of the acute anti-viral response. This priming of microglia has been implicated in the cognitive impairments induced by LPS in animals with prior pathology (Murray et al., 2012). The current study has shown that by placing prior pathology in the basal forebrain cholinergic system, systemic LPS produces acute and transient cognitive deficits only in mu-p75-sap-lesioned animals. It is therefore important to investigate whether such a lesion of the cholinergic system was sufficient to prime microglia to produce exaggerated responses to subsequent LPS treatment.

The transcription of a number of inflammatory cytokine mRNA species was examined in both the medial septum (Figure 3.13) and hippocampus (Figure 3.14) of PBS control and mu-p75-sap-lesioned animals (40 days post-surgery) 3 hours following systemic challenge with LPS (100 µg/kg, i.p.). Systemic LPS induced robust up-regulation of the pro-inflammatory genes IL-1 β , TNF- α , IL-6 and CXCL1, and of downstream effector genes TNFAIP2 and PTX3 in both the medial septum/diagonal band and in the hippocampus of normal animals and those with cholinergic lesions. Two-way ANOVA analyses revealed a main effect of LPS in each case ($F \geq 10.23$, df 1,15, $p \leq 0.006$). There were no significant differences in these inductions with respect to normal animals similarly challenged.

This indicates that there is no evidence of microglial priming 40 days after surgical induction of a basal forebrain cholinergic lesion of deliberately limited extent (0.08 µg mu-p75-sap). Systemic LPS challenge induces CNS inflammation but microglia are not primed to produce an exaggerated response to this subsequent stimulation.

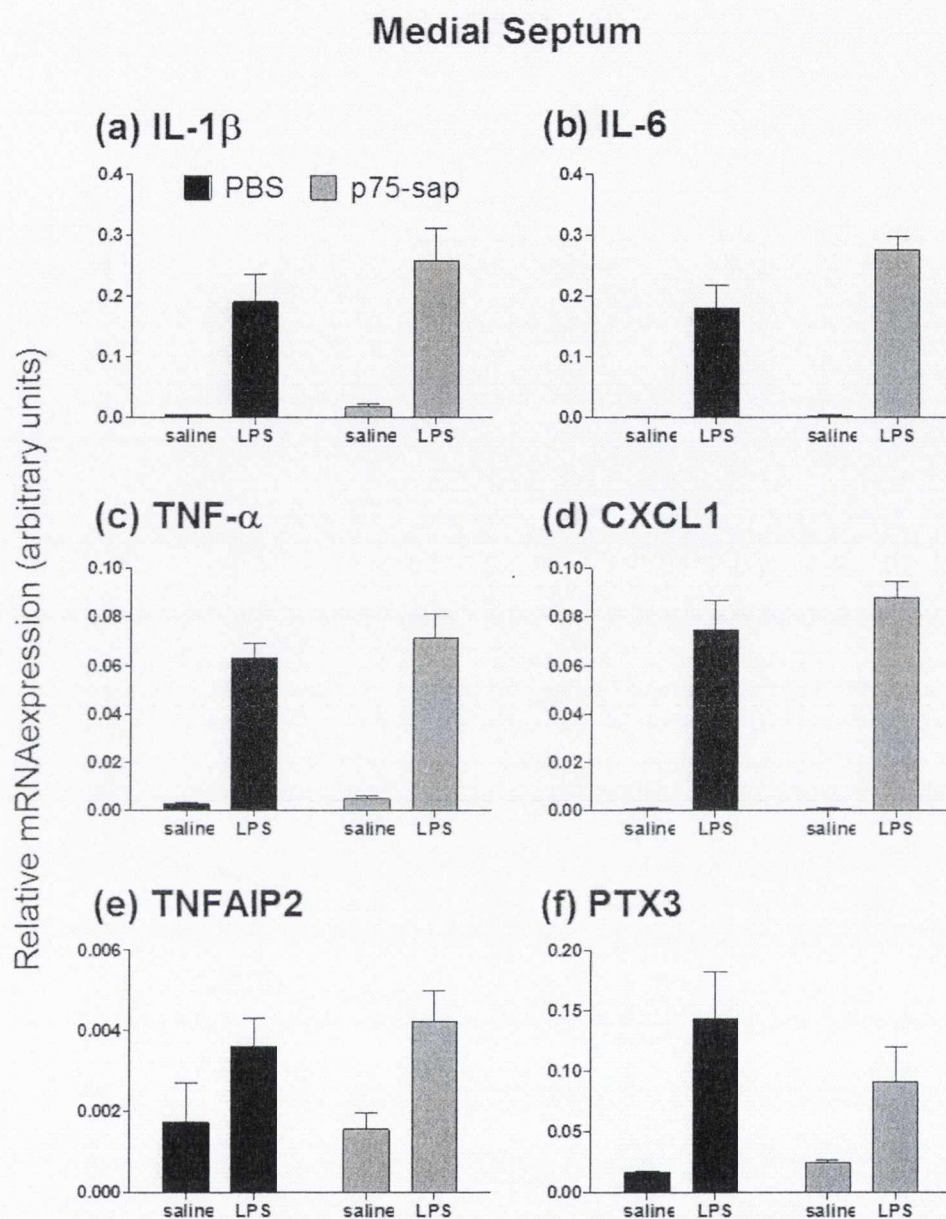


Figure 3.13 *Inflammatory response to systemic LPS in the medial septum of lesioned animals.* PBS-injected controls and mu-p75-sap-lesioned animals (0.08 μ g) 40 days post-lesion were challenged systemically with LPS (100 μ g/kg, i.p.) or sterile saline. Expression of pro-inflammatory genes IL-1 β , TNF- α , IL-6 and CXCL1, and downstream effectors genes TNFAIP2 and PTX3 were examined in the medial septum/diagonal band 3 hours post-challenge. Two-way ANOVA revealed main effects of LPS but no differences in lesioned animals with respect to PBS control animals similarly challenged with LPS Data expressed as mean $\pm$ SEM. (n = 4 for PBS + saline and n = 5 for all other groups).

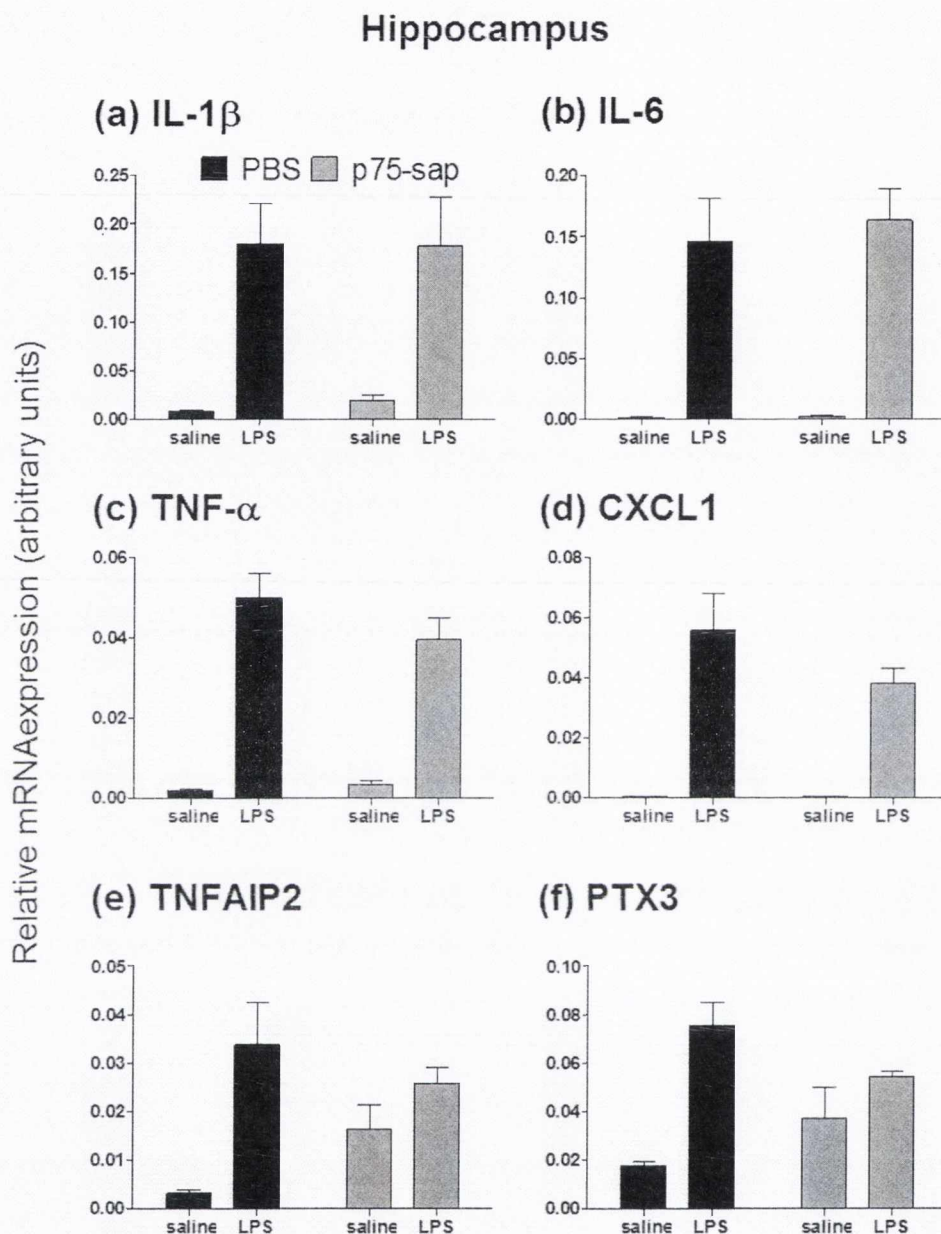


Figure 3.14 *Inflammatory response to systemic LPS in the hippocampus of lesioned animals.* PBS-injected controls and mu-p75-sap-lesioned animals (0.08 μ g) 40 days post-lesion were challenged systemically with LPS (100 μ g/kg, i.p.) or sterile saline. Expression of pro-inflammatory genes IL-1 β , TNF- α , IL-6 and CXCL1, and downstream effectors genes TNFAIP2 and PTX3 were examined in the hippocampus 3 hours post-challenge. Two-way ANOVA revealed main effects of LPS but no differences in lesioned animals with respect to PBS control animals similarly challenged. Data expressed as mean $\pm$ SEM. (n = 4 for PBS + saline and n = 5 for all other groups)

3.2.11 Is the T-maze working memory task dependent on central cholinergic function?

To assess whether the T-maze working memory task used here requires normal functioning of the cholinergic system, a group of naïve female C57BL/6 mice were first trained on the T-maze as earlier described. These animals were then challenged with either the muscarinic cholinergic receptor antagonist, scopolamine hydrobromide (1 mg/kg i.p.), or sterile saline. Animals were tested in the T-maze one hour following systemic challenge. Scopolamine induced a marked impairment in working memory compared to saline-treated control animals (Figure 3.15). Two-way repeated measures ANOVA analysis revealed a significant main effect of trial block ($F = 16.74$, $df\ 1, 21$, $p = 0.0005$) and of treatment ($F = 20.18$, $df\ 1, 21$, $p = 0.0002$), with an interaction between these factors ($F = 12.65$, $df\ 1, 21$, $p = 0.0019$). Bonferroni post-hoc tests showed that there was a significant impairment in the ability to perform this working memory task following treatment with scopolamine ($p < 0.001$). Thus, the T-maze working memory alternation task is dependent on a functional muscarinic cholinergic signalling system.

5.2.12 Cholinesterase inhibition partially protects against LPS-induced cognitive deficits

The ability of the cholinesterase inhibitor, donepezil, to rescue the working memory deficits induced by LPS was assessed on the T-maze working memory task. Animals were trained and challenged systemically with LPS as before but then received a systemic injection of donepezil hydrochloride (1 mg/kg i.p.) or vehicle (endotoxin-free water) one hour after LPS. Donepezil afforded significant, though incomplete, protection against the LPS-induced working memory deficits (Figure 3.16) but had no effect on normal animals. Two-way repeated measures ANOVA analysis of the LPS-treated groups revealed significant main effects of treatment ($F = 12.17$, $df\ 1, 132$, $p = 0.0021$). Bonferroni post-hoc tests revealed a significant difference between mu-p75-sap-lesioned animals challenged with LPS and those that received both LPS and donepezil, at 3 hours post-LPS challenge ($p < 0.05$).

Thus, this demonstrates that acetylcholinesterase inhibition partially protects against the LPS-induced cognitive deficits in animals with existing cholinergic hypofunction.

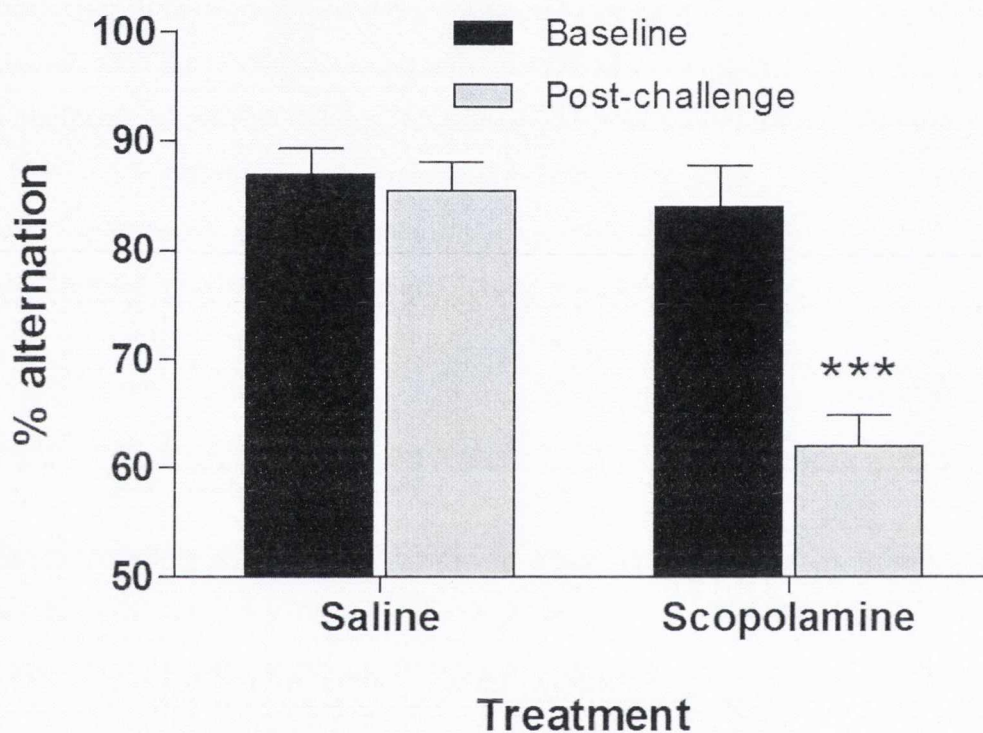


Figure 3.15 *Normal cholinergic function is necessary for working memory.* T-maze dependence on a functional cholinergic system was verified by systemic challenge with either scopolamine hydrobromide (1 mg/kg, i.p., $n = 10$) or sterile saline ($n = 13$). Animals were tested in the T-maze one hour after systemic challenge. Significant differences between saline and scopolamine by Bonferroni post-hoc tests after significant interactions and main effects by two-way repeated measures ANOVA are indicated by *** $p < 0.001$. Data are expressed as mean $\pm$ SEM.

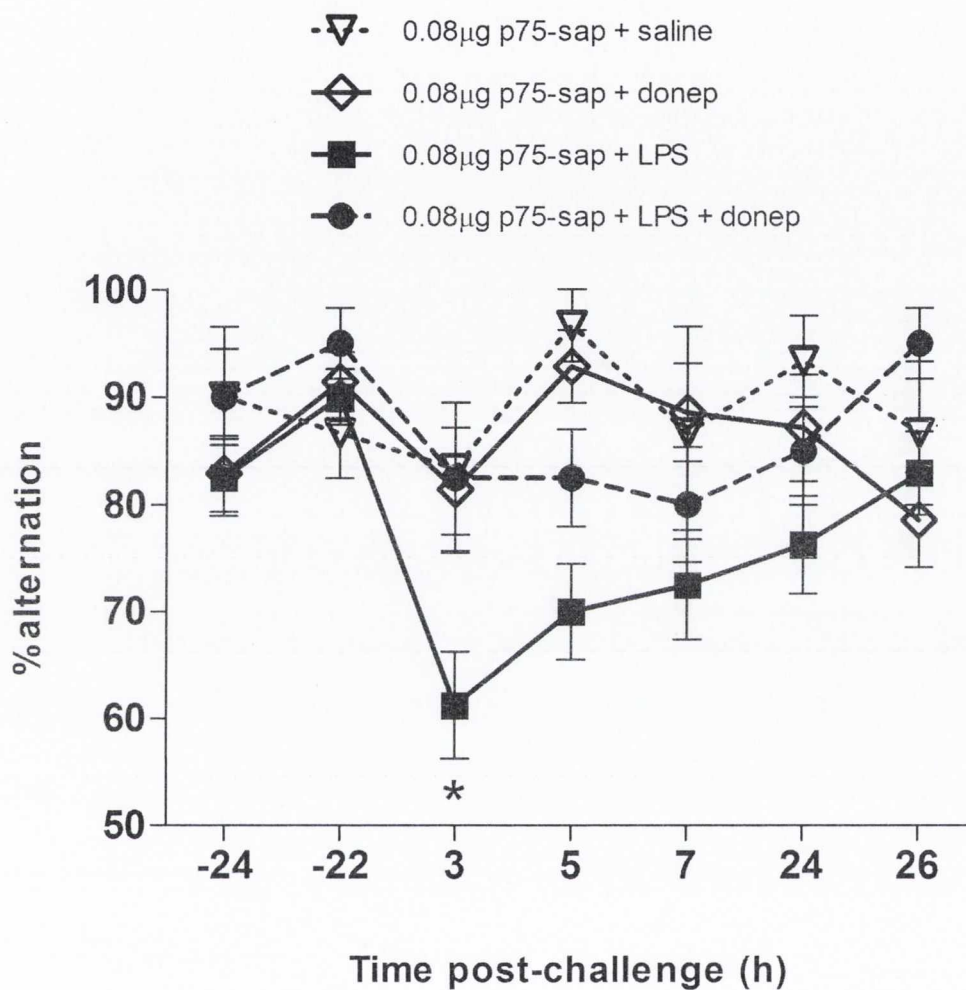


Figure 3.16 Cholinesterase inhibition protects against LPS-induced cognitive deficits. LPS-induced working memory deficits were partially protected against by the acetylcholinesterase inhibitor donepezil (1 mg/kg, i.p.), administered 1 hour after LPS. Statistically significant differences by Bonferroni post-hoc test after significant main effects by two-way repeated measures ANOVA are denoted $*p < 0.05$. Data are expressed as mean $\pm$ SEM. (mu-p75-sap + saline $n = 6$; mu-p75-sap + donepezil $n = 14$; mu-p75-sap + LPS $n = 16$; mu-p75-sap + LPS + donepezil $n = 8$).

3.2.13 Microglial priming following severe cholinergic depletion and acutely post-lesion

The current study has thus far demonstrated that microglia in neither the basal forebrain nor the hippocampus are primed by basal forebrain cholinergic neurodegeneration to produce exaggerated inflammatory responses to subsequent systemic inflammatory insult. This lack of priming could be attributed to two potential sources. Firstly, since evidence for priming was assessed at 40 days post-lesion, it is possible that the critical window during which microglia remain primed has passed at this point. Secondly, due to the deliberately limited nature of the lesion, the cholinergic loss produced by the dose of mu-p75-sap used (0.08 µg) might not have been sufficient to prime microglia in the first instance. This finding prompted subsequent experiments that were designed specifically to investigate whether microglia in these regions could be primed to produce exaggerated responses to subsequent inflammatory challenge by (1) a more severe lesion of the basal forebrain cholinergic system (1.2 µg mu-p-75 sap), or (2) by examination of the inflammatory response at a more acute time-point following lesion (14 days) such that the microglia would remain primed by the ongoing/recent cholinergic neuronal death.

3.2.13.1 Peripheral cytokine response to systemic LPS in 1.2 µg mu-p75-sap-lesioned animals, 40 days post-lesion

Animals were intracerebroventricularly injected with 1.2 µg mu-p75-sap to induce a more severe cholinergic lesion than described earlier. At 40 days post-lesion, animals were challenged systemically with LPS (100 µg/kg i.p.), to determine if severe hypocholinergia primes the brain to produce amplified responses to subsequent inflammatory stimulation, even at a time-point distal to the acute insult.

Prior reports by Tracey and colleagues have shown that the vagus nerve provides tonic inhibition of peripheral inflammation via a cholinergic mechanism and that removal of this by vagotomy leads to exaggerated peripheral inflammatory responses to endotoxin (Borovikova et al., 2000). Thus the current study has measured circulating cytokine levels to determine whether basal forebrain cholinergic depletion impacted upon the peripheral immune response to subsequent inflammatory stimulation. Two way ANOVA analysis of plasma TNF-α (Figure 3.17a) revealed a main effect of LPS ($F = 11.52$, $df\ 1,16$, $p = 0.0037$), but no effect of cholinergic lesion and no interaction. Bonferroni post-hoc tests

revealed that mu-p75-sap-lesioned animals showed similar levels of circulating TNF- α to PBS controls following systemic LPS ($p > 0.05$).

Similarly, plasma levels of IL-6 were not differentially induced by LPS in cholinergic lesioned compared to PBS controls (Figure 3.17b). Two-way ANOVA analysis revealed a main effect of LPS ($F = 52.00$, $df\ 1,14$, $p < 0.0001$), but no effect of cholinergic lesion and no interaction. Bonferroni post-hoc tests revealed that levels of circulating IL-6 following systemic LPS was not significantly different in mu-p75-sap-lesioned animals compared to PBS controls ($p > 0.05$).

Plasma CXCL1 was induced to similar levels in both mu-p75-sap and PBS animals challenged with LPS (Figure 3.17c). Two-way ANOVA analysis revealed a main effect of LPS ($F = 104.32$, $df\ 1,14$, $p < 0.0001$), but no effect of cholinergic lesion and no interaction. Bonferroni post-hoc tests revealed that levels of circulating CXCL1 following systemic LPS were not significantly different in mu-p75-sap-lesioned animals compared to PBS controls ($p > 0.05$).

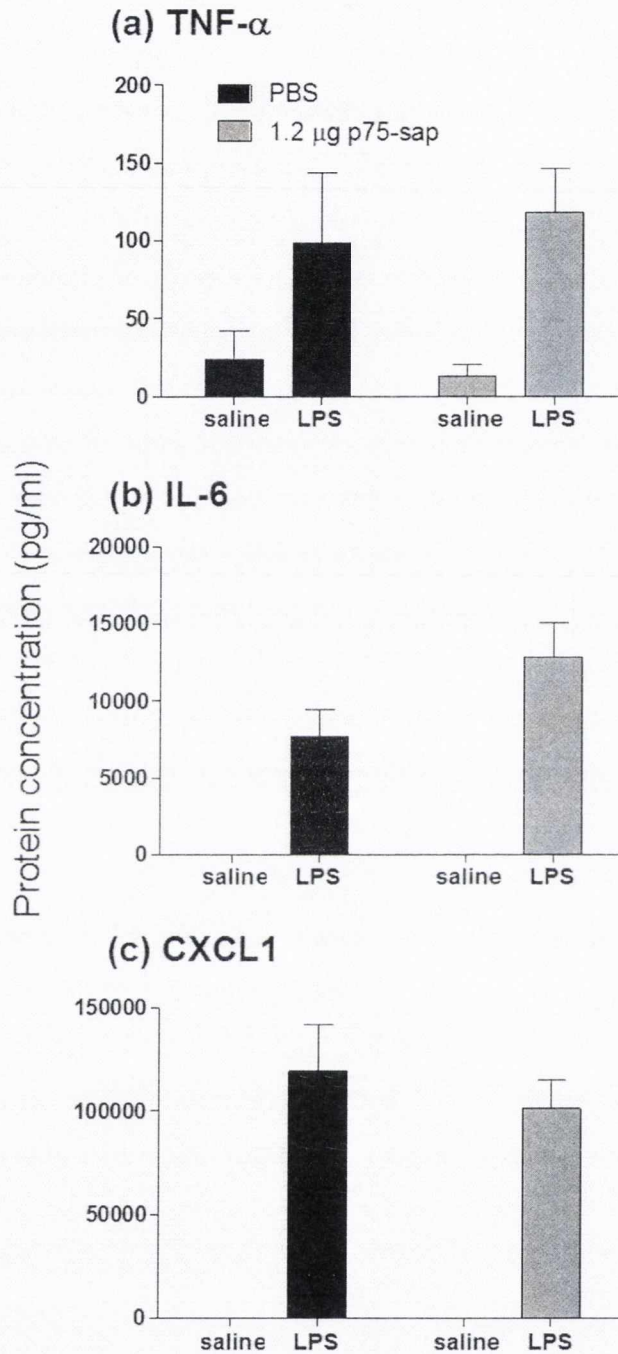


Figure 3.17 Plasma cytokine profile following systemic LPS challenge. Plasma levels of (a) TNF- α , (b) IL-6, and (c) CXCL1 were measured by sandwich ELISA in PBS- and 1.2 μ g mu-p75-sap-injected animals, 40 days post-insult and 3 hours after systemic LPS challenge (100 μ g/kg). Two-way ANOVA followed by Bonferroni post-hoc tests revealed main effect of LPS but no differences between cholinergic lesioned animals and PBS controls ($p > 0.05$). Data are expressed as mean $\pm$ SEM. ($n = 4$ for PBS, $n = 6$ for mu-p75-sap).

3.2.13.2 Cholinergic depletion in 1.2 µg mu-p75-sap-lesioned animals, 40 days post-lesion

To assess the degree of basal forebrain cholinergic depletion produced by this higher dose of mu-p75-sap (1.2 µg), mRNA transcripts for a number of cholinergic markers were assessed by qPCR 40 days after the initial neurotoxic insult. Transcripts were measured in the medial septum, where cholinergic neurons are degenerating, and the hippocampus, a major projection area for this neuronal population. It is likely that hippocampal expression of acetylcholine receptors would be diminished following depletion of basal forebrain cholinergic neurons, since cells that these neurons synapse on would lose their innervation.

Student's *t*-test analysis revealed that ChAT expression was significantly decreased in the medial septum of mu-p75-sap-injected animals compared to PBS controls ($t = 5.655$, $df = 18$, $p < 0.0001$) (Figure 3.18a). Given that earlier experiments (Figure 3.7) indicated that septal ChAT mRNA expression predicted ChAT-positive neuronal loss, the decrease in ChAT mRNA here suggests that this mu-p75-sap dose induce approximately 50% loss of cholinergic cells in the medial septum and diagonal band.

Hippocampal expression of two key receptors for acetylcholine, the $\alpha 7$ -nAChR and the M1-mAChR, was assessed to investigate the impact of decreased basal forebrain cholinergic innervation.

Student's *t*-test analysis revealed that hippocampal $\alpha 7$ -nAChR expression was significantly decreased in mu-p75-sap-injected animals compared to PBS controls ($t = 6.103$, $df = 18$, $p < 0.0001$) (Figure 3.18b).

Similarly, Student's *t*-test analysis revealed that hippocampal M1-mAChR expression was significantly decreased in mu-p75-sap-injected animals compared to PBS controls ($t = 3.453$, $df = 18$, $p = 0.0028$) (Figure 3.18c).

Thus, a more severe lesion of the basal forebrain cholinergic system produced approximately 50% loss of ChAT expression in the medial septum along with decreased expression of acetylcholine receptors in the hippocampus.

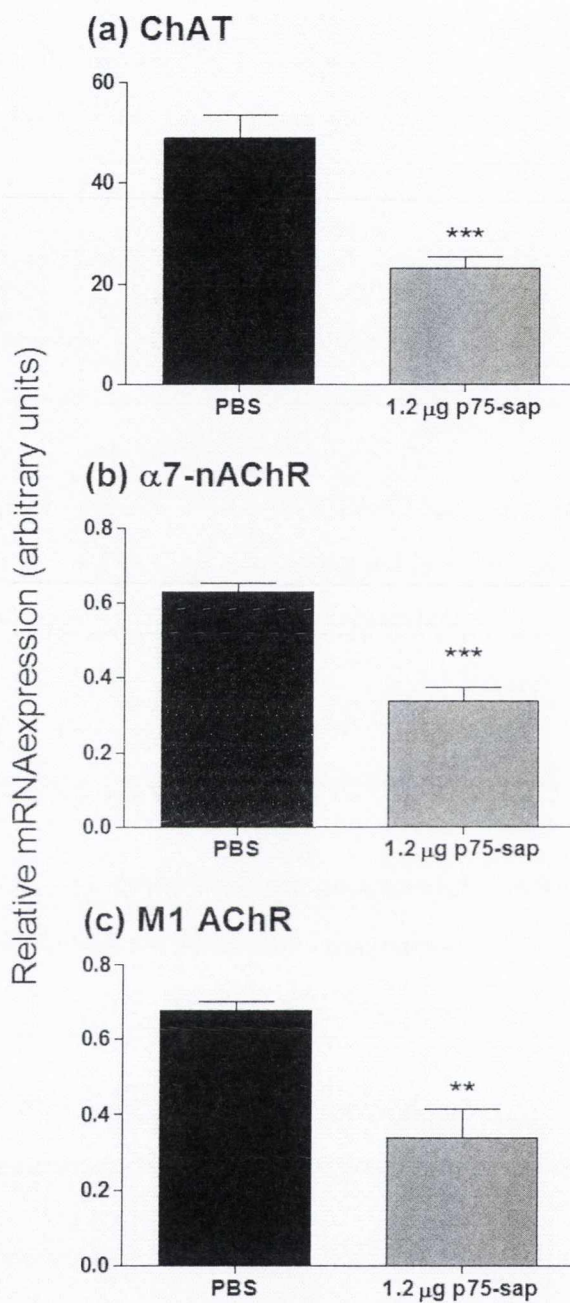


Figure 3.18 Basal forebrain cholinergic depletion in lesioned animals. Degeneration of basal forebrain cholinergic neurons in mu-p75-sap-injected animals (1.2 μg) was assessed by measuring expression of mRNA transcripts for (a) ChAT in the medial septum, (b) α7-nAChR in the hippocampus and (c) M1 AChR in the hippocampus. Significant differences to PBS-injected controls by Student's *t*-test are denoted by ***p* < 0.01 and ****p* < 0.001. Data are expressed as mean ± SEM (n = 8 for PBS, n = 12 for mu-p75-sap)

3.2.13.3 Inflammatory response to systemic LPS in the basal forebrain of 1.2 μ g mu-p75-sap-lesioned animals, 40 days post-lesion

To assess whether microglia in the medial septum were primed by existing cholinergic neuropathology to produce amplified responses to subsequent inflammation, mu-p75-sap (1.2 μ g) animals, 40 days post-lesion, were challenged peripherally with LPS (100 μ g/kg i.p.) and a number of inflammatory transcripts were measured by qPCR, 3 hours post-LPS.

Expression of IL-1 β was markedly induced in the medial septum by LPS and to a greater degree in animals with cholinergic deficits compared to PBS-injected controls (Figure 3.19a). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 12.10$, df 1,15, $p = 0.0034$), and of LPS ($F = 38.22$, df 1,15, $p < 0.0001$) and an interaction between these two factors ($F = 9.96$, df 1,15, $p = 0.0065$). Bonferroni post-hoc comparisons revealed that IL-1 β was induced to significantly greater levels by LPS in mu-p75-sap-lesioned animals compared to PBS controls ($p < 0.001$).

TNF- α expression was not induced to different levels in medial septum of lesioned versus non-lesioned animals (Figure 3.19b). Two-way ANOVA analysis revealed a main effect of LPS ($F = 72.60$, df 1,15, $p < 0.0001$) but not of cholinergic lesion. There was no interaction between these two factors. Bonferroni post-hoc comparisons revealed that there were no significant differences in TNF- α induction by LPS in mu-p75-sap-lesioned animals compared to PBS ($p > 0.05$).

There was no differential induction of IL-6 expression in the medial septum of mu-p75-sap versus PBS animals following systemic LPS challenge (Figure 3.19c). Two-way ANOVA analysis revealed a main effect of LPS ($F = 18.77$, df 1,15, $p = 0.0006$) but not of cholinergic lesion. There was no interaction between these two factors. Although there appeared to be a trend towards greater IL-6 induction after LPS in mu-p75-sap-lesioned animals, Bonferroni post-hoc comparisons revealed this was not significantly different to PBS animals similarly challenged ($p > 0.05$).

CXCL1 was induced by LPS but to similar levels in both mu-p75-sap and PBS animals (Figure 3.19d). Two-way ANOVA analysis revealed a main effect of LPS ($F = 29.15$, df 1,15, $p < 0.0001$) but not of cholinergic lesion and no interaction. Bonferroni post-hoc comparisons revealed no significant differences between LPS challenged animals ($p > 0.05$).

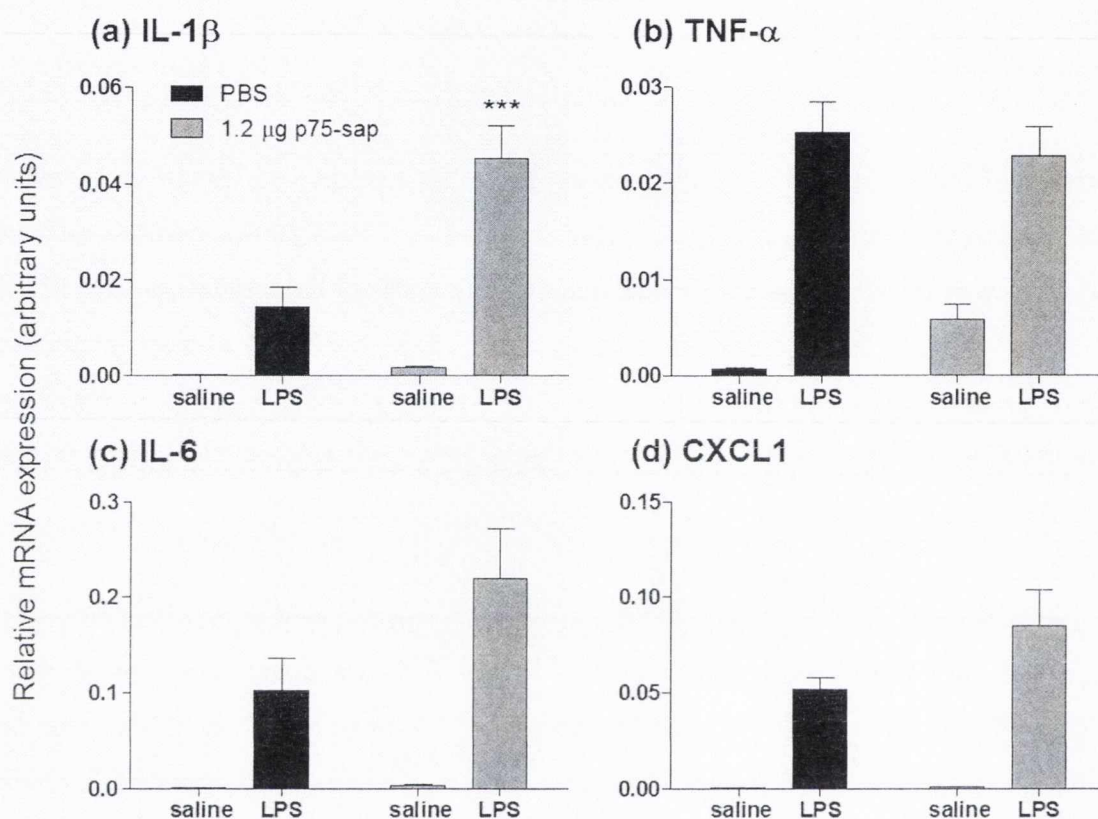


Figure 3.19 LPS-induced inflammatory response in the medial septum/diagonal band of mu-p75-sap-lesioned animals. mRNA expression of (a) IL-1 β , (b) TNF- α , (c) IL-6 and (d) CXCL1 was measured by qPCR in the medial septum of PBS- and 1.2 μ g mu-p75-sap-injected animals, 40 days post-insult and 3 hours after systemic LPS challenge (100 μ g/kg). Bonferroni post-hoc tests following significant difference revealed by two-way ANOVA analyses are denoted ***p < 0.001. Data are expressed as mean $\pm$ SEM (n = 4 for PBS, n = 6 for mu-p75-sap)

3.2.13.4 Inflammatory response to systemic LPS in the hippocampus of 1.2 µg mu-p75-sap-lesioned animals, 40 days post-lesion

The above analyses in the basal forebrain indicated that more robust degeneration in this region produced microglial priming only to a limited degree, with moderately increased IL-1 β transcription in lesioned compared to normal animals. However, it was hypothesised that the loss of cholinergic input to the hippocampus, in the same animals, would prime microglia of this region to produce exaggerated responses to subsequent inflammatory stimuli. Thus, in the hippocampi of the same animals (1.2 µg mu-p75-sap, 40 days post-lesion, challenged peripherally with LPS (100 µg/kg i.p.)) we assessed whether microglia were primed by basal forebrain cholinergic depletion. Inflammatory gene transcription was measured by qPCR and ELISA, 3 hours post-LPS.

IL-1 β transcription was induced by systemic LPS in the hippocampus of mu-p75-sap-lesioned animals to a markedly greater degree than in PBS animals (Figure 3.20a). Two-way ANOVA analysis revealed a main effect of LPS ($F = 4.64$, $df\ 1,16$, $p = 0.048$) and of cholinergic lesion ($F = 5.58$, $df\ 1,16$, $p = 0.0312$) but no interaction between these two factors. Bonferroni post-hoc comparisons revealed that IL-1 β was induced to significantly greater levels by LPS in mu-p75-sap-lesioned animals compared to PBS controls ($p < 0.05$).

Systemic LPS induced exaggerated expression of TNF- α in the hippocampus of mu-p75-sap animals with respect to LPS-treated PBS animals (Figure 3.20b). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 9.93$, $df\ 1,16$, $p = 0.0062$), and of LPS ($F = 5.33$, $df\ 1,16$, $p = 0.0346$) but no interaction between these two factors. Bonferroni post-hoc comparisons revealed that TNF- α induction was significantly greater after LPS in mu-p75-sap-lesioned animals compared to PBS controls ($p < 0.05$).

Hippocampal IL-6 transcription showed a much greater induction in mu-p75-sap animals compared to PBS animals after systemic LPS challenge (Figure 3.20c). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 5.53$, $df\ 1,16$, $p = 0.0318$) and of LPS ($F = 11.24$, $df\ 1,16$, $p = 0.004$) and an interaction between these two factors ($F = 5.23$, $df\ 1,16$, $p = 0.0361$). Bonferroni post-hoc comparisons revealed that IL-6 induction was significantly greater after LPS in mu-p75-sap-lesioned animals compared to PBS controls ($p < 0.01$).

Transcription of CXCL1 was also more robustly induced in the hippocampus of mu-p75-sap animals compared to PBS following systemic LPS challenge (Figure 3.20d). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 8.34$, $df\ 1,16$, $p = 0.0107$) and of LPS ($F = 11.96$, $df\ 1,16$, $p = 0.0032$) and an interaction between these two factors ($F = 6.69$, $df\ 1,16$, $p = 0.0199$). Bonferroni post-hoc comparisons revealed that induction of CXCL1 was significantly elevated in mu-p75-sap-lesioned animals compared to PBS controls after LPS challenge ($p < 0.01$).

PTX3 induction in the hippocampus of mu-p75-sap animals was quite variable although there appeared to be a trend towards a differential response to LPS in mu-p75-sap versus PBS animals (Figure 3.20e). Two-way ANOVA analysis revealed no main effects or interactions. A further one way ANOVA analysis ($F = 4.27$, $df\ 3,16$, $p = 0.0215$) followed by selected Bonferroni post-hoc comparisons revealed that PTX3 was induced to significantly greater levels by LPS in mu-p75-sap-lesioned animal compared to PBS controls ($p < 0.05$).

Hippocampal expression of TNFAIP2 was induced more robustly by LPS in animals with a basal forebrain cholinergic lesion than in normal animals (Figure 3.20f). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 6.02$, $df\ 1,16$, $p = 0.026$) but not of LPS. There was no interaction between these two factors. Bonferroni post-hoc comparisons revealed that TNFAIP2 was induced to significantly greater levels by LPS in mu-p75-sap-lesioned animals compared to PBS controls ($p < 0.05$).

IL-1 β protein production was found to be differentially induced by LPS in the hippocampus of mu-p75-sap-lesioned animals compared to PBS controls (Figure 3.21). Two-way ANOVA analysis revealed main effects of LPS ($F = 10.03$, $df\ 1,14$, $p = 0.0055$) and cholinergic lesion ($F = 11.32$, $df\ 1,14$, $p = 0.0046$) and an interaction between these two factors ($F = 7.83$, $df\ 1,14$, $p = 0.0142$). Bonferroni post-hoc comparisons revealed that IL-1 β protein levels were induced to significantly greater levels by systemic LPS in mu-p75-sap animals compared to PBS controls ($p < 0.01$).

Thus it appears that loss of basal forebrain cholinergic input to the hippocampus primes the brain to produce exaggerated inflammatory responses to subsequent peripheral LPS challenge, even at 40 days after the induction of the cholinergic lesion.

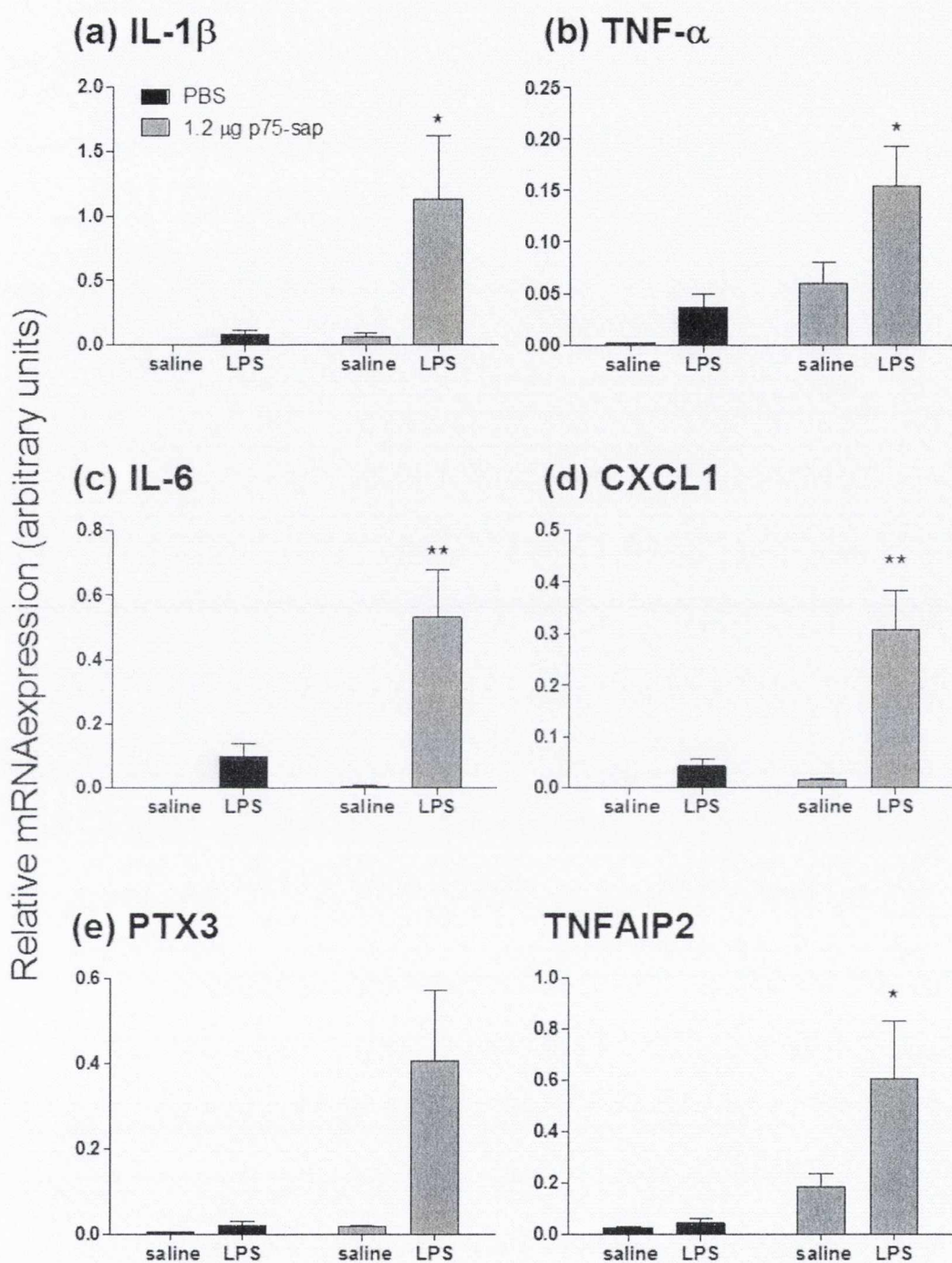


Figure 3.20 LPS-induced inflammation in the hippocampus of mu-p75-sap-lesioned animals. mRNA expression of (a) IL-1 β , (b) TNF- α , (c) IL-6, (d) CXCL1, (e) PTX3 and (f) TNFAIP2 was measured by qPCR in the hippocampus of PBS- and 1.2 μ g mu-p75-sap-injected animals, 40 days post-insult and 3 hours after systemic LPS challenge (100 μ g/kg). Bonferroni post-hoc tests following two-way ANOVA analyses are denoted * p < 0.05 and ** p < 0.01. Data are expressed as mean $\pm$ SEM (n = 4 for PBS, n = 6 for mu-p75-sap)

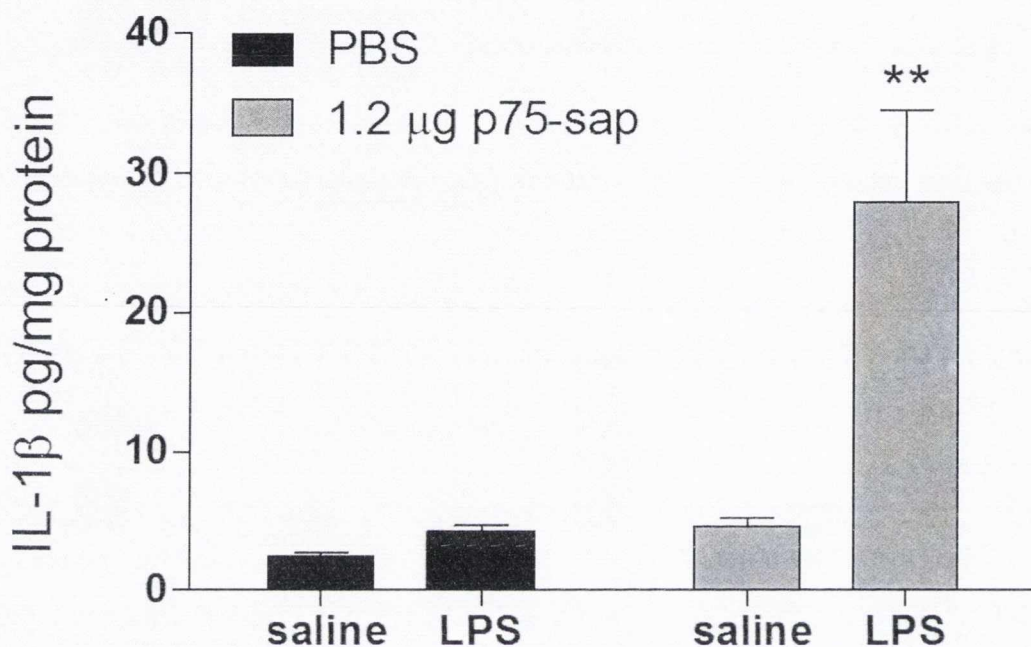


Figure 3.21 Systemic LPS induced IL-1 β protein in hippocampus of mu-p75-sap-lesioned animals. Hippocampal IL-1 β protein production was assessed in PBS- and 1.2 μ g mu-p75-sap-injected animals, 40 days post-lesion and 3 hours after systemic LPS challenge (100 μ g/kg i.p.). Bonferroni post-hoc tests following two-way ANOVA analysis are denoted ** $p < 0.01$. Data are expressed as mean $\pm$ SEM (PBS + saline $n = 3$, PBS + LPS $n = 4$, p75-sap + saline $n = 6$ and p75-sap + LPS $n = 5$)

3.2.13.5 Microglial activation in 1.2 µg mu-p75-sap animals, 40 days post-lesion

The CNS microglial profile was examined in the medial septum and hippocampus, 40 days following a 1.2 µg mu-p75-sap-induced lesion of the basal forebrain cholinergic neurons. The upregulation of myeloid-specific scavenger receptors markers that are expressed on microglia were analysed by qPCR, 3 hours following systemic LPS challenge (100 µg.kg i.p.).

Expression of the scavenger receptor A2 (SRA2) was induced in the medial septum by LPS and by cholinergic lesion, with greatest expression in lesioned animals subsequently challenged peripherally with LPS (Figure 3.22a). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 9.81$, df 1,16, $p = 0.064$) and of LPS ($F = 12.84$, df 1,16, $p = 0.025$). There was no interaction between these two factors. Bonferroni post-hoc comparisons revealed that SRA2 was induced to significantly greater levels by LPS in mu-p75-sap-lesioned animals compared to PBS controls ($p < 0.05$).

SRA2 transcription in the hippocampus was induced by cholinergic lesion but was not further affected by LPS challenge (Figure 3.22b). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 7.83$, df 1,16, $p = 0.0129$). There was no effect of LPS and no interaction.

Expression of the microglial activation marker, CD68, was induced in the medial septum by basal forebrain cholinergic lesion and unaffected by LPS challenge (Figure 3.22c). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 40.43$, df 1,16, $p < 0.0001$). There was no effect of LPS and no interaction between the two factors.

Hippocampal expression of CD68 was robustly induced by mu-p75-sap-induced cholinergic lesion but was not further affected by LPS (Figure 3.22d). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 12.94$, df 1,16, $p = 0.024$). There was no effect of LPS and no interaction between the two factors.

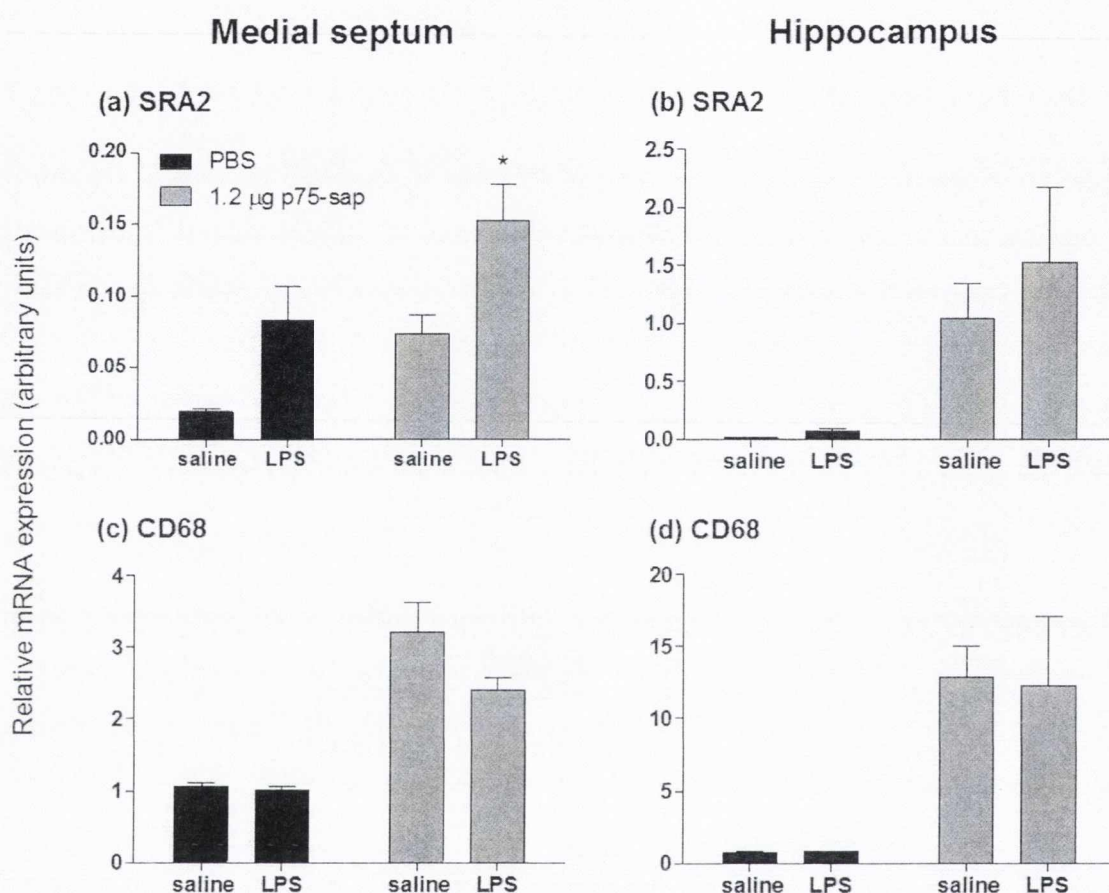


Figure 3.22 *CNS microglial activation following systemic LPS in mu-p75-sap-lesioned animals.* mRNA expression of SRA2 (a-b) and CD68 (c-d) was measured by qPCR in the medial septum and hippocampus of PBS- and 1.2 µg mu-p75-sap-injected animals, 40 days post-insult and 3 hours after systemic LPS challenge (100 µg/kg). Bonferroni post-hoc tests following two-way ANOVA analyses are denoted * $p < 0.05$. Data are expressed as mean $\pm$ SEM ($n = 4$ for PBS, $n = 6$ for mu-p75-sap)

3.2.13.6 Peripheral cytokine response to systemic LPS in 0.4 µg mu-p75-sap-lesioned animals, 14 days post-lesion

We have shown above that more robust degeneration in the basal forebrain cholinergic system leads to robust priming of the microglia of the hippocampus, even 40 days after lesioning. It was clear that more limited lesions (0.08 µg) did not lead to microglial priming at 40 days (Figure 3.13), but it was of interest to know whether lower levels of degeneration would lead to priming at earlier time points post-lesion. Animals were intracerebroventricularly injected with 0.4 µg mu-p75-sap and challenged systemically with LPS (100 µg/kg i.p.) 14 days following surgery to induce the cholinergic lesion in order to investigate whether a limited cholinergic lesion examined at a time proximal to the initial neurotoxic insult could prime microglia to produce exaggerated responses to subsequent inflammatory challenge. All experiments were analysed 3 hours post-LPS challenge.

Circulating cytokine levels were measured to determine if basal forebrain cholinergic neurodegeneration impacted upon the peripheral immune response to subsequent inflammatory stimulation. Two way ANOVA analysis of plasma TNF- α (Figure 3.23a) revealed a main effect of LPS ($F = 27.72$, $df\ 1,15$, $p < 0.0001$), but no effect of cholinergic lesion and no interaction. Bonferroni post-hoc tests revealed that mu-p75-sap-lesioned animals showed similar levels of circulating TNF- α to PBS controls following systemic LPS ($p > 0.05$).

Similarly, plasma levels of IL-6 were not differentially induced by LPS in cholinergic lesioned compared to PBS controls (Figure 3.23b). Two-way ANOVA analysis revealed a main effect of LPS ($F = 20.63$, $df\ 1,13$, $p = 0.0006$), but no effect of cholinergic lesion and no interaction. Bonferroni post-hoc tests revealed that levels of circulating IL-6 following systemic LPS were not significantly different in mu-p75-sap-lesioned animals compared to PBS controls ($p > 0.05$).

Plasma CXCL1 was induced to similar levels in both mu-p75-sap and PBS animals challenged with LPS (Figure 3.23c). Two-way ANOVA analysis revealed a main effect of LPS ($F = 53.17$, $df\ 1,14$, $p < 0.0001$), but no effect of cholinergic lesion and no interaction. Bonferroni post-hoc tests revealed that levels of circulating CXCL1 following systemic LPS were not significantly different in mu-p75-sap-lesioned animals compared to PBS controls ($p > 0.05$).

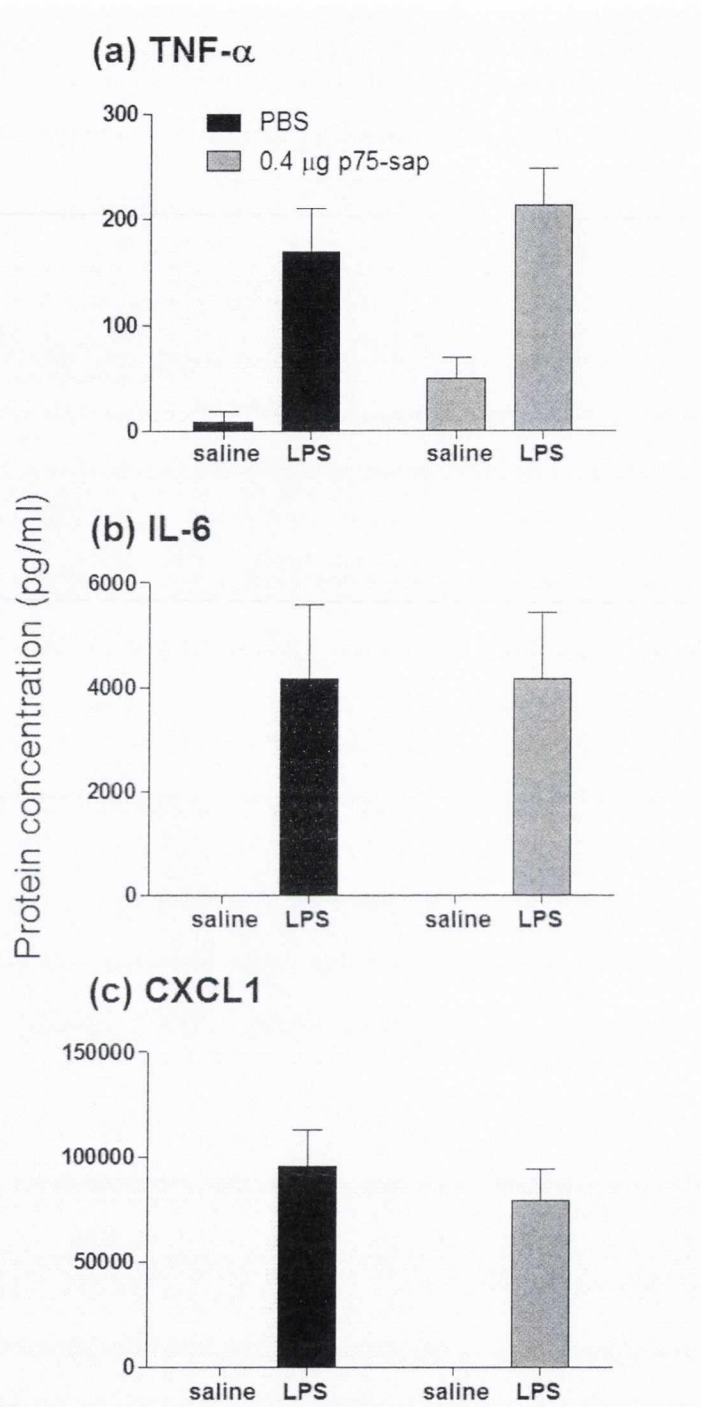


Figure 3.23 Plasma cytokine profile following systemic LPS challenge. Plasma levels of (a) TNF- α , (b) IL-6, and (c) CXCL1 were measured by sandwich ELISA in PBS- and 0.4 μ g mu-p75-sap-injected animals, 14 days post-insult and 3 hours after systemic LPS challenge (100 μ g/kg). Two-way ANOVA followed by Bonferroni post-hoc tests revealed that peripheral cytokine responses to LPS were not exaggerated in cholinergic lesioned animals ($p > 0.05$). Data are expressed as mean $\pm$ SEM ($n = 4$ for PBS groups, $n = 5$ for mu-p75-sap + saline, $n = 6$ for mu-p75-sap + LPS)

3.2.13.7 Cholinergic depletion in 0.4 µg mu-p75-sap-lesioned animals, 14 days post-lesion

The extent of basal forebrain cholinergic depletion produced by this more limited lesion (0.4 µg mu-p75-sap) was assessed 14 days post-injection by qPCR for mRNA transcripts for a number of cholinergic markers. Transcripts were measured in the medial septum and the hippocampus.

Student's *t*-test analysis revealed that ChAT expression was significantly decreased in the medial septum of mu-p75-sap-injected animals compared to PBS controls ($t = 2.55$, $df = 17$, $p = 0.0207$) (Figure 3.24a).

Hippocampal expression of the acetylcholine receptors, $\alpha 7$ -nAChR and M1-mAChR, were found to be unchanged at 14 days following 0.4 µg mu-p75-sap lesion. Student's *t*-test analysis revealed that hippocampal $\alpha 7$ -nAChR expression was not significantly altered by basal forebrain cholinergic loss ($t = 0.7294$, $df = 17$, $p = 0.4757$) (Figure 3.24b). Similarly, Student's *t*-test analysis revealed that hippocampal M1-mAChR expression was not affected by cholinergic lesion ($t = 1.047$, $df = 17$, $p = 0.3099$) (Figure 3.24c). It is, however, of interest that there was a trend towards increased M1-mAChR expression in these animals at 14 days post-lesion when higher doses decreases expression of this receptor at 40 days (Figure 3.18).

Thus, 0.4 µg mu-p75-sap caused approximately 30% loss of ChAT expression in the medial septum without significant reduction in acetylcholine receptor expression in the hippocampus.

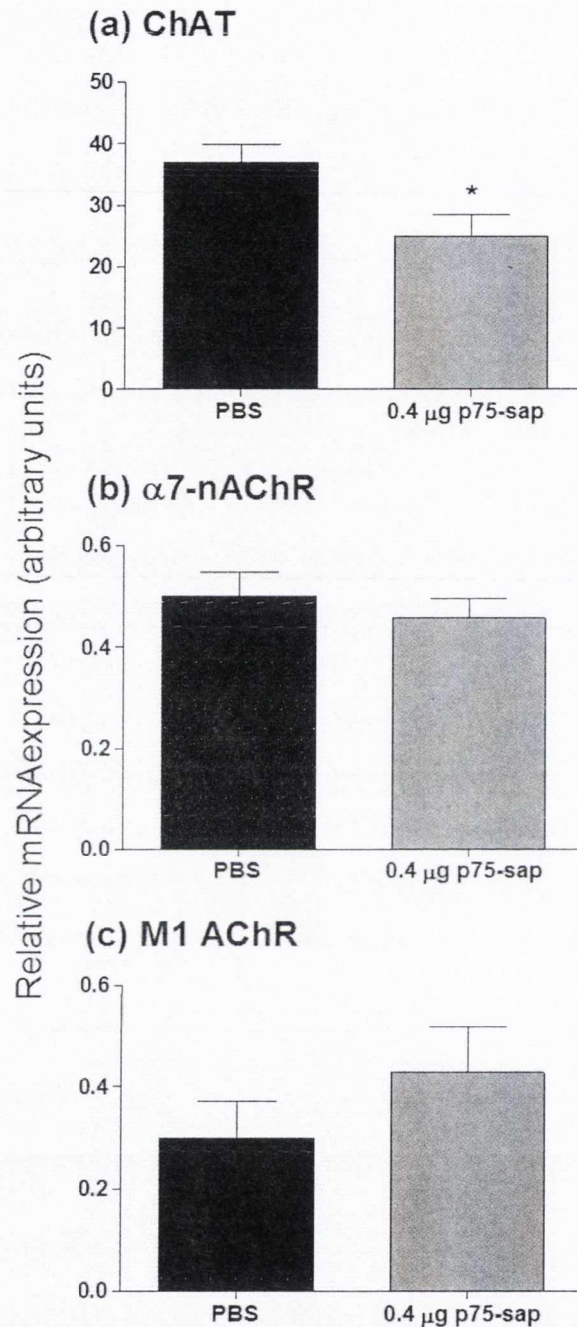


Figure 3.24 Basal forebrain cholinergic depletion in lesioned animals. Degeneration of basal forebrain cholinergic neurons in mu-p75-sap-injected animals (0.4 μg) was assessed 14 days post-lesion by measuring expression of mRNA transcripts for (a) ChAT in the medial septum, (b) α7-nAChR in the hippocampus and (c) M1 AChR in the hippocampus. Significant differences to PBS-injected controls by Student's *t*-test are denoted **p* < 0.05. Data are expressed as mean ± SEM (n = 8 for PBS, n = 11 for mu-p75-sap)

3.2.13.8 Inflammatory response to systemic LPS in the basal forebrain of 0.4 µg mu-p75-sap-lesioned animals, 14 days post-lesion

Following the observation that this more limited lesion produced approximately 30% depletion of cholinergic cells, evidence for microglial priming in the medial septum/diagonal band was assessed in these animals (0.4 µg mu-p75-sap, 14 days post-lesion, 100 µg/kg LPS). A number of inflammatory transcripts were measured by qPCR 3 hours post-LPS.

Transcription of IL-1 β was robustly induced in the medial septum in animals with cholinergic deficits compared to PBS-injected controls (Figure 3.25a). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 4.84$, $df\ 1,15$, $p = 0.0439$), and of LPS ($F = 8.59$, $df\ 1,15$, $p = 0.0103$). There was no interaction between these two factors. Bonferroni post-hoc comparisons revealed that IL-1 β was induced to significantly greater levels by LPS in mu-p75-sap-lesioned animals compared to PBS controls ($p < 0.05$).

TNF- α expression did not appear to be induced to different levels in medial septum of lesioned versus non-lesioned animals (Figure 3.25b). Two-way ANOVA analysis revealed a main effect of LPS ($F = 10.07$, $df\ 1,15$, $p = 0.0063$) but not of cholinergic lesion. There was no interaction between these two factors. Bonferroni post-hoc comparisons revealed that there were no significant differences in TNF- α induction by LPS in mu-p75-sap-lesioned animals compared to PBS ($p > 0.05$).

There was no differential induction of IL-6 expression in the medial septum of mu-p75-sap versus PBS animals following systemic LPS challenge (Figure 3.25c). Two-way ANOVA analysis revealed a main effect of LPS ($F = 24.07$, $df\ 1,15$, $p = 0.0002$) but not of cholinergic lesion. There was no interaction between these two factors. Bonferroni post-hoc comparisons revealed IL-6 induction in LPS-challenged mu-p75-sap and PBS animals was not significantly different ($p > 0.05$).

CXCL1 transcription was induced by LPS to similar levels in both mu-p75-sap and PBS animals (Figure 3.25d). Two-way ANOVA analysis revealed a main effect of LPS ($F = 55.44$, $df\ 1,15$, $p < 0.0001$) but not of cholinergic lesion and no interaction. Bonferroni post-hoc comparisons revealed no significant differences between LPS challenged animals ($p > 0.05$).

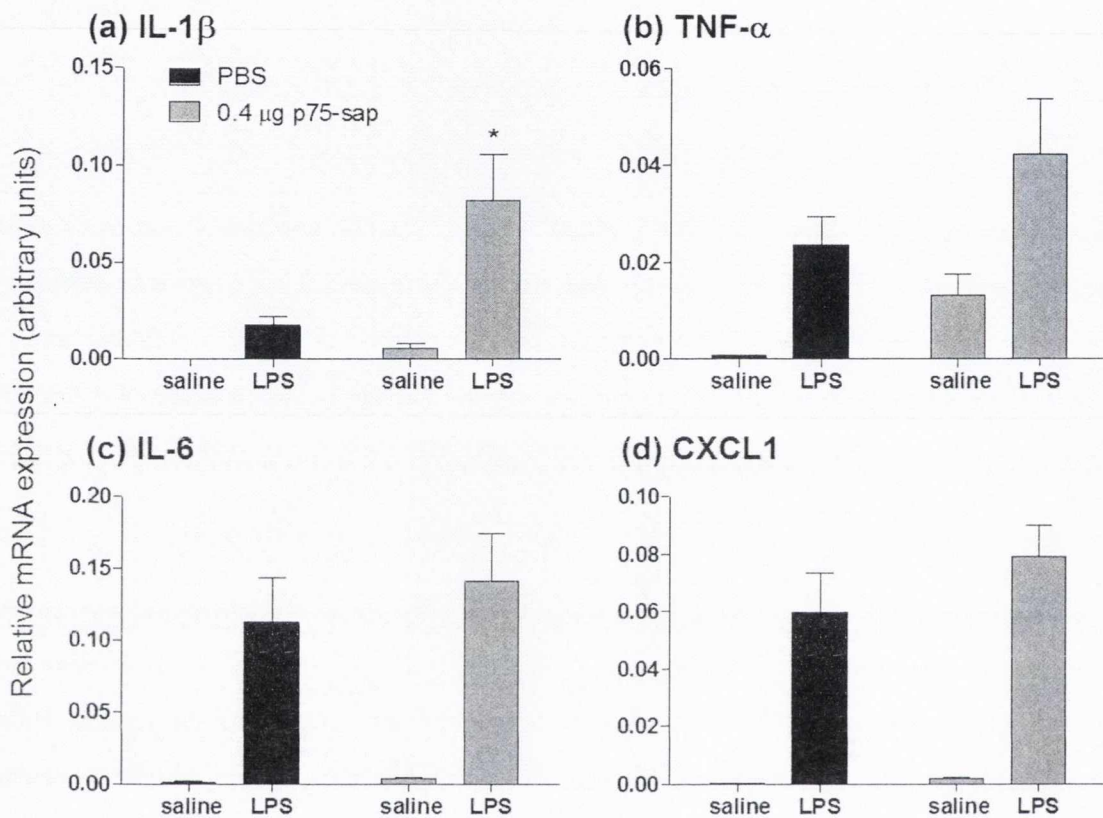


Figure 3.25 Inflammatory response to systemic LPS in the medial septum of 0.4 μg mu-p75-sap-lesioned animals. mRNA expression of (a) IL-1β, (b) TNF-α, (c) IL-6 and (d) CXCL1 was measured by qPCR in the medial septum of PBS- and 0.4 μg mu-p75-sap-injected animals, 14 days post-insult and 3 hours after systemic LPS challenge (100 μg/kg). Bonferroni post-hoc tests following two-way ANOVA analyses are denoted * $p < 0.05$. Data are expressed as mean $\pm$ SEM (n = 4 for PBS groups, n = 5 for mu-p75-sap. + saline, n = 6 for mu-p75-sap + LPS)

3.2.13.9 Inflammatory response to systemic LPS in the hippocampus of 0.4 µg mu-p75-sap-lesioned animals, 14 days post-lesion

The above analysis in the basal forebrain indicated that this more limited lesion (0.4 µg) produced microglial priming at an acute time-point following lesion, but only to a limited degree, with moderately increased IL-1 β transcription in lesioned compared to normal animals. It was hypothesised that loss of cholinergic innervation in the hippocampus, the major projection area from the basal forebrain, would prime microglia in this region to produce exaggerated responses to subsequent inflammatory stimuli. Thus, in the hippocampi of the same animals (0.4 µg mu-p75-sap, 14 days post-lesion, challenged peripherally with LPS (100 µg/kg i.p.)) we assessed whether microglia were primed by basal forebrain cholinergic depletion. Inflammatory gene transcription was measured by qPCR and ELISA, 3 hours post-LPS.

Hippocampal IL-1 β mRNA expression after systemic LPS appeared to be induced more robustly in mu-p75-sap-lesioned animals than in PBS animals (Figure 3.26a). Two-way ANOVA analysis revealed a main effect of LPS ($F = 4.77$, df 1,15, $p = 0.0453$) and of cholinergic lesion ($F = 4.60$, df 1,15, $p = 0.0487$) but no interaction between these two factors. Although IL-1 β induction was greater in mu-p75-sap-lesioned animals compared to PBS controls, there was considerable variability and Bonferroni post-hoc comparisons revealed this difference was not statistically significant ($p < 0.05$).

Systemic LPS did not induce exaggerated expression of TNF- α in the hippocampus of mu-p75-sap animals compared to PBS (Figure 3.26b). Two-way ANOVA analysis revealed no main effects and no interactions.

Hippocampal IL-6 transcription was induced to similar levels by LPS in mu-p75-sap and PBS controls (Figure 3.26c). Two-way ANOVA analysis revealed a main effect of LPS ($F = 12.79$, df 1,15, $p = 0.028$) but no effect of cholinergic lesion and no interaction. Bonferroni post-hoc comparisons revealed that IL-6 induction was not significantly different after LPS in mu-p75-sap-lesioned animals compared to PBS controls ($p > 0.05$).

Transcription of CXCL1 was not differentially induced in the hippocampus of mu-p75-sap animals compared to PBS following systemic LPS (Figure 3.26d). Two-way ANOVA analysis revealed a main effect of LPS ($F = 8.34$, df 1,16, $p = 0.0107$) but not of cholinergic lesion and no interaction between these two factors. Bonferroni post-hoc

comparisons revealed that induction of CXCL1 was not significantly elevated in mu-p75-sap-lesioned animals compared to PBS controls after LPS challenge ($p > 0.05$).

PTX3 induction after systemic LPS in the hippocampus of mu-p75-sap animals appeared higher than in PBS animals but this induction was not significant (Figure 3.26e). Two-way ANOVA analysis revealed no main effects or interactions.

Similarly, hippocampal expression of TNFAIP2 appeared to be induced more robustly by LPS in mu-p75-sap animals compared to PBS, but this induction was not significant (Figure 3.26f). Two-way ANOVA analysis revealed no main effects or interactions.

Hippocampal IL-1 β protein production appeared to be induced by LPS to greater levels in mu-p75-sap-lesioned animals compared to PBS controls, although this was not statistically significant (Figure 3.27). Two-way ANOVA analysis revealed no main effects or interaction. It seems likely that the variability in response accounts for the lack of statistical significance but it does seem plausible that IL-1 β protein is increased in lesioned animals to levels that could be deleterious to the brain.

It appears that a limited basal forebrain cholinergic lesion (0.4 μ g mu-p75-sap) does not prime the brain to produce exaggerated responses to subsequent peripheral LPS challenge. 14 days after neurotoxic insult. There is a trend towards an amplified response in lesioned animals but statistics do not bear this out, possibly due to variability in the lesion.

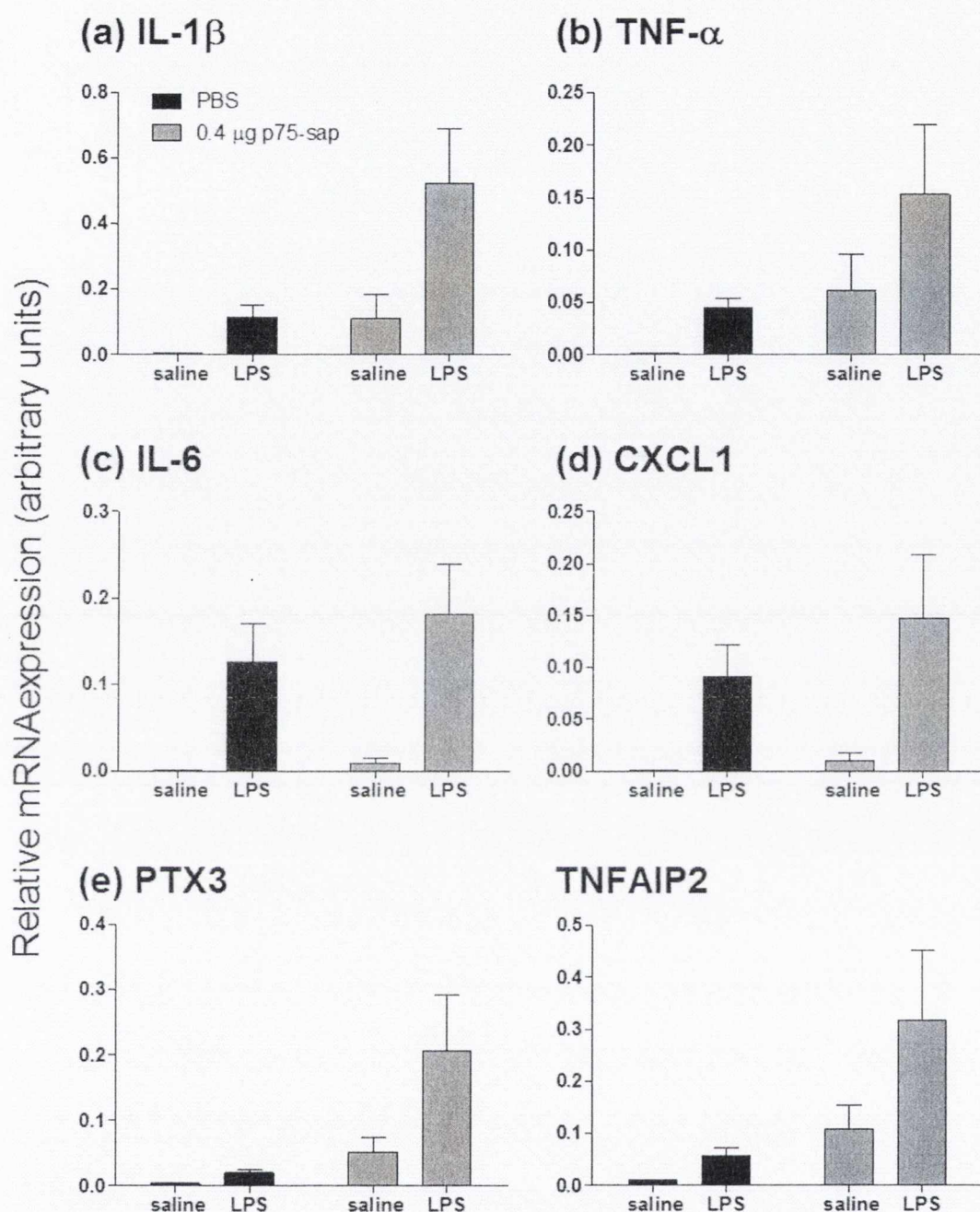


Figure 3.26 LPS-induced inflammation in the hippocampus of 0.4 µg mu-p75-sap-lesioned animals. mRNA expression of (a) IL-1 β , (b) TNF- α , (c) IL-6, (d) CXCL1, (e) PTX3 and (f) TNFAIP2 was measured by qPCR in the hippocampus of PBS- and 0.4 µg mu-p75-sap-injected animals, 14 days post-insult and 3 hours after systemic LPS challenge (100 µg/kg). Two-way ANOVA analyses are described in the main text. Data are expressed as mean $\pm$ SEM (n = 4 for PBS groups, n = 5 for mu-p75-sap + saline, n = 6 for mu-p75-sap + LPS)

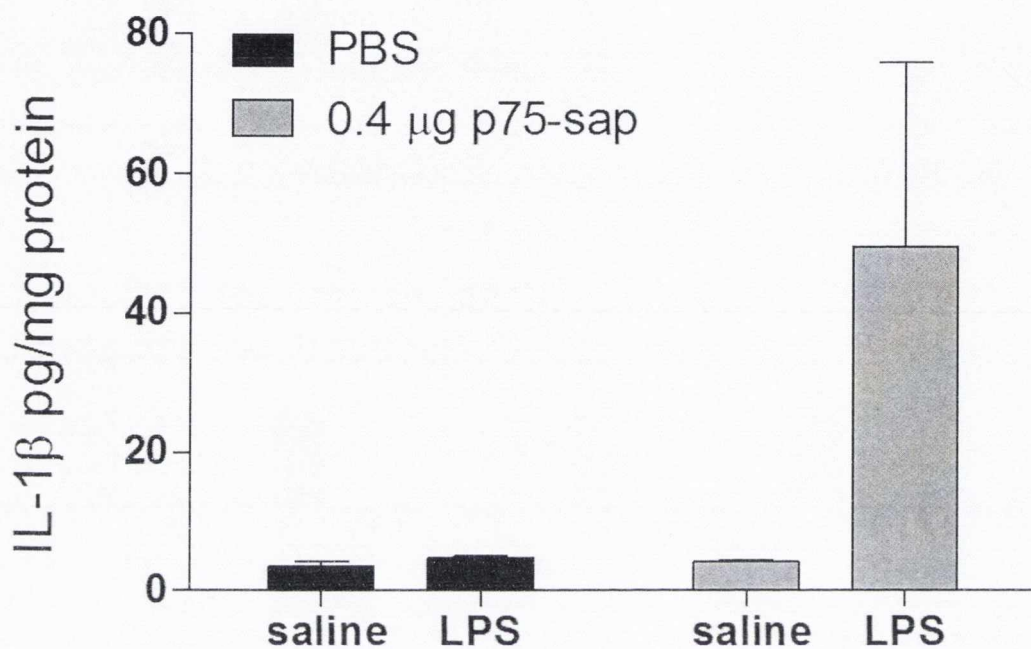


Figure 3.27 Systemic LPS induced IL-1 β protein in hippocampus of mu-p75-sap-lesioned animals. Hippocampal IL-1 β protein production was assessed in PBS- and 0.4 μ g mu-p75-sap-injected animals, 40 days post-lesion and 3 hours after systemic LPS challenge (100 μ g/kg i.p.). Two-way ANOVA analysis is described in the main text. Data are expressed as mean $\pm$ SEM (PBS + saline n = 4, PBS + LPS n = 4, p75-sap + saline n = 5 and p75-sap + LPS n = 6)

3.2.13.10 Microglial activation in 0.4 µg mu-p75-sap animals, 14 days post-lesion

The acute CNS microglial profile was examined in the medial septum and hippocampus, 14 days following a 0.4 µg mu-p75-sap-induced lesion of the basal forebrain cholinergic neurons. The upregulation of myeloid-specific scavenger receptors markers that are expressed on microglia were analysed by qPCR, 3 hours following systemic LPS challenge (100 µg.kg i.p.).

Expression of SRA2 appeared to be induced by cholinergic lesion in the medial septum, with no further induction following peripheral LPS (Figure 3.28a). Two-way ANOVA analysis, however, revealed no main effects or interactions. The lack of main effect of lesion despite a clear trend towards an increased microglial response could be a result of variability in quality of cholinergic lesions.

SRA2 transcription in the hippocampus was induced by cholinergic lesion but was not further affected by LPS challenge (Figure 3.28b). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 5.03$, df 1,15, $p = 0.0405$). There was no effect of LPS and no interaction.

Expression of the microglial activation marker, CD68, was induced in the medial septum by basal forebrain cholinergic lesion and unaffected by LPS challenge (Figure 3.28c). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 5.82$, df 1,15, $p = 0.0291$). There was no effect of LPS and no interaction between the two factors.

Hippocampal expression of CD68 was induced by mu-p75-sap-induced cholinergic lesion but was not further affected by LPS (Figure 3.28d). Two-way ANOVA analysis revealed a main effect of cholinergic lesion ($F = 7.88$, df 1,15, $p = 0.0133$). There was no effect of LPS and no interaction between the two factors.

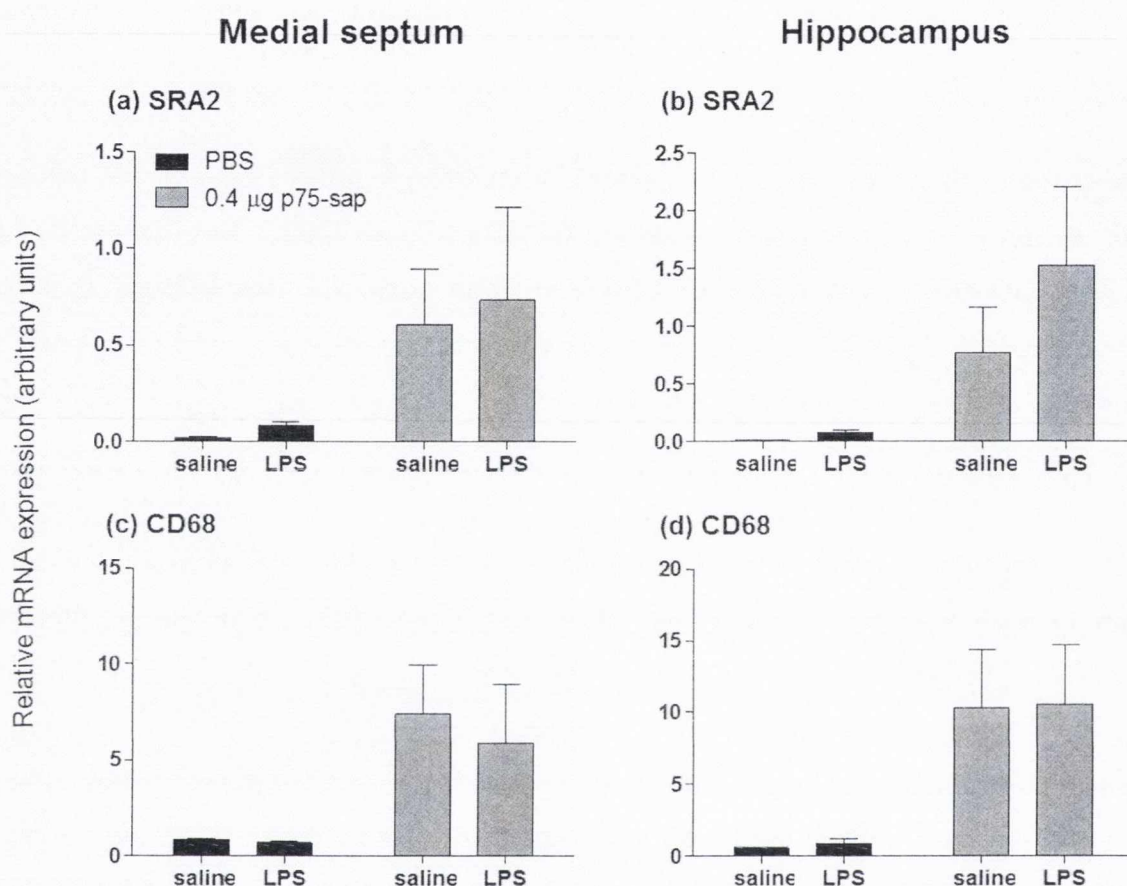


Figure 3.28 Microglial activation following systemic LPS in *mu-p75-sap*-lesioned and normal animals. mRNA expression of SRA2 (a-b) and CD68 (c-d) was measured by qPCR in the medial septum and hippocampus of PBS- and 0.4 µg *mu-p75-sap*-injected animals, 14 days post-insult and 3 hours after systemic LPS challenge (100 µg/kg). Data were analysed by two-way ANOVA and are expressed as mean ± SEM (n = 4 for PBS groups, n = 5 for *mu-p75-sap* + saline, n = 6 for *mu-p75-sap* + LPS)

3.2.13.11 Microglial production of IL-1 β following systemic LPS in 0.4 μ g mu-p75-sap animals, 14 days post-lesion

A small subset of 0.4 μ g mu-p75-sap-lesioned animals, 14 days post-lesion, were challenged peripherally with LPS (100 μ g/kg i.p.) or sterile saline and examined immunohistochemically for production of IL-1 β , 3 hours following systemic challenge. There was no detectable production of IL-1 β in the medial septum of mu-p75-sap animals challenged with saline (Figure 3.29a). IL-1 β was however, detectable by immunohistochemical labelling in the medial septum of mu-p75-sap-lesioned animals challenged with LPS (Figure 3.29b). IL-1 β -positive cells showed microglial morphology (inset in Figure 3.29b).

This preliminary investigation with small numbers of animals supports the brain ELISA data that is suggestive of increased IL-1 β protein in the medial septum following systemic inflammatory challenge, but only in those animals with a prior cholinergic lesion. Further studies with increased animal numbers and different severity of lesion at various time-points will be necessary to confirm this finding.

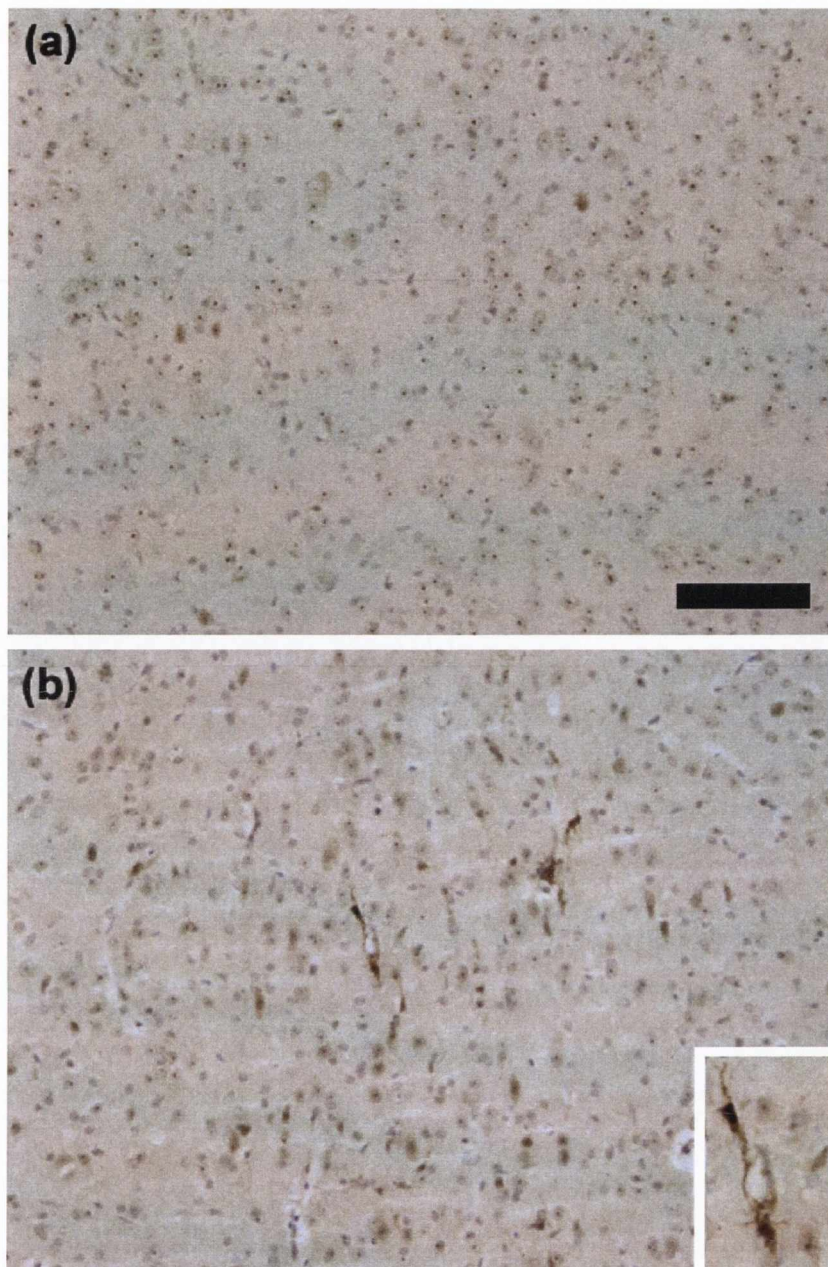


Figure 3.29 Systemic LPS induces microglial IL-1 β production in *mu-p75-sap*-lesioned animals. Representative photomicrographs of immunohistochemical labelling for IL-1 β in the medial septum of 0.4 μ g p75-sap-injected animals challenged systemically with either (a) sterile saline or (b) LPS (100 μ g/kg i.p.). Immunohistochemistry was performed 14 days post-insult and 3 hours after systemic LPS challenge. Inset highlights the apparent microglial morphology of IL-1 β -positive cells. Scale bar = 100 μ m.

3.3 Discussion

The aim of this study was to produce a lesion of the basal forebrain cholinergic system of limited extent in order to mimic the early stages of dementia-associated cholinergic neuronal loss. Using this background of existing neuropathology as a pre-disposing risk factor, the aim was to create a novel animal model of inflammation-induced delirium. The current study has shown that moderate cholinergic hypofunction confers increased susceptibility to systemic inflammation-induced acute working memory deficits. These deficits present independent of microglial priming and can be protected against by acetylcholinesterase inhibition. The presence or absence of microglial priming is dependent on both the severity of cholinergic depletion along with the time post-surgery. Thus, these data demonstrate an important interaction between prior cholinergic pathology and subsequent systemic inflammatory insults, and offer a novel animal model in which to study aspects of the pathophysiology of delirium during dementia, with implications for the reconciliation of inflammatory and cholinergic hypotheses of delirium.

3.3.1 Limited lesions of the basal forebrain cholinergic system

Much of the previous work from our laboratory has used the ME7 strain of murine prion disease as a model in which to study the interaction of systemic inflammation with chronic neurodegenerative disease. This has also provided a key platform to study progressive neurodegenerative disease as a risk factor for developing delirium induced by inflammatory insults. The phenomenon of microglial priming was first described in the ME7 model (Cunningham et al., 2005b), whereby exaggerated CNS responses to moderate systemic inflammation with LPS manifest during chronic neurodegeneration and this has been repeated with systemic ‘viral’ stimulation with poly I:C in chapter 4. This has also facilitated the development of the first animal model of delirium during dementia (Murray et al., 2012) along with investigation into the mechanisms that underlie the acute cognitive deficits that occur when peripheral inflammation is superimposed upon existing neurodegenerative disease. The ME7 model of prion disease represents one of the best animal models available with which to examine the microglial response to genuine progressive neurodegenerative disease with robust amyloidosis, synaptic loss, neuronal loss as well as progressive affective, cognitive and neurological deficits and eventual death. However, it has become important to take our observations on microglial priming and on susceptibility to cognitive deficits out of the prion disease setting and replicate them in a neuropathological setting which more closely mirrors other aspects of dementia in the

geriatric patient population. To address this, a major aim of the current study was to produce, in an animal, one key aspect of neuropathology that occurs in a very large proportion of human dementia cases and that could be then used to create a susceptible state for infection-induced delirium. Thus, the current body of work aimed to produce limited selective lesions of the basal forebrain cholinergic system, a region which shows significant degeneration in dementia and AD (Davies and Maloney, 1976, Whitehouse et al., 1982).

To achieve this, the mu-p75-sap immunotoxin was used to selectively lesion p75-neurotrophin receptor-expressing cholinergic neurons of the basal forebrain. This novel version of the ribosomal toxin, saporin, is bound to an antibody directed against the mouse p75-neurotrophin receptor and thus selectively targets basal forebrain cholinergic neurons in mice (Berger-Sweeney et al., 2001). This toxin has been most widely used to ablate this population of neurons to study the role of the basal forebrain cholinergic system in cognitive function (Hunter et al., 2004, Moreau et al., 2008). Groups that have used higher doses of this toxin (3.6 μ g and 7.1 μ g) have reported some non-selective cell loss and cerebellar Purkinje cell damage associated with impairments in motor function (Berger-Sweeney et al., 2001), which could prove to be confounding factors for cognitive tasks. Indeed, these higher doses of toxin destroy about 80-90% of ChAT-positive neurons but also prove lethal to mice. The current study therefore tested muscle strength and motor coordination to ensure that the dose of mu-p75-sap used here was not producing lesions so severe as to induce such motor deficits. Motor deficits could confound the interpretation of many cognitive tasks. However, the T- and Y-maze paradigms used here have been specifically designed to allow us to assess cognitive performance simply by correct and incorrect responses rather than speed of responding or other parameters that could complicate the interpretation of data arising from animals experiencing acute sickness behaviour (post-LPS) or experiencing motor deficits (due to non-specific saporin-induced neuronal loss). Thus we have addressed both the severity of lesions and the nature of cognitive tasks to ensure that our cognitive measures are robust for the model system in use. The selectivity of mu-p75-sap was also assessed by performing immunohistochemical labelling and cell counting for the neuronal nuclei marker, NeuN, in the intermediate lateral septum, which is not known to be populated by cholinergic neurons. This region lies proximal to the ventricular system into which the immunotoxin is injected and is therefore directly exposed to the circulating toxin. Neuronal numbers were not different between PBS-injected controls and mu-p75-sap-lesioned animals, confirming that the dose used in

the current study did not produce measureable non-selective neuronal loss in proximal regions. Since the aim of the current body of work was to mimic the early stages of dementia-associated cholinergic loss, a limited lesion of basal forebrain cholinergic neurons was induced, using a significantly lower dose of this toxin than used elsewhere in the literature.

To verify the extent of the lesions produced, evidence for loss of cholinergic neurons has been provided by three distinct methods. Decreased ChAT-positive cell numbers were reported in the medial septum and ventral diagonal band, along with decreased ChAT mRNA expression, thus confirming depletion of this neuronal population. It is worth noting that the ChAT mRNA levels mirror the quantified loss of ChAT-positive cells quite well, thus indicating that these mRNA levels provide a reasonable estimate of the degree of neuronal loss, using a technique (qPCR) that is significantly more time-efficient than by traditional neuropathology. AChE enzyme activity was also quantified in the hippocampus, where the axons of septo-hippocampal cholinergic neurons terminate (Mesulam et al., 1983). Since the hippocampus receives considerable input from the basal forebrain, ChAT pre-synaptic terminal fields were also examined in mu-p75-sap-lesioned animals and preliminary qualitative assessment revealed clear decreases in synaptic ChAT, indicative of reduced acetylcholine production and cholinergic input (Perry et al., 1977). Previous lesion studies of basal forebrain cholinergic system in rodents have ablated the entire population of cholinergic cells in this brain region (Berger-Sweeney et al., 2001, Vuckovich et al., 2004, Nag et al., 2009). The novelty of the current model lies in the creation of a lesion of limited extent, such that it is more biologically relevant to early human dementia than other studies. The current data illustrate approximately 20% depletion of cholinergic neurons in the basal forebrain, following mu-p75-saporin lesion (0.08 µg). The success of this limited cholinergic lesion is further supported by the loss of hippocampal AChE enzymatic activity.

Animals with moderate cholinergic hypofunction (20% depletion) retain the ability to perform cognitive tests of visuo-spatial and working memory, consistent with prior reports of selective cholinergic lesions in rats (Wrenn et al., 1999). Only with lesions showing greater than 50% loss of cholinergic function do cognitive impairments manifest in the Morris water maze task (Berger-Sweeney et al., 2001). This study from the Baxter group also reported a positive correlation between hippocampal ChAT activity and spatial learning ability. Consistent with this, the current study showed a positive correlation

between ChAT-positive neuronal numbers in the medial septum and visuo-spatial reference memory capability in the Y-maze. Even with the higher dose of the mu-p75-sap immunotoxin (0.4 µg/mouse), animals are still capable of learning the Y- and T-maze tasks, although they appear to do so at a slightly slower rate. By design, this work aimed to create a limited lesion, that of itself would not impair cognitive function, and therefore this higher dose was not used for subsequent cognitive studies. This higher dose will continue to be used however, for mechanistic studies investigating the acute inflammatory response to cholinergic neuronal death and the influence of cholinergic tone on microglial activation due to the production of more robust cholinergic loss.

The higher dose of mu-p75-sap (0.4 µg) used in these cognitive studies remains considerably less than the doses used in the majority of the literature (Berger-Sweeney et al., 2001, Vuckovich et al., 2004, Moreau et al., 2008, Nag et al., 2009). Indeed, much of the cholinergic lesion literature in both the mouse and the rat has used high doses of toxin to completely ablate the basal forebrain cholinergic nuclei in order to examine its function in various aspects of learning and memory (Baxter et al., 1995). Though the current chapter showed robust cholinergic neuronal loss, both quantitative neuropathology and expression of ChAT mRNA in the later priming studies indicated that 0.4 µg of mu-p75-sap only produced approximately 30% depletion of ChAT levels. Thus it is likely that such moderate depletion, while capable of impeding the rate of learning does not actually impair the ability to learn the Y- and T-maze tasks.

By design, the Y-maze reference memory task is a simple hippocampal-dependent task that essentially all animals are capable of learning, given sufficient training. If an animal were to show impairments in Y-maze acquisition, then it is very likely that this animal would not be capable of learning the more challenging hippocampal-dependent T-maze working memory task. The current studies demonstrated that cholinergic-lesioned animals at both low and higher doses successfully learned the Y-maze reference memory task, despite the higher dose animals requiring more training time. This suggested that both groups would also be capable of T-maze acquisition, as was also demonstrated.

That the T-maze did not demonstrate any statistically significant differences between animal groups with varying levels of cholinergic depletion may also relate to the task itself. Most previous T-maze alternation tasks have been based on food reward (Deacon and Rawlins, 2006), however our task requires the mouse to alternate for escape from shallow

water in the T maze. Alternation for escape rather than for food is probably not an innate behaviour in mice and requires a considerable period of training, such that even some normal animals do not reach the defined learning criteria (as stated in methods section 2.2.5.4). This introduces a level of variability in the learning curves for all treatment groups. Since figure 3.2 shows all animals that were trained in the T-maze, it includes animals in each of the three groups that ultimately did not learn the task and therefore dissecting differences due to relatively subtle differences in cholinergic contribution becomes more difficult. Only animals reaching criterion were challenged in subsequent acute experiments.

The other obvious explanation for a lack of impairment in T-maze acquisition following a cholinergic lesion is the presence of compensatory mechanisms, whereby another brain system adopts the role of the pathways compromised by the cholinergic degeneration. For example there is evidence that noradrenaline, which also projects to the hippocampal formation, is involved in attentional, learning and memory processes (Murchison et al., 2004). Nonetheless, given the assumed role of the basal forebrain cholinergic system in learning, memory and attention (Baxter and Chiba, 1999, Hasselmo, 2006), it has been surprising that immunolesions have had relatively mild and indeed variable effects on learning and memory in experimental studies. A key review from the Baxter group has suggested that while the septohippocampal cholinergic pathway is indeed involved in learning and memory, it might not be absolutely necessary (Parent and Baxter, 2004). A re-evaluation of the role of the basal forebrain cholinergic system in these processes is thus necessary. Some groups have reported impairments following cholinergic lesion in mice (Moreau et al., 2008) while other studies have failed to show deficits in learning and memory in rats (Kirby and Rawlins, 2003), even with 90% reduction in hippocampal ChAT activity (Baxter et al., 1995). Clearly, the basal forebrain cholinergic system is involved in learning and memory, however with complete lesions, and a consequent absence of cholinergic input, it seems likely that compensatory mechanisms can play a role. Thus the cholinergic system may become redundant for our T-maze working memory task. However, if animals learn the T-maze with a mild cholinergic lesion that leaves the basal forebrain cholinergic system relatively intact, it is likely that the cholinergic system still modulates working memory and that any subsequent acute disruption in function of the basal forebrain cholinergic system may still have significant effects on performance of the task, as was observed from the subsequent experiments where lesioned animals were challenged with LPS.

One important experiment to conduct will be to train intact animals on the T-maze alternation task, subsequently lesion the cholinergic system and then assess post-operative working memory. Since the current study employs a training strategy commencing approximately 20 days following mu-p75-sap injection, it is possible that T-maze acquisition would be modulated by non-cholinergic systems that compensate for the cholinergic degeneration as discussed above. Training animals prior to induction of cholinergic lesion and then returning the animals the T-maze a short time after surgery (4-7 days) would allow us to assess the importance of these basal forebrain cholinergic pathways in this working memory paradigm. This would allow for the delineation of the cholinergic requirement for both learning the basis of correct responding in this maze and for continued working memory performance, and should allow insights into how the brain adapts in the absence of such a system.

3.3.2 Acute inflammatory response to cholinergic lesions

It would be predicted that a discrete lesion of any population of neurons would result in an acute inflammatory response to the dying cells, likely characterised by activation of the microglial population proximal to these neurons and elevated cytokine synthesis. Thus the innate inflammatory response to a selective lesion of basal forebrain cholinergic neurons was investigated. This has been conducted over a number of different time-points following stereotaxic lesions, in order to construct a temporal profile of inflammatory changes that occur. There is an acute innate immune response to cholinergic neuronal death in the medial septum, and this is characterised by elevated expression of pro-inflammatory mediators IL-1 β , TNF- α , IL-6 and CXCL1. This is consistent with activation of innate immune mediators during any type of CNS trauma or acute injury (Rothwell and Luheshi, 2000, Lucas et al., 2006). Interestingly, there is also a marked type I IFN response to acute cholinergic neurodegeneration in the mu-p75-sap model. There is clear induction of IFN- β along with IRF7, Mx1 and PKR, whose expression is directly dependent on type I IFN production (Staeheli et al., 1986, Hovanessian, 1989, Honda and Taniguchi, 2006). Typically associated with the immune response to viral infections, type I IFNs are generally viewed as beneficial anti-inflammatory mediators in the brain due to their therapeutic efficacy during MS (Billiau, 2006) but as will be discussed in chapters 4 and 5, an increased type I IFN response can be associated with deleterious consequences for the brain. The current data demonstrate that the type IFN response is also induced by sterile neurotoxic cell death, while further data in chapter 5 demonstrate an increase in type I

IFNs associated with a chronic infectious neurodegenerative disease. This supports recent data elsewhere that have demonstrated induction of the type I IFN response in microglia following sterile axonal injury (Khorrooshi and Owens, 2010, Hosmane et al., 2012). Given the anti-inflammatory role for type I IFNs, it seems plausible that in the context of acute cholinergic neuronal death and concurrent increases in pro-inflammatory gene transcription, that these mediators are acting to restore the basal inflammatory phenotype in the brain.

There is also evidence of increased microglial activation following induction of basal forebrain cholinergic lesions. Increased expression of the macrophage scavenger receptor SRA2 and the endosomal/lysosomal marker CD68 was observed in the acute stages following cholinergic lesion, indicative of microglial phagocytosis of neuronal debris. This is consistent with previous data in an animal model of Wallerian degeneration, in which transected axons degenerate and local microglia become phagocytically active to effect removal of axonal and myelin debris (Palin et al., 2008). A recent study demonstrated that microglial phagocytosis of damaged brain tissue is mediated by TRIF (Hosmane et al., 2012), and given the type I IFN response reported currently, this might also be the case here. Previous studies have also demonstrated evidence of acute microglial activation in response to cholinergic lesions in the medial septum 3 to 7 days after immunotoxic lesion in both rats and mice (Rossner et al., 1995, Hunter et al., 2004). This observation is repeated here, as demonstrated by IBA-1 immunolabelling which showed morphological evidence of microglial activation in both the medial septum and dentate gyrus region of the hippocampus. This activation was detected acutely (6 days) only in mu-p75-sap-lesioned animals and showed resolution in both regions by 40 days when the 0.4µg dose of mu-p75-saporin was used. It is important to note that microglial activation is not restricted to the lesion site, but extends to the hippocampus, where there is degeneration of synaptic terminal fields of septo-hippocampal projection neurons.

The acute inflammatory response evoked by basal forebrain cholinergic lesions was evident at 3 and 7 days post-lesion and showed full resolution to baseline levels by 35-40 days. This timecourse of inflammation is consistent with reports that the neuronal death produced by mu-p75-sap occurs between day 3 and day 14 post-injection (Hunter et al., 2004). Cholinergic neuronal depletion has been confirmed by the three measures described above and not through the use of immunohistochemistry for apoptotic markers due to the subtle nature of the lesion produced. One would not expect to produce large synchronous

cell death events as might be observed with more severe lesions. The observation that acute inflammation shows resolution by 28-35 days is important since cognitive experiments have been conducted at this time-point following lesion. Thus the current study has shown that there is an acute inflammatory reaction mediated by microglia, in response to dying cholinergic neurons that has resolved by the time of the LPS-induced cognitive deficits.

3.3.3 Cognitive deficits without microglial priming – what is the underlying mechanism?

It has previously been shown, in an animal model of progressive neurodegenerative disease, namely the ME7 model of murine prion disease, that microglia are primed by the underlying pathology such that subsequent inflammatory stimulation with LPS induces an exaggerated CNS response (Cunningham et al., 2005b), and this observation has been repeated with poly I:C in chapter 4 of this thesis. Our laboratory has developed one of the first animal models of delirium, whereby peripheral administration of LPS induces acute and transient working memory deficits only in those animals with underlying neuropathology (Murray et al., 2012). This has recapitulated key facets of the delirium present in vulnerable populations that suffer subsequent systemic inflammatory events. Furthermore, recent data emerging from our laboratory have highlighted the role of IL-1 β in such acute onset cognitive dysfunction, whereby an IL-1 receptor antagonist protects against LPS-induced deficits and systemic IL-1 β itself can induce cognitive deficits in animals with existing prion pathology (Skelly et al., submitted).

The current study has also demonstrated that systemic LPS produced acute and transient cognitive impairment, but only in those animals with prior neurodegeneration. In this case, however, the degeneration is cholinergic. This is an important finding since it indicates that cholinergic hypofunction constitutes a risk factor for systemic inflammation-induced cognitive deficits and that we can now use this model system to ask a number of pertinent questions about the impact of systemic inflammation on the hypocholenergic brain. This is highly analogous to clinical populations such as AD patients that exhibit robust cholinergic neuronal degeneration (Davies and Maloney, 1976, Whitehouse et al., 1982) and that are known to be more susceptible to development of delirious episodes (Fick et al., 2009). The dose of LPS used (100 μ g/kg) is designed to mimic a mild to moderate peripheral infection that induces sickness behaviour but does not cause multi-organ failure associated with severe sepsis. Though sepsis is capable of causing delirium and cognitive dysfunction in

almost any individual (Ebersoldt et al., 2007), much milder inflammation can induced delirium in vulnerable populations such as the elderly and demented (Fong et al., 2009b). Thus the current work adds acute cholinergic hypofunction to chronic neurodegenerative disease (Murray et al., 2012) as a risk factor for inflammation-induced cognitive impairment.

Despite an acute induction of inflammatory markers and microglia activation in response to cholinergic lesions, this shows resolution longitudinally. The septal and hippocampal mRNA expression of inflammatory cytokines after systemic LPS treatment indicates that there is no evidence that the microglia of the basal forebrain or hippocampus were primed by the cholinergic deficit, at the time-point at which the animals were challenged with LPS – 40 days post surgical lesion. Thus either the critical period in which microglia remain primed has passed at this distal time-point from surgery, or the loss of cholinergic function with the dose used for cognitive experiments (0.08 µg) was not great enough to produce a primed response in the first place. Immunohistochemical analysis of microglia also indicates that they are not morphologically activated by the time cognitive experiments are performed, although there is evidence of acute microglial activation which is supported by prior studies (Hunter et al., 2004, Rossner et al., 1995). As will be discussed further below, we now present evidence for both of these possibilities: more severe lesions produce primed microglia and shorter time post-injury means that even at lower doses, microglia become primed for at least a short period.

However, the results from the current study make an important prediction: whilst primed microglia may increase the likelihood of deleterious inflammation-induced cognitive (Murray et al., 2012) and neurological impairments (Cunningham et al., 2009), this exaggerated CNS inflammatory response to peripheral immune stimulation is not essential to produce such deficits. The independence from microglial priming does not imply a lack of CNS inflammatory response to systemic LPS. Rather, it simply means that microglia appear to respond equally to systemic inflammation in ‘limited lesion’ and normal animals. This implies that the vulnerability in these animals represents a neuronal susceptibility to disruption of function. In the absence of a primed response, the loss of cholinergic modulation becomes the obvious candidate for the key predisposing factor and indeed the cholinergic deficiency hypothesis of delirium has previously been proposed (Trzepacz, 2000, Hsieh et al., 2008). Indeed, early animal models of delirium produced cognitive impairments and EEG changes akin to those observed in delirious patients through the

administration of the muscarinic cholinergic antagonist, atropine (Trzepacz et al., 1992, Leavitt et al., 1994). These observations have been repeated more recently by inducing delirium-like behavioural and EEG changes using the anti-cholinergic agent biperiden (Tamura et al., 2006). Cholinergic neuromodulation is important in normal cognitive function (Benarroch, 2010) and is involved in regulation of many of the processes that are disturbed during delirium. Thus, loss of cholinergic neuromodulation can lead to deficits in attention and general cognition (McGaughy et al., 2000). Several lines of evidence have suggested that anti-cholinergic medications can induce delirium in the elderly population (Tune et al., 1981, Han et al., 2001) and serum anti-cholinergic activity has been found to be increased in patients with delirium (Golinger et al., 1987, Flacker et al., 1998, Mussi et al., 1999). One prospective study of elderly patients undergoing elective hip replacement surgery also found that low plasma cholinesterase activity was indicative of reduced cholinergic function and predictive of post-operative delirium (Cerejeira et al., 2011). A recent longitudinal study of delirium in the elderly hip fracture patients reported that despite a delirium-associated increase in serum anti-cholinergic activity, this effect was actually absorbed by the risk factor (surgery) (van Munster et al., 2012). This suggests that an independent relationship of serum anti-cholinergic activity to occurrence of delirium may not be possible and that underlying pre-disposing risk factors remain central to the incidence of delirium.

It is known that prior cognitive impairment and dementia are major risk factors for delirium (Inouye, 2006) and the current data suggest that a loss of cholinergic modulation can leave these individuals more susceptible to the CNS effects of systemic inflammation. Previous studies from our laboratory have shown that increased susceptibility is associated with a significant loss of pre-synaptic terminals (Murray et al., 2012) and this is also a key feature of AD (DeKosky and Scheff, 1990). The current study has importantly highlighted that this increased susceptibility can be associated with different pathologies; in this case with the selective loss of basal forebrain cholinergic tone and consequent loss of input to the hippocampus. It was therefore important to address whether boosting synaptic acetylcholine levels and therefore cholinergic tone would offer protection against the deleterious effects of systemic inflammation on cognitive function in vulnerable individuals.

3.3.4 Cognitive protection by acetylcholinesterase inhibition

We have previously shown that our novel T-maze working memory task is dependent on hippocampal integrity (Murray et al., 2012). Since the current study was based around cholinergic function and loss of tonic inhibition of the inflammatory response it was important to assess whether performance on the T-maze working memory task was dependent on a functional cholinergic system. Antagonism of the M₁ muscarinic cholinergic receptor with scopolamine revealed impairment in T-maze working memory performance, indicating that this task also requires a functional muscarinic cholinergic system. This is in line with prior reports that show cognitive impairments following scopolamine treatment (Spowart-Manning and van der Staay, 2004, Riedel et al., 2009). Although several studies have reported increased locomotor activity following systemic scopolamine administration (Riedel et al., 2009, Klinkenberg and Blokland, 2010), the T-maze alternation task has been designed specifically to simply report correct or incorrect responses, thus minimising the effects of locomotor speed, activity, motivation and anxiety which could otherwise confound such a task.

Acetylcholinesterase inhibitors are a class of pharmacological agents that have been shown to improve cognition and global function in patients with mild to moderate AD (Rogers et al., 1998), and donepezil is currently one of the principal therapeutic options for treating some of the cognitive deficits present in this neurodegenerative disease (Birks, 2006). Animal studies have also demonstrated that short-term memory deficits induced by scopolamine can be reversed by donepezil (Riedel et al., 2009) and that donepezil and physostigmine can ameliorate the behavioural deficits associated with the Tg2576 mouse model of AD (Dong et al., 2005). In the current study, administration of donepezil offered protection against the systemic inflammation-induced working memory deficits in mu-p75-sap-lesioned animals. This suggests that decreased central cholinergic tone may constitute a risk factor for acute cognitive deficits triggered by systemic inflammatory events. It will be important to identify the mechanisms that underpin these deficits, but the data are consistent with the rather straightforward interpretation that hypocholinergic animals have decreased cholinergic reserve, but levels that are nonetheless sufficient for normal cognitive function. However, upon systemic inflammatory challenge they become acutely impaired due, perhaps, to further direct effects of inflammation upon acetylcholine levels. Bolstering these levels with acetylcholinesterase inhibition was sufficient to offer significant protection.

Prior evidence has revealed that both systemic (Rada et al., 1991) and centrally administered (Taepavarapruk and Song, 2010) IL-1 β can directly diminish acetylcholine outflow in the hippocampus. Two studies have shown that this decrease in hippocampal acetylcholine can lead to impairments in working memory performance (Matsumoto et al., 2001, Taepavarapruk and Song, 2010) but the observed deficits can be reversed by cholinesterase inhibition (Matsumoto et al., 2001). The current study shows increased hippocampal IL-1 β mRNA following systemic LPS treatment, with no differential effect in normal and cholinergic lesioned animals. Thus, if central IL-1 β can decrease acetylcholine levels in normal animals (Matsumoto et al., 2001, Taepavarapruk and Song, 2010), and if mu-p75-sap-lesioned animals already have existing cholinergic depletion, we could predict that a further acetylcholine decrease could contribute to the observed cognitive deficits. The current data support this hypothesis as they show a protection against inflammation-induced working memory deficits using donepezil, which increases the availability of synaptic acetylcholine. In the context of the interaction between systemic inflammation and existing neurodegeneration these cognitive deficits are robustly induced by much milder systemic inflammation than are required to induce cognitive changes in normal animals.

It is also plausible that regulation of central inflammation and microglial activation is modulated by tonic inhibition of basal cholinergic function, similar to the cholinergic anti-inflammatory pathway proposed for regulation of peripheral inflammation (Tracey, 2007) and indeed we do show evidence for exaggerated CNS inflammatory responses in cholinergic depleted animals, but it is important to stress that this exaggerated response is not necessary for the cognitive impairments seen with the 0.08 μ g dose used in the cognitive studies. Such microglial priming is, however, predicted to significantly exacerbate cognitive dysfunction.

The interface between systemic inflammation and cholinergic function has become the subject of much debate (Berg and Moller, 2011, de Pont et al., 2011, Frolich and Forstl, 2011, Gusmao-Flores et al., 2011, Opdam et al., 2011) in light of the recent clinical trial of the acetylcholinesterase inhibitor rivastigmine as a treatment for delirium in intensive care patients (van Eijk et al., 2010). This study showed no evidence of resolution of delirium in rivastigmine-treated patients and was stopped prematurely due to increased mortality. The basis for testing rivastigmine was founded both on the well-established neuromodulatory role of acetylcholine, along with its ability to suppress peripheral macrophage function via

the stimulation of the $\alpha 7$ subunit of the nicotinic receptor through the vagus nerve (Pavlov et al., 2006). This work by Tracey and colleagues has shown that loss of inhibitory cholinergic input to the viscera causes more severe TNF- α and nitric oxide responses to systemic LPS. The $\alpha 7$ subunit of the nicotinic acetylcholine receptor has been found to be expressed on microglia (Shytle et al., 2004) and agonism at this receptor subunit has been shown to down-regulate microglial activation (De Simone et al., 2005). It was therefore reasonable to hypothesise that increasing synaptic acetylcholine with an acetylcholinesterase inhibitor could protect the brain from the deleterious effects of systemic inflammation. Since the rivastigmine trial was conducted on critically ill intensive care patients, many of the patients were suffering multi-organ failure from complications of severe sepsis (van Eijk et al., 2010). In such patients, without an existing hypocholinergic disposition, increasing the anti-inflammatory effects of acetylcholine could further suppress the immune response and contribute to the increased mortality, greater severity and longer duration of delirium observed. In short, it is hard to imagine that during a change in homeostasis as severe as septic shock that increasing neuromodulation via the cholinergic system would have very significant effects. Conversely, what the current data illustrates is that with approximately 20% depletion of hippocampal cholinergic innervation, systemic inflammation induces acute and transient cognitive deficits that can be protected against via acetylcholinesterase inhibition, with possible implications for delirium treatment in the geriatric population who show pre-existing cholinergic hypofunction.

3.3.5 Robust cholinergic depletion induces microglial priming.

Systemic inflammation produced acute cognitive deficits only in animals with underlying hypocholinergic neuropathology. These deficits occurred in the absence of microglial priming. While there were robust systemic and CNS inflammatory responses in all LPS-treated animals there was no evidence in hypocholinergic animals of an exaggerated CNS inflammatory response to the systemic insult, supporting the idea that decreased cholinergic reserve and neuromodulation underpins the inflammation-induced deficits. Despite the lack of evidence for microglial priming with using mu-p75-sap at a dose of 0.08 μ g per mouse, we predicted that priming was merely being missed because analyses occurred after resolution of post-lesion microglial responses or that the loss of cholinergic tone was insufficiently robust to induce a lasting state of microglial priming. The findings in this chapter showed that cholinergic lesions do indeed induce acute inflammation in

response to dying cholinergic neurons. This is characterised by elevated pro-inflammatory and type I IFN gene transcription and microglial activation, which resolves by the time cognitive testing was conducted. Thus the reasons for absence of priming might be two-fold: (i) the critical period in which microglia remain primed following acute activation has likely passed at the time of LPS-induced deficits (40 days post-lesion), and (ii) the loss of cholinergic signalling produced with the dose of mu-p75-sap used (0.08 μ g) might not be great enough to induce priming in the first place. This led to two experiments to address firstly whether microglia could be primed by a more robust cholinergic lesion using a higher dose of toxin (1.2 μ g/mouse), and secondly whether priming was detectable upon examination of the responses to LPS at an earlier time-point post-lesion when microglia might still shown evidence of acute activation (14 days).

Tracey's group have previously proposed the cholinergic anti-inflammatory pathway, whereby cholinergic signalling through the efferent vagus nerve provides tonic inhibition of peripheral inflammation (Tracey, 2007, Tracey, 2009). This modulation of inflammation is mediated by several steps, the first being vagal cholinergic signalling to the celiac ganglion, from which the splenic nerve sends noradrenergic projections to the spleen (Rosas-Ballina et al., 2008). These noradrenergic neurons, rather than synapse on another set of neurons, actually signal to a subset of acetylcholine-producing T cells in the spleen (Rosas-Ballina et al., 2011), and it is from here that the anti-inflammatory influence of acetylcholine takes effect via signal transduction through the $\alpha 7$ -nAChR which is expressed on peripheral macrophages (Wang et al., 2003), leading to inhibition of cytokines such as IL-1 β and TNF- α . The hypothesis of the current work was that this cholinergic tonic inhibition of microglia and inflammation would also occur in the brain, since the $\alpha 7$ -nAChR is reported to be expressed on microglia (Shytle et al., 2004, Suzuki et al., 2006). Thus, depletion of central cholinergic signalling would predispose to exacerbation of neuroinflammation following systemic immune stimulation with LPS.

At the two doses of mu-p75-sap used in the current study, ChAT depletion in the medial septum was assessed by mRNA analysis, and found was found to decrease by approximately 50% in 1.2 μ g and 30% in 0.4 μ g mu-p75-sap-injected mice, roughly consistent with the higher doses of immunotoxin used elsewhere (Hunter et al., 2004, Moreau et al., 2008), although one study by Baxter's group reported complete loss of ChAT-positive neurons in the medial septum with a 1.2 μ g dose (Nag et al., 2009).

The peripheral cytokine response to systemic LPS was not different between controls and lesioned animals at either dose of mu-p75-sap, and showed robustly elevated levels of plasma TNF- α , IL-6 and CXCL1. Given the vagus nerve-mediated cholinergic modulation of peripheral inflammation reported by Tracey's group, the current study investigated whether central cholinergic tone in the basal forebrain might also exert neuromodulatory influence on peripheral inflammatory mechanisms. The current data showed that there is no differential peripheral response, indicating that the basal forebrain cholinergic system is not involved in central control of peripheral inflammation, either by control of the vagus nerve or any other route. While projecting to many regions of the brain (Benarroch, 2010), the basal forebrain cholinergic neurons show no reported modulation of vagal tone. Indeed, Tracey's group have reported that a centrally acting acetylcholinesterase inhibitor can suppress peripheral inflammation via the cholinergic anti-inflammatory pathway (Pavlov et al., 2009). That acetylcholinesterase inhibition protects against sepsis-induced peripheral cytokine production suggests that this might also be the case for the donepezil data in the current study. Thus, it will be important to assess whether donepezil exerts an anti-inflammatory neuromodulatory influence on the hypocholinergic brain following LPS. The absence of a peripheral primed response also replicates previous findings from our laboratory using the ME7 model of murine prion disease that demonstrated similar levels of circulating cytokines in NBH and ME7 animals following systemic administration of LPS or poly I:C (Cunningham et al., 2005a, Field et al., 2010, Murray et al., 2012).

The central cytokine response to systemic LPS was assessed in the regions of cholinergic neuropathology produced by mu-p75-sap lesions, the medial septum and hippocampus. In the more severe lesion (1.2 μ g), the inflammatory response in both the medial septum and hippocampus was primed by the underlying cholinergic neuropathology to produce an exaggerated response to subsequent LPS stimulation. This amplified inflammation was characterised by increased expression of classical pro-inflammatory mediators IL-1 β , TNF- α , IL-6 and CXCL1, with downstream activation of TNF- α also indicated by exaggerated hippocampal expression of TNFAIP2. These robust increases in mRNA expression were consistent with the priming observed in the ME7 model of chronic neurodegenerative disease (Cunningham et al., 2009, Cunningham et al., 2005b) along with that observed in models of ageing (Godbout et al., 2005), PD (Pott Godoy et al., 2008) and MS (Ramaglia et al., 2012). Furthermore, the presence of IL-1 β protein was also detectable in the brains of LPS-challenged lesioned animals, thus recapitulating one of the key characteristics of the original description of priming in the ME7 model (Combrinck et al., 2002). The

heightened expression of the endosomal/lysosomal markers SRA2 and CD68 in lesioned animals is indicative of microglial activation (Palin et al., 2008, Hughes et al., 2010). Though these cells would appear morphologically activated, it is only upon further stimulation that they produce and secrete various pro-inflammatory mediators. This confirmed that even at a time-point distal to the initial surgical production of cholinergic lesion (40 days), that a robust lesion of the basal forebrain cholinergic system, with consequent decreasing of cholinergic tone can prime microglia to produce exaggerated responses to mild systemic inflammatory stimulation.

The presence of a primed microglial response to cholinergic neurodegeneration was also examined using a lower dose of mu-p75-sap but at a time-point more acute to the initial lesion (14 days) when microglia might still be present and activated following the initial neurotoxic insult. Although the overall pattern of the neuroinflammatory response to systemic LPS in the septum and hippocampus was similar to that observed with the more severe lesion at 40 days, the effect was less marked and more variable. The amplified expression of IL-1 β was repeated in the medial septum. Although this effect was not significantly different to LPS-challenged controls in the hippocampus due to some variability, the data were clearly indicative of a primed response. This pattern of IL-1 β expression was also true for measures of protein production, where despite some variability the data were suggestive of a primed response to systemic immune challenge. A preliminary immunohistochemical investigation also demonstrated evidence of septal IL-1 β production which appeared to be of microglial origin, supporting the primed IL-1 β response demonstrated in prior reports (Cunningham et al., 2005b, Cunningham et al., 2009, Murray et al., 2012) and this will further demonstrated with immune stimulation with a viral mimetic in chapter 4.

Thus the current data have demonstrated that a more severe state of hypocholinergia (1.2 μ g mu-p75-sap), and one that may represent the moderate to severe stages of AD-associated cholinergic degeneration (Whitehouse et al., 1982), primes the brain and its microglia to produce an exaggerated response to systemic immune stimulation. Similarly, a less robust lesion but examined at an early time-point following surgical lesion also displays evidence of microglial priming. This demonstrates that although priming is not essential for the production of inflammation-induced cognitive deficits, cholinergic hypofunction can indeed cause priming, allowing for the prediction that the presence of priming would only add to the severity and duration of the acute deficits earlier described.

There exists a cholinergic anti-inflammatory pathway that modulates systemic inflammatory response to invading pathogens via a vagus nerve reflex arc (Tracey, 2007, Tracey, 2009). This pathway modulates inflammation through the $\alpha 7$ -nAChR as discussed above (Wang et al., 2003). Microglia are known to express $\alpha 7$ -nAChR (Shytle et al., 2004) and agonism of this receptor, using nicotine has been shown to down-regulate aspects of microglial activation (De Simone et al., 2005). However, the current study demonstrates, for the first time, that when acetylcholine is depleted due to degenerative changes the loss of basal forebrain cholinergic tone leads to priming of the microglial population. Thus, we provide direct evidence that the basal forebrain cholinergic cells provide tonic inhibition of inflammation which when removed results in an exaggerated response to subsequent inflammatory challenge. It seems likely that depletion of cholinergic function below a certain threshold, in a chronic neurodegenerative condition, would lead to an ongoing state of increased microglial activation and contribute to exaggerated responses to secondary insults such as infection, injury, surgery and other co-morbidities common in the aging population.

Thus acute and transient working memory deficits akin to delirium can manifest in animals with a hypocholinergic predisposition independent of microglial priming. More severe cholinergic depletion primes microglia to produce amplified central inflammatory responses to relatively mild peripheral inflammation. If cognitive testing was performed at a time when microglia are primed, the cognitive deficits would likely be considerably more profound in severity and duration. This has relevance for geriatric and demented patients that suffer from delirium but that might experience different symptoms based on the severity of the underlying pathology, and once again highlights the importance of elucidating further understanding of the mechanisms that underpin this debilitating and poorly understood condition. Although the motivation of the current studies was to model and interrogate molecular mechanisms of inflammation-induced delirium, the findings on cholinergic control of microglial activation have considerable implications for neurodegeneration and neuroinflammation in a general sense.

3.3.6 Conclusion

The results presented here illustrate that loss of cholinergic innervation confers increased susceptibility to the cognitive effects of systemic inflammation. Since the nature of these deficits are acute and transient, with impairments in attention, recall and short-

term/working memory, this model displays some of the key core features of delirium as defined by ICD-10 and DSM-IV criteria (WHO, 1992, APA, 1994). The current data also demonstrate that in animals with a pre-existing cholinergic deficit, acetylcholinesterase inhibition is partially protective against inflammation-induced cognitive dysfunction, and it is predicted that the same would be true for patients. However, it is also hypothesised that pro-cholinergic strategies in sepsis, in individuals lacking a “hypocholinergic predisposition”, may be less effective than in those on a background of progressive dementia, since interfering with cholinergic function in patients with multi-organ failure due to sepsis might have deleterious consequences for this already vulnerable group.

The neuroinflammatory hypothesis of delirium has often been viewed as a distinct pathophysiological route to delirium (Cerejeira et al., 2010) but the findings of the current study go some distance towards reconciling this with the cholinergic deficiency hypothesis (Hsieh et al., 2008). The population at most risk to developing delirium are the aged and demented (Inouye, 2006), a group that often exhibit multiple co-morbidities and thus a single mechanism that underpins the consequent delirium seems less likely. The frequency of infection in this population is quite high (Estes et al., 2006) which, coupled with dementia-associated cholinergic loss, could provide a plausible explanation for subsequent transition to delirium. Given that a single episode of delirium is predictive of long-term cognitive decline (MacLulich et al., 2009), acceleration of dementia (Fong et al., 2009a) and also hastens the time to permanent institutionalisation and death (Rockwood et al., 1999, Witlox et al., 2010), delineating mechanisms that underpin this devastating condition are sorely needed.

Our laboratory has previously shown that as neurodegenerative pathology becomes more global so too does the breadth of CNS changes induced by systemic inflammation (Cunningham et al., 2009). While the current model predicts only a subset of symptoms based on its deliberately limited extent, it provides an excellent paradigm in which to interrogate the interaction between systemic inflammation and cholinergic function, relevant to delirium. This novel animal model can therefore contribute significantly to our understanding of delirium pathophysiology, which is currently rather limited in both intensive care and geriatric settings.

Chapter 4

The impact of systemic poly I:C on the ME7 model of
murine prion disease

3.1 Introduction

Inflammation has long been thought to be involved in the neurodegenerative disease process but whether it has a causative role or is actually a consequence of progressing disease remains unclear. However, some role for inflammation seems clear from studies showing that long-term treatment with NSAIDs for conditions such as rheumatoid arthritis affords significant protection against the subsequent development of AD and PD (in t' Veld et al., 2001, Chen et al., 2005).

Ageing remains the single biggest risk factor for neurodegenerative disease and the ever increasing ageing population brings with it, the need to gain better understanding of the mechanisms underlying neurodegeneration. Aged and demented patients and those suffering from AD may be exposed to infections or inflammatory events which interact with the underlying neuropathology to elicit detrimental consequences such as episodes of delirium (Simone and Tan, 2010). A single delirious episode can lead to institutionalisation and ultimately shorten the time to death (Rockwood et al., 1999). Recent studies have suggested that the aged and demented population are particularly susceptible to the negative consequences (functional and cognitive decline) associated with such systemic inflammatory events (Holmes et al., 2009), however the mechanisms that underpin these detrimental consequences remain poorly understood.

Our laboratory has used the ME7 model of murine prion disease as a model of progressive neurodegenerative disease since it displays robust progressive and irreversible neuronal death, synaptic loss, extracellular amyloidosis and microglial activation, along with progressive neurological and cognitive changes that are also apparent in human neurodegenerative disease (Betmouni et al., 1996, Betmouni et al., 1999, Cunningham et al., 2003). Using this model, it has been shown that systemic immune stimulation with bacterial endotoxin (LPS) evokes exaggerated sickness behaviour (Combrinck et al., 2002), exacerbates CNS inflammation and apoptosis (Cunningham et al., 2005b) and accelerates disease progression (Cunningham et al., 2009). These studies have also been the first to demonstrate that the brain's microglia are primed by the underlying neuropathology to produce exaggerated responses to subsequent central or systemic inflammatory stimulation. This microglial priming phenomenon is not limited to the ME7 model and has been replicated in animal models of AD (Sly et al., 2001), PD (Pott Godoy et al., 2008), ageing (Barrientos et al., 2006, Godbout et al., 2005) and multiple sclerosis

(Ramaglia et al., 2012), while systemic inflammation-induced disease exacerbation has been reported in models of AD (Kitazawa et al., 2005), amyotrophic lateral sclerosis (Nguyen et al., 2004) and stroke (McColl et al., 2007).

Most studies investigating the effect of systemic inflammation on neurodegenerative disease have used bacterial LPS to mimic acute bacterial infection, but the effect of viral infection has been relatively overlooked, a surprising fact given the high frequency of viral infections in the aged and demented population (Falsey et al., 2005, Estes et al., 2006). Although some studies have shown that viral infection is associated with more rapid cognitive decline in patients (Aiello et al., 2006, Strandberg et al., 2003), their effect on the degenerating brain remains poorly understood and merits investigation.

The aim of the current chapter is to examine the effect of systemic stimulation with a viral mimetic, namely the synthetic double-stranded RNA, poly I:C, on chronic neurodegenerative disease (ME7 prion disease). Poly I:C is a potent activator of the type I interferon response (Jacobs and Langland, 1996, Matsumoto and Seya, 2008) via sensing by TLR3 (Alexopoulou et al., 2001) and other dsRNA sensors (Yoneyama et al., 2005, Kato et al., 2006), and has been used extensively in the immunology field to mimic viral infection. There has been limited investigation of its effects on the brain, however, with a small number of studies examining sickness behavioural changes (Cunningham et al., 2007), sleep patterns (Traynor et al., 2006) and inflammatory gene transcription (Konat et al., 2009) in normal animals. One further study has recently demonstrated that central poly I:C treatment and TLR3 stimulation can impair working memory and constrain hippocampal neurogenesis (Okun et al., 2010), which could be important in the context of viral infection during ageing and dementia.

Prior reports from our laboratory demonstrated that microglia in the diseased brain are primed to produce exaggerated CNS responses to peripheral LPS (Cunningham et al., 2005b). Here we hypothesised that this would also hold for systemic viral mimetics using poly I:C (12 mg/kg i.p.). Furthermore, inflammatory processes that occur in the diseased brain following poly I:C treatment were examined to assess whether they were distinct from those activated in response to LPS. This chapter also aimed to assess the effect of acute antiviral stimulation on measures of acute sickness during neurodegenerative disease and whether there are deleterious consequences for progression of disease following systemic poly I:C challenge, and if so, investigate what mechanisms might underpin the

exacerbation of disease. Thus, this work will be important to gain a better understanding of the processes that underpin the exacerbation of clinical symptoms in vulnerable populations like the aged and demented that frequently suffer from viral infection.

4.2 Results

4.2.1 CNS priming following systemic poly I:C

We previously established that there is a robust increase in plasma cytokine levels (IFN-β, TNF-α, IL-6) in response to systemic poly I:C in normal animals and these levels are not significantly different in ME7 prion-diseased mice (Field et al., 2010; Table 4.1).

Table 4.1 Plasma inflammatory cytokines

Cytokine	NBH + saline	NBH + poly I:C	ME7 + saline	ME7 + poly I:C
IFN-β	ND	2567 ± 410	ND	2737 ± 694
TNF-α	ND	132 ± 24	ND	121 ± 36
IL-6	ND	1837 ± 374	ND	1739 ± 569

Systemic cytokine levels 6 hours following poly I:C or sterile saline (pg/ml). ND = not detectable.

Systemic poly I:C induced differential CNS responses in NBH and ME7 animals. Though systemic poly I:C induced TNF-α mRNA in normal animals and TNF-α mRNA was markedly induced in ME7-inoculated animals *per se*, poly I:C actually suppressed TNF-α transcription in ME7 animals (Figure 4.1a). One-way ANOVA ($F = 46.03$, $df\ 5,26$, $p < 0.0001$) with selected Bonferroni post-hoc tests revealed that ME7 + saline was significantly different to NBH + saline ($p < 0.001$). Systemic challenge with poly I:C (12 mg/kg) induces opposite effects on TNF-α in NBH and ME7 animals. Levels in NBH + poly I:C were clearly increased but levels in ME7 + poly I:C animals were actually depressed at 4 hours with respect to ME7 animals and statistically significantly lower 6 hours following systemic challenge ($p < 0.001$ by one-way ANOVA with Bonferroni post-hoc test).

Poly I:C induced very marked increases in IL-6 mRNA by 4 hours in the hippocampus of both NBH and ME7 animals (Figure 4.1b). The increase was, however, more marked in ME7 animals. A significant one-way ANOVA ($F = 65.01$, $df\ 5,26$, $p < 0.0001$) with selected Bonferroni post-hoc tests revealed no difference between IL-6 levels in NBH + saline and ME7 + saline animals ($p > 0.05$), but showed that ME7 + poly I:C at 4 hours was significantly different to NBH + poly I:C ($p < 0.001$) and these levels decreased somewhat by 6 hours.

IL-1β mRNA was clearly induced in the hippocampus of ME7 animals 4 hours post-poly I:C challenge and returned to near baseline levels by 6 hours in normal animals. The poly

I:C-induced increase was markedly higher in ME7 animals (Figure 4.1c). One-way ANOVA ($F = 25.32$, $df\ 5,26$, $p < 0.0001$) followed by selected Bonferroni post-hoc comparisons revealed that ME7 + saline animals showed significantly greater expression of IL-1 β than NBH + saline ($p < 0.05$), and that the IL-1 β induction after poly I:C challenge was considerably more marked in ME7 than NBH animals ($p < 0.001$).

IFN- β , whose expression is dependent on IRF3 activation, was induced more markedly in the hippocampus of ME7 animals treated with poly I:C (Figure 4.1d) and appeared to peak at 4 hours. A significant one-way ANOVA ($F = 19.43$, $df\ 5,26$, $p < 0.0001$) followed by Bonferroni post-hoc tests revealed that IFN- β induction in ME7 animals challenged with poly I:C was significantly higher than in NBH animals receiving the same treatment at their peak values ($p < 0.01$), but ME7 and NBH animals given saline were not significantly different ($p > 0.05$).

PTX3, an NF κ B-dependent gene with no reported regulation by IRF3, and thought to be downstream of IL-1 β activation showed an exaggerated induction in the hippocampus of ME7 animals challenged with poly I:C when compared to NBH animals similarly challenged. Levels of this transcript were markedly increased 6 hours following systemic challenge (Figure 4.1e), distinct from the NF κ B-dependent primary response genes IL-1 β , TNF- α and IL-6 (Figure 4.1a, b and c) and consistent with induction secondary to IL-1 β synthesis and action. Selected Bonferroni post-hoc comparisons following a significant one-way ANOVA ($F = 10.03$, $df\ 5,26$, $p < 0.0001$) revealed that PTX3 induction in saline-treated ME7 and NBH animals was not significantly different. PTX3 transcript levels were significantly higher in poly I:C-challenged ME7 animals compared to poly I:C-challenged NBH animals at 6 hours post-challenge ($p < 0.01$).

Hippocampal expression of the NF κ B- and IRF3-dependent gene interferon inducible protein 10 (IP-10) was also examined. This chemokine mRNA showed a very similar temporal expression profile of induction to the other primary response genes studied (Figure 4.1f), peaking at 4 hours and decreasing thereafter, making it unlikely that it is directly induced by IFN- β . After a significant one-way ANOVA ($F = 67.76$, $df\ 5,23$, $p < 0.0001$), Bonferroni post-hoc tests showed that induction of IP-10 in ME7 + poly I:C animals was significantly higher than that in NBH + poly I:C at both 4 and 6 hours ($p < 0.05$) but ME7 + saline was not significantly different to NBH + saline ($p > 0.05$).

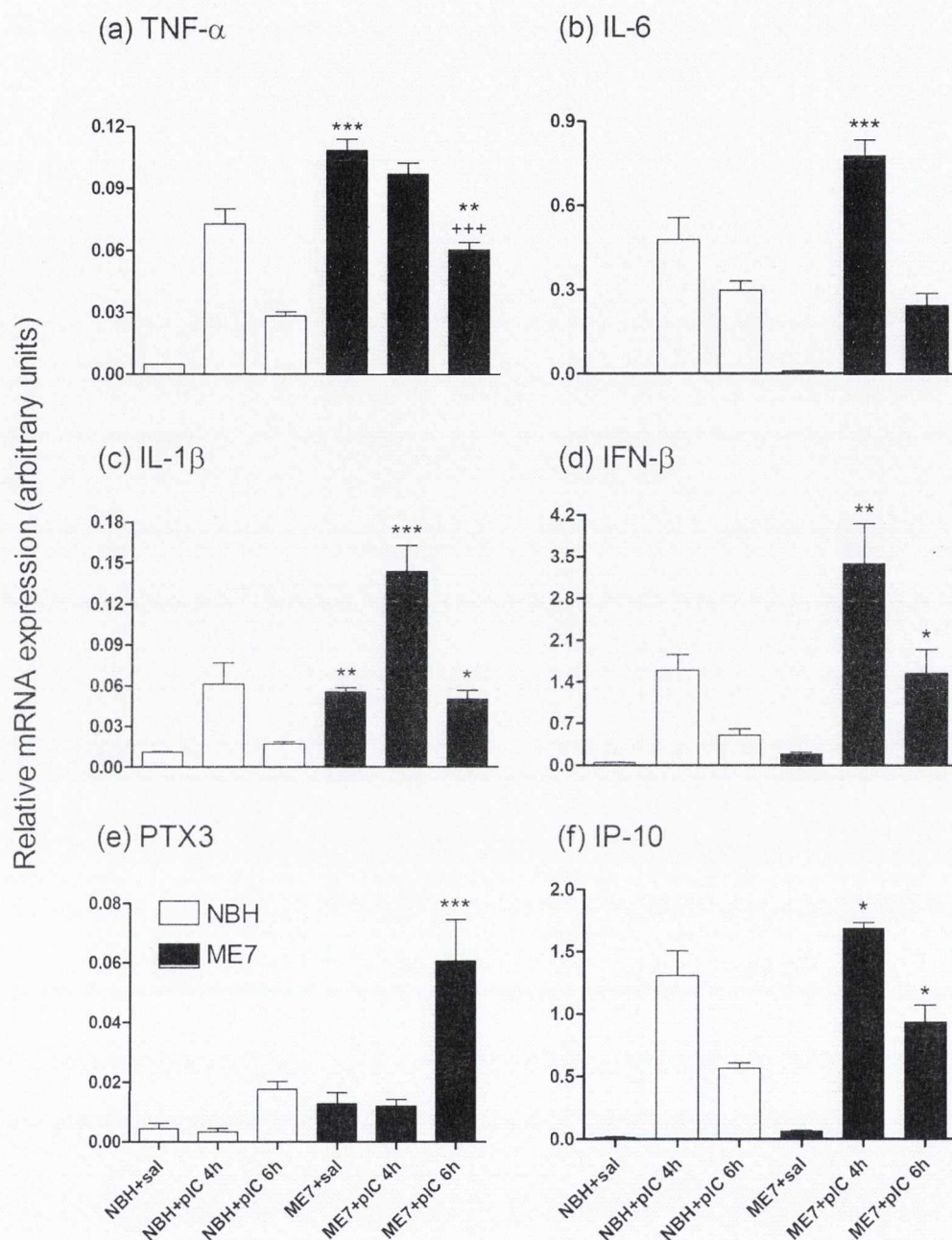


Figure 4.1 *Inflammatory cytokine and NF κ B- and IRF3-dependent gene transcription.* Hippocampal expression of mRNA for (a) TNF- α , (b) IL-6, (c) IL-1 β , (d) IFN- β , (e) PTX3 and (f) IP-10 at 4 and 6 h post-challenge with poly I:C (12 mg/kg) in NBH and ME7 animals 18 weeks post-inoculation. Cytokine levels were measured by quantitative PCR. Significant differences, by Bonferroni post-hoc analysis after significant one-way ANOVA are denoted by * $p < 0.05$, ** $p < 0.01$ and ***/+ $p < 0.001$. Comparisons between ME7 and NBH are represented by *, and comparisons between saline and poly I:C are denoted +. Data are expressed as mean $\pm$ SEM; $n \geq 3$ for all groups.

4.2.2 Is there evidence for exaggerated TNF- α production in poly I:C-challenged diseased animals?

Despite a decrease in transcription of TNF- α mRNA, we investigated whether this could be explained by increased translation to active protein. Therefore, we assessed TNF- α -responsive transcripts using qPCR and performed ELISA for TNF- α protein in the hippocampus to address this possibility (Figure 4.2).

TNF- α -converting enzyme, ADAM17 (or TACE) has been shown to be a key protein involved in processing the release of soluble TNF- α from its membrane-bound precursor (Black et al., 1997) and has also been shown to be induced by TNF- α (Black, 2002, Bartsch et al., 2010). The transcription of this gene was found to be upregulated in prion disease but not further altered by systemic poly I:C challenge (Figure 4.2a). There was a main effect of disease ($F = 65.95$, df 1,14, $p < 0.0001$) but no significant effects of poly I:C and no interaction of these factors.

The TNF- α inducible gene ADAM8 was not induced by either disease ($F = 1.84$, df 1,14, $p = 0.1967$) or systemic poly I:C ($F = 1.64$, df 1,14, $p = 0.2205$) and there was no interaction of these factors ($F = 2.60$, df 1,14, $p = 0.1292$) (Figure 4.2b).

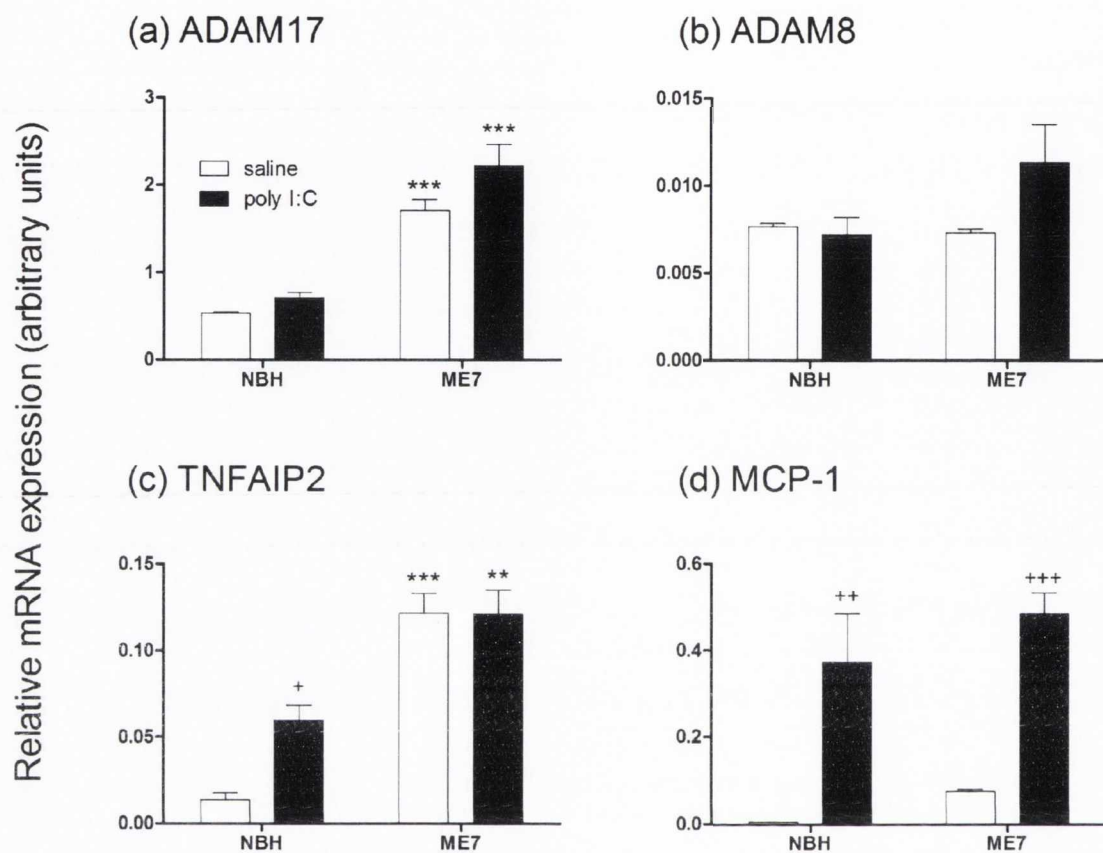
Tumour necrosis factor- α -inducible protein-2 (TNFAIP2) expression was elevated in the hippocampus of NBH animals challenged with poly I:C but not in ME7 animals similarly challenged (Figure 4.2c). There was a main effect of disease ($F = 51.45$, df 1,13, $p < 0.0001$) but not of poly I:C ($F = 3.81$, df 1,13, $p = 0.0728$) and there was no interaction ($F = 4.01$, df 1,13, $p = 0.0666$) although this was close to reaching significance. Bonferroni post-hoc tests showed that poly I:C significantly induced TNFAIP2 transcription in NBH animals ($p < 0.05$).

Transcription of the chemokine, monocyte chemotactic protein-1 (MCP-1 or CCL2), was elevated in response to poly I:C in both animal groups but was not more robustly affected in ME7 than NBH (Figure 4.2d). There was a main effect of poly I:C ($F = 31.54$, df 1,14, $p < 0.0001$) but not of disease ($F = 1.68$, df 1,14, $p = 0.2154$) and no interaction of these factors ($F = 0.09$, df 1,14, $p = 0.7722$).

Investigation of TNF- α protein synthesis was examined by ELISA in the hippocampus of NBH and ME7 animals following systemic poly I:C challenge (12 mg/kg). LPS-injected

brain tissue (1 µg intra-hippocampal) was also included here for positive control purposes, since detection of cytokine protein in the brain following systemic immune stimulation can often prove difficult: even when biologically active, cytokines such as IL-1 β and TNF- α may be below the reliable detection limits of ELISA kits. There was no detectable TNF- α protein in brains of systemically challenged animals (Figure 4.2e) but intra-cerebral LPS challenge induced robust TNF- α protein production in the brain. Bonferroni post-hoc comparisons following one-way ANOVA analysis ($F = 1644$, df 4,15, $p < 0.0001$) revealed that TNF- α levels were significantly higher after intra-cerebral LPS compared to all other groups ($p < 0.001$).

Thus, there is not robust evidence for increased TNF- α protein expression in the brain of diseased animals in response to systemic poly I:C and patterns of TNF- α inducible gene transcription are consistent with the primary transcriptional response, i.e. that TNF- α mRNA is elevated by disease and by systemic poly I:C, but the combination of these insults leads to a suppression of disease-associated TNF- α transcription.



(e)

Cytokine	NBH + sal	NBH + pIC	ME7 + sal	ME7 + pIC	LPS i.c.
TNF- α	ND	ND	ND	ND	1.95 $\pm$ 0.13***

Figure 4.2 *TNF- α -dependent gene transcription.* Expression of mRNA for the TNF-releasing gene ADAM17 (a) and the TNF-responsive genes ADAM8 (b), TNFAIP2 (c) and MCP-1 (d) was measured in the hippocampus of NBH or ME7 animals 18 weeks post-inoculation and 6 hours post-challenge with poly I:C (12 mg/kg). Bonferroni post-hoc tests after two-way ANOVA are represented by */+ $p < 0.05$, **/+ $p < 0.01$, ***/+++ $p < 0.001$. Comparisons between ME7 and NBH are represented by *, and comparisons between saline and poly I:C are denoted +. (e) Hippocampal TNF- α protein levels (pg/mg of protein) following systemic poly I:C and intra-cerebral LPS challenge. ND signifies that levels were below detection limits. Bonferroni post-hoc tests after one-way ANOVA are denoted by *** $p < 0.05$. Data are expressed as mean $\pm$ SEM ($n = 3$ for NBH + saline and $n = 5$ for all other groups)

4.2.3 Core body temperature response to poly I:C in prion disease

Poly I:C induced differential CNS cytokine profiles in normal and ME7 animals and since CNS cytokines are thought to have a key role in thermoregulatory responses, we decided to assess the core body temperature response of these animals in order to make an initial assessment of differential physiological CNS outcomes of diseased and normal animals to the same systemic challenge. At 18 weeks post-inoculation with either ME7 or NBH, animals were challenged systemically with either poly I:C (12 mg/kg) or sterile saline and core body temperature was measured by rectal probe to determine the effect that systemic viral mimetics have on the temperature response in the neurodegenerative state. Poly I:C induced a mild hyperthermic response in normal animals (NBH) but induced a marked hypothermia in prion-diseased animals (Figure 4.3). Repeated measures ANOVA revealed a significant effect of time ($F = 3.25$, df 3,78, $p = 0.0261$) and treatment ($F = 7.07$, df 3,78, $p = 0.0013$) and an interaction of treatment and time ($F = 5.12$, df 9,78, $p < 0.0001$). Bonferroni post-hoc tests revealed that ME7 + poly I:C animals were significantly different to NBH + poly I:C animals at 4 hours ($p < 0.01$), 9 hours and 13 hours ($p < 0.001$) following systemic poly I:C challenge and were significantly different to ME7 + saline animals at 13 hours post-challenge ($p < 0.01$). Conversely ME7 + saline were not found to be different to NBH + saline at any time point ($p > 0.05$).

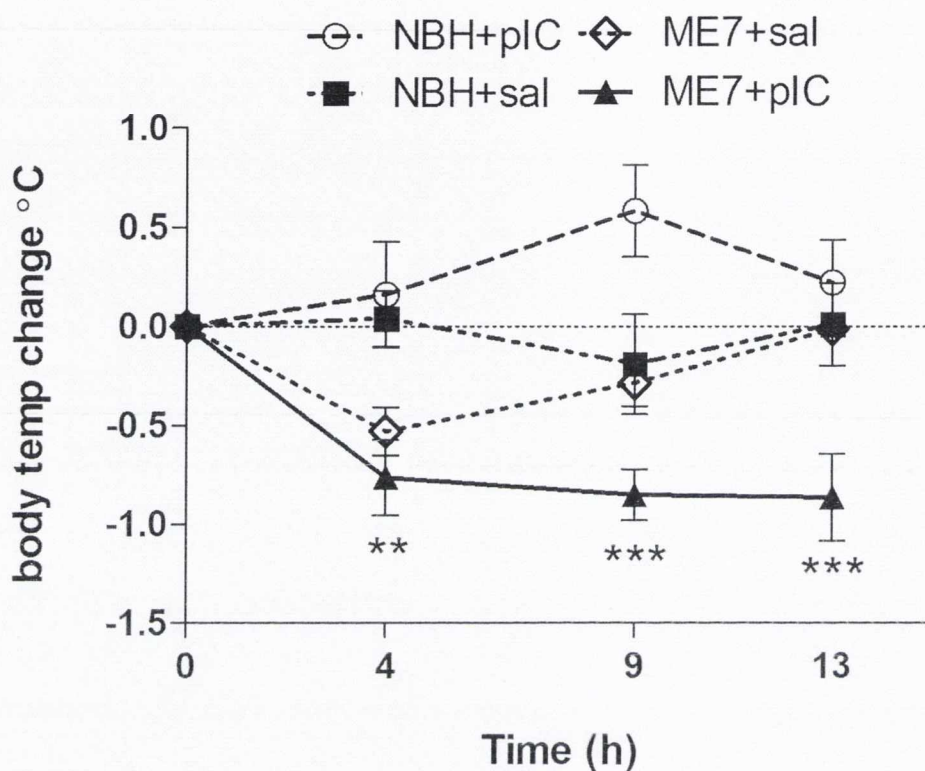


Figure 4.3 Core body temperature response to poly I:C in ME7 animals. Animals were treated systemically with poly I:C (12 mg/kg) at 18 weeks post-inoculation with ME7 or NBH. Core body temperature was taken by rectal probe at 0, 4, 9 and 13 h post-poly I:C or sterile saline. Significant differences between ME7 + poly I:C and corresponding NBH + poly I:C time point by Bonferroni post hoc test, after significant two-way repeated measures ANOVA, are denoted by ** $p < 0.01$ and *** $p < 0.001$. Data are expressed as mean $\pm$ SEM (n = 10 for NBH + saline, n = 5 for NBH + poly I:C, n = 7 for ME7 + saline, n = 8 for ME7 + poly I:C)

4.2.4 Histological analysis of cellular changes

Following confirmation of differential CNS transcriptional and physiological responses to systemic poly I:C challenge in normal and diseased animals, we sought to characterise inflammatory changes at a histological level in an attempt to identify key cellular events. Inflammatory changes in ME7 animals challenged systemically with poly I:C were assessed by a number of immunohistochemical labelling methods. IBA-1 labelling of microglia 3 hours following systemic challenge illustrated clear morphological evidence of microglial activation (Figure 4.4a-c) in ME7 animals treated with either saline or poly I:C compared to NBH animals that received poly I:C. NBH animals treated with saline are shown in Figure 4.5a and not different from poly I:C-challenged animals. One-way ANOVA ($F = 37.53$, $df\ 2,7$, $p < 0.0002$) followed by Bonferroni post-hoc tests revealed significantly increased numbers of IBA-1-positive activated microglia ($p < 0.001$; Figure 4.6a) in ME7 animals compared to NBH with no further increase following administration of poly I:C ($p > 0.05$).

These morphologically activated microglia, however, remain negative for IL-1 β immunolabelling in saline-treated ME7 animals. Upon systemic challenge with poly I:C these microglial cells, in the periventricular and dentate gyrus regions, synthesise detectable levels of IL-1 β (Figure 4.4i) in ME7 but not NBH animals. IL-1 β positive cells were found to be significantly higher in number in ME7 animals challenged with poly I:C than all other groups ($p < 0.05$ by Kruskal-Wallis test for nonparametric data followed by Dunn's test for multiple comparisons; Figure 4.6b). This IL-1 β labelling is indicative of a phenotypic switch in microglial function without a further morphological change and demonstrates that microglia are primed to respond more robustly to systemic stimulation with poly I:C.

Immunohistochemistry for TNF- α was also conducted to assess TNF- α protein production in ME7 animals following systemic poly I:C. Despite evidence of microglial TNF- α production after intra-hippocampal poly I:C challenge (2 μ g; Figure 4.5c), there was no evidence of TNF- α -positive cells present in the brains of ME7 animals after systemic poly I:C treatment (Figure 4.5d). The lack of positive labelling for TNF- α , shown for ME7 + poly I:C, was also true of ME7 + saline and NBH + poly I:C animals (not shown). This is consistent with the mRNA and protein data for TNF- α presented earlier.

There was not robust evidence of nuclear translocation of the transcription factor nuclear factor kappa B (NFκB) in NBH and ME7 animals 3 hours following systemic poly I:C challenge (Figure 4.5g,i). Conversely, there was clear nuclear translocation of NFκB in the brain following intra-hippocampal poly I:C challenge (2 µg; Figure 3.5e) and this corresponded with complete disappearance of cytosolic NFκB in the positive cells. There was evidence of cytoplasmic NFκB in ME7 animals (Figure 4.5h,i), which was more obvious and robust than that observed in NBH animals (Figure 4.5g), but nuclear translocation of some, as opposed to all, cytoplasmic NFκB is not straightforward to demonstrate (ie where animals have received intra-hippocampal poly I:C, effectively all cytoplasmic NFκB is translocated).

The cerebrovascular endothelial cell layer was assessed for activation, as determined by synthesis of COX-2, the biosynthetic enzyme for the production of prostaglandins. Endothelial COX-2 was detectable in response to systemic poly I:C in both NBH and ME7 animals (Figure 4.4d,f) but not in NBH or ME7 animals that received saline (Figure 4.4e; Figure 4.5b). Quantification of individual COX-2-labelled cells is not straightforward in the tightly apposed endothelial cell layer of hippocampal vessels, but it is clear that the vast majority of hippocampal vessels are positively labelled after systemic poly I:C challenge in NBH and ME7 animals, while those in the ME7 animals challenged with saline are not.

Numerous cells in periventricular and perivascular areas and around the dentate gyrus also showed positive labelling for the transcription factor IRF3 (Figure 4.4j-l), which is involved in the initial production of IFN-β in response to TLR3 activation. There was evidence of more intense and more frequent staining of nuclei in the hippocampus and thalamus, the regions of prior ME7-associated pathology, consistent with nuclear translocation of IRF3 and consistent with the consequent stimulation of type I interferon induction.

Immunohistochemistry for immunoglobulin G (IgG) was performed to assess the integrity of the blood-brain barrier, which, if compromised could allow circulating inflammatory mediators or indeed poly I:C access to the brain parenchyma. IgG labelling was absent from the brain parenchyma and only localized to some small blood vessels but this was present in all animals, independent of disease state or systemic inflammatory insult. This suggests that the BBB is not significantly compromised in ME7 prion-diseased animals *per se* or those challenged systemically with poly I:C, although this technique may not be

optimal in formalin-fixed tissue and perhaps could be better addressed using fresh frozen tissue.

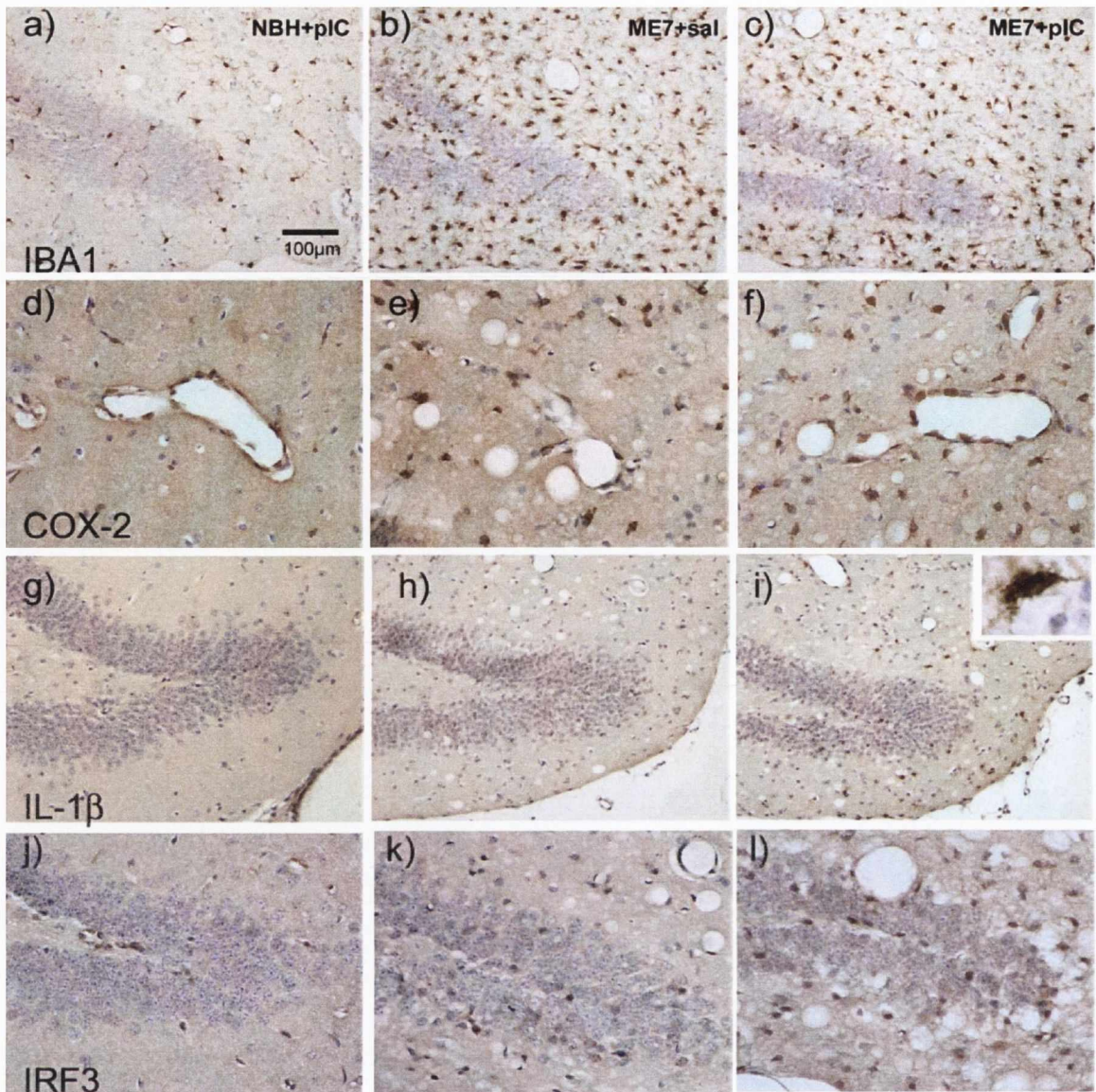


Figure 4.4 *Microglia are primed with respect to responses to systemic poly I:C.* Microglial priming and endothelial activation was assessed 3 hours after systemic challenge with either poly I:C (12 mg/kg) or saline in NBH and ME7 animals 18 weeks post-inoculation. (a-c) IBA-1 labelling of microglial cells in NBH + poly I:C, ME7 + saline and ME7 + poly I:C animal groups, showing more condensed morphology and increased numbers in ME7 with respect to NBH. (d-f) COX-2 labelling shows few microglia in NBH but activated endothelium after poly I:C. Conversely, ME7 + saline shows increased microglia but absent endothelial staining, while ME7 + poly I:C animals show both populations as COX-2 positive. (g-i) IL-1 β labelling is absent in both NBH + poly I:C and ME7 + saline animals but ME7 + poly I:C (i) shows positive cells of microglial morphology (inset) in periventricular and perivascular areas around the dentate gyrus. (j-l) IRF3 labelling in scattered cells in NBH + poly I:C and in ME7 + saline and in greater numbers in periventricular areas and around the dentate gyrus in ME7 + poly I:C animals. Scale bar = 100 μ m in (a-c) and (g-i) and 50 μ m in (d-f) and (j-l).

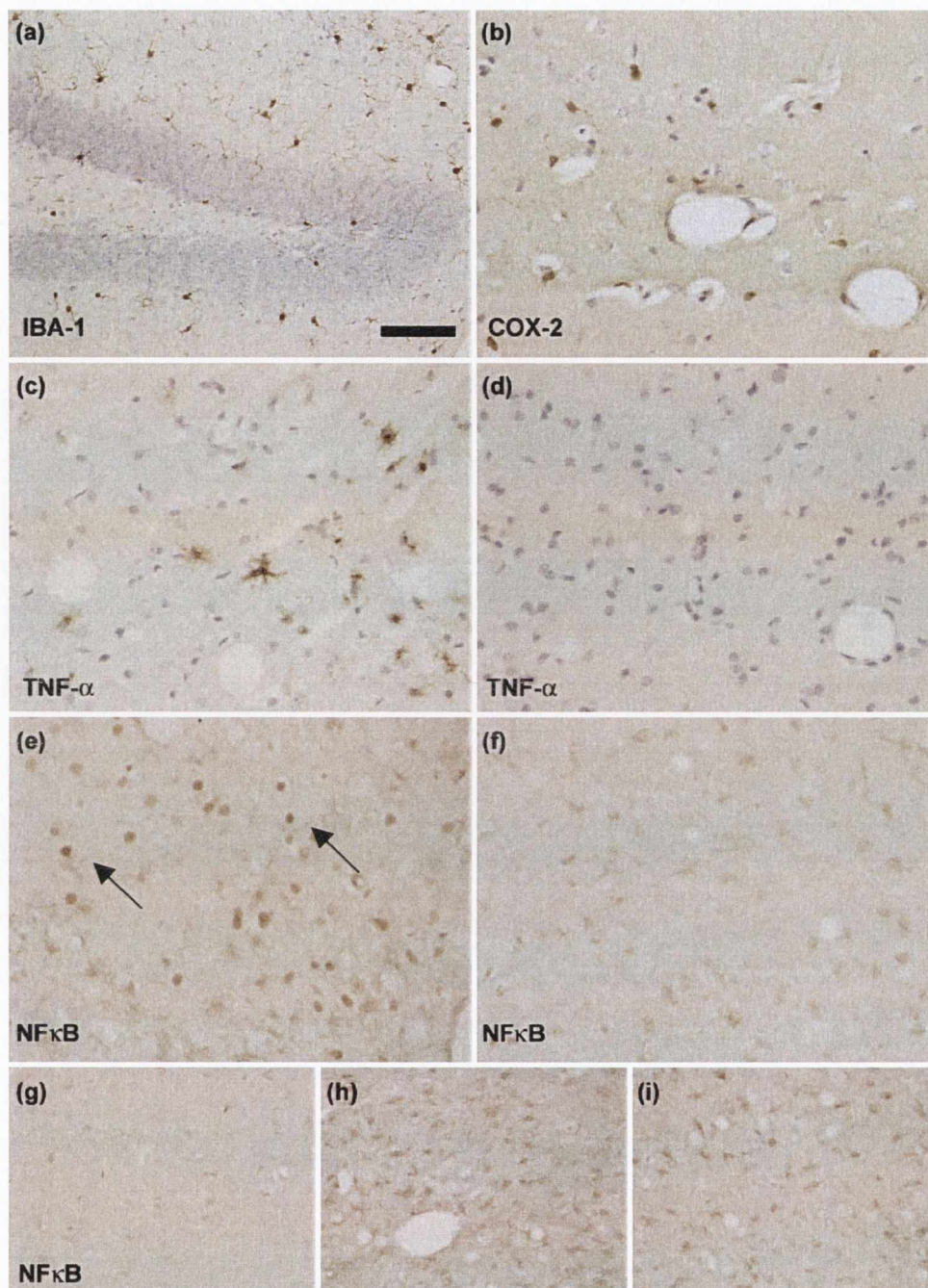
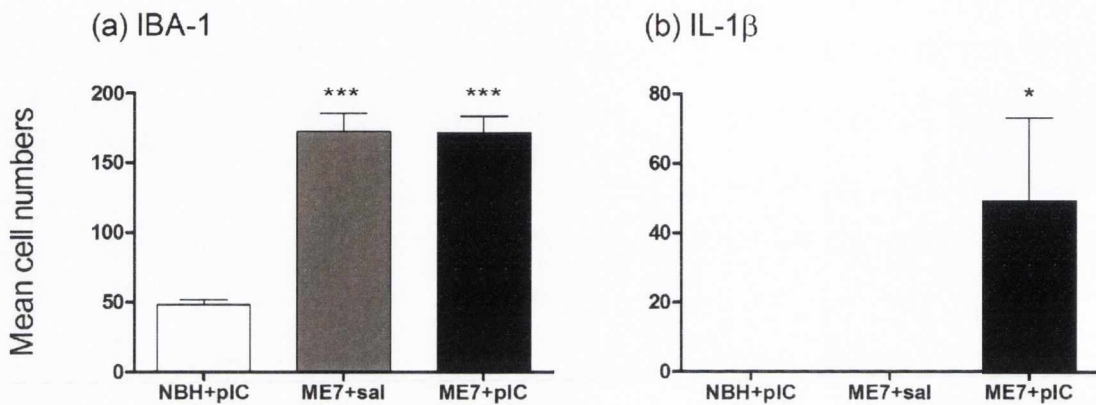


Figure 4.5 *Cellular changes after peripheral and central poly I:C.* (a) IBA-1 microglial labelling and (b) COX-2 labelling in NBH + saline (i.p.) animals showing few microglia and absent endothelial activation. (c,d) Microglial TNF- α labelling is present after intra-hippocampal poly I:C but not after systemic poly I:C in ME7 animals. (e) Nuclear translocation (arrows) of NF κ B after intra-hippocampal poly I:C in ME7 animals. (f) Cytoplasmic NF κ B labelling in the non-injected side after intra-hippocampal poly I:C in ME7 animals. (g-i) NF κ B labelling in NBH + poly I:C, ME7 + saline, and ME7 + poly I:C, respectively after peripheral challenge. Increased cytoplasmic labelling in ME7 but nuclear translocation is not obvious. In all cases ME7 were 18 weeks post-inoculation. Poly I:C dose: 2 μ g intra-hippocampal, 12 mg/kg i.p. Scale bar = 100 μ m in (a) and 50 μ m in (b-i).



Immunohistochemical quantification

IHC label	NBH + poly I:C	ME7 + saline	ME7 + poly I:C
IBA-1	48 $\pm$ 5	172 $\pm$ 15***	172 $\pm$ 14***
IL-1 β	ND	ND	49 $\pm$ 29*

Figure 4.6 Immunohistochemical quantification of microglia and IL-1 β -producing cells.

(a) IBA-1 cell counts were performed in a 0.62 mm x 0.47 mm region of the hippocampus. (b) IL-1 β counts were performed in the hippocampus & thalamus in 10 μ m coronal sections, 3 h after poly I:C administration. ND signifies that positive cells were not detectable. Significant differences to NBH + poly I:C controls by one way ANOVA and Bonferroni post-hoc tests are signified by * p < 0.05 and *** p < 0.001. Data are presented as mean $\pm$ SEM.

4.2.5 Evidence for exaggerated IFN- β production and action in the CNS

Increased IFN- α/β action in the hippocampus was assessed by examining expression of transcripts for IRF7, OAS1a, Mx1 and PKR (Figure 4.7). These genes are known to be both IFN- β -responsive and STAT1/2-dependent and are not induced directly by TLR3 signalling or by IRF3 activation (Honda and Taniguchi, 2006). IRF7 transcription was clearly induced by poly I:C 6 hours following challenge in both NBH and ME7 animals (Figure 4.7a). There was a main effect of poly I:C ($F = 231.16$, df 1,14, $p < 0.0001$) and a main effect of disease ($F = 39.84$, df 1,14, $p < 0.0001$) and a significant interaction between these two factors ($F = 23.98$, df 1,14, $p = 0.0002$).

Similarly, 2'-5'-oligoadenylate synthetase-1a (OAS1a) showed a much higher induction in ME7 + poly I:C animals than in NBH + poly I:C animals (Figure 4.7b). There were main effects of disease ($F = 43.96$, df 1,14, $p < 0.0001$) and of poly I:C ($F = 79.41$, df 1,14, $p < 0.0001$) and an interaction between these two factors ($F = 21.32$, df 1,14, $p = 0.0004$).

The Mx1 protein in C57 mice is known to be non-functional due to a deletion in exons 9 through 11 in the *Mx1* gene (Staeheli and Sutcliffe, 1988, Jin et al., 1998). Thus, for the current study, Mx1 was assessed at the exon 2-exon 3 junction which is unaffected by the deletion. Mx1 showed a comparable exaggerated induction in ME7 animals treated with poly I:C compared to poly I:C-challenged NBH animals (Figure 4.7c). There were main effects of disease ($F = 7.70$, df 1,14, $p < 0.0149$) and of poly I:C ($F = 45.29$, df 1,14, $p < 0.0001$) and an interaction of these of factors ($F = 5.87$, df 1,14, $p = 0.0295$).

Finally, transcription of dsRNA-dependent protein kinase (PKR) was more robustly induced by poly I:C in ME7 animals than in NBH animals (Figure 4.7d). There were main effects of disease ($F = 9.51$, df 1,14, $p = 0.0081$) and of poly I:C ($F = 55.12$, df 1,14, $p < 0.0001$) but no significant interaction in this case ($F = 0.89$, df 1,14, $p = 0.3622$). Bonferroni post-hoc tests after significant one-way ANOVA, however, show that expression of PKR in ME7 + poly I:C is significantly higher than in NBH + poly I:C ($p < 0.05$).

Thus, there is exaggerated type I IFN action in the CNS of ME7 animals challenged with poly I:C by comparison to NBH animals similarly challenged.

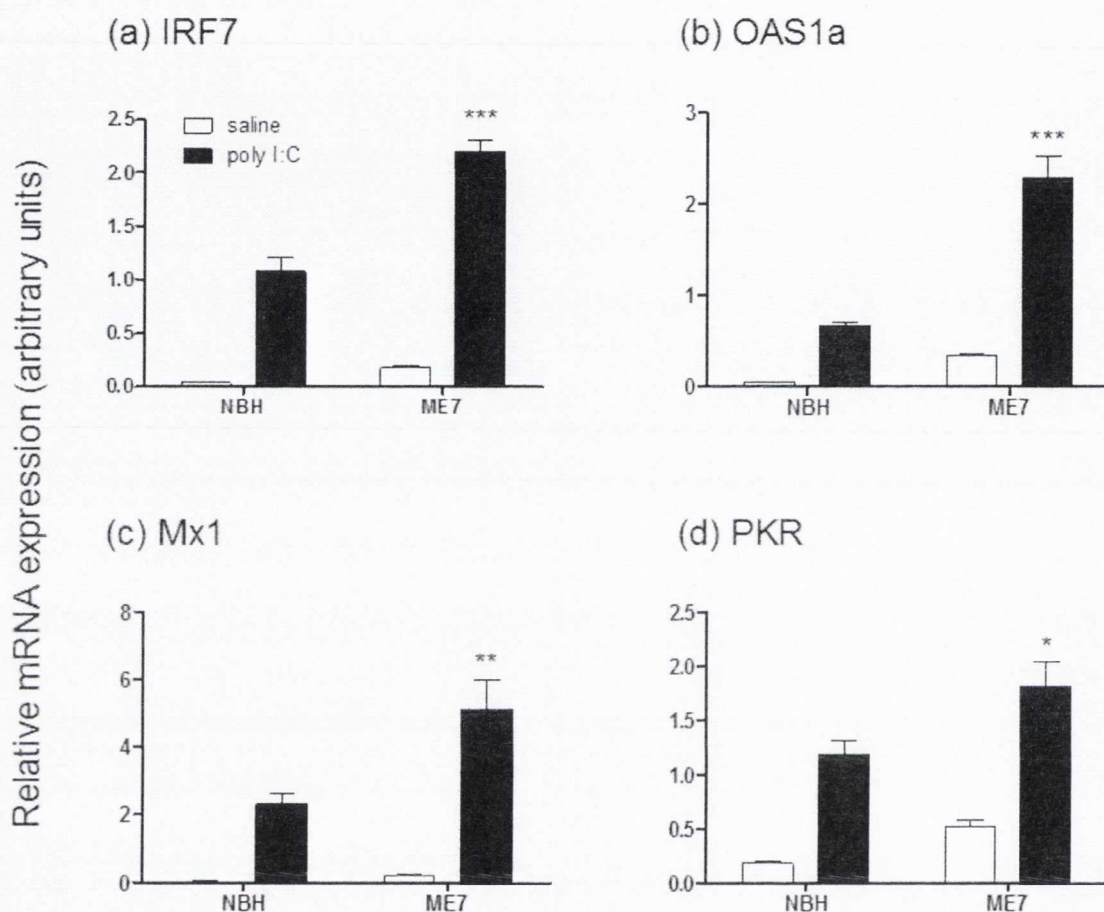


Figure 4.7 *IFN-responsive, STAT1/2-dependent gene transcription.* Expression of mRNA for the IFN- β -responsive genes (a) IRF7, (b) OAS1a, (c) Mx1 and (d) PKR was measured in the hippocampus of NBH or ME7 animals 18 weeks post-inoculation and 6 h post-challenge with poly I:C (12 mg/kg i.p.) or saline. Significant differences by Bonferroni post-hoc tests after two-way ANOVA are represented by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Bonferroni comparisons are between ME7 and NBH. Data are expressed as mean $\pm$ SEM ($n = 3$ for NBH + saline and $n = 5$ for all other groups).

4.2.6 Evidence of anti-inflammatory activation: additional IFN- β effects?

Since IFN- β is typically viewed as an anti-inflammatory cytokine, a number of gene transcripts were examined to assess the possible anti-inflammatory profile IFN- β might induce in the CNS.

Hippocampal IL-10 expression was markedly increased by systemic poly I:C challenge in ME7 animals compared to NBH controls (Figure 4.8a). Two-way ANOVA analysis revealed a main effect of disease ($F = 10.84$, $df\ 1,14$, $p = 0.0053$) and of poly I:C ($F = 15.94$, $df\ 1,14$, $p = 0.0013$) and a significant interaction of these two factors ($F = 5.75$, $df\ 1,14$, $p = 0.0310$). Bonferroni post-hoc tests showed that ME7 + poly I:C animals demonstrated considerably more marked induction than NBH + poly I:C ($p < 0.01$) or ME7 + saline animals ($p < 0.001$).

The transcription of the myeloid restricted anti-inflammatory marker, triggering receptor expressed on myeloid cells (TREM2), was markedly induced by disease and induced further by poly I:C in these animals (Figure 4.8b). Two-way ANOVA revealed a main effect of disease ($F = 84.63$, $df\ 1,10$, $p < 0.0001$) but not of poly I:C ($F = 4.03$, $df\ 1,10$, $p = 0.0726$). However there was an interaction between these two factors ($F = 5.61$, $df\ 1,10$, $p = 0.0394$) with Bonferroni post-hoc tests showing TREM2 to be markedly more elevated in ME7 + poly I:C than either ME7 + saline ($p < 0.01$) or NBH + poly I:C ($p < 0.001$).

Investigation of the expression of the signalling type I interferon receptor, IFNAR2, revealed that its expression was significantly increased in ME7 animals compared to NBH controls (Figure 4.8c). Two-way ANOVA revealed a main effect of disease ($F = 92.94$, $df\ 1,11$, $p < 0.0001$) on CNS expression of IFNAR2 but without effect of poly I:C treatment ($F = 0.32$, $df\ 1,11$, $p = 0.5827$) or an interaction. Bonferroni post-hoc tests showed increased expression of the receptor during disease ($p < 0.001$) with no further change following poly I:C treatment.

4.2.7 Classical pathways of inflammatory activation

Given the balance of pro- and anti-inflammatory transcripts present in the brain following systemic poly I:C, some further measures of robust pro-inflammatory activation were assessed that might indicate whether deleterious/damaging inflammatory pathways were being induced by this stimulation.

The expression of the p40 subunit of IL-12 was examined and shown to be increased by disease but downregulated by systemic poly I:C treatment (Figure 4.9a). Two-way ANOVA revealed a main effect of disease ($F = 93.81$, $df\ 1,14$, $p < 0.0001$) and of poly I:C ($F = 18.89$, $df\ 1,14$, $p = 0.0007$) along with an interaction between these two factors ($F = 99.35$, $df\ 1,14$, $p = 0.0006$). Bonferroni post-hoc analysis revealed that poly I:C did not induce IL-12 expression in NBH animals and that ME7 prion disease *per se* significantly increased its expression ($p < 0.001$). Systemic poly I:C treatment, however, significantly decreased this IL-12 expression in diseased animals ($p < 0.001$), but levels of this transcript still remained higher than the corresponding NBH control animals.

IFN- γ was induced in ME7 animals (main effect of disease; $F = 21.34$, $df\ 1,14$, $p = 0.0004$) but was decreased by poly I:C challenge (main effect of poly I:C, $F = 6.30$, $df\ 1,14$, $p = 0.0250$). There was no interaction of these two factors (Figure 4.9b).

CNS expression of the inducible form of nitric oxide synthase (iNOS), which can ultimately give rise to the generation of neurotoxic free radical species, was increased by poly I:C in NBH animals but was not induced by poly I:C in ME7 animals (Figure 4.9c). Two-way ANOVA revealed no main effects but revealed an interaction between disease and poly I:C treatment ($F = 5.22$, $df\ 1,14$, $p = 0.0385$). Bonferroni post-hoc analysis showed that poly I:C significantly increases iNOS expression in NBH animals but does not affect its transcript levels in ME7 mice. This is consistent with the earlier observed absence of CNS up-regulation of TNF- α following systemic poly I:C in ME7 animals.

The expression of matrix metalloproteinase-9 (MMP9) was very low and was not altered by disease or poly I:C treatment, with no statistically significant changes (Figure 4.9d).

A number of gene transcripts indicative of cytotoxic T cell or natural killer cell action in the brain had previously been assessed following poly I:C challenge in ME7 animals (Field et al., 2010). The expression of CD8, perforin, granzyme A and MHC class I was found to be somewhat induced by disease but not further altered by systemic poly I:C challenge, suggesting a lack of induction of cytotoxic T cell recruitment and infiltration into the brain, although this was examined only at early time points after poly I:C treatment.

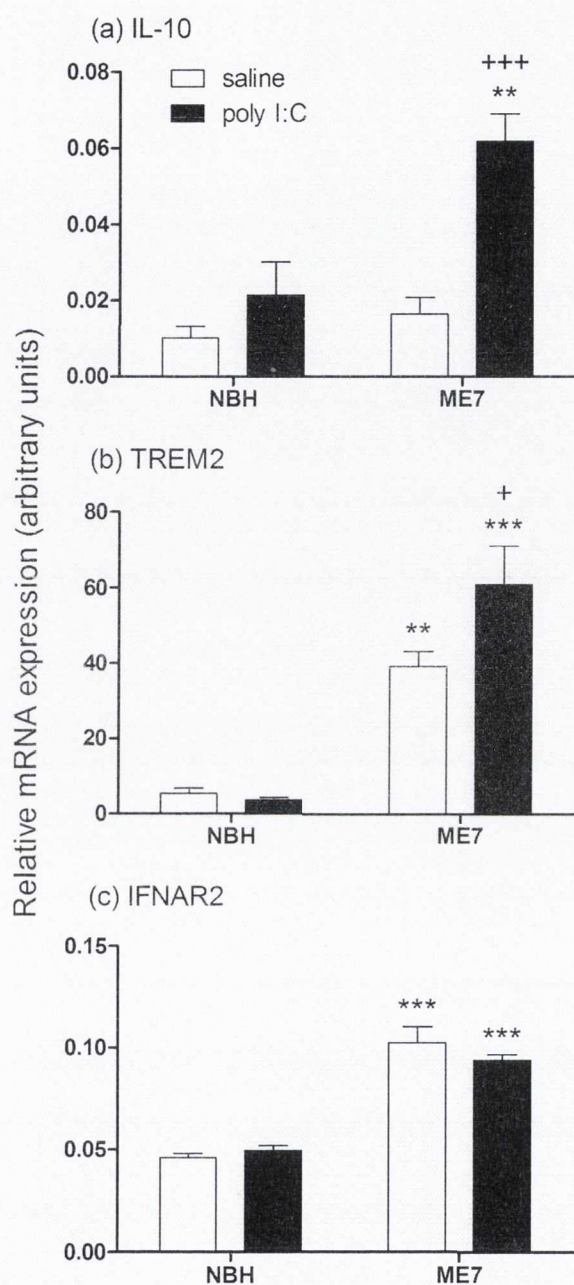


Figure 4.8 Evidence of anti-inflammatory profile following *IFN- β* induction. Expression of mRNA for (a) IL-10, (b) TREM2 and (c) IFNAR2 as assessed by quantitative PCR 6 h following poly I:C (12 mg/kg i.p.) or sterile saline in ME7 or NBH animals at 18 weeks post-inoculation. Significant differences by Bonferroni post-hoc tests after significant two-way ANOVA are represented by $+p < 0.05$, $**p < 0.01$, $***/+p < 0.001$. Comparisons between ME7 and NBH are represented by *, and comparisons between saline and poly I:C are denoted +. Data are expressed as mean $\pm$ SEM ($n = 3$ for NBH + saline and $n = 5$ for all other groups)

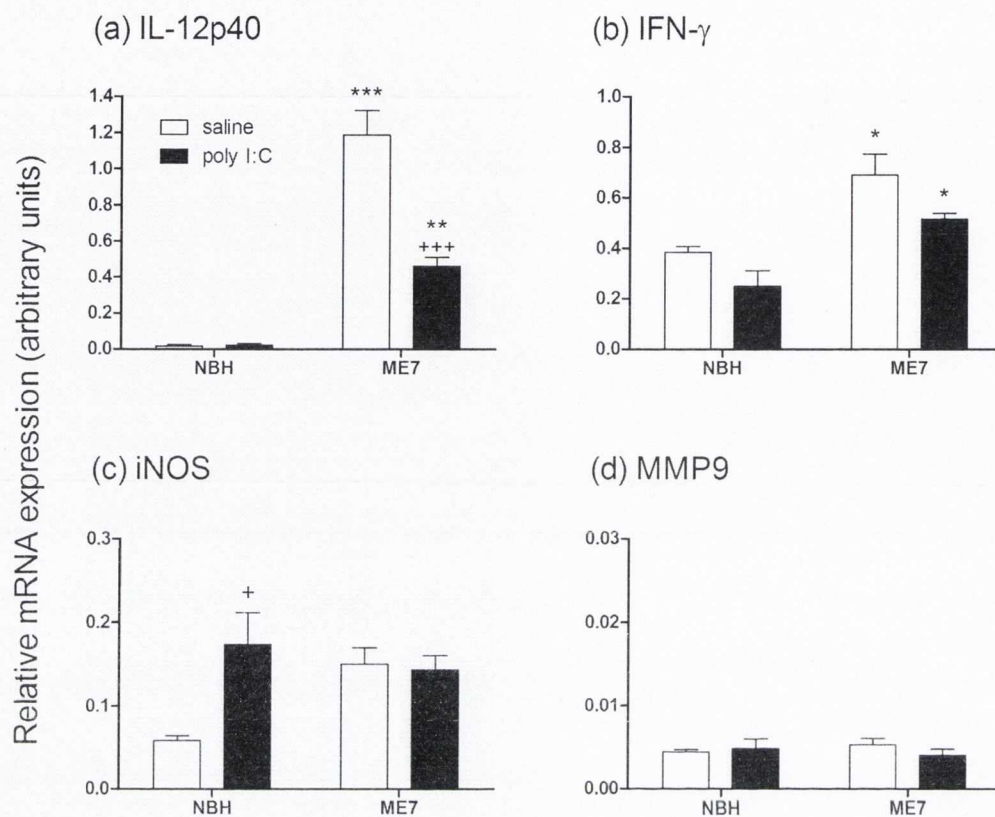


Figure 4.9 Absence of other pro-inflammatory mediator expression following IFN- β induction. Expression of mRNA for (a) IL-12 subunit p40, (b) IFN- γ , (c) iNOS and (d) MMP9 as assessed by quantitative PCR 6 h following poly I:C (12 mg/kg i.p.) or sterile saline in ME7 or NBH animals at 18 weeks post-inoculation. Significant differences by Bonferroni post-hoc tests after significant two-way ANOVA are represented by $*/+p < 0.05$, $**p < 0.01$, $***/+++p < 0.001$. Comparisons between ME7 and NBH are represented by *, and comparisons between saline and poly I:C are denoted +. Data are expressed as mean $\pm$ SEM ($n = 3$ for NBH + saline and $n = 5$ for all other groups).

4.2.8 Neurological consequences after systemic inflammation during disease

To examine the effect of multiple systemic inflammatory events that activate the acute anti-viral response on the long-term progression of neurodegenerative disease, animals were challenged three times with systemic poly I:C (12 mg/kg) or saline at 14, 16 and 18 weeks post-inoculation with ME7 or NBH. Animals were assessed for performance on motor co-ordination and muscle strength tasks (horizontal bar and inverted screen), which are known to deteriorate with progression of the ME7 strain of prion disease but to be intact at 16 weeks post-inoculation (Betmouni et al., 1996, Cunningham et al., 2005a).

Systemic poly I:C significantly impaired performance of ME7 animals on both the horizontal bar and inverted screen acutely at 16 weeks post-inoculation. Neither co-ordination nor muscle strength were acutely affected in poly I:C-treated NBH animals or in ME7 animals given saline. Two-way repeated measures ANOVA analysis of acute effects on the horizontal bar revealed a significant interaction between treatment and time ($F = 3.03$, $df\ 8,156$, $p = 0.0034$), with main effects of treatment ($F = 11.86$, $df\ 2,156$, $p < 0.0001$) and of time ($F = 3.34$, $df\ 4,156$, $p = 0.0118$). Bonferroni post-hoc tests showed that ME7 + poly I:C animals were significantly impaired with respect to both other groups 6 hours ($p < 0.05$), 14 hours ($p < 0.001$) and 24 hours ($p < 0.05$) following systemic challenge.

Similarly, on the inverted screen there was a significant interaction of treatment and time ($F = 2.58$, $df\ 8,156$, $p = 0.0113$) and main effect of treatment ($F = 13.19$, $df\ 2,156$, $p < 0.0001$) and time ($F = 5.04$, $df\ 4,156$, $p < 0.0008$). Bonferroni post-hoc tests showed that ME7 + poly I:C animals were significantly impaired compared to ME7 + saline at 6 and 14 hours ($p < 0.001$), and were also impaired compared to NBH + poly I:C at 6 ($p < 0.05$) and 14 hours ($p < 0.01$) following challenge.

However, longitudinal analysis of performance on bar and screen tasks showed that repeated challenge with poly I:C (at 14, 16 and 18 weeks) resulted in more rapid development of permanent loss of function on these tasks. Two-way repeated measures analysis of weekly performance in the same animals revealed clearly exacerbated neurological decline as measured by both tasks. There was a significant interaction of treatment and time ($F = 8.88$, $df\ 12,228$, $p < 0.0001$) with main effects of both treatment ($F = 17.12$, $df\ 2,228$, $p < 0.0001$) and of time ($F = 29.09$, $df\ 6,228$, $p < 0.0001$) on horizontal bar performance. Bonferroni post-hoc tests revealed significant differences between ME7

+ poly I:C and ME7 + saline from 17 weeks onwards ($p < 0.05$ at 17 weeks and $p < 0.001$ at 18 and 19 weeks). Similar analysis of inverted screen data revealed an interaction between treatment and time ($F = 15.62$, $df\ 12,228$, $p < 0.0001$) and main effects of treatment ($F = 30.35$, $df\ 2,228$, $p < 0.0001$) and of time ($F = 59.45$, $df\ 6,228$, $p < 0.0001$). Bonferroni post-hoc tests showed significant differences between ME7 + poly I:C and ME7 + saline at 17 and 19 weeks ($p < 0.001$). Thus poly I:C induced acute neurological impairments and also exacerbated progression of disease in ME7 animals, without effect in normal animals.

These data are presented as acute systemic inflammatory events superimposed on long-term progression of disease (Figure 4.10). Systemic poly I:C induces acute neurological impairments only in prion-diseased animals with no significant effect in NBH mice similarly challenged or in ME7 animals treated with saline. The magnitude of neurological impairment is dependent on the degree to which prion disease has progressed (time post-inoculation). Longitudinal analysis of performance on bar and screen tasks showed that repeated challenge with poly I:C (at 14, 16 and 18 weeks) resulted in more rapid development of permanent loss of function on these tasks. Thus poly I:C induced acute neurological impairments and also exacerbated progression of disease in ME7 animals, without effect in normal animals. These data demonstrate that repeated anti-viral acute phase responses have a negative influence on the progression of neurodegenerative disease, and suggest that systemic viral infections will have deleterious consequences for the progression of disease.

[Data in Figure 4.10 provided by Dr. Colm Cunningham (full data in Field et al., 2010)]

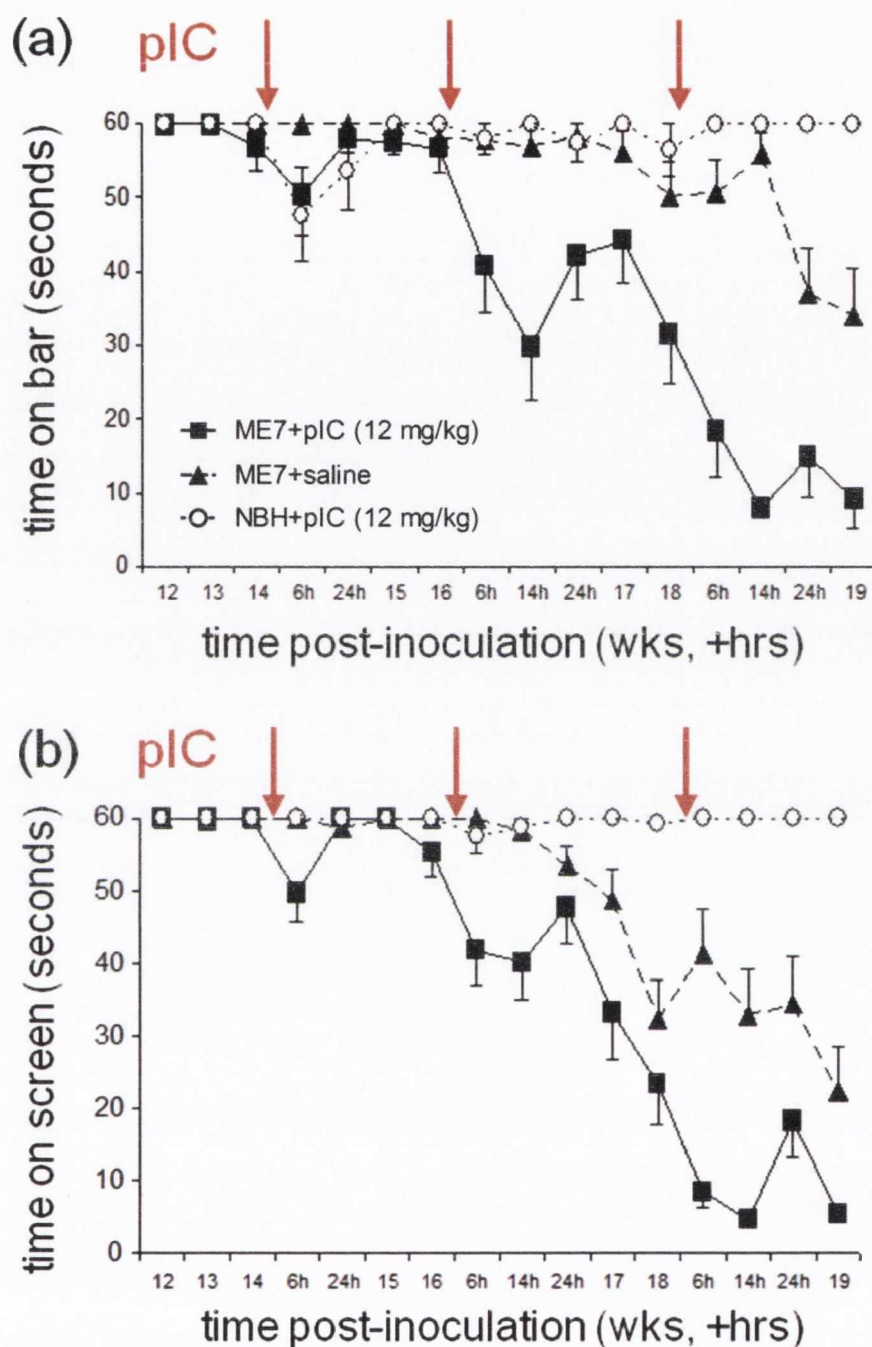


Figure 4.10 *Acute and longitudinal changes in disease progression following systemic poly I:C.* The acute and weekly time point assessments on the bar and screen tests for motor co-ordination and muscle strength are shown to depict the course of neurological impairments when underlying disease and systemic inflammatory events combine. Systemic challenge with poly I:C (12 mg/kg) is indicated by red arrows. Animals received three systemic challenges with poly I:C. Data expressed as mean $\pm$ SEM ($n = 12$ for NBH + poly I:C and $n = 15$ for all other groups)

4.2.9 Peripheral and central inflammatory response to multiple poly I:C challenges

The current chapter reported that poly I:C caused acute exacerbations of neurological function and longitudinally accelerated neurodegenerative disease. Since the animals showing this exacerbation of disease were challenged with poly I:C on three separate occasions, we also conducted preliminary investigations into the effects of multiple challenges with poly I:C on the systemic and CNS inflammatory response in normal animals. Thus, plasma levels of IFN- β , IL-1 β , TNF- α and IL-6 (Figure 4.11), and hippocampal mRNA transcription of IRF7, IL-1 β , TNF- α and IL-6 (Figure 4.12) were examined following one, two or three doses of poly I:C (12 mg/kg i.p.) to determine whether repeated systemic administration has a tolerising or sensitising effect on responses to subsequent stimulation. Normal animals received either one, two or three doses of poly I:C, each subsequent dose administered two weeks following the previous one and in each case mice were euthanized for plasma measurements 3 hours following the final dose of poly I:C. Each dose was administered two weeks apart to ensure full recovery from the prior systemic inflammatory response and to reflect the repeated course of poly I:C in the longitudinal disease progression experiments.

Plasma levels of IFN- β were elevated with each poly I:C challenge but were not different with one, two or three systemic challenges (Figure 4.11a). One-way ANOVA analysis ($F = 4.724$, $df\ 3,11$, $p = 0.0236$) followed by selected Bonferroni post-hoc comparisons revealed that plasma IFN- β was significantly elevated after systemic poly I:C with respect to saline ($p < 0.05$) but the three poly I:C treatment groups were not different to each other ($p > 0.05$).

Similarly, plasma levels of IL-1 β were elevated following poly I:C challenge but were not different after one, two or three challenges (Figure 4.11b). One-way ANOVA analysis ($F = 4.371$, $df\ 3,11$, $p = 0.0295$) followed by selected Bonferroni post-hoc comparisons revealed that plasma IL-1 β was significantly elevated after systemic poly I:C with respect to saline ($p < 0.05$) but the three poly I:C treatment groups were not different to each other ($p > 0.05$).

Plasma levels of TNF- α (Figure 4.11c) and IL-6 (Figure 4.11d) showed similar patterns of induction and each showed variability in the response following one, two or three challenges with poly I:C. One-way ANOVA analyses revealed that there was no difference in plasma levels of TNF- α ($F = 2.5$, $df\ 3,11$, $p = 0.1137$) and IL-6 ($F = 0.3381$, $df\ 3,11$, $p =$

0.0579) following one, two or three systemic challenges with poly I:C, although there appeared to be a trend towards an increase in the peripheral response in both of these cytokines following the third dose of poly I:C.

Hippocampal expression of IRF7 mRNA was measured by qPCR as a readout of type I interferon induction and was induced by poly I:C to similar levels in each of the three treatment groups (Figure 4.12a). One-way ANOVA analysis ($F = 7.308$, $df\ 3,10$, $p = 0.007$) followed by selected Bonferroni post-hoc comparisons revealed that poly I:C significantly induced IRF7 following one, two or three doses ($p < 0.05$) but that this induction was not different with each dose ($p > 0.05$).

IL-1 β mRNA expression was variable following systemic poly I:C challenge and appeared slightly but not significantly elevated (Figure 4.12b). One-way ANOVA analysis ($F = 1.27$, $df\ 3,10$, $p = 0.3367$) revealed that there were no differences in IL-1 β mRNA expression following one, two or three doses of poly I:C.

Poly I:C induced hippocampal mRNA expression of TNF- α to similar levels following one, two or three doses of poly I:C (Figure 4.12c). One-way ANOVA analysis ($F = 5.111$, $df\ 3,10$, $p = 0.0212$) followed by selected Bonferroni post-hoc comparisons revealed that TNF- α mRNA expression was significantly induced following one, two or three doses of poly I:C ($p < 0.05$) but that this induction was not different with each dose ($p > 0.05$).

Hippocampal IL-6 mRNA expression appeared to be induced by each dose of poly I:C but was not induced to different levels each time (Figure 4.12d). One-way ANOVA analysis ($F = 4.346$, $df\ 3,10$, $p = 0.0333$) followed by selected Bonferroni post-hoc comparisons revealed that IL-6 was significantly induced by poly I:C only after three doses of poly I:C ($p < 0.05$) and that this induction was not different to the expression after one or two doses.

Thus, this preliminary investigation into the effects of multiple challenges with poly I:C that activate the acute anti-viral response, seems to confirm that the systemic and central type I interferon response to systemic poly I:C neither becomes tolerant to repeated challenge, nor shows exaggerated responses upon multiple challenges. The inflammatory cytokine response is less clear due to the variability inherent in a study with small numbers of subjects such as this. There is some suggestion, albeit not statistically significant, that the peripheral induction of TNF- α and IL-6 may become amplified upon multiple viral

challenges. The mRNA expression in the brain, however, appears equivalent after one, two or three challenges. The preliminary findings observed here remain important given the use of the repeated poly I:C challenge in the initial disease progression experiments, and given the propensity for the aged and demented population to experience multiple co-morbidities and infections one after the other.

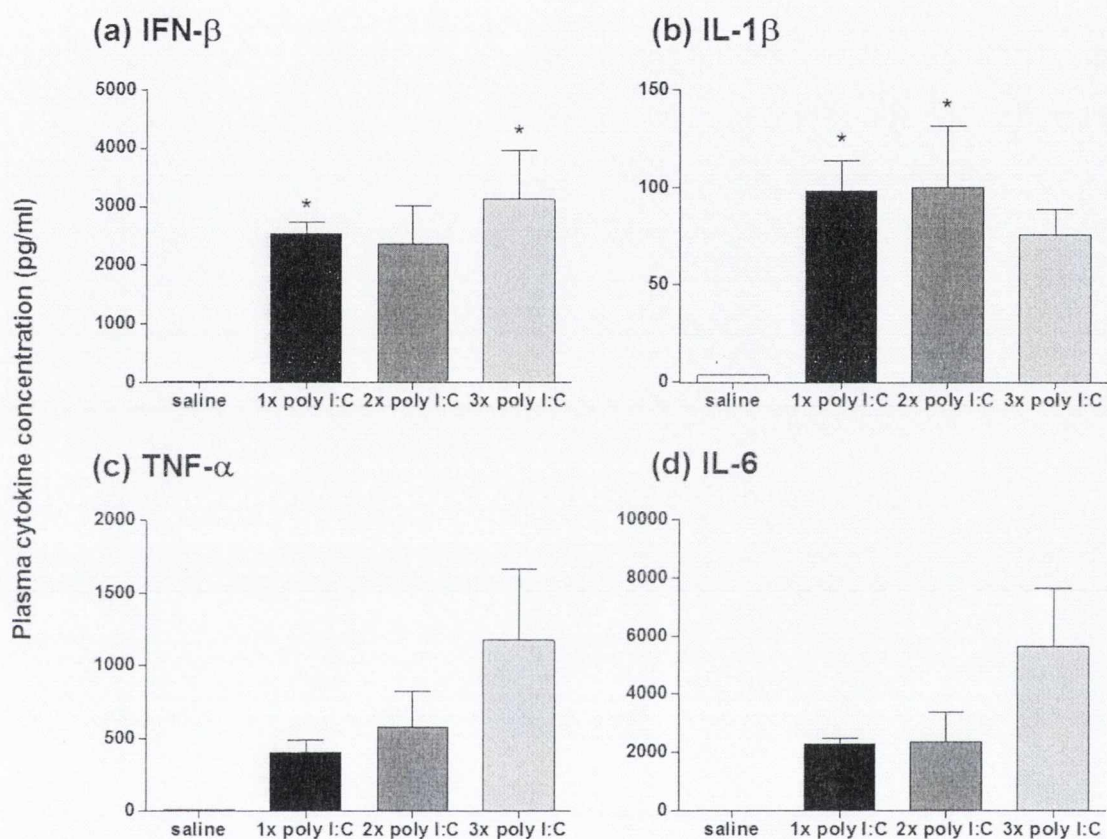


Figure 4.11 Plasma cytokine levels after repeated systemic poly I:C challenge. Plasma levels of (a) IFN- β , (b) IL-1 β , (c) TNF- α and (d) IL-6 protein 3 hours following one, two or three challenges with poly I:C (12 mg/kg i.p.). Each systemic challenge was made two weeks apart. Significant differences to saline as determined by Bonferroni post-hoc tests after one-way ANOVA are represented by * $p < 0.05$. No significant differences were observed between the each of the poly I:C treatment groups. Data are expressed as mean $\pm$ SEM ($n = 3$ for saline, $n = 4$ for each poly I:C group)

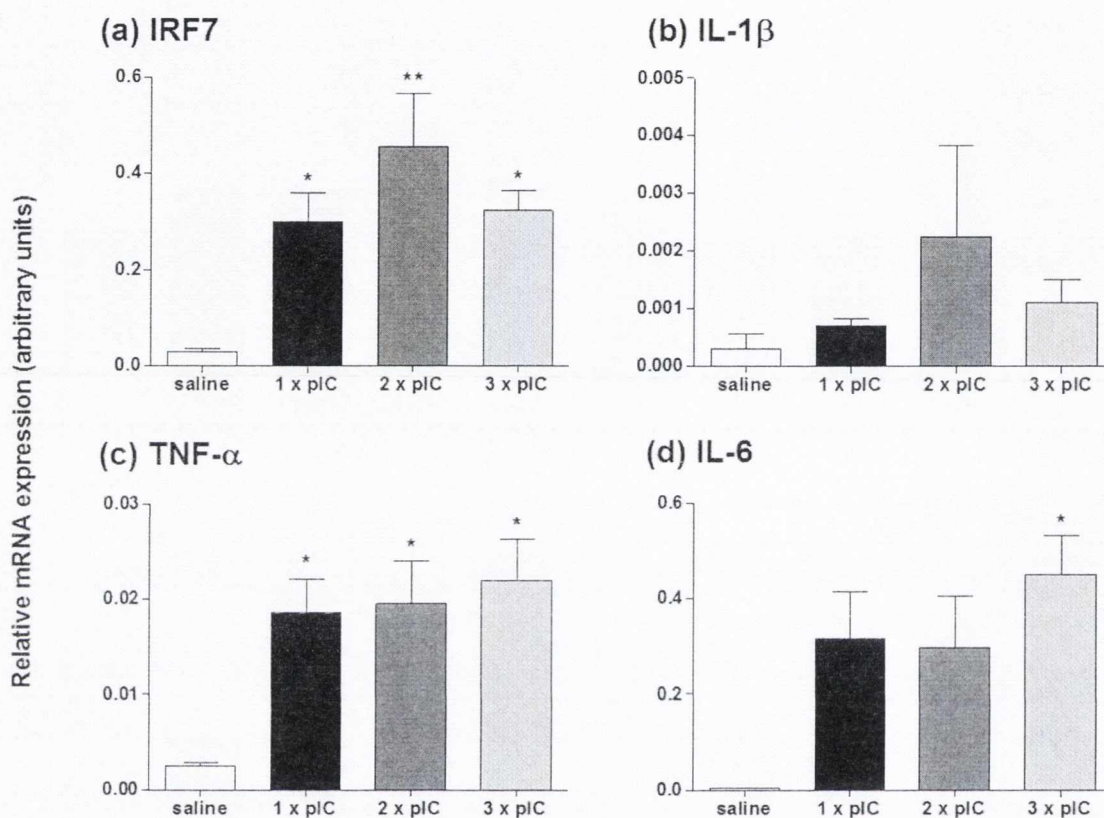


Figure 4.12 *Hippocampal cytokine mRNA expression after repeated systemic poly I:C challenge.* Hippocampal expression of (a) IRF7, (b) IL-1 β , (c) TNF- α and (d) IL-6 mRNA as measured by qPCR, 3 hours following one, two or three challenges with poly I:C (12 mg/kg i.p.). Each systemic challenge was made two weeks apart. Significant differences to saline as determined by Bonferroni post-hoc tests after one-way ANOVA are represented by * $p < 0.05$ and ** $p < 0.01$. No significant differences were observed between the each of the poly I:C treatment groups. Data are expressed as mean $\pm$ SEM ($n = 3$ for saline and 2 x pIC, $n = 4$ for the other pIC groups)

4.2.10 Is increased amyloid burden responsible for disease exacerbation?

Since a ramping up of peripheral and central inflammatory responses does not obviously occur upon multiple challenges with poly I:C, we sought other mechanisms that might underpin the poly I:C-induced exacerbation of neurodegenerative disease. An exacerbation of amyloid burden in ME7 animals following multiple systemic poly I:C challenges might contribute to accelerated progression of disease. We investigated this by assessing the deposition of PrP^{Sc}-positive amyloid in the hippocampus.

Deposition of the aberrant, infectious form of prion protein, PrP^{Sc}, is not significantly altered by repeated systemic poly I:C stimulation (Figure 4.13). Animals inoculated with NBH or ME7 were challenged systemically with poly I:C (12 mg/kg) or saline at 14, 16 and 18 weeks post-inoculation. Immunohistochemistry was performed at the level of the dorsal hippocampus at 20 weeks post-inoculation to assess proteinase K-resistant prion protein (PrP^{Sc}) deposition. NBH brain tissue is completely devoid of PrP^{Sc} deposition (Figure 4.13a). ME7 animals showed profound PrP^{Sc} labelling in the hippocampus and the density and distribution of this labelling is not significantly different in those ME7 animals receiving three systemic challenges with poly I:C (Figure 4.13b,c). Thus, systemic administration of poly I:C does not appear to influence disease-associated PrP^{Sc} deposition and does not increase amyloid burden.

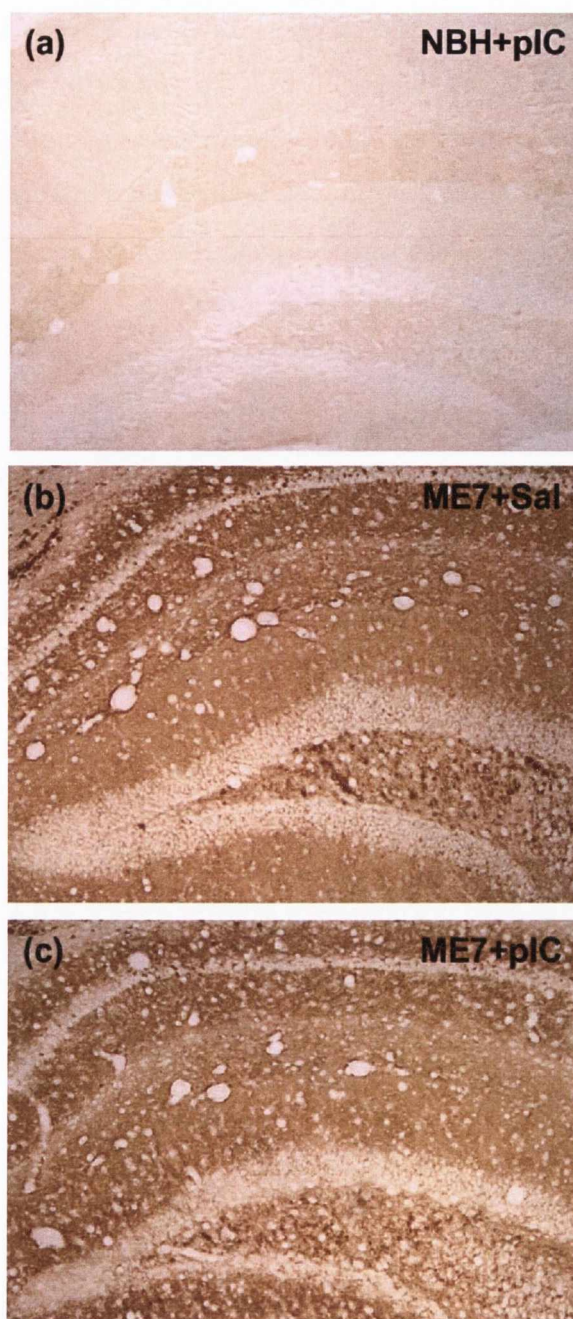


Figure 4.13 *PrP^{Sc} deposition in ME7 animals following repeated poly I:C stimulation.* Immunohistochemistry for the aberrant form of prion protein, PrP^{Sc}, was performed on coronal sections at the level of the hippocampus in NBH and ME7 animals that received three systemic challenges with either poly I:C (12 mg/kg) or saline at 14, 16 and 18 weeks post-inoculation. (a) NBH tissue was devoid of PrP^{Sc}. (b) ME7 animals showed profound PrP^{Sc} accumulation throughout the hippocampus and the density and distribution of this labelling is not significantly different in those ME7 animals which received three poly I:C challenges.

4.2.11 Apoptosis

We investigated whether there was evidence of increased cell death in ME7 animals challenged with poly I:C. Small numbers of activated caspase-3-positive cells and larger numbers of TUNEL-positive cells were observed in ME7 animals 15 hours after treatment with saline or poly I:C (12 mg/kg i.p.). Examples of both activated caspase-3 and TUNEL-positive cells are shown in Figure 4.14 (a) and (b). Quantification of caspase-3 positive cells is impractical due to the small number of detected cells positive for this protein. The larger number and smaller size of TUNEL-positive cells reflects the later stage of cell-degeneration, as previously shown after LPS treatment of ME7 animals (Cunningham et al., 2005b).

TUNEL-positive apoptotic cells (positive TUNEL labelling plus evidence of nuclear condensation) were counted in the areas of pathology (the hippocampus and thalamus) in 10 μ m sections of animals 15 hours following challenge with either poly I:C or saline (Figure 4.14c). ME7 animals challenged with poly I:C had significantly higher numbers of apoptotic cells per 10 μ m section than those treated with saline (12 ± 3 versus 6 ± 1 ; $p < 0.05$ by one-way ANOVA with Bonferroni post-hoc test). NBH animals challenged with poly I:C showed very low number of apoptotic cells (1 ± 1 per 10 μ m section).

The expression of the pro-apoptotic genes PKR, Fas and Bax were also examined (Figure 4.14d,e,f) and a clear poly I:C-induced increase in PKR and Fas mRNA expression was observed. Bax was induced somewhat in ME7 animals, but not elevated further by poly I:C treatment. Two time-points are provided to provide temporal information about the changes in pro-apoptotic genes.

The expression of PKR (Figure 4.14d) is influenced by disease and poly I:C as revealed by a significant one-way ANOVA ($F = 20.80$, df 5,26, $p < 0.0001$). Bonferroni post-hoc comparisons revealed that while NBH and ME7 were not significantly different, PKR expression in ME7 animals challenged with poly I:C was significantly higher than similarly challenged NBH animals at both 4 and 6 hours post-challenge ($p < 0.05$).

Examination of hippocampal expression of Bax revealed an increase with disease but no further change with poly I:C (Figure 4.14e). One-way ANOVA analysis ($F = 10.78$, df 5,26, $p < 0.0001$) followed by selected Bonferroni post-hoc comparisons revealed that Bax expression in ME7 animals was significantly increased compared to NBH controls ($p <$

0.001). No further induction of Bax expression was observed with poly I:C treatment and in fact, expression appeared to be decreased 6 hours following poly I:C in ME7 animals compared to ME7 *per se*.

Hippocampal expression of the death receptor Fas, which is involved in initiation of apoptosis, was also examined (Figure 4.14f). One-way ANOVA analysis ($F = 61.99$, $df = 5,26$, $p < 0.0001$) with selected Bonferroni post-hoc comparisons revealed that Fas expression was significantly induced by ME7 disease compared to NBH ($p < 0.001$), and also that 4 hours following poly I:C treatment, ME7 animals showed significantly increased expression of Fas compared to both ME7 *per se* ($p < 0.001$) and NBH + poly I:C ($p < 0.001$).

Thus the current data show that there is both increased apoptosis along with amplified expression of some of the genes that make up the pro-apoptotic machinery, in ME7 animals challenged systemically with poly I:C.

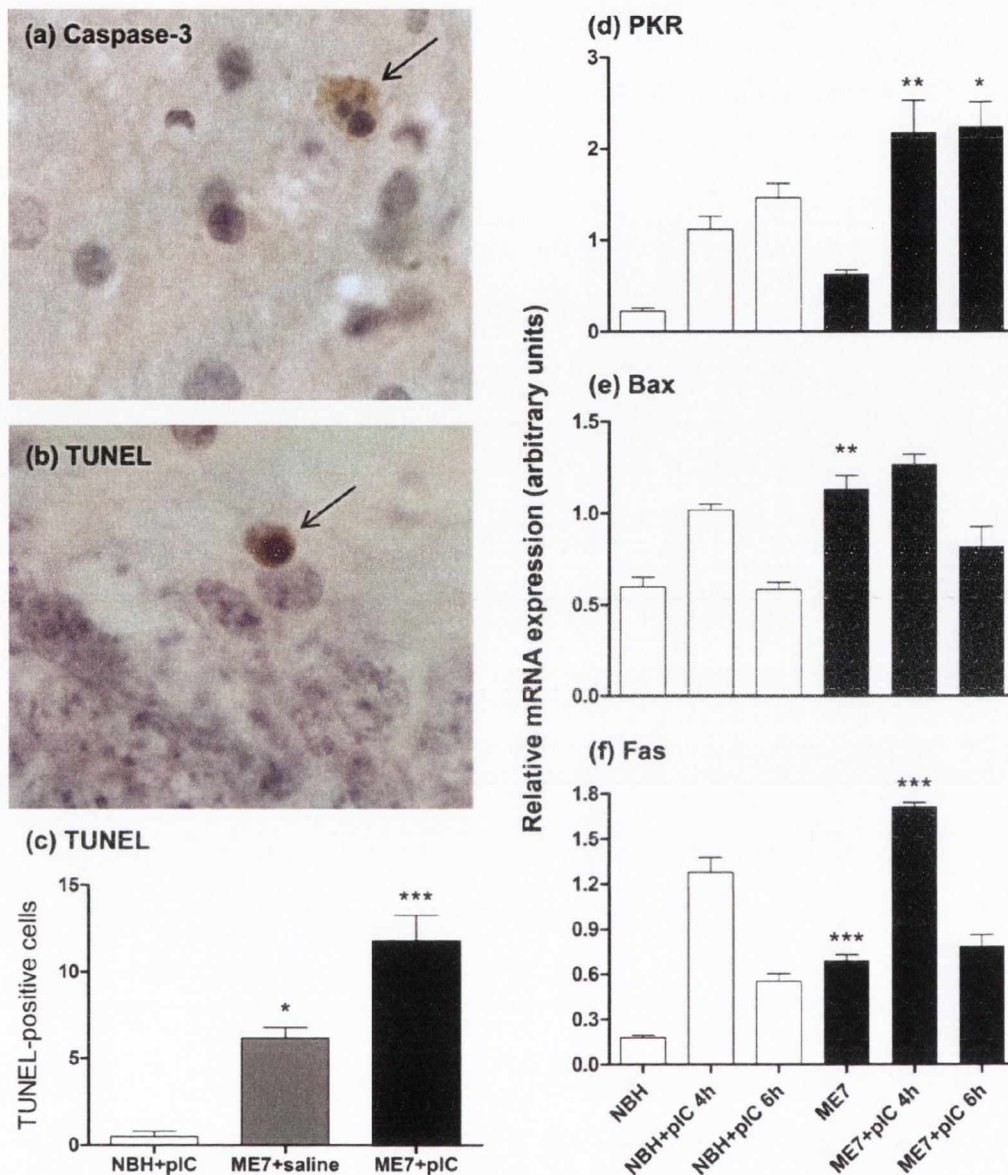


Figure 4.14 Systemic poly I:C activates pro-apoptotic pathways and increases apoptosis in ME7 animals. Examples of immunohistochemically labelled activated caspase-3 and TUNEL-positive apoptotic cells are indicated by arrows in (a) and (b) respectively. (c) TUNEL-positive cell numbers present in the hippocampus and thalamus 15 h following systemic poly I:C (12 mg/kg i.p.). Significant differences to NBH + poly I:C controls by one-way ANOVA and Bonferroni post-hoc tests indicated by * $p < 0.05$ and *** $p < 0.001$. (d-f) Analysis of mRNA expression of PKR, Bax and Fas at different time points following poly I:C challenge (12 mg/kg i.p.). Significant differences by Bonferroni post-hoc tests after significant one way ANOVA are indicated by ** $p < 0.01$ and *** $p < 0.001$. Data are expressed as mean $\pm$ SEM ($n = 3$ for NBH, $n = 4$ for NBH and ME7 + poly I:C 4 h and $n = 5$ for all other groups)

4.3 Discussion

This chapter has investigated the impact of acute systemic inflammatory stimulation with dsRNA (poly I:C), to mimic the acute phase of the anti-viral response, on chronic neurodegenerative disease. Repeated peripheral poly I:C challenges produce acute neurological deficits and longitudinally accelerate progression of neurodegenerative disease without lasting effects in normal animals. Systemic poly I:C challenge also causes an exaggerated hypothermic response in ME7 prion-diseased animals in which there is underlying pathology. Concurrent with these exacerbations of disease symptoms, systemic poly I:C produced amplified CNS type I interferon and IL-1 β responses, indicating that the degenerating brain is primed to produce exaggerated responses to subsequent systemic immune stimulation. Despite an apparent anti-inflammatory post-priming phenotype, pro-apoptotic gene expression was up-regulated with evidence of increased apoptotic cell death in diseased animals administered poly I:C, thus indicating that systemic stimulation of the acute antiviral response exacerbates existing chronic neurodegeneration.

4.3.1 Microglial priming and exaggerated CNS inflammation

The synthetic dsRNA, poly I:C, has been widely used in the field of inflammation to mimic the acute antiviral response, since most viruses generate dsRNA at some point during their life cycle. Poly I:C can evoke an inflammatory response through its action as a PAMP for the pattern recognition receptor TLR3. Although there are a number of other receptors that recognise poly I:C and viral dsRNA, such as retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated protein 5 (MDA-5) and PKR (Honda and Taniguchi, 2006, Kato et al., 2006, Yoneyama et al., 2004), TLR3 remains one of the key responders to this type of stimulation (Alexopoulou et al., 2001) and agonism of this PRR has been shown to induce a much more robust antiviral response than TLR4 stimulation (Doyle et al., 2003). Poly I:C is known to be a potent activator of the TLR3-mediated antiviral response through the robust induction of type I interferons α and β and other inflammatory cytokines, and has therefore been used extensively to study the acute phase of viral infection (Jacobs and Langland, 1996, Matsumoto and Seya, 2008). Recent studies have confirmed that microglial cells express TLR3 and respond to poly I:C (Melton et al., 2003, Olson and Miller, 2004) and are dependent on this PRR for responses to intracerebroventricularly administered poly I:C (Town et al., 2006). It has also been reported that TLR3 is expressed on astrocytes (Farina et al., 2005) and endothelial cells

(Tissari et al., 2005), providing further routes for stimulation of the TLR3-mediated type I interferon response. The effect of poly I:C on the brain, however, has received quite limited attention (Katafuchi et al., 2003, Traynor et al., 2006, Cunningham et al., 2007, Konat et al., 2009) and remains in its infancy.

The current study has provided the first demonstration of microglial priming following stimulation of the acute anti-viral response using poly I:C. In animals primed by ongoing neurodegeneration, systemic challenge with poly I:C induces exaggerated IL-1 β and IFN- β responses in the brains of ME7 prion-diseased animals. This is demonstrated by increased mRNA transcripts for these genes in the hippocampus and thalamus, the primary regions of pathology during prion disease, and also by immunohistochemical labelling for IL-1 β and IRF3. The presence of an exaggerated TNF- α response was also investigated but this proved to be absent, as indicated by decreased mRNA transcripts, non-detectable protein levels and a complete absence of immunolabelling for this cytokine. Previous studies have illustrated that primed microglia undergo a phenotypic switch without further morphological change upon subsequent central or systemic immune stimulation with LPS (Cunningham et al., 2005b). In the current study we show that this exaggerated response also occurs upon poly I:C stimulation, inducing clear evidence of IL-1 β activation with microglial morphology in the periventricular region of the hippocampus and around the dentate gyrus. This activation is only present in ME7 animals challenged systemically with poly I:C and is not detectable in normal animals receiving poly I:C or in prion disease *per se*. Since the numbers of microglia do not change following systemic poly I:C challenge, one might predict that it is some underlying facet of disease that makes these cells more susceptible to systemic inflammatory insults rather than merely having increased numbers of cells to produce inflammatory mediators. Indeed a recent study has identified that the complement system, specifically the C3 or factor B proteins, is necessary for microglial priming in the EAE model of MS (Ramaglia et al., 2012). This is one of the first studies to identify a discrete priming factor. We have also seen from chapter 3 that cholinergic hypofunction is also sufficient to prime microglia to produce exaggerated responses to subsequent inflammatory stimulation. Given that the current study has demonstrated an amplified type I IFN response, it was therefore important to address whether these inflammatory mediators are the “priming factor” in ME7 model and chapter 5 will aim to interrogate this possibility.

IRF3 labelling also showed similar localisation to the periventricular and dentate gyrus areas and exhibited evidence of nuclear translocation, predictive of subsequent activation of transcription of IFN- α/β . This increased IL-1 β and IRF3 activation could be a consequence of activation of the cerebrovascular endothelial cell layer by poly I:C, which is demonstrated by the immunohistochemical labelling of COX-2. That is, the cells that are positive for IRF3 do not appear to be endothelial cells. Thus, systemic poly I:C might act directly on the endothelial layer and transduce its inflammatory signal via induction of mediators of inflammation into the parenchyma, as is reported in numerous studies on the routes of transduction of systemic inflammation to the brain (Gosselin and Rivest, 2008, Uchikado et al., 2004, Zhou et al., 2009). However, it also remains possible that poly I:C itself or circulating inflammatory mediators could have some access to the brain parenchyma. This might be possible since prior reports have shown that systemic poly I:C itself can compromise the integrity of the BBB (Wang et al., 2004) although in this case not until 24 hours after challenge. Moreover, there is evidence that suggests the BBB is already somewhat compromised in areas of prion disease pathology (Chung et al., 1995, Wisniewski et al., 1983) and as such the poly I:C-induced disruption could exhibit its effect at an earlier time-point thus providing access to the parenchyma. The current study suggests that that poly I:C or its induced systemic inflammatory mediators are not afforded access to the parenchyma since the IgG labelling was not present the brain parenchyma. However, this technique may be less sensitive to formalin fixation and would likely need further optimisation in fresh-frozen tissue to definitively address this issue. Ongoing work in the laboratory has prepared fresh-frozen tissue to address this issue.

Previous studies in normal animals have shown a robust induction of circulating cytokines IFN- β , IL-6, IL-1 β and TNF- α in response to peripheral poly I:C challenge (Cunningham et al., 2007, Konat et al., 2009). There was also significant up-regulation of these mRNA transcripts in the hippocampus and hypothalamus following poly I:C treatment of normal animals. We have also shown that systemic poly I:C induces up-regulation of antiviral transcripts, IFN- α/β , TLR3 and RIG-I in both the hippocampus and hypothalamus of normal animals (Field et al., 2010). An exaggerated or primed transcription of these genes upon systemic poly I:C challenge is present in ME7 prion-diseased mice, but this is not caused by exaggerated peripheral cytokine production: levels of circulating cytokines are similar in both NBH and ME7 animals. The primed response in the brain however, is limited to the region of underlying pathology in prion disease, the hippocampus and thalamus, and is not evident in the hypothalamus which does not display obvious prion-

related pathology (Field et al., 2010). Thus, it seems that the priming phenomenon occurs specifically in regions where there is existing brain pathology.

The TLR3 response to viral dsRNA or poly I:C initiates signal transduction via transcription factors NF κ B and interferon regulatory factor 3 (IRF3), through the recruitment of the adaptor molecule TRIF (Toll/IL-1R-domain-containing adaptor protein inducing IFN- β) (Akira and Takeda, 2004, Kawai and Akira, 2010). TRIF can recruit TNF receptor associated factor-6 (TRAF6) and activate transforming growth factor- β activated kinase-1 (TAK1), leading to activation of NF κ B, which facilitates its nuclear translocation, and stimulation of NF κ B-dependent gene transcription. Such NF κ B-dependent genes include the classical pro-inflammatory cytokines IL-6, TNF- α and IL-1 β . If TRIF recruits TRAF3 and TBK1, however, phosphorylation of IRF3 is the result, leading to its nuclear translocation and up-regulation of gene transcription for IRF3-dependent genes such as IFN- β (Honda and Taniguchi, 2006).

The production of type I IFN results in signalling at the type I interferon receptor (IFNAR), which forms a positive-feedback loop mechanism whereby production of IFN- α/β drives further increases in these mediators. Binding at the IFNAR leads to activation of IFN-stimulated gene factor 3 (ISGF3; a heterotrimer of signal transducer and activator of transcription 1 (STAT1), STAT2 and IRF9), which translocates to the nucleus and induces transcription of IRF7. This drives further IFN- α/β production, eliciting the full antiviral response and activates many key antiviral transcripts such as PKR, OAS1a and Mx1 (Honda and Taniguchi, 2006). The current study reported robust transcription of all of these genes and demonstrates that IFN- α/β is produced in the CNS, at mRNA and protein levels, and is active in the brain. Levels of all of these transcripts are markedly increased by systemic challenge with poly I:C and this occurs to a much higher level in ME7 animals, despite similar systemic responses. Thus, the finding that the CNS produces an exaggerated type I IFN response to systemic poly I:C, provides further evidence that the degenerating brain is primed by the underlying neurodegeneration to produce amplified responses to subsequent stimulation of the antiviral immune response. Although we have provided evidence of increased type I IFN in the brain, the cell type that releases IFN- β in the brain remains unclear. Immunohistochemical analysis has, so far, proved inconclusive but the antibodies tested are suggestive of astrocyte labelling and parallel work in the lab does indicate that astrocytes of the diseased brain are also primed to produce exaggerated responses to inflammatory stimulation (Hennessy et al., unpublished observations). It

remains an important priority to identify what cells in the brain are responsible for IFN- β secretion.

There are limited reports in the literature of an increased interferon response during prion disease *per se* (Baker et al., 2004, Riemer et al., 2004, Stobart et al., 2007) and also during sterile neurodegenerative insults (Khorrooshi and Owens, 2010, Hosmane et al., 2012). The latter studies have both demonstrated evidence of type I IFN response in the brain, and specifically microglia, following sterile axonal injury via transection of entorhinal afferent or dorsal root axotomy. Since the type I IFN is principally thought to be produced following viral infection and stimulation of PRRs that recognise nucleic acids, it is certainly surprising that this response seems to be induced also by neurodegeneration that is not of infectious aetiology. It seems plausible that given the widely reported anti-inflammatory nature of IFN- α/β that such an increased response in neurodegenerative conditions might be part of the brain's attempt to control deleterious inflammation associated with neuronal injury. Chapter 5 will investigate whether this increased type I IFN response is also a characteristic of our ME7 model of murine prion disease. Thus it became important to address whether there is an increased type I IFN response in the ME7 model of prion disease. Given that type I IFNs have previously been shown to be capable of inducing priming in peritoneal macrophages (Vadiveloo et al., 2000) it is possible that these molecules act as the microglial priming factor in ME7 prion disease. Consequently, chapter 5 will investigate the role the effects of systemic poly I:C on the primed response in the absence of type I IFN signalling using IFNAR1^{-/-} mice and will also examine disease progression in these animals.

4.3.2 Pro- and anti-inflammatory effects

Type I IFNs are principally involved in orchestrating the immune response to viral infection but have typically been viewed as anti-inflammatory mediators in the brain (Prinz et al., 2008). Given that these molecules often serve to control and reduce inflammatory responses, a primed or exaggerated IFN- α/β response might be beneficial to the degenerating brain. A number of well described pro- and anti-inflammatory pathways were investigated following poly I:C treatment to examine the central inflammatory effects of inducing a systemic anti-viral response during chronic neurodegeneration and whether it differs from that observed in response to LPS/TLR4 stimulation (Cunningham et al., 2005b).

The classical inflammation-inducible enzymes, iNOS and the matrix metalloproteinase MMP9, showed no further induction in ME7 animals following peripheral poly I:C challenge, while the expression of IFN- γ , TNF- α and the p40 (40-kDa) subunit of IL-12 was suppressed after poly I:C in ME7 mice. Expression of TNF-inducible genes ADAM8 and TNFAIP2 confirmed the lack of TNF- α induction in poly I:C-treated ME7 mice. There was also an absence of detectable protein levels of TNF- α by ELISA and immunolabelling despite positive control tissue (LPS i.c.) showing evidence of TNF- α induction. This inflammatory profile, concurrent with increases in transcription IL-1 β , IL-6, IFN- β , IL-10 and TREM2, and increased IL-1 β and IRF3 immunolabelling, represents a post-priming inflammatory phenotype that is somewhat different to that described after LPS challenge (Cunningham et al., 2005b). This might well come as a result of the anti-inflammatory effects of type I IFN, since these molecules are the principal component of the anti-viral response and IFN- β remains one of the main pharmacological treatments for MS. Indeed, IFN- β therapy has been shown to reduce expression of TNF- α and IFN- γ (Gayo et al., 1999) but promote induction of IL-10 (Rudick et al., 1998) in peripheral blood mononuclear cells of patients with relapsing-remitting MS. Thus, the poly I:C-induced changes in these cytokines in ME7 animals could be directly due to an increased IFN- β response in the diseased brain. Furthermore, CNS anti-inflammatory effects of IFN- β are also reported, with type I IFNs shown to limit leukocyte infiltration to the brain (Prinz et al., 2008) and reduce the central expression of pro-inflammatory cytokines such as TNF- α , IL-12 and IL-17 (Makar et al., 2008, Chen et al., 2009) and indeed, it has been proposed that IFN- β might evoke its therapeutic effects through inhibition of IL-12 (Karp et al., 2000). IL-12 is typically produced by macrophages to stimulate T cell proliferation, favouring a T_H1 profile (Trinchieri, 1994), and as such, increased CNS expression of IL-12 is reported in EAE models that show marked T cell infiltration (Issazadeh et al., 1995, Bright et al., 1998). Thus, amplified IFN- β following poly I:C might inhibit microglial production of IL-12 and further limit T cell infiltration into the diseased brains.

Type I interferon treatment has also been shown to reduce disruption of the BBB in animal models and MS patients (Billiau, 2006), providing a possible mechanism for neuroprotection in MS. This could also provide a plausible explanation for the lack of IgG infiltration into the brain parenchyma in the current study. A less compromised BBB would in theory protect the parenchyma from exposure to poly I:C or other inflammatory mediators in the circulation, perhaps offering insight into the mechanism by which the inflammatory signal is transduced from periphery to brain. Rather than dsRNA crossing a

damaged BBB, a likely route is via endothelial activation, which was confirmed by COX-2 labelling, and subsequent production of damaging inflammatory cytokines by these cells or perivascular macrophages and/or microglia. In addition to this, loss of endogenous IFN- β exacerbates inflammation and pathology in the EAE mouse model of MS, further supporting its anti-inflammatory effects in the brain (Teige et al., 2003). The apparent anti-inflammatory profile observed in the current study following IFN- β induction may be explained simply by exaggerated production of IFN- β but also may be influenced by increased hippocampal expression of the type I interferon receptor subunit 2 (IFNAR2) in prion disease *per se*. IFN- α/β may therefore engage more receptors, such that subsequent anti-viral stimulation, which induces large increases in type I interferon expression, could propagate a larger IFN- α/β -dependent signal.

Despite the reported anti-inflammatory influences of IFN- β , ME7 animals challenged systemically with poly I:C show elevated IL-1 β at mRNA and protein levels in the microglia. This is in contrast to reports elsewhere which demonstrated that poly I:C-induced type I interferon diminished IL-1 β production in response to fungal pathogens (Guarda et al., 2011), although neurodegenerative disease might cause some as yet unknown alteration in these anti-inflammatory mechanisms. The production of IL-1 β after poly I:C treatment in diseased animals could have negative consequences for the diseased brain and induce deleterious mechanisms, such as apoptosis or increased synaptic degeneration, and indeed, IL-1 β has been implicated in the neuronal loss following ischaemic, hypoxic and excitotoxic insults (Rothwell et al., 1997). Thus, it will be important to address the role of this increased poly-I:C-mediated IL-1 β production in prion disease.

4.3.3 Exaggerated hypothermia and acceleration of disease

Despite the apparent anti-inflammatory influences of type I IFN responses in the brain, it is clear that stimulation of the anti-viral response with poly I:C had negative consequences for the progression of chronic neurodegenerative disease in the ME7 model. The current study has shown that systemic poly I:C (12 mg/kg i.p.) induces a mild hyperthermic response in normal (NBH) animals, consistent with prior reports (Cunningham et al., 2007). However, these data have also demonstrated that a single systemic challenge with poly I:C induces a marked *hypothermic* response in prion-diseased animals when compared to normal animals similarly challenged. The novelty of the current data lies in

the fact that the temperature response during neurodegenerative disease is driven in a different direction than that in normal animals. A differential response to peripheral inflammatory insults during neurodegenerative disease has been reported elsewhere with the demonstration that systemic infection can produce divergent effects on core body temperature in young and aged rats (Barrientos et al., 2009). In this study the authors report a hyperthermic response in young animals but aged animals exhibit an initial marked hypothermia followed by elevated core body temperature that persists for 3 days. A further study in aged rodents also demonstrated exaggerated sickness behaviour in aged animals following peripheral LPS administration (Godbout et al., 2005). The current findings are also consistent with previously ME7 studies which demonstrated that peripheral inflammatory challenge with bacterial endotoxin (LPS, 500 µg/kg i.p.) induces exaggerated sickness behaviour in prion-diseased animals as measured by a hypothermic response and decreased locomotor activity (Combrinck et al., 2002).

The febrile response in rodents has been reported elsewhere to be influenced by IL-1 β (Watkins et al., 1995, Chai et al., 1996). There is however, some evidence of IL-1 β -induced hypothermia in mice (Wang et al., 1997) and indeed recent data from our laboratory has also demonstrated this (Skelly et al., unpublished observations). Thus, it is important to assess the role of IL-1 β in this abnormal hypothermic response. Chapter 5 will assess the role of IL-1 β in the hypothermic response by employing ME7-inoculated mice deficient in the signalling IL-1 receptor type I (IL-1RI $^{-/-}$). Prior to this confirmation that the kinetics of prion disease remain similar in wild type and IL-1RI $^{-/-}$ mice must be conducted.

Since poly I:C is known to be a potent inducer of the type I interferon antiviral response (Field et al., 1967, Matsumoto and Seya, 2008), it is possible that the observed hypothermia in ME7 animals is a result of the reported increase in type I IFN signalling in the brain, in a way similar to the increased sickness behaviour evident in patients receiving IFN- α treatment for cancer therapy (Capuron et al., 2002). Increased sickness and neuropsychiatric complications such as delirium and acute cognitive dysfunction have also been reported with IFN- β treatment for multiple sclerosis (Neille et al., 1996, Goeb et al., 2003). Thus, it has become important to identify the origins of the differential responses in prion-diseased animals and chapter 5 of this thesis will examine the CNS response to systemic poly I:C challenge in prion-diseased animals that are lacking the type I interferon receptor (IFNAR1 $^{-/-}$) and are thus unresponsive to the type I interferons (IFN- α/β). This

will be important in addressing whether the type I IFNs are key to the primed microglial response in ME7 animals.

The current study has shown that prion-diseased animals receiving three systemic challenges with poly I:C show acute exacerbations of neurological function concurrent with an acceleration of disease progression. This is the first study to report exacerbation of disease following mimicry of a viral infection and is highly relevant to the ageing population that are frequently exposed to viral infections, and often suffer from multiple consecutive inflammatory insults. It is known from previous studies that repeated challenges do not produce behavioural tolerance to poly I:C (Cunningham et al., 2007) and the current data provide preliminary evidence that in normal animals, the peripheral and central inflammatory response to poly I:C stimulation, does not appear to be different after one, two or three challenges, each administered two weeks apart. This will be further discussed below but demonstrates that the inflammatory response neither exhibits a ramping up, nor a dampening down, following repeated acute stimulation of the antiviral immune reaction, an important observation given the use of multiple poly I:C challenges in the longitudinal disease progression experiments.

Repeated acute systemic inflammatory events superimposed upon longitudinal decline allow for the illustration of the impact of repeated antiviral immune responses on the precise course of progressive disease. The acute exacerbations of neurological function are followed by recovery, but as disease progresses, the animals do not achieve 100% recovery. That is, they recover to a new, lower baseline level of performance. It is plausible that the magnitude of the acute exacerbation and the acceleration of disease progression might be due to the severity of the underlying pathology that increases with the burden of disease. Thus, at a more advanced stage in disease, animals are at risk of suffering from greater inflammation-induced neurological exacerbations. One would expect this to be true also for cognitive function, whereby increased disease-associated synaptic loss and microglial activation confer increased susceptibility to suffering from the cognitive effects of systemic inflammation. Progression of disease/dementia seems to be a risk factor for systemic inflammation-induced decline, and it has been shown that elderly patients (mean age, 80 years) with cardiovascular disease show increased cognitive decline that is related to viral or bacterial burden (Strandberg et al., 2003). These observations are similar to those found previously where a single peripheral challenge with bacterial LPS (500 µg/kg) at 14-15 weeks post-inoculation caused an accelerated appearance of late-stage

signs of neurodegenerative disease (Cunningham et al., 2009). Thus, it is now apparent that this is not limited to bacterial inflammation, and that the anti-viral mimic, poly I:C, can also exacerbate neurological function and accelerate progression of disease. This finding is important in its own right, but mechanistic elucidation is now an important priority. The current chapter examined three initial possibilities: 1) that one poly I:C challenge sensitizes the animal to subsequent poly I:C challenges, thus ‘ramping up’ inflammatory responses, 2) that repeated poly I:C induces accelerated amyloidosis, and 3) that poly I:C acutely increases cell death.

4.3.4 Cytokine profiles and PrP^{Sc} deposition in response to repeated poly I:C stimulation

Prion-diseased animals exhibited acute exacerbation and longitudinal neurological decline upon receiving three challenges with poly I:C. Subsequent challenges were made two weeks after the previous ones to ensure the prior acute inflammatory response had resolved but each new challenge was superimposed onto an increased burden of disease (ie 2 weeks later each time). Thus, it was important to assess, under normal conditions, whether repeated poly I:C challenge produced differential inflammatory responses due to tolerance or sensitisation. The peripheral and CNS inflammatory response to multiple challenges with such a viral mimetic has not previously been investigated.

Although our laboratory has previously demonstrated that mice do not exhibit behavioural tolerance to repeated challenge with poly I:C, the inflammatory responses *in vivo* have not been investigated. This is an important area of investigation since the phenomenon of endotoxin tolerance exists for the TLR4 immune response evoked by bacterial LPS (Biswas and Lopez-Collazo, 2009). In this scenario, repeated TLR4 stimulation with LPS eventually triggers a “dampening down” of the inflammatory response. This phenomenon may have its origins in conditions of chronic low-grade inflammation whereby the host enters into an unresponsive state to endotoxin to offer protection from the deleterious effects of “ramping up” the inflammatory response. Upon multiple challenges with systemic LPS, systemic responses become significantly weaker but CNS responses can be maintained and in some cases amplified (Puentener et al., 2012).

The current study has demonstrated that in normal animals, the peripheral cytokine response to activation of the acute anti-viral response with poly I:C, does not appear to be different with multiple challenges of this nature, as measured by circulating levels of IFN-

β , IL-1 β , TNF- α and IL-6. In this preliminary investigation relatively small numbers were used and there was considerable variability in some of the experimental groups. Although the IFN- β and IL-1 β responses appear quite consistent, the peripheral induction of TNF- α and IL-6 is rather variable. Nonetheless, animals that received just a single systemic challenge with poly I:C were most consistent and it is with subsequent challenges that the variability within treatment groups appears to become more apparent. There was a trend towards increased TNF- α and IL-6 responses following three poly I:C challenges.

The CNS response to multiple poly I:C challenges also appears relatively clear with respect to type I interferon response, with similar induction of IRF7 across all three challenges. The expression of mRNA transcripts for TNF- α and IL-6 appears to follow a similar pattern of induction to IRF7 in the brain, although IL-1 β is less obvious in this instance, with considerable variability apparent in the animals challenged twice.

Thus, the type I IFN response to repeated stimulation of the antiviral immune reaction with poly I:C does not appear to exhibit a ramping up nor a dampening down of the response. This is in contrast to the widely reported induction of tolerance to multiple LPS challenges, both *in vitro* and *in vivo* (Beeson, 1947, Medvedev et al., 2000, Medvedev et al., 2006). Is it possible that there is a divergence between the IRF3 and NF κ B signalling pathways activated by TLR3 and TLR4 stimulation that might be responsible for the lack of tolerance in one instance and the induction of tolerance in another. The fact that the IL-1 β , TNF- α , and IL-6 responses are more variable in the periphery and the CNS could be as a result of this. The nature of this preliminary study might also account for some of the variability here and a more comprehensive analysis of the effects of multiple challenges would be desirable in the future. Nonetheless, the current data do suggest that there is neither a robust tolerising nor a robust sensitising effect of multiple poly I:C challenges in normal animals. This represents important information in the context of exacerbation and acceleration of neurodegenerative disease following multiple systemic inflammatory events. This raises the possibility that the increased magnitude of neurological and sickness responses to each subsequent challenge during disease can be attributed to the increasing underlying disease burden rather than priming by the repeated challenges themselves. These data could therefore provide novel information relating to the inflammatory milieu in vulnerable patient populations that can experience numerous viral infections.

As for effects on amyloidosis after three poly I:C challenges, there was no apparent cumulative effect. There are numerous studies in the AD field suggesting that multiple LPS challenges can alter amyloid processing and lead to exacerbated amyloid deposition (Brugg et al., 1995, Lee et al., 2008), but we do not observe similar changes here. However, there is clear evidence that extracellular PrP^{Sc} deposition can continue despite halting of prion disease progression (Mallucci et al., 2003) suggesting a mismatch between degree of amyloidosis and severity of disease stage. Likewise in AD patients, clearance of amyloid plaques by immunotherapy did not lead to clinical benefits (Holmes et al., 2008). Conversely, it is well established that sepsis induces neuronal death, albeit in relatively severe systemic inflammatory states (Semmler et al., 2005, Semmler et al., 2007). Thus we propose that induction of *de novo* neuropathology is a plausible way in which systemic inflammation may exacerbate disease. Recent longitudinal studies have shown that patients who suffer episodes of delirium, which are often induced by systemic inflammation, have a greatly increased risk of developing dementia and in these cases there was no obvious association between their dementia and typical neuropathological features such as amyloid plaques and Tau tangles (Davis et al., 2012)

4.3.5 Type I interferons and apoptosis

Despite the potential anti-inflammatory influences of the type I IFN response in the brain, systemic stimulation with poly I:C can clearly still be deleterious to disease progression, as seen with the poly I:C-induced acceleration of disease. The use of IFN- α in cancer therapy has shown that increased levels of circulating type I IFN leads to sickness behaviour (Capuron et al., 2002) and several prospective studies have also reported neuropsychiatric complications such as depression and delirium in patients receiving IFN- α (Meyers et al., 1991, Schaefer et al., 2002, Raison et al., 2005). There is also evidence showing that systemic injection of IFN- α can induce interferon-responsive genes in the hypothalamus of mice (Wang et al., 2008). Furthermore, a recent study by Okun and colleagues has provided new data demonstrating that TLR3 signalling negatively regulates hippocampal-dependent learning and memory and suppresses neurogenesis (Okun et al., 2010). Taken together, these data, along with the finding from the current study, demonstrate that TLR3 stimulation and action of type I IFN in the brain, despite anti-inflammatory influence, can be associated with negative consequences for brain function.

The data in the current study, through upregulation of IFN-responsive genes IRF7, OAS1a, Mx1 and PKR, indicate that type I IFN are secreted and active in the degenerating brain after systemic poly I:C challenge. Although there is evidence that transgenic (Campbell et al., 1999), or viral encephalitis-induced (Sas et al., 2009) expression of IFN- α can produce CNS neuropathology there has been limited investigation into the pathological effects of acute type I interferon responses in the brain.

Evidence for apoptotic cell death was investigated in the brains of ME7 prion-diseased mice since it was hypothesised that increased cell death might play a role in the poly I:C-mediated exacerbation of neurodegenerative disease. The current data show increased activated caspase-3 and TUNEL-positive cell death by immunohistochemistry following poly I:C challenge in prion-diseased animals, concurrent with elevated expression of the death receptor, Fas, and the type I IFN-inducible gene PKR. There is evidence that IFN- α/β is a potent pro-apoptotic stimulus. The marked type I interferon-dependent up-regulation of PKR in ME7 animals in response to systemic poly I:C could initiate apoptotic pathways, leading to increased cell death which might impact upon the kinetics of prion disease progression. Prior reports have demonstrated that increased activity of PKR can induce apoptosis (Balachandran et al., 1998, Balachandran et al., 2000) with up-regulation of Fas and Bax thought to be involved, along with activation of caspase-3. Previous studies using the transgenic Tg(PG14) form of prion disease have shown that neuronal degeneration in this model is modulated by activation of these pathways (Chiesa et al., 2005). Further studies have also shown PKR to impair long-term potentiation (LTP) and memory consolidation (Jiang et al., 2010) through phosphorylation and consequent deactivation of the eukaryotic translation initiation factor, eIF2 α . Phosphorylation of eIF2 α results in inhibition of protein synthesis and it is possible that this could result in the depletion of key proteins that might be involved in suppression of apoptotic cascades or indeed suppression of other proteins key to normal cellular function. Given that PKR is robustly induced by type I IFNs, the observed increase in apoptotic cell death might be directly caused by the poly I:C-induced elevated type I IFN response.

IL-1 β has also been reported to be involved in the induction of apoptosis *in vivo* and *in vitro*, via caspase-3-mediated mechanisms. In light of this role for IL-1 β , it is possible that the amplified production of IL-1 β following systemic poly I:C treatment in ME7 animals is responsible for the observed increase in apoptotic cell death. The presence of activated caspase-3-positive apoptotic cells in the current study could be indicative of IL-1 β -

dependent apoptosis as prior reports have shown decreased caspase-3 activation and apoptosis following treatment with IL-1 receptor antagonist (Nesic et al., 2001). This pro-inflammatory cytokine has also been implicated in the apoptotic cell death observed in acute brain injury (Wang et al., 2005) and in the normal ageing brain (Martin et al., 2002). There is also evidence of neuronal apoptosis mediated by IL-1 β in viral encephalitis (Takikita et al., 2001), an important observation given the use of poly I:C to mimic viral infection in the current study. It will be important to identify whether the increased apoptotic cell death in poly I:C challenge ME7 animals is mediated by IL-1 β and chapter 5 will aim to address this through the use of ME7-inoculated IL-1R1^{-/-} mice.

Thus, the current data have shown increased apoptotic cell death along with up-regulation of pro-apoptotic machinery in ME7 prion-diseased animals challenged systemically with poly I:C. Regardless of the mechanism that underpins this apoptosis, type I IFN or IL-1 β , this increased cell death is likely to be detrimental to the already vulnerable brain during neurodegeneration. In light of this, it seems that the exacerbation and acceleration of disease demonstrated earlier could be attributed to increased cell death upon stimulation of the acute anti-viral response.

4.3.6 Conclusion

This chapter has illustrated that stimulation of the acute anti-viral response with poly I:C, when superimposed onto prior neurodegenerative pathology, causes exaggerated IL-1 β and type I IFN responses in the CNS and demonstrates that the microglial population of the areas of prion-associated neuropathology are primed to produce exaggerated responses to systemic poly I:C challenges. These challenges also trigger acute neurological exacerbations and accelerate neurodegenerative disease. Previous work on chronic neurodegenerative disease has shown that microglia are primed by underlying disease to produce exaggerated sickness and CNS inflammatory responses to subsequent systemic stimulation with the TLR4 agonist LPS (Combrinck et al., 2002, Cunningham et al., 2005b). This priming phenomenon and exacerbation of pathology is not limited to prion disease and has been reported in other models of neurodegeneration including AD (Kitazawa et al., 2005, Sly et al., 2001), PD (Pott Godoy et al., 2008), amyotrophic lateral sclerosis (Nguyen et al., 2004), ageing (Barrientos et al., 2006, Godbout et al., 2005), multiple sclerosis (Ramaglia et al., 2012) and stroke (McColl et al., 2007). The current body of work demonstrates that stimulation of the peripheral anti-viral response with poly

I:C is also sufficient to activate microglia that have previously been primed by existing neurodegenerative disease. Thus, the hyper-reactivity exhibited by primed microglia is not specific to LPS challenge, and adds TLR3 (and perhaps other anti-viral PRRs) to the list of PRRs that are capable of initiating the exacerbation of neurodegenerative disease. While this may have been predicted from previous work with LPS/TLR4 (Cunningham et al., 2009), the demonstration that systemic poly I:C can produce amplified CNS responses during neurodegenerative disease has not been shown before and is not a trivial undertaking. Given the high incidence of viral infection in the aged and demented population (Estes et al., 2006) and the dearth of investigation into the effects of systemic viral infections on the degenerating brain, this constitutes an important study that should open exciting new research avenues.

These findings are highly relevant to the clinical setting where studies have revealed that a single acute episode of delirium, can lead to long-term cognitive decline and hasten the time to death (Rockwood et al., 1999). There are also implications for the recent study that showed that increased acute and chronic peripheral inflammation (serum TNF- α) was associated with worsening of dementia and cognitive decline in AD (Holmes et al., 2009). In particular, the observation that multiple systemic inflammatory events (in this case poly I:C challenge) produce progressively more severe and less reversible deficits appears to mimic the fluctuating rate of neurological decline apparent in AD patients (Hope et al., 1999, Holmes and Lovestone, 2003).

This study has begun to identify some of the neuropathological changes that may underpin inflammation-induced deterioration of neurological function in the vulnerable CNS and has prompted specific questions about the involvement of exaggerated IL- β and type I IFN responses in these pathological changes: what role do type IFNs play in the primed response to systemic poly I:C during neurodegenerative disease, is neurodegeneration exacerbated by poly I:C in animals deficient in IL-1 or type I IFN signalling. Chapter 5 will begin to address some of these issues.

Given the high frequency of viral infection in the aged and demented population (Falsey et al., 2005, Estes et al., 2006), and the evidence that higher viral burden increases the incidence of dementia and rate of cognitive decline (Strandberg et al., 2003, Aiello et al., 2006), the current findings have therapeutic implications for targeting these systemic

exacerbations in susceptible patient populations and could offer novel opportunities to slow disease progression.

Chapter 5

The role of IL-1RI and IFNAR1 in the CNS inflammatory state and progression of disease in the ME7 model of prion disease

5.1 Introduction

Inflammatory processes have long been implicated in the pathogenesis of neurodegenerative disease. The pro-inflammatory cytokine IL-1 β , has been shown to be elevated in the brains of AD patients (Griffin et al., 1989) and other clinical studies have demonstrated that long-term NSAID therapy for chronic inflammatory conditions is associated with reduced risk of development of AD and PD (in t' Veld et al., 2001, Chen et al., 2005). Furthermore, there is also an inflammatory component to prion disease, with evidence of increased inflammatory gene transcripts and microglial activation (Cunningham et al., 2005b, Baker et al., 2004). As with AD, whether this is a cause, or consequence of progressive neuronal death remains unclear (Wyss-Coray, 2006).

Neurodegenerative disease is characterised by the progressive loss of neurons, often leading to multi-domain brain dysfunction including impairments in global cognition, memory and movement, and ultimately death. The most common neurodegenerative diseases are AD, PD, ALS and MS. The group of diseases known as transmissible spongiform encephalopathies (TSEs) or prion diseases are another class of neurodegenerative diseases that received attention during the 1990s in the UK and Ireland as a result of the outbreak of variant Creutzfeldt-Jakob disease (vCJD) – the “human form” of bovine spongiform encephalopathy. Initially thought of as viral infections, early studies of prion disease in mice attempted to treat and slow the progression by boosting the type I interferon response (Field et al., 1969, Worthington, 1972, Gresser et al., 1983) but this avenue proved unfruitful. This viral aetiology hypothesis was eventually supplanted by Prusiner who demonstrated that TSEs caused a conformational change in the ubiquitous cellular prion protein (PrP^C) to the aberrant “scrapie” form (PrP^{Sc}) (Prusiner, 1982). The mechanism by which this misfolded protein causes neurotoxicity remains poorly understood.

Our laboratory have utilised the ME7 strain of murine prion disease to study the brain macrophage (microglial) response to chronic neurodegeneration. This progressive disease is characterised by extracellular amyloidosis, synaptic loss, neuronal death and irreversible cognitive decline (Betmouni et al., 1999, Cunningham et al., 2003), thus recapitulating many key facets of the human demented population. The ME7 strain of prion disease also exhibits a distinctive inflammatory profile with morphologically activated microglia (Betmouni et al., 1996) and moderately increased mRNA transcripts for IL-1 β and TNF- α

(Cunningham et al., 2005c). There have also been reports of and increased type I IFN response in other models of murine prion disease (Baker et al., 2004, Riemer et al., 2004, Stobart et al., 2007).

Chapter 4, along with previous clinical research, has highlighted that systemic inflammatory events can exacerbate dementia and accelerate disease progression (Holmes et al., 2009), but elucidation of the inflammatory pathway(s) that underpin the inflammation-induced decline has not yet been identified. As has been shown in chapter 4, systemic stimulation of the peripheral anti-viral response using the synthetic dsRNA, poly I:C, produces amplified IL-1 β and IFN- β responses in the degenerating brain, concomitant with acceleration of disease progression. Along with these inflammatory responses was exaggerated hypothermia, increased apoptosis and microglial priming. Thus the role of IL-1 β and type I IFN in these processes became an important area of investigation. Furthermore, it was both necessary and relatively convenient to examine the impact of these genes in the progression of prion disease *per se*.

Since type I IFNs have been reported to be capable of inducing priming in peripheral macrophages (Vadiveloo et al., 2000) similar to classical macrophage priming by IFN- γ (Johnson et al., 1983), it was important to investigate whether there was a type I IFN response in ME7 prion disease *per se* and also whether the primed response observed in chapter 4 was dependent on type I IFNs. Thus ME7 animals lacking the type I IFN receptor subunit 1 (IFNAR1^{-/-}) were challenged systemically with poly I:C to assess the CNS inflammatory profile and microglial phenotype in the absence of type I IFN signalling.

Prior to examining the effects of poly I:C stimulation in prion diseased immune knockout animals, it was important to assess disease progression in these animals to verify that the kinetics of disease were not significantly altered since interpretation of effects of systemic challenges would be significantly confounded if underlying disease state was fundamentally different in different strains. Previous research on the progression of the ME7 prion disease identified changes in affective behaviours, such as reduced burrowing activity and hyperactivity in the open field (Deacon et al., 2001, Cunningham et al., 2003), from approximately 12 and 14 weeks post-inoculation respectively, along with cognitive decline in the T-maze task (Skelly et al, in preparation). Impairments in motor function (balance, co-ordination and muscle strength) then manifest toward the late stages of

disease (17-18 weeks onwards), followed by characteristic clinical signs such as piloerection, weight loss, incontinence and eventual death at about 23 weeks (Felton et al., 2005). Thus the current chapter will use IL-1RI^{-/-} and IFNAR1^{-/-} mice to examine the effect of endogenous IL-1 and type I IFN signalling on the progression of ME7 prion disease per se (i.e. without further systemic inflammatory insults).

It is plausible that inflammation and particular inflammatory mediators may be important in the disease process per se, but may alternatively play a role only when the individual is subject to systemic inflammatory insults. Such inflammatory insults are common in the ageing and demented population but the dissociation of the underlying disease and superimposed co-morbidity is not easily studied in human populations. That is to say, it is difficult to dissect inflammation inherent to the disease from inflammation occurring in individuals as a result of some co-morbidity (such as atherosclerosis, obesity, arthritis, diabetes and many others). The ME7 model facilitates examination of progression of neurodegenerative disease without further immune stimulation but also the impact of systemic insults and particular inflammatory molecules on altered trajectory of disease or exacerbation of function.

Thus the objectives of the current chapter were as follows:

- 1.) Determination of the progression of ME7 prion disease in IL-1RI^{-/-} and IFNAR1^{-/-} mice
- 2.) Assessment of the role of IL-1 signalling in exaggerated hypothermic responses and apoptosis following systemic poly I:C
- 3.) Assessment of the role of IFNAR signalling in controlling microglial phenotype in ME7 prion disease upon systemic poly I:C stimulation.

5.2 Results

5.2.1 Early behavioural changes in wild type and IL-1RI^{-/-} ME7 mice

Early changes in affective behaviours are characteristic of the ME7 strain of prion disease (Deacon et al., 2001, Cunningham et al., 2003) and as such, burrowing activity was assessed longitudinally in prion-inoculated wild type and IL-1RI^{-/-} mice.

Measurements of burrowing behaviour for a two-hour period were taken weekly from 10 to 14 weeks post-inoculation (Figure 5.1a). There was no difference in burrowing activity between wild-type and IL-1RI^{-/-} ME7 mice. Two-way repeated measures ANOVA analysis of ME7 animals only, revealed a main effect of weeks post-inoculation ($F = 26.13$, df 4,136, $p < 0.0001$), no effect of strain but an interaction between these two factors ($F = 2.74$, df 4,136, $p = 0.0311$). Bonferroni post-hoc tests revealed that IL-1RI^{-/-} mice were not significantly different to wild-type mice at any of the time points examined. Two-way repeated measures ANOVA analysis of NBH animals revealed that there were no differences in burrowing behaviour between normal wild-type and IL-1RI^{-/-} mice.

Locomotor activity was assessed longitudinally in the open field arena in NBH- and ME7 prion-inoculated wild type and IL-1RI^{-/-} mice. Previous studies by our laboratory have reported disease-associated hyperactivity in prion animals with onset appearing at approximately 14 to 15 weeks post-inoculation (Cunningham et al., 2005a, Felton et al., 2005). Open field measurements were recorded weekly commencing at week 11 to chart the longitudinal changes and demonstrate the disease-associated hyperactivity.

Distance travelled in the open field was assessed by counting the number of squares crossed in the 3 minute testing period and the percentage change from the baseline measurement (11 weeks post-inoculation) was charted (Figure 5.1b). Two way repeated measures ANOVA analysis of percentage change from baseline distance travelled in ME7 animals revealed a main effect of strain ($F = 65.56$, df 1,344, $p < 0.0001$), a main effect of weeks post-inoculation ($F = 18.68$, df 8,344, $p < 0.0001$) and an interaction between these two factors ($F = 3.71$, df 8,344, $p = 0.0004$). Bonferroni post-hoc tests revealed that IL-1RI^{-/-} ME7 mice were significantly different to wild-type ME7 mice only at 19 weeks post-inoculation ($p < 0.05$). Two-way repeated measures ANOVA analysis of NBH revealed no effect of strain on the open field activity. The absolute distance travelled has also been

shown to highlight the baseline hyperactivity that appears in NBH IL-1RI^{-/-} mice and that is more marked in ME7 animals (Figure 5.1c).

Thus, the absence of the signalling type 1 IL-1 receptor (IL-1RI^{-/-}) in prion-diseased mice has no effect on the onset or magnitude of the disease-associated dysfunction in burrowing behaviour that is apparent in wild-type ME7 animals. Prion-diseased IL-1RI^{-/-} mice display a similar temporal profile of onset of locomotor hyperactivity to wild-type ME7 animals. IL-1RI^{-/-} mice also show a somewhat more hyperactive phenotype in the open field compared to wild-type animals.

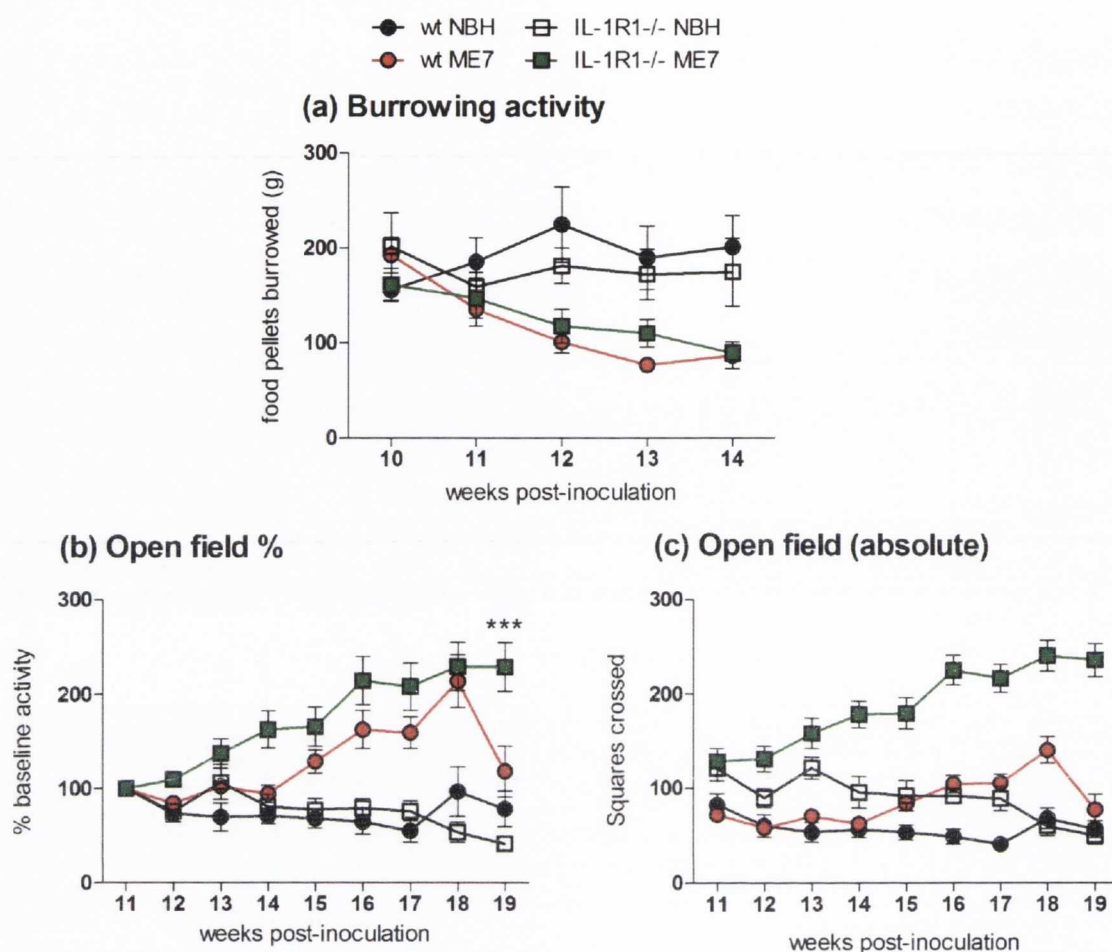


Figure 5.1 *Burrowing in prion-diseased wild type and IL-1R1^{-/-} mice.* (a) NBH and ME7-inoculated wild-type and IL-1R1^{-/-} mice were assessed weekly to measure disease-associated changes in burrowing behaviour. Two-way repeated measures ANOVA revealed no differences in burrowing behaviour between strains, in either NBH or ME7. Data are expressed as mean $\pm$ SEM; n = 10 wt NBH, n = 6 IL-1R1^{-/-} NBH, n = 30 wt ME7, n = 15 for IL-1R1^{-/-}ME7. (b) NBH and ME7-inoculated wild-type and IL-1R1^{-/-} mice were assessed weekly in the open field arena to examine locomotor activity as disease progresses. Percentage change from baseline distance travelled was calculated from the number of squares crossed in the open field over a 3 minute period. Significant differences between ME7 groups by Bonferroni post hoc tests after two-way repeated measures ANOVA, are denoted *** $p < 0.001$. (c) Open field distance presented as absolute values to show baseline hyperactivity. Data are expressed as mean $\pm$ SEM; n = 5 wt NBH, n = 5 IL-1R1^{-/-} NBH, n = 20 wt ME7, n = 25 for IL-1R1^{-/-}ME7.

5.2.2 Longitudinal neurological scores in wild type and IL-1RI^{-/-} ME7 mice

Neurological function was measured longitudinally in NBH- and ME7-inoculated wild type C57 and IL-1RI^{-/-} mice using the horizontal bar and inverted screen tests for motor co-ordination and muscle strength (Kondziella, 1964). Statistical analyses were only performed on the ME7 animals so as to compare the effect of IL-1RI^{-/-} deletion on the progression of prion disease and since there was an a priori prediction that NBH animals in both strains would maintain essentially maximal performance throughout. Prion-diseased animals deficient in IL-1RI showed an approximate one to two-week delay in the onset of neurological impairment (Figure 5.2).

Two-way repeated measures ANOVA analysis of the horizontal bar scores (Figure 5.2a) revealed a main effect of strain ($F = 12.08$, df 1,110, $p = 0.0021$), a main effect of weeks post-inoculation ($F = 39.42$, df 5,110, $p < 0.0001$), and an interaction between these two factors ($F = 5.02$, df 5,110, $p = 0.0004$). Bonferroni post-hoc comparisons revealed that ME7-inoculated IL-1RI^{-/-} mice were significantly different from their wild-type controls at 17, 18 and 19 weeks post-inoculation ($p < 0.05$).

Analysis of the inverted screen scores (Figure 5.2b) by two-way repeated measures ANOVA revealed a main effect of strain ($F = 16.34$, df 1,110, $p = 0.0005$), a main effect of weeks post-inoculation ($F = 52.15$, df 5,110, $p < 0.0001$) and an interaction between these two factors ($F = 4.95$, df 5,110, $p = 0.0004$). Bonferroni post-hoc comparisons revealed that ME7-inoculated IL-1RI^{-/-} mice were significantly different from their wild-type controls at 18, 19 and 20 weeks post-inoculation ($p < 0.05$).

Thus, prion-diseased animals deficient in the signalling IL-1 receptor, type I (IL-1RI^{-/-}) showed an approximate one to two-week delay in onset of neurological dysfunction, as measured by tests for motor co-ordination and muscular strength.

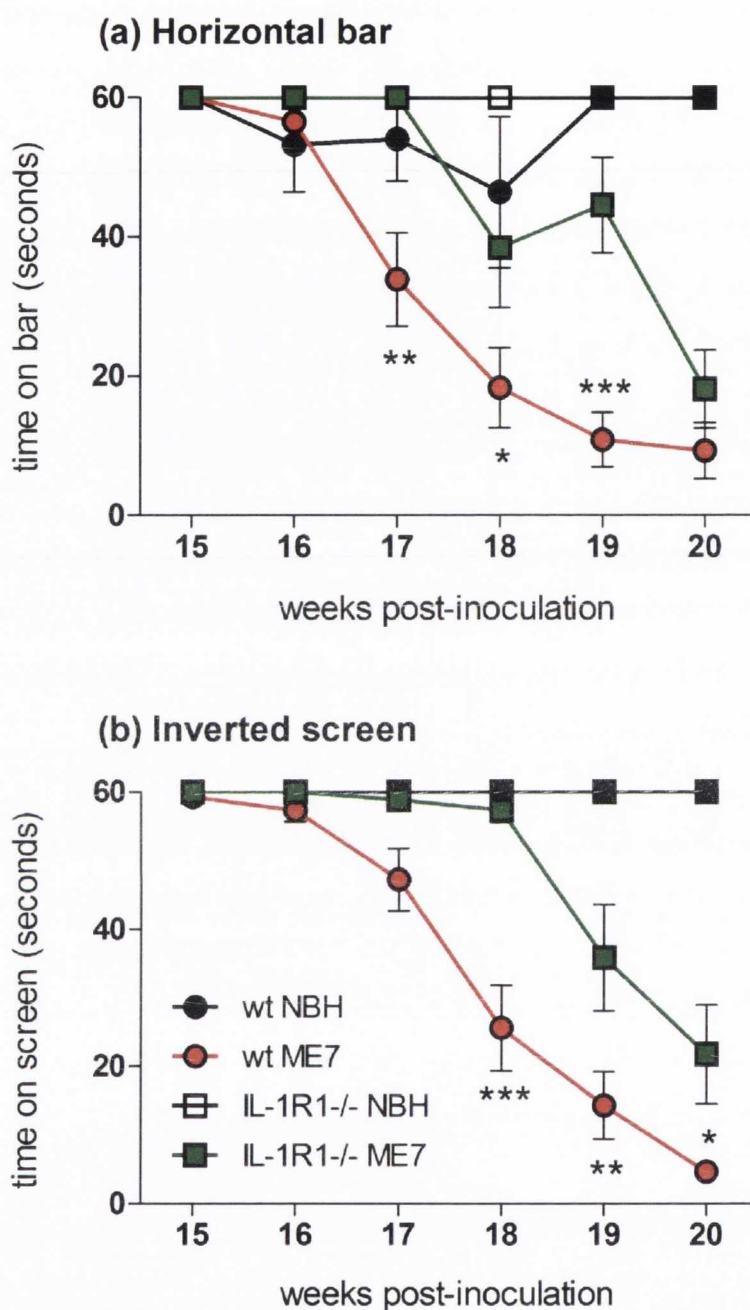
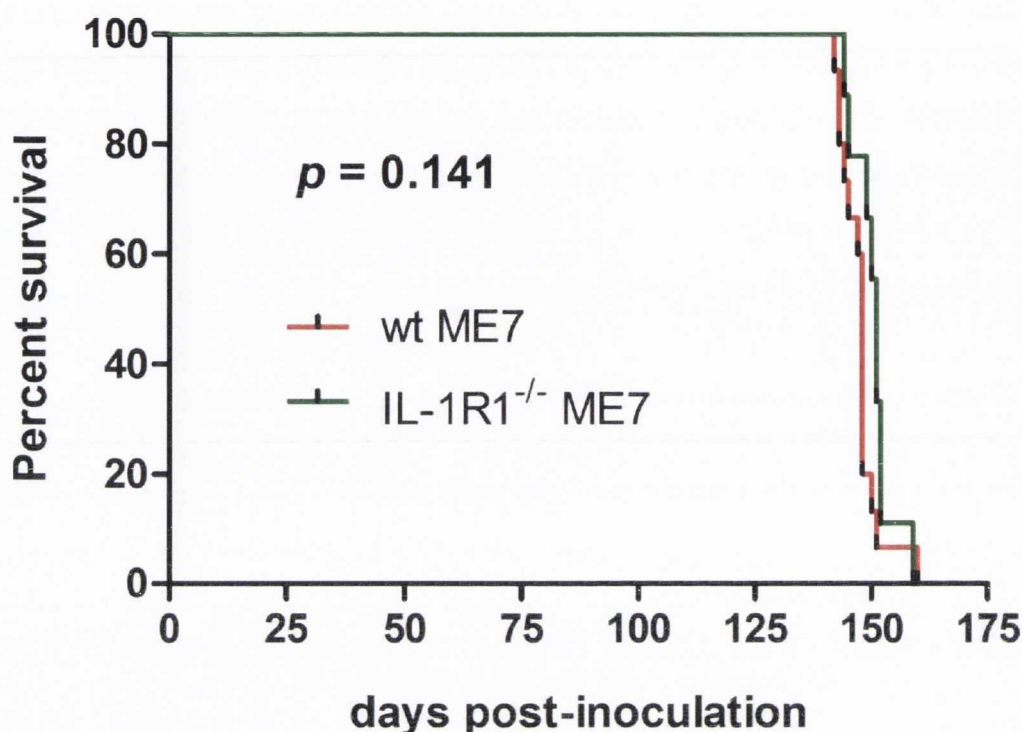


Figure 5.2 *Neurological scores in prion-diseased wild type and IL-1R1^{-/-} mice.* NBH and ME7-inoculated wild-type and IL-1R1^{-/-} mice were assessed weekly on the horizontal bar and inverted screen tests for motor co-ordination and muscle strength. Significant differences between ME7 groups on the horizontal bar (a) and the inverted screen (b) following Bonferroni post hoc tests after two-way repeated measures ANOVA, are denoted by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Data are expressed as mean $\pm$ SEM; $n = 5$ wt NBH, $n = 5$ IL-1R1^{-/-} NBH, $n = 15$ wt ME7, $n = 9$ for IL-1R1^{-/-}ME7.

5.2.3 Survival of prion-diseased IL-1RI^{-/-} mice

The effect of deletion of the signalling IL-1 receptor, type I on the long-term outcome of prion disease was assessed by examining survival of ME7-inoculated IL-1RI^{-/-} mice compared to wild-type prion diseased mice (Figure 5.3). After onset of late-stage clinical signs and before terminal symptoms of disease, animals were euthanized based on a humane end point, defined as the loss of 15% of an animal's peak body weight. Survival was calculated as days post-inoculation and Kaplan-Meier log-rank survival analysis revealed that there was no significant difference in survival times in ME7-inoculated IL-1RI^{-/-} mice when compared to wild-type control. Median survival for IL-1RI^{-/-} mice was 151 days compared to 148 days in wild-types.

Thus, absence of IL-1RI and consequent loss of IL-1 signalling does not impact upon survival of the ME7 strain of murine prion disease, despite delaying some late stage signs.



Strain	Median survival post-inoculation (days)
Wild-type C57BL/6	148
IL-1R1 ^{-/-}	151

Figure 5.3 *Survival of ME7 prion diseased IL-1R1^{-/-} mice.* Survival analysis plot for ME7-inoculated wild-type and IL-1R1^{-/-} mice. Mice were euthanized based on a humane end point defined by 15% decrease from peak body weight. Kaplan-Meier log-rank survival analysis revealed that survival of prion-disease IL-1R1^{-/-} mice is not significantly different compared to wild-type mice. Median survival times are shown in the table. n = 15 for wild-type and n = 9 for IL-1R1^{-/-} mice.

5.2.4 The role of IL-1 β in acute sickness and apoptosis

Following the observation in chapter 3 that systemic poly I:C induced a marked hypothermia and increased apoptotic cell death in the brains of prion-diseased animals with respect to NBH animals, a possible role for IL-1 β in these processes was investigated using mice deficient in the IL-1 receptor, type I (IL-1RI^{-/-}). This is the signalling receptor that is involved in transduction of the IL-1 β -mediated inflammatory response. It was hypothesised that the increased IL-1 β message and protein reported in chapter 4 might contribute to the poly I:C-induced deleterious consequences during neurodegeneration. Given that those behavioural changes that we know to be hippocampal dependent (burrowing and open field) do not show different times of onset in the wild type and IL-1RI^{-/-} mice, the current experiment is not confounded by a different course of disease. However it is difficult to rule out underlying differences in pathology without formal testing.

5.2.4.1 Thermoregulatory changes after poly I:C

To determine if the hypothermic response reported in chapter 4 was dependent on IL-1 β , ME7-inoculated wild-type mice and IL-1RI^{-/-} mice were challenged systemically with poly I:C (12 mg/kg i.p.) and their core body temperature measured by rectal probe at 4, 9 and 13 hours post-challenge (Figure 5.4). Two-way repeated measures ANOVA analysis using strain and time post-challenge as factors (with time as the repeated measure), found that there was a main effect of time post-challenge ($F = 8.88$, $df\ 3,42$, $p = 0.0001$) but not of strain and no interaction between these two factors. Since prior experiments demonstrated peak difference between poly I:C and controls 13 hours post-challenge, a Student's t -test was performed at this time point but was not significant ($t = 1.909$, $df\ 14$, $p = 0.077$).

Although there is an apparent effect of IL-1RI deletion on the poly I:C-induced hypothermic response, this effect is not statistically significant. This could be due to the variability in groups which might be minimized by increasing numbers of animals. Thus the current study indicates that IL-1 β might have a role in the observed hypothermia but further experiments will be needed to fully address this.

5.2.4.2 Apoptosis

TUNEL-positive apoptotic cells were counted in the regions of pathology (hippocampus and thalamus) in 10 μ m sections. In both strains of mouse, ME7 animals showed increased numbers of apoptotic cells compared to NBH, but there was no significant difference in the

number of TUNEL-positive cells in wild-type mice compared to IL-1RI^{-/-} animals (Figure 5.5). In this experiment systemic poly I:C treatment did not induce statistically significantly increased numbers of apoptotic cells in ME7 animals compared to saline-treated ME7 animals, although the poly I:C treated group clearly showed more variability and a trend towards higher scores than the ME7 saline groups, which showed very consistent scores. Bonferroni post-hoc tests after one-way ANOVA analysis ($F = 19.14$, $df = 5,32$, $p < 0.0001$) revealed no significant difference in apoptotic cell numbers in prion-diseased mice of either strain 15 hours following systemic poly I:C challenge ($p > 0.05$).

Since systemic poly I:C treatment in this instance did not appear to induce greater numbers of TUNEL-positive apoptotic cells, it is difficult to infer whether IL-1 β is involved in the increased apoptosis reported in chapter 4. Although there were no significant differences, it does appear that despite the variability, the loss of IL-1 signalling may actually lead to increased apoptosis, suggestive of a possible protective role of IL-1 β .

The variability in apoptotic cell numbers in ME7 animals challenged with poly I:C might be important and reflective of changes in the pro- and anti-inflammatory balance after poly I:C during neurodegeneration. The current findings suggest that the induction of apoptosis during neurodegenerative disease may not simply be due to increased pro-inflammatory signalling and the balance between pro- and anti-inflammatory effects may be important in these cell death mechanisms.

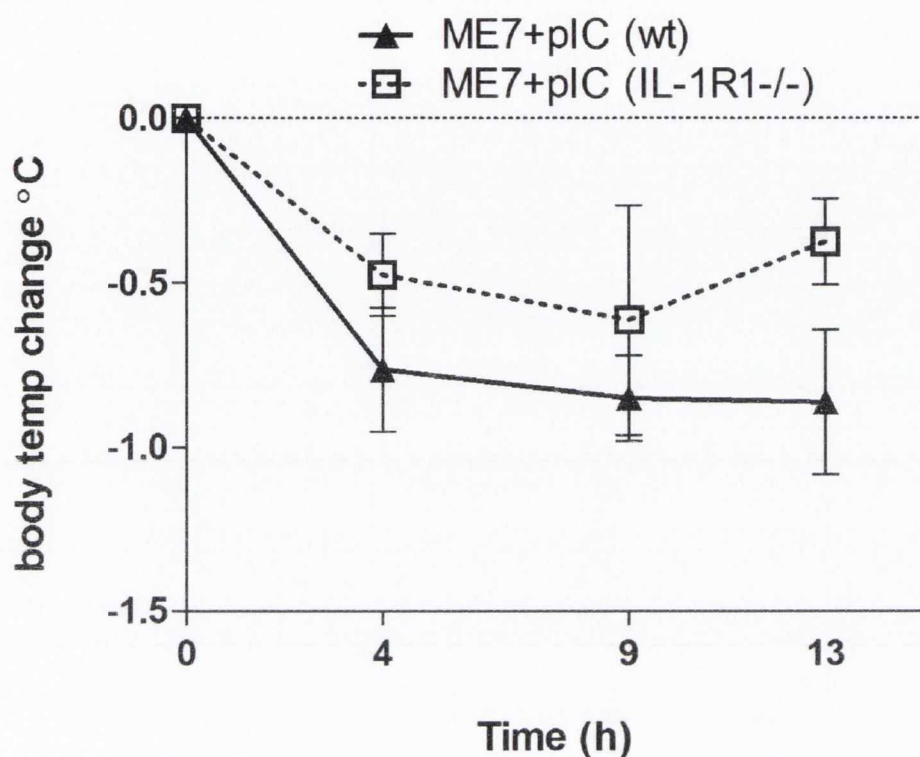


Figure 5.4 Core body temperature response to poly I:C in *IL-1R1*^{-/-} ME7 animals. Wild-type mice (wt) and mice deficient in the IL-1 receptor type 1 (*IL-1R1*^{-/-}) were treated systemically with poly I:C (12 mg/kg) at 18 weeks post-inoculation with ME7. Core body temperature was taken by rectal probe at 0, 4, 9 and 13 h post-poly I:C. Data were analysed by two-way repeated measures ANOVA, main effects are described in the main text. Data are expressed as mean $\pm$ SEM; $n = 8$ for both groups.

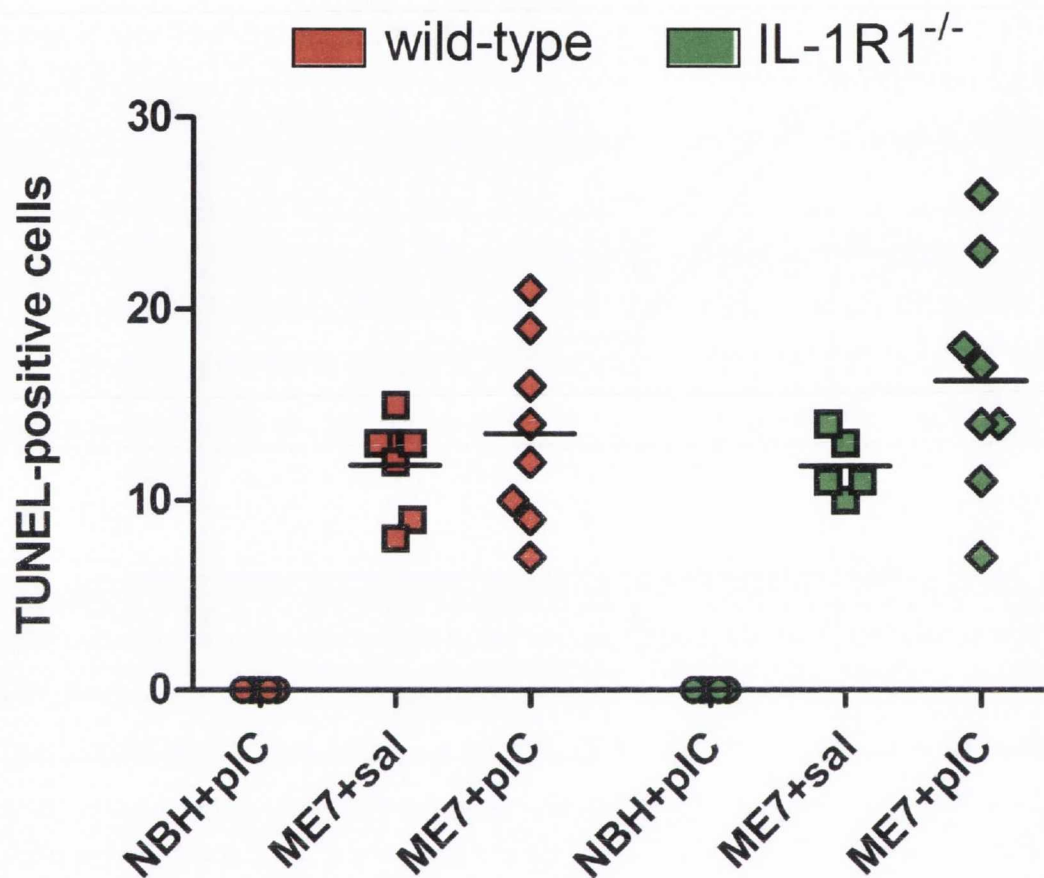


Figure 5.5 Apoptosis in prion diseased wild type and IL-1R1^{-/-} mice after systemic poly I:C. Wild-type mice and mice lacking the IL-1 receptor type 1 (IL-1R1^{-/-}) were treated systemically with poly I:C (12 mg/kg) at 18 weeks post-inoculation with NBH or ME7. TUNEL-positive apoptotic cells were counted in the hippocampus and thalamus of all animals 15 hours following systemic challenge. Data were analysed by one-way ANOVA and are expressed as mean ± SEM. n = 5 for all NBH + poly I:C, n = 7 for wt ME7 + sal, n = 5 for IL-1R1^{-/-} ME7 + sal, n = 8 for all ME7 + poly I:C.

5.2.5 Type I IFNs in ME7 prion disease

Chapter 4 reported that systemic poly I:C challenge accelerated the progression of ME7 prion disease concomitant with amplified type I IFN responses. Thus if exaggerated type I IFN production could be deleterious for the diseased brain it was important to investigate if an increased type I IFN response is present in prion disease per se. Since this work began, type I IFN responses have been reported to be induced in models of sterile neurodegeneration. Two recent such studies showed increased IFN- α/β following transection of entorhinal afferent fibres (Khorrooshi and Owens, 2010) and dorsal root axotomy (Hosmane et al., 2012). Hippocampal expression of IFN- β and several of its dependent genes have therefore been examined by qPCR in ME7 versus NBH animals, 18 weeks post-inoculation.

IFN- β expression was elevated in the hippocampus of ME7 prion-diseased animals compared with NBH animals (Figure 5.6a). Student's *t*-test analysis revealed that hippocampal IFN- β expression was significantly increased in ME7 compared to NBH ($t = 2.773$, $df\ 12$, $p = 0.0169$). Hippocampal expression of the IFNAR2 subunit of the type I IFN receptor was elevated in ME7 animals compared with NBH (Figure 5.6b). Student's *t*-test analysis revealed that hippocampal IFNAR2 expression was significantly increased in ME7 compared to NBH ($t = 4.509$, $df\ 5$, $p = 0.0063$). IRF7 expression was increased in the hippocampus of ME7 animals compared with NBH (Figure 5.6c). Student's *t*-test analysis revealed that hippocampal IRF7 expression was significantly increased in ME7 animals compared to NBH ($t = 5.455$, $df\ 6$, $p = 0.0016$). Hippocampal expression of Mx1 was increased in ME7 animals compared with NBH (Figure 5.6d). Student's *t*-test analysis revealed that hippocampal Mx1 expression was significantly increased in ME7 animals compared to NBH ($t = 6.435$, $df\ 6$, $p = 0.0007$). OAS1a expression was elevated in the hippocampus of ME7 animals compared with NBH (Figure 5.6e). Student's *t*-test analysis revealed that hippocampal OAS1a expression was significantly increased in ME7 animals compared to NBH ($t = 12.46$, $df\ 6$, $p < 0.0001$). PKR transcripts were increased in the hippocampus of ME7 animals compared with NBH (Figure 5.6f). Student's *t*-test analysis revealed that hippocampal PKR expression was significantly increased in ME7 animals compared to NBH ($t = 3.472$, $df\ 6$, $p = 0.0133$).

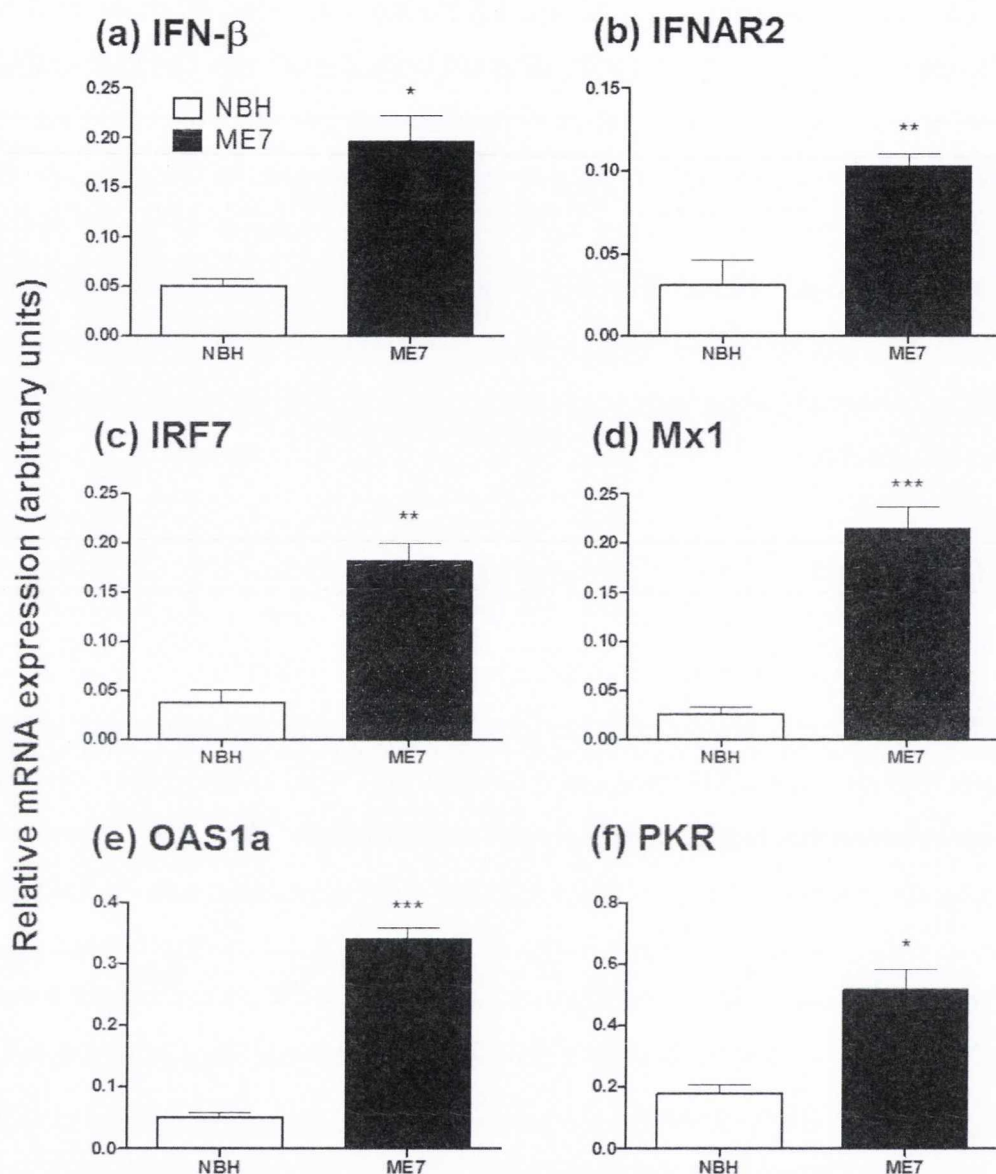


Figure 5.6 Type I IFN response in ME7 prion disease. Hippocampal expression of mRNA for (a) IFN- β , (b) IFNAR2, (c) IRF7, (d) Mx1, (e) OAS1a and (f) PKR in ME7 and NBH animals, 18 weeks post-inoculation. Significant differences by Student's *t*-test are denoted * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Data are expressed as mean $\pm$ SEM; $n = 3$ for NBH, $n = 5$ for ME7

5.2.6 Early behavioural changes in wild type and IFNAR1^{-/-} ME7 mice

Disease-associated changes in burrowing were assessed longitudinally in ME7-inoculated wild-type and IFNAR1^{-/-} mice. Measurements of burrowing were taken weekly for a two hour period from 10 to 14 weeks post-inoculation (Figure 5.7a). Two-way repeated measures ANOVA analysis of the ME7 animals only, revealed a main effect of weeks post-inoculation ($F = 30.20$, $df\ 4,172$, $p < 0.0001$) but no effect of strain ($F = 0.3$, $df\ 1,172$, $p = 0.5882$) and no interaction between these two factors ($F = 1.26$, $df\ 4,172$, $p = 0.2890$). Bonferroni post-hoc tests revealed no differences between wild-type or IFNAR1^{-/-} at any of the time points examined. Similarly, two-way repeated measures ANOVA analysis of NBH animals revealed no differences in burrowing behaviour over time between wild-type and IFNAR1^{-/-} mice.

Locomotor activity in the open field arena was also assessed longitudinally in NBH- and ME7 prion-inoculated wild type and IFNAR1^{-/-} mice. Previous studies by our laboratory have reported disease-associated hyperactivity in prion animals with onset appearing at approximately 14 to 15 weeks post-inoculation (Cunningham et al., 2005a, Felton et al., 2005). Measurements were recorded weekly from week 11 to chart the longitudinal changes and demonstrate the hyperactivity. The 12 week measurement was omitted due to clashes with other behavioural testing.

Distance travelled in the open field was assessed by counting the number of squares crossed in the 3 minute testing period and the percentage change from the baseline measurement (11 weeks post-inoculation) was charted (Figure 5.7b). Two way repeated measures ANOVA analysis of percentage change from baseline distance travelled in ME7 animals revealed a main effect of weeks post-inoculation ($F = 29.13$, $df\ 8,264$, $p < 0.0001$) but no effect of strain ($F = 0.17$, $df\ 1,264$, $p = 0.6837$) and no interaction between these two factors ($F = 0.17$, $df\ 8,264$, $p = 0.9949$). Bonferroni post hoc test revealed that IFNAR1^{-/-} ME7 animals were not significantly different to wild-type ME7 at any time in disease ($p > 0.05$).

Thus, the absence of type I interferon signalling in IFNAR1^{-/-} mice has no effect on the onset or magnitude of the prion disease-associated dysfunction in burrowing behaviour that is apparent in wild-type animals. ME7-inoculated IFNAR1^{-/-} mice also display a similar temporal profile of onset of locomotor hyperactivity from 15 weeks post-inoculation compared to wild-type ME7 animals.

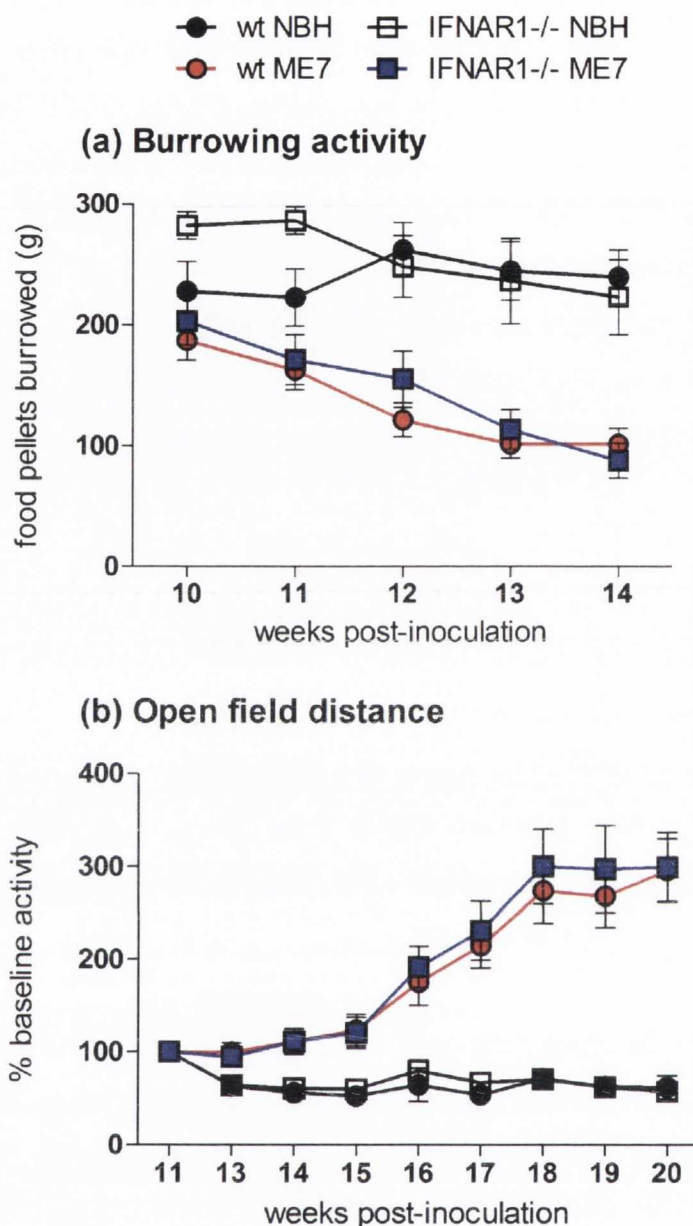


Figure 5.7 Early behavioural changes in prion-diseased wild type and IFNAR1^{-/-} mice. (a) NBH and ME7-inoculated wild-type and IFNAR1^{-/-} mice were assessed weekly to measure disease-associated changes in burrowing activity. Two-way repeated measures ANOVA revealed no differences in burrowing between strains, in either NBH or ME7. Data are expressed as mean $\pm$ SEM; $n = 10$ wt NBH, $n = 6$ IFNAR1^{-/-} NBH, $n = 30$ wt ME7, $n = 15$ for IFNAR1^{-/-}ME7. (b) NBH and ME7-inoculated wild-type and IFNAR1^{-/-} mice were assessed weekly to examine locomotor activity as disease progresses. Percentage change from baseline distance travelled was calculated from the number of squares crossed in the open field over a 3 minute period. Two-way repeated measures ANOVA revealed no difference between strains. Data are expressed as mean $\pm$ SEM; $n = 5$ wt NBH, $n = 6$ IFNAR1^{-/-} NBH, $n = 20$ wt ME7, $n = 15$ for IFNAR1^{-/-}ME7.

5.2.7 Longitudinal neurological scores in wild type and IFNAR1^{-/-} ME7 mice

Neurological function was measured longitudinally in NBH- and ME7-inoculated wild type C57 and IFNAR1^{-/-} mice using the horizontal bar and inverted screen tests for motor co-ordination and muscle strength. Statistical analyses were only performed on the ME7 animals so as to compare the effect of IFNAR1 deletion on the progression of prion disease as there was an *a priori* prediction that NBH animals in both strains of mouse would maintain effectively maximal performance throughout. Prion-diseased animals deficient in IFNAR1 showed an approximate one to two week delay in the onset of neurological impairment (Figure 5.8).

Two-way repeated measures ANOVA analysis of the horizontal bar scores (Figure 5.8a) revealed a main effect of strain ($F = 4.74$, df 1,165, $p = 0.0367$), a main effect of weeks post-inoculation ($F = 64.86$, df 5,165, $p < 0.0001$), and an interaction between these two factors ($F = 2.53$, df 5,165, $p = 0.031$). Bonferroni post-hoc comparisons revealed that ME7-inoculated IFNAR1^{-/-} mice were significantly different from their wild-type controls at both 17 and 18 weeks post-inoculation ($p < 0.05$).

Analysis of the inverted screen (Figure 5.8b) scores by two-way repeated measures ANOVA revealed a main effect of strain ($F = 27.81$, df 1,165, $p < 0.0001$), a main effect of weeks post-inoculation ($F = 70.61$, df 5,165, $p < 0.0001$), and an interaction between these two factors ($F = 9.13$, df 5,165, $p < 0.0001$). Bonferroni post-hoc comparisons revealed that ME7-inoculated IFNAR1^{-/-} mice were significantly different from their wild-type controls at 18, 19 and 20 weeks post-inoculation ($p < 0.01$).

Thus, prion-diseased animals deficient in the IFNAR1 gene showed a one to two week delay in onset of neurological dysfunction, as measured by tests for motor co-ordination and muscular strength.

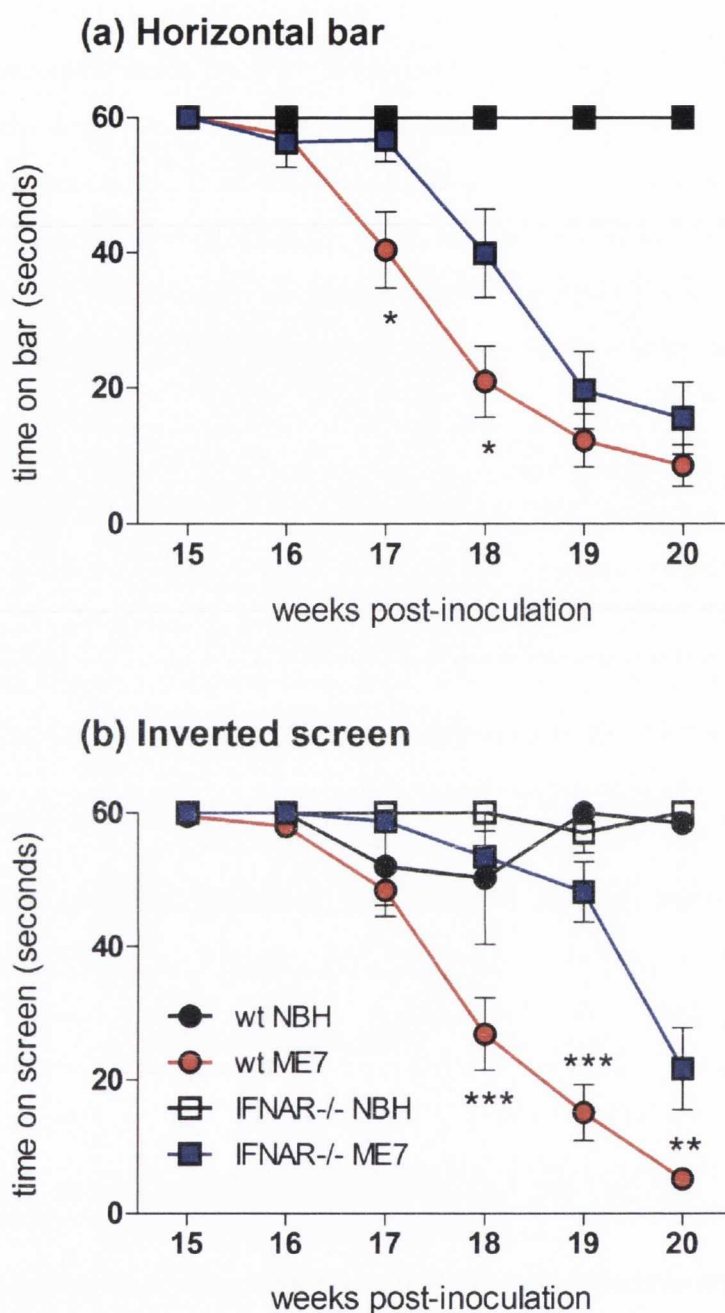
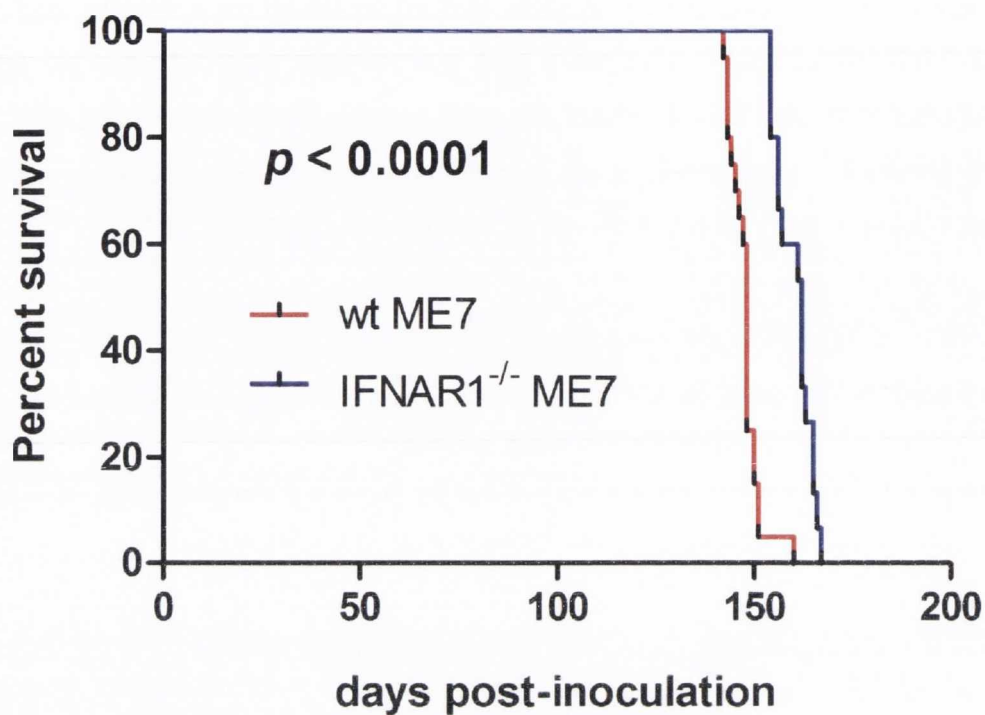


Figure 5.8 *Neurological scores in prion-diseased wild type and IFNAR1^{-/-} mice.* NBH and ME7-inoculated wild-type and IFNAR1^{-/-} mice were assessed weekly on the horizontal bar and inverted screen tests for motor co-ordination and muscle strength. (a) Significant differences between ME7 groups on the horizontal bar following Bonferroni post hoc tests after two-way repeated measures ANOVA, are denoted by * $p < 0.05$. (b) Significant differences between ME7 groups on the inverted screen following Bonferroni post hoc tests after two-way repeated measures ANOVA, are denoted by ** $p < 0.01$ and *** $p < 0.001$. Data are expressed as mean $\pm$ SEM; $n = 5$ wt NBH, $n = 6$ IFNAR1^{-/-} NBH, $n = 20$ wt ME7, $n = 15$ for IFNAR1^{-/-} ME7.

5.2.8 Susceptibility of IFNAR1^{-/-} mice to prion disease – survival analysis

To examine the influence of type I interferons on the long-term outcome of ME7 prion disease, survival of ME7-inoculated IFNAR1^{-/-} mice was analysed and compared to wild-type prion diseased mice (Figure 5.9). After onset of late-stage clinical signs and before terminal symptoms of disease, animals were euthanized based on a humane end point, defined as the loss of 15% of an animal's peak body weight. Survival was calculated as days post-inoculation and Kaplan-Meier log-rank survival analysis revealed that ME7-inoculated IFNAR1^{-/-} mice survived on average 2 weeks longer than their wild-type counterparts (median survival 162 days versus 148 days; $p < 0.0001$).

Thus, absence of IFNAR1 and consequent loss of signalling of the type I interferons confers a slightly longer survival rate (approximately 2 weeks) in prion-diseased animals.



Strain	Median survival post-inoculation (days)
Wild-type C57BL/6	148
IFNAR1 ^{-/-}	162

Figure 5.9 Increased survival of ME7 prion diseased IFNAR1^{-/-} mice. Survival analysis plot for ME7-inoculated wild-type and IFNAR1^{-/-} mice. Mice were euthanized based on a humane end point defined by 15% decrease from peak body weight. Kaplan-Meier log-rank survival analysis demonstrated a significantly increased survival time in IFNAR1^{-/-} mice ($p < 0.0001$). Median survival times as days post-inoculation are shown in the table. $n = 20$ for wild-type and $n = 15$ for IFNAR1^{-/-} mice.

5.2.9 Systemic inflammatory response to poly I:C in prion-diseased wild-type and IFNAR1^{-/-} mice

Chapter 4 reported that the degenerating brain is primed to produce exaggerated IL-1 β and type I IFN responses to systemic poly I:C challenge, despite similar peripheral cytokine induction. This led to the current study which posed the question of whether the observed priming is mediated by type I IFN, since there is evidence that type I IFN can induce priming of peripheral macrophages (Vadiveloo et al., 2000, Thomas et al., 2006). Thus ME7- and NBH-inoculated IFNAR1^{-/-} mice were challenged systemically with poly I:C to investigate the inflammatory profile following acute stimulation of the anti-viral response in the absence of type I IFN signalling.

The systemic inflammatory response was examined in NBH- and ME7-inoculated wild-type and IFNAR1^{-/-} mice following peripheral challenge with poly I:C (12 mg/kg i.p.). Plasma levels of IFN- β , TNF- α , IL-1 β and IL-6 were measured by ELISA 4 hours after poly I:C challenge.

Plasma levels of IFN- β were induced to similar levels by systemic poly I:C in NBH animals of both wild-type and IFNAR1^{-/-} mice. IFN- β was induced to slightly greater levels by poly I:C in wild-type ME7 animals than IFNAR1^{-/-} (Figure 5.10a). Three-way ANOVA analysis revealed there to be a main effect of disease ($F = 7.09$, df 1,31, $p = 0.0122$) and treatment ($F = 237.08$, df 1,31, $p < 0.0001$) but not of strain. The only interactions present were found between disease and treatment ($F = 7.09$, df 1,31, $p = 0.0099$). Bonferroni post-hoc comparisons revealed that wild-type NBH mice challenged with poly I:C did not have significantly different circulating levels of IFN- β when compared with IFNAR1^{-/-} NBH mice similarly challenged ($p > 0.05$). Poly I:C induced IFN- β in both wild-type and IFNAR1^{-/-} ME7 animals but the levels in wild-type mice were significantly higher ($p < 0.05$).

Plasma TNF- α was induced by poly I:C to slightly lower levels in IFNAR1^{-/-} mice compared to wild-type controls (Figure 5.10b). Three-way ANOVA analysis revealed main effects of strain ($F = 6.93$, df 1,31, $p = 0.0131$), disease ($F = 11.18$, df 1,31, $p = 0.0022$) and treatment ($F = 156.24$, df 1,31, $p < 0.0001$) and an interaction between strain and treatment ($F = 6.70$, df 1,31, $p = 0.0145$), and between disease and treatment ($F = 10.85$, df 1,31, $p = 0.0025$). Selected Bonferroni post-hoc comparisons revealed that plasma TNF- α levels were significantly higher in wild-type ME7 animals challenged with poly I:C

compared to IFNAR1^{-/-} ME7 animals similarly challenged. A similar pattern was observed in NBH animals but this difference did not reach significance.

Levels of plasma IL-1 β was induced to similar levels by systemic poly I:C in NBH and ME7 of both wild-type and IFNAR1^{-/-} animals (Figure 5.10c). Three-way ANOVA analysis revealed a main effect of poly I:C ($F = 137.18$, df 1,31, $p < 0.0001$) and no interactions. Bonferroni post-hoc tests found no significant differences across all groups challenged with poly I:C.

IL-6 was markedly induced in the plasma of wild-type NBH and ME7 animals challenged with poly I:C but this induction was almost totally abolished in IFNAR1^{-/-} animals (Figure 5.10d). Three-way ANOVA analysis revealed main effects of strain ($F = 24.34$, df 1,31, $p < 0.0001$) and treatment ($F = 41.92$, df 1,31, $p < 0.0001$), and an interaction between strain and treatment ($F = 24.28$, df 1,31, $p < 0.0001$). Bonferroni post-hoc tests revealed that IL-6 induction in wild-type NBH mice challenged with poly I:C was significantly higher than that in IFNAR1^{-/-} NBH mice challenged with poly I:C ($p < 0.05$). Similarly, induction of IL-6 following systemic poly I:C challenge was significantly higher in wild-type ME7 animals compared to IFNAR1^{-/-} ME7 animals ($p < 0.01$).

Thus, the systemic inflammatory response in normal wild-type and IFNAR1^{-/-} mice is similar with respect to induction of IFN- β , TNF- α and IL-1 β . Induction of IL-6, however, is almost completely abolished, which suggests that IL-6 production after immune stimulation with poly I:C is dependent upon type I interferon signalling and IRF3 activation. The response in ME7 prion-diseased animals is broadly similar although the induction of IFN- β and TNF- α is not as robust in prion animals.

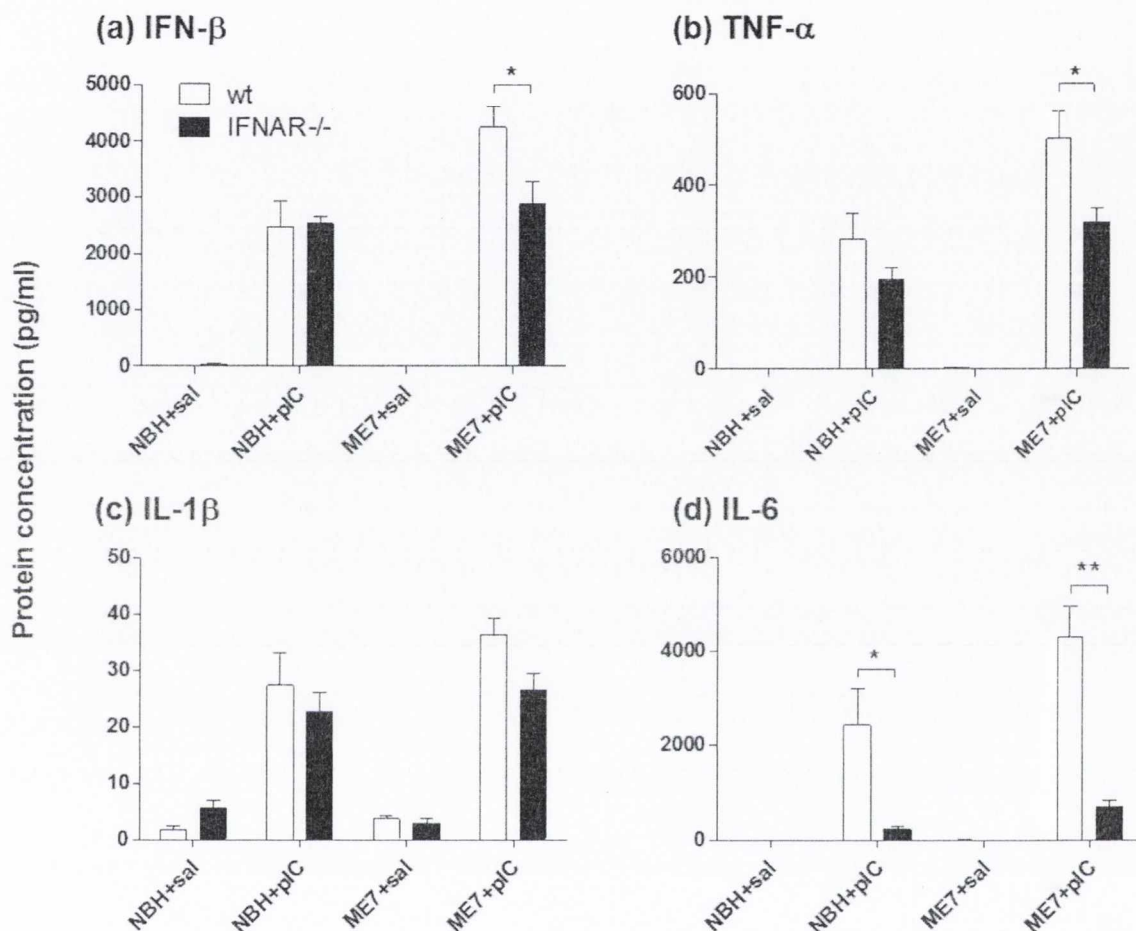


Figure 5.10 *Peripheral inflammatory response to systemic poly I:C in wild type and IFNAR^{-/-} mice.* Plasma levels of (a) IFN-β, (b) TNF-α, (c) IL-1β and (d) IL-6 in NBH- and ME7-inoculated wild-type and IFNAR^{-/-} mice 4 hours following systemic challenge with poly I:C (12 mg/kg i.p.). Significant differences by Bonferroni post-hoc tests after three-way ANOVA analysis are denoted by * $p < 0.05$ and ** $p < 0.01$. Data are expressed as mean $\pm$ SEM; $n = 4$ wt NBH + sal; $n = 5$ wt NBH + pIC; $n = 5$ wt ME7 + sal; $n = 6$ wt ME7 + pIC; $n = 4$ IFNAR NBH + sal; $n = 4$ IFNAR NBH + pIC; $n = 6$ IFNAR ME7 + sal; $n = 5$ IFNAR ME7 + pIC.

5.2.10 Hippocampal type I interferon response to systemic poly I:C in prion diseased wild-type and IFNAR1^{-/-} mice

The type I interferon response was examined in the CNS of NBH- and ME7-inoculated wild-type and IFNAR1^{-/-} mice after systemic poly I:C challenge (12 mg/kg i.p.). Hippocampal mRNA transcription of type I interferon-dependent genes was measured by qPCR 4 hours following peripheral challenge.

Hippocampal mRNA expression of IRF7 was markedly induced in wild-type NBH and ME7 animals in response to systemic poly I:C but was not induced in IFNAR1^{-/-} mice (Figure 5.11a). Three-way ANOVA analysis revealed main effects of strain ($F = 81.41$, df 1,31, $p < 0.0001$), disease ($F = 6.28$, df 1,31, $p = 0.0177$) and treatment ($F = 42.42$, df 1,31, $p < 0.0001$), with interactions between strain and disease ($F = 6.09$, df 1,31, $p = 0.0192$), and between strain and poly I:C ($F = 37.39$, df 1,31, $p < 0.0001$). Selected Bonferroni post-hoc comparisons revealed significant differences between wild-type and IFNAR1^{-/-} NBH mice challenged with poly I:C ($p < 0.01$), and also between wild-type and IFNAR1^{-/-} ME7 mice challenged with systemic poly I:C ($p < 0.01$).

Hippocampal induction of Mx1 was increased by systemic poly I:C challenge in both wild-type NBH and ME7 animals but was not induced at all in IFNAR1^{-/-} mice (Figure 5.11b). Three-way ANOVA analysis revealed main effects of strain ($F = 58.54$, df 1,31, $p < 0.0001$), disease ($F = 4.92$, df 1,31, $p = 0.034$) and treatment ($F = 43.43$, df 1,31, $p < 0.0001$), with interactions between strain and disease ($F = 4.99$, df 1,31, $p = 0.0327$), and between strain and poly I:C ($F = 42.63$, df 1,31, $p < 0.0001$). Selected Bonferroni post-hoc comparisons revealed significant differences between wild-type and IFNAR1^{-/-} NBH mice challenged with poly I:C ($p < 0.01$), and also between wild-type and IFNAR1^{-/-} ME7 mice challenged with systemic poly I:C ($p < 0.01$).

Expression of PKR mRNA in the hippocampus was induced by systemic poly I:C challenge in both wild-type NBH and ME7 animals but was not induced in IFNAR1^{-/-} mice (Figure 5.11c). PKR expression also appeared to be induced by ME7 *per se* in wild-type animals although this did not reach significance. Three-way ANOVA analysis revealed main effects of strain ($F = 57.11$, df 1,31, $p < 0.0001$), disease ($F = 13.76$, df 1,31, $p = 0.0008$) and treatment ($F = 19.85$, df 1,31, $p = 0.0001$), with interactions between strain and disease ($F = 8.91$, df 1,31, $p = 0.0055$), and between strain and poly I:C ($F = 18.96$, df 1,31, $p = 0.0001$). Selected Bonferroni post-hoc comparisons revealed significant

differences between wild-type and IFNAR1^{-/-} NBH mice challenged with poly I:C ($p < 0.01$), and also between wild-type and IFNAR1^{-/-} ME7 mice challenged systemically with poly I:C ($p < 0.01$).

These results confirm that acute *in vivo* induction of IRF7, Mx1 and PKR in the brain following systemic poly I:C challenge cannot occur in the absence of type I IFN signalling in IFNAR1^{-/-} mice.

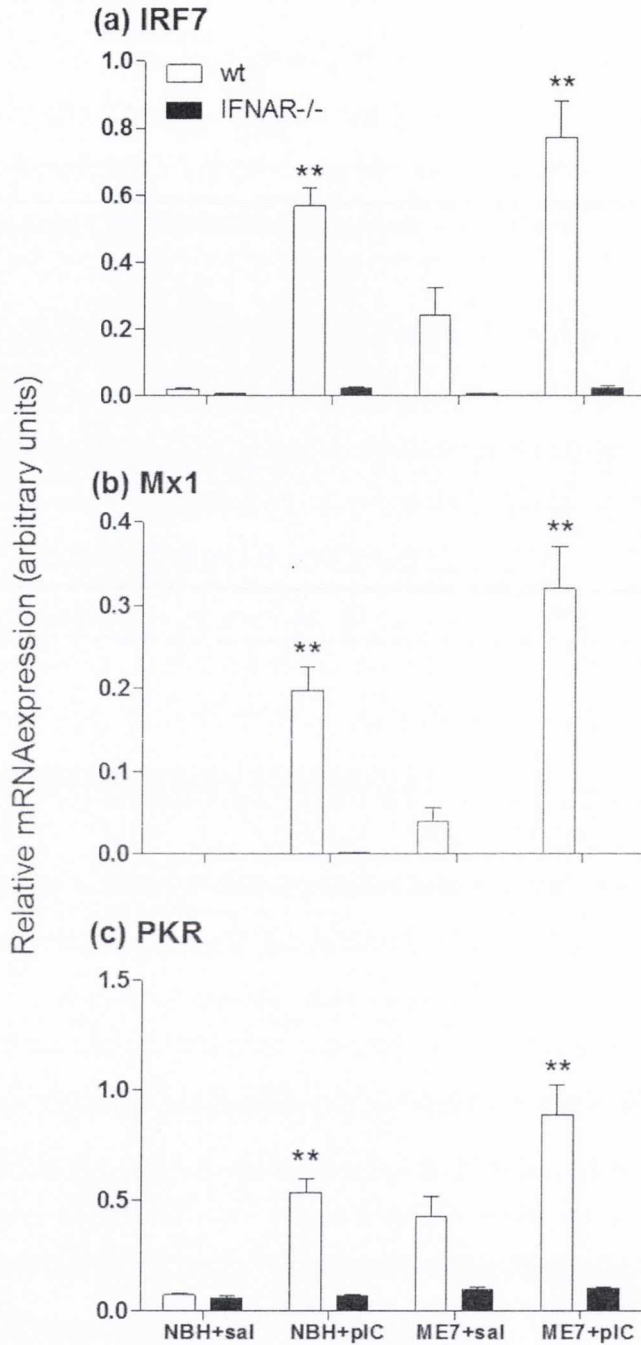


Figure 5.11 Hippocampal type I IFN response after systemic poly I:C in wild type and *IFNAR1*^{-/-} mice. Hippocampal mRNA expression of (a) IRF7, (b) Mx1 and (c) PKR in NBH- and ME7-inoculated wild-type and *IFNAR1*^{-/-} mice 4 hours following systemic challenge with poly I:C (12 mg/kg i.p.). Significant differences to corresponding *IFNAR1*^{-/-} group by Bonferroni post-hoc tests after three-way ANOVA analysis are denoted ** $p < 0.01$. Data are expressed as mean $\pm$ SEM; $n = 4$ wt NBH + sal; $n = 5$ wt NBH + pIC; $n = 5$ wt ME7 + sal; $n = 6$ wt ME7 + pIC; $n = 4$ *IFNAR* NBH + sal; $n = 4$ *IFNAR* NBH + pIC; $n = 6$ *IFNAR* ME7 + sal; $n = 5$ *IFNAR* ME7 + pIC.

5.2.11 Hippocampal inflammatory profile following systemic poly I:C in prion diseased wild-type and IFNAR1^{-/-} mice

Chapter 4 reported that the degenerating brain and its microglia are primed to produce exaggerated IL-1 β to systemic poly I:C challenge. Given the increased type I IFN response in prion disease *per se*, the exaggerated response to systemic poly I:C and the previously reported ability of these molecules to induce priming in peripheral macrophages, the current study aimed to address whether the priming observed in chapter 4 is mediated by type I IFN or whether type I IFN would instead act in an anti-inflammatory capacity. Thus, the inflammatory cytokine response to systemic poly I:C (12 mg/kg, i.p.) was examined by qPCR in the hippocampus of NBH- and ME7-inoculated IFNAR1^{-/-} mice, 4 hours following peripheral challenge.

IL-1 β mRNA expression in the hippocampus was clearly induced 4 hours following systemic poly I:C in wild-type ME7 animals but was induced to greater levels in the absence of type I IFN signalling in IFNAR1^{-/-} mice (Figure 5.12a). Three-way ANOVA analysis revealed a main effect of strain ($F = 5.74$, df 1,31, $p = 0.0228$), disease ($F = 28.67$, df 1,31, $p < 0.0001$) and of poly I:C ($F = 27.83$, df 1,31, $p < 0.0001$). There were significant interactions between strain and poly I:C ($F = 5.59$, df 1,31, $p = 0.0245$), and also between disease and poly I:C ($F = 13.03$, df 1,31, $p = 0.0011$). Bonferroni post-hoc tests confirmed that poly I:C significantly elevated IL-1 β expression in IFNAR1^{-/-} ME7 animals compared to wild-type ME7 animals ($p < 0.05$).

Hippocampal mRNA expression of the anti-inflammatory cytokine IL-10 was slightly induced by poly I:C in wild-type NBH animals and was markedly increased in wild-type ME7 animals. This IL-10 expression was clearly reduced in IFNAR1^{-/-} mice, whether NBH or ME7 (Figure 5.12b). Three-way ANOVA analysis revealed a main effect of strain ($F = 12.56$, df 1,31, $p = 0.0014$), disease ($F = 10.59$, df 1,31, $p = 0.0029$) and of poly I:C ($F = 9.89$, df 1,31, $p = 0.0038$). There were significant interactions between strain and disease ($F = 6.29$, df 1,31, $p = 0.018$), and also between strain and poly I:C ($F = 5.46$, df 1,31, $p = 0.0265$). Bonferroni post-hoc tests confirmed that IL-10 induction was significantly attenuated following systemic poly I:C in IFNAR1^{-/-} ME7 animals compared to wild-type ME7 animals ($p < 0.01$).

Poly I:C induced marked increases in IL-6 in wild-type NBH and ME7 animals which were profoundly attenuated in both NBH and ME7 IFNAR1^{-/-} animals (Figure 5.13a).

Three-way ANOVA analysis revealed a main effect of strain ($F = 26.58$, $df\ 1,31$, $p < 0.0001$) and of poly I:C treatment ($F = 49.15$, $df\ 1,31$, $p < 0.0001$), along with a significant interaction between strain and poly I:C ($F = 25.75$, $df\ 1,31$, $p < 0.0001$). Bonferroni post-hoc tests confirmed that the poly I:C-induced increase in IL-6 mRNA expression in wild-type NBH and ME7 was significantly attenuated in both NBH and ME7 IFNAR1^{-/-} animals ($p < 0.01$).

IL-12 is typically produced by macrophages to stimulate T_H1 cell proliferation. Transcription of the p40 subunit of IL-12 was induced by prion disease *per se* in wild-type but considerably lower in IFNAR1^{-/-} animals. Poly I:C had no effect on IL-12 expression in IFNAR1^{-/-} ME7 mice but appeared to decrease mRNA levels in wild-type ME7 animals (Figure 5.13b). Three-way ANOVA analysis revealed a main effect of strain ($F = 9.49$, $df\ 1,31$, $p = 0.0043$) and of disease ($F = 23.33$, $df\ 1,31$, $p < 0.0001$), along with a significant interaction between strain and disease ($F = 6.62$, $df\ 1,31$, $p = 0.0151$). Bonferroni post-hoc tests revealed that the ME7-induced increase in IL-12 in wild-type animals was abolished in IFNAR1^{-/-} mice ($p < 0.01$).

Hippocampal mRNA expression of TNF- α was induced by prion disease *per se* in wild-type and IFNAR1^{-/-} animals but was induced to a lesser extent in the knockout mice, suggesting that IFN-I somehow supports TNF- α expression in disease. As previously found in chapter 4, in wild-type animals, poly I:C induced TNF- α expression in NBH but decreased its expression in ME7 animals. Conversely, in IFNAR1^{-/-} animals, TNF- α expression was increased in ME7 following systemic poly I:C (Figure 5.14a). Three-way ANOVA analysis revealed a main effect of disease ($F = 53.74$, $df\ 1,31$, $p < 0.0001$) and of poly I:C treatment ($F = 15.86$, $df\ 1,31$, $p = 0.0004$). There was also a three way interaction between strain, disease and poly I:C ($F = 6.39$, $df\ 1,31$, $p = 0.0168$). Bonferroni post-hoc tests revealed that the ME7-induced increase in TNF- α mRNA expression in wild-type animals was significantly reduced in both IFNAR1^{-/-} animals ($p < 0.01$). Systemic poly I:C also significantly increased TNF- α in IFNAR1^{-/-} ME7 mice relative to saline-treated controls ($p < 0.05$). These data suggest that type I IFN, when induced by poly I:C suppresses CNS TNF- α .

Hippocampal expression of iNOS was also induced to greater levels in IFNAR1^{-/-} ME7 animals compared to wild-type ME7 animals. In fact, like TNF- α , transcription of iNOS mRNA appeared to be decreased by poly I:C in wild-type ME7 mice (Figure 5.14b).

Three-way ANOVA analysis revealed a main effect of strain ($F = 5.71$, $df\ 1,31$, $p = 0.0231$) and of disease ($F = 32.72$, $df\ 1,31$, $p < 0.0001$). There was also a significant interaction between strain and poly I:C ($F = 13.77$, $df\ 1,31$, $p = 0.0008$). Bonferroni post-hoc tests revealed that iNOS expression in IFNAR1^{-/-} ME7 animals challenged with poly I:C was not different to saline controls but was significantly elevated when compared to wild-type ME7 animals ($p < 0.05$).

Thus, the inflammatory profile in ME7-inoculated IFNAR1^{-/-} mice following systemic poly I:C is distinct from that in wild-type mice. Most notable, is the increased expression of TNF- α and IL-1 β which suggests that the increased type I IFN response in wild-type ME7 animals restrains the production of these cytokines following poly I:C. Concurrent with this, there is decreased IL-6 and IL-10 expression suggesting that their induction is dependent on type I IFN and indeed the latter may be the key factor in suppression of IL-1 β and TNF- α in wild type ME7 animals challenged with poly I:C. That is to say, even though IL-1 β is induced in ME7 + poly I:C animals, to a higher level than in NBH + poly I:C animals, in wild type animals the concurrent expression of IFN- β appears to restrain this response. When this inhibition is lifted, in IFNAR1^{-/-} animals IL-1 β reaches rather high levels in a CNS context. Overall, the data are suggestive of a less anti-inflammatory CNS milieu in IFNAR1^{-/-}, which lifts the inhibition on some pro-inflammatory processes.

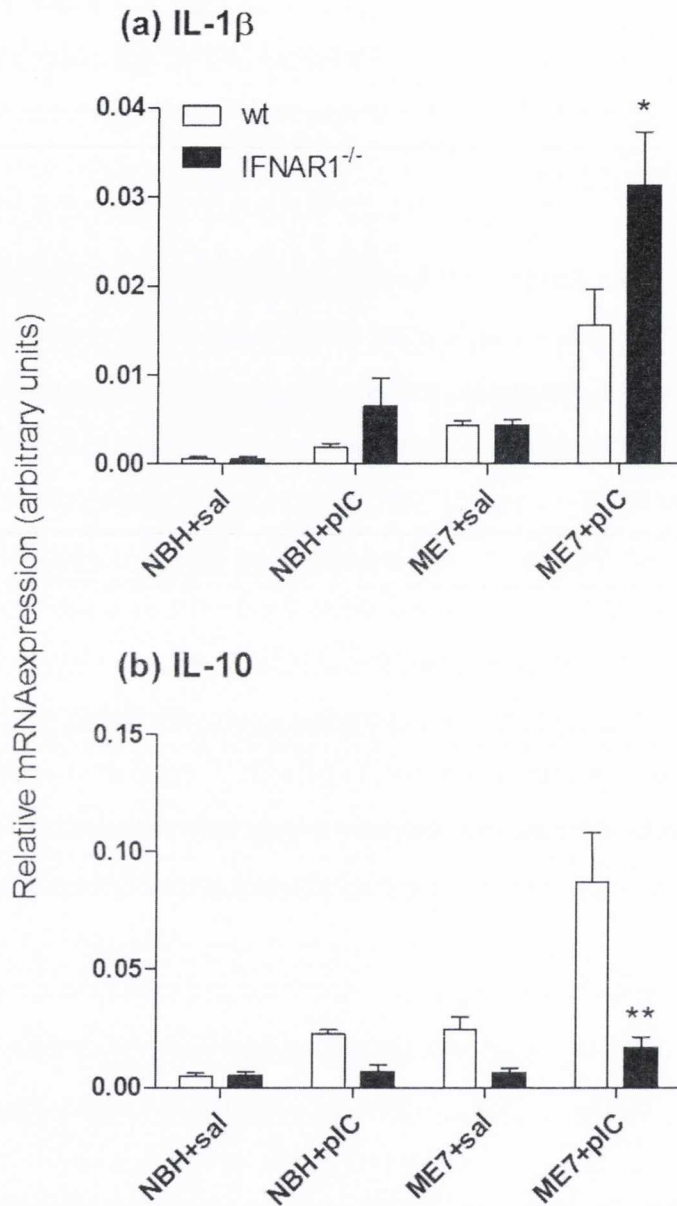


Figure 5.12 Hippocampal expression of IL-1 β and IL-10 after systemic poly I:C in wild type and IFNAR1^{-/-} mice. Hippocampal mRNA expression of (a) IL-1 β and (b) IL-10 in NBH- and ME7-inoculated wild-type and IFNAR^{-/-} mice 4 hours following systemic challenge with poly I:C (12 mg/kg i.p.). Significant differences by Bonferroni post-hoc tests after three-way ANOVA analysis are denoted * $p < 0.05$ and ** $p < 0.01$. Data are expressed as mean $\pm$ SEM; $n = 4$ wt NBH + sal; $n = 5$ wt NBH + pIC; $n = 5$ wt ME7 + sal; $n = 6$ wt ME7 + pIC; $n = 4$ IFNAR NBH + sal; $n = 4$ IFNAR NBH + pIC; $n = 6$ IFNAR ME7 + sal; $n = 5$ IFNAR ME7 + pIC.

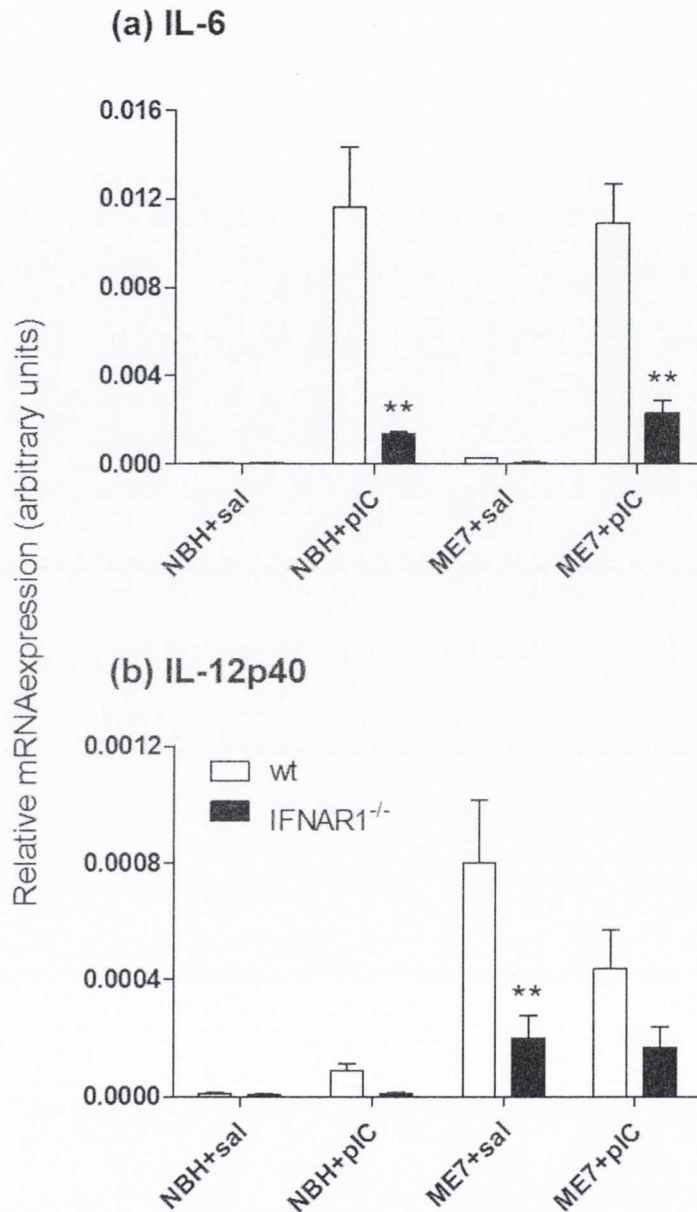


Figure 5.13 Hippocampal *Il-6* and *Il-12* mRNA expression after poly systemic I:C in wild type and *IFNAR1*^{-/-} mice. Hippocampal mRNA expression of (a) IL-6 and (b) IL-12p40 in NBH₊ and ME7-inoculated wild-type and *IFNAR1*^{-/-} mice 4 hours following systemic challenge with poly I:C (12 mg/kg i.p.). Significant differences by Bonferroni post-hoc tests after three-way ANOVA analysis are denoted ** $p < 0.01$. Data are expressed as mean $\pm$ SEM; $n = 4$ wt NBH + sal; $n = 5$ wt NBH + pIC; $n = 5$ wt ME7 + sal; $n = 6$ wt ME7 + pIC; $n = 4$ IFNAR NBH + sal; $n = 4$ IFNAR NBH + pIC; $n = 6$ IFNAR ME7 + sal; $n = 5$ IFNAR ME7 + pIC.

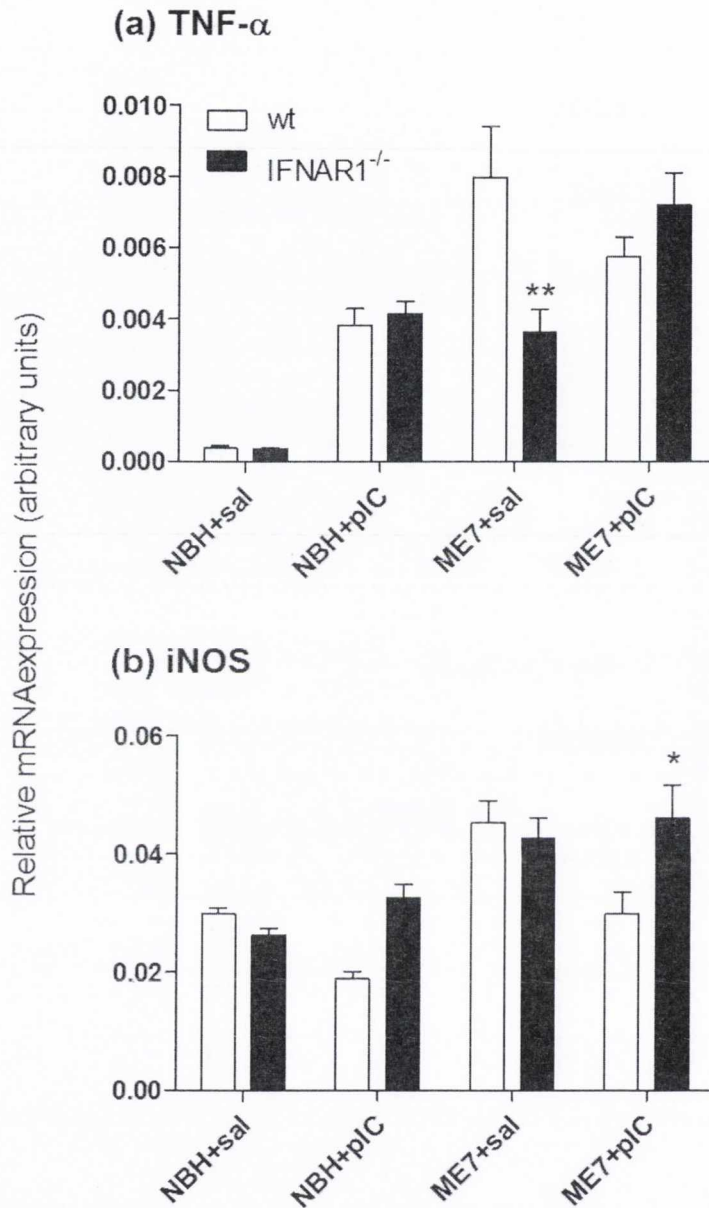


Figure 5.14 Hippocampal $\text{TNF-}\alpha$ and iNOS mRNA expression after systemic poly I:C in wild type and $\text{IFNAR1}^{-/-}$ mice. Hippocampal mRNA expression of (a) $\text{TNF-}\alpha$ and (b) iNOS in NBH- and ME7-inoculated wild-type and $\text{IFNAR1}^{-/-}$ mice 4 hours following systemic challenge with poly I:C (12 mg/kg i.p.). Significant differences by Bonferroni post-hoc tests after three-way ANOVA analysis are denoted $*p < 0.05$ and $**p < 0.01$. Data are expressed as mean $\pm$ SEM; $n = 4$ wt NBH + sal; $n = 5$ wt NBH + pIC; $n = 5$ wt ME7 + sal; $n = 6$ wt ME7 + pIC; $n = 4$ IFNAR NBH + sal; $n = 4$ IFNAR NBH + pIC; $n = 6$ IFNAR ME7 + sal; $n = 5$ IFNAR ME7 + pIC.

5.2.12 Hippocampal expression of pro-apoptotic factors following systemic poly I:C in prion diseased wild-type and IFNAR1^{-/-} mice

The expression of mRNA transcripts for pro-apoptotic proteins was examined by qPCR in the hippocampus of NBH- and ME7-inoculated wild-type and IFNAR1^{-/-} mice, 4 hours following peripheral challenge with poly I:C (12 mg/kg, i.p.).

The expression of the death receptor, Fas, was induced by systemic poly I:C in the hippocampus of wild-type and IFNAR1^{-/-} NBH and ME7 animals, although induction in ME7 animals appeared slightly lower in IFNAR1^{-/-} mice (Figure 5.15a). Three-way ANOVA analysis revealed a main effect of strain ($F = 5.07$, df 1,31, $p = 0.0316$), disease ($F = 24.21$, df 1,31, $p < 0.0001$) and of poly I:C treatment ($F = 119.69$, df 1,31, $p < 0.0001$). There were no significant interactions between factors. Bonferroni post-hoc tests revealed that poly I:C significantly induced Fas expression in both wild-type and IFNAR1^{-/-} ME7 animals ($p < 0.01$) but that the induction was not different between strains ($p > 0.05$).

Hippocampal mRNA expression of Fas ligand (FasL) induced by disease but not by systemic poly I:C challenge in wild-type animals. This disease-associated induction of FasL was not present in IFNAR1^{-/-} animals (Figure 5.15b). Three-way ANOVA analysis revealed a main effect of strain ($F = 10.27$, df 1,31, $p = 0.031$) and disease ($F = 18.65$, df 1,31, $p = 0.0001$), and an interaction between these two factors ($F = 11.29$, df 1,31, $p = 0.0021$). Bonferroni post-hoc tests revealed that ME7 prion disease significantly induced FasL expression in wild-type ($p < 0.05$) but not in IFNAR1^{-/-} animals ($p > 0.05$), and that poly I:C had no effect on FasL mRNA transcription in either strain.

Thus the current data demonstrate that there are decreases in some pro-apoptotic transcripts during ME7 prion disease in IFNAR1^{-/-} animals.

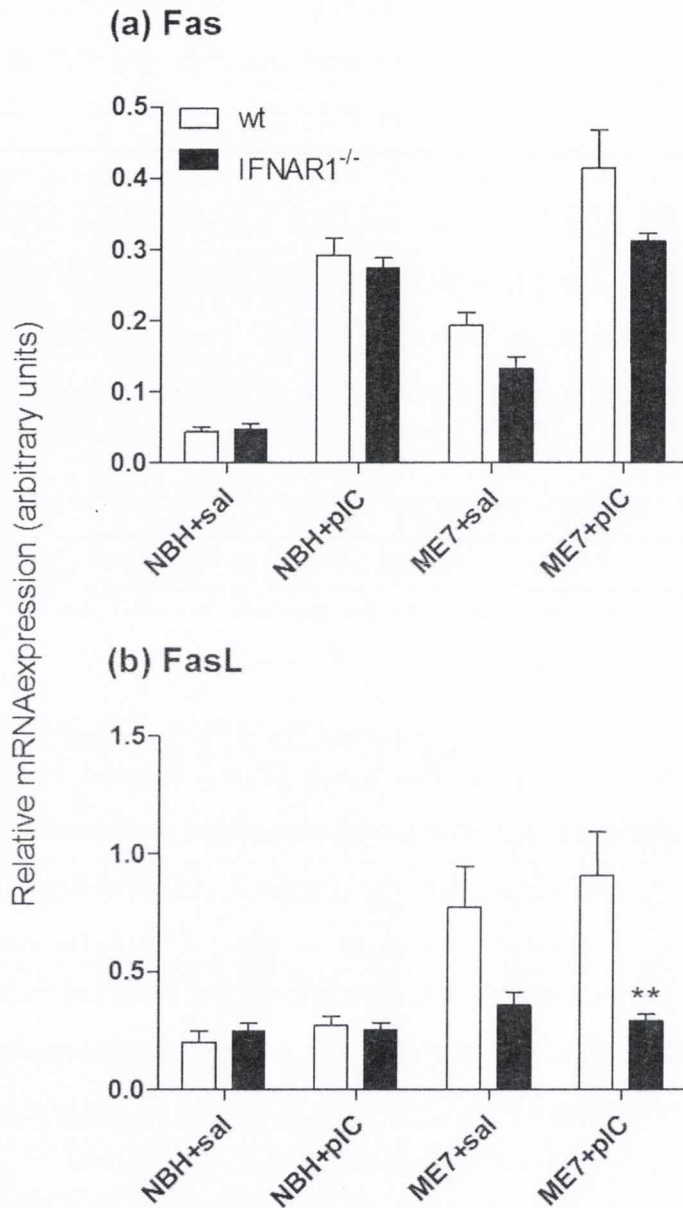


Figure 5.15 Hippocampal mRNA expression of pro-apoptotic factors after systemic poly I:C in wild type and *IFNAR1*^{-/-} mice. Hippocampal mRNA expression of (a) Fas and (b) Fas ligand in NBH- and ME7-inoculated wild-type and *IFNAR*^{-/-} mice 4 hours following systemic challenge with poly I:C (12 mg/kg i.p.). Significant differences by Bonferroni post-hoc tests after three-way ANOVA analysis are denoted ** $p < 0.01$. Data are expressed as mean $\pm$ SEM; $n = 4$ wt NBH + sal; $n = 5$ wt NBH + pIC; $n = 5$ wt ME7 + sal; $n = 6$ wt ME7 + pIC; $n = 4$ IFNAR NBH + sal; $n = 4$ IFNAR NBH + pIC; $n = 6$ IFNAR ME7 + sal; $n = 5$ IFNAR ME7 + pIC.

5.3 Discussion

The current chapter aimed to investigate the role of IL-1 β and type I IFN signalling in the pathogenesis of ME7 prion disease progression per se and also their roles in the CNS consequences of systemic inflammation following poly I:C stimulation. Prion-diseased IL-1RI^{-/-} mice showed no difference in onset of early affective and behavioural signs. Absence of IL-1 signalling resulted in a one to two week delay in onset of late-stage neurological impairments but there was no difference in overall survival. ME7 prion-diseased IFNAR1^{-/-} mice showed no difference in early symptoms but displayed a one to two week delay in onset of motor symptoms and these mice survived about 2 weeks longer. Thus removal of type I IFN signalling appeared to slightly slow disease progression. The systemic inflammatory response to poly I:C in prion-diseased IFNAR1^{-/-} mice was largely unaffected although the IL-6 response was abolished. The type I IFN response in the CNS during prion disease seems to restrain the poly I:C-induced IL-1 β and TNF- α responses since the induction of these cytokines is markedly increased in IFNAR1^{-/-} mice. Conversely, type I IFN seem to be essential for both IL-6 and IL-10 induction in the brain following systemic poly I:C. There was also lesser induction the pro-apoptotic factor FasL in the brains of ME7 IFNAR1^{-/-} mice, suggesting a possible protection against the deleterious consequences of an amplified type I IFN during prion disease. These represent completely novel data on the role of type I IFN responses during neurodegenerative disease.

5.3.1 Progression of ME7 prion disease

There has been much research that has investigated the pathogenesis of prion disease and the possible biological mechanisms that might underpin these progressive neurodegenerative conditions. This group of diseases known as TSEs, although relatively uncommon in humans, share many key aspects of the more common age-related disease such as AD and PD, including extracellular amyloidosis and microglial activation (Miyazono et al., 1991, Prusiner, 1998). Our laboratory has utilised the ME7 strain of murine prion disease to study the host inflammatory response during neurodegenerative disease along with how systemic inflammatory events may impact upon progressive neurodegenerative disease. The current study investigated the role of inflammatory mediators, IL-1 and type I IFNs, and their removal on the progression of ME7 prion disease.

5.3.1.1 IL-1RI^{-/-} and prion progression

Transduction of the IL-1-mediated inflammatory response occurs through the IL-1 receptor, type I (IL-1RI) via a MyD88-dependent mechanism, and many of the detrimental effects elicited by IL-1 are thought to occur through this receptor (Dinarello, 1996). The current chapter used mice deficient in IL-1RI (IL-1RI^{-/-}) to examine the progression of ME7 prion disease in the absence of IL-1 signalling (summarised in Table 5.1). In the CNS, basal expression of IL-1 β is low but appears to be physiologically active and many studies have highlighted its influence on neuro-immune interactions (Gosselin and Rivest, 2007). Despite an absence of detectable IL-1 β protein in the brains of ME7 mice (Walsh et al., 2001), this inflammatory cytokine might still contribute to the progression of disease since low levels which are often undetectable, have been shown to be involved in the pathogenesis of myriad clinical diseases (Dinarello, 1996). Moreover, expression of IL-1RI is upregulated in the brains of prion disease mice (Schultz et al., 2004, Murray et al., 2012) and as such, an increase in the functional receptor for IL-1 β should allow for a greater transduction of the IL-1-mediated inflammatory signal, even with low levels of this cytokine. Thus, this could be sufficient to contribute to neuropathological processes.

ME7-inoculated IL-1RI^{-/-} mice displayed a similar profile of early behavioural changes to that of wild-type prion animals, as measured by burrowing activity and locomotor hyperactivity in the open field arena. Previous studies have shown that the tendency of ME7 prion-diseased mice to burrow food pellets decreases by week 12 post-inoculation (Deacon et al., 2001) and this is repeated here, with IL-1RI^{-/-} mice showing no difference to wild-types. Normal healthy IL-1RI^{-/-} mice display no overt phenotypic abnormalities but have been reported to show decreased anxiety-like behaviours, spending more time in the open arms of an elevated plus maze and increased latency to enter the dark portion of a light-dark box paradigm (Koo and Duman, 2009). Ongoing studies within our own laboratory have also demonstrated these decreased anxiety-like behaviours. These studies have also revealed evidence of locomotor hyperactivity in the open field in IL-1RI^{-/-} mice which is in contrast to reports elsewhere (Koo and Duman, 2009). The current study also reported a general hyperactivity in IL-1RI^{-/-} mice with ME7 even more active than wild type controls. The open field data in the current study have been presented as percentage change from baseline in order to eliminate any differences in activity that might merely be due to strain hyperactivity rather than disease-associated changes. Thus the ME7-induced locomotor changes can be directly compared between wild-type and IL-1RI^{-/-} mice. Consistent with prior reports, the onset of prion-induced hyperactivity appeared at around

14 weeks post-inoculation in wild-type ME7 mice (Cunningham et al., 2003, Felton et al., 2005) and although IL-1RI^{-/-} mice appeared more generally hyperactive, the time of onset of ME7-induced changes was not altered by IL-1RI deletion.

Neurological impairments in ME7 animals typically begin to manifest from about 17 weeks post-inoculation onwards, with animals showing deficits on the horizontal bar and inverted screen tasks of motor co-ordination and muscle strength. Wild-type ME7 animals in the current study follow the same timeline of changes that have been previously reported (Cunningham et al., 2009) although IL-1RI^{-/-} ME7 mice appear to show an approximate one week delay on onset of these motor impairments. In addition, the median survival time of ME7-inoculated IL-1RI^{-/-} mice was not different to wild-type animals (151 vs 148 days) so despite a slight divergence in progression of neurological impairments, overall survival is unaltered. This is in contrast to prior reports showing increased survival times in prion-diseased animals lacking the IL-1RI (Schultz et al., 2004). This study used the 139A strain of prion disease and demonstrated that disruption of IL-1 signalling conferred an approximate 3 week increase in survival along with a slower rate of accumulation of the aberrant prion protein PrP^{Sc}. A prior study by the group of Adriano Aguzzi showed that the kinetics of prion disease progression was not different in MyD88^{-/-} mice compared to wild-type controls (Prinz et al., 2003), and given that TLR and IL-1 signalling is dependent on MyD88 activation, this study is consistent with the current work which suggests that IL-1β is not a key driver of prion disease pathogenesis. However, it is important to note that most studies of prion disease simply assess disease progression by the time to very late stage signs and death whereas the current studies actually chart the longitudinal progression of disease via a number of measures. Thus the current studies offer a more comprehensive analysis of the kinetics of prion disease progression than other groups have provided with similar knockout models.

Despite the low levels of IL-1β in ME7 animals and the similar disease progression profile in IL-1RI^{-/-} ME7 mice, it remains possible that this cytokine could still play a role in exacerbation of progression upon systemic inflammatory insult. It is known from other studies in our laboratory that systemic inflammation-induced increases in CNS IL-1β production are associated with exacerbation of the central inflammatory response in prion disease and induction of hippocampal-dependent working memory impairment (Skelly et al, in preparation). Furthermore ME7-inoculated IL-1RI^{-/-} animals challenged systemically with LPS exhibit impairments in the T-maze working memory paradigm and show similar

deficits to wild-type mice (Skelly, in preparation). These findings suggest that while IL-1 β is sufficient, it is not essential for the production of inflammation-induced cognitive deficits.

This argues that other cytokines, such as TNF- α may compensate when IL-1 signalling is deficient during development (Bluthe et al., 2000). Thus, although the current data show that IL-1 signalling is not vital for prion disease progression, this pro-inflammatory mediator might still contribute to the exacerbation of symptoms associated with systemic immune stimulation by poly I:C.

Table 5.1 Summary of prion disease progression in IL-1RI^{-/-}

Behavioural dysfunction/pathology	Week of onset	Effect of IL-1RI deletion
Impaired food burrowing	12/13	No change in onset or magnitude of disease-associated burrowing impairment
Locomotor hyperactivity	16/17	No change in onset; hyperactivity exaggerated compared to wild-type
Motor deficits	17/18	Delayed onset by ~2 weeks
Clinical symptoms/survival	20/21	No change in onset and survival
Overt clinical signs/terminal symptoms – kyphosis, incontinence, hunched posture, ruffled fur, $\geq$ 15% decrease from peak body weight		

5.3.1.2 Role of IL-1 β in acute sickness and apoptosis during prion disease

Chapter 4 reported marked hypothermia following systemic poly I:C challenge in ME7 animals which was not apparent in NBH mice similarly challenged. Thus, the current study employed IL-1RI^{-/-} mice to address whether the thermoregulatory alterations reported in chapter 4 could be attributed to increased production and action of IL-1 β , either in the periphery or in the brain. Using ME7-inoculated IL-1RI^{-/-} mice, the current data suggest that IL-1 β might be responsible for the hypothermic response observed after systemic poly I:C challenge (12 mg/kg) in wild-type ME7 animals. The hypothermia in IL-1RI^{-/-} ME7 animals appears less profound than that in wild-type animals but the small numbers and variability across time-points result in changes that are not statistically significant. Further studies will be needed to definitively address whether IL-1 β is the key driver of the hypothermic response in ME7 animals following systemic poly I:C.

The use of knockout animals brings some limitations as there may be some compensatory mechanisms employed by a host organism that inherently lacks the ability to respond to IL-1 β . Indeed other groups have reported that, in response to LPS treatment TNF- α can assume the role of IL-1 β in its absence (Bluthe et al., 2000, Rijneveld et al., 2001). Our laboratory has recently demonstrated that this is also true for the cognitive effects of IL-1 β with a series of experiments which have shown that IL-1 β was sufficient but not essential to induce working memory deficits in mice with underlying neurodegeneration (Skelly et al., in preparation). The febrile (i.e. hyperthermic) response in rodents has been reported elsewhere to be influenced by IL-1 β (Watkins et al., 1995, Chai et al., 1996). There is however, some evidence of IL-1 β -induced hypothermia in mice (Wang et al., 1997) and indeed recent data from our laboratory has also demonstrated this hypothermic response (Skelly et al., unpublished observations). Although IL-1 β is generally viewed as the classical endogenous pyrogen in the healthy individual (see Dinarello, 1999 for review), it is plausible that this pro-inflammatory molecule might have a different effect if there is dysregulation of thermoregulatory systems in the diseased brain. If IL-1 is not responsible for the hypothermic response in poly I:C-challenged ME7 animals, TNF- α would become the obvious candidate given its reported cryogenic properties. However, as was reported in chapter 4, there is no detectable TNF- α protein in the prion brain following systemic poly I:C, and mRNA expression is actually suppressed. Thus the molecular mechanisms that underpin the exaggerated hypothermia remain unclear.

IL-1 β has been reported to be involved in the induction of apoptosis in numerous experimental conditions *in vivo* and *in vitro*, including experimental acute myocardial infarction (Abbate et al., 2008) and neuronal cell culture studies (Fankhauser et al., 2000). This pro-inflammatory cytokine has also been implicated in the apoptotic cell death observed in acute CNS injury (Nesic et al., 2001, Wang et al., 2005) and in the normal ageing brain (Martin et al., 2002). There is also evidence of neuronal apoptosis mediated by IL-1 β in viral encephalitis (Takikita et al., 2001). IL-1-mediated neuronal apoptosis seems to occur via caspase-3 dependent mechanisms (Nesic et al., 2001). Given that chapter 4 reported that systemic poly I:C challenge in ME7 animals was associated with an amplified IL-1 β response and presence of activated caspase-3-positive cells, one might predict that IL-1 is responsible for the apoptosis that contributes to the poly I:C-induced exacerbation of neurodegenerative disease. Thus, the influence of IL-1 β on apoptosis was examined in ME7-inoculated IL-1RI^{-/-} mice challenged systemically with poly I:C.

Chapter 3 demonstrated an increase in TUNEL-positive apoptotic cell numbers in ME7 animals challenged with poly I:C compared to saline. However these findings were not replicated in ME7-inoculated wild-type animals that were used as controls for the current IL-1RI^{-/-} study. In fact, the number of TUNEL-positive cells in ME7 *per se* without further inflammatory challenge were greater in the current study compared to those in chapter 4 (11 versus 6 positively labelled cells). Numbers of TUNEL-positive cells were equivalent in both wild-type and IL-1RI^{-/-} ME7 animals challenged with saline, with very little variability within these groups. Systemic poly I:C did not induce significantly increased apoptotic cell death in ME7 animals of either strain of mouse, making it difficult to infer what effect IL-1 β might play in the ME7-associated apoptosis. It is important to note, however, that although there were no significant changes in mean numbers of apoptotic cells, systemic poly I:C challenges induced greater variability in the apoptotic response in both wild-type and IL-1RI^{-/-} ME7 animals and if anything, there was a trend towards increased apoptosis in the knockout mice.

As reported in chapter 4, poly I:C induces both pro- and anti-inflammatory responses in an individual and the neuropathological response to poly I:C may depend on the balance between these two responses in a particular individual. In this context, and given the possible differential inter-individual responses to poly I:C, this could account for the variability in apoptotic responses. Despite the variability in apoptotic cell numbers following poly I:C, it does appear that the loss of IL-1 signalling may lead to increased apoptosis since all but two of the IL-1RI^{-/-} ME7 mice challenged with poly I:C have apoptotic cell numbers higher than the average for wild-type mice. This would be suggestive of a protective role of IL-1 β , and might indicate that type I interferon-dependent mechanisms have some role in apoptotic cell death. The current findings, along with the reported contribution of type I IFN to apoptosis (Balachandran et al., 2000), suggests that the induction of apoptosis during chronic neurodegenerative disease may not simply be due to increased pro-inflammatory signalling and that the relationship between poly I:C, IL-1 β and apoptosis is not straightforward. The inter-relationship between IL-1 β and type I IFN will be further discussed below.

It should also be noted that the exacerbation of disease reported in chapter 4 may not necessarily be due to increased apoptotic cell death. A recent study using the triple transgenic AD mouse model (3xTg-AD; encoding APP_{Swe}, TauP and PS1) reported exacerbated neuropathology in these mice following systemic poly I:C challenge (Krstic et

al., 2012). These mice demonstrated a strikingly similar pathology to that observed post-mortem in human patients with AD. Related to the current study this poly I:C treatment in 3xTg-AD mice induced synaptic dysfunction and axonal damage that contributed to exacerbated neuropathology that may not depend on apoptosis. Thus it could be possible that synaptic dysfunction and axonal damage in the current study could underpin the exacerbation of function observed in chapter 4 and that apoptosis is not required

5.3.1.3 Type IFNs in prion disease and *IFNARI*^{-/-} prion progression

Chapter 4 of this thesis demonstrated that that peripheral stimulation of the acute anti-viral response with poly I:C induced amplified IL-1 β and type I IFN responses in ME7 prion-diseased animals. This was associated with exacerbation of neurological impairment and acceleration of disease. A major aim of the current work was to investigate if this primed response was mediated by type I IFNs, since these molecules have been shown to induce priming in peripheral macrophages. Furthermore, if an exaggerated type I IFN response is associated with deleterious consequences, it was important to address whether a type I IFN response is a feature of prion disease *per se* and what is the effect on disease progression in the absence of such a response.

Here it was demonstrated that the ME7 strain of murine prion disease, independent of systemic challenges, is associated with upregulation of IFN- β and several type I IFN-dependent genes that are typically induced during the acute phase of viral infections. The presence of type I IFN-dependent genes IRF7, Mx1, OAS1a and PKR is indicative of increased type I IFN secretion and action in the prion-diseased brain, independent of any exogenous inflammatory stimulus. This is consistent with prior reports that demonstrate induction of some type I IFN-responsive genes in other mouse models of prion disease (Baker et al., 2004, Riemer et al., 2004, Stobart et al., 2007). Type I IFN responses have also recently been associated with non-infectious, sterile neurodegenerative insults *in vivo*. One such study reported upregulation of type I IFN-responsive genes in the hippocampus following transection of entorhinal afferents and consequent axonal degeneration (Khorrooshi and Owens, 2010). This study also demonstrated that the microglia were the cells responsible for the injury-induced type I IFN response. A further study which performed dorsal root axotomy in mice, also reported that the resulting axonal degeneration induces microglial type I IFN responses in a TRIF-dependent manner and that this is essential for clearance of neuronal debris (Hosmane et al., 2012). These data

suggest a novel role for type I IFN signalling during neurodegeneration, independent of viral infection. In this context, it is significant that we also observed a robust type I IFN response in the mu-p75-sap model of cholinergic neurodegeneration (Chapter 3). Thus the type I IFN response is induced by both infectious (ME7) and sterile (saporin) degenerative insults.

Perhaps this type I IFN induction is caused by PRR stimulation by some facet of acute/chronic neurodegenerative insults. For example if the neuronal degeneration is causing deleterious processes such as necrosis or DNA damage, it is likely that some of these patterns (DAMPs?) would be recognised by PRRs that might initiate the type I IFN response. A recent study has shown that TLR3 can act as an endogenous sensor of necrosis independent of viral infection (Cavassani et al., 2008). Given that there is evidence for necrotic cell death in both prion disease (Hur et al., 2002) and saporin-induced neurodegeneration (Seeger et al., 1997), and that TLR3 can act as an endogenous sensor of necrosis (Cavassani et al., 2008), type I IFN induction could be the result of this type of TLR3 activation. The Hosmane study reported that type I IFN induction following dorsal root axotomy was dependent on TRIF, which acts as an adaptor for TLR3 and TLR4. Thus, it seems likely that one of these PRRs is activated by some moiety produced during neurodegenerative insult. A further study reported that mRNA can act as an endogenous ligand for TLR3, such that RNA escaping from damaged tissue or contained within endocytosed cells (phagocytic microglia) could stimulate a type I IFN response via TLR3 activation.

In light of this induction of a type I IFN response in ME7 prion disease, it thus became important to investigate the role of such a response in the progression of prion disease and what the consequences are for disease when this system is removed. As such, the progression of ME7 prion disease was examined in mice lacking the functional type I IFN receptor, subunit 1 (IFNAR1^{-/-}) and is summarised in Table 5.2.

The profile of early affective behavioural changes in ME7 prion-diseased animals was assessed in IFNAR1^{-/-} mice and was found to be similar to that observed in wild-type controls. The deficits in burrowing activity in both wild-type and IFNAR1^{-/-} mice were as previously reported (Deacon et al., 2001), with no difference in onset between strains. Consistent with prior reports, prion disease-induced open-field hyperactivity were first manifest at about 15 weeks post-inoculation in wild-type ME7 animals (Cunningham et al.,

2005a, Felton et al., 2005), and IFNAR1^{-/-} mice were no different. Similar to IL-1RI^{-/-} mice, normal IFNAR1^{-/-} animals displayed a mildly hyperactive phenotype so data was presented as percentage change from baseline in order to eliminate any differences that might arise from this rather than disease-associated changes. Thus, although there is a slight difference in locomotor activity between wild-type and IFNAR1^{-/-} animals, the magnitude of changes in ME7 mice was comparable in both strains. There are no reported phenotypic or developmental abnormalities in IFNAR1^{-/-} mice (Muller et al., 1994). Since studies investigating the behavioural characteristics of this strain have not, up until now, been conducted, the current data provides novel information on the general behavioural characteristics of normal IFNAR1^{-/-} mice along with analysis during neurodegeneration.

Prion disease-induced neurological impairments of muscle strength and motor function were manifest from about 17 weeks in wild-type ME7 animals and the timeline of these impairments was as previously reported. The onset of neurological impairment in IFNAR1^{-/-} ME7 animals, however, was delayed by approximately one to two weeks, with these mice still exhibiting significantly better performance on the inverted screen at 20 weeks post-inoculation. Survival analysis of prion-disease IFNAR1^{-/-} mice revealed that these mice survive on average two weeks longer than wild-type controls (median survival 162 vs 148 days). Such analysis of prion disease in the absence of a functional type I IFN system has not previously been conducted. A humane end-point for terminally diseased animals was set such that they were euthanized upon reaching 15% loss of their peak body weight.

Of course, one key question to address is whether the neuropathology of these IFNAR1^{-/-} ME7 animals differs from wild-type ME7 animals. This question has not been addressed here due to time constraints. It is likely that in the animals that were euthanized at terminal stages, one might reasonably expect the neuropathology to be the same in both wild-type and IFNAR1^{-/-} since all are at terminal stages. However, one might predict that these strains would show different pathology at the time when they showed divergence on non-invasive markers of disease progression (18 weeks post-inoculation of neurological testing of motor function). Indeed such studies were initiated in the course of this thesis but had not been completed at the time of writing. These experiments should allow for the delineation of some of the processes that confer the increased survival when type I IFN signalling is absent.

Table 5.2 Summary of prion disease progression in IFNAR1^{-/-}

Behavioural dysfunction/pathology	Week of onset	Effect of IFNAR1 deletion
Impaired food burrowing	12/13	No change in onset or magnitude of disease-associated burrowing impairment
Locomotor hyperactivity	16/17	No change in onset; hyperactivity exaggerated compared to wild-type
Motor deficits	17/18	Delayed onset by ~2 weeks
Clinical symptoms/survival	20/21	Delayed onset by ~ 2 weeks
Neuropathology	?	<i>Predict no difference in end stage pathology but perhaps differences earlier in disease</i>

Overt clinical signs/terminal symptoms – kyphosis, incontinence, hunched posture, ruffled fur, ≥ 15% decrease from peak body weight

The current data demonstrate that the type I IFN response is upregulated in ME7 prion disease and that these inflammatory mediators have a detrimental effect during the later stages of disease. This symptomatic stage of disease progresses somewhat more slowly in IFNAR1^{-/-} mice and they survive for longer. One might predict that removal of type I IFN signalling through deletion of the IFNAR1 subunit of the cognate receptor might accelerate disease progression since type I IFN has widely reported beneficial anti-inflammatory effects in viral infections (Isaacs and Lindenmann, 1957, Pestka et al., 1987). Prior reports have shown that IFNAR1^{-/-} mice are highly susceptible to the lethal effects of viral infections (Hwang et al., 1995, Muller et al., 1994). Indeed, IFN- α is commonly used for treatment of hepatitis C (Liang et al., 2000, Myers et al., 2002) and for various types of cancer (Ferrantini et al., 2007), while IFN- β remains the most widely recommended therapeutic option for treatment of relapsing-remitting MS (Goodin et al., 2002). In this context, the fact that deletion of IFNAR1 and consequent loss of type I IFN signalling in prion-diseased mice confers increased survival upon diseased animals might be considered a surprising finding. One might predict that upregulation of IFN-dependent pathways in prion disease could be the brain's attempt to control the possible deleterious pro-inflammatory processes mediated by microglia and astrocytes that are triggered by progressive neuronal death. However, as has been shown here, removal of the type I IFN signal has proved beneficial.

It remains possible that an increased type I IFN response in disease could itself, be associated with some of the pathophysiology of the disease. It is known that type I IFNs can induce apoptosis through upregulation of PKR-mediated cascades (Balachandran et al., 1998, Balachandran et al., 2000) and clinical studies have reported neuropsychiatric complications, including depression and delirium, in patients receiving either IFN- α for cancer (Capuron et al., 2002) and IFN- β for MS (Goeb et al., 2003). Further animal studies have shown that transgenic overexpression of IFN- α in the brains of mice, although protective against neurotropic viruses, is associated with increased inflammation and neurodegeneration (Akwa et al., 1998, Campbell et al., 1999). Although more typically associated with IFN- γ , there are also reports of type I IFN induction of reactive oxygen species (ROS) via activation of NADPH oxidase during the respiratory burst response (Ellermann-Eriksen et al., 1989, Little et al., 1994). Production of ROS can cause damage to cell by oxidative stress and thus might provide a way in which type I IFN activation is deleterious in the brain. It appears that while type I IFNs clearly are necessary and important in fighting viral infection, and can be anti-inflammatory, their regulation must be tightly controlled. Thus, the current study has shown that removal of the type I IFN response in murine prion disease is protective and confers increased survival upon diseased animals. Therefore, while these mediators may suppress other pro-inflammatory processes, their increased expression in prion disease seems to be detrimental to the degenerating brain. Type I IFN-dependent mechanisms in the inflammatory response in prion disease will be discussed below.

5.3.2 Systemic poly I:C and central inflammatory changes in prion-diseased IFNAR1^{-/-} mice – does priming still occur?

Chapter 4 of this thesis demonstrated that peripheral stimulation of the type I IFN anti-viral response using poly I:C in animals with existing neurodegeneration results in acute exacerbation and long-term acceleration of neurodegenerative disease. This was accompanied by further activation of primed microglia, resulting in amplified IL-1 β and type I IFN responses in the brains of prion diseased mice. Thus, the effect of systemic poly I:C on the CNS inflammatory profile in ME7 prion disease was examined in IFNAR1^{-/-} mice. Removal of the type I IFN response via genetic knockout of IFNAR1 was predicted to elicit a dysregulation of many cellular processes that are dependent on IFN- α/β and to alter the CNS inflammatory response to systemic poly I:C.

5.3.2.1 Peripheral inflammation after poly I:C

Previous studies using IFNAR1 or IFNAR2 knockout mice have mainly served to explore the role of type I IFN in viral infection (Muller et al., 1994, Hwang et al., 1995, Meissner et al., 2005). The effects of the type I IFN response on brain function and inflammation during neurodegeneration have not been widely investigated and have mostly been limited to studies of EAE. In this context type I IFNs have been reported to be beneficial since mice devoid of IFNAR1 developed exacerbated EAE and increased neuroinflammation (Prinz et al., 2008). The previous chapter demonstrated that the IFN- α/β inducer, poly I:C, has deleterious consequences for neurodegenerative disease. The current chapter has shown that the systemic inflammatory response to poly I:C IFNAR1^{-/-} animals is largely unaltered, with the exception of circulating IL-6; plasma concentrations of IFN- β , TNF- α and IL-1 β do not show significant differences between wild-type and IFNAR1^{-/-} animals but the IL-6 response is almost totally abolished. Circulating IL-6 levels following systemic poly I:C in normal wild-type animals were consistent with that reported previously (Cunningham et al., 2007). These data therefore suggest that IL-6 production following dsRNA stimulation is dependent on type I IFN signalling, rather than on a MyD88/NF κ B pathway as has been reported upon LPS stimulation of TLR4 signalling (Libermann and Baltimore, 1990). Since this pattern of IL-6 expression was also present in the CNS, further discussion will follow below. The systemic cytokine response in ME7 mice challenged with poly I:C is as previously reported (Field et al., 2010) with a slightly lower response on IFNAR1^{-/-} mice. The lack of peripheral IL-6 induction remains present in ME7 animals challenged with poly I:C and the implications of this will be discussed below.

5.3.2.2 CNS response to systemic poly I:C

The levels of circulating IFN- β in response to systemic poly I:C administration confirmed that primary induction of type I IFNs themselves are not altered by genetic knockout of IFNAR1, at least not 4 hours following challenge. It seems likely that at this time-point, the observed IFN- β induction is generated from the initial response to TLR3 stimulation and that the later phase of IFN- β -induced IFN- β would be somewhat reduced in IFNAR1^{-/-} mice. This has recently been observed in ongoing work which showed decreased IFN- β induction 5 hours post-poly I:C challenge (CC, unpublished observations).

A panel of known type I IFN-responsive genes, IRF7 (Honda and Taniguchi, 2006), Mx1 (Staeheli et al., 1986) and PKR (Hovanessian, 1989), was examined in the brains of wild-

type and IFNAR1^{-/-} NBH and ME7 animals to confirm that the type I IFN response was indeed absent in knockout mice. Hippocampal expression of these genes was markedly upregulated in response to systemic poly I:C in wild-type animals but was absent in IFNAR1^{-/-} mice. Since the induction of these genes in the brain is directly dependent on IFN- α/β signalling, their absence provides clear *in vivo* evidence that the type I IFN response is non-functional in the brains of IFNAR1^{-/-} mice.

Having established that the type I IFN response was absent in IFNAR1^{-/-} mice, a selection of inflammatory transcripts were examined in the brain in order to investigate the effect of removing IFN- α/β signalling on the CNS inflammatory profile following systemic activation of the acute anti-viral response during neurodegenerative disease. The inflammatory response in wild-type ME7 animals was as previously reported in chapter 4 but there were some key differences in the response in IFNAR1^{-/-} ME7 mice. Neuroinflammatory changes have never previously been examined in response to systemic “viral” challenge during neurodegeneration in animals devoid of type I IFN signalling.

The inflammatory profile in ME7 prion disease *per se* (i.e. without further immune stimulation) in IFNAR1^{-/-} mice will first be discussed. ME7 prion disease robustly induced TNF- α and IL-12p40. Induction of TNF- α and IL-12 was halved in IFNAR1^{-/-} ME7 animals, suggesting that although these cytokines remain increased by disease *per se*, their induction is partially dependent on type I IFN. Given that the current study has reported a type I IFN response in ME7 prion disease, the removal of this in IFNAR1^{-/-} mice is the likely reason for the reduced expression of TNF- α and IL-12. A dependence on IFN- α/β for TNF- α production would not have been expected since under normal conditions, type I IFNs are typically thought to mediate anti-inflammatory effects and inhibit pro-inflammatory cytokines such as IL-1 β and TNF- α (Coclet-Ninin et al., 1997). The neurodegenerative brain, however, constitutes a very different environment from the normal healthy brain, with widespread amyloidosis, microglial activation and neuronal death. There seems to be an altered immune phenotype that accompanies neurodegenerative disease which might account for the possible role of type I interferon signalling in disease-associated TNF- α induction. To the best of my knowledge, there is no reported role for type I IFN in the induction of TNF- α , and studies to investigate this possibility are lacking, such that the current data which show type I IFN induced-TNF- α during neurodegenerative disease, are completely novel.

IL-12 is a pro-inflammatory cytokine produced by macrophages and is principally involved in cytolytic activity through stimulation of the proliferation of T cells (Trinchieri, 2003). Favours a T_H1 profile, IL-12 is also said to provide the link between innate and adaptive immunity (Hsieh et al., 1993). Increased IL-12 induction in the brains of prion-disease animals would be predictive of T cell recruitment, which is indeed known to occur during the pathogenesis of human and murine prion disease (Betmouni et al., 1996, Lewicki et al., 2003). However, the numbers of T cells are quite limited in the brains of prion diseased animals and in fact these cells have been shown to play no part in the progression of prion disease: using both severe combined immune deficient (SCID) and RAG-1^{-/-} mice that lack T and B lymphocytes, prion progression was shown to be unaffected in immune compromised mice (Klein et al., 1997). Previous studies have demonstrated that type I IFN signalling is required for TLR-induced secretion of IL-12 by dendritic cells (Gautier et al., 2005). That study showed that following TLR3/4 ligation, IL-12 was not produced by bone marrow derived dendritic cells (BM-DCs) from STAT1^{-/-} mice and that BM-DCs from IFNAR1^{-/-} mice did not phosphorylate STAT1 and consequently did not produce IL-12. Thus, it seems likely that reduced induction of IL-12 in IFNAR1^{-/-} ME7 animals is due to the absence of type I IFN signalling.

The principal aim of the current study was to determine whether the prion-diseased brain remains primed to produce exaggerated inflammatory responses to systemic poly I:C stimulation in the absence of type I IFN signalling in IFNAR1^{-/-} mice. Thus the central inflammatory response to poly I:C in ME7 animals was examined in IFNAR1^{-/-} mice. The effects of systemic poly I:C on the CNS inflammatory profile in prion-diseased IFNAR1^{-/-} animals are markedly different to those in wild-type animals. Chapter 4 demonstrated microglial priming in ME7 animals following systemic poly I:C stimulation, characterised by amplified IFN- β , IL-1 β , IL-6 and IL-10 induction while poly I:C decreased induction of TNF- α . The current study in prion-diseased IFNAR1^{-/-} animals shows that the IL-1 β response to systemic poly I:C is markedly greater in IFNAR1^{-/-} animals than in wild-type animals. Therefore it appears that in wild-type ME7 animals, that the type I IFN system actually restricts IL-1 β synthesis upon poly I:C stimulation. In the absence of type I IFN signalling, there is less control of IL-1 β expression and therefore it is increased further in IFNAR1^{-/-} ME7 animals. Thus type I IFNs are not responsible for “priming” of microglia and these mediators do not contribute to their hypersensitivity to inflammatory stimulation. It remains plausible that the presence of IFN- α/β does contribute to maintaining the muted pro-inflammatory profile during prion disease *per se*.

There is very little induction of IL-1 β mRNA or protein in the brain of prion-diseased animals (Walsh et al., 2001) and it is only following subsequent inflammatory challenge, central or systemic, that IL-1 β becomes synthesised to detectable levels (Chapter 4; Combrinck et al., 2002). In ME7 prion disease *per se*, where IL-1 β is low, IFN- β might promote induction of TNF- α transcription. Following systemic poly I:C challenge however, where IL-1 β is upregulated, it appears that IFN- β might then limit or restrain TNF- α induction in this scenario. There is no evidence in the literature for this binary regulation of TNF- α but this is a robust finding and constitutes a novel aspect of microglial regulation in neurodegenerative disease.

Without type I IFN signalling in IFNAR1^{-/-} ME7 animals, the inhibitory effect of IFN- α/β is absent and increased induction of both IL-1 β and TNF- α is the result. The anti-inflammatory influence of type I IFNs are well established and what little we know about the effect of these molecules in the brain has come from research on the mechanism of action of IFN- β therapy for MS. Indeed, in the EAE animal model of MS, clinical disease is exacerbated by either removal of type I IFN signalling through genetic deletion of IFNAR1 (Prinz et al., 2008) or deletion of the IFN- β gene (Teige et al., 2003), underlining the beneficial effects of this system. There is also a body of evidence highlighting the ability of IFN- α/β to inhibit and down-regulate pro-inflammatory mediators such as IL-1 β (Guarda et al., 2011), TNF- α (Abu-Khabar et al., 1992, Gayo et al., 1999) and IL-12 (Wang et al., 2000). Thus, it appears that in wild-type ME7 animals challenged systemically with poly I:C, IFN- β exerts an anti-inflammatory influence on both IL-1 β and TNF- α , where further induction of IL-1 β is restrained and induction of TNF- α is attenuated. The absence of anti-inflammatory type I IFN signalling therefore accounts for the amplified induction of IL-1 β and TNF- α in IFNAR1^{-/-} ME7 animals.

We have seen from chapter 4 that the prion-diseased brain also produces an exaggerated IL-10 response to systemic poly I:C challenge, and the current body of work has repeated this finding. This IL-10 response is absent in IFNAR1^{-/-} ME7 mice and is thus totally dependent on IFN- α/β for its induction. IL-10 is typically produced by T_H2 cells and is involved in differentiation of B cells and inhibition of T_H1 cell cytokine synthesis (Fiorentino et al., 1989, Moore et al., 2001). This “anti-inflammatory” cytokine is also reported to inhibit cytokine production in monocytes, macrophages and dendritic cells (Donnelly et al., 1999). Given that the prion brain only shows limited T cell infiltration it appears unlikely that the exaggerated IL-10 production originates from this population and

more likely comes from microglia; indeed macrophages (and therefore likely microglia) have been shown to produce IL-10 in response to inflammatory stimulation (Saraiva and O'Garra, 2010). IL-10 induction is mediated following IFN- α/β induction by both STAT3 and phosphoinositide 3-kinase (Ziegler-Heitbrock et al., 2003, Wang et al., 2011). Since IL-10 also signals through STAT3 homodimers, it is possible that this anti-inflammatory mediator can further propagate its own signal to dampen the influence of pro-inflammatory cytokines that might have detrimental effects. Indeed IL-10 has been shown to induce more IL-10 in macrophages in an autocrine manner via STAT3 activation (Staples et al., 2007). Much of the anti-inflammatory action of type I IFN signalling is mediated through its ability to produce IL-10. Studies have shown that the LPS-induced induction of IL-10 is mediated by type I IFN signalling (Chang et al., 2007) and clinical data has reported increased IL-10 associated with IFN- β therapy for MS (Rudick et al., 1998). Furthermore, a recent study of IFN- β treatment for relapsing-remitting MS reported increased IL-10 in patients that responded to therapy and decreased levels in non-responders (Graber et al., 2007), indicating that IL-10 might play a key role in maintaining inflammatory homeostasis in the brain during degenerative disease.

In the context of the exaggerated IL-1 β response to poly I:C in IFNAR1^{-/-} ME7 animals and the shift in direction of TNF- α induction, IL-10 might underpin these changes, although since both IL-1 β and IL-10 are exaggerated in wild-type ME7 animals challenged with poly I:C, IL-10 might merely be preventing further uncontrolled IL-1 β -mediated neuroinflammation. A recent study has demonstrated that type I IFNs can decrease IL-1 β production in bone marrow-derived macrophages, and also in human primary monocytes (Guarda et al., 2011). This is mediated by IFNAR-dependent activation of STAT3 which induces IL-10. This IL-10 then signals through its receptor to further activate STAT3 in an autocrine manner, which was ultimately responsible for reduction of IL-1 β production. It therefore seems likely that the absence of IL-10 following poly I:C in IFNAR1^{-/-} animals is due to lack of STAT3 activation by IFNAR and that the consequent increases in TNF- α and IL-1 β could be explained by this attenuated IL-10 response. Indeed prior reports have demonstrated that IL-10 can suppress macrophage TNF- α production (Bogdan et al., 1992). A further study demonstrated that prion disease progression is accelerated in IL-10^{-/-} mice (Thackray et al., 2004). These mice show a markedly shortened incubation time concurrent with an early increase in TNF- α expression that is presumably prevented by IL-10 in wild-type mice. It would be interesting to examine, in wild-type ME7 animals, the role of IL-10 in controlling inflammatory processes. Whether augmenting the anti-

inflammatory response by administration of exogenous IL-10 alongside poly I:C challenge can protect against the previously observed amplified inflammation and exacerbation of neurodegeneration would be an important question to address. Similarly, an experiment using an IL-10 blocking antibody could prove useful to this investigation as it might be expected that the central inflammatory response would be exacerbated by inhibition of IL-10 similar to that demonstrated in the Thackray report. This Thackray study is notable since removal of IL-10 accelerates prion disease by exacerbation of inflammation, yet removal of IFNAR signalling slows the progression of disease. Given that the action of type I IFN has been reported to be mediated through IL-10 activation, it seems that there may be some divergence of IFN- α/β and IL-10 action in prion disease. The principal effects of type I IFN on disease may not be through their anti-inflammatory role and what controls the apoptosis and the inflammatory phenotype in prion disease remains an important question to answer.

Another important observation from the current study was the complete absence of IL-6 induction in IFNAR1^{-/-} mice. The absence of an IL-6 response reported earlier in the periphery was replicated in the brain of both NBH and ME7 animals. This lack of IL-6 induction following poly I:C challenge in IFNAR1^{-/-} mice suggests that IL-6 is directly regulated by the type I IFN signalling cascade and not via NF κ B nuclear translocation as has been reported (Libermann and Baltimore, 1990). Indeed there is some prior evidence of IL-6 regulation through IFNAR: one study showed reduced IL-6 production in murine embryonic fibroblasts lacking the type I IFN receptor (Mitani et al., 2001) and a further study has reported a diminished IL-6 response in the liver of IFNAR1^{-/-} mice following liver ischemia-reperfusion injury (Zhai et al., 2008). Yamamoto and colleagues have previously demonstrated that IL-6 is induced by a TRIF-dependent mechanism following LPS treatment (Yamamoto et al., 2003). Thus, LPS-induced IL-6 does not seem to be dependent on MyD88 activation of NF κ B but is produced as part of the later phase of NF κ B activation which is mediated by TRIF (Akira and Takeda, 2004). It might have been expected that IL-6 induction following poly I:C challenge would occur via TLR3-mediated TRIF activation of NF κ B but this does not seem to be the case here, since the IL-6 response is completely abolished in IFNAR1^{-/-} mice and other NF κ B-dependent pathways are intact. Preliminary studies from our laboratory with LPS in IFNAR1^{-/-} mice have not reported this decreased IL-6, suggesting that with LPS/TLR4 stimulation, IL-6 is produced via TRIF-NF κ B activation. The current data however, indicate that IL-6 induction following TLR3 stimulation with poly I:C occurs downstream of type I IFN signalling.

IL-6 is known to be markedly induced in both periphery and brain during sepsis (Remick et al., 2005, Meyer et al., 1997) and has also been associated with cognitive impairment (Heyser et al., 1997) and delirium (de Rooij et al., 2007). Given that other groups have reported that IL-6 facilitates memory impairment after peripheral LPS (Sparkman et al., 2006) and that systemic poly I:C has been associated with cognitive impairments in the contextual fear conditioning paradigm (Kranjac et al., 2012), the IFNAR1^{-/-} ME7 model could provide a novel platform with which to study the role of the IL-6/IFN-I axis in delirium. One might predict that the absence of an IL-6 response following poly I:C in IFNAR1^{-/-} ME7 mice would provide protection against inflammation-induced cognitive deficits.

A recent study on neuroinflammation during sepsis identified that LPS induction of IL-6 was abrogated by inhibition of STAT3 via knockdown or pharmacological targeting (Beurel and Jope, 2009). Thus, if IL-6 is activated by STAT3 and also transduces its signal via the IL-6 receptor activation of STAT3, it is possible that a positive feedback mechanism could be initiated whereby IL-6 promotes its own induction. Furthermore, IFN- α/β signalling via IFNAR is capable of activating the STAT3 homodimer. This typically promotes anti-inflammatory and anti-proliferative activity along with IL-10 induction as discussed above (Gonzalez-Navajas et al., 2012), with have beneficial influences by attenuation of the pro-inflammatory response during an infection or insult (Ito et al., 1999). Consequently, if this activation of STAT3 is also responsible for induction of IL-6, it is then plausible that the failure of poly I:C to induce an IL-6 response in IFNAR1^{-/-} mice is due to the failure to effect IFNAR-dependent STAT3 activation.

5.3.2.3 Apoptotic factors in IFNAR1^{-/-} ME7 animals

Type I IFNs have been implicated in the induction of apoptotic cascades. Signalling through IFNAR can also promote activation of the STAT1 homodimer to induce a pro-inflammatory response that favours recruitment of immune cells to the site of inflammation. This is also the principal mechanism of type II IFN signalling by its sole member, IFN- γ (Platanias, 2005). This activation of STAT1 is also associated with upregulation of pro-apoptotic factors (Kim and Lee, 2007). Accordingly, removal of the type I IFN response may block STAT1-dependent cell infiltration or apoptotic cascades. The current study has examined transcription of the death receptor Fas and its ligand, Fas ligand (FasL), in the brains of IFNAR1^{-/-} ME7 animals.

The Fas-FasL system is one of the best characterised mechanisms of apoptotic cell death. Fas triggers apoptosis when bound to FasL (Nagata, 1997, Ashkenazi and Dixit, 1998). Generally, Fas expression is quite ubiquitous, with expression of FasL more restricted to the immune system. FasL binds to Fas, which is expressed at the cell surface of the target cell. Binding of FasL to Fas leads to recruitment and activation of the cytoplasmic adaptor protein FADD (Fas-associated death domain-containing protein). Activated FADD then targets caspase-8 which when activated, signals to the execution caspases-7 and 3 to trigger proteolysis and cell death.

The Fas-FasL system is known to contribute to the tightly regulated immune phenotype in the normal healthy brain. Many cells of the CNS can express Fas and FasL, including neurons, astrocytes, microglia, oligodendrocytes and vascular endothelium (Choi and Benveniste, 2004). Indeed, as well as mediating apoptosis, it appears that endothelial expression of FasL provides important protection from extravasation of immune cells into the brain parenchyma (Sata and Walsh, 1998, Walsh and Sata, 1999). Constitutive FasL expression on endothelial cells targets adherent mononuclear cells for apoptosis and thus prevents extravasation into surrounding tissue. Although expression of FasL in the periphery is more restricted to immune cells, it is reported to be constitutively expressed in brain, with an apparent contribution to the “immune-privileged” nature of this environment (Nieder Korn, 2006). Constitutive CNS expression of FasL allows the brain to quickly respond to any infiltrating and possible damaging cell, targeting it for apoptosis and thus maintaining the suppressed immune status in the brain. Although there are also reports of constitutive Fas expression in the CNS, its basal expression is so low that it seems to be non-functional (Tan et al., 2001). Inducible expression of Fas receptors in the CNS can elicit bystander damage via apoptosis of neurons and glia and as such the Fas-FasL system may be considered a double-edged sword; contributing to the muted immune status in normal brain and inducing neuronal death in the compromised brain. Thus the cells of the CNS must be tightly regulated to avoid unexpected and unwanted cell death that could have deleterious consequences at times when both Fas and FasL are expressed. It has been reported that endothelia and astrocytes are resistant to Fas-mediated cell death (Becher et al., 1998, Saas et al., 1999, Lee et al., 2000), presumably to prevent unnecessary apoptosis of key components of the BBB.

The current study has shown increased Fas and FasL mRNA transcription in ME7 animals, indicating that the prion disease-associated cell death observed in both the previous and the current chapter could occur via Fas-FasL interactions as has been reported elsewhere (Jamieson et al., 2001). Consistent with this, expression of the Fas-FasL system have been reported to be elevated in the compromised CNS, including AD (de la Monte et al., 1997, Ferrer et al., 2001) and prion disease (Jamieson et al., 2001, Puig and Ferrer, 2001). Although Fas expression seems to be inducible by systemic poly I:C, FasL remains unchanged following systemic challenge. Fas appears to be further induced by poly I:C in ME7 animals and while FasL is not further affected, the increased availability of the death receptor could provide the ligand with more opportunity to bind and thus transduce the apoptotic signalling cascade. Since the BBB is known to be compromised in prion disease (Wisniewski et al., 1983), and since FasL plays a role in endothelial regulation of extravasation of cells (Walsh and Sata, 1999, Sata et al., 2000), disease-associated dysregulation of the BBB could result in infiltration of peripheral immune cells which might account for the increased Fas-FasL expression.

The expression of Fas in IFNAR1^{-/-} NBH and ME7 animals is very similar to that in wild-type mice. Given that PKR has been shown to induce expression of Fas mRNA (Balachandran et al., 1998, Donze et al., 1999), and that PKR expression is attenuated in IFNAR1^{-/-} mice, a decrease in CNS expression of Fas might have been expected, although there are several other mechanisms that mediate expression of Fas *in vivo* (Sharma et al., 2000). FasL expression however is not induced by ME7 prion disease in IFNAR1^{-/-} mice and remains at basal levels, suggesting that type I IFN signalling is responsible for the disease-associated upregulation of FasL expression. It will be important to identify the cell type which expresses FasL in the brain of ME7 animals and to establish whether it is one discrete cell population that decreases its FasL expression in IFNAR1^{-/-} mice. This lack of FasL expression could be indicative of decreased apoptosis in ME7 animals, and might underpin the slower disease progression observed earlier and would merit further investigation.

It remains possible that prion disease-associated increased apoptosis may be controlled by the increased type I IFN response that is present in ME7 animals. Since PKR is induced by type I IFN, and has been associated with induction of apoptosis (Balachandran et al., 1998, Balachandran et al., 2000), activation of PKR could underpin these processes. Poly I:C-induced exacerbation of neurodegeneration reported in chapter 4 could also be mediated by

PKR activation, through either apoptotic pathways or perhaps via translational inhibition. It is well established that PKR activation results in phosphorylation of the α -subunit of eukaryotic translation initiation factor 2 (eIF2 α), which acts to inhibit protein translation (Levin and London, 1978).

The amplified type I IFN response induced by poly I:C in ME7 animals may increase PKR-mediated eIF2 α phosphorylation. Though this typically results in translational inhibition, some processes show resistance to this inhibition, including Fas and caspase-3-mediated apoptotic pathways (Donze et al., 1999). Thus, apoptosis may still proceed despite phosphorylation of eIF2 α . A recent study has suggested that the neurodegeneration evident during prion disease is mediated by sustained translational repression by phosphorylated eIF2 α , although the mechanism of neuronal death could not be successfully identified, with neither apoptosis nor necrosis evident in the regions of pathology in that study (Moreno et al., 2012). This study also demonstrated that endoplasmic reticulum (ER) stress via PrP accumulation can lead to phosphorylation of protein kinase-like ER kinase (PERK) which, like PKR, can also phosphorylate eIF2 α and inhibit protein synthesis. Thus although apoptosis might be involved in the poly I:C-induced exacerbation of disease, it might not be necessary, as the shutdown of protein synthesis via translation inhibition by eIF2 α phosphorylation is capable of mediating loss of neurons. Given that prion disease progresses more slowly in IFNAR1^{-/-} mice and that the PKR response is depressed in these mice, this suggests that eIF2 α phosphorylation might also be responsible for neurodegeneration in ME7 prion disease. Further research will be necessary to investigate what role, if any, PKR-induced phosphorylation of eIF2 α might play in the increased neurodegeneration present following poly I:C stimulation in ME7 prion disease. In light of the data presented here and taking account of the reported apoptotic role of PKR, we predict that activation of the type I interferon response and consequent induction of PKR can promote pro-apoptotic cascades that could contribute to the observed acceleration of and exacerbation of neurodegenerative disease. Before the completion of this thesis, a large study in wild-type and IFNAR1^{-/-} mice was initiated to investigate the induction of PKR, the phosphorylation of eIF2 α and the induction of apoptosis and other features of pathology following systemic poly I:C in ME7 animals, and due to time constraints was not completed. This study should provide important mechanistic information about how type I IFN contributes to prion disease progression and impact upon neuronal death, along with identification of cell types that produce active PKR and contribute to translational repression.

Thus, the current preliminary investigations suggest that there is a down-regulation of apoptotic machinery in prion-disease animals that lack functional type I IFN signalling, which could be responsible for the increased survival demonstrated earlier. This would again suggest that while IFN α/β can be beneficial to some aspects of neurodegenerative disease, these “anti-inflammatory” mediators can also contribute to deleterious processes. A comprehensive study will be important to investigate mechanisms of cell death in prion diseased IFNAR1^{-/-} animals to fully address this issue.

5.3.3 Conclusion

The current chapter demonstrated the role of IL-1 and type I IFN signalling in the progression of ME7 prion disease using IL-1RI^{-/-} and IFNAR1^{-/-} genetic knockout mice. Removal of IL-1 signalling slightly altered the course of ME7 prion disease with delayed onset of neurological deficits, but total survival time post-inoculation was unaffected. This suggests that IL-1, while clearly capable of having deleterious and degenerative influences on the brain (Simi et al., 2007), is not necessary for pathogenesis of neurodegeneration in the ME7 model of prion disease.

Conversely, type I IFNs are typically viewed as anti-inflammatory due to their ability to decrease the pro-inflammatory response. Their effects on brain function however, remain relatively unknown. The current chapter has demonstrated that there is an increased type I IFN response associated with prion disease itself and that genetic deletion of the IFNAR1 gene and consequent disruption of type I IFN signalling is actually somewhat protective against ME7 prion disease. Onset of neurological symptoms occurs later in disease and survival time post-inoculation is increased, suggesting that contrary to their protective effects in MS in humans (Goodin et al., 2002) and EAE in mice (Brod and Burns, 1994, Brod and Khan, 1996), type I IFN signalling is actually contributing to progression of prion disease in the ME7 mouse.

Recent studies have implicated a sustained repression of translation and shutdown of protein synthesis in prion disease-associated neurodegeneration through ER stress-mediated phosphorylation of eIF2 α (Moreno et al., 2012). Type I IFNs seem to be a central feature of the local response to sterile neuronal injury (Khoroshii and Owens, 2010, Hosmane et al., 2012) and this has now been shown to be the case for neurodegeneration in ME7 prion disease also. Thus the type I IFN response seems to be a general feature of

inflammation during neurodegeneration. Given that PKR is a key responder to type I IFN signalling, and that this molecule mediates phosphorylation of eIF2 α , this could be a major mechanism for the poly I:C-induced exacerbation of neurological impairments and acceleration of disease progression in prion animals. An important question arising from the current body of work is whether systemic immune stimulation with poly I:C produces exacerbation of neurological function in prion-diseased IFNAR1^{-/-} mice. An investigation of neuropathological changes alongside this will also provide insight into the mechanisms of type I IFN-associated disease progression and this work is currently being undertaken in the laboratory..

The current work has implications for the aged and demented population who frequently suffer from viral infection and therefore have an increased type I IFN inflammatory profile. Although they have never been directly implicated in the pathophysiology of delirium, type I IFNs might yet constitute an important trigger of this acute and transient brain dysfunction given that they are the key mediators produced during viral infection, and also given that infection and inflammation remain some of the principal risk factors for development of delirium in the aged and demented population. The high frequency of viral infections in this population (Falsey et al., 2005, Estes et al., 2006) makes it likely that infection-induced delirium would commonly be caused by viruses in susceptible individuals. Given that delirious episodes are predictive of long-term decline and poor outcomes it therefore becomes very important to try to understand the key mediators or processes that underpin this acute brain dysfunction that can exacerbate and accelerate disease and dementia.

Chapter 6

General Discussion

6. General discussion

The overall aim of this thesis was to study the interaction between systemic inflammation and neurodegeneration, and to gain a better understanding of how CNS function is affected by peripheral inflammatory insults. Despite the long-standing dogma of immune-privilege, we now know that the brain does not lack immune function but rather, it is very tightly regulated. There has been considerable research over the last 20 years on immune-to-brain interaction and much of the current understanding has come from the sickness behaviour literature (Dantzer and Kelley, 2007). There have been relatively few studies investigating the impact of peripheral inflammation on the brain and its microglial population during chronic neurodegeneration. Delirium during dementia is frequently caused by inflammation due to infection or surgery (Fong et al., 2009), and our laboratory has proposed that studying the processes underpinning delirium offer important insights into the consequences of systemic inflammatory insults on the degenerating or vulnerable brain. This thesis has developed and characterised a novel animal model of inflammation-induced delirium on a background of prior cholinergic hypofunction, similar to that apparent in the early stages of AD. Furthermore, this work has also demonstrated that chronic neurodegenerative disease shows acute exacerbation and longitudinal acceleration upon stimulation of the anti-viral innate immune response with poly I:C. Thus the current body of work demonstrates that there are multiple levels by which systemic inflammation and prior pathology can interact, with negative consequences for the individual (Figure 6.1). These studies have implications for geriatric populations that frequently suffer from systemic inflammatory events and are most susceptible to the neurological/cognitive effects of this inflammatory stimulation

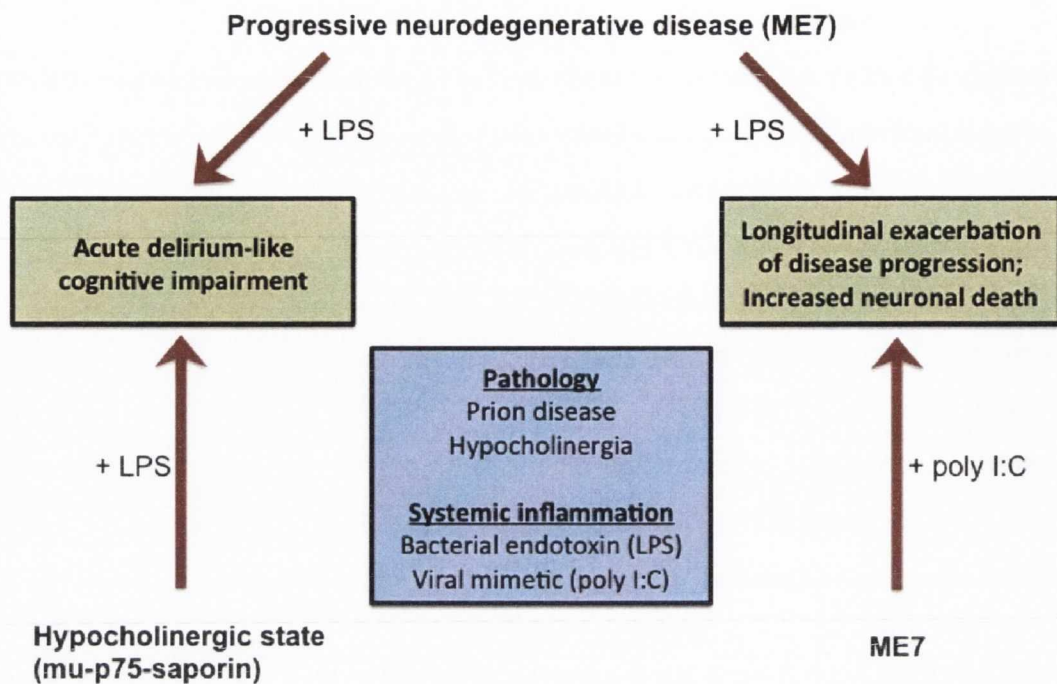


Figure 6.1 *Multiple levels of interaction between systemic inflammation and prior neuropathology.* Prior CNS pathology of multiple aetiologies constitutes a vulnerable state and a risk for the deleterious effects of systemic inflammatory stimuli. Thus, peripheral inflammation may interact with prior pathology on multiple levels.

6.1 A novel model of delirium

One of the major achievements of this thesis was the development of a novel animal model of inflammation-induced delirium as occurs during dementia. Our laboratory previously described the first such animal model using ME7 murine prion disease to model progressive dementia onto which superimposition of systemic inflammation with bacterial LPS produced acute and transient impairments in cognition (Murray et al., 2012). This demonstrated that prior neurodegenerative pathology (prion disease) constitutes a risk factor for inflammation-induced acute cognitive deficits similar to delirium in elderly demented patients. The current body of work was aimed at creating a neuropathology distinct from prion disease so as to address, and potentially reconcile, two current hypotheses in the delirium literature: the cholinergic deficiency hypothesis and the neuroinflammatory hypothesis. These are two leading proposals in the clinical literature (van Gool et al., 2010), but there is no clinical or fundamental data linking these hypotheses. By placing “prior pathology” in the basal forebrain cholinergic system, it was possible to replicate a key aspect of early AD – degeneration of cholinergic neurons of the

basal forebrain (Davies and Maloney, 1976), and to subsequently assess the vulnerability of these animals to systemic inflammatory insult.

In order to create a valid animal model of a neuropsychiatric disorder, a number of criteria should be met: 1) construct validity, 2) face validity and 3) predictive validity (Nestler and Hyman, 2010). Construct validity states that the animal model must recreate an aetiological process that causes disease in humans. Face validity ensures that a model recreates biochemical, neuropathological or behavioural features of the human condition, although in practice it can be difficult to satisfy all of these criteria since many of these aspects are the very processes that animal models are attempting to delineate. Finally, predictive validity states that a model should respond to treatments in a way that can predict the effects of those treatments in humans.

Using the selective cholinergic immunotoxin, mu-p75-saporin, the current study created a hypocholinergic state upon which systemic LPS was superimposed, to replicate the infections that are commonplace in elderly and demented patients. Peripheral LPS induced acute and transient working memory deficits, but only in those animals that had existing cholinergic dysfunction. Thus this model has recapitulated key core aspects of delirium as presented in vulnerable individuals and we can conclude that that prior hypocholinergia confers increased susceptibility to the cognitive effects of systemic inflammation. Consequently this novel model demonstrates construct validity with neuronal loss as a risk factor and inflammation as precipitating factor. We can also provide the behavioural aspect of face validity by creating cognitive deficits that are acute and transient, with impairments in attention, recall, and short-term/working memory that are key characteristics of delirium as defined by the DSM-IV (APA, 1994) and ICD-10 (WHO, 1992).

While it is difficult to state with confidence that the combination of prior hypocholinergia and superimposed systemic inflammation actually produces delirium, it is clear that there are key similarities between the current model and clinical delirium and that the combination of insults is essential. This model system then allows one to pose a number of questions. Among the first of these was whether microglial priming is necessary for these acute deficits and the data were relatively clear on this question: there was no differential inflammatory response to LPS in lesioned versus control animals. Microglia are not primed by the dose of mu-p75-sap used here (0.08 µg/mouse) and at the time-point examined (40 days post-lesion) and thus acute cognitive dysfunction in the hypocholinergic animal can

occur in the absence of microglial priming. However, it was also demonstrated that hippocampal microglia are primed by the loss of cholinergic tone 40 days after more severe lesions and even at earlier time points after more moderate lesions. We can therefore make the prediction that the observed deficits would be more severe at a time where the microglial population are primed, either in the acute stages following cholinergic degeneration, or at later times after more severe cholinergic depletion. Performing these experiments would necessitate altering the model to allow naive mice to be trained on the T-maze paradigm before surgery to produce the cholinergic lesion. Placing these mice back in the T-maze shortly following surgery (5-7 days), verifying performance in the absence of systemic inflammation and then administering LPS at 10-14 days would allow for assessment of acute deficits at a time point at which microglial priming would be present. Perhaps the more clinically relevant experiment would be to perform more severe lesions and challenge these animals with LPS 40 days later. In this scenario the microglial priming that is manifest arises primarily from the loss of cholinergic tone rather than some injury-related acute microglial activation. Basal forebrain cholinergic loss reaches approximately 75% in moderate/severe AD (Whitehouse et al., 1982) and thus, even the highest dose of mu-p75-saporin used in this study produces clinically relevant cholinergic loss.

The lack of microglial priming in the cognitive experiments performed in this thesis suggested that dysfunctional cholinergic neuromodulation most likely underpinned the inflammation-induced cognitive deficits. Indeed the current work demonstrated that treatment with the acetylcholinesterase inhibitor, donepezil, protected against the LPS-induced impairments. It is possible to make the prediction that donepezil might alleviate the symptoms of delirium in those individuals with a hypocholinergic predisposition. Since there are currently no licensed treatments for delirium, the current study thus brings predictive validity to this novel model. The mechanism that underpins this protection will be an important avenue to investigate. Donepezil functions by inhibiting acetylcholine degradation and thus there is more neurotransmitter available at the synapse. Accordingly, cognitive protection might work by a) bolstering cholinergic reserve and raising the threshold required to produce cognitive deficits or b) by reducing inflammation via increased anti-inflammatory acetylcholine signalling.

This work has implications for the recent clinical trial of rivastigmine treatment for delirium in the intensive care unit, which reported increased mortality, greater severity and

longer duration of delirium in patients treated with rivastigmine (van Eijk et al., 2010). This trial was conducted on critically ill patients, many of whom were suffering multi-organ failure from complications of severe sepsis, and were not likely to have had an existing hypocholinergic disposition. Thus boosting acetylcholine could further suppress the immune response with negative consequences for these patients. From the current thesis however, we can now make the prediction that acetylcholinesterase inhibition could prove useful in preventing delirium in those individuals with existing hypocholinergia in early dementia and AD. This could offer improvement in quality of life in such patients and also slow the progression of cognitive and functional decline that often follows delirious episodes.

6.2 A central cholinergic anti-inflammatory pathway

Over the last 10 years, the control of peripheral inflammation via the vagus nerve-mediated cholinergic anti-inflammatory pathway has received much attention (Tracey, 2002, Tracey, 2007, Tracey, 2009, Waldburger and Firestein, 2010). This is dependent on cholinergic signalling at the $\alpha 7$ -nAChR on peripheral macrophages which acts to inhibit NF κ B-dependent inflammatory gene transcription. The existence of such a pathway in the brain has never been investigated although recent confirmation that microglia express $\alpha 7$ -nAChR (Shytle et al., 2004, De Simone et al., 2005) was suggestive of the existence of tonic cholinergic inhibition of inflammation and microglial activation in the brain.

The current studies have demonstrated that a robust cholinergic lesion that produces more severe hypocholinergia (~50% depletion) than that apparent in the cognitive studies is sufficient to induce microglial priming. The absence of priming in the cognitive studies likely reflects both the deliberately limited nature of the lesion (0.08 μ g mu-p75-sap per mouse) along with the time-point examined (40 days post-lesion) which allowed for resolution of the initial inflammatory response and microglial activation that accompanies the acute cell death. Thus with a more robust lesion (1.2 μ g/mouse), there was an exaggerated CNS inflammatory response to immune stimulation with systemic LPS, characterised by increased pro-inflammatory transcripts in the medial septum and hippocampus, along with increased hippocampal IL-1 β production. The current work has also shown that a moderate cholinergic lesion (0.4 μ g/mouse), examined at a time point close to the initial cell death and where microglia would still be activated (14 days), primes microglia to produce amplified IL-1 β responses to subsequent peripheral LPS stimulation.

This thesis has demonstrated direct evidence that the basal forebrain cholinergic neurons provide tonic inhibition of inflammation which when removed results in an exaggerated response to subsequent inflammatory challenge, and that severe hypocholinergia also reduces $\alpha 7$ -nAChR expression in the hippocampus. Thus, direct evidence has been provided for a CNS cholinergic anti-inflammatory pathway similar to that observed in the periphery (Tracey, 2007). There have been previous studies demonstrating the presence of the $\alpha 7$ -nAChR on microglia (Shytle et al., 2004) and illustrating the ability of nicotine to act in an anti-inflammatory manner (De Simone et al., 2005), but the current data provide the first clear evidence that a disease-relevant decrease in cholinergic tone can affect the activation and the responsiveness of microglia to subsequent challenges. Based on the current findings, it seems likely that depletion of cholinergic function below a certain threshold, in a chronic neurodegenerative condition, would lead to an ongoing state of increased microglial activation and contribute to exaggerated responses to secondary insults such as infection, injury, surgery and other co-morbidities common in the ageing population. Thus in the later stages of dementia where microglia are primed by a more severe hypocholinergic predisposition, one would predict that inflammation-induced delirious episodes would also be more severe and possibly be of longer duration, with negative consequences for the individual.

6.3 Type I IFNs and neurodegeneration

This thesis identified that type I IFN induction is a common theme in neurodegeneration, regardless of the type of insult/injury. Investigations into the acute inflammatory response to mu-p75-sap-induced cholinergic neuronal death in the basal forebrain reported induction of IFN- β and increased expression of type I IFN-dependent genes. Furthermore, induction of the type I IFN response was also demonstrated in the ME7 model of murine prion disease. Thus the current work has revealed that the type I IFN response is upregulated by both sterile neurotoxic (saporin) and chronic infectious (prion) neurodegeneration.

There is an emerging literature describing type I IFN induction with neurodegeneration. A recent study from Trevor Owens' group demonstrated evidence of a type I IFN response in the CNS following sterile injury (Khorrooshi and Owens, 2010). This study reported increased microglial IRF7 production in the hippocampus of mice after transection of entorhinal afferents and consequent axonal degeneration. Similarly, a further study has recently reported a type I IFN response in microglia following dorsal root axotomy, and

that microglial phagocytosis of neuronal debris is TRIF-dependent (Hosmane et al., 2012). Both of these studies confirmed that microglia were responsible for type I IFN production. Thus it seems likely that microglia are the mediators of the type I IFN response in the mu-p75-sap and ME7 models of neurodegeneration, although it will be important to definitively address what population produce type I IFN in our models, both in response to acute/chronic neuronal death and in the inflammatory studies using poly I:C.

Given that type I IFNs are the principal immune responders to viral infection, induction of these mediators during neuronal death in the absence of viral stimulation represents novel data. Type I IFNs are typically viewed as anti-inflammatory in the CNS due to their therapeutic use in the treatment of relapsing-remitting MS. Thus one might imagine that their role in neurodegeneration in the current study would be to limit further inflammation that could be deleterious to the brain, via inhibition of pro-inflammatory mediators and maintenance of the muted microglial phenotype. However, the removal of type I IFN signalling (IFNAR1^{-/-} mice) increased survival time in ME7 prion disease, suggesting that type I IFNs are actually having deleterious effects in the brain during chronic neurodegeneration. It is possible that an increased type I IFN response could contribute to neuronal death and dysfunction either via promotion of pro-apoptotic pathways or through eIF2 α phosphorylation and inhibition of protein synthesis. The Hosmane study however, reported that absence of TRIF/type I IFN signalling reduces phagocytic clearance of neuronal debris and limits axonal outgrowth, suggesting a beneficial influence of type I IFN in this context. Dorsal root axotomy and hippocampal entorhinal deafferentation are relatively acute denervation studies as is the mu-p75-saporin model of acute neuronal destruction (and consequent deafferentation) while the prion model is a chronic progressive neurodegenerative disease in which neurons die after 16-18 weeks after a long period of synaptic loss and axonal degeneration. Thus it seems likely that type I IFNs may have different influences on the degenerative process depending on the type of neuronal death. The protective role of IFN- β in MS and animal models thereof may be significantly different since this is a primary immune-mediated condition. It will be important to identify what effect these molecules have during cholinergic degeneration in the mu-p75-sap model; whether they contribute to the degeneration or limit the further deleterious effects of IL-1 β or TNF- α . Since our animal studies have shown this type I IFN response associated with neurodegeneration, it would be interesting to investigate whether this is also a feature of AD in humans.

Following the observation of a type I IFN response in prion disease per se, this thesis demonstrated that systemic poly I:C induced amplified type I IFN and microglial IL-1 β responses in diseased animals. The impact of type I IFN signalling on the primed microglial state was then examined using IFNAR1^{-/-} ME7 animals. Removal of type I IFN signalling resulted in elevated IL-1 β and TNF- α induction after systemic poly I:C challenge compared to wild-type prion-diseased animals. Interestingly, poly I:C-induced IL-10 was dependent on IFN- α/β and was abolished in IFNAR1^{-/-} mice. Thus it is apparent that type I IFNs are not responsible for induction of the primed response and actually seem to restrict further inflammatory gene expression following poly I:C treatment as indicated by the elevated IL-1 induction. Though not required for microglial priming, it is likely that type I IFN signalling and its IL-10 induction is involved in maintenance of the muted pro-inflammatory profile in prion disease.

6.4 Microglial priming in two discrete models: cholinergic degeneration and prion disease

Microglial priming, whereby some aspect of neurodegenerative disease makes these cells hyper-sensitive to subsequent inflammatory stimulation, eliciting exaggerated CNS inflammatory responses, was first described in the ME7 model of prion disease (Cunningham et al., 2005). Other groups have since reported priming in ageing (Godbout et al., 2005), and in neurodegenerative models of AD (Sly et al., 2001), PD (Pott Godoy et al., 2008) and MS (Ramaglia et al., 2012). This thesis has also provided evidence of microglial priming in a model of cholinergic neuronal degeneration (mu-p75-sap). As such, there is direct relevance to dementia and AD, in which there is robust degeneration of basal forebrain cholinergic neurons (Davies and Maloney, 1976). Indeed microgliosis has been reported in the hippocampus and cortex of AD patients (McGeer et al., 1987), which could result from loss of cholinergic innervation along with progressive neuronal death. The current studies have shown that loss of innervation to the hippocampus results in microglial priming and that there is exaggerated IL-1 β production following systemic LPS challenge. Thus microglial priming is not limited to progressive/chronic/infectious neurodegeneration and can also occur with permanent loss of acetylcholine innervation, but only where the cholinergic depletion is sufficiently robust.

In addition to showing that there are multiple routes to the primed state, this thesis has now added viral stimulation (poly I:C) via TLR3/MDA5 to the triggers that can further activate

the primed microglial cell. This is highly relevant to the aged and demented population due to the high incidence of viral infection in these individuals (Estes et al., 2006) along with the fact that viral infections tend to be more common than those of bacterial aetiology. One obvious avenue of investigation arising from the current studies would be to assess the impact of a real infection (eg. mouse-adapted human influenza virus H1N1) on the microglial and CNS inflammatory profile during neurodegeneration. This would draw more parallels with human disease and viral infection. Recent mouse cognitive studies with mouse-adapted influenza have shown remarkably limited cognitive deficits (Jurgens et al., 2012), but these studies were carried out in the young healthy mouse and the results of this thesis would certainly suggest that these effects would be considerably more robust in animals with prior vulnerabilities such as advanced age or prior neurodegenerative disease.

Although this thesis has demonstrated two new variants on the microglial priming theme, the existence of a single “priming factor” seems unlikely since human neurodegenerative disease processes often present with a multitude of neuropathologies. The original macrophage priming literature stated that priming was achieved by exposure to IFN- γ with full activation triggered by subsequent LPS treatment (Johnson et al., 1983), thus placing IFN- γ first in the discoveries of priming factors. Our laboratory and this thesis have thus far demonstrated priming of microglial cells by chronic neurodegenerative disease in which there is widespread synaptic loss and amyloidosis, and also by cholinergic denervation. A recent study of EAE has also suggested that a dysfunctional complement system, specifically the C3 protein, is responsible for induction of priming (Ramaglia et al., 2012). Numerous other mechanisms that produce priming have also been identified including exposure to amyloid-beta protein, α -synuclein, glucocorticoids; loss of microglial inhibition via TREM2, CD200R, and fractalkine receptor; and also via activation of the inflammasome machinery (see Cunningham (2012) for a comprehensive review). The existence of multiple routes to microglial priming might indicate that there are subtle differences between cells primed by different stimuli. Given that the peripheral macrophage literature has classified these cells into different states of activation (Martinez et al., 2008), it seems likely that a similar classification might also hold true for microglia. Perhaps rather than constituting a separate entity, microglial priming is actually just a continuum on the spectrum of the different states of macrophage activation.

6.5 Systemic viral mimetics accelerate neurodegenerative disease

Another major observation of the current work was the acute exacerbation of neurological function and acceleration of disease progression following repeated systemic stimulation of the acute anti-viral response using poly I:C. These deficits become progressively more severe as disease progresses and animals recover to a lower baseline with each subsequent challenge. This follows from previous observations where bacterial LPS accelerates disease progression in ME7 animals (Cunningham et al., 2009). The findings from the current studies are directly relevant to the geriatric population that frequently suffer from viral infections that can contribute to cognitive decline (Strandberg et al., 2003). There are also implications for the recent study in which increased circulating levels of TNF- α was associated with acceleration of dementia and cognitive decline in AD patients (Holmes et al., 2009).

The use of multiple poly I:C challenges has particular relevance for the aged and demented population since these individuals rarely suffer from a single isolated systemic inflammatory event. In reality, the geriatric population are exposed to many inflammogens, and suffer from repeated infections, injuries and traumas as the immune system deteriorates with age. Thus it seems likely that the current observations with repeated poly I:C challenge can be applied to these vulnerable populations.

Stimulation of the acute anti-viral response in the diseased brain also results in amplified IL-1 β and type I IFN induction. Since IL-1 β has been shown to be deleterious to the injured brain (Simi et al., 2007), this cytokine seemed a likely contributor to the acceleration of disease and exacerbation of function shown here. However, if anything the ME7+poly I:C IL-1RI^{-/-} animals showed more apoptosis than their ME7+poly I:C wild-type controls. Given the somewhat conflicting evidence for increased apoptosis in chapters 4 and 5, it remains unclear whether apoptosis underpins the exacerbation. Failing this, type I IFN stimulation of PKR and consequent phosphorylation of eIF2 α becomes an obvious line of investigation. Translational inhibition mediated by eIF2 α phosphorylation has recently been shown to contribute to progression of prion disease (Moreno et al., 2012). Thus the presence of increased phosphorylation of eIF2 α following systemic poly I:C treatment in ME7 animals must be investigated to assess the role of translational repression in the viral mimetic-induced exacerbation and acceleration of neurodegenerative disease, as well as a possible mechanism for the role of type I IFN in disease progression per se.

6.6 Summary

The studies performed in this thesis have important implications for diverse fields of neuroscience, including CNS inflammation, psychoneuroimmunology, neurology and old age psychiatry along with relevance for intensive care unit medicine. Delirium during dementia represents a prominent manifestation of the deleterious influences of activating the peripheral immune response in vulnerable individuals. With this condition comes acute and longitudinal neurological decline. Given that the world's ageing population is predicted to double by 2030, there are important medical, social and economic imperatives to adequately address the effects of systemic inflammatory insults in the brains of this uniquely susceptible cohort. This thesis has begun to offer some mechanistic insights into the deleterious effects of peripheral inflammation on the compromised brain and will provide a platform for future studies in this field.

Chapter 7

Bibliography

7. Bibliography

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