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Investigation into possible modulators of microglial activation

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

2010

Kevin J. Murphy
I Declaration of Authorship

I declare that this thesis is entirely my own work with the following exceptions: certain results were kindly donated to me by Drs. Rachael Clarke and Anne-Marie Miller. This work has not been previously submitted for a degree to this or any other university. I give my permission to the library to lend or copy this thesis.

Kevin Murphy
II Acknowledgements

Firstly I would like to thank my supervisor Professor Marina Lynch for running the HRB Integrated programme in Neuroscience in TCIN and also for giving me the opportunity to do my research project in her lab, but in particular I want to thank Marina for the support, encouragement, and guidance given to me over the last 4 years, and of course, the kicks up the backside when needed!

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III Abstract

Ageing is associated with an increase in inflammatory changes and a decline in biological function. Inflammatory changes in the brain are primarily the result of activated microglia, the immune cells of the brain, and it appears that there may be several states of activated microglia associated with ageing. These stages of activation serve to function in various capacities, including increased motility, antigen presentation, phagocytosis and production of neurotoxic factors. Microglial activation is known to be potently induced by the pro-inflammatory cytokine interferon-gamma (IFN-γ), a macrophage-activating factor. However the cell source of IFN-γ in the central nervous system (CNS) is not known, and therefore the trigger(s) leading to age-related microglial activation are not known.

One of the functional outcomes of activated microglia is the release of pro-inflammatory cytokines, which transmit signals that can lead to a deficit in synaptic function and eventual neurodegeneration. Ageing is the most significant risk factor in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, which is typified by a selective and progressive degeneration of neurons in the CNS. Such neurons are incapable of regeneration due, in part, to the inhibitory actions of the Nogo protein. The aims of this study were to examine the existence of multiple activation states of microglia in the brain of aged rats, determine a possible activator(s) for these microglia, investigate the possibility that infiltrating NK cells might be a source of immunomodulatory molecules in the age-associated activation of microglia and assess whether or not Nogo may be a potential immune modulatory molecule in the aged and Aβ-treated brain.

The data reveal that there are many activation states of microglia in the brain of aged rats; this activated microglia may interact with other immune cells like T cells, carry out phagocytic functions and/or secrete immunomodulatory molecules like pro-inflammatory cytokines. It was demonstrated that IFN-γ acts as a potent activator of microglia, and that Natural Killer (NK) cells, peripheral immune cells that are a significant source of IFN-γ, may infiltrate the CNS via an age-compromised blood-brain barrier and thus provide the brain with a source of microglia-activating IFN-γ. It was also demonstrated that HMGB1 can induce an activated microglial profile in vitro, although no age-related changes in HMGB1 were identified.

The findings from this study also indicate there are parallel age-related increases in Nogo-B and the activities of caspases -8 and -3, two markers of deterioration in neuronal function, and that these changes were accompanied by a decrease in the synaptic vesicle protein, synaptophysin, which is a marker of synaptic contacts. Moreover, this study demonstrates that Nogo-B enhances the activation of caspases -8 and -3 in a dose-dependent manner and is capable of inducing the expression of markers of microglial activation.

Due to the association of neuroinflammatory age-related and Aβ-induced changes with Nogo-B expression, the possible interaction of Aβ and Nogo-A was investigated in terms of microglial activation and neuronal deterioration. The evidence reveals a complex interaction between Aβ and Nogo given that Nogo-A and Nogo-B exacerbate the effects of Aβ on neurons at low doses, but attenuate those effects at a higher dose. Interestingly, the presence of Aβ did not modulate the effect of Nogo-A or
Nogo-B on microglial activation. Further research is required to elucidate the role of the Nogo protein in Aβ-induced changes.

In the last few years the concept that microglia exist in either a resting or activated state has been challenged. The data presented here supports the emerging evidence that several activated states are likely to exist. Several factors can modulate microglial activation and the present findings suggest a likely source of the archetypical activator IFN-γ is infiltrating NK cells. Importantly a key finding is that the microenvironment significantly affects microglial activation and this is typified by the demonstrated dose-dependent interaction between Aβ and Nogo.
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VII List of Abbreviations

The following abbreviations have been used:

Aβ  amyloid-beta
AD  Alzheimer's disease
ALS amyotrophic lateral sclerosis
ANOVA analysis of variance
AP  alkaline-phosphatase
APP amyloid precursor protein
BACE1 beta-site APP cleaving enzyme-1
BBB blood brain barrier
BCA bicinchoninic acid
BSA bovine serum albumin
C-terminal/terminus carboxyl-terminal/terminus
CD  cluster of differentiation
cDNA complementary DNA
CNS central nervous system
COS cells simian CV-1 cells carrying SV40 virus gene
CSPG chondroitin sulphate proteoglycans
°C degrees Celsius
DAMPS damage-associated molecular patterns
DC  Dulbecco's modified eagle medium
DISC death-inducing signalling complex
DMSO dimethylsulphoxide
DNA deoxyribonucleic acid
DNase deoxyribonuclease
dNTP deoxyribonucleotide triphosphate
EAE experimental autoimmune encephalomyelitis
ECL enhanced chemiluminescence
ELISA enzyme-linked immunosorbent assay
ER  endoplasmic reticulum
EST(s) expressed sequence tag(s)
EtBr  ethidium bromide
EtOH  ethanol
FACS  fluorescence-activated cell sorting
FBS  fetal bovine serum
g  gram(s)
x g  times G-force
GM-CSF  granulocyte macrophage colony stimulating factor
GPI  glycosylphosphatidylinositol
GST  glutathione s-transferase
h  hour(s)
HRP  horseradish peroxidase
HGNC  HUGO nomenclature committee
HMGB1  High Motility Group Box 1
HUGO  Human Genome Organisation
ICAM-1  intracellular adhesion molecule-1
icv  intracerebroventricular
Ig  immunoglobulin
IFN-γ  Interferon-γ
IL-1β  Interleukin-1-beta
IL-2  Interleukin-2
IL-6  Interleukin-6
IL-12  Interleukin-12
IL-15  Interleukin-15
IL-18  Interleukin-18
IL-23  Interleukin-23
iNOS  inducible nitric oxide synthase
ip  intraperitoneal
JAK  Janus kinase
JNK  c-jun N-terminal kinase
kDa  kilo-Daltons
kg  kilograms
LAMP(s)  lysosomal-associated membrane protein(s)
LDS  lithium dodecyl sulphate
LLT1  lectin-like transcript 1
<table>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LRR</td>
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<td>LRRCT</td>
<td>LRR-type C-terminal flanking domain</td>
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<td>NSF</td>
<td>N-ethylmaleimide sensitive factor</td>
</tr>
<tr>
<td>N-terminal/terminus</td>
<td>amino-terminal/terminus</td>
</tr>
<tr>
<td>OMgp</td>
<td>oligodendrocyte-myelin glycoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OPC(s)</td>
<td>oligodendrocyte precursor cell(s)</td>
</tr>
<tr>
<td>PAMPS</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>pg</td>
<td>picogram(s)</td>
</tr>
<tr>
<td>pmol</td>
<td>picomole(s)</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>QPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>sec</td>
<td>second(s)</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute-1640 medium</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RTN</td>
<td>reticulon</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>sAPP</td>
<td>soluble APP</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble NSF attachment protein receptors</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Strep-HRP</td>
<td>HRP-conjugated streptavidin</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate/ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>TBS-Tween</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th cell</td>
<td>helper T cell</td>
</tr>
<tr>
<td>Th1 cell</td>
<td>Type 1 Th cell</td>
</tr>
<tr>
<td>TLR(s)</td>
<td>toll-like receptor(s)</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethyl benzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>-----</td>
<td>-------------------</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1 The Brain

1.1.1 Blood Brain Barrier

The brain is a highly complex organ and the most important of the body. It is the centre for controlling all the other organs of the body in their function. Because of its importance, its protection from infectious agents and lethal substances is paramount, and therefore the brain is treated as a privileged organ in comparison to other organs of the body; this protective advantage is derived from the blood brain barrier (BBB).

The BBB maintains a stable environment for brain cells to function effectively without the risk of invading pathogens interfering with their normal ability. It is composed of endothelial cells that limit the passive diffusion of substances across vessel walls. This anatomic function gives the BBB a selective control so only the metabolites necessary for brain growth and function are transported across the barrier. The BBB has a relative deficiency in vesicular transport capabilities. Instead (1) diffusion of lipid-soluble substances, (2) facilitative and energy-dependent receptor-mediated transport of specific water-soluble substances and (3) ion channels are the means employed by the BBB to enable entry of molecules, such as energy substrates, essential amino acids and peptides from the blood to the brain and, at the same time, remove unwanted metabolites from the brain.

Endothelial cells are interconnected by a complex array of tight junctions that block diffusion across the vessel wall. However, these junctions normally have a low resistance (5-10Ω/cm²) but, in the BBB, the resistance is very high (2000Ω/cm²) and small molecules, such as K⁺ ions, are excluded from passing across the barrier. Problems can arise though when this resistance is reduced, which can be caused from a “leaky” BBB. Various pathological conditions and diseases, such as Alzheimer’s disease (AD), Multiple Sclerosis (MS), Parkinson’s disease (PD) and malignant brain cancer, are associated with altered BBB function. Certain inflammatory responses, such as the release of pro-inflammatory cytokines (see section 1.8), can cause a partial breakdown of the BBB, therefore increasing the risk of infection and damaging foreign materials invading into the brain. It is widely known that neuroinflammation involves important roles of different types of glia in the brain.
1.2 Glia in the CNS

1.2.1 Microglia

Microglial cells are the brain’s main resident immunocompetent cells (Benveniste, 1997; Farber & Kettenmann, 2005). Their functions include antigen presentation, phagocytosis and chemotaxis (Szczepanik et al., 2001). They make up 10-20% of all cells in the central nervous system (CNS) and are predominantly found in grey matter (Lawson et al., 1990; Lawson et al., 1992; Kim & de Vellis, 2005).

Microglia were first described by Franz Nissel in 1899 but it was in 1927 when they were first defined as a specific class of brain cell by neuroanatomist Pío del Río-Hortega using his silver carbonate staining method. Microglia were characterised as having branched processes on their surfaces. However, subsequent work by Río-Hortega described microglia as having a resting ramified (quiescent) morphology but with the ability to transform into amoeboid-like macrophages (Río-Hortega, 1932).

Their precise origins are relatively unknown, however one theory is that microglia are derived from early embryonic macrophage-like mesodermal precursor cells, which enter the brain from the periphery during embryonic development upon where they differentiate into microglia (Chan et al., 2007). Alternatively, some speculate that microglia arise from circulating blood monocytes, which invade the CNS during embryonic, fetal or perinatal stages (Kim & de Vellis, 2005).

Over time it was established that microglia respond quickly to their microenvironment and display various functional capabilities such as migration, phagocytosis and antigen presentation (Laurenzi et al., 2001). In line with this range of functions, microglia have four classification types based on their activation status: a quiescent state, an amoeboid phagocytic state, an activated non-phagocytic state and an activated phagocytic state (Sasaki et al., 1993; Kreutzberg, 1996). However it has become clear that this may be an oversimplification with many activated states likely (Perry et al., 2007). It is now known that when the CNS is challenged by an inflammatory insult or infection, microglia transform from their resting state to an activated status (Streit, 2000, 2002).

Microglia govern the inflammatory response in the CNS and, via the actions of released inflammatory molecules and expressed surface markers, they can arrange for
the infiltration of peripheral immune cells into the CNS; allowing them to contribute to the inflammatory response (Kato et al., 1996).

1.2.2 Astrocytes

In addition to microglia, astrocytes too can coordinate an innate immune response in the CNS. They have a star-shaped appearance visualised by the presence of several branched processes, which radiate in all directions from the cell body.

Astrocytes originate from the neuroectoderm, which makes up the neuroepithelial cells of the neural tube (Morest & Silver, 2003). They are typified by intermediate filaments called glial filaments, which largely consist of polymers of glial fibrillary acidic protein. Based on distribution and morphology, there have been two main classes of astrocytes identified: fibrous astrocytes and protoplasmic astrocytes. The former has many glial filaments that extend long, unbranching, cylindrical processes through nerve fibres and are generally localised to white matter. Protoplasmic astrocytes have less glial filaments and they extend short, thick, branched sheet-like processes through nerve cells. They can be found mainly in grey matter (Miller & Raff, 1984).

Astrocytes have been shown to have several functions. They help provide support for microglia, neurons and the vascular endothelium; their processes have a ubiquitous distribution throughout the parenchyma in the CNS reflecting their importance in granting support for the neural network. Astrocytes also contribute to the maintenance of the BBB, where many of their processes end in expansions ('perivascular feet') mostly covering the external surface of blood capillaries (Hirano & Llena, 2006).

Astrocytes also serve as a form of electrical insulation by separating axon terminals from other nerve cells; hence preventing axon terminals from inappropriately influencing neighbouring neurons (Temburni & Jacob, 2001). Astrocytes also have roles in glycogen storage, neuronal development, ion homeostasis, neurotransmitter re-uptake, phagocytosis of degenerating synaptic axon terminals and neurons, and maintaining the functional integrity of synapses (Ridet et al., 1997; Temburni & Jacob, 2001).
It has been shown that astrocytes become activated during several CNS pathologies such as stroke, trauma, tumour growth and neurodegenerative disease; a process called ‘reactive gliosis’ (Pekny & Nilsson, 2005). During reactive gliosis, astrocytes have been reported to upregulate the expression of cell surface proteins, while they present antigen to T cells, and secrete pro-inflammatory cytokines, chemokines and adhesion molecules that can modulate the immune response (Frohman et al., 1989; Lieberman et al., 1989; Gourmala et al., 1997; Pekny & Nilsson, 2005). Astrocytes have also been found to produce protective factors and secrete growth factors and neurotrophins (Lieberman et al., 1989; Rudge et al., 1992).

1.2.3 Oligodendrocytes

In the CNS, oligodendrocytes form myelin, the tight wrappings of multilamellar insulating membrane layers that ensheath nerve cell axons, which serves as electrical insulation that speeds up the propagation of action potentials down the long nerve cell process.

The origins of oligodendrocytes are thought by some to be closely linked with astrocytes in a process termed gliogenesis that occurs during late embryonic development and continues into postnatal stages. Gliogenesis generates both astrocytes and oligodendrocytes via a shared common glial progenitor (Mayer-Proschel et al., 1997; Rao & Mayer-Proschel, 1997; Rao et al., 1998).

Another hypothesis suggests that oligodendrocytes either originate independently or are more closely related to motor neurons and that these cells derive from motor neuron/oligodendrocyte precursor cells (Briscoe et al., 2000; Richardson et al., 2000; Stiles, 2003). Oligodendrocyte precursor cells (OPCs) arise from the neuroepithelium of the ventricular/subventricular zone of the brain and migrate from this region into the developing white matter, at which point they exit the cell cycle, become non-migratory and differentiate into myelin-forming oligodendrocytes (Baumann & Pham-Dinh, 2001).

Because oligodendrocytes form myelin, and thus serve to promote the conduction velocity of nerve signals along a neuronal axon, oligodendrocyte dysfunction has consequently been implicated in several demyelinating diseases such as MS.
1.3 Ageing

1.3.1 Introduction

Until the middle of the last century, the ageing process was considered to be governed by unknown laws. It was in 1956 that Harmon first put forward a proper theory of ageing (Harman, 1956). He stated that ageing was the result of an accumulation of endogenous oxygen free-radicals generated in cells by aerobic metabolism, which causes damage to DNA, protein and lipids. This theory was termed the ‘free-radical theory’ of ageing. The theory incorporates the two main important aspects of the ageing process: the characteristic progressive decline in biological function over time and the decreased resistance to various forms of stress and disease. Studies have since shown that ageing is associated with increased generation of oxidative species and a decline in the vigor of defense and repair, which leads to oxidative damage (Cutler, 1991; Bowling & Beal, 1995; Beckman & Ames, 1998).

Over the years there have been several other theories of ageing, including ones involving antagonistic pleitrophy, genetic mutation accumulation (Bowles, 2000) and mitochondrial dysfunction as a critical control component of ageing (Hamilton et al., 2001; Hagen, 2003; Wallace, 2005). Additionally a ‘nitric oxide theory’ exists, which states that the accumulation of nitric oxide (NO) in the brain results in ageing as a consequence (McCann et al., 2005). Another theory, the ‘multiple hormone deficiency theory’, proposes that the underlying cause of ageing is a chaotic endocrine system (Hertoghe, 2005).

However there is a theory that encompasses many of the above-mentioned theories into the one, the ‘molecular inflammatory theory of ageing’. This states that the activation of redox sensitive transcriptional factors by age-related oxidative stress causes an upregulation of pro-inflammatory responses (Kregel & Zhang, 2007).

Fully understanding ageing has always been confounded by the fact that it can be hard to dissociate the effects of normal ageing from those of age-related disease.
1.3.2 Ageing and Neuroinflammation

There is much evidence supporting the belief that changes in the immune system that lead to increased inflammation or reduction in immune function are tied to ageing (Renshaw et al., 2002; Licastro et al., 2005; Nikolich-Zugich, 2005). It has been shown that the ability of macrophages, innate immune cells, to phagocytose is altered with age (Plowden et al., 2004). Increased expression of pro-inflammatory cytokine production and a downregulation of toll-like receptors (TLRs) are associated with age (Renshaw et al., 2002; Licastro et al., 2005; Johnson, 2006).

Much work supports the idea that it is the hippocampus that is mostly affected by ageing in the brain. And so ageing is accompanied by deficiencies in learning and memory (Driscoll et al., 2003). Some studies have reported deficiency in long term potentiation (LTP), the cellular model of learning and memory, in the hippocampus with age and this is associated with inflammatory changes (Lynch, 1998; McGahon et al., 1999; Miller & O’Callaghan, 2005; Griffin et al., 2006). Behaviour tests in ageing animals show age-related impairments (Liu et al., 2005), and, based on mounting evidence, it has been suggested that impairments in LTP and behaviour are a consequence of increased inflammatory changes in the hippocampus (Lynch & Lynch, 2002; Maher et al., 2005; Griffin et al., 2006). Therefore with all this evidence connecting age to inflammation, it is not surprising to learn that age-related inflammation is considered to be linked to an increased susceptibility in the onset of neurodegenerative diseases, such as AD (Heininger, 2000; Streit, 2004; Licastro et al., 2005).

1.4 Alzheimer’s disease

1.4.1 Introduction

Alois Alzheimer first described what we know as AD in 1906, having discovered lesions in the brain of a patient with dementia (Alzheimer et al., 1995). Today AD is the most common progressive age-related neurodegenerative disorder, with 20-30 million individuals around the world affected with the disease (Selkoe,
Onset generally occurs late in life establishing age as a major risk factor for the disease.

It is typified by a progressive loss of cognitive memory function and dementia that can span a period of 5-20 years (Streit, 2004; Griffin et al., 2006; Simard et al., 2006). Clinical symptoms include impairment in memory, motor skills and language, personality changes, increased anxiety, and the deterioration of other higher cognitive functions. Despite the employment of various neurological diagnostic testing, brain pathology remains the only definite diagnosis of AD.

AD is characterised primarily by extracellular amyloid-β (Aβ) deposits known as senile plaques, intracellular neurofibrillary tangles that consist of hyperphosphorylated tau protein and a profound loss of basal forebrain cholinergic neurons that innervate the hippocampus and the neocortex (Selkoe, 2003). The presence of Aβ plaques and neurofibrillary tangles are widely distributed in the normal aged brain (Heininger, 2000), however it is their overexpression that has been associated with AD.

1.4.2 AD and Neuroinflammation

Acetylcholinesterase inhibitors (Doody, 1999) and N-methyl-D-asparate antagonists (Farlow, 2005) are currently the primary forms of treatment for the disease. However these drugs only serve to lessen the extent to which the symptoms will become evident. It has been noted that, when taken over long periods for conditions, such as rheumatoid arthritis, the use of non-steroidal anti-inflammatory drugs (NSAIDS) reduce the risk of developing AD (Breitner et al., 1995) and delay the onset of the disease (Rich et al., 1995), and consequently several clinical trials have been undertaken to assess their effectiveness in established disease. There is little evidence that this treatment strategy is effective, at least in late disease.

There is no present treatment whereby the disease can be regressed or halted from further progression. It is apparent that further understanding of the disease and the processes involved is required before a long-term preventive treatment can be established.

Studies show that inflammation plays an active role in the development and progression of AD, and is not just a consequence of the disease itself (Szczechanik et al.,
2001). The presence of activated microglia and astrocytes has been shown to surround neuritic plaques in AD (Duffy et al., 1980; McGeer et al., 1987; Kim & de Vellis, 2005; Rogers et al., 2007), and in addition, an upregulation of cytokines in amyloid plaques has been reported (Bauer et al., 1991; Cacabelos et al., 1994). Moreover activated microglia have been shown to release neurotoxic factors and pro-inflammatory cytokines that can result in neuronal damage (McGeer & McGeer, 2003). In fact it has been suggested that these inflammatory molecules establish a self-perpetuating cycle which aids plaque formation and promotes the progression of AD (Griffin et al., 1998). Recent in vivo and in vitro studies support this hypothesis, whereby Aβ-treated rats and glial cultures resulted in the production of pro-inflammatory cytokines (Minogue et al., 2003; Lyons et al., 2007b).

1.4.3 Amyloid Cascade Hypothesis

It is believed by many that the onset of AD is a combination of the effects of neuroinflammation and the amyloid cascade (Allan & Rothwell, 2001; Sastre et al., 2006; Wyss-Coray, 2006). The Amyloid Cascade Hypothesis states that the build-up of Aβ peptide is the predominant factor driving AD pathogenesis (Masters et al., 1985; Hardy & Selkoe, 2002).

The senile plaques consist of accumulated aggregated hydrophobic Aβ (Glenner & Wong, 1984; Masters et al., 1985), which is a 4kDa protein that is derived from the proteolytic process of amyloid precursor protein (APP), a large type I membrane-spanning glycoprotein (Hardy & Selkoe, 2002) of 770 residues. APP appears critical for normal neuronal function (Tomita et al., 1998; Yan et al., 2003) and research has indicated roles for APP fragments in axonal transport (Kamal et al., 2000), cell adhesion, cell survival, cholesterol metabolism, gene transcription and cognitive processes (Turner et al., 2003). Aβ formation, and its accumulation over time, in neurons has been referred to as the key event in the pathogenesis of AD, with in vitro studies showing that Aβ peptides are toxic to neurons (see Walsh & Selkoe, 2004).
Figure 1.1. Amyloid Precursor Protein Proteolysis.

Aβ originates from a series of cleavages of APP (Figure 1). APP proteolysis is catalysed by first either β-secretase (BACE1; beta-site APP cleaving enzyme-1) or α-secretase, followed by γ-secretase. The α- and BACE1 cleavages seem to be mutually exclusive events, with each liberating a large extracellular domain of the APP protein, which differ by only 17 amino acids at the carboxyl (C)-terminus. The remaining C-terminal fragment of APP is attached to the membrane and is referred to as either C83 when cleaved by α-secretase or C99 by BACE1, reflecting the number of amino acids from the intracellular C-terminal where cleavage occurs. Following α- and BACE1 cleavage, γ-secretase either cleaves C99 or C83 to generate the principal Aβ fragments or a shortened Aβ-like fragment known as p3, respectively. Two different types of Aβ, Aβ1-40 or Aβ1-42, are formed depending on whether γ-secretase cleaves between residues 40 and 41 or between 42 and 43. Following both types of sequential proteolysis, a degradation product, soluble APP (sAPPα or sAPPβ), is also formed.

Normally both forms of the Aβ peptide are present in a healthy individual, but there is significantly more Aβ1-40 than Aβ1-42. Compared with Aβ1-40, Aβ1-42 is deposited earlier in AD pathogenesis (Saido et al., 1995) and it has been shown in vitro that Aβ1-42 assembles into filaments much more quickly (Jarrett et al., 1993). Mutations in APP can lead to an increase in the BACE1 and γ-secretase proteolytic pathway, by altering the site preference of γ-secretase. This causes an overexpression in the production of Aβ1-42.
which has been shown to lead to AD pathogenesis (Nonomura et al., 1996; Bogdanovic et al., 2002; Pasalar et al., 2002; Cruts et al., 2003).

1.5 Neuronal Function and Neurodegeneration

1.5.1 Neurons

Neurons are the basic structural and functional units of the nervous system and in the CNS, they are found in grey matter. They consist of dendrites, a cell body, and an axon ensheathed by myelin, and an axon terminus; although there are variations related to their function. The functions of neurons include receiving electrical signals called action potentials, integrating these action potentials, and transmitting these signals to other neurons or effector organs, such as muscles and glands.

Neurons differentiate from neuroepithelial precursor cells (McKay, 1988), but it has also been shown that radial glia, a type of glial cell in the CNS, have the ability to serve as neuronal progenitors and as a scaffold upon which newborn neurons migrate (Mo et al., 2007). Some early studies highlighted cell-cell interaction as having an important role in defining neuron type once they were generated. The analysis stated that the specific cellular fate of newly differentiated neurons depends on the cells they interact with in their environment (Ready et al., 1976; Lawrence & Green, 1979).

Most neurons can be classified as one of three structural and functional neurons types; which type a neuron is depends upon the number of dendrites extending from the cell body. The majority of the neurons in the CNS are multipolar neurons which have several dendrites, and serve principally as motor neurons conducting impulses away from the CNS (efferent) to muscles and glands. Bipolar neurons can be found in the sense organs and have only two processes, a single dendrite and an axon on opposite ends of the cell body. Lastly, unipolar neurons, or sensory neurons, are devoid of any dendrites and have a single axon, conducting impulses to the CNS (afferent). However there are other unique neuronal types identified according to their location and shape. Interneurons are responsible for receiving, relaying, integrating, and sending nerve impulses between sensory neurons and motor neurons. They are found exclusively in the CNS and account for almost 99% of all the nerve cells in the body.
1.5.2 Function

The unique morphological and intracellular structure of the neuron is dedicated to the efficient and rapid transmission of neural signals. Within the neuron, the neuronal signal travels electrically. When a nerve impulse reaches the synaptic knob at the axon terminus, neuronal signals are conveyed chemically by a limited number of chemical molecules, known as neurotransmitters. These neurotransmitters are released into the synaptic gap, communicating with a dendrite or cell body of another neuron; the point of contact being a synapse. Specialised parts of the neuron facilitate the production, release, binding, and uptake of these neurotransmitters.

Neurons are supported by and interact with various glial cells of the CNS. For instance, it is known that an interaction with astrocytes is critical for signalling, energy metabolism, extracellular ion homeostasis, volume regulation, and neuroprotection in the CNS. Astrocytes also participate in the detection, propagation, and modulation of excitatory synaptic signals, provide metabolic support to active neurons, separate axon terminals from other nerve cells, and contribute to functional hyperaemia in active brain tissue (Temburni & Jacob, 2001; see Benarroch, 2005). Disturbances of these neuron-astrocyte interactions are likely to play an important role in neurologic disorders including cerebral ischemia, neurodegeneration, migraine, cerebral edema, and hepatic encephalopathy. Oligodendrocytes are also critical to the normal functioning of neurons. As producers of myelin in the CNS, they are responsible for ensheathing the axon and thus enable rapid propagation of neural signals along the axon.

1.5.3 Neuronal Cell Deterioration

There is a finite supply of neurons in the CNS and due to a lack of regenerative capabilities in the adult CNS any neuronal cells damaged or destroyed through injury, disease or ageing cannot be repaired or replaced. Research has shown that the onset of various neurodegenerative disorders, such as AD, PD, MS, has been associated with a significant loss of viable neurons in the CNS (Ruberg et al., 1997; Vogt et al., 2009; Yu et al., 2010). Dysfunction in various glial cell types can have an adverse effect on the normal functioning of neurons. For instance, astrocytic damage could result in a disruption in ion homeostasis. It has been shown that oligodendrocyte dysfunction has
been implicated in several demyelinating disorders, including MS. Neuronal damage can result in a loss of function and damages neurons may consequently undergo apoptosis, the process of programmed cell death. Several proteins are now accepted as markers of cell deterioration in neurons, some of which will be discussed in more detail in section 1.5.4. These include important components involved in the apoptotic pathway and a synaptic vesicle membrane protein which is important in normal synaptic transmission.

1.5.4 Indicators of Cell Death

1.5.4.1 Caspase-3

Caspase-3 is a key enzyme in the apoptotic pathway, and is a marker of deterioration of cell function and, in some cases, cell death in neurons. Caspases are divided into three group types depending on the prodomain structure of the inactive enzyme, and the enzyme function. Caspase-3 belongs to group III, which corresponds to the effector (or executioner) caspases (Lavrik et al., 2005). Caspase-3 consists of a short amino (N)-terminal prodomain (20-30 amino acids) followed by a large, p20 (20kDa), and a small, p10 (10kDa), subunit (Thornberry & Lazebnik, 1998).

All effector caspases are activated by initiator caspases (group II). Caspase-3 can be activated via intrinsic or extrinsic pathways (Scaffidi et al., 1998); which pathway occurs is determined by the levels of the death-inducing signalling complex (DISC). When there are low levels of DISC, there tends to be lower levels of active caspase-8, an initiator caspase, and so signalling requires an additional amplification loop. This scenario initiates the intrinsic pathway and it involves the cleavage of the Bcl-2 family protein Bid by caspase-8, which in turn generates tBid. This leads to a tBid-mediated release of cytochrome c from the mitochondria (Korsmeyer et al., 2000), that results in the activation of pro-caspase-9 (Jiang & Wang, 2000), another initiator caspase, via the formation of the apoptosome. Active caspase-9 cleaves the downstream effector caspase-3, which initiates the death cascade. This involves caspase-3 proteolytically cleaving a range of substrates, which leads to the dismantling of the dying cell (Fischer et al., 2003).
However when there are high levels of DISC and thus there are increased amounts of active caspase-8 available, the extrinsic pathway is initiated, whereby caspase-8 acts directly on caspase-3 to become activated.

1.5.4.2 Caspase-8

Caspase-8 is another marker of deterioration of cell function and, in some cases, cell death but it belongs to the second group of caspases (group II), which are characterised by their ability to initiate apoptosis (Lavrik et al., 2005). Caspase-8 is similar in structure to caspase-3 but it also contains two tandem death effector domains between its prodomain and subunits (Muzio et al., 1996; Sprick et al., 2002). These are responsible for the recruitment of initiator caspases into death- or inflammation-inducing signalling complexes (Lavrik et al., 2005).

As described above, caspase-8 is capable of directly or indirectly activating caspase-3 to commence apoptosis. Caspase-8 has also been implicated in the regulation of cell migration and adhesion. It was reported by Frisch and colleagues that the loss of caspase-8 leads to reduced cellular motility (Helfer et al., 2006).

1.5.4.3 Sphingomyelinase

In addition to the activation of Bid via direct enzymatic means, which utilizes caspase-8, Bid can also be activated by another pathway; a pathway involving the enzymatic activity of sphingomyelinase. One sequence of events, which involves sphingomyelinase and leads to cell death, is as follows.

The cytokine, tumour necrosis factor-α (TNF-α) binds to the p55 TNF receptor death domain (Schwandner et al., 1998), which results in rapid receptor internalisation (Schutze et al., 1999; Guicciardi et al., 2000). This leads to the activation of endosomal acidic sphingomyelinase. This can occur directly, or indirectly via TNF receptor-dependent activation of caspase-8 (Schotte et al., 1998; Heinrich et al., 2004). Sphingomyelinase subsequently induces production of ceramide (Perry & Hannun, 1998), a potent pro-apoptotic second messenger lipid, which activates and mediates the translocation of the aspartate protease cathepsin D from the lumen of the endosomal compartment into the cytosol (Heinrich et al., 1999; Heinrich et al., 2004). Once in the
cytoplasm cathepsin D activates tBid by proteolysis of Bid, resulting in mitochondrial apoptosis signalling (Heinrich et al., 2004).

Due to the importance of sphingomyelinase in this type of activation pathway, it shall be used in this study as a potential indicator of cell death.

1.5.4.4 Synaptophysin

Synaptophysin is a 38kDa major integral membrane protein of synaptic vesicles, constituting ~7% of total vesicle protein and containing four TM regions (Jahn et al., 1985; Wiedenmann & Franke, 1985; Leube et al., 1987; Sudhof et al., 1987). It is found in practically all neurons in the CNS (McMahon et al., 1996).

Based on several studies, it has been reported that synaptophysin is required for exocytosis in neuronal synapses (Alder et al., 1992a; Alder et al., 1992b; Shibaguchi et al., 2000), however a study by McMahon and colleagues demonstrated that synaptophysin is not essential for synaptic transmission (McMahon et al., 1996). Another study suggested that synaptophysin is not absolutely required but may serve as a positive modulator of exocytosis (Mitter et al., 2003).

Synaptophysin forms a high molecular weight complex in the vesicle membrane consisting of four synaptophysin subunits and synaptobrevin, a low molecular protein of 18kDa (Johnston & Sudhof, 1990; Calakos & Scheller, 1994; Edelmann et al., 1995). Synaptobrevin also forms another complex, the SNARE (soluble NSF (N-ethylmaleimide sensitive factor) attachment protein receptors) complex (Edelmann et al., 1995), with two other synaptic proteins, SNAP-25 (soluble synaptosomal-associated protein of 25 kDa) and syntaxin (Rizo & Sudhof, 2002), and this SNARE complex has been shown to be responsible for exocytosis in synaptic vesicles (Sutton et al., 1998; Jahn & Sudhof, 1999; Lin & Scheller, 2000). A suggested function of the synaptophysin-synaptobrevin complex is to enable synaptophysin to control the availability of synaptobrevin to enter the SNARE complex, subsequently impacting on neuronal transmission (Edelmann et al., 1995; Mitter et al., 2003). Additionally another research group also identified synaptophysin as having a potential role in regulating activity-dependent synapse formation (Tarsa & Goda, 2002).

Due to its ubiquitous expression in axon terminals, synaptophysin is now accepted as a marker for synaptic density in neurons, and is used in assessing synaptogenesis (Masliah & Terry, 1993; Thiel, 1993; Eastwood et al., 1995; Eastwood...
& Harrison, 1995; Calhoun et al., 1996; Sarnat & Born, 1999; Tarsa & Goda, 2002; Valtorta et al., 2004). It has been reported that synaptophysin is decreased in hippocampus of aged animals suggesting a reduction in the abundance of synaptic terminals (Davies et al., 2003), and, in this study, it will be used as a marker of synaptic loss.

In addition to synaptic vesicle membranes, synaptophysin can also be found in spinal cord, retina, the vesicles of adrenal medulla, neuromuscular junctions and endocrine cells (Wiedenmann et al., 1986; Obendorf et al., 1988; Valtorta et al., 1988; Calhoun et al., 1996; Zhang & Rosenblum, 1996; Nag & Wadhwa, 2001). It is also expressed by neuroendocrine cells throughout the body, both normal and neoplastic, and is considered a marker in the diagnosis and prognosis of neuroendocrine pulmonary tumours (Wiedenmann et al., 1986; Stridsberg, 1995; Kasprzak et al., 2007).

1.5.5 Post-Axonal Injury

1.5.5.1 CNS versus PNS

"Once development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated"

Santiago Ramón y Cajal, 1928.

Axons are generally believed to be incapable of regeneration in the adult CNS and originally it was thought that this inability of mature axons to regenerate was due to a loss of an intrinsic regenerative capacity in adult neurons (Ramón y Cajal, 1928; Schwab & Bartholdi, 1996). In the CNS, as neuronal networks form in the mammalian developing nervous system, axons progressively cease to grow. Lesions that occur at or around the perinatal period can trigger some degree of regeneration, while the majority of lesioned axons in a postnatal organism are not repaired, which can result in devastating and irreversible functional deficits. In the early 20th century observations by Tello (1911) suggested that this incapacity to regenerate possibly involved the adult CNS environment (Tello, 1911; Ramón y Cajal, 1928). Tello demonstrated that the inability of lesioned adult CNS neurons to extend axonal processes could be overcome
by introducing the permissive environment of a peripheral nerve, given that axons of the peripheral nervous system (PNS) are capable of regeneration in the adult.

Many decades later, this influential work was replicated by Aguayo and collaborators using novel techniques showing that CNS neurons can form long growth projections through peripheral nerve grafts (Richardson et al., 1980; David & Aguayo, 1981; Benfey & Aguayo, 1982; Richardson et al., 1984). It was also observed that only a few fibres grew out from the peripheral grafts, and their growth soon stopped after re-entering the spinal cord; demonstrating that the failure to regenerate is not a deficit of CNS neurons, but a case of blockage by the CNS environment. In 2000, Woolf and colleagues also showed that replacing the CNS environment with peripheral nerve grafts, Schwann cells or olfactory ensheathing glia encourages axonal growth (Behar et al., 2000).

1.5.5.2 Inhibitors of Axonal Regeneration

It has now been established that central myelin, produced by oligodendrocytes, is a powerful suppressor of axonal growth, a concept first proposed by Berry (Berry, 1982). In a study by Schwab and Thoenen, axons of cultured neurons were shown to not grow into explants of adult CNS tissue despite the administration of nerve growth factor (NGF); however they did grow in peripheral nerve explants containing myelin (Schwab & Thoenen, 1985). They showed that when neurons are grown on or exposed to central myelin extracts, neurite outgrowth is significantly reduced and growth cones collapse occurs. In addition, other researchers have further demonstrated that it is CNS myelin, and not PNS myelin (derived from Schwann cells), that possesses neurite inhibitory activity (Carbonetto et al., 1987; Crutcher, 1989; Savio & Schwab, 1989; Khan et al., 1990; Sagot et al., 1991). Experiments involving co-cultures of dissociated glial cells and neurons have shown that mature oligodendrocytes were strictly avoided by neurons and any growth cones that came into contact with oligodendrocytes subsequently collapsed and retracted (Caroni & Schwab, 1988a; Bandtlow et al., 1990; Moorman, 1996).

Two main sites have been considered as the source of the CNS inhibitory factors that limit axon regeneration. One site is at the fibrous scar that forms at the central area of necrosis following CNS injury. This area is infiltrated by glial cells (primarily astrocytes) and other non-neuronal cells. In most instances, it has been reported that
regenerating nerve fibres either bypass or stop at the neuroglial scar that arises from the lesioned tissue, which appears to act as a mechanical barrier to axons. Several studies have demonstrated that the glial scar contains components that can inhibit axon growth, including the glycoprotein tenascin-C (TEN-C), semaphorin-3A and chondroitin sulphate proteoglycans (CSPG) (Letourneau et al., 1994; Davies et al., 1999; Pasterkamp et al., 1999; Probstmeier et al., 2000; Asher et al., 2001; Morgenstern et al., 2002; Sandvig et al., 2004). The expression of several different CSPGs is increased following CNS injury and their removal from lesion sites has been shown to improve axon regeneration and functional recovery in various lesion models.

In addition to the glial-scar inhibitors, CNS myelin represents another major source of inhibitors of axon regeneration. After axonal injury in the PNS, myelin is rapidly cleared by macrophages as the axons degenerate. However in the CNS, myelin is cleared a lot more slowly, which allows demyelinating axons and the proximal stumps of cut axons to become exposed to myelin-associated inhibitors (Filbin, 2003; Yiu & He, 2003; He & Koprivica, 2004). To date, three distinct inhibitory components of myelin have been identified: myelin-associated glycoprotein (MAG) (McKerracher et al., 1994), oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002b) and Nogo (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000).

1.5.6 Myelin-Associated Inhibitors

1.5.6.1 MAG

MAG is a transmembrane (TM) protein with five immunoglobulin-like domains in its extracellular domain that is expressed in both the PNS and CNS (Lai et al., 1987; Salzer et al., 1987). While attempting to identify myelin-associated inhibitors using fractionation by ion exchange chromatography, McKerracher and colleagues monitored myelin proteins via a neurite outgrowth assay and discovered MAG has high inhibitory activity (McKerracher et al., 1994). Further work by Filbin and colleagues showed that MAG is an inhibitory molecule for many types of mature neurons in vitro (Mukhopadhyay et al., 1994; DeBellard et al., 1996). Investigators established that MAG is bi-functional, whereby depending on the age of the neuron, MAG can either stimulate or inhibit neurite outgrowth in embryonic or postnatal neurons, respectively.
(Salzer et al., 1990; DeBellard et al., 1996; Turnley & Bartlett, 1998). Even though it has been shown that axon regeneration has improved in the PNS in MAG knockout mice, axon regeneration in the mature PNS occurs despite the presence of MAG. One explanation for this may be due to the rapid and more efficient clearance of myelin debris after an injury in the PNS, which is not the case in the CNS (David et al., 1990).

1.5.6.2 OMgp

OMgp was identified independently by two groups. When McKerracher and colleagues used chromatographic separation to identify myelin-associated inhibitors, two major peaks of inhibitory activity were recorded. The first of these resulted from the presence of MAG. Using PNA-agarose chromatography to further separate the second peak of inhibitory activity, OMgp was subsequently identified as an inhibitor (Kottis et al., 2002). OMgp was also identified separately by the He group during a search for glycosylphosphatidylinositol (GPI)-linked inhibitors in CNS myelin (Wang et al., 2002b). OMgp contains a leucine-rich repeat (LRR) domain and is a GPI-anchored protein (Mikol et al., 1990) that can be detected on the surface of oligodendrocytes and in myelin layers adjacent to axons (Mikol & Stefansson, 1988; Mikol et al., 1990; Habib et al., 1998). Its expression in oligodendrocytes correlates with the onset of myelination (Habib et al., 1998; Vourc'h et al., 2003). It has been shown that OMgp has potent growth cone collapsing and neurite outgrowth inhibitory activities in vitro (Wang et al., 2002b; Barton et al., 2003). Using OMgp knockout mice, it was demonstrated that a loss of OMgp promotes functional recovery through axon regeneration after spinal cord injury (SCI) in vivo (Ji et al., 2008a).

1.5.6.3 Nogo

Nogo was the long sought-after high molecular weight myelin-associated inhibitor that was first reported and characterised by three independent groups. Due to the relevance of Nogo to my studies, its discovery, characterisation, function and scientific significance will be examined in detail in section 1.6.
1.6 Nogo

1.6.1 History

Over 20 years ago, Martin Schwab and colleagues carried out sodium dodecyl sulphate polyacrylamide gel electrophoresis experiments designed to fractionate rat brain myelin proteins. Two membrane protein fractions, NI-35 and NI-250, containing peptides of molecular weight 35kDa and 250kDa, respectively, were identified to have nonpermissive substrate properties for neurite growth (Caroni & Schwab, 1988b). A monoclonal antibody, IN-1, was raised against NI-250 and bound to NI-250 and NI-35, blocking the inhibitory action of the two protein fractions and myelin (Caroni & Schwab, 1988a). Using methods of biochemical purification, the Schwab group purified the bovine homolog of NI-250 and were able to acquire the amino acid sequence for six peptides from NI-250 (Spillmann et al., 1998). This information was used to search databases of mammalian genes and expressed sequence tags (ESTs). This enabled Schwab's group (Chen et al., 2000) and two other research laboratory groups from the labs of Stephen Strittmatter and Frank Walsh (GrandPre et al., 2000; Prinjha et al., 2000) to successfully clone the full-length mammalian gene, incorporating all six homologous peptide sequences, which was designated Nogo.

1.6.2 Structure

Through alternate RNA splicing and promoter usage, three transcripts of the nogo (ASY) gene can be generated, which translate into three major isoforms of the Nogo protein. These are termed Nogo-A (1,162 amino acids; 250kDa), Nogo-B (373 amino acids; 55kDa) and Nogo-C (199 amino acids; 25kDa). The human nogo gene is localised to position 16.3 of the short arm of chromosome 2.

As seen in Figure 2, the 3' segments denote exons 4-9, and are shared by all three major isoforms. The 5' segment, exon 1A, is shared by Nogo-A and Nogo-B. Exons 2 and 3 are specific to Nogo-A, although they can be found on some minor Nogo transcripts (Hunt et al., 2002; Oertle et al., 2003a). Exon 1C is specific to Nogo-C.
Figure 1.2. The transcript structure of the three major isoforms of Nogo.

All three major products of nogo splicing share an isoform-shared sequence of 188 amino acids C-terminal reticulon homology domain (encoded by exons 4-9). This sequence has a detectable homology to a small family of reticulon (RTN1, RTN2 and RTN3) genes. Nogo is the fourth member of this family of genes, and has been assigned the alias ‘RTN4’ by the HUGO (Human Genome Organisation) nomenclature committee (HGNC). Expressed RTN proteins are predominantly found in the endoplasmic reticulum (ER) membranes (Chen et al., 2000; Hamada et al., 2002; Oertle & Schwab, 2003) and share a highly evolutionarily conserved 188 amino acid C-terminal domain that can be found in all eukaryotes (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Oertle et al., 2003b). The C-terminal tail contains a consensus sequence that may serve as an ER retention signal (van de Velde et al., 1994; Geisler et al., 1998).

All RTN proteins have two large hydrophilic domains (encoded by exon 4, and exons 6 and 7, respectively) near the C-terminus of the protein (encoded by exons 8 and 9, along with a 3’ untranslated region). These TM domains are 35 and 36 amino acids each in length and can span the membrane twice (Chen et al., 2000; GrandPre et al., 2000; Novak & Tallerico, 2006). Within the second TM domain exists a leucine-zipper-like motif, suggesting potential binding via leucine dimerisation. In contrast to the C-terminus, the amino (N)-terminal domain varies in its length for the different reticulons and shows less conservation (Di Scala et al., 2005). Most of the known RTN proteins have a relatively short N-terminal sequence, including Nogo-B and Nogo-C. However, the most recognised RTN, Nogo-A, has a very long N-terminal sequence mainly due to
the inclusion of exon 3 in its genetic sequence, as can be seen in Figure 3. All three Nogo isoforms share an extracellular 66 amino acid subdomain ('Nogo-66') that is located within a 94 amino acid connecting loop between the two large TM domains near the C-terminus (Chen et al., 2000; Prinjha et al., 2000; Fournier et al., 2001; Fournier et al., 2002b; GrandPre et al., 2002; Oertle et al., 2003c). Though early models of Nogo suggest that both its N- and C-terminus are cytoplasmic (GrandPre et al., 2000), subsequent work shows that at least a part of the N-terminus of Nogo is extracellular (Oertle et al., 2003c; Acevedo et al., 2004; Hu et al., 2005; Miao et al., 2006).

![Figure 1.3. The protein structure of Nogo-A, Nogo-B and Nogo-C.](image)

In terms of expression, Nogo-A is highly localized to oligodendrocytes in the adult CNS (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), and on some central and peripheral neurons; whereas it is not expressed in astrocytes or Schwann cells (Josephson et al., 2001; Huber et al., 2002; Wang et al., 2002c). It is found in the innermost and outermost loops of the myelin membrane (Huber et al., 2002), and its expression has also been reported in peripheral tissues such as heart and testis (Chen et al., 2000). Nogo-B expression has a ubiquitous pattern throughout the PNS and CNS. It is highly expressed in endothelial cells and in smooth muscle cells of intact blood vessels, and can also be found in skeletal muscle, heart, spleen and testis tissue (Josephson et al., 2001; Oertle et al., 2003a). Nogo-C has been mainly found to be expressed in various peripheral tissues, especially in skeletal muscle and including kidney, liver, heart and adipose tissue (Morris et al., 1999), but has also been reported in the brain. Protein and mRNA levels of all three isoforms have been detected in the
spinal cord, and cerebral cortex, hippocampus and cerebellum brain regions (Chen et al., 2000; Huber et al., 2002).

1.6.3 Nogo-66 Receptor

Using an alkaline-phosphatase (AP)-Nogo-66 fusion protein, an approach described by Flanagan and Cheng (Flanagan & Cheng, 2000), initial studies were carried out demonstrating the high-affinity of binding of the AP-Nogo-66 fusion protein to neurons. This binding protein was associated with growth cone collapse and inhibited neurite outgrowth. The fusion protein was then used to screen pools of a cDNA expression library transfected into COS (simian CV-1 cells carrying the genetic material of the SV40 virus) cells. This resulted in the identification of cDNA that conferred high-affinity binding to the fusion protein. The encoded protein was named Nogo-66 receptor (NgR) (Fournier et al., 2001). Strittmatter and colleagues also investigated whether Nogo-66 and NgR interact directly; the two were confirmed to form a protein complex when a myc-tagged NgR was tested for binding to a glutathione S-transferase (GST)-tagged Nogo-66 in cell extracts (Fournier et al., 2002a).

The cDNA sequence for NgR encodes a protein 443 amino acids in length, containing a LRR-type N-terminal domain translocation signal sequence, followed by eight LRR domains, a cysteine-rich LRR-type C-terminal flanking domain (LRRCT), a unique C-terminal region, and a GPI-anchorage site, as is illustrated in Figure 4 (Fournier et al., 2001; Barton et al., 2003; He et al., 2003). Fournier and colleagues also demonstrated that all of the NgR LRR domains are required to bind with Nogo (Fournier et al., 2002a), which has a leucine-zipper-like motif in its second TM domain.

![Figure 1.4](image)

Figure 1.4. The protein structure of the Nogo-66 Receptor.

NgR is expressed in the brain and principally on neurons. It has been shown to be expressed on macrophages (Fry et al., 2007) and on activated microglia/macrophages (unpublished; referred to in David, et al., 2008).
In addition to Nogo, NgR binds with the neurite growth inhibitory myelin proteins MAG and OMgp (Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002b). NgR forms a ternary receptor complex with the LRR TM protein LINGO-1 (Wang et al., 2002a; Wong et al., 2002; Mi et al., 2004) and either the neurotrophin receptor p75NTR or the orphan TNF receptor family member TAJ/TROY (Park et al., 2005; Shao et al., 2005).

1.6.4 Functions

The receptor complex formed by NgR and its co-receptors, LINGO-1 and either TROY or p75NTR, mediates GTPase Rho-A activation after the binding of NgR to one of its ligands, such as Nogo-A, the larger Nogo isoform. The activation of small GTPases, such as Rho, modulates cytoskeletal changes, and is therefore an important factor in determining shape and motility of a cell. It has been reported that the activation of Rho-A in axonal growth cones by the NgR complex leads to growth cone collapse and the prevention of neurite growth (Dubreuil et al., 2003). In 2008, López-Vales and colleagues (David et al., 2008) speculated that in SCI the ability of NgR to deter axon growth into a lesion area may, in part, be an unfortunate consequence of an otherwise beneficial role. It was discovered that microglia/macrophages express NgR; because of the pivotal role of these cells in the immune response it is possible that the marked expression of NgR on microglia/macrophages may modulate the inflammatory response and secondary damage after SCI. Microglia/macrophages are rapidly recruited to a lesion site upon injury and it was suggested that NgR-expressing cells are repulsed by normal myelin and their associated inhibitors, preventing infiltration of macrophages into adjacent undamaged areas, limiting the size of the lesion but also consequently inhibiting the growth of axons into the injured site (David et al., 2008).

Much research has been done on Nogo-A as a neurite growth inhibitor to date (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Kim et al., 2003; Simonen et al., 2003). Three distinct regions of Nogo-A have been reported to be associated with its ability to inhibit axon growth (Fournier et al., 2001; Oertle et al., 2003c); one of these is the Nogo-66 loop found between its two TM domains. It has also been demonstrated that Nogo-A possesses a segment in its N-terminus, which has a high affinity for NgR binding (Hu et al., 2005).
In 2004, it was reported that the N-terminus of Nogo-B (which is also shared by Nogo-A) is associated with a vascular remodeling function upon injury. Nogo-B promotes the migration of endothelial cells but inhibits that of vascular smooth muscle cells (Acevedo et al., 2004). A few years later it was discovered that a receptor for this region of Nogo-B was identified on endothelial cells (Miao et al., 2006).

Some findings have suggested other more cell-autonomous functions of Nogo (see Teng & Tang, 2008). For instance, Rapoport and colleagues reported that Nogo-A may have an important, although non-essential, role in modulating the shape and organization of the ER tubular network (Voeltz et al., 2006). Also, a role in membrane trafficking has been suggested, where it has been reported that Nogo-B and Nogo-C may negatively modulate BACE1-mediated Aβ production, possibly by altering the trafficking itinerary of BACE1 (Nakajima et al., 2004; He et al., 2006; Murayama et al., 2006; Wojcik et al., 2007).

Nogo-B has also been implicated in apoptosis, particularly in some cancer cell lines. The over-expression of its gene (dubbed ASY) appeared to amplify apoptosis in cancer cell lines compared to non-tumourigenic cell lines (Li et al., 2001). Previous to this finding, it was shown that Nogo-B (dubbed RTN-xs), along with RTN1-C, binds to the anti-apoptotic proteins, Bcl-2 and Bcl-XL, and in doing so diminishes their anti-apoptotic activity; possibly by sequestering these proteins away from the mitochondria (Tagami et al., 2000). Additionally, Qi and colleagues showed that the over-expression of Nogo-B induces an ER stress response that was accompanied with resulting apoptosis (Kuang et al., 2005; Kuang et al., 2006). It has also been shown that the over-expression of Nogo-C induces HEK293 cell apoptosis through a mechanism involving the JNK-c-Jun pathway (Chen et al., 2006b).

However, based on some indirect evidence, it has been speculated that at least in the case of Nogo-A, a moderate and sustained expression of Nogo may serve to be neuroprotective in function by preconditioning neurons and glia against ER stress and increasing their resistance to apoptotic insults during CNS injury (see Teng & Tang, 2008).
1.6.5 Scientific Relevance

There is significant literature supporting the crucial role of myelin-inhibitors in both limiting plasticity in the healthy brain and in preventing CNS regeneration during injury (Caroni & Schwab, 1988a). Nogo has been of interest in the last decade due to its involvement in the inhibition of neurite outgrowth in damaged neurons and its involvement in various neurodegenerative disorders and injury, including SCI, AD, MS, amyotrophic lateral sclerosis (ALS), epilepsy and stroke.

1.7 Microglial Activation

1.7.1 Introduction

Under normal conditions, resting ramified microglia are found ubiquitously throughout the CNS, surveying their environment for any evidence of physiological disturbances (Chan et al., 2007). Their cell body remains motionless but their long branching processes constantly move and monitor the surrounding area. This allows for the detection and initiation of a microglial-driven immune response in local areas of the CNS, all the while preserving an environment of immunological serenity.

Resting microglia are sensitive to any changes in their environment and infection or injury, their soma enlarges and processes shorten, assuming an amoeboid-like state. This allows for free movement around the CNS and these amoeboid microglia migrate to the damaged sites where they proliferate (activated non-phagocytic state) in preparation for full activation. This process is called 'gliosis' (Stoll & Jander, 1999; Acarin et al., 2000; Benveniste et al., 2004). Activated non-phagocytic microglia are capable of presenting antigen and mediating inflammatory signalling, but are unable to carry out phagocytosis on damaged or foreign material (Kreutzberg, 1996). When the microglia are presented with degenerating tissue or foreign material, they enter a state of activation where they are phagocytic, retract their cellular processes, and exhibit an enlarged cell body, are amoeboid in morphology and are motile. They are capable of antigen presentation because of increased expression of cell surface proteins and the production of pro-inflammatory cytokines and neurotoxic factors including reactive oxygen and nitrogen species (Aloisi, 2001). Even though the expression of some of the
expressed surface markers is not wholly exclusive to microglia, documenting the expression of a number of these markers in tandem can be used as a means in identifying microglial activation; thus in this study, five markers of activation were used.

1.7.2 Cell-surface Markers of Inflammation

1.7.2.1 MHC II

Major histocompatibility complex class II (MHC II) is a cell surface antigen that is produced in the rough ER. It consists of two glycoproteins, a 35kDa α-chain and a 30kDa β-chain, each bearing a specific TM region and cytoplasmic tail. Their expression on microglia (Matsumoto & Fujiwara, 1986; Hickey & Kimura, 1988; McGeer et al., 1993) has been reported to be increased by interferon-gamma (IFN-γ; see section 1.8.2.2), a pro-inflammatory cytokine (Vass & Lassmann, 1990; Maher et al., 2006; Lyons et al., 2007b).

MHC II antigens expressed on activated cells have the ability, when in conjunction with co-stimulatory molecules (Chambers, 2001), to interact with other cells, particularly helper T cells (Th-cells), lymphocytes that play a central role in cell-mediated immunity (Watts, 1997; Sedgwick et al., 1998). An MHC II antigen is recognised by a T cell receptor (TCR) found on the surface of T cells and their interaction results in the activation of the T cell (Brown et al., 1993; Germain & Margulies, 1993; Cresswell, 1994; Germain, 1994; Bentley et al., 1995; Fields et al., 1995; Watts, 1997; Davis et al., 1998). It has been shown that prolonged loss in the interaction between MHC II and TCR leads to defects in T cell activation (Fischer et al., 2007). Work from a number of groups has revealed that Type I Th cells (Th1 cells) can also stimulate microglia to express MHC II and other inflammatory mediators, indicating positive feedback in MHC II signalling (Aloisi, 1999, 2001; Seguin et al., 2003).

There is some evidence that MHC II antigens on microglia modulates phagocytosis, which is supported by the finding that MHC II expression localises with CD68 (see section 1.7.4.1), a lysosomal marker of phagocytic activity during inflammation (Schmitt et al., 2000; Cho et al., 2006). Research has established that
MHC II is upregulated with age (Griffin et al., 2006) and in several CNS diseases (Streit et al., 1999).

1.7.2.2 CD11b

CD11b is principally expressed on microglia and monocytes, and it has been shown that its expression is considerably increased following cell activation (Streit et al., 1999; Rock et al., 2004). Along with CD18, CD11b forms the heterodimeric integrin complement receptor-3 (also known as macrophage-1 antigen; MAC-1), which is involved in several different functions of the inflammatory process, such as cell-mediated cytotoxicity, motility, cellular activation and chemotaxis (Weber et al., 1997; Nagai et al., 2005; Solovjov et al., 2005). It acts as a binding protein for intracellular cell adhesion molecule-1 (ICAM-1), a TM glycoprotein that mediates cell-cell interactions and thus allows for signal transduction (Schwarz et al., 2002).

Pahan and colleagues showed that NO is instrumental in increasing CD11b expression in microglia. Using different inducers of NO production, such as lipopolysaccharide (LPS) and IFN-γ, microglia were stimulated to express CD11b via NO (Roy et al., 2006). It has also been shown that CD11b expression is upregulated in response to LPS (Ji et al., 2008b) and Aβ (Jana et al., 2008). It was also reported that there was an age-related increase in CD11b mRNA expression in the mouse hippocampus (Sandhir et al., 2008) and the evidence suggests that MAC-1 plays an important role in myelin phagocytosis in MS and experimental autoimmune encephalomyelitis (EAE) (Smith, 2001; Reichert & Rotshenker, 2003; Rotshenker et al., 2008).

1.7.2.3 CD40

CD40 is a member of the TNF receptor family that has been shown to be expressed on immune cells, astrocytes and endothelial cells (Harnett, 2004; Chen et al., 2006a). Its expression is increased by LPS and the inflammatory cytokines IFN-γ and TNF-α (Nguyen & Benveniste, 2000; Tan et al., 2002; Qin et al., 2005), and IFN-γ-induced expression of CD40 has been shown to enhance the ability of microglia to activate and restimulate T cells (Benveniste et al., 2004).
The ligand for CD40 is CD154, a 39kDa type II TM protein of the TNF superfamily, which is expressed on activated CD4^+ T cells (Danese et al., 2004). The binding of CD40 with CD154 leads to the production of pro-inflammatory cytokines, such as TNF-α, chemokines and other co-stimulatory molecules like MHC II (Stout & Suttles, 1996; Chen et al., 2006a). The CD40-CD154 complex also initiates a signalling cascade that involves the activation of transcription factors, such as nuclear factor-kappa B (NF-κB), and kinases like mitogen-activated protein (MAP) kinases (Tan et al., 2002; Chen et al., 2006a).

CD154 is also known to be expressed on astrocytes and CD4^+ T cells and a marked increase in its expression has been reported in AD and in some brain injuries (Calingasan et al., 2002; Benveniste et al., 2004), however there are no data showing any potential interaction of microglia with astrocytes via a CD40-CD154 interaction. Disruption of the CD40-CD154 interaction has also been implicated in MS demonstrating beneficial effects (Gerritse et al., 1996).

1.7.2.4 TLR2

TLR2 is a member of a germline, TM pattern recognition receptor family, which is expressed on microglia (Rivest, 2003; Olson & Miller, 2004; Kielian, 2006), and it has a central role in initiating the immune response. TLRs recognise conserved structural motifs on a wide array of pathogens, which are referred to as pathogen-associated molecular patterns (PAMPS) (Kirk & Bazan, 2005; Akira et al., 2006). TLR2 plays an essential role in recognising structural components of various gram-positive bacteria, fungi, and protozoa, and subsequent activation of microglia (Kielian et al., 2002; Qureshi & Medzhitov, 2003; Kielian et al., 2005; Kirk & Bazan, 2005; Akira et al., 2006).

It has been shown that TLR2 expression is increased by insult (Glezer et al., 2007). Due to a recent finding, TLR2 signalling has been implicated in AD whereby fibrillar Aβ was shown to activate microglia via TLR2 (Jana et al., 2008). The activation of TLR2 has also been demonstrated to trigger phagocytic activity in murine microglia (Ribes et al., 2009) and BV-2 cells, a murine microglial cell-line (Tahara et al., 2006). This is supported by a study involving APP transgenic mice, whereby a deficiency in TLR2 was associated with a rapid increase in Aβ formation (Richard et al., 2008).
1.7.2.5 TLR4

TLR4, another member of the TLR family, is similar to TLR2 in being instrumental in detecting microbial infection; however TLR4 recognises structural components of gram-negative bacteria, such as the endotoxin LPS, which is a major component of the outer membrane (Qureshi & Medzhitov, 2003).

TLR4 signalling has been shown to be associated with microglial activation and neuronal death (Fernandez-Lizarbe et al., 2009), oligodendrocyte injury (Lehnardt et al., 2002), apoptosis of activated microglia (Liu et al., 2001; Jung et al., 2005), and neurodegeneration (Lehnardt et al., 2003).

1.7.3 Implications of Microglial Activation

Microglial activation is a critical component of the innate immune response in the CNS and, under normal conditions, activated microglia serve as a neuroprotective element, reacting to and disposing of lethal pathogens and neurotoxic proteins, and aiding repair after cell injuries. However when microglia are chronically activated, their released factors, which include pro-inflammatory cytokines, chemokines, reactive oxygen species (ROS), complement factors, proteases, eicosanoids, neurotoxic secretory products, free radical species and NO, all contribute to neuronal dysfunction and cell death (Griffin et al., 1998). It has been suggested that microglia are probable co-ordinators of the inflammatory response due to their ability to arrange the infiltration of peripheral immune cells into the CNS (Kato et al., 1996). Microglial activation, and the subsequent increase in inflammatory mediators, have been implicated in various neurodegenerative conditions but it is still unknown what it is that elicits this immune response.

1.7.3.1 Microglial Activation and Age

It has been shown several times that activated microglia release various cytokines and chemokines in the aged brain (Murray & Lynch, 1998; Hauss-Wegrzyniak et al., 1999; Sloane et al., 1999; Kullberg et al., 2001; Maher et al., 2005), and additional work has shown age-related increases in inflammatory cytokines TNF-α,
interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Sheng et al., 1998; Conde & Streit, 2006; Baune et al., 2009). In parallel expression of markers of microglial activation, such as MHC II, ICAM and CD86 are increased with age (Rozovsky et al., 1998; Godbout et al., 2005; Griffin et al., 2006; Lynch et al., 2007; Lyons et al., 2007a; Clarke et al., 2008; Downer et al., 2008). These data are supported by research carried out using microarray analysis reporting an age-related increase in genes involved in the immune response (Godbout et al., 2005; Rowe et al., 2007).

1.7.3.2 Microglial Activation and Aβ

Deposition of Aβ plaques is one of the two post-mortem characteristics of AD, and several studies have shown that activated microglia surround these neuritic plaques in AD brains (McGeer et al., 1987; Kim & de Vellis, 2005; Rogers et al., 2007). This suggests that microglia may be playing a role in the etiology of the disease, and some theories have proposed that activated microglia may initiate an immune response in AD. One hypothesis states that it is the interaction of plaque-specific material reacting with the surrounding reactive microglia that elicits a response, while another suggests that microglia interacts with certain structural components of amyloid to trigger a response (Yates et al., 2000). However it should be noted that injured and dying cells are known to secrete complement components, lysosomal enzymes, cellular DNA, glycation end products and nucleotides, which are all capable of activating microglia (Yan et al., 1996; Minghetti, 2005; Farber & Kettenmann, 2006; Okura et al., 2008). Therefore it has also been proposed that microglial activation is not a cause, but a consequence of the neuronal damage, which occurs in AD (Lynch, 2009).

1.7.4 Phagocytic Activity

Phagocytosis is an essential aspect of many biological processes, including normal tissue turnover (Han et al., 1993), embryological development and tissue remodelling (Cohen, 1991; Hopkinson-Woolley et al., 1994), but it also plays a key part in the resolution of inflammation (Savill et al., 2002), as it involves the clearance of apoptotic inflammatory cells debris and pathogens in the CNS. During phagocytosis, particles are recognised and bound to the surface of the cell. Once phagocytic receptors
are engaged, numerous signalling pathways are activated. Signals co-ordinate the rearrangement of the cytoskeleton, which guides the movement of the membrane to internalise the bound particles and form a ‘phagosome’ around the particles that engulfs the material (Stuart & Ezekowitz, 2008). The phagosome is then fused with the lysosome for degradation.

Microglia are the principal phagocytes in the CNS under normal conditions, but macrophages and dendritic cells (DC) also play a role following their infiltration. It has recently been reported that TLRs can exert positive and autonomous control on internalisation and on phagosome maturation in a phagocyte (Blander, 2008).

1.7.4.1 CD68

CD68 is a 110kDa highly glycosylated type I TM glycoprotein and is a lysosomal marker of phagocytic activity (Micklem et al., 1989; Ramprasad et al., 1996; Cho et al., 2006). CD68 belongs to the LAMP (lysosomal-associated membrane protein) family of glycoproteins. A role for CD68 in antigen processing and the protection of lysosomal membranes against lysosomal hydrolases has been suggested (Holness et al., 1993; Holness & Simmons, 1993). CD68 was originally thought to be expressed only by monocytes and macrophages (Holness & Simmons, 1993), but a recent study investigating CD68 mRNA expression shows CD68 is also expressed on other cell types including microglia (Schmitt et al., 2000). This expression on phagocytic cells and particularly an increase in expression during phagocytosis has led to interest in this molecule as a marker of microglial activation and of phagocytic microglia (da Silva & Gordon, 1999). Fibroblasts, endothelial cells and tumour cells also express CD68 (Kunz-Schughart et al., 2003; Gottfried et al., 2008).

1.8 Cytokines

1.8.1 Introduction

Cytokines are signalling molecules that are released from activated microglia and astrocytes in response to injury or infection. An increase in their release is accepted as a marker of cell activation in the CNS (Neumann, 2001; Moynagh, 2005). Once
released they carry signals locally within the CNS that can regulate the functions of cells. Cytokines bind to targeted receptor sites on cells enabling the intracellular domain of the receptor to interact with accessory molecules, which leads to the activation of downstream effectors and protein kinases that serve to phosphorylate various protein substrates (Foster, 2001).

Two types of cytokine exist, pro- and anti-inflammatory. Pro-inflammatory cytokines are responsible for initiating systemic inflammation, whereas anti-inflammatory cytokines invoke regulatory suppression of the actions of pro-inflammatory cytokines. The inflammatory response incorporates a combination of these two types of cytokines to exact an effect; this depends on the growth and activation state of cells, cytokine concentrations, tissue responsiveness, ligand occupation at targeted receptors and the availability of synergistic molecules in the surrounding microenvironment (Dinarello, 1998).

Some cytokines, such as IL-1β, have been shown to activate genes responsible for initiating apoptosis in glia, neurons and endothelial cells (Martin et al., 2002; Wang et al., 2005). They can affect the functions of neurons and ultimately contribute to neuronal dysfunction (Benveniste, 1992). Several studies have shown that certain cytokines like IL-1β can cause excessive neuronal activation, seizures, the release and impaired uptake of neurotransmitters, intracellular entry of calcium and the release of NO and free radicals, and disrupt ion homeostasis (Murray et al., 1997; Panegyres & Hughes, 1998; Vereker et al., 2001). Inflammatory cytokines have been shown to play a role in the pathogenesis of several neurodegenerative conditions, including AD, MS, EAE, PD and ageing (Kania et al., 1995; Merrill & Benveniste, 1996; Griffin et al., 1998; Lynch, 1998; Paolisso et al., 1998; Bruunsgaard et al., 1999; Dobbs et al., 1999; Bruunsgaard et al., 2000; Yates et al., 2000; Chabas et al., 2001; Lynch & Lynch, 2002; Maher et al., 2005; Puttaparthi & Elliott, 2005; Lyons et al., 2007b). They have also been implicated in ‘sickness behaviour’, whose symptoms range from lethargy, depression, anxiousness, fever, and increased sleep (Dantzer, 2004).

However overexpression of the cytokine IL-1β in an Alzheimer’s disease transgenic mouse model resulted in a reduction of Aβ plaques (Shaftel et al., 2007), and it has also been implicated in maintaining normal cognitive function (Avital et al., 2003).
1.8.2 Pro-inflammatory Cytokines

The pro-inflammatory cytokines TNF-α, IFN-γ, High Motility Group Box 1 (HMGB1), interleukin-12 (IL-12) and interleukin-18 (IL-18) will be focused on for the purposes of this study.

1.8.2.1 Tumour Necrosis Factor-α (TNF-α)

TNF-α is a potent pro-inflammatory cytokine and a member of the TNF superfamily of ligands (Wallach et al., 1999; Wajant et al., 2003; Shen & Pervaiz, 2006). TNF-α was originally discovered in mouse serum during endotoxemia and recognized for its anti-tumor activity (Carswell et al., 1975), although its role in the CNS was not observed until the 1980s, when it was shown that microglia were found to produce TNF-α (Frei et al., 1987). It can be synthesised in the CNS by microglia (Nakajima et al., 2004), astrocytes (Brenner et al., 1993), and some populations of neurons (Lieberman et al., 1989; Morganti-Kossman et al., 1997; Chung et al., 2005), as a 26kDa membrane-bound polypeptide precursor that is cleaved by proteolysis to release a 17kDa subunit; mediated by the proteinase TNF-α converting enzyme (Black et al., 1997; Moss et al., 1997; Solomon et al., 1997).

TNF-α signalling has been shown to have several important functions within the CNS including injury-mediated microglial and astrocyte activation, and regulation of BBB permeability, febrile responses, glutamatergic transmission, and synaptic plasticity and synapse scaling (Selmaj et al., 1990; Merrill, 1991; Sedgwick et al., 2000; Beattie et al., 2002; Leon, 2002; Pickering et al., 2005). TNF-α signalling can induce a variety of cellular responses including the activation of a number of intracellular signalling pathways including NF-κB, p38, c-jun N-terminal kinase (JNK), and the ceramide/sphingomyelinase signalling pathway, resulting in a number of responses including inflammation, proliferation, cell migration, apoptosis, and necrosis (Eissner et al., 2000; Harashima et al., 2001; Eissner et al., 2004; Heinrich et al., 2004; Ware, 2005).

There is a robust and rapid increase in TNF-α expression levels in the CNS both after acute insults and in a number of chronic neurodegenerative disorders including AD, PD, ischemia and traumatic brain injury (Mogi et al., 1994; Viviani et al., 1998; Allan & Rothwell, 2001; Tobinick et al., 2006). In keeping with the belief that TNF-α
is a chief regulator of the immune response, it has been shown in TNF-α neutralisation studies that TNF-α has the ability to regulate expression of other pro-inflammatory cytokines (Maini et al., 1995).

1.8.2.2 Interferon-γ (IFN-γ)

1.8.2.2.1 Biological Functions

IFN-γ is the sole member of the type II family of interferons, proteins that were identified for their ability to interfere with viral replication (Isaacs & Lindenmann, 1957). It is a biologically-active non-covalently linked 34kDa homodimer and produced predominantly by NK cells but it is also secreted by CD4^+ and CD8^+ cytotoxic T cells, DC and Natural Killer T cells (NKT cells) (Schoenborn & Wilson, 2007; Uemura et al., 2009). It has been described as having important roles in promoting host defense and immunopathologic processes (Farrar & Schreiber, 1993).

IFN-γ is one of the most potent activators of microglia, and it has been shown that IFN-γ can increase cell surface markers of microglial activation, such as CD40, ICAM-1 and MHC II (Vass & Lassmann, 1990; Molina & Huber, 1991; Bach et al., 1997; Nguyen & Benveniste, 2000; Benveniste et al., 2004; Lynch et al., 2007), and the production of pro-inflammatory cytokines (Nguyen & Benveniste, 2000; Hausler et al., 2002; Gasic-Milenkovic et al., 2003; Maher et al., 2006; Lyons et al., 2007b). It has also been shown to increase the expression of inducible nitric oxide synthase (iNOS) (Harris et al., 1995) and caspase-1 (Hausler et al., 2002; Kim et al., 2002). In light of these findings, it is suggested that IFN-γ plays a role in neurodegeneration (Blasko et al., 2001; Hausler et al., 2002; Mastrangelo et al., 2009), which is consistent with the finding that it is upregulated in the hippocampus of the aged brain in which a compromise in neuronal plasticity has been reported (Maher et al., 2006; Clarke et al., 2008).

1.8.2.2.2 IFN-γ-induced Signalling

IFN-γ mediates its effects on cells through a high-affinity IFN-γ receptor, which is composed of an α- and a β-chain (Farrar & Schreiber, 1993). When IFN-γ binds with its receptor, these chains oligomerise into a heterotetramer, which consists of two α-
chains and two β-chains (Farrar & Schreiber, 1993). The receptor is universally expressed on all nucleated cells (Farrar & Schreiber, 1993). IFN-γ-dependent signal transduction requires three intracellular proteins that belong to the JAK-STAT (Janus kinases-Signal Transducers and Activators of Transcription) signalling pathway. Two of these proteins are JAK-1 and JAK-2, which are activated upon ligation of the IFN-γ receptor and promote receptor phosphorylation and activation. Dimerisation, of the third component, a latent cytosolic transcription factor called STAT1 occurs (Shuai et al., 1992; Muller et al., 1993; Watling et al., 1993; Shuai et al., 1994) and STAT1 homodimerises and translocates to the nucleus to promote the transcriptional activation of IFN-γ-inducible genes that contain the γ-activating site motif (Shuai et al., 1993; Durbin et al., 1996; Meraz et al., 1996).

1.8.2.3 High Motility Group Box 1 (HMGB1)

HMGB1 (Semino et al., 2007) is a non-histone nuclear DNA binding protein that promotes protein assembly on specific DNA targets (Scaffidi et al., 2002). HMGB1 has been found to be expressed by various cell types of the CNS, including microglia, astrocytes, neurons and NK cells (Wang et al., 1999; Rendon-Mitchell et al., 2003; Semino et al., 2007; Andersson et al., 2008; Enokido et al., 2008). HMGB1 was initially categorised as a transcriptional regulatory molecule due to its ability to bind to DNA causing DNA to bend and unwind from the nucleosomes, subsequently enabling the molecular processes of transcription, recombination, genome stability and replication. Its ubiquitous expression in mammalian tissues is indicative of its function as a nuclear protein (Mosevitsky et al., 1989). Although it has been shown that HMGB1 also has a significant immunomodulatory role, whereby it was recognised that HMGB1 can activate a chronic inflammatory response once it has been released into the extracellular environment, it has been reported that this response occurs in a delayed mediatory fashion (Wang et al., 1999). HMGB1 can also trigger sickness behaviour (Agnello et al., 2002) and is a part of a family of damage-associated molecular pattern molecules (DAMPs), which trigger and maintain an immune response (Rubartelli & Lotze, 2007).

The primary function of HMGB1 in the brain is not fully understood but evidence suggests HMGB1 is critical in regulating DNA repair systems (Yuan et al., 2004; Prasad et al., 2007). It has also been shown that HMGB1 mediates the signalling
between NK cells and DC (Lotze & Tracey, 2005). Several research groups have demonstrated the ability of HMGB1 to induce the expression of several other inflammatory cytokines (Agnello et al., 2002; Scaffidi et al., 2002; O'Connor et al., 2003; DeMarco et al., 2005; Kim et al., 2006). HMGB1 can induce NK cells to produce IFN-γ (DeMarco et al., 2005), a cytokine that has been shown to have the ability to cause the translocation of HMGB1 from the nuclei to the cytoplasm; a process that appears to require the activity of TNF-α (Rendon-Mitchell et al., 2003).

HMGB1 is released passively from necrotic cells (Scaffidi et al., 2002), or actively, from macrophages for example, following inflammatory stimuli (Bonaldi et al., 2003). Kim and colleagues demonstrated the ability of HMGB1, which was released from stress-induced neurons, to activate microglia (Kim et al., 2006), thus activating them to produce various neurotoxic factors that impact upon the normal functioning of the CNS. Research has shown that upon release HMGB1 interacts with RAGE, TLR2 and TLR4, further supporting HMGB1 as an activator of microglia (Yu et al., 2006; Lotze et al., 2007; Yamada & Maruyama, 2007). Interestingly, HMGB1 has also been shown to bind to fibrillar Aβ1-42, subsequently interfering with the ability of microglia to clear Aβ, thus leading to enhanced neurotoxicity to neurons (Takata et al., 2004).

1.8.2.4 Interleukin-12 (IL-12)

IL-12 is a 70kDa heterodimer protein consisting of two disulphide subunits, p35 and p40. It is mainly produced by antigen-presenting cells and phagocytic cells, such as monocytes, macrophages, DC, neutrophils and B-cells (Watford et al., 2003). It has multi-potent effects, inducing a Th1 response, enhancing the CD8+ T cell response, activating natural killer cells and inducing production of IFN-γ (Trinchieri, 2003; Watford et al., 2003).

IL-12 has been used therapeutically due to its ability to induce an efficient anti-tumour effect on primary or metastatic tumours in various murine models and humans (Colombo & Trinchieri, 2002; Del Vecchio et al., 2007). These anti-tumour effects include anti-proliferative effects, anti-angiogenic effects (Wigginton et al., 2001; Lee et al., 2002) and cytotoxic effects of effector lymphocytes. It has been shown that various effector cells are required for IL-12-mediated anti-tumour effects, including CD8+ T cells (Brunda et al., 1993), NKT cells (Cui et al., 1997), CD4+ T cells (Zilocchi et al., 1998) and NK cells (Kodama et al., 1999). It was subsequently shown that IFN-γ
production is essential for most of the anti-tumour effects induced by IL-12 administration (Ogawa et al., 1998; Subleski et al., 2006). IL-12 is known to stimulate a range of immune cells to produce IFN-γ, including NK cells (Lauwerys et al., 1999), T cells (Kubin et al., 1994) and B-cells (Yoshimoto et al., 1997).

The IL-12 receptor is expressed on NK cells, T cells and DC (Grohmann et al., 1998). Binding of IL-12 to its receptor results in the activation of the JAK-STAT signalling pathway in a similar fashion to that described in section 1.8.2.2.2, however IL-12 receptor activation involves JAK2, TRK2 and STAT4 (Uemura et al., 2009). It has also been shown that IL-12 can activate the MAP kinase, p38 (Gollob et al., 1999).

### 1.8.2.5 Interleukin-18 (IL-18)

IL-18 is a member of the IL-1 family of pro-inflammatory cytokines. It is synthesised as a 24kDa precursor protein that is cleaved by caspase-1 into its active form (18kDa) (Ghayur et al., 1997; Gu et al., 1997). In the CNS, IL-18 is expressed in a wide range of cells including macrophages and DC (Stoll et al., 1998), and IL-18 mRNA has been reported on astrocytes, microglia, T cells and B-cells (Akira, 2000). IL-18 has been shown to induce a range of inflammatory cytokines but it is its ability to stimulate IFN-γ that it was first recognized; and so it was previously known as IFN-γ-inducing factor (IGIF) (Nakamura et al., 1989; Puren et al., 1998; Akira, 2000).

IL-18 shares biological properties with IL-12, such as its ability to enhance NK cell cytotoxicity and stimulate Th1 cell differentiation (Okamura et al., 1995; Micallef et al., 1996; Kohno et al., 1997). It has been demonstrated that IL-18 can work in true synergy with other Th1-related cytokines, such as IL-12, IL-2, IL-15 and IL-23, to produce IFN-γ (Micallef et al., 1996; Robinson et al., 1997; Nakahira et al., 2002; Okamoto et al., 2002; Okazawa et al., 2004). It is also an activator of polarised Th1 cells and can induce lymphocyte proliferation (Robinson et al., 1997; Lebel-Binay et al., 2000).
1.9 NK cells

1.9.1 Introduction

NK cells are a large population of lymphocytes characterised by their potent cytotoxic activities against tumours and virally-infected cells (Biron et al., 1999; Kim et al., 2000; Solana & Mariani, 2000). They are considered to be an important component of the innate immune response due to their ability to kill certain lymphoid tumour cells in absence of any stimulation. NK cells are derived from pluripotent hematopoietic stem cells, developing in the bone marrow (Carlyle & Zuniga-Pflucker, 1998; Raulet, 1999).

In addition to producing various pro-inflammatory cytokines such as IFN-γ and TNF-α (Solerte et al., 2000; Uemura et al., 2009), NK cells are known to secrete High Motility Group Box 1 (HMGB1) (Semino et al., 2007), which is a DNA binding protein that promotes protein assembly on specific DNA targets (Scaffidi et al., 2002). It has been shown to have a significant immunomodulatory role, mediating between NK cells and DC (Lotze & Tracey, 2005). HMGB1 has been reported to interact with TLR2 and TLR4 (Yu et al., 2006), increase proinflammatory cytokine production in the brain, and trigger sickness behaviour (Agnello et al., 2002; O'Connor et al., 2003).

1.9.2 Identification and Signalling

The ability of NK cells to function is finely regulated by a series of inhibitory or activating receptors. NK cells target cells that have undergone an expression-altering transformation, such as with tumours, or have been infected with a virus (Walzer et al., 2005). NK cells carry out their cytotoxic functions by two different mechanisms, utilizing either their activating or inhibitory receptors.

Many studies have revealed that NK cells recognise MHC I molecules via a number of expressed surface receptors as a method of preventing NK cell cytotoxicity (Ljunggren & Karre, 1990; Moretta et al., 1990; Ciccone et al., 1992; Moretta et al., 1992; Colonna et al., 1993; Moretta et al., 1993; Yokoyama & Seaman, 1993). When a cell has lost the ability to express MHC I or is only capable of expressing insufficient amounts, NK cells elicit a cytotoxic attack on the cell. Conversely, when an inhibitory
receptor on the surface of a NK cell binds with sufficient MHC I of a target cell, the registered signal that occurs prevents the cell from cell death. It is in this regard that NK cells can differentiate between normal cells and abnormal cells (Moretta et al., 1996). Activating receptors are 'switched on' upon interaction with a potential target cell, and unless signals are received by the inhibitory receptors indicating an adequate expression of MHC I, the target cell is labelled for destruction (Moretta & Moretta, 2004). When activated for killing, NK cells generate and release cytotoxic granules onto the surface of the bound target cell, and effector proteins and proteolytic enzymes contained within penetrate the target cell membrane and induce programmed cell death (Biron, 1998; Solana & Mariani, 2000).

The major activating receptors, also known as natural cytotoxicity receptors (NCRs), responsible for NK cell triggering are NKp46 (Sivori et al., 1997; Pessino et al., 1998), NKp30 (Pende et al., 1999), NKp44 (Vitale et al., 1998; Cantoni et al., 1999) and NKG2D (Cerwenka & Lanier, 2001; Diefenbach & Raulet, 2001). NKG2D has been shown to be also expressed on cytolytic T cells (Moretta & Moretta, 2004), but the expression of NKp46, NKp30 and NKp44 is restricted to NK cells; thus representing the most accurate surface markers for identifying NK cells.

Additionally there are several other characteristic phenotypic NK cell markers, such as CD161, CD56, CD16, CD94 and CD57 (Tarazona et al., 2000). From the markers available, CD161 and NKp30 were chosen to be used as markers to identify NK cells in this study.

1.9.2.1 CD161

CD161 molecules are type II TM C-type lectin-like receptors expressed in the cell membrane as disulphide homodimers (Pozo et al., 2006). CD161 is expressed on the majority of NK cells and on a subset of CD4^ and CD8^ T cells (Lanier et al., 1994; Poggi et al., 1997). CD161 is thought to be implicated in triggering NK cell cytotoxicity by contributing to target cell recognition (Ryan et al., 1995; Seaman, 2000). Braud and colleagues reported that one ligand for the CD161 receptor is lectin-like transcript 1 (LLT1) molecule. When CD161 on NK cells engages with LLT1 expressed on target cells, NK cell-mediated cytotoxicity and IFN-γ secretion was inhibited (Aldemir et al., 2005).
1.9.2.2 NKp30

NKp30 is a 30kDa triggering receptor found exclusively on all resting and activated NK cells (Pende et al., 1999). NKp30 ligation has been implicated in the activation of the NF-κB pathway in NK cells (Pandey et al., 2007), and is involved in the crosstalk between NK cells and DC (Moretta et al., 2006). A ligand, NKp30L, has since been discovered by Watzl and colleagues, who assessed its expression in 30 cell lines of different origin and found NKp30L is ubiquitously expressed in transformed and non-transformed cell lines (Byrd et al., 2007).

1.9.3 NK cells and Inflammation

In addition to their cytotoxic activities, NK cells can, when stimulated, secrete cytokines and chemokines (Walzer et al., 2005; Semino et al., 2007; Uemura et al., 2009). They also express receptors for a number of cytokines and chemokines and so are capable of responding to various stimuli (Solana & Mariani, 2000). NK cells have been shown to be involved in crosstalk with DC (Fernandez et al., 1999), whereby NK cell effector functions are stimulated via contact with activated DC. DC have been shown to provide NK cells with a source of cytokines for their activation when stimulated by pathogens or TLR ligands (Iwasaki & Medzhitov, 2004). NK cell and their cytotoxic effects have been associated with neuroinflammatory disorders like AD (Solerte et al., 1998) and MS (Segal, 2007), as well age-related alterations in NK cell phenotype and function in peripheral blood (Borrego et al., 1999).
1.10 Objectives

The aims of this project were:

- to assess microglial activation in age-related inflammation.
- to examine whether particular cytokines may be responsible for the activation of microglia in the brain.
- to investigate whether NK cells, a source of inflammatory cytokines, are present in the brain of aged rats, and may therefore contribute to age-related microglial activation.
- to determine whether Nogo expression is altered with age and whether Nogo can modulate microglial and/or neuronal function.
- to assess whether Aβ treatment exerts any effect on Nogo expression and changes in microglial and neuronal function.
Chapter 2

Methodology and Statistical Methods
2.1 Animals

Male Wistar rats aged 2–4 months (young), 15-18 months (middle-aged) and 18-25 months (aged) were used for the studies described herein. Young, middle aged and aged rats weighed between 250g-420g, 450-550g and 560g-710g, respectively. The rats were supplied by Harlan (UK). Rats were housed in groups of 4 and maintained in the BioResources Unit, Trinity College, Dublin 2.

All animals were maintained under veterinary supervision at an ambient temperature of 22°–23°C and under a 12h light-dark cycle and food (normal laboratory chow) and water were available: ad libitum. All animal experimentation was performed under a license granted by the Minister for Health and Children (Ireland), with approval from the local ethical committee and in compliance with the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EC, and every effort was made to minimise stress to the animals.

2.2 Animal Treatments

There were 3 main studies undertaken in this project. All rats were anaesthetised by intraperitoneal (ip) injection with urethane (1.5g/kg, 33% w/v), the depth of anaesthesia was determined by the absence of a pedal reflex, and urethane was administered to a maximum dose of 2.5g/kg. Rats implanted with minipumps were anaesthetised with ketamine (75mg/kg) and xylazine (10mg/kg).

In one study, tissue prepared by Drs Alessia Piazza and Anne-Marie Miller was used. In this study young (2-3 months) rats were randomly assigned to 4 treatment groups. Animals in groups I and II (n=6 in each group) received an intracerebroventricular (icv) injection (5μl) of sterile saline or Aβ1-40/1-42 (25μM/25μM; Biosource, Belgium) respectively, and were killed 4h after treatment. Animals in groups III and IV (n=6 in each group) were implanted subcutaneously with osmotic mini-pumps (model 2004, Alzet, USA) in the mid-scapular region. The pump was attached via polyvinylchloride tubing (Alzet, 0.69mm diameter) to a chronic indwelling cannula (Alzet, Infusion Kit II), which was positioned stereotaxically in the lateral ventricle (0.9mm posterior to bregma, 1.3mm lateral to the midline and 3.5mm ventral to the dura). The
cannula was affixed to the skull using cryanoacetate gel and was secured in place by a smooth covering of dental cement (Stoelten, USA). The pump was implanted containing $\text{Aβ}_{40-1}$ (63.8μM) or $\text{Aβ}_{1-40/1-42}$ (26.9μM/36.9μM) respectively; $\text{Aβ}$ was infused at a rate of 6μl/day for 8 days (total of 48μl of respective $\text{Aβ}$). Post-operative care included a subcutaneous injection of the analgesic Rimadil (5mg/kg). At the end of this period rats were sacrificed and hippocampal tissue was harvested for analysis.

In a second study, groups of young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) rats were anaesthetised with isoflurane (4% induction, 1.5-2% maintenance) in 100% oxygen, and secured in a custom-built Perspex cradle with ear and tooth bars to support the head. A series of MR imaging experiments designed to assess T1 and T2 relaxation time were performed on the animals. Respiration rate and temperature were monitored using purpose-built MRI-compatible monitoring equipment (S.A. Instruments, USA) during the MRI acquisition. Rats were allowed to recover for 48h before being anaesthetised by ip injection with urethane. Rats underwent transcardial perfusion before cortical and hippocampal tissue were harvested.

In a further study, young (3-4 months) and aged (18-22 months) were sacrificed and their cortical and hippocampal tissue harvested for further analysis.

### 2.3 Tissue Preparation

#### 2.3.1 In vivo

##### 2.3.1.1 Transcardial Perfusion

Animals undergoing transcardial perfusion were first anaesthetised by ip injection with urethane. While the anaesthetic was taking effect, the perfusion pump was set up; attaching the perfusion tubing and needle. The open end of the perfusion tube was placed in a 2L beaker filled with ice-cold saline. Using the pump 100ml of saline was run through the tubing to flush out any air bubbles or residue. The rate of flow was set to 20ml/min before turning off the pump until needed. A surgery site was set up with scissors, scalpel, forceps, clamps and guillotine. A rack was placed over a catch container for fluids. Once the animal was anaesthetized, it was placed on a rack.
with the abdomen exposed. The pinch-response method was used to determine the depth of anaesthesia. Confident that the animal was unresponsive, the perfusion procedure was carried out. Using a scalpel, an incision was made through the abdomen the length of the diaphragm. The connective tissue at the bottom of the diaphragm was cut through with sharp scissors to allow access to the rib cage. The ribs were cut through just left of the rib cage midline with a large scissors, blunt side down, opening up the thoracic cavity. The thoracic cavity was clamped open, exposing the heart and providing drainage for blood and fluids. While holding the beating heart steady, an incision was made in the left ventricle and the perfusion needle was inserted directly into the protrusion extending in about 5mm. Caution was taken not to pierce the interior wall of the heart during this. The needle position was secured by clamping in place at the point of entry to the aorta. The pump was started and a slow steady flow of saline was allowed to flow. Caution was taken to not introduce air into the flow of saline. The volume needed to sufficiently perfuse the animal was scaled according to the size of the animal, however 200-300ml was found to be adequate for each animal. Perfusion continued until a pale discolouration was observed in the organs of the animal, indicating the blood was being cleared from the body (a lightened colour of the liver was the best indicator). Upon completion of the perfusion procedure, the pump was turned off and the animal was decapitated and brain tissue was dissected.

2.3.1.2 Preparation of Tissue

Animals were killed by decapitation and in some studies brains were hemi sectioned, and 1 half of the brain was covered in Tissue-Tek® OCT, frozen in isopentane on dry-ice, and stored at -80°C for later preparation of cryostat sections. The hippocampi and cerebral cortices from the other half of the brain were stored at -80°C for later analysis. In other experiments hippocampus and cortex was dissected free from whole brain. Tissue was stored for later analysis by Polymerase Chain Reaction (PCR), or for Enzyme-Linked ImmunoSorbent Assay (ELISA), other assays and western immunoblotting.

The cerebellum and pre-frontal cortex were dissected free from the brain tissue and discarded. The remaining brain was bisected along the midline of the brain from the anterior to the posterior. All brain tissue was dissected free from the cortex and hippocampus. Using forceps and scalpel, the hippocampal tissue from each remaining
hemi-section was carefully peeled away from the remaining cortex of each hemi-section. These tissues were appropriately prepared for storage.

For PCR, ~30μg of hippocampal or cortical tissue was frozen in 500μl of RNALater by submerging RNA storage tubes in 50ml of isopentane chilled over dry ice. Frozen tissue was stored at -80°C for later analysis of messenger ribonucleic acid (mRNA). For ELISA, other assays and western immunoblotting hippocampi and cortices were sliced bi-directionally to a thickness of 350μm using a McIlwain tissue chopper and rinsed in 500μl of Krebs solution containing CaCl₂ (2mM) (Krebs-Ca²⁺ solution). The slices were allowed to settle and were rinsed twice more in Krebs-Ca²⁺ solution and finally rinsed in 500μl of Krebs solution containing 10% dimethylsulphoxide (DMSO) and stored in 500μl of this solution at -80°C until required for further analysis. For flow cytometry and Magnetic-Activated Cell Sorting (MACS), fresh tissue was prepared as a single cell suspension and prepared for immediate analysis (see sections 2.4.6 and 2.4.7).

2.3.1.3 Tissue Fractionation

Cortical tissue underwent fractionation to allow the assessment of activation of sphingomyelinase (see section 2.4.2) in membrane and cytosolic fractions and assessment of cytochrome c in the cytosolic fractions (see section 2.4.5). Cortical tissue (200μg) was washed in 500μl of ice-cold phosphate-buffered saline (PBS) and added to liquid nitrogen to snap-freeze the samples. The frozen tissue was ground using a sterile glass homogeniser, and transferred into a tube containing cell lysis buffer (1ml).

Samples were homogenised in 1 burst of 20sec using the Kinematica Polytron® System PT 1200E homogeniser. The samples were centrifuged at 500 x g for 15min at 4°C and the supernatant was kept. The pellet was resuspended in 1ml lysis buffer and homogenised for 20sec. Samples were centrifuged at 500 x g for 15min at 4°C, the resulting pellet was discarded and the previous supernatant and the newly spun supernatant were pooled. The pooled supernatants were centrifuged at 45,000 x g for 15min. The resulting pellet is the membrane-bound fraction and the supernatant is the cytosolic fraction. The pellet was washed twice in 1ml lysis buffer, and resuspended in Krebs-Ca²⁺ solution (300μl). Samples were stored at -80°C.
2.3.1.4 Protein Quantification

All glass pipettes and homogenisers were baked in tin-foil overnight at 200°C in an oven. All the glass pipettes and homogenisers, and the polytron homogeniser attachment were washed between samples with deionised water (dH$_2$O).

The stored hippocampal and cortical aliquots were thawed on ice and rinsed 3 times in 500µl of ice-cold Krebs-Ca$^{2+}$ solution. The slices were allowed to settle and then either homogenised in either Krebs-Ca$^{2+}$ solution (1ml) in a 2ml Eppendorf tube using the Kinematica Polytron® System PT 1200E homogeniser in 2 x 6sec bursts, or in a 1ml glass homogeniser by 40 up-and-down strokes in Krebs-Ca$^{2+}$ solution.

Protein concentration was assessed in the hippocampal and cortical homogenates, and in the cytosolic and membrane fractions using the bicinchoninic acid (BCA) protein assay kit (Perbio Pierce Thermo Scientific, USA). Standards (0-2000µg/ml) were prepared with bovine serum albumin (BSA) standard (2mg/ml) by diluting to various concentrations in Krebs-Ca$^{2+}$ solution. Triplicate standards and samples (25µl) were pipetted into a 96-well plate to which diluted working reagent (200µl; BCA Protein Assay Kit; Perbio Pierce Thermo Scientific, UK) was added; the plate was covered and incubated at 37°C for 30min. The absorbance was read at 570nm and the standard curve was plotted allowing the protein concentration of each sample to be calculated. Protein concentrations were equalised by dilution in ice-cold Krebs-Ca$^{2+}$ solution to a concentration of 1mg/ml, and stored at -80°C.

2.3.2 In vitro

2.3.2.1 Coverslips

The following procedures were carried out in a laminar flow fume hood (Advanced Biosafety Cabinet Class II; AGB Scientific Ltd., Ireland). Glass coverslips (10mm diameter) were sterilised in 70% ethanol and dried overnight under UV light in a sterile fume hood. The following day, the coverslips were submerged in a poly-L-lysine solution (1mg/ml; diluted in sterile PBS) and placed in an incubator for 2h at 37°C, dried in a sterile fume hood and used in 24-well cell culture plates.
2.3.2.2 Neuronal Preparation

Primary cortical neurons were prepared from 1 day old male Wistar rats (BioResources Unit, Trinity College, Dublin). The neonates were decapitated, the cerebral cortices were dissected and the meninges were removed. They were bi-directionally chopped with a sterile scalpel (Schwann-Mann, UK) and then incubated in 2ml sterile PBS (Biosera, UK) containing 0.3% trypsin (Sigma-Aldrich, UK) at 37°C for 25min with 5% CO₂: 95% air in a Nuaire Flow CO₂ incubator (Jencons, UK). Brain tissue was gently triturated 5 times in 4ml of PBS containing 0.1% trypsin inhibitor (Sigma-Aldrich, UK), DNase (0.2mg/ml; Sigma-Aldrich, UK) and MgSO₄ (0.1M). Cell suspensions were passed through a sterile nylon mesh filter and pelleted by centrifugation at 1200rpm for 5min at 20°C using a Sorvall Legend RT centrifuge (Perbio Pierce Thermo Scientific, UK). The pellet was resuspended in 1.5ml of NBM supplemented with 10% heat-inactivated horse serum (Gibco, UK), penicillin (100U/ml), streptomycin (100U/ml), glutamax (2mM; Gibco, UK) and B-27 (1:50 dilution; Gibco, UK). The resuspended neurons were counted, equalised to a density of 1x10⁶ cells/ml and plated (55μl/well) on poly-L-lysine (60μg/ml) coated coverslips in 24-well plates and incubated at 37°C for 2h before the addition of B27-supplemented NBM (400μl) to each well. Cells were grown for 48h. The medium was then replaced with NBM containing cytosine arabinofuranoside (5μg/ml; Sigma-Aldrich, UK) to prevent proliferation of non-neuronal cells. Arabinofuranoside-supplemented media was removed after 24h and replaced with complete NBM (400μl/well). After a further 2 days incubation cells were ready for treatment.

2.3.2.3 Mixed Glia Preparation

Primary cortical mixed glia were prepared from 1 day old male Wistar rats (BioResources Unit, Trinity College, Dublin). The neonates were decapitated, the cerebral cortices were dissected and the meninges were removed. Tissue was bi-directionally chopped with a sterile scalpel (Schwann-Mann, UK) and placed in warm filter sterilised DMEM (2ml; Gibco, UK) supplemented with 10% heat-inactivated foetal calf serum (Sigma-Aldrich, UK), streptomycin (100U/ml; Gibco, UK) and
penicillin (100U/ml; Gibco, UK). The tissue was incubated at 37°C for 20min with 5% CO₂:95% air in a Nuaire Flow CO₂ incubator (Jencons, UK). Tissue was tritutrated 5 times using a sterile plastic Pasteur pipette (Sarstedt, Ireland); the suspension was filtered through a sterile mesh filter (40µm; BD Biosciences, France), centrifuged at 2000 x g for 3min at 20°C (Sorvall Legend RT) and the pellet was resuspended in warmed 1.5ml of DMEM.

The resuspended mixed glia were counted, equalised to a density of 1x10⁶ cells/ml, plated (60µl/well) on poly-L-lysine (60µg/ml) coated coverslips in 24-well plates and incubated at 37°C for 2h before the addition of warm DMEM (400µl) to each well. Cells were grown at 37°C in a humidified 5% CO₂:95% air environment and media was changed every 3 days for 12-14 days until cells reached greater than 70% confluency, whereupon cells are ready for treatments.

2.3.2.4 Preparation of Purified Glial Cultures

Primary cortical microglia were prepared from 1 day old male Wistar rats (BioResources Unit, Trinity College, Dublin) using the same method described above (section 2.3.2.3). Once the pellet was resuspended in DMEM, the cells obtained from each neonate were plated into 2 separate T25 flasks using a sterile plastic Pasteur pipette. The glia were incubated at 37°C for 2h to allow them to adhere to the surface of flasks before each flask was flooded with 8ml of warm DMEM. Cells were grown at 37°C in a humidified 5% CO₂:95% air environment and, the following day, the media was replaced with 8ml of culture media containing mononuclear phagocyte colony stimulating factor (M-CSF; 20ng/ml) and granulocyte macrophage colony stimulating factor (GM-CSF; 10ng/ml). Culture media was replaced with fresh culture media supplemented with M-CSF and GM-CSF 7 days later.

On day 12-14, the flasks were wrapped with parafilm to make them air-tight. They were placed on an orbital shaker and shaken at 110rpm for 2h at room temperature (RT). The flasks were taken back to the hood and tapped about 10 times. This separated non-adherent microglia from the astrocytes. The non-adherent microglia were pooled from the different flasks, poured into a 50ml Falcon tube and centrifuged at 2000rpm for 5min at 20°C. The astrocytes were incubated with 1ml of Trypsin-EDTA at 37°C for 15min and tapped about 10 times. This separated the astrocytes from
the surface of the flasks. The astrocyte cell suspension was poured into a new 50ml Falcon tube and centrifuged at 2000rpm for 5min at 20°C.

The supernatant was removed and the resulting pellet was resuspended in 1ml DMEM. The cells were counted and equalized to a cell density of 4x10⁵ cells/ml and 1x10⁶ cells/ml for microglia and astrocytes, respectively. Microglial cells were plated (60μl/well) on poly-L-lysine coated coverslips (60μg/ml) in 24-well plates, and astrocytes were plated similarly but without the use of coverslips. Cells were incubated for 2h at 37°C before the addition of warm DMEM (400μl) to each well. Cells were allowed to grow until they reached greater than 70% confluency before treatment.

### 2.3.2.5 NK Cell Preparation

Primary spenic NK cells were prepared from male Wistar rats (BioResources Unit, Trinity College, Dublin) by MACS described in section 2.4.6. Isolated NK cells were centrifuged at 2000 x g for 3min at 20°C and the pellet was resuspended in RPMI media (1ml).

NK cells were counted, equalised to a density of 1x10⁶ cells/ml, added to 15ml Falcon tubes (400μl/tube) and incubated at 37°C for 2h before the addition of warm RPMI (2.5ml) to each well. Cells were grown at 37°C in a humidified 5% CO₂:95% air environment overnight before treatments.

### 2.3.2.6 Culture Treatment Protocols

Solutions used for treating cells were diluted to the required concentration in pre-warmed supplemented media and were filtered through a syringe with a 0.2μm cellulose acetate membrane filter (acidisc syringe filters; Pall Corporation, UK). Once cells were confluent they were treated with Nogo-A, Nogo-B, Aβ, HMGB1, IFN-γ, TNF-α, IL-12 and IL-18 as follows for 24h:

Nogo-A and Nogo-B (Abcam, UK and Alpha Diagnostic, USA, respectively) were prepared from a stock solution of 100μg/ml (0.01μg/ml, 0.1μg/ml or 1μg/ml). Aβ (Invitrogen, USA) was prepared as a stock solution (1mg/ml in 1:5, dH₂O: PBS) and was incubated at 37°C for 24-48h to aggregate the peptide. Neurons or glia were treated with Aβ (10μM) for 24h. HMGB1 (Biochemistry Dept., Trinity College, Dublin) peptide was prepared from a stock solution of 216.6μg/ml to its final concentration
IFN-γ (R&D Systems, UK) and TNF-α (R&D Systems, UK) were prepared in DMEM since they were used to treat glia only. The stock solutions in each case were 100μg/ml and 10μg/ml, respectively and the final concentration for both was 50ng/ml. IL-12 recombinant protein (R&D Systems, UK) and IL-18 recombinant protein (R&D Systems, UK) were prepared in RPMI since they were used to treat NK cells only. The stock solutions in each case were 10μg/ml and 25μg/ml, respectively and the final concentrations were 10ng/ml and 10ng/ml, respectively.

2.3.2.7 Cell Harvesting

Following treatment periods, supernatants were removed using a sterile Pasteur pipette and aliquoted into fresh tubes and stored at -80°C until required for analysis. Cells were either harvested for protein or RNA analysis.

For later analysis of protein, cell lysates were incubated in cell lysis buffer for 15min at RT, scraped from coverslips and stored at -80°C. For later RNA analysis, lysates were washed once in 500μl of ice-cold PBS and frozen in cell lysis mastermix (Nucleospin RNA II, Macherey-Nagel, Ireland) and stored at -80°C.

2.3.2.8 Cell Counting

Cell counts were performed by diluting cells (1:10) in 100μl trypan blue (Sigma-Aldrich, UK). A sample of cell suspension (10μl) was loaded onto a disposable haemocytometer (Hycor Biomedical, UK). Viable cells (i.e. those which did not stain and appear light under a light microscope) were counted.

2.4 Methods of Analysis

2.4.1 Analysis of Cytokine Expression by ELISA

Commercially available ELISA kits were used to measure the concentration of IFN-γ (R&D Systems, UK). High-binding certified 96-well microtitre plates (NUNC Immuno, Denmark) were incubated overnight at RT on a shaker with anti-rat IFN-γ capture antibody in PBS (pH 7.3; 100μl/well; 2μg/ml). Plates were washed 3 times with
wash buffer (200μl/well; PBS containing 0.05% Tween-20) and non-specific binding sites were blocked in assay diluent (300μl/well; PBS containing 1% BSA; pH 7.3) by incubating the plates on a shaker for 1h at RT.

Standard curves were prepared for IFN-γ (0-2500pg/ml; R&D Systems, UK) in assay diluent. The plates were washed 3 times in wash buffer (200μl/well), and samples (100μl; ~1mg/ml) and cytokine standards were added to in duplicate/triplicate and incubated on a shaker for 2h at RT.

Plates were washed 3 times with wash buffer (200μl/well) and incubated on a shaker for 2h at RT with biotinylated anti-rat IFN-γ detection antibody in assay diluent (100μl/well; 150μg/ml).

Plates were washed 3 times with wash buffer (200μl/well) and incubated for 20-30min at RT in the dark with horseradish peroxidase-conjugated streptavidin (Strep-HRP; 100μl/well in assay diluent; 1:200 dilution) and incubated for 20-30min at RT in the dark with substrate solution (100μl; 1:1 H₂O₂: tetramethyl benzidine (TMB); R&D Systems, UK) or until colour developed.

The reaction was quenched by adding a stop solution (50μl; 1M H₂SO₄) to each well and absorbance was read at 450nm using a 96-well plate reader (Labsystem Multiskan RC, UK). Cytokine concentrations were calculated by reference to the appropriate standard curve; concentrations were corrected for protein concentration where appropriate and expressed as pg/mg protein (GraphPad Prism v4.0; GraphPad Software, USA).

2.4.2 Analysis of Sphingomyelinase Activity

Sphingomyelinase activity (Molecular Probes, UK) was analysed in homogenate, and membrane preparations and cytosolic fractions prepared from cortex of young and aged rats. The cortical homogenate was equalised (~1mg/ml) for protein concentration in reaction buffer (0.5M Tris-HCl, 50mM MgCl₂). Sphingomyelinase stock solution (10U/ml) was diluted in reaction buffer to produce a 0.04U/ml positive control and H₂O₂ (20mM) was used as a second positive control. Reaction buffer without sphingomyelinase served as a negative control. A working solution was prepared [Amplex Red Reagent (100μM; 100μl), HRP (2U/ml; 100μL), choline oxidase


(0.2U/ml.; 100μL), alkaline phosphatase (8U/ml; 200μl) and sphingomyelin (0.5mM; 1μl) in reaction buffer (8.5ml) and 100μl was incubated with 100μl of each sample and each control for 30min at 37°C in the dark. Fluorescence was measured in a Fluoroskan Ascent FL Reader at 0, 5, 15, 20, 30, 60 and 120min at an excitation wavelength of 544nm and an emission wavelength of 590nm with background fluorescence subtracted.

2.4.3 Analysis of Caspase Activity

Activities of caspase-8 and caspase-3 were analysed in cortical and hippocampal homogenate prepared from young and aged rats, and in cortical neurons prepared from 1-day old Wistar rats using a colorimetric method (Biomol, UK). Assay kit stock solutions for caspase-8 and caspase-3 were diluted 1/50 in the kit assay buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM EDTA, 10% glycerol; pH7.4). All controls and samples were made up in a total reaction volume of 100μl. Assay buffer (25μl) was added to the positive control and each test sample well, and 50μl was added to the negative control. The plate was incubated for 10min at RT. Cortical sample and the diluted caspase enzyme (25μl) was added to test and positive control wells respectively, giving a final reaction volume of 50μl. Caspase-8 substrate (Ac-IETD-p-nitroanilide; pNA) and caspase-3 substrate (Ac-DEVD-pNA) were prepared to provide for a final concentration of 200μM. The reaction was started by the addition of 50μl of the substrate solution and absorbance was measured continuously from 0-20min at 405nm. Background absorbance was subtracted. Measurements were also taken for the pNA calibration standard (100μl) and background absorbance for the pNA standard was also subtracted. These measurements allowed the enzyme activity in the samples to be expressed as pmol/min. Data were corrected for protein and plotted as pmol/min/mg.
2.4.4 Analysis of RNA Expression

2.4.4.1 RNA Isolation

For all RNA work, standard precautions were taken to keep reagents and equipment free from RNases. RNA was extracted from *in vivo* snap-frozen cortical and hippocampal tissue and *in vitro* harvested lysates using the Nucleospin® RNA II – Macherey-Nagel RNA Isolation kit. Cortical and hippocampal tissue (~30mg) and *in vitro* samples harvested for RNA analysis were thawed on ice. Tissue samples were homogenised in cell lysis buffer (353.5μl; 350μl RA1 buffer contained in the kit + 3.5μl β-mercaptoethanol) using the Kinematica Polyton® System PT 1200E homogeniser (Kinematica Inc., USA) for 2 x 5sec bursts. The lysis buffer and homogenised tissue was transferred to autoclaved RNase-free 2ml Eppendorf tubes. Samples obtained from *in vitro* experiments were stored in cell lysis buffer and when needed thawed for analysis.

Nucleospin® Filter units were placed in a collecting tube and homogenised samples or thawed samples from *in vitro* experiments were applied. Samples were centrifuged at 11,000 x g for 1min. The filter units were discarded and 70% ethanol (350μl; Sigma-Aldrich, UK) was added to the samples and mixed by pipetting up and down 3 times. Nucleospin® RNA II columns were placed in a 2ml centrifuge tube, samples were loaded and centrifuged at 8,000 x g for 30sec in order to bind the DNA to the column. The columns were placed in fresh collecting tubes and Membrane Desalting Buffer (350μl) was added. The columns and collecting tubes were centrifuged at 11,000 x g for 1min to dry the silica column membrane. A DNase reaction mixture (95μl) was added directly onto the centre of the silica membrane and incubated for 15min at RT. The membrane was subsequently washed and dried 3 times with RA2 buffer (200μl; provided in the kit) and RA3 buffer (600μl; provided in the kit) at 8,000 x g for 30sec. The third wash was carried out using RA3 buffer (250μl) at 11,000 x g for 2min. The flow-through was discarded each time. The columns were transferred into pre-labelled nuclease-free 1.5ml microcentrifuge tubes supplied with the kit. Pure mRNA was eluted from the columns by adding 60μl of RNase-free H₂O to each column and centrifuging at 11,000 x g for 1min. For a higher yield and concentration, the eluate was reapplied to the column and centrifuged again. All mRNA
samples were stored at -80°C until required. RNA concentration was quantified using a Nanodrop ND-1000 Spectrophotometer (Perbio Pierce Thermo Scientific, UK).

2.4.4.2 RNA Integrity Check

Gel electrophoresis was used to separate mRNA products, in order to check the purity and integrity of the mRNA samples isolated. In order to visualise mRNA, ethidium bromide (EtBr), which intercalates between the nucleotides of the mRNA and fluoresces when illuminated by ultraviolet light, was added to the gel. RNA samples were run on a 1% agarose gel, consisting of 1.3g of agarose (Sigma-Aldrich, UK) dissolved in Tris-borate/ethylenediaminetetraacetate (TBE; 130ml) buffer, which was heated to aid the dissolution. Once the agarose had cooled sufficiently (~40°C), EtBr (1.3µl, 10mg/ml; Sigma-Aldrich, UK) was added to a final concentration of 1µg/ml. The contents of the flask were poured into a mini-gel electrophoresis tank, which was fitted with a well comb and dividers, and allowed to set.

Once solid, the comb and supports were removed and TBE was added to the tank until the surface of the gel was submerged. Equal quantities (3µl) of mRNA samples were added to 6x gel loading dye (2µl; Promega, UK) and diethyl pyrocarbonate RNase-free water (7µl). DNA ladder (10µl; Promega, USA) was mixed with 6x gel loading buffer (2µl; Promega, UK). The mRNA samples and the DNA ladder (10µl) were loaded onto the gel and separated by application of 90V for 60min. Migration of the bromophenol blue dye towards the bottom of the gel indicated separation of samples.

RNA was visualised under a UV light and photographed by means of a Gel-Doc-It Imaging System Ultraviolet Transilluminator (Labworks™ Image Acquisition and Analysis; Version 4.6; UVIP BioImaging Systems Ltd., UK). Successful preparation of mRNA is displayed by separation of the mRNA into 2 predominant bands of small ~2kb and large ~5kb ribosomal RNA (28S:18S rRNA), the ratio of intensities of these bands should be 1-2:1.

2.4.4.3 cDNA Synthesis

RNA was reverse transcribed into cDNA using a high-capacity cDNA archive kit (Applied Biosystems, Germany) according to the protocol provided by the
manufacturer. The amount of mRNA used as template for first strand or copy DNA (cDNA) synthesis was sample-specific; between 0.5 and 2.5μg RNA in 50μl was used. If less than 50μl mRNA was required the template shortfall was made up to the 5μl volume using RNase-free water.

A 2x mastermix was prepared containing 10μl of 10x reverse transcriptase buffer, 4μl of 25x dNTPs, 10μl of 10x random primer and 5μl of multiscribe reverse transcriptase (50U/μl) per mRNA sample being reverse transcribed into cDNA. The 2x mastermix (50μl) was added to the RNA and RNase-free water to give a total volume of 100μl per sample to be transcribed. Tubes were incubated for 10min at 25°C followed by 2h at 37°C on a thermocycler (PTC-200, Peltier Thermal Cycler, MJ Research, Biosciences Ireland).

2.4.4.4 Quantitative Real Time Polymerase Chain Reaction (QPCR)

QPCR primers and probes were delivered as lPre-Developed TaqMan® Gene Expression Assays for the rat genes listed in Table 2.1. QPCR was performed on Applied Biosystems ABI Prism 7300 Real Time Sequence Detection System v1.3.1 in 96-well format with 25μl reaction volume per well. cDNA (10μl) was diluted 1:4 with RNase-free water, and 10μl of this was mixed with 12.5μl of Taqman Universal PCR Mastermix (Applied Biosystems, Germany), 1.25μl of the respective target gene assay and 1.25μl of the endogenous control β-actin (Part No. 4352340E, Applied Biosystems, Germany), resulting in a total volume of 25μl. Each sample was measured in either duplicate or triplicate in a single QPCR run. The following conditions were run for 40 cycles: 2min at 50°C, 10min at 95°C and for each cycle: 15sec at 95°C for denaturation and 1min at 60°C for transcription. Gene expression values were calculated using the efficiency-corrected comparative CT method, determining target gene expression relative to β-actin endogenous control expression and relative to the control sample.
### Table

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<td>IFN-γ</td>
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**Supplier:** Applied Biosystems, Germany

#### 2.4.5 Western Immunoblotting

**2.4.5.1 Preparation of Samples for Immunoblotting**

In some studies, the protein expression of Nogo-A, Nogo-B, NgR, HMGB1 and synaptophysin were analysed. Samples were prepared from either cortical or hippocampal prepared homogenates. All samples were diluted to equalise for protein concentration. Aliquots (100µl; ~1mg/ml) were added to NuPAGE® lithium dodecyl sulphate (LDS) sample buffer (25µl; 7% lauryl alcohol sulphate 7%, 13% lithium salt) and NuPAGE® reducing agent (10µl; 15% DTT; Invitrogen, UK). Sample preparations were denatured at 70°C for 10min, and stored at -20°C until required for immunoblotting.

#### 2.4.5.2 SDS - Polyacrylamide Electrophoresis (SDS-PAGE)

NuPAGE® Novex Bis-Tris pre-cast gel cassettes (Invitrogen, UK) containing a sodium dodecyl sulphate (SDS) concentration of either 4%, 10%, 12% or 4-12% were used in these experiments. They were inserted in an electrophoretic unit (XCell SureLock™ Mini-Cell System, Invitrogen, UK) and the upper and lower chambers of the unit were filled with 200ml and 600ml 1X NuPAGE® MOPS SDS Running Buffer (Invitrogen, UK), respectively. Due to the addition of the reducing agent to the samples,
NuPAGE® antioxidant (500μl) was added to the running buffer in the upper buffer chamber.

Denatured samples (10μl) were loaded into the wells of the gels. Pre-stained molecular weight markers (5μl; Biorad Dual Colour Standard, UK and Biorad Kaleidoscope Standard, UK) were added to each gel. The lid of the Mini-Cell System was aligned on the buffer core and a constant current of 170V for 70min was applied.

2.4.5.3 Western Transfer Protocol

For each gel run, 2 pieces of filter paper (Whatman, UK), 2 blotting pads (Invitrogen, UK) and 1 piece of Protran® Nitrocellulose Transfer Membrane (Whatman Schleicher and Schnell, Germany) were pre-soaked in 1X NuPAGE® Transfer Buffer (Invitrogen, UK). Following protein separation, the gel was placed onto a pre-soaked nitrocellulose membrane, and the 2 filter papers were placed either side of the gel and membrane forming a ‘sandwich’. The sandwich was placed in the XCell II™ Blot Module and into the XCell transferring unit. The blot module was filled with transfer buffer (NUPAGE) containing the suggested volume of NuPAGE® antioxidant (1ml/1L transfer buffer) until the gel/membrane assembly was covered. The outer buffer chamber was filled with 650ml distilled water. The lid of the unit was aligned and a constant current of 30V was applied to the transferring unit for 65min.

2.4.5.4 Western Immunoblot Analysis

The membranes were blocked in 20ml of Tris-buffered saline (TBS-T) containing either 5% BSA or Marvel Milk on a ‘rock and roller’ shaker for 2h at RT or overnight at 4°C. Blocked membranes were washed 3 times in 10ml of TBS-T for 15min and incubated for 2h in the presence of primary antibody (10ml; see Table 2.2) that specifically targeted the molecule of interest on the ‘rock and roller’ shaker. The membranes were washed 3 times in 10ml of TBS-T for 15min and incubated for 1h with the secondary antibody (10ml; see Table 2.2) on the ‘rock and roller’ shaker. The antibodies were diluted as summarised in Table 2.2. The membranes were washed 6 times in TBS-T (10ml) for 10min.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Size</th>
<th>Primary</th>
<th>Secondary</th>
<th>Exposure</th>
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57
Table 2.2. Antibody Dilution Factors for Western Immunoblotting.

### 2.4.5.5 Development of Western Immunoblot

Enhanced Chemiluminescence (ECL) (Amersham, UK) or SuperSignal West Dura Extended Duration Substrate (Perbio Pierce Thermo Scientific, USA) was used to visualise the protein complexes. After 5min incubation at RT with either of the detection systems, the immunoblots were exposed to 5 X 7 inches photographic CL-Xposure™ Clear Blue X-ray Film (Perbio Pierce Thermo Scientific, USA) at RT in the dark in order to detect the immunoreactive bands on the membranes. The films were exposed to the membrane for varying lengths of time to obtain the optimal image. The X-ray films were processed with the use of an AGFA CP1000 Developer.

Results from the development of the films were photographed and quantified using the Gel-Doc-It Imaging System Ultraviolet Transilluminator. Protein band intensity was assessed by densitometry using the Gel-Pro application within the software package Labworks™ Image Acquisition and Analysis (Version 4.6; UVP BiolImaging Systems Ltd., UK). All blots were stripped by incubating for 5min in Reblot Plus Strong Solution (Chemicon International, USA) on the rock and roller shaker and blots were incubating in actin antibody (10ml; see Table 2.2) which served as loading controls. The final value was a ratio of the specific protein band to the loading control.

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<tr>
<th></th>
<th>(kDa)</th>
<th>1:100 1% Marvel Milk</th>
<th>1:1000 α-rabbit, 1% Marvel Milk</th>
<th>Time</th>
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</thead>
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<td>1:1000 α-mouse, 1% BSA</td>
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**Supplier:** See Appendix II
2.4.6 Cell Sorting by MACS

Using magnetic beads and a PE-conjugated primary antibody for NKp30, a cell surface marker for NK cells, NK cells were magnetically sorted from rat spleens by MACS using a autoMACS™ Separator (Miltenyi Biotec, Germany). Tissue was passed through a cell strainer (70μm) with MACS buffer to obtain a single cell suspension. The cells were centrifuged at 170 x g for 10min, the pellet was resuspended in collagenase D (1mg/ml) and DNase I (200μg/ml) in 5ml of PBS for 30min at 37°C and cells were counted. A minimum of $1 \times 10^7$ cells/ml was used.

The cells were centrifuged at 300 x g for 10min. The supernatant was aspirated and the pellet was resuspended in ice-cold MACS buffer (100μl per $1 \times 10^7$ cells/ml). NKp30 fluorescently-tagged antibody (1:30 dilution) was added to this volume. Cell suspensions were mixed and incubated for 15min at 4°C in the dark. Excess antibody was removed by washing each sample twice in MACS buffer (1-2mls MACS buffer per $1 \times 10^7$ cells/ml) and samples were centrifuged at 300 x g for 10min between washes. The supernatant was aspirated and the pellet was resuspended in ice-cold MACS buffer (80μl per $1 \times 10^7$ cells/ml). Anti-PE microbeads (20μl per $1 \times 10^7$ cells/ml; Miltenyi Biotec, Germany) were added to the resuspended cells, mixed and incubated for 15min at 4°C in the dark, washed in MACS buffer (1-2mls MACS buffer per $1 \times 10^7$ cells/ml) and centrifuged at 300 x g for 10min between washes. The supernatant was aspirated and the pellet was resuspended up to $1 \times 10^8$ cells/ml in 500μl of MACS buffer. Samples were run through the autoMACS™ Separator and the resulting cells were used.

2.4.7 Flow Cytometry

Cell surface markers were assessed by flow cytometry (DAKO CyANADP flow cytometer) in NK cells, and also in microglia prepared from brain tissue of young, middle aged and aged rats. The flow cytometer was calibrated using Flow-Check Fluorospheres (Beckman Coulter, Ireland). Brain tissue was passed through a cell strainer (70μm) with DMEM to obtain a single cell suspension. The cells were centrifuged at 170 x g for 10min, and the pellet was resuspended in collagenase D
(1mg/ml) and DNase I (200µg/ml) in 5ml of PBS for 30min at 37°C. After 30min cells were centrifuged at 170 x g for 5min. Pellets were resuspended in 9ml of 1.088g/ml Percoll. This was underlaid with 5ml of 1.122g/ml Percoll, and overlaid with 9ml of 1.072g/ml Percoll. The latter was overlaid with 9ml of 1.030g/ml Percoll and finally this upper layer was overlaid with 9ml of PBS. Samples were centrifuged at 1250 x g for 45min at 18°C. The layer found between the 1.088:1.072 gradient barrier interface and 1.072:1.030 gradient barrier interface, which corresponds to mononuclear cells, was carefully removed and centrifuged at 170 x g for 5min. The sample pellets were washed 3 times by resuspending them in 6ml of ice-cold FACS buffer and centrifuged at 170 x g for 5min. The pellets were resuspended in 6ml of FACS block, incubated for 15min at 4°C in the dark, and washed twice. Pellets were resuspended in 6ml of FACS buffer; 500µl cell suspension was added to FACS tubes and washed. The volume remaining after the FACS buffer was decanted from the tubes was 100µl.

Cells were incubated in fluorescent-tagged antibodies (Table 2.3) for CD161a and NKp30 (NK cell markers), and OX6, CD45 and CD11b (markers of microglial activation). Cell suspensions were incubated for 20-30min at RT in the dark, washed in FACS buffer (5ml/tube) and centrifuged at 170 x g for 5min between washes. Compensation beads were used to compensate between fluorescent channels. Immunofluorescence analysis was then performed and the results analysed using Summit software.

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD161a</td>
<td>1:30</td>
</tr>
<tr>
<td>NKp30</td>
<td>1:30</td>
</tr>
<tr>
<td>OX6</td>
<td>1:100</td>
</tr>
<tr>
<td>CD11b</td>
<td>1:100</td>
</tr>
<tr>
<td>CD45</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Supplier: See Appendix II

Table 2.3. Antibody Dilution Factors for Flow Cytometry.
2.5 Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Data were analysed, where appropriate, using a 1-tailed or 2-tailed Student’s t-test for independent means or a 1-way analysis of variance (ANOVA) to determine whether significant differences existed between conditions. If the analysis by ANOVA indicated significance (when $p<0.05$), a post-hoc Student Newman-Keuls test analysis was used to determine which conditions were significantly different from each other (Graphpad Prism, USA).
Chapter 3

Assessment of microglial activation states and their instigators
3.1 Introduction

There is mounting evidence associating ageing with an increase in inflammatory changes and a decline in biological function (Murray & Lynch, 1998; Renshaw et al., 2002; Licastro et al., 2005; Nikolich-Zugich, 2005). Inflammatory changes in the brain are predominantly caused by the activation of microglia and their subsequent release of pro-inflammatory cytokines. These cytokines can transmit signals that can lead to an impairment of synaptic function and eventual neurodegeneration. The expression of markers of microglial activation has been demonstrated in various studies (Rozovsky et al., 1998; Godbout et al., 2005; Griffin et al., 2006; Lynch et al., 2007; Lyons et al., 2007a; Clarke et al., 2008; Downer et al., 2008). Additionally, research has confirmed that activated microglia secrete inflammatory cytokines and chemokines in the aged brain (Murray & Lynch, 1998; Hauss-Wegrzyniak et al., 1999; Sloane et al., 1999; Kullberg et al., 2001; Maher et al., 2005), and subsequent work has observed an increase in several inflammatory cytokines with age (Sheng et al., 1998; Conde & Streit, 2006; Baune et al., 2009). Moreover, it appears ageing can cause alterations in the phagocytic activity of macrophages (Plowden et al., 2004). Impairments in synaptic plasticity as revealed by deficits in LTP and poor performance in spatial learning tasks have been associated with increases in inflammatory changes in the aged brain (Lynch & Lynch, 2002; Maher et al., 2005; Griffin et al., 2006). It appears that ageing can have adverse effects on a wide range of biological functions, many of which have been associated with microglial activation. Therefore, the first aim of this study was to assess microglial activation in age-related inflammation.

Based on the considerable evidence pertaining to a link between age and inflammation, it is believed that age-related inflammation is coupled with an increased susceptibility in the onset of neurodegenerative diseases (Heininger, 2000; Streit, 2004; Licastro et al., 2005). However, it is not fully understood what is/are the causative element(s) of microglial activation and subsequent neurodegeneration. The second aim of this study was to investigate whether or not particular cytokines could be responsible for the activation of microglia in the brain.
3.2 Methods

Groups of young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) rats (n>6) were anaesthetised with isoflurane (4% induction, 1.5-2% maintenance) in 100% oxygen for the purpose of MR imaging experiments. Rats were allowed to recover for 48h before being anaesthetised by ip injection of urethane (1.5g/kg, 33% w/v), and transcardially perfused with saline. Brains were removed from the rats and a hemi-section of brain was used to isolate cells for FACS analysis, and cortical and hippocampal tissue was harvested for later mRNA analysis. Cortical mixed glia and purified microglia were prepared from 1-day old Wistar rats. Glial preparations were cultured in DMEM for 12-14 days at 37°C before treatment (see section 2.3.2). Treatments included incubation of IFN-γ and/or TNF-α at concentrations of 50ng/ml for 24h at 37°C in 5% CO₂: 95% air before harvesting.

3.3 Results

The objective of this study was to establish whether the effect of age can result in an inflammatory profile, and to determine whether resultant mRNA and protein expression are paralleled in both the cortex and hippocampus. The mean values ± SEM for all parameters are presented in tables 3.1, 3.2, 3.3 and 3.4.

*Effect of age on MHC II mRNA expression in the rat cortex and hippocampus.*

During microglial activation, microglia undergo a morphological transformation, which is associated with the upregulation of cell surface receptors, such as the antigen presenting molecule, MHC II. The present data demonstrate that MHC II mRNA expression was similar in cortical tissue prepared from young, middle-aged and aged, rats (Figure 3.1a). However MHC II mRNA expression was significantly increased in hippocampal tissue from aged rats, compared with middle-aged, rats (*p<0.05; ANOVA; Figure 3.1b).

The percentage of OX-6⁺ cells isolated from a population of monocytic cells gated on size and granularity were assessed in hemisections of brain tissue, and the
mean data shows that there was a significant increase in aged, compared with young, rats (*p<0.05; Student’s t-test for independent means; Figure 3.2).

Effect of age on CD68 mRNA, TLR-2 mRNA and TLR-4 mRNA expression in the rat cortex and hippocampus.

There is some evidence suggesting that there are several activation states of microglia, such as one with phagocytic properties. Phagocytosis involves the digestive removal of foreign bodies or cell debris, and it is known that activation of lysosomes occurs following phagocytosis. One marker of phagocytic microglia is the lysosomal protein, CD68 (Ramprasad et al., 1996) and in this study, CD68 mRNA expression was significantly increased in cortical (a) and hippocampal (b) tissue prepared from aged, compared with young (*p<0.05; Student’s t-test for independent means; **p<0.01; ANOVA) or middle-aged (†p<0.05; †††p<0.001; ANOVA; Figure 3.3), rats.

Certain TLRs, found on the surfaces of activated microglia, have also been associated with phagocytosis (Doyle et al., 2004). Here mRNA expression of TLR2 and TLR4 were also assessed in snap-frozen cortical and hippocampal tissue prepared from young, middle-aged and aged rats. TLR2 mRNA expression was similar in cortical tissue prepared from young, middle-aged and aged rats, but expression was significantly increased in hippocampal tissue prepared from aged, compared with middle-aged, rats (*p<0.05; Student’s t-test for independent means; Figure 3.4). In contrast, TLR4 mRNA expression was significantly decreased in cortical tissue prepared from aged or middle-aged, compared with young, rats (*p<0.05; ANOVA; Figure 3.5a) although expression was significantly increased in hippocampal tissue prepared from aged, compared with middle-aged, rats (*p<0.05; Student’s t-test for independent means; Figure 3.5b).

Effect of age on IFN-γ mRNA in the rat cortex and hippocampus.

IFN-γ mRNA expression was assessed in snap-frozen cortical and hippocampal tissue prepared from young, middle-aged and aged rats. IFN-γ mRNA was similar in cortical tissue prepared from young, middle-aged or aged rats (Figure 3.6a). In contrast, IFN-γ mRNA expression was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (*p<0.05; Student’s t-test for independent means; Figure 3.6b).
Effect of age on TNF-α mRNA in the rat cortex and hippocampus.

It is widely accepted that glial cells are the primary source of pro-inflammatory cytokines such as TNF-α in the CNS. It is also known that TNF-α is a key instigator in immune-mediated inflammation. TNF-α mRNA was assessed in snap-frozen cortical and hippocampal tissue prepared from young, middle-aged and aged rats. Expression was significantly increased in cortical tissue prepared from aged, compared with middle-aged, rats (*p<0.05; Student’s t-test for independent means; Figure 3.7a;) and similarly there was a significant increase in TNF-α mRNA expression in hippocampal tissue prepared from aged, compared with young (*p<0.05; ANOVA) or middle-aged, rats (+p<0.05; ANOVA; Figure 3.7b).

<table>
<thead>
<tr>
<th>Target</th>
<th>Young</th>
<th>Middle-Aged</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II mRNA</td>
<td>0.63 ± 0.04</td>
<td>0.70 ± 0.04</td>
<td>0.84 ± 0.09</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD68 mRNA</td>
<td>0.50 ± 0.19</td>
<td>0.62 ± 0.08</td>
<td>1.19 ± 0.04</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2 mRNA</td>
<td>0.21 ± 0.07</td>
<td>0.15 ± 0.02</td>
<td>0.32 ± 0.08</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4 mRNA</td>
<td>0.62 ± 0.08</td>
<td>0.32 ± 0.02</td>
<td>0.44 ± 0.05</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ mRNA</td>
<td>0.29 ± 0.06</td>
<td>0.34 ± 0.03</td>
<td>0.61 ± 0.29</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α mRNA</td>
<td>0.89 ± 0.15</td>
<td>0.49 ± 0.03</td>
<td>1.53 ± 0.32</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Statistical tests performed were either 1-way ANOVA or Student’s t-test for independent means.

Table 3.1. Effect of age on several markers in cortical tissue. Data are expressed as means ± standard error of the mean (n≥5).
### Table 3.2. Effect of age on several markers in hippocampal tissue. Data are expressed as means ± standard error of the mean (n=6).

<table>
<thead>
<tr>
<th>Target</th>
<th>Young</th>
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<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II mRNA</td>
<td>0.78 ± 0.04</td>
<td>0.70 ± 0.04</td>
<td>0.93 ± 0.06</td>
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<tr>
<td>(arbitrary units)</td>
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</tr>
<tr>
<td>CD68 mRNA</td>
<td>0.18 ± 0.04</td>
<td>0.31 ± 0.04</td>
<td>0.56 ± 0.09</td>
</tr>
<tr>
<td>(arbitrary units)</td>
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<td></td>
</tr>
<tr>
<td>TLR2 mRNA</td>
<td>1.39 ± 0.54</td>
<td>1.25 ± 0.16</td>
<td>2.59 ± 0.54</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4 mRNA</td>
<td>0.45 ± 0.17</td>
<td>0.24 ± 0.01</td>
<td>0.57 ± 0.12</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ mRNA</td>
<td>1.19 ± 0.06</td>
<td>1.48 ± 0.28</td>
<td>2.06 ± 0.27</td>
</tr>
<tr>
<td>(arbitrary units)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α mRNA</td>
<td>0.56 ± 0.11</td>
<td>0.57 ± 0.04</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Statistical tests performed were either 1-way ANOVA or Student's t-test for independent means.

### Table 3.3. Effect of age on OX-6^ cells in whole brain tissue. Data are expressed as percentage means ± standard error of the mean (n≥6).

<table>
<thead>
<tr>
<th>Target</th>
<th>Young</th>
<th>Middle-Aged</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX-6^ cells (%)</td>
<td>1.21 ± 0.18</td>
<td>1.82 ± 0.41</td>
<td>1.75 ± 0.11</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Statistical test performed was the Student's t-test for independent means.
**Effect of IFN-γ and TNF-α on markers of microglial activation in vitro**

It has been reported that IFN-γ is a potent activator of microglia (Nguyen & Benveniste, 2002) while neuronally-derived TNF-α also stimulates microglial activation (Janelbins et al., 2008). Since age-related increases in these cytokines were observed, it was considered that they may act alone or together to contribute to this activation. Therefore their effect was assessed *in vitro* in mixed glia and purified microglia on cell surface markers. Both IFN-γ and TNF-α, alone and in combination, significantly increased expression of MHC II mRNA (a) and CD11b mRNA (b) in mixed glial cultures (*p<0.05; Student’s t-test for independent means; **p<0.01; ***p<0.001; ANOVA; Figure 3.8) but no additive effect was observed.

IFN-γ significantly increased expression of CD40 mRNA (b) but not CD11b (a) in purified microglia (***p<0.001; ANOVA; Figure 3.9) whereas TNF-α significantly increased mRNA expression of both CD11b and CD40 (***p<0.001; ANOVA; Figure 3.9). While no additive effect was observed on expression of CD11b, IFN-γ and TNF-α synergised to further significantly increase expression of CD40 mRNA (***p<0.001; versus IFN-γ alone or TNF-α alone; Figure 3.9b).

<table>
<thead>
<tr>
<th>Target</th>
<th>Cell Type</th>
<th>Control</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IFN-γ + TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II mRNA</td>
<td>Cortical</td>
<td>0.751 ±</td>
<td>1.414 ±</td>
<td>1.536 ±</td>
<td>1.549 ±</td>
</tr>
<tr>
<td></td>
<td>mixed glia</td>
<td>1.128</td>
<td>0.044</td>
<td>0.094</td>
<td>0.135</td>
</tr>
<tr>
<td>CD11b mRNA</td>
<td>Cortical</td>
<td>0.861 ±</td>
<td>1.417 ±</td>
<td>5.320 ±</td>
<td>4.597 ±</td>
</tr>
<tr>
<td></td>
<td>mixed glia</td>
<td>0.165</td>
<td>0.145</td>
<td>0.723</td>
<td>1.227</td>
</tr>
<tr>
<td>CD11b mRNA</td>
<td>Cortical</td>
<td>0.970 ±</td>
<td>1.062 ±</td>
<td>1.588 ±</td>
<td>1.618 ±</td>
</tr>
<tr>
<td></td>
<td>microglia</td>
<td>0.074</td>
<td>0.091</td>
<td>0.067</td>
<td>0.108</td>
</tr>
<tr>
<td>CD40 mRNA</td>
<td>Cortical</td>
<td>0.911 ±</td>
<td>1.541 ±</td>
<td>1.745 ±</td>
<td>2.773 ±</td>
</tr>
<tr>
<td></td>
<td>microglia</td>
<td>0.075</td>
<td>0.059</td>
<td>0.147</td>
<td>0.078</td>
</tr>
</tbody>
</table>

Table 3.4. Effect of IFN-γ and TNF-α *in vitro*. Data are expressed as means ± standard error of the mean (n=6).
Cortical and hippocampal tissue was dissected free from young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) male Wistar rats that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, homogenised and assessed for MHC II mRNA by QPCR. MHC II mRNA was similar in cortical tissue prepared from young, middle-aged and aged rats (a) but was significantly increased in hippocampal tissue prepared from aged, compared with middle-aged, rats (b; *p<0.05; ANOVA). Data are expressed as a ratio of MHC II mRNA:β-actin mRNA, and means ± SEM (n=6) are presented.
(a) **Cortex**

![Bar chart showing MHC II mRNA levels in young, middle-aged, and aged groups for cortex.](image)

(b) **Hippocampus**

![Bar chart showing MHC II mRNA levels in young, middle-aged, and aged groups for hippocampus.](image)

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Figure 3.2. Effect of age on OX-6 expression in the rat brain.

Monocytes were isolated from Percoll gradients carried out on male Wistar rat brain hemispheres that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, and assessed for a marker of activated microglial cells in percentage of total monocytes by flow cytometry. OX-6$^+$ cells were significantly increased in hemi-sectioned brain prepared from aged (22-25 months), compared with young (3-4 months), rats (*p<0.05; Student’s t-test for independent means). Data are presented as means ± SEM (n≥6).
Figure 3.3. Effect of age on CD68 mRNA in rat cortex and hippocampus.

Cortical and hippocampal tissue was dissected free from young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) male Wistar rats that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, homogenised and assessed for CD68 mRNA by QPCR.

(a) CD68 mRNA was significantly increased in cortical tissue prepared from aged, compared with young (**p<0.01; ANOVA) or middle-aged (***p<0.001; ANOVA) rats.

(b) CD68 mRNA was significantly increased in hippocampal tissue prepared from aged, compared with young (*p<0.05; Student’s t-test for independent means), or middle-aged (*p<0.05; ANOVA), rats. Data are expressed as a ratio of CD68 mRNA:β-actin mRNA, and means ± SEM (n=6) are presented.
(a) **Cortex**

![Graph showing CD68 mRNA levels in young, middle-aged, and aged groups for cortex. The graph indicates a significant increase in CD68 mRNA levels in the aged group compared to the young and middle-aged groups.]  

(b) **Hippocampus**

![Graph showing CD68 mRNA levels in young, middle-aged, and aged groups for hippocampus. The graph indicates a significant increase in CD68 mRNA levels in the aged group compared to the young and middle-aged groups.]
Figure 3.4. Effect of age on TLR2 mRNA in rat cortex and hippocampus.
Cortical and hippocampal tissue was dissected free from young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) male Wistar rats that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, homogenised and assessed for TLR2 mRNA by QPCR. TLR2 mRNA was similar in cortical tissue prepared from young, middle-aged and aged rats (a) but was significantly increased in hippocampal tissue prepared from aged, compared with middle-aged, rats (b; *p<0.05; Student’s t-test for independent means). Data are expressed as a ratio of TLR2 mRNA:β-actin mRNA, and means ± SEM (n≥5) are presented.
Figure 3.5. Effect of age on TLR4 mRNA in rat cortex and hippocampus.

Cortical and hippocampal tissue was dissected free from young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) male Wistar rats that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, homogenised and assessed for TLR4 mRNA by QPCR. TLR4 mRNA was significantly decreased in cortical tissue prepared from aged or middle-aged compared with young, rats (a; *p<0.05; **p<0.01; ANOVA), whereas expression was significantly increased in hippocampal tissue prepared from aged, compared with middle-aged, rats (*p<0.05; Student’s t-test for independent means). Data are expressed as a ratio of TLR4 mRNA:β-actin mRNA, and means ± SEM (n≥5) are presented.
(a) **Cortex**

TLR4 mRNA

Young | Middle Aged | Aged

(b) **Hippocampus**

TLR4 mRNA

Young | Middle Aged | Aged

*Significant difference

**Highly significant difference
Figure 3.6. Effect of age on IFN-γ mRNA in rat cortex and hippocampus.

Cortical and hippocampal tissue was dissected free from young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) male Wistar rats that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, homogenised and assessed for IFN-γ mRNA by QPCR. IFN-γ mRNA was similar in cortical tissue prepared from aged, middle-aged and young rats (a), but was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (b; *p<0.05; Student’s t-test for independent means). Data are expressed as a ratio of IFN-γ mRNA:β-actin mRNA, and means ± SEM (n=6) are presented.
(a) Cortex

IFN-\(\gamma\) mRNA

<table>
<thead>
<tr>
<th>Age</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>0.0</td>
</tr>
<tr>
<td>Middle Aged</td>
<td>0.5</td>
</tr>
<tr>
<td>Aged</td>
<td>1.0</td>
</tr>
</tbody>
</table>

(b) Hippocampus

IFN-\(\gamma\) mRNA

<table>
<thead>
<tr>
<th>Age</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>0.0</td>
</tr>
<tr>
<td>Middle Aged</td>
<td>1.5</td>
</tr>
<tr>
<td>Aged</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Note: * indicates a significant difference.
Figure 3.7. Effect of age on TNF-α mRNA in rat cortex and hippocampus.

Cortical and hippocampal tissue was dissected free from young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) male Wistar rats that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, homogenised and assessed for TNF-α mRNA by QP/CY. TNF-α mRNA was significantly increased in cortical tissue (a; *p<0.05; Student’s t-test for independent means) and hippocampal tissue prepared from aged, compared with young or middle-aged, rats (a; *p<0.05; **p<0.05 ANOVA). Data are expressed as a ratio of TNF-α mRNA:β-actin mRNA, and means ± SEM (n=6) are presented.
(a) Cortex

(b) Hippocampus
Cortical mixed glia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with IFN-γ (50ng/ml) and/or TNF-α (50ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for MHC II mRNA and CD11b mRNA by QPCR. MHC II mRNA (a) and CD11b mRNA (b) were significantly increased in rat cortical mixed glial cells treated with either IFN-γ (**p<0.01; ANOVA; *p<0.05; Student’s t-test for independent means), TNF-α (***p<0.001; **p<0.01; ANOVA) or both IFN-γ and TNF-α (***p<0.01. ANOVA) compared to control. Data are expressed as a ratio of MHC II mRNA:β-actin mRNA and CD11b mRNA:β-actin mRNA, respectively, and means ± SEM (n=6) are presented.
Figure 3.9. Effect of IFN-γ and TNF-α on CD11b and CD40 mRNA in purified microglia.

Purified cortical microglia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with IFN-γ (50ng/ml) and/or TNF-α (50ng/ml) for 24h at 37°C in 5% CO₂:95% air. Once harvested, lysates were assessed for CD11b mRNA and CD40 mRNA by QPCR.

(a) CD11b mRNA was not affected by IFN-γ but was significantly increased in purified microglia prepared from neonatal rats by TNF-α and by a combination of IFN-γ + TNF-α compared with control (**p<0.001; ANOVA).

(b) CD40 mRNA was significantly increased by IFN-γ and TNF-α alone and in combination (**p<0.001; ANOVA). The effect of the combination of cytokines was significantly greater than either alone (***p<0.001; ANOVA). Data are expressed as a ratio of CD11b mRNA:β-actin mRNA and CD40 mRNA:β-actin mRNA, respectively, and means ± SEM (n=6) are presented.
(a) CD11b mRNA

Control  IFN-γ  TNF-α  IFN-γ + TNF-α

(b) CD40 mRNA

Control  IFN-γ  TNF-α  IFN-γ + TNF-α
3.4 Discussion

The aim of this work was to investigate age-related changes in microglial activation. The data show that several markers of microglial activation are upregulated with age and that IFN-γ and/or TNF-α may be instrumental in stimulating this activation.

In order to establish whether there was a presence of activated microglia in the aged brain or not, the mRNA expression of MHC II, an antigen presentation molecule, was assessed in young and aged rats, and its expression was found to be significantly increased with age within the hippocampus. Numerous studies by this lab and others have reported a similar age-related increase in MHC II (Perry et al., 1993; Morgan et al., 1997; Godbout et al., 2005; Griffin et al., 2006; Lynch et al., 2007; Lyons et al., 2007a; Clarke et al., 2008; Downer et al., 2008; Martin et al., 2009). This data is further supported by the finding that the number of OX-6^ cells increases with age.

The expression of MHC II in the aged brain indicates an increase in potential binding and activation of T cells. The MHC II antigen serves to present antigen by interacting with a TCR found on the surface of T cells, and when accompanied by co-stimulatory molecules, this engagement activates the T cell and triggers a cascade of signalling events critical to the immune response (Brown et al., 1993; Germain & Margulies, 1993; Cresswell, 1994; Germain, 1994; Bentley et al., 1995; Fields et al., 1995; Rhode et al., 1996; Watts, 1997; Davis et al., 1998; Chambers, 2001). Some of these events include phosphorylation of receptor subunits, docking of receptor-associated signalling and adapter proteins, activation of cytoplasmic signalling cascades, and upregulation of several gene products (Cantrell, 1996; Qian & Weiss, 1997).

The presence and upregulation of MHC II in the aged brain indicates the presence of microglia in an activated state (Kreutzberg, 1996; Stoll & Jander, 1999; Aloisi, 2001; Benveniste et al., 2004), however we now know there are several states of activation in microglia. It has been suggested that microglia with antigen presenting capabilities can be classified into one of two types: microglia in an activated non-phagocytic state or those in an activated phagocytic state. The former may be a graded response, which may progress to the latter state and clearly the phenotype of the cells
differ in that the graded activation state represents a cell without phagocytic capabilities. Phagocytosis is a necessary process within the brain and is necessary during tissue turnover, development and tissue remodelling (Cohen, 1991; Han et al., 1993; Hopkinson-Woolley et al., 1994), and in this study the expression of CD68, a phagocytic marker, was assessed to determine whether the activated microglia in the aged brain have indeed a fully activated phagocytic profile. The age-related increases in MHC II accompanied by an increase in the mRNA expression of CD68, suggests that the activated microglia in the aged brain are either in the amoeboid state or the fully activated phagocytic state, as both have phagocytic capabilities (Ferrer et al., 1990; Kreutzberg, 1996; Aloisi, 2001; Christensen et al., 2006). However these two types of microglial activation states differ in that amoeboid microglia have been proposed not to be capable of antigen presentation or capable of producing inflammatory cytokines like other activated microglia (Kreutzberg, 1996). Amoeboid microglia survey the microenvironment phagocytosing apoptotic debris during development when brain rewiring leads to apoptosis of some cells. Fearn and colleagues suggested that phagocytosis in the aged rat brain may be a result of continuing dendritic or synaptic remodeling (Perry et al., 1993). Interestingly, a recent study has observed that, distinct from the transformation of resting microglia into activated microglia in the white matter of the brain, the activation of resting microglia after degeneration in grey matter was shown to involve CD68 but without any morphological transformation (McKay et al., 2007). This may suggest an additional microglial activation state in the conversion from the resting state to the motile activated amoeboid-like state.

Many findings support the dual expression of MHC II and CD68 in activated microglia, which is reported in this study, and increases in both markers were observed with age (Vaughan & Peters, 1974; Graeber et al., 1990; da Silva & Gordon, 1999; Wong et al., 2005). Interestingly, the interaction of CD68 and MHC II has been highlighted by the observation that MHC II colocalises with CD68 (Schmitt et al., 2000; Cho et al., 2006). Recent work from this lab demonstrated by flow cytometry that phagocytic activity by CD11b-positive cells is increased with age and is accompanied by an increase in the expression of CD68 and other cell surface markers of microglial activation, such as CD11b and CD40 (O’Reilly et al, unpublished).

In addition to the association of CD68 with phagocytic activity in the brain, various TLRs have also been implicated in the phagocytic process. Generally TLR signalling is known to initiate the transcription of cytokines and co-stimulatory
molecules via the recruitment and interaction of the adaptor protein myeloid differentiation factor 88 (MyD88) after the recognition of PAMPS, which leads to further recruitment of kinases that subsequently enables the translocation of NF-κB to the nucleus, mediating an increase in pro-inflammatory responses (Liew et al., 2005). However, TLR signalling has additionally been shown to be involved in the regulation of the phagosome-lysosome endocytic pathway by enhancing antigen presentation and host defence (Blander & Medzhitov, 2004; Blander, 2007b, a). Blander and Medzhitov detailed evidence demonstrating the ability of TLR2 and TLR4 signalling to control several aspects of phagocytosis, including antigen presentation, the enhancement of internalisation, and phagolysosomal fusion, thus enabling an inducible form of phagosome maturation (Blander & Medzhitov, 2004, 2006). So in light of the literature and the evidence for phagocytosis in this study, TLR2 mRNA expression was assessed in tissue from young and aged rats and the data confirm significant increases in hippocampus with age. This finding is supported by in vitro studies describing the ability of activated TLR2 to trigger phagocytosis in microglia (Tahara et al., 2006; Ribes et al., 2009). Microglial phagocytosis has been associated with the removal of Aβ deposits in models of AD (Okura et al., 2008), and recent work suggested that there was an increase in the formation of Aβ in APP transgenic mice when TLR2 was lacking (Richard et al., 2008). Furthermore TLR2 has been reported to be present on the membrane of phagosomes forming around Saccharomyces cerevisiae (Underhill et al., 1999).

In the periphery it has been demonstrated that TLR4 is necessary for phagocytosis (Neal et al., 2006), so the expression of TLR4 mRNA was also assessed in this study, and the data show that there is a significant increase with age in hippocampus. TLR4 signalling has been implicated in various processes involved in neurodegeneration including microglial activation and neuronal death (Lehnardt et al., 2003; Fernandez-Lizarbe et al., 2009). Evidence has also shown that TLR4 signalling can induce apoptosis of activated microglia, while TLR2 does not (Liu et al., 2001; Jung et al., 2005). This was shown to occur during overexpression of activated microglia, and it was suggested that it may be a self-regulated necessity in order to prevent or limit bystander killing of vulnerable neurons by released immune modulators. However, due to the inherent importance of microglia and their limited replenishment mechanism, depleting microglia in the brain could have significant
consequences in terms of hindering inflammatory challenges and tissue repair. The data also show a significant decrease with age in cortex; however the values of the young group may be unreliable. This lack of confidence in the young group stems from doubt over the accuracy of their age based on above average body weights.

The data thus far suggest the presence of several states of microglial activation in the aged brain. These states confer capabilities that include the upregulation of T cell recruitment and phagocytic activity. Having established an upregulation in relation to cell surface markers of microglial activation and also phagocytosis, the question of whether there were any changes in pro-inflammatory cytokines arose; several inflammatory cytokines are known to impact on neurons to induce neurodegeneration (Benveniste, 1992; Banati et al., 1993; Blasko et al., 2001; Martin et al., 2002; Wang et al., 2005). The mRNA expression of two pro-inflammatory cytokines, IFN-γ and TNF-α were examined in this study and both were found to be significantly increased with age. This is not surprising based on the numerous reports documenting microglial activation coinciding with an increase in production of pro-inflammatory cytokines (Aloisi, 2001; Minagar et al., 2002). It is also provides further evidence for an activated microglial profile in the aged brain, as particular pro-inflammatory cytokines, such as TNF-α, are considered to be indicators of a heightened activation state in microglia and astrocytes (Lynch, 1998; Bruunsgaard et al., 2001; Neumann, 2001; Moynagh, 2005; Nolan et al., 2005; Lynch, 2009). Both activated non-phagocytic and activated phagocytic microglia have the ability to produce inflammatory molecules.

It has been stated many times how the activation of microglia can provide an immune response in the brain and ultimately lead to the neuronal loss associated with neurodegeneration. However, determining what might trigger the microglial activation states in this study was considered, and it was proposed that either IFN-γ or TNF-α, alone, or perhaps both working together could initiate this activated microglial response. The findings of the present study show that IFN-γ and TNF-α, both on their own and when present together, can induce the expression of cell surface markers, MHC II and CD11b in mixed glia. However, the presence of both inflammatory cytokines did not exacerbate the increases observed by either cytokine treated alone. Research has shown that CD11b is primarily expressed on microglia and monocytes and an upregulation in its expression is indicative of cell activation (Streit et al., 1999; Rock et al., 2004). Evidence from the literature supports this finding where IFN-γ has
been shown to have the ability to stimulate microglia to increase the expression of CD11b (Roy et al., 2006). TNF-α has also been found to increase CD11b expression (Steadman et al., 1996). CD11b is known to form an antigen, MAC-1, with CD18 that is involved in chemotaxis, cell-mediated cytotoxicity and motility (Weber et al., 1997; Nagai et al., 2005; Solovjov et al., 2005), and functions as a binding protein for ICAM-1, a protein that is associated with facilitating cell-cell interactions and subsequently signal transduction (Schwarz et al., 2002). ICAM-1 has also been implicated in phagocytosis in MS and EAE (Smith, 2001; Reichert & Rotshenker, 2003; Rotshenker et al., 2008).

As this assessment was undertaken in mixed glia, which contain a significant percentage of astrocytes, it cannot be irrefutably concluded that the increases in the expression markers of microglial activation were purely associated with microglia. So for the purpose of establishing whether these increases were attributed to microglia or not, the mRNA expression of CD11b and another surface marker of activated microglia, CD40, was assessed in pure microglia, and the data show both markers are significantly increased by IFN-γ and/or TNF-α. When IFN-γ and TNF-α accompany each other in tandem, the data also shows that they seem work to induce a further significant increase in CD40. Various studies support the contention that IFN-γ and TNF-α increase the expression of CD40 (Nguyen & Benveniste, 2000; Tan et al., 2002; Qin et al., 2005). When CD40 binds to its ligand, CD154, the interaction leads to the production of pro-inflammatory cytokines, such as TNF-α, thus creating a positive feedback mechanism (Stout & Suttles, 1996; Chen et al., 2006a). CD40 can enhance the ability of microglia to activate and restimulate T cells when induced by IFN-γ (Benveniste et al., 2004). The engagement of CD40 with CD154 promotes the upregulation of various co-stimulatory molecules and cytokines. As CD154 is mainly found on CD4^+ T cells, binding with CD40 expressed on antigen presenting cells, such as microglia, encourages the functional interactions between the cell types. So the data provides evidence that the presence of IFN-γ and TNF-α can induce an activated profile of microglia in the brain.

It seems reasonable to suggest that several activation states of microglia exist, including ones that increase interaction with other cells like T cells, which depends on increased cell surface markers, ones that upregulate phagocytic activation and ones that increase production of immunomodulatory molecules. The evidence presented here
suggests that microglia obtained from the brain of aged animals are primed to
upregulate interaction with T cells as MHC II is increased, are phagocytic since CD68
increases, and are capable of producing immunomodulatory molecules due to observed
increases in TNF-α. The evidence presented suggests that one possible stimulatory
factor is IFN-γ since it alone and in combination with TNF-α, increases expression of
specific cell surface markers on microglia.
Chapter 4

An investigation into the causative source of IFN-γ-induced microglial activation in ageing
4.1 Introduction

Inflammatory changes in the brain appear to contribute to the deficits in neuronal function associated with ageing and neurodegenerative disorders, and fundamental to these changes is an increase in microglial activation, which is potently stimulated by the pro-inflammatory cytokine IFN-\(\gamma\). However a cell source of IFN-\(\gamma\) in the CNS remains unknown. IFN-\(\gamma\) is predominantly produced by NK cells and Th1 cells (Schoenborn & Wilson, 2007; Uemura et al., 2009); however both cells are, under normal conditions, found in the PNS. The presence of the BBB serves to prevent invading pathogens entering the brain and interfering with normal functioning, but it has been demonstrated that the BBB can become compromised in neurodegenerative disorders and ageing (Pahnke et al., 2009; Popescu et al., 2009; Stolp & Dziegielewksa, 2009). When this occurs, the selective control of the BBB is diminished, which literally opens up the brain to infiltrating cells, immunomodulatory molecules and potentially infectious agents that normally would be excluded from the brain. Here it was hypothesised that NK cells infiltrate into the CNS via a compromised BBB and are responsible for the presence of microglia-inducing IFN-\(\gamma\). Therefore the central aim of this study was to determine if NK cells are present in the brain of aged rats and if they have the means to modulate microglial activation.

Aside from IFN-\(\gamma\)-induced microglial activation, there are several other inflammatory cytokines that can interact with microglia, activating them to produce neurotoxic factors that cause changes to the normal functioning of the CNS. One such cytokine is HMGB1, which was initially classified as a transcriptional regulatory molecule but it now appears to have a roles in neuroinflammation having been associated with inflammatory processes in the brain and with AD. There is evidence that HMGB1 is a mediator in a delayed inflammatory response (Wang et al., 1999). Perhaps because of its ability to interact with TLR2 and TLR4, a second aim of this study was to investigate HMGB1 for changes with age and possible interaction with IFN-\(\gamma\) in relation to microglial activation in glial cells.
4.2 Methods

Groups of young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) rats (n≥6) were anaesthetised with isoflurane (4% induction, 1.5-2% maintenance) in 100% oxygen for the purpose of MR imaging experiments. Rats were allowed to recover for 48h before being anaesthetised by ip injection of urethane (1.5g/kg, 33% w/v), and transcardially perfused with saline. The brain tissue harvested from the rats used in the in vivo experiments in chapter 3 were the same used in the in vivo experiments in this chapter, with the exception of any experiments by MACS or data kindly provided by Dr. Rachael Clarke. Brains were removed from the rats and a hemi-section of brain was used to isolate cells for FACS analysis, and cortical and hippocampal tissue was harvested for later mRNA and protein analysis, namely QPCR and western immunoblot/ELISA, respectively. Cortical mixed glia, purified microglia or purified astrocytes were prepared from 1-day old Wistar rats, and cultured in DMEM for 12-14 days at 37°C before treatment. Splenic NK cells were prepared from 2-3 month old Wistar rats by MACS, and cultured in RPMI overnight at 37°C before treatment (see section 2.3.2). Treatments included incubation of either HMGB1 (10ng/ml, 100ng/ml or 1000ng/ml), IFN-γ (50ng/ml), TNF-α (50ng/ml), IL-12 (10ng/ml) or IL-18 (10ng/ml) for 24h at 37°C in 5% CO₂: 95% air before harvesting.

4.3 Results

The objective of this study was to investigate whether NK cells are present in the brain of aged rats, and may therefore contribute to microglial activation which occurs with age. The mean values ± SEM for all parameters are presented in tables 4.1, 4.2 and 4.3.

*Effect of age on NK cell number in the whole rat brain.*

Freshly-dissociated monocytic cells were prepared from hemisections of brain and assessed by FACS for evidence of microglial activation. Cells were gated according to size and granularity, and the population of OX-6+ cells which also stained positively
for CD11b and CD45 was assessed in preparations obtained from young, middle-aged and aged rats. The percentage of these cells in the total number of monocytes was significantly increased in freshly-dissociated preparations obtained from middle-aged and aged rats compared with young rats (*p<0.05; **p<0.01; Student’s t-test for independent means; Figure 4.1.

Activated microglia are the primary source of pro-inflammatory changes in the brain and among the factors which potently activate these cells is IFN-γ (Nguyen & Benveniste, 2002). Analysis of IFN-γ indicated that there was no age-related change in cortical tissue (Figure 4.2a) but its concentration was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (*p<0.05; ANOVA; Figure 4.2b). This finding parallels the significant increase in IFN-γ mRNA in hippocampal tissue shown in Figure 3.6. However the cell source of IFN-γ in the CNS is not known. IFN-γ is produced by Natural Killer (NK) cells, which are traditionally found in the periphery. Using two known markers for NK cells, NKp30 and CD161a, the presence of NK cells was assessed in hemisections of brain tissue prepared from young, middle-aged and aged rats. NKp30^CD161a^ cells were isolated from a population of monocytic cells gated on size and granularity. The mean data show that there was a significant increase in the percentage of NKp30^CD161a^ cells in hemisections of brain tissue prepared from aged, compared with middle-aged, rats (*p<0.05; Student’s t-test for independent means; Figure 4.3). Due to above average body weights in the young group of rats, doubt was expressed over the accuracy of their age and so their data in the assessment of NK cell numbers was deemed potentially unreliable. For this reason, the data is presented with and without the young group included.

**Effect of cytokines on IFN-γ production in NK cells.**

It has been shown that IL-12 can induce NK cells to release IFN-γ, and IL-18 can be synergistic with IL-12 in this role (Ortaldo & Young, 2003). Rat splenic NK cells were isolated by MACS, stimulated *in vitro* in the presence and absence of IL-12 and IL-18 and assessed for IFN-γ concentration. IFN-γ concentration was significantly increased in splenic NK cells, which were stimulated with IL-18 (10ng/ml) or IL-12 (10ng/ml) and IL-18 compared with control (*p<0.05; Student’s t-test for independent means; Figure 4.4a). IFN-γ concentration was similar in NK cells treated with IL-12 compared with control. In addition to this, data (kindly provided by Dr. Rachael
Clarke) indicated that IL-2 stimulated IFN-γ release from a human NK cell line and also from a preparation of CD161⁺ cells prepared from rat brain (*p<0.05; **p<0.001; Student’s t-test for independent means; Figures 4.4b and 4.4c).

**Effect of age on HMGB1 expression in the rat cortex and hippocampus.**

It has been recently shown that NK cells also release a protein known as High-Motility Group Box 1 (HMGB1) (Semino *et al.*, 2007), which is known to have a significant immunomodulatory role (Lotze & Tracey, 2005). HMGB1 expression was assessed in snap-frozen cortical and hippocampal tissue prepared from young, middle-aged and aged rats. However the data show that HMGB1 expression was similar in cortical and hippocampal tissue prepared from young, middle-aged and aged rats. This is reflected in the sample immunoblots and also in the mean densitometric data in Figure 4.5.

<table>
<thead>
<tr>
<th>Target</th>
<th>Tissue</th>
<th>Young</th>
<th>Middle-Aged</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX-6⁺CD11b⁺ CD45⁻ cells (%)</td>
<td>Whole brain</td>
<td>13.75 ± 2.14</td>
<td>31.60 ± 4.67</td>
<td>22.91 ± 1.34</td>
</tr>
<tr>
<td>NKp30⁺CD161a⁺ cells (%)</td>
<td>Whole brain</td>
<td>24.52 ± 5.03</td>
<td>14.15 ± 1.83</td>
<td>21.17 ± 1.77</td>
</tr>
<tr>
<td>IFN-γ (pg/mg)</td>
<td>Cortical</td>
<td>40.92 ± 4.54</td>
<td>37.95 ± 3.72</td>
<td>38.07 ± 4.91</td>
</tr>
<tr>
<td>HMGB1 (arbitrary units)</td>
<td>Cortical</td>
<td>0.051 ± 0.007</td>
<td>0.035 ± 0.005</td>
<td>0.041 ± 0.005</td>
</tr>
<tr>
<td>IFN-γ (pg/mg)</td>
<td>Hippocampal</td>
<td>38.33 ± 6.47</td>
<td>50.62 ± 7.79</td>
<td>71.12 ± 10.41</td>
</tr>
<tr>
<td>HMGB1 (arbitrary units)</td>
<td>Hippocampal</td>
<td>0.037 ± 0.005</td>
<td>0.029 ± 0.004</td>
<td>0.04 ± 0.006</td>
</tr>
</tbody>
</table>

Statistical tests performed were either 1-way ANOVA or Student’s t-test for independent means.

Table 4.1. Effect of age on several markers in whole brain, cortical and hippocampal tissue. Data are expressed as means ± standard error of the mean (n≥6).
Table 4.2. Effect of cytokines on IFN-γ in NK cells. Data are expressed as means ± standard error of the mean (n>6; *n=2).

Effect of HMGB1 on MHC II mRNA and CD11b mRNA in rat cortical mixed glia.

It has been reported that HMGB1 can mediate the response to infection, injury and inflammation (Yang et al., 2002; Li et al., 2003; O’Connor et al., 2003; Alleva et al., 2005; Yang et al., 2005; Kim et al., 2006), and therefore its effect on markers of microglial activation in cultured mixed glia was examined. HMGB1 significantly increased the expression of MHC II mRNA (1000ng/ml; *p<0.05; Student’s t-test for independent means; 10ng/ml; **p<0.01; ANOVA; Figure 4.6a), but not CD11b mRNA (Figure 4.6b) in cortical mixed glia, compared with control.

Effect of IFN-γ and HMGB1 on markers of microglial activation in glial cultures.

In vitro experiments were carried out to assess the effect of IFN-γ and HMGB1 co-treatment on markers of microglial activation, MHC II mRNA and CD11b mRNA, in rat cortical mixed glia.

IFN-γ (50ng/ml) significantly increased the expression of MHC II mRNA and CD11b mRNA in cortical mixed glia compared with control (*p<0.05; **p<0.01; Student’s t-test for independent means; Figure 4.7). HMGB1 (10ng/ml) significantly increased MHC II mRNA but not CD11b mRNA (**p<0.01; ANOVA; Figure 4.7a). Whereas there was no effect of IFN-γ and HMGB1 on MHC II mRNA, the combination
of these factors significantly increased CD11b mRNA compared with control (**p<0.01; ANOVA; Figure 4.7b).

The effect of IFN-γ and HMGB1 on CD40 mRNA and CD11b mRNA was also assessed in purified microglia prepared from neonatal rats. IFN-γ significantly increased CD40 mRNA expression alone and in the presence of HMGB1 (**p<0.01; ANOVA; Figure 4.8a). However neither IFN-γ nor HMGB1, alone or in combination, affected CD11b mRNA expression (Figure 4.8b). In contrast, IFN-γ alone, and in combination with HMGB1, significantly increased CD40 mRNA expression in purified astrocytes (**p<0.001; ANOVA; Figure 4.9).

<table>
<thead>
<tr>
<th>Target</th>
<th>Cell Type</th>
<th>Control</th>
<th>HMGB1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10ng/ml</td>
</tr>
<tr>
<td>MHC II mRNA</td>
<td>Cortical mixed glia</td>
<td>1.053 ± 0.068</td>
<td>1.743 ± 0.139</td>
</tr>
<tr>
<td>CD11b mRNA</td>
<td>Cortical mixed glia</td>
<td>1.612 ± 0.624</td>
<td>2.452 ± 0.508</td>
</tr>
<tr>
<td>CD40 mRNA</td>
<td>Cortical microglia</td>
<td>0.966 ± 0.061</td>
<td>1.541 ± 0.059</td>
</tr>
<tr>
<td>CD11b mRNA</td>
<td>Cortical microglia</td>
<td>1.030 ± 0.054</td>
<td>0.984 ± 0.060</td>
</tr>
<tr>
<td>CD40 mRNA</td>
<td>Cortical astrocytes</td>
<td>1.473 ± 0.186</td>
<td>3.586 ± 0.416</td>
</tr>
</tbody>
</table>

Statistical tests performed were either 1-way ANOVA or Student’s t-test for independent means.

Table 4.3. Effect of HMGB1 and IFN-γ in vitro. Data are expressed as means ± standard error of the mean (n=6).
Figure 4.1. Effect of age on the activated microglia in the rat brain.
Monocytes were isolated from Percoll gradients carried out on male Wistar rat brain hemispheres that were pre-anaesthetised with urethane (1.5 g/kg, 33% w/v) and transcardially perfused with saline, and assessed for markers of microglial activation in percentage of total monocytes by flow cytometry. OX-6^CD45^CD11b^+ cells were significantly increased in hemi-sectioned brain prepared from aged (22-25 months) or middle-aged (15-18 months), compared with young (3-4 months), rats (*p<0.05; **p<0.01; Student’s t-test for independent means). Data are presented as means ± SEM (n≥6). Sample FACS plots are shown.
Young Middle Aged Aged

OX-6^-CD45^+CD11b^+ (% of total monocytes)

Young Middle Aged

** *
Figure 4.2. Effect of age on IFN-γ in rat cortex and hippocampus. Cortical and hippocampal tissue was dissected free from young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) male Wistar rats that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, homogenised and assessed for IFN-γ by ELISA. IFN-γ concentration was similar in cortical tissue prepared from young, middle-age and aged rats (a) but was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (b; *p<0.05; ANOVA). Data are expressed as pg IFN-γ/mg protein, and means ± SEM (n≥6) are presented.
(a) Cortex

(b) Hippocampus
Figure 4.3. Effect of age on the presence of NK cells in the rat brain.
Monocytes were isolated from Percoll gradients carried out on male Wistar rat brain hemispheres that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, and assessed for markers of NK cells in percentage of total monocytes by flow cytometry. NKp30*CD161* cells were significantly increased in hemi-sectioned brain prepared from aged (22-25 months), compared with middle-aged (15-18 months), rats (*p<0.05; Student's t-test for independent means). Data are presented as means ± SEM (n>6). Sample FACS plots are shown.

NOTE: Due to inconsistencies in the body weight of the animals used in the young group, there is doubt over the accuracy of their age, and so this data has been presented with and without the young group.
CD161⁺NKp30⁺ (% of total monocytes)

YOUNG

MIDDLE-AGED

AGED

Young Middle Aged Aged

CD161⁺NKp30⁺ (% of total monocytes)

Middle Aged Aged

103
Figure 4.4. Effect of IL-2, IL-12 and IL-18 on IFN-γ in rat splenic NK cells.

Splenic cells were isolated by MACS from young (2-3 months) male Wistar rats that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and killed. Splenic cells were grown in RPMI for 24h at 37°C before incubation of cells with IL-12 (10ng/ml) and/or IL-18 (10ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for IFN-γ. IFN-γ concentration was significantly increased in rat splenic NK cells treated with IL-18 (10ng/ml) or IL-12 (10ng/ml) or IL-18 + IL-12 compared with control (a; *p<0.05; Student’s t-test for independent means; n=2). IL-2 significantly increased IFN-γ from an NK cell line and also from CD161⁺ cells prepared from rat cortical tissue (b, c; *p<0.05; ***p<0.001; Student’s t-test for independent means). Data are expressed as pg IFN-γ/mg and means ± SEM (n=4) are presented.

NOTE: Data in (b) and (c) were kindly provided by Dr. Rachael Clarke.
(a) Splenic NK cells

![Graph showing IFN-γ (pg/mg) for control, IL-12, IL-18, and IL-12+ IL-18 treatments.]

(b) NK cell line

![Graph showing IFN-γ (pg/mg) for control and IL-2 treatments.]

(c) CD161+ cells (cortex)

![Graph showing IFN-γ (pg/mg) for control and IL-2 treatments.]

**Notes:**
- IFN-γ (Interferon-gamma) levels are measured in pg/mg.
- The graphs compare control treatments with stimulate treatments (IL-12, IL-18, IL-12+ IL-18, IL-2).
- Asterisks indicate statistical significance: **p < 0.001, *p < 0.05**.
Figure 4.5. Effect of age on HMGB1 expression in rat cortex and hippocampus.

Cortical and hippocampal tissue was dissected free from young (3-4 months), middle-aged (15-18 months) and aged (22-25 months) male Wistar rats that were pre-anaesthetised with urethane (1.5g/kg, 33% w/v) and transcardially perfused with saline, homogenised and assessed for HMGB1 expression by western immunoblot. HMGB1 expression were similar in rat cortical and hippocampal tissue prepared from aged, compared with middle-aged or young, or from middle-aged, compared with young, rats. Data are expressed as a ratio of HMGB1:β-actin, and means ± SEM (n=6) are presented. Sample immunoblots are shown.
(a) Cortex

Cortex

HMGB1 (29kDa)

β-actin (43kDa)

Young Middle-Aged Aged

(b) Hippocampus

Hippocampus

HMGB1 (29kDa)

β-actin (43kDa)

Young Middle-Aged Aged
Figure 4.6. Effect of HMGB1 on MHC II mRNA and CD11b mRNA in rat cortical mixed glia.

Cortical mixed glia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with HMGB1 (10ng/ml, 100ng/ml or 1000ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for MHC II mRNA and CD11b mRNA by QPCR.
(a) MHC II mRNA was significantly increased in mixed glial cells treated with HMGB1 (10ng/ml) or HMGB1 (1000ng/ml) compared with control (**p<0.01; ANOVA, *p<0.05; Student’s t-test for independent means).
(b) CD11b mRNA was similar in rat cortical mixed glial cells treated with HMGB1 (10ng/ml, 100ng/ml or 1000ng/ml) compared with control. Data are expressed as a ratio of MHC II mRNA:β-actin mRNA and CD11b mRNA:β-actin mRNA, respectively, and means ± SEM (n=6) are presented.
Figure 4.7. Effect of IFN-γ and HMGB1 on MHC II mRNA and CD11b mRNA in rat cortical mixed glia.

Cortical mixed glia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with IFN-γ (50ng/ml) and/or HMGB1 (10ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for MHC II mRNA and CD11b mRNA by QPCR.

(a) MHC II mRNA was significantly increased in mixed glial cells treated with IFN-γ (**p<0.01; Student’s t-test for independent means) or HMGB1 (**p<0.01; ANOVA) compared with control.

(b) CD11b mRNA significantly increased in mixed glial cells treated with IFN-γ (*p<0.05; Student’s t-test for independent means) or both IFN-γ and HMGB1 (***p<0.01; ANOVA) compared with control. Data are expressed as a ratio of MHC II mRNA:β-actin mRNA and CD11b mRNA:β-actin mRNA, respectively, and means ± SEM (n=6) are presented.
Figure 4.8. Effect of IFN-γ and HMGB1 on CD40 mRNA and CD11b mRNA in purified microglia prepared from rat cortex.

Purified cortical microglia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with IFN-γ (50 ng/ml) and/or HMGB1 (10 ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for CD40 mRNA and CD11b mRNA by QPCR.

(a) CD40 mRNA was significantly increased in microglia treated with IFN-γ and IFN-γ + HMGB1 (**p<0.01; ANOVA) but not HMGB1 alone.
(b) CD11b mRNA was unchanged with treatment. Data are expressed as a ratio of CD40 mRNA:β-actin mRNA and CD11b mRNA:β-actin mRNA, respectively, and means ± SEM (n=6) are presented.
(a) CD40 mRNA

(b) CD11b mRNA

Control  IFN-γ  HMGB1  IFN-γ + HMGB1

Control  IFN-γ  HMGB1  IFN-γ + HMGB1

**
Figure 4.9. Effect of IFN-$\gamma$ and HMGB1 on CD40 mRNA in purified astrocytes prepared from rat cortex.

Purified cortical astrocytes were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with IFN-$\gamma$ (50ng/ml) and/or HMGB1 (10ng/ml) for 24h at 37°C in 5% CO$_2$: 95% air. Once harvested, lysates were assessed for CD40 mRNA by QPCR. CD40 mRNA was significantly increased in astrocytes treated with IFN-$\gamma$ and IFN-$\gamma$ + HMGB1 (**p<0.001; ANOVA) but not HMGB1 alone. Data are expressed as a ratio of CD40 mRNA:β-actin mRNA and means ± SEM (n=6) are presented.
4.4 Discussion

The aim of this study was to investigate if NK cells were present in the brain of aged rats and could therefore potentially be responsible for the proposed IFN-γ-induced activation of microglia. The data show that NK cells are present in the brain, and that their number is significantly increased with age, and they can upregulate the production of IFN-γ, which increases microglial activation.

With the aim of assessing the presence of an activated microglial profile in this study, cell populations positive for OX-6, CD11b and CD45 (a TM protein found on microglia and lymphocytes (Cosenza-Nashat et al., 2006)) were isolated by FACS and the number of OX-6\(^+\)CD11b\(^+\)CD45\(^+\) cells was found to significantly increase with age. This finding supports the body of research, which reports that microglial activation increases with age. Microglia are activated by several cytokines (Aloisi, 2001), but IFN-γ is probably their most powerful activator and this is a cytokine, which is also a crucial component in regulating immune and inflammatory processes (Boehm et al., 1997).

The expression of IFN-γ with age was assessed and a significant age-related upregulation in IFN-γ was observed; this finding is coupled with the increase seen in activated microglia with age and the increase in IFN-γ mRNA in chapter 3. Resting microglia were shown to constitutively express the IFN-γ receptor and when an intrathecal injection of IFN-γ was administered into the rat CNS a significant increase in the number of MHC II antigen was observed (Vass & Lassmann, 1990). When IFN-γ binds to its receptor, the interaction triggers the transcription of many IFN-γ-inducible immune genes via the classical IFN-γ-induced JAK-STAT signal transduction cascade (Bach et al., 1997; Nguyen & Benveniste, 2000). IFN-γ is known to increase cell surface markers of microglial activation like MHC II, CD40 and ICAM-1 (Vass & Lassmann, 1990; Molina & Huber, 1991; Bach et al., 1997; Nguyen & Benveniste, 2000; Benveniste et al., 2004; Lynch et al., 2007), result in production of pro-inflammatory cytokines (Gautam et al., 1992; Nguyen & Benveniste, 2000; Temburni & Jacob, 2001; Hausler et al., 2002; Gasic-Milenkovic et al., 2003; Maher et al., 2006; Lyons et al., 2007b) and increase the expression of other neurotoxic factors such as iNOS (Harris et al., 1995) and caspase-1 (Hausler et al., 2002; Kim et al., 2002); all of
these factors can affect functions of neuronal and glial cells (Popko et al., 1997; Hanisch, 2002).

An issue arises in that IFN-γ is predominantly produced by immune cells of the periphery, in particular NK cells (Obara et al., 2005), which are a large population of lymphocytes that reside in the PNS that are potently cytotoxic against tumours and virally-infected cells (Biron et al., 1999; Kim et al., 2000; Solana & Mariani, 2000). Circulating immune cells like NK cells are prevented from entering the brain due to the presence of the BBB, which also impedes the entry of pathogens. However if the BBB is compromised, its ability to control, for example entry of circulating cells, is reduced. Ironically, a 'leaky' BBB can hinder the effective delivery of treatments for brain infections, often leading to severe consequences. On the contrary BBB disruption assists the delivery of immunotherapies, which target specific regions of the brain for particular brain disorders (Pahnke et al., 2009).

Here it was hypothesised that NK cells infiltrate the CNS with age, whereby they actively produce IFN-γ to activate microglia. Double-positive NKp30 and CD161a cells were isolated by FACS and the data shows the presence of NK cells in the aged brain. The data also show that there is a marked age-related increase in the number of NK cells in the brain between middle-aged and aged rats. This evidence correlates with the increase in IFN-γ, suggesting the rise in IFN-γ expression may be a result of the increase in NK cells in the brain. These findings support the perception that NK cells cross an age-compromised BBB to cause IFN-γ-induced microglial activation in the brain, subsequently leading to neurodegeneration. Numerous studies have implicated a breach in the BBB in contributing to neurodegenerative disorders (Pahnke et al., 2009; see Popescu et al., 2009; see Stolp & Dziegielewska, 2009), such as MS (Sospedra & Martin, 2005; Kebir et al., 2007), AD (Zlokovic, 2004; Deane et al., 2005; Messier & Teutenberg, 2005; Pahnke et al., 2009) and PD (Kortekaas et al., 2005; Chao et al., 2009), but a compromised BBB has also been associated with ageing. Hosokawa and Ueno reported on the impairment of the BBB with age in a senescence-accelerated mouse model (Hosokawa & Ueno, 1999). The literature documents that the infiltration of NK cells into the CNS has been reported following peripheral axotomy (Hammarberg et al., 2000) and in cases of autoimmune CNS inflammation (Matsumoto et al., 1998; Huang et al., 2006). In EAE models where the BBB is disrupted (Dousset et al., 2006; Leech et al., 2007), it was estimated that NK cells comprise 10-20% of CNS infiltrates (Matsumoto et al., 1998; Huang et al., 2006), so there is evidence to
support the proposal that NK cells can infiltrate into the CNS with age, and that these cells can initiate an immune response in the brain.

To evaluate the possible triggers of IFN-γ release from NK cells, splenic NK cells were isolated from rat and incubated in the presence of the pro-inflammatory cytokines IL-12 and IL-18. The data show that NK cells are stimulated by both cytokines in vitro to produce significant levels of IFN-γ. It has been shown that IL-12 and IL-18 are both potent inducers of IFN-γ from NK cells, stimulating the production of IFN-γ independently and synergistically (Nakamura et al., 1989; Coughlin et al., 1998; Puren et al., 1998; Akira, 2000; Zhang et al., 2001; Trinchieri, 2003; Watford et al., 2003; Rodriguez-Galan et al., 2005). Interleukin-2 (IL-2) is also known to induce NK cells to produce IFN-γ (Handa et al., 1983; Ye et al., 1995; Pintaric et al., 2008; Duluc et al., 2009). Data, kindly provided by Dr. Rachael Clarke, confirm that IL-2 stimulates the production of IFN-γ from an NK cell line and cortical CD161^ cells.

Recent work has reported that NK cells also produce a pro-inflammatory cytokine called HMGB1 (Semino et al., 2007), and that it can induce NK cells to produce IFN-γ (DeMarco et al., 2005). HMGB1 was discovered many decades ago but, until recently, it was primarily considered to regulate transcription as a nuclear non-histone DNA binding protein (Lotze & Tracey, 2005). In addition to activated NK cells, HMGB1 has also been found to be expressed by many cells of the CNS, including astrocytes, neurons and microglia (Wang et al., 1999; Rendon-Mitchell et al., 2003; Andersson et al., 2008; Enokido et al., 2008). Indeed HMGB1 appears to be ubiquitously distributed in all cells in mammalian tissues, which is likely due to its function as a nuclear protein (Mosevitsky et al., 1989).

HMGB1 binds to DNA in the minor groove without sequence specificity causing DNA bending, unwinding DNA from nucleosomes and thus facilitating transcription, recombination, genome stability and replication. However HMGB1 is now recognised as having a significant immunomodulatory role in inflammation. It was Wang and colleagues that first noticed HMGB1 activates an inflammatory response once it is released into the extracellular milieu, although in a late mediatory fashion (Wang et al., 1999). The authors showed that LPS, TNF-α or IL-1-stimulated monocytes/macrophages secreted HMGB1 as a delayed inflammatory response. It is now known that HMGB1 belongs to a family of DAMPs, which initiate and perpetuate an immune response (Rubartelli & Lotze, 2007). Thus it is not surprising to learn that
HMGB1 has been shown to mediate signalling between NK cells and DC (Lotze & Tracey, 2005), and be associated with sickness behaviour (Agnello et al., 2002). HMGB1 is released passively from cells which are undergoing necrosis (Scaffidi et al., 2002), or actively, from macrophages for example, following inflammatory stimuli (Bonaldi et al., 2003).

HMGB1 expression was assessed in this study and the data shows that there was no change in expression levels with age. This finding contrasts with a previous report, which demonstrates that hippocampal and cortical expression were slightly increased in 24 month old mice compared with 14 month old mice, yet decreased in 14 month old, compared with 0.5 month old, mice (Enokido et al., 2008). Interestingly, HMGB1 protein levels change with age in a cell-specific manner; expression decreases in neurons with age yet it increases in astrocytes (Enokido et al., 2008).

Despite the present finding that there are similar expression levels of HMGB1 with age, HMGB1 may still play a role in neurodegenerative disorders. The main function of HMGB1 in the brain is not fully understood and there is evidence suggesting HMGB1 is critical in regulating DNA repair systems (Yuan et al., 2004; Prasad et al., 2007). Evidence shows that HMGB1 can induce chronic inflammation (Scaffidi et al., 2002; O'Connor et al., 2003; Wang et al., 2004; Kim et al., 2006; Yamada & Maruyama, 2007; Andersson et al., 2008), which is a hallmark of neurodegeneration, so the ability of HMGB1 to regulate the expression of markers of microglial activation was assessed in vitro. The data show that, in mixed glia, HMGB1 significantly induces MHC II, but not CD11b. This work is supported by findings by Kim and colleagues who demonstrated the ability of HMGB1 to activate microglia using extracellular HMGB1 released from endotoxin-treated neurons (Kim et al., 2006). HMGB1 is also capable of inducing several inflammatory cytokines (Agnello et al., 2002; Scaffidi et al., 2002; O'Connor et al., 2003; Kim et al., 2006). The assessment of MHC II needs to be repeated in purified glial cultures in order to determine whether the effect seen by HMGB1 occurs in microglia and/or astrocytes.

It has been suggested that HMGB1 and IFN-γ function synergistically to increase NO production (Kim et al., 2006). Here cultured mixed glia were assessed for markers of activated microglia following stimulation with HMGB1 and IFN-γ; the data highlight significant IFN-γ-induced increases in MHC II and CD11b, however, HMGB1 only induced the expression of MHC II. Co-stimulation with HMGB1 and IFN-γ triggered an increase in CD11b, although there was no evidence of an additive
effect. Interestingly, co-stimulation of HMGB1 and IFN-γ exerted no effect on MHC II, while it was seen that both cytokines can induce an effect alone. This suggests that IFN-γ and HMGB1 negatively impact on their ability to induce the expression of MHC II in glia. However whether this effect occurs in purified glial cultures or not requires further study before any conclusions can be made. IFN-γ also potently increased CD40 mRNA but HMGB1 failed to do so in pure microglia and pure astrocytes and there was no evidence of an additive effect. These findings contrast with previous data, which demonstrated that IFN-γ does not require the presence of HMGB1 to induce microglial activation, but it was considered that the IFN-induced effects may be exacerbated by HMGB1. Both IFN-γ and HMGB1 failed to elicit a response from pure microglia in terms of CD11b expression, contrasting with findings in mixed glia. However the effects seen in mixed glia may be due to astrocytic presence, and thus these investigations need to be repeated in purified astrocyte cultures to determine. Although it is more likely that a failure to see an effect in CD11b in purified microglia is due to contamination as IFN-γ is known to induce the activation of microglia (Benveniste et al., 2004). It can be observed that the control group values in this experiment were very high, suggesting possible contamination of wells during treatment. While HMGB1 can induce IFN-γ production in NK cells (DeMarco et al., 2005), IFN-γ has been shown to cause HMGB1 translocation from the nuclei to the cytoplasm (Rendon-Mitchell et al., 2003). The authors also demonstrated that inhibition of TNF-α activity, using TNF-α neutralising antibodies, and TNF knockout animals, led to an inhibition of this IFN-γ-mediated HMGB1 translocation, indicating a role for TNF-α in the regulation of HMGB1.

Work carried out by Gardella and colleagues suggests that, in monocytes, translocated HMGB1 is released from the cytoplasm into the extracellular milieu by exocytosis (Gardella et al., 2002). Once HMGB1 is released it is known to interact with RAGE, TLR2 and TLR4, which supports the role of HMGB1 as an initiator of microglial activation (Lotze et al., 2007; Yamada & Maruyama, 2007). Interestingly, HMGB1 has been shown to bind to fibrillar Aβ1-42, inhibiting its clearance by microglia and therefore enhancing neurotoxicity to neurons, a suggestion supported by an observed release of HMGB1 from dying neurons (Takata et al., 2004).

This study set out to identify a possible source for IFN-γ in the brain, as it has always been unclear, which cells are responsible for the release of this potent activator of microglia. The evidence presented suggests NK cells infiltrate the CNS because the
BBB is compromised with age and that these cells are stimulated by cytokines, such as IL-12 or IL-18, to produce IFN-γ. The expression of another cytokine produced by NK cells, HMGB1, did not change with age and despite upregulating MHC II expression in vitro, it showed no further evidence of activating microglia, or acting in conjunction with IFN-γ to stimulate microglia.
Chapter 5

An investigation of immunomodulatory molecules in ageing
5.1 Introduction

Ageing is the most significant risk factor in the pathogenesis of AD. The hallmark neurodegeneration is characterised by a selective and progressive degeneration of neurons in the CNS, which alters the functioning of neuronal circuits. When microglia are chronically activated, they secrete various factors that contribute to the activation of signalling pathways, which lead to neuronal dysfunction and cell death. These include pro-inflammatory cytokines, chemokines, ROS, complement factors, proteases, eicosanoids, neurotoxic secretory products, free radical species and NO (Kreutzberg, 1996; Griffin et al., 1998; Takeuchi et al., 2005; Suzumura et al., 2006). For instance, the pro-inflammatory cytokine TNF-α is known to initiate the caspase cascade that results in neuronal cell dysfunction (Schwandner et al., 1998; Guicciardi et al., 2000; Heinrich et al., 2004).

Several molecules, including those with inflammatory properties, can initiate and/or fuel the events leading up to neuronal deterioration. Due to the recognised involvement of the Nogo protein in inhibiting axonal regeneration (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), it was considered whether Nogo-A, and/or one of its splice variants, might impact on particular aspects of neuroinflammation, and thereby contribute to neurodegeneration. One aim of this study was to assess changes in microglial and neuronal function with age by examining the expression levels of markers of microglial activation and deterioration of cell function. The second objective was to examine whether Nogo expression changes with age and to investigate if Nogo can modulate microglial and/or neuronal function.
5.2 Methods

Groups of young (3-4 months) and aged (18-22 months) rats (n=8) were anaesthetised by ip injection of urethane (1.5g/kg, 33% w/v) and sacrificed by decapitation before cortical and hippocampal tissue was harvested for mRNA and protein analysis. Activities of caspases -8 and -3, and sphingomyelinase were assessed in cortical and hippocampal samples using commercially-available assay kits, expression of markers of microglial activation were assessed by QPCR and western immunoblotting was used to evaluate Nogo and synaptophysin (see section 2.4). Some cortical tissue was fractionated in order to assess cytosolic and membrane-bound sphingomyelinase activity. Cortical neurons, mixed glia, purified microglia or purified astrocytes were prepared from 1-day old Wistar rats. Neuronal preparations were cultured in NBM for 5-7 days at 37°C before treatment and glial preparations were cultured in DMEM for 12-14 days at 37°C before treatment (see section 2.3.2). Treatments included incubation of Nogo-A or Nogo-B at concentrations of 10ng/ml, 100ng/ml or 1000ng/ml for 24h at 37°C in 5% CO₂: 95% air before harvesting.

5.3 Results

The objective of this study was to assess age-related changes in microglial and neuronal function, to establish whether Nogo expression is altered with age and to determine whether Nogo modulates microglial and/or neuronal function. The mean values ± SEM for all parameters are presented in tables 5.1, 5.2, 5.3 and 5.4.

Effect of age on markers of microglial activation in cortex and hippocampus.

Activated microglia are typified by cell surface expression of the antigen presenting molecule, MHC II, and the antigens, CD11b and CD40. These markers were assessed in snap-frozen cortical and hippocampal tissue prepared from young and aged rats. MHC II mRNA and CD11b mRNA expression were significantly increased in cortical tissue prepared from aged, compared with young, rats (*p<0.05; **p<0.01; Student’s t-test for independent means; Figure 5.1a, b) but expression of CD40 mRNA was unchanged (Figure 5.1c). However a significant increase in expression of all
markers was observed in hippocampal tissue prepared from aged, compared with young, rats (*p<0.05; ***p<0.001; Student's t-test for independent means; Figure 5.2).

Effect of age on activities of caspase-8 and caspase-3 in the rat cortex and hippocampus.

It has been shown that an increase in microglial activation is associated with neuronal dysfunction (Kreutzberg, 1996; Griffin et al., 1998; Takeuchi et al., 2005; Suzumura et al., 2006), and since the activities of caspase-8 and caspase-3 are indicators of deterioration in cell function, these measures were assessed in cortical and hippocampal tissue prepared from young and aged rats. Activities of caspase-8 and caspase-3 were significantly increased in cortical tissue prepared from aged, compared with young, rats (*p<0.05; ***p<0.001; Student's t-test for independent means; Figure 5.3). Similarly, activities of both enzymes were significantly increased in hippocampal tissue prepared from aged, compared with young, rats (***p<0.001; Student's t-test for independent means; Figure 5.4).

Effect of age on the activation of sphingomyelinase in the rat cortex and hippocampus.

It has been reported that caspase-8 activity can lead to the cleavage of caspase-3 directly, but it is also known that activation of caspase-3 can occur downstream of caspase-8 activation indirectly via activation of sphingomyelinase. Therefore the activity of sphingomyelinase in whole homogenate, and in membrane-bound and cytosolic fractions prepared from cortex of young and aged rats was assessed in this study. However the data show that sphingomyelinase activity was similar in tissue obtained from young and aged rats irrespective of the preparation analysed (Figure 5.5). Sphingomyelinase activity was also unchanged with age in homogenate prepared from hippocampal tissue (Figure 5.6). These data indicate that activation of sphingomyelinase did not contribute to the age-related increase in caspase-3 activity.

Effect of age on synaptophysin and Nogo-B in the rat cortex and hippocampus.

Synaptophysin is an integral membrane protein of synaptic vesicles and its expression is considered to be an indication of healthy synaptic contacts (Masliah & Terry, 1993; Thiel, 1993; Tarsa & Goda, 2002; Valtorta et al., 2004). The present data indicate that synaptophysin expression was similar in cortical tissue prepared from aged and young rats (Figure 5.7a); this is shown in the sample immunoblots and also in the
mean densitometric data. However it was significantly decreased in hippocampal tissue prepared from aged, compared with young, rats (*p<0.05; Student’s t-test for independent means; Figure 5.7b).

<table>
<thead>
<tr>
<th>Target</th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II mRNA</td>
<td>0.93 ± 0.04</td>
<td>1.05 ± 0.03</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
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<tr>
<td>CD11b mRNA</td>
<td>1.02 ± 0.14</td>
<td>3.38 ± 0.74</td>
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<tr>
<td>(arbitrary units)</td>
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<tr>
<td>CD40 mRNA</td>
<td>0.71 ± 0.07</td>
<td>0.60 ± 0.08</td>
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<tr>
<td>(arbitrary units)</td>
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<td></td>
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<tr>
<td>Caspase-8 activity</td>
<td>627.4 ± 7.75</td>
<td>651.3 ± 4.61</td>
</tr>
<tr>
<td>(pmol/min/mg)</td>
<td></td>
<td></td>
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<tr>
<td>Caspase-3 activity</td>
<td>388.0 ± 2.49</td>
<td>429.2 ± 1.02</td>
</tr>
<tr>
<td>(pmol/min/mg)</td>
<td></td>
<td></td>
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<tr>
<td>Sphingomyelinase activity:</td>
<td>0.01 ± 0.001</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>whole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(fluorescence U/ml)</td>
<td></td>
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<tr>
<td>Sphingomyelinase activity:</td>
<td>0.02 ± 0.002</td>
<td>0.02 ± 0.002</td>
</tr>
<tr>
<td>cytosolic</td>
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<tr>
<td>(fluorescence U/ml)</td>
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<tr>
<td>Sphingomyelinase activity:</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
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<tr>
<td>membrane-bound</td>
<td></td>
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<tr>
<td>(fluorescence U/ml)</td>
<td></td>
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<tr>
<td>Synaptophysin</td>
<td>1.79 ± 0.22</td>
<td>1.78 ± 0.16</td>
</tr>
<tr>
<td>(arbitrary units)</td>
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<tr>
<td>Nogo-B</td>
<td>0.13 ± 0.02</td>
<td>0.12 ± 0.02</td>
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<tr>
<td>(arbitrary units)</td>
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</table>

Table 5.1. Effect of age on several markers in cortical tissue. Data are expressed as means ± standard error of the mean (n≥7).

The data show that Nogo-B was similar in cortical tissue and significantly increased in hippocampal tissue prepared from aged, compared with young, rats.
In contrast Nogo-A expression was similar in samples of hippocampal tissue prepared from young and aged rats (Figure 5.9). These findings are reflected in the sample immunoblots presented and also in the mean densitometric data in these Figures 5.8 and 5.9.

<table>
<thead>
<tr>
<th>Target</th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II mRNA</td>
<td>0.95 ± 0.03</td>
<td>1.11 ± 0.05</td>
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<td>(arbitrary units)</td>
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<tr>
<td>CD11b mRNA</td>
<td>1.06 ± 0.09</td>
<td>3.56 ± 0.18</td>
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<tr>
<td>(arbitrary units)</td>
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<tr>
<td>CD40 mRNA</td>
<td>0.99 ± 0.04</td>
<td>1.38 ± 0.13</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
<td></td>
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<tr>
<td>Caspase-8 activity</td>
<td>215.8 ± 2.13</td>
<td>354.3 ± 3.03</td>
</tr>
<tr>
<td>(pmol/min/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-3 activity</td>
<td>377.8 ± 1.26</td>
<td>515.5 ± 1.77</td>
</tr>
<tr>
<td>(pmol/min/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphingomyelinase activity: whole</td>
<td>0.01 ± 0.001</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>(fluorescence U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>1.32 ± 0.03</td>
<td>1.23 ± 0.02</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
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<tr>
<td>Nogo-B</td>
<td>0.16 ± 0.03</td>
<td>0.42 ± 0.11</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
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<tr>
<td>Nogo-A</td>
<td>0.30 ± 0.04</td>
<td>0.30 ± 0.05</td>
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<tr>
<td>(arbitrary units)</td>
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</table>

Statistical test performed was the Student’s t-test for independent means.

Table 5.2. Effect of age on several markers in hippocampal tissue. Data are expressed as means ± standard error of the mean (n≥6).

Effect of Nogo-A and Nogo-B on activities of caspase-8 and caspase-3 in rat cortical neurons.

It has been reported, at least in cancer cell lines, that Nogo-B may increase caspase-3 (Li et al., 2001) and it was considered that it may exert a similar effect on
neurons. Therefore in vitro experiments were carried out to assess the effect of Nogo-A and Nogo-B on the activities of caspase-8 and -3 in rat cortical neurons prepared from 1-day old rats. Incubation of neurons in the presence of Nogo-A significantly increased the activities of caspase-8 and caspase-3 in a dose-dependent manner in cortical neurons (**p<0.001; ANOVA; Figure 5.10). Caspase-8 activity was significantly decreased in cortical neurons treated with Nogo-A (10ng/ml) compared with control (**p<0.001; ANOVA; Figure 5.10a).

Nogo-B also significantly increased the activities of caspase-8 and caspase-3 in cortical neurons prepared from 1-day old rats (**p<0.001; ANOVA; Figure 5.11). However the highest concentration of Nogo-B (1000ng/ml) significantly reduced activities of caspase-8 and caspase-3 compared with the effect of 100ng/ml Nogo-B (**p<0.001; ANOVA). Caspase-3 activity was significantly decreased in cortical neurons treated with Nogo-B (10ng/ml) compared with control (**p<0.001; ANOVA; Figure 5.11b).

Effect of Nogo-A and Nogo-B on MHC II mRNA and CD11b mRNA in rat cortical mixed glia.

It has been shown that NgR is upregulated in (Teng & Tang, 2008) inflammatory conditions with evidence indicating increased expression on activated microglia models of SCI, AD, stroke and MS (GrandPre et al., 2002; Li & Strittmatter, 2003; Lee et al., 2004; Satoh et al., 2005; Zhu et al., 2007). However a role for Nogo-B has not been reported in inflammation. Here, the possibility that Nogo-A or Nogo-B might lead to microglial activation was considered and therefore their effects on markers of microglia activation in cultured mixed glia were examined. Nogo-A (10ng/ml and 100ng/ml) exerted no significant effect on expression of MHC II mRNA or CD11b mRNA in cortical mixed glia (Figure 5.12). In contrast, MHC II mRNA and CD11b mRNA expression were significantly increased in rat cortical mixed glia treated with Nogo-B (100ng/ml) compared with control (**p<0.01; Student’s t-test for independent means; Figure 5.13).

The stimulatory effect of IFN-γ on microglia is reported here (Figures 3.9 and 4.8) and elsewhere (Nguyen & Benveniste, 2002) and it was considered that it may synergize with Nogo-B to further enhance microglial activation. To assess this, the effect of IFN-γ alone and in combination with Nogo-B was assessed on MHC II mRNA and CD11b mRNA. The data show that while IFN-γ and Nogo-B separately
significantly increased expression of both markers (*p<0.05; **p<0.01; ***p<0.001; ANOVA; Figure 5.14), there was no evidence of an additive or synergistic effect.

Effect of Nogo-B on CD40 mRNA in rat cortical microglia and astrocytes.

Microglia and astrocytes were separated from a mixed glial culture, treated with Nogo-B (100ng/ml), and assessed for expression of CD40 mRNA, another marker of activated microglia and astrocytes. Activation of this co-stimulatory molecule and cell surface receptor is known to lead to the induction of pro-inflammatory cytokines, chemokines and other co-stimulatory molecules (Stout & Suttles, 1996). The data presented here show that CD40 expression was significantly increased in microglia and astrocytes treated with Nogo-B (**p<0.01; ***p<0.001; Student’s t-test for independent means; Figure 5.15).

<table>
<thead>
<tr>
<th>Target</th>
<th>Cell Type</th>
<th>Control</th>
<th>Nogo-A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10ng/ml</td>
</tr>
<tr>
<td>Caspase-8 activity</td>
<td>Cortical</td>
<td>788.90 ±</td>
<td>451.40 ±</td>
</tr>
<tr>
<td>(pmol/min/mg)</td>
<td>neurons</td>
<td>16.44</td>
<td>14.21</td>
</tr>
<tr>
<td>Caspase-3 activity</td>
<td>Cortical</td>
<td>1265.00 ±</td>
<td>1682.00 ±</td>
</tr>
<tr>
<td>(pmol/min/mg)</td>
<td>neurons</td>
<td>16.91</td>
<td>15.47</td>
</tr>
<tr>
<td>MHC II mRNA</td>
<td>Cortical</td>
<td>1.34 ±</td>
<td>1.40 ±</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td>mixed glia</td>
<td>0.15</td>
<td>0.27</td>
</tr>
<tr>
<td>CD11b mRNA</td>
<td>Cortical</td>
<td>1.31 ±</td>
<td>1.23 ±</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td>mixed glia</td>
<td>0.14</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Statistical tests performed were either 1-way ANOVA or Student’s t-test for independent means.

Table 5.3. Effect of Nogo-A in vitro. Data are expressed as means ± standard error of the mean (n=6).
<table>
<thead>
<tr>
<th>Target</th>
<th>Cell Type</th>
<th>Control</th>
<th>Nogo-B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10ng/ml</td>
<td>100ng/ml</td>
</tr>
<tr>
<td>Caspase-8 activity</td>
<td>Cortical neurons</td>
<td>235.30 ±</td>
<td>283.20 ±</td>
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<tr>
<td>(pmol/min/mg)</td>
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<td>4.25</td>
<td>3.89</td>
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<tr>
<td>Caspase-3 activity</td>
<td>Cortical neurons</td>
<td>200.10 ±</td>
<td>128.3 ±</td>
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<tr>
<td>(pmol/min/mg)</td>
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<td>6.07</td>
<td>9.80</td>
</tr>
<tr>
<td>MHC II mRNA</td>
<td>Cortical neurons</td>
<td>0.96 ±</td>
<td>1.00 ±</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td>mixed glia</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>CD11b mRNA</td>
<td>Cortical neurons</td>
<td>1.03 ±</td>
<td>0.95 ±</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td>mixed glia</td>
<td>0.07</td>
<td>0.09</td>
</tr>
<tr>
<td>CD40 mRNA</td>
<td>Cortical neurons</td>
<td>0.97 ±</td>
<td>Not done</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td>microglia</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>CD40 mRNA</td>
<td>Cortical neurons</td>
<td>1.47 ±</td>
<td>Not done</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td>astrocytes</td>
<td>0.19</td>
<td></td>
</tr>
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<td>MHC II mRNA</td>
<td>Cortical neurons</td>
<td>1.12 ±</td>
<td>1.67 ±</td>
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<tr>
<td>(arbitrary units)</td>
<td>mixed glia</td>
<td>0.05</td>
<td>0.06</td>
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<tr>
<td>CD11b mRNA</td>
<td>Cortical neurons</td>
<td>0.99 ±</td>
<td>1.46 ±</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td>mixed glia</td>
<td>0.04</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Statistical tests performed were either 1-way ANOVA or Student’s t-test for independent means.

Table 5.4. Effect of Nogo-B *in vitro*. Data are expressed as means ± standard error of the mean (n=6).
Figure 5.1. Effect of age on markers of microglial activation in rat cortex.

Cortical tissue was dissected free from young (3-4 months) and aged (18-22 months) male Wistar rats, homogenised and assessed for MHC II mRNA, CD11b mRNA and CD40 mRNA by QPCR. MHC II mRNA (a) and CD11b mRNA (b) were significantly increased in cortical tissue prepared from aged, compared with young, rats (*p<0.05; **p<0.01; Student’s t-test for independent means) but CD40 mRNA expression (c) was unchanged. Data are expressed as a ratio of MHC II mRNA:β-actin mRNA, CD11b mRNA:β-actin mRNA and CD40 mRNA: β-actin mRNA respectively, and means ± SEM (n≥8) are presented.
Figure 5.2. Effect of age on markers of microglial activation in rat hippocampus.

Hippocampal tissue was dissected free from young (3-4 months) and aged (18-22 months) male Wistar rats, homogenised and assessed for MHC II mRNA, CD11b mRNA and CD40 mRNA by QPCR. MHC II mRNA (a), CD11b mRNA (b) and CD40 mRNA (c) were significantly increased in hippocampal tissue prepared from aged, compared with young, rats (*p<0.05; ***p<0.001; Student's t-test for independent means). Data are expressed as a ratio of MHC II mRNA:β-actin mRNA, CD11b mRNA:β-actin mRNA and CD40 mRNA:β-actin mRNA, respectively, and means ± SEM (n=7) are presented.
Figure 5.3. Effect of age on activities of caspase-8 and caspase-3 in rat cortex.

Cortical tissue was dissected free from young (3-4 months) and aged (18-22 months) male Wistar rats, homogenised and assessed for activities of caspase-8 and caspase-3 by assay kit. Caspase-8 (a) and caspase-3 (b) activity were significantly increased in cortical tissue prepared from aged, compared with young, rats (*p<0.05; ***p<0.001; Student’s t-test for independent means). Data are expressed as pmol/min/mg and are means ± SEM (n=8).
Figure 5.4. Effect of age on activities of caspase-8 and caspase-3 in rat hippocampus.

Hippocampal tissue was dissected free from young (3-4 months) and aged (18-22 months) male Wistar rats, homogenised and assessed for activities of caspase-8 and caspase-3 by assay kit. Caspase-8 (a) and caspase-3 (b) activity were significantly increased in hippocampal tissue prepared from aged, compared with young, rats (**p<0.001; Student’s t-test for independent means). Data are expressed as pmol/min/mg and are means ± SEM (n=8).
Figure 5.5. Effect of age on the activation of sphingomyelinase in rat cortex.
Cortical tissue was dissected free from young (3-4 months) and aged (18-22 months) male Wistar rats, homogenised and assessed for sphingomyelinase activity by assay kit. Some cortical tissue was fractionated in order to assess cytosolic and membrane-bound sphingomyelinase activity. Sphingomyelinase activity was similar in homogenate prepared from cortical tissue (a), as well as in a cytosolic fraction (b) and membrane fraction (c) prepared from the cortical tissue of aged, compared with young, rats. Data are expressed as fluorescence U/ml means ± SEM (n=8).
Figure 5.6. Effect of age on the activation of sphingomyelinase in rat hippocampus.

Hippocampal tissue was dissected free from young (3-4 months) and aged (18-22 months) male Wistar rats, homogenised and assessed for sphingomyelinase activity by assay kit. Sphingomyelinase activity was similar in hippocampal tissue of aged, compared with young, rats. Data are expressed as fluorescence U/ml means ± SEM (n≥7).
Figure 5.7. Effect of age on synaptophysin expression in rat brain.
Cortical and hippocampal tissue was dissected free from young (3-4 months) and aged (18-22 months) male Wistar rats, homogenised and assessed for synaptophysin expression by western immunoblot.
(a) Synaptophysin expression was similar in cortical tissue prepared from aged, compared with young, rats (n≥7).
(b) Synaptophysin expression was significantly decreased in hippocampal tissue prepared from aged, compared with young, rats (*p<0.05; Student’s t-test for independent means; n=6). Data are expressed as a ratio of synaptophysin:β-actin, and means ± SEM are presented. Sample immunoblots are shown.
Synaptophysin/β-actin
Arbitrary Units

(a) Cortex

(b) Hippocampus

Young
Aged

β-actin (43kDa)
Synaptophysin (38kDa)
Figure 5.8. Effect of age on Nogo-B expression in rat cortex and hippocampus.

Cortical and hippocampal tissue was dissected free from young (3-4 months) and aged (18-22 months) male Wistar rats, homogenised and assessed for Nogo-B expression by western immunoblot.

(a) Nogo-B expression was similar in cortical tissue prepared from aged, compared with young, rats.

(b) Nogo-B expression was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (**p<0.01, Student's t-test for independent means). Data are expressed as a ratio of Nogo-B:β-actin, and means ± SEM (n≥7) are presented. Sample immunoblots are shown.
Figure 5.9. Effect of age on Nogo-A expression in rat hippocampus.
Hippocampal tissue was dissected free from young (3-4 months) and aged (18-22 months) male Wistar rats, homogenised and assessed for Nogo-A expression by western immunoblot. Nogo-A expression was similar in hippocampal tissue prepared from aged, compared with young, rats. Data are expressed as a ratio of Nogo-A:β-actin, and means ± SEM (n≥11) are presented. Sample immunoblots are shown.
Nogo-A/β-actin
Arbitrary Units

0.4

0.2

0.0

Nogo-A (5kDa)
β-actin (43kDa)

Young

Dead

147
Figure 5.10. Effect of Nogo-A on activities of caspase-8 and caspase-3 in cortical neurons.

Cortical neurons were prepared from 1-day old Wistar rats and cultured in NBM for 5-7 days at 37°C before incubation of cells with Nogo-A (10ng/ml, 100ng/ml or 1000ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for activities of caspase-8 and caspase-3 by assay kit.

Nogo-A significantly increased activities of caspase-8 (a) and caspase-3 (b) in a dose-dependent manner (***p<0.001 versus control; ++p<0.001 versus Nogo-A (10ng/ml); ###p<0.001 versus Nogo-A (100ng/ml); ANOVA). Caspase-8 activity was significantly decreased in cortical neurons treated with Nogo-A (10ng/ml) compared to control (***p<0.001; ANOVA). Data are expressed as pmol/min/mg and are means ± SEM (n=6).
Caspase-3 (pmol/min/mg) Caspase-8 (pmol/min/mg)

(a)

(b)

Nogo-A (ng/ml)

Caspase-3 (pmol/min/mg)

Caspase-8 (pmol/min/mg)
Figure 5.11. Effect of Nogo-B on activities of caspase-8 and caspase-3 in cortical neurons.

Cortical neurons were prepared from 1-day old Wistar rats and cultured in NBM for 5-7 days at 37°C before incubation of cells with Nogo-B (10ng/ml, 100ng/ml or 1000ng/ml) for 24h at 37°C in 5% CO₂; 95% air. Once harvested, lysates were assessed for activities of caspase-8 and caspase-3 by assay kit.

Nogo-B significantly increased activities of caspase-8 (a) and caspase-3 (b) (**p<0.001 versus control; ***p<0.001 versus Nogo-B (10ng/ml); ANOVA). Caspase-8 and caspase-3 activity were significantly reduced in cortical neurons treated with Nogo-B (1000ng/ml) compared with Nogo-B (100ng/ml) (###p<0.001; ANOVA). Caspase-3 activity was significantly decreased in cortical neurons treated with Nogo-B (10ng/ml) (**p<0.001; ANOVA). Data are expressed as pmol/min/mg and are means ± SEM (n=6).
Figure 5.12. Effect of Nogo-A on MHC II mRNA and CD11b mRNA in rat cortical mixed glia.

Cortical mixed glia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with Nogo-A (10ng/ml or 100ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for MHC II mRNA and CD11b mRNA by QPCR.

MHC II mRNA (a) and CD11b mRNA (b) were similar in rat cortical mixed glia treated in the presence or absence of Nogo-A. Data are expressed as a ratio of MHC II mRNA:β-actin mRNA and CD11b mRNA:β-actin mRNA, respectively, and means ± SEM (n=6) are presented.
(a) MHC II mRNA

(b) CD11b mRNA

Nogo-A (ng/ml)

Nogo (ng/ml)
Figure 5.13. Effect of Nogo-B on MHC II mRNA and CD11b mRNA in rat cortical mixed glia.

Cortical mixed glia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with Nogo-B (10ng/ml or 100ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for MHC II mRNA and CD11b mRNA by QPCR.

MHC II mRNA (a) and CD11b mRNA (b) was significantly increased in mixed glial cells treated with Nogo-B (100ng/ml) compared with control (**p<0.01; ANOVA, Student’s t-test for independent means). Data are expressed as a ratio of MHC II mRNA:β-actin mRNA and CD11b mRNA:β-actin mRNA, respectively, and means ± SEM (n=6) are presented.
(a)

![Bar chart for MHC II mRNA expression](chart_a.png)

(b)

![Bar chart for CD11b mRNA expression](chart_b.png)
Figure 5.14. Effect of Nogo-B alone or in combination with IFN-γ on MHC II mRNA and CD11b mRNA in rat cortical mixed glia.

Cortical mixed glia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with IFN-γ (50ng/ml) and/or Nogo-B (100ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for MHC II mRNA and CD11b mRNA by QPCR. MHC II mRNA (a) and CD11b mRNA (b) were significantly increased in mixed glial cells treated with IFN-γ, Nogo-B or IFN-γ + Nogo-B (*p<0.05; **p<0.01; ***p<0.001; ANOVA), but no significant additive effect was observed. Data are expressed as a ratio of MHC II mRNA:β-actin mRNA and CD11b mRNA:β-actin mRNA, respectively, and means ± SEM (n=6) are presented.
(a) MHC II mRNA

Control  IFN-γ  Nogo-B  IFN-γ + Nogo-B

(b) CD11b mRNA

Control  IFN-γ  Nogo-B  IFN-γ + Nogo-B
Figure 5.15. Effect of Nogo-B on CD40 mRNA in cortical microglia and astrocytes.

Purified cortical microglia and astrocytes were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with Nogo-B (100ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for CD40 mRNA by QPCR. Nogo-B significantly increased CD40 mRNA in microglia (a) and astrocytes (b) prepared from neonatal rat brain (***p<0.001; **p<0.01; Student’s t-test for independent means). Data are expressed as a ratio of CD40 mRNA:β-actin mRNA, and means ± SEM (n=6) are presented.
5.4 Discussion

The aim of this study was to determine any age-related changes in Nogo-A and one of its splice variants, Nogo-B, in the brain and to examine whether these changes parallel neuroinflammatory change and to assess the possibility of a functional link between expression of Nogo and inflammation. The data indicate that Nogo-B is upregulated in the brain of aged rats and that it may modulate an inflammatory response.

Microglial activation has been consistently shown to precede neurodegeneration in the brain. Giulian was the first to report that activated microglia have the ability to kill neurons (Giulian et al., 1993a; Giulian et al., 1993b). The authors suggested that release of cytotoxic agents, such as pro-inflammatory cytokines, from reactive microglia and invading macrophages can potentiate neurotoxicity in the brain. As evident from data presented thus far, with age comes an associative increase in activated microglia. Once stimulated, microglia can initiate signalling events that set in motion a series of interactions that ultimately are detrimental to the survival of neurons.

In this study, the association between activated microglia and neuronal dysfunction was first examined. The data shown supports previous findings that there are age-related increases in markers of microglial activation, MHC II, CD11b and CD40, both in the cortex and hippocampus. However the age-related increase in MHC II in the cortex contrasts with the previous finding from chapter 3 (Figure 3.1); this may be due to a variability in the animals used in the two separate in vivo studies. An age-related increase in MHC II expression has been reported consistently in the literature (Griffin et al., 2006; Lynch et al., 2007) and an increase in MHC II has also been implicated in several CNS diseases, such as AD and MS (Streit et al., 1999). MHC II expression is indicative of stimulated microglia (Rozovsky et al., 1998) and is critical in the ability of activated microglia to interact with T cells (Brown et al., 1993; Germain & Margulies, 1993; Cresswell, 1994; Germain, 1994; Bentley et al., 1995; Fields et al., 1995; Rhode et al., 1996; Watts, 1997; Davis et al., 1998; Sedgwick et al., 1998; Chambers, 2001; Fischer et al., 2007). This, in turn, can stimulate further expression of MHC II on microglia (Aloisi, 2001; Seguin et al., 2003), increasing T cell activation in the brain and subsequent neuroinflammation. Some evidence suggests MHC II has a
function in modulating phagocytosis and this proposal was made following the observation that it co-localises with CD68, the lysosomal protein, which is a proposed phagocytic marker (Schmitt et al., 2000; Cho et al., 2006).

CD11b is primarily expressed on microglia and monocytes (Streit et al., 1999) and its upregulation indicates a proliferation of these cell types. An increase in microglia is generally associated with an increase in microglia that adopt the amoeboid state. The microglia are motile and migrate to a site of damage and proliferate in preparation for graded activation (Kreutzberg, 1996). CD11b serves as a binding protein for ICAM-1, which mediates cell-cell interactions and subsequent signal transduction (Schwarz et al., 2002). The increase in CD11b expression with age observed here is supported by a similar increase in CD11b mRNA in the mouse hippocampus (Sandhir et al., 2008). Importantly, CD11b expression in microglia has been shown to be upregulated by IFN-γ (Roy et al., 2006), which is increased with age and associated with microglial activation as described in chapter 3. Along with CD18, CD11b is known to form MAC-1, an antigen, which appears to be involved in cellular activation, cell-mediated cytotoxicity, motility and chemotaxis (Weber et al., 1997; Nagai et al., 2005; Solovjov et al., 2005) It may also have a role in phagocytic activity in MS and EAE (Smith, 2001; Reichert & Rotshenker, 2003; Rotshenker et al., 2008).

The present finding of an age-related increase in CD40 is also supported by previous studies carried out in this laboratory (Lynch et al., 2007; Martin et al., 2009). Even though the expression of CD40 is not restricted to microglia, and can be found on other immune cells and brain cell types like astrocytes and endothelial cells, it is commonly used as a marker of microglial activation. The interaction between CD40 and its ligand, CD154, can lead to the upregulation of co-stimulatory molecules like CD80 and CD86 as well as cytokines and MHC II, which are symptomatic of microglial activation (Chen et al., 2006a). It has been shown that IFN-γ potently increases expression of CD40, which has been interpreted as an indicator of microglial activation and that interaction between microglia and T cells ensues (Benveniste et al., 2004); this finding was somewhat predictable since its ligand is found on activated CD4⁺ T cells (Danese et al., 2004). Upregulation of CD40 and its interaction with T cells has been implicated in neurodegenerative conditions, such as AD, and also in brain injuries (Calingasan et al., 2002; Benveniste et al., 2004). Indeed Aβ-induced microglial activation requires CD40-CD154 interaction, which leads to neuronal injury (Tan et al., 1999). Work has also suggested the involvement of CD40 in phagocytosis
where it was shown that a loss in CD40 decreased the phagocytosis of Aβ deposits (Nikolic et al., 2008).

Having established an age-related inflammatory profile in this study, I sought to establish whether this was accompanied by evidence of neuronal cell dysfunction as it is known that activated microglia produce TNF-α (Frei et al., 1987; Nakajima et al., 2004) and that TNF-α signalling can induce a variety of cellular responses including the activation of the ceramide/sphingomyelinase signalling pathway (Heinrich et al., 1999; Heinrich et al., 2004). This pathway results in mitochondrial apoptosis signalling, which involves various caspase enzymes, important molecules in the apoptotic pathway in neurons (Jiang & Wang, 2000; Fischer et al., 2003; Heinrich et al., 2004). An upregulation of particular caspases, such as caspase-8 and caspase-3, which leads to diminished synaptic plasticity, can denote deterioration in neuronal cell function, and even cell death in some cases. Here activities of caspase-8 and -3 were found to be increased in cortex and hippocampus prepared from aged rats. Caspase-8 is an important enzyme found upstream of caspase-3 in the caspase cascade (Fischer et al., 2003). It is known as an initiator caspase due to its ability to cleave effector caspases, such as caspase-3, to their active form. There are no reports of age-related increases in caspase-8 in the literature. However, Clark and colleagues carried out a study investigating caspase-8 and its proteolysis after TBI, whereby age was a variable considered and the authors found no discernible age-related differences in caspase-8 expression in non-specific brain tissue (Zhang et al., 2003). The age-related increase in caspase-8 described here is consistent with the view that there is neuronal deterioration.

Activation of caspase-8 can induce activation of caspase-3 and here the data show that parallel activation of both caspases occurred in hippocampus and cortex of aged rats. Active caspase-3 is involved in the proteolytic cleavage of various substrates that culminate in DNA damage and the destruction of a cell (Fischer et al., 2003). The activation of caspase-3 is an essential aspect of the death cascade; one function of caspase-3 is to cleave PARP-1, a protein that facilitates DNA repair, therefore inactivating PARP and hindering the survival of the cell. An additive effect of inactivating PARP is an increase in free adenosine-5'-triphosphate (ATP) as the DNA repair pathway is energetically expensive. The ATP that is subsequently available is used for the execution of apoptosis (Fischer et al., 2003). The increases in caspase-3 with age are supported by studies reporting an age-related expression of caspase-3 in
the retina (Jiang et al., 2009), hippocampus (Lynch & Lynch, 2002; Zhang et al., 2004),
the motor-, visual- and prefrontal cortex, and amygdala (Zhang et al., 2004). An age-
related activation of caspase-3 in neurons by glutamate has also been reported (Brewer
et al., 2005).

Caspase-8 can induce the cleavage of procaspase-3 to its active form by several
signalling pathways. One pathway involves the activation of endosomal acidic
sphingomyelinase by caspase-8, which in turn initiates apoptosis signalling that
involves mitochondria. Activated sphingomyelinase instigates the production of a pro-
apoptotic lipid, ceramide, which prompts the release of cathepsin D and generates
translocation of tBid to the mitochondria; this leads to the release of cytochrome c and
ultimately to the activation of caspase-3 (Perry & Hannun, 1998; Heinrich et al., 1999;
Heinrich et al., 2004). To assess whether this pathway was involved in caspase-3
activation here, cortical tissue from young and aged rats was fractionated and whole
homogenate, cytosolic and membrane-bound fractions were assessed for activation of
sphingomyelinase. The data show no apparent changes in activity of this enzyme with
age, and similar lack of change was also observed in the hippocampus. There are no
data in the literature which address this issue, however a study concerning sphingolipid
metabolism during development and ageing suggests increases in sphingomyelinase
activity in the brain with age (Sacket et al., 2009). Recent work from this laboratory
describes age-related increases in caspase-8 and caspase-3 activity in the hippocampus,
and demonstrates these data are age-associated with an increase in sphingomyelinase
activation in cytosolic and membrane-bound fractions (Kelly et al., unpublished).
Significantly these changes were accompanied by impairment in LTP, suggesting
sphingomyelinase activity and subsequent mitochondrial apoptosis signalling are
contributory elements in apparent deficits of synaptic plasticity. However the activation
of caspase-3 in this study is apparently a result of an alternative signalling pathway
involving caspase-8.

Synaptophysin is a major integral membrane protein found in all neurons in the
CNS and is considered a marker of synaptic density (Masliah & Terry, 1993; Thiel,
1993; Eastwood et al., 1995; Eastwood & Harrison, 1995; Calhoun et al., 1996; Sarnat &
Born, 1999; Tarsa & Goda, 2002; Valtorta et al., 2004). Here, synaptophysin was
used to assess synaptic contacts in tissue prepared from young and aged rats, and the
data show that expression of synaptophysin was significantly decreased with age in the
hippocampus. This finding mirrors the previously-reported age-related decrease in
synaptophysin expression in the hippocampus (Chen et al., 1998; Davies et al., 2003). A number of studies using human tissue have examined age-related changes in synaptophysin mRNA and found a significant age-related decline in expression in the parahippocampal gyrus (Eastwood et al., 2006) and in the left temporal cortex of post-mortem schizophrenic brains (Tcherepanov & Sokolov, 1997). Similar data have been obtained in multiple AD mouse models, where synaptophysin-immunoreactive presynaptic boutons were shown to be significantly decreased with age in various brain regions (Rutten et al., 2005).

One of the characteristics of the adult CNS, which contrasts with the PNS, is that, once neurons become damaged and deteriorate in function, they can not undergo regeneration. This functional difference is, in part, a result of inhibitors like Nogo, which are associated with the myelin ensheathing the axon of the neuron and their ability to hamper neurite outgrowth (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). It was considered here that, in addition to its function as an inhibitor of axonal regeneration, Nogo might contribute to inflammatory stages and thereby impact on neurodegeneration. So the expression of Nogo-A and Nogo-B, the two major isoforms of the Nogo protein, were assessed in this study. The data show that Nogo-B expression was significantly increased in the hippocampus with age; but that Nogo-A expression did not change. This significant upregulation in Nogo-B expression is being reported here for the first time. There are also no age-related differences in Nogo-B expression reported in the literature to date. However, due to the localisation of Nogo to the ER of the cell, it has recently been suggested that Nogo has some ER-autonomous functions (see Teng & Tang, 2008), and it was shown that the over-expression of Nogo-B induces an ER stress response, which results in apoptosis (Kuang et al., 2005; Kuang et al., 2006). This is consistent with work showing that the over-expression of the Nogo-B gene amplifies apoptosis in several cancer cell lines compared to non-tumourigenic cell lines (Li et al., 2001). Nogo-B was also found to bind to anti-apoptotic proteins, Bcl-2 and Bcl-X, diminishing their anti-apoptotic ability (Tagami et al., 2000). A very recent study reports an upregulation of Nogo-B during ischaemia but that it was necessary for subsequent blood flow recovery, macrophage infiltration and tissue repair (Yu et al., 2009). Similarly earlier findings associated Nogo-B with a vascular remodelling function upon injury, where Nogo-B promoted the migration of endothelial cells (Acevedo et al., 2004; Miao et al., 2006). The present finding that Nogo-A was similar in cortical and hippocampal tissue prepared from young and aged
rats is in contrast with the literature where studies report that there is a selective decline of Nogo-A expression with age in the hippocampus (Gil et al., 2006; Trifunovski et al., 2006).

Nogo-A and its isoform, Nogo-B, were assessed for their ability to modulate the activation of caspase-8 and -3 in neurons. In separate in vitro experiments, cortical neurons were treated with either Nogo-A or Nogo-B and the findings show that both forms can indeed alter the activity of these caspases. Nogo-A significantly increases the activity of caspase-8 and caspase-3 in a dose-dependent fashion, and while Nogo-B also increased the activation of both enzymes, the maximum effect was observed at lower concentrations than that induced by Nogo-A. The parallel age-associated increases in Nogo-B, caspase-8 and caspase-3 suggest that Nogo contributes to neurodegenerative changes in the aged brain.

The ability of Nogo-A and Nogo-B to initiate microglial activation was assessed in mixed glia, and interestingly, the data show that Nogo-B and not Nogo-A can significantly activate the expression of two markers of microglial activation. Both MHC II and CD11b expression was significantly increased upon exposure to Nogo-B. These novel findings, taken with previous data, highlight the fact that Nogo-B influences inflammatory functions in microglia.

In light of this finding, glia were incubated with Nogo-B in the presence and absence of IFN-γ to determine whether these factors could act synergistically or additively on expression of MHC II or CD11b. The data supports the previous findings in that both Nogo-B and IFN-γ significantly increase these markers of microglial activation but co-treatment did not result in any additive effect on these markers. Analysis of the effect of Nogo-B on CD40 expression in isolated microglia and astrocytes established that Nogo-B significantly increases the expression of CD40 mRNA in both cell types.

This work provides evidence of coupled neuroinflammation and neurodegenerative changes in cortex and hippocampus of aged, compared with young, rats and these are exemplified by increased expression of markers of microglial activation, MHC II, CD40 and CD11b, together with activation of caspases -8 and -3. The question was asked, does Nogo have a neuroinflammatory role in the brain? An age-related increase in the expression of Nogo-B was demonstrated for the first time; it coupled with increases in the markers of microglial activation and impairment in
neuronal function and, significantly, incubation of glia or neurons in the presence of Nogo-B revealed that it exerted direct effects on these functions, at least \textit{in vitro}. 
Chapter 6

Role of Nogo in Aβ-induced neuroinflammation
6.1 Introduction

It is accepted that neuroinflammatory changes occur in the brain of AD patients and that these are associated with neurodegenerative changes. In spite of being the focus of much research in the past decade or more, the processes involved in triggering these events are not known. However it is considered that Aβ peptides play a significant role in driving both processes and consequently analysis of changes induced by Aβ treatment is valuable in attempting to gain greater understanding. Like AD, neuroinflammation and neurodegenerative changes occur in the aged brain and in the previous chapter it was observed that Nogo-B is increased in the brain with age and that is capable of increasing microglial activation and also inducing changes in activity of caspases -8 and -3.

In this study the possibility that Nogo-B might be increased in brain tissue prepared from Aβ-treated rats was considered. Therefore the aims of this study were to examine the effects of acute and chronic Aβ-related changes in Nogo-A, Nogo-B and NgR, to evaluate whether any changes were accompanied by changes in microglial activation or activation of caspases and to determine whether Aβ-induced changes on mixed glia were amplified by Nogo-B.
6.2 Methods

Groups of young (2-3 months) rats (n=6) were either anaesthetised by ip injection of urethane (1.5g/kg, 33% w/v), received an icv injection (5μl) of sterile saline or Aβ1-40/1-42 (25μM/25μM) and sacrificed 4h post treatment, or anaesthetised by ip injection of ketamine (75mg/kg) and xylazine (10mg/kg), and implanted subcutaneously in the mid-scapular region with an Alzet 2004 osmotic minipump containing Aβ40-1 (26.9μM/36.9μM) or Aβ1-40/1-42 (26.9μM/36.9μM). The pump infused Aβ to a cannula positioned in the lateral ventricle (0.9mm posterior to bregma, 1.3mm lateral to the midline and 3.5mm ventral to the dura) at a rate of 6μl/day for 8 days (total of 48μl), at which time the rats were sacrificed. Hippocampal tissue was harvested for mRNA and protein analysis. Activity of caspase-3 was assessed using commercially-available assay kits, expression of markers of microglial activation were assessed by QPCR and western immunoblotting was used to evaluate Nogo-A, Nogo-B and NgR (see section 2.4). Cortical neurons and mixed glia were prepared from 1-day old Wistar rats. Neuronal preparations were cultured in NBM for 5-7 days at 37°C before treatment and mixed glial preparations were cultured in DMEM for 12-14 days at 37°C before treatment (see section 2.3.2). Treatments included incubation of Aβ (aggregated at 37°C for 24-48h), Nogo-A or Nogo-B at concentrations of 10μM, 10ng/ml or 100ng/ml, respectively, for 24h at 37°C in 5% CO2: 95% air before harvesting.

6.3 Results

The objective of this study was to assess whether treatment of rats with Aβ had any effect on Nogo expression and caspase-3 activation, and using in vitro techniques, to establish whether Nogo interacts with Aβ to modulate microglial or neuronal function. The mean values ± SEM for all parameters are presented in tables 6.1, 6.2, 6.3 and 6.4.
Effect of Aβ on Nogo-B in the rat hippocampus.

The data show that Nogo-A protein expression was similar in hippocampal tissue prepared from control-treated rats and rats which received a single icv injection of Aβ (1-40/1-42; 50μM; 4h; Figure 6.1a). Similarly, rats that were infused with Aβ (1-40/1-42; 63.8μM) via an implanted osmotic minipump for 8 days exerted no significant effect on hippocampal Nogo-A (Figure 6.1b). Analysis of Nogo-B indicated that while acute Aβ treatment exerted no significant effect on hippocampal Nogo-B (Figure 6.2a), chronic treatment of rats for 8 days resulted in a significant increase in its expression (*p<0.05; Student’s t-test for independent means; Figure 6.2b). Expression of NgR was also assessed in hippocampal tissue prepared from control- and Aβ-treated rats and neither acute nor chronic treatment exerted any effect on this measure (Figure 6.3). These data are reflected in the sample immunoblots in Figures 6.1, 6.2 and 6.3.

Effect of Aβ on MHC II mRNA and caspase-3 activity in the rat hippocampus.

MHC II mRNA expression and activity of caspase-3 were assessed by Dr. Anne-Marie Miller in the same hippocampal tissue in which analysis of changes in Nogo-A, Nogo-B and NgR were investigated. The data (which were kindly provided by Dr. Anne-Marie Miller) revealed that MHC II mRNA was significantly increased in hippocampal tissue prepared from rats which received a single icv injection of Aβ (*p<0.05; Student’s t-test for independent means; Figure 6.4a) and in rats which received an infusion of Aβ for 8 days (*p<0.05; 1-tailed Student’s t-test for independent means; Figure 6.4b). Caspase-3 activity was increased in both experimental paradigms; a single icv injection of Aβ, as well as infusion of Aβ for 8 days resulted in a significant increase in enzyme activity in hippocampus (***p<0.001; Student’s t-test for independent means; Figures 6.4c and 6.4d).
<table>
<thead>
<tr>
<th>Target</th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nogo-A (arbitrary units)</td>
<td>0.729 ± 0.099</td>
<td>0.726 ± 0.107</td>
</tr>
<tr>
<td>Nogo-B (arbitrary units)</td>
<td>1.019 ± 0.048</td>
<td>1.045 ± 0.102</td>
</tr>
<tr>
<td>NgR (arbitrary units)</td>
<td>0.386 ± 0.053</td>
<td>0.429 ± 0.080</td>
</tr>
<tr>
<td>MHC II mRNA (arbitrary units)</td>
<td>0.230 ± 0.031</td>
<td>0.345 ± 0.036</td>
</tr>
<tr>
<td>Caspase-3 activity (pmol/min/mg)</td>
<td>771.0 ± 6.255</td>
<td>809.7 ± 5.096</td>
</tr>
</tbody>
</table>

Statistical test performed was the Student’s t-test for independent means.

Table 6.1. Effect of acute Aβ treatment on several markers in hippocampal tissue. Data are expressed as means ± standard error of the mean (n>6).

<table>
<thead>
<tr>
<th>Target</th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nogo-A (arbitrary units)</td>
<td>0.119 ± 0.040</td>
<td>0.133 ± 0.049</td>
</tr>
<tr>
<td>Nogo-B (arbitrary units)</td>
<td>0.159 ± 0.024</td>
<td>0.231 ± 0.024</td>
</tr>
<tr>
<td>NgR (arbitrary units)</td>
<td>0.606 ± 0.049</td>
<td>0.661 ± 0.139</td>
</tr>
<tr>
<td>MHC II mRNA (arbitrary units)</td>
<td>0.226 ± 0.030</td>
<td>0.365 ± 0.057</td>
</tr>
<tr>
<td>Caspase-3 activity (pmol/min/mg)</td>
<td>604.0 ± 21.70</td>
<td>799.5 ± 11.68</td>
</tr>
</tbody>
</table>

Statistical test performed was the Student’s t-test for independent means.

Table 6.2. Effect of chronic Aβ treatment on several markers in hippocampal tissue. Data are expressed as means ± standard error of the mean (n>5).
Effect of Aβ alone, and in combination with Nogo-B, on markers of microglial activation in mixed glia.

In an effort to assess the potential of Aβ, alone and in combination with Nogo-B to stimulate microglial activation, mixed glia were incubated in the presence of Aβ (10μM) and/or Nogo-A (10ng/ml)/Nogo-B (100ng/ml), and the expression of MHC II and CD11b was assessed. The data shows both Aβ and Nogo-A significantly increase MHC II expression in rat cortical mixed glia (*p<0.05; Student’s t-test for independent means; Figure 6.5a). CD11b expression is significantly upregulated by Aβ, and Aβ and Nogo-A in combination, although there is no significant modulation of CD11b by Nogo-A alone (*p<0.05; Student’s t-test for independent means; Figure 6.5b).

The data indicate that both Aβ and Nogo-B, alone and in combination, significantly increased expression of MHC II mRNA (*p<0.01; Student’s t-test for independent means; Figure 6.6a) and CD11b mRNA (**p<0.01; Student’s t-test for independent means; ***p<0.001; ANOVA; Figure 6.6b). However there was no evidence of an additive effect on either marker of microglial activation.

Effect of Aβ alone, or in combination with either Nogo-A or Nogo-B on activity of caspase-3 in rat cortical neurons.

The data presented in Figures 6.7 and 6.8 indicate that Aβ (10μM) exerted no significant effect on caspase-3 activity in cultured neurons. As shown in Figure 5.10b and in Figure 6.6, Nogo-A increased caspase-3 activity in a dose-dependent manner so that both 10ng/ml and 100ng/ml Nogo-A significantly increased caspase-3 activity compared with control (**p<0.01; ***p<0.001; ANOVA; Figures 6.7a and 6.7b). Addition of Aβ significantly increased the effect of 10ng/ml Nogo-A so that a marked synergistic effect of the 2 agents was observed (***p<0.001, versus control; +++p<0.001, versus Aβ alone and Nogo-A alone; ANOVA; Figure 6.7a). However, the effect of 100ng/ml Nogo-A and Aβ together decreased caspase-3 activity relative to Nogo-B alone (###p<0.001; ANOVA; Figure 6.7b); although it remained significantly greater than control value (**p<0.01; ANOVA) or Aβ alone (+++p<0.001; ANOVA; Figure 6.7b).

In contrast to the effect of Nogo-A, 10ng/ml Nogo-B significantly decreased caspase-3 activity (**p<0.001; ANOVA; Figure 6.7a), whereas 100ng/ml Nogo-B
significantly increased it (**p<0.001; ANOVA; Figure 6.8b). The combination of 10ng/ml Nogo-B and Aβ resulted in an increase in caspase-3 activity (**p<0.01, versus control; +++p<0.01 versus Aβ alone and Nogo-B alone; Anova; Figure 6.8a). Like Nogo-A, the effect of 100ng/ml Nogo-B and Aβ together decreased caspase-3 activity relative to Nogo-B alone (###p<0.001; ANOVA; Figure 6.8b); although it remained significantly greater than control value (**p<0.01; ANOVA) or Aβ alone (++p<0.01; ANOVA; Figure 6.8b).

<table>
<thead>
<tr>
<th>Target</th>
<th>Cell Type</th>
<th>Control</th>
<th>Aβ</th>
<th>Nogo-B (100ng/ml)</th>
<th>Aβ + Nogo-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II mRNA</td>
<td>Cortical mixed glia</td>
<td>0.907 ± 0.070</td>
<td>1.283 ± 11.99</td>
<td>1.288 ± 14.76</td>
<td>1.209 ± 11.82</td>
</tr>
<tr>
<td>CD11b mRNA</td>
<td>Cortical mixed glia</td>
<td>1.026 ± 0.072</td>
<td>2.034 ± 0.188</td>
<td>1.337 ± 0.049</td>
<td>2.364 ± 0.175</td>
</tr>
<tr>
<td>Caspase-3 activity</td>
<td>Cortical neurons</td>
<td>200.10 ± 6.07</td>
<td>208.50 ± 11.50</td>
<td>128.30 ± 9.80</td>
<td>251.50 ± 6.27</td>
</tr>
<tr>
<td>Caspase-3 activity</td>
<td>Cortical neurons</td>
<td>200.10 ± 6.07</td>
<td>208.50 ± 11.50</td>
<td>432.20 ± 13.13</td>
<td>255.50 ± 5.81</td>
</tr>
</tbody>
</table>

Statistical test performed was 1-way ANOVA.

Table 6.3. Effect of Nogo-B alone and in combination with Aβ in vitro. Data are expressed as means ± standard error of the mean (n≥6).
<table>
<thead>
<tr>
<th>Target</th>
<th>Cell Type</th>
<th>Control</th>
<th>Aβ</th>
<th>Nogo-A (10ng/ml)</th>
<th>Aβ + Nogo-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC II mRNA</td>
<td>Cortical mixed glia</td>
<td>0.768 ± 0.084</td>
<td>1.297 ± 0.184</td>
<td>1.284 ± 0.204</td>
<td>1.093 ± 0.175</td>
</tr>
<tr>
<td>CD11b mRNA</td>
<td>Cortical mixed glia</td>
<td>0.700 ± 0.112</td>
<td>1.302 ± 0.195</td>
<td>1.225 ± 0.220</td>
<td>1.305 ± 0.236</td>
</tr>
<tr>
<td>Caspase-3 activity</td>
<td>Cortical neurons</td>
<td>1265 ± 16.91</td>
<td>1199 ± 25.15</td>
<td>1702 ± 23.93</td>
<td>7192 ± 177.4</td>
</tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-3 activity</td>
<td>Cortical neurons</td>
<td>1265 ± 16.91</td>
<td>1199 ± 25.15</td>
<td>2327 ± 34.79</td>
<td>1381 ± 17.36</td>
</tr>
</tbody>
</table>

Statistical test performed was 1-way ANOVA.

Table 6.4. Effect of Nogo-A alone and in combination with Aβ in vitro. Data are expressed as means ± standard error of the mean (n≥6).
Figure 6.1. Effect of Aβ on Nogo-A in the rat hippocampus.

Hippocampal tissue was dissected free from young (2-3 months) male Wistar rats that were either anaesthetised by ip injection of urethane (1.5g/kg, 33% w/v), received an icv injection (5µl) of sterile saline or Aβ_{1-40/1-42} (25µM/25µM; acute treatment) and sacrificed 4h post treatment, or anaesthetised by ip injection of ketamine (75mg/kg) and xylazine (10mg/kg), and implanted subcutaneously with an osmotic minipump containing Aβ_{40-1} (26.9µM/36.9µM) or Aβ_{1-40/1-42} (26.9µM/36.9µM; chronic treatment), which infused Aβ at a rate of 6µl/day for 8 days (total of 48µl). At this point, the rats were sacrificed by decapitation. Hippocampi were homogenised and assessed for Nogo-A expression by western immunoblot.

(a) Nogo-A expression was similar in hippocampal tissue prepared from rats acutely treated with Aβ compared with control.

(b) Nogo-A expression was similar in hippocampal tissue prepared from rats chronically treated with Aβ, compared with tissue obtained from control rats. Data are expressed as a ratio of Nogo-A:β-actin, and means ± SEM (n=6) are presented. Sample immunoblots are shown.
Figure 6.2. Effect of Aβ on Nogo-B in the rat hippocampus.

Hippocampal tissue was dissected free from young (2-3 months) male Wistar rats that were either anaesthetised by ip injection of urethane (1.5g/kg, 33% w/v), received an icv injection (5μl) of sterile saline or Aβ_{1-40/1-42} (25μM/25μM; acute treatment) and sacrificed 4h post treatment, or anaesthetised by ip injection of ketamine (75mg/kg) and xylazine (10mg/kg), and implanted subcutaneously with an osmotic minipump containing Aβ_{40,1} (26.9μM/36.9μM) or Aβ_{1-40/1-42} (26.9μM/36.9μM; chronic treatment), which infused Aβ at a rate of 6μl/day for 8 days (total of 48μl). At this point, the rats were sacrificed by decapitation. Hippocampi were homogenised and assessed for Nogo-B expression by western immunoblot.

(a) Nogo-B expression was similar in hippocampal tissue prepared from rats acutely treated with Aβ, compared with control-treated rats.

(b) Nogo-B expression was significantly increased in hippocampal tissue prepared from rats chronically treated with Aβ, compared with control-treated rats (*p < 0.05, 1-tailed Student's t-test for independent means).

Data are expressed as a ratio of Nogo-B/β-actin, and means ± SEM (n=6) are presented. Sample immunoblots are shown.
Figure 6.3. Effect of Aβ on NgR in the rat hippocampus.
Hippocampal tissue was dissected free from young (2-3 months) male Wistar rats that were either anaesthetised by ip injection of urethane (1.5g/kg, 33% w/v), received an icv injection (5μl) of sterile saline or Aβ$_{1-40/1-42}$ (25μM/25μM; acute treatment) and sacrificed 4h post treatment, or anaesthetised by ip injection of ketamine (75mg/kg) and xylazine (10mg/kg), and implanted subcutaneously with an osmotic minipump containing Aβ$_{40}$ (26.9μM/36.9μM) or Aβ$_{1-40/1-42}$ (26.9μM/36.9μM; chronic treatment), which infused Aβ at a rate of 6μl/day for 8 days (total of 48μl). At this point, the rats were sacrificed by decapitation. Hippocampi were homogenised and assessed for NgR expression by western immunoblot.
(a) NgR expression was similar in hippocampal tissue prepared from rats acutely treated with Aβ, compared with control-treated rats.
(b) NgR expression was similar in hippocampal tissue prepared from rats chronically treated with Aβ, compared with control-treated rats. Data are expressed as a ratio of NgR:β-actin, and means ± SEM (n=6) are presented. Sample immunoblots are shown.
(a) **Acute Aβ**

- **NgR (51kDa)**
- **β-actin (43kDa)**

Comparison between Control and Aβ.

(b) **Chronic Aβ**

- **NgR (51kDa)**
- **β-actin (43kDa)**

Comparison between Control and Aβ.
Hippocampal tissue was dissected free from young (2-3 months) male Wistar rats that were either anaesthetised by ip injection of urethane (1.5g/kg, 33% w/v), received an icv injection (5μl) of sterile saline or \( \text{Aβ}_{40/42} \) (25μM/25μM; acute treatment) and sacrificed 4h post treatment, or anaesthetised by ip injection of ketamine (75mg/kg) and xylazine (10mg/kg), and implanted subcutaneously with an osmotic minipump containing \( \text{Aβ}_{40/42} \) (26.9μM/36.9μM) or \( \text{Aβ}_{1-40/1-42} \) (26.9μM/36.9μM; chronic treatment), which infused Aβ at a rate of 6μl/day for 8 days (total of 48μl). At this point, the rats were sacrificed by decapitation. Hippocampi were homogenised and assessed for MHC II mRNA and caspase-3 activity by QPCR and assay kit, respectively.

(a, b) MHC II mRNA was significantly increased in hippocampal tissue prepared from rats acutely (a; *p<0.05; Student’s t-test for independent means) and chronically (b; *p<0.05; 1-tailed Student’s t-test for independent means) treated with Aβ.

(c, d) Caspase-3 activity was significantly increased in hippocampal tissue prepared from rats acutely (c) and chronically (b) treated with Aβ (** p<0.001; Student’s t-test for independent means). Data are expressed as a ratio of MHC II:β-actin or as pmol/min/mg, and means ± SEM (n>5) are presented.

**NOTE:** These data were kindly provided by Dr. Anne-Marie Miller and were obtained from the same tissue in which analysis of Nogo was undertaken.
(a) Acute Aβ

(b) Chronic Aβ

(c) Acute Aβ

(d) Chronic Aβ
Figure 6.5. Effect of Nogo-A alone or in combination with Aβ on MHC II mRNA and CD11b mRNA in rat cortical mixed glia.

Cortical mixed glia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with Aβ (10μM; aggregated for 24-48h at 37°C) and/or Nogo-A (10ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for MHC II mRNA and CD11b mRNA by QPCR.

(a) MHC II mRNA expression was significantly increased in mixed glia treated with Aβ or Nogo-A alone.

(b) CD11b mRNA expression was significantly increased in mixed glial cells treated with Aβ or Aβ + Nogo-A (*p<0.05; ANOVA), but no significant additive effect was observed. Data are expressed as a ratio of MHC II mRNA:β-actin mRNA and CD11b mRNA:β-actin mRNA, respectively, and means ± SEM (n>6) are presented.
(a) MHC II mRNA

Control  Aβ  Nogo-A  Aβ + Nogo-A

(b) CD11b mRNA

Control  Aβ  Nogo-A  Aβ + Nogo-A
Cortical mixed glia were prepared from 1-day old Wistar rats and cultured in DMEM for 12-14 days at 37°C before incubation of cells with Aβ (10μM; aggregated for 24-48h at 37°C) and/or Nogo-B (100ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for MHC II mRNA and CD11b mRNA by QPCR. MHC II mRNA (a) and CD11b mRNA (b) was significantly increased in mixed glia treated with Aβ or Nogo-B alone or in combination (*p<0.05; Student’s t-test for independent means; **p<0.01; ***p<0.001; ANOVA), but no significant additive effect was observed. Data are expressed as a ratio of MHC II mRNA:β-actin mRNA and CD11b mRNA:β-actin mRNA, respectively, and means ± SEM (n=12) are presented.
Figure 6.7. Effect of Nogo-A alone or in combination with Aβ on the activation of caspase-3 in cortical neurons.

Cortical neurons were prepared from 1-day old Wistar rats and cultured in NBM for 5-7 days at 37°C before incubation of cells with Aβ (10μM; aggregated for 24-48h at 37°C) and/or Nogo-A (10ng/ml or 100ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for caspase-3 by assay kit. Aβ exerted no effect on caspase-3 in cultured neurons whereas Nogo-A (10ng/ml; a, and 100ng/ml; b) significantly increased activity of caspase-3 (**p<0.01; ***p<0.001 versus control; ANOVA). The combination of Aβ and 10ng/ml Nogo-A significantly increased caspase-3 activity (**p<0.001 versus control; +++p<0.001 versus Aβ or Nogo-A alone; ANOVA), whereas the combination of Aβ and 100ng/ml Nogo-A decreased caspase-3 activity relative to Nogo-A alone (b; ###p<0.001; ANOVA) although it remained significantly greater than control value (*p<0.05; ANOVA) or Aβ alone (+++p<0.001; ANOVA). Data are expressed as pmol/min/mg and are means ± SEM (n=6).
Figure 6.8. Effect of Nogo-B alone or in combination with Aβ on the activation of caspase-3 in cortical neurons.

Cortical neurons were prepared from 1-day old Wistar rats and cultured in NBM for 5-7 days at 37°C before incubation of cells with Aβ (10μM; aggregated for 24-48h at 37°C) and/or Nogo-B (10ng/ml or 100ng/ml) for 24h at 37°C in 5% CO₂: 95% air. Once harvested, lysates were assessed for caspase-3 by assay kit. Aβ exerted no effect on caspase-3 in cultured neurons whereas Nogo-B (10ng/ml; a) significantly decreased it and Nogo-B (100ng/ml; b) significantly increased activity of caspase-3 (***p<0.001 versus control; ANOVA). The combination of Aβ and 10ng/ml Nogo-B significantly increased caspase-3 activity (a; **p<0.01 versus control; ††p<0.01 versus Aβ; ‡‡‡p<0.001 versus Nogo-B; ANOVA), whereas the combination of Aβ and 100ng/ml Nogo-B decreased caspase-3 activity relative to Nogo-B alone (b; ‡‡‡p<0.001; ANOVA) although it remained significantly greater than control value (***p<0.01; ANOVA) or Aβ alone (††p<0.01; ANOVA). Data are expressed as pmol/min/mg and are means ± SEM (n=6).
6.4 Discussion

The aim of this study was to assess whether acute or chronic treatment of rats with Aβ affected Nogo expression in the hippocampus and, using an in vitro approach, to determine whether the presence of Aβ modulated the effect of Nogo on microglial activation or affected caspase-3 activity in neurons. The data show that intracerebral infusion of Aβ for 8 days increased hippocampal expression of Nogo-B, although it did not affect Nogo-A. Nogo-B increased expression of markers of microglial activation in a mixed glial culture and modulated caspase-3 activity in cultured neurons, but the interaction with Aβ on these two measures is complex.

In this study two in vivo models were used to further investigate the role of Nogo protein in the brain. In one model rats were injected intracerebroventricularly with a single injection of Aβ40/1-42 and killed 4 hours later, while in the second model rats were chronically infused with Aβ40/1-42 for 8 days. The expression levels of Nogo-A, -B and its receptor, NgR were assessed in the hippocampus. No changes in the expression levels of Nogo-A, Nogo-B or NgR were observed after acute treatment. Similarly chronic treatment exerted no effect on expression of Nogo-A and NgR. However the expression of Nogo-B was significantly increased upon chronic administration of Aβ. To my knowledge this Aβ-related increase in Nogo-B is reported for the first time here. There is evidence to suggest that Nogo-B has an important role in modulating the production of Aβ. It has been shown in various studies that Nogo-B interacts with BACE1, the β-secretase enzyme that is responsible for APP processing that culminates in the production of Aβ (Nakajima et al., 2004; He et al., 2006; Murayama et al., 2006; Wojcik et al., 2007). It was shown that the overexpression of Nogo-B resulted in a reduction of Aβ production, suggesting Nogo-B negatively modulates BACE1-mediated Aβ production. The suggested mechanism involves Nogo-B-inducing changes in the trafficking capabilities of the enzyme. Some of the authors involved in these studies have since recently confirmed this inhibitory effect in normal BACE1 functioning (Kume et al., 2009). On one hand the finding that Aβ upregulates Nogo-B may be interpreted as a potentially neuroprotective response. It has been suggested that Nogo-A and NgR may act in a neuroprotective capacity; Nogo-A may be involved in the preconditioning of neurons and glia against ER stress, thus increasing
their resistance to apoptotic insults after injury in the CNS (see Teng & Tang, 2008). Also a recent study on ALS reports the transient overexpression of Nogo-A and NgR in motor neurons; suggesting a survival reaction of these cells under stressful conditions (Miyazaki et al., 2009).

Despite the lack of modulation in Nogo-A expression with Aβ described here, Nogo-A has been reported to be overexpressed around and within Aβ-stained deposits (Gil et al., 2006), and so it is possible that exposure of rats to Aβ for 8 days was insufficient to increase Nogo-A and that a longer exposure may be necessary to increase its exposure. It has been found that Nogo-A expression significantly increases in neurons in a mouse model for ALS (Dupuis et al., 2002), another neurodegenerative disease, typified by selective degeneration of motor neurons. This finding was also shown to be positively correlated with severity of symptoms in patients (Jokic et al., 2005).

Like Nogo-B, NgR has also been implicated in reducing the production of Aβ. It has been shown to interact physically with APP and Aβ, and the deletion of the NgR gene results in increased Aβ levels and plaque deposition in a double transgenic AD mouse model, where injection of soluble NgR produces the opposite effect (Park et al., 2006a; Park et al., 2006b). This suggests functions for NgR other than its role in inhibiting axonal regeneration. Increased NgR immunoreactivity has been associated with Aβ plaques and due to a similar distribution to activated microglia, it was suggested that some of the immunoreactivity could indicate NgR+ activated microglia (Simard et al., 2006). This theory seems a reasonable one as the expression of NgR in activated macrophages has been reported in different nervous system disorders, such as peripheral nerve injury, SCI and MS (see David et al., 2008). The association of NgR with Aβ is consistent with the reported presence of Nogo-A in Aβ deposits (Gil et al., 2006). However, despite the associative findings of NgR with AD in the literature, there were no changes in the expression of NgR with Aβ treatment in the present study.

The data presented in chapter 5 demonstrated that Nogo-B expression was associated with microglial activation in the brain of aged rats and that it also increased activation of caspases-8 and -3. It was of interest to assess whether parallel changes occurred in animals treated with Aβ and therefore expression of MHC II and activation of caspase-3 were assessed in aliquots of the same tissue in which Nogo expression was investigated. These experiments were undertaken by Dr Anne-Marie Miller and she provided the data presented in Figure 6.4; overall, these data reveal that acute and
chronic Aβ treatment increased both measures in hippocampal tissue. The data show a significant increase in MHC II mRNA with both acute and chronic treatment of Aβ. Interestingly the literature supports the association of AD and an upregulated MHC II expression (Parachikova et al., 2007). The data also show a significant upregulation in the activity of caspase-3 in both the acute and chronic models of AD. This work is supported by the literature where it has been found that the chronic administration of Aβ increases the activation of caspase-3 (Miller et al., 2009). Various other studies support these findings (Selznick et al., 1999; Stadelmann et al., 1999; Behl, 2000).

It was shown in chapter 5 that Nogo-B can induce the expression of markers of microglial activation and here, it was considered that Aβ in combination with Nogo-A or Nogo-B might alter the ability of these proteins to express markers of microglial activation. Mixed glia were treated with Nogo-A/Nogo-B alone and in combination with Aβ in vitro, and assessed for the mRNA expression of MHC II and CD11b. The data show that incubation of mixed glia with Aβ increased expression of MHC II and CD11b indicating that it upregulated microglial activation. Several groups (Yates et al., 2000; Streit, 2004; Rogers et al., 2007; Jana et al., 2008), including this group (Lyons et al., 2007b) have reported that Aβ increased microglial activation and, in particular these data are consistent with the finding that Aβ increases MHCII mRNA in mixed glia (Lyons et al., 2007b). The mechanism by which Aβ exerts this effect is not clear but there are several possible binding proteins which appear to act as receptors for Aβ including RAGE, class A scavenger receptors, the P2X7 receptor and the ApoE receptor (El Khoury et al., 1996; Yan et al., 1996; El Khoury et al., 1998; Lue et al., 2001; Sanz et al., 2009); any one of these may be activated to produce the effect described here.

Both Nogo-A and Nogo-B increased microglial activation as indicated by upregulation of MHC II mRNA and, in regards to Nogo-B, CD11b mRNA and this is consistent with the Nogo-B data presented in chapter 5. While it was not explored in this study, it is assumed that this action is mediated by interaction with NgR, based on recent reports revealing the expression of NgR on microglia and activated macrophages (David et al., 2008). Here, the interaction between Aβ and Nogo-A or Nogo-B on these markers of microglial activation was assessed and there was no evidence of an additive effect. It could be speculated that in addition to the known receptors for Aβ and Nogo-A/Nogo-B on microglia (El Khoury et al., 1996; Yan et al., 1996; El Khoury et al., 1998; David et al., 2008), there may be another receptor yet to be discovered or fully characterised that both Aβ and Nogo protein are capable of binding, resulting in an
activated microglial profile. Alternatively, Aβ and Nogo may share an already known receptor that one of them is already associated with binding. Either speculation would imply potential competition for the receptor in question explaining a possible reason why there was no additive effect seen here, although further studies are required in order to investigate these suggested reasons for a lack of an additive effect. It is possible that the kinetics of the respective actions of Nogo-B and Aβ are different and that assessment of changes at different time points is required to identify optimal conditions and subsequently to uncover any possible additive effect. It is also possible that the lack of an interaction reflects a ceiling effect. Further evaluation is necessary to fully explore any additive or synergist effect of Nogo-B and Aβ on these markers of microglial activation.

Because caspase-3 activity was increased in hippocampal tissue prepared from Aβ-treated rats in which Nogo-B was also increased, it was considered that Aβ might interact with Nogo protein to modulate its effect on caspase-3. The findings show that Nogo-A increased caspase-3 activity in a dose-dependent manner while Aβ at the concentration used here (10μM) exerted no effect. The combination of the lower dose of Nogo-A and Aβ markedly increased caspase-3 activity with clear evidence of a synergistic effect; this suggests that any potential damaging effect of Aβ is exacerbated in circumstances in which Nogo-A is decreased. However this effect is dependent on the dose of Nogo-A since the higher concentration had the opposite effect. Nogo-B also exerted dose-related changes in caspase-3 activity in cultured neurons and the interaction with Aβ was also dose-dependent; whereas the combination of the lower concentration of Nogo-B and Aβ markedly increased caspase-3 activity compared with either agent alone, the higher concentration of Nogo-B had the opposite effect. The lower dose of Nogo-B significantly decreased caspase-3 activity; however the control group values were quite high. So in this instance, the decrease in caspase-3 activity by Nogo-B at its lower dose, and the failure of Aβ to induce a response may simply be due to a contaminated control group, as the treatment wells for Aβ were adjacent to the control wells. It is uncertain why Aβ (10μM) did not exert an effect on caspase activity in neurons, however possible reasons may include insufficient aggregation of the Aβ peptide prior to its use or the use of a suspect batch of Aβ peptide. There is also the possibility that the concentration of 10μM was not sufficient to elicit a response, however when incubated in combination with Nogo protein, there appeared to be a
significant response in caspase activity. However, the presence of Nogo protein may have provided just enough toxicity in conjunction with the effect of Aβ to significantly alter caspase activity. Alternatively there may have been a failure to deliver the sufficient concentration of Aβ to the culture plate wells. These experiments need to be repeated and carried out in conjunction with an assessment of cell viability. This requires further study to fully understand the nature of the interaction between Aβ and Nogo-A, and between Aβ and Nogo-B, and a detailed assessment of the changes in concentration of Nogo-A or Nogo-B under different circumstances in vivo is necessary to appropriately evaluate the interaction.

Under chronic conditions, Nogo-B expression was significantly upregulated in a model of AD, which was shown to have a profile consistent with activated microglia and neuronal dysfunction; further providing evidence for the involvement of Nogo-B in neurodegeneration. It was subsequently shown that, despite any upregulation in markers of microglia by Nogo-A or Nogo-B, the presence of Aβ did not augment this. Further work is needed to fully elucidate the modulatory effect of Nogo on Aβ-induced change. However it appears that both Nogo-A and Nogo-B may exacerbate the inflammatory effects of Aβ on neurons at low doses, but attenuate those effects at a higher dose. However neuronal cell viability needs to be assessed during these incubations before it can be said Nogo protein has any neuroprotective roles.
Chapter 7
Overview
7.1 General Discussion

The data presented here indicate that there is age-related increase in microglial activation and that there are at least two potential activators. The first is IFN-γ and the data indicate that this may be derived from NK cells, which are increased in the brain of aged rats. The second possible activator is Nogo-B, which is also increased in the brain of aged rats and which, not only increases microglial activation but also triggers an increase in caspase-3 activity.

The brain in the past was considered immune privileged due to the presence of the BBB, which, under normal conditions, functions to prevent infectious agents and circulating immune cells from entering the brain. However if the BBB becomes compromised, its ability to control trafficking into the brain diminishes, which can lead to the infiltration of peripheral immune cells including NK cells. Numerous studies have suggested that an increase in the permeability of the BBB contribute to neuroinflammation in several neurodegenerative disorders and in ageing (Hosokawa & Ueno, 1999; Pahnke et al., 2009; see Popescu et al., 2009; see Stolp & Dziegielewska, 2009). Inflammatory mediators have multiple actions on the neurovascular unit, which result in increased permeability of the BBB. Pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α produced systemically or within the brain by activated microglia can signal through the endothelial cells, of which the BBB consists, to cause an alteration in the tight junction structure, leading to increased permeability of the BBB (see Stolp & Dziegielewska, 2009). The increased permeability and elevation of inflammatory instigators can subsequently result in damage to brain parenchyma, glia, neurons or axons. A central doctrine of this thesis is the belief that ageing is associated with an increase in the activation of microglia and associated neuroinflammation in the brain and these changes are known to play a role in neurodegenerative changes in the CNS. It is proposed that the BBB becomes compromised during ageing and in this study it was revealed not only that NK cells are present in the brain of aged rats but also that there is an age-related increase in the number of NK cells. This finding indicates first, that the BBB is compromised in the aged brain and the second that NK cells by releasing IFN-γ may be involved in initiating the age-related activation of microglia in the brain of aged animals. This result also parallels the finding that TNF-α is increased with age,
implicating TNF-α as a potential causative agent in increasing the permeability of the BBB (Saija et al., 1995; Abraham et al., 1996; Pan & Kastin, 2001). Interestingly, it is also worth noting that Nogo-B has been shown to promote the migration of endothelial cells (Acevedo et al., 2004) and a Nogo-B-specific receptor has been found to be expressed on endothelial cells (Miao et al., 2006). Taking the interaction of Nogo-B with endothelial cells into account, one could speculate that, under particular circumstances, Nogo-B may negatively impact on the permeability of the BBB via this receptor; however this concept would require further study. Nogo-B and its involvement in neuroinflammation will be discussed in more detail later.

Due to the characteristic cytotoxic activities of NK cells, their presence in the brain can have adverse effects in the CNS. These cells are the principal producers of IFN-γ (Obara et al., 2005), and so it is not surprising that the age-related increase in NK cells is correlated with an increase in the expression of IFN-γ in the brain. In vitro data in this study reveal IFN-γ as a potent activator of microglia, as demonstrated by an IFN-γ-induced increase in the expression of MHC II, CD40 and CD11b. The present findings also show that IFN-γ can work concomitantly with TNF-α, another pro-inflammatory cytokine that can be produced by NK cells; this combination can further enhance the expression of CD40, a receptor whose binding with its ligand can lead to further production of pro-inflammatory cytokines like TNF-α. Interestingly the age-related increase in NK cells in the brain was shown to parallel an increase in OX-6^CD11b^CD45^ cells, indicating an upregulation in the presence of activated microglia in the brain.

Together these data have established an association between ageing and the infiltration of NK cells into the brain during ageing, where they actively produce IFN-γ to activate microglia. The concept of infiltrating NK cells into the brain is consistent with data from studies reporting the infiltration of NK cells into the brain in neurodegenerative disorders like MS and the animal model of MS, EAE (Matsumoto et al., 1998; Hammarberg et al., 2000; Dousset et al., 2006; Huang et al., 2006; Leech et al., 2007).

In addition to IFN-γ, NK cells are known to produce another pro-inflammatory cytokine, HMGB1 (Semino et al., 2007), which is capable of inducing NK cells to produce IFN-γ (DeMarco et al., 2005). However HMGB1 expression does not appear to change with age, which is in contrary to the literature where it was reported that
HMGB1 expression is slightly increased in the hippocampus and cortex of aged, compared with, middle-aged mice (Enokido et al., 2008). However despite evidence showing that HMGB1 can induce chronic inflammation (Scaffidi et al., 2002; O'Connor et al., 2003; Wang et al., 2004; Kim et al., 2006; Yamada & Maruyama, 2007; Andersson et al., 2008), it was shown that the stress-induced secretion of HMGB1 by monocytes/macrophages is in fact a delayed inflammatory response (Wang et al., 1999). So despite no age-related changes in HMGB1 expression seen here, HMGB1 may play a role in ageing and neurodegenerative disorders, which should be assessed in a time-dependent manner in an in vivo study. In vitro data presented here demonstrate the ability of HMGB1 to induce an increase in MHC II expression. Data in the literature support HMGB1 as an activator of microglia where it has been shown that released HMGB1 interacts with TLR2, TLR4 and RAGE (Lotze et al., 2007; Yamada & Maruyama, 2007). There is evidence proposing HMGB1 and IFN-γ function synergistically to increase NO production (Kim et al., 2006), but in contrast to this suggestion, co-incubation in mixed glia, pure microglia or pure astrocytes in the presence of IFN-γ and HMGB1 did not provide evidence of an additive effect with respect to their ability to alter the expression of markers of microglial activation.

These data establish an association between age, microglial activation, an increase in IFN-γ expression and NK cell presence in the brain. It has been shown that once activated, microglia can initiate neuroinflammatory changes through their ability to present antigen, and increase the production of inflammatory cytokines (Benveniste et al., 2004). It is also known that there are several states of microglial activation, each having their own combination of functional capabilities. In this study, the evidence presented suggests the possible presence of many states of activation in the brain of aged rats. Various markers of microglial activation were assessed in order to ascertain the potential states of activation present with age. The data presented show age-related increases in the expression of MHC II, CD68 and TLRs-2 and -4 and these findings provide evidence for the potential presence of multiple microglial activation states in the brain of aged rats.

Here the upregulated expression of MHC II found in the brain of aged rats indicated an activated state of microglia with increased antigen presentation capabilities (Kreutzberg, 1996; Stoll & Jander, 1999; Aloisi, 2001; Benveniste et al., 2004). The presence of the MHC II antigen on activated microglia enables their interaction with T
cells, activating the T cells and thus initiating an immune response (Brown et al., 1993; Germain & Margulies, 1993; Cresswell, 1994; Germain, 1994; Bentley et al., 1995; Fields et al., 1995; Rhode et al., 1996; Watts, 1997; Davis et al., 1998; Chambers, 2001).

An age-related increase in the expression of CD68, which is a proposed marker of phagocytic activity, suggests the presence of an amoeboid activated microglial state and/or a fully activated phagocytic state (Ferrer et al., 1990; Kreutzberg, 1996; Aloisi, 2001; Christensen et al., 2006). The functional capabilities of the amoeboid activation state of microglia are restricted when compared with the activated phagocytic state; it has been proposed that they are incapable of antigen presentation or secreting inflammatory cytokines (Kreutzberg, 1996). However in addition to these known phagocytic states, there is now evidence from a recent study suggesting other activated microglial states exist, which include phagocytic ability during the transition from a resting microglial profile to the amoeboid-like state (McKay et al., 2007). The present data reveal concomitant increases in the expression of TLR2 and TLR4, which further supports the presence of phagocytic microglia in the aged brain. Evidence has highlighted the ability of TLR2 and TLR4 to control numerous stages of the phagocytic process and data from in vivo and in vitro studies has demonstrated the involvement of these TLRs in triggering phagocytosis (Blander & Medzhitov, 2004; Tahara et al., 2006; Blander, 2007b, a; Okura et al., 2008; Richard et al., 2008; Ribes et al., 2009). An increase in TLR signalling has also been shown to be involved in the transcription of cytokines and co-stimulatory molecules involved in the immune response (Liew et al., 2005). The present findings in which parallel changes in TLR expression and expression of TNF-α and IFN-γ is consistent with this.

In this study the age-related increase in microglial activation is associated with evidence of deterioration in neuronal function as indicated by an upregulation in the activities of caspases -8 and -3 in tissue prepared from the aged brain. Increases in caspase-8 and caspase-3 signify the initiation of the caspase cascade that culminates in DNA damage and the possible destruction of a cell, and a decrease in synaptophysin expression implies a reduction in the abundance of synaptic terminals, which would be indicative of a loss in neurons.

Once a neuron is damaged, it is incapable of regenerative abilities, in part, due to the presence of myelin-associated inhibitors, such as Nogo. In addition to the
inhibitory actions of Nogo, evidence from the literature has also implicated Nogo and its various isoforms in aspects of the neuroinflammatory process. The findings presented here confirm the involvement of the Nogo protein in age-related neuroinflammation for the first time. The second largest isoform of the Nogo protein, Nogo-B, was reported here to increase with age, paralleling the evidence presented on neuronal cell dysfunction and microglial activation. Significantly in vitro experiments confirm the ability of Nogo-B to alter the activity of caspases -8 and -3 in cultured neurons and its greatest effect was seen at a lower concentration. Like Nogo-B, Nogo-A can also alter the activation of these enzymes. Even though to date there are no reports of any age-related changes in Nogo-B expression, evidence demonstrating the ability of Nogo-B to induce apoptosis has been presented, where the overexpression of Nogo-B induces an ER response that led to apoptosis (Kuang et al., 2005; Kuang et al., 2006). Research has also shown that Nogo-B can amplify apoptosis in cancer cell lines (Li et al., 2001), while it binds to anti-apoptotic proteins, effectively diminishing their anti-apoptotic ability (Tagami et al., 2000).

The findings presented here demonstrate that Nogo-B can initiate an activated microglial profile in mixed glia and also in purified cultures of microglia and astrocytes but Nogo-A fails to do so. An investigation into the ability of Nogo-B to interact with IFN-γ to activate microglia in vitro showed that these two potent activators do not act in a synergistic manner. These findings collectively confirm Nogo-B as a significant immunomodulatory protein in upstream and downstream events of neurodegeneration, and may play a role in age-associated neurodegeneration.

Since ageing is considered a significant risk factor in the pathogenesis of neurodegenerative diseases like AD, and neuroinflammation is known to precede this eventual neurodegeneration, it seemed reasonable to suggest that Nogo-B expression is tied to the neuroinflammatory process. Consistent with this proposal was a novel finding showing Nogo-B expression was markedly increased in Aβ-treated rats, and that this increase in expression coincided with an upregulation in microglial activation and neuronal dysfunction. Despite a lack of change in Nogo-A expression in this study, its expression has been reported to be increased around and within Aβ deposits (Gil et al., 2006) and its expression is also increased in post-mortem brain tissue obtained from patients with the neurodegenerative disorder, ALS (Dupuis et al., 2002). It is possible that a longer exposure to Aβ may lead to overexpression of Nogo-A than the 8 day-
infusion of Aβ used here, and that Nogo-A may still play a role in modulating changes in the neuroinflammatory process.

In light of previous in vitro findings concerning the Nogo protein, and the parallel changes seen in this AD study with Nogo-B and evidence of microglial activation and neuronal dysfunction, it seemed logical to investigate whether the presence of Aβ can alter the ability of Nogo to exert effects on microglia and neurons. Interestingly, although it appears Nogo-B can initiate the activation of microglia in vitro, the co-incubation of cells in the presence of Aβ with Nogo-B did not result in an additive response. It is worth considering that Aβ and Nogo-A/Nogo-B may bind a shared receptor on microglia through which an activated profile can be induced and thus any interaction between them is negated by the competition for this receptor. This would explain the lack of interaction between Nogo-A/Nogo-B and Aβ in inducing an activated microglial response. This receptor may be one that is already known to have binding properties with either Nogo or Aβ, or it is perhaps a receptor yet to be discovered or fully characterised. Further investigations into these speculations are required. Despite the fact that it is unclear how Aβ exerts its effect on microglia, there are several binding proteins that act as receptors for Aβ (El Khoury et al., 1996; Yan et al., 1996; El Khoury et al., 1998; Lue et al., 2001; Sanz et al., 2009). A recent report confirms the expression of NgR on microglia and activated macrophages (David et al., 2008), and so it is generally considered that the Nogo-B- and Nogo-A-induced activation of microglia is facilitated through the NgR. However, a novel Nogo-B-specific receptor was discovered to be expressed on endothelial cells (Miao et al., 2006), although at this time there is no evidence of its expression on glia. It should also be considered that the lack of an additive effect of Aβ and Nogo may be due to a ceiling effect in the expression of MHC II and CD11b.

The present in vitro findings indicate that Nogo-B and Nogo-A increased the activity of caspase-3 dose-dependently in neurons and can also induce a synergistic effect with Aβ. It appears that both Nogo-B and Nogo-A can enhance the Aβ-induced activity of caspase-3 when incubated in low doses but high doses failed to exert this effect and actually attenuated the Aβ-induced change. Thus, on the one hand, the detrimental effect exerted by Aβ can be exacerbated in conditions where Nogo-A/Nogo-B is expressed although this is clearly a dose-dependent effect. On the other hand, a potentially neuroprotective effect of Nogo-A/Nogo-B was suggested and evidence in the literature supports such an effect. It has been shown that Nogo-B
interacts with BACE1, inhibiting its production of Aβ; it was suggested this interaction interferes with the trafficking capabilities of BACE1 (Nakajima et al., 2004; He et al., 2006; Murayama et al., 2006; Wojcik et al., 2007; Kume et al., 2009). However, the cell viability of the neurons used in these incubations needs to be assessed before the Nogo protein can be confirmed as having any neuroprotective roles. Further assessment of the functions of Nogo in vivo is needed to fully elucidate the involvement of Nogo in the immunomodulatory interactions during age- and Aβ-associated inflammation in the brain. Interestingly, the ability of Nogo-A/Nogo-B to attenuate activity of caspase-3 when present in high doses suggests that they may be possible immuno-targets in the treatment of AD, although additional investigations addressing dose concentrations and kinetics are vital to fully understand their interaction with Aβ.

The data presented in this thesis provide evidence supporting the hypothesis that, during ageing, NK cells infiltrate from the periphery across a possible compromised BBB into the brain, whereby they produce IFN-γ to activate microglia; this, in turn, initiates signalling pathways to induce neuroinflammatory changes and eventual neurodegeneration. The data couple Nogo-B expression with age-related, as well as Aβ-associated, evidence of microglial activation and neuronal deterioration in vivo, and verifies its ability to modulate both microglial and astrocytic activation and neuronal dysfunction in vitro. Importantly it has been demonstrated that Nogo can interact with Aβ to induce neuroinflammatory changes in a somewhat complex dose-dependent manner. These novel findings give further insight into brain inflammation and present new avenues of focus in mediating neuroinflammation. The modulatory effects of the components involved in neuroinflammation investigated here are summarised in Figure 7.1
Figure 7.1. Summary schematic.
Chapter 8

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VIII Appendix I: Company Addresses

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AbD Serotec
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AGB Scientific
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TX 78244,
USA

Applied Biosystems
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BD Biosciences Europe
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BD Biosciences France
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ZI des Iles - BP4
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Gibco
See Invitrogen UK

GraphPad Software
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HYCOR Biomedical
An Agilent Technologies Division,
Pentlands Science Park,
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Penicuik,
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Scotland

Invitrogen UK
Invitrogen Ltd.,
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Linchinnnan Drive,
Paisley,
PA4 9RF
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Invitrogen USA
EvoQuest™ Laboratory Services,
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Jencons
VWR International Ltd (Export/FMS Team),
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Kinematica
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Macherey-Nagel
Labquip (Ireland) Ltd.,
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Fonthill Industrial Park,
Clondalkin,
Dublin 22.,
Ireland
S.A. Instruments
SA Instruments, Inc.,
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Sarstedt
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Drinagh,
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Wicklow,
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Sigma-Aldrich UK
Sigma-Aldrich Company Ltd.,
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Ultra-Violet Products
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Unit 1
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Hullfield Road,
Cambridge,
CB4 1TG,
United Kingdom

Whatman
Whatman Plc.
Whatman House,
St. Leonard’s Road,
Maidstone,
Kent,
ME16 0LS
United Kingdom
The following solutions were used:

**Phosphate-buffered saline (PBS)**
8g 137mM Sodium chloride (NaCl),
0.2g 2.7mM potassium chloride (KCl),
1.15g 8.1mM di-sodium hydrogen phosphate (Na₂HPO₄),
0.2g 1.5mM potassium dihydrogen phosphate (KH₂PO₄), pH 7.3,
dissolved in 1L dH₂O (1X), pH 7.3, and filtered.

**Tris-buffered saline (TBS)**
31.52g 20mM Tris-HCl; pH 7.5,
87.66g 150mM NaCl,
dissolved in 1L dH₂O (10X) or 10L dH₂O (1X), pH 7.6.

**TBS-Tween (TBS-T)**
TBS Buffer (1X) with 0.05% Tween.

**Krebs solution**
7.948g NaCl,
0.189g KCl,
0.16g KH₂PO₄,
0.27g magnesium sulphate (MgSO₄),
1.344g sodium hydrogen carbonate (NaHCO₃),
1.8g glucose,
dissolved and filtered in 1L dH₂O, pH 7.3.
**Calcium Chloride (2mM, CaCl₂)**

1.47g CaCl₂ dissolved in 10ml dH₂O.

**Krebs solution containing CaCl₂ (Krebs-Ca²⁺ solution)**

200μl CaCl₂ dissolved in 100ml Krebs solution.

**ELISA stopping solution (1M)**

1M Hydrogen sulphide (H₂SO₄): 980μl concentrated H₂SO₄ to 9.02ml dH₂O. (Add acid last).

**Tissue-Tek® OCT**

Polyvinyl Alcohol <11%, Carbowax <5%, nonreactive ingredients >85%, manufactured by Sakura® Finetek, NL. Product # 4583.

**Cell Lysis Buffer**

10mM Tris-HCl,  
50mM sodium chloride (NaCl),  
10mM sodium pyrophosphate (Na₄P₂O₇),  
50mM sodium fluoride (NaF),  
dissolved and filtered in 1L dH₂O, pH 7.4.  
+  
1% IGEPAL (NP-40),  
1mM sodium orthovanadate (Na₃VO₄),  
1mM phenylmethylsulphonyl fluoride (PMSF),  
1mM protease inhibitor cocktail.
Fluorescence Activated Cell Sorting (FACS) Buffer

PBS (IX; Gibco, UK),
2% Foetal Bovine Serum (FBS),
0.1% sodium azide (NaN₃)

FACS Block

50% FACS Buffer
50% FBS

Magnetic-Activated Cell Sorting (MACS) Buffer

PBS (IX; pH 7.2; Gibco, UK, Biosera, UK),
0.5% Bovine Serum Albumin (BSA),
2mM Ethylenediaminetetraacetic acid (EDTA)

MACS Rinsing Solution

PBS (IX; pH 7.2; Gibco, UK),
2mM Ethylenediaminetetraacetic acid (EDTA)

Gel Electrophoresis Reagents and Materials

- BSA came from Sigma-Aldrich Laboratories.
- The MOPS SDS Running Buffer, MOPS SDS Transfer Buffer, NuPAGE® Sample Reducing Reagent (10X), 4%, 10%, 12% and 4-12% NuPAGE® Novex Bis-Tris Gels, XCell SureLock™ Mini-Cell, XCell II™ Blot Modules are manufactured by Invitrogen, UK.
- Reblot Plus Strong Solution (10X) from Chemicon International, USA.
- Protran® Nitrocellulose Transfer Membrane BA83 (0.2μM), size 300mm X 3.0 M from Whatman Schleicher and Schnell, Germany were used for transferring Western blots from NuPAGE® Novex Bis-Tris Gels.
• Enhanced Chemiluminescence from Amersham Western Blotting Detection Reagents, GE Healthcare, UK.

• SuperSignal West Dura Extended Duration Substrate manufactured by Perbio Pierce Thermo Scientific, USA. Product # 34075.

• CL-Xposure™ Clear Blue X-ray Film, 5 X 7 inches manufactured by Perbio Pierce Thermo Scientific, USA. Product # 34090.

Assay Kits

• BCA Protein Assay Kit using bicinchoninic acid Acid, Product# 23227 manufactured by Perbio Pierce Thermo Scientific, USA. (Plates used were Microtest 96-well plates, flat bottom, manufactured by Sarstedt, Ireland).

• Amplex® Red Sphingomyelinase Assay Kit manufactured from Molecular Probes, Invitrogen Detection Technologies, UK. Product # A12220.

• Caspase-8 Drug Discovery Kit manufactured by BIOMOL® International, USA. Product # AK-715.

• Caspase-3 Drug Discovery Kit manufactured by BIOMOL® International, USA. Product # AK-703.

Beads

• Anti-mouse Ig κ CompBeads. BD Biosciences, UK. Product # 552843.

• Anti-PE MACS Microbeads. Miltenyi Biotec, Germany. Product # 130-048-801

Genetic Taqman Assays

• All taqman assays used were supplied by Applied Biosystems, Germany.
Antibodies

Immunoblot Primary Antibodies

- **Actin** – mouse IgG1 monoclonal antibody recognises an epitope located on the N-terminal end of the β-isoform of actin. Sigma-Aldrich Ireland Ltd., Ireland. Product # A5441.

- **Nogo-A** – rabbit polyclonal antibody mapping in the middle region of the putative extracellular domain of rat Nogo-A. Chemicon International, USA. Product # AB5664P.

- **Nogo-A** – rabbit polyclonal IgG immunised with a synthetic peptide conjugated to KLH derived from within residues 1100 to the C-terminus of rat Nogo-A. Abcam, UK. Product # ab32298.

- **Nogo-B** – rabbit polyclonal antibody mapping within the N-terminus by the splice site of Nogo-A from the putative extracellular domain of rat Nogo-B. Chemicon International, USA. Product # AB5668P.

- **Nogo-B** – rabbit polyclonal IgG immunised with a synthetic peptide of 12 amino acids within the N-terminus by the splice site of Nogo-A from the putative extracellular domain of rat Nogo-B (KLH coupled). Abcam, UK. Product # ab26398.

- **Nogo-R (NgR)** – rabbit polyclonal IgG immunised with a synthetic peptide conjugated to KLH derived from within residues 150-250 of rat Nogo receptor. Abcam, UK. Product # ab26291.

- **HMGB1 (High-Mobility Group Box 1)** – rabbit polyclonal IgG immunised with a synthetic peptide conjugated to KLH derived from within residues 150 to C-terminus of human HMGB1. Abcam, UK. Product # ab18256.

- **Synaptophysin** – mouse monoclonal IgG1 derived from the hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunised mouse. Sigma-Aldrich, UK. Product # S5768.

Immunoblot Secondary Antibodies

- Anti-mouse IgG1 (whole molecule) peroxidase conjugated antibody by Sigma-Aldrich, UK. Developed in the mouse and absorbed with rat serum proteins.
• Anti-rabbit IgG\(_1\) (whole molecule) peroxidase conjugated antibody by Sigma-Aldrich, UK. Developed in the rabbit and absorbed in human IgG.

**Flow Cytometry Fluorescent Tagged Antibodies**

- **CD161a** – monoclonal IgG\(_1\) \(k\) antibody conjugated with a FITC fluorescent tag. BD Biosciences, UK. Product # 555008.
- **NKp30 (CLH9)** – mouse monoclonal antibody conjugated with a PE fluorescent tag; raised against rat NKp30-Fc protein. Santa Cruz Biotechnology Inc., USA. Product # sc-33647.
- **OX-6** – monoclonal IgG\(_1\) \(k\) antibody conjugated with a PE fluorescent tag. BD Biosciences, UK. Product # 554929.
- **CD11b** – mouse IgG2a monoclonal antibody conjugated with an Alexa Fluor® 647 fluorescent tag; recognises the rat equivalent of human CD11b, the receptor for the iC3b component of complement. AbD Serotec, UK. Product # MCA275A647.
- **CD45** – monoclonal IgG\(_1\) \(k\) antibody conjugated with a PE-CY™5 fluorescent tag. BD Bioscience, UK. Product # 559135.

**Cell Culture Media**

- Neurobasal medium (NBM) was supplemented with 10% heat activated (56°C for 60min) FBS, 100mM L-Glutamine (Gibco, UK), 100\(\mu\)g/ml penicillin/streptomycin (Gibco, UK).
- Dulbecco’s Modified Eagles Medium (DMEM) (Gibco, UK) was supplemented with 10% heat activated (56°C for 60min) FBS, 100mM L-Glutamine (Gibco, UK), 100\(\mu\)g/ml penicillin/streptomycin (Gibco, UK).
- Roswell Park Memorial Institute (RPMI)-1640 medium ( Biosera, UK) was supplemented with 10% heat activated (56°C for 60min) FBS, 100mM L-Glutamine (Gibco, UK), 100\(\mu\)g/ml penicillin/streptomycin (Gibco, UK).
**In vitro Treatments**

- **Nogo-A** – Synthetic peptide derived from within residues 1100 to the C-terminus of Rat Nogo A. Abcam, UK. Product # ab32297. Note: Catalogued as ‘Nogo’.

- **Nogo-B** – 12 amino acid peptide within the N-terminus and at the splice site of Nogo-A from the putative extracellular domain of rat Nogo-B, conjugated to KLH. Alpha Diagnostic, USA. Product # NogoB21-P.

- **Aβ1-42** – 85.1% peptide content, supplied as trifluoroacetate salt. Biosciences, Ireland. Product # 03-112.

- **IFN-γ** – N-terminal methionyl form of rat IFN-γ containing 135 amino acid residues. R&D Systems, UK. Product # 585-IF.

- **TNF-α** – N-terminal methionyl form of rat TNF-α containing 145 amino acid residues. R&D Systems, UK. Product # 510-RT.

- **HMGB1** – expressed and purified by the Biochemistry Dept., Trinity College Dublin, Ireland.

- **IL-12r** – a disulfide-linked heterodimeric protein consisting of the C-terminal 6 histidine-tagged p35 and p40 subunits. R&D Systems, UK. Product # 1760-RL.

- **IL-18** – Methionyl form of recombinant rat IL-18 containing 159 amino acid residues. R&D Systems, UK. Product # 521-RL.