UNIVERSITY OF LONDON THESIS

Degree PhD Year 2007 Name of Author PINTEAUX-JONES, Fleur

A Study of Microglial Metabotropic Glutamate Receptor Modulation of Inflammation

A thesis submitted for the degree of Doctor of Philosophy

By

Fleur Pinteaux-Jones

Department of Neuroinflammation
Institute of Neurology
University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I, Fleur Pinteaux-Jones, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Microglia are the immune cell of the central nervous system and become reactive in response to infection or trauma. In Multiple Sclerosis (MS) microglia show early reactivity which may contribute to subsequent neurotoxicity. Glutamate has been implicated in the neurodegeneration in MS. Microglia express subtypes of metabotropic glutamate receptors (mGluRs), the activation of which can lead to microglial evoked neurotoxicity (group II) or neuroprotection (group III). This suggests that release of glutamate from neurones or glial cells may activate microglia via these receptors.

Increased microglial reactivity during disease is possibly due to an imbalance of mGluR expression with neurotoxic group II increased above neuroprotective group III mGluRs. Microglial expression of mGluR subtypes was therefore investigated on activated compared with untreated cells. It was found that microglia exposed to myelin expressed increased levels of group II and mGluR1 and 8 subtypes compared with control cells.

MS is characterised by lesions in the CNS due to localised myelin breakdown. This research investigated the effects of microglial exposure to myelin and demonstrated that myelin induced microglial activation, upregulation of iNOS expression, and increased TNFα and nitrite release. Conditioned medium from these cells was toxic to neurones in culture. Microglia phagocytose myelin *in vitro* leading to cytokine release and increased affinity for oligodendrocytes, inducing their death by cell contact through TNF receptors. This study examined whether microglial phagocytosis could be modulated by stimulation of mGluRs and demonstrated that blocking group II or stimulation of group I or III mGluRs significantly reduced phagocytic activity. Since myelin induced toxicity was also reduced by blocking microglial group II or stimulating group III mGluRs, these results suggest that modulation of microglial mGluRs may be of therapeutic benefit in MS.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AD	**Alzheimer's disease**
ADA	**trans-azetidine-2,4-dicarboxylic acid**
AIDA	**1-aminoindan-1,5-dicarboxylic acid**
AMPA	**α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid**
AP4	**2-amino-4-phosphobutyric acid**
APC	**antigen presenting cell**
APP	**amyloid precursor protein**
ATP	**adenosine triphosphate**
Aβ	**β-Amyloid**
BBB	**blood brain barrier**
BSA	**bovine serum albumin**
Ca^{2+}	**calcium**
CaMK	**calcium-calmodulin-dependent protein kinase**
cAMP	**cyclic- adenosine monophosphate**
CGA	**chromogranin A**
CGC	**cerebellar granule cell**
CNS	**central nervous system**
CR	**complement receptor**
CSF	**cerebro-spinal fluid**
DAG	**diacylglycerol**
DAPI	**4'-6-Diamidino-2-phenylindole**
DBI	**diffuse brain injury**
DCGIV	**L-2-(2,3-dicarboxycyclopropyl)-glycine**
DEPC	**diethylpyrocarbonate**
DHPG	**3,5-Dihydroxyphenylglycine**
DIV	**days in vitro**
DNA	**deoxyribonuleic acid**
dNTP	**deoxyribonucleoside triphosphate**
DTT	**dithiothrietol**
EAE	**experimental allergic encephalomyelitis**

EBSS	**Earle's balanced salt solution**
ECL	**enhanced chemiluminescence**
ECM	**extracellular matrix**
EM	**electron microscope**
ER	**endoplasmic reticulum**
FAD	**flavine adenine dinucleotide**
FADD	**fas associated death domain**
F-PBS	**Formaldehyde-phosphate buffered saline**
GDP	**guanosine diphosphate**
G-protein	**guanosine triphosphate (GTP)-binding protein**
GTP	**guanosine triphosphate**
HRP	**horse radish peroxidase**
iGluR	**ionotropic glutamate receptor**
IL	**interleukin**
iNOS	**inducible nitric oxide synthase**
IP_3	**inositol-1,4,5-triphosphate**
K^+	**potassium**
LPS	**lipopolysaccharide**
MAG	**myelin-associated glycoprotein**
MAP4	**(S)-2-amino-2-methyl-2-(2'carboxycyclopropyl)glycine**
MAPK	**mitogen activated protein kinase**
MBP	**myelin basic protein**
MCCG	**2S,1'S,2'S-2-methyl-2-(2'carboxycyclopropyl)glycine**
MEK	**MAPK kinase**
MEM	**Minimum Essential Medium**
mGluR	**metabotropic glutamate receptor**
mRNA	**messenger ribonucleic acid**
MGCM	**microglial conditioned medium**
MHC	**major histocompatibility complex**
MMLV	**moloney murine leukaemia virus**
MOG	**myelin oligodendrocyte glycoprotein**
MS	**multiple sclerosis**
Na^+	**sodium**
NAAG	**N-acetylaspartylglutamate**

NADPH	**nicotinamide-adenine-dinucleotide phosphate**
NMDA	**N-methyl-D-aspartate**
NO	**nitric oxide**
NT-3	**neurotrophin 3**
OL	**oligodendrocyte**
OPC	**oligodendrocyte precursor cell**
PAMP	**pathogen associated molecular pattern**
PBS	**phosphate buffered saline**
PCD	**programmed cell death**
PD	**Parkinson's disease**
PLP	**proteolipid protein**
PKA	**protein kinase A**
PKC	**protein kinase C**
PPMS	**primary progressive multiple sclerosis**
PRMS	**progressive relapsing multiple sclerosis**
PRR	**pattern recognition receptor**
RANTES	**from-regulated upon activation of normal T cells expressed and secreted**
mRNA	**ribonucleic acid**
ROS	**reactive oxygen species**
RRMS	**relapsing remitting multiple sclerosis**
SDS	**sodium dodecylsulphate**
SDS-PAGE	**SDS-Polyacrylamide gel electrophoresis**
SMAC	**second microglial derived activator of caspases**
SPMS	**secondary progressive multiple sclerosis**
TBE	**Tris-borate-EDTA**
TBS	**tris buffered saline**
TGF	**transforming growth factor**
TNF	**tumour necrosis factor**
TTBS	**tween 20-tris buffered saline**

ACKNOWLEDGEMENTS

I would like to thank my supervisors Doctor Jennifer Pocock and Professor David Baker for all their support and guidance throughout my PhD. I would also like to acknowledge Annabella Polito and Anne Palser from the lab of Professor Richard Reynolds, Imperial College, who kindly provided me with rat EAE sections, and Gareth Pryce in the department of Neuroinflammation for the EAE tissue he provided.

A special thanks also to my colleagues at Wakefield Street and my family and friends who have provided me with constant encouragement over the last three years.

Finally I would also like to thank AIMS2CURE for their generous funding of this research.

1.0

INTRODUCTION

1.1 Cells of the Central Nervous system

1.1.1 General Introduction

The cells of the central nervous system (CNS) comprise neurones and glia. These broad categories encompass several different types of cells that vary based on their structure, chemistry and function. The CNS consists of around 10^{12} neurones which facilitate synaptic transmission and communication across the CNS (Bear et al., 2001). Action potentials carry electrical information from one neurone to another along their axons. Neurones, however, are outnumbered by non-neuronal cellular elements collectively referred to as 'neuroglia' which make up around 90% of the cells in the brain. The term neuroglia 'nerve glue', was employed by Rudolph Virchow in 1859 who believed glia to function as an inactive connective tissue to hold neurones together (Bear et al., 2001). For a long time this view of glia as simple supporting cells providing trophic support to neurones presided but it has now become apparent that glial cells have a far more diverse function in the CNS. Using silver staining, Del Rio Hortega was first able to distinguish between the main types of supporting cell in the CNS which include astrocytes, oligodendrocytes and macrophages (Barron, 1995).

1.1.2 Astrocytes

Astrocytes are stellate process-bearing cells with even distribution throughout the CNS. Embryonically, astrocytes develop from radial glial cells, which retract their processes as they mature, thus serving as astrocyte progenitors (Ransom and Sontheimer 1992; Chanas-Sacre et al., 2000; Mori et al., 2005). Neurones and oligodendrocytes are enveloped as astrocytes extend processes to the pia mater and the ependyma to form the glia limitans, thus providing an interface where neurones and astrocytes may interact.

Astrocytes are connected to each other by gap junctions which allow ions to diffuse across the brain parenchyma.

Astrocytes are responsible for initiating and maintaining the endothelial tight junctions of the blood brain barrier (BBB) and also produce several growth factors which regulate the morphology, differentiation or survival of neurones. They provide the link between vasculature and neurones, transporting and processing glucose from the blood stream to produce lactate which is the main energy substrate for neurones (Tsacopoulos and Magistretti 1996; Magistretti and Pellerin 1999). Astrocytes also function as buffer cells at the synaptic cleft. Due to their high potassium permeability, astrocytes can take up excess potassium and store it to protect neighbouring neurones from depolarisation (Newman and Reichenbach 1996). Expression of transporter proteins on their cell surface membranes also allows astrocytes to buffer neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA) from the synapse (Kimelberg and Katz 1985; Kimelberg and Norenberg 1989; Schousboe et al., 2004), thus preventing excessive stimulation or excitotoxicity (Ransom et al., 1995).

Astrocytes are the largest of the glial cells (40-50μ m) and make up approximately 30-40% of the glial population (Aldskogius and Kozlova, 1998). Astrocytes have unique morphological features including their stellate shape and the presence of glial end feet on capillaries. Astrocytes are the only glial cells to contain intermediate filaments made of glial fibrillary acidic protein (GFAP) and are therefore easily identifiable (reviewed by: Eng et al., 2000).

1.1.3 Oligodendrocytes

The myelin sheath is formed by specialised glial cells. Oligodendrocytes and Schwann cells are responsible for the myelination of CNS and peripheral nervous system (PNS) axons respectively. These cells wrap myelin sheaths, produced from

extensions of their cell membranes, around axons (Figure 1.1). Oligodendrocytes are capable of forming up to 50 sheath segments therefore allowing myelination of several axons from the same cell (Compston et al., 1997). Oligodendrocytes also regulate extracellular potassium ion concentrations and are able to secrete neurotrophic factors including nerve growth factor (NGF), brain derived nerve factor (BDNF), and neurotrophin 3 (NT-3) (Dai et al., 2001).

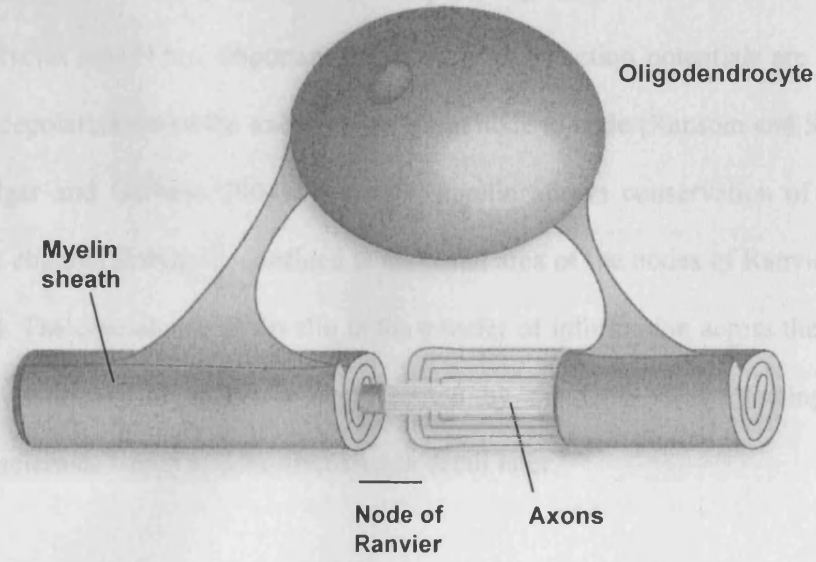


Figure 1.1 Schematic diagram of an oligodendrocyte cell.
Oligodendrocytes extrude processes which wrap themselves around portions of surrounding axons. As each process wraps itself around, it forms layers of myelin. Each process thus becomes a segment of the axon's myelin sheath. Areas of exposed axon between myelin sheaths are known as the Nodes of Ranvier.

1.1.3a Myelin

Oligodendrocyte membrane extensions are wrapped around axons resulting in apposition between the intracellular or extracellular surface membranes. The intracellular apposition is referred to as the major dense line while the extracellular apposition is named the intraperiod line. The areas of myelin free axon between the

myelin sheaths are known as the nodes of Ranvier which facilitate the fast transmission of action potentials.

Myelin contains a high lipid content of around 70-75%, which helps with its function as an axonal insulator, and around 25% protein. The main myelin proteins are proteolipid protein (PLP) which makes up 50%, and myelin basic protein (MBP) isoforms which make up 30% of total myelin protein. Myelin associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG) are present to a much lesser extent making up about 1.5% of total myelin protein (Cuzner and Norton 1996).

Myelin serves two important functions; Firstly, action potentials are conducted faster as depolarisation of the axon 'jumps' from node to node (Ransom and Sontheimer 1992; Edgar and Garbern 2004). Secondly, myelin allows conservation of metabolic energy as channel activity is confined to the small area of the nodes of Ranvier (Bear et al., 2001). The crucial role of myelin in the transfer of information across the brain and normal functioning of axons is demonstrated by the CNS demyelinating disorder multiple sclerosis which will be discussed in detail later.

1.1.4 Macrophages

These cells are derived from bone marrow promonocytes, which then differentiate into circulating blood monocytes and finally localise throughout the body as mature macrophages where they make up the mononuclear phagocyte system (Figure 1.2). Macrophages contain considerable rough-surfaced endoplasmic reticulum and mitochondria. They can be localised throughout connective tissue and around the basement membrane of small blood vessels and in particular in the liver (Kupffer cells), lungs (alveolar macrophages), and lymph node medullary sinuses where they can fulfil their role as peripheral immune cells (Roitt and Delves, 2003). Some macrophages in the brain are known as perivascular macrophages although other macrophage

populations are also known to infiltrate the CNS after blood brain barrier breakdown. Resident microglial cells can adopt similar morphology and functions as macrophages, these cells will be discussed in detail later.

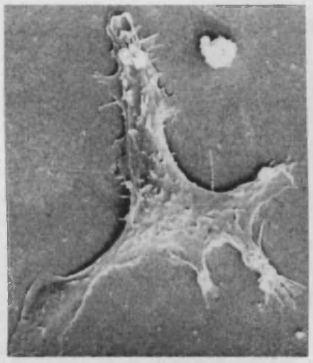


Figure 1.2 EM photograph of a macrophage cell.

Image taken from www.malp-research.de/

1.1.5 The blood brain barrier

The CNS is protected to a certain extent from circulating blood borne solutes. Without a barrier mechanism, circulating blood molecules, for instance hormones which can act as neurotransmitters, could interfere with synaptic transmission and cell interactions (Pardridge et al., 1987). The CNS is therefore separated from circulating haematopoietic cells by the tight junctions formed by capillary and venule endothelial cells (Reese and Karnovski, 1967). Endothelial cells together with perivascular microglia and notably astrocytes, constitute the BBB thus creating the unique microenvironment of the CNS. However, due to the high metabolic requirements of the brain, the BBB has transport mechanisms for the specific nutrients required for normal brain function (Cornford and Landon, 1985) including molecular exclusion pumps such as p-glycoprotein that maintain gradients between the blood and the brain (Tsuji et al., 1992; Tatsuta et al., 1992).

The original idea of a barrier mechanism separating the brain from blood circulation came from studies of dye injections into the circulation. In the early twentieth century, Goldman (1909, 1913), showed that while the brain could be stained

with trypan blue when injected into the brain ventricular system, intravenous injection led to staining of all organs with the exception of the brain and spinal cord (Reviewed by Ribatti et al., 2006). It was also observed that transplanted tissue grafts in the brain were tolerated for relatively long periods in the brain without evidence of an immune response (Widner et al., 1989; Young et al., 1989). Indeed, endothelial tight junctions and the fact that the brain has no conventional lymphatic drainage system results in highly restricted access to blood and lymph borne immune cells such as T and B lymphocytes and monocytes (Aloisi et al., 2000).

It is now known that immune regulatory functions can still preside in the CNS despite the BBB as demonstrated by several studies. These include Medawar et al., (1948) who showed that allogenic skin grafts implanted into the brains of sensitised rabbits were rejected. Investigation into the pathogenesis of multiple sclerosis has also highlighted the permeability of the BBB. T-cell infiltration into the CNS occurs in viral and autoimmune encephalitis (Weiner et al., 1984), and has also been demonstrated in the brains of MS patients (Hauser et al., 1986), although the extent of lymphocyte infiltration has been shown to be dependent on lesion location (Bo et al., 2003). The induction of a successful immune response within the CNS is dependent on T cell contact with the endothelial cell wall and penetration of the cells through the BBB (Naparstek et al., 1984). It has been suggested that microglial cells and astrocytes act as regulatory cells at the interface between the brain and the components of the immune system controlling the action of cellular and humoral elements of the immune response (Frei et al., 1988).

Increased permeability of the BBB is central to several neuropathological conditions including MS. Generally, disruption of the BBB leads to the accumulation of blood derived fluids around brain blood vessels. In MS, T-cells and macrophages invade the brain through the disrupted BBB, initiating inflammatory cascades.

1.2 Microglia

Microglia are a major glial component of the central nervous system (CNS), and constitute 5-15% of the total brain cell population (Carson, 2002). Microglial cells are expressed ubiquitously throughout the brain and spinal cord, although there are regional differences in cell number and a larger proportion of microglia in the grey compared with the white matter (Lawson et al., 1990). While microglia are a distinct cell type expressing a unique ramified morphology at rest, certain stimuli cause them to transform into activated cells, which then display the morphological features and functions of macrophages (Kreutzberg, 1996; Stence et al., 2001).

The hallmark of microglia is their ability to become reactive with a graded response to pathological challenge. Microglia possess many functional properties including migration, proliferation, displacement of synaptic boutons (Ling 1981), and also act as brain macrophages, antigen presenting cells, and immunoeffector cells involved in local inflammatory processes in the brain (Guilian, 1987; Streit et al., 1988).

1.2.1 Origins of microglia

It has been suggested that microglia enter the brain as monocytes during embryonic development, then differentiate into glial cells displaying many of the cell surface antigens of macrophages (Lee et al., 2002), (Figure 1.3). There is now much evidence pointing to the monocytic origin of microglia. Carbon particles used to label monocytes injected into the blood stream of postnatal rats were later localised in cells resembling mature microglia (Ling et al 1980). Furthermore, both resting and reactive microglia can be visualised by B4 isolectin which is a marker also used for labelling of blood monocytes (Streit and Kreutzberg 1987), and immunocytochemical labelling of macrophages infiltrating the CNS has been used to chart the change from rounded cells

to highly specialised microglia (Hume et al, 1983; Perry et al., 1985). Indeed several microglial cell surface antigens are either associated or exclusive to cells of monocytic lineage such as the Fc receptors (CD16a and CD16b) and complement type 3 receptors (CD11b) (Perry et al., 1985, Perry and Gordon, 1987). Studies using bone marrow chimeras have also shown that the CNS becomes populated with cells expressing donor specific markers (Hickey and Kimura, 1988).

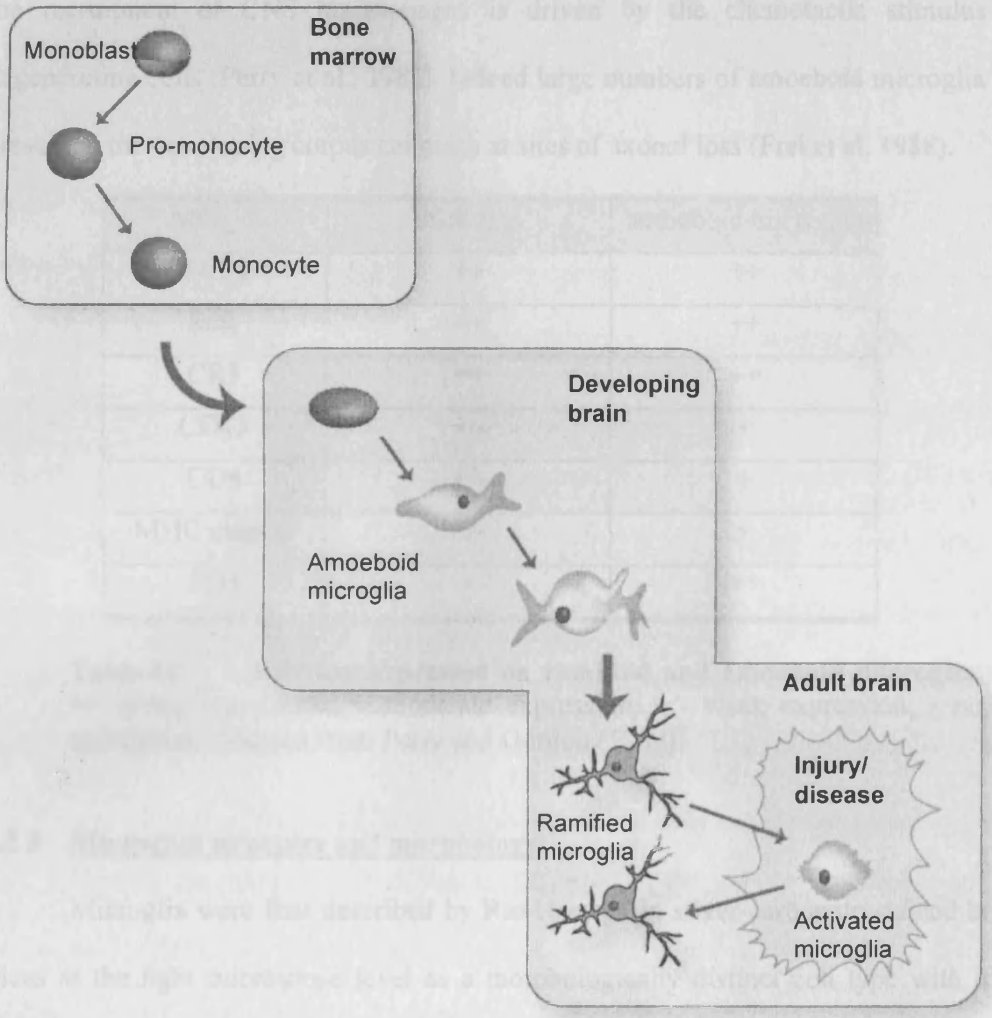


Figure 1.3 Origins and development of microglia.
Microglia develop as monocytes from bone marrow before entering the developing brain as amoeboid microglia. In the adult brain microglia maintain a ramified state, which is thought to maintain neuronal health. During disease microglia may become activated which can be harmful to neurones and surrounding cells. Adapted from Kazuyuki et al., (2001).

1.2.2 Microglia in the developing brain

Large numbers of CNS macrophages are found in the developing brain during the late stages of embryogenesis at time points that correlate with the rapid upregulation in naturally occurring neuronal apoptotic cell death, which is a vital feature of development (Oppenheim, 1981). These large populations of macrophages generally disappear by the late postnatal period (Ling and Wong, 1993). It has been suggested that the recruitment of CNS macrophages is driven by the chemotactic stimulus of degenerating cells (Perry et al., 1985). Indeed large numbers of amoeboid microglia are present in the developing corpus callosum at sites of axonal loss (Frei et al, 1988).

Antigen	microglia	amoeboid microglia
F4/80	++	++
FcR	++	++
CR3	++	++
CD45	+/-	+
CD4	+/-	+
MHC class II	+/-	+
ED1	-	++

Table 1.1 Antigens expressed on ramified and amoeboid microglia. ++ strong expression, + moderate expression, +/- weak expression, - no expression. Adapted from Perry and Gordon (1989).

1.2.3 Microglial structure and morphology

Microglia were first described by Rio-Hortega in silver-carbonate stained brain slices at the light microscope level as a morphologically distinct cell type with long branched processes (see Ling and Wong, 1993; Banati and Graeber 1994). This downregulated immunophenotype is indicative of microglial cells 'at rest' (Figure 1.4A). Microglial activation is associated with cell transformation to macrophages and is a continuous rather than an all or nothing response causing a range of morphological

changes from retraction of processes to full conversion into condensed, granular amoeboid cells (Figure 1.4B). The functional activation state of microglia is dependent on several factors including the extracellular matrix substrate and cytokine composition of the microenvironment (Milner and Cambell 2003). Different patterns of microglial activation will in turn have distinct consequences for surrounding tissues (Morgan et al., 2005). Microglial morphology mirrors different functional states including cytokine and chemokine release, altered ion channel and cell surface antigen expression, chemotactic movement and phagocytic behaviour (Hanisch, 2002).

Amoeboid or immature microglia are found in perinate and neonate brains. Amoeboid cells transform into ramified cells during postnatal development (Ling, 1981). Amoeboid microglia express increased expression of CD4 in rats and leukocyte common antigens (CD45), which may be a reflection of plasma protein infiltration into the cerebrospinal fluid (CSF) in immature brains as both plasma protein concentration in CSF, and CD4 and CD45 expression, decrease in the maturing CNS (Perry and Gordon, 1987). This suggests that damage to the BBB could result in upregulation of macrophage antigens on microglia. It has become clear that changes in the CNS microenvironment regulate the morphology and expression of cell surface antigens on microglia, thus influencing their expression of cytoplasmic enzymes and secretory factors (Aloisi et al., 2000; 2001).

Resting microglia have rod shaped cell somata, indeed the first description of microglia by Franz Nissl in 1899 used the term 'rod cells' to depict their ramified morphology. Observation by electron microscopy (EM) shows that ramified microglia have flattened or angular nuclei (Ling and Wong, 1993). Resting microglial cells extend long processes from their somata, which collectively form a network that covers the parenchyma, although it is rare that microglial processes overlap or touch. Ramified microglia lack hydrolytic enzymes and the capacity to engulf particles. Interestingly,

recent studies using two photon microscopy have shown that resting microglia are not as quiescent as was previously assumed (Davalos et al., 2005; Nimmerjahn et al., 2005). Although cell somata remained fixed, the branching microglial processes are extremely motile and directly contact astrocytes, neurones and blood vessels (Nimmerjahn et al., 2005), which further suggests that resting microglia maintain and monitor the CNS microenvironment.

Microglial activation is a graded response to pathogenic insult. Activation stimulates proliferation and migration to the site of injury and results in morphological, immunophenotypic and functional changes (Kreutzberg, 1996). Morphologically, activation causes microglia to retract processes and become more rounded and granular resembling amoeboid cells. Observation by EM shows rounded nuclei and large amounts of cytoplasm containing Golgi apparatus, rough endoplasmic reticulum (ER) and numerous lysosomal and phagosomal granules (Ling and Wong 1993). Immunophenotypic changes include upregulation of immune molecules such as the CR3 complement receptors (CD11b) and the major histocompatibility complex (MHC) class I and II antigens (Graeber at al., 1988, 1989, Streit et al 1989) required for phagocytic and antigen presenting functions respectively. Functionally, activated microglia release a number of inflammatory mediators including reactive oxygen species, nitric oxide, glutamate, cytokines such as interleukin 1 (IL-1β), tumour necrosis factor α (TNF-α), interferon γ (IFN-γ) and transforming growth factor β (TGF-β) (Gerbicke-Haerter, 1996).

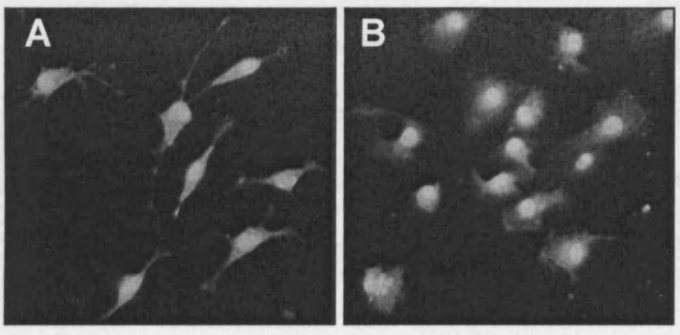


Figure 1.4 Morphology of ramified and activated primary microglia
Primary microglia were stained with fluorescein diacetate to show **(A)**. resting/ ramified and **(B).** activated microglia (x400 magnification). Photographs: Dr. J M Pocock

Most scientists agree that microglia are a distinct cell type. Indeed microglia, unlike macrophages have a distinctive ramified morphology and the capacity to proliferate in vitro (Giulian and Baker, 1986). Microglia have also been shown to express spikes on their membranes in comparison to the smooth or ruffed appearance of macrophage membranes (Giulian et al., 1995). However, upon activation microglia and macrophages share most phenotypical markers and can exert similar effector functions. The differentiation between macrophages that have infiltrated the CNS due to BBB breakdown, perivascular macrophages and resident microglia is therefore very difficult and a primary concern when using brain sections. While most of the work in this study used primary microglia cultured *in vitro*, the lack of a purely microglial marker made differentiation between microglia and macrophages in brain sections impossible.

1.2.4 Substances released from activated microglia

Activated microglia constitutively and inducibly express or release a number of CNS signalling ligands, which facilitate the rapid induction of inflammation and can be deleterious to neuronal survival. These substances have been implicated in neuronal-glial interactions through activation and inhibition of pathways that regulate cell death or survival, proliferation, and activation.

1.2.4a Cytokines

Many cellular interactions between tissues in the body rely on the production and transfer of cytokines and chemokines. These signalling proteins are produced by numerous cell types and control cell differentiation, growth and survival (Hanisch, 2002), and furthermore, control the processes and outcome of traumatic infections or degenerative insults (Hopkins and Rothwell, 1995). Activated microglia have been shown to release a number of cytokines. These include IL-1β, which has been shown to

enhance activation of the immune response including the astrocytic contribution to inflammatory processes (Giulian et al., 1987), FasL (Badie et al., 2000; Terrazzino et al., 2002), and TNFα (Hanisch et al., 2002, Taylor et al., 2005).

Fas (CD95) is a member of a large family of cytokines that includes TNFβ (lymphotoxin) and tumour necrosis factor alpha (TNFα). These cytokines exert effects on the inflammatory response, cell death and proliferation (Vilcek and Lee, 1991; Gebicke-Haerter, 2001). The death inducing receptor Fas is involved in the apoptotic pathway and is released by activated microglia. Both Fas ligand (FasL) and its receptor Fas show upregulated expression in acute and chronic neurobiological conditions (Dowling et al., 1996; D'Souza et al., 1996; Felderhoff-Mueser et al., 2002), and may contribute to neurotoxicity by inducing apoptosis (Griffith et al., 1995; Nagata et al., 1999). Furthermore, recent studies have demonstrated that the neurotoxic effects of microglial TNFα release are dependent on the co-release of FasL (Taylor et al., 2005).

TNFα binds two receptors, p55 (TNFR1) and p75 (TNFR2) with equal affinity. These receptors are linked to different signal transduction pathways (Mattson et al., 1997). Both receptors are expressed by microglia *in vitro* (Dopp et al., 1997). TNFα stimulation of TNFR1 has been shown to induce apoptosis and activation of $NF\text{-}_kB$ thus leading to induction of proinflammatory and immunomodulatory genes (Tartaglia and Goeddel, 1992), whereas stimulation of TNFR2 can be protective (Yang et al., 2002). The inflammatory response has enormous capacity for amplification therefore necessitating in-built mechanisms for its modulation and suppression. It has recently been shown that cerebellar granule cells (CGCs) in culture constitutively express higher amounts of apoptosis inducing TNFR1, thus rendering them particularly sensitive to microglial TNFα secretion (Taylor et al., 2005).

TNFα is primarily produced by macrophages and microglia and is constitutively released at very low levels by ramified unstimulated microglia (Lee et al., 2002; Taylor

et al., 2005). Low levels of TNFα can also induce neuroprotective effects (Carlson et al., 1999), such as enhancing the survival of cultured hippocampal and cortical neurones (Cheng et al., 1994). However TNFα is also associated with early release from microglia following activation (Taylor et al., 2005). TNFα in turn activates microglia potentiating the release of further neurotoxic substances (Gebicke-Haerter, 2001). Although not directly neurotoxic to neurones in culture (Ciesielski-Treska et al., 1998; Taylor et al., 2005), TNFα becomes neurotoxic when released as a glial-derived inflammatory response and has been shown to act in concert with FasL (Taylor et al., 2005) and glutamate release (Floden et al., 2005).

1.2.4b Reactive oxygen and nitrogen species

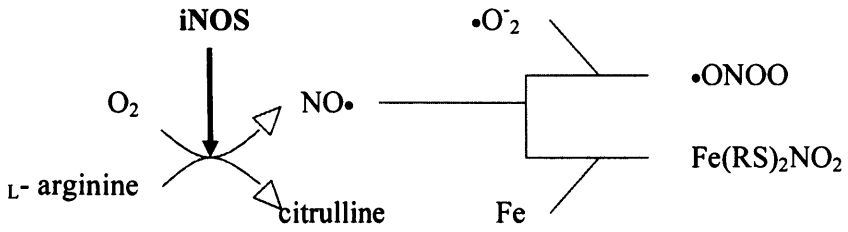
Initiation of microglial phagocytosis results in upregulation of nicotinamide-adenine-dinucleotide phosphate (NADPH) (Ueyama et al., 2004). Electrons from NADPH are transferred by the flavocytochrome oxidase enzyme to molecular oxygen to form reactive oxygen species (ROS). The reaction catalysed by NADPH can be summarised by

$$NADPH + O_2 \longrightarrow NADP^+ + \bullet O_2^-$$

The small superoxide anion can then be converted to hydrogen peroxide by superoxide dismutase and then to hydroxyl radicals (•OH). Each of these reactive oxygen products are highly reactive and are released as part of the inflammatory response to kill invading pathogens especially those engulfed by phagocytosis (Roitt and Delves, 2003).

Nitric oxide (NO), as well as performing as a physiologic mediator (Chen et al., 2000), is involved in the innate inflammatory response and can operate against invading

microorganisms in the cytosol. Inducible nitric oxide synthase (iNOS) is the enzyme responsible for production of NO from the terminal nitrogen atom of L-arginine in the presence of NADPH and O_2 (Minghetti et al., 1997).



Inducible nitric oxide is implicated in the pathogenesis of inflammatory demyelinating diseases, as NO and peroxynitrite are toxic to oligodendrocytes, axons and neurones, inhibiting mitochondrial respiratory chain complexes, reducing ATP levels, and causing Na/Ca influx (Bolaños et al., 1994, Ding et al., 1997). Insult induced activation of microglia initiates the production of NO and reactive oxygen intermediates (Taupenot et al., 1996; Kingham et al., 1999), which are toxic to neurones in culture (Thery et al., 1991). Furthermore, NO can react with the superoxide free radicals ($O_2^{\cdot}$) that are also released by microglia (Woodroofe et al., 1989) and can form the highly reactive peroxynitrite ($ONOO^{\cdot -}$), which can be toxic to neurones (Kim and Ko., 1998). Cytokines also have an effect on iNOS expression and NO release, indeed it has been shown that TNFα and NO may mutually provoke the release of the other *in vitro* (Sun et al, 1998). Although it has now been suggested that the toxic effects ascribed to NO may in fact be due to peroxynitrite and other metabolic derivatives, it is clear that either directly or indirectly, NO release can have detrimental consequences for tissues in the CNS.

1.2.4c Glutamate

Glutamate is the primary excitatory transmitter in the mammalian CNS and is responsible for fast synaptic transmission. Glutamate is one of the 20 amino acids in the body that are the 'building blocks' of protein and is therefore abundant in all cells of the body including neurones. Glutamate is released from presynaptic vesicles by Ca^{2+} dependent mechanisms. However, excessive glutamate release can lead to excitotoxicity. High extracellular glutamate has been associated with influx of Na^{+} and Ca^{2+} through both ligand and voltage gated channels leading to raised intracellular Ca^{2+} activation of a range of enzymes and cell death (Meldrum and Garthwaite, 1990).

Although low levels of glutamate agonists have been shown to be neuroprotective against subsequent excitotoxic neurotoxicity (Chuang et al., 1992), it has also been demonstrated that glutamate can induce N-methyl-$_D$-aspartate (NMDA) mediated neurotoxic effects (Piani et al, 1991, Piani and Fontana 1994, Kingham et al., 1999). Activated microglia release glutamate, furthermore it has been demonstrated that glutamate is a major component of activation induced microglial neurotoxicity (Piani et al., 1991, 1992; Patrizio and Levi 1994). Glutamate release from microglia occurs via the cysteine-glutamate antiporter and through a bafilomycin A-sensitive mechanism (Kingham et al., 1999), which may in turn lead to activation of microglial metabotropic glutamate receptors (mGluRs). Excess glutamate can also prevent the uptake of cysteine, a necessary building block of glutathione. Glutathione is known to afford significant protection from injury associated with oxidative stress (Ishida et al., 2006; Shih et al., 2006).

1.3 Glutamate receptors

Glutamate is the primary excitatory transmitter in the mammalian CNS and it acts through a number of ionotropic (ligand gated cation channels) and metabotropic receptors. Metabotropic receptors are coupled to guanosine triphosphate (GTP)-binding proteins more commonly known as G-proteins. Fast synaptic transmission is mediated primarily by ionotropic glutamate receptors (iGluRs) but metabotropic glutamate receptors (mGluRs) also contribute by coupling with G-proteins and thus controlling membrane enzymes and ion channels.

1.3.1 Ionotropic glutamate receptors

There are three types of iGluRs which are named after the agonists that were originally found to activate them (Figure 1.5). They are NMDA, α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (Kainate) receptors, which share common structural features that place them within one superfamily. AMPA and NMDA receptors mediate the majority of fast excitatory transmission in the brain.

AMPA receptors are permeable to Na^+ and K^+ and are impermeable to Ca^{2+}. At negative membrane potentials, activation of AMPA receptors leads to influx of Na^+ ions into the cell causing depolarisation. NMDA receptor activation also results in Na^+ influx but in contrast to AMPA receptors, NMDA receptors are permeable to Ca^{2+} ions and the inward ionic current is voltage dependant. This is caused by blockade of the receptor by Mg^{2+} ions which block the pore at normal negative membrane potentials (Mayer et al., 1984; Nowak et al., 1984). Only when the membrane becomes depolarised, usually following AMPA receptor activation, are these Mg^{2+} ions removed. This results in

influx of Ca^{2+} through the NMDA receptor leading to intracellular signalling cascades, which can induce long lasting changes in synaptic transmission (Nakanishi et al., 1998).

Excessive activation of iGluRs, however causes neuronal death through excitotoxicity as they are the main routes for Ca^{2+} entry into the neurone. Damage occurs due to Ca^{2+} induced stimulation of intracellular enzymes that break down vital neuronal proteins, lipids and nucleic acids (Doble, 1995). The targeting of iGluRs for therapeutic use has therefore several limitations, namely interference with fast synaptic transmission or increased risk of excitotoxic damage. Modulation of mGluR activation has thus become an increasingly important treatment strategy as this route minimises these adverse side effects. Indeed, mounting evidence suggests that stimulation of mGluR subtypes mediate distinct facilitatory or inhibitory actions on neuronal excitation and excitability and may therefore be of therapeutic benefit in neurodegenerative processes (Nicoletti et al., 1996).

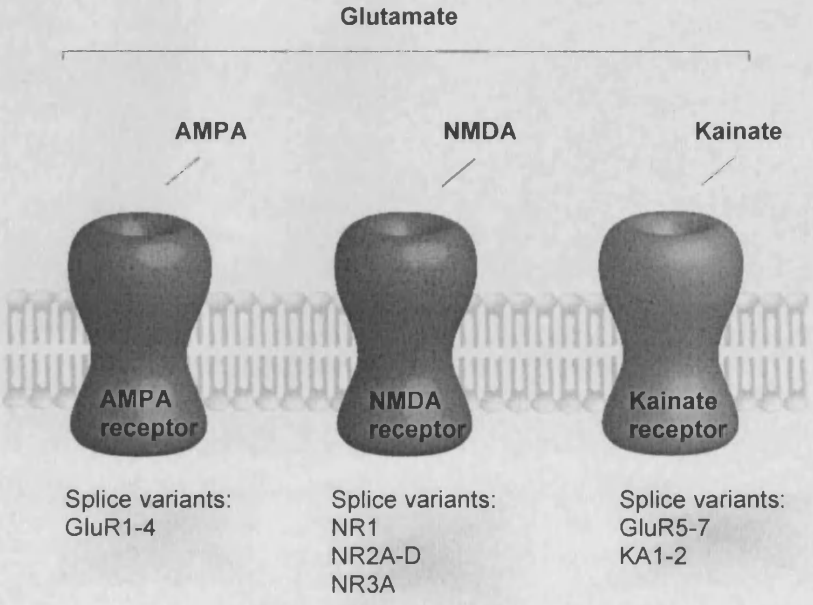


Figure 1.5 Neuropharmacology of iGluR synaptic transmission.
Each ionotropic glutamate receptor subtype is named after the original chemical agonist found to activiate them. Each iGluR subtype also has several splice variants. Adapted from Watkins, (2000).

1.3.2 G-protein coupled receptors

Most signalling between cells depends upon the release of soluble molecules that bind to receptors on a target cell subsequently inducing a signalling cascade. G-proteins are frequently used to communicate extracellular signals received at the cell surface receptor into intracellular responses. Most G-protein coupled receptors consist of a polypeptide with seven membrane spanning alpha helices. Two of the extracellular loops of the polypeptide form the transmitter binding sites, while two of the intracellular loops can bind to and activate G-proteins. Structural variations on the intracellular loops determine which G-proteins and therefore which effector systems are activated. The intracellular C-terminus regulates receptor activity by interacting with proteins including calmodulin (Bhave et al., 2003; Pin et al., 2003).

1.3.2a G-protein coupled effector mechanisms

Each G-protein has three subunits α β and γ. Unactivated G-proteins have a guanosine diphosphate (GDP) molecule bound to their α subunit and the whole complex is mobile on the intracellular membrane. When the G-protein binds to an activated metabotropic receptor, the GDP is exchanged for a guanosine triphosphate (GTP) molecule thus activating the complex resulting in a conformational change in the Gα subunit which decreases its affinity to the Gβγ subunit. The dissociated Gα + GTP and Gβγ subunits can then stimulate or inhibit specific effector pathways (Bear et al., 2001). These include direct activation of potassium channels or the production of intracellular second messengers such as adenylate cyclase (AC) (Rodbell et al., 1971) and phosholipase C (PLC) (Abbracchio et al., 1995; Baek et al., 1996). Other second messenger pathways activated by G proteins include MAPK (Blaukat et al., 2000) and ion channels (Postma et al., 1996; Komwatana et al., 1998). G-proteins can be either stimulatory or inhibitory and consequently modulate positive or negative effects on

downstream signalling (Bear et al., 2001). The signal is terminated by GTPase activity of the Gα subunit which hydrolyses the bound GTP to GDP causing re-association with Gβγ allowing the cycle to begin again (Figure 1.6). There are four major subfamilies of G-proteins; these are G_s, G_i, G_q, G_{12}, which vary in the composition of the α subunit (Fields and Casey, 1997) (Table 1.2).

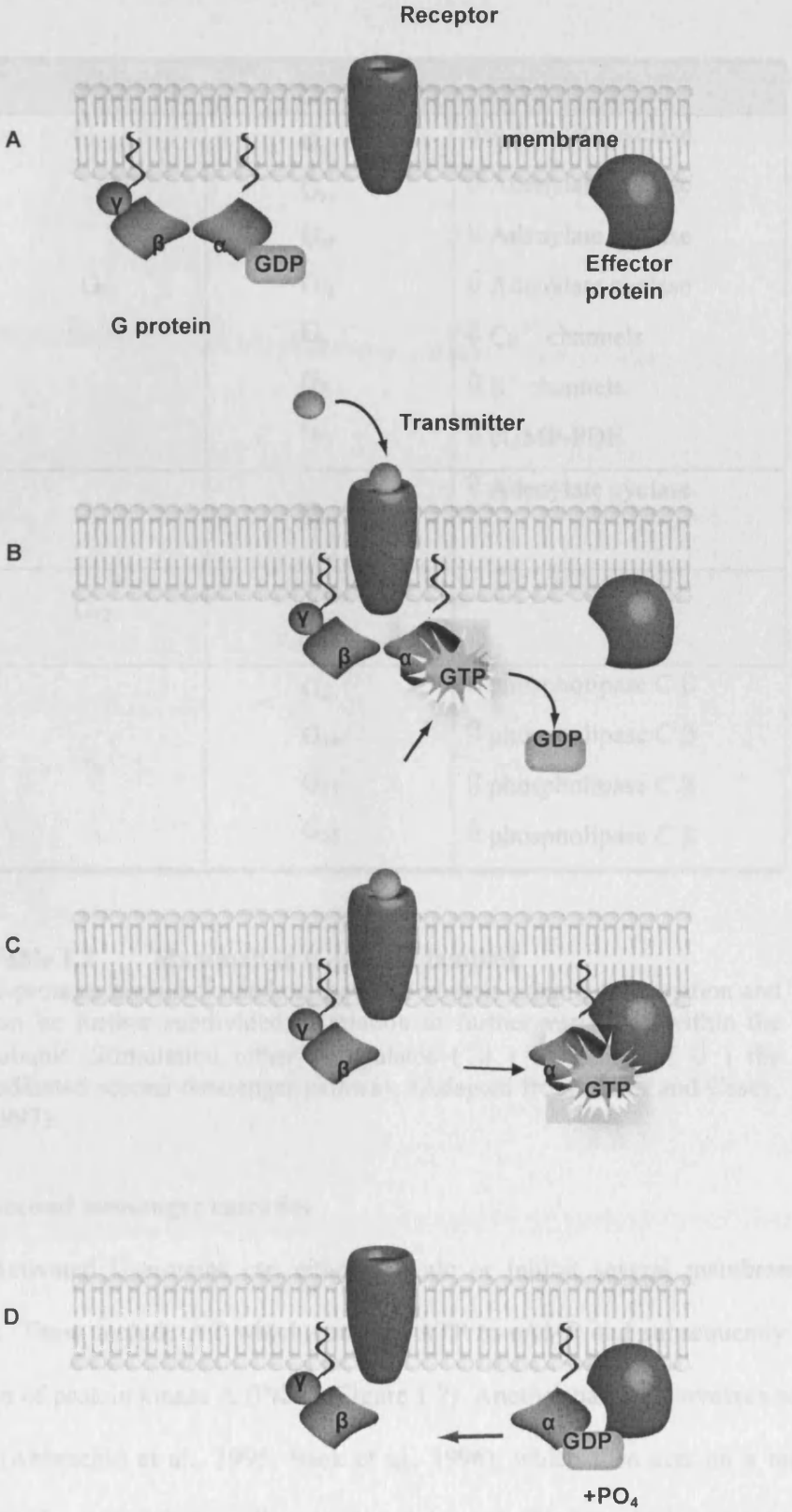


Figure 1.6 Mode of activation of G proteins.
(A) In its inactive state the α subunit of the G protein binds GDP. **(B)** When a transmitter activates the G-protein coupled receptor the GDP is exchanged for GTP. **(C)** Activation causes the G protein to split and the Gα with GTP subunit activates an effector protein. **(D)** The Gα subunit removes the phosphate (PO_4) from GTP thus converting it back to GDP and terminating activity and allowing Gα and Gβγ subunits to rejoin allowing the cycle to begin again. Adapted from Bear et al., (2001).

subfamily	G_α subunit	Signalling pathway
G_i	G_z	⇓ Adenylate cyclase
	G_{i1}	⇓ Adenylate cyclase
	G_{i2}	⇓ Adenylate cyclase
	G_{i3}	⇓ Adenylate cyclase
	G_o	⇓ Ca^{2+} channels
	G_k	⇑ K^+ channels
	G_t	⇑ cGMP-PDE
G_s	G_s	⇑ Adenylate cyclase
G_{12}	G_{12}	
	G_{13}	
G_q	G_q	⇑ phospholipase C β
	G_{14}	⇑ phospholipase C β
	G_{15}	⇑ phospholipase C β
	G_{16}	⇑ phospholipase C β

Table 1.2 Mammalian G-protein families.
G-proteins are subdivided on the basis of their subunit composition and can be further subdivided in relation to further variations within the subunit. Stimulation either upregulates (⇑) or inhibits (⇓) the indicated second messenger pathway. (Adapted from Fields and Casey, 1997).

1.3.2b Second messenger cascades

Activated G-proteins can either activate or inhibit several membrane bound enzymes. These include AC which converts ATP to cAMP and subsequently leads to activation of protein kinase A (PKA) (Figure 1.7). Another pathway involves activation of PLC (Abbrachio et al., 1995, Baek et al., 1996), which then acts on a membrane phospholipid causing it to split into diacylglycerol (DAG) which activates protein kinase C (PKC), and inositol-1,4,5-triphosphate (IP_3), a water soluble molecule (Figure 1.8). Diffusion of IP_3 into the cytosol allows binding to IP_3 gated calcium channels on

organelles resulting in Ca^{2+} release, which can trigger activation of calcium-calmodulin-dependent protein kinase (CaMK) (Berridge, 1984). Activation of protein kinases triggers phosphorylation of proteins. This process changes the conformation of a given protein and therefore affects its function. Indeed the phosphorylation of ion channels results in an altered functional state (Gudenmann et al., 1997).

Second messenger cascades are important for several reasons. Primarily, signal cascades allow amplification of a given signal. Also, the use of small diffusible messengers permits signalling over a distance instead of in localised areas. Furthermore, the generation of more long lasting chemical changes is possible and the many stages of a second messenger cascade allow downstream regulation or interaction from other systems (Bear et al., 2001).

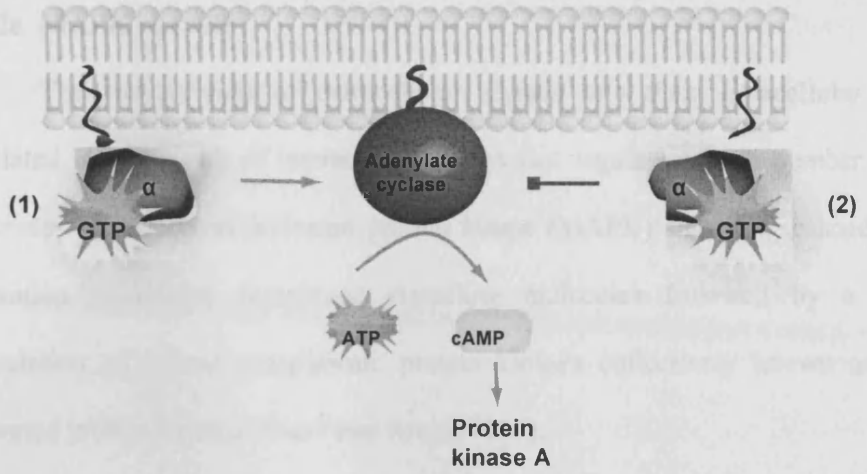


Figure 1.7 Stimulatory or inhibitory effects of G proteins coupled to adenylate cyclase. (1) Binding of transmitter to stimulatory G proteins (G_s) leads to activation (green arrows) of adenylate cyclase, which generates cAMP, which in turn activates the downstream enzyme protein kinase A. **(2)** Binding of transmitter to inhibitory G proteins (G_i) leads to inhibition (red lines) of adenylate cyclase activity. Adapted from Bear et al., (2001).

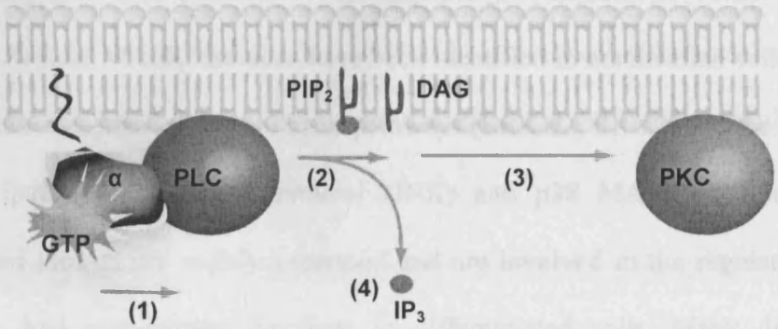


Figure 1.8 Second messengers generated by stimulation of phospholipase C (1) Various G-proteins stimulate the enzyme PLC which acts on membrane phospholipid PIP_2. **(2)** PIP_2 is split into DAG and IP_3. **(3)** DAG stimulates the downstream enzyme PKC. **(4)** IP_3 stimulates IP_3 gated Ca^{2+} channels on smooth ER and other organelles inducing release of Ca^{2+} from intracellular stores which can go on to stimulate downstream enzymes. Adapted from Bear et al., (2002).

1.3.2c MAPK cascade

The transmission of extracellular signals into their intracellular targets is mediated by a network of interacting proteins that regulate a large number of cellular processes. The mitogen activated protein kinase (MAPK) signalling cascade involves activation of several membrane signalling molecules followed by a sequential stimulation of several cytoplasmic protein kinases collectively known as mitogen-activated protein kinases (Seger and Krebs, 1995).

Mitogen-activated protein kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis (Pearson et al., 2001). MAPK pathways are linked to many G-protein linked cell surface receptors and as such, a host of signalling molecules including cytokines and neurotransmitters can selectively activate these cascades via receptor activation of intracellular second messengers. All MAPK pathways operate through sequential phosphorylation events to phosphorylate transcription factors and regulate gene expression (Tamura et al., 2002).

Several MAPK cascades have been identified in mammalian cells, including the extracellular signal-regulated kinase pathways (ERK1/2, ERK5) and the stress activated kinase pathways, c-Jun N terminal (JNK) and p38 MAPK. Extracellular signal-regulated kinases are widely expressed and are involved in the regulation of meiosis, mitosis, and post-mitotic functions in differentiated cells. Many different stimuli activate the ERK pathway. Phosphorylation of ERKs leads to the activation of their kinase activity (Boulton and Cobb, 1991).

Activated Ras activates the protein kinase activity of RAF kinase, a serine/threonine-selective protein kinase. RAF kinase phosphorylates and activates MEK, another serine/threonine kinase. MEK phosphorylates and activates extracellular signal-regulated kinase. The series of kinases from RAF to MEK to ERK is an example

of a protein kinase cascade. Such series of kinases provide opportunities for feedback regulation and signal amplification (Avruch et al., 2001).

1.3.3 Metabotropic glutamate receptors

Functional mGluRs possess a bi-lobed extra-cellular N terminal domain which contains the glutamate binding site. In the absence of ligands or when antagonised, the bi-lobed extracellular domain of the homodimer are separated into an open formation (Kunishima et al., 2000). Once activated by a selective agonist they close together thus triggering intracellular signal transduction. An extracellular cysteine rich region links mGluRs to a G-protein coupled receptor transmembrane heptahelical domain that mediates G-protein activation (Kunishima et al., 2000).

To date, eight members of the mGluR family have been identified (mGluR1-8). These have been divided into subgroups based on sequence homology, second messenger coupling and pharmacology. Group I subtypes include mGluR1 (splice variants a, b, c, d, e and g) and 5 (splice variants a and b), group II subtypes include mGluR2 and 3, and group III subtypes which include mGluR4 (splice variants a and b) mGluR6, mGluR7 (splice variants a and b) and mGluR8. All mGluRs belong to the family C G-protein coupled receptors. Group I mGluRs couple via the G_q/G_{11} to PLC (De Blasi et al., 2001). Group I mGluRs are unlike other subgroups in that the splice variants, mGluR1a and mGluR5a and b, are directly coupled to the IP_3 receptors on endoplasmic reticulum by homer proteins (Xiao et al., 1998; Tu et al., 1998), which are involved in mGluR trafficking (Ango et al., 2002). The group II and III receptors couple via G_i-protein to inhibit AC activity and are negatively coupled to forskolin sensitive cAMP production. (Reviewed by Kew and Kemp, 2005).

A number of agonists and antagonists for metabotropic glutamate receptors are highly selective for members within each group. Agonists bind the receptor and mimick

the action of endogenous ligands while antagonists block receptors from activation by agonists. Although a number of splice variants have been shown to exist for many receptors, these generally involve changes in amino acid structure down-stream from the N-terminal domain, and therefore don't generally affect the pharmacology of the receptor for agonist or antagonist binding (O'Hara et al., 1993).

The compounds used in this study (Table 1.3) included ADA a group I selective mGluR agonist with more potency at mGluR5 than mGluR1 (Klein et al., 1997). A relatively potent and selective antagonist of group I mGluRs, AIDA, was used which had no effect on group II or group III receptors nor on ionotropic glutamate receptors (Moroni et al., 1997). The group II receptor agonists included DCIIV a highly potent agonist for group II mGluRs (Brabet et al., 1998), and NAAG a highly selective agonist for the subtype mGluR3 with very weak activation of NMDA receptors. MCCG, a selective antagonist of group II mGluRs (Thomas et al., 2000) was used to block activation of the group II subtype. The group III agonist used, AP4, was selective for group III receptors alone (Tones et al., 1995). However, MAP4, although a selective antagonist for group III mGluRs, has also been shown to have group II and III agonist properties in some neurochemical systems (Sekiyama et al., 1996).

	Subtypes	Selective Agonists	Antagonists
Group I	mGlu1, mGlu5	DHPG ADA	AIDA
Group II	mGlu2, mGlu3	DCG-IV L-CCG NAAG *only mGlu3*	MCCG
Group III	mGlu4, mGlu6, mGlu7, mGlu8	AP4	MAP4

Table 1.3 Agonists and antagonists used for specific groups of mGluRs
To date eight subtypes of the mGluR family have been identified. These have been divided into subgroups based on sequence homology, second messenger coupling and pharmacology. Subgroups of mGluRs can be specifically stimulated or inhibited by selective agonists and antagonists.

1.3.3a Microglial metabotropic glutamate receptors

Microglia possess functional group I, II and III mGluRs. To date, microglia have been shown to express mGluR5 (Biber et al., 1999) mGluR2 and mGluR3 (Taylor et al., 2002) mGluR4, mGluR6 and mGluR8 (Taylor et al., 2002, 2003). Glial cells play a vital role in maintaining glutamate homeostasis and are also a primary source of glutamate released during excitotoxic stress in pathological conditions. This suggests that excess glutamate released from either neurones or microglia themselves may stimulate microglial mGluRs culminating in a cycle of activation and neurotoxicity (Taylor et al., 2002). As such, control of microglial reactivity by modulation of microglial mGluRs may be a potential therapeutic strategy in neurodegenerative diseases where microglial activation is implicated.

1.3.3b Neuronal metabotropic glutamate receptors

Metabotropic glutamate receptors can influence fast synaptic transmission by activation of slow excitatory postsynaptic potentials but in general they have a more modulatory role that includes the regulation of neuronal excitability, synaptic transmission and plasticity. Neuronal group I mGluRs are typically found post synaptically in somatodendritic neurones and are thought to increase neuronal excitation thus reinforcing excitatory synaptic transmission (Sourdet et al., 2003). In contrast, neuronal group II and III receptors are localised at presynaptic terminals away from the active zone of neurotransmitter release (Nolan and Logan, 1998). Activation of these receptors decreases neuronal excitation and glutamate release, but only in response to concentrations of glutamate high enough to spread back to presynaptic regions (Holscher et al., 1999; Cartmell and Schoepp, 2000). Stimulation of neuronal mGluRs has been shown to induce neuroprotective effects although these results remain open to conjecture.

Hippocampal (Blaabjerg et al., 2003) and CGC (Pizzi et al., 1996) neurones could be protected from subsequent excitotoxicity by pre-incubation with the group I agonist 3,5-Dihydroxyphenylglycine (DHPG) which resulted in reduced NMDA mediated inward currents suggesting inhibition of NMDA-receptor function. The DHPG conferred neuroprotection was inhibited by mGluR1 but not mGluR5 antagonism suggesting that this effect was due to activation of the mGluR1 subtype alone (Blaabjerg et al., 2003).

It has been found that Alzheimer's disease (AD) patients show upregulation of neuronal group II mGluR2 expression, which was closely associated with hyperphosphorylated tau deposition and correlated with the severity of neurofibrillary pathology (Lee et al., 2004). However, activation of group II neuronal mGluRs has also been shown to be neuroprotective, where group II mGluR agonists reduced neuronal degeneration induced by hypoxia and glucose deprivation (Buisson and Choi., 1995), and similarly reduced the death of neurones *in vitro* after exposure to cytotoxic concentrations of NMDA (Bruno et al., 1994; Battaglia et al., 1998). It is possible that increased mGluR2 expression in AD is a compensatory response to neuronal damage attempting to reduce excitability and subsequent excitotoxicity.

Stimulation of group III neuronal mGluRs can also be neuroprotective. Using a model of diffuse brain injury (DBI) in rats, it has been demonstrated that injection of the group III agonist L-AP4 protects against neuronal damage and promotes improved motor and cognitive performance compared with controls. Group III expression was also markedly upregulated after DBI especially in the area of impact, again suggesting a compensatory response (Zhou et al., 2004).

It has been proposed that the timing of mGluR agonist treatment is the crucial mediator of neuroprotection. Indeed pre-treatment of neuronal cultures is thought to transiently increase calcium influx thus inducing sensitisation or internalisation of

neuronal NMDA receptors that in turn causes a weakened response to subsequent exposure to NMDA (Baskys and Blaabjerg 2005).

1.3.3c Oligodendroglial metabotropic glutamate receptors

Metabotropic glutamate receptors 3 and 5 are highly expressed in oligodendrocyte progenitor cells (OPC) and mature oligodendrocytes (OL), although in mature OLs mGluR5 expression is markedly reduced (Deng et al., 2004; Luvt et al., 2006). Activation of group I mGluRs on OLs reduces their vulnerability to excitotoxic insult (Kellans et al., 2001) by reducing downstream oxidative stress and inhibiting ROS accumulation and intracellular glutathione loss (Deng et al., 2004). The down regulation of mGluR5 in mature OLs results in a notably lowered response to the group I agonist compared with OPCs (Luvt et al., 2006), which may explain the vulnerability of mature OLs to excitotoxic damage.

1.3.3d Astrocytic metabotropic glutamate receptors

Astrocytes express subtypes from all three groups of mGluRs including mGluR1 (Agrawal et al., 1998), mGluR5 (Biber et al., 1999), and mGluR2, 3, 4, 6 and 7 (Yao et al., 2005). Studies have shown that activation of group II and group III mGluRs on astrocytes can be neuroprotective via restoration of the capacity for glutamate uptake and increased glutathione levels (Zhou et al., 2006; Yao et al., 2005), and release of TGFβ (D'Onofrio et al., 2001; Bruno et al., 1998). The role of group I mGluRs, however remains uncertain. Activation of astrocytic group I mGluRs in rat astrocytes induced a significant upregulation in their capacity to take up aspartate through activation of glutamate transporter 1 (GLT-1) (Vermeiren et al., 2005). The same agonist in human astrocyte cultures, however, caused a significant down regulation of

both glutamate-aspartate transporter (GLAST) and GLT-1 protein (Aronica et al., 2003).

1.4 Neuronal glial interactions

Signalling between neurones and microglia is central to the regulation of microglial reactivity and neuronal survival (Cuzner and Woodroofe, 2002). The plethora of receptors for CNS signalling molecules expressed by microglia suggests a complex role in the maintenance of the CNS microenvironment. Ramified microglia in normal conditions are known to actively interact with surrounding glial cells and neurones, thereby fulfilling important neurotrophic roles (Streit, 2002; Davalos et al., 2005; Nimmerjahn et al., 2005). Indeed 'resting' microglia have recently been shown to be surprisingly active with highly motile processes functioning as monitors of CNS microenvironment and possibly facilitating rapid responses to brain injury (Davalos et al., 2005; Nimmerjahn et al., 2005).

Normal neuronal activity is signalled by the release of fractalkine and ATP, an activity associated transmitter, stimulating the release of neurotrophic factors by neighbouring microglia (Hanisch 2002). Indeed substances released by healthy neurones have been shown to neutralise the effects of microglial activators such as LPS, and IFN-γ (reviewed by Aloisi et al., 2001) and inhibit microglial TNFα production (Mott et al., 2004). It has recently been demonstrated that interaction of the neuronal membrane protein CD200 with the myeloid cell receptor CD200R can promote microglial ramification and that CD200 deficient mice show increased microglial activation in the resting CNS (Hoek et al, 2000). Other glial cells such as astrocytes have also been shown to down regulate microglial activation (Aloisi et al., 1997).

Neurotrophins such as NGF, BDNF and NT-3 are capable of inhibiting microglial expression of MHC class II antigens and the co-stimulatory molecules implicated in antigen presentation (Neumann et al., 1998), which lead to initiation of the inflammatory response. However, high extracellular ATP levels associated with tissue damage and in particular high ATP release from astrocytes induces microglial activation and migration towards the injury site (Davalos et al., 2005). The release of neurotoxic factors may in turn activate more microglia, this cycle is also exacerbated by disruption of 'calming' inputs from damaged neurones (Figure 1.9).

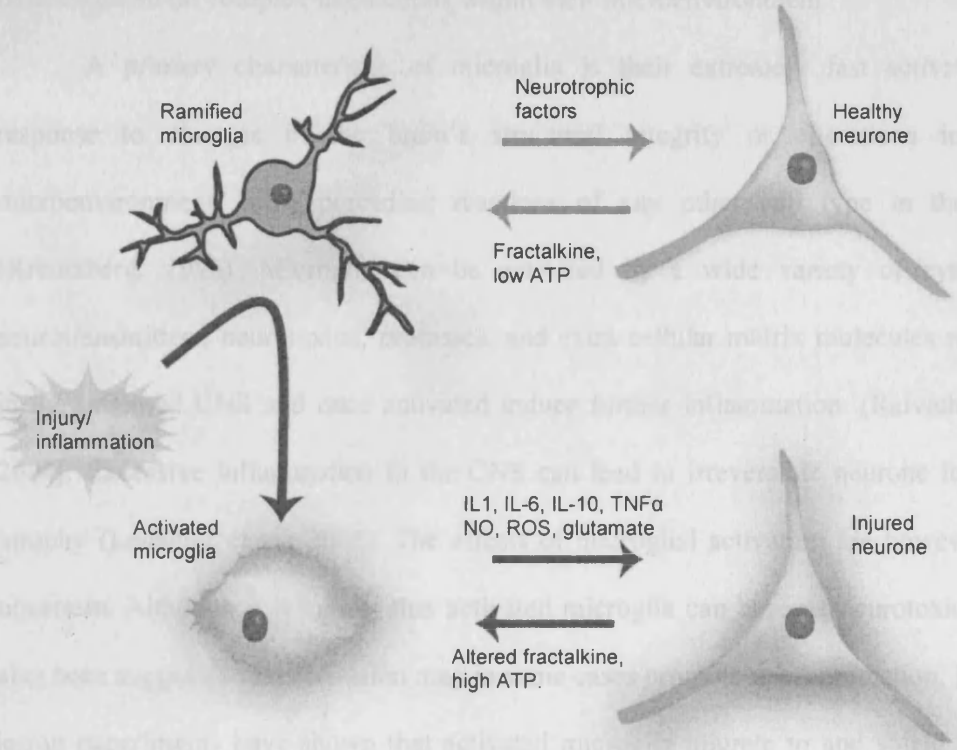


Figure. 1.9 Microglial and neuronal interactions
Microglia release neurotrophic compounds at rest which are thought to promote neuronal survival. Neurones are also thought to release substances which limit microglial activation. Upon activation, microglia release neurotoxic factors which induce neuronal death therefore upsetting the neurotrophic communication between cells. Adapted from Hanisch et al., (2002).

1.4.1 Microglial functions- cytotoxic or neuroprotective role?

Although microglia monitor and maintain neuronal health, promoting growth and development (Hanisch, 2002), few pathological disturbances in the CNS occur without their involvement (Kreutzberg, 1996). Microglial cells are extremely sensitive to changes in CNS homeostasis and become activated under most neuropathological circumstances including peripheral nerve injury, trauma and stroke, inflammatory disease and neurotoxic injury (Streit, 2000). Involvement of microglia in the inflammatory response or homeostatic maintenance seems to hang in a precarious balance based on complex interactions within their microenvironment.

A primary characteristic of microglia is their extremely fast activation in response to changes in the brain's structural integrity or alterations in CNS microenvironment, often preceding reactions of any other cell type in the CNS (Kreutzberg, 1996). Microglia can be activated by a wide variety of cytokines, neurotransmitters, neurotoxins, proteases, and extra-cellular matrix molecules released in the inflamed CNS and once activated induce further inflammation. (Raivich et al., 2004). Excessive inflammation in the CNS can lead to irreversible neurone loss and atrophy (Lehnardt et al., 2003). The effects of microglial activation are however still uncertain. Although it is known that activated microglia can become neurotoxic it has also been suggested that activation may in some cases promote neuroprotection. Indeed, lesion experiments have shown that activated microglia migrate to and shield injured areas by extension of their processes (Nimmerjahn et al., 2005).

Microglial involvement in the inflammatory response has become an important area of research as it has become increasingly apparent that many neurodegenerative disorders are accompanied by immune reactions and microglial activation (Mattson et al., 1997). Chronic microglial activation has been implicated in multiple sclerosis (MS), AD and Parkinson's disease (PD) (Gebicke-Haerter, 2001). It has been shown that

activated microglia release a number of potentially neurotoxic factors which can induce neuronal damage. Reducing microglial reactivity and neurotoxicity could therefore be of therapeutic benefit in a number of CNS disorders (Nicoletti et al., 1996; Pocock and Liddle, 2001).

1.4.2 Cell death

Two structurally and biochemically distinct types of cell death, apoptosis and necrosis were described by Kerr et al., (1972). Necrosis is pathological cell death induced by departures from physiological conditions. Disruptions to the structural and functional integrity of the plasma membrane can lead to abnormalities in intracellular Na^+ and Ca^{2+} levels and inflammation (Martin et al., 1999). The other form of cell death, apoptosis, refers to the ability of cells to undergo programmed cell death (PCD). Apoptosis is generally seen as physiological cell death and involves active intrinsic mechanisms controlling a cell death pathway (Payne et al., 1995). Each form of cell death can be recognised morphologically by several distinct characteristics (Table 1.4).

	Apoptosis	Necrosis
Energy requirement	ATP required for active process of apoptosis	No ATP required
Cells affected	Isolated single cells	Clusters of cells
Cell volume Homeostasis	Preserved in early stages	Lost in early stages
Plasma membrane	Preservation of integrity, blebbing of cell surface	Loss of integrity
Cytoplasmic organelles	Compaction and contraction, formation of apoptotic bodies	Swelling of structures
Nuclear chromatin	Condensation and fragmentation	Karyolysis and pyknosis
DNA	Internucleosomal fragmentation	Random degradation
Cellular response	No inflammation or neutrophilic infiltration	Marked inflammatory response with Leukocyte infiltration
Rate of progression	Normally 'all or none' phenomenon	Normally a 'dose dependent' phenomenon

Table 1.4 Cellular features of apoptotic and necrotic cell death. Adapted from Sathasivam et al., 2001.

Laboratory assays for apoptosis include the TUNEL assay where broken deoxyribonucleic acid (DNA) ends are labelled preferentially although this method can also detect necrotic cell death. The characteristic bands caused by DNA laddering can also be viewed by agarose gel electrophoresis (Otsuki and Shibata, 2003). However, the method used in this study was direct inspection of pyknotic condensed nuclei using a fluorescence microscope. This is generally agreed to be a safe way of discerning between apoptotic and necrotic cells (Figure 1.10). We also used a caspase assay which uses inhibitors of caspases that bind covalently to active caspases and have a green fluorescence. Any unbound compound diffuses out of the cell so the fluorescent signal is a direct marker of how much active caspase 3 or 7 is expressed by the cells.

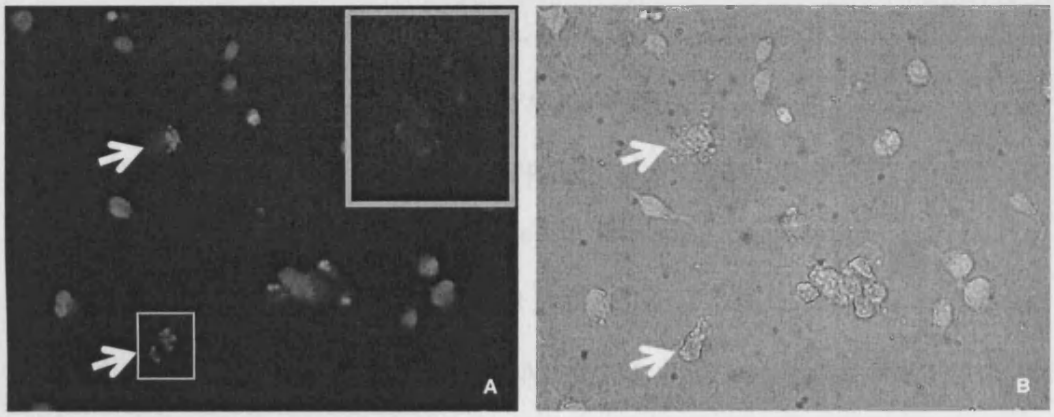


Figure 1.10 Morphology of apoptotic nuclei
(A) Oligodendrocyte cells stained with Hoechst which is a nuclear stain. White arrows show apoptotic nuclei which are clearly condensed and pyknotic. **(B)** Phase contrast image of the same field. x400 magnification.

1.4.3 Molecules involved in apoptosis

Apoptosis can be mediated by both intrinsic and extrinsic pathways. Extrinsic pathways require ligation of a receptor complex. The most documented extrinsic pathways include members of the TNF superfamily, TNF and Fas. TNF binds with TNFR1 which induces activation of effector caspases (Chen et al., 2002). The interaction between Fas and FasL results in activation of the death inducing signalling complex which includes the Fas associated death domain (FADD) and activation of caspases 8 and 10 (Wajant et al., 2002). Intrinsic apoptosis is usually a response to stress. Mitochondria contain apoptogenic factors such as cytochrome c and second mitochondrial derived activator of caspases (SMAC), which when released into the cytosol lead to activation of caspases (Zamzami et al., 1998). Members of the Bcl2 family which are either pro-apoptotic (Bax, Bak, Bad) or anti-apoptotic (Bcl-xL, Bcl2) control the release of these factors (Murphy et al., 2000).

Caspases belong to a family of proteases and are the effector cells of apoptosis (Figure 1.11). As with other multicomponent cascades, caspases are activated by proteolytic cleavage of the proenzyme next in the chain. Caspases function as initiators of cell disassembly in response to pro-apoptotic stimuli, or as effectors with an active role in cell destruction through regulation of proteolytic cleavages (Slee et al., 1999). The caspase cascade terminates with the rapid nuclear fragmentation caused by a Ca-dependent endonuclease which acts on the DNA between nucleosomes (Hudig et al., 1993). The relative expression of these proteins within cells determines the threshold for apoptosis and may explain the vulnerability of certain cell types to death inducing signals (reviewed by Bergman and Blaydes, 2006).

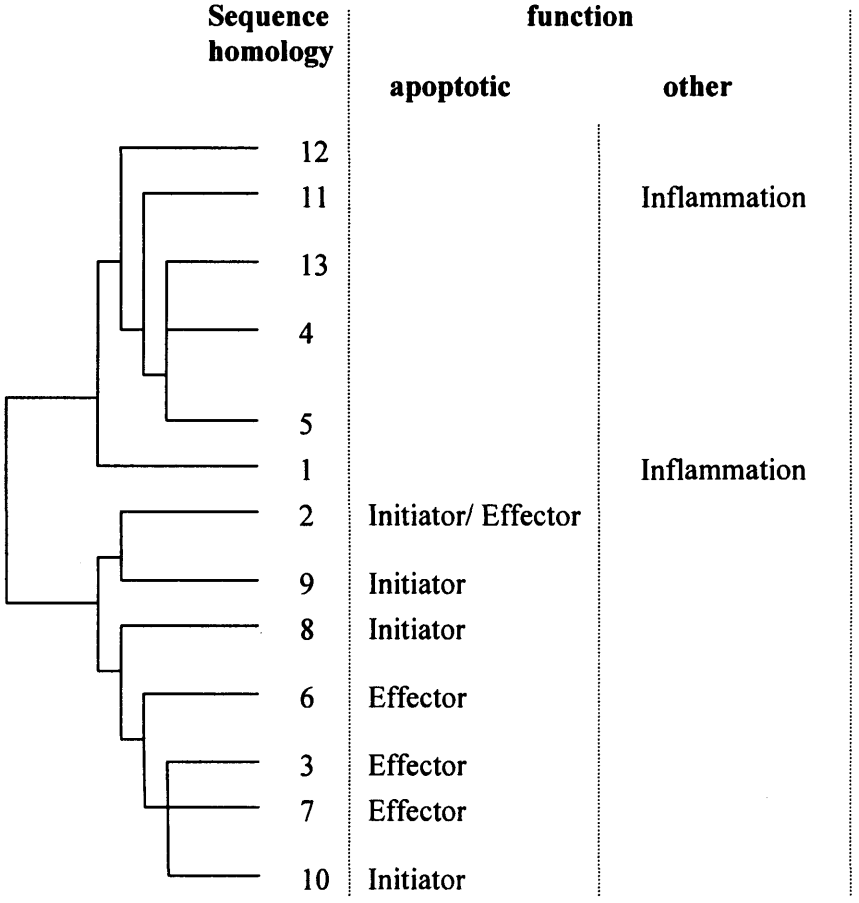


Figure 1.11 Sequence homology and function of caspases. Caspases are the effectors of apoptosis and are divided into initiator caspases, which cleave inactive proforms of effector caspases, and effector caspases, which cleave other protein substrates resulting in apoptosis. Adapted from Thornberry and Lazebnik (1998).

1.5 Immunity

The word immunity is derived from the Latin word *immunitas,* which means 'freedom from.' Humans have developed a series of defence mechanisms designed to keep us free from infection. The first line of defence is our skin, which is impermeable to most infectious agents. Membranes lining the inner surfaces of the body exposed to bacteria also produce mucus, which is designed to prevent bacterial adherence to epithelial cells. Microbial agents are trapped and removed by movement of cilia (Roitt and Delves, 2003).

The immune response involves two parts: A specific or acquired response to a particular antigen and a non-specific innate, inflammatory response. These two arms of the immune response are referred to as the acquired and the innate immune responses respectively and both result in inflammation.

1.5.1 Innate immunity

Innate immunity is an evolutionarily ancient system providing organisms with immediate defence mechanisms against infection. In contrast to acquired immunity, innate immunity does not have the capability to specifically recognise antigens. However, phagocytic cells are able to recognise a small number of highly conserved structures known as pathogen-associated molecular patterns (PAMPs) present on many different microorganisms including the bacterial cell wall component lipopolysaccharide (LPS). Recognition of PAMPs leads to a signalling cascade triggering phagocytosis and release of inflammatory mediators thus inducing a localised inflammatory response (Janeway et al., 2002).

1.5.2 The role of microglia in innate immunity

Microglia are often referred to as the resident immune cells of the CNS due to their ability to proliferate and their active migration towards sites of injury. Innate immunity revolves around the initiation of an inflammatory response against invading pathogens and is presided by microglia. Microglial cells express several receptors on their cell membranes, including toll like receptors and complement receptors, which indicates their complex role as immune regulatory cells in the CNS (Aloisi, 2001).

1.5.2a Toll-like receptors

Cells of the immune system recognise the molecular patterns expressed on the surface of invading pathogens (PAMPs), which are expressed by a large number of infectious agents and differ from self patterns, through activation of genetically conserved receptors known as toll-like receptors (TLRs) (Medzhitov and Janeway, 2000; Janeway et al., 2002). Lipopolysaccharide, a major constituent of bacterial wall in gram-negative bacteria, is bound by plasma LPS-binding protein, which is then captured by microglial CD14 acting as a pattern recognition receptor (PRR). This complex is then internalised activating Toll like receptors which in turn stimulate an NFκB transcription factor pathway culminating in initiation of the phagocytic process and upregulation of several proinflammatory genes including IL1, IL6, IL 12, TNFα and B7 the main co-stimulatory molecule (reviewed by Aloisi, 2001). LPS has been shown to induce upregulation of microglial CD14 (Becher and Antel, 1996). It has been shown that LPS induced microglial neurotoxicity can be prevented by inhibition of TLR4 on microglia therefore indicating a mechanistic link between innate immunity, microglia and neurotoxicity (Lehnardt et al., 2003).

1.5.2b Complement receptors

The complement system is one of the triggered enzyme cascade systems found in plasma and serves to attract phagocytic cells to invading pathogens. The most abundant component C3, is split by a convertase enzyme formed from its own cleavage product C3b and factor B, and stabilised against breakdown by association with microbial surfaces (Roitt and Delves, 2003). Microglia express the CR1, CR3 and CR4 receptors for C3bi which are primarily responsible for clearance of complexes labelled with C3 (Mosley and Cuzner, 1996). Activation of microglial CR3 receptor also increases release of cytotoxic TNFα further contributing towards the inflammatory cascade (Williams et al., 1994).

1.5.3 Acquired immunity

The cells of the acquired immune response are a type of leukocyte known as lymphocytes. B lymphocytes and T lymphocytes are generated in the bone marrow by stem cells. T cells then travel to and develop in the thymus. Peripheral lymphoid tissue contains both B and T cells in three different stages of maturation, these are naïve cells, effector cells and memory cells.

Immature lymphocytes with many different antigen receptors are screened for self antigens during development to minimise the risk of autoimmune attack. Cells expressing receptors for self antigen and cells with a weak association for MHC antigen undergo programmed cell death before maturation. T cells surviving screening are positive for either CD4 or CD8 depending on whether their T cell receptor recognises MHC-I presented antigens (CD8 +ve) or MHC-II presented antigens (CD4 +ve) (Roitt and Delves, 2003).

Invading exogenous pathogens are phagocytosed by antigen presenting cells (APCs) such as dendritic cells (Inaba et al., 1983; Banchereau et al., 1998), or microglia

(Williams et al., 1994), which then process and express parts of the pathogen in the context of the MHC-II antigens. Endogenous antigens such as viruses within host cells are expressed in the context of the MHC-I antigens. Mature lymphocytes leave the thymus and spread through the body to the lymph nodes but remain inactive or 'naïve' unless they bind with their specific antigen in the context of an MHC class I or II protein. This under the right conditions will induce activation and clonal expansion producing effector cells capable of mounting an acquired immune response (Roitt and Delves, 2003).

CD8 +ve or cytotoxic T cells (Tc) are capable of killing infected cells by releasing cytotoxins. Activation of Tc cells is dependent on several interactions between the T cell and the APC. A strong reaction between the T cell receptor and the MHC-I presented antigen is required along with stimulation by interaction between CD28 on the Th cell and the proteins CD80 (B7.1) or CD86 (B7.2) on the APC without which the T cells become anergic (Mueller et al., 1989; Liu and Linsley, 1992) and apoptotic (Liu and Janeway, 1990). Activation induced clonal expansion of Tc cells also requires IL-2 produced by the activation and proliferation of T helper (Th) cells. Dependence on co-stimulatory molecules and Th cell activation are in built mechanisms to protect against T cell autoimmunity (Liu and Janeway, 1990).

CD4 +ve T cells or T helper (Th) cells do not have phagocytic or cytotoxic capability but they mediate these responses in other cell types. Th cells recognise antigen presented in the context of MHC-II antigen which provides a signal for activation. Th cell activation is also dependent on co-stimulatory molecules. Once activated the Th cell will proliferate by producing T cell growth factor IL-2, into Th effector and Th memory cells. Effector T cells differentiate into two major subtypes, Th1 and Th2 cells defined by the cytokines they produce (reviewed by: Constant and Bottomly, 1997). Antigen presenting cells and the cytokines released during an

inflammatory event influence the differentiation towards Th1 or Th2 subtype. It has been proposed that Th1 cells produce IFN-γ and TGFβ and are important in maximising the activity of macrophages and microglia and the proliferation of Tc cells (Constant and Bottomly, 1997). IL-12 released from macrophages and microglia, encourages development of Th1 cells which secrete cytokines IL-1, IFNγ and TNF, known to further promote microglial activation and inflammation (Abbas et al., 1996). Th2 cells are thought to produce other cytokines including IL-4 and IL-5 which have a feedback effect promoting Th2 activity and have important roles in humoral immunity promoting B cell proliferation and antibody production (Roitt and Delves, 2003).

B cells play an important role in the humoral immune response. B cell receptors recognise specific antigen in their native form. Receptor binding with specific antigen induces B cell differentiation into effector molecules known as plasma cells. However, like T cells, B cells need additional signals to induce activation. Some antigens are capable of delivering both antigen and second signals to the B cell, whereas most are T-dependent, meaning that an additional signal from a Th2 cell is required for maximal antibody production from B cells. Antibodies mark specific antigens for phagocytosis and can trigger the complement cascade (Roitt and Delves, 2003).

1.5.4 The role of microglia in acquired immunity

Microglia express MHC class II antigens (Bo et al., 1994) and under inflammatory conditions this expression is rapidly up-regulated, thus suggesting that microglia participate in the antigen presentation occurring during inflammation (Xu and Ling, 1994, 1995). Microglia also express B7 (CD80 and CD86) molecules which are the co-stimulatory receptors crucial for T cell activation therefore confirming the professional APC status of these cells (De-simone et al., 1995; Menendez-Iglesias et al.,

1997; Wolf et al., 2001). Furthermore, Th1 cell activation also depends on IL-12 which is released by APCs including microglia, upon contact with T cells (Aloisi et al 1997; Becher et al., 2000), allowing microglia to regulate the T cell response. The production of IL-12 however is regulated by TNFα release, and Becher et al., (1996, 1999, 2000), have shown a causal connection between TNFα and IL-12 secretion by microglia. Microglial release of inflammatory cytokines including TNFα and IL-1 has also been implicated in the facilitation of leukocyte entry into the CNS via upregulation of adhesion molecules and chemokines (Sedgewick et al., 2000).

Innate immune mechanisms influence acquired immunity thus linking the two arms of immune response. Indeed, the optimal function of cellular acquired immunity is dependent upon the activity of the innate system with APC stimulation and cytokine release (Unanue, 1997). Microglia, as the immune cells of the CNS clearly have the potential to modulate the T cell driven acquired immunity, whilst also playing an active role in the innate arm of the immune response, (Figure 1.12).

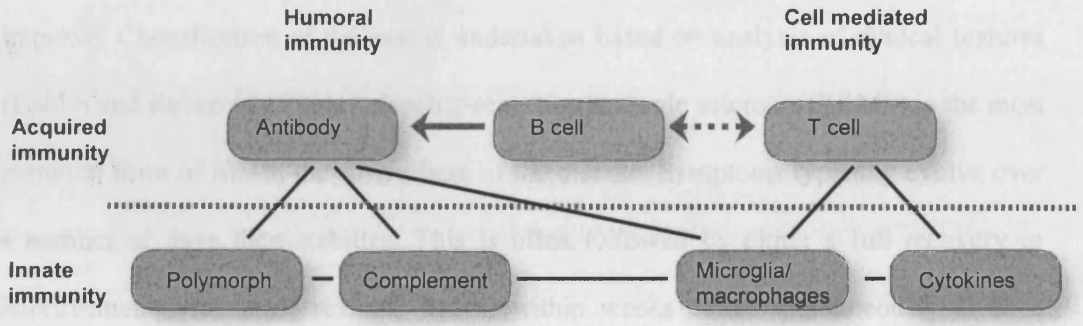


Figure 1.12 Relationship between acquired and innate immunity
Microglia play an important role in innate immunity, releasing inflammatory mediators once activated including a number of cytokines. They also transform into phagocytosing cells capable of clearing debris and pathogens marked with complement. At the same time the cytokines released by activated microglia are important in the acquired immune response and are thought to act as an attractant to primed T cells migrating through the BBB. T cells are then re-stimulated by microglia which can also function as APCs. (Adapted from Roitt and Delves, 2003).

1.6 Multiple sclerosis

Multiple sclerosis is the major human demyelinating disease of the central nervous system affecting around 85,000 people in the UK. Disease onset typically occurs between the ages 29-33 years making MS the most common neurological disease of young adults with twice as many women affected as men (Steinman 1996). MS is a multifaceted inflammatory disease of CNS white matter characterised by T cell, B cell and macrophage infiltration whose antigenic target is thought to be common myelin antigens (Hafler 2004). In MS the myelin sheaths are damaged leading to localised demyelinated lesions in fact, the name sclerosis is derived from the Greek word meaning 'hardening' which was an early description of the multiple plaques observed in the MS brain.

1.6.1 Clinical course of MS

A characteristic feature of MS is that symptoms tend to worsen suddenly then improve. Classification of patients is undertaken based on analysis of clinical features (Lublin and Reingold 1996). Relapsing-remitting multiple sclerosis (RRMS) is the most common form of MS in the early phase of the disease. Symptoms typically evolve over a number of days then stabilize. This is often followed by either a full recovery or improvement with some residual deficit within weeks either spontaneously or as a response to corticosteroid treatment. The efficacy of corticosteroid treatment in RRMS often decreases with time (Noseworthy et al., 2000).

An initial RRMS disease course frequently deteriorates to secondary progressive multiple sclerosis (SPMS) when disease symptoms progress between relapses and baseline neurologic disability increases (Bjartmar and Trapp, 2001). Response to immunotherapy dwindles and eventually disappears. The development of SPMS is

associated with significantly fewer gadolinium enhanced lesions and a reduction in brain parenchymal volume (Hafler et al., 2004).

In approximately 10% of patients, MS begins with a primary progressive phase (PPMS) (Zamvil and Steinmann, 2003), which is characterised by a progressive clinical course from the onset of disease with less inflammation but acute neurodegeneration. PPMS patients typically do not suffer acute attacks but undergo gradual clinical decline. Progressive relapsing multiple sclerosis (PRMS) follows a progressive clinical course from onset of disease with clear acute relapses sometimes with recovery (Figure 1.13).

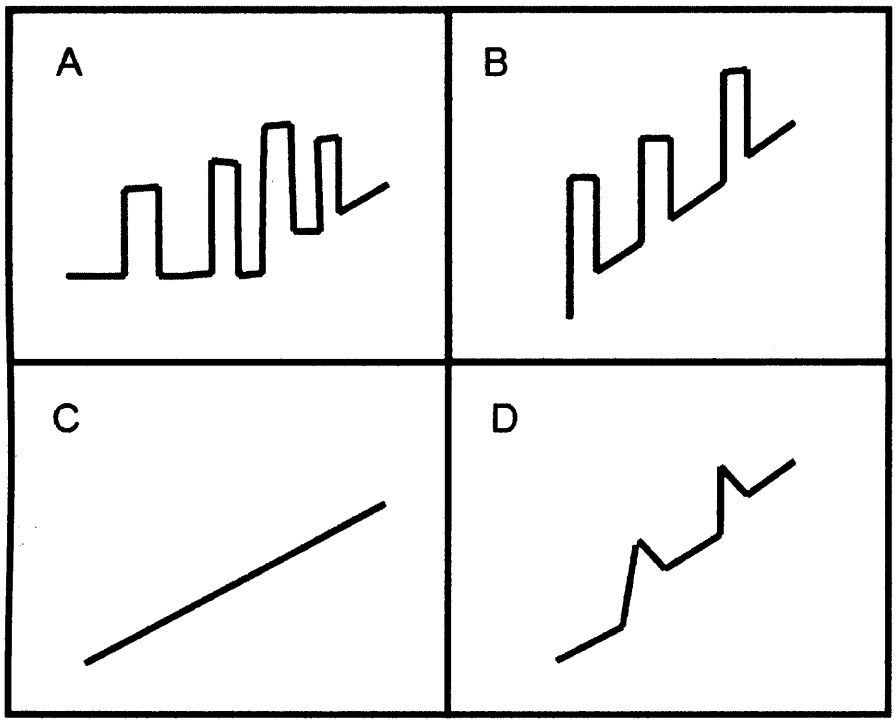


Figure 1.13 Schematic diagram of the clinical progression of MS
The bold line indicates clinical disability (A) shows RRMS with clinical relapses with return to baseline neurological function but eventual progression to SPMS. (B) SPMS when disease symptoms progress between relapses and baseline neurological disability increases. (C) Shows PPMS which is characterised by progressive disease course from onset. (D) Shows PRMS which follows a progressive course from onset but has obvious relapses where recovery may or may not occur. (Lublin and Reingold, 1996).

These distinctions, however, are very fluid; in the region of 40% of RRMS sufferers will eventually stop having relapses instead developing the progressive neurodegenerative symptoms of SPMS. It has been suggested that disease progression in MS is a continuum with acute inflammatory events early on followed by secondary induction of an underlying neurodegenerative process refractory to immunotherapy (Hafler 2004).

1.6.2 Pathological features of MS

The pathology of MS is well documented. On a macroscopic level, MS is characterised by multiple lesions or plaques occurring predominantly in the white matter of the brain and spinal cord. Lesions are most commonly found around blood vessels, and correspond to local myelin destruction or demyelination (Conlon et al., 1999; Noseworthy et al., 2000). These lesions comprise a well demarcated area characterised by a loss of myelin with relative preservation of axons and the subsequent formation of astrocytic scars.

At the cellular level, early active lesions are characterised by infiltration of inflammatory lymphocytes, macrophages and microglia, and oedema formation (Adams et al., 1989; Bruck, 2005a). Macrophages and microglia are frequently found around the active edges of the lesion containing lipid filled vesicles of myelin debris (Adams et al., 1989). Infiltrating macrophages are mainly derived from resident microglia (Li et al., 1996). Chronic lesions are characterised by lower numbers of leukocytes extensive demyelination and astrogliosis (Lucchinetti et al., 1996). Furthermore, in PP or SPMS there is notably less inflammatory activity and more axonal degeneration (Lassmann 1998; Bruck, 2005b).

1.6.3 Pathogenesis of MS

Although the autoimmune nature of MS has not yet been proven, the importance of immunopathological events has been widely documented (Lassmann 1998; Hemmer et al., 2002; Neumann et al., 2002). The inflammatory response, involving migration of activated T lymphocytes, is central to the pathogenesis of demyelination. The main candidate CNS auto-antigens are thought to be myelin proteins (Zamvil and Steinman, 2003). The immune system has several internal methods designed to avoid the development of autoimmune responses against the CNS. Indeed many of the components of myelin sheaths such as MBP and PLP, are expressed in the thymus thus allowing deletion of developing T cells capable of reacting with these molecules (Klein et al., 2000). It is possible that in genetically susceptible people myelin auto-antigens remain unchecked or are more readily activated, thus causing a predisposition to MS.

The initiating event for the presence or activation of auto-reactive T cells remains unknown, a 'molecular mimicry' theory has been postulated whereby common microbes activate APCs through the normal acquired immune response but also express cell surface protein sequences cross reactive with self myelin antigens (Conlon et al., 1999). Another possibility is that immunoregulatory defects in MS patients allows activation of autoreactive T cells. Initial activation of T cells is regulated by dendritic cells in the blood, which are recognised as primary antigen presenting cells (Becher et al., 2006), inducing proliferation and activation of T cells which subsequently circulate around the body, finally adhering to endothelial cells and altering the properties of the BBB to allow penetration (Hafler, 2004). T lymphocyte infiltration depends on alterations in the expression of cell surface molecules on activated T cells as well as endothelial cells, together with the secretion of enzymes that degrade the extracellular matrix (Carrithers et al., 2000). Within the CNS, T cells are re-stimulated by antigen presented by microglia and macrophages (Shrikant and Benveniste, 1996; Aloisi et al.,

2000) amplifying the immune response. Th1 cytokines are released initiating an inflammatory cascade which further activates microglia (reviewed by Noseworthy 2000; Hafler 2004), (Figure 1.14). Disease progression and demyelination are thought to induce production of new myelin epitopes which leads to recruitment of more T cells, further exacerbating the inflammatory cascade (Vanderlugt and Miller, 2002).

These concepts have been reinforced by experimental animal models such as experimental allergic encephalomyelitis (EAE) where immunisation with myelin or myelin proteins with adjuvant induces a CD4 +ve T cell mediated destruction of CNS myelin very similar to that seen in MS. Indeed, the importance of lymphocyte migration into the CNS in MS was highlighted recently during trials for the humanised antibodies natizulab and campath IH. These antibodies target the antigens α_4 integrins and CD54, responsible for the adhesion and migration of lymphocytes through the BBB, and reduced relapses and the formation of new lesions in MS patients (Miller et al., 2003; Coles et al., 2004). Furthermore T cell clones isolated from MS patients often show specifity for the p85-99 epitope of the myelin basic protein (Pette et al., 1990; Martin et al., 1990). The possibility that epitope spreading occurs after T cell activation has been highlighted by the fact that most MS patients display T cell reactivity to several myelin antigens (Ota et al., 1990), which is compatible with the theory that MS is initiated by invading infectious antigens cross reactive with myelin proteins.

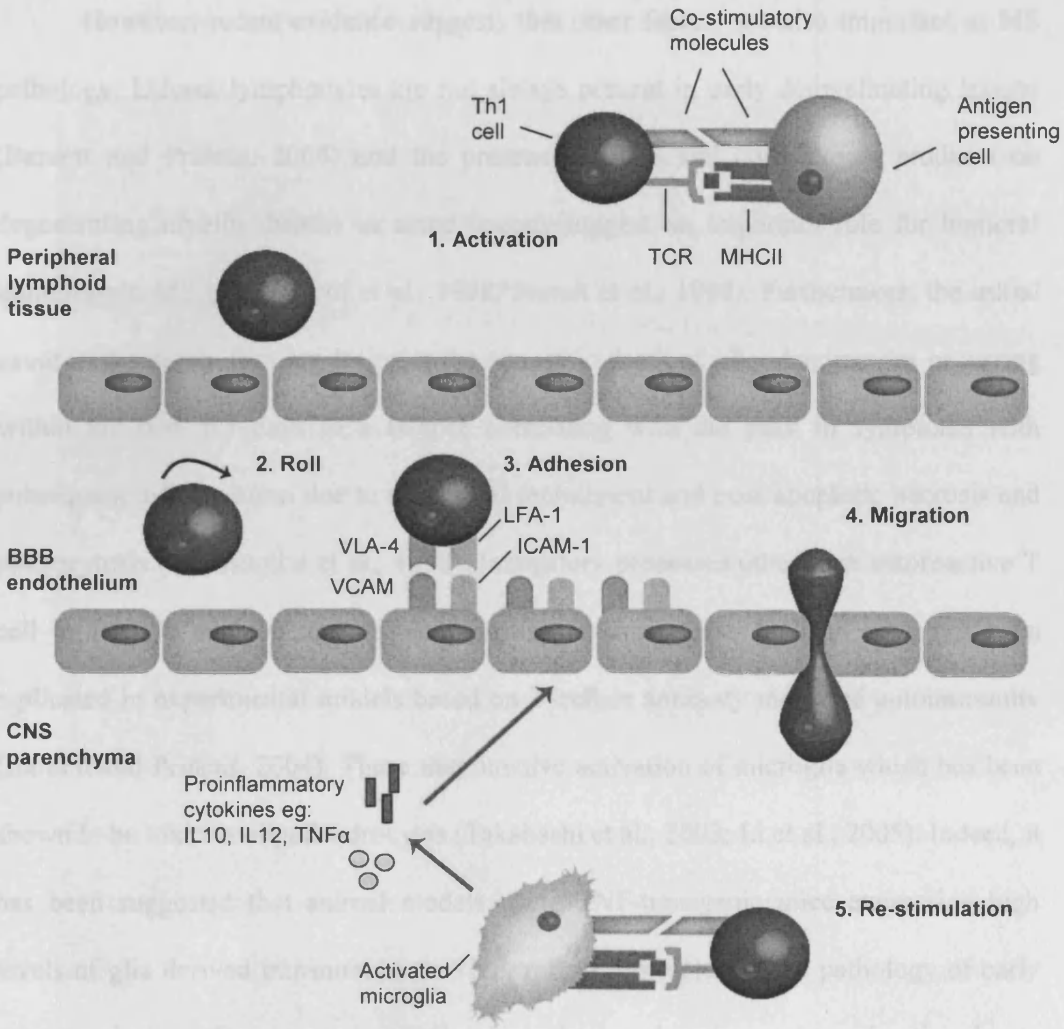


Figure 1.14 T cell migration and regulation of CNS T-cell response by microglia

(1) Th1 cells are activated in the peripheral lymphoid system by antigen presenting cells such as dendritic cells. **(2)** Following activation they enter the blood stream in search of their target antigen. **(3)** Adhesion of T cells to the endothelium occurs after binding of ICAM or VCAM with corresponding T cell receptors LFA-1 and VLA-4. **(4)** This allows cross linking of ICAM leading to Rho activation and cytoskeletal rearrangement which therefore allows migration of T cells through the blood brain barrier. **(5)** Once in the CNS parenchyma, T cells interact with microglia which display upregulated MHC-II following activation by inflammatory stimuli. The microglia then re-stimulate the T cells and secrete a variety of mediators which further exacerbate the inflammatory response and also leads to upregulation of molecules such as ICAM and VCAM on the endothelial cell surface which ultimately encourages further infiltration of T cells. Adapted from Vanderlugt and Miller, (2002).

However, recent evidence suggests that other factors are also important in MS pathology. Indeed, lymphocytes are not always present in early demyelinating lesions (Barnett and Prineas, 2004) and the presence of IgG and complement products on degenerating myelin sheaths in acute lesions suggest an important role for humoral immunity in MS (Lucchinetti et al., 1998; Storch et al., 1998). Furthermore, the initial event in the newly forming lesion is the apoptotic death of oligodendrocytes occurring within the first 1-3 days of a relapse correlating with the peak in symptoms with subsequent inflammation due to microglial recruitment and post apoptotic necrosis and phagocytosis (Akassoglou et al., 1998). Instigatory processes other than autoreactive T cell infiltration may be responsible for relapses since these findings have not been replicated in experimental models based on T-cell or antibody mediated autoimmunity (Barnett and Prineas, 2004). These may involve activation of microglia which has been shown to be toxic to oligodendrocytes (Takahashi et al., 2003; Li et al., 2005). Indeed, it has been suggested that animal models using TNF-transgenic mice expressing high levels of glia derived transmembrane TNF, reflect more closely the pathology of early preactive lesions (Barnett et al., 2006), which show early primary demyelination due to oligodendrocyte apoptosis and microglial activation, followed later by mononuclear infiltration, axonal loss and gliosis (Akassoglou et al., 1998).

A variety of mechanisms are involved in the pathogenesis of demyelination but the precise initiating event remains open to conjecture. Microglia have been implicated in the demyelination process due to their ability to phagocytose myelin debris, release cytotoxic substances and act as APCs. MS is a disease with extreme heterogeneity in clinical course due to differing appearance and location of lesions. The fact that essentially different processes have been implicated in the induction of MS-like plaques such as autoimmunity or viral infection suggests that MS may be a disease with heterogeneous pathogenic factors. Indeed, detailed analysis of actively demyelinating

lesions has shown fundamental differences in the pattern of demyelination between patients and found that most lesions can be divided into two main groups. One with characteristically high infiltration of inflammatory cells such as macrophages and microglia and localisation of activated complement and IgG on degenerating myelin sheaths more suggestive of T-cell or humoral inflammatory mediated damage. The other presenting with primary oligodendrocyte dystrophy and notable absence of IgG deposition is suggestive of viral or toxin induced demyelination (Lucchinetti et al., 2000). However, lesion differences between patients have also been attributed to lesional evolution rather than pathogenic heterogeneity (Barnett et al., 2006).

As previously discussed, the immune system has evolved several effector mechanisms designed to destroy invading pathogens. Unfortunately these mechanisms can also damage host tissue in autoimmune mediated disease which means that a multitude of immune regulated pathways may mediate demyelination. However, the same inflammatory mediator may affect different cells in varying degrees depending on functional properties of surrounding cells, which may lead to primary demyelination in some cases and axonal degeneration in others. It is also probable that genetically determined susceptibility of target cells regulates the nature and magnitude of tissue damage caused by immune mediated pathways (Compston et al., 2006). These factors may be responsible for the heterogeneity in MS lesions which develop in the context of a complex interaction of antigen dependent and independent mechanisms.

1.6.4 Axonal damage and inflammation

The ability of high dose steroids to reverse MS symptoms suggests that the acute oedema and inflammation and its subsequent resolution underlie relapse and remission. Indeed, therapies for MS at present are anti-inflammatory. Studies using MRI and evoked potentials have shown that acute relapses are caused by focal inflammatory

demyelination (McDonald, 2000). Clinical deficits are therefore due to nerve conduction blocks caused by inflammation and associated demyelination. Remission is probably the result of attenuated inflammatory response allowing some remyelination to occur thus restoring saltatory conduction. Remyelination and recovery after relapse often declines as disease progresses. This has been attributed to the on-going inflammatory activity in localised areas which may impede recovery processes and damage denuded axons (Figure 1.15).

Recently attention has become more focussed on axonal pathology in MS (Trapp et al., 1998). The close proximity of areas of inflammatory activity and areas with notable axonal loss suggest that axonal loss may be correlated to inflammatory activity in demyelinating lesions (Bruck, 2005), and that recurrent inflammation is the cause of permanent axonal damage (McDonald 2000). It has been proposed that the build up of axonal loss is what leads to secondary progressive form of MS and is responsible for clinical outcome (Owens, 2003; Neumann 2003). These observations were mirrored in a recent study which found that in acute EAE, axonal loss, demyelination and inflammation all correlated with clinical scores indicating a contributory role for microglia and other immune cells in the pathology of early disease. However in chronic EAE animals, only axonal loss correlated with clinical scores suggesting that irreversible neurological deficit was due to axonal loss (Papadopoulos et al., 2006).

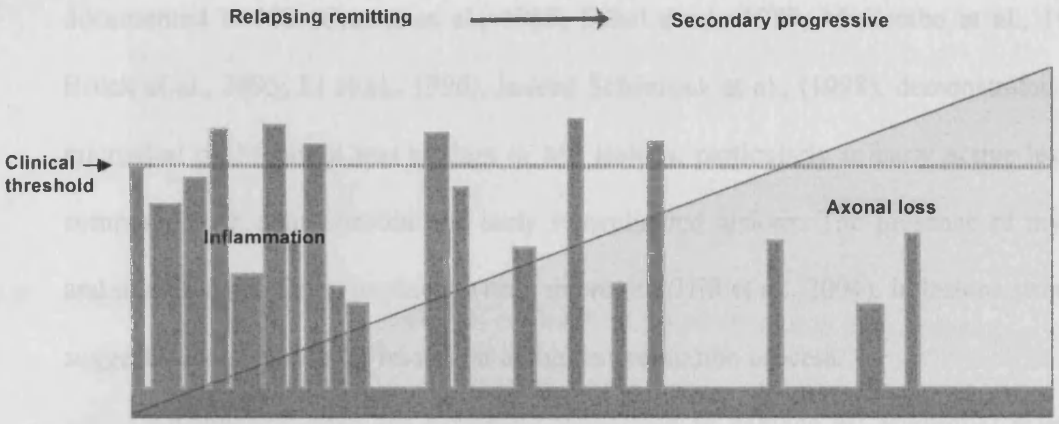


Figure 1.15 Course of inflammation and axonal damage in MS
Inflammatory episodes (shown in red) are frequent in relapsing remitting MS although not all episodes are noticeable at a clinical level (green dashed line). It is probable that disease progression is a result of continued inflammatory activity with demyelination and increased axonal damage. Progressive disease is characterised by infrequent inflammation but increased axonal loss and gliosis. Adapted from Compston and Coles (2002).

1.7 The role of microglia in multiple sclerosis

Although the immune response is meant as a protective mechanism, excessive inflammation is a common cause of damage in the CNS. It is becoming more widely accepted that CNS inflammation plays a role in most neurodegenerative disorders. Indeed microglial and macrophage activation and associated upregulation of MHC-II have been implicated in MS (Woodroofe et al., 1986; Hayes et al., 1987; Gehrmann et al., 1995), suggesting the involvement of the innate immune response. Strategies attenuating underlying inflammatory activity could therefore be of therapeutic benefit to MS patients.

Rapid recruitment of microglia occurs in both MS and EAE. Indeed, the high levels of inflammatory infiltrates including T cells and B cells, and the rapid upregulation of macrophages and microglia in lesions are consistent changes

documented in MS (Cuzner et al., 1988; Sobel et al., 1989; McCombe et al., 1994; Bruck et al., 1995; Li et al., 1996). Indeed Schonrock et al., (1998), demonstrated that microglial proliferation was evident in MS lesions, particularly in early active lesions compared with demyelinated and early remyelinated lesions. The presence of myelin and myelin degradation products within microglia (Hill et al., 2004), in lesions strongly suggests an active role for microglia in the demyelination process.

Oligodendrocytes are extremely vulnerable to damage by microglial released factors associated with the immune response including pro-inflammatory cytokines, ROS and NO, and glutamate which will be discussed later. Activated microglia also show increased capacity for binding of oligodendrocytes and phagocytosis of myelin compared with that of macrophages (Mosley et al., 1996). Although it has been demonstrated that the degree of oligodendrocyte loss in MS lesions is not homogenous and differs between MS patients, it is known that capacity for remyelination is closely correlated to the extent of oligodendrocyte survival (Luccinetti et al., 1999). Microglial toxicity to oligodendrocytes is therefore a relevant concern in the pathogenesis of MS.

Microglia also function as effector cells in the acquired immune response acting as APCs as already discussed. It has been postulated that T-cell infiltration occurs in response to cytokine induced up-regulation of endothelial cell adhesion molecules (Tomimoto et al., 1993; Sedgewick et al., 2000). It is possible that T cells are then presented with a potential MS antigen in the context of microglial MHC II molecules, inducing T cell activation and proliferation and stimulating the adaptive response thus exacerbating BBB damage and subsequent T cell infiltration. Indeed activated microglia show upregulated MHC class II molecules (Matsumoto et al., 1992; Bo et al., 1994), and co-stimulatory molecule B7 expression allowing maximal activation of T cells and subsequent immune response (Windhagen et al., 1995).

Positron emission tomography mapping of microglial activation in MS using the radioligand [^{11}C] (R)-PK11195, which binds invading blood borne cells of monocyte lineage and activated microglial cells, detects signal even in white matter regions that would normally be classified as 'normal appearing' and during remission of disease (Banati et al., 2000). These findings are in accordance with those of Barnett at al., (2004), which put forward the importance of the humoral response in preactive lesions and furthermore suggest an underlying role of microglia in the ultimate progressive nature of MS. However, the exact role of microglia in MS is still not fully understood since it is becoming increasingly evident that microglia also secrete substances with anti-inflammatory properties such as TGFβ and IL-10 which are thought to be potential mediators of remyelination or disease remission (Diemel et al., 1998; Ledeen and Chakraborty, 1998).

1.7.1 Inflammatory mediators in MS

The inflammatory reaction is associated with upregulation of a number of pro-inflammatory mediators which have been identified within MS lesions including the cytokines IFN-γ, TNFα and TGFβ (Brosnan et al 1996), NO (Liu et al., 2001; Broholm et al., 2004; Hill et al., 2004), and glutamate (Werner et al., 2001; Srinivasan et al., 2005). This project has focussed on regulation of TNFα, iNOS and glutamate production by microglial cells, their role in MS will therefore be discussed in more detail (Figure 1.16). While the diversity of interactions combined with the abundance of inflammatory mediators in MS makes it difficult to pin point a direct link between these particular molecules and tissue damage, there is substantial evidence to suggest that they participate in the demyelination process.

1.7.1a TNFα

High levels of TNFα expression both at mRNA and protein levels have been demonstrated in active MS lesions (Olsson 1995, Brosnan et al., 1995). This expression being particularly localised to microglial cells (Renno et al., 1995). Myelin phagocytosis by microglia occurs *in vitro* and induces microglial activation, leading to cytokine release and increased affinity for oligodendrocytes, inducing their death by cell contact through TNF receptors (Zajicek et al., 1992; Williams et al., 1994). Findings indicating that microglial expression of cell surface TNFα could directly induce oligodendrocyte damage (Merill et al., 1993), and that transmembrane TNFα can induce demyelination when produced by astrocytes but not neurones (Akassoglou et al., 1998), suggest that appropriate cellular contact appears to be an important factor in the toxicity of TNFα. Oligodendrocyte apoptosis in mixed glial cultures induced by TNFα was prevented by blockade of AMPA/Kainate receptors therefore indicative of a cytokine induced failure of the glutamate buffering function of astrocytes (Takahashi et al., 2003).

Mice over expressing TNFα suffered from chronic inflammatory demyelinating disease (Probert et al., 1995). However, the role of TNFα in MS pathology, remains unclear, studies using animal models of MS have also shown that TNF is not required for induction of disease and furthermore that treatment with TNF reduced the severity of disease (Liu et al., 1998). Furthermore, MS patients who received neutralizing antibodies to TNFα did not show improved clinical scores compared with those taking antibodies to IFN (Skurkovich et al., 2001). Interestingly, oligodendrocytes express both TNFR1 and TNFR2 and as in neuronal cultures (Taylor et al., 2005), it has been proposed that apoptosis inducing TNFR1 is upregulated on oligodendrocytes in MS lesions as a result of chronic inflammation (Tchelingerian et al., 1995). Stimulation of p75 TNFR2 receptor, however, has been shown to promote oligodendrocyte precursor

growth in chemically induced demyelination which may explain the seemingly dual role of TNFα in inflammation and suggests a role for TNF in repair and remyelination (Arnett et al., 2001).

1.7.1b Nitric oxide

There has also been much interest in the role of NO in MS. NO is a non-specific inflammatory mediator released from microglia and macrophages which are prolific in early lesions, indeed the iNOS expressed in chronic active plaques is amplified in areas of higher inflammation (reviewed by Smith et al., 1999), and is associated with CD64 (Okuda et al., 1995; Ruuls et al 1995; Hill et al., 2004). Expression of iNOS is also upregulated in astrocytes and macrophages in post mortem studies of patients with MS (Bo et al., 1994, DeGroot et al., 1997). Furthermore, MBP fragments are found in microglial cells expressing iNOS suggesting a direct involvement in the demyelination process in active plaques (Hill et al., 2004). NO may also induce cerebral vasodilation which could facilitate the entry of leukocytes through the BBB by reducing the velocity of the surrounding blood flow (Smith and Lassmann, 2002).

Since both neurones and oligodendrocytes have high metabolic requirements, NO and reactive nitrogen species may contribute to neurodegeneration and demyelination via impairment of mitochondrial energy metabolism and subsequent ATP depletion (Bolanos et al., 1995). Indeed, oligodendrocytes are more susceptible to NO mediated damage than either astrocytes or microglia (Merrill et al., 1993). Furthermore, axons exposed to NO *in vivo* can undergo continued conduction block, especially when electrically active during NO exposure (Smith et al., 2001), which is more likely in MS lesions due to demyelination. Sodium and calcium channel blockers can reduce NO mediated axonal degeneration by preventing the accumulation of these ions, which is

the result of Na^+ /K^+ pump failure and reversal of the Na^+/Ca^{2+} exchanger due to NO mediated impairment of mitochondrial ATP production (Kapoor et al., 2003).

At the clinical level, patients with RRMS display significantly higher expression of nitrate and nitrite, the stable derivatives of NO, in the CSF, serum and urine compared with controls (Giovannoni, 1998; Acar et al., 2003; Rejdak et al., 2004). Studies using animal models of MS have also demonstrated that in diseased animals, microglia and macrophages secrete significantly increased amounts of nitrate and nitrite, (MacMicking et al., 1992). Furthermore, Inhibitors of iNOS have been shown to reduce the inflammation and demyelination in animal models of MS (Rose et al., 1998), which further suggests a central role for NO in demyelination.

However, the actual role of NO in MS pathology remains unclear. Other studies using animal models have shown that iNOS inhibitors worsened disease course (Ruuls et al., 1996), and that iNOS knockout led to increased susceptibility to initiation of EAE (Sahrbacher et al., 1998). Furthermore, NO production from microglia has been shown to inhibit T cell proliferation by modulating the inhibitory effect of interferon gamma receptor on the proliferation of encephalitogenic T cells (Willenborg et al., 1999). The role of iNOS in MS is at present unclear but it is possible that at different stages of disease, NO mediates different effects ranging from cytotoxic to immunomodulatory. It has been suggested that the pro-oxidant or antioxidant effects of NO are dependent on the concentrations and ratio of superoxide to NO. This divergence in function may explain the different effects of NO inhibition in *in vivo* models (Smith and Lassman 2002).

1.7.1c Glutamate in MS

Evidence suggests that glutamate excitotoxicity may also play a role in the neuronal and oligodendrocyte damage in MS and EAE (Smith et al., 2000; Werner et

al., 2001; Matute et al., 2001; Groom et al., 2003). Studies using magnetic resonance spectroscopy have shown that levels of glutamate are significantly higher in acute MS lesions and NAWM when compared with control subjects (Srinivasan et al., 2005). Furthermore, microglia and macrophages express high levels of glutamine, a marker for glutamate production, in areas of white matter inflammation close to dystrophic neurones in MS compared with control patients (Werner et al., 2001). Oligodendrocytes are responsible for the maintenance of glutamate homeostasis in the white matter and have been shown to express glutamate transporters (Pitt et al., 2003). However, it has been suggested that oligodendrocyte metabolism of glutamate in active and chronic MS lesions is impaired (Werner et al., 2001).

Oligodendrocytes and axons are sensitive to oxidative stress as a result of their high metabolic requirements. They also express AMPA/ kainate receptors which mediate excitotoxicity stimulated by glutamate (McDonald et al., 1998). Indeed stimulation of AMPA receptors is toxic to oligodendrocytes *in vitro* (Matute et al., 1997; McDonald et al., 1998), inducing rapid Ca^{2+} influx (Fern and Moller 2000; Yoshioka et al., 2000), competitive inhibition of cystine uptake and oxidative stress (Rosin et al., 2004). Agonist concentrations similar to those affecting neuronal and oligodendrocyte cultures, however, do not induce astrocytic death (McDonald et al., 1998).

Microglial products IL-1β and TNFα induce oligodendrocyte death in mixed glial cultures which is prevented by AMPA receptor blockade thus pointing to a link between microglial activation, excess extracellular glutamate and oligodendrocyte death (Takahashi et al., 2003). TNFα has also been shown to reduce oligodendrocyte glutamate transporters and inhibit glutamate uptake (Pitt et al., 2003), therefore impeding homeostatic function and leading to cell death.

The role of glutamate in MS is highlighted by the distinctly reduced oligodendrocyte death and axonal damage and subsequent amelioration of disease recorded after treatment of EAE mice with antagonists to NMDA and AMPA/kainate receptors (Achiron et al., 2000; Pitt et al., 2000; Smith et al., 2000). Furthermore, the glutamate receptor antagonist and sodium channel blocker, riluzole, suppresses inflammation and demyelination in EAE (Gilgun-sherki et al., 2003) and slows the progression of CNS atrophy in PPMS (Killestein et al., 2005).

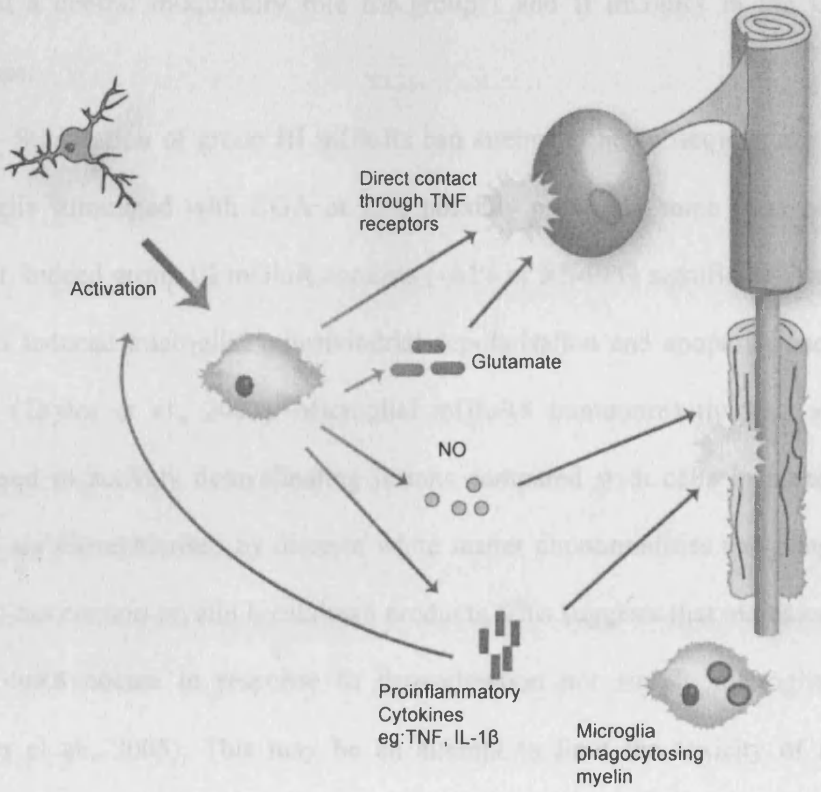


Figure 1.16 Possible mechanisms of degeneration during inflammation
T cells re-stimulated in the CNS activate local cells including microglia which trigger an inflammatory cascade. Activated microglia release a number of inflammatory mediators which may damage oligodendrocytes and exposed axons. NO can cause axonal conduction block while TNFα and glutamate are toxic to oligodendrocytes and neurones. Activated microglia also phagocytose myelin which may promote demyelination.

1.7.2 Metabotropic glutamate receptors in MS

Stimulation of group II mGluRs has been shown to promote microglial reactivity and neurotoxicity; conditioned medium from microglia treated with group II agonists DCGIV or $_{L}$-CCG induced increased apoptosis when applied to neuronal cultures and the group II antagonist MCCG significantly reduced apoptosis induced by chromogranin A (CGA) (Taylor et al., 2002). Microglia in chronic active MS plaques display increased expression of both groups I and II mGluRs (Geurts et al., 2003). Recently, group II and mGlu1 immunoreactivity has been detected in activated but not ramified microglia of chronic active MS lesions (Guerts et al., 2003). These findings suggest a central modulatory role for group I and II mGluRs in the inflammatory response.

Stimulation of group III mGluRs can attenuate the subsequent neurotoxicity of microglia stimulated with CGA or LPS possibly providing some form of therapeutic benefit. Indeed group III mGluR agonists $_{L}$-AP4 or RS-PPG significantly reduced CGA or LPS induced microglial mitochondrial depolarisation and apoptosis back to control levels (Taylor et al., 2003). Microglial mGluR8 immunoreactivity is also strongly increased in actively demyelinating lesions compared with cells in preactive lesions which are charachterised by discrete white matter abonormalities and phagocytic cells that do not contain myelin breakdown products. This suggests that increased expression of mGluR8 occurs in response to demyelination not simply microglial activation (Geurts et al., 2005). This may be an attempt to limit the toxicity of microglia to oligodendrocytes.

Astrocyte secretion of RANTES, a T cell chemoattractant that has been implicated in MS and EAE, showing increased expression correlated with the intensity of inflammation (Ransohoff. 1999), was significantly reduced by co incubation with L-AP4 a neuroprotective (Taylor et al., 2003), group III mGluR agonist. Treatment of

EAE mice with L-AP4 lowered RANTES mRNA expression and significantly increased the rate of recovery (Besong et al., 2002). Together, these findings suggest that microglial mGluR expression is modulated in MS lesions which may in turn affect the inflammation and subsequent degeneration which are features of this disease.

1.8 Aims

Microglia clearly play an important role in MS pathology as general mediators of inflammation through secretion of toxic inflammatory products and also at a more specific level with a distinct role in demyelination through toxicity to oligodendrocytes and active phagocytosis of myelin. It is also possible that microglia are a part of the initial events in MS pathogenesis, acting as APCs and releasing chemoattractants which attract autoreactive T cells into the CNS or initiating oligodendrocyte apoptosis through release of toxic factors.

Microglia express mGluRs which can be neurotoxic (group II) or protective (group III). Microglial neurotoxicity during disease progression could be explained by increased expression or activation of one subtype over another. Loss of microglial regulation during the progression of MS could therefore be due to changes in mGluR expression. This project will analyse expression of mGluR subtypes on activated compared with unstimulated microglia and also assess the expression of microglial mGluRs during disease progression using an animal model of MS.

Since microglia are known to phagocytose myelin, this study will aim to determine the effects of myelin on microglia by assessing levels of activation, expression and release of inflammatory mediators and subsequent toxicity to neurones. Given that microglial mGluR stimulation has been shown to modulate the reactivity of

microglia we will also investigate whether microglial reactivity after incubation with myelin can be modulated by stimulation of their mGluRs since this may have implications for therapeutic strategies in MS. Phagocytosing microglia are thought to be 'super activated' displaying similar morphology and functions to activated macrophages, it is possible that in this state they release substances deleterious to the survival of surrounding cells. As such this study will also explore whether myelin phagocytosis can be modulated by stimulation of microglial mGluRs.

2.0

MATERIALS AND METHODS

2.1 Materials

This section lists that materials used and the company that supplied them. All analytical reagent grade chemicals used in this study were from Sigma, (Dorset, UK).

MATERIAL	SUPPLIER
Ampicillin	Sigma (Dorset, UK)
AMT HCL	Sigma (Dorset, UK)
ARA-C	Sigma (Dorset, UK)
Avidin-Biotin Complex	Sigma (Dorset, UK)
Benzamadine	Sigma (Dorset, UK)
Bradford protein assay reagent kits	PerbioScience (Cheshire, UK)
BSA	Sigma (Dorset, UK)
Buffer saturated phenol	Invitrogen (Paisley, UK)
CaspaTag	Chemicon (Hampshire, UK)
Citifluor® antifade reagent	Citifluor Ltd (London, UK)
CO_2 Gas, tissue culture grade	BOC gases (Guilford, UK)
DAPI	Sigma (Dorset, UK)
Dextran beads	Invitrogen (Paisley, UK)
DiI fluorescent labelling	Invitrogen (Paisley, UK)
DNA ladder (100bp)	Promega (Paisley, UK)
dNTP (100mM)	Invitrogen (Paisley, UK)
Donkey anti-goat IgG (HRP)	Autogen Bioclear (Wiltshire UK)
DPX mountant	VWR International (Leicester UK)
Dulbecco's modified Eagles' medium (DMEM)	Sigma (Dorset, UK)

Earle's balanced salt solutions (EBSS)	Life Technologies (Paisley, UK)
Enhanced chemiluminescence reagent (ECL)	Amersham (Buckinghamshire, UK)
Foetal calf serum	Life technologies (Paisley, UK)
Folin- Ciocalteu reagent	VWR International (Leicester UK)
Formaldehyde	VWR International (Leicester, UK)
Fraction V Albumin	Sigma (Dorset, UK)
Glass coverslips (13mm)	Scientific Laboratory Supplies (Nottingham, UK)
Glutamate dehydrogenase	Sigma (Dorset, UK)
Goat anti-mouse (HRP)	Autogen Bioclear (Wiltshire,UK)
Goat anti-rabbit (HRP)	Autogen Bioclear (Wiltshire,UK)
Goat anti rabbit (TRITC)	Sigma (Dorset, UK)
Greiss reagent	Sigma (Dorset, UK)
Hoechst 33342 (2'[epoxyphenyl]-5 -[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazol)	Sigma (Dorset, UK)
Ipegal	Sigma (Dorset, UK)
Lipopolysaccharide (LPS)	Sigma (Dorset, UK)
Methanol	VWR International (Leicester, UK)
Minimum essential medium	Life technologies (Paisley, UK)
mGlu1,5, 2,3,4,6,7,8 primers	Sigma (Dorset, UK)
Mouse anti-βactin	Sigma (Dorset, UK)
Mouse anti-ED-1	Serotec (Oxford, UK)
Mouse anti-Pan Cadherin	Abcam (Cambridge, UK)
Mouse anti-PERK	Autogen Bioclear (Wiltshire,UK)
New born calf serum	Invitrogen (Paisley, UK)
Oligo $(dT)_{12-18}$ primer	Invitrogen (Paisley, UK)

OX-42	Autogen Bioclear (Wiltshire, UK)
Penicillin	Sigma (Dorset, UK)
Percoll	Sigma (Dorset, UK)
Propidium Iodide	Sigma (Dorset, UK)
Protein A/G agarose beads	Autogen Bioclear (Wiltshire, UK)
PP2	Sigma (Dorset, UK)
PVDF membrane (Immobilon-P-polyvinylidene di-fluoride)	Sigma (Dorset, UK)
Rabbit anti-iNOS	BD Biosciences (Oxford, UK)
Rabbit anti-total-ERK	New England Biolabs (Herts, UK)
Rabbit anti-pan Cadherin	Abcam (Cambridge, UK)
Rabbit anti-mGluR1	Upstate (Buckinghamshire, UK)
Rabbit anti-mGluR 2	Upstate (Buckinghamshire, UK)
Rabbit anti-mGluR 2/3	Upstate (Buckinghamshire, UK)
Rabbit anti-mGluR 4	Upstate (Buckinghamshire, UK)
Rabbit anti-mGluR 5	Upstate (Buckinghamshire, UK)
Rabbit anti-mGluR 7	Upstate (Buckinghamshire, UK)
Rabbit anti-mGluR 8	Upstate (Buckinghamshire, UK)
SDS polyacrylamide gels (10%)	Bio-Rad (Hertfordshire, UK)
Sheep anti mouse (TRITC)	Sigma (Dorset, UK)
Sheep anti mouse (FITC)	Sigma (Dorset, UK)
Soybean trypsin inhibitor	Sigma (Dorset, UK)
Taq DNA polymerase	Invitrogen (Paisley, UK)
TBE buffer (10x)	Invitrogen (Paisley, UK)
Tissue culture plasticware	Scientific Laboratory Supplies (Nottingham, UK)

TNFα Quantikine ELISA kits	R&D systems (Oxford, UK)
Trypsin	Sigma (Dorset, UK)
Trizol reagent	Invitrogen (Paisley, UK)
Tween 20	Sigma (Dorset, UK)
U0126	Promega (Southampton, UK)
Wistar rats (bred and reared in house)	Charles River UK (Kent, UK)

2.2 Methods

2.2.1 Isolation and culture of primary microglia

Microglial cells were isolated from 3-14 day old Wistar rat pups. Animals were killed by cervical dislocation and decapitation in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. 4-6 Brains were transferred into 40 mls of ice cold phosphate-buffered saline (PBS, 140mM NaCl, 5mM KCl, 25mM Na_2HPO_4, 2.9mM $Na_2HPO_4.2H_2O$, 11mM glucose, 0.2% BSA, pH 7.4). The brains were homogenised with a glass hand held homogeniser and teflon pestle (Thomas B841 homogeniser with number S 947 teflon pestle, USA) until smooth, and centrifuged at 500g for 10 minutes at RT (Eppendorf 5804R centrifuge using rotor A-4-44 with swing out buckets). The pellet was then resuspended in 70% isotonic Percoll diluted with PBS, this was overlaid with 35% isotonic Percoll and finally with PBS. This gradient was centrifuged at 1250g for 45 minutes at RT. After removal of myelin, cells at the 35/70% interface (Figure 2.1) were collected and washed in PBS by centrifugation at 500g for 10 minutes at RT.

The pellet was then resuspended in minimum essential medium (MEM) supplemented with Earle's salts (25mM KCl, 30mM glucose, 25mM $NaHCO_3$, 1mM glutamine,10% heat inactivated foetal calf serum, 50U/ml penicillin, 50µg/ml streptomycin, 3.3µg/ml ampicillin) and cell number calculated using a haemocytometer. Trypan blue (0.001%) was used to ensure viability of microglial cells. Cells were plated on 13mm glass coverslips at 9×10^4 cells per coverslip. Forty minutes after plating, the cells were washed in medium to remove debris, adhered cells were left in fresh medium. Cultures were maintained at 37°C in 5% CO_2. Cells were routinely used at 1 day *in vitro* (*DIV*). After 1 *DIV,* microglia exhibited a resting, ramified morphology with only a small number of cells staining for ED-1 (see Immunolocalisation 2.2.12), an antibody

that recognises a lysosomal protein (Graeber et al., 1990; Slepko and Levi, 1996). ED-1 binds with an antigen expressed on the membranes of cytoplasmic granules, like phagolysosomes, as well as on the cell surface, and expression correlates with phagocytic activity of the respective cell type (Damoiseaux et al., 1994), therefore high levels of ED-1 staining are associated with macrophages or activated microglia while low ED-1 expression is related to resting, ramified microglia (Graeber et al., 1990; Slepko and Levi, 1996).

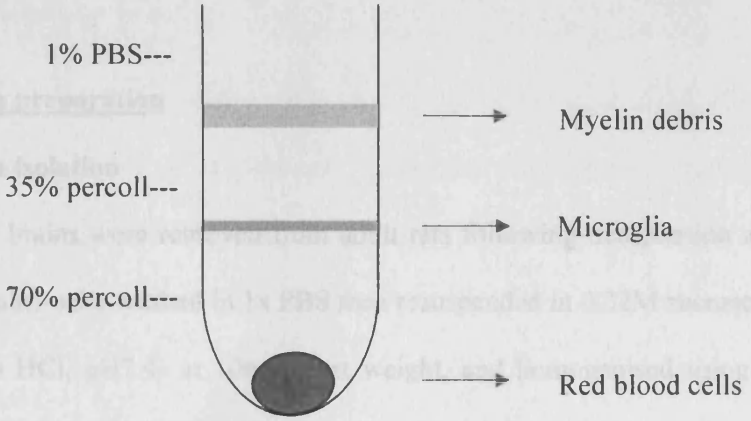


Figure 2.1 Schematic diagram of a percoll gradient
Following centrifugation, red blood cells are pelleted at the bottom of the tube while microglia collect at the 70/35% percoll interface. Myelin is collected and removed from the 35% percoll-PBS interface

2.2.2 Growth and maintenance of N9 microglial cell line

The N9 cell line was kindly provided by Dr. Paola Ricciardi-Castagnoli and initially derived from embryonic day 13 mouse microglial cultures (Corradin et al., 1993). Responses from these cells were similar to those from primary cultures of rat microglia (Kingham and Pocock, 2000). Stocks of N9 cells were preserved in N9 freezing medium (10% DMSO, 90% FCS) and stored at -80°C. N9 cells were cultured in DMEM supplemented with 4.4mM $NaHCO_3$, 50μM β-mercaptoethanol, 5% foetal

calf serum, 50U/ml penicillin, 50μg/ml streptomycin, 3.3μg/ml ampicillin, 50mg/ml kanamycin and 50mg/ml gentamycin, at 37°C in a humidified atmosphere of 5% carbon dioxide in air and passaged regularly with a sterile cell scraper. N9s were plated at a density of 4 x 10^4 cells per 13mm coverslip or at 2.5 x 10^5 cells per lysate dish and routinely used after 1 *DIV*. N9 cells were found to express different mGluR profiles to primary microglia and were therefore not used for toxicity, modulatory or phagocytosis experiments. Throughout this thesis these cells will be referred to as N9 cells to differentiate them from the primary microglia.

2.2.3 Myelin preparation

2.2.3a Myelin isolation

Whole brains were removed from adult rats following decapitation and placed on ice. The brains were washed in 1x PBS then resuspended in 0.32M sucrose (made up in 10mM Tris HCl, pH7.4) at 10ml/g wet weight, and homogenised using an Ultra-Turrax homogeniser (Janke and Kunkel Ika Werk) for 2 minutes. The resulting suspension was layered onto 0.8M sucrose in 10mM Tris-HCl and centrifuged at 20,000 rpm for 30 minutes at RT (in a Beckman J2-21 centrifuge with rotor JS-4.2, USA). The interfaces were then removed, pooled and made up to 240mls in 10mM Tris-HCl then centrifuged at 20,000 rpm for 10 minutes at RT. Pellets were retained and exposed to osmotic shock in dH_20 for 30 minutes at 4°C, after which samples were centrifuged and washed in 10mM Tris-HCl before repeating the whole process again. Finally the sample was resuspended in Tris-HCl for analysis of protein concentration (Lowry et al., 1951; Norton and Podulso, 1973).

2.2.3b Myeln labelling

Myelin was labelled using DiI fluorescent labelling dye from Invitrogen. Myelin was incubated with DiI fluorescent labelling dye at a concentration of 12.5µg per ml of myelin sample for 30 minutes at 37°C with constant end over end roatation. Labelled myeln was then washed three times in sterile PBS to remove any unbound dye.

2.2.4 Measurement of protein in myelin samples

The total protein concentration of myelin samples was determined by the Folin phenol method (Lowry et al., 1951), which is based on a two-step colour reaction. In an alkaline solution copper associates with protein and subsequently reduces the Folin reagent to form a blue coloured product. To maximise the colour resulting from the reduction, 0.4M sodium hydroxide is present to neutralise any acid released on addition of the Folin's solution and sodium carbonate is added to buffer the reagents to an optimal pH of 10. The optical density, read at 750nm, is proportional to the copper bound protein.

For protein analysis, an aliquot of each sample was thawed on ice and diluted to 1 in 30 using 0.05M NaOH. Standards of bovine serum albumin (BSA) fraction V were prepared (0, 5, 10, 15, 20 and 30 µg/ml) in a volume of 150µl. Samples and standards were assayed in duplicate. 50µl of each diluted sample was placed in a 10ml conical tube (Fisher Scientific, Loughborough, UK) with 100µl distilled water. 50µl 0.4M NaOH was added to each tube (standards and samples), followed by 1ml of freshly prepared solution X (2% Na_2CO_3, 0.02% K_2 Tartrate in 0.1M NaOH added to 1% $CuSO_4.5H_2O$ in a ratio of 99:1). All tubes were vortexed, and left for 15min. 100µl of Folin and Ciocalteu's reagent (diluted 1:1 with water; VWR) was added to each tube. After vortex mixing the tubes, they were incubated in the dark for a further 30 min. The absorbance of each sample was read on an Ultraspec 2000 spectrophotometer

(Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), with water as a reference reading repeated in between samples. The generation of a linear standard curve allowed conversion of optical density to μg of protein per sample, any samples with concentrations above the level of the standard curve were re-assayed with a 1:50 dilution.

2.2.5 Treatment of microglial cultures

Microglia were routinely used one day after plating. Microglial cells were exposed to Lipopolysaccharide (LPS) or myelin at defined concentrations for stipulated times. (For concentrations see Table 2.1). In experiments modulating mGluRs, microglia were pre-treated with the appropriate mGluR agonist or antagonist for 30 minutes at 37°C before incubation with an activator. For inhibition of iNOS, microglia were incubated with the selective iNOS inhibitors AMT-HCl (150nm) (Kingham et al., 1999) for 1 hour prior to addition of activators. In experiments modulating phosphorylation of ERK proteins, U0126 (5μM) (Favata et al., 1998) was applied to cultures for 30 minutes preceding microglial activation.

At specified times, conditioned medium was collected from the microglia (MGCM) and centrifuged at 500g (in a Jouan centrifuge, St Herblain, France), for 5 minutes to allow removal of debris or floating cells. The supernatants were then frozen in 1ml aliquots to minimise excessive freeze thaw cycles, and stored at -20°C for future measurement of particular substances (eg: TNFα, glutamate, nitrite) or stored in sterile eppendorfs for application to neurones and subsequent assessment of apoptosis. The cells were then fixed in 4% formaldehyde- phosphate-buffered saline (F-PBS, 4% formaldehyde, 140mM NaCl, 5mM KCl, 25mM Na_2HPO_4, 2.9mM $Na_2HPO_4.2H_2O$, 11mM glucose, 0.2% BSA, pH 7.4) and stored at 4°C for immunolocalisation. Fixed

cells were later probed for mGluR or ED-1 expression (Table 2.2). Microglia were also lysed for western blotting or mRNA was extracted for reverse transcription.

	Agonist/ compound	Concentration	Antagonist	Concentration
Group I	ADA	250μM	AIDA	250 μM
Group II	DCGIV	500nM	MCCG	500μM
Group III	AP4	100μM	MAP4	500μM
Activators	LPS	1μg/ml		
	Myelin	0.001- 10μg/ml		
iNOS inhibitor	AMT-HCL	150nM		
MEK inhibitor	U0126	1μM- 5μM		
Rho kinase inhibitor	Y-27632	100nM		
Src kinase inhibitor	PP2	50-100nM		

Table 2.1 Concentrations of mGluR agonists, antagonists and activators used
These compounds were added at given concentrations to microglia or CGCs *in vitro*. Concentrations were selected based on previous studies.

2.2.6 Assessment of microglial phagocytic activity

Microglia were incubated at 37°C with 20μg/ml fluorescently labelled dextran beads or 2 μg/ml fluorescently labelled myelin for 2, 4 or 24 hours. Subsets of cells were pre-treated with mGluR agonists or antagonists, U0126 or PP2 for 30 minutes prior to incubation with either dextran beads or fluorescent myelin (Table 2.1). Microglia that were exposed to the rho kinase inhibitor Y-27632 were incubated with this compound for 24 hours before exposure to labelled myelin as described above (Table 2.1). To ensure that the observed fluorescence was due to phagocytosis and not binding, subsets of microglia were pre-treated with 154mM sodium azide in sterile PBS for 5 minutes at 37°C before exposure to labelled myelin (Nesti et al., 2004), or incubated with labelled myelin at 4°C. Coverslips were then removed from the wells,

washed in warm basic medium (120mM NaCl, 3.5mM KCl, 0.4mM KH_2PO_4, 20mM N-Tris, 5mM $NaHCO_3$, 1.2mM $NaSO_4$, 5mM glucose) to remove any free dextran beads or fluorescent myelin from the cells, and immediately observed in a fluorescence microscope (Zeiss, Oberkochen Germany). To ascertain that un-phagocytosed myelin was removed from the microglia, a subset of cells were incubated with cold stripping wash (0.5M sodium chloride, 0.2M acetic acid in sterile PBS) for 2 minutes which strips substances bound to cells (Nesti et al., 2004). Fluorescence from 40kDa beads was captured with a FITC filter set and manual exposure of 20000ms. Fluorescence from 10kDa beads was captured with a rhodamine filter set and manual exposure of 15000ms. The fluorescence from cells incubated with labelled myelin was measured with a rhodamine filter set and manual exposure of 2000ms. A phase contrast image of the same field was also acquired for quantification of phagocytosing cells.

2.2.7 Preparation of cerebellar granule neurones

Cerebellar granule neurones (CGCs) were prepared from 3-6 day old Wistar rats (Evans and Pocock, 1999). Animals were killed by cervical dislocation and decapitation in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom). Cerebella were removed into chilled Solution B (100mM phosphate buffered salts Dulbecco's formula (PBS), 10mM glucose, 0.3% fatty acid free BSA, 0.38% $MgSO_4.7H_2O$) and mechanically dissociated with a sterile razor blade then incubated in 0.25% trypsin for 20 minutes at 37°C with regular agitation. Solution W (16% solution C made up in solution B, 20mls) was then added to the cell suspension and cells were pelleted by centrifugation at 500g for 5 minutes and resuspended in solution C (100mM PBS, 0.3% fatty acid free BSA, 10mM glucose, 0.38% $MgSO_4.7H_2O$, 100U/ml DNAse, 50μg/ml soybean trypsin inhibitor). This solution was pipetted on top of EBSS/BSA (Earle's balanced salt solution with 26mM $NaHCO_3$, 3mM $MgSO_4.7H_2O$, 4% fatty acid

free albumin; 5mls) and centrifuged for 10 minutes at 500g. After removal of EBSS solution from the pellet, cells were resuspended in culture medium and plated at a density of 6 x 10^5 on 13mm coverslips. Cultures were maintained at 37°C in 5% CO_2 in MEM (with Earle's salts supplemented with 25mM KCl, 30mM glucose, 25mM $NaHCO_3$, 1mM glutamine, 10% foetal calf serum, 50U/ml penicillin, and 50μg/ml streptomycin, 3.3 μg/ml ampicillin). This medium was the same as that used to culture microglia thus allowing us to add conditioned medium from the microglia to the neurones.

Coverslips were prepared beforehand. This involved soaking in ethanol for 7 days with regular agitation before baking at 180°C for a further 7 days. Coverslips were then soaked in Poly-D-lysine (15mg/l) for 1 hour at 37°C before use. CGCs were incubated for 1 *DIV* at 37°C in a humidified atmosphere of 5% CO_2 in air before addition of medium supplemented with arabinofuranoside (ARA-C: 10μM) to prevent glial cell proliferation. After a further 2 *DIV* 100μl sterile dH_2O was added to each well. CGCs were routinely used 7-9 days after plating. Cultures contained few contaminating cells indicated by positive immunoreactivity to the microglial marker OX-42 (Kingham and Pocock 1999) that recognises the CR3 complement receptor (CD11b/CD18) (Graeber et al 1989).

2.2.8 Treatment of cerebellar granule neurones

CGCs were routinely used after 7 *DIV*. At this time, half of the CGC medium was removed leaving 200μl in the wells. This was replaced with microglial conditioned medium which was added to each well for a 50:50 dilution (200μl) and incubated for 24 hours at 37°C for assessment of subsequent apoptosis and death.

2.2.9 Assessment of cell viability

2.2.9a Assessment of apoptosis using Hoechst 33342 staining

Apoptotic cells were counted using the fluorescent dye 2'[epoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'bi-1H-benzimidazol (Hoechst 33342) (Rivera et al., 1998; Kingham et al., 1999) which binds to the minor groove of DNA with preference for AT sequences (Portugal and Waring, 1988). Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (F-PBS) (4% formaldehyde, 140mM NaCl, 5mM KCl, 25mM Na2HPO$_4$, 2.9mM Na2HPO$_4$.2H$_2$O, 11mM glucose, 0.2% BSA, pH 7.4) and incubated with Hoechst 33342 (17.8µM) for 10 minutes in the dark. Cells were viewed at excitation 365nm with emission over 490nm recorded using a fluorescence microscope (Zeiss, Oberkochen Germany). Apoptotic cells presented with condensed pyknotic nuclei which were more intensely stained than healthy living cells that exhibit much weaker staining (Kingham and Pocock. 1999) Apoptotic cells were counted as a percentage of the total number of cells per field.

2.2.9b Assessment of caspase expression and cell death

The expression of active caspase-3 was quantified in live cells using CaspaTag Caspase3 (DEVD) activity kit. Caspase 3 belongs to the subset of caspases known as effector caspases that cleave selected substrates thus leading to the typical changes associated with apoptosis, and was therefore used as a marker for cells undergoing apoptosis. All cells were counterstained with propidium iodide (PI) which only permeates cells with disrupted plasma membranes where it intercalates between the bases of DNA, and is therefore a marker for cell death.

After removal of 200µl of CM, 3µl of CaspaTag solution was added with the PI (5µg/ml) and incubated for 30 minutes at 37°C. Coverslips were then removed from the wells, washed in warm basic medium (120mM NaCl, 3.5mM KCl, 0.4mM KH$_2$PO$_4$,

20mM N-Tris, 5mM $NaHCO_3$, 1.2mM $NaSO_4$, 5mM glucose) and immediately observed in a fluorescence microscope. CaspaTag fluorescence was captured with a FITC filter set and manual exposure of 20000ms. PI fluorescence was captured with a rhodamine filter set and a manual exposure of 500ms. A phase contrast image of the same field was also acquired for quantification of viable compared with dead cells as a percentage of total cells per field.

2.2.10 Enzyme-linked immunosorbant assays

The enzyme-linked Immunosorbant assay (ELISA) is a method which allows the amount of a given antigen in a sample to be quantified. This method employs a quantitative sandwich enzyme immunoassay technique. The wells of a 96 well plate are coated with a monoclonal antibody specific for a given protein, any of this protein present in the samples will bind to the immobilised antibody. A second polyclonal antibody linked with a chromogenic enzyme is then incubated prior to addition of a substrate solution which causes an enzyme reaction producing colour in the wells. The intensity of the colour measured is proportional to the amount of protein bound in the initial step.

For the quantitative determination of rat Tumor Necrosis Factor alpha (TNFα) concentration in cell culture supernatants, Quantikine M Rat TNFα Immunoassay kits (R&D systems, Oxford UK), were used according to the manufacturer's instructions. The plates are pre-coated with a specific monoclonal antibody for TNFα. After pipetting 50μl of assay diluent to each well, 50μl of the supernatant collected from treated or untreated microglia, or 50μl of standard were added in duplicate and incubated at room temperature for 2 hours. Following 5 washes with the wash buffer provided, rat TNFα conjugate was added to the wells and incubated at room temperature for 2 hours. After a final 5 washes, 100μl of substrate solution was added to the wells and incubated for 30

minutes at room temperature in the dark. Stop solution (1M hydrochloric acid) was then added to the wells in the same order, the optical density of each well was determined within 30 minutes using a microplate reader set to 450nm with a reference filter of 540nm. TNFα concentrations in cell supernatants were determined using the standard curve from each plate (see Figure 2.2)

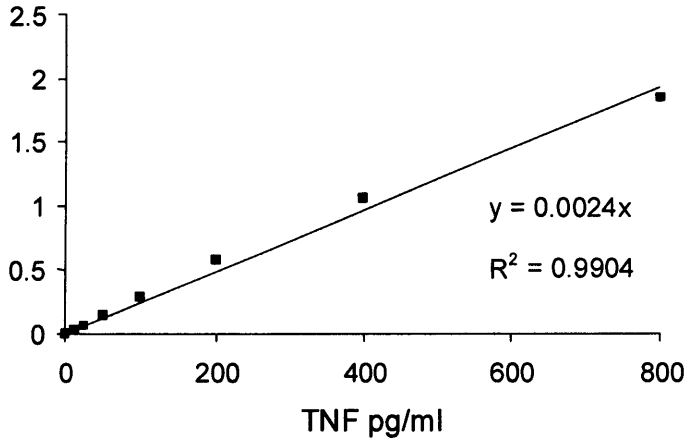


Figure 2.2 Representative graph of a TNFα standard curve

The optical density of the colour reaction from known concentrations of TNFα were measured to construct a standard curve by which other samples could be measured.

2.2.11 Glutamate measurement

The presence of glutamate in MGCM was analysed by fluometric quantification of NADPH, which is produced though the reaction of glutamate with $NADP^+$ in the presence of glutamate dehydrogenase (Nicholls and Sihra, 1986). This reaction yields one mol of NADPH for every mol of glutamate meaning that NADPH levels measured are directly proportional to glutamate levels in the supernatant.

MGCM (100µl) was diluted in basic medium (120mM NaCl, 3.5mM KCl, 0.4mM KH_2PO_4, 20mM N-Tris, 5mM $NaHCO_3$, 1.2mM $NaSO_4$, 5mM glucose; 900µl) then incubated with $NADP^+$ (1mM) and glutamate dehydrogenase (32.4U/ml) for 8

minutes at 37°C in the dark. NADPH levels were then measured using a Perkin Elmer fluorimeter with excitation set at 340nm and emission greater than 400nm was collected. The concentration of glutamate was determined against a standard curve (Figure 2.3). Glutamate experiments were performed in duplicate on samples collected from three separate experiments.

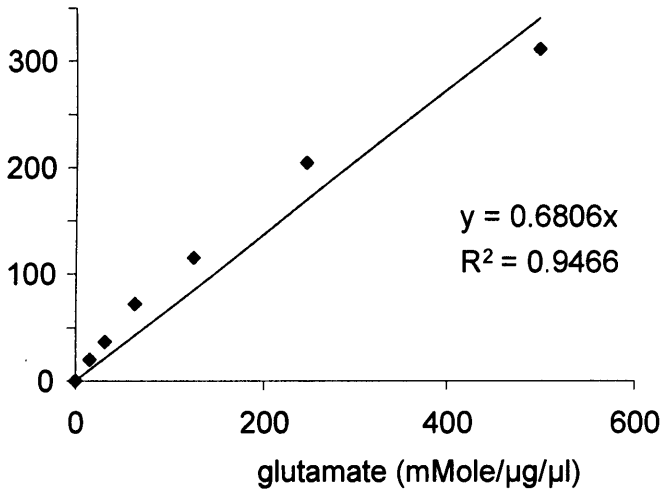


Figure 2.3 Representative graph of a glutamate standard curve
The optical density of known glutamate samples were measured to construct a standard curve by which other samples could be measured

2.2.12 Immunolocalisation

2.2.12a Fluorescent staining of primary cells

This method localises the expression of labelled proteins throughout the cell thus allowing the sub-cellular expression of a given protein to be analysed under different stimuli. Double immuncytochemical staining was performed using immunofluorescence. See table 2.2 for a list of proteins probed for and respective blocking and antibody conditions used.

Microglia were activated after 1 *DIV* then fixed in 4% F-PBS and stored at 4°C until ready for staining. Cells were permeabilised in methanol at -20°C for 10 minutes. After washing in PBS, cells were blocked for 30-60 minutes in appropriate blocking serum (eg: 4% Normal goat serum in 1x PBS) followed by addition of primary antibody which was incubated with the cells overnight at 4°C (Table 2.2). A negative control in which primary antibody was omitted for each condition was left in blocking serum overnight to test the specifity of staining. Cells were washed in three changes of PBS then incubated with the appropriate secondary antibody for one hour at room temperature (Table 2.2). All incubations were performed in a humidified chamber to prevent excessive evaporation. After a final three washes the nuclear stain 4'-6-Diamidino-2-phenylindole (DAPI) was applied for 90 seconds after which cells were washed in distilled water and mounted with DPX mountant.

2.2.12b Fluorescent staining of fixed sections.

Spinal cord sections were kindly supplied by Prof. Reynolds' lab. Spinal cords were removed from rats at the acute initial disease episode (22-27 days post MOG injection) and the long-term chronic phase of disease (>100 days post MOG injection). Tissue was perfusion and post-fixed in 4% parafomaldehyde. Cords were then cut in transverse sections of 6-8 microns. Lesions in tissue were identified by the clusters of MHC-II labelled cells which are present in inflammatory plaques

Sections were blocked with 5% normal goat serum for 1 hour at RT. A mixture of two antibodies (rabbit anti- mGluR, 1:50; and an antibody raised against MHC class II antigens, mouse anti- OX6 directly conjugated to FITC, 1:50) in PBS were added to the sections and incubated for 1 hour at RT. After 3x 5 minute washes in PBS, sections were incubated with a fluorescent secondary antibody (goat anti-rabbit (TRITC conjugated), 1:50) to detect cells labelled for mGluRs, for 1 hour at RT. Following

another washing stage, sections were incubated for 90 seconds with DAPI and washed once more in PBS before being mounted in Citifluor® antifade reagent. Sections were examined under a fluorescent microscope (Zeiss, Oberkohen Germany).

Primary Antibody	Cell specificity	Dilution and incubation time	Source and reference	Secondary antibody, dilution and incubation time
Immunolocalisation				
Rabbit α mGluR	C terminus of metabotropic glutamate receptor	1:100; overnight 4°C	Upstate Taylor et al., 2002, 2003)	Sigma Goat-anti-rabbit TRITC conjugated; 1:50; 1h RT
Mouse α ED-1	lysosomal protein on macrophages and activated microglia	1:100; overnight 4°C	Sigma Graeber et al., 1990; Slepko and Levi. 1996	Sigma Sheep-anti-mouse TRITC conjugated; 1:100; 1h RT
Mouse α cadherin	Cadherin, calcium dependent cell adhesion molecules, predominantly membranous signal	1:50; overnight 4°C	Abcam; Grunwald et al., 1993	Sigma Sheep-anti-mouse FITC conjugated; 1:100; 1h RT
Mouse α MOG	Raised against rat myelin oligodendrocyte protein (MOG)	1:100 Overnight 4°C	Prof. David Baker	Sigma Sheep anti mouse TRITC conjugated; 1:100 1h RT
Western Blotting				
Mouse α β-actin	β-actin, cytoskeletal protein implicated in cell motility	1:500; 2h RT	Sigma (Dorset, UK)	Sigma Rabbit-anti-mouse HRP conjugated; 1:500; 2h RT
Mouse α PERK	Phosphorylated-44-42 MAPK	1:750; 2h RT	Autogen Bioclear (Wiltshire, UK)	Autogen Rabbit-anti-mouse HRP conjugated; 1:1000; 2h RT
Rabbit α iNOS	Inducible nitric oxide synthase	1:5000; 2 h RT	BD biosciences (Oxford, UK)	Sigma Goat-anti-rabbit HRP conjugated; 1:500; 1h RT

Table 2.2 Antibody concentrations used for Immunolocalisation and western blotting experiments.

2.2.13 Western Blotting

Western blotting is a technique for quantifying specific protein levels in a given sample. This technique involves running the protein in a sample through a gel under high electrical current to separate proteins by molecular weight, smaller proteins will travel further than large proteins in a given time. These are then transferred on to a membrane and probed with antibodies raised against the protein of interest.

2.2.13a Preparation of cell lysates

Following treatment, microglial cells were scraped into 10µl ice cold RIPA buffer (1% NP-40 (Ipegal), 0.5% sodium deoxycholate, 0.1% SDS, 1mM benzamadine, 4µg/ml leupeptin – in 50mM Tris (pH 7.5)). This was pooled from 3 coverslips to give approximately 30µl of sample. Lysates were incubated on ice for approximately 1 hour before being centrifuged (in a Jouan centrifuge, France), at 13500g for 5 minutes this allowed removal of un-lysed protein and debris.

2.2.13b Bradford Protein Assay

The protein concentration of the cell lysates was calculated by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as standard (Figure 2.4). Samples (1µl each) to be tested were pipetted into glass tubes in duplicate then mixed with Bradford reagent (1ml). After 20 minutes the optical density was measured at 595nm against a Bradford reagent blank. The protein concentration in 1µl of cell lysate was determined from the standard curve and the amount of sample required to give appropriate protein concentration (45-60ug/ml) was calculated.

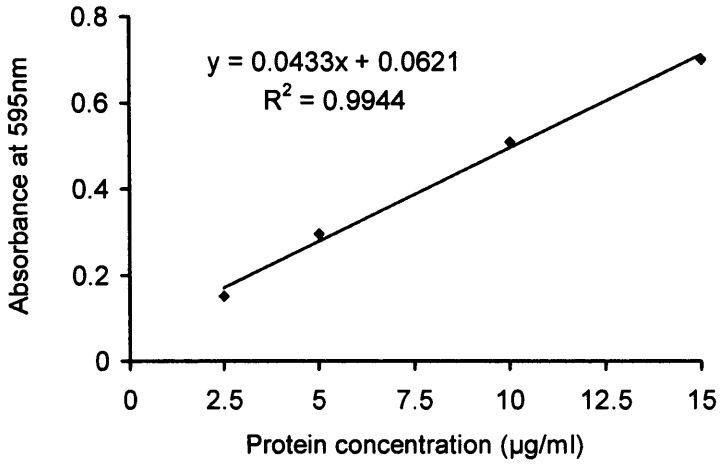


Figure 2.4 Representative standard curve for protein assay

The optical density (measured at 595nm), of BSA samples of 0-15µg/ml were measured to construct a standard curve by which other samples could be measured

2.2.13c SDS-Polyacrylamide gel electrophoresis

Protein in the cell lysates was separated by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) in BioRad mini protean II electrophoresis cells (BioRad, UK). For western blot analysis of iNOS, phospho ERK or actin expression, samples were resolved on a 10% sodium dodecyl sulphate (SDS)- polyacrylamide gel and transferred to Immobilon P polyvinylidene difluoride (PVDF) membranes.

Prior to electrophoresis, samples were boiled for 5 minutes in 10µl laemelli sample buffer (1% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 125mM Tris/HCl, 1% bromophenol blue, pH 6.8; 30-40µg/well). BioRad SDS-PAGE pre-cast Tris-HCl gels (10%) were prepared and washed in running buffer (125 mM Tris, pH 8.3, 1M Glycine, 0.01% SDS) then placed in the running stand ensuring a good seal was formed to prevent leaking of the inner chamber. Molecular weight markers were added to one lane of each gel followed by remaining samples in the other lanes. The remaining running buffer was then poured first into the inner chamber without disturbing the loaded samples, then the outer chamber until the tank was filled. The gels were

electrophoressed at 180V for approximately 40 minutes or until the samples had reached the bottom of the gel.

2.2.13d Electrophoretic Protein transfer

Transfer buffer (25mM Tris, 192mM Glycine, 20% methanol, 0.01% SDS) was prepared while the gel was running then put in the freezer to cool. The glass plates holding the gel were separated and the gel was cut in the bottom left hand corner on the side of the molecular weight markers. The gel was equilibrated in transfer buffer for 10 minutes as were 2 pieces of 3MM paper cut to the size of the gel. Meanwhile, the PVDF (Immobilon P) membrane was activated in chilled methanol for 1 minute then equilibrated in cold transfer buffer for a further 5 minutes. The gel was transferred to a cassette by removing the transfer buffer, placing a 3MM sheet on top and inversing the tray. The membrane was laid on the gel without bubbles followed by the remaining 3MM sheet. The loaded cassette was then slotted between the blank cassette and the ice pack in the transfer tank which was then filled with the remaining transfer buffer and a stir bar. The gel was transferred at 80V for 2 hours or 22V for 16 hours.

2.2.13e Immunoblotting

The membrane was removed from the transfer tank and washed for 10 minutes in Tween-20 Tris buffered saline (TTBS; 10mM Tris/HCl, 150mM NaCl, 0.5% Tween-20, pH 7.4) before being immersed in blocking buffer (TTBS, 5% non-fat dried milk) for up to 2 hours at room temperature to minimise non-specific binding. The appropriate primary antibody was then diluted in blocking buffer and incubated with the membrane for 1-2 hours. After washing with three changes of T-TBS, the membrane was incubated with the corresponding secondary antibody conjugated to HRP for a further 1-2 hours at room temperature (Table 2.2). Following a final three washes with T-TBS, the proteins

of interest were detected by enhanced chemiluminescence. To ascertain equal protein loading, membranes were re-probed for mouse anti- β-actin. western blots were performed in triplicate therefore blots shown are representative of a single experiment. Gel-Pro software (version 3.1) was used to determine optical density and subsequent quantification of protein. iNOS and phospho ERK expression were therefore calculated in relation to β-actin.

2.2.14 Reverse transcriptase polymerase chain reaction (RT-PCR)

Control of gene transcription whereby a gene's DNA sequence is used as a template for DNA synthesis, is critical in the regulation of gene expression. Gene transcription levels are altered in response to many signals that occur during normal physiological function but also in response to other factors including disease. This leads to changes in levels of individual mRNAs. RT-PCR is used to analyse gene transcripts, first mRNA is extracted from cells then used as a template for reverse transcription to complimentary DNA (cDNA). Specially designed primers are then used to amplify a selected cDNA region using the polymerase chain reaction (PCR).

2.2.14a mRNA extraction

Microglia destined for mRNA extraction and PCR were plated in lysate dishes at 450,000 cells per lysates and used after 1 *DIV*. Microglia were washed once in medium to remove dead cells and remaining debris, then lysed in 1ml Trizol reagent per condition (2-6 wells) for 10 minutes at room temperature and transferred to sterile 1.5ml eppendorfs. Neat chloroform (200μl per sample) was then added and samples mixed thoroughly then allowed to settle before being centrifuged at 1500g for 10 minutes at 4°C (eppendorf S415R, with rotor F45-24-11). Extracted mRNA remained in the upper aqueous phase which was collected by a sterile glass pipette into a fresh tube, care was

taken not to aspirate any of the white interfase, then precipitated with isopropyl alcohol (500µl per sample) for 10 minutes at room temperature. mRNA was pelleted by centrifugation at 1200g at 4°C for 10 minutes then washed once in 75% ethanol. After removal of supernatant any remaining ethanol was left to evaporate before re-suspending the pellet in 40µl DEPC-treated water and heating at 60°C for 10 minutes to aid dissolving. The mRNA suspension was then frozen and thawed before removing 3µl into 400µl sterile water for measurement of total mRNA quantified by optical density readings at 260 nm. Purity was estimated by the 260/280nm ratio and only samples with an mRNA ratio over 1.8 were used.

.

2.2.14b Reverse Transcription

The cDNA template for PCR is synthesised from mRNA by reverse transcription. Moloney Murine Leukaemia virus (M-MLV) reverse transcriptase is an mRNA-dependant DNA polymerase which uses single stranded mRNA in the presence of Oligo d(T) primer to catalyse the first strand of cDNA.

mRNA (3µg) was added to sterile tubes with 40U RNase inhibitor to prevent degradation of mRNA and made up with sterile water to 13.5µl. Oligo d(T) primer (250µg) was added to mRNA solution and heated at 70°C for 10 minutes. After quenching on ice, samples were reverse transcribed into single-stranded cDNA in 14.5µl reaction mixture (5x RT buffer [50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$], 10mM 0.1M dithiothrietol (DTT), 40U RNase inhibitor, 0.5mM deoxyribonucleoside triphosphates (dNTPs), 600U MMLV) at 37°C for 80 minutes. Incubation at 70°C for 10 minutes terminated the reverse transcription reaction. Resultant single stranded cDNA was diluted with sterile water for a final concentration of 20ng/µl then boiled for 5 minutes to denature before freezing.

2.2.15c Polymerase chain reaction (PCR)

PCR is a technique for amplifying areas of DNA using complementary fragments of DNA flanking the region of interest (oligonucleotide primers). Primer design requires knowledge of the DNA sequence of a gene. Complementary sequences are chosen at sites on the DNA, which are most unique to reduce the chance of primers binding to other regions on the template DNA. A primer length of 17-28 bp is required to ensure uniqueness and is usually composed of 50-60% G + C content. The first stage in PCR involves separation of the DNA double helix into single strand segments using a high temperature, usually between 91 and 97°C for 1 min, following which primers anneal to the single stranded DNA at specific temperatures related to their basic structure. The melting temperature (T_m) of primers depends on their length and composition of bases. The annealing temperature (T_a) is directly related to the Tm (Lewin, 1994). These parameters are calculated by the following formulae:

$T_m = 4\ (G + C) + 2\ (A + T)\ °C$

$T_a = T_m - 4\ °C$

Most annealing temperatures are in the range of about 50-65 °C. A higher temperature means the reaction is more stringent, with less chance of primer mismatch, however if the temperature is too high, then less product will be made. When using a combination of primers, the annealing temperature should be optimised to ensure the formation of non-specific low molecular weight products are minimised. After the annealing stage, the temperature is then changed to allow activation of a heat-tolerant DNA polymerase, which extends the primer in a 5' to 3' direction using surplus free deoxyribonucleoside triphosphates (dNTPs). A third temperature change denatures the DNA strands and the cycle can repeat, allowing the number of cycles to determine the

degree of DNA amplification. The amplification is exponential up to a point then plateaus out, with DNA polymerase being the rate limiting factor. Taq DNA polymerase (from *Thermus aquaticus* bacteria) is heat stable and therefore ideal for this application. It has a half life of 30 min at 95 °C thus the number of amplification cycles shouldn't exceed 30 cycles without reducing the denaturation time. Taq polymerase requires the presence of free magnesium ions to function, with the magnesium acting as a cofactor. Lower concentrations of magnesium chloride ($MgCl_2$) increase the stringency of the reaction, thus $MgCl_2$ titrations should be performed with each new primer combination.

As mGluRs are expressed at low levels on microglia, titrations were performed for both $MgCl_2$ and optimum annealing temperatures (OATs) in order to optimise the protocol. Results showed that for mGluR1 (Figure 2.5) a concentration of 2.5mM $MgCl_2$ and an OAT of 65°C produced the clearest band. For mGluR5 (Figure 2.5) the optimal concentration of $MgCl_2$ was 2.5mM and annealing temperature was 65°C. Primers for group II mGluR subtypes 2 and 3 worked best with 2.5 and 2 mM $MgCl_2$ respectively and were both annealed at 61°C (Figure 2.5). The optimal annealing temperature for all group III mGluR primers was 56°C (Figure 2.6). The $MgCl_2$ concentrations that worked best for mGluR4 and 6 was 1.5mM while mGluR 7 and 8 showed clearer bands using a higher concentration of 2mM.

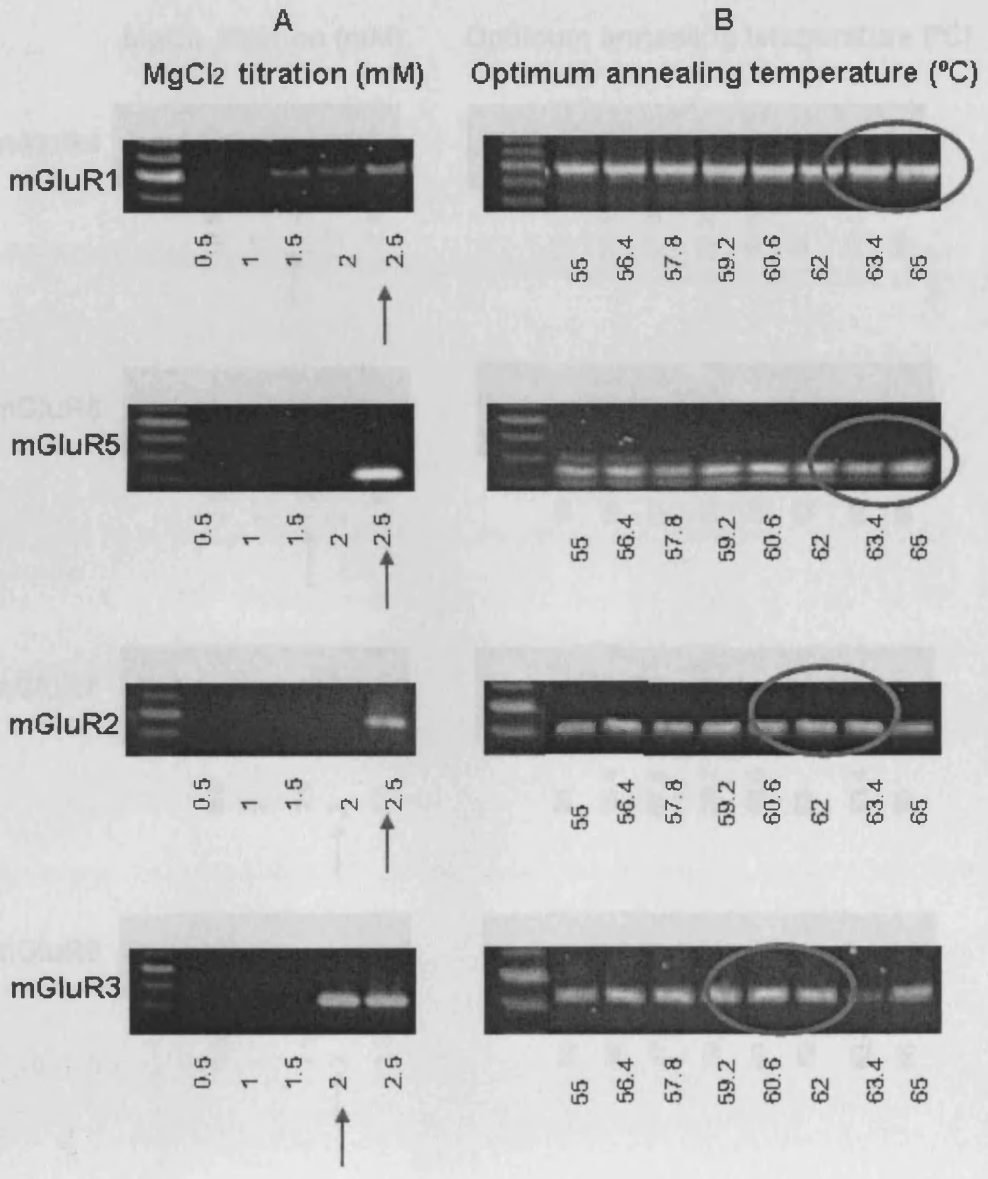


Figure 2.5 Optimisation of PCR protocol for group I and II metabotropic glutamate receptors.

(A) Various concentrations of $MgCl_2$ (from 0.5-2.5mM) were tested to investigate which were optimal for each set of primers. The arrows indicate $MgCl_2$ concentration used for each set of primers. **(B)** Primer pairs were also tested at different annealing temperatures (from 55-65°C). The Circled bands indicate the optimum annealing temperature used for each primer pair, these were chosen as at optimum temperatures the bands were notably clearer.

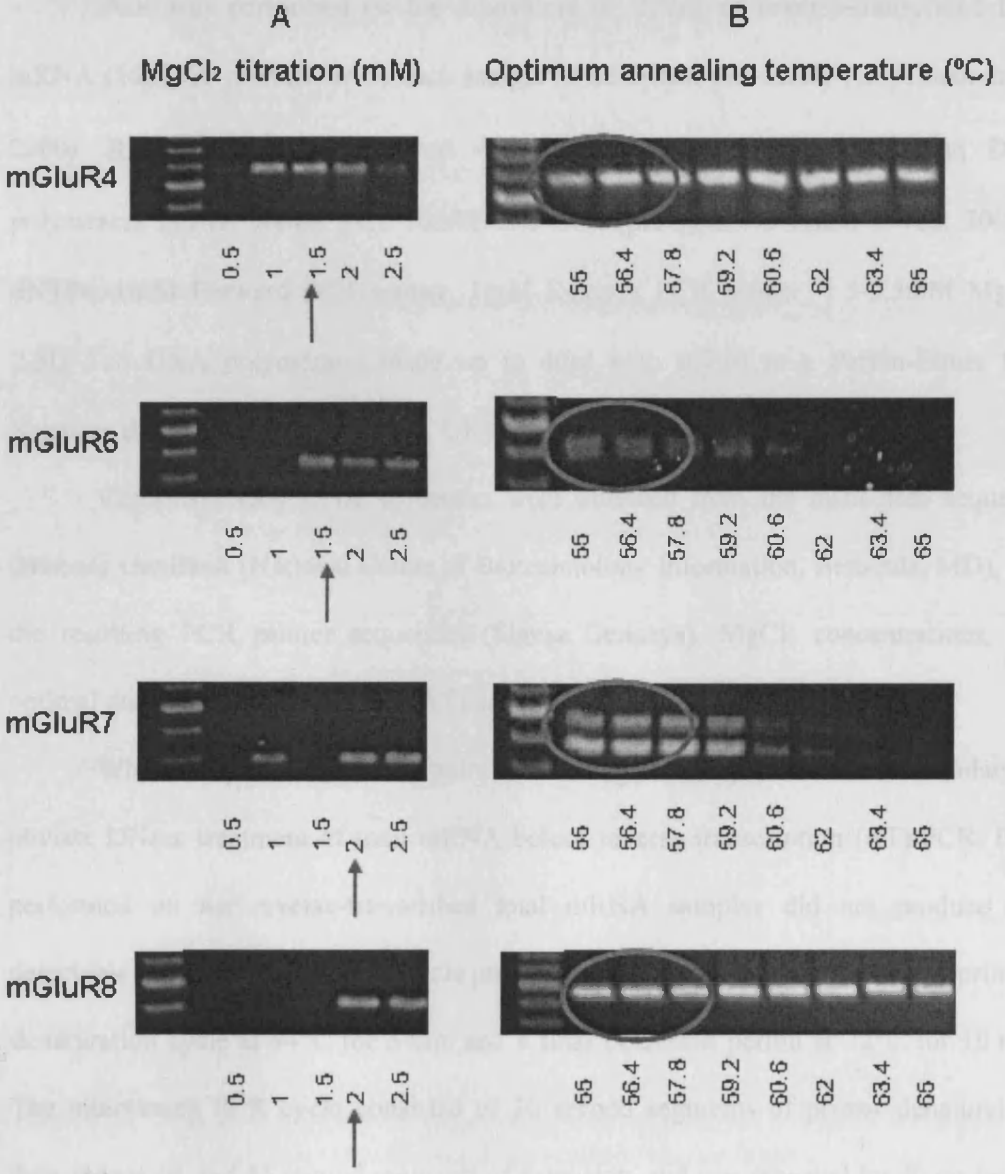


Figure 2.7 Optimisation of PCR protocol for group III metabotropic glutamate receptors.

(A) Various concentrations of $MgCl_2$ (from 0.5-2.5mM) were tested to investigate which were optimal for each set of primers. The arrows indicate $MgCl_2$ concentration used for each set of primers. **(B)** Primer pairs were also tested at different annealing temperatures (from 55-65°C). The Circled bands indicate the optimum annealing temperature used for each primer pair, these were chosen as at optimum temperatures the bands were notably clearer.

PCR was performed on the equivalent of 200ng of reverse-transcribed total mRNA (10μl RT product) from each sample as described previously (Copelman et al., 2000). RT product was incubated with 40μl reaction mixture (10x Taq DNA polymerase buffer: 50mM KCl, 10mM Tris-HCl, (pH 9), 0.1% Triton X-100; 200μM dNTPs, 1mM Forward PCR primer, 1mM Reverse PCR primer, 1.5-2.5mM $MgCl_2$, 2.5U Taq DNA polymerase; made up to 40μl with dH_20) in a Perkin-Elmer Life Sciences thermocycler (Warrington, UK).

Complementary DNA sequences were obtained from the nucleotide sequence database GenBank (National Centre of Biotechnology Information, Bethesda, MD), and the resulting PCR primer sequences (Sigma Genosys), $MgCl_2$ concentrations, and optimal annealing temperatures (OAT) are shown (Table 2.3).

Wherever possible, primer pairs were designed to span an intron boundary to obviate DNase treatment of total mRNA before reverse transcription (RT)-PCR. PCR performed on non-reverse-transcribed total mRNA samples did not produce any detectable products. The thermal cycle profile for each set of primers included a primary denaturation cycle at 94°C for 5 min and a final extension period at 72°C for 10 min. The intervening PCR cycle consisted of 30 second segments of primer denaturation, then annealing, and 45 second segments of extension, and was repeated for 40 cycles for each primer pair.

mGluR	Primer	$MgCl_2$ (mM)	OAT (°C)
mGluR1	Forward: 5'-TGGCTCCGAACAGAGCATCGAAT-3' Reverse: 5'-TTGACTATGTCGAGCATCGCCCTT -3'	2.5	64
mGluR5	Forward: 5'-GTCCTTCTGTTGATCCTGTC -3' Reverse: 5'-ATGCAGCATGGCCTCCACTC -3'	2.5	64
mGluR2	Forward: 5'-GCCCCCTTTCGCCCAGCAGATAC-3' Reverse: 5'-CAGGCAGGCGATGGTGACAGGT-3'.	2.5	62
mGluR3	Forward: 5'-GCTCCAACATCCGCAAGTCCTA-3' Reverse: 5'-TGTCCATGGCCAGGTGCTTGTC-3'	2	60
mGluR4	Forward: 5'-TGAGCTACGTGCTGCTGGCG-3' Reverse: 5'-TGTCGGCTGACTGTGAGGTG-3'	1.5	56
mGluR6	Forward: 5'-CAAGTAGCAAGGTTGAGTGT-3' Reverse: 5'-GGTTGTAGTGTTGGATCAAG-3'	1.5	56
mGluR7	Forward: 5'-GAACTCTGTGAAAATGTAGACC-3' Reverse: 5'-TTAGGGAGTCCAGAATTACAG-3'	2	56
mGluR8	Forward: 5'-GAGGGTTATAACTACCAGGT-3' Reverse: 5'-TAGGTGCTGTGACAGATTTCT-3'	2	56
GAPDH	Forward: 5'-TGGTGCCAAAAGGGTCATCATCTCC-3' Reverse: 5'-GCCAGCCCCAGCATCAAAGGTG-3'	2	60

Table 2.3 Forward and reverse primer sequences for each mGluR with optimal MgCl concentrations and annealing temperatures

2.2.14d 1% Agarose Gel Electrophoresis

Once amplified the PCR product can be analysed by size separation using ethidium bromide stained agarose gel electrophoresis. Ethidium bromide intercalates between the bases of double stranded DNA helices and these complexes fluoresce under ultraviolet light.

1% agarose was added to 1x Tris-borate-EDTA (TBE) buffer (10x TBE from Invitrogen diluted with dH_20) and heated on medium power in a microwave for 3

minutes. After cooling, ethidium bromide was added to the gel solution (0.5μg/ml) which was then poured and allowed to set. Gels were submerged in running buffer (1x TBE). Sucrose loading dye (6x stock: 30% w/v ultra pure sucrose and .35% orange dye) was added to equivalent amounts of PCR product (20μl) in order to weigh samples down in the wells. Samples were loaded by submerging pipette tip into the wells and slowly releasing the product avoiding air bubbles. Product size was verified by running the samples against a 100 bp DNA ladder (10μl). The gel was run at 110V for 2 hours then viewed under UV light and analysed using GelPro image analysis package (Media Cybernectics, Berkshire, UK).

2.2.15 Statistical analysis

Data was analysed with the GraphPad Prism computer package (GraphPad Software, San Diego California, USA). A normality and quality of variance test was carried out on all data to determine which test was appropriate. A t-test, for normally distributed data sets was used with significance level set at 95%. If variances of data sets were significantly different, then Welch's correction was applied to correct for this. All values are indicated as the mean ± standard error of the mean (SEM). P-values were taken as an indicator of statistical significance with the following nomenclature: * - $P < 0.05$, ** - $P < 0.01$ and *** - $P < 0.001$. Individual n-numbers are given in each results chapter.

3.0

EXPRESSION OF MICROGLIAL METABOTROPIC GLUTAMATE RECEPTORS

3.1 Introduction

Metabotropic glutamate receptors have important roles in both neuronal and glial functions including regulation of neurotransmitter release and neuronal excitation (Holscher et al., 1999; Cartmell and Schoepp, 2000). In the CNS, mGluRs are expressed in regional and cellular specific patterns which may alter their modulatory functions (Biber et al., 1999; Sahara et al., 2001; Defagot et al., 2002; Hubert et al., 2004). Microglia possess functional group I, II and III mGluRs. To date, microglia have been shown to express mGluR5 (Biber et al., 1999) mGluR2 and mGluR3 (Taylor et al., 2002) mGluR4, mGluR6 and mGluR8 (Taylor et al., 2002, 2003). Furthermore, stimulation of microglial group II mGluRs has been shown to induce a neurotoxic phenotype wheras group III microglial mGluRs can promote neuroprotection (Taylor et al., 2002, 2003, 2005)

Microglial cells play a vital role in maintaining glutamate homeostasis and are also a primary source of glutamate released during excitotoxic stress in pathological conditions. This suggests that excess glutamate released from either neurones or microglia themselves may stimulate microglial mGluRs culminating in a cycle of activation and neurotoxicity (Taylor et al., 2002). As such, control of microglial reactivity by modulation of microglial mGluRs may be a potential therapeutic strategy in neurodegenerative diseases where microglial activation is implicated.

Trafficking of mGluRs occurs between the nucleus and plasma membrane. This process has been associated with the functional activation states of the receptors, mGluRs localised around the nucleus and cytoplasm are thought to be pre-active while trafficking to the membrane has been associated with a functionally reactive state (Roche et al., 1999; Ciruela et al., 1999). It is possible that loss of microglial regulation

and subsequent reactivity and neurotoxicity during disease progression could be the result of increased expression or activation of one mGluR subtype over another.

To this end, primary and N9 microglial mGluR expression was examined in activated compared with untreated control cells by immunocytochemistry or RT-PCR, to determine whether activation induced changes in mGluR profile. Tissue taken form the animal model of MS, EAE, or from control animals was also analysed either by immunohistochemistry or RT-PCR to assess mGluR expression through disease progression.

3.2 Results

3.2.1 Microglia express group I metabotropic glutamate receptors

Immunocytochemical determination of mGluR1 expression on microglia using an antibody raised against mGluR1 (1:100, see table 2.2), showed that control untreated microglia expressed mGluR1 (Figure 3.1). In all immunocytochemistry experiments the specifity of the secondary antibody for the primary antibody was verified by a negative control in which primary antibody was omitted (Figure 3.17A). This control however, did not test the reliability of the primary antibody which could be achieved by peptide blocking.

Microglia exposed to myelin (1μg/μl) and LPS (1μg/μl) for 24 hours showed a similar level of mGluR1 expression to that seen in untreated cells. Microglial mGluR1 expression was localised around the nucleus with very little staining of processes which were positive for cadherin. RT-PCR of microglial mRNA (Figure 3.2A) showed that microglia express mGluR1. This was detected as a 473bp single stranded DNA product. Exposure to myelin increased microglial mGluR1 mRNA while LPS treated cells showed similar levels to control untreated microglia. The N9 microglial cell line was also analysed by RT-PCR for mGluR1 expression (Figure 3.2B). In contrast to primary microglia, this expression was not altered by exposure to either myelin or LPS.

Immunocytochemical detection of mGluR5 using an antibody raised against mGluR5 (1:100) showed that microglia constitutively expressed mGluR5 (Figure 3.3). Incubation of microglia with myelin and to a lesser extent LPS for 24 hours increased the expression of mGluR5. In contrast to mGluR1, mGluR5 expression was not present around the nucleus but was localised to the membrane and processes shown by co-staining with the cadherin antibody although this was more obvious in the myelin treated microglia than in the control or LPS treated cells. RT-PCR of microglial mRNA

(Figure 3.4A) showed that microglia express mGluR5 which was detected as a 210bp single stranded DNA product. Interestingly, microglia incubated with LPS expressed less mGluR5 mRNA than untreated cells and microglia treated with myelin showed no mGluR5 mRNA expression. This was unexpected so repeated using different primers which showed the same result. The N9 microglial cell line was also analysed by RT-PCR for mGluR5 expression (Figure 3.4B). Although N9 cells expressed mGluR5, in contrast to primary microglia this was not altered by exposure to either myelin or LPS. See table 3.1 for a summary of group I mGluR protein and mRNA expressed by microglia and N9 cells.

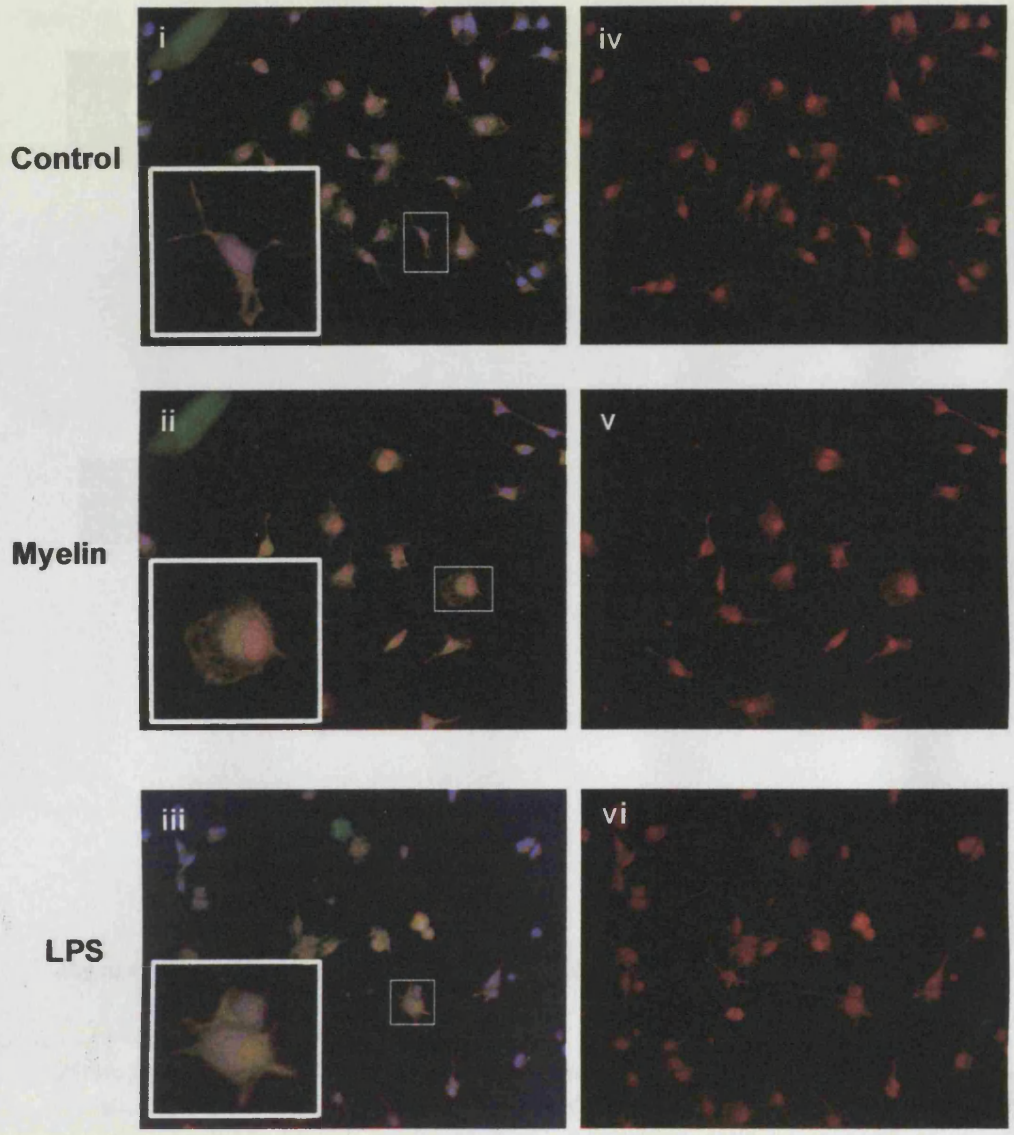


Figure 3.1 Expression and localisation of mGluR1 protein in untreated and activated primary microglia.

Microglia were either left untreated (i and iv) or incubated with myelin (1μg/ml) (ii and v) or LPS (1μg/ml) (iii and vi) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were fixed in 4% F-PBS. Microglia were co-stained with antibodies to mGluR1 and cadherin, a plasma membrane protein. Fluorescent secondary antibodies conjugated with TRITC and FITC were used to detect cells stained with mgluR and cadherin respectively. DAPI, a nuclear stain (blue), was also used toaid localisation of mGluR1 expression. Figures (i-iii), show representative images of microglia co-stained for mGluR1, cadherin and DAPI. Figures (iv-vi), show the same field but only the mGluR1 staining. These figures are representative images of staining repeated in three separate experiments and showed that microglia expressed mGluR1 protein which appeared more localised to the nucleus. Activation of cells with myelin or LPS did not obviously alter mGluR1 protein immunolocalisation. (x400

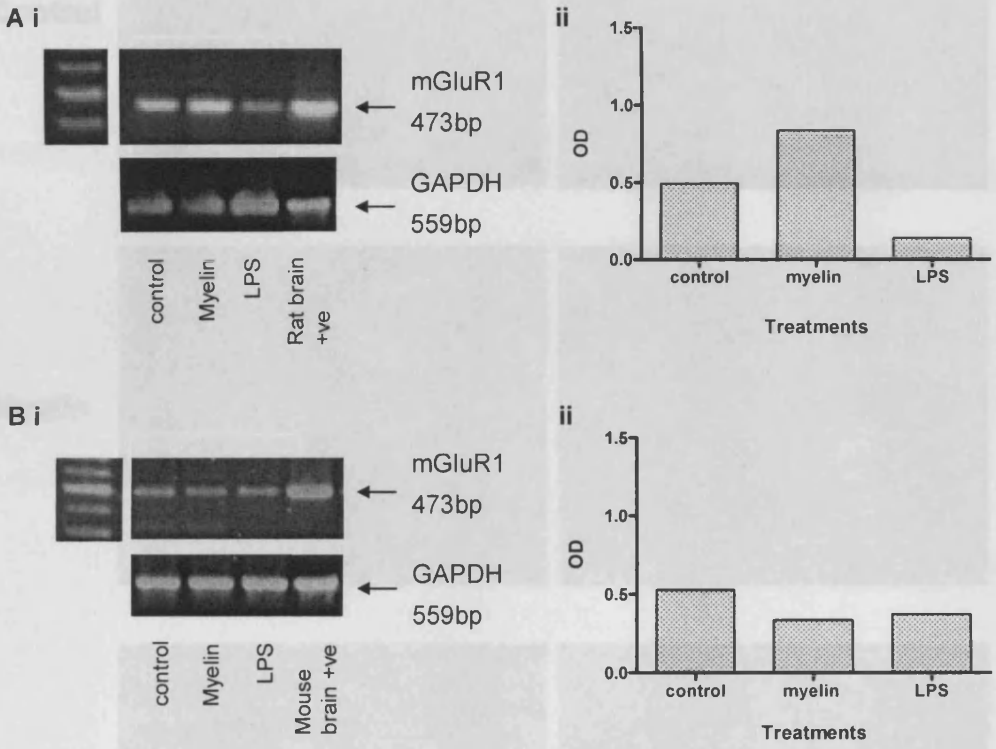


Figure 3.2 Expression of mGluR1 mRNA in primary microglia or N9 cell lines activated with myelin or LPS.

Microglia **(A)** or N9 cells **(B)** were analysed for mGluR1 mRNA expression (predicted 473bp). Cells were either left untreated or incubated with myelin (1μg/ml) or LPS (1μg/ml) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were lysed in trizol for mRNA extraction and RT-PCR. Equal amounts of each PCR product (200ng single stranded DNA) were size fractionated on a 1% agarose, ethidium bromide stained gel. Equivalent amounts of ssDNA taken from whole mouse or rat brains were also run on the same gels and used as a positive control (Mouse/Rat brain +ve). A 100bp DNA ladder marker was run where the brightest band is equal to 500bp. GAPDH expression (predicted 559bp) for equivalent amounts of mRNA from each condition are shown below. Bands were quantified by densitometry using gelpro 3.1 software which allowed the correction of each sample against their respective GAPDH levels. Graphs show the OD for microglia (Aii) and N9s (Bii), OD represents mGluR1/GAPDH. Results shown are the average taken from two separate experiments and indicate that in microglia, incubation with myelin increased expression of mGluR1 while incubation with LPS had no significant effect compared with control. N9 cells also expressed mGluR1 but levels were not altered by exposure to myelin or LPS.

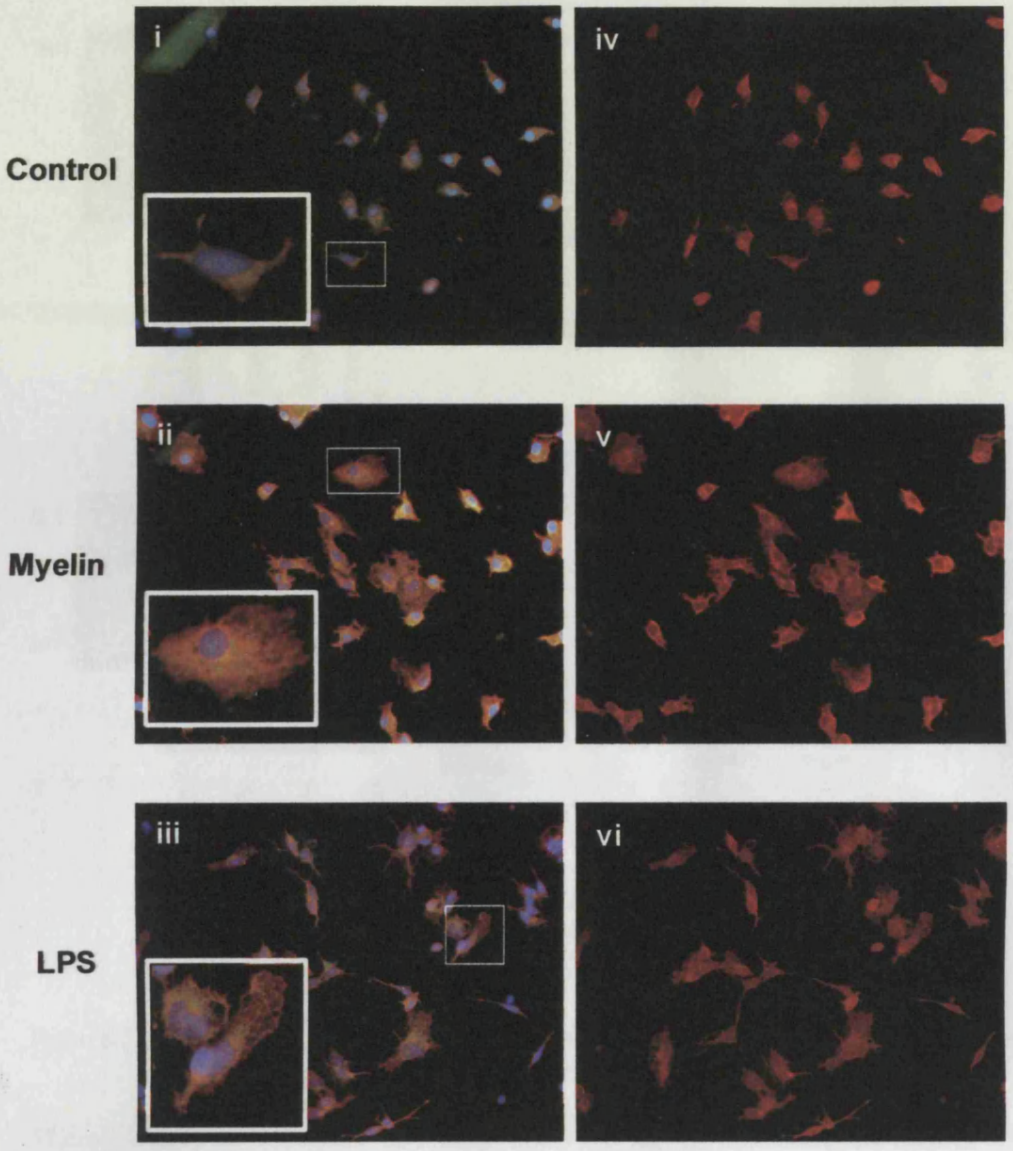


Figure 3.3 Expression and localisation of mGluR5 protein in untreated and activated primary microglia.

Microglia were either left untreated (i and iv) or incubated with myelin (1μg/ml) (ii and v) or LPS (1μg/ml) (iii and vi) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were fixed in 4% F-PBS. Microglia were co-stained with antibodies to mGluR5 and cadherin, a plasma membrane protein. Fluorescent secondary antibodies conjugated with TRITC and FITC were used to detect cells stained with mGluR and cadherin respectively. DAPI, a nuclear stain (blue), was used to aid localisation mGluR5 expression. Figures i-iii, show representative images of microglia co-stained for mGluR5, cadherin and DAPI. Figures iv-vi, show the same field but only the mGluR5 staining. These figures are representative images of staining repeated in three separate experiments and showed that microglia expressed mGluR5 protein which appeared localised to the membrane. Activation of cells with myelin or LPS increased the expression of mGluR5 protein in the membrane compared with untreated cells. (x400 magnification).

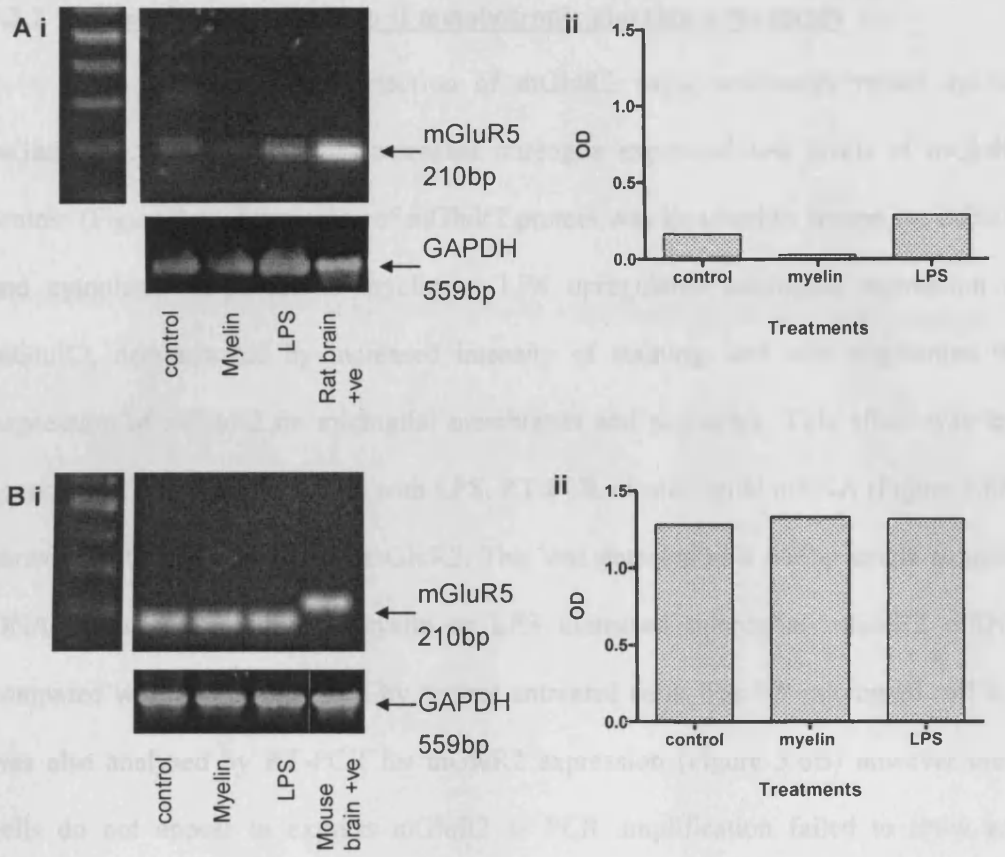


Figure 3.4 Expression of mGluR5 mRNA in primary microglia or N9 cell lines activated with myelin or LPS.

Microglia **(A)** or N9 cells **(B)** were analysed for mGluR5 mRNA expression (predicted 210bp). Cells were either left untreated or incubated with myelin (1μg/ml) or LPS (1μg/ml) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were lysed in trizol for mRNA extraction and RT-PCR. Equal amounts of each PCR product (200ng single stranded DNA) were size fractionated on a 1% agarose, ethidium bromide stained gel. Equivalent amounts of ssDNA taken from whole mouse or rat brains were also run on the same gels and used as a positive control. A 100bp DNA ladder marker was run where the brightest band is equal to 500bp. GAPDH expression (predicted 559bp) for equivalent amounts of mRNA from each condition are shown below. Bands were quantified by densitometry using gelpro 3.1 software which allowed correction each sample against their respective GAPDH levels. Graphs show the OD for microglia (Aii) and N9s (Bii), OD represents mGluR5/GAPDH. Results shown are the average taken from two separate experiments and indicate that in microglia, incubation with myelin decreased the expression of mGluR5 while incubation with LPS had no significant effect compared with control. Interestingly N9 cells expressed notably higher levels of mGluR5 mRNA than any other subtype and compared with microglial expression although levels were not altered by activation.

3.2.2 Microglia express group II metabotropic glutamate receptors

Immunocytochemical detection of mGluR2 using antibodies raised against mGluR2 (1:100), showed that untreated microglia expressed low levels of mGluR2 protein (Figure 3.5). Expression of mGluR2 protein was localised to around the nucleus and cytoplasm. Exposure to myelin or LPS upregulated microglial expression of mGluR2, demonstrated by increased intensity of staining, and also augmented the expression of mGluR2 on microglial membranes and processes. This effect was less pronounced in microglia treated with LPS. RT-PCR of microglial mRNA (Figure 3.6A) showed that microglia express mGluR2. This was detected as a 444bp single stranded DNA product. Exposure to myelin or LPS increased microglial mGluR2 mRNA compared with levels expressed by control untreated cells. The N9 microglial cell line was also analysed by RT-PCR for mGluR2 expression (Figure 3.6B) however these cells do not appear to express mGluR2 as PCR amplification failed to show any evidence of mRNA expression. This was not due to problems with primer design or PCR protocol as mGluR2 was expressed in both rat and mouse positive controls, obtained from whole rat and mouse brains respectively, which were run alongside the N9 samples.

Expression of mGluR3 was also assessed using immunocytochemistry (Figure 3.7), the antibody used has been shown to bind with both mGluR2 and mGluR3 subtypes but with a higher affinity for mGluR3. Untreated cells showed low levels of mGluR3 expression which was localised around the nucleus and cytoplasm. Microglia exposed to myelin appeared to express more mGluR3 although this was still restricted to the cytoplasm. Analysis of microglial mRNA by RT-PCR confirmed these results with untreated microglia expressing low levels of mGluR3 which was markedly increased in the samples incubated with either myelin or LPS (Figure 3.8A). N9 mRNA from cells treated in the same conditions was also assessed (Figure 3.8B). N9 cells did

not contain mGluR3 mRNA. This was not due to problems with primer design or PCR protocol as mGluR3 was expressed in the mouse positive control run alongside the N9 samples. See table 3.1 for a summary of group II mGluR protein and mRNA expressed by microglia and N9 cells.

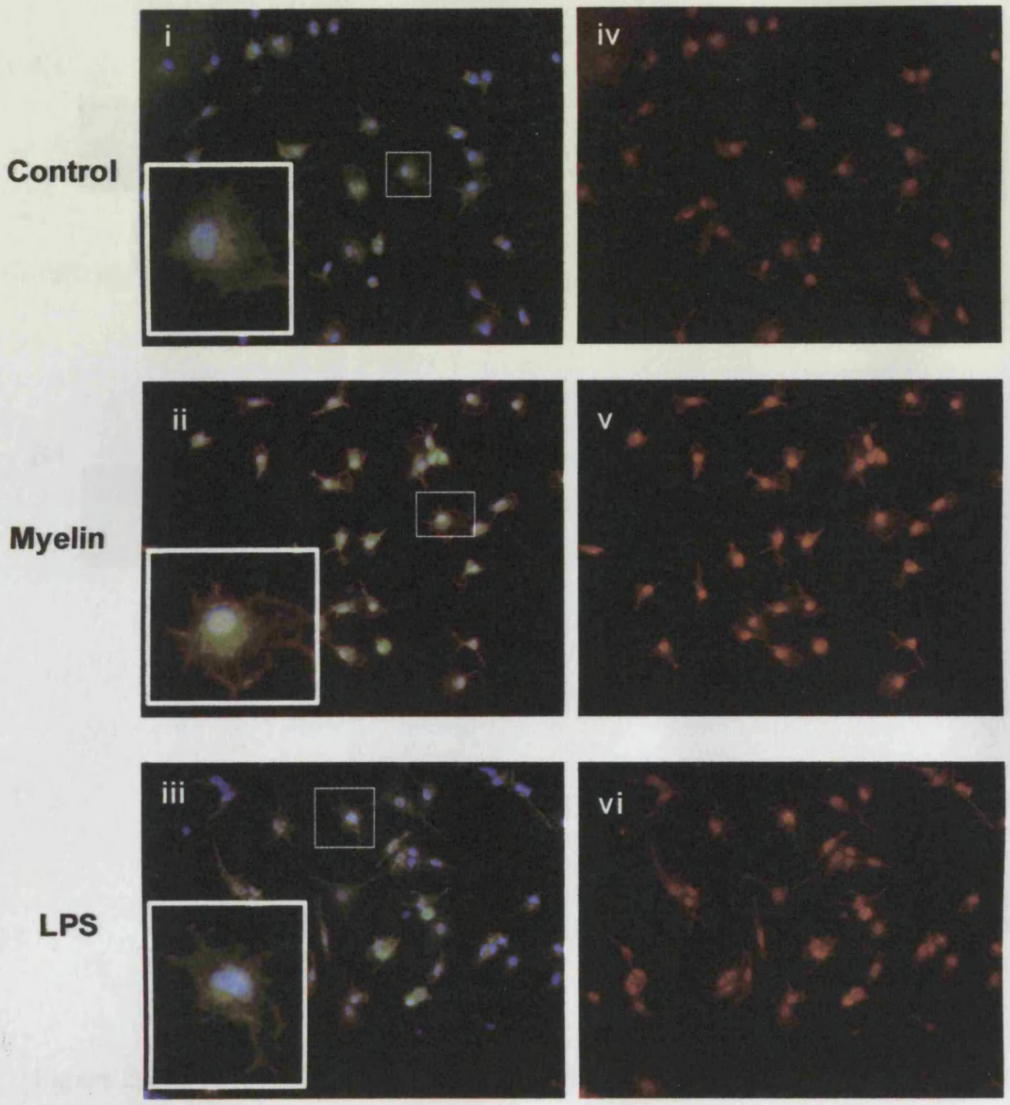


Figure 3.5 Expression and localisation of mGluR2 protein in untreated and activated primary microglia.

Microglia were either left untreated (i and iv) or incubated with myelin (1μg/ml) (ii and v) or LPS (1μg/ml) (iii and vi) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were fixed in 4% F-PBS. Microglia were co-stained with antibodies to mGluR2 and cadherin, a plasma membrane protein. Fluorescent secondary antibodies conjugated with TRITC and FITC were used to detect cells stained with mGluR and cadherin respectively. DAPI, a nuclear stain (blue), was also used to aid localisation of mGluR2 expression. Figures i-iii, show representative images of microglia co-stained for mGluR2, cadherin and DAPI. Figures iv-vi, show the same field but only the mGluR2 staining. These figures are representative images of staining repeated in three separate experiments and showed that microglia expressed mGluR2 protein which appeared localised to both the nucleus and the membrane. Activation of cells with myelin or LPS appeared to increase mGluR2 protein expression. (x400 magnification).

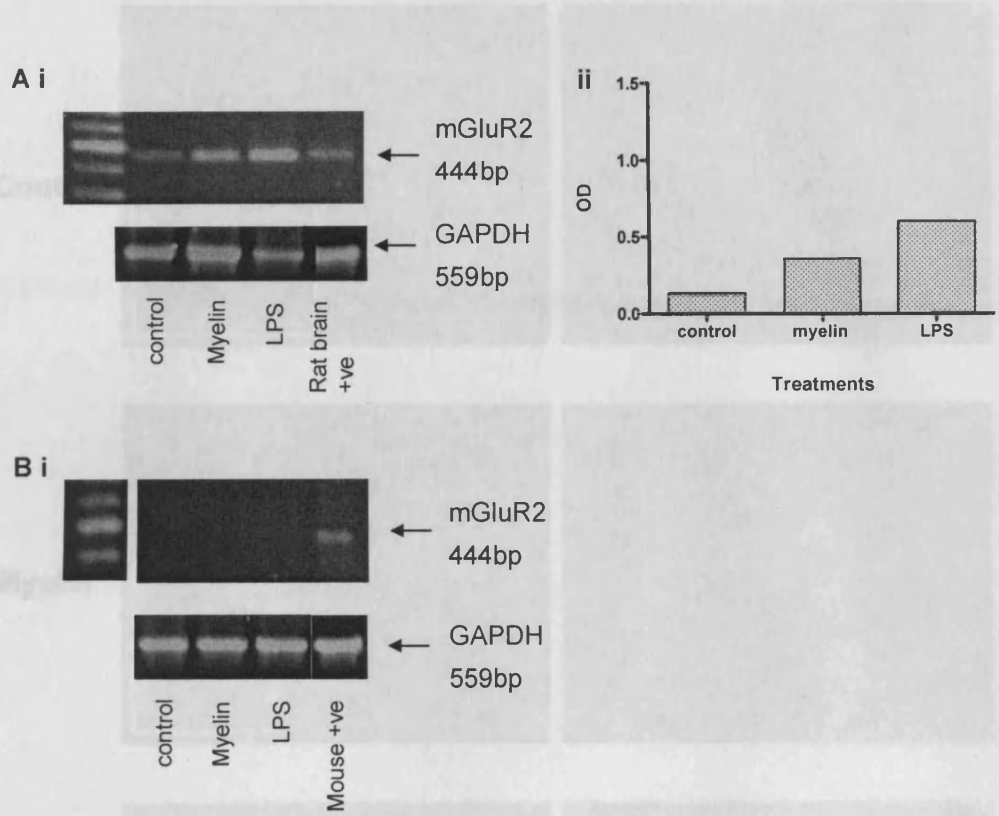


Figure 3.6 Expression of mGluR2 mRNA in primary microglia or N9 cell lines activated with myelin or LPS.

Microglia **(A)** or N9 cells **(B)** were analysed for mGluR2 mRNA expression (predicted 444bp). Cells were either left untreated or incubated with myelin (1μg/ml) or LPS (1μg/ml) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were lysed in trizol for mRNA extraction and RT-PCR. Equal amounts of each PCR product (200ng single stranded DNA) were size fractionated on a 1% agarose, ethidium bromide stained gel. Equivalent amounts of ssDNA taken from whole mouse or rat brains were also run on the same gels and used as a positive control (Rat/mouse +ve). A 100 bp DNA ladder marker was run where the brightest band is equal to 500bp. GAPDH expression (predicted 559bp) for equivalent amounts of mRNA from each condition are shown below. Bands were quantified by densitometry using gelpro 3.1 software which allowed correction each sample against their respective GAPDH levels. Graphs show the OD for microglia (Aii) and N9s (Bii), OD represents mGluR2/GAPDH. Results shown are the average taken from two separate experiments and indicate that in microglia, incubation with myelin or LPS increased expression of mGluR2. Using these primers, N9 cells did not express mGluR2.

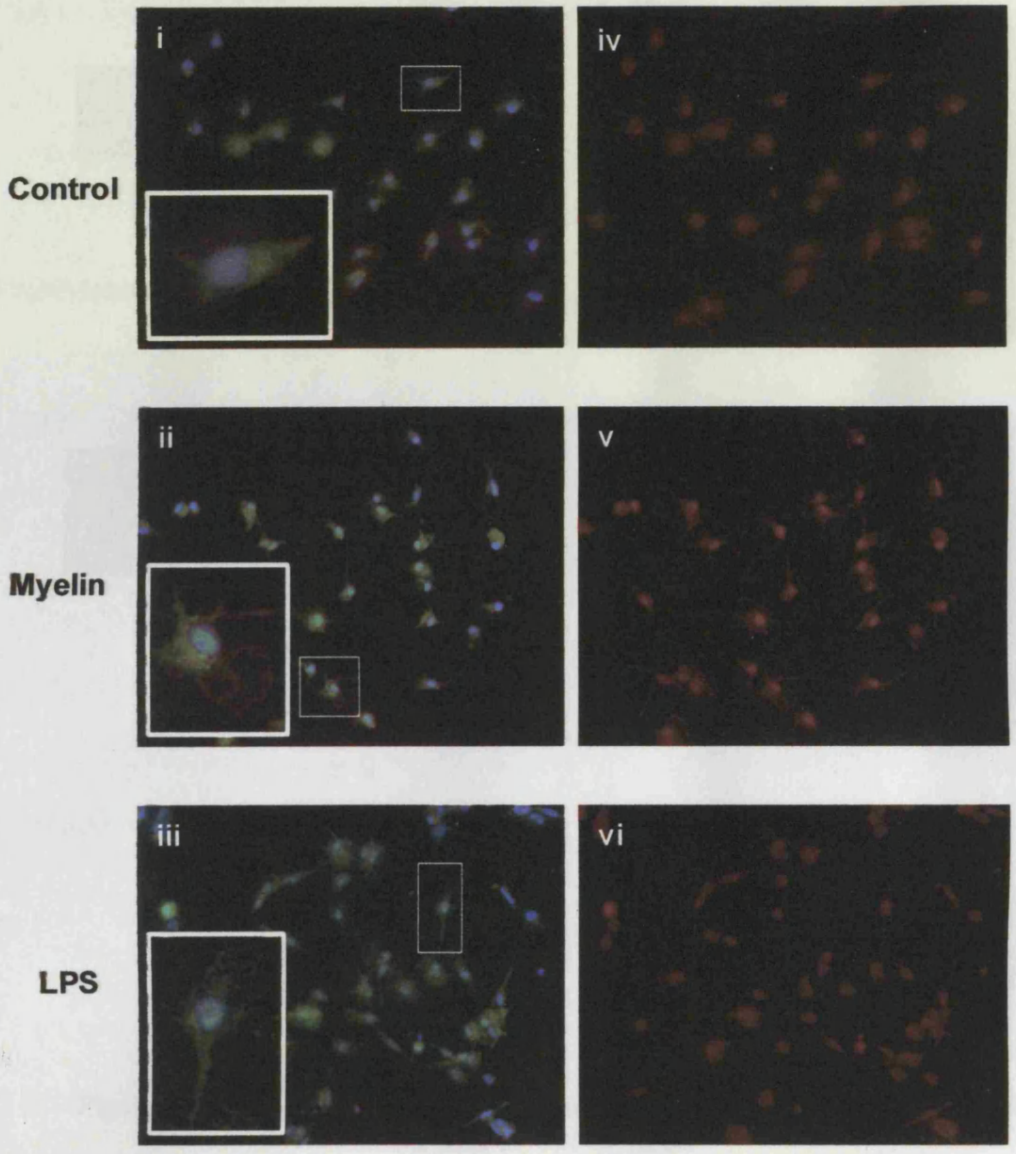


Figure 3.7 Expression and localisation of mGluR3 protein in untreated and activated primary microglia.

Microglia were either left untreated (i and iv) or incubated with myelin (1μg/ml) (ii and v) or LPS (1μg/ml) (iii and vi) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were fixed in 4% F-PBS. Microglia were co-stained with antibodies to mGluR3 and cadherin, a plasma membrane protein. Fluorescent secondary antibodies conjugated with TRITC and FITC were used to detect cells stained with mGluR and cadherin respectively. DAPI, a nuclear stain (blue), was also used to aid localisation of mGluR3 expression. Figures i-iii, show representative images of microglia co-stained for mGluR3, cadherin and DAPI. Figures iv-vi, show the same field but only the mGluR3 staining. These figures are representative images of staining repeated in three separate experiments and showed that microglia expressed mGluR3 protein which appeared localised to both the nucleus and the membrane. Activation of cells with myelin or LPS appeared to increase mGluR3 protein expression.

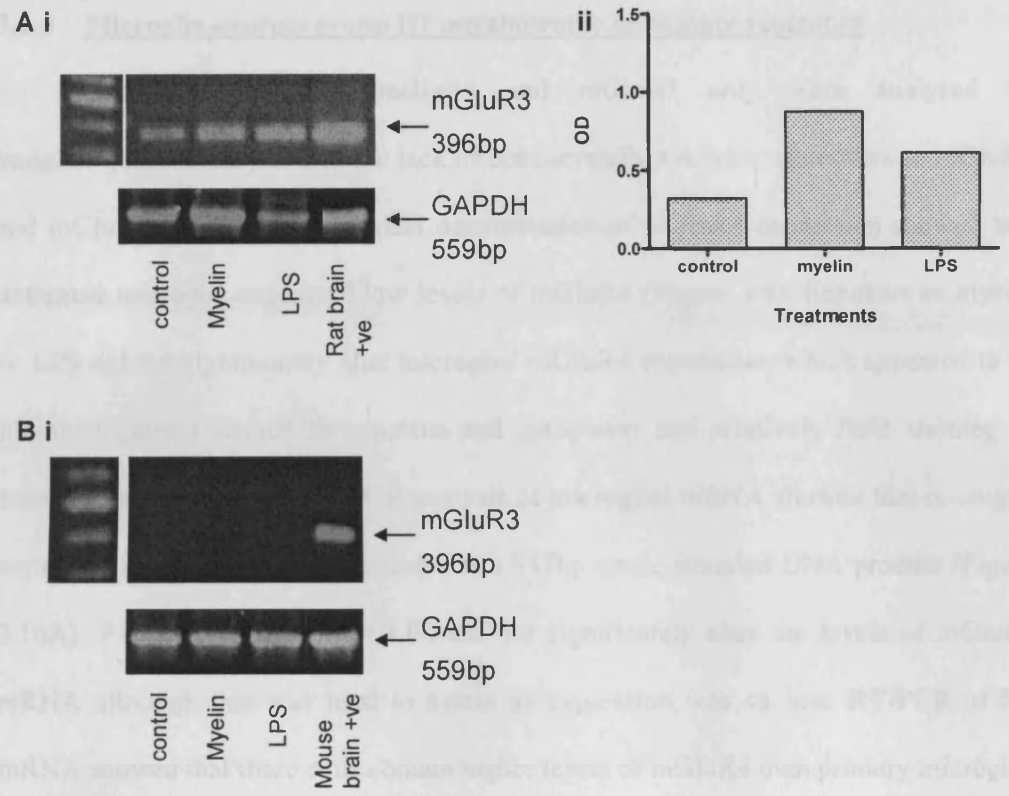


Figure 3.8 Expression of mGluR3 mRNA in primary microglia or N9 cell lines activated with myelin or LPS.

Microglia **(A)** or N9 cells **(B)** were analysed for mGluR3 mRNA expression (predicted 396bp). Cells were either left untreated or incubated with myelin (1μg/ml) or LPS (1μg/ml) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were lysed in trizol for mRNA extraction and RT-PCR. Equal amounts of each PCR product (200ng single stranded DNA) were size fractionated on a 1% agarose, ethidium bromide stained gel. Equivalent amounts of ssDNA taken from whole mouse or rat brains were also run on the same gels and used as a positive control (rat/mouse +ve). A 100bp DNA ladder marker was run where the brightest band is equal to 500bp. GAPDH expression (predicted 559bp) for equivalent amounts of mRNA from each condition are shown below. Bands were quantified by densitometry using gelpro 3.1 software which allowed correction each sample against their respective GAPDH levels. Graphs show the OD for microglia (Aii) and N9s (Bii), OD represents mGluR3/GAPDH. Results shown are the average taken from two separate experiments and indicate that in microglia, incubation with myelin or LPS slightly increased expression of mGluR3. N9 cells did not express mGluR3.

3.2.3 Microglia express group III metabotropic glutamate receptors

Group III subtypes mGluR4 and mGluR7 only were analysed by immunocytochemistry due to the lack of commercially available antibodies to mGluR6 and mGluR8. Immunocytochemical determination of mGluR4 expression showed that untreated microglia expressed low levels of mGluR4 (Figure 3.9). Exposure to myelin or LPS did not significantly alter microglial mGluR4 expression, which appeared to be mainly localised around the nucleus and cytoplasm and relatively little staining of membranes or processes. RT-PCR analysis of microglial mRNA showed that microglia expressed mGluR4. This was detected as a 547bp single stranded DNA product (Figure 3.10A). Exposure to myelin or LPS did not significantly alter the levels of mGluR4 mRNA although this was hard to assess as expression was so low. RT-PCR of N9 mRNA showed that these cells contain higher levels of mGluR4 than primary microglia, however this expression was not affected by incubation with either myelin or LPS (Figure 3.10B).

Microglia also expressed mGluR6 mRNA, detected as a 363bp single stranded DNA product (Figure 3.11A). Expression was very low which made it difficult to assess whether incubation with different activators had any effect. Microglia expressed very low levels mGluR6 which were unaffected by exposure to myelin but appeared to be knocked out by LPS. We were unable to detect mGluR6 in the N9 cell line although this may be due to problems with primer design as while the rat brain positive control contained mGluR6, the mouse brain positive control failed to show mGluR6 expression (Figure 3.11B).

Immunolocalisation of mGluR7 protein showed no expression above negative control (data not shown) this was in accordance with previous data (Taylor et al., 2003). Analysis of microglial mRNA by RT-PCR demonstrated that microglia did not express mRNA for mGluR7 either in untreated cells or those incubated with myelin or LPS

(Figure 3.12A). RT-PCR of N9 mRNA confirmed these findings as PCR amplification failed to show any evidence of mGluR7 mRNA (Figure 3.12B). This was not due to problems with primer design or PCR protocol as mGluR7 was expressed in both rat and mouse positive controls which were run alongside the N9 samples.

Expression of mGluR8 mRNA was analysed by RT-PCR. Microglial cells constitutively expressed low levels of mGluR8 (Figure 3.13A).which was detected as a 440bp single stranded DNA product. Exposure to myelin increased mGluR8 expression while LPS appeared to have the opposite effect. Analysis of the N9 cell line showed that these cells do not express mGluR8 as PCR amplification failed to indicate any evidence of mGluR8 in the samples (Figure 3.13B). We verified that this was not due to problems with primer design or PCR protocol by running positive controls of whole mouse and rat brain samples which both came up positive. See table 3.1 for a summary of group III mGluR protein and mRNA expressed by microglia and N9 cells.

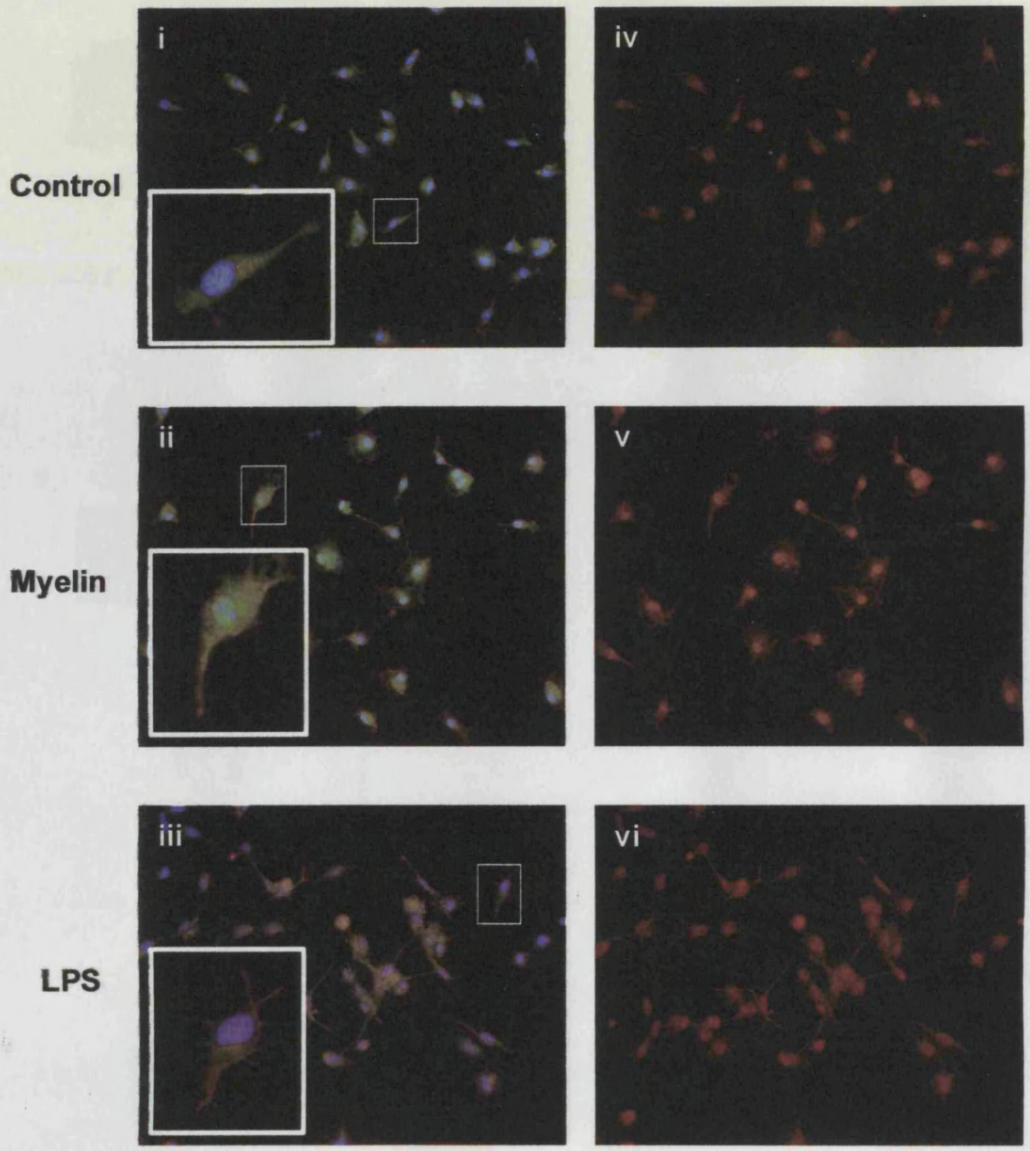


Figure 3.9 Expression and localisation of mGluR4 protein in untreated and activated primary microglia.

Microglia were either left untreated (i and iv) or incubated with myelin (1μg/ml) (ii and v) or LPS (1μg/ml) (iii and vi) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were fixed in 4% F-PBS. Microglia were co-stained with antibodies to mGluR4 and cadherin, a plasma membrane protein. Fluorescent secondary antibodies conjugated with TRITC and FITC were used to detect cells stained with mGluR and cadherin respectively. DAPI, a nuclear stain (blue), was also used to aid localisation of mGluR4 expression. Figures i-iii, show representative images of microglia co-stained for mGluR4, cadherin and DAPI. Figures iv-vi, show the same field but only the mGluR4 staining. These figures are representative images of staining repeated in three separate experiments and showed that microglia expressed mGluR4 protein which appeared localised to the nucleus. Activation of cells with myelin or LPS did not noticeably alter mGluR4 protein expression.

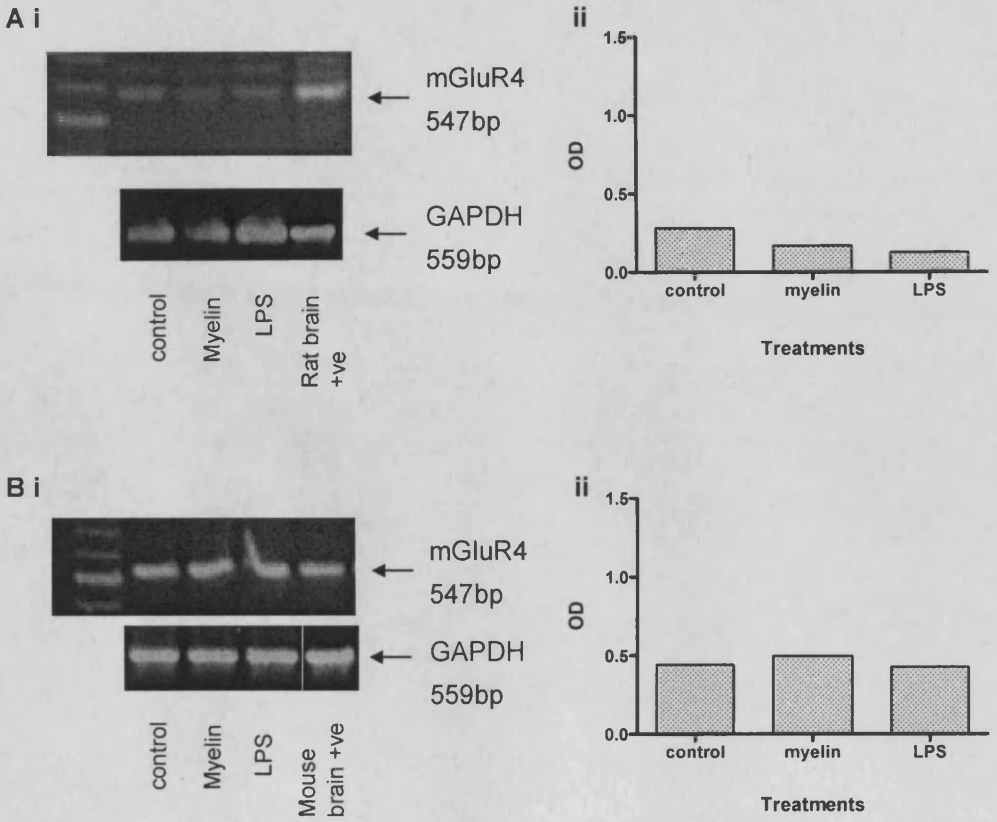


Figure 3.10 Expression of mGluR4 mRNA in primary microglia or N9 cell lines activated with myelin or LPS.

Microglia **(A)** or N9 cells **(B)** were analysed for mGluR4 mRNA expression (predicted 547bp). Cells were either left untreated or incubated with myelin (1μg/ml) or LPS (1μg/ml) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were lysed in trizol for mRNA extraction and RT-PCR. Equal amounts of each PCR product (200ng single stranded DNA) were size fractionated on a 1% agarose, ethidium bromide stained gel. Equivalent amounts of ssDNA taken from whole mouse or rat brains were also run on the same gels and used as a positive control (rat/mouse +ve). A 100bp DNA ladder marker was run where the brightest band is equal to 500bp. GAPDH expression (predicted 559bp) for equivalent amounts of mRNA from each condition are shown below. Bands were quantified by densitometry using gelpro 3.1 software which allowed correction each sample against their respective GAPDH levels. Graphs show the OD for microglia (Aii) and N9s (Bii), OD represents mGluR4/GAPDH. Results shown are the average taken from two separate experiments and indicate that microglia expressed mGluR4 mRNA but that levels of mGluR4 were not altered by activation. N9 cells also expressed mGluR4 mRNA but again these levels remained the same after activation.

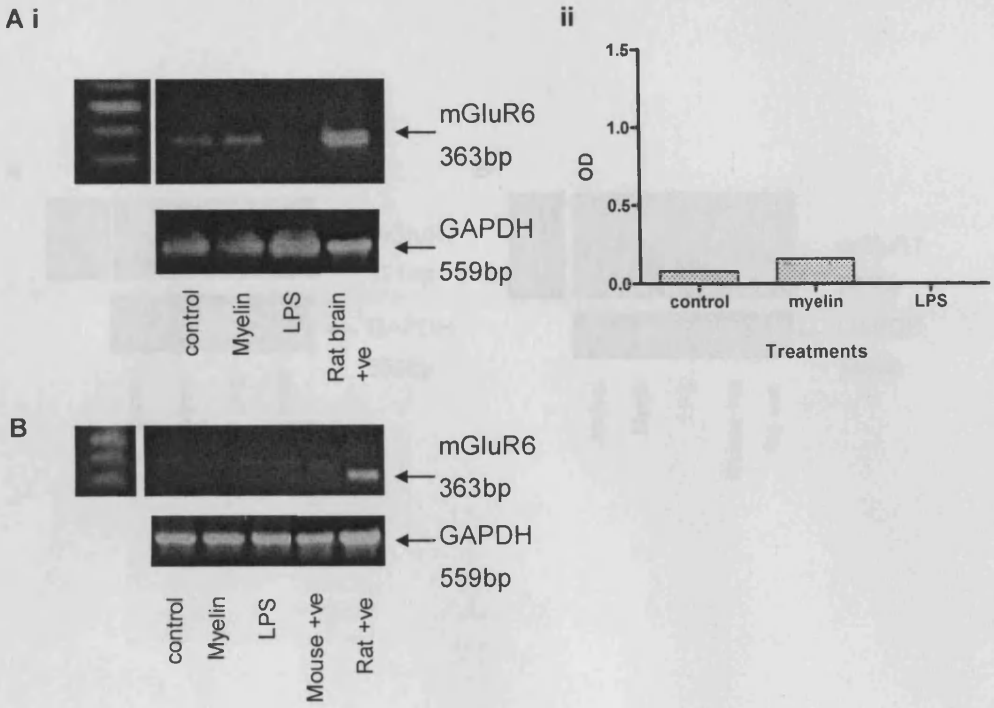


Figure 3.11 Expression of mGluR6 mRNA in primary microglia or N9 cell lines activated with myelin or LPS.

Microglia **(A)** or N9 cells **(B)** were analysed for mGluR6 mRNA expression (predicted 363bp). Cells were either left untreated or incubated with myelin (1μg/ml) or LPS (1μg/ml) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were lysed in trizol for mRNA extraction and RT-PCR. Equal amounts of each PCR product (200ng single stranded DNA) were size fractionated on a 1% agarose, ethidium bromide stained gel. Equivalent amounts of ssDNA taken from whole mouse or rat brains were also run on the same gels and used as a positive control (rat/mouse +ve). A 100bp DNA ladder marker was run where the brightest band is equal to 500bp. GAPDH expression (predicted 559bp) for equivalent amounts of mRNA from each condition are shown below. Bands were quantified by densitometry using gelpro 3.1 software which allowed correction each sample against their respective GAPDH levels. Graphs show the OD for microglia (Aii) OD represents mGluR6/GAPDH. Results shown are the average taken from two separate experiments and indicate that microglia expressed extremely low levels of mGluR6 mRNA. N9 cells did not express mGluR6 but this may have been a problem in primer design since the mouse brain positive was also negative for mGluR6.

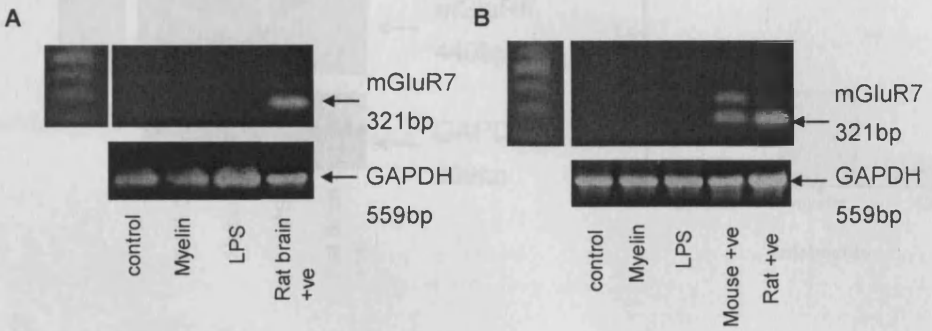


Figure 3.12 Expression of mGluR7 mRNA in primary microglia or N9 cell lines activated with myelin or LPS.

Microglia **(A)** or N9 cells **(B)** were analysed for mGluR7 mRNA expression (predicted 321bp). Cells were either left untreated or incubated with myelin (1μg/ml) or LPS (1μg/ml) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were lysed in trizol for mRNA extraction and RT-PCR. Equal amounts of each PCR product (200ng single stranded DNA) were size fractionated on a 1% agarose, ethidium bromide stained gel. Equivalent amounts of ssDNA taken from whole mouse or rat brains were also run on the same gels and used as a positive control (rat/mouse +ve). A 100bp DNA ladder marker was run where the brightest band is equal to 500bp. GAPDH expression (predicted 559bp) for equivalent amounts of mRNA from each condition are shown below. Results shown are representatives from two separate experiments and indicate that neither microglia nor N9s expressed mGluR7 mRNA. This was not due to problems with PCR protocol or primer design as mGluR7 mRNA was expressed in both rat and mouse whole brain samples.

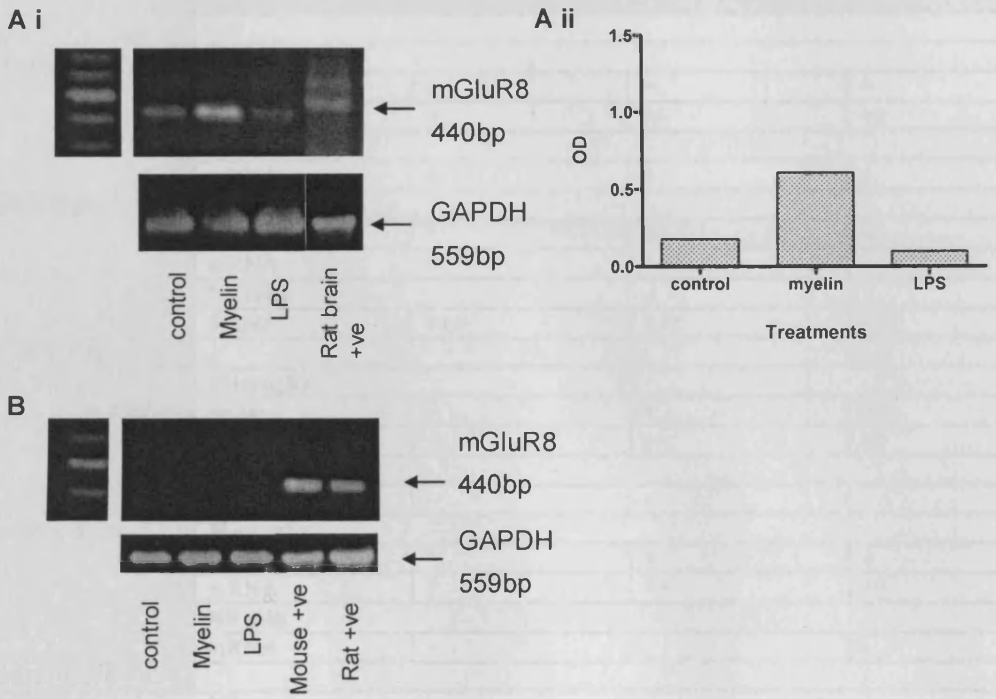


Figure 3.13 Expression of mGluR8 mRNA in primary microglia or N9 cell lines activated with myelin or LPS.

Microglia **(A)** or N9 cells **(B)** were analysed for mGluR8 mRNA expression (predicted 440bp). Cells were either left untreated or incubated with myelin (1μg/ml) or LPS (1μg/ml) for 24 hours at 37°C. Untreated cells were used as negative controls. Cells were lysed in trizol for mRNA extraction and RT-PCR. Equal amounts of each PCR product (200ng single stranded DNA) were size fractionated on a 1% agarose, ethidium bromide stained gel. Equivalent amounts of ssDNA taken from whole mouse or rat brains were also run on the same gels and used as a positive control (rat/mouse +ve). A 100bp DNA ladder marker was run where the brightest band is equal to 500bp. GAPDH expression (predicted 559bp) for equivalent amounts of mRNA from each condition are shown below. Bands were quantified by densitometry using gelpro 3.1 software which allowed correction each sample against their respective GAPDH levels. Graphs show the OD for microglia (Aii) OD represents mGluR8/GAPDH. Results shown are the average taken from two separate experiments and indicate that microglia expressed extremely low levels of mGluR8 mRNA which was increased by exposure to myelin. N9 cells did not express mGluR8 mRNA but this was not due to problems in primer design or PCR protocol since the mouse brain positive was positive for mGluR8.

		CONTROL	LPS	MYELIN
GROUP I				
mGluR 1	**Microglia**			
	protein	+	+	+
	mRNA	+	+/-	++
	N9 cells			
	mRNA	+	+	+
mGluR 5	**Microglia**			
	protein	+	++	++
	mRNA	+	++	-
	N9 cells			
	mRNA	+++	+++	+++
GROUP II				
mGluR 2	**Microglia**			
	protein	+	+	++
	mRNA	+	++	++
	N9 cells			
	mRNA	-	-	
mGluR 3	**Microglia**			
	protein	+	+	+
	mRNA	+	++	++
	N9 cells			
	mRNA	-	-	-
GROUP III				
mGluR 4	**Microglia**			
	protein	+	+	+
	mRNA	+/-	+/-	+/-
	N9 cells			
	mRNA	+	+	+
mGluR 6	**Microglia**			
	mRNA	+/-	+/-	-
	N9 cells			
	mRNA	-	-	-
mGluR 7	**Microglia**			
	mRNA	-	-	-
	N9 cells			
	mRNA	-	-	-
mGluR 8	**Microglia**			
	mRNA	+	+/-	++
	N9 cells			
	mRNA	-	-	-

Table 3.1 Summary table of mGluR protein and mRNA expression on microglia and N9 cells either left untreated or incubated with LPS or myelin.

+++ very strong expression, ++ strong expression, + some expression, +/- weak expression, - no expression.

3.2.4 Macrophages express metabotropic glutamate receptors

Rat peritoneal macrophages were fixed in 4% F-PBS after 1 hour, 24 hours or 7 days *in vitro* and analysed for the expression of group I, II and III mGluRs by immunocytochemistry. Macrophages expressed mGluR1 protein (Figure 3.14A). In accordance with the microglial staining, macrophage mGluR1 expression appeared to be localised aound the nucleus and cytoplasm, this remained the case after 24 hours. After 7 days, mGluR1 expression remained at a similar level and although still mainly localised around the nucleus there was more evidence of staining in the cytoplasm. Macrophages expressed higher levels of mGluR5 than mGluR1 protein (Figure 3.14B). After 1 hour mGluR5 expression was localised around the nucleus, this remained the case after 24 hours, however after 7 days mGluR5 protein was more localised to the cell membranes and cytoplasm which was consistent with our findings in primary microglia.

Expression of group II mGluR2 and 2/3 was noticeably less intense than either mGluR1 or 5 at all time points. At each time point, both mGluR2 (Figure 3.15A) and 2/3 (Figure 3.15B) remained localised around the nucleus and cytoplasm with no visible translocation to the membrane. Staining for mGluR 2/3 was barely visible above the negative control (Figure 3.17B), it is therefore possible that macrophages express little if any mGluR3 protein.

Group III mGluR4 was also expressed by macrophages (Figure 3.16A). After 1 hour this expression was localised around the nucleus and cytoplasm and after 24 hours was clearly more cytoplasmic. After 7 days translocation of mGluR4 protein to the membrane had occurred with very little staining around the nucleus. Levels of mGluR7 protein were noticeably lower than mGluR4 and not visible until after 24 hours when the expression was clearly NW_expressed in the cytoplasm (Figure 3.16B). After 7 days, mGluR7 protein levels were still very low but had visibly translocated to the

membrane. These findings are in contrast with those observed in microglial cultures since microglia did not express mGluR7.

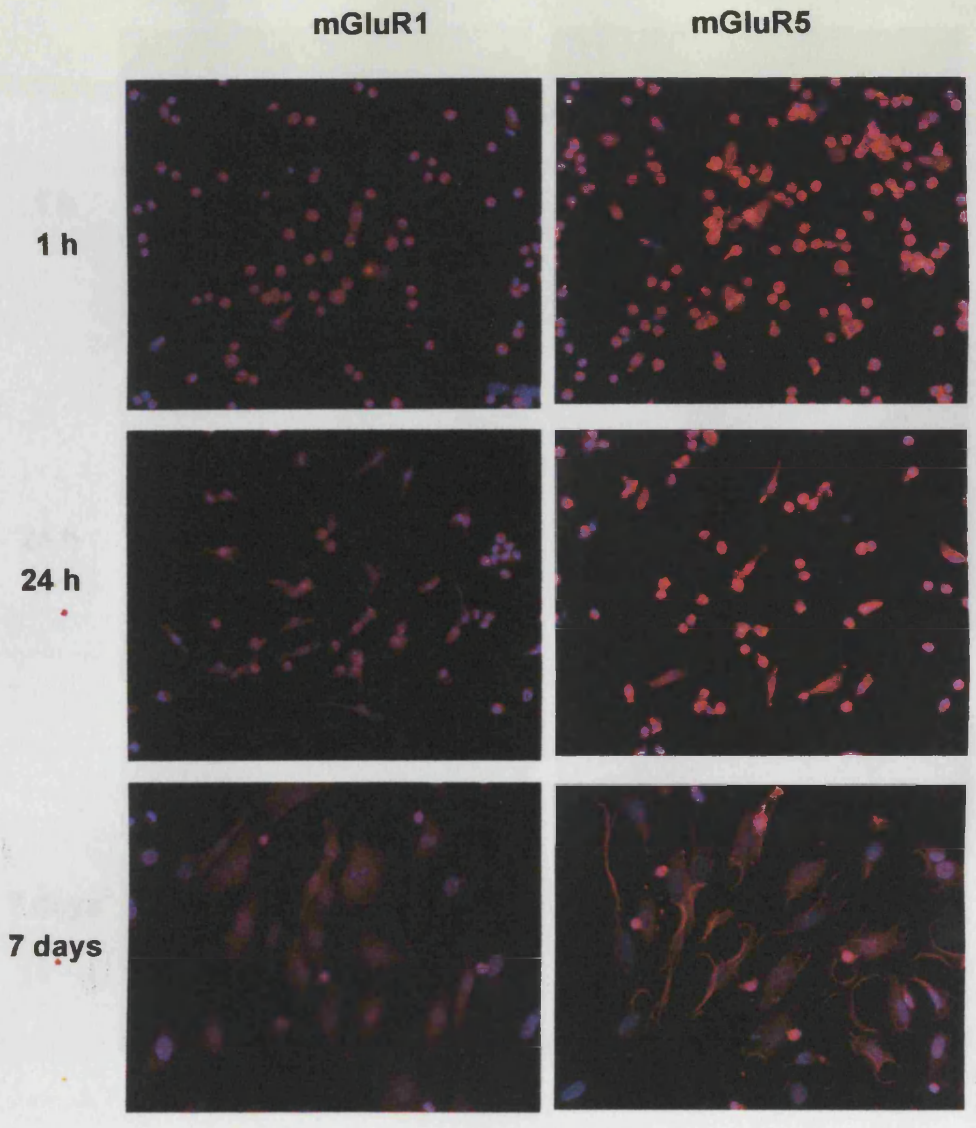


Figure 3.14 Expression and localisation of group I mGluRs in primary macrophage cells after 1h, 24h and 7 days.

Primary macrophages were cultured and left *in vitro* for 1hr, 24 hrs or 7 days before being fixed in 4% F-PBS. Cells were labelled with antibodies to mGluR1 or mGluR5. A secondary antibody conjugated to TRITC was used to detect mGluR labelled cells. The nuclear marker DAPI (blue staining) was used to localise mGluR staining. These figures are representative images of the staining repeated in three separate experiments. The staining was very similar to that seen in the microglia with macrophages after 7 days expressing mGluR1 remaining more localised to the nucleus and mGluR5 more localised to membrane. It was also noticeable that between the 24 hour and 7 day timepoints, mGluR5 and to some extent mGluR1 expression appeared to be trafficked away from the nucleus. (x400 magnification)

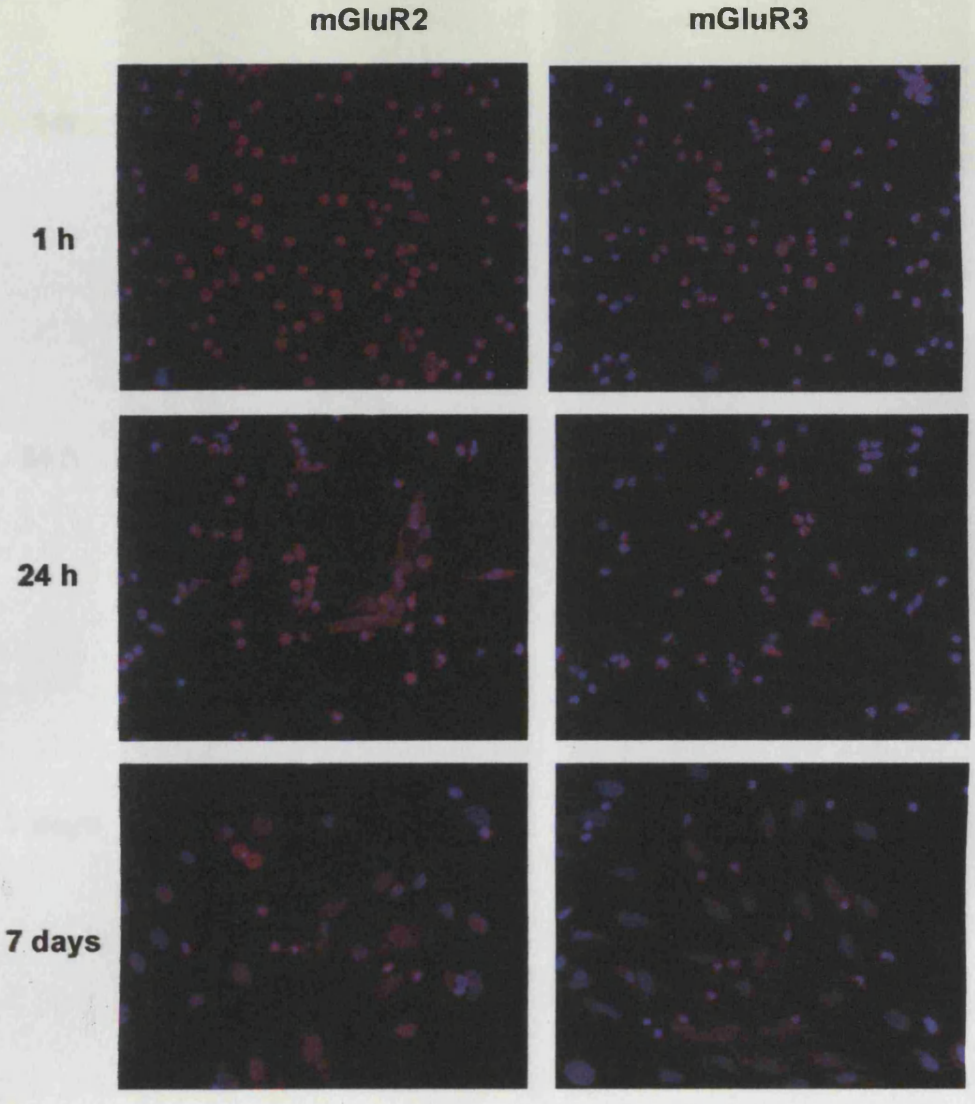


Figure 3.15 Expression and localisation of group I mGluRs in primary macrophage cells after 1h, 24h and 7 days.

Primary macrophages were cultured and left in vitro for 1h, 24h or 7 days before being fixed in 4% F-PBS. Cells were co-stained with antibodies to mGluR2 or mGluR3 (both red staining) and the nuclear membrane marker DAPI (blue staining). These figures are representative images of the staining repeated in three separate experiments. These figures are representative images of the staining repeated in three separate experiments. The staining shows that macrophages express more mGluR2 than mGluR3 protein which is similar in microglia although the mGluR2 remains more localised in the nucleus in the macrophages. (x400 magnification)

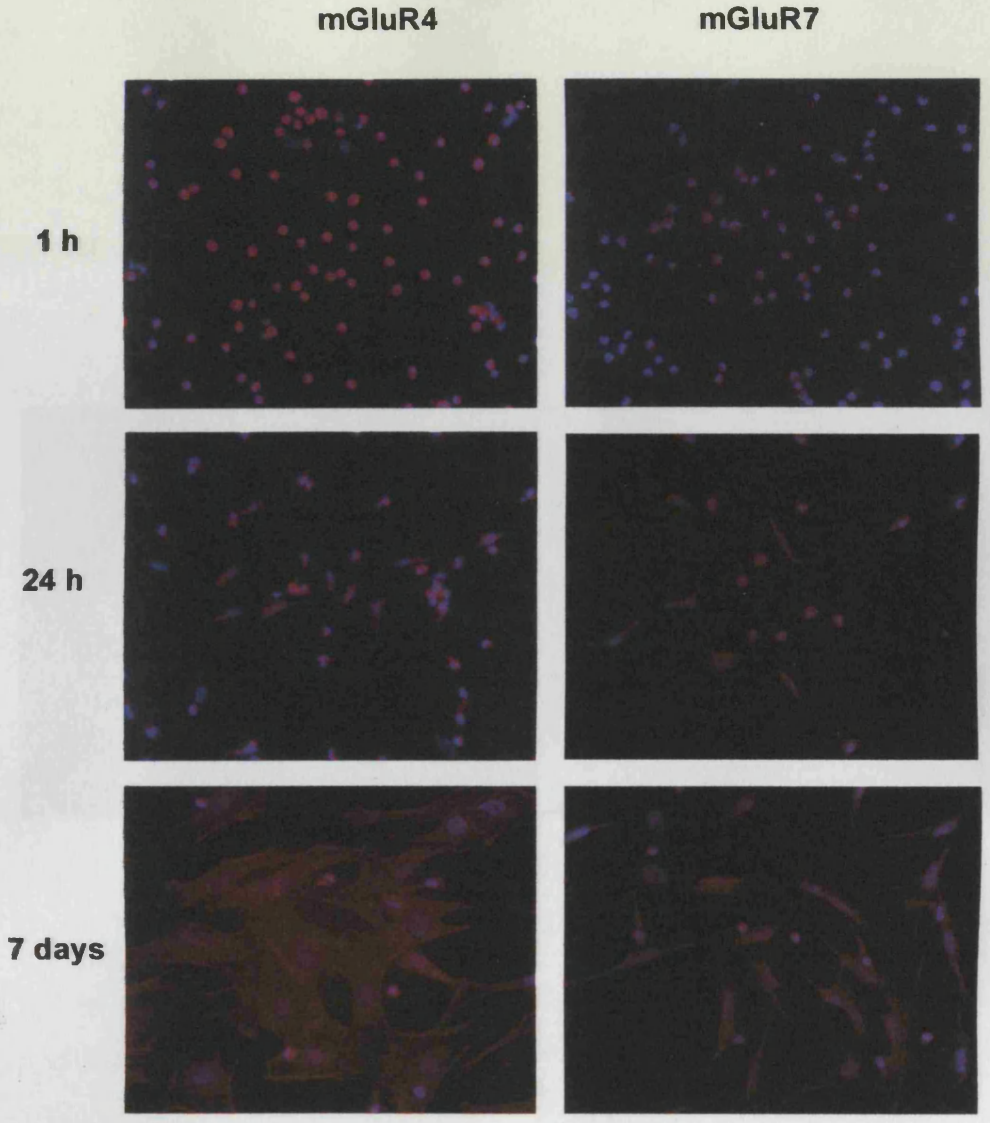


Figure 3.16 Expression and localisation of group I mGluRs in primary macrophage cells after 1h, 24h and 7 days.

Primary macrophages were cultured and left in vitro for 1h, 24h or 7 days before being fixed in 4% F-PBS. Cells were co-stained with antibodies to mGluR 1 or mGluR5 (both red staining) and the nuclear membrane marker DAPI (blue staining). These figures are representative images of the staining repeated in three separate experiments. The staining shows that macrophages express very little mGluR7. Interestingly, mGluR4 expression increases in 7 day old macrophages and appears to be more localised to the membrane. (x400 magnification)

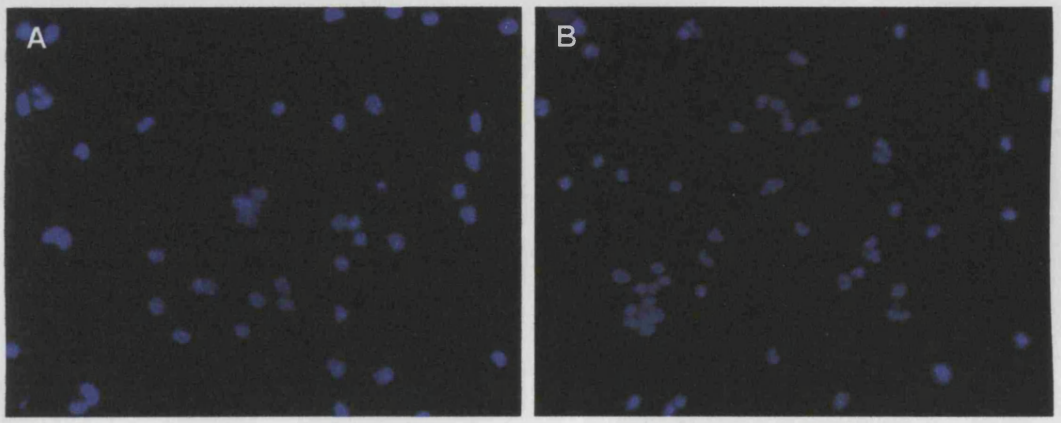


Figure 3.17 Negative controls for microglial and macrophage staining

Primary microglia **(A)** or macrophages **(B)** were cultured and left *in vitro* for 24 hours before being fixed in 4% F-PBS. Primary antibody was omitted from these cells but secondary antibodies conjugated to TRITC were applied as per previous experiments with the nuclear stain DAPI (blue staining). These figures are representative images of staining repeated on three separate occasions. There was no visible positive staining which suggests that the staining of mGluR protein on both macrophages and microglia was specific. (x400 magnification)

3.2.5 Group I mGluR expression in EAE

For the following experiments, expression of mGluRs was examined in both a mouse and a DA rat model of MS. Serial spinal cord sections removed from rats during the acute and chronic phases of MOG induced EAE disease and from normal adjuvant injected control rats were generously supplied by Annabellla Polito and Anne Palser from Professor Reynolds lab and were used for immunohistochemistry due to the lack of commercially available mGluR antibodies which react with mouse protein. Sections were double stained with antibodies raised against specific mGluRs and an antibody raised against MHC class II antigens (OX-6) directly conjugated to FITC to detect activated microglia and macrophages. As discussed, the differentiation of activated microglia from macrophages is very difficult without the use of several markers so in these experiments OX-6 labelled cells will be reffered to as microglia/ macrophages (MG/Ø).

RT-PCR, however, was performed on whole brains and spinal cords taken from mice in the first acute phase of EAE where most glial infiltration occurs and also adjuvant injected control mice. Samples were analysed for mGluR mRNA by RT-PCR as described. Both the brain and spinal cord homogenates showed relatively high expression of mGluR1 mRNA but there was no visible difference in expression between control and EAE animals either in the brain or the spinal cord, this was confirmed by densitometric analysis (Figure 3.18A).

Spinal cord sections from control animals showed very low expression of MG/Ø and equally low positive staining for mGluR1 (Figure 3.19A) which suggests that the detected mGluR1 mRNA expression in control animals may be neuronal or astrocytic. In spinal cord sections from EAE acute phase animals there was a dramatic increase in the presence of MG/Ø especially localised in and around perivascular cuffs. There was also increased staining for mGluR1 but this was not for the most part co-localised with

microglia (Figure 3.19B). In sections from the chronic phase of EAE there were markedly reduced numbers of microglia although these cells were positive for mGluR1 (Figure 3.19C marked by arrow).

Brain homogenates analysed for mGluR5 mRNA revealed that there was no significant difference between mGluR5 expression in control or EAE brain tissue. Analysis of spinal cord homogenates, however, revealed a significant reduction of mGluR5 mRNA in the EAE animals compared with control (Figure 3.18B).

Sections analysed as above but for mGluR5 expression showed a similar pattern of staining. Control sections had noticeably fewer DAPI positive and no OX-6 positive cells confirming the absence of any inflammatory cells (Figure 3.20A) which again suggests that the mGluR5 mRNA expression observed in control cords may be neuronal or from other glial cell types. Sections from the acute phase of EAE had a noticeably increased number of MG/Ø compared with sections from control amimals, expression of mGluR5 in these sections was also increased although this was not co-localised to microglia (Figure 3.20B). Chronic phase sections however showed a notable upregulation of mGluR5 expression with some localisation to microglial cells (Figure 3.20C marked by arrow).

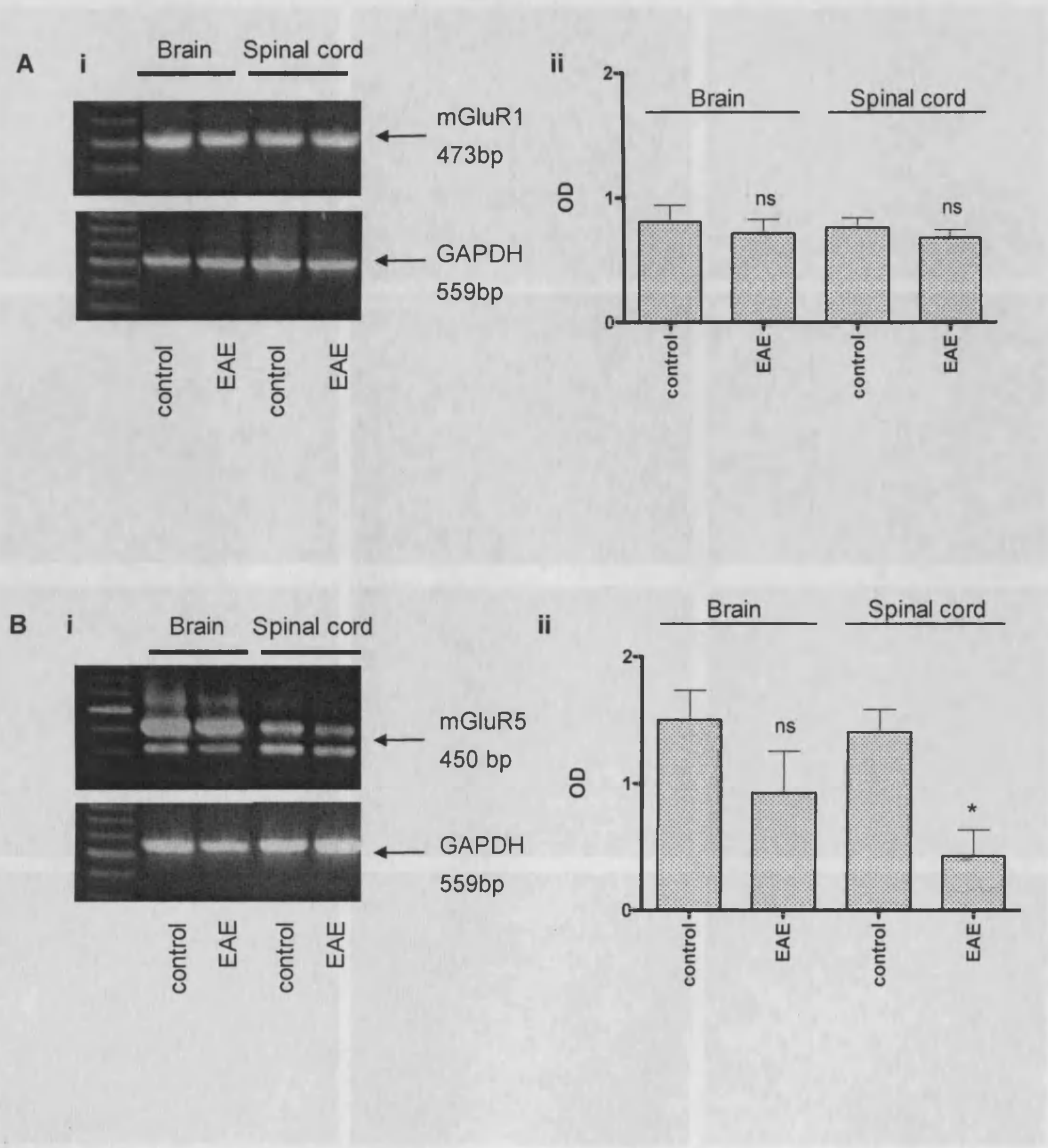


Figure 3.18 Expression of mGluR1 and 5 mRNA in brain and spinal cord homogenates from EAE and control mice

Brains and spinal cords were removed from mice at the acute phase of disease and from adjuvant injected control mice at the same time point. Levels of mGluR1 **(Ai and ii)** and 5 **(Bi and ii)** mRNA were measured in the brain and spinal cord lysates by RT-PCR. Representative PCR gels are shown (Ai and Bi). GAPDH levels for each sample were also measured to ensure equal loading. Samples were quantitatively analysed by densitometry scanning. Results are shown as arbitrary densitometry units ± SEM for total mGluR/GAPDH mRNA (Aii and Bii). Expression of mGluR1 mRNA remained the same in both the brain and spinal cord of control and EAE animals. Levels of mGluR5 mRNA were decreased in EAE spinal cord compared with control although they remained the same in brain tissue. n=4, *P<0.05.

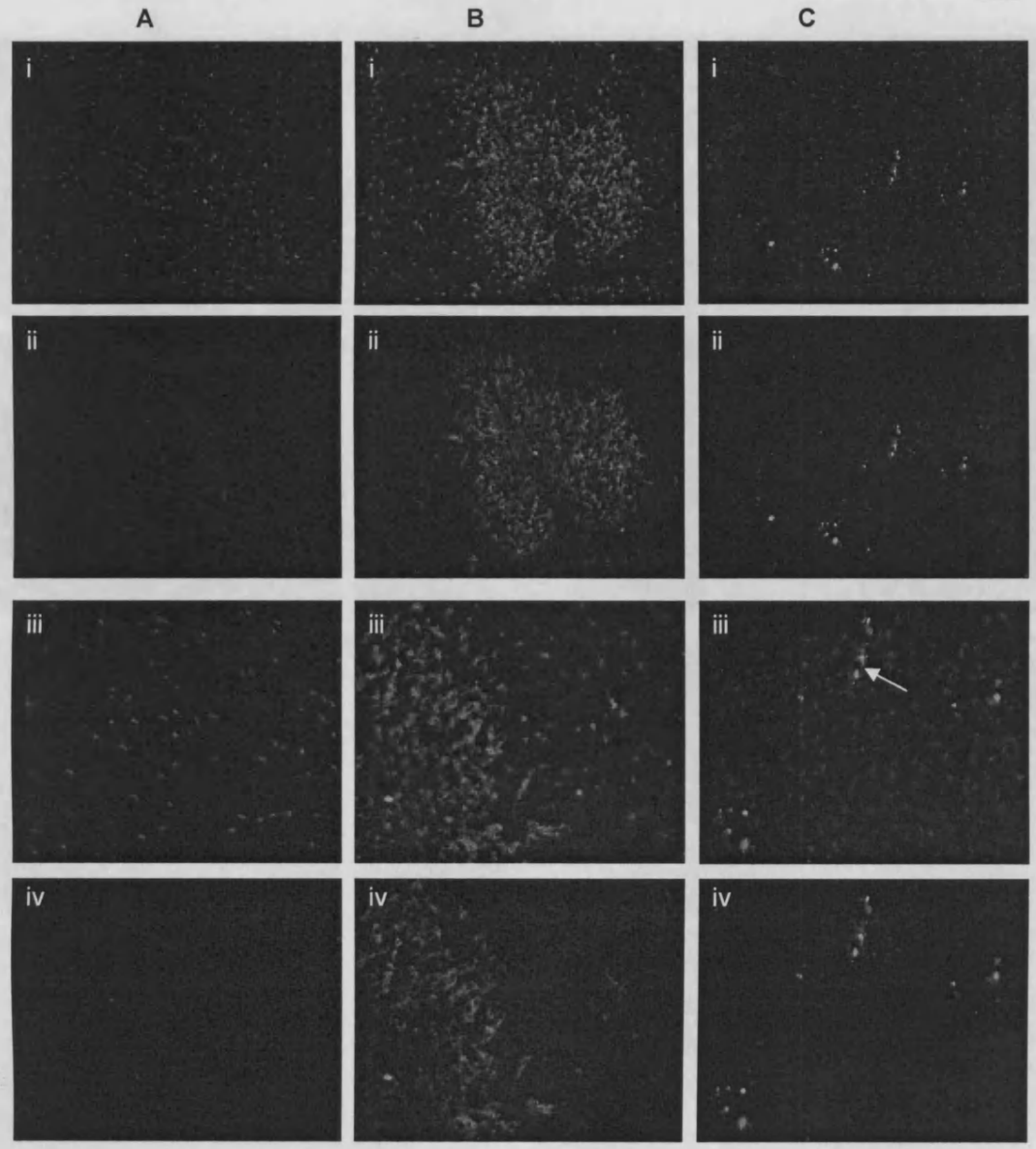


Figure 3.19 Expression of mGluR1 protein in spinal cord sections from acute and chronic phase EAE and adjuvant treated control rats.

Spinal cord sections were supplied by Prof. Reynolds' lab, cords were removed from rats at the acute and chronic phase of disease, cut in transverse sections then fixed in parafomaldehyde. Sections were double stained with an antibody raised against mGluR1 and an antibody raised against MHC class II antigens (OX-6) directly conjugated to FITC. A fluorescent secondary antibody (TRITC) was used to detect cells labelled with mGluR in sections taken from **(A)** adjuvant controls, **(B)** acute phase or **(C)** chronic phase animals. DAPI was used to detect nuclei. Overlaid images of all staining are shown (i and iii) as well as images with only mGluR and OX-6 staining (ii and iv). Original magnification x200 (i and ii) and x400 (iii and iv).

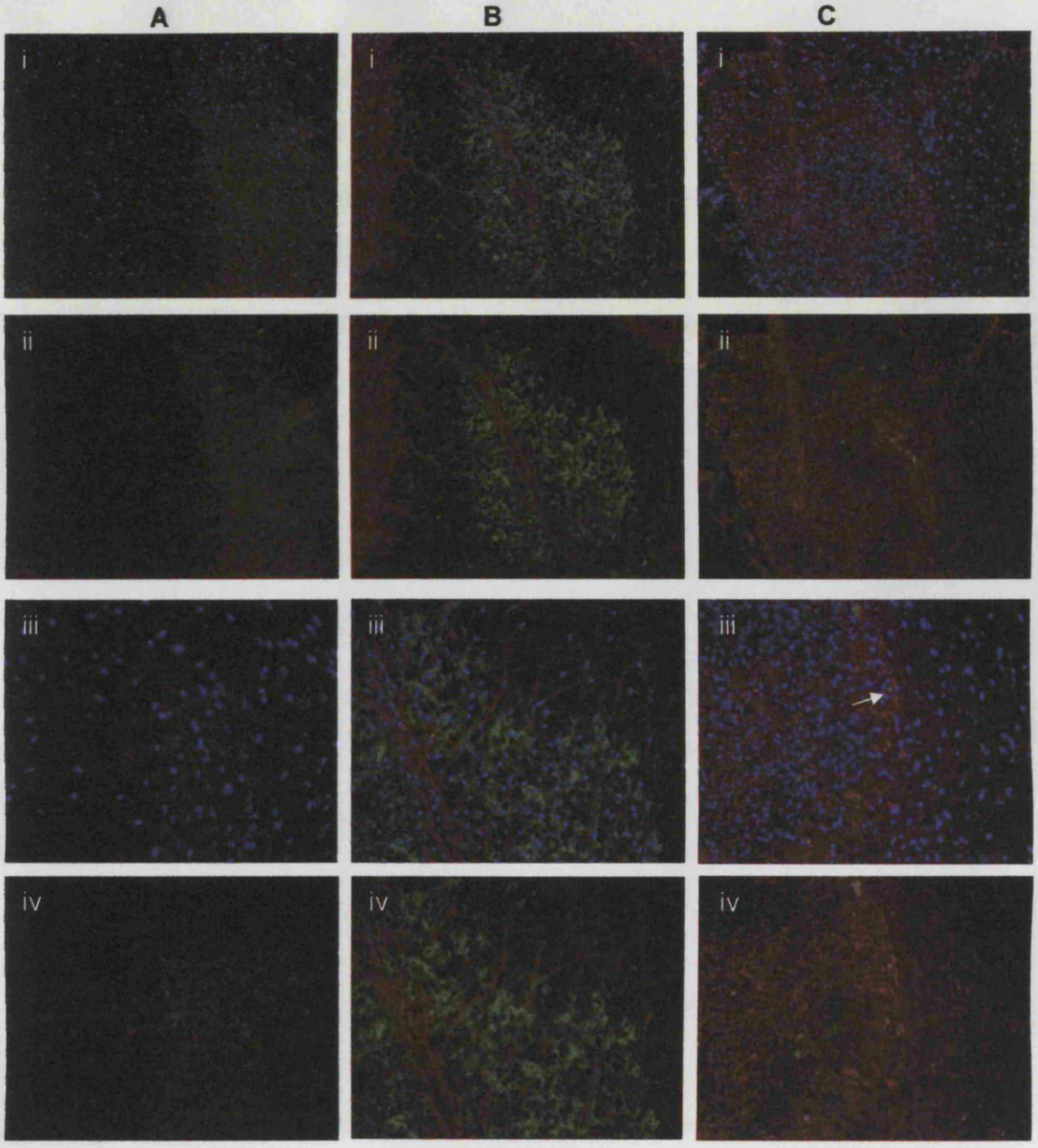


Figure 3.20 Expression of mGluR5 protein in spinal cord sections from acute and chronic phase EAE and adjuvant treated control rats.

Spinal cord sections were supplied by Prof. Reynolds' lab, cords were removed from rats at the acute and chronic phase of disease, cut in transverse sections then fixed in parafomaldehyde. Sections were double stained with an antibody raised against mGluR5 and an antibody raised against MHC class II antigens (OX-6 directly conjugated to FITC). A fluorescent secondary antibody (TRITC) was used to detect cells labelled with mGluR in sections taken from **(A)** adjuvant controls, **(B)** acute phase or **(C)** chronic phase animals. DAPI was used to detect nuclei. Overlaid images of all staining are shown (i and iii) as well as images with only mGluR and OX-6 staining (ii and iv). Original magnification x200 (i and ii) and x400 (iii and iv).

3.2.6 Group II mGluR expression in EAE

Group II mGluR expression was analysed as above. RT-PCR analysis showed that mGluR2 mRNA was expressed in the brains of both control and EAE mice and there was no significant difference between them (Figure 3.21A). Interestingly, the levels of mGluR2 in the spinal cord were notably lower than in the brain. In addition, cords taken from acute phase EAE animals showed significantly reduced levels of mGluR2 mRNA compared with control, almost to the point of complete absence ($P<0.05$).

Spinal cord sections from control animals demonstrated no specific MHC class II or mGluR2 staining suggesting no expression of glial cells, again the mGluR2 mRNA expression in control spinal cords may therefore be attributed to neuronal cells (Figure 3.22A). Acute EAE sections did have increased mGluR2 staining although not as noticeable as the upregulation of group I receptor staining, and again not localised to microglia (Figure 3.22B). Sections from the spinal cords of chronic EAE animals had low numbers of microglia compared with acute sections but did show elevated mGluR2 expression which in some cases was localised to the microglia (Figure 3.22C marked by arrow).

Analysis of mGluR3 mRNA in brain homogenates demonstrated no significant change between control and EAE conditions (Figure 3.21B). EAE spinal cord homogenates, however, had significantly lower levels of mGluR3 mRNA than controls cords ($P<0.05$). Stained spinal cord sections from control animals again indicated no positive staining for either MHC-II or mGluR3 (Figure 3.23A). Furthermore, acute EAE sections did not show upregulation of mGluR3 (Figure 3.23B). Sections from chronic animals also showed little mGluR3 staining although this did seem to be localised to the few microglia present (Figure 3.23C marked by arrow).

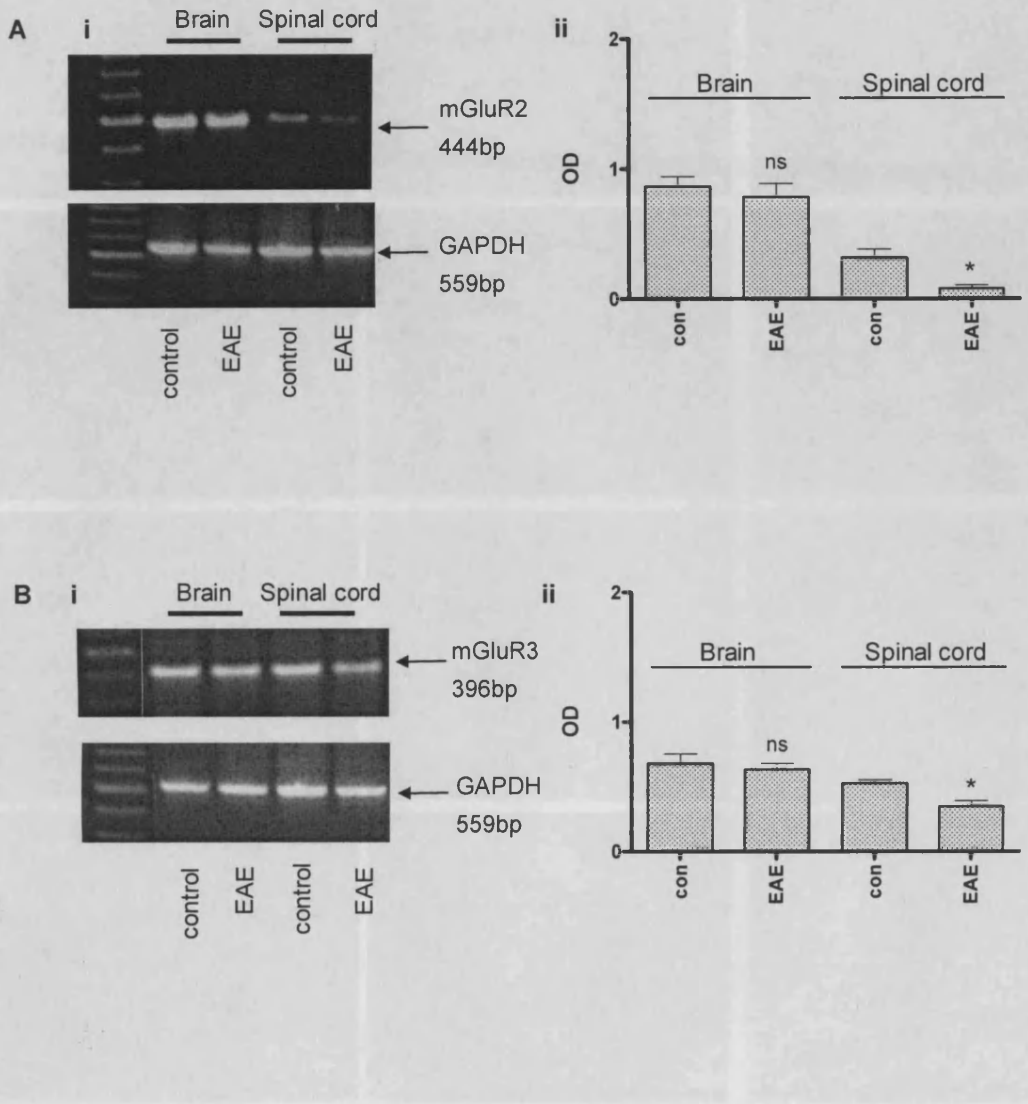


Figure 3.21 Expression of mGluR2 and 3 mRNA in brain and spinal cord homogenates from EAE and control mice

Brains and spinal cords were removed from mice at the acute phase of disease and from adjuvant injected control mice at the same time point. Levels of mGluR2 **(Ai and ii)** and 3 **(Bi and ii)** mRNA were measured in brain and spinal cord lysates by RT-PCR. Representative PCR gels are shown (Ai and Bi). GAPDH levels for each sample were also measured to ensure equal loading. Samples were quantitatively analysed by densitometry scanning. Results are shown as arbitrary densitometry units ± SEM for total mGluR/GAPDH mRNA (Aii and Bii). Expression of mGluR2 and 3 mRNA remained the same in brain lysates of control and EAE animals. Both mGluR2 and 3 mRNA levels decreased in EAE spinal cord lysates compared with control. n=4, *P<0.05.

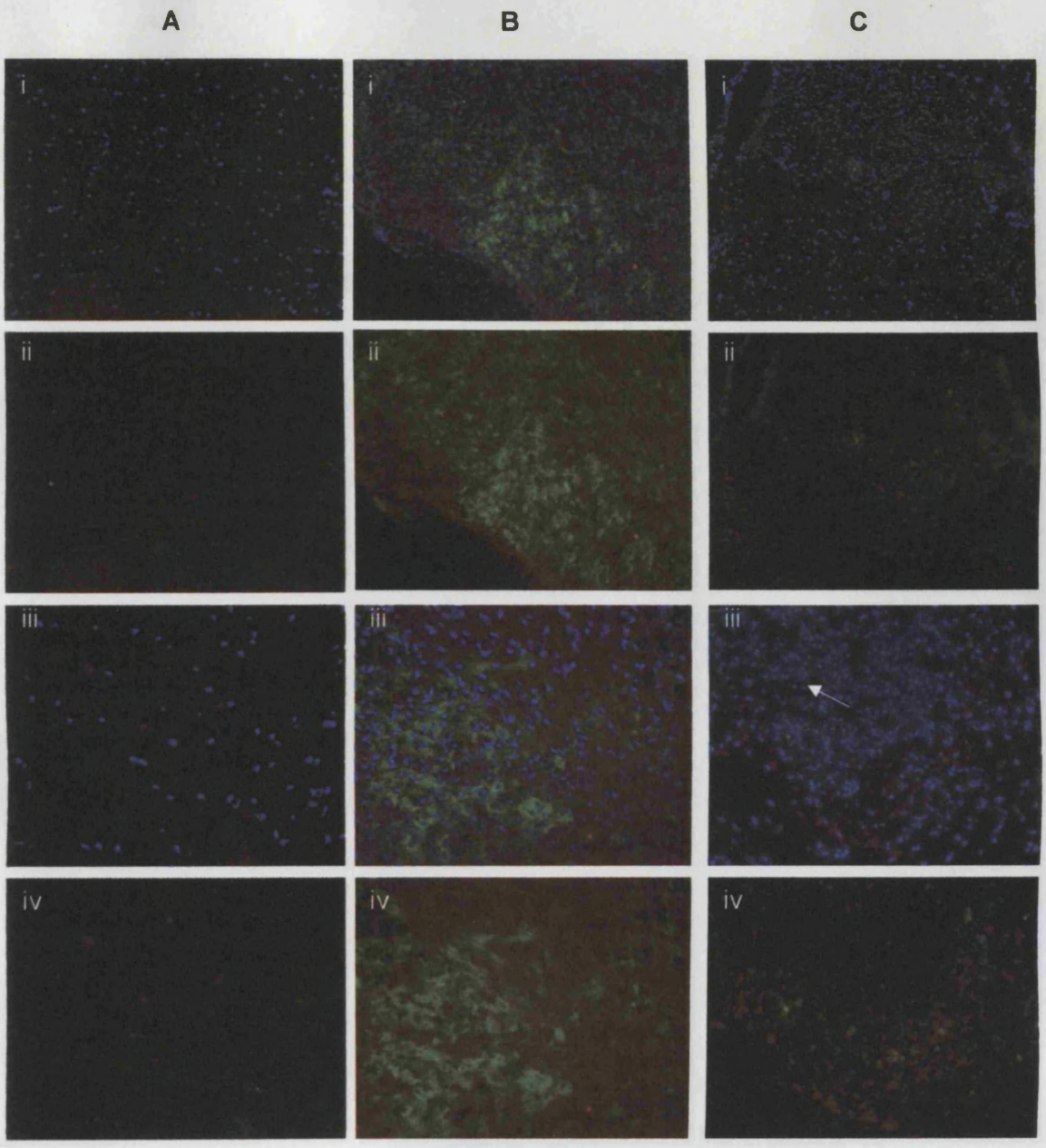


Figure 3.22 Expression of mGluR2 protein in spinal cord sections from acute and chronic phase EAE and adjuvant treated control rats.

Spinal cord sections were supplied by Prof. Reynolds' lab, cords were removed from rats at the acute and chronic phase of disease, cut in transverse sections then fixed in parafomaldehyde. Sections were double stained with an antibody raised against mGluR2 and an antibody raised against MHC class II antigens (OX-6 directly conjugated to FITC). A fluorescent secondary antibody (TRITC) was used to detect cells labelled with mGluR in sections taken from **(A)** adjuvant controls, **(B)** acute phase or **(C)** chronic phase animals. DAPI was used to detect nuclei. Overlaid images of all staining are shown (i and iii) as well as images with only mGluR and OX-6 staining (ii and iv). Original magnification x200 (i and ii) and x400 (iii and iv).

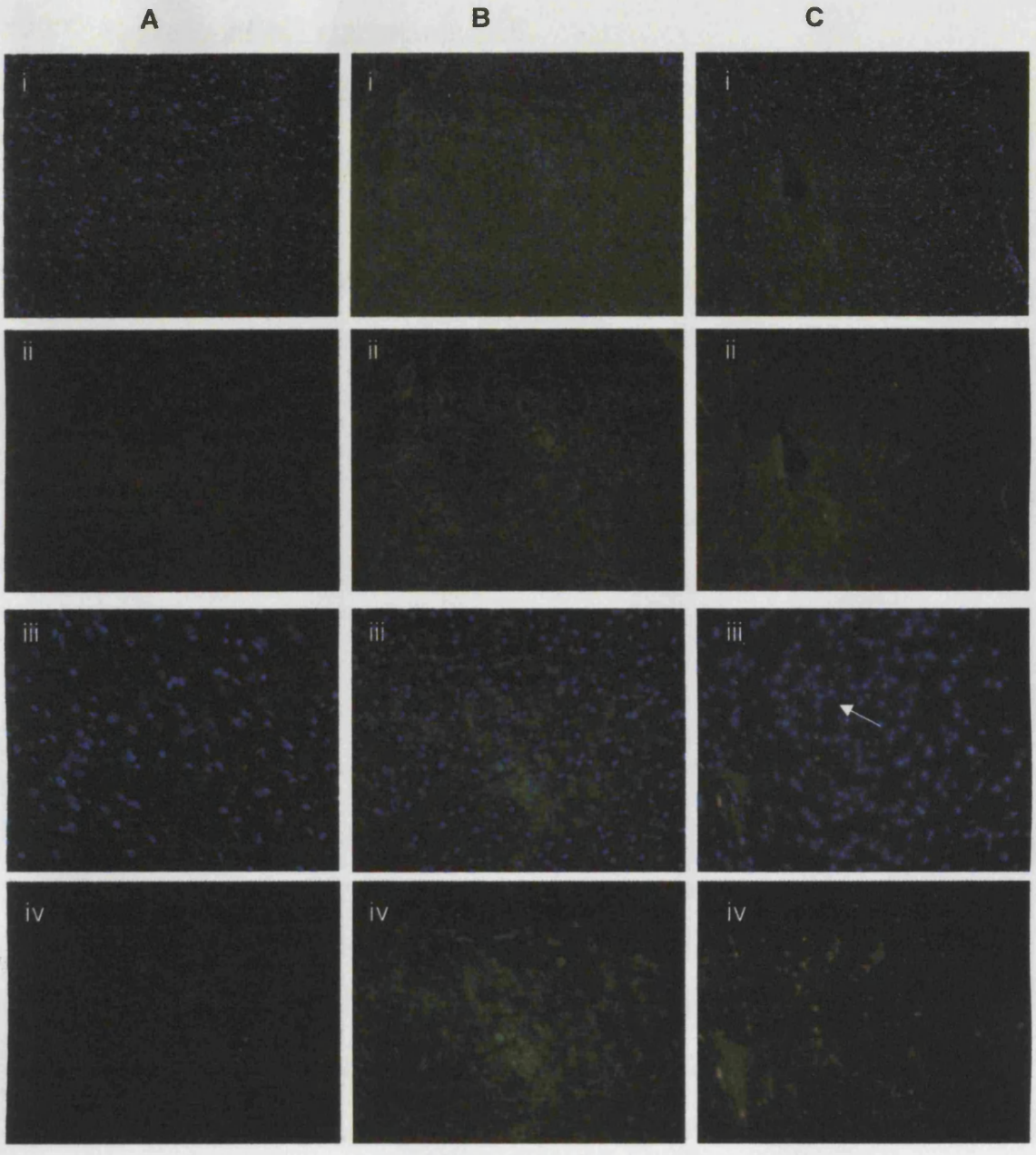


Figure 3.23 Expression of mGluR3 protein in spinal cord sections from acute and chronic phase EAE and adjuvant treated control rats.

Spinal cord sections were supplied by Prof. Reynolds' lab, cords were removed from rats at the acute and chronic phase of disease, cut in transverse sections then fixed in parafomaldehyde. Sections were double stained with an antibody raised against mGluR3 and an antibody raised against MHC class II antigens (OX-6 directly conjugated to FITC). A fluorescent secondary antibody (TRITC) was used to detect cells labelled with mGluR in sections taken from **(A)** adjuvant controls, **(B)** acute phase or **(C)** chronic phase animals. DAPI was used to detect nuclei. Overlaid images of all staining are shown (i and iii) as well as images with only mGluR and OX-6 staining (ii and iv). Original magnification x200 (i and ii) and x400 (iii and iv).

3.2.7 Group III mGluR expression in EAE

Group III mGluR expression was analysed as above. Homogenates were analysed for mGluR4 mRNA by RT-PCR. Brain homogenates taken from both control and EAE acute phase animals both expressed similar levels of mGluR4 mRNA (Figure 3.24A). Interestingly, expression of mGluR4 mRNA was significantly lower in spinal cord than in brain homogenates although there was no significant change between control and EAE conditions.

Spinal cord sections from control animals showed a very low number of cells positive for MHC class II antigen, which were also stained for mGluR4 (Figure 3.25A). Although the intensity of staining was extremely weak, this may imply that the few microglia in control cords express low levels of mGluR4. Acute EAE sections showed slight upregulation of mGluR4 staining which seemed to be co-localised to microglia though again this staining was very weak (Figure 3.25B). Microglial cells in chronic EAE sections were also positive for mGluR4 (Figure 3.25C).

mRNA for mGluR8 was expressed in brain homogenates although this was unchanged in EAE and control conditions (Figure 3.24B). Spinal cord homogenates also expressed mGluR8 mRNA which appeared to be lower in EAE cords compared with controls though this was not significant. Sections taken from the spinal cords of control animals expressed extremely low levels of MHC class II staining. Cells that were stained appeared very ramified suggestive of microglial cells at rest (Figure 3.26A). Acute EAE sections showed upregulation of mGluR8 staining which was localised to a small number of microglial cells (Figure 3.26B). Sections taken from cords of chronic EAE animals showed very weak staining for mGluR8 though this was localised to MHC class II staining (Figure 3.26C marked by arrow).

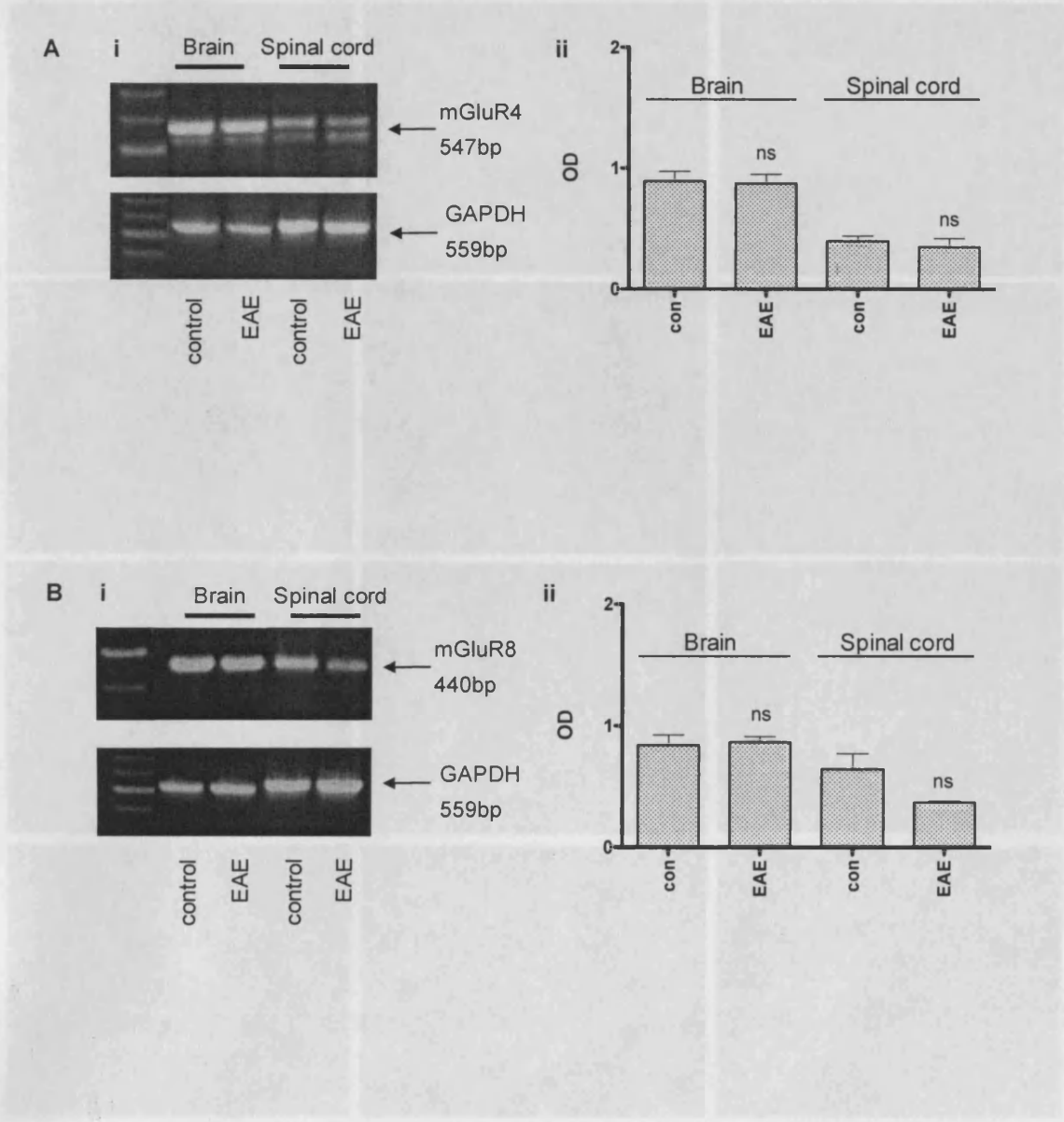


Figure 3.24 Expression of mGluR4 and 8 mRNA in brain and spinal cord homogenates from EAE and control mice

Brains and spinal cords were removed from mice at the acute phase of disease and from adjuvant injected control mice at the same time point. Levels of mGluR4 **(Ai and ii)** and 8 **(Bi and ii)** mRNA were measured in brain and spinal cord lysates by RT-PCR. Representative PCR gels are shown (Ai and Bi). GAPDH levels for each sample were also measured to ensure equal loading. Samples were quantitatively analysed by densitometry scanning. Results are shown as arbitrary densitometry units ± SEM for total mGluR/GAPDH mRNA (Aii and Bii). Expression of mGluR4 and 8 mRNA remained the same in both brain and spinal cord lysates of control and EAE animals. n=4, *P<0.05.

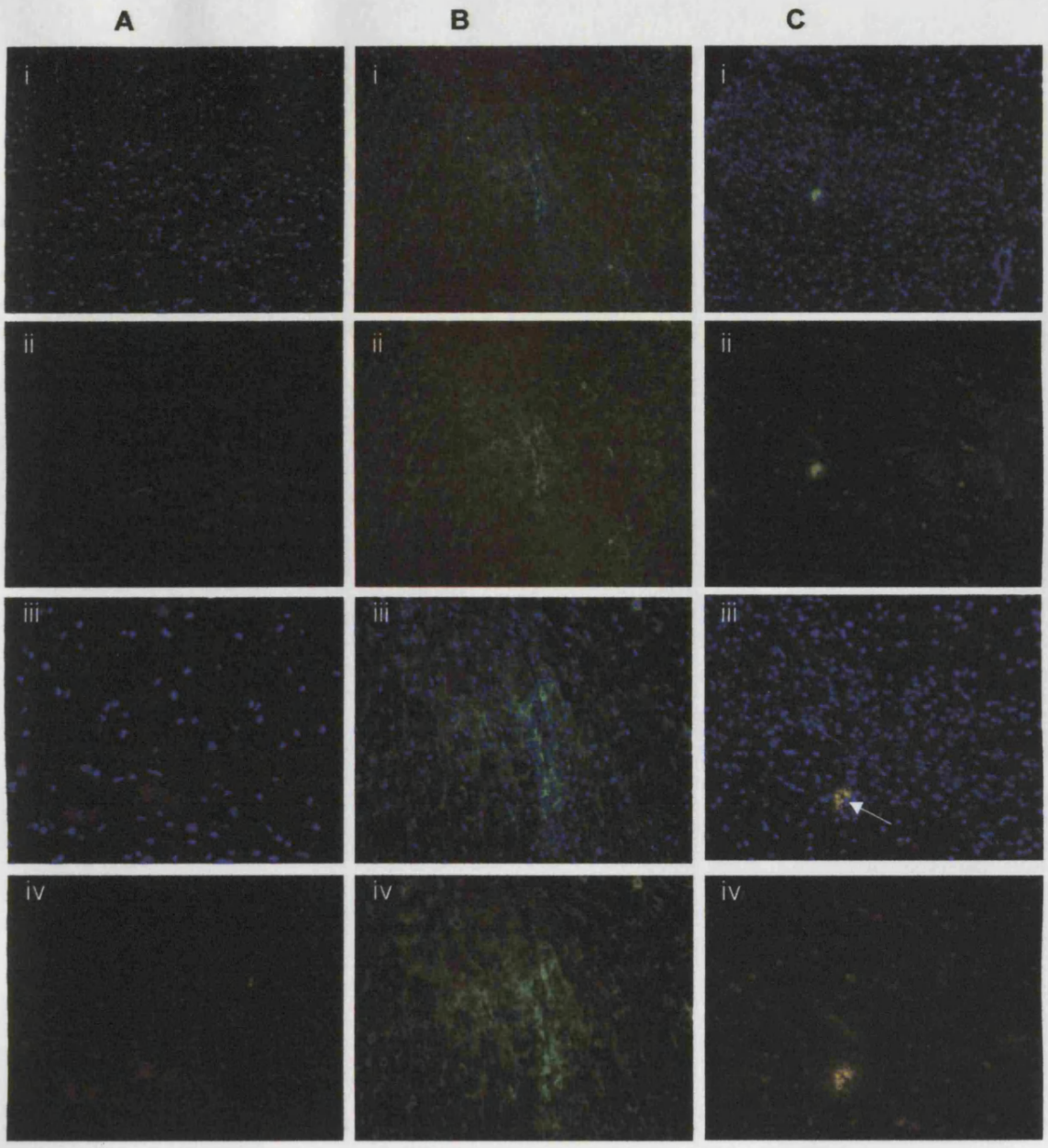


Figure 3.25 Expression of mGluR4 protein in spinal cord sections from acute and chronic phase EAE and adjuvant treated control rats.

Spinal cord sections were supplied by Prof. Reynolds' lab, cords were removed from rats at the acute and chronic phase of disease, cut in transverse sections then fixed in parafomaldehyde. Sections were double stained with an antibody raised against mGluR4 and an antibody raised against MHC class II (OX-6 directly conjugated to FITC). A fluorescent secondary antibody (TRITC) was used to detect cells labelled with mGluR in sections taken from **(A)** adjuvant controls, **(B)** acute phase or **(C)** chronic phase animals. DAPI was used to detect nuclei. Overlaid images of all staining are shown (i and iii) as well as images with only mGluR and OX-6 staining (ii and iv). Original magnification x200 (i and ii) and x400 (iii and iv).

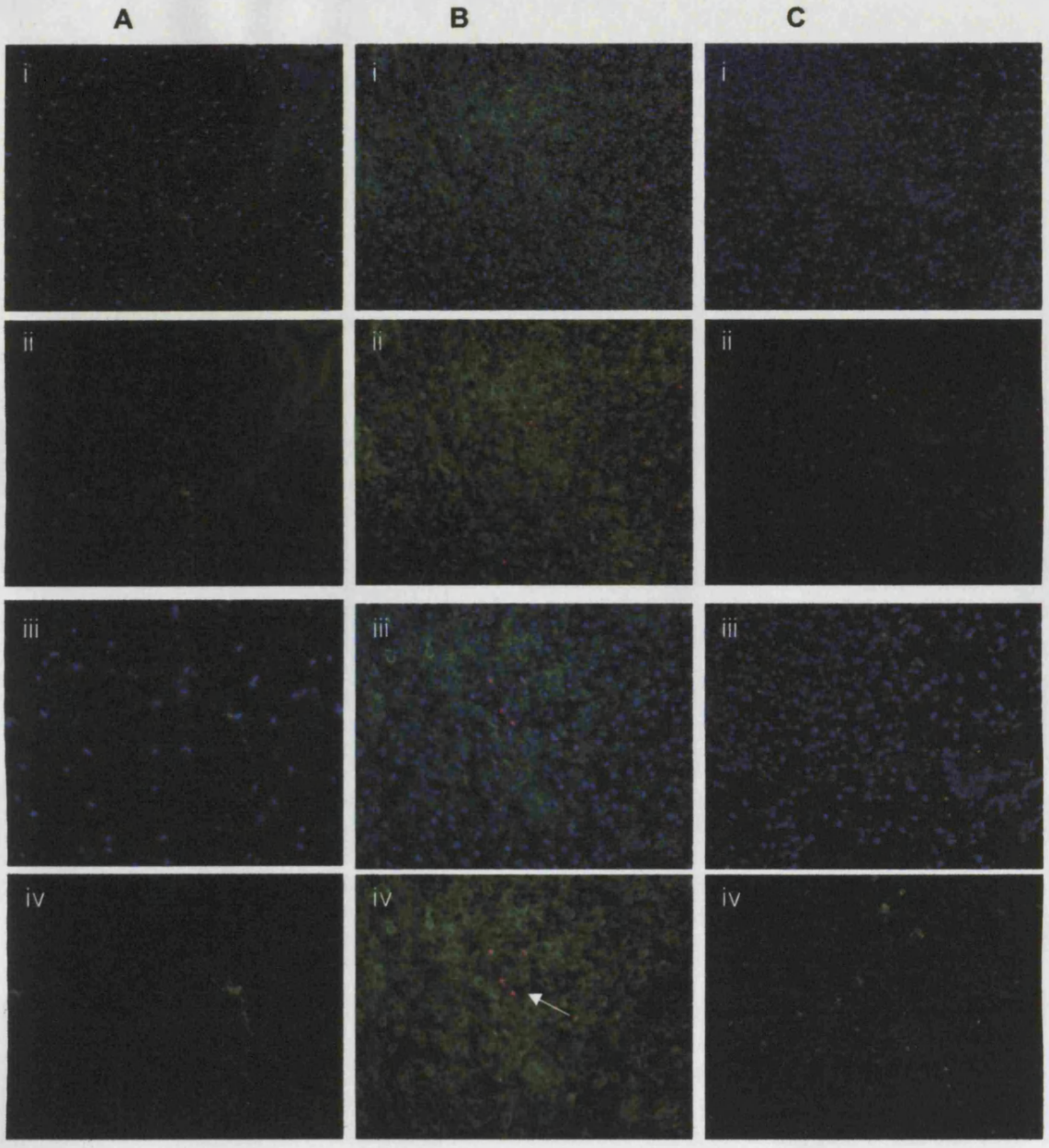


Figure 3.26 Expression of mGluR8 protein in spinal cord sections from acute and chronic phase EAE and adjuvant treated control rats.

Spinal cord sections were supplied by Prof. Reynolds' lab, cords were removed from rats at the acute and chronic phase of disease, cut in transverse sections then fixed in parafomaldehyde. Sections were double stained with an antibody raised against mGluR8 and an antibody raised against MHC class II (OX-6 directly conjugated to FITC). A fluorescent secondary antibody (TRITC) was used to cells labelled with mGluR in sections taken from **(A)** adjuvant controls, **(B)** acute phase or **(C)** chronic phase animals. DAPI was used to detect nuclei. Overlaid images of all staining are shown (i and iii) as well as images with only mGluR and OX-6 staining (ii and iv). Original magnification x200 (i and ii) and x400 (iii and iv).

3.3 Discussion

Astrocyte mGluR expression is modulated in response to different types of brain injury (Aronica et al., 2000, 2001; Ferraguti et al., 2001), and microglial mGluR subtypes are modulated in MS (Geurts et al., 2003, 2005). It is known that stimulation of group II microglial mGluRs induces a neurotoxic microglial phenotype and that stimulation of group III mGluRs on microglia can be neuroprotective (Taylor et al., 2002, 2003, 2005). The expression of mGluR subtypes on microglia is therefore of particular interest as microglial toxicity during disease could be due to upregulation of one subtype over another. These findings demonstrate that activation of microglia induced changes in their mGluR profile compared with unstimulated cells.

Confirming the findings of Biber et al., (1999), we showed that microglia constitutively express group I mGluRs. Expression of mGluR1 was clearly more localised to the nucleus while mGluR5 expression was concentrated on membranes and processes. This contradicts the findings of Hubert et al., (2001) who found most mGluR1 labelling in substantia nigra neurones located to the plasma membrane, whereas mGluR5 was primarily expressed in intracellular compartments, and serves to highlight the differences in mGluR expression between different cell types. It has been suggested that pre-active mGluRs are localised in the cytoplasm while trafficking to the membrane denotes a functionally active state. Indeed, activity-regulated increases in homer protein 1a expression disrupt binding of homer protein 1b to mGluRs, leading to increased trafficking of metabotropic receptors to the plasma membrane and additional functional surface receptors (Roche et al., 1999; Ciruela et al., 1999). Another possibility is that some functions necessitate a large intracellular pool of mGluRs which can be readily inserted into the membrane without the need for new receptors to be synthesized and transported (Szekeres et al., 1998).

It was found that microglial mGluR5 protein was more strongly expressed in both untreated and activated conditions, than any other subtype and was also primarily expressed in membranes and processes with little staining in the nucleus suggesting a functionally active state. Interestingly, analysis of mRNA expression by RT-PCR showed only a very weak signal for mGluR5 in contrast to other subtypes. Indeed activation with myelin which noticeably increased intensity of staining compared with control cells induced almost complete knock-down at the mRNA level. This may be due to mRNA levels being reduced once proteins are trafficked away to the membrane, it is possible that increases in mGluR5 mRNA did occur but were transient due to high turnover (Eliakim et al., 1997).

Microglia but not N9 cells constitutively expressed low levels of mGluR2. These results demonstrate that unstimulated microglia possessed mGluR2 protein that was visibly localised in the nucleus. Activation of microglia increased the intensity of staining in the cytoplasm but also induced expression of mGluR2 protein in the membrane and processes which may indicate mGluR trafficking and activation. This was confirmed by RT-PCR results, which showed that levels of microglial mGluR2 mRNA were increased by exposure to myelin and to a greater extent by LPS. These results are of interest as microglial mGluR2 stimulation induces microglial activation and a neurotoxic phenotype (Taylor et al; 2002, 2005). It is possible that the increased reactivity of microglia during disease or insult could be due to increased expression of 'neurotoxic' mGluR2. Indeed, group II mGluRs are upregulated in activated microglia surrounding MS lesions (Geurts et al., 2002).

MGluR3 was also found to be expressed in microglia but not in the N9 cell line. Microglia constitutively expressed higher levels of mGluR3 compared with mGluR2 in control untreated cells. It has been suggested that the mGluR3 receptor subtype may be neuroprotective as stimulation of mGluR3 in astrocytes induces release of TGFβ (Bruno

et al., 1998). Interestingly however, levels of mGluR3 were increased by exposure to LPS. This is possibly in response to cell activation perhaps to minimise damage by toxic substances released and balance the increase in mGluR2 expression.

Of the group III mGluRs (mGluRs 4, 6, 7 and 8) N9 cells only expressed mGluR4 subtype while microglia expressed all subtypes bar mGluR7. Immunolocalisation of mGluR4 demonstrated that microglial expression of this protein is localised in the cytoplasm around the nucleus suggesting a pre-active state. Analysis of group III mRNA levels showed that mGluR4 and 6 were extremely low so it was difficult to accurately assess whether activation induced any changes in expression. Microglial mGluR8 expression, however, was upregulated by exposure to myelin. It would therefore be of interest to see which subtype of group III mGluR was responsible for enhancing the neuroprotective phenotype of microglia. Studies using mGluR4 knock out mice have shown that selective activation of mGluR4 in cortical cultures affords neuroprotection against excitotoxic cell death (Bruno et al., 2000). Stimulation of microglial group III mGluRs can also be neuroprotective (Taylor et al., 2003), as such, the low constitutive expression of these receptors in resting microglia may explain their early reactivity in response to infection or injury.

The differences in mGluR phenotype between primary mcroglia and N9 cells were to be expected since N9s are an immortalised cell line and cultured for longer periods of time. Indeed, culturing microglia has been shown to change the pattern of a number of receptors and ion channels (Kettenmann et al., 1993; Eder et al., 1997), and furthermore, differences in culture conditions and age are known to alter mGluR mRNA expression (Catania et al., 1994; Schools and Kimelberg, 1999).

Analysis of mGluR mRNA expression in control and EAE mice showed no significant changes in mGluR mRNA in the brains of EAE compared with control mice. This was expected since most pathology in the EAE model is found in the spinal cord.

As such, there were distinct and significant reductions in the levels of mGluR5, mGluR2 and mGluR3 mRNA in EAE spinal cords compared with controls. Since sections of acute EAE showed dramatic increases in the number of MG/Ø, the decreased mGluR expression recorded in EAE spinal cord homogenates most likely reflects decreased neuronal or other glial mGluR mRNA, which as demonstrated in recent literature have different functions compared with microglial mGluRs (Bruno et al., 1995; Buisson and Choi, 1996; Attwell et al., 1998; Allen et al., 1999; Taylor et al., 2002, 2005). Immunohistochemistry on spinal cord sections however, demonstrated a notable increase in mGluR1, mGluR5 and mGluR2 expression in the acute phase of EAE which is more likely attributable to glial cells in line with the findings of Geurts et al., (2003), and also since these subtypes were either down-regulated (mGluR5 and 2) or unaltered (mGluR1) in the EAE spinal cord homogenates. Although this positivity was not frequently co-localised to microglia, it is possible that the staining observed was astrocytic.

Interestingly, some co-localisation of microglial and mGluR8 staining was observed in acute EAE sections, which is in accordance with previous work showing microglial mGluR8 expression in human actively demyelinating MS lesions (Geurts et al., 2005). These results are consistent with the upregulation of microglial mGluR8 mRNA induced by exposure to myelin and the finding that mGluR8 was generally not observed in human pre-active lesions (Geurts et al., 2005) which suggests that upregulation of this subtype is not simply the result of activation but possibly influenced by demyelination and may indicate an attempt to limit microglial toxicity.

The low expression of mGluRs on microglia, as evidenced by PCR data in primary cultures, probably make it difficult to attain positive results when staining sections. Furthermore, modulation of microglial mGluR expression in EAE is unlikely to be visible in PCR analysis of whole brain and spinal cord homogenates due to the

relatively small proportion of microglia to neurones and other glial cells, which is compounded by the low expression of mGluRs on microglia. Further work is required to elucidate the microglial expression of mGluRs in different stages of EAE, this could possibly be achieved by isolating microglia from EAE animals and would allow analysis of microglial mGluR expression through disease progression.

4.0

MYELIN INDUCES A REACTIVE MICROGLIAL PHENOTYPE WHICH CAN BE MODULATED BY METABOTROPIC GLUTAMATE RECEPTORS

4.1 Introduction

Microglia are implicated in the pathology of MS and have been localised in active lesions (Hill et al., 2004). Since myelin breakdown is a feature of MS and microglia have been shown to actively phagocytose myelin (Mosley et al., 1996), the effects of myelin on microglial reactivity and subsequent neurotoxicity were examined. Reactivity of microglia was assessed by upregulation of ED-1, a marker for the lysosomal compartment which is expressed by activated phagocytic microglia. Microglial neurotoxicity was determined by applying microglial conditioned media to CGCs followed by quantification of apoptotic cells by Hoechst staining.

The inflammatory mediators TNFα and iNOS may also be involved in demyelinating pathology. Indeed reactive microglia in active lesions have been shown to express iNOS (Hill et al 2004), the enzyme responsible for NO production which is toxic to oligodendrocytes (Merrill et al., 1993). TNFα has also been implicated in the death of oligodendrocytes and has been linked with NO mediated toxicity (Mitrovik et al., 1994), which implies a pivotal role for both TNFα and iNOS in lesion formation. Since myelin debris and microglia are both present in MS lesions, microglial release of TNFα and expression of iNOS was examined after incubation with myelin.

It has been shown that modulation of group II microglial mGluRs can be neurotoxic (Taylor et al., 2002) and that activation of group III mGluRs can be neuroprotective (Taylor et al., 2003). This work aimed to determine whether modulation of microglial mGluRs could regulate myelin induced microglial neurotoxicity. The mechanisms of mGluR modulation of microglial toxicity were also examined. Since

mGluRs are not the primary mediators of fast synaptic transmission, the modulation of these receptors may represent a therapeutic strategy in MS.

4.2 Results

4.2.1 Myelin induces a reactive microglial phenotype

In the following experiments microglia were plated at 90,000 cells per coverslip as described and incubated at 37°C for 24 hours before use. Myelin was added to microglia at concentrations ranging from 1ng/ml to 10μg/ml. Microglial activation was assessed in a number of ways including quantification of ED-1 expression, TNFα, glutamate and nitrite release, and expression of iNOS.

Localisation of myelin within and around microglial processes was confirmed by double immunocytochemical detection of myelin oligodendrocyte glycoprotein (MOG) and isolectin B4 a microglial marker (Streit and Kreutzberg, 1987) (Figure 4.1A). Myelin was stained with an antibody raised in mice against rat MOG protein kindly provided by Professor David Baker. The secondary antibody was specific for the primary antibody as no staining was observed in negative controls in which primary antibody was ommited (Figure 4.1B). Ideally. the specifity of the primary antibody should also have been tested by peptide blocking.

Activation of microglia incubated with myelin was assessed by positive staining with enhanced ED-1. Representative figures for microglial ED-1 staining are shown (Figure 4.2). ED-1 positive cells were quantified as a percentage of total number of cells per field as determined by DAPI staining (Figure 4.3). Untreated microglial cultures expressed very low (<20% ± 0.53) levels of ED-1 protein. Exposure of microglia to 0.01μg/ml myelin induced significantly higher levels of ED-1 expression than those observed in untreated cells ($P<0.05$). Greatest ED-1 expression was induced by 10μg/ml myelin ($P<0.01$). There was no significant difference, however, between the ED-1 levels induced by 10μg/ml and 1μg/ml myelin ($P=0.1709$).

To observe the timecourse of microglial reactivity these experiments were repeated exposing microglia to 1μg/ml myelin for 1, 4, 8, 24 and 48 hours at 37°C. The percentage of cells expressing ED-1 was analysed at these timepoints (Figure 4.4). Quantification of ED-1 expression as described above showed that basal levels of ED-1 found in control untreated microglia were similar to those found in previous experiments and were lower than 20% (±1.1%) (Figure 4.5). There was no significant difference between ED-1 expression in control cells and those exposed to myelin for under 8hrs ($P=0.7752$). However, after 24 hours incubation with myelin, over 70% (± 3.6%) of microglia were positive for ED-1 which was significantly higher than levels found in untreated cultures ($P<0.01$). Continued exposure to myelin for a further 48 hours did not significantly increase the expression of ED-1 compared with expression after 24 hours ($P=0.0584$). This was probably due to cells dying and floating off the coverslips (Figure 4.5) since there were consistently less microglia per field after incubation with myelin for 48 hours (observation, data not shown).

Western blotting for microglial iNOS protein was performed and revealed a prominent band at the predicted molecular weight for iNOS protein of 110 kDa (Figure 4.6B). Treatment with myelin for 8 hours induced very low levels of iNOS expression in microglia and this was further increased by myelin exposure for 24 and 48 hours. Microglial lysates were also analysed for phospho-ERK protein, these data suggest that untreated cells do not express phospho-ERK while incubation with myelin for 24-48 hours induced phospho-ERK expression above control levels (Figure 4.6A). The levels of nitrite released by microglia were also assessed by analysis of total nitrite in microglial conditioned medium (MGCM) collected from the cells after 24 hours incubation with myelin (Figure 4.6C). Untreated cells released small amounts of nitrite at levels consistently less than 100nM/μg/μl. As expected, nitrite levels were significantly increased in MGCM from cells treated with myelin ($P<0.05$) although

exposure to LPS caused very high amounts of nitrite release ($P<0.001$) which were above the levels induced by myelin.

Since TNFα and glutamate have both been implicated in the pathology of MS and are known to be toxic to oligodendrocytes in vitro, we also collected MGCM for analysis of release of these substances by microglia treated with myelin for 1-48 hours. We confirmed previous findings that non-activated microglia release less than 50pg/ml TNFα (Ciesielski- Treska et al., 1998; Kim et al., 2004; Taylor et al., 2005). These levels did not increase after 24 or 48 hours (Figure 4.7A). Both myelin and LPS potently induced early TNFα release in microglia after 4 hours. Treatment with myelin for 4 hours induced TNFα release of ~400pg/ml ($P<0.001$) which increased to ~1000pg/ml by 24 hours ($P<0.001$). After 48 hours, TNFα release plateaued and the increases observed were not statistically significant compared with TNFα release after 24 hours. Levels of microglial glutamate release were also increased by incubation with myelin after 24 hours and both myelin and LPS after 48 hours (Figure 4.7B). Untreated microglia consistently released ~10μM/μg/μl glutamate while treatment with myelin for 24 hours caused an increased release of ~22μM/μg/μl ($P<0.05$).

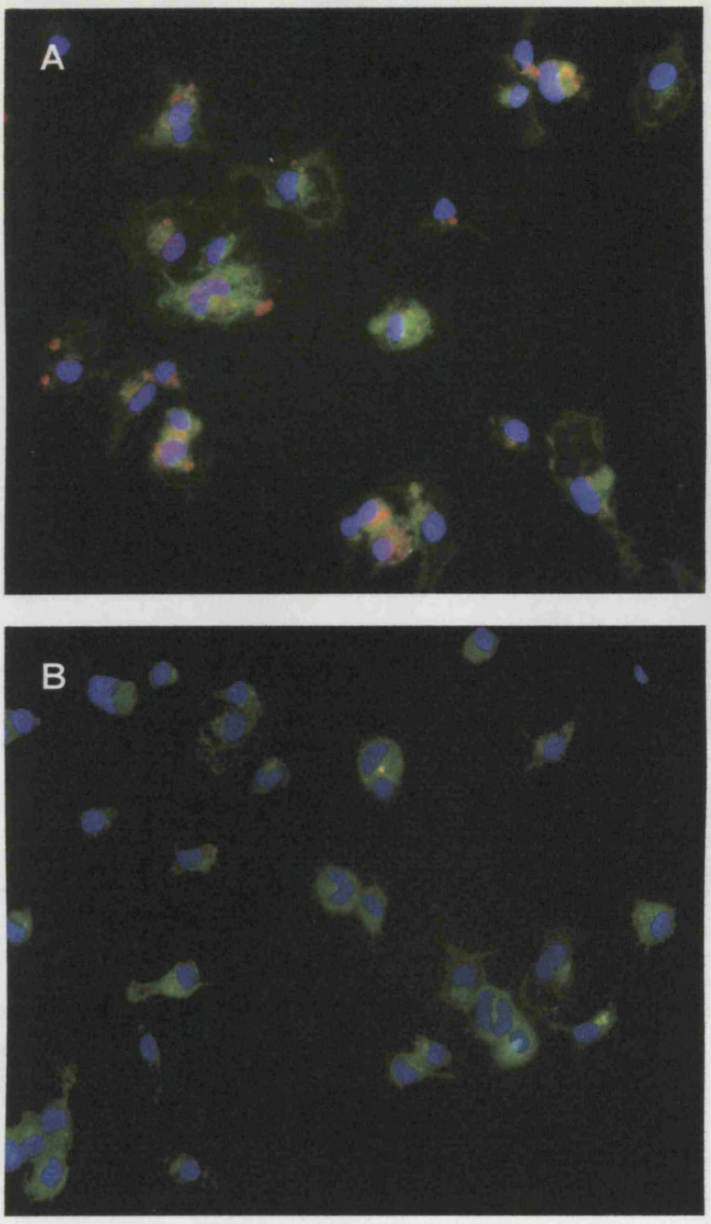


Figure 4.1 Myelin incubated with microglia for 24 hours is co-localised to microglial processes.

Microglia were incubated with myelin (1μg/ml) for 24 hours before being fixed in 4% F-PBS. **(A)** Microglia were co-stained with antibodies raised against myelin oligodendrocyte glycoprotein (MOG). A secondary antibody conjugated to TRITC was used (red staining). Isolectin B4 which is a marker for microglia and directly conjugated to FITC (green staining) and DAPI a nuclear marker (blue staining) were also used. There was noticeable co-localisation of staining for MOG and isolectin B4 showing myelin in and around microglial processes. **(B)** Negative control in which primary antibody against MOG was omitted. There was no TRITC staining which indicates that the positive staining in figure A was specific.

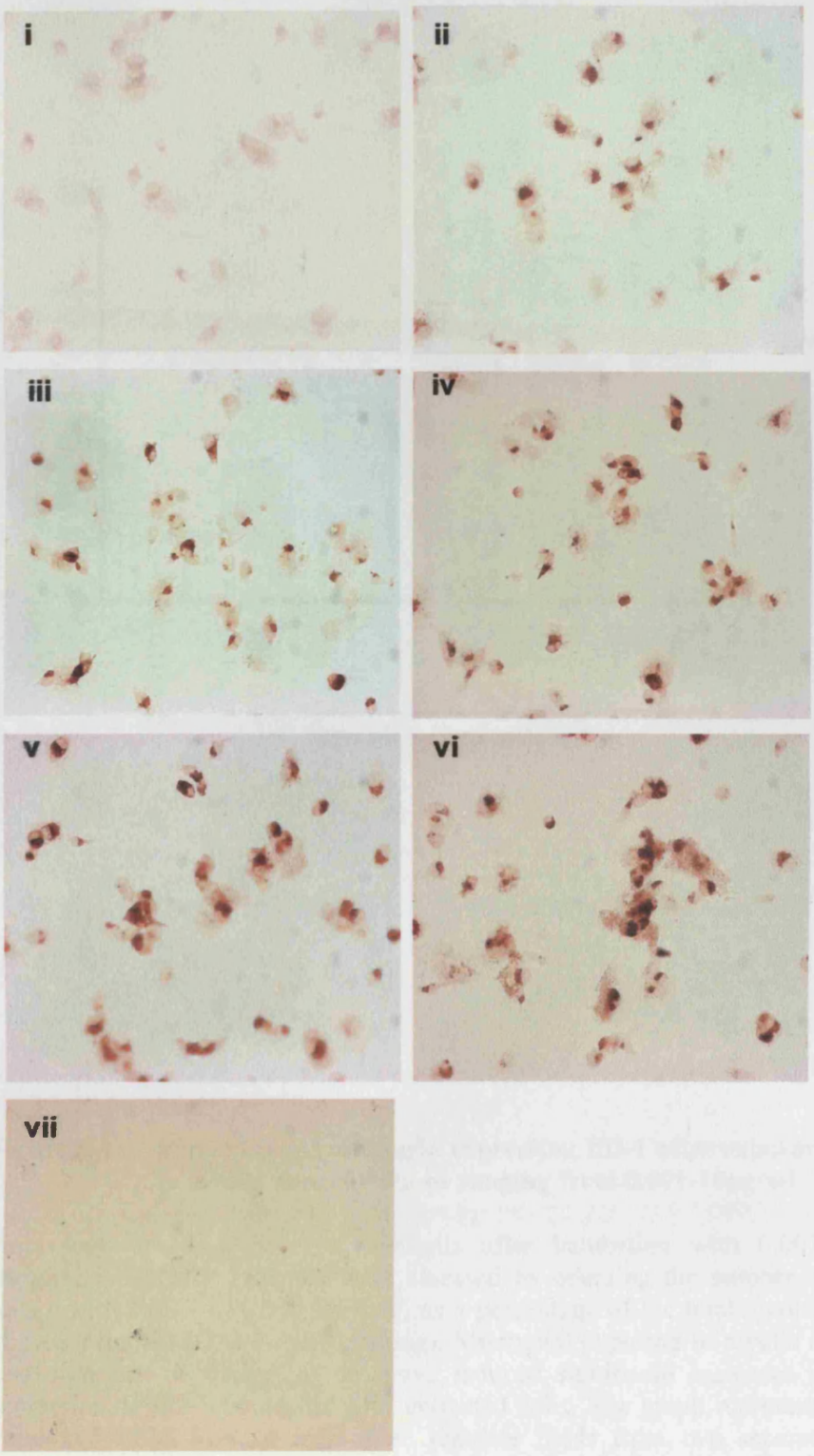


Figure 4.2 Increasing microglial ED-1 expression following exposure to myelin concentrations ranging from 0.001- 10µg/ml

Cultured microglia were either left untreated (i) or incubated with 0.001 (ii) 0.01 (iii) 0.1 (iv) 1 (v) or 10 (vi) µg/ml myelin for 24 hours then fixed in 4% F-PBS. Microglia were co-stained with an antibody raised against ED-1 antigen, a marker for activation and phagocytosis, followed by diaminobenzadine tetrahydrochloride (DAB), and DAPI, a nuclear stain (not shown). A negative control (vii) where primary antibody was omitted shows no positive staining which shows that ED-1 staining was specific.

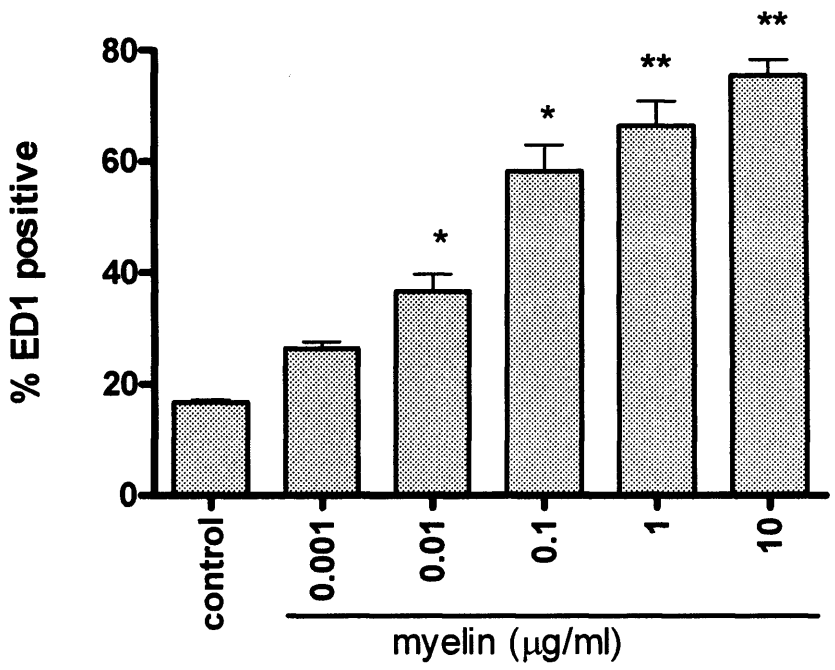


Figure 4.3 Percentage of microglia expressing ED-1 after exposure to myelin concentrations ranging from 0.001-10μg/ml

Percentage of ED-1 positive microglia after incubation with 0.001-10μg/ml myelin for 24 hours were assessed by counting the number of cells positive for ED-1 (red staining) as a percentage of the total number of cells per field (DAPI –blue staining). Microglial exposure to myelin at concentrations of 0.01μg/ml or above induced significant increases in expression of ED-1 compared with untreated cells. The graph represents mean and SEM from at least three separate fields from two separate coverslips repeated in at least two different experiments. *P<0.05, **P<0.01 versus control.

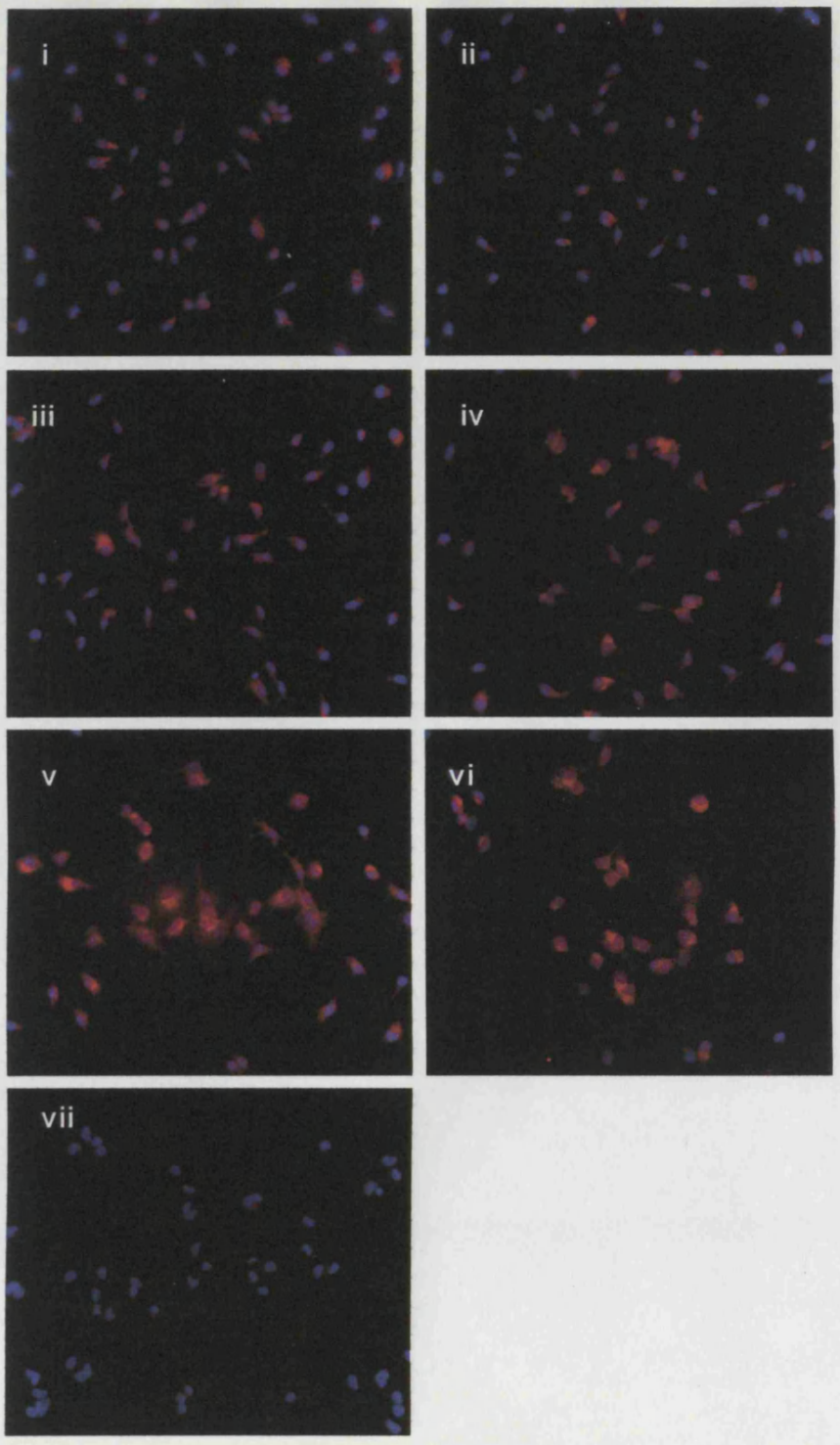


Figure 4.4 Microglial ED-1 expression following exposure to 1μg/ml myelin for 1-48 hours

(A) Cultured microglia were either left untreated (i) or incubated with myelin (1μg/ml) for 1h (ii) 4h (iii) 8h (iv) 24h (v) or 48h (vi) then fixed in 4% F-PBS. Microglia were then co-stained with an antibody raised against ED-1 antigen, a marker for activation and phagocytosis. Positive labelling of ED-1 was detected by a fluorescent secondary antibody conjugated to TRITC. Microglia were also stained with DAPI, a nuclear stain (blue staining). A negative control where primary antibody was omitted is shown (vii) and there was no obvious red staining indicating that the positive staining in the other conditions is specific for ED-1.

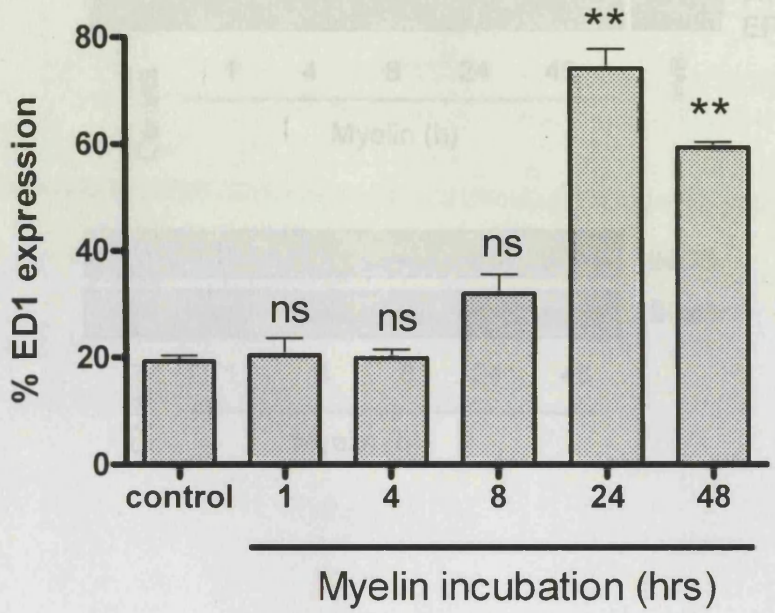


Figure 4.5 Percentage of microglia expressing ED-1 following exposure to 1μg/ml myelin for 1-48 hours.

Percentage of microglia expressing ED-1 after incubation with 1μg/ml myelin for 1, 4, 8, 24, and 48 hours were assessed by counting the number of cells positive for ED-1 as a percentage of the total number of cells per field (DAPI). Incubation of microglia with myelin for over 24 hours obviously increased expression of ED-1 on the cells. The graph represents mean and SEM from at least three separate fields from two separate coverslips repeated in at least two different experiments. **P<0.01 versus control.

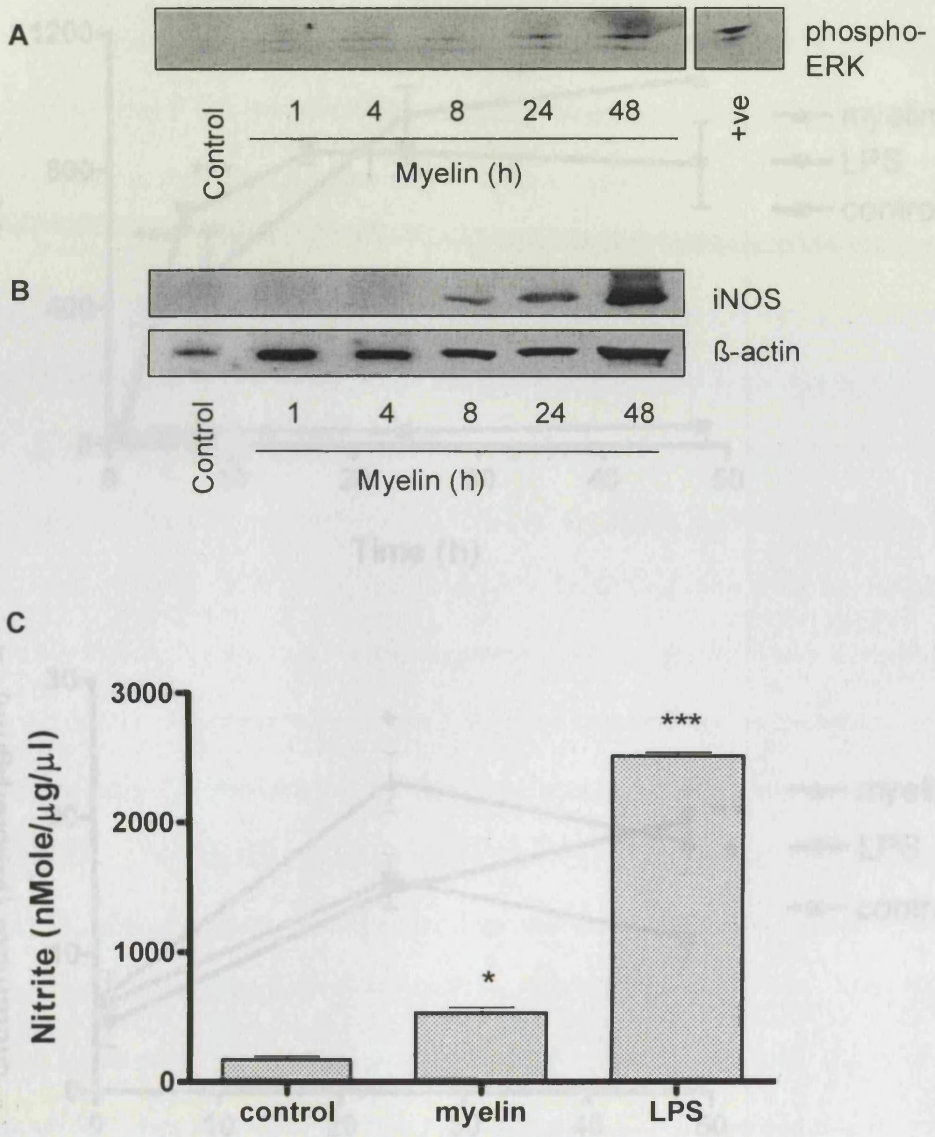


Figure 4.6 Expression of phospho-ERK and iNOS protein and release of nitrite from microglia exposed to myelin for 1-48 hours

Microglia were incubated with 1µg/ml myelin for 1- 48 hours after which protein lysates were collected. Phosphorylated ERK **(A)** and iNOS **(B)** expression were analysed by western blotting. Unstimulated microglia were used as a negative control and an IFNγ+LPS treated macrophage lysate was used as a positive control. The Phospho-ERK blot is representative of lysates taken from a single experiment. iNOS blots were repeated at least twice. **(C)** Conditioned medium was collected from microglia either exposed to myelin or LPS, or left untreated for 24 hours and analysed for total nitrite release. Myelin induced significant increase in the release of nitrite from microglia compared with untreated cells. Results represent mean and SEM from at least two separate experiments *P<0.05, ***P<0.001 versus control.

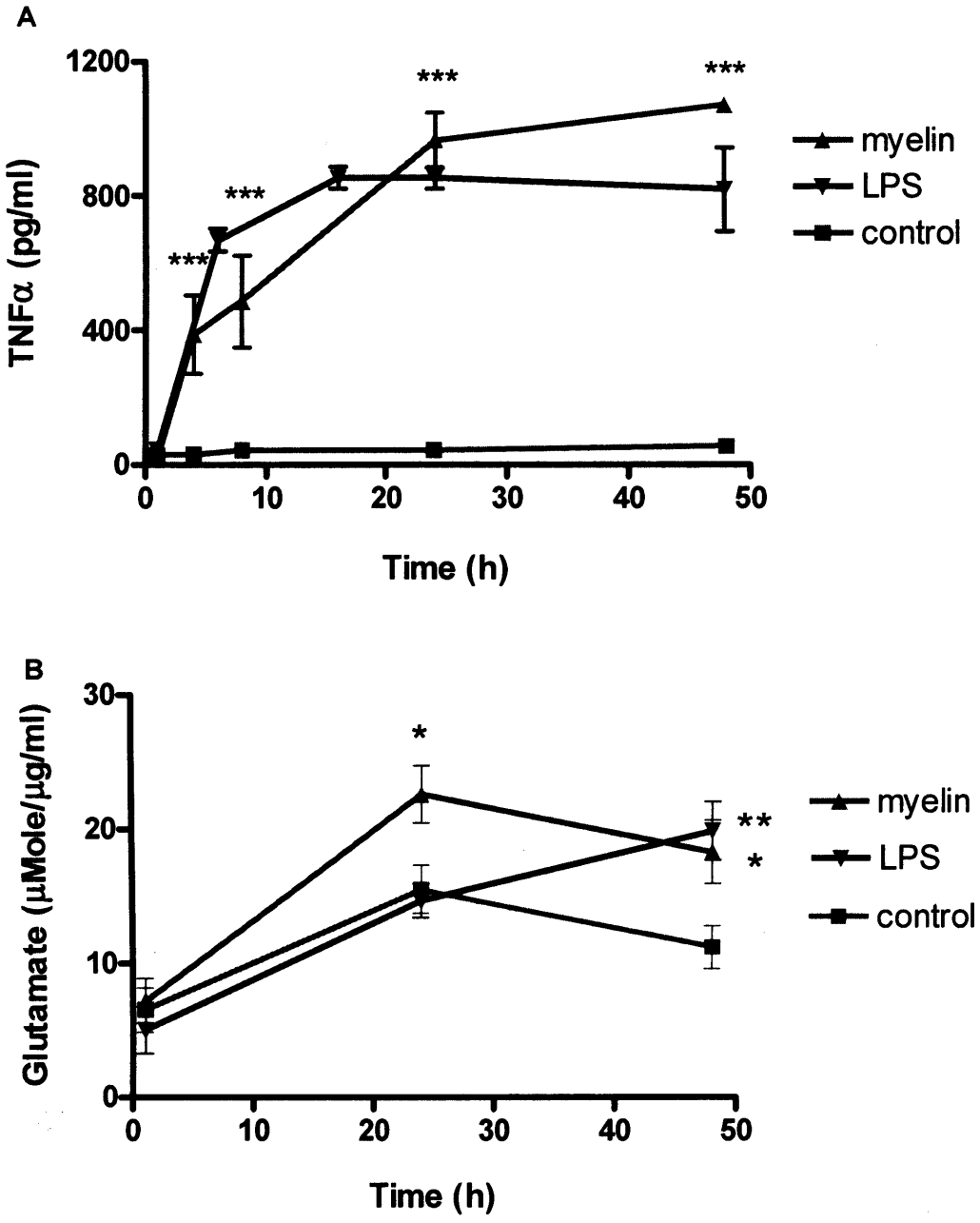


Figure 4.7 Microglial exposure to myelin or LPS for 1-48 hours induces significant release of TNFα and glutamate after 24 hours.

Microglia were either left untreated or incubated with 1µg/ml myelin or LPS for 1-48 hours, conditioned medium was collected at specified time points. **(A)** Conditioned medium was analysed for release of TNFα by ELISA. Incubation of microglia with myelin or LPS induced significantly increased TNFα release after 4 hours compared with untreated cells. **(B)** Glutamate release from microglia was assessed, after 24 hours microglia incubated with myelin released significantly higher amounts of glutamate compared with untreated cells. Values represent means +SEM taken from three different coverslips per condition and repeated in at least two separate experiments. *P<0.05, **P<0.01, ***P<0.001 versus control at the same timepoint.

4.2.2 Myelin causes microglia to become neurotoxic

Neurotoxicity of microglia was assessed by adding the CM from microglial cultures to neurones *in vitro* for 24 hrs at 37°C. Resultant neuronal apoptosis was quantified by Hoechst staining where apoptotic nuclei appear condensed, pyknotic and brightly stained and can be counted as a percentage of total viable cells per field. CM collected from untreated microglia (used as control CM), or microglia exposed to 0.001-10μg/ml myelin, was applied to neurones in culture (Figure 4.8). Neurones left in their own medium were used as negative controls to assess basal levels of apoptosis. There was no significant difference between the levels of apoptosis in basal conditions and those caused by control CM ($P=0.2682$) which induced less than 10% (± 1.6%) of neurones to become apoptotic, suggesting that control microglia do not release neurotoxic factors. Microglia treated with 0.01μg/ml myelin became neurotoxic as at this concentration CM induced significantly increased apoptosis compared with CM from untreated cells ($P<0.05$). CM from microglia exposed to 10μg/ml myelin induced over 40% (± 3.2%) apoptosis ($P<0.001$) but there was no significant difference between apoptosis induced by 10μg/ml or 1μg/ml myelin MGCM ($P=0.7586$). CM from microglia treated with LPS was used as a positive control for neurotoxicity and both 0.1 and 1μg/ml LPS induced around 40% (± 2.1%) apoptosis in neuronal cultures, this was not significantly different from the apoptosis caused by 1μg/ml myelin ($P=0.6411$). These results parallel data from ED-1 experiments which demonstrated that myelin caused microglia to become activated and neurotoxic at concentrations of 1μg/ml and above although increasing concentrations above 1μg/ml did not significantly increase neurotoxicity.

To observe the timecourse of microglial neurotoxicity these experiments were repeated applying CM from microglia exposed to myelin for 1, 4, 8 and 24 hours to neurones in culture (Figure 4.9A). Again there was no significant difference in the

percentage of apoptosis between the neurones left untreated and those incubated with control CM (P=0.0824). After 1 hour incubation with myelin however, CM from microglia induced a significant increase in neuronal apoptosis (P<0.05) although this was still less than 20% (± 1.4%). CM from microglia exposed to myelin for 24 hours induced ~50% (± 1.3%) apoptosis (P<0.001). Interestingly microglia incubated with myelin for 4 hours induced ~30% (± 1.4%) neurones to become apoptotic, despite the fact that no iNOS was detected at this time point. However, potent release of TNFα occurred after 4 hours which suggests an important role in subsequent neurotoxicity.

Myelin was applied directly to neurones to ensure that the toxicity observed was not due to any direct toxic effects of myelin on neurones (Figure 4.9B). Adding myelin to neurones for 24 hours at the same concentration as in MGCM did not induce any significant apoptosis above levels induced by control CM (P=0.3636). Furthermore, myelin added directly to neurones in the presence of control CM did not have any deleterious effect on neuronal survival thus demonstrating that the presence of microglia and myelin together are required for neurotoxicity.

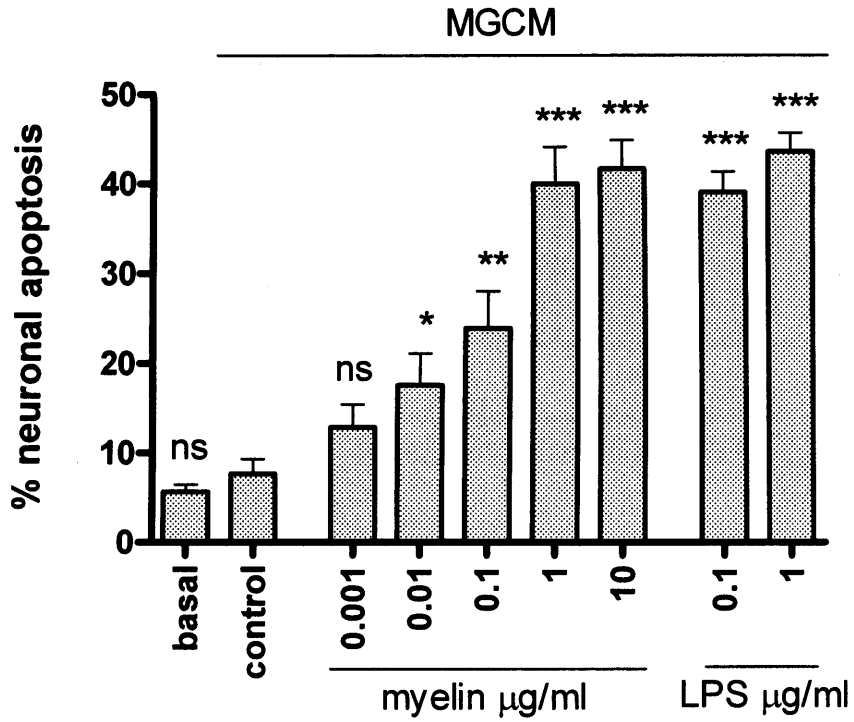


Figure 4.8 Microglia incubated with myelin releae factors which are neurotoxic

Microglia were either left untreated (control) or incubated with 0.001-10μg/ml myelin or 0.1-1μg/ml LPS for 24 hours. Conditioned medium was then collected from the microglia and applied to neurones in culture for a further 24 hours. A subset of neurones were left untreated for 24 hours to asses basal levels of apoptosis. Neurones were then fixed in 4% F-PBS and apoptosis measured by Hoechst 33342 nuclear staining. Apoptotic nuclei were condensed and pyknotic and counted as a percentage of live cells per field. MGCM from cells incubated with 0.01μg/ml myelin or above induced significantly higher levels of apoptosis in neurones compared with CM from untreated control microglia. Values represent mean +SEM from five separate fields per condition repeated three times in at least three separate experiments. *P<0.05, **P<0.01, ***P<0.001versus control CM.

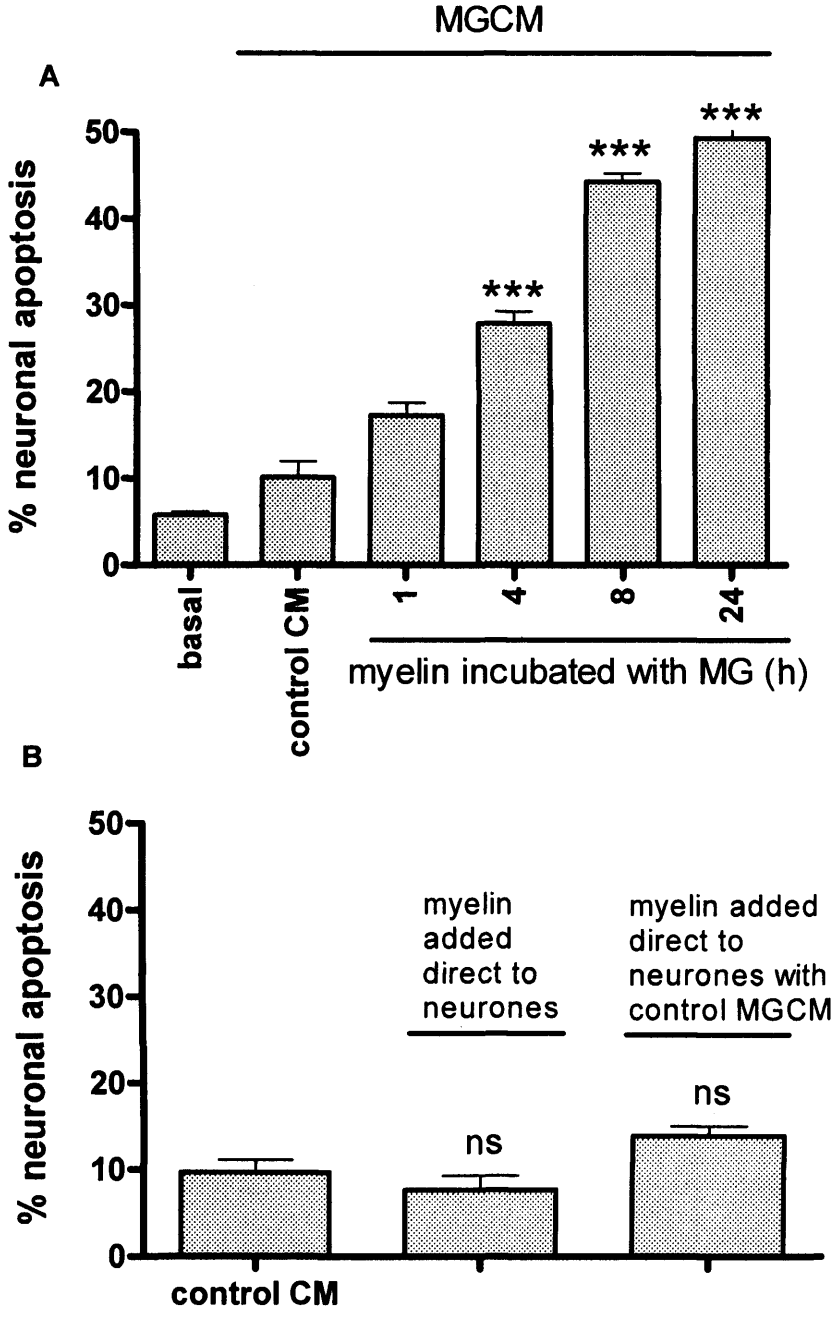


Figure 4.9 Microglia incubated with myelin for over 4 hours release factors which are neurotoxic

(A) Microglia were either left untreated or incubated with myelin for 1, 4, 8 and 24 hours. Conditioned medium was then collected from the microglia and applied to neurones in culture for a further 24 hours. A subset of neurones were left untreated for 24 hours to asses basal levels of apoptosis. CM from microglia incubated with myelin for 4 hours or more was significantly more neurotoxic than CM from untreated control microglia. **(B)** As a control, myelin was added directly to neurones in culture either alone or in the presence of control microglial CM for 24 hours to negate any direct toxicity to neurones. Neurones were then fixed in 4% F-PBS and apoptosis measured by Hoechst 33342 nuclear staining. Apoptotic cells contained pyknotic nuclei and were counted as a percentage of live cells per field. Values represent mean +SEM from at least five separate fields per coverslip on three different coverslips repeated in at least three separate experiments. ***P<0.001 versus control CM.

4.2.3 Myelin induced microglial toxicity is due to TNFα and NO release

Since myelin induced the expression of phospho-ERK in microglia, the role of phospho-ERK in the process of myelin induced microglial neurotoxicity was examined. The above experiments were repeated, but microglia were treated with the MEK inhibitor U0126 (5μM) for 30 minutes before incubation with myelin. Microglia were also treated with myelin or U0126 alone, or left untreated as positive and negative controls respectively. The CM from microglia was assessed for presence of TNFα (Figure 4.10A) and nitrite (Figure 4.10B). Microglia incubated with myelin released significantly increased levels of TNFα compared with control cells as discussed above. Treatment of microglia with U0126 alone did not induce significant TNFα release compared with release from untreated cells. However, pre-treatment with this compound significantly reduced the microglial TNFα release induced by exposure to myelin ($P<0.001$) by around 75%. Exposure to myelin caused microglia to release 160nM/μg/μl nitrite (± 17.3nM/μg/μl). Prior treatment with U0126 significantly reduced the amount of nitrite released by microglia incubated with myelin alone ($P<0.05$) down to 94nM/μg/μl (± 12.4nM/μg/μl), although these levels were still significantly above the nitrite released by untreated cells ($P<0.05$).

The neurotoxicity of CM from microglia treated with U0126 prior to incubation with myelin was assessed (Figure 4.10C). U0126 afforded significant protection against the neurotoxicity induced by myelin ($P<0.001$). Moreover, the levels of apoptosis induced by CM from microglia treated with U0126 prior to incubation with myelin were not significantly different from those induced by CM from untreated cells.

Since myelin induced iNOS expression in microglia, the role of iNOS in the process of myelin induced toxicity was examined. Microglial cells were treated with AMT-HCl (150nM), a potent iNOS inhibitor, for 1 hour prior to incubation with myelin for a further 24 hours. A subset of cells were left untreated as controls or incubated with

AMT-HCl or myelin alone as positive and negative controls respectively. The effect of iNOS inhibition on TNFα (Figure 4.11A) or glutamate (Figure 4.11B) release were assessed. Microglia incubated with AMT-HCl alone did not release significant levels of TNFα above control (P=0.3781). However, AMT-HCl failed to have any effect on the TNFα release induced by myelin (P=0.3824). In the same vein, AMT-HCl also had no significant effect on the glutamate release induced by myelin (P=0.7292).

The neurotoxicity of CM from microglia treated with AMT-HCl before incubation with myelin was also assessed (Figure 4.11C). CM from microglia treated with AMT-HCl alone did not have any detrimental effect on neuronal survival compared with control CM (P=0.1830). CM from microglia treated with myelin alone induced 45% (±1.7%) apoptosis consistent with previous data. Pre-treatment of microglia with AMT-HCl afforded significant protection against the neurotoxicity induced by myelin alone (P<0.001) bringing the levels of apoptosis down to 20% (± 2%). However this was still significantly above the levels of apoptosis caused by control CM (10% ± 0.7%). Interestingly, AMT-HCl had no effect on TNFα (Figure 4.11A) or glutamate (Figure 4.11B) release, this suggests that other factors may be involved in the toxicity of myelin MGCM and would explain why the microglial toxicity induced by myelin was not completely blocked by this compound. This was in contrast to the protection afforded by pre-treatment with U0126 which completely abrogated myelin induced neuronal apoptosis. Since U0126 significantly reduced both the microglial nitrite and TNFα release while AMT-HCl only inhibited iNOS expression, these data suggest that both iNOS and TNFα have important roles in the microglial toxicity induced by myelin.

Direct application to neuronal cultures of AMT-HCl or U0126 in the presence of control CM had no significant effect on neuronal survival (Figure 4.12A). Furthermore when CM from microglia treated with myelin was added to neuronal cultures with

direct application of AMT-HCl, there was no significant modulation of apoptosis (Figure 4.12B) demonstrating that the neuroprotection afforded by AMT-HCl was not attributable to a direct action of iNOS inhibition on neurones but instead by inhibiting microglial iNOS expression. Inhibition of microglial iNOS expression may reduce microglial toxicity by preventing feedback activation of microglia by NO release . Direct application of U0126 in the presence of myelin CM, however, significantly reduced the levels of apoptosis induced by myelin CM alone (Figure 4.12B). This may suggest that some of the protection afforded by U0126 is due to a direct action on neurones and not purely due to a reduction of microglial toxixicty.

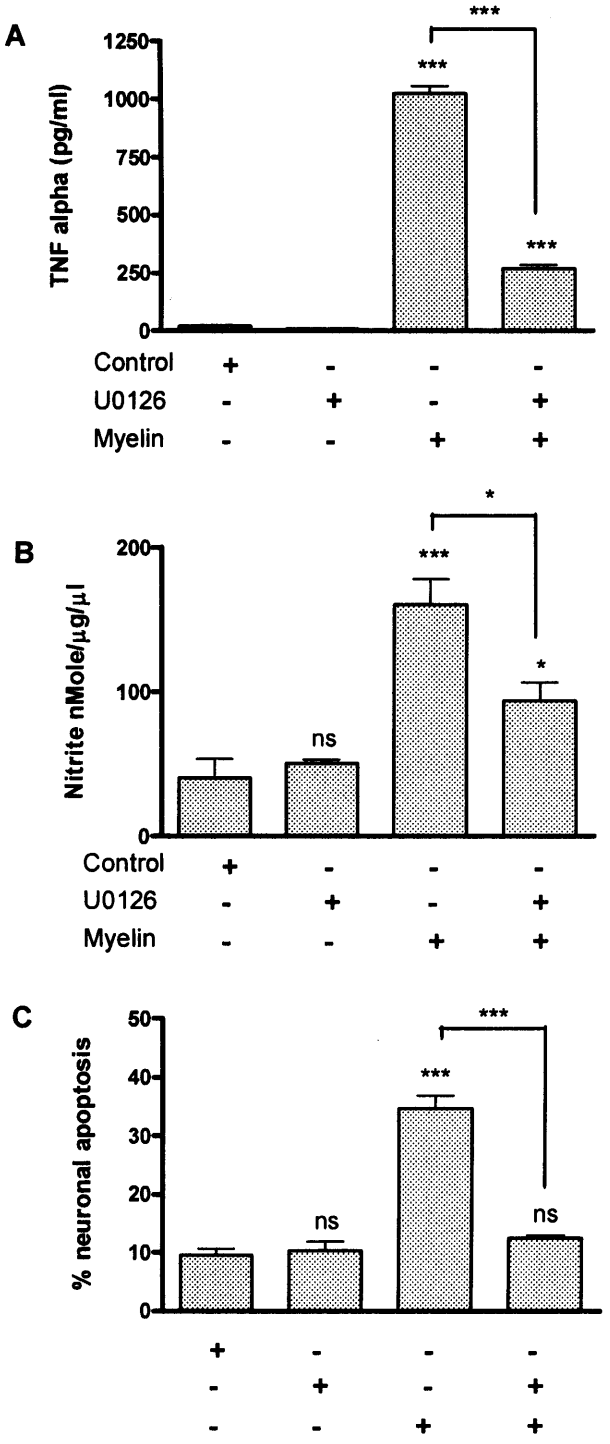


Figure 4.10 Modulation by U0126 of the toxic factors released by microglia after exposure to myelin

Microglia were incubated with MEK inhibitor U0126 (5µM) for 1hr before incubation with myelin for a further 24hrs. Conditioned medium from either microglia either left untreated or incubated with U0126 or myelin alone were used as controls. **(A)** Conditioned medium was collected and assessed for TNFα release. Incubation of microglia with U0126 prior to incubation with myelin significantly reduced the TNFα release induced by myelin alone. **(B)** CM was also analysed for nitrite levels. Nitrite release induced by myelin was significantly reduced by prior incubation with U0126. **(C)** CM was also applied to neurone cultures and levels of apoptosis were assessed by nuclear staining with Hoechst 33342. CM from microglia incubated with U0126 prior to exposure to myelin induced significantly less apoptosis than that induced by CM from microglia incubated with myelin alone. Values represent mean +SEM from five separate fields per coverslip from three different coverslips repeated in at least two separate experiments. *P<0.05, **P<0.01, ***P<0.001 versus control unless indicated by a bar.

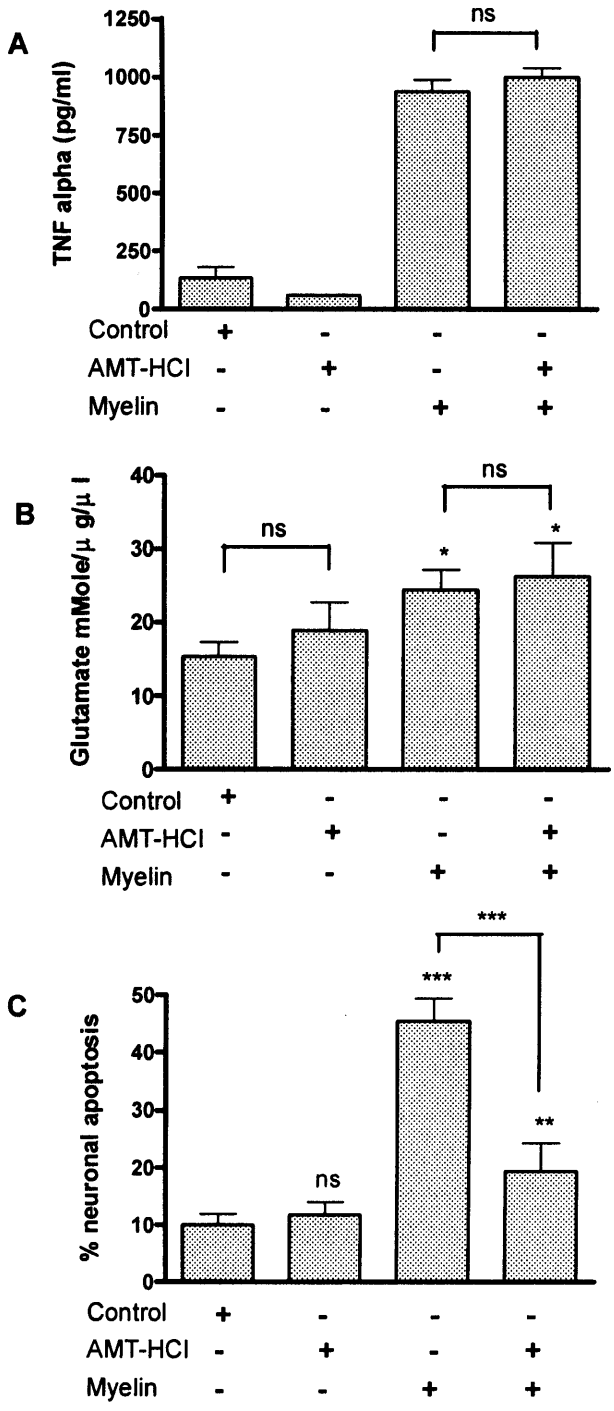


Figure 4.11 Modulation by AMT-HCl of the toxic factors released by microglia after exposure to myelin

Microglia were incubated with iNOS inhibitor AMT-HCl (150nM) for 1hr before incubation with myelin for a further 24hr. CM from microglia either left untreated or incubated with AMT-HCl or myelin alone were used as controls. **(A)** CM from microglia was collected and assessed for TNFα release. Incubation of microglia with AMT-HCl prior to incubation with myelin did not reduce the TNFα release induced by myelin alone. **(B)** MGCM was also analysed for glutamate levels. Glutamate release was not affected by prior incubation with AMT-HCl **(C)** MGCM was also applied to neurone cultures and levels of apoptosis were assessed by nuclear staining with Hoechst 33342. CM from microglia incubated with AMT-HCl prior to exposure to myelin induced significantly less apoptosis than that induced by CM from microglia incubated with myelin alone. Values represent mean +SEM from five separate fields per coverslip from three different coverslips repeated in at least two separate experiments. *P<0.05, **P<0.01, ***P<0.001 versus control unless indicated by a bar.

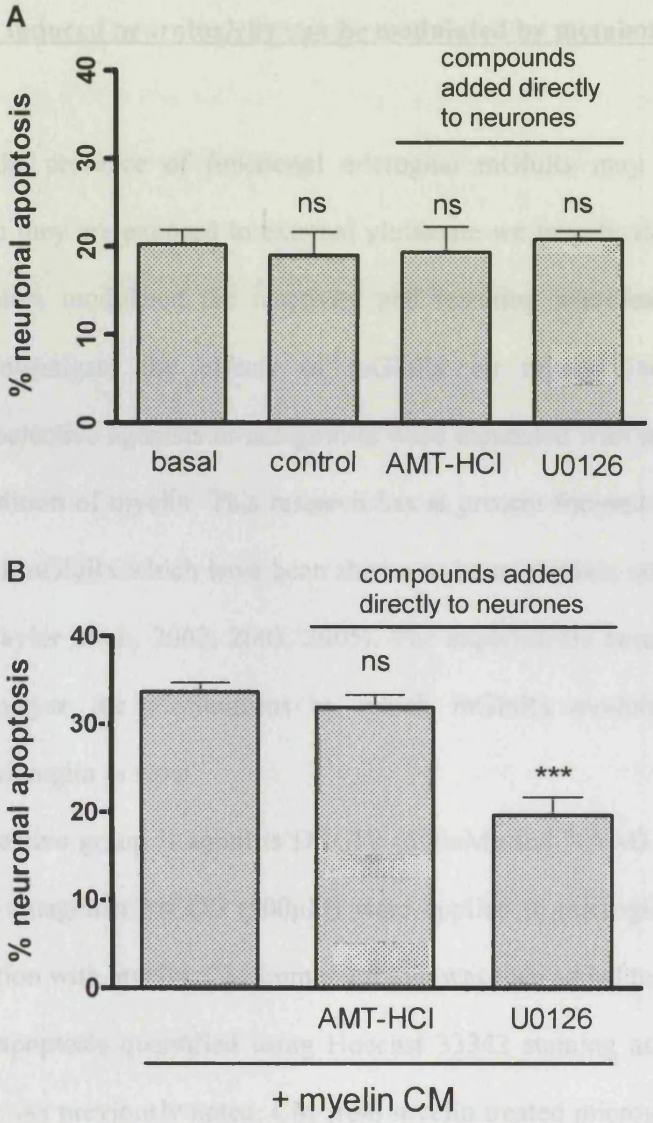


Figure 4.12 Direct application of AMT-HCl has no effect on neuronal while direct application of U0126 affords significant neuroprotection

(A) iNOS inhibitor AMT-HCl or ERK inhibitor U0126 were added directly to neurones in culture for 24 hours with control MGCM. Neurones were then fixed in 4% F-PBS and stained with Hoechst to detect apoptotic cells. Direct addition of AMT-HCL or U0126 to neurones had no significant effect on neuronal survival. **(B)** Since both AMT-HCL and U0126 reduced the toxicity of microglia when applied prior to myelin, these compounds were added directly to neurones in the presence of myelin MGCM to ascertain that the protection afforded by either AMT-HCl or U0126 was not a direct effect on the neurones themselves. Direct application of AMT-HCl had no significant effect on the neuronal death induced by myelin. Direct addition of U0126 however afforded significant neuroprotection which may suggest that the protective effects of U0126 are due to a direct effect on neurones. Values represent mean and SEM from 5 different fields per condition repeated 3 times in at least two separate experiments. ***P<0.001 versus myelin CM.

4.2.4 Myelin induced neurotoxicity can be modulated by metabotropic glutamate receptors

Since the presence of functional microglial mGluRs may affect microglial reactivity when they are exposed to external glutamate we investigated how activation of these receptors modulated the reactivity and resulting neurotoxicity induced by myelin. To investigate the effects of mGluRs on myelin induced microglial neurotoxicity, selective agonists or antagonists were incubated with microglial cultures prior to the addition of myelin. This research has at present focused on modulation of group II and III mGluRs which have been shown to be neurotoxic and neuroprotective respectively (Taylor et al., 2002, 2003, 2005). The experiments described above were repeated to analyse the mechanisms by which mGluRs modulated the reactive phenotype of microglia *in vitro.*

The selective group II agonists DCGIV (500nM) and NAAG (50μM- selective for mGlu3) or antagonist MCCG (500μM) were applied to microglia for 30 minutes prior to incubation with myelin. CM from microglia was then added to CGC cultures for 24 hours and apoptosis quantified using Hoechst 33342 staining as described above (Figure 4.13A). As previously noted, CM from myelin treated microglia induced death of cultured neurones by apoptosis. CM from microglia treated with DCGIV alone was neurotoxic ($P<0.05$) leading to 25% (± 5%) apoptosis in neuronal cultures. This confirms the findings described by Taylor et al. (2002). Furthermore, when incubated with microglia prior to exposure to myelin, DCGIV significantly increased apoptosis compared with that caused by myelin CM alone ($P<0.05$). Treatment of microglia with NAAG did not induce a neurotoxic phenotype since CM from these cells did not lead to increased levels of apoptosis compared with CM from control cells. Indeed exposure to NAAG prior to incubation with myelin reduced the toxicity of microglia treated with myelin alone ($P<0.01$). Similarly, CM from microglia exposed to MCCG did not have

any significant effect on neuronal survival compared with medium from untreated cells. Moreover, medium from cells incubated with MCCG and myelin resulted in significant attenuation of neuronal apoptosis compared with medium from microglia exposed to myelin alone ($P<0.001$). However, levels of apoptosis induced by CM from microglia treated with MCCG and myelin were still significantly above those caused by control CM ($P<0.05$) suggesting that other factors not influenced by the modulation of group II mGluRs are also responsible for myelin induced microglial neurotoxicity.

These experiments were repeated for analysis of active caspase 3 expression and cell death by PI uptake (Figure 4.14). As with Hoechst experiments, the percentage of dead cells or cells expressing active caspase were assessed as a percentage of total cells per field (Figure 4.13B). As expected, results mirrored the Hoechst findings. Microglia pre-treated with NAAG or MCCG before incubation with myelin showed reduced caspase 3 and PI staining compared with cells exposed to myelin alone, which suggests that factors released by microglia in CM induce neuronal apoptosis via activation of caspase 3, and that inhibition of group II mGluR2 with MCCG or activation of mGluR3 with NAAG prevents this.

These observed changes in levels of neuronal apoptosis were due to substances released from microglia in the CM since direct application of receptor agonists or antagonists to CGCs had no significant effect on neuronal survival (Figure 4.15A). Furthermore when CM from microglia treated with myelin was added to neuronal cultures with direct application of either NAAG or MCCG there was no significant modulation of apoptosis (Figure 4.15B) demonstrating that the neuroprotection afforded by these compounds was not attributable to a direct action on group II receptors on neurones but instead by modulating the toxicity of the microglia.

Since treatment with NAAG did not afford the same level of neuroprotection as treatment with MCCG, further experiments concentrated on the effects of DCGIV and

MCCG alone. This research has shown that TNFα, glutamate and iNOS have been implicated in the toxicity of microglial medium and as such the release and expression of these compounds was examined after treatment of microglia with DCGIV or MCCG either alone or in the presence of myelin. Interestingly, the TNFα release from microglia induced by myelin was not significantly altered by prior treatment with either DCGIV or MCCG ($P=0.9978$ and $P=0.7221$ respectively) (Figure 4.16A). Analysis of glutamate concentrations in MGCM showed that although myelin induced a significant glutamate release above controls, so too did DCGIV and MCCG. However, incubation of microglia with these compounds preceding incubation with myelin seemed to potentiate glutamate release above levels induced by myelin alone, particularly with co-incubation with DCGIV ($P<0.01$) (Figure 4.16B).

Finally the expression of phospho-ERK (Figure 4.16C) and iNOS protein (Figure 4.16D and E) was determined under the conditions described above. Microglia left untreated or incubated with DCGIV or MCCG alone did not express phospho-ERK above control levels. The phospho-ERK expression in microglia was upregulated after incubation with myelin as shown, furthermore this expression was reduced by pre-treatment with MCCG (Figure 4.16C). Similarly, neither untreated microglia nor those treated with DCGIV or MCCG alone expressed any iNOS protein. As discussed, myelin treated microglia expressed iNOS after 24 hours. Pre-treatment with DCGIV did not have any effect on this expression but treatment with MCCG visibly reduced the iNOS levels induced by myelin alone (Figure 4.16D). These data suggest that the beneficial effect on neuronal survival mediated by pre-treatment with MCCG could be due to reduced expression of iNOS protein consistent with the neuroprotection afforded by iNOS inhibitors (Figure 4.11C).

The above experiments were repeated with the group III agonist AP4 and antagonist MAP4. CM from microglia treated with AP4 (500μM) or MAP4 (500μM)

either with or without the presence of myelin for 24 hours was applied to neurones for assessment of induced apoptosis (Figure 4.17A), caspase 3 expression and death (Figure 4.17B). Exposure of microglia to AP4 or MAP4 did not induce a neurotoxic phenotype since levels of apoptosis were not significantly different from those induced by control CM (P=0.2903 and P=0.0997 respectively). As such the stimulation of group III mGluRs unlike stimulation of group II mGluRs does not result in the transformation of microglia to a neurotoxic phenotype. CM taken from microglia incubated with MAP4 together with myelin had no significant effect on neuronal apoptosis compared with levels induced by myelin alone (P=0.9016). However, medium from microglia incubated with AP4 together with myelin showed significantly attenuated neurotoxicity compared with medium taken from microglia incubated with myelin alone (P<0.01). Interestingly, the neuroprotective effect of AP4 was not as potent as that induced by the group II receptor antagonist MCCG. These data suggest that microglial reactivity is primarily mediated by group II mGluRs and that blocking this reactivity rather than stimulating a protective phenotype is a more beneficial way of reducing the microglial toxicity induced by myelin.

Analysis of active caspase 3 expression and PI uptake in CGCs mirrored these results (Figure 4.17B) although CM from microglia incubated with MAP4 alone slightly but significantly increased caspase expression above levels induced by control CM. This suggests that although no significant increases in apoptosis or cell death were visible, apoptotic mechanisms were upregulated. As shown in previous experiments, the CM from microglia incubated with myelin induced neurones to express significantly elevated caspase 3 expression and PI uptake (P<0.001 and P<0.01 respectively). Furthermore, AP4 afforded significant neuroprotection and reduction of the caspase 3 expression and PI uptake induced by myelin alone (For representative images see Figure 4.18).

The observed changes in neuronal apoptosis were due to factors released from microglia into the CM since direct application of receptor agonists or antagonists had no significant effect on neuronal survival (Figure 4.19A). Furthermore when CM from microglia treated with myelin was added to neuronal cultures with direct application of AP4, there was no significant modulation of apoptosis (Figure 4.19B) demonstrating that the neuroprotection afforded by AP4 was not attributable to a direct action of group III receptors on neurones but instead by modulating the toxicity of microglia.

CM and cell lysates were assessed for TNFα and glutamate release, and iNOS expression respectively from untreated microglia or cells treated with AP4 or MAP4 either with or without the presence of myelin for 24 hours. Incubation of AP4 or MAP4 alone did not cause microglia to release any significant levels of TNFα above control (P=0.3229 and P=0.6942 respectively) (Figure 4.20A). Treatment of microglia with AP4 or MAP4 together with myelin however, potentiated TNFα release above levels induced by myelin alone which suggests that TNFα may not be a factor in the neuroprotection mediated by AP4. Glutamate levels in media were also assessed (Figure 4.20B). We found that as with the group II receptors, treatment of microglia with AP4 induced significant increases in glutamate release to levels similar to those induced by myelin alone. Again, incubation of microglial AP4 or MAP4 together with myelin seemed to potentiate the release of glutamate, however, the increases observed were not statistically significant (P=0.2443 and P=0.0589 respectively) when compared with the release induced by incubation with myelin alone.

Microglial lysates were analysed for phospho-ERK (Figure 4.20C) and iNOS protein (Figure 4.20D and E) expression by western blotting. While phospho-ERK was not expressed in control microglia or those treated with AP4 alone, expression seemed to be upregulated by exposure to MAP4 alone or by myelin. In contrast to the modulation of phospho-ERK expression seen by group II antagonists, neither AP4 nor

MAP4 appeared to alter phospho-ERK expression induced by myelin (Figure 4.20C). Microglia left unstimulated or cells treated with AP4 or MAP4 alone did not express any iNOS protein. Microglia treated with AP4 prior to addition of myelin reduced the expression of iNOS induced by myelin alone but this effect was consistently not as pronounced as the effect induced by MCCG (Figure 4.16D).

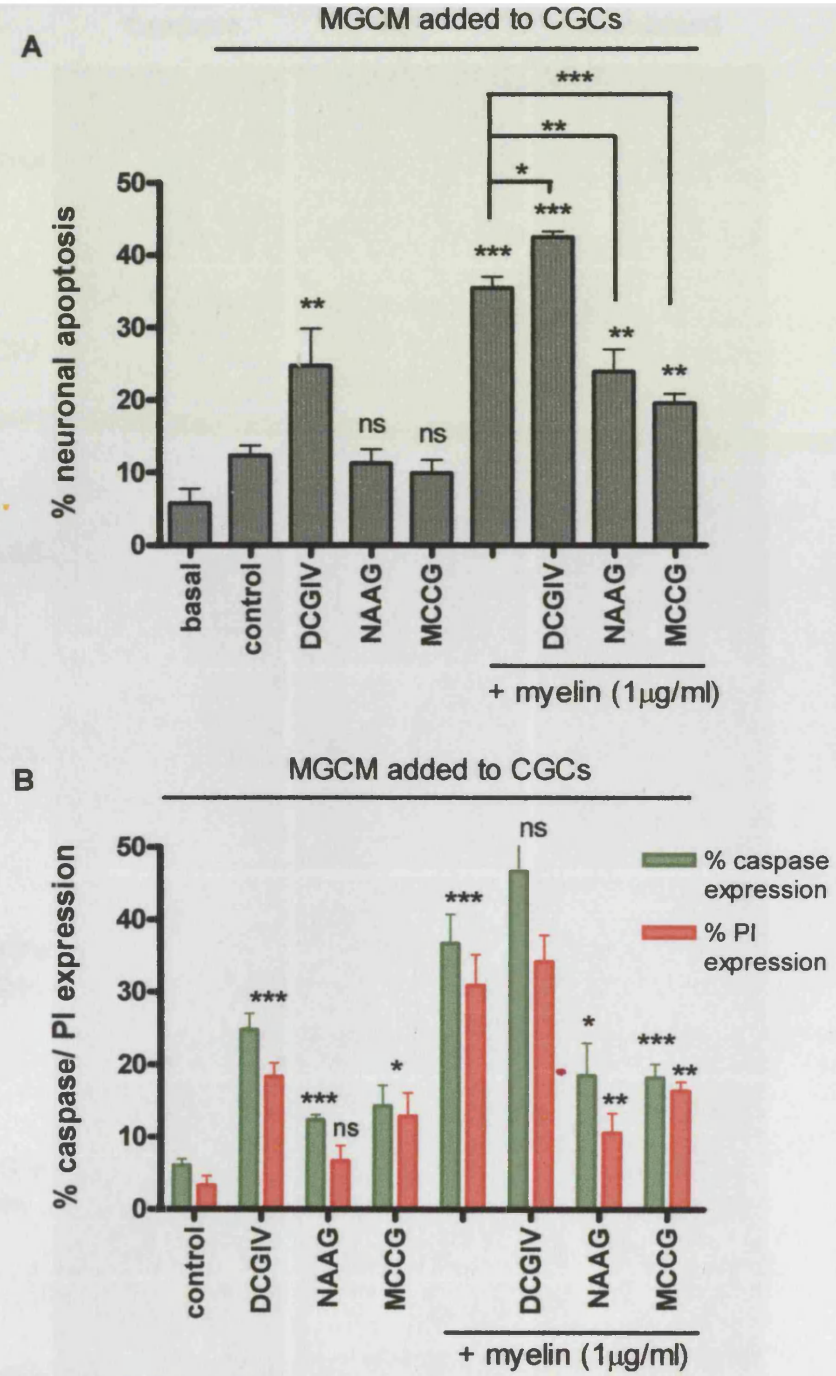


Figure 4.13 Stimulation of microglial group II mGluRs induces microglial neurotoxicity.

Conditioned medium from microglia either untreated or stimulated with group II mGluR agonists (DCGIV 500nM, NAAG 50μM) or antagonists (MCCG 500μM) in the presence or absence of myelin was applied to neurones in culture. **(A)** Apoptosis was assessed by Hoechst 33342 nuclear staining and subsequent counting of condensed pyknotic nuclei. **(B)** Caspase-3 activity and cell death were determined by staining with caspatag and PI respectively. Untreated neurones left without microglial CM (basal) and neurones incubated with medium from untreated microglia (control CM) were used as negative controls. Neurones incubated with medium from microglia treated with DCGIV suffered significant apoptosis above that induced by control CM or MCCG CM. Toxicity of myelin CM was reduced by pre-treating microglia with NAAG or MCCG but increased by pre-treating with DCGIV. Medium from MG treated with DCGIV alone also induced increased neuronal caspase expression and death. Neuronal caspase expression and cell death induced by CM from MG incubated with myelin was reduced by pre-treating MG with NAAG or MCCG. Values represent mean and SEM from 5 different fields per condition repeated 3 times in at least three separate experiments. *P<0.05, **P<0.01, ***P<0.001 **(A)** versus control CM unless indicated by a bar. **(B)** versus control for DCGIV, NAAG, MCCG and myelin conditions, versus myelin for

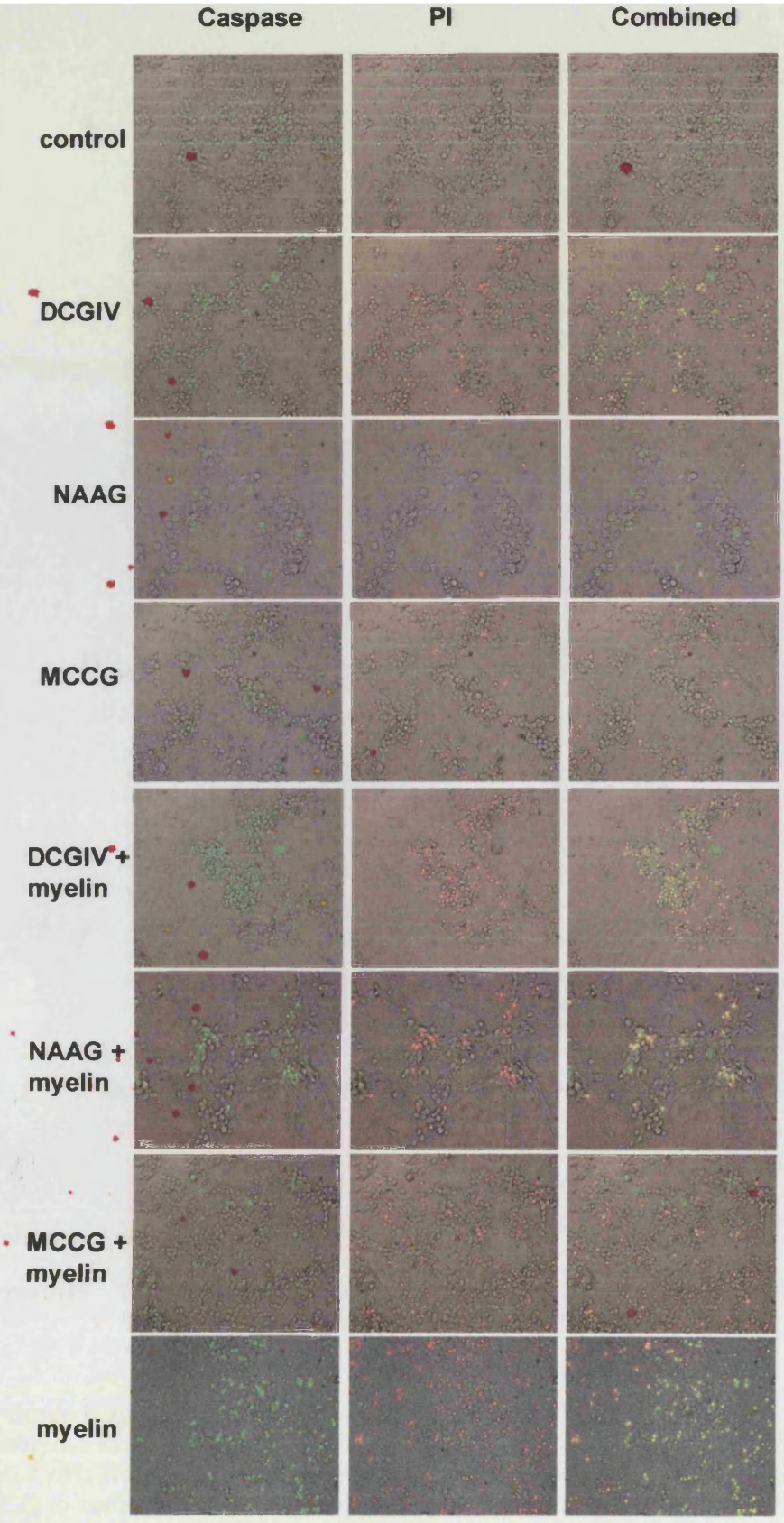


Figure 4.14 Caspase and PI expression induced by myelin is modulated by group II mGluRs

Caspase-3 activity (green) and PI staining (red) were assessed in neuronal cultures exposed for 24 hours to microglial CM from microglia treated as described in Figure 4.10. Cells were incubated with CaspaTag and PI for 30 min, and staining was viewed using a fluorescence microscope. Where CaspaTag staining and PI staining overlap, cells appear yellow. The number of caspase-3- or PI-positive cells were counted and expressed as a percentage of the total cell number per field.

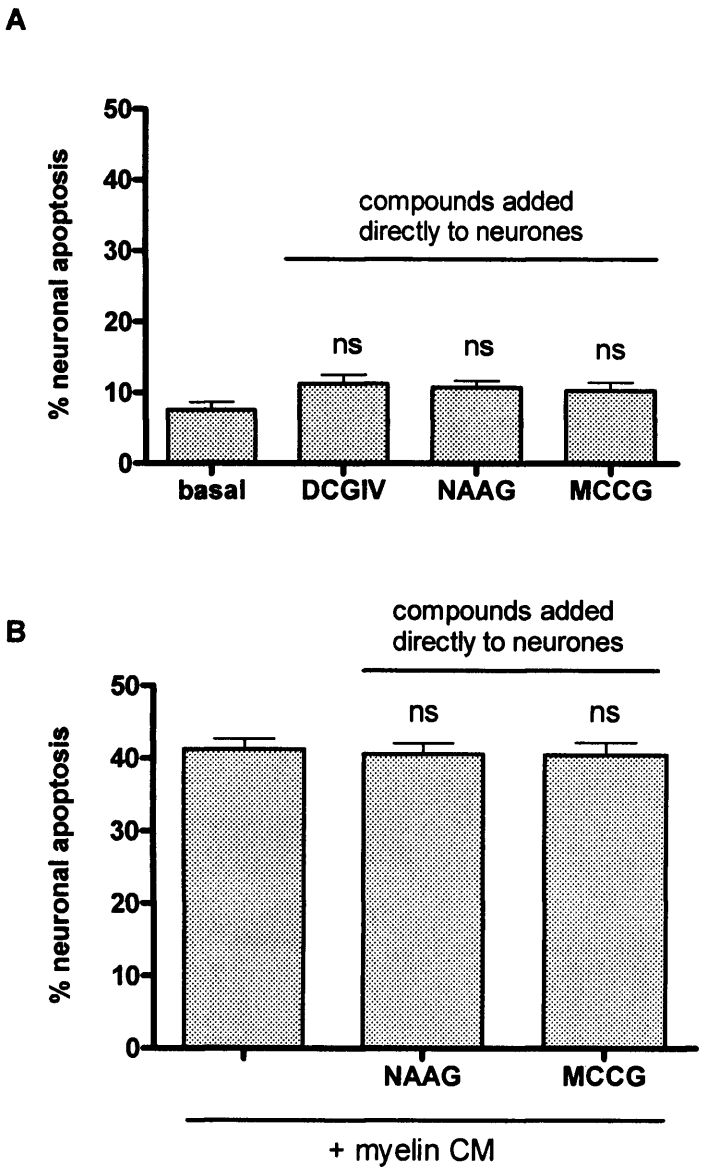


Figure 4.15 Direct application of group II agonists or antagonists to neurones has no effect on neuronal survival following exposure to myelin MGCM

(A) Group II agonists DCGIV or NAAG and antagonist MCCG were added directly to neurones in culture for 24 hours. Neurones were then fixed in 4% F-PBS and stained with Hoechst to detect apoptotic cells. Direct addition of DCGIV, NAAG or MCCG to neurones had no significant effect on neuronal survival. **(B)** Since both NAAG and MCCG reduced the toxicity of microglia when applied prior to myelin. NAAG or MCCG were added directly to neurones in the presence of myelin CM to ascertain that the protection afforded by these compounds was not a direct effect on the neurones themselves. Values represent mean and SEM from 5 different fields per condition repeated 3 times in at least three separate experiments.

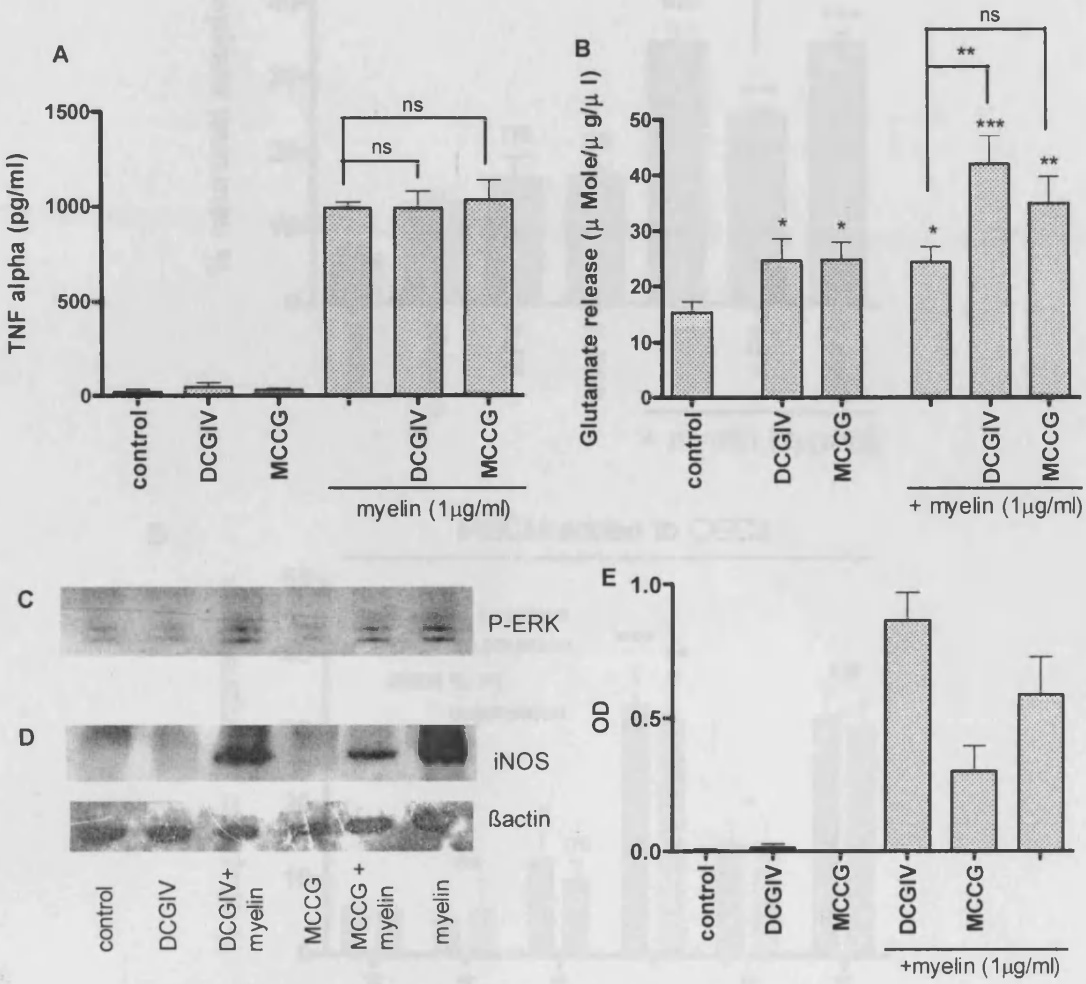


Figure 4.16 Modulation of microglial iNOS expression and glutamate release but not TNFα release by group II mGluRs.

Microglia were either left untreated or incubated with group II mGluR agonist DCGIV (500nm) or antagonist MCCG (500µM) with or without the presence of myelin, as a positive control microglia were incubated with myelin alone. **(A)** The CM from microglia was analysed for TNFα and showed that neither DCGIV nor MCCG altered the TNFα release induced by myelin alone. **(B)** CM was also analysed for glutamate levels. Stimulation with both agonists and antagonists increased glutamate release above control levels. Interestingly only DCGIV had a significant effect on the levels of glutamate release induced by myelin. MG lysates from the above conditions were analysed for **(C)** phospho-ERK protein and **(D)** iNOS protein by Western blotting The first lane shows non stimulated microglia as a negative control for iNOS expression. **(E)** Densitometric analysis of iNOS expression in rat microglia incubated with myelin as above, values were normalised by the amount of ß-actin protein and represent the means of three separate blots. *P<0.05, **P<0.01, ***P<0.001 versus control unless indicated by a bar.

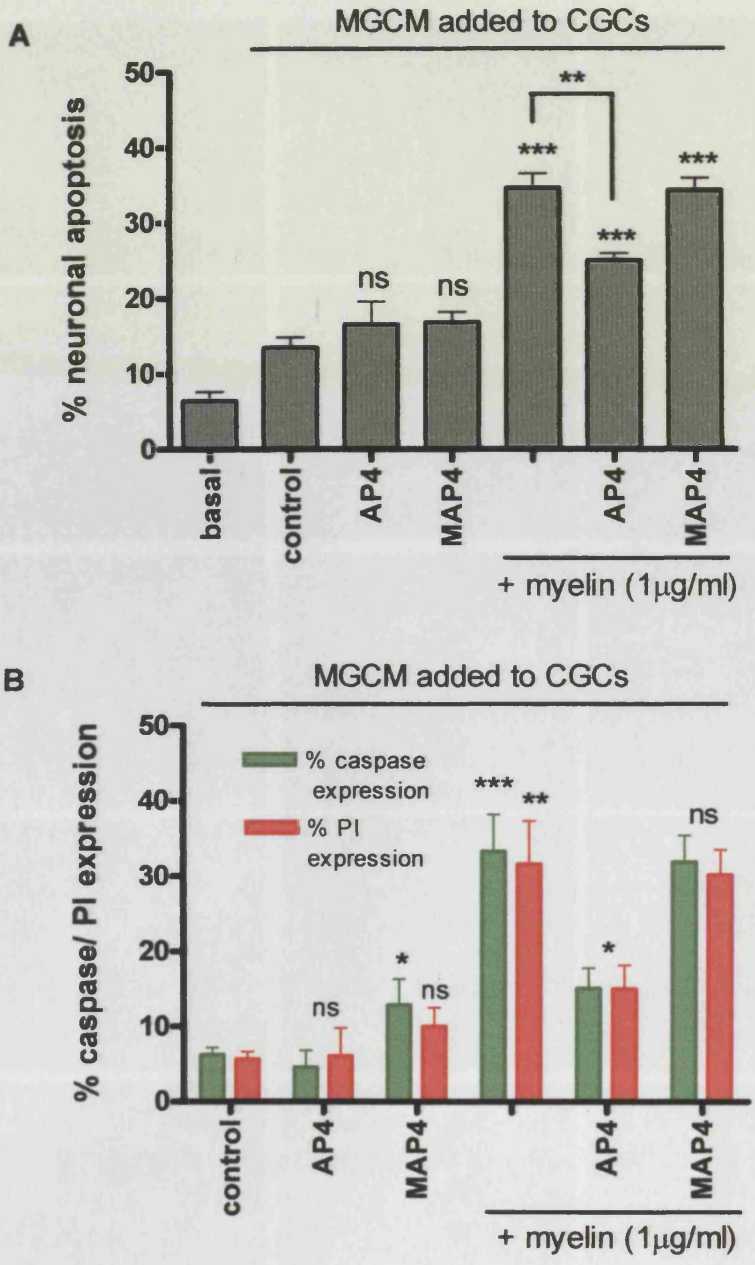


Figure 4.17 Stimulation of microglial group III mGluRs promotes neuronal survival.

Conditioned medium from microglia either untreated or stimulated with group III mGluR agonists (AP4 500μM) or antagonists (MAP4 500μM) in the presence or absence of myelin was applied to neurones in culture. Untreated neurones left without microglial CM (basal) and neurones incubated with medium from untreated microglia (control CM) were used as negative controls. **(A)** Apoptosis was assessed by Hoechst 33342 staining and subsequent counting of condensed pyknotic nuclei. **(B)** Caspase-3 activity and cell death were determined by staining with caspatag and PI respectively. Values represent mean and SEM from 5 different fields per condition repeated 3 times in at least three separate experiments. *P<0.05, **P<0.01, ***P<0.001 **(A)** versus control CM unless indicated by a bar. **(B)** versus control CM for AP4, MAP4 and myelin conditions, versus myelin for compounds+ myelin conditions.

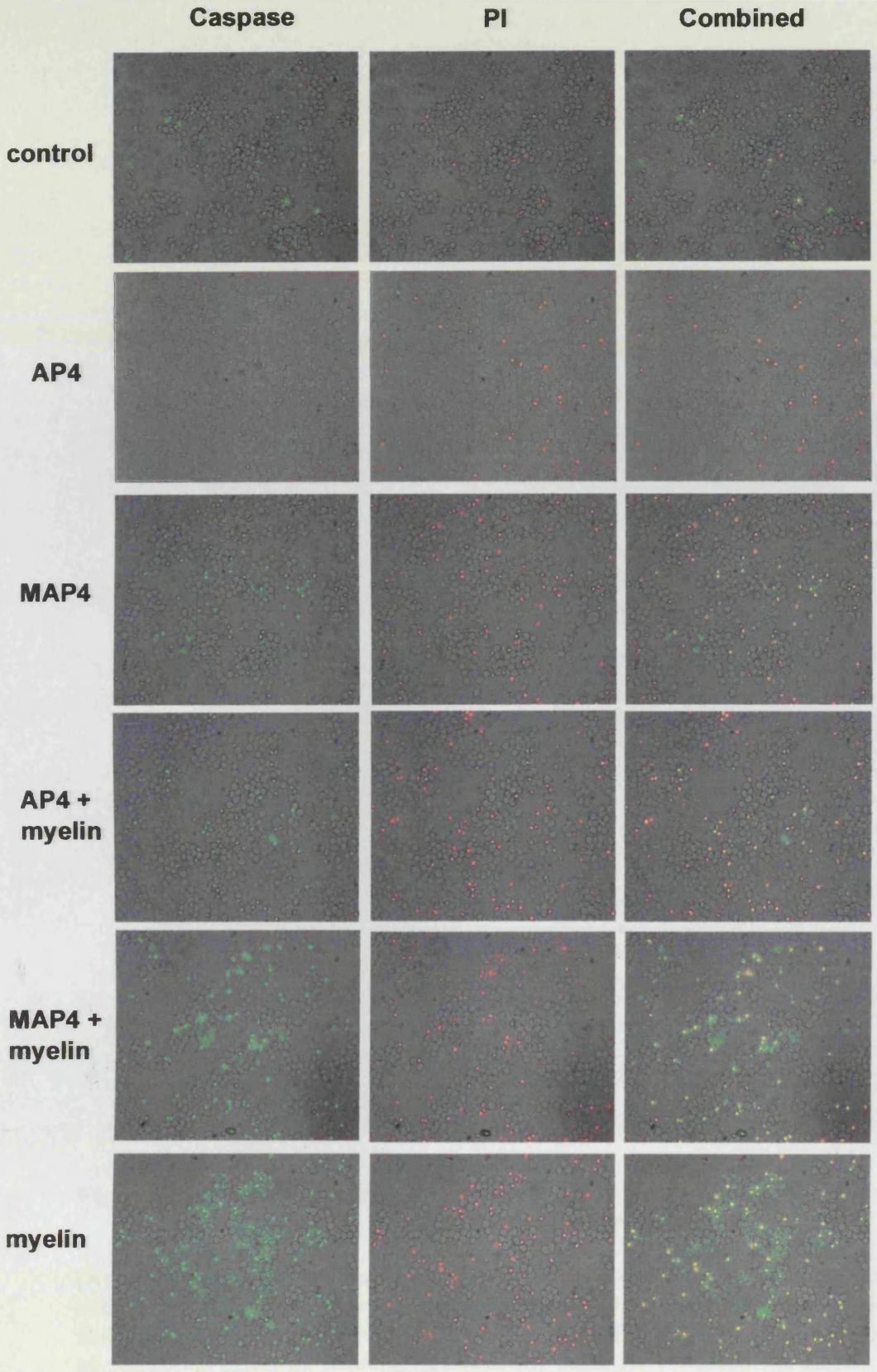


Figure 4.18 Caspase and PI expression induced by myelin is modulated by group III mGluRs

Caspase-3 activity (green) and PI staining (red) were assessed in neuronal cultures exposed for 24 hours to MGCM from microglia treated as labelled. Cells were incubated with CaspaTag and PI for 30 min, and staining was viewed using a fluorescence microscope. Where CaspaTag staining and PI staining overlap, cells appear yellow. The number of caspase-3- or PI-positive cells were counted and expressed as a percentage of the total cell number per field

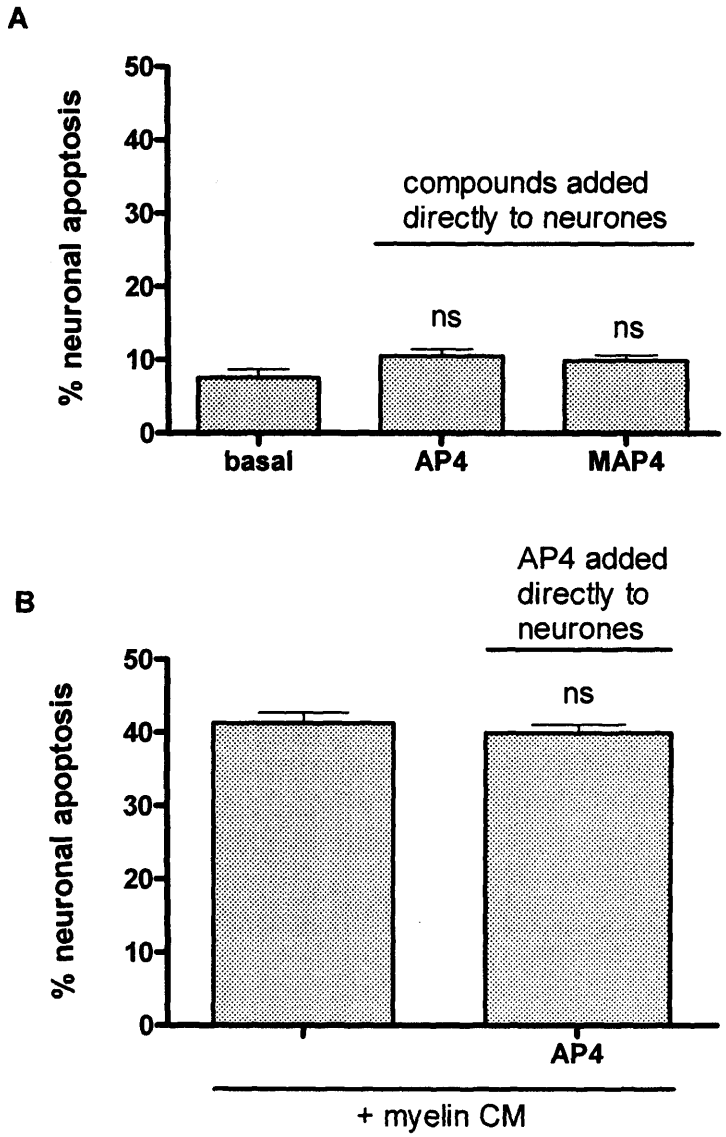


Figure 4.19 Direct application of group III agonists or antagonists to neurones has no effect on neuronal survival

(A) Group III agonist AP4 and antagonist MAP4 were added directly to neurones in culture for 24 hours. Neurones were then fixed in 4% F-PBS and nuclei stained with Hoechst to detect apoptotic cells. Direct addition of AP4 or MAP4 to neurones had no significant effect on neuronal survival. **(B)** Since AP4 reduced the toxicity of microglia when applied prior to myelin this compound was added directly to neurones in the presence of myelin CM to ascertain that the protection afforded by AP4 was not a direct effect on the neurones themselves. Values represent mean and SEM from 5 different fields per condition repeated 3 times in at least three separate experiments.

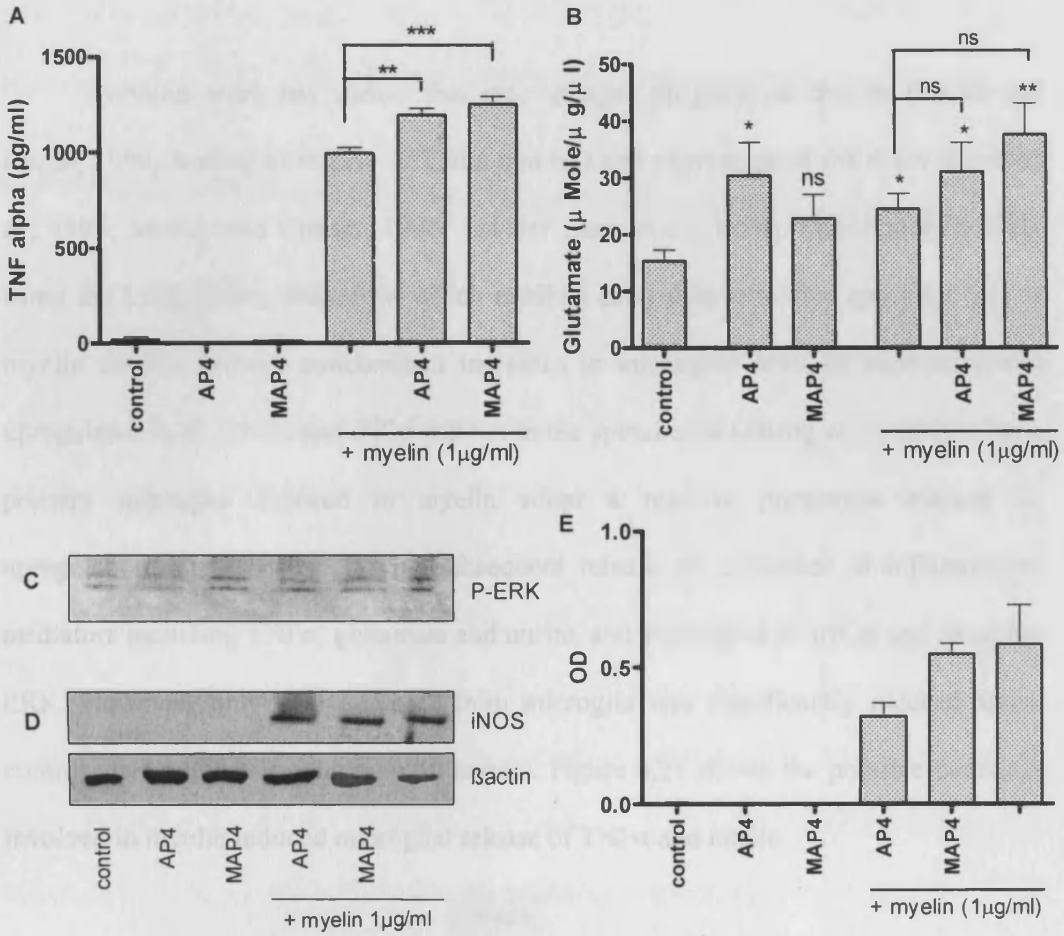


Figure 4.20 Modulation of microglial iNOS expression but not glutamate or TNFα release by group III mGluRs

Microglia were either left untreated or incubated with group III mGluR agonist AP4 (500nm) or antagonist MAP4 (500μM) with or without the presence of myelin, as a positive control microglia were incubated with myelin alone. **(A)** The CM from microglia was analysed for TNFα and showed that both AP4 and MAP4 increased the TNFα release induced by myelin alone. **(B)** CM was also analysed for glutamate levels. Stimulation with AP4 increased glutamate release above control levels. Interestingly neither AP4 nor MAP4 had any significant effect on the levels of glutamate release induced by myelin. MG lysates from the above conditions were analysed for **(C)** Phospho-ERK and **(D)** iNOS protein by western blotting The first lane shows non stimulated microglia as a negative control for iNOS expression. Microglial iNOS levels stimulated by incubation with myelin were reduced by blocking group II mGluRs. **(D)** Densitometric analysis of iNOS expression in rat microglia incubated with myelin as above, values were normalised by the amount of ß-actin protein and represent the means of three separate blots. *P<0.05, **P<0.01, ***P<0.001 versus control unless indicated by a bar.

4.3 Discussion

Previous work has shown that macrophages phagocytose myelin (Bruck and Friede, 1990) leading to release of TNFα and NO and expression of iNOS (Williams et al., 1994; Mosley and Cuzner, 1996; van der Laan et al., 1996). Furthermore a study using the Long Evans Shaker rat which exhibits early demyelination and later loss of myelin sheaths showed concomitant increases in microglial MHC-II expression and upregulated IL1β, TNFα and iNOS mRNA in the spinal cord (Zhang et al., 2001). Here, primary microglia exposed to myelin adopt a reactive phenotype marked by upregulation of ED-1 protein and subsequent release of a number of inflammatory mediators including TNFα, glutamate and nitrite, and expression of iNOS and phospho-ERK. However, only TNFα release from microglia was significantly induced above control after 4 hours incubation with myelin. Figure 4.21 shows the possible pathways involved in myelin induced microglial release of TNFα and nitrite.

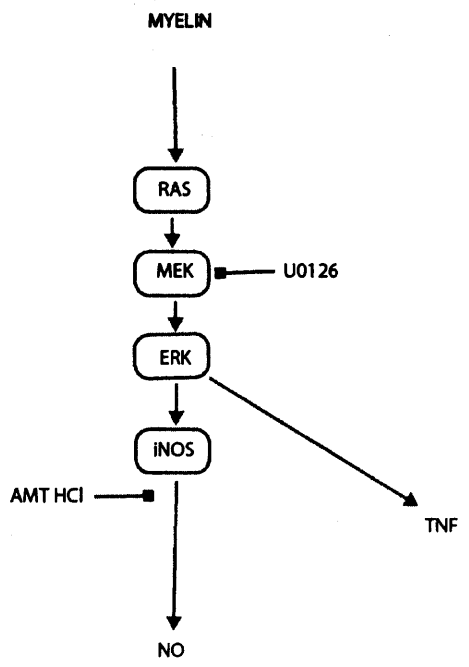


Figure 4.21 Myelin induces TNFα and nitrite release coupled to ERK in microglia

Myelin induced microglia to release TNFα and express iNOS. The MEK inhibitor U0126 prevented myelin induced TNFα and iNOS expression while inhibiting iNOS activity with AMT-HCl prevented NO and downstream products but had no effect on TNFα release.

The factors released by microglia exposed to myelin were deleterious to neuronal survival since incubation of cultured neurones with CM from microglia treated with myelin induced significantly increased levels of apoptosis. This was attributable to myelin induced factors released by microglia, since direct application of myelin to neurones either alone or in the presence of microglial control CM had no adverse effect. These findings have important implications in the study of MS pathology since removal of myelin debris by microglia in MS plaques has been well documented (Lee et al., 1990; Li et al., 1996; Hill et al., 2004; Tanuma et al., 2006) and could therefore have detrimental consequences for neuronal survival by bystander damage.

The CM from microglia exposed to myelin for 4 hours was toxic to neurones in culture which suggests a major role for TNFα as a mediator of neurotoxicity since TNFα release but no iNOS expression were observed at this time point. Indeed, TNFα release from microglia has been shown to induce neuronal death *in vitro* (Combs et al., 2001; Floden et al., 2005). Furthermore incubation of the MEK inhibitor U0126 with microglia prior to exposure to myelin significantly reduced both the TNFα and nitrite release induced by myelin and as such dramatically reduced the neuronal apoptosis compared with that induced by CM from microglia exposed to myelin alone.

It is also possible that iNOS and downstream products such as nitric oxide and nitrite have a role in the microglial toxicity induced by myelin since pre-treatment with the iNOS inhibitor AMT-HCl also attenuated the neuronal apoptosis induced by myelin CM. This, however, contradicts findings which have shown that co-cultured or CM from activated microglia does not induce NO or peroxynitrite mediated cell death (Ciesielski-Treska et al., 1998; Kingham et al., 1999; Taylor et al., 2005). However, other studies have demonstrated nitric oxide toxicity to neurones in co-culture systems (Bal-Price and Brown 2001; Xie et al., 2002; Katsuki et al., 2006; Gibbons and Dragunow, 2006). Although we used MGCM instead of co-culture, it may be possible

that nitrite and nitrate in the CM from myelin stimulated microglia is reduced to nitric oxide when applied to cultured neurones (Millar, 1995; Tsuchiya et al., 2005). Experiments using direct application of NO or its derivatives to neurones in culture would determine the toxicity of these compounds. It is more probable that the inhibition of iNOS and downstream NO release had neuroprotective effects through reducing the overall feedback activation of microglia caused by these substances. It has also recently been shown that blocking NO activity prevented upregulation of CR3 through modulation of guanylate cyclase and cyclic GMP pathways (Roy et al., 2006). CR3 is the receptor by which most myelin is phagocytosed (Reichert et al., 2001; Reichert and Rotshenker, 2003). As such the toxic downstream effects of myelin phagocytosis by microglia may be blocked by inhibition of iNOS activity which may explain the neuroprotective effect of iNOS inhibition in microglial cultures.

It has previously been demonstrated that activation of microglial group II mGluRs is neurotoxic (Taylor et al, 2002). These data confirm these findings and further show that myelin induced neurotoxicity can also be reduced by blocking group II mGluRs prior to incubation with myelin. Furthermore, CM from microglia incubated with DCGIV prior to exposure to myelin induced significantly elevated levels of apoptosis in neuronal cultures compared with CM from microglia incubated with myelin alone. These data suggest that the reactive phenotype stimulated by myelin mediates its effects through the activation of group II mGluRs, specifically the mGluR2 subtype since stimulation of mGluR3 subtype by NAAG did not induce a neurotoxic microglial phenotype and furthermore reduced the neurotoxicity of microglia incubated with myelin alone.

Stimulation of microglial group III mGluRs can be neuroprotective (Taylor et al., 2003). Here, microglial group III stimulation also decreased the microglial neurotoxicity induced by myelin. Blockade of microglial group III receptors however,

had no significant effect on the toxicity of CM stimulated with myelin which suggests that group III receptors do not mediate the toxicity of myelin but may in some way promote a neuroprotective phenotype in microglia which reduces the toxic effect of subsequent exposure to myelin. Furthermore, the neuroprotection afforded by stimulation of group III mGluRs was significantly less than that induced by block of group II mGluRs which may suggest that the neurotoxic group II pathway is more important in myelin mediated neurotoxicity. It has also been proposed that group III mGluRs have a lower coupling efficiency to adenylate cyclase (Kemp et al., 1996).

CM from microglia exposed to myelin also induced neuronal expression of active caspase 3 which was reduced by pre-treatment with MCCG, NAAG or AP4. This may suggest that the neuronal apoptosis induced by factors released from myelin stimulated microglia was mediated by caspase 3, although further work analysing expression of different activator caspases will elucidate the apoptotic mechanism more clearly. These findings demonstrate that the microglial activation and neurotoxicity induced by myelin can be suppressed by modulation of mGluRs which may prove a putative therapeutic strategy in MS.

Further work is necessary to elucidate the mechanisms of mGluR modulation of microglial toxicity. Microglial TNFα release induced by myelin is not attenuated by modulation of mGluRs which may indicate that TNFα release is not a factor in mGluR mediated neuroprotection. It is possible that the role of TNFα in myelin-induced neuronal death is as a mediator of downstream inflammatory events rather than a directly neurotoxic molecule, indeed it has been demonstrated that TNFα alone is not toxic to neurones but acts in concert with FasL to exert neurotoxic effects (Taylor et al., 2005). The fact that both AP4 and MCCG, shown here to promote neuronal survival, induced the same release of glutamate as microglia incubated with myelin alone suggests that at these levels, glutamate is not the primary mediator of neurotoxicity

caused by myelin. Interestingly however, treatment of microglia with DCGIV potentiated the release of glutamate induced by myelin alone which may explain the increased neurotoxicity of DCGIV-myelin CM. The expression of iNOS protein induced by exposure to myelin was reduced by MCCG and to a lesser extent AP4, which may indicate that attenuated nitric oxide activity is involved in the neuroprotection mediated by these compounds. This is consistent with the neuroprotection afforded by iNOS inhibitors.

The mechanisms of mGluR mediated neuroprotection remain unclear but it has been shown in astrocytes that stimulation of mGluRs can reduce IFNγ induced iNOS expression (Murphy et al., 1995) so it may be possible that stimulation of group III mGluRs mildly activates microglia which could then desensitise them to further activation. In other systems, activation of group III mGluRs in bone marrow stromal cells reduced intracellular Ca^{2+} levels and NO production (Foreman et al., 2005).

Future studies repeating these experiments using co-culture would shed some light on the compounds that are directly involved in toxicity to neurones. Further work is required to understand the mechanisms involved in microglial toxicity and neuroprotection. Studies of the effects of myelin and mGluRs on the signalling pathways upstream of iNOS induction and TNFα release, and how these pathways interact, will help to elucidate the mechanisms of myelin mediated toxicity and mGluR mediated neuroprotection.

5.0

MODULATION OF MICROGLIAL MYELIN PHAGOCYTOSIS BY METABOTROPIC GLUTAMATE RECEPTORS

5.1 Introduction

Microglia show early reactivity in response to insult and microglial activation has been associated with release of inflammatory mediators and neurotoxicity. Activation of microglia occurs as a graded response with super activated cells adopting a phagocytic macrophage like morphology. Microglia have been shown to phagocytose myelin *in vitro*. Indeed, activated microglia show increased capacity for binding of oligodendrocytes and phagocytosis of myelin compared with that of macrophages (Mosley at el., 1996). Furthermore, the presence of myelin and myelin degradation products within microglia/MØ in MS lesions (Hill et al., 2004) strongly suggests an active role for microglia in the demyelination process.

Phagocytosis is an actin dependent mechanism and is initiated by the binding of a receptor on the phagocyte with ligands on the cell surface of a particle. There are several receptors capable of inducing phagocytosis including pattern recognition receptors, Fc receptors and complement receptors. Fc mediated phagocytosis involves recognition by the phagocyte of the Fc region of antibody labbeled pathogens which leads to SRC/SYK mediated polymerisation of actin and phagocytosis. Complement mediated phagocytosis is a biochemical cascade activated by the binding of the C1 complex to antibodies or antigen leading to production of C3 onvertase and C3 hyrolysis which in turn activates a cascade of cleavage and activation culminating in internalisation and phagocytosis. The myelin used in this project was not opsonised so the method of phagocytosis in this case is uncertain as antibodies or complement were unlikely to have been involved. However, phagocytosis is a complex not a linear process influenced by a range of factors including the expression of several receptors capable of mediating phagocytosis,and additional signals from complex particles. It is

possible that complex particles can activate several receptors which may interact in different ways to influence the phagocytic mechanism.

Here we have shown that incubation with myelin induces microglia to release a number of potentially cytotoxic molecules which can cause neuronal death. Since myelin induced microglial reactivity can be modulated by stimulation of mGlu receptors this study investigated whether myelin phagocytosis could also be modulated in the same way. Dextran beads with weights of 10kDa and 40kDa, or labelled myelin, were incubated with microglia for 2, 4 or 24 hours to investigate the timecourse and capacity of microglia for phagocytic activity.

5.2 Results

5.2.1 Microglial phagocytosis is upregulated by prior exposure to myelin

In the following experiments microglia were plated at 90,000 cells per coverslip as described and incubated at 37°C for 24 hours before use. Microglia were incubated with 20μg/ml of both 10kDa (TRITC conjugated) and 40kDa (FITC conjugated) dextran beads either alone or after prior incubation for 30 minutes with either 1μg/ml myelin or mGluR agonists or antagonists. Levels of phagocytosis were assessed by imaging with a Zeiss fluorescence microscope (Oberkochen, Germany). Phagocytosis was quantified by analysing the number of cells containing fluorescent beads as a percentage of the total cells per field. These experiments were repeated after 2, 4 and 24 hours.

Phagocytosis of dextran beads occurred in untreated control microglial cultures after 2 hours suggesting that microglia undergo phagocytosis 'at rest' (Figure 5.1). It was found that 26% (± 3.1%) of microglia had engulfed the 10kDa dextran beads after 2 hours incubation while only 18% (± 3.3%) contained 40kDa dextran beads which suggests that compounds of a smaller molecular weight were more readily phagocytosed in resting microglia. Incubation with myelin for 30 minutes prior to addition of 10 and 40 kDa beads induced a highly significant increase in the percentage of cells undergoing phagocytosis ($P<0.001$) to 60% (± 3.4%) and 53% (± 4.2%) respectively. Treatment of group I mGluRs with ADA (250μM) had no significant effect on the phagocytosis of either 10kDa or 40kDa dextran beads ($P=0.3471$ and $P=0.2813$ respectively). Blockade of group I mGluRs with the antagonist AIDA (250μM), however, did induce slightly increased levels of phagocytosis of the 10kDa beads compared with untreated cells ($P<0.05$) but not of the 40kDa beads ($P=0.1861$). Interestingly, treatment of group II mGluRs with agonist DCGIV (500nM) clearly

promoted a phagocytic phenotype in microglia, inducing 55% (± 4.6%) of cells to phagocytose 10kDa beads and 50% (± 3.4%) of cells to phagocytose 40kDa beads. The mGluR3 subtype-specific agonist NAAG (50μM) however did not induce significant increases in the percentage of phagocytosing microglia which suggests that the mGluR2 subtype is responsible for upregulated phagocytosis. Incubation with group III agonists or antagonists prior to incubation with dextran beads had no effect on the levels of phagocytosis induced by the beads alone ($P=0.7139$ and $P=0.2222$ respectively).

These experiments were repeated after 4 and 24 hours to determine whether induced increases in microglial phagocytic activity remained elevated after longer periods of time. After 4 hours incubation with 10 and 40kDa beads, levels of phagocytosis in cultures incubated with the beads alone had risen to 33% (± 3.2%) and 26% (± 4.9%) respectively (Figure 5.2). However, prior exposure to myelin (1μg/ml) for 30 minutes still induced significantly elevated microglial phagocytosis compared with untreated cells ($P<0.01$). Neither stimulation nor blockade of group I mGluRs prior to incubation with the beads altered the levels of phagocytosing cells detected in untreated cultures. Microglia treated with DCGIV before incubation with beads however, maintained upregulated levels of phagocytosis compared with untreated cells while stimulation of mGluR3 receptors still had no significant effects. Blockade of group II mGluRs with antagonist MCCG (500μM) preceding treatment with the beads seemed to reduce the basal levels of phagocytosing microglia but these results were not significant. Both stimulation and blockade of group III mGluRs induced significant increases in the levels of phagocytosis compared with those seen in untreated microglia this may be explained by the partial agonist effects of MAP4.

Further incubation with 10 and 40kDa beads for 24 hours resulted in a basal level of 64% (± 11.2%) and 34% (± 4%) phagocytosis respectively (Figure 5.3). As such, pre-treatment with either myelin or any of the mGluR agonists or antagonists did

not significantly alter phagocytosis of the 10kDa beads compared with basal levels. This suggests that although phagocytosis of beads of this molecular weight can be modulated over a period of 2-4 hours, after 24 hours the phagocytic response to substances of this weight is saturated. It is interesting however that antagonism of group II receptors did not reduce basal levels of dextran bead phagocytosis, and suggests that there is no tonic stimulation of group II receptors under basal conditions.

The percentage of untreated microglia phagocytosing 40kDa beads, however, remained relatively low after 24 hours (Figure 5.3), which indicates that at rest, microglia are less likely to phagocytose compounds of a higher molecular weight. As found after 2 and 4 hours, pre-treatment of the cells with myelin still induced significant upregulation of phagocytosis of 40kDa beads after 24 hours compared with basal levels ($P<0.01$). Prior treatment with group I agonists or antagonists had no significant effect on the phagocytosis of 40kDa beads. However, stimulation of group II receptors with DCGIV still maintained an increase in phagocytosis above basal levels after 24 hours incubation. In accordance with the results found after 2 and 4 hours, NAAG or MCCG had no significant effects on levels of phagocytosis after 24 hours. Interestingly pre-treatment of microglia to either AP4 or MAP4 still induced more cells to phagocytose 40kDa beads compared with basal levels after 24 hours.

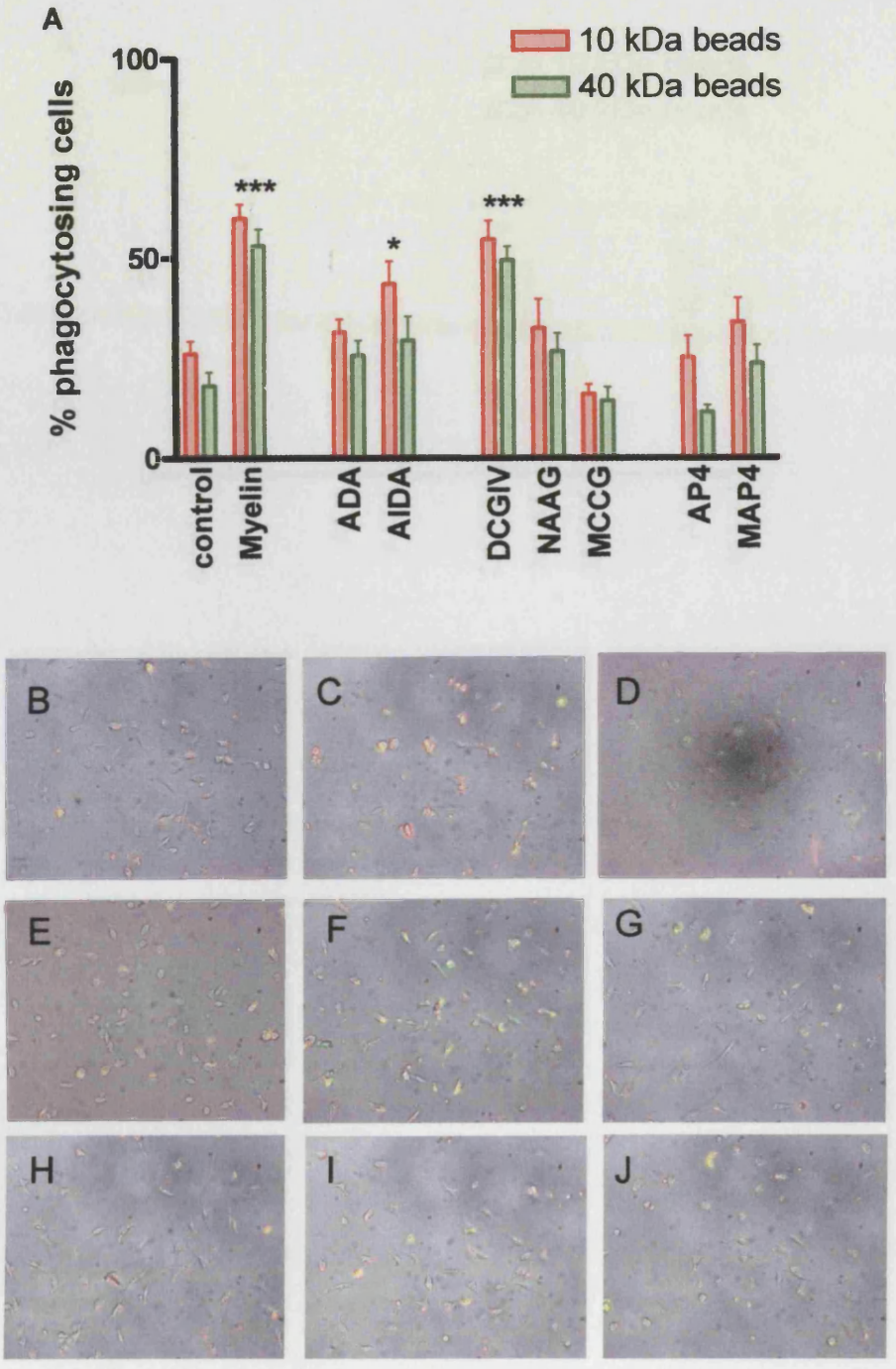


Figure 5.1 Phagocytosis of dextran beads by microglia after 2 hours is increased by exposure to myelin, DCGIV or AIDA.

Cultured microglia (1 *DIV*) were incubated with 20µg/ml of both 10 kDa (TRITC conjugated) and 40kDa (FITC conjugated) dextran beads for 2 hours at 37°C. Control cells were left untreated before incubation with the beads. A subset of cells were stimulated with either myelin or mGluR agonists or antagonists prior to incubation with the beads. Live cells were imaged and captured by phase contrast. **(A)** Phagocytosing cells were counted as a percentage of total cells per field. These data represent the mean and SEM of at least three fields per condition repeated in at least three separate experiments. **Figures B-J**. Representative images of microglial bead phagocytosis. Control microglia treated with beads alone (B) or microglia stimulated with myelin (C), ADA (D), AIDA (E), DCGIV (F), NAAG (G), MCCG (H), AP4 (I), MAP4 (J) prior to incubation with beads.

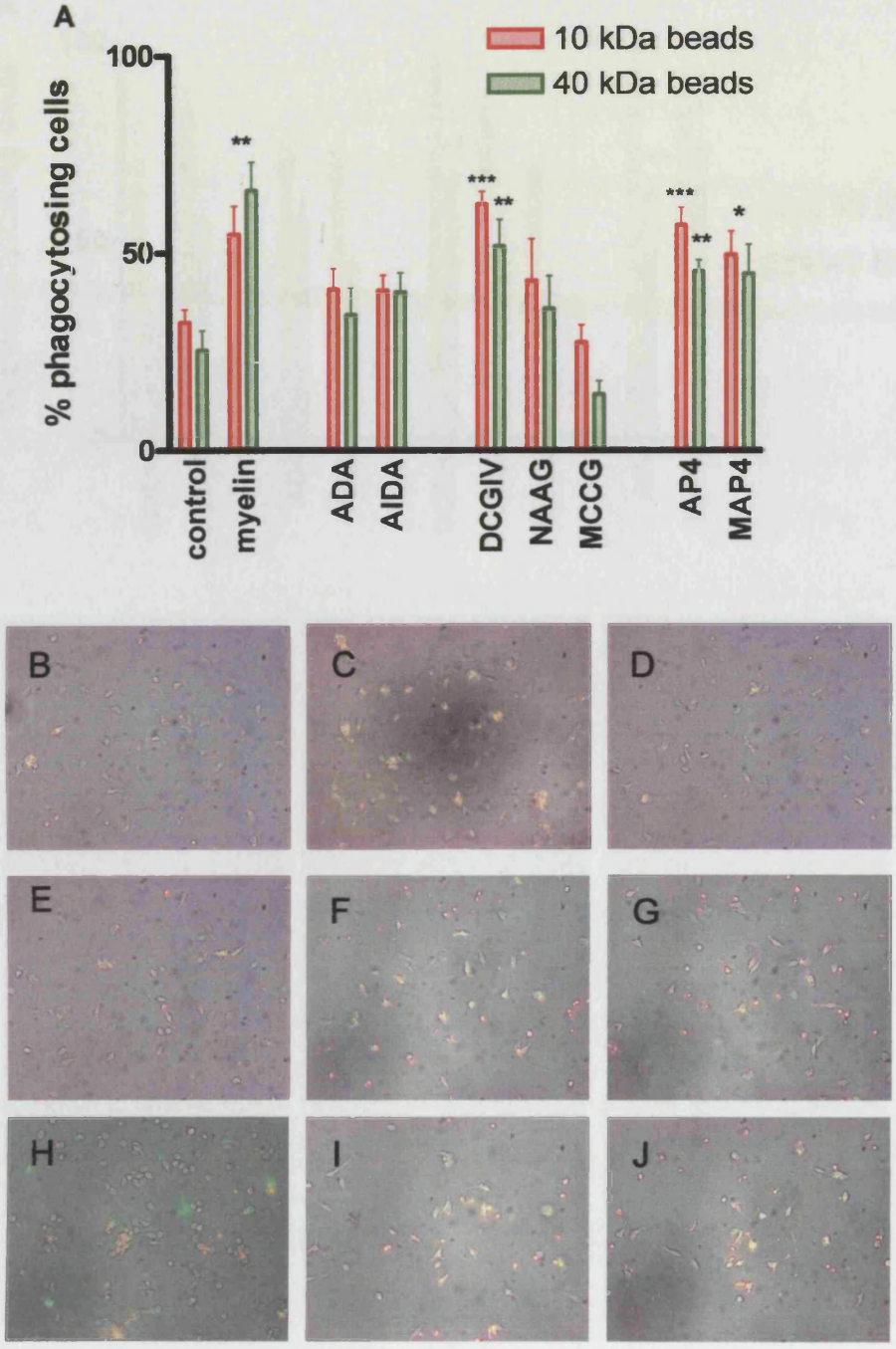


Figure 5.2 Phagocytosis of dextran beads by microglia after 4 hours is increased by exposure to myelin, DCGIV, AP4 or MAP4.

Cultured microglia (1 *DIV*) were incubated with 20µg/ml of both 10 kDa (TRITC conjugated) and 40kDa (FITC conjugated) dextran beads for 4 hours at 37°C. Control cells were left untreated before incubation with the beads. A subset of cells were stimulated with either myelin or mGluR agonists or antagonists prior to incubation with the beads. Live cells were imaged and captured by phase contrast. **(A)** Phagocytosing cells were counted as a percentage of total cells per field. These data represent the mean and SEM of at least three fields per condition repeated in at least three separate experiments. **Figures B-J** Representative images of microglial bead phagocytosis. Control microglia treated with beads alone (B) or microglia stimulated with myelin (C), ADA (D), AIDA (E), DCGIV (F), NAAG (G), MCCG (H), AP4 (I), MAP4 (J) prior to incubation with beads.

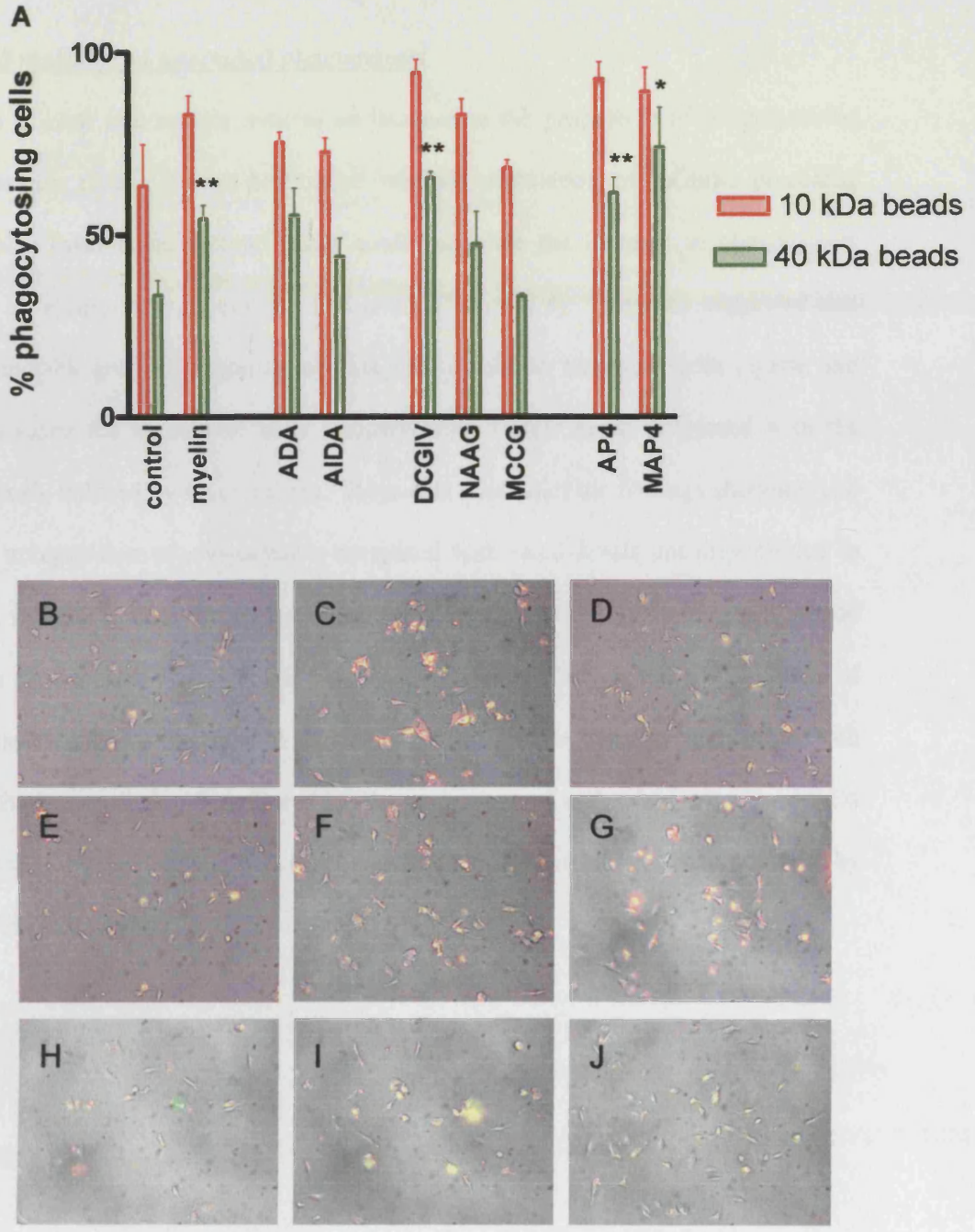


Figure 5.3 **Phagocytosis by microglia of 40kDa dextran beads after 24 hours was increased by exposure to myelin, DCGIV, AP4 and MAP4.**

Cultured microglia (1 *DIV*) were incubated with 20μg/ml of both 10 kDa (TRITC conjugated) and 40kDa (FITC conjugated) dextran beads for 24 hours at 37°C. Control cells were left untreated before incubation with the beads. A subset of cells were stimulated with either myelin or mGluR agonists or antagonists prior to incubation with the beads. Live cells were imaged and captured by phase contrast. **(A)** Phagocytosing cells were counted as a percentage of total cells per field. These data represent the mean and SEM of at least three fields per condition repeated in at least three separate experiments. **Figures B-J**. Representative images of microglial bead phagocytosis. Control microglia treated with beads alone (B) or microglia stimulated with myelin (C), ADA (D), AIDA (E), DCGIV (F), NAAG (G), MCCG (H), AP4 (I), MAP4 (J) prior to incubation with beads.

5.2.2 Modulation of microglial phagocytosis

It is clear that myelin induces an increase in the proportion of phagocytosing cells. For this reason it was determined whether stimulation of mGluRs preceding exposure to myelin and dextran beads could modulate the increase in phagocytosis induced by exposure to myelin for two hours (Figure 5.4). This data suggested that incubation with group III agonists or antagonists prior to treatment with myelin and beads, reduced the amount of cells phagocytosing 10kDa beads compared with the phagocytosis induced by myelin alone. These data contradict the findings showing AP4 induced upregulation of phagocytosis compared with basal levels but may be due to different signalling mechanisms induced by exposure to myelin. Interestingly blockade of group II receptors with MCGC had no significant effect on the phagocytosis of 10kDa beads induced by myelin. Treatment with MCCG prior to incubation with myelin did however significantly reduce the percentage of cells phagocytosing 40kDa beads. These results suggest that myelin induced phagocytosis can be modulated by stimulation of mGluRs.

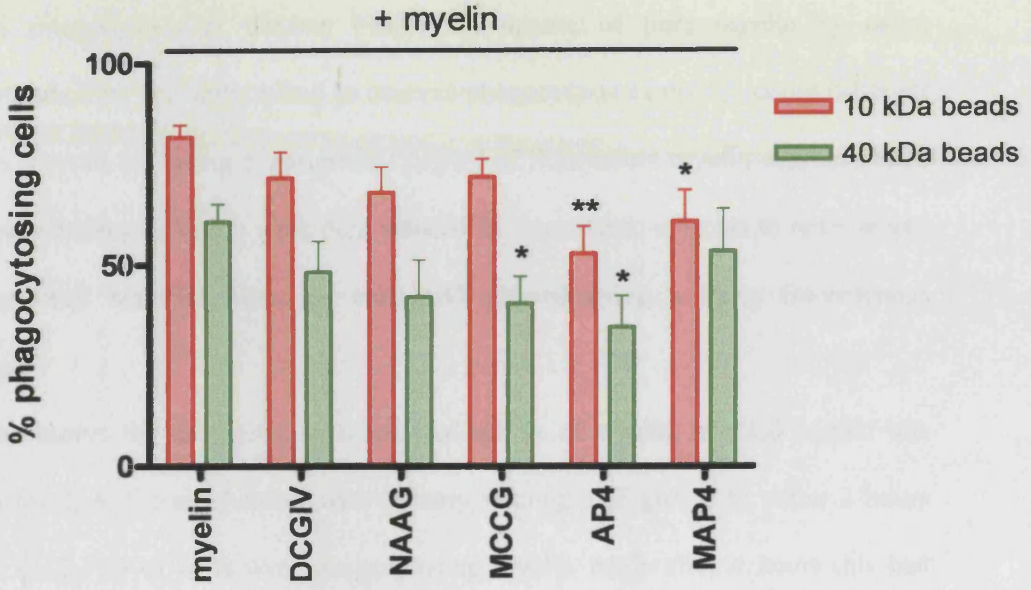


Figure 5.4 Prior exposure of microglia to MCCG or AP4 reduces the phagocytosis of 40kDa dextran beads induced by incubation with myelin.

Cultured microglia (1 *DIV*) were incubated with 20μg/ml of both 10 kDa (TRITC conjugated) and 40kDa (FITC conjugated) dextran beads for 4 hours at 37°C. Cells were stimulated with mGluR agonists or antagonists then incubated with myelin prior to addition of dextran beads. Positive controls omitted stimulation with mGluR agonists or antagonists. Live cells were imaged and captured by phase contrast. Phagocytosing cells were counted as a percentage of total cells per field. These data represent the mean and SEM of at least three fields per condition repeated in at least two separate experiments.

5.2.3 Myelin is phagocytosed by primary microglia

Since myelin and the group II agonist DCGIV consistently upregulated microglial phagocytosis of dextran beads, the uptake of pure myelin by using fluorescent labelling was determined to observe phagocytosis of myelin under different conditions. For all following experiments, 2μg/ml of fluorescent myelin was incubated with primary microglia which were then washed in warm basic solution to remove any un-phagocytosed material from the cells and viewed using a Zeiss fluorescence microscope.

To observe the timecourse of microglial uptake of myelin, labelled myelin was incubated for 2, 4, 6 and 24 hours with primary microglia (Figure 5.5). After 2 hours only 17% (± 3.7%) of cells were phagocytosing myelin while after 4 hours this had risen to 42% (± 3.1%). After 24 hours the percentage of cells phagocytosing myelin had reached 80% (± 2.4%). Modulation of dextran bead phagocytosis after 24 hours was less prominent than at earlier timepoints, so for experiments using labelled myelin, an incubation time of 4 hours was used since there was no significant increase between myelin uptake after 4 and 6 hours (P=0.054).

To ascertain that myelin was being phagocytosed and was not simply binding to microglia, cells were treated with myelin while incubating at 4°C since at this temperature the phagocytic but not the binding mechanism is inhibited (Figure 5.6). Cells were also pre-treated with 154mM sodium azide in PBS as described, which acted to metabolically poison the cells and block receptor mediated endocytosis. Levels of phagocytosis in cultures treated with myelin alone and incubated at 37°C reached 59% (± 2.5%). Cells incubated at 4°C however, did not show elevated levels of phagocytosis with only 8% (±1.7%) cells positive for TRITC staining. This indicates that only a low amount of the fluorescence counted as phagocytosis may be due to binding. Blocking endocytosis significantly reduced the amount of phagocytosing cells compared to

conditions treated with myelin alone ($P<0.001$) However the levels of phagocytosis were still significantly higher than those recorded after incubation at 4°C so it may be possible that sodium azide treatment reduces but does not completely block receptor mediated endocytosis or more likely that myelin phagocytosis is also mediated by different mechanisms. A subset of cells were also treated with a stripping wash following incubation with myelin. This served to remove myelin on the cell surface allowing detection of internalised fluorescence only. Cultures treated with the acid wash post incubation with myelin still showed an average of 57% (± 3.4%) phagocytosis which was not significantly different from levels detected in cells treated with myelin alone then washed in basic medium ($P=0.5625$). This demonstrated that excess myelin not taken up by microglia was effectively removed by washing cells prior to observation and that acid-stripping of un-internalised substances was unnecessary in this case.

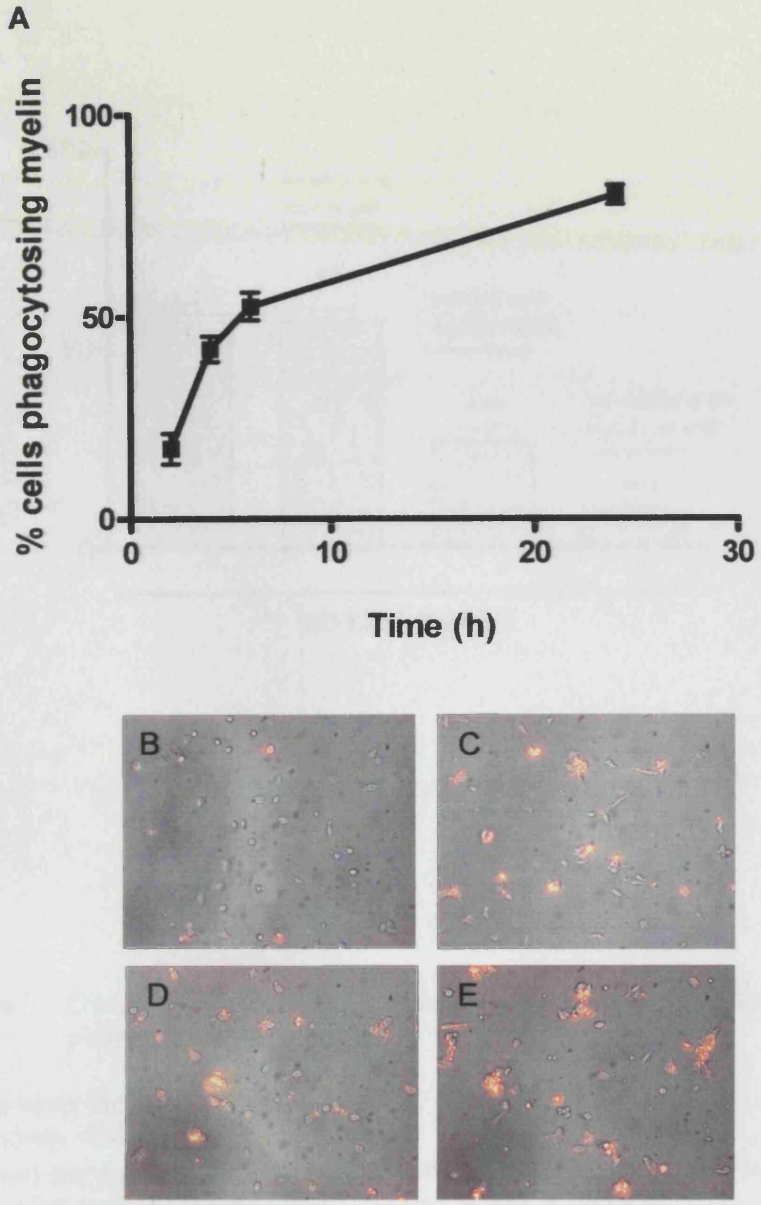


Figure 5.5 Timecourse of fluorescent myelin phagocytosis

Microglia were incubated with myelin (TRITC labelled) for 2, 4 , 6, and 24 hours at 37°C and live cells were imaged and captured by phase contrast. **(A)** The number of cells phagocytosing myelin were counted as a percentage of the total cells per field. Representative images of microglia incubated with labelled myelin for 2 **(B)**, 4 **(C)**, 6 **(D)**, 24 **(E)** hours. These data represent mean and SEM of at least four fields per condition repeated in at least two separate experiments.

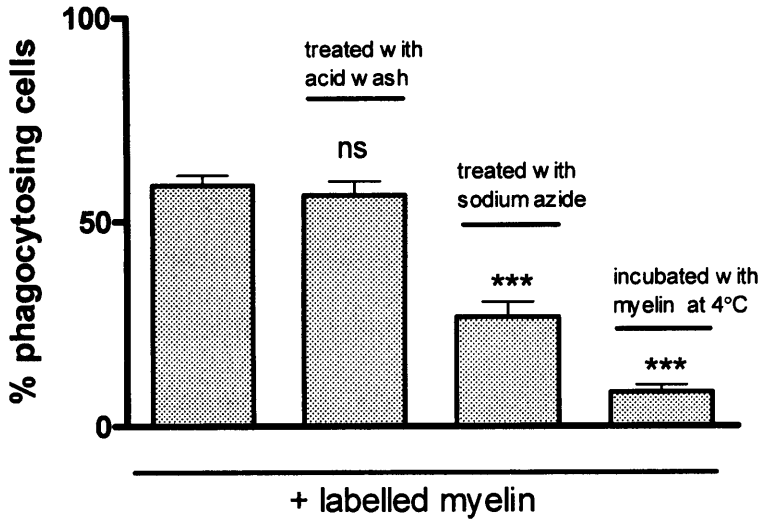


Figure 5.6 Observed microglial ingestion of fluorescent myelin is due to phagocytosis not binding.

Microglia were incubated with myelin (TRITC labelled) either alone or after prior exposure to sodium azide (154mM in PBS; which blocks cellular endocytosis) for 4 hours at 37°C. A subset of microglia were treated with an acid wash after incubation with myelin to remove any un-internalised myelin on the cell surface. A further subset of cells were incubated with fluorescent myelin for 4 hours at 4°C to eliminate the possibility that observed fluorescence was due to binding. Live cells were imaged and captured by phase contrast. The number of cells phagocytosing myelin were counted as a percentage of the total cells per field. These data represent mean and SEM of at least four fields per condition repeated in at least two separate experiments.

5.2.4 Microglial myelin phagocytosis is modulated by metabotropic glutamate receptors

Treatment with mGluRs modulated the phagocytosis of dextran beads and furthermore, the increased phagocytosis of beads induced by myelin could be reduced by pre-treatment with group II antagonists and group III agonists. As such it was determined whether myelin phagocytosis could also be reduced by modulation of mGluRs (Figure 5.7). For these experiments microglia were pre-treated with specific mGluR agonists or antagonists for 30 minutes prior to incubation with myelin for a further 4 hours as described above.

Treatment with the group I agonist ADA reduced the phagocytosis of myelin by 20% ($P<0.001$) while blockade of this receptor had no significant effect. The group II agonist DCGIV which induced increases in the phagocytosis of dextran beads compared with basal levels, slightly increased the percentage of cells phagocytosing myelin although this was not significant. This may suggest that the microglial phagocytic response was overwhelmed by the concentrations of myelin used. Blockade of group II receptors with MCCG significantly reduced levels of myelin phagocytosis ($P<0.001$). Treatment of the mGluR3 with subtype-specific group II agonist NAAG, however, did not lead to any significant changes in levels of myelin phagocytosis which indicates that phagocytosis is modulated by the mGluR2 subtype. Interestingly the group III agonist AP4, which caused increased phagocytosis of dextran beads when incubated with myelin alone, induced a 20% reduction in myelin phagocytosis ($P<0.001$) while blockade of group III receptors induced no significant change in the phagocytosis of myelin. These data are in accordance with the dextran bead data which showed that myelin induced upregulation of phagocytosis could be reduced by prior treatment with MCCG or AP4.

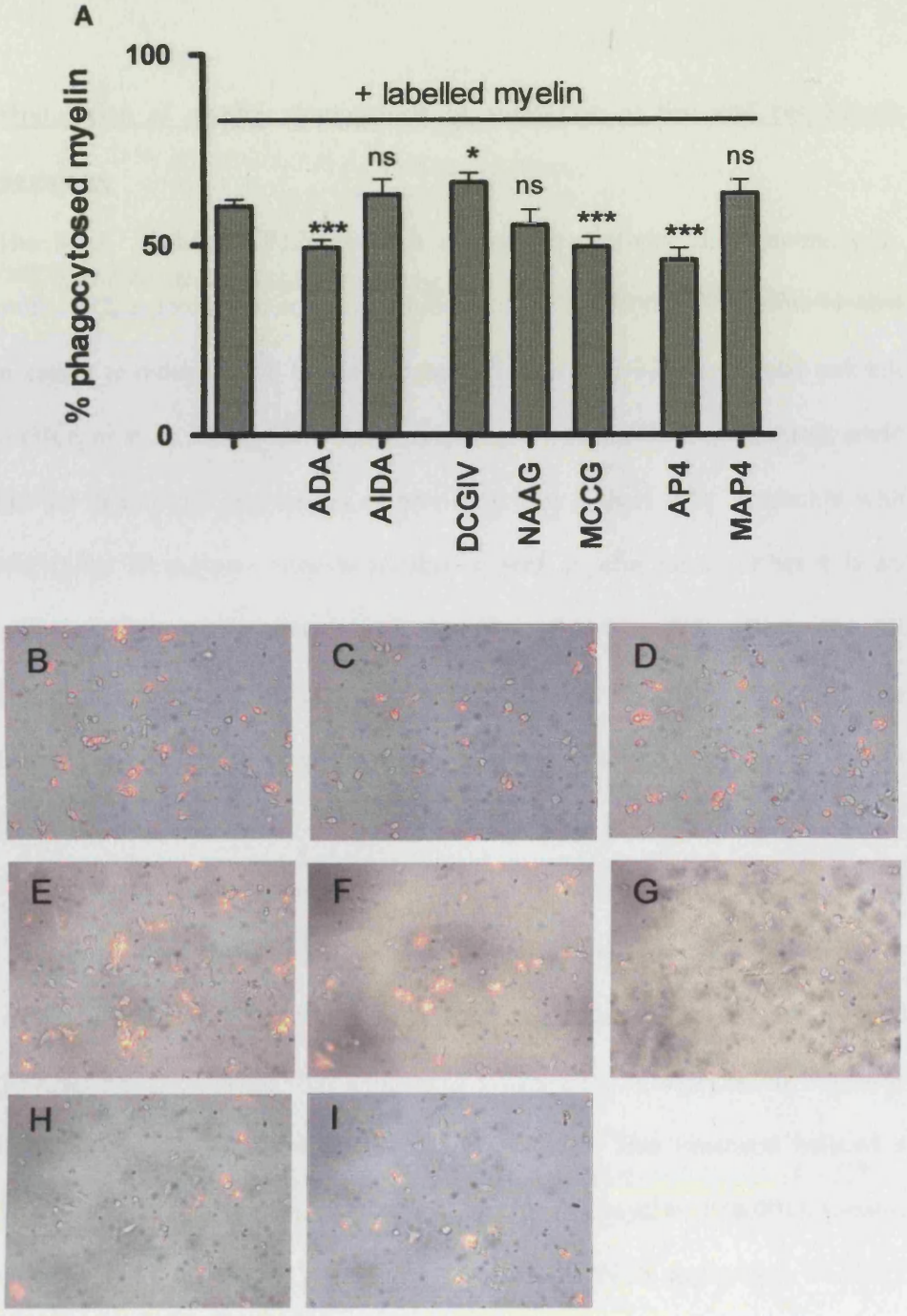


Figure 5.7 Phagocytosis of fluorescent myelin by microglia is reduced by prior exposure to ADA, MCCG or AP4

Microglia were stimulated with group II and III mGluR agonists or antagonists prior to incubation with fluorescently myelin (TRITC labelled) for 4 hours at 37°C. A subset of cells were exposed to myelin alone as a positive control. **(A)** The number of microglia phagocytosing myelin were counted as a percentage of total cells per field. These data represent the mean plus SEM of at least three fields per condition repeated in at least three separate experiments. **Figures B-I:** Representative images of microglia incubated with labelled myelin alone (B) or cells first stimulated with ADA (C), AIDA (D), DCGIV (E), NAAG (F), MCCG (G), AP4 (H), MAP4 (I) before incubation with myelin are shown. Labelled myelin can be seen within the microglia (captured by phase contrast).

5.2.5 Modulation of myelin phagocytosis by inhibition of Src and rho kinase signalling

The MEK inhibitor U0126 reduced microglial reactivity and neurotoxicity. Furthermore, PP2, a potent and selective inhibitor of the src family of tyrosine kinases has been shown to reduce iNOS expression and albumin induced intracellular calcium increases (Hooper et al., 2005). Here it was determined whether these compounds could also affect the phagocytic mechanism of myelin uptake (Figure 5.8). Treatment with PP2 (100nM) for 30 minutes prior to incubation with myelin for a further 4 hours significantly reduced myelin phagocytosis by 20% ($P<0.01$). This effect was not detected using a lower concentration of 50nM. Incubation of U0126 with microglia before further treatment with labelled myelin, however, had no significant effect on myelin phagocytosis.

Rho-GTPases act as intracellular switches and have important roles in cell motility. As such, their involvement in myelin phagocytosis was analysed here. Y-27632 inhibits the small GTPase RhoA or its downstream target Rho-associated coiled kinase (ROCK). Microglial cells were exposed to Y-27632 for 24 hours before exposure to labelled myelin for a further 4 hours (Figure 5.9 A-C). This treatment induced a potent reduction (50%) in the number of cells phagocytosing myelin ($P<0.001$). Lysates of microglia treated with Y-27632 were also assessed for iNOS expression. Y-27632 notably reduced levels of iNOS induced by exposure to myelin alone (Figure 5.9D), which suggests that the mechanisms involved in iNOS expression and phagocytosis may be linked.

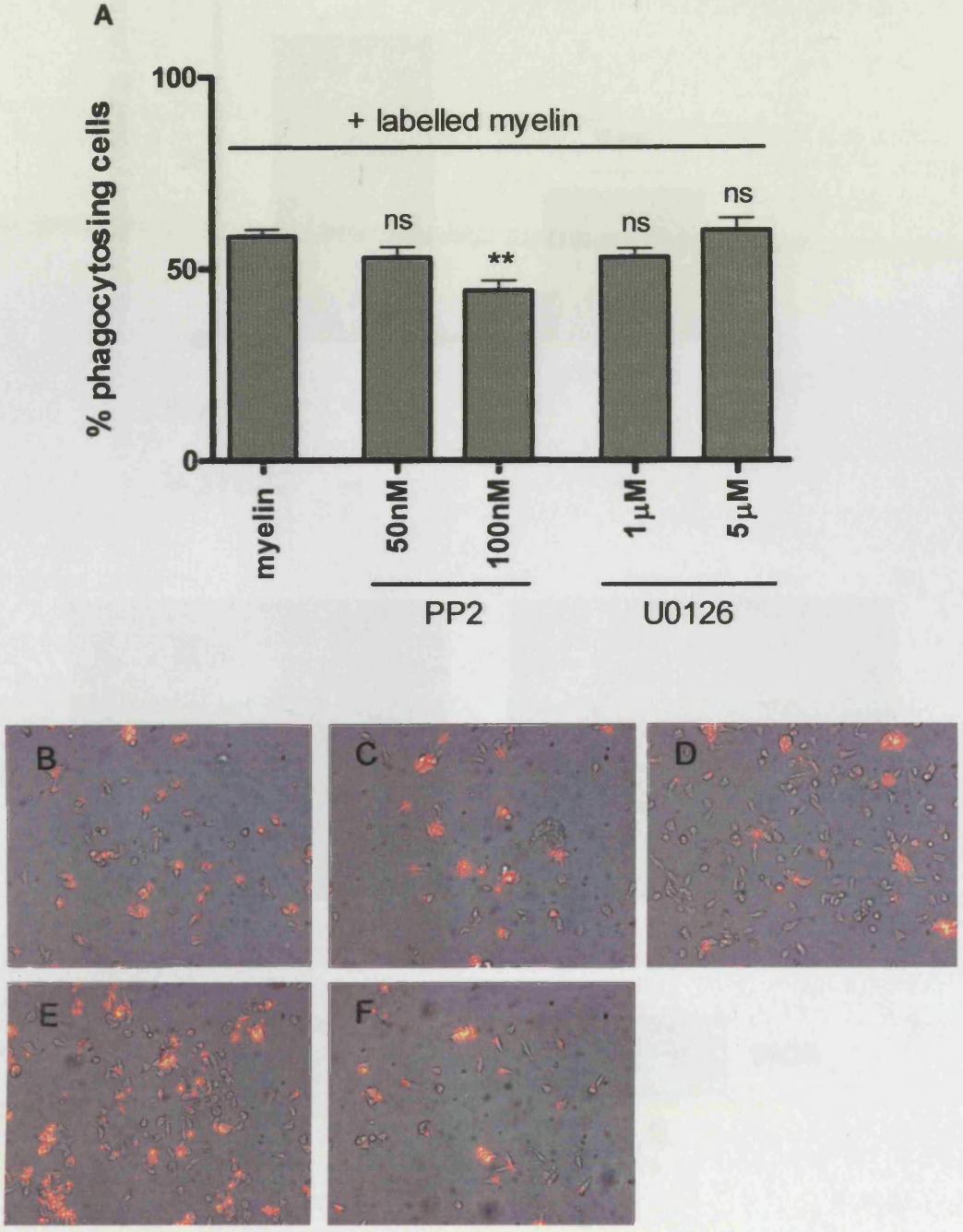


Figure 5.8 Phagocytosis of fluorescent myelin by microglia is reduced by prior incubation with PP2

Microglia were stimulated with inhibitors PP2 or U0126 for 30 minutes prior to incubation with fluorescently labelled myelin for 4 hours at 37°C. A subset of cells were exposed to myelin alone as a positive control. **(A)** The number of microglia phagocytosing myelin were counted as a percentage of total cells per field. These data represent the mean plus SEM of at least three fields per condition repeated in at least three separate experiments. **Figures B-F:** Representative images of microglia incubated with labelled myelin alone (B) or cells first stimulated with PP2 50nM (C), PP2 100nM (D), U0126 1μM (E), U0126 5μM (F), before incubation with myelin are shown. Labelled myelin can be seen within the microglia (captured by phase contrast).

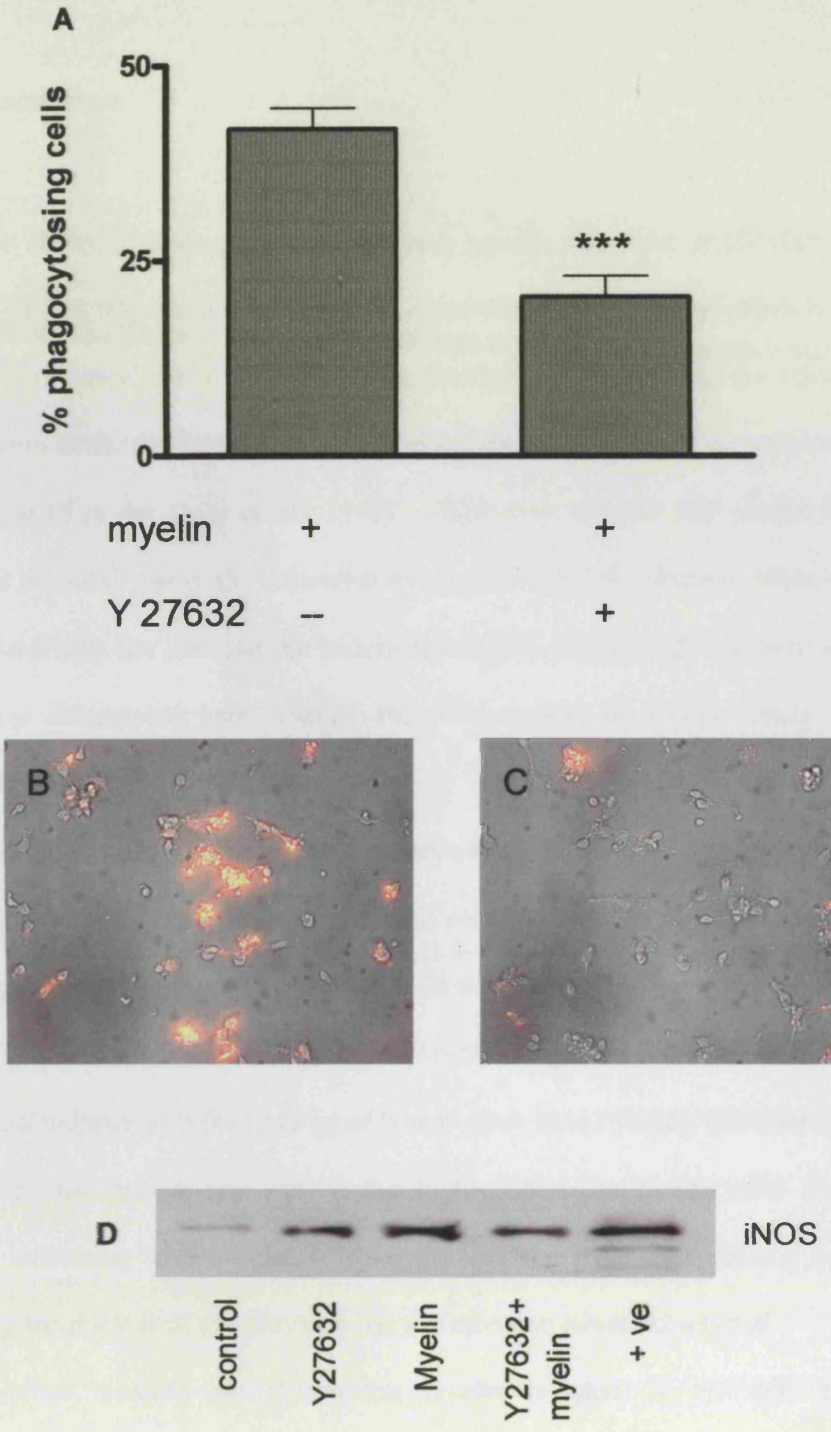


Figure 5.9 Phagocytosis of fluorescent myelin by microglia is reduced by prior exposure to rho-kinase inhibitor Y-27637

Microglia were incubated with labelled myelin either alone as a positive control, or after prior treatment with Y-27632 for 24 hours at 37°C. **(A)** The number of microglia phagocytosing myelin were counted as a percentage of total cells per field. These data represent the mean plus SEM of at least three fields per condition repeated in at least three separate experiments. Representative images of microglia incubated with labelled myelin alone **(B)** or microglia pre-treated with Y-27632 before incubation with myelin **(C)**. **(D)** iNOS expression in cells treated as described was assessed by western blotting. This blot represents preliminary findings but shows that Y-27632 reduces the iNOS expression induced by myelin alone.

5.3 Discussion

The ability of microglia to phagocytose myelin may have implications in the pathology of MS. Myelin phagocytosis has been shown here and by others to induce release of cytokines and nitric oxide from microglia. Furthermore, the monoclonal antibodies most effective in promoting EAE also induced the greatest increase in myelin phagocytosis (Van der Goes et al., 1999) which may suggest that phagocytosis of myelin has a central role in the inflammatory processes in EAE disease. Modulation of microglial mGluRs can attenuate the toxicity induced by myelin and other activators. As such it was determined here whether the phagocytosis of myelin could also be modulated in this way.

Microglial phagocytosis of dextran beads was first assessed to allow analysis of phagocytic activity of otherwise unstimulated cells. Microglial cells incubated solely with dextran beads underwent phagocytosis at rest suggesting that microglia can be functionally active while maintaining ramified morphology and non-reactive phenotype. This is in accordance with the findings of Nimmerjhan et al., (2005), who demonstrated that 'resting' microglia *in vivo* were in fact highly active and motile cells. As such it would be interesting to determine whether the medium from unstimulated microglia undergoing basal levels of phagocytosis has any effect on neuronal survival.

Previous research has shown that myelin is taken up by microglia and macrophages via receptor mediated phagocytosis involving complement receptor 3 (CR3) (Bruck and Friede, 1990b, 1992; Van der Laan et al., 1996), scavenger receptor AI/II (SRAI/II) and the Fcγ receptor (FcγR) (Reichert and Rotshenker, 2003, Makranz et al., 2004, 2006) although phagocytosis by CR3 dominates (Reichert et al., 2001; Reichert and Rotshenker, 2003). It was found here that myelin induced potent increases in the number of cells phagocytosing dextran beads which suggests that activation

primes microglia to become phagocytic. These results are in accordance with recent work which found that LPS (Vallieres et al., 2006) and IFNγ (Chan et al., 2001) stimulated phagocytosis.

Consistently increased phagocytosis of dextran beads was observed over timepoints ranging from 2 to 24 hours in both microglial and N9 cultures treated with the group II mGluR agonist DCGIV. Current research has shown that CR3 and to a lesser extent SRAI/II mediated phagocytosis are modulated by intracellular levels of cAMP with normal levels activating and high levels inhibiting the phagocytic response (Makranz et al., 2004, 2006), DCGIV is known to activate group II mGluRs which are negatively coupled to AC, therefore reducing or maintaining normal intracellular cAMP levels. This mechanism may have a role in DCGIV-induced increases in phagocytic activity compared with untreated cells. Blockade of this receptor contained or reduced levels of phagocytosis compared with control levels.

Since dextran beads are not physiologic compounds, microglia were also treated with mGluR agonists or antagonists prior to incubation with labelled myelin to determine whether myelin phagocytosis could be modulated in the same way. Stimulation of group II receptors did not increase levels of myelin phagocytosis, this may be due to myelin induced saturation of the phagocytic response. However, blockade of group II mGluRs significantly reduced the phagocytosis of myelin. This is possibly due to lack of inhibition of adenylate cyclase (AC) thus allowing build up of cAMP levels and subsequent inhibition of phagocytosis (Makranz et al., 2006)

Interestingly pre-treatment with AP4 prior to incubation with labelled myelin also induced a reduction in phagocytic activity. This was in contrast to our findings with dextran beads which showed AP4-induced upregulation of phagocytosis. However, the type of inflammatory stimuli received by both macrophages (Slobodov et al., 2001) and microglia (Reichert et al., 2001) affects the functional response of these cells inducing

distinct patterns of phagocytosis. Furthermore, certain treatments have been found to block myelin phagocytosis while having no effect on the phagocytosis of dextran beads suggesting involvement of different or divergent pathways (Bruck and Friede 1990a). This may explain the differential effects of AP4 on the phagocytosis of dextran beads and pure myelin since the beads represent non-physiologic stimuli.

Like group II mGluRs, group III mGluRs are negatively coupled to AC which suggests that attenuation of myelin phagocytosis in this case may not be mediated by intracellular cAMP levels. However, cAMP levels are regulated by the balance between production by AC and degradation by phosphodiesterases (Patel et al., 2001; Cooper et al., 2003) which are also subject to modulation. Furthermore the differential regulation of ligand and voltage gated ion channels such as potassium or calcium channels by groups II and III mGluRs has been shown in other cell types which demonstrates the possibility of divergent functions in these two groups (Conn and Pin, 1997; De Blasi et al., 2001).

Activation of PKC has also been implicated in the modulation of CR3 and SRAI/II mediated phagocytosis, DAG which is a second messenger that stimulates PKC, has a dose dependent dual effect, activation of phagocytosis at low concentrations and inhibition at high concentrations (Cohen et al., 2006). Stimulation of group I mGluRs significantly reduced myelin phagocytosis. This is possibly due to PKC sensitive mechanisms since group I receptors are positively coupled via G_q to PLC and as such their stimulation results in upregulation of intracellular DAG which may subsequently inhibit phagocytosis.

We analysed phagocytic transduction pathways by using specific inhibitors of cell signalling pathways. Pre-treatment with PP2, a potent and selective inhibitor of the src family of tyrosine kinases, prior to incubation with labelled myelin significantly reduced myelin phagocytosis. This is consistent with Srk/Syk involvement in early

FcγR signalling and suggests modulation via the FcγR (Song et al., 2004). Treatment of microglia with PP2, however, did not completely abrogate myelin phagocytosis which suggests involvement of other pathways consistent with the observation that most myelin phagocytosis is mediated via CR3. Treatment with the MEK inhibitor U0126, however, had no effect on myelin phagocytosis. These results are in accordance with the findings of Song et al., (2004) who showed that ERK was involved in activation induced microglial expression of macrophage inflammatory protein but not in FcγR mediated phagocytosis.

Rho GTPases act as intracellular molecular switches transducing extracellular signals to the actin cytoskeleton and therefore have important roles in cell motility. Inhibition of microglial rho kinase prior to incubation with myelin induced significant reduction in phagocytic activity. This may be explained in part by the importance of rho GTPase in rearrangement of the cytoskeleton required during the phagocytic process. Indeed, it has been demonstrated that rho activity is required for actin assembly in the phagocytic cup for subsequent particle engulfment in CR3 but not FcγR mediated phagocytosis (Caron and Hall, 1998; Olazabel et al., 2002). We found that inhibition of Rho kinase activity with Y27632 was most effective in preventing the phagocytosis of myelin by microglia which further suggests that myelin phagocytosis is primarily mediated by CR3.

CR3 mediated phagocytosis has been shown to induce NO production in macrophages (Goodrum et al., 1994) and microglia (Corradin et al., 1993; Van der laan et al., 1996) consistent with their role in innate immunity. Furthermore, NO induces upregulation of CR3 expression (Roy et al., 2006) which suggests a possible positive feedback mechanism. It is possible that the neuroprotection afforded by blocking the action of group II or stimulation of group III mGluRs on microglia could be the result of a reduced capacity for phagocytosis and subsequent reduction of myelin induced iNOS

expression. Interestingly, blocking rho kinase activity, known to be vital in CR3 mediated phagocytosis, also reduced iNOS expression compared with levels induced by myelin alone.

Future work will involve repeating these experiments using western blotting for MBP or MOG within cells, or fluorescent activated cell sorting (FACS) analysis for more quantitative results. FACS analysis would allow investigation of the total myelin phagocytosed for different conditions instead of the percentage of cells undergoing phagocytosis. This would probably reveal larger differences between conditions since purely counting the number of phagocytosing cells negates the possibility of differences in cellular myelin load. It is also possible that the levels of phagocytosis recorded for the 40kDa beads were influenced by the fact that the cells were always incubated with both 10kDa and 40kDa beads together. If the phagocytic response is potentiated by activation then perhaps the presence of 10kDa beads promoted the phagocytosis of 40kDa beads. Further experiments incubating the beads separately would answer these questions. It would also be of interest to measure the effect of iNOS inhibitors such as AMT-HCL on myelin phagocytosis since this compound has been shown to reduce the myelin induced microglial neurotoxic phenotype.

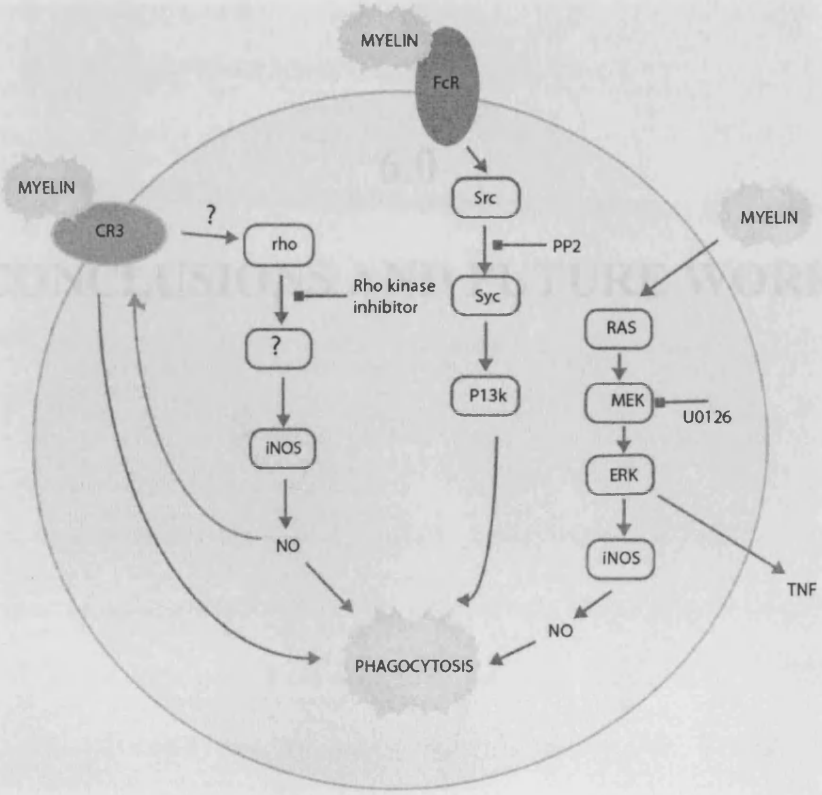


Figure 5.10 Possible signalling pathways regulating microglial myein phagocytosis

Microglial phagocytosis of myelin is regulated by inhibition of rho kinase which is responsible for reorganisation of the actin cytoskeleton and as such must be central to the phagocytic mechanism, it has also been shown that inhibition of rho-kinase activity can reduce iNOS expression, this may also play a part in regulation of phagocytosis since NO regulates CR3 expression in microglia. Inhibition of Src activity also reduced phagocytosis, this is consistent with the role of Src in FcR signalling and suggests that not all myelin is phagocytosed by CR3 (Song et al., 2004).

6.0

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

Metabotropic glutamate receptors in the CNS are expressed in regionally and cellular specific patterns that govern their modulatory functions. Microglia express subtypes from group I, II and III mGluRs. shown- Stimulation of group II microglial mGluRs has been shown to be neurotoxic while stimulation of group I mGluRs can be neuroprotective. As such, microglial reactivity and toxicity during disease progression may be the result of increased expression of one subtype of mGluR over another. This study found that activation of microglia induced changes in mGluR phenotype compared with untreated cells. Both group I (mGluR1) and II (mGluR2 and 3) mGluRs were upregulated after exposure to myelin. Interestingly group II subtype mGluR2 has been shown to induce a neurotoxic microglial phenotype (Taylor et al., 2002, 2005) while mGluR3 stimulation has been associated in astrocytes with neuroprotection via TGFβ release (Bruno et al., 1998). It is possible that microglial upregulation of both subtypes may reflect an attempt to regulate the toxic effects of activation. In line with these findings, we also show here that myelin induced upregulation of microglial mGluR8. These findings are in agreement with work by Guerts et al., (2003) who found that microglia/MØ in MS plaques also showed increased expression of this subtype. Given that group III microglial mGluRs can promote neuroprotection, it is possible that upregulation of mGluR8 is in an attempt to control the toxic effects of activation. These results suggest that functional microglial phenotype regulates the mGluR profile and as such may modulate reactivity.

Microglial exposure to myelin induced a reactive phenotype involving release of inflammatory mediators including TNFα, glutamate and nitrite, and upregulated expression of iNOS and Phospho-ERK. Treatment with myelin induced microglia to become neurotoxic since medium from these cells was deleterious to neuronal survival

in culture. This was attributable to factors released by microglia since direct application of myelin to neurones induced no such effect. It is possible that myelin induced microglial neurotoxicity is due to the high TNFα release or the downstream products of iNOS expression since compounds blocking these mediators were neuroprotective. Indeed TNFα is toxic to neurones when released from microglia in concert with FasL (Taylor et al., 2005) and glutamate (Floden et al., 2005) possibly mediating NO dependent death in neurones (Combs et al., 2001). Microglial reactivity after exposure to myelin has important implications in the study of MS pathology where microglial phagocytosis of myelin debris is known to occur and as such could be cauing by-stander damage to neurones and other supporting cells thus further exacerbating the inflammatory cascade. These data highlight the importance of strategies that reduce microglial reactivity as a potential treatment in MS.

Interestingly, myelin induced microglial neurotoxicity was modulated by microglial mGluRs and could be attenuated by blocking group II or stimulating group III mGluRs. Furthermore, modulation of microglial mGluRs reduced the iNOS expression induced by myelin, consistent with the neuroprotection afforded by inhibition of microglial iNOS. The phagocytosis of myelin is thought to induce respiratory burst activity in microglia where NADPH oxidase, which is normally dormant in resting phagocytes, is activated and subsequenty catalyses the production of superoxide from oxygen. Superoxide readily reacts with NO to form peroxynitrite which recent studies have suggested is more toxic to neurones and oligodendrocytes than NO alone (Li et al., 2005) It is possible that reduced NO therefore leads to reduced $ONOO^-$ preduction which may reduce the overall feedback activation of microglia.

Interestingly the phagocytosis of myelin could also be similarly attenuated by blocking group I or II mGluRs and stimulating group III mGluRs. It has been shown that microglial and macrophage phagocytosis mediated by CR3 induces NO release

(Van der laan et al., 1996; Roy et al., 2006) which would be expected as part of an innate immune response. It is possible that the reduced neurotoxicity and iNOS expression shown here by modulation of group II and III mGluRs could therefore be a result of attenuation of the inflammatory phagocytic response mediated by myelin.

Taken together, these findings indicate that changes in microglial mGluR expression may be of relevance in the microglial activation in MS. Furthermore, modulation of microglial mGluRs may therefore represent possible pharmacological targets for therapeutic interventions in multiple sclerosis.

6.2 Future work

Attenuation of microglial neurotoxicity and reactivity by modulation of mGluRs may be of therapeutic benefit in MS. As such, future work will involve elucidation of precisely which mGluR subtypes are most effective at promoting a protective phenotype by repeating neurotoxicity experiments using a wider range of potent and selective mGluR agonists and antagonists. Microglial neurotoxicity experiments will also be repeated after stimulation of group I microglial mGluRs which have been shown here to modulate phagocytosis. The signalling pathways involved in attenuation of microglial toxicity mediated by mGluRs will also be investigated using co-culture systems which will provide data more closely related to *in vivo* situations. Microglial activation was shown here to modulate mGluR expression, future work determining non-toxic factors capable of upregulating neuroprotective microglial mGluR phenotype would therefore be of therapeutic relevance for inflammatory diseases of the CNS.

This work has shown that mGluR expression is markedly altered in the EAE mouse model of MS compared with untreated controls. Since mGluR expression varies in different cell types, the use of brain and spinal cord homogenates could only provide a broad overview of the general mGluR profile. Further research will involve the isolation of primary microglia from an EAE model which will allow the specific analysis of microglial mGluR expression during disease progression. This would be of particular interest as expression of microglial mGluR subtypes could be compared during inflammatory attack, remission or the progressive chronic phase of disease. Further work will also involve the development of a single cell RT-PCR protocol, this would not only enable quantitative analysis of microglial mGluR expression but also

allow region specific analysis of microglial mGluR profiles in preactive or chronic plaques compared with normal appearing white matter.

Quantitative determination of microglial phagocytic activity will also be analysed using western blotting for MOG proteins, and FACS analysis. This will be interesting since this study, analysing the percentage of phagocytosing cells, could not properly account for differences in myelin load within cells. Further investigations into the signalling pathways involved in myelin phagocytosis and consequent reactive phenotype are necessary to understand how myelin induces microglia to become neurotoxic. This could be explored using specific inhibition of CR3 or FcR signalling and subsequent assessment of phagocytic activity and neurotoxicity. Specific iNOS inhibition will also allow analysis of the role of iNOS in phagocytosis. Inducing effective phagocytic activity and clearance without this associated reactivity will also be an important avenue of research for the future.

7.0 REFERENCE LIST

Abbas A. K., Murphy K. M. and Sher A. (1996) Functional diversity of helper T lymphocytes. *Nature* **383,** 787-793.

Abbott N. J. (2002) Astrocyte-endothelial interactions and blood-brain barrier permeability*. *Journal of Anatomy* **200,** 629-638.

Acar G., Idiman F., Idiman E., Kirkali G., Cakmakci H. and Ozakbas S. (2003) Nitric oxide as an activity marker in multiple sclerosis. *J. Neurol.* **250,** 588-592.

Achiron A., Miron S., Lavie V., Margalit R. and Biegon A. (2000) Dexanabinol (HU-211) effect on experimental autoimmune encephalomyelitis: implications for the treatment of acute relapses of multiple sclerosis. *J Neuroimmunol.* **102,** 26-31.

Adams C. W., Poston R. N. and Buk S. J. (1989) Pathology, histochemistry and immunocytochemistry of lesions in acute multiple sclerosis. *J. Neurol. Sci.* **92,** 291-306.

Agrawal S. K., Theriault E. and Fehlings M. G. (1998) Role of group I metabotropic glutamate receptors in traumatic spinal cord white matter injury. *J. Neurotrauma* **15,** 929-941.

Akassoglou K., Bauer J., Kassiotis G., Pasparakis M., Lassmann H., Kollias G. and Probert L. (1998) Oligodendrocyte apoptosis and primary demyelination induced by local TNF/p55TNF receptor signaling in the central nervous system of transgenic mice: models for multiple sclerosis with primary oligodendrogliopathy. *Am. J. Pathol.* **153,** 801-813.

Aldskogius H. and Kozlova E. N. (1998) Central neuron-glial and glial-glial interactions following axon injury. *Prog. Neurobiol.* **55,** 1-26.

Allen J. W., Ivanova S. A., Fan L., Espey M. G., Basile A. S. and Faden A. I. (1999) Group II metabotropic glutamate receptor activation attenuates traumatic neuronal injury and improves neurological recovery after traumatic brain injury. *J Pharmacol. Exp. Ther.* **290,** 112-120.

Aloisi F., Penna G., Cerase J., Menendez I. B. and Adorini L. (1997) IL-12 production by central nervous system microglia is inhibited by astrocytes. *J. Immunol.* **159,** 1604-1612.

Aloisi F., Serafini B. and Adorini L. (2000) Glia-T cell dialogue. *J. Neuroimmunol.* **107,** 111-117.

Aloisi F. (2001) Immune function of microglia. *Glia* **36,** 165-179.

Arduini A., Sullivan S. G. and Stern A. (1985) Comparative oxidative damage in red cells and myelin. *Prog. Clin. Biol. Res.* **195,** 123-133.

Arnett H. A., Mason J., Marino M., Suzuki K., Matsushima G. K. and Ting J. P. (2001) TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat Neurosci.* **4,** 1116-1122.

Aronica E., van Vliet E. A., Mayboroda O. A., Troost D., da Silva F. H. and Gorter J. A. (2000) Upregulation of metabotropic glutamate receptor subtype mGluR3 and

mGluR5 in reactive astrocytes in a rat model of mesial temporal lobe epilepsy. *Eur. J Neurosci.* **12,** 2333-2344.

Aronica E., Catania M. V., Geurts J., Yankaya B. and Troost D. (2001) Immunohistochemical localization of group I and II metabotropic glutamate receptors in control and amyotrophic lateral sclerosis human spinal cord: upregulation in reactive astrocytes. *Neuroscience* **105,** 509-520.

Aronica E., Gorter J. A., Ijlst-Keizers H., Rozemuller A. J., Yankaya B., Leenstra S. and Troost D. (2003) Expression and functional role of mGluR3 and mGluR5 in human astrocytes and glioma cells: opposite regulation of glutamate transporter proteins. *Eur. J. Neurosci.* **17,** 2106-2118.

Attwell P. J., Koumentaki A., bdul-Ghani A. S., Croucher M. J. and Bradford H. F. (1998) Specific group II metabotropic glutamate receptor activation inhibits the development of kindled epilepsy in rats. *Brain Res.* **787,** 286-291.

Badie B., Schartner J., Vorpahl J. and Preston K. (2000) Interferon-gamma induces apoptosis and augments the expression of Fas and Fas ligand by microglia in vitro. *Exp. Neurol.* **162,** 290-296.

Baek S. H., Seo J. K., Chae C. B., Suh P. G. and Ryu S. H. (1996) Identification of the peptides that stimulate the phosphoinositide hydrolysis in lymphocyte cell lines from peptide libraries. *J. Biol. Chem.* **271,** 8170-8175.

Bal-Price A. and Brown G. C. (2001) Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. *J Neurosci.* **21,** 6480-6491.

Banati R. B. and Graeber M. B. (1994) Surveillance, intervention and cytotoxicity: is there a protective role of microglia? *Dev. Neurosci.* **16,** 114-127.

Banati R. B., Newcombe J., Gunn R. N., Cagnin A., Turkheimer F., Heppner F., Price G., Wegner F., Giovannoni G., Miller D. H., Perkin G. D., Smith T., Hewson A. K., Bydder G., Kreutzberg G. W., Jones T., Cuzner M. L. and Myers R. (2000) The peripheral benzodiazepine binding site in the brain in multiple sclerosis: quantitative in vivo imaging of microglia as a measure of disease activity. *Brain* **123 (Pt 11),** 2321-2337.

Banchereau J. and Steinman R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245-252.

Barnett M. H. and Prineas J. W. (2004) Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann. Neurol.* **55,** 458-468.

Barnett M. H., Henderson A. P. and Prineas J. W. (2006) The macrophage in MS: just a scavenger after all? Pathology and pathogenesis of the acute MS lesion. *Mult. Scler.* **12,** 121-132.

Barron K. D. (1995) The microglial cell. A historical review. *J Neurol. Sci.* **134 Suppl,** 57-68.

Baskys A. and Blaabjerg M. (2005) Understanding regulation of nerve cell death by mGluRs as a method for development of successful neuroprotective strategies. *J. Neurol. Sci.* **229-230,** 201-209.

Battaglia G., Bruno V., Ngomba R. T., Di G. R., Copani A. and Nicoletti F. (1998) Selective activation of group-II metabotropic glutamate receptors is protective against excitotoxic neuronal death. *Eur. J. Pharmacol.* **356,** 271-274.

Bear MF, connors BW and Paradiso MA (2001) *Neuroscience- exploring the brain.* Lippincott williams and wilkins.

Becher B., Dodelet V., Fedorowicz V. and Antel J. P. (1996) Soluble tumor necrosis factor receptor inhibits interleukin 12 production by stimulated human adult microglial cells in vitro. *J. Clin. Invest* **98,** 1539-1543.

Becher B. and Antel J. P. (1996) Comparison of phenotypic and functional properties of immediately ex vivo and cultured human adult microglia. *Glia* **18,** 1-10.

Becher B., Blain M., Giacomini P. S. and Antel J. P. (1999) Inhibition of Th1 polarization by soluble TNF receptor is dependent on antigen-presenting cell-derived IL-12. *J. Immunol.* **162,** 684-688.

Becher B., Blain M. and Antel J. P. (2000) CD40 engagement stimulates IL-12 p70 production by human microglial cells: basis for Th1 polarization in the CNS. *J. Neuroimmunol.* **102,** 44-50.

Becher B., Bechmann I. and Greter M. (2006) Antigen presentation in autoimmunity and CNS inflammation: how T lymphocytes recognize the brain. *J. Mol. Med* **84,** 532-543.

Berridge M. J. and Irvine R. F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312,** 315-321.

Besong G., Battaglia G., D'Onofrio M., Di M. R., Ngomba R. T., Storto M., Castiglione M., Mangano K., Busceti C. L., Nicoletti F. R., Bacon K., Tusche M., Valenti O., Conn P. J., Bruno V. and Nicoletti F. (2002) Activation of group III metabotropic glutamate receptors inhibits the production of RANTES in glial cell cultures. *J. Neurosci.* **22,** 5403-5411.

Bhave G., Nadin B. M., Brasier D. J., Glauner K. S., Shah R. D., Heinemann S. F., Karim F. and Gereau R. W. (2003) Membrane topology of a metabotropic glutamate receptor. *J. Biol. Chem.* **278,** 30294-30301.

Biber K., Laurie D. J., Berthele A., Sommer B., Tolle T. R., Gebicke-Harter P. J., van C. D. and Boddeke H. W. (1999) Expression and signaling of group I metabotropic glutamate receptors in astrocytes and microglia. *J. Neurochem.* **72,** 1671-1680.

Bjartmar C. and Trapp B. D. (2001) Axonal and neuronal degeneration in multiple sclerosis: mechanisms and functional consequences. *Curr. Opin. Neurol.* **14,** 271-278.

Blaabjerg M., Baskys A., Zimmer J. and Vawter M. P. (2003) Changes in hippocampal gene expression after neuroprotective activation of group I metabotropic glutamate receptors. *Brain Res. Mol. Brain Res.* **117,** 196-205.

Bo L., Dawson T. M., Wesselingh S., Mork S., Choi S., Kong P. A., Hanley D. and Trapp B. D. (1994) Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains. *Ann. Neurol.* **36,** 778-786.

Bo L., Mork S., Kong P. A., Nyland H., Pardo C. A. and Trapp B. D. (1994) Detection of MHC class II-antigens on macrophages and microglia, but not on astrocytes and endothelia in active multiple sclerosis lesions. *J. Neuroimmunol.* **51,** 135-146.

Bo L., Vedeler C. A., Nyland H., Trapp B. D. and Mork S. J. (2003) Intracortical multiple sclerosis lesions are not associated with increased lymphocyte infiltration. *Mult. Scler.* **9,** 323-331.

Bolanos J. P., Peuchen S., Heales S. J., Land J. M. and Clark J. B. (1994) Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *J. Neurochem.* **63,** 910-916.

Broholm H., Andersen B., Wanscher B., Frederiksen J. L., Rubin I., Pakkenberg B., Larsson H. B. and Lauritzen M. (2004) Nitric oxide synthase expression and enzymatic activity in multiple sclerosis. *Acta Neurol. Scand.* **109,** 261-269.

Brosnan C. F., Cannella B., Battistini L. and Raine C. S. (1995) Cytokine localization in multiple sclerosis lesions: correlation with adhesion molecule expression and reactive nitrogen species. *Neurology* **45,** S16-S21.

Bruck W. and Friede R. L. (1990) Anti-macrophage CR3 antibody blocks myelin phagocytosis by macrophages in vitro. *Acta Neuropathol. (Berl)* **80,** 415-418.

Bruck W. and Friede R. L. (1990) L-fucosidase treatment blocks myelin phagocytosis by macrophages in vitro. *J Neuroimmunol.* **27,** 217-227.

Bruck W., Porada P., Poser S., Rieckmann P., Hanefeld F., Kretzschmar H. A. and Lassmann H. (1995) Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann. Neurol.* **38,** 788-796.

Bruck W. (2005) Inflammatory demyelination is not central to the pathogenesis of multiple sclerosis. *J. Neurol.* **252 Suppl 5,** v10-v15.

Bruck W. (2005) The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage. *J. Neurol.* **252 Suppl 5,** v3-v9.

Bruno V., Copani A., Battaglia G., Raffaele R., Shinozaki H. and Nicoletti F. (1994) Protective effect of the metabotropic glutamate receptor agonist, DCG-IV, against excitotoxic neuronal death. *Eur. J. Pharmacol.* **256,** 109-112.

Bruno V., Battaglia G., Copani A., Giffard R. G., Raciti G., Raffaele R., Shinozaki H. and Nicoletti F. (1995) Activation of class II or III metabotropic glutamate receptors protects cultured cortical neurons against excitotoxic degeneration. *Eur. J Neurosci.* **7,** 1906-1913.

Bruno V., Battaglia G., Casabona G., Copani A., Caciagli F. and Nicoletti F. (1998) Neuroprotection by glial metabotropic glutamate receptors is mediated by transforming growth factor-beta. *J. Neurosci.* **18,** 9594-9600.

Bruno V., Battaglia G., Ksiazek I., van der P. H., Catania M. V., Giuffrida R., Lukic S., Leonhardt T., Inderbitzin W., Gasparini F., Kuhn R., Hampson D. R., Nicoletti F. and Flor P. J. (2000) Selective activation of mGlu4 metabotropic glutamate receptors is protective against excitotoxic neuronal death. *J Neurosci.* **20,** 6413-6420.

Buisson A. and Choi D. W. (1995) The inhibitory mGluR agonist, S-4-carboxy-3-hydroxy-phenylglycine selectively attenuates NMDA neurotoxicity and oxygen-glucose deprivation-induced neuronal death. *Neuropharmacology* **34,** 1081-1087.

Buisson A., Yu S. P. and Choi D. W. (1996) DCG-IV selectively attenuates rapidly triggered NMDA-induced neurotoxicity in cortical neurons. *Eur. J Neurosci.* **8,** 138-143.

Carlson N. G., Wieggel W. A., Chen J., Bacchi A., Rogers S. W. and Gahring L. C. (1999) Inflammatory cytokines IL-1 alpha, IL-1 beta, IL-6, and TNF-alpha impart neuroprotection to an excitotoxin through distinct pathways. *J. Immunol.* **163,** 3963-3968.

Caron E. and Hall A. (1998) Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* **282,** 1717-1721.

Carrithers M. D., Visintin I., Kang S. J. and Janeway C. A., Jr. (2000) Differential adhesion molecule requirements for immune surveillance and inflammatory recruitment. *Brain* **123 (Pt 6),** 1092-1101.

Cartmell J. and Schoepp D. D. (2000) Regulation of neurotransmitter release by metabotropic glutamate receptors. *J. Neurochem.* **75,** 889-907.

Catania M. V., Landwehrmeyer G. B., Testa C. M., Standaert D. G., Penney J. B., Jr. and Young A. B. (1994) Metabotropic glutamate receptors are differentially regulated during development. *Neuroscience* **61,** 481-495.

Chan A., Magnus T. and Gold R. (2001) Phagocytosis of apoptotic inflammatory cells by microglia and modulation by different cytokines: mechanism for removal of apoptotic cells in the inflamed nervous system. *Glia* **33,** 87-95.

Chanas-Sacre G., Rogister B., Moonen G. and Leprince P. (2000) Radial glia phenotype: origin, regulation, and transdifferentiation. *J. Neurosci. Res.* **61,** 357-363.

Chen A., Kumar S. M., Sahley C. L. and Muller K. J. (2000) Nitric oxide influences injury-induced microglial migration and accumulation in the leech CNS. *J. Neurosci.* **20,** 1036-1043.

Cheng B., Christakos S. and Mattson M. P. (1994) Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. *Neuron* **12,** 139-153.

Chuang D. M., Gao X. M. and Paul S. M. (1992) N-methyl-D-aspartate exposure blocks glutamate toxicity in cultured cerebellar granule cells. *Mol. Pharmacol.* **42,** 210-216.

Ciesielski-Treska J., Ulrich G., Taupenot L., Chasserot-Golaz S., Corti A., Aunis D. and Bader M. F. (1998) Chromogranin A Induces a Neurotoxic Phenotype in Brain Microglial Cells. *J. Biol. Chem.* **273,** 14339-14346.

Ciruela F., Soloviev M. M. and McIlhinney R. A. (1999) Co-expression of metabotropic glutamate receptor type 1alpha with homer-1a/Vesl-1S increases the cell surface expression of the receptor. *Biochem. J* **341 (Pt 3),** 795-803.

Cohen G., Makranz C., Spira M., Kodama T., Reichert F. and Rotshenker S. (2006) Non-PKC DAG/phorbol-ester receptor(s) inhibit complement receptor-3 and nPKC inhibit scavenger receptor-AI/II-mediated myelin phagocytosis but cPKC, PI3k, and PLCgamma activate myelin phagocytosis by both. *Glia* **53,** 538-550.

Coles A., Deans J. and Compston A. (2004) Campath-1H treatment of multiple sclerosis: lessons from the bedside for the bench. *Clin. Neurol. Neurosurg.* **106,** 270-274.

Combs C. K., Karlo J. C., Kao S. C. and Landreth G. E. (2001) beta-Amyloid stimulation of microglia and monocytes results in TNFalpha-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. *J. Neurosci.* **21,** 1179-1188.

Compston A, Confavreux C, Lassmann H, McDonald I, Miller D, Noseworthy J, Smith K and Wekerle H (2006) *Multiple Sclerosis. Chpt 10.* Churchill Livingstone.

Compston A., Zajicek J., Sussman J., Webb A., Hall G., Muir D., Shaw C., Wood A. and Scolding N. (1997) Glial lineages and myelination in the central nervous system. *J. Anat.* **190 (Pt 2),** 161-200.

Compston A. and Coles A. (2002) Multiple sclerosis. *Lancet* **359,** 1221-1231.

Conlon P., Oksenberg J. R., Zhang J. and Steinman L. (1999) The immunobiology of multiple sclerosis: an autoimmune disease of the central nervous system. *Neurobiol. Dis.* **6,** 149-166.

Conn P. J. and Pin J. P. (1997) Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol.* **37,** 205-237.

Cooper D. M. (2003) Regulation and organization of adenylyl cyclases and cAMP. *Biochem. J* **375,** 517-529.

Cornford E. M. and Landon K. P. (1985) Blood-brain barrier transport of CI-912: single-passage equilibration of erythrocyte-borne drug. *Ther. Drug Monit.* **7,** 247-254.

Corradin S. B., Mauel J., Donini S. D., Quattrocchi E. and Ricciardi-Castagnoli P. (1993) Inducible nitric oxide synthase activity of cloned murine microglial cells. *Glia* **7,** 255-262.

Cuzner M. L., Hayes G. M., Newcombe J. and Woodroofe M. N. (1988) The nature of inflammatory components during demyelination in multiple sclerosis. *J. Neuroimmunol.* **20,** 203-209.

Cuzner M. L. and Norton W. T. (1996) Biochemistry of demyelination. *Brain Pathol.* **6,** 231-242.

D'Onofrio M., Cuomo L., Battaglia G., Ngomba R. T., Storto M., Kingston A. E., Orzi F., De B. A., Di I. P., Nicoletti F. and Bruno V. (2001) Neuroprotection mediated by

glial group-II metabotropic glutamate receptors requires the activation of the MAP kinase and the phosphatidylinositol-3-kinase pathways. *J. Neurochem.* **78,** 435-445.

D'Souza S. D., Bonetti B., Balasingam V., Cashman N. R., Barker P. A., Troutt A. B., Raine C. S. and Antel J. P. (1996) Multiple sclerosis: Fas signaling in oligodendrocyte cell death. *J. Exp. Med.* **184,** 2361-2370.

Dai X., Qu P. and Dreyfus C. F. (2001) Neuronal signals regulate neurotrophin expression in oligodendrocytes of the basal forebrain. *Glia* **34,** 234-239.

Davalos D., Grutzendler J., Yang G., Kim J. V., Zuo Y., Jung S., Littman D. R., Dustin M. L. and Gan W. B. (2005) ATP mediates rapid microglial response to local brain injury in vivo. *Nat. Neurosci.* **8,** 752-758.

De Groot C. J., Ruuls S. R., Theeuwes J. W., Dijkstra C. D. and Van D., V (1997) Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. *J. Neuropathol. Exp. Neurol.* **56,** 10-20.

De B. A., Conn P. J., Pin J. and Nicoletti F. (2001) Molecular determinants of metabotropic glutamate receptor signaling. *Trends Pharmacol. Sci.* **22,** 114-120.

De S. R., Giampaolo A., Giometto B., Gallo P., Levi G., Peschle C. and Aloisi F. (1995) The costimulatory molecule B7 is expressed on human microglia in culture and in multiple sclerosis acute lesions. *J. Neuropathol. Exp. Neurol.* **54,** 175-187.

Defagot M. C., Villar M. J. and Antonelli M. C. (2002) Differential localization of metabotropic glutamate receptors during postnatal development. *Dev. Neurosci.* **24,** 272-282.

Diemel L. T., Copelman C. A. and Cuzner M. L. (1998) Macrophages in CNS remyelination: friend or foe? *Neurochem. Res.* **23,** 341-347.

Ding M., St Pierre B. A., Parkinson J. F., Medberry P., Wong J. L., Rogers N. E., Ignarro L. J. and Merrill J. E. (1997) Inducible nitric-oxide synthase and nitric oxide production in human fetal astrocytes and microglia. A kinetic analysis. *J. Biol. Chem.* **272,** 11327-11335.

Doble A. (1995) Excitatory amino acid receptors and neurodegeneration. *Therapie* **50,** 319-337.

Dopp J. M., kenzie-Graham A., Otero G. C. and Merrill J. E. (1997) Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. *J. Neuroimmunol.* **75,** 104-112.

Dowling P., Shang G., Raval S., Menonna J., Cook S. and Husar W. (1996) Involvement of the CD95 (APO-1/Fas) receptor/ligand system in multiple sclerosis brain. *J. Exp. Med.* **184,** 1513-1518.

Eder C., Klee R. and Heinemann U. (1997) Distinct soluble astrocytic factors induce expression of outward K+ currents and ramification of brain macrophages. *Neurosci. Lett.* **226,** 147-150.

Edgar J. M. and Garbern J. (2004) The myelinated axon is dependent on the myelinating cell for support and maintenance: molecules involved. *J. Neurosci. Res.* **76,** 593-598.

Eliakim A., Moromisato M., Moromisato D., Brasel J. A., Roberts C., Jr. and Cooper D. M. (1997) Increase in muscle IGF-I protein but not IGF-I mRNA after 5ádays of endurance training in young rats. *Am J Physiol Regul Integr Comp Physiol* **273,** R1557-R1561.

Felderhoff-Mueser U., Sifringer M., Pesditschek S., Kuckuck H., Moysich A., Bittigau P. and Ikonomidou C. (2002) Pathways leading to apoptotic neurodegeneration following trauma to the developing rat brain. *Neurobiol. Dis.* **11,** 231-245.

Fern R. and Moller T. (2000) Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop. *J. Neurosci.* **20,** 34-42.

Ferraguti F., Corti C., Valerio E., Mion S. and Xuereb J. (2001) Activated astrocytes in areas of kainate-induced neuronal injury upregulate the expression of the metabotropic glutamate receptors 2/3 and 5. *Exp. Brain Res.* **137,** 1-11.

Fields T. A. and Casey P. J. (1997) Signalling functions and biochemical properties of pertussis toxin-resistant G-proteins. *Biochem. J.* **321 (Pt 3),** 561-571.

Floden A. M., Li S. and Combs C. K. (2005) Beta-amyloid-stimulated microglia induce neuron death via synergistic stimulation of tumor necrosis factor alpha and NMDA receptors. *J. Neurosci.* **25,** 2566-2575.

Foreman M. A., Gu Y., Howl J. D., Jones S. and Publicover S. J. (2005) Group III metabotropic glutamate receptor activation inhibits Ca2+ influx and nitric oxide synthase activity in bone marrow stromal cells. *J Cell Physiol* **204,** 704-713.

Frei K., Siepl C., Groscurth P., Bodmer S. and Fontana A. (1988) Immunobiology of microglial cells. *Ann. N. Y. Acad. Sci.* **540,** 218-227.

Gebicke-Haerter P. J., Spleiss O., Ren L. Q., Li H., Dichmann S., Norgauer J. and Boddeke H. W. (2001) Microglial chemokines and chemokine receptors. *Prog. Brain Res.* **132,** 525-532.

Geurts J. J., Wolswijk G., Bo L., Van D., V, Polman C. H., Troost D. and Aronica E. (2003) Altered expression patterns of group I and II metabotropic glutamate receptors in multiple sclerosis. *Brain* **126,** 1755-1766.

Geurts J. J., Wolswijk G., Bo L., Redeker S., Ramkema M., Troost D. and Aronica E. (2005) Expression patterns of Group III metabotropic glutamate receptors mGluR4 and mGluR8 in multiple sclerosis lesions. *J Neuroimmunol.* **158,** 182-190.

Gibbons H. M. and Dragunow M. (2006) Microglia induce neural cell death via a proximity-dependent mechanism involving nitric oxide. *Brain Res.* **1084,** 1-15.

Gilgun-Sherki Y., Panet H., Melamed E. and Offen D. (2003) Riluzole suppresses experimental autoimmune encephalomyelitis: implications for the treatment of multiple sclerosis. *Brain Res.* **989,** 196-204.

Giulian D. and Baker T. J. (1986) Characterization of ameboid microglia isolated from developing mammalian brain. *J Neurosci.* **6,** 2163-2178.

Giulian D. (1987) Ameboid microglia as effectors of inflammation in the central nervous system. *J. Neurosci. Res.* **18,** 155-3.

Goodrum K. J., McCormick L. L. and Schneider B. (1994) Group B streptococcus-induced nitric oxide production in murine macrophages is CR3 (CD11b/CD18) dependent. *Infect. Immun.* **62,** 3102-3107.

Graeber M. B., Streit W. J. and Kreutzberg G. W. (1988) Axotomy of the rat facial nerve leads to increased CR3 complement receptor expression by activated microglial cells. *J. Neurosci. Res.* **21,** 18-24.

Graeber M. B., Banati R. B., Streit W. J. and Kreutzberg G. W. (1989) Immunophenotypic characterization of rat brain macrophages in culture. *Neurosci. Lett.* **103,** 241-246.

Griffith T. S., Brunner T., Fletcher S. M., Green D. R. and Ferguson T. A. (1995) Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270,** 1189-1192.

Groom A. J., Smith T. and Turski L. (2003) Multiple sclerosis and glutamate. *Ann. N. Y. Acad. Sci.* **993,** 229-275.

Hafler D. A. (2004) Multiple sclerosis. *J. Clin. Invest* **113,** 788-794.

Hanisch U. K. (2002) Microglia as a source and target of cytokines. *Glia* **40,** 140-155.

Hauser S. L., Bhan A. K., Gilles F., Kemp M., Kerr C. and Weiner H. L. (1986) Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. *Ann. Neurol.* **19,** 578-587.

Hayes G. M., Woodroofe M. N. and Cuzner M. L. (1987) Microglia are the major cell type expressing MHC class II in human white matter. *J. Neurol. Sci.* **80,** 25-37.

Hemmer B., Archelos J. J. and Hartung H. P. (2002) New concepts in the immunopathogenesis of multiple sclerosis. *Nat Rev. Neurosci.* **3,** 291-301.

Hickey W. F. and Kimura H. (1988) Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science* **239,** 290-292.

Hill K. E., Zollinger L. V., Watt H. E., Carlson N. G. and Rose J. W. (2004) Inducible nitric oxide synthase in chronic active multiple sclerosis plaques: distribution, cellular expression and association with myelin damage. *J Neuroimmunol.* **151,** 171-179.

Hoek R. M., Ruuls S. R., Murphy C. A., Wright G. J., Goddard R., Zurawski S. M., Blom B., Homola M. E., Streit W. J., Brown M. H., Barclay A. N. and Sedgwick J. D. (2000) Down-regulation of the macrophage lineage through interaction with OX2 (CD200). *Science* **290,** 1768-1771.

Holscher C., Gigg J. and O'Mara S. M. (1999) Metabotropic glutamate receptor activation and blockade: their role in long-term potentiation, learning and neurotoxicity. *Neurosci. Biobehav. Rev.* **23,** 399-410.

Hopkins S. J. and Rothwell N. J. (1995) Cytokines and the nervous system. I: Expression and recognition. *Trends Neurosci.* **18,** 83-88.

Hubert G. W., Paquet M. and Smith Y. (2001) Differential subcellular localization of mGluR1a and mGluR5 in the rat and monkey Substantia nigra. *J Neurosci.* **21,** 1838-1847.

Hubert G. W. and Smith Y. (2004) Age-related changes in the expression of axonal and glial group I metabotropic glutamate receptor in the rat substantia nigra pars reticulata. *J. Comp Neurol.* **475,** 95-106.

Hudig D., Ewoldt G. R. and Woodard S. L. (1993) Proteases and lymphocyte cytotoxic killing mechanisms. *Curr. Opin. Immunol.* **5,** 90-96.

Hume D. A., Perry V. H. and Gordon S. (1983) Immunohistochemical localization of a macrophage-specific antigen in developing mouse retina: phagocytosis of dying neurons and differentiation of microglial cells to form a regular array in the plexiform layers. *J. Cell Biol.* **97,** 253-257.

Inaba K., Granelli-Piperno A. and Steinman R. M. (1983) Dendritic cells induce T lymphocytes to release B cell-stimulating factors by an interleukin 2-dependent mechanism. *J. Exp. Med.* **158,** 2040-2057.

Inaba K., Steinman R. M., Voorhis W. C. V. and Muramatsu S. (1983) Dendritic Cells are Critical Accessory Cells for Thymus-Dependent Antibody Responses in Mouse and in Man. *PNAS* **80,** 6041-6045.

Ishida Y., Nagai A., Kobayashi S. and Kim S. U. (2006) Upregulation of protease-activated receptor-1 in astrocytes in Parkinson disease: astrocyte-mediated neuroprotection through increased levels of glutathione peroxidase. *J. Neuropathol. Exp. Neurol.* **65,** 66-77.

Janeway C. A. and Medzhitov R. (2002) INNATE IMMUNE RECOGNITION. *Annual Review of Immunology* **20,** 197-216.

Katsuki H., Okawara M., Shibata H., Kume T. and Akaike A. (2006) Nitric oxide-producing microglia mediate thrombin-induced degeneration of dopaminergic neurons in rat midbrain slice culture. *J Neurochem.* **97,** 1232-1242.

Kemp M. C., Jane D. E., Tse H. W. and Roberts P. J. (1996) Agonists of cyclic AMP-coupled metabotropic glutamate receptors in adult rat cortical slices. *Eur. J Pharmacol.* **309,** 79-85.

Kerr J. F., Wyllie A. H. and Currie A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26,** 239-257.

Kettenmann H., Banati R. and Walz W. (1993) Electrophysiological behavior of microglia. *Glia* **7,** 93-101.

Kew J. N. and Kemp J. A. (2005) Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology (Berl)* **179,** 4-29.

Killestein J., Kalkers N. F. and Polman C. H. (2005) Glutamate inhibition in MS: the neuroprotective properties of riluzole. *J Neurol. Sci.* **233,** 113-115.

Kim W. K. and Ko K. H. (1998) Potentiation of N-methyl-D-aspartate-mediated neurotoxicity by immunostimulated murine microglia. *J. Neurosci. Res.* **54,** 17-26.

Kim W. K., Hwang S. Y., Oh E. S., Piao H. Z., Kim K. W. and Han I. O. (2004) TGF-beta1 represses activation and resultant death of microglia via inhibition of phosphatidylinositol 3-kinase activity. *J Immunol.* **172,** 7015-7023.

Kimelberg H. K. and Katz D. M. (1985) High-affinity uptake of serotonin into immunocytochemically identified astrocytes. *Science* **228,** 889-891.

Kimelberg H. K. and Norenberg M. D. (1989) Astrocytes. *Sci. Am.* **260,** 66-72, 74, 76.

Kingham P. J., Cuzner M. L. and Pocock J. M. (1999) Apoptotic pathways mobilized in microglia and neurones as a consequence of chromogranin A-induced microglial activation. *J. Neurochem.* **73,** 538-547.

Klein L., Klugmann M., Nave K. A., Tuohy V. K. and Kyewski B. (2000) Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. *Nat Med* **6,** 56-61.

Konat G. W. and Wiggins R. C. (1985) Effect of reactive oxygen species on myelin membrane proteins. *J. Neurochem.* **45,** 1113-1118.

Kreutzberg G. W. (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* **19,** 312-318.

Kunishima N., Shimada Y., Tsuji Y., Sato T., Yamamoto M., Kumasaka T., Nakanishi S., Jingami H. and Morikawa K. (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **407,** 971-977.

Lassmann H. (1998) Neuropathology in multiple sclerosis: new concepts. *Mult. Scler.* **4,** 93-98.

Lawson L. J., Perry V. H., Dri P. and Gordon S. (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience* **39,** 151-170.

Ledeen R. W. and Chakraborty G. (1998) Cytokines, signal transduction, and inflammatory demyelination: review and hypothesis. *Neurochem. Res.* **23,** 277-289.

Lee H. G., Ogawa O., Zhu X., O'Neill M. J., Petersen R. B., Castellani R. J., Ghanbari H., Perry G. and Smith M. A. (2004) Aberrant expression of metabotropic glutamate receptor 2 in the vulnerable neurons of Alzheimer's disease. *Acta Neuropathol. (Berl)* **107,** 365-371.

Lee S. C., Moore G. R., Golenwsky G. and Raine C. S. (1990) Multiple sclerosis: a role for astroglia in active demyelination suggested by class II MHC expression and ultrastructural study. *J Neuropathol. Exp. Neurol.* **49,** 122-136.

Lee Y. B., Nagai A. and Kim S. U. (2002) Cytokines, chemokines, and cytokine receptors in human microglia. *J. Neurosci. Res.* **69,** 94-103.

Lehnardt S., Massillon L., Follett P., Jensen F. E., Ratan R., Rosenberg P. A., Volpe J. J. and Vartanian T. (2003) Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proc. Natl. Acad. Sci. U. S. A* **100,** 8514-8519.

Lewin B (1994) *Genes V.* Oxford University Press.

Li H., Cuzner M. L. and Newcombe J. (1996) Microglia-derived macrophages in early multiple sclerosis plaques. *Neuropathol. Appl. Neurobiol.* **22,** 207-215.

Li J., Baud O., Vartanian T., Volpe J. J. and Rosenberg P. A. (2005) Peroxynitrite generated by inducible nitric oxide synthase and NADPH oxidase mediates microglial toxicity to oligodendrocytes. *Proc. Natl. Acad. Sci. U. S. A* **102,** 9936-9941.

Ling E. A., Penney D. and Leblond C. P. (1980) Use of carbon labeling to demonstrate the role of blood monocytes as precursors of the 'ameboid cells' present in the corpus callosum of postnatal rats. *J. Comp Neurol.* **193,** 631-657.

Ling E. A. (1981) Ultrastructure and peroxidase cytochemistry of macrophages present in the retina of postnatal rats. *Arch. Histol. Jpn.* **44,** 167-176.

Ling E. A. and Wong W. C. (1993) The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. *Glia* **7,** 9-18.

Liu J. S., Zhao M. L., Brosnan C. F. and Lee S. C. (2001) Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am J Pathol.* **158,** 2057-2066.

Liu Y. and Janeway C. A., Jr. (1990) Interferon gamma plays a critical role in induced cell death of effector T cell: a possible third mechanism of self-tolerance. *J. Exp. Med.* **172,** 1735-1739.

Liu Y. and Linsley P. S. (1992) Costimulation of T-cell growth. *Curr. Opin. Immunol.* **4,** 265-270.

Lublin F. D. and Reingold S. C. (1996) Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology* **46,** 907-911.

Lucchinetti C., Bruck W., Parisi J., Scheithauer B., Rodriguez M. and Lassmann H. (1999) A quantitative analysis of oligodendrocytes in multiple sclerosis lesions. A study of 113 cases. *Brain* **122 (Pt 12),** 2279-2295.

Lucchinetti C., Bruck W., Parisi J., Scheithauer B., Rodriguez M. and Lassmann H. (2000) Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann. Neurol.* **47,** 707-717.

Lucchinetti C. F., Bruck W., Rodriguez M. and Lassmann H. (1996) Distinct patterns of multiple sclerosis pathology indicates heterogeneity on pathogenesis. *Brain Pathol.* **6,** 259-274.

Lucchinetti C. F., Brueck W., Rodriguez M. and Lassmann H. (1998) Multiple sclerosis: lessons from neuropathology. *Semin. Neurol.* **18,** 337-349.

MacMicking J. D., Willenborg D. O., Weidemann M. J., Rockett K. A. and Cowden W. B. (1992) Elevated secretion of reactive nitrogen and oxygen intermediates by inflammatory leukocytes in hyperacute experimental autoimmune encephalomyelitis: enhancement by the soluble products of encephalitogenic T cells. *J. Exp. Med.* **176,** 303-307.

Magistretti P. J. and Pellerin L. (1999) Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging. *Philos. Trans. R. Soc. Lond B Biol. Sci.* **354,** 1155-1163.

Makranz C., Cohen G., Baron A., Levidor L., Kodama T., Reichert F. and Rotshenker S. (2004) Phosphatidylinositol 3-kinase, phosphoinositide-specific phospholipase-Cgamma and protein kinase-C signal myelin phagocytosis mediated by complement receptor-3 alone and combined with scavenger receptor-AI/II in macrophages. *Neurobiol. Dis.* **15,** 279-286.

Makranz C., Cohen G., Reichert F., Kodama T. and Rotshenker S. (2006) cAMP cascade (PKA, Epac, adenylyl cyclase, Gi, and phosphodiesterases) regulates myelin phagocytosis mediated by complement receptor-3 and scavenger receptor-AI/II in microglia and macrophages. *Glia* **53,** 441-448.

Martin R., Jaraquemada D., Flerlage M., Richert J., Whitaker J., Long E. O., McFarlin D. E. and McFarland H. F. (1990) Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *J. Immunol.* **145,** 540-548.

Matsumoto Y. (1994) Role of microglia in autoimmune encephalomyelitis. *Neuropathol. Appl. Neurobiol.* **20,** 196-198.

Mattson M. P., Barger S. W., Furukawa K., Bruce A. J., Wyss-Coray T., Mark R. J. and Mucke L. (1997) Cellular signaling roles of TGF beta, TNF alpha and beta APP in brain injury responses and Alzheimer's disease. *Brain Res. Brain Res. Rev.* **23,** 47-61.

Matute C., Sanchez-Gomez M. V., Martinez-Millan L. and Miledi R. (1997) Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc. Natl. Acad. Sci. U. S. A* **94,** 8830-8835.

Matute C., Alberdi E., Domercq M., Perez-Cerda F., Perez-Samartin A. and Sanchez-Gomez M. V. (2001) The link between excitotoxic oligodendroglial death and demyelinating diseases. *Trends Neurosci.* **24,** 224-230.

Mayer M. L., Westbrook G. L. and Guthrie P. B. (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature* **309,** 261-263.

McCombe P. A., de J. J. and Pender M. P. (1994) Inflammatory cells, microglia and MHC class II antigen-positive cells in the spinal cord of Lewis rats with acute and

chronic relapsing experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **51,** 153-167.

McDonald J. W., Althomsons S. P., Hyrc K. L., Choi D. W. and Goldberg M. P. (1998) Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. *Nat Med* **4,** 291-297.

McDonald W. I. (2000) Relapse, remission, and progression in multiple sclerosis. *N. Engl. J. Med* **343,** 1486-1487.

McDonald W. I., Compston A., Edan G., Goodkin D., Hartung H. P., Lublin F. D., McFarland H. F., Paty D. W., Polman C. H., Reingold S. C., Sandberg-Wollheim M., Sibley W., Thompson A., van den N. S., Weinshenker B. Y. and Wolinsky J. S. (2001) Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* **50,** 121-127.

Medawar P (1948) Immunity to homologous grafted skin III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue and to the anterior chamber of the eye. *British Journal of Experimental Pathology* **29,** 59-68.

Medzhitov R. and Janeway C., Jr. (2000) The Toll receptor family and microbial recognition. *Trends Microbiol.* **8,** 452-456.

Meldrum B. and Garthwaite J. (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **11,** 379-387.

Menendez I. B., Cerase J., Ceracchini C., Levi G. and Aloisi F. (1997) Analysis of B7-1 and B7-2 costimulatory ligands in cultured mouse microglia: upregulation by interferon-gamma and lipopolysaccharide and downregulation by interleukin-10, prostaglandin E2 and cyclic AMP-elevating agents. *J. Neuroimmunol.* **72,** 83-93.

Merrill J. E. (1992) Tumor necrosis factor alpha, interleukin 1 and related cytokines in brain development: normal and pathological. *Dev. Neurosci.* **14,** 1-10.

Merrill J. E., Ignarro L. J., Sherman M. P., Melinek J. and Lane T. E. (1993) Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J. Immunol.* **151,** 2132-2141.

Millar J. (1995) The nitric oxide/ascorbate cycle: how neurones may control their own oxygen supply. *Med Hypotheses* **45,** 21-26.

Miller D. H., Khan O. A., Sheremata W. A., Blumhardt L. D., Rice G. P., Libonati M. A., Willmer-Hulme A. J., Dalton C. M., Miszkiel K. A. and O'Connor P. W. (2003) A controlled trial of natalizumab for relapsing multiple sclerosis. *N. Engl. J Med* **348,** 15-23.

Minghetti L., Nicolini A., Polazzi E., Creminon C., Maclouf J. and Levi G. (1997) Inducible nitric oxide synthase expression in activated rat microglial cultures is downregulated by exogenous prostaglandin E2 and by cyclooxygenase inhibitors. *Glia* **19,** 152-160.

Mitrovic B., Martin F. C., Charles A. C., Ignarro L. J., Anton P. A., Shanahan F. and Merrill J. E. (1994) Neurotransmitters and cytokines in CNS pathology. *Prog. Brain Res.* **103,** 319-330.

Mori T., Buffo A. and Gotz M. (2005) The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis. *Curr. Top. Dev. Biol.* **69,** 67-99.

Mosley K. and Cuzner M. L. (1996) Receptor-mediated phagocytosis of myelin by macrophages and microglia: effect of opsonization and receptor blocking agents. *Neurochem. Res.* **21,** 481-487.

Mueller D. L., Jenkins M. K. and Schwartz R. H. (1989) Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* **7,** 445-480.

Murphy C. A., Hoek R. M., Wiekowski M. T., Lira S. A. and Sedgwick J. D. (2002) Interactions between hemopoietically derived TNF and central nervous system-resident glial chemokines underlie initiation of autoimmune inflammation in the brain. *J. Immunol.* **169,** 7054-7062.

Murphy K. M., Streips U. N. and Lock R. B. (2000) Bcl-2 inhibits a Fas-induced conformational change in the Bax N terminus and Bax mitochondrial translocation. *J. Biol. Chem.* **275,** 17225-17228.

Murphy S., Lin H. L. and Park S. K. (1995) Cytokine-induced expression of type II nitric oxide synthase in astrocytes is downregulated by ATP and glutamate. *Glia* **15,** 77-82.

Nagata S. (1999) FAS LIGAND-INDUCED APOPTOSIS. *Annual Review of Genetics* **33,** 29-55.

Nakanishi K. and Kukita F. (1998) Functional synapses in synchronized bursting of neocortical neurons in culture. *Brain Res.* **795,** 137-146.

Naparstek Y., Cohen I. R., Fuks Z. and Vlodavsky I. (1984) Activated T lymphocytes produce a matrix-degrading heparan sulphate endoglycosidase. *Nature* **310,** 241-244.

Neumann H., Misgeld T., Matsumuro K. and Wekerle H. (1998) Neurotrophins inhibit major histocompatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor. *Proc. Natl. Acad. Sci. U. S. A* **95,** 5779-5784.

Neumann H., Medana I. M., Bauer J. and Lassmann H. (2002) Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends Neurosci.* **25,** 313-319.

Neumann H. (2003) Molecular mechanisms of axonal damage in inflammatory central nervous system diseases. *Curr. Opin. Neurol.* **16,** 267-273.

Newman E. and Reichenbach A. (1996) The Muller cell: a functional element of the retina. *Trends Neurosci.* **19,** 307-312.

Nicoletti F., Bruno V., Copani A., Casabona G. and Knopfel T. (1996) Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? *Trends Neurosci.* **19,** 267-271.

Nimmerjahn A., Kirchhoff F. and Helmchen F. (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308,** 1314-1318.

Nolan M. F. and Logan S. D. (1998) Metabotropic glutamate receptor-mediated excitation and inhibition of sympathetic preganglionic neurones. *Neuropharmacology* **37,** 13-24.

Noseworthy J. H., Lucchinetti C., Rodriguez M. and Weinshenker B. G. (2000) Multiple sclerosis. *N. Engl. J. Med.* **343,** 938-952.

Nowak L., Bregestovski P., Ascher P., Herbet A. and Prochiantz A. (1984) Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* **307,** 462-465.

Okuda Y., Nakatsuji Y., Fujimura H., Esumi H., Ogura T., Yanagihara T. and Sakoda S. (1995) Expression of the inducible isoform of nitric oxide synthase in the central nervous system of mice correlates with the severity of actively induced experimental allergic encephalomyelitis. *J. Neuroimmunol.* **62,** 103-112.

Olazabal I. M., Caron E., May R. C., Schilling K., Knecht D. A. and Machesky L. M. (2002) Rho-kinase and myosin-II control phagocytic cup formation during CR, but not FcgammaR, phagocytosis. *Curr. Biol.* **12,** 1413-1418.

Olsson T. (1995) Critical influences of the cytokine orchestration on the outcome of myelin antigen-specific T-cell autoimmunity in experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol. Rev.* **144,** 245-268.

Oppenheim R. W. (1981) Cell death of motoneurons in the chick embryo spinal cord. V. Evidence on the role of cell death and neuromuscular function in the formation of specific peripheral connections. *J. Neurosci.* **1,** 141-151.

Ota K., Matsui M., Milford E. L., Mackin G. A., Weiner H. L. and Hafler D. A. (1990) T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* **346,** 183-187.

Otsuki Y., Li Z. and Shibata M. A. (2003) Apoptotic detection methods--from morphology to gene. *Prog. Histochem. Cytochem.* **38,** 275-339.

Owens T. (2003) The enigma of multiple sclerosis: inflammation and neurodegeneration cause heterogeneous dysfunction and damage. *Curr. Opin. Neurol.* **16,** 259-265.

Papadopoulos D., Ewans L., Pham-Dinh D., Knott J. and Reynolds R. (2006) Upregulation of alpha-synuclein in neurons and glia in inflammatory demyelinating disease. *Mol. Cell Neurosci.* **31,** 597-612.

Patel T. B., Du Z., Pierre S., Cartin L. and Scholich K. (2001) Molecular biological approaches to unravel adenylyl cyclase signaling and function. *Gene* **269,** 13-25.

Patrizio M. and Levi G. (1994) Glutamate production by cultured microglia: differences between rat and mouse, enhancement by lipopolysaccharide and lack effect of HIV coat protein gp120 and depolarizing agents. *Neurosci. Lett.* **178,** 184-189.

Payne C. M., Bernstein C. and Bernstein H. (1995) Apoptosis overview emphasizing the role of oxidative stress, DNA damage and signal-transduction pathways. *Leuk. Lymphoma* **19,** 43-93.

Pereira C. F. and Oliveira C. R. (2000) Oxidative glutamate toxicity involves mitochondrial dysfunction and perturbation of intracellular Ca2+ homeostasis. *Neurosci. Res.* **37,** 227-236.

Perry V. H., Hume D. A. and Gordon S. (1985) Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience* **15,** 313-326.

Perry V. H. and Gordon S. (1987) Modulation of CD4 antigen on macrophages and microglia in rat brain. *J. Exp. Med.* **166,** 1138-1143.

Pette M., Fujita K., Wilkinson D., Altmann D. M., Trowsdale J., Giegerich G., Hinkkanen A., Epplen J. T., Kappos L. and Wekerle H. (1990) Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple-sclerosis patients and healthy donors. *Proc. Natl. Acad. Sci. U. S. A* **87,** 7968-7972.

Piani D., Frei K., Do K. Q., Cuenod M. and Fontana A. (1991) Murine brain macrophages induced NMDA receptor mediated neurotoxicity in vitro by secreting glutamate. *Neurosci. Lett.* **133,** 159-162.

Piani D., Spranger M., Frei K., Schaffner A. and Fontana A. (1992) Macrophage-induced cytotoxicity of N-methyl-D-aspartate receptor positive neurons involves excitatory amino acids rather than reactive oxygen intermediates and cytokines. *Eur. J. Immunol.* **22,** 2429-2436.

Piani D. and Fontana A. (1994) Involvement of the cystine transport system xc- in the macrophage-induced glutamate-dependent cytotoxicity to neurons. *J. Immunol.* **152,** 3578-3585.

Pin J. P. and Acher F. (2002) The metabotropic glutamate receptors: structure, activation mechanism and pharmacology. *Curr. Drug Targets. CNS. Neurol. Disord.* **1,** 297-317.

Pitt D., Werner P. and Raine C. S. (2000) Glutamate excitotoxicity in a model of multiple sclerosis. *Nat Med* **6,** 67-70.

Pitt D., Nagelmeier I. E., Wilson H. C. and Raine C. S. (2003) Glutamate uptake by oligodendrocytes: Implications for excitotoxicity in multiple sclerosis. *Neurology* **61,** 1113-1120.

Pizzi M., Galli P., Consolandi O., Arrighi V., Memo M. and Spano P. F. (1996) Metabotropic and ionotropic transducers of glutamate signal inversely control cytoplasmic Ca2+ concentration and excitotoxicity in cultured cerebellar granule cells: pivotal role of protein kinase C. *Mol. Pharmacol.* **49,** 586-594.

Probert L., Akassoglou K., Pasparakis M., Kontogeorgos G. and Kollias G. (1995) Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-specific expression of tumor necrosis factor alpha. *Proc. Natl. Acad. Sci. U. S. A* **92,** 11294-11298.

Raivich G. and Banati R. (2004) Brain microglia and blood-derived macrophages: molecular profiles and functional roles in multiple sclerosis and animal models of autoimmune demyelinating disease. *Brain Res. Brain Res. Rev.* **46,** 261-281.

Ransohoff R. M. (1999) Mechanisms of inflammation in MS tissue: adhesion molecules and chemokines. *J. Neuroimmunol.* **98,** 57-68.

Ransom B. R. and Sontheimer H. (1992) The neurophysiology of glial cells. *J. Clin. Neurophysiol.* **9,** 224-251.

Ransom C. B. and Sontheimer H. (1995) Biophysical and pharmacological characterization of inwardly rectifying K+ currents in rat spinal cord astrocytes. *J. Neurophysiol.* **73,** 333-346.

Reese T. S. and Karnovsky M. J. (1967) Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* **34,** 207-217.

Reichert F., Slobodov U., Makranz C. and Rotshenker S. (2001) Modulation (inhibition and augmentation) of complement receptor-3-mediated myelin phagocytosis. *Neurobiol. Dis.* **8,** 504-512.

Reichert F. and Rotshenker S. (2003) Complement-receptor-3 and scavenger-receptor-AI/II mediated myelin phagocytosis in microglia and macrophages. *Neurobiol. Dis.* **12,** 65-72.

Rejdak K., Eikelenboom M. J., Petzold A., Thompson E. J., Stelmasiak Z., Lazeron R. H., Barkhof F., Polman C. H., Uitdehaag B. M. and Giovannoni G. (2004) CSF nitric oxide metabolites are associated with activity and progression of multiple sclerosis. *Neurology* **63,** 1439-1445.

Ribatti D., Nico B., Crivellato E. and Artico M. (2006) Development of the blood-brain barrier: a historical point of view. *Anat. Rec. B New Anat.* **289,** 3-8.

Roche K. W., Tu J. C., Petralia R. S., Xiao B., Wenthold R. J. and Worley P. F. (1999) Homer 1b regulates the trafficking of group I metabotropic glutamate receptors. *J Biol. Chem.* **274,** 25953-25957.

Roitt IM and Delves PJ (2003) *Essential Immunology*. Blackwell publishing.

Rose J. W., Hill K. E., Wada Y., Kurtz C. I., Tsunoda I., Fujinami R. S. and Cross A. H. (1998) Nitric oxide synthase inhibitor, aminoguanidine, reduces inflammation and demyelination produced by Theiler's virus infection. *J. Neuroimmunol.* **81,** 82-89.

Rosin C., Bates T. E. and Skaper S. D. (2004) Excitatory amino acid induced oligodendrocyte cell death in vitro: receptor-dependent and -independent mechanisms. *J. Neurochem.* **90,** 1173-1185.

Roy A., Fung Y. K., Liu X. and Pahan K. (2006) Up-regulation of microglial CD11b expression by nitric oxide. *J Biol. Chem.* **281,** 14971-14980.

Ruuls S. R., Bauer J., Sontrop K., Huitinga I., 't Hart B. A. and Dijkstra C. D. (1995) Reactive oxygen species are involved in the pathogenesis of experimental allergic encephalomyelitis in Lewis rats. *J. Neuroimmunol.* **56,** 207-217.

Ruuls S. R., Van Der L. S., Sontrop K., Huitinga I. and Dijkstra C. D. (1996) Aggravation of experimental allergic encephalomyelitis (EAE) by administration of nitric oxide (NO) synthase inhibitors. *Clin. Exp. Immunol.* **103,** 467-474.

Sahara Y., Kubota T. and Ichikawa M. (2001) Cellular localization of metabotropic glutamate receptors mGluR1, 2/3, 5 and 7 in the main and accessory olfactory bulb of the rat. *Neurosci. Lett.* **312,** 59-62.

Sahrbacher U. C., Lechner F., Eugster H. P., Frei K., Lassmann H. and Fontana A. (1998) Mice with an inactivation of the inducible nitric oxide synthase gene are susceptible to experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* **28,** 1332-1338.

Sathasivam S., Ince P. G. and Shaw P. J. (2001) Apoptosis in amyotrophic lateral sclerosis: a review of the evidence. *Neuropathol. Appl. Neurobiol.* **27,** 257-274.

Schonrock L. M., Kuhlmann T., Adler S., Bitsch A. and Bruck W. (1998) Identification of glial cell proliferation in early multiple sclerosis lesions. *Neuropathol. Appl. Neurobiol.* **24,** 320-330.

Schools G. P. and Kimelberg H. K. (1999) mGluR3 and mGluR5 are the predominant metabotropic glutamate receptor mRNAs expressed in hippocampal astrocytes acutely isolated from young rats. *J Neurosci. Res.* **58,** 533-543.

Schousboe A., Sarup A., Bak L. K., Waagepetersen H. S. and Larsson O. M. (2004) Role of astrocytic transport processes in glutamatergic and GABAergic neurotransmission. *Neurochem. Int.* **45,** 521-527.

Sedgwick J. D., Riminton D. S., Cyster J. G. and Korner H. (2000) Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol. Today* **21,** 110-113.

Shih A. Y., Erb H., Sun X., Toda S., Kalivas P. W. and Murphy T. H. (2006) Cystine/glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation. *J. Neurosci.* **26,** 10514-10523.

Shrikant P. and Benveniste E. N. (1996) The central nervous system as an immunocompetent organ: role of glial cells in antigen presentation. *J. Immunol.* **157,** 1819-1822.

Slee E. A., Harte M. T., Kluck R. M., Wolf B. B., Casiano C. A., Newmeyer D. D., Wang H. G., Reed J. C., Nicholson D. W., Alnemri E. S., Green D. R. and Martin S. J. (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J. Cell Biol.* **144,** 281-292.

Slobodov U., Reichert F., Mirski R. and Rotshenker S. (2001) Distinct inflammatory stimuli induce different patterns of myelin phagocytosis and degradation in recruited macrophages. *Exp. Neurol.* **167,** 401-409.

Smith T, Antony G, Bin Z and Lechoslaw T (2000) Autoimmune encephalomyelitis ameillorated by AMPA antagonists. *Nat Med* **6,** 62-66.

Smith K. J., Kapoor R., Hall S. M. and Davies M. (2001) Electrically active axons degenerate when exposed to nitric oxide. *Ann. Neurol.* **49,** 470-476.

Smith K. J. and Lassmann H. (2002) The role of nitric oxide in multiple sclerosis. *Lancet Neurol.* **1,** 232-241.

Sobel R. A. (1989) T-lymphocyte subsets in the multiple sclerosis lesion. *Res. Immunol.* **140,** 208-211.

Song X., Tanaka S., Cox D. and Lee S. C. (2004) Fcgamma receptor signaling in primary human microglia: differential roles of PI-3K and Ras/ERK MAPK pathways in phagocytosis and chemokine induction. *J Leukoc. Biol.* **75,** 1147-1155.

Sourdet V., Russier M., Daoudal G., Ankri N. and Debanne D. (2003) Long-term enhancement of neuronal excitability and temporal fidelity mediated by metabotropic glutamate receptor subtype 5. *J Neurosci.* **23,** 10238-10248.

Srinivasan R., Sailasuta N., Hurd R., Nelson S. and Pelletier D. (2005) Evidence of elevated glutamate in multiple sclerosis using magnetic resonance spectroscopy at 3 T. *Brain* **128,** 1016-1025.

Steinman L. (1996) Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* **85,** 299-302.

Stence N., Waite M. and Dailey M. E. (2001) Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia* **33,** 256-266.

Storch M. K., Piddlesden S., Haltia M., Iivanainen M., Morgan P. and Lassmann H. (1998) Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. *Ann. Neurol.* **43,** 465-471.

Streit W. J. and Kreutzberg G. W. (1987) Lectin binding by resting and reactive microglia. *J Neurocytol.* **16,** 249-260.

Streit W. J., Graeber M. B. and Kreutzberg G. W. (1988) Functional plasticity of microglia: a review. *Glia* **1,** 301-307.

Streit W. J., Graeber M. B. and Kreutzberg G. W. (1989) Peripheral nerve lesion produces increased levels of major histocompatibility complex antigens in the central nervous system. *J. Neuroimmunol.* **21,** 117-123.

Streit W. J. (2000) Microglial response to brain injury: a brief synopsis. *Toxicol. Pathol.* **28,** 28-30.

Streit W. J. (2002) Microglia as neuroprotective, immunocompetent cells of the CNS. *Glia* **40,** 133-139.

Streit W. J. (2002) Microglia and the response to brain injury. *Ernst. Schering. Res. Found. Workshop* 11-24.

Sun D., Coleclough C., Cao L., Hu X., Sun S. and Whitaker J. N. (1998) Reciprocal stimulation between TNF-alpha and nitric oxide may exacerbate CNS inflammation in experimental autoimmune encephalomyelitis. *J Neuroimmunol.* **89,** 122-130.

Szekeres P. G., Koenig J. A. and Edwardson J. M. (1998) Involvement of receptor cycling and receptor reserve in resensitization of muscarinic responses in SH-SY5Y human neuroblastoma cells. *J Neurochem.* **70,** 1694-1703.

Takahashi J. L., Giuliani F., Power C., Imai Y. and Yong V. W. (2003) Interleukin-1beta promotes oligodendrocyte death through glutamate excitotoxicity. *Ann. Neurol.* **53,** 588-595.

Tanuma N., Sakuma H., Sasaki A. and Matsumoto Y. (2006) Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis. *Acta Neuropathol. (Berl)* **112,** 195-204.

Tartaglia L. A. and Goeddel D. V. (1992) Tumor necrosis factor receptor signaling. A dominant negative mutation suppresses the activation of the 55-kDa tumor necrosis factor receptor. *J. Biol. Chem.* **267,** 4304-4307.

Tatsuta T., Naito M., Oh-hara T., Sugawara I. and Tsuruo T. (1992) Functional involvement of P-glycoprotein in blood-brain barrier. *J. Biol. Chem.* **267,** 20383-20391.

Taupenot L., Ciesielski-Treska J., Ulrich G., Chasserot-Golaz S., Aunis D. and Bader M. F. (1996) Chromogranin A triggers a phenotypic transformation and the generation of nitric oxide in brain microglial cells. *Neuroscience* **72,** 377-389.

Taylor D. L., Diemel L. T., Cuzner M. L. and Pocock J. M. (2002) Activation of group II metabotropic glutamate receptors underlies microglial reactivity and neurotoxicity following stimulation with chromogranin A, a peptide up-regulated in Alzheimer's disease. *J. Neurochem.* **82,** 1179-1191.

Taylor D. L., Diemel L. T. and Pocock J. M. (2003) Activation of microglial group III metabotropic glutamate receptors protects neurons against microglial neurotoxicity. *J. Neurosci.* **23,** 2150-2160.

Taylor D. L., Jones F., Kubota E. S. and Pocock J. M. (2005) Stimulation of microglial metabotropic glutamate receptor mGlu2 triggers tumor necrosis factor alpha-induced neurotoxicity in concert with microglial-derived Fas ligand. *J. Neurosci.* **25,** 2952-2964.

Tchelingerian J. L., Monge M., Le S. F., Zalc B. and Jacque C. (1995) Differential oligodendroglial expression of the tumor necrosis factor receptors in vivo and in vitro. *J. Neurochem.* **65,** 2377-2380.

Terrazzino S., Bauleo A., Baldan A. and Leon A. (2002) Peripheral LPS administrations up-regulate Fas and FasL on brain microglial cells: a brain protective or pathogenic event? *J. Neuroimmunol.* **124,** 45-53.

Thery C., Chamak B. and Mallat M. (1991) Cytotoxic Effect of Brain Macrophages on Developing. *Eur. J. Neurosci.* **3,** 1155-1164.

Thornberry N. A. and Lazebnik Y. (1998) Caspases: enemies within. *Science* **281,** 1312-1316.

Tomimoto H., Akiguchi I., Akiyama H., Kimura J. and Yanagihara T. (1993) T-cell infiltration and expression of MHC class II antigen by macrophages and microglia in a heterogeneous group in leukoencephalopathy. *Am. J. Pathol.* **143,** 579-586.

Trapp B. D., Peterson J., Ransohoff R. M., Rudick R., Mork S. and Bo L. (1998) Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med* **338,** 278-285.

Tsacopoulos M. and Magistretti P. J. (1996) Metabolic coupling between glia and neurons. *J. Neurosci.* **16,** 877-885.

Tsuchiya K., Kanematsu Y., Yoshizumi M., Ohnishi H., Kirima K., Izawa Y., Shikishima M., Ishida T., Kondo S., Kagami S., Takiguchi Y. and Tamaki T. (2005) Nitrite is an alternative source of NO in vivo. *Am J Physiol Heart Circ. Physiol* **288,** H2163-H2170.

Tsuji A., Terasaki T., Takabatake Y., Tenda Y., Tamai I., Yamashima T., Moritani S., Tsuruo T. and Yamashita J. (1992) P-glycoprotein as the drug efflux pump in primary cultured bovine brain capillary endothelial cells. *Life Sci.* **51,** 1427-1437.

Tu J. C., Xiao B., Yuan J. P., Lanahan A. A., Leoffert K., Li M., Linden D. J. and Worley P. F. (1998) Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron* **21,** 717-726.

Ueyama T., Lennartz M. R., Noda Y., Kobayashi T., Shirai Y., Rikitake K., Yamasaki T., Hayashi S., Sakai N., Seguchi H., Sawada M., Sumimoto H. and Saito N. (2004) Superoxide production at phagosomal cup/phagosome through beta I protein kinase C during Fc gamma R-mediated phagocytosis in microglia. *J. Immunol.* **173,** 4582-4589.

Unanue ER. (1997) Studies in Listeriosis show the strong symbiosis between the innate cellular system and the T cell response. *Immunol Rev* **158,** 11-25.

Vallieres N, Berard J, David S and Lacroix S (2006) Systemic injections of lipopolysaccharide accelerates myelin phagocytosis during Wallerian degeneration in the injured mouse spinal cord. *Glia* **53,** 103-113.

van der Laan L. J., Ruuls S. R., Weber K. S., Lodder I. J., Dopp E. A. and Dijkstra C. D. (1996) Macrophage phagocytosis of myelin in vitro determined by flow cytometry: phagocytosis is mediated by CR3 and induces production of tumor necrosis factor-alpha and nitric oxide. *J Neuroimmunol.* **70,** 145-152.

Van der G. A., Brouwer J., Hoekstra K., Roos D., van den Berg T. K. and Dijkstra C. D. (1998) Reactive oxygen species are required for the phagocytosis of myelin by macrophages. *J Neuroimmunol.* **92,** 67-75.

Van der G. A., Kortekaas M., Hoekstra K., Dijkstra C. D. and Amor S. (1999) The role of anti-myelin (auto)-antibodies in the phagocytosis of myelin by macrophages. *J Neuroimmunol.* **101,** 61-67.

Vermeiren C., Najimi M., Vanhoutte N., Tilleux S., de H., I, Maloteaux J. M. and Hermans E. (2005) Acute up-regulation of glutamate uptake mediated by mGluR5a in reactive astrocytes. *J. Neurochem.* **94,** 405-416.

Vilcek J. and Lee T. H. (1991) Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem.* **266,** 7313-7316.

Vilhardt F., Plastre O., Sawada M., Suzuki K., Wiznerowicz M., Kiyokawa E., Trono D. and Krause K. H. (2002) The HIV-1 Nef protein and phagocyte NADPH oxidase activation. *J. Biol. Chem.* **277,** 42136-42143.

Wajant H. (2002) The Fas signaling pathway: more than a paradigm. *Science* **296,** 1635-1636.

Wajant H., Pfizenmaier K. and Scheurich P. (2002) TNF-related apoptosis inducing ligand (TRAIL) and its receptors in tumor surveillance and cancer therapy. *Apoptosis.* **7,** 449-459.

Weiner H. L., Hafler D. A., Fallis R. J., Johnson D., Ault K. A. and Hauser S. L. (1984) Altered blood T-cell subsets in patients with multiple sclerosis. *J. Neuroimmunol.* **6,** 115-121.

Werner P., Pitt D. and Raine C. S. (2001) Multiple sclerosis: altered glutamate homeostasis in lesions correlates with oligodendrocyte and axonal damage. *Ann. Neurol.* **50,** 169-180.

Widner H., Brundin P., Bjorklund A. and Moller E. (1989) Survival and immunogenicity of dissociated allogeneic fetal neural dopamine-rich grafts when implanted into the brains of adult mice. *Exp. Brain Res.* **76,** 187-197.

Willenborg D. O., Fordham S. A., Staykova M. A., Ramshaw I. A. and Cowden W. B. (1999) IFN-gamma is critical to the control of murine autoimmune encephalomyelitis and regulates both in the periphery and in the target tissue: a possible role for nitric oxide. *J. Immunol.* **163,** 5278-5286.

Williams K., Ulvestad E. and Antel J. (1994) Immune regulatory and effector properties of human adult microglia studies in vitro and in situ. *Adv. Neuroimmunol.* **4,** 273-281.

Windhagen A., Newcombe J., Dangond F., Strand C., Woodroofe M. N., Cuzner M. L. and Hafler D. A. (1995) Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J. Exp. Med* **182,** 1985-1996.

Wolf S. A., Gimsa U., Bechmann I. and Nitsch R. (2001) Differential expression of costimulatory molecules B7-1 and B7-2 on microglial cells induced by Th1 and Th2 cells in organotypic brain tissue. *Glia* **36,** 414-420.

Woodroofe M. N., Bellamy A. S., Feldmann M., Davison A. N. and Cuzner M. L. (1986) Immunocytochemical characterisation of the immune reaction in the central nervous system in multiple sclerosis. Possible role for microglia in lesion growth. *J. Neurol. Sci.* **74,** 135-152.

Woodroofe M. N., Hayes G. M. and Cuzner M. L. (1989) Fc receptor density, MHC antigen expression and superoxide production are increased in interferon-gamma-treated microglia isolated from adult rat brain. *Immunology* **68,** 421-426.

Xiao B., Tu J. C., Petralia R. S., Yuan J. P., Doan A., Breder C. D., Ruggiero A., Lanahan A. A., Wenthold R. J. and Worley P. F. (1998) Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. *Neuron* **21,** 707-716.

Xie Z., Wei M., Morgan T. E., Fabrizio P., Han D., Finch C. E. and Longo V. D. (2002) Peroxynitrite mediates neurotoxicity of amyloid beta-peptide1-42- and lipopolysaccharide-activated microglia. *J Neurosci.* **22,** 3484-3492.

Xu J. and Ling E. A. (1994) Upregulation and induction of surface antigens with special reference to MHC class II expression in microglia in postnatal rat brain following intravenous or intraperitoneal injections of lipopolysaccharide. *J. Anat.* **184 (Pt 2),** 285-296.

Xu J. and Ling E. A. (1995) Induction of major histocompatibility complex class II antigen on amoeboid microglial cells in early postnatal rats following intraperitoneal injections of lipopolysaccharide or interferon-gamma. *Neurosci. Lett.* **189,** 97-100.

Yang L., Lindholm K., Konishi Y., Li R. and Shen Y. (2002) Target depletion of distinct tumor necrosis factor receptor subtypes reveals hippocampal neuron death and survival through different signal transduction pathways. *J. Neurosci.* **22,** 3025-3032.

Yao H. H., Ding J. H., Zhou F., Wang F., Hu L. F., Sun T. and Hu G. (2005) Enhancement of glutamate uptake mediates the neuroprotection exerted by activating group II or III metabotropic glutamate receptors on astrocytes. *J. Neurochem.* **92,** 948-961.

Yoshioka A., Yamaya Y., Saiki S., Kanemoto M., Hirose G., Beesley J. and Pleasure D. (2000) Non-N-methyl-D-aspartate glutamate receptors mediate oxygen--glucose deprivation-induced oligodendroglial injury. *Brain Res.* **854,** 207-215.

Young M. J., Rao K. and Lund R. D. (1989) Integrity of the blood-brain barrier in retinal xenografts is correlated with the immunological status of the host. *J. Comp Neurol.* **283,** 107-117.

Zajicek J. P., Wing M., Scolding N. J. and Compston D. A. (1992) Interactions between oligodendrocytes and microglia. A major role for complement and tumour necrosis factor in oligodendrocyte adherence and killing. *Brain* **115 (Pt 6),** 1611-1631.

Zamvil S. S. and Steinman L. (2003) Diverse targets for intervention during inflammatory and neurodegenerative phases of multiple sclerosis. *Neuron* **38,** 685-688.

Zamzami N., Brenner C., Marzo I., Susin S. A. and Kroemer G. (1998) Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. *Oncogene* **16,** 2265-2282.

Zhang S. C., Goetz B. D., Carre J. L. and Duncan I. D. (2001) Reactive microglia in dysmyelination and demyelination. *Glia* **34,** 101-109.

Zhao W., Xie W., Le W., Beers D. R., He Y., Henkel J. S., Simpson E. P., Yen A. A., Xiao Q. and Appel S. H. (2004) Activated microglia initiate motor neuron injury by a nitric oxide and glutamate-mediated mechanism. *J. Neuropathol. Exp. Neurol.* **63,** 964-977.

Zhou F., Yao H. H., Wu J. Y., Yang Y. J., Ding J. H., Zhang J. and Hu G. (2006) Activation of Group II/III metabotropic glutamate receptors attenuates LPS-induced astroglial neurotoxicity via promoting glutamate uptake. *J. Neurosci. Res.* **84,** 268-277.