Microglial glutathione and glutamate: Regulation mechanisms

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I, Victoria Fry, 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, the immune cells of the central nervous system (CNS), are important in the protection of the CNS, but may be implicated in the pathogenesis of neuroinflammatory disease. Upon activation, microglia produce reactive oxygen and nitrogen species; intracellular antioxidants are therefore likely to be important in their self-defence. Here, it was confirmed that cultured microglia contain high levels of glutathione, the predominant intracellular antioxidant in mammalian cells. The activation of microglia with lipopolysaccharide (LPS) or LPS + interferon-γ was shown to affect their glutathione levels. GSH levels in primary microglia and those of the BV-2 cell line increased upon activation, whilst levels in N9 microglial cells decreased.

Microglial glutathione synthesis is dependent upon cystine uptake via the x_c^- transporter, which exchanges cystine and glutamate. Glutamate is an excitatory neurotransmitter whose extracellular concentration is tightly regulated by excitatory amino acid transporters, as high levels cause toxicity to neurones and other CNS cell types through overstimulation of glutamate receptors or by causing reversal of x_c^- transporters. Following exposure to LPS, increased extracellular glutamate and increased levels of messenger ribonucleic acid (mRNA) for xCT, the specific subunit of x_c^-, were observed in BV-2 and primary microglial cells, suggesting upregulated GSH synthesis. An activation-induced decrease in N9 GSH levels suggests that this cell line is more susceptible to oxidative damage, and may be less able to upregulate GSH synthesis. Albumin, to which microglia may be exposed following blood-brain barrier damage, increased iNOS expression, glutamate release, xCT mRNA levels and intracellular levels of GSH and ATP in BV-2 and primary microglia. Primary and BV-2 microglial conditioned medium contained low levels of GSH, suggesting that microglia may release GSH.

Modulation of microglial metabotropic glutamate receptors (mGluRs) may alter microglial activation and neurotoxicity. Here, stimulation of the neuroprotective mGluR5 and group III mGluRs caused a decline in GSH levels in BV-2 and N9 microglia, respectively. In contrast mGluR1 stimulation may increase BV-2 GSH levels. The work presented in this thesis therefore extends current knowledge regarding microglial GSH and its regulation, and contributes to the understanding of microglial neurotoxicity and neuroprotection.

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Abbreviations

γGC	γ-glutamylcysteine
γGT	γ-glutamyl transpeptidase
(2R,4R)-APDC	(2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate
[^{3}H]-methoxy-PEPy	[^{3}H]-3-methoxy-5-(pyridin-2-ylethynyl)-pyridine
4AP	4-aminopyridine
4F2hc	the non-specific glycoprotein subunit of x_c^-; also known as CD98
AAA	aminoadipic acid
ABC	ATP-binding cassette
AC	adenylyl cyclase
ACPD	1-aminocyclopentane-1,3-dicarboxylic acid
AD	Alzheimer's disease
AIDA	(RS)-1-aminoindan-1,5-dicarboxylic acid
AIDS	acquired immune deficiency syndrome
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
APA	aminopimelic acid
APICA	(RS)-1-amino-5-phosphonoindan-1-carboxylic acid
ApN	aminopeptidase N
ApoE	apolipoprotein E
APP	β amyloid precursor protein
ATP	adenosine triphosphate
Aβ	β amyloid
$Aβ_{x-y}$	residues x-y of β amyloid peptide (e.g. 1-42, 25-35)
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BM	basic medium
BSO	L-buthionine sulfoximine
cAMP	cyclic adenosine monophosphate
CaR	Ca^{2+}-sensing receptor

CD	cluster of differentiation
CDPPB	3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide
CGA	chromogranin A
CGC	cerebellar granule cell (neurone)
CHO	Chinese hamster ovary (cell line)
CHPG	(RS)-2-chloro-5-hydroxyphenylglycine
CM	conditioned medium
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
COX	cyclooxygenase
CPG	(S)-4-carboxyphenylglycine
CSF	cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
dbcAMP	dibutyryl-cyclic adenosine monophosphate
DCG IV	(2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine
DHK	dihydrokainate
DHPG	(RS)-3,5-dihydroxyphenylglycine
DIV	days *in vitro*
D-MEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
EA	ethacrynic acid
EAAC1	excitatory amino acid carrier 1
EAAT	excitatory amino acid transporter
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ERK	extracellular signal-regulated kinase
FasL	Fas ligand
FBS	foetal bovine serum
fig.	figure
GABA	γ-aminobutyric acid
Gβγ	G protein βγ subunit
GCL	glutamate-cysteine ligase
GCP II	glutamate carboxypeptidase II

GDH	glutamic dehydrogenase
GDP	guanine diphosphate
GFAP	glial fibrillary acidic protein
GLAST	L-glutamate/L-aspartate transporter
GLT-1	L-glutamate transporter 1
GPCR	G protein-coupled receptor
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione; also used as a general term for glutathione
GSSG	oxidised glutathione
GST	glutathione S-transferase
GTP	guanine triphosphate
h	hour(s)
HEK	human embryonic kidney (cell)
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HNE	4-hydroxy-2-nonenal
HPLC	high-performance liquid chromatography
IFNγ	interferon-γ
iGluR	ionotropic glutamate receptor
IL	interleukin
iNOS	inducible nitric oxide synthase
IP3	inositol triphosphate
IP-10	IFNγ-inducible protein-10
KA	kainic acid
L-AP4	L-(+)-2-amino-4-phosphonobutyric acid
LBP	LPS-binding protein
L-CCG-I	(2S,1'S,2'S)-2-(carboxycyclopropyl)glycine
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
L-SOP	L-serine-O-phosphate
LTD	long-term depression

LTD_4	leukotriene D_4; cysteinylglycine leukotriene
LTP	long-term potentiation
LY 367385	(+)-2-methyl-4-carboxyphenylglycine
MAP4	(S)-2-amino-2-methyl-4-phosphonobutanoic acid
MBP	myelin basic protein
MCB	monochlorobimane
MCCG	2S,3S,4S-2-methyl-2-(carboxycyclopropyl)glycine
MCP-1	monocyte chemoattractant protein 1
M-CSF	macrophage colony-stimulating factor
MEM	minimum essential medium
MG	microglia(l)
mGluR	metabotropic glutamate receptor
MHC	major histocompatibility complex
min	minute(s)
MIP	macrophage inflammatory protein
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine
MMP	matrix metalloproteinase
MPP^+	1-methyl-4-phenylpyridinium
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
Mrp	multidrug resistance-associated protein
MS	multiple sclerosis
MTEP	3-((2-methyl-1,3-thiazol-4-yl)ethynyl)-pyridine
n.s.	not significant
NAAG	N-acetyl-L-aspartyl-L-glutamic acid
NAC	N-acetyl-cysteine
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NCS	newborn calf serum
NFT	neurofibrillary tangle

NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
O_2^-	superoxide
$ONOO^-$	peroxynitrite
OPA	orthophosphoric acid
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDL	poly-D-lysine
PG	prostaglandin
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PNS	peripheral nervous system
PRR	pattern recognition receptor
PSD	post-synaptic density
RANTES	regulated upon activation, normal T cell expressed and secreted
RNS	reactive nitrogen species
ROS	reactive oxygen species
RR(MS)	relapsing-remitting (multiple sclerosis)
(RS)-PPG	(RS)-4-phosphonophenylglycine
SCM	serum-containing medium
s.e.m.	standard error of the mean
SFM	serum-free medium
SIB-1757	6-methyl-2-(phenylazo)-3-pyridinol
tADA	trans-azetidine-2,4-dicarboxylic acid
TES	N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid
TLR	Toll-like receptor

TNFα	tumour necrosis factor α
VIP	vasoactive intestinal peptide
$VPAC_2$	VIP receptor 2
x_c^-	x_c^- glutamate/cystine antiporter system
xCT	system x_c^- transporter related protein; the specific subunit of x_c^-

Chapter 1

Introduction

1.1. The Central Nervous System

The mammalian brain and spinal cord constitute the central nervous system (CNS), which contains four main groups of resident cells; neurones, astrocytes, microglia and oligodendrocytes. Non-neuronal cells are collectively known as glia, which, in the human CNS, far outnumber the neurones (Hawkins and Olszewski 1957; Pfrieger and Barres 1995). The CNS circulation is separated from the main circulation by the blood-brain barrier (BBB), which acts as a filter, preventing large or potentially hazardous molecules from entering the CNS.

1.1.1. Neurones

Neurones transmit impulses within the CNS and the peripheral nervous system (PNS). Afferent or sensory neurones detect information throughout the body and transmit this information, in the form of a wave of depolarisation called an action potential, to the CNS. Efferent or motor neurones transmit responses from the CNS to effector tissues in the periphery. Interneurones are those which are fully contained within the CNS, often transmitting impulses between neurones in different regions.

Neurones consist of a cell body of between 4 and 100 μm in diameter, containing the nucleus and the majority of the organelles, which has branched extensions called dendrites; these receive the majority of the inputs to the cell. Most neurones also have one or more axons which are long, thin structures usually surrounded by myelin sheaths, allowing fast transmission of an action potential. The axon terminal is also branched, allowing each neurone to synapse with a number of different effector neurones. At the synapse, the arrival of an action potential causes influx of calcium ions (Ca^{2+}) and fusion of neurotransmitter vesicles with the plasma membrane. The neurotransmitter released diffuses across the synapse, to be detected by specific receptors in the dendrites of the effector cell, thus eliciting an action potential in the postsynaptic neurone. Neurotransmitters are diverse compounds, and certain monoamines, amino acids, purines and peptides have all been shown to mediate neurotransmission at synapses. A second compound may be co-released with the primary neurotransmitter and act upon its own specific receptor population, thus

further diversifying neurotransmission at the synapse and potential effects upon the postsynaptic neurone. In addition the receptors at the postsynaptic membrane may be modified by other compounds and transition metal ions present extracellularly, which may enhance or reduce their activity. The expression of neurotransmitter receptors may also be modified in response to the environment, as detected by the postsynaptic neurone.

Neurones are also functionally coupled through gap junctions, rendering the cytoplasm of the two cells continuous through nanometre-sized channels, and thus allowing bidirectional transmission of ions and other small molecules. Electrical impulses can therefore be transmitted at these electrical synapses without the need for a neurotransmitter (Connors and Long 2004; Hormuzdi *et al.* 2004).

1.1.2. Astrocytes

Astrocytes are the largest of the glial cells, and support neurones structurally and metabolically. Astrocytes associate closely with axon terminals (Spacek 1985), with one astrocyte in direct contact with up to 140 000 synapses (Bushong *et al.* 2002). Independent control of individual synapses is permitted by the existence of functionally distinct microdomains within each astrocyte (Grosche *et al.* 1999). The importance of bidirectional signalling between neurones and astrocytes in normal synaptic function has become apparent within the last decade, and synapses are now often considered as tripartite in recognition of this (Araque *et al.* 1999). Astrocytes respond to the presence of neurotransmitters and intercellular signalling molecules, and can release neuromodulatory compounds.

Other functions of astrocytes include supplying energy to neurones under conditions of hypoglycaemia or intense neural activity (Brown and Ransom 2007), buffering of CNS K^+ (Kofuji and Newman 2004), promotion of myelination in response to neuronal activity (Ishibashi *et al.* 2006), and activity-dependent control of brain microcirculation (Zonta *et al.* 2003). Astrocytes are also crucial for functional synapse development (Ullian *et al.* 2001).

Astrocytes communicate with one another through propagation of waves of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Cornell-Bell *et al.* 1990; Schipke *et al.* 2002), transmitted extracellularly by adenosine triphosphate (ATP) release and detection (Guthrie *et al.* 1999; Arcuino *et al.* 2002; Schipke *et al.* 2002). Such astrocyte ATP release also appears to affect microglia, acting upon their purinergic receptors (Schipke *et al.* 2002).

1.1.3. Oligodendrocytes

The main function of oligodendrocytes is the insulation of axonal conductance by the formation of the myelin sheath. The myelin sheath comprises layers of plasma membrane extensions, from which the cytoplasm has largely been extruded, which wrap around the axon. The membranes which constitute myelin are lipid-rich, with high levels of glycosphingolipids and cholesterol, and contain myelin-specific proteins. Myelin sheaths in the CNS each insulate about 1 mm of axon. A fully-myelinated axon therefore has many myelin sheaths, with small gaps, or nodes of Ranvier, between successive sheaths. The myelination of axons in this way allows saltatory conduction, first demonstrated by Lillie (1925), in which the wave of depolarisation "jumps" from one node to the next.

Oligodendrocytes are highly vulnerable to glutamate excitotoxicity and oxidative damage (McTigue and Tripathi 2008). The functional unit formed by the axon and the myelinating oligodendrocyte is such that oligodendrocyte death and subsequent demyelination may lead to axonal damage and loss of function and even neuronal death.

1.1.4. Microglia

Like many mammalian organ systems, the CNS contains resident macrophages which protect the largely immune-privileged CNS. These are known as microglia, and as the focus of this thesis, are described in detail in section 1.2 below.

1.1.5. The Blood-Brain Barrier

The blood-brain barrier (BBB) separates the circulation of the brain from the rest of the circulatory system, protecting the CNS against pathogens and foreign molecules, and helping to regulate the environment for the vulnerable cells of the CNS. The BBB is important in the context of microglial function as endogenous components of whole blood, as well as foreign particles carried in the circulation, may activate microglia.

The physical barrier function is mediated by the tight junctions present between adjacent endothelial cells. These junctions consist of transmembrane proteins such as claudins (Furuse *et al.* 1998), occludin (Furuse *et al.* 1993) and junctional adhesion molecule (Martin-Padura *et al.* 1998), which are anchored to the cytoskeleton and prevent the paracellular passage of solutes. Transcellular transport is therefore the only method by which substances can enter the brain. Only small, lipophilic molecules are able to diffuse through the nonfenestrated endothelial cells (Ballabh *et al.* 2004). Specific transporters exist for other molecules essential for brain function, such as amino acids and glucose, and larger molecules are transported by means of receptor-mediated endocytosis (Ballabh *et al.* 2004). Conversely, transporters such as P glycoprotein and multidrug resistance-associated protein remove certain substances from the brain by actively pumping them through the luminal plasma membrane into the circulation (Cordon-Cardo *et al.* 1989; Huai-Yun *et al.* 1998). The endothelial cells also provide a metabolic barrier, biochemically altering certain molecules entering the cell from the blood to control their entry into the brain (Minn *et al.* 1991).

Leukocyte entry into the brain is mediated by cytokines and complementary adhesion molecules (Pryce *et al.* 1997; Imhof and Aurrand-Lions 2004). The process of BBB penetration begins with chemoattraction and rolling of the cells, followed by firm adhesion on the luminal surface of the endothelium. Leukocyte transmigration was initially thought to occur at the tight junction, through specific protein-protein interactions (Bianchi *et al.* 1997), but more recent evidence suggests that under some circumstances at least, leukocytes may migrate through endothelial cells, without disrupting tight junctions (Wolburg *et al.* 2005; Carman and Springer 2008). Finally,

proteases secreted by the infiltrating leukocyte degrade the basal lamina surrounding the endothelial cells of the BBB and gain access to the brain (Yadav *et al.* 2003).

The endothelial cells of the BBB are closely associated with pericytes and astrocyte end-feet. Such accessory cells provide biochemical and trophic support to the endothelial cells of the BBB, thus ensuring that the barrier is maintained (Ballabh *et al.* 2004). Conversely, activated microglia may enhance the permeability of the BBB and upregulate the expression of cytokines, chemokines and adhesion molecules by the endothelial cells, through the secretion of proinflammatory cytokines such as tumour necrosis factor-α (TNFα) (Prat *et al.* 2001).

The BBB presents a problem for the pharmacological treatment of CNS disorders. Compounds must not only be able to transverse the barrier, but also avoid both enzymatic degradation and removal from the CNS by the multidrug transporters.

1.2. Microglia

Microglia, the resident immune cell of the CNS, were first described in 1919 by del Rio-Hortega (del Rio-Hortega 1919; see Kreutzberg 1996). During the development of the CNS, microglia are important in axonal pruning, as well as for the clearance of debris and apoptotic cells (Ashwell 1990; Brockhaus *et al.* 1996; Mallat *et al.* 2005). Microglia have a role throughout life in the protection of the immune-privileged CNS against foreign entities, and represent the link between the CNS and the immune system. Like other elements of the CNS, such as neuronal dendrites and astrocyte processes, microglial processes are constantly motile, allowing microglia to monitor the environment and respond accordingly with changes in their morphology and gene expression profile (Davalos *et al.* 2005; Nimmerjahn *et al.* 2005). Microglia may also be mobile within the CNS and able to migrate as a response to tissue damage, or to specific chemoattractants released by CNS or immune cells (Carbonell *et al.* 2005).

1.2.1. Origin

Microglia bear a certain resemblance to the resident cells of the mononuclear phagocyte lineage which exist in other tissues. Indeed, after decades of debate, it is now widely accepted that microglia too are of haematopoietic origin and derive from infiltrating monocytes during early development (Ling and Wong 1993; Kreutzberg 1996). However, unlike other resident monocyte populations, microglia appear to constitute a self-renewing population throughout adulthood, separated from the bone marrow-derived monocyte lineage. The large increase in the local microglial population (microgliosis) following CNS insult was until fairly recently believed to originate at least partly from bone marrow-derived progenitors (Hickey and Kimura 1988; Flugel *et al.* 2001; Djukic *et al.* 2006). However, the latest studies have utilised novel methodology to generate data suggesting that in mouse models of CNS disease and injury, microgliosis is due entirely to proliferation of resident cells (Ajami *et al.* 2007; Mildner *et al.* 2007).

1.2.2. Morphology of microglia

During early postnatal development, monocytes infiltrate the brain and transform into amoeboid microglia, characterised morphologically by their large cell body and short processes (Ling and Wong 1993). This allows them to move within the brain, pruning axons where appropriate and phagocytosing apoptotic cells and other debris associated with CNS development (Ashwell 1990; Brockhaus *et al.* 1996; Mallat *et al.* 2005). Within weeks of their appearance in the CNS, microglia adopt a more ramified, downregulated phenotype (Ling and Wong 1993).

Under normal circumstances, microglia in the adult CNS exist in this branched state. Such ramified microglia were until recently often referred to as "resting" microglia. The recent discovery that microglia are in fact constantly active, monitoring the CNS environment, has prompted a re-evaluation of the role of ramified microglia (Davalos *et al.* 2005; Nimmerjahn *et al.* 2005). At a given moment, individual microglia appear to be responsible for specific portions of brain volume; adjacent microglia are closely opposed, and the boundaries between them may move over time, but their processes mutually repel one another (Nimmerjahn *et al.* 2005). Little is known regarding the true characteristics of ramified microglia *in vivo*, as any manipulation or investigation is likely to activate microglia to some extent (Streit *et al.* 1999).

Microglia adopt an activated phenotype when exposed to substances not usually present in the CNS environment (Kreutzberg 1996). Activated microglia are morphologically similar to the amoeboid microglia present during development (Dheen *et al.* 2007). Microglial activation is also associated with changes in the release profile of microglia and altered receptor expression. Under conditions of neuronal degeneration, microglia may transform into ovoid phagocytic cells, also known as foamy macrophages (Kreutzberg 1996), distinguished by the presence of phagosomes (Streit and Kreutzberg 1988; Rieske *et al.* 1989).

The different morphologies adopted by microglia, and the relationships between them, are illustrated in figure 1.1. It is important to note that this is a simplified view,

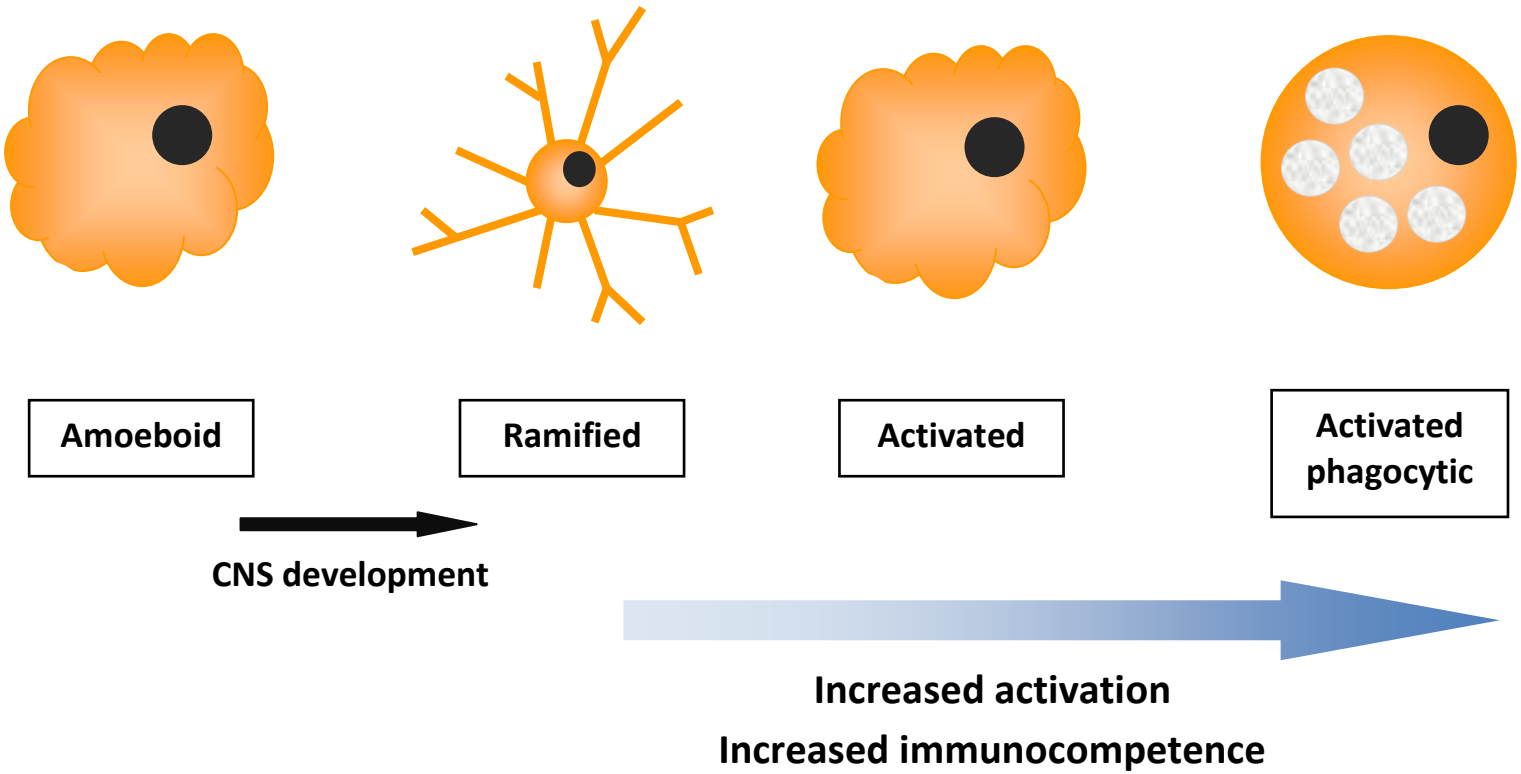


Figure 1.1. *Diagrammatic representation of microglial morphology. During CNS development, monocytes infiltrate the brain and transform into amoeboid microglia, and later ramified microglia. In the adult CNS, microglia exist in a ramified state and assume a rounded phenotype only when activated. Microglia may become phagocytic under conditions of neuronal death and degeneration. Microglial activation is associated with increased release of cytokines and expression of cytokine receptors and MHC antigens. Adapted from Kreutzberg (1996).*

and that there is more likely a morphological continuum, with different triggers having subtly different effects upon microglia.

1.2.3. Microglial activation

Microglia may be activated by a whole host of apparently structurally unrelated compounds. Microglia are activated in infectious diseases of the CNS, and pathogens and pathogen components activate microglia *in vitro* (Aloisi 2001). Microglia detect pathogens by means of pattern recognition receptors (PRRs) on the cell surface, which recognise so-called pathogen-associated molecular patterns (PAMPs), molecular structures associated with pathogens but not with host cells (Medzhitov and Janeway, Jr. 2000). Most PRRs are expressed only at low levels on ramified microglia, if at all, and are upregulated upon microglial activation (Aloisi 2001).

The most commonly used compound to stimulate microglial activation experimentally *in vitro* and *in vivo* is the bacterial endotoxin lipopolysaccharide (LPS), the major mediator of shock induced by gram-negative bacteria (Rietschel *et al.* 1994). This therefore mimics gram-negative bacterial infection of the brain, which is prevented under normal circumstances by the BBB. The PRRs Toll-like receptor 4 (TLR4) (Poltorak *et al.* 1998; Qureshi *et al.* 1999) and cluster of differentiation (CD) 14 (Wright *et al.* 1990; Haziot *et al.* 1996) expressed by microglia and other cells of the monocyte/macrophage lineage are involved in the detection of LPS, along with the accessory proteins MD2 (Shimazu *et al.* 1999) and LPS-binding protein (LBP) (Tobias *et al.* 1989; Schumann *et al.* 1990).

Other PRRs expressed by microglia include TLR2, TLR9 and the mannose receptor. In addition, microglial receptors exist allowing detection and phagocytosis of other pathogens which have been opsonised (coated) by soluble components of the immune system (Aloisi 2001). The receptors expressed by microglia which allow detection of pathogens are summarised in table 1.1.

Receptor	Pathogen-associated molecular pattern recognised	References
TLR2	gram-positive bacterial peptidoglycans and lipoteichoic acids	Schwandner *et al.(* 1999); Yoshimura *et al.* (1999)
TLR4	gram-negative bacterial endotoxin, LPS	Poltorak *et al.(* 1998); Qureshi *et al.* (1999)
TLR9	bacterial DNA	Hemmi *et al.* (2000)
CD14	LPS	Wright *et al.* (1990); Haziot *et al.* (1996)
Mannose receptor	pathogen-specific oligosaccharides	Linehan *et al.* (2000)
Fc receptors	pathogens opsonised by immunoglobulins	Aloisi (2001)
Complement receptors	pathogens opsonised by complement	Aloisi (2001)
Complement receptor 3	- pathogens opsonised by complement component C3b - diverse protein and non-protein pathogen components	Ehlers (2000)

Table 1.1. *Receptors expressed by microglia allowing the detection of pathogens. CD, cluster of differentiation; DNA, deoxyribonucleic acid; LPS, lipopolysaccharide; TLR, Toll-like receptor.*

Microglia react rapidly to local tissue damage, suggesting that a signal from damaged cells may be sufficient to activate microglia (Gehrmann *et al.* 1991; Morioka *et al.* 1992). Accordingly, ATP, large amounts of which are released mainly by astrocytes under pathological conditions (Ciccarelli *et al.* 2001), has been shown *in vitro* to affect microglial morphology (Honda *et al.* 2001), to cause elevation of intracellular Ca^{2+} (McLarnon *et al.* 1999), and release of cytokines (Inoue 2002), and to have a chemotactic effect (Honda *et al.* 2001; Davalos *et al.* 2005). These effects, characteristic of microglial activation, may be mediated via the P2Y purinergic receptor (Honda *et al.* 2001; Davalos *et al.* 2005), or the $P2X_7$ ATP-gated ion channel (Monif *et al.* 2009). Elevated extracellular K^+, also indicative of tissue damage, depolarises the membrane potential of microglia and may enhance aspects of microglial activation (Colton *et al.* 1994a; Abraham *et al.* 2001). In addition, inwardly-rectifying K^+ channels expressed by microglia (Kettenmann *et al.* 1990; Norenberg *et al.* 1994), current through which is enhanced under conditions of elevated K^+, may be involved in cytokine-dependent proliferation and differentiation (Schlichter *et al.* 1996; Shirihai *et al.* 1996).

The antibody ED1 may be used experimentally to detect activated microglia. ED1 recognises the rat homologue of the lysosomal glycoprotein CD68 (Holness and Simmons 1993; Damoiseaux *et al.* 1994), the expression of which is upregulated following microglial activation (Tran *et al.* 1998).

1.2.4. Microglia and the immune system

Like other cells of the mononuclear phagocyte lineage, microglia are equipped to phagocytose pathogens, dead cells and other debris (Giulian and Baker 1986; Streit and Kreutzberg 1988; Rieske *et al.* 1989), and to communicate with other cells of the immune system. The most evident microglial phagocytosis is undertaken by ovoid foamy macrophages (Streit and Kreutzberg 1988; Rieske *et al.* 1989). Phagocytosis of pathogen components and subsequent processing leads to the presentation of antigens by the major histocompatibility complex (MHC) molecules for T cell activation (Cash *et al.* 1993). This is accompanied by an upregulation of other molecules associated with activation, including a number of cell adhesion molecules, which allow microglia to further detect and internalise diverse debris (Raivich *et al.*

1999). The removal of apoptotic cells and debris is important in the homeostasis of the CNS environment. The process of phagocytosis leads to antigen presentation and may induce a general pro-inflammatory phenotype in the microglia.

The main mode of communication between microglia and other cells of the immune system is through the secretion of cytokines and the expression of cytokine receptors (see fig. 1.2). This may mediate recruitment of further microglia and peripheral immune cells, and affect the activation state of such cells. Cytokines are broadly grouped into pro-inflammatory and anti-inflammatory mediators. Microglial activation may induce the release of cytokines of both groups, and alter cytokine receptor expression, depending on the type and intensity of the stimulus.

Microglia express receptors for the lymphocyte-derived cytokine interferon γ (IFNγ) and respond to it both *in vitro* and *in vivo* with an upregulation in microglial expression of MHC molecules, suggesting enhanced antigen presenting ability (Vass and Lassmann 1990; Loughlin *et al.* 1992; Panek and Benveniste 1995; Deckert-Schluter *et al.* 1999). IFNγ appears to amplify the pro-inflammatory effects of microglial activators (Colton *et al.* 1994b; Hausler *et al.* 2002) and is often used *in vitro* as a co-stimulator for LPS-mediated activation.

The effects of anti-inflammatory cytokines may be largely attributed to the downregulation of pro-inflammatory cytokines or functional antagonism of their effects (Hanisch 2002). For example, LPS has been shown to induce transforming growth factor β (TGFβ) production by microglia, through a tumour necrosis factor α- (TNFα-) dependent pathway. TGFβ was then shown to inhibit LPS-stimulated TNFα production (Chao *et al.* 1995). A self-regulating mechanism may therefore exist, with the potential to control inflammation.

1.2.5. Expression and release profiles of activated microglia

Different microglial receptors and signalling pathways are involved in the detection of and response to different molecules which activate microglia (Pocock and Liddle 2001). It is therefore likely that different microglial activators cause the upregulation of specific genes and therefore lead to slightly different activated phenotypes. The

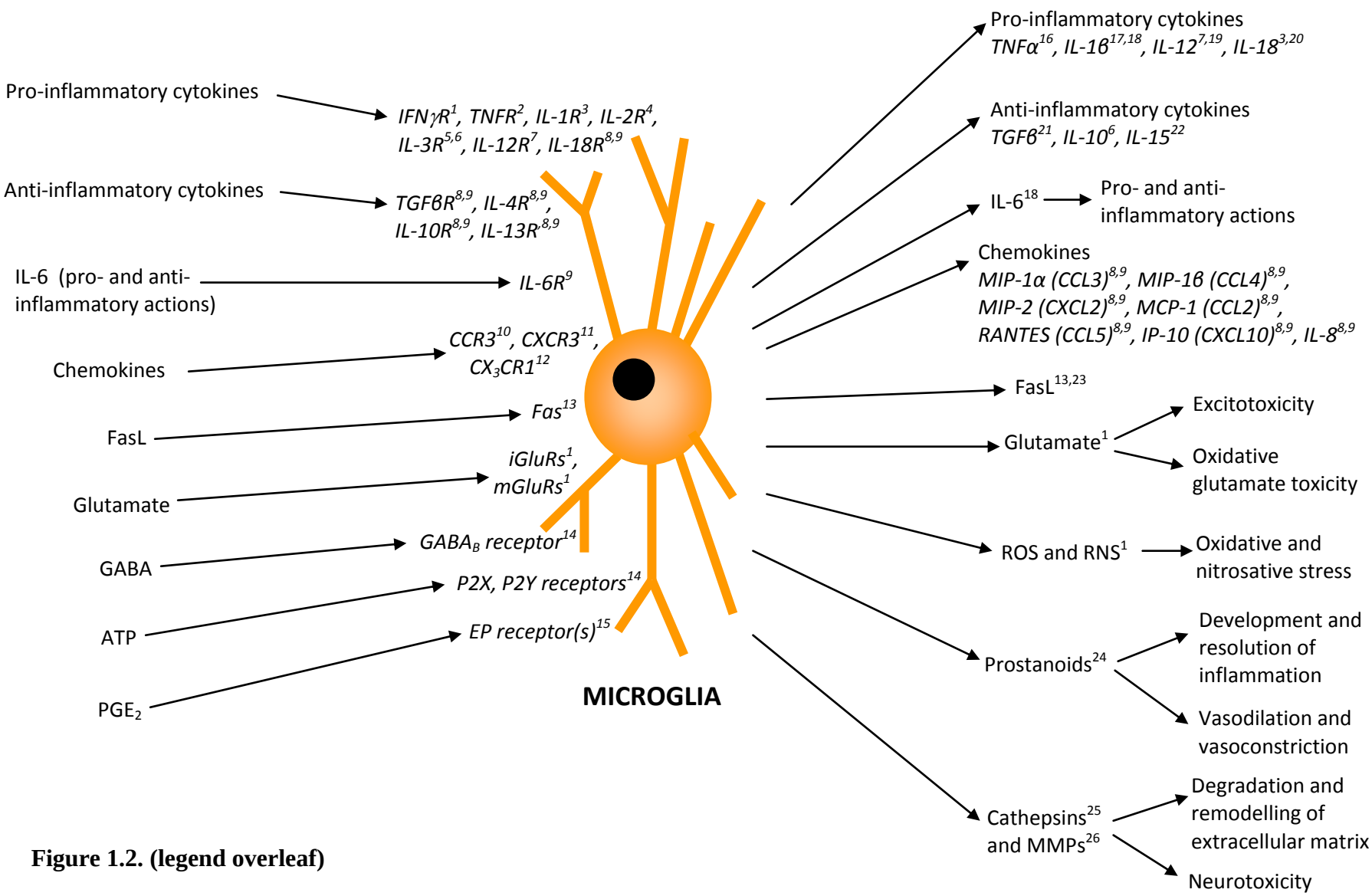


Figure 1.2. (legend overleaf)

Figure 1.2. (previous page) *Microglial expression and release profiles. Microglia express receptors allowing the detection of cytokines, chemokines, neurotransmitters and other signalling molecules, and may release cytokines, chemokines, other pro- and anti-inflammatory molecules and cytotoxic compounds. ATP, adenosine triphosphate; EP receptor, E-prostaglandin receptor; FasL, Fas ligand; GABA, γ-aminobutyric acid; IFNγ, interferon-γ; iGluR, ionotropic glutamate receptor; IL, interleukin; IP-10, IFNγ-inducible protein-10; MCP, monocyte chemoattractant protein; mGluR, metabotropic glutamate receptor; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; P2X receptor, metabotropic purinergic receptor; P2Y receptor, purinergic ligand-gated ion channel; PGE_2, prostaglandin E2; R, receptor; RANTES, regulated upon activation, normal T cell expressed and secreted; RNS, reactive nitrogen species; ROS, reactive oxygen species; TGFβ, transforming growth factor β; TNFα, tumour necrosis factor α.*[1]*see text;* [2]*Dopp et al. (1997);* [3]*Prinz and Hanisch (1999);* [4]*Sawada et al. (1995);* [5]*Appel et al. (1995);* [6]*Kitamura et al. (2000);* [7]*Suzumura et al. (1998);* [8]*Aloisi (2001);* [9]*Hanisch (2002);* [10]*He et al. (1997);* [11]*Biber et al. (2002), Dijkstra et al. (2004);* [12]*Maciejewski-Lenoir et al. (1999), Hulshof et al. (2003);* [13]*Spanaus et al. (1998), White et al. (1998), Terrazzino et al. (2002);* [14]*Pocock and Kettenmann (2007);* [15]*Nakano et al. (2008);* [16]*Chen and Goeddel (2002);* [17]*Conti et al. (1999);* [18]*Lee et al. (1993), Horvath et al. (2008);* [19]*Becher et al. (1996);* [20]*Conti et al. (1999);* [21]*Constam et al. (1992), da Cunha et al. (1993), Peress and Perillo (1995);* [22]*Hanisch et al. (1997);* [23]*Dowling et al. (1996), Bechmann et al. (1999), Frigerio et al. (2000), Badie et al. (2001), Taylor et al. (2005);* [24]*Minghetti and Levi (1995), Giulian et al. (1996), Hoozemans et al. (2002), Mohri et al. (2006), Zhang et al. (2006), Kim et al. (2008);* [25]*Petanceska et al. (1996), Liuzzo et al. (1999), Kingham and Pocock (2001), Gan et al. (2004), Kim et al. (2007), Sakamoto et al. (2008);* [26]*Gottschall et al. (1995), Cross and Woodroofe (1999), Kauppinen and Swanson (2005), Liuzzi et al. (2007), Milner et al. (2007), Crocker et al. (2008), Woo et al. (2008).*

receptors which may be expressed, and compounds which may be released by microglia are summarised in figure 1.2. Microglial activation may lead to the expression of the so-called death receptor, Fas, and its ligand FasL, and the release of reactive oxygen and nitrogen species (ROS and RNS), proteolytic enzymes, prostanoids and glutamate. Microglia may release glutamate through increased expression or activity of the cystine/glutamate antiporter x_c^- or the reversal of excitatory amino acid transporters. The regulation of these transporters and implications for CNS disease are discussed in detail in section 1.4.2 below.

The release of ROS and RNS by activated microglia represents a cytotoxic attack mechanism against invading pathogens. Activated microglia may express inducible nitric oxide synthase (iNOS), allowing the production of the vasodilator and non-specific inflammatory mediator nitric oxide (NO) from L-arginine (Chao *et al.* 1992; Ding *et al.* 1997; Possel *et al.* 2000). Microglial activation may also induce the rapid generation of superoxide by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, known as the respiratory burst (Colton and Gilbert 1987; Klegeris and McGeer 1994; Sankarapandi *et al.* 1998). Either NO or superoxide may be cytotoxic alone; alternatively they may combine to form the highly reactive species peroxynitrite (Ischiropoulos *et al.* 1992; Szabo *et al.* 1997; Noack *et al.* 1999). ROS and RNS have the potential to cause bystander damage and death, by reacting with proteins, nucleic acids and lipids of neighbouring cells (Valko *et al.* 2007). Peroxynitrite has dual effects upon mitochondrial energy metabolism; its actions causing the collapse of the mitochondrial membrane potential and dysregulation of mitochondrial respiration, leading to energy crisis and cell death, are well-characterised (Bolaños *et al.* 1995). However, in some cell types peroxynitrite may be protective through the upregulation of the pentose phosphate pathway, leading to enhanced NADPH levels to regenerate the reduced form of the antioxidant glutathione (Almeida *et al.* 2005). Alternatively, NO may cause an upregulation of glycolysis, leading to compensatory ATP production and maintenance of the mitochondrial membrane potential (Almeida *et al.* 2001). This suggests that under some conditions ROS and RNS may have a role in the control of energy metabolism (Bolaños *et al.* 2008).

1.2.6. Neurone-microglia interactions: neuroprotection and neurotoxicity

The different resident cell types of the CNS are all closely associated *in vivo*, and intercellular communication mechanisms between cells are likely to be complex. As the only resident immune cell in the otherwise largely immune-privileged CNS (Aloisi *et al.* 2001), microglia are crucial in the protection of neurones from potentially damaging insults (Streit 2002). As described previously, damaged neurones are able to elicit a microglial response by the release of ATP and K^+, as well as chemokines such as fractalkine (CX_3CL1) (Chapman *et al.* 2000). Excessive inflammation may however be harmful, and the CNS may provide a general anti-inflammatory environment through the production of anti-inflammatory cytokines and neuropeptides, and the expression of FasL to induce apoptosis of Fas-expressing immune cells crossing the BBB (Bechmann *et al.* 1999; Aloisi *et al.* 2001). Evidence also exists that neurones may directly downregulate the level of microglial activation. This may occur by direct contact between neuronal CD200 and the inhibitory CD200 receptor expressed by microglia (Hoek *et al.* 2000), or by release of soluble mediators by neurones. Soluble neurone-derived CD22 has been shown to bind to microglial CD45 and inhibit LPS-induced TNFα production (Mott *et al.* 2004), and the neurotrophins nerve growth factor (NGF) and neurotrophin-3 act upon microglial neurotrophin receptors to decrease expression of MHC class II molecules (Neumann *et al.* 1998). The lack of such neurone-derived inhibitory stimuli may indicate neuronal damage and may be an important step in the initiation of microglial activation.

The phagocytic capability of microglia makes them important in the everyday maintenance of homeostasis in the CNS environment, clearing apoptotic cells and other debris, without necessarily transforming to a rounded phenotype (Streit *et al.* 1999). During the resolution of inflammation, microglia are important in the promotion of repair. Microglia make direct contact with stressed neurones and perform synaptic stripping (Neumann *et al.* 2006; Trapp *et al.* 2007), phagocytose damaged or dead cells, and release anti-inflammatory cytokines such as TGFβ (Chao *et al.* 1995; Kreutzberg 1996). In addition, microglia may enhance neuronal survival; unstimulated microglia have been shown to release neurotrophic factors such as PDGF, epidermal growth factor, basic fibroblast growth factor, hepatocyte growth

factor and brain-derived neurotrophic factor (BDNF) *in vitro* (Araujo and Cotman 1992; Presta *et al.* 1995; Hamanoue *et al.* 1996; Nakajima *et al.* 2001a; Morgan *et al.* 2004).

However, it is clear from the previous discussions that by eliciting and amplifying an immune response, microglia may directly or indirectly cause neuronal damage. Indeed a number of microglial release products are directly neurotoxic. Activated microglia produce NO and superoxide as an anti-microbial defence (Colton and Gilbert 1987; Chao *et al.* 1992), but neurones are susceptible to oxidative damage, particularly the inhibition of mitochondrial energy metabolism (Bolaños *et al.* 1995). The neuronal antioxidant capacity is relatively low (Wang *et al.* 2003a), and neurones lack the ability to upregulate glycolysis to maintain the mitochondrial membrane potential (Almeida *et al.* 2001). Enhanced release of glutamate by activated microglia may be toxic to neurones through overactivation of glutamate-gated ion channels and the subsequent intracellular pathways (Piani and Fontana 1994; Barger and Basile 2001). FasL and TNFα released by activated microglia have the ability to activate apoptotic cascades in cells expressing the appropriate receptors (Tartaglia *et al.* 1993; Kischkel *et al.* 1995). *In vitro* studies consistently demonstrate that the neurotoxicity of LPS is entirely microglia-dependent (Boje and Arora 1992; Kim *et al.* 2000; Taylor *et al.* 2003; Zujovic and Taupin 2003), and mice lacking TLR4, the LPS recognition receptor, which is only expressed by microglia in the CNS did not suffer LPS-induced neuronal injury (Lehnardt *et al.* 2003).

1.2.7. Microglia in ageing, neuroinflammatory and neurodegenerative disease

Proteins which are upregulated in neurological diseases characterised by an inflammatory component have also been found to activate microglia *in vitro*. These include components of the blood which may be encountered at elevated concentrations by microglia following BBB damage, such as albumin (Si *et al.* 1997; Hooper *et al.* 2005, 2009) and thrombin (Choi *et al.* 2003), as well as proteins present in the lesions characteristic of particular neurodegenerative diseases, such as β amyloid (Aβ) and chromogranin A (CGA) in Alzheimer's disease (Taupenot *et al.* 1996; McDonald *et al.* 1997; Kingham *et al.* 1999; Combs *et al.* 2001; Le *et al.* 2001), and Prion proteins in spongiform encephalopathies (Brown *et al.* 1996;

Combs *et al.* 1999; Fabrizi *et al.* 2001). Indeed, *in vitro* evidence suggests that the neurotoxicity of albumin (Hooper *et al.* 2009) and CGA (Kingham *et al.* 1999; Taylor *et al.* 2002) is dependent upon the presence of microglia. Such observations clearly implicate microglia and the downstream consequences of microglial activation in disease pathogenesis.

In chronic neuroinflammatory diseases, microglia are continually exposed to such disease-related activators. This leads to chronic activation of microglia as they persistently attempt to clear these entities through the release of toxic compounds, the initiation and maintenance of an inflammatory response and an upregulated phagocytic activity (Eikelenboom *et al.* 2002). The release of chemokines and pro-inflammatory cytokines recruits and activates further microglia by a positive feedback mechanism (Aloisi 2001; Hanisch 2002) and stimulates microglial proliferation (Graeber *et al.* 1988; Ajami *et al.* 2007), thus causing microglial expansion at the site of the original lesion.

Such an enlarged population of chronically activated microglia may cause bystander damage to neurones and other cells and structures within the CNS, thus contributing to pathology (Lehnardt *et al.* 2003; Heneka and O'Banion 2007). Microglial release products such as TNFα, glutamate, proteases and ROS and RNS derived from NO and superoxide have all been implicated in such bystander damage. Interestingly however, a recent paper has demonstrated a neuroprotective effect of microglia-derived TNFα following ischaemia (Lambertsen *et al.* 2009), challenging the widely-accepted view that TNFα has neurotoxic actions in the pathogenesis of neuroinflammatory diseases. This also highlights the complexity of microglial involvement in neurological disease.

In ageing mouse brain, an upregulation of genes associated with inflammation was observed (Lee *et al.* 2000), including some factors associated with microglial activation. Microglia *in vivo* tend to become progressively more activated with age (Streit *et al.* 1999), indeed, an age-related microglial dystrophy has been reported in human brain (Streit *et al.* 2004). These observations may have implications for age-related neuroinflammatory and neurodegenerative diseases.

The main neuroinflammatory conditions relevant to this thesis are multiple sclerosis (MS) and Alzheimer's disease (AD). These diseases, and specifically the role of microglia in disease, are discussed in more detail in the next section.

1.3. Neuroinflammatory disease

Although microglia are implicated to some extent in the vast majority of CNS diseases, particularly those involving inflammation, this thesis focuses largely upon their role in multiple sclerosis and Alzheimer's disease. Detailed descriptions will therefore be limited to these conditions.

1.3.1. Multiple sclerosis

Multiple sclerosis (MS) is thought to be an autoimmune disorder of the CNS (Steinman 1996; Zamvil and Steinman 2003). It consists of inflammation and neurodegeneration and is characterised by areas of focal demyelination and axonal damage within the white matter (Conlon *et al.* 1999; Compston and Coles 2002). Most cases of MS begin with a relapsing-remitting (RR) disease. RRMS consists of acute inflammatory attacks, with some axonal loss but little obvious functional deterioration between relapses. RRMS usually develops into a chronic secondary progressive disease with time, characterised by a loss of the relapsing-remitting pattern, less inflammation and more axonal degeneration, along with clear accumulating disability (Bjartmar and Trapp 2001). In approximately 10% of cases MS takes a primary progressive course, a chronic disease characterised by less inflammation, more neurodegeneration, clear functional deterioration and a lack of acute attacks (Zamvil and Steinman 2003).

There is approximately a 25% concordance rate of MS among monozygotic twins (Ebers *et al.* 1986; Mumford *et al.* 1994), suggesting that both genetic and environmental factors influence disease development. The major histocompatibility complex (MHC) has consistently been shown to be associated with MS (Haines *et al.* 1996; Sawcer *et al.* 1996, 2005; Hafler *et al.* 2007). This genetic locus contains the genes for the human leukocyte antigen (HLA), responsible for antigen

presentation to $CD4^+$ T cells, as well as those for TNFα, components of the complement cascade and myelin oligodendrocyte glycoprotein, all of which are involved in MS pathogenesis (Zamvil and Steinman 2003). Environmental factors hypothesised to have a role in the development of MS include infectious agents, particularly Epstein-Barr virus (EBV), sunlight exposure and vitamin D levels, and increased sanitation levels, which, by limiting exposure of the immune system to pathogenic antigens, may lead to compromised development of the immune system and predispose to autoimmunity (Fleming and Cook 2006; Giovannoni and Ebers 2007).

Current MS treatments are mainly immunomodulatory and immunosuppressive, and act on the inflammatory stage, decreasing the duration and frequency of relapses. Immunosuppression may however have serious side effects. Some patients, particularly those with progressive MS, do not respond to these treatments, and although they reduce inflammation, they do not appear to prevent subsequent neurodegeneration associated with the later progressive stages of disease.

1.3.1.1. MS pathology and pathogenesis

The white matter lesions characteristic of MS correspond to areas of focal demyelination, a consequence of immune attack against myelin antigens (Conlon *et al.* 1999). Such lesions are characterised by the presence of inflammatory lymphocytes, as well as macrophages and activated microglia containing phagocytic vesicles of myelin debris (Adams *et al.* 1989). During the inflammatory phase of MS, a limited amount of remyelination by oligodendrocytes may occur, giving rise to shadow plaques (Prineas *et al.* 1993), so-called because of their reduced myelin density, and therefore less prominent myelin-specific staining compared with normal white matter. Axonal damage only occurs in the presence of demyelination, and may be due to the lack of protection and trophic support by the myelin sheath (Kornek *et al.* 2000; Wilkins *et al.* 2001; Compston and Coles 2002), or to an independent process initiated by inflammatory mediators (Coleman and Perry 2002; Centonze *et al.* 2009). Following damage to axons, the distal portion of the neurone is subject to Wallerian degeneration, and the cell body may also die due to lack of connections with other neurones (Coleman and Perry 2002). Some lesions have been observed

which lack significant inflammatory pathology but are instead characterised by oligodendrocyte apoptosis, perhaps representing a different disease stage or heterogeneity in the mechanisms of demyelination (Lucchinetti *et al.* 2000; Barnett and Prineas 2004).

In the initial stages of MS and the animal model of MS, experimental autoimmune encephalomyelitis (EAE), T cells specific for myelin proteins encounter their antigens in the CNS, undergo clonal expansion and recruit other immune cells, initiating an inflammatory cascade. The epitope spread theory suggests that as inflammation and demyelination progress, new, previously cryptic, myelin epitopes appear, which recruit their own specific T cells and exacerbate inflammation (Lehmann *et al.* 1992).

There are two theories relating to the apparent link between MS development and viral or bacterial infection. The most long-standing of these is that of molecular mimicry. Peptides derived from EBV can activate human myelin basic protein- (MBP-) specific T cells (Wucherpfennig and Strominger 1995) and bind MBP-specific autoantibodies (Wucherpfennig *et al.* 1997), indicating portions of structural homology and suggesting that following EBV infection, presentation of particular EBV antigens could induce an autoimmune response. Superantigens, viral and bacterial exotoxins which bind to the T cell receptor and non-specifically activate T cells (Marrack and Kappler 1990), have also been implicated in MS development and relapse rate. Bacterial superantigens have the ability to activate T cells specific for myelin proteins, and may modulate the induction, symptoms and relapse rate of EAE (Brocke *et al.* 1993; Matsumoto and Fujiwara 1993; Schiffenbauer *et al.* 1993; Soos *et al.* 1995; Zhang *et al.* 1995).

1.3.1.2. Microglia in MS

As the resident immune cell of the brain, it is not surprising that microglia are implicated in the pathogenesis of a disease with such a clear immune involvement as MS. Indeed, MS lesions are characterised by infiltration of activated microglia and macrophages, which phagocytose myelin breakdown products and are consequently filled with lipid-containing vesicles (Adams *et al.* 1989). Microglia express MHC

class II molecules and are thought to participate in the initiation of inflammation and demyelination in MS by presenting the autoantigen to $CD4^+$ T cells (Matsumoto *et al.* 1992; Bo *et al.* 1994). The identification of MHC class II molecules as a genetic risk factor for MS (Sawcer *et al.* 2005; Hafler *et al.* 2007) may indicate the importance of this interaction in MS pathogenesis.

Soluble factors released by microglia may also be important in the pathogenesis of MS. The cytokine TNFα is expressed at high levels by activated microglia in EAE and MS lesions (Selmaj *et al.* 1991; Baker *et al.* 1994; Renno *et al.* 1995), and is implicated in oligodendrocyte death and myelin damage (Selmaj and Raine 1988; D'Souza *et al.* 1995; Akassoglou *et al.* 1998). Microglial TNFα has been shown to induce oligodendrocyte death through direct contact between cell surface TNFα and oligodendrocyte TNF receptor 1 (Zajicek *et al.* 1992; Merrill *et al.* 1993), which couples to a signal transduction pathway culminating in apoptosis (Tartaglia *et al.* 1991, 1993; Chinnaiyan *et al.* 1996). Oligodendrocyte TNF receptor 1 may be upregulated in response to stress (Tchelingerian *et al.* 1995) and in the presence of TNFα (Dopp *et al.* 1997). TNFα is also implicated in leukocyte chemotaxis via upregulation of chemokine expression (Sedgwick *et al.* 2000). TNFα induces microglial proliferation *in vitro* in the presence of astrocytes, suggesting a role in amplification of the immune response (Dopp *et al.* 1997). Mice overexpressing TNFα suffered a chronic inflammatory demyelinating disease (Probert *et al.* 1995), and injection of TNFα worsens the clinical course of EAE (Kuroda and Shimamoto 1991; Crisi *et al.* 1995). Administration of antibodies against TNFα or soluble TNF receptors significantly inhibits the development of EAE (Ruddle *et al.* 1990; Baker *et al.* 1994; Martin *et al.* 1995; Selmaj *et al.* 1995; Klinkert *et al.* 1997). More recently, studies with mice lacking TNF receptor 1 have suggested that this receptor is essential for the development of clinical signs of EAE (Gimenez *et al.* 2006). Some studies have however reported protective effects of TNFα in EAE (Willenborg *et al.* 1995; Frei *et al.* 1997; Liu *et al.* 1998a). This protective role may be due to a proliferative effect of oligodendrocyte precursor cell TNF receptor 2 stimulation, leading to remyelination (Arnett *et al.* 2001).

Microglia are both a source and a target of chemokines (Hanisch 2002; Sanders and De Keyser 2007), which mediate the recruitment of cells to the lesion site (Sellebjerg

and Sorensen 2003). Microglia also secrete anti-inflammatory cytokines such as TGFβ and IL-10, which play a regulatory role in EAE and MS, downregulating the immune response (Imitola *et al.* 2005). Accordingly, the presence of activated microglia appears to be important for remyelination (Diemel *et al.* 1998; Kotter *et al.* 2001; Li *et al.* 2005).

Activated microglia associated with lesions in MS and EAE express iNOS and release NO (Okuda *et al.* 1995; Hill *et al.* 2004), which has been implicated in a number of elements of disease pathogenesis. Indeed, inhibition of iNOS has been shown to reduce inflammation, demyelination and axonal necrosis in a mouse model of MS (Rose *et al.* 1998). By dilating cerebral vessels and reducing the velocity of blood flow, NO may facilitate leukocyte binding and migration across the BBB (Smith and Lassmann 2002). Oligodendrocytes are highly susceptible to NO-mediated damage *in vitro* (Merrill *et al.* 1993; Mitrovic *et al.* 1994). The NO derivative peroxynitrite may cause neuronal death by inhibiting the mitochondrial respiratory chain, leading to ATP depletion (Bolaños *et al.* 1995). Like neurones, oligodendrocytes have high rates of oxidative metabolism (Juurlink 1997) and may have low levels of the antioxidant glutathione (Thorburne and Juurlink 1996; Juurlink *et al.* 1998; Almazan *et al.* 2000; Fragoso *et al.* 2004); evidence suggests that oligodendrocytes may also be susceptible to peroxynitrite-mediated mitochondrial dysfunction (Scott *et al.* 2003). NO exposure during periods of electrical activity has been demonstrated to cause a block of axonal conduction (Smith *et al.* 2001), with demyelinated and recently remyelinated axons particularly susceptible (Redford *et al.* 1997). The mechanism involved in this conduction block is currently unclear. NO may however also have immunomodulatory effects, beneficial in the context of MS, such as the inhibition of antigen presentation and T cell proliferation, and the promotion of T cell apoptosis in the clearance of inflammation (Smith and Lassmann 2002).

Glutamate levels have been found to be elevated in the CSF of MS patients (Stover *et al.* 1997) and in active MS lesions (Srinivasan *et al.* 2005). Elevated extracellular glutamate may cause ionotropic glutamate receptor-mediated excitotoxicity towards oligodendrocytes and neurones; indeed, oligodendrocytes have been shown to be vulnerable to kainate-mediated toxicity *in vitro* and *in vivo* (Matute *et al.* 1997;

McDonald *et al.* 1998). In addition, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) / kainate receptor antagonists prevented neurone and oligodendrocyte death, reduced disease pathology and ameliorated clinical symptoms in EAE (Pitt *et al.* 2000; Smith *et al.* 2000). Receptor-independent oxidative glutamate toxicity may also be involved, in which elevated extracellular glutamate competitively inhibits oligodendrocyte cystine uptake via the x_c^- glutamate/cystine antiporter, limiting cystine supply for synthesis of the antioxidant glutathione, and increasing the susceptibility of the cell to oxidative stress (Rosin *et al.* 2004). As explained previously, a high metabolic rate and low glutathione content renders oligodendrocytes highly vulnerable to oxidative stress.

Oligodendrocytes contribute to the maintenance of glutamate homeostasis in the white matter, and evidence suggests that oligodendrocyte glutamate transporters may be downregulated in MS (Werner *et al.* 2001; Pitt *et al.* 2003; Domercq *et al.* 2005). In addition, mitochondrial dysfunction and energy failure, such as may occur following oxidative glutamate toxicity or peroxynitrite exposure in MS, may lead to a reversal of glutamate transporters due to a loss of the Na^+ and K^+ gradients across the plasma membrane (Nicholls and Attwell 1990), further elevating extracellular glutamate levels. Interestingly, TNFα has been shown to downregulate oligodendrocyte glutamate transporters and inhibit glutamate uptake (Pitt *et al.* 2003), and the toxicity of TNFα towards oligodendrocytes was prevented by AMPA receptor antagonists (Takahashi *et al.* 2003). These findings seem to suggest a pathway, in which TNFα-mediated downregulation of oligodendrocyte glutamate transporters elevates extracellular glutamate and causes excitotoxic cell death of oligodendrocytes through AMPA receptor stimulation, and which may be involved in the pathogenesis of MS. However, since other cytokines and chemokines and NO have also been implicated in MS pathogenesis, alongside a clear immune involvement, the pathway leading to the pathology associated with MS is likely to be much more complicated, involving oligodendrocytes, neurones, microglia and infiltrating immune cells.

1.3.1.3. Albumin as an *in vitro* model of BBB damage in MS

By its nature, the BBB excludes large molecules including blood-borne proteins from the brain. Albumin, the most abundant plasma protein, is present at a concentration of 35 – 50 $mg.ml^{-1}$ in the plasma, but at an approximately thousandfold lower concentration in the CNS under normal circumstances (Hooper *et al.* 2005). Thus, a damaged or dysfunctional BBB may expose the brain to abnormally high levels of proteins such as albumin. MS lesions usually occur in close proximity to blood vessels (Adams *et al.* 1989), and have been found to be associated with BBB dysfunction (Gay and Esiri 1991; Vos *et al.* 2005), suggesting an influential role for a blood-derived factor in MS pathogenesis. Indeed, extravasation of albumin into the CNS in chronic relapsing EAE, a model of MS, was shown to correlate with inflammation and clinical signs of disease (Butter *et al.* 1991). Albumin has been shown to cause microglial activation and proliferation *in vitro* (Si *et al.* 1997; Hooper *et al.* 2005, 2009), suggesting a role of albumin in the initiation of an inflammatory response at the site of the plaque.

Compromised BBB integrity and elevated CSF albumin levels are also associated with other acute and chronic neurodegenerative conditions including Alzheimer's disease and vascular dementia (Alafuzoff *et al.* 1983; Skoog *et al.* 1998; Fiala *et al.* 2002), ischaemic stroke (Hornig *et al.* 1983) and human immunodeficiency virus (HIV) infection of the CNS (Kim *et al.* 2003b; Kanmogne *et al.* 2007).

Partially purified, "fraction V" albumin (Cohn *et al.* 1944) contains bound fatty acids and coagulation factors, but has been shown to have very similar effects upon microglia as pure albumin (Hooper *et al.* 2005). Fraction V albumin is used to activate microglia *in vitro* in this thesis, to further investigate the effects of albumin upon microglia in the context of disease pathogenesis.

1.3.2. Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder associated with advanced age and characterised by memory loss and cognitive impairment. The characteristic histopathological features of AD are extracellular plaques of insoluble β amyloid (Aβ) (Masters *et al.* 1985) and intracellular accumulations of hyperphosphorylated tau, known as neurofibrillary tangles (NFTs) (Goedert *et al.* 1988), both of which are predominantly found in the hippocampus and cortex. These aggregates are accompanied by activated microglia and reactive astrocytes, neuronal cell death and vascular damage. Definite diagnosis of AD is only possible post-mortem, with confirmation of the presence of the plaques and tangles characteristic of the disease.

1.3.2.1. Aβ and the amyloid cascade hypothesis

The main constituent of extracellular plaques in AD, Aβ, is produced by proteolytic cleavage of β amyloid precursor protein (APP) (see fig. 1.3). APP may be cleaved by three protease complexes, termed α, β and γ secretase (Checler 1995). Cleavage of the extracellular domain of APP by α secretase occurs within the Aβ peptide region, releasing an apparently harmless fragment which cannot yield Aβ. An alternative cleavage of APP by the sequential actions of β and γ secretase produces the Aβ peptide (Checler 1995). Whilst α and β secretases usually cleave APP at a specific dipeptide bond, γ secretase cleavage does not appear to be as precise, and the resulting Aβ peptide may be 39 to 42 amino acids in length (Checler 1995). The presenilins, mutations of which have been linked with AD susceptibility (Rogaev *et al.* 1995; Sherrington *et al.* 1995; Wisniewski *et al.* 1995), have been shown to form part of the γ secretase complex, directly implicating these proteins in Aβ production (Wolfe *et al.* 1999; Kimberly *et al.* 2000). Aβ may exist as soluble monomers or aggregate into insoluble oligomers, as found in Aβ plaques (Soto 1999). It has been demonstrated *in vitro* that the peptide of 42 amino acids ($A\beta_{1\text{-}42}$) has a higher propensity to aggregate and is the predominant species found in Aβ plaques (Burdick *et al.* 1992; Jarrett *et al.* 1993).

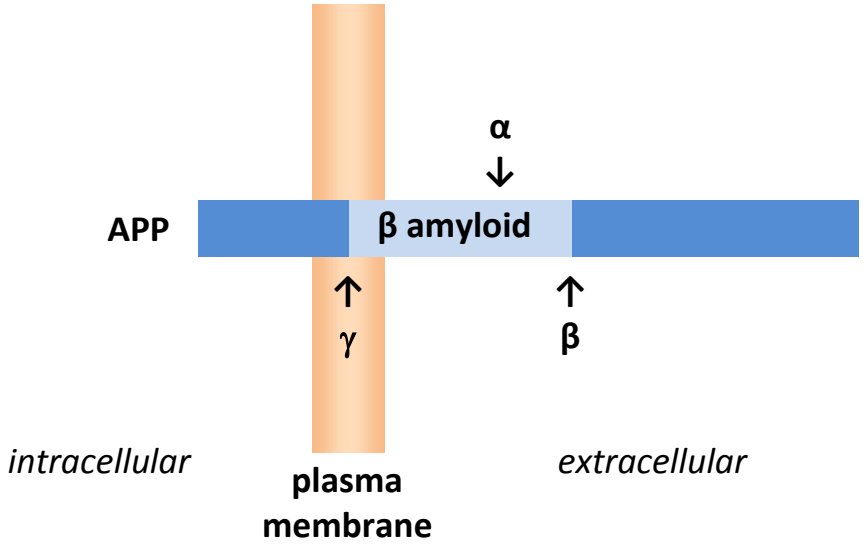


Figure 1.3. *Diagrammatic representation of the cleavage of amyloid precursor protein by secretases. Amyloid precursor protein (APP) is a transmembrane protein, which may be cleaved by α secretase or by β and γ secretase. Only β and γ secretase cleavage leads to the production of β amyloid peptide. The approximate cleavage sites of α, β and γ secretase are indicated by α, β and γ, respectively.*

The amyloid cascade hypothesis is probably the most well-known and influential theory regarding AD pathogenesis. This hypothesis states that Aβ produced by abnormal APP processing is the causative agent in AD (Hardy and Higgins 1992; Hardy and Selkoe 2002). The formation of Aβ plaques is said to precede the appearance of NFTs, neuronal death, vascular damage and cognitive impairment (Hardy and Higgins 1992). Indeed, familial AD is associated with mutations affecting the processing of APP and favouring the production of $A\beta_{1-42}$ (Chartier-Harlin *et al.* 1991; Goate *et al.* 1991; Murrell *et al.* 1991; Hendriks *et al.* 1992; Mullan *et al.* 1992; Scheuner *et al.* 1996).

The neurotoxicity of aggregated Aβ has been demonstrated *in vitro* (Yankner *et al.* 1990; Rapoport *et al.* 2002), and may be due to the loss of antioxidant and neuroprotective properties of monomeric Aβ (Zou *et al.* 2002), or the binding of transition metal ions and catalysis of oxygen radical generation (Huang *et al.* 1999; Zou *et al.* 2002). The amyloid cascade hypothesis of AD pathogenesis is however not universally accepted; it has been hypothesised that Aβ plaques may be a consequence of the disease process rather than the primary aetiological factor (Smith *et al.* 2002; Lee *et al.* 2004a). This is supported by reports of Aβ plaques in apparently cognitively normal individuals (Vehmas *et al.* 2003). Thus, the sequestering of redox-active metal ions by Aβ has been suggested to be a neuroprotective measure (Lovell *et al.* 1998a; Dong *et al.* 2003). In addition, observations suggest that neurones containing NFTs may be protected from immediate cell death (Morsch *et al.* 1999).

1.3.2.2. Oxidative stress in AD

Whether or not plaques and tangles cause the cognitive decline associated with AD, oxidative stress undoubtedly plays an important role in its pathogenesis. Oxidative stress increases with advancing age, and all known risk factors for AD enhance the production of ROS and/or compromise the antioxidant capacity of the CNS (Nunomura *et al.* 2006). The inhibition of ROS production and the promotion of antioxidant capabilities have been found to decrease the incidence of AD (Nunomura *et al.* 2006).

The protein apolipoprotein E (ApoE) constitutes a genetic polymorphism which has been identified as a risk factor for late-onset AD (Saunders *et al.* 1993). ApoE is important in lipoprotein transport and metabolism (Mahley 1988), and of the cells of the CNS, ApoE is released at appreciable levels by astrocytes and microglia (Pitas *et al.* 1987; Saura *et al.* 2003; Mori *et al.* 2004; Qin *et al.* 2006). There are three ApoE alleles, ApoE2, ApoE3 and ApoE4 (Mahley 1988), and the expression of one or two ApoE4 alleles is associated with increased risk of late-onset AD (Alzheimer's Disease Collaborative Group 1993; Noguchi *et al.* 1993; Payami *et al.* 1993; in 't Veld *et al.* 2001). The difference between the three alleles relates to the presence of cysteine or arginine at two positions in the primary structure of the protein. ApoE has been shown to possess allele-specific antioxidant activity, the level of which is inversely correlated with AD risk (Miyata and Smith 1996). Thus, ApoE2 has the highest level of antioxidant activity and ApoE4, the AD-risk allele, the lowest. The differing antioxidant protection offered by the ApoE alleles may be due to the reducing power of the sulfhydryl group of the cysteine residues, as ApoE2 contains two cysteines and ApoE4 two arginines (Ramassamy *et al.* 1999). Alternatively, different metal ion-binding abilities may be implicated (Ramassamy *et al.* 1999).

1.3.2.3. Microglia in AD

Microglia and reactive astrocytes are found in the vicinity of Aβ plaques in AD; moreover, microglial migration and activation occurs early in the disease process (Cagnin *et al.* 2001; Vehmas *et al.* 2003). Inflammation has therefore been proposed to play a part in the pathogenesis of AD (Blasko *et al.* 2004; Griffin 2006). Indeed, the process of ageing is itself associated with a shift in innate immunity towards a more pro-inflammatory state (Blasko *et al.* 2004). Genetic polymorphisms of the inflammatory cytokines TNFα and IL-1, both of which may be produced by microglia, have been shown to be risk factors for the development of AD (Grimaldi *et al.* 2000; Nicoll *et al.* 2000; Perry *et al.* 2001). However, immunoglobulins and T cells are not detectable in AD brain, and adhesion molecules on the endothelial cells of the BBB which allow leukocyte migration are not upregulated in AD, suggesting a lack of involvement of the peripheral immune system (Eikelenboom *et al.* 2002). The extent of the inflammatory response in AD therefore appears to be much more localised compared with that in MS, for example.

Aβ has been shown to be chemotactic for microglia *in vitro* (Davis *et al.* 1992; Yan *et al.* 1996; Nakai *et al.* 1998; Le *et al.* 2001), and may stimulate microglial proliferation (Goodwin *et al.* 1995). Microglia may be activated by Aβ (McDonald *et al.* 1997; Combs *et al.* 2001; Le *et al.* 2001) and by the secretory protein chromogranin A (CGA), which is upregulated in Aβ plaques (Taupenot *et al.* 1996; Kingham *et al.* 1999). Activated microglia localised to plaques in AD brain may have both beneficial and deleterious effects.

Some studies have implicated microglia in the production of Aβ at the site of senile plaques. Aβ has been shown to be present in endoplasmic reticulum in microglia associated with plaques, a location associated with protein synthesis and secretion rather than phagocytosis (Frackowiak *et al.* 1992). In addition, exposure to $A\beta_{25-35}$ or proinflammatory stimuli enhanced Aβ release by microglia of the BV-2 cell line (Bitting *et al.* 1996). Activated microglia in rat brain following nerve injury showed enhanced APP synthesis (Banati *et al.* 1993), which the authors interpreted as providing increased substrate for Aβ production and thereby contributing to plaque formation. However the function of uncleaved APP is unknown, although recent proposals include a role in the regulation of synaptic function (Hoe et al., 2009), or activity as a damage-response protein (Hardy 2009). This latter hypothesis suggests that upregulated APP synthesis may be a protective measure rather than an amyloidogenic process.

Microglia may also have detrimental effects through their inflammatory function. Indeed, *in vitro* studies have demonstrated that microglia exposed to Aβ peptides are neurotoxic (Meda *et al.* 1995; Combs *et al.* 1999, 2001; Qin *et al.* 2006). Microglia stimulated with $A\beta_{25-35}$ or $A\beta_{1-40}$ upregulate TNFα (Meda *et al.* 1995; Yan *et al.* 1996; Yates *et al.* 2000; Combs *et al.* 2001; Lue *et al.* 2001). TNFα induces microglial glutamate release (Piani and Fontana 1994; Takeuchi *et al.* 2006) and subsequent neurotoxicity (Takeuchi *et al.* 2006). Exposure of microglia to Aβ peptides also causes NO release, which appears to be downstream of TNFα upregulation and may be dependent upon the priming of microglia by the lymphocyte-derived cytokine IFNγ (Goodwin *et al.* 1995; Meda *et al.* 1995; Hellendall and Ting 1997; Combs *et al.* 2001). Notably, microglia isolated from AD

brain released higher levels of nitrate, a stable metabolite of NO, than did those from control aged brain (Lue *et al.* 2001).

Aβ peptides can also cause the production of superoxide by the respiratory burst in microglia (McDonald *et al.* 1997; Combs *et al.* 1999) and macrophages (Klegeris and McGeer 1997). If simultaneously produced, NO and superoxide can combine to form the highly reactive anion peroxynitrite which causes oxidative damage to proteins, lipids and nucleic acids. Accordingly, hippocampal neurones from AD brain show enhanced nitration of tyrosine residues within cellular proteins, indicative of peroxynitrite-mediated damage (Good *et al.* 1996; Smith *et al.* 1997).

Microglia stimulated with Aβ peptides may also release increased levels of the proinflammatory cytokine IL-1 (Walker *et al.* 1995; Yates *et al.* 2000; Lue *et al.* 2001). IL-1 has been shown to increase expression of APP (Fogal and Hewett 2008) and has been associated with the formation of both Aβ plaques and NFTs in AD (Griffin 2006). IL-1 released by microglia also enhances astrocyte activation and proliferation (Giulian *et al.* 1986), and, in combination with IFNγ may lead to microglial iNOS expression and NO release (Ding *et al.* 1997).

Aggregated Aβ has also been shown to stimulate microglial release of the chemokines IL-8, MCP-1 (CCL2) and MIP-1α (CCL3) and the macrophage/microglial growth factor macrophage colony-stimulating factor (M-CSF) (Lue *et al.* 2001). This suggests that microglia at the site of the plaque amplify the inflammatory response by recruiting further microglia and macrophages and stimulating their proliferation. Microglia isolated from AD brain also release higher levels of M-CSF and the complement pathway component C1a than those from control aged brain (Lue *et al.* 2001).

Aβ peptides have been shown to induce microglial glutamate release by increased activity of the x_c^- cystine/glutamate antiporter system (Qin *et al.* 2006), and by reversal of the excitatory amino acid transporters normally responsible for high-affinity glutamate uptake (Noda *et al.* 1999). Interestingly, secreted APP, the result of APP cleavage by α or β secretase alone, has also been shown to cause microglial

neurotoxicity through upregulation of the x_c^- transporter system and subsequent enhanced glutamate release (Barger and Basile 2001).

Chromogranin A (CGA), which is also upregulated in Aβ plaques, causes microglial and neuronal apoptosis *in vitro* (Kingham *et al.* 1999; Hooper and Pocock 2007). In microglia, CGA has been shown to induce iNOS expression and NO release (Taupenot *et al.* 1996; Kingham *et al.* 1999; Kingham and Pocock 2000), glutamate release (Kingham *et al.* 1999), and cathepsin B release (Kingham and Pocock 2001), all of which contributed to neurotoxicity. However, whilst the neurotoxicity of CGA appears to be entirely microglia-dependent (Kingham *et al.* 1999; Taylor *et al.* 2002), Aβ is directly neurotoxic in addition to its effects upon microglia (Taylor *et al.* 2002, 2003).

Further evidence for a role of inflammation in AD pathogenesis comes from epidemiological studies, which have demonstrated that chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a decreased risk of developing AD (McGeer *et al.* 1996; Stewart *et al.* 1997; in 't Veld *et al.* 2001). Although a history of chronic NSAID use did not appear to affect the abundance of senile plaques in non-demented elderly individuals, it was associated with fewer activated microglia at the site of plaques, suggesting that NSAIDs may decrease AD risk by reducing microglial activation (Mackenzie and Munoz 1998). Accordingly, ibuprofen was found to reduce the numbers of activated microglia associated with Aβ plaques and the levels of IL-1β in the brain in a transgenic mouse model of AD (Lim *et al.* 2000). A reduction in plaque numbers in ibuprofen-treated animals was also observed (Lim *et al.* 2000). Ibuprofen and certain other NSAIDs have been shown to decrease Aβ production by cytokine-stimulated neurones *in vitro* (Blasko *et al.* 2001; Sastre *et al.* 2003), and to decrease $A\beta_{1\text{-}42}$ levels in a mouse model of AD, perhaps due to effects upon γ secretase activity (Weggen *et al.* 2001; Eriksen *et al.* 2003). Evidence exists to suggest that this may be mediated both by COX inhibition (Qin *et al.* 2003), and independently of it (Weggen *et al.* 2001; Eriksen *et al.* 2003). It therefore appears that NSAIDs may decrease the production of the amyloidogenic forms of Aβ as well as reducing microglial activation, to lower the risk of developing AD.

On the other hand, microglia may protect against disease progression and cognitive decline through the phagocytosis of Aβ (Shaffer *et al.* 1995; Paresce *et al.* 1996), which has been recently shown *in vivo* to be dependent upon microglial TLR2 expression (Richard *et al.* 2008). In AD brain, microglia have been shown to be immunoreactive for tau protein, suggesting that they may also phagocytose degenerating NFT-containing neurites (Vehmas *et al.* 2003). *In vitro* studies have suggested that microglia are the predominant producers of ApoE (Saura *et al.* 2003; Mori *et al.* 2004), which appears to have antioxidant properties, and is neuroprotective (Qin *et al.* 2006). A recent study demonstrated that the ability of macrophages to degrade Aβ *in vitro* varied according to the ApoE allele expressed. Thus, macrophages expressing ApoE4, a known risk factor for AD development, were less efficient than those expressing ApoE2 or ApoE3 at clearing Aβ from brain slices derived from APP transgenic mice (Zhao *et al.* 2009). MMP-2 and 9, both of which may be released by microglia, have also been shown *in vitro* to possess Aβ-degrading properties (Roher *et al.* 1994; Backstrom *et al.* 1996).

TGFβ is produced by microglia and upregulated under inflammatory conditions (Constam *et al.* 1992; da Cunha *et al.* 1993; Peress and Perillo 1995). Elevated TGFβ levels have been detected in the serum and CSF of AD patients (Chao *et al.* 1994), and Aβ plaques and NFTs show TGFβ immunoreactivity (van der Wal *et al.* 1993; Peress and Perillo 1995). TGFβ has been identified as a microglial chemoattractant (Yao *et al.* 1990), and is generally recognised as having neuroprotective and neurotrophic properties (Finch *et al.* 1993; Flanders *et al.* 1998). TGFβ has been demonstrated to be associated with a lower Aβ plaque load, but increased vascular amyloid in aged transgenic mice expressing human APP and in AD (Wyss-Coray *et al.* 2001). TGFβ appeared to enhance microglial activation in transgenic mice and to enhance Aβ clearance by microglia *in vitro* (Wyss-Coray *et al.* 2001). However, TGFβ has also been demonstrated to enhance Aβ production and deposition in animal models (Frautschy *et al.* 1996; Wyss-Coray *et al.* 1997; Lesne *et al.* 2003), suggesting that the actions of this cytokine may be rather more complicated.

Because Aβ peptides and CGA have been shown to cause microglial activation, and both are associated with senile plaques in AD, the effects of both these peptides upon aspects of microglial function are investigated in this thesis. $A\beta_{25-35}$ has been shown

to replicate the effects of $A\beta_{1-42}$ *in vitro* (Yankner *et al.* 1990), and is used to activate microglia here.

1.3.2.4. Treatment of AD

Current treatment of AD consists largely of acetylcholinesterase inhibitors, to improve cognitive function, alongside drugs for the management of mood disorders, agitation and psychosis associated with the disease (Citron 2002). However such drugs purely treat the symptoms of AD rather than modifying the disease course and whilst they may slow disease progression, there is currently no known preventative or cure for AD. As described above, long-term use of certain NSAIDs may reduce the risk of the development of AD, through reduction of microglial activation and amyloidogenic Aβ production. Interestingly, certain statins, often prescribed to reduce blood cholesterol levels in patients at risk of cardiovascular disease, also appear to reduce AD risk (Wolozin *et al.* 2007).

AD treatment strategies currently in development include those inhibiting the production of Aβ (Petit *et al.* 2001; Lanz *et al.* 2003; Silvestri 2009), and preventing abnormal protein-protein interactions which may be involved in plaque formation (McLaurin *et al.* 2006; Wright 2006). In addition, either active or passive vaccination against Aβ may ameliorate disease, although the mechanism by which this occurs is unknown (Schenk *et al.* 1999; Bard *et al.* 2000; DeMattos *et al.* 2001; Melnikova 2007).

1.4. Glutamate

The amino acid L-glutamate is the most abundant excitatory neurotransmitter in the mammalian CNS. Released at synapses to mediate neurotransmission, its effects are mediated through ionotropic and metabotropic glutamate receptors. Glutamate transporters expressed by neurones and glia remove glutamate from the extracellular environment, thus controlling extracellular glutamate levels and the activation of glutamate receptors.

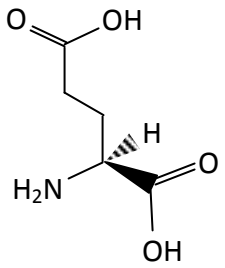


Figure 1.4. *The structure of L-glutamate*

Extracellular glutamate concentrations have generally been reported to be between 0.5 and 5 µM in the rat brain (Benveniste *et al.* 1984; Lerma *et al.* 1986; Phillis *et al.* 1994; Wahl *et al.* 1994; Miele *et al.* 1996; Del Arco *et al.* 1999; Baker *et al.* 2002; Montiel *et al.* 2005), as measured by microdialysis and analysis of artificial CSF superfusate. However, more recent studies have demonstrated extracellular glutamate concentrations in the range 25 – 90 nM in rat hippocampal slices by analysis of basal N-methyl-D-aspartate (NMDA) currents (Cavelier and Attwell 2005; Herman and Jahr 2007; Le Meur *et al.* 2007). This is closer to the theoretical minimum glutamate concentration of ~2 nM calculated based upon the analysis of K^+, Na^+ and H^+ ion fluxes and their coupling to glutamate transport (Zerangue and Kavanaugh 1996).

Since the studies of John Olney in the 1970s (see Olney 1982; Rothman and Olney 1986), evidence for the involvement of glutamate toxicity in neurological conditions has been rapidly accumulating. Elevated extracellular glutamate levels have been

measured directly in animal models following ischaemia (Benveniste *et al.* 1984; Drejer *et al.* 1985; Hagberg *et al.* 1985; Lekieffre *et al.* 1992; O'Regan *et al.* 1997; Ritz *et al.* 2004; Homola *et al.* 2006) and traumatic brain injury (Faden *et al.* 1989; Liu *et al.* 1991). CSF glutamate levels significantly above control have been detected in patients suffering from ALS, viral meningitis, acute MS and myelopathy (Rothstein *et al.* 1990; Stover *et al.* 1997). Glutamate toxicity is implicated to some extent in the vast majority of acute and chronic neurological conditions, including stroke, epilepsy, AD, Parkinson's disease, MS and acquired immune deficiency syndrome (AIDS) dementia (Lipton and Rosenberg 1994; Doble 1999; Pitt *et al.* 2000).

1.4.1. Glutamate receptors

Glutamate receptors fall into two classes, the ionotropic glutamate receptors, which are ligand-gated cation channels, and the metabotropic glutamate receptors, G protein-coupled receptors with heptahelical transmembrane domains. Figure 1.5 summarises the glutamate receptor subtypes.

1.4.1.1. Ionotropic glutamate receptors

Ionotropic glutamate receptors (iGluRs) are usually found post-synaptically, where they mediate fast synaptic transmission by initiating an action potential in the post-synaptic neurone upon synaptic glutamate release. iGluRs are separated into three subtypes according to the selective ligands originally described: NMDA receptors, AMPA receptors and kainate receptors.

In the presence of glutamate, iGluRs mediate entry of Na^+, K^+ and/or Ca^{2+} into the cell. All iGluRs form functional tetramers (Wu *et al.* 1996; Mano and Teichberg 1998; Rosenmund *et al.* 1998), with NMDA receptors consisting of a combination of NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B subunits, and AMPA and kainate receptors consisting of GluR1-4 subunits and GluR5-7 and KA1 and KA2 subunits, respectively (see fig. 1.5) (Dingledine *et al.* 1999; Kew and Kemp 2005). The exact combination of subunits confers specific properties upon iGluRs, such as the Ca^{2+} permeability (Burnashev *et al.* 1995; Matsuda *et al.* 2002), channel

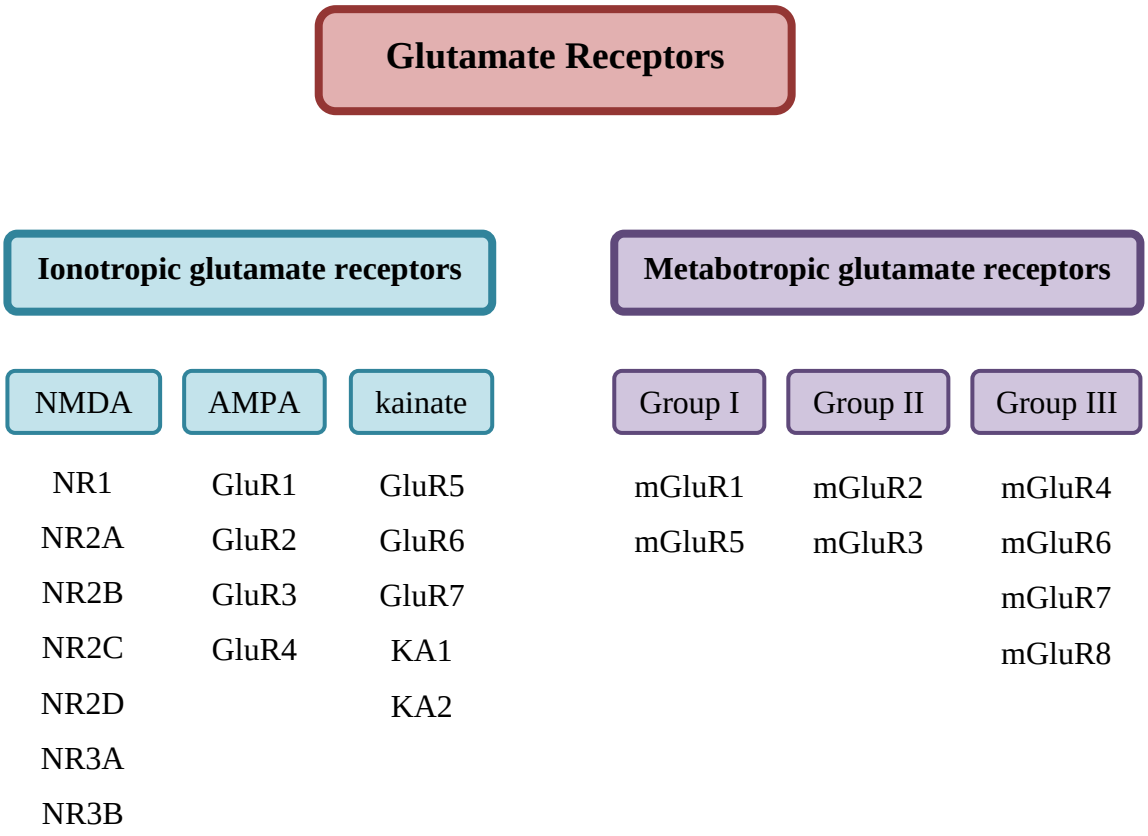


Figure 1.5. *Glutamate receptor subtypes. The ionotropic glutamate receptors, classified according to the selective ligands originally described, are ligand-gated ion channels. The metabotropic glutamate receptors are G protein-coupled receptors and are divided into three groups according to their specific properties.*

conductance (Ciabarra *et al.* 1995; Howe 1996; Swanson *et al.* 1996) desensitisation kinetics (Herb *et al.* 1992) and pharmacology (Partin *et al.* 1993; Ishii *et al.* 1993; Alt *et al.* 2004).

The activation of postsynaptic iGluRs is crucial for long-term potentiation (LTP) and long-term depression (LTD), two forms of synaptic plasticity important in learning and memory. LTP is defined as an activity-dependent increase in synaptic strength (Bliss and Lomo 1973), while LTD is an activity-dependent decrease in synaptic strength (Lynch *et al.* 1977). However, prolonged activation of iGluRs, as may occur in pathological situations where extracellular glutamate levels are elevated, causes an NMDA receptor-dependent phenomenon known as excitotoxicity (Choi *et al.* 1988; Frandsen *et al.* 1989). The high levels of Ca^{2+} influx caused by continuous NMDA receptor stimulation lead to the inappropriate continual activation of cellular processes regulated by Ca^{2+}, including the activity of enzymes such as phospholipases, endonucleases and proteases, which can damage cell structures (Choi 1988; Tymianski and Tator 1996). Excess intracellular Ca^{2+} may also lead to the opening of the mitochondrial permeability transition pore (Schinder *et al.* 1996). Effects of the ensuing loss of the mitochondrial membrane potential include mitochondrial swelling (Reed and Savage 1995; Zamzami *et al.* 1996), release of reactive oxygen species (Zamzami *et al.* 1995; Schinder *et al.* 1996) and apoptosis-inducing factor (Susin *et al.* 1996), and the uncoupling of ATP synthesis (Vayssiere *et al.* 1994; Reed and Savage 1995; Schinder *et al.* 1996). Excitotoxicity ultimately leads to cell death, and is implicated in the pathogenesis of a number of neurological conditions.

Primary cultured microglia and microglial cell lines have been shown to express AMPA (Noda *et al.* 2000; Mayer *et al.* 2001; Hagino *et al.* 2004; Christensen *et al.* 2006; Liu *et al.* 2006), kainate (Noda *et al.* 2000; Yamada *et al.* 2006) and NMDA (Liu *et al.* 2006) receptor subunits. Agonists of iGluRs have been shown to cause electrophysiological responses (Noda *et al.* 2000; Hagino *et al.* 2004; Liu *et al.* 2006), immediate early gene expression (Eun *et al.* 2004), TNFα release (Noda *et al.* 2000; Mayer *et al.* 2001; Hagino *et al.* 2004), ATP release (Liu *et al.* 2006) and cytoskeletal remodelling (Christensen *et al.* 2006) in microglia *in vitro*.

Microglia have also been shown to express AMPA and NMDA receptor subunits *in vivo* during rat CNS development (Ong *et al.* 1996; Kaur *et al.* 2005), in the rat following transient forebrain ischaemia (Gottlieb and Matute 1997), and within active demyelinating plaques in human MS tissue (Newcombe *et al.* 2008). Co-localisation of kainate receptor subunits with the microglial marker OX-42 has been demonstrated in the rat hippocampus following ischaemia (Yamada *et al.* 2006).

1.4.1.2. Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are G protein-coupled glutamate receptors (GPCRs) which are found both pre- and post-synaptically, as well as on glial cells. G proteins are so-called because of their interaction with the guanine nucleotides guanine triphosphate (GTP) and guanine diphosphate (GDP). Ligation of a GPCR by an agonist causes displacement of the G protein-associated GDP by GTP, thus activating the G protein and allowing it to interact with target effector proteins. Intrinsic GTPase activity of the G protein hydrolyses the GTP to GDP to terminate the signal transduction activity. Because of the slower kinetics of metabotropic receptors, these are believed to have more of a modulatory role upon synaptic transmission.

To data, eight mGluR subtypes have been identified (Houamed *et al.* 1991; Masu *et al.* 1991; Abe *et al.* 1992; Tanabe *et al.* 1992; Nakajima *et al.* 1993; Okamoto *et al.* 1994; Saugstad *et al.* 1994; Duvoisin *et al.* 1995). These are divided into three groups on the basis of their sequence homology, preferred G protein coupling and pharmacology. Group I consists of mGluRs 1 and 5; group II, mGluRs 2 and 3; and group III, mGluRs 4, 6, 7 and 8 (fig. 1.5).

1.4.1.2.1. Structure

mGluRs are the largest sub-group of the class C GPCR family, which also includes the Ca^{2+}-sensing receptor (CaR) (Brown *et al.* 1993), the $GABA_B$ receptor (Kaupmann *et al.* 1997), pheromone (Herrada and Dulac 1997; Matsunami and Buck 1997) and taste receptors (Hoon *et al.* 1999). Family C GPCRs are considerably larger than other known GPCRs.

The eight known mGluRs all have a similar structure, illustrated in figure 1.6, and exist as functional homodimers (Kunishima *et al.* 2000). Although mGluRs have heptahelical transmembrane domains typical of GPCRs (Bhave *et al.* 2003), there is little homology between mGluR heptahelical domains and those of other GPCRs outside of class C. The glutamate binding site of mGluRs is contained within a large bi-lobed N-terminal domain (O'Hara *et al.* 1993; Okamoto *et al.* 1998; Han and Hampson 1999; Kunishima *et al.* 2000; Peltekova *et al.* 2000; Malherbe *et al.* 2001; Rosemond *et al.* 2002; Sato *et al.* 2003), and agonist binding is thought to trigger signal transduction by causing a conformational change and altering interactions between the receptors forming the dimer (Kunishima *et al.* 2000; Tsuchiya *et al.* 2002; Sato *et al.* 2003). The C termini of mGluRs vary widely in length and amino acid sequence, and are thought to mediate interactions with other proteins. The second and third intracellular loops and the intracellular C terminus have all been implicated in G protein coupling (Pin *et al.* 1994; Gomeza *et al.* 1996; Francesconi and Duvoisin 1998; Chang *et al.* 2000).

1.4.1.2.2. Localisation

All mGluRs are found in the CNS. mGluR6 was thought to be exclusively retinal (Nakajima *et al.* 1993), although its expression has since been reported in astrocytes and microglia *in vitro* (Faden *et al.* 1997; Wroblewska *et al.* 1998; Taylor *et al.* 2003; Yao *et al.* 2005; Pinteaux-Jones 2007), and *in vivo* in certain areas of adult rat brain (Faden *et al.* 1997; Hoang and Hay 2001).

Neuronal group I mGluR expression is predominantly postsynaptic (Martin *et al.* 1992), generally being localised to the periphery of the post-synaptic density (Baude *et al.* 1993; Nusser *et al.* 1994; Vidnyanszky *et al.* 1994, 1996; Lujan *et al.* 1996, 1997). Group II mGluRs are implicated in inhibition of neurotransmission (Hayashi *et al.* 1993; Macek *et al.* 1996; Kew *et al.* 2002; Mateo and Porter 2007), and tend to be located extrasynaptically (Ohishi *et al.* 1994; Petralia *et al.* 1996; Yokoi *et al.* 1996; Lujan *et al.* 1997; Shigemoto *et al.* 1997), suggesting their involvement in heterosynaptic modulation (Mitchell and Silver 2000; Chen and Bonham 2005). Group III mGluRs are predominantly located at the presynaptic active zone

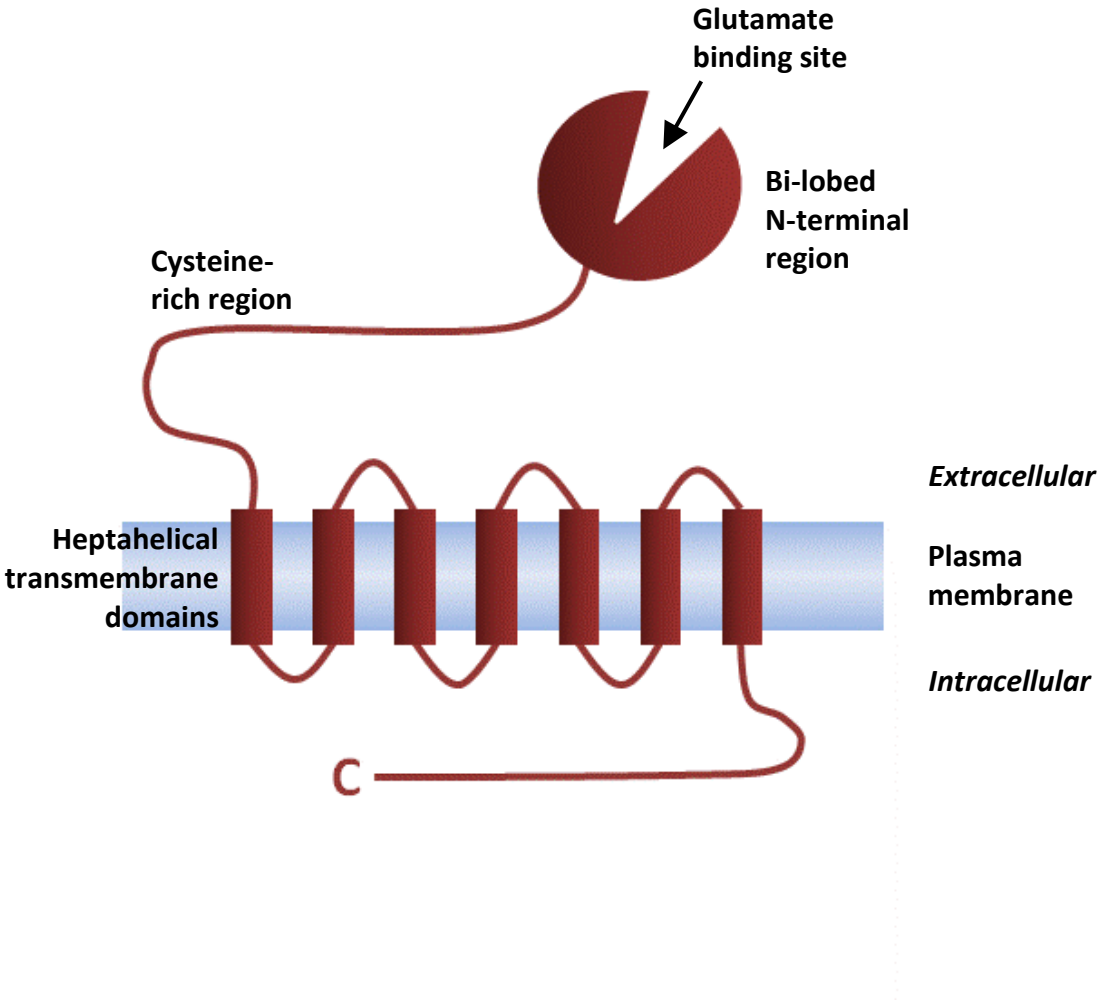


Figure 1.6. *Diagrammatic representation of the structure of mGluRs. The glutamate binding site resides within the large bi-lobed N-terminal region, which is linked to the heptahelical transmembrane domains typical of G protein-coupled receptors by a cysteine-rich region. The intracellular C-terminal domain varies in length and amino acid sequence between mGluR subtypes. Adapted from Kew and Kemp (2005).*

(Shigemoto *et al.* 1996, 1997), where they act as inhibitory autoreceptors (Trombley and Westbrook 1992; Tanabe *et al.* 1993; Jane *et al.* 1994; Takahashi *et al.* 1996).

Glial cells have also been shown to express mGluRs. Astrocytes express mGluR3 and mGluR5 (Tanabe *et al.* 1993; Petralia *et al.* 1996; Biber *et al.* 1999; Janssens and Lesage 2001; Aronica *et al.* 2003, 2005; Geurts *et al.* 2003, 2005), and possibly other subtypes at lower levels (Biber *et al.* 1999; Janssens and Lesage 2001; Yao *et al.* 2005). Oligodendrocytes and their precursors may express mGluR1, mGluR3 and mGluR5 (Luyt *et al.* 2003, 2006; Deng *et al.* 2004).

Microglia in culture have been shown to express mGluRs of all three groups. Of the group I mGluRs, the expression of mGluR5 appears to be more prominent (Biber *et al.* 1999; Byrnes *et al.* 2009), although mGluR1 mRNA and protein expression have also been demonstrated (Pinteaux-Jones 2007; Byrnes *et al.* 2009). Microglia have also been shown to express mRNA and protein for all group II and III mGluRs except mGluR7 (Taylor *et al.* 2002, 2003; Pinteaux-Jones 2007).

1.4.1.2.3. Signalling

Group I mGluRs preferentially couple to phosphoinositide hydrolysis by phospholipase C through the $G_{q/11}$ type of G protein (Houamed *et al.* 1991; Abe *et al.* 1992; Daggett *et al.* 1995; Joly *et al.* 1995), whilst mGluRs of groups II and III are negatively coupled to adenylate cyclase and cAMP via the G proteins $G_{i/o}$ (Tanabe *et al.* 1992, 1993; Nakajima *et al.* 1993; Okamoto *et al.* 1994; Saugstad *et al.* 1994; Duvoisin *et al.* 1995). These preferred signalling pathways are illustrated in figure 1.7.

It has become clear that in reality mGluR signalling is more complex and heterogeneous. Coupling of mGluRs to individual signalling pathways may depend upon the expression and co-localisation of the relevant proteins within the cell. mGluRs can couple to G proteins other than their "preferred" type (Aramori and Nakanishi 1992; Joly *et al.* 1995; Sortino *et al.* 1996; McCool *et al.* 1998; Francesconi and Duvoisin 2000; Thandi *et al.* 2002), and may also mediate signalling independent of G proteins (Heuss *et al.* 1999; Benquet *et al.* 1999; Gee

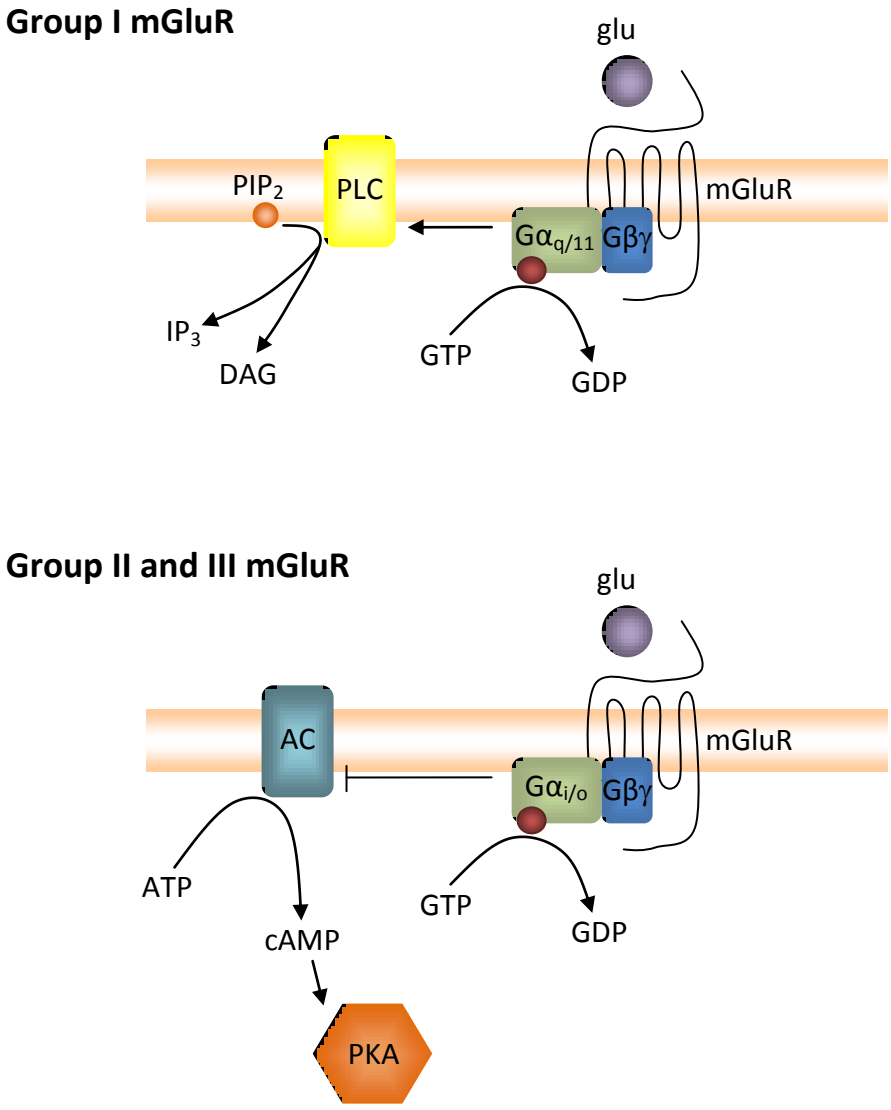


Figure 1.7. *The preferred signalling pathways of group I (top) and group II and III (bottom) mGluRs. Upon binding of glutamate, mGluRs associate with a G protein, and cause binding of GTP to the GTP/GDP binding site. This activates the G protein, allowing it to dissociate into Gα and Gβγ subunits, and interact with target enzymes. Intrinsic GTPase activity of the Gα subunit hydrolyses GTP to GDP + P_i, which causes deactivation and reassociation of the G protein. Group I mGluRs preferentially signal through $G_{q/11}$, activating PLC, which causes conversion of PIP_2 to the second messengers IP_3 and DAG. Group II and III mGluRs preferentially signal through $G_{i/o}$, which inhibits the activity of AC and therefore decreases cAMP production and PKA activation. AC, adenylate cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; Gα, G protein α subunit; Gβγ, G protein βγ subunit; glu, glutamate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; IP3, inositol triphosphate; mGluR, metabotropic glutamate receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PLC, phospholipase C.*

and Lacaille 2004). Certain sequences within the C terminal region of mGluRs have been shown to mediate interactions with other proteins, such as scaffolding and cytoskeletal proteins, signalling proteins, and other cell surface receptors (for review see Enz 2007). Such interactions are likely to be important in targeting the receptor to the correct region of the plasma membrane, anchoring the protein to the cytoskeleton, and determining interactions with other signalling proteins.

Stimulation of microglial mGluR5 has been demonstrated to lead to increases in cAMP levels (Byrnes *et al.* 2009), suggesting positive coupling to adenylate cyclase via a G_s G protein, and also to increases in phosphatidylinositol hydrolysis (Byrnes *et al.* 2009) and elevated intracellular Ca^{2+} (Biber *et al.* 1999), suggesting coupling via a $G_{q/11}$ G protein. The latter pathway appears to mediate mGluR5-dependent inhibition of LPS-induced microglial activation (Byrnes *et al.* 2009). Agonists of group II mGluRs decreased forskolin-stimulated cAMP production in microglia, suggesting negative coupling to adenylate cyclase via a $G_{i/o}$ G protein (Taylor *et al.* 2002). Downstream effects include increased staining with ED1 antibody, suggesting microglial activation (Taylor *et al.* 2002), NFκB activation (Kaushal and Schlichter 2008), release of TNFα (Taylor *et al.* 2005; Kaushal and Schlichter 2008) and FasL (Taylor *et al.* 2005), and *in vivo*, increased BDNF mRNA expression (Venero *et al.* 2002), suggesting microglial release of this factor. Stimulation of group III mGluRs on microglia also decreased forskolin-stimulated cAMP production (Taylor *et al.* 2003), suggesting that this receptor is also coupled via $G_{i/o}$. Increased microglial ED1 staining compared with control was observed, demonstrating a degree of microglial activation (Taylor *et al.* 2003).

1.4.1.2.4. Group I mGluRs

mGluR5 is co-localised with the NMDA receptor post-synaptically (Alagarsamy *et al.* 2002), through protein-protein interactions at the post-synaptic density (PSD) (Naisbitt *et al.* 1999; Tu *et al.* 1999). Stimulation of mGluR5 induces excitatory currents and potentiates NMDA receptor currents and associated neuronal responses *in vitro* and *in vivo* (Pisani *et al.* 1997; Ugolini *et al.* 1999; Awad *et al.* 2000; Salt and Binns 2000), and evidence suggests an involvement in LTP (Riedel *et al.* 1995; Manahan-Vaughan *et al.* 1996; Lu *et al.* 1997; Naie and Manahan-Vaughan 2004;

Neyman and Manahan-Vaughan 2008). Group I mGluR and mGluR5-specific antagonists have been shown to be neuroprotective against a range of insults *in vitro* and *in vivo* (Buisson and Choi 1995; Henrich-Noack *et al.* 1998; Bruno *et al.* 1999, 2000b; Battaglia *et al.* 2001, 2002), likely due to a downregulation of NMDA receptor activity and therefore a reduced likelihood of excitotoxicity.

Activation of group I mGluRs has been shown to increase APP metabolism to a soluble nonamyloidogenic fragment (Lee *et al.* 1995), and mGluR5 stimulation has been demonstrated to be neuroprotective against Aβ toxicity in neuronal cultures and a neuronal cell line (Pizzi *et al.* 2005). Evidence exists for an increase in cortical neuronal mGluR5 expression in AD and aged Down's syndrome patients (Oka and Takashima 1999), which could be considered to be a protective measure to counteract Aβ toxicity. However, another group reported a decrease in cortical mGluR expression in AD, and a decrease in group I mGluR expression in Lewy body disease which correlated with AD-like changes (Albasanz *et al.* 2005), perhaps implicating decreased mGluR expression in AD pathogenesis.

Group I mGluR expression is upregulated in astrocytes associated with MS lesions (Geurts *et al.* 2003; Newcombe *et al.* 2008). Activated microglia have been shown to cause a downregulation of astrocyte mGluR5, which may be due to microglia-derived TNFα and/or IL-1β (Kiefer *et al.* 1994; Lehrmann *et al.* 1998; Aronica *et al.* 2005; Tilleux *et al.* 2007). Activation of astrocyte group I mGluRs has been shown both to upregulate (Vermeiren *et al.* 2005, 2006) and to downregulate (Gegelashvili *et al.* 2000; Aronica *et al.* 2003) their glutamate transporter expression and activity.

Microglial mGluR5, found to be upregulated in activated microglia associated with MS lesions (Geurts *et al.* 2003), may be neuroprotective. Stimulation of mGluR5 inhibited a number of aspects of LPS-induced microglial activation *in vitro*, including ROS and NO production, microglial proliferation, TNFα release, expression of the phagocytosis-related protein galectin-3 and neurotoxicity (Byrnes *et al.* 2009).

1.4.1.2.5. Group II mGluRs: neurotoxicity of mGluR2 and neuroprotection by mGluR3

Group II mGluR agonists are protective against a range of neurotoxic paradigms in mixed cultures and *in vivo* (Bruno *et al.* 1994, 1995, 1997, 1998b; Buisson and Choi 1995; Buisson *et al.* 1996; Copani *et al.* 1995; Henrich-Noack *et al.* 1998; Matarredona *et al.* 2001; Battaglia *et al.* 2003; Yao *et al.* 2005; Zhou *et al.* 2006). The presence of astrocytes was shown to be necessary for this neuroprotection *in vitro* (Copani *et al.* 1995; Bruno *et al.* 1998b; Yao *et al.* 2005; Zhou *et al.* 2006), and neuroprotection was found to be due to mGluR3 stimulation (Bruno *et al.* 1998b; Thomas *et al.* 2001, 2003; Berent-Spillson *et al.* 2004; Corti *et al.* 2007). Release of TGFβ (Bruno *et al.* 1998a; Corti *et al.* 2007), enhancement of glutamate uptake (Aronica *et al.* 2003; Yao *et al.* 2005; Zhou *et al.* 2006) and inhibition of glutamate release via x_c^- (Baker *et al.* 2002; Xi *et al.* 2002) have all been implicated in astrocyte-dependent mGluR3-mediated neuroprotection. Group II mGluR expression by astrocytes has been shown to increase following injection of kainic acid into the mouse hippocampus to induce neuronal injury (Ferraguti *et al.* 2001), as well as in active MS lesions (Geurts *et al.* 2003; Newcombe *et al.* 2008), suggesting an intrinsic neuroprotective mechanism.

Neuronal group II mGluRs, in this case specifically mGluR2, have been implicated in mechanisms of neurotoxicity (Corti *et al.* 2007). In AD, increased mGluR2 expression has been demonstrated in neurones and dendritic processes in hippocampal CA1 and CA3 regions, areas associated with pathology. mGluR2 staining co-localised with phosphorylated tau, suggesting that it is the affected neurones which have increased mGluR2 expression (Lee *et al.* 2004b). It is however unclear whether the increased mGluR2 expression is involved in the pathogenesis or is a consequence of the pathology.

Microglial mGluR2 activation has been found to be neurotoxic and to underlie the microglial neurotoxicity of the Alzheimer's disease-associated peptide CGA (Taylor *et al.* 2002). Glutamate excitotoxicity was not responsible for neurotoxicity (Taylor *et al.* 2002), and the mechanism was subsequently shown to involve microglial release of TNFα and FasL, which bind to TNFR1 and Fas, respectively, on CGCs

(Taylor *et al.* 2005). In contrast, the mGluR3-specific agonist NAAG was completely non-toxic.

Microglia were also neurotoxic when activated with myelin (Pinteaux-Jones *et al.* 2008). Neurotoxicity was attenuated by the group II mGluR antagonist MCCG, suggesting involvement of group II mGluRs in this toxicity (Pinteaux-Jones *et al.* 2008). Interestingly, toxicity was also attenuated by NAAG, suggesting a neuroprotective role of microglial mGluR3 in this case (Pinteaux-Jones *et al.* 2008). Myelin-induced increases in extracellular glutamate levels and TNFα release were not reversed by MCCG or NAAG, suggesting that the mechanism of myelin neurotoxicity may be slightly different from that of microglia-dependent CGA neurotoxicity (Pinteaux-Jones *et al.* 2008). Increased microglial group II mGluR expression has been shown in activated and amoeboid microglia associated with MS lesions (Geurts *et al.* 2003), but since a non-discriminatory mGluR2/3 antibody was used, and neurotoxicity and neuroprotection appear to be receptor subtype-specific, it is unclear whether this upregulation would increase microglial neurotoxicity.

DCG IV has also been demonstrated to activate microglia *in vivo* (Matarredona *et al.* 2001), but in this case led to microglial expression of BDNF mRNA (Matarredona *et al.* 2001; Venero *et al.* 2002), suggesting a neuroprotective microglial phenotype. The mGluR subtype responsible was however not determined, so it is possible that BDNF expression may be an mGluR3-mediated effect. DCG IV injection also caused neurotoxicity (Venero *et al.* 2002), however it was reversed by NMDA antagonists and not mGluR antagonists and was therefore probably due to the low-affinity NMDA agonist property of DCG IV (Hayashi *et al.* 1993).

Thus, it appears that the group II mGluRs, mGluR2 and mGluR3, have distinct functions. mGluR3 is responsible for neuroprotection by astrocytes, whilst mGluR2 expressed by neurones and microglia is involved in mechanisms of neurotoxicity.

1.4.1.2.6. Group III mGluRs: neuroprotective

Group III mGluRs appear to be universally neuroprotective. Group III mGluR agonists protected against a number of neurotoxic paradigms *in vitro* (Bruno *et al.*

1995, 1996, 2000a; Copani *et al.* 1995; Faden *et al.* 1997; Gasparini *et al.* 1999; Yao *et al.* 2005; Zhou *et al.* 2006) and against NMDA excitotoxicity *in vivo* (Gasparini *et al.* 1999; Bruno *et al.* 2000a). Whilst one group demonstrated the necessity for astrocytes in this group III mGluR-mediated neuroprotection (Yao *et al.* 2005; Zhou *et al.* 2006), some studies have shown neuroprotection by group III mGluR agonists in pure or enriched neuronal cultures (Graham and Burgoyne 1994; Copani *et al.* 1995; Maiese *et al.* 1995). Evidence suggests that the neuroprotective effect of group III mGluR agonists is predominantly due to mGluR4 stimulation (Bruno *et al.* 2000a; Maj *et al.* 2003), and may simply reflect the autoreceptor function of neuronal group III mGluRs (Bruno *et al.* 2000a). Alternatively, the neuroprotective mechanism may involve a rescue of depleted astrocyte glutamate uptake (Yao *et al.* 2005; Zhou *et al.* 2006). It was suggested that via restoration of the levels of the intracellular antioxidant glutathione, group III mGluR stimulation may counteract the effects of NO and other ROS and RNS, which may include downregulation of glutamate transporter activity (Yao *et al.* 2005; Zhou *et al.* 2006). An alternative explanation for a group III mGluR-mediated enhancement of glutamate uptake by astrocytes involves the classical $G_{i/o}$ signalling pathway. Protein kinase C (PKC) has been shown to decrease the cell surface expression of the glutamate transporter excitatory amino acid transporter 1 (EAAT1), and protein kinase A (PKA), the cAMP-dependent protein kinase, has been found to induce PKC activity. Thus, a downregulation of PKA activity may downregulate PKC activity and release a PKC-mediated inhibition of EAAT1 expression (Zhou *et al.* 2006).

Microglial group III mGluRs have also been found to be neuroprotective. Despite apparently being coupled to the same class of G protein as microglial group II mGluRs, activation of group III mGluRs was protective against microglial neurotoxicity due to LPS, CGA, $A\beta_{25\text{-}35}$ or myelin (Taylor *et al.* 2003; Pinteaux-Jones *et al.* 2008). The presence of microglia was necessary for the mGluR-mediated protection (Taylor *et al.* 2003; Pinteaux-Jones *et al.* 2008), and in the case of myelin at least, group III mGluR agonists did not reverse the myelin-induced increases in extracellular glutamate levels (Pinteaux-Jones *et al.* 2008), suggesting that protection mediated by microglial group III mGluRs occurs via a distinct mechanism from that seen in astrocyte/neurone co-cultures and pure neuronal cultures.

Thus, group III mGluRs appear to be universally neuroprotective. In active MS lesions, the expression of mGluR4 and mGluR8 by astrocytes, and the expression of mGluR8 by microglia, have been shown to be upregulated (Geurts *et al.* 2005). This suggests that activated glia in MS lesions may have a neuroprotective function.

1.4.1.2.8. Specific mGluR agonists and antagonists used in this thesis

The mGluR agonists and antagonists utilised to selectively stimulate and block specific microglial receptor subtypes in this study are listed in table 1.2.

Group I-selective compounds

Compound	Activity	Concentration used	References
DHPG	Orthosteric agonist or partial agonist	100 µM	Ito *et al.* (1992); Brabet *et al.* (1995)
tADA	Orthosteric agonist	250 µM	Manahan-Vaughan *et al.* (1996)
CDPPB	Allosteric mGluR5-specific agonist	500 nM	Lindsley *et al.* (2004); Kinney *et al.* (2005)
AIDA	Competitive antagonist	250 µM	Pellicciari *et al.* (1995); Moroni *et al.* (1997)
MTEP	Non-competitive mGluR5-specific antagonist	100 nM	Cosford *et al.* (2003b)
SIB-1757	Non-competitive mGluR5-specific antagonist	50 µM	Varney *et al.* (1999)

Group II-selective compounds

DCG IV	Orthosteric agonist	500 nM	Hayashi *et al.* (1993); Ishida *et al.* (1993); Brabet *et al.* (1998)
NAAG	Orthosteric mGluR3-specific agonist	50 µM	Wroblewska *et al.* (1997)
MCCG	Competitive antagonist	500 µM	Jane *et al.* (1994); Knopfel *et al.* (1995); Salt and Eaton (1995)
APICA	Competitive antagonist or inverse agonist	200 µM	Ma *et al.* (1997)

Group III-selective compounds

L-AP4	Orthosteric agonist	100 µM	Nakanishi (1992); Bushell *et al.* (1995)
(RS)-PPG	Orthosteric agonist	100 µM	Gasparini *et al.* (1999)
MAP4	Competitive antagonist or partial agonist	500 µM	Jane *et al.* (1994); Bushell *et al.* (1995); Knopfel *et al.* (1995); Salt and Eaton (1995)

Table 1.2. *Summary of selective mGluR agonists and antagonists used in this thesis. AIDA, (RS)-1-aminoindan-1,5-dicarboxylic acid; APICA, (RS)-1-amino-5-phosphonoindan-1-carboxylic acid; CDPPB, 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide; DCG IV, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; DHPG, (RS)-3,5-dihydroxyphenylglycine; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; MAP4, (S)-2-amino-2-methyl-4-phosphonobutanoic acid; MCCG, 2S,3S,4S-2-methyl-2-(carboxycyclopropyl)glycine; MTEP, 3-((2-methyl-1,3-thiazol-4-yl)ethynyl)-pyridine; NAAG, N-acetyl-L-aspartyl-L-glutamic acid; (RS)-PPG, (RS)-4-phosphonophenylglycine; SIB-1757, 6-methyl-2-(phenylazo)-3-pyridinol; tADA, trans-azetidine-2,4-dicarboxylic acid.*

1.4.2. Glutamate transporters

The two main glutamate transporter types are the excitatory amino acid transporter (EAAT) family and the x_c^- glutamate/cystine antiporter system, which are represented in figure 1.8.

1.4.2.1. Excitatory amino acid transporters

The electrogenic Na^+-dependent EAATs mediate high-affinity glutamate uptake. The Na^+ gradient, maintained by ATPases in the plasma membrane, allows glutamate uptake to occur against its concentration gradient. One glutamate molecule is cotransported with three Na^+ and one proton, whilst one K^+ is transported in the opposite direction (see fig. 1.8) (Zerangue and Kavanaugh 1996; Levy *et al.* 1998; Owe *et al.* 2006). The EAATs prevent inappropriate receptor activation, especially important in the case of synaptic iGluRs, of which prolonged stimulation can lead to excitotoxicity and neuronal death.

EAAT1 (Storck *et al.* 1992; Tanaka 1993) and EAAT2 (Pines *et al.* 1992) (also known as L-glutamate/L-aspartate transporter, GLAST and L-glutamate transporter 1, GLT-1, respectively) are primarily astrocytic transporters responsible for the maintenance of extracellular glutamate below neurotoxic levels (Danbolt *et al.* 1992; Rosenberg *et al.* 1992; Rothstein *et al.* 1994, 1996; Lehre *et al.* 1995). EAAT1 is expressed at its highest level in the cerebellar cortex, and appears to be expressed at a lower level than EAAT2 throughout the rest of the CNS (Rothstein *et al.* 1994; Lehre *et al.* 1995). EAAT2 is thought to be responsible for more than 90 % glutamate transporter activity under normal circumstances *in vivo* (Danbolt *et al.* 1992; Haugeto *et al.* 1996; Tanaka *et al.* 1997).

EAAT3 (Kanai and Hediger 1992) (also known as excitatory amino acid carrier 1, EAAC1), is primarily expressed by neurones (Rothstein *et al.* 1994; Conti *et al.* 1998; Kugler and Schmitt 1999; Plachez *et al.* 2000), and appears to be responsible for the uptake of cysteine for neuronal glutathione synthesis (Chen and Swanson 2003; Himi *et al.* 2003; Aoyama *et al.* 2006). EAAT4 is predominantly expressed in the cerebellar Purkinje cells (Fairman *et al.* 1995; Lin *et al.* 1998) and EAAT5 is

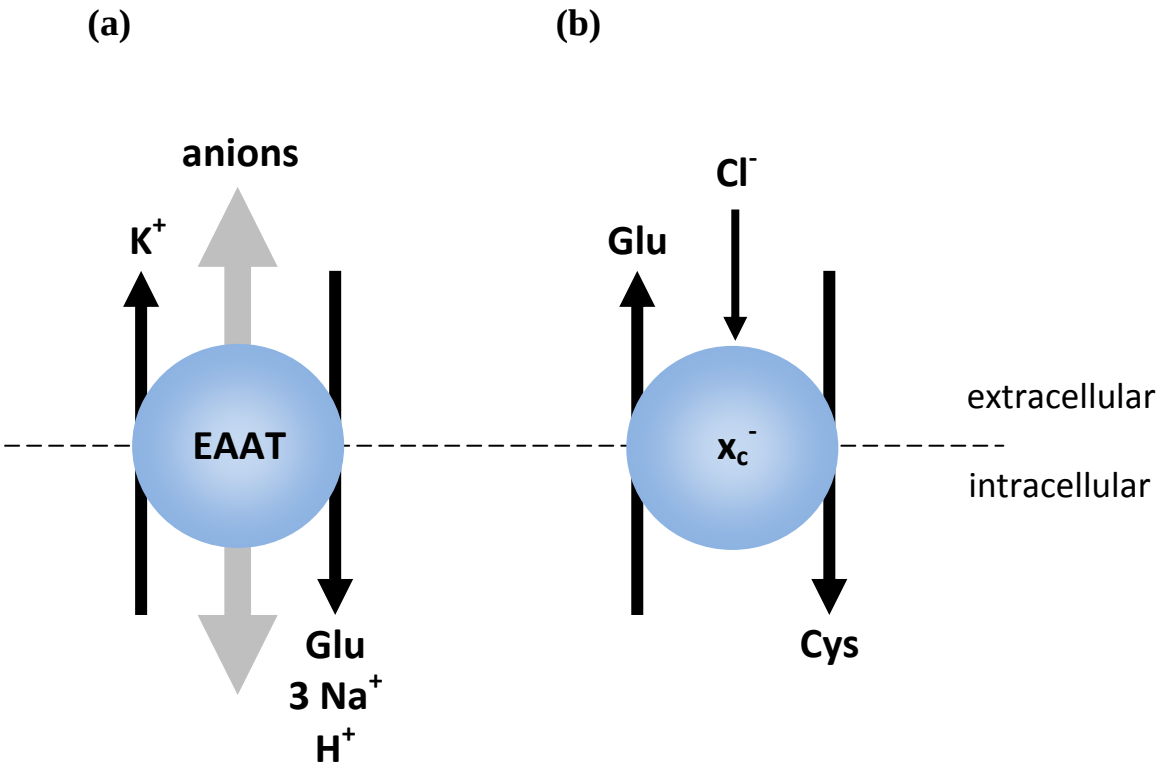


Figure 1.8. *Glutamate transporters: (a) excitatory amino acid transporters (EAATs) and (b) the x_c^- cystine/glutamate transporter system. The EAATs cotransport one molecule of glutamate and three sodium ions; K^+/H^+ exchange also occurs. The anion channel function of EAATs is most pronounced in EAAT4 and EAAT5, which primarily transport chloride ions. The x_c^- transporter system usually imports cystine in exchange for glutamate, and appears to be Cl^--dependent at low cystine concentrations.*

expressed primarily in the retina (Arriza *et al.* 1997). Although all the EAATs allow a degree of anion transport (Wadiche *et al.* 1995), this is most pronounced in EAAT4 and EAAT5, which act as chloride channels.

1.4.2.2. x_c^- glutamate/cystine transporter system

The x_c^- glutamate/cystine transporter system is an electroneutral antiporter which, under normal conditions, imports cystine for glutathione synthesis in exchange for glutamate (Bannai 1986) (fig. 1.8). x_c^--mediated transport appears to be partially Cl^--dependent at low cystine concentrations (Murphy *et al.* 1989). The x_c^- system is expressed at the plasma membrane as a heterodimer consisting of the 4F2hc glycoprotein, which is associated with a number of transport systems, and the specific protein xCT (Sato *et al.* 1999; Bassi *et al.* 2001).

The x_c^- transporter system appears to have a significant impact upon the homeostasis of both of its physiological substrates, glutamate and cystine. Pharmacological inhibition of x_c^- reduced the striatal glutamate concentration in the rat by ~ 60 %, suggesting that the majority of extracellular glutamate in the normal CNS is a product of x_c^- activity (Baker *et al.* 2002). Mice lacking the xCT gene had increased plasma levels of cystine/GSSG and related disulphides, and decreased plasma levels of GSH, although the GSH content of all tissues examined, including some CNS areas, was not significantly different between wild-type and mutant mice (Sato *et al.* 2005). Fibroblasts derived from xCT-deficient animals only survived in culture when supplemented with exogenous antioxidants (Sato *et al.* 2005).

Although under normal circumstances, x_c^- mediates cystine uptake and glutamate release, high concentrations of glutamate have been shown to inhibit the uptake of cystine (Bannai 1986; Watanabe and Bannai 1987; Sato *et al.* 1999; Tomi *et al.* 2002; Patel *et al.* 2004). Therefore, under conditions of elevated extracellular glutamate, it is possible that the x_c^- transporter may reverse and therefore function as a glutamate uptake transporter.

Of the cells of the CNS, the x_c^- system is expressed by microglia (Qin *et al.* 2006; Barger *et al.* 2007; Domercq *et al.* 2007) and astrocytes (Allen *et al.* 2001;

Gochenauer and Robinson 2001; Pow 2001), but not by neurones (Pow 2001; Qin *et al.* 2006).

1.4.2.3. Microglial glutamate transporters

Microglia express EAAT2 (Kondo *et al.* 1995; Lopez-Redondo *et al.* 2000; Nakajima *et al.* 2001b; Jacobsson *et al.* 2006; O'Shea *et al.* 2006), and possibly also EAAT1 (Kondo *et al.* 1995; O'Shea *et al.* 2006). Microglial EAAT expression appears to be increased following infection or trauma. *In vitro*, microglia demonstrated increased EAAT2 protein expression and glutamate uptake capacity following exposure to LPS (Persson *et al.* 2005; Jacobsson *et al.* 2006; O'Shea *et al.* 2006), which was found to be TNFα-dependent (Persson *et al.* 2005). Microglia also showed increased levels of TNFα production and EAAT2 protein expression following infection with Herpes simplex virus (Persson *et al.* 2007). *In vivo*, microglial EAAT2 expression was increased in the rat facial motor nucleus following facial nerve axotomy (Lopez-Redondo *et al.* 2000), in the cerebral parenchyma and in perivascular microglia of simian immunodeficiency virus-infected macaques (Chretien *et al.* 2002), and in MS lesions (Werner *et al.* 2001). Following traumatic brain injury in the rat, an increase in EAAT1 and EAAT2-expressing microglia was detected in all brain areas studied except the trauma zone itself (van Landeghem *et al.* 2001). Marked upregulation of EAAT1 expression has been demonstrated in activated microglia in patients suffering with Prion disease (Chretien *et al.* 2004) and HIV (Vallat-Decouvelaere *et al.* 2003). Following ischaemia, microglial EAAT1 expression appeared to be restricted to early and intermediate stages of activation and was not present on phagocytosing microglia/macrophages or blood-derived infiltrating monocytes (Beschorner *et al.* 2007).

Microglia robustly express the x_c^- transporter, and microglial x_c^- function is considered to be important in the supply of cystine for microglial glutathione synthesis (McBean 2002; Barger *et al.* 2007). However the concomitant glutamate release has been implicated in excitotoxic damage to neurones (Piani and Fontana 1994; Barger and Basile 2001; Qin *et al.* 2006) and oligodendrocytes (Domercq *et al.* 2007). Microglial x_c^- expression and activity is upregulated following LPS

treatment (Barger *et al.* 2007; Domercq *et al.* 2007). Increased microglial glutamate release, attributed to x_c^- activity, has also been shown following treatment with soluble APP (Barger and Basile 2001) or Aβ (Qin *et al.* 2006). Through this upregulation of x_c^-, microglial activation may therefore enhance microglial antioxidant defence with the consequence of endangering neighbouring cells.

Microglial EAAT activity is much lower compared with that of astrocytes (Leonova *et al.* 2001; Persson *et al.* 2005). However under conditions of oxidative stress and hypoxia, the glutamate uptake capacity of astrocytes may decrease (Volterra *et al.* 1994; Miralles *et al.* 2001; Dallas *et al.* 2007) and microglial EAAT-mediated glutamate uptake may partially replace astrocyte glutamate uptake. Alternatively, the concurrent upregulation of microglial EAAT and x_c^- following microglial activation may indicate complementary functions. Microglial EAAT activity may represent a mechanism to take up glutamate released by x_c^- and therefore prevent excitotoxicity, or a mechanism by which x_c^- activity is driven by provision of free intracellular glutamate.

Following traumatic brain injury (van Landeghem *et al.* 2001) or in MS (Newcombe *et al.* 2008), microglia may express EAAT3, the neuronal transporter responsible for cysteine uptake for the synthesis of glutathione (Chen and Swanson 2003; Himi *et al.* 2003; Aoyama *et al.* 2006). Microglia are thought to rely on L-cystine uptake by the x_c^- transporter system to supply cysteine for glutathione synthesis (Barger *et al.* 2007). The function of microglial EAAT3 expression is therefore unclear, but is suggested to be a further mechanism of increasing microglial glutamate uptake under conditions of downregulated astrocyte glutamate uptake and elevated extracellular glutamate (van Landeghem *et al.* 2001; Newcombe *et al.* 2008).

1.4.2.4. Glutamate transporters in AD and MS

Extracellular glutamate levels are elevated in a number of neurological conditions, and may be toxic through excitotoxicity or oxidative glutamate toxicity, a phenomenon in which elevated extracellular glutamate inhibits cystine uptake by the x_c^- system, thus limiting the supply of cystine for glutathione synthesis (Murphy *et al.* 1989). Alterations in the expression or activity of the high affinity EAATs could

therefore contribute to the pathogenesis of neurological diseases; these transporters may represent a potential target for drugs to treat such diseases (Sheldon and Robinson 2007).

Brains from patients suffering with Alzheimer's disease have a lower glutamate uptake capacity compared with controls, as assessed by D-aspartate binding (Cross *et al.* 1987; Cowburn *et al.* 1988; Masliah *et al.* 1996). Moreover, glutamate uptake in the frontal cortex was inversely correlated with cognitive decline (Masliah *et al.* 1996). Decreased neuronal glutamate transporter density was observed in post-mortem AD brain compared with control (Scott *et al.* 1995), and EAAT2 protein expression in the frontal cortex was found to be lower in AD than in control brains (Li *et al.* 1997). Astrocytes derived from AD cortex were deficient in glutamate uptake and had lower EAAT1 and EAAT2 protein expression than controls (Liang *et al.* 2002). However, one study demonstrated *de novo* neuronal EAAT1 expression associated with AD, the expression of EAAT1 being colocalised with tau (Scott *et al.* 2002). Platelets and fibroblasts derived from AD patients have been shown to have decreased EAAT1 expression compared with age-matched controls (Zoia *et al.* 2004, 2005), as well as decreased glutamate uptake capacity (Ferrarese *et al.* 2000; Begni *et al.* 2004). Fibroblast EAAT1 protein expression was found to be inversely correlated with disease severity (Zoia *et al.* 2005). In addition, APP transgenic mice, a model of AD, had decreased affinity and surface expression of glutamate transporters, in combination with decreased protein expression of EAAT1 and EAAT2 in the neocortex (Masliah *et al.* 2000). There was however no change in the mRNA levels of any of the transporters, suggesting a post-transcriptional regulation (Masliah *et al.* 2000).

The activity of EAAT1, EAAT2 and EAAT3 may be modulated by the cellular redox potential. Glutamate uptake activity has been shown to be downregulated by oxygen free radicals and thiol oxidising compounds, and upregulated by thiol reducing compounds (Volterra *et al.* 1994; Trotti *et al.* 1997a, 1997b). It is suggested that disulphide bond formation within or between functional EAATs inhibits their activity (Trotti *et al.* 1998). Accordingly, the lipid peroxidation product HNE increased ROS levels and decreased the glutamate uptake capacity of fibroblasts derived from AD patients, but not those derived from control subjects (Begni *et al.*

2004). Exogenous antioxidants have been shown to reverse a depleted glutamate uptake capacity (Blanc *et al.* 1998; Begni *et al.* 2004).

In multiple sclerosis, the expression of the high-affinity glutamate transporters, particularly EAAT2, may be downregulated (Werner *et al.* 2001; Pitt *et al.* 2003). Inhibition of oligodendrocyte glutamate transporters led to oligodendrocyte apoptosis due to AMPA and kainate receptor activation, and consequent axonal damage (Domercq *et al.* 2005), implicating the downregulation of glutamate transporters in MS pathogenesis. However, increased glutamate transporter expression and glutamate uptake in MS tissue has also been reported (Vallejo-Illarramendi *et al.* 2006). Following EAE, the animal model of MS, spinal cord synaptosomes also showed an increased glutamate uptake capacity; EAAT1 and EAAT2 expression was decreased whilst EAAT3 expression was transiently upregulated (Ohgoh *et al.* 2002).

1.5. Glutathione

Glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH) is a tripeptide thiol, consisting of L-glutamate, L-cysteine and L-glycine.

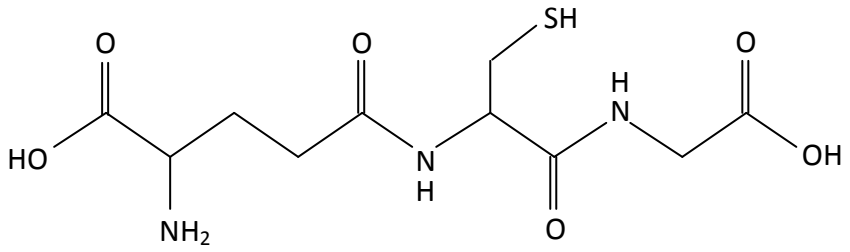


Figure 1.9. *The structure of glutathione*

1.5.1. Functions of glutathione

Probably the best known and most extensively studied function of GSH is its antioxidant property, which it possesses by virtue of the thiol group of the cysteine residue. GSH reduces reactive oxygen species and free radicals, as well as maintaining the thiol redox potential in cells by ensuring sulfhydryl groups of cellular proteins are in the reduced state (Klebanoff 1957). Upon oxidation, the disulphide GSSG is produced, and GSH is regenerated in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) by the action of glutathione reductase (GR; EC 1.8.1.7) (Rall and Lehninger 1952). GSH has a role in the regeneration of antioxidant vitamins. Redox coupling between GSH and ascorbic acid (vitamin C) maintains the latter in its active reduced state (Meister 1994; Winkler *et al.* 1994), and a second redox coupling between ascorbic acid and tocopherol (vitamin E) regenerates the active reduced form of tocopherol (Packer *et al.* 1979; Mukai *et al.* 1991; Sharma and Buettner 1993). GSH is also a transport and storage form of the amino acid cysteine, and acts as a cofactor in some isomerisation reactions within the cell (Meister and Anderson 1983).

In the immune system, GSH appears to be crucial for the proliferation of lymphocytes during activation (Hamilos *et al.* 1989; Suthanthiran *et al.* 1990; Smyth 1991). Evidence suggests that this may be due to thiol-mediated cell cycle control (Iwata *et al.* 1994); a number of transcription factors are redox-sensitive (Powis *et al.* 1995; Arrigo 1999; Morel and Barouki 1999). Reflecting its importance in the immune response, depletion of lymphocyte and plasma GSH is associated with HIV/AIDS (Buhl *et al.* 1989; Roederer *et al.* 1991; Staal *et al.* 1992a, 1992b); indeed, GSH levels appear to be a key determinant of patient survival (Herzenberg *et al.* 1997).

Due to its γ-glutamyl group as well as its ability to modulate proteins through the formation of disulphide bonds, GSH may act as a neuromodulator at the CaR, NMDA, AMPA, kainate, μ-opioid and neurokinin-1 receptors (Varga *et al.* 1989, 1997; Yoneda *et al.* 1990; Leslie *et al.* 1992; Liu and Quirion 1992; Janaky *et al.* 1993, 2007, 2008; Ogita *et al.* 1995; Jenei *et al.* 1998; Wang *et al.* 2006). A number of studies have identified two GSH binding sites on CNS tissues (Ogita and Yoneda 1987, 1988; Guo *et al.* 1992; Janaky *et al.* 2000), where binding at one site cannot be inhibited by glutamate receptor ligands, and is postulated to be a GSH-specific site, thus potentially identifying GSH as a novel neurotransmitter (Guo *et al.* 1992; Guo and Shaw 1992; Lanius *et al.* 1994; Janaky *et al.* 2000).

1.5.2. Glutathione synthesis and consumption

Glutamate-cysteine ligase (GCL, also known as γ-glutamylcysteine synthase; EC 6.3.2.2) catalyses the formation of γ-glutamylcysteine (γGC) from glutamate and cysteine (Strumeyer and Bloch 1960), the rate limiting step in GSH biosynthesis (Meister and Anderson 1983). This enzymatic reaction may be limited by the availability of cysteine, and is regulated by GSH, the end product of the pathway, which can competitively inhibit GCL by interacting with the glutamate binding site (Richman and Meister 1975). GSH synthase (EC 6.3.2.3) then catalyses the formation of GSH from γGC and glycine (Snoke *et al.* 1953). Each of the two enzymatic steps are coupled to ATP hydrolysis (Snoke and Bloch 1952; Snoke *et al.* 1953).

The simple reduction of free radicals and reactive oxygen species and oxidised sulfhydryl groups by GSH, producing GSSG, occurs in the presence of GSH peroxidase (GPx; EC 1.11.1.9) (Mills 1957), although in the case of the highly reactive radicals, oxidation may occur spontaneously in the absence of the enzyme (Andersson *et al.* 1982; Moldeus *et al.* 1982). As described above, GSH is readily regenerated in the presence of NADPH by the action of GR (Rall and Lehninger 1952). Under normal circumstances, the ratio of GSSG to GSH is very low (Hwang *et al.* 1992), due to the reducing environment of the cytosol and the efficiency of GSH reductase. GSH may also be irreversibly consumed by conjugation to other molecules by glutathione S-transferase (GST; EC 2.5.1.18) (Gillham 1971). This pathway is involved in the detoxification of xenobiotics (Smith *et al.* 1977; Younes *et al.* 1980), as well as in normal metabolic processes (Elce and Harris 1971; Soderstrom *et al.* 1985). GSH may also be released by the cell, either as the thiol or as a glutathione-S-conjugate following GST activity. In these situations, it is important that GSH utilisation is balanced by *de novo* synthesis, especially considering that cellular depletion of GSH is associated with apoptotic cell death (Kane *et al.* 1993; Merad-Boudia *et al.* 1998; Anderson *et al.* 1999; Higuchi and Matsukawa 1999; Wullner *et al.* 1999; Higuchi 2004).

The pathways described here are summarised in figure 1.10.

1.5.3. Glutathione in the CNS

Although GSH is present in almost all cell types, it is especially important in the CNS, where metabolic activity and therefore free radical production is high, and levels of other antioxidants are relatively low (Dringen 2000). The concentration of GSH in the brain is in the range 1 – 3 mM (Dringen 2000); however its distribution is not homogeneous. Histochemical and immunofluorescent staining of brain sections have demonstrated that neurones contain low levels of GSH whilst the surrounding glial cells have a much higher GSH content (Slivka *et al.* 1987; Philbert *et al.* 1991; Pearce *et al.* 1997).

In culture, rather a wide range of values for GSH concentrations of each cell type have been reported. Neurones have intracellular GSH levels between 10 and 30

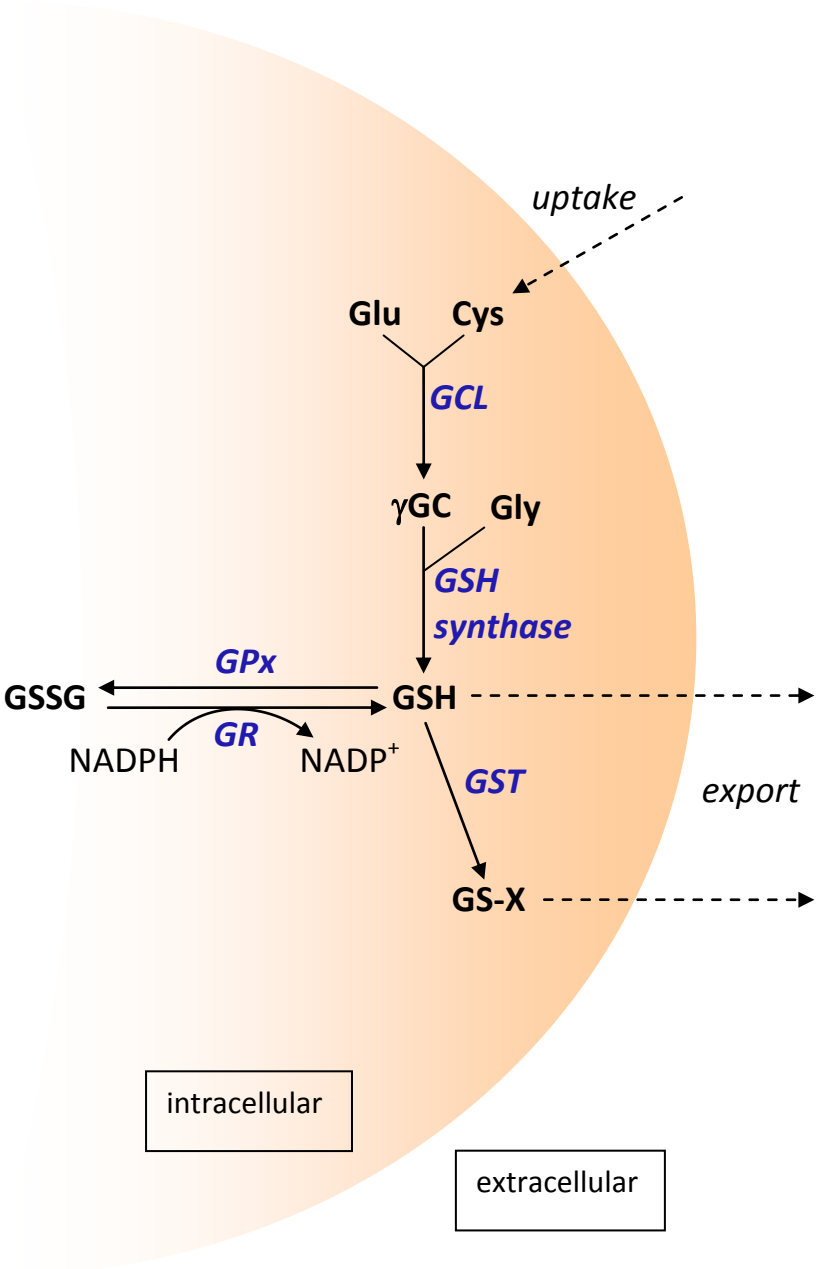


Figure 1.10. *Diagrammatic representation of the cellular synthesis and consumption of glutathione. Glutathione is synthesised from its constituent amino acids by the sequential actions of the enzymes GCL and GSH synthase, is reversibly consumed by oxidation and irreversibly consumed by conjugation and export. Cys, cysteine; γGC, γ-glutamylcysteine; Glu, glutamate; Gly, glycine; GSH, reduced glutathione; GSSG, oxidised glutathione; GS-X, glutathione S-conjugate. Enzymes (shown in blue), GCL, glutamate-cysteine ligase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH synthase, glutathione synthase; GST, glutathione-S-transferase.*

nmol.mg^{-1} protein (Bolaños *et al.* 1996; Dringen *et al.* 1999a, 1999b; Wullner *et al.* 1999; Hirrlinger *et al.* 2002c; Sebastia *et al.* 2003; Gegg *et al.* 2005), and most studies report astrocyte GSH levels in the region 20 – 60 nmol.mg^{-1} protein (Raps *et al.* 1989; Yudkoff *et al.* 1990; Dringen and Hamprecht 1996, 1998; Juurlink *et al.* 1996; Sagara *et al.* 1996; Dringen *et al.* 1999b; Wang and Cynader 2000; Hirrlinger *et al.* 2002c; Muyderman *et al.* 2004; Minich *et al.* 2006; Vargas *et al.* 2006), with a few reports of lower levels around 10 nmol.mg^{-1} protein (Chatterjee *et al.* 1999; García-Nogales *et al.* 1999; Gegg *et al.* 2005). Where both cell types have been studied in parallel, astrocytes were consistently found to contain higher levels than neurones (Raps *et al.* 1989; Dringen *et al.* 1999a, 1999b; Hirrlinger *et al.* 2000, 2002b, 2002c; Wang and Cynader 2000; Keelan *et al.* 2001).

Microglial cells in culture have a GSH level of 25 – 40 nmol.mg^{-1} protein (Hirrlinger *et al.* 2000, 2002c; Persson *et al.* 2006), although one study reported a much higher level of 110 nmol.mg^{-1} protein (Chatterjee *et al.* 1999). Evidence suggests that microglia contain the highest GSH levels amongst the different cell types in the CNS (Chatterjee *et al.* 1999, 2000; Hirrlinger *et al.* 2000, 2002b; Noack *et al.* 2000). Oligodendrocytes are usually reported to contain very low levels of GSH ($\leq$ 10 nmol.mg^{-1} protein) (Thorburne and Juurlink 1996; Juurlink *et al.* 1998; Almazan *et al.* 2000; Fragoso *et al.* 2004), although some comparative studies have found levels similar to or even higher than those of microglia and astrocytes (Hollensworth *et al.* 2000; Hirrlinger *et al.* 2002b, 2002c). These same studies reported that oligodendrocyte GPx activity was the highest amongst the different brain cells in culture (Hollensworth *et al.* 2000; Hirrlinger *et al*. 2002b), strengthening their conclusion that oligodendrocytes have a high antioxidant capacity.

The variability in reported GSH concentrations of cultured cells is probably due to different methods used to measure GSH, or to differences in culture conditions; indeed GSH levels in neurones were shown to more than double when the culture medium was supplemented with particular amino acid/dipeptide combinations (Dringen *et al.* 1999b). Such an effect of culture conditions upon GSH levels indicates that any data relating to the GSH content of cultured cells should be interpreted with caution.

GSH is present extracellularly in the brain at a concentration of approximately 2 μM (Yang *et al.* 1994; Lada and Kennedy 1997), and in CSF in the range 0.5 – 6 μM (Anderson *et al.* 1989; Do *et al.* 2000; Wang and Cynader 2000; Calabrese *et al.* 2002, 2003; Kawakami *et al.* 2006).

1.5.4. Glutathione and related enzymes in ageing, AD and MS

Oxidative stress has long been implicated in the ageing process and in a number of CNS diseases, especially those associated with ageing (Harman 1956; Ames *et al.* 1993; Halliwell 2006). The levels of antioxidants such as glutathione in these situations are therefore of considerable interest.

The gene expression profile of the ageing mouse brain compared with that of the young adult mouse is indicative of increased oxidative stress with age (Lee *et al.* 2000). In accordance with this, there exists a clear, well-documented age-related decrease in GSH, increase in GSSG and consequent decrease in GSH:GSSG ratio in a number of brain regions, as demonstrated in aged rodents compared with young animals (Ravindranath *et al.* 1989; Iantomasi *et al.* 1993; Favilli *et al.* 1994; Sasaki *et al.* 2001; Liu 2002; Rebrin *et al.* 2003, 2007; Wang *et al.* 2003b; Suh *et al.* 2004; Zhu *et al.* 2006; Parihar *et al.* 2008). This has been shown to be accompanied by a shift in the glutathione redox potential in favour of the pro-oxidising state (Rebrin *et al.* 2003, 2007).

An age-related increase in the activities of the glutathione-consuming enzymes GPx, GST and the ectoenzyme γ glutamyl transpeptidase (γGT) has been demonstrated in rat brain (Iantomasi *et al.* 1993; Favilli *et al.* 1994; Kim *et al.* 2003a; Zhu *et al.* 2006). Conversely, GCL expression and activity has been widely reported to decline with age (Iantomasi *et al.* 1993; Favilli *et al.* 1994; Liu and Choi 2000; Liu 2002; Wang *et al.* 2003b; Zhu *et al.* 2006). This appears to be due to a decline in the expression of the regulatory subunit of the enzyme (Liu 2002; Zhu *et al.* 2006), important in lowering the affinity of GCL for GSH (Huang *et al.* 1993), which inhibits activity (Richman and Meister 1975). Accordingly, an age-associated decrease in the affinity of GCL for the substrate cysteine has been reported (Suh *et al.* 2004).

Although AD is also associated with increased oxidative stress, there do not appear to be clear, well-documented alterations in the GSH system in AD brain compared with controls. This may reflect a disease-associated increase in the generation of oxidative stress rather than a decrease in antioxidant capacities. Evidence does however exist for altered peripheral GSH status in AD, suggesting that the oxidative stress associated with AD may extend further than the affected areas of the CNS (Ceballos-Picot *et al.* 1996; Liu *et al.* 2005; Bermejo *et al.* 2008).

GSH levels have been reported to be higher in the midbrain and hippocampus of AD patients than age-matched controls (Adams, Jr. *et al.* 1991), although other studies have failed to show a significant difference in total glutathione and thiol content of a number of regions in control and AD brain (Perry *et al.* 1987; Aksenov and Markesbery 2001) or CSF (Konings *et al.* 1999). Lower protein-bound thiol levels were reported in AD hippocampus compared with control (Aksenov and Markesbery 2001), as were increased levels of GSH conjugates of the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) in a number of areas of AD brain (Volkel *et al.* 2006), both of which are suggestive of increased oxidative damage. AD patients have also been shown to have increased GR and GPx gene expression in the hippocampus, parahippocampal gyrus and amygdala, compared with control (Lovell *et al.* 1995; Aksenov and Markesbery 2001), perhaps suggestive of a protective upregulation of the GSH/GSSG redox reactions. The activity of GST in a number of brain regions and in ventricular CSF were found in one study to be lower in AD patients compared with controls (Lovell *et al.* 1998b). However, in another case there was no difference in GST activity in frontal cortex or substantia innominata between control and AD brains (Perry *et al.* 1987). Polymorphisms in certain GST genes have been found to affect age at onset and clinical progression of AD (Li *et al.* 2003, 2006; Kolsch *et al.* 2004; Bernardini *et al.* 2005; Spalletta *et al.* 2007).

CSF from MS patients has been shown to have decreased GSH and increased GSSG levels compared with controls (Calabrese *et al.* 2002). In addition, increased levels of the lipid peroxidation product malondialdehyde in MS CSF suggest increased levels of oxidative stress (Calabrese *et al.* 1994). CSF from MS patients also had increased GR activity and decreased GPx activity (Calabrese *et al.* 1994). In contrast to the situation in CSF, in the plasma of MS patients, GSH levels and the

GSH:GSSG ratio were found to be increased compared with controls (Calabrese *et al.* 2002).

1.5.5. Astrocytes release GSH

The release of GSH by astrocytes is very well-characterised (Yudkoff *et al.* 1990; Juurlink *et al.* 1996; Sagara *et al.* 1996; Dringen *et al.* 1999b; Stewart *et al.* 2002). GSH is released via the ATP-dependent transporter, multidrug resistance-associated protein 1 (Mrp1) (Hirrlinger *et al.* 2002c; Minich *et al.* 2006), and subsequently cleaved by the astrocyte-associated ectoenzyme γGT (EC 2.3.2.2) to produce cysteinylglycine (Dringen *et al.* 1997). This provides a supply of GSH precursors for neurones. The neurone-associated ectoenzyme aminopeptidase N (ApN) cleaves the astrocyte-derived cysteinylglycine to produce L-cysteine (Dringen *et al.* 2001), which is taken up via EAAT3 (Aoyama *et al.* 2006), thus providing neurones with the limiting substrate for GSH synthesis. Accordingly, the GSH level of neurones co-cultured with astrocytes has been demonstrated to be much higher than in neurones cultured alone (Bolaños *et al.* 1996; Dringen *et al.* 1999b; Gegg *et al.* 2005). Depletion of astrocyte GSH leads to lower levels of GSH in co-cultured neurones and increased neuronal sensitivity to nitric oxide toxicity (Gegg *et al.* 2005), illustrating the importance of this supply for maintenance of neuronal GSH levels and neuronal survival. The pathway described here is illustrated in figure 1.11.

1.5.6. Microglial glutathione

As in all cell types, the main, continuous source of ROS in microglia is the mitochondrial electron transport chain, as a by-product of cellular respiration. In addition, microglial activation causes expression of iNOS and consequent production of NO (Chao *et al.* 1992; Ding *et al.* 1997; Possel *et al.* 2000), as well as the generation of superoxide by NADPH oxidase (Colton and Gilbert 1987; Klegeris and McGeer 1994; Sankarapandi *et al.* 1998). Either of these species may be damaging alone; alternatively they may combine to form peroxynitrite (Ischiropoulos *et al.* 1992; Szabo *et al.* 1997; Noack *et al.* 1999). The production of ROS and RNS during normal microglial function is potentially cytotoxic, and indicates that antioxidants such as GSH are likely to be important for microglial protection.

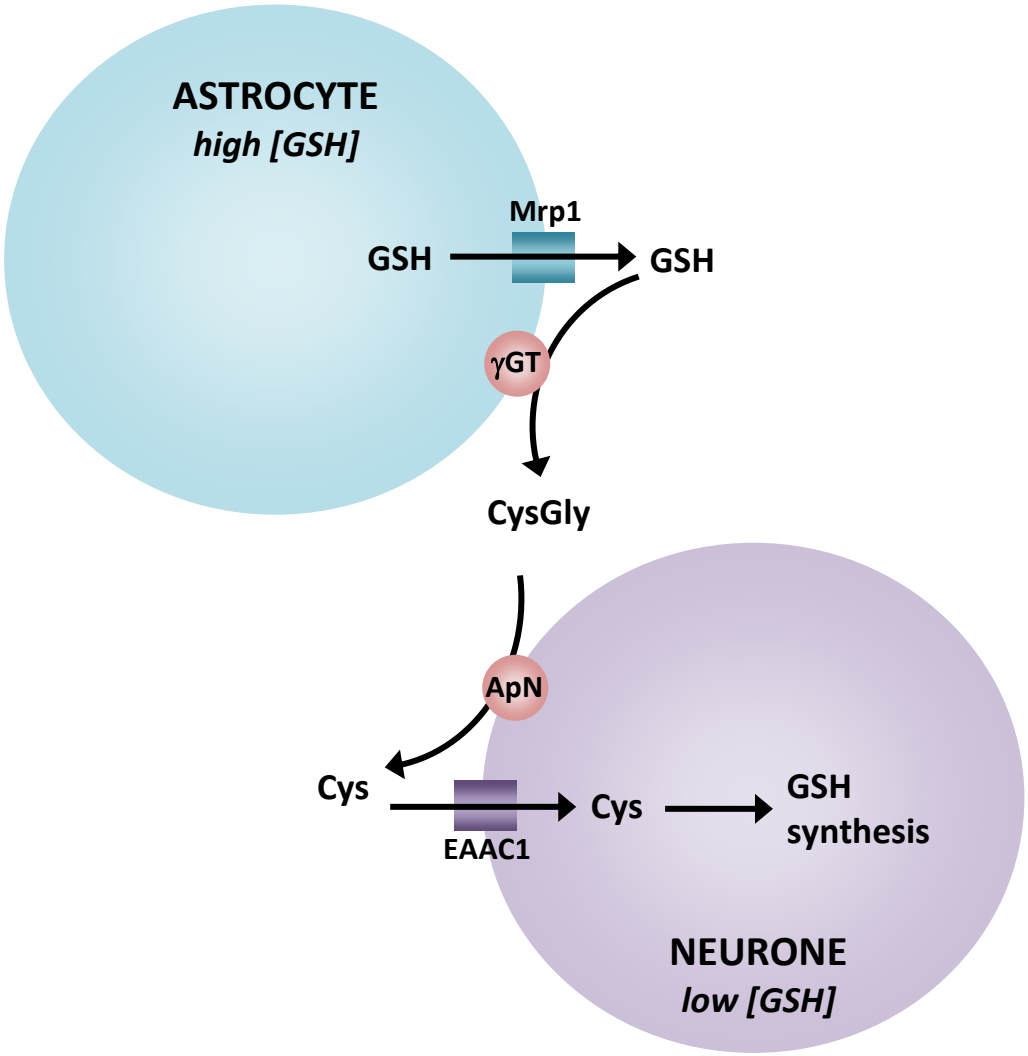


Figure 1.11. *Astrocytes release GSH to supply neurones with cysteine for GSH synthesis. GSH is released by astrocytes via Mrp1, and is cleaved sequentially by the astrocyte-associated enzyme γGT and the neurone-associated enzyme ApN to yield cysteine, which is taken up via neuronal EAAC1. ApN, aminopeptidase N; Cys, cysteine; CysGly, cysteinylglycine; EAAC1, excitatory amino acid carrier 1; GSH, reduced glutathione; γGT, γ-glutamyl transpeptidase; Mrp1, multidrug resistance protein 1.*

Accordingly, microglia have a prominent GSH system, containing the highest levels of GSH amongst the cells of the CNS (Chatterjee *et al.* 1999, 2000; Hirrlinger *et al.* 2000, 2002b; Noack *et al.* 2000), found to be in the region of 25 – 40 nmol.mg^{-1} protein *in vitro* (Hirrlinger *et al.* 2000, 2002c; Persson *et al.* 2006). GPx immunoreactivity appeared to be largely confined to microglia in the rat (Lindenau *et al.* 1998) and human CNS (Hirato *et al.* 2003). In culture, microglial GPx activity was significantly higher than that of neurones (Hirrlinger *et al.* 2000), similar to that of astrocytes (Hirrlinger *et al.* 2000; Hollensworth *et al.* 2000), but lower than that of oligodendrocytes (Hollensworth *et al.* 2000; Hirrlinger *et al.* 2002b).

Cultured microglia also demonstrated robust GR immunoreactivity (Gutterer *et al.* 1999), and GR activity was significantly higher in microglia than in neurones or astrocytes (Hirrlinger *et al.* 2000), but significantly lower than in oligodendrocytes (Hirrlinger *et al.* 2002b). Inhibition of GCL by L-buthionine sulfoximine (BSO) depleted microglial GSH by 70 % in 6 hours in N9 microglial cells (Roychowdhury *et al.* 2003), suggesting a relatively high rate of continual GSH synthesis under normal circumstances. Microglia are thought to rely on L-cystine uptake by the x_c^- transporter system to supply cysteine for glutathione synthesis (Barger *et al.* 2007). This cystine is imported in exchange for glutamate, and glutamate is imported by EAATs which are expressed at low levels by microglia.

A number of studies report alterations in microglial GSH levels in response to microglial activation; however, a consensus of the effect of activation upon intracellular GSH is yet to be reached. In mixed glial cultures containing 10 – 20% microglia, fluorescence of monochlorobimane (MCB), a marker of GSH, declined by around 40% in microglia following treatment with LPS and IFNγ (Chatterjee *et al.* 2000; Noack *et al.* 2000), whilst GSH levels in the co-cultured astrocytes remained stable. Inhibition of iNOS blocked the GSH decline. GSH levels in the BV-2 and N11 microglial cell lines were also shown to decline in the presence of LPS and IFNγ, which was prevented by iNOS inhibition in the case of N11 cells, but not BV-2 cells (Moss and Bates 2001). Biochemical determination of GSH levels in N9 cells also demonstrated a decrease following LPS and IFNγ treatment (Roychowdhury *et al.* 2003), which was shown to be limited to the cytosolic portion of GSH, while

mitochondrial GSH levels remained stable. Conversely, Dopp *et al.* (2002) demonstrated an increase in microglial GSH levels over time in primary rat microglia in the presence of TNFα. This result was corroborated by Persson *et al.* (2006) in primary rat microglia treated with LPS or TNFα, where GSH levels were determined by two independent methods.

Macrophages have been shown to release thiols (Watanabe and Bannai 1987), and more recently, GSH specifically (Sato *et al.* 2001). Although one recent study (Hirrlinger *et al.* 2002c) failed to detect GSH release by microglia, the conditions used were optimised for astrocytes (Dringen *et al.* 1997, 1999b; Stewart *et al.* 2002; Gegg *et al.* 2005; Minich *et al.* 2006; Frade *et al.* 2008) and may not have favoured the detection of microglial GSH release. Microglia have been shown to express functional Mrps (Ballerini *et al.* 2002; Dallas *et al.* 2003); microglial expression of Mrp1 is well documented (Ballerini *et al.* 2002; Hirrlinger *et al.* 2002a; Dallas *et al.* 2003), and other Mrp subtypes may also be expressed (Ballerini *et al.* 2002; Hirrlinger *et al.* 2002a). Mrp1 can transport GSSG (Leier *et al.* 1996), glutathione conjugates (Loe *et al.* 1996) and drugs in cotransport with GSH (Zaman *et al.* 1995; Loe *et al.* 1998), as well as some drugs alone or conjugated or cotransported with other molecules (Konig *et al.* 1999; Leslie *et al.* 2001). Indeed, vincristine export by microglia of the MLS-9 cell line has been shown to depend upon intracellular GSH, suggesting cotransport via Mrp1 (Dallas *et al.* 2003). Microglia therefore may have the ability to release GSH or glutathione conjugates via Mrp1. Unlike astrocytes, microglial γGT activity is low or absent (Murata *et al.* 1997; Ruedig and Dringen 2004), suggesting that should microglia release GSH, it may have a different function from astrocyte-derived GSH.

1.6. Aims

Microglia are implicated in the pathogenesis of neuroinflammatory conditions such as AD and MS. However, microglial activation may have positive or negative outcomes in terms of the disease course and neuronal fate. The GSH system of microglia is yet to be fully understood. Microglial GSH levels may impact upon microglial survival, in that GSH depletion may leave the cell vulnerable to oxidative damage and death. This may affect the neuroprotective or neurotoxic properties of microglia; for example the death of microglia chronically producing ROS and RNS may limit the production of such species and therefore the associated neurotoxicity. Alternatively microglial GSH depletion and death may be detrimental to neurones.

Studies upon the effect of activation upon microglial glutathione levels have produced conflicting results; one aim of this thesis is to resolve this and to extend the investigation to proteins associated with neuroinflammatory disease, as published reports have limited study to the effects of LPS and IFNγ, and TNFα. Whether or not microglia have an ability to release GSH is also disputed, although the likely function of such release is unclear as unlike astrocytes, microglia do not possess the transferase γGT to provide neuronal GSH precursors. A second aim of this thesis is to clarify whether or not microglial glutathione release occurs *in vitro* and to investigate the effect of microglial activation upon release.

Changes in microglial GSH levels may relate to changes in the expression of glutamate transporters, as these supply glutamate and cystine for microglial GSH synthesis. However, alterations in glutamate transporter expression or activity may have effects upon the amino acid constitution of the extracellular environment and consequences for other cells. Therefore a third aim of this thesis is to investigate the expression of glutamate transporters by microglia *in vitro* in the presence of LPS and disease-related microglial activators, and the consequences of such expression upon glutamate levels.

Modulation of microglial mGluRs has been shown to affect the neuroprotective or neurotoxic nature of microglia *in vitro*. The final aim of this thesis is to investigate

whether such mGluR modulation also affects microglial GSH levels, transporter expression and glutamate release, as this may provide further insight into the mechanisms of neuroprotection and neurotoxicity.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Animals

Wistar rat pups were obtained from Institute of Neurology in-house colonies and Sprague-Dawley rat pups were obtained from University College London in-house colonies.

2.1.2 Reagents

Gibco brand D-MEM and MEM powder, phenol red-free D-MEM, FBS, NCS, amphotericin B fungicide and EBSS were obtained from Invitrogen (Paisley, UK). DHPG, tADA, AIDA, DCG IV, MCCG, L-AP4, MAP4, (RS)-PPG, SIB-1757, APICA, DHK, CNQX, MK-801 and AMT-HCl were obtained from Tocris (Bristol, UK). Orthophosphoric acid, concentrated HCl, methanol and Y27632 were obtained from VWR International (Leicestershire, UK). Absolute ethanol was obtained from the in-house pharmacy or from VWR. CDPPB, MTEP and L-cystine were obtained from Calbiochem, Merck Chemicals (Nottingham, UK). IFNγ was obtained from R&D Systems (Oxfordshire, UK). CGA was obtained from Scientific Marketing Associates (Herts, UK). $A\beta_{25-35}$ was obtained from Bachem (Merseyside, UK). Pierce BSA standard for the protein assay was obtained from ThermoFisher Scientific (Loughborough, UK). The GSH-Glo glutathione assay kit was obtained from Promega UK Ltd. (Southampton, UK) and the ATP assay kit from Roche Diagnostics (West Sussex, UK). Rabbit anti-mouse iNOS antibody was obtained from BD Transduction Laboratories (Oxford, UK) and goat-anti rabbit TRITC-conjugated antibody was obtained from Sigma-Aldrich (Dorset, UK). Vectashield mountant was obtained from Vector Laboratories (Peterborough, UK). TRIzol® reagent, Tris/borate/EDTA buffer, MMLV reverse transcriptase, $oligo(dT)_{12-18}$ primer, RNase OUT, dNTPs and *Taq* DNA polymerase was obtained from Invitrogen and DNA ladders and gel loading dye from Promega. Oligonucleotide primers for β-actin, rat EAAT1, rat EAAT2 and mouse xCT were a kind gift from Dr Marcus Rattray, Kings College London. Duplicates of these, and other primers were obtained from Sigma Genosys. Antibiotics for tissue culture, Percoll®, poly-D-

lysine, accutase®, LPS, fraction V albumin, NAAG, N-acetyl-L-cysteine, L-cysteine, fibrin, fibrinogen, aminopimelic acid, aminoadipic acid, 1400W, GSH, GSSG, glutathione reductase, monochlorobimane, ethacrynic acid, L-glutamate dehydrogenase, pyruvate kinase, phosphoenol pyruvate, GABA, ATP, ADP, lactate dehydrogenase, propidium iodide, agarose, Coomassie reagent for Bradford protein assay, and all other chemicals were obtained from Sigma Aldrich.

2.1.3 Consumables

Filtration units for cell culture, syringes, glass Pasteur pipettes, 13 mm glass coverslips, 0.5 ml tubes and TriGene Green disinfectant were obtained from Scientific Laboratory Supplies (SLS; Nottingham, UK). Sterile Petri dishes for cell culture were obtained from Marathon Laboratory Supplies (London, UK). Six and 24 well tissue culture plates and tissue culture flasks were obtained from Triple Red (Bucks, UK). Sterile 50 ml centrifuge tubes were obtained from Triple Red and SLS. Cell scrapers were obtained from Marathon and Helena Biosciences (Tyne & Wear, UK). Razor blades were obtained from Blademail (Norfolk, UK). Glass slides, syringe filters, sterile serological pipettes, sterile 15 ml centrifuge tubes, 96 well plates, 1.5 ml tubes and latex gloves were obtained from VWR International (Leicestershire, UK). Pipette tips were obtained from VWR International, SLS or Anachem (Luton, UK). RNase-free pipette tips were obtained from Starlab (UK) Ltd. (Milton Keynes, UK). Eppendorf brand safe-lock 1.5 ml and 2 ml tubes for molecular biology were obtained from Helena Biosciences. PCR 0.2 ml tubes and caps in strips were obtained from Applied Biosystems (Warrington, UK). Molecular BioProducts brand RNase away RNase inhibitor spray for surfaces and gloves was obtained from ThermoFisher Scientific (Loughborough, UK). Chromacol brand vials and lids for HPLC were obtained from VWR International.

2.2 Methods

2.2.1 Cell culture

All cell medium was made up using distilled water (dH_2O) which was filtered to 0.2 μm and UV-treated. Once prepared, medium was filtered to 0.2 μm again to sterilise. A Heraeus HeraSafe class II safety cabinet and Heraeus HeraCell Incubators were used for cell culture. Cell culture cabinets and incubators were cleaned regularly with TriGene disinfectant and all items placed in hoods and incubators were sprayed with 70% ethanol to maintain the sterile environment.

2.2.1.1 Culture of primary rat microglia

All tools used in the dissection of brains during preparation of primary cells were autoclaved and then sterilised under UV light before use. Razor blades were flamed thoroughly until glowing red-hot and only used once.

2.2.1.1.1 Percoll gradient method

Initially, primary rat microglial cells were obtained by a method devised in this laboratory (Kingham *et al.* 1999). Post-natal day 3-5 Wistar rat pups were killed by cervical dislocation and decapitation in accordance with the Scientific Procedures Act 1986 (United Kingdom). Brains were removed and all but the cerebellae were transferred immediately to ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 5.37 mM KCl, 5.65 mM $NaH_2PO_4.H_2O$, 13.3 mM $Na_2HPO_4.7H_2O$, 11.1 mM D-glucose, 0.02% bovine serum albumin (BSA), 100 $units.ml^{-1}$ penicillin, 0.1 $mg.ml^{-1}$ streptomycin, 3 $\mu g.ml^{-1}$ ampicillin, pH 7.4, made up in filtered, UV-treated dH_2O). Brains were rinsed and homogenised in PBS and split between 50 ml centrifuge tubes such that there were approximately 3 brains per tube. Each tube was topped up to 40 ml with PBS and centrifuged in an Eppendorf 5804R benchtop centrifuge at 4500 rpm (3645 *g*) for 10 min. Supernatants were removed and discarded and the pellets were resuspended in 70% Percoll in PBS. A Percoll gradient was set up with 15 ml 70% Percoll in PBS, overlaid with 12 ml 30% Percoll in PBS and finally 5 ml

PBS, again with the equivalent of approximately 3 brains per tube. The gradients were centrifuged at 4000 rpm (2880 *g*) for 50 min to separate the microglia from the rest of the brain matter. Following this centrifugation, microglia were collected from the interface between the 70% Percoll and 30% Percoll layers (see fig. 2.1) and transferred to fresh PBS.

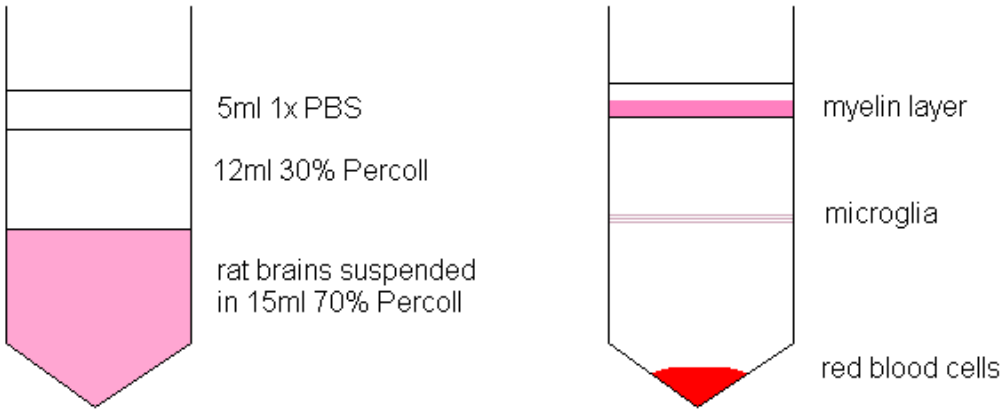


Figure 2.1. *Separation of microglia from the rest of the brain matter using a Percoll gradient.*

Microglia were pelleted by centrifuging at 4800 rpm (4147 *g*) for 10 min and pellets were resuspended in microglial medium (glutamine-free minimum essential medium (MEM) with Earle's salts supplemented with 10% foetal bovine serum (FBS), 21.5 mM KCl, 33.3 mM D-glucose, 2.05 mM glutamine, 100 units.ml^{-1} penicillin, 100 μg.ml^{-1} streptomycin, 100 μg.ml^{-1} kanamycin and 50 μg.ml^{-1} gentamicin). Cells were counted with trypan blue and plated at 1.2 x 10^5 cells on 13 mm glass coverslips or 4.8 x 10^5 cells per well in 35 mm culture dishes in a small volume (100 μl on 13 mm coverslips or 400 μl in 35 mm dishes). Cells were left to adhere in an incubator at 37°C in a humidified atmosphere of 6% CO_2 in air for 45 minutes, then washed twice with microglial medium (as above) and replaced in the incubator with a larger volume of medium; approximately 500 μl MEM per 13 mm coverslip or 2 ml MEM per 35 mm dish. Two to 3 hours later microglia were washed again, as before, and

left in the incubator overnight. Cells were washed once more the following morning and used within 48 hours.

It has previously been demonstrated by immunocytochemistry, using antibodies directed against the microglial antigen OX-42 and the astrocyte antigen glial fibrillary acidic protein (GFAP), as well as the microglial marker *Bandeiraea simplicifolia* isolectin B4, that up to 4 days *in vitro* (DIV), primary microglia cultures prepared using the Percoll gradient method devised in this laboratory (Kingham *et al.* 1999) contain consistently > 90% microglia, with the majority of the contaminating cells being astrocytes (Kingham *et al.* 1999;Morgan *et al.* 2004). In addition it was shown that at 2 DIV < 20% microglia stain positively with the ED1 antibody, indicating a low level of basal activation (Morgan *et al.* 2004).

2.2.1.1.2. Mixed glial culture method

The above method, utilising a Percoll gradient to isolate microglia, has been used in this laboratory for almost ten years. However, in order to obtain oligodendrocytes from the same tissue, we have devised another protocol, following the more widely-used method involving establishing a mixed glial culture and then removing the microglia by shaking. This also has the advantage of a higher microglial yield. The method used here is loosely based upon that devised by (Armstrong 1998). Oligodendrocytes can be removed after the microglia by further, more vigorous shaking.

2.2.1.1.2.1 Coating culture flasks with poly-D-lysine

Four millilitres of poly-D-lysine (PDL) at 100 $\mu g.ml^{-1}$ in sterile filtered, UV-treated dH_2O was added to each 75 cm^2 tissue culture flask and incubated at room temperature in a tissue culture hood for 2 hours. The excess liquid was then aspirated and the flasks left in the tissue culture hood with their caps off until completely dry. PDL-coated flasks were stored at -20°C and placed in the incubator at 37°C approximately one hour before use.

2.2.1.1.2.2 Preparation of primary mixed glial cultures

Eight post-natal day 2 Sprague-Dawley rat pups were killed by cervical dislocation and decapitation in accordance with the Scientific Procedures Act 1986 (United Kingdom). Brains were removed and immediately transferred to a dish of ice-cold L-glutamine-free MEM with Earle's salts, supplemented with 100 units.ml^{-1} penicillin, 100 μg.ml^{-1} streptomycin and 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (HEPES-MEM). Brains were then dissected one at a time in a separate dish. The cerebellum and brainstem were removed and discarded and the brain divided into the two hemispheres. The mid-brain was also removed and discarded and the two cortical hemispheres placed in a fresh dish of HEPES-MEM. The cortices were then mechanically dissociated using a syringe and needles of progressively smaller gauges (19G, 23G, 25G). Once fully homogenised, the tissue was placed in a 50 ml centrifuge tube and made up to 50 ml with HEPES-MEM. This was centrifuged in an Eppendorf 5804R benchtop centrifuge for 10 minutes at 230 *g* to loosely pellet the cells. The pellet was resuspended in pyruvate-free Dulbecco's Modified Eagle Medium (D-MEM) containing 4500 mg/l D-glucose, supplemented with 10% FBS, 1 mM sodium pyruvate and 25 μg.ml^{-1} gentamicin. The cells were divided between three PDL-coated 75 cm^2 tissue culture flasks (see 2.2.1.1.2.1 above), each containing approximately 15 ml D-MEM (as before). Cultures were maintained at 37°C in a humidified atmosphere of 6.0% CO_2 in air. The medium on the glial cultures was changed on day 3, day 6 and day 10.

2.2.1.1.2.3 Isolation of microglia

By day 13, microglia were clearly visible as the smallest, brightest cells within the glial cultures. Microglia were removed by shaking at 125 rpm on a Stuart Mini Orbital Shaker model SSM1 for 4-5 hours in a Hybaid Midi Dual 14 oven maintained at 37°C (Sierra *et al.* 2008). The medium was then removed and centrifuged at 4500 rpm (3645 *g*) for 5 min and the pellet resuspended in a small volume of D-MEM (as before). Cells were counted using a haemocytometer and plated at 1 x 10^5 cells on 13 mm glass coverslips or at 5-6 x 10^5 cells per well in 35 mm culture dishes or at 1 x 10^6 cells per well in 60 mm culture dishes in a small

volume (50 µl on 13 mm coverslips, 400 µl in 35 mm dishes, 800 µl in 60 mm dishes) and placed in the incubator to adhere for 1 hour. Further D-MEM was then added to give approximately 500 µl per 13 mm coverslip or 2 ml per 35 mm dish. The following day, the medium was changed and compounds added as appropriate. Primary microglia obtained in this manner were always used within 3 days of removal from the mixed glial culture.

Staining using the microglial marker *Bandeiraea simplicifolia* isolectin B4 demonstrated that microglia obtained from three different preparations were 96.3 ± 1.2 % pure, with the majority of the contaminating cells being of the oligodendrocyte lineage (Ioanna Sevastou, personal communication).

2.2.1.2. Microglial cell lines

Because of the difficulties of obtaining large numbers of primary microglia and culturing them in a "ramified" state, and as a means to minimise the use of animals in experiments, immortalised cell lines are often used to represent cultured microglia. The mouse microglial cell lines BV-2 and N9 are two of the most commonly used as models of primary microglia in culture. Of the two, the BV-2 cell line appears to be more frequently used in published studies, particularly in recent years (128 references found for BV-2 microglial cells in NCBI PubMed in 2008 and 2009 to date, compared with 21 references for N9 cells).

The BV-2 cell line was originally derived from mouse microglial cultures obtained from newborn animals of the C57BL/6 strain, transformed with the v-raf and v-myc oncogenes of the J2 retrovirus (Blasi *et al.* 1990). BV-2 cells have been shown to resemble primary microglia morphologically, electrophysiologically and functionally. BV-2 cells phagocytose inactivated yeast cells and latex beads and maintain the constitutive and inducible secretory properties of primary microglia. In addition, this phenotype is maintained over time in *in vitro* culture (Blasi *et al*. 1990; Bocchini *et al.* 1992). A more recent study strengthened these original findings, demonstrating that the genes upregulated by LPS in BV-2 microglia mirror those upregulated in primary microglia, although the upregulation in BV-2 cells was often less pronounced (Lund et al. 2006).

The N9 cell line was derived originally from microglial cultures from embryonic day 13 outbred CD1 mice, transformed with the v-myc oncogene from the 3RV retrovirus (Corradin *et al.* 1993). N9 microglia have been shown to respond to classical microglial activating stimuli such as LPS, IFNγ and TNFα with NFκB expression, and release of NO, TNFα and chemokines, in a similar way to primary microglia (Corradin *et al.* 1993; Meda *et al.* 1995; Bonaiuto *et al.* 1997). Although it has been claimed that N9 cells may not be phagocytically active (Zhang *et al.* 2003), phagocytosis of E. Coli bioparticles (Stefano *et al.* 2009), latex beads (Bruce-Keller *et al.* 2001), dextran beads and myelin (Pinteaux-Jones 2007) by N9 microglia has been demonstrated in recent years.

Both BV-2 (Moss and Bates 2001) and N9 (Roychowdhury *et al.* 2003) cell lines have been previously utilised in studies investigating microglial glutathione levels, although in neither case were results compared with those for primary microglia.

2.2.1.2.1. Culture of the BV-2 mouse microglial cell line

The BV-2 mouse microglial cell line was a kind gift from Dr. Claudie Hooper (MRC Centre for Neurodegenerative Research, Institute of Psychiatry, Kings College London, UK) and originally obtained from Dr. FS Tzeng (Department of Life Sciences, National Cheng Kung University, Taiwan). BV-2 microglial cells were cultured in pyruvate-free D-MEM with 4500 $mg.L^{-1}$ glucose, supplemented with 10% FBS, 2 mM glutamine, 100 $units.ml^{-1}$ penicillin, 100 $\mu g.ml^{-1}$ streptomycin and 125 $ng.ml^{-1}$ amphotericin B fungicide, at 37°C in a humidified atmosphere of 6.0% CO_2 in air. Medium was changed at least weekly and when confluent, cells were removed from the flask using 2 ml 400-600 $U.ml^{-1}$ accutase in Dulbecco's PBS containing 0.5 mM EDTA and 3 $mg.L^{-1}$ phenol red. Cells were pelleted by centrifugation in an Eppendorf 5804R benchtop centrifuge at 4500 rpm (3645 *g*) for 5 min. Cells were counted using a haemocytometer and plated as required at 0.5-2 x 10^4 cells on 13 mm glass coverslips and at 5-10 x 10^4 cells per well in 35 mm culture dishes, and typically used after 2-3 DIV.

2.2.1.2.2. Culture of the N9 mouse microglial cell line

The N9 microglial cell line was a kind gift from Dr. Paola Ricciardi Castagnoli (CNR Cellular and Molecular Pharmacology Centre, Milan, Italy). N9 mouse microglial cells were cultured in pyruvate- and $NaHCO_3$-free Dulbecco's modified Eagle medium (D-MEM) with 4500 $mg.L^{-1}$ glucose, supplemented with 5% newborn calf serum (NCS), 4.4 mM $NaHCO_3$, 57.2 μM β-mercaptoethanol, 50 $units.ml^{-1}$ penicillin, 50 $\mu g.ml^{-1}$ streptomycin, 100 $\mu g.ml^{-1}$ kanamycin and 50 $\mu g.ml^{-1}$ gentamicin. Medium was changed at least weekly and when confluent cells were scraped from the flask and pelleted by centrifugation in an Eppendorf 5804R benchtop centrifuge at 4500 rpm (3645 *g*) for 5 min. Cells were counted using a haemocytometer and plated as required at 1-2 x 10^4 cells on 13 mm glass coverslips and at 8-10 x 10^4 cells per well in 35 mm culture dishes, and typically used after 2-3 DIV.

2.2.2 Determination of intracellular reduced glutathione levels with reverse-phase HPLC

2.2.2.1 Principle

Reduced glutathione (GSH) was separated from other constituents of samples by means of reverse-phase high performance liquid chromatography (HPLC). In this method the mobile phase, here 15 mM orthophosphoric acid (OPA), is forced through a chromatography column (the stationary phase) under high pressure. The column contains C_{18} chains (octadecasilyl) bonded to microscopic silicone beads. Hydrophobic interactions with the C_{18} chains retard the progress of molecules as they are forced through the column. Time taken for a given molecule to be eluted from the column (its retention time) depends upon its hydrophobicity and is identical under the same conditions.

An electrochemical method of detection was used to quantify GSH levels of samples (Riederer *et al.* 1989). Eluent from the column passes into the electrical cell (model 5010), part of the Coulochem II electrochemical detector (ESA Analytical,

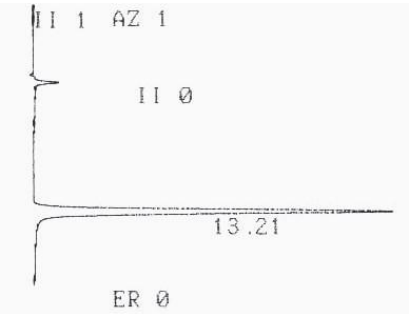
Aylesbury, UK). The porous graphite analytical cell contains two electrodes in series, set to constant voltages optimised for the molecule being studied. At each electrode, molecules within the eluent are oxidised, and the electrons released produce a current. The voltage at the upstream electrode (E1) is below that which the molecule of interest will oxidise to eliminate the more readily oxidised species within the sample. The downstream electrode (E2) is set to a voltage at which the molecule of interest readily oxidises, and is connected to the integrator, which draws a chromatograph representing the current produced at E2 in real time. Molecules are identified by their retention time; the peak height at this time corresponds to the current they induce at E2, which is directly proportional to the concentration of the molecule of interest. Figure 2.2 shows some typical chromatograms.

A suitable voltage at E2 for a molecule of interest is determined by means of a voltammogram; a standard concentration of the molecule of interest is run through the column at a range of different voltages, and peak height is plotted against voltage (fig. 2.3). The optimal voltage is that which gives a maximal or almost maximal peak height whilst keeping background current low and producing a clean, narrow peak on the chromatograph.

2.2.2.2 Preparation of cells

Cells were cultured in 35 mm dishes, and following treatment of cells as desired, medium was removed, and cells were gently rinsed with phosphate-buffered saline (PBS; 154 mM NaCl, 1.84 mM KH_2PO_4, 9.81 mM $K_2HPO_4.3H_2O$, pH adjusted to 7.2-7.3 with further addition of $KH_2PO_4/K_2HPO_4.3H_2O$ as necessary). Cells were detached by incubation for 5 minutes with 200 µl 400-600 $U.ml^{-1}$ accutase in Dulbecco's PBS containing 0.5 mM EDTA and 3 $mg.L^{-1}$ phenol red, transferred to 1.5 ml tubes and pelleted by centrifugation in a Jouan A14 microcentrifuge at 7000 rpm (2465 *g*) for 10 minutes. Pellets were resuspended in Earle's balanced salt solution (EBSS), typically 20 µl, 5 µl of which was then removed for determination of protein content (see 2.2.11). The remainder was snap frozen on dry ice and stored at -80°C.

A



B

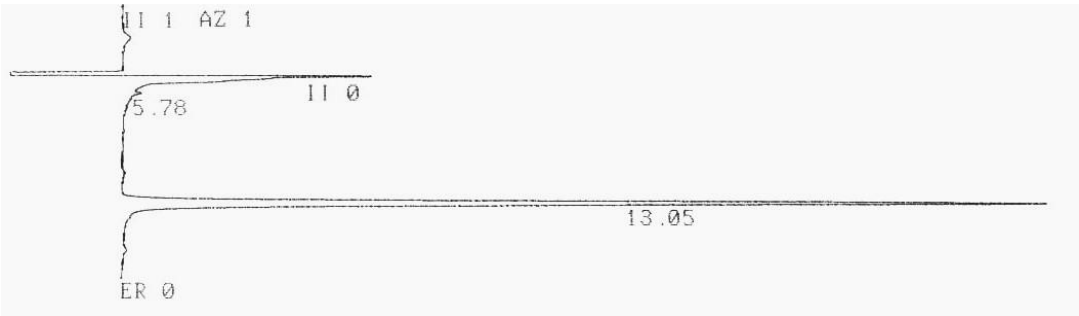


Figure 2.2. *Examples of chromatograms produced following injection of 5 µM reduced glutathione (**A**) and a sample from activated BV-2 cells (**B**), with E1=100mV and E2=600mV. The retention time of reduced glutathione is just over 13 minutes. The peak at around 5 minutes in (**B**) may be due to components present in the Earle's balanced salt solution (EBSS) in which the BV-2 sample is resuspended. Note also the slightly less stable baseline in (**B**) which may be due to small amounts of other compounds in the sample.*

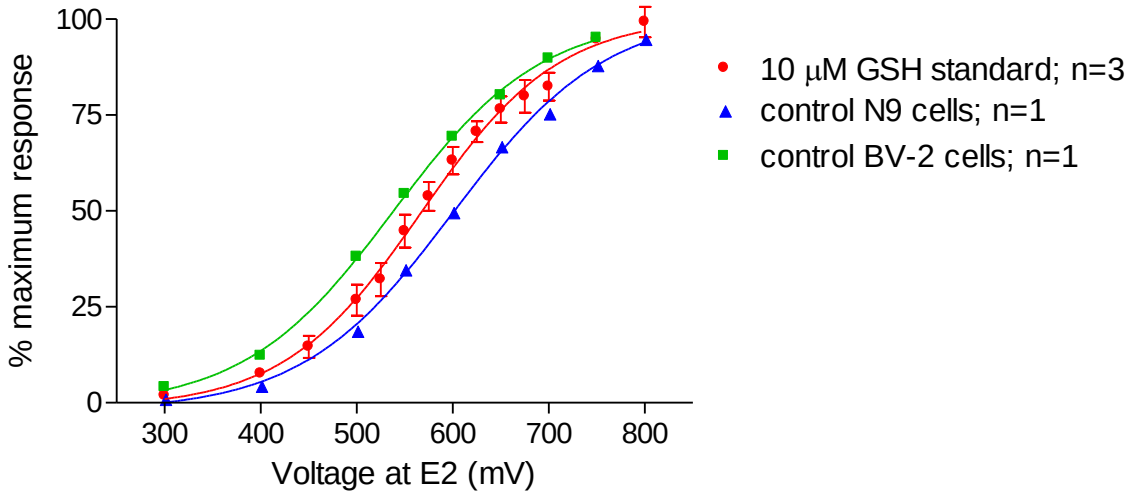


Figure 2.3. *Voltammogram constructed using 10 μM reduced glutathione (GSH) standard (red). GSH chromatograms with a sample from each of control N9 cells (blue) and BV-2 cells (green) are also shown to demonstrate that the shape of the chromatogram for GSH with this setup remains the same. The voltage at E1 was maintained at 100 mV throughout. Here the optimum voltage at E2 is 650 mV as this gives a near-maximal response, and at higher voltages the background current was too high to be used routinely and the chromatography became noisy.*

2.2.2.3 Measurement of reduced glutathione

The GSH concentrations of samples were measured electrochemically following separation by reverse-phase HPLC, using a method based on that of Riederer *et al.* (1989) (Gegg *et al.* 2005). The mobile phase was 15mM OPA, made up in water purified to an electrical resistance of 18.2 Mohms.cm^{-1} (PureLab Maxima, Elga, Derbyshire, UK). Samples were thawed and GSH was extracted by the addition of a suitable volume of 15 mM OPA (185 µl-985 µl) followed by thorough vortexing. Protein was pelleted by centrifugation in an Eppendorf 5415R centrifuge at 10 000 *g* for 5 minutes. The supernatant was injected into the HPLC, either by means of a Hamilton syringe (Sigma Aldrich, Dorset, UK) and Rheodyne manual injector (20 µl injection loop) or a Jasco autosampler (model AS-2055 Plus; Jasco, Essex, UK), which injected 50 µl. In either case the samples passed through a 3 mm x 10 mm guard column, and into a 4.6 mm x 250 mm analytical column packed with 5 µM octadecasilyl-silica (HPLC Technology Co Ltd, purchased from Chromacol Ltd, Hertfordshire, UK). Both columns were housed in a block heater (model 7970; Jones Chromatography, Mid Glamorgan, UK) and maintained at 30°C. The mobile phase was pumped through the system at 0.5 ml.min^{-1} by a Jasco PU-1580 pump (Jasco, Essex, UK). With this setup the pressure in the column was in the range 50 - 70 kg.cm^{-2}. With a new column, the retention time for GSH was around 13.7 minutes, electrode potentials were set at E1=100 mV and E2=450 mV, and the total run time used was 18 minutes. A Chromjet integrator (model SP4400; ThermoFinnigan, Thermo Fisher Scientific, Hemel Hempstead, UK), connected to the downstream electrode, recorded the generated current and produced chromatograms in real-time. Standards of two or three concentrations of GSH in 15 mM OPA were run within each experiment (fig. 2.4) to allow GSH concentrations of the samples to be calculated by comparison. These were corrected for sample dilution, normalised for protein content, and expressed as nmol.mg^{-1} protein.

Data produced by this method were relatively consistent in terms of percentage change from control with test conditions. However the actual values in nmol.mg^{-1} protein did show some degree of variation. This echoes the variability in reported values as mentioned in section 1.5.3. In any case, the importance of absolute values

of glutathione content of cells in culture is questionable, as it has been shown to be affected by culture conditions (Dringen *et al*. 1999b). Data are presented wherever possible in nmol.mg^{-1} protein, but values are not necessarily comparable between experiments. Therefore all necessary controls and comparison values were run in every experiment, and are not shared between figures. For example, where LPS was added in combination with other compounds, an LPS-only control was run in the same experiment rather than referring to earlier results for LPS-treated cells.

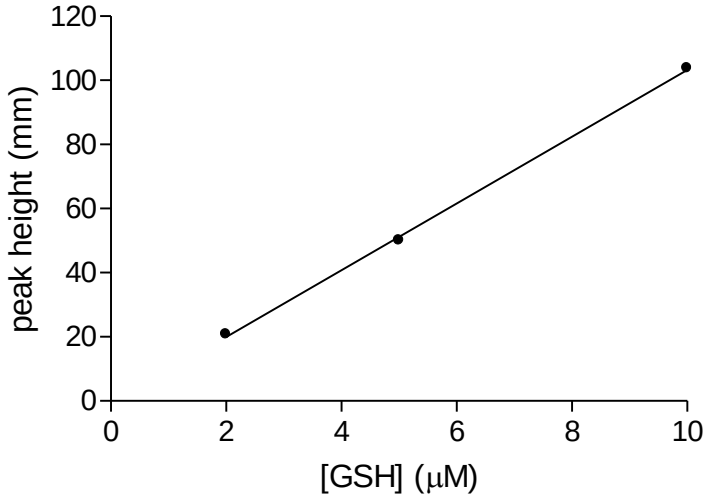


Figure 2.4. *Example of a typical reduced glutathione (GSH) reverse-phase HPLC standard curve constructed with 2 µM, 5 µM and 10 µM GSH in 15 mM OPA. In this case, r^2=0.9991.*

To check GSH recovery, some cells were scraped as normal and having been resuspended in EBSS, the suspension was split in two. To half was added a known concentration of GSH and to the other half an equivalent volume of EBSS. The two samples were then run through the assay. The efficiency of the GSH method is given by:

$$\text{Recovery} = \frac{\text{concentration difference according to chromatogram}}{\text{actual concentration difference}} \times 100$$

GSH recovery using this method was calculated to be 77.5 ± 3.4 % (n=6).

2.2.2.4 Measurement of oxidised and total glutathione

As well as the reduced form, GSH, oxidised glutathione (GSSG) may also be present in cells. To calculate total glutathione levels (i.e. GSH + GSSG), from which GSSG levels can be calculated, cells were prepared for HPLC as above, but following resuspension in EBSS samples were split in half. One half of each sample was used to measure GSH, as above. The other half was treated with glutathione reductase (GR; EC 1.6.4.2), in the presence of reduced nicotinamide adenosine dinucleotide phosphate (NADPH), in order to convert GSSG to GSH (Stewart *et al.* 2002). Samples were incubated at 37°C for 10 minutes, in a Techne Dri-Block model DB-2D block heater, with an equal volume of 100 mM KH_2PO_4, 3.4 mM EDTA buffer, pH 7.6, containing 500 μM NADPH and 2 units GR. Samples were snap-frozen on dry ice and GSH levels determined by HPLC exactly as above. The difference between total glutathione concentration and GSH concentration was used to calculate the concentration of GSSG.

The efficiency of the GR protocol in converting GSSG to GSH was calculated by preparing standards containing known concentrations of GSSG and following the above protocol. Alongside, GSH standards were prepared, frozen and run, following the protocol for samples for GSH measurement. These were used to calculate GSH method efficiency for the experiment (see above). GR protocol efficiency can then be calculated as follows:

$$\text{Efficiency} = \frac{\text{Percentage of GSSG detected by HPLC}}{\text{Efficiency of GSH protocol within same experiment}} \times 100\%$$

The efficiency of the GR method as used here was calculated to be 85.4 ± 4.8 % (n=4).

2.2.3 Determination of intracellular glutathione levels by imaging with monochlorobimane

Imaging of monochlorobimane (MCB) fluorescence was carried out to determine the cellular levels of GSH and as a comparative technique to HPLC determination (Chatterjee *et al.* 1999; Keelan *et al.* 2001). MCB is a non-fluorescent bimane, freely permeable across plasma membranes. It is conjugated to GSH by the endogenous enzyme glutathione S-transferase (GST; EC 2.5.1.18) to produce a fluorescent adduct, with an excitation wavelength of 380 nm and an emission wavelength of 485 nm. This provides a way to visualise GSH within live cells and to quantify the GSH concentration by measuring fluorescence intensity.

Cells were plated on coverslips in 24 well plates and allowed to reach no more than 50% confluency. An optimised concentration of MCB (50 μM for BV-2 microglial cells, 100 μM for primary microglia) was added directly to the media in the wells and the cells were returned to the incubator (37°C, 6.0% CO_2) for 30 minutes. Coverslips were carefully removed and rinsed gently in warmed basic medium (153 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES), 5 mM $NaHCO_3$, 1.2 mM Na_2SO_4, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, 5 mM glucose). An Olympus (Tokyo, Japan) IX70 inverted fluorescence microscope, with an Olympus UApo 40 x Oil Iris objective, was used in combination with Perkin Elmer Imaging Suite v4.0 software to quantify the fluorescence for analysis. The excitation wavelength used was 360 nm and emission > 490 nm was recorded. Identically treated coverslips were viewed with a Zeiss Axioskop 2 fluorescence microscope (Oberkochen, Germany), using a 40 x Neofluar objective, with an excitation wavelength of 365 nm and emission > 490 nm recorded. Images (as presented in figure 3.5B) were captured with a Zeiss AxioCam HRc camera and Zeiss Axiovision 3.1 software.

To check the specificity of MCB for GSH over other thiols, ethacrynic acid (EA), an inhibitor of GST, was used to block GS-MCB formation (Ploemen *et al.* 1990; Keelan *et al.* 2001). EA (500 μM) was added to BV-2 cells 10 minutes prior to MCB addition. Due to the higher MCB concentration used for primary microglial cells, a

higher EA concentration (1 mM) was necessary to inhibit MCB binding and eliminate fluorescence.

2.2.4 Determination of glutathione levels in cell medium using the GSH-Glo glutathione assay kit

The GSH-Glo glutathione assay kit (Promega) uses a two-step reaction system to quantify reduced glutathione using a luminescent signal (fig. 2.5). Luciferin is produced from luciferin-NT, a luciferin derivative, by glutathione S-transferase (GST; EC 2.5.1.18), but only in the presence of reduced glutathione (GSH). In the second reaction, stabilised luciferase from *Photinus pyralis* (EC 1.13.12.7) causes release of light by luciferin.

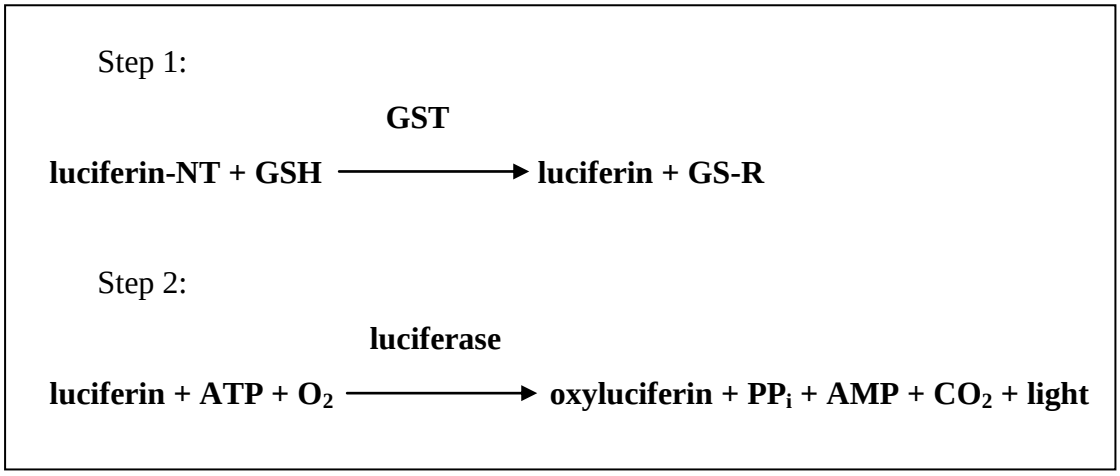


Figure 2.5. *The GSH-Glo glutathione assay uses a two-step process*

The experiment was performed according to the manufacturer's instructions. Microglial cells were plated and cultured until sufficiently confluent and medium was changed to serum-free, phenol red-free medium (which was otherwise identical to that used for routine culture), before being treated experimentally as required. At the end of the incubation period, medium was removed and centrifuged at 10 000 rpm (5031 *g*) for 2 minutes in a Jouan A14 microcentrifuge to pellet any cell debris.

50 µl of sample was combined with 50 µl of GSH-Glo reagent (supplied with kit), which contains luciferin-NT and glutathione S-transferase in 50 mM tricine buffer pH 7.9, in a well of an opaque white Packard 96-well polystyrene OptiPlate. This was mixed briefly on a plate shaker and incubated at room temperature for 30 minutes. One hundred microlitres of luciferin detection reagent (reconstituted with luciferin detection buffer according to the manufacturer's instructions) was then added to each well, before the plate was briefly shaken once more and incubated at room temperature for a further 15 minutes. Luminescence was recorded using a Tecan GENios plate reader. Glutathione concentrations of samples in each experiment were calculated using a standard curve which was run alongside samples (fig. 2.6).

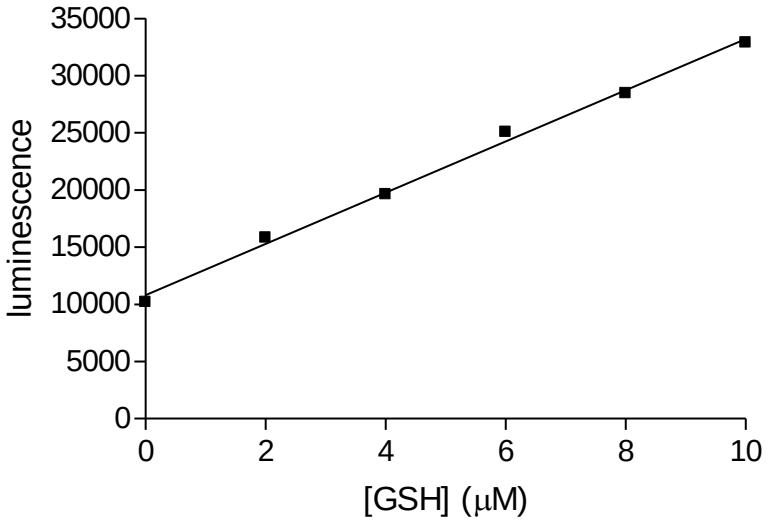


Figure 2.6. *Typical GSH standard curve produced using the GSH-Glo assay kit (r^2=0.9956).*

2.2.5. A comparison of the three methods used to measure glutathione

HPLC is often used to measure GSH concentrations in biological samples. It is well-known as being a sensitive and reliable method of quantification, quoted in one paper as being able to detect 0.5 µM GSH (Kamencic *et al.* 2000), and found in experiments conducted for this thesis, and elsewhere (Stewart *et al.* 2002; Frade *et al.* 2008) to reliably detect concentrations of 1 µM and below. This level of sensitivity allows robust detection of GSH in microglial cell extracts. However, the method is rather time-consuming, especially where an autosampler is unavailable. When measuring the GSH content of cultured cells, relatively large amounts of protein are required for each measurement, such as is easily obtainable when immortalised cell lines are used, but can pose a problem in the case of primary microglia.

It is difficult to assess the sensitivity of the monochlorobimane imaging method of GSH measurement in whole cells, as fluorescence is only expressed as arbitrary fluorescence units or relative to relevant controls. However, in previous studies microglial GSH has been robustly detected using MCB (Chatterjee *et al.* 1999, 2000). In tissue homogenates, a MCB fluorescence method has been shown to detect 0.5 µM GSH, suggesting a sensitivity comparable to that of HPLC (Kamencic *et al.* 2000). Imaging using MCB has the advantage of allowing simultaneous assessment and comparison of GSH levels in different cell types in co-culture (Chatterjee *et al.* 1999). It is however important to note that intracellular GS-MCB fluorescence may be affected by export via multidrug resistance-associated proteins (Mrps) (Waak and Dringen 2006).

According to the manufacturer, the GSH-Glo assay kit is able to detect GSH concentrations as low as 3 nM, and only small volumes of samples are required. Additionally, the assay is quick and simple to conduct. However, the running costs of such a kit-form assay are much higher compared with those of the other methods utilised here. Therefore the GSH-Glo assay kit is only used in this thesis to measure GSH levels in microglial conditioned medium, where it was not possible to use HPLC or MCB imaging.

2.2.6. Determination of glutamate levels in cell medium

2.2.6.1 Principle

Glutamate is the most abundant excitatory neurotransmitter in the mammalian nervous system. Its location must however be tightly regulated as continuous exposure of neurones to high levels of extracellular glutamate leads to excitotoxicity (Frandsen *et al.* 1989). Astrocytes play the major role in uptake of released glutamate via excitatory amino acid transporters (EAATs), (Okamoto and Quastel 1972; Storck *et al.* 1992; Pines *et al.* 1992). However, microglia have also been shown to have an impact on extracellular glutamate levels, (e.g. Noda *et al.* 1999; Nakajima *et al.* 2001b).

In vivo the enzyme L-glutamate dehydrogenase (EC 1.4.1.3) mediates the interconversion of L-glutamate and α-ketoglutarate (Fisher 1985) (fig. 2.7). The conversion of L-glutamate to α-ketoglutarate, by removal of the amino group, is part of the pathway converting nitrogen-containing proteins into suitable substrates for the tricarboxylic acid cycle in respiration. The reverse reaction allows incorporation of nitrogen into organic molecules to allow protein formation, and is the only source of *de novo* amino acid synthesis in mammals. Regulation of this reversible reaction can occur allosterically through relative abundance of adenine and guanine nucleotides; higher levels of ADP and GDP suggest energy depletion and therefore drive the catabolism of glutamate, while higher levels of ATP and GTP favour the inclusion of nitrogen for protein formation (Frieden 1959b; Cross and Fisher 1970).

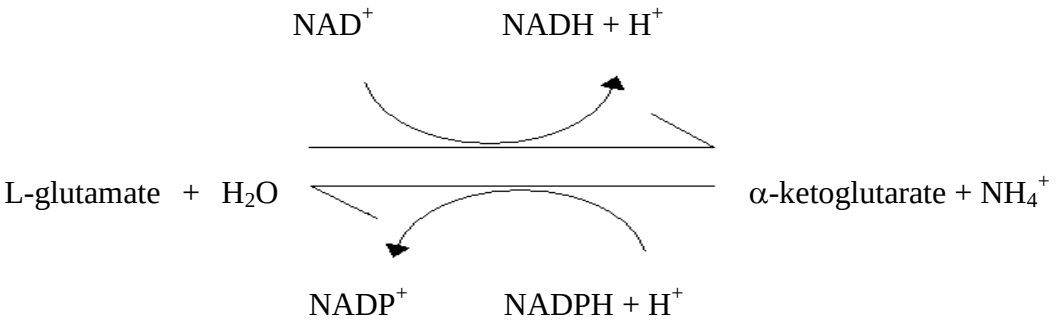


Figure 2.7. *L-glutamate dehydrogenase catalyses the conversion of L-glutamate into α-ketoglutarate.*

The preferred cofactor in the catabolism of glutamate is NAD^+, however $NADP^+$ can also be utilised (Frieden 1959a). When excited with light of wavelength 340 nm, NADH and NADPH fluoresce, whilst NAD^+ and $NADP^+$ do not. This phenomenon can be used to quantify the concentration of glutamate in a sample. Following a short incubation at 37°C in the presence of an excess of NAD^+ (or $NADP^+$) and L-glutamate dehydrogenase (GDH), fluorescence is directly proportional to the concentration of glutamate (Nicholls and Sihra 1986).

2.2.6.2 Optimisation of the existing method

Before using this assay it is important that it be validated in this laboratory for use with our samples of microglial conditioned medium (CM).

Standards of glutamate were prepared in basic medium (BM; 120 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES), 5 mM $NaHCO_3$, 1.2 mM Na_2SO_4, 5 mM glucose) and in serum-free culture medium (SFM). One hundred microlitres of each of these or of CM was incubated with 1 mM NAD^+ or $NADP^+$ and 10 – 50 U GDH at 37ºC for 2 - 12 minutes, made up to a total reaction volume of 1 ml with BM. A Techne Dri-Block model DB-2D block heater was used to maintain the incubation temperature. NADH or NADPH levels were determined using a Perkin-Elmer LS-2 B fluorimeter with excitation at 340 nm; emission greater than 430 nm or 460 nm was recorded.

Although the original assay was run using NAD^+ as the cofactor (Nicholls and Sihra 1986), a number of recent publications report the use of $NADP^+$ (Sim *et al.* 2006; Yang and Wang 2008; Kilbride *et al.* 2008), therefore these cofactors were first compared in the assay. Both were used at a concentration of 1 mM as this is commonly reported in the literature. Emission greater than 460 nm was recorded. Ten, 25 or 50 U GDH was used in 1 ml total volume. Fifty units per ml appears to be most commonly used for the fluorimetric detection of glutamate (Syed *et al.* 2007; Yang and Wang 2008), however 10 $U.ml^{-1}$ GDH has been used successfully in previous versions of this assay in this laboratory (Pinteaux-Jones *et al.* 2008).

It appears that either $NADP^+$ or NAD^+ may be used as a cofactor to give a standard curve with good linearity, and that in either case 1 mM is sufficient within the range of glutamate concentrations used (fig. 2.8). The regression coefficient (r^2) was consistently higher at each GDH concentration with NAD^+ rather than $NADP^+$. In addition, the signal in the presence of NAD^+ was also slightly higher. Therefore the assay was validated and used with NAD^+ (1 mM) as the cofactor. Interestingly, r^2 was also consistently higher with lower GDH concentrations. Although the fluorescence is higher with more GDH, the basal fluorescence is also increased. The reason for this is unclear; any glutamate present in the enzyme preparation would likely be metabolised by GDH prior to utilisation in the experiment. However, the inclusion of a control lacking glutamate, but including GDH, and the use of such a control as "zero" would eliminate the problem of any increase in the basal fluorescence.

Some samples of CM from control BV-2 microglia were also tested with the same three GDH concentrations (table 2.1). It is possible that some component of the medium could inhibit the enzyme or that GDH levels could become a limiting factor and mask higher glutamate concentrations. Treatment of BV-2 cells with LPS for 24 hours appeared to increase their glutamate release so that extracellular concentrations are 44-50% higher than control. Although there are differences between the glutamate concentrations calculated with different GDH concentrations, there does not appear to be a trend in either absolute concentration or in % increase with LPS treatment with increasing amounts of GDH, suggesting that these differences are not due to different GDH levels. Indeed, such differences due to sampling error are likely considering that this data derives from a single experiment. This data shows that 10 U GDH is sufficient for such samples, as using more of the enzyme had no effect on the outcome.

Although 460 nm is reported to be the peak of the emission of NADH (Lakowicz *et al.* 1992), the original paper on which this method is based (Nicholls and Sihra 1986) records emission above 430 nm. Therefore standard curves were run in parallel with emission above 460 nm and 430 nm recorded (fig. 2.9). The resulting curves have similar r^2 values, but the signal is substantially higher at 460 nm. Therefore 460nm will continue to be used in this assay.

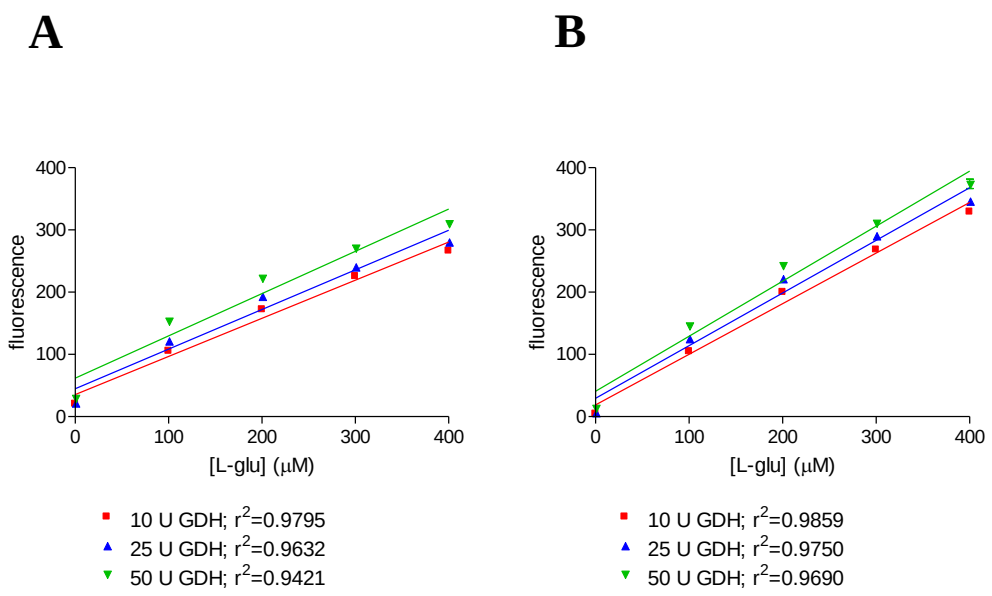


Figure 2.8. *L-glutamate standard curves prepared using different amounts of GDH in the presence of 1 mM $NADP^+$ (**A**) or 1 mM NAD^+ (**B**). Standard concentrations of L-glutamate (0 – 400 µM) were prepared in basic medium and 100 µl was incubated for 8 minutes at 37℃ with 1 mM $NADP^+$ and 10, 25 or 50 U GDH (final volume 1 ml). Samples were excited with light of wavelength 340 nm and emission greater than 460 nm was recorded. Data represent the results of two independent experiments, each consisting of two samples per condition.*

[GDH] (U)	[L-glu] in control CM (µM)	[L-glu] in LPS CM (µM)	% increase in [L-glu] with LPS
10	188.9	278.9	47.6
25	213.3	308.0	44.4
50	176.8	264.5	49.6

Table 2.1. *L-glutamate levels in samples of CM from control BV-2 cells and those treated with LPS (1 µg.ml^{-1}) for 24 hours, determined in each case by standard curves constructed using the same amount of GDH (see fig. 2.8). Samples (100 µl) were incubated for 8 minutes at 37ºC with 1 mM NAD^+ and 10 – 50 U GDH (total volume 1 ml). Data represent the results of a single experiment, consisting of two samples per condition.*

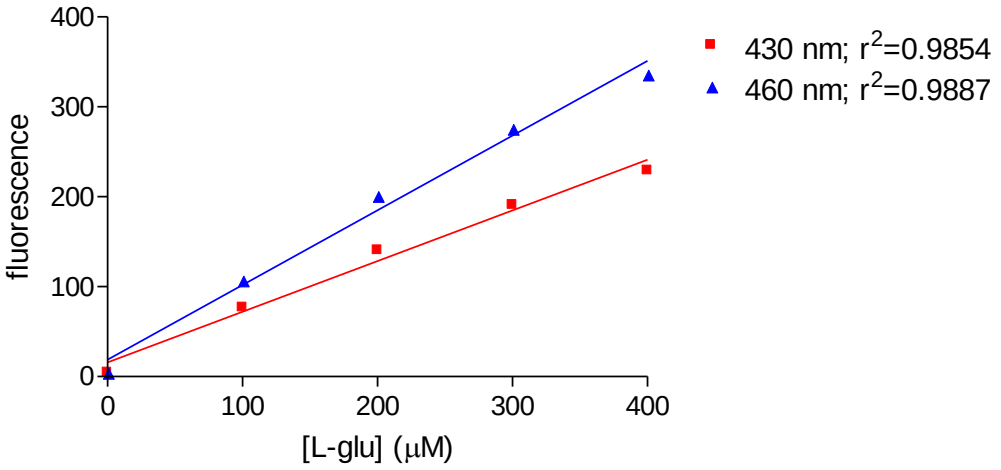


Figure 2.9. *L-glutamate standard curves prepared using emission wavelengths of 430 nm and 460 nm after excitation at 340 nm. Standard concentrations of L-glutamate were prepared in basic medium and 100 µl was incubated for 8 minutes at 37 ℃ with 1 mM NAD^+ and 10 U GDH (final volume 1 ml). Data represent the results of a single experiment, consisting of two samples per condition.*

It is also important to check that the 8 minute incubation used historically in this laboratory is sufficiently long to allow the reaction to run to completion and the fluorescence to plateau. Figure 2.10 shows a time course experiment run using a blank (BM with GDH and NAD^+), 200 µM glutamate, control BV-2 CM and CM from LPS-treated BV-2 cells (1 µg.ml^{-1} for 24 hours). This demonstrates that in all cases, 8 minutes is indeed a sufficient incubation time for the experiment.

The issue about the medium in which glutamate standards should be made also needs to be addressed, as it is not clear whether BM or culture medium has been previously used in this laboratory, and there is a chance that the components of culture medium, which would be present in test samples, could have a slight effect upon fluorescence. Therefore parallel standard curves were prepared in BM and serum-free BV-2 culture medium (SFM) (fig. 2.11), using all the standard conditions determined above. The standard curves are of approximately the same linearity and the slopes are very slightly different. SFM alone has a slightly higher fluorescence than BM

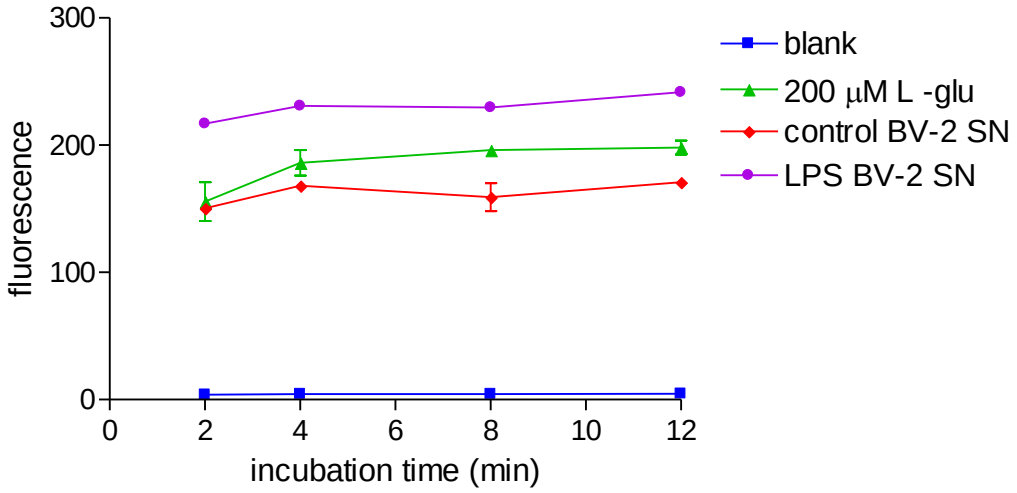


Figure 2.10. *Time course of GDH-catalysed reaction for samples of CM from control BV-2 cells and those treated with LPS (1 µg.ml^{-1}) for 24 hours. Samples or standards (100 µl) were incubated with 1 mM NAD$^+$ and 10 U GDH for 2 - 12 minutes at 37℃ (total assay volume, 1 ml). Samples were excited with light of wavelength 340 nm and emission greater than 460 nm was recorded. Data represent the results of a single experiment, consisting of two samples per condition.*

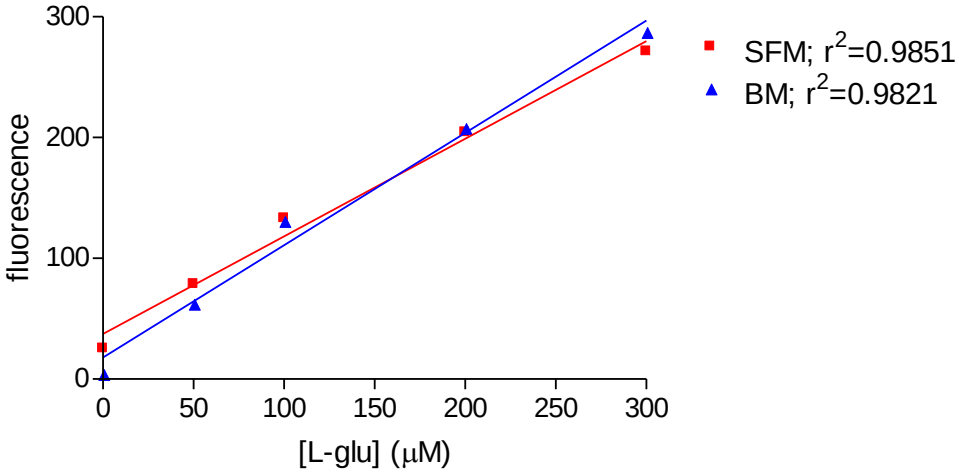


Figure 2.11. *L-glutamate standard curves prepared in SFM and BM. Samples (100 µl) were incubated with 1 mM NAD$^+$ and 10 U GDH for 8 minutes at 37℃ (total assay volume, 1 ml). Samples were excited with light of wavelength 340 nm and emission with a wavelength greater than 460 nm was recorded. Data represent the results of a single experiment, consisting of two samples per condition.*

alone, possibly due to the phenol red present. BM containing higher (300 μM) concentrations of glutamate gave a slightly higher reading than SFM containing the same glutamate concentration. However, the standard curves do lie along very similar lines, especially between 100 μM and 200 μM glutamate, the range in which most samples of conditioned medium tend to lie. Therefore it is probably equally valid to use a standard curve prepared in basic medium and one prepared in serum-free BV-2 culture medium.

It is important to note that serum-free culture medium has been used here. If samples of serum-containing CM are used, a sample of identical fresh culture medium needs should be taken in order to measure basal levels of glutamate. Serum contains glutamate and the amount probably varies between preparations of medium.

2.2.6.3 Optimised protocol

The presence of glutamate in culture medium was assessed by fluorometric quantification of NADH. Samples of conditioned medium from cultured cells were centrifuged at 10 000 rpm (5031 *g*) for 2 minutes in a Jouan A14 microcentrifuge to pellet any cell debris, and transferred to fresh tubes. Wherever possible, samples were assayed fresh, immediately upon removal from cells, but if necessary they were stored at -20°C. One hundred microlitres of each sample was incubated with 1 mM NAD^+ and 10 U GDH for 8 minutes at 37°C, made up to a total volume of 1 ml with BM. NADH levels were determined using a Perkin-Elmer LS-2 B fluorimeter with excitation at 340 nm; emission greater than 460 nm was recorded. A glutamate standard curve, constructed in basic medium, was run with each experiment. Blanks, consisting of basic medium with NAD^+ and GDH, allowed all other measurements to be corrected for any glutamate contamination of the reagents or basal fluorescence. In addition, a control sample of fresh medium identical to that in which cells were cultured was assayed for comparison with test samples. Triplicate readings were taken from each sample to increase accuracy and in most cases conditions were run in duplicate in each experiment.

2.2.7 Determination of nitrate/nitrite levels in cell medium as an indicator of iNOS activity

Nitric oxide (NO) is a highly reactive gas with diverse functions in mammalian cells. Originally described as endothelium-derived relaxing factor (Furchgott and Zawadzki 1980), it is a vasodilator in the cardiovascular system (Ignarro *et al.* 1987; Palmer *et al.* 1987), and, being small and readily diffusible, also acts as a signalling molecule, both intra- and intercellularly (e.g. Wood *et al.* 1990). As it is a highly reactive free radical, it is also employed defensively, being produced by macrophages and microglia in response to the presence of certain pathogenic components, such as LPS (Stuehr and Marletta 1985, 1987; Stuehr *et al.* 1989). NO is produced by the enzyme nitric oxide synthase (NOS; EC 1.14.13.39) through the oxidation of L-arginine to L-citrulline (Moncada *et al.* 1989). There are three different isoforms of this enzyme, the predominant one in microglia being inducible NOS (iNOS) (Forstermann *et al.* 1991; Hevel *et al.* 1991). NO is highly unstable, and undergoes a series of reactions with molecules present in biological fluids, with the stable end-products nitrate (NO_3^-) and nitrite (NO_2^-) (Marletta *et al*. 1988):

$2\ NO + O_2 \rightarrow 2\ NO_2$

$2\ NO_2 \rightarrow N_2O_4 \qquad N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^-$

$NO_2 + N_2O_4 + H_2O \rightarrow NO + 2\ NO_3^-$

$NO + NO_2 \rightarrow N_2O_3 \qquad N_2O_3 + H_2O \rightarrow 2\ NO_2^-$

The relative amounts of nitrate and nitrite produced depend upon the particular reactions taking place. Measuring the total nitrate and nitrite levels therefore represents the best indication of NO concentration, and may be viewed as an indirect indication of NOS activity.

Here nitrate was reduced to nitrite with acidified vanadium (III) chloride and nitrite was detected using the Griess reaction using an adaptation of the method of Miranda *et al.* (2001).

Samples of conditioned medium from cultured cells were centrifuged at 10 000 rpm (5031 *g*) for 2 minutes in a Jouan A14 microcentrifuge to pellet any cell debris, transferred to fresh tubes and stored at –20°C until needed. The reaction was run in 96 well plate format and standard curves of 0-100 µM sodium nitrate and sodium nitrite were run on each plate (fig. 2.12). Nitrate concentrations of samples were corrected for the efficiency of conversion of nitrate to nitrite, as calculated from the standard curves. All samples and standards were run in triplicate. Ten microlitres of 80 $mg.ml^{-1}$ vanadium (III) chloride in 10 M HCl was added to 200 µl of standard/sample in each well. One hundred microlitres of Greiss reagent (0.1% (w/v) N-(-1-naphyl)-ethylenediamine and 1% (w/v) sulphanilamide in 5% H_3PO_4) which reacts with nitrite to give a pink colour, was added to each well and plates were incubated at room temperature for 30-45 minutes in the dark. Absorbance was measured at 540 nm using an Anthos HT11 microplate reader.

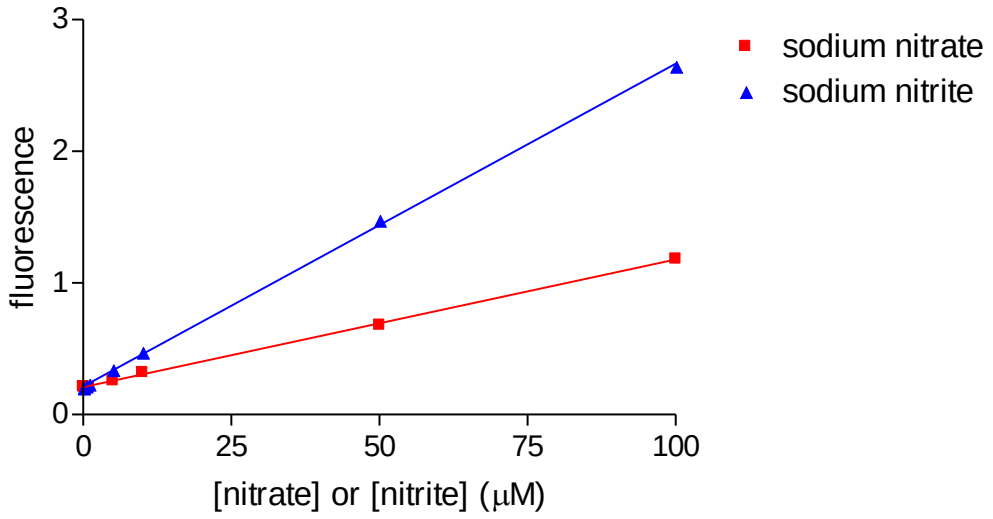


Figure 2.12. *Typical sodium nitrate and nitrite standard curve (r^2=0.998 for nitrate; r^2=0.9995 for nitrite). Here there is an average conversion efficiency of nitrate to nitrite of 39.8%.*

2.2.8 Measurement of intracellular ATP and ADP levels

2.2.8.1 Measurement of ATP levels

ATP is the universal energy store, providing energy for all active cellular processes through the cleavage of its two high energy phosphoanhydride bonds. There is a high turnover of ATP in the cell, and it is continually regenerated from ADP and AMP. ATP depletion is characteristic of cells under stress.

The luciferase from *Photinus pyralis* (EC 1.13.12.7) catalyses the following reaction:

ATP + D-luciferin + O_2 → oxyluciferin + PP_i + AMP + CO_2 + light

Light output of the reaction can be measured quantitatively using a luminometer. At low ATP concentrations, the light output is directly proportional to the concentration of ATP in the sample.

Cells were plated on 35 mm dishes and cultured until sufficiently confluent. The culture medium was changed for serum-free medium (unless otherwise stated) at least several hours prior to addition of test compounds. Following treatment with these compounds as required, the medium was removed and the cells rinsed gently with phosphate-buffered saline (PBS; 154 mM NaCl, 1.84 mM KH_2PO_4, 9.81 mM $K_2HPO_4.3H_2O$, pH adjusted to 7.2-7.3 with further addition of $KH_2PO_4/K_2HPO_4.3H_2O$ as necessary). The cells were then scraped in 50 µl of PBS, transferred to a 1.5 ml tube, snap frozen on dry ice and stored at -20ºC until needed.

An ATP assay kit (Roche Diagnostics) was used to determine ATP concentrations, run broadly in line with the manufacturer's instructions. One hundred and seventy microlitres of cell lysis reagent (supplied with the kit) was added to cell samples immediately upon defrosting and samples were incubated at room temperature for 5 minutes. Fifty microlitres of this treated sample was combined with 100 µl dilution buffer (supplied with the kit). To this mixture was added 50 µl luciferase reagent (made up in dilution buffer according to the manufacturer's instructions) and the luminescence was measured immediately using a Labtech Jade luminometer. Three

readings were taken at 5 second intervals and the average of these was taken as the luminescence measurement. Each sample was run in triplicate and each test condition was repeated three times in each experiment. Concentrations of ATP were determined using a standard curve (fig. 2.13), normalised for protein content (see 2.2.11) and expressed as nmol.mg^{-1} protein.

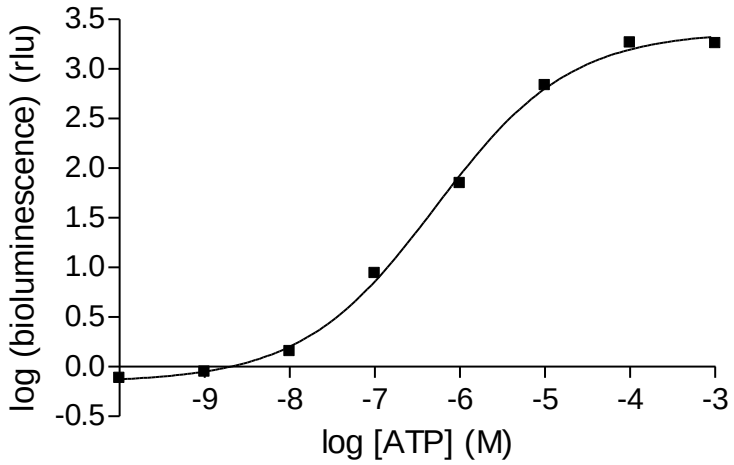


Figure 2.13. *Typical ATP standard curve. When the log_{10} of the bioluminescence reading is plotted against the log_{10} of the ATP concentration, a sigmoidal curve is produced (r^2=0.9956). This can then be used to calculate the ATP concentrations of samples.*

2.2.8.2 Measurement of ADP levels and ATP:ADP ratios

Measurement of the ADP levels within a sample and calculation of the ATP:ADP ratio can give more detailed information about the state of the cell. With a small alteration to the basic protocol, ATP and ADP levels can be measured successively in the same sample (Pocock and Nicholls 1998; Kingham and Pocock 2000). In the presence of pyruvate kinase (EC 2.7.1.40), ATP can regenerated from ADP with the conversion of phosphoenolpyruvate (PEP) to pyruvate:

phosphoenolpyruvate + ADP → pyruvate + ATP

Cell samples were diluted with cell lysis reagent and incubated as before, and 50 µl of this was combined with 100 µl dilution buffer containing 8 U pyruvate kinase. The ATP level was measured as before. PEP (in a small volume; final concentration 20 µM) was then added to the mixture to begin the ATP regeneration reaction and the sample was incubated for 1 minute at room temperature. Three readings were taken at 5 second intervals as before, and the average of these was taken as an estimate of the total ATP + ADP luminescence measurement. In the presence of ADP alone, a small, dose-dependent luminescent signal was seen before addition of PEP, however this was < 2% luminescence observed after ATP regeneration from ADP by pyruvate kinase and PEP, and < 2% luminescence observed with ATP of the same concentration, and was therefore considered negligible.

2.2.9 Measurement of lactate dehydrogenase release

Lactate dehydrogenase (LDH; EC 1.1.1.27) is a soluble cytosolic enzyme which mediates the interconversion of lactate and pyruvate, being especially important under hypoxic conditions. The loss of membrane integrity which occurs during both apoptosis and necrosis leads to the release of cytosolic components, including LDH, which is stable for at least 3 days in serum-free culture medium (Koh and Choi 1987). The LDH content of cell medium following treatment is therefore commonly used as an indicator of cell viability and has been used successfully as such in microglia and other monocytic cells (Decker and Lohmann-Matthes 1988; Kingham *et al.* 1999).

LDH levels were determined using a method based on that of (Dringen *et al.* 1998). Cells were plated in 16 mm culture dishes and medium was changed to serum-free medium before cells were treated as required. The medium was removed and centrifuged at 10 000 rpm (5031 *g*) for 1 minute in a Jouan A14 microcentrifuge to pellet any cell debris. Ten microlitres of the resulting supernatant was combined with 90 µl of 80 mM Tris-HCl buffer pH 7.2 containing 200 mM NaCl in a well of a 96 well test plate. To this was added 100 µl of 80 mM Tris-HCl buffer pH 7.2 containing 200 mM NaCl, 3.2 mM sodium pyruvate and 0.4 mM NADH. The conversion of NADH to NAD^+ (coupled to the conversion of pyruvate to lactate)

was followed by measuring the absorbance at 340 nm of the samples every minute for six minutes, using a Tecan GENios plate reader. A decrease in the absorbance represents a loss of NADH, and the more LDH in the sample, the more rapid the decrease. The rate of decline of absorbance was then normalised, with 0% representing the decline when the test sample was serum-free medium alone, and 100% representing the decline in the presence of a sample from cells treated for 30 minutes with 1% Triton X-100. Each sample was assayed in triplicate and each test condition repeated at least twice in each experiment. An example of the raw data generated in this experiment is presented in figure 2.14.

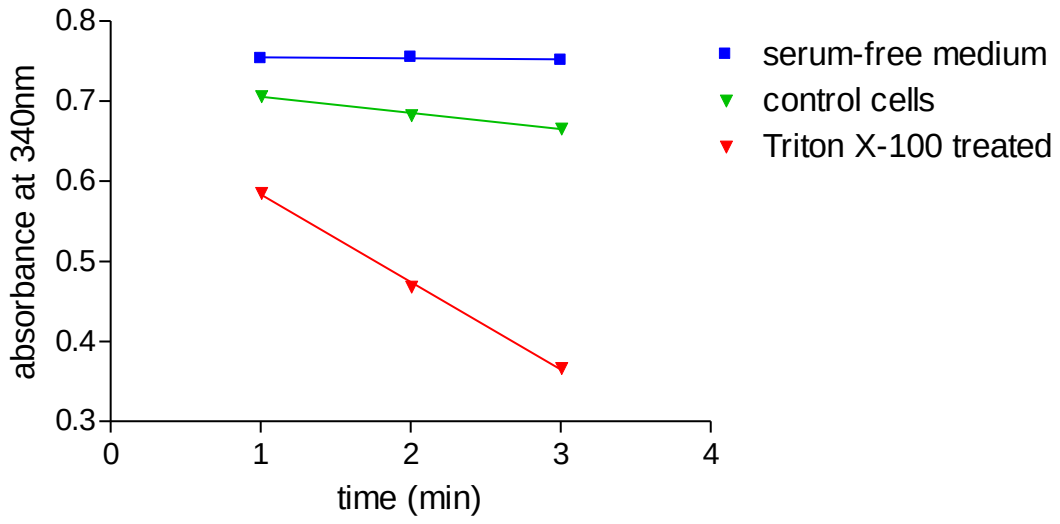


Figure 2.14. *Typical result of the LDH assay, demonstrating the declines in absorbance at 340 nm observed with serum-free medium, conditioned medium from control BV-2 cells and conditioned medium from BV-2 cells treated with 1% Triton X-100 for 30 minutes. The proportion of LDH release by the control cells, and therefore plasma membrane disruption, was calculated to be 17.6% in this case.*

2.2.10 Fluorescence Microscopy

2.2.10.1 The fluorescence microscope

A Zeiss Axioskop 2 fluorescence microscope (Oberkochen, Germany) was used to obtain images of iNOS immunocytochemistry, and of nuclear stains to assess cell death. In all cases, cells were viewed with a 40 x Neofluar objective. Images were captured using a Zeiss AxioCam HRc camera and Zeiss Axiovision 3.1 software.

2.2.10.2 Immunocytochemistry to identify iNOS expression

BV-2 microglial cells and primary microglia were cultured on 13 mm glass coverslips and treated as required, and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; 154 mM NaCl, 1.84 mM KH_2PO_4, 9.81 mM $K_2HPO_4.3H_2O$, pH adjusted to 7.2-7.3 with further addition of $KH_2PO_4/K_2HPO_4.3H_2O$ as necessary) and stored at 4ºC until required for immunofluorescence. Cells were permeabilised in 100% ice cold methanol for 20 minutes at 20ºC and then washed three times with PBS. Cells were blocked with 4% normal goat serum (NGS) for 30 minutes at room temperature. Following removal of NGS, 200 µl rabbit anti-mouse iNOS antibody at 1:250 dilution in PBS was added to each coverslip and incubated in a wet box at 4ºC overnight. The following day, coverslips were washed three times with PBS and incubated with 200 µl goat anti-rabbit tetramethyl rhodamine iso-thiocyanate (TRITC) conjugated antibody at 1:500 dilution in PBS for 2 hours in the dark at room temperature. Coverslips were washed a further three times with PBS (in the dark) and incubated with 200 µl DNA-staining dye 4',6-Diamidino-2-Phenylindole (DAPI) at 1:1000 dilution in PBS for 1 minute in the dark at room temperature. Coverslips were washed once more with PBS, rinsed with dH_2O and mounted on a glass slide with Vectashield mountant. The edges of each coverslip were sealed to prevent the cells drying out and slides were stored in the dark at -20ºC.

Cells were first viewed under an excitation wavelength of 365 nm, with emission over 490 nm recorded to visualise DAPI staining. Images were captured with automatic exposure time. TRITC immunofluorescence in the same field was

captured by excitation with light of wavelength 485 nm with emission over 530 nm recorded. In this case, all images were captured with an exposure time of 20 seconds. Brightness, contrast and colour balance were adjusted identically in all images to allow fair comparison between treatments. Negative controls were performed in which the primary antibody was replaced with PBS, to check for non-specific binding of the secondary antibody.

2.2.10.3 Measurement of cell death using Hoechst-33342 and propidium iodide

2´-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5´-bi-1H-benzimidazole (Hoechst-33342) and propidium iodide (PI) both bind DNA, but Hoechst-33342 is lipophilic and readily crosses plasma membranes, while PI is impermeant to functional plasma membranes, and can only bind the nuclear DNA when membrane integrity is compromised. Additionally, when excited with ultraviolet light (wavelength 350nm), bound Hoechst-33342 fluoresces blue, with maximum emission at 461 nm (Latt and Stetten 1976), whilst bound PI fluoresces red (617 nm maximum emission) when excited with light of wavelength 494 nm. Therefore, the addition of Hoechst-33342 and PI together to live (i.e. not fixed) cells allows live and dead cells to be distinguished. All nuclei stain blue with Hoechst-33342, whilst only the nuclei of dead cells stain red with PI. The % cell death can therefore be calculated (Bonfoco *et al.* 1995; Kingham and Pocock 2001).

Cells were plated on 13 mm coverslips in cell culture plates, media changed and cells treated as required. Cells were incubated in the dark at 37ºC with 1.25 $\mu g.ml^{-1}$ PI for 30 minutes and 4 $\mu g.ml^{-1}$ Hoechst-33342 for 15 minutes, added directly to the medium. Coverslips were removed from plates and rinsed gently in warmed basic medium (153 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES), 5 mM $NaHCO_3$, 1.2 mM Na_2SO_4, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, 5 mM glucose). Each coverslip was placed face-down on a glass slide and viewed immediately. Cells were first viewed under an excitation wavelength of 365 nm, with emission over 490 nm recorded to visualise Hoechst-33342 staining. PI staining the same field was captured by excitation with light of wavelength 485 nm with emission over 530 nm recorded.

2.2.11 Measurement of gene expression by evaluation of mRNA levels using reverse transcription and polymerase chain reaction

2.2.11.1 Principle

The reverse transcriptase polymerase chain reaction (RT-PCR) is a method allowing investigation of gene expression through quantitative analysis of mRNA expression. The method consists of four distinct steps. First, the RNA from the cells or tissue of interest must be isolated. Next, a complementary DNA (cDNA) copy is produced of all of the RNA extracted from the cells using an RNA-dependent DNA polymerase enzyme (reverse transcriptase; EC 2.7.7.49). The PCR itself involves the amplification of the DNA complimentary to the mRNA of interest only. This is possible since the DNA polymerase enzyme responsible for this amplification requires an oligonucleotide primer complimentary to a small section of cDNA in order to initiate synthesis. Therefore if primers are designed of a sequence which exists only within the mRNA/cDNA of interest, the PCR becomes specific for this gene. Two primers are required for each gene of interest, both of which have unique sequences and which span (ideally) a 200-600 base pair portion of the mRNA of interest.

PCR is controlled by temperature changes; first, a 94°C dissociation phase separates double-stranded DNA to allow primers to anneal in the second phase. The optimum temperature for the annealing phase (optimum annealing temperature, OAT) is worked out for each individual primer and averaged for each primer pair. It depends on the relative cytosine/guanine and adenine/thymine nucleotide content of the primers and is given by:

OAT = Tm - 4°C

Where Tm is the melting temperature, given by:

Tm = 4(G+C) + 2(T+A) °C

Where G is the number of guanine nucleotides, C is the number of cytosine nucleotides, T is the number of thymine nucleotides and A is the number of adenine nucleotides in the primer.

However the annealing temperature should not exceed 65°C, so as to be sufficiently different from the temperature used in the third phase, elongation. In this phase a complementary strand of DNA is synthesised for each existing strand by means of the *Thermophilus aquaticus (Taq)* DNA polymerase (EC 2.7.7.7). *Thermophilus aquaticus* is a bacterium, first discovered in thermal springs, which can withstand high temperatures, such as that used for the dissociation phase here. The elongation phase is conducted at 72°C, the optimum temperature for *Taq* DNA polymerase.

Each time the dissociation-annealing-elongation cycle is repeated each existing strand is copied and therefore the number of copies of the cDNA of interest doubles, in an exponential manner. As reagents are consumed the reaction slows and then at some point stops altogether. The result is that over time the number of copies of the cDNA of interest takes on an S-shaped growth curve (fig. 2.15).

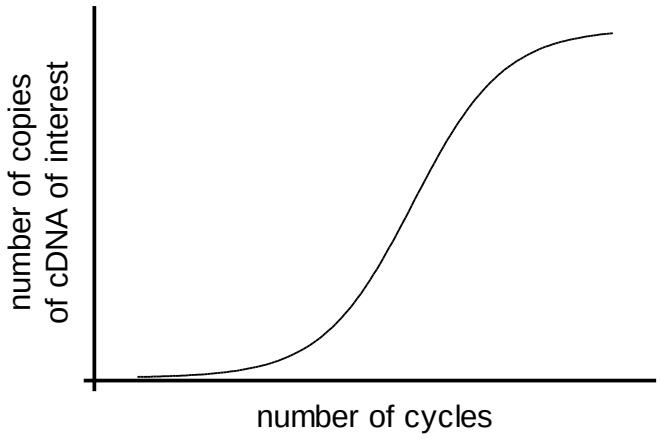


Figure 2.15. *The number of copies of cDNA of interest increases exponentially before slowing and levelling off due to some limiting factor.*

It is important with each gene of interest to optimise the number of cycles used so that this falls within the maximum growth phase and differences in expression can be clearly seen. A more recent development is quantitative real-time PCR, in which a fluorescent dye is used to visualise DNA. After each cycle the DNA can be quantified by measuring fluorescence so that the S-shaped curve can be followed and compared between samples.

The final step in RT-PCR allows visualisation of the amount of DNA produced in the PCR, and comparison between samples. PCR products are run on an agarose gel stained with ethidium bromide, which intercalates into DNA and fluoresces under UV light. The densities of the bands on the ethidium bromide gel correspond directly to the amount of mRNA in the original sample.

As a control to test for equal amounts of total mRNA within the original sample, RT-PCR is also performed for a gene whose expression is thought to remain constant. The density of the bands for the gene of interest on the gel can then be corrected for the density of the bands for the control genes. Here two control genes were used, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), an enzyme involved in glycolysis, and the cytoskeletal protein β-actin.

2.2.11.2 Extraction of mRNA from cells

RNase-free consumables were used throughout and surfaces and gloves were regularly sprayed with RNase inhibitor. Cells were scraped using TRIzol reagent according to manufacturer's instructions. Following removal of all medium, cells were rinsed with PBS and then scraped into 1 ml TRIzol using a plastic scraper. The TRIzol was transferred into autoclaved RNase-free safelock tubes and incubated at room temperature for at least 5 minutes. Two hundred microlitres of chloroform were added and tubes were shaken vigorously for 15 seconds and then incubated at room temperature for a further 2-3 minutes. The tubes were spun at 11 200 rpm (11 600 g) in an Eppendorf 5415R benchtop centrifuge for 15 minutes at 4°C and the clear upper aqueous phase was transferred into new tubes, taking care not to contaminate this with material from the lower phases. RNA was precipitated by mixing with 0.5 ml isopropyl alcohol and incubating at room temperature for 10

minutes. RNA was then pelleted by spinning at 11 200 rpm (11 600 *g*) for 10 minutes at 4ºC. The resulting supernatant was removed and RNA pellets were washed with 1 ml 75% ethanol, vortexed and spun at 8200 rpm (6200 *g*) for 5 minutes at 4°C. The supernatant was removed and the RNA pellet was allowed to completely dry before being dissolved in an appropriate volume (20-100 µl, depending on the size of the pellet) of dH_2O treated with the nuclease inhibitor diethyl pyrocarbonate (DEPC). Tubes were incubated at 60°C for 10 minutes to aid dissolving. Pellets were then frozen (-80°C) and defrosted to read the absorbance at 260 nm (A_{260}) and at 280 nm (A_{280}) on a Pharmacia Biotech Ultraspec 2000 spectrophotometer. The ratio A_{260} / A_{280} indicates the purity of the RNA, where pure RNA gives a ratio of 2.

The Beer-Lambert law states that

A_{260} = RNA extinction coefficient (25 µl.µg^{-1}.cm^{-1} x RNA concentration (µg.µl^{-1}) x path length (1 cm)

So, RNA concentration (µg.ml^{-1}) = A_{260} x 40 x dilution factor

In this case, [RNA] (µg.µl^{-1}) $= A_{260} \times \frac{40}{1000} \times \frac{400}{3} = A_{260} \times \frac{16}{3}$

2.2.11.3 Reverse transcription

Reverse transcription was carried out using Moloney murine leukaemia virus (MMLV) reverse transcriptase (RT). Up to 3 µg (≤13.5 µl) RNA was reverse transcribed. This was made up to 13.5 µl with DEPC-treated dH_2O, and to this was added 0.25 µg (1 µl of 0.25 µg.µl^{-1}) oligo$(dT)_{12\text{-}18}$ primer and 40 units (1 µl) RNaseOUT recombinant ribonuclease inhibitor. This mixture was incubated at 70°C for 10 minutes before being quenched on ice. Fourteen and a half microlitres of RT mastermix were then added to each tube, containing per tube, 6 µl 5X buffer (supplied with the MMLV RT enzyme), 3 µl 0.1 M dithiothreitol (DTT), a further 40 units (1 µl) RNaseOUT, 1.5 µl 10 mM deoxynucleotide triphosphates (dNTPs) and 3

μl (600 units) MMLV RT. Final concentrations in the reaction mixture were 10 mM DTT, 2.67 units.μl^{-1} RNaseOUT, 0.5 mM dNTPs, 8.33 ng.μl^{-1} oligo(dT)$_{12-18}$ primer, 20 units.μl^{-1} MMLV RT; final volume, 30 μl. Tubes were vortexed and centrifuged briefly and incubated at 37°C for 1 hour 20 minutes. The reaction was terminated by heating to 70°C for 10 minutes and tubes were then transferred to ice. DEPC-treated dH_2O was added to give a concentration equivalent to 20 ng reverse transcribed RNA.μl^{-1} (for example, if 3 μg RNA was reverse transcribed, 120 μl DEPC-treated dH_2O was added to give a total volume of 150 μl). Finally the MMLV reverse transcriptase was destroyed with a 5 minute incubation at 100°C, then the samples were transferred to ice and stored at –20°C. A negative control without reverse transcriptase was run alongside every reverse transcribed sample. To check for contamination with cellular DNA, these negative control samples were subsequently run through a PCR for a control housekeeping gene.

2.2.11.4 Polymerase chain reaction

A PCR was run on the product of 0.125 ng reverse transcribed RNA (6.25 μl). To each tube was added 18.75 μl PCR mastermix containing, per tube, 2.5 μl 10X *Taq* DNA polymerase buffer (supplied with the enzyme), 0-1.5 μl 50 mM $MgCl_2$ (concentration optimised for each primer by experiment; see table 2.2), 1 μl of each of the relevant forward and reverse primers at 2.5 μM, 0.125 μl 10 mM dNTPs, 0.625 units *Taq* DNA polymerase (0.125 μl of 5 units.μl^{-1}) and 12.5-14 μl DEPC-treated dH_2O to make up the volume. Final concentrations of reagents were as follows: 0-3 mM $MgCl_2$ as optimised, 0.1 μM each of forward and reverse primers, 50 μM dNTPs, 0.025 units.μl^{-1} *Taq* DNA polymerase; final volume, 25 μl. The reaction consists of an initial dissociation at 94°C for 5 minutes, followed by an optimised number of cycles (see table 2.2) of a 30 second dissociation at 94°C, a 30 second annealing phase at the OAT (calculated for each primer, see table 2.2 and section 2.2.10.1 above) and a 30 second elongation phase at 72°C. The reaction ends with a further 7 minute elongation phase, and then the samples are cooled to 4°C. PCRs were run on control genes (here GAPDH and β-actin) which are believed to be

	Species	Forward sequence (5'–3')	Reverse sequence (5'–3')	Amplicon size (bp)	Optimum annealing temperature (OAT; °C)	Optimum [$MgCl_2$] (mM)	Number of PCR cycles	Source	Reference
β-actin	rat, mouse	ATC GTG GGC CGC CCT AGG CAC	TGG CCT TAG GGT TCA GAG GGG C	330 (genomic) / 243 (cDNA)	65	2	22	Dr Rattray, Kings College London*	Tortarolo *et al.* (2004)
GAPDH	rat, mouse	TGG TGC CAA AAG GGT CAT CAT CTC C	GCC AGC CCC AGC ATC AAA GGT G	559	60	2	22	Sigma Genosys	Taylor *et al.* (2003)
r EAAT1	rat, mouse	TCC TCA TTC ATG CCG TCA TCG TCC	TCT TGG TTT CGC TGT CTG GCA CG	653	65	1.5	35	Dr Rattray, Kings College London*	Tortarolo *et al.* (2004)
r EAAT2	rat, mouse	AGC CGT GGC AGC CAT CTT CAT AGC	ATG TCT TCG TGC ATT CGG TGT TGG G	326	65	1.5	35	Dr Rattray, Kings College London	Tortarolo *et al.* (2004)
m xCT	mouse	TAC CAC CAT CAG TGC GGA GG	GTA TCG AAG ATA AAT CAG TCC TGC	~350-400	55	1.5	35	Dr Rattray, Kings College London	
r xCT	rat, mouse	CCT GGC ATT TGG ACG CTA CAT	TCA GAA TTG CTG TGA GCT TGC A	182	64	2.5	35	Sigma Genosys	Tomi *et al.* (2002)

Table 2.2. *Polymerase Chain Reaction (PCR) primers and their properties. bp, base pairs; EAAT, excitatory amino acid transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; m, mouse; r, rat; xCT, the specific subunit of the x_c^- transporter. *duplicates purchased from Sigma Genosys.*

expressed at the same level irrespective of the conditions, alongside the genes of interest.

2.2.11.5 Agarose gel electrophoresis

Following PCR 5 µl of 6 x gel loading dye was added to each 25 µl sample and 15 µl of sample + dye was loaded into each lane of a 1% w/v agarose gel in Tris/borate/ EDTA buffer, containing 0.5 $\mu g.ml^{-1}$ ethidium bromide. 10 µl (1 µg) 100 bp DNA ladder was loaded alongside. The gel was run for approximately 2 hours at 100-120 V. Gels were de-stained in dH_2O for 15 minutes and then viewed under UV light using a Gel-Pro Imager system. Images were captured using a CoolSNAP-Pro camera and band densities were determined using Gel-Pro Analyzer v 3.1.

2.2.12 Determination of protein levels using the Bradford assay

Protein concentration was determined using the Bradford assay (Bradford 1976), 96 well plate format. An appropriate volume (< 10 µl) of sample or standard was added to each well, followed by 200 µl Coomassie reagent. Plates were incubated at room temperature for 5-10 minutes and absorbance at 595 nm was measured using a Tecan GENios plate reader. A standard curve with 0-6 $mg.ml^{-1}$ BSA was run on each plate (fig. 2.16). All samples and standards were run in triplicate.

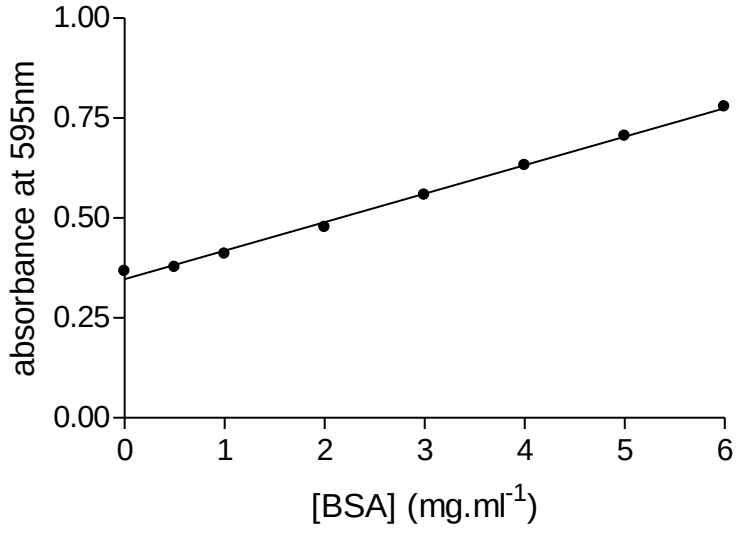


Figure 2.16. *Typical standard curve for Bradford protein assay, constructed with 0-6 $mg.ml^{-1}$ bovine serum albumin (BSA). A standard curve in triplicate was run on each plate. $r^2=0.9934$.*

2.2.13 Data analyses

Data presented represent at least n=3, unless otherwise indicated. Microsoft Excel and GraphPad Prism 4 were used to analyse and present data and statistical analyses were run in GraphPad Prism. Unless otherwise stated, graphs are presented, and results quoted, as mean ± standard error of the mean (s.e.m.). Student's t tests were used in cases where only two groups were compared. A one-way analysis of variance (ANOVA) was used to establish statistical significance between three or more data sets. Dunnett's post-hoc test was employed when comparing all groups with one control group, whilst the moderately conservative Tukey's 'Honest Significant Difference' post-hoc test was used where multiple comparisons were required. These post-hoc tests were selected due to the fact that they correct for multiple comparisons, so that the likelihood of a type I error (false positive result) does not increase for larger data sets. A two-way ANOVA was used to establish significance in cases where two independent treatment variables were investigated, with Bonferroni's post-hoc tests as appropriate. A p value smaller than 0.05 was considered statistically significant; *p<0.05, **p<0.01, ***p<0.001, n.s., not significant (p>0.05).

Chapter 3

Results I:
Microglial glutathione levels and glutathione release following microglial activation

3.1. Introduction and summary of results

Activated microglia release nitric oxide (NO) and superoxide as a cytotoxic attack mechanism against invading pathogens (Colton and Gilbert 1987; Chao *et al.* 1992; Klegeris and McGeer 1994; Ding *et al.* 1997; Sankarapandi *et al.* 1998; Possel *et al.* 2000). However reactive oxygen and nitrogen species (ROS and RNS) derived from NO and superoxide may also cause local cellular damage by reacting with proteins, lipids and nucleic acids (Valko *et al.* 2007) and by inhibiting mitochondrial energy metabolism, preventing the production of adenosine triphosphate (ATP) (Bolaños *et al.* 1995). Microglial antioxidants are therefore likely to be important in protecting microglia from such oxidative damage. Indeed, microglia have been shown to contain levels of glutathione significantly higher than in astrocytes or neurones (Chatterjee *et al.* 1999; Hirrlinger *et al.* 2000), and measured in primary cultures containing 90% microglia in the range 25 - 40 $nmol.mg^{-1}$ protein (Hirrlinger *et al.* 2000; Persson *et al.* 2006), and in the BV-2 and N11 microglial cell lines as 55 - 60 $nmol.mg^{-1}$ protein (Moss and Bates 2001).

It is less clear how the glutathione levels in microglia are modified by microglial activation. Reduced glutathione (GSH) levels in the BV-2 and N11 microglial cell lines have been shown to decline in the presence of lipopolysaccharide (LPS) and interferon-γ (IFNγ) (Moss and Bates 2001), and in the case of N11 cells, the decrease was blocked by inhibition of inducible nitric oxide synthase (iNOS) (Moss and Bates 2001). Biochemical determination of GSH levels in N9 cells also demonstrated a decrease following LPS and IFNγ treatment (Roychowdhury *et al.* 2003). Additionally, in mixed glial cultures containing 10 – 20% microglia, fluorescence of monochlorobimane (MCB), a marker of GSH, was found to decline by around 40% in microglia following treatment with LPS and IFNγ (Chatterjee *et al.* 2000), whilst the GSH content of the co-cultured astrocytes remained stable. Preincubation with the iNOS inhibitor N-iminoethyl-lysine blocked this decline. It therefore appears that the production of NO following microglial activation causes a decline in cellular GSH levels. Conversely, in primary cultures of rat microglia, intracellular GSH levels have been found to increase in the presence of LPS or tumour necrosis factor-

α (TNFα) (Dopp *et al.* 2002; Persson *et al.* 2006), determined by MCB fluorescence, an enzymatic method, and high performance liquid chromatography (HPLC).

It is hypothesised that the measurement of GSH in primary microglia and the BV-2 and N9 cell lines here will reveal significant effects of microglial activation upon intracellular GSH. However, based upon previous studies it is unclear whether microglial activation will lead to an increase or a decrease in GSH levels. A NO-dependent decrease in intracellular GSH as found previously (Chatterjee *et al.* 2000; Moss and Bates 2001) may be due to increased utilisation of GSH as an antioxidant (Chatterjee *et al.* 2000), or to inhibition of mitochondrial ATP production and therefore reduced availability of ATP for GSH synthesis (Moss and Bates 2001). An increase in GSH levels following microglial activation may indicate the presence of a compensatory pathway, upregulating GSH levels in anticipation of an oxidative insult. Previous studies have suggested that upregulation of the x_c^- transporter, which imports cystine for GSH synthesis, may underlie such an increase (Persson *et al.* 2006). Another possible target is glutamate-cysteine ligase (GCL), which catalyses a potentially rate-limiting step in GSH synthesis (Meister and Anderson 1983); indeed, enhanced GCL expression has been demonstrated in astrocytes upon exposure to NO (Gegg *et al.* 2003).

It is also unclear whether microglia release GSH. Microglia have been shown to express functional multidrug resistance-associated protein 1 (Mrp1) (Ballerini *et al.* 2002; Dallas *et al.* 2003), and GSH was shown to be necessary for vincristine export from the Mrp1-expressing MLS-9 microglial cell line (Dallas *et al.* 2003). However, a study in which robust GSH release by astrocytes was demonstrated, microglial GSH release could not be detected (Hirrlinger *et al.* 2002c).

Here, the GSH levels of two commonly-used mouse microglial cell lines, BV-2 and N9, were determined, and the effects of a number of compounds associated with microglial activation upon GSH levels were studied. BV-2 microglia had a control GSH concentration of around 50 $nmol.mg^{-1}$ protein, which was transiently increased by microglial activation by LPS or fraction V albumin in a concentration-dependent manner, being significantly different from control levels after 16 – 24 hours' incubation. The effect of LPS was not altered by pre-incubation of BV-2 cells with

iNOS inhibitors. N9 microglia had a higher basal GSH concentration of around 80 nmol.mg^{-1} protein. Activation of this cell line with the same compounds led to concentration-dependent decreases in GSH levels, which were maintained over time. Due to practical considerations, it was not possible to assess the impact of iNOS inhibition upon the GSH content of N9 cells here, although iNOS inhibition has been shown elsewhere to prevent declines in the GSH levels in cells of the N11 cell line (Moss and Bates 2001), another cloned line produced alongside N9 cells (Righi *et al.* 1989), which appears to behave similarly (Ricciardi-Castagnoli and Paglia 1992; Corradin *et al.* 1993). Neither the Alzheimer's disease- (AD-) associated protein chromogranin A (CGA) nor residues 25-35 of the β amyloid peptide ($A\beta_{25-35}$) significantly altered the GSH content of either cell line. Analysis of intracellular GSH of primary rat microglia revealed a GSH concentration of around 30 nmol.mg^{-1} protein, which was elevated by microglial activation with LPS alone or in combination with IFNγ, and fraction V albumin, although to a lesser extent than in BV-2 cells. BV-2 cells therefore represent a more suitable model for microglia in such investigations.

Conditioned medium from BV-2 and primary microglial cultures was found to consistently contain GSH, demonstrating a low level of GSH release by microglia. This appears to be the first demonstration of GSH release by primary microglia. The role of microglia-derived GSH is as yet undetermined.

3.2. Microglia express iNOS and release NO upon activation

Expression of iNOS was detected directly by means of immunocytochemistry, and also by measurement of the release of nitrate and nitrite, the stable metabolites of NO. In the absence of activators, just 8.5 ± 5.5 % of primary microglia in culture were positively stained for iNOS (fig. 3.1). Treatment of microglia with LPS, IFNγ, LPS and IFNγ in combination or fraction V albumin significantly increased the number of cells expressing iNOS. LPS (1 μg.ml^{-1}; fig. 3.1B) or fraction V albumin (2 mg.ml^{-1}; fig. 3.1E) caused 65.5 ± 12.5 % and 57.8 ± 12.0 % of cells, respectively, to express iNOS. Enlarged amoeboid cells were visible, particularly in the presence

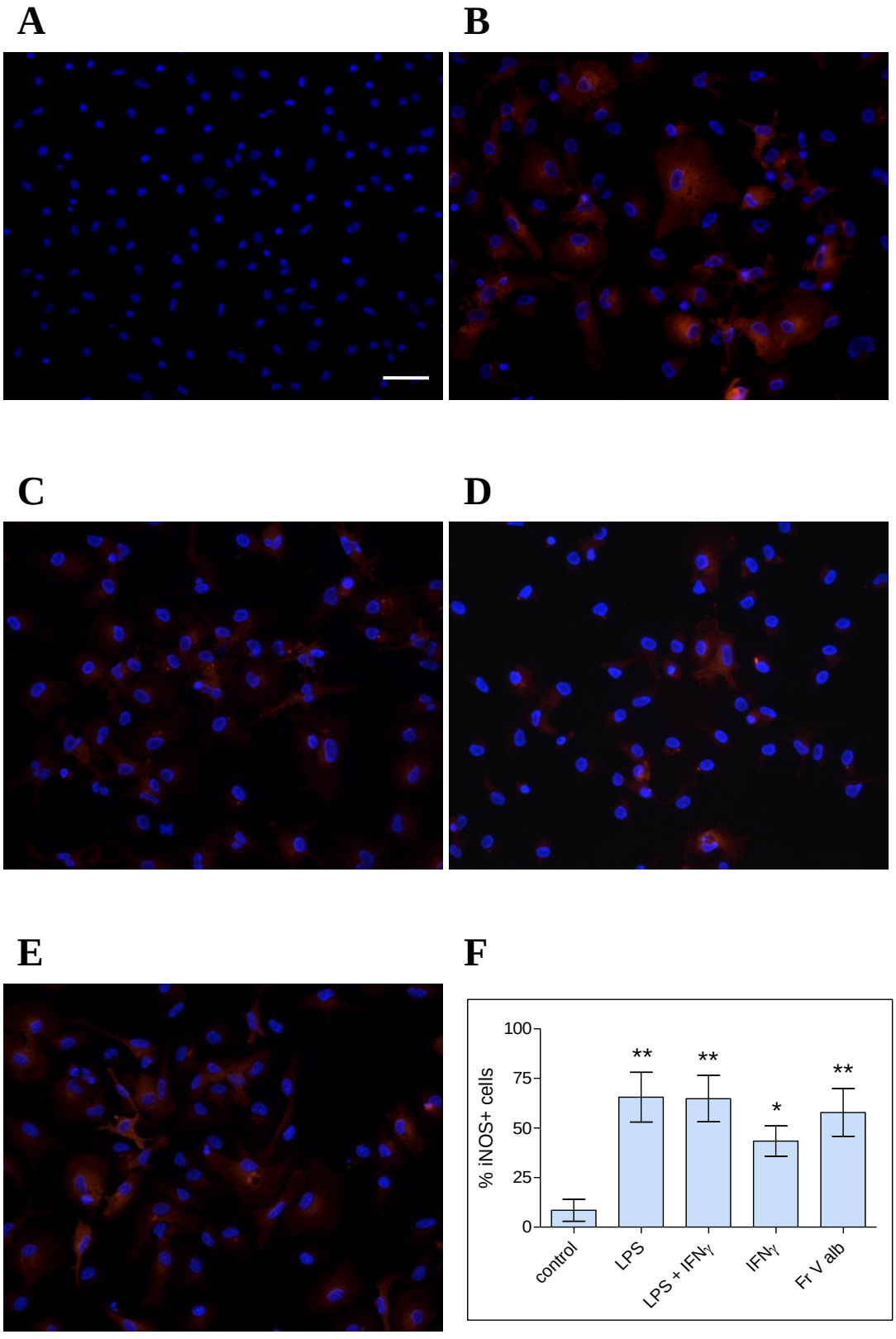


Figure 3.1. (legend overleaf)

Figure 3.1. (previous page) *iNOS expression by primary microglia under control (**A**) and activated (**B**-**E**) conditions. Cells were incubated with (**B**) LPS (1 µg.ml^{-1}), (**C**) LPS + IFNγ (100 U.ml^{-1}), (**D**) IFNγ or (**E**) fraction V albumin (2 mg.ml^{-1}) for 24 hours before fixation. iNOS (red) was visualised using immunocytochemistry, and nuclei were visualised using 4',6-diamidino-2-phenylindole (DAPI; blue). Cells were counted by eye; any cell with visible cytoplasmic red staining was deemed to be positive for iNOS. Representative images are shown. **F**, Data represent the mean ± s.e.m. of two (**C**-**E**) or three (**A**, **B**) independent experiments, each consisting of two coverslips per condition, with at least three fields viewed per coverslip. This represents a total of approximately 1200 (**C**-**E**) or 1800 (**A**, **B**) cells per condition. Data were analysed with a one-way ANOVA (p=0.0002), with Dunnett's post-test (*p<0.05, **p<0.01, compared with control). Magnification, 40X. Scale bar, 20 µm.*

of LPS. Treatment with LPS in combination with IFNγ (100 $U.ml^{-1}$) led to a similar high proportion of iNOS-positive cells (64.7 ± 11.7 %), but as demonstrated in figure 3.1B and C, the staining was less striking and the presence of IFNγ seemed to reduce the appearance of large amoeboid cells. Following treatment with IFNγ alone, 43.4 ± 7.6 % microglial cells expressed iNOS (fig. 3.1D), and very few microglia demonstrated the enlarged amoeboid phenotype.

BV-2 cells also had a low level of basal iNOS expression (fig. 3.2A), with 13.1 ± 4.8 % control cells positively stained. In contrast to the situation in primary microglia, immunohistochemical iNOS detection in BV-2 cells demonstrated that LPS + IFNγ gave the most prominent iNOS expression, with 79.8 ± 12.8 % cells strongly expressing iNOS throughout their cytoplasm (fig. 3.2C). As illustrated by the images presented here, there were consistently far fewer cells adhered to the coverslip following treatment with LPS + IFNγ (fig. 3.2C), and to a lesser extent, IFNγ alone (fig. 3.2D). Just 27.6 ± 4.4 % of cells expressed iNOS following LPS treatment (fig. 3.2B), which was not significantly different from control, but 68.9 ± 10.3 % of cells treated with IFNγ alone expressed iNOS (fig. 3.2D). Treatment with fraction V albumin caused 68.6 ± 10.5 % of BV-2 cells to express iNOS, with the cells appearing smaller and more rounded (fig. 3.2E).

These results in BV-2 cells reflect published data demonstrating that LPS alone causes little or no nitrate and nitrite production by BV-2 cells, whilst LPS + IFNγ significantly increase nitrate/nitrite (Moss and Bates 2001; Horvath *et al.* 2008). In addition, IFNγ alone has been shown to cause a similar level of nitrate/nitrite production to LPS + IFNγ (Shen *et al.* 2005); the two treatments were found here to lead to iNOS expression in a similar proportion of BV-2 cells (fig. 3.2F).

In the case of N9 microglia, iNOS expression was inferred from measurement of nitrate and nitrite levels in conditioned medium (fig. 3.3). LPS + IFNγ was the only treatment to significantly increase nitrate/nitrite release by N9 cells, giving levels 30-fold higher than control. This is in accordance with published data, demonstrating increased iNOS protein expression and activity and increased nitrite production, following LPS + IFNγ treatment of N9 cells (Mayo and Stein 2007). Although no

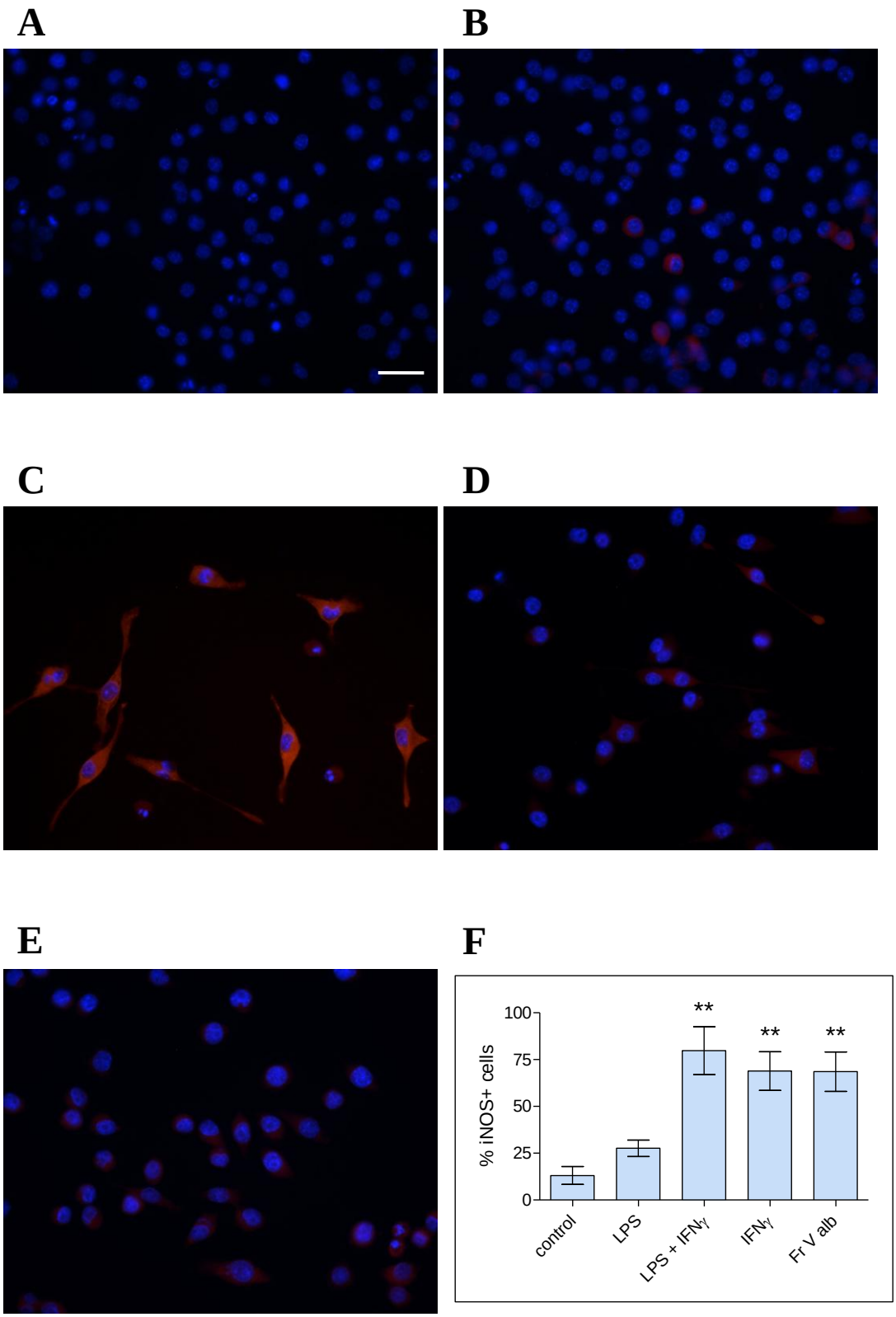


Figure 3.2. (legend overleaf)

Figure 3.2. (previous page) *iNOS expression by BV-2 microglia under control (**A**) and activated (**B**-**E**) conditions. Cells were incubated with (**B**) LPS (1 µg.ml^{-1}), (**C**) LPS + IFNγ (100 U.ml^{-1}), (**D**) IFNγ or (**E**) fraction V albumin (2 mg.ml^{-1}) for 24 hours before fixation. iNOS (red) was visualised using immunocytochemistry, and nuclei were visualised using 4',6-diamidino-2-phenylindole (DAPI; blue). Cells were counted by eye; any cell with visible cytoplasmic red staining was deemed to be positive for iNOS. Representative images are shown. **F**, Data represent the mean ± s.e.m. of three independent experiments, each consisting of two coverslips per condition, with three fields viewed per coverslip. This represents a total of 1100 cells per condition on average. Data were analysed with a one-way ANOVA ($p<0.0001$), with Dunnett's post-test (**$p<0.01$ compared with control). Magnification, 40X. Scale bar, 20 µm.*

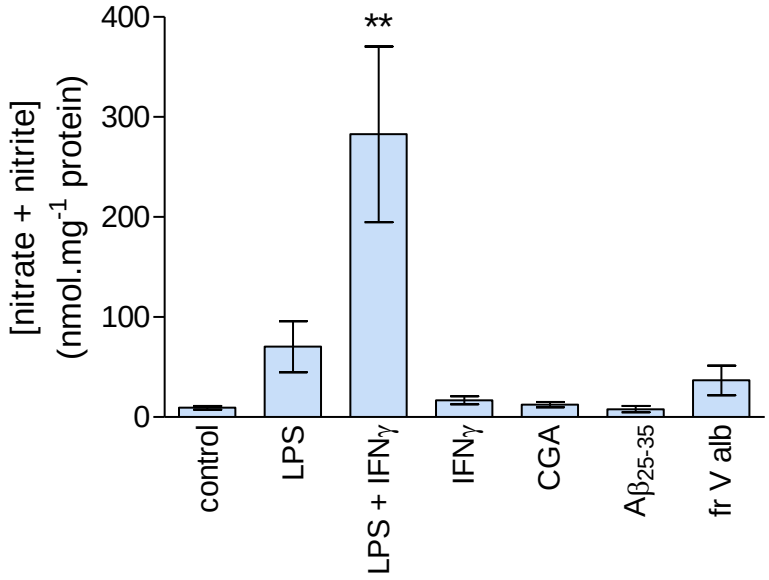


Figure 3.3. *The effect of a number of compounds associated with microglial activation upon nitrate and nitrate levels in N9 microglial conditioned medium. N9 cells were cultured in the presence of LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), CGA (500 nM), Aβ$_{25-35}$ (25 µM), fraction V albumin (2 mg.ml^{-1}), or a combination, for 24 hours before nitrate and nitrite levels in conditioned medium were determined by the Griess assay. Data represent the mean ± s.e.m. of at least three independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA (p<0.0001) with Dunnett's post-test (**p<0.01 compared with control).*

other conditions were statistically significant, nitrate/nitrite levels following LPS treatment were more than 7-fold higher than control, and following fraction V albumin treatment were almost 4-fold higher. By contrast, IFNγ alone, $A\beta_{25-35}$ (25 μM) or CGA (500 nM) failed to even double control nitrate/nitrite levels.

3.3. ATP levels in BV-2 microglia upon activation

Figure 3.4Ai demonstrates that ATP levels in plated BV-2 microglial cells showed a general decline over time, regardless of whether or not the culture medium contained serum. Fraction V albumin significantly increased ATP levels following 8 or 24 hours' incubation. Although there was also a trend towards an increase in the presence of LPS, this failed to reach statistical significance. In contrast, 24 hours' exposure to IFNγ alone or in combination with LPS produced ATP levels slightly, but not significantly lower than the serum-free medium (SFM) control. The effect of the treatments relative to SFM control is demonstrated in figure 3.4Aii.

Preliminary data suggested that the ratio of adenosine diphosphate (ADP) to ATP in plated BV-2 cells was high (fig. 3.4C), and that total ATP + ADP levels (fig. 3.4B) and the ADP/ATP ratio (fig. 3.4C) declined over time in all cells cultured in SFM, but not those cultured in medium containing 10% foetal bovine serum (FBS) (serum-containing medium, SCM).

3.4. Measuring intracellular reduced glutathione using two methods: reverse-phase high performance liquid chromatography and imaging using monochlorobimane

Reverse-phase high performance liquid chromatography (HPLC) and quantification of monochlorobimane (MCB) fluorescence for measurement of intracellular GSH were used to measure intracellular GSH levels of BV-2 microglial cells (fig. 3.5). BV-2 cells were incubated with LPS (1 $\mu g.ml^{-1}$), LPS + IFNγ (100 $U.ml^{-1}$) or fraction V rat albumin (2 $mg.ml^{-1}$) for 24 hours before GSH analysis. Using HPLC

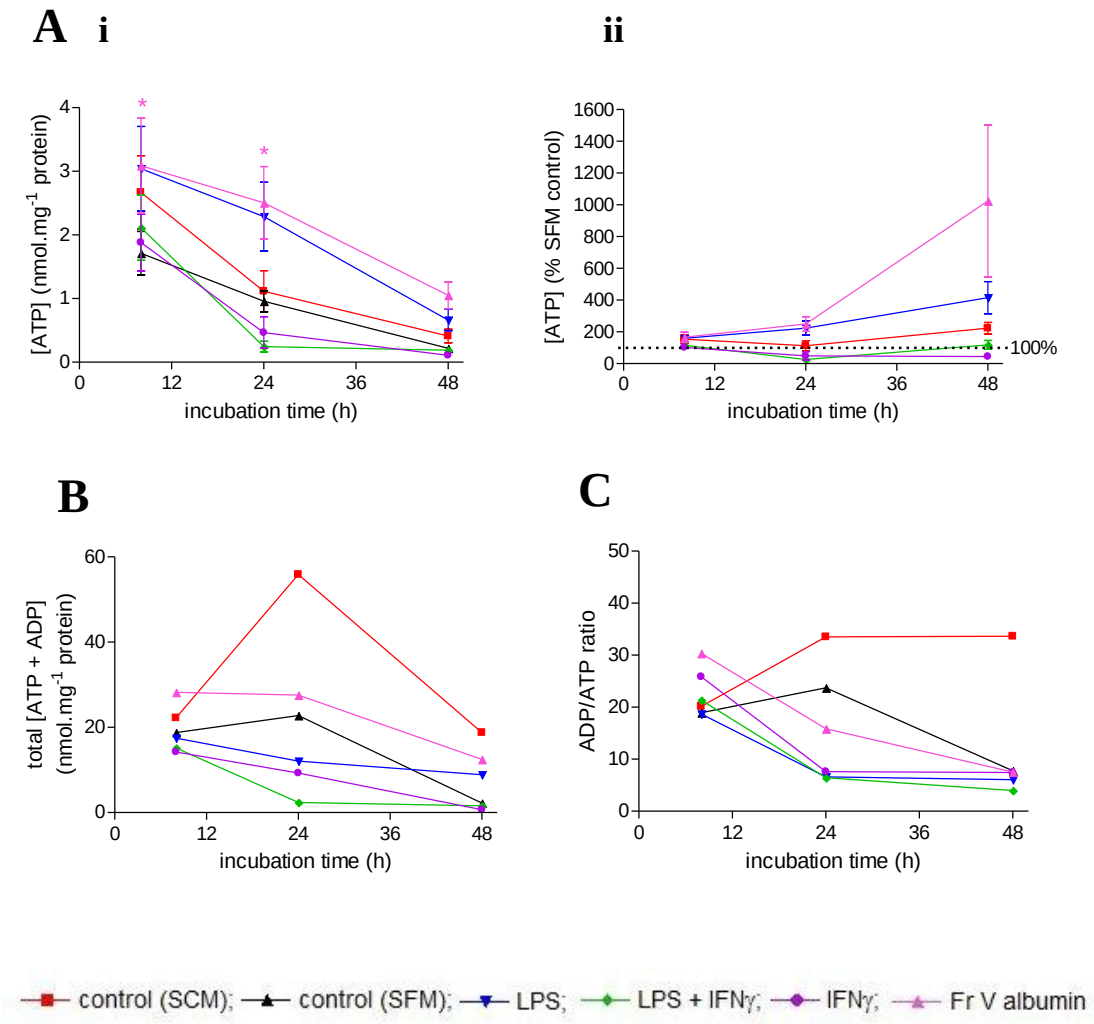


Figure 3.4. *The effect of microglial activators upon ATP levels, total ATP + ADP levels and the ADP/ATP ratio over time. BV-2 cells were cultured in SCM, SFM, or in SFM in the presence of LPS (1 μg.ml^{-1}), IFNγ (100 U.ml^{-1}), fraction V albumin (2 mg.ml^{-1}) or a combination for 8, 24 or 48 hours. Cells were scraped and ATP and ADP levels determined with a luminescence-based ATP assay kit. Data represent the results of one experiment (**B**, **C**) or three independent experiments (**A**), each consisting of three samples per condition. **A**i, ATP levels, expressed as nmol.mg^{-1} protein, were analysed with a two-way ANOVA, which showed a highly significant effect ($p<0.001$) of time and a highly significant effect ($p<0.001$) of treatment upon ATP levels, with no significant interaction between time and treatment. Bonferroni's post-tests were conducted to compare all conditions with the SFM control (▲) at each time point (*$p<0.05$). **A**ii, The same data expressed as a percentage of SFM control at each time point highlights the comparative effects of the treatments.*

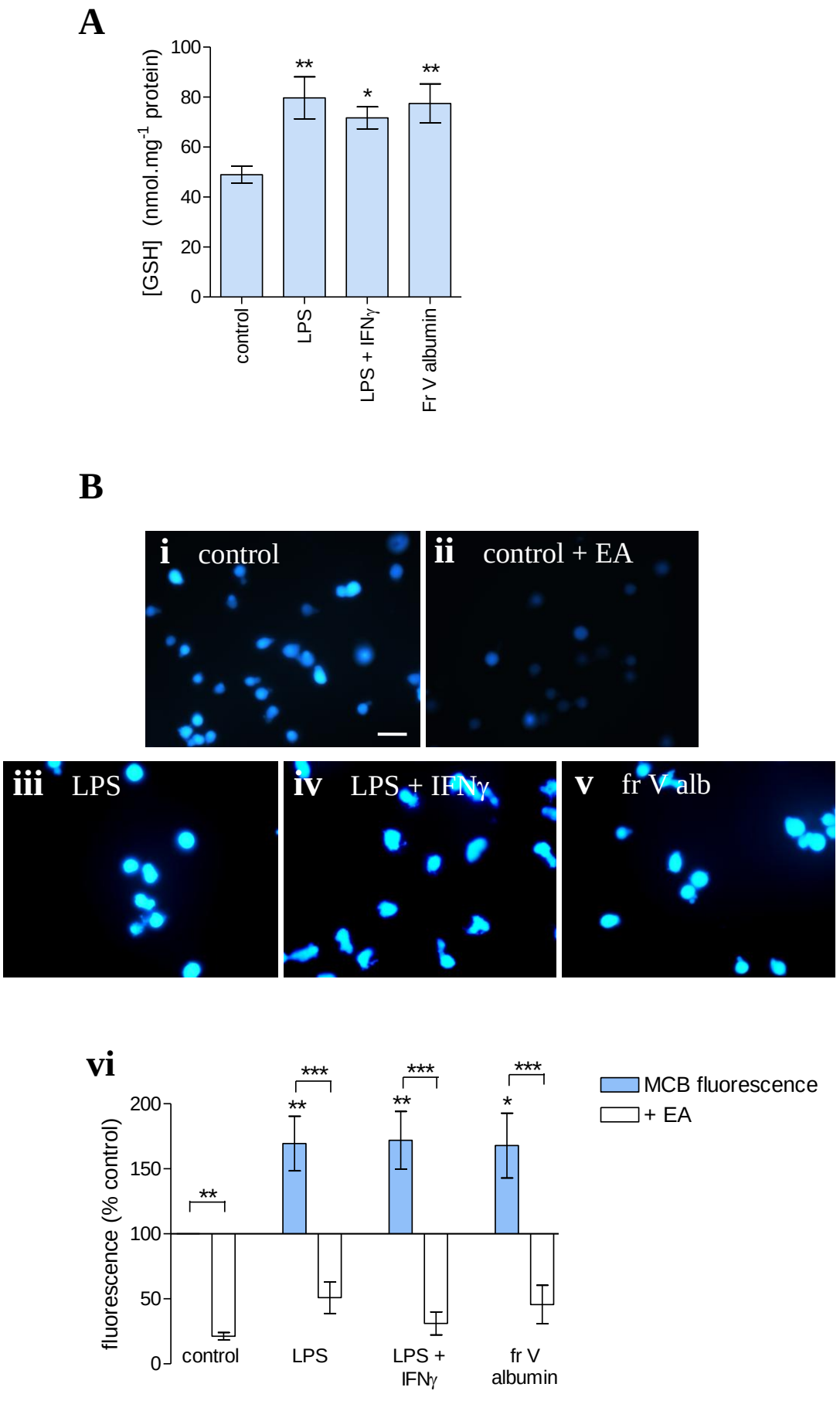


Figure 3.5. (legend overleaf)

Figure 3.5. (previous page) *The effect of microglial activation upon GSH levels in BV-2 microglial cells, as measured with reverse-phase HPLC (**A**) and quantitative analysis of monochlorobimane imaging (**B**). **A**, BV-2 cells were cultured in medium containing 10% FBS in the presence of LPS (1 µg.ml^{-1}), LPS + IFNγ (100 U.ml^{-1}) or fraction V albumin (2 mg.ml^{-1}) for 24 hours. Data represent the mean ± s.e.m. of nine independent experiments, each consisting of two samples per condition. Dunnett's multiple comparison post-test was used to determine the significance of the effects of individual treatments (*p<0.05, **p<0.01, compared with control). **B**, BV-2 cells were cultured in SFM in the presence of LPS (1 µg.ml^{-1}) (**iii**), LPS + IFNγ (100 U.ml^{-1}) (**iv**) or fraction V albumin (2 mg.ml^{-1}) (**v**) for 24 hours. Cells were incubated with 50 µM MCB for 30 minutes before imaging. Image **i** represents control cells. For image **ii** and where indicated in **vi**, 500 µM ethacrynic acid (EA) was added 10 minutes prior to MCB. **i-v**, Representative images are shown. Magnification, 40X. Scale bar, 20 µm. **vi**, Data are expressed as % average control fluorescence in each experiment. Each data set was analysed with a one-way ANOVA (p<0.0001 in each case). Data represent the mean ± s.e.m. of 3 – 6 independent experiments, each consisting of one or two coverslips per condition, with typically six fields analysed per coverslip. Tukey's multiple comparison post-test was used to establish significance of the effects of individual treatments (*p<0.05, **p<0.01, ***p<0.001, compared with control or as indicated).*

the concentration of GSH in control cells was calculated to be 48.9 ± 3.4 nmol.mg^{-1} protein. Both methods demonstrated a significant increase in intracellular GSH following all three treatments. LPS caused a 62.8 ± 17.2 % increase according to the HPLC method and a 69.4 ± 21.0 % increase according to MCB fluorescence quantification. Following treatment with LPS + IFNγ, intracellular GSH levels increased by 46.7 ± 9.0 % and 71.9 ± 22.2 % according to HPLC and MCB fluorescence, respectively. Fraction V albumin had a similar effect to LPS, causing a 58.3 ± 15.9 % increase in intracellular GSH according to HPLC and a 67.9 ± 24.9 % increase according to quantification of MCB fluorescence. The glutathione S-transferase (GST) inhibitor ethacrynic acid (EA; 500 μM) significantly blocked MCB fluorescence under each condition, suggesting that the majority of the MCB fluorescence is due to conjugation of the bimane to GSH rather than any other cellular thiols. There was no significant difference between any of the conditions when EA was added prior to MCB, suggesting that these compounds do not affect the binding of MCB to other thiols, and that the differences are indeed due to changes in GSH levels. Both these methods measure only the reduced form of glutathione, and theoretically an increase or decrease in GSH could simply indicate an equal and opposite change in the level of oxidised glutathione (GSSG). By incubating samples with glutathione reductase (GR), GSSG is converted to GSH so that subsequent HPLC analysis measures the total glutathione levels in a cell. Table 3.1 shows that following a number of different treatments, there was no difference between intracellular GSH and total glutathione in BV-2 cells, suggesting that all intracellular glutathione is present in the reduced form.

3.5. Different effects of microglial activation upon reduced glutathione levels in BV-2 and N9 microglial cells

By means of reverse-phase HPLC, the GSH levels of another mouse microglial cell line, N9, were determined, to compare with BV-2 GSH levels. BV-2 and N9 cells were incubated with LPS (1 μg.ml^{-1}), LPS + IFNγ (100 U.ml^{-1}), IFNγ, fraction V rat albumin (2 mg.ml^{-1}), CGA (500 nM) or Aβ_{25-35} (25 μM) for 24 hours before GSH analysis (fig. 3.6). The intracellular GSH concentration of control N9 cells was 70%

	[GSH] (nmol.mg^{-1} protein)	[GSH + GSSG] (nmol.mg^{-1} protein)
control	35.9 ± 1.93	35.9 ± 1.99
LPS	56.1 ± 5.52	55.1 ± 4.59
LPS + IFNγ	83.3 ± 13.95	82.6 ± 13.26
IFNγ	40.5 ± 5.83	40.0 ± 5.81
fr V albumin	61.7 ± 9.60	59.6 ± 7.93
A$\beta_{25\text{-}35}$	66.6 ± 11.36	65.2 ± 10.91
CGA	33.7 ± 2.58	33.3 ± 2.63
AIDA	23.2 ± 0.81	22.5 ± 0.93

Table 3.1. *A comparison of GSH and total glutathione (GSH + GSSG) in BV-2 microglial cells under a number of conditions. BV-2 cells were cultured in the presence of LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), fraction V albumin (2 mg.ml^{-1}), A$\beta_{25\text{-}35}$ (25 µM), CGA (500 nM), myelin (10 mg.ml^{-1}), AIDA (250 µM) or a combination. GSSG was converted to GSH using GR, and GSH was detected with reverse-phase HPLC. Data represent the mean ± s.e.m. of three independent experiments, each consisting of two samples per condition. Student's t tests were used to compare [GSH] with [GSH + GSSG] under each condition and there was no significant difference under any condition tested.*

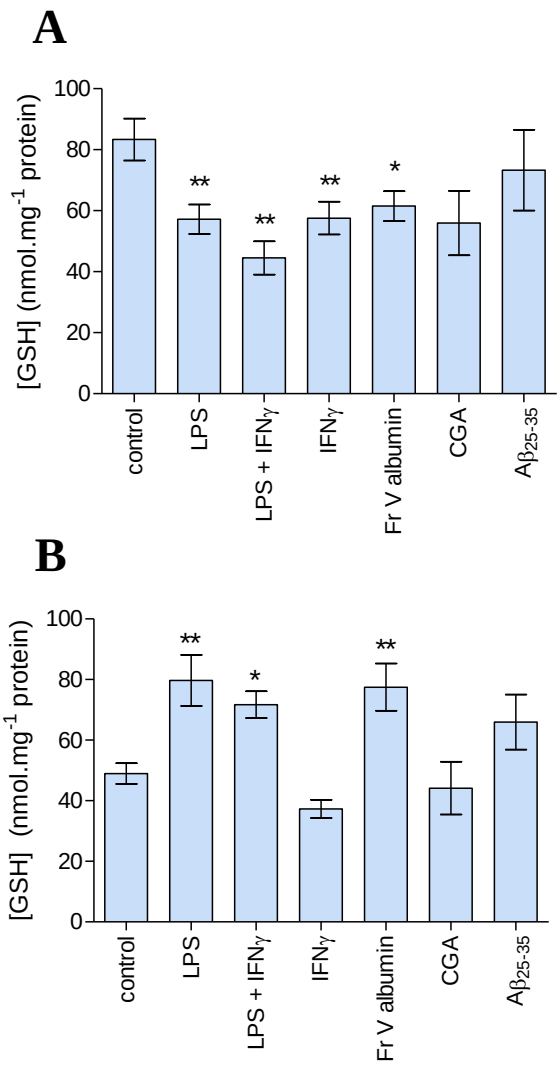


Figure 3.6. *The effect of microglial activation with various compounds upon GSH levels in N9 (**A**) or BV-2 (**B**) microglial cells. N9 or BV-2 cells were cultured for 24 hours in the presence of LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), fraction V albumin (2 mg.ml^{-1}), CGA (500 nM), Aβ$_{25-35}$ (25 µM) or a combination for 24 hours, and intracellular GSH levels were determined by reverse-phase HPLC.* **A,** *Data represent the mean ± s.e.m. of three (CGA, Aβ$_{25-35}$), six (fraction V albumin) or seven (control, LPS, LPS + IFNγ, IFNγ) independent experiments, each consisting of at least two samples per condition.* ***B****, Data represent the mean ± s.e.m. of three (CGA, Aβ$_{25-35}$) or nine (control, LPS, LPS + IFNγ, IFNγ, fraction V albumin) independent experiments, each consisting of two samples per condition. Each data set was analysed using a one-way ANOVA, which concluded that treatments had a highly significant effect upon GSH level in both cell lines (p=0.0003 for N9,* ***A****; p<0.0001 for BV-2,* ***B****). Dunnett's multiple comparison post-test was used to determine the significance of the effects of individual treatments (*p<0.05, **p<0.01, compared with control).*

higher than that of control BV-2 cells, at 83.4 ± 6.9 nmol.mg^{-1} protein. In addition, while microglial activation with LPS, LPS + IFNγ and fraction V albumin significantly increased GSH levels in BV-2 cells, by 62.8 ± 17.2 %, 46.7 ± 9.0 % and 58.3 ± 15.9 % respectively (fig. 3.6B), N9 GSH levels were decreased under the same conditions, by 31.3 ± 5.8 %, 46.6 ± 6.5 % and 26.2 ± 5.8 % respectively (fig. 3.6A). The condition having the most similar effect upon GSH levels in BV-2 and N9 cell lines was treatment with IFNγ alone, which caused a statistically significant 30.9 ± 6.4 % decrease in N9 cells and a (non-significant) 23.8 ± 6.1 % decrease in BV-2 cells. Treatment with $A\beta_{25-35}$ or CGA had no significant effect upon intracellular GSH in either cell line.

3.6. The differences between BV-2 and N9 microglial cells were not due to differential sensitivity to the activators

The different effects of the compounds associated with microglial activation upon GSH levels in BV-2 and N9 microglial cells may be due to different sensitivities to the activators, and these different responses may represent different parts of a common biphasic concentration-dependent response. Therefore intracellular GSH levels of BV-2 and N9 cells were determined with a range of concentrations of LPS (1 ng.ml^{-1} – 10 μg.ml^{-1}) and fraction V albumin (0.1 – 2 mg.ml^{-1}).

Figure 3.7 demonstrates that LPS caused a concentration-dependent decrease in GSH levels in N9 microglial cells (fig. 3.7A) and a concentration-dependent increase in BV-2 microglial GSH levels (fig. 3.7B). The effect upon BV-2 cells was gradual and became significant at 1 μg.ml^{-1} LPS, while in N9 cells 0.1 μg.ml^{-1} LPS had a significant effect. The effect in N9 cells was also more abrupt; there appears to be a threshold between 10 ng.ml^{-1} and 0.1 μg.ml^{-1} LPS, at which the intracellular GSH concentration drops from around 130 nmol.mg^{-1} protein to around 80 nmol.mg^{-1} protein.

Fraction V albumin caused a trend towards a concentration-dependent decrease in GSH levels in N9 cells (fig. 3.8A) and a trend towards a concentration-dependent

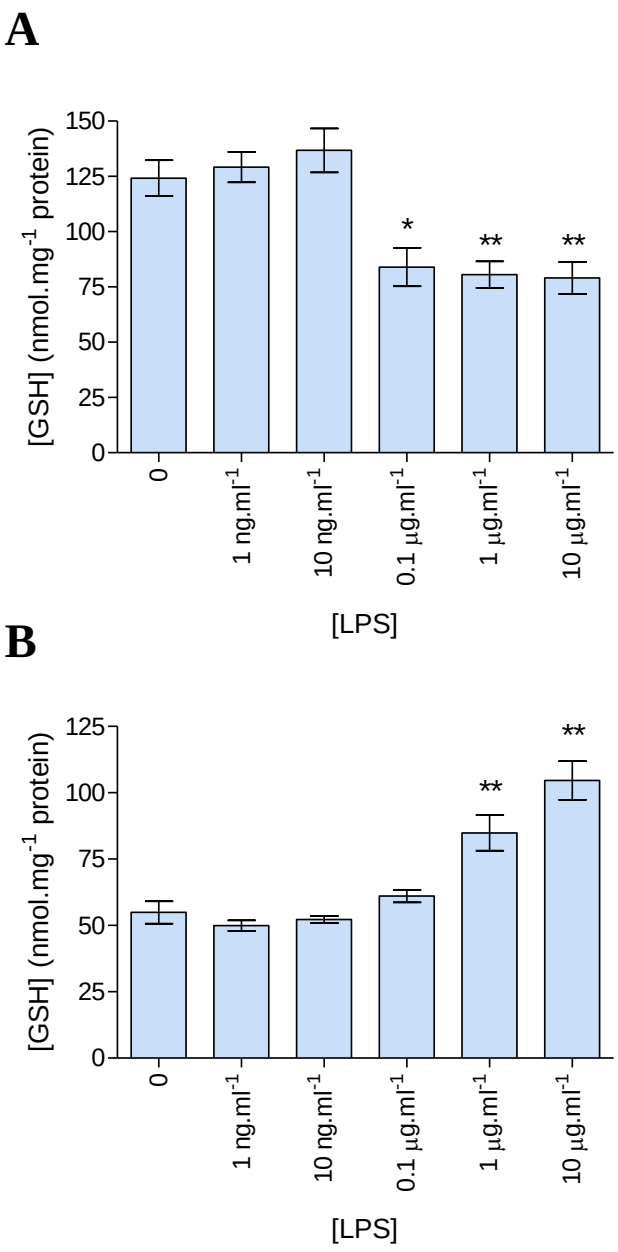


Figure 3.7. *The effect of LPS upon GSH levels in N9 (**A**) or BV-2 (**B**) microglial cells. Cells were cultured for 24 hours in the presence of LPS (1 ng.ml^{-1} – 10 µg.ml^{-1}), and intracellular GSH levels were determined by reverse-phase HPLC. Data represent the mean ± s.e.m. of three independent experiments, each consisting of two samples per condition. Each data set was analysed with a one-way ANOVA, which showed a highly significant effect of LPS concentration upon GSH levels in each case (p<0.0001). Dunnett's multiple comparison post-test was used to determine the significance of the effect of each concentration (*p<0.05, **p<0.01, compared with control).*

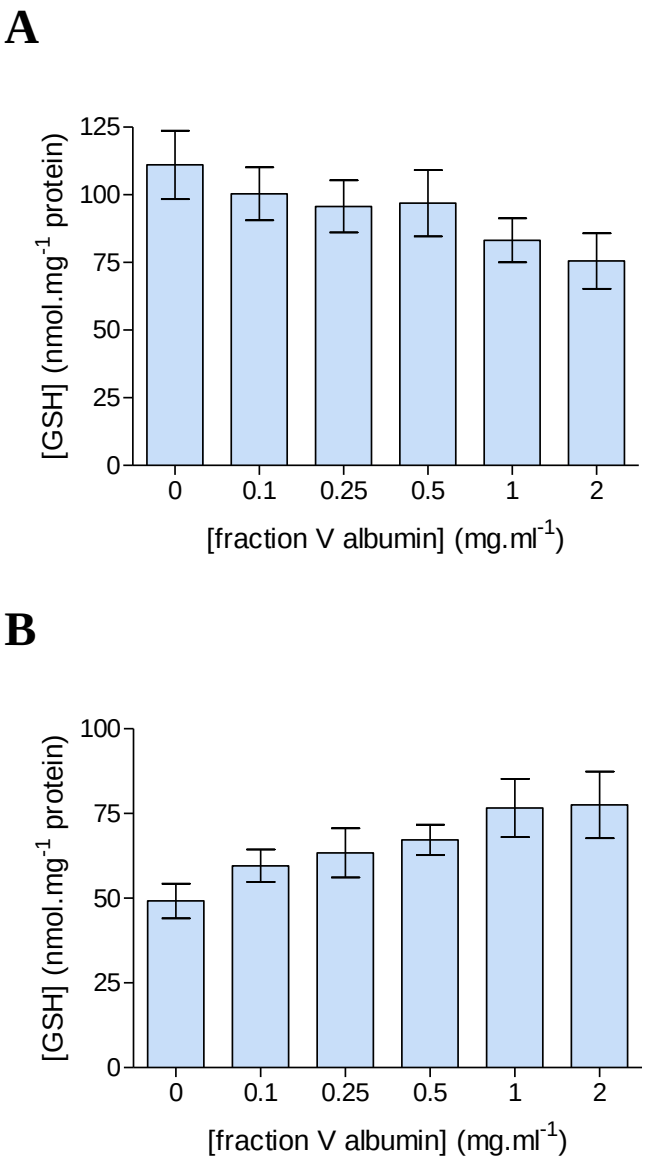


Figure 3.8. *The effect of fraction V albumin upon GSH levels in N9 (**A**) or BV-2 (**B**) microglial cells. Cells were cultured for 24 hours in the presence of fraction V albumin (0.1 – 2 mg.ml^{-1}), and intracellular GSH levels were determined by reverse-phase HPLC. Data represent the mean ± s.e.m. of three independent experiments, each consisting of two samples per condition. Each data set was analysed with a one-way ANOVA, which showed the effect of fraction V albumin concentrations upon GSH levels to be non-significant in either case (p=0.2404 for N9, **A**; p=0.0625 for BV-2, **B**).*

increase in GSH levels in BV-2 cells (fig. 3.8B), although in this case the effect was not found to be statistically significant in either cell line.

In an attempt to enhance the effect of fraction V albumin, and because serum contains a certain amount of albumin, the medium on plated BV-2 microglia was changed to SFM several hours before the addition of fraction V albumin. SFM is used when preparing samples for a number of other experiments but is not routinely used when preparing BV-2 cells for HPLC because the cells begin to detach in the absence of serum, reducing the number available for GSH determination. Comparing the effect of fraction V albumin upon GSH levels in BV-2 cells cultured in SCM with those cultured in SFM does indeed reveal a significant difference ($p<0.0001$, fig. 3.9). Importantly, the GSH content in control cells in the different media did not significantly differ (49.2 ± 5.1 nmol.mg^{-1} protein with SCM, 54.3 ± 3.9 nmol.mg^{-1} protein with SFM), although adding as little as 0.25 mg.ml^{-1} fraction V albumin to BV-2 cells in SFM led to a significant increase in intracellular GSH levels. The effect of fraction V albumin on GSH levels in BV-2 cells in SFM became highly significant compared with control ($p < 0.001$) at 1 mg.ml^{-1}. Interestingly, with 0.1 – 1 mg.ml^{-1} fraction V albumin, BV-2 cells in SFM consistently contained 33 % more GSH than those cultured in SCM. With 2 mg.ml^{-1} fraction V albumin, the difference declined to 19 %.

3.7. The differences between BV-2 and N9 microglial cells were not due to differences in the temporal responses to the activators

The apparent opposite effects of microglial activation upon GSH levels in BV-2 and N9 microglial cell lines could be due to temporal aspects of the response. A time course analysis of GSH levels in N9 cells (fig. 3.10) showed that control GSH levels remained fairly constant over at least 48 hours, and suggested that GSH levels in cells treated with LPS, LPS + IFNγ, CGA or $A\beta_{25-35}$ remained fairly consistently below control levels from 4 hours onwards. However, due to technical considerations, LPS + IFNγ and $A\beta_{25-35}$ could not be repeated a sufficient number of times to allow statistical analyses. The effect of LPS was found to be statistically

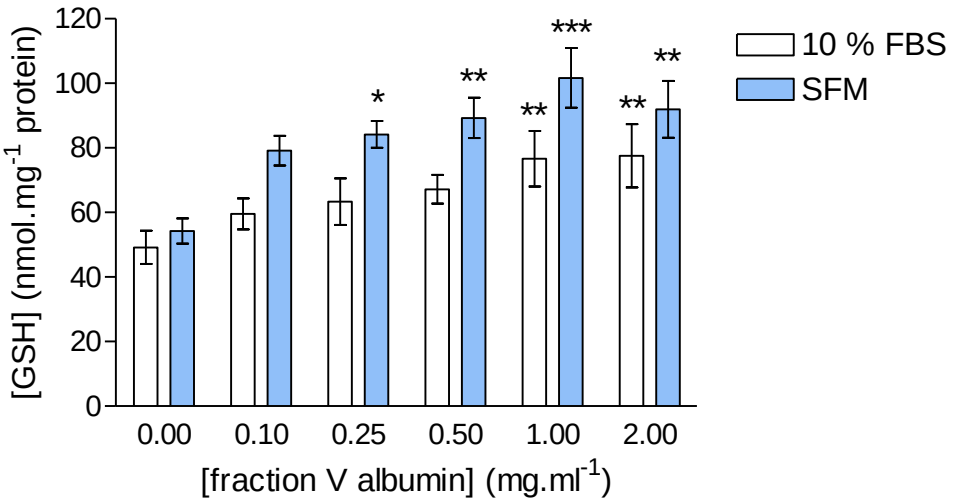


Figure 3.9. *The effect of fraction V albumin upon BV-2 microglial GSH levels in SCM or SFM. BV-2 cells were plated in SCM, which was changed either to fresh SCM or to SFM before cells were exposed to fraction V albumin (0 – 2 mg.ml^{-1}) for 24 hours. GSH levels were determined by reverse-phase HPLC. Data represent the mean ± s.e.m. of two (SFM) or three (10 % FBS) independent experiments, each consisting of two samples per condition. Data were analysed by means of a two-way ANOVA, which concluded that the effect of fraction V albumin concentration and the effect of the type of medium in which the cells were cultured were both highly significant (p<0.0001), with no significant interaction between the two (p=0.7767). Bonferroni's post-tests were carried out to determine the significance of the effect of each concentration of fraction V albumin, in each type of medium (*p<0.05, **p<0.01, ***p<0.001, compared with relevant control).*

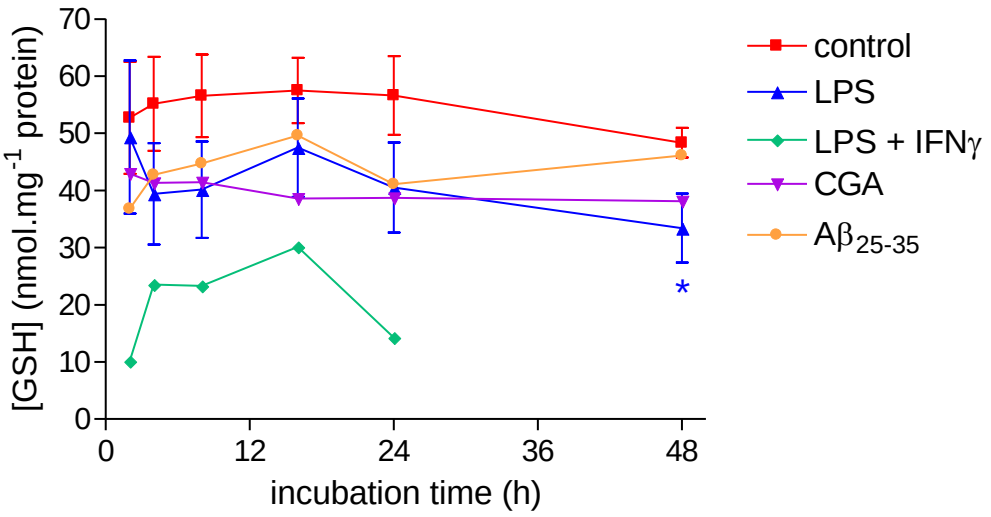


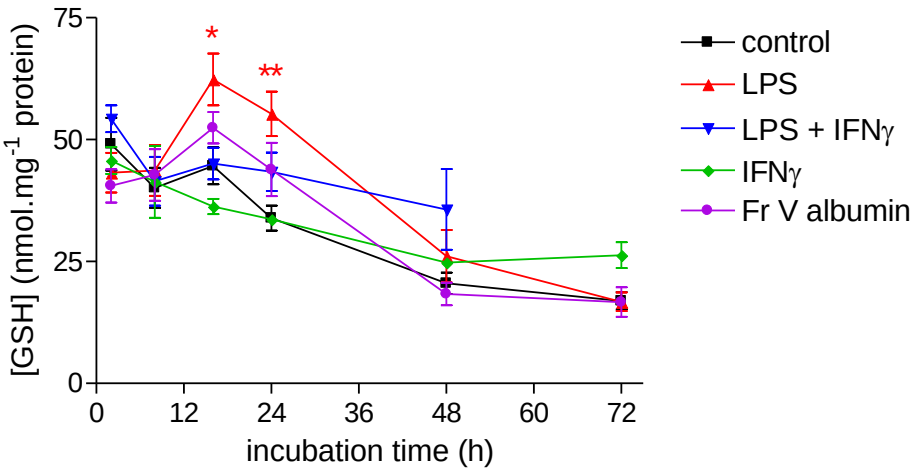
Figure 3.10. *Time course of changes in GSH levels in N9 microglial cells. N9 cells were cultured for 2, 4, 8, 16, 24 or 48 hours in the presence of LPS (1 µg.ml^{-1}), LPS + IFNγ (100 U.ml^{-1}), CGA (500 nM) or Aβ$_{25-35}$ (25 µM), and reduced glutathione (GSH) levels were determined using reverse-phase HPLC. Data represent the mean ± s.e.m. (where appropriate) of one (LPS + IFNγ, Aβ$_{25-35}$), two (CGA), three (LPS) or four (control) independent experiments, each consisting of two samples per condition. A one-way ANOVA was carried out on control, LPS and CGA data at each time point. Stastistical significance (i.e. p<0.05) was only established at 48 hours (p=0.0439); Dunnett's post test was used to compare the LPS and CGA treatment with control at this time point (*p<0.05 compared with control).*

significant only following 48 hours' incubation. The lack of statistical significance at earlier time points may be due to the relatively high levels of inter-experiment variability.

The time course of the experiment in BV-2 microglial cells is clearly different (fig. 3.11A). Intracellular GSH levels in plated BV-2 cells declined steadily over time, with a GSH concentration in control cells at 72 hours only 35 % of that at 2 hours. Treatment of BV-2 cells with LPS caused intracellular GSH levels to rise to a maximum at 16 - 24 hours and then to decline sharply and return to control levels by around 48 hours. Fraction V had a similar effect, although this was less pronounced and therefore did not reach statistical significance. LPS + IFNγ appeared to slightly stabilise the time-dependent decline in GSH levels between 16 and 48 hours. This treatment caused cells to detach from the plate over time, and a 72 hour measurement was not possible. There was no significant effect of IFNγ alone at any time point.

The effects of the iNOS inhibitors 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT-HCl; 150 nM) (Nakane *et al.* 1995) and N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride (1400W; 10 μM) (Garvey *et al.* 1997) upon the time course of the effect of LPS upon BV-2 microglial GSH levels were also investigated (fig. 3.11B). When tested alone, neither AMT-HCl nor 1400W gave intracellular GSH levels significantly different from control at 2, 24 or 72 hours. When BV-2 cells were exposed to AMT-HCl or 1400W for 1 hour prior to LPS addition, intracellular GSH levels were not significantly different at any time point to those found when LPS alone was used. In addition, the intracellular GSH concentration in BV-2 cells following exposure to AMT-HCl was not significantly different to that following exposure to 1400W, in either the absence or presence of LPS. As demonstrated by figure 3.11C, these concentrations of AMT-HCl and 1400W inhibited 35.1 ± 2.2 % and 78.8 ± 1.2 %, respectively, of the LPS-induced nitrate and nitrite production by primary microglial cells.

A



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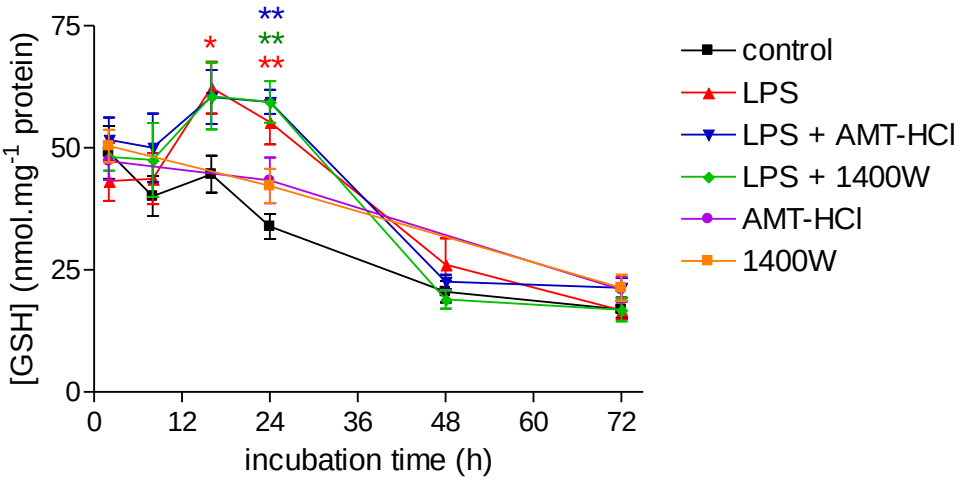

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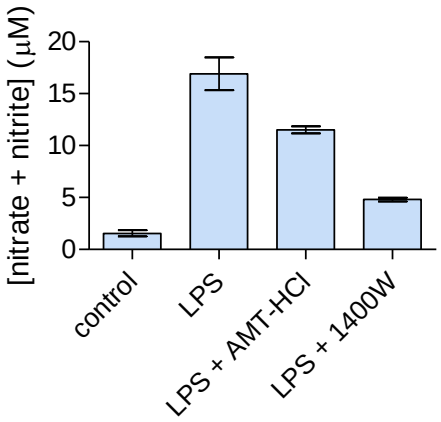


Figure 3.11. (legend overleaf)

Figure 3.11. (previous page) ***A***, ***B***, *Time course of changes in GSH levels in BV-2 microglial cells. BV-2 cells were cultured for 2, 8, 16, 24, 48 or 72 hours in the presence of LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), fraction V albumin (2 mg.ml^{-1}), AMT-HCl (150 nM), 1400W (10 µM), or a combination, and GSH levels were determined using reverse-phase HPLC. Where used in combination, cells were pre-incubated with AMT-HCl or 1400W for 1 hour before addition of LPS. Data represent the mean ± s.e.m of five (control, LPS, fraction V albumin) or three (all other data sets) independent experiments, each consisting of two samples per condition. Data were analysed as one set with a one-way ANOVA at each time point, which showed significant differences between treatments at 16 hours ($p<0.01$) and at 24 hours ($p<0.0001$). At these time points, Tukey's multiple comparison post-tests were performed (*$p<0.05$, **$p<0.01$ compared with control).* ***C****, The effect of iNOS inhibitors upon microglial NO production as measured by nitrate and nitrite release. Primary microglial cells were preincubated with AMT-HCl (150 nM) or 1400W (10 µM) for 1 hour before addition of LPS (1 µg.ml^{-1}). Conditioned medium was removed after 24 hours for nitrate/nitrite measurement. Data represent mean ± s.e.m. of four samples per condition, from one experiment.*

3.8. Activation of primary microglia led to changes in GSH levels similar to those seen in BV-2 cells

The effect of microglial activation upon intracellular GSH levels is clearly different in BV-2 and N9 microglial cells, and this does not appear to be explained by different sensitivities to the compounds or by temporal differences. Therefore, GSH levels in primary rat microglia were determined by means of reverse-phase HPLC (fig. 3.12) and quantitative analysis of MCB fluorescence (fig. 3.13). Control primary rat microglia were found to contain GSH at a concentration of 28.1 ± 2.0 $nmol.mg^{-1}$ protein (fig. 3.12) as determined by reverse-phase HPLC. Following 24 hours' incubation with 1 $\mu g.ml^{-1}$ LPS, this was increased by 29.7 ± 20.1 % to 36.4 ± 5.6 $nmol.mg^{-1}$ protein. The GSH concentration following 24 hours' incubation with 2 $mg.ml^{-1}$ fraction V albumin was 32.6 ± 4.0 $nmol.mg^{-1}$ protein, representing a 16.0 ± 14.4 % increase above control. However, due to the relatively high level of variation under each condition, neither of the changes observed was statistically significant.

Quantitative analysis of MCB fluorescence in primary microglia suggested that LPS, LPS + IFNγ (100 $U.ml^{-1}$) and fraction V albumin all significantly increased microglial GSH content (fig. 3.13). Incubation of microglia with LPS for 24 hours resulted in a GSH level 31.9 ± 9.2 % above control level, incubation with LPS + IFNγ gave a 49.5 ± 11.9 % increase above control, and incubation with fraction V albumin gave a 40.5 ± 5.7 % increase above control. Incubation with IFNγ alone, however, did not significantly alter MCB fluorescence. EA (1 mM) significantly blocked MCB fluorescence under each condition, suggesting that the majority of the MCB fluorescence is due to conjugation of the bimane to GSH rather than any other cellular thiols. There was no significant difference between any of the conditions when EA was added prior to MCB, suggesting that these treatments do not affect the binding of MCB to other thiols, and that the differences are indeed due to changes in GSH levels.

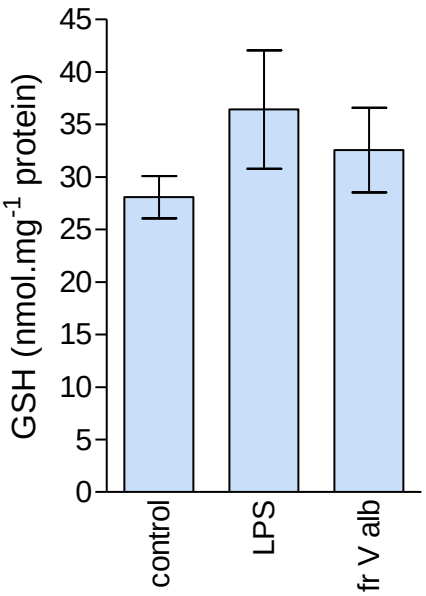


Figure 3.12. *The effect of microglial activation upon GSH levels in primary rat microglia, as measured by reverse-phase HPLC. Primary rat microglia were cultured for 24 hours in the presence of LPS (1 µg.ml^{-1}) or fraction V albumin (2 mg.ml^{-1}). Data represent the mean ± s.e.m. of four independent experiments, each consisting of one or two samples per condition. Data were analysed with a one-way ANOVA, which failed to demonstrate a significant effect of treatment upon GSH levels (p=0.31).*

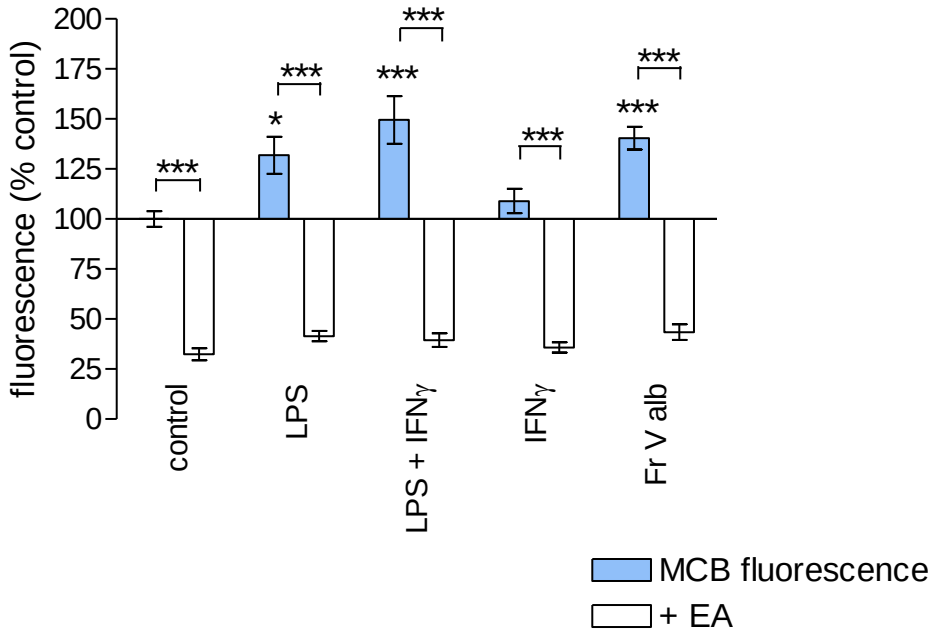


Figure 3.13. *The effect of microglial activation upon microglial GSH levels, as measured with quantitative analysis of MCB imaging. Primary rat microglial cells were cultured in SFM in the presence of LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), fraction V albumin (2 mg.ml^{-1}), or a combination for 24 hours. Cells were incubated with 100 µM MCB for 30 minutes before imaging. Where indicated, 1 mM ethacrynic acid was added 10 minutes prior to MCB. Data represent the mean ± s.e.m. of four independent experiments, each consisting of 1 – 4 (typically 3) coverslips per condition, with typically six fields analysed per coverslip. Data are expressed as % average control fluorescence in each experiment, and was analysed with a one-way ANOVA which showed there to be highly significant differences between conditions (p<0.0001). Tukey's multiple comparison post-test was used to establish significance between individual data sets (*p<0.05, ***p<0.001, when compared with control or between test conditions as indicated).*

3.9. GSH release by BV-2 microglial cells

It has not been firmly established whether microglia release GSH. Because components of the culture medium (Dulbecco's modified Eagle medium, D-MEM) interfere with the electrochemical detection used with HPLC, BV-2 microglial conditioned medium was analysed for GSH content by means of a luminescent GSH detection assay. In five independent experiments BV-2 conditioned medium contained micromolar levels of GSH. Following 8 hours' incubation, very little GSH was detected in microglial conditioned medium, and there was no difference in the release by control and activated cells (fig. 3.14A). Following 16 hours' incubation, control conditioned medium contained 4.9 ± 1.6 μM GSH, and LPS (1 $\mu g.ml^{-1}$) conditioned medium contained 25.1 ± 13.2 μM GSH, representing a significant 417 ± 272 % increase above control (fig. 3.14A). In contrast, conditioned medium from BV-2 cells treated with LPS + IFNγ (100 $U.ml^{-1}$) contained just 7.1 ± 2.5 μM GSH (fig. 3.14A). Following 24 hours' incubation, control conditioned medium contained 6.9 ± 1.9 μM GSH, LPS conditioned medium contained 20.0 ± 6.0 μM GSH, and LPS + IFNγ conditioned medium contained 8.6 ± 2.2 μM GSH (fig. 3.14A). Data therefore suggest that very little GSH release occurs for the first 8 hours of incubation, that LPS-induced GSH release occurs mostly between 8 and 16 hours' incubation, and that LPS + IFNγ treatment leads to a GSH release profile very similar to that of untreated cells throughout (fig. 3.14A).

The import of cystine via the x_c^- transporter may be a rate-limiting factor in GSH synthesis. Therefore the effects of supplementary cystine (to a total of 1 mM) and the x_c^- inhibitor aminopimelic acid (APA) (2.5 mM) upon BV-2 GSH release were investigated. As shown in figure 3.14B, supplementary cystine enhanced BV-2 GSH release by around 50% in control and LPS-treated cells. Such an effect was not evident in the case of LPS + IFNγ-treated cells. In contrast, APA consistently decreased GSH release, by 59.2 ± 5.8 %, 79.3 ± 5.2 % and 61.8 ± 16.0 %, for control, LPS-treated and LPS + IFNγ-treated cells, respectively. When the data set was analysed as a whole, a two-way analysis of variance (ANOVA) failed to demonstrate a significant effect of cystine/APA in the medium (p=0.0918) or of LPS/IFNγ treatment (p=0.0749). However, analysis of the control and APA data

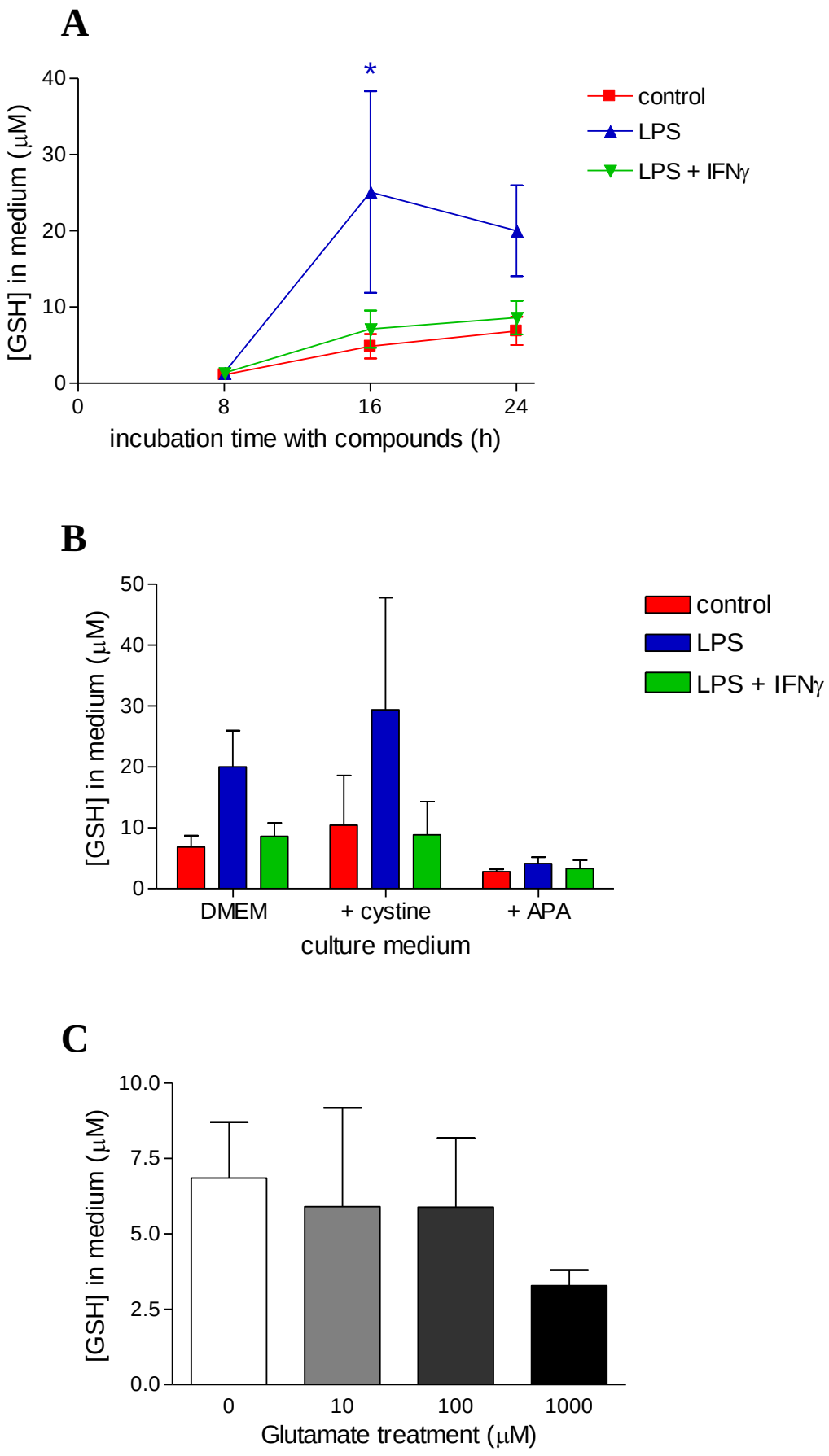


Figure 3.14. (legend overleaf)

Figure 3.14. (previous page) *GSH is present in BV-2 microglial conditioned medium.* ***A****, BV-2 cells were cultured in the presence of LPS (1 µg.ml^{-1}) or IFNγ (100 U.ml^{-1}) for 8, 16 or 24 hours.* ***B****, BV-2 cells were cultured for 1 hour in serum-free DMEM containing supplementary L-cystine (to a total of 1 mM) or aminopimelic acid (APA; 2.5 mM) before the addition of LPS and IFNγ for 24 hours.* ***C****, BV-2 cells were cultured in the presence of L-glutamate (10 µM – 1 mM) for 24 hours. Conditioned medium was analysed for GSH content using the GSH-Glo assay kit.* ***A****, Data represent the mean ± s.e.m. of four (8h, 16h) or five (24h) independent experiments. Data were analysed with a two-way ANOVA, which demonstrated a significant effect of incubation time (p=0.0167) and treatment (p=0.0172) upon GSH release. Bonferroni's post-test was used to test for significant differences between treatments at each time point; *p<0.05 compared with control.* ***B****, Data represent the results of three (cystine, APA) or five (DMEM) independent experiments. Data were analysed with a two-way ANOVA, which failed to demonstrate a significant effect of cystine/APA presence (p=0.0918) or LPS/IFNγ treatment (p=0.0749) upon GSH release.* ***C****, Data represent the results of three (10 µM, 100 µM, 1000 µM glutamate) or five (0 µM glutamate) independent experiments. Data were analysed with a one-way ANOVA (p=0.6979, n.s.).*

sets, without the cystine data, with a two-way ANOVA found the effect of APA to be significant (p=0.0112), but not that of LPS/IFNγ treatment (p=0.1304). Analysis of the control and cystine data sets, without the APA data, failed to demonstrate significant effects of cystine (p=0.4632) or of LPS/IFNγ treatment (p=0.0596). The lack of significance of the effect of cystine is likely to be due to the high levels of inter-experiment variability.

The effect of exogenous glutamate (10 µM – 1 mM) upon GSH release was also investigated. Treatment with 1 mM glutamate led to 52.1 ± 7.5 % lower GSH levels in conditioned medium compared with control conditioned medium (fig. 3.14C). However, a one-way ANOVA failed to demonstrate this to be significant (p=0.6979), again probably due to the variability of the data.

Levels of lactate dehydrogenase (LDH) in conditioned medium were determined, as a measure of cell stress and lysis, to ascertain whether the GSH release detected may be due to toxicity of the compounds, causing cell lysis, rather than specific release of GSH. Control cells released 12.6 ± 1.14 % of the total LDH. Figure 3.15 shows that the only treatments to elicit significant LDH release above this were LPS (1 $\mu g.ml^{-1}$) in combination with IFNγ (100 $U.ml^{-1}$), which caused 23.3 ± 2.05 % LDH release at 16 and 39.8 ± 3.98 % release at 24 hours, and IFNγ alone, which only caused significant release (23.4 ± 3.79 %) after 24 hours' incubation (fig. 3.15A). In contrast, after 24 hours' incubation, LPS alone, fraction V albumin (2 $mg.ml^{-1}$) , cystine (to a total of 1 mM) or APA (2.5 mM) did not cause significant LDH release above control levels (fig. 3.15B).

3.10. GSH release by primary microglial cells

Primary microglial conditioned medium was also analysed for GSH content, to establish whether primary microglia may also release GSH. Low micromolar concentrations of GSH were detected in primary microglial conditioned medium in four independent experiments. The effect of microglial activation with LPS and IFNγ as well as the effect of cystine and the x_c^- inhibitor APA upon GSH release were

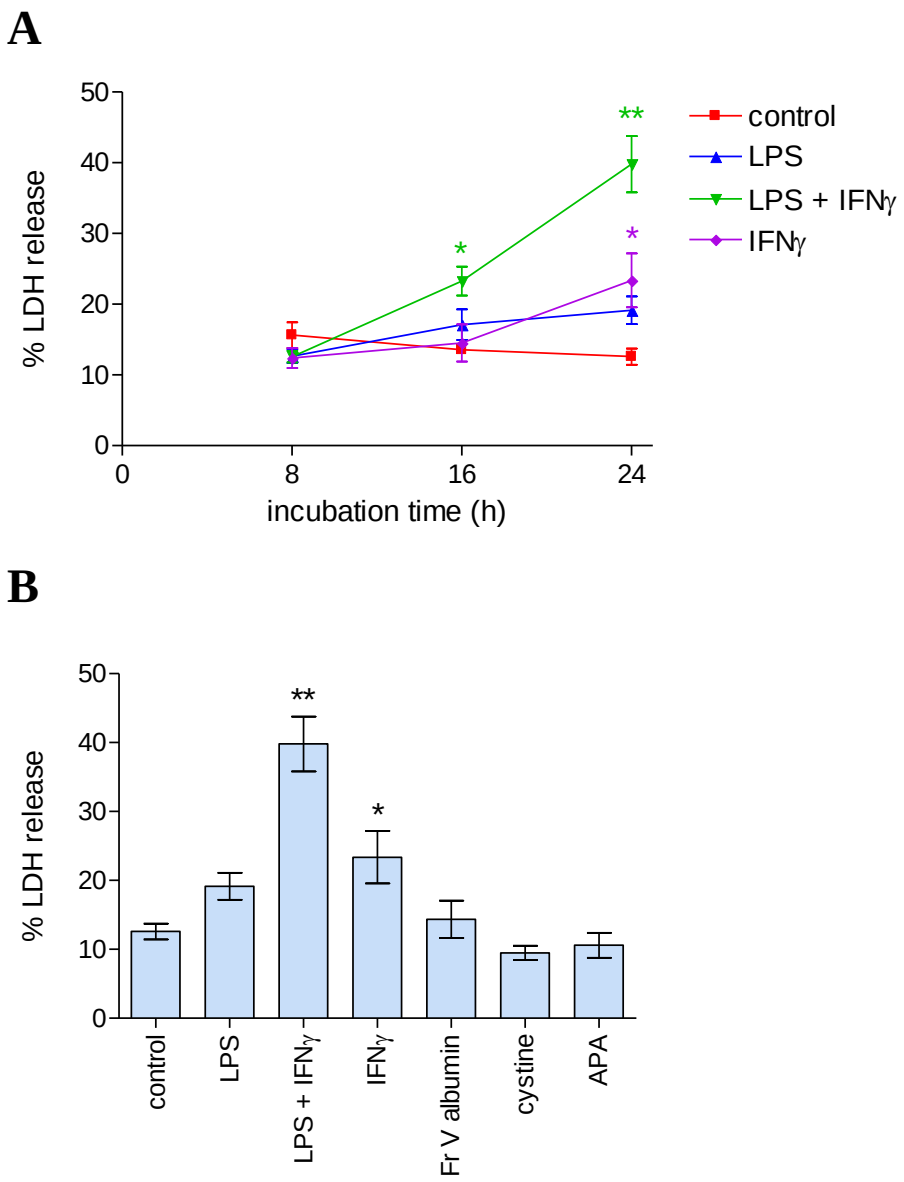


Figure 3.15. *LDH release by BV-2 microglial cells as an indicator of cell viability. BV-2 microglia were cultured in the presence of LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), fraction V albumin (2 mg.ml^{-1}), SIB-1757 (50 µM), supplementary L-cystine (to a total of 1 mM), APA (2.5 mM) or a combination for 8, 16 or 24 hours and conditioned medium was assayed for lactate dehydrogenase (LDH) content. 0% represents the LDH content of serum-free medium and 100% the LDH content of conditioned medium following 30 minutes' incubation with 1% Triton X-100. Data represent the mean ± s.e.m. of three independent experiments, each consisting of two or three samples per condition. Data were analysed with a one-way ANOVA with Dunnett's multiple comparison post-test, *p<0.05, **p<0.01 compared with control.*

investigated (fig. 3.16). Because BV-2 microglia did not demonstrate appreciable GSH release following 8 hours' incubation, primary microglial conditioned medium was only analysed following 16 and 24 hours' incubation. Individual one-way ANOVAs at each time point failed to demonstrate a significant effect of LPS/IFNγ treatment upon GSH release in the absence or presence of cystine or APA ($p > 0.65$ in each case). Indeed, there did not even appear to be a consistent trend towards an effect of either LPS or LPS + IFNγ treatment.

3.11. Discussion

3.11.1. GSH content of BV-2 and N9 cells is affected differently by identical activating stimuli; BV-2 cells are more like primary microglia

Using reverse-phase HPLC, GSH levels of BV-2 and N9 microglial cells under control and activated conditions were determined in parallel. Under control conditions, BV-2 and N9 cells contained 48.9 ± 3.4 and 83.4 ± 6.9 $nmol.mg^{-1}$ protein, respectively. A number of studies upon microglia appear to report GSH concentrations normalised to a control (Dopp *et al.* 2002; Roychowdhury *et al.* 2003), but the GSH concentrations determined here are similar to other reported values for microglial cell lines (Moss and Bates 2001).

Strikingly, where LPS, LPS + IFNγ and fraction V albumin caused significant increases in BV-2 GSH levels, identical treatments caused significant decreases in N9 GSH levels (fig. 3.6). In N9 cells (fig. 3.6A), LPS or IFNγ alone had similar effects, and the two compounds appeared to have an additive effect. In BV-2 cells (fig. 3.6B), while LPS significantly increased GSH levels, treatment with IFNγ had no significant effect. When measured by HPLC, the GSH content of BV-2 cells following treatment with LPS and IFNγ in combination represented an intermediate between the GSH content observed following incubation with each of the compounds alone. Different effects of these microglial activators upon the GSH levels in these microglial cell lines could indicate differences in the signalling pathways following activation. This could have important implications, since LPS is

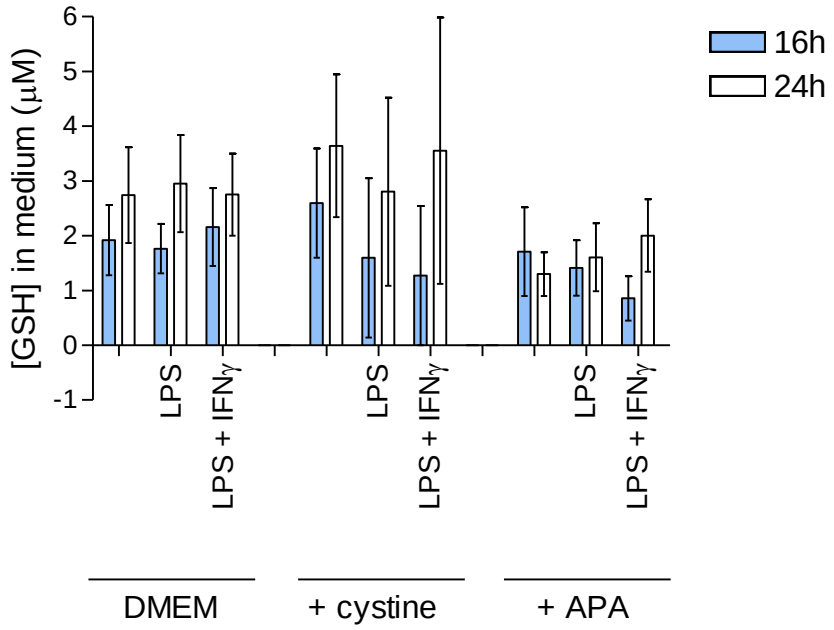


Figure 3.16. *GSH is present in primary microglial conditioned medium. Primary rat microglia were pre-incubated for 1 hour in serum-free DMEM containing supplementary L-cystine (to a total of 1 mM) or aminopimelic acid (APA; 2.5 mM), before the addition of LPS (1 μg.ml^{-1}) and IFNγ (100 U.ml^{-1}) for 16 or 24 hours. Conditioned medium was analysed for GSH content using the GSH-Glo assay kit. Data represent the mean ± s.e.m. of three (cystine) or four (DMEM, APA) independent experiments. Data were analysed with individual one-way ANOVAs, which failed to demonstrate an effect of LPS/IFNγ treatment in serum-free DMEM or in the presence of cystine or APA at either time point ($p > 0.65$ in all cases, n.s.).*

commonly used in both cell lines as a model of microglial activation, often in the investigation of activation-associated intracellular signalling pathways.

The effects of microglial activation with LPS, IFNγ and fraction V albumin upon GSH levels in primary microglia were also investigated, using both reverse-phase HPLC and quantitative analysis of MCB imaging (fig. 3.12 and 3.13). The intracellular GSH level of primary microglia was found to be 28.1 ± 2.0 nmol.mg^{-1} protein, in agreement with previous reports (Hirrlinger *et al.* 2000; Persson *et al.* 2006). Activation of microglia with LPS (1 μg.ml^{-1}) for 24 hours caused around a 30 % increase in GSH levels. This result only reached statistical significance for the MCB imaging data, likely due to the fact that large numbers of coverslips can be imaged and analysed using this method, reducing the indicators of variation, whilst large numbers of cells are required for HPLC analysis and therefore fewer measurements could be taken. Exposure of primary microglia to fraction V albumin (2 mg.ml^{-1}) led to a 40.5 % increase in GSH levels, as measured by MCB imaging but only a non-significant 16.0 % increase when determined by HPLC. This may be due to the fact that cells prepared for HPLC were cultured in medium containing 10 % FBS to prevent detachment and maintain maximum protein content, so as to ensure the best possible signal on HPLC. In contrast, the medium on cells used for MCB imaging was changed to SFM prior to the addition of activators. FBS in the medium is likely to contain a certain amount of albumin, however as demonstrated here (fig. 3.9), although this may mask to some extent the effects of exogenous albumin, it does not appear to affect the basal GSH levels. The data presented here suggest that, in terms of the effects of microglial activation upon GSH levels, BV-2 cells are more akin to primary microglia than are N9 cells. The high numbers of cells required for HPLC analysis, and the low signal to noise ratio observed when measuring primary microglial GSH levels by HPLC justify the use of the BV-2 cell line as a microglial model in this situation. The data produced must however be interpreted with caution.

3.11.2. The two methods of GSH quantification: advantages and disadvantages

Here reverse-phase HPLC and quantification of MCB fluorescence were used to measure the intracellular GSH levels of BV-2 microglial cells. Similar results were

produced with the two methods, with LPS, LPS + IFNγ and fraction V albumin significantly increasing intracellular GSH levels in both cases (fig. 3.5). HPLC is renowned as a highly sensitive and accurate measure (Riederer *et al.* 1989; Frade *et al.* 2008), and has the advantage of yielding absolute GSH levels, while MCB imaging gives data in arbitrary fluorescence units which can be normalised as appropriate. Relatively large amounts of protein are however required for HPLC determination, whereas an imaging method such as that using MCB allows analysis of many more conditions utilising fewer cells. The latter method is therefore particularly useful in the case of primary microglial cells whose quantity is limited.

Potential drawbacks of the MCB imaging method are centred on the fact that it relies upon endogenous GST to conjugate the bimane to GSH. Should any treatment of the cells alter GST expression or activity, this could result in a change in MCB-GSH conjugation efficiency and therefore affect the fluorescence levels at the end of the incubation period, an outcome which would be interpreted as a change in GSH concentration. Indeed, a 1.7-fold upregulation of GST protein has been demonstrated in BV-2 microglia following 24 hours' exposure to 100 $ng.ml^{-1}$ LPS, as has a 1.2-fold upregulation of GST gene expression in primary microglial cultures following 4 hours' exposure (Lund *et al.* 2006). LPS has also been shown to upregulate mRNA and protein expression for the GSTπ isoform in macrophages (Xue *et al.* 2005). Cadmium treatment of primary microglia, which increased ROS levels and was neurotoxic in mixed neurone/glia cultures, led to up to 2.7-fold increased GSTπ mRNA expression levels (Yang *et al.* 2007). Conversely, viral infection of N9 cells was shown to significantly downregulate GSTπ gene expression (McKimmie *et al.* 2006). GST expression and activity has been shown to decrease in AD (Lovell *et al.* 1998b), although cell-specific expression was not identified. Immunohistochemical observations have however demonstrated an upregulation of GSTμ isoform in microglia in AD (Tchaikovskaya *et al.* 2005).

Nevertheless, regardless of the rate, the GST-catalysed reaction should achieve a steady state given sufficient incubation time, provided MCB does not become limiting. For this reason it is important to follow the time course of the reaction and select an appropriate MCB incubation time. However some cell types, notably astrocytes, rapidly export the GS-MCB conjugate via Mrp1 (Waak and Dringen

2006), a phenomenon characterised by a rapid decrease in fluorescence after a relatively short period of incubation (Chatterjee *et al.* 1999). In these instances, fluorescence intensity may never attain the "steady state" enabling determination of GSH concentration; the fluorescence profile over time would more likely reflect the relative rates of the activities of GST and Mrp1.

There are a number of different GST isoforms, with different cell types having different expression profiles. Microglia have been shown to express GSTμ (Tchaikovskaya *et al.* 2005) and GSTπ (McKimmie *et al.* 2006; Yang *et al.* 2007). Affinity for MCB varies between GST isoforms (Cook *et al.* 1991; Ublacker *et al.* 1991), therefore it is not necessarily valid to compare different cell types under the same conditions (Chatterjee *et al.* 1999, 2000; Keelan *et al.* 2001), unless their GST isoform profiles have been verified as being similar. It should also be noted that the MCB-GSH adduct has inhibitory effects upon GST-catalysed reactions, with an IC_{50} in the tens of micromolar range (Cook *et al.* 1991), indicating a possibility of end-product inhibition of the reaction.

EA is used to inhibit the conjugation of MCB to GSH. EA also forms a (non-fluorescent) conjugate with MCB, with both EA and the MCB-EA conjugate inhibiting GST activity (Ploemen *et al.* 1990; Awasthi *et al.* 1993). The data presented here (fig 3.5B) demonstrate that when pre-treated with 500 μM EA, BV-2 cells under different conditions have a fluorescence level 21 – 51% of that of MCB-treated control cells. A higher concentration of EA may have been more efficacious, but due to its inhibition of GST and depletion of mitochondrial GSH, EA itself has deleterious effects upon microglia in culture (Roychowdhury *et al.* 2003). Indeed, higher concentrations or longer incubations tested in this study caused the microglia to die and detach from the coverslip, rendering imaging impossible.

In addition to the GST-catalysed specific conjugation of MCB, the bimane non-enzymatically and non-specifically conjugates to thiols including GSH and cysteine, producing around 20% of the fluorescence observed for GSH in the presence of GST (Fernandez-Checa and Kaplowitz 1990). Such non-enzymatic conjugation could therefore account for a large proportion of the fluorescence detected following EA treatment. Another factor affecting the efficacy of EA could be the existence of

different GST isoforms, which may have different affinities for EA as well as for MCB (Ploemen *et al.* 1990). As described previously, selective upregulation of specific GST isoforms may occur in microglia under certain conditions (Tchaikovskaya *et al.* 2005; McKimmie *et al.* 2006; Yang *et al.* 2007). It is therefore not unreasonable to suggest that treatments applied to microglia here may have selectively upregulated an isoform of GST with a lower affinity for EA.

3.11.3. Integration of results with published data

The difference observed here between primary microglia and BV-2 cells on the one hand, and N9 microglial cells on the other, may go some way towards explaining the dichotomy in the literature. The two previous studies detecting an increase in GSH levels following microglial activation (Dopp *et al.* 2002; Persson *et al.* 2006) both utilised microglia-rich primary cultures. LPS has also been shown to increase the GSH levels of macrophages in culture (Sato *et al.* 1995, 2001). However, Chatterjee *et al.* (2000) demonstrated a decrease in microglial GSH within a mixed glial culture following LPS + IFNγ treatment, with little or no effect upon co-cultured astrocytes. This result clearly contradicts findings for primary microglia, as presented here and elsewhere, but one cannot exclude the possibility that this may be due to interactions between the astrocytes and microglia in the culture. Direct GSH interactions between astrocytes and neurones have been well-characterised; astrocytes release GSH (Yudkoff *et al.* 1990; Sagara *et al.* 1996; Dringen *et al.* 1999b) via Mrp1 (Hirrlinger *et al.* 2002c; Minich *et al.* 2006), which is broken down by astrocyte- and neurone-associated ectoenzymes (Dringen *et al.* 1997, 2001) to provide the limiting substrate for neuronal GSH synthesis, L-cysteine. Intercellular GSH interactions involving microglia have not yet been demonstrated, although microglia have been shown to express Mrp1 (Ballerini *et al.* 2002; Hirrlinger *et al.* 2002a; Dallas *et al.* 2003) and it has been demonstrated here and elsewhere (Dallas *et al.* 2003) that microglia may have an ability to release GSH.

Astrocytes and microglia functionally express the x_c^- transporter (Allen *et al.* 2001; Pow 2001; McBean 2002), and maintenance of intracellular GSH levels is dependent upon x_c^--mediated cystine uptake (Cho and Bannai 1990; Barger *et al.* 2007). If astrocytic x_c^- activity exceeds that of microglia, cystine levels in the medium may be

depleted by astrocytes, resulting in a reduced availability for microglial uptake and GSH synthesis. Alternatively astrocytes may modify microglial behaviour in a more indirect way, through bidirectional intercellular communication. For example, activated microglia release cytokines such as TNFα and interleukin 1β (IL-1β) (Hanisch 2002), which can alter the expression profile of genes involved in immunity and signalling in astrocytes (Falsig *et al.* 2006). Subsequent release products of astrocytes could potentially modulate aspects of microglial behaviour, perhaps affecting GSH synthesis or inducing GSH release via microglial Mrp1 transporters.

The results presented here are in agreement with previous studies of N9 cells and N11 cells, documenting a 50-85% decrease in GSH levels following activation with LPS + IFNγ (Moss and Bates 2001; Roychowdhury *et al.* 2003). N11 cells represent one of the other clones produced alongside N9 cells (Righi *et al.* 1989), and the two have been shown to be very similar (Ricciardi-Castagnoli and Paglia 1992; Corradin *et al.* 1993). However, using an identical reverse-phase HPLC method to that used here (Riederer *et al.* 1989), Moss and Bates (2001) also reported that the GSH levels in BV-2 cells decreased by approximately 40% following LPS + IFNγ exposure. There is a slight difference between the preparation of samples for HPLC analysis between this study and that of Moss and Bates (2001), which could plausibly effect the efficiency of GSH quantification, for example, but which would not be expected to lead to directly opposing results. The availability of cystine in the culture medium may have implications for GSH synthesis, but both here and in the previous study (Moss and Bates 2001), BV-2 cells were cultured in D-MEM, which contains 200 μM cystine as standard (Dulbecco and Freeman 1959), supplemented with 10 % FBS.

3.11.4. GSH content as a balance between cystine import and protection against oxidative damage

LPS added to microglia or macrophages has been shown to enhance L-glutamate release (Piani and Fontana 1994; Sato *et al.* 1995; Domercq *et al.* 2007) by increased expression of the x_c^- transporter system (Sato *et al.* 2001; Barger *et al.* 2007). Since the x_c^- transporter imports cystine in exchange for glutamate, enhanced glutamate

release would be accompanied by increased cystine import. There is however some evidence to suggest that the LPS-stimulated upregulation of x_c^- and consequent glutamate release becomes desensitised at particularly high LPS concentrations (Piani and Fontana 1994; Sato *et al.* 1995, 2001) and over extended periods of time (Sato *et al.* 1995). High concentrations of glutamate competitively inhibit cystine uptake (Bannai 1986; Watanabe and Bannai 1987; Sato *et al.* 1999; Patel *et al.* 2004), so sustained glutamate release in the presence of LPS may eventually inhibit cystine uptake, perhaps explaining the apparent desensitisation of glutamate release. In addition, in macrophages at least, IFNγ appears to attenuate the LPS-induced enhancement of glutamate release (Sato *et al.* 1995), thus further limiting cystine uptake via x_c^-. Decreased cystine import may cause depletion of intracellular cysteine, therefore compromising *de novo* GSH synthesis. LPS and IFNγ both cause iNOS expression and NO release in BV-2 cells, but the effect of the combination of LPS and IFNγ has a much greater effect than either compound alone (fig. 3.2; Blasi *et al.* 1995; Chang *et al.* 2008), creating a higher demand for antioxidants such as GSH. GSH may be used simply as an electron donor, in which case it is regenerated by GR, or it may be irreversibly conjugated to electrophilic centres, such as in the detoxification of peroxidised lipids. The intracellular GSH content at any one point is likely to reflect the balance between the rate of GSH synthesis, which may be determined by that of cystine uptake via x_c^-, and the demand upon GSH as an antioxidant following NO- (and superoxide-) mediated oxidative damage. This is summarised in fig. 3.17.

Moss and Bates (2001) used a high LPS concentration of 10 $\mu g.ml^{-1}$, in combination with IFNγ to bring about the decrease in GSH levels. Here 10 $\mu g.ml^{-1}$ LPS alone led to an increase in GSH levels. LPS (1 $\mu g.ml^{-1}$) + IFNγ also resulted in an increase in GSH levels relative to control, but a slight decrease relative to 1 $\mu g.ml^{-1}$ LPS alone, perhaps due to the associated enhancement of iNOS expression and NO production and consequent increased demand on GSH. It is not implausible that the presence of IFNγ with 10 $\mu g.ml^{-1}$ LPS could have much greater effects. The tenfold higher LPS concentration in combination with IFNγ could have a substantially higher effect upon iNOS expression and NO production, or upon the production of other reactive species, so that the rate of GSH consumption exceeds the rate of synthesis. Since 10

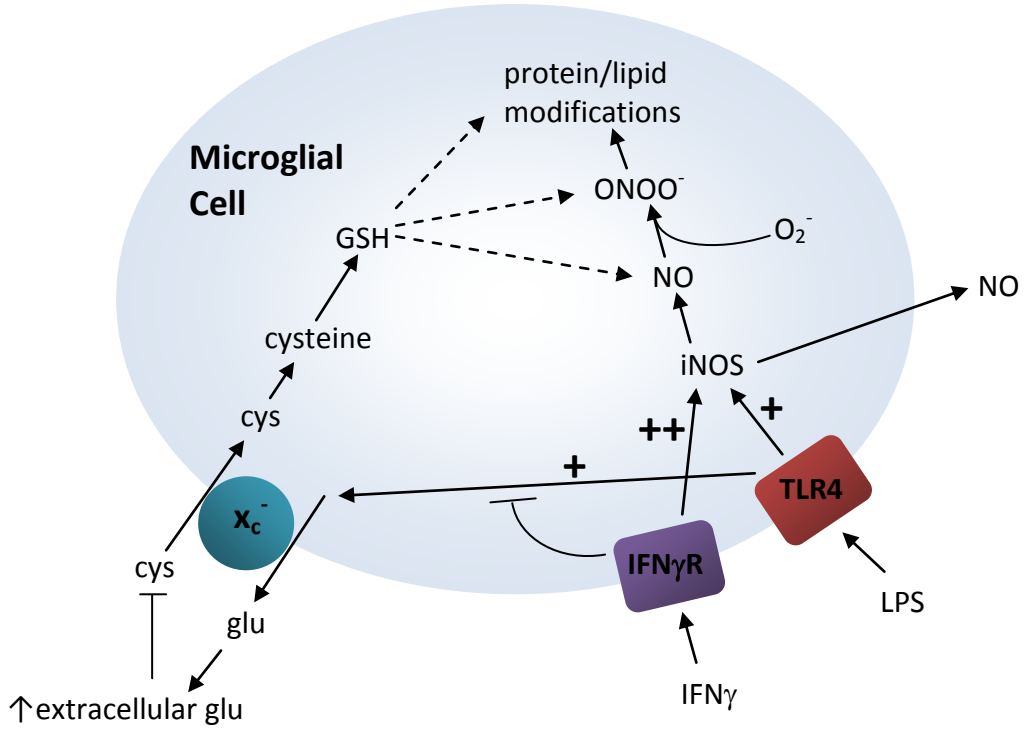


Figure 3.17. *A summary of the effects of LPS and IFNγ upon x_c^- expression and activity and GSH levels in microglia. LPS and IFNγ both induce iNOS expression and consequent NO production, with the combined effect of the two being greater than that of either compound alone. GSH is consumed by the cell in protecting against oxidative stress, to which NO may contribute. LPS also may enhance cystine export via the x_c^- transporter system, which may represent the rate-limiting step in GSH synthesis. IFNγ may attenuate the LPS-induced x_c^- upregulation. This hypothesis may explain the differences between the results obtained in this study and those obtained in a published study (Moss and Bates 2001). Cys, L-cystine; glu, L-glutamate; GSH, reduced glutathione; IFNγ, interferon-γ; IFNγR, IFNγ receptor; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; O_2^-, superoxide; $ONOO^-$, peroxynitrite; TLR4, toll-like receptor 4; x_c^-, x_c^- L-glutamate/L-cystine antiporter system.*

$\mu g.ml^{-1}$ LPS + IFNγ was not tested here, and Moss and Bates (2001) did not publish results obtained with lower LPS concentrations, it is not possible to say for certain whether these data are truly contradictory or evidence for a biphasic effect of LPS in the presence of IFNγ upon GSH levels in BV-2 microglia.

Temporal analysis of the effect of activation upon GSH levels in BV-2 microglia demonstrated an overall decline in BV-2 GSH levels over time, which was less pronounced in the presence of IFNγ (fig. 3.11A). Since the cells were plated and exposures carried out in medium containing 10 % FBS, which allows the cells to proliferate, this decrease over time may be related to the increasing density of BV-2 cells in the culture plate. Indeed, iNOS expression in both control and activated BV-2 cells is slightly elevated when the plated cells are more confluent (Dr. Claudie Hooper and Ioanna Sevastou, personal communication). Consequent increases in NO production may place extra demands upon GSH without upregulating its synthesis. However, two different iNOS inhibitors, which were shown to attenuate the release of nitrate and nitrite by microglia (fig. 3.11C), had no effect upon GSH levels in either control or LPS-treated BV-2 cells (fig. 3.11B), and did not prevent the decline in GSH levels over time. This suggests that in BV-2 cells under the conditions investigated here, GSH levels were not significantly affected by NO. However, the slight upregulation of iNOS expression may be indicative of a low level of activation in densely populated BV-2 cultures (Dr. Claudie Hooper, personal communication), therefore there may also be increased release of glutamate by the cells. Since glutamate is a competitive inhibitor of cystine uptake via x_c^- (Bannai 1986; Patel *et al.* 2004), glutamate accumulation over time may slow the rate of GSH synthesis by decreasing cystine availability. This could be exaggerated if cystine levels in the medium significantly deplete over time due to cellular uptake and utilisation.

The time course of the effect of LPS upon GSH levels bears a certain resemblance to the time course of the effect of LPS upon the uptake of cystine by macrophages (Sato *et al.* 1995). In this study, cystine uptake was very low in control macrophages and increased dramatically with incubation with 1 $ng.ml^{-1}$ LPS, up to 12 hours. After 24 hours' incubation with LPS, cystine uptake had begun to decline and by 48 hours, it was about one-third of that at 12 hours. An adaptation of figure 1 from Sato *et al.*

(1995) is presented alongside the time course of the effect of LPS on GSH content in figure 3.18. The implications of this similarity are limited by the fact that although microglia and macrophages are of the same lineage and share many characteristics, they are fundamentally different cell types. In addition, different LPS concentrations were used in the two studies. This observation is nonetheless interesting, and may suggest that in the presence of LPS, the GSH concentration in BV-2 cells is highly dependent upon cystine import via x_c^-.

Conversely, in the case of N9 cells, GSH levels appeared to remain fairly stable over time in control and activated cells (fig. 3.10). Microglial activation does not appear to transiently upregulate GSH synthesis as may be the case for BV-2 cells. It is likely that treatment of N9 microglia with LPS and/or IFNγ induces the production of NO (and possibly other ROS and RNS), which places an increased demand upon GSH as an antioxidant, and thus causes a sustained decrease in GSH levels. This hypothesis is supported by published data in the N11 cell line, similar to the N9 line, which demonstrates that GSH depletion can be prevented by iNOS inhibition (Moss and Bates 2001).

3.11.5. Fundamental differences between BV-2 and N9 cell lines

The data presented here clearly demonstrate a very significant difference in the regulation of GSH levels in N9 and BV-2 microglial cells. Although the data concerning GSH levels presented by Moss and Bates (2001) deviates from that found here, some differences were found between BV-2 and N11 cells, the latter of which are very similar to N9 cells as used here (Righi *et al.* 1989; Ricciardi-Castagnoli and Paglia 1992; Corradin *et al.* 1993). For example, decreases in the GSH levels of N11 cells were prevented by inhibition of iNOS, whilst decreases in the GSH levels of BV-2 cells were not (Moss and Bates, 2001). The extent of mitochondrial complex inhibition by microglial activation also differed slightly between the two cell lines (Moss and Bates, 2001).

There are a number of fundamental differences in the production and immortalisation of the N9 and BV-2 cell lines, which are summarised in table 3.2.

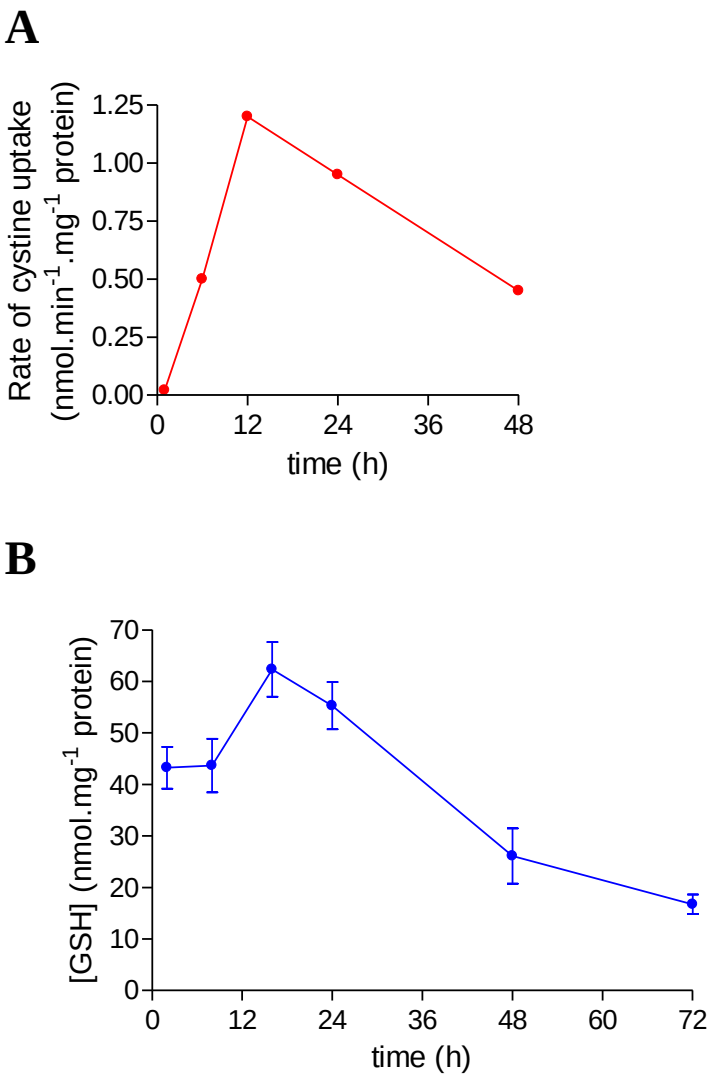


Figure 3.18. *The time course of the rate of cystine uptake by LPS-treated macrophages shows similarity to the time course of the GSH content of LPS-treated microglia.* ***A****, The effect of 1 ng.ml*$^{-1}$ *LPS upon the rate of cystine uptake by peritoneal macrophages, adapted from Sato et al. (1995).* ***B****, The effect of 1 µg.ml*$^{-1}$ *LPS upon the GSH content of BV-2 microglial cells, from figure 3.11.*

	N9	BV-2
Mouse strain	CD1	C57BL/6
Age of mouse	ED 12-13	Newborn
Brain areas	Ventral mesencephalon & cerebral cortex	Cerebral cortex
Primary culture method	Serum free astrocyte conditioned medium on polyornithine-coated dishes	Shaking and selective plastic adherence of mixed brain cultures
Oncogenes	v-myc (3RV retrovirus)	v-raf/v-myc (J2 retrovirus)

Table 3.2. *Differences in the generation of N9 and BV-2 mouse microglial cell lines.*

The CD1 mouse strain is an outbred line, not genetically defined, whereas the C57BL/6 strain is inbred, with well-defined characteristics. C57BL/6 mice exhibit a Th1-dominant immune response, characterised by T cell IFNγ release, which stimulates macrophages to release NO (Mills *et al.* 2000). Microglia harvested from this strain therefore may exhibit enhanced iNOS/NO responses to activation. Indeed, in one study incubation of N9 cells with 1 $\mu g.ml^{-1}$ LPS for 18 hours induced < 10 µM nitrite release, while incubation of BV-2 cells with the same LPS concentration for 24 hours induced > 25 µM nitrite release (Chang *et al.* 2008). Clearly, these results are not directly comparable due to the slightly longer incubation period with BV-2s. However, few studies have been published which directly compare the two cell lines.

In addition, there are likely to be differences between microglia at embryonic day 12-13 and those of a newborn mouse. Microglia are derived from monocytes and begin entering the brain at around embryonic day 5. Initially microglia assume an amoeboid phenotype and have an active role in phagocytosing debris from apoptotic

neurones and degenerating processes during central nervous system (CNS) development. Amoeboid microglia differentiate into ramified microglia and by around post-natal day 15 few amoeboid cells remain (Ferrer *et al.* 1992; Ling and Wong 1993). Therefore, embryonic microglia may have a more pro-inflammatory phenotype as a result of their amoeboid morphology at this developmental stage. The higher basal GSH levels in N9 microglia relative to BV-2 microglia could reflect their embryonic origin; additionally it is possible that amoeboid microglia in the developing brain simply react differently to activation compared with more ramified post-natal microglia. Indeed, a comparison of primary microglia obtained from neonatal mouse and rat brain with primary microglia obtained from adult mouse and rat brain demonstrated significant age-related differences in NO and cytokine release (Schell *et al.* 2007). Primary microglia used in this study were prepared from post-natal day 2-5 rats, therefore the similarity of BV-2 cells rather than N9 cells to these primary microglia could simply be a reflection of the developmental stage.

A difference between the culture conditions of BV-2 and N9 cells concerns the fact that N9 culture medium contains the antioxidant β-mercaptoethanol (βME; 57.2 μM). βME reduces disulphide bonds and therefore may be likely to convert cystine into cysteine in the culture medium, thus preventing cystine uptake for GSH synthesis. However, βME has been reported to increase cystine uptake and intracellular GSH levels in peripheral monocytes (Messina and Lawrence 1992), spleen cells (Burger *et al.* 1982), insulin-secreting cells (Janjic and Wollheim 1992), and embryonic cells (Takahashi *et al.* 2002), through the formation of a cystine – βME disulphide which is taken up in these cell types by the Na^+-independent system of neutral L-amino acid transport (Ishii *et al.* 1981; Ohmori and Yamamoto 1983). Since there are no reports of microglial expression of this uptake system (Bode 2001; Wagner *et al.* 2001), it is unclear what effect βME may have upon N9 microglia in culture, and whether formation of the cystine – βME disulphide would enhance cystine uptake or prevent it. βME (50 μM) was also used for N9 cell culture by Roychowdhury *et al.* (2003), whose investigations demonstrated decreases in GSH content upon LPS + IFNγ treatment. However, there is no record of βME use in another study documenting activation-induced decreases in GSH levels in BV-2 and N11 microglial cell lines (Moss and Bates 2001), suggesting that βME was not responsible for the observed declines in GSH levels.

3.11.6. Other potential differences between GSH metabolism in BV-2 and N9 cell lines

The differences between N9 and BV-2 cells could lead to differences in one or more of a number of elements related to GSH metabolism. For example, N9 cells may have a reduced capacity for GSH regeneration following oxidation. In BV-2 cells GSSG levels were negligible under a number of different conditions (table 3.1). GSSG levels in N9 cells were however not determined, but a failure of GR to convert GSSG back to GSH at an adequate rate during oxidative stress could increase GSSG levels and explain the decreased GSH levels following microglial activation.

GSH may be irreversibly consumed through conjugation to other molecules in the detoxification of xenobiotics (Smith *et al.* 1977; Younes *et al.* 1980) as well as in some normal metabolic processes (Elce and Harris 1971; Soderstrom *et al.* 1985). For example, leukotriene C4, an inflammatory mediator which may be released by microglia (Matsuo *et al.* 1995), is formed by conjugation of GSH to leukotriene A4 (Soderstrom *et al.* 1985). A decrease in GSH levels, as seen here in N9 cells, could therefore represent enhanced leukotriene C4 production and release.

3.11.7. End-product inhibition of glutamate-cysteine ligase

Glutamate-cysteine ligase (GCL, also known as γ-glutamylcysteine synthase) catalyses the formation of γ-glutamylcysteine (γGC) from glutamate and cysteine, the rate limiting step in GSH biosynthesis (Meister and Anderson 1983). GSH, the end product of the pathway, can competitively inhibit GCL by interacting with the glutamate binding site, with an apparent K_i of 2.3 mM (Richman and Meister 1975). The molar concentration of GSH in astrocytes was calculated as approximately 3.2 mM (Chatterjee *et al.* 1999), and microglia are reported to have higher intracellular GSH levels than astrocytes (Chatterjee *et al.* 1999; Hirrlinger *et al.* 2000; Noack *et al.* 2000). This suggests that the GSH concentration within microglia is in the range for feedback inhibition upon GCL. The GSH level in control N9 microglial cells was found here to be 70% higher than that in control BV-2 cells (fig. 3.6). Perhaps the

greater abundance of GSH in N9 cells provides a tonic inhibition of GSH synthesis, therefore reducing the rate at which the cells are able to replace GSH in the presence of ROS and RNS which may place demands upon GSH as an antioxidant. The possibility also exists of indirect regulation of GCL by microglial activation. Indeed, in astrocytes, NO donors caused an upregulation of GCL expression and activity and an increase in intracellular GSH levels (Gegg *et al.* 2003).

3.11.8. ATP and GSH levels in BV-2 microglia

It was found here that ATP levels in BV-2 cells decline over time in culture. This is at odds with the stable ATP levels observed in N9 cells over 24 hours (Kingham and Pocock 2000), and occurred whether cells were cultured in SFM or SCM, suggesting that it did not represent a deleterious effect of serum removal. Most published studies have measured ATP levels at one time point (e.g. Moss and Bates 2001; Chenais *et al.* 2002), or if a number of time points were used, data were normalised to control levels at the same time point (e.g. Yang *et al.* 2002), therefore it cannot necessarily be assumed that ATP levels remained constant over time in such studies. Preliminary experiments here suggested a high ADP/ATP ratio in BV-2 cells, and a decline in total ATP + ADP levels over time following removal of serum, perhaps suggesting either an accumulation of AMP or a release of ATP.

Intracellular accumulation of AMP and an increased AMP:ATP ratio is a sign of energy depletion and may upregulate a number of aspects of cellular energy metabolism through the activation of 5'-AMP-dependent protein kinase (Bolaños *et al.* 2008). Microglial ATP release has been demonstrated in activated N9 microglial cells (Ferrari *et al.* 1997) and macrophages (Sperlagh *et al.* 1998). Extracellular ATP has been shown *in vitro* to affect microglial morphology (Honda *et al.* 2001), to cause elevation of intracellular Ca^{2+} (McLarnon *et al.* 1999), and release of cytokines (Inoue 2002), and to have a chemotactic effect (Honda *et al.* 2001; Davalos *et al.* 2005), suggesting that microglial ATP release may cause chemotaxis and activation of further microglia, amplifying any microglial response.

Fraction V albumin was the only compound to significantly affect the ATP concentration in BV-2 microglia, more than doubling it after 24 hours' incubation.

This may relate to the ability of albumin to cause proliferation of microglia (Hooper *et al.* 2005). Published data have demonstrated decreases in ATP levels in BV-2 and N11 microglial cells after 16 – 20 hours' incubation with LPS + IFNγ (Moss and Bates 2001; Chenais *et al.* 2002), and a delayed decrease following exposure of primary microglia or N9 cells to CGA for at least 22 hours (Kingham and Pocock 2000). Here, LPS + IFNγ and IFNγ alone led to slightly lower ATP levels at 24 hours, although this was not found to be statistically significant. Conversely, LPS alone caused a trend towards an increase in ATP levels, although the effect was less pronounced than that of fraction V albumin.

NO and its derivative peroxynitrite inhibit mitochondrial ATP synthesis (Bolaños *et al.* 1995; Brookes *et al.* 1999; Chenais *et al.* 2002), and are widely reported to be anti-proliferative in a number of cell types both *in vitro* and *in vivo* (Garg and Hassid 1989; Kawahara *et al.* 2001; Gibbs 2003). However more recent studies have shown the relationship between NO and ROS production, and ATP levels and proliferation to be more complicated. The gram-positive bacterial cell wall component lipoteichoic acid (LTA), which increased iNOS immunoreactivity (Kinsner *et al.* 2005), had proliferative effects upon microglia at low doses, but decreased viability at higher concentrations (Jiang-Shieh *et al.* 2005). Similarly, the cytokine interleukin-5 (IL-5) increased nitrite production by microglia whilst also stimulating proliferation (Liva and de Vellis 2001). There is also some evidence for involvement of superoxide-derived ROS in control of cell proliferation, and therefore perhaps ATP levels (Jekabsone *et al.* 2006; Mander *et al.* 2006).

Protective functions of NO and peroxynitrite have been demonstrated in some cell types. NO may upregulate the glycolytic pathway, leading to compensatory ATP production and maintenance of the mitochondrial membrane potential (Almeida *et al.* 2001), whilst peroxynitrite may increase the activity of the pentose phosphate pathway, leading to enhanced NADPH levels to regenerate the reduced form of the antioxidant glutathione (García-Nogales *et al.* 2003). Such a regulation of energy metabolism may be implicated here, especially in the case of fraction V albumin treatment of BV-2 cells, where iNOS expression occurred alongside elevated intracellular ATP and GSH levels, and proliferation was evident (Hooper *et al.* 2005).

Studies have shown that LPS and IFNγ stimulate iNOS expression and NO release through the activation of distinct signalling pathways in BV-2 cells (Shen *et al.* 2005), and that while IFNγ potentiates the LPS-induced release of some cyto- and chemokines, it attenuates the LPS-induced release of others (Hausler *et al.* 2002). It is therefore quite plausible that LPS and IFNγ could have different effects upon ATP levels, despite the fact that they increase iNOS expression to a similar extent.

ATP is required for the GCL-catalysed synthesis of γGC from cysteine and glutamate, and for the GSH synthase-catalysed formation of GSH from γGC and glycine (Snoke and Bloch 1952; Snoke *et al.* 1953). Should energy depletion occur following microglial activation, *de novo* GSH synthesis would likely be compromised. Indeed, the effects of different treatments of BV-2 cells upon ATP levels appears to mirror to some extent their effects upon intracellular GSH levels. The time course of changes in ATP and GSH levels under different conditions is presented in figure 3.19. In control cells and in the presence of LPS and fraction V albumin, changes in ATP and GSH levels are clearly correlated, with the decline in ATP level preceding the decline in GSH level in each case. The ATP levels of BV-2 microglial cells may therefore be an important determinant of intracellular GSH levels. However, in the case of IFNγ alone or in combination with LPS, a striking decline in ATP levels was seen, whilst GSH levels remained rather stable. This implicates the involvement of other factors in determining GSH levels under these conditions. For comparison, all the graphs in figure 3.19 are drawn on identical axes.

ATP levels of N9 microglia were not tested in this study, although a previous report from this laboratory indicated that ATP levels of plated N9 cells remain relatively stable over time (Kingham and Pocock 2000), not unlike intracellular GSH levels of N9 cells as determined in this study (fig. 3.10).

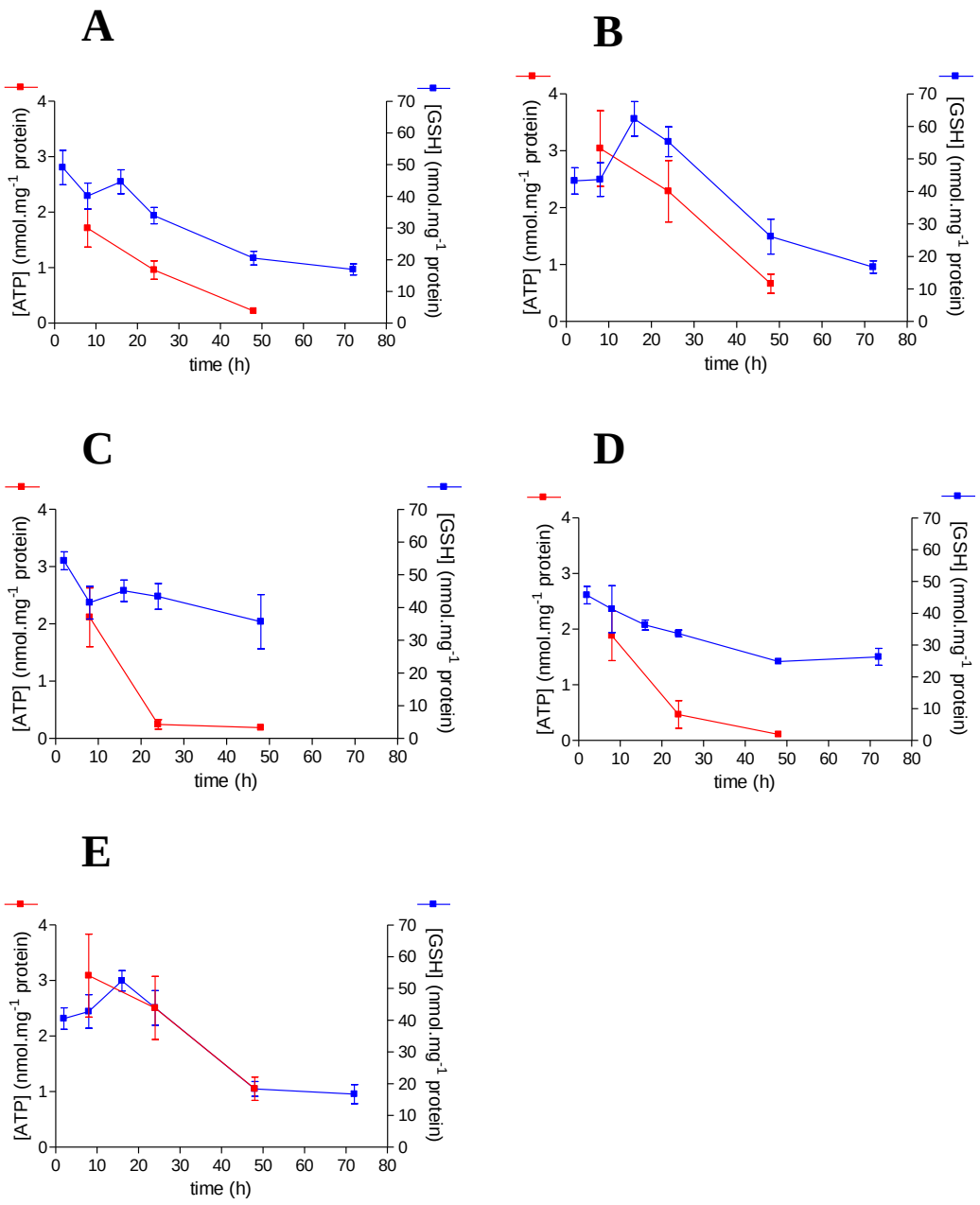


Figure 3.19. *Comparison of the time course of changes in ATP (■) and GSH (■) levels in BV-2 cells. Data taken from figures 3.4 and 3.11 and plotted according to treatment (**A**, control; **B**, LPS; **C**, LPS + IFNγ; **D**, IFNγ; **E**, fraction V albumin) demonstrate a correlation between the changes in ATP and GSH levels over time and following different treatments.*

3.11.9. Implications for neuroinflammatory disease

Albumin is the most abundant plasma protein, but due to its size, is only present at high levels in the CNS under conditions of blood-brain barrier (BBB) dysfunction, as may be found in neuroinflammatory conditions (Hooper *et al.* 2005). As shown here and elsewhere (Hooper *et al.* 2009), fraction V albumin induces iNOS expression and NO release by microglia. Albumin has also been found to induce microglial superoxide production (Si *et al.* 1997). Such ROS and RNS may be harmful to the microglia themselves as well as to neighbouring cells; alternatively they may have a role in the control of mitochondrial respiration, as seen in other cell types including astrocytes (Almeida *et al.* 2001). The enhanced ATP and GSH content of fraction V albumin-treated microglia demonstrated here may be indicative of an enhanced metabolism, in accordance with the proliferative effect of albumin (Hooper *et al.* 2005). As demonstrated in figure 3.19, the ATP content of fraction V albumin-treated cells is higher relative to their GSH content than under any other condition.

Treatment of microglia with the AD-related proteins Aβ or CGA causes microglial neurotoxicity (Kingham *et al.* 1999; Taylor *et al.* 2002). Here neither protein was found to enhance the release of nitrate and nitrite by N9 microglia. However, other studies have demonstrated enhanced iNOS expression and NO production by microglia treated with CGA (Taupenot *et al.* 1996; Kingham *et al.* 1999; Kingham and Pocock 2000; Hooper and Pocock 2007). In all cases primary microglia were used, and the difference between nitrate/nitrite levels in control and CGA-treated cells was shown to be less pronounced in the case of N9s (Kingham and Pocock 2000). CGA also causes microglial apoptosis (Kingham *et al.* 1999; Kingham and Pocock 2000; Hooper and Pocock 2007), but this is not likely to be due to GSH depletion as CGA did not significantly alter the GSH content of either BV-2 or N9 microglial cells.

Aβ peptides have been shown to be neurotoxic directly, as well as through their effects upon microglia (Taylor *et al.* 2002, 2003). Here $A\beta_{25-35}$ did not significantly alter nitrate and nitrite release by N9 microglia, in accordance with other studies (Goodwin *et al.* 1995; Meda *et al.* 1995) which demonstrate that priming by the cytokine IFNγ is required to stimulate iNOS expression and NO release. $A\beta_{1-40}$ has

been previously shown to increase microglial glutamate release via the x_c^- transporter (Qin *et al.* 2006), suggesting that microglia may be taking up cystine at a higher rate, allowing a higher rate of GSH synthesis. As Aβ peptides including $A\beta_{25-35}$ upregulate microglial superoxide production (McDonald *et al.* 1997; Combs *et al.* 1999), enhanced rates of cystine uptake and GSH synthesis may be necessary to balance the demand upon GSH and maintain GSH levels close to control level, as found in both BV-2 and N9 microglial cell lines in the present study.

3.11.10. Microglial GSH release

Here low micromolar levels of GSH were consistently detected in conditioned medium from BV-2 and primary rat microglial cells following 16 – 24 hours' incubation, indicating that microglia are capable of releasing small amounts of GSH. Macrophages have been shown to release thiols (Watanabe and Bannai 1987), and more recently, GSH specifically (Sato *et al.* 2001). Vincristine export by cells of the rat MLS-9 microglial cell line has been demonstrated to depend upon intracellular GSH, suggesting cotransport via Mrp1 (Dallas *et al.* 2003). However, this appears to be the first demonstration of GSH release by primary microglial cells.

In BV-2 cells, but not in primary microglia, LPS increased GSH release. LPS in combination with IFNγ did not enhance GSH release in either primary or BV-2 microglia. This may be related to the relative iNOS expression levels induced by LPS and LPS + IFNγ treatment in the two cell types. As shown here (figs. 3.1 and 3.2) and elsewhere (Shen *et al.* 2005; Horvath *et al.* 2008), LPS alone leads to relatively low levels of iNOS expression and NO release by BV-2s, but high levels of iNOS expression and NO release by primary microglia. LPS + IFNγ treatment leads to a much higher level of iNOS expression in BV-2s. LPS treatment of BV-2 cells may therefore be the anomaly here, in that it may cause upregulation of GSH synthesis without high levels of NO production and the associated demand upon GSH as an antioxidant. This would also fit with the large increase in intracellular GSH in BV-2 cells following LPS treatment. In contrast, LPS + IFNγ treatment of BV-2 cells and LPS treatment of primary microglia are likely to increase GSH

utilisation as well as its synthesis; less striking increases in intracellular GSH are seen under these conditions.

The x_c^- inhibitor APA seemed to decrease microglial release of GSH and reduced the LPS-mediated enhancement of GSH release in BV-2 cells, although statistical significance was not attained in these preliminary experiments. A difference may suggest that GSH release is somewhat dependent upon cystine import via x_c^-, and may implicate the intracellular GSH pool as the source of the released GSH.

In the case of BV-2 cells at least, the GSH release detected was not due simply to lysis of the cells, as LPS markedly elevated the GSH content of conditioned medium, but did not elevate the LDH content, a widely-used measure of cell lysis. In contrast, the highest LDH content of conditioned medium occurred following LPS + IFNγ treatment, a condition which did not significantly alter GSH content. However, significant LDH release following treatment with LPS + IFNγ or IFNγ alone has important implications for previous or subsequent data relating to other release products of BV-2 microglia under such activated conditions. This finding suggests that regulated release may be overestimated, unless measurements of release are corrected for lysis by simultaneous LDH release measurement.

In a previous study investigating GSH release by different CNS cell types in primary cultures, microglial GSH release was not detected (Hirrlinger *et al.* 2002c). However, GSH release was only monitored for 6 hours, whilst in the present study the GSH content of the medium was measured over 24 hours. In accordance with the published study (Hirrlinger *et al.* 2002c), during the first 8 hours very little GSH appeared to be released by BV-2 cells. GSH release in the aforementioned study was measured by culturing cells in a salt solution consisting of 44 mM $NaHCO_3$, 110 mM NaCl, 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.92 mM NaH_2PO_4 and 5 mM D-glucose (Hirrlinger *et al.* 2002c). This solution is routinely used for the measurement of GSH release by astrocytes (Dringen *et al.* 1997, 1999b; Stewart *et al.* 2002; Gegg *et al.* 2005; Minich *et al.* 2006; Frade *et al.* 2008), however when BV-2 cells were cultured in such a solution here, they displayed retracted processes and ruffled membranes and a larger proportion of microglia became detached than those cultured in D-MEM. In addition, GSH release was barely detectable when the

salt solution was used, and modulation of release by LPS was not apparent, in contrast to cells cultured in phenol red-free D-MEM. A second simple medium, designated basic medium (BM), consisting of 5 mM $NaHCO_3$, 153 mM NaCl, 2.6 mM $CaCl_2$, 3.5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM Na_2SO_4, 0.4 mM KH_2PO_4, 20 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) and 5 mM D-glucose, which is used widely in this laboratory, was less detrimental towards microglial cells. However, a standard curve made up in BM was not linear and again, LPS could not be shown to modulate GSH release. It therefore appears that modulation of microglial GSH release by LPS requires a component of D-MEM not present in these simple media, perhaps specific amino acids. As the x_c^- inhibitor APA appeared to decrease BV-2 GSH release to a low level and prevent its modulation by LPS, a lack of cystine in the medium could have a similar effect. Although the use of phenol red-free D-MEM does considerably reduce the signal to noise ratio of the assay, the coefficient of determination for the standard curve was consistently $\geq$ 0.99, and of those tested here, this appeared to be the only condition under which GSH release and its modulation could be reliably detected.

The likely role of microglial-derived GSH is unclear. GSH released by astrocytes supplies cysteine for neuronal GSH synthesis, but in order for released GSH to be utilised by neurones it first needs converting to cysteinylglycine by the ectoenzyme γ-glutamyl transpeptidase (γGT). The detection of GSH in conditioned medium here in the absence of the γGT inhibitor acivicin (Dringen *et al.* 1997) suggests that microglial γGT activity is low or absent, in accordance with other reports (Murata *et al.* 1997; Ruedig and Dringen 2004). Neuronal γGT activity is also low (Shine and Haber 1981; Philbert *et al.* 1995; Ruedig and Dringen 2004), therefore a direct GSH transfer from microglia to neurones seems unlikely. However, astrocytes have high γGT activity (Ruedig and Dringen 2004), and could perhaps process microglial-derived, as well as astrocyte-derived GSH for neuronal use. Under conditions of persistent stress, astrocyte GSH is likely to become depleted; perhaps microglia act as a secondary source of GSH to ensure continued neuroprotection. Astrocytes have also been shown to take up GSH (Kannan *et al.* 2000); therefore microglia may supply GSH to astrocytes so that their neuroprotective function is not compromised.

Microglia have been shown to express functional Mrps (Ballerini *et al.* 2002; Dallas *et al.* 2003), proteins associated with the efflux of xenobiotics. In particular, microglia have been shown to express Mrp1 (Ballerini *et al.* 2002; Hirrlinger *et al.* 2002a; Dallas *et al.* 2003), which can transport GSSG (Leier *et al.* 1996), glutathione conjugates (Loe *et al.* 1996) and drugs in cotransport with GSH (Zaman *et al.* 1995; Loe *et al.* 1998), as well as some drugs alone or conjugated or cotransported with other molecules (Konig *et al.* 1999; Leslie *et al.* 2001). The presence of GSH in microglial conditioned medium suggests that of these options, cotransport may most likely be occurring; GSH release by microglia may therefore simply be a by-product of Mrp-mediated export of other molecules.

GSH has been detected extracellularly in the brain and in the CSF at micromolar concentrations (Anderson *et al.* 1989; Yang *et al.* 1994; Lada and Kennedy 1997; Do *et al.* 2000; Wang and Cynader 2000; Calabrese *et al.* 2002, 2003; Kawakami *et al.* 2006), indicating that not all GSH released is broken down by ectoenzymes. Microglia may be responsible for maintaining an extracellular GSH level for antioxidant or other functions.

There is significant evidence supporting a role of GSH as a neuromodulator. GSH may be capable of binding to the glutamate-binding sites of the N-methyl-D-aspartate (NMDA) (Yoneda *et al.* 1990; Leslie *et al.* 1992; Ogita *et al.* 1995; Varga *et al.* 1997; Jenei *et al.* 1998) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Varga *et al.* 1989, 1997; Jenei *et al.* 1998), and may have consequences for glutamate, NMDA and AMPA-stimulated pathways (Janaky *et al.* 1993, 2007, 2008). Extracellular GSH may also form disulphide bonds with the μ-opioid, neurokinin-1 and kainate receptors and affect their ligand-binding properties (Liu and Quirion 1992). GSH also potentiates activity of the Ca^{2+}-sensing receptor through interaction with the amino acid binding site, which is distinct from the Ca^{2+} binding site (Wang *et al.* 2006). In addition, GSH may enhance glutamate uptake (Varga *et al.* 1994). A number of studies have identified two GSH binding sites on CNS tissues (Ogita and Yoneda 1987, 1988; Guo *et al.* 1992; Janaky *et al.* 2000), where binding at one site cannot be inhibited by glutamate receptor ligands, and is postulated to be a GSH-specific site, thus perhaps

identifying GSH as a novel neurotransmitter (Guo *et al.* 1992; Guo and Shaw 1992; Lanius *et al.* 1994; Janaky *et al.* 2000).

3.11.11. Conclusion

Microglial activation upregulates the production of ROS and RNS by microglia, suggesting that antioxidants such as GSH are likely to be important in protecting microglia from oxidative damage. The effect of microglial activation by LPS and fraction V albumin upon intracellular GSH is different in BV-2 and N9 mouse microglial cell lines. The effect upon BV-2 cells is a transient increase in GSH levels, whilst in N9 cells, GSH levels below control are established following activation and maintained over time. GSH levels are a result of a balance between the rate of GSH synthesis and the demands upon GSH as an antioxidant as well as reactions which irreversibly consume GSH. It is proposed that in the case of BV-2 cells, microglial activation leads to a transient upregulation in GSH synthesis, which may be related to increased x_c^- activity or ATP levels. In N9 cells however, GSH utilisation appears to have the greatest impact upon GSH levels, leading to a sustained decrease in cellular GSH content. Whatever the difference between the cell lines, BV-2 cells appear to be more similar to primary microglia. BV-2 cells will therefore be used in further experiments concerning GSH levels and x_c^- where it is not possible to use primary microglia, particularly in GSH quantification by HPLC where relatively large amounts of protein are required. The AD-related proteins CGA and $A\beta_{25-35}$ did not significantly alter the GSH levels of BV-2 or N9 microglial cells. This suggests that GSH demand and utilisation are balanced by GSH regeneration and *de novo* synthesis effectively under these conditions.

Contrary to one previous report (Hirrlinger *et al.* 2002c), but in accordance with another (Dallas *et al.* 2003), microglia appear to release low levels of GSH, and such release appears to depend somewhat upon the activity of the x_c^- transporter system. This appears to be the first demonstration of GSH release by primary microglia. The function of microglia-derived GSH is yet to be established; it may enable transfer of the antioxidant between cell types, maintain an extracellular GSH pool, or represent a signalling role of GSH.

Chapter 4

Results II:
Microglial glutathione and glutamate transporters

4.1. Introduction and summary of results

Elevated extracellular glutamate levels, leading to excitotoxicity and oxidative glutamate toxicity, have been implicated in the pathogenesis of many neurological diseases. A decrease in glutamate uptake and decreased astrocyte expression of excitatory amino acid transporters (EAATs) 1 and 2 have been reported in Alzheimer's disease (AD) (Cross *et al.* 1987; Cowburn *et al.* 1988; Scott *et al.* 1995; Masliah *et al.* 1996; Li *et al.* 1997; Liang *et al.* 2002). A decline in the expression of EAAT1 and 2 have also been described in multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) (Werner *et al.* 2001; Ohgoh *et al.* 2002; Pitt *et al.* 2003).

Microglia have been shown to express EAATs and the x_c^- transporter system, which, under normal circumstances, mediate the import and export of glutamate, respectively (Kondo *et al.* 1995; Lopez-Redondo *et al.* 2000; Nakajima *et al.* 2001b; McBean 2002; Jacobsson *et al.* 2006; O'Shea *et al.* 2006; Barger *et al.* 2007). In the previous chapter, compounds associated with microglial activation were found to affect the reduced glutathione (GSH) content of BV-2, N9 and primary microglial cells. One of the potentially limiting factors in GSH synthesis is cystine import via the glutamate/cystine antiporter system x_c^-, thus upregulated GSH synthesis may be coupled with enhanced glutamate release. Concurrent glutamate uptake by microglial EAATs may limit or reverse enhanced levels of glutamate. Although millimolar levels of glutamate are present intracellularly, EAATs and x_c^- may be located in close vicinity to one another so that EAAT-mediated glutamate uptake may be directly coupled to x_c^- activity (Igo and Ash 1998; Rimaniol *et al.* 2001; Persson *et al.* 2006). Changes in the expression of microglial glutamate transporters upon activation or under conditions of disease may therefore affect extracellular glutamate levels. The effects of lipopolysaccharide (LPS), the standard *in vitro* microglial activator, albumin, to which microglia may be exposed upon blood-brain barrier (BBB) damage, and chromogranin A (CGA) and β amyloid peptide (residues 25-35; $A\beta_{25\text{-}35}$), proteins upregulated in Alzheimer's disease (AD), upon microglial glutamate release and glutamate transporter expression were therefore investigated.

Microglia were found to express mRNA for EAAT1, EAAT2 and the specific subunit of x_c^-, xCT. However, the microglial cells lines BV-2 and N9 expressed the EAATs at much lower levels than primary microglia; their EAAT mRNA expression was close to the limit of detection under the conditions used. LPS and fraction V albumin enhanced glutamate release and upregulated EAAT2 and xCT mRNA expression. However CGA and $A\beta_{25-35}$ were not found to have any effects upon the extracellular glutamate levels or upon microglial transporter expression.

Inhibition of x_c^- by aminoadipic acid (AAA) and aminopimelic acid (APA) caused a decline in microglial GSH content, highlighting the importance of cystine uptake via this transporter for GSH synthesis. The x_c^- transporter appeared to have a dominant effect in BV-2 cells, endowing these cells with an ability to buffer the extracellular glutamate concentration to 170 - 200 μM, approximately equal to the extracellular cystine concentration. However, high levels of extracellular glutamate may compromise GSH synthesis through decreased cystine uptake via x_c^-. In contrast, low (1 – 10 μM) levels of exogenous glutamate increased intracellular GSH levels. These observations may indicate an involvement of microglia in glutamate release in neuroinflammatory and neurodegenerative disease, and may also have implications for microglial GSH levels, and consequently microglial survival under conditions of elevated extracellular glutamate.

4.2. Microglial glutamate release increases following microglial activation

Primary microglia were exposed to LPS, interferon-γ (IFNγ), LPS and IFNγ in combination or fraction V albumin for 24 hours in serum-free medium (SFM) (fig. 4.1). Fresh SFM was found to contain 15.8 ± 6.3 μM, and conditioned medium from control cells contained 14.0 ± 0.9 μM, implying that untreated primary microglia did not modify the glutamate content of the medium (fig. 4.1). However, 24 hours' treatment with LPS (1 $\mu g.ml^{-1}$) significantly elevated the glutamate content of conditioned medium by 222.7 ± 47.4 % to 45.2 ± 6.6 μM, and treatment with fraction V albumin (2 $mg.ml^{-1}$) significantly elevated the glutamate content by 257.0

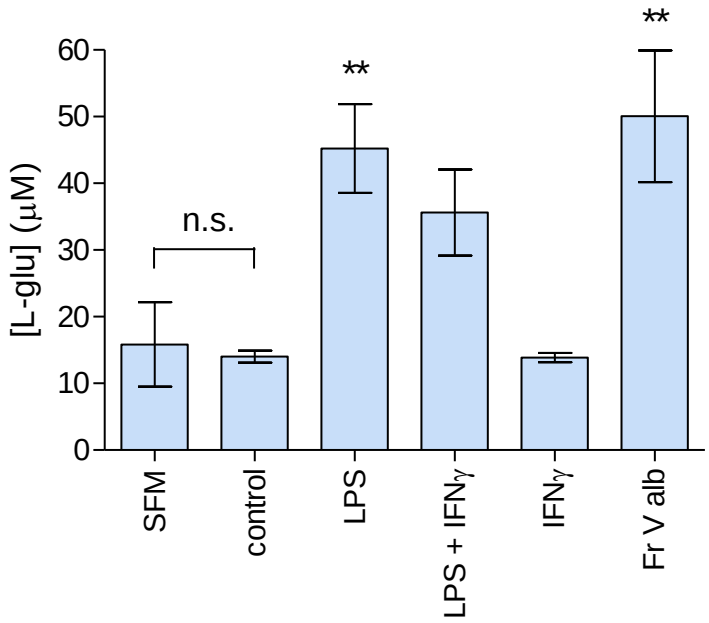


Figure 4.1. *The effect of microglial activation upon L-glutamate levels in microglial conditioned medium. Primary rat microglia were plated at 1 x 10^5 cells per 13mm coverslip, culture medium was changed to SFM and cells were cultured in the presence of LPS (1 μg.ml^{-1}), IFNγ (100 U.ml^{-1}), fraction V albumin (2 mg.ml^{-1}) or a combination for 24 hours, before L-glutamate levels in the supernatant were determined. A sample of fresh SFM was also tested for comparison. Data represent the mean ± s.e.m. of three independent experiments, each consisting of three replicates per condition. Data were analysed using a one-way ANOVA ($p<0.0001$), with Dunnett's multiple comparison post-test to compare all treatments with control. (**$p<0.01$, n.s. $p>0.05$, $n=3$).*

± 70.5 % to 50.1 ± 9.9 μM (fig. 4.1). A combination of LPS and IFNγ more than doubled the glutamate content of conditioned medium to 35.6 ± 6.5 μM, but this was not found to be significantly different from control (fig. 4.1). IFNγ alone had no effect; conditioned medium contained 13.9 ± 0.7 μM following 24 hours' treatment (fig. 4.1). These results suggest that LPS and fraction V albumin increase glutamate release by primary microglia.

Similar experiments were carried out using BV-2 (figs. 4.2 and 4.3) and N9 cells (fig. 4.4). Conditioned medium from control BV-2 cells contained 121.7 ± 3.7 μM glutamate when cultured in serum-containing medium (SCM) comprising 10 % foetal bovine serum (FBS) (fig. 4.2A), and 174.9 ± 18.2 μM glutamate when cultured in SFM. Whether the experiment was conducted using SCM (fig. 4.2A) or SFM (fig. 4.2B), conditioned medium from BV-2 cells exposed to LPS alone or in combination with IFNγ had significantly higher glutamate concentrations than control, suggesting enhanced glutamate release under these conditions. Conditioned medium from LPS-treated cells had 53.3 ± 16.7 % higher glutamate compared to control when the experiment was conducted with SCM (fig. 4.2A), and 59.5 ± 17.6 % higher glutamate in SFM (fig. 4.2B). LPS in combination with IFNγ caused a 46.1 ± 17.2 % increase in glutamate in SCM (fig. 4.2A), and a 74.1 ± 22.3 % increase in SFM (fig. 4.2B). Fraction V albumin caused a significant 39.9 ± 15.1 % increase in the glutamate content of SCM (fig. 4.2A). In SFM, fraction V albumin treatment increased glutamate levels by 83.5 ± 22.3 % (fig. 4.2B), but due to the variation in the data and the fact that these data derive from three independent experiments while the other points on figure 4.2B derive from seven or fourteen experiments, this result did not prove to be statistically significant. When BV-2 cells were exposed to IFNγ alone or the AD-associated protein CGA in SCM, there were no changes in the glutamate content of the conditioned medium (fig. 4.2A). $A\beta_{25-35}$ treatment caused a 21.1 ± 3.7 % increase in glutamate in BV-2 conditioned medium, but this was not found here to be significantly different from control levels (fig. 4.2A).

The time course of glutamate release by BV-2 microglia exposed to LPS or fraction V albumin was investigated. Culture medium was changed to SFM before the addition of compounds because of the variable glutamate concentration of serum,

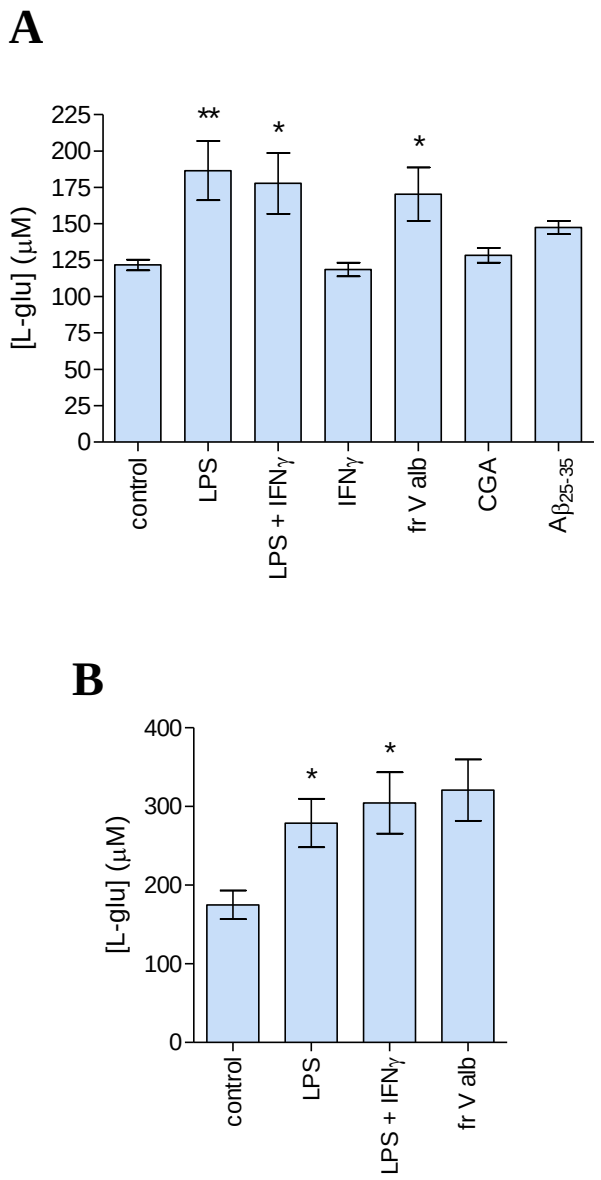


Figure 4.2. *The effect of microglial activation upon L-glutamate levels in BV-2 cell conditioned medium. BV-2 microglial cells were cultured in medium containing 10% FBS (**A**) or SFM (**B**) and exposed to LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), CGA (500 nM), fraction V albumin (2 mg.ml^{-1}), $A\beta_{25\text{-}35}$ (25 µM), or a combination for 24 hours, before L-glutamate levels in the supernatant were determined.* ***A****, Data represent the mean ± s.e.m. of two (IFNγ, CGA, $A\beta_{25\text{-}35}$), three (LPS, LPS + IFNγ, fraction V albumin) or five (control) independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA (p=0.0029), followed by Dunnett's multiple comparison post-test (*p<0.05, **p<0.01, compared with control).* ***B****, Data represent the mean ± s.e.m. of three (fraction V albumin), seven (LPS + IFNγ) or fourteen (control, LPS) independent experiments. Data were analysed with a one-way ANOVA (p=0.0075), followed by Dunnett's multiple comparison post-test (*p<0.05 compared with control).*

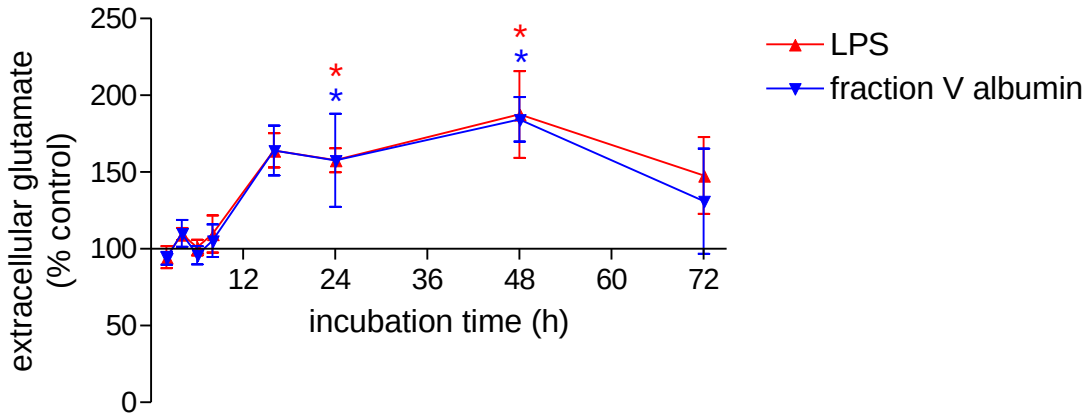


Figure 4.3. *Time course of the effects of LPS and fraction V albumin upon L-glutamate levels in BV-2 microglial cell conditioned medium. BV-2 cells in serum-free medium were exposed to LPS (1 µg.ml^{-1}) or fraction V albumin (2 mg.ml^{-1}) for 2 – 72 hours, before L-glutamate levels in the supernatant were determined. Data are presented as % control value at each time point and represents the mean ± s.e.m. of three independent experiments, except for control and LPS at 24 hours, where data represent the mean ± s.e.m. of thirteen independent experiments. Raw data at each time point were analysed using a one-way ANOVA (p=0.983 at 2h; p=0.976 at 4h; p=0.9773 at 6h; p=0.9736 at 8h; p=0.2125 at 16h; p=0.0078 at 24h; p=0.0396 at 48h; p=0.5134 at 72h); where p<0.05 (24h, 48h) Dunnett's multiple comparison post-test was carried out (*p<0.05 compared with control).*

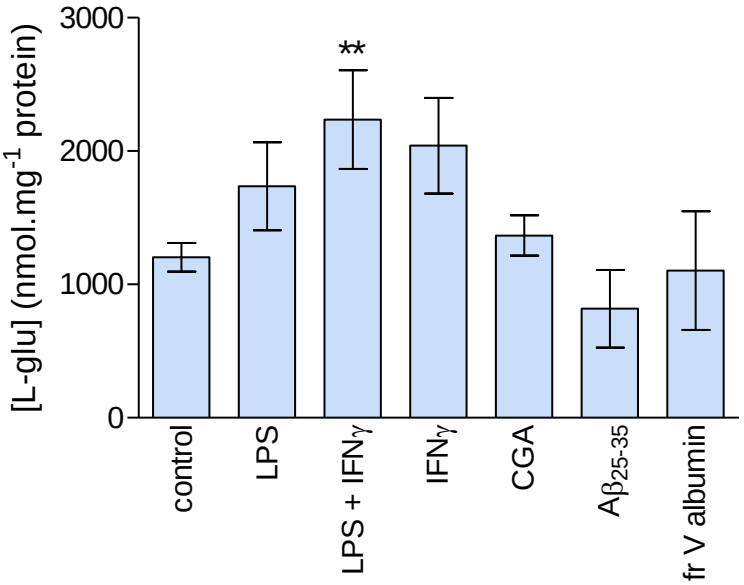


Figure 4.4. *The effects of microglial activation upon L-glutamate levels in N9 microglial cell conditioned medium. N9 cells were cultured in medium containing 5% newborn calf serum and exposed to LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), CGA (500 nM), Aβ$_{25-35}$ (25 µM), fraction V albumin (2 mg.ml^{-1}) or a combination for 24 hours, before L-glutamate levels in the supernatant were determined. Data represent the mean ± s.e.m. of three (LPS, IFNγ, Aβ$_{25-35}$, fraction V albumin), five (CGA), six (LPS + IFNγ) or eight (control) independent experiments, each consisting of 2 – 4 replicates per condition. Data were analysed with a one-way ANOVA (p=0.0043) with Dunnett's multiple comparison post-test to compare all treatments with control (**p<0.01).*

and because the changes following treatment were consistently more pronounced in SFM (fig. 4.2). In addition, as serum promotes cell proliferation, over longer periods of time enhanced or reduced glutamate release could reflect the effects of densely populated cells rather than the treatments themselves. Figure 4.3 shows the glutamate concentration of the culture medium following 2 – 72 hours' exposure to LPS or fraction V albumin. The glutamate content of control conditioned medium in this experiment was 164.5 ± 11.1 μM (n=35). The two treatments had similar effects over time. No changes in the glutamate concentration of the conditioned medium were found until after 8 hours' exposure. After 16 hours' incubation, LPS caused the glutamate content of conditioned medium to increase by 64.1 ± 11.1 %, and fraction V albumin caused it to increase by 64.0 ± 16.2 %, however neither was found to be statistically significant compared with control. Glutamate release was significant in both cases following 24 and 48 hours' exposure; at 48 hours the glutamate levels in the conditioned medium of LPS-treated cells were 87.6 ± 28.2 % above control, and those in the conditioned medium of fraction V albumin-treated cells were 84.4 ± 14.6 % above control. Extracellular glutamate levels appeared to reach a plateau at this point, and may have even declined slightly between 48 and 72 hours.

The glutamate content of control N9 microglial conditioned medium was 1204 ± 106.4 nmol.mg^{-1} protein. In the case of N9 cells, of the compounds tested, only a combination of LPS and IFNγ significantly increased the glutamate level of the conditioned medium by 85.8 ± 30.7 (fig. 4.4). LPS and IFNγ alone increased levels by 44.2 ± 27.4 % and 69.5 ± 29.8 % respectively, but neither was found to be significantly different from control (fig. 4.4). In contrast to the results seen with primary microglia and BV-2 cells (figs. 4.1, 4.2 and 4.3), treatment of N9 cells with fraction V albumin did not cause any change in the glutamate levels of N9 conditioned medium (fig. 4.4). CGA treatment was also without effect, and exposure of N9 cells to $A\beta_{25-35}$ caused a non-significant 32.1 ± 24.2 % decrease in the glutamate content of conditioned medium (fig. 4.4).

4.3. Microglial expression of mRNA for EAAT1

Primary rat microglia were found to consistently express mRNA for the glutamate transporter EAAT1 (GLAST). Figure 4.5 demonstrates a representative agarose gel of PCR products, with the relevant gel for β-actin mRNA for comparison. As seen in figure 4.5, LPS (1 $\mu g.ml^{-1}$) and fraction V albumin (2 $mg.ml^{-1}$) appear to downregulate the expression of EAAT1 mRNA. The AD-related proteins CGA and $A\beta_{25-35}$ did not however appear to alter the levels of EAAT1 mRNA in primary microglia (fig. 4.5B).

A robust band on the agarose gel following PCR run on a sample of mRNA extracted from whole mouse brain demonstrated that the primer recognises mouse as well as rat EAAT1 (fig. 4.6A), making it suitable for use with the BV-2 and N9 cell lines. As demonstrated by figures 4.6 and 4.7, BV-2 and N9 microglia expressed EAAT1 mRNA at very low levels. In some cases, faint but definite bands were detected on the agarose gel (e.g. figs. 4.6A and 4.7), whilst in other cases bands were hardly detected (e.g. fig. 4.6B). Such low expression, at the limit of detection, is likely to compromise the accuracy of the data. In the case of BV-2 cells, no bands were detectable for 5 of 9 control samples. However, when BV-2 cells were treated with fraction V albumin, four samples, from four independent experiments, contained detectable amounts of EAAT1 mRNA. In two of these four experiments, no EAAT1 mRNA was detected in control cells. This could represent an interesting finding, although as mentioned above, data must be interpreted with caution when mRNA levels are close to the limit of detection. In the case of N9 cells, no bands were detected on the agarose gel for 3 of 5 samples from control cells, and for 4 of 5 samples from LPS + IFNγ-treated cells.

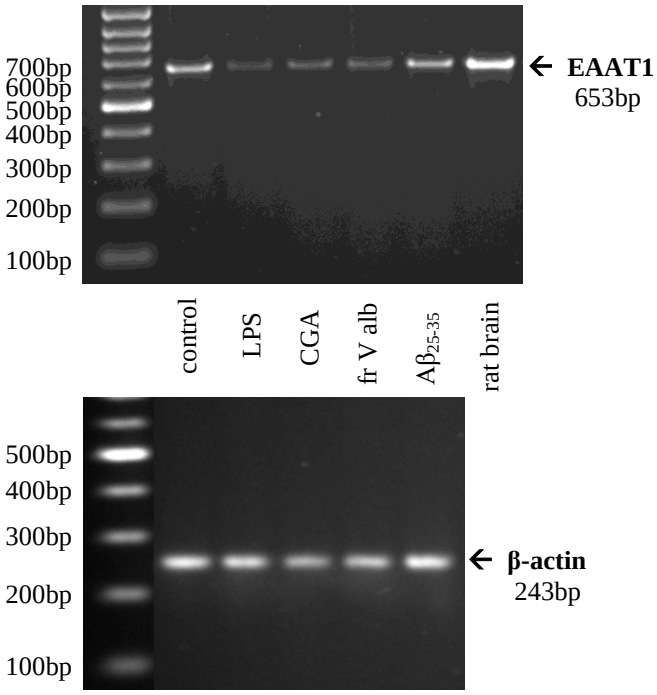


Figure 4.5. *The effect of microglial activation upon EAAT1 mRNA expression by primary rat microglia. Plated primary microglia were exposed to LPS (1 µg.ml^{-1}), CGA (500 nM), fraction V albumin (2 mg.ml^{-1}) or Aβ$_{25-35}$ (25 µM) for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for EAAT1and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Bands for EAAT1 were consistently detectable. Gels shown are representative of three (Aβ$_{25-35}$), four (LPS, fr V albumin, CGA), or seven (control) independent experiments.*

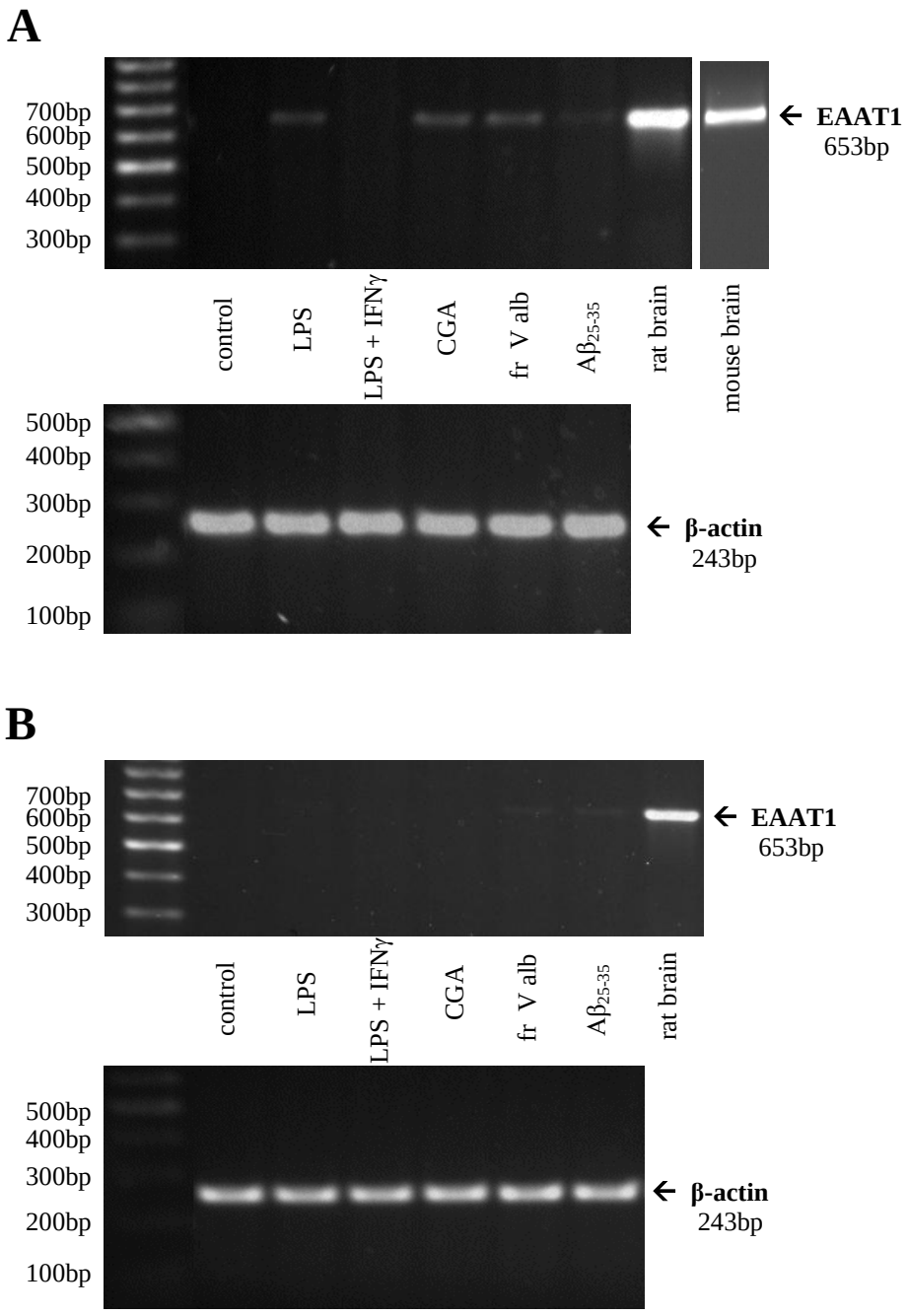


Figure 4.6. *The effect of microglial activation upon EAAT1 mRNA expression by BV-2 microglia. Plated BV-2 cells were exposed to LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), CGA (500 nM), fraction V albumin (2 mg.ml^{-1}), Aβ$_{25-35}$ (25 µM), or a combination, for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for EAAT1 and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Four (LPS + IFNγ, CGA, Aβ$_{25-35}$, fr V albumin), five (LPS) or seven (control) independent experiments were carried out. Results from two such experiments are presented, illustrating inter-experiment variability.*

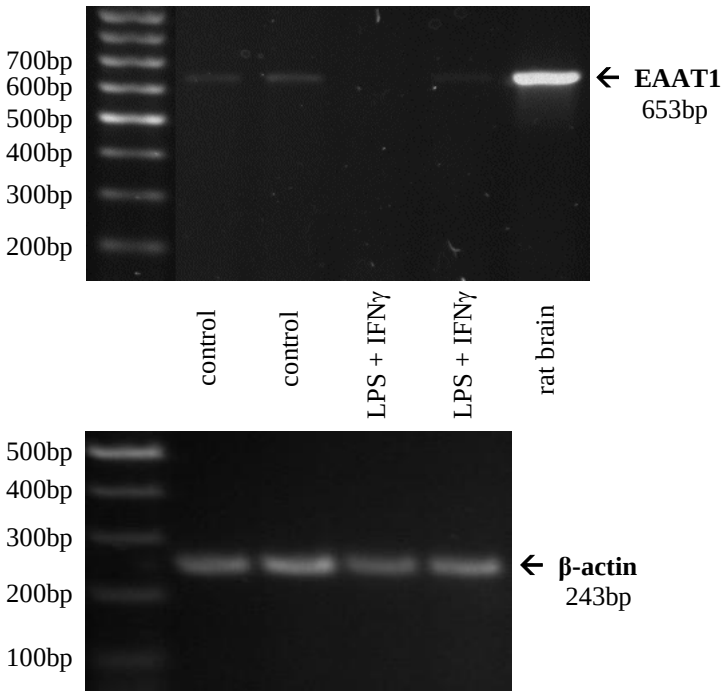


Figure 4.7. *The effect of microglial activation upon EAAT1 mRNA expression by N9 microglia. Plated N9 cells were exposed to LPS (1 µg.ml^{-1}) and IFNγ (100 U.ml^{-1}) for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for EAAT1 and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Gels shown are representative of two independent experiments, one consisting of two, and one of three replicates per condition.*

4.4. Microglial expression of mRNA for EAAT2

Primary rat microglia also expressed EAAT2 mRNA (fig. 4.8). EAAT2 mRNA expression was undetectable in four of seven samples from control cells. In the presence of LPS or fraction V albumin, primary microglia were consistently found to express mRNA for EAAT2. In contrast, neither CGA nor $A\beta_{25-35}$ appeared to alter the level of EAAT2 mRNA expression in primary microglial cells (fig. 4.8).

In a similar situation to that for EAAT1, BV-2 and N9 cells were only found to express mRNA for EAAT2 at a very low level (fig. 4.9 and 4.10). The positive controls again confirm that the primers recognise mouse EAAT2 mRNA (fig. 4.10), and show that the PCR conditions used were adequate to give a good signal where EAAT2 was expressed (figs. 4.9 and 4.10). A band on the agarose gel was not detectable in 7 of 9 samples of control BV-2 cells (fig. 4.9) and in 4 of 7 samples of control N9 cells (fig. 4.10). In the case of BV-2 cells, LPS treatment may have slightly increased the levels of EAAT2 mRNA, as an increase was consistently seen (fig. 4.9), and only 2 of 7 samples of LPS-treated BV-2 cells did not produce a detectable band upon agarose gel electrophoresis. LPS + IFNγ, fraction V albumin and $A\beta_{25-35}$ did not appear to affect EAAT2 mRNA levels of BV-2 microglia (fig. 4.9). Following treatment with CGA, EAAT2 bands were consistently not detected in five independent experiments (fig. 4.9). However, as the mRNA levels were close to the limit of detection of the experiment, all such observations must be interpreted with caution. In addition, treatment of N9 microglial cells with LPS + IFNγ did not appear to alter the expression of mRNA for EAAT2, and the limited results for fraction V albumin, CGA and $A\beta_{25-35}$ did not indicate any striking effects of these compounds (fig. 4.10).

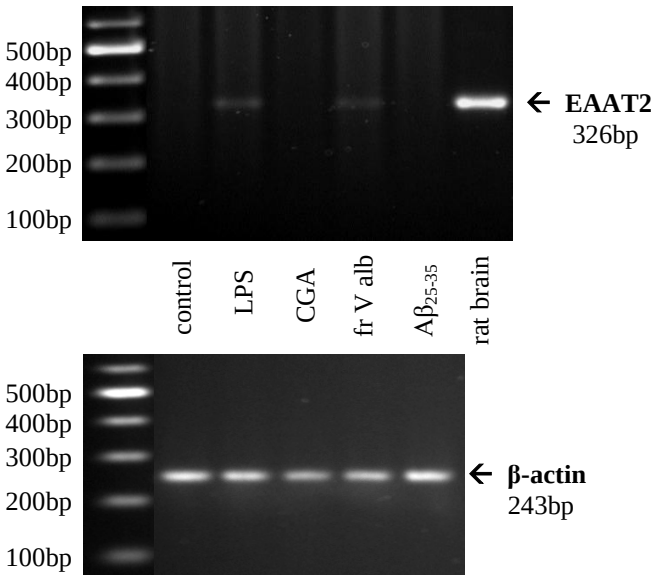


Figure 4.8. *The effect of microglial activation upon EAAT2 mRNA expression by primary rat microglia. Plated primary microglia were exposed to LPS (1 µg.ml^{-1}), CGA (500 nM), fraction V albumin (2 mg.ml^{-1}) or Aβ$_{25-35}$ (25 µM) for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for EAAT2 and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Gels shown are representative of three (Aβ$_{25-35}$), four (LPS, fr V albumin, CGA), or seven (control) independent experiments.*

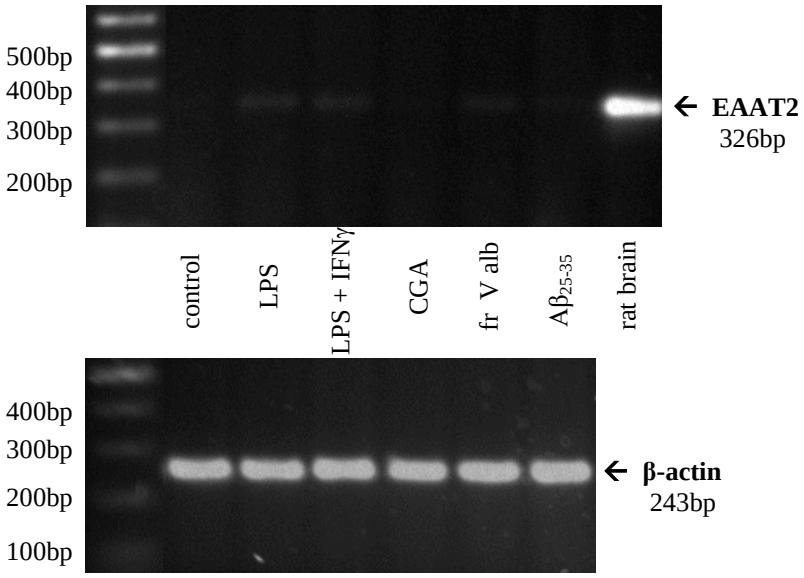


Figure 4.9. *The effect of microglial activation upon EAAT2 mRNA expression by BV-2 microglia. Plated BV-2 cells were exposed to LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), CGA (500 nM), fraction V albumin (2 mg.ml^{-1}), $A\beta_{25-35}$ (25 µM), or a combination, for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for EAAT2 and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Gels shown are representative of five (LPS + IFNγ, CGA, $A\beta_{25-35}$, fr V albumin), six (LPS) or eight (control) independent experiments.*

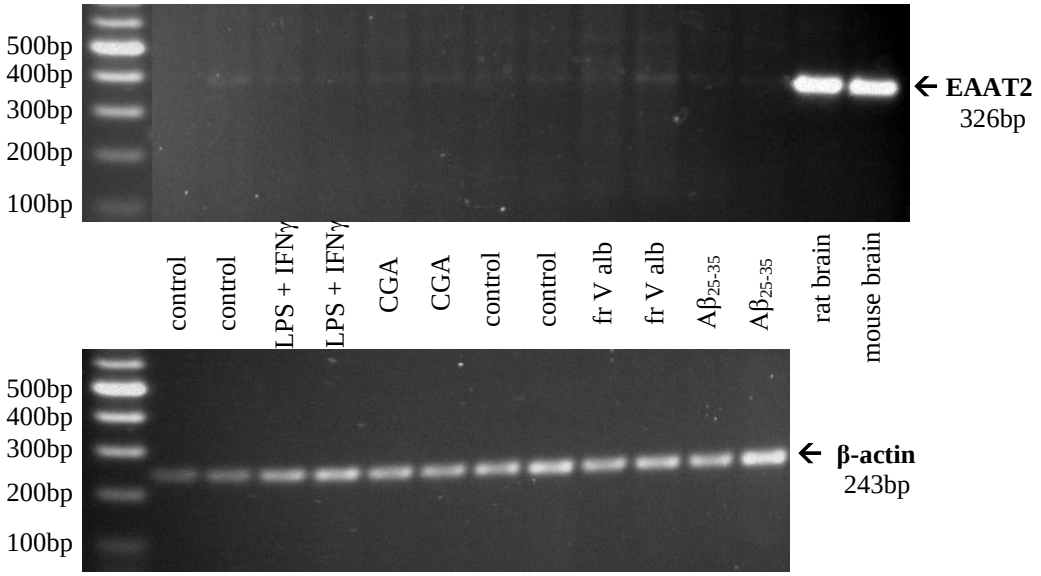


Figure 4.10. *The effect of microglial activation upon EAAT2 mRNA expression by N9 microglia. Plated N9 cells were exposed to LPS (1 μg.ml^{-1}), IFNγ (100 U.ml^{-1}), CGA (500 nM), fraction V albumin (2 mg.ml^{-1}), $Aβ_{25-35}$ (25 μM), or a combination, for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for EAAT2 and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat and mouse brains were run as positive controls. Gels shown are from a single experiment, consisting of two replicates per condition (fraction V albumin, CGA, $Aβ_{25-35}$) or representative of three independent experiments, one consisting of one, and two consisting of two replicates per condition (control, LPS + IFNγ).*

4.5. Microglial expression of mRNA for the x_c^- transporter system

The glutamate/cystine antiporter x_c^- is expressed at the plasma membrane as a heterodimer consisting of the 4F2hc glycoprotein, which is associated with a number of transport systems, and the specific protein xCT (Sato *et al.* 1999; Bassi *et al.* 2001). Here the expression of x_c^- was assessed by measuring mRNA levels for the specific xCT subunit. xCT mRNA expression was consistently detected in all samples of primary microglia and BV-2 and N9 microglial cells.

The expression of xCT mRNA in primary rat microglia was rather variable, although LPS and fraction V albumin consistently upregulated xCT mRNA expression (fig. 4.11). The AD-related proteins CGA and $A\beta_{25-35}$ did not appear to affect the expression of mRNA for the xCT subunit of x_c^- (fig. 4.11). A primer designed against mouse xCT detected robust expression of xCT mRNA by BV-2 and N9 cells, as well as mouse brain positive controls (figs. 4.12 and 4.13). Treatment with LPS, LPS + IFNγ or fraction V albumin also appeared to upregulate xCT mRNA levels in BV-2 cells (fig. 4.12). As was the case for primary microglia, neither CGA nor $A\beta_{25-35}$ seemed to have an effect upon BV-2 cell expression of xCT mRNA (fig. 4.12). In the case of N9 microglial cells however, there was little evidence for any effect of LPS + IFNγ, fraction V albumin, CGA or $A\beta_{25-35}$ upon the expression of xCT mRNA (fig. 4.13).

4.6. x_c^- inhibition decreases GSH levels in BV-2 microglial cells

Cystine import via x_c^- is thought to be a limiting factor for GSH synthesis in microglia, and glutamate taken up via EAATs may drive cystine uptake via x_c^-. The effects of compounds modulating x_c^- and EAAT2 activity upon GSH levels in BV-2 and N9 microglial cells were therefore investigated, by means of reverse-phase high performance liquid chromatography (HPLC) with electrochemical detection of GSH. D-MEM, in which the cells were cultured, contains 200 μM cystine (Dulbecco and Freeman 1959). Increasing the cystine content to 300 μM or 600 μM had no significant effect upon intracellular GSH levels of BV-2 cells (fig. 4.14A). The

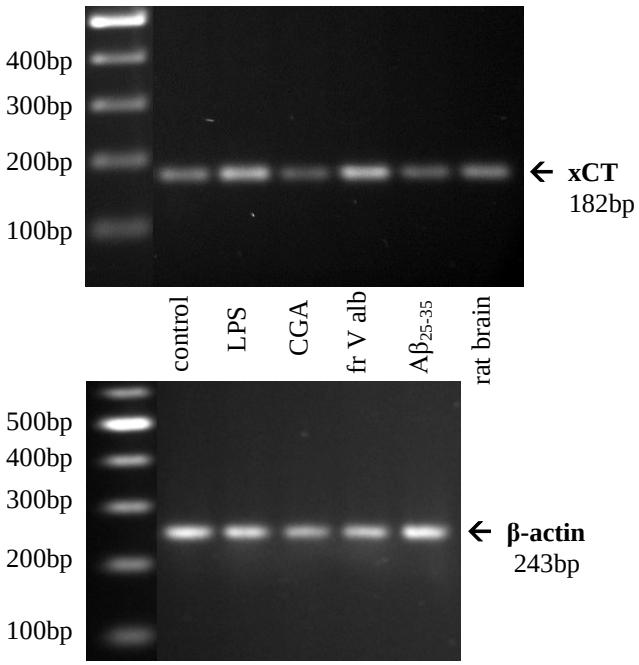


Figure 4.11. *The effect of microglial activation upon xCT mRNA expression by primary rat microglia. Plated primary microglia were exposed to LPS (1 µg.ml^{-1}), CGA (500 nM), fraction V albumin (2 mg.ml^{-1}) or $A\beta_{25-35}$ (25 µM) for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for xCT and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Bands for xCT were consistently detectable. Gels shown are representative of two ($A\beta_{25-35}$), three (fr V albumin, CGA), four (LPS) or seven (control) independent experiments.*

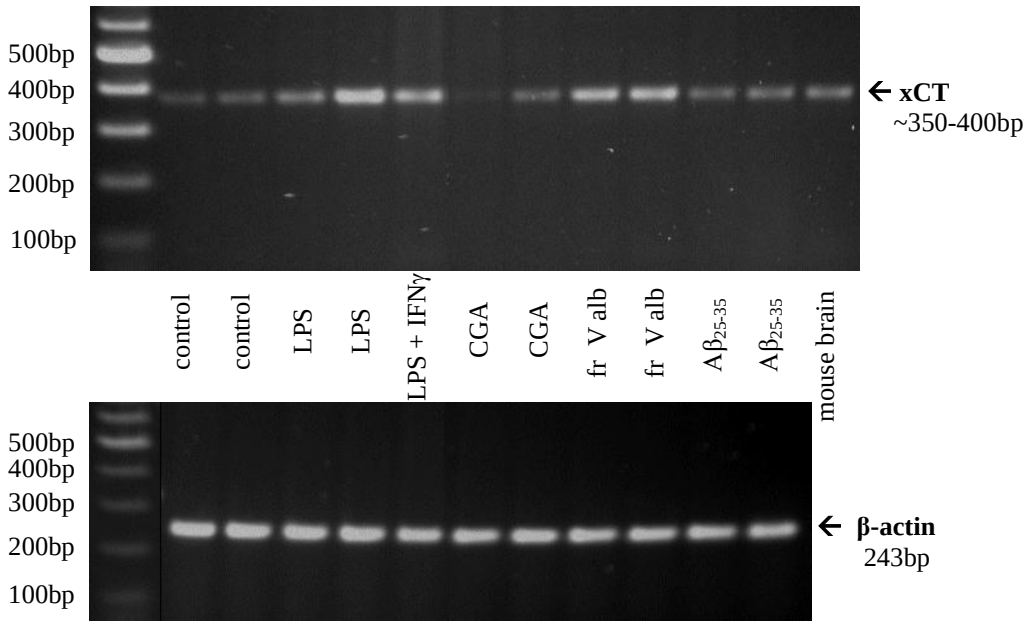


Figure 4.12. *The effect of microglial activation upon xCT mRNA expression by BV-2 microglia. Plated BV-2 cells were exposed to LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), CGA (500 nM), fraction V albumin (2 mg.ml^{-1}), $A\beta_{25-35}$ (25 µM), or a combination, for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for xCT and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole mouse brain was run as a positive control. Bands for xCT were consistently detectable. Gels shown are representative of five (LPS + IFNγ, CGA, fraction V albumin, $A\beta_{25-35}$), six (LPS) or eight (control) independent experiments.*

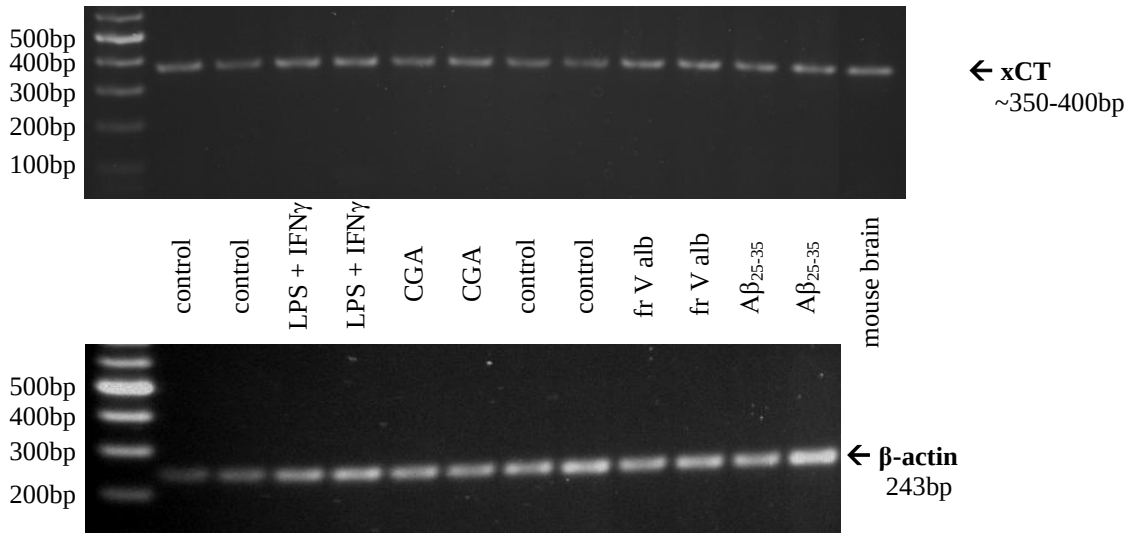


Figure 4.13. *The effect of microglial activation upon xCT mRNA expression by N9 microglia. Plated N9 cells were exposed to LPS (1 µg.ml^{-1}), IFNγ (100 U.ml^{-1}), CGA (500 nM), fraction V albumin (2 mg.ml^{-1}), $A\beta_{25-35}$ (25 µM), or a combination, for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for xCT and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat and mouse brain were run as positive controls. Bands for xCT were consistently detectable. Gels shown are from a single experiment, consisting of two replicates per condition (fraction V albumin, CGA, $A\beta_{25-35}$), or representative of three independent experiments, two consisting of two, and one of three replicates per condition (control, LPS + IFNγ).*

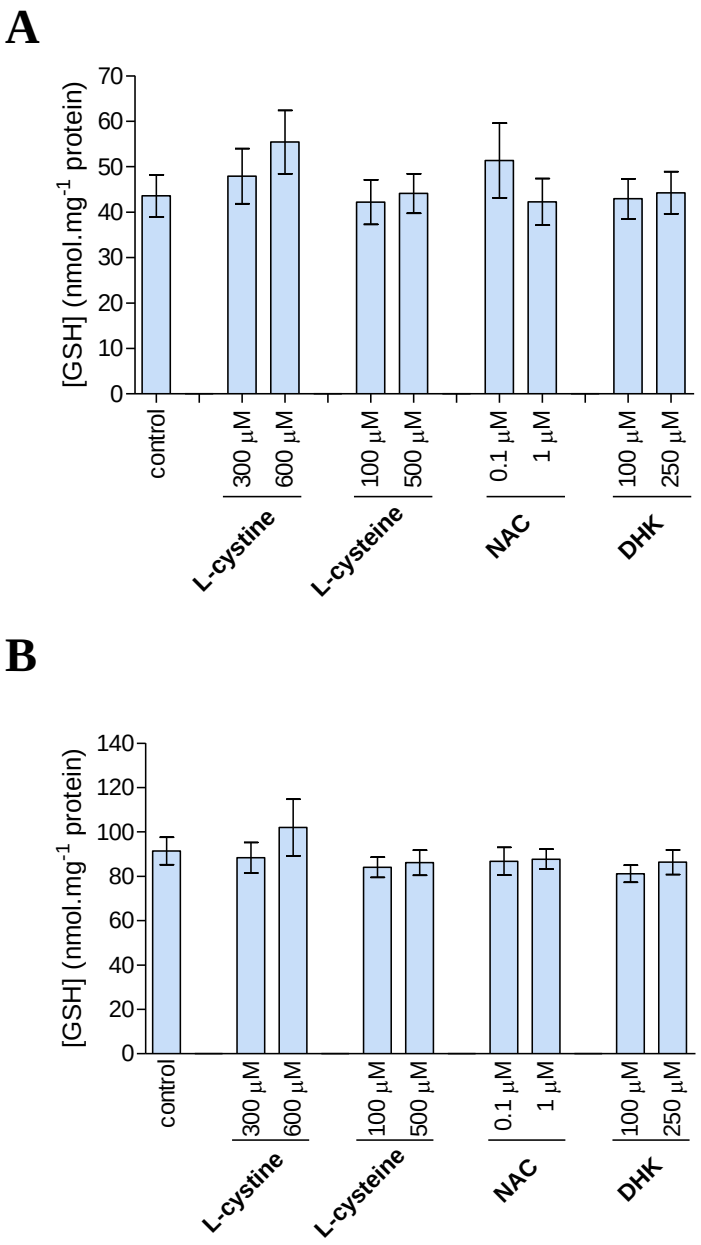


Figure 4.14. *The effect of compounds acting at the x_c^- and EAAT2 transporters upon BV-2 (**A**) and N9 (**B**) microglial GSH levels. BV-2 or N9 cells were cultured for 24 hours in the presence of supplementary L-cystine (to give a total of 300 or 600 µM), L-cysteine (100 or 500 µM), N-acetyl-cysteine (NAC; 0.1 or 1 µM) or dihydrokainate (DHK; 100 or 250 µM), and GSH levels were determined by reverse-phase HPLC. Data represent the mean ± s.e.m. of three independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA (**A**, p=0.71, n.s.; **B**, p=0.6327, n.s.).*

reduced analogue of cystine, cysteine (100 or 500 μM), to which cystine is converted intracellularly following import, also had no effect when added to the culture medium (fig. 4.14A). Similarly, the x_c^- substrate agonist and antioxidant N-acetyl-cysteine (NAC; 0.1 or 1 μM), which can be deacetylated to cysteine intracellularly and thus act as a GSH precursor (Magnusson *et al.* 1989; Sjodin *et al.* 1989), was without effect (fig. 4.14A). Blockade of the EAAT2 glutamate transporter with dihydrokainate (DHK; 100 or 250 μM) (Johnston *et al.* 1979; Pocock *et al.* 1988; Robinson *et al.* 1991; Arriza *et al.* 1994) also had no effect upon BV-2 microglial GSH levels (fig. 4.14A). Identical concentrations of supplementary cystine, cysteine, NAC and DHK were also without significant effect upon the GSH content of N9 microglial cells (fig. 4.14B).

Because BV-2 cells appear to respond more similarly to primary microglia in terms of the effect of microglial activation upon the GSH content (chapter 3) and xCT gene expression (figs. 4.11 and 4.12), further investigation of the impact of the x_c^- transporter upon GSH levels, under control and activated (1 $\mu g.ml^{-1}$ LPS) conditions, was limited to BV-2 cells (fig. 4.15). In this experiment, control cells were found to contain 29.7 ± 1.7 $nmol.mg^{-1}$ protein GSH, and LPS treatment increased levels by 66.3 ± 10.5 % to 49.3 ± 3.1 $nmol.mg^{-1}$ protein (fig. 4.15). A higher concentration of cystine (to give a total of 1 mM) had no significant effect upon GSH levels in either non-activated or activated cells, although a trend towards an increase was seen in non-activated cells, where cystine increased the GSH level to 40.3 ± 3.4 $nmol.mg^{-1}$ protein (an increase of 35.8 ± 11.6 %), and attenuated the difference between GSH levels in non-activated and activated cells (fig. 4.15). Aminopimelic acid (APA; 2.5 mM) (Qin *et al.* 2006), an inhibitor of the x_c^- transporter system (Bannai 1986; Watanabe and Bannai 1987), significantly decreased the GSH level in non-activated BV-2 cells by 53.7 ± 2.0 % to 13.7 ± 0.6 $nmol.mg^{-1}$ protein (fig. 4.15). Whilst APA did not significantly alter GSH levels in activated cells (n.s. when compared to LPS alone), APA prevented LPS from significantly increasing GSH levels compared with control (hatched bar), whilst in all other cases LPS treatment led to GSH levels significantly higher than control levels. In the presence of supplementary cystine and APA, GSH levels in activated and non-activated cells were almost identical to those in non-cystine/APA-treated activated and non-activated cells. There were significant differences between non-activated cells (but not activated cells) in the presence of

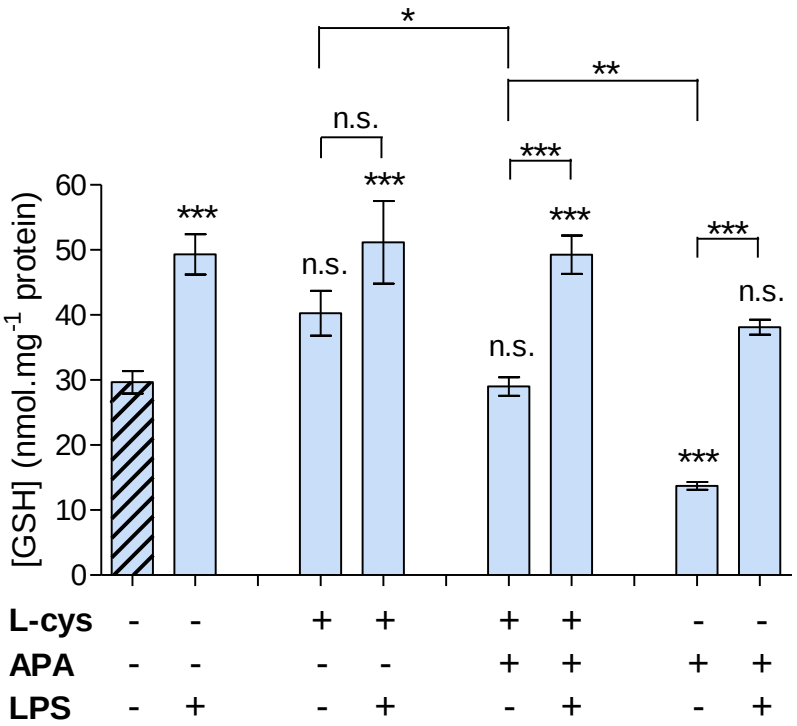


Figure 4.15. *Further investigation into the effect of compounds acting at the x_c^- transporter upon GSH levels in BV-2 microglial cells. BV-2 cells were cultured for 24 hours in the presence of supplementary L-cystine (to give a total of 1 mM), APA (2.5 mM) or a combination, before the addition of LPS (1 µg.ml^{-1}). After a further 24 hours, GSH levels were determined by reverse-phase HPLC. Data represent the mean ± s.e.m. of at least three independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA (p<0.0001), with Tukey's multiple comparison post-test (*p<0.05, **p<0.01, ***p<0.001, n.s. p>0.05, compared with control (hatched bar) or as indicated).*

cystine and APA compared with either of the treatments alone, thus the addition of cystine slightly reversed the APA-induced decrease in GSH levels, and the addition of APA blocks x_c^- to some extent, even in the presence of a high cystine concentration.

4.7. Effects of x_c^- antagonism upon microglial glutamate release

In initial experiments, it appeared that the x_c^- antagonist APA (250 µM – 2.5 mM) dose-dependently decreased the elevated extracellular glutamate observed in the presence of LPS (1 $\mu g.ml^{-1}$) and LPS + IFNγ (100 $U.ml^{-1}$) (fig. 4.16A). This compound was initially prepared in 80% acetic acid, according to the information supplied with the product. Although the acid was neutralised in the medium before it was added to cells, it appeared to have deleterious effects upon cells, so alternative solvents in which APA is soluble were investigated. It was found that APA was soluble in equimolar NaOH, for example 250 mM APA was soluble in 250 mM NaOH. As shown in figure 4.16B, APA (2.5 mM) dissolved in equimolar NaOH did not affect the glutamate levels in BV-2 conditioned medium, under control or activated (LPS, LPS + IFNγ) conditions.

Aminoadipic acid (AAA) is also a x_c^- transporter inhibitor (Bannai 1986; Watanabe and Bannai 1987; Piani and Fontana 1994). In contrast to APA, AAA (2.5 mM) (Kingham *et al.* 1999; Qin *et al.* 2006; Domercq *et al.* 2007) significantly decreased LPS and LPS + IFNγ-mediated increases in extracellular glutamate, although the resulting glutamate concentrations were still above that of non-activated cells (fig. 4.17). Supplementary cystine (to give a total of 1 mM) did not have any significant effects upon glutamate levels in conditioned medium (fig. 4.17). Interestingly, there was a significant difference between extracellular glutamate levels following LPS treatment of cells cultured in the presence of cystine and those cultured in the presence of cystine and APA, but not between LPS-treated cells with cystine and those with cystine and AAA (fig. 4.17).

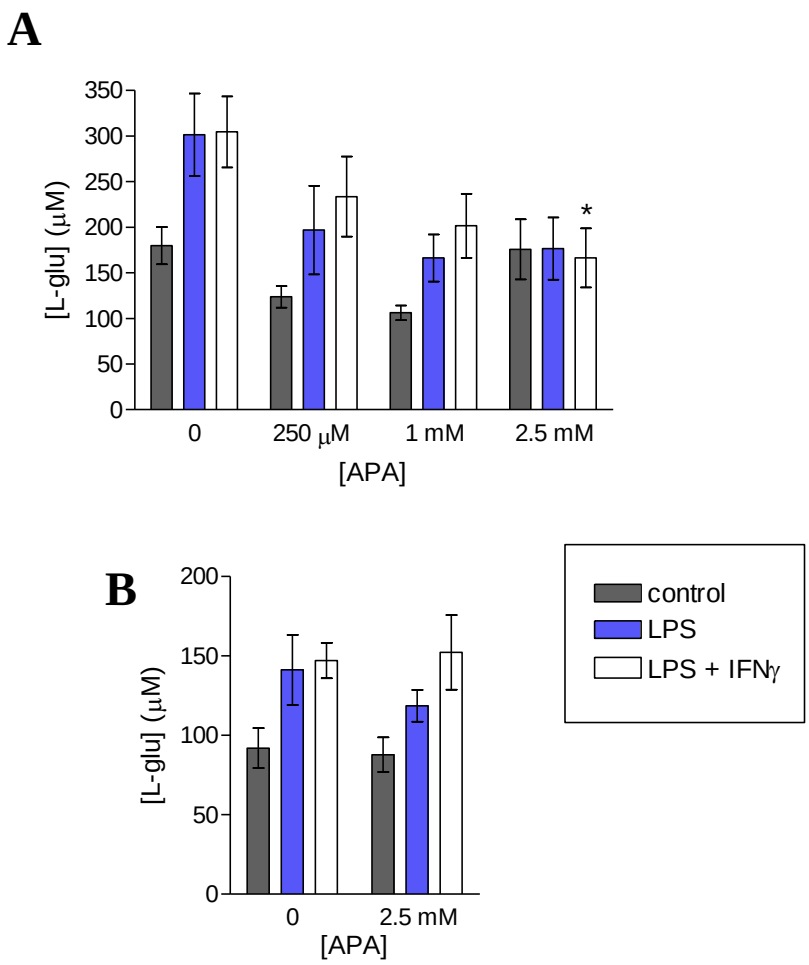


Figure 4.16. *The effect of pre-incubation with APA, in two different solvents, upon L-glutamate levels in conditioned medium from control BV-2 microglial cells and those treated with LPS or LPS + IFNγ. Plated BV-2 microglia were cultured in SFM containing APA (250 μM - 2.5 mM) for 24 hours before the addition of LPS (1 μg.ml^{-1}) alone or in combination with IFNγ (100 U.ml^{-1}). After a further 24 hours, L-glutamate levels in conditioned medium were determined.* ***A****, APA made up in 80% acetic acid, neutralised in the medium. Data represent the mean ± s.e.m. of three (at each APA concentration) or seven (in the absence of APA) independent experiments. Data were analysed with a two-way ANOVA, which showed a significant effect of LPS/IFNγ (p=0.0267) and a significant effect of APA (p=0.0041), but no significant interaction between the variables (p=0.67).* ***B****, APA made up in equimolar NaOH. Data represent the mean ± s.e.m. of three independent experiments. Data were analysed with a two-way ANOVA, which showed a significant effect of LPS/IFNγ (p=0.0254), but no significant effect of APA (p=0.6), and no significant interaction (p=0.69). For both data sets, Bonferroni's post-tests were carried out to compare all concentrations of APA with non-APA treated cells in the absence and presence of LPS and LPS + IFNγ (*p<0.05 compared with the same treatment in the absence of APA).*

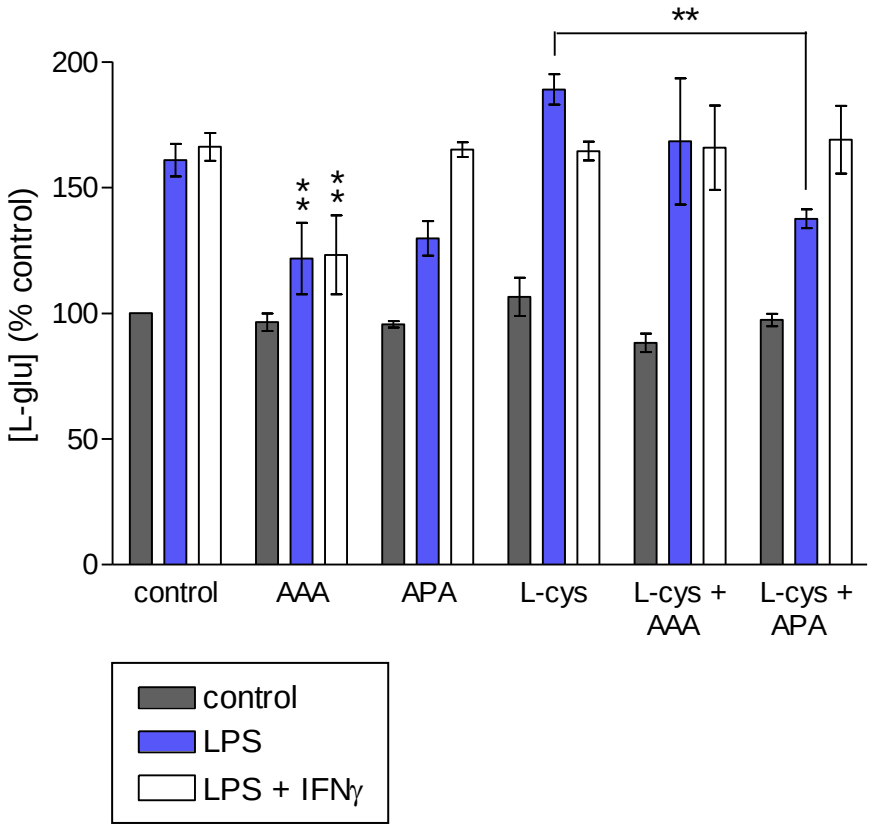


Figure 4.17. *The effect of pre-incubation with AAA, APA and L-cystine upon L-glutamate levels in conditioned medium from control BV-2 microglial cells and those treated with microglial activators. Plated BV-2 microglia were cultured in SFM containing AAA (2.5 mM), APA (2.5 mM), supplementary L-cystine (to a total of 1 mM), or a combination, for 24 hours before the addition of LPS (1 µg.ml^{-1}) or LPS + IFNγ (100 U.ml^{-1}). After a further 24 hours L-glutamate levels in conditioned medium were determined. Data represent the mean ± s.e.m. of at least three independent experiments for each condition. Data are presented as % control to highlight trends as there was a high level of variability when values were expressed in µM. Control [L-glutamate] in this experiment was 160.3 ± 20.4 µM. Data were analysed with a two-way ANOVA, which showed a significant effect of AAA/APA/L-cystine ($p=0.0005$) and a significant effect of LPS/IFNγ ($p<0.0001$) upon final extracellular L-glutamate levels. There was a less significant interaction between the variables ($p=0.0427$). Bonferroni's post test was carried out to compare the effects of AAA/APA/L-cystine in the absence and presence of LPS and LPS + IFNγ (**$p<0.01$, compared with the same condition in the absence of AAA/APA/L-cystine, or as indicated).*

4.8. BV-2 cells partially buffer exogenous glutamate in culture medium

By formulation, serum-free D-MEM in which BV-2 cells were plated contains no glutamate (Dulbecco and Freeman 1959). Addition of up to 100 μM glutamate to the culture medium had very little effect upon the glutamate levels in conditioned medium (fig. 4.18). Conditioned BV-2 cell medium following 24 hours' culture in the presence of 0 – 100 μM glutamate contained between 170 μM and 200 μM glutamate (fig. 4.18), indicating that the presence of BV-2 microglia elevated the extracellular glutamate levels. However, in the presence of 1 mM glutamate, BV-2 cells reduced the extracellular glutamate concentration from 598 ± 38 μM to 364 ± 55 μM over the 24 hour incubation (fig. 4.18). The difference between 1 mM glutamate as added and 598 μM as detected in SFM likely reflects the degradation of glutamate; the cell-free SFM sample was prepared at the same time as the glutamate-supplemented culture medium and kept under identical conditions for the duration of the incubation. Under conditions of elevated glutamate, LPS (1 $\mu g.ml^{-1}$) appeared to have a less pronounced effect upon glutamate levels, with a significant 71.7 ± 13.3 % increase (representing a difference of 122.7 ± 22.7 μM) in the absence of exogenous glutamate, and only a (non-significant) 15.8 ± 7.3 % increase (a difference of 57.7 ± 26.7 μM) in the presence of 1 mM exogenous glutamate (fig. 4.18).

As shown in figure 4.19, there was little effect of either the x_c^- transporter antagonist APA (2.5 mM) or the EAAT2 antagonist DHK (250 μM) upon the glutamate-buffering effect of BV-2 microglia. In the presence of 1 mM glutamate both APA and DHK slightly (but significantly) attenuated the microglia-mediated reduction in glutamate levels, but only in the presence of LPS. The difference between LPS treatment in the presence of APA or DHK and LPS treatment alone was only around a quarter of that between LPS treatment and the cell-free SFM control.

Cultured microglia *in vitro* have been shown to express α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Noda *et al.* 2000; Mayer *et al.* 2001; Hagino *et al.* 2004; Christensen *et al.* 2006; Liu *et al.* 2006), kainate (Noda *et al.*

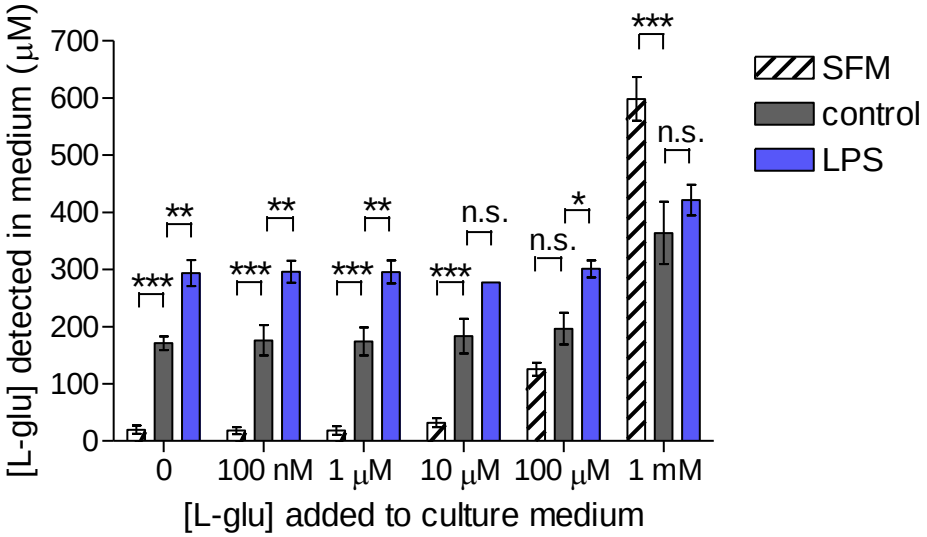


Figure 4.18. *BV-2 microglial cells buffer extracellular L-glutamate levels. BV-2 microglial cells were plated, medium was changed to SFM and cells were cultured in the presence of L-glutamate (100 nM – 1 mM) for 2 hours before the addition of LPS (1 μg.ml^{-1}). After 24 hours conditioned medium was removed and L-glutamate levels determined. Cell-free controls were also prepared in SFM and maintained under the same conditions (SFM). Data represent the mean ± s.e.m. of three independent experiments. Data were analysed with a two-way ANOVA, which demonstrated a highly significant effect of BV-2 cell presence and LPS, and of exogenous L-glutamate upon final extracellular L-glutamate levels, and also that there was a significant interaction between the variables ($p<0.0001$ in each case). Bonferroni's post-test was used to compare cell-free controls with control cells, and control cells with LPS-treated cells, at each L-glutamate concentration (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s. $p>0.05$, as indicated).*

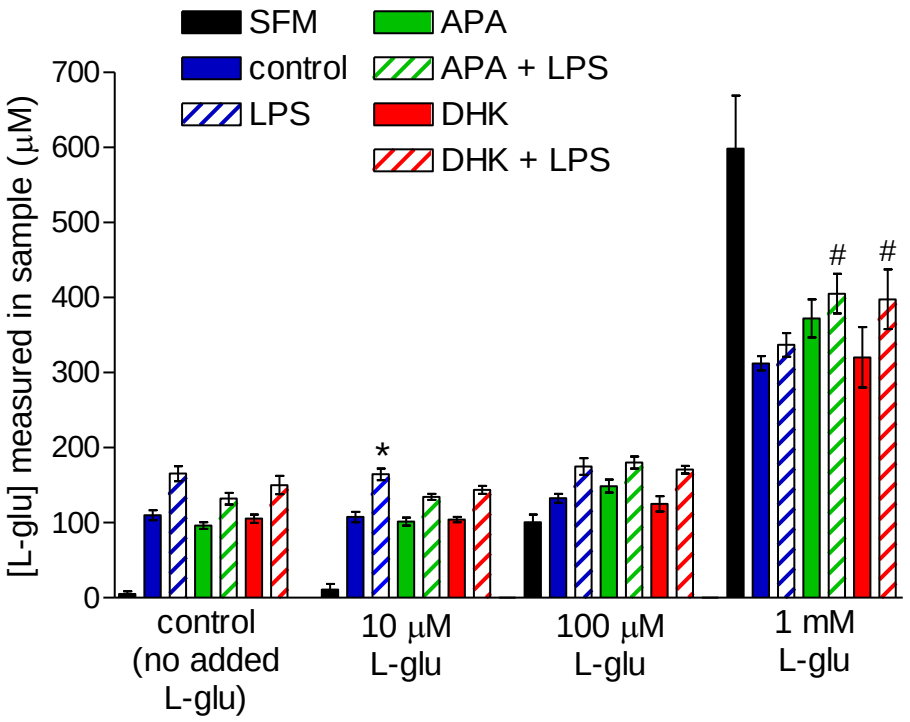


Figure 4.19. *Neither APA or DHK appear to be able to inhibit the ability of BV-2 cells to counter high concentrations of exogenous L-glutamate added to the culture medium. BV-2 microglial cells were cultured with SFM containing APA (2.5 mM) or DHK (250 μM) where indicated. L-glutamate (100 nM – 1mM) was added after 24 hours, and LPS (1 μg.ml^{-1}) after a further 2 hours. After 24 hours' incubation, conditioned medium was removed and L-glutamate levels determined. Data represent the mean ± s.e.m. of three (SFM, APA, APA + LPS, DHK, DHK + LPS) or four (control, LPS) independent experiments, each consisting of two replicates per condition. Data were analysed with a two-way ANOVA, which showed there to be a significant effect of exogenous L-glutamate concentration, and of APA/DHK/LPS treatment upon final extracellular L-glutamate levels, and a significant interaction between the variables ($p<0.0001$ in all cases). Bonferroni's post-tests were used to compare treatments within each L-glutamate concentration (*$p<0.05$ compared with control at each L-glutamate concentration; #$p<0.05$ compared with LPS alone at each L-glutamate concentration).*

2000) and N-methyl-D-aspartate (NMDA) (Liu *et al.* 2006) receptor subunits and to respond physiologically to AMPA/kainate receptor agonists (Noda *et al.* 2000; Mayer *et al.* 2001; Eun *et al.* 2004; Hagino *et al.* 2004; Christensen *et al.* 2006; Liu *et al.* 2006). Microglia have also been shown to express AMPA and NMDA receptor subunits *in vivo* under certain conditions (Ong *et al.* 1996; Gottlieb and Matute 1997; Kaur *et al.* 2005; Yamada *et al.* 2006; Newcombe *et al.* 2008). It was considered that Ca^{2+} entry through such iGluRs may alter microglial glutamate uptake by altering the expression or activity of glutamate transporters. For example, the transcription factor cAMP-response element binding protein as well as a number of members of the basic helix-loop-helix transcription factor family may be activated by calmodulin in the presence of Ca^{2+} (Hermann et al. 1998; West et al. 2001). Therefore, the NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine (MK-801; 10 µM) (Wong *et al.* 1986) and the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 µM) (Honore *et al.* 1988) were added to microglia prior to the addition of exogenous glutamate to assess whether iGluRs are involved in the microglial buffering of glutamate levels in culture medium. As demonstrated in figure 4.20, neither MK-801 nor CNQX affected the ability of microglia to buffer exogenous glutamate.

4.9. Primary microglia partially buffer moderate levels of exogenous glutamate in culture medium

Primary rat microglia were cultured in the presence of exogenous glutamate, to assess the ability of primary microglia to buffer glutamate added to the culture medium. Figure 4.21 shows that primary microglia did not cause an elevation in the glutamate concentration of conditioned medium in the absence of exogenous glutamate in the way that BV-2 microglia did (fig. 4.18). Thus, when no glutamate was added, the glutamate concentration found in cell-free controls ("SFM") and conditioned medium was almost identical. However, when 100 µM glutamate was added to the cells for 24 hours, the cells halved the apparent glutamate concentration, reducing it from 110.2 ± 12.4 µM to 55.3 ± 9.7 µM. When 1 mM exogenous

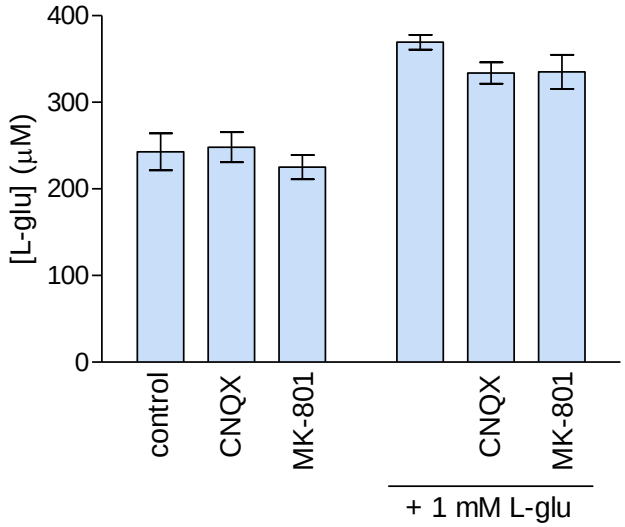


Figure 4.20. *Ionotropic glutamate receptor antagonists have no effect upon L-glutamate levels in BV-2 cell conditioned medium. BV-2 microglia were cultured in SFM in the presence of CNQX (20 µM) or MK-801 (10 µM) for 24 hours, before L-glutamate levels of the conditioned medium were determined. Where indicated, 1 mM exogenous L-glutamate was added to cultures 2 hours after addition of antagonists. Data represent the mean ± s.e.m. of three independent experiments, each consisting of two replicates per condition. Data were analysed with a two-way ANOVA, which showed that exogenous L-glutamate had a significant effect upon final extracellular L-glutamate concentrations ($p<0.0001$) but that there was no significant effect of the glutamate receptor antagonists ($p=0.288$), nor was there any significant interaction between the two variables ($p=0.4545$).*

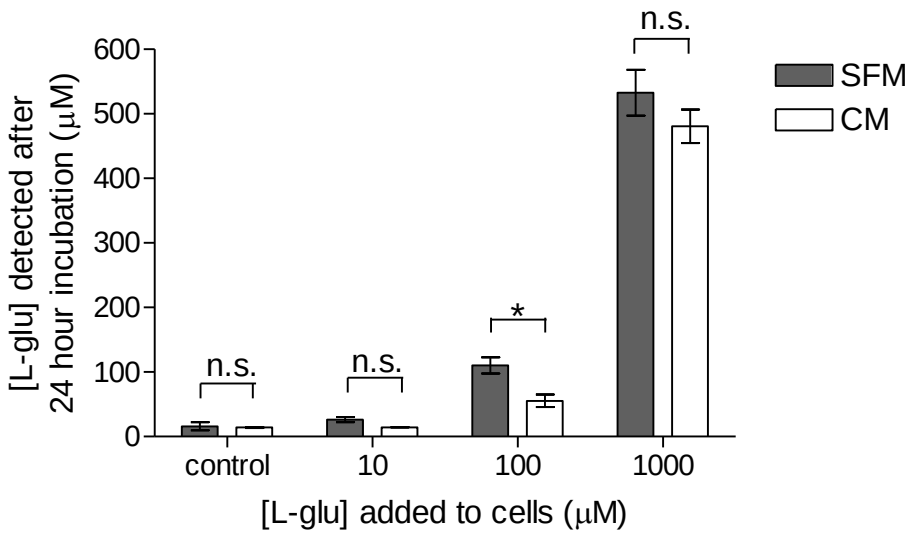


Figure 4.21. *Primary microglial cells buffer extracellular L-glutamate levels to some extent. L-glutamate levels in culture medium containing supplementary L-glutamate before and after 24 hours' incubation with microglia. Primary rat microglia were plated at 1 x 10^5 cells per 13 mm coverslip, culture medium was changed to SFM and cells were exposed to exogenous L-glutamate (10 μM – 1 mM) for 24 hours, before L-glutamate levels in the conditioned medium were determined (CM). Cell-free controls were also prepared in SFM and maintained under the same conditions (SFM). Data represent the mean ± s.e.m. of three independent experiments, each consisting of three replicates per condition. Data were analysed with Student's t tests to compare conditions in pairs, as indicated (*p<0.05, n.s. p>0.05).*

glutamate was added to the cultures, microglia slightly attenuated it, but this was not significant.

4.10. Exogenous glutamate in culture medium may affect GSH levels

BV-2 microglial cells were incubated with 1 nM – 1 mM glutamate for 24 hours before GSH levels were determined by quantitative analysis of monochlorobimane (MCB) imaging. This demonstrated a highly significant effect of exogenous glutamate upon the GSH content of BV-2 microglia, as assessed by one-way ANOVA (p<0.0001) (fig. 4.22). Micromolar concentrations of glutamate were found to significantly increase GSH levels, with a 35.2 ± 9.9 % increase in BV-2 GSH levels following treatment with 1 µM glutamate and a 58.2 ± 18.5 % increase following treatment with 10 µM glutamate (fig. 4.22). In addition, in the presence of 1 mM glutamate, a (non-significant) 25.7 ± 4.6 % decrease in GSH levels compared with control was detected (fig. 4.22).

4.11. Discussion

4.11.1. Microglia express glutamate transporters

Glutamate transport across the cell surface of microglia is bidirectional. Under normal circumstances, microglia take up glutamate by means of the EAATs (Lopez-Redondo *et al.* 2000; Nakajima *et al.* 2001b) and release glutamate in exchange for cystine via the x_c^- transporter system (Piani and Fontana 1994; Qin *et al.* 2006). Glutamate levels of microglial conditioned medium were determined, as an indication of the relative activity of these transporters. In the case of primary microglia there was no difference between the glutamate concentration of SFM and that of control conditioned medium, suggesting either that the activity of these transporters was very low, or that the levels of glutamate release and uptake were approximately equal. However in both the N9 and BV-2 cell line, control conditioned medium contained higher concentrations of glutamate than fresh

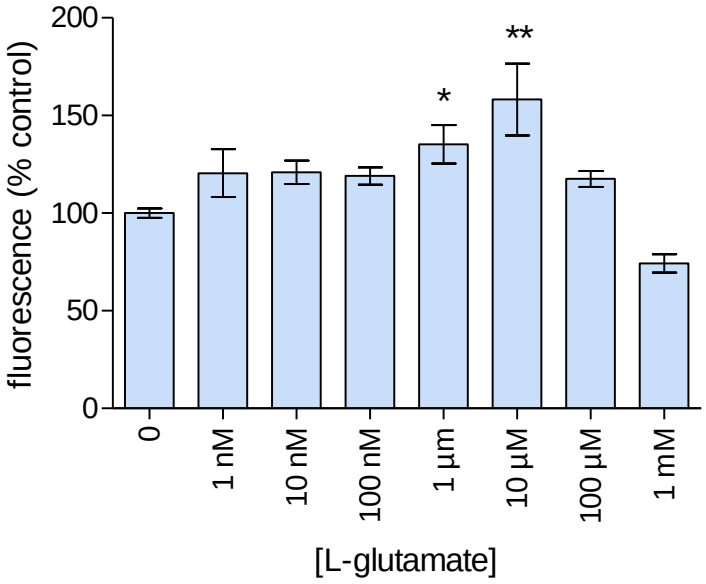


Figure 4.22. *The effect of L-glutamate upon BV-2 microglial GSH levels. BV-2 cells were cultured in the presence of L-glutamate (1 nM – 1 mM) for 24 hours. GSH levels were determined by quantitative analysis of MCB imaging. Data represent the mean ± s.e.m. of five (10 nM), six (100 nM, 1 mM) or seven (0, 1 nM, 1 µM, 10 µM, 100 µM) experiments, each consisting of between one and six replicates per condition. Data were analysed with a one-way AVOVA ($p<0.0001$), with Dunnett's post-test (*$p<0.05$, **$p<0.01$, compared with control). The data were obtained by Ruth Faram under my supervision.*

medium, suggesting a net glutamate release by N9 and BV-2 cells. Indeed, in the case of BV-2 cells, the glutamate concentration of control conditioned medium was similar whether the cells were cultured in SFM or SCM, despite the fact that fresh SFM contains lower levels of glutamate. This suggests that BV-2 cells maintain an extracellular glutamate concentration in the region of 100 – 200 μM. This may be due to net release up to a certain point, which is then balanced by high affinity uptake by EAATs and/or glutamate competition with cystine for uptake via x_c^- (Bannai 1986; Sato *et al.* 1995, 1999; Rimaniol *et al.* 2001; Patel *et al.* 2004).

EAATs 1 and 2 were originally believed to be exclusively astrocytic transporters responsible for the maintenance of extracellular glutamate below neurotoxic levels (Danbolt *et al.* 1992; Rothstein *et al.* 1994, 1996; Lehre *et al.* 1995). Later studies demonstrated expression of these EAATs by microglia (Swanson *et al.* 1997; Nakajima *et al.* 2001b) as well as macrophages (Rimaniol *et al.* 2000). In accordance with this, expression of both EAAT1 and EAAT2 mRNA was demonstrated here in primary rat microglia, and low levels of EAAT1 and EAAT2 mRNA expression were detected in BV-2 and N9 microglial cell lines. The expression of both EAATs by the microglial cell lines was very low, close to the limit of detection, and therefore any observations from these experiments should be interpreted with caution. As well as the standard *in vitro* microglial activators LPS and IFNγ, the effects of albumin, CGA and $A\beta_{25-35}$ upon the expression of EAAT1 and EAAT2 by microglia were investigated. This appears to be the first report investigating the effects of these neuroinflammation-related proteins upon microglial EAAT expression. The data obtained in the PCR experiments would be strengthened by studies of EAAT protein expression and determination of transporter function in primary microglia, as post-transcriptional events, as well as modifications of the protein *in situ*, may affect its true glutamate uptake ability. However, due to the large amounts of protein that would likely be required, especially considering the low level of EAAT expression by microglia, it was not possible to carry out such investigations here.

4.11.2. The effects of fraction V albumin upon glutamate transport is similar to that of LPS

It is demonstrated here that LPS causes increased glutamate levels in microglial conditioned medium, suggestive of glutamate release by microglia. This is in agreement with published data (Piani and Fontana 1994; Nakamura *et al.* 2003; Domercq *et al.* 2007; Barger *et al.* 2007). In N9 cells, co-stimulation with IFNγ was necessary to give a significant increase in glutamate levels, and in BV-2 cells LPS alone or in combination with IFNγ gave similar significant increases in glutamate levels.

In addition, the data presented here show enhanced microglial glutamate release following treatment with fraction V albumin in primary microglia and BV-2 microglial cells, but not in N9 cells. Albumin induces superoxide production by primary microglia (Si *et al.* 1997; Nakamura *et al.* 2000) and was shown in the previous chapter and elsewhere (Hooper *et al.* 2009) to elevate iNOS expression in primary and BV-2 microglial cells. There is evidence for the involvement of superoxide (Barger *et al.* 2007; Jin *et al.* 2007) and NO (Li *et al.* 1999; Nakamura *et al.* 2003) in the pathways leading to enhanced glutamate release. Indeed, oxygen free radicals have been shown to downregulate EAAT-mediated glutamate uptake without affecting transporter expression (Volterra *et al.* 1994), probably by inducing disulphide bond formation between functional EAATs (Trotti *et al.* 1997b; Blanc *et al.* 1998). Albumin-induced glutamate release may therefore be mediated through a pathway involving NO or superoxide, or both of these reactive species.

It should also be noted that albumin has been shown to induce microglial proliferation (Hooper *et al.* 2005), and since the glutamate release data for primary and BV-2 microglia were not corrected for protein levels, proliferation may have amplified an increased release rate of glutamate. LPS however, has an antiproliferative effect upon microglia (Gebicke-Haerter *et al.* 1989; Bianco *et al.* 2006), so the increased glutamate release recorded may have actually been an underestimate. In addition, LPS + IFNγ and IFNγ alone cause significant LDH release after 24 hours in BV-2 cells at least (fig. 3.16), suggesting that glutamate

release may be due simply to cell lysis. However in BV-2 cells, glutamate release following incubation with LPS + IFNγ was no higher than that following incubation with LPS alone, and IFNγ did not have any effect (fig. 4.2). Similar results were obtained for primary microglia (fig. 4.1).

The time course of LPS-induced glutamate release in BV-2 cells shows clear similarities with that presented by Nakamura *et al.* (2003) for primary microglia. In this published study, inhibitors of NOS and of the x_c^- transporter were found to prevent the enhancement of glutamate release. Both LPS and albumin-induced glutamate release appeared to have reached a plateau by 48 hours, suggesting that the rapid phase of glutamate release is somewhat transient. In macrophages at least, evidence exists for a more transient upregulation of x_c^- expression and cystine uptake upon LPS treatment (Sato *et al.* 1995, 2001; see fig. 3.18A). Indeed, between 12 and 48 hours' exposure to LPS, the rate of cystine uptake was shown to decline sharply, so that by 48 hours, the cystine uptake rate was only around one third of the maximum observed rate (Sato *et al.* 1995). Since the x_c^- transporter exchanges cystine and glutamate in a 1:1 ratio (Bannai 1986), this implies a simultaneous decrease in glutamate release rate.

To complement these glutamate release data, the expression of the glutamate transporters EAAT1, EAAT2 and the x_c^- system were investigated in microglia, by means of PCR. In the case of the x_c^- system, mRNA levels of its specific subunit, xCT, were measured. The effects of LPS, particularly upon microglial expression of EAAT2 and xCT, are fairly well characterised. Here, the effects of fraction V albumin (as well as the AD-related proteins CGA and $A\beta_{25\text{-}35}$) upon transporter mRNA expression were investigated alongside LPS.

The expression of mRNA for EAAT1 by primary microglia appeared to decrease in the presence of LPS. This is at odds with a previous study, which failed to demonstrate any effect of LPS upon the expression of EAAT1 protein by rat microglia (O'Shea *et al.* 2006). Other published studies demonstrate increased microglial EAAT1 expression *in vivo* following traumatic brain injury in the rat (van Landeghem *et al.* 2001) and in patients suffering with Prion disease (Chretien *et al.* 2004) and HIV (Vallat-Decouvelaere *et al.* 2003). However, all these published

studies measure protein rather than mRNA expression, therefore post-translational modifications could account for this discrepancy. Alternatively, the differences between this study and the *in vivo* studies mentioned above could relate to the differences between microglia *in vitro* and *in vivo*. A recent *in vivo* study found that following ischaemia, microglial EAAT1 expression appeared to be restricted to the earlier stages of activation and was not present on more highly activated, phagocytosing microglia (Beschorner *et al.* 2007). It has been suggested that the process of isolating and culturing microglia leads to a low level of microglial activation (Rosenstiel *et al.* 2001). Microglia in culture may therefore represent those in an early stage of activation, and the addition of LPS may increase the level of activation to the stage at which EAAT1 gene expression becomes downregulated. Microglial EAAT1 mRNA expression appeared to similarly decrease in the presence of fraction V albumin.

In the presence of LPS, primary microglia were found to upregulate their expression of mRNA for EAAT2, in accordance with published data (Persson *et al.* 2005; Jacobsson *et al.* 2006; O'Shea *et al.* 2006). Increased microglial EAAT2 expression has also been demonstrated following viral infection *in vitro* (Persson *et al.* 2007) and *in vivo* (Chretien *et al.* 2002), and following brain and neuronal injury (Lopez-Redondo *et al.* 2000; van Landeghem *et al.* 2001). Fraction V albumin also caused a consistent increase in EAAT2 mRNA expression.

It is interesting that the data presented here suggest a decrease in EAAT1 mRNA expression, but an increase in EAAT2 expression following LPS treatment. This suggests that microglia may consistently express one EAAT subtype, but that under stressful conditions EAAT1 expression may be replaced by that of EAAT2. Under conditions of oxidative stress and hypoxia, the glutamate uptake capacity of astrocytes may decrease (Volterra *et al.* 1994; Miralles *et al.* 2001; Dallas *et al.* 2007) and microglial EAAT-mediated glutamate uptake may partially replace astrocyte glutamate uptake. A review which collated data for kinetic parameters of uptake showed that the average reported K_m of rat EAAT2 for glutamate was 8-fold lower than that of EAAT1 (Gegelashvili and Schousboe 1998). Thus, replacement of EAAT1 with EAAT2 under stressful situations may be associated with an increased affinity for glutamate. Alternatively, it may be that the microglial capacity for

functional EAAT2 expression is greater than that of EAAT1, so that a switch from EAAT1 to EAAT2 would enhance the glutamate uptake capacity. A low level of microglial EAAT1 protein expression has been reported previously (O'Shea *et al.* 2006); such low expression of EAAT1 may explain the lack of detection of microglial EAAT1 protein in other studies (Nakajima *et al.* 2001b; Persson *et al.* 2005; Jacobsson *et al.* 2006).

A constitutive expression of xCT mRNA by primary microglia and cells of the BV-2 and N9 cells lines was observed here. In primary microglia and BV-2 cells, LPS consistently increased xCT mRNA expression. LPS has previously been shown to increase the expression of xCT mRNA (Barger *et al.* 2007) and protein (Domercq *et al.* 2007), with a concomitant increase in glutamate release (Barger *et al.* 2007; Domercq *et al.* 2007). In addition, fraction V albumin consistently increased primary and BV-2 microglial xCT mRNA expression. This observation fits with recent data demonstrating that the increased extracellular glutamate following treatment of microglia with fraction V or pure albumin was prevented by the x_c^- inhibitor AAA (Hooper *et al.* 2009).

Coincident upregulation of microglial EAAT2 and xCT mRNA expression, which has been confirmed at the protein level following LPS treatment (Persson *et al.* 2005; Jacobsson *et al.* 2006; O'Shea *et al.* 2006; Domercq *et al.* 2007), may indicate complementary functions of these proteins. For example, an enhancement of glutamate uptake via EAAT2 might counteract the enhanced glutamate release occurring as a consequence of increased x_c^- activity. Alternatively, glutamate imported via EAAT2 may provide glutamate in the direct vicinity of x_c^- transporters and thereby contribute to the maintenance of sufficient cystine uptake for GSH synthesis (Igo and Ash 1998; Rimaniol *et al.* 2001; Persson *et al.* 2006).

4.11.3. Implications for Alzheimer's disease

The proteins CGA and $A\beta_{25-35}$ are upregulated in AD, and microglia may come into contact with them during disease pathogenesis. Both have been shown to activate microglia *in vitro* (Taupenot *et al.* 1996; McDonald *et al.* 1997; Kingham *et al.* 1999; Combs *et al.* 2001; Le *et al.* 2001). In the present study, neither protein was

found to significantly alter the extracellular glutamate levels of BV-2 or N9 microglial cells, despite the fact that slightly lower concentrations of CGA have previously been shown to have effects including the elevation of glutamate release in primary microglial cells (Kingham *et al.* 1999; Hooper and Pocock 2007), and similar concentrations of $A\beta_{25-35}$ have other effects upon microglia *in vitro* (Klegeris and McGeer 1997; Noda et al. 1999; Taylor *et al.* 2002). Only the full-length Aβ peptide $A\beta_{1-40}$ appears to have been found to enhance microglial glutamate release in cultured microglia (Klegeris and McGeer 1997; Qin *et al.* 2006), which was suggested to be via increased x_c^- activity. Conversely, electrophysiological data suggest that $A\beta_{25-35}$ may increase the activity of microglial glutamate transporters of the EAAT type (Noda *et al.* 1999).

PCR for the EAATs demonstrated little effect of CGA or $A\beta_{25-35}$ upon microglial EAAT1 or EAAT2 mRNA expression. In addition, neither CGA nor $A\beta_{25-35}$ appeared to alter the expression of xCT mRNA, despite the fact that an increase in extracellular glutamate following microglial treatment with $A\beta_{1-40}$ was prevented by the x_c^- inhibitors AAA and APA (Qin *et al.* 2006). The regulation of cell surface x_c^- expression and activity may however occur at stages downstream of gene transcription; an LPS-induced enhancement of glutamate release has been shown to occur in the presence mRNA or protein synthesis inhibitors suggesting that increased activity of existing transporters or increased trafficking to the plasma membrane may be involved (Barger *et al.* 2007).

BBB damage is associated with AD as well as MS and other neurological conditions (Alafuzoff *et al.* 1983; Skoog *et al.* 1998; Fiala *et al.* 2002). Therefore microglia may be exposed to albumin during disease pathogenesis, which may affect microglial expression of glutamate transporters and enhance glutamate release, even if proteins specifically associated with AD, such as CGA and $A\beta_{25-35}$ do not. In addition, AD is associated with increased levels of oxidative stress (Nunomura *et al.* 2006). The redox status of the cell has been shown to affect EAAT activity independently of transporter expression, with oxygen free radicals downregulating, and antioxidants upregulating EAAT-mediated glutamate uptake, through inducing and reducing disulphide bonds between functional EAATs (Volterra *et al.* 1994; Trotti *et al.*

1997b; Blanc *et al.* 1998; Begni *et al.* 2004). Such post-translational modifications may therefore affect microglial glutamate uptake in AD.

4.11.4. x_c^- inhibition affects microglial GSH content but has less of an effect upon glutamate release

There was no effect of DHK upon GSH levels in BV-2 or N9 microglial cells. This may reflect a low level of EAAT2 expression, as suggested by low mRNA levels, or it may suggest that EAAT2 activity simply has little effect upon GSH levels under non-activated conditions. Exogenous cystine, cysteine or NAC also did not affect the cellular GSH content of either cell line, and was also shown in chapter 3 not to affect microglial GSH release. This suggests that the levels of cystine already present in the medium (200 μM) are not limiting for GSH synthesis. Inhibition of the x_c^- transporter in BV-2 cells did however decrease GSH levels, confirming that x_c^--mediated cystine uptake is crucial for microglial GSH synthesis. Paradoxically, x_c^- transporter inhibition had little effect upon extracellular glutamate levels. However, AAA is a competitive transportable inhibitor (Tsai *et al.* 1996; Pow 2001), therefore the uptake of AAA is likely to be coupled to the export of glutamate or other x_c^- substrate. The small decline in extracellular glutamate in the presence of AAA under some conditions may reflect the replacement of a proportion of the glutamate export with re-export of the imported AAA. The mechanism of x_c^- inhibition by APA is likely to be identical to that of AAA given that the compounds are both glutamate analogues, the only difference between the two being that APA contains a 7-carbon chain where AAA has a 6-carbon chain, and considering their similar inhibition characteristics (Bannai 1986; Watanabe and Bannai 1987; Piani and Fontana 1994). Therefore this explanation for a lack of significant alteration of extracellular glutamate despite x_c^- inhibition can be extended to APA.

Rather high concentrations (2.5 mM) of AAA and APA were used here, in accordance with published work (Kingham *et al.* 1999; Qin *et al.* 2006; Domercq *et al.* 2007). However, as AAA and APA are glutamate analogues, such high concentrations may have non-specific effects. AAA may also be a competitive substrate for Na^+-dependent glutamate transport by the EAATs (Tsai *et al.* 1996), and an inhibitor of glutamine synthetase (McBean 1994), thus potentially affecting

other aspects of glutamate uptake and metabolism in microglia besides the x_c^- transporter. APA, on the other hand, is also a low-affinity NMDA antagonist (Scanziani *et al.* 1997). It should therefore be considered that different non-specific effects of the compounds could explain the slightly different effects of AAA and APA upon microglial glutamate release.

4.11.5. Microglial buffering of extracellular glutamate

BV-2 cells, and to some extent, primary microglia, demonstrated a "buffer" function for glutamate levels in the culture medium. When high levels of glutamate were added to the medium, this was decreased in the presence of microglia over 24 hours. Although glutamate did appear to degrade in the medium, this is not likely to explain this result as the cell-free controls were prepared at the same time and kept in identical conditions. The difference between cell-free control and conditioned medium therefore appears to be due to an action of the cells. In the case of BV-2s, the extracellular glutamate concentration was raised to 170 – 200 µM even in the absence of exogenous glutamate, and following addition of up to 100 µM glutamate. Interestingly, this is very similar to the cystine concentration in D-MEM (200 µM), lending weight to the idea that this buffering action is purely due to the action of x_c^-, and competition between glutamate and cystine for the transporter. This would also explain the decrease in extracellular glutamate following addition of 1 mM glutamate. In this light, it would be interesting to investigate this buffering effect in the presence of different concentrations of extracellular cystine.

Surprisingly however, APA was not able to prevent the buffering effect seen at any glutamate concentration. The EAAT inhibitor DHK similarly had no effect. Since APA and DHK were not tested in combination, it is a possibility that EAATs could mediate this effect in the absence of adequate x_c^- function. Alternatively, the glutamate buffering capacity of BV-2 microglia may be due to a glutamate receptor-mediated upregulation of the x_c^- transporter, such that it may be partially inhibited by APA, but still sufficiently available to glutamate to mediate the changes observed within 24 hours. Interestingly, 1 mM glutamate has been shown to increase microglial TNFα production through stimulation of AMPA/kainate receptors (Noda *et al.* 2000), and TNFα has been shown to upregulate EAAT2 expression and

glutamate uptake (Persson *et al.* 2005). However in the case of BV-2 cells here, the AMPA/kainate receptor antagonist did not alter the extracellular glutamate concentration either in the absence of exogenous glutamate or following addition of 1 mM glutamate, although this was not tested in combination with x_c^- inhibition. Prolonged exposure to such a high concentration of glutamate may however cause desensitisation of glutamate receptors (Dingledine *et al.* 1999); the use of more physiological micromolar concentrations of glutamate or specific glutamate receptor agonists would be beneficial to clarify the situation and allow elucidation of the mechanisms involved.

In contrast to BV-2 cells, primary microglia did not increase extracellular glutamate in the absence of exogenous glutamate. Primary microglia were also unable to counter the highest (1 mM) glutamate concentration. These effects are most likely to be due to differences in transporter expression. The less pronounced "buffer" effect in primary microglia may reflect a lower impact of x_c^- upon glutamate levels relative to the EAATs. As described above, stimulation of microglial AMPA/kainate receptors by 1 mM glutamate leads to TNFα production (Noda *et al.* 2000), which may mediate EAAT2 upregulation (Persson *et al.* 2005), and therefore increase the glutamate uptake capacity of microglia. An AMPA/kainate receptor-dependent upregulation of EAATs by 1 mM glutamate has also been demonstrated in the rat optic nerve (Vallejo-Illarramendi *et al.* 2006). However 1 mM glutamate has also been shown to inhibit microglial glutamate uptake by EAATs, to a similar level as DHK (Persson *et al.* 2005). It is therefore proposed that in the case of primary microglia, high levels of exogenous glutamate may inhibit EAAT-mediated uptake, while the x_c^- transporter is not expressed at a level sufficient to significantly alter the extreme glutamate concentrations that BV-2 cells modulated. These hypotheses relating to the glutamate-buffering ability of microglia are illustrated in figure 4.23.

4.11.6. The buffering of exogenous glutamate affects microglial GSH content

If the ability of microglia to attenuate high levels of glutamate in the culture medium is due to x_c^- mediated transport, glutamate would be imported in place of cystine, thus potentially compromising GSH synthesis. Therefore the effect of exogenous

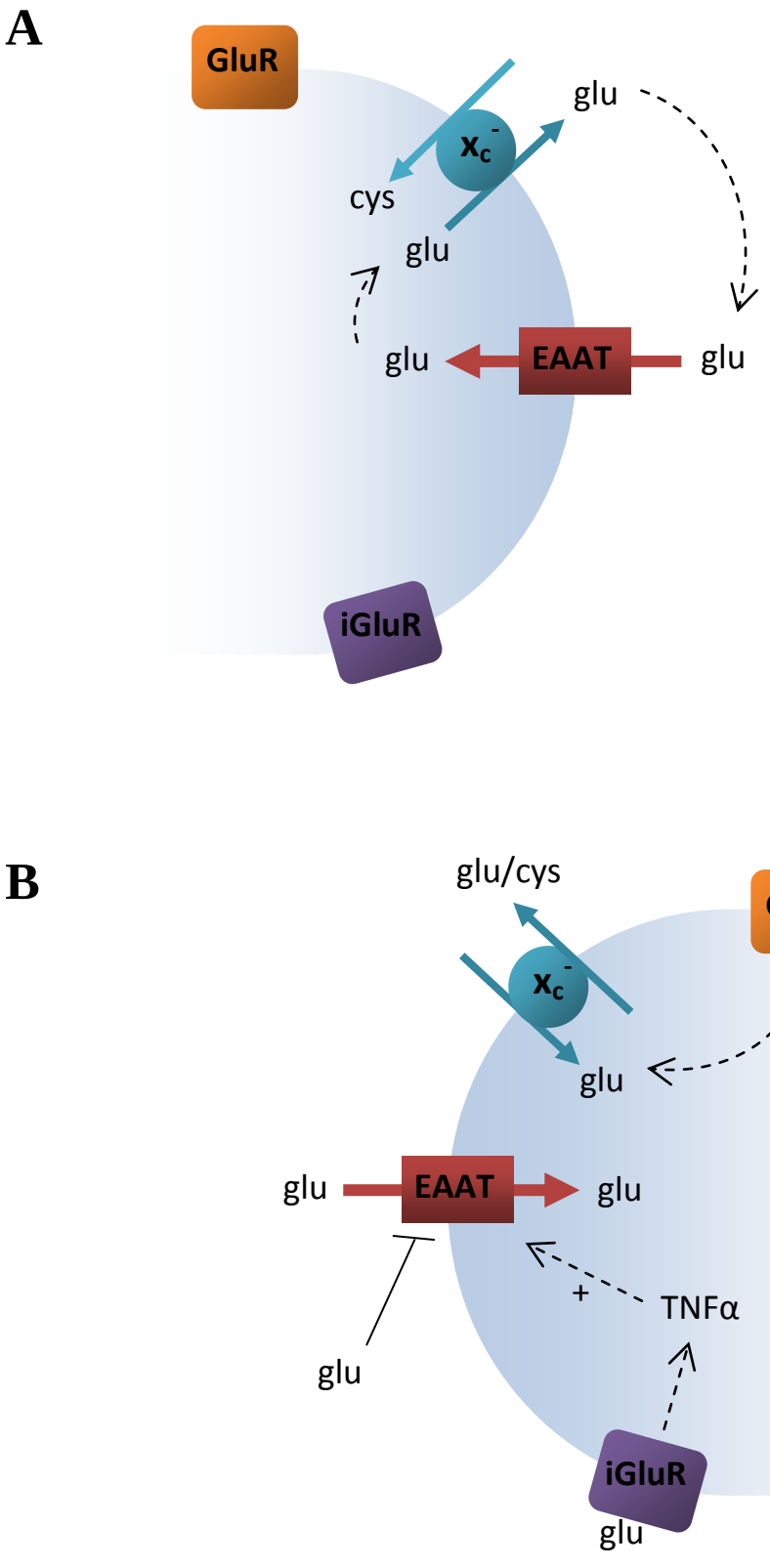


Figure 4.23. (legend overleaf)

Figure 4.23. (previous page) *Hypotheses to explain the glutamate-buffering capability of microglia.* ***A****, Under normal conditions, EAATs mediate glutamate uptake whilst* x_c^- *releases glutamate in exchange for cystine. This allows the development and maintenance of a constant extracellular glutamate concentration. In BV-2 microglia, the effect of* x_c^- *appears to dominate such that the extracellular glutamate concentration attained is approximately equivalent to the extracellular cystine concentration. In primary microglia, the activity of* x_c^- *and the EAATs appears to be approximately equal.* ***B****, Under situations of elevated glutamate, glutamate may compete with cystine for uptake via* x_c^- *(Bannai 1986; Watanabe and Bannai 1987; Sato et al. 1999; Tomi et al. 2002; Patel et al. 2004). The increased glutamate concentration may also increase the rate of EAAT-mediated glutamate uptake. In addition, extracellular glutamate acting upon glutamate receptors of the AMPA/kainate subtypes may increase the expression of EAATs (Vallejo-Illarramendi et al. 2006), possibly through the stimulation of TNFα production (Noda et al. 2000), which has been shown to lead to enhanced EAAT expression and glutamate uptake (Persson et al. 2005). Glutamate acting upon other glutamate receptors may cause upregulation of* x_c^-*. However, high levels of glutamate may inhibit EAATs and thus limit microglial glutamate-buffering capacity (Persson et al. 2005). cys, cystine; EAAT, excitatory amino acid transporter; glu, glutamate; GluR, glutamate receptor; iGluR, ionotropic glutamate receptor; TNFα, tumour necrosis factor α;* x_c^-*;* x_c^- *glutamate/cystine antiporter system.*

glutamate upon intracellular GSH levels was investigated. It was found that 1 – 10 μM glutamate caused a significant increase in GSH levels, as determined by quantitative analysis of MCB fluorescence. Glutamate has previously been shown to cause increases in GSH levels in macrophages, an effect which was blocked by EAAT inhibitors (Rimaniol *et al.* 2000, 2001). However, high concentrations of glutamate have also been shown to reduce the rate of microglial glutamate uptake by EAAT2 (Persson *et al.* 2005). The uptake of cystine, the limiting amino acid in GSH synthesis, has repeatedly been shown to be competitively inhibited by high concentrations of glutamate (Bannai 1986; Watanabe and Bannai 1987; Sato *et al.* 1999; Tomi *et al.* 2002; Patel *et al.* 2004). In the presence of sufficient cystine, it is likely that optimum GSH synthesis is possible at a glutamate concentration which allows sufficient glutamate uptake via EAATs to provide glutamate in the direct vicinity of x_c^- transporters and thereby allow a high rate of cystine uptake for GSH synthesis (Igo and Ash 1998; Rimaniol *et al.* 2001; Persson *et al.* 2006), without being high enough to inhibit either transporter (fig. 4.24). Indeed, in retinal Müller glial cells, it was demonstrated that the presence of glutamate and cystine, and functional EAATs and x_c^- system were necessary to maintain the GSH level (Reichelt *et al.* 1997).

4.11.7. Conclusion

It is demonstrated here that microglia express mRNA for glutamate transporters, and that this expression may be modified by microglial activation. LPS upregulates the expression of EAAT2 and xCT, the specific subunit of the x_c^- transporter, but may downregulate the expression of EAAT1. Albumin may have similar effects. Both LPS and albumin were also shown to increase the glutamate levels of conditioned medium. However, CGA and $A\beta_{25-35}$, two proteins upregulated in AD, could not be found to affect microglial glutamate transport. BV-2 cells only express low levels of EAAT mRNA and the effects of the glutamate/cystine antiporter system x_c^- appear to dominate. This endows BV-2 cells with the ability to buffer extracellular glutamate concentrations, through competition between cystine and glutamate for transport. Primary microglia were also able to buffer high extracellular glutamate concentrations to a limited extent, although it is unclear how important this would be *in vivo*, where astrocytes robustly express EAATs and have a much higher glutamate

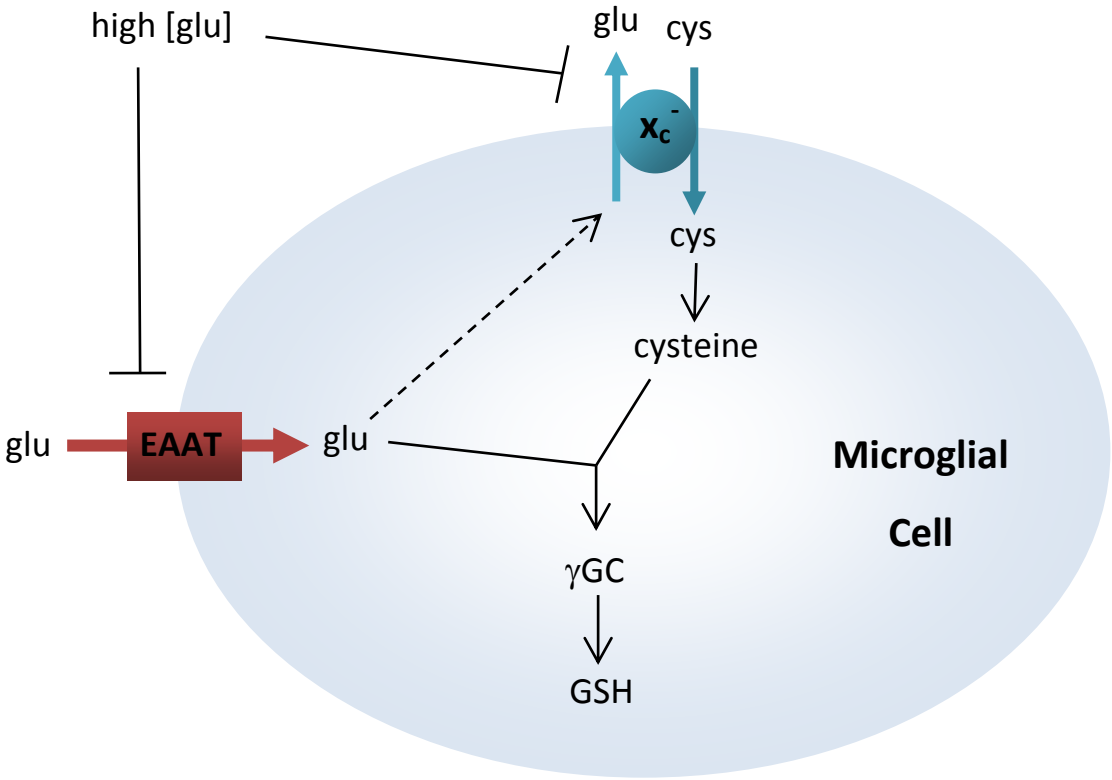


Figure 4.24. *Summary of the effects of glutamate upon amino acid uptake for GSH synthesis. Extracellular glutamate stimulates EAAT-mediated uptake, which drives cystine import via x_c^-, and thus GSH synthesis. However, high levels of glutamate inhibit EAATs and compete with cystine for x_c^--mediated transport. cys, L-cystine; EAAT, excitatory amino acid transporter; γGC, γ-glutamylcysteine; glu, L-glutamate; GSH, glutathione; x_c^-, x_c^- L-glutamate/L-cystine antiporter system.*

uptake capacity than microglia (Leonova *et al.* 2001; Persson *et al.* 2005), and are thought to be primarily responsible for maintaining the extracellular glutamate concentration below neurotoxic levels (Rosenberg *et al.* 1992; Rothstein *et al.* 1996). Perhaps microglial glutamate uptake would become significant only under exceptional circumstances. The consequences of such glutamate uptake would depend upon the transporter(s) involved, but should the x_c^- transporter be involved, this may compromise GSH synthesis through decreased cystine supply, and may be detrimental towards the microglia, especially in the event of CNS challenge.

Chapter 5

Results III:
The effects of metabotropic glutamate receptor modulation upon microglial glutathione content and glutamate release

5.1. Introduction and summary of results

In the previous chapter, it was shown that L-glutamate has effects upon microglial GSH levels, and it was concluded that this is likely to be due to direct effects upon glutamate transporters, particularly the x_c^- glutamate/cystine antiporter system. However since microglia express ionotropic glutamate receptors (iGluRs) (Ong *et al.* 1996; Gottlieb and Matute 1997; Noda *et al.* 2000; Mayer *et al.* 2001; Hagino *et al.* 2004; Kaur *et al.* 2005; Christensen *et al.* 2006; Liu *et al.* 2006; Yamada *et al.* 2006; Newcombe *et al.* 2008) and metabotropic glutamate receptors (mGluRs) (Biber *et al.* 1999; Taylor *et al.* 2002, 2003; Geurts *et al.* 2003, 2005; Byrnes *et al.* 2009), alterations in extracellular glutamate concentration are also likely to affect microglia through these receptors. Indeed, mGluR2 activation has been implicated in microglial neurotoxicity (Taylor *et al.* 2002, 2005; Pinteaux-Jones *et al.* 2008), and activation of group III mGluRs (Taylor *et al.* 2003; Pinteaux-Jones *et al.* 2008) as well as mGluR5 (Byrnes *et al.* 2009) has been shown to attenuate microglial activation and to bring about a neuroprotective microglial phenotype. Thus, mGluRs of all three groups may have an impact upon the neurotoxic or neuroprotective potential of microglia.

As shown in chapter 3 of this thesis and elsewhere (Chatterjee *et al.* 1999; Hirrlinger *et al.* 2000; Moss and Bates 2001; Persson *et al.* 2006), microglia contain high levels of the antioxidant glutathione (GSH). This is crucial for protecting microglia against oxidative stress, and thus ensuring their survival. However the benefit of microglial survival *in vivo* clearly depends upon the state of the microglia and whether they are expressing a neuroprotective or neurotoxic phenotype.

In astrocytes, stimulation of group II or III mGluRs has been found to rescue an LPS-induced decline in intracellular GSH (Zhou *et al.* 2006). Group II mGluR agonists also rescued GSH depletion in dorsal root ganglion neurones and Schwann cells in co-culture following glucose-induced oxidative injury (Berent-Spillson and Russell 2007). This was accompanied by a reduction in the levels of reactive oxygen species (ROS) and superoxide and by neuroprotection (Berent-Spillson and Russell 2007). In HT22 hippocampal neurones, group I mGluR agonists protected against

oxidative glutamate toxicity, and partially rescued glutamate-induced GSH depletion (Sagara and Schubert 1998); indeed, increased mGluR1 expression was associated with resistance to oxidative glutamate toxicity (Sahin *et al.* 2006). Enhanced expression of group I mGluRs by primary cultures of cortical neurones was found in the presence of elevated extracellular glutamate or cystine deprivation, conditions under which GSH synthesis may be compromised (Sagara and Schubert 1998). Group I mGluR stimulation was also shown to be protective against kainate and oxygen-glucose deprivation toxicity in oligodendrocytes, by attenuation of ROS levels and restoration of the intracellular GSH level (Deng *et al.* 2004).

The modulation of mGluRs may therefore have an effect upon cellular antioxidant defences, and thus cell fate, in a number of CNS cell types, although there do not appear to be any published accounts of the effects of mGluR modulation upon the GSH levels of microglia. It was postulated that, in addition to the known effects of microglial mGluRs, they may have the potential to affect the GSH system of microglia. This may include effects upon GSH synthesis, altering the rate of cystine uptake via x_c^- (Berent-Spillson and Russell 2007), or the expression of glutamate-cysteine ligase (GCL) (Sagara and Schubert 1998; Sahin *et al.* 2006), both of which are potentially rate-limiting steps in the GSH synthesis pathway. Increased or decreased GSH synthesis would likely affect intracellular GSH levels and therefore have implications for the fate of the cell. Alternatively, mGluR stimulation may have direct, GSH-independent effects upon mitochondrial ROS production, for example via the production of the antiapoptotic protein Bcl-2, as shown in HT22 cells (Sahin *et al.* 2006). Such an effect would reduce the demand upon GSH as an antioxidant, preserving cellular GSH for continual protection.

In addition to the effects upon microglial GSH content, the effects of mGluR modulation upon glutamate release and nitric oxide (NO) production (measured by quantification of nitrate and nitrite, stable NO metabolites) were examined, and preliminary investigations were carried out into the effects of mGluR modulation upon microglial glutamate transporter messenger ribonucleic acid (mRNA) expression. The effects of iGluR antagonists upon microglial GSH content and glutamate release were also investigated, to ascertain the impact of microglial iGluR expression upon glutamate/cystine balance and GSH levels.

In cells of the BV-2 microglial cell line, the use of specific antagonists showed that mGluR1α stimulation increased intracellular GSH levels, while mGluR5 stimulation decreased GSH levels. The effects of the same antagonists upon glutamate release were less striking. In accordance with this, modulation of the x_c^- transporter system had little effect upon group I mGluR-mediated alterations in GSH levels, and in preliminary experiments group I mGluR compounds did not appear to have effects upon x_c^- mRNA expression. It is proposed that mGluR1 and mGluR5 may have opposing effects upon certain aspects of the behaviour of microglia, and perhaps different effects upon neurotoxicity or neuroprotection.

In N9 microglial cells, the group III mGluR agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) decreased GSH levels. This compound has also been shown to enhance microglial neuroprotection (Taylor *et al.* 2003; Pinteaux-Jones *et al.* 2008), and explanations were sought to explain these findings concurrently. Group III mGluR stimulation may promote GSH consumption through its utilisation as an antioxidant, thus detoxifying reactive oxygen species more efficiently and protecting neurones from oxidative stress. Alternatively, activation of group III mGluR may decrease the expression or activity of the x_c^- transporter, an effect observed with the group III agonist (RS)-4-phosphonophenylglycine ((RS)-PPG) in preliminary investigations into xCT mRNA expression in N9 cells. This would decrease the availability of cystine for GSH synthesis but reduce glutamate release and the likelihood of oxidative glutamate toxicity towards neurones. Finally, it was considered that group III mGluR stimulation may promote release of GSH, its oxidised form GSSG, or cysteinylglycine (or a conjugate thereof) which may be taken up by neurones to supply cysteine for neuronal GSH synthesis.

This appears to be the first demonstration of mGluR-mediated effects upon microglial GSH levels, showing that the effects of extracellular glutamate upon GSH levels may not be limited to direct effects upon glutamate transporters, and further highlighting the importance and therapeutic potential of microglial mGluRs.

5.2. The effects of ionotropic glutamate receptor antagonists upon GSH levels in BV-2 cells

BV-2 microglial cells were treated with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Honore *et al.* 1988) and the N-methyl-D-aspartate (NMDA) receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine (MK-801) (Wong *et al.* 1986) prior to determination of intracellular GSH levels. Antagonists were tested alone, as well as in the presence of 10 μM and 1 mM glutamate. Figure 5.1A demonstrates that MK-801 had no effect, either in the absence or presence of exogenous glutamate.

Antagonism of non-NMDA receptors with CNQX also did not have a statistically significant effect at any glutamate concentration, although CNQX did appear to enhance the differences between the GSH content in the presence of different concentrations of glutamate. Thus, 10 μM glutamate was found to enhance the GSH content of BV-2 microglial cells from 37.3 ± 2.1 nmol.mg^{-1} protein to 46.2 ± 7.5 nmol.mg^{-1} protein, but in the presence of CNQX, 10 μM glutamate enhanced the GSH content from 25.4 ± 5.9 nmol.mg^{-1} protein to 51.3 ± 12.1 nmol.mg^{-1} protein, which was found to be statistically significant. Likewise, the difference between the GSH content of cells exposed to 10 μM glutamate and those exposed to 1 mM glutamate was not significant, whilst the difference between the effects of 10 μM glutamate + CNQX, and 1 mM glutamate + CNQX was statistically significant. Neither CNQX nor MK-801 had any effect upon glutamate levels in BV-2 microglial conditioned medium (fig. 5.1B).

5.3. The group III mGluR agonist L-AP4 causes a significant decrease in N9 GSH levels

The effects of specific mGluR agonists upon GSH levels in N9 cells were investigated. The group III mGluR agonist L-AP4 caused a 49.1 ± 2.9 % decrease in the GSH content of N9 microglial cells (fig. 5.2). In addition, slight decreases in N9

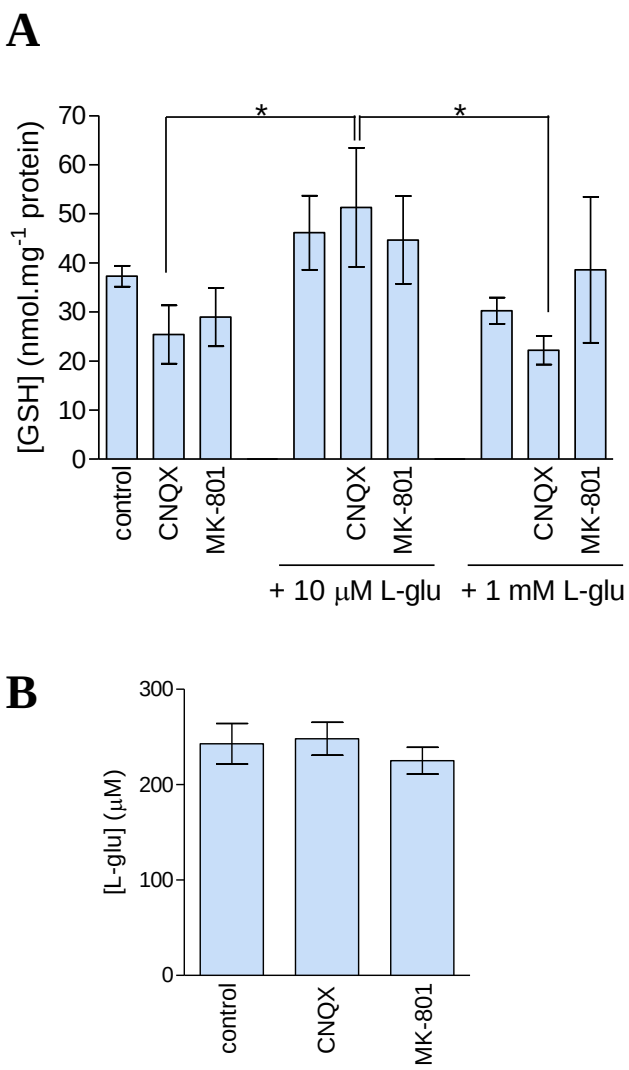


Figure 5.1. *The effect of glutamate receptor antagonists upon (**A**) BV-2 microglial GSH levels and (**B**) L-glutamate levels in conditioned medium. BV-2 microglia were cultured in the presence of CNQX (20 μM) or MK-801 (10 μM) for 2 hours before the addition of L-glutamate (10 μM or 1 mM where indicated). After 24 hours, L-glutamate levels in culture medium were determined and GSH levels were measured by reverse-phase HPLC.* ***A****, Data represent the mean ± s.e.m. of three (all CNQX, MK-801 conditions), five (10 μM glu), seven (1 mM glu) or fifteen (control) experiments, each consisting of two replicates per condition. Results in the absence of exogenous L-glutamate were analysed with a one-way ANOVA (p=0.08; n.s.), and the entire data set was analysed with a two-way ANOVA which showed that overall, exogenous L-glutamate had a significant effect upon GSH levels (p=0.0023), while the antagonists did not (p=0.6125), nor was there any significant interaction between the two variables (p=0.3695). Bonferroni's post-test was used to compare identical treatments in the presence of different L-glutamate concentrations (*p<0.05 as indicated, in all other cases p>0.05).* ***B****, Data represent the mean ± s.e.m. of three independent experiments, each consisting of two replicates per conditions. Data were analysed with a one-way ANOVA (p=0.6393; n.s.).*

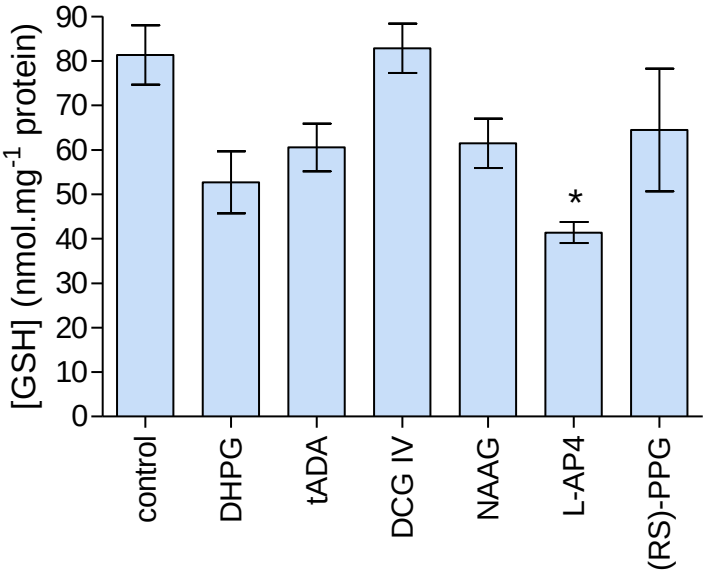


Figure 5.2. *The effects of specific mGluR agonists upon GSH levels in N9 microglial cells. N9 cells were cultured in the presence of DHPG (100 µM), tADA (250 µM), DCG IV (500 nM), NAAG (50 µM), L-AP4 (100 µM) or (RS)-PPG (100 µM) for 24 hours and intracellular GSH levels were determined by reverse-phase HPLC. Data represent the mean ± s.e.m. of three (DHPG, tADA, NAAG, L-AP4, (RS)-PPG), six (DCG IV) or eight (control) independent experiments, each consisting of at least two replicates per condition. Data were analysed with a one-way ANOVA ($p=0.0117$), with Dunnett's multiple-comparison post-test (*$p<0.05$ compared with control).*

GSH levels were demonstrated following 24 hours' incubation with the group I mGluR agonists (RS)-3,5-dihydroxyphenylglycine (DHPG) (35.3 ± 8.6 % decrease) and trans-azetidine-2,4-dicarboxylic acid (tADA) (25.6 ± 6.6 % decrease), the group II agonist N-acetyl-L-aspartyl-L-glutamic acid (NAAG) (24.4 ± 6.8 % decrease) and the group III agonist (RS)-PPG (20.8 ± 16.9 % decrease), although these were not shown to be statistically significant.

There was no significant effect of any mGluR agonists or antagonists upon the LPS + IFNγ-induced decrease in GSH levels in N9 cells (fig. 5.3A). However, L-AP4 slightly enhanced the decline in the GSH content of N9 cells caused by LPS + IFNγ (fig. 5.3A) and fraction V albumin (fig. 5.3B), and further experiments may find this to be a consistent, significant effect.

5.4. mGluR agonists have no effect upon GSH levels in BV-2 microglia

In contrast to the modulation detected in N9 cells (fig. 5.2), specific mGluR agonists had no effect upon the GSH levels in BV-2 microglial cells (fig. 5.4.).

5.5. mGluR1α antagonism decreases BV-2 GSH levels and mGluR5 antagonism increases BV-2 GSH levels, while antagonism of group II and III mGluRs has no effect

The effects of specific mGluR antagonists upon BV-2 GSH levels were investigated, in the absence and presence of exogenous glutamate. Whilst the group II mGluR antagonist (RS)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA) and the group III mGluR antagonist (S)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4) had no effect upon GSH levels, selective group I mGluR antagonism appeared to modulate BV-2 GSH levels (fig. 5.5). Treatment with 250 μM (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA), an mGluR1α-selective competitive antagonist led to a 32.5 ± 7.4 % decrease in BV-2 GSH levels, and the mGluR5-

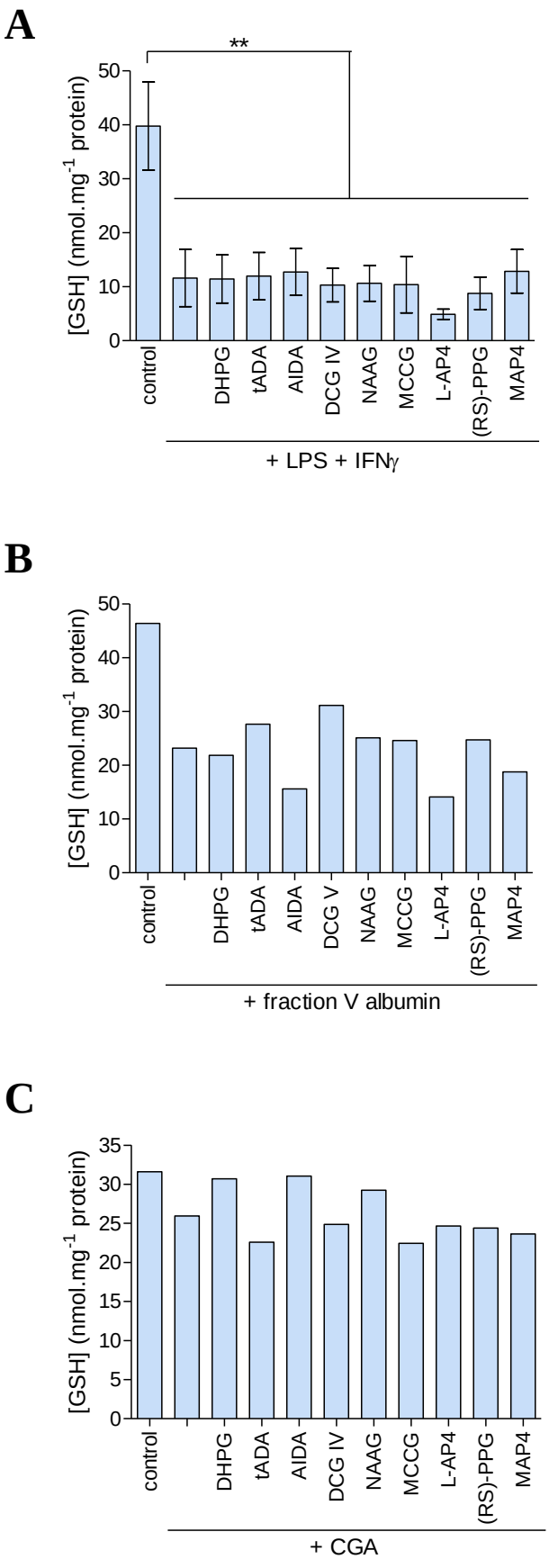


Figure 5.3. (legend overleaf)

Figure 5.3. (previous page) *Preliminary investigations into the effect of mGluR modulation upon activation-induced changes in GSH levels in N9 microglial cells. N9 cells were pre-incubated with DHPG (100 µM), tADA (250 µM), AIDA (250 µM), DCG IV (500 nM), NAAG (50 µM), MCCG (500 µM), L-AP4 (100 µM), (RS)-PPG (100 µM) or MAP4 (500 µM) for 30 minutes before the addition of (**A**) LPS (1 µg.ml^{-1}) + IFNγ (100 U.ml^{-1}), (**B**) fraction V albumin (2 mg.ml^{-1}) or (**C**) CGA (500 nM). Cells were then cultured for 24 hours before intracellular reduced glutathione (GSH) levels were determined by reverse-phase HPLC. **A**, Data represent the mean ± s.e.m. of two independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA (p=0.0012), with Tukey's post-test. All treatments were found to significantly decrease GSH levels compared with control (***p<0.001), but no mGluR compounds were found to significantly alter the LPS + IFNγ-induced decline in GSH content. **B**, **C**, Data represent the results of one experiment, consisting of two replicates per condition.*

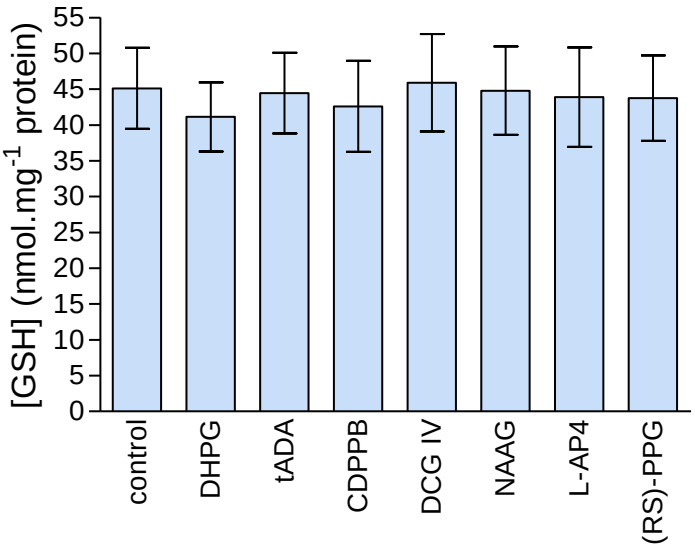


Figure 5.4. *The effects of specific mGluR agonists upon BV-2 microglial GSH levels. BV-2 cells were cultured in the presence of DHPG (100 µM), tADA (250 µM), CDPPB (500 nM), DCG IV (500 nM), NAAG (50 µM), L-AP4 (100 µM) or (RS)-PPG (100 µM) for 24 hours, and GSH levels were determined by reverse-phase HPLC. Data represent the mean ± s.e.m. of three independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA (p=0.9996, n.s.).*

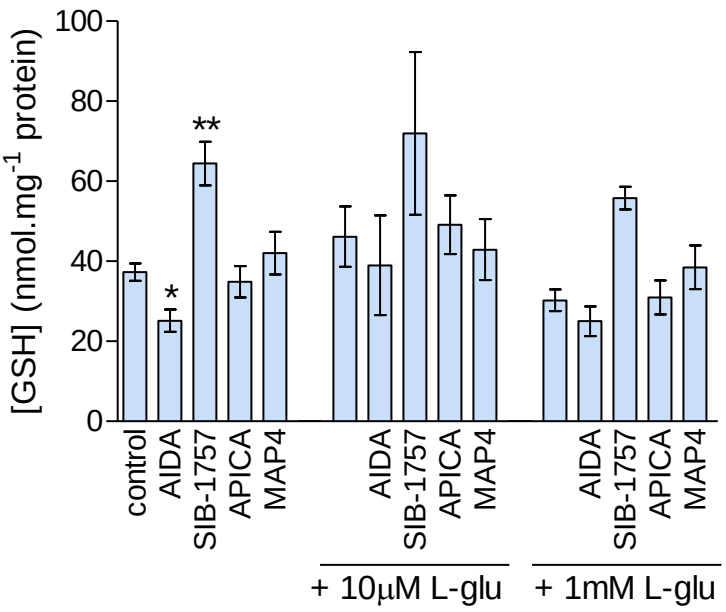


Figure 5.5. *The effect of specific mGluR antagonists, in the absence and presence of elevated L-glutamate levels, upon BV-2 microglial GSH levels. BV-2 microglia were cultured in the presence of AIDA (250 μM), SIB-1757 (50 μM), APICA (200 μM) or MAP4 (500 μM) for 2 hours before the addition of L-glutamate (10 μM or 1 mM). After 24 hours, GSH levels were determined by reverse-phase HPLC. Data represent the mean ± s.e.m. of three (APICA; 10 μM glu + AIDA, SIB-1757, APICA, MAP4; 1 mM glu + AIDA, SIB-1757, APICA, MAP4), five (SIB-1757, 10 μM glu), six (MAP4), seven (1 mM glu), nine (AIDA) or fifteen (control) independent experiments, each consisting of two replicates per condition. Results in the absence of exogenous L-glutamate were analysed with a one-way ANOVA ($p<0.0001$) with Dunnett's post-test to compare treatments with control (*$p<0.05$, **$p<0.01$). The entire data set was analysed with a two-way ANOVA which showed that overall, exogenous L-glutamate had a significant effect upon GSH levels ($p=0.0062$), as did the mGluR antagonists ($p<0.0001$), but there was no significant interaction between the two variables ($p=0.9704$). Bonferroni's post-test was used to compare identical conditions in the presence of different L-glutamate concentrations but no significant differences were found.*

selective non-competitive antagonist 6-methyl-2-(phenylazo)-3-pyridinol (SIB-1757) (50 µM) induced a 72.8 ± 14.6 % increase in GSH levels. Both the effect of glutamate and the effect of mGluR antagonists was significant, as tested with a two-way ANOVA. However, no differences were observed with each antagonist in the presence of different glutamate concentrations (0, 10 µM, 1 mM).

The effects of group I mGluR modulation upon GSH levels in BV-2 cells were further investigated (fig. 5.6). AIDA concentration-dependently decreased GSH levels, with 1 mM AIDA giving levels just 40.4 ± 3.6 % of control. There was no effect of adding the group I mGluR agonists DHPG or tADA upon the AIDA-induced decrease in GSH levels. The SIB-1757-mediated increase in BV-2 GSH levels did not appear to be modulated by increasing the L-cystine concentration in the medium or by the addition of the x_c^- transporter inhibitor aminopimelic acid (APA). In addition, neither 250 µM AIDA nor 50 µM SIB-1757 altered the LPS-induced increase in GSH levels. Surprisingly, another mGluR5-selective non-competitive antagonist, 3-((2-methyl-1,3-thiazol-4-yl)ethynyl)-pyridine (MTEP) (100 nM), was not found to have a significant effect upon GSH levels in BV-2 cells.

5.6. mGluR agonists and antagonists have no effect upon L-glutamate levels in BV-2 or N9 conditioned medium

Because evidence suggests a link between glutamate transport and GSH synthesis, mGluR agonists and antagonists were also tested for an effect on extracellular glutamate levels. Preliminary data suggested that there was little effect of mGluR modulation upon the glutamate concentration of BV-2 cell conditioned medium (fig. 5.7). Indeed, the effects of AIDA, DCG IV, NAAG, APICA, L-AP4, (RS)-PPG and MAP4 were not found to be statistically significant. Other compounds also appeared to be without effect, with the exception of the mGluR5-specific non-competitive antagonist SIB-1757, which may have caused a slight increase in extracellular glutamate levels.

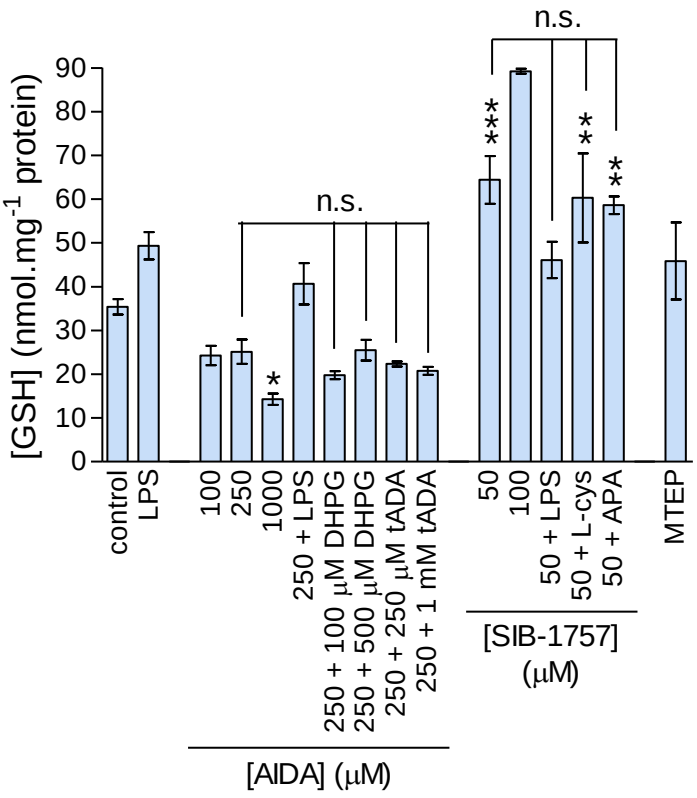


Figure 5.6. *Further investigation of the effect of group I-specific mGluR antagonists upon BV-2 microglial GSH levels. BV-2 cells were cultured in the presence of AIDA (100 – 100 µM), SIB-1757 (50 or 100 µM), LPS (1 µg.ml^{-1}), DHPG (100 or 500 µM), tADA (250 µM or 1 mM), supplementary L-cystine (to give a total of 1 mM), APA (2.5 mM), MTEP (100 nM), or a combination for 24 hours before GSH levels were determined by reverse-phase HPLC. Where DHPG or tADA were used, these were added to cells 30 minutes prior to AIDA treatment. Where L-cystine and APA were used, these were added 24 hours prior to SIB-1757 treatment. Data represent the mean ± s.e.m. of one (100 µM SIB-1757), five (LPS, 50 µM SIB-1757), nine (250 µM AIDA), twenty (control) or three (all other conditions) independent experiments, each consisting of two replicates per condition. All data except those for 100 µ M SIB-1757 were analysed using a one-way ANOVA ($p<0.0001$), with Tukey's multiple comparison post-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s., $p>0.05$, compared with control or as indicated).*

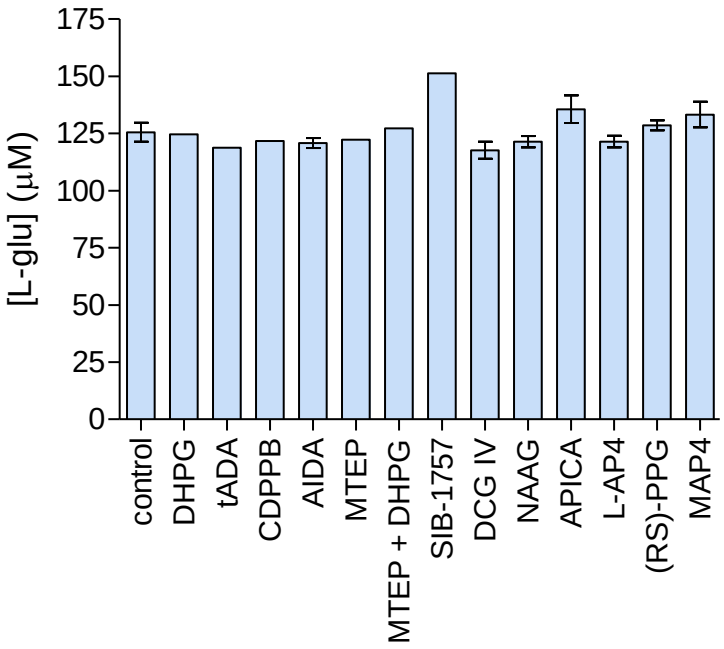


Figure 5.7. *The effect of mGluR modulation upon L-glutamate levels in BV-2 cell conditioned medium. BV-2 microglial cells were cultured in medium containing 10% FBS in the presence of DHPG (100 µM), tADA (250 µM), CDPPB (500 nM), AIDA (250 µM), MTEP (100 nM), SIB-1757 (50 µM), DCG IV (500 nM), NAAG (50 µM), APICA (200 µM), L-AP4 (100 µM), (RS)-PPG (100 µM), MAP4 (500 µM), or a combination for 24 hours, before L-glutamate levels in the supernatant were determined. Data represent the results of one experiment, consisting of two replicates per condition (DHPG, tADA, CDPPB, MTEP, MTEP + DHPG, SIB-1757), or the mean ± s.e.m. of two (AIDA, NAAG, L-AP4, (RS)-PPG), three (DCG IV, APICA), four (MAP4) or eight (control) independent experiments, each consisting of two replicates per condition. Data for control, AIDA, DCG IV, NAAG, APICA, L-AP4, (RS)-PPG and MAP4 were analysed with a one-way ANOVA (p=0.2414, n.s.).*

Specific mGluR agonists were also tested for effects on glutamate levels in N9 microglial conditioned medium (fig. 5.8). Whilst no compounds had a significant effect, slight increases were observed in the presence of the group II agonists DCG IV and NAAG and the group III agonists L-AP4 and (RS)-PPG (fig. 5.8). In particular, L-AP4 increased the glutamate concentration by 63.2 ± 34.2 %, although this was not significant due to the variability of the data. In addition, there was no significant effect of any mGluR agonists or antagonists upon the LPS + IFNγ-induced increase in glutamate levels in N9 conditioned medium (fig 5.9).

5.7. High concentrations of the mGluR5 antagonist SIB-1757 increase L-glutamate levels in BV-2 conditioned medium, but at this level SIB-1757 is toxic to BV-2 cells

Because SIB-1757 and AIDA had effects upon GSH levels in BV-2 cells, and because SIB-1757 showed a slight tendency towards increasing glutamate levels in conditioned medium in preliminary experiments, the effects of these compounds upon extracellular glutamate levels were investigated in more detail. As demonstrated in figure 5.10A, treatment of cells with the mGluR5-specific antagonist SIB-1757 concentration-dependently increased the glutamate content of BV-2 conditioned medium. A concentration of 500 µM was necessary to cause a significant increase, approximately doubling the control glutamate concentration. SIB-1757 is an orange coloured compound. To check that the apparent enhancement of glutamate was not due to SIB-1757 autofluorescence, identical fresh dilutions of the compound in SFM were assayed, and the fluorescence seen was not different from SFM alone. Zero point five percent dimethyl sulfoxide (DMSO), the final DMSO content of cell medium following treatment with 500 µM SIB-1757, did not have an effect upon glutamate levels in BV-2 conditioned medium. SIB-1757 and LPS were found to have an additive effect; SIB-1757 (50 µM) in combination with LPS gave a glutamate concentration 81.3 ± 19.5 % above control, while 50 µM SIB-1757 alone increased glutamate levels by 26.5 ± 5.0 % and LPS alone increased levels by 44.8 ± 13.0 %. This suggests that antagonism of microglial mGluR5 enhanced the LPS-induced increase in extracellular glutamate.

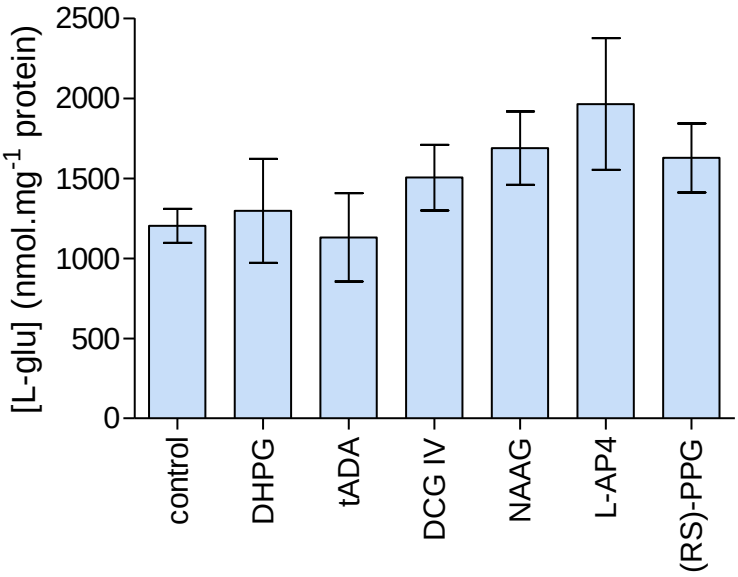


Figure 5.8. *The effects of specific mGluR agonists upon L-glutamate levels in N9 microglial cell conditioned medium. N9 cells were cultured in medium containing 5% newborn calf serum and exposed to DHPG (100 µM), tADA (250 µM) DCG IV (500 nM), NAAG (50 µM), L-AP4 (100 µM) or (RS)-PPG (100 µM) for 24 hours, before L-glutamate levels in the supernatant were determined. Data represent the mean ± s.e.m. of eight (control) or three (all other conditions) independent experiments, each typically consisting of two replicates per condition. Data were analysed with a one-way ANOVA (p=0.1411, n.s.).*

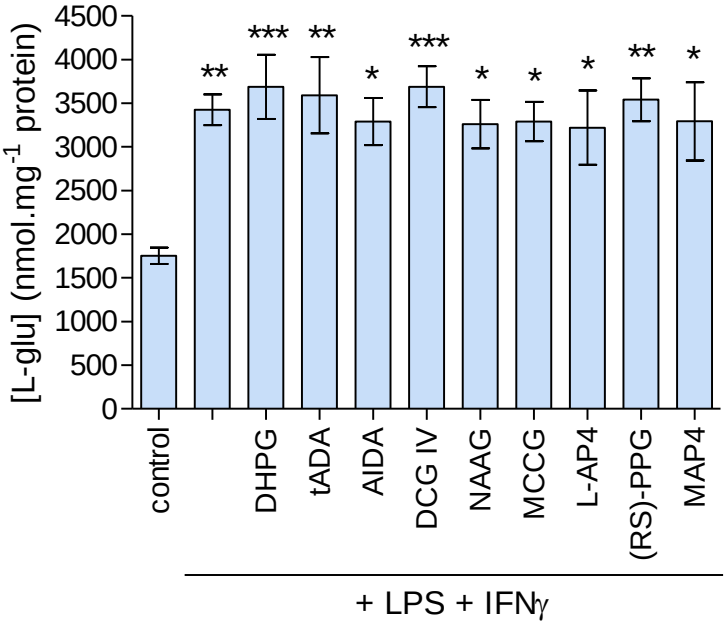


Figure 5.9. *The effect of mGluR modulation in the presence of LPS + IFNγ upon L-glutamate levels in N9 microglial cell conditioned medium. N9 cells were pre-incubated with DHPG (100 µM), tADA (250 µM), AIDA (250 µM), DCG IV (500 nM), NAAG (50 µM), MCCG (500 µM), L-AP4 (100 µM), (RS)-PPG (100 µM) or MAP4 (500 µM) for 30 minutes before the addition of LPS (1 µg.ml^{-1}) + IFNγ (100 U.ml^{-1}). Cells were then cultured for 24 hours before L-glutamate levels in the supernatant were determined. Data represent the mean ± s.e.m. of two independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA (p=0.0006), with Tukey's post-test (*p<0.05, **p<0.01, ***p<0.001 compared with control). There was no significant effect of any mGluR compound upon the LPS + IFNγ-induced enhancement of glutamate release.*

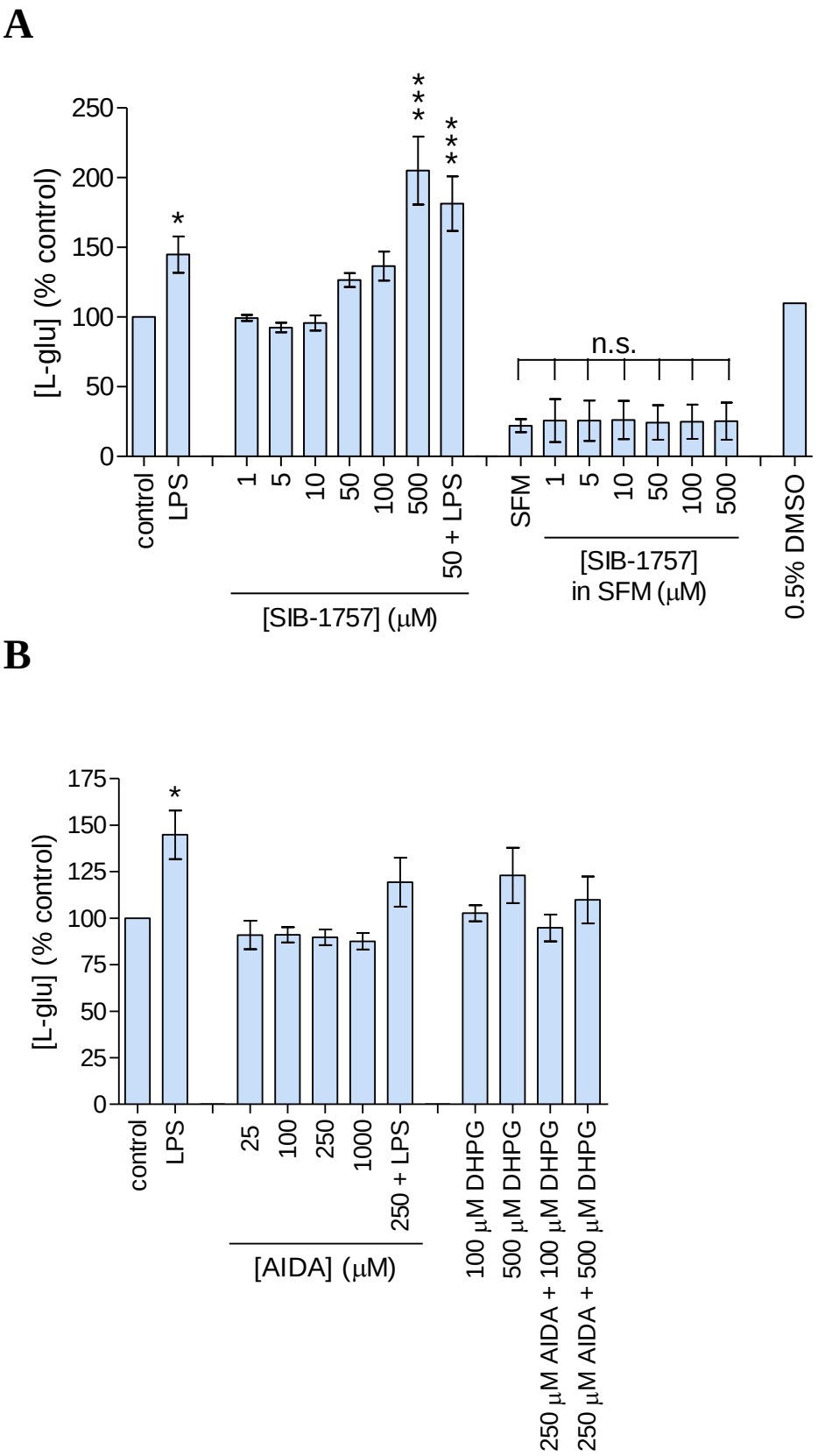


Figure 5.10. (legend overleaf)

Figure 5.10. (previous page) *The effects of AIDA and SIB-1757 upon L-glutamate levels in BV-2 cell conditioned medium. BV-2 microglial cells were cultured in SFM for 24 hours in the presence of LPS (1 μg.ml^{-1}), SIB-1757 (1 – 500 μM), DMSO (0.5%), AIDA (25 – 1000 μM), DHPG (100 or 500 μM), or a combination, before L-glutamate levels of conditioned medium were determined. Data are expressed as % control due to variation between absolute values. Control [L-glutamate] in this experiment was 127.7 ± 17.7 μM.* ***A****, Data represent the mean ± s.e.m. of one (DMSO), two (all concentrations of SIB-1757 in SFM), three (1 μM, 5 μM, 10 μM, 100 μM, 500 μM SIB-1757) or four (control, LPS, 50 μM SIB-1757, 50 μM SIB-1757 + LPS, SFM) independent experiments. All data except those for DMSO were analysed with a one-way ANOVA (p<0.0001) with Tukey's multiple-comparison post tests (*p<0.05, ***p<0.001, compared with control; n.s., not significant as indicated).* ***B****, Data represent the mean ± s.e.m. of two (25 μM AIDA), three (100 μM AIDA, 1000 μM AIDA, 100 μM DHPG, 500 μM DHPG, 250 μM AIDA + 100 μM DHPG, 250 μM AIDA + 500 μM DHPG) or four (control, LPS, 250 μM AIDA, 250 μM AIDA + LPS) independent experiments. Data were analysed with a one-way ANOVA (p=0.0029) with Tukey's multiple-comparison post tests (*p<0.05 compared with control).*

In contrast, the mGluR1α antagonist AIDA had no effect upon glutamate levels in BV-2 microglial conditioned medium (fig. 5.10B). However 250 μM AIDA in combination with LPS gave a glutamate concentration 19.4 ± 13.1 % above control, which was not significant, whereas LPS alone caused a significant 44.8 ± 13.0 % increase in glutamate release. This suggests that AIDA may attenuate the LPS-induced glutamate increase, although the difference between results with LPS + AIDA and LPS alone was not found to be statistically significant.

BV-2 cell death was assessed by means of staining with Hoechst-33342 and propidium iodide (PI) and by measurement of lactate dehydrogenase (LDH) release. Figure 5.11A demonstrates that 500 μM SIB-1757, which induced significant increases in glutamate in conditioned medium, is toxic to BV-2 cells, causing 43.4 ± 10.7 % of cells to stain with PI, indicating compromised plasma membrane function and therefore cell death. By contrast, 50 μM SIB-1757 caused only 14.7 ± 4.3 % BV-2 cell death, compared to 5.3 ± 0.6 % of control cells which stained with PI. LPS (1 $\mu g.ml^{-1}$), AIDA (250 μM) and 0.5% DMSO were similarly non-toxic to BV-2 microglial cells. Determination of LDH levels in conditioned medium showed that 50 μM SIB-1757 did not significantly elevate LDH above control levels (fig. 5.11B), confirming that SIB-1757 is non-toxic at this concentration.

Supplementary cystine (to a total of 1 mM) in the medium and the x_c^- inhibitors aminoadipic acid (AAA) (2.5 mM) and APA (2.5 mM) were used to investigate whether the potentiation of LPS-induced glutamate release by SIB-1757 involves an effect upon the x_c^- transporter. As shown in figure 5.12, AAA significantly attenuated the increases in extracellular glutamate with LPS (1 $\mu g.ml^{-1}$) and LPS + SIB-1757, and there was a significant difference between LPS-induced glutamate release in the presence of supplementary cystine and in the presence of cystine and APA, as demonstrated previously (see chapter 4). However under all conditions, there was consistently a slightly higher level of glutamate in the presence of SIB-1757 compared with control cells, and in the presence of SIB-1757 + LPS compared with LPS-treated cells.

Inducible nitric oxide synthase (iNOS) expression and consequent NO release is often associated with microglial glutamate release and cell death. However,

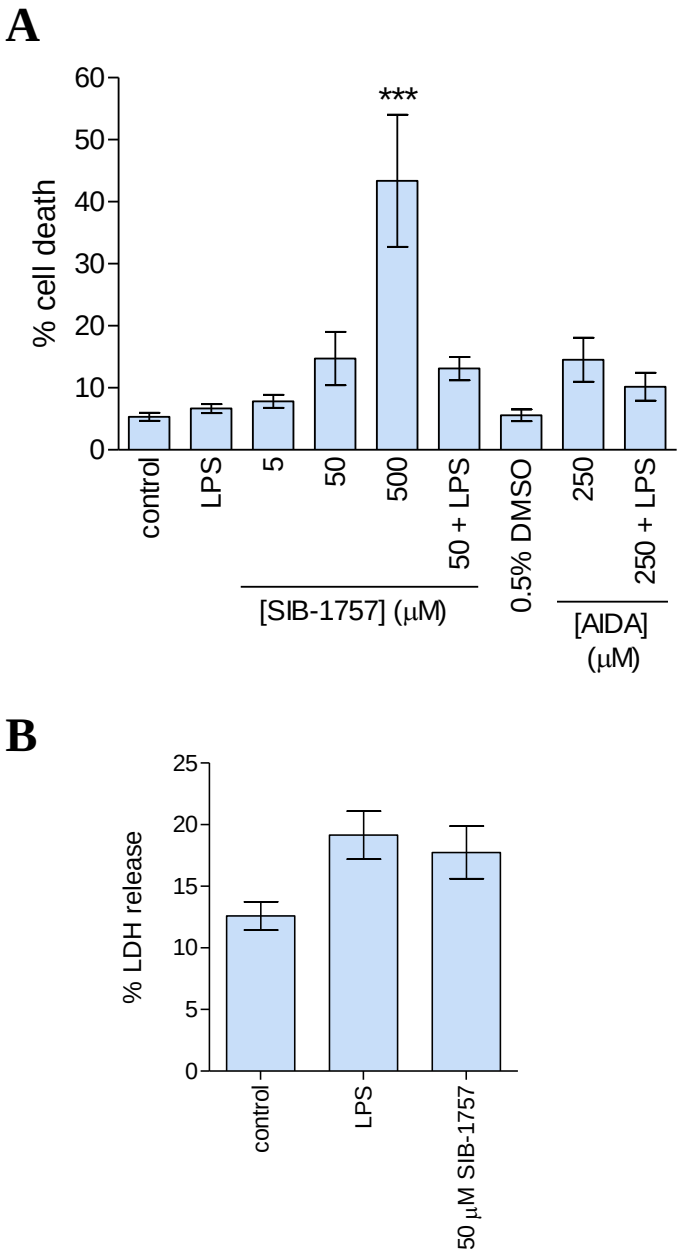


Figure 5.11. *BV-2 microglial cell death following exposure to LPS, SIB-1757 and AIDA. BV-2 cells were cultured for 24 hours in the presence of LPS (1 μg.ml^{-1}), SIB-1757 (5 – 500 μM), AIDA (250 μM), or a combination, and cell death was assessed by Hoechst-33342 and propidium iodide staining (**A**) or LDH release (**B**). **A**, Data represent the mean ± s.e.m. of three independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA ($p<0.0001$) with Tukey's multiple comparison post-test (***$p<0.001$ compared with control; all other conditions n.s.). **B**, Data represent the mean ± s.e.m. of four independent experiments, each consisting of two or three replicates per condition. Data were analysed with a one-way ANOVA ($p=0.0665$, n.s.).*

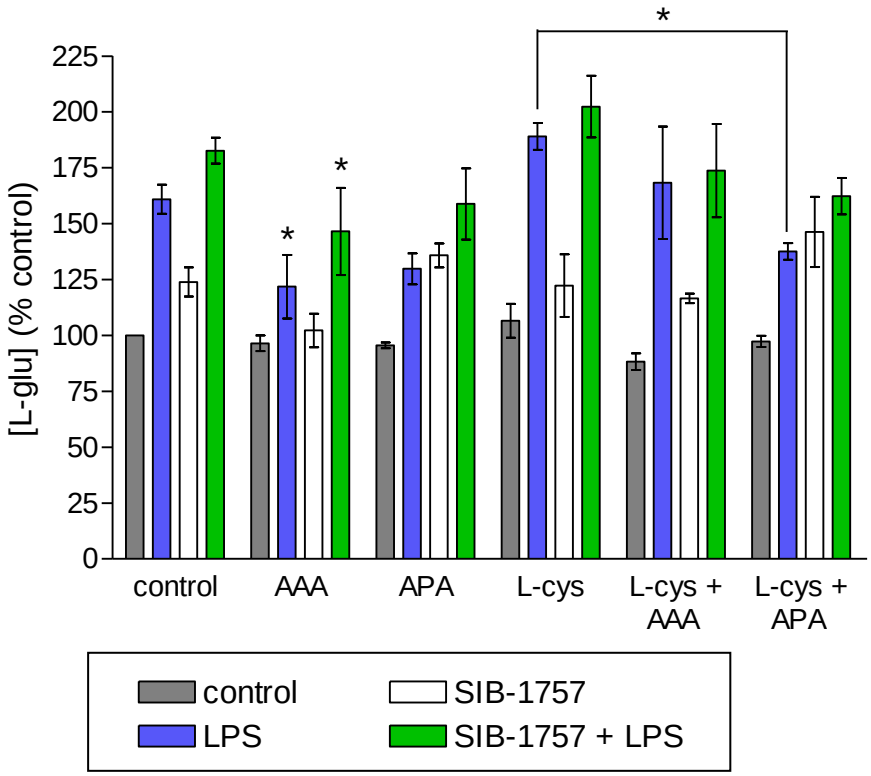


Figure 5.12. *The effect of pre-incubation with AAA, APA and L-cystine upon L-glutamate levels in conditioned medium from control BV-2 microglial cells and those treated with SIB-1757 and LPS. Plated BV-2 microglia were cultured in SFM containing AAA (2.5 mM), APA (2.5 mM), supplementary L-cystine (to a total of 1 mM), or a combination, for 24 hours before the addition of LPS (1 µg.ml^{-1}) and SIB-1757 (50 µM). After a further 24 hours L-glutamate levels in conditioned medium were determined. Data represent the mean ± s.e.m. of at least three independent experiments for each condition. Data are presented as % control to highlight trends as there was a high level of variability when values were expressed in µM Control [L-glutamate] in this experiment was 160.3 ± 20.4 µM. Data were analysed with a two-way ANOVA, which showed both the presence of AAA/APA/L-cystine and the exposure to LPS/SIB-1757 to have a significant effect upon L-glutamate levels (p=0.0003 and p<0.0001 respectively). There was no significant interaction between the variables (p=0.0845). Bonferroni's post test was carried out to compare the effects of AAA/APA/L-cystine under each pre-tested condition. (*p<0.05, compared to the same condition in the absence of AAA/APA/L-cystine, or as indicated).*

immunocytochemistry for iNOS in BV-2 cells in three independent experiments showed that cells treated with 50 μM SIB-1757 did not express iNOS any more highly than control cells (fig. 5.13).

5.8. mGluR-mediated modulation of transporter mRNA expression

In the search for an explanation for the effects of mGluRs agonists and antagonists upon the GSH content and the glutamate release/uptake balance of microglia, the effects of a number of mGluR agonists and antagonists upon glutamate transporter mRNA were investigated. Due to the effects of the group I antagonists AIDA and SIB-1757 in BV-2 microglial cells, these investigations were focussed on the effects of group I mGluRs.

As previously demonstrated in chapter 4 of this thesis, the expression of mRNA for EAAT1 and EAAT2 by microglia of the BV-2 and N9 cell lines was very low and close to the limit of detection, such that in some experiments mRNA could not be detected at all. The group I mGluR antagonists AIDA and SIB-1757 did not appear to have an effect upon the expression of mRNA for EAAT1 (fig. 5.14) or EAAT2 (fig. 5.15) by BV-2 microglial cells. In the case of EAAT2, mRNA was not detected in 7 of 9 samples of control cells, and EAAT2 mRNA could not be detected following treatment of BV-2 cells with any mGluR agonist or antagonist (fig. 5.15). Low levels of mRNA for EAAT2 were consistently detected in samples from N9 cells, and in a single experiment, there was no evidence for any effect of specific agonists or antagonists of group I, II or III mGluRs upon EAAT2 mRNA levels (fig. 5.16). In the case of primary microglial cells, preliminary data suggested there was no effect of group I mGluR-specific compounds upon the expression of EAAT2 mRNA (fig. 5.17B), but some compounds may have had effects upon the expression of EAAT1. As shown in figure 5.17A, the mGluR5-specific allosteric antagonist MTEP (100 nM) appeared to downregulate and MTEP in combination with the mGluR5 positive allosteric modulator 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) (500 nM) appeared to upregulate the expression of EAAT1

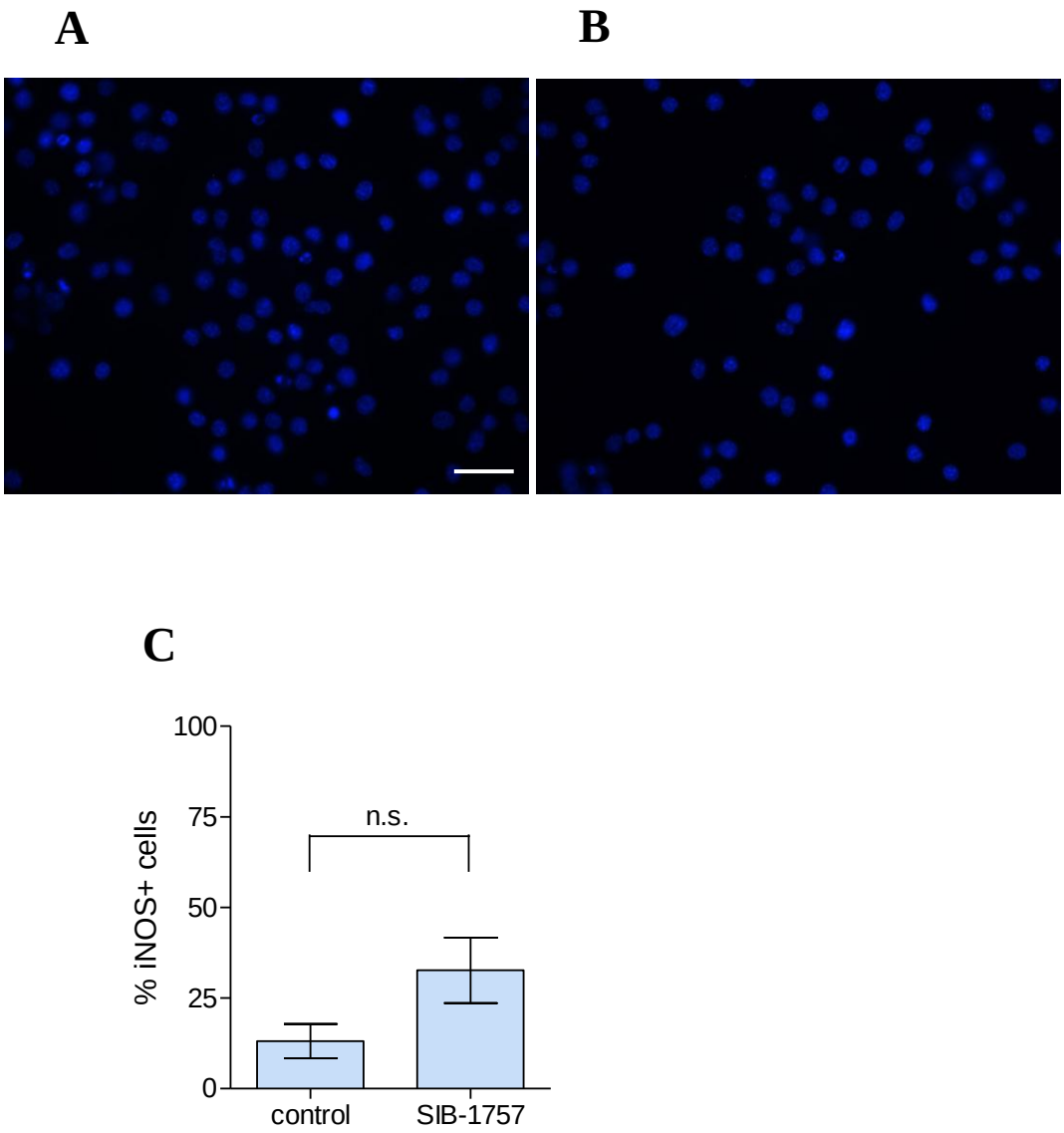


Figure 5.13. *iNOS expression by BV-2 microglia following 24 hours incubation with (**B**) or without (**A**) SIB-1757 (50 µM). Cells were fixed and iNOS was visualised using immunocytochemistry. Representative images are shown. LPS + IFNγ, used as a positive control in each experiment, consistently led to high levels of iNOS expression (>75 % cells postively stained). Magnification, 40X. Scale bar, 20 µm.* ***C****, Data represent the mean ± s.e.m. of three independent experiments, each consisting of two coverslips per condition, with three fields viewed per coverslip. Data were analysed with a Student's t test, which failed to demonstrate a significant difference in iNOS expression between control and SIB-1757-treated cells (p=0.0852).*

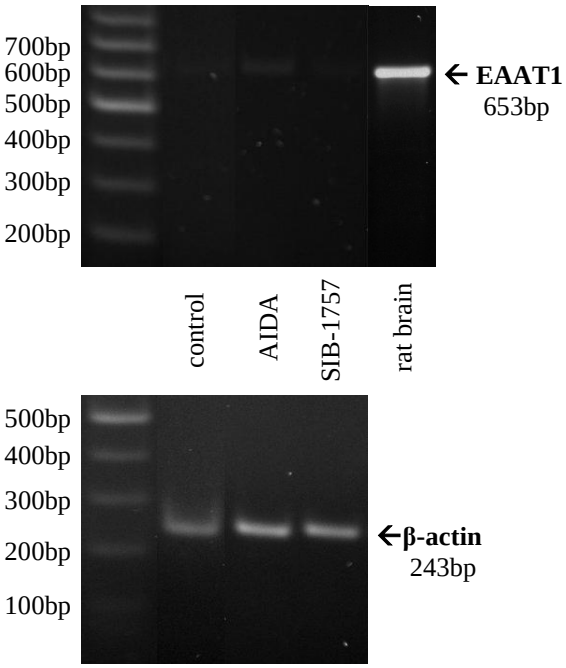


Figure 5.14. *The effect of group I mGluR antagonists upon EAAT1 mRNA expression by BV-2 microglia. Plated BV-2 cells were exposed to AIDA (250 μM) or SIB-1757 (50 μM) for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for EAAT1 and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Gels shown are representative of three (AIDA, SIB-1757) or seven (control) independent experiments.*

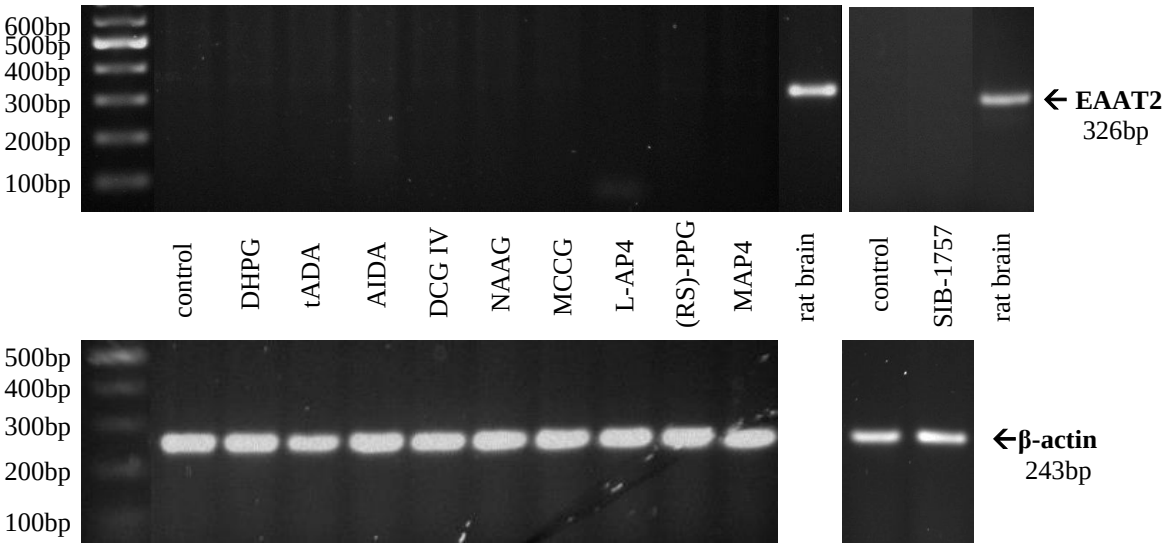


Figure 5.15. *The effect of mGluR modulation upon EAAT2 mRNA expression by BV-2 microglia. Plated BV-2 cells were exposed to DHPG (100 µM), tADA (250 µM), AIDA (250 µM), CDPPB (500 nM), MTEP (100 nM), SIB-1757 (50 µM), DCG IV (500 nM), NAAG (50 µM), MCCG (500 µM), L-AP4 (100 µM), (RS)-PPG (100 µM), or MAP4 (500 µM) for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for EAAT2 and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Gels shown are representative of three (AIDA, SIB-1757) or seven (control) independent experiments. For all other conditions, one experiment was conducted; EAAT2 mRNA expression was not detected under any condition.*

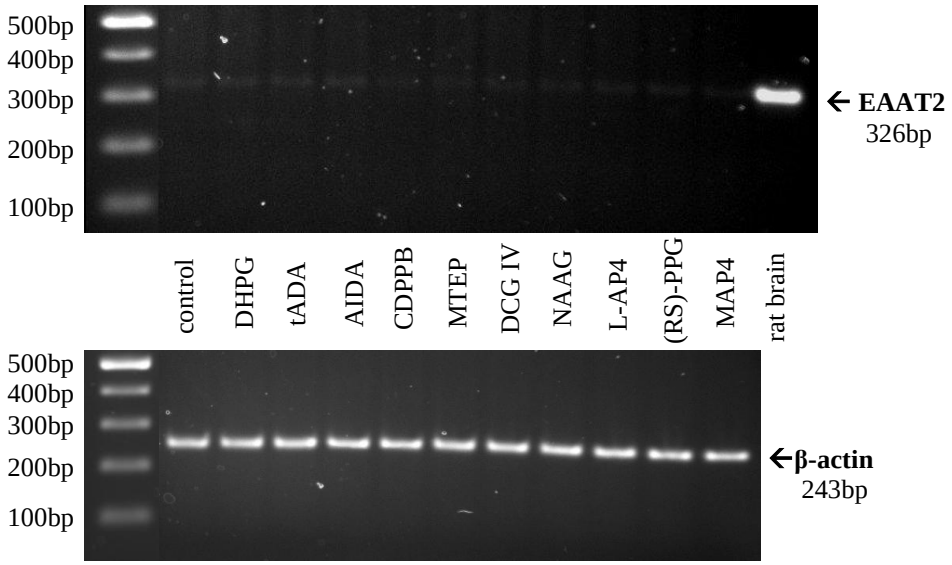


Figure 5.16. *Preliminary investigation into the effect of mGluR modulation upon EAAT2 mRNA expression by N9 microglial cells. Plated N9 microglia were exposed to DHPG (100 µM), tADA (250 µM), AIDA (250 µM), CDPPB (500 nM), MTEP (100 nM), DCG IV (500 nM), NAAG (50 µM), L-AP4 (100 µM), (RS)-PPG (100 µM), or MAP4 (500 µM) for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for xCT and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Data represent the results of one experiment.*

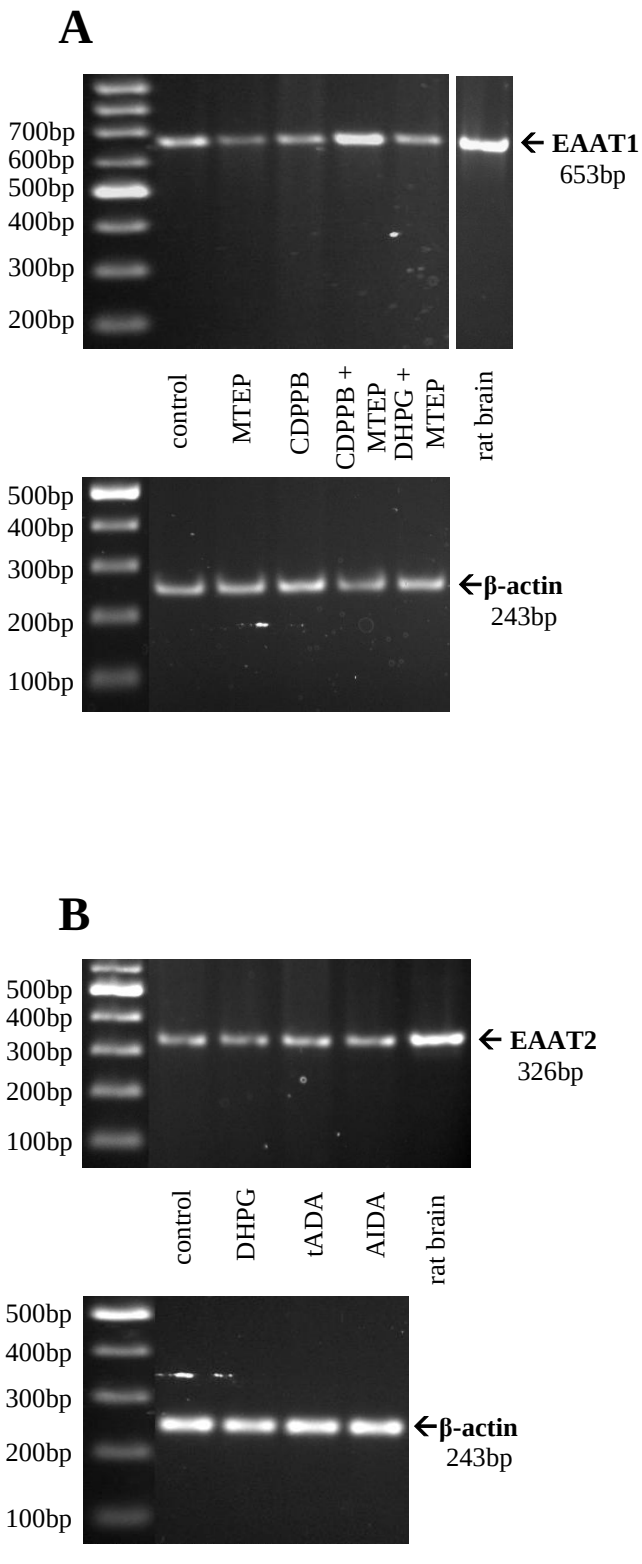


Figure 5.17. (legend overleaf)

Figure 5.17. (previous page) *Preliminary investigation into the effect of group I mGluR modulation upon (**A**) EAAT1 and (**B**) EAAT2 mRNA expression by primary microglial cells. Plated microglia were exposed to DHPG (100 µM), tADA (250 µM), AIDA (250 µM), CDPPB (500 nM), MTEP (100 nM), or a combination, for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for EAAT1 (**A**), EAAT2 (**B**) and β-actin (**A**,**B**), according to optimised conditions (35, 35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Data represent the results of one experiment.*

mRNA. As these data derive from a single experiment, further replicates would be required to substantiate these findings.

Primary microglial cells, and microglia of the BV-2 and N9 cell lines, were consistently found to express mRNA for xCT, the specific subunit of the x_c^- transporter (figs. 5.18, 5.19 and 5.20, and previously shown in chapter 4). The effects of specific group I mGluR agonists and antagonists upon xCT mRNA expression in primary microglia was investigated. The group I mGluR antagonist AIDA appeared to slightly increase xCT mRNA, although the data is derived from just one experiment (fig. 5.18). In an attempt to verify this finding, the effects of AIDA and SIB-1757, the mGluR5-specific antagonist which was previously shown to increase the GSH content and glutamate release by BV-2 cells, were tested upon BV-2 xCT mRNA expression. However, data from three independent experiments failed to demonstrate an effect of either AIDA or SIB-1757 (fig. 5.19). In addition, in a single preliminary experiment, no striking effect of any of a number of specific group I, II and III mGluR agonists and antagonists was found (fig. 5.19). However, in the case of N9 cells, data from a single experiment suggested that the expression of mRNA for xCT may decrease following treatment with the group II mGluR agonist NAAG (50 μM), the group III agonist (RS)-PPG (100 μM) or the group III antagonist MAP4 (500 μM) (fig. 5.20).

5.9. Consistent elevation of nitrate and nitrite release from N9 microglial cells by the group I mGluR agonist DHPG

To complement the data relating to GSH content and glutamate levels in conditioned medium, nitrate/nitrite release was measured following treatment of N9 microglial cells with specific mGluR agonists, as an indication of iNOS activity. As demonstrated in figure 5.21, none of the agonists tested significantly altered nitrate/nitrite levels in N9 conditioned medium, although the group I mGluR agonist DHPG caused a 72.1 ± 32.9 % increase. For comparison, under the same conditions, LPS + IFNγ treatment led to nitrate/nitrite levels 30-fold higher than control (fig. 3.3). Preliminary measurements of nitrate/nitrite release in the presence of

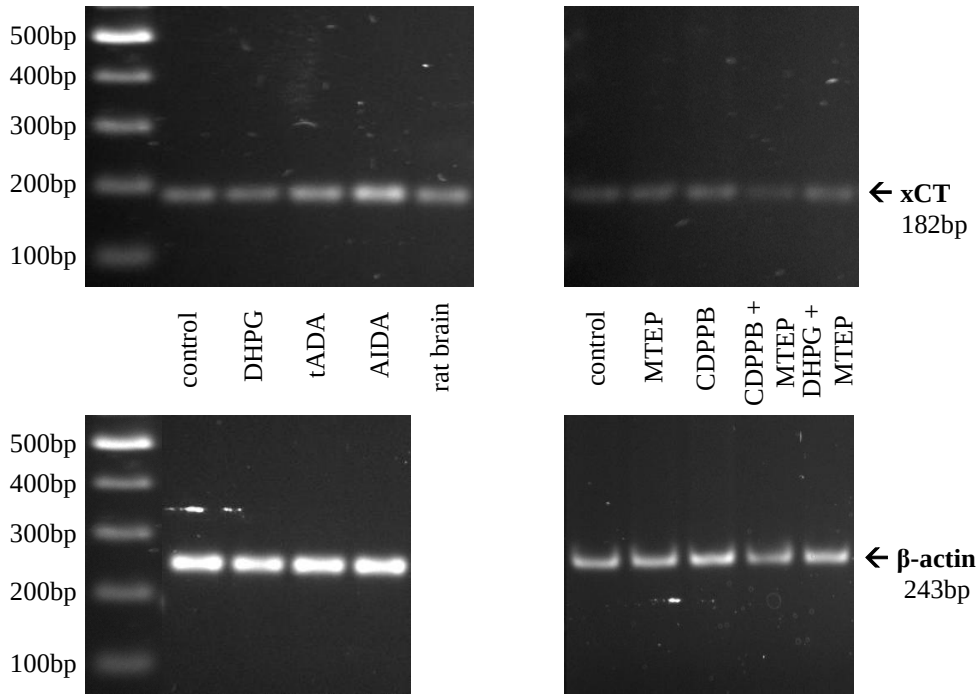


Figure 5.18. *Preliminary investigation into the effect of group I mGluR modulation upon xCT mRNA expression by primary microglial cells. Plated microglia were exposed to DHPG (100 µM), tADA (250 µM), AIDA (250 µM), CDPPB (500 nM), MTEP (100 nM), or a combination, for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for xCT and β-actin, according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Data represent the results of one experiment.*

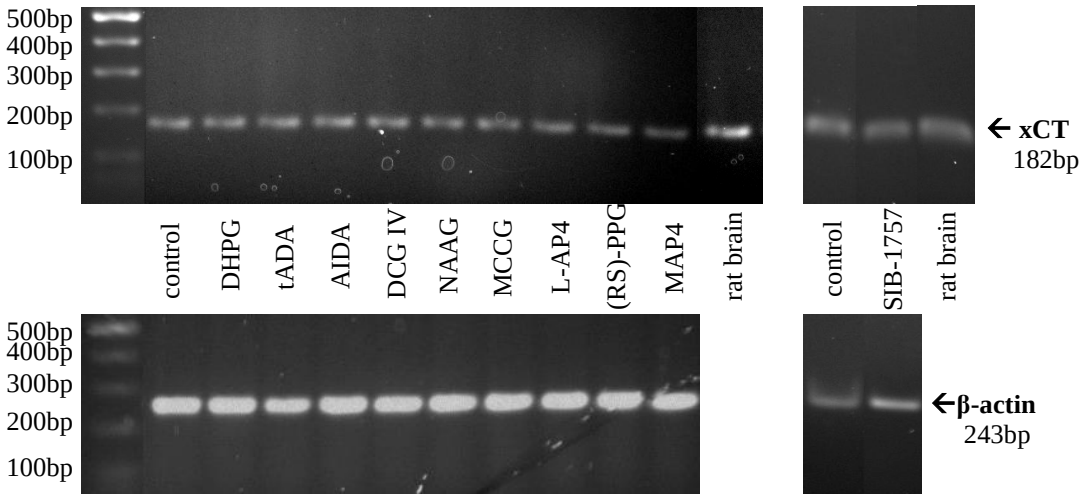


Figure 5.19. *Preliminary investigations into the effect of mGluR modulation upon xCT mRNA expression by BV-2 microglial cells. Plated BV-2 microglia were exposed to DHPG (100 µM), tADA (250 µM), AIDA (250 µM), CDPPB (500 nM), MTEP (100 nM), SIB-1757 (50 µM), DCG IV (500 nM), NAAG (50 µM), MCCG (500 µM), L-AP4 (100 µM), (RS)-PPG (100 µM), or MAP4 (500 µM) for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for xCT and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. In most cases, data represent the results of one experiment, except under control conditions or in the presence of AIDA or SIB-1757, where, data represent the results of three (AIDA, SIB-1757) or eight (control) independent experiments, and representative gels are shown.*

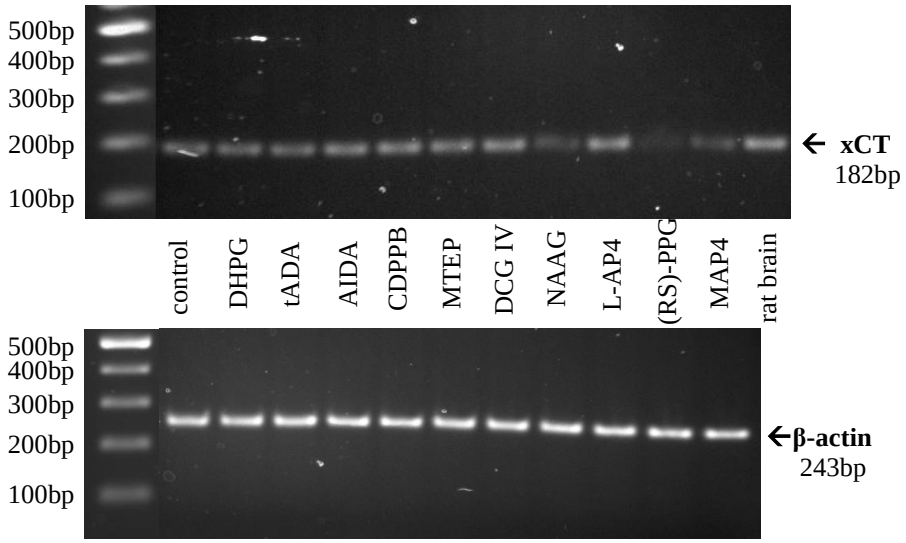


Figure 5.20. *Preliminary investigation into the effect of mGluR modulation upon xCT mRNA expression by N9 microglial cells. Plated N9 microglia were exposed to DHPG (100 µM), tADA (250 µM), AIDA (250 µM), CDPPB (500 nM), MTEP (100 nM), DCG IV (500 nM), NAAG (50 µM), L-AP4 (100 µM), (RS)-PPG (100 µM), or MAP4 (500 µM) for 24 hours before extraction of RNA. Following reverse-transcription, PCR was performed for xCT and β-actin according to optimised conditions (35 and 22 cycles respectively; see Materials and Methods for further details). PCR products were run on 1% agarose gel with ethidium bromide, and bands visualised under UV light. mRNA extracted from whole rat brain was run as a positive control. Data represent the results of one experiment.*

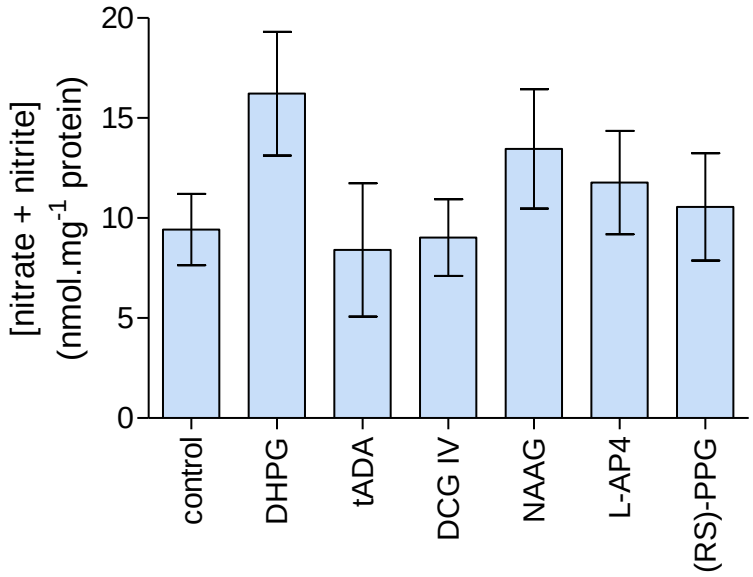


Figure 5.21. *The effect of specific mGluR agonists upon nitrate and nitrate levels in N9 microglial conditioned medium. N9 cells were cultured in the presence of DHPG (100 µM), tADA (250 µM), DCG IV (500 nM), NAAG (50 µM), L-AP4 (100 µM) or (RS)-PPG (100 µM) for 24 hours before nitrate and nitrite levels in conditioned medium were determined. Data represent the mean ± s.e.m. of at least three independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA (p=0.4349, n.s.).*

compounds associated with microglial activation in combination with mGluR agonists and antagonists were also carried out (fig. 5.22). DHPG consistently elevated the nitrate/nitrite levels in N9 microglial conditioned medium in the presence of LPS + IFNγ (fig. 5.22A), fraction V albumin (fig. 5.22B) and CGA (fig. 5.22C). Interestingly, in the presence of LPS + IFNγ, DHPG had the effect of elevating nitrate/nitrite levels by 264.6 ± 36.9 $nmol.mg^{-1}$ protein, while in non-activated cells the difference between the nitrate/nitrite levels of conditioned medium from control and DHPG-treated cells was just 6.8 ± 3.1 $nmol.mg^{-1}$ protein.

5.10. Discussion

In the previous chapter, glutamate was shown to affect microglial GSH levels and to modulate glutamate uptake/release balance. The most direct way in which these effects may be mediated is through modulation of glutamate transporters, however since microglia have been shown to express iGluRs and mGluRs, alterations in glutamate levels would change the activation of these receptors and may have an impact upon GSH levels. The effects of agonists and antagonists of glutamate receptors upon GSH levels were therefore investigated. Studies upon glutamate release and nitrate/nitrite production and preliminary investigations of transporter expression were also carried out to complement the GSH data and give more of an insight into potential modulation mechanisms.

5.10.1. The effects of ionotropic glutamate receptors

Whilst the NMDA antagonist MK-801 was without effect, the AMPA/kainate antagonist CNQX (Honore *et al.* 1988) had a slight, but interesting effect upon GSH levels in the presence of different concentrations of glutamate. In the presence of CNQX, GSH levels in the presence of 10 μM glutamate were significantly higher than those in the absence of glutamate or in the presence of 1 mM glutamate; an effect not seen in the absence of CNQX. CNQX does not cross-react with mGluRs (Pin and Duvoisin 1995) or EAATs (Aprico *et al.* 2004; Wersinger *et al.* 2006), so this effect is attributable to its actions at iGluRs. *In vitro*, primary microglia and BV-

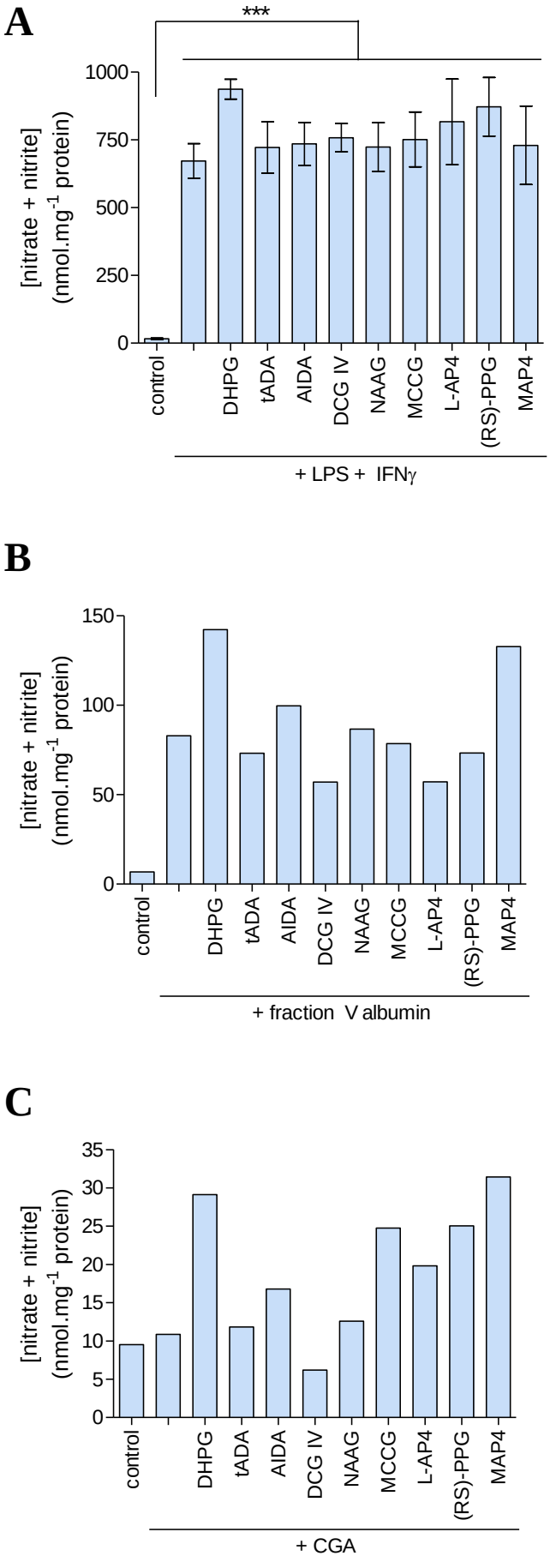


Figure 5.22. (legend overleaf)

Figure 5.22. (previous page) *Preliminary investigations upon the effect of mGluR modulation in the presence of microglial activators upon nitrate and nitrite levels in N9 microglial cell conditioned medium. N9 cells were pre-incubated with DHPG (100 µM), tADA (250 µM), AIDA (250 µM), DCG IV (500 nM), NAAG (50 µM), MCCG (500 µM), L-AP4 (100 µM), (RS)-PPG (100 µM) or MAP4 (500 µM) for 30 minutes before the addition of (**A**) LPS (1 µg.ml^{-1}) + IFNγ (100 U.ml^{-1}), (**B**) fraction V albumin (2 mg.ml^{-1}) or (**C**) CGA (500 nM). Cells were then cultured for 24 hours before nitrate and nitrite levels in conditioned medium were determined. **A**, Data represent the mean ± s.e.m. of two independent experiments, each consisting of two replicates per condition. Data were analysed with a one-way ANOVA ($p<0.0001$), with Tukey's post-test. All treatments were found to significantly elevate nitrate/nitrite levels compared with control (***$p<0.001$), but no mGluR compounds were found to significantly alter the LPS + IFNγ-induced nitrate/nitrite elevation. **B**, **C**, Data represent the results of one experiment, consisting of two replicates per condition.*

2 cells have been shown to express functional AMPA/kainate receptors (Noda *et al.* 2000; Mayer *et al.* 2001; Eun *et al.* 2004; Hagino *et al.* 2004; Christensen *et al.* 2006; Liu *et al.* 2006). In addition, microglia have been shown to possess AMPA/kainate receptor subunits *in vivo* during CNS development (Ong *et al.* 1996; Kaur *et al.* 2005), following transient forebrain ischaemia (Gottlieb and Matute 1997), and in multiple sclerosis (MS) tissue within active plaques (Newcombe *et al.* 2008), suggesting that AMPA/kainate receptor-mediated effects upon microglial GSH levels may be relevant under certain conditions *in vivo*.

The data presented here suggest that functional AMPA/kainate receptors may have a stabilising effect upon GSH levels in the presence of exogenous glutamate, as AMPA/kainate receptor inhibition with CNQX enhanced the differences between GSH levels at different glutamate concentrations. AMPA/kainate receptors may therefore have a role physiologically in maintaining microglial GSH levels in the event of altered extracellular glutamate levels. Indeed, microglial AMPA/kainate receptor subunit expression has been shown to be upregulated *in vivo* under pathological conditions (Gottlieb and Matute 1997; Newcombe *et al.* 2008) when extracellular glutamate levels may be elevated (Buryakova and Sytinsky 1975; Benveniste *et al.* 1984; Hagberg *et al.* 1985; Faden *et al.* 1989; Perry and Hansen 1990; Rothstein *et al.* 1990; Liu *et al.* 1991; O'Regan *et al.* 1997; Stover *et al.* 1997; Ritz *et al.* 2004; Homola *et al.* 2006). The intracellular signalling pathways through which AMPA/kainate receptor stimulation may stabilise GSH levels are currently unclear. However, microglial AMPA/kainate receptor stimulation may lead to the release of tumour necrosis factor α (TNFα) (Noda *et al.* 2000; Mayer *et al.* 2001; Hagino *et al.* 2004), which has been previously shown to have effects upon microglial EAAT expression (Persson *et al.* 2005), x_c^- activity (Piani and Fontana 1994; Sato *et al.* 1995), and GSH levels (Dopp *et al.* 2002; Persson *et al.* 2006).

5.10.2. The effect of group I metabotropic glutamate receptors

Here, effects of group I mGluR modulation were observed upon GSH content, glutamate release/uptake balance and potentially upon nitrate/nitrite production by microglial cells.

The mGluR5-specific non-competitive antagonist SIB-1757 (Varney *et al.* 1999) increased BV-2 GSH levels, and although it also dose-dependently increased glutamate release, this was only significant at concentrations which were toxic to the cells. At a non-toxic concentration (50 µM), SIB-1757 had a highly significant effect upon GSH levels but no significant effect upon the glutamate content of BV-2 conditioned medium. SIB-1757 had no effect upon the LPS-induced increase in GSH levels but appeared to potentiate the LPS-induced increase in glutamate release. Surprisingly, a second mGluR5-specific non-competitive antagonist, MTEP (Cosford *et al.* 2003b) did not significantly increase BV-2 GSH levels. The concentration used (100 nM) was selected as it is an order of magnitude above *in vitro* K_d and IC_{50} values but well below that at which non-selective binding occurs (Cosford *et al.* 2003b). However, recent studies have utilised higher concentrations of MTEP (Lindstrom *et al.* 2008; Salah and Perkins 2008) and since only one concentration was tested in this study, the results are not conclusive.

Primary microglia express functional mGluR5 (Biber *et al.* 1999; Pinteaux-Jones 2007; Byrnes *et al.* 2009), and the striking effect of the specific, non-competitive mGluR5 antagonist SIB-1757 upon BV-2 cells here suggests that these cells also express mGluR5. A recent study found that activation of microglial mGluR5 with (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) inhibited a number of aspects of LPS-induced microglial activation, including reactive oxygen species (ROS) and NO production, microglial proliferation, TNFα release, expression of the phagocytosis-related protein galectin-3 and neurotoxicity (Byrnes *et al.* 2009). mGluR5-mediated protection appeared to be through the prototypical group I mGluR signalling pathway, involving phospholipase C (PLC), increases in intracellular Ca^{2+} and PKC. The effects of SIB-1757 seen here do seem to complement this study, as inhibition of mGluR5 slightly potentiated LPS-induced glutamate release, suggesting that a tonic mGluR5 stimulation was limiting glutamate release. The increase in GSH levels following SIB-1757 treatment may be a consequence of other effects, such as altered glutamate transport, or a direct effect of blocking the mGluR5 signal transduction pathway. In BV-2 cells, SIB-1757 and LPS both increased microglial GSH levels, but their effects did not appear to be additive.

The mGluR1α-specific antagonist AIDA (Pellicciari *et al.* 1995; Moroni *et al.* 1997) dose-dependently decreased GSH levels in BV-2 microglial cells, with GSH levels following treatment with 1 mM AIDA only 40% of control levels. In contrast, AIDA did not affect glutamate levels in BV-2 conditioned medium, but showed a trend towards attenuation of LPS-induced increases in both GSH content and glutamate release. The small effects of AIDA observed here are consistent with low-level receptor expression (Geurts *et al.* 2003; Byrnes *et al.* 2009), although the marginal significance of 250 μM AIDA and greater effect of 1 mM upon GSH levels could reflect cross-reactivity of the compound with mGluR5 (Moroni *et al.* 1997), or with mGluR2, where it acts as a low-affinity agonist (Moroni *et al.* 1997). This does however seem unlikely since SIB-1757 caused an increase rather than a decrease in GSH levels, and selective group II mGluR agonists had no effect upon GSH levels (fig. 5.4).

Modulation of group I mGluRs has been shown to have effects upon GSH levels in other CNS-associated cell types, lending support to the apparent effects of group I-selective compounds upon microglial GSH levels. Glutamate toxicity in the HT22 neuronal cell line was reduced by DHPG and potentiated by AIDA (Luo and DeFranco 2006) and group I mGluR stimulation rescued glutamate-induced decreases in GSH (Sagara and Schubert 1998; Luo and DeFranco 2006). HT22 cells selected for resistance to glutamate toxicity had 5.3-fold increased mGluR1 expression, as well as increased vasoactive intestinal peptide (VIP) receptor 2 ($VPAC_2$) expression. Synergistic actions of these receptors when stimulated by glutamate and VIP respectively increased Bcl-2 expression, which increased intracellular GSH and thereby provided protection against oxidative glutamate toxicity (Sahin *et al.* 2006). DHPG protected against KA, oxygen/glucose deprivation and cystine starvation-induced GSH depletion in oligodendrocyte precursor cells (OPCs) in OPC-enriched cultures prepared from mixed glial cultures (Deng *et al.* 2004). DHPG also elevated GSH levels in striatal slices (Maccarrone *et al.* 2008).

It appears that the two group I mGluR subtypes have opposite effects upon GSH levels in BV-2 microglial cells. An effect of mGluR antagonists in the absence of specific agonists, and a lack of an effect of the group I mGluR agonists DHPG and

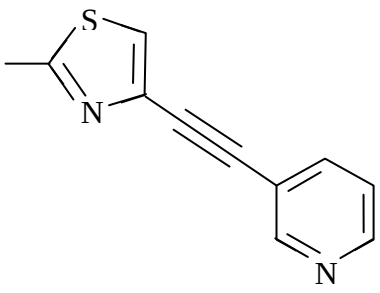
tADA and the mGluR5 positive allosteric modulator CDPPB suggest that the receptors are activated under control conditions. This may reflect the effect of the micromolar levels of glutamate present in the medium, of low levels of glutamate released by microglia, particularly BV-2 cells, under basal conditions (see chapter 4), or constitutive activation of these receptors (Prezeau *et al.* 1996; Pagano *et al.* 2000; Ango *et al.* 2001; Muhlemann *et al.* 2005). Thus, mGluR5 activation apparently decreases intracellular GSH levels, whilst activation of mGluR1α increases GSH levels.

There are a number of ways in which the effects of mGluR modulation upon GSH levels may be mediated. In terms of effects upon GSH synthesis, mGluRs could alter the expression or activity of EAATs, thus altering extracellular glutamate levels and affecting the activity of other glutamate receptors, as well as impacting the intracellular glutamate pool which drives cystine uptake via x_c^-. Signalling from mGluRs could also directly affect x_c^- expression or activity, thus affecting intracellular availability of cystine, which may be a limiting factor in GSH synthesis. Although here the antagonists only had small, non-significant effects upon extracellular glutamate levels, suggesting that the effects upon GSH levels are independent of glutamate transporter, the medium used in the glutamate assay was devoid of serum, cells were cultured in serum-containing medium (SCM) prior to GSH determination. Collation of control BV-2 medium samples tested throughout this study shows that SCM contains 98.8 ± 4.6 μM (n=10). Serum-free D-MEM does not contain any glutamate by formulation (Dulbecco and Freeman 1959). Therefore with serum-free medium there was likely to have been lower basal levels of mGluR activation, and less scope for the action of antagonists. This is supported by the fact that in the presence of LPS, which causes increased glutamate release and therefore would enhance mGluR activation, the antagonists had more pronounced effects.

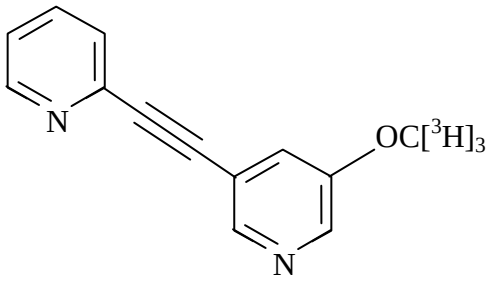
The expression of mRNA for EAAT1 and EAAT2 by the BV-2 and N9 microglial cell lines was very low, close to the limit of detection; data must therefore be interpreted with caution. However, neither AIDA nor SIB-1757 appeared to have a striking effect upon mRNA levels of either transporter in BV-2 cells, and initial investigations did not provide any evidence of modulation of expression by other specific agonists and antagonists of mGluRs in either cell line.

In primary microglial cells, preliminary experiments suggested that the non-competitive mGluR5 antagonist MTEP (Cosford *et al.* 2003b) may downregulate, and a combination of MTEP and a positive allosteric modulator of mGluR5, CDPPB (Lindsley *et al.* 2004; Kinney *et al.* 2005) may upregulate EAAT1 mRNA levels. This finding is at odds with studies in astrocytes, where stimulation of mGluR5 has been demonstrated to downregulate EAAT1 expression (Gegelashvili *et al.* 2000; Aronica *et al.* 2003). However, it is possible that different signalling pathways exist in different cell types. Clearly further experiments would be required to substantiate this finding, especially as CDPPB alone did not increase, and may have even slightly decreased the levels of mRNA for EAAT1. Allosteric interactions do however tend to be more complex than orthosteric interactions. Although the structure of CDPPB is notably different to that of MTEP and [^{3}H]-3-methoxy-5-(pyridin-2-ylethynyl)-pyridine ([^{3}H]-methoxy-PEPy), which are rather similar (fig. 5.23), both MTEP and CDPPB can antagonise [^{3}H]-methoxy-PEPy binding, although with markedly different affinities (Cosford *et al.* 2003b; Kinney *et al.* 2005). Recent data indicate that the binding of CDPPB and [^{3}H]-methoxy-PEPy is competitive (Chen *et al.* 2007), suggesting that the binding sites of CDPPB and MTEP may overlap. A positive cooperative interaction of glutamate and CDPPB is suggested as an explanation for the difference between the EC_{50} for potentiation of a response to glutamate by CDPPB and its much higher dissociation constant with respect to [^{3}H]-methoxy-PEPy (Kinney *et al.* 2005; Chen *et al.* 2007). The unexpected effects of a combination of MTEP and CDPPB compared with either compound alone may reflect a complex interaction between the binding of these two ligands to mGluR5 in the presence of glutamate (serum-containing medium contains ~100 µM glutamate) in the modulation of EAAT1 expression.

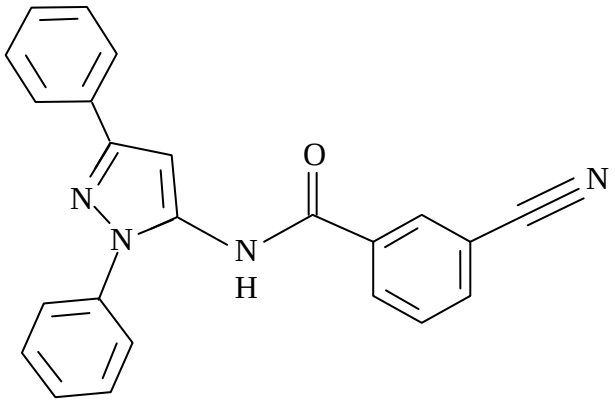
Alterations in the expression or activity of x_c^- do not appear to be responsible for the changes in GSH content of BV-2 microglia. The increase in GSH levels seen in the presence of the mGluR5 antagonist SIB-1757 was not altered in the presence of elevated extracellular cystine levels or by the x_c^- inhibitor APA, and its effects upon glutamate release were not modulated by supplementary cystine, AAA or APA. In addition, there did not appear to be any effects of group I mGluR agonists or antagonists upon xCT mRNA expression in N9 or BV-2 cell lines or in primary



MTEP



[^{3}H]-methoxy-PEPy



CDPPB

Figure 5.23. *The structures of the mGluR5 allosteric ligands 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]-pyridine (MTEP) (Cosford et al. 2003b), [^{3}H]-3-methoxy-5-(pyridin-2-ylethynyl)-pyridine ([^{3}H]-methoxy-PEPy) (Cosford et al. 2003a), and 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) (Lindsley et al. 2004): MTEP and [^{3}H]-methoxy-PEPy share similar structures, while that of CDPPB is somewhat different.*

microglial cells. Although AIDA has been reported to inhibit cystine uptake in rat striatal slices (Baker *et al.* 2002) and in dibutyryl-cyclic adenosine monophosphate-(dbcAMP-) treated astrocytes (Gochenauer and Robinson 2001), the concentration used (1 mM) in these studies may be sufficient to cause non-specific effects, such as mGluR2 agonism (Moroni *et al.* 1997).

The effects of mGluR activity upon GSH levels could also be due to effects upon the intracellular GSH synthesising enzymes, glutamate-cysteine ligase (GCL) or GSH synthase. GCL is the rate-limiting enzyme in GSH synthesis (Meister and Anderson 1983), therefore changes in its expression or activity are likely to impact upon cellular GSH levels. GCL upregulation has been demonstrated in a number of cell types, and was often associated with stressful stimuli, such as hydrogen peroxide (Ochi 1995), ROS (Ochi 1996), the lipid peroxidation product 4-hydroxy-2-nonenal (Liu *et al.* 1998b; Dickinson *et al.* 2002) and proinflammatory cytokines such as TNFα (Urata *et al.* 1996; Morales *et al.* 1997; Rahman *et al.* 1999; Takamura *et al.* 2006). Such conditions may also be associated with microglial activation. GCL upregulation has been demonstrated in a glioma cell line (Gomi *et al.* 1997), but has not so far been shown in microglia. It is possible that signalling pathways occurring following stimulation of mGluR1α or mGluR5 may upregulate or downregulate, respectively, microglial GCL expression or activity.

The effects of group I mGluR antagonists upon GSH levels in BV-2 microglia could be independent of GSH synthesis and simply be due to alteration of the rate of GSH consumption. SIB-1757 increased the GSH content of BV-2 cells, and it follows that the underlying stimulation of mGluR5 decreased the GSH content. However mGluR5 stimulation has also been shown to decrease the LPS-induced production of reactive oxygen and nitrogen species (Byrnes *et al.* 2009), and is therefore likely to diminish rather than enhance the demand upon GSH as an antioxidant.

The data here also suggest that the group I mGluR agonist DHPG (Ito *et al.* 1992; Brabet *et al.* 1995) may enhance nitrate/nitrite production by N9 microglial cells. DHPG alone caused a slight, but non-significant increase in nitrate/nitrite levels, and preliminary data showed a consistent potentiation of the effects of the microglial activators LPS + IFNγ, fraction V albumin and CGA by DHPG. This does not appear

to be in agreement with a recent study demonstrating an mGluR5-mediated inhibition of aspects of LPS-induced microglial activation, including NO production (Byrnes *et al.* 2009). This could be due to differences in receptor subtype expression. Byrnes *et al.* (2009) do not detect mGluR1α surface expression, whilst in N9 cells, mGluR1α and mGluR5 may be coexpressed (Pinteaux-Jones 2007), and may signal through different pathways, as appears to be the case in BV-2 cells. Even if mGluR1α is expressed at a lower level, this does not necessarily indicate that its effects are less potent than those of mGluR5, and there could be a dominant effect of mGluR1α in enhancing activation-induced NO production.

It therefore appears that, to some extent at least, the two subtypes of group I mGluRs, mGluR1α and mGluR5, may have distinct effects in microglia. Different responses following mGluR1 and mGluR5 activation have previously been demonstrated in a number of systems. When heterologously expressed in CHO cells, mGluR1α and mGluR5 both couple to extracellular signal-related kinase (ERK), but the mGluR1α pathway is pertussis toxin-sensitive, suggesting coupling via a $G_{i/o}$ G protein rather than the $G_{q/11}$ G protein typical of group I mGluRs (Thandi *et al.* 2002). In human embryonic kidney (HEK) cells, mGluR1α stimulation was found to lead to a single burst of Ca^{2+} mobilisation, whilst mGluR5α caused intracellular Ca^{2+} oscillations (Kawabata *et al.* 1996). In a later study, mGluR1 and mGluR5 expressed in HEK cells were demonstrated to cause different frequencies of inositol triphosphate (IP_3) and Ca^{2+} oscillations, leading to different frequencies of repetitive translocation of PKCβII between the plasma membrane and the cytosol (Dale *et al.* 2001). The group I mGluR subtypes were found to utilise different sources of Ca^{2+} for their signalling in hippocampal inhibitory neurones; mGluR1α stimulation caused Ca^{2+} influx and release from intracellular stores, whilst mGluR5 stimulation only led to intracellular release of Ca^{2+} (Topolnik *et al.* 2006). It is entirely possible that similar differences in group I mGluR signalling could lead to distinct effects of mGluR1 and mGluR5 in microglia.

Figure 5.24 summarises the apparent opposing actions of mGluR1α and mGluR5 in microglia, based upon results from BV-2 and N9 cells presented here, in combination with the recently-published study in primary microglia (Byrnes *et al.* 2009).

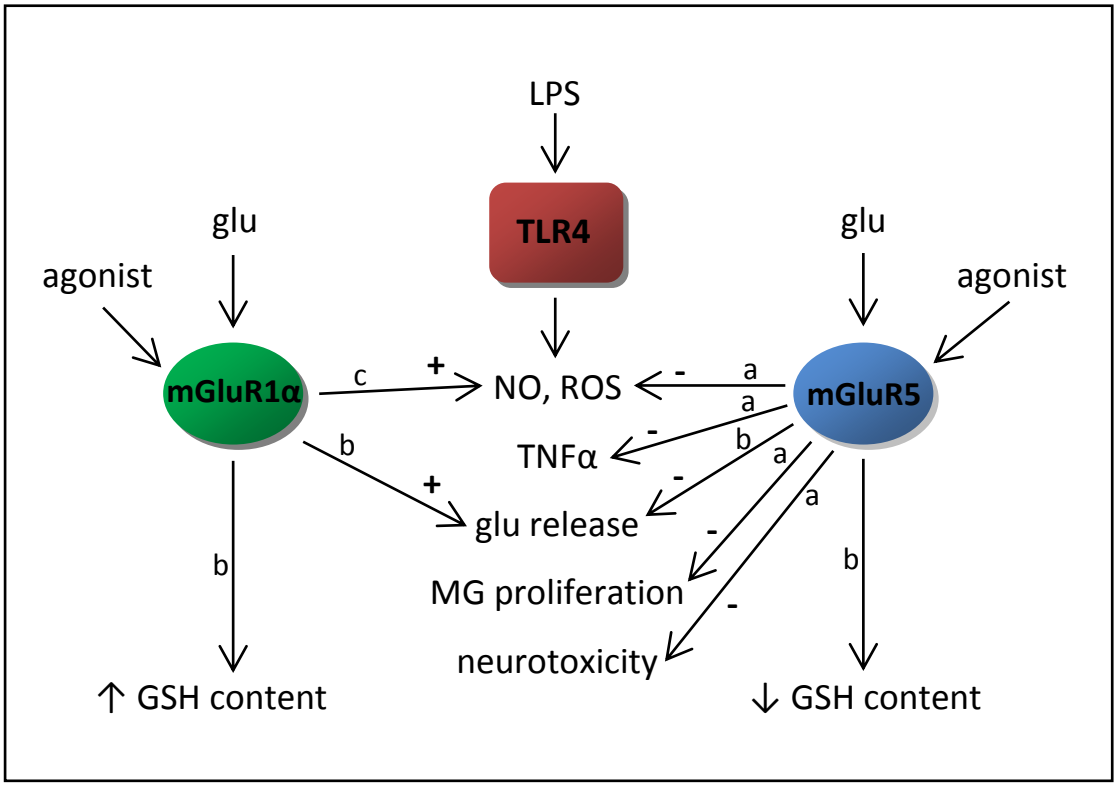


Figure 5.24. *Microglial group I mGluRs appear to have opposing actions upon aspects of microglial activation. mGluR1α activation appears to enhance the release of NO and ROS, whilst mGluR5 activation inhibits NO and ROS release, TNFα production, glutamate release, microglial proliferation and neurotoxicity. In addition, mGluR1α increases, and mGluR5 decreases microglial GSH content. Glu, L-glutamate; GSH, glutathione; LPS, lipopolysaccharide; MG, microglia(l); mGluR1α, metabotropic glutamate receptor 1α; mGluR5, metabotropic glutamate receptor 5; NO, nitric oxide; ROS, reactive oxygen species; TLR4, toll-like receptor 4; TNFα, tumour necrosis factor α; +, enhancement of LPS-induced characteristic; -, inhibition of LPS-induced characteristic; a, as demonstrated by (Byrnes et al. 2009); b, as suggested by BV-2 data; c, as suggested by preliminary N9 data.*

5.10.3. The effect of group II metabotropic glutamate receptors

In this study, preliminary experiments suggested that the mGluR3 agonist NAAG appeared to decrease xCT mRNA expression in preliminary experiments in N9 cells. This is in agreement with *in vivo* microdialysis studies, which demonstrated an autoregulatory mechanism whereby group II mGluR stimulation reduced glutamate release by decreasing x_c^- transporter activity (Baker *et al.* 2002; Xi *et al.* 2002). Interestingly, NAAG is an endogenous specific mGluR3 agonist of the mammalian CNS (Curatolo *et al.* 1965; Zaczek *et al.* 1983; Wroblewska *et al.* 1997). Such an effect of this peptide may therefore have *in vivo* relevance. Further experiments are however necessary in order to substantiate this finding.

5.10.4. The effect of group III metabotropic glutamate receptors

Microglia in culture express group III mGluRs (mGluR4, 6 and 8) (Taylor *et al.* 2003) and microglial mGluR8 expression has been demonstrated in demyelinating and chronic MS lesions (Geurts *et al.* 2005). In the present study, the group III mGluR agonist L-AP4 (Nakanishi 1992; Bushell *et al.* 1995) decreased GSH levels in N9 cells. L-AP4 may also have caused a slight increase in glutamate release, in agreement with an earlier report (Pinteaux-Jones *et al.* 2008), although due to inter-experiment variability in the present study the increase was not found to be statistically significant. In preliminary experiments, L-AP4 did not affect the glutamate release induced by LPS + IFNγ. It is interesting that an effect of L-AP4 was observed at all, given that N9 medium contains 5 % serum, and thus approximately 50 µM glutamate. This concentration of glutamate is within a similar range to that reported for the EC_{50}/K_d of mGluR4 and mGluR6 (Schoepp *et al.* 1999), and may therefore be expected to result in approximately half-maximal activation of these receptors. L-AP4 is a more potent agonist at all group III mGluRs than glutamate (Schoepp *et al.* 1999), and at a concentration of 100 µM, as used here, would be expected to fully activate the receptors. The difference between control and L-AP4-treated cells may therefore represent the difference between half and full activation of mGluR4 or mGluR6. High concentrations of G protein-coupled receptor agonists are often associated with receptor desensitisation and internalisation, however the desensitisation of mGluR4, at least, has been shown to

be agonist-independent (Mathiesen and Ramirez 2006), suggesting that an effect of L-AP4 following a 24 hour exposure is entirely possible.

Increased extracellular glutamate levels, as may have occurred in the presence of L-AP4, suggest increased operation of the x_c^- transporter or decreased activity or reversal of EAATs. However, preliminary data here suggested that the group III agonist (RS)-PPG may have decreased the levels of mRNA for xCT in N9 microglia, although L-AP4 did not appear to have the same effect, despite the fact that both compounds would be expected to act as agonists at microglial mGluRs 4, 6 and 8 at the concentrations tested (Conn and Pin 1997; Gasparini *et al.* 1999; Schoepp *et al.* 1999). It may appear somewhat contradictory that the group III mGluR antagonist MAP4 also appeared to decrease xCT mRNA expression, however the agonist/antagonist profile of MAP4 is more complicated. Although its inhibition of L-AP4-mediated effects is well-characterised (Jane *et al.* 1994; Bushell *et al.* 1995; Knopfel *et al.* 1995; Salt and Eaton 1995; Vignes *et al.* 1995; Taylor *et al.* 2003), it has also been reported to act as a weak mGluR4a (Knopfel *et al.* 1995) and mGluR6 (Laurie *et al.* 1997; Sekiyama *et al.* 1996) agonist. MAP4 may therefore be a group III mGluR partial agonist (Laurie *et al.* 1997), perhaps explaining its apparent ability to emulate the effects of (RS)-PPG. Glutamate also has concentration-dependent effects upon x_c^- and EAAT operation (see fig. 4.24); it may therefore have effects upon these transporters at several different levels. Alterations in extracellular glutamate levels due to changes in transporter function provide a potential for positive or negative feedback in this system.

The decrease in xCT mRNA levels following group III mGluR stimulation suggested by preliminary PCR results would fit with the decrease in the GSH content of N9 cells, as GSH synthesis is dependent upon cystine import via x_c^-. The concurrent increase in extracellular glutamate may therefore be due to other effects of group III mGluR stimulation, perhaps upon the EAAT glutamate transporters. This may be an effect upon EAAT expression at the mRNA level; the effects of mGluR compounds other than those acting upon group I receptors upon EAAT mRNA expression were not investigated in primary microglia in the present study and the expression of EAAT mRNA by BV-2s and N9s was very low, such that changes may not have been detected. Alternatively, post-transcriptional regulation of EAAT expression or

activity may be involved. *In vivo*, a group III mGluR antagonist prevented downregulation of EAAT2 protein in the hippocampal CA1 region following transient ischaemia (Chen *et al.* 2005), implicating group III stimulation in EAAT2 downregulation. In astrocytes *in vitro*, dbcAMP upregulated EAAT1 and EAAT2 mRNA and protein expression, and increased glutamate uptake capacity (Eng *et al.* 1997; Swanson *et al.* 1997; Schlag *et al.* 1998). Microglial group III mGluRs have been shown to couple via $G_{i/o}$ (Taylor *et al.* 2003), therefore the agonist L-AP4 would be expected to decrease the availability of cAMP, thus perhaps limiting EAAT2 expression. In addition, a decrease in EAAT activity may hinder x_c^- activity by limiting its access to intracellular glutamate to drive cystine import, thus potentially decreasing the rate of GSH synthesis, and providing another explanation for the decreased GSH content found following treatment with L-AP4. A hypothesis explaining the effects of group III mGluRs upon GSH levels, glutamate release and xCT expression is illustrated in figure 5.25.

Neuroprotection via stimulation of group III mGluRs on both astrocytes and microglia has been demonstrated previously. In microglia, L-AP4 prevented microglial neurotoxicity following stimulation with LPS, CGA or myelin (Taylor *et al.* 2003; Pinteaux-Jones *et al.* 2008), but in the case of myelin at least, L-AP4 did not have any effect upon myelin-induced iNOS expression, or glutamate or TNFα expression (Pinteaux-Jones *et al.* 2008). The protective effects of L-AP4 were dependent upon the presence of microglia, which presumably released neuroprotective factors into the medium. Microglial group III mGluRs, when stimulated with L-AP4 or (RS)-PPG, are negatively coupled to AC (Taylor *et al.* 2003), as are microglial group II mGluRs (Taylor *et al.* 2002), but these intracellular signalling pathways diverge at some point following the cAMP decrease, and stimulation of group II mGluRs causes neurotoxicity (Taylor *et al.* 2002, 2005) whilst group III mGluRs are neuroprotective (Taylor *et al.* 2003; Pinteaux-Jones *et al.* 2008).

Stimulation of microglial group III mGluRs therefore appears to promote neuroprotection in the presence of a decrease in intracellular GSH levels. Perhaps the mechanism of neuroprotection depletes microglial GSH. For example, group III mGluRs may upregulate the activity of antioxidant defences to allow the microglia to

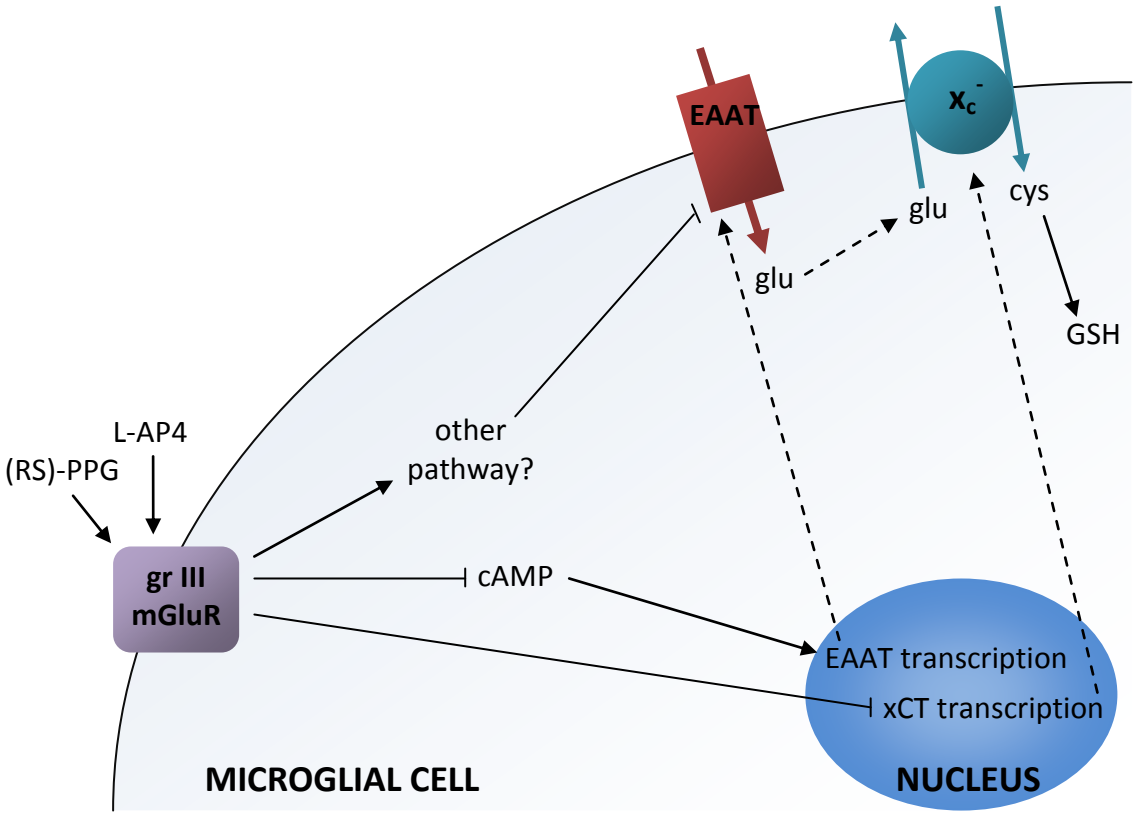


Figure 5.25. *Hypothesis to explain concurrent GSH depletion, increased extracellular glutamate and decreased xCT mRNA levels, as found in the case of N9 microglial cells following group III mGluR stimulation. Activation of group III mGluRs may downregulate EAAT expression or activity through inhibition of a cAMP-mediated enhancement of EAAT gene transcription or otherwise (Eng et al. 1997;Swanson et al. 1997;Schlag et al. 1998;Chen et al. 2005). A decrease in EAAT expression would lead to less glutamate uptake and therefore increased extracellular glutamate levels. Data in this study suggested that group III mGluR stimulation may downregulate xCT mRNA levels, which would likely decrease x_c^- expression and activity and consequently compromise GSH synthesis. cAMP, cyclic adenosine monophosphate; cys, L-cystine; EAAT, excitatory amino acid transporter; glu, L-glutamate; gr III mGluR, group III metabotropic glutamate receptor; GSH, glutathione; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; (RS)-PPG, (RS)-4-phosphonophenylglycine; x_c^- x_c^- glutamate/cystine antiporter system; xCT, the specific subunit of x_c^-.*

detoxify potentially damaging reactive species, thus consuming GSH but protecting against neuronal damage. Since GSSG levels in the presence of L-AP4 were not tested, there is a possibility that this could account for the apparent depletion of GSH. As mentioned above, GCL, the rate-limiting enzyme in GSH synthesis (Meister and Anderson 1983), has been noted to be upregulated in hepatocytes, fibroblasts, endothelial and epithelial cells under a number of conditions which may also be associated with microglial activation (Ochi 1995, 1996; Urata *et al.* 1996; Morales *et al.* 1997; Liu *et al.* 1998b; Rahman *et al.* 1999; Dickinson *et al.* 2002; Takamura *et al.* 2006). It may be that in preventing proinflammatory effects, group III mGluR activation removes or counteracts such potential GCL-upregulatory stimuli.

Activity of the x_c^- transporter has been shown *in vitro* to be associated with oxidative glutamate toxicity towards neurones (Piani and Fontana 1994; Barger and Basile 2001; Qin *et al.* 2006; Barger *et al.* 2007) and oligodendrocytes (Domercq *et al.* 2007; Matute 2007), therefore downregulation of the expression of this transporter, as suggested by preliminary PCR data, may promote neuronal and oligodendroglial survival. Microglial glutamate release may however have less of an impact *in vivo*, where astrocytes express EAATs at a high level, and are capable of dealing with high concentrations of extracellular glutamate (Rothstein *et al.* 1996). Decreased microglial x_c^- expression would likely limit cystine uptake and therefore decrease the rate of GSH synthesis, an effect demonstrated here in N9 cells treated with L-AP4.

An alternative explanation for microglial GSH depletion in combination with neuroprotection may be the supply of cysteine to neurones, thus enabling neuronal GSH synthesis. This function is normally performed by astrocytes, which release GSH (Yudkoff *et al.* 1990; Sagara *et al.* 1996; Dringen *et al.* 1999b) through multidrug resistance protein 1 (Mrp1) (Hirrlinger *et al.* 2002c; Minich *et al.* 2006). Microglia may also release GSH (Dallas *et al.* 2003; see chapter 3), and have been shown to express functional Mrp1, as well as other Mrp subtypes (Ballerini *et al.* 2002; Hirrlinger *et al.* 2002a; Dallas *et al.* 2003). However, in order to provide cysteine for neuronal uptake and GSH synthesis, the released GSH must be broken down by γGT (Dringen *et al.* 1997) which is expressed by astrocytes (Dringen *et al.* 1997) but not at significant levels by microglia or neurones (Shine and Haber 1981;

Philbert *et al.* 1995; Murata *et al.* 1997; Ruedig and Dringen 2004), suggesting that such an interaction between microglia and neurones would need to involve astrocytes or be γGT-independent. GSSG is also a substrate for Mrp1 (Leier *et al.* 1996; Heijn *et al.* 1997), and GSSG released by astrocytes was noted to be broken down in a γGT-independent manner (Minich *et al.* 2006). Microglial GSSG release may therefore represent a γGT-independent cysteine supply route for neurones. Alternatively, there is the possibility that microglia release a different GSH precursor for neurones, such as cysteinylglycine or cysteine, thus eliminating the need for extracellular γGT. Since the release of either of these molecules would deplete microglial cyst(e)ine, intracellular GSH levels would be expected to fall as a consequence. Macrophages have been shown to release cysteine (Gmunder *et al.* 1990; Yeh *et al.* 2000), but it is questionable whether microglia would release cysteine as part of a neuroprotective mechanism as it is also an agonist at NMDA receptors, mediating neuronal excitotoxicity at high concentrations (Olney *et al.* 1990; Lipton *et al.* 1993; Yeh *et al.* 2000). Cysteinylglycine is therefore a more likely candidate. Indeed, Mrp1-mediated transport of cysteinylglycine conjugates of isothiocyanates (Callaway *et al.* 2004) and the cysteinylglycine leukotriene LTD_4 (Schaub *et al.* 1991; Leier *et al.* 1994; Jedlitschky *et al.* 1996) have been reported. It is therefore plausible that cysteinylglycine or a cysteinylglycine conjugate may be exported by one of the Mrps expressed by microglia, to supply cysteine for neuronal GSH synthesis.

5.10.5. Conclusion

Further to published data demonstrating microglial neuroprotection and neurotoxicity following differential mGluR stimulation, data are presented here showing modulation of microglial GSH levels by antagonists of group I mGluRs and the group III mGluR agonist L-AP4 in mouse microglial cell lines. This appears to be the first demonstration of mGluR-mediated modulation of microglial GSH. Interestingly, stimulation of mGluR5 or of group III mGluRs, both of which have been shown to enhance microglial neuroprotection (Taylor *et al.* 2003; Pinteaux-Jones *et al.* 2008; Byrnes *et al.* 2009), also depleted microglial GSH levels. Stimulation of mGluR5 appeared to decrease microglial glutamate release, whilst

group III mGluR stimulation enhanced glutamate release, suggesting that different pathways may be involved. Microglial GSH is crucial in the defence against oxidative stress, and depleted GSH may lead to compromised microglial function. Neuroprotection following microglial mGluR modulation may involve increased GSH utilisation or a decrease in the rate of GSH synthesis; alternatively decreased GSH levels and neuroprotection may be unrelated consequences of the mGluR stimulation. In any case, GSH depletion over time may compromise the neuroprotective ability of microglia. Further study is required to confirm that the effects noted here also occur in primary microglia and to elucidate the signalling pathways involved. Because elevated extracellular glutamate levels and glutamate toxicity are implicated to some extent in the majority of neurodegenerative and neuroinflammatory diseases, an understanding of microglial mGluRs and GSH is important to fully understand the role of microglia in these circumstances. A better understanding of microglia may also allow development of therapies to target the harmful effects of microglia without compromising their normal function.

Chapter 6

General discussion

General discussion

The work presented here centred upon the investigation of the glutathione (GSH) levels of microglial cells *in vitro*, under conditions associated with microglial activation and neuroinflammatory disease, as well as in the presence of specific agonists and antagonists of metabotropic glutamate receptors (mGluRs). In addition, any concomitant changes in glutamate transporter expression and extracellular glutamate levels were investigated, in order to help explain any changes in the GSH content of microglia.

Microglia are the immune cells of the CNS, having a crucial role in detecting and removing foreign molecules which enter the brain. This involves their transient activation to a more immunocompetent phenotype, allowing the release of antimicrobial compounds and phagocytosis of debris. Microglia are therefore fundamentally a functional part of the CNS, adapted to protect the more vulnerable cells, particularly neurones, from damage. Microglial dysfunction, in the form of chronic activation, may be implicated in neuroinflammatory conditions. However, the majority of observations of microglial behaviour are likely to have physiological, as well as pathological, significance. This often appears to be overlooked in studies focussing on the potential role of microglia in pathogenesis.

Microglia, like all metabolically active cells, constantly produce reactive oxygen species (ROS) during mitochondrial respiration. In addition, microglial activation leads to the production of nitric oxide (NO) and superoxide, which may give rise to a number of other reactive species, including peroxynitrite. This suggests that antioxidants such as GSH are likely to be important in protecting microglia from oxidative damage. Indeed, microglia have high levels of GSH and the enzymes involved in its metabolism (Chatterjee *et al.* 1999, 2000; Hirrlinger *et al.* 2000, 2002b; Hollensworth *et al.* 2000; Noack *et al.* 2000). Previous literature has suggested that microglial GSH levels may be altered by microglial activation by LPS, IFNγ and TNFα, although reports exist both of increases and decreases in GSH levels (Chatterjee *et al.* 2000; Noack *et al.* 2000; Moss and Bates 2001; Dopp *et al.* 2002; Roychowdhury *et al.* 2003; Persson *et al.* 2006). Here, it was found that GSH

levels in the BV-2 mouse microglial cell line increased following treatment with the classical microglial activator lipopolysaccharide (LPS) alone and in combination with interferon-γ (IFNγ), whilst N9 microglial GSH levels decreased under identical conditions. The differences between the responses of the cell lines did not appear to be due to different sensitivities of the cell lines to the activators, nor to a temporal difference in the response. Parallel experiments performed in primary rat microglia suggested that in this situation, the BV-2 cell line more closely modelled primary microglia, with an increase in the GSH content following microglial activation.

The major function of intracellular GSH is its antioxidant property. It is therefore clear that increased production of ROS and reactive nitrogen species (RNS) would place increased demand upon GSH, and in the absence of adequate synthesis may lead to a declined GSH content. However, intracellular signalling pathways may exist which allow the maintenance of intracellular GSH through enhanced synthesis. As glutamate and glycine are present at high levels intracellularly, cystine availability may limit the rate of GSH synthesis. In glial cells, cystine for GSH synthesis is supplied via the x_c^- glutamate/cystine antiporter; studies have demonstrated LPS-induced x_c^- upregulation in microglia (Barger *et al.* 2007; Domercq *et al.* 2007), perhaps increasing supply of this potentially limiting substrate. The rate-limiting enzyme in GSH synthesis is thought to be GCL (Meister and Anderson 1983), which has been shown in astrocytes to be upregulated in the presence of NO (Gegg *et al.* 2003). However, as both enzymes involved in GSH synthesis are ATP dependent, a compromised cellular energy status under conditions of oxidative stress may prevent adequate GSH synthesis to meet demand. In some cell types NO and peroxynitrite have been shown to upregulate glycolysis or the pentose-phosphate pathway, resulting in a rescue of ATP or NADPH production, respectively (Almeida *et al.* 2001, 2005). Whilst ATP might allow the continued synthesis of GSH, NADPH allows reduction of oxidised glutathione to maintain GSH levels. The potential effects of microglial activation upon intracellular GSH levels are illustrated in figure 6.1. There may be differences in the underlying cell biology and signalling pathways of BV-2 and N9 cells, which somehow leads to the difference in the effects of activation upon the GSH levels in these cell lines.

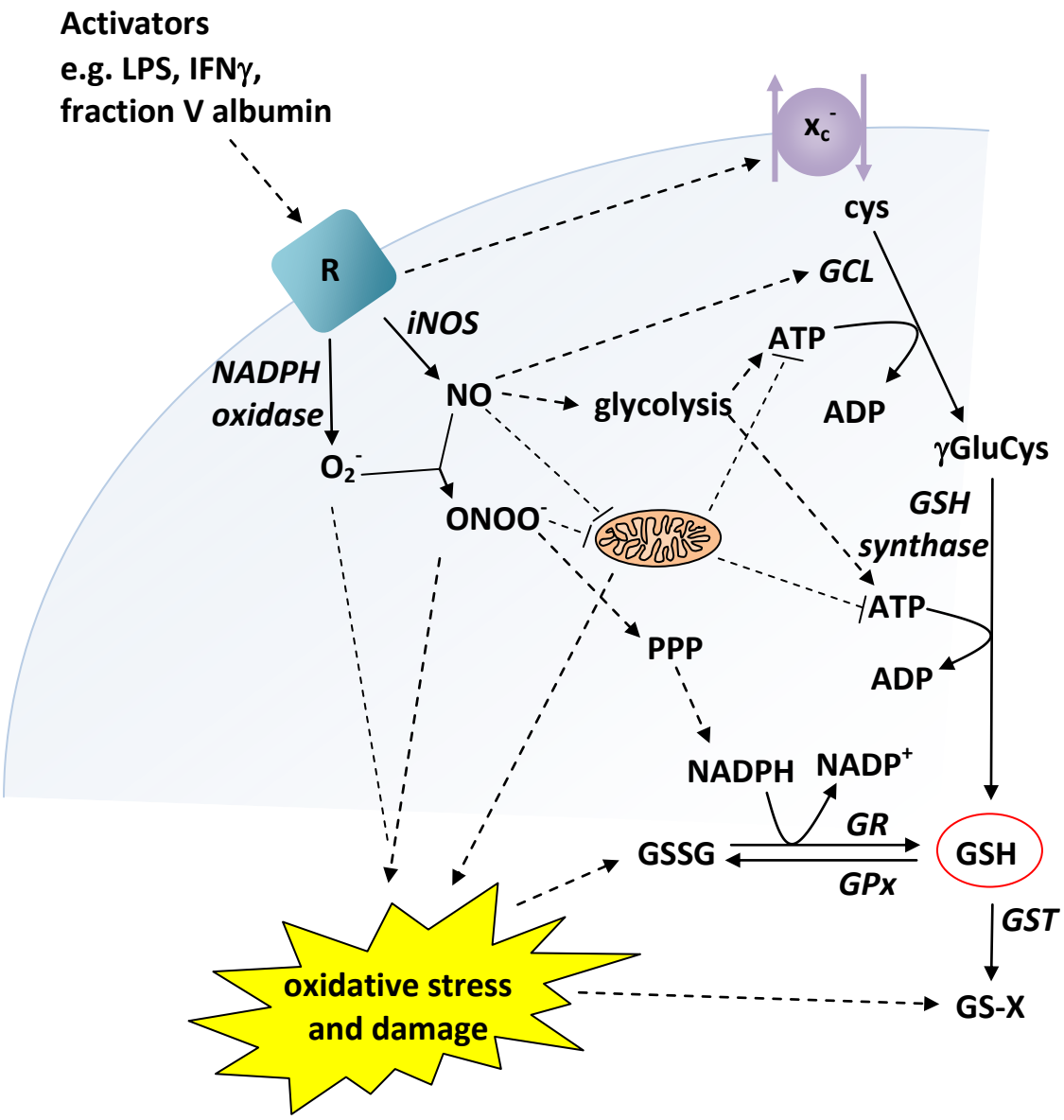


Figure 6.1. *Mechanisms by which microglial activators such as LPS, IFNγ or fraction V albumin may affect levels of intracellular GSH (circled). Solid arrows indicate reactions occurring; the enzymes responsible are in italics alongside. Dotted arrows indicate potential stimulatory/upregulatory effects, whilst dotted lines blocked at the end indicate potential inhibitory/downregulatory effects. γGluCys, γ-glutamylcysteine; ADP, adenosine diphosphate; ATP, adenosine triphosphate; cys, L-cystine; GCL, glutamate-cysteine ligase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione S-transferase; GSSG, oxidised glutathione; GS-X, S-conjugates of glutathione; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NADP$^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; O_2^-, superoxide; ONOO$^-$, peroxynitrite; PPP, pentose-phosphate pathway; R, cell surface receptor; x_c^-, x_c^- glutamate/cystine antiporter system.*

LPS or LPS + IFNγ treatment of BV-2 or primary microglia leads to ROS and RNS production, which is likely to cause oxidative stress and increase GSH consumption. There must therefore be a highly significant upregulation of GSH synthesis in BV-2 and primary microglial cells, in order to cause a net increase in intracellular GSH. An increase in extracellular glutamate, as well as elevated levels of messenger ribonucleic acid (mRNA) for both the excitatory amino acid transporter (EAAT2) and xCT, the specific subunit of the x_c^- transporter, were found to be associated with LPS-induced GSH increases in BV-2 and primary microglial cells. Elevated x_c^- activity enhances the cystine uptake capacity of microglia and therefore may represent the mechanism by which LPS increases microglial GSH levels. However the x_c^- transporter releases glutamate in exchange for cystine, suggesting that increased microglial cystine uptake and GSH synthesis may cause toxicity to neighbouring cells. A concurrent elevation of EAAT2 may indicate a mechanism to counteract the elevated glutamate release. Although millimolar levels of glutamate are present intracellularly, EAATs and x_c^- may be located in close vicinity to one another so that EAAT-mediated glutamate uptake may be directly coupled to x_c^- activity (Igo and Ash 1998; Rimaniol *et al.* 2001; Persson *et al.* 2006). The overall increase in extracellular glutamate under conditions of microglial activation suggested that x_c^- activity is likely to be more highly upregulated than that of EAAT2, unless EAAT2 transporters undergo reversal and contribute to glutamate release. In addition, the time course of GSH levels of BV-2 cells closely resembled that of their ATP levels, in both control and activated cells, perhaps reflecting the importance of *de novo* GSH synthesis in maintaining intracellular GSH.

In contrast, it appears that N9 cells are either more susceptible to ROS than BV-2s, or are unable to upregulate GSH synthesis to the same degree. Therefore treatment of N9 cells with LPS or LPS + IFNγ led to a decrease in intracellular GSH as oxidative damage in the cell was dealt with. Indeed, a decrease in the intracellular GSH levels of N9 cells was also shown by Moss and Bates (2001), who also demonstrated that the decrease could be prevented by inhibition of iNOS, clearly implicating NO in the GSH decline.

The experiments presented here failed to find significant effects of two peptides upregulated in Alzheimer's disease (AD), chromogranin A (CGA) and β amyloid

peptide (residues 25-35; $A\beta_{25-35}$) upon the GSH content of microglia, the glutamate content of conditioned medium or the expression of mRNA for glutamate transporters. This is surprising, as previous studies have demonstrated effects of CGA and Aβ peptides at concentrations similar to those used here. The issue of LPS contamination of compounds used to treat microglia has recently been raised by Weinstein *et al.* (2008), a finding which should prompt a re-evaluation of any studies involving the addition of compounds to microglia *in vitro*. Aβ or CGA contaminated with LPS would activate microglia, which may then be attributed to an action of the compound studied rather than potential contamination. A further consideration in this study is the use of the amyloidogenic portion of Aβ, $A\beta_{25-35}$, rather than the full-length peptide. Although the two have been shown to have similar effects in microglia (Yankner *et al.* 1990), it should be considered that differences in their actions may exist. In addition, the age of the $A\beta_{25-35}$ and CGA used here and the number of freeze-thaw cycles the compounds may have been exposed to may go towards explaining their lack of an effect.

However, albumin, to which microglia may be exposed following blood-brain barrier (BBB) damage in a number of neuroinflammatory and neurodegenerative conditions, including AD and multiple sclerosis (MS), was found to affect microglial GSH and glutamate regulation. Albumin causes microglial NO (Hooper *et al.* 2009) and superoxide (Si *et al.* 1997) production, which might be expected to inhibit mitochondrial respiration and deplete GSH. However, GSH levels were found to increase upon treatment with "fraction V" (partially purified) albumin. The intracellular levels of ATP were also found to increase following fraction V treatment, and a correlation between ATP and GSH levels were observed. Thus, it appears that the NO produced upon fraction V treatment may be able to upregulate the glycolytic pathway in BV-2 microglia. The enhancement of ATP levels in BV-2 cells and the GSH content of BV-2 and primary microglia in the presence of fraction V albumin may be related to the ability of albumin to cause microglial proliferation (Hooper *et al.* 2005). The upregultation of both ATP and GSH in BV-2 microglia was somewhat transient, suggesting that the cells might only be equipped to deal with transient oxidative insults, and that longer insults may be more detrimental. It is however possible that this is an artifact resulting from longer periods of time in culture, which may involve depletion of certain components of the cell medium or a

build up of toxic release products. Albumin-induced increases in intracellular GSH and iNOS expression have been shown to be unaffected by the LPS neutralising agent polymyxin B, suggesting that although these effects are similar to those observed in the presence of LPS, they are not due to LPS contamination of the fraction V albumin (Hooper *et al.* 2009).

Fraction V albumin treatment also led to an increase in the glutamate content of microglial conditioned medium, suggesting an altered glutamate uptake/release balance, although this may have been amplified by cell proliferation over the incubation period. Some slight changes in the glutamate transporter expression of primary microglia at the mRNA level were however detected, suggesting a real effect upon glutamate release; in particular, fraction V albumin consistently increased xCT mRNA levels, potentially explaining both the enhanced GSH content, and the neurotoxicity of albumin-treated microglia *in vitro* (Hooper *et al.* 2009).

Due to the limited availability of primary microglia, a number of the findings presented here only relate to the microglial cell lines BV-2 and N9, especially in the case of GSH determination by high performance liquid chromatography (HPLC), where large amounts of protein are required; indeed, on the few occasions where HPLC was used to assess the GSH content of primary microglia the effect of any treatment was small compared with the variability of the data. Therefore an important follow-up to this project would be the extension of observations in the cell lines into primary microglia. Due to the difficulties of using the HPLC determination method, it may be necessary to use alternative means of measuring primary microglial GSH content, such as the monochlorobimane imaging method used in this thesis, or a biochemical assay, such as the GSH-Glo assay used here to determine the GSH content of microglial conditioned medium.

It is possible that the expression of mRNA for subtypes of glutamate transporters may not accurately reflect their functional expression or activity. As well as the possibility of post-transcriptional regulation, modulation of the activity of EAATs by the cellular redox potential has been demonstrated (Volterra *et al.* 1994; Trotti *et al.* 1997a, 1997b, 1998; Blanc *et al.* 1998; Begni *et al.* 2004). This is clearly an important consideration in this study, given that microglial activation increases

oxidative stress. The findings regarding the expression of mRNA for glutamate transporters would therefore be greatly strengthened by investigations of protein expression, by Western blotting to evaluate absolute protein levels and immunofluorescence to determine the localisation of the protein. The activity of transporters could be investigated by uptake or release assays utilising radiolabelled substrate molecules.

Primary microglia and those of the BV-2 cell line were also demonstrated to release low levels of GSH into the culture medium. This appears to be the first demonstration of GSH release by primary microglial cells. Microglial GSH release is likely to occur through multidrug resistance-associated proteins (Mrps), probably Mrp1, as microglial expression of functional Mrp1 has been demonstrated (Ballerini *et al.* 2002; Hirrlinger *et al.* 2002a; Dallas *et al.* 2003), and export of the Mrp1 substrate vincristine by microglia of the MLS-9 cell line was found to depend upon intracellular GSH (Dallas *et al.* 2003). Whether this microglial GSH release simply represents cotransport with exported molecules, or whether it fulfils a function in itself is yet to be determined. GSH released by astrocytes is broken down by astrocyte and neurone-associated ectoenzymes to provide cysteine which is taken up by neurones for GSH synthesis (Dringen 2000). It is possible that microglial GSH release may have a similar function, although microglia lack the ectoenzyme γ-glutamyl transpeptidase (γGT) expressed by astrocytes which initiates the extracellular processing of GSH (Murata *et al.* 1997; Ruedig and Dringen 2004). Uncleaved GSH released by microglia may act as an extracellular antioxidant; GSH is present extracellularly *in vivo* (Yang *et al.* 1994; Lada and Kennedy 1997) and may be important in detoxifying ROS and RNS released by CNS cells, especially microglia. Alternatively, GSH may act as a neuromodulator, either due to binding of the γ-glutamyl residue of GSH to glutamate binding sites (Varga *et al.* 1989, 1997; Yoneda *et al.* 1990; Leslie *et al.* 1992; Janaky *et al.* 1993, 2007, 2008; Ogita *et al.* 1995; Jenei *et al.* 1998; Wang *et al.* 2006), or to the formation of disulphide bonds with the receptor (Liu and Quirion 1992). Evidence also exists suggesting that GSH may even represent a novel neurotransmitter (Guo *et al.* 1992; Guo and Shaw 1992; Lanius *et al.* 1994; Janaky *et al.* 2000).

Microglial mGluRs have been found to alter microglial behaviour, making them neuroprotective (Taylor *et al.* 2003; Pinteaux-Jones *et al.* 2008; Byrnes *et al.* 2009) or neurotoxic (Taylor *et al.* 2002, 2005; Pinteaux-Jones *et al.* 2008), depending upon the particular receptors involved. Concomitant effects upon microglial glutamate release have been documented (Pinteaux-Jones *et al.* 2008). It was therefore proposed that mGluR modulation may have effects upon the GSH system of microglia, perhaps by direct or indirect effects upon ATP levels, ROS levels, GCL activity or the activity of glutamate transporters. mGluR5 and has been shown to enhance the neuroprotective ability of microglia (Byrnes *et al.* 2009), and was found here to cause declines in microglial GSH levels. This may reflect an upregulated rate of GSH utilisation for detoxification of ROS and RNS, which may be responsible for the enhanced neuroprotection. The GSH decline was however accompanied by lower levels of glutamate release, but no detectable alterations in transporter expression at the mRNA level, suggesting decreased activity of x_c^-, perhaps due to post-transcriptional downregulation or decreased protein expression at the plasma membrane. Such a downregulation of x_c^- activity could explain the decreased GSH levels through compromised synthesis, but how this might be related to neuroprotection is unclear.

It was interesting that mGluR1 was found here to have opposite effects to mGluR5; AIDA, an antagonist at mGluR1, decreased both extracellular glutamate and intracellular GSH levels, suggesting that an underlying stimulation of mGluR1 was having a positive modulatory effect upon both. A recent paper evaluates the effect of group I mGluR stimulation upon LPS-stimulated microglia (Farso *et al.* 2009), concluding that group I stimulation reduces microglial activation. However, upon closer inspection of the data, it appears that whilst DHPG, a group I agonist which may have a slight preference for mGluR5 (Schoepp *et al.* 1999), does reduce markers of microglial activation, AIDA, an mGluR1-preferring antagonist (Moroni *et al.* 1997), does not always reverse the effects of DHPG. In particular, DHPG reduces microglial glutamate release, and AIDA potentiates the effect. Assuming that the response to DHPG is mGluR5-mediated and the response to AIDA is mGluR1-mediated, such a finding is in direct accordance with the data presented in this thesis, and lends weight to the hypothesis that mGluR1 and mGluR5 may have some opposing actions in microglia. Indeed, microglial group I mGluRs have been

demonstrated to couple to two different G proteins, $G_{q/11}$, the prototypical group I coupling, leading to phosphatidylinositol hydrolysis and Ca^{2+} mobilisation, and G_s, leading to adenylate cyclase activation and increased cyclic adenosine monophosphate levels (Biber et al. 1999; Byrnes et al. 2009). Alternatively, even if both microglial group I mGluRs couple via $G_{q/11}$, divergence in the signalling pathway may be attained by eliciting different profiles of Ca^{2+} release (Kawabata *et al.* 1996; Dale *et al.* 2001), or utilising different sources of Ca^{2+} for this signalling (Topolnik *et al.* 2006).

Group III mGluRs are also neuroprotective *in vitro* (Taylor *et al.* 2003), and in this study also caused a decline in the intracellular GSH levels of N9 cells. The pathway associated with the group III mGluR-mediated decrease in GSH content may be different from that involved in the effects of mGluR5, as the decrease was associated with slightly elevated extracellular glutamate levels. This data could suggest a group III mGluR-mediated upregulation of microglial antioxidant function; a downregulation of intracellular GSH levels suggests an increased rate of GSH utilisation, whilst increased extracellular glutamate may indicate an upregulation of x_c^- to increase the supply of cystine for GSH synthesis. However, preliminary data suggested a downregulation of xCT expression at the mRNA level following group III mGluR stimulation, a finding which, if substantiated in further experiments, would prompt a re-evaluation of the pathway involved.

An alternative explanation for neuroprotection in the presence of GSH depletion, as demonstrated following both mGluR5 and group III mGluR stimulation, is the release of GSH to protect the more vulnerable CNS cell types. Astrocytes participate a pathway whereby they release GSH to supply cysteine to neurones, but microglia have not as yet been shown to do so. Microglial GSH release has been demonstrated here, but as microglia lack the ectoenzyme γGT, the supply of cysteine to neurones would necessarily involve astroctyes or other γGT-expressing cells. Alternatively, perhaps microglial GSH release maintains or enhances extracellular GSH levels, allowing the detoxification of ROS and RNS released by microglia, before they are able to have detrimental effects upon the more vulnerable cells of the CNS. The GSH-Glo assay, used successfully here to detect GSH in microglial conditioned medium, could be employed to test this hypothesis. By using co-cultures, where

purified neurones and purified microglia share the same culture medium, either by the use of inserts or with two coverslips placed side-by-side in a larger dish, it would be possible to test whether mGluR stimulation (or other treatments) affect neuronal GSH levels. Treating the co-culture and then processing the coverslips separately to determine the GSH levels in each cell type, would allow greater insight into the GSH interactions between microglia and neurones, if any such interactions occur. Experiments such as this may also aid the elucidation of the function of microglial GSH release. A better understanding of the mechanisms involved in neuroprotection by microglial mGluR5 and those of group III would be advantageous, as microglial mGluRs represent a possible target for future neuroprotective strategies in neuroinflammatory disease.

Preliminary data suggested a possible decrease in xCT mRNA expression by N9 cells following treatment with the specific mGluR3 agonist N-acetyl-L-aspartyl-L-glutamic acid (NAAG). NAAG is an endogenous peptide (Curatolo *et al.* 1965; Zaczek *et al.* 1983; Wroblewska *et al.* 1997), thus, any effects upon microglia *in vitro* may reflect *in vivo* activities of the peptide. NAAG has been shown to be somewhat neuroprotective against myelin-induced microglial neurotoxicity (Pinteaux-Jones *et al.* 2008), paralleling the neuroprotective effect of astrocyte mGluR3 stimulation (Corti *et al.* 2007). This contrasts with the neurotoxic effect of microglial mGluR2 stimulation (Taylor *et al.* 2002). Given further time and funding, further investigation of the neuroprotective and neurotoxic properties of microglia induced by mGluR modulation, particularly with relevance to glutamate transport and GSH homeostasis, would be a priority.

Alterations in microglial GSH levels may have implications in pathological situations. Enhanced microglial GSH levels under conditions of microglial activation would protect the microglia from the damaging reactive oxygen and nitrogen species produced during activation, but may allow the cell to persist in a situation where the detrimental bystander damage of such reactive species is more significant than the protective effect conferred. In addition, an enhancement of GSH levels is likely to involve enhanced cystine import via x_c^-, with an accompanying enhancement of glutamate release. Conversely, a decline in microglial GSH levels may lead to

oxidative stress and microglial cell death, but whether this would be advantageous or deleterious for overall brain health would depend upon the particular situation.

Should the GSH content of microglia be altered following activation, it is important to consider the effect this might have on other cell types, particularly neurones, *in vivo*. An *in vitro* model of intercellular interactions could be achieved by the use of co-culture or by transferring microglial conditioned medium onto neuronal cultures. The effect of microglial GSH depletion upon neuronal survival could be assessed by culturing microglia in the absence of cystine, which over time should deplete their GSH. This could be controlled for by adding cystine to the cystine-depleted microglial conditioned medium before transferring it to separate neuronal cultures. It would also be of interest to assess the effect of such artificial GSH depletion in combination with microglial activation or mGluR modulation, to investigate any contribution of microglial GSH content to the neuroprotective or neurotoxic potential of microglia.

A number of *in vitro* studies from this lab and others report that microglia are toxic to neurones and oligodendrocytes by co-culture studies or transfer of microglial conditioned medium (Piani and Fontana 1994; Kingham *et al.* 1999; Barger and Basile 2001; Kingham and Pocock 2001; Taylor *et al.* 2002; Domercq *et al.* 2007; Jin *et al.* 2007; Noda *et al.* 2007). This neurotoxicity is often at least partly attributed to microglial glutamate release following increased activity of the x_c^- transporter. However, *in vivo,* astrocytes are responsible for the majority of glutamate uptake in the CNS (Rothstein *et al.* 1996), and are able to remove the high levels of glutamate released during synaptic activity and under normal circumstances prevent glutamate toxicity. The levels of glutamate released by microglial x_c^- activity are likely to be much lower than those released synaptically, so the activity of astrocytic EAATs may prevent glutamate released by microglia from ever reaching neuronal or oligodendrocyte receptors or transporters to cause excitotoxicity or oxidative glutamate toxicity. Published rates of astrocyte and microglial EAAT activity and microglial x_c^- activity vary enormously due to culture and experimental differences, so it is difficult to compare these and arrive at a valid conclusion. However, a recent *in vitro* co-culture study demonstrated that astrocytes can protect neurones from cell

death induced by 100 μM exogenous glutamate and LPS-treated microglial conditioned medium (Liang *et al.* 2008).

Glutamate transporters expressed by astrocytes *in vivo* are however not uniformly expressed across the plasma membrane; a number of studies have shown their expression to be enriched in the vicinity of synapses and in response to synaptic activity, and is has been suggested that factors released by neurones induce astrocyte EAAT expression (Theodosis *et al.* 2008). Whether or not astrocyte EAATs can take up glutamate released by microglia may therefore depend upon the interactions between these cell types and the relative localisation of astrocyte EAATs and microglial x_c^-. Cultured microglia and astrocytes certainly associate *in vitro* and may affect one another's behaviour (DeWitt *et al.* 1998). A splice variant of EAAT2 with identical kinetics to EAAT2 found parasynaptically has been found to be localised to astrocyte cell bodies and processes which are not associated with synapses (Sullivan *et al.* 2004). Such a transporter is likely to be responsible for regulating the glutamate concentration of the general extracellular milieu, perhaps dealing with low level continuous glutamate release as well as transient higher level release, due to spillover from synapses and perhaps from activated microglia with upregulated x_c^- activity. Since microglial glutamate release is likely to be continuously occurring at a low level, being upregulated under certain conditions, there is likely to be an intrinsic mechanism to remove such glutamate. Whether microglial EAATs are sufficiently expressed to have this function under normal conditions, or whether astrocytes are necessary, is unclear.

Upregulation of microglial EAAT2 following microglial activation, and the ability of x_c^- to import glutamate at high extracellular glutamate concentrations suggest that under conditions of elevated extracellular glutamate, microglia may play a part in countering elevated glutamate release and perhaps limit glutamate-mediated damage. This may be particularly important considering that the glutamate uptake capacity of astrocytes may decrease under conditions of oxidative stress and hypoxia (Volterra *et al.* 1994; Miralles *et al.* 2001; Dallas *et al.* 2007).

In summary, this thesis has extended current knowledge regarding microglial GSH and the mechanisms by which its intracellular levels may be affected by activation

and by mGluR stimulation. Microglial conditioned medium was found to contain GSH, suggesting microglial release; this being the first such observation in primary microglial cultures. This work has also uncovered a number of areas which merit further investigation, to enable a better understanding of the significance of alterations in microglial GSH, and whether these may play a part in the mechanisms behind microglial neuroprotection and toxicity.

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