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An investigation of the role of c-Jun N-terminal Kinase (JNK) in inflammation-associated cell deterioration in rat hippocampus.

By

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

Submitted March 2005

Department of Physiology, Trinity College, Dublin

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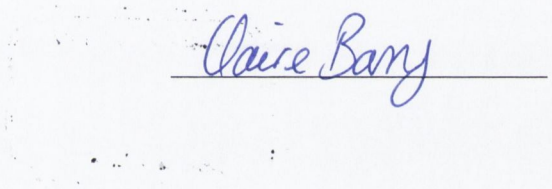
I Declaration

I declare that this thesis is entirely my own work with the following exceptions: certain Western immunoblotting results were kindly donated to me by Nelson Rebola, Niall Kavanagh and Rachael Clarke, and are acknowledged within this thesis. This work has not been previously submitted for a degree to this or any other university. I give my permission to the library to lend or copy this thesis.



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III Abstract

c-Jun N-terminal kinase (JNK) is a mitogen-activated protein kinase which is activated in response to various types of stress (e.g. ischemia, heat shock, osmotic stress, UV irradiation, and hypoxia). JNK is strongly implicated in mediating detrimental aspects of inflammatory change across a range of cell types, including that associated with neuroinflammation. However, definitive evidence of JNK involvement is still lacking, therefore the objective of these studies was to assess the role of JNK in inflammatory models *in vivo*, in particular, its involvement in the impairment of long-term potentiation (LTP) observed under these conditions. By using direct inhibition of the kinase and two agents reputed to have antiinflammatory properties, it was intended that the relevance of JNK and its associated signalling in mediation of inflammatory change would be determined.

These studies indicate that JNK plays a significant role in mediating the central effects of lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, which is a potent initiator of inflammation. Pretreatment with the JNK inhibitor D-JNKI1, attenuated the depressive effects of LPS on LTP, and also rescued hippocampal cells from LPS-induced apoptosis. The biochemical steps by which JNK exerts its influence are less apparent. It was demonstrated that following LPS challenge, many parameters are upregulated such as, activated JNK, c-jun phosphorylation, bcl-2 phosphorylation, as well as indicators of apoptosis such as increased DNA fragmentation, and caspase 3 activation. JNK inhibition attenuated these changes. However, D-JNKI1 treatment did not reduce the LPS-induced increase in IL-1β, suggesting it is upstream of JNK activation. In addition the LPS-induced increase in cytochrome *c* translocation to the cytosol was not effected by D-JNKI1; perhaps some other factor limited by JNK is required for induction of cell death by this route.

Atorvastatin (ATV) is one of a family of lipid lowering agents called statins. Statins are widely prescribed synthetic HMG-CoA reductase inhibitors, which therefore inhibit the rate limiting step of cholesterol synthesis. Much evidence exists to suggest that statins have beneficial properties not associated with cholesterol lowering, and that they are implicated in antiinflammatory signalling. The present studies indicated that ATV treatment modestly attenuated the inhibitory effects of LPS on LTP, concurrent with downregulating the associated JNK signalling. ATV also effectively protected against the impairment in LTP associated with age.

A2A adenosine receptor blockade has been associated with neuroprotective effects and consistently, the present studies show that treatment with the adenosine A2A receptor antagonist, SCH58261, effectively reduced the LPS-induced impairment of LTP, along with the associated increases in IL-1β expression, JNK activity, and caspase 3 activity.

It seems reasonable to conclude that JNK plays a significant role in mediating neuroinflammatory effects associated with LPS treatment. Its inhibition attenuates the depressive effects of LPS on LTP, and also prevented LPS-induced apoptosis. Like JNK inhibition, HMG-CoA reductase inhibition by atorvastatin also exerted protective effects in animals treated with LPS, as well as in aged animals. The mechanisms involved in statin-induced protective effects are complex and probably quite numerous due to the wide range of cell signalling molecules impacted by inhibition of the cholesterol synthesis pathway. However, data presented in these studies indicate that the beneficial effect could be linked to a down regulation in JNK signalling. Similarly, the A2A adenosine receptor antagonist SCH58261, prevented a LPS-induced impairment of LTP and associated increase in a cell death marker in the hippocampus, and this too was allied with downregulation of JNK signalling. Therefore JNK may be a point of convergence in cell death signalling associated with inflammation, and as such represents a potent target for research into neuroprotective strategies.

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VI List of Abbreviations

°C	Degrees Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
A2A receptor	Adenosine 2A receptor
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AFC	7-amino-4-(trifluoromethyl) coumarin
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ANOVA	Analysis of Variance
ANT	Adenine nucleotide translocator
AP-1	Activator protein-1
AP-5	D-2-amino-5-phosphonopentanoate
Apaf-1	Apoptotic protease activation factor-1
APC	Antigen presenting cell
ATF2	Activating transcription factor-2
ATV	Atorvastatin
Aβ	β-amyloid
BBB	Blood brain barrier
BDNF	Brain-derived growth factor
BH	bcl-2 homology
BSA	Bovine serum albumin
CA	Cornus Ammon
Ca^{2+}	Calcium ions
CaMKII	Ca^{2+}/calmodulin dependent kinase II
cAMP	Cyclic adenosine monophosphate
$CD4^{+}$	CD4 positive
CIITA	MHC Class II transactivator
CNS	Central nervous system
CREB	cAMP response element binding protein
DAG	Diacylglycerol
DED	Death effector domain

DISC	Death inducing signalling complex
D-JNKI1	c-Jun N-terminal Kinase Peptide Inhibitor 1, D-stereoisomer
DMEM	Dulbeccos modified eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAE	Experimental allergic encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol bis (β-aminoethylether) N, N tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
E-LTP	Early phase-LTP
eNOS	Endothelial-derived nitric oxide synthase
epsp	Excitatory post synaptic potential
ERK	Extracellular signal regulated kinase
EtOH	Ethanol
FADD	Fas associated death domain
FasL	Fas Ligand
FGF	Fibroblast growth factor
g	gram
GluR	Glutamate receptor
HDL	High density lipoprotein
HFS	High frequency stimulation
HIV	Human immunodeficiency virus
HMG-CoA	Hydroxyl-3-methylglutaryl Coenzyme A
HPA	Hypothalamo-pituitary-adrenal axis
HRP	Horse radish peroxidase
Hz	Hertz
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
ICAM	Intracellular adhesion molecule
ICE	IL-1β converting enzyme
IDL	Intermediate density lipoprotein
IFN	Interferon

IGF	Insulin-like growth factors
IL-	Interleukin-
IL-1R1	IL-1 type I receptor
IL-1ra	IL-1 receptor antagonist
IL-1RAcP	IL-1 receptor accessory protein
iNOS	Inducible nitric oxide synthase
IP3	Inositol-4,5-bisphsphate
IRAK	IL-1 receptor associated kinase
JBD	JNK binding domain
JIP	JNK interacting protein
JNK	c-jun N-terminal protein kinase
K^+	Potassium ions
kDa	Kilodalton
KDO	2-keto-3-deoxyoctonic acid
kg	Kilogram
LBP	LPS-binding protein
LDL	Low density lipoprotein
LFA-1	Leukocyte-functional antigen-1
LFS	Low frequency stimulation
L-JNKI1	c-Jun N-terminal Kinase Peptide Inhibitor 1, L-stereoisomer
L-LTP	Late-phase LTP
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
mg	milligram
Mg^{2+}	Magnesium ions
MHC	Major histocompatibility complex
min	Minute
ml	millilitre
MLK	Mixed-lineage kinase
mm	millimetre
mM	millimolar
MMP	Matrix metalloproteinase

Th	T helper cell
Thr	Threonine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF6	TNF receptor associated kinase
TUNEL	Tdt-mediated dUTP nick end-labelling
UV	Ultra violet
VDAC	Voltage dependent anion channel
VLDL	Very low density lipoprotein
w/v	weight per volume

Chapter 1

Introduction

1.1 Inflammation

Over the past 10-15 years, inflammation in the central nervous system (CNS) has emerged as a critical area for research as the processes involved seem to underlie detrimental age related changes, as well as at least some of the changes associated with Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis and many other neurodegenerative diseases.

The inflammatory reaction is part of the non specific immune defence of the body, is triggered by irritation, injury or infection, and is classically characterised by heat and swelling usually accompanied by redness and pain. This initial response is designed to increase blood flow to the affected area thereby bringing more white blood cells (leukocytes) to fight an infection i.e. adherence and invasion of leukocytes to injured or infected tissue. Different types of leukocytes combat pathogens in different ways; macrophages act mainly by phagocytosis, while lymphocytes act via antigen-antibody reactions to destroy compromised cells and pathogens. The purpose of the innate immune response is to coordinate a defensive response to the detected pathogen. There are many inflammatory mediators which modulate the immune response, these include proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-8, and IL-12, as well as other mediators such as prostaglandins, platelet activating factor, free radicles (Cohen, 2002), kinins, and substance P. These contribute to efficient control of growth, and destruction of pathogens. These potent signalling molecules can eventually lead to changes in transcription that decide the fate of cells. The brain is unique in its inflammatory response; due to the confines of space, swelling or oedema cannot be tolerated, and the sensitive nature of neuronal cells means that neuroinflammation must be strictly controlled. Neuroinflammation occurs in response to a wide variety of insults to the brain, and can be defined here as an increase in activation of microglia and a change in cytokine profile, particularly an increase in proinflammatory cytokines.

In the context of the present studies the inflammatory process is discussed as a negative event, but it is an essential part of the innate immune response, and is critical in the efficient defence of the body from invading pathogens. Inflammation is primarily a protective process, but excessive or uncontrolled production of inflammatory mediators causing chronic inflammation is often degenerative (Fujihara *et al.*, 2003). This is true both peripherally and centrally.

1.2 Inflammation in the CNS

1.2.1 Resident brain cell contribution to CNS inflammation

Traditionally the CNS has been thought of as immunologically shielded by way of the blood brain barrier (BBB), with very few if any resident immune cells. However, it is now apparent that the BBB can be breached by immuno-active cells (for example activated T-cells; Hickey *et al.*, 1991). Perhaps more importantly it has become apparent that the resident cells of the CNS, namely glia and neurons, are active in mediating central inflammatory responses.

Until recently, neurons had been considered to play a passive role in inflammatory reactions. It now appears that neurons have an immune function; evidence demonstrates that neurons can express major histocompatibility complex (MHC) class I (Neumann *et al.*, 1997a), produce a range of cytokines including IL-1β (Minogue *et al.*, 2003), and IFN-γ (Neumann *et al.*, 1997b). Electrical activity of nerve cells appears to regulate MHC class II expression on surrounding glia (Neumann *et al.*, 1996) as well as MHC class I expression on neurons themselves (Neumann *et al.*, 1997a). It would appear then, that compromised nerve cells may have the ability to change the local microenvironment, by interaction with surrounding glia, to facilitate the recruitment of immune cells.

Oligodendrocytes, astrocytes and microglia are the three main classes of glial cell in the CNS, with the latter two actively participating in immune reactions. The role of astrocytes is to preserve normal nerve cell functioning by 1) provision of a support frame, 2) clearance of diffusing transmitter substances, 3) maintenance of ion homeostasis, and 4) production of neurotrophic factors (Piehl and Lidman, 2001). They also become activated in response to CNS insult, undergoing hypertrophy (astrogliosis), proliferation, and migration towards the site of injury, forming an insulating glial scar within weeks. They have the ability to express MHC class II, produce the regulatory mediator TGF-β, and induce T-cell apoptosis, suggesting a role in modulating/ restricting the inflammatory response in the CNS. Astrocytes can also secrete a number of neuroprotective components, to aid cell repair and growth. However the astrocytic response is slow compared with the activation of microglia.

Microglia/ CNS macrophages are thought to originate from the mesoderm, are similar to monocytes, and play an important role in immune surveillance and host defence. Microglia are extremely sensitive to changes in their microenvironment and are activated in all conditions that disrupt normal neuronal functioning (Kreutzberg, 1996). Activated microglia undergo morphological changes such as hypertrophy and proliferation, as well as

functional differentiation with upregulation of MHC antigens and other cell surface receptors. When activated, microglia secrete an array of inflammatory regulators such as cytokines, and chemokines, as well as more non-specific inflammatory mediators e.g. reactive oxygen species (ROS) and nitric oxide (NO). Some of the secreted factors are potentially neuroprotective, but the majority are proinflammatory and neurotoxic, such as IL-1β and TNF-α. At the end stage of activation microglia are phagocytic and act as the scavenger cells of the CNS. The neurotoxic potential of activated microglia is evidenced by their detection in post mortem analysis of AD and Parkinson's diseased brains in areas most affected by neurodegeneration (Rogers *et al.*, 1988; McGeer *et al.*, 1988), as well as their association with the pathogenesis of multiple sclerosis, amyotrophic lateral sclerosis, and other autoimmune disorders. In addition, the neurotoxic properties of many of the products of activated microglia are well documented.

1.2.2 Peripheral contribution to CNS inflammation

It is now well documented that peripheral cytokines can affect a central behavioural and biochemical response but how this is mediated is unclear. These cytokines have the ability to induce what is collectively described as sickness behaviour; fever, lethargy, increased sleep, reduced social activity, reduced mobility, decreased learning, anorexia, and decreased libido (Rothwell, 1991; Wilson *et al.*, 2002), as well as inducing complex intracellular signalling cascades. Centrally-acting cytokines have a central or peripheral source as they are either produced within the brain (due to peripheral stimulation), or transported from the periphery into the CNS. Both mechanisms are likely, as peripheral immune cells such as monocytes, macrophages, and neutrophils, as well as resident brain cells such as neurons and glia are sources.

Peripheral immune cells such as macrophages are a major source of cytokines, and they are stimulated by injury, insult or infection. Macrophages are potently activated by binding of lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria. The classical view of action is that a cytokine is released into the systemic circulation, and exerts central actions by entry into the CNS from the periphery. Cytokines and most immunomodulators are rather large molecules, and are unlikely to pass through the tight vasculature of the BBB under normal circumstances, however, it has been demonstrated that direct entry can occur at circumventricular regions (organum vasculosum lateralis terminalis) where the BBB is less stringent. Secondly, cytokines or cytokine producing cells could gain access to the CNS in circumstances when the BBB is

compromised. It has been suggested that cytokines affect the BBB, causing increased permeability and disruption. For example, binding of cytokines to receptors on endothelial cells of brain vasculature causes release of other mediators (endothelial cell adhesion molecules, chemokines, nitric oxide and prostaglandins) which lead to impairment of BBB integrity (Wong *et al.*, 1996; Anthony *et al.*, 1997). This increased permeability may therefore allow access of activated macrophages or cytokines into the CNS. In addition, active transport mechanisms across the BBB have been reported for IL-1 and TNF (Dunn, 1992; Gutierrez *et al.*, 1993). Entry of activated T-cells into the CNS is also documented (Hickey *et al.*, 1991), and as these cells are sources and modulators of cytokine production this is significant.

Peripheral stimulation of the vagal nerve causing induction of central responses has also been implicated as a mechanism contributing to entry of cytokines into the CNS (Dantzer *et al.*, 1998). Therefore there are a number of ways in which peripheral inflammation or stimulation can contribute to CNS inflammation.

1.2.3 T-cells and cytokines

T-cells are important sources of cytokines. T-cells are specialised white blood cells (leukocytes) which direct the ongoing immune response through the secretion of cytokines, which act as growth and differentiation factors for themselves and other cell types (Szabo *et al.*, 2003). The T helper (Th) cells are critical for appropriate immune response and can be classified into two major subsets, Th1 and Th2, although others have been described recently. Th1 cells secrete IL-2, IFNγ, TNFα and TNFβ and mediate a cellular immune response; fighting viruses and intracellular pathogens, as well as eliminating cancerous cells. Th2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13 and potentiate a humoral response for fighting extracellular organisms, and mediate allergic type reactions. Th1 and Th2 cells develop from naïve Th cells. T-cell differentiation is initiated when the T-cell receptor (TCR) on a naïve Th cell binds to its antigen bound to MHC class II molecules on an antigen presenting cell (APC). APCs include dendritic cells, tissue macrophages, and B-cells. The Th1 response is often viewed as proinflammatory; conversely Th2 is viewed as anti-inflammatory. Many factors influence the differentiation process toward the Th1 or 2 lineage, including the type of APC, the concentration of the antigen (duration and strength of signal), the ligation of select costimulatory molecules, and the local cytokine environment (Szabo *et al.*, 2003). It appears that IL-4 predominantly induces Th2 differentiation, while IFNγ, IL-12, IL-18, IL-23 and IL-27 are important in Th1

differentiation. So in essence, cytokines present in the local microenvironment of injury or infection determine what cytokines will be produced by influencing T-cell differentiation. Consequently, the cytokine profile in the CNS is determined by the production by microglia and neurons, as well as that of infiltrating T-cells.

1.3 Cytokines

Cytokines are a family of about 30 recognised non-antibody proteins that can be produced by a wide range of cell types and have a multitude of actions. They include the interleukins (IL-1 to IL-24), tumour necrosis factors (TNFs), transforming growth factors (TGF beta 1-3) and interferons (IFN) and generally act through specific receptors. They are potent signalling molecules and lead to changes in transcription that determine the outcome of cell survival or programmed cell death.

Various cytokines have been reported to be constitutively expressed in the brain under normal conditions, and also show a spatial variation across brain regions. Cytokines present in the brain include TNFα, IFNs, IL-1, -2, -3, -4, -6, -10, -12, -15, -18, transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factors (IGF), nerve growth factor (NGF) and brain-derived growth factor (BDNF; Hanisch, 2002). Cytokines appear to have a physiological role outside of coordinating responses to pathological stimuli (reviewed in Vitkovic *et al.*, 2000); these include neuromodulatory roles in CNS development and repair (e.g. IL-1, IL-6, and TNF-α), and modulation of synaptic plasticity in the developing brain (IL-1; Zhao *et al.*, 1998).

Cytokines can broadly be divided into pro- and anti- inflammatory cytokines. However there is much overlap in function of different cytokines, and the effects of a particular cytokine may change over time with early expression after an insult assisting in repair and survival, and later expression contributing to ongoing pathology, alternatively, the converse may occur (Allan and Rothwell, 2003). Indeed many cytokines are beneficial at low concentration but exhibit neurotoxic properties at higher concentration.

TNFα is a proinflammatory cytokine, and can be produced in the CNS by a number of cell types including neurons, astrocytes and microglia, and is observed after injury, ischaemia, or bacterial infection as well as in multiple sclerosis and AD. The harmful effects of TNFα relate to its ability to promote inflammation and oedema (see Reimold, 2002), in addition to direct toxic effects on neurons (Gelbard *et al.*, 1993; Talley *et al.*, 1995). However it appears that TNFα is important in promoting cell survival; animals

deficient in TNFα are more vulnerable to neuronal damage, possibly linked to an impaired NGF induction (Bruce *et al.*, 1996). So it seems that low levels of TNFα are neuroprotective and necessary but high levels can be destructive (Hanisch, 2002). IL-6 is another proinflammmatory mediator, and can be induced by IL-1β, and can cause induction of acute phase proteins, increases in vascular permeability, lymphocyte activation, and reactive gliosis. Mice deficient in IL-6 show sensory impairments and impaired axonal regeneration, while over expression induces ataxia, tremor, motor impairment, seizures, upregulation of ICAM1, TNF-α, IL-1β, and impairment in synaptic plasticity associated with a defect in avoidance learning (Hanisch, 2002). It is apparent using these two 'proinflammatory' cytokines as examples that their presence is necessary for normal cell functioning and that therapies should aim to control the excessive or chronic expression without compromising basal levels.

IL-4, IL-10, IL-13 and TGFβ conversely exhibit antiinflammatory, immunosuppressive and neuroprotective actions, mainly by down-regulating inflammatory cytokines (Dinarello, 1997a). IL-4 and IL-13 also interfere with IL-1 bioactivity as they promote IL-1ra synthesis (Dinarello, 1997b). These cytokines also influence the expression of microglial cell surface molecules, as well as having more specific roles in influencing B-cells, mast cells and thymocytes (Hanisch, 2002).

In terms of the inflammatory response leading to neuronal damage, cytokines have been shown to activate glial cells, cause self-induction, initiate the complement cascade, cause excitotoxic glutamate receptor-mediated damage, change Ca^{2+} entry and neurotransmitter release in neurons, cause oxidative stress, and induce nitric oxide.
Increases in TNFα and IL-1β as well as other inflammatory cytokines are observed in response to neuronal insult, however presence after injury does not prove a detrimental role. More convincing is the fact that treatment with TNFα or IL-1β exacerbates injury already present and that specific inhibition attenuates the amount of cell death that occurs (Viviani, 2004).

1.4 Interleukin 1β (IL-1β)

1.4.1 IL-1β

One of the most important cytokines produced in the CNS in terms of proinflammatory reaction is IL-1β. IL-1β is one of a family of cytokines produced by immunoactive cells, such as microglia, mediating systemic host responses. The IL-1 family comprises of the two agonists IL-1α and IL-1β, and the receptor antagonist IL-1ra,

as well as a host of newly-described family members (Smith *et al.*, 2000). IL-1β is formed as its precursor pro-IL-1β (33 kDa), which is inactive. It is cleaved to its active mature form (17.5kDa) by IL-1β converting enzyme (ICE; also known as caspase 1). Actions of IL-1 are believed to be mediated through actions of a transmembrane 80kDA type I receptor which has a similar affinity for IL-1β and IL-1α. The type I receptor has the ability to initiate intracellular events upon IL-1 binding, but requires an accessory protein for signal transduction (Rothwell, 1999). A second receptor is the type II receptor which is a 68kDa protein, and was thought to have no ability to initiate intracellular signal transduction events, and probably serve as a 'decoy' receptor, however this is now under dispute as IL-1 signalling has been shown to occur in IL-1R1 deficient mice (Parker *et al.*, 2002). A number of other IL-1 receptors have recently been described (Born *et al.*, 2000).

It is believed that cytokines exert a number of neuromodulatory effects within the CNS under both physiological and pathological conditions. The actions of IL-1β centrally are manifold, including modulation of EEG and neuronal activity, induction of NGF, self induction (of IL-1β), astrogliosis, increased metabolic rate, peripheral IL-6 release, and leukocytosis, as well as a number of effects that can be collectively described as sickness behaviour; fever, lethargy, increased sleep, reduced social activity, reduced mobility, decreased learning, anorexia, and decreased libido (Rothwell, 1991; Wilson *et al.*, 2002). Maximal change in body temperature in response to IL-1 occur at lower doses after injection into the brain than when injected peripherally; in conscious rats, peripheral injection of 1-2μg recombinant IL-1 elicits maximal increases in body temperature and metabolic rate, but doses of only 5-10ng are required if injected centrally (Rothwell, 1991). Concurrent with the idea of IL-1β as a neuromodulator is the finding that IL-1 receptors have been shown to be present in many brain regions with high concentrations in the hippocampus and hypothalamus (Ban *et al.*, 1991; Wong and Licinio, 1994), while IL-1R1 mRNA expression has been demonstrated in perivascular cells and some neuronal cell groups (Ericsson *et al.*, 1995).

IL-1β seems to be the main IL-1 agonist induced in the CNS in response to systemic (e.g. injury, infection, peripheral administration of LPS), or local (e.g. injury or stroke) insults (Rothwell, 1999). IL-1β can elicit an array of responses which can potentially either inhibit, exacerbate or induce neuronal damage and death. These include induction of fever and oedema, damage to the cerebral vasculature, activation of glia, induction of neurotrophins, growth factors/ phospholipase A_2, cyclooxygenase, β-amyloid precursor protein, adhesion molecules and corticotrophin-releasing factor, release of nitric

oxide and other free radicals, activation of complement and modification of calcium homeostasis (Rothwell, 1999). Some of these responses serve to enable tissue repair to take place in an efficient manner, while some cause more tissue damage. However it should be stressed that infusion of IL-1β into the brain of normal rats or application to neuronal cultures does not cause overt damage or death to cells (Rothwell, 1999). It appears that IL-1β exacerbates damage to compromised neuronal cells, and that it may depend on other factors present in the damaged brain (Viviani, 2004; see Rothwell, 1999). Although the etiology of CNS disorders vary, IL-1*β* does seem to be a common factor in processes leading to neurodegeneration.

1.4.2 IL-1*β* signalling

The outcome of IL-1 signalling is a change in gene expression (O' Connor and Coogan, 1999); IL-1β stimulation can result in upregulation of a great many genes such as cytokines, growth factors, and acute-phase proteins. When IL-1 binds to IL-1R1, the IL-1 receptor accessory protein (IL-1RAcP) is recruited to form a high affinity receptor complex. This receptor complex in association with MyD88 can recruit several kinases; IL-1 receptor associated kinase (IRAK), or TGFβ activated kinase (TAK). IRAK and TAK can go on to associate with tumour necrosis factor (TNF) receptor associated kinase (TRAF6), causing activation of the transcription factors NF-κB (Cao *et al.*, 1996) and AP-1 as well as mitogen-activated protein kinases (MAPKs; Baud *et al.*, 1999). NF-κB activation is caused by activation of IκB kinase which phosphorylates IκB, marking it for degradation. IκB usually sequesters the p50 p65 hetrodimer NF-κB in the cytosol, thereby inactivating it. When IκB is degraded, NF-κB is released and can translocate to the nucleus, bind to its nuclear κB site and activate gene transcription, leading to expression of proteins such as IL-6, cyclooxygenase-2, inducible nitric oxide synthase, IL-1β, TNF and interferons (O'Neill and Kaltschmidt, 1997). IL-1*β* can also activate a number of protein kinase cascades. One family of protein kinases that have been very strongly implicated in IL-1 signalling are the MAPKs (Vereker *et al.*, 2000b).

1.5 Mitogen Activated Protein Kinases (MAPK)

There are three families of MAP kinase; the extracellular signal regulated kinases (ERKs); the p38 kinases; and the c-jun N-terminal protein kinases (JNKs). The ERKs are activated in response to growth factors and are believed to be mainly involved in cell growth and differentiation, and are often associated with a neuroprotective role. The p38

kinases and the JNKs are both activated in response to stressful situations e.g. deprivation of trophic factors, or exposure to free radicals or LPS, as well as ischemia, heat shock, osmotic stress, ultra violet (UV) irradiation, and hypoxia, and are usually associated with a neurodegenerative role (Mielke and Herdegen, 2000). p38 is undoubtedly important in mediating cellular responses to stress or inflammation, however for the purpose of these studies, JNK and related signalling will be examined in more detail.

1.6 c-Jun N-terminal Kinase (JNK)

1.6.1 JNK

JNKs are a family of kinases of the mitogen-activated protein kinase group. JNKs are also termed stress-activated protein kinases (SAPK), as they were first described as being activated in response to various environmental stresses e.g. deprivation of trophic factors, or exposure to free radicals or LPS, as well as ischemia, heat shock, osmotic stress, UV irradiation, and hypoxia (Mielke and Herdegen, 2000). JNK is activated in response to environmental stress, by the engagement of several classes of cell surface receptors, including cytokine receptors, serpentine receptors, and tyrosine kinase receptors (Whitmarsh and Davis, 1996).

As a rule MAPK activation (phosphorylation) is mediated by a MAPK kinase (MAPKK/ MKK), which is itself activated by phosphorylation by a MAPKK kinase (MAPKKK/ MEKK), creating a kinase-signalling cascade. These pathways however may be stimulated at any point along the cascade. The JNK pathway consists of MEKK1 or 4, MKK4 or 7 (Yang *et al.*, 1997a; Tournier *et al.*, 1999), and JNK sequentially.

JNK is so called because it binds the NH_2-terminal activation domain of the transcription factor c-Jun, and phosphorylates it on Ser63 and Ser73. However JNK has a wide variety of substrates, both transcription factors and non-nuclear proteins (see Meilke and Herdegen, 2000). JNK is encoded by three different genes: jnk1, jnk2, and jnk3. While jnk1 and 2 are ubiquitously expressed, jnk3 appears to be restricted to the brain, testis and heart (Gupta *et al.*, 1996). There are many splice variants giving a total of 10 JNK variants (four JNK1s, four JNK2s, and two JNK3s). Whilst differences between the 10 protein products are not defined, it is suggested that the isoforms differentially regulate and phosphorylate their transcription factors. It is important to note that in the brain all three isoforms are predominantly present in their activated forms; under normal conditions basal JNK activity is 15-30 fold higher in the brain than in other tissues (Borsello and Bonny, 2004). This high basal level of activity suggests that JNK has a physiological role

in the nervous system, however the function of JNKs have yet to be defined but gene knockout studies are creating a clearer picture.

JNK1 or 2 knockout mice have been shown to be deficient in T-helper (CD4+) cell function (Bogoyevitch *et al.*, 2004). More specifically loss of JNK1 resulted in T-cells that hyperproliferated, showed less activation-induced apoptosis, and differentiated preferentially into helper cell Th2 antigen-presenting cells (Dong *et al.*, 1998). Loss of JNK2 resulted in impaired interferon production by Th1 cells and also impaired T-cell activation and apoptosis (Yang *et al.*, 1998; Sabapathy *et al.*, 1999). Compound JNK1 and 2 knockouts are not viable due to deregulation of apoptosis in the developing brain (Kuan *et al.*, 1999). It appears that JNK1 and 2 have both pro- and anti-apoptotic roles in the developing brain. JNK3 appears not to be important for neuronal development, as JNK3 knockouts are born normally with no apparent abnormal phenotype. However, JNK3 knockout mice have been shown to be more resistant to kainic acid-induced excitotoxic death (Yang *et al.*, 1997b) as well as exhibiting a reduced stress-induced JNK activity, and decreased brain injury following cerebral ischaemia-hypoxia (Kuan *et al.*, 2003). This would support the concept of selectively targeting JNK3 in prevention of neurotoxicity

1.6.2 JNK and inflammation

JNK is strongly implicated in inflammatory processes, in particular that associated with autoimmune disease. Increased JNK activation is reported in synoviocytes and synovium of joints suffering rheumatoid arthritis (RA; Han *et al.*, 1999; Han *et al.*, 2001), oligodendrocytes at the edge of active multiple sclerosis lesions (Bonetti *et al.*, 1999), and in T-cells, microglia and astrocytes in experimental autoimmune encephalomyelitis (EAE; Shin *et al.*, 2003). JNK also appears to play a role in allergic cellular inflammation (Eynott *et al.*, 2003) and is implicated in other neurodegenerative disorders, often linked to chronic inflammation; for example, JNK activation is increased in degenerating neurons in AD (Zhu *et al.*, 2001), while elimination of JNK prevented neurodegeneration in a mouse MPTP model of Parkinson's disease (Hunot *et al.*, 2004).

1.6.3 JNK-mediated signalling

JNK is of particular interest due to its undefined involvement in both the cell death pathway, and cell survival, as well as inflammatory processes. JNK activation was first correlated with apoptosis in 1995 in the model of apoptosis resulting from nerve growth factor withdrawal from the PC12 neuronal-like cell line (Xia *et al.*, 1995).

Activation of JNK leads to the modification of many substrates. Cytoplasmic targets include bcl-2 (Maundrell *et al.,* 1997), tau (hyperphosphorylation of which is a hallmark of neurodegenerative disease e.g. AD; Reynolds *et al.*, 1997), and glucocorticoid receptors (phosphorylation by JNK causes inactivation, halting glucocorticoid receptor mediated transcriptional activation). Active JNK also has nuclear targets, namely transcription factors including c-Jun (a target unique to JNK; Gupta *et al.*, 1996), activating transcription factor 2 (ATF2) as well as other activator protein 1 (AP-1) proteins, ELK1, c-Myc, and the tumour suppressor gene p53 (Fuchs *et al.,* 1998). In addition, JNK can regulate the turnover of p53 by stabilising it, resulting in an increase in Fas and bax expression, a decrease in bcl-2 expression, and possible increases in ROS, all of which have been linked to apoptosis.

JNK and c-Jun can be linked with both neurodegeneration and neuroprotection (Herdegen *et al.*, 1997; Ip and Davis, 1998), and the eventual outcome seems to be dependent on parallel cascade activation (e.g. ERK), availability of other transcription factors, and other physico-chemical parameters that alter the balance of pro and anti degeneration intracellular programmes. Recently much emphasis has been put on the role of JNK in apoptosis as sustained activation is associated with apoptosis in many cell types such as Jurkat T cells (Chen *et al.*, 1996), endothelial cells (Hu *et al.*, 1999), HeLa cells (Wang *et al.*, 1998), and rat mesangial cells (Guo *et al.*, 1998). Moreover, JNK activation is increased in degenerating neurons in Alzheimer's disease (Zhu *et al.*, 2001). Further evidence of JNK involvement in cell death pathways is that inhibition of JNK prevents apoptosis of multiple myeloma cells (Xu *et al.*, 1998), death of sympathetic neurons upon NGF withdrawal (Eilers *et al.*, 2001), and IL-1 induced apoptosis of pancreatic β-cells (Bonny *et al.*, 2001), while more recently JNK inhibition protected against NMDA-mediated excitotoxicity *in vitro*, and decreased lesion volume from cerebral ischaemia *in vivo*, with an associated improved performance in a rotarod test (Borsello *et al.*, 2003). Although JNK is known to be important in this process its precise role is as yet undefined.

1.7 Cell Death pathways

1.7.1 Apoptosis

Apoptosis is a complex biochemical process, which can be stimulated by a plethora of agents. However all apoptotic pathways are believed to converge on the activation of caspases which lead to the characteristic morphological changes associated with apoptosis first described in the 70's. These changes include condensation and fragmentation of the

nucleus, shrinkage of the cytoplasm, and formation of apoptotic bodies. Apoptosis refers both to the actual process and the morphological changes, and occurs in all tissues. This process, in contrast to necrosis, does not induce inflammation. Although many factors contribute to the progression of apoptosis, for the purpose of these studies the focus shall be on how JNK may influence this process. The cell death pathway can roughly be divided into a mitochondrial pathway and a mitochondrial independent pathway.

1.7.2 Mitochondrial pathway

Permeability of the mitochondrial membrane is a key issue in regulation of apoptosis. Certain mediators of cell death reside within mitochondria and can be released through disruption in the outer mitochondrial membrane. It is these mediators which then proceed to activate the caspases leading to the DNA degradation which is characteristic of apoptosis. The bcl-2 family of proteins are cytosolic proteins associated with the outer mitochondrial membrane that have the ability to form homo and heterodimers. These proteins can be divided into repressors (e.g. bcl-2, bcl-X_L, Mcl-1, bcl-w, boo and A1) and promoters (e.g. bax, bcl-X_S, bak, bid, bim, bad, bok, Diva, bik, hrk, Blk, Bnip3 and Bnip3) of apoptosis, and their function appears to centre around their ability to influence membrane permeability, however the mechanisms by which they act remain controversial (for review see Harris and Thompson, 2000). A number of models of action have been proposed, such as the formation or modulation of specific channels or alternatively, induction of non-specific rupture of the outer membrane due to matrix swelling.

The voltage dependent anion channel (VDAC) spans the outer mitochondrial membrane, and while it is highly permeable to molecules up to 5000 daltons while open, the outer mitochondrial membrane is still impermeable to the apoptosis promoting proteins. One proposed mechanism of action of bcl-2 family members relates to their interaction with VDAC; one study showed that bax association with VDAC resulted in formation of a channel with a larger pore size than either VDAC or bax alone, and that had increased permeability to pro-apoptotic substances (Shimizu *et al.*, 2000). There is also evidence that bcl-2 family members (e.g. bax and bid) can form independent channels in lipid membranes capable of releasing cytochrome c, the most recognised of these being bax in a multimeric state (Jurgensmeier *et al.*, 1998), although this has yet to be observed *in vivo*.

Another possibility is that bcl-2 family members influence the permeability transition (PT) pore, which is made up of the VDAC in the outer membrane and the

adenine nucleotide translocator (ANT) in the inner membrane, opening of which causes matrix swelling. Matrix swelling, which leads to non-specific membrane rupture and subsequent non-specific release of apoptotic mediators, can be caused by a number of apoptotic stimuli, and may be both caused and alleviated by bcl-2 family members.

The bcl-2 family members share a similar structure and have between 1 and 4 bcl-2 homology (BH) domains. A subclass of the pro-apoptotic bcl-2 family, the BH3-only proteins, appear to have a modulatory role acting on their multi-domain relatives. They can act on bax and bak promoting their proapoptotic activity (e.g. bid and bim), or they can act on bcl-2 or bcl-XL blocking their antiapoptotic activity (e.g. bad and bik; Kuwana and Newmeyer, 2003). As suggested by these authors, it is tempting to hypothesize that the response to each type of apoptotic stimulus is mediated through the differential activation of the wide range of BH3-only proteins, determining the effectiveness of the multidomain proteins.

However the regulation process occurs, it is generally accepted that pro-apoptotic bcl-2 proteins promote release of pro-apoptotic mediators and that the anti-apoptotic members prevent this release. While it has been shown that bcl-2 /bcl-xL and bax/bik can exert their effects independently, it is thought that in normal circumstances bcl-2 outnumbers the pro-apoptotic members and forms heterodimers with them, thereby inactivating their pro-apoptotic effect; this association promotes decreased permeability. Therefore, in theory, increased permeability could be caused by an increase in expression of pro-apoptotic members, a decrease in bcl-2, or an alteration in bcl-2 that interferes with dimerisation. JNK could affect this in a number of ways. JNK stabilises and activates p53 (transcription factor; Fuchs *et al.*, 1998), which suppresses bcl-2 expression and enhances bax expression (Miyashita *et al.*, 1994). It has also been shown that activated JNK can cause phosphorylation of bcl-2 (Maundrell *et al.*, 1997) and that this antagonises the anti-apoptotic action of bcl-2 (Park *et al.*, 1997; Halder *et al.*, 1995; Yamamoto *et al.*, 1999). However it should be noted that the result of bcl-2 phosphorylation remains controversial; while the studies just mentioned correlate bcl-2 phosphorylation with apoptosis, some studies in other cell lines correlate bcl-2 phosphorylation with its antiapoptotic function (Deng *et al.*, 2001; Ito *et al.*, 1997; Horiuchi *et al.*, 1997). There are suggestions that bim, a BH-3 only member of the bcl-2 family, is also an important participant in JNK-dependent apoptosis in neurons; following NGF withdrawal, bim contributes to bax-dependent cytochrome c release and apoptosis of neurons, while under these conditions

dominant negative c-Jun decreases bim expression and promotes neuronal survival (Whitfield *et al.*, 2001).

With increased permeability of the mitochondrial membrane, substances that normally reside in the intermembrane space can translocate into the cytosol. Proteins of note that normally reside within the mitochondrion include cytochrome c, Smac/ DIABLO and apoptosis inducing factor. Once in the cytosol, cytochrome c associates with ATP, apoptotic protease activation factor-1 (Apaf-1) and the precursor of caspase 9, causing production of the active form of caspase 9, a mediator caspase, initiating the caspase cascade, which ultimately leads to apoptosis.

1.7.3 Mitochondrial independent pathway

JNK also has the ability to activate the mitochondrial independent cell death pathway; Phosphorylation of the transcription factor c-Jun leads to expression of c-jun, and Fas ligand (FasL). Fas, also known as Apo-1 or CD95, is a death domain-containing member of the tumour necrosis factor receptor superfamily. Fas is constitutively expressed in several different cell types and modulated by cytokines, but FasL is tightly regulated, induced only under certain circumstances, except in immune-privileged areas (Zimmermann *et al.*, 2001). Fas and FasL are involved in the attenuation of immune responsiveness through the deletion of activated peripheral lymphocytes (Ju *et al.*, 1995), and mutations of these molecules result in autoimmune diseases. They are also strongly implicated in cell death signalling,

When FasL binds to its receptor complex (Fas) this induces trimerisation. This causes formation of the Death Inducing Signalling Complex (DISC). First the adaptor protein Fas Associated Death Domain (FADD) binds via its death domain to the death domain of the Fas receptor complex. FADD also has a death effector domain (DED) which allows it to couple to downstream effectors (see Figure 1.1). The DED recruits procaspase-8. The aggregation of two or more procaspase-8 molecules allows processing to the mature, active form of caspase 8 (Zimmermann *et al.*, 2001). Caspase 8 can then proceed to trigger the apoptotic caspase cascade. Caspase 8 can also lead to activation of the mitochondrial pathway as it has the ability to cleave Bid (a Bcl2 family member) and thus induce its translocation to mitochondria where it triggers cytochrome *c* release (Li *et al.*, 1998). FADD can also bind the Fas death domain associated protein (Daxx). The recruitment of Daxx has been shown to lead to activation of JNK and downstream apoptosis (Yang *et al.*, 1997c), completing a positive feedback loop.

1.7.4 Caspase cascade

Active caspase 8 or 9 causes activation of caspase 3, an effector caspase. Activation of effector caspases (2, 3, and 7) generally results in an irreversible commitment to cell death. Caspases degrade cytoskeletal structures (such as actin, lamin, and alpha-fodrin), nuclear proteins (including the DNA repair enzymes poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase), as well as activating two factors that lead to DNA fragmentation; DNA fragmentation factor and an inhibitor of the caspase-activated DNAse (reviewed in Blatt and Glick, 2001). Caspase 3 can also activate other caspases, as well as p21-activated kinase (leading to further activation of JNK), and can cleave members of the bcl-2 family (see Blatt and Glick, 2001). The increase of DNA fragmentation and decreased cell integrity eventually lead to the appearance of the characteristic signs of apoptosis. The debris created by this process may further potentiate the activation of microglia, thereby sustaining the chronic aspect of inflammation.

1.8 Inflammation and cognitive decline

It might be predicted that these degenerative effects of sustained inflammation, would have some functional effect on actual cognition. This is indeed the case as observed in various models of inflammation; IL-1β has been shown to disrupt the acquisition of spatial learning information in the Morris water maze (Oitzl *et al.*, 1993). For instance, IL-1β mediated memory impairment (Palin *et al.*, 2004), IL-6 overexpression was associated with deficits in avoidance learning (Heyser *et al.*, 1997), while a decline in cognitive function with age, including memory related functions, is well documented (Lynch, 1998). Moreover, the well-known dementia exhibited by AD patients is linked to the inflammatory processes of reactive astrogliosis and microglial activation (Licastro and Chiappelli, 2003). Normal cognitive functioning is reliant on synaptic efficacy. One way to assess synaptic efficacy is by analyzing the ability of neurons to sustain a type of synaptic plasticity called long-term potentiation, often proposed as a model for memory formation (Bliss and Colingridge, 1993; Lynch, 2004).

1.9 Long-Term Potentiation (LTP)

1.9.1 LTP

Synaptic plasticity is a term used to describe a change in synaptic function or efficacy in response to some stimulus, and can be defined as a change or modulation in synaptic transmission at a particular synapse. This modulation is a feature of the adult

brain as well as the developing brain and it is generally accepted that information is acquired (learning) and retained (memory) in this way. Long-term potentiation (LTP) is one form of synaptic plasticity, and as the name suggests it is a long lasting 'strengthening' of the synaptic transmission (hours to weeks). This 'strengthening' can be represented by assessing the effect of tetanic stimulation on excitatory postsynaptic potentials (epsps: the postsynaptic change in electrical potential following afferent stimulation of a synapse). Low frequency stimulation (LFS; e.g. 0.33Hz) of the afferent input for extended periods of time elicits a synaptic response (epsp) that remains stable in terms of slope and amplitude. High frequency stimulation (HFS; 250Hz) for a short period of time (often there are 3 or 4 trains of stimulation [theta bursts] in one second) induces a short-lived tetanus (i.e. summation of individual epsps to a high membrane potential). On return to LFS, an increase in the amplitude and slope of the epsp shows that the synaptic response has been strengthened, i.e. LTP has been induced. This is true if recording is made from individual cells or populations of cells. LTP can be induced by a number of stimuli including high frequency stimulation/ titanic stimulation (Bliss and Lomo, 1973), patterned stimulation (Larson *et al.*, 1986), or by chemical means such as ATP (Wieraszko and Seyfried, 1989), an intracellular increase in calcium (Malenka *et al.*, 1988), and BDNF perfusion (Messaoudi *et al.*, 2002).

1.9.2 LTP and the hippocampus

The hippocampal formation lies below the cortical structures and consists of the dentate gyrus, hippocampus, and subiculum, and is part of the limbic system. There are 3 major pathways of neuronal projection associated with the hippocampus. The perforant pathway projects from the entorhinal cortex of the temporal lobe and synapses with the dendrites of granule cells of the dentate gyrus. The mossy fibres of the dentate gyrus project to the pyramidal cells of the Cornus Ammon (CA) 3 region. These cells input to the CA1 pyramidal cells via the Schaffer collateral tract, which in turn project to the subiculum. (See Figure 1.2)

LTP was first characterised and found to be sustained at the synapses formed between the perforant path and the granule cells in the dentate gyrus of the hippocampus of anaesthetised rabbits (Bliss and Lomo, 1973). Since then LTP has been shown to occur in a wide variety of brain regions (e.g. amygdala, subiculum, cortex, and striatum), and in the other two main afferent pathways of the hippocampus; the mossy fibres projection from

the dentate gyrus to the CA3 region, and the Schaffer collateral tract from CA3 to CA1 region.

It is interesting that such a robust and stable form of LTP is found to occur in the hippocampus, a brain region long associated with learning and memory. Since 1957, when Scoville and Milner observed that damage to the hippocampus caused impairment in learning and memory in humans, the hippocampus has been known to be very important in memory formation. Early research showed a particular role in spatial learning and memory (Barnes, 1988; Richter-Levin *et al.*, 1995), and evidence still indicates a role in memory (See review Lynch, 2004, Martin and Morris, 2002). Several factors discussed below led to consideration of LTP as a cellular model of memory formation.

1.9.3 LTP and Memory

LTP, like memory, is associated with hippocampal function, is induced rapidly, and shows a lasting incremental response to short bursts of activity at specific synapses; these properties are all consistent with a natural memory mechanism (Eichenbaum, 1995). Importantly, the three basic properties of LTP, i.e. co-operativity, associativity and input-specificity point towards a possible involvement in memory (Bliss and Collingridge, 1993). Co-operativity means that for LTP to occur two things must happen at the same time, namely depolarisation of the postsynaptic membrane and neurotransmitter stimulation at the synapse. Strong depolarisation occurs due to frequent, fast stimulation leading to activation of a large number of afferent fibres but not with stimulation which affects only a few afferent fibres. Therefore evidence that there is an intensity threshold for induction exists (McNaughton *et al.*, 1978). Associativity means that a 'weak' input can induce LTP if it occurs at the same time as a 'strong' tetanus at a separate but convergent input on the same neuron (McNaughton *et al.*, 1978). This is essentially a cellular analogue of Hebbian classical conditioning. Finally, LTP is input-specific because only the inputs active at the time of tetanus are potentiated (Anderson *et al.*, 1977; Lynch *et al.*, 1977).

In further support of the role of LTP as a biological substrate for memory is the observation that synchronised firing patterns at frequencies similar to those required to induce LTP have been recorded in the hippocampus during learning and exploratory behaviour (see Bliss and Collingridge, 1993) and that many factors that inhibit LTP in the hippocampus also inhibit learning (see review Lynch, 2004).

There is evidence that similar mechanisms underlie both LTP and memory. These include the requirement for activation of NMDA receptors (Morris *et al.*, 1986; Tsien *et al.*, 1996; Kentros *et al.*, 1998). Furthermore, an increase in glutamate release, an increase in PIP_2 hydrolysis and an increase in aspartate in the CA1, CA3, and dentate gyrus of the hippocampus (Richter-Levin *et al.*, 1995; Laroche *et al.*, 1990; Lynch *et al.*, 1990; Redini-Del Negro and Laroche, 1993) as well as involvement of CaMKII, ERK, and PKC (see section 1.9.6) are associated with both LTP and memory (for review see Lynch, 2004).

Interestingly the temporal aspect of memory and LTP are also comparable. Short term memory lasts for several hours and is independent of protein synthesis, while long term memory lasts for days or longer and is linked to and dependent on gene expression, protein synthesis, and new synaptic associations (Grecksch and Matthies, 1980; Quevedo *et al.*, 2004). LTP also comprises of distinct phases; the early phase (E-LTP) lasts several hours and is independent of protein synthesis, while long lasting LTP (L-LTP) persists for days to weeks and requires protein synthesis (Krug *et al.*, 1984; Stanton and Sarvey, 1984).

1.9.4 Mechanisms of LTP

LTP has been proposed to occur due to an increase in efficiency of synaptic transmission as well as an increase in the excitability of the postsynaptic cell indicating the involvement of both pre and post -synaptic mechanisms. For ease of discussion LTP is divided into two components, its induction and its maintenance/ expression. The induction of LTP describes the changes that take place during tetanisation (or other appropriate stimuli) that cause the effect to be established, while the maintenance describes the underlying mechanisms which maintain LTP over longer periods. For the purpose of these studies the focus is on LTP sustained in the hippocampus.

1.9.5 Induction of LTP

1.9.5.1 NMDA receptor

The induction of LTP depends on strong depolarisation of the postsynaptic membrane at the same time as synaptic stimulation. It is thought that a major role is played by the NMDA receptor (ionotropic), one of 3 subtypes of glutamate receptors. The NMDA receptor is found to be distributed densely in the CA1 field, and its associated ion channel has been shown to have a high Ca^{2+} permeability as well as Na^{+} and K^{+} permeability (MacDermott *et al.*, 1986). The NMDA receptor is made up of glutamate binding sites with an associated cation channel, plus a glycine binding site and polyamine

binding site. However, it is unusual because it is both voltage dependent *and* agonist dependent. Binding of its agonist causes a conformational change in the channel from closed state to open state, however at resting membrane potential (-70mV) any movement of ions through the channel is blocked by the presence of Mg^{2+}. However, if agonist binding is coupled with sufficient depolarisation of the membrane, the Mg^{2+} block is removed allowing Ca^{2+} and Na^{+} into the cell and K^{+} out of the cell (MacDermott *et al.*, 1986). The co-operativity threshold therefore results from the need for depolarisation to remove the Mg^{2+} block of the channel. Associativity is accounted for in this model – depolarisation can arise from a separate input, allowing induction of LTP at a weakly stimulated synapse. Input specificity is also accounted for by the need for a sufficient concentration of glutamate to be present at a specific synapse to activate adequate numbers of NMDA receptors. This model therefore correlates with observed requirements for LTP induction.

Interest in this receptor began when it was observed that D-2-amino-5-phosphonopentanoate (AP-5), a NMDA antagonist, induced learning deficits and blocked induction of LTP (Collingridge *et al.*, 1983), and subsequently activation of NMDA receptors was shown to be required for LTP (Morris *et al.*, 1986; Tsien *et al.*, 1996; Kentros *et al.*, 1998). However NMDA blockade appeared to have no effect on normal transmission or even maintenance of previously established LTP indicating that NMDA receptors mediate LTP induction but not maintenance. LTP was shown to be NMDA receptor dependent in all subfields of the hippocampus except the mossy fibres from the dentate gyrus to CA3 (Bliss and Collingridge, 1993). While NMDA receptor activation is not involved in all forms of LTP, an increase in intracellular Ca^{2+} is a common factor.

1.9.5.2 Calcium involvement

It is thought that the key signal for LTP induction is influx of Ca^{2+}. Thus LTP induction was blocked by an intracellular injection of the Ca^{2+} chelator EGTA into hippocampal pyramidal cells (Lynch *et al.*, 1983), or if Ca^{2+} was removed from the extracellular fluid (Dunwiddie and Lynch, 1979). Furthermore, sudden high intracellular Ca^{2+} concentrations, facilitated by photolysis of nitr-5 injected into CA1 hippocampal cells, were sufficient to induce LTP (Malenka *et al.*, 1988). Ca^{2+} influx is most likely augmented by release of Ca^{2+} from intracellular stores as evidenced by thapsigargin treatment which depletes intracellular Ca^{2+} and was shown to impair LTP (Harvey and Collingridge, 1992). Intracellular Ca^{2+} release could result from activation of the

metabotropic glutamate receptors which can lead to G-protein linked activation of phospholipase C (PLC), and the formation of the second messenger IP_3, which in turn causes the intracellular release of Ca^{2+}.

1.9.6 Maintenance of LTP

Maintenance of LTP has been proposed to occur due to an increase in efficiency of synaptic transmission as well as an increase in the excitability of the post synaptic cell suggesting both post and pre-synaptic mechanisms. Presynaptically, this could functionally occur via enhancement of neurotransmitter release or an increase in the probability of release. Postsynaptically, modifications such as an increase in receptor numbers or sensitivity, increases in conduction of particular channels, increases in number of functional synapses, or other morphological or metabolic modifications could account for the observed changes. It is likely to be a combination of these factors. But how are these long-term changes induced?

As LTP induction results in an increase in Ca^{2+} levels, it is not surprising that numerous Ca^{2+} dependent enzymes have been proposed to play a part in converting the Ca^{2+} signal into persistent modifications of synaptic efficacy. Two of the more extensively studied proteins include protein kinase C (PKC) and Ca^{2+}/calmodulin dependent kinase II (CaMKII).

PKC inhibitors block LTP (see Bliss and Collingridge, 1993), and PKC activation, following blockade of NMDA receptors, rescues LTP in anaesthetised mice (Kleschevnikov and Routtenberg, 2001), pointing towards an important role for PKC. However, PKC is not sufficient to induce LTP and its precise role remains to be elucidated although it has been shown to influence the ERK signalling pathway (Corbit *et al.*, 2000).

CaMKII has been extensively studied in relation to LTP. CaMKII is strongly expressed in postnatal forebrain structures such as the hippocampus and cortex both pre- and post- synaptically. CaMKII activation has been shown to be associated with LTP and its active form is sufficient to induce potentiated synaptic transmission and occlude LTP (Pettit *et al.*, 1994), additionally, persistent activation is observed following induction of LTP and this is attributed to autophosphorylation on the Thr286 residue (Giese *et al.*, 1998). The significance of CaMKII involvement is also evidenced by the fact that animals treated with inhibitors of CaMKII (Malenka *et al.*, 1989), transgenic mice deficient in CaMKII (Silva *et al.*, 1992) and animals with mutated Thr286 (Giese *et al.*, 1998) all exhibit impaired LTP and spatial learning. CaMKII may cause a postsynaptic increase in

excitability directly as it has the ability to phosphorylate non-NMDA receptors (at position 831 on the GluR1 subunit), increasing the channel conductance (Derkach *et al.*, 1999). CaMKII phosphorylates a number of cytoskeletal proteins, such as neurofilament-L (Hashimoto *et al.*, 2000) possibly underlying the morphological changes observed with LTP. CaMKII has also been implicated in the delivery of AMPA receptors to the spine following LTP induction as outlined in the silent synapse theory (see below).

The silent synapse theory proposes that numbers of synapses are not electrophysiologically functional (i.e. silent) due to the fact that they have only NMDA receptors post synaptically. So presynaptic activation causes glutamate release but, due to the properties of NMDA receptors, no synaptic transmission occurs if the post-synaptic membrane is at resting potential. Normal synapses coexist with these silent synapses and can mediate depolarisation via non-NMDA receptors (e.g. AMPA). If this depolarisation is sufficiently strong and concurrent with glutamate release at the silent synapses, the NMDA receptor channels can be activated. This influx of Ca^{2+} is thought to trigger the trafficking of AMPA receptors from the endoplasmic reticulum to the post synaptic membrane, converting the synapse from a silent to an active synapse. This model can account for some properties of LTP.

Ca^{2+} influx leading to elevated intracellular Ca^{2+} can also activate the protein kinase A (PKA) signalling pathway via Ca^{2+}-sensitive adenylyl cyclases producing cAMP, which can bind to, and activate, PKA. Active PKA can phosphorylate many targets including the NMDA receptor, the cAMP response element binding protein (CREB transcription factor), and the GluR1 subunit of the AMPA receptors. There is much evidence that the PKA pathway is highly involved in elements of LTP (Matynia *et al.*, 2002)

Furthermore, cAMP can activate extracellular regulated kinase (ERK; Patterson *et al.*, 2001). The importance of ERK has been demonstrated; ERK inhibitors suppress LTP (English and Sweatt, 1997), and ERK activation occurs following LTP induction (McGahon *et al.*, 1999). It has a wide range of targets including cytoskeletal proteins, and transcription factors.

The endpoint of activation of many signalling pathways is the transcription of particular genes to induce long-term changes, and protein synthesis has been shown to be necessary for the long-term maintenance of LTP (as well as memory consolidation; Frey *et al.*, 1996; Otani *et al.*, 1989). Protein synthesis would be necessary for any morphological changes, such as those suggested to be involved in LTP; increases in area of boutons and

spines, number of spines, width of spine neck, and number of shaft synapses as well as increases in neurotransmitter, and associated vesicles. Metabolic changes both pre- and post-synaptically may also require new protein synthesis. Presynaptically, it was shown that LTP resulted in an increase in glutamate release (Lynch *et al.*, 1990) and increased expression of associated vesicle proteins (Lynch *et al.*, 1994a). LTP is also associated with increased phospholipase activity, with subsequent increase in membrane phospholipid turnover, including conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG; Farooqui and Horrocks, 1994). These substances are potent signalling molecules.

As LTP induction appears to occur postsynaptically and at least some elements of maintenance require presynaptic modification, it follows that there should be some postsynaptically produced factor that stimulates presynaptic changes; this is referred to as a retrograde messenger. There are a number of candidates such as nitric oxide (NO; Bon *et al.*, 1992) and arachidonic acid (Williams *et al.*, 1989), both of which are produced during LTP induction.

It is apparent that LTP induction process is coupled with activation of many signalling pathways, leading to the short and long-term functional changes involved. It is also clear that the signalling processes involved in LTP induction and maintenance are complex, and much is yet to be understood.

1.10 Inflammation and LTP

Here it is proposed that LTP is representative of memory or effective cognitive function, which can be impaired in response to inflammation, therefore it should follow that LTP is impaired in inflammatory situations. This is indeed the case as the proinflammatory cytokine IL-1β has been shown to impair LTP in CA1 and CA3, and in the dentate gyrus (Curran *et al.*, 2003; Lynch, 1998; Cunningham *et al.*, 1996). The latter study showed that a decrease in Ca^{2+} influx (pre or post synaptically) was concurrent with IL-1β-induced LTP inhibition. The investigators suggested that IL-1β acts to undermine Ca^{2+} influx, which is essential for LTP induction and expression. IL-1β in the dentate gyrus was shown to cause a large depression of pharmacologically isolated NMDA receptor epsps (Coogan and O' Connor, 1997), which were fully antagonised by IL-1ra. This effect of IL-1β on the NMDA receptor may impair the Ca^{2+} influx, which impairs LTP. IL-1β has been shown to inhibit glutamate release from rat hippocampal synaptosomes (Murray *et al.*, 1997), leading to the assertion that IL-1β impairs LTP by this

manner. It has been observed that IL-1β results in the increased formation of ROS, and concurrently increased lipid peroxidation. This increased lipid peroxidation leads to a decrease in membrane arachidonic acid concentration, and therefore a decrease in membrane fluidity, and therefore an impairment of hippocampal function, including LTP (Lynch, 1998). It can be seen therefore that there are a number of ways in which IL-1β could lead to the impairment of LTP, as it has a large range of targets and mediators. There is evidence that effects of IL-1β are mediated through activation of JNK; for example, *in vitro* perfusion of the JNK inhibitor SP6000125 reversed the IL-1β-induced inhibition of LTP (Curran *et al.*, 2003).

Furthermore, the inflammatory cytokines IL-18 and TNFα impaired LTP in the rat dentate gyrus *in vitro* (Curran *et al.*, 2003), while *in vivo*, in a chronic brain inflammation model where animals received a chronic infusion of LPS, LTP was impaired in dentate gyrus (Hauss-Wegrzyriak *et al.*, 2002). Likewise, β-amyloid injection-induced inflammation was associated with impaired LTP and learning deficits (Stéphan *et al.*, 2003; Minogue *et al.*, 2003). The process of ageing causes the brain to exhibit many of the hallmarks of chronic neuroinflammation, and age is associated with a reduced ability to sustain LTP (Lynch, 2004).

1.11 Lipopolysaccharide (LPS)

1.11.1 LPS

Lipopolysaccharide (LPS) is a complex glycolipid, a component of the cell wall of gram-negative bacteria (e.g. Escherichia *coli* and Bordetella *pertusis*), and plays a role in inducing gram-negative disease states, including the potentially fatal condition of toxic shock syndrome/septic shock. It is estimated that 150,000 people die each year in the United States of toxic shock syndrome. LPS is one of the most potent microbial initiators of inflammation (Cohen, 2002), and acts to activate the innate immune response, the purpose of which is to coordinate a defensive response to the detected pathogen. This involves the secretion of proinflammatory cytokines as well as other mediators (Cohen, 2002), designed to efficiently control growth of pathogens, and destroy pathogens. Therefore LPS administration upregulates many inflammatory parameters; TNFα, IL-1β (Vereker *et al.*, 2000b), NF-κB (Zhang and Ghosh, 2000) and many others, resulting in activation of microglia and other immunoactive cells. As a result of its potent and widespread effects that mimic an inflammatory reaction, LPS treatment is often used as a model of inflammation.

Predictably with this model, both LTP and memory/ cognitive functioning are impaired; chronic or acute LPS administration results in inhibition of LTP in the dentate gyrus (Hauss-Wegrzyriak *et al.*, 2002; Vereker *et al.*, 2000a), inhibition of spatial learning (Shaw *et al.*, 2001), diminished performance in a passive avoidance task and the elevated maze (Jain *et al.*, 2002), and impairment of memory consolidation in contextual fear conditioning (Pugh *et al.*, 1998).

LPS is made up of three regions: (i) Lipid A; which consists of disaccharide, phosphoryl groups and fatty acid, (ii) Core region; consisting of an outercore and an innercore containing heptose and 2-keto-3-deoxyoctonic acid (KDO), and (iii) O antigenic side chain. The Lipid A region and KDO are essential for cytokine induction.

In animal studies LPS has been shown to induce systemic effects of 'sickness behaviour', such as a decrease in feeding, activity, and social behaviour (Bluthé *et al.*, 1992; Dantzer *et al.*, 1998; Lacosta *et al.*, 1999), as well as disrupting various types of learning and synaptic function (see above). These effects appear to be mediated centrally, through induction of an inflammatory response. Therefore it is evident that LPS can have a broad range of effects in the central nervous system. LPS has also been shown to be associated with apoptosis in a wide variety of cell types, such as in mouse lymphocytes (Norimatsu *et al.*, 1995), rat hippocampal tissue (Vereker *et al.*, 2000a), and human endothelial cells (Haendeler *et al.*, 1996); The LPS-induced inflammatory and apoptotic changes in hippocampus may explain the decrease in synaptic efficacy observed.

In order to exert their intracellular effects LPS and LPS containing particles (including intact bacteria) form complexes with a plasma protein known as LPS binding protein (LBP; Schumann *et al.*, 1990). This complex is then delivered to the cell surface receptor CD14. CD14 is a 55kDa glycoprotein which has no trans-membrane domain and therefore cannot directly mediate LPS signaling. The formation of the CD14/LPS complex significantly reduces (100-1000 fold) the concentration of LPS required for activation of macrophages as compared with LPS alone (Fujihara *et al.*, 2003). CD14 deficient mice are highly resistant to LPS-induced shock (Haziot *et al.*,1996) and predictably antibodies to CD14 block the LBP dependant activation of macrophages by LPS (Wright *et al.*, 1990). What CD14 appears to do is concentrate LPS and present it to the signalling receptor, Toll-like receptor (TLR) 4 (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). The TLRs are a family of protein receptors that recognise bacterial products by the pathogen-associated molecular patterns that are highly conserved and invariant among microbes. There are at least 10 members of the TLR family with TLR4 as the main LPS receptor. Mutations of the TLR4

gene cause hyporesponsiveness to LPS, and defective LPS signaling (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999, Hoshino *et al.*, 1999). Also essential for LPS-induced signalling is the physically associated accessory protein MD-2. Macrophages from MD-2 deficient mice do not produce inflammatory cytokines in response to LPS, and, similar to TLR4 deficient mice, these mice survive endotoxic shock, but are vulnerable to Salmonella *typhimurium* infection (Nagai *et al.*, 2002). MD-2 was also demonstrated to be necessary for LPS activation of the MAPK pathways (Yang *et al.*, 2000). MD-2 plays a role in regulating the cellular distribution of TLRs as well as in LPS recognition (Nagai *et al.*, 2002). Intracellularly the TLR4-MD-2 complex interacts with a number of adaptor proteins to mediate signaling changes (e.g. MyD88). In addition to the MAPKs, protein kinase A and C, as well as src related kinases have been implicated in these signal cascades. It is thought that this is one route through which LPS proceeds to initiate intracellular signal cascades and ultimately influence gene expression, thereby producing its manifold effects.

LPS is able to induce most cell types to synthesise a range of chemical messengers including cytokines. It is thought that it is the downstream effects of these messengers that mediate the disease states and in the context of this study it also appears that the actions of LPS in the hippocampus are mediated by cytokine production. This innate immune response to invading pathogens is a central part of our natural defence system against infecting micro-organisms, but may also be responsible for the observed impairment in LTP (Henderson *et al.*, 1996), as excessive or uncontrolled production of these inflammatory cytokines and mediators may lead to tissue damage and serious systemic complications (Fujihara *et al.*, 2003)

1.11.2 LPS and IL-1β

One of the main cytokines produced in response to LPS is IL-1β and much evidence suggests that many LPS-induced effects are mediated by IL-1β. Consistently, IL-1ra, an IL-1β antagonist, attenuated the LPS-induced decrease in feeding (Swiergiel *et al.*, 1997), and impairment in learning and memory (Pugh *et al.*, 1998). IL-1ra also attenuated the depressive effect of peripheral LPS administration on social exploration and body weight (Bluthé *et al.*, 1992). These results suggest that IL-1β and/ or IL-1α stimulation of their receptors is necessary for LPS to have its effects. It is well documented that peripheral administration of LPS results in increased IL-1β concentration in the brain. There is evidence that peripheral administration of LPS increases expression of IL-1β at

the mRNA level and protein level in the spleen, pituitary, and brain of mice (Goujon *et al.*, 1996), and also in rat microglia (Van Dam *et al.*, 1992). The latter study also showed the appearance of IL-1 in macrophage cells in the choroid plexus and the meninges. It was shown that LPS administration results in an increased cerebrovascular permeability, including disruption of the BBB as evidenced by cerebral spinal fluid leukocytosis (Boje, 1996), suggesting that immunoactive cells and IL-1β may gain access to the CNS. Intracerebral injection of neutralising antibodies to IL-1β lead to an inhibition of the central actions of IL-1β, reducing the effects of LPS administration. To inhibit the same LPS induced effects, intravenous injection requires up to 100-fold greater dose of IL-1β antibody. This indicates that IL-1β within the brain mediates the effects of LPS (Rothwell, 1991).

In addition to inducing IL-1β centrally which can activate the JNK and p38 signalling pathway (Vereker *et al.*, 2000b), LPS may itself directly activate JNK; LPS has been shown to activate MKK4 (Swantek *et al.*, 1997), and numerous studies have shown that LPS causes an increase in JNK activity (Hambleton *et al.*, 1996; Pyo *et al.*, 1998; Loscher *et al.*, 2000).

1.12 Age

Aging is often defined as the decline in various body systems and functions that occur with the passage of time, although there is great variability in degree of deterioration that occurs, particularly in relation to the CNS. The changes which occur are manifold and can be broadly described as either physical, biochemical, or functional. Pertinent to quality of life are the functional changes observed such as impaired memory.

In terms of physical age-related changes that occur, early work suggested neuronal loss or a decrease in neuronal density in a number of brain regions including hippocampus, however, the counting methods utilised required assumptions about size, shape, and distribution of cells, which can introduce bias so may not be reliable. Newer unbiased stereological evidence provides mixed reports, but points to no cell loss occurring in the hippocampus (Rapp and Gallagher, 1996) or the entorhinal cortex (Merrill *et al.*, 2001) with age, although a change in synaptic density may still play a role (Miller and O' Callaghan, 2003). The other residential cells of the CNS are also affected; increased astrogliosis is observed with age (O' Callaghan and Miller, 1991) however this does not appear to be linked to disease or cognitive impairment. Morphology of microglia undergoing healthy aging was examined in samples taken from cognitively normal elderly

donors; microglia prepared from aged subjects exhibited several abnormalities including deramification, spheroid formation, gnarling and fragmentation of processes (Streit *et al.*, 2004) and these changes were different to those observed in neurodegenerative disorders.

Functionally, an age-related memory decline is well documented across most species. Aged rats show impaired spatial learning in a circular platform task, a T-maze test, an eight arm radial maze, and in a Morris water maze (reviewed by Rosenzweig and Barnes 2003). The authors also describe synaptic plasticity, such as LTP, being defective with age under certain conditions. Impairment of LTP with age is variable, but this parallels performance i.e. not all aged animals show a deficit in learning or LTP. Many studies show cognitive deficits in aged rats, with spatial information processing appearing to be most affected. Significantly, impaired learning, demonstrated by poor performance in the Barnes circular maze, was positively correlated to a decreased ability to sustain LTP in CA1 (Bach *et al.*, 1999). It has been postulated that aged animals possess the mechanisms necessary for memory formation, and that age-related memory deficits result from more subtle changes in memory process for memory storage or maintenance (Foster, 1999), and the author suggests this is due to a change in the threshold of synaptic modulation , mediated by age-related changes in Ca^{2+} homeostasis.

Age related cognitive impairments could be linked to changes in signalling, information encoding, and plastic, electrophysiological, or neurochemical properties of neurons or glia (Miller and O' Callaghan, 2003). A wide range of cellular signalling and processes have been reported to change centrally with age and may underlie the cognitive decline observed. For example, hippocampal pyramidal cells exhibit reduced responsiveness to acetylcholine and other neurotransmitters (Haigler *et al.*, 1986), while reduced potassium currents, decreased microvascular integrity, and decreased glucose metabolism have all been observed in the hippocampus of aged subjects. As reviewed by Lynch (2004), decreased phosphoinositide turnover (Nicolle *et al.*, 1999; Lynch *et al.*, 1994b), decreased PKC activity (Colombo and Gallagher, 2002), increased DNA and RNA oxidation (Liu *et al.*, 2002), and decreased arachidonic acid concentration in the membrane (Ulmann *et al.*, 2001) are all associated with an age-related decline in spatial memory. Additionally, treatment with rolipram, a cAMP phosphodiesterase inhibitor, enhanced performance of aged rats in a Barnes circular maze, suggesting that a decrease in cAMP signalling contributes to the age related change (Bach *et al.*, 1999). Increased ROS production with age is well documented and can be attributed to the increase in superoxide dismutase (SOD) activity in the absence of a concomitant increase in glutathione

peroxidase or catalase activity. ROS exert oxidative stress and can increase IL-1β concentration, as well as causing lipid peroxidation, with a resultant decrease in membrane polyunsaturated fatty acids. This leads to decreased membrane fluidity, impacting on normal functioning of membrane associated proteins (e.g. receptors) and predictably impairing LTP (Lynch, 2004). Furthermore, ROS are linked to signs of inflammation. Aged hippocampus also exhibits a decrease in cell survival signalling molecule activity such as ERK (McGahon *et al.*, 1999), PI3K (Maher *et al.*, 2004), and Trk (Mullany *et al.*, 1996), as well as an increase in markers of cell deterioration such as caspase 3 and TUNEL (Martin *et al.*, 2002). Taken together, these changes suggest that the aged brain is, in fact, a stressed environment. This is supported by evidence that immunological functioning, the intrinsic system for dealing with stressors, is upregulated with age, and significantly, age is characterised by a shift within innate immunity towards a proinflammatory status (Blasko *et al.*, 2004). However as the "stress" of age by its nature cannot be rectified, age leads to chronic activation of inflammatory responses, further perpetuating this neurodegenerative cycle. Proinflammatory cytokines such as TNFα, IL-1β, and IL-6 chronically produced at high concentrations are toxic to neruons (Jeohn *et al.*, 1998), and therefore this inflammatory change may underlie the deficits in synaptic function. Additionally, these cytokines have been shown to affect metabolism of APP to increase amyloidogenc peptides. This disturbed balance between production and degradation of β-amyloid triggers chronic inflammatory processes in microglia and astrocytes (Blasko *et al.*, 2004). Further evidence that inflammatory processes are involved in age-related changes is that anti-inflammatory treatments showed beneficial effects. Therefore chronic treatment with non-steroidal anti-inflammatory drugs restored functioning of aged rats in learning of passive avoidance task and elevated maze (Jain *et al.*, 2002), while chronic treatment of rats with aspirin which combats inflammation markedly improves spatial learning (Smith *et al.*, 2002).

1.13 Neuroprotection

Protection of effective functioning of neurons in various inflammatory paradigms is obviously a target of research, and has huge potential benefit across a broad range of disorders. There are a number of approaches to take when looking for compounds or therapies with a neuroprotective effect; one can either target specific pathways which are implicated in neurodegeneration, or one can look at epidemiological studies for a treatment strategy showing evidence of neuroprotective effects. Both approaches have been

undertaken here; inhibition of JNK was targeted due to the evidence suggesting the importance of JNK in inflammatory processes; the lipid lowering agent atorvastatin was used due to epidemiological evidence of reduced incidence of dementia and generalised neuroprotective effects; the Adenosine A2A receptor antagonist, SCH58261, was utilised because of epidemiological evidence suggesting that caffeine was protective against Parkinson's disease.

1.14 JNK inhibition

It is clear that JNK activation and its downstream effects can be associated with inflammation and apoptosis, and that JNK signalling could be an important therapeutic target for prevention of unwanted apoptosis in the nervous system, particularly that associated with inflammation and neurodegeneration.

Recently some specific JNK inhibitors were identified. CEP-1347 (KT 7515) is a small molecule adapted from the naturally occurring compound K252a. It inhibits JNK activity but not p38 activity, probably through inhibition of kinases upstream of JNK, the mixed lineage kinases (MLK; Maroney *et al.*, 1998) by acting as a direct competitive inhibitor of ATP. Therefore there are problems apparent with this inhibitor; MLKs have the potential to affect other targets and signalling molecules and conversely JNK may be activated in some circumstances by MLK-independent pathways.

SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) is an ATP-competitive inhibitor of equal potency towards the three JNK gene products (Han *et al.*, 2001), and is only soluble in DMSO. While low concentrations appear selective, higher concentrations of SP600125 may have broad effects; SP600125 has some ability to inhibit kinases upstream in the JNK pathway and even the p38 pathway, specifically MKK4 and MKK7 were inhibited as well as MKK3 and MKK6 (Bain *et al.*, 2003).

Recently, JNK interacting proteins 1 and 2 (JIP-1, 2; also known as IB-1 and 2) were identified as cytoplasmic scaffold proteins that have the ability to bind selectively to JNK and several components of the JNK signalling pathway (the MAPKK MKK7, and the MAPKKKs MLKs). Importantly however they did not bind to other MAPKs, including p38, (Dickens *et al.*, 1997). It was also found that over-expression of JIP-1 caused cytoplasmic retention of JNK and thereby inhibited JNK related gene expression. It has since been suggested that JIP-1 facilitates the MAPK cascade by bringing the components into close proximity (Whitmarsh *et al.*, 1998). However overexpression would mean binding of the component parts of the pathway to separate scaffold molecules thereby

decreasing JNK signalling. In any case JIP-1 binds to numerous proteins, but a specific JNK binding domain (JBD) was identified at the N terminus of the JIP proteins and over-expression of this segment has been shown to be sufficient to prevent the NGF-withdrawal induced phosphorylation of c-Jun, the subsequent apoptosis in sympathetic neurons (Harding *et al.*, 2001; Eilers *et al.*, 2001), and to preserve mitochondrial function (Harding *et al.*, 2001). Interestingly, in the MPTP/MPP+ model of Parkinson Disease, over-expression of JBD has been shown to decrease the associated phosphorylation of JNK and c-Jun, decrease apoptosis, decrease caspase activation and to exert beneficial behavioural effects (Xia *et al.*, 2001).

To facilitate treatments without transfection or adenovirus techniques, bioactive cell-permeable peptide inhibitors of JNK were engineered by linking the minimal 20-amino acid inhibitory domains of the JIP proteins to the 10-amino acid HIV-TAT sequence that rapidly translocates inside cells and these inhibitors were shown to reduce IL-1 induced apoptosis of pancreatic β-cells (Bonny *et al.*, 2001). This gave rise to L-JNKI1 (c-Jun N-terminal Kinase Peptide Inhibitor 1, L-stereoisomer), and D-JNKI1 (c-Jun N-terminal Kinase Peptide Inhibitor 1, D-stereoisomer) target-competitive inhibitors of JNK, actions of which are reviewed by Borsello and Bonny (2004). Although D-JNKI1 demonstrated a 15-fold reduction in its ability to inhibit JNK/c-Jun interaction compared with L-JNKI, it was still able to prevent c-Jun phosphorylation inside cells and protect against apoptosis, and has the advantage of being more resistant to neuronal proteases (Bonny *et al.*, 2001).

D-JNKI1 was selected as the inhibitor for these studies; its mode of action is at least partly understood (Barr *et al.*, 2002), it has a long half-life in neuronal tissue, it is rapidly taken up into cells and is water-soluble. D-JNKI1 was effective in neuroprotection in an ischaemic model *in vivo*, and also prevented excitotoxicity *in vitro* (Borsello *et al.*, 2003). Similarly, D-JNKI1 exerted a protective effect on lesion size and functional outcome in mice undergoing severe cerebral ischaemia *in vivo*, and also protected against cell death and associated decrease in amplitude of the evoked potential response in newborn rat hippocampal slices *in vitro* (Hirt *et al.*, 2004).

1.15 Statins

1.15.1 Statins

Statins are a family of widely prescribed synthetic lipid/cholesterol lowering agents. Structurally similar to hydroxyl-3-methylglutaryl Coenzyme A (HMG-CoA), a

precursor of cholesterol, statins act as competitive inhibitors of HMG-CoA reductase which converts HMG-CoA to mevalonate, thereby inhibiting the rate limiting step of cholesterol synthesis. The decrease in hepatic cholesterol synthesis results in a compensatory enhancement of uptake and catabolism of low density lipoprotein (LDL) by increasing hepatic LDL receptor expression. These effects result in decreased plasma and hence intracellular cholesterol levels.

Cholesterol is an indispensable component of cell membrane and a precursor for steroid hormones and bile acids and other essential compounds, as well as an important source of energy. Cholesterol circulates in the blood as high density lipoprotein (HDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL). LDL accounts for 60-75% of total blood cholesterol and is generally considered the "bad" cholesterol. This is because high levels of LDL cholesterol are associated with increased risk of atherosclerosis, cardiovascular disease (Brewer *et al.*, 1985), and Alzheimer's disease (Lesser *et al.*, 2001). In contrast HDL is considered "good" cholesterol as it allows removal of excess cholesterol to the liver for recycling and excretion and is thought to be beneficial, linked to longevity, and decreased risk of cardiovascular disease (Barter, 2004). Reduction of LDL cholesterol by statin treatment has proven to be effective in reducing coronary morbidity and mortality, attributed to its ability to decrease atherosclerosis, and decrease cardiovascular disease. Commonly-prescribed statins include atorvastatin (ATV), simvastatin, fluvastatin, pravastatin, and lovastatin. Several clinical studies confirm that the incidence of stroke is reduced with statin treatment but epidemiological evidence, suggests that statins may also exert a broader neuroprotective effect; for example statin treatment reduced the incidence of dementia by 60-73%, compared with other cholesterol lowering agents (Wolozin *et al.*, 2000); Similarly, patients receiving statin treatment were 71% less likely to be diagnosed with dementia or AD than controls or patients receiving other cholesterol lowering medication (Jick *et al.*, 2000). In addition pre-treatment with statins reduced infarct volume and conferred protection from neurological deficits in mouse models of cerebral ischaemia (Endres *et al.*, 1998; Laufs *et al.*, 2002) and also protected cultured cortical neurons from NMDA induced excitotoxicity (Zacco *et al.*, 2003).

1.15.2 Cholesterol-independent effects of statins

In general, statin treatment reduces LDL cholesterol by 25-50% (Vaughan, 2003), however, as it was noted that statin treatment appeared to be having effects beyond its lipid lowering capabilities, investigation began into other effects statins may have. Improved endothelial function as evidenced by increased levels of eNOS was observed at a time point prior to significant reduction in cholesterol levels following statin treatment (Kwak *et al.*, 2003; Shovman *et al.*, 2001). Reduction in instances of stroke and thrombosis was not linked to a change in size or number of atherosclerotic plaques, but rather was attributed to the apparent ability of statins to stabilise existing plaques (Cucchiara and Kasner, 2001). It is when vascular plaques rupture that they cause stroke or thrombosis, and this is determined in part by macrophage activity and T-cell infiltration at the plaque, associated with cytokine production and a consequential increase in leukocyte proliferation, migration, and adhesion, as well as increased levels of ROS and MMPs, which reduce plaque stability (Kwak *et al.*, 2003; Vaughan, 2003). These data indicate that statins may exert anti-inflammatory effects independent of the beneficial effects associated with lowering of cholesterol. Extensive study of the effects of statin treatment has revealed a wide range of immunomodulatory roles.

1.15.3 Depletion of intermediary compounds of cholesterol synthesis

The conversion of HMG CoA to mevalonate is only one of the first steps in cholesterol synthesis, and some pleiotropic effects of statins may be attributed to the depletion of the many intermediary substances (See Figure 1.3). These downstream products of the mevalonate pathway are biologically active in mediating the immune response as cotreatment with exogenous mevalonate appears to reverse the anti-inflammatory or protective actions of statin treatment (Zacco *et al.*, 2003; Youssef *et al.*, 2002). Of intermediate metabolites, with the potential to modulate inflammatory response, the isoprenoids are prime candidates as they are involved in post translational modification (isoprenylation) of numerous proteins central to normal cell function. The isoprenoids (including, 3,3dimethylpyrophosphate, geranylgeranylpyrophosphate, farnesylpyrophosphate, and geranylpyrophosphate) have been shown to have a role in inflammation via regulation of components of intracellular second messenger systems, adhesion molecules and proliferation of leukocytes (Cucchiara and Kasner, 2001).

One protein which requires modulation by such compounds is the small GTPase Rho, where prenylation by geranylgeranylpyrophosphate allows its association with

cellular membranes. Rho is important in cell growth, mitogenic pathways and various signalling cascades, such as that associated with intracellular adhesion molecule-1 (ICAM-1), which is central in mediating transvascular lymphocyte migration. Further evidence for this pathway is that abolition of Rho prenylation was reported to be effective in restricting transvascular lymphocyte migration into the CNS and attenuated neuroinflammatory disease (Walters *et al.*, 2002). Interestingly extravasation of lymphocytes across other vascular beds was less affected by inhibition of Rho prenylation, suggesting selective benefit for neuroinflammation rather than peripheral inflammation. Expression of other adhesion molecules can be modulated by statin treatment via isoprenoid depletion. Rho functionality is also important in the control of the eNOS system (see NO availability; section 1.15.4). Lastly, Rho and another GTP protein, Ras, are central in controlling the NF-κB transduction pathway, a transcription factor critically involved in inflammatory events such as cytokine production. ATV treatment appeared to stabilise the protein IκB which inhibits NF-κB, again through isoprenoid depletion as mevalonate partially reversed its inhibition (Ortego *et al.*, 1999).

In the context of this study, the fact that lovastatin was shown to inhibit UV-C-induced caspase 3 activation and associated cell death, by a JNK-dependent mechanism, is particularly significant. It was shown that this was because of inhibition of the Rac-MKK4 pathway probably due to attenuation of isoprenylation of Rho GTPases (Bardeleben *et al.*, 2003).

Isoprenoids are emerging as potential novel targets for immunomodulation, although investigation into the effects of reduced isoprenoid activity is still at relatively early stages. Thus it may be possible to target more specific pathways than those effected by the broader actions of statin treatment. However, caution should be used as many proteins involved in regulation of cell proliferation and survival require isoprenylation and this may account for the findings of some studies which indicated that statin treatment induced apoptosis in neuronal cells in culture (Tanaka *et al.*, 2000; Michikawa and Yanagisawa, 1999).

1.15.4 NO availability

Statins can modulate NO, which regulates endothelial function; its actions include inhibition of leukocyte and platelet adhesion, control of vascular tone and growth, and maintenance of a thromboresistant interface between the bloodstream and vessel wall (Vaughan, 2003). The source and extent of NO production seems to determine its

biological effects. Activation of inducible nitric oxide synthase (iNOS) is implicated in a number of CNS disorders; ischaemia, AD, Parkinson's disease, and also tumours and trauma (Cucchiara and Kasner, 2001), as well as in the pathogenesis of experimental allergic encephalomyelitis (EAE; Stanislaus *et al.*, 1999). Its production can be attenuated with treatment of statins (lovastatin in the case of EAE), and this shows beneficial effects. In contrast, NO produced by endothelial NOS (eNOS) is critical in regulating the homeostatic functions of endothelia. eNOS is linked to a protective role and enhanced NO bioavailability, allowing normal functioning. Statins appear to have beneficial effects by a dual role of increasing eNOS and decreasing iNOS. But how is this mediated? Cholesterol synthesis intermediaries depleted by statin treatment play a significant role in controlling eNOS. Mevalonate inhibits the survival pathway PI3K, which would otherwise activate Akt leading to upregulation of eNOS. Accordingly a reduction in mevalonate leads to enhanced activity of Akt and eNOS. Further downstream, Rho which requires isoprenylation by geranylgeranylpyrophosphate (isoprenoid; see section 1.15.3), downregulates eNOS as well as reducing eNOS mRNA stability via effects on cytoskeleton (Vaughan, 2003). In the case of iNOS, it is well documented that statins reduce proinflammatory cytokine production such as TNFα, and IFNγ, potent inducers of iNOS in the CNS (Stanislaus *et al.*, 1999).

1.15.5 Antioxidant effects

ROS are known to lead to vascular damage, and their production is thought to be involved in the pathogenesis of atherosclerosis and endothelial dysfunction. ROS are also produced following an ischemic event or brain injury, and are upregulated in the aged brain. ROS induce lipid peroxidation, protein oxidation and denaturation of nucleic acids, leading to neuronal and endothelial damage (Cucchiara and Kasner, 2001). ROS can elicit direct cellular damage and mitogenicity, serve as intracellular second messengers and scavenge vasoprotective NO (Wassmann *et al.*, 2002). Therefore it is clear that antioxidant activity which acts to decrease free radicals and ROS may be beneficial. ATV has been shown to have antioxidant properties; ATV decreased expression of essential subunits of the NAD(P)H oxidase enzyme system, the main producer of ROS; ATV upregulated catalase, an enzyme important in elimination of free radicals (Wassmann *et al.*, 2002). Therefore ATV has the ability to decrease ROS production and increase ROS elimination resulting in reduced ROS levels. Other statins have been shown to have antioxidant effects i.e. lovastatin, pravastatin, and simvastatin have an inhibitory effect on superoxide anion

formation, while fluvastatin has radical scavenging ability (Shovman *et al.*, 2001). *In vitro*, fluvastatin, ATV, pravastatin and simvastatin were shown to have intrinsic antioxidant activity with both anti-hydroxyl and peroxyl radical activity (Franzoni *et al.*, 2003).

1.15.6 Effects on leukocyte function

One of the most striking effects of statins is modulation of leukocyte function. This is mediated in a number of ways and has a significant impact on immune function. It appears that most aspects of leukocyte function can be disrupted by statin treatment, including migration/ chemotaxis, surface protein expression, adhesiveness, proliferation, and differentiation.

1.15.6.1 Effects on migration and adhesion

Statin modulation of chemotaxis and factors controlling leukocyte migration is well documented. Monocyte chemotactic protein (MCP)-1 is produced by macrophages and monocytes and is one factor responsible for chemotaxis; Statins (lovastatin, simvastatin and pravastatin) have been shown to reduce MCP-1 mRNA *in vivo* and *in vitro* (Romano *et al.*, 2000). Additionally *in vitro*, pravastatin reduced chemotaxis of the human monocyte cell line U937 (Kreuzer *et al.*, 1991). *In vivo*, lovastatin and atorvastatin decreased leukocyte infiltration into CNS in EAE (Stanislaus *et al.*, 2001; Atkas *et al.*, 2003; Youssef *et al.*, 2002) while lovastatin, pravastatin, and simvasatin significantly reduced carrageenan and LPS-induced pouch leukocyte recruitment at site of infection (Diomede *et al.*, 2001). The latter study also demonstrated that this effect was due to the inhibition of mevalonate derived isoprenoid synthesis; exogenous mevalonate completely reversed the anti-inflammatory effect of statins, while inhibition of sterol synthesis had no anti-inflammatory effect. This effect was attributed to a reduction in inflammatory cytokines, specifically IL-6, and the chemotactic protein MCP-1. Rho functionality is critical for migration of leukocytes so statin-induced inhibition of isoprenoids leads to decreased migration into brain parenchyma. This could be affected through Rho in a number of ways. Modulation of the NF-κB pathway by reduced Rho signalling results in a change in cytokine and chemokine profile e.g. MCP-1, IL-6, IL-1β, as well as effecting adhesion molecules and enzymes, while reduced prenylation of mediators of the ICAM-1 signal transduction pathway causes impairment of ICAM-1 functioning. Generally, endothelial and glial activation due to insult results in expression of adhesion molecules such as ICAM-1, VCAM-1 and E selectin, and these factors influence adhesiveness of

immunoactive cells, recruitment of lymphocytes to the area and are essential for tranvascular migration of T-cells. Fluvastatin reduced leukocyte-endothelium interactions (Kimura *et al.*, 1997), while other statins appear to down regulate leukocyte-functional antigen-1 (LFA-1; the counter receptor for endothelial ICAM-1) essential for appropriate leukocyte interaction and tranvascular migration of T-lymphocytes. While it is suggested that statins also directly block LFA-1 by binding to the L-site, thereby inhibiting binding of lymphocytes to ICAM (Vaughan, 2003), it is unlikely that this plays a major role, as in most cases the effects of statins on neuroinflammation can be reversed by addition of exogenous mevalonate (Adamson and Greenwood, 2003).

As well as a decreased chemotactic influence due to a reduction in cytokines, the decreased activity and recruitment of macrophage/ monocytes results in reduced matrix metalloproteinase (MMP) production which have also been shown to be important for CNS infiltration of macrophage (Wong *et al.*, 2001). Statins have been shown to inhibit production of MMP-1, MMP-9 and MMP-3 by macrophages *in vivo* and *in vitro* (Bellosta *et al.*, 1998)

1.15.6.2 Effects on proliferation, differentiation and T-cell function

Statins have a well-documented effect on leukocyte proliferation, for example ATV reduced T-cell proliferation (Kwak *et al.*, 2000). In the first stages of neuroinflammatory disease, leukocyte response to antigen relies on clonal expansion of specific T-cell populations (Adamson and Greenwood, 2003), and statin treatment has been shown to result in an inability of T and B-cells to proliferate in response to a number of stimuli. Leukocyte proliferation was decreased *in vitro* and *in vivo* by ATV in a dose dependant manner (Youssef *et al.*, 2002), and this was correlated in a later study (Atkas *et al.*, 2003), with both studies reporting prevention of development of EAE in a mouse model. The former study also assessed splenocyte cultures for Th1 cytokines (IL-2, IL-12, IFNγ, TNFα) and Th2 cytokines (IL-4, IL-5, IL-10, TGFβ) and showed an ATV induced Th2 bias, where Th1 response was blocked, and Th2 response was stimulated. The latter study also mirrored this showing a blockade of the Th1 response (Atkas *et al.*, 2003). It was also noted that ATV induced Th2 differentiation of naïve Th0 cells and that adoptive transfer of these Th2 leukocytes protected recipient animals from developing EAE (Youssef *et al.*, 2002). In terms of mechanism, they reported that ATV induced activation of the Th2 inducing STAT6 transcription factor and inhibited the Th1 inducing STAT4 transcription

factor, resulting in a switch from a proinflammatory Th1 cytokine profile to an antiinflammatory Th2 cytokine profile.

Another way in which statins may modulate T-cell activation and differentiation is through changes in major histocompatibility complex class II (MHC-II) expression. MHC-II molecules are necessary for T-cell activation and control of the immune response (Vaughan, 2003; Youssef *et al.*, 2002). MHC-II are required for antigen presentation and T-cell activation through the TCR, activation of which triggers proliferation, and differentiation into the two distinct effector cell populations (Th1 and Th2; Shovman *et al.*, 2001). ATV, pravastatin, and lovastatin suppressed MHC-II expression in a dose dependant manner, reversible by application of mevalonate, with ATV the most potent in its effects (Kwak *et al.*, 2000). Interestingly, only expression of inducible MHC-II was effected, not constitutive expression in specialised antigen presenting cells (APC) such as B-lymphocytes. It was determined that MHC-II was decreased via suppression of the transactivator CIITA (MHC Class II transactivator), specifically inhibition of the inducible promoter IV (Kwak *et al.*, 2000). The ultimate result of down regulation of MHCII by statins is to decrease T-cell activation *in vivo* and therefore inhibits release of proinflammatory cytokines (Kwak *et al.*, 2000; Vaughan, 2003). This is indeed the case as significant decreases in proinflammatory cytokine production have been reported with reductions in IL-1β, TNFα, IL-6, and IFNγ (Stanislaus *et al.*, 1999; Cucchiara and Kasner, 2001; Vaughan , 2003).

1.15.7 Selection of ATV

For the purpose of these studies ATV was the statin that was selected; it is commercially available, well tolerated, relatively inexpensive, has a high safety profile, and is one of the most widely-prescribed statins. ATV was the most powerful inhibitor of MHC-II which as described can directly control the immune response (Kwak *et al.*, 2000). ATV was also effective as an inflammatory mediator in an EAE mouse model, reducing infiltration of inflammatory cells into the CNS (Aktas *et al.*, 2003). As our studies required modulation of neuroinflammation this was an important factor. NF-κB activation has been shown to be upregulated in the stressed brain; ATV was shown to inhibit NF-κB activation and resulting chemokine expression in vascular smooth muscle cells and mononuclear cells (Ortego *et al.*, 1999). These data suggested that ATV may be a suitable statin to use in our inflammatory models.

ATV does not cross the BBB, and this may be an important factor as debate exists as to the beneficial effects of a statin which does cross the BBB. This is reviewed by Sparks and colleagues (Sparks *et al.*, 2002), and they postulate that a statin which crosses the BBB may be detrimental. This position results from the fact that statin treatment *in vitro* can result in neuronal death (Tanaka *et al.*, 2000; Michikawa and Yanagisawa, 1999). In addition, they put forward that evidence suggests that excess blood-borne cholesterol inhibits cholesterol synthesis and increases Aβ production in cells. Since Aβ has a detrimental effect in cells, in part by inhibiting cholesterol synthesis, it would follow that it would not be beneficial to further reduce cholesterol synthesis at the level of the neuron. Given that it was suggested that it is excess cholesterol outside a neuron that stimulates AD pathogenesis (increased Aβ production), it would make sense to reduce peripheral levels of cholesterol, thereby using the blood as a sponge to reduce extracellular levels of cholesterol in the brain. This issue is still not resolved and is not aided by the fact that effects on cognitive ability seems very mixed across the forms of statins, with both an improvement and a decline in function reported. Lovastatin, simvastatin, and cerivastatin, cross the BBB, while atorvastatin, prevastatin and fluvastatin do not.

1.16 A2A Adenosine receptor antagonist SCH58261

Epidemiological studies have established an association between the common consumption of coffee or other caffeinated beverages and a reduced risk of developing Parkinson's disease (Chen *et al.*, 2001), a disorder associated with the degeneration of a specific subset of dopaminergic neurons (substantia nigra). While there are 4 distinct receptors for the nucleoside neuromodulator adenosine (A1, A2A, A2B, and A3), pharmacological studies show the A1 and A2 subtypes of adenosine receptors are the most likely targets for caffeine in brain (Fredholm *et al.*, 1999), which acts in an antagonistic manner on these receptors. Interestingly, A2A receptor expression is largely restricted to the striatum (Svenningsson *et al.*, 1999) the major target of the dopaminergic neurons that degenerate in Parkinson's disease. In a recent study Chen and colleagues showed that in a MPTP murine model of Parkinson's disease, caffeine attenuated the MPTP-induced loss of striatal dopamine and dopamine transporter binding sites. A2A specific antagonists mimicked this protective effect, but an A1 specific antagonist did not. A2A knockout mice also showed a blunted MPTP-induced neurotoxicity, confirming the role of the A2A receptor in mediating MPTP toxicity and supporting its contribution to the neuroprotective effects of caffeine (Chen *et al.*, 2001). However, another study demonstrated that animals

deficient in A2A adenosine receptor showed extensive tissue damage, prolonged and higher levels of proinflammatory cytokines, and death in response to a dose of inflammatory stimulus that causes minimal tissue damage in wild type mice (Ohta and Sitkovsky, 2001). The authors suggested that A2A receptors are required for negative feedback mechanism promoting the limitation and termination of inflammatory responses. It is important to note that this latter study was carried out in the periphery, with an emphasis on liver damage, and one could postulate that central actions of A2A receptors may be different. Further evidence for central neuroprotective effects exist; A2A receptor antagonists exerted a protective effect in two models of Parkinson's disease, KW-6002 preserved dopaminergic neurons in 6-OHDA treated rats, while both KF18446 and KW-6002 prevented the loss of functional dopaminergic nerve terminals in striatum and the ensuing gliosis caused by MPTP in mice (Ikeda *et al.*, 2002). β-amyloid toxicity on rat cultured cerebellar granule neurons was prevented by coapplication with caffeine or A2A receptor blockade (Dall'Inga *et al.*, 2003), and the A2A receptor antagonist DMPX prevented frontal cortex EEG abnormalities in the quinolinic acid model of Huntington's disease (Reggio *et al.*, 1999). Additionally mice lacking A2A receptors are less vulnerable to ischaemia and neuronal damage (Chen *et al.*, 1999)

It has been suggested that there is a regulatory connection between adenosine receptors and inflammatory cytokines; IL-1β and TNFα have been shown to upregulate expression and function of A2A receptors in human monocytic THP-1 cell lines (Khoa *et al.*, 2001) and neuronal cell lines (Trincavelli *et al.*, 2002). Taken with the accumulating evidence, this may represent a novel way in which proinflammatory cytokines can predispose cells for neurodegeneration. In PC12 cells IL-1β and TNFα caused cell death, as did adenosine, an adenosine receptor agonist, and the A2A agonist CGS21680. The specific A2A antagonist SCH58261 was protective against adenosine and the adenosine receptor agonist (NECA) induced cell death, but the A2B antagonist MRS1706 was ineffective (Trincavelli *et al.*, 2003).

In summary A2A receptor upregulation and activation appears to play a role in neurodegeneration in different neurotoxic situations in the brain with blockade of these receptors showing a protective effect. However, the precise mechanism by which A2A receptor activation leads to cell death has not been determined.

1.17 Objectives

Given that the literature to date suggests a role for JNK activation in mediating LPS-induced inflammatory change, but definitive evidence is still lacking, the objective of these studies was to assess the role of JNK in inflammatory models *in vivo*, in particular, its involvement in the impairment in LTP observed under these conditions. By using direct inhibition of the kinase and two agents reputed to have antiinflammatory properties, it was intended that the relevance of JNK and its associated signalling would be determined. The mechanisms by which these substances may mediate cellular changes were also to be investigated by *ex vivo* and *in vitro* analysis.

Therefore the main objectives of this study are:

1) To assess the ability of the JNK inhibitor D-JNKI1 to attenuate the central effects of i.p. LPS administration, specifically the effects leading to cell degeneration and decreased synaptic efficacy, and therefore determine the role of JNK in mediating inflammatory effects.
2) To assess the ability of atorvastatin to attenuate the central effects of i.p. LPS administration, specifically the effects leading to cell degeneration and decreased synaptic efficacy
3) To assess the ability of atorvastatin to affect changes in primary cultured glia, in particular to reverse the effects of LPS treatment.
4) To assess the ability of atorvastatin to reverse the age-related impairment in synaptic efficacy.
5) To assess the ability of SCH58261 for the ability to attenuate the central effects of i.p. LPS administration, specifically the effects leading to cell degeneration and decreased synaptic efficacy.

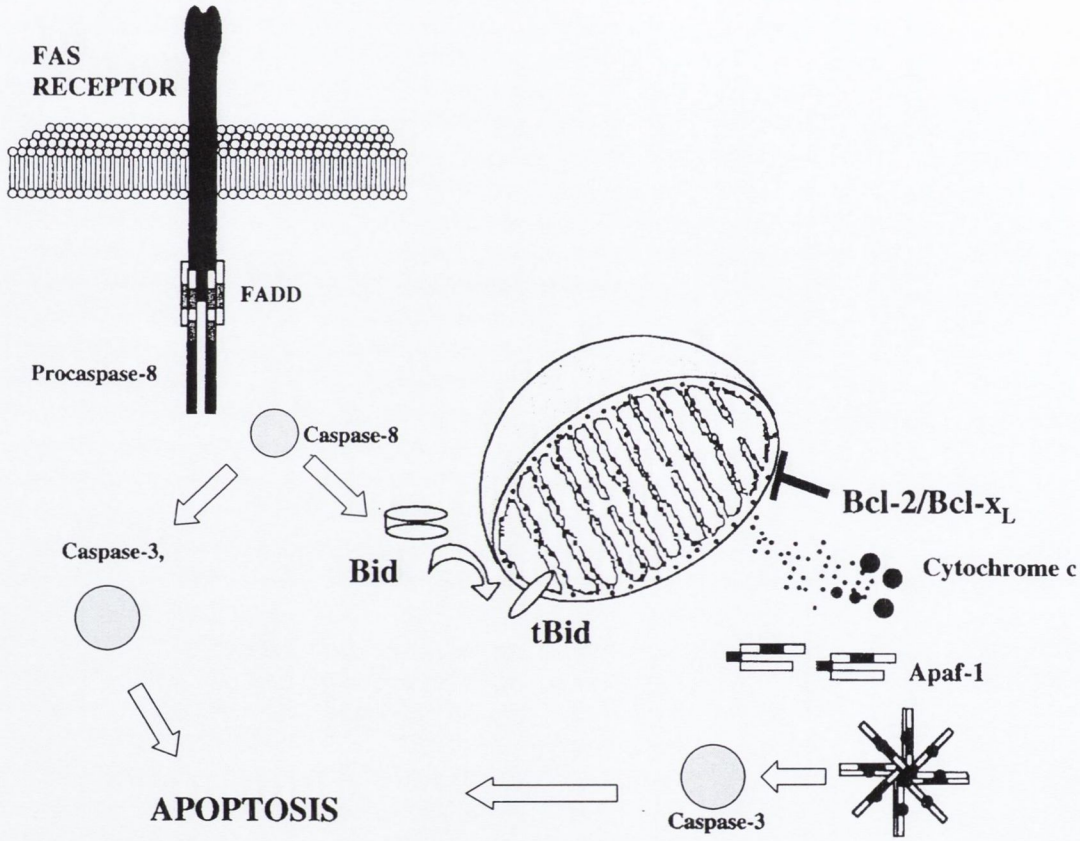


Figure 1.1 Diagram of Fas Signalling

Adapted from Zimmermann K. C., Bonzon C., and Green D. R. (2001) The machinery of programmed cell death. *Pharmacological Therapeutics* **92(1)**; 57-70.

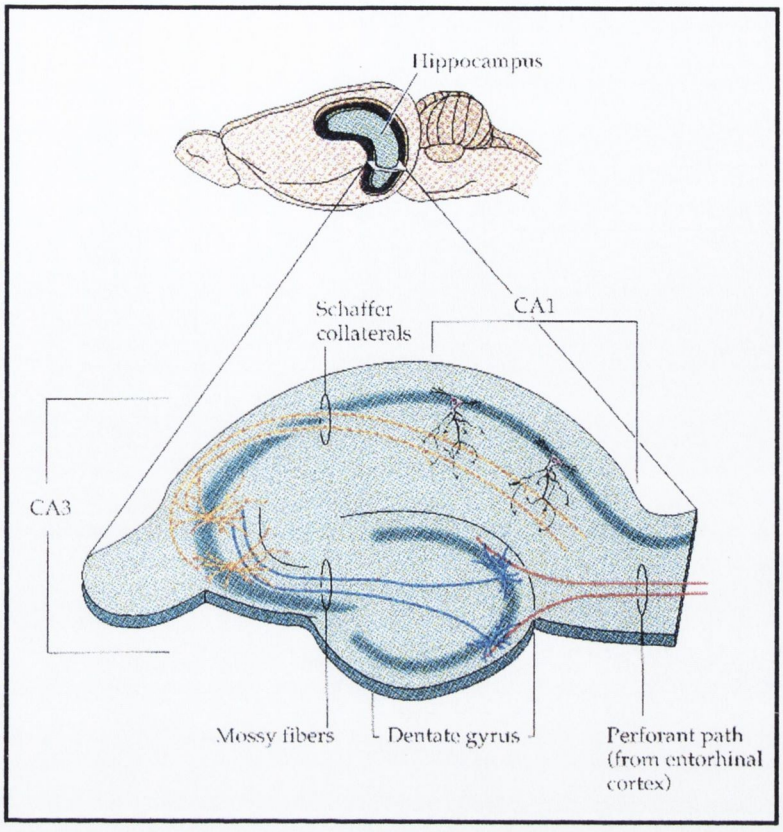


Figure 1.2 Diagram of a section through the rodent hippocampus showing the major excitatory pathways and their synaptic connections.

Taken from Purves D., Augustine G. J., Fitzpatrick D., Katz L. C., LaMantia A., McNamara J. O., (1997), Neuroscience, p441, Sinauer Associates Inc.

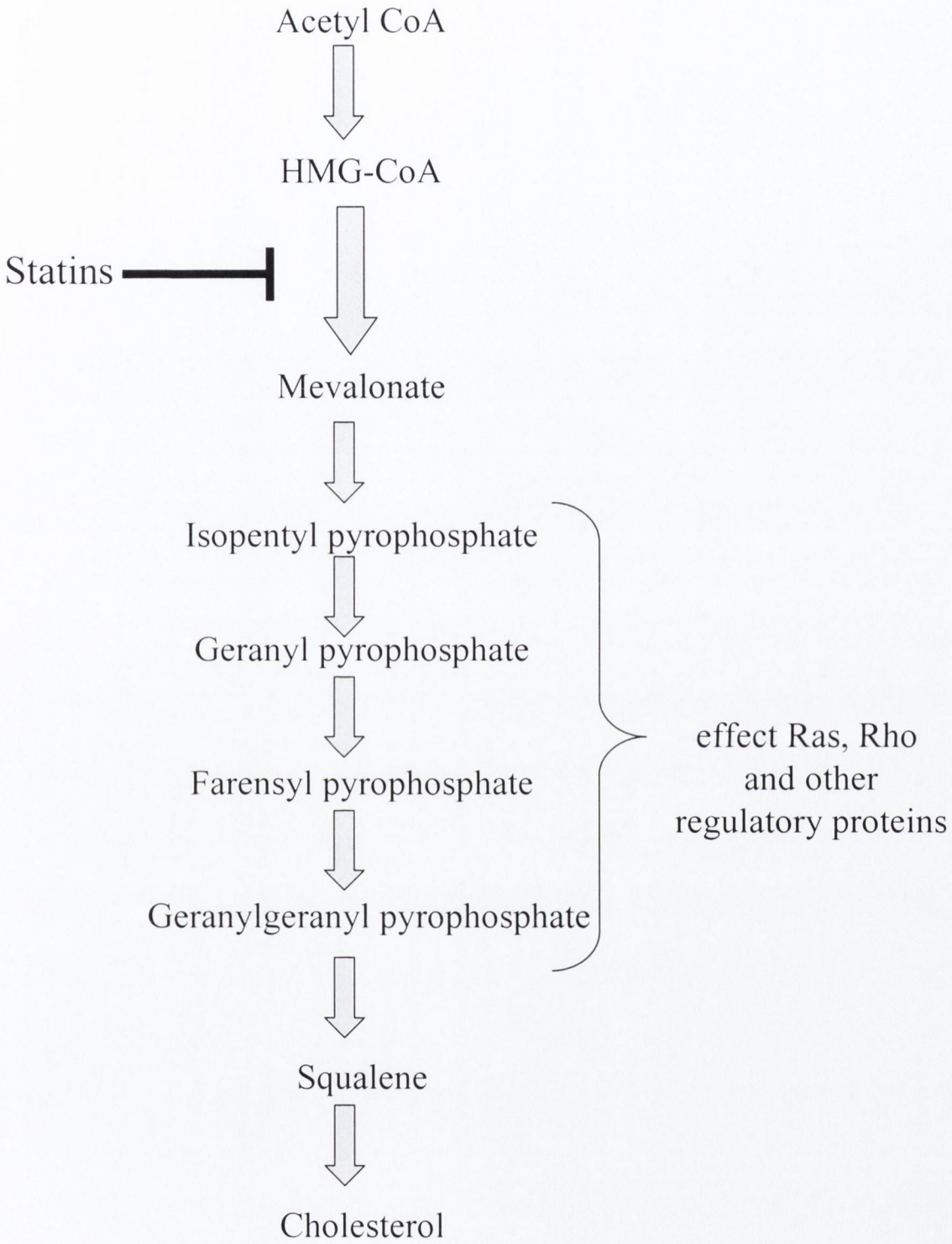


Figure1.3 Diagram of the Cholesterol biosynthetic pathway.

Chapter 2

Materials and Methods

2.1 Animals

All animals used in *in vivo* studies were male Wistar rats. Young animals were an inbred strain (2-4months old; BioResources Unit, Trinity College, Dublin2, Ireland), weighing between 250g and 350g. Aged animals were an inbred strain (23months old; Bantham and Kingham, UK), weighing between 450g and 600g. Animals were housed in groups of 3-6 in a controlled environment (temperature: 20 - 22°C; 12:12 hours light/ dark cycle), with free access to water and food (normal laboratory chow unless otherwise stated).

Primary cultures were prepared from 1 day-old Wistar rat pups (BioResources Unit, Trinity College, Dublin 2, Ireland).

All animal experimentation was performed under a licence granted by the Minister for Health and Children (Ireland) under the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EC.

2.2 Dietary manipulation

In two series of experiments rats were fed on specific diets (see below). Animals were housed in groups of 3 (aged) or groups of 6 (young). In both cases, food intake was measured for 2 weeks prior to start of the dietary period to establish daily food intake. Sufficient chow and supplemented chow was prepared for 2-3 days at a time, and rats were offered their full daily requirement each day. Animals were weighed regularly before, during, and once after dietary manipulation.

In one study young and aged animals were randomly divided into groups which were fed for 8 weeks with an experimental diet of normal laboratory chow supplemented with atorvastatin (5mg/kg body weight/day; Lipitor; Pfizer-Parke Davis, Ireland) or a control diet of normal laboratory chow. Following dietary manipulation, animals were assessed for their ability to sustain LTP.

In the second study, young animals were randomly divided into groups which were fed for 3 weeks with an experimental diet of normal laboratory chow supplemented with atorvastatin (5mg/kg body weight/day; Lipitor; Pfizer-Parke Davis, Ireland) or a control diet of normal laboratory chow. Following dietary manipulation, animals received an intraperitoneal (i.p.) injection of either saline (1ml/kg) or lipopolysaccharide (LPS; 100μg/kg; Sigma-Aldrich, UK). 3h after the i.p. injection animals were assessed for their ability to sustain LTP.

2.3 Animal preparation and pretreatment; intracerebroventricular (i.c.v.) and intraperitoneal (i.p.) injection

Animals were anaesthetized by i.p. administration of 33% w/v urethane (1.5g/kg), and kept warm while awaiting procedures. The absence of a pedal reflex was used to confirm deep anaesthesia and, if needed, a further top-up dose was administered (to a maximum of 2g/kg). Fur on the scalp was clipped and animals were placed in a head holder in a stereotaxic frame (ASI Instruments, USA). A midline incision was made by a scalpel and the skin pulled back to reveal the skull. The periosteum was scraped away and a dental drill was used to drill a small hole at 2.5mm posterior and 0.5mm lateral to bregma to facilitate an intracerebroventricular (i.c.v.) injection. The syringe was inserted to a depth of 3.5mm.

In some experiments 5µl of either saline (control) or D-JNKI1 (500µM; Alexis Biochemicals, UK) was administered by i.c.v. injection, 1h prior to an i.p. injection of either saline (1ml/kg) or lipopolysaccharide (LPS; 100µg/ kg; Sigma-Aldrich, UK). 3h after the i.p. injection animals were assessed for their ability to sustain LTP.

In a second series of experiments 5µl of either vehicle (PBS; 0.00043% DMSO) or SCH58261 (50nM; in PBS, 0.00043% DMSO) was administered by i.c.v. injection, 30min prior to an i.p. injection of either saline (1ml/kg) or lipopolysaccharide (LPS; 100µg/ kg; Sigma-Aldrich, UK). 3h after the i.p. injection, animals were assessed for their ability to sustain LTP.

See table 2.1 for treatment schedule for the different *in vivo* studies.

Table 2.1 Summary of *in vivo* studies

	Age (no.)	Diet	i.c.v. injection	i.p. injection	LTP
Study 1 (a)	Young (36)	N/a	5μl saline or D-JNKI1 (200μM or 500μM)	1h post icv, saline (1ml/kg) or LPS (100μg/ kg)	N/a
Study 1 (b)	Young (24)	N/a	5μl saline or D-JNKI1 (500μM)	1h post icv, saline (1ml/kg) or LPS (100μg/ kg)	Assessed 3h post ip
Study 1 (c)	Young (24)	N/a	5μl saline or D-JNKI1 (500μM)	1h post icv, saline (1ml/kg) or LPS (100μg/ kg)	N/a
Study 2	Young (24)	3 weeks control diet or diet + atorvastatin (5mg/kg body weight/day)	N/a	saline (1ml/kg) or LPS (100μg/ kg)	Assessed 3h post ip
Study 3	Young (12) Aged (18)	8 weeks control diet or diet + atorvastatin (5mg/kg body weight/day)	N/a	N/a	Assessed after diet
Study 4	Young (25)		5μl vehicle or SCH58261 (50nM)	30min post icv, saline (1ml/kg) or LPS (100μg/ kg)	Assessed 3h post ip

2.4 Induction of LTP *in vivo*

2.4.1 Preparation

Animals were anaesthetized by intraperitoneal (i.p.) administration of 33% w/v urethane (1.5g/kg), and kept warm while awaiting procedures. The absence of a pedal reflex was used to confirm deep anaesthesia and, if needed, a further top-up dose was administered (to a maximum of 2g/kg). The recording chamber consisted of a stereotaxic unit attached to the laboratory bench and surrounded by a Faraday cage to isolate it from interference from the external environment. All instruments in the cage were grounded to eliminate 50Hz cycle noise. Fur on the scalp was clipped and rats were placed in a head holder in the stereotaxic frame. A midline incision was made by a scalpel and the skin pulled back to reveal the skull. The periosteum was scraped away and a dental drill was used to remove a window of skull, and the dura mater was peeled away to expose the brain.

2.4.2 Electrode implantation

Bipolar stimulating electrodes and unipolar recording electrodes (Clark Electromedical, UK) were used in this study. The electrodes were lowered to the brain surface with the stimulating electrode positioned 4.4mm lateral to lambda, and the recording electrode positioned 2.5mm lateral and 3.9mm posterior to bregma. The stimulating and recording electrodes were lowered through the cortical and hippocampal layers to the perforant path (angular bundle) and the inner molecular layer (granule cell layer) of the dentate gyrus, respectively. This progress was monitored by a pulse (0.1Hz frequency, 0.1ms duration, 2ms delay, 4V) achieved by passing a single square wave of current generated by a constant isolation unit (IsoFlex, UK), to the bipolar stimulating electrode. The evoked response was transmitted via a pre-amplifier (DAM 50; Differential Amplifier; gain 75, World Precision Instruments, USA) to an analogue-to-digital converter (Maclab/2e, Analog Digital Instruments). This was a digitised system linked to an Apple Macintosh computer (Performa 200), which interfaced with the converter via a specifically written software package (Scope, Version 3.36). This was customised to control both the generation of the square wave pulses and recording of the evoked potentials which were displayed on the screen. The stimulating electrode was lowered in increments into the perforant path. The recording electrode was lowered into the dentate gyrus until the characteristic perforant path – granule cell synapse response was observed. Electrode depth was adjusted to maximize the slope of the field excitatory postsynaptic potential (epsp) and the stimulating intensity adjusted until amplitude of the potential was approximately 1mV. The final depth of the recording electrode was between 2.5 and 3.5mm and for the stimulating electrode was between 2.5 and 3mm.

2.4.3 LTP induction and recording

After a period of stabilisation, test shocks at low frequency (0.033Hz, 0.1s, 2ms delay) were recorded for a 15min control period to establish stable baseline recordings. This was followed by tetanic stimulation (three trains of stimuli; 250Hz for 200ms; 30s interval). Recording at test shock frequency then resumed for 45min. This protocol has been shown to induce saturated LTP in perforant path-granule cell synapses in anaesthetized rats. The field epsps were displayed on-line and were analysed on completion of the experiments. The slope of the population field excitatory post-synaptic potential (field epsp) was used as a measure of excitatory synaptic transmission in the dentate gyrus.

2.5 Preparation of tissue for storage

At the end of the recording period rats were killed by decapitation. The brain was dissected rapidly on ice. The hippocampus, dentate gyrus, entorhinal cortex and cortex were dissected free. This procedure took approximately 2min. In some cases quarter or half brains were covered in OCT and frozen onto cork discs for cryostat sectioning at a later date.

The brain tissue was sliced bidirectionally (350μm thick) using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., UK) and placed in microfuge tubes containing Krebs solution (composition in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_20$ 1.18, $NaHCO_3$ 16, glucose 10) to which $CaCl_2$ (2mM) was added. The tissue samples were washed twice with Krebs solution containing $CaCl_2$ (2mM), and washed twice and then frozen in Krebs solution containing $CaCl_2$ (2mM) and 10% dimethyl sulphoxide (DMSO), and stored at – 80°C until required.

2.6 Preparation of Primary Cultures

2.6.1 Aseptic technique and preparation

Aseptic technique was adhered to for all cell manipulations. All glassware, pipette tips, dissection instruments, deionised H_2O and microfuge tubes (Sarstedt, Ireland) were wrapped in aluminum foil and autoclave tape (Sigma-Aldrich, UK) and autoclaved at 121°C for 20min before being wiped down with 70 % ethanol (EtOH) and placed in the laminar flow hood. All equipment used in the dissection procedure was also exposed to ultraviolet (UV) light, ensuring sterility.

All cell culture work was carried out in a laminar flow hood (Astec-Microflow, USA) the interior of which was sterilized by wiping down all accessible surfaces with 70% EtOH, followed by a 10 min exposure to UV light. Disposable latex gloves (sprayed with 70% EtOH) were worn at all times when cell manipulations were being carried out in the hood. Gloves were changed regularly to avoid contamination.

Solutions and media used were hand filtered through a syringe with an attached 0.2μm cellulose acetate membrane syringe filter (Pall Gelman Sciences Inc., USA) into autoclaved glass bottles or sterile 50ml plastic tubes (Sarstedt, Ireland).

Prior to dissection and preparation of neurons or glia, 13mm diameter glass coverslips (Lennox, Ireland) were soaked in 70% EtOH, followed by an overnight exposure to UV light. Sterile coverslips were then coated with poly-L-lysine (40μg/ml in sterile dH_20; Sigma-Aldrich, UK) in a final volume of 25ml for 1h at 37°C, so as to provide a suitable surface to which dissociated neurons would adhere. Coated coverslips were then air dried, placed in sterile 24-well plates (Cruinn Diagnostics, Ireland) and stored at 4°C until required for use (maximum 2 week storage).

2.6.2 Neuronal preparation procedure

Primary hippocampal or cortical neurons were isolated and prepared from postnatal one-day-old Wistar rats and maintained in neurobasal medium (Gibco BRL, Scotland). Rats were decapitated, the hippocampi or cortices dissected and the meninges were removed. The dissected hippocampi or cortices were cross-chopped and incubated in sterile Dulbecco's phosphate buffered saline (PBS; Sigma-Aldrich, UK) containing trypsin (0.3mg/ml; Sigma-Aldrich, UK) for 25min at 37°C. The tissue was then triturated (7×) in PBS containing soyabean trypsin inhibitor (0.1mg/ml; Sigma-Aldrich, UK), $MgSO_4$ (10mM; Sigma-Aldrich, UK) and DNAse (0.2 mg/ml; Sigma-Aldrich, UK) and gently filtered through a sterile mesh filter (40μm; BD Biosciences, USA). Following centrifugation (2000×*g* for 3min at 20°C), the pellet was resuspended in neurobasal medium (Gibco BRL, Scotland), supplemented with heat inactivated horse serum (10%; Gibco BRL, Scotland), penicillin (1%; Gibco BRL, Scotland), streptomycin (1%; Gibco BRL, Scotland) and glutamax (2 mM, 1%; Gibco BRL, Scotland) and B27 (1%; Gibco BRL, Scotland). Suspended cells were plated out in a 24-well plate (Cruinn Diagnostics, Ireland) on circular 13mm diameter poly-L-lysine coated (40 μg/ml; Sigma-Aldrich, UK) coverslips (Lennox, Ireland), and incubated in a humidified atmosphere containing 5% CO_2: 95% air at 37°C. After 48h, B27 was omitted from the supplemented culture medium and cytosine-arabinofuranoside (5 μg/ml; Sigma-Aldrich, UK) was included for 12-24h to prevent proliferation of non-neuronal cells. The cells then received their appropriate treatments.

2.6.3 Glial preparartion procedure

Primary cortical mixed glia were isolated and prepared from postnatal one-day-old Wistar rats and maintained in Dulbeccos modified eagles medium (DMEM F-12; Gibco BRL, Scotland). Rats were decapitated, cortices dissected and the meninges were removed. The

dissected cortices were cross-chopped and placed in DMEM F-12 supplemented with foetal calf serum (10%; Gibco BRL, Scotland), penicillin (1%; Gibco BRL, Scotland), and streptomycin (1%; Gibco BRL, Scotland). The tissue was then triturated (7×) and gently filtered through a sterile mesh filter (40μm; BD Biosciences, USA). Following centrifugation (2000×g for 3min at 20°C), the pellet was resuspended in supplemented DMEM. Suspended cells were plated out in a 24-well plate (Cruinn Diagnostics, Ireland) on circular 13mm diameter poly-L-lysine coated (40μg/ml; Sigma-Aldrich, UK) coverslips (Lennox, Ireland), and incubated in a humidified atmosphere containing 5% CO_2: 95% air at 37°C. Media was changed every 2-3 days, and cells were allowed at least 10 days before treatment.

2.6.4 Treatments

The appropriate supplemented media was prewarmed and the treatments added to the media at the following final concentrations: D-JNKI1 (1μM; Alexis Biochemicals, UK); Lipopolysaccharide (LPS; 100ng/ml; Sigma-Aldrich, UK); Atorvastatin (ATV; 10μM; Calbiochem, UK; 0.2% DMSO); Vehicle (0.2% DMSO). For treatment schedule for different studies, see Table 2.2

Table 2.2 Summary of *in vitro* studies

	Culture	Pretreatment	Treatment	Harvest
Study 1	Hippocampal Neurons	N/a	Control or LPS (100ng/ml) 24h	Fixed for staining
Study 2 LPS Timecourse	Cortical Neurons	N/a	Control or LPS (100ng/ml) 3,6,18,24 h	S/n, WCL, Fractions: Cyt, M
Study 3	Cortical Neurons	30min Control or D-JNKI1 (1μM)	18h Control or LPS (100ng/ml) ±D-JNKI1 (1μM)	S/n, WCL, Fractions: Cyt, M
Study 4	Cortical Glia	24h Control or Atorvastatin (ATV; 10μM)	24h Control or LPS (100ng/ml) ±ATV (10μM)	S/n

2.6.5 Harvesting of treated cells

At the end of the treatment period, the 24-well plates were removed from the incubator, placed directly onto ice, the supernatant media was removed, and in some instances stored for analysis, and the coverslips washed with phosphate buffered saline (PBS; composition in mM; NaCl 100, Na_2HPO_4 80, NaH_2PO_4 20; pH7.4).

To harvest the cells for preparation of a whole cell lysate, 100µl of Lysis buffer (composition in mM: HEPES 25, $MgCl_2$ 5, DTT 5, EDTA 5, PMSF 2, 10µg/ml leupeptin, 10µg/ml pepstatin A, pH 7.4) was added to each well and rocked on ice for 15min. A 1ml syringe insert was used to scrape the cells from the coverslips and the lysis buffer cell suspension was collected and frozen until further analysis.

To harvest the cells for preparation of crude fractions, coverslips were washed in PBS and then incubated with 75-100µl of permeabilisation buffer (composition in mM: sucrose 250; KCl 70; NaCl 137; Na_2HPO_4 4.5; KH_2PO_4 1.4; PMSF 100µM; leupeptin; 10µg/ml aprotinin 2µg/ml; digitonin 200µg/ml; pH7.2) and rocked on ice for 5min. The permeabilisation buffer was collected as a cytosolic fraction, and the cells then incubated with a mitochondrial buffer (composition in mM: Tris Base 50; NaCl 150; EGTA 2; EDTA 2; PMSF 100µM; leupeptin; 10µg/ml aprotinin 2µg/ml; Triton-X 0.2%; Igepal P-40 0.3%; pH7.2) A 1ml syringe insert was used to scrape the cells from the coverslips and the mitochondrial buffer cell suspension was collected as a mitochondrial enriched fraction and frozen until later analysis.

To fix the cells for immunocytochemistry, the coverslips were washed in PBS before incubation for 30min with 4% paraformaldehyde in PBS (w/v). The paraformaldehyde was replaced with PBS, the 24-well plates wrapped in cling-film and stored at 4°C until coverslips were required for staining.

2.7 Protein quantification using the Bradford protein assay

In the case of *in vivo* studies, thawed tissue slices were washed 3 times in Krebs solution containing $CaCl_2$ (2mM). Tissue was homogenized using a 1ml glass homogeniser (Jencons, UK) by 20-30 up and down strokes (solution and volume used depended on procedure to follow). Samples derived from cultures, also underwent protein quantification. Protein quantification was assessed according to Bradford (1976). Aliquots

of samples (10µl/5µl) were added to a 96-well plate (microtest plate; Sarstedt, Ireland) in triplicate. Standards were prepared from a stock solution of 1000µg/ml bovine serum albumin (BSA; Sigma-Aldrich, UK). This was diluted in dH_2O to prepare a range of standards (including a blank of dH_2O) ranging from 1000µg/ml to 15.625µg/ml. Triplicate aliquots (10µl) of standard were added to the 96-well plate and filtered Bio-Rad dye reagent (1:5 dilution in dH_2O; Bio-Rad, UK) was added to both samples and standards (final volume 200µl). Absorbance was assessed at 595nm using a 96-well plate reader (Labsystems Multiskan RC). A regression line was plotted (Instat or Prism) and the concentration of protein was calculated and converted to mg protein/ml.

2.8 Sample preparation for gel electrophoresis

Hippocampal slices were thawed rapidly, washed 3 times in Krebs solution containing $CaCl_2$ (2mM) and homogenized. In most cases, samples homogenized (15 up and down strokes in a 1ml glass homogeniser; Jencons, UK) in 500µl Krebs solution or lysis buffer, were analysed for protein (See section 2.7), diluted to equal protein concentration, and aliquots were added to a volume of sample buffer (Tris-HCl, 0.5mM, pH6.8; glycerol 20% (v/v); sodium dodecyl sulphate (SDS), 2% (w/v); ß-mercaptoethanol, 5% (v/v); bromophenol blue, 0.05% w/v)), and boiled for 10min.

Cytosolic and membrane-enriched fractions were prepared by gently homogenizing (2 up and down strokes in a 1ml glass homogeniser; Jencons, UK) samples in 400µl Krebs, followed by incubation in a permeabilisation buffer (composition in mM: sucrose 250, KCl 70, NaCl 137, Na_2HPO_4 4.5, KH_2PO_4 1.4, PMSF 0.1, with leupeptin 10 µg/ml, aprotinin 2µg/ml, digitonin 200µg/ml) for 10min on ice, and centrifugation (10,000xg for 15min at 4°C). The resultant supernatant was taken as the cytosolic fraction, and the pellet was resuspended in a mitochondrial buffer (composition in mM Tris-Base 50, NaCl 150, EGTA 2, EDTA 2, PMSF 0.1, with Triton 0.2%, Igepal P-40 0.3%, leupeptin 10 µg/ml, aprotinin 2µg/ml), and this suspension was taken as a membrane-enriched fraction. These fractions were equalized for protein concentration (see section 2.7) and aliquots were added to an equal volume of sample buffer, and boiled for 10min. Samples were stored at -80°C until required.

Subsequently, a method which was considered superior was adopted for our fractionation. In this instance, hippocampal slices were thawed rapidly, washed 3 times in Krebs solution

containing $CaCl_2$ (2mM) and homogenized in 500µl lysis buffer (composition in mM: HEPES 25, $MgCl_2$ 5, DTT 5, EDTA 5, PMSF 2, 10µg/ml leupeptin, 10µg/ml pepstatin A, pH 7.4). Homogenate was then centrifuged at 750xg for 10min at 4°C. The resultant supernantant was removed and placed into a fresh microfuge tube; the pellet was resuspended in lysis buffer and served as the nuclear fraction. The supernatant underwent centrifugation at 10,000xg for 10min at 4°C, giving a further supernatant (cytosolic fraction) and a pellet (which when resuspended in lysis buffer served as the mitochondrial fraction). These fractions were equalized for protein concentration (see section 2.7) and aliquots were added to an equal volume of sample buffer, and boiled for 10min. Samples were stored at -80°C until required.

2.9 SDS-polyacrylamide gel electrophoresis

2.9.1 Preparation of polyacrylamide gels

Polyacrylamide separation gels (1mm thick) of concentration of either 10%, 12%, or 15%, and 4% stacking gels were cast between 10cm wide glass plates (Sigma-Aldrich, UK) and mounted on an electrophoresis unit (Sigma Techware, UK) using spring clamps. The upper and lower reservoirs of the unit were filled with electrode running buffer (25mM Tris-base; 200mM glycine; 17mM SDS). Samples (10µl) were loaded into the wells using a Hamilton MicroLiter syringe. Pre-stained molecular weight standards (10µl; Sigma-Aldrich, UK or Santa Cruz Biotechnology Inc., USA) were also loaded. Proteins were fractionated by application of a constant current (between 26mA and 32mA) to the gel apparatus and migration of the bromophenol blue was monitored. The current was switched off when the blue dye band reached the bottom of the gel (approximately 25-30 min).

2.9.2 Semi-dry electrophoretic blotting of proteins

The gel was removed from the gel apparatus and immersed in transfer buffer (25mM Tris-base; 192mM glycine; 20% methanol (v/v); 0.05% SDS (w/v)). It was placed on top of a sheet of nitrocellulose membrane (0.45µm pore size; Sigma-Aldrich, UK) moistened in the transfer buffer. One piece of filter paper (Standard Grade No. 3, Whatman, UK) was placed on top of the gel and one piece was placed beneath the nitrocellulose paper forming a sandwich. The sandwich was soaked in transfer buffer and placed on the platinum-coated titanium electrode (anode) of a Biometra semi-dry blotter (Sigma-Aldrich, UK). Air bubbles were removed from the sandwich by gently rolling a Pasteur pipette over it. The lid of the blotter (stainless steel cathode) was placed down firmly on top of the sandwich. The uncovered portion of the cathode was shielded with a mylar cut-out (Sigma-Aldrich,

UK), ensuring all applied current passed directly through the sandwich. A constant current of 225mA for approximately 75 min was applied

2.10 Western immunoblotting

Generally, the nitrocellulose membrane was blocked for non-specific binding of protein and probed with an antibody raised against the protein sought. The nitrocellulose membrane was washed in PBS-Tween or Tris buffered saline (TBS)-Tween (composition in mM; Tris-HCl 20; NaCl 150; pH 7.6, 0.05% Tween) and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody. See Table 2.3 for details and summary of antibodies used. A chemiluminescent detection agent which binds to HRP was added and the membrane was exposed to 5 x 7 inch photographic film (Hyperfilm ECL, Amersham, UK) and developed using a Fuji X-ray processor.

2.10.1 Analysis of JNK phosphorylation

To assess phosphorylation of JNK, nitrocellulose membranes were blocked in 2% BSA in TBS overnight at 4°C, and then immunoblotted with a mouse monoclonal antibody (1:300 dilution TBS containing 0.1% BSA, Santa Cruz Biotechnology Inc., USA) 2h at room temperature. Nitrocellulose membranes were washed before incubation with an anti-mouse IgG HRP linked antibody (1:400 dilution TBS containing 0.1% BSA, Sigma-Aldrich, UK) for 1h at room temperature.

2.10.2 Analysis of total JNK

To assess expression of JNK1, nitrocellulose membranes were blocked in 2% BSA in TBS overnight at 4°C, and then immunoblotted with a mouse monoclonal antibody (1:300 dilution TBS containing 0.1% BSA, Santa Cruz Biotechnology Inc., USA) 2h at room temperature. Nitrocellulose membranes were washed before incubation with an anti-mouse IgG HRP linked antibody (1:400 dilution TBS containing 0.1% BSA, Sigma-Aldrich, UK) for 1h at room temperature.

2.10.3 Analysis of Actin

To assess expression of actin, nitrocellulose membranes were blocked in 2% bovine serum albumin (BSA) in TBS overnight at 4°C and then immunoblotted with a mouse monoclonal antibody (1:400 dilution in TBS containing 0.1% BSA; Santa Cruz Biotechnology Inc., USA) for 2h at room temperature. Nitrocellulose membranes were

washed before incubation with an anti-mouse IgG HRP linked antibody (1:500 dilution in TBS containing 0.1% BSA, Sigma-Aldrich, UK) for 1h at room temperature.

2.10.4 Analysis of c-Jun phosphorylation

To assess phosphorylation of c-Jun, nitrocellulose membranes were blocked in 6% non-fat dried milk in PBS-Tween overnight at 4°C, and then immunoblotted with a mouse monoclonal antibody (1:200 dilution in PBS-Tween containing 2% non-fat dried milk; Santa Cruz Biotechnology Inc., USA) for 2h at room temperature. Nitrocellulose membranes were washed before incubation with an anti-mouse IgG HRP linked antibody (1:400 dilution in PBS-Tween containing 2% non-fat dried milk, Sigma-Aldrich, UK) for 1 h at room temperature.

2.10.5 Analysis of total c-Jun

To assess expression of total c-Jun, nitrocellulose membranes were blocked in 2% BSA in TBS-Tween overnight at 4°C, and then immunoblotted with a rabbit monoclonal antibody (1:1000 dilution in TBS-Tween containing 0.1% BSA; Cell Signalling Technology, USA) overnight at 4°C. Nitrocellulose membranes were washed before incubation with an anti-rabbit IgG HRP linked antibody (1:1000 dilution in TBS-Tween containing 0.1% BSA, Sigma-Aldrich, UK) for 1 h at room temperature.

2.10.6 Analysis of bcl-2 phosphorylation

To assess expression of phosphorylated bcl-2 (p-bcl-2), nitrocellulose membranes were blocked in 2% BSA in TBS overnight at 4°C, and then immunoblotted with a rabbit polyclonal antibody (1:250 dilution TBS containing 0.1% BSA; Oncogene, USA) overnight at 4°C. Nitrocellulose membranes were washed before incubation with an anti-rabbit IgG HRP linked antibody (1:500 dilution TBS containing 0.1% BSA, Sigma-Aldrich, UK) for 1h at room temperature.

2.10.7 Analysis of bax

To assess expression of bax, nitrocellulose membranes were blocked in 2% BSA in TBS overnight at 4°C and then immunoblotted with a mouse monoclonal antibody (1:200 dilution in TBS containing 0.1% BSA; Santa Cruz Biotechnology Inc., USA) overnight at 4°C. Nitrocellulose membranes were washed before incubation with an anti-mouse IgG HRP linked antibody (1:400 dilution in TBS containing 0.1% BSA, Sigma-Aldrich, UK) for 1h at room temperature.

2.10.8 Analysis of cytosolic Cytochrome *c*

To assess cytochrome *c* expression, nitrocellulose membranes were blocked in 6% non-fat dried milk in PBS-Tween (0.05% Tween 20) overnight at 4°C, and then immunoblotted with a rabbit monoclonal antibody (1:250 dilution PBS-Tween containing 2% non-fat dried milk; Santa Cruz Biotechnology Inc., USA) overnight at 4°C. Nitrocellulose membranes were washed before incubation with an anti-rabbit IgG HRP linked antibody (1:1000 dilution in PBS-Tween containing 2% non-fat dried milk, Sigma-Aldrich, UK) for 1h at room temperature.

2.10.9 Analysis of Fas Ligand

To assess expression of Fas Ligand, nitrocellulose membranes were blocked in 2% BSA in TBS overnight at 4°C, and then immunoblotted with a rabbit polyclonal antibody (1:500 dilution in TBS containing 0.1% BSA; Santa Cruz Biotechnology Inc., USA) for 2h at room temperature. Nitrocellulose membranes were washed before incubation with and anti-rabbit IgG HRP-linked antibody (1:2500 dilution TBS containing 0.1% BSA; Sigma-Aldrich, UK) for 1h at room temperature.

2.10.10 Analysis of ERK phosphorylation

To assess phosphorylation of ERK, nitrocellulose membranes were blocked in 2% BSA in TBS overnight at 4°C, and then immunoblotted with a mouse monoclonal antibody (1:300 dilution in TBS containing 0.1% BSA; Santa Cruz Biotechnology Inc., USA) for 2h at room temperature. Nitrocellulose membranes were washed before incubation with an anti-mouse IgG HRP linked antibody (1:800 dilution in TBS containing 0.1% BSA, Sigma-Aldrich, UK) for 1h at room temperature.

2.10.11 Analysis of CD40

To assess expression of CD40, nitrocellulose membranes were blocked in 2% BSA in TBS overnight at 4°C, and then immunoblotted with a mouse monoclonal antibody (1:250 dilution in TBS containing 0.1% BSA; Santa Cruz Biotechnology Inc., USA) for 2h at room temperature. Nitrocellulose membranes were washed before incubation with an anti-mouse IgG HRP linked antibody (1:500 dilution in TBS containing 0.1% BSA, Sigma-Aldrich, UK) for 1h at room temperature.

Table 2.3 Summary of western immunoblotting

	BSA/ Marvel	Primary Antibody	Source	Secondary Antibody	Source
Actin	BSA/TBS	1:400; mouse monoclonal; 2h	Santa Cruz, USA	1:500; ant-mouse IgG; 1h	Sigma, UK
bax	BSA/TBS	1:200; mouse monoclonal; O/n	Santa Cruz, USA	1:400; anti-mouse IgG; 1h	Sigma, UK
p-bcl-2	BSA/TBS	1:250; rabbit polyclonal; O/n	Oncogene, USA	1:500; anti-rabbit IgG; 1h	Sigma, UK
CD40	BSA/TBS	1:250; mouse monoclonal; 2h	Santa Cruz, USA	1:500; anti-mouse IgG; 1h	Sigma, UK
Cytochrome *c*	Marvel/PBS	1:250; rabbit monoclonal; O/n	Santa Cruz, USA	1:1000 anti-rabbit IgG; 1h	Sigma, UK
Total-c-jun	BSA/TBS	1:1000; rabbit monoclonal; O/n	Cell Signalling, USA	1:1000 anti-rabbit IgG; 1h	Sigma, UK
p-c-jun	Marvel/PBS	1:200; mouse monoclonal; 2h	Santa Cruz, USA	1:400; anti-mouse IgG; 1h	Sigma, UK
p-ERK	BSA/TBS	1:300; mouse monoclonal; 2h	Santa Cruz, USA	1:800; anti-mouse IgG; 1h	Sigma, UK
Fas Ligand	BSA/TBS	1:500; rabbit polyclonal; 2hr	Santa Cruz, USA	1:2500; anti-rabbit IgG; 1hr	Sigma, UK
Total-JNK	BSA/TBS	1:300; mouse monoclonal; 2h	Santa Cruz, USA	1:400; anti-mouse IgG; 1h	Sigma, UK
p-JNK	BSA/TBS	1:300; mouse monoclonal; 2h	Santa Cruz, USA	1:400; anti-mouse IgG; 1h	Sigma, UK

2.10.12 Stripping and reprobing

In some cases nitrocellulose membranes were probed with a number of antibodies. Following development of blots, the nitrocellulose membranes were washed in PBS, placed in stripping solution (Re-Blot Plus Strong Solution; Chemicon, UK) for 7-10min at room temperature, washed in PBS and then blocked in the appropriate medium for subsequent immunoblotting. Blots were stripped and reprobed a maximum of 3 times.

2.11 Development of blots and quantification of protein expression

Following these incubations and further washing, the immunocomplexes were detected using enhanced chemiluminescence (Pierce No. 34075 Supersignal ® West Dura Extended Duration Substrate; 5min treatment), and exposure to 5 x 7 inch photographic film (Hyperfilm ECL, Amersham, UK), and developed using a Fuji X-ray processor. Pictures were taken using a White/UV Transilluminator (Ultra Violet Products (UVP), UK) and the Annotating Image Capturing System Grab it 2.0, and subsequently quantified by densitometry using Gelworks ID Advanced version 2.51. This gives values based on the height, width, and intensity of the defined bands, relative to the other bands on the particular gel. The resultant figures were normalised between separate gels by bringing all the control values (e.g. saline treated) to a particular number, and then the rest of the values from that gel underwent the same manipulation. Values are expressed as arbitrary units.

2.12 ATP assay

ATP concentration was assessed by a luciferase driven bioluminescence kit (ATP Bioluminescence Assay Kit CLS II, Roche Molecular Biochemicals, UK), Slices of hippocampal tissue were washed three times in Krebs solution containing $CaCl_2$ (2mM), and homogenised in reaction buffer (500µl; composition in mM: Tris Base 100; EDTA 4, pH 7.5). A 100µl aliquot of each sample was placed in a fresh microfuge tube and 9 volumes of boiling reaction buffer added, and samples incubated at 100°C for a further 2min. Samples were transferred onto ice and centrifuged at 1000xg for 60sec at 4°C and the resultant supernatant samples were quantified and equalized for protein. Triplicate samples, standards, and blanks (50µl) were added to black 96-well plates. Luciferase reagent (50µl; Roche Molecular Biochemicals, UK) was added, the plate placed in the luminometer (Fluoroskan Ascent FL, Labsystems, Medical Supply Company, Ireland) and the light signal was integrated for 5sec. The blank value was subtracted from the raw data and ATP concentrations were calculated with reference to a log-log plot of the standard curve data (10^{-5} – 10^{-10} M ATP). Values are expressed as nM ATP.

2.13 Caspase 3 activation assay

Cleavage of the caspase-3 substrate (Ac-DEVD-AFC, Alexis Biochemicals, UK) to its fluorescent product was used as a measure of caspase-3 activity. Slices of hippocampal

tissue were washed, homogenised in lysis buffer (400µl; 25mM HEPES, 5mM $MgCl_2$, 5mM dithiothreitol, 5mM EDTA, 2mM phenylmethylsulfonyl fluoride, 10µg/ml leupeptin, 6.25µg/ml pepstatin, pH 7.4), incubated on ice for 20min, quantified and equalised for protein concentration. Triplicate samples (50µl) were added to reaction buffer (50µl; 50mM HEPES, 2mM EDTA, 20% glycerol, 10mM dithiothreitol, pH 7.4) with and without Ac-DEVD-AFC peptide (10µM) in 96-well plates, and incubated for 1h at 37°C. Fluorescence was assessed (excitation 390nM; emission 510nM) and enzyme activity was calculated with reference to a standard curve of AFC (0-10µM); values were expressed as nmol AFC/mg protein/min.

2.14 Immunohistochemistry

2.14.1 Cytospin preparation

Cells were prepared from frozen slices of hippocampus. Thawed slices of tissue were washed three times in fresh Krebs solution containing $CaCl_2$ (2mM). Slices were incubated in a collagenase PBS solution for 45min on ice. Washed slices were resuspended in 750µl PBS, triturated with a glass pasteur pipette, and passed through a fine nylon mesh to remove tissue clumps. Aliquots (100µl) of the cell suspension were spun (600rpm for 2min; Shandon Cytospin 3) onto glass slides, allowed to dry, fixed in methanol, and stored at room temperature in the dark until further analysis.

2.14.2 Brain Slice preparation

Half or quarter brains were covered in OCT and frozen by liquid nitrogen onto cork discs, and stored at -80°C. Sections (10µm) were cut in a cryostat (Leica, UK), and preliminary staining was carried out to determine when the sections included the hippocampus. Once in the hippocampus the slices were adhered to subbed slides in a sequential order, such that each slide had three slices each 30 sections apart. Slides were stored at -20°C.

2.14.3 TUNEL staining

The DeadEnd Colorimetric Apoptosis Detection System (Promega, USA) was used and this system end-labels the fragmented DNA of apoptotic cells using a modified TUNEL (TdT-mediated dUTP Nick-End Labelling) assay. All procedures were carried out at room temperature unless otherwise stated. This procedure was carried out on cells on coverslips and cytospun cells. Coverslips were removed from 24-well plates, and on slides, cytospun cells were surrounded with Pap Pen. The tissue was rehydrated in saline solution for 5min,

washed twice in PBS for 5min and fixed for 20min with 4% paraformaldehyde, and washed. The cells were then permeabilised with Proteinase K (20μg/ml) for 10min, washed, and refixed in 4% paraformaldehyde for 5min, and washed. Slides were then immersed in equilibration buffer (50μl; composition in mM: potassium cacodylate 200 (pH 6.6 at 25°C); Tris-HCl 25 (pH 6.6 at 25°C); DTT 0.2; cobalt chloride 2.5) containing 0.25mg/ml BSA for 10min, and then incubated at 37°C for 60min in terminal deoxynucleotidyl transferase (TdT) mix, containing TdT enzyme, biotinylated nucleotide mix and equilibration buffer. The reaction was terminated by immersing the cells in 2X sodium chloride/sodium citrate buffer for 15min. Cells were washed three times in PBS to remove unincorporated biotinylated nucleotides and endogenous peroxidases were blocked by immersion in 0.3% H_2O_2 for 5min. Cells were washed, incubated with streptavidin horseradish peroxidase (1:500 in PBS) for 30min, washed again and staining was developed by addition of the chromogene 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 15-20min which stains apoptotic nuclei brown. This reaction was terminated by washing in distilled water. Cells were counterstained with toluidine blue to identify TUNEL negative cells, dehydrated through alcohol to xylene, mounted in DPX and viewed under a light microscope under x40 magnification. In the case of cytospun cells, the proportion of positively stained cells were calculated and expressed as the percentage of the total cells counted.

2.14.4 Active caspase 3 staining

All procedures were carried out at room temperature unless otherwise stated. Brain sections were allowed to thaw, and surrounded by Pap Pen. The tissue was rehydrated in saline solution for 5min, washed twice in PBS for 5min and fixed for 20min with 4% paraformaldehyde, and washed. The cells were permeabilised with Proteinase K (20μg/ml), TritonX (0.2%) in PBS for 10min, washed, and refixed in 4% paraformaldehyde for 5min, and washed again. Endogenous peroxidases were blocked by immersion in 0.3% H_2O_2 in 0.3%NGS in PBS for 15min. Following washing the sections were incubated with a blocking buffer (10% Normal goat serum (NGS) in 4% BSA in PBS) for 2h, and then incubated with an anti-active caspase 3 antibody (1:100; Promega, USA; in 5% NGS in 2% BSA in PBS) overnight at 4°C. Sections were washed before incubation with an anti-rabbit IgG biotinylated antibody (1:100; Vector, UK; in 2% BSA in PBS). Following washing, sections were incubated with reagent mix (2 drops reagent A, 2 drops reagent B, in 5ml PBS; ABC *Elite* Kit; Vector, UK) for 1h at room temperature,

and washed. Staining was developed by addition of the chromogene DAB for 10-15min. This reaction was terminated by immersion in distilled water. Cells were counter stained with toluedene blue, dehydrated through alcohol to xylene, mounted in DPX and viewed under a light microscope under x40 magnification.

2.15 Immunofluorescent staining

Immunofluorescent staining was carried out on cultured neurons fixed to coverslips.

2.15.1 Active caspase 3 staining

The cells were permeabilised with Proteinase K (20μg/ml) in 0.1% TritonX100 in TBS (w/v) for 8min, washed, refixed in 4% paraformaldehyde for 10min, washed, blocked with blocking buffer (10% NGS in TBS; v/v) for 2h and incubated with an active caspase 3 rabbit IgG antibody (1:200 in 2.5% NGS in TBS, Promega, USA) overnight at 4°C. Cells were stained by incubating in fluorescently tagged secondary antibody for 1h at 37°C in darkness; goat anti rabbit IgG L-Rhodamine linked antibody (1:100 in 2.5% NGS in TBS, BioSource, UK). Coverslips were sealed using fluorescent mounting medium (Vectastain, Vector, UK) and stored in the dark at 4°C until viewed under a Zeiss fluorescence microscope with the appropriate filter (L-Rhodamine – excitation 574nm and emission 602nm).

2.15.2 Phosphorylated JNK staining

The cells were permeabilised with Proteinase K (20μg/ml) in 0.1% TritonX100 in TBS (w/v) for 8min, washed, refixed in 4% paraformaldehyde for 10min, washed, blocked with blocking buffer (10% NGS in TBS; v/v) for 2h and incubated with a phosphorylated JNK mouse IgG antibody (1:200 in 2.5% NGS in TBS, Santa Cruz Biotechnology Inc., USA) overnight at 4°C. Cells were stained by incubating in fluorescently tagged secondary antibody for 1h at 37°C in darkness; goat anti mouse IgG and IgM FITC linked antibody (1:100 in 2.5% NGS in TBS, BioSource, UK). Coverslips were sealed using fluorescent mounting medium (Vectastain, Vector, UK) and stored in the dark at 4°C until viewed under a Zeiss fluorescence microscope with the appropriate filter (FITC – excitation 495nm and emission 525nm)

2.15.3 GFAP staining

To ensure purity of glial and neuronal primary cultures, coverslips with fixed cells from a standard preparation for each cell type under went immunofluorescent staining for GFAP, an astrocytic cell marker. The micrographs in Figure 2.1 demonstrate that glial cultures showed positive staining for the glial marker while neuronal cultures did not. The cells were permeabilised with Proteinase K (20μg/ml) in 0.1% TritonX100 in TBS (w/v) for 8min, washed, refixed in 4% paraformaldehyde for 10min, washed, blocked with blocking buffer (10% NGS in TBS; v/v) for 2h and incubated with a GFAP antibody (1:800 in 2.5% NGS in TBS, Chemicon, UK) overnight at 4°C. Cells were stained by incubating in fluorescently tagged secondary antibody for 1h at 37°C in darkness; goat anti rabbit IgG L-Rhodamine linked antibody (1:100 in 2.5% NGS in TBS, BioSource, UK). Coverslips were sealed using fluorescent mounting medium (Vectastain, Vector, UK) and stored in the dark at 4°C until viewed under a Zeiss fluorescence microscope with the appropriate filter (L-Rhodamine – excitation 574nm and emission 602nm).

2.16 Enzyme Linked Immunosorbent Assay (ELISA)

2.16.1 IL-1β (Duoset, R&D Systems)

All procedures were carried out at room temperature. 96-well plates (Nalge Nunc International, UK) were coated in the capture antibody (100μl; anti-rat IL-1β; R&D Systems, UK; 0.8μg/ml in PBS [composition for ELISAs in mM: NaCl 137; KCl 2.7; Na_2HPO_4 8.1; KH_2PO_4 1.5; pH 7.4]) and incubated overnight. The plates were washed 3 times in PBS Tween (0.05%), a blocking buffer was added to the wells (280μl; 1%BSA, 5% sucrose, 0.05% NaN_3 in PBS) and the plates incubated for 1h. Standards (0 – 2000pg/ml) were prepared from recombinant rat IL-1β (R&D Systems, UK) in diluent (1%BSA in PBS). The plates were washed 3 times in PBS Tween and 100μl of standard or sample were incubated for 2h. Following further washing, detection antibody (100μl; biotinylated anti-rat IL-1β; R&D Systems, UK; 350ng/ml in 2% NGS in diluent) was incubated for 2h. The plates were washed 3 times in PBS Tween and streptavidin-HRP conjugate (100μl) was added and incubated for 20min. The plates were washed 3 times in PBS Tween and substrate solution (100μl; 1:1 reagent A (H_2O_2): reagent B (TMB); R&D Systems, UK) was added to the wells and incubation continued in the dark for 20min which created a colour change to blue. Stop solution of 1M H_2SO_4 (50μl) was added and the plates read at 450nm. A standard curve was constructed by plotting the standards

against their absorption. Results are expressed in pg IL-1β/mg tissue corrected for protein or pg/ml.

2.16.2 IL-4 (Duoset, R&D Systems)

All procedures were carried out at room temperature. 96-well plates (Nalge Nunc International, UK) were coated in the capture antibody (100µl; anti-rat IL-4; R&D Systems, UK; 2µg/ml in PBS) and incubated overnight. The plates were washed 3 times in PBS Tween (0.05%), a blocking buffer was added to the wells (280µl; 1%BSA, 5% sucrose, 0.05% NaN_3 in PBS) and the plates incubated for 1h. Standards (0 – 1000pg/ml) were prepared from recombinant rat IL-4 (R&D Systems, UK) in diluent (1%BSA in PBS). The plates were washed 3 times in PBS Tween and 100µl of standard or sample were incubated for 2h. Following further washing, detection antibody (100µl; biotinylated anti-rat IL-4; R&D Systems, UK; 50ng/ml in diluent) was incubated for 2h. The plates were washed 3 times in PBS Tween and streptavidin-HRP conjugate (100µl) was added and incubated for 20min. The plates were washed 3 times in PBS Tween and substrate solution (100µl; 1:1 reagent A (H_2O_2): reagent B (TMB); R&D Systems, UK) was added to the wells and incubation continued in the dark for 20min which created a colour change to blue. Stop solution of 1M H_2SO_4 (50µl) was added and the plates read at 450nm (plate reader). A standard curve was constructed by plotting the standards against their absorption. Results are expressed in pg IL-4/mg tissue corrected for protein or pg/ml.

2.16.3 IFNγ (Duoset, R&D Systems)

All procedures were carried out at room temperature. 96-well plates (Nalge Nunc International, UK) were coated in the capture antibody (100µl; anti-rat IFNγ; R&D Systems, UK; 2µg/ml in PBS) and incubated overnight. The plates were washed 3 times in PBS Tween (0.05%), a blocking buffer was added to the wells (280µl; 1%BSA, 5% sucrose, 0.05% NaN_3 in PBS) and the plates incubated for 1h. Standards (0 – 1250pg/ml) were prepared from recombinant rat IFNγ (R&D Systems, UK) in diluent (1%BSA in PBS). The plates were washed 3 times in PBS Tween and 100µl of standard or sample were incubated for 2h. Following further washing, detection antibody (100µl; biotinylated anti-rat IFNγ; R&D Systems, UK; 150ng/ml in diluent) was incubated for 2h. The plates were washed 3 times in PBS Tween and streptavidin-HRP conjugate (100µl) was added and incubated for 20min. The plates were washed 3 times in PBS Tween and substrate

solution (100µl; 1:1 reagent A (H_2O_2): reagent B (TMB); R&D Systems, UK) was added to the wells and incubation continued in the dark for 20min which created a colour change to blue. Stop solution of 1M H_2SO_4 (50µl) was added and the plates read at 450nm (plate reader). A standard curve was constructed by plotting the standards against their absorption. Results are expressed in pg IL-4/mg tissue corrected for protein or pg/ml.

2.16.4 IL-10 (Cytoset, Biosource)

All procedures were carried out at room temperature unless otherwise stated. 96-well plates (Nalge Nunc International, UK) were coated in the capture antibody (100µl; anti-rat IL-10; Biosource, UK; 1.25µg/ml in PBS) and incubated overnight at 4°C. The plates were washed 3 times in PBS Tween (0.05%), a blocking buffer was added to the wells (280µl; 0.5%BSA in PBS) and the plates incubated for 2h. Standards (0 – 2000pg/ml) were prepared from recombinant rat IL-10 (Biosource, UK) in diluent (0.1% Tween-20 in block buffer). The plates were washed 3 times in PBS-Tween and 100µl of standard or sample were incubated for 1.5h. Following further washing, detection antibody (100µl; biotinylated anti-rat IL-10; Biosource, UK; 125ng/ml in 5% foetal bovine serum in diluent) was incubated for 1h. The plates were washed 3 times in PBS Tween and streptavidin-HRP conjugate (100µl; 0.2µl/ml) was added and incubated for 45min. The plates were washed 3 times in PBS Tween and Tetramethylbenzidine (TMB; 100µl;) was added to the wells and incubation continued in the dark for 30min which created a colour change to blue. Stop solution of 1M H_2SO_4 (50µl) was added and the plates read at 450nm (plate reader). A standard curve was constructed by plotting the standards against their absorption. Results are expressed in pg IL-10/mg tissue corrected for protein or pg/ml.

2.17 Statistical analysis

Data are expressed as the mean ± standard error of the means (SEM). One-way analysis of variance (ANOVA) was performed to determine whether significant differences existed between conditions. When this analysis indicated significance (at the 0.05 level), *post hoc* student Newmann-Keuls test analysis was used to determine which conditions were significantly different from each other (GraphPad Prism). Where appropriate, student's t-test was carried out. Correlation analysis was assessed by a Pearson correlation.

2.18 Materials

2.18.1 Reagents

ABC *Elite* Kit	Vector, UK
Ac-DEVD-AFC	Alexis Biochemicals, UK
Amonium persulfate (APS)	Sigma- Aldrich, UK
Aprotinin	Sigma- Aldrich, UK
Atorvastatin Lipitor	Pfizer-Parke Davis, Ireland
Atorvastatin calcium	Calbiochem, UK
ATP Bioluminescence Assay Kit CLS II	Roche Molecular Biochemicals, UK
B27	Gibco BRL, Scotland
Bio-Rad dye reagent	Bio-Rad, UK
Bis-acrylamide	Sigma-Aldrich, UK
Bovine serum albumin (BSA)	Sigma- Aldrich, UK
Bromophenol blue	Sigma-Aldrich, UK
Calcium Chloride $CaCl_2$	Lennox, Ireland
Caspase-3 substrate Ac-DEVD-AFC,	Alexis Biochemicals, UK
Cytosine-arabinofuranoside	Sigma- Aldrich, UK
DeadEnd Colorimetric Apoptosis Detection System	Promega, USA
Diaminobenzidine tetrahydrochloride (DAB)	Dakocytomation
Digitonin	Sigma-Aldrich, UK
D-JNKI1	Alexis Biochemicals, UK
DMSO	Sigma-Aldrich, UK
DNAse	Sigma- Aldrich, UK
DPX	BDH lab supplies
DTT dithiothreitol	Sigma-Aldrich, UK
Dulbecco's phosphate buffered saline	Sigma- Aldrich, UK
Dulbeccos modified eagles medium DMEM F-12	Gibco BRL, Scotland
EDTA	Sigma-Aldrich, UK
EGTA	Sigma-Aldrich, UK
Ethanol	
Fluorescent mounting medium	Vector, UK
Foetal calf serum	Gibco BRL, Scotland
Glucose	Lennox, Ireland
Glutamax	Gibco BRL, Scotland
Glycerol	Sigma-Aldrich, UK
Glycine	Sigma-Aldrich, UK
Heat inactivated horse serum	Gibco BRL, Scotland
HEPES	Sigma-Aldrich, UK
Hydrochloric acid	Lennox, Ireland
Hydrogen Peroxide H_2O_2	Sigma-Aldrich, UK
Igepal P-40	Sigma-Aldrich, UK
IL-1β	R&D systems, UK
IL-4	R&D systems, UK
Leupeptin	Sigma-Aldrich, UK
Lipopolysaccharide (LPS: e. coli)	Sigma-Aldrich, UK

Luciferase reagent	Roche Molecular Biochemicals, UK
β-Mercaptoethanol	Sigma-Aldrich, UK
Magnesium Chloride MgCl2	Sigma-Aldrich, UK
Magnesium sulphate $MgSO_4.7H_20$	Sigma-Aldrich, UK
Methanol	Lennox, Ireland
Methyl green	Sigma-Aldrich, UK
Neurobasal medium	Gibco BRL, Scotland
Nitrocellulose membrane 0.45μm pore size;	Sigma- Aldrich, UK
Non-fat dried milk (Marvel)	
Normal goat serum	Vector, UK
OCT Compound Tissue Tek	R. A. Lamb Ltd, UK
Paraformaldehyde	Riedel-de-Haën
Penicillin /streptomycin	Gibco BRL, Scotland
Pepstatin A	Sigma-Aldrich, UK
PMSF phenylmethylsulfonyl fluoride	Sigma-Aldrich, UK
Poly-l-lysine	Sigma-Aldrich, UK
Potassium chloride KCl	Sigma-Aldrich, UK
Potassium hydroxide	Sigma-Aldrich, UK
Potassium phosphate KH_2PO_4	Sigma-Aldrich, UK
Pre-stained molecular weight standard	Santa Cruz, USA
Pre-stained molecular weight standard	Sigma-Aldrich, UK
Proteinase K	Promega, USA
ReBlot Plus strong antibody stripping solution	Chemicon, UK
SCH58261	Gift
Sodium azide NaN_3	Sigma-Aldrich, UK
Sodium carbonate	Sigma-Aldrich, UK
Sodium chloride NaCl	Sigma-Aldrich, UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, UK
Sodium hydrogen carbonate $NaHCO_3$	Lennox, Ireland
Sodium hydroxide	Lennox, Ireland
Sodium phosphate (dibasic) Na_2HPO_4	Sigma-Aldrich, UK
Sodium phosphate (monobasic) NaH_2PO_4	Sigma-Aldrich, UK
Substrate solution reagent A(H_2O_2): reagent B (TMB);	R&D systems, UK
Sucrose	Lennox, Ireland
Sulphuric Acid H_2SO_4	Lennox, Ireland
Supersignal ® West Dura Extended Duration Substrate	Pierce, USA
Temed	Sigma-Aldrich, UK
Toluidine blue	Gurrs, UK
Tris Base	Sigma-Aldrich, UK
Tris-Hcl	Sigma-Aldrich, UK
TritonX	Sigma-Aldrich, UK
Trypsin	Sigma- Aldrich, UK
Trypsin inhibitor soyabean	Sigma- Aldrich, UK
Tween-20	Lennox, Ireland
Urethane	Sigma-Aldrich, UK

2.18.2 Equipment

24-well plates	Cruinn Diagnostics, Ireland
Autoclave tape	Sigma-Aldrich, UK

Biometra semi-dry blotter	Sigma-Aldrich, UK
Bipolar stimulating, and unipolar recording electrodes	Clark Electromedical, UK
Electrophoresis unit	Sigma Techware, UK
Filter paper Standard Grade No. 3,	Whatman, Kent, UK
Fluoroskan Ascent FL Labsystems	Medical Supply Company, Ireland
Fujifilm FPM800A X-ray processor	Fujifilm
Glass coverslips (13mm diameter)	Lennox, Ireland
Glass homogeniser (1ml)	Jencons, UK
Glass plates (10cm wide)	Sigma- Aldrich, UK
Hyperfilm ECL (5 x 7 inch photographic film)	Amersham, UK
Laminar flow hood.	Astec-Microflow, USA
Leica Cryostat	Laboratory instruments supplies Ltd, Ireland
McIlwain tissue chopper	Mickle Laboratory Engineering Co., UK
Mesh filter (40μm)	BD Biosciences, USA
Microtest plate (96-well)	Sarstedt, Ireland
Mylar cut-out	Sigma-Aldrich, UK
Nalge Nunc International plates (96-well)	Bio Sciences, Ireland
Plate reader (96-well)	Labsystems Multiskan RC
Shandon Cytospin 3	Shandon
Sigma Laboratory Centrifuge 2K15C	AGB Scientific, Ireland
Stereotaxic frame	ASI Instruments
Syringe filter- cellulose acetate membrane (0.2 μm)	Pall Gelman, USA
Syringes	BD Plastipak

2.18.3 Antibodies

Actin, mouse monoclonal antibody	Santa Cruz, USA
active caspase 3 rabbit IgG antibody	Promega, USA
anti-active caspase 3 antibody	Promega, USA
anti-mouse IgG biotinylated antibody raised in horse	Vector, UK
anti-mouse IgG HRP linked antibody	Sigma-Aldrich, UK
anti-rabbit IgG biotinylated antibody	Vector, UK
anti-rabbit IgG HRP linked antibody	Sigma-Aldrich, UK
bax, mouse monoclonal antibody	Santa Cruz, USA
cytochrome *c*, rabbit monoclonal antibody	Santa Cruz, USA
Fas Ligand rabbit polyclonal antibody	Santa Cruz, USA
GFAP antibody	Chemicon, UK
goat anti mouse IgG and IgM FITC linked antibody	BioSource, UK
goat anti rabbit IgG L-Rhodamine linked antibody	BioSource, UK
JNK1, mouse monoclonal antibody	Santa Cruz, USA
phosphorylated bcl-2, rabbit polyclonal antibody	Oncogene, USA
phosphorylated c-Jun, mouse monoclonal antibody	Santa Cruz. USA
phosphorylated ERK, mouse monoclonal antibody	Santa Cruz, USA
phosphorylated JNK mouse IgG antibody	Santa Cruz, USA
phosphorylated JNK, mouse monoclonal antibody	Santa Cruz, USA

A) B)

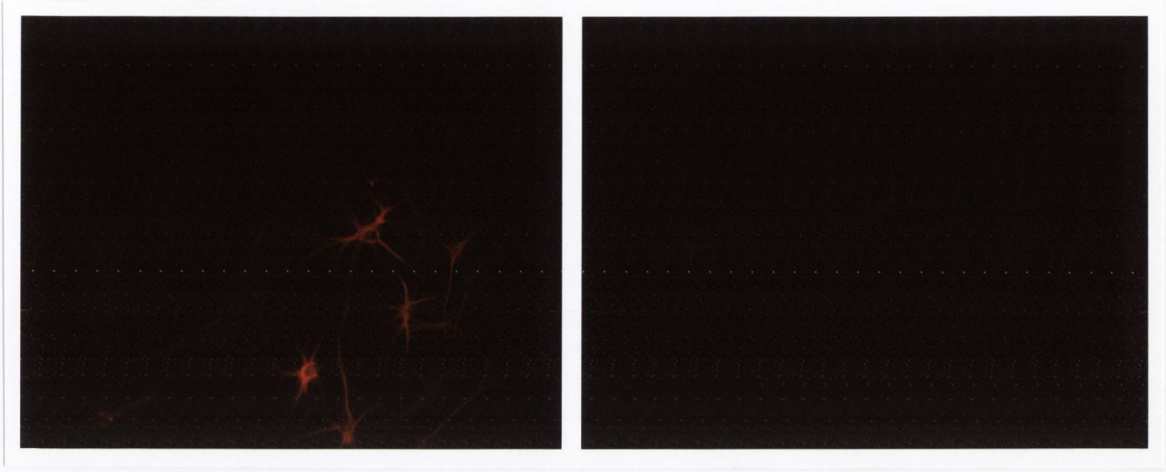


Figure 2.1
Immunofluorescent GFAP staining in (A) a primary glial culture, and (B) a primary neuronal culture.

Chapter 3

Results

3. 1. Analysis of LPS-induced effects on hippocampus of rats pretreated with the JNK inhibitor D-JNKI1

3. 1. 1. LPS administration increased activity of JNK and caspase 3 *in vitro* and *in vivo*.

LPS injection is often used as a model of acute inflammation, therefore the ability of LPS to induce detrimental changes was assessed *in vitro* and *in vivo*. For the *in vitro* study, primary cultured hippocampal neurons were treated with either control media or LPS-containing media (100ng/ml) for 24h. In the case of the *in vivo* study, male Wistar rats received an i.p. injection of either saline or LPS (100μg/kg) and were killed after a 3h period.

LPS-treated primary hippocampal neurons (100ng/ml; 24h) exhibited increased fluorescent staining for phosphorylated JNK compared with control-treated neurons (Figure 3.1A). Hippocampal tissue prepared from control-treated and LPS-treated rats was examined by SDS-PAGE and Western Immunoblotting. The sample immunoblot in Figure 3.1B indicates that JNK phosphorylation was increased in hippocampus of LPS-treated rats (lane 2) compared with control-treated rats (lane 1). Assessment of mean values obtained from densitometric analysis (Figure 3.1C) indicated JNK phosphorylation (arbitrary units ±SEM) was significantly increased in hippocampus of LPS-treated rats (1766.68 ±87.47) compared with control-treated rats (1447.75 ±41.5; *$p<0.05$; unpaired student t test; n=5).

Concurrent with the increase in JNK phosphorylation, LPS-treated primary hippocampal neurons (100ng/ml; 24h) exhibited increased fluorescent staining for active caspase 3 compared with control-treated neurons (Figure 3.2A). Hippocampal tissue prepared from control-treated and LPS-treated rats was examined for caspase 3 activity by a fluorometric assay (Figure 3.2B). Caspase 3 activity (nmol AFC/mg/min ±SEM) was significantly increased in hippocampus of LPS-treated rats (2.64 ±0.43) compared with control-treated rats (1.60 ±0.27; *$p<0.05$; unpaired student t test; n=5).

3. 1. 2. D-JNKI1 (200μM or 500μM) did not attenuate the LPS-induced increase in JNK phosphorylation, but did attenuate the LPS-induced increase in c-Jun phosphorylation in a dose dependent manner.

As the JNK signalling pathway has been shown to be important in inflammatory processes, the JNK inhibitor, D-JNKI1 was assessed for its ability to attenuate LPS-induced JNK activation at both 200μM and 500μM concentration. Male Wistar rats received an i.c.v. injection of either saline or D-JNKI1 (5μl; 200μM or 500μM), 1h later received an i.p. injection of either saline or LPS (100μg/kg), and were killed following a further 3h period.

The active form of JNK is phosphorylated, therefore, using an antibody specific for the phosphorylated form of JNK, hippocampal tissue was examined by SDS-PAGE and Western Immunoblotting. The sample immunoblots in Figure 3.3A and B indicate that JNK phosphorylation was increased in hippocampus of control-treated rats which received LPS i.p. (lane 2; A and B), rats pretreated with D-JNKI1 which received saline i.p. (lane 3; 200μM and 500μM; A and B respectively), and D-JNKI1-treated rats which received LPS i.p. (lane 4; 200μM and 500μM; A and B respectively) compared with control-treated rats which received saline i.p. (lane 1). Assessment of mean values obtained from densitometric analysis (Figure 3.3C) indicated JNK phosphorylation (arbitrary units ±SEM) was significantly increased in hippocampus of control-treated rats which received LPS i.p. (1969 ±137.9) compared with control-treated rats which received saline i.p. (1497 ±102.9; *$p<0.05$; Newmann-Keuls; n=10). The expression of JNK phosphorylation was similarly increased in hippocampus of rats pretreated with D-JNKI1 (200μM and 500μM) which received LPS i.p. (1778 ±108.6 and 2513±363.8 respectively), and in D-JNKI1-treated rats (200μM and 500μM) which received saline i.p. (1797 ±62.8 and 2156 ±330.3 respectively), such that mean values in all groups (n=6) were significantly increased compared with values for saline-treated controls (*$p<0.05$; Newmann-Keuls).

As it was apparent that D-JNKI1 did not prevent the phosphorylation of JNK caused by LPS, we went on to investigate the effect of D-JNKI1 on the ability of JNK to phosphorylate its targets. The transcription factor c-Jun is phosphorylated by active JNK, so an antibody specific for the phosphorylated form of c-Jun was used to assess JNK inhibition by D-JNKI1, in hippocampal tissue examined by SDS-PAGE and Western Immunoblotting. The sample immunoblot in Figure 3.4A indicates that c-Jun phosphorylation was increased in hippocampus of control-treated rats which received LPS i.p. (lane 2) compared with control-treated rats which received saline i.p. (lane 1). It was

also marginally increased in samples prepared from rats pretreated with 200μM or 500μM D-JNKI1 which received saline i.p. (lane 3 and 5 respectively). Assessment of mean values obtained from densitometric analysis (Figure 3.4B) indicated c-Jun phosphorylation (arbitrary units ±SEM) was increased in hippocampus of control-treated rats which received LPS i.p. (1984 ±126.7) compared with control-treated rats which received saline i.p. (1549 ±43.7; +$p<0.01$; student t test; n=12). The expression of c-Jun phosphorylation in hippocampus of rats pretreated with 200μM or 500μM D-JNKI1 which received LPS i.p. was decreased (1542 ±238.1; n=6 and 1377 ±173.6; n=10 respectively) compared with control-treated rats which received LPS i.p., with the 500μM pretreatment showing a significant effect (*$p<0.05$ Newmann-Keuls). Pretreatment with 200μM or 500μM D-JNKI1 in rats which received saline i.p. resulted in a modest non-significant increase in c-Jun phosphorylation (1723 ±200; n=6 and 1793 ±104.5; n=10 respectively).

These data indicate that 500μM D-JNKI1 is effective in rats following i.c.v injection, therefore it was assessed for its ability to reverse the central effects of i.p. LPS administration, a model of acute inflammation, specifically the detrimental effects leading to apoptosis and decreased synaptic efficacy. Male Wistar rats received an i.c.v. injection of either saline or D-JNKI1 (5μl; 500μM), 1h later received an i.p. injection of either saline or LPS (100μg/kg), and, following a further 3h period, were assessed for their ability to sustain LTP, then killed.

3. 1. 3. D-JNKI1 abrogated the inhibitory effects of LPS on LTP.

The effect of i.p. LPS injection on the induction and maintenance of LTP in D-JNKI1-treated and control-treated animals was examined in the perforant path – granule cell synapses. The mean stimulus strength (mV; ±SEM) required to induce a spike in control-treated rats which received saline i.p. and LPS i.p. was 2.022 (±0.56) and 1.005 (±0.25) respectively. The corresponding values for D-JNKI1-treated rats were 1.837 (±0.79) and 1.695 (±0.49). There was no significant difference between these values.

Figure 3.5A and B show that tetanus-induced LTP was blocked in perforant path – granule cell synapses of control-treated rats which received LPS i.p. (n=9) compared with control-treated rats which received saline i.p. (n=12); the mean percentage change in epsp slope (±SEM) in the 2min immediately following tetanic stimulation (compared with that in the 5min immediately prior to stimulation) was 123.2 (±2.2), which was significantly decreased from that observed in control-treated rats which received saline i.p.; 155.4 (±3.3; ***$p<0.001$; Newmann-Keuls). Similarly, the mean percentage change in epsp slope

(±SEM) in the last 5 minutes of the experiment was significantly decreased from 116.0 (±1.4) in control-treated rats which received saline i.p., to 97.44 (±0.6) in control-treated rats which received LPS i.p. (***$p<0.001$; Newmann-Keuls).

This impairment was suppressed by pretreatment with D-JNKI1; rats pretreated with D-JNKI1 which received LPS i.p. (n=6) sustained LTP, as did D-JNKI1-treated rats which received saline i.p. (n=6). The mean percentage changes in epsp slope (± SEM) in the 2 minutes immediately following tetanic stimulation in D-JNKI1-treated rats which received saline i.p. and LPS i.p. were 134.2 (±1.5) and 124.8 (±0.8) respectively. Therefore D-JNKI1 failed to reverse the LPS-attenuation of the initial increase in epsp slope and, alone, caused a significant decrease compared with saline-treated controls (++$p<0.01$; Newmann-Keuls). However pretreatment with D-JNKI1 abrogated the effect of LPS at the later time-point; mean percentage changes in epsp slope (±SEM) in the last 5 minutes of the experiment were 110.3 (±0.3) and 112.4 (±0.8) for D-JNKI1-treated rats which received saline i.p. and LPS i.p. respectively. Nevertheless it should be noted that the LTP sustained by the saline-treated control group was significantly greater than that of either of the D-JNKI1 treated groups (**$p<0.01$; Newmann-Keuls).

3. 1. 4. D-JNKI1 did not attenuate the LPS-induced increase in IL-1β concentration or JNK phosphorylation.

As IL-1β has previously been shown to be increased in response to LPS (Vereker *et al.*, 2000a), IL-1β was assessed in hippocampal tissue by ELISA. Figure 3.6 demonstrates that IL-1β concentration (pg/mg protein ±SEM) was increased in hippocampus of control-treated rats which received LPS i.p. (710 ±103.2; n=6) compared with control-treated rats which received saline i.p. (602 ±55.9; n=6), although this did not reach statistical significance. IL-1β concentration in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. was similarly increased (805 ±108.7; n=6), while that of D-JNKI1-treated rats which received saline i.p. was similar to controls (605 ±66.8; n=5).

The sample immunoblot in Figure 3.7A indicates that JNK phosphorylation was increased in hippocampus of control-treated rats which received LPS i.p. (lane 2), compared with control-treated rats which received saline i.p. (lane 1). Assessment of mean values obtained from densitometric analysis (Figure 3.7B) indicated mean JNK activity, expressed as a ratio of phosphorylated JNK to total JNK1 (±SEM; n=6 for all groups), was significantly increased in hippocampus of control-treated rats which received LPS i.p. (1.16 ±0.082) compared with control-treated rats which received saline i.p. (0.90 ±0.076;

*p<0.05; student t test). JNK activity in hippocampus of rats pretreated with D-JNKI1 which received LPS or saline i.p. was similarly increased (1.15 ±0.159 and 1.24 ±0.187 respectively), such that mean values in both groups were significantly increased compared with saline-treated controls. These data indicate that these events are upstream of the action of D-JNKI1.

3. 1. 5. D-JNKI1 attenuated the LPS-induced increase in c-Jun and bcl-2 phosphorylation.

The sample immunoblot in Figure 3.8A indicates that c-Jun phosphorylation was increased in hippocampus of control-treated rats which received LPS i.p. (lane 2) compared with control-treated rats which received saline i.p. (lane 1). Assessment of mean values obtained from densitometric analysis (Figure 3.8B) indicated that mean c-Jun phosphorylation (arbitrary units ±SEM; n=6 for all groups) was significantly increased in hippocampus of control-treated rats which received LPS i.p. (2127 ±205.6) compared with control-treated rats which received saline i.p. (1492 ±62.83; *p<0.05; Newmann-Keuls). This LPS-induced increase in c-Jun phosphorylation was attenuated by D-JNKI1 pretreatment so that the mean value in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. (1728 ±198.4) was not significantly different to that of saline-treated controls, similarly in D-JNKI1-treated rats which received saline i.p. (1721 ±161.4).

The mitochondrial associated protein bcl-2 is a target substrate for JNK, therefore, rat hippocampal tissue was examined by SDS-PAGE and Western Immunoblotting. The sample immunoblot in Figure 3.9A indicates that bcl-2 phosphorylation was increased in the membrane-enriched fraction prepared from hippocampus of control-treated rats which received LPS i.p. (lane 2) compared with control-treated rats which received saline i.p. (lane 1). Assessment of mean values obtained from densitometric analysis (Figure 3.9B) indicated mean bcl-2 phosphorylation (arbitrary units ±SEM; n=6 for all groups) was significantly increased in the membrane-enriched fraction from hippocampus of control-treated rats which received LPS i.p. (2212 ±175.5) compared with control-treated rats which received saline i.p. (1730 ±122.5; *p<0.05; Newmann-Keuls). This LPS-induced increase in bcl-2 phosphorylation was attenuated by D-JNKI1 pretreatment so that the mean value in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. (1681 ±135.1) was similar to that in tissue prepared from saline-treated controls, and significantly less than the value in samples prepared from control-treated rats which

received LPS i.p. (+$p<0.05$; Newmann-Keuls), similarly in D-JNKI1-treated rats which received saline i.p. (1399 ±127.6; ++$p<0.01$; Newmann-Keuls).

Figure 3.10 shows that a significant positive correlation existed between hippocampal c-Jun phosphorylation and membrane-enriched hippocampal bcl-2 phosphorylation ($p<0.01$). This suggests that c-Jun phosphorylation contributes to bcl-2 phosphorylation, however, the r^2 was 0.35, indicating that other factors most likely have an impact too.

3. 1. 6. Bax expression and ATP concentration in hippocampal tissue was unaffected by LPS or D-JNKI1 administration.

Expression of bax, a mitochondrial protein linked to apoptosis, was investigated; the sample immunoblot in Figure 3.11A indicates that bax expression was similar in the membrane-enriched fraction prepared from hippocampus of all experimental groups. Assessment of mean values obtained from densitometric analysis (Figure 3.11B) indicated no significant difference in mean bax expression (arbitrary units ±SEM; n=6 for all groups) between any of the groups examined; 1495 ±48.1; 1439 ±105.7; 1409 ±101.9; and 1445 ±54.2 respectively.

Since mitochondrial associated proteins impact on mitochondrial function, and ATP production is dependent on normal mitochondrial functioning, hippocampal ATP concentration was assessed by a fluorometric assay. Figure 3.12 demonstrates that mean ATP concentration (nM ±SEM; n=6 for all groups) was similar in all groups; control-treated rats which received saline i.p. (9.97 ±0.526); control-treated rats which received LPS i.p. (10.04 ±0.676); rats pretreated with D-JNKI1 which received saline i.p. (8.52 ±0.450); and D-JNKI1-treated rats which received LPS i.p. (9.32 ±0.711).

3. 1. 7. D-JNKI1 did not affect the LPS-induced increase in cytochrome c in the cytosolic fraction.

Cytochrome c is released into the cytosol when the mitochondrial membrane is compromised, therefore its presence was investigated in a cytosolic fraction prepared from hippocampus of experimental animals. The sample immunoblot in Figure 3.13A indicates that cytochrome c was increased in the cytosolic fraction prepared from hippocampus of control-treated rats which received LPS i.p. (lane 2) and D-JNKI1-treated rats which received LPS i.p. (lane 4) compared with control-treated rats which received saline i.p. (lane 1) and rats pretreated with D-JNKI1 which received saline i.p. (lane 3). Assessment

of mean values obtained from densitometric analysis (Figure 3.13B) indicated mean cytochrome c expression (arbitrary units ±SEM; n=6 for all groups) was significantly increased in the cytosolic fraction from hippocampus of control-treated rats which received LPS i.p. (2057 ±192.4) compared with control-treated rats which received saline i.p. (1559 ±62.8; *$p<0.05$; student t test). This LPS-induced increase in cytosolic cytochrome c was only slightly attenuated by D-JNKI1 pretreatment so the mean value in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. (1930 ±158.1) was not significantly different from control-treated rats which received LPS i.p. or control-treated rats which received saline i.p. D-JNKI1-treated rats which received saline i.p. showed no change in cytosolic cytochrome c (1554 ±104.7) compared with control-treated rats which received saline i.p.

3. 1. 8. D-JNKI1 attenuated the LPS-induced increase in hippocampal cells positively stained for activated caspase 3 in cryostat sections.

Caspase 3 is a marker of cell degeneration and its activation was assessed by immunohistochemical staining of cryostat sections of rat brain. As demonstrated in Figure 3.14 positive staining for activated caspase 3 was markedly increased in the hippocampal region of cryostat sections prepared from control-treated rats which received LPS i.p. when compared with that prepared from control-treated rats which received saline i.p. as indicated by the sample sections (panel B and A respectively). Panel A shows toluidine blue-stained healthy cells, whereas, panel B exhibits darker brown activated caspase 3 positive stained cells (black arrows) as well as an altered morphology; condensed, more angular cells. The LPS-induced increase in activated caspase 3 staining was attenuated by pre-treatment with D-JNKI1 (panel D) and there was no substantial effect on activated caspase 3 staining in rats pretreated with D-JNKI1 which received saline i.p. (panel C). The pictures are representative of staining obtained in sections prepared from 4 animals for each treatment group.

3. 1. 9. D-JNKI1 reduced the LPS-induced increase in TUNEL positive stained cells.

TUNEL stains the ends of nicked or fragmented DNA and is also a marker of cell degeneration and was assessed in cytospun hippocampal cells. Figure 3.15A shows sample micrographs of hippocampal cells, adhered to a slide by a cytospin technique, which have undergone TUNEL staining. A sample prepared from a control-treated rat which received saline i.p. shows toluidine blue-stained healthy cells (left panel), whereas, a sample

prepared from a control-treated rat which received LPS i.p. exhibits dark brown TUNEL positive stained cells (right panel; black arrows). TUNEL positive cells were counted and expressed as a percentage of total cell number (Figure 3.15B) and analysis indicated that mean percentage TUNEL staining (% ±SEM, n=6 for all groups) was significantly increased in hippocampus of control-treated rats which received LPS i.p. (13.7 ±0.99) compared with control-treated rats which received saline i.p. (7.8 ±1.69; **$p<0.01$; Newmann-Keuls). This LPS-induced increase in TUNEL staining was attenuated by D-JNKI1 pretreatment so that the mean value in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. (9.7 ±1.42) was similar to saline-treated controls, and significantly less than that in hippocampal cells prepared from control-treated rats which received LPS i.p. (+$p<0.05$; Newmann-Keuls). A similar pattern was observed in D-JNKI1-treated rats which received saline i.p. (8.3 ±0.81)+$p<0.05$; Newmann-Keuls).

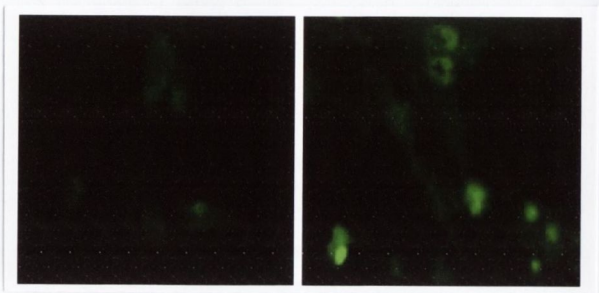
Figure 3.16 shows that a significant inverse correlation exists between the percentage of TUNEL positive stained hippocampal cells and the ability to sustain LTP (expressed as the percentage increase in epsp slope; $p<0.05$; $r^2=0.28$) This suggests that the number of cells with nuclei which exhibit fragmented DNA influences to the ability to sustain LTP, however, the r^2 was 0.28, indicating that the contribution is small and that there are other factors involved.

3.1.10. Fas Ligand expression in hippocampal tissue was unaffected by LPS or D-JNKI1 administration.

Fas Ligand expression has been shown to be upregulated following various inflammatory stimuli, and was therefore assessed by SDS-PAGE and Western Immunoblotting. The sample immunoblot in Figure 3.17A indicates that Fas Ligand expression was similar in hippocampal tissue prepared from all groups. Assessment of mean values obtained from densitometric analysis (Figure 3.17B) indicated an increase in mean Fas Ligand expression (arbitrary units ±SEM; n=6 for all groups) in hippocampal tissue prepared from control-treated rats which received LPS i.p. (1822 ±180.2), compared with control-treated rats which received saline i.p. (1491 ±68.1), but this did not reach statistical significance. Similarly there was a slight increase with rats pretreated with D-JNKI1 which received saline i.p. (1987 ±179.8), and D-JNKI1-treated rats which received LPS i.p. (1887 ±184.6).

Figure 3.1 LPS administration increased activity of JNK *in vitro* and *in vivo*
A) LPS-treated primary hippocampal neurons (100ng/ml; 24h) exhibited increased fluorescent staining for phosphorylated JNK compared with control-treated neurons. B) The sample immunoblot indicates that JNK phosphorylation was increased in hippocampus of LPS-treated rats (100μg/kg; 3h; i.p.; lane 2) compared with control-treated rats (lane 1). C) Assessment of mean values obtained from densitometric analysis indicated that JNK phosphorylation was significantly increased in hippocampus of LPS-treated rats compared with control-treated (*$p<0.05$; unpaired student t test). Data are expressed as means ±SEM of 5 values in each group.

A)



B)

C)

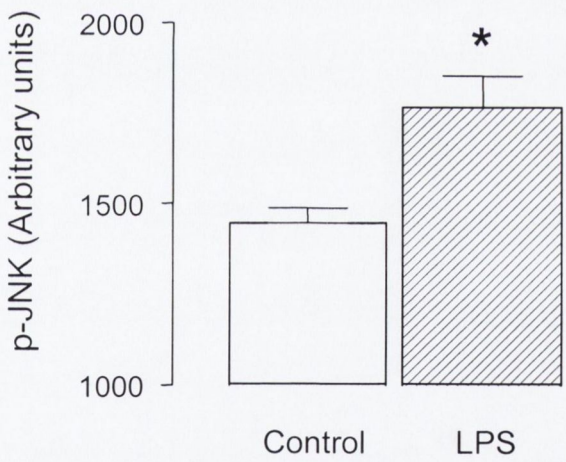
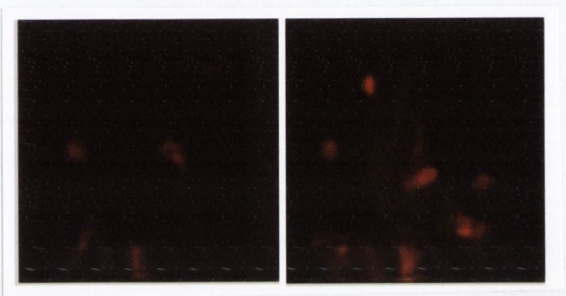


Figure 3.2 LPS administration increased activity of Caspase 3 *in vitro* and *in vivo*
A) LPS-treated primary hippocampal neurons (100ng/ml; 24h) exhibited increased fluorescent staining for active caspase 3 compared with control-treated neurons. B) Caspase 3 activity was significantly increased in hippocampus of LPS-treated rats (100μg/kg; 3h; i.p.) compared with control-treated rats (*$p<0.05$; unpaired student t test) as assessed by a fluorometric assay. Data are expressed as means ±SEM of 5 values in each group.

A)



B)

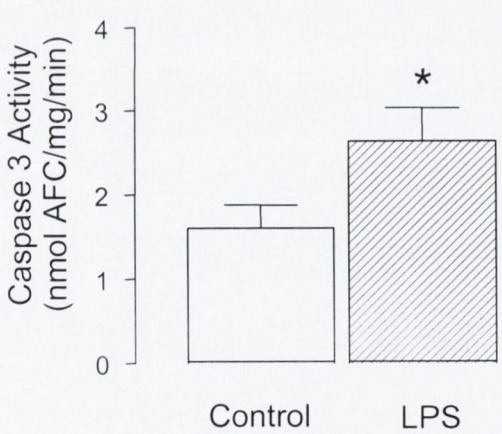


Figure 3.3 D-JNKI1 (200μM or 500μM) did not attenuate the LPS-induced increase in JNK phosphorylation.

The sample immunoblots in Figure 3.3 A and B indicate that JNK phosphorylation was increased in hippocampus of control-treated rats which received LPS i.p. (lane 2), rats pretreated with D-JNKI1 which received saline i.p. (lane 3; 200μM and 500μM; A and B respectively), and D-JNKI1-treated rats which received LPS i.p. (lane 4; 200μM and 500μM; A and B respectively) compared with control-treated rats which received saline i.p. (lane 1). Assessment of mean values obtained from densitometric analysis (Figure 3.3 C) indicated JNK phosphorylation was significantly increased in hippocampus of control-treated rats which received LPS i.p. compared with control-treated rats which received saline i.p. (*$p<0.05$; Newmann-Keuls; n=10). The expression of phosphorylated JNK was similarly increased in hippocampus of rats pretreated with D-JNKI1 (200μM and 500μM; n=6 in each case) which received LPS i.p. or saline i.p., such that all groups were significantly increased compared with saline-treated controls (*$p<0.05$; Newmann-Keuls). Data are expressed as means ±SEM.

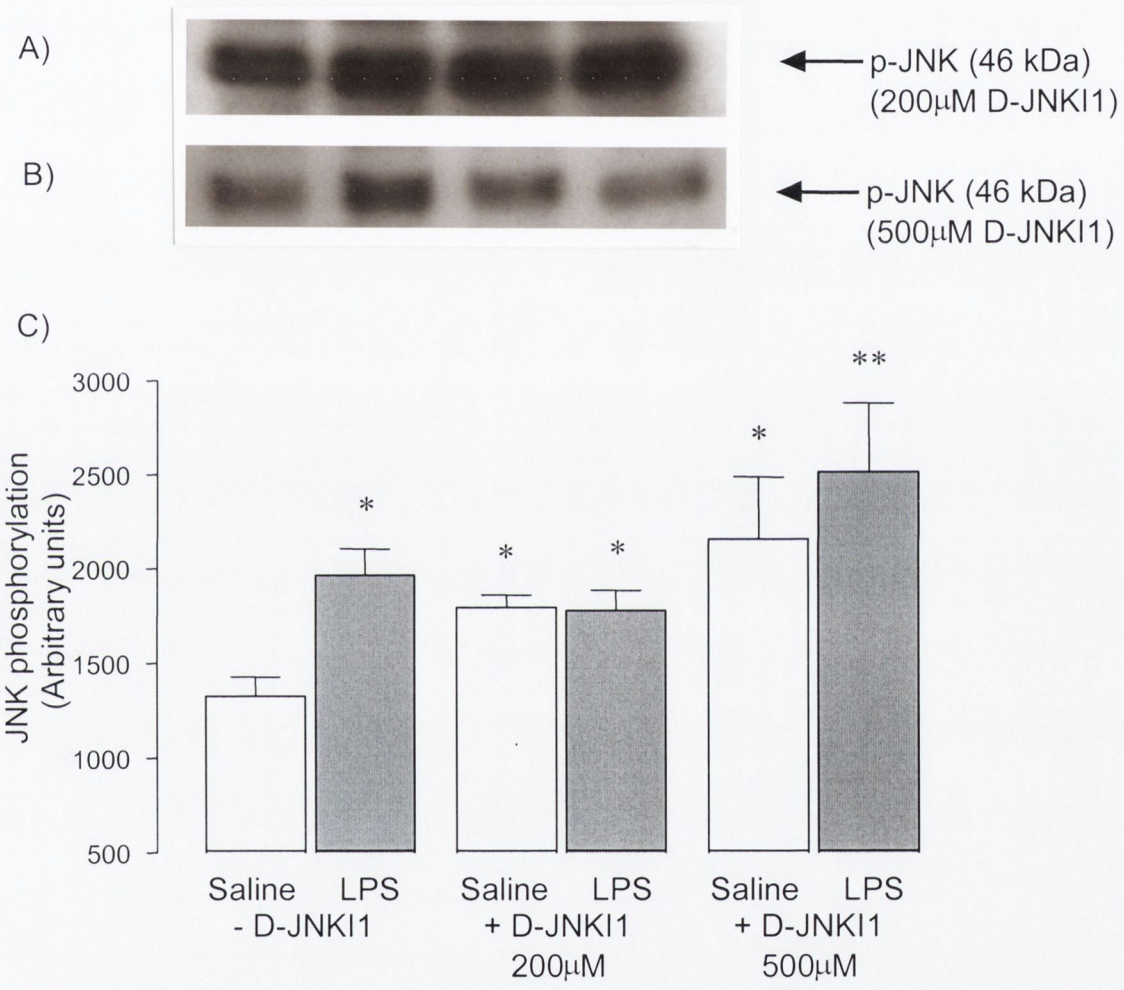

A)
B)
p-JNK (46 kDa)
(200μM D-JNKI1)
p-JNK (46 kDa)
(500μM D-JNKI1)
C)
JNK phosphorylation
(Arbitrary units)
3000
2500
2000
1500
1000
500
*
*
*
*
**
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1
200μM
Saline
LPS
+ D-JNKI1
500μM

Figure 3.4 D-JNKI1 attenuated the LPS-induced increase in c-Jun phosphorylation in a dose dependent manner.

A) The sample immunoblot indicates that c-Jun phosphorylation was increased in hippocampus of control-treated rats which received LPS i.p. (lane 2) compared with control-treated rats which received saline i.p. (lane 1). B) Assessment of mean values obtained from densitometric analysis indicated c-Jun phosphorylation was increased in hippocampus of control-treated rats which received LPS i.p. compared with control-treated rats which received saline i.p. (+$p<0.01$; student t test; n=12). The expression of phosphorylated c-Jun in hippocampus of rats pretreated with 200μM (n=6) or 500μM (n=10) D-JNKI1 which received LPS i.p. was decreased compared with control-treated rats which received LPS i.p., with the 500μM pretreatment showing a significant effect (*$p<0.05$ Newmann-Keuls; n=10). Pretreatment with 200μM (n=6) or 500μM (n=10) D-JNKI1 in rats which received saline i.p. resulted in a modest non-significant increase in c-Jun phosphorylation. Data are expressed as means ±SEM.

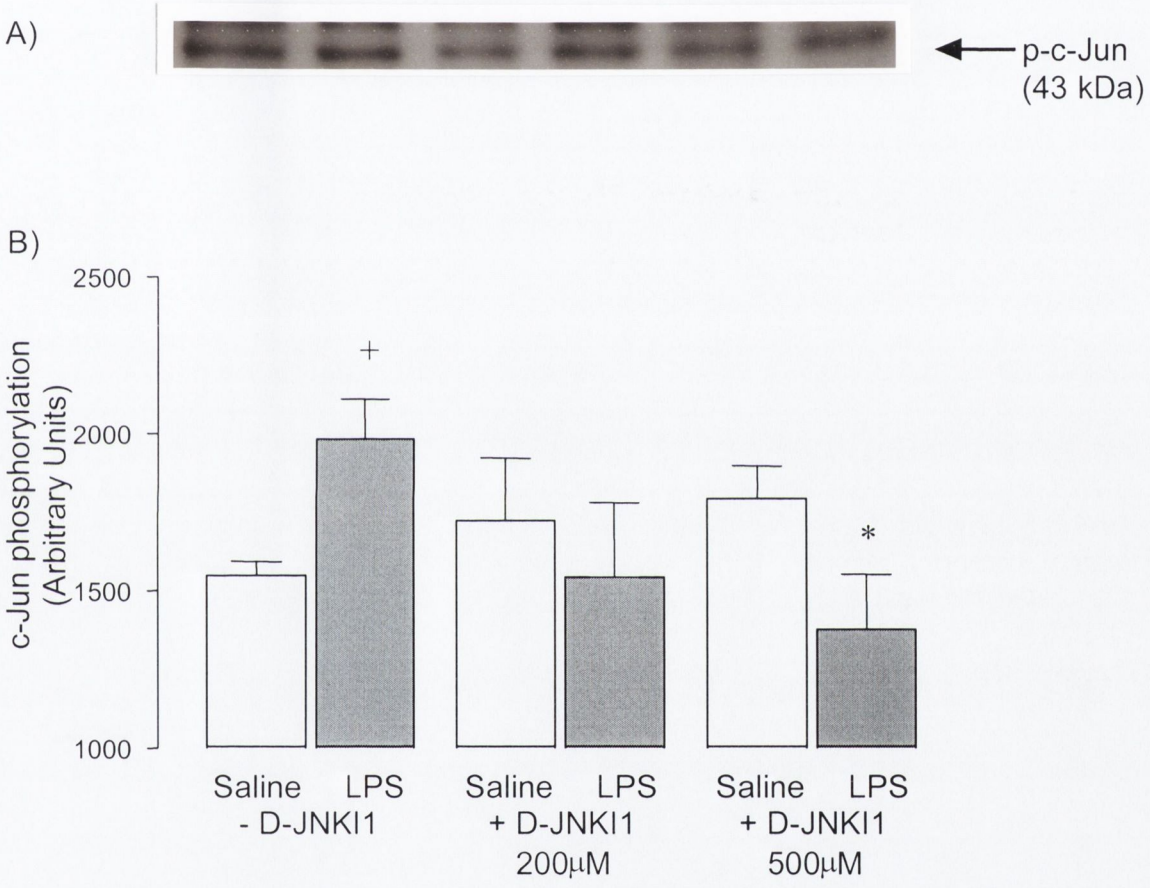

A)
p-c-Jun
(43 kDa)
B)
c-Jun phosphorylation
(Arbitrary Units)
2500
2000
1500
1000
+
*
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1
200μM
Saline
LPS
+ D-JNKI1
500μM

Figure 3.5 D-JNKI1 abrogated the inhibitory effects of LPS on LTP.
A) Tetanus-induced LTP was blocked in perforant path – granule cell synapses of control-treated rats which received LPS i.p. (n=9) compared with control-treated rats which received saline i.p. (n=12). This impairment was suppressed by pretreatment with D-JNKI1; rats pretreated with D-JNKI1 which received LPS i.p. (n=6) sustained LTP, as did D-JNKI1-treated rats which received saline i.p. (n=6). Data are expressed as the mean percentage change in population epsp slope (relative to the mean epsp slope in the 5min immediately prior to tetanic stimulation). Values are means and bars denoting ±SEM are included at 5min intervals.

A)

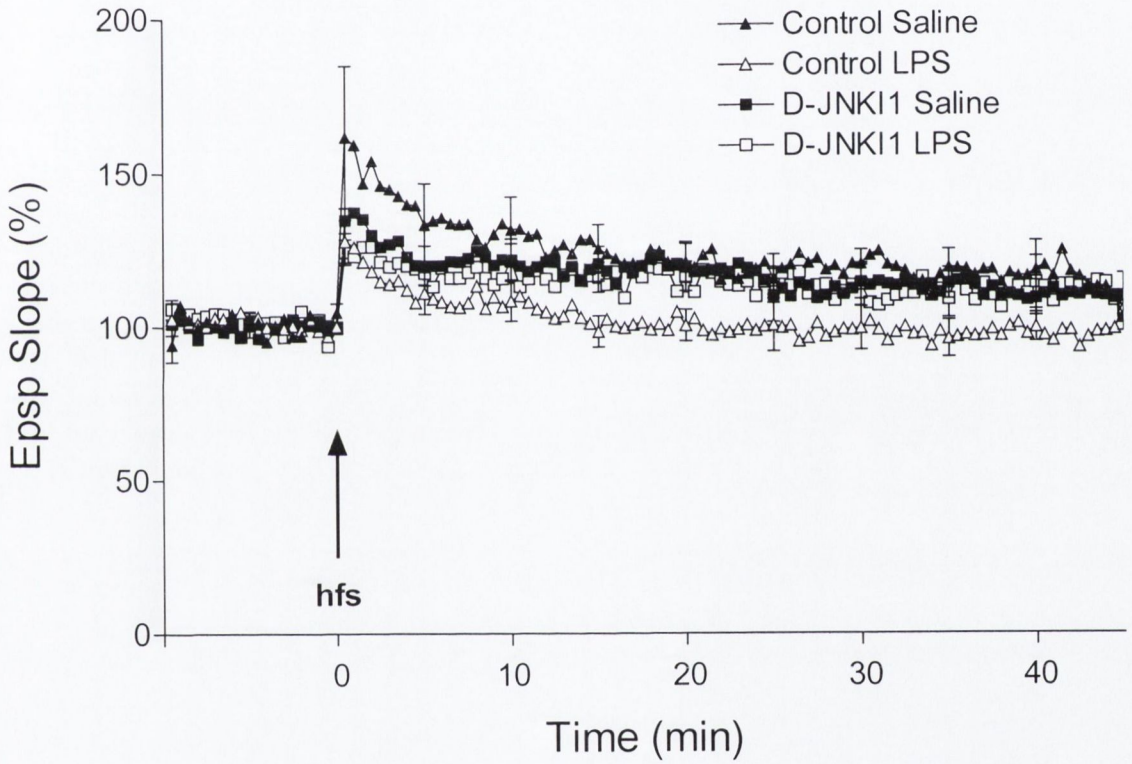

Control Saline
Control LPS
D-JNKI1 Saline
D-JNKI1 LPS
Epsp Slope (%)
200
150
100
50
0
hfs
0
10
20
30
40
Time (min)

Figure 3.5 D-JNKI1 abrogated the inhibitory effects of LPS on LTP.
B) Mean percentage population epsp slope was assessed in the 2min and 40-45min following tetanic stimulation, and analysis revealed that there was a significant inhibitory effect of LPS on LTP at both time points (***$p<0.001$; Newmann-Keuls in each case). Pretreatment with D-JNKI1 abrogated the effect of LPS at the later timepoint, however the LTP sustained by the saline-treated control group was significantly greater than that of either of the D-JNKI1 treated groups (**$p<0.01$; Newmann-Keuls). D-JNKI1 failed to reverse the LPS-induced attenuation of the initial increase in epsp slope, and alone caused a significant decrease compared with saline-treated controls (++$p<0.01$; Newmann-Keuls).

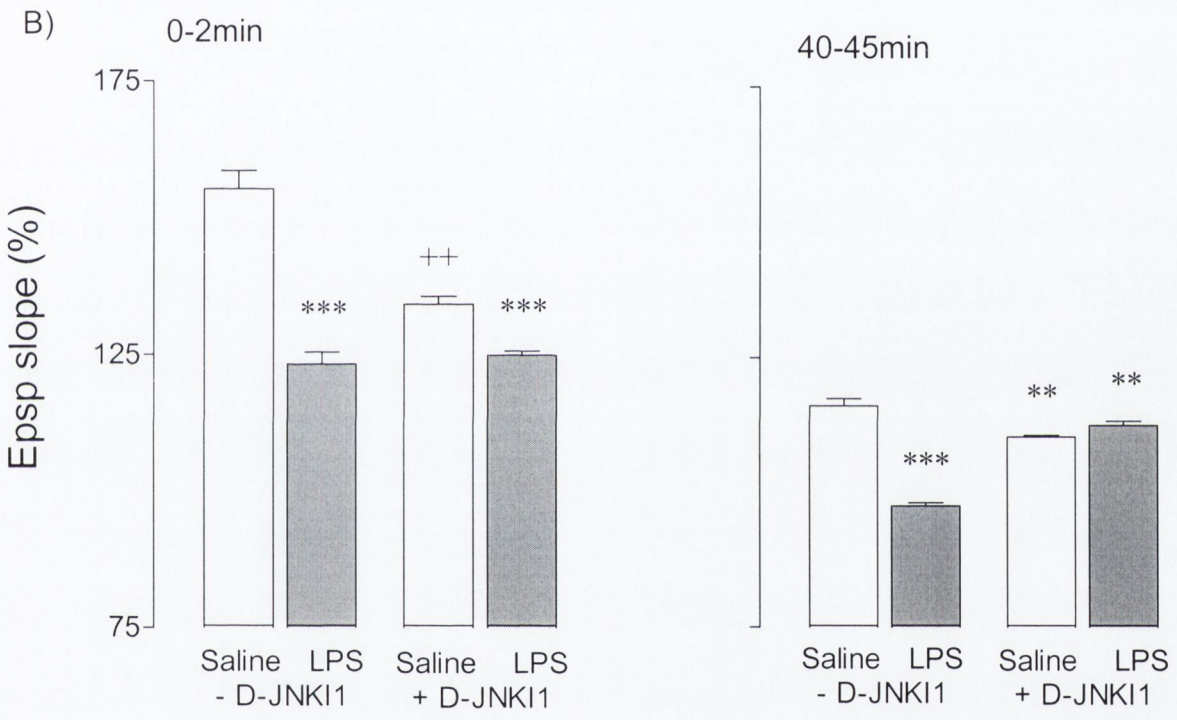

B)
0-2min
40-45min
Epsp slope (%)
175
125
75
***
++
***
**
***
**
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1

Figure 3.6 D-JNKI1 did not attenuate the LPS-induced increase in IL-1β concentration.

Mean IL-1β concentration was nonsignificantly increased in hippocampus of control-treated rats which received LPS i.p. (n=6) compared with control-treated rats which received saline i.p. (n=6). Mean IL-1β concentration in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. (n=6) was similarly increased, while that of D-JNKI1-treated rats which received saline i.p. (n=5) was similar to controls. Data are expressed as means ±SEM; all samples were analysed in triplicate.

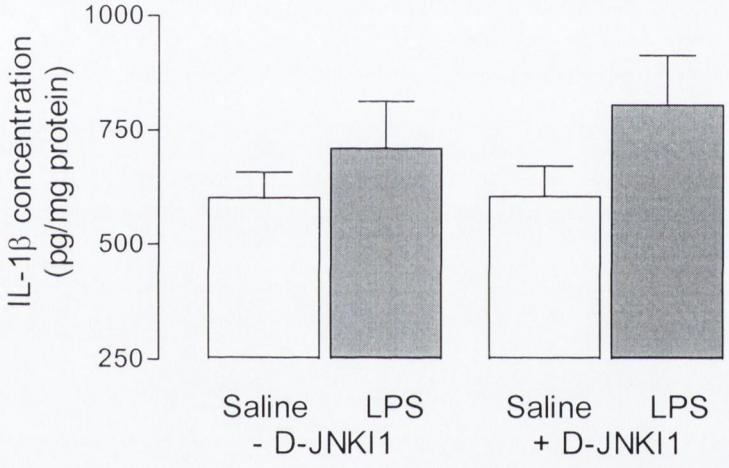

IL-1β concentration
(pg/mg protein)
1000
750
500
250
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1

Figure 3.7 D-JNKI1 did not attenuate the LPS-induced increase in JNK phosphorylation.

A) The sample immunoblot indicates that JNK phosphorylation was increased in hippocampus of control-treated rats which received LPS i.p. (lane 2), and rats pretreated with D-JNKI1 which received saline i.p. (lane 3) or LPS i.p. (lane 4) compared with control-treated rats which received saline i.p. (lane 1). B) Assessment of mean values obtained from densitometric analysis indicated JNK activity expressed as a ratio of phosphorylated JNK to total JNK1 was significantly increased in hippocampus of control-treated rats which received LPS i.p. compared with control-treated rats which received saline i.p. (*$p<0.05$; student t test). JNK activity in hippocampus of rats pretreated with D-JNKI1 which received LPS or saline i.p. was similarly increased, such that both groups were increased compared with saline-treated controls. Data are expressed as means ±SEM of 6 values in all groups.

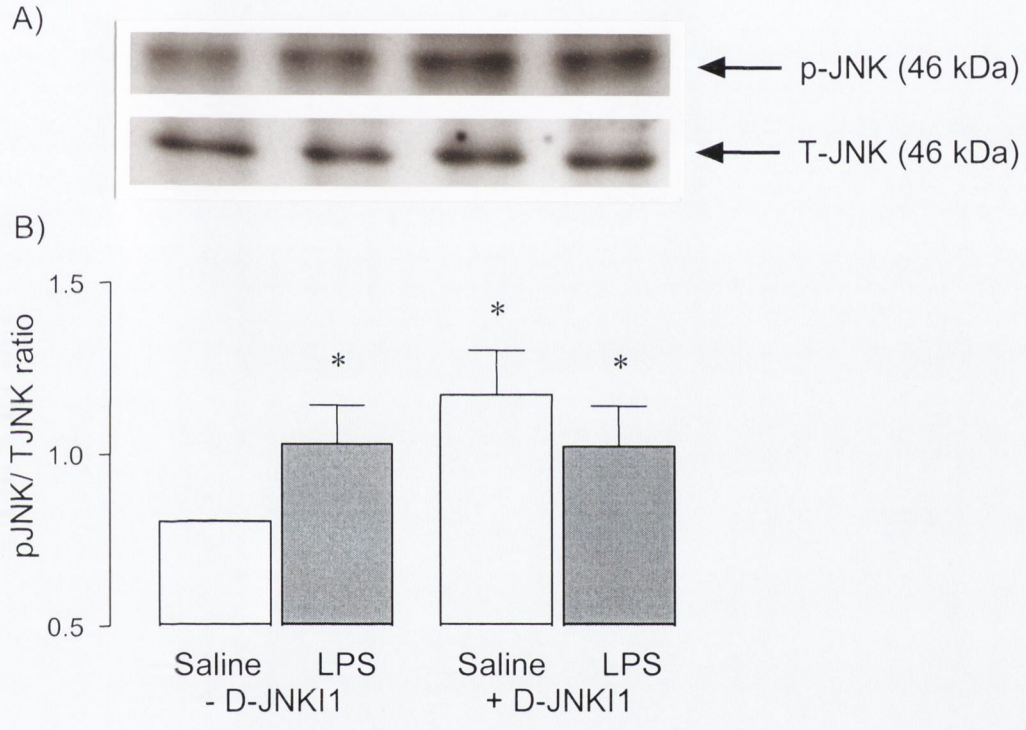

A)
p-JNK (46 kDa)
T-JNK (46 kDa)
B)
pJNK/ TJNK ratio
1.5
1.0
0.5
*
*
*
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1

Figure 3.8 D-JNKI1 attenuated the LPS-induced increase in c-Jun phosphorylation. A) The sample immunoblot indicates that c-Jun phosphorylation was increased in hippocampus of control-treated rats which received LPS i.p. (lane 2) compared with control-treated rats which received saline i.p. (lane 1). B) Assessment of mean values obtained from densitometric analysis indicated that c-Jun phosphorylation was significantly increased in hippocampus of control-treated rats which received LPS i.p. compared with control-treated rats which received saline i.p. (*$p<0.05$; Newmann-Keuls). This LPS-induced increase in c-Jun phosphorylation was attenuated by D-JNKI1 pretreatment so that the mean value in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. was not significantly different to saline-treated controls, similarly in D-JNKI1-treated rats which received saline i.p. Data are expressed as means ±SEM of 6 values in all groups.

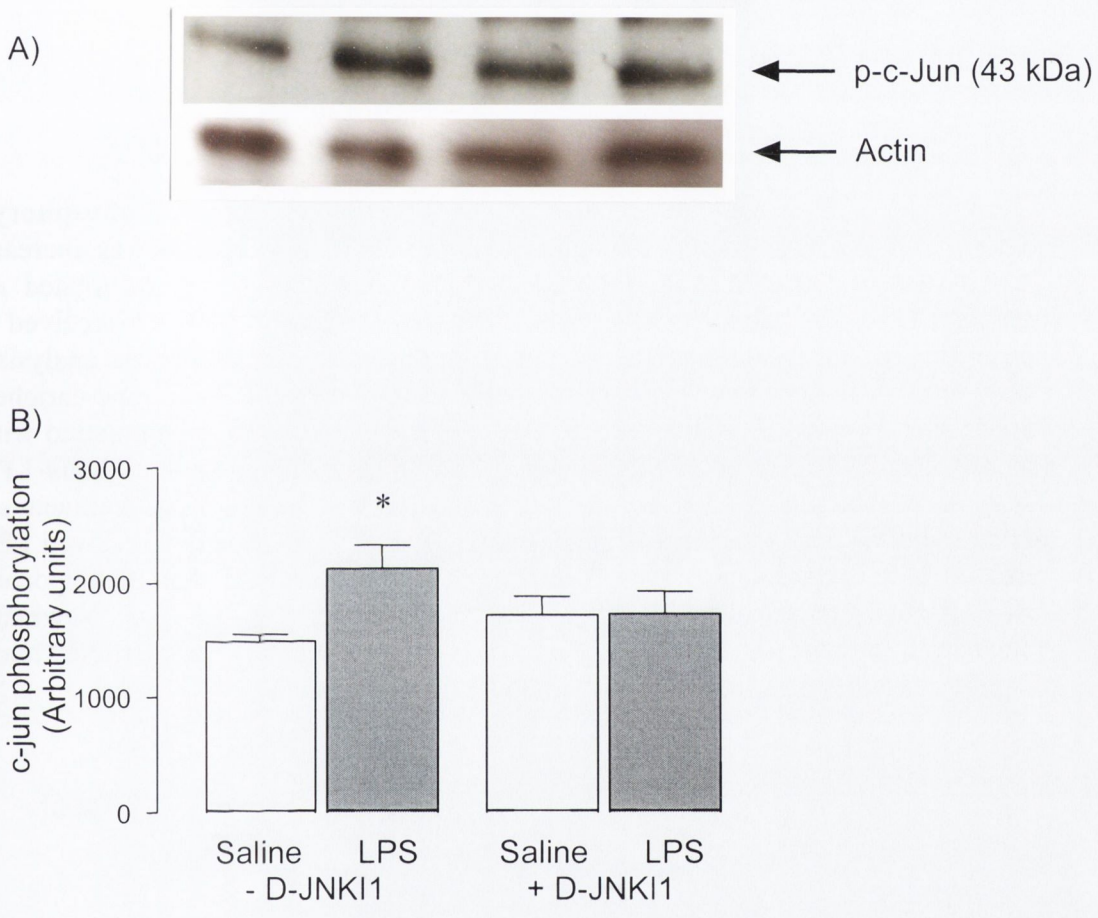

A)
p-c-Jun (43 kDa)
Actin
B)
c-jun phosphorylation
(Arbitrary units)
3000
2000
1000
0
*
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1

Figure 3.9 D-JNKI1 attenuated the LPS-induced increase in bcl-2 phosphorylation. A) The sample immunoblot indicates that bcl-2 phosphorylation was increased in the membrane-enriched fraction prepared from hippocampus of control-treated rats which received LPS i.p. (lane 2) compared with control-treated rats which received saline i.p. (lane 1). B) Assessment of mean values obtained from densitometric analysis indicated that bcl-2 phosphorylation was significantly increased in the membrane-enriched fraction from hippocampus of control-treated rats which received LPS i.p. compared with control-treated rats which received saline i.p. (*$p<0.05$; Newmann-Keuls). This LPS-induced increase in bcl-2 phosphorylation was attenuated by D-JNKI1 pretreatment so that the mean value in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. was similar to saline-treated controls, and significantly less than that in hippocampal cells prepared from control-treated rats which received LPS i.p. (+$p<0.05$; Newmann-Keuls), similarly in D-JNKI1-treated rats which received saline i.p. (++$p<0.01$; Newmann-Keuls). Data are expressed as means ±SEM of 6 values in all groups.

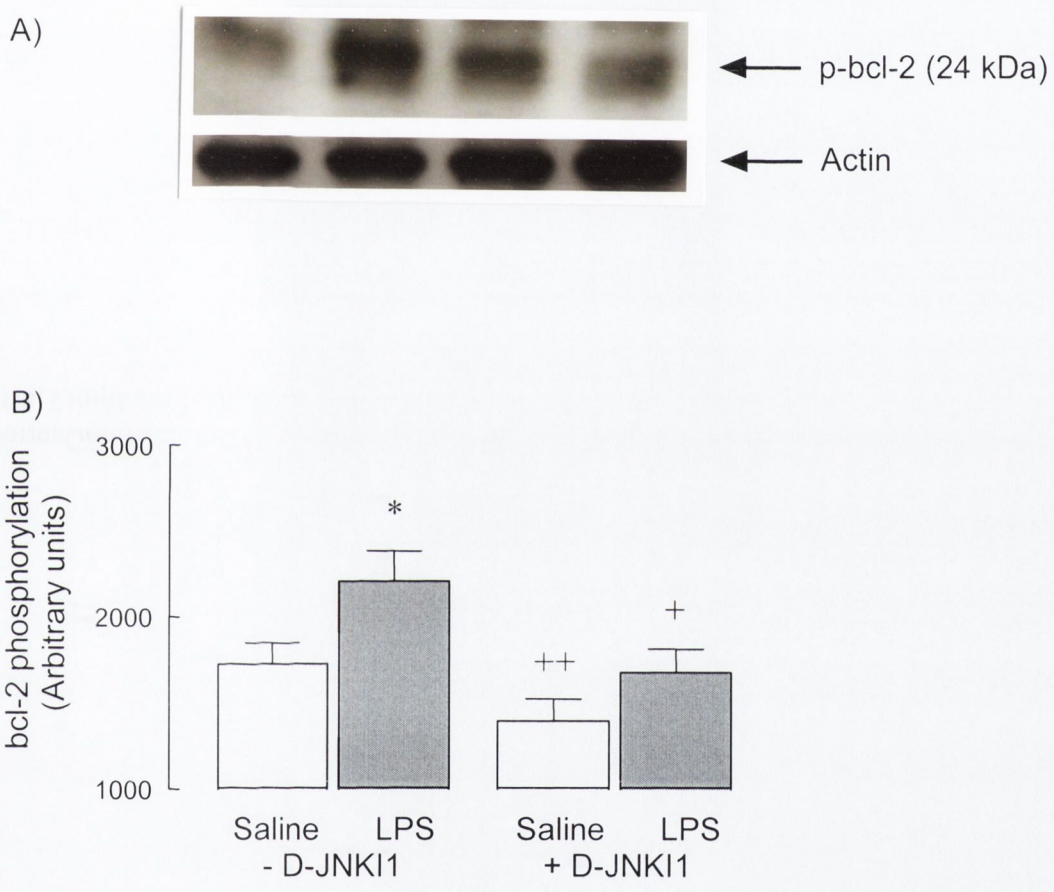

A)
p-bcl-2 (24 kDa)
Actin
B)
bcl-2 phosphorylation
(Arbitrary units)
3000
2000
1000
*
++
+
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1

Figure 3.10 Bcl-2 phosphorylation is positively correlated to c-Jun phosphorylation. A significant positive correlation exists between hippocampal c-Jun phosphorylation and membrane-enriched hippocampal bcl-2 phosphorylation ($p<0.01$; $r^2=0.35$)

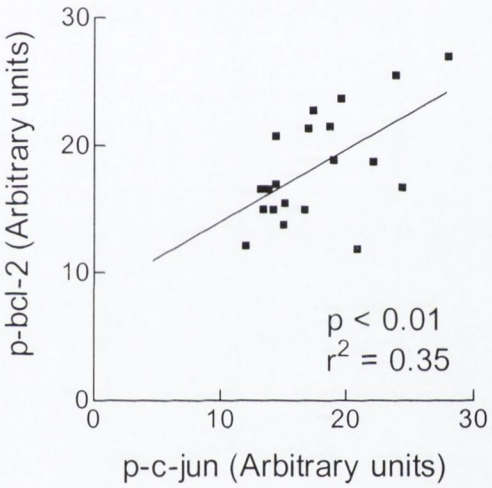

p-bcl-2 (Arbitrary units)
30
20
10
0
0
10
20
30
p-c-jun (Arbitrary units)
p < 0.01
r2 = 0.35

Figure 3.11 Bax expression in hippocampal tissue was unaffected by LPS or D-JNKI1 administration.

A) The sample immunoblot indicates that bax expression was similar in the membrane-enriched fraction prepared from hippocampus of rats of each treatment group. B) Assessment of mean values obtained from densitometric analysis indicated no significant difference in mean bax expression between any of the groups examined. Data are expressed as means ±SEM of 6 values in all groups.

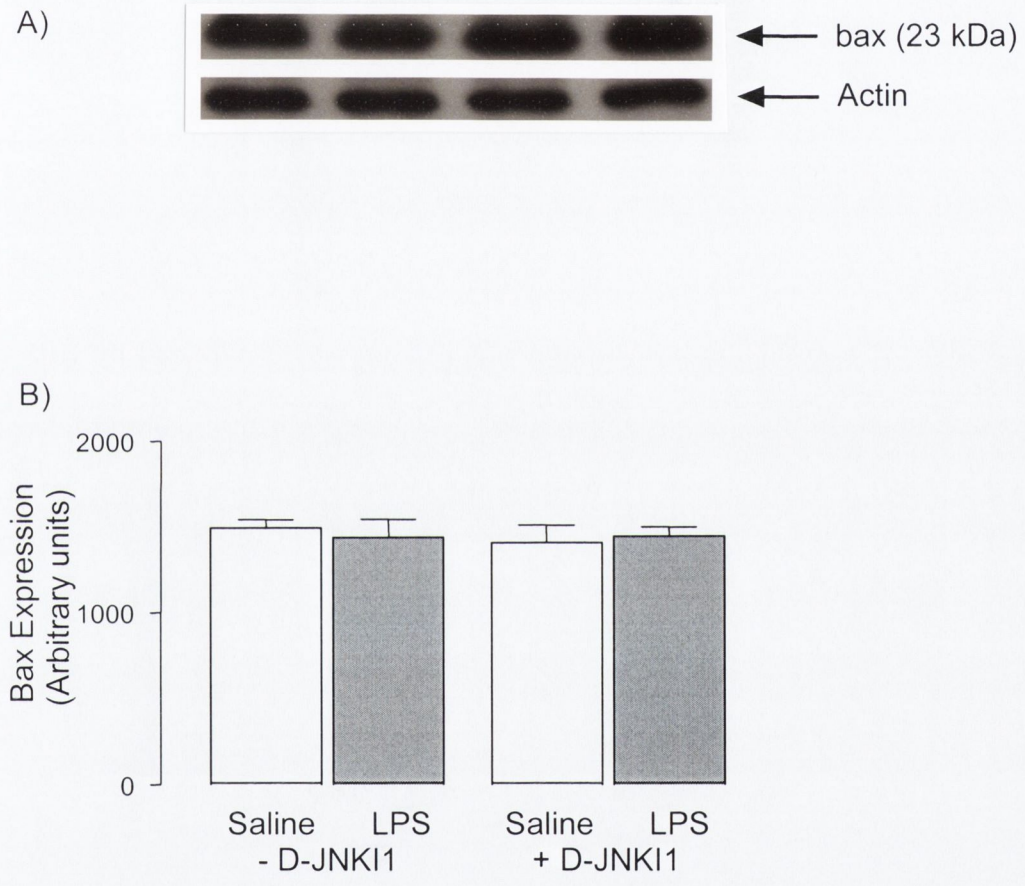

A)
bax (23 kDa)
Actin
B)
Bax Expression
(Arbitrary units)
2000
1000
0
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1

Figure 3.12 ATP concentration in hippocampal tissue was unaffected by LPS or D-JNKI1 administration.

ATP concentration was similar in hippocampal tissue prepared from control-treated rats which received saline i.p. or LPS i.p., and rats pretreated with D-JNKI1 which received saline i.p. or LPS i.p. Data are expressed as means ±SEM of 6 values in all groups.

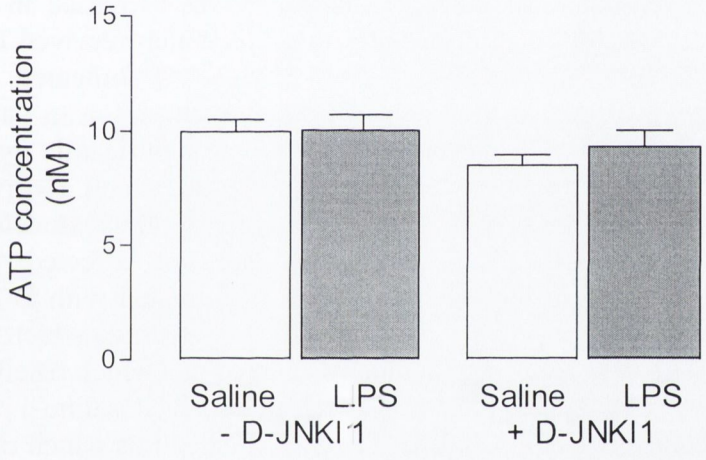

ATP concentration (nM)
15
10
5
0
Saline
LPS
- D-JNKI 1
Saline
LPS
+ D-JNKI1

Figure 3.13 D-JNKI1 did not affect the LPS-induced increase in cytochrome c in the cytosolic fraction.

A) The sample immunoblot indicates that cytochrome c was increased in the cytosolic fraction prepared from hippocampus of control-treated rats which received LPS i.p. (lane 2) and D-JNKI1-treated rats which received LPS i.p. (lane 4) compared with control-treated rats which received saline i.p. (lane 1). B) Assessment of mean values obtained from densitometric analysis indicated that cytochrome c was significantly increased in the cytosolic fraction from hippocampus of control-treated rats which received LPS i.p. compared with control-treated rats which received saline i.p. (*$p<0.05$; student t test). This LPS-induced increase in cytosolic cytochrome c was not affected by D-JNKI1 pretreatment so the mean value in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. was not significantly different to control-treated rats which received LPS i.p. but significantly increased compared with control-treated rats which received saline i.p. (*$p<0.05$; student t test). D-JNKI1-treated rats which received saline i.p. showed no change in cytosolic cytochrome c compared with control-treated rats which received saline i.p. Data are expressed as means ±SEM of 6 values in all groups.

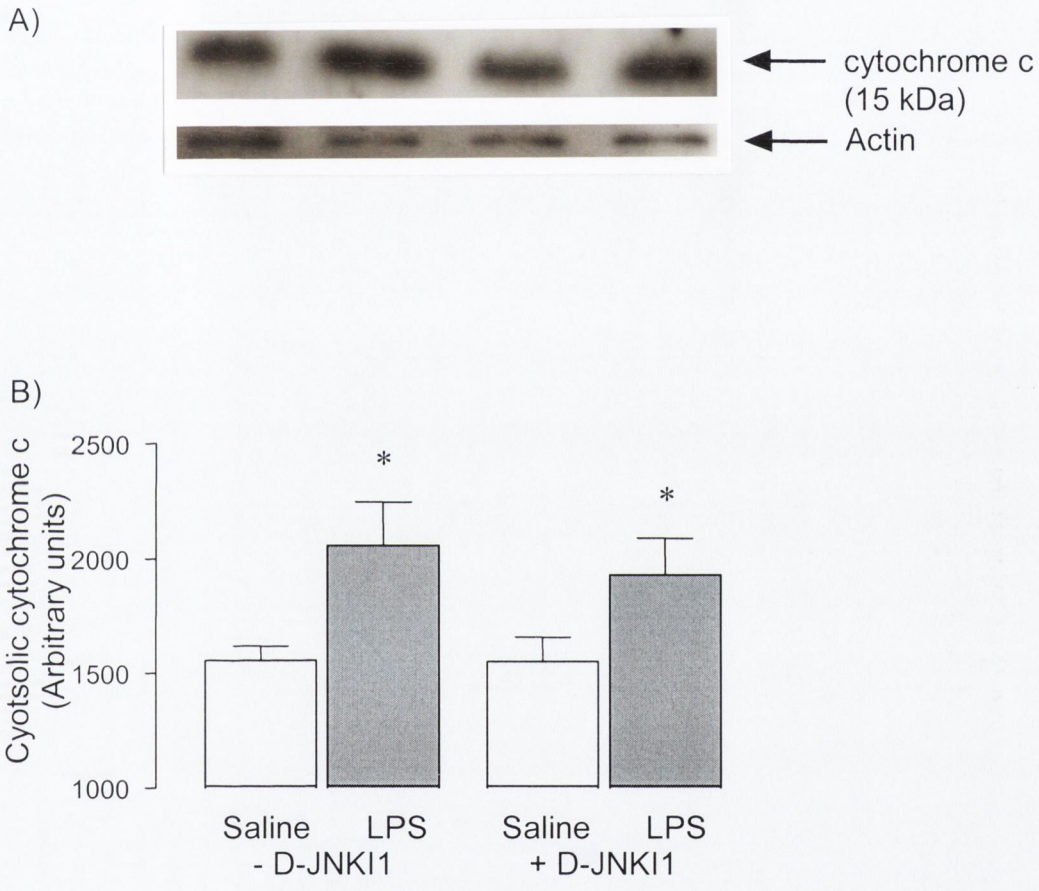

A)
cytochrome c
(15 kDa)
Actin
B)
Cyotsolic cytochrome c
(Arbitrary units)
2500
2000
1500
1000
*
*
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1

Figure 3.14 D-JNKI1 attenuated the LPS-induced increase in hippocampal cells positively stained for activated caspase 3 in cryostat sections.
Positive staining for activated caspase 3 was markedly increased in the hippocampal region of cryostat sections prepared from control-treated rats which received LPS i.p. when compared with that prepared from control-treated rats which received saline i.p. as indicated by the sample sections (panel B and A respectively). Panel A shows toluidine blue-stained healthy cells, whereas, panel B exhibits darker brown activated caspase 3 positive stained cells (black arrows) as well as an altered morphology. The LPS-induced increase in activated caspase 3 staining was attenuated by pre-treatment with D-JNKI1 (panel D) and there was no substantial effect on activated caspase 3 staining in rats pretreated with D-JNKI1 which received saline i.p. (panel C). Magnification X40 and the bar in panel B represents 20μm. Pictures are representative of staining obtained in sections prepared from 4 animals for each treatment group.

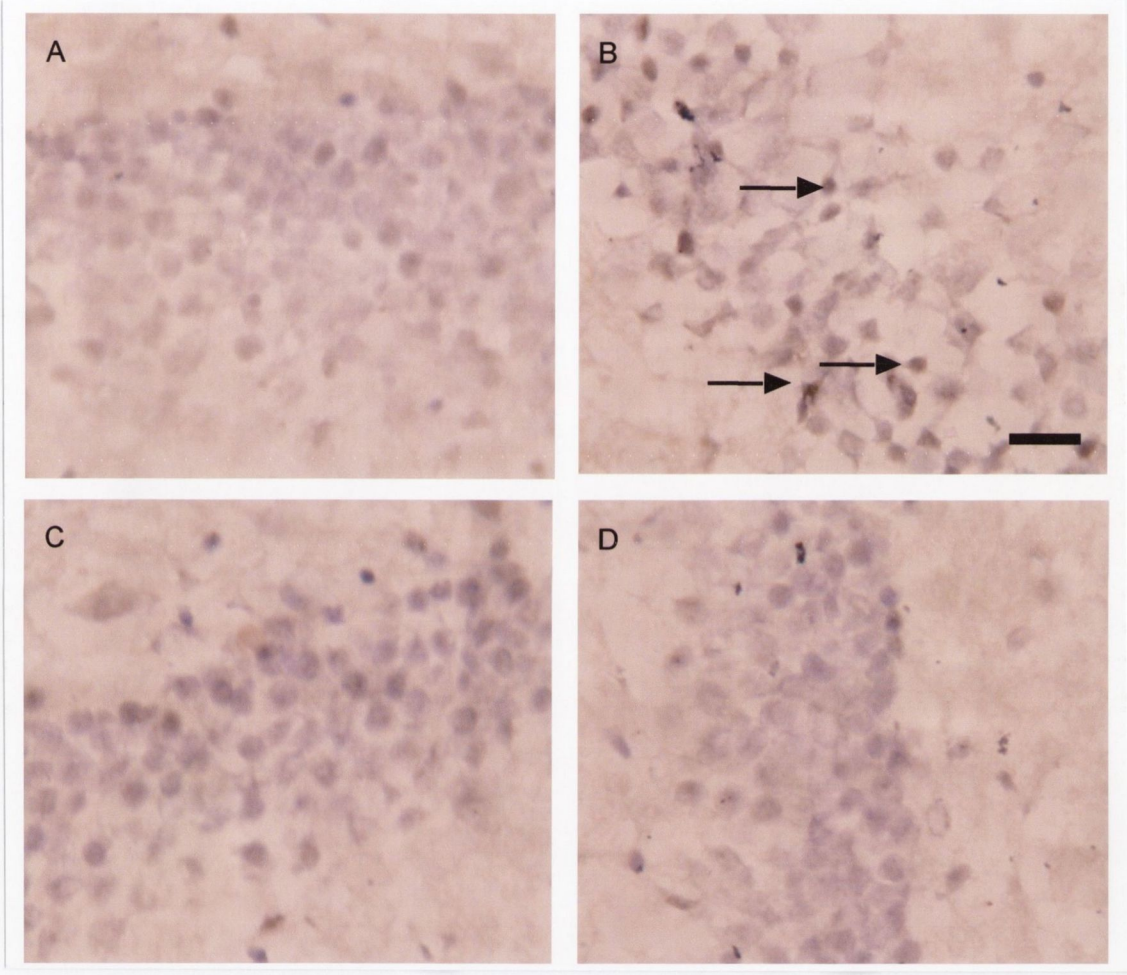
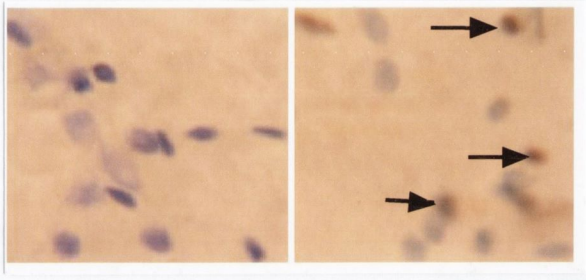

A
B
C
D

Figure 3.15 D-JNKI1 reduced the LPS-induced increase in TUNEL positive stained cells.

A) Two sample micrographs of cytospun hippocampal cells which have undergone TUNEL staining are shown. A sample prepared from a control-treated rat which received saline i.p. shows toluidine blue-stained healthy cells (left panel), whereas, a sample prepared from a control-treated rat which received LPS i.p. exhibits dark brown TUNEL positive stained cells (right panel; black arrows). B) TUNEL positive cells were counted and expressed as a percentage of total cell number and analysis indicated that TUNEL staining was significantly increased in hippocampus of control-treated rats which received LPS i.p. compared with control-treated rats which received saline i.p. (**$p<0.01$; Newmann-Keuls). This LPS-induced increase in TUNEL staining was attenuated by D-JNKI1 pretreatment so that the mean value in hippocampus of rats pretreated with D-JNKI1 which received LPS i.p. or saline i.p. was similar to saline-treated controls, and significantly less than that in hippocampal cells prepared from control-treated rats which received LPS i.p. (+$p<0.05$; Newmann-Keuls in each case). Data are expressed as means ±SEM of 6 values in all groups.

A)



Saline LPS

B)

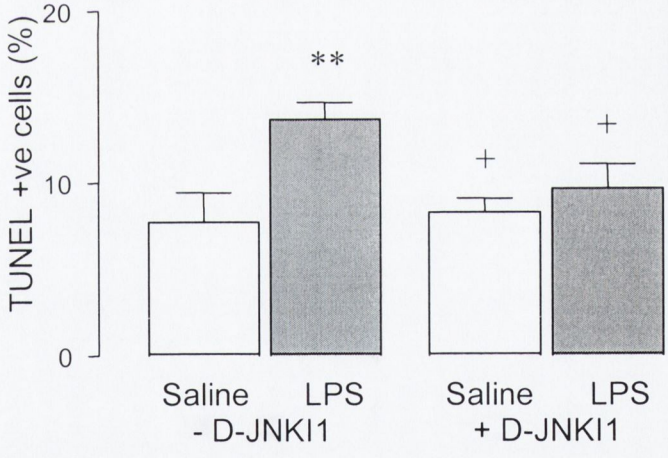


Figure 3.16 The percentage of TUNEL-positive stained cells are inversely correlated to the ability to sustain LTP.

A significant negative correlation exists between the percentage of TUNEL positive stained hippocampal cells and the ability to sustain LTP expressed as the percentage increase in epsp slope ($p<0.05$; $r^2=0.28$)

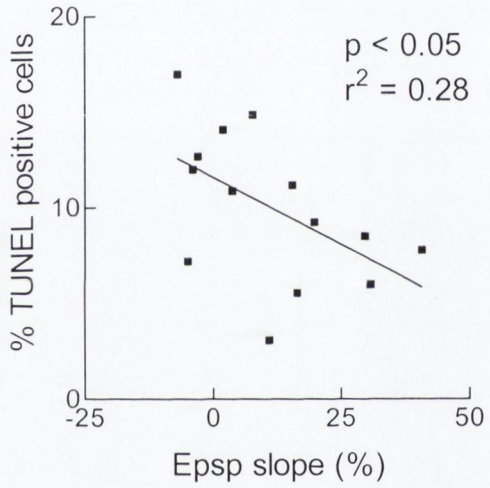
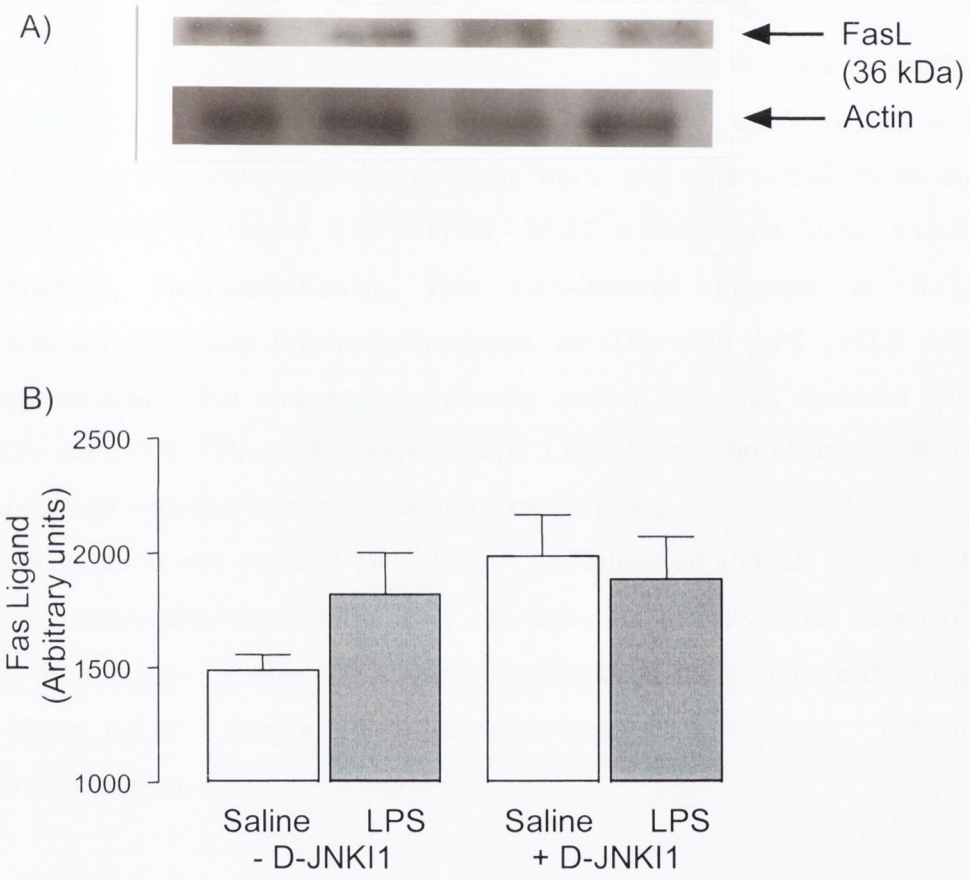

20
10
0
-25
0
25
50
% TUNEL positive cells
Epsp slope (%)
p < 0.05
r2 = 0.28

Figure 3.17 Fas Ligand expression in hippocampal tissue was unaffected by LPS or D-JNKI1 administration.

A) The sample immunoblot indicates that Fas Ligand expression was similar in hippocampal tissue prepared from all treatment groups. B) Assessment of mean values obtained from densitometric analysis indicated an increase in mean Fas Ligand expression in hippocampal tissue prepared from control-treated rats which received LPS i.p. compared with control-treated rats which received saline i.p., but this did not reach statistical significance. Similarly there was a slight increase with rats pretreated with D-JNKI1 which received saline i.p. or LPS i.p. Data are expressed as means ±SEM of 6 values in all groups.


A)
FasL
(36 kDa)
Actin
B)
Fas Ligand
(Arbitrary units)
2500
2000
1500
1000
Saline
LPS
- D-JNKI1
Saline
LPS
+ D-JNKI1

3. 2. Analysis of the temporal aspects of LPS-induced effects on cultured cortical neurons.

Primary cultured cortical neurons were treated with either media alone or media supplemented with LPS (100ng/ml). LPS treatment was for 3h, 6h, 18h or 24 h.

3. 2. 1. IL-1β concentration in supernatant collected from cultured cortical neurons was increased following 18h and 24h incubation with LPS, while IFNγ was not detectable.

IL-1β concentration was assessed by an ELISA performed on supernatant collected from the treated cultured cells, and each sample was analysed in triplicate. Figure 3.18 demonstrates that mean IL-1β concentration in supernatant prepared from neurons (pg/ml ±SEM; n=5 for all groups) was significantly increased following treatment for 18h with LPS compared with that from cells treated with media alone, and cells treated for 3h and 6h with LPS (116.3 ±21.18 versus 22.7 ±11.44; 20.47 ±14.88; and 20.61 ±12.61 respectively; *$p<0.05$; Newmann-Keuls). This LPS-induced increase in IL-1β concentration was still observed following treatment for 24h with LPS (142.2 ±38; **$p<0.01$; Newmann-Keuls) but was not significantly greater than that observed with treatment for 18h with LPS. 3h or 6h treatment with LPS affected no change in IL-1β concentration compared with that from cells receiving media alone.

IFN-γ concentration was assessed by an ELISA performed on aliquots of the same. Figure 3.19 shows mean absorbance; thus IFNγ concentration did not reach detectable levels in any of the treatment groups. Data points expressed on the graph are the mean absorbance at 450nm for each sample; all samples were analysed in triplicate. The line represents the limit of detection.

3. 2. 2. JNK phosphorylation in whole cell lysate prepared from cultured cortical neurons was increased after 3h, 6h, and 18h incubations with LPS, while bcl-2 phosphorylation was increased only at 18h.

The sample immunoblot in Figure 3.20A indicates that JNK phosphorylation was increased in whole cell lysate prepared from cultured cortical neurons incubated for 3h, 6h, 18h and 24h with LPS (lane 2, 3, 4, and 5 respectively) compared with neurons receiving media alone (lane 1). Assessment of mean values obtained from densitometric analysis (Figure 3.20B) indicated mean JNK phosphorylation (arbitrary units ±SEM; n=4 for all groups) was increased in whole cell lysate from cultured cortical neurons incubated for 3h,

6h, 18h and 24h with LPS when compared with neurons treated with media alone; 2379 ±229.3, 2211 ±239.8, 2871 ±753, and 2222 ±461.1 versus 1421 ±84.2 respectively; *$p<0.05$; student t test. The increase was maximal at the 18h timepoint.

The sample immunoblot in Figure 3.21A indicates that bcl-2 phosphorylation was increased in the mitochondrial fraction prepared cultured cortical neurons incubated for 18h with LPS (lane 4) compared with neurons receiving media alone (lane 1) and those incubated for 3h, 6h, and 24 h with LPS (lane 2, 3, and 5 respectively). Assessment of mean values obtained from densitometric analysis (Figure 3.23B) indicated mean bcl-2 phosphorylation (arbitrary units ±SEM; n=5 for all groups) was increased in the mitochondrial fraction prepared from cultured cortical neurons incubated for 18h with LPS (4032 ±1291) compared with media alone, 3h, 6h and 24 h treatment groups (1528 ±21.6, 1315 ±332.7, 1705 ±335.7, and 1493 ±533.1 respectively). However this increase was not significant ($p=0.0676$; ANOVA).

3. 2. 3. Cytosolic cytochrome c of cultured cortical neurons was modestly increased in a time dependent manner following incubation with LPS.

The sample immunoblot in Figure 3.22A and subsequent assessment of mean values obtained from densitometric analysis (Figure 3.22B) indicated mean cytochrome c expression (arbitrary units ±SEM) in the cytosolic fraction prepared from cultured cortical neurons which received media alone (968 ±31.9; n=3), cells treated for 3h (1119 ±53.9; n=3), 6h (1102 ±88.1; n=4), 18h (1270 ±82.6; n=3), and 24h (1260 ±128.3; n=4) with LPS increased modestly at each successive timepoint up to 18h. A significant increase was observed only at the 18h timepoint when compared with treatment of media alone (*$p<0.05$; student t test).

3. 2. 4. Caspase 3 activity of whole cell lysate prepared from cortical neurons was not effected following any of the incubation periods with LPS.

Figure 3.23 demonstrates that caspase 3 activity (nmol AFC/mg/min ±SEM; n=6 for all groups) was similar in whole cell lysate prepared from cells in all groups; cells receiving media alone (15.0 ±2.18), cells treated for 3h (10.7 ±2.16), 6h (12.1 ±1.80), 18h (12.5 ±1.03), and 24h (13.8 ±2.25) with LPS.

Figure 3.18 IL-1β concentration in supernatant collected from cultured cortical neurons was increased following 18h and 24h incubation with LPS.
Mean IL-1β concentration in supernatant prepared from neurons was significantly increased following treatment for 18h with LPS compared with that from cells receiving media alone, cells treated for 3h and 6h with LPS (*$p<0.05$; Newmann-Keuls). This LPS-induced increase in mean IL-1β concentration was still observed following treatment for 24h with LPS (**$p<0.01$; Newmann-Keuls) but was not significantly greater than that observed with the 18h treatment. Treatment for 3h or 6h with LPS affected no change in IL-1β concentration compared with that from cells receiving media alone. Data are expressed as means ±SEM of 5 values in all groups; all samples were analysed in triplicate.

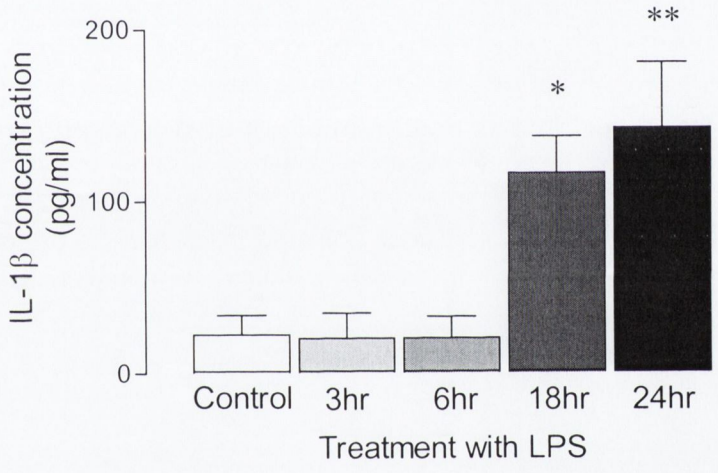

IL-1β concentration
(pg/ml)
200
100
0
*
**
Control
3hr
6hr
18hr
24hr
Treatment with LPS

Figure 3.19 IFN-γ was not detectable in supernatant collected from cultured cortical neurons following any of the incubation periods with LPS.
IFN-γ concentration did not reach detectable levels in supernatant prepared from cells receiving media alone, or following LPS treatment. Data points expressed on the graph are the mean absorbance at 450nm for each sample; all samples were analysed in triplicate. The line represents the limit of detection.

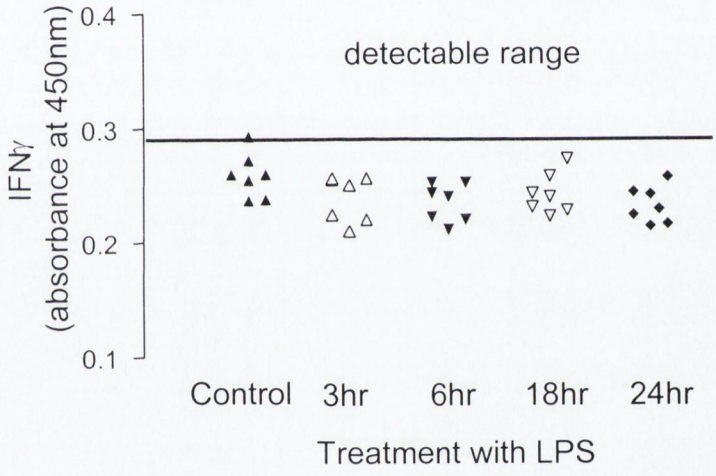

IFNγ
(absorbance at 450nm)
0.4
0.3
0.2
0.1
detectable range
Control
3hr
6hr
18hr
24hr
Treatment with LPS

Figure 3.20 JNK phosphorylation in whole cell lysate prepared from cultured cortical neurons was increased after 3h, 6h, 18h and 24h incubations with LPS

A) The sample immunoblot indicates that JNK phosphorylation was increased in whole cell lysate prepared from cultured cortical neurons incubated for 3h, 6h, 18h and 24h with LPS (lane 2, 3, 4, and 5 respectively) compared with neurons receiving media alone (lane 1). B) Assessment of mean values obtained from densitometric analysis indicated mean JNK phosphorylation was increased in whole cell lysate from cultured cortical neurons incubated for 3h, 6h, 18h and 24h with LPS when compared with neurons receiving media alone (*$p<0.05$; student t test). The increase was maximal at the 18h timepoint. Data are expressed as means ±SEM of 4 values in all groups.

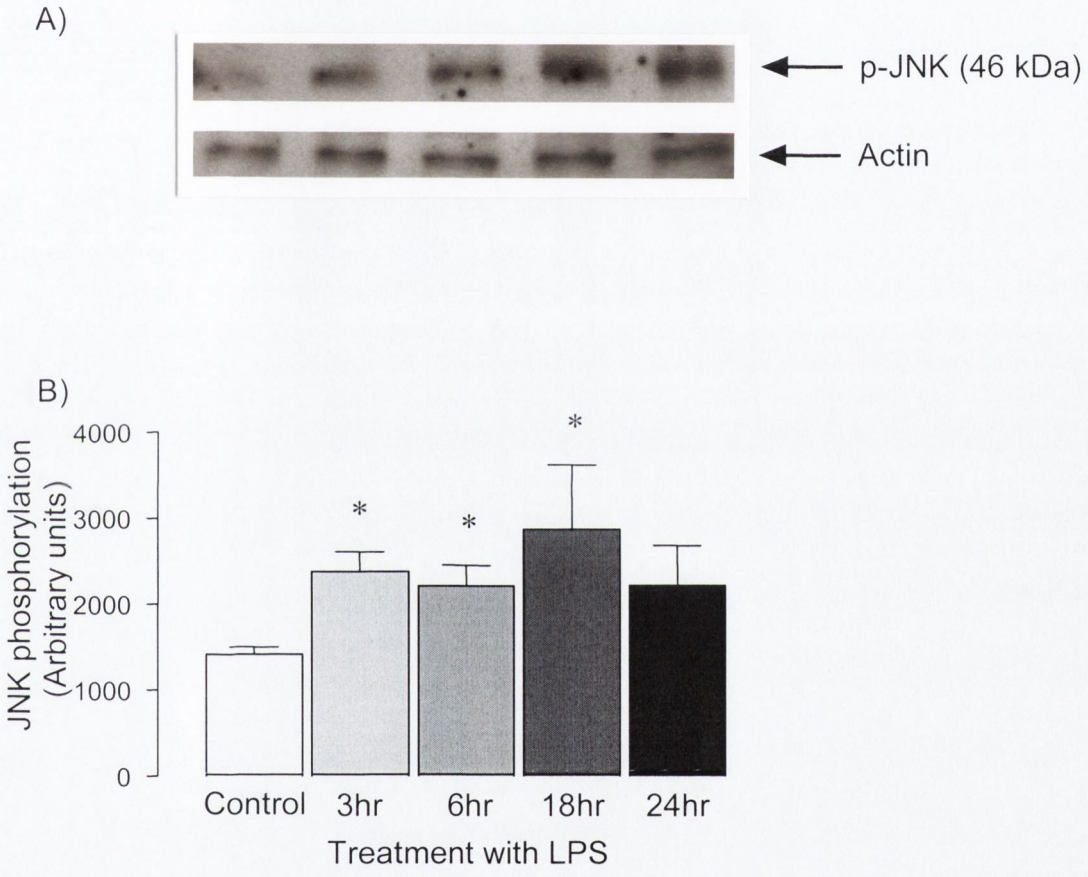

A)
p-JNK (46 kDa)
Actin
B)
JNK phosphorylation
(Arbitrary units)
4000
3000
2000
1000
0
*
*
*
Control
3hr
6hr
18hr
24hr
Treatment with LPS

Figure 3.21 Bcl-2 phosphorylation in mitochondrial fraction prepared from cultured cortical neurons was increased following an 18h incubation with LPS.
A) The sample immunoblot indicates that bcl-2 phosphorylation was increased in the mitochondrial fraction prepared from cultured cortical neurons incubated for 18h with LPS (lane 4) compared with neurons receiving media alone (lane 1). B) Assessment of mean values obtained from densitometric analysis indicated that bcl-2 phosphorylation was increased in the mitochondrial fraction prepared from cultured cortical neurons incubated for 18h with LPS compared with all other treatment groups. However this increase was not quite significant ($p=0.0676$; ANOVA). Data are expressed as means ±SEM of 5 values in all groups.

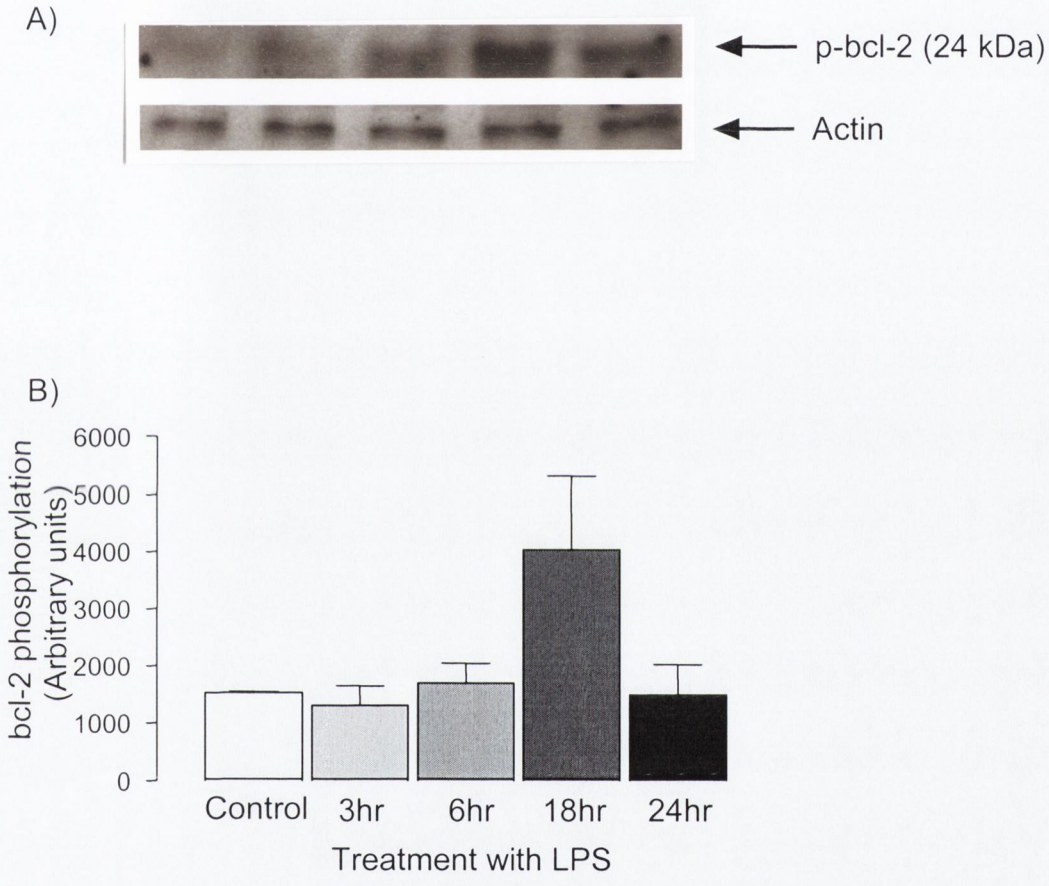

A)
p-bcl-2 (24 kDa)
Actin
B)
bcl-2 phosphorylation
(Arbitrary units)
6000
5000
4000
3000
2000
1000
0
Control
3hr
6hr
18hr
24hr
Treatment with LPS

Figure 3.22 Cytosolic cytochrome c of cultured cortical neurons was modestly increased in a time dependent manner following incubation with LPS.
A) The sample immunoblot and B) subsequent assessment of mean values obtained from densitometric analysis indicated that cytochrome c in the cytosolic fraction prepared from cultured cortical neurons which received media alone (n=3), treatment for 3h (n=3), 6h (n=4), 18h (n=3), and 24h (n=4) with LPS, increased modestly at each successive timepoint. A significant increase was observed only the 18h timepoint when compared with treatment of media alone (*$p<0.05$; student t test). Data are expressed as means ±SEM.

A)

cytochrome c
(15 kDa)

B)

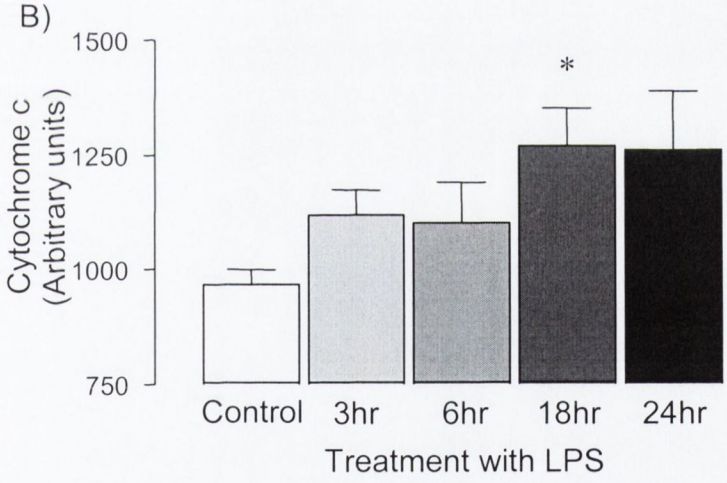
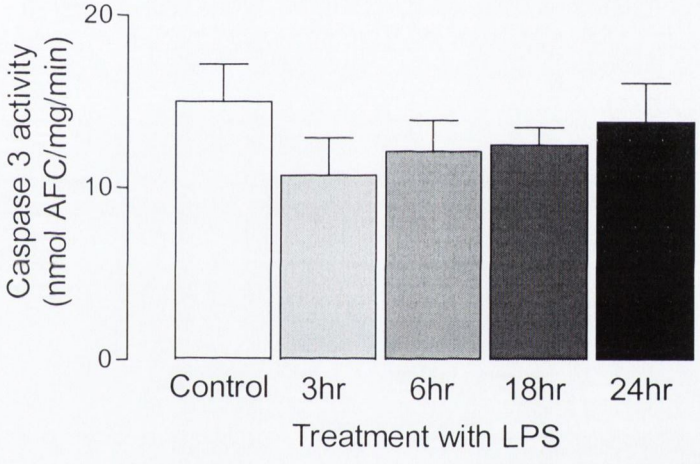

1500
1250
1000
750
Cytochrome c
(Arbitrary units)
*
Control
3hr
6hr
18hr
24hr
Treatment with LPS

Figure 3.23 Caspase 3 activity of whole cell lysate prepared from cortical neurons was not affected following any of the incubation periods with LPS.

Caspase 3 activity as assessed by a fluorometric assay was similar in whole cell lysate prepared from cells receiving media alone, or following treatment for 3h, 6h, 18h, and 24h with LPS. Data are expressed as means ±SEM of 6 values in all groups; all samples were analysed in triplicate.


Caspase 3 activity
(nmol AFC/mg/min)
20
10
0
Control
3hr
6hr
18hr
24hr
Treatment with LPS

3. 3. Analysis of LPS-induced effects on cultured cortical neurons pretreated with the JNK inhibitor D-JNKI1

D-JNKI1 was assessed for its ability to reverse some effects of LPS treatment on cultured cortical neurons. As the previous timecourse study indicated that LPS-induced signalling occurred maximally after 18h incubation, neurons were pretreated for 30min with media supplemented with D-JNKI1 (1μmol/l) or media alone, and then coincubated with media supplemented with LPS (100ng/ml) ± D-JNKI1 or media alone ± D-JNKI1 for 18h.

3. 3. 1. D-JNKI1 did not affect the LPS-induced increase in IL-1β concentration in supernatant collected from cultured cortical neurons.

Figure 3.24 demonstrates that mean concentration of IL-1β in supernatant prepared from neurons (pg/ml ±SEM; n=6 for all groups) was significantly increased following treatment for 18h with LPS compared with that from cells treated with media alone (387 ±43.8 and 151 ±27.1 respectively; ***$p<0.001$; Newmann-Keuls). This LPS-induced increase in IL-1β concentration was still observed in supernatant prepared from cells pretreated with D-JNKI1 and receiving LPS treatment (350 ±26.4; ***$p<0.001$; Newmann-Keuls), while the mean value from cells pretreated with D-JNKI1 and receiving control treatment (113 ±8.9) did not show any significant change compared with the mean value from cells receiving media alone.

3. 3. 2. D-JNKI1 modulated the LPS-induced increase in bcl-2 phosphorylation in mitochondrial fraction prepared from cultured cortical neurons.

The sample immunoblot in Figure 3.25A indicates that bcl-2 phosphorylation was increased in the mitochondrial fraction prepared from control-treated cultured cortical neurons which received LPS (lane 2) compared with control-treated neurons which received media alone (lane 1). Assessment of mean values obtained from densitometric analysis (Figure 3.25B) indicated mean bcl-2 phosphorylation (arbitrary units ±SEM; n=4 for all groups) was increased in the mitochondrial fraction from cultured cortical neurons which received LPS compared with neurons receiving media alone (2314 ±401.4 and 1557 ±45.9 respectively) but this did not reach statistical significance. This LPS-induced increase in bcl-2 phosphorylation was reduced by D-JNKI1 pretreatment so that the mean value in neurons pretreated with D-JNKI1 which received LPS or media alone (1798 ±318.3 and 1828 ±447.9 respectively) was less than that in control-treated neurons which received LPS.

Figure 3.24 D-JNKI1 did not affect the LPS-induced increase in IL-1β concentration in supernatant collected from cultured cortical neurons.

Mean IL-1β concentration in supernatant prepared from neurons was significantly increased following treatment for 18h with LPS compared with that from cells receiving media alone (***$p<0.001$; Newmann-Keuls). This LPS-induced increase in IL-1β concentration was still observed in supernatant prepared from cells pretreated with D-JNKI1 and receiving LPS treatment (***$p<0.001$; Newmann-Keuls), while the mean value from cells pretreated with D-JNKI1 and receiving control treatment did not show any significant change compared with the mean value from cells receiving media alone. Data are expressed as means ±SEM of 6 values in all groups; all samples were analysed in triplicate.

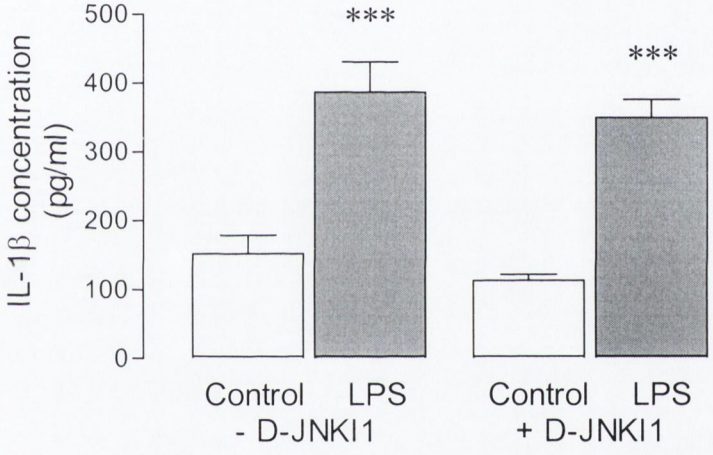
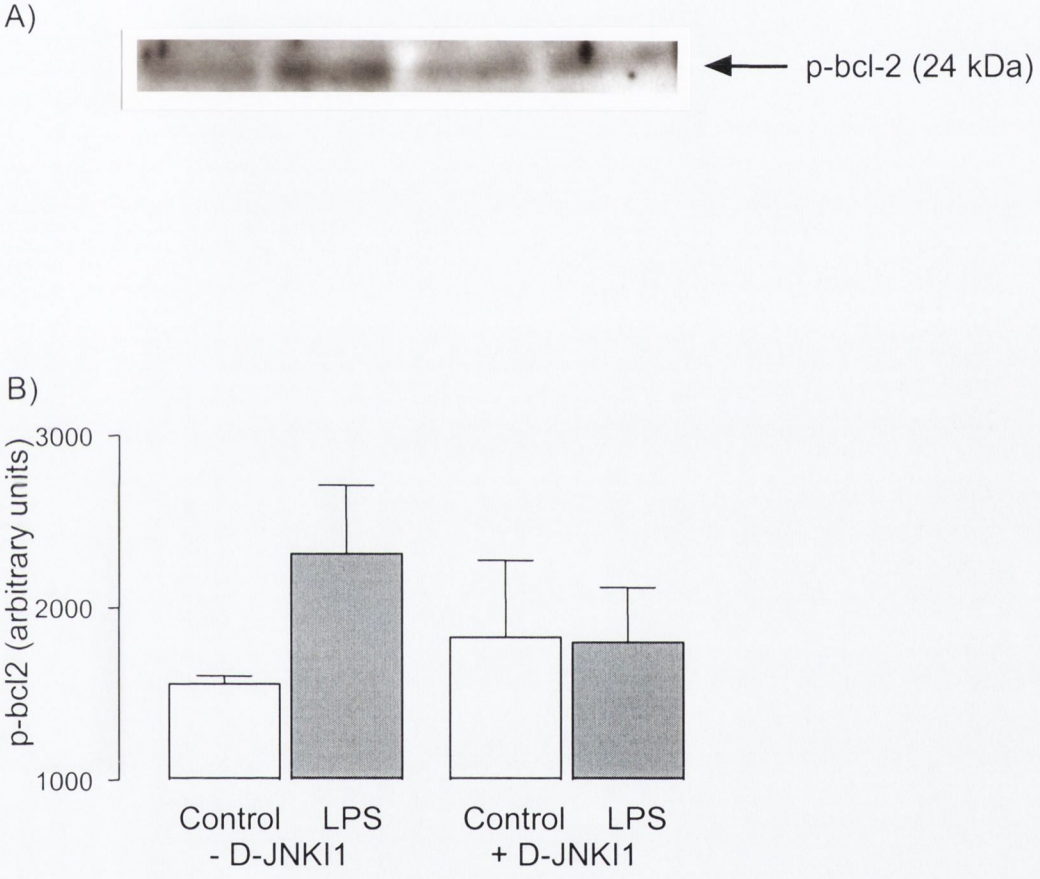

IL-1β concentration (pg/ml)
500
400
300
200
100
0
***
***
Control
LPS
- D-JNKI1
Control
LPS
+ D-JNKI1

Figure 3.25 D-JNKI1 attenuated the modest LPS-induced increase in bcl-2 phosphorylation in a mitochondrial fraction prepared from cultured cortical neurons. A) The sample immunoblot indicates that bcl-2 phosphorylation was increased in the mitochondrial fraction prepared from control-treated cultured cortical neurons which received LPS (lane 2) compared with control-treated neurons which received control media (lane 1). B) Assessment of mean values obtained from densitometric analysis indicated that bcl-2 phosphorylation was increased in the mitochondrial fraction from cultured cortical neurons which received LPS compared with neurons receiving media alone, but this did not reach statistical significance. This LPS-induced increase in bcl-2 phosphorylation was reduced by D-JNKI1 pretreatment so that the mean value in neurons pretreated with D-JNKI1 which received LPS or media alone was less than that in control-treated neurons which received LPS. Data are expressed as means ±SEM of 4 values in all groups.


A)
p-bcl-2 (24 kDa)
B)
p-bcl2 (arbitrary units)
3000
2000
1000
Control
LPS
- D-JNKI1
Control
LPS
+ D-JNKI1

3. 4. Analysis of LPS-induced effects on hippocampus of rats which received a diet supplemented with atorvastatin.

Since epidemiological evidence suggests that atorvastatin may have anti-inflammatory effects, atorvastatin was assessed for its ability to reverse the central effects of i.p. LPS administration, specifically the detrimental effects leading to apoptosis and decreased synaptic efficacy. Male Wistar rats treated with ATV (5mg/kg/day) or untreated (control) for 3weeks, after which they received an i.p. injection of either saline or LPS (100μg/kg), and following a 3h period were assessed for their ability to sustain LTP, then killed. Rachael Clarke, a current Ph.D. student, assisted in the dietary treatment of these animals.

Figure 3.26 indicates mean body weight (g ±SEM; n=6 for all groups) of control-treated rats was significantly greater at the end of the treatment period compared with values before treatment (**$p<0.01$; Newmann-Keuls) in both the saline and LPS treated groups (295 ±8.7 versus 219 ±11.4; 313 ±12.5 versus 226 ±10.0 respectively; a percentage increase of 35% and 38% respectively). This significant increase in body weight at the end of the treatment period compared with values before start of treatment was not observed in ATV-treated rats in either the saline or LPS treated groups (269 ±15.5 versus 225 ±12.0; 258 ±20.2 versus 208 ±10.6 respectively; but still represented a percentage increase of 20% and 24% respectively). There was no significant difference in mean body weight between the treatment groups following the treatment period.

3. 4. 1. Atorvastatin modestly attenuated the inhibitory effects of LPS on LTP.

The effect of i.p. LPS injection on the induction and maintenance of LTP in ATV or control-treated animals was examined in the perforant path – granule cell synapses. The mean stimulus strength (mV; ±SEM) required to induce a spike in control-treated rats which received saline or LPS was 3.85 (±1.037) and 4.05 (±1.042) respectively. The corresponding values for ATV-treated rats which received saline or LPS were 3.36 (±0.987) and 5.68 (±1.258). There was no significant difference between these values.

Figure 3.27A and B show that tetanus-induced LTP was blocked in perforant path – granule cell synapses of control-treated rats which received LPS (n=10) compared with control-treated rats which received saline. (n=7); the mean percentage changes in epsp slope (±SEM) in the 2min immediately following tetanic stimulation (compared with that in the 5min immediately prior to stimulation) was 123.8 (±1.09), which was significantly decreased from that observed in control-treated rats which received saline; 160.8 (±2.97;

p<0.001; Newmann-Keuls). Similarly, the mean percentage change in epsp slope (±SEM) in the last 5 minutes of the experiment was significantly decreased from 123.9 (±1.74) in control-treated rats which received saline, to 95.1 (±1.00) in control-treated rats which received LPS (p<0.001; Newmann-Keuls).

This impairment was mildly suppressed by treatment with ATV; ATV-treated rats which received saline or LPS (n=5) did not fully sustain LTP. Mean percentage epsp at 0-2min was significantly decreased in ATV-treated rats which received saline or LPS (126.2 ±2.70 and 122.7 ±1.98, respectively) when compared with that of control-treated rats which received saline (***p<0.001; Newmann-Keuls in each case). Similarly, at 40-45min, mean percentage epsp was significantly decreased in ATV-treated rats which received saline or LPS (102.9 ±2.65 and 105.5 ±2.36, respectively) when compared with that of control-treated rats which received saline (***p<0.001; Newmann-Keuls in each case). However, these values were significantly greater than that of control-treated rats which received LPS (++p<0.01; Newmann-Keuls in each case).

3. 4. 2. Atorvastatin attenuated the LPS-induced JNK activation and c-Jun phosphorylation in hippocampal tissue.

Rachael Clarke carried out the assessment of JNK phosphorylation, and provided additional n numbers for the assessment of c-Jun phosphorylation. The sample immunoblot in Figure 3.28A indicates that JNK phosphorylation was increased in hippocampus of control-treated rats which received LPS compared to control-treated rats which received saline. Assessment of mean values obtained from densitometric analysis (Figure 3.28B) indicated mean JNK activity, (arbitrary units ±SEM; n=6 for all groups), was significantly increased in hippocampus of control-treated rats which received LPS (7018 ±465.5) compared with control-treated rats which received saline (5176 ±403.0; p<0.05; Newmann-Keuls). This LPS-induced increase in JNK activity was attenuated by ATV pretreatment so that the mean value in hippocampus of rats pretreated with ATV which received LPS i.p. (5866 ±221.6) was not significantly different to that of saline-treated controls, similarly in ATV-treated rats which received saline i.p. (5932 ±459.5).

The sample immunoblot in Figure 3.29A indicates that c-Jun phosphorylation was increased in hippocampus of control-treated rats which received LPS compared to control-treated rats which received saline. Assessment of mean values obtained from densitometric analysis (Figure 3.29B) indicated mean c-Jun phosphorylation, expressed as a ratio of phosphorylated c-Jun to total c-Jun (±SEM; n=6 for all groups), was increased in

hippocampus of control-treated rats which received LPS compared with control-treated rats which received saline (1.12 ±0.113 and 0.80 ±0.058 respectively) but this did not reach statistical significance (p=0.0595; ANOVA). The expression of c-Jun phosphorylation in hippocampus of ATV-treated rats which received saline or LPS (0.90 ±0.077 and 1.01 ±0.062 respectively) were reduced compared to that of control-treated rats which received LPS.

3. 4. 3. ERK phosphorylation in hippocampal tissue was not affected by atorvastatin or LPS treatment.

The sample immunoblot in Figure 3.30A indicates that ERK phosphorylation was similar in hippocampus of all treatment groups. Assessment of mean values obtained from densitometric analysis (Figure 3.30B) indicated no significant difference in mean ERK phosphorylation, expressed as a ratio of phosphorylated ERK to actin, (±SEM; n=6 for all groups) between any of the groups examined; 0.30 ±0.002; 0.31 ±0.033; 0.28 ±0.022; and 0.27 ±0.018 respectively.

Figure 3.26 Body weights

Mean body weight of control-treated rats was significantly greater at the end of the treatment period compared with values before treatment (**$p<0.01$; Newmann-Keuls) in both saline and LPS treated groups. This increase was not observed in ATV-treated rats in either the saline or the LPS treated groups, however there was no significant difference in rat body weight between the treatment groups following dietary manipulation. Data are expressed as means ±SEM of 6 values in all groups.

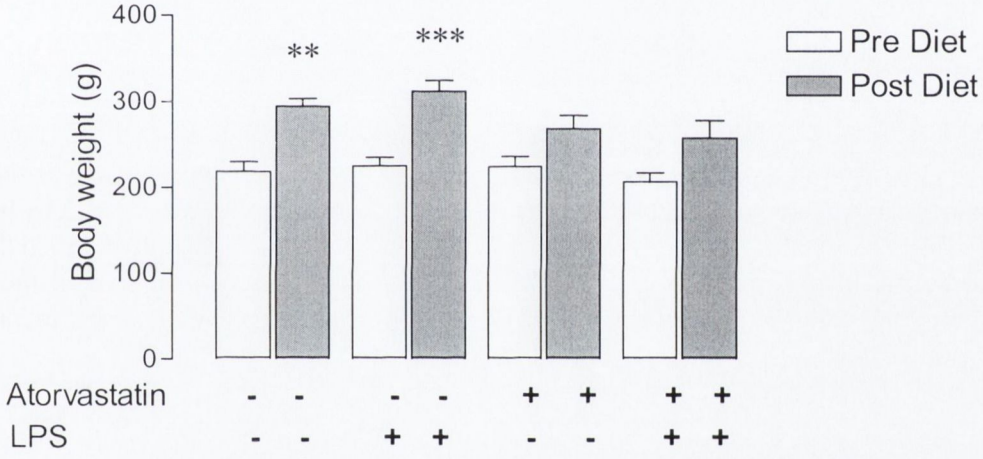

400
300
200
100
0
Body weight (g)
**
***
Pre Diet
Post Diet
Atorvastatin - - - - + + + +
LPS - - + + - - + +

Figure 3.27 Atorvastatin modestly attenuated the inhibitory effects of LPS on LTP.
A) Tetanus-induced LTP was blocked in perforant path – granule cell synapses of control-treated rats which received LPS (n=10) compared with control-treated rats which received saline. (n=7). This impairment was also seen following treatment with ATV; ATV-treated rats which received saline or LPS (n=5 in each case) did not fully sustain LTP. Data are expressed as the mean percentage change in population epsp slope (relative to the mean epsp slope in the 5min immediately prior to tetanic stimulation). Values are means and bars denoting ±SEM are included at 5min intervals.

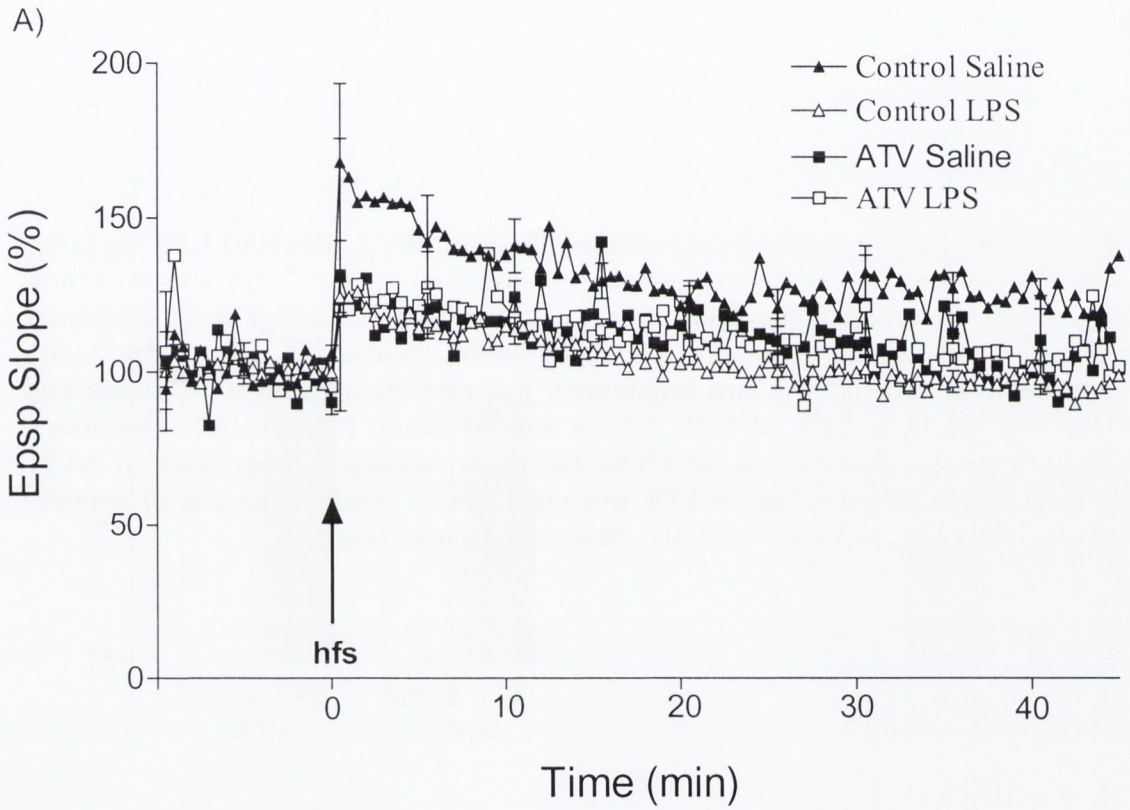

A)
Control Saline
Control LPS
ATV Saline
ATV LPS
Epsp Slope (%)
200
150
100
50
0
hfs
0
10
20
30
40
Time (min)

Figure 3.27 Atorvastatin modestly attenuated the inhibitory effects of LPS on LTP. B) Mean percentage population epsp slope was assessed in the 2min and 40-45min following tetanic stimulation, and analysis revealed that there was a significant inhibitory effect of LPS on LTP at both time points in control-treated animals (***$p<0.001$; Newmann-Keuls in each case). This impairment was also observed in ATV-treated rats which received saline or LPS, at both 0-2min and 40-45min (***$p<0.001$; Newmann-Keuls in each case). However, at 40-45min the mean percentage epsp slope in ATV-treated rats which received saline or LPS was significantly greater than that of control-treated rats which received LPS (**$p<0.01$; Newmann-Keuls in each case).

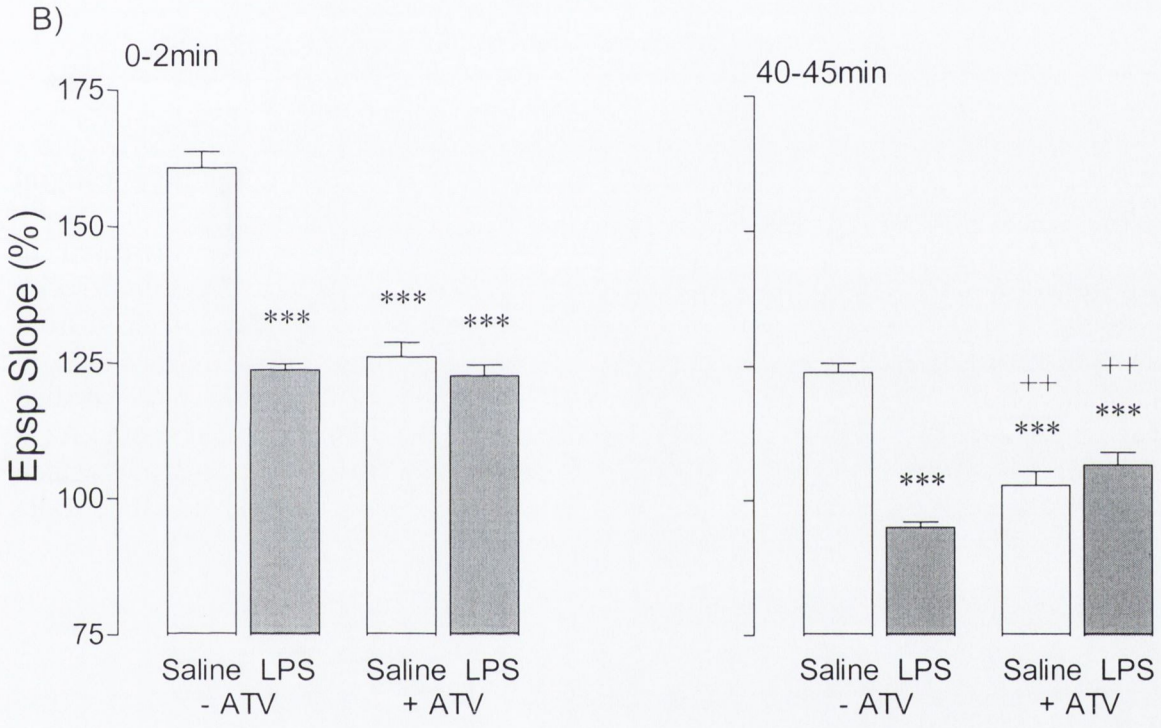

B)
0-2min
40-45min
Epsp Slope (%)
175
150
125
100
75
***
***
***
***
***
++
++
Saline LPS
- ATV
Saline LPS
+ ATV
Saline LPS
- ATV
Saline LPS
+ ATV

Figure 3.28 Atorvastatin attenuated the LPS-induced JNK activation in hippocampal tissue.

A) The sample immunoblot indicates that JNK phosphorylation was increased in hippocampal tissue prepared from control-treated rats which received LPS compared with control-treated rats which received saline. B) Assessment of mean values obtained from densitometric analysis indicated that mean JNK activity was significantly increased in hippocampus of control-treated rats which received LPS compared with control-treated rats which received saline (*$p<0.05$; Newmann-Keuls). Pre-treatment with ATV attenuated this increase so that JNK activity in hippocampus of ATV-treated rats which received saline or LPS was similar to control-treated animals which received saline. Data are expressed as means ±SEM of 6 values in all groups.

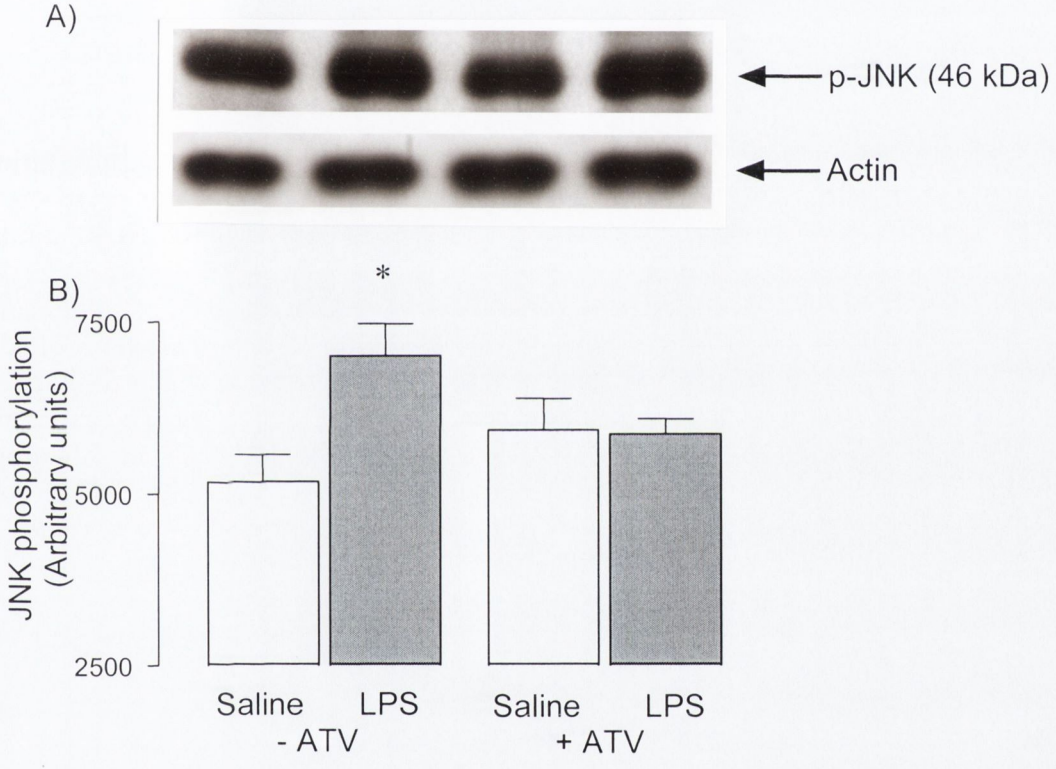

A)
p-JNK (46 kDa)
Actin
B)
*
JNK phosphorylation
(Arbitrary units)
7500
5000
2500
Saline
LPS
- ATV
Saline
LPS
+ ATV

Figure 3.29 The effect of Atorvastatin and LPS on c-Jun phosphorylation in hippocampal tissue.

A) The sample immunoblot indicates that c-Jun phosphorylation was similar in hippocampal tissue prepared from all treatment groups. B) Assessment of mean values obtained from densitometric analysis indicated that mean c-Jun phosphorylation, expressed as a ratio of phosphorylated c-Jun to total c-Jun, was increased in hippocampus of control-treated rats which received LPS compared with control-treated rats which received saline although this did not reach statistical significance ($p=0.0595$; ANOVA). The expression of phosphorylated c-Jun in hippocampus of ATV-treated rats which received saline or LPS was also not significantly changed, but were similar to mean values in that of control-treated rats which received LPS. Data are expressed as means ±SEM of 6 values in all groups.

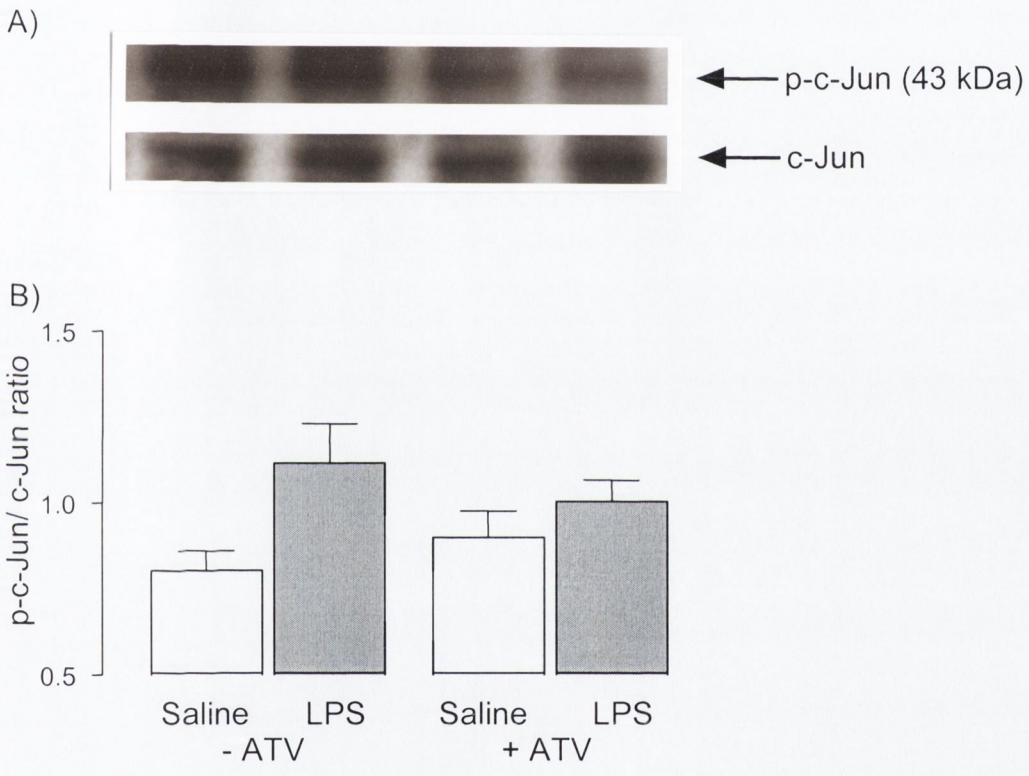
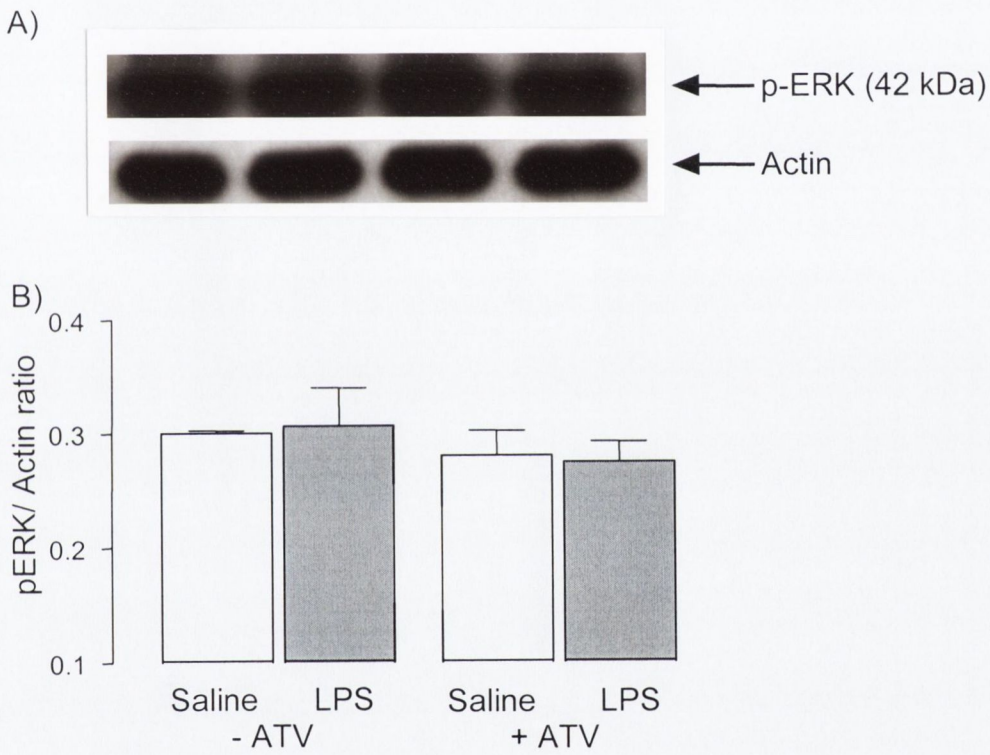

A)
p-c-Jun (43 kDa)
c-Jun
B)
p-c-Jun/ c-Jun ratio
1.5
1.0
0.5
Saline
LPS
- ATV
Saline
LPS
+ ATV

Figure 3.30 ERK phosphorylation in hippocampal tissue was not affected by atorvastatin or LPS treatment.

A) The sample immunoblot indicates that ERK phosphorylation was similar in hippocampal tissue prepared from all treatment groups. B) Assessment of mean values obtained from densitometric analysis indicated that there was no significant difference in mean ERK phosphorylation, expressed as a ratio of phosphorylated ERK to actin, between any of the groups examined. Data are expressed as means ±SEM of 6 values in all groups.


A)
p-ERK (42 kDa)
Actin
B)
pERK/ Actin ratio
0.4
0.3
0.2
0.1
Saline
LPS
- ATV
Saline
LPS
+ ATV

3. 5. Analysis of LPS-induced effects on cultured cortical glia pretreated with atorvastatin.

Atorvastatin was assessed for its ability to reverse the effects of LPS treatment on cultured cortical glia. Glial cells received a 24h pretreatment of media supplemented with ATV (10μM) or media alone, and then received treatment of media supplemented with LPS (100ng/ml) or media alone for 24h. Assessment of CD40 expression by PAGE and western immunoblotting was carried out by Niall Kavanagh as part of his final year project, and he also assisted in the analysis of IL-10 and IL-4 concentration by ELISA.

3. 5. 1. Atorvastatin did not affect the LPS-induced increase in IL-1β concentration in supernatant collected from cultured cortical glial cells.

Figure 3.31 demonstrates that mean concentration of IL-1β in supernatant prepared from glia (pg/ml ±SEM; n=6 for all groups) was significantly increased following treatment for 24h with LPS compared with that from cells receiving media alone (226 ±35.6 and 33 ±12.8 respectively; ***$p<0.001$; Newmann-Keuls). This LPS-induced increase in IL-1β concentration was still observed in samples prepared from cells pretreated with ATV and receiving LPS treatment but not to the same extent (146 ±50.26; *$p<0.05$; Newmann-Keuls). Samples prepared from cells pretreated with ATV and receiving control treatment did not show any significant change compared with cells receiving media alone (7 ±4.6).

3. 5. 2. Atorvastatin attenuated the LPS-induced increase in CD40 in cultured glial cells.

CD40 is a cell surface receptor and has been implicated in inflammatory signalling. The sample immunoblot in Figure 3.32A indicates that CD40 expression was increased in whole cell lysate of glial cells which received LPS (lane 2) compared with glial cells treated with media alone (lane 1). Assessment of mean values obtained from densitometric analysis (Figure 3.32B) indicated that mean CD40 expression (arbitrary units ±SEM; n=6 for all groups) was significantly increased in whole cell lysate of LPS-treated glia compared with glia treated with media alone (2223 ±160.6 and 1499 ±0.6 respectively; *$p<0.05$; Newmann-Keuls). Mean CD40 expression in LPS or media alone treated glial cells pretreated with ATV were similar to controls and significantly less than that of LPS-treated glia (1534 ±265.9 and 1441 ±240.1 respectively; +$p<0.05$; Newmann-Keuls).

3. 5. 3. Atorvastatin attenuated the LPS-induced increase in IL-10 concentration in supernatant collected from cultured cortical glial cells, while IL-4 was not detectable.

Figure 3.33 demonstrates that mean concentration of IL-10 in supernatant prepared from glia (pg/ml ±SEM; n=6 for all groups) was significantly increased following treatment for 24h with LPS compared with that from cells treated with media alone (299 ±34.6 and 8 ±4.3 respectively; ***$p<0.001$; Newmann-Keuls). An LPS-induced increase in IL-10 concentration was still observed in samples prepared from cells pretreated with ATV and receiving LPS treatment (216 ±43.3; ***$p<0.001$; Newmann-Keuls), although this was significantly less than that observed in LPS-treated controls (+$p<0.05$; Newmann-Keuls). Samples prepared from cells pretreated with ATV and receiving control treatment did not show any significant change compared with cells receiving media alone (1 ±0.9).

IL-4 concentration was assessed by an ELISA in aliquots of the same. Figure 3.34 shows mean absorbance; thus IL-4 concentration did not reach detectable levels in any of the treatment groups. Data points expressed on the graph are the mean absorbance at 450nm for each sample; all samples were analysed in triplicate. The line represents the limit of detection.

Figure 3.31 Atorvastatin did not affect the LPS-induced increase in IL-1β concentration in supernatant collected from cultured cortical glial cells.
Mean IL-1β concentration in supernatant prepared from glia was significantly increased following treatment for 24h with LPS compared with that from cells receiving media alone (***$p<0.001$; Newmann-Keuls). This LPS-induced increase in IL-1β concentration was still observed samples prepared from cells pretreated with ATV and receiving LPS treatment (*$p<0.05$; Newmann-Keuls). Samples prepared from cells pretreated with ATV and receiving control treatment did not show any significant change compared with cells receiving media alone. Data are expressed as means ±SEM of 6 values in all groups; all samples were analysed in triplicate.

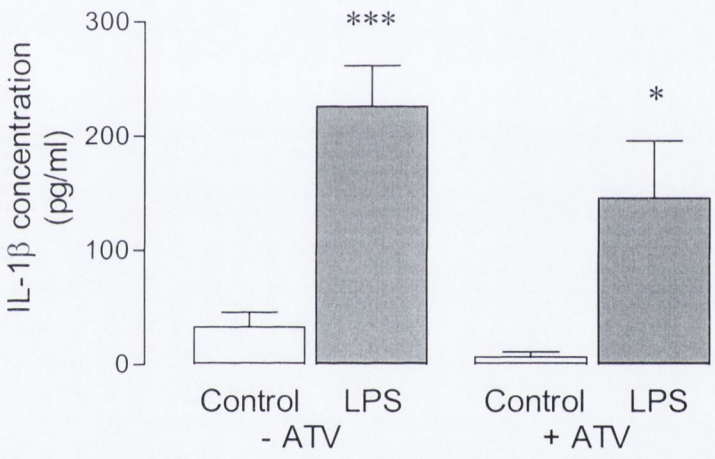

IL-1β concentration (pg/ml)
300
200
100
0
***
*
Control
LPS
- ATV
Control
LPS
+ ATV

Figure 3.32 Atorvastatin attenuated the LPS-induced increase in CD40 in cultured glial cells.

A) The sample immunoblot indicates that CD40 expression was increased in whole cell lysate of glial cells which received LPS (lane 2), compared with control-treated glial cells (lane 1). B) Assessment of mean values obtained from densitometric analysis indicated that CD40 expression was significantly increased in whole cell lysate of LPS-treated glia compared with glia treated with media alone (*$p<0.05$; Newmann-Keuls). CD40 expression in glial cells pretreated with ATV which received LPS or control media was similar to that of glia receiving media alone, and significantly less than that of glia which received LPS (+$p<0.05$; Newmann-Keuls). Data are expressed as means ±SEM of 6 values in all groups.

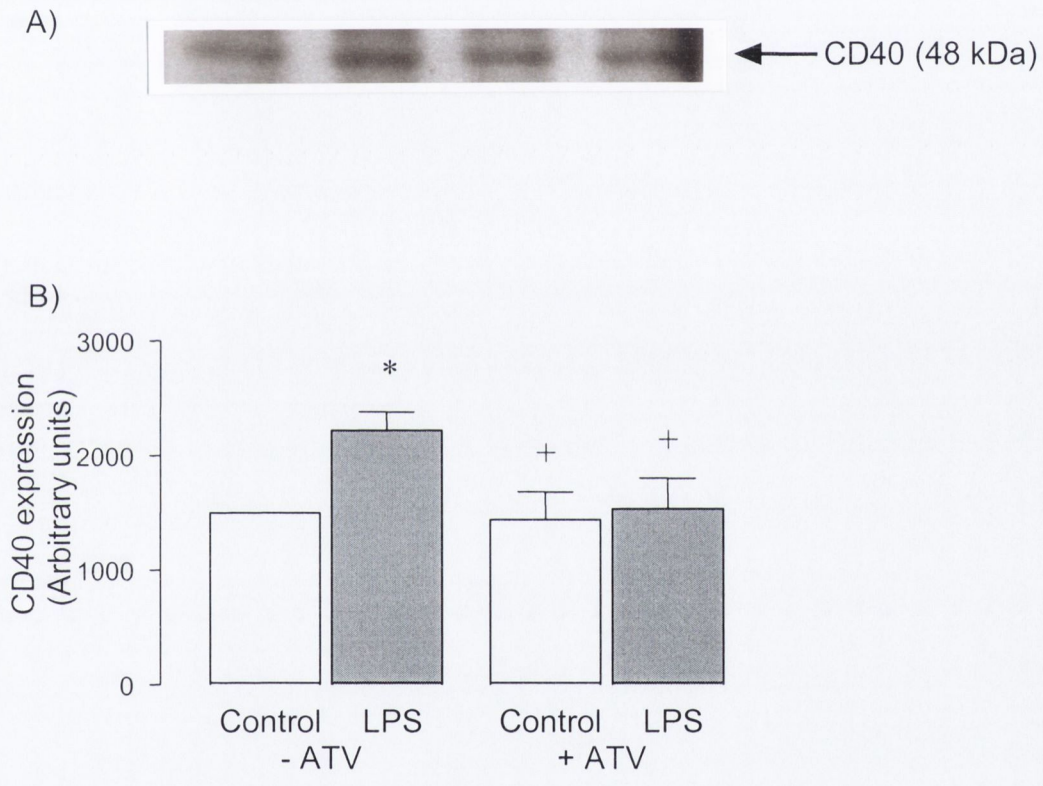

A)
CD40 (48 kDa)
B)
CD40 expression
(Arbitrary units)
3000
2000
1000
0
*
+
+
Control
LPS
- ATV
Control
LPS
+ ATV

Figure 3.33 Atorvastatin attenuated the LPS-induced increase in IL-10 concentration in supernatant collected from cultured cortical glial cells.
Mean IL-10 concentration in supernatant prepared from glia was significantly increased following treatment for 24h with LPS compared with that from cells receiving media alone (***$p<0.001$; Newmann-Keuls). An LPS-induced increase in IL-10 concentration was still observed in samples prepared from cells pretreated with ATV and receiving LPS treatment (***$p<0.001$; Newmann-Keuls), although this was significantly less than that observed in LPS-treated controls (+$p<0.05$; Newmann-Keuls). Samples prepared from cells pretreated with ATV and receiving control treatment did not show any significant change compared with cells receiving media alone. Data are expressed as means ±SEM of 6 values in all groups; all samples were analysed in triplicate.

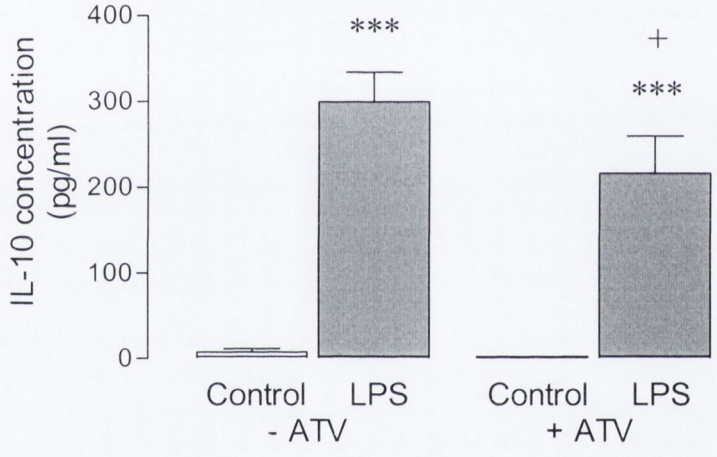
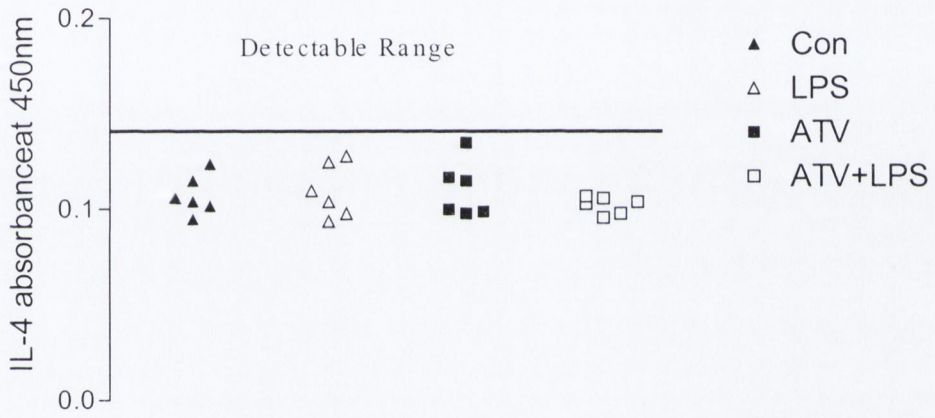

IL-10 concentration (pg/ml)
400
300
200
100
0
***
+
***
Control
LPS
- ATV
Control
LPS
+ ATV

Figure 3.34 IL-4 was not detectable in media collected from cultured cortical glial cells.

IL-4 concentration did not reach detectable levels in any of the treatment groups. Data points expressed on the graph are the mean absorbance at 450nm for each sample; all samples were analysed in triplicate. The line represents the limit of the detection.


IL-4 absorbanceat 450nm
0.2
0.1
0.0
Detectable Range
Con
LPS
ATV
ATV+LPS

3. 6. Analysis of the effects of ageing on hippocampus of rats which received a diet supplemented with atorvastatin.

As age is a model of chronic inflammation, atorvastatin was assessed for its ability to reverse the effects of ageing, specifically the detrimental effects leading to apoptosis and decreased synaptic efficacy. Aged (22-24 month) and young (3-4 month) male Wistar rats were treated with ATV (5mg/kg/day) or were untreated (controls) for 8weeks, after which they were assessed for their ability to sustain LTP, then killed. Rachael Clarke assisted in the dietary treatment of these animals.

Figure 3.35 indicates that mean body weight (g ±SEM) was significantly greater at the end of the treatment period compared with values before treatment in control-treated young rats as well as ATV-treated young rats (374 ±14.0 versus 294 ±13.0; 363 ±6.3 versus 286 ±9.28 respectively; *$p<0.05$; Newmann-Keuls; n=6). There was no significant difference in mean young rat body weight between the treatment groups before or after the treatment period. Mean body weight of control-treated or ATV-treated aged rats (n=9 per group) remained unchanged throughout the treatment period (534 ±17.4 versus 527 ±15.5; 577 ±21.6 versus 551 ±22.1).

3. 6. 1. Atorvastatin abrogated the inhibitory effects of age on LTP.

The effect of ageing on the induction and maintenance of LTP in animals which received ATV supplemented or normal laboratory chow was examined in the perforant path – granule cell synapses. The mean stimulus strength (mV; ±SEM) required to induce a spike in control-treated young and aged rats was 2.95 (±0.502) and 2.07 (±0.402) respectively. The corresponding values for ATV-treated young and aged rats was 3.72 (±0.336) and 1.77 (±0.474). There was no significant difference between these values.

Figure 3.36A and B show that tetanus-induced LTP was blocked in perforant path – granule cell synapses of control-treated aged rats (n=9) compared with control-treated young rats (n=5); the mean percentage changes in epsp slope (±SEM) in the last 5 minutes of the experiment (compared with that in the 5min immediately prior to stimulation) was significantly decreased from 114.1 (±0.53) in control-treated young rats, to 103.8 (±0.61) in control-treated aged rats (***$p<0.001$; Newmann-Keuls). However the mean percentage change in epsp slope in the 2min immediately following tetanic stimulation was similar in control-treated young and aged rats; 125.1 (±4.06) and 128.6 (±0.89) respectively.

This impairment was suppressed by dietary supplementation of ATV; ATV-treated aged rats (n=8) sustained LTP, as did ATV-treated young rats (n=4). ATV treatment

abrogated the effect of age at 40-45min so that the mean percentage epsp slope in ATV-treated aged rats (118.7 ±1.41) was significantly greater than that of both control-treated aged and young rats (+++$p<0.001$; Newmann-Keuls in each case). ATV-treated aged rats also showed a significant increase in mean percentage epsp slope at the 0-2min time point (134.9 ±2.33; *$p<0.05$; Newmann-Keuls). ATV-treated young rats exhibited a significant increase in mean percentage epsp slope compared with control-treated young rats (128.6 ±0.89; **$p<0.01$; Newmann-Keuls) at the 0-2min time point, but showed a significant decrease at the 40-45min time point (109.0 ±0.69; ***$p<0.001$; Newmann-Keuls).

Figure 3.35 Body weights
Mean body weight of ATV and control-treated young rats was significantly greater at the end of the treatment period compared with values before treatment (*$p<0.05$; Newmann-Keuls; n=6). There was no significant difference in mean young rat body weight between the treatment groups before or after the treatment period. Mean body weight of ATV and control-treated aged rats (n=9 per group) remained unchanged. Data are expressed as means ±SEM.

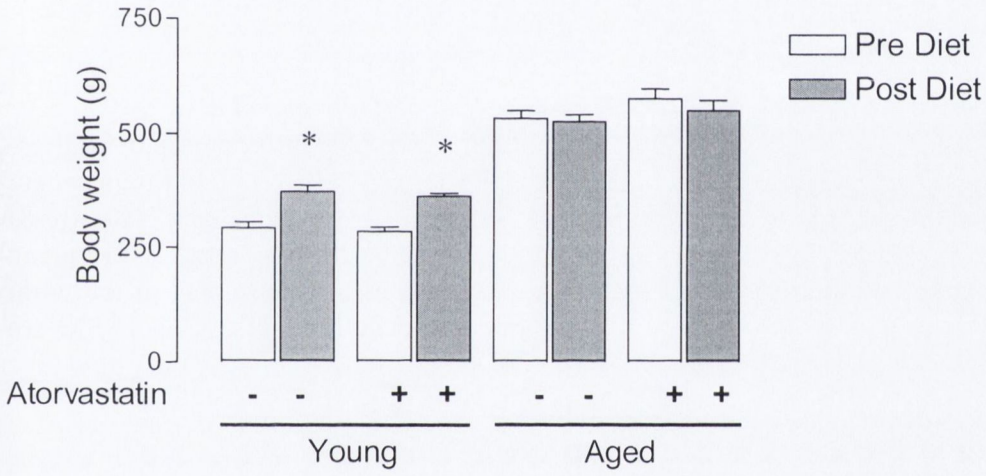

750
500
250
0
Body weight (g)
*
*
Pre Diet
Post Diet
Atorvastatin
- - + + - - + +
Young
Aged

Figure 3.36 Atorvastatin abrogated the inhibitory effects of age on LTP.
A) Tetanus-induced LTP was blocked in perforant path – granule cell synapses of control-treated aged rats (n=9) compared with control-treated young rats (n=5). This impairment was suppressed by dietary supplementation of ATV; ATV-treated aged rats (n=8) sustained LTP, as did ATV-treated young rats (n=4). Data are expressed as the mean percentage change in population epsp slope (relative to the mean epsp slope in the 5min immediately prior to tetanic stimulation). Values are means and bars denoting ±SEM are included at 5min intervals.

A)

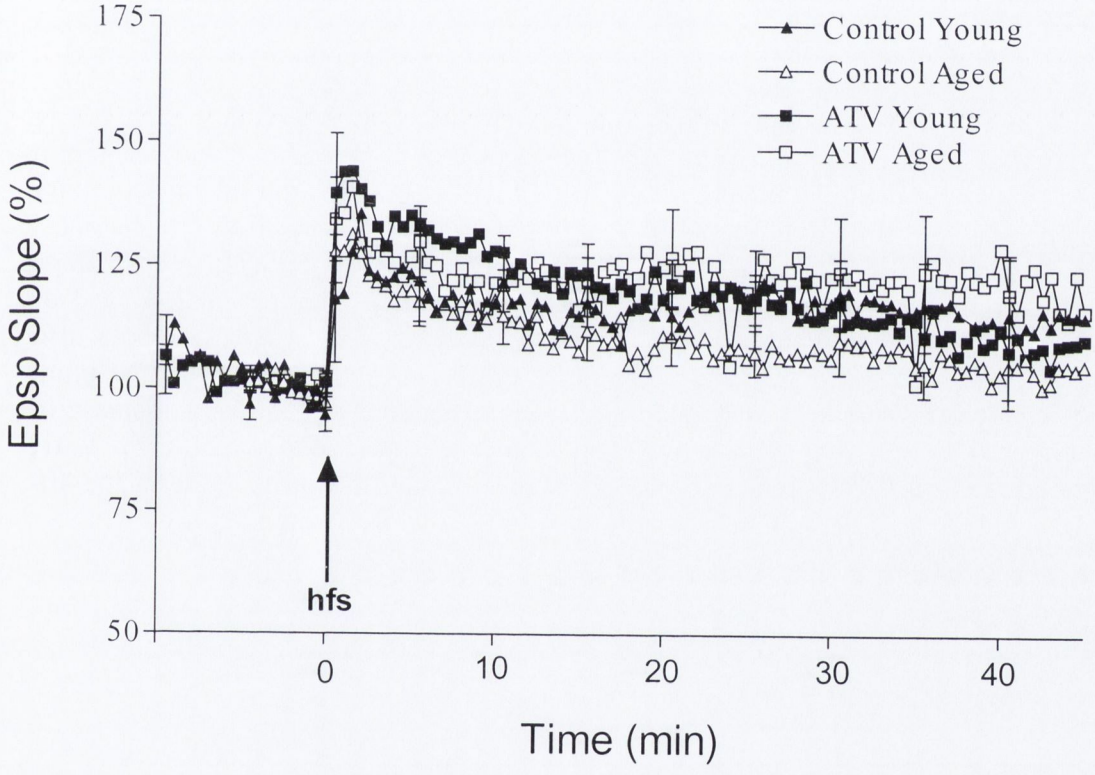
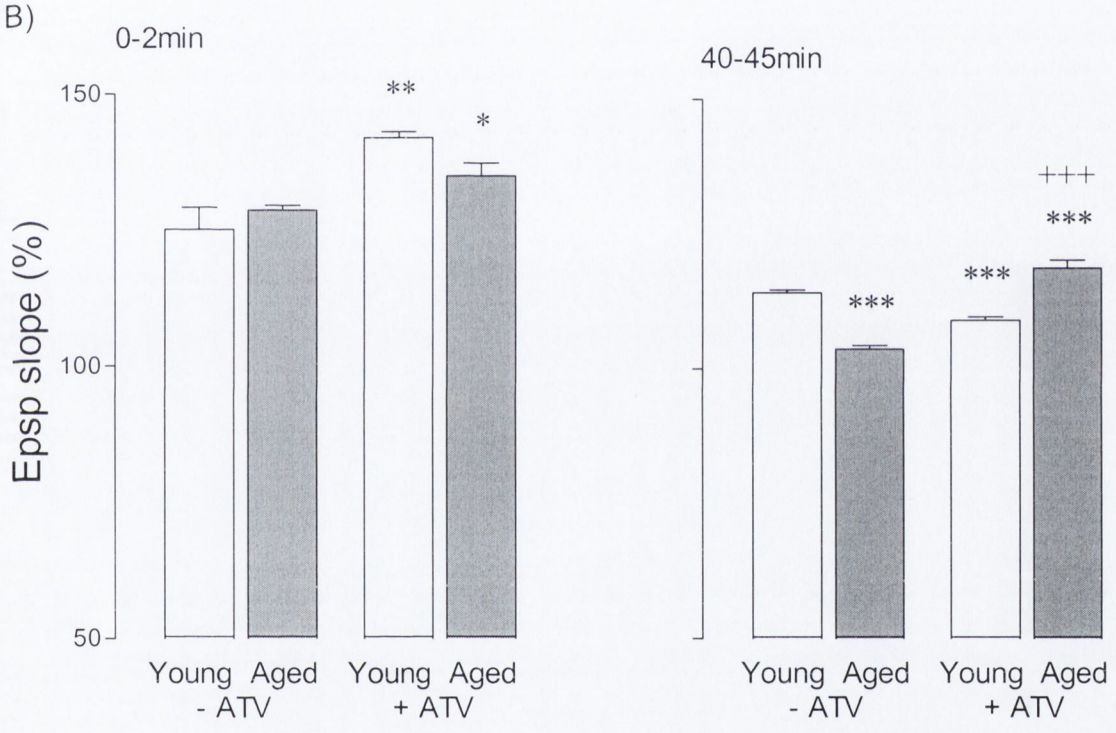

175
150
125
100
75
50
Epsp Slope (%)
Control Young
Control Aged
ATV Young
ATV Aged
hfs
0
10
20
30
40
Time (min)

Figure 3.36 Atorvastatin abrogated the inhibitory effects of age on LTP.
B) Mean percentage population epsp slope was assessed in the 2min and 40-45min following tetanic stimulation, and analysis revealed that there was a significant inhibitory effect of age on LTP at the later time point (***$p<0.001$; Newmann-Keuls). The ATV treatment abrogated the effect of age at 40-45min so that the mean percentage epsp slope in ATV-treated aged rats was significantly greater than that of control-treated aged or young rats (+++$p<0.001$; Newmann-Keuls in each case). ATV-treated aged rats also showed a significant increase in mean percentage epsp slope at the 0-2min time point (*$p<0.05$; Newmann-Keuls). ATV-treated young rats exhibited a significant increase in mean percentage epsp slope compared with control-treated young rats (**$p<0.01$; Newmann-Keuls) at the 0-2min time point, but showed a significant decrease at the 40-45min time point (***$p<0.001$; Newmann-Keuls).


B)
0-2min
40-45min
Epsp slope (%)
150
100
50
**
*
***
***
***
+++
Young
Aged
- ATV
Young
Aged
+ ATV
Young
Aged
- ATV
Young
Aged
+ ATV

3. 7. Analysis of LPS-induced effects on hippocampus of rats which were pretreated with SCH58261.

SCH58261, an A2A adenosine receptor antagonist, has also been reported to have anti-inflammatory effects centrally. SCH58261 was assessed for its ability to reverse the central effects of i.p. LPS administration, specifically the detrimental effects leading to apoptosis and decreased synaptic efficacy. Male Wistar rats received an i.c.v. injection of either vehicle or SCH58261 (5µl; 50nM), 30min later received an i.p. injection of either saline or LPS (100µg/kg), and, following a further 3h period, were assessed for their ability to sustain LTP, then killed.

3. 7. 1. SCH58261 attenuated the inhibitory effects of LPS on LTP.

The effect of i.p. LPS injection on the induction and maintenance of LTP in SCH58261-treated and vehicle-treated animals was examined in the perforant path – granule cell synapses. The mean stimulus strength (mV; ±SEM) required to induce a spike in vehicle-treated rats which received saline i.p. and LPS i.p. was 2.3 (±0.34) and 3.3 (±0.47) respectively. The corresponding values for SCH58261-treated rats were 3.0 (±0.53) and 2.9 (±0.32). There was no significant difference between these values.

Figure 3.37A and B show that tetanus-induced LTP was blocked in perforant path – granule cell synapses of vehicle-treated rats which received LPS i.p. (n=8) compared with control-treated rats which received saline i.p. (n=6); the mean percentage change in epsp slope (±SEM) in the 2min immediately following tetanic stimulation (compared with that in the 5min immediately prior to stimulation) was 115.1 (±1.08), which was significantly decreased from that observed in vehicle-treated rats which received saline i.p.; 126.8 (±1.01; ***$p<0.001$; Newmann-Keuls). Similarly, the mean percentage changes in epsp slope (±SEM) in the last 5 minutes of the experiment was significantly decreased from 105.2 (±0.51) in vehicle-treated rats which received saline i.p., to 99.0 (±0.47) in vehicle-treated rats which received LPS i.p. (***$p<0.001$; Newmann-Keuls).

This impairment was suppressed by pretreatment with SCH58261; Rats pretreated with SCH58261 which received LPS i.p. (n=6) sustained LTP, as did SCH58261-treated rats which received saline i.p. (n=5). Pretreatment with SCH58261 abrogated the effect of LPS at both 0-2min and 40-45min post tetanus, so that the mean percentage epsp slope from rats pretreated with SCH58261 which received LPS i.p. (130.2 ±0.74 and 104.6 ±0.64 respectively) was similar to vehicle-treated rats which received saline i.p., and significantly greater than that in vehicle-treated rats which received LPS i.p. (+++$p<0.001$; Newmann-

Keuls). SCH58261-treated rats which received saline i.p. exhibited a significant increase in mean epsp slope compared with vehicle-treated rats which received saline i.p. at both 0-2min (115.1 ±1.08; *p<0.05; Newmann-Keuls) and 40-45min (111.5 ±1.10; ***p<0.001; Newmann-Keuls) post tetanic stimulation.

3. 7. 2. SCH58261 attenuated the LPS-induced increase in IL-1β concentration, JNK phosphorylation, and caspase 3 activity

The IL-1β ELISA and the caspase 3 assay was carried out with the assistance of Nelson Rebola, while the assessment of JNK phosphorylation was carried out by him in its entirety. Figure 3.38 demonstrates that mean concentration of IL-1β (pg/mg ±SEM; n=6 for all groups) was significantly increased in samples prepared from hippocampus of vehicle-treated rats which received LPS i.p. compared with vehicle-treated rats which received saline i.p. (529.9 ±32.46 and 381.9 ±23.56 respectively; *p<0.05; Newmann-Keuls). This LPS-induced increase in IL-1β concentration was attenuated by SCH58261 pretreatment so that the mean value in hippocampus of SCH58261-treated rats which received LPS i.p. was similar to vehicle-treated controls, and significantly less than that in hippocampal cells prepared from vehicle-treated rats which received LPS i.p. (391.7 ±51.28; +p<0.05; Newmann-Keuls), similarly in SCH58261-treated rats which received saline i.p. (383.8 ±24.68; +p<0.05; Newmann-Keuls).

Assessment of mean values obtained from densitometric analysis (Figure 3.39) indicated mean JNK activity, (arbitrary units ±SEM; n=6 for all groups), was significantly increased in hippocampus of control-treated rats which received LPS (1337 ±111.5) compared with control-treated rats which received saline (1000 ±0.0; *p<0.05; Newmann-Keuls). This LPS-induced increase in JNK activity was attenuated by SCH58261 pretreatment so that the mean value in hippocampus of rats pretreated with SCH58261 which received LPS i.p. (1026 ±114.1) was not significantly different to that of saline-treated controls, but significantly reduced when compared to that of vehicle-treated animals which received LPS (*p<0.05; Newmann-Keuls), similarly in D-JNKI1-treated rats which received saline i.p. (980 ±68.2; *p<0.05; Newmann-Keuls).

Figure 3.40 demonstrates that mean caspase 3 activity (nmol AFC/mg/min ±SEM; n=6 for all groups) was significantly increased in hippocampus of vehicle-treated rats which received LPS i.p. compared with vehicle-treated rats which received saline i.p. (23.6 ±2.28 and 13.5 ±2.54 respectively; *p<0.05; Newmann-Keuls). This LPS-induced increase in caspase 3 activity was attenuated by SCH58261 pretreatment so that the mean

value in hippocampus of SCH58261-treated rats which received LPS i.p. was similar to saline-treated controls, and significantly less than that in hippocampal cells prepared from vehicle-treated rats which received LPS i.p. (8.1 ±1.51; +++$p<0.001$; Newmann-Keuls), similarly in SCH58261-treated rats which received saline i.p. (13.7 ±2.44; ++$p<0.01$; Newmann-Keuls).

Figure 3.37 SCH58261 attenuated the inhibitory effects of LPS on LTP.
A) Tetanus-induced LTP was blocked in perforant path – granule cell synapses of vehicle-treated rats which received LPS i.p. (n=8) compared with vehicle-treated rats which received saline i.p. (n=6). This impairment was suppressed by pretreatment with SCH58261; rats pretreated with SCH58261 which received LPS i.p. (n=6) sustained LTP, as did SCH58261-treated rats which received saline i.p. (n=5). Data are expressed as the mean percentage change in population epsp slope (relative to the mean epsp slope in the 5min immediately prior to tetanic stimulation). Values are means and bars denoting ±SEM are included at 5min intervals.

A)

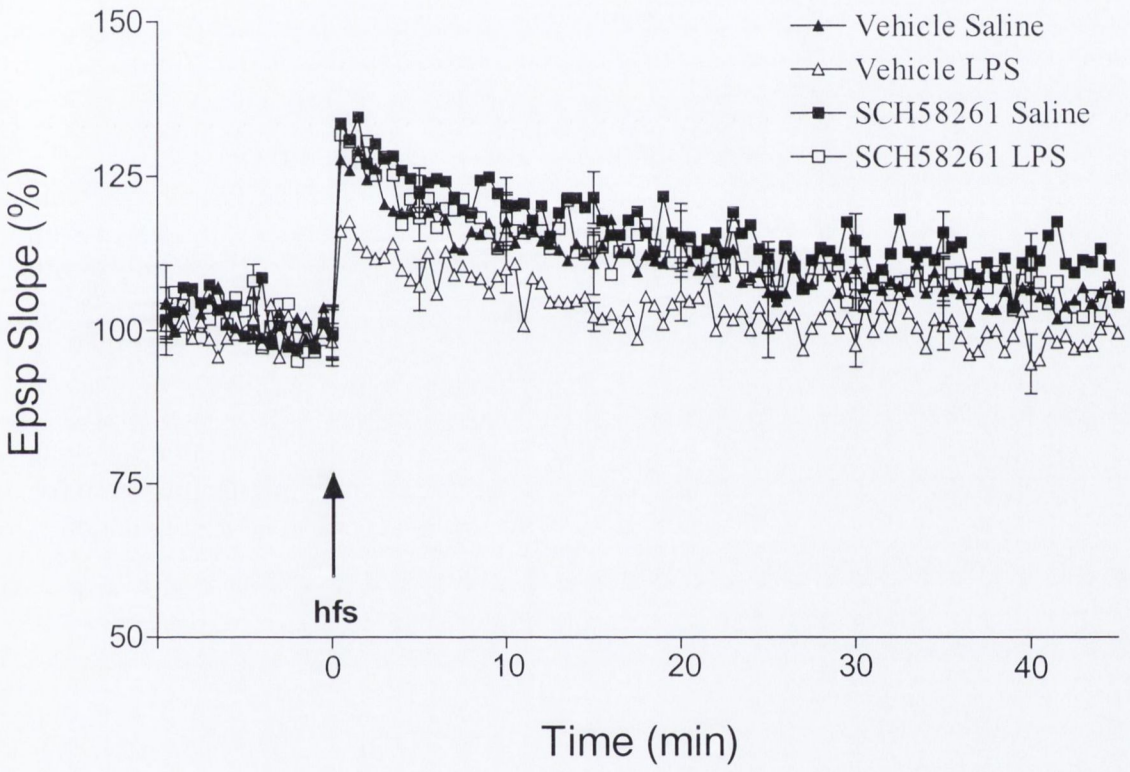

Vehicle Saline
Vehicle LPS
SCH58261 Saline
SCH58261 LPS
Epsp Slope (%)
150
125
100
75
50
hfs
0
10
20
30
40
Time (min)

Figure 3.37 SCH58261 attenuated the inhibitory effects of LPS on LTP.
B) Mean population epsp slope was assessed in the 2min and 40-45min following tetanic stimulation, and analysis revealed that there was a significant inhibitory effect of LPS on LTP at both time points (***$p<0.001$; Newmann-Keuls in each case). Pretreatment with SCH58261 abrogated the effect of LPS at both time points, so that the mean percentage epsp slope from rats pretreated with SCH58261 which received LPS i.p. was similar to vehicle-treated rats which received saline i.p., and significantly greater than that in vehicle-treated rats which received LPS i.p. (+++$p<0.001$; Newmann-Keuls). SCH58261-treated rats which received saline i.p. exhibited a significant increase in mean epsp slope compared with vehicle-treated rats which received saline i.p. at both 0-2min (*$p<0.05$; Newmann-Keuls) and 40-45min (***$p<0.001$; Newmann-Keuls) post tetanic stimulation.

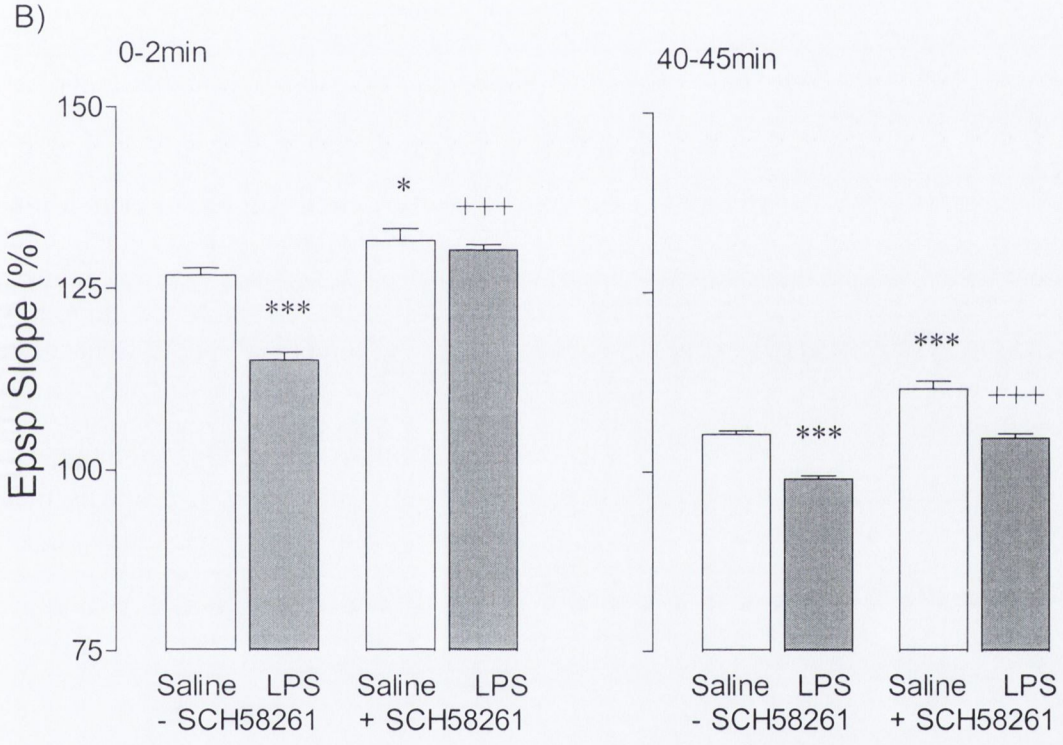

B)
0-2min
40-45min
Epsp Slope (%)
150
125
100
75
*
+++
***
***
***
+++
Saline
LPS
- SCH58261
Saline
LPS
+ SCH58261
Saline
LPS
- SCH58261
Saline
LPS
+ SCH58261

Figure 3.38 SCH58261 attenuated the LPS-induced increase in IL-1β concentration
Mean IL-1β concentration in was significantly increased in hippocampus of vehicle-treated rats which received LPS i.p. compared with vehicle-treated rats which received saline i.p. (*$p<0.05$; Newmann-Keuls). This LPS-induced increase in IL-1β concentration was attenuated by SCH58261 pretreatment so that the mean value in hippocampus of rats pretreated with SCH58261 which received LPS i.p. was similar to saline-treated controls, and significantly less than that in hippocampal cells prepared from vehicle-treated rats which received LPS i.p. (+$p<0.05$; Newmann-Keuls), similarly in SCH58261-treated rats which received saline i.p. (+$p<0.05$; Newmann-Keuls). Data are expressed as means ±SEM of 6 values in all groups.

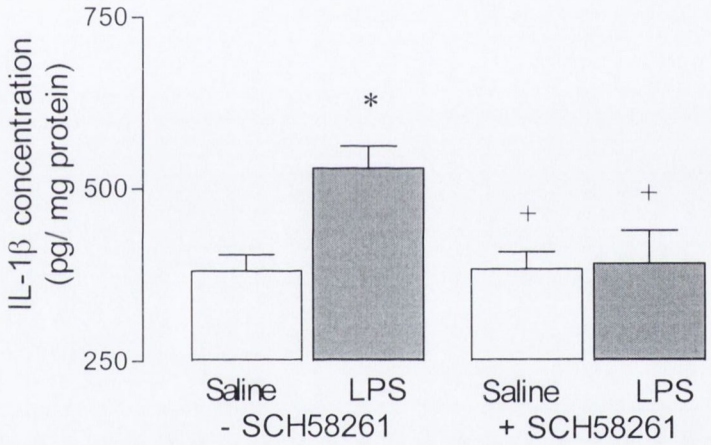

IL-1β concentration
(pg/ mg protein)
750
500
250
*
+
+
Saline
LPS
Saline
LPS
- SCH58261
+ SCH58261

Figure 3.39 SCH58261 attenuated the LPS-induced increase in JNK phosphorylation
Assessment of mean values obtained from densitometric analysis indicated that mean JNK activity was significantly increased in hippocampus of vehicle-treated rats which received LPS compared with vehicle-treated rats which received saline (*$p<0.05$; Newmann-Keuls). Pre-treatment with SCH58261 attenuated this increase so that mean JNK activity in hippocampus of SCH58261-treated rats which received saline or LPS was similar to that of vehicle-treated animals which received saline, and significantly less than that in hippocampal tissue prepared from vehicle-treated rats which received LPS i.p. (+$p<0.05$; Newmann-Keuls). Data are expressed as means ±SEM of 6 values in all groups.

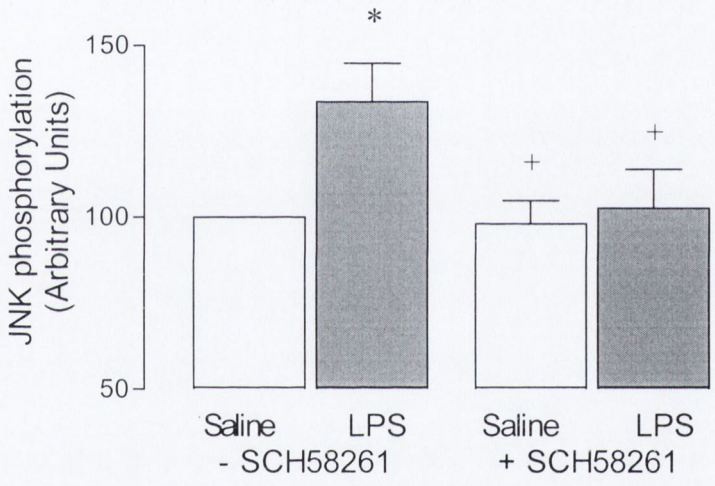
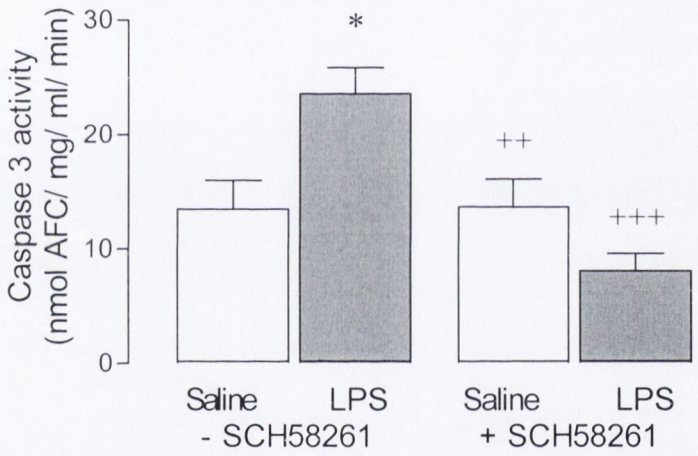

JNK phosphorylation
(Arbitrary Units)
150
100
50
*
+
+
Saline
LPS
- SCH58261
Saline
LPS
+ SCH58261

Figure 3.40 SCH58261 attenuated the LPS-induced increase in caspase 3 activity
Caspase 3 activity was significantly increased in hippocampus of vehicle-treated rats which received LPS i.p. compared with vehicle-treated rats which received saline i.p. (*$p<0.05$; Newmann-Keuls). This LPS-induced increase in caspase 3 activity was attenuated by SCH58261 pretreatment so that the mean value in hippocampus of rats pretreated with SCH58261 which received LPS i.p. was similar to saline-treated controls, and significantly less than that in hippocampal cells prepared from vehicle-treated rats which received LPS i.p. (+++$p<0.001$; Newmann-Keuls), so too in SCH58261-treated rats which received saline i.p. (++$p<0.01$; Newmann-Keuls). Data are expressed as means ±SEM of 6 values in all groups.


Caspase 3 activity
(nmol AFC/ mg/ ml/ min)
30
20
10
0
*
++
+++
Saline
LPS
- SCH58261
Saline
LPS
+ SCH58261

Chapter 4

Discussion

This study set out to investigate the effects of LPS administration in hippocampus, with the emphasis on an analysis of activation of JNK as a potential pivotal mediator in the deficits produced by LPS.

The data show that peripheral injection of LPS induced JNK activation in the rat hippocampus, and this effect was mirrored *in vitro* in cultured cortical and hippocampal neurons. This is supported by previous evidence that LPS causes JNK activation/phosphorylation in a variety of different cell types (Hambleton *et al.*, 1996; Pyo *et al.*, 1998; Loscher *et al.*, 2000), and in hippocampus following peripheral administration (Vereker *et al.*, 2000a). LPS has been shown to activate the mitogen-activated protein kinase (MAPK) pathways (Swantek *et al.*, 1997), specifically the upstream JNK MAPKK; MKK4 (Swantek *et al.*, 1997). Here the data show that, concomitant with the increase in JNK activation, peripheral injection of LPS induced caspase 3 activation in the rat hippocampus, an effect also mirrored *in vitro* in cultured hippocampal neurons. A LPS-induced increase in caspase 3 was previously documented in hippocampal cells (Lynch *et al.*, 2004). As caspase 3 is a mediator of cell deterioration, the current findings provide support that LPS can initiate apoptotic changes centrally. This is consistent with evidence that LPS can induce detrimental effects/ apoptosis in various cell types (Norimatsu *et al.*, 1995; Haendeler *et al.*, 1996), as well as centrally following peripheral administration (Vereker *et al.*, 2000a, Lynch *et al.*, 2004). Interestingly co-localisation of active JNK and active caspase 3 has been observed (Lynch and Lynch, 2001) and although activation of these factors did not always coincide, it may suggest a causal role of JNK in mediating the LPS-induced activation of caspase 3. Nevertheless, it is important to note that a recent finding indicates that caspase 3 activation in neurons is not always linked to cell death (Garnier *et al.*, 2004). The implication of these data is that JNK may have a role in LPS-induced detrimental effects

These data raise the question of how LPS induces effects in these circumstances. Much evidence suggests that LPS signalling is mediated through the LPS receptor TLR4 (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999) and mRNA for the LPS receptors CD14 and TLR4 have been found within the brain (Lacroix *et al.*, 1998; Laflamme and Rivest, 2001 respectively). Recent work identified microglia as the major LPS-responsive cell in the CNS, and showed that microglia also express TLR4 (Lehnardt *et al.*, 2003). Interestingly this study also demonstrated that neurons did not express TLR4 when assessed by PCR or a fluorescent LPS binding method, however one explanation for the *in vitro* data presented here is that neurons do indeed express TLR4. It also raises the issue of possible glial

contamination in the culture, but as the culture methods were evaluated using staining with a glial marker, this is unlikely. In any case, LPS signalling through TLR4 *in vivo* would depend on entry of LPS into the CNS which in normal circumstances would be restricted by the BBB. It has been shown that LPS administration results in increased cerebrovascular permeability, including disruption of the BBB as evidenced by cerebrospinal fluid leukocytosis (Boje, 1996), and that binding of LPS-induced cytokines to receptors on endothelial cells in brain vasculature, causes release of other mediators (endothelial cell adhesion molecules, chemokines, nitric oxide and prostaglandins), which leads to impairment of BBB integrity (Wong *et al.*, 1996; Anthony *et al.*, 1997). This increased permeability may therefore allow access of LPS, and/or activated macrophages and/or cytokines into the brain. Alternatively, LPS may mediate its effects through IL-1β signalling. IL-1β, an inflammatory cytokine, is rapidly increased in the brain in response to peripheral injection of LPS; IL-1β mRNA and protein is increased in the pituitary and brain of mice (Goujon *et al.*, 1996), and also in rat microglia (Van Dam *et al.*, 1992). Evidence that LPS exerts its effects through IL-1β exists in that IL-1ra, an IL-1β antagonist, attenuated the LPS-induced decrease in feeding (Swiergiel *et al.*, 1997), the impairment in learning and memory (Pugh *et al.*, 1998), as well as the depressive effect of peripheral LPS administration on social exploration and body weight (Bluthé *et al.*, 1992). The dependence of the LPS-induced response on IL-1β release could be true *in vitro*; although glial cells are thought to be the main CNS producer of inflammatory cytokines (Hanisch, 2002), cultured neurons have been shown to produce IL-1β in response to certain stimuli including β-amyloid (Minogue *et al.*, 2003). The data presented here demonstrated that neurons produced IL-1β in response to LPS. In support of a mediatory role for IL-1β in LPS signalling, IL-1β has been shown to activate similar pathways to LPS such as the SAPKs (Vereker *et al.*, 2000a and b). IL-1β may be produced by resident brain cells, or originate from the periphery via immune cells such as monocytes, macrophage and neutrophils (Rothwell, 1991) which may gain entry through the compromised BBB. Whichever way the effects are mediated it is apparent that LPS is capable of affecting changes in neuronal cells *in vivo* and *in vitro*. Since LPS administration increases JNK activation and concomitantly impairs LTP (Vereker *et al.*, 2000a; Nolan *et al.*, 2002), it was postulated that JNK may play a role in mediating these effects. This hypothesis is supported by the findings that JNK activation is coupled to deficits in LTP, for example in aged rats (Martin *et al.*, 2002), rats exposed to γ-irradiation (Lonergan *et al.*, 2002) and IL-1β-treated (Vereker *et al.*, 2000b) and β-amyloid-treated (Minogue *et al.*, 2003) rats. JNK

is linked to apoptosis in a variety of cell types (Chen *et al.*, 1996; Hu *et al.*, 1999; Wang *et al.*, 1998; Guo *et al.*, 1998; Zhu *et al.*, 2001; Eilers *et al.*, 2001) but definitive evidence for a pivotal role of JNK in cell death signalling is lacking, therefore this study was designed to address this issue.

A JNK inhibitor, D-JNKI1, made up of the minimal 20-amino acid inhibitory domains (JNK binding domain; JBD) of the JIP proteins and the 10-amino acid HIV-TAT sequence that rapidly translocates inside cells, was used in these studies. Of the two isomers available, D-JNKI1 demonstrated a 15-fold reduction in its ability to inhibit JNK/c-Jun interaction compared with L-JNKI, but it was still able to prevent c-Jun phosphorylation inside cells and protect against apoptosis, and has the advantage of being more resistant to neuronal proteases (Bonny *et al.*, 2001). JNK is activated when it is phosphorylated, however 200μM or 500μM i.c.v. injections of D-JNKI1 did not attenuate the LPS-induced increase in the phosphorylation of JNK itself but did decrease c-Jun phosphorylation, one of the main consequences of JNK activation. This can be explained due to the described nature of the inhibitor D-JNKI1, as it acts as a target-competitive inhibitor of JNK (Barr *et al.*, 2002). Targets of JNK have similar binding domains but the affinity of JNK for the binding domain of the JIP1 protein is much greater, therefore the inhibitor interferes with the binding of JNK to its substrates. Hence in the presence of D-JNKI1, JNK phosphorylation may still occur but prevents JNK from acting on its substrates. Interestingly D-JNKI1 alone appeared to cause an increase in JNK phosphorylation, although the reasons for this are unclear. Perhaps binding of JNK to D-JNKI1 somehow promotes interaction with upstream kinases thereby effectively marking JNK for phosphorylation. In fact the JIP protein, from which the JBD of the JNK inhibitor is derived, is a scaffold protein which facilitates the MAPK cascade by bringing the components into close proximity (Whitmarsh *et al.*, 1998). In any case, the effectiveness of D-JNKI1 in decreasing phosphorylation of c-Jun, a target unique to JNK (Gupta *et al.*, 1996), was confirmed, with a significant inhibitory effect at the 500μM dose. Therefore in accordance with other studies, the effect of D-JNKI1 was localised downstream of JNK phosphorylation, at the level of JNK interaction with its substrates (Hirt *et al.*, 2004; Borsello *et al.*, 2003).

LPS is a potent inducer of inflammatory mediators (Cohen, 2002), and can induce numerous changes centrally. Centrally-mediated changes such as decreases in feeding, motor activity, and social behaviour (Bluthé *et al.*, 1992; Dantzer *et al.*,1998; Lacosta *et al.*,1999), as well as central changes in biochemical signalling (Loscher *et al.*, 2000) have

been reported following intraperitoneal LPS treatment. This suggests that peripherally administered LPS induces a challenge in the brain, potentially impacting on synaptic efficacy. Previous studies in this laboratory showed that injection of LPS inhibits LTP in the dentate gyrus (Vereker *et al.*, 2000a; Kelly *et al.*, 2003), and, predictably, LPS is also reported to impair hippocampal-dependant spatial learning (Shaw *et al.*, 2001), diminish performance in a passive avoidance task and the elevated maze (Jain *et al.*, 2002), and disrupt memory consolidation in contextual fear conditioning (Pugh *et al.*, 1998). The hypothalamo-pituitary-adrenal axis (HPA) may play a role as its activation has been shown to occur in response to various types of stress, and usually results in the production of corticosteroids from the adrenal cortex. This is of consequence as the hippocampus, effective functioning of which is particularly vulnerable to stress, has the highest concentration of corticosterone receptors in the brain (see McEwen, 1994), and circulating levels of glucocorticoids correlate to an ability to sustain LTP; high concentrations inhibited LTP, while low levels enhanced LTP (Diamond *et al.*, 1994; Diamond *et al.*, 1996; Kim and Diamond, 2002; Pavlides *et al.*, 1993). As expected, HPA activation due to fear impairs memory (Diamond *et al.*, 1999), and administration of high concentrations of corticosterones disrupt spatial learning, (Luine, 1994). This raises the question of whether the manipulations involved in the rat undergoing anaesthesia and the subsequent procedure increase glucocorticoids and thereby influence LTP. In the current experiments stress levels for the rat should be at a minimum, however it has been demonstrated that even handling 15 minutes post tetanus caused an increase in glucocorticoids, resulting in an impaired LTP (Korz and Frey, 2003). However, earlier studies indicate that chronic or acute treatment with corticosterone was able to decrease LTP in the dentate gyrus below that of baselines recorded from rats under urethane anaesthesia (Pavlides et al., 1993), suggesting that the procedure itself does not excessively increase corticosterones.

The proinflammatory cytokine IL-1β is also expressed in the brain in response to stress and LPS, and has been reported to activate the HPA (Melik Parsadaniantz *et al.*, 1999), as well as result in secretion of corticotrophin releasing factor from hypothalamus (Sapolsky *et al.*, 1987). Indeed the effects of LPS may be mediated more directly through IL-1β signalling as evidenced by work with the antagonist IL-1ra (Swiergiel *et al.*, 1997; Pugh *et al.*, 1998; Bluthé *et al.*, 1992). Significantly, IL-1β is known to activate JNK and p38. There is evidence linking these protein kinases to cell deterioration, possibly accounting for the LPS-induced decrease in synaptic efficacy. Activation of p38 appears to play a significant role in LPS and IL-1β-induced signalling (Kelly *et al.*, 2003), however

until recently, the lack of a specific JNK inhibitor has made determination of the role of JNK difficult, and it has been implicated in both apoptotic processes and cell survival. It was hypothesised that, as peripherally administered LPS inhibits LTP and concomitantly increases JNK activation, JNK could be a mediating factor.

The LPS-induced inhibition of both the induction and the maintenance of LTP in dentate gyrus observed here, coupled with LPS-induced activation of JNK are in accordance with previous findings (Vereker *et al.*, 2000a; Nolan *et al.*, 2002). LPS has also been reported to block LTP in other brain regions such as the CA1 (Mizuno *et al.*, 2004). Recently, inhibition of p38 was shown to effectively reverse the LPS-induced impairment of LTP (Kelly *et al.*, 2003) and parallel data for JNK inhibition are presented here. Thus pretreatment with D-JNKI1 reduced the LPS-induced impairment of LTP, although it failed to effect the attenuation of the initial increase in epsp slope. Interestingly the JNK inhibitor itself caused a significant decrease in initial potentiation of the epsp slope, although not to the same degree as LPS implying that JNK could have a small but not critical role in LTP induction. This is not unreasonable as the high basal level of JNK activity in the CNS could point towards a physiological role rather than just a pathological one (Borsello and Bonny, 2004). However the difference observed could be due to the large individual variation recorded at this time point in control animals. Late LTP expression was not impaired in D-JNKI1-treated animals which received LPS compared with D-JNKI1-treated animals which received saline showing that JNK inhibition prevented LPS-induced impairment. Nevertheless the LTP sustained by the control saline treated group was significantly greater than that of either of the D-JNKI1-treated groups. This means that while JNK inhibition had a protective role with challenged animals, the same level of JNK inhibition also attenuated full LTP expression. Implications of this finding could be that basal levels of JNK may be required for normal LTP induction and expression. This could indeed be the case since activation of JNK can be linked with cell growth/ survival and could be necessary for the morphological changes that take place with LTP; similarly activation of p38 has been linked to the induction of BDNF triggered LTP (Ying *et al.*, 2002). It has been shown that treatment with a p38 inhibitor resulted in a greater change in epsp slope at induction and during expression than control animals (Kelly *et al.*, 2003). This might suggest that p38 is activated during LTP induction as an endogenous limiting step. However, given the evidence presented here JNK is unlikely to play a similar role. The fact that D-JNKI1 has the ability to abrogate the LPS-induced

impairment of LTP points to a central role for JNK signalling in mediating LPS-associated detrimental changes.

The effects of LPS could be mediated through TLR4 signalling or IL-1β mediated signalling. Significantly the timecourse of the study, in which assessment of ability to sustain LTP took place 3h post LPS injection, has been shown to be sufficient for IL-1β induction centrally (Vereker *et al.*, 2000a) while Singh and Jiang (2004) detected IL-1β mRNA centrally after 0.5h with a peak 4h after injection. Consistently, IL-1β treatment has been shown to result in impaired LTP, while use of an IL-1β inhibitor (ICE inhibitor) blocked the effects of peripheral LPS administration within this time frame (Vereker *et al.*, 2000a). The present *in vivo* studies indicate that IL-1β was slightly, though not significantly, increased in response to LPS within this timeframe. Pretreatment with D-JNKI1 did not induce a change in basal levels of IL-1β and was not able to prevent the modest LPS-induced increase in IL-1β. The trend observed in this study is strengthened by previous work that showed IL-1β inhibition prevented JNK phosphorylation in response to LPS (Vereker *et al.*, 2000a), implying that IL-1β induction is upstream of JNK activation. In the present study the effect of D-JNKI1 on the LPS-induced expression of IL-1β was also assessed *in vitro*. Cortical neurons treated for 18h with LPS showed a substantial significant increase in IL-1β production, and pre-treatment with D-JNKI1 was unable to prevent this. While glia are thought to be the main producers of cytokines centrally, this present finding is in line with previous data showing neurons producing IL-1β in response to β-amyloid (Minogue *et al.*, 2003).

The inability of D-JNKI1 to affect LPS-provoked IL-1β induction suggests that IL-1β gene transcription is not dependent on JNK activation and related signalling. This further supports IL-1β being upstream of JNK activation. It may also suggest that the effects of LPS are mediated through pathways other than the JNK pathway and that compensatory/concurrent mechanisms also induce IL-1β. This is likely as LPS induces many signalling molecules, such as TNFα, which may impact upon IL-1β expression, for example, via NF-κB signalling. Obviously a direct comparison between *in vivo* and *in vitro* work must be approached with reservation, not least because of the variation in conditions; animals received a 100μg/kg dose of LPS and tissue was assessed 3h later, while primary cultured neurons were treated with a 100ng/ml dose of LPS in media for 18h. The environment in brain in situ would comprise of neurons, glia, and astrocytes as well as any infiltrating peripheral immune cells and circulating cells, while the *in vitro* environment is much more regulated consisting of purely neuronal cells. Additionally, the concentration of

LPS resulting in the CNS as a result of peripheral administration has not been assessed and may not be comparable to the *in vitro* situation. One significant difference between the *in vivo* and *in vitro* studies relates to timing of events. In the current studies *in vivo*, LPS-induced IL-1β expression was shown to occur centrally after 3h, and *in vitro* work presented here indicates that IL-1β induction does not occur in neurons until an 18h timepoint. Interestingly, both the *in vivo* and *in vitro* work presented here show associated detrimental changes at these respective timepoints. Tissue homogenate from *in vivo* studies includes both neurons and glia, and it may be the case that glia are the principal producers of IL-1β *in vivo* and may be able to do so in a more rapid manner than cultured neurons, possibly explaining the disparity in time required for IL-1β induction. Data in the present studies show an upregulation in IL-1β expression by cultured mixed glial cells following treatment with LPS for 24h, while cultured microglia have been shown to express IL-1β at the mRNA and protein level after LPS treatment (Mizuno *et al.*, 2004), although this was after a 48h incubation. However a mouse macrophage cell line (RAW 264.7) did show an upregulation of IL-1β after only a 4h incubation with LPS (Blonska *et al.*, 2003). However no timecourse study of glial production of IL-1β in response to LPS has been carried out to date.

One objective of this study was to determine the cellular changes which accompany the LPS-induced impairment of LTP. While increases in reactive oxygen species (ROS) concentration have been implicated in LPS-mediated changes, and may indeed play an important role, this pathway was not investigated further in these studies. Consistent with previous findings it was shown that intraperitoneal (i.p.) LPS administration significantly increased JNK phosphorylation in hippocampal tissue. This effect was paralleled *in vitro* as JNK phosphorylation in whole cell lysate prepared from cultured cortical neurons was increased in response to treatment with LPS. Interestingly, there was a significant increase after 3h incubation with a further increase at the 18h timepoint, possibly representing a biphasic response. Since only LPS exposure for 18h was linked to detrimental changes such as an increase in IL-1β, increased bcl-2 phosphorylation, and increased cytochrome c in the cytosol, one could postulate that the initial increase in JNK phosphorylation may not be detrimental, but the prolonged activation results in initiation of cellular damage. It is possible that the factors causing phosphorylation may change over time and that these shifts in stimulation cause differential actions. Perhaps the later increase in JNK phosphorylation represents JNK activation by IL-1β, which may have the ability to cause cellular degeneration, but that the earlier JNK activation by other means may not.

Alternatively, perhaps there is a threshold for JNK activation above which detrimental changes occur. Of note from this *in vitro* work is the fact that JNK activation appears to occur prior to detectable increases in IL-1β. This could imply that JNK activation leads to induction of IL-1β, not the other way around. However JNK inhibition did not downregulate the LPS-induced IL-1β *in vivo* or *in vitro*, suggesting that other LPS-activated pathways may be more critical for IL-1β production. In agreement with previous data *in vivo*, D-JNKI1 did not cause an inhibition of the phosphorylation of JNK caused by LPS.

JNK activation has manifold downstream effects, including phosphorylation of the inducible transcription factor c-jun, which has been linked with both neurodegeneration and neuroprotection (Herdegen *et al.*, 1997; Ip and Davis, 1998). Recent evidence points toward c-Jun as a mediator of JNK-induced degeneration and apoptosis, for example, in degenerating hippocampal neurons following application of kainic acid (Yang *et al.*, 1997b). Since c-Jun is believed to be a target unique to JNK (Gupta *et al.*, 1996), its phosphorylation is often used as a marker of JNK activity. Consistently then, the increase in JNK activity *in vivo* reported in the present studies, was associated with an increase in c-jun phosphorylation in hippocampal tissue prepared from control-treated rats which received LPS compared with control-treated rats which received saline. This is in accordance with previous literature (Arndt *et al.*, 2004, Nolan *et al.*, 2002). The data presented indicate tissue prepared from D-JNKI1-treated animals which received LPS did not show this increase. Therefore, in parallel with the LTP findings, D-JNKI1 attenuated other effects of LPS; D-JNKI1 treated rats which received LPS did show a modest though statistically insignificant increase in c-Jun phosphorylation. This confirms an attenuation of c-Jun phosphorylation by JNK with use of the JNK inhibitor D-JNKI1. This is of consequence because JNK activation and resulting c-Jun phosphorylation are correlated to an inability to sustain LTP (Lonergan *et al.*, 2004).

c-Jun has also been implicated in activation of other aspects of the AP-1 pathway. AP-1 is a transcriptional activator and is formed from dimers of a number of proteins belonging to the Jun, Fos, Maf and ATF families. JNKs can activate c-Jun, the most potent transcription factor of its group, but have also been implicated in phosphorylation of ATF2 (Gupta *et al.*, 1995). The AP-1 proteins have long been linked to cell death, but the complex interaction of these proteins and varied transcriptional responses depending on cell type, or developmental stage, makes their roles hard to define (Shaulian and Karin, 2002). However they are implicated in regulation of gene transcription in response to LPS

(Guha and Mackman, 2001), which includes the induction of IL-1β and other proinflammatory cytokines, chemokines, and other transcription factors. This means that c-Jun activity may be linked to IL-1β induction, although this appears unlikely to play a major role as D-JNKI1 was unable to attenuate the LPS-induced increase in IL-1β either *in vivo* or *in vitro*. Interestingly, inhibition of p38, the other SAPK, resulted in a reduced induction of IL-1β in response to LPS (Lee *et al.*, 1994), so may play a more upstream role than JNK in mediation of LPS-induced effects.

A second reported substrate for JNK is the mitochondrial associated protein bcl-2. The bcl-2 family of proteins appear to modulate the permeability of the mitochondrial membrane, thereby controlling a key aspect in the regulation of apoptosis. Mediators of cell death reside within mitochondria and can be released through disruptions in the outer mitochondrial membrane, and trigger activation of caspase cascades. The bcl-2 family can be divided into repressors (e.g. bcl-2, bcl-X_L,) and promoters (e.g. bax, bcl-X_S, bik) of apoptosis, but the mechanisms by which the bcl-2 family act remain controversial (Harris and Thompson, 2000). It has been shown that activated JNK can cause phosphorylation of bcl-2 (Maundrell *et al.*, 1997) and that this antagonises the anti-apoptotic action of bcl-2 (Park *et al.*, 1997; Halder *et al.*, 1995; Yamamoto *et al.*, 1999). In the current study, an increase in bcl-2 phosphorylation in a membrane enriched fraction of hippocampal tissue was associated with LPS injection for the first time. This appeared to be mediated by JNK as tissue prepared from animals pretreated with D-JNKI1 did not show this increase when challenged with LPS. However it should be noted that while previous studies and the current study correlate bcl-2 phosphorylation with apoptosis, others have correlated bcl-2 phosphorylation with its antiapoptotic function (Deng *et al.*, 2001; Ito *et al.*, 1997; Horiuchi *et al.*, 1997). It is possible that phosphorylation of different residues on bcl-2 mediated by distinct factors could account for this disparity or it may be that the ratio of active pro and anti-apoptotic family members is the key issue.

There was a significant positive correlation between c-Jun phosphorylation and bcl-2 phosphorylation indicating that JNK may act on these two proteins with a similar affinity in a similar way. Alternatively, it may implicate c-Jun as a mediator of JNKs effects on bcl-2, however no evidence as yet exists to link these two proteins. Another bcl-2 family member which has been shown to be upregulated during LPS-induced degenerative change is bax (Munshi *et al.*, 2002), however the present data indicate no change in bax expression following LPS treatment. It may be the case that inactivation of bcl-2 is sufficient to cause apoptotic effects by a derepression of bax activity, without an overall change in bax

expression. A modest LPS-induced increase in bcl-2 phosphorylation was also observed *in vitro* after an 18hr incubation and the attenuating effect of D-JNKI1 on bcl-2 phosphorylation *in vitro* was similar to that recorded *in vivo*. There are suggestions that bim, a BH-3 only member of the bcl-2 family, is also an important participant in JNK-dependent apoptosis in neurons; following NGF withdrawal, bim contributes to bax-dependent cytochrome c release and apoptosis of neurons, while under these conditions dominant negative c-Jun decreases bim expression and promotes neuronal survival (Whitfield *et al.*, 2001).

If the antiapoptotic properties of bcl-2 are compromised by its phosphorylation, we would expect a tendency for cells to exhibit signs of apoptosis or mitochondrial dysfunction following LPS injection. Hence, ATP concentration was assessed in hippocampus, on the premise that ATP production is dependent on effective mitochondrial functioning. No change was observed following treatment with LPS or D-JNKI1. While some studies link a decrease in ATP levels to apoptosis (Bachelet *et al.*, 2002), it was previously shown that ATP levels did not always change during apoptosis (Davis and Johnson, 1999), although $[^{32}P]P_i$ incorporation into ATP did decrease, indicating a possible decrease in synthesis. Interestingly it has been shown that apoptotic cell death as opposed to necrotic cell death is dependent on a sufficient level of ATP (Leist *et al.*, 1997; Nicotera, 2002). Therefore it may not be unexpected that no change in ATP concentration was observed in these studies.

The effect of impaired functioning of bcl-2 is most likely manifested by an increase in permeability of the outer mitochondrial membrane. With increased permeability of the outer mitochondrial membrane, substances that normally reside in the intermembrane space can translocate into the cytosol, such as cytochrome *c*. Once in the cytosol, cytochrome *c* associates with ATP, Apaf-1 and the precursor of caspase 9, causing production of the active form of caspase 9, a mediator caspase, initiating the caspase cascade, which ultimately leads to apoptosis. The present data show an increase in expression of cytochrome *c* in the cytosolic fraction of hippocampal tissue following LPS injection. This effect was mirrored *in vitro* with a significant increase in cytochrome c in a cytosolic preparation from cortical cultured neurons after 18h incubation with LPS. These data concur with previous reports (Vereker *et al.*, 2000a). However, D-JNKI1 did not prevent the LPS-induced increase in cytochrome c, but only fractionally reduced the LPS-induced increase in cytochrome *c*.

Cytochrome c release from the mitochondria usually triggers the caspase cascade resulting in activation of caspase 3. Caspase 3 is an effector caspase, activation of which generally results in an irreversible commitment to cell death. Caspases degrade cytoskeletal proteins, nuclear proteins, including DNA repair enzymes, as well as activating other caspases, p21-activated kinase, and members of the bcl-2 family (see Blatt and Glick, 2001). The resulting increased DNA fragmentation and decreased cell integrity eventually lead to the appearance of the characteristic signs of apoptosis. In agreement with previous findings (Nolan *et al.*, 2003; Alikhani *et al.*, 2003; Cai *et al.*, 2003), LPS increased caspase 3 activity. Positive staining for the active form of caspase 3 was increased in the hippocampal region of cryostat sections prepared from brains of LPS-treated compared with saline-treated rats. The hippocampal cells from the LPS-treated brains also displayed morphology consistent with apoptotic cell death, exhibiting condensed nuclei and a more fragmented appearance. Pretreatment with D-JNKI1 abolished this LPS-induced increase in caspase 3 positive staining, indicating a protective effect. Congruent with this, is the effect of D-JNKI1 on LPS-induced TUNEL staining in hippocampal cells. Decreased DNA repair which occurs downstream of caspase activation, results in an increase in fragmented DNA as the constitutive break down of DNA with normal functioning is not rectified. The percentage of TUNEL positive stained cells was significantly increased in LPS-treated compared with saline-treated rats, while animals pretreated with D-JNKI1 did not show this increase when challenged with LPS, effectively returning the mean percentage value of LPS-treated animals to a value similar to that in control-treated animals, indicating that D-JNKI1 pretreatment effectively rescued cells from apoptotic death. Therefore one could postulate that LPS-induced apoptotic cell death is dependent on JNK mediated signalling and gene transcription. Significantly, the percentage of TUNEL positive stained cells showed an inverse correlation to the ability to sustain LTP, implying that these detrimental changes may be the cause of the observed LPS-induced deficits.

So it appears from this that cytochrome c release does not determine if cell degeneration occurs. Perhaps some other factor dependent on JNK activation is required for induction of cell death by this route. Alternatively, another factor that can be released from the mitochondria may be more critical in leading to degeneration, and perhaps inhibition of JNK can influence the release or action of these. For example SMAC/Diablo release has been shown to be dependent on JNK activation (Chauhan *et al.*, 2003). Could the level of JNK activity influence a differential permeability of the outer mitochondrial

membrane, allowing release of some substances but not others? It is likely that LPS administration can impact upon many factors which influence mitochondrial membrane integrity; while LPS-induced bcl-2 phosphorylation was attenuated by D-JNKI1 treatment, other members of the bcl-2 family may be similarly modulated by proteins not affected by the inhibitor, leading to increased permeability of the mitochondrial membrane via an alternate route. Therefore inhibition of JNK may not be sufficient to prevent release of cytochrome c from mitochondria. Nevertheless, D-JNKI1 did appear to be sufficient to prevent apoptotic cell death as presented here by assessment of two cell death markers.

The D-JNKI1-induced abrogation of c-Jun phosphorylation is of great significance, particularly in relation to cell death signalling (Ham *et al.*, 2000; Eilers *et al.*, 2001; Herdegen *et al.*, 1998). The transcription factor c-Jun, when phosphorylated, translocates to the nucleus where it modulates gene transcription activity. This includes induction of Fas L (Morishima *et al.*, 2001; Le-Niculescu *et al.*, 1999), which can initiate the mitochondrial independent cell death pathway (Herdegen *et al.*, 1998). The involvement of the mitochondrial cell death pathway in JNK-mediated LPS-induced cell death was not confirmed in this study. While D-JNKI1 did decrease LPS-induced bcl-2 phosphorylation, it did not affect a change on cytochrome c expression in the cytosol or hippocampal ATP concentration, thus other possibilities were considered. Fas L which has previously been shown to be upregulated in response to c-Jun phosphorylation (Herdegen *et al.*, 1998), and has been liked to non-mitochondrial cell death signalling (Zimmermann *et al.*, 2001), in fact showed no significant change in response to LPS treatment or D-JNKI1. Hence the exact mechanism of JNK-mediated changes remains unclear. It may be of interest to assess the impact of JNK inhibition on the activation of the other AP-1 proteins (c-Fos and ATF-2), and the transcription factor Elk1, as well as looking at the impact on gene expression controlled by these factors.

Although JNK inhibition did not appear to impact upon LPS-induced IL-1β production, it is possible that it mediates a beneficial outcome via more generalised effects on inflammatory responses. In support of this hypothesis it has been shown that T-cells from mice deficient in JNK1 were hypoproliferative and biased toward Th2 cell production (Constant *et al.*, 2000) which is associated with an anti-inflammatory cytokine profile. Many factors influence the differentiation of naïve T-cells to Th1 or Th2, including the type of APC, the concentration of the antigen (duration and strength of signal), the ligation of select costimulatory molecules, and the local cytokine environment (Szabo *et al.*, 2003). The molecular mechanism by which these factors control T-cell differentiation is unclear

but, as JNK can modulate the activity of transcription factors responsible for cytokine gene production (e.g. c-Jun and AP-1), it is possible that these factors may influence JNK activity and hence impact on T-cell differentiation.

Using the same model of inflammation, the protective potential of atorvastatin treatment was investigated. Atorvastatin is one of a family of synthetic lipid/ cholesterol lowering agents which are structurally similar to hydroxyl-3-methylglutaryl Coenzyme A (HMG-CoA), a precursor of cholesterol. Statins act as competitive inhibitors of HMG-CoA reductase which converts HMG-CoA to mevalonate, thereby inhibiting the rate limiting step of cholesterol synthesis. Epidemiological evidence, suggests that statins may also exert a broader neuroprotective effect, reducing incidence of dementia (Wolozin *et al.*, 2000) and AD (Jick *et al.*, 2000). There are a number of ways statins are proposed to exert their protective effects, including a reduction of isoprenoid synthesis (Zacco *et al.*, 2003; Youssef *et al.*, 2002; Ortego *et al.*, 1999), a decrease in NF-κB-associated signalling (Ortego *et al.*, 1999), an increase in production of eNOS and a decrease in iNOS production (Stanislaus *et al.*, 1999). In addition statins have reported antioxidant activity *in vivo* (Shovman *et al.*, 2001; Wassmann *et al.*, 2002) and *in vitro* (Franzoni *et al.*, 2003). It appears that most aspects of leukocyte function can be disrupted by statin treatment; migration/ chemotaxis, surface protein expression, adhesiveness, proliferation, and differentiation (Kwak *et al.*, 2000; Romano *et al.*, 2000; Diomede *et al.*, 2001). Taken together these effects suggest that statins may be able to exert an anti-inflammatory effect. Given that JNK appeared to play an important role in mediating the inflammatory changes associated with LPS administration, this study was designed to assess if atorvastatin could (1) preserve synaptic efficacy following LPS administration, and (2) modulate the JNK pathway.

The LPS-induced inhibition of both the induction and the maintenance of LTP in dentate gyrus reported in the literature and observed in the earlier study with D-JNKI1, is consistent; LPS administration significantly decreased LTP in dentate gyrus in this study. Pretreatment of rats for 3 weeks with atorvastatin modestly attenuated the LPS-induced impairment of LTP. While ATV failed to affect the immediate depression in epsp slope induced by LPS, it significantly reduced its effect at the later timepoint. However LTP was still diminished when compared to control values. This protective effect of ATV is consistent with evidence of positive effects of ATV and other statins on outcome of the multiple sclerosis model experimental allergic encephalomyelitis (Stanislaus *et al.*, 1999;

Youssef *et al.*, 2002; Aktas *et al.*, 2003). Youssef and colleagues assessed secretion of Th1 cytokines (IL-2, IL-12, IFNγ, TNFα) and Th2 cytokines (IL-4, IL-5, IL-10, TGFβ) in splenocytes isolated from statin-treated and control mice and showed an ATV-induced Th2 bias, where Th1 response was decreased, and Th2 response was stimulated. Similar findings were observed by Atkas and colleagues (2003). In terms of mechanism, it was reported that ATV induced activation of the Th2-inducing STAT6 transcription factor and inhibited the Th1-inducing STAT4 (Youssef *et al.*, 2002).

Protective effects of statin treatment have also been reported *in vitro*. It has been shown in rat primary astrocytes that lovastatin inhibited LPS and cytokine mediated production of iNOS, and NF-κB activation. Similarly lovastatin inhibited LPS-induced expression of IL-1β, TNFα, IL-6 in rat primary astrocytes, microglia and macrophages (Pahan *et al.*, 1997), while ATV treatment reduced NF-κB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells (Ortego *et al.*, 1999). These studies indicate that statins act to induce an anti-inflammatory response, inhibiting inflammatory changes. This could be the case in the present studies. Significantly it has been shown that a number of statins protected cultured neurons from excitotoxic death induced by NMDA (Zacco *et al.*, 2003).

ATV treatment alone exerted a significant negative effect on LTP, suggesting an overall detrimental outcome of HMG-CoA reductase inhibition. This might not be entirely unexpected when evidence from other *in vitro* studies is taken into consideration. For example, primary cortical neurons underwent apoptosis in response to statin treatment in a time and dose dependent manner. This was identified to be a consequence of downregulation of certain isoprenoids resulting in induction of p53 and bax, leading to cell death (Tanaka *et al.*, 2000). Pravastatin, simvastatin, and lovastatin all caused cell death at 100μM concentration but it is noteworthy that pravastatin failed to cause cell death at lower doses. Another study demonstrated that while statin (compactin) treatment of primary cultured neurons induced neuronal death in dose dependent manner, this was shown to be dependent on cholesterol depletion not isoprenoid depletion (Michikawa and Yanagisawa, 1999). However it should be noted that the doses used by Zacco and colleagues which protected against excitotoxic cell death was at least 300-fold less than that used in the latter study. Additionally, statin treatment potentiated apoptotic cell death via caspase 3 activation in a murine hippocampal cell line, but was not sufficient to induce apoptosis on its own (Föcking *et al.*, 2004). Therefore evidence of statin-induced cell death does exist, but consistency in underlying mechanisms accounting for this is lacking and

consequences of statin treatment may critically depend on the dose used. However it does appear that statin treatment may be detrimental in some cases *in vivo*, as evidenced by reported statin-associated memory loss (Wagstaff *et al.*, 2003), and cognitive decline (Wardle *et al.*, 2000). Statins may exert a detrimental effect by the simple decrease in cholesterol synthesis, impacting on membrane fluidity and normal cell functioning.

Consistent with their positive effect *in vitro*, one particular statin, simvastatin, protected against behavioural and morphological consequences of brain ischemia in neonate rats (Balduini *et al.*, 2001), while pravastatin was reported to reduce delayed neuronal death following transient forebrain ischemia in adult rat hippocampus (Daimon *et al.*, 2004). Furthermore ATV provided beneficial effects on neurological functional deficits, neuronal survival and synaptogenesis in experimental traumatic brain injury (Lu *et al.*, 2004). Therefore the data presented in this study supports the growing evidence that ATV is a neuroprotectant. Reported effects of statins on cognitive function are varied; some reports indicate a link between statin treatment and memory loss, while some report small beneficial effects, and many report no change in cognitive ability (reviewed by Wagstaff *et al.*, 2003).

The highest recommended clinical dose of atorvastatin for the treatment of hypercholesterolemia is 80mg/day, i.e. approx 1.1mg/kg. Pharmacokinetic data in rodents indicate that larger doses (up to 10mg/kg/day) are required to achieve similar circulating concentrations. In the present study a dose of 5mg/kg was administered, but the bioavailability of ATV from administration by the method used here has yet to be assessed. The lipid profile of tissue from these animals is currently undergoing analysis by collaborators, and the outcome of this will give a better indication of the effectiveness of statin treatment. It may be the case that lower doses of statins, while not being effective cholesterol reducing agents, may exert protective antiinflammatory effects in a safer manner.

As JNK activation and associated signalling appeared to be central to some of the inflammatory effects mediated by LPS, JNK phosphorylation was assessed in this study. JNK phosphorylation was significantly upregulated by LPS and this was attenuated but not completely reversed by ATV treatment. This profile of JNK activation was predictably mirrored by changes in c-Jun phosphorylation. Therefore LTP impairment was associated with JNK and c-Jun activation while ATV has some ability to down regulate the JNK pathway, possibly underlying the modest protective influence on LTP. How atorvastatin mediates it effects is still undetermined, however, the conversion of HMG CoA to

mevalonate is only one of the first steps in cholesterol synthesis, and downstream products of the mevalonate pathway are biologically active, therefore depletion of these intermediary compounds may play a role. Notably, cotreatment with exogenous mevalonate appears to reverse the anti-inflammatory or protective actions of statin treatment (Zacco *et al.*, 2003; Youssef *et al.*, 2002). Hence isoprenoids, produced downstream of mevalonate, have been implicated as they are involved in post translational modification (isoprenylation) of numerous proteins central to normal cell function (Cucchiara and Kasner, 2001). One protein which requires isoprenylation for effective functioning is the small GTPase Rho. Rho is important in cell growth, mitogenic pathways and various signalling cascades. Indeed this pathway may represent a direct link between statin treatment and diminished JNK signalling; lovastatin was shown to inhibit UV-C-induced caspase 3 activation and associated cell death, and this was attributed to a decrease in JNK activation. It was shown that this was because of inhibition of the Rac-MKK4 pathway, probably due to attenuation of isoprenylation of Rho GTPases (Bardeleben *et al.*, 2003). Cerivastatin was shown to inhibit β-adrenergic receptor-stimulated apoptosis as well as inhibiting JNK activation and the associated cytochrome c release and caspase 3 activation in rat ventricular myocytes; this effect was also attributed to inhibition of a GTPase, Rac1 (Ito *et al.*, 2004). Furthermore, lovastatin inhibited JNK1 and NF-κB activation in Chinese hamster ovary cells in response to UV irradiation, and this was shown to be due to inactivation of Rho (Gnad *et al.*, 2000). PI3K and Akt are mediators of a cell survival signalling pathway, linked to the activation of ERK. Simvastatin was shown to inhibit thrombin-induced Rho A activation and resulting Akt inactivation in human endothelial cells (Eto *et al.*, 2002), while ATVs protective effect on myocardium was dependent on activation of PI3K and Akt (Bell and Yellon, 2003). Therefore it was postulated that ATV may exert an effect through upregulation of ERK activity as well as down regulation of JNK activity, however, no significant change in ERK phosphorylation in response to LPS or atorvastatin was shown in the present study.

In order to determine if ATV could be inducing protective effects through modulation of activity of glial cells, *in vitro* studies using primary cultured cortical glia were performed. LPS increased IL-1β secretion by glia while ATV pretreatment attenuated but did not abolish this effect. Therefore, unlike D-JNKI1, ATV may exert its effect upstream of IL-1β. As it has been previously shown that ATV may influence the cytokine profile produced in response to inflammatory stimuli *in vivo* (Youssef *et al.*, 2002, Aktas *et al.*, 2003), two antiinflammatory cytokines IL-10 and IL-4 were examined

in supernatant collected from cultured glial cells exposed to ATV and LPS. Interestingly, IL-10 showed a similar release profile as IL-1β, while IL-4 was not detectable. It is possible that the LPS-induced increase in IL-10 represents initiation of a protective response, however the fact that statin treatment reduced this LPS-induced increase is inconsistent with this idea. This is in contrast to the finding that increased hippocampal concentration of IL-10 *in vivo* was associated with protection against the damaging effects of IL-1β (Lynch *et al.*, 2004). In addition, IL-10 release has previously been shown not to be effected by LPS administration *in vivo* (Nolan *et al.*, 2004), however some *in vitro* studies show an LPS-induced increase in IL-10 expression in human monocytic cells (Gee *et al.*, 2002). Previous work in this laboratory has revealed a consistent finding that LPS does not effect IL-10 *in vivo* but does *in vitro* (Moore, *pers. comm.*). It is likely that the *in vitro* studies on a particular cell type would not correlate well to the *in vivo* situation as discussed above. Moreover, as ATV reputedly does not cross the BBB *in vivo* (Sparks *et al.*, 2002), the direct application to glia *in vitro* may not be comparable. However the reasons for this pattern of IL-10 induction are still unclear.

CD40 is a 45-50 kDa transmembrane protein that is a member of the TNF/ NGF receptor family. A wide range of antigen presenting cells express CD40 including dendritic cells, B-cells, monocytes and macrophages, while its ligand CD40L is found on the surface of T-lymphocytes. The CD40/CD40L interaction plays a role in activation, differentiation, and proliferation of immune cells, and regulation of apoptosis, and it has been implicated in a range of immune diseases such as systemic lupus erthematosus, rheumatoid arthritis, and atherosclerosis (see Tan *et al.*, 2002a). On binding of CD40L, the cytoplasmic tail of CD40 is thought to interact with various TRAFs to initiate signalling cascades, which, depending on cell type and stage of development, can result in activation of the JNK pathway or transcription factors like NF-κB. Microglia constitutively express low levels of CD40 (Tan *et al.*, 1999), but when challenged with IFNγ, expression is increased (Tan *et al.*, 1999). Additionally LPS has been shown to induce increased CD40 expression in human fetal astrocytes (Abdel-Haq *et al.*, 1999) and in primary cultured microglia (Kim *et al.*, 2002). Moreover, recent evidence demonstrated that astrocytes exhibit increased levels of CD40L *in vivo* in response to brain injury or in AD (Calingasan *et al.*, 2002), while neurons were demonstrated to express functional CD40 *in vitro* and *in vivo*, which could be increased following treatment with neurotoxic stressors (Tan *et al.*, 2002b). Therefore CD40 has been implicated in various aspects of the immune response and following noxious stimuli. In the present study it is demonstrated that primary cultured glial cells

express CD40, and that this expression is increased following treatment with LPS. Furthermore, pre-treatment with ATV prevented the LPS-induced increase in CD40 expression, however the mechanism by which this occurs remains to be elucidated. In line with these data, a previous study established that ATV suppressed IFNγ-induced expression of CD40 in microglia (Youssef *et al.*, 2002). This may represent another way in which statin treatment could have an impact on JNK signalling.

The protective effect of ATV treatment in aged rats which is a more chronic model of inflammation was also assessed here. An age related decline in ability to sustain LTP was observed, in accordance with previous studies in this laboratory (Lynch and Lynch, 2001; O'Donnell *et al.*, 2000) and by others (Bach *et al.*, 1999; Diana *et al.*, 1995). Treatment with ATV induced a beneficial effect in the early and later response to tetanic stimulation in aged animals such that ATV-treated aged rats showed a significant increase in mean percentage epsp when compared with control-treated young or aged rats at both timepoints. The effect of ATV treatment on LTP in young animals was more complicated. At 0-2min post tetanic stimulation, ATV appeared to have an enhancing effect on mean percentage epsp slope when compared to that of control-treated young animals, but this effect was not sustained for the duration of the experiment; ATV-treated young rats exhibited an impaired LTP at 40-45min post tetanic stimulation compared to control-treated young rats. This initial beneficial effect is inconsistent when looked in conjunction with the observed effects on LTP in the ATV-treated young animals in the LPS study which received saline, although the end result of impaired maintenance of LTP is similar in both studies. Hence there is a disparity between the effects of ATV on young and aged animals. Perhaps an altered cholesterol metabolism associated with age means that statin treatment can be beneficial, while inhibition of cholesterol synthesis in the younger animals is detrimental. Exploration of this differential effect was outside the scope of these studies, but highlights that caution should be exercised when treatment of young individuals with statins is indicated.

The differences in response to ATV treatment between the two studies may be due to a number of factors. For example the longer treatment of 8weeks in the age study may be expected to exert a more significant effect than the 3week treatment in the LPS study. In addition, acute LPS treatment represents a very different challenge to the CNS compared with the more chronic challenge of age. Interestingly, the epidemiological evidence supporting a neuroprotective role for statins are associated with prevention of effects linked to chronic inflammation rather than an acute insult.

The neuroprotective effects of SCH58261, an A2A adenosine receptor antagonist were investigated. Epidemiological studies have established an association between caffeine consumption and a reduced risk of developing Parkinson's disease (Chen *et al.*, 2001). Pharmacological studies show the A1 and A2 subtypes of adenosine receptors are the most likely targets for caffeine in the brain (Fredholm *et al.*, 1999) and that caffeine acts in an antagonistic manner on these receptors. The A2A receptor specifically is strongly implicated in mediation of neurodegeneration, as its expression is largely restricted to the striatum (Svenningsson *et al.*, 1999), the major target of the dopaminergic neurons that degenerate in Parkinson's disease. Caffeine, and A2A specific antagonists exerted a protective effect in a MPTP murine model of Parkinson's disease, but an A1 specific antagonist did not (Chen *et al.*, 2001). Moreover, A2A receptor knockout mice showed a blunted MPTP-induced neurotoxicity, as well as being less vulnerable to ischemia and neuronal damage (Chen *et al.*, 1999). In this study SCH58261, a selective A2A receptor antagonist, was assessed as a potential neuroprotective agent. Given that JNK appeared to play an important role in mediating the inflammatory changes associated with LPS administration, this study was designed to assess if SCH58261 could (1) preserve synaptic efficacy following LPS administration, (2) modulate the JNK pathway, and (3) protect against apoptotic cell death.

The LPS-induced inhibition of both the induction and the maintenance of LTP in dentate gyrus reported in the literature and observed in the two earlier studies described here was replicated in this study. Evidence is presented here which demonstrates that pretreatment with SCH58261 protects animals from LPS-induced impairment in LTP; both the early and late effects of LPS were inhibited. Therefore blockade of A2A receptors by the antagonist SCH58261, exerted a protective effect. This indicates for the first time that A2A adenosine receptors play a role in mediating detrimental changes induced by LPS. Support for a central neuroprotective role of A2A antagonists exist; A2A receptor antagonists exerted a protective effect in 6-OHDA-treated rats, and MPTP-treated mice, two models of Parkinson's disease (Ikeda *et al.*, 2002), while A2A receptor blockade (or caffeine treatment) prevented β-amyloid-induced toxicity on rat cultured cerebellar granule neurons (Dall'Inga *et al.*, 2003). Furthermore, the A2A receptor antagonist DMPX prevented frontal cortex EEG abnormalities in the quinolinic acid model of Huntington's disease (Reggio *et al.*, 1999). However, how this blockade may lead to these protective effects is less apparent.

Interestingly, treatment with SCH58261 alone led to a significant enhancement of LTP suggesting that blockade of A2A receptors may have a beneficial effect in the absence of a challenge. Indeed it has been reported that caffeine, as well as reversing memory disruption induced by MPTP in rats (Gevaerd *et al.*, 2001; Da Cuhna *et al.*, 2002), had a beneficial effect on memory retention in spatial learning in a Morris water maze when administered post trial (Angelucci *et al.*, 2002). It should be noted that the mean percentage increase in epsp slope exhibited by the vehicle-treated rats in this study did not reach previously observed values. This was probably due to the requirement for DMSO in the vehicle solution in order to solubilise the compound. Although the final concentration of DMSO was very low (0.00043%), DMSO has been shown to cause a decrease in neurotransmitter release at very low concentrations (Lynch, personal communication) and could therefore directly impact on the induction and expression of LTP, possibly accounting for the diminished LTP results.

This study sought to elucidate the biochemical changes underlying the protective influence of A2A adenosine receptor blockade. The well-documented LPS-induced increase in IL-1β was attenuated completely by pretreatment with SCH58261. Interestingly, recent evidence indicates that A2A agonists and IL-1β act to cause apoptosis through a similar mechanism (Trincavelli *et al.*, 2003), so perhaps A2A receptor blockade can play some role in blocking the self-induction of IL-1β. If IL-1β is down regulated then it might be predicted that IL-1β-dependent downstream signalling would be decreased. In support of this, the data show the LPS-induced increase in JNK activation was blocked by SCH58261. Adenosine receptors have been linked to MAP kinase signalling including the SAPKs p38 and JNK (for review see Schulte and Fredholm, 2003). Therefore, in parallel with the LTP findings, SCH58261 attenuated other effects of LPS. Predictably then, the level of caspase 3 activation in hippocampal tissue was significantly increased in LPS-treated rats, and, consistent with the idea of JNK playing a central role in this detrimental change, the SCH58261-induced inhibition of JNK was linked to attenuation of caspase 3 activation. This indicates that activation of A2A adenosine receptors may be critical in initiation of LPS-induced apoptotic cell death, and can be abrogated by SCH58261. This is supported by the demonstration that the proinflammatory cytokines IL-1β and TNFα, induce apoptotic cell death in a similar manner to adenosine and specific A2A receptor agonists (Trincavelli *et al.*, 2003). It may be suggested therefore that A2A receptor blockade may reduce LPS-induced apoptosis by down regulating IL-1β signalling, by attenuating IL-1β expression and possibly interfering with other downstream mediators.

Low levels of expression of A2A adenosine receptors have been detected on excitatory neurons (Sebastio and Ribiero, 1996) and on glial cells (Brodie *et al.*, 1998). As it was demonstrated in the present study that LPS induces IL-1β, it is noteworthy that IL-1β has been shown to cause an increase in A2A adenosine receptor expression in neuronal cell lines (Trincavelli *et al.*, 2002), while TNFα upregulates the expression and function of A2A AR in human monocytic THP-1 cell lines (Khoa *et al.*, 2001). The function of this upregulation is not clear, as it could be a cell defence mechanism or conversely lead to cell death. As it has been shown on numerous occasions that A2A stimulation enhances glutamate release (Pintor *et al.*, 2001), one potential method of neuroprotection by SCH58261 is blockade of excitotoxicity. This is indeed the case as use of the A2A antagonist SCH58261 inhibited glutamate release induced by quinolinic acid (Popoli *et al.*, 2002), as well as lesion size and the resulting gliosis. Alternatively, A2A receptor antagonists may have a protective effect by influencing glial activation; decreased gliosis in response to MPTP treatment was observed following A2A receptor blockade (astrocytic activation) (Ikeda *et al.*, 2002) while conversely administration of an A2A agonist induces gliosis *in vivo* (Fiebich *et al.*, 1996).

The physiological role of the endogenous agonist, adenosine, is wide-ranging; it has been shown to modulate proliferation, survival and apoptosis of many different cell types (Jacobson *et al.*, 1999) and this may be mediated by entry of the nucleoside into the cells or by activation of the transmembrane adenosine receptors.

While the mechanism of action of the A2A antagonist SCH58261 is still unclear, the data presented here indicate that A2A receptor activation does play a central role in mediating the effects of LPS, particularly those leading to apoptotic cell death. It is demonstrated for the first time that A2A receptor blockade can protect against the LPS-induced impairment in LTP, and the associated cell degeneration.

The evidence presented here suggests that JNK plays a significant role in mediating inflammatory effects, induced by LPS. Its inhibition attenuates the depressive effects of LPS on LTP, and rescues cells from LPS-induced apoptosis, although the biochemical steps by which JNK exerts this effect remains to be clearly elucidated. Like JNK inhibition, HMG-CoA reductase inhibition by atorvastatin also exerted protective effects in animals treated with LPS, as well as in aged animals. The mechanisms involved in statin-induced protective effects are complex and probably numerous due to the wide range of cell signalling molecules impacted by inhibition of the cholesterol synthesis pathway. However, data presented here indicate that the beneficial effect could be linked to a down

regulation in JNK signalling. Similarly, the A2A receptor antagonist, prevented an impairment of LTP and associated cell death markers in the hippocampus following LPS treatment, and this too was allied with down regulation of JNK signalling. Therefore, I conclude that JNK is a point of convergence in cell death signalling associated with inflammation, and as such represents a potent target for research into neuroprotective strategies.

Future work:

Several questions arise from the data presented here. For example, do hippocampal neurons express TLR4? It would be interesting to ascertain the expression of TLR4 in different brain regions, particularly those susceptible to insult, as well as in the different cell types, (microglia, astrocytes, oligodendrocytes and neurons) in these regions.

LPS induces differential responses *in vivo* and in neurons *in vitro* in respect to timing of events; LPS-induced IL-1β induction occurred at 3h and 18h respectively. It may be pertinent to study more carefully the timecourse of LPS-induced events *in vitro*, particularly to investigate if glial cells could have a more rapid or greater response, and therefore help elucidate the relative contributions of neurons and glia to LPS-induced effects *in vivo*. Also of interest would be the clarification of LPS-induced signalling in neurons and in types of glia, as it is likely that different intracellular cascades are initiated.

One unanswered question arising from this study is the precise mechanism by which JNK inhibition can protect against LPS-induced impairment of LTP and apoptotic cell death. It has yet to be ascertained whether the mitochondrial or non-mitochondrial pathways are more central to JNK-mediated LPS-induced cell death. It was interesting to note that while JNK inhibition resulted in protective effects, cytochrome c release was still observed; perhaps some other factor limited by JNK is required for induction of cell death by this route. Clarification may come from the study of a number of proteins of the bcl-2 family, or from investigation of other factors released from the mitochondria. It may be that aspects of the non-mitochondrial cell death pathway are more significant for JNK mediated cell death, for example, JNK inhibition may impact on Elk-1 activation, as well as AP-1 proteins other than c-Jun, such as ATF-2 and c-Fos.

Atorvastatin treatment appears to have varied effects both *in vivo* and *in vitro*. These discrepancies need to be addressed before ATV can be effectively used as a neuroprotectant. A dose response study would be useful to assess the potential positive and negative effects of ATV treatment in aged and young animals, and clarify if there is a

differential effect with age. It would also be informative to assess the effects of ATV treatment on cognitive function, i.e. learning and memory. For example could ATV treatment protect against LPS- or age-associated impairment in performance in a spatial learning task? In addition, the mechanism of action of ATV warrants further investigation. Is the decrease in NF-κB signalling reported *in vitro* likely to have bearing on the *in vivo* situation? Assessment of the cytokine profile *in vivo* would also be beneficial. The finding that CD40 expression can be downregulated by ATV is of consequence and merits more study.

A2A receptor blockade by SCH58261 confers protection against LPS-induced impairment of LTP as well as apoptotic cell death, however, the mechanism of action remains unclear. Since the LPS-induced increase in IL-1β was decreased it may be of benefit to assess microglial activation as well as the production of antiinflammatory cytokines which could downregulate IL-1β.

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VII Appendix I Mean Data

A) Analysis of LPS-induced effects on hippocampus of rats

Parameter	Treatment		Units
	Saline	LPS	
pJNK	1448± 41.53	1767± 87.47	Arbitrary units
Caspase 3 activity	1.604± 0.2753	2.637± 0.4027	nmol AFC/mg/min

B) Analysis of LPS-induced effects on hippocampus of rats pretreated with the JNK inhibitor D-JNKI1 at two different concentrations

Parameter	Treatment				Units
	Control		D-JNKI1		
	Saline	LPS	Saline	LPS	
pJNK	1328± 102.8	1969± 137.9	200μM 1797± 62.80	200μM 1778± 108.6	Arbitrary Units
pJNK			500μM 2156± 330.3	500μM 2513± 363.8	Arbitrary Units
p-c-Jun	1549± 43.7	1984± 126.9	200μM 1723± 200.1	200μM 1542± 238.1	Arbitrary Units
p-c-Jun			500μM 1793± 104.5	500μM 1377± 173.6	Arbitrary Units

C) Analysis of LPS-induced effects on hippocampus of rats pretreated with the JNK inhibitor D-JNKI1

Parameter	Treatment				Units
	Control		D-JNKI1		
	Saline	LPS	Saline	LPS	
% epsp slope 0-2min	155.4± 3.30	123.2± 2.20	134.2± 1.50	124.8± 0.80	% epsp
% epsp slope 40-45min	116.0± 1.4	97.4± 0.6	110.3± 0.3	112.4± 0.8	% epsp
IL-1β	603± 55.8	711± 103.2	606± 66.8	806±108.7	pg/mg protein
PJNK/TJNK	0.8± 0.00	1.03± 0.113	1.17± 0.128	1.02± 0.118	ratio
p-c-Jun	1492± 63	2127± 206	1721± 161	1728± 198	Arbitrary Units
p-bcl-2	1730± 123	2212± 176	1399± 128	1681± 135	Arbitrary Units
bax	1495± 48.1	1439± 5.7	1409± 1.9	1445± 54.2	Arbitrary Units
ATP	9.98± 0.53	10.0± 0.68	8.5± 0.45	9.3± 0.71	nM
Cytochrome c	1559± 63	2057± 192	1554± 105	1930± 158	Arbitrary Units
TUNEL stained cells	7.76± 1.7	13.7± 1.0	8.32± 0.8	9.7± 1.4	% TUNEL positive cells
Fas Ligand	1491± 0.1	1822± 0.2	1987± 0.8	1887± 4.6	Arbitrary Units

D) Analysis of the temporal aspects of LPS-induced effects on cultured cortical neurons.

Parameter	Treatment					Units
	Control	3h LPS	6h LPS	18h LPS	24h LPS	
IL-1β	22.7±11.44	20.5± 14.88	20.6± 12.61	116.3± 21.18	142.2± 38.0	pg/ml
IFNγ	Not detectable					
pJNK	1421± 84.20	2379± 229.3	2211± 239.8	2871± 753.0	2222± 461.1	Arbitrary Units
p-bcl-2	1528± 21.59	1315± 332.7	1705± 335.7	4032± 1291	1493± 533.1	Arbitrary Units
Cytochrome c	1001± 1.262	1164± 93.64	1125± 66.32	1270± 82.64	1290± 118.1	Arbitrary Units
Caspase 3 activity	15.0± 2.18	10.7± 2.16	12.1± 1.80	12.5± 1.03	13.8± 2.25	nmol AFC /mg/min

E) Analysis of LPS-induced effects on cultured cortical neurons pretreated with the JNK inhibitor D-JNKI1

Parameter	Treatment				Units
	Control		D-JNKI1		
	Control	LPS	Control	LPS	
IL-1β	151.4± 27.09	387.0± 43.82	113.2± 8.853	349.8± 26.38	pg/ ml
p-bcl-2	1557± 45.9	2314± 401.4	1828± 447.9	1798± 318.3	Arbitrary Units

F) Body weights of animals treated with atorvastatin and LPS

								Units
Control				Atorvastatin				
Saline		LPS		Saline		LPS		
Pre diet	Post Diet	Pre diet	Post Diet	Pre diet	Post Diet	Pre diet	Post Diet	
219± 11.4	295± 8.8	226± 10.0	313± 12.5	225± 12.0	269± 15.5	208± 10.6	258± 20.2	g

G) Analysis of LPS-induced effects on hippocampus of rats which received a diet supplemented with atorvastatin.

Parameter	Treatment				Units
	Control		Atorvastatin		
	Saline	LPS	Saline	LPS	
%epsp slope 0-2min	160.8± 2.97	123.8±1.09	126.2± 2.70	122.7± 1.98	% epsp
epsp slope 40-45min	123.9± 1.7	95.1± 1.0	102.9± 2.60	106.7± 2.37	% epsp
pJNK	5176±403.0	7018± 465.5	5932± 459.5	5866± 221.6	Arbitrary Units
p-c-Jun/c-Jun	0.8± 0.06	1.1± 0.11	0.9± 0.08	1.0± 0.06	Ratio
pErk/Actin	0.3± 0.0	0.31± 0.03	0.28± 0.02	0.27± 0.02	Ratio

H) Analysis of LPS-induced effects on cultured cortical glia pretreated with atorvastatin.

Parameter	Treatment				Units
	Control		Atorvastatin		
	Control	LPS	Control	LPS	
IL-1β	33.2± 12.80	226.2± 35.63	7.04± 4.566	146.0± 50.26	pg/ml
CD40	1500± 0.6	2224± 160.7	1441± 240.1	1534± 266.0	Arbitrary Units
IL-10	7.6± 4.25	299.3± 34.64	0.96± 0.93	216.2± 43.34	pg/ml
IL-4	Not detectable				

I) Body Weights of young and aged animals treated with atorvastatin

								Units
Control				Atorvastatin				
Young		Aged		Young		Aged		
Pre diet	Post Diet	Pre diet	Post Diet	Pre diet	Post Diet	Pre diet	Post Diet	
294± 13.0	374± 13.9	534± 17.4	526± 15.5	287± 9.2	363± 6.2	577± 21.6	551± 22.1	g

J) Analysis of the effects of ageing on hippocampus of rats which received a diet supplemented with atorvastatin.

Pararameter	Treatment				Units
	Control		Atorvastatin		
	Young	Aged	Young	Aged	
% epsp slope 0-2min	125.1± 4.06	128.6± 0.89	141.9± 1.14	134.9± 2.33	% epsp
% epsp slope 40-45min	114.1± 0.53	103.8± 0.61	109.0± 0.69	118.7± 1.41	% epsp

K) Analysis of LPS-induced effects on hippocampus of rats which were pretreated with SCH58261.

Pararameter	Treatment				Units
	Vehicle		SCH58261		
	Saline	LPS	Saline	LPS	
% epsp slope 0-2min	126.8± 1.01	115.1± 1.08	131.5± 1.68	130.2± 0.74	% epsp
% epsp slope 40-45min	105.2± 0.51	98.9± 0.47	111.5± 1.10	104.6± 0.64	% epsp
IL-1β	381.9± 23.56	529.9± 32.46	383.8± 25.00	391.7± 47.81	pg/mg protein
pJNK	1000±0.00	1337.0± 111.5	980.9± 68.20	1026.0± 114.10	Arbitrary Units
Caspase 3	13.5± 2.54	23.6±2.28	13.7± 2.44	8.1± 1.51	nmol AFC /mg/min

VIII Appendix II Addresses

Alexis Biochemicals,
P.O. Box 6757
Bingham
Nottingham NG13 8LS
UK

Amersham Biosciences UK Limited
Pollards Wood
Nightingales Lane
Chalfont St.Giles
Bucks HP8 4SP
UK

BD Biosciences (Falcon)
2350 Qume Drive
San Jose
California
USA

Bio-Rad Laboratories Ltd.
Bio-Rad House
Maylands Avenue
Hemel Hempstead
Herts HP2 7TD
UK

BioSource United Kingdom
Rue de L'industrie, 8
B-1400 Nivelles
Belgium

Calbiochem (Merck)
European Distribution centre
Boulevard Industrial Park
Nottingham NG91BR
UK

Cell Signaling Technology, Inc.
166B Cummings Centre
Beverly
MA 01915
USA

Chemicon
The Science Centre
Eagle Close
Chandlers Ford
Hampshire SO53 4NF
UK

Cruinn Diagnostics Ltd.
Unit 5b, 6b, Hume Centre
Park West Industrial Estate
Dublin 12
Ireland

Gibco BRL
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF
Scotland
UK

Lennox
John F Kennedy Drive
Naas Rd
Dublin12
Ireland

Medical Supply Company Ltd.
Damastown
Mulhuddart
Dublin 15
Ireland

Nalge (Europe) Limited
Unit 1a
Thorn Business Park
Hereford HR2 6JT
UK

Oncogene Science
Bayer HealthCare LLC
Subsidiary of Bayer Corporation
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USA

Pall Gelman Sciences Inc.
2200 Northern Boulevard East Hills
NY 11548
USA

Pfizer-Parke Davis
Parkway House
Ballymount Road Lower
Dublin 12
Ireland

Pierce Biotechnology, Inc.
Customer Service Department
P.O. Box 117
Rockford, IL. 61105
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2800 Woods Hollow Road
Madison
WI 53711
USA

R&D Systems Europe Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, Oxon OX14 3NB
United Kingdom

Roche Diagnostics Ltd.
Bell Lane, Lewes
East Sussex BN7 1LG
UK

Santa Cruz Biotechnology Inc.
2161 Delaware Ave
Santa Cruz
CA 95060
USA

Sarstedt Ltd.
Sinnottstown Lane
Drinagh - Wexford
Ireland

Sigma-Aldrich
Fancy Rd.
Poole
Dorset BH124WQH
UK

Vector Laboratories, Ltd.
3, Accent Park
Bakewell Road
Orton Southgate
Peterborough PE2 6XS
UK

IX Appendix III Solutions

ATP assay reaction buffer (pH 7.5)
Tris Base, 100mM
EDTA, 4mM

Electrode running buffer
Tris base, 25mM
Glycine, 200mM
SDS, 17mM

Equilibration buffer
Potassium cacodylate, 200mM (pH 6.6 at 25°C)
Tris-HCl, 25mM (pH 6.6 at 25°C)
DTT 0.2mM
Cobalt chloride, 2.5mM

Krebs solution containing $CaCl_2$
NaCl, 136mM
KCl, 2.54mM
KH_2PO_4, 1.18mM
$MgSO_4$, 1.18mM
$NaHCO_3$, 16mM
Glucose, 10mM
Containing $CaCl_2$, 2mM

Lysis buffer for caspase3 assay, pH 7.4
HEPES, 25mM
$MgCl_2$, 5mM
DTT, 5mM
EDTA, 5mM
PMSF, 2mM
Leupeptin, 10μg/ml
Pepstatin, 6.25μg/ml

Lysis buffer for preparation of a whole cell lysate (pH 7.4)
HEPES, 25mM
$MgCl_2$, 5mM
DTT, 5mM
EDTA, 5mM
PMSF, 2mM
leupeptin, 10μg/ml
pepstatin A, 10μg/ml

Mitochondrial buffer (pH7.2)
Tris Base, 50mM
NaCl, 150mM
EGTA, 2mM
EDTA, 2mM
PMSF, 100μM
Leupeptin, 10μg/ml
Aprotinin, 2μg/ml
Triton-X 0.2%
Igepal P-40 0.3%

Permeabilisation buffer for preparation of crude fractions (pH7.2)
Sucrose, 250mM
KCl, 70mM
NaCl, 137mM
Na_2HPO_4, 4.5mM
KH_2PO_4, 1.4mM
PMSF 100μM
Leupeptin, 10μg/ml
Aprotinin, 2μg/ml
Digitonin, 200μg/ml

Phosphate buffered saline (PBS), pH 7.4
Na_2HPO_4, 80mM
NaH_2PO_4, 20mM
NaCl, 100mM

Phosphate buffered saline (PBS), pH 7.4 for ELISA
NaCl, 137mM
KCl, 2.7mM
Na_2HPO_4, 8.1mM
KH_2PO_4, 1.5mM

Reaction buffer for caspase 3 assay (pH 7.4)
HEPES, 50mM
EDTA, 2mM
glycerol, 20%
dithiothreitol, 10mM

Sample buffer
Tris-HCl, 0.05M, pH 6.8
Glycerol 20% (v/v)
SDS 2% (w/v)
β-Mercaptoethanol 5% (v/v)
bromophenol blue 0.05% (w/v)

Separating gel (12%), pH 8.8
Acrylamide/bis-acrylamide (30% stock), 40% (v/v)
dH_2O, 33% (v/v)
Tris-HCl, 0.05M, pH 6.8, 25% (v/v)
SDS (10% w/v stock), 1% (v/v)
APS (10% w/v stock), 0.5% (v/v)
TEMED, 0.05% (v/v)

Separating gel (10%), pH 8.8
Acrylamide/bis-acrylamide (30% stock), 33% (v/v)
dH_2O, 40% (v/v)
Tris-HCl, 0.05M, pH 6.8, 25% (v/v)
SDS (10% w/v stock), 1% (v/v)
APS (10% w/v stock), 0.5% (v/v)
TEMED, 0.05% (v/v)

Stacking gel (4%), pH 6.8
Acrylamide/bis-acrylamide (30% stock), 13% (v/v)
dH_2O, 60% (v/v)
Tris-HCl, 0.05M, pH 6.8, 25% (v/v)
SDS (10% w/v stock), 1% (v/v)
APS (10% w/v stock), 0.5% (v/v)
TEMED, 0.5% (v/v)

Transfer buffer, pH 8.3
Tris base, 25mM
Glycine, 192mM
Methanol, 20% (v/v)
SDS, 0.05% (w/v)

Tris buffered saline (TBS), pH 7.6
Tris-HCl, 20mM
NaCl, 150mM

X List of Publications

C. E. BARRY, Y.M. NOLAN, AND M. A. LYNCH. (2003) Activation of c-Jun N-terminal Kinase mediates lipopolysaccharide-induced cell deterioration in rat hippocampus. *Society for Neuroscience Abstracts* 690.15

C. E. BARRY, AND M. A. LYNCH (2003) The role of c-Jun N-terminal Kinase (JNK) in lipopolysaccharide-induced cell deterioration in rat hippocampus. *J Physiol* 551P C26

BARRY C. E. AND LYNCH M. A. (2003) The role of c-Jun N-terminal Kinase (JNK) in LPS-induced cell deterioration in rat hippocampus. *Brit. Neurosci. Assoc. Abstr.*, Vol 17, p52

REBOLA N., BARRY C., OLIVEIRA C. R., LYNCH M. A., & CUNHA R. A. (2004) Blockade of adenosine A2A receptors attenuates inflammatory responses in the central nervous system. *FENS Forum Abstracts, volume 2,* A023.11

BARRY C. E., NOLAN Y., CLARKE R., LYNCH A. M., AND LYNCH M. A. (2005) Activation of c-Jun-N-terminal kinase is critical in mediating lipopolysaccharide-induced changes in the rat hippocampus. *J Neurochem,* accepted for publication, manuscript number 03011.

C. E. BARRY, Y.M. NOLAN, AND M. A. LYNCH. (2003) Activation of c-Jun N-terminal Kinase mediates lipopolysaccharide-induced cell deterioration in rat hippocampus. *Society for Neuroscience Abstracts* 690.15

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, exerts manifold effects in the central nervous system, causing an immune challenge in the brain. It has been reported that intraperitoneal injection of LPS inhibits long-term potentiation (LTP) in dentate gyrus, although the mechanism by which this is affected remains unclear. LPS administration is associated with activation of stress-activated protein kinases such as c-Jun N-terminal protein kinase (JNK) and this could account for the LPS-induced decrease in synaptic efficacy as there is much evidence linking activation of JNK to cell deterioration. Here we report that intracerebroventricular administration of the specific JNK inhibitor, D-JNKI1, attenuated the LPS-induced inhibition of LTP in perforant path-dentate gyrus granule cell synapses in urethane-anaesthetised male Wistar rats. Activation of JNK by LPS triggered downstream signalling cascades, including activation of the transcription factor c-Jun and bcl-2 phosphorylation. These changes were coupled with increased cytosolic cytochrome *c* expression, and Tdt-mediated dUTP Nick-End Labelling (TUNEL) in cytospun cells, suggesting that cells underwent apoptosis. Pre-treatment with D-JNKI1 attenuated these changes. The evidence is consistent with a pivotal role for JNK in mediating the effects of LPS.

C. E. BARRY, AND M. A. LYNCH (2003) The role of c-Jun N-terminal Kinase (JNK) in lipopolysaccharide-induced cell deterioration in rat hippocampus. *J Physiol* 551P C26

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, exerts manifold effects in the central nervous system. It has been reported that i.p. injection of LPS inhibits LTP in dentate gyrus. While the mechanism by which this is affected remains unclear, LPS administration is associated with activation of stress-activated protein kinases such as c-Jun N-terminal protein kinase (JNK). There is evidence linking these protein kinases to cell deterioration, possibly accounting for the LPS-induced decrease in synaptic efficacy. Here we report that i.c.v. administration of the specific JNK inhibitor, D-JNKI1 (500μM; 5μl), attenuated the LPS-induced (100μg/kg) inhibition of LTP in perforant path-dentate gyrus granule cell synapses in urethane-anaesthetized male Wistar rats. Following electrophysiological recording, rats were decapitated, and hippocampus was dissected and frozen until further analysis. LPS induced an increase in phosphorylated JNK expression in hippocampal tissue (1767 arbitrary units (±88;SEM) compared to 1448 (±41) in saline treated rats ($p<0.05$; student t test; n=5). Table 1 shows that this activation of JNK by LPS triggers downstream signaling cascades, including activation of the transcription factor c-Jun and bcl-2 phosphorylation. These changes are coupled with increased cytosolic cytochrome *c* expression, and Tdt-mediated dUTP Nick-End Labelling (TUNEL) in cytospun cells, indicative of apoptosis. Pre-treatment with D-JNKI1 attenuated these changes.

Table 1. D-JNKI1 attenuated central effects of LPS. (Values are means (±SEM))

Parameter measured(units)	Control Saline	Control LPS	D-JNKI1 Saline	D-JNKI1 LPS
Phosphorylated c-Jun (AU)	1492 ±63	2127 ±206	1721 ±161	1728 ±198
Phosphorylated bcl-2 (AU)	1730 ±123	2212 ±176	1399 ±128	1681 ±135
Cytochrome c in cytosol	1559 ±63	2057 ±192	1554 ±105	1930 ±158
TUNEL stained cells (%)	7.76 ±1.7	13.7 ±1.0	8.32 ±0.8	9.7 ±1.4
LTP 40-45min (% epsp slope)	116.0 ±1.4	97.4 ±0.6	110.3 ±0.3	112.4 ±0.8
$p<0.05$; ANOVA; n 6; cf Control-Saline $p<0.05$; ANOVA; n=6; cf Control-LPS AU = Arbitrary Units				

In Summary, the evidence is consistent with a pivotal role for JNK in mediating the effects of LPS

Supported by grants from the Health Research Board, and Enterprise Ireland.

BARRY C. E. AND LYNCH M. A. (2003) The role of c-Jun N-terminal Kinase (JNK) in LPS-induced cell deterioration in rat hippocampus. *Brit. Neurosci. Assoc. Abstr.*, Vol 17, p52

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, exerts manifold effects in the central nervous system. It has been reported that i.p. injection of LPS inhibits long-term potentiation (LTP) in dentate gyrus. While the mechanism by which this is affected remains unclear, LPS administration is associated with stimulation of stress-activated protein kinases such as c-Jun N-terminal protein kinase (JNK). There is evidence linking these protein kinases to cell deterioration, possibly accounting for the LPS induced decrease in synaptic efficacy, however until now, the lack of a specific JNK inhibitor has made determination of the role of JNK difficult. Recently a specific JNK inhibitor (D-JNKI1; Alexis Biochemicals) was engineered and here we report that JNK inhibition attenuated the LPS-induced inhibition of LTP in urethane-anaesthetized rats. It was demonstrated that LPS activated JNK, stimulating downstream signaling cascades, including activation including activation of the transcription factor c-Jun. It was also shown that LPS leads to bcl-2 phosphorylation, and subsequently induces apoptosis, as suggested by increases in cytochrome *c* in the cytosol, TUNEL staining in cytospun cells. Pre-treatment with D-JNKI1 reduced these changes. In summary the evidence is consistent with the idea that LPS mediated effects are dependant at least in part on JNK activity.

REBOLA N., BARRY C., OLIVEIRA C. R., LYNCH M. A., & CUNHA R. A. (2004) Blockade of adenosine A2A receptors attenuates inflammatory responses in the central nervous system. *FENS Forum Abstracts, volume 2, A023.11*

Recent observations have highlighted the neuroprotective impact of A2A receptor blockade in stressful brain conditions (Curr. Top. Med. Chem. 3:413) but the mechanism by which this occurs remains elusive. One of the mechanisms proposed is the control by A2A receptors of neuro-inflammation, since A2A receptors control inflammation in peripheral systems (Nature 414:916). Thus, we now tested the effect of intracerebroventricular (icv) injection of a selective A2A antagonist (SCH 58261, 50 nM) in rats injected intraperitoneally (ip) with lypopolysaccharide (LPS, 200 microg/kg), a trigger of inflammation. Focusing on the hippocampus we evaluated: in vivo long-term potentiation (LTP), IL-1beta levels and caspase-3 activity. In control rats, IL-1beta levels were 382±24 pmol/mg protein and the activity of caspase-3 was 22±6 nmol AFC (arbitrary fluorescent counts) /mg/min (n=5). LPS increased IL-1beta levels (530±32 pmol/mg protein; n=6, $P<0.05$) and caspase-3 activity (49±11 nmol AFC/mg/min, n=5, $P<0.05$), both effects being prevented by SCH 58261 (IL-1beta levels: 392±48 pmol/mg protein; caspase-3 activity: 15±4 nmol AFC/mg/min, n=5), whereas SCH 58261 alone did not modify IL-1beta levels and caspase-3 activity compared to control. In control rats, LTP evaluated 30 min after the tetanic train was 112±0.7% (n=4) and was abolished by LPS treatment (103±0.8%). SCH 58261 did not affect LTP (115±0.9%) but abolished the ability of LPS to depress LTP (113±0.4%). These results indicate that adenosine A2A receptors may play an important role in controlling CNS inflammatory responses and support the possibility that the neuroprotective effect of adenosine A2A receptor antagonists may involve the control of neuro-inflammation. These results also raise the possibility of using A2A receptors antagonists as a therapeutic strategy in several neurodegenerative diseases where CNS inflammatory processes have already been implicated, like, for example, Alzheimer's disease. (Supported by FCT).

BARRY C. E., NOLAN Y., CLARKE R., LYNCH A. M., AND LYNCH M. A. (2005) Activation of c-Jun-N-terminal kinase is critical in mediating lipopolysaccharide-induced changes in the rat hippocampus. *J Neurochem,* accepted for publication, manuscript number 03011.

Lipopolysaccharide (LPS) exerts a myriad of effects in rat hippocampus; it increases the concentration of the proinflammatory cytokine, interleukin-1β (IL-1β), and signalling through the IL-1 Type I receptor (IL-1RI) resulting in phosphorylation of the stress-activated protein kinase, c-jun-N-terminal kinase (JNK) and impairment in long-term potentiation (LTP). The present study was designed to establish whether activation of JNK was a pivotal event in mediating the effects of LPS in hippocampus and therefore LPS-treated rats were injected intracerebroventricularly with saline, the JNK inhibitor D-JNKI1, or with the anti-inflammatory cytokine IL-4, which antagonizes the effects of IL-1β upstream of JNK activation. We report that IL-4 blocked the LPS-induced increase in IL-1RI expression and associated increases in phosphorylation of JNK and c-jun, while D-JNKI1 inhibited the LPS-induced phosphorylation of c-jun. Both IL-4 and D-JNKI1 inhibited the increase in caspase-3 staining which was associated with LPS treatment, and both abrogated the LPS-induced inhibition of LTP in perforant path-granule cell synapses. The data presented are consistent with the proposal that JNK activation, probably as a result of increased IL-1RI activation, is a critical step in mediating the detrimental effects of LPS in hippocampus.

Key words: Lipopolysaccharide, LTP, c-jun-N-terminal kinase (JNK), hippocampus, IL-1β, IL-4.

Activation of c-Jun-N-terminal kinase is critical in mediating lipopolysaccharide-induced changes in the rat hippocampus

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ABSTRACT

Lipopolysaccharide (LPS) exerts a myriad of effects in rat hippocampus; it increases the concentration of the proinflammatory cytokine, interleukin-1β (IL-1β), and signalling through the IL-1 Type I receptor (IL-1RI) resulting in phosphorylation of the stress-activated protein kinase, c-jun-N-terminal kinase (JNK) and impairment in long-term potentiation (LTP). The present study was designed to establish whether activation of JNK was a pivotal event in mediating the effects of LPS in hippocampus and therefore LPS-treated rats were injected intracerebroventricularly with saline, the JNK inhibitor D-JNKI1, or with the anti-inflammatory cytokine IL-4, which antagonizes the effects of IL-1β upstream of JNK activation. We report that IL-4 blocked the LPS-induced increase in IL-1RI expression and associated increases in phosphorylation of JNK and c-jun, while D-JNKI1 inhibited the LPS-induced phosphorylation of c-jun. Both IL-4 and D-JNKI1 inhibited the increase in caspase-3 staining which was associated with LPS treatment, and both abrogated the LPS-induced inhibition of LTP in perforant path-granule cell synapses. The data presented are consistent with the proposal that JNK activation, probably as a result of increased IL-1RI activation, is a critical step in mediating the detrimental effects of LPS in hippocampus.

Key words: Lipopolysaccharide, LTP, c-jun-N-terminal kinase (JNK), hippocampus, IL-1β, IL-4.

Running Title: LPS-induced JNK activation leads to impaired LTP

INTRODUCTION

The findings of several studies have indicated that peripherally-administered lipopolysaccharide (LPS), a component of the cell wall of gram negative bacteria, exerts marked effects on neuronal function. These effects include fever, changes in sleep pattern and anorexia (Bluthé *et al.*, 1992; Linthorst and Reul, 1998; Dantzer *et al.*, 1998; Lacosta *et al.*, 1999). LPS has also been shown to inhibit various forms of learning (Pugh *et al.*, 1998; Shaw *et al.*, 2001; Jain *et al.*, 2002) and to disrupt long-term potentiation (LTP) in hippocampus (Vereker *et al.*, 2000a; Kelly *et al.*, 2003; Lynch *et al.*, 2004).

Although LPS, by binding to CD14 leads to activation of Toll-like receptor 4 and the associated signalling cascades (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999) much evidence suggests that its inhibitory effect on LTP is mediated by the proinflammatory cytokine, interleukin-1β (IL-1β), which is increased in rat brain following treatment with LPS (Quan *et al.*, 1994; Van Dam *et al.*, 1998; Ilyin *et al.*, 1998). Consistent with this idea are the findings that IL-1β, like LPS, inhibits LTP and that LPS-induced inhibition of LTP is abrogated by pre-treatment of rats with an inhibitor of interleukin-1β converting enzyme (Vereker *et al.*, 2000a).

One of the several changes which results from LPS treatment and which occurs downstream of the interaction of IL-1β with its signalling receptor, IL-1RI, is increased phosphorylation of the stress-activated protein kinase, c-jun N-terminal kinase (JNK; Hambleton *et al.*, 1996; Nick *et al.*, 1996; Pyo *et al.*, 1998; Mackichan and Defranco, 1999; Loscher *et al.*, 2000; Vereker *et al.*, 2000a; Nolan *et al.*, 2002). Several studies have coupled increased JNK activation with deficits in LTP in hippocampus, for example in aged rats (Martin *et al.*, 2002), rats exposed to γ-irradiation (Lonergan *et al.*, 2002) and IL-1β-treated (Vereker *et al.*, 2000b; O'Donnell *et al.*, 2000) and Aβ-treated (Minogue *et al.*, 2003) rats. Interestingly, phosphorylation of JNK and its substrate, c-jun, has been linked with neurodegenerative changes (Herdegen *et al.*, 1997; Ip and Davis, 1998; Zhu *et al.*, 2001) and JNK activation has been shown to trigger apoptosis in many cell types (Xia *et al.*,

1995; Chen *et al.*, 1996; Hu *et al.*, 1999). Recent findings from this laboratory have indicated that JNK activation is coupled with degenerative changes in cells of the entorhinal cortex (Vereker *et al.*, 2000a) and hippocampus (Minogue *et al.*, 2003; Lonergan *et al.*, 2004); consistently, inhibition of JNK prevents neurodegenerative changes and/or apoptosis in certain cells including neurons (Eilers *et al.*, 2001; Minogue *et al.*, 2003). Evidence suggests that JNK-induced activation of the transcription factor, c-jun (Gupta *et al.*, 1996) can trigger sequential activation of caspase-8 and caspase-3, which are have been identified as reliable markers of cell stress (Zimmerman *et al.*, 2001). It is interesting that, although caspase-3 activation has been considered to be a reliable marker for apoptotic cell death (Green and Reed, 1998), it has recently been reported that activation of caspase-3 in neurons is not always associated with evidence of cell death (Garnier et al., 2004)

Although JNK activation has been coupled with deficits in LTP, definitive evidence for a pivotal role for JNK is lacking and therefore this study was undertaken to address this issue. We decided to use two approaches to impact on LPS-activated JNK phosphorylation. First, we argued that if IL-1RI activation was pivotal in mediating the effect of LPS on JNK activation and thence leading to inhibition of LTP, then decreasing receptor expression may be an effective modulator of LPS-stimulated changes. Key anti-inflammatory actions of IL-4 result from its ability to antagonize IL-1β-induced changes (Brown and Hural, 1997; te Velde *et al.*, 1990; Vannier *et al.*, 1992); here we considered the possibility that these actions might include decreasing expression of IL-1RI. Second, we argued that JNK may exert its effect on LTP primarily by activating c-jun, and to investigate this, we assessed the effect of the inhibitor, D-JNKI1, on LPS-induced changes.

The data presented indicate that the inhibitory effect of LPS on LTP was blocked by the anti-inflammatory cytokine, IL-4, and also by D-JNKI1. The evidence is consistent with the view that IL-4 downregulates IL-1β-induced activation of JNK by reducing expression of IL-

1RI, while D-JNKI1 blocks activation of c-jun. These findings are consistent with the hypothesis that activation of JNK and the downstream consequences of this are critical steps in the LPS-triggered attenuation of LTP.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (2-4months old; BioResources Unit, Trinity College Dublin), weighing between 250g and 350g, were housed in groups of 3-6 in a controlled environment (temperature: 20 - 22°C; 12:12h light/ dark cycle), with free access to food and water. In one series of experiments, urethane-anaesthetized rats (1.5g urethane/kg) were injected intraperitoneally with either saline or lipopolysaccharide (LPS from *Escherichia coli* serotype 0111:B4; 100μg/kg; Sigma, UK) and 3h later killed by stunning and decapitation and hippocampus dissected free and stored for later analysis (see below). In two further series of experiments, animals were anaesthetized by intraperitoneal administration of urethane (1.5g/kg), and injected intracerebroventricularly with saline (5μl) or the peptide inhibitor, D-JNKI1 (500μM; 5μl; Alexis Biochemicals), or alternatively with saline (5μl) or IL-4 (20ug/ml; 5μl; R & D Systems, UK). Intracerebroventricular injections were given through a small burr hole drilled in the skull 2.5mm posterior to Bregma and 0.2mm lateral to the midline at a depth of 3.5mm. Rats treated with IL-4 were concurrently injected intraperitoneally with saline or LPS (100μg/kg; Sigma, UK), while D-JNKI1 pretreated animals received the intraperitoneal injection 1h later. In addition to these animals, we used one-day-old Wistar rats for preparation of neuronal cultures. Animals were maintained under veterinary supervision and all experiments were carried out under licence from the Department of Health and Children (Ireland) and in accordance with local ethical guidelines.

Induction of LTP *in vivo*

Animals were assessed for their ability to sustain long-term potentiation (LTP) in perforant path-granule cell synapses as previously described (Martin *et al.*, 2002) three hours after the intraperitoneal injection. Briefly, a bipolar stimulating electrode and a unipolar recording electrode were stereotaxically positioned in the perforant path (4.4mm lateral to lambda) and dorsal cell body region of the dentate gyrus (2.5mm lateral and 3.9mm posterior to Bregma) respectively of urethane-anaesthetized rats. Test shocks were delivered at 30sec intervals, and recorded for 10min before and 40min after tetanic stimulation (3 trains of stimuli; 250Hz for 200msec; 30sec intertrain interval).

Preparation of tissue

In the initial study, rats were killed 3h after saline or LPS treatment and in the studies in which LTP was assessed, rats were killed at the end of the recording period. In both cases, rats were killed by decapitation and the brains were rapidly removed. The brain was hemisected, coated in OCT compound, mounted onto cork and immersed in isopentane in a liquid nitrogen bath. Tissue was stored and later used to prepare cryostat sections. The other half of the hippocampus was cross chopped using a McIlwain tissue chopper (350 x 350μm), frozen in Krebs solution containing $CaCl_2$ (2mM) and 10% DMSO at –80°C as previously described (Martin *et al.*, 2002) and stored at -80°C until required for analysis.

Analysis of IL-4 concentration

The concentration of IL-4 was assessed by ELISA (R & D Systems, UK) in supernatants obtained from hippocampal homogenates prepared from the frozen samples prepared following assessment of LTP as described above; the method has been described in detail previously (Maher *et al.*, 2004). Briefly, hippocampal slices were thawed, rinsed three times in ice-cold Krebs solution and then homogenized in 500μl ice-cold Krebs solution a 1ml glass homogenizer by 20 up-and-down strokes. Protein concentrations in homogenates were equalized (Bradford, 1976) and triplicate aliquots (100μl) were added to antibody-coated 96-well plates (360μg/ml mouse anti-rat IL-4 diluted in phosphate-

buffered saline (PBS: composition in mM: NaCl 137; KCl 2.7; Na_2HPO_4 8.1; KH_2PO_4 1.5; pH 7.3), incubated overnight at room temperature, washed several times with PBS containing 0.05% Tween 20, blocked for 1h at room temperature with blocking buffer, (PBS; 5% sucrose, 1% bovine serum albumin (BSA), 0.05% NaN_3) and incubated with standards (0-1000pg/ml) or samples for 2h at room temperature. Wells were washed with PBS, incubated with secondary antibody (9μg/ml biotinylated goat anti-rat antibody diluted in PBS containing 1% BSA) for 2h at room temperature, washed again and incubated in horseradish peroxidase-conjugated streptavidin (1:200 dilution in PBS containing 1% BSA) for 20min at room temperature. Substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) was added, incubation continued at room temperature in the dark for 30min and the reaction was stopped using 1M H_2SO_4. Absorbance was read at 450nm. Values were corrected for protein concentration and expressed as pg/mg protein.

Expression of IL-1RI, phosphorylated JNK and phosphorylated c-jun

Phosphorylated JNK and phosphorylated c-jun were analysed in samples of hippocampal homogenate tissue as previously described (Lonergan *et al.*, 2002). Tissue homogenates were diluted to equalize for protein concentration and aliquots (100µl, 1mg/ml) were added to 100µl sample buffer (5µl; Tris-HCl, 0.5mM, pH 6.8; glycerol 10%; SDS, 10%; β-mercaptoethanol, 5%; bromophenol blue, 0.05% w/v), boiled for 5 min and aliquots (10µl) were loaded onto 12% SDS gels. IL-1RI expression was assessed in a membrane-enriched fraction Hippocampal slices were homogenized in Krebs solution, incubated for 10min on ice in permeabilisation buffer (composition in mM: sucrose 250, KCl 70, NaCl 137, Na_2HPO_4 4.5, KH_2PO_4 1.4, PMSF 0.1, containing leupeptin (10 µg/ml), aprotinin (2µg/ml) and digitonin (200µg/ml)), and centrifuged at 10,000 x g for 15min at 4°C. The resulting pellet (membrane fraction) was resuspended in lysis buffer, equalized for protein and added to half the volume of sample buffer (final concentration 0.5mg/ml). Preparations were boiled for 5min and aliquots (10µl) were loaded onto 10% SDS gels. In all cases, proteins were

fractionated by application of a 26mA constant current for 25-30min, transferred onto nitrocellulose strips (225mA for 75min) and immunoblotted with the appropriate antibody. IL-1RI membrane protein expression was assessed by blocking overnight at 4°C in PBS-Tween-20 (0.1%; PBS-T; PBS composition in mM; NaCl 100, Na_2HPO_4 80, NaH_2PO_4 20; pH 7.4) containing 6% non-fat dried milk. After washing (5x10min washes in PBS-T), membranes were incubated in the primary antibody (rabbit anti-rat IL-1RI IgG (Santa Cruz Biotechnology Inc, USA; 1:1,000) in PBS-T containing 2% non-fat dried milk) for 45min at room temperature and 45min at 37°C. To assess phosphorylated JNK, proteins were immunoblotted with an antibody which specifically targets phosphorylated JNK (G-7, Santa Cruz Biotechnology Inc, USA; 1:300) in TBS-T containing 0.1% BSA for 2h at room temperature. To assess phosphorylation of c-jun, nitrocellulose membranes were blocked in 6% non-fat dried milk in PBS-Tween overnight at 4°C, and immunoblotted with a mouse monoclonal antibody (1:200 dilution in PBS-Tween containing 2% non-fat dried milk; Santa Cruz Biotechnology Inc., USA) for 2h at room temperature. The secondary antibodies used were: for IL-1RI, an anti-rabbit IgG HRP-linked antibody, (1:2,000 dilution in PBS-T containing 2% non-fat milk, Amersham, UK) for 1h at room temperature and 45min at 37°C and washed (6x10min); for p-JNK, an anti-mouse IgG horse radish peroxidase (HRP)-linked antibody (1:400 dilution TBS containing 0.1% BSA, Sigma, UK) for 1h at room temperature; and for p-c-jun, an anti-mouse IgG HRP-linked antibody (1:400 dilution in PBS-Tween containing 2% non-fat dried milk, Sigma, UK) for 1h at room temperature.

Nitrocellulose membranes were probed with a number of antibodies and, to ensure equal protein loading blots which were assessed for pJNK were stripped and reprobed to assess total JNK and those blots which were assessed for p-c-jun were stripped and reprobed to assess for actin. The stripping procedure was as follows. Following development of blots, the nitrocellulose membranes were washed in PBS, placed in

stripping solution (Re-Blot Plus Strong Solution; Chemicon, UK) for 7-10min at room temperature, washed in PBS and then blocked in the appropriate medium for subsequent immunoblotting. Blots were stripped and reprobed a maximum of 3 times.

The nitrocellulose blots were stripped and reprobed for total JNK (mouse monoclonal antibody, F-3; 1:300 in Tris-buffered saline (TBS) containing 0.1% BSA Santa Cruz Biotechnology Inc., USA), and/or actin (mouse monoclonal antibody; 1:400 dilution in TBS containing 0.1% BSA; Santa Cruz Biotechnology Inc., USA). The secondary antibodies used were: for JNK1, anti-mouse IgG HRP linked antibody (1:400 dilution TBS containing 0.1% BSA, Sigma, UK) for 1h at room temperature; and for actin, an anti-mouse IgG HRP-linked antibody (1:500 dilution in TBS containing 0.1% BSA, Sigma, UK) for 1h at room temperature.

Protein complexes were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce, USA). Immunoblots were exposed to film (Amersham, UK) for 1 to 10s and processed using a Fuji X-ray processor. Protein bands were quantitated by densitometric analysis using two software packages, Grab It (Grab It Annotating Grabber 2.04.7, Synotics; UVP Ltd., UK) and Gelworks (Gelworks ID, Version 2.51; UVP Ltd., UK) for photography and densitometry respectively. Gelworks provides a single value (in arbitrary units) representing the density of each blot and the values presented here are means of data generated from several separate experiments.

Preparation of hippocampal neurons

Primary hippocampal neurons were isolated and prepared from postnatal one-day-old Wistar rats and maintained in neurobasal medium (NBM; Gibco BRL, UK; Nolan *et al.*, 2004). Rats were decapitated, the hippocampi dissected and the meninges were removed. The dissected hippocampi were cross chopped and incubated in sterile Dulbecco's PBS (Sigma, UK) containing trypsin (0.3mg/ml) for 25min at 37 °C. The tissue was then triturated (7×) in PBS containing soyabean trypsin inhibitor (0.1mg/ml), DNAse (0.2 mg/ml), and

$MgSO_4$ (10mM, Sigma, UK) and filtered through a sterile mesh filter (40μm). Following centrifugation (2000×g for 3min at 20 °C), the pellet was resuspended in NBM, supplemented with heat inactivated horse serum (10%), penicillin (100 units/ml), streptomycin (100 units/ml), glutamax (2 mM) and B27 (1%). Suspended cells were plated out in a 96-well plate at a density of 0.25×10^6 cells on circular 13 mm diameter poly-L-lysine coated (40 μg/ml) coverslips, and incubated in a humidified atmosphere containing 5% CO_2: 95% air at 37 °C and allowed adhere for 2h before wells were topped up with supplemented media. After 48h, B27 was omitted and 5ng/ml cytosine-arabinofuranoside was included in the culture medium for 12-24hrs to prevent proliferation of non-neuronal cells. Neurons were incubated in the absence or presence of LPS (100 ng/ml in NBM). In some experiments, cells were pretreated for 2h with IL-4 (100 ng/ml in NBM) or for 30min with D-JNKI1 (1μM; Alexis Biochemicals, UK). 24h after addition of LPS, supernatant was removed, cells were rinsed in TBS and fixed in 4% paraformaldehyde in TBS for immunohistochemical assessment of IL-1RI or phosphorylated JNK.

Immunohistochemistry

Neurons were stained for IL-1RI and phosphorylated JNK as previously described (Nolan *et al.*, 2004). Briefly, cells were washed with TBS, fixed in 4% paraformaldehyde, washed in TBS were permeabilised with Proteinase K (20μg/ml) in 0.1% TritonX100 in TBS (w/v) for 8min. Cells were washed, refixed in 4% paraformaldehyde for 10min, washed, blocked with blocking buffer (10% NGS in TBS; v/v) for 2h and incubated with a phosphorylated JNK mouse IgG antibody (1:200 in 2.5% NGS in TBS, Santa Cruz Biotechnology Inc., USA) overnight at 4°C, or IL-1RI (1:200 in 2.5% NGS; Santa Cruz Biotechnology Inc., USA). Cells were stained by incubating in fluorescently tagged secondary antibody for 1h at 37°C in darkness; goat anti mouse IgG and IgM FITC linked antibody (1:100 in 2.5% NGS in TBS, BioSource, UK) for analysis of p-JNK. Biotinylated anti-rabbit secondary antibody (1:50 in 2.5% NGS; Vector Laboratories, USA) was incubated

with cells probed for IL-1RI for 2h at room temperature, washed in TBS followed by an incubation in the dark for 2h with ExtrAvidin FITC conjugated antibody (1:50; Sigma, UK). All cells were then washed in TBS, and coverslips were sealed using fluorescent mounting medium (Vectastain, Vector, UK) and stored in the dark at 4°C until viewed under a Zeiss fluorescence microscope with the appropriate filter (FITC – excitation 495nm and emission 525nm)

Frozen cryostat sections were prepared for assessment of activated caspase-3 staining. All procedures were carried out at room temperature unless otherwise stated. Brain sections were allowed to thaw, and surrounded by Pap Pen. The tissue was rehydrated in saline solution for 5min, washed twice in PBS for 5min and fixed for 20min with 4% paraformaldehyde, and washed. The cells were permeabilised with Proteinase K (20μg/ml), TritonX (0.2%) in PBS for 10min, washed, and refixed in 4% paraformaldehyde for 5min, and washed again. Endogenous peroxidases were blocked by immersion in 0.3% H_2O_2 in 0.3%NGS in PBS for 15min. Following washing the sections were incubated with a blocking buffer (10% NGS in 4% BSA in PBS) for 2h, and then incubated with an anti-active caspase-3 antibody (1:100; Promega, USA; in 5% NGS in 2% BSA in PBS) overnight at 4°C. Sections were washed before incubation with an anti-rabbit IgG biotinylated antibody (1:100; Vector, UK; in 2% BSA in PBS). Following washing, sections were incubated with reagent mix (2 drops reagent A, 2 drops reagent B, in 5ml PBS; ABC *Elite* Kit; Vector, UK) for 1h at room temperature, and washed. Staining was developed by addition of the chromogene DAB for 10-15min. This reaction was terminated by immersion in distilled water. Cells were counter stained with toluedene blue, dehydrated through alcohol to xylene, mounted in DPX and viewed under a light microscope under x40 magnification.

Statistical Analysis

Statistical evaluation was carried out using a student's t-test for independent means, or when appropriate, using one-way analysis of variance. When the latter analysis indicated significance (at the 0.05 level), post hoc student Newmann-Keuls test analysis was used to determine which conditions were significantly different from each other.

RESULTS

Intraperitoneal administration of LPS significantly increased IL-1RI expression in hippocampus ($p < 0.05$; student's t-test for independent means; Figure 1a) and this was mirrored by an LPS-induced increase in staining for IL-1RI in hippocampal neurons (compare (ii; LPS-treated cells) with (i; controls)). In addition to the LPS-induced increase in IL-1RI expression, we also confirmed earlier findings by showing that LPS significantly increased IL-1mRNA (from 0.72 (± 0.02; SEM; arbitrary units) in control tissue, to 0.92 (± 0.04) in hippocampal tissue prepared from LPS-treated rats) and IL-1β protein (from 182pg/mg (± 35.9; SEM) in control tissue, to 352.7 (± 21.25) in hippocampal tissue prepared from LPS-treated rats. These changes were accompanied by increased expression of phosphorylated JNK (pJNK; $p < 0.05$; student's t-test for independent means; Figure 1b) and in hippocampal neurons (compare (iv; LPS-treated cells) with (iii; controls)). We analysed the correlation between expression of pJNK and LTP (as assessed by the mean percentage change in the population epsp slope 35-40min following tetanic stimulation) from previously published studies (Vereker *et al.*, 2000a; Lonergan *et al.*, 2002); there was a significant inverse relationship between the measures ($p < 0.001$; Figure 1c) suggesting that activation of JNK significantly contributed to the LPS-triggered attenuation of LTP. In addition to these changes, we observed that LPS injection led to a significant decrease in hippocampal concentration of IL-4 ($p < 0.05$; student's t-test for independent means; Figure 2a). We considered that this might contribute to the LPS-induced increases in IL-1RI expression and JNK phosphorylation and therefore assessed the ability of IL-4 to modulate these LPS-

induced changes. The data indicate that the LPS-induced increase in IL-1RI expression on hippocampal neurons (Figure 2b; compare (ii; LPS-treated cells) with (i; controls)) was markedly reduced when cells were coincubated in the presence of IL-4 (Figure 2b; (iv)) while IL-4 alone exerted no appreciable effect (iii). IL-4 also abrogated the LPS-induced increase in expression of pJNK; thus while JNK phosphorylation was significantly increased in hippocampal tissue prepared from LPS-treated rats compared with saline-treated control rats ($p < 0.05$; ANOVA; Figure 2c), the mean value in tissue prepared from animals which received both LPS and IL-4 was similar to that in controls. In parallel with the effect of LPS on JNK phosphorylation, we demonstrate that phosphorylation of c-jun (p-c-jun) was significantly increased in tissue prepared from LPS-treated, compared with saline-treated, rats ($p < 0.05$; ANOVA; Figure 2d). IL-4 partially reversed this effect so that the residual increase in expression of p-c-jun which was observed in tissue prepared from rats which were treated with LPS and IL-4 was not statistically different from the control value.

Our hypothesis is that JNK phosphorylation was necessary for LPS-induced inhibition of LTP and therefore we predicted that, since IL-4 prevented the LPS-induced increase in JNK phosphorylation, it would also prevent the effect of LPS on LTP. Here we demonstrate that tetanic stimulation of the perforant path resulted in an immediate and marked increase in the slope of the epsp in rats which received saline intraperitoneally and intracerebroventricularly, but saline-treated rats which received LPS exhibited a marked impairment in LTP (Figure 3a). The LPS-induced deficit in LTP was attenuated by pre-treatment with IL-4; the mean percentage change (± SEM) in the 2min period immediately following tetanic stimulation in saline-treated and LPS-treated rats were 162.30 (± 3.35) and 130.30 (± 1.70; $p < 0.001$; ANOVA), whereas the corresponding value in LPS-treated rats which received IL-4 was 166.20 (± 2.78; $p < 0.001$ compared with the value in the rats treated with LPS). The mean percentage changes in the last 5 min of the experiment were 126.60 (± 0.97), 104.50 (± 0.85) and 127.90 (± 0.96) in the saline-treated control group, the

group treated with LPS and the groups treated with LPS and IL-4 respectively, which represented a significant difference between saline- and LPS-treated rats ($p < 0.001$; ANOVA) and between LPS-treated rats which received saline and IL-4 ($p < 0.001$; ANOVA). IL-4 treatment alone exerted only a small effect of the mean slope of the epsp; the values in the 2min immediately following the tetanus and the last 5min of the experiment were 148.60 (± 2.94) and 122.40 (± 0.87) respectively.

These data couple the LPS-induced impairment in LTP with an increase in activation of JNK and to further investigate this interaction, we assessed the effect of the JNK inhibitor, D-JNKI1, on LPS-induced changes in hippocampus. The primary action of D-JNKI1 is to block downstream actions of JNK since it contains the minimal 20-amino acid inhibitory domains of JNK-interacting protein (JIP) that competitively blocks the interaction between JNK and its targets. Here we report that D-JNKI1 abrogates the LPS-induced increase in c-jun phosphorylation; this is demonstrated in the sample immunoblot (Figure 4) and confirmed by analysis of the mean data obtained from densitometric assessment, which indicated that mean expression of phosphorylated c-jun was significantly increased in tissue prepared from saline-treated rats which received LPS intraperitoneally compared with rats which received saline only ($p < 0.05$; ANOVA). Pretreatment of saline-injected rats with D-JNKI1 inhibited the effects of LPS so that the value was similar in tissue prepared from this group of rats and rats which received saline only. D-JNKI1 pretreatment did not attenuate the LPS-induced changes in JNK phosphorylation, which is consistent with the view that the inhibitor acts downstream of JNK activation (data not shown).

If c-jun activation and the consequent downstream effects of c-jun activation are critical in mediating the inhibitory effect of LPS on LTP, then it must be predicted that pre-treatment of rats with D-JNKI1 will abrogate the LPS-induced attenuation of LTP. The data shown in Figure 5 indicate that tetanic stimulation of the perforant path resulted in an immediate and marked increase in the slope of the epsp in control-treated rats which received saline

intraperitoneally, but control-treated rats which received LPS intraperitoneally (n = 12) exhibited a marked impairment in LTP. Analysis of the mean epsp slopes in the 2min immediately following tetanic stimulation indicated that there was a significant difference in the response of these two groups of rats to the tetani ($p < 0.001$; ANOVA). Thus the mean percentage changes in population epsp slopes (± SEM), compared with the epsp slope in the 5min immediately prior to tetanic stimulation were 155.4 (± 3.3) and 124.8 (± 0.8) respectively (Figure 5b). The mean percentage change in population epsp slope 35-40 min post tetanus in saline-treated rats was 116.0 (± 1.4) which was significantly different from that observed in control-treated rats which received LPS i.p (97.44; ± 0.6; $p < 0.001$; ANOVA; Figure 5c). Pretreatment of rats which received LPS intraperitoneally with D-JNKI1 slightly attenuated the early effect of LPS and reversed the later effect; thus the mean percentage changes in population epsp slopes in the 2 min immediately following tetanic stimulation and in the last 5 minutes of the experiment were 134.2 (± 1.5) and 112.4 (± 0.8) respectively, which in the latter case represented a statistically significant difference from the value obtained in control-treated rats which received LPS i.p ($p < 0.01$; ANOVA). The mean percentage changes in population epsp slopes in the 2 min immediately following tetanic stimulation and in the last 5 minutes of the experiment in D-JNKI1-treated rats which received saline intraperitoneally were 134.2 (± 1.5) and 110.3 (± 0.3) respectively; thus the early response to tetanic stimulation was attenuated in these rats compared with saline-treated control rats ($p<0.001$; ANOVA) but the later values were similar in the two groups.

In an effort to provide support for the finding that LPS induces cell stress, as indicated by the increase in activation of JNK and c-jun, we assessed activation of caspase-3 in cryostat sections obtained from hippocampus of rats from each treatment group. Consistent with previous reports, we demonstrate that JNK activation was associated with marked staining for activated caspase-3 in granule cells of the dentate gyrus; thus LPS

increased the number of cells which stained positively for activated caspase-3 (Figures 6; compare (ii; LPS-treated) with (i; saline-treated)). Cotreatment with LPS and D-JNKI1 (Figure 6(iv)) or IL-4 (Figure 6(vi)) prevented the LPS-induced effect of caspase-3, while there was no substantial effect with either D-JNKI1 (Figure 6(iii)) or IL-4 (Figure 6(v)) alone.

DISCUSSION

We set out to investigate whether activation of JNK was critical in mediating the detrimental effects of LPS in hippocampus; accordingly we adopted 2 strategies which we considered would address the question and investigated the ability of the JNK inhibitor D-JNKI1, and the ability of IL-4 which attenuates IL-1β-associated changes, to reverse the effects of intraperitoneally-administered LPS. The data indicate that inhibition of JNK by either means blocked the LPS-induced inhibition of LTP and identify activation of JNK and c-jun as pivotal events.

The data presented show that intraperitoneal injection of LPS increases IL-1RI expression in hippocampus; this change accompanied increases in IL-1β mRNA and protein in hippocampus which have been previously reported (Kelly *et al.*, 2003; Lynch *et al.*, 2004) and which were also observed in this study. LPS injection also resulted in increased activation of JNK and this was paralleled by evidence of a similar change *in vitro*; thus the LPS-induced increase in JNK phosphorylation, which has been reported in several cells and tissues including hippocampus (Vereker *et al.*, 2000a; Loscher *et al.*, 2000; Nolan *et al.*, 2002), also occurs following exposure of hippocampal neurons to LPS. Here we also report that these changes are associated with a decrease in hippocampal concentration of the anti-inflammatory cytokine, IL-4. This finding suggests that the inflammatory changes associated with LPS injection, which have commonly been attributed to increased production of IL-1β, may be compounded by the decrease in IL-4

concentration. However it must be acknowledged that several effects of LPS, which might contribute to the increase in JNK activation have been reported; for example, LPS increases production of reactive oxygen species in hippocampus and cortex (Nolan et al., 2003) and this has been linked with increased activation of JNK (Lonergan et al., 2002).

We argued that if IL-1β played a significant role in triggering the LPS-induced changes then blocking its subsequent actions would abrogate the effects of LPS. Similarly, if the decrease in IL-4 concentration was a contributory factor, then administration of IL-4 might abrogate the effects of LPS. The data show that IL-4 inhibited the increase in IL-1RI expression associated with LPS treatment, and the associated increase in activation of JNK. Consistent with the commonly-held view that c-jun is activated as a consequence of JNK activation, we also observed that IL-4 partially abrogated the LPS-induced increase in c-jun phosphorylation. One of the most significant findings of the present study is that the modulatory effect of IL-4 on LPS-induced activation of JNK and c-jun was accompanied by restoration of the synaptic response to tetanic stimulation. Thus the LPS-induced attenuation of LTP was abrogated by IL-4 and this effect is very similar to our recent result which showed that IL-4 also suppressed the IL-1β-induced inhibition of LTP (Nolan *et al.*, unpublished). In addition to its effects on LTP, LPS disrupts memory consolidation and inhibits various forms of learning including spatial learning (Pugh *et al.*, 1998; Shaw *et al.*, 2001; Jain *et al.*, 2002). It also induces sickness behaviour (Bluthé *et al.*, 1992; Dantzer *et al.*, 1998; Lacosta *et al.*, 1999) and, in this case, it has been reported that the LPS-induced effect is blocked by IL-4 (Bluthé *et al.*, 2002). The question of the mechanism of action of IL-4 in these circumstances is an important one and several studies have demonstrated that its key anti-inflammatory actions result from its ability to inhibit expression, release and downstream actions of proinflammatory cytokines (Brown and Hural, 1997; te Velde *et al.*, 1990; Vannier *et al.*, 1992). Concurring with these findings, the present data suggests that,

in hippocampus at least, it decreases expression of IL-1RI and consequently downregulates IL-1β-stimulated signalling cascades.

IL-4 receptors have been identified on haematopoetic, endothelial and epithelial cells (Nelms *et al.*, 1999), while reports of receptor expression on brain-derived cells has been confined to cultured glial cells (Brodie *et al.*, 1998; Molina-Holgado *et al.*, 2001; Szczepanik *et al*,. 2001). However, recent findings using immunohistochemistry in cryostat sections prepared from hippocampus and in hippocampal neurons in culture demonstrated the presence of IL-4 receptors on neurons and this has been confirmed by gel electrophoresis and immunoblotting (Maher *et al.*, 2004; Nolan *et al.*, unpublished). In hippocampus, as in other tissues, the interaction of IL-4 with its receptor leads to activation of the JAK-STAT pathway (Maher *et al.*, 2004; Nolan *et al.*, unpublished). It remains to be established whether the action of IL-4 in the present study relies on IL-4-receptor activation of the JAK/STAT pathway; in this regard it is interesting to note that intracerebroventricular injection of IL-4 resulted in increased activation of JAK1 and STAT6 in hippocampus and that these changes were attenuated in rats which were injected with both IL-4 and LPS (data not shown).

There is broad agreement in the literature that, in haemapoetic cells, IL-4 can act to abrogate the increase in JNK activation induced by inflammatory stimuli like TNF, possibly by downregulating TNF receptors (Chan and Riches, 1998; Manna and Aggarwal, 1998), which is analogous to the effect of IL-4 on expression of IL-1RI which is described here. Our present findings, as well as earlier data which pinpointed a potentially pivotal role for JNK phosphorylation in inhibiting LTP, led us to assess the effect of D-JNKI1 and here we demonstrate that the inhibition of LTP in perforant path granule cell synapses induced by LPS is abrogated by pre-treatment of rats with D-JNKI1. It can therefore be proposed that JNK activation and the downstream consequences play a pivotal role in inhibition of LTP. This is consistent with several reports which revealed an inverse

correlation between JNK activation and inhibition of LTP in IL-1β-treated (Vereker *et al.*, 2000b, Kelly *et al.*, 2001), LPS-treated (Vereker *et al.*, 2000a, Nolan *et al.*, 2002), and Aβ-treated (Minogue *et al.*, 2003) rats, as well as aged rats (O'Donnell *et al.*, 2000) and rats exposed to irradiation (Lonergan *et al.*, 2002). Predictably the LPS-associated increase in c-jun activation was inhibited by D-JNKI1, an effect which can be explained by the fact that the inhibitor competitively blocks the interaction between JNK and its targets. Although these data identify a clear role for activation of JNK and c-jun in inhibiting LTP, the question of how these changes lead to impairment in LTP remains open. One possibility is that a cascade of reactions is triggered which lead to deterioration, or even death, of cells (Herdegen *et al.*, 1998; Behrens *et al.*, 1999; Le-Niculescu *et al.*, 1999; Minogue *et al.*, 2003; Lonergan *et al.*, 2004). Here we show that, in parallel with the increases in JNK and c-jun activation, LPS markedly increases the number of hippocampal cells, specifically granule cells of the dentate gyrus, which stain positively for activated caspase-3, a reliable marker of cell stress and/or cell death (Green and Reed, 1998; Kuan *et al.*, 1999). Thus the cell stress induced in hippocampal cells by LPS administration may contribute to the impairment in synaptic function which we report here. Significantly, both IL-4 and D-JNKI1 pretreatment inhibit the LPS-induced activation of JNK and caspase-3, and also the impairment in LTP. A similar set of parallel changes have been described in hippocampus of aged rats (Lynch and Lynch, 2002), Aβ-treated rats (Minogue *et al.*, 2003) and rats which were exposed to irradiation (Lonergan *et al.*, 2002), strengthening the argument of a causal interaction between these cellular events.

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Figure 1

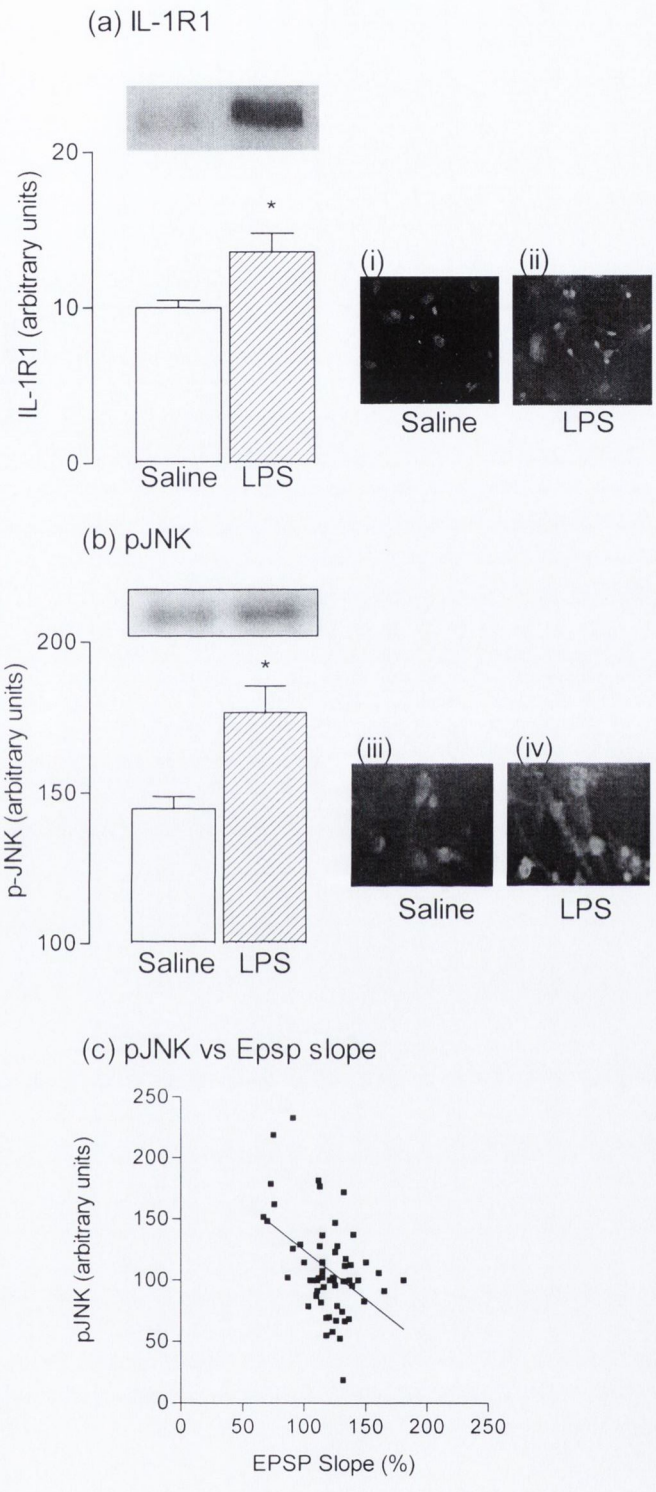
(a) IL-1R1
IL-1R1 (arbitrary units)
20
10
0
*
Saline
LPS
(i)
(ii)
Saline
LPS
(b) pJNK
p-JNK (arbitrary units)
200
150
100
*
Saline
LPS
(iii)
(iv)
Saline
LPS
(c) pJNK vs Epsp slope
pJNK (arbitrary units)
250
200
150
100
50
0
0
50
100
150
200
250
EPSP Slope (%)

Figure 2

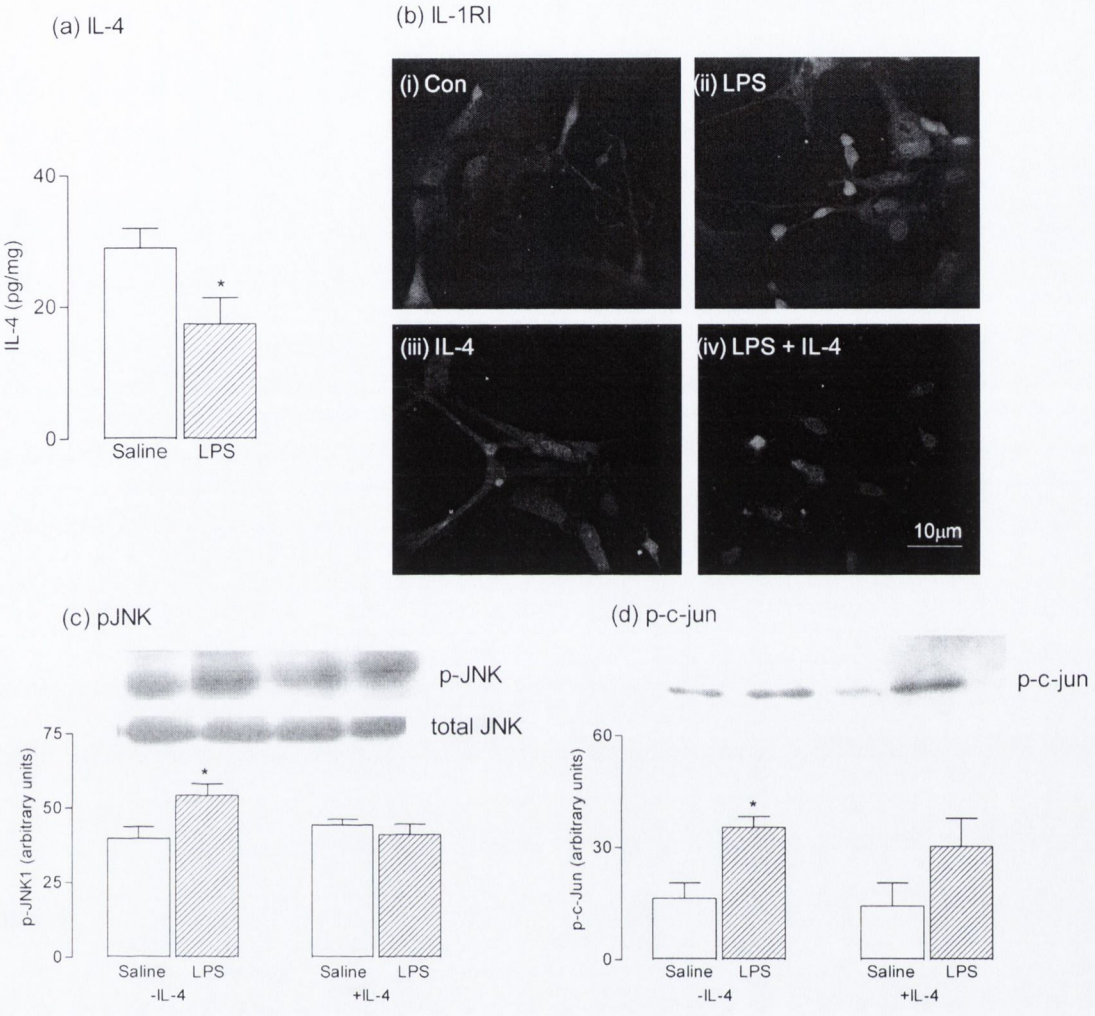
(a) IL-4
IL-4 (pg/mg)
40
20
0
Saline
LPS
*
(b) IL-1RI
(i) Con
(ii) LPS
(iii) IL-4
(iv) LPS + IL-4
10μm
(c) pJNK
p-JNK
total JNK
p-JNK1 (arbitrary units)
75
50
25
0
Saline
LPS
-IL-4
+IL-4
(d) p-c-jun
p-c-jun
p-c-Jun (arbitrary units)
60
30
0

Figure 3

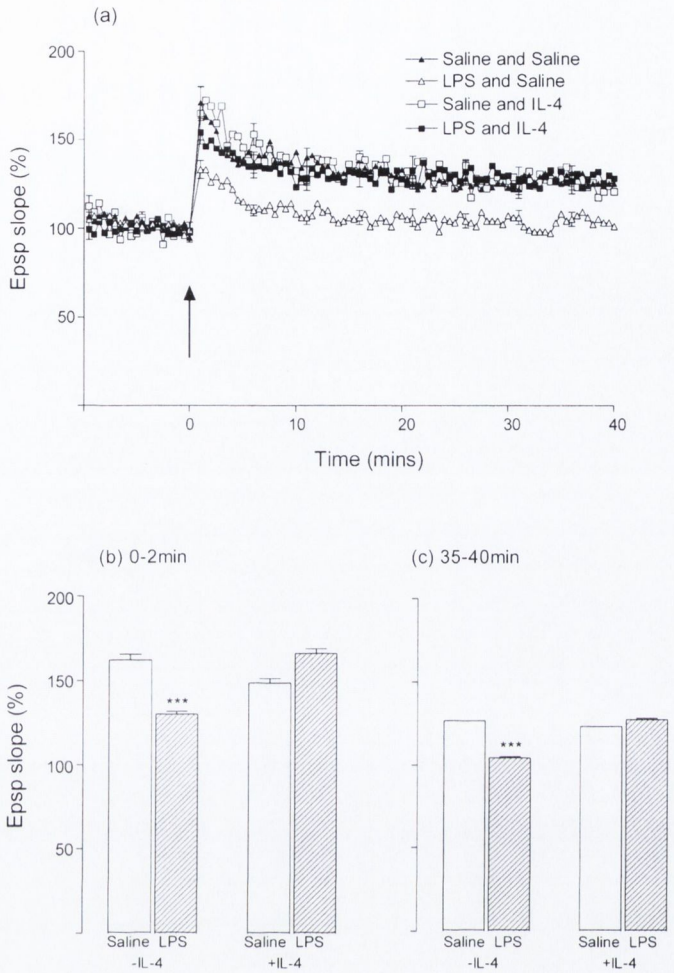

Figure 4

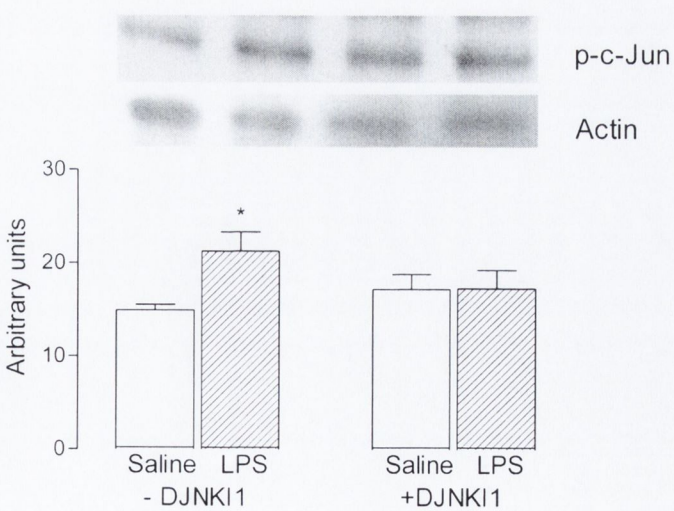

Figure 5

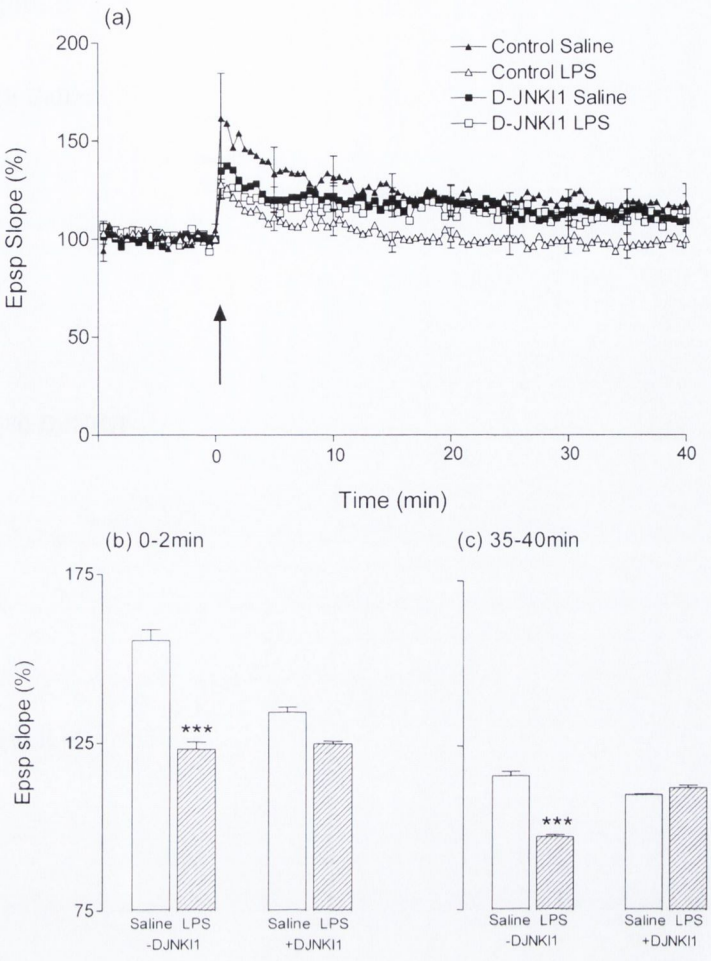
(a)
Control Saline
Control LPS
D-JNKI1 Saline
D-JNKI1 LPS
Epsp Slope (%)
200
150
100
50
0
0
10
20
30
40
Time (min)
(b) 0-2min
(c) 35-40min
Epsp slope (%)
175
125
75
***
***
Saline LPS
-DJNKI1
Saline LPS
+DJNKI1
Saline LPS
-DJNKI1
Saline LPS
+DJNKI1

Figure 6

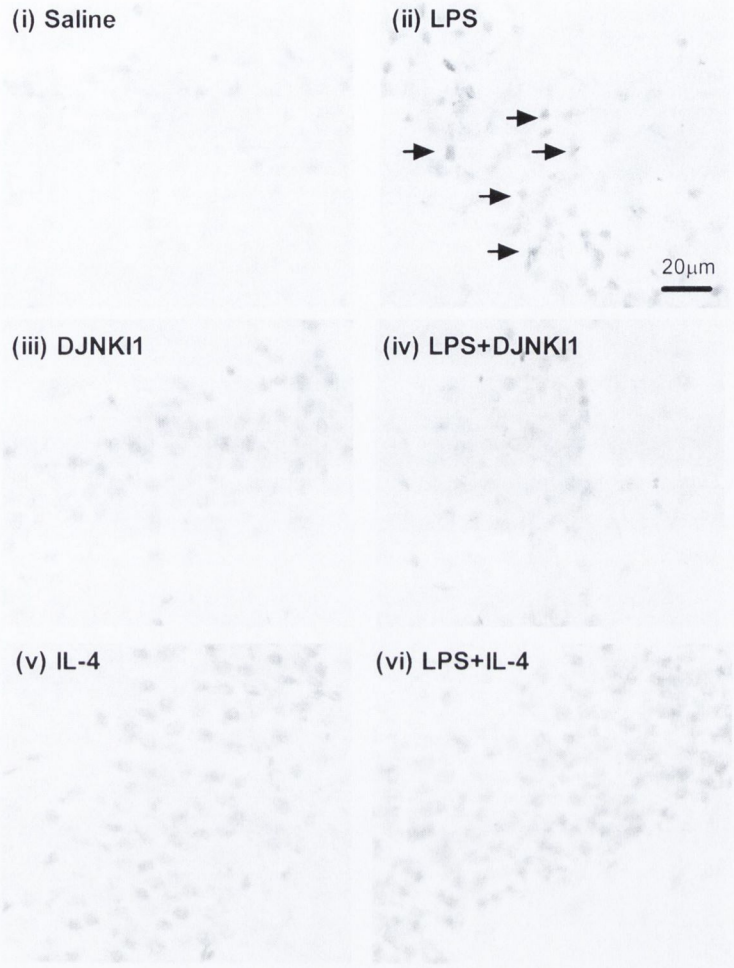

Figure 7

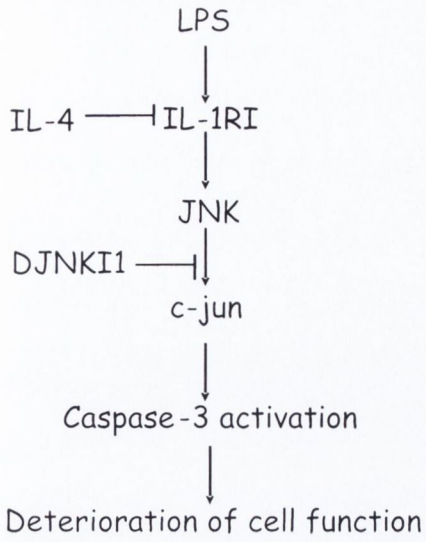
LPS
IL-4
IL-1RI
JNK
DJNKI1
c-jun
Caspase-3 activation
Deterioration of cell function

Figure legends

Figure 1 LPS administration increases expression of IL-1R1 and activity of JNK. Intraperitoneal injection of LPS (100μg/kg) resulted in a significant enhancement of (a) IL-1R1 expression and (b) JNK phosphorylation in hippocampus as shown by a sample immunoblot and analysis of the mean data obtained from densitometric analysis (*$p<0.05$; student t test, in each case; n=6 and n=5 respectively). Similarly, primary hippocampal neurons exposed to LPS (100ng/ml) for 24hr exhibited increased levels of fluorescent staining for (a) IL-1R1 and (b) phosphorylated JNK compared to controls. (c) An increase in JNK phosphorylation was inversely correlated with the ability to sustain LTP ($p<0.001$).

Figure 2 IL-4 administration attenuates the LPS-induced effects on IL-1R1 expression, JNK phopshorylation and c-Jun phosphorylation. (a) Intraperitoneal injection of LPS (100μg/kg) resulted in a significant decrease in IL-4 concentration in hippocampus (*$p<0.05$; student's t-test; n=6). (b) Treatment with IL-4 attenuated the LPS-induced increase in IL-1R1 expression in primary cultured neurons. LPS significantly enhanced expression of (c) JNK phosphorylation and (d) c-Jun phosphorylation in hippocampus as shown by sample immunoblots and analysis of the mean data obtained from densitometric analysis (*$p<0.05$; ANOVA; n=6), while intracerebroventricular injection of IL-4 attenuated these LPS-induced effects.

Figure 3 IL-4 abrogates the inhibitory effects of LPS on LTP. (a) Intraperitoneal injection of LPS blocked tetanus-induced LTP in perforant path – granule cell synapses, but this effect was suppressed by intracerebroventricular injection of IL-4. Data are expressed as the mean percentage change in population epsp slope (relative to the mean epsp slope in the 5min immediately prior to tetanic stimulation). Values are means (n=6 for each group) and bars denoting ±SEM are included at 5min intervals. The mean

percentage changes in epsp slope in the 2min (b) and 35-40min (c) following tetanic stimulation indicate the significant inhibitory effect of LPS on LTP (***$p<0.001$; ANOVA in each case). Co-treatment with IL-4 abrogated the inhibitory effects of LPS on LTP.

Figure 4 D-JNKI1 pretreatment attenuates the LPS-induced increase in c-Jun phosphorylation. LPS significantly increased c-Jun phosphorylation in hippocampus as shown by the sample immunoblot and analysis of the mean data obtained from densitometric analysis ($p<0.05$; ANOVA; $n=6$). Intracerebroventricular injection of D-JNKI1 reduced the LPS-induced increase in c-Jun phosphorylation.

Figure 5 D-JNKI1 abrogates the inhibitory effects of LPS on LTP. (a) Intraperitoneal injection of LPS blocked tetanus-induced LTP in perforant path – granule cell synapses, but this effect was suppressed by i.c.v. injection of D-JNKI1. Data are expressed as the mean percentage change in population epsp slope (relative to the mean epsp slope in the 5min immediately prior to tetanic stimulation). Values are means ($n \geq 6$ for each group) and bars denoting ±SEM are included at 5min intervals. The mean percentage changes in epsp slope in the 2min (b) and 35-40min (c) following tetanic stimulation indicate the significant inhibitory effect of LPS on LTP (***$p<0.001$; ANOVA). Pretreatment with D-JNKI1 attenuated the later effect of LPS (c), however LTP sustained by the control saline group was greater than that sustained by either of the D-JNKI1-treated groups ($p<0.01$; ANOVA).

Figure 6 D-JNKI1 and IL-4 both attenuated the LPS-induced increase in hippocampal cells positively stained for activated caspase 3 in cryostat sections.

Positive staining for activated caspase 3 was markedly increased in the hippocampal region of cryostat sections prepared from control-treated rats which received LPS when compared