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Analysis of the pro- and anti-inflammatory signalling in the aged hippocampus: effect of dexamethasone and vitamin D_3

By

Michelle E. Moore

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

For my Mam and Dad

I. Declaration

I declare that this thesis is entirely my own work with the following exceptions: certain photomicrographs were kindly donated by Yvonne Nolan and Yvonne Mc Cartney. 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.

Michelle Moore

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II. Summary

The objectives of this study were to establish the cellular events and alterations in signalling associated with ageing and to determine the effectiveness of two anti-inflammatory agents, dexamethasone and vitamin D_3, in attenuating the age-related changes in the hippocampus. The findings of this study report an increase in interleukin (IL)-1β concentration and as it is postulated that a balance exists between the pro- and anti-inflammatory signalling in the brain, a concurrent decrease in IL-10 concentration was also observed in the aged hippocampus. As a consequence, it is not surprising that the activity of downstream IL-1β signalling mediators, namely c-Jun N-terminal kinase (JNK) and caspase-3, were upregulated, while IL-10 signalling, as evidenced by the phosphorylation of Janus kinase1 (JAK1) and signal transducers and activators of transcription-3 (STAT-3), was diminished. The data presented indicate cell deterioration in the aged rat hippocampus as suggested by the increased cleavage of poly (ADP-ribose) polymerase, and this concurs with the impairment in long-term potentiation (LTP) associated with age. This thesis investigated whether the effects of treatment with dexamethasone and vitamin D_3 in the periphery were mimicked in the aged hippocampus. These data report abrogation of the age-related imbalance in inflammatory signalling in the hippocampus following treatment with dexamethasone and vitamin D_3.

An investigation into the cellular source of IL-1β was undertaken; this elucidated microglia as the main producers of IL-1β in the aged brain. Interferon (IFN)-γ, whose concentration was augmented in the aged hippocampus, was found to consistently stimulate microglial release of IL-1β. Infiltration of the aged brain by natural killer cells was observed and these cells were shown to release IFN-γ upon stimulation. These data, along with the finding that IFN-γ release from neurons and glia was undetectable, suggest that the source of IFN-γ in the aged hippocampus is natural killer cells.

The most significant finding of this study is that dexamethasone and vitamin D_3 can restore the balance between pro- and anti-inflammatory signalling in the aged

hippocampus, as evidenced by the attenuation of impairment in synaptic function, characterized by LTP.

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Finally, thanks to my family and Eoghan for their support and understanding over the last three years.

IV. Table of Contents

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VI. List of Abbreviations

The following abbreviations are used:

^0C	Degrees Celsius
µg	microgram
µl	microlitre
µM	micromolar
$1,25(OH)_2D_3$	1, 25-dihydroxyvitamin D_3
Aβ	Amyloid-β
ACTH	Adrenocorticotropin hormone
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Antigen presenting cell
ATF	Activating transcription factor
BBB	Blood-brain barrier
BSA	Bovine albumin serum
$CD4^+$	CD4 positive
$CD161^+$	CD161 positive
CIITA	MHC Class II transactivator
CNS	Central nervous system
COX	Cycloxygenase
CR	Caloric restriction
CRF	Corticotrophin releasing factor
CSF	Cerebrospinal fluid
DC	Dendritic cell
DJNK1I	c-Jun N-terminal kinase peptide inhibitor 1, D-stereoisomer
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme linked immunosorbent assay

EPA	Eicosapentanoic acid
EPSP	Excitatory postsynaptic potential
g	gram
GR	Glucocorticoid receptor
HFS	High frequency stimulation
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary axis
HRP	Horseradish peroxidase
IBD	Inflammatory Bowel Disease
ICAM	Intracellular adhesion molecule
ICE	Interleukin-1β converting enzyme
ICV	Intracerebroventricular
IL-1β	Interleukin-1β
IL-1RI	Interleukin-1 type I receptor
IL-1RII	Interleukin-1 type II receptor
IL-1ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-10R	Interleukin-10 receptor
IL-12	Interleukin-12
IFN-γ	Interferon-γ
iNOS	Inducible nitric oxide
IRF	Interferon regulating factor
JAK	Janus kinase
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
LFS	Low-frequency stimulation
LPS	Lipopolysaccharide

LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
mg	milligram
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein-1
mM	millimolar
mRNA	messenger Ribonucleic acid
ms	millisecond
mtDNA	mitochondrial DNA
mV	millivolt
MS	Multiple Sclerosis
NFkB	Nuclear factor kB
ng	nanogram
NGF	Nerve growth factor
NK	Natural killer
nM	nanomolar
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly- (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-tween
PGE	Prostaglandin E
PLA_2	Phospholipase 2
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulphate
STAT	Signal transducers and activators of transcription

TBS	Tris buffered saline
TBS-T	Tris buffered saline-tween
TGF β	Transforming growth factor β
Th	T helper cell
TNF- α	Tumour necrosis factor-α
UV	Ultraviolet
VDR	Vitamin D_3 receptor
w/v	Weight per volume

Chapter 1

Introduction

1.1 Ageing

1.1.1 The Ageing Body

Ageing is an intrinsic property and is discernible at all levels of a living organism. Consistent in all aged cells are their loss of division capacity, functional decline and failure of homeodynamics. These features are evident in genetically altered or environmentally challenged cells prior to their development in normal cells (Kaul *et al.*, 2003). The ageing process does not occur because the living organism is equipped with specific genes to cause ageing, but as a result of imperfect maintenance and repair mechanisms both at the genetic and cellular level (Kaul *et al.*, 2003). Cellular signaling pathways are a necessity for the normal function of the cell and the entire living system, however a slight alteration in this pattern, disturbing homeostasis of the body, will lead to the dysfunction and dysregulation which are manifestations of the ageing processes (Kaul *et al.*, 2003).

1.1.2 Theories of Ageing

Because the central nervous system is crucial in maintaining homeostasis, it is suggested that it is a major target for disruption during ageing process. In fact, hippocampal formation is one of the most susceptible regions during ageing (Hasan and Glees, 1973). Many neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease manifest only in the aged brain, indicating neural dysfunction and susceptibility which develops with age. Several changes have been described with age which probably contribute to the deficits in function observed. For example, changes in receptor expression and function have been noted e.g. NMDA receptor binding decreases with age (Ingram et al., 1992; Bonhaus et al., 1990; Shigenaga *et al.*, 1994) and is paralleled by cognitive decline (Barnes, 1988). Deficits observed in aged experimental animals are closely linked to loss of dopamine (Roth and Joseph, 1994; Shigenaga *et al.*, 1994) and 5HT2 receptors (Slotkin *et al.*, 2005; Shigenaga *et al.*, 1994). Decreases in neurotransmitters such as acetylcholine, dopamine and noradreneline (see Gottfries, 1990; Trollor and Valenzuela, 2001) have been shown, while the loss of particular neurons, especially cholinergic neurons, has been linked with functional deficits (Gu *et al.*, 2000). Neurotrophins modulate LTP (Lynch, 2004) and thus, it is not surprising that

the age-related decline in function is mirrored by a decrease in NGF release (Kelly *et al.*, 2000), and related signalling molecules, namely trk B (Croll *et al.*, 1998) and ERK expression (Hu *et al.*, 1998) in the aged brain.

Lipid peroxidation in the aged brain alters the functioning of receptors, membrane-associated enzymes, ion channels and pumps and consequent signal transduction. A decline in immune competence, with compromised innate and acquired immunological responses, is characteristic of the ageing body (Smith *et al.*, 2001). Evidence of these changes, along with many others, contribute to the ageing process and development of neurodegenerative diseases. Several theories of ageing proposed and supported by experimental findings include "The free radical theory of ageing", "The membrane hypothesis of ageing" and "The immune theory of ageing".

1.1.3 The Free Radical Theory of Ageing

Harman proposed that accumulation of detrimental changes in cells and tissues of the body which occur during a lifetime can hinder function and eventually terminate in cell death (Harman, 2001). Specifically, he suggested that accumulation of reactive oxygen species (ROS) may play a significant role. ROS is produced as a by-product of aerobic metabolism (Shigenaga *et al.*, 1994), which is part of the normal physiological and metabolic processes necessary for cell viability and function. Some of the most common examples of ROS are the superoxide radical ($O_2^{\cdot-}$), H_2O_2 and the hydroxyl radical ($OH^{\cdot}$; Shigenaga *et al.*, 1994; Schipper, 2004). These molecules target macromolecules such as proteins and lipids and generate subsequent radicals from these attacked molecules, thereby establishing a positive feedback loop for their own propagation (Troen, 2003).

Cells are equipped with "antioxidant defenses" which balance the damaging effects of ROS with their essential and inevitable production (Yu, 1994). These defenses are in the form of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidases as well as non-enzymatic scavengers such as ascorbate and tocopherol (Schipper, 2004). SOD is regarded as being the primary defense against oxidative damage, because it terminates the generation of further free radicals by dismutation of the

superoxide radical. It is active principally intracellularly and localised within the cytoplasm and mitochondrial compartments, however it does exert its effects extracellularly as well (Yu, 1994). Interestingly, transgenic flies with overexpression of SOD have been found to live longer than their wildtype counterparts (Troen, 2003). Further intrinsic defense is provided by the action of glutathione peroxidase. This is found intracellularly within the cytosol and mitochondrial matrix and catalyses the conversion of H_2O_2 into H_2O (Yu, 1994). The amount of oxidative stress that the cell is exposed to is determined by the balance between ROS production and ROS scavagening (Bokov *et al.*, 2004).

As mitochondria are present within virtually all cells of the body, a failure in the normal functioning of mitochondria can impact negatively on the ability of the cell to cope with physiological stresses (Shigenaga *et al.*, 1994). Mitochondrial dysfunction has been suggested as being a prominent factor in propagating the ageing process (see Trollor and Valenzuela, 2001; Shigenaga *et al.*, 1994). It is agreed that mitochondria are the greatest source of ROS and are also the prime target for oxidative damage (Shipper, 2004; Shigenaga *et al.*, 1994; Yu, 1994). De la Asuncion and colleagues (1996) illustrated the link between oxidative damage and mitochondrial dysfunction associated with age. ROS targets mitochondrial DNA (mtDNA) which has been found to be particularly susceptible to oxidative damage (Schipper, 2004). Furthermore, mutations in mtDNA accumulate as a function of age in the brain and this is perhaps a contributory factor in the development of age-related neurodegenerative diseases (Shigenaga *et al.*, 1994; Troen, 2003; Schipper, 2004; Forster et al., 1996). Studies testing the cognitive ability (Forster *et al.*, 1996; Pellmar *et al.*, 1991) and motor activity (Forster *et al.*, 1994; de la Asuncion *et al.*, 1996) in aged animals revealed a correlation between the impairment of these functions and oxidative damage.

In addition to the intrinsic defense mechanisms which deal with ROS, interventions such as caloric restriction (CR; Bokov *et al.*, 2004) and supplementation with vitamins C and E (Yu, 1994) succeed in preventing accumulation of oxidative damage and in the case of CR, increasing life span and retardation of the ageing process (Bokov *et al.*, 2004).

1.1.4 The membrane hypothesis of ageing

Normal functioning of the brain is considered to depend greatly on the high content of lipids. Lipids exert influence over activities of the brain as they act as enzyme effectors, antioxidants and as substrates for the production of metabolites and they also determine the biophysical nature of membranes, especially membrane stability and fluidity. Clearly, deviations from the optimal lipid content can impact on a host of cellular functions (Zhang *et al.*, 1996b). Youdim and colleagues (2000) stated that the lipid bilayer tends to exist at an optimum transition point between gel and liquid crystal, and the maintenance of this state, often referred to as fluidity, is of paramount importance and depends on the nature of membrane lipids.

In the aged brain, changes in membrane fluidity occur due to alterations in the lipid content of the brain (Zhang *et al.*, 1996b). Increased membrane concentration of cholesterol (Yehuda *et al.*, 2002) coupled with increases in both dicholol (Zhang *et al.*, 1996b) and sphingomyelin (Giusto *et al.*, 1992) during ageing, disturb the membrane fluidity index, leading to membrane rigidity. Furthermore, decreases in phospholipid metabolism, fatty acid incorporation into the membrane and alterations in the saturation of phopholipid acyl chains have been suggested as factors augmenting membrane rigidity (see Lynch, 1998). Gabbita and colleagues (1997) believe that lipid peroxidation, as a result of oxidative damage, is to be regarded as a contributor to the decline in membrane fluidity. This finding compounds the original hypothesis of Harman, which posits that free radicals contribute to the age-related changes in the CNS. Consistent with this idea is the finding that ROS is increased in the aged brain (Murray and Lynch 1998; see McGahon *et al.*, 1999) and that arachidonic acid and other polyunsaturated fatty acids are targets for peroxidation (Murray *et al.*, 1999). Studies show that aged rats which have increased peroxidation, increased ROS or decreased arachidonic acid content (all of which have been shown to result in membrane rigidity) display impairments in synaptic function e.g. LTP (McGahon *et al.*,1999; Murray and Lynch, 1998; Lynch and Voss ,1994) and neurostransmitter function (Murray *et al.*, 1997; Mullany *et al.*, 1996).

The brain is particularly susceptible to oxidative stress and resultant lipid peroxidation for a number of reasons. Firstly, the content of polyunsaturated fatty acids in the brain is very high and these are primary targets for oxidation. The brain contains limited antioxidant defenses in the form of either enzymatic or non-enzymatic molecules. Furthermore, the brain utilises 25% of the total O_2 intake and thus has a greater production of oxygen radicals by weight than any other organ of the body. Unlike other organs which undergo mitosis to replenish and regenerate itself following damage, the brain is postmitotic and therefore differentiation and cellular repletion does not occur (Gabbita *et al.*, 1997; Murray and Lynch, 1998b). However, all brain areas are not affected equally with respect to membrane fluidity. Several studies identify the hippocampus as one of the most vulnerable and susceptible areas to changes in fluidity (see Yehuda *et al.*, 2002). This is affirmed by the fact that the cholesterol/phospholipid ratio is highest in the hippocampus (Zhang *et al.*, 1996b).

1.1.5 The Immune Theory of Ageing

In 1962, Walford posited the immune theory of ageing which states that the normal process of ageing in man and all animals is pathogenically related to faulty immunological process and may be analogous to autoimmune phenomena involving all body tissues (Meredith and Walford, 1979; Effros, 2005). This aspect of the ageing process is thought to be responsible for development of age-related diseases such as cancer, autoimmune and cardiovascular disease. Numerous studies support the correlations drawn between alterations in immune functions and mortality. For example, C.elegans with increased resistance to bacteria, demonstrate an elongated lifespan (Garsin *et al.*, 2003).

This theory puts forward a number of ways in which ageing impacts on immune system functions. Firstly, it is suggested that the ageing process results from injury to the immune system so that autoantibodies are produced and self attack occurs instead of attack on non-self organisms (Ali, 2005). Cellular injury results in alterations in function and culminates in cell death. Walford also proposed that thymic dysfunction is central to the decline of the immune system; more recently, the link between these two functions

has been elucidated by Fabris. Based on the fact that thymic hormones influence the proliferation and differentiation of T cells, Fabris suggested that as thymic hormone concentrations decrease with age, so does the stimulus for the conversion of stem cells into T cells (Fabris, 1992). Depletion studies and other pharmacological manipulations of noradreneline innervation show that the age-related decline in noradrenergic nerve fibres is associated with altered T and B cell activity as well as decreased IL-2 production (see ThyagaRajan and Felten, 2002). A similar reduction in IL-2 production was observed in aged mice in vivo (Smith *et al.*, 2001). This group also reported an age-related defect in generation of type 2 responses and interestingly lymphocytes from these aged mice produced increased amounts of type 1 cytokines such as IFN-γ and IL-12. This study suggests an increase in pro-inflammatory response and a suppression of an anti-inflammatory response in the ageing body.

A link between the free radical theory of ageing and the immune theory of ageing exists. ROS production is accompanied by cellular damage and in response to this, an imflammatory response is initiated to dispose of injured cells in order to limit further damage to neighbouring cells. In response to this inflammation, heat shock proteins and damage-related molecules such as fibrinectin and fibrinogen are upregulated. Heat shock proteins are associated with the recruitment of macrophages and other cells involved in the inflammatory process, all of which secrete cytokines, chemokines and adhesion molecules. Ongoing and increasing oxidative stress which occurs during ageing, induces constant expression of these molecules, the outcome of which is chronic inflammation (Land, 2004).

1.2 Immunity

Ageing is an innate biological phenomenon. It is the inevitable fate of living creatures and is exemplified by a decline in the activity of essential organs and systems of the body including the immune system (Chakravarti and Chakravarti, 2004).

The tissues and organs of the immune system, which defend the body against foreign and abnormal cells, are part of an extraordinarily complex system that relies on a dynamic and elaborate communication network. The system is under the control of hormonal and

neural signals with their own internal regulators, cytokines (McEwen *et al.*, 1997). In mid to late embryogenesis, migration of pluripotent haematopoetic stem cells from the yolk sac to the liver, spleen and bone marrow is the first step in development of immunocytes. At this stage, their differentiation and development into granulocytes, erythrocytes, lymphocytes or monocytes, which is dependent on signals from the microenvironment, occurs (McEwen *et al.*, 1997).

The innate and the adaptive immune responses achieve protection via two distinct but mutually dependent forms of immunity and differ because of the timing and selectivity of their actions. Inflammation is a form of innate immunity which is responsible for initiating the first line of defense against injury or infection. This reaction of the microcirculation is characterized by increased blood flow to the site of trauma which facilitates the transport of serum proteins and leukocytes and the concomitant upregulation of expression of adhesion molecules and release of chemotactic factors (Lawrence *et al.*, 2002). These actions manifest in the cardinal signs of inflammation – heat, redness, swelling, pain and loss of tissue function. As a result of inflammation, neutrophils are the first cell type present at the site of injury. These are followed by monocytes which eventually mature into macrophages and phagocytose the foreign material and apoptotic cells. They release hydrolytic and proteolytic enzymes which eliminate and destroy the invading organisms, ultimately leading to restitution of normal tissue structure and function (Lawrence *et al.*, 2002). In the brain, termination of an injurious stimulus and phagocytosis of an invading molecule is executed by microglia, the resident macrophages of the brain.

In addition to its roles described above, the innate response is responsible for instructing the adaptive immune response to coordinate a further defense against an injury or insult. Adaptive immunity, in contrast with innate immunity, shows specificity in response and a capacity to distinguish between self and non-self. It is typified by its ability to establish immunological memory which results in an enhanced response upon secondary exposure (McEwen *et al.*, 1997). Adaptive immunity employs antibody-mediated immunity provided by B cells and cell-mediated immunity involving T cells and the cytokines

which they release. These B and T cells, which have differentiated from prelymphocytes and undergone maturation in the bone marrow, wait in lymphoid tissues throughout the body for their recruitment to the immune response.

Inflammation is a beneficial response of the host to an invading pathogen, foreign insult or tissue injury and ultimately leads to the restoration of tissue structure and function (Lawrence *et al.*, 2002). However, inflammation in the brain is a serious and potentially detrimental event. Owing to the susceptibly of neural cells, the limited space and the importance of the brain, cardinal signs of inflammation such as swelling and loss of function cannot be endured. Prolonged, extensive or non-regulated inflammation ceases to be advantageous and contributes to the pathogenesis of diseases. The impact of inflammation on the genesis and development of age-related diseases such as Alzheimer's disease and Parkinson's disease and other diseases such as multiple sclerosis and rheumatoid arthritis is being documented with an accumulating body of work (Bodles and Barger, 2004). Similarly, inflammation in the aged brain is viewed as a detrimental process, involving the production of excessive amounts of pro-inflammatory cytokines and resulting in decreasing amounts of anti-inflammatory cytokines.

1.3 Inflammation of the Central Nervous System

Over the past years, the central nervous system (CNS) was regarded as an "immune-privileged" site, however recently this view has been challenged. It has been suggested that the CNS should be regarded as an immunologically specialized site and consistent with this view, resident cells of the CNS, namely neurons and glia have been found to be responsible for mediating immunoreactivity.

1.3.1 Resident inflammatory cells of the CNS

It is estimated that there are 100 billion neurons in the CNS, however this only constitutes approximately 10% of the total number of cells. Glia are the predominant cell type in the brain. Traditionally, neurons are regarded as being the most important cell type involved in functions such as perception, learning and memory. Recently, glial responsibility in these processes, along with their role in neurodevelopment has been recognized (Fossel,

2004). The role of neurons in the immune response is evidenced by their expression of major histocompatibility complex (MHC) class I genes in vitro (Neumann *et al.*, 1997a). Neurons are involved in cytokine physiology; interleukin-1β (IL-1β) has shown to be released from neurons following γ-irradiation (Lynch *et al.*, 2003) and lipopolysaccharide (LPS) challenge (Nolan *et al.*, 2004). Interleukin-10 (IL-10) has also been detected in neuronal supernatant (Nolan *et al.*, 2004). Pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and IL-1β can influence neuronal behaviour (Neumann *et al.*, 1997b); in addition to this, IL-1 receptor type I (IL-1RI) has been identified in neuronal cultures (Nolan *et al.*, 2004).

Oligodendrocytes, astrocytes and microglia are the three types of glial cells in the brain. Oligodendrocytes are necessary for efficient transmission of electrical current across neurons, however it is astrocytes and microglia that comprise the highly reactive parenchymal cell populations which mount the immune response against infectious and inflammatory stimuli (Aloisi *et al.*, 2000a). Both astrocytes and microglia have been found to release cytokines and because of this, it is thought that they provide a link for communication between the CNS and peripheral immune cells (Aschner, 1998). Astrocytes provide support for microglial growth; thus microglia have been shown to expand *in vitro* in the presence of astrocytes, as a result of astrocytic release of growth factors, namely macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (Aloisi *et al.*, 2000b).

Astrocytes are activated following brain inflammation resulting in reactive gliosis and formation of a glial scar, aiding the healing process (Ledeboer *et al.*, 2002; Tuppo and Arias, 2005). Initially, it was thought that astrocytes were capable of antigen presentation because expression of MHC class I and II molecules was evident on their surface following stimulation with interferon-γ (IFN-γ; Aloisi *et al.*, 1998). However, further investigation revealed that astrocytes did not support T cell proliferation following MHC class II expression (see review Correale and Villa, 2004). The traditional astrocytic role in the immune response was questioned again following studies showing an LPS-induced increase in IL-10 mRNA in astrocytes (Ledeboer *et al.*, 2002) and a suppression of

microglial secretion (Aloisi *et al.*, 2001) of IL-1β owing to astrocytic-derived signal (Vincent et al., 1997). As a result of these actions, astrocytes may limit the degenerative and detrimental effects of inflammation.

1.3.2 Microglia

Microglia modulate CNS inflammation and their activation is a marker of the intracerebral inflammatory response, which is observed in neurodegenerative diseases (Ledeboer *et al.*, 2002) such as Alzheimer's disease. Microglia are small, capable, protean cells which readily divide and are ubiquitous in the nervous system (Streit, 2000). They derive from meosdermal precursor cells of haemapoietic lineage (Vilhardt, 2005; Tuppo and Arias, 2005) and are known to divide into old age (see Fossel, 2004). During embryogenesis, activated microglia are present at sites of axonal growth after which time they disappear and reappear again in the adult brain during infection or injury (Giulian and Baker, 1986). Determining the fate of neurons during development is another function of these cells, evidenced by the death of developing Purkinje cells caused by the presence of microglia (see Vilhardt, 2005). The brain parenchyma is dotted with microglia existing in a ramified state, an adaptation for the normal microenvironment of the CNS and electron microscopy has shown the surfaces of ramified microglia covered with spines (Vilhardt, 2005). Once an injury or insult has been detected, resting microglia are attracted to the site of insult, where they undergo morphological changes, involving the contraction of their spines and development of an ameboid shape, and proliferation in number (von Bernhardi and Eugenin, 2004). Their ability to respond quickly to an injury suggests that their quiescent, resting form represents a state of vigilance to changes in the CNS microenvironment (Kreutzberg, 1996).

Microglia play a role in host defense by secreting cytokines such as IL-1β, IL-6 and TNF-α (Tuppo and Arias, 2005; Vilhardt, 2005; Ke and Gibson, 2004); however, when the concentrations of these substances rise above innate low levels, the action of microglia is no longer neuroprotective. To aid the development of ameboid microglia from those in the quiescent phase, receptors for pathogen-associated molecules and cytokine receptors have been found on the surface of microglia (Aloisi, 2001). For example, the IFN-γ

receptor is constitutively expressed on resting microglia (see Aloisi, 2001). Microglia have been found to act as antigen presenting cells (APC) as MHC class II molecules are expressed constitutively on resting microglia (Iglesias *et al.*, 1997). Upregulation of these molecules occurs in diseased states such as Alzheimer's disease (Tuppo and Arias, 2005), following IFN-γ and/or LPS stimulation in vitro (Aloisi *et al.*, 1998) and also following intracerebral injection of IFN-γ (see Aloisi, 2001). Chemokines, which facilitate the recruitment of leukocytes from the blood into the CNS, are produced intracerebrally by microglia (Aloisi, 2001). T cells, dendritic cells and macrophages are recruited to the CNS during an immune response by the action of macrophage inflammatory protein-1 (MIP-1) and monocyte chemoattractant protein-1 (MCP-1), which are both produced by microglia (Tuppo and Arias, 2005; Vilhardt, 2005; Aloisi, 2001; Aloisi *et al.*, 2000b). MHC class II molecules, present on microglia, mediate the interaction between APCs and T cells during the immune response (Iglesias *et al.*, 1997). Studies using microglia which have been activated by IFN-γ or LPS, demonstrate the ability of microglia to process and present antigen to T cells (Aloisi *et al.*, 1998), resulting in the polarisation of naïve T cells.

Age-related neurodegenerative diseases are associated with the changes in microglial functions described above. A study by Kullberg and colleagues (2001) shows a strong region-specific activation of microglia which concurs with behavioural sensorimotor disturbances of aged rats. Microglial activation increases as a product of age in the normal ageing rat (Ogura *et al.*, 1994). Moreover, primary cultures of microglia isolated from aged rats showed a dramatic increase in proliferation, GFAP levels, ameboid morphology and antigen expression (Rozovsky *et al.*, 1998).

Microglial activation can be limited by a number of immunosuppresssing agents such as transforming growth factor-β (TGF-β), IL-10 and prostaglandin E_2 (PGE_2); Aloisi *et al.*, 1998). TGF-β succeeds in suppressing microglial proliferation in young rats, however this ability is ineffective in ageing rats (Rozovsky *et al.*, 1998). In activated microglia, IL-10 can inhibit MHC class II expression and production of cytokines and chemokines, all of which are essential for antigen presentation and T cell priming (Ledeboer *et al.*,

2002; Aloisi, 2001; Iglesias *et al.*, 1997). PGE_2 attenuates cerebral inflammation by acting on a number of targets including microglia. Its inhibitory actions on microglia are similar to those of IL-10 (Aloisi, 2001).

1.3.3 Blood-Brain Barrier

Separation of the components of the peripheral immune system from those of the CNS is thought to be achieved through the presence of endothelial tight junction of the blood-brain barrier (BBB), the absence of intentional connections with the peripheral immune system and because of the controls in place in the CNS to limit immune reactions (Aloisi, 2000b). The blood-cerebrospinal fluid (CSF) barrier also aids the BBB in its defense of the CNS; the choroid plexus is vascularised with capillaries that are fenestrated and lack tight junctions, however CPE cells which comprise the choroid plexus have tight junctions and prevent serum proteins form gaining access to the CSF. The BBB is also endowed with active transporters which allow efflux of noxious substances out of the brain while facilitating the movement of nutrients into the brain through influx transporters. Despite these control measures, suggestions that peripheral cells such as leukocytes can gain access to the brain have been described (Hickey *et al.*, 1991). Three possible routes of access have been documented (Ransohoff *et al.*, 2003). Firstly, leukocytes travel through the fenestrated endothelial cells of the choroids plexus stroma, migrate across the villi and cross the epithelial barrier of the choroid plexus with the help of chemokines and adhesion molecules. In this manner, they can merge with the CSF at its formation stage. It is thought that this is the process by which leukocytes can enter the brain under normal physiological conditions as was demonstrated in a study using healthy mice injected intravenously with fluorescently-labelled lymphocytes (see Ransohoff *et al.*, 2003). Furthermore, Kivisakk and colleagues (2003) found that central memory $CD4^+$ T cells travel into the CSF through the choroid plexus or meningeal vessels in humans. The second route involves specialized perivascular spaces called Virchow-Robin spaces (Brown, 2001). This area is in direct contact with the CSF, and as a result penetrating arteries from the periphery can access the CSF also. However, it is thought that this route can only be exploited when the CNS is compromised, as happens in diseased states, as leukocytes rolling on the capillaries involved was absent in healthy mice (see Ransohoff

et al., 2003). The final route involves passing from the blood into the parenchymal perivascular spaces. From the network of arterioles and capillaries, leukocytes must extravasate through postcapillary venules directly into the parenchyma. Via this route, crossing of the BBB and the endothelial basal lamina must be achieved; studies by Hickey and colleagues (1991) and Wekerle and colleagues (1985) show that T cells that have been activated can access the brain by extravasation.

A number of peripheral cell types have been detected in the CNS. Mononuclear phagocytes are the main components of leukocytes in the brain and $CD8^+$ T cells have been found to be clonally expanded in the CNS of MS patients. Dendritic cells are capable of entering the choroids plexus and meninges, however they haven't been detected in the parenchyma as yet. It has also been suggested that natural killer (NK) cells may be involved during experimental autoimmune encephalitis (EAE) (see Ransohoff *et al.*, 2003).

1.4 Periperhal Inflammation

1.4.1 T Cells

T cells are a subgroup of leukocytes which mediate the immune response by releasing both pro- and anti-inflammatory cytokines upon stimulation. An insight into functioning of the immune system was gained following the discovery of 2 subsets of mouse $CD4^+$ T cell clones which had separate and conflicting functional abilities (Kim *et al.*, 1985) and cytokine profiles. Based on these findings, classification of these distinct cell types was made; Th1 cells were thought to be involved in cell-mediated immunity as seen in delayed-type hypersensitivity and Th2 cells were supposed to stimulate antibody release from B cells by the action of their secreted cytokines. However since then, T cell function and their pattern of cytokine release has been elucidated; Th1 cells produce cytokines such as IL-1β, IFN-γ and TNF-α and β whereas Th2 cells produce IL-4, -5, -6, -10, -13. Typically, Th1 cells are viewed as mounting a pro-inflammatory response to infection or injury and in contrast, Th2 cells act in an anti-inflammatory manner. Th1 and Th2 cells differentiate from naïve T cells following stimulation. Evidence suggests that the cytokine environment has a profound effect on T cell polarization; studies have shown

that IFN-γ and IL-12 are the most important cytokines in stimulating Th1 proliferation (Constant and Bottomly., 1997). It has been suggested that IFN-γ achieves this by preventing polarisation to the Th2 phenotype whereas IL-12 acts directly on increasing Th1 differentiation (Pagenstecher *et al.*, 2000). In the case of Th2 cell proliferation, IL-4 and IL-10 have been found to be the pivotal cytokines; IL-4 augments Th2 proliferation directly *in vitro* whereas IL-10 contributes to increasing Th2 polarisation indirectly by suppressing Th1 differentiation (Constant and Bottomly, 1997).

Activation of naive T cells also depends on antigen presentation. Th1 and Th2 cell proliferation is determined by specificity of the antigen which is presented by MHC class II molecules on various antigen-presenting cells such as dendritic cells and microglia. Activated T cells have been found to enter the CNS by crossing the BBB (Hickey *et al.*, 1991; Wong *et al.*, 1999; Pryce *et al.*, 1997). It has been suggested that T cells in the periphery become activated following CNS injury or infection by the passing of CNS antigens into the lymphoid tissues via the CSF (Ransohoff *et al.*, 2003). It is thought that activation is necessary for migration as naïve T cells preferentially circulate through lymphoid organs rather than cross the BBB into nonlymphoid tissue (Aloisi *et al.*, 2000b). Once lymphocytes have been activated, entry into the brain depends on the recognition of antigen on blood vessel walls and also on the presence of adhesion molecules such as ICAM-1 (Dietrich, 2002) and CD40 (Omari and Dorovini, 2003). These surface molecules differentially regulate the entry of T cells into the CNS. Microglia, which are the resident antigen-presenting cells of the CNS, constitutively express MHC class II, ICAM-1 and CD40, suggesting a role for them in attracting T cells into the CNS (Aloisi *et al.*, 2000b; Aloisi *et al.*, 1998; Gimsa *et al.*, 2001).

Another type of $CD4^+$ T cell are T regulatory cells with the phenotype $CD4^+CD25^+$ and these constitute approximately 10% of all $CD4^+$ cells (Thompson and Powrie, 2004). These cells provide protection after CNS injury by producing neurotrophins and cytokines which alter the functioning of microglia and ultimately result in the protection of neurons (Kipnis *et al.*, 2004). These cells are thought to mediate this protective effect by facilitating the transcription of anti-inflammatory cytokines such as IL-4, IL-10 and

TGF-β (see Groux, 2001).

1.4.2 Natural Killer cells

Another central component of the immune system are natural killer (NK) cells. Even though some NK cell functions may be similar to those of T lymphocytes, NK cells provide and maintain a primary immunosurveillance system of their own (Mariani and Facchini, 2003), while in addition, providing an initial response to viral and pathogenic infection. NK cells are described as large granular lymphocytes, first recognized because of their ability to lyse tumor cells quickly (see Seaman, 2000). They are bigger than other lymphocytes and lack surface markers seen on B and T cells such as $CD4^+$. NK cells express a number of receptors on their surface for example CD16, 2B4, CD56 (an isoform of neural cell adhesion molecule) and receptors for various cytokines such as IL-2, IL-1 and IL-15, all of which influence NK cell function. NK cell activity is controlled also by the expression of MHC class I expression on target cells (Yamasaki, 2004). Between 75-90% of NK cells express the CD161 receptor, a representative of a family of transmembrane C-type lectin-like receptors, which is used as a marker for NK cell presence in tissue. NK cells reside in the lungs, gastrointestinal tract and liver and do not circulate through the lymphatic system until activated (Seamen, 2000).

Once activated, NK cells can release cytokines such as IFN-γ and as a consequence of this, have the ability to influence cell functioning, especially immune cells (Mariani and Facchini, 2003). NK cells become activated following stimulation by cytokines such as IL-2 (Borrego *et al.*, 1999), IL-1β and IL-12, which induce the secretion of IFN-γ. Cooper and colleagues (2001) provide evidence for the role of IL-1β and IL-12 costimulation in the secretion of IFN-γ from NK cells in vitro. The IL-2 receptor is constitutively expressed on NK cells (Cooper *et al.*, 2001; Krishnaraj, 1997) and following binding of its ligand, NK cell proliferation, cytokine secretion and cytotoxic activities are enhanced. Dendritic cells (DC) are a source of IL-2; studies indicate a role for DC-derived cytokines, including IL-2 in the stimulation of NK cell function and proliferation (see Delgi-Esposti and Smyth, 2005; Marcenaro *et al.*, 2005). In particular, IFN-γ secretion is upregulated following DC-induced stimulation of NK cells (Yu et al.,

2001). Induction of NK cell activity can be achieved via direct cell-cell contact with DC (Fernandez *et al.*, 1999; Kamath *et al.*, 2005). Immature DCs are thought to induce activation through binding of CD48 and CD70, which are constitutively expressed on their surface, to NK cell activating receptors 2B4 and CD27 (see Delgi-Esposti and Smyth, 2005). These ligands are also used by mature DCs along with costimulatory molecules in order to activate NK cells by direct contact (see Delgi-Esposti and Smyth, 2005).

The issue of age-related changes in NK cell activity is controversial. A number of studies report an increase in basal activity with age, some a decrease while others find no change (Kutza and Murasko., 1994). However a study by Kutza and Murasko (1994) attributes these discrepancies to varied experimental protocols and procedures. As a result, they carried out experiments using methodolgies which have been accepted as "standard". Conclusions drawn from these experiments indicate that ageing is associated with an increase in basal NK activity. This finding was corroborated more recently by Borrego and colleagues (1999) and Solona and colleagues (1999). Interestingly, NK cell function and activity induced by IL-2 is preserved in aged subjects (see Solana *et al.*, 1999; Kutza and Murasko, 1994).

1.5 Cytokines

Trauma to the central nervous system triggers a variety of inflammatory responses which include activation of macrophages at an injury site, release of cytokines and trophic factors, secretion of proteases (reviewed by Mocchetti and Wrathall, 1995) and activation of glial cells. Cytokines are a family of proteins, consisting of interleukins, interferons, growth factors and chemokines (Foster, 2001), which mediate the action of the components of the immune system. They represent the chief mediators responsible for the communication between T cells, macrophages and other immune cells during the mounting of an immune response to antigens and infectious agents (Belardelli, 1995). They have the ability to stimulate growth and differentiation of both haemapoietic and non-haemapoietic cells. Cytokine expression is transient and strictly controlled; following insult, a cell must be activated prior to the release and expression of a given cytokine

(Foster, 2001). Research has focused on separating the plethora of cytokines into distinct groups based on whether they provide protection for the central nervous system or initiate cell death pathways. However, this has proved to be a complicated exercise as the cellular effects of a particular cytokine are influenced by the type of cell on which it is acting, the neighbouring cells, combinations of other cytokines present simultaneously, and the concentration of the cytokine (Viviani *et al.*, 2004).

The biological activities of cytokines are mediated via signal transduction which is initiated by specific membrane receptors expressed on most cell types. The cytokine binds to its receptor to create the signal which will be propagated through the entire cell. Although the precise mechanism is not entirely understood, it is thought that cytokine binding induces a conformational change or oligomerisation of multiple receptors which allows activation of a cascade involving intracellular proteins specific for the cytokine. Ultimately, alterations in gene expression and protein synthesis result.

Cytokine expression is upregulated in a number of diseases such as HIV-associated dementia, cerebral ischemia, Alzheimer's disease, heart stroke, diabetes and stroke. The delicate balance between the pro- and anti-inflammatory responses are responsible for disease development and progression (Dinarello, 1997).

1.5.1 Pro-inflammatory Signalling

A major focus of biological research is interleukin-1β as it has implications both *in vitro* and *in vivo*. The mammalian mitogen-activated protein (MAP) kinase-induced signal transduction pathway, of which IL-1β is a part, has a role in a number of pathological conditions. All eukaryotic cells possess multiple MAP kinase pathways, each of which is preferentially recruited by distinct sets of stimuli, thereby allowing the cell to respond co-ordinately to diverging inputs (Kyriakis *et al.*, 2001). The MAP kinase pathway can be stimulated by many types of extracellular signals and depending on the type of stimulus, either Extracellular Regulated Kinase (ERK) or Stress-Activated Protein Kinase (SAPK) pathway will be utilised. Following stimulation by UV irradiation, heat and osmotic shock, genotoxic agents and ageing, the SAPK pathway will be activated, which involves

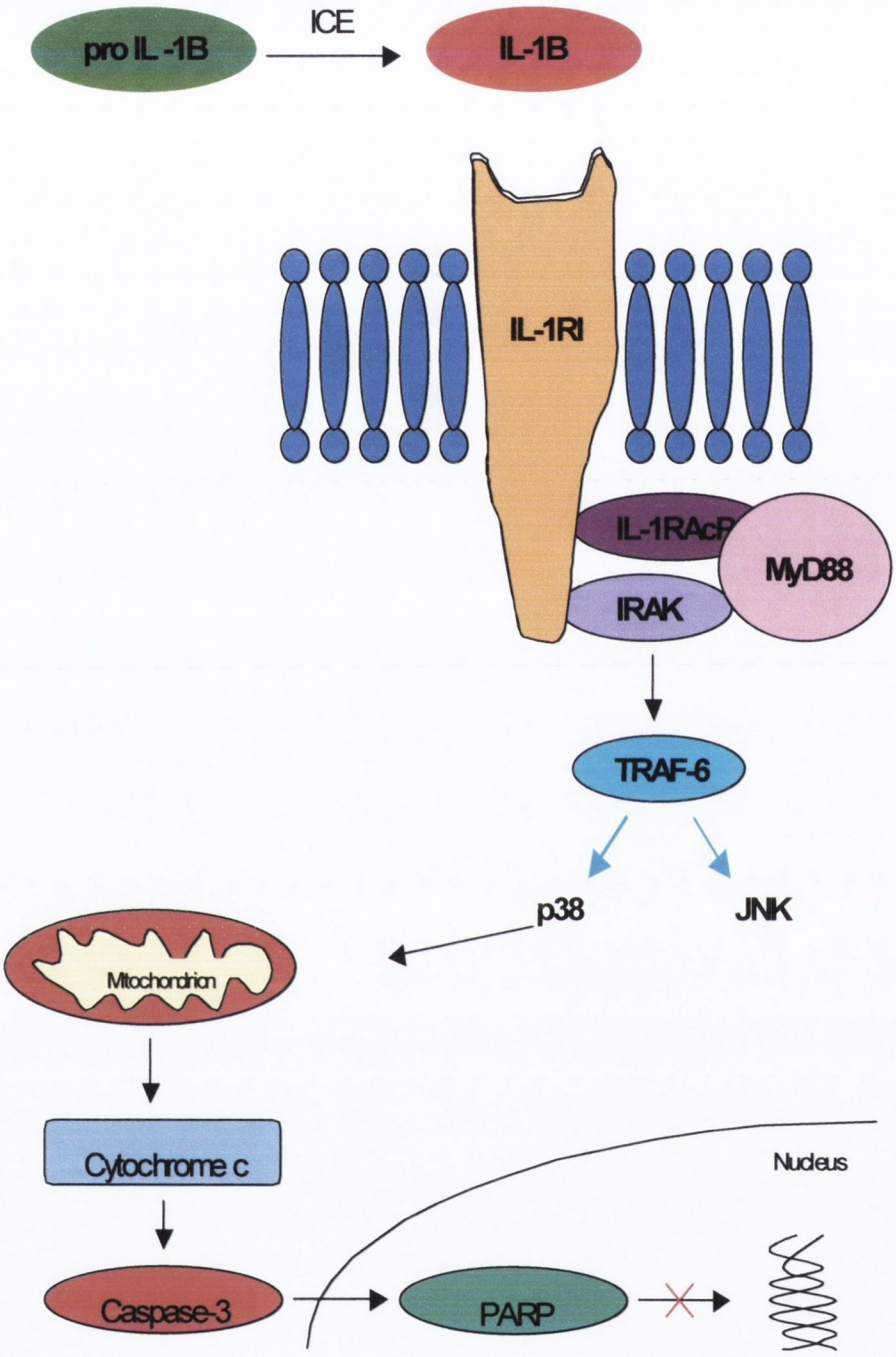


Figure 1.1 IL-1β signalling pathway

c-Jun NH_2-terminal kinase (JNK; Figure 1.1).

1.5.1.1 IL-1β

The IL-1 family consists of ten proteins including the three most studied – IL-1α, IL-1β and IL-1 ra, the endogenous receptor antagonist. These all have a good degree of homology (approximately 45% between IL-1α and IL-1β) but they derive from separate genes. IL-1α and IL-1β have synergistic functions but IL-1ra is the only naturally occurring inhibitor of IL-1 in the brain and periphery. These three members of the IL-1 family are produced as inactive entities and therefore need to be converted into the active form. In the case of IL-1β, cleavage of pro-IL-1β (31kDa) by an IL-1β converting enzyme (ICE) leads to production of the active cytokine (17kDa). IL-1 exerts its many biological effects through its plasma membrane receptors, IL-1RI (80 kDa); IL-1RII (68kDa) has no cytoplasmic domain and is not involved in signalling. The extracellular domains of these receptors show a 28% homology in the amino acid sequence but it has become apparent that they function very differently. That is to say that IL-1RI is the mediator for transduction but IL-1RII is thought to regulate extracellular IL-1 levels and it does this by acting as a decoy. IL-1RI has three Ig-like domains, which it uses to entrap the IL-1 molecule. The member of the IL-1 family that this work focuses on is IL-1β. It has a pleiotropic nature and acts both locally and systemically. Its role in inflammation is as an endogenous pyrogen, which causes an increase in body temperature. As with other cytokines, limited exposure to low concentrations of IL-1β may be neuroprotective but prolonged exposure to higher concentrations may induce neurodegeneration.

Glucocorticoids have been established as important regulators of IL-1 formation. IL-1 acts on the hypothalamus to induce the release of corticotropin-releasing factor; corticotrophin-releasing factor in turn stimulates the release by the anterior pituitary of adrenocorticotropic hormone, which leads to the secretion by the adrenal cortex of glucocorticoids (Amano *et al.*, 1992; Besedovsky *et al.*, 1986). Corticosteroids inhibit IL-1 expression and production in a murine model (Synder *et al.*, 1982). In addition, dexamethasone, a synthetic glucocorticoid, blocks accumulation of IL-1β mRNA in U937 cells following incubation with toxic shock supernatants as shown by Knudsen and

colleagues (1987). The mechanism by which glucocorticoids exert their anti-inflammatory effects is not yet understood but possible mechanisms have been suggested. Firstly, it has been postulated that they could act by inhibiting transcription and secretion of IL-1β (Kern *et al.*, 1988), however these suggestions have been rejected by findings of Amano and colleagues (1992). In contrast, Amano and colleagues (1992) have evidence showing the decrease in stability of IL-1β mRNA by dexamethasone. Another possible mechanism has been put forward by Knudsen and colleagues (1987) which suggests that glucocorticoids induce the synthesis and release of lipomodulin, an inhibitor of phospholipase A_2. In this way, release of free arachidonic acid from phospholipid side chains is inhibited and a decrease in inflammation results (Knudsen *et al.*, 1987).

1.5.1.2 IL-1β-induced signalling

JNK mediates intracellular signals originating from diverse extracellular stimuli, including growth factors, cytokines and various stresses (Park *et al.*, 1997). These are an evolutionary-conserved family of serine-threonine protein kinases, first identified in 1990 following the discovery of a 54 kDa protein kinase induced by intraperitoneal injection of the protein synthesis inhibitor cycloheximide (Barr and Bogoyevitch., 2001).

JNK kinases are highly conserved with an amino acid homology of more than 90% in mammals and more than 70% between mammals and Drosphilia. JNK kinases contain 11 protein kinase domains and it is these motifs that constitute the conserved features of protein kinases, such as the 3-dimensional fold and the binding of ATP and peptide substrates. As is the case for all protein kinases, the small terminal lobe allows orientation and binding of ATP and the role of the larger C terminal lobe is in peptide substrate recognition (Barr and Bogoyevitch, 2001). JNKs are encoded by 3 genes namely jnk1, jnk2, which are ubiquitously expressed, and jnk3 which is restricted to the brain, testes and heart. Differential mRNA splicing further diversifies the expression of each JNK-encoding gene. Type 1 and type 2 result from splicing within the catalytic domain at a region spanning subdomains IX and X, whereas splicing at the extreme COOH terminus produces 54kDa and 46kDa polypeptides (Kyriakis and Avruch, 2001). The isoforms of JNK3, 48kDa and 57kDa polypeptides, are larger than the corresponding JNK1 and

JNK2 proteins; this is a consequence of an extended N-terminus in JNK3. It is noteworthy that both JNK1 and JNK2 undergo further splicing of their substrate recognition regions and it is thought that this represents a more finely-tuned control over JNK substrates, most notably c-Jun (Mielke and Herdegen, 2000).

Cellular effects of JNK will be mediated by their direct substrates, mainly c-jun, whose phosphorylation by JNK was the reason for their identification. JNK upregulates c-jun activity by phosphorylating Ser 63 and Ser 73 on its N-terminal activation domain; this phosphorylation also stabilises c-Jun, leading to a slight increase in its steady state level (Barr and Bogoyevitch, 2001). JNKs catalyse a variety of nuclear and cytoplasmic substrates besides c-jun and the transcriptional actions of c-jun can be independent of its N-terminal phosphorylation as demonstrated for mitosis in fibroblasts. Nevertheless, recent findings clearly highlight that degeneration and apoptosis by JNK is most likely mediated by c-jun e.g. degenerating hippocampal neurons following application of kainic acid (Yang *et al.*, 1997). Other JNK substrates include ATF2 and ELK-1. ATF2 stimulates expression of c-jun gene as a result of heterodimerisation with c-jun. ELK-1 mediates the induction of c-fos gene, whose product in association with c-jun, forms the AP-1 heterodimer. In both these ways, JNK can regulate the abundance and activity of c-jun and demonstrates its potential to regulate transcriptional activity at multiple levels (Barr and Bogoyevitch, 2001).

A feature of signal transduction pathways that is coming to light at the moment is the use by certain signalling cascades of scaffolding proteins. JNK-interacting protein (JIP-1) regulates the translocation of activated JNK from the cytoplasm into the nucleus. As with all scaffolding proteins, JIP-1 propagates signals through a specific set of kinases, in this case with final activation of JNK (Mielke and Herdegen, 2000), however it does not possess enzymatic activity. The key to JIP's ability to ensure efficient translation is the fact that it restricts that components of the pathway involving JNK to a particular confined area of the cell. JIP mRNA has been found in high concentrations in cerebral cortex and hippocampus.

Work carries out by Xia and colleagues (1995) illustrates the fact that the stimulation of JNK was a prerequisite for cell death under various conditions and blockade of JNK activation prevented cell death. Compounding this was the finding that hippocampal neurons from JNK3 knockout mice were protected against excitotoxic death (Yang *et al.*, 1997). To corroborate this, further observations in a range of cell types, including Jurkat T cells, HeLa cells, endothelial cells and rat mesangial cells, have illustrated the link between JNK activation and cell death. Suppression of apoptosis in response to UV-radiation and anisomycin has been recorded in mice deficient in both JNK1 and 2 (Barr and Bogoyevitch, 2001). These findings imply a role played by JNK in an intracellular pathway which terminates with cell death. Another pathway followed by JNK when mediating apoptosis is through the mitochondria. Mitochondria release apoptogenic factors, such as cytochrome c, upon stimulation. Cytochrome c is a soluble protein located in the intermembrane space of the mitochondria, however once released, it acts as a co-factor in the presence of dATP to stimulate the aggregation of Apaf-1. Apaf-1 recruits and activates caspase-9 and the downstream caspase cascade. Xie and colleagues (1991) showed a feedback amplification of cytochrome c release during stress involving the activated caspases. Caspase-9 in turn cleaves pro-caspase-3 to form caspase-3 (Green, 1998). Caspase-3 has a number of cellular substrates including actin, fodrin, lamin and poly-ADP polymerase (PARP; Enari *et al.*, 1998). PARP is involved in the maintenance of DNA integrity and following cleavage by caspase-3, apoptosis has been shown to ensue.

1.5.1.3 Interferon-γ

Interferon-γ (IFN-γ) is another pro-inflammatory cytokine which is stimulated in response to stress and more notably, to tumours and viruses. Interferons were first discovered in supernatant released from cells which were exposed to a virus. Isaacs and Lindemann (1957) found that cells which were challenged with a virus were protected following subsequent exposure to another virus and that this protection resulted from the presence of a substance they called "interferon". When it was first characterised, interferon was described as the first line of defense against viral infections (DeMaeyer and DeMaeyer-Guignard, 1988); however since then, its effects have extended from viral

inhibition to the regulation of the cells of the immune system. The role of IFN-γ is exemplified in mice with mutations in genes encoding several components of the cellular response to IFN-γ, namely IFN-γ itself, part of its receptor and STAT1, one of its signalling molecules. These mice display abnormalities in their lymphoid system and also less resistance to infections (Boehm *et al.*, 1997).

IFN-γ is released from T helper cells of the Th1 subset and $CD8^+$ cytotoxic T cells, following stimulation with specific antigens or mitogens such as staphylococcal entertoxin A and B or phytohemagglutinin (Pestka *et al.*, 2004) and from NK cells, when stimulated by macrophage-derived cytokines such as TNF-α, IL-12 (Boehm *et al.*, 1997) and IL-2 (Borrego *et al.*, 1999). IFN-γ released from NK cells results in the display of its antiviral activity (Mahalingam *et al.*, 1999) and creation of a positive feedback loop, leading to its autostimulation (see Boehm *et al.*, 1997). Two types of interferon exist, type I and type II. Type I interferons include IFN-α, IFN-β, IL-28 and IL-29. The type II interferon group comprise IFN-γ only. Following X-ray analysis, IFN-γ was found to exist as a 34kDa homodimer.

1.5.1.4 IFN-γ-induced Signalling

Generation of an intracellular signal which is necessary for eliciting a physiological response is achieved through establishment of ligand-receptor binding. In accordance with this, IFN-γ binds to a specific receptor which is found ubiquitously on nucleated cells. The IFN-γ receptor is composed of an α-chain which is responsible for ligand binding and a β-chain which mediates downstream signalling (Figure 1.2. The α-chain is constitutively linked with JAK1 and the β-chain with JAK2. Following IFN-γ binding to its receptor, the α-chains dimerise. Phosphorylation of the two α-chains and two β-chains occurs following association of the β-chains with the IFN-γ-α-chain complex. Consequently, the respective JAK molecules become activated, leading to the phosphorylation of the tyrosine residues on the α-chains. This creates binding sites for the latent cytoplasmic molecules STAT-1, which becomes phosphorylated upon binding. Following phosphorylation, STAT-1 becomes dissociated from the receptor complex and

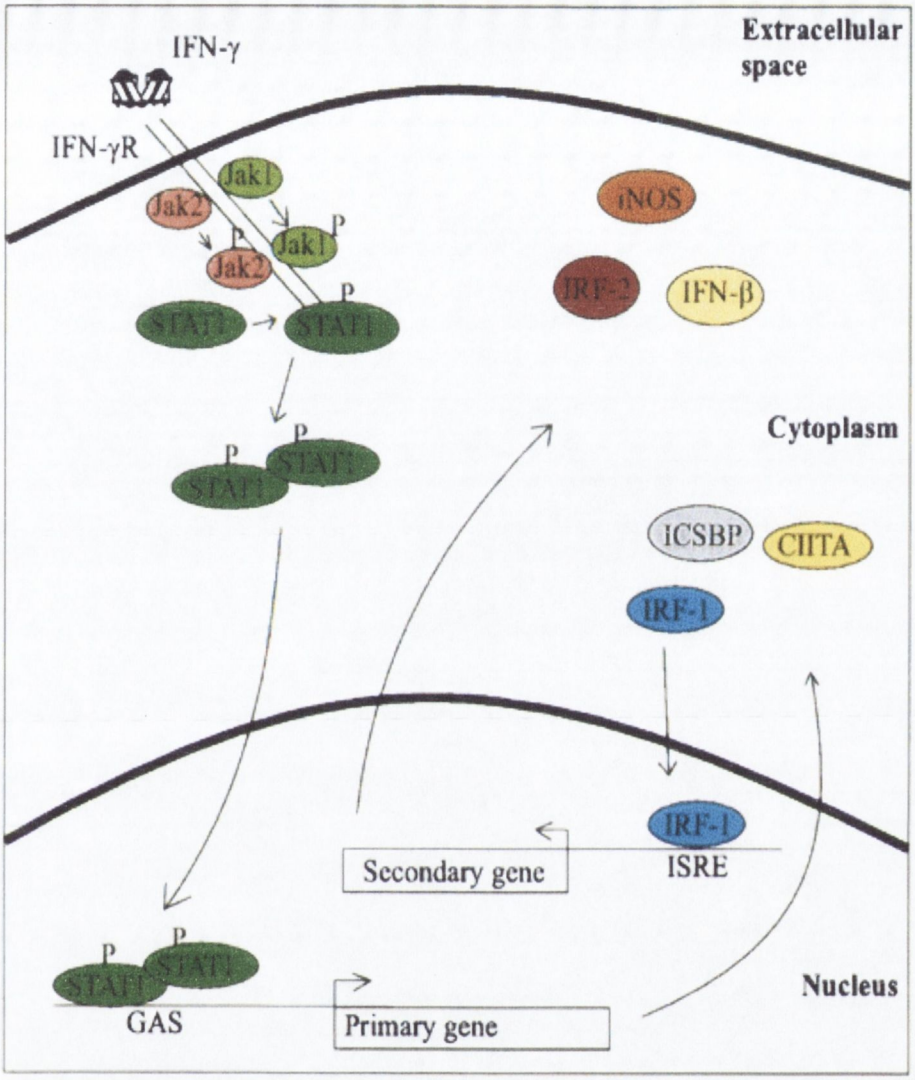


Figure 1.2 The Interferon-γ signalling pathway

Adapted from Puladan S.R. (1998). Interleukin-4 and interferon-γ: The quintessence of a mutual antagonistic relationship. *Scandanavian Journal of Immunology*, **48(5)**, 459-468.

forms homodimers which are also called gamma-interferon activation factors (GAF). Translocation to the nucleus takes place, where initiation of transcription of various genes begins following binding of GAF to particular DNA sequences contained in the promoter region of target genes termed gamma activated sites (GAS; Lew *et al.*, 1991). STAT-1 plays a pivotal role in the transduction of the IFN-γ signal as Decker and colleagues (1991) found that the proteins which comprise the GAF were present in the cytoplasm of fibroblasts before IFN-γ treatment. They are consequently ready and capable of receiving input when IFN-γ binds to its receptor and also to translocate to the nucleus, where it binds the GAS region and modifies gene transcription. Interestingly, mice with mutations in the STAT-1 gene do not register any response to IFN-γ challenge (see Boehm *et al.*, 1997). During this signalling process, it is thought that the IFN-γ-receptor complex is internalised (DeMaeyer and DeMaeyer-Guignard, 1988; see Boehm *et al.*, 1997). Following transduction, degradation of IFN-γ by lysosomes has been found to occur following experiments using chloroquine, an inhibitor of lysosomal activity (DeMaeyer and DeMaeyer-Guignard, 1988).

Over 200 genes have been discovered whose transcription is altered by IFN-γ. Following stimulation of a number of cell types with IFN-γ, the mRNA of these genes are induced, repressed, stabilised and destabilised (Boehm *et al.*, 1997). One family of transcription factors which are regulated by IFN-γ are interferon regulating factors (IRF-1, IRF-2, p48 and ICSBP). Expression of all four members can be modified by IFN-γ treatment. In particular, IRF-1 expression is enhanced by IFN-γ and participates in apoptotic signalling within a given cell following stimulation (Kim *et al.*, 2002). IRF-1 may be deemed important as its constitutive expression renders cells remarkably resistant to three RNA virus families. Furthermore, IRF-1 knockout mouse fibroblasts are more susceptible to EMCV than their wildtype counterparts (see Boehm *et al.*, 1997). IRF-2 is constitutively expressed in many cell types and following stimulation with IFN-γ, it antagonises the effects of IRF-1 and inhibits transcription. ICSBP is expressed exclusively in cells of the immune system and negatively regulates IRF-1 targets.

1.5.1.5 Functions of IFN-γ

The role of IFN-γ in the immune system is very broad, however some of its most important functions include macrophage activation (Belardelli, 1995), Th cell differentiation and polarisation, leukocyte-endothelial interactions and apoptosis (Boehm *et al.*, 1997). The interrelationship between Th1 cells and IFN-γ is extremely important in primary defense against invading organisms, as evidenced by the fact that clearance of pathogens such as Leishmania, Taxoplasma and Listeria is prevented when the Th1 response and IFN-γ production or function is tampered with (see Boehm *et al.*, 1997). Data suggest that IFN-γ plays a pivotal role, along with IL-12 in the determination of T helper cell polarisation; these two cytokines shift the balance towards a Th1 stabilisation. However, it is thought that IFN-γ potentiates the differentiation stimulated by IL-12 as IFN-γ knockout mice do not display great disruption in Th1 cell numbers (see Boehm *et al.*, 1997). IFN-γ also acts by inhibiting Th2 differentiation from naïve T cells (Gajewski and Fitch, 1988). One of the first biological effects of IFN-γ was the induction of expression of MHC class II molecules. The purpose of these molecules is to present antigen to immune cells such as T cells and microglia, to promote their polarisation and activation. This increase in expression is mediated by the enhanced functioning of various intermediaries which contribute to the antigen presentation by MHC class II molecules. IFN-γ has been demonstrated to increase MHC class II expression on microglia (Neumann *et al.*, 1996) and on normal human bronchial epithelial cells (Pawliczak *et al.*, 2005). Following polarisation of naïve T cells, Th1 cells are recruited to the site of invasion or inflammation. To aid this recruitment, substances called chemokines are released. Fractalkine is a chemokine belonging to the CX3C chemokine family and is involved in both cell adhesion and as a chemoattractant. Following IFN-γ stimulation, endothelial cells, fibroblasts and keratinocytes have been found to have increased fractalkine production (Sukkar *et al.*, 2004). Similarly, MCP-1, another chemokine, has shown increased mRNA and protein following IFN-γ stimulation in human bronchial epithelial cells (Pawliszak *et al.*, 2005). Another molecule involved in the leukocyte-endothelium interaction is intracellular adhesion molecule-1 (ICAM-1). ICAM-1 gene induction on epithelial cells (see Boehm *et al.*, 1997) as well as ICAM-

protein expression on endothelial cells (Renkonen *et al.*,1992) are augmented following IFN-γ treatment. IFN-γ treatment can also lead to DNA damage and results in apoptosis by increasing caspase-3 cleavage in NOD mice; inhibition of caspase-3 attenuated cell death (Augstein *et al.*, 2004). Another target of IFN-γ in the caspase family of proteins is caspase-1. Kim and colleagues (2002) demonstrated that inhibitors of caspase-1 decrease cell death initiated by IFN-γ and IRF-1.

1.5.2 Anti-inflammatory signalling

Establishment and maintenance of controlled responses to stress, pathogens and injury is achieved through the expression of anti-inflammatory cytokines such as IL-10, IL-4 and IL-5. These molecules antagonise the effects of pro-inflammatory cytokines to limit the inflammatory response which, if left uncontrolled, would develop into chronic inflammation. In this situation, cells would be destroyed and neurodegeneration would ensue.

1.5.2.1 IL-10

IL-10, initially described as cytokine synthesis inhibitory factor, is a cytokine produced by activated T cells, B cells, monocytes, macrophages and other cells that have a wide range of functions important for the inflammatory response (Howard *et al.*, 1992). IL-10 is a pleiotropic cytokine that regulates a variety of functions of haemapoietic cells and non-haemapoietic cells. Its principal function is to limit and eventually terminate inflammatory responses; in effect, infectious organisms are destroyed with minimal damage to host tissues. IL-10, first recognised for its ability to inhibit activation and effector function of T cells, monocytes and macrophages, is a multifunctional cytokine with varied effects on most cells types. The inhibitory effects of IL-10 on pro-inflammatory cytokine production and physiology of individual cell types suggest that it could have potent anti-inflammatory activities *in vivo* (Moore *et al.*, 2001).

In response to a stimulus, IL-10 expression is increased in a diverse array of cells. This expression is controlled by different mechanisms depending on the cell type. Experiments have shown that the transcription factors Sp1 and Sp3, which regulate IL-10

transcription, are expressed constitutively by a variety of cells. Taken together with fact that IL-10 mRNA is regulated at the post-transcriptional level, it can be suggested that IL-10 is transcribed constitutively and therefore its expression is regulated by the action of the RNA degradation mechanisms (Moore *et al.*, 2001). This is a quick and efficient method of controlling IL-10 expression. Human IL-10 is the only one for which a 3-dimensional structure has been constructed. From this structure, it is known that IL-10 is constituted of a V-shaped dimer, with each arm of the dimer consisting of six α-helices. Four of the helices (A-D) form a typical bundle that is also seen in IL-4. The C-terminal of the other two helices (E, F) penetrates into the N-terminal of the four helices bundle. This structure is similar to that of IFN-γ (Fickenscher *et al.*, 2002).

The structural relationship between IL-10 and IFN-γ is highlighted also in the IL-10 receptor structure; that is to say that at least two subunits of this receptor are members of the interferon receptor family. Immunoprecipitation studies reveal the molecular size of the IL-10R1 to be 90-120kDa and it is thought that helix A, the AB loop, part of helix B and C-terminal helices E and F of IL-10 were responsible for contact with IL-10R1 (Moore *et al.*, 2001; Fickenscher *et al.*, 2002). IL-10R1 expression varies depending on the cell type. Generally, the receptor is constitutively expressed in haematopoietic cells. For example, activation of monocytes is accompanied by an upregulation of IL-10R1 expression and thereby demonstrating IL-10's inhibitory effect on these cells (see Moore *et al.*, 2001). However, in non-haematopoietic cells IL-10R1 expression can be induced; this was evident in fibroblasts stimulated by LPS and epidermal cells and keratinocytes stimulated by glucocorticoids and dihydroxyvitamin D_3 (Moore *et al.*, 2001). The second part of IL-10R is described as an accessory subunit or an additional membrane spanning receptor chain, IL-10R2 and is expressed constitutively in cells. The IL-10/IL-10 receptor complex formation occurs in two steps: firstly, IL-10 binds with high affinity to the larger receptor chain, IL-10R1, followed by IL-10R2 binding with lower affinity. Studies have elucidated the structure of the IL-10/IL-10R1 complex; in this complex, the dimeric IL-10 molecule binds symmetrically to two soluble extracellular IL-10R1 chains demonstrating similarities with the IFNγ/IFNγR1 complex. However following gel filtration experiments and crystallography, evidence for a larger complex has been found.

Based on these observations it was suggested that IL-10R1 and IL-10R2 recognise the same binding site on IL-10 (Fickensher *et al.*, 2002). Supporting this, IL-10R2 knockout mice develop severe chronic enterocolitis and treatment with anti-hIL-10R2 antibodies eliminate IL-10 responses (Moore *et al.*, 2001).

IL-10 modulates expression of cytokines with important consequences for their ability to activate and sustain immune and inflammatory responses. IL-10 potently inhibits production of IL-1 (Haddad *et al.*, 2003; Mesples *et al.*, 2003; Sawada *et al.*, 1999) and TNF-α, a cytokine that plays a crucial role in the development of sepsis syndrome (Gesser *et al.*, 1997). These are one of most important inhibitory effects of IL-10, as these cytokines mediate further downstream activities of inflammatory pathways and processes by inducing secondary mediators such as chemokines and prostaglandins (Moore *et al.*, 2001). IL-10 and its anti-inflammatory effects were shown to be mediated by the Ras MAPK pathway by Geng and colleagues (1994). Their work was carried out in monocytes stimulated by LPS and the results clearly identified the Ras/MAPK signalling pathway as a component of intracellular signalling in this cell type. However, the detailed mechanism by which IL-10 targets MAPK signalling is currently unknown, although it is possible that IL-10 signalling interferes directly with the activation of MAPKs. For example, LPS-induced phosphorylation and consequently activation of p38 in monocytes was shown to be inhibited by IL-10 (Haddad *et al.*, 2003). p38 is believed to be the factor through which IL-10 can exert its effects on PGE_2. It has been demonstrated by Williams and colleagues (2000) that PGE_2 can only succeed in elevating IL-10 levels in cells in the presence of activated p38. Interestingly, TNFα can induce tyrosine phosphorylation of p38. It has also been suggested that IL-10 may work via Bcl-2, which is part of the MAPK pathway stimulated by IL-1. Increased expression of Bcl-2 is associated with survival of human B cells treated with IL-10 (Moore *et al.*, 2001). Also, engagement of the IL-10 receptor has been shown to activate the JAK/STAT pathway. There is evidence that points towards the requirement for JAK1/STAT3 in TNFα suppression and more specifically, evidence is available that identifies the 3'AU-rich elements of TNFα mRNA translation as the target of IL-10 signalling (Kontyiannas *et al.*, 2001). For example, serum levels of TNFα were reduced following administration of IL-10 in Balb/c mice

treated with LPS to induce toxic shock. Similarly, inhibition of TNFα production in baboons and humans, resulting from experimental endotoxemia, was also found following IL-10 administration (for review see Moore *et al.*, 2001).

In addition to IL-10's effect on pro-inflammatory cytokines, it controls inflammation by increasing expression of various cytokine antagonists. Castella and colleagues (1994) have shown that IL-10 increased IL-1 receptor antagonist production by LPS-stimulated human polymorphonuclear leukocytes and consequently blocked IL-1's effect on the target cells. Sawada and colleagues (1999) have demonstrated that IL-10 functions as an inhibitory regulator of cytokine activity in the CNS cytokine network. Their study focused on microglia and they suggest that IL-10 may play an important role in CNS inflammation by affecting microglial function. Glial cells, which include astrocytes and microglia, produce a variety of immunoregulatory cytokines. Microglia, which are very similar both phenotypically and functionally to macrophages, produce pro-inflammatory cytokines such as IL-1 and TNF-α and the anti-inflammatory cytokines IL-10 and TGF-β. IL-10, as well as TGF-β, has been shown to inhibit cytokine production by monocytes-type cells including microglia (Mizuno *et al.*, 1994; Mesples *et al.*, 2003). IL-10 has also been shown to have a negative effect on its close relation IFN-γ. IFN-γ is a pro-inflammatory cytokine and IFN-γ-induced class II MHC mRNA expression on microglia has been shown to be down regulated by IL-10 (Mizuno *et al.*, 1994).

The principal effect of IL-10 is amelioration of inflammation. Its effects can be seen in disease states such as Inflammatory Bowel Disease (IBD) and EAE. For example, IL-10 knockout mice develop chronic IBD because they cannot initiate an immune response to intestinal flora (Mocellin *et al.*, 2003). The recovery of rats and mice from EAE was associated with expansion of Th_2-like cells producing IL-10. IL-10 was observed in low concentrations in relapsing EAE. Finally, in IL-10 knockout mice the severity of TNF-α-induced EAE was greater than in normal mice (Moore *et al.*, 2001). These cases suggest a direct role for IL-10 in CNS inflammatory diseases.

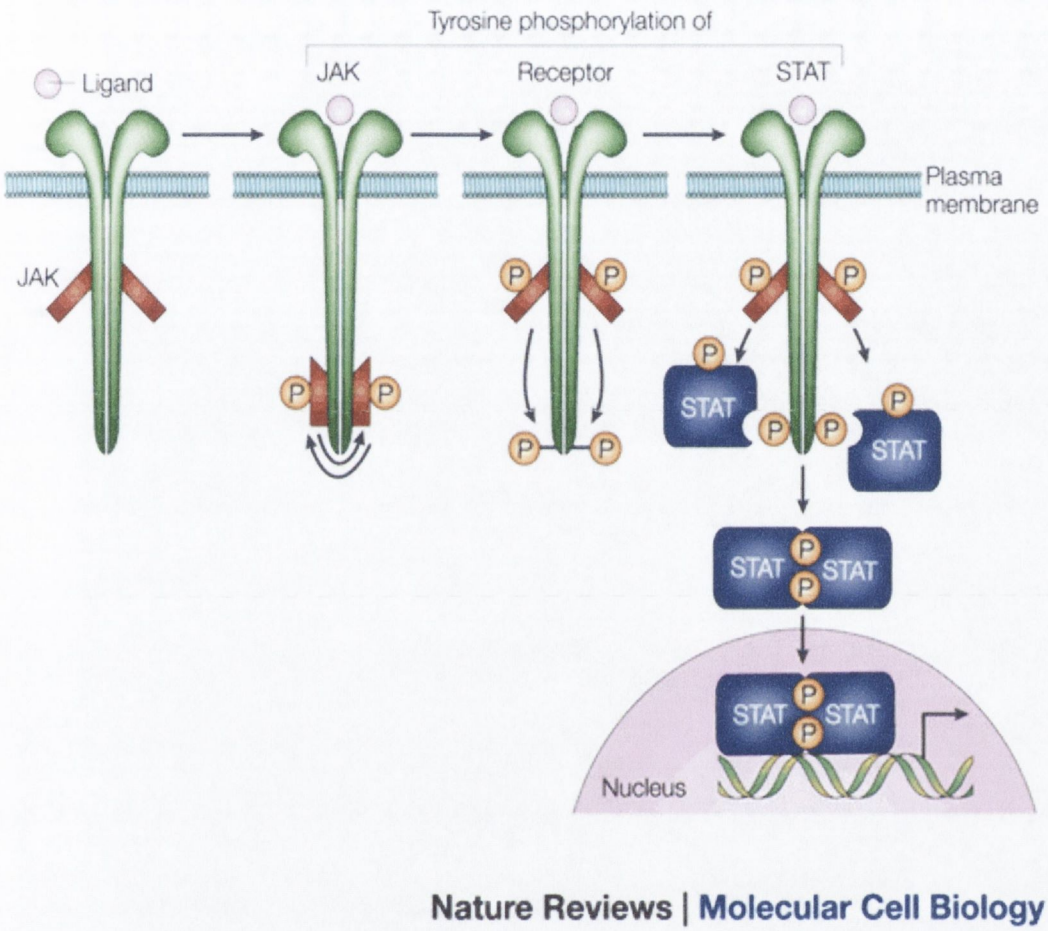


Figure 1.3 The IL-10 signalling pathway.

Adapted from Levy D.E. and Darnell J.E. (2002). STATS: Transcriptional control and biological impact.*Nature Reviews Molecular Cell Biology* **3**, 651-662.

1.5.2.2 IL-10-induced signalling

Polypeptide cell signalling molecules such as cytokines impact on a diverse range of biological events in the cell through interaction with their specific receptors at the cell surface. Ligand binding induces sequential interactions between the cytoplasmic domain of the receptor and specific kinases, which ultimately results in alterations in gene expression (Park *et al.*, 1996). The JAK/STAT pathway, which is an example of this type of signalling, is employed to propagate signals from a variety of growth factors and cytokines such as IL-10 (Figure 1.3). Treatment of T cells and monocytes with IL-10 results in the tyrosine phosphorylation of JAK1 and STAT3 (Finbloom *et al.*, 1995).

Unlike growth factor receptors, cytokine receptors lack intrinsic catalytic activity, consequently they are forced to signal through the Janus kinases (JAKs). JAKs physically associate with cytokine receptor subunits and are essential for cytokine signalling therefore fulfilling the criteria for classification as mediators of cytokine signalling (O'Shea, 1997). Four mammalian JAKs have been identified, JAK1, 2, 3 and Tyk 2. They comprise approx. 1150 amino acids with apparent molecular weights of about 120-130kDa. Jak 1,2 and Tyk2 are ubiquitously expressed whereas JAK 3 has more regulated and restricted tissue expression. The distinguishing characteristic of the JAK family is the existence of both kinase and pseudokinase domains. Several segments of homology are discovered between JAKs and have been called JAK homology domains (JH). In total seven JH domains have been identified however, the function of only the JH1 catalytic domain has been elucidated. Regulation of the catalytic function of JAKs is controlled by the tyrosine residues in their catalytic domains. Following structural studies, a model was produced in which a blockade of the active site of the enzyme was provided by the activation loop when the JAK is unphosphorylated, however, following phosphorylation of the tyrosine residue, substrate accessibility is facilitated. Only JAKs have a pseudokinase domain which contains all the subdomains that correspond to those in the active tyrosine kinase catalytic domains, but they deviate from the typical motifs. The function of this domain has not been determined however, several lines of evidence indicate a role for it in the regulation of catalytic function. For example, in the growth hormone receptor/JAK complex, knockout of the pseudokinase domain led to more

pronounced signalling, therefore suggesting the catalytic domain as a target for JAK kinase inhibition. Another function of the catalytic domain is in facilitating association with STATs (Leonard and O'Shea., 1998).

Information about JAK substrates is quite limited. The cytokine receptors are one of the established substrates of JAKs (Levy *et al.*, 2002). Phosphorylation of these receptors provides a docking site for proteins with phosphotyrosine binding domains, which consequently become JAK substrates. Among these are STATs, Grb2, Vav and Stam (Leonard and O'Shea, 1998). Attenuation of JAK signalling is poorly understood however, two mechanisms have been described. JAK2 phosphorylation has been found to be suppressed following association of the SH2 containing tyrosine phosphatase with cytokine receptors (O'Shea, 1997). Recently, an inhibitor of JAK catalytic activity, called SOCS-1 has been identified (Leonard and O'Shea, 1998; Levy *et al.*, 2002). More investigation into this area is warranted.

Gene activation stimulated by cytokines is mediated by the modification of transcription activators present in the cytoplasm such as STATs. There are seven known mammalian STAT proteins namely STAT1, 2,3,4,5a, 5b and 6 and are approx 750 to 850 amino acids in length. Initially, it was thought that STAT proteins existed as a monomer pool in the cytoplasm of the cell. However, recently the discovery that the bulk of these STAT proteins exist in protein assemblies in the size range of 200-400kDa to 1-2 MDa suggests that the initiation and transmission of STAT signalling through the cytosolic compartment includes novel regulatory, scaffolding and chaperone proteins (Ndubuisi *et al.*, 1999).

Following JAK recruitment to the receptor, the latent STATs present in the cytoplasm become activated by phosphorylation on a tyrosine residue. An upstream highly conserved region of amino acids, called the Src homology 2 (Sh2), attaches to the intracellular domain of the ligand-receptor-kinase complex at specific phosphotyrosine residues, thereby facilitating the interaction between the STAT proteins and the receptor-kinase complex. Following STAT phosphorylation, the Sh2 domain aids the dimerisation

of the activated STATs (Horvath and Darnell, 1997; Strehlow *et al.*, 1998; Leonard and O'Shea, 1998). The mechanism by which the newly phosphorylated STAT molecule leaves the receptor-kinase complex in order to dimerise has not been identified as yet (Horvath and Darnell, 1997). The STAT proteins can form homo or heterodimers via binding of the Sh2 domain on one STAT molecule and the phosphorylated tyrosine on the other. Others are formed in deference to binding with the receptor-kinase complex, as each STAT possesses both an Sh2 domain and a phosphorylated tyrosine, thus forming stable bivalent molecules. The specificity of the Sh2 domain and the microenvironment of the phosphorylated tyrosine determines the establishment of either homo or heterodimers (Leonard and O'Shea., 1998). For example, STAT 1 and 3 can form heterodimers and STAT1, 3,4,5a and 5b can form homodimers. Two variations in STAT-containing complexes broaden their potential role. Firstly, STAT1 and 2 and STAT1 and 3 can form heterodimers and also STAT 1,3 and 5 all have at least one protein variant produced by differential splicing (Darnell, 1996; Zhong *et al.*, 1994). Following dimerisation, the dimer translocates to the nucleus and can bind directly to the DNA. In theory, transcription factors have the potential to activate or repress the transcription of target genes; however at the moment, evidence suggests that STATs are only involved in the setting of gene activation and increasing transcription (Leonard and O'Shea, 1998).

Negative regulation of STATs is achieved through a number of possible mechanisms. Firstly, dephosphorylation of the tyrosine or serine residue, in the case of a few STAT proteins, would disrupt dimerisation and hamper its binding with DNA. Degradation of STAT by a proteasome would be effective. Thirdly, the SOCS family of proteins (Strehlow *et al.*, 1998; Levy *et al.*, 2002) can inhibit signalling using a negative feedback loop and finally Bcl-6 is a protein which can compete with STAT for the binding domain on a target (Leonard and O'Shea, 1998).

1.6 The Hippocampus

From early adulthood onward, the average weight of the human brain decreases and the hippocampus is no exception. In numerous studies the hippocampus has been shown to be particularly vulnerable to stress and ageing. This cerebral organ was termed

hippocampus (Greek. Hippokampos) as Aranzi (1564) thought that its dissected form resembled a seahorse. The hippocampal formation is located on the medial aspect of each hemisphere and consists of the dentate gyrus, the hippocampus itself and the parahippocampal gyrus. The dentate gyrus lies between the parahippocampal gyrus and the hippocampus. The hippocampus is formed by an infolding of the inferomedial part of the temporal lobe into the lateral ventricle and is subdivided into 3 distinct areas according to Lorente de No (1934), namely the CA1, CA2 and CA3. There are 3 major pathways in the hippocampus. The perforant pathway projects from the entorhinal cortex of the temporal lobe and connects with the granule cells of the dentate gyrus. The mossy fibres of the granule layer form excitatory connections with pyramidal cells of the CA3 area. The Schaffer collateral pathway is responsible for the projection of the pyramidal cells of the CA3 to the CA1.

Originally, the hippocampus was thought to be responsible for various sensations including, the sense of smell and emotion. The importance of the hippocampus in memory was discovered following the observations of Milner and colleagues (1966). A study of patient H.M. revealed impairments in anterograde memory following the bilateral removal of various medial-temporal lobe structures including the hippocampus as a treatment for epileptic seizures. Presently, new techniques have been employed to study memory; for example, control lesions have been created in animal models, recordings of the activity of single or groups of neurons have been made during execution of memory tasks by experimental animals and most recently, techniques such as functional magnetic resonance imaging and positron emission tomography (PET) scans monitor the brain's metabolic activity and blood flow during task completion in human subjects.

1.7 Long-Term Potentiation

1.7.1 Mechansims of Long-Term Potentiation

It has been suggested that the changes leading to the generation of long-term potentiation (LTP) might reflect the biological changes which occur during learning and memory. In

1973, the first description of LTP was made by Bliss and Lomo following an experiment which demonstrated that trains of high frequency stimulation applied to the perforant path of an anaesthetized rabbit resulted in the increased efficiency of synaptic transmission in the granule cells of the dentate gyrus, represented by an increase in amplitude of the excitatory postsynaptic potential (EPSP) of the cells (Figure 1.4). Studies following this original description of LTP consolidated Bliss and Lomo's findings and provided support for the hypothesis that LTP and learning and memory processes rely on the same synaptic modifications. In support of this, it was found that the basic properties of LTP paralleled the requirements for learning and memory set down in Hebb's postulate. Hebb (1949) hypothesized that information storage would be achieved through increasing the efficiency of a synapse following input from two neurons simultaneously. The three basic properties of LTP are cooperativity, associativity and input specificity (Bliss and Collingridge, 1993). Cooperativity means that increasing the frequency of stimulation will decrease the stimulation strength required for LTP induction. Associativity refers to the fact that LTP can be induced in synapses which are receiving a weak input, once another set of synapses on the same neuron are being stimulated above threshold simultaneously. Input specificity means that when LTP is being induced in a set of synapses on a postsynaptic cell, adjacent synapses which are receiving no input will not undergo LTP.

In addition to the induction process, two phases of LTP exist; the early phase which lasts 2-3 hours and does not require protein synthesis and the late phase, which is more persistent, lasting weeks relies on protein synthesis (see Lynch, 2004). Induction of LTP occurs when the calcium concentration in the postsynaptic cell is increased; experiments involving the injection of EGTA (Lynch *et al.*, 1983) and BAPTA (Mulkey and Malenka, 1992) blocked LTP. As a result, it has been accepted that an increase in postsynaptic calcium concentration is a requirement for LTP induction. The NMDA receptor facilitates this elevation of calcium concentration. When glutamate binds to the receptor and the postsynaptic membrane is depolarised, the calcium channel on the postsynaptic cell is opened, allowing the influx of calcium (Robertson, 2002; Lynch 2004). The importance of the receptor in LTP induction is highlighted in experiments using receptor

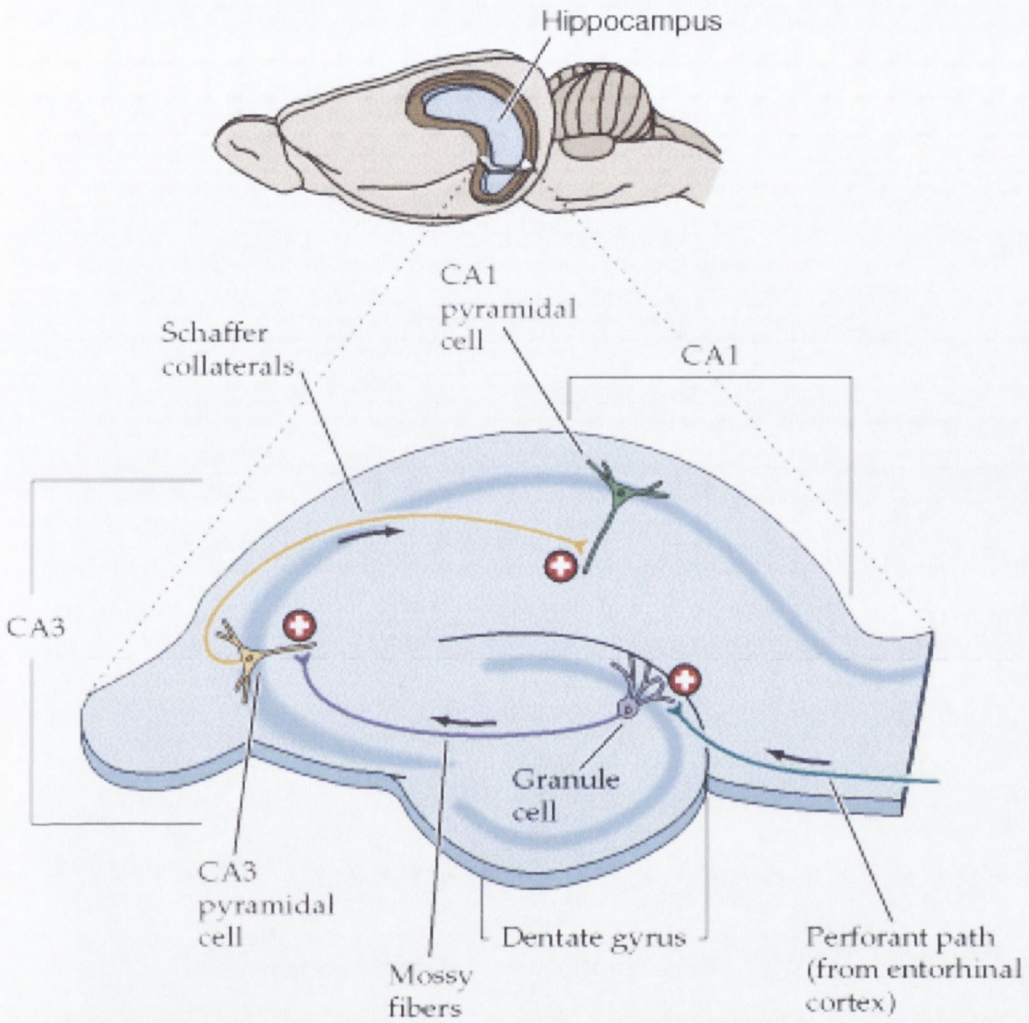


Figure 1.4 Diagram of a section through the rodent hippocampus showing the major excitatory pathways and synaptic connections.

Adapted from Purves D., Augustine G.J., Fitzpatrick D., Katz L.C., LaMantia A. and McNamara O.J., (2001). Neuroscience, 2nd Edition. Sinnauer Associates Inc.

blockers and transgenic mice which lack an intrinsic part of the receptor (see Robertson, 2002). The maintenance of LTP is associated with changes in protein synthesis pre- and post-synaptically. Pre-synaptically increased efficiency in synaptic transmission, possibly due to alterations in transmitter release, contributes to the maintenance of LTP. Evidence from Lynch and colleagues (1994) demonstrate an increase in synthesis of synapsin, synaptotagmin and synaptophysin, all of which are involved in vesicular fusion and trafficking. Post-synaptically, protein synthesis leads to morphological changes such as increased surface area (Desmond and Levy, 1988), spine number (Chang and Greenough, 1984) and spine area (Fifkova and Vanharreveld, 1977).

1.7.2 LTP and Stress

Hippocampal functions, including LTP, are altered by stress. Stress is manifested in a number of forms including behavioural stress, oxidative stress, irradiation stress and stress resulting from ageing. Stress can be described as a disturbance of physiological and psychological homeostasis ultimately controlled by the hypothalamic-pituitary axis, which results in the release of corticosteroids form the adrenal cortex (Lynch, 2004). The hippocampus is particularly susceptible to stress as it contains the highest concentration of corticosteroid receptors in the brain (McEwen, 1994). Levels of glucocorticoids found in stressed animals have an inhibitory effect on hippocampal cell activity (Talmi *et al.*, 1993), however, low levels beneficially affect activity (Joels *et al.*, 1995); consistent with this is the effect on LTP (see Lynch, 2004). Sapolsky and colleagues (1987) report that IL-1β stimulates secretion of corticotrophin releasing factor from the hypothalamus and this is consistent with the finding that IL-1β expression in the brain is increased with behavioural stress (Murray and Lynch, 1998b). Isolation stress has been shown to lead to an accumulation of ROS and this results in impairment in LTP (Murray and Lynch, 1998b; Vereker *et al.*, 2001). Data suggest that this occurs as a result of increased IL-1β concentration, which leads to increased activation of superoxide dismutase. Age is associated with an impairment in LTP (Martin *et al.*, 2002) and interestingly, glucocorticoids, IL-1β and ROS have been shown to increase in the aged hippocampus (Murray and Lynch., 1998a; McGahon *et al.*, 1999; O'Donnell *et al.*, 2000).

1.8 Treatment Strategies for Ageing

One focus of ongoing research is attempting to identify strategies which might ameliorate the undesirable effects of the ageing process on brain function through the development of new drugs and testing the ability of drugs whose primary function may not be linked with CNS inflammation. The destruction caused by an uncontrolled inflammatory response is partly responsible for the alterations in cell function seen in the ageing brain and therefore actions of these drugs and treatments should downregulate this inflammatory response. Protection of the cells which are impacted by inflammation are also potential targets for interventions. Innate anti-inflammatory defenses, such as antioxidants, are unable to cope with the microenvironment of the ageing brain and administration with some of these intermediaries involved in these defenses may provide relieve from ageing.

Studies have shown that eicosapentanoic acid (EPA) can inhibit the age-related increase in IL-1β and its downstream signalling effectors such as p38 (Martin *et al.*, 2002). Functional deficits are consequently restored with this treatment as evidenced in aged rats which maintained LTP following EPA treatment (McGahon *et al.*, 1999b). α-tocopherol and ascorbate are naturally occurring antioxidants in the body but in the aged brain, the extent of reactive oxygen species accumulation exceeds the body's innate defenses' ability to deal with it. Treatment with vitamin E and C in the diet of aged and young rats reversed age-related neuronal changes (O'Donnell and Lynch, 1998) and the age-related impairment in LTP (Murray and Lynch, 1998a). Statins are synthetic lipid/cholesterol lowering drugs, prescribed widely throughout the world. Treatment of rats with Atorvastatin reduced the age-induced impairment of LTP and increased IL-10 release from glia (Clarke *et al.*, personal communication).

Inhibitors of the pro-inflammatory pathways driven by the inflammatory response provide a means by which the ageing process can be augmented. Barry and colleagues (2005) demonstrated that administration of a JNK inhibitor blocks the LPS-induced increase in active caspase-3. Minocycline, which is a member of the tetracycline class of

antibiotics, has been shown in numerous studies to reduce neural inflammation and cell death (see Stirling *et al.*, 2005). In particular, it prevents microglial activation and cell death (Zemke and Majid, 2004).

1.8.1 Dexamethasone Treatment

Glucocorticoids are extremely potent anti-inflammatory and immunosuppressive agents. These steroid hormones are capable of preventing apoptosis through induction of survival genes such as the Bcl-2 family and NFκB, depending on cell type. In contrast, apoptosis is encouraged by glucocorticoids in cells of the haematopoietic system which are involved in inflammation, namely monocytes, macrophages and T lymphocytes (Amsterdam *et al.*, 2002). Glucocorticoids, the final effector product of the hypothalamic-pituitary-adrenal (HPA) axis, can inhibit the actions of the HPA axis by establishing a feedback loop and they can also control immune response at many different levels (Spencer *et al.*, 1993).

Dexamethasone, a stable and potent glucocorticoid hormone analogue, acts in a similar manner to the endogenous adrenal steroids; the primary one being corticosterone. These steroids bind to two receptor subtypes found in the central nervous system: type I or mineralocorticoid receptor and type II or glucocorticoid receptor. The presence of the receptors throughout the body determines the selectivity of corticosterone; as it is a systemic hormone, its effects must be controlled. The heterogeneity of the receptor expression is evident in the brain and immune tissues. For example, the type I receptor is highly concentrated in the hippocampus and undetectable in the thymus whereas the ubiquitous type II receptor is seen throughout the brain and particularly abundant in thymus, spleen and lymph nodes. DeKloet and Reul (1987) found that the hippocampus receives a tonic input at all times of the day from corticosterone acting via the type I receptor. The role of the type II receptor is thought to be central to the negative feedback affects of corticosterone on the HPA axis activity (Spencer *et al.*, 1993).

It is thought that the majority of glucocorticoid effects are achieved by signalling through the glucocorticoid receptor (GR). Recently two isoforms of the GR were identified.

Although the GRα isoform is believed to possess all the receptor activity, the GRβ form can inhibit the action of GRα; however, its role in glucocorticoid-induced modulation of apoptosis has not yet been elucidated (Amsterdam *et al.*, 2002). Preceding activation of GR by glucocorticoids, it forms a heterocomplex with heat shock proteins hsp90, hsp56 and hsp70, which is located primarily in the cytosol and partially in the nucleus. The hormone enters the cell without the help of a carrier protein and binds with the receptor, inducing a conformational change. The heat shock proteins dissociate and allow the hormone-receptor complex to proceed to the nucleus. Once inside the nucleus, the complex can modulate the expression of genes, some of which are involved in apoptosis, by binding to a certain DNA sequence called the GR element (Amsterdam *et al.*, 2002; Goppelt-Struebe, 1997).

Three possible mechanisms by which GRs can modulate gene transcription exist. Firstly, it can exert its action through direct enhancement of transcription. Proteins involved in protein and carbohydrate metabolism, such as aspartate aminotransferase, are affected in this manner. Binding to the negative GR element in a target gene can inhibit transcription. IL-2, IL-6 and IL-8 are controlled this way. The final way in which GR can influence gene regulation is by indirect modulation of gene expression. This involves the use of an intermediate GR-sensitive gene which proceeds to manipulate a glucocorticoid-sensitive target gene. The NFκB family was discovered recently to be affected this way (Goppelt-Struebe, 1997).

Dexamethasone is a clinically widely used glucocorticoid administered in order to reduce inflammation in a wide range of tissues. Its precise mechanism of action is not clear presently, however some suggestions as to its mode of action have been put forward.

Members of the Bcl-2 family of genes, such as Bcl-2 and Bcl-X_S, have conflicting role in the process of apoptosis (Amsterdam *et al.*, 2002). In T lymphocytes and ovaria granulose cells it has been shown that glucocorticoids have a positive effect on expression of Bcl-2, the antiapoptotic member of the family (Sasson *et al.*, 2001). In TMK-1 human gastric cancer cells, dexamethasone suppressed the pro-apoptotic Bcl-X_S

upregulation and reciprocally augmented the basal levels of Bcl-X_L (Chang *et al.*, 1997). Enhancement of the anti-apoptotic members of the Bcl-2 family prevents cytochrome c translocation, the caspase cascade and PARP cleavage which are steps in the process of cell death.

The effect and the mechanism of action of dexamethasone is determined by the cell type on which it is acting. Injection of dexamethasone into mammary gland inhibits the usual targets genes of AP-1, namely c-jun and stomelysin-1. It has been suggested that an interaction between steroid hormone receptors and AP-1 were responsible for this impairment in AP-1 activity and subsequent inhibition of apoptosis (Amsterdam *et al.*, 2002). Further anti-inflammatory effects of dexamethasone have been observed in ovaries. In this case, prevention of apoptosis in granulosa cells is mediated by deamethasone-induced enhancement of cell contacts and intracellular communication, stabilization of the actin cytoskeleton and by upregulation of Bcl-2 expression (Amsterdam *et al.*, 2002). Experiments carried out on human and rat hepatocytes have revealed further protective effects of glucocorticoids against apoptosis.

Another proposed mechanism of action of glucocorticoids is achieved through regulation of prostaglandin (PG) biosynthesis. It is widely accepted that prostaglandins are targets of glucocorticoid action. PGs, especially PGE_2, are known to act in a pro-inflammatory fashion. Glucocorticoids induce their effect on de novo protein biosynthesis (Flower *et al.*, 1979) caused by a range of stimuli including IL-1β. Prostanoid synthesis begins with production of phospholipase A_2 (PLA_2) as a result of various stressors and stimuli, including age. Three forms of mammalian PLA_2 are recognized at the moment:- pancreatic secretory PLA_2, non-pancreatic secretory PLA_2, and cytosolic PLA_2 (Murakami *et al.*, 1997). PLA_2 exerts its effects on phospholipids in the cell membrane thereby causing release of arachidonic acid. Prostaglandin H synthase catalyses the conversion of arachidonic acid to prostaglandin H_2. Finally prostaglandin H_2 can be converted into other prostanoids such as prostaglandin E_2, prostaglandin $F_{2\alpha}$, prostaglandin I_2 and thromboxane A_2, depending on the enzymes active in the stimulated cells (Kawamura *et al.*, 2000). Recently, two isoforms of prostaglandin H synthase have

been discovered. Prostaglandin H synthase-1 is expressed constitutively in most cell types. Pro-inflammatory cytokines and growth factors such as IL-1β and TNF-α are responsible for upregulation of prostaglandin H synthase-2 gene expression and this has been shown to be suppressed by dexamethasone (Herschman, 1996). Barnes and Adcock (1993) demonstrated that the expression of IL-1β and TNF-α were down regulated with dexamethasone and therefore it is suggested that the down-regulation of prostaglandin H synthase-2 expression may be an indirect consequence of dexamethsone's upstream effects. Goppelt-Struebe (1997) has shown a reduction in the induction of non-pancreatic secretory PLA_2 following administration of glucocorticoids. They have also shown that cytosolic PLA_2 mRNA expression is increased when incubated with cytokines and that this effect can be prevented by glucocorticoids. As mentioned earlier, PGE_2 is the primary metabolite of PLA_2. When prostaglandin E synthase is present in a cell, PLA_2 will be converted into PGE_2. Jakobsson and colleagues (1999) have identified the fact that the expression of the synthase is regulated by dexamethasone. Kawamura and colleagues (2000) have shown that overall prostanoid levels in pleural exudates are reduced by dexamethasone.

Cyclooxygenase participates in the inflammatory response as it mediates prostaglandin synthesis. Two isoforms of cyclooxygenase exist; cyclooxygenase-1 (COX-1) is constitutively expressed and remains unaffected by most stimuli in cell culture systems whereas expression of cyclooxygenase-2 (COX-2) gene is enhanced by several kinds of extracellular stimuli (Rosen *et al.*, 1989; Xie *et al.*, 1991). During monocytic differentiation, COX-1 expression was found to be sensitive to inhibition by glucocorticoids, however it is not known if this is due to the negative GR element in the COX-1 gene. Inflammatory stimuli usually affect COX-2 expression without any changes in COX-1 mRNA or protein expression (Goppelt-Struebe, 1997). Evidence from Lacroix and Rivest (1998) demonstrate that transcription of the gene encoding COX-2, but not COX-1, is stimulated within specific cell populations of the rat brain in response to different experimental models of systemic inflammation and the pro-inflammatory cytokines IL-1β and TNF-α. Confirmation of COX-2 as a target of glucocorticoid modulation was received by *ex vivo* studies; following adrenalectomy, COX-2 levels

were increased in macrophages of rats when compared with their normal counterparts and interestingly, dexamethasone replacement treatment returned these levels to those seen in normal rats (Goppelt-Struebe, 1997). This has been corroborated by the findings of Niki and colleagues (1997). Their evidence shows that antigen-induced elevations in COX-2 levels were inhibited by treatment with dexamethasone and COX-1 levels in this granulation tissue were unchanged. The same effects of dexamethasone have been observed in leukocytes and in a cell culture system (O'Bannion *et al.*, 1992).

Regulation of COX-2 expression has been investigated at three different levels, namely mRNA translation, mRNA stability and gene transcription. Only a limited amount of research has been carried out at the transitional level. Because mRNA levels of COX-2 were not seen to be as sensitive to inhibition by glucocorticoids as its protein expression, it was concluded that glucocorticoids exerted their effects at the translational level. IL-1 has been shown to stabilise COX-2 mRNA and in contrast dexamethasone can suppress mRNA levls even when the maximal transcriptional rate has been reached (Goppelt-Struebe, 1997). Various functional studies have shown that glucocorticoids interfere with COX-2 gene transcription. In serum-stimulated fibroblasts, dexamethasone was observed to decrease transcriptional activity (DeWitt and Meade, 1993). Unpublished data cited by Goppelt-Struebe (1997) has demonstrated that in mesangial cells stimulated with platelet derived growth factor, reduced COX-2 mRNA levels could not be attributed to destabilization of message RNA and therefore implying that glucocorticoids interfere with gene transcription.

1.8.2 Vitamin D Treatment

Until the 1980s, the thought that vitamin D may have a part to play in the regulation of the immune system had never been considered. The only function or purpose of vitamin D in the human body was with regard to bone metabolism, and thereby influencing calcium and phosphate levels (DeLuca and Cantorna, 2001). During the last few years, it has been established that the hormonal form of vitamin D_3 has a much wider biological role than was previously thought (Yu *et al.*, 1991). Research resulted in the finding that vitamin D metabolism begins in the liver with its hydroxylation to form 25-

hydroxyvitamin D_3 (25-OH-D_3). In order for it to be metabolically active, further conversion of 25-OH-D_3 to 1,25$(OH)_2D_3$ must take place in the convoluted tubule of the kidney (DeLuca and Cantorna, 2001).

The first indication that vitamin D_3 might regulate immune function was the finding that peripheral blood cells have 1,25$(OH)_2D_3$ receptors. Furthermore, when 1,25$(OH)_2D_3$ is added to mitogen-stimulated human peripheral blood lymphocytes in vitro, inhibition of their proliferation, their immunoglobin synthesis and their accumulation of transcripts for the interleukins 1, 2 and 6, tumour necrosis factors α and β and interferon-γ ensues (Cantorna *et al.*, 1996). Radiolabelling of 1,25 $(OH)_2D_3$ was performed and its action tracked within various tissues. The results demonstrated that the hormone localises in the nucleus of its target tissues and also that localisation occurred in tissues such as keratinocytes of skin, islets cells of pancreas and lymphocytes, none of which had been investigated or considered as targets for vitamin D_3 (DeLuca and Cantorna, 2001). The hormone was found localised with a receptor-like binding protein (Yu *et al.*, 1991), now named the vitamin D receptor (VDR). Manolagas and colleagues (1985) discovered that lymphocytes contain large quantities of VDR, however it is only expressed upon activation. In contrast, VDR is expressed constitutively in monocytes (Yu *et al.*, 1991). Experiments have shown that VDR is highly regulated so that it is expressed at distinct stages of maturation (Yu *et al.*, 1991) thereby increasing or suppressing proliferation of the lymphocytes depending on the cell type (DeLuca and Cantorna, 2001). It has been suggested that VDR expression is controlled by signals arising from antigen receptors on T cells, the protein kinase C pathway and monocyte-derived intercellular mediators, which exert their influences at the transcriptional and post-transcriptional levels (Yu *et al.*, 1991).

Work carried out by Yang and colleagues (1993) highlighted vitamin D_3's control over T-cell mediated immunity. It was shown that the delayed hypersensitivity response to dinitrobenzene was blunted in mice given supplemental vitamin D_3. Thus vitamin D_3 became known as an immunosuppressant. Further evidence showed that this action of vitamin D_3 appears to be specific as the animals' ability to respond to an opportunistic

infection was not hindered. Experiments demonstrated that vitamin D_3 attenuates the effects of inflammatory bowel disease (IBD) (Cantorna *et al.,* 2000). Results showed that vitamin D-deficient IL-10 knockout mice developed symptoms of IBD within 6-8weeks; consequently this draws a link between vitamin D_3 and the anti-inflammatory cytokines. Further evidence corroborating this link was discovered in the murine model of multiple sclerosis. The name given to this model is experimental encephalomyelitis (EAE) which is an autoimmune disorder characterised by inflammation and demyelination in the CNS (Racke *et al.*, 1991). The sequence of neural degeneration observed in human multiple sclerosis can be recreated by injection of myelin basic protein in mice. Cantorna and colleagues (1998) found following vitamin D_3 administration that transforming growth factor (TGF-β) and IL-4 transcripts were markedly increased and the pro-inflammatory cytokines interferon -γ and TNF-α gene expression was reduced (DeLuca and Cantorna, 2001).

$1,25(OH)_2D_3$ has been shown to be a positive regulator of the two cytokines TGF-β and IL-4 (Cantorna *et al.*, 1998). TGF-β belongs to a family of peptides that appears to have a profound immunosuppressive effects, including inhibition of T cell activation and proliferation, down regulation of IFN-γ-induced class II MHC expression and decrease in the generation of cytotoxic lymphocytes (Racke *et al.*, 1991; Chantry *et al.,* 1989). The mechanisms of TGF-β actions have been elucidated to some degree using the EAE model. There are two possible mechanisms by which vitamin D_3 attenuates the severity of EAE. The first possibility is that the hormone is suppressing Th1 cell proliferation and consequently the pro-inflammatory cytokines they secrete. In addition, vitamin D_3 may enhance the numbers of Th2 cells and the anti-inflammatory cytokines they produce. It is possible that these two mechanisms are interdependent and therefore an enhancement of proliferation of one T cell type will result in the suppression of the other (Cantorna *et al.,* 1998). Experiments have shown that TGF-β plays a pivotal role in the treatment of inflammatory diseases. In fact, treatment of murine EAE with TGF-β has been shown to be beneficial, whereas neutralisation of TGF-β *in vivo* allows progression in the severity of EAE (Cantorna *et al.*,1998). Similarly, work carried out by Racke and colleagues (1991) illustrated a marked decrease in inflammation in the CNS in TGF-β-treated mice.

Santambrogia and colleagues (1993) detail similar results.

It is interesting to note that Cantorna and colleagues (1998) revealed that TGF-β release stimulated by vitamin D_3 supplementation was originating from macrophages and not T cells. Following insult or injury, macrophages are activated thereby initiating inflammation before T cell mediated immunity. It can be postulated that it is through the immediate release of TGF-β from macrophages that TGF-β is able to suppress the Th1 response. Th1 suppression by vitamin D_3, via TGF-β, in the murine EAE model can be suggested because of the fact that the Th1 response in the control animals was twice that of the animals treated with vitamin D_3, although a direct link has not been found. The Th1 response cytokine IFN-γ has been shown to be inversely correlated with TGF-β production. For example, the natural cytotoxicity of rat microglia toward oligodendrocytes *in vitro* can be upregulated by IFN-γ and inhibited by TGF-β (Santambrogia *et al.*, 1993). Also exposure of host endothelial cells, microglia and astrocytes to TGF-β will infer resistance on these cells to IFN-γ-induced class II MHC molecule upregulation and subsequently affect antigen presentation to T cells (Racke *et al.*, 1991). Suggestions have been made as to the mechanism of IL-4 increase in response to vitamin D_3 supplementation. Evidence from Cantorna and colleagues (1998) showed that vitamin D_3's effect on IL-4 is indirect. They hypothesised that the elevation of IL-4 concentration may be due to the fact that TGF-β can drive Th2 differentiation by manipulation of the cellular environment, in order to make it more conducive to Th2.
Based on the ability of TGF-β to ameliorate the effect of various inflammatory diseases, it seems likely that some of the anti-inflammatory effects of vitamin D_3 are mediated by this cytokine.

1.9 Objectives

Evidence suggests that inflammation is central to the decline in functional capacity of the aged brain. The hypothesis proposed is that the balance between pro- and anti-inflammatory cytokine concentrations in the hippocampus is a key component leading to the age-related deficits of the brain. The aim of this study was to test this hypothesis and to establish whether dexamethasone and vitamin D_3 alone or in combination might act in

the CNS as they do in the periphery and restore the proposed imbalance in cytokines and consequently the deficit in synaptic function as tested by analysis of LTP.

Chapter 2

Materials and Methods

2.1 Materials

Acrylamide	Sigma
Actin antibody	Santa Cruz
Agarose	Promega
Ammonium persulfate	Sigma
Anti-active caspase-3 antibody	Promega
Anti-goat IgG HRP	Vector
Anti-mouse IgG HRP	Sigma
Anti-rabbit IgG	Biosource
Anti-rat PARP antibody	Biosource
B27	Gibco BRL
Bio-Rad	Bio-Rad Laboratories
Bis-Acrylamide	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
Calcium Chloride	Lennox
Caspase-3 Antibody	Biosource
Chloroform	Sigma
Cytosine-arabino-furanoside	Sigma
Dexamethasone	Sigma
Diaminobenzidine tetrahydrochloride	DAKO
Diethyl Pyrocarbonate	Sigma
Dimethyl sulphoxide	Sigma
Dithiothreitol (DTT)	Promega
Dnase	Sigma
DNase-RNase Free Water	Sigma
DPX	BDH Lab Supplies
Dulbecco's phosphate buffered saline	Sigma
Dulbecco's modified Eagle media	Gibco
Enhanced Chemiluminesence Detection Kit	Amersham
Ethanol	Lennox

Ethidium Bromide	Sigma
Foetal Calf Serum	Vector
Glucose	Lennox
Glutamax	Gibco BRL
Glycerol	Sigma
Glycine	Sigma
Heat-inactivated horse serum	Gibco BRL
Hydrochloric acid	Lennox
Hydrogen peroxide	Sigma
Hyperfilm	Amersham
IFN-γ Duoset	R&D Systems
IL-1β Duoset	R&D Systems
IL-10 Duoset	R&D Systems
Isopropanol	Sigma
JNK1 Antibody	Santa Cruz
Lipopolysaccharide	Sigma
Magnesium sulphate	Lennox
Magnesium Chloride	Sigma
β-Mercaptoethanol	Sigma
Methanol	Lennox
Neurobasal Medium	Gibco BRL
Nitrocellulose membrane	Sigma
Non-fat dried milk (Marvel)	
Normal goat serum	Vector
dNTP mix	Promega
OCT Compound Tissue Tek	R.A. Lamb Ltd. UK
Oligo dT Primer	Invitrogen
Ox-6 Antibody	Serotec
Ox-42 Antibody	Serotec
p-JNK Antibody	Santa Cruz
p-JAK1 Antibody	Biosource

p-STAT3 Antibody	Biosource
Paraformaldehyde	Sigma
PARP Antibody	Biosource
Penicillin/Streptomycin	Gibco BRL
Phosphate buffered saline(10X)	Sigma
Poly-l-lysine	Sigma
Potassium chloride	Sigma
Potassium hydroxide	Sigma
Potassium phosphate	Sigma
Prestained molecular weight standard	Santa Cruz
Prestained molecular weight standard(broad range)	Sigma
Primers	MWG Biotech
ReBlot Plus strong antibody stripping solution	Chemicon
Ribonuclease Inhibitor	Promega
Sodium azide	Sigma
Sodium carbonate	Sigma
Sodium chloride	Sigma
Sodium dodecylsulphate	Sigma
Sodium hydrogen carbonate	Sigma
Sodium hydroxide	Lennox
Sodium phosphate(monobasic)	Sigma
Sodium phosphate(dibasic)	Sigma
Standard grade No.3 filter paper	Whatman
Streptomycin	Gibco BRL
Substrate Reagent Pack	R&D Systems
Sucrose	Lennox
Sulphuric acid	Lennox
Superscript II	Invitrogen
Supersignal West Dura Extended Duration Substrate	Pierce
Taq polymerase	Promega
Tetramethylbenzidine	R&D Systems

Tri Reagent	Sigma
Tris base	Sigma
Tris HCl	Sigma
Triton X-100	Sigma
Trypsin	Sigma
Trypsin Inhibitor	Sigma
Tween-20	Lennox
Urethane	Sigma
Vectastain ABC Kit Standard	Vector
Vitamin D_3	Sigma
Whatman filter paper	Lennox

2.2 Animals

2.2.1 Housing of animals

The male Wistar rats that were used in this study were housed in the BioResources Unit in Trinity College, Dublin. The young rats were an inbred strain, aged between 2 and 4 months and weighed approximately 250g-350g. The aged rats were an inbred strain, aged between 22 and 24 months old and weighed between 500-600g (Bantham and Kingham, UK). The animals were housed in groups of 2 and they were kept on a 12-hour light-dark cycle with the ambient temperature maintained between 22°C and 23°C. Food (normal laboratory chow) and water were available *ad libitium.*

Primary cultures were prepared from one-day old Wistar pups (BioResources Unit, trinity College, Dublin2, Ireland).

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

2.2.2 Dexamethasone and vitamin D_3 treatment

Groups of aged and young rats were subdivided into a further four treatment groups. The first group acted as controls and therefore did not receive any treatments. The remaining three groups received dexamethasone (1μg/ml; 30μg/rat/day), vitamin D_3 (0.1μg/ml; 3μg/rat/day) or a combination of both in their drinking water. All treatments were administered for two weeks. The water intake of the rats was monitored for one week before the experimental treatments were started in order to establish daily water intake. The rats received their full water and food requirement each day. The rats were observed daily and were under veterinary supervision. During this period, the treatments were prepared freshly everyday.

2.3 Tissue preparation

2.3.1 Dissection

In the first study, the rats were euthanised by cervical dislocation followed by decapitation. The brain was removed rapidly and dissected on ice into its two

hemispheres. The hippocampus and cortex were dissected free from one hemisphere; the second hemisphere was snap frozen and used for preparation of cryostat sections (2.3.2)

In the second study, the animals were anaesthetized, LTP was assessed and at the end of electrophysiological recording, the rats were killed by decapitation. The brains were removed rapidly and dissected into hippocampus, dentate gyrus and cortex.

2.3.2 Preparation of slices for freezing

In the first study, one hemisphere was coated in tissue-tek and was snap frozen in liquid nitrogen. In the second study, one quarter of the hippocampus was snap frozen in liquid nitrogen for assessment of RNA.

The remaining tissue in both studies was sliced bi-directionally to a thickness of 350μm using a McIlwain tissue chopper (Mickle Laboratory Engineering Co.,UK) and was divided between 2 or 5 microfuge tubes containing Krebs solution (composition of Krebs solution in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, Glucose 10, $CaCl_2$ 1.13). The slices were allowed to settle and were rinsed once more with Krebs solution. The slices were rinsed a further two times with Krebs solution containing 10% dimethyl sulphoxide and stored at –80°C until needed.

2.3.3 Protein quantification

Tissue was thawed rapidly and washed 3 times in Krebs solution (composition of Krebs solution in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13). Tissue was homogenised in a 1ml glass homogeniser (Jencons, UK). Protein quantification was assessed according to a previously described method (Bradford, 1976). Standards were prepared from a stock solution of 1000μg/ml bovine albumin serum (BSA; Sigma, UK). This was diluted in dH_2O to prepare a range of standards from 500μg/ml to 0μg/ml. Triplicate aliquots of standards and samples (10μl) were added to a 96 well plate (Sarstedt, Ireland). Filtered Bio-Rad dye reagent (200μl; 1:5 dilution in dH_2O; Bio-Rad, UK) was added and absorbance was assessed at 595nm using a 96-well plate reader (Labsystems Multiskan RC). A regression line was plotted (Instat) and the protein concentration was calculated.

2.4 Induction of LTP *in vivo*

2.4.1 Preparation of rats

Rats were anaesthetised by intraperitoneal injection of urethane (1.5g/kg; 33% w/v). 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 2.5g/kg). Animals were kept warm. The recording chamber consisted of a stereotaxic unit attached to the laboratory bench and surrounded by a Faraday cage to isolate it from interference in the external environment. All instruments in the cage were grounded to eliminate 50Hz cycle noise. Fur on the scalp was clipped and the head was positioned in a head holder in a stereotaxic frame (ASI Instruments, UK). A midline incision was made with a scalpel to reveal the skull. The periosteum was scrapped clear and a dental drill was used to remove a window of skull to allow placement of electrodes. 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. Electrodes were lowered to the surface of the brain, with the stimulating electrode placed at 4.4mm lateral to lambda and the recording electrode 2.5mm and 3.9mm posterior to bregma. The electrodes were lowered through the cortical and hippocampal layers until the stimulating electrode was positioned in the perforant path and the recording electrode entered the granule cell layer of the dentate gyrus. This was monitored by passing a 0.1msec duration, 2ms delay, 4V pulse through the stimulating electrode at a frequency of 0.1Hz. The evoked response was transmitted through a pre-amplifier (DAM 50; Differential Amplifier; gain 75, World Precision Intruments, USA) to an analogue-to-digital converter (MacLab/2e, Analog Digital Instruments) and displayed on an Apple Mac computer (Performa 200). The computer 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 the recording of the evoked potentials. The depth of the electrodes was attuned to ensure maximum excitatory postsynaptic potential (EPSP) slope and the stimulating intensity

was adjusted until an amplitude of approximately 1mV was reached. The final depth of the recording electrode was between 2.5 and 3.5mm and the stimulating electrode was between 2.5 and 3mm.

2.4.3 LTP Induction and recording

After a period of stabilisation, stable baseline recordings were established by applying test shocks at a low frequency (0.033Hz, 0.1s, 2ms delay) for 15mins. This was followed by tetanic stimulation consisting of 3 trains of stimuli (250Hz for 200ms) at 30s intervals. Recording at test shock frequency was resumed for 45mins. The field epsps were displayed online and could be analysed at time of recording or and a later date. The slope of the epsp was taken as an indicator of the excitatory synaptic transmission in the dentate gyrus.

2.5 Analysis of Cytokines

2.5.1 Preparation of samples

Before analysis was carried out, the hippocampal slices were thawed rapidly, washed 3 times in ice-cold Krebs solution (composition of Krebs solution in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13) and homogenized (X60 strokes) in Krebs solution (800µl) using a 1ml glass homogeniser. Protein concentrations were assessed (see section 2.3.3) and the samples were equalized with Krebs solution. Samples were stored at -80°C.

2.5.2 Analysis of interleukin-1β concentration

Interleukin-1β (IL-1β) concentration was determined using an Enzyme Linked Immunosorbent Assay (ELISA). 96-well plates (Nunc-Immuno plate with Maxisorp surface) were coated with capture antibody (100µl per well; 0.8µg/ml; goat anti-rat IL-1β in PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4 and 1.5mM KH_2PO_4, pH 7.2-7.4; R&D Systems, USA) and incubated overnight at room temperature (RT). The plates were washed 3 times in PBS containing 0.05 % Tween (PBS-T; pH 7.2-7.4), and were incubated for 1hr at RT in a blocking buffer (300µl; PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3). A serial dilution of recombinant rat IL-1β (R&D Systems,

USA) in PBS containing 1% BSA was performed to produce the standards (0-2000pg/ml). The plates were washed 3 times in PBS-T and 100µl of standards and samples were incubated for 2hrs at RT. At the end of the incubation period, the plates were washed 3 times in PBS-T and the detection antibody (100µl; biotinylated anti-rat IL-1β in PBS containing 2% normal goat serum; R&D Systems, USA) was added and incubated for 2hrs at RT. The plates were washed 3 times and were incubated for 20mins at RT with streptavidin-horseradish peroxidase conjugate (100µl; 1:200 dilution in PBS containing 1% BSA; R&D Systems, USA). The plates were washed 3 times in PBS-T and the substrate solution (100µl; 1:1 dilution of reagent A (H_2O_2) and reagent B (tetramethylbenzidine); R&D Systems, USA) was added to the wells and allowed to incubate in the dark for 1hr or until a blue colour appeared. The reaction was terminated using the stop solution (1M H_2SO_4) and the plates were read at 450nm immediately (Multiskan RC). A standard curve was constructed by plotting the standards against their absorption and the results were expressed as pg IL-1β/mg tissue corrected for protein.

2.5.3 Analysis of interleukin-10 concentration

Interleukin-10 (IL-10) concentration was determined using an ELISA. 96-well plates (Nunc-Immuno plate with Maxisorp surface) were coated with capture antibody (100µl per well; 1.25µg/ml; goat anti-rat IL-10 in coating buffer ($NaHCO_3$ and Na_2CO_3, pH 9.4) and incubated overnight (min 18hr) at 4^0C. The plates were washed 3 times in PBS-T (pH 7.2-7.4), and were incubated for 2hrs at RT in a blocking buffer (300µl; NaCl, $Na_2HPO_4.2H_2O$, KH_2PO_4, KCl, BSA). A serial dilution of recombinant rat IL-10 (BioSource, UK) in block buffer containing 0.1% Tween was performed to produce the standards (0-2000pg/ml). The plates were washed 3 times in PBS-T and 100µl of standards and samples were incubated for 1.5hrs at RT. At the end of the incubation period, the plates were washed 3 times in PBS-T and the detection antibody (100µl; biotinylated anti-rat IL-10 in PBS containing 5% fetal bovine serum; BioSource, UK) was added and incubated for 1hr at RT. The plates were washed 3 times and were incubated for 45mins at RT with streptavidin-horseradish peroxidase conjugate (100µl; 0.2 µl/ml in block buffer containing 0.1% Tween). The plates were washed 3 times in

PBS-T and 100µl of tetramethylbenzidine (Sigma, UK) was added to the wells and allowed to incubate in the dark for 30mins or until a blue colour appeared. The reaction was terminated using the stop solution (1M H_2SO_4) and the plates were read at 450nm immediately (Multiskan RC). A standard curve was constructed by plotting the standards against their absorption and the results were expressed as pg IL-10/mg tissue corrected for protein.

2.5.4 Analysis of interferon-γ concentration

Interferon-γ (IFN-γ) concentration was determined using an ELISA. 96-well plates (Nunc-Immuno plate with Maxisorp surface) were coated with capture antibody (100µl per well; 2µg/ml; monoclonal mouse anti-rat in PBS) and incubated overnight (min 18hr) at RT. The plates were washed 3 times in PBS-T (pH 7.2-7.4), and were incubated for 2hrs at RT in a blocking buffer (300µl; PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3). A serial dilution of recombinant rat IFN-γ (R&D Systems, USA) in PBS containing 1% BSA was performed to produce the standards (0-2500pg/ml). The plates were washed 3 times in PBS-T and 100µl of standards and samples were incubated for 2hrs at RT. At the end of the incubation period, the plates were washed 3 times in PBS-T and the detection antibody (100µl; 150ng/ml; biotinylated goat anti-rat IFN-γ in PBS containing 1% BSA; R&D Systems, USA) was added and incubated for 2hrs at RT. The plates were washed 3 times and were incubated for 1hr at RT with streptavidin-horseradish peroxidase conjugate (100µl; 1:200 dilution in PBS containing 1% BSA; R&D Systems, USA). The plates were washed 3 times in PBS-T and 100µl of substrate solution (1:1 mixture of reagent A (H_2O_2) and reagent B (tetramethylbenzidine); R&D Systems, USA) was added to the wells and the plates allowed to incubate in the dark for 30mins or until a blue colour appeared. The reaction was terminated using the stop solution (50µl; 1M H_2SO_4) and the plates were read at 450nm immediately (Multiskan RC). A standard curve was constructed by plotting the standards against their absorption and the results were expressed as pg IFN-γ /mg tissue corrected for protein.

2.5.5 Analysis of human interferon-γ concentration

Interferon-γ (IFN-γ) concentration was determined using an ELISA. 96-well plates (Nunc-Immuno plate with Maxisorp surface) were coated with capture antibody (100µl per well; 4µg/ml; monoclonal mouse anti-human in PBS and incubated overnight (min 18hr) at RT. The plates were washed 3 times in PBS-T (pH 7.2-7.4), and were incubated for 1hr at RT in a blocking buffer (300µl; PBS containing 1% BSA and 0.05% NaN_3). A serial dilution of recombinant human IFN-γ (R&D Systems, USA) in TBS (20mM Trizma base, 150mM NaCl) containing 0.1% BSA and 0.05% Tween was performed to produce the standards (0-1000pg/ml). The plates were washed 3 times in PBS-T and 100µl of standards and samples were incubated for 2hrs at RT. At the end of the incubation period, the plates were washed 3 times in PBS-T and the detection antibody (100µl; 100ng/ml; biotinylated goat anti-human IFN-γ in TBS containing 0.1% BSA and 0.05% Tween; R&D Systems) was added and incubated for 2hrs at RT. The plates were washed 3 times and were incubated for 20mins at RT with streptavidin-horseradish peroxidase conjugate (100µl; 1:200 dilution in PBS containing 0.1% BSA and 0.05% Tween ; R&D Systems, USA). The plates were washed 3 times in PBS-T and 100µl of substrate solution (1:1 mixture of reagent A (H_2O_2) and reagent B (tetramethylbenzidine); R&D Systems, USA) was added to the wells and allowed to incubate in the dark for 30mins or until a blue colour appeared. The reaction was terminated using the stop solution (50µl; 1M H_2SO_4) and the plates were read at 450nm immediately (Multiskan RC). A standard curve was constructed by plotting the standards against their absorption and the results were expressed as pg IFN-γ /ml.

2.5.6 Analysis of IL-2 concentration

IL-2 was determined using an ELISA kit (BioSource, UK). A serial dilution of rat IL-2 in standard diluent buffer was performed to produce the standards (0-1500pg/ml). Pre-coated 96-well plates were incubated with standard diluent buffer (150µl for sample wells; 100µl for standard wells), standards (100µl), samples (50µl) and biotinylated anti-IL-2 (50µl) for 2hours at RT. The plates were washed 4 times in wash buffer and the streptavidin-horseradish peroxidase (100µl) was added and incubated for 30minutes at

RT. Plates were washed 4 times and were incubated with stabilised chromagen (100µl) in the dark for 30minutes or until a blue colour developed. The reaction was terminated using stop solution (100µl) and the plates were read at 450nm immediately (Multiskan RC). A standard curve was constructed by plotting the standards against their absorption and the results were expressed as pgIL-2 /mg corrected for protein.

2.5.7 Analysis of Blood Serum for Corticosterone

Blood was collected from the trunk of rats and after incubation for 24hours at 2-8^{0}C, the serum was isolated and centrifuged at 10,000g for 10 minutes. From this, the serum was isolated and stored at –20^{0}C until required for analysis and the pellet was discarded. Analysis of corticosterone was carried out according to the instructions included in the enzymeimmunoassay kit for corticosterone (IDS, USA). Samples, which were diluted 1:10 with PBS containing horse serum, were incubated in a 96-well plate pre-coated with a polyclonal rabbit anti-corticosterone antibody and an enzyme conjugate, which contains horseradish peroxidase, for 16-24 hours at 2-8^{0}C. Wells were washed with PBS containing Tween and incubated with tetramethylbenzidine and hydrogen peroxide until the colour developed. The reaction was terminated upon addition of 0.5M HCL. Absorbance was read at 450nm and values were expressed as ng/ml.

2.6 SDS-polyacrylamide gel electrophoresis

2.6.1 Preparation of samples

Before analysis was carried out, the hippocampal slices were thawed rapidly at 37°C by agitation and inversion and washed 3 times in ice-cold Krebs solution (composition of Krebs solution in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13) and homogenized (X60 strokes) in Krebs solution using a 1ml glass homogeniser. Protein concentrations were assessed (see section 2.3.3) and the samples were equalized accordingly with Krebs solution. An equal volume of sample buffer (0.5M Tris-HCl pH 6.8; 10% glycerol (v/v); 2% SDS (w/v); 5%

β-mercaptoethanol (v/v); 0.05% bromophenol blue (w/v)) was added to the samples. In the case of samples used for CD161 analysis, samples were diluted with an equal volume of NuPage lithium dodecyl sulfate sample buffer (Invitrogen, UK) containing NuPage reducing agent (Invitrogen, UK). Samples were boiled for 10 minutes and samples were stored at -80°C until needed.

2.6.2 Gel electrophoresis

Polyacrylamide separation gels with an SDS concentration of 7.5%, 10% or 12% were cast between two glass gel plates (1mm thick) with a 4% stacking gel (See Appendix). This was mounted on an electrophoresis unit (Sigma Techware, UK), the upper and lower reservoirs of which were filled with electrode running buffer (25mM Tris-base; 200mM glycine; 17mM SDS). Aliquots of samples (10μl) and a pre-stained molecular weight marker (10μl; Sigma, UK or Santa Cruz, USA or Biorad, UK) were loaded into the wells using a Hamilton Microliter syringe. Protein separation was achieved through application of a 32mA current and the migration of the bromophenol blue-stained samples was monitored. The separation was terminated when the blue band reached the bottom of the gel (approximately 30mins).

In the case of CD161, NuPage Bis-Tris pre-cast gels with an SDS concentration of 10% were mounted into the electrophoresis unit (Xcell II Surelock Mini Cell System, Invitrogen, UK). The unit was filled with NuPage running buffer (Invitrogen, UK). Aliquots of samples (10μl) and a pre-stained molecular weight marker (10μl Biorad, UK) were loaded into the wells using a Hamilton Microliter syringe. A current of 200V for 30mins was passed across the unit to separate the proteins.

2.6.3 Western immunoblotting

Gels were removed from the gel apparatus and placed on top of a piece of nitrocellulose paper (0.45μm pore size; Sigma, UK), which was previously soaked in transfer buffer (25mM Tris-base; 192mM glycine; 20% methanol (v/v); 0.05% SDS (w/v)). Filter paper (Standard Grade No.3, Whatman, UK) was placed on top and beneath the nitrocellulose and the gel forming a sandwich. The sandwich was soaked in transfer buffer and placed on the graphite electrode (anode) of a semi-dry blotter (Biometra, UK),

which had been moistened with transfer buffer. Air bubbles were removed by gently running a glass pipette over the sandwich. The lid of the blotter (cathode) was placed firmly on top of the sandwich and a constant current (225mA) was directly through the sandwich for 90mins.

The NuPage wet blotting system (Invitrogen, UK) was used in the case of immunoblotting for CD161. The gel was placed onto pre-soaked nitrocellulose. A piece of filter paper (Standard Grade No.3, Whatman, UK) and 2 blotting pads (Invitrogen, UK), all of which were soaked in transfer buffer (Invitrogen, UK), were placed either side of the gel and membrane, forming a sandwich. The sandwich was placed with the blotting module and was filled with transfer buffer. This was then placed into the transferring unit, which was filled with distilled water. A constant current (30mV) was directly through the sandwich for 60mins.

The nitrocellulose membrane was isolated and blocked for non-specific binding using either non-fat dried milk or BSA. The membrane was probed for an antibody raised against the appropriate protein followed by a horseradish peroxidase (HRP) conjugated secondary antibody. These were washed off and a chemiluminesent detection agent (Supersignal Ultra; Pierce, The Netherlands) or Enhanced Chemiluminesence (ECL; Amersham, UK) was added. The membrane was exposed to 5x7 inch photographic film and developed using a Fuji X-ray processor.

2.6.4 Analysis of JNK Activity

In the case of pJNK expression, the membranes were blocked for non-specific binding by incubation in Tris-buffered saline-Tween (0.05% Tween-20; TBS-T) containing 2% BSA overnight at $4^{O}C$. The membranes were washed in TBS-T 3 times in 15mins. Immunoblotting with a mouse monoclonal IgG1 antibody raised against a peptide corresponding to a short amino acid sequence phosphorylated on Thr-183 and tyr-185 of JNK1 of human origin (1:400 TBS-T containing 0.1% BSA; Santa Cruz, USA) was carried out at RT for 2hrs. The membranes were washed in TBS-T 3 times for 15mins and the secondary antibody, HRP conjugated anti-mouse IgG (1:400 TBS-T containing 0.1% BSA; Sigma, UK) was added and the incubation continued for 1hr at RT. The membranes were washed in TBS-T 3 times for 15mins. Immunoreactive bands

were detected following incubation for 5mins with Super Signal (Pierce, USA). The membranes were exposed to photographic film for 5s in the dark after which the film was developed.

The membranes probed for pJNK expression were stripped using a stripping solution (10ml per blot; 1:10 dilution in dH_20; Reblot Plus Antibody Stripping Solution, Chemicon, California, USA) and reprobed for total JNK expression. The membranes were blocked for non-specific binding by incubation in TBS-T containing 2% BSA overnight at 4^OC. and were washed in TBS-T 3 times for 15mins. Immunoblotting with a mouse monoclonal IgG1 raised against amino acids 1-384 representing full length JNK1 of human origin (1:300 TBS-T containing 0.1% BSA; Santa Cruz, USA) was carried out at RT for 2hrs. The membranes were washed in TBS-T 3 times for 15mins and the secondary antibody, HRP conjugated anti-mouse IgG (1:400 TBS-T containing 0.1% BSA; Sigma, UK) was added and the incubation continued for 1hr at RT. The membranes were washed in TBS-T 3 times in 15mins. Immunoreactive bands were detected following incubation for 5mins with Super Signal (Pierce, USA). The membranes were exposed to photographic film for 5s in the dark after which the film was developed.

2.6.5 Analysis of caspase-3 cleavage

To assess caspase-3 cleavage, the membranes were blocked for non-specific binding by incubation in TBS-T containing 2% BSA overnight at 4^OC. The membranes were washed in TBS-T 3 times for 15mins and were immunoblotted with a rabbit polyclonal antibody that targets the full length (32kDa) pro-caspase-3 (BioSource, USA; diluted 1:200 TBS-T containing 0.1% BSA) for 2hrs at RT. The membranes were washed in TBS-T 3 times and incubated with a HRP conjugated anti-rabbit IgG (1:2000 TBS-T containing 0.1% BSA; Sigma) for 1hr at RT. The membranes were washed in TBS-T 3 times for 15mins. Immunoreactive bands were detected following incubation for 5 minutes with Super Signal (Pierce, USA). The membranes were exposed to photographic film for 5s in the dark after which time, the film was developed.

2.6.6 Analysis of PARP cleavage

PARP cleavage was assessed by analysing the expression of the 85kDa fragment. The membranes were blocked for non-specific binding by incubation in phosphate-buffered saline PBS-T containing 6% non-fat dried milk overnight at $4^{O}C$. The membranes were washed in PBS-T 3 times for 15mins. Immunoblotting with a rabbit antibody raised against a peptide corresponding to the PARP cleavage site (1:500 PBS-T containing 2% non-fat dried milk; BioSource, UK) was carried out at RT for 2hrs. The membranes were washed in PBS-T 3 times for 15mins and the secondary antibody, HRP conjugated anti-rabbit IgG (1:2000 PBS-T containing 2% non-fat dried milk; BioSource, UK) was added and the incubation continued for 2hrs at RT. The membranes were washed in PBS-T 6 times in 45mins. Immunoreactive bands were detected following incubation for 5mins with Super Signal (Pierce, USA). The membranes were exposed to photographic film for 5s in the dark after which the film was developed.

2.6.7 Analysis of JAK1 phosphorylation

JAK1 phosphorylation was assessed by blocking the nitrocellulose membranes in TBS-T containing 2% BSA overnight at $4^{O}C$. The membranes were washed in TBS-T 3 times for 15mins. Immunoblotting with a rabbit antibody raised against a peptide corresponding to tyrosine phosphorylated JAK1 protein (1:750 TBS-T containing 0.1% BSA; Biosource, USA) was carried out at RT for 2hrs. The membranes were washed in TBS-T 3 times for 15mins and the secondary antibody, HRP conjugated anti-rabbit IgG (1:1500 TBS-T containing 0.1% BSA; Sigma, UK) was added and the incubation continued for 1hr at RT. The membranes were washed in TBS-T 3 times for 15mins. Immunoreactive bands were detected following incubation for 3mins with ECL (Amersham, Buckinghamshire, UK). The membranes were exposed to photographic film for 30s in the dark after which the film was developed.

2.6.8 Analysis of STAT-3 phosphorylation

To assess STAT-3 phosphorylation, the membranes were blocked for non-specific binding by incubation in TBS-T containing 2% BSA overnight at $4^{O}C$. The membranes were washed in TBS-T 3 times for 15mins. Immunoblotting with a rabbit antibody raised

against a peptide corresponding to phosphorylated tyrosine 705 on the STAT-3 protein (1:500 TBS-T containing 0.1% BSA; Biosource, USA) was carried out at RT for 2hrs. The membranes were washed in TBS-T 3 times for 15mins and the secondary antibody, HRP conjugated anti-rabbit IgG (1:3000 TBS-T containing 0.1% BSA; Sigma, UK) was added and the incubation continued for 1hr RT. The membranes were washed in TBS-T 4 times in 1hr. Immunoreactive bands were detected following incubation for 5mins with Supersignal (Pierce, USA). The membranes were exposed to photographic film for 20s in the dark after which the film was developed.

2.6.9 Analysis of CD161 expression

Assessment of CD161 expression was carried out by blocking nitrocellulose membranes in TBS-T containing 5% BSA for 2hrs at RT. The membranes were washed in TBS-T 3 times for 15mins and were immunoblotted with a mouse antibody that targets the CD161 protein found on rat NK and T cell subpopulations (Serotec, UK; diluted 1:250 TBS-T containing 2% BSA) overnight at $4^{O}C$. The membranes were washed in TBS-T 3 times and incubated with a HRP conjugated anti-mouse IgG (1:1000 TBS-T containing 2% BSA; Sigma) for 1hr at RT. The membranes were washed in TBS-T 3 times for 15mins. Immunoreactive bands were detected following incubation for 5mins with Super Signal (Pierce, USA). The membranes were exposed to photographic film for 10s in the dark after which the film was developed.

2.6.10 Analysis of actin expression

Following western immunoblotting for caspase-3, phosphorylated JAK-1, Stat-3 and CD161, nitrocellulose membranes were stripped using a stripping solution (10ml per blot; 1:10 dilution in dH_2O; Reblot Plus Antibody Stripping Solution, Chemicon, California, USA) and reprobed for total actin expression to assess loading of protein. Membranes were blocked for non-specific binding by incubation in TBS-T containing 2% BSA overnight at $4^{O}C$ and were washed in TBS-T 3 times for 15mins. Immunoblotting with a mouse monoclonal IgG antibody corresponding to amino acid sequence mapping at the carboxy terminus of actin (1:200 TBS-T containing 0.1% BSA; Santa Cruz, California, USA) was carried out at RT for 2hrs. The membranes were

washed in TBS-T 3 times for 15mins and the secondary antibody, HRP conjugated anti-mouse IgG (1:400 TBS-T containing 0.1% BSA; Sigma, UK) was added and the incubation continued for 1hr at RT. The membranes were washed in TBS-T 3 times for 15mins. Immunoreactive bands were detected following incubation for 5mins with Super Signal (Pierce, USA). The membranes were exposed to photographic film for 5s in the dark after which the film was developed.

2.6.11 Densitometry

Densitometric analysis using the ZERO-Dscan Image Analysis System (Scanalytics, USA) was used to quantify protein bands. Values are expressed in arbitrary units.

2.7 Analysis of mRNA

2.7.1 Precautions

RNAses, which easily degrade RNA, are ubiquitous in the environment and therefore all solutions were treated before use with diethyl pyrocarbonate (DEPC; 0.1%(v/v) Sigma, UK), which inactivates RNases. Solutions containing amines such as Tris were prepared with DEPC-treated water because they cannot be treated directly with DEPC. Carrying out the procedures on ice prohibited degradation of RNA by endogenous RNAses.

2.7.2 RNA extraction from tissue

A modified version of the single step method of acid guanidine thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) was used to extract RNA from tissue. Hippocampal tissue, which was snap frozen upon dissection, was homogenized in Tri reagent (Sigma, UK). To remove any insoluble tissue, the tubes were centrifuged for 10mins at 12000 x *g* and the resulting supernatants were placed in clean eppendorf tubes and allowed to stand at RT for 5mins. Phase separation occurred following incubation with 0.2ml of chloroform (Sigma, UK) per 1ml of Tri reagent for 15mins at RT. The mixture was centrifuged at 12000 x *g* for 15mins and this resulted in the formation of three distinct layers:- a lower pink, phenol-chloroform phase, the

interphase which contains DNA and the upper aqueous layer which contains the RNA. The aqueous layer was transferred to clean eppendorf tubes containing isopropanol (Sigma, UK), 0.5ml per 1 ml Tri reagent. Samples were incubated at RT for 10mins and then centrifuged for at 12000 x *g* for 10mins at 4^0C. The supernatant was removed and the RNA pellet was washed with 75% ethanol (Sigma, UK) and mixed well. Samples were centrifuged at 7500 x *g* for 5mins and then resuspended in DNase-RNase free H_20 (Sigma, UK). RNA samples were stored at -80^0C until required.

2.7.3 Analysis of isolated RNA by gel electrophoresis

To check the purity and integrity of the isolated RNA, samples were run on a 1% (w/v) agarose gel. 1.3g of agarose was dissolved in 100ml of 1X tris borate EDTA (TBE) buffer (0.08M Tris, 0.04M boric acid, 1mM EDTA; pH 8.3) by boiling. Ethidium bromide (Sigma, UK) was added to give a final volume of 0.5μg/ml and the gel was set in a horizontal gel system. RNA samples (3μl) were mixed with H_2O (2μl) and 6X loading dye (1μl; Promega, USA). Samples (5μl) were loaded into the wells and a voltage of 90V was applied to the gel. Migration of the dye towards the bottom of the gel indicated separation of samples. The gel was visualized under UV light and photographed using a UV transilluminator (Ultra Violet Products Ltd., UK).

2.7.4 Reverse transcription

10μl of sample RNA was incubated with 1μl oligo dT Primer (Invitrogen, USA) and 1μl of dNTP mix (containing 10mM each of dATP, dTTP, dCTP and dGTP; Promega, USA) at 65^0C for 5mins and then chilled quickly on ice. 4μl of 5X first-strand buffer, 2μl 0.1M dithiotreitol (DTT) and 1μl ribonuclease inhibitor (Invitrogen, UK) were added to the mixture which was incubated at 42^0C for 2mins. 1μl of Superscript II was added and this mixture was incubated for 50mins at 42^0C for cDNA synthesis and then for 10mins at 75^0C to inactivate the reverse transcriptase.

2.7.5 Polymerase chain reaction

A mastermix PCR mixture (final volume 25μl) contained 16.5μl of DNase-RNase free water, 2.5μl of 10X PCR buffer, 1.5μl magnesium chloride, 1μl dNTP mix (Promega

Corporation, Madison, USA), 0.5µl-1µl of upstream and downstream primers (MWG Biotech, Germany) and 0.5µl Taq polymerase (Promega Corporation, Madison, USA). cDNA sample (2µl)was added to the mastermix and the PCR was run with a denaturing step of 95^{0}C for 1min, followed by 25-35 cycles consisting of a denaturing step of 95^{0}C for 1min, an annealing step of 50-55^{0}C (see table 2.1 for optimal annealing temperatures) for 1min and an extension step of 72^{0}C for 1min 30s. A final extension step of 72^{0}C for 10mins was carried out to ensure complete extension of the PCR product. The products and a 100bp ladder were mixed with a loading buffer (ratio 1:6) and were loaded onto a 1.5% (w/v) agarose gel containing ETBR (0.5µg/ml) and visualised under a UV light and photographed using a UV transilluminator (Ultra Violet Products Ltd., UK).

2.7.6 Densitometry

Densitometric analysis using the ZERO-Dscan Image Analysis System (Scanalytics, USA) was used to quantify protein bands. Values are expressed as arbitrary units.

Table 2.1

Target Gene	Primer Sequence	Annealing Temperature (^{0}C)	Fragment Size
β-actin	For 5'-AGAAGAGCTATGAGCTGCCTGACG-3' Rev 5'-CTTCTGCATCCTGTCAGCGATGC-3'	65	236
IL-1β	For 5'-GCACCTTCTTTTCCTTCATC-3' Rev 5'-CTGATGTACCAGTTGGGGAA-3'	59	447
IL-10	For 5'-TGCCAAGCCTTGTCAGAAATGATCAAG-3' Rev 5'-GTATCCAGAGGGTCTTCAGCTTCTCTC-3'	65	127
MHCII	For 5'-CAGTCACAGAAGGCGTTTATG-3' Rev 5'-GATCGCAGGCCTTGAATGATG-3'	58	245
IL-1RI	For 5'-AGATGGAAGGACCTATGATG-3' Rev 5'-TGCAGCATCTGACGACAGGA-3'	54	640

2.8 Culture of primary cells and cell lines

2.8.1 Preparation of sterile coverslips

Glass coverslips (13mm diameter; Chance Propper, UK) were sterilized overnight in 70% ethanol, followed by an overnight exposure to UV light. Sterile coverslips were incubated for 1hr at 37°C in a sterile solution of poly-L-lysine (40μg/ml in sterile dH_2O) so as to provide a suitable surface to which cells could adhere. Coated coverslips were air dried and placed in 24-well plates (Cruinn Diagnostics, Ireland) and stored at 4^0C until required (maximum storage of 2 weeks).

2.8.2 Preparation of hippocampal neurons

Primary hippocampal neurons were established from postnatal one-day old Wistar rats, supplied by the BioResources Unit in Trinity College, Dublin. Rats were decapitated, the hippocampii were dissected free and the meninges were removed. The hippocampi were chopped using a sterile disposable scalpel and incubated in 0.3% trypsin in PBS (Sigma, UK) for 25mins at 37°C. The tissue was triturated in PBS containing 0.1% soybean trypsin inhibitor (Sigma, UK), DNase (0.2mg/ml; Sigma, UK) and $MgSO_4$ (0.1M; Sigma, UK) and filtered through a sterile mesh filter (40μm; BD Biosciences, USA). The suspension was centrifuged at 2000 x *g* for 3mins at RT and the pellet was resuspended in warm neurobasal medium (NBM; Gibco, UK), supplemented with penicillin (100U/ml; Gibco, UK), heat-activated horse serum (10%; Gibco, UK), streptomycin (100U/ml; Gibco, UK), glutamax (2mM; Gibco, UK) and B27 (1%; Gibco, UK). Resuspended neurons were plated onto each poly-L-lysine coated glass coverslip at a density of $0.25x10^6$ cells and allowed to adhere for at least 2hrs in a humidified incubator containing 5% CO_2 and 95% air at 37°C. Warmed supplemented NBM (500μl) containing B27 was added to each well and the neurons were incubated for 3 days. After this, the medium was replaced with supplemented NBM containing 5ng/ml cytosine-arabino-furanoside (ARA-C; Sigma-Aldrich, England) in order to prevent proliferation of non-neuronal cells. After 24hrs, the media was replaced with warmed supplemented NBM until the cells were ready to be treated.

2.8.3 Preparation of cortical glia

Primary cortical mixed glia were established from postnatal one-day old Wistar rats, supplied by the BioResources Unit in Trinity College, Dublin. Rats were decapitated, the cortices were dissected free and the meninges were removed. The cortices were chopped and incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal calf serum (10%; Gibco, UK), penicillin (100U/ml; Gibco, UK) and streptomycin (100U/ml; Gibco, UK) for 25mins at 37^0C. Tissue was triturated and filtered through a nylon mesh filter (40μm; BD Biosciences, USA). After centrifugation, the pellet was resuspended in supplemented DMEM. Resuspended glia were plated onto each poly-L-lysine coated glass coverslip and allowed to adhere for at least 2hrs in a humidified incubator containing 5% CO_2 and 95% air at 37°C. Warmed supplemented DMEM (500μl) was added to each well. Media was changed every 2-3 days and glia were grown for 10 days before treatment.

2.8.4 Treatment of cultured cells

All treatments were diluted to the required concentration in pre-warmed supplemented media. All solutions were sterilely filtered through a syringe with a 0.2μm cellulose acetate membrane filter (Pall Gelman Sciences Inc., USA) attached before treatment.

Recombinant IL-1β (R&D Systems, UK) was prepared as a stock solution in sterile PBS and diluted to a final concentration of 10ng/ml in media. Cells were treated for 24hrs with IL-1β.

Lipopolysaccharide (LPS; Sigma, UK) was prepared as a stock solution in sterile PBS and diluted to a final concentration of 100ng/ml in media. Cells were treated with LPS for 24hrs.

IFN-γ (Chemicon, UK) was prepared as a stock solution in sterile PBS and diluted to a final concentration of 10ng/ml in media. Cells were treated for 24hrs.

Recombinant IL-10 (R&D Systems, UK) was prepared as a stock solution in sterile PBS and diluted to a final concentration of 50ng/ml in media. Cells were treated for 1hr with IL-10 before IL-1β/LPS / IFNγ treatment.

Dexamethasone (Sigma, UK) was reconstituted in sterile H_2O and diluted to a final concentration of 6.8 x 10^{-11}mol/L in media. Vitamin D_3 was dissolved in ethanol (final concentration: 0.0001% ethanol) and diluted to a working concentration of 10^{-6}mol/L in media. Cells were treated with dexamethasone and vitamin D_3 in combination for 1hr prior to IL-1β/LPS treatment.

Following the treatments, supernatants were removed and stored at -80°C until further analysis was carried out. For immunohistochemistry, cells were fixed by washing 3 times in TBS followed by incubation with 4% paraformaldehyde (w/v; Sigma, UK) for 30mins. The cells were stored in TBS at 4°C until required for assessment.

2.8.5 Culture of Natural Killer cells

IL-2 dependent human NK cells were grown in RPMI with glutamax (Gibco, UK) supplemented with fetal calf serum (10%; Gibco, UK), penicillin (100U/ml; Gibco, UK) and streptomycin (100µg/mg; Gibco, UK) and IL-2 (20ng/ml; R&D System, UK). Media used for treatments contained reduced fetal calf serum concentration (2%). Cells were split every 2 or 3 days when cell density was 0.5-0.6 x 10^6/ml.

2.8.6 Treatment of cultured cells

IL-2 was washed out before treatments began. The cell suspension was centrifuged at 1200x*g* for 10minutes at room temperature after which the pellet was suspended in pre-warmed supplemented reduced serum media. This was repeated 3 times, after which the cells were incubated in pre-warmed reduced serum media without IL-2 for 24hours. All treatments were diluted to the required concentration in pre-warmed supplemented media without IL-2. All solutions were sterile filtered through a syringe with a 0.2µm cellulose acetate membrane filter (Pall Gelman Sciences Inc., USA) attached, before treatment. Cells were incubated with the treatments in 24-well plates.

Recombinant IL-2 (R&D Systems, UK) was prepared as a stock solution in sterile PBS and BSA (0.2%) and diluted to a final concentration of 20ng/ml in media. Cells were treated for 24hours with IL-2.

Dexamethasone (Sigma, UK) was reconstituted in sterile H_20 and diluted to final concentrations of 6.8 x 10^{-11}mol/L, 1.36 x 10^{-10}mol/L and 6.8 x 10^{-10}mol/L in media.

Vitamin D_3 (Sigma-Aldrich,UK) was dissolved in ethanol (final conc.: 0.0001% ethanol) and diluted to working concentrations of 10^{-6}mol/L, 2 x 10^{-6}mol/L and 10^{-5}mol/L in media. Cells were treated with dexamethasone and vitamin D_3 in combination for 24hours prior to IL-2 treatment.

Recombinant IL-10 (R&D Systems, UK) was prepared as a stock solution in sterile PBS and diluted to a final concentration of 500ng/ml in media. Cells were treated for 24hours with IL-10 before IL-2 treatment

Following the treatments, the cell suspensions were centrifuged at 14000xg for 2minutes. The supernatants were removed and stored at -80^0C until required for analysis.

2.9 Immunohistochemistry

2.9.1 Preparation of Brain Slices

Sections were cut from half brains which were coated in OCT, frozen in liquid nitrogen and stored at -80^0C. Sections (10μm) were cut using a cryostat (Leica, UK) and toluidine blue was used to determine the presence of the hippocampus in the sections. Upon identification of the hippocampus, sections were placed on subbed slides. Each slide contained three slices, 30 sections apart. Slides were stored at -20^0C.

2.9.2 Immunostaining for activated microglia

Cryostat slices were sectioned from the frozen hemispheres taken on decapitation of the rats. Sections were fixed in ice-cold ethanol prior to being washed in PBS twice for 5 minutes. Slices were incubated in blocking serum which consisted of 10% normal horse serum and 4% BSA in PBS for 30 minutes at room temperature. The blocking serum was removed and the incubation continued overnight at 4^0C with OX-42 (1:100 in PBS (v/v); Serotec, UK) in a humidified chamber. Sections were washed 3 times in 15 minutes and incubated with biotinylated horse anti-mouse IgG (1:200 dilution in PBS) for 2 hours at room temperature. Sections were washed prior to incubation with 0.3% H_2O_2 in TBS (v/v) for 15 minutes. The sections were washed and incubated for 30 minutes in ABC (Vectastain ABC kit standard; Vector, UK). Cells were washed again before developing the colour using 3, 3'-diaminobenzidine (DAB; DAKO Corporation, USA) containing H_2O_2 (1:500 dilution; Sigma, UK). Rinsing the cells in dH_2O terminated the

reaction. The cells were counterstained with methylene green before being dehydrated in increasing volumes of alcohol and mounted in DPX onto glass slides.

Sections were fixed in ice-cold ethanol prior to being washed in TBS twice for 5 minutes. Slices were incubated in blocking serum which consisted of 10% normal horse serum and 4% BSA in TBS for 30 minutes at room temperature. The blocking serum was removed and the incubation continued overnight at 4^0C with OX-6 (1:100 in TBS containing 2% BSA (v/v); Serotec, UK) in a humidified chamber. Sections were washed 3 times in 15 minutes and incubated with biotinylated horse anti-mouse IgG (1:200 dilution in TBS containing 2% BSA) for 2 hours at room temperature. Sections were washed prior to incubation with 0.3% H_2O_2 in TBS (v/v) for 15 minutes. The sections were washed and incubated for 30 minutes in ABC (Vectastain ABC kit standard; Vector, UK). Cells were washed again before developing the colour using 3, 3'-diaminobenzidine (DAB; DAKO Corporation, USA) containing H_2O_2 (1:500 dilution; Sigma, UK). Rinsing the cells in dH_2O terminated the reaction. The cells were counterstained with toluidine blue before being dehydrated in increasing volumes of alcohol and mounted in DPX onto glass slides.

OX-6 staining was carried out on cortical glia fixed to coverslips. The same procedure as above was implemented in this case. Coverslips were mounted using DPX onto glass slides.

2.9.3 Immunostaining for $CD4^+$ T cells

Cryostat slices were sectioned from the frozen hemispheres taken on decapitation of the rats. Sections were fixed in ice-cold ethanol prior to being washed in PBS twice for 5 minutes. Slices were incubated in blocking serum which consisted of 10% normal goat serum and 4% BSA in PBS for 30 minutes at room temperature. The blocking serum was removed and the incubation continued overnight at 4^0C with CD4 (1:100 in PBS (v/v); Serotec, UK) in a humidified chamber. Sections were washed 3 times in 15 minutes and incubated with biotinylated goat anti-mouse IgG (1:100 dilution in PBS) for 2 hours at room temperature. Sections were washed prior to incubation with 0.3% H_2O_2 in TBS (v/v) for 15 minutes. The sections were washed and incubated for 30 minutes in ABC (Vectastain ABC kit standard; Vector, UK). Cells were washed again before developing

the colour using 3, 3'-diaminobenzidine (DAB; DAKO Corporation, USA) containing H_2O_2 (1:500 dilution; Sigma, UK). Rinsing the cells in dH_2O terminated the reaction. The cells were counterstained with toluidine blue before being dehydrated in increasing volumes of alcohol and mounted in DPX onto glass slides.

2.9.4 Immunostaining for CD161 expression

Cryostat slices were sectioned from the frozen hemispheres taken on decapitation of the rats. Sections were fixed in ice-cold ethanol prior to being washed in PBS twice for 5 minutes. Slices were incubated in blocking serum which consisted of 10% normal horse serum and 4% BSA in PBS for 30 minutes at room temperature. The blocking serum was removed and the incubation continued overnight at 4^0C with CD161 (1:100 in PBS (v/v); Serotec, UK) in a humidified chamber. Sections were washed 3 times in 15 minutes and incubated with biotinylated horse anti-mouse IgG (1:100 dilution in PBS) for 2 hours at room temperature. Sections were washed prior to incubation with 0.3% H_2O_2 in TBS (v/v) for 15 minutes. The sections were washed and incubated for 30 minutes in ABC (Vectastain ABC kit standard; Vector, UK). Cells were washed again before developing the colour using 3, 3'-diaminobenzidine (DAB; DAKO Corporation, USA) containing H_2O_2 (1:500 dilution; Sigma, UK). Rinsing the cells in dH_2O terminated the reaction. The cells were counterstained with toluidine blue before being dehydrated in increasing volumes of alcohol and mounted in DPX onto glass slides.

2.9.5 Immunostaining for IL-10R expression

Cryostat slices were sectioned from the frozen hemispheres taken on decapitation of the rats. Sections were fixed in ice-cold ethanol prior to being washed in PBS twice for 5 minutes. Slices were incubated in blocking serum which consisted of 10% normal goat serum and 4% BSA in PBS for 30 minutes at room temperature. The blocking serum was removed and the incubation continued overnight at 4^0C with IL-10R rabbit polyclonal IgG antibody (1:50; Santa Cruz Biotechnology, USA) in a humidified chamber. Sections were washed 3 times in 15 minutes and incubated with biotinylated anti-rabbit IgG antibody (1:50; Vector, UK) for 2 hours at room temperature. Sections were washed prior to incubation with 0.3% H_2O_2 in TBS (v/v) for 15 minutes. The

sections were washed and incubated for 30 minutes in ABC (Vectastain ABC kit standard; Vector, UK). Cells were washed again before developing the colour using 3, 3'-diaminobenzidine (DAB; DAKO Corporation, USA) containing H_2O_2 (1:500 dilution; Sigma, UK). Rinsing the cells in dH_2O terminated the reaction. The cells were counterstained with toluidine blue before being dehydrated in increasing volumes of alcohol and mounted in DPX onto glass slides.

Cultured hippocampal neurons were washed in TBS for 5 minutes prior to being fixed in 4% paraformaldehyde (w/v; Sigma, UK) for 30 minutes. The cells were washed twice in 10 minutes and permeabilised in 0.1% Triton in TBS (v/v) containing proteinase K (1:500 dilution; Sigma, UK) for 15 minutes. Cells were washed 3 times in 15 minutes, refixed in 4% paraformaldehyde for 15 minutes and washed again 3 times in 15 minutes before addition of 10% normal goat serum in TBS ((v/v); Vector, UK) for 2 hours at room temperature. Cells were treated overnight in a humidified chamber at 4°C with IL-10R rabbit polyclonal IgG (1:200 in 2.5% normal goat serum; Santa Cruz Biotechnology). Cells were washed 3 times in 15 minutes and incubated in 0.3% H_2O_2 in TBS (v/v) for 15 minutes. Cells were washed again before incubation with biotinylated anti-rabbit secondary antibody (1:50 in 2.5% normal goat serum; Vector, UK) for 2 h at room temperature, washed again, followed by an incubation in the dark for 2 h with ExtrAvidin conjugated to fluoroscein (1:50; Sigma). After washing, all cells were mounted with an aqueous mounting medium (Vector, UK), sealed and examined under a Zeiss fluorescence microscope at an excitation wavelength of 495 nm and photomicrographs were taken at 40X magnification.

2.10 Immunocytochemistry

Cultured neurons and glia were fixed as described in Section 2.8.4 and stored at 4^0C until required for staining.

2.10.1 Phosphorylated JNK Staining

Neuronal cells were washed once in TBS for 5 minutes prior to being fixed in 4% paraformaldehyde (w/v; Sigma, UK) for 30 minutes. The cells were washed twice in 10

minutes and permeabilised in 0.1% Triton in TBS (v/v) containing proteinase K (1:500 dilution; Sigma, UK) for 15 minutes. Cells were washed 3 times in 15 minutes, refixed in 4% paraformaldehyde for 15 minutes and washed again 3 times in 15 minutes before addition of 10% normal horse serum in PBS ((v/v); Vector, UK) for 2 hours at room temperature. The blocking serum was removed and the incubation continued overnight at 4^0C with p-JNK (1:100 TBS-T containing 2.5% normal horse serum (v/v); Santa Cruz, USA) in a humidified chamber. Cells were washed 3 times in 15 minutes and incubated in 0.3% H_2O_2 in TBS (v/v) for 15 minutes. Cells were washed again before incubation with biotinylated horse anti-mouse IgG (1:50 dilution in TBS) for 2 hours at room temperature. The cells were washed and incubated for 15 minutes in ABC (Vectastain ABC kit standard; Vector). Cells were washed again before developing the colour using 3, 3'-diaminobenzidine (DAB; DAKO Corporation, USA) containing H_2O_2 (1:100 dilution; Sigma, UK). Rinsing the cells in dH_2O terminated the reaction. The cells were counterstained with methylene green before being dehydrated in increasing volumes of alcohol and mounted in DPX onto glass slides.

2.10.2 Immunostaining for Caspase-3 Activity

Cryostat slices were sectioned from the frozen hemispheres taken on decapitation of the rats. Slices and neuronal cells were washed once in TBS for 5 minutes prior to being fixed in 4% paraformaldehyde (w/v; Sigma, UK) for 30 minutes. The cells were washed twice in 10 minutes and permeabilised in 0.1% Triton in TBS (v/v) containing proteinase K (1:500 dilution; Sigma, UK) for 15 minutes. Cells were washed 3 times in 15 minutes, refixed in 4% paraformaldehyde for 15 minutes and washed again 3 times in 15 minutes before addition of 10% normal goat serum in PBS ((v/v); Vector, UK) for 2 hours at room temperature. The blocking serum was removed and the incubation continued overnight at 4^0C with anti-active caspase-3 (1:100 TBS-T containing 2.5% normal goat serum (v/v); Promega, USA) in a humidified chamber. Cells were washed 3 times in 15 minutes and incubated in 0.3% H_2O_2 in TBS (v/v) for 15 minutes. Cells were washed again before incubation with biotinylated goat anti-rabbit IgG (1:50 dilution in TBS) for 2 hours at room temperature. The cells were washed and incubated for 15 minutes in ABC (Vectastain ABC kit standard; Vector, UK). Cells were washed again

before developing the colour using 3, 3'-diaminobenzidine (DAB; DAKO Corporation, USA) containing H_2O_2 (1:100 dilution; Sigma, UK). Rinsing the cells in dH_2O terminated the reaction. The cells were counterstained with methylene green before being dehydrated in increasing volumes of alcohol and mounted in DPX onto glass slides.

2.11 Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was performed to determine whether significant differences existed between conditions. If this indicated significance (at the 0.05 level), a post-hoc student Newmann-Keuls test analysis was used to determine which conditions were significantly different from each other. Where appropriate, a student's t test for independent means was used (GraphPad Prism, USA).

Chapter 3

Results

3.1 Age is associated with an increase in IL-1β protein and mRNA

The concentration of the pro-inflammatory cytokine IL-1β was assessed in hippocampal homogenate prepared from young and aged rats. Figure 3.1 shows that IL-1β concentration was increased significantly in hippocampal homogenate prepared from aged rats (127.89 ± 18.79pg/mg; n=7) compared with tissue prepared from young rats (77.39 ± 8.45pg/mg; n=6; *$p<0.05$, Student's t test for independent means). Mean IL-1β concentration was decreased significantly in dexamethasone-treated (60.56 ± 15.49pg/mg; n=6), compared with control-treated, aged rats (+$p<0.05$, ANOVA). Dexamethasone treatment did not elicit any effect in young rats (75.23 ± 20.23pg/mg; n=6). Mean IL-1β concentration was decreased in tissue prepared from vitamin D_3-treated (57.11 ± 15.0pg/mg; n=6), compared with control-treated, aged rats (++$p<0.01$, Student's t test for independent means). Vitamin D_3 treatment in young rats slightly reduced the mean IL-1β concentration (41.22 ± 11.0; n=6). Treatment with dexamethasone and vitamin D_3 in combination (34.53 ± 10.44pg/mg; n=6) reduced the age-related increase in IL-1β concentration most effectively (+++$p<0.001$; ANOVA). This treatment also slightly decreased IL-1β concentration in young rats (51.30 ± 9.10; n=6).

To further investigate this age-related change, expression of IL-1β mRNA was measured. This was assessed in the groups that received dexamethasone and vitamin D_3 in combination as these were found to have the greatest effect on IL-1β protein concentration. Figure 3.2 (A) shows a sample gel illustrating IL-1β mRNA expression in young (lane 1) and aged (lane 3) control-treated rats and young (lane 2) and aged (lane 4) dexamethasone and vitamin D_3-treated rats. Figure 3.2 (B) represents the mean data obtained from statistical analysis; this revealed a significant increase in IL-1β mRNA in aged (1.24 arbitrary values ± 0.21; n=7), compared with young (0.51 arbitrary values ± 0.20; n=7), rats (*$p<0.05$, Student's t test for independent means). Dexamethasone and vitamin D_3 was also associated with an increase in IL-1β mRNA in aged animals (1.75 arbitrary values ± 0.18; n=6). No substantial effect on IL-1β mRNA expression was observed in dexamethasone and vitamin D_3-treated (0.70 arbitrary values ± 0.11; n=3), compared with control-treated, young rats.

3.2 Age is associated with increased signalling downstream of IL-1β

Signalling by IL-1β is initiated by binding to its receptor, IL-1R1. Expression of IL-1β mRNA was assessed in the groups which received dexamethasone and vitamin D_3 in combination, as this was found to have the greatest effect on IL-1β concentration at the protein level. Figure 3.3(A) shows a sample gel illustrating IL-1RI mRNA expression in young (lane 1) and aged (lane 3) control-treated rats and young (lane 2) and aged (lane 4) dexamethasone and vitamin D_3-treated rats. Figure 3.3(B) represents mean data obtained from densitometric analysis; this revealed a significant increase in IL-1RI mRNA expression in aged (1.18 arbitrary values ± 0.19; n=7), compared with young (0.2664644 arbitrary values ± 0.07549056; n=6), rats (***$p<0.001$, Student's t test for independent means). Dexamethasone and vitamin D_3 significantly attenuated the age-related increase in IL-1RI mRNA expression (0.2370 arbitrary values ± 0.10; n=3; ++$p<0.01$, ANOVA). A small but insignificant decrease in IL-1RI mRNA expression was seen in young rats treated with dexamethasone and vitamin D_3 (0.06 arbitrary values ± 0.02; n=4).

Activation of JNK was assessed by gel electrophoresis and western immunoblotting using antibodies which specifically identifies JNK phosphorylation on threonine 183 and tyrosine 185 and activation is expressed as a ratio of pJNK to total JNK. Figure 3.4 illustrates the mean data obtained from densitometric analysis; a significant increase in JNK activity, expressed as a ratio of pJNK to total JNK expression, was observed in hippocampal tissue prepared from aged (0.947000 arbitrary units ± 0.050000; n=5), compared with young (0.578800 arbitrary units ± 0.12000; n=5), control-treated rats (*$p<0.05$, Student's t test for independent means). Treatment with dexamethasone decreased JNK activity (0.7640 arbitrary units ± 0.080; n=5) compared with control-treated aged rats, however the difference did not reach statistical significance. JNK activity was similar in dexamethasone-treated (0.63 arbitrary units ± 0.15; n=5) and control-treated, young rats. As indicated by the sample immunoblot, JNK activity was unaffected in the hippocampus prepared from vitamin D_3-treated (0.898770 arbitrary units ± 0.1120; n=5), compared with control-treated, aged rats. An increase in JNK activity was seen in vitamin D_3-treated (0.89 arbitrary units ±0.16; n=5), compared with

control-treated, young rats. JNK activity was decreased by dexamethasone and vitamin D_3 in aged rats (0.770 arbitrary units ± 0.130; n=5) when compared with control-treated aged rats. No substantial effect of dexamethasone and vitamin D_3 treatment was observed in young rats (0.69 arbitrary units ± 0.10; n=5).

Data from several studies indicate that signalling downstream of JNK involves the activation of the caspase cascade and cleavage of the DNA repair enzyme PARP. This results in accumulation of fragmented DNA and cell degeneration. Mean data obtained from densitometric analysis of intact caspase-3 (32kDa) is shown in Figure 3.5; this revealed a significant decrease in intact caspase-3 in hippocampal tissue prepared from aged (0.008 arbitrary units ± 0.003; n=4), compared with young (0.10 arbitrary units ± 0.03; n=4; *$p<0.05$, Student's t test for independent means) rats, suggesting that caspase-3 cleavage occurs with age. Increased expression of the intact form of caspase-3 was observed in dexamethasone-treated (0.04 arbitrary units ± 0.01; n=6), compared with control-treated, aged rats (*$p<0.05$, Student's t test for independent means). Treatment with vitamin D_3 alone (0.06 arbitrary units ± 0.03; n=6) and in combination with dexamethasone (0.04 arbitrary units ± 0.01; n=6) increased the expression of intact caspase-3 in aged rats, however this did not reach a statistically significant level. Dexamethasone (0.03 arbitrary units ± 0.01; n=5), vitamin D_3 (0.06 arbitrary units ± 0.02; n=5), and a combination of both (0.04 arbitrary units ± 0.01; n=5) decreased expression of intact caspase-3 in young rats.

In parallel, expression of the activated form of caspase-3 was assessed by immunohistochemistry (Figure 3.6). Positive staining for activated caspase-3 was observed in the hippocampal region of cryostat sections prepared from aged control-treated rats (v), whereas healthy toluidine blue stained cells were present in a representative section from the group of young control-treated rats. Attenuation in the age-related increase in activated caspase-3 staining was observed in sections from dexamethasone (vi), vitamin D_3 (vii) and dexamethasone and vitamin D_3 (viii)-treated rats. Treatment of young rats with dexamethasone (ii), vitamin D_3 (iii) and a combination of both (iv) did not have an effect on activated caspase-3 staining.

Expression of the 85kDa form of PARP indicates its cleavage by an upstream molecule and leads to its inability to repair nicked DNA. A sample immunoblot illustrating PARP cleavage in control-treated (lane 1), dexamethasone-treated (lane 2), vitamin D_3-treated (lane 3) and dexamethasone and vitamin D_3-treated (lane 4), young rats and control-treated (lane 5), dexamethasone-treated (lane 6), vitamin D_3-treated (lane 7) and dexamethasone and vitamin D_3-treated (lane 8), aged rats is shown in Figure 3.7 (A). Treatment with dexamethasone (lane 6), vitamin D_3 (lane 7) and a combination of both (lane 8) attenuated the age-related increase in PARP cleavage. The above treatments had no effect in young rats (lane 2, 3, 4). Assessment of mean values obtained from densitometric analysis (Figure 3.7(B) indicated that PARP cleavage was significantly increased in tissue prepared from aged (91.70 arbitrary units ± 5.25; n=5), compared with young, control-treated rats (50.57620 arbitrary units ± 7.6020; n=5; ***$p<0.001$, ANOVA). Dexamethasone treatment attenuated the age-related increase in PARP cleavage (61.84 arbitrary units ± 2.57; n=5; ++$p<0.01$, ANOVA). A decrease in the 85kDa form of PARP was found in tissue prepared from vitamin D_3-treated (56.67 arbitrary units ± 9.59; n=5), compared with control-treated, aged rats (++$p<0.01$, ANOVA). PARP cleavage was decreased in hippocampal tissue prepared from dexamethasone and vitamin D_3-treated (44.49 arbitrary units ± 12.58; n=5), compared with control-treated, aged rats (+$p<0.05$, ANOVA). Treatment with dexamethasone (50.58 arbitrary units ± 7.30; n=5), vitamin D_3 (59.69 arbitrary units ± 4.41; n=5) and a combination of both (46.60 arbitrary units ± 16.02; n=5) did not have an effect on PARP cleavage.

3.3 Age is associated with impairment in LTP

Figure 8 shows an increase in the mean percentage change in population excitatory post synaptic potential (EPSP) slope in all groups, following a delivery of a high frequency train of stimuli to the perforant path (at time 0). Figure 3.9(A) illustrates that the mean percentage change in EPSP slope was decreased in aged (118.33 ± 2.48; n=4), compared with young (128.65 ± 1.51; n=4), control-treated rats (**$p<0.01$, ANOVA). Treatment with dexamethasone and vitamin D_3 increased the mean EPSP slope in young (146.59 ±

1.8; n=4; +++p<0.001, ANOVA) and aged rats (136.82 ± 2.38; n=5; +++p<0.001, ANOVA), compared with age-matched control-treated groups. The maintenance of LTP was assessed by analysing the mean change in EPSP slope in the last 5 minutes of recording. Figure 3.9(B) shows a decrease in EPSP slope in aged (92.66 ± 0.84; n=10), compared with young (128.14 ± 0.41; n=10), control-treated rats (***p<0.001, ANOVA). Treatment with dexamethasone and vitamin D_3 increased the change in EPSP slope in young (134.43 ± 1.56; n=10; ++p<0.01, ANOVA) and aged rats (117.77 ± 2.08; n=10; +++p<0.001, ANOVA), compared with age-matched control-treated groups.

3.4 Age is associated with an increase in corticosterone concentration

Corticosterone is the primary glucocorticoid found in the rat and numerous studies have shown that an increase in its concentration has an inhibitory effect on activity and learning and memory. Figure 3.10 shows that corticosterone concentration was increased significantly in serum from aged (248.05 ± 59.0ng/ml; n=6), compared with young (102.19 ± 31.48ng/ml; n=6; **p<0.01, ANOVA), rats. Treatment with dexamethasone and vitamin D_3 (39.7 ± 7.22ng/ml; n=7) attenuated this age-related increase in corticosterone concentration (+++p<0.001, ANOVA).

3.5 Age is associated with a decrease in IL-10 protein and mRNA

The concentration of the anti-inflammatory cytokine IL-10 was assessed in hippocampal homogenate prepared from young and aged rats. Figure 3.11 shows that IL-10 concentration was decreased significantly in hippocampal homogenate prepared from aged rats (105.81pg/mg ± 18.11; n=10) compared with tissue prepared from young rats (164.03180pg/mg ± 10.670; n=10; *p<0.05, ANOVA). Dexamethasone treatment slightly, but insignificantly, decreased IL-10 concentration in hippocampal tissue prepared from young (119.60pg/mg ± 10.76; n=4) or aged (91.48235pg/mg ± 24.220; n=4), rats. Mean IL-10 concentration was decreased in tissue prepared from vitamin D_3-treated (50.86pg/mg ± 16.64; n=3), compared with control-treated, aged rats. Vitamin D_3 did not elicit a substantial effect on IL-10 concentration (190.29 pg/mg ± 16.26; n=2) in

young rats. Treatment with dexamethasone and vitamin D_3 in combination (244.67pg/mg ± 13.55; n=4) attenuated the age-related decrease in IL-10 concentration (+++$p<0.001$; ANOVA). This treatment decreased IL-10 concentration in young rats (80.05pg/mg ± 37.28; n=4).

Because dexamethasone and vitamin D_3, given in combination, reversed the age-related decrease in IL-10 concentration, the expression of IL-10 mRNA was assessed only in animals which received this treatment. Figure 3.12 (A) comprises a sample gel indicating a decrease in IL-10 mRNA expression in aged (lane 3), compared with young (lane 1), rats. Treatment with dexamethasone and vitamin D_3 abrogated the age-related decrease in IL-10 mRNA expression (lane 4). Dexamethasone and vitamin D_3 decreased IL-10 mRNA expression in young rats (lane 2). Mean data obtained from densitometric analysis is illustrated in Figure 3.12(B); it shows a decrease in IL-10 mRNA expression in aged (0.31 arbitrary values ± 0.02; n=7), compared with young, rats (0.42 arbitrary values ± 0.03; n=8; *$p<0.05$, ANOVA). Following treatment with dexamethasone and vitamin D_3, IL-10 mRNA expression levels in aged animals (0.41 arbitrary values ± 0.06; n=7) were increased compared with control-treated aged rats. A decrease in IL-10 mRNA expression was observed in dexamethasone and vitamin D_3-treated (0.21 arbitrary values ± 0.01; n=5), compared with control-treated, young rats (+$p<0.05$, ANOVA).

3.6 IL-10 receptor is present in the hippocampus

To propagate downstream signalling, a cytokine must interact with its receptor and facilitate the recruitment of kinases and accessory proteins. In this manner, gene transcription can be altered. As a result of our finding that IL-10 concentration was increased in hippocampal tissue, it was reasonable to investigate the presence of the IL-10 receptor (IL-10R) in the hippocampus. Figure 3.13 shows positive staining for IL-10R in the hippocampal region of cryostat sections and in cultured hippocampal neurons. Positively stained cells are pictured at a magnification of 4x. A negative control is also shown.

3.7 Age is associated with a decrease in signalling through JAK1 and STAT-3

As a result of the decreased concentration of IL-10 in the aged brain, an investigation into whether its downstream signalling molecules were downregulated with age was undertaken. JAK1 is a kinase recruited to the IL-10 receptor following IL-10 binding. Mean data obtained from densitometric analysis are illustrated in Figure 3.14; these revealed a significant decrease in JAK1 phosphorylation in aged (0.98 arbitrary units ± 0.008; n=6), compared with young (2.12 arbitrary units ± 0.33; n=6), control-treated rats (*$p<0.05$, ANOVA). Dexamethasone treatment attenuated the age-related decrease in JAK1 phosphorylation (1.46 arbitrary units ± 0.13; n=6; ++$p<0.01$, Student's t test for independent means). An increase in JAK1 phosphorylation was found in vitamin D_3-treated (1.37 arbitrary units ± 0.08; n=6), compared with control-treated, aged rats (+++$p<0.001$, ANOVA). JAK1 phosphorylation was increased in hippocampal tissue prepared from dexamethasone and vitamin D_3-treated (1.57 arbitrary units ± 0.26; n=6), compared with control-treated, aged rats (+$p<0.05$, Student's t test for independent means). Treatment with dexamethasone (1.83 arbitrary units ± 0.29; n=6), vitamin D_3 (1.77 arbitrary units ± 0.32; n=6) and a combination of both (1.97 arbitrary units ± 0.37; n=6) in young rats did not alter JAK phosphorylation, compared with control-treated young rats.

Subsequent to JAK recruitment, STAT-3 binds the receptor complex which facilitates its phosphorylation; therefore phosphorylation of STAT-3 was investigated in hippocampal homogenate prepared from young and aged rats. Assessment of mean data obtained from densitometric analysis (Figure 3.15) indicated that STAT-3 phosphorylation was significantly decreased in hippocampal tissue prepared from aged (0.38 arbitrary units ± 0.02; n=6), compared with young (0.48 arbitrary units ± 0.04; n=6), control-treated rats (**$p<0.01$, Student's t test for independent means). Dexamethasone treatment attenuated the age-related decrease in STAT-3 phosphorylation (0.41 arbitrary units ± 0.04; n=6). No effect of dexamethasone treatment was observed in young rats (0.53 arbitrary units ± 0.05; n=6). An increase in STAT-3 phosphorylation was found in vitamin D_3-treated (0.56 arbitrary units ± 0.10; n=6), compared with control-treated, aged rats. STAT-3

phosphorylation was increased in hippocampal tissue prepared from dexamethasone and vitamin D_3-treated (0.45 arbitrary units ± 0.06; n=6), compared with control-treated, aged rats. Vitamin D_3 (0.65 arbitrary units ± 0.10; n=6) and dexamethasone and vitamin D_3 (0.77 arbitrary units ± 0.14; n=6) increased STAT-3 phosphorylation in young rats.

3.8 Dexamethasone and vitamin D_3 stimulated IL-10 release from neurons following treatment with IL-1β *in vitro*

To investigate a possible source of IL-10, its concentration was measured in supernatant from hippocampal neurons treated with dexamethasone and vitamin D_3 in the presence or absence of IL-1β. Figure 3.16 illustrated that IL-10 concentration was unchanged in cells treated only with IL-1β (84.90pg/ml ± 10.90; n=6), compared with control-treated (72.41pg/ml ± 22.11; n=6), hippocampal neurons. However, neurons treated with dexamethasone and vitamin D_3 prior to IL-1β treatment resulted in increased IL-10 concentrations in supernatant (133.00pg/ml ± 16.50; n=6), compared with IL-1β-treated neurons which received no pre-treatment (+$p<0.05$, Student's t test for independent means). IL-1β concentration from neurons which were treated with dexamethasone and vitamin D_3 only (87.21 pg/ml ± 13.90; n=6) was similar to the concentration in control-treated neurons.

3.9 Dexamethasone and vitamin D_3 and IL-10 attenuate IL-1β-induced signalling *in vitro*

To further address the possibility that dexamethasone and vitamin D_3 elicited their anti-inflammatory effects through IL-10 in the aged brain, primary cultures of hippocampal neurons were treated with a combination of dexamethasone and vitamin D_3 and IL-10. Following this pre-treatment, neurons were treated with IL-1β, to mimic the environment in the aged brain. Figures 3.17(A) and 3.18(A) show two photomicrographs which are representative of staining in cultures prepared from 6 neonatal rats. Figures 3.17(A)(i) and 3.18(A)(i) show positively and negatively stained hippocampal neurons stained for p-JNK. Similarly, Figures 3.17(A)(ii) and 3.18(A)(ii) show staining for caspase-3. The

mean percentage of positively stained cells for p-JNK was increased in IL-1β-treated (42.80% ± 1.87; n=6), compared with control-treated (24.54% ± 2.67; n=6; ***p<0.001, ANOVA; Figures 3.17(B) and 3.18(B)), hippocampal neuronal cultures. Treatment with dexamethasone and vitamin D_3 decreased the IL-1β-induced increase in p-JNK staining (26.29% ± 2.47; n=6; +++p<0.001, Student's t test for independent means; Figure 3.17(B)). Similar to the effect of dexamethasone and vitamin D_3, IL-10 caused a decrease in the percentage of p-JNK positively stained cells in IL-1β-treated neurons (29.00% ± 0.84; n=6; +++p<0.001, ANOVA). Dexamethasone and vitamin D_3 (24.66% ± 2.84; n=6) and IL-10 (23.95% ± 1.16; n=6) did not elicit an effect on p-JNK staining in control-treated neurons. The mean percentage of positively stained cells for activated caspase-3 was increased in IL-1β-treated (42.25% ± 2.30; n=6), compared with control-treated (18.25% ± 1.32; n=6; ***p<0.001, Student's t test for independent means Figures 3.17(C) and 18(C)), hippocampal neuronal cultures. Treatment with dexamethasone and vitamin D_3 decreased the IL-1β-induced increase in caspase-3 staining (22.75% ± 0.88; n=6; +++p<0.001, ANOVA; Figure 3.17(C)). Figure 3.18(C) demonstrates that IL-10 treatment also decreased the percentage of positively stained cells for caspase-3 in IL-1β-treated neurons (24.79% ± 1.05; n=6; +++p<0.001, ANOVA). Neurons treated with dexamethasone and vitamin D_3 (18.33% ±1.49; n=6) and IL-10 (17.9% ± 0.81; n=6) had similar amounts of caspase-3 staining compared with control-treated neurons.

3.10 Age is associated with an increase in IFN-γ

Evidence suggests that in the aged brain, the cytokine balance is shifted towards the increased production of pro-inflammatory cytokines; this was verified by the increase in IL-1β concentration shown here. The concentration of another pro-inflammatory cytokine, IFN-γ, was measured. Figure 3.19 indicates that the concentration of IFN-γ was increased in aged (1791.87pg/mg ± 242.32; n=6), compared with young (983.98pg/mg ± 232.95; n=5), rats (*p<0.05, Student's t test for independent means). This concentration was decreased in dexamethasone-treated (1484.90pg/mg ± 162.41; n=6), compared with control-treated, aged rats, however it did not reach statistical significance. Vitamin D_3 treatment did not reduce the age-related increase in IFN-γ (1620.96pg/mg ± 206.57; n=6).

Mean IFN-γ concentration was decreased in dexamethasone and vitamin D_3-treated (1312.01pg/mg ± 259.99; n=5), compared with control-treated, aged rats. Dexamethasone (1114.81pg/mg ± 345.05; n=6), vitamin D_3 (1607.46pg/mg ± 388.58; n=6) or dexamethasone and vitamin D_3 in combination (1108.85pg/mg ± 87.99; n=6) did not substantially affect IFN-γ concentration in young rats.

3.11 Glia are the main source of IL-1β

To elucidate the mechanism by which dexamethasone and vitamin D_3 elicited their anti-inflammatory effect on IL-1β, the source of IL-1β needed to be established. Neurons and glia were treated with LPS, which has been shown to induce inflammation similar to that seen in the aged brain, and IFN-γ which has been shown above to be increased in the aged brain. Mean IL-1β concentration was increased in supernatant obtained from neurons treated with LPS (66.7520pg/mg ± 17.248; n=4; *$p<0.05$, ANOVA) and IFN-γ (37.7180pg/mg ± 3.853; n=3; +$p<0.05$, Student's t test for independent means), compared with control-treated neurons (11.5720pg/mg ± 4.325; n=3; Figure 3.20(A)). Glia treated with IFN-γ (217.353pg/mg ± 42.383; n=11; +++$p<0.001$, ANOVA) released a greater amount of IL-1β compared with LPS-treated glia (120.846pg/mg ± 18.822; n=11; *$p<0.05$, ANOVA); however, both treatments increased IL-1β release compared with the control treatment (42.743pg/mg ± 3.242; n=11; Figure 3.20(B)). Although both cell types released IL-1β, glia did so to a greater extent than neurons and IL-1β concentration in supernatant prepared from untreated glia was significantly increased compared with that in supernatant from untreated neurons ($p<0.001$; Student's t test for independent means).

3.12 Microglial activation correlates with age

When microglia receive a stimulus, they become activated and release cytokines such as IL-1β. OX-6 is regarded as a marker of microglia which have been activated and therefore OX-6 mRNA and protein were investigated here. Figure 3.21(A) shows a sample gel for OX-6 mRNA; it reveals that OX-6 mRNA expression was increased in

tissue prepared from aged (lane 3), compared with young (lane 1), rats. Treatment with dexamethasone and vitamin D_3 attenuated this age-related increase in expression (lane 4). Figure 3.21(B) was constructed from mean data obtained from densitometric analysis. Expressed as a ratio of OX-6 to β-actin, mean OX-6 expression was increased in aged (0.15 arbitrary values ± 0.04; n=5), compared with young (0.0086 arbitrary values ± 0.005; n=5), rats (**$p<0.01$, ANOVA). Expression was decreased in dexamethasone and vitamin D_3-treated (0.06 arbitrary values ± 0.02; n=5), compared with control-treated, aged rats (+$p<0.05$, ANOVA) but expression was unaltered in dexamethasone and vitamin D_3-treated young rats (0.0022 arbitrary values ± 0.0004; n=5).

Microglial activation was also assessed by immunohistological staining in cryostat sections prepared from rat brain (Figure 3.22). The first photomicrograph shown in Figure 3.22 is representative of what was observed in young rats; it shows limited brown staining within the hippocampal formation. In contrast, a section prepared from an aged animal shows increased brown staining and altered cell morphology. An example of a section prepared from an aged dexamethasone and vitamin D_3-treated rat demonstrates less intense staining than in control-treated aged rats. Treatment with dexamethasone and vitamin D_3 did not have any substantial effect in young rats. The pictures are representative of staining obtained in sections prepared from 6 animals for each treatment group.

3.13 IFN-γ acts as a stimulus for microglial activation

Data from several studies suggest that microglia possess receptors for IFN-γ on their surface and are, consequently, activated by this cytokine. As a result of the findings that IFN-γ concentration and microglial activation were both increased with age, an experiment to investigate the possibility that IFN-γ might act as a stimulus for microglial activation was carried out. Figure 3.23 shows the mean data obtained from densitometric analysis; it reveals that OX-6 mRNA expression, expressed as a ratio of OX-6 to β-actin, was increased in cultured glia treated with IFN-γ (0.255 arbitrary values ± 0.0653; n=6), compared with control-treated cells (0.025 arbitrary values ± 0.0212; n=6; **$p<0.01$,

Student's t test for independent means). As IL-10 prevented the IFN-γ-induced OX-6 expression and therefore microglial activation, its effect on IL-1β release from IFN-γ-treated cortical glial cells was investigated (Figure 3.24). Mean IL-1β concentration was increased in IFN-γ-treated (217.35pg/mg ± 42.38; n=11), compared with control-treated (42.74pg/mg ± 3.24; n=11), cultured glia (***$p<0.001$, ANOVA). This concentration was decreased in IFN-γ-treated glia which were pre-treated with IL-10 (60.42pg/mg ± 6.90; n=4), compared with untreated cells (+$p<0.05$, ANOVA). The concentration of IL-1β in glia pre-treated with IL-10 (62.27pg/ml ± 11.85; n=5) was similar to that in control-treated glia which received no pre-treatment.

3.14 $CD4^+$ cells are found in the hippocampus

It is known that peripheral cells produce IFN-γ however, evidence suggests that resident cells of the brain, such as neurons and glia, do not have this ability. Figure 3.25 shows undetectable IFN-γ release from cultured glia treated with or without LPS (n=5). As a result, it is postulated here that peripheral cells with this capability, such as $CD4^+$ T cells can cross the blood-brain barrier of an aged animal and elicit various effects such as releasing IFN-γ. Figure 3.26 shows photomicrographs taken of hippocampal cells of cryostat sections stained for $CD4^+$ cells. Some weak positive staining was present in sections from both young and aged rats however, there was slightly more staining in the aged rat. Dexamethasone and vitamin D_3-treated, young and aged animals showed positive staining for $CD4^+$ cells.

Data obtained from densitometric analysis reveals that mean CD4+ expression was increased in hippocampal homogenate prepared from aged (0.40 arbitrary values ± 0.06; n=4), compared with young (0.29 arbitrary values ± 0.06; n=5), rats, however it did not reach statistical significance (Figure 3.27). Expression was unchanged in dexamethasone and vitamin D_3-treated (0.40 arbitrary values ± 0.08; n=5), compared with control-treated, aged rats. Young rats treated with dexamethasone and vitamin D_3 showed an increased expression of $CD4^+$ cells (0.61 arbitrary values ± 0.22; n=5).

3.15 CD161$^+$ cells are present in the hippocampus and increased with age

Another peripheral cell type that releases IFN-γ are NK cells; for this reason, immunohistochemical staining and western immunoblotting for these cells were carried out. Figure 28 reveals the expression of CD161 positive cells in the hippocampal region of cryostat sections prepared from young and aged rats. CD161 expression, as evidenced by brown staining on the outer surface of cells, was increased in aged (ii), compared with young (i), control-treated rats. Dexamethasone and vitamin D_3-treated aged rats (iv) show decreased positive staining compared with control-treated aged rats. Expression in dexamethasone and vitamin D_3-treated young rats (ii) was similar to control-treated young rats.

Figure 3.29(A) shows a sample immunoblot indicating an increase in CD161 expression in hippocampal homogenate prepared from aged (lane 3), compared with young, rats (lane 1). The expression was decreased in dexamethasone and vitamin D_3-treated (lane 4), compared with control-treated, aged rats. Similar expression was observed in dexamethasone and vitamin D_3-treated (lane 2) and control-treated young rats. Data from densitometric analysis (Figure 3.29(B)) shows an increase in CD161 expression in aged (1.43 arbitrary values ± 0.15; n=6), compared with young (0.89 arbitrary values ± 0.10; n=5; **$p<0.01$, ANOVA), rats. A decrease in expression was found in dexamethasone and vitamin D_3-treated (0.78 arbitrary values ± 0.11; n=4), compared with control-treated, aged rats (+$p<0.05$, ANOVA). Dexamethasone and vitamin D_3 treatment did not elicit any effect in young rats (0.75 arbitrary values ± 0.15; n=5) compared with control-treated young rats.

3.16 IL-2 is present in the hippocampus

Evidence suggests that IL-2 stimulates NK cells to release IFN-γ. Because of this IL-2 concentration was assessed. Figure 3.30 shows that mean IL-2 concentration was similar in young (286.53 arbitrary values ± 13.82; n=7) and aged (305.74 arbitrary values ± 41.98; n=7) control-treated rats. Dexamethasone and vitamin D_3 treatment (257.91

arbitrary values ± 13.10; n=7) did not significantly alter IL-2 concentration in aged rats but slightly, although not significantly, increased IL-2 concentration in young rats (376.18 arbitrary values ± 36.35; n=8).

3.17 IL-2 induces IFN-γ release from NK cells in vitro; abrogated by dexamethasone and vitamin D_3

Figure 3.31 shows that treatment of NK cells in vitro with IL-2 induced a 6-fold increase in IFN-γ concentration (n=6 for both groups).

The inhibitory effect of dexamethasone and vitamin D_3 on IL-2-induced IFN-γ release from NK cells was assessed (Figure 3.32). The data show that there was a concentration-dependent effect, with the lowest concentration (6.8×10^{-11}mol/L dexamethasone and 10^{-6}mol/L vitamin D_3) exerting a minimal effect and inhibiting the IL-2 induced stimulation to 96%. The intermediate (1.36×10^{-10}mol/L dexamethasone and 2×10^{-6}mol/L vitamin D_3) and highest (6.8×10^{-10}mol/L dexamethasone and 10^{-5}mol/L vitamin D_3) concentrations reduced the IL-2-induced stimulation to 88% and 80% respectively.

Figure 3.33 shows that mean IFN-γ concentration released from IL-2-treated (820.17pg/ml ± 28.26; n=5) was increased significantly, compared with release from control-treated (138.32pg/ml ± 5.66; n=6), NK cells (***$p<0.001$, ANOVA). Il-2-induced mean IFN-γ concentration was decreased significantly by pre-treatment with dexamethasone and vitamin D_3 (6.8×10^{-10}mol/L dexamethasone and 10^{-5}mol/L vitamin D_3; 649.95pg/ml ± 9.66; n=6; +++$p<0.001$, ANOVA). Treatment with IL-10 did not alter the IL-2-induced IFN-γ release from NK cells (819.96pg/ml ± 20.66; n=5). Pre-treatment with dexamethasone and vitamin D_3 (108.6pg/ml ± 7.83; n=6) and IL-10 (155.46pg/ml ± 9.6; n=6) did not alter unstimulated IFN-γ concentration.

Figure 3.1 Age is associated with an increase in IL-1β concentration

IL-1β concentration was increased significantly in the hippocampus of aged, compared with young, rats (n=7 and 6 respectively; *$p<0.05$, Student's t test for independent means). This change was significantly decreased in hippocampal tissue prepared from dexamethasone-treated, compared with control-treated, aged rats (n=6 and 7 respectively; +$p<0.05$, ANOVA). IL-1β concentration was significantly decreased in tissue prepared from vitamin D_3-treated, compared with control-treated, aged rats (n=6 and 7 respectively; ++$p<0.01$, ANOVA). IL-1β concentration was significantly decreased in tissue prepared from dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats (n=6 and 7 respectively; +++$p<0.001$, ANOVA).

Values are expressed as pg IL-1/mg protein and are expressed as means ± SEM.

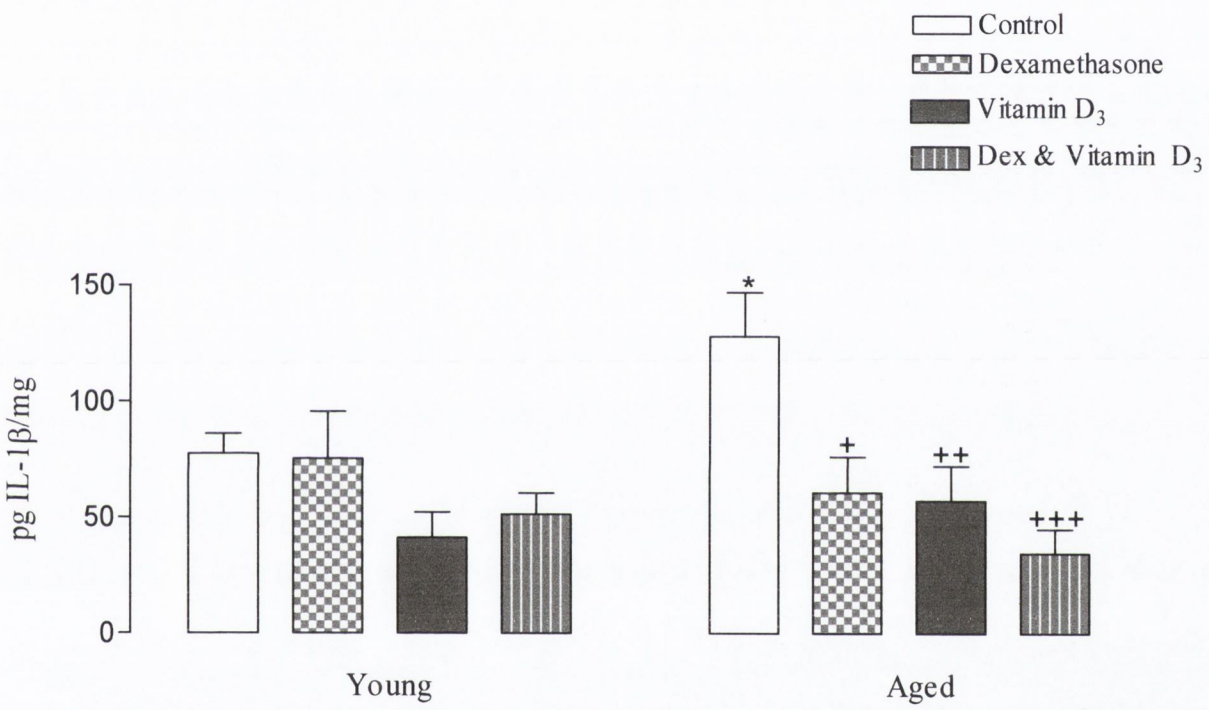

Control
Dexamethasone
Vitamin D_3
Dex & Vitamin D_3
pg IL-1β/mg
150
100
50
0
*
+
++
+++
Young
Aged

Figure 3.2 IL-1β mRNA expression is increased with age.

(A) The sample gel shows the molecular expression of IL-1β in control-treated (lane 1) and dexamethasone and vitamin D_3-treated (lane 2), young rats and also in control-treated (lane 3) and dexamethasone and vitamin D_3-treated (lane 4), aged rats.

(B) IL-1β expression, expressed as a ratio of IL-1β to β-actin, was increased significantly in the hippocampus of aged, compared with young, rats (n=7 for both groups; *$p<0.05$, Student's t test for independent means). Dexamethasone and vitamin D_3 treatment exerted no significant effect in young or aged rats (n=3 and n=6 respectively).
Values are expressed as arbitrary values and as means ± SEM.

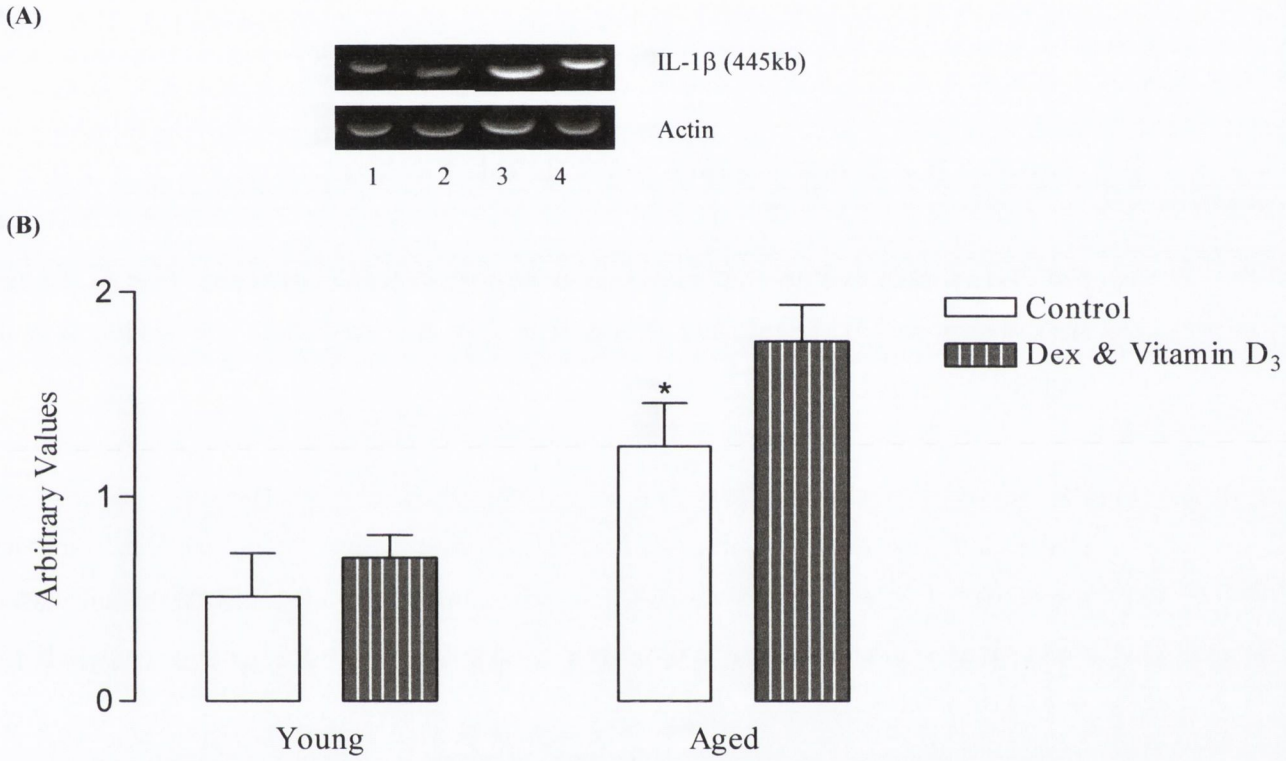
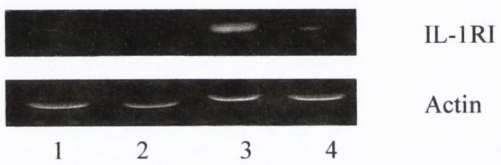

(A)
IL-1β (445kb)
Actin
1 2 3 4
(B)
Arbitrary Values
2
1
0
Young
Aged
*
Control
Dex & Vitamin D3

Figure 3.3 IL-1RI mRNA expression is increased with age.

(A) The sample gel shows the molecular expression of IL-1RI in young (lane 1) and aged (lane 3) control-treated rats and young (lane 2) and aged (lane 4) dexamethasone and vitamin D_3-treated rats.

(B) IL-1RI expression, expressed as a ratio of IL-1RI to β-actin, was increased significantly in the hippocampus of aged, compared with young, rats (n=7 and n=6 respectively; ***$p<0.001$, ANOVA). This was significantly attenuated in dexamethasone and vitamin D_3-treated, compared to control-treated, aged rats (n=3 and n=7 respectively; ++$p<0.01$, ANOVA).
Values are expressed as arbitrary values and as means ± SEM.

(A)


IL-1RI
Actin
1
2
3
4

(B)

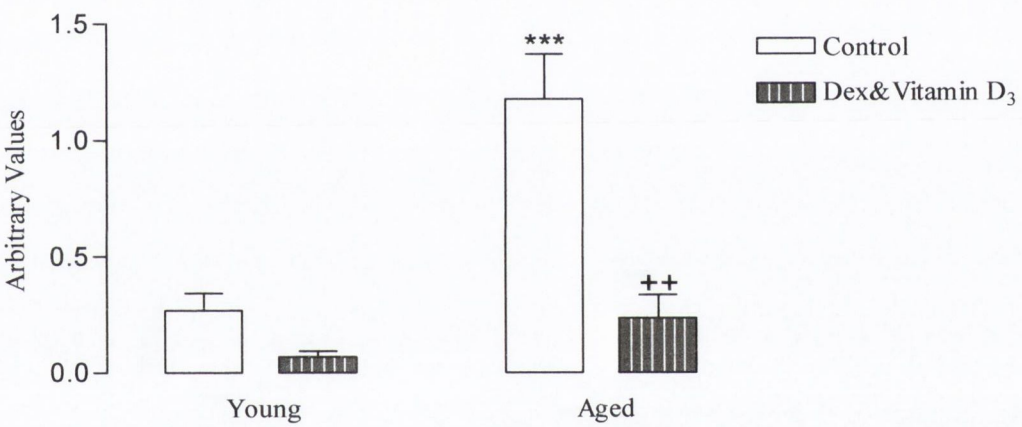

1.5
1.0
0.5
0.0
Arbitrary Values
***
++
Control
Dex& Vitamin D_3
Young
Aged

Figure 3.4 JNK activity is upregulated with age

JNK activity, expressed as a ratio of pJNK to total JNK expression, was increased significantly in the hippocampus of aged, compared to young, rats (n=5 for both groups; *$p<0.05$, Student's t test for independent means). There was a decrease in JNK activity in the hippocampus of dexamethasone-treated, compared with control-treated, aged rats, however this did not reach statistical significance (n=5 for both treatment groups). There was no change in JNK activity in hippocampal tissue of vitamin D_3-treated, compared with control-treated, aged rats (n=5 for both groups). JNK activity was slightly, but not significantly, decreased in hippocampal tissue of dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats, however this did not reach statistical significance (n=5 for both groups).

Data are expressed as arbitrary units and as mean ± SEM.

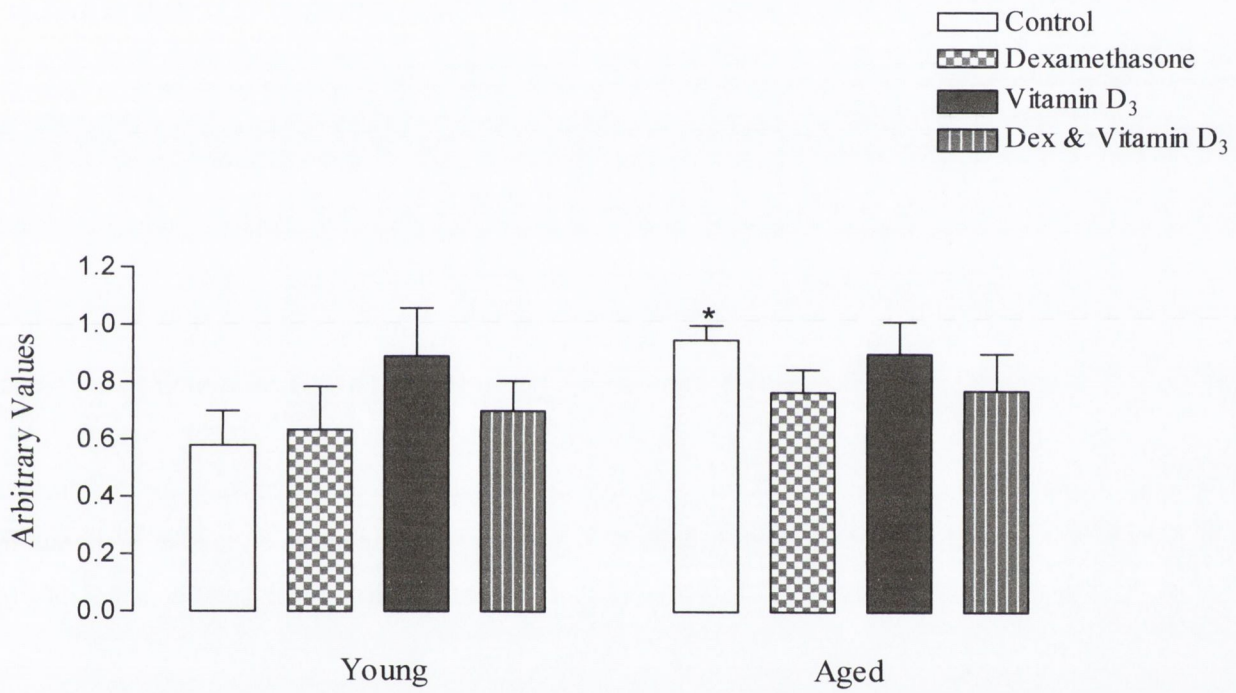

Control
Dexamethasone
Vitamin D_3
Dex & Vitamin D_3
Arbitrary Values
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Young
Aged

Figure 3.5 Age is associated with a decrease in intact caspase-3 (32kDa) expression

Expression of the uncleaved form of caspase-3 was decreased in the hippocampus of aged, compared with young, rats (n=4 and n=4 respectively; *$p<0.05$, Student's t test for independent means). Expression of the uncleaved form of caspase-3 was increased in dexamethasone-treated, compared with control-treated, aged rats (n=6 and n=4 respectively; *$p<0.05$, Student's t test for independent means). Expression of the uncleaved form of caspase-3 was slightly, but insignificantly, increased in tissue prepared from vitamin D_3-treated, compared with control-treated, aged rats, however this did not reach statistical significance (n=6 and n=4 respectively). Expression of the uncleaved form of caspase-3 was slightly, but insignificantly, increased in tissue prepared from dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats (n=6 and n=4 respectively).

Data are expressed as arbitrary units and as mean ± SEM.

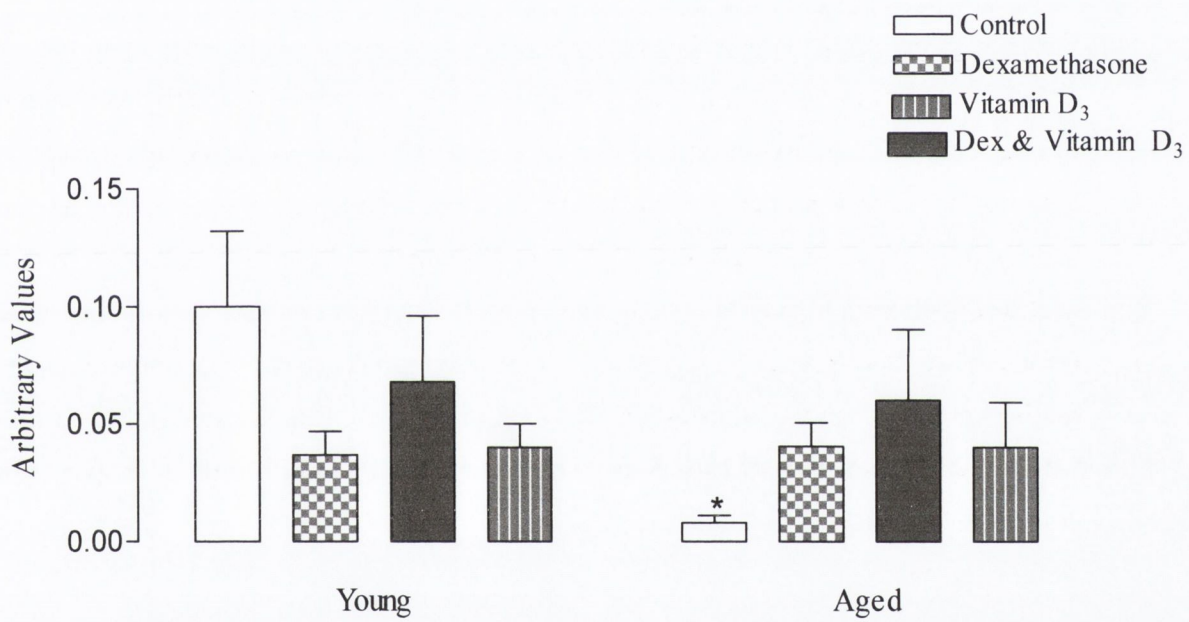

Control
Dexamethasone
Vitamin D_3
Dex & Vitamin D_3
Arbitrary Values
0.15
0.10
0.05
0.00
*
Young
Aged

Figure 3.6 Age is associated with an increase in activated caspase-3

Positive staining for activated caspase-3 was obtained in the hippocampal region of cryostat sections prepared from aged control-treated rats, whereas healthy toluidine blue stained cells only were present in sections prepared from young control-treated rats. Staining in sections prepared from dexamethasone, vitamin D_3 and dexamethasone and vitamin D_3-treated, aged rats show a lesser amount of activated caspase-3. Treatment with dexamethasone, vitamin D_3 and a combination of both in young rats did not have an effect on activated caspase-3 staining. Pictures are representative of staining obtained in sections prepared from 6 animals for each treatment group. Counterstaining was achieved with toluidine blue. Magnification 40x. The scale bar represents 10μm.

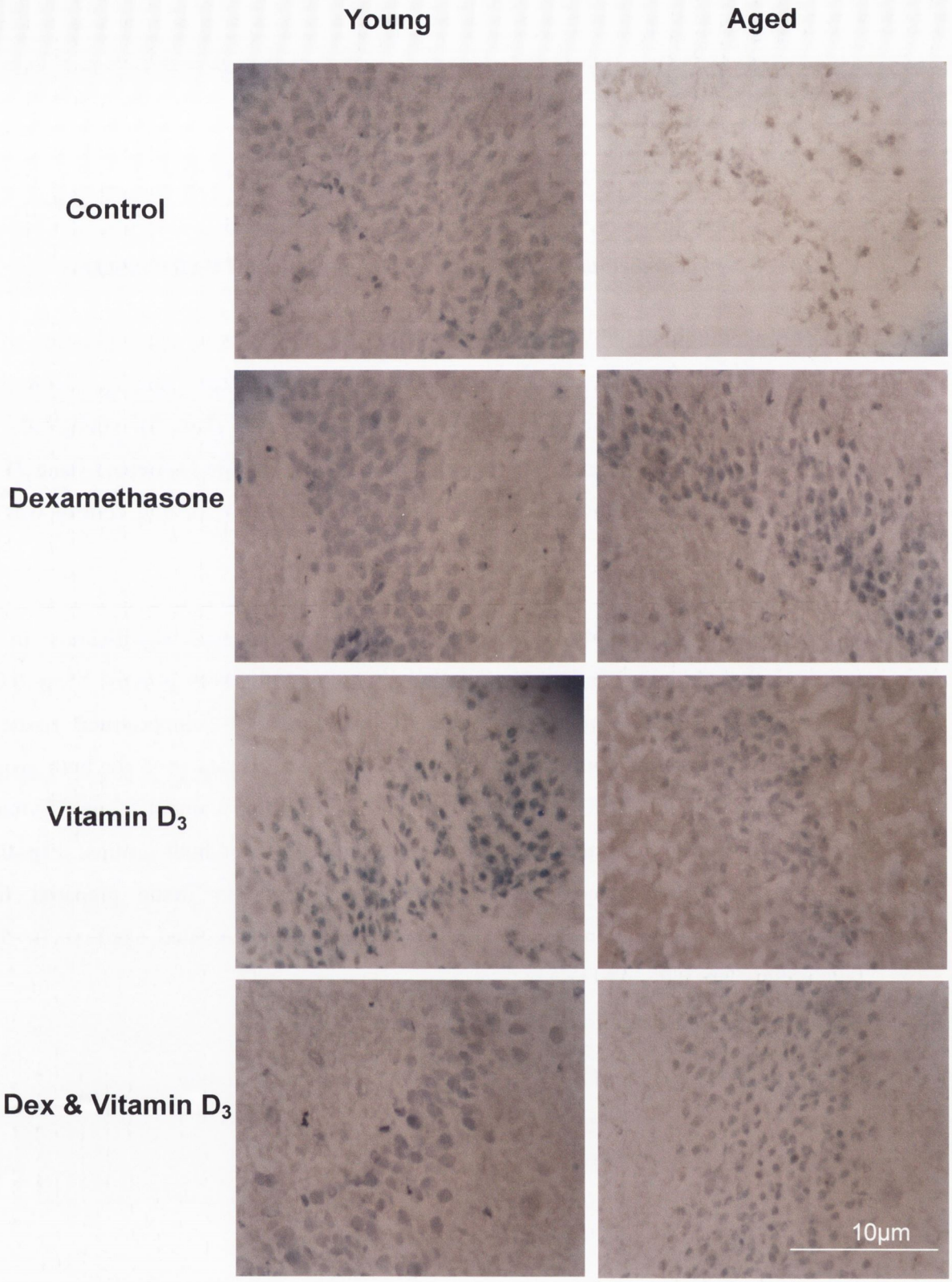

Young
Aged
Control
Dexamethasone
Vitamin D_3
Dex & Vitamin D_3
10μm

Figure 3.7 Age is associated with an increased expression of PARP (85kDa)

(A) The sample immunoblot shows PARP cleavage, as shown by the expression of the 85kDa form, in control-treated (lane 1), dexamethasone-treated (lane 2), vitamin D_3-treated (lane 3) and dexamethasone and vitamin D_3-treated (lane 4), young rats and control-treated (lane 5), dexamethasone-treated (lane 6), vitamin D_3-treated (lane 7) and dexamethasone and vitamin D_3-treated (lane 8), aged rats. A loading control was not performed for this analysis.

(B) Expression of the 85kDa, cleaved PARP was increased significantly in the hippocampus of aged, compared with young, rats (n=5 for both groups; ***$p<0.001$, ANOVA). PARP cleavage was decreased significantly in hippocampal tissue of dexamethasone-treated, compared with control-treated, aged rats (n=5 for both groups; ++$p<0.01$, ANOVA). PARP cleavage was decreased in hippocampal tissue of vitamin D_3-treated, compared with control-treated, aged rats (n=5 for both groups; ++$p<0.01$, ANOVA). PARP cleavage was decreased significantly in tissue prepared from dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats (n=5 for both groups; +$p<0.05$, ANOVA).

Data are expressed as arbitrary units and as mean ± SEM.

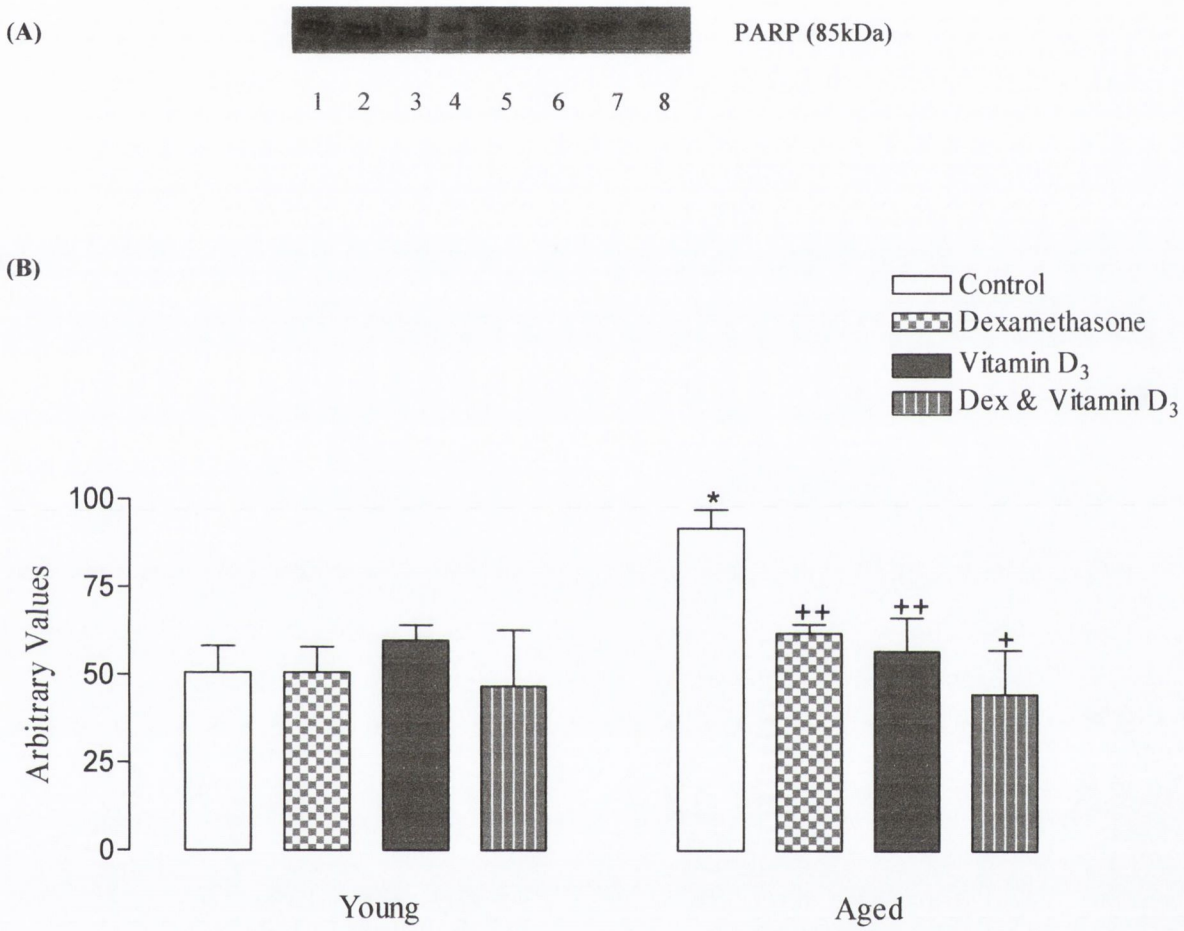

(A)
PARP (85kDa)
1 2 3 4 5 6 7 8
(B)
Control
Dexamethasone
Vitamin D3
Dex & Vitamin D3
Arbitrary Values
100
75
50
25
0
*
++
++
+
Young
Aged

Figure 3.8 Dexamethasone and vitamin D_3 attenuates the inhibitory effect of age on LTP.

LTP was inhibited in the dentate gyrus of aged (n=7), compared with young (n=6), rats. This impairment was prevented in dexamethasone and vitamin D_3-treated (n=5), compared to control-treated, aged rats. Data are expressed as the mean percentage change in population EPSP slope relative to the mean EPSP slope in the 5 minutes immediately prior to tetanic stimulation. Values are expressed as means ± SEM. SEM values are included for every 10^{th} response.

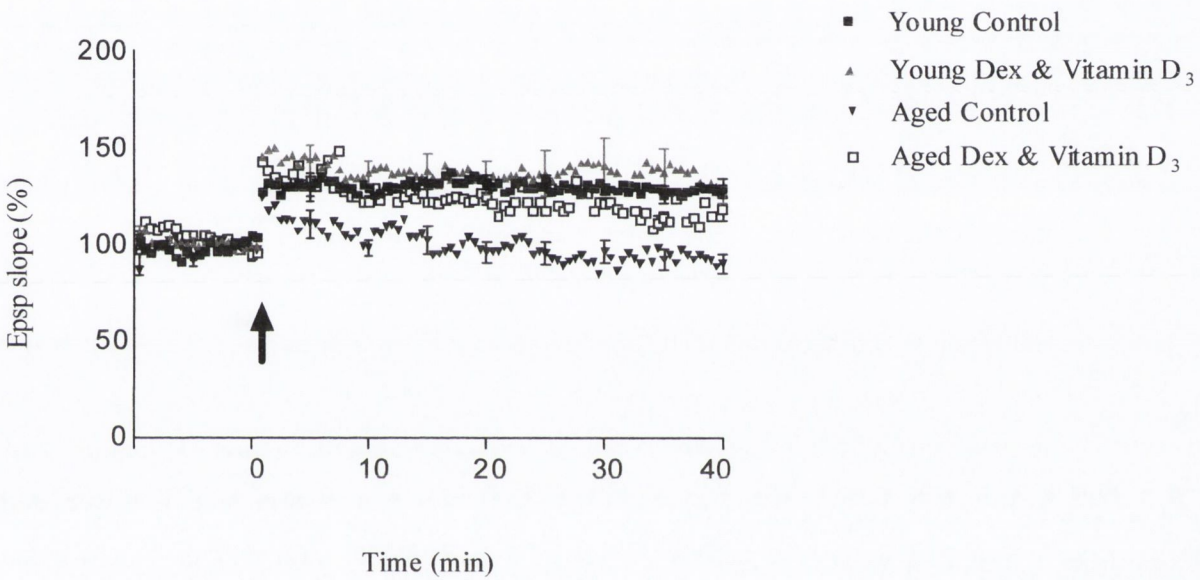

Young Control
Young Dex & Vitamin D 3
Aged Control
Aged Dex & Vitamin D 3
Epsp slope (%)
200
150
100
50
0
0
10
20
30
40
Time (min)

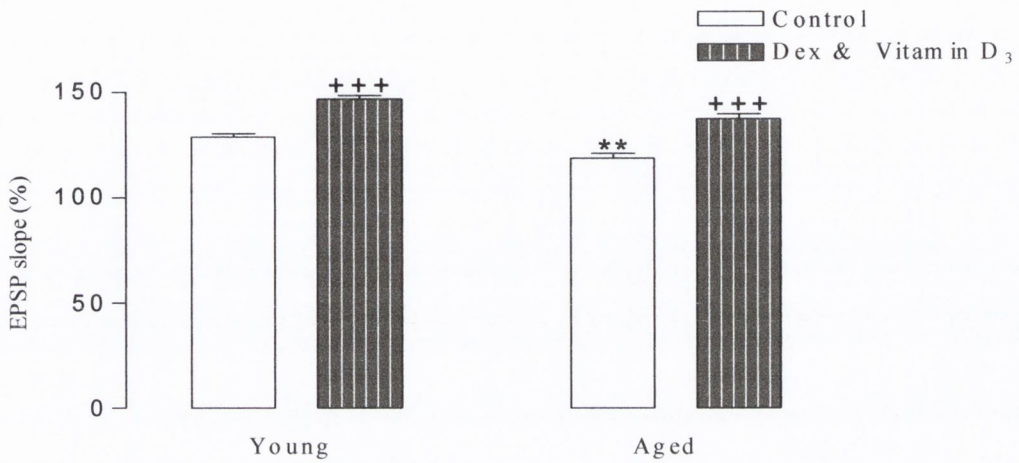
Figure 3.9 Dexamethasone and vitamin D_3 restore LTP

(A) Mean percentage change in EPSP slope in the first 2 minutes post tetanic stimulation was decreased significantly in aged, compared with young, rats (n=4 for both groups; **p<0.01, ANOVA). The mean value was increased in dexamethasone and vitamin D_3-treated aged rats compared with control-treated aged rats (n=10 for both groups; +++p<0.001, ANOVA). Dexamethasone and vitamin D_3 treatment significantly increased the EPSP slope in young rats (n=4; +++p<0.001, ANOVA).

(B) Mean percentage change in EPSP slope in the last 5 minutes of recording post tetanic stimulation was decreased significantly in aged, compared with young, rats (n=7 and n=6 respectively; ***p<0.001, ANOVA). Dexamethasone and vitamin D_3 treatment of aged rats restored LTP so that the mean EPSP slope was comparable with control-treated aged rats (n=5 and n=7 respectively; +++p<0.001, ANOVA). Dexamethasone and vitamin D_3 treatment significantly increased the EPSP slope in young rats (n=4; ++p<0.001, ANOVA).

Values are expressed as means ± SEM.

(A)


Control
Dex & Vitamin D3
EPSP slope (%)
150
100
50
0
+++
**
+++
Young
Aged

(B)

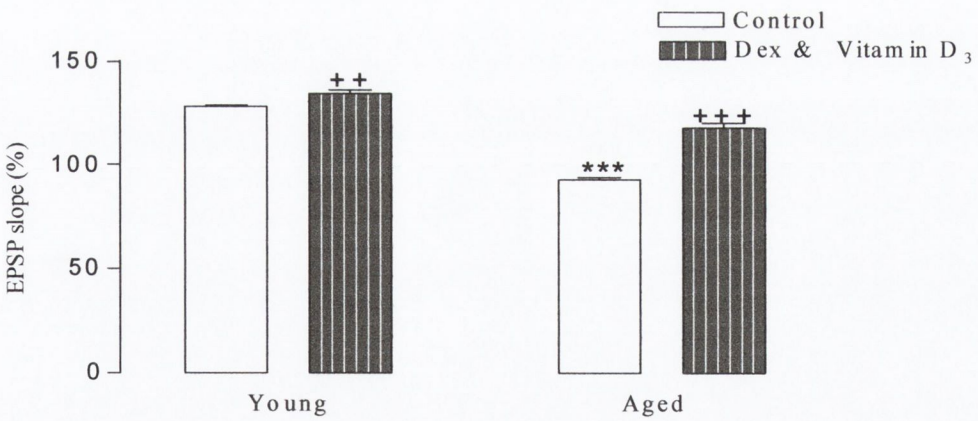

Control
Dex & Vitamin D3
EPSP slope (%)
150
100
50
0
++
***
+++
Young
Aged

Figure 3.10 Corticosterone concentration is increased with age.

Corticosterone concentration is increased in serum from aged, compared with young, control-treated rats (n=6 for both groups;**$p<0.01$, ANOVA). This change was significantly decreased in dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats (n=7 and n=6 respectively; +++$p<0.001$, ANOVA).

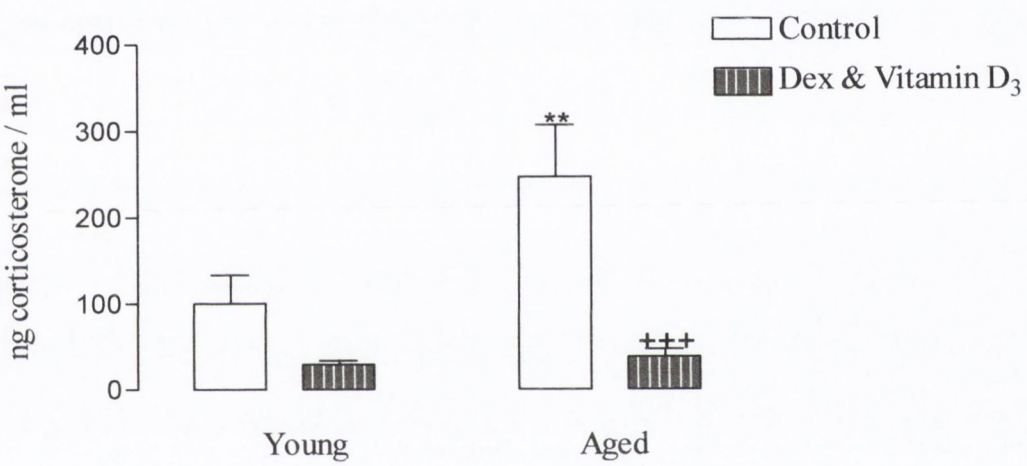

Control
Dex & Vitamin D_3
ng corticosterone / ml
400
300
200
100
0
**
+++
Young
Aged

Figure 3.11 Age is associated with a decrease in IL-10 concentration

IL-10 concentration was decreased significantly in the hippocampus of aged, compared with young, rats (n=10 for both groups; *$p<0.05$, ANOVA). Dexamethasone had no effect on IL-10 concentration in hippocampal tissue of dexamethasone-treated, compared with control-treated, aged rats (n=4 and n=10 respectively). IL-10 concentration was slightly, but insignificantly, decreased in tissue prepared from vitamin D_3-treated, compared with control-treated, aged rats (n=3 and n=10 respectively). IL-10 concentration was increased significantly in tissue prepared from dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats (n=4 and n=10 respectively; +++$p<0.001$, ANOVA).

Data are expressed as arbitrary units and as mean ± SEM.

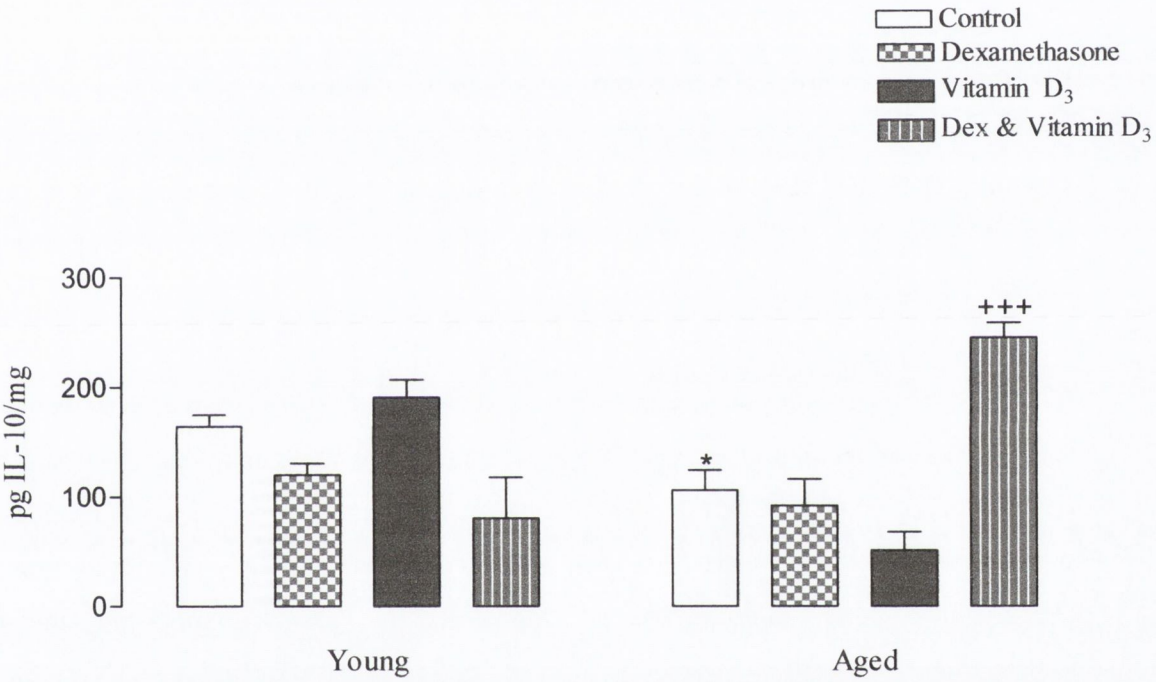
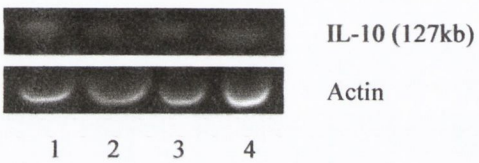

Control
Dexamethasone
Vitamin D_3
Dex & Vitamin D_3
pg IL-10/mg
300
200
100
0
*
+++
Young
Aged

Figure 3.12 IL-10 mRNA expression is decreased with age.

(A) The sample gel shows the molecular expression of IL-10 in young (lane 1) and aged (lane 3) control-treated rats and young (lane 2) and aged (lane 4) dexamethasone and vitamin D_3-treated rats.

(B) IL-10 mRNA expression, expressed as a ratio of IL-10 to β-actin, was decreased significantly in the hippocampus of aged, compared with young, rats (n=7 and n=8 respectively; *$p<0.05$, ANOVA). This expression was increased in dexamethasone and vitamin D_3-treated, compared to control-treated, aged rats (n=7 for both groups). Dexamethasone and vitamin D_3 treatment decreased IL-10 mRNA expression, compared to the control treatment, in young rats (n=5 and n=8 respectively; +$p<0.05$, ANOVA). Values are expressed as arbitrary values and as means ± SEM.

(A)


IL-10 (127kb)
Actin
1 2 3 4

(B)

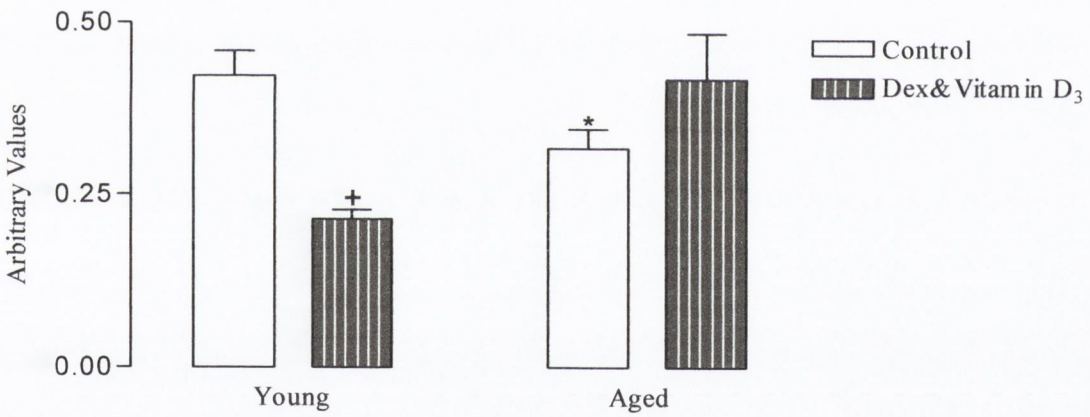
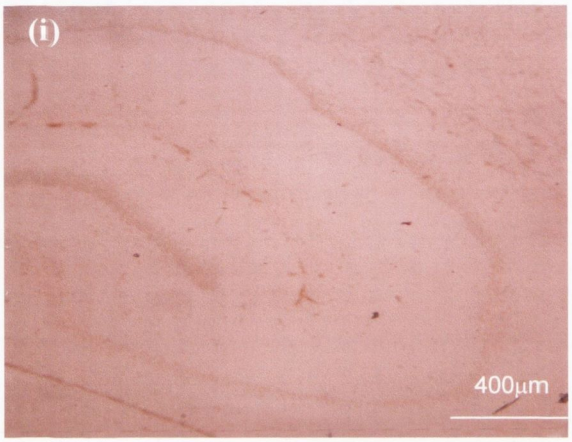
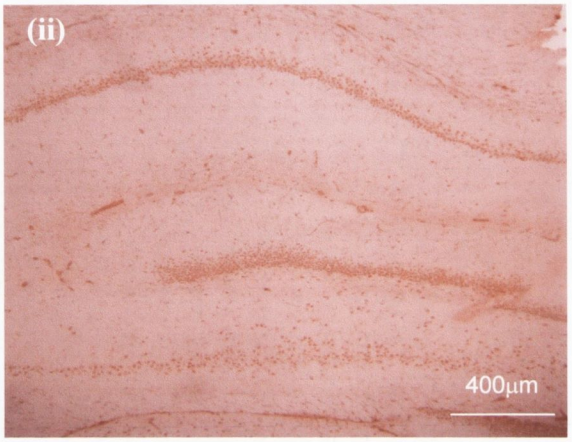

0.50
0.25
0.00
Arbitrary Values
+
*
Young
Aged
Control
Dex& Vitamin D_3

Figure 3.13 IL-10 receptor is present in the hippocampus

Photomicrographs indicate the presence of the IL-10 receptor in hippocampal cells in cryostat slices. Positively stained cells are shown at 4x magnification in panel (ii) and the scale bar represents 400μm. A negative control was prepared by omitting the primary antibody (i). Panel (iii) shows hippocampal neurons fluorescently stained for IL-10R. This work was carried out by Y. Nolan.



-ve control



IL-10 receptor (4x)

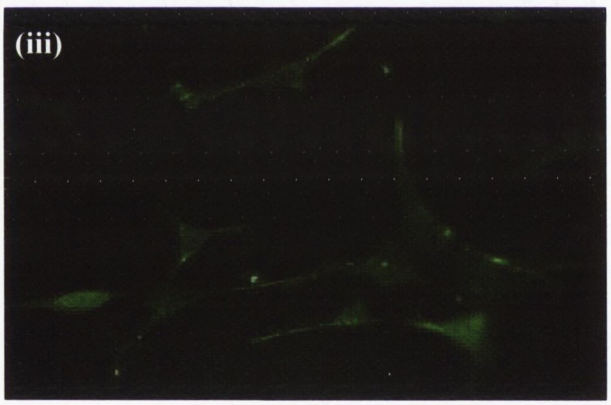


Figure 3.14 JAK1 phosphorylation is decreased with age

JAK1 phosphorylation, expressed as a ratio of p-JAK1 to actin, was decreased significantly in the hippocampus of aged, compared with young, rats (n=6 for both groups; *$p<0.05$, ANOVA). JAK1 phosphorylation was increased significantly in tissue prepared from dexamethasone-treated, compared with control-treated, aged rats (n=6 for both groups; ++$p<0.01$, Student's t test for independent means). JAK1 phosphorylation was increased significantly in tissue prepared from vitamin D_3-treated, compared with control-treated, aged rats (n=6 for both groups; +++$p<0.001$, Student's t test for independent means). JAK1 phosphorylation was significantly increased in tissue prepared from dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats (n=6 for both groups; +$p<0.05$, Student's t test for independent means).

Data are expressed as arbitrary units and as mean ± SEM.

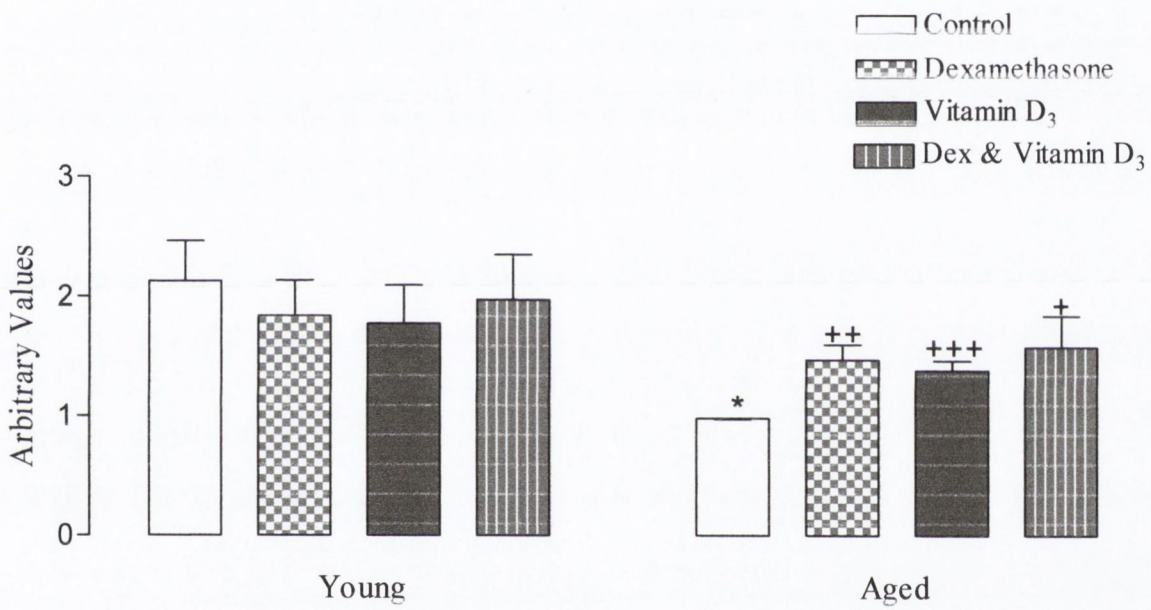

Control
Dexamethasone
Vitamin D_3
Dex & Vitamin D_3
Arbitrary Values
3
2
1
0
*
++
+++
+
Young
Aged

Figure 3.15 Age is associated with a decrease in STAT-3 phosphorylation

STAT-3 phosphorylation was decreased significantly in the hippocampus of aged, compared with young, rats (n=6 for both groups; **$p<0.01$, Student's t test for independent means). STAT-3 phosphorylation was slightly, but not significantly, increased in tissue prepared from dexamethasone-treated, compared with control-treated, aged rats (n=6 for both groups). STAT-3 phosphorylation was slightly, but not significantly, increased in tissue prepared from vitamin D_3-treated, compared with control-treated, aged rats (n=6 for both groups). STAT-3 phosphorylation slightly, but not significantly, increased in tissue prepared from dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats (n=6 for both groups).

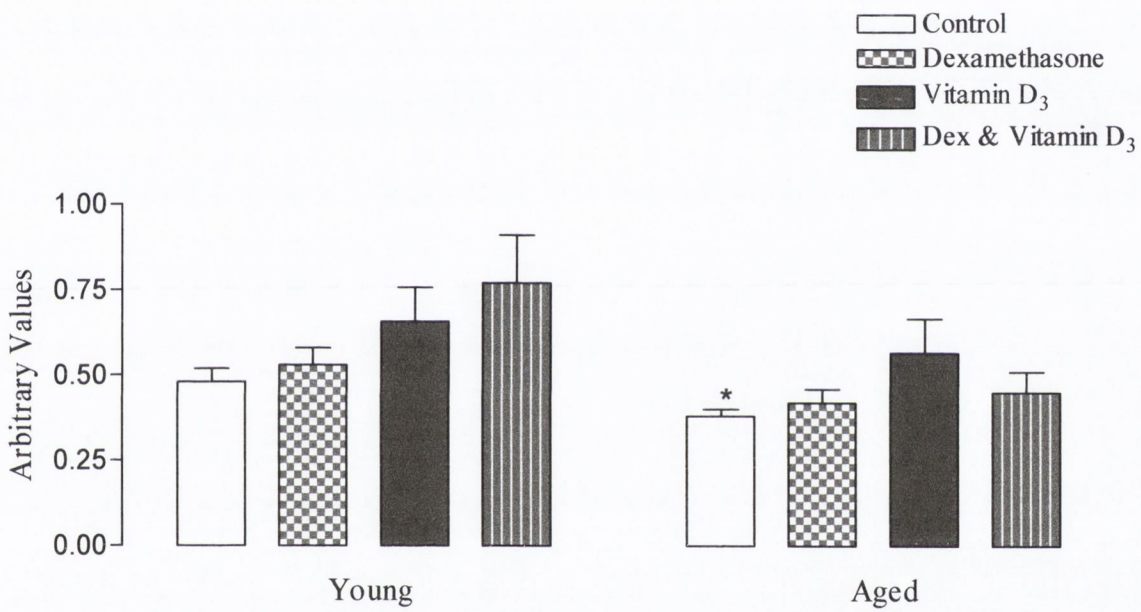

Control
Dexamethasone
Vitamin D_3
Dex & Vitamin D_3
Arbitrary Values
1.00
0.75
0.50
0.25
0.00
*
Young
Aged

Figure 3.16 Dexamethasone and vitamin D_3 increase IL-10 release from IL-1β-stimulated hippocampal neurons

IL-10 concentration in supernatant from hippocampal neurons treated with IL-1β, compared with control-treated cells (n=6 for both treatment groups) was unaltered. Dexamethasone and vitamin D_3 treatment increased IL-10 release from IL-1β-treated, compared with control-treated hippocampal neurons (n=6 for both groups; $+p<0.05$, Student's t test for independent means).

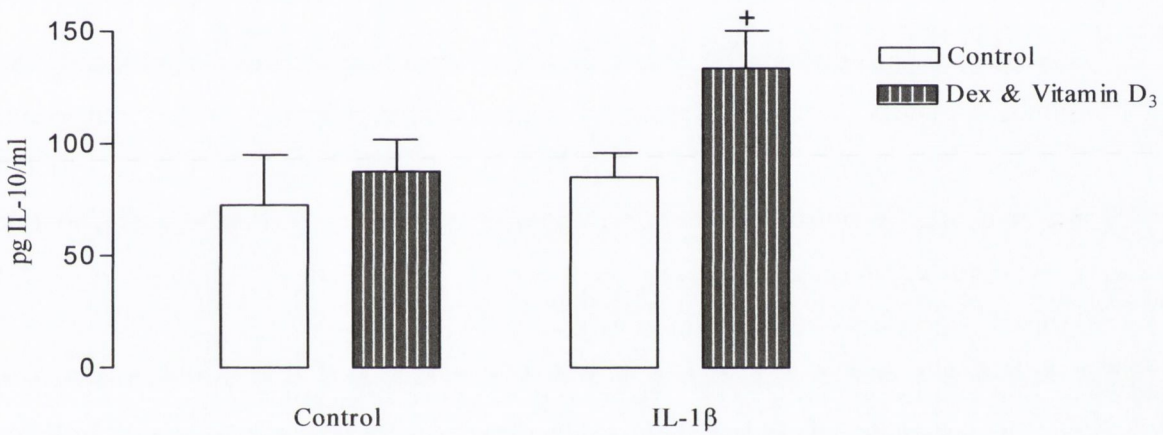

150
100
50
0
pg IL-10/ml
+
Control
Dex & Vitamin D_3
Control
IL-1β

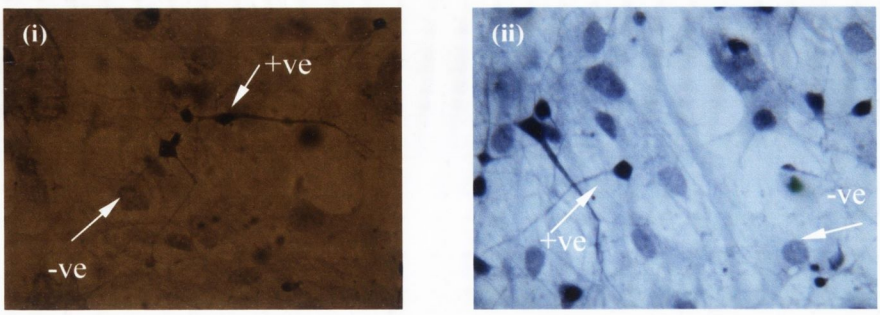
Figure 3.17 IL-1β-induced increase in p-JNK and caspase-3 staining was attenuated by dexamethasone and vitamin D_3

(A) The sample photomicrographs are representative of p-JNK (i) and caspase-3 (ii) staining obtained in hippocampal neurons prepared from 6 animals. A positively and a negatively stained cell is indicated in each photograph. Counterstaining was achieved with methylene green.

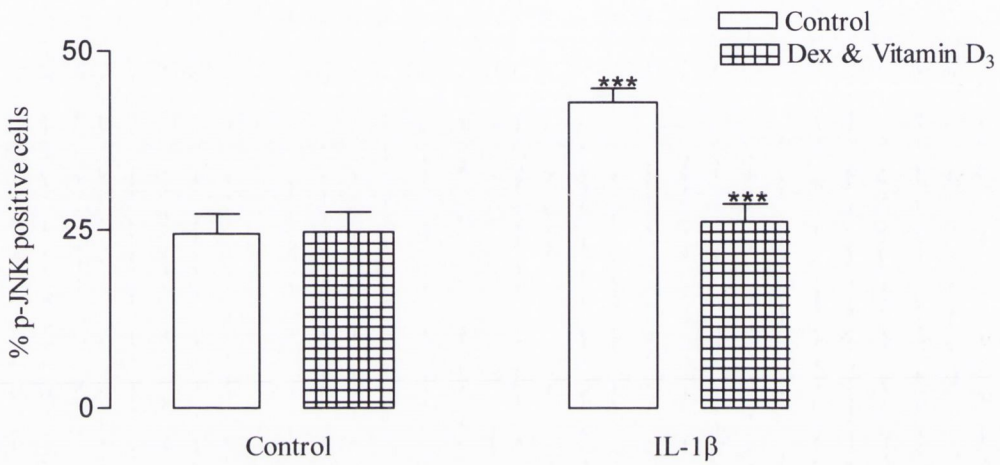
(B) The percentage of p-JNK positively stained cells was increased significantly in IL-1β-treated, compared with control-treated, hippocampal neurons (n=6 for both treatment groups; ***$p<0.001$, Student's t test for independent means). This IL-1β-induced increase was attenuated in dexamethasone and vitamin D_3-treated, compared with control-treated, hippocampal neurons (n=6 for both treatment groups; +++$p<0.001$, Student's t test for independent means).

(C) The percentage of caspase-3 positively stained cells was increased significantly in IL-1β-treated, compared with control-treated, hippocampal neurons (n=6 for both treatment groups; ***$p<0.001$, ANOVA). This IL-1β-induced increase was attenuated in dexamethasone and vitamin D_3-treated, compared with control-treated, hippocampal neurons (n=6 for both treatment groups; +++$p<0.001$, ANOVA).
Values represent the number of positively stained cells expressed as a percentage of the total; approx. 400 were counted per slide.

(A)


(i)
+ve
-ve
(ii)
+ve
-ve

(B)


Control
Dex & Vitamin D3
% p-JNK positive cells
50
25
0
***
***
Control
IL-1β

(C)

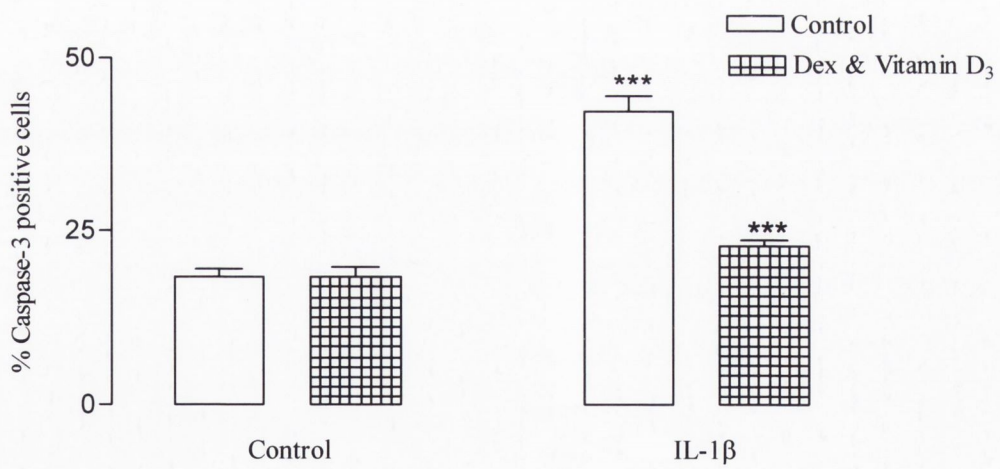

Control
Dex & Vitamin D3
% Caspase-3 positive cells
50
25
0
***
***
Control
IL-1β

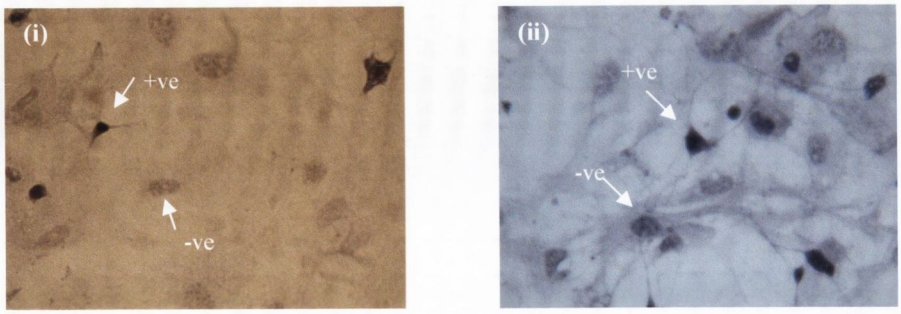
Figure 3.18 IL-1β-induced increase in p-JNK and caspase-3 staining was attenuated by IL-10

(A) The sample photomicrographs are representative of p-JNK (i) and caspase-3 (ii) staining obtained in hippocampal neurons prepared from 6 animals. A positively and a negatively stained cell is indicated in each photograph. Counterstaining was achieved with methylene green.

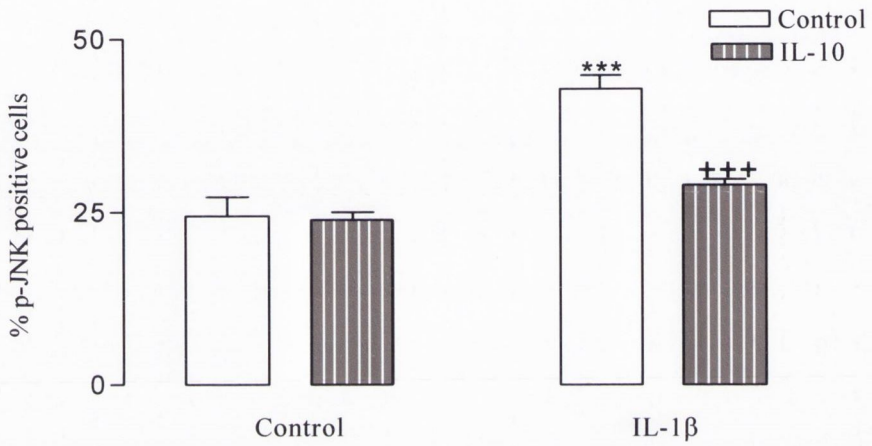
(B) The percentage of p-JNK positively stained cells was increased significantly in IL-1β-treated, compared with control-treated, hippocampal neurons (n=6 for both treatment groups; ***$p<0.001$, Student's t test for independent means). This IL-1β-induced increase was attenuated by IL-10 treatment (n=6 for both treatment groups; +++$p<0.001$, ANOVA).

(C) The percentage of caspase-3 positively stained cells was increased significantly in IL-1β-treated, compared with control-treated, hippocampal neurons (n=6 for both treatment groups; ***$p<0.001$, ANOVA). This IL-1β-induced increase was attenuated by IL-10 treatment (n=6 for both treatment groups; +++$p<0.001$, ANOVA).
Values represent the number of positively stained cells expressed as a percentage of the total; approx. 400 were counted per slide.

(A)


(i)
+ve
-ve
(ii)
+ve
-ve

(B)


Control
IL-10
% p-JNK positive cells
50
25
0
***
+++
Control
IL-1β

(C)

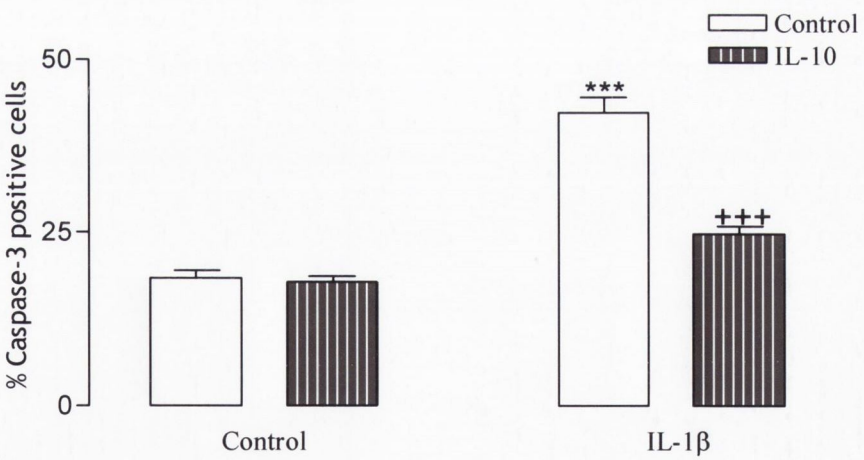

Control
IL-10
% Caspase-3 positive cells
50
25
0
***
+++
Control
IL-1β

Figure 3.19 Age is associated with an increase in IFN-γ concentration

IFN-γ concentration was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (n=6 and n=5 respectively; *$p<0.05$, Student's t test for independent means). IFN-γ concentration was slightly, but insignificantly, decreased in tissue prepared from dexamethasone-treated, compared with control-treated, aged rats, however this did not reach statistical significance (n=6 for both groups). IFN-γ concentration was slightly, but insignificantly, decreased in tissue prepared from vitamin D_3-treated, compared with control-treated, aged rats, however this did not reach statistical significance (n=6 for both groups). IFN-γ concentration slightly, but insignificantly, decreased in hippocampus of dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats, however this did not reach statistical significance (n=6 and n=5 respectively).

Values are expressed as pg IFN-γ/mg protein and are expressed as means ± SEM.

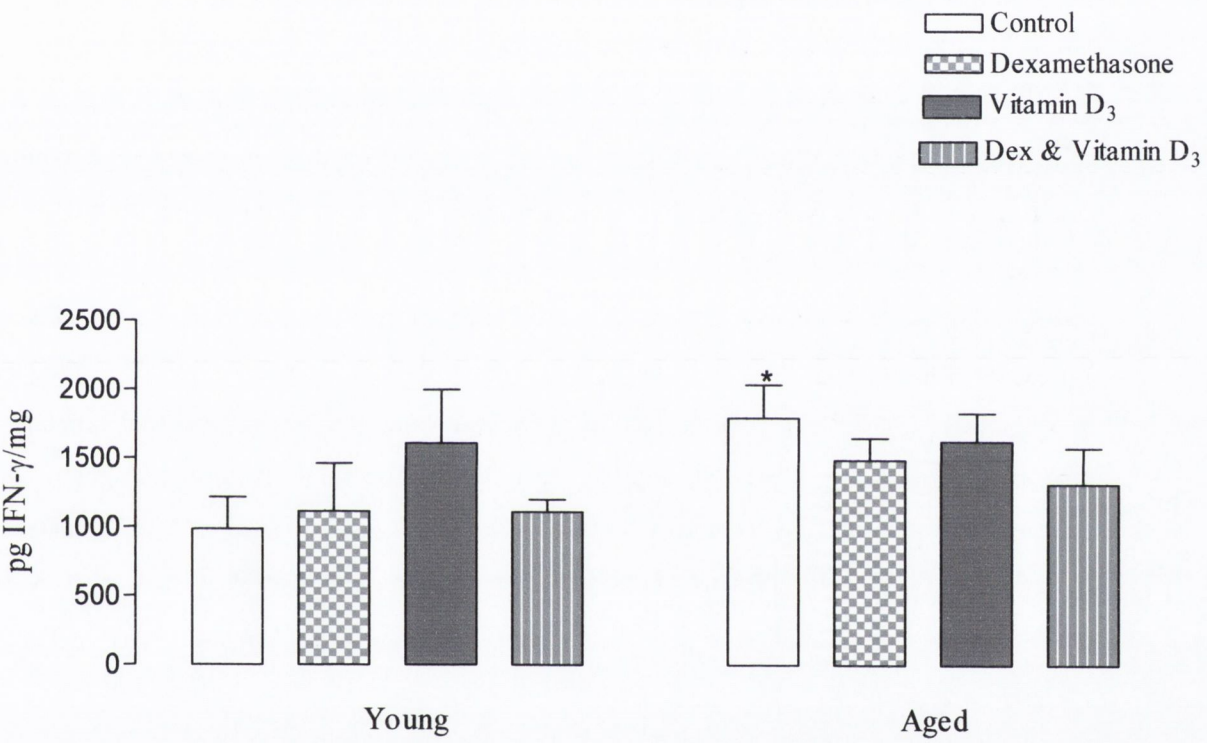
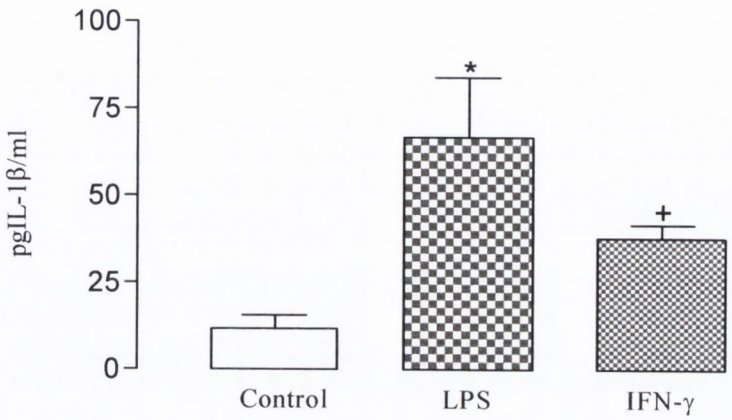

Control
Dexamethasone
Vitamin D_3
Dex & Vitamin D_3
pg IFN-γ/mg
2500
2000
1500
1000
500
0
*
Young
Aged

Figure 3.20 Glia release greater amounts of IL-1β than neurons

(A) IL-1β concentration was increased significantly in hippocampal neurons treated with LPS (100ng/ml; n=4; *$p<0.05$ ANOVA) and IFN-γ (10ng/ml) compared with control-treated cells (n=3 for both groups; +$p<0.05$, Student's t test for independent means).

(B) IL-1β concentration was increased significantly in cortical glia treated with LPS and IFN-γ compared with control-treated cells (n=11 for all groups; *$p<0.05$, ANOVA; +++$p<0.001$, ANOVA). Mean IL-1β concentration was significantly greater in unstimulated glia ($p<0.001$; Student's t test for independent means), compared with neurons, and also in LPS-treated ($p<0.001$; Student's t test for independent means) and IFN-γ-treated ($p<0.001$; Student's t test for independent means) cells.

(A)


100
75
50
25
0
pgIL-1β/ml
*
+
Control
LPS
IFN-γ

(B)

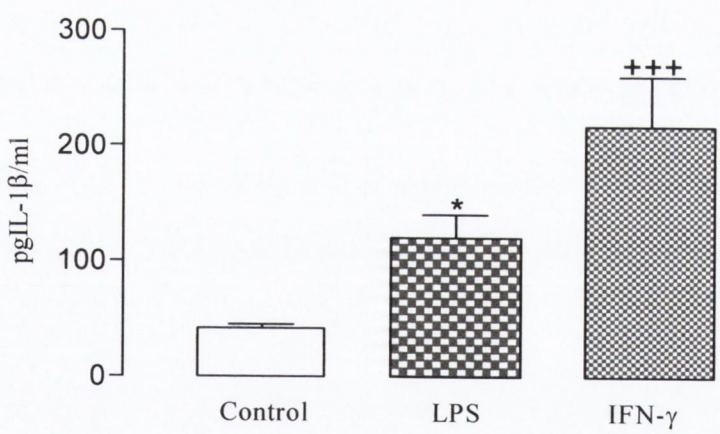
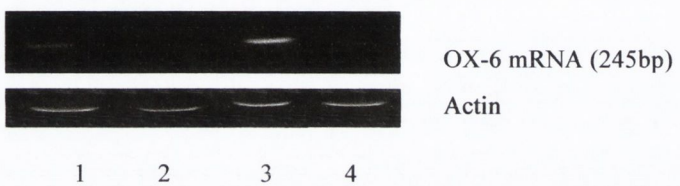

300
200
100
0
pgIL-1β/ml
*
+++
Control
LPS
IFN-γ

Figure 3.21 OX-6 mRNA expression is increased with age.

(A) The sample gel shows the molecular expression of OX-6 in young (lane 1) and aged (lane 3) control-treated rats and young (lane 2) and aged (lane 4) dexamethasone and vitamin D_3-treated rats.

(B) OX-6 mRNA expression, expressed as a ratio of OX-6 to β-actin, was increased significantly in the hippocampus of aged, compared with young, rats (n=5 for both groups; $^{**}p<0.01$, ANOVA). This expression was significantly decreased in dexamethasone and vitamin D_3-treated, compared to control-treated, aged rats (n=5 for both groups; $^{*}p<0.05$, ANOVA).
Values are expressed as arbitrary values and as means ± SEM.

(A)


OX-6 mRNA (245bp)
Actin
1
2
3
4

(B)

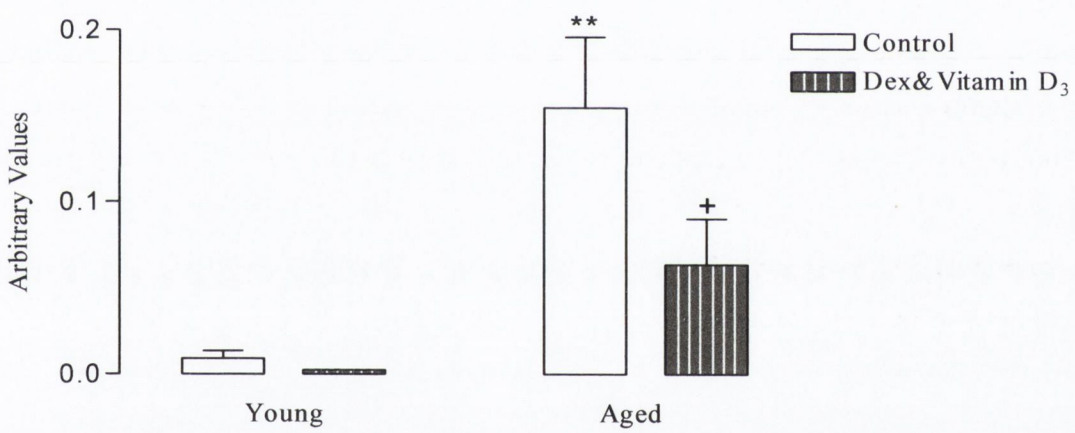

Arbitrary Values
0.2
0.1
0.0
**
+
Control
Dex& Vitamin D3
Young
Aged

Figure 3.22 Increased OX-6 expression indicates microglia activation in the aged brain.

Microglial activation, assessed by OX-6 expression, was increased in hippocampal slices of aged, compared with young, rats indicated by the brown staining. Microglial activation was decreased in hippocampal slices of dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats. Basal levels of activated microglia are present in hippocampal slices of dexamethasone and vitamin D_3-treated young rats. Pictures are representative of staining obtained in sections prepared from 6 animals for each treatment group.

Counterstaining was achieved with methyl green. Magnification 10x. The scale bar represents 10μm.

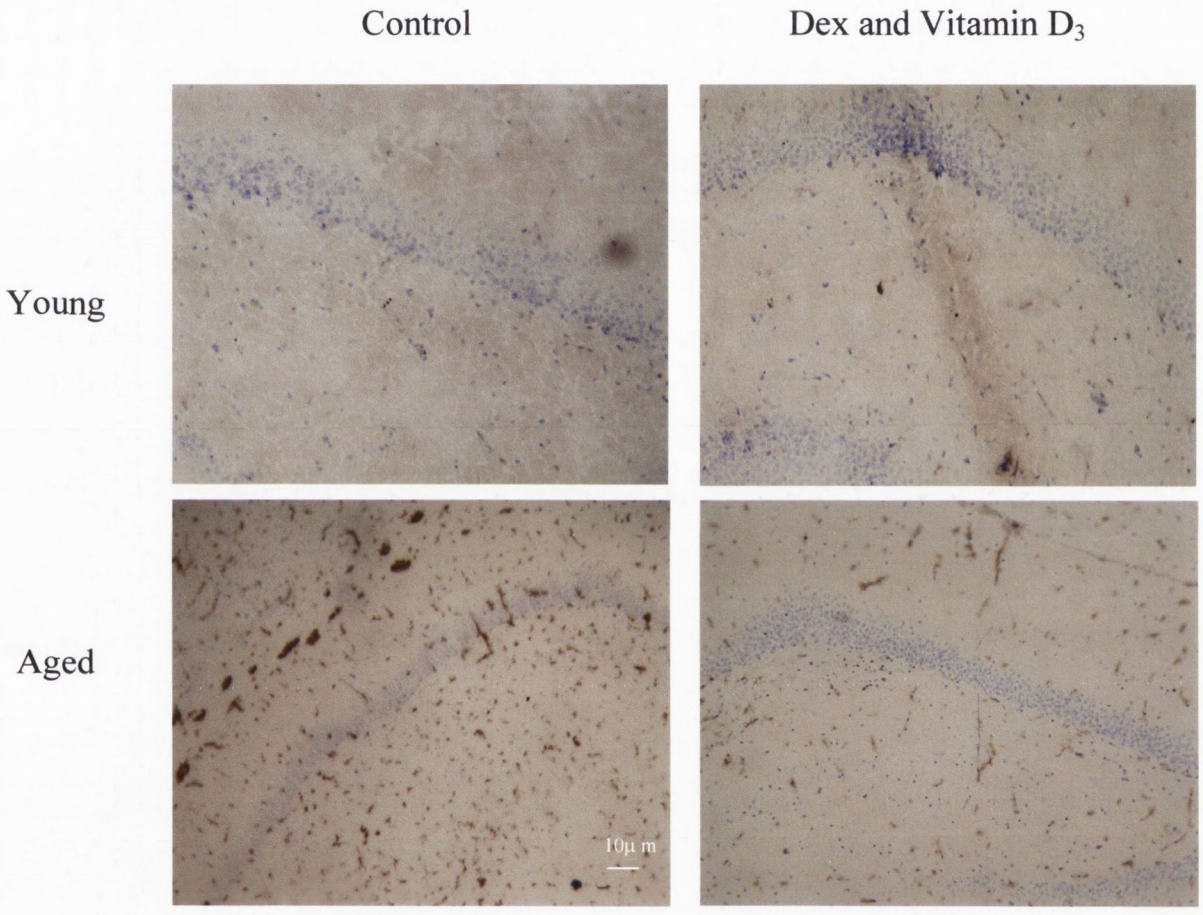

Control
Dex and Vitamin D_3
Young
Aged
10μ m

Figure 3.23 IFN-γ induces OX-6 mRNA expression in cultured glial cells

OX-6 mRNA expression, expressed as a ratio of OX-6 to β-actin, was increased significantly in cortical glial cells treated with IFN-γ compared to control-treated cells (n=6 for both groups; **$p<0.01$, Student's t test for independent means).
Values are expressed as arbitrary values and means ± SEM.

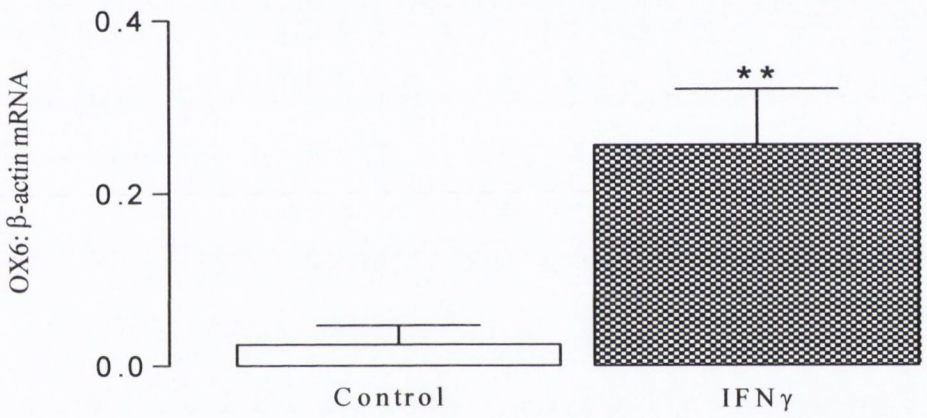

0.4
0.2
0.0
OX6: β-actin mRNA
**
Control
IFNγ

Figure 3.24 IFN-γ stimulates release of IL-1β from glia

IL-1β concentration was increased significantly in cortical glia treated with IFN-γ compared with control-treated cells (n=11 for both groups; ***$p<0.001$, ANOVA). This IL-1β concentration was decreased significantly in IFN-γ and IL-10-treated, compared with IFN-γ-treated, glia (n=4 and n=11 respectively; +$p<0.05$, ANOVA).
Values are expressed as pg IL-1β/ml and are expressed as means ± SEM.

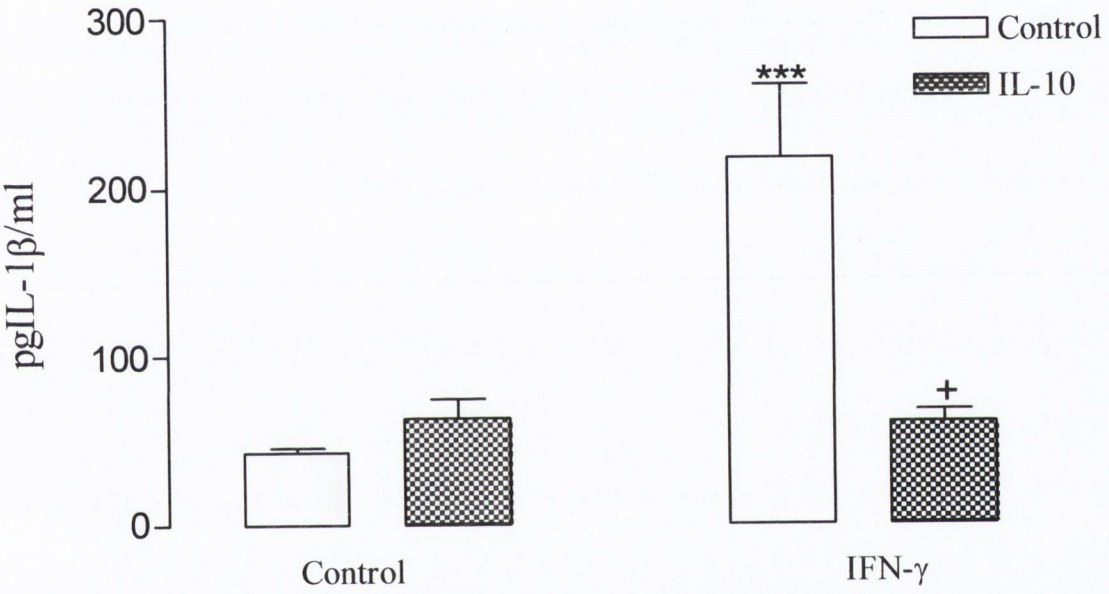

300
200
100
0
pgIL-1β/ml
Control
IL-10
***
+
Control
IFN-γ

Figure 3.25 IFN-γ release from cultured cortical glia is undetectable

IFN-γ concentration in supernatant collected from cultured cortical glia was undetectable in both the control-treated and LPS-treated groups (n=5).

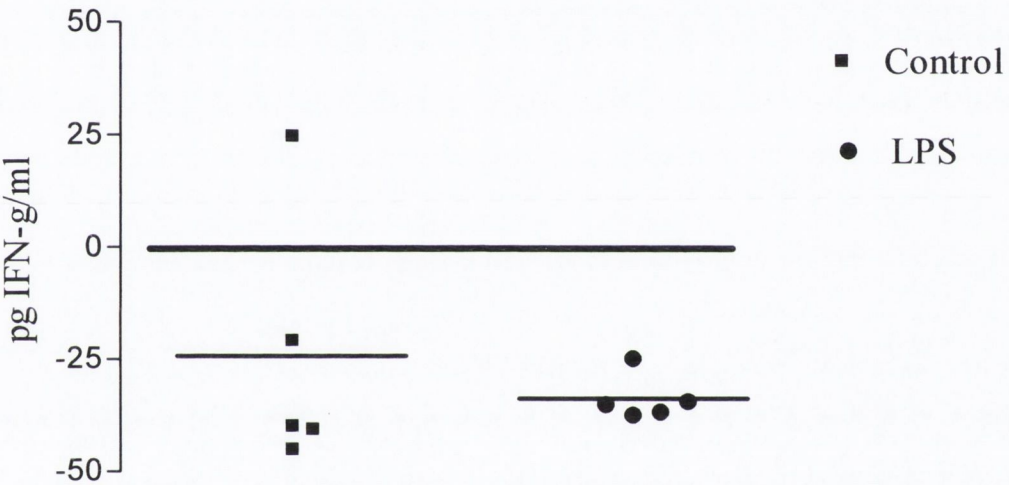

50
25
0
-25
-50
pg IFN-g/ml
Control
LPS

Figure 3.26 Positively stained $CD4^+$ cells are present in the hippocampus.

$CD4^+$ cells were present in hippocampal slices from aged rats (iii). Slices prepared from young rats (i) contained $CD4^+$ cells, however this was to a lesser degree compared with aged rats. The insets show magnified representative $CD4^+$cells. Hippocampal slices from dexamethasone and vitamin D_3-treated young (ii) and aged (iv) rats showed the presence of $CD4^+$ cells. Pictures are representative of staining obtained in sections prepared from 6 animals for each treatment group. Counterstaining was achieved with toluidine blue. Magnification 10x. The scale bar represents 10μm.

Figure 3.27 $CD4^+$ cells are present in hippocampal homogenate.

Data from densitometric analysis indicated that mean CD4 expression was similar in all treatment groups (n=4 for young control-treated group; n=5 for aged control-treated and young and aged dexamethasone and vitamin D_3-treated groups). Data are expressed as arbitrary units and as means ± SEM.

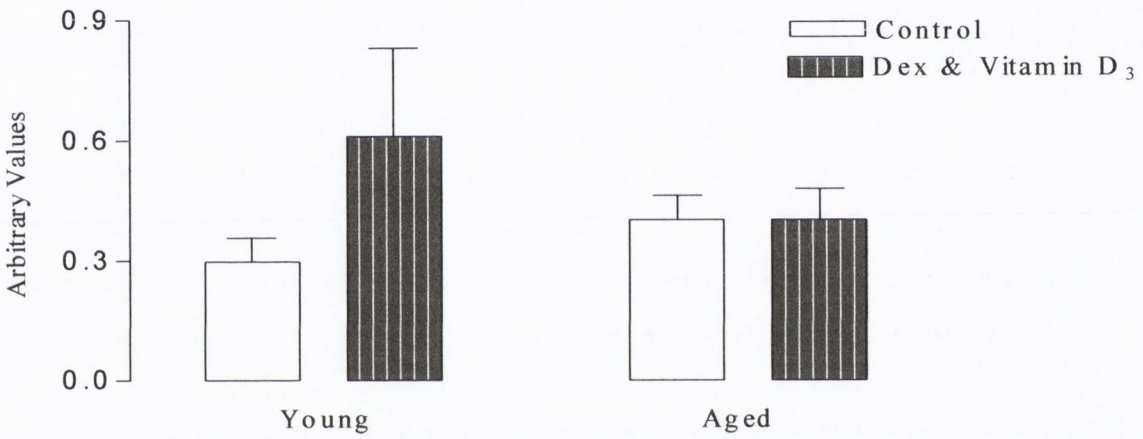

0.9
0.6
0.3
0.0
Arbitrary Values
Control
Dex & Vitamin D3
Young
Aged

Figure 3.28 Age is associated with an increase in hippocampal cells positively stained for CD161.

$CD161^+$ cells were present to a greater degree in hippocampal slices from aged, compared with young, control-treated rats. Cells positively stained for CD161 were present to a lesser degree in dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats. Staining in dexamethasone and vitamin D_3-treated young rats was similar to that of control-treated young rats. Pictures are representative of staining obtained in sections prepared from 6 animals for each treatment group.
Counterstaining was achieved with toluidine blue. Magnification 40x.

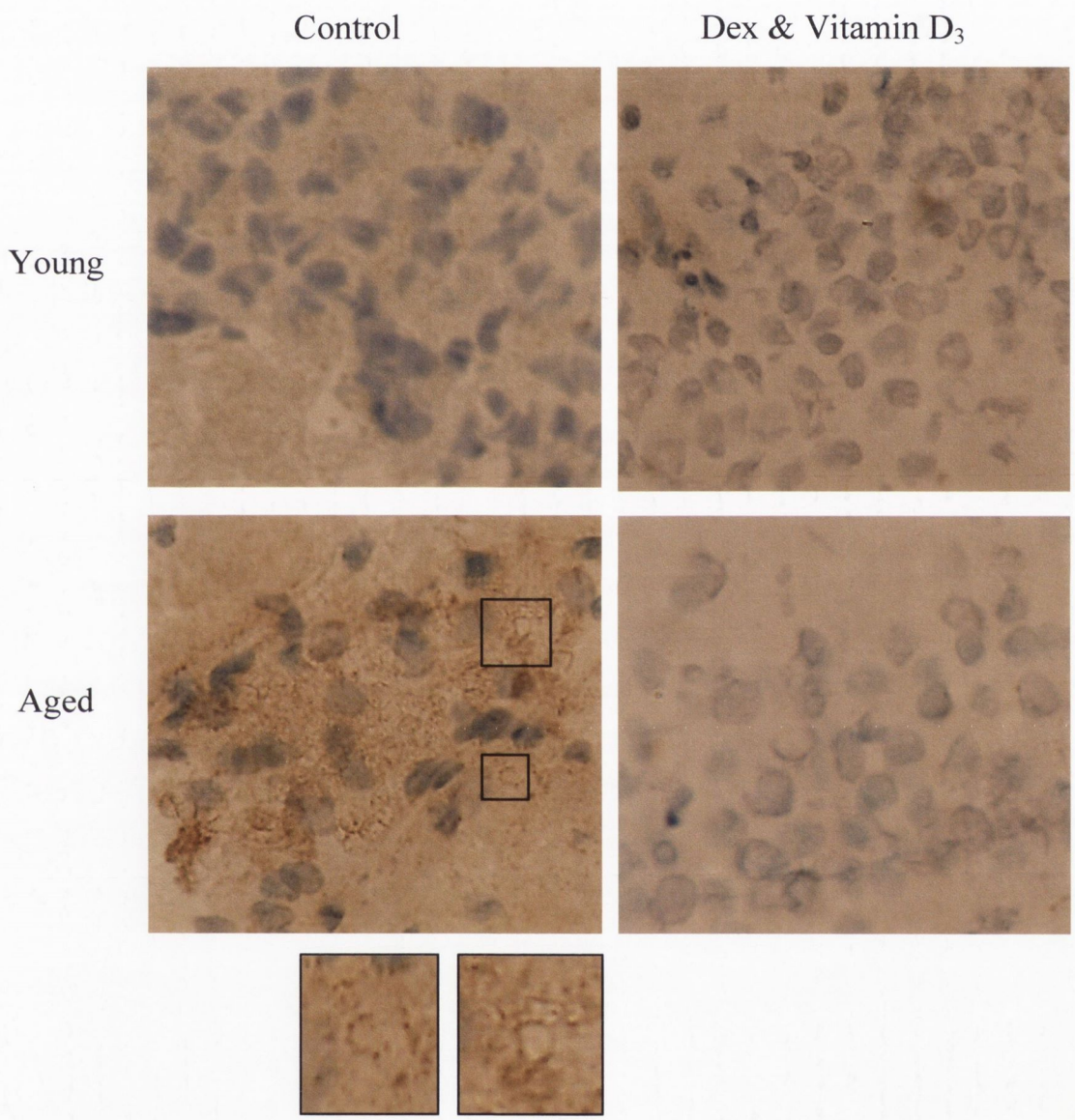
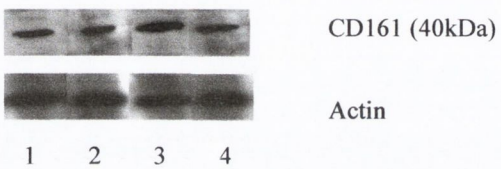

Control
Dex & Vitamin D_3
Young
Aged

Figure 3.29 CD161 expression is increased with age.

(A) The sample immunoblot shows CD161 expression in young (lane 1) and aged (lane 3) control-treated rats and young (lane 2) and aged (lane 4) dexamethasone and vitamin D_3-treated rats.

(B) CD161 expression, expressed as a ratio with actin, was increased significantly in the hippocampus of aged, compared with young, rats (n=6 and n=5 respectively; **$p<0.01$, ANOVA). This expression was decreased in dexamethasone and vitamin D_3-treated, compared with control-treated, aged rats (n=4 and n=6 respectively; +$p<0.05$, ANOVA). There was no change in CD161 expression in dexamethasone and vitamin D_3-treated, compared with control-treated, young rats (n=5 for both treatment groups).

(A)


CD161 (40kDa)
Actin
1
2
3
4

(B)

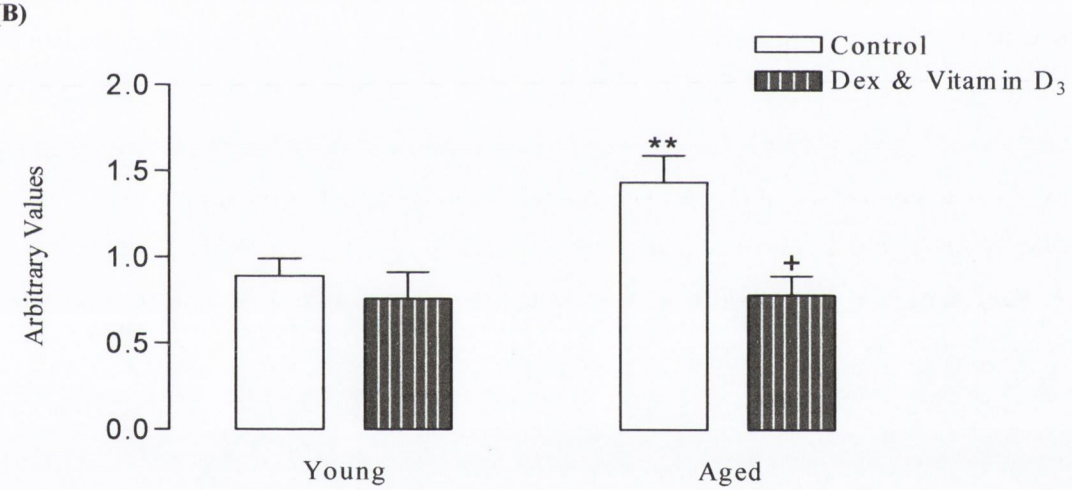

Control
Dex & Vitamin D3
Arbitrary Values
2.0
1.5
1.0
0.5
0.0
**
+
Young
Aged

Figure 3.30 IL-2 is present in the hippocampus but its concentration is unaffected by age.

Mean IL-2 (20ng/ml) concentration was similar in hippocampal tissue prepared from all treatment groups (n=7 for young and aged control-treated and aged dexamethasone and vitamin D_3-treated groups; n=8 for young dexamethasone and vitamin D_3-treated group). Values are expressed as pg IL-2/mg protein and are expressed as means ± SEM.

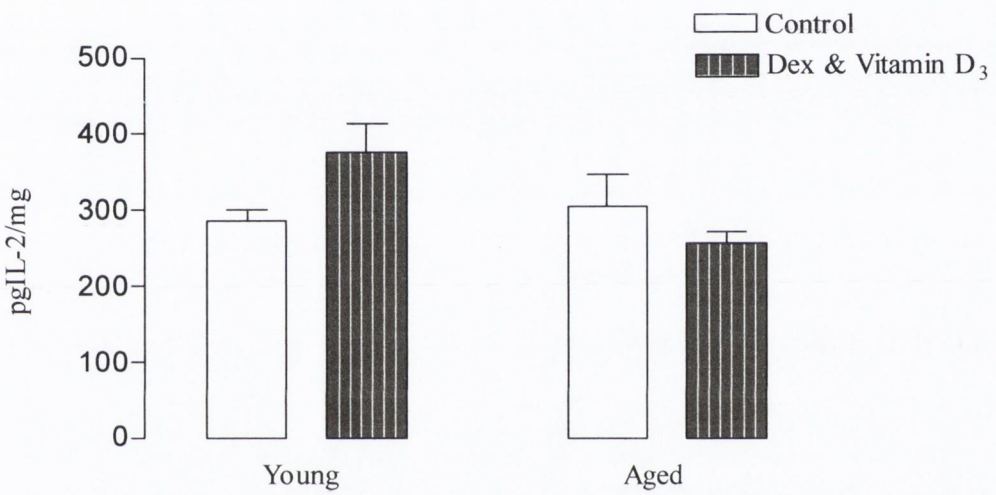

Control
Dex & Vitamin D_3
500
400
300
200
100
0
pgIL-2/mg
Young
Aged

Figure 3.31 IL-2 induces IFN-γ release from NK cells *in vitro*

Release of IFN-γ was increased 6-fold from NK cells treated with IL-2 (20ng/ml) compared with control-treated NK cells (n=6 for both treatment groups). Basal concentration of IFN-γ was 138.32pg/ml.

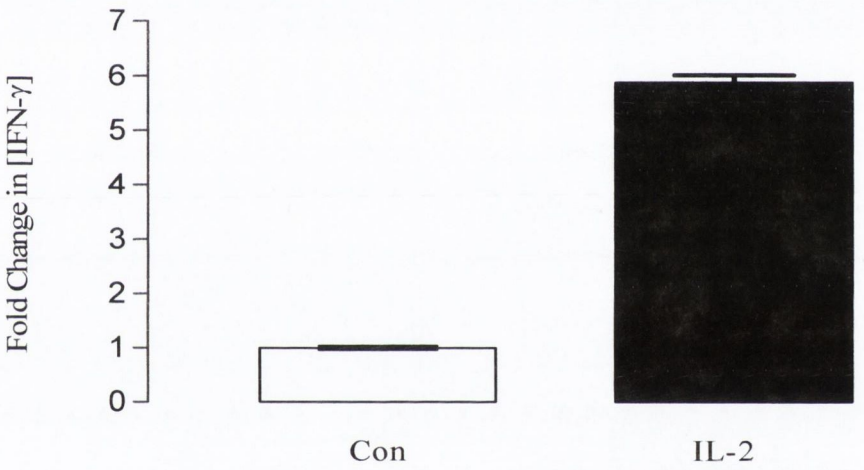

Fold Change in [IFN-γ]
7
6
5
4
3
2
1
0
Con
IL-2

Figure 3.32 Dexamethasone and vitamin D_3 attenuated IL-2-induced IFN-γ release from NK cells

Stimulation of NK cells with IL-2 (20ng/ml; n=5) is represented by 100% release of IFN-γ. Increasing concentrations of dexamethasone and vitamin D_3 pre-treatment (6.8×10^{-11}mol/L dexamethasone and 10^{-6}mol/L vitamin D_3; 1.36×10^{-10}mol/L dexamethasone and 2×10^{-6}mol/L vitamin D_3; 6.8×10^{-10}mol/L dexamethasone and 10^{-5}mol/L vitamin D_3) decreased the percentage release of IFN-γ (n=6 for all treatment groups). Basal concentration of IFN-γ was 138.32pg/ml.

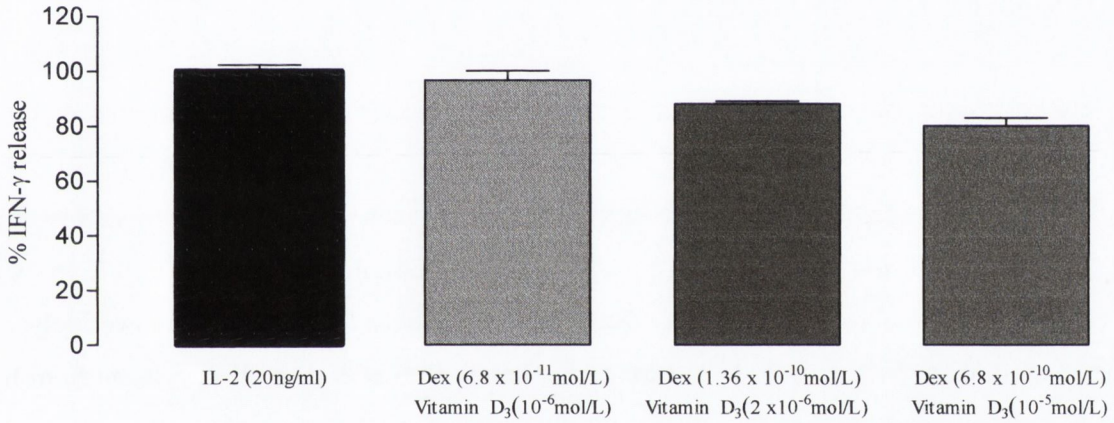
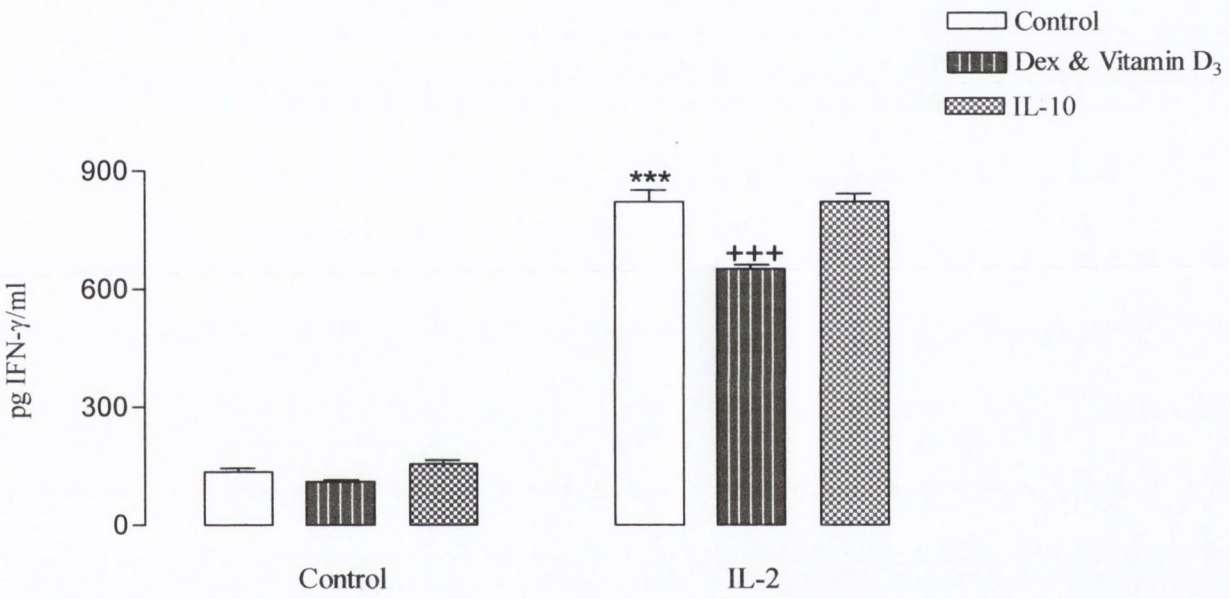

% IFN-γ release
120
100
80
60
40
20
0
IL-2 (20ng/ml)
Dex (6.8 x 10-11mol/L)
Vitamin D3(10-6mol/L)
Dex (1.36 x 10-10mol/L)
Vitamin D3(2 x10-6mol/L)
Dex (6.8 x 10-10mol/L)
Vitamin D3(10-5mol/L)

Figure 3.33 IL-2 increases IFN-γ release from NK cells *in vitro*; abrogated by dexamethasone and vitamin D_3

Mean IFN-γ concentration was increased significantly in IL-2-treated, compared with control-treated, NK cells (n=5 and n=6 respectively; ***$p<0.001$, ANOVA). This IFN-γ concentration was decreased significantly in IL-2-stimulated NK cells pre-treated with dexamethasone and vitamin D_3 (6.8×10^{-10} mol/L dexamethasone and 10^{-5}mol/L vitamin D_3), compared with those which received no pre-treatment (n=6 and n=5 respectively; +++$p<0.001$, ANOVA). IL-10 pre-treatment induced no effect on IFN-γ release from IL-2-stimulated NK cells (n=6).

Values are expressed as pg IFN-γ/mg protein and are expressed as means ± SEM.


Control
Dex & Vitamin D_3
IL-10
pg IFN-γ/ml
900
600
300
0
***
+++
Control
IL-2

Chapter 4

Discussion

The objectives of this study were to establish the cellular events and alterations in signalling associated with ageing and to determine the effectiveness of two anti-inflammatory agents, dexamethasone and vitamin D_3 in attenuating the age-related changes in the hippocampus. The findings suggest that the anti-inflammatory effects of dexamethasone and vitamin D_3, which have been described in the periphery, extend to the brain and act to decrease hippocampal IL-1β concentration, increase IL-10 concentration and restore the ability of the aged brain to sustain LTP.

Direct communication between the CNS and the immune and endocrine systems is facilitated by cytokines, such as IL-1β. Upregulation of IL-1β has been reported in numerous studies following exposure to LPS (Vereker *et al.*, 2000a), traumatic brain injury (see O'Connor and Coogan, 1999), γ-irradiation (Lonergan *et al.*, 2002) and oxidative stress (Kelly *et al.*, 2001). Moreover, IL-1β is implicated in brain pathologies exacerbated by inflammation (see Rothwell and Luheshi, 2000), degenerative diseases such as Parkinson's disease (see Arimoto and Bing, 2003) and experimental models of neurodegenerative diseases (Bruccoleri *et al.*, 1998; Pearson *et al.*, 1999; Vezzani *et al.*, 1999).

One hallmark of ageing is a disruption in the balance of pro- and anti-inflammatory cytokines. Data from this study revealed an age-related increase in IL-1β concentration in the hippocampus, confirming earlier similar findings (Martin *et al.*, 2002; Murray and Lynch, 1998b). Interestingly, brain expression of IL-1 increases as a factor of age in neurologically intact humans (see Wilson *et al.*, 2002). Consistent with evidence linking IL-1β and neurodegenerative disease is the finding that aged rats exhibited impaired cytokine and behavioural responses to intracerebroventricular (i.c.v.) injection of IL-1β (Gayle *et al.*, 1999), thus suggesting that the age-related alterations in cytokine balance and more specifically an increase in pro-inflammatory cytokines, may render the CNS particularly susceptible to cytokine-driven neurodegenerative diseases. In addition to the age-related increase in IL-1β protein, this study has demonstrated an increase in IL-1β mRNA in aged, compared with young, rats. To my knowledge, this effect has not been

reported before, however IL-1β mRNA has been found to be increased transiently in the hippocampus of mice following peripheral administration of LPS (O'Connor and Coogan, 1999). Upregulation was also observed following traumatic brain injury in the hippocampus (Fan *et al.*, 1995) and hypothalamic IL-1β mRNA was induced by acute restraint stress and tail shock (see Wilson *et al.*, 2002).

Because pro-IL-1β needs to be cleaved in order for it to be biologically active (Hailer *et al.*, 2005), another point at which regulation can occur exists. Caspase-1 facilitates the activation of IL-1β and consequently, caspase-1 is a target for many of the stimuli which impact on IL-1β concentration within the brain. The observed age-related increase in IL-1β was paralleled by an increase in caspase-1 activity (Lynch and Lynch, 2001). Similarly, LPS challenge which results in elevated hippocampal IL-1β concentration is coupled with an increase in caspase-1 activity (Vereker *et al.*, 2000a) and caspase-1 mRNA (Lindberg *et al.*, 2004). Several studies report a hypoxia-induced expression of caspase-1 (Kim *et al.*, 2003; Chu and Xin, 2005). Of note is the fact that neuronal injury, apoptosis and neurodegeneration associated with elevation in IL-1β concentration, are prevented in caspase-1 deficient mice (Felderhoff-Mueser et al., 2005; see Akama and Eldik, 1999). Additionally, inhibitors of caspase-1 attenuated the potassium-induced apoptosis of rat cerebellar-granule neurons (Lynch *et al.,* 1997), amyloid β-induced apoptosis (Egashira *et al.*, 2002) and the LPS-induced increase in IL-1β and cell degeneration in the enterohinal cortex (Campbell *et al.*, 2000). The mechanism leading to the increase in IL-1β in the hippocampus of aged rats is partly elucidated by these findings.

Binding to the type I receptor (IL-1RI) facilitates the downstream signalling and behavioural effects resulting from elevations in the concentration of IL-1β in the brain. Because of the pleiotropic nature of IL-1β and other cytokines, the presence of unique cell membrane receptors is necessary to ensure specificity of the cytokine response (Wilson *et al.*, 2002). Studies demonstrate that in unstimulated hippocampii, IL-1RI expression is found in the granule cells of the dentate gyrus and the pyramidal cells of

CA1 and CA3 (Ban *et al.*, 1991; Wong and Licinio, 1994; Yabuuchi *et al.*, 1994; Ericsson *et al.*, 1995). In fact, the highest density of IL-1 receptors is found in the hippocampus of the rodent brain (see Wilson *et al.*, 2002). In addition to this, IL-1RI mRNA was detected in the hippocampus using in situ hybridisation (Wong and Licinio, 1994) and this is consistent with the finding of this study which shows that basal levels of IL-1RI mRNA were present in the hippocampus obtained from young rats. Consistent with the finding of this study that IL-1RI mRNA expression was upregulated with age are the results of Lynch and Lynch (2001) and Murray and Lynch (1998b). Induction of IL-1RI mRNA and protein expression was observed in inflammatory conditions and following administration of substances which cause inflammation. For example, injection of LPS increased IL-1RI mRNA (Ilyin *et al.*, 1998) and protein in the hippocampus (Lynch and Lynch, 2001) and parenchyma of the brain (Stern *et al.*, 2000). Furthermore, LPS upregulated IL-1RI expression in monocytes (Penton-Rol *et al.*, 1999) and osteoarthritis is associated with increased IL-1RI expression (Shlopov *et al.*, 2000). Interestingly, blockade of IL-1RI by the receptor antagonist (IL-1ra), ameliorated LPS-induced sickness behaviour (Wilson *et al.*, 2002) and mice with sciatic nerve injury displayed reduced hyperalgesia following administration of a neutralising antibody for IL-1RI (Sommer *et al.*, 1999). These results taken together with the finding that IL-1β and IL-1RI mRNA can be induced by IL-1β (Ilyin and Plata-Salaman, 1996a, b; Docagne *et al.*, 2005), may explain the parallel increases in IL-1β and IL-1RI in the aged hippocampus.

In many of the inflammatory conditions in which IL-1β concentration is increased, a number of other cytokines and free radicals are upregulated also. For example, IL-6 expression in the whole brain, as well as the hippocampus, was reported to be increased in aged mice (Ye and Johnson, 2001a). Studies have shown that the increase in ROS and iNOS observed in these conditions may not only accompany the increase in IL-1β, but may actually be caused by IL-1β. For example, IL-1β stimulated iNOS production in astrocytes following Aβ stimulation (Akama and van Eldik, 2000). Similarly, Arimoto and Bing (2003) demonstrated an LPS-induced increase in IL-1β expression which was followed 2 hours later by an increase in iNOS production, thus suggesting the production

of the cytokine was, at least, partly responsible for the upregulation of the free radical. In the aged brain, ROS accumulation has been observed (see Lynch, 2004; Murray and Lynch, 1998a) and it has been suggested that downstream effects of IL-1β are mediated by ROS (Raingeaud *et al.*, 1995). Consistent with this are results from O'Donnell and Lynch (1998) which showed that IL-1β can induce ROS production in hippocampal tissue *in vitro*. Also, LPS, which increases IL-1β, concomitantly increased ROS production (Vereker *et al.*, 2000b). In fact, a causal interaction between ROS production and caspase-1 activity has been proposed as both are upregulated following LPS stimulation (Verker *et al.*, 2000a). One of the downstream effects of IL-1β, mediated by ROS, is the activation of JNK. IL-1β has been reported to augment JNK activity in a number of cell types, including human glomerular mesangial (Uciechowski *et al.*, 1996), HeLa (Raingeaud *et al.*, 1995) and hippocampal (Nolan *et al.*, 2004) cells and this effect can be mimicked by the treatment of hippocampal cells with hydrogen peroxide, *in vitro* (Vereker *et al.*, 2000b). These findings are consistent with other experimental conditions which induce ROS and JNK activation such as UV irradiation (Zhang *et al.*, 1997) and osmotic stress (Qin *et al.*, 1999).

In this study, ageing was associated with increased activation of JNK *in vivo* and this was paralleled by a similar change *in vitro* following stimulation with IL-1β. Thus, the age-related increase in JNK phosphorylation which has been reported in the hippocampus *in vivo* (Nolan *et al.*, 2005; O'Donnell *et al.*, 2000; Lynch and Lynch, 2001; Martin *et al.*, 2002), also occured in neurons following exposure to IL-1β. These findings corroborate those of other laboratories which have demonstrated an increase in JNK activation following IL-1β stimulation in glial cells (Parker *et al.*, 2002; Zhang *et al.*, 1996a), human neuroma fibroblasts (Lu *et al.*, 1997) and stromal cells (Rizzo and Carlo-Stella, 1996). These data also support previous findings from this laboratory which demonstrated that i.c.v. injection of IL-1β upregulated JNK activity in the hippocampus of rats (Vereker *et al.*, 2000b; Kelly *et al.*, 2001). Other mitogenic stimuli which increase IL-1β, also result in the stimulation of JNK activity, namely γ-irradiation (Lynch *et al.*, 2003; Lonergan *et al.*, 2002), LPS (Lonergan *et al.*, 2004; Lynch *et al.*, 2004), amyloid

β (Minogue *et al.*, 2003) and ischemia (Ferrer *et al.*, 1997). Indeed, administration of a peptide inhibitor of caspase-1 has been shown to lead to an attenuation in JNK activity observed in LPS-treated rats (Vereker *et al.*, 2000a) and hippocampal neurons exposed to irradiation (Lynch *et al.*, 2003). These results compound the link between the age-related increase in IL-1β concentration and consequent increase in JNK activity.

Neuronal loss is a hallmark of the ageing brain as evidenced by the increased TUNEL staining in cortex from aged, compared with young, rats (Martin *et al.*, 2002). A key mediator of this apoptotic pathway is thought to be IL-1β. JNK activation is also critically involved in paradigms of apoptosis as seen in NGF-deprived PC12 cells (Xia *et al.*, 1995), inflammation (Ip and Davis, 1998), ischemia (Ferrer *et al.*,1997), Bortezomib-treated Jurkat cells (Yu *et al.*, 2004) and PC12 cells exposed to UV irradiation and oxidative stress (Maroney *et al.*, 1999). Inhibition of JNK in sympathetic neurons prevented apoptosis induced by NGF deprivation (Eilers *et al.*, 2001) and similarily, cortical neurons exposed to amyloid β were rescued from cell death (Bozyczko-Coyne *et el.*, 2001). Knockout studies have indicated that JNK activity is associated with the propagation of inflammation through the augmentation of the release of pro-inflammatory cytokines (Dong *et al.*, 1998). It must be noted that JNK activity alone is not sufficient for mediating cell death as it is present in the normal brain (Carletti *et al.*, 1995) and is involved in neuronal differentiation in PC12 cells (Eiler *et al.*, 1998). Therefore, perhaps activation of the downstream mediators of the IL-1β signal, such as caspase-3 and PARP, is necessary for the cell deterioration observed in the aged brain.

In an effort to elucidate the downstream changes induced by the age-related increase in IL-1β concentration, caspase-3 expression was assessed. In keeping with the age-related increase in JNK activity, expression of the active form of caspase-3 was increased in the hippocampus of aged rats and this was mirrored by an increase in the number of hippocampal neurons positively stained for caspase-3, following *in vitro* IL-1β stimulation. These data are supported by the work of Martin and colleagues (2002) and Lynch and Lynch (2001) who reported an age-related increase in caspase-3 activity along with enhanced staining for caspase-3, both in the hippocampus. Similarly, sections

prepared from cortex of aged rats displayed more intense caspase-3 positive staining than that observed in the young cortex (Maher *et al.*, 2004). Reports of increases in caspase-3 activation in aged humans, particularly in whole brain and T lymphocytes exist (Aggarwal *et al.*, 1999; Su *et al.*, 2001). As a result of the increase in active caspase-3 in the aged brain, the pro form of caspase-3 is virtually absent in the brain as the rat ages (see Zhang *et al.*, 2004). While several upstream activators may contribute to the increase in caspase-3 activity, it has been consistently shown that IL-1β can act as a stimulator. Thus, analysis of caspase-3 activation in cortical and hippocampal tissue revealed an IL-1β-stimulated increase (Lynch and Lynch, 2001; Martin *et al.*, 2002), which corroborates findings of the current study. LPS and Aβ, both of which have been shown to increase IL-1β concentration, also induced caspase-3 activation in the hippocampus (Barry *et al.*, 2005; Minogue *et al.*, 2003).

Cleavage of 32kDa caspase-3 marks its proteolytic activation and is a key milestone in the process leading to apoptotic cell death (see Zhang *et al.*, 2004; Green and Reed, 1998). Apoptosis mediated by caspase-3 has been shown to be induced by a number of stimuli including tetrahydrocannibanoids (Downer *et al.*, 2003), γ-irradiation (Lynch *et al.*, 2003; Lonergan *et al.*, 2002), p53 (Inamura *et al.*, 2001), hypoxia (Gozal *et al.*, 2003; Hu *et al.*, 2005) and TNF-α (Aggarwal *et al.*, 1999). In humans, apoptosis is common and widespread during neurogenesis (Chan *et al.*, 2002), as evidenced by an increase in caspase-3 expression during embryonic and early postnatal development (Srinivasan *et al.*, 1998; Shimohama *et al.*, 1999). However, in the adult brain, apoptosis is an indicator of brain pathology, as in the case of Alzheimer's disease and is linked with upregulation of caspase-3 (Shimohama *et al.*, 1999). In the aged rat, the increased IL-1β concentration, paralleled by an upregulation of caspase-3 activity and enhanced cell death, suggests that neuronal apoptosis in the CNS may be mediated by the IL-1β-induced caspase-3 activity (Lynch and Lynch, 2001). The role of caspase-3 in cell death is underlined by the finding that caspase-3-deficient mice exhibited reduced apoptosis, which correlated with a doubling of brain size. Further evidence that inhibition of caspase-3 proffers protection was provided by the finding that sympathetic neurons were spared UV-induced apoptosis following administration of a caspase-3 inhibitor (Park *et al.*, 1998).

PARP is a protein-modifying nucleotide-polymerising nuclear enzyme that is able to synthesise poly (ADP-ribose) units from nicotinamide adenine dinucleotide and which can catalyse their transfer to substrate proteins and in this manner, it repairs damaged or nicked DNA (Hatip-Al-Khatib *et al.*, 2004). It is agreed that PARP is a cellular substrate for caspases, particularly caspase-3 and it is its DXXD motif which facilitates this cleavage (Kannan and Jain, 2000). The data presented here reveal that PARP cleavage is enhanced in tissue prepared from aged, compared with young, rats, consolidating the suggestion that the pathway leading to apoptosis, involving IL-1β and its downstream mediators, is activated in the aged hippocampus. Evidence from this laboratory corroborates this finding (Martin *et al.*, 2002), while others have reported that PARP cleavage is augmented with age in various brain areas. For example, an age-related decrease in PARP activity was reported in the cerebellum (Malanga *et al.*, 2005), in the hippocampus (Strosnajder *et al.*, 2000) and in the cortex (Hiona *et al.*, 2004) and most specifically, Dorszewska and colleagues (2004) demonstrated a decline in PARP activity and expression between the 12th and 24th month of a rat's life. Similarly, an inverse correlation exists, in human fibroblasts, between poly (ADP-ribosyl)ation and the age of the donor (Dell'Orco and Anderson, 1997). To extend these findings, DNA fragmentation has also been found to be increased in old rats (White and Barone, 2001).

Other experimental paradigms can increase PARP cleavage; for instance it has been shown in brain and spleen cells following γ-irradiation (Ushakova *et al.*, 2004; Lonergan *et al.*, 2002; Lynch *et al.*, 2003), in hippocampal and renal tubular cells following LPS treatment (Jo et al., 2002; Vereker *et al.*, 2000a; Lynch *et al.*, 2004) and in the hippocampus *in vivo* following Aβ administration (Minogue *et al.*, 2003). It is noteworthy that all these insults increase IL-1β concentration. In fact, the link between IL-1β and PARP is also highlighted by concurrent increases in IL-1β mRNA and PARP cleavage in the pontine reticular formation following spinal cord transection in the rat (Wu *et al.*, 2003).

The importance of maintenance of the uncleaved form of PARP and the consequent

protection is underlined following knockout studies. Mice, which were genetically modified in order to eliminate the full activated form of PARP, presented with higher incidences of hepatocellular carcinomas in old age (Tong *et al.*, 2002). Conversely, PARP "knock-in" mice, which express the uncleaved form, showed resistance to inflammation and LPS (Petrilli *et al.*, 2004). In addition, Petrilli and colleagues (2004) administered a caspase inhibitor to these "knock-in" mice and observed protection in response to renal ischemia-reperfusion. PARP expression seems to be correlated with JNK activity - a balance exists between the activity of one and the cleavage of the other. Thus, genetic ablation of PARP has been shown to facilitate an upregulation of basal JNK activity (Andreone *et al.*, 2003). It is suggested that PARP is required to maintain a balance between basal JNK activity and its phosphorylation in response to stress, as evidenced by a hybrid bacterial system in which PARP interacts with JNK (unpublished data Zingarelli and O'Connor- cited in Andreone *et al.*, 2003).

It is proposed here that the cell deterioration, a feature of the aged brain, is mediated by the sequential activation of IL-1β, JNK and caspase-3 and PARP cleavage. Previous studies have shown that vasointestinal peptide, a JNK inhibitor (Kelly *et al.*, 2001), decreased IL-1β-stimulated caspase-3 activation *in vitro* (Lynch and Lynch, 2001). Treatment with two JNK inhibitors, CEP-1347 and SP600125, successfully reduced caspase-3 activity and cell death in cortical neurons stimulated with Aβ (Bozycko-Coyne *et al.*, 2001) and U937 cells stimulated with cantharidin (Huh *et al.*, 2004). Additionally, alterations in JNK gene coding prevented the release of cytochrome c (Tournier *et al.*, 2000) and consequently, augmented caspase-3 activity (see Bozycko-Coyne *et al.*, 2001). Generation of antisense, which lead to the depletion of JNK proteins, attenuated the Aβ-induced activation of caspase-3, PARP cleavage and sequential DNA fragmentation (Fogarty *et al.*, 2003). In addition, similar results to those in the current study were presented by Lynch and colleagues (2003) who proposed that LPS treatment induced caspase-3 activation and PARP cleavage following activation by upstream kinases including JNK. Moreover, a caspase-1 inhibitor prevented the LPS-induced increase in IL-1β concentration, JNK activation and consequent PARP cleavage (Vereker *et al.*, 2000a).

This study revealed an age-related decrease in LTP in the hippocampus of rats. This result concurs with data from a study by Martin and colleagues (2002), which reported a similar marked attenuation in the response of aged animals to tetanic stimulation. Several other studies report a similar change (Murray and Lynch, 1998b; deToledo-Morrell and Morrell, 1985; McGahon *et al.*, 1999a, b; O'Donnell *et al.*, 2000; Maher *et al.*, 2005). The underlying cause of this impairment in the ability of aged rats to sustain LTP has not been unequivocally established. However, evidence suggests that the age-related increase in IL-1β may contribute significantly to this impairment (Nolan *et al.*, 2004). In numerous studies, LTP has been found to be impaired under experimental conditions which increase IL-1β. For example, stressed rats do not sustain LTP (Vereker *et al.*, 2001; Murray and Lynch, 1998b) while those which are exposed to γ-irradiation (Lynch *et al.*, 2003) or treated with LPS (Vereker *et al.*, 2000a) similarly fail to sustain LTP. Furthermore, IL-1ra, which disrupts IL-1β signalling, has been found to induce a larger LTP than observed in control slices (Bellinger *et al.*, 1993) and following co-application with IL-1β, IL-1ra antagonised the inhibition of LTP seen in the dentate gyrus following IL-1β application alone (Cunningham *et al.*, 1996). A pivotal role for IL-1β in stress-induced inhibition of hippocampal dependent learning has been provided by the finding that IL-1ra prevents the impairment in contextual fear conditioning and memory consolidation induced by IL-1β (Pugh *et al.*, 1999; Depino *et al.*, 2004). IL-1β also inhibited learning in the Morris water maze (Gibertini, 1998) and mediates the *Legionella pneumophilia*-induced impairment in navigational learning (Gibertini *et al.*, 1995). Interestingly, auditory-cue fear conditioning, which is a hippocampal-independent form of learning was unaffected by IL-1β. Ageing is associated with increased IL-1β expression in the hippocampus (Martin *et al.*, 2002) and this, taken together with the finding that LTP is inhibited in the ageing dentate gyrus (Murray and Lynch, 1998b), substantiates the suggestion that IL-1β is negatively correlated with, and is one of the factors which contribute to the age-related impairment in LTP.

However other factors are likely to contribute; one of these is corticosterone. Following stimulation, the hypothalamus releases corticotrophin releasing factor (CRF); this elicits

the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary, which culminates in the secretion of glucocorticoids from the adrenal cortex (Besedovsky *et al.*, 1986; Amano *et al.*, 1992; Tsigos and Chrousos, 2002). A significant finding of this study is that serum corticosterone was increased with age. The findings of several studies concur with this data (Vallee *et al.*, 1999; Dellu *et al.*, 1996; De Kloet *et al.*, 1991; Patel and Finch, 2002). The mechanism by which corticosterone is increased in the aged animal is unknown; however a suggestion as to the identity of the mediators of this increase has been made. In 1986, Besedovsky and colleagues suggested that IL-1β is this mediator following an experiment in which an antibody directed against IL-1 neutralised the capacity of supernatants from stimulated leukocytes to increase corticosterone levels in the rat. More recently, Buckingham and colleagues (1994) found a similar elevation *in vivo* in response to orally and peripherally administered IL-1β. Additionally, numerous studies have demonstrated that IL-1 is capable of stimulating serum corticosterone release by acting at various points along the HPA axis. For example, CRF was released from the hypothalamus subsequent to IL-1β application *in vitro* (Tsagarakis *et al.*, 1989). This central action of IL-1β in the brain to stimulate CRF secretion can sequentially elicit the release of ACTH (Haddad *et al.*, 2002) and corticosterone as evidenced by the study in which IL-1β was administered i.c.v. and resulted in the immediate augmentation of plasma ACTH and corticosterone levels (Brown *et al.*, 1991).

Activation of the HPA axis can be achieved by LPS administration (see Haddad *et a*l., 2002) and this leads to an increase in plasma ACTH levels. Interestingly, antibodies against the IL-1 receptor prevented this LPS-induced effect (Rivier *et al.*, 1989). It is interesting to note that Alzheimer's disease is associated with a greater concentration in serum cortisol (see Jacobson and Sapolsky, 1991). The link between these two observations may be IL-1β and therefore it may be suggested that an experimental paradigm which results in an increase in IL-1β can lead to an elevation in plasma corticosterone. To extend these findings, it might be postulated that the age-related increase in IL-1β observed in this study is the mediator of the enhanced serum corticosterone levels in the aged rats.

During ageing, the negative feedback influence on the HPA axis is downregulated, resulting in increased levels of circulating glucocorticoids. Neuronal damage in the ageing hippocampus (Nichols *et al.*, 2001), possibly due to the augmented pro-inflammatory signalling, can eliminate the negative feedback elicited by the hippocampus. Physiological and pharmacological studies have identified the presence of corticosteroid receptors in brain regions (Rosenfeld *et al.*, 1993) including the hippocampus (see Jacobson and Sapolsky, 1991; De Kloet *et al*, 1991) and an age-related increase in glucocorticoid levels has been shown to impact on hippocampal functioning (Nichols *et al.*, 2001). A correlation exists between elevated glucocorticoid levels and the impairment in memory in elderly subjects (Lupien *et al.*, 1994, 1998) and old rats (Landfield *et al.*, 1978; see Hibberd *et al.*, 2000). Specifically, Vallee and colleagues (1999) observed impairments in spatial recognition memory in the Y maze and spatial working memory in the radial maze. Likewise, LTP was inversely correlated with serum corticosterone levels (see Patel and Finch, 2002; Nishiyama, 2001). Furthermore, one single injection of corticosterone or injections over 21 days demonstrate that acute and chronic exposure to corticosterone can reduce LTP in the dentate gyrus (see Lynch, 2004).

Up to this point in the current study, the results suggest that the age-related increase in IL-1β signalling, involving the sequential activation of JNK and caspase-3 and cleavage of PARP, and the enhanced serum corticosterone levels suggest that cell deterioration is occurring and that all these factors contribute to the impairment in LTP seen in aged rats.

As it is postulated that a balance exists between pro- and anti-inflammatory cytokine production and expression in the brain, it is significant that the increase in IL-1β concentration is accompanied by an age-related decrease in IL-10 concentration. This is the first indication of this age-induced change in the hippocampus however, a decline in IL-10 secretion in coronal brain sections and glia from aged, compared with young, mice was observed previously (Ye and Johnson, 2001b). Similarly, in the periphery, aged mice demonstrated impairment in type 2 cytokine responses following schistosome egg challenge; specifically, a reduction in IL-10 concentration was found (Smith *et al.*, 2001).

Interestingly, patients with Alzheimer's disease, in which an increase in IL-1 has been reported (Griffin *et al.*, 1989), have decreased IL-10 production (DeLuigi *et al.*, 2001) and individuals with "low" IL-10 genotype are thought to have a higher predisposition to development of Alzheimer's disease (Ma *et al.*, 2005). Numerous studies underline the significant contribution of IL-10 in the anti-inflammatory response by genetically altering IL-10 biology in mice. As a consequence of IL-10 knockout, overproduction (Berg *et al.*, 1995) or prolonged increased activation (Agnello *et al.*, 2000) of pro-inflammatory such as TNF-α and IL-6 occur. Following administration of a low dose of LPS, IL-10 knockout mice show heightened and prolonged fever compared to wild-type counterparts (Leon *et al.*, 1998) and chronic inflammatory bowel disease develops in the absence of IL-10 (Kuhn *et al.*, 1993). An identical reduction in IL-10 mRNA expression was found in the hippocampus of aged rats. To my knowledge this has not been reported previously, although IL-10 mRNA expression has been observed in other regions of the CNS including the hypothalamus and pituitary (Rady *et al.*, 1995).

The effects of IL-10 are mediated by its interaction with a high affinity surface receptor (IL-10R; Moore *et al.*, 2001; Donnelly *et al.*, 1999). Here, the presence of the IL-10R is shown in the granule cells of the dentate gyrus *in vivo* and the hippocampal neurons *in vitro*. Although it is assumed that this receptor is required for signalling, it is not explicitly demonstrated. *In vivo* analysis has elucidated the presence of IL-10R in the rat brain, particularly in the cortex, cerebellum, hippocampus, hypothalamus and pituitary (Ward *et al.*, 2001) and *in vitro* studies reveal its expression in glial cells by RT-PCR and immunocytochemistry (Ledeboer *et al.*, 2002).

Evidence suggests that IL-10 signalling is mediated through JAK1 and STAT-3 (Williams *et al.*, 2004; Strle *et al.*, 2001; Donnelly *et al.*, 1999; Moore *et al.*, 2001). In accordance with this, the current study demonstrated that the age-related decrease in IL-10 concentration and mRNA expression was paralleled by age-related reductions in phosphorylation of JAK1 and STAT-3. Obligate roles for JAK1 and STAT-3 in IL-10's anti-inflammatory actions have been established (Riley *et al.*, 1999; see Williams *et al.*, 2004); disruption of the gene encoding JAK1 in mice lead to the elimination of the

characteristic ability of IL-10 to prevent macrophage production of TNF-α, following LPS stimulation (Rodig *et al.*, 1998). IL-10 promoter activity is blocked by a dominant negative form of STAT-3 (Benkhart *et al.*, 2000) and STAT-3 deficient mice do not register a response to IL-10 (Riley *et al.*, 1999). In parallel with IL-10 and IL-10R abilities to prevent apoptosis, STAT-3 blocks apoptosis in cells lines (Bromberg *et al.*, 1999).

As it is agreed that IL-10 is neuroprotective; activation of IL-10R can inhibit apoptosis (Strle *et al.*, 2002) which is consistent with findings that IL-10 aided the survival of cortical and cerebellar granule neurons (Bachas *et al.*, 2001; Grilli *et al.*, 2000), astrocytes (Pahan *et al.*, 2000) and oligodendrocytes (Molina-Holgado *et al.*, 2001). Therefore, the consequences of the age-related reduction in IL-10 concentration and signalling are significant. IL-10 antagonises mediators of inflammation in the brain as evidenced by its blockade of TNF-α and IL-1 production (Strle *et al.*, 2001; see Bluthe *et al.*, 1999; Pahan *et al.*, 2000; Knoblach and Faden, 1998) in response to various stimuli including endotoxin (Donnelly *et al.*, 1999) and experimentally induced traumatic brain injury (Fiorentino *et al.*, 1991a). Another inflammatory cytokine, IL-6, which has been shown to be upregulated with age (Godbout and Johnson, 2004) is decreased by IL-10 in astrocytes at the protein and mRNA levels (Pousset *et al.*, 1999) and furthermore enhanced degradation of IL-6 mRNA in PBMCs occurs following IL-10 administration (Wang *et al.*, 1994). IL-10 has been shown to abrogate the LPS-induced changes in cellular signalling (Kelly *et al.*, 2001) and behavioural effects (Bluthe *et al.*, 1999) while Aβ-induced cytokine expression (Szczepanik *et al.*, 2003) and *M. tuberculosis*-induced apoptosis (Rojas *et al.*, 1999) are also blocked following IL-10 administration.

In addition to IL-10's suppressing effect on IL-1β production (Strle *et al.*, 2001; Pahan *et al.*, 2000), IL-10-associated downstream signalling is consequently antagonised. The data from this study showed that IL-1β-induced JNK and caspase-3 staining in hippocampal neurons was attenuated following IL-10 treatment. This effect on JNK activity was also observed in synaptosomes which were treated with IL-1β (Kelly *et al.*, 2001) and dendritic cells treated with TNF-α (Sato *et al.*, 1999). Existence of this negative

correlation between JNK activity and IL-10 is supported by a study using T cells. When T cells underwent anergy, an inhibition of JNK activity along with enhanced IL-10 release was observed (Chou *et al.*, 1998). Consistent with the result of this study is the finding of Lynch and colleagues (2003) who reported attenuation in caspase-3 activation in dissociated cells treated with IL-10. Furthermore, cerebellar granule cells were spared death following IL-10-mediated blockade of caspase-3 activity (Bachis *et al.*, 2001). The suggestion that IL-10 acts as an antioxidant (Kelly *et al.*, 2001) may explain its reversal of the IL-1β-induced caspase-3 activation. Caspases are redox sensitive and therefore IL-1β treatment may have induced ROS accumulation in these neurons, as seen previously (Vereker *et al.*, 2000b). It must be noted that expression and activation of other factors involved in IL-1β signalling namely, caspase-1, IL-1RI and IL-1ra are altered in response to IL-10 treatment (Rojas et al., 1999; unpublished data cited in Pousset *et al.*, 2001; Strle *et al.*, 2001; Bluthe *et al.*, 1999; Williams *et al.*, 2004). To extend these findings, the IL-1β-induced impairment in LTP was also abrogated by IL-10 (Lynch *et al.*, 2004).

These findings demonstrate that IL-10 antagonises the effects of IL-1β in the brain and as a consequence, it may be postulated that IL-10 can counteract the IL-1β-induced changes in the aged brain. It can also be suggested that any treatment which increases IL-10 concentration in the brain may also abrogate IL-1β signalling and any changes which occur as a result. Because of their ability to increase IL-10 in peripheral cells (Richards *et al.*, 2000; Pedersen *et al.*, 2004), dexamethasone and vitamin D_3 were administered alone and in combination to young and aged rats. The present findings demonstrate that the age-related decrease in hippocampal IL-10 concentration was abrogated following treatment with dexamethasone and vitamin D_3, as were the age-related decreases in JAK1 and STAT-3 phosphorylation. To my knowledge, this is the first study to present the evidence indicating that dexamethasone and vitamin D_3 restore IL-10 concentration in the hippocampus of aged rats and that, in parallel, lead to attenuation of the age-induced alterations in IL-10 signalling.

In addition to these anti-inflammatory effects, dexamethasone and vitamin D_3 affect IL-1β signalling, as is consistent with the theory that anti- and pro-inflammatory responses

exist in a state of inverse correlation. The age-induced increases in IL-1β concentration and mRNA expression were corrected by dexamethasone and vitamin D_3 treatment. These results are broadly consistent with previous studies which reported an inhibitory effect of dexamethasone on IL-1β production *in vivo* (Besedovsky *et al.*, 1986) and *in vitro* (Amano *et al.*, 1992). Dexamethasone can impact on IL-1β mRNA expression and stability in monocytes and in a U937 cell line (Amano *et al.*, 1992; Knudsen *et al.*, 1987). Moreover, increased levels of serum glucocorticoids inhibited secretion and functional effects of IL-1 (Kovalovsky *et al.*, 2000). Alterations in receptor functionality have been observed; in the current study, a decrease in IL-1RI mRNA expression following treatment with dexamethasone and vitamin D_3 was observed Others document an increase in production of IL-1ra (Arzt *et al.*, 1994); however, both of these changes have the same end product- a decrease in IL-1β signalling. Thus, regulation of IL-1β signalling by glucocorticoids is complex and occurs at multiple levels; inhibition of de novo synthesis of IL-1 protein, secretion, transcription and mRNA stability have been observed (see Kovalovsky *et al.*, 2000; see McEwen *et al.*, 1997).

Another significant finding of this study is the attenuation of downstream IL-1β signalling, in parallel with the abrogation of the age-related increase in IL-1β concentration, following dexamethasone and vitamin D_3 treatment. A decrease in the age-related increase in phosphorylation of JNK was observed; groups of aged rats treated with dexamethasone and vitamin D_3 showed reduction sin hippocampal JK activity. While elucidating the mechanism underlying the therapeutic effects of glucocorticoids in lung disease, Pelaia and colleagues (2001) discovered that this treatment could block IL-1β-induced JNK activity and perhaps this is one way in which dexamethasone and vitamin D_3 are exerting their anti-inflammatory effect in the aged brain. As MAPK substrates such as AP-1 and ATF-1 command a central role in modulation of many genes involved in inflammation, blockade of specific members of MAPK family, for example JNK, by glucocorticoids allows control at a critical step in the process. Dexamethasone suppressed JNK activation in a number of tissue types, including endothelial cell lines (Pelaia *et al.*, 2001) and murine macrophages (Swantek *et al.*, 1997); here the results show that in combination with vitamin D_3, dexamethasone can inhibit JNK

phosphorylation *in vivo* and *in vitro*, as evidenced by reduced staining in cultured hippocampal neurons.

An anti-apoptotic role for dexamethasone has been elucidated (Amsterdam *et al.*, 2002) and it is suggested that this is achieved by negatively altering expression of bcl-x_s (Chang *et al.*, 1997) while stimulating the expression of bcl-2, the anti-apoptotic member of the family (Sasson *et al.*, 2001). Here, this anti-apoptotic role is extended to show that the age-related increases in active caspase-3 and PARP cleavage, which are thought to be indicative of cell death (Martinou *et al.*, 1996), are ameliorated in groups of aged rats treated with dexamethasone and vitamin D_3. As previously discussed, the age-related impairment in LTP might be attributed to the upregulation of IL-1β and its signalling mediators, JNK and caspase-3, and consistently amelioration of these changes by treatment with dexamethasone and vitamin D_3 resulted in restoration of LTP in aged animals to a level that was comparable with young rats. This supports previous work from this laboratory which suggests that inflammation negatively correlates with synaptic plasticity (Lynch, 2004). Significantly, dexamethasone and vitamin D_3 lead to a decrease in corticosterone levels in the serum of aged rats, perhaps as a consequence of the increase in IL-1β concentration (see above).

The protective effects of glucocorticoids extend beyond its antagonising of IL-1β-induced signalling, as evidenced by their ability to inhibit cytokine production stimulated by Aβ (Szczepanik *et al.*, 2005). It is noteworthy that in this paradigm, IL-10 mimics the effects of dexamethasone on cytokine production. A similar situation was observed in this study; the inhibitory effect of dexamethasone and vitamin D_3 on p-JNK and active caspase-3 staining in hippocampal neurons was mirrored by IL-10 treatment. Furthermore, work by A. Piazza (personal communication) reflected the similarity between these two treatment regimes; both have been shown to attenuate the LPS-induced IL-1β secretion from cultured glia. The dexamethasone and vitamin D_3-induced increase in IL-10 release from hippocampal neurons seen in this study is comparable to further results of A. Piazza, indicating treatment-induced IL-10 protein release and mRNA expression in cultured glia. The hypothesis that the anti-inflammatory effects of

dexamethasone and vitamin D_3 are mediated by IL-10 is supported by other studies. For example, dexamethasone and IL-10 exerted similar effects on cell proliferation and differentiation (Pousset *et al.*, 1999). Dexamethasone augmented IL-10 release from T cells (Richards *et al.*, 2000), while vitamin D_3 inhibited secretion of pro-inflammatory cytokines such as IFN-γ from Th1 cells (Lemire *et al.*, 1995). In combination, dexamethasone and vitamin D_3 aid the polarisation of naive T cells into Th2 cells (Barrat *et al.*, 2002; O'Garra and Barrat, 2003). Modulation of dendritic cell (DC) maturation (Xing *et al.*, 2002) and development of tolerogenic DC function by the synergistic effects of dexamethasone and vitamin D_3 result in the production of IL-10 (Pedersen *et al.*, 2004).

The results of this study are in agreement with the effects of dexamethasone and vitamin D_3 in the periphery. Pro-inflammatory cytokine production was inhibited by dexamethasone and vitamin D_3 (Cantorna *et al.*, 1998, 2000; Barrat *et al.*, 2002), while both treatments administered separately ameliorated EAE (Mattner *et al.*, 2000; Wilckens and De Rijk., 1997). In fact, it has been suggested that chronic inflammation, as seen in EAE, can be caused, in part, by defective innate glucocorticoid homeostasis and in turn, administration of glucocorticoids can alter the path of the disease dramatically (Wilckens and De Rijk, 1997). EAE is associated with the development of Th1 cells from stimulated naive T cells and perhaps a mechanism of action of dexamethasone and vitamin D_3 is by preventing development of Th1 cells and also by skewing polarisation towards a Th2 response.

Indeed, glucocorticoids are significant factors in the development of the Th1/Th2 pattern (Wilckens and De Rijk, 1997) as detailed by the glucocorticoid-induced upregulation of Th2 cells and their cytokines (Ramirez *et al.*, 1996). It can be suggested that it is because of the effect of dexamethasone on DC activity that the shift from a Th1 to a Th2 cytokine profile occurs, as reported by both Franchimont and colleagues (1998) and Elenkov and colleagues (1999). Antigen processing and presentation, required for stimulation of T cell responses, is carried out by DCs (Steinmann, 1991) and therefore, it is not surprising that dexamethasone has been found to suppress DC functional activity (see Xing *et al.*, 2002).

DCs must undergo maturation in order to possess the ability to activate T cells and this maturation process is dependent on exposure to inflammatory products (Xing *et al.*, 2002). Once DCs are stimulated to undergo maturation, they express cell surface markers required for antigen-presentation and consequent T cell polarisation (Penna *et al.*, 2000). Two of the markers, MHCII and CD80/86 were observed as being decreased following treatment of bone marrow cultures with dexamethasone (Xing *et al.*, 2002). A secretory product of DCs is IL-12 which is also necessary for T cell differentiation and activation. The presence of this cytokine determines the outcome of the polarisation process; that is to say, whether an anti- or pro-inflammatory response will be initiated. Diminished capacity of DCs to produce and release IL-12 was found in mouse bone marrow (Pan *et al.*, 2001) and splenocytes (DeKruyff *et al.*, 1998) following incubation with dexamethasone. Vitamin D_3 also exerts an inhibitory effect at this level. It acts at two distinct stages in the life of a DC; it inhibited differentiation from its precursors and thus decreased cell number and blocked terminal maturation, therefore hindering its ability to act as an APC (Piemonti *et al.*, 2000).

As the vitamin D receptor (VDR) is expressed on T cells, it was proposed that it may act as an immunosuppressant (Bunce *et al.*, 1997). Once T cells leave the thymus and enter the circulation, VDR expression is lost. However, following stimulation by a mitogen, expression is reinduced (Hewison *et al.*, 1997; Bhalla *et al.*, 1983; Provvedini *et al.*, 1983). Since its first discovery, the VDR has been found in the rat hippocampus (Langub *et al.*, 2001). The direct effect of vitamin D_3 on T cells was demonstrated in a study where an enhancement of Th2 polarisation in the absence of an antigen was achieved following co-incubation with vitamin D_3 (Boonstra *et al.*, 2001). Specifically, this study detailed an overall alteration in the Th1/Th2 pattern, caused by a blockade of Th1 development while favouring Th2 proliferation by vitamin D_3 treatment. Like dexamethasone, vitamin D_3 can reduce expression of co-stimulatory molecules and IL-12, key requirements for Th1 development (Penna *et al.*, 2000).

Combined treatment with dexamethasone and vitamin D_3 prevented monocyte differentiation and maturation of DCs (Piemonti *et al.*, 2000; Xing *et al.*, 2002). Vitamin

D_3, in conjunction with glucocorticoids, enhanced development of IL-10-producing regulatory T cells (Barrat *et al.*, 2002). Compounding this, dexamethasone acted additively with vitamin D_3 to suppress lymphocyte proliferation and inhibit Th1 cytokine production (Jirapongsananuruk *et al.*, 2000). This complimentary relationship is highlighted further by the finding that glucocorticoids profoundly increased 1α-hydroxylase production (Spanos *et al.*, 1977). The findings of that study were mirrored by the evidence that prednisone administration in man resulted in enhanced production of vitamin D_3 (Avioli *et al.*, 1968). Combined treatment represents the most beneficial therapeutic profile as vitamin D_3 is limited by risk of hypocalcaemia and the harmful effect of high doses of glucocorticoids are well documented.

In an effort to elucidate the source of IL-1β in the brain, cultures of both neurons and glia were prepared and stimulated with LPS and IFN-γ. It is generally accepted that IL-1β is released from glia *in vitro* (Dello Russo *et al.*, 2004; Nolan *et al.*, 2004) and in agreement, it is demonstrated that IL-1β release from glia is increased following treatment with LPS and IFN-γ. In addition, neurons have been shown on numerous occasions to release IL-1β (Orzylowska *et al.*, 1999; Lynch *et al.*, 2003; Nolan *et al.*, 2004; Minogue *et al.*, 2003) along with other inflammatory mediators such as IL-6 (Murphy *et al.*, 1999) and TNF-α (Breder *et a*l., 1993). Results from LPS and IFN-γ-stimulated neurons are similar; although both cell types release IL-1β, it is clear from this and other studies (Davies *et al.*, 1999; Li *et al.*, 2003; Minogue *et al.*, 2003) that glia are the primary source; even under basal conditions, the concentration of IL-1β released from cultured glia is significantly greater than that from neurons. In an effort to identify the cellular source of IL-1β in the aged hippocampus, it was necessary to investigate the status of glia in the aged brain.

Coupled with the age-related increase in IL-1β in the aged brain is evidence of microglial activation, *in vivo* and *in vitro*. Increased MHCII expression and enhanced pro-inflammatory cytokine secretion are manifestations of microglial activation (Terao *et al.*, 2002). Here the evidence indicate increased microglial activation in the hippocampus of

aged rats, which tallies with previous observations in rats (Ogura *et al.*, 1994; Lu *et al.*, 1994) and monkey (Sloane *et al.*, 1999; Sheffield and Berman, 1998). This finding extends to alterations induced by age, as assessed by MHCII mRNA expression, in this study and also by others (Frank *et al.*, 2005). Stimuli which increase IL-1 such as LPS (Vereker *et al.*, 2000b), also trigger microglial activation (Nolan *et al.*, 2004; Xu and Ling, 1994a; Hauss-Wegrzynick *et al.*, 2002). Similarly, microglial activation, accompanied by increased IL-1, are well documented characteristics of Alzheimer's disease (Blum-Degen *et al.*, 1995; Griffin *et al.*, 1994; Sheng *et al.*, 2000). Treatment of aged rats with dexamethasone and vitamin D_3 attenuated the age-related increase in microglial activation. No previous studies have identified this effect of combined treatment; however dexamethasone alone has been documented on several occasions as having such inhibition. Markers of microglial activation, such as ED1, OX-42 and CD11b in various paradigms of stress - entrohinal lesions (Woods *et al.*, 1999) and an experimental model of glioma (Badie *et al.*, 2000) - were eliminated by dexamethasone. Dexamethasone is also reported to reduce axotomy-induced MHCII expression on microglia (Kiefer and Kreutzberg, 1991) and DCs (Xing *et al.*, 2002). As presentation of antigen to naive T cells, in order to induce polarisation, is dependent on MHCII upregulation, it is noteworthy that dexamethasone and vitamin D_3 have been found to inhibit the immune response at both of these stages.

Identification of the stimuli involved in the regulation of microglial activation (Aloisi *et al.*, 1999) is a significant issue when attempting to limit the destructive changes occurring with age. The opposing actions of IL-10, which reduces APC function in the periphery (Fiorentino *et al.*, 1991b) and IFN-γ, which primes microglia to secrete pro-inflammatory cytokines (Beneviste, 1997), on microglial activation have been assessed in this study. Induction of MHCII expression occurred *in vivo* following intraperitoneal injection of IFN-γ (Lu *et al.*, 1994; Xu and Ling, 1994b; Grau *et al.*, 1997; Ng and Ling, 1997). Using immunohistochemistry and RT-PCR, the present results demonstrated increased OX-6 expression in glial cell cultures following incubation with IFN-γ, which is consistent with previous *in vitro* studies (Lauro *et al.*, 1995; Lee et al., 1994; Steiniger and van der Meide, 1998). On the other hand, IL-10 suppressed the expression of MHCII

transcriptional activator (CIITA) and by doing so the transcription of MCHII was downregulated in the aged brain (Frank *et al.*, 2005). Here the data show that IL-10 blocked the IFN-γ-induced upregulation of MCHII expression on glial cells. Consistently, IL-10 has been shown to curtail microglial activation centrally (Balasingam and Yong, 1996) and peripherally (Fiorentino *et al.*, 1991b). In fact, when administered peripherally (Rott *et al.*, 1994) or centrally (Willenborg *et al.*, 1995) or when it is overexpressed (Bettelli *et al.*, 1998), IL-10 blocks development of EAE in mice and rats.

The hypothesis presented here is that the balance between pro- and anti-inflammatory responses is skewed in the aged brain and this is strengthened by the finding that IL-10 protein and mRNA were reduced while MCHII expression was increased. Similarly, Frank and colleagues (2005) have observed an age-induced decrease in the ratio of IL-10 to IFN-γ. Additionally, IFN-γ suppressed IL-10 gene expression and IL-10, along with IL-1ra, production in microglia (Aloisi *et al.*, 1999; Aloisi, 2001). An enhancement of IL-1β secretion from cultured glia in response to IFN-γ is described here and this is broadly consistent with findings that IFN-γ induced secretion of pro-inflammatory cytokines from microglia (Meeuwsen *et al.*, 2005). The present data show that IL-10 blocked the IFN-γ-induced IL-1β secretion from cultured glia.

In parallel with the age-related decrease in IL-10 and increase in IL-1β concentrations, is the finding that IFN-γ is increased in the aged brain, *in vivo*. This is corroborated by work from this laboratory in the hippocampus (Minogue and Lynch, unpublished) and from endothelial cells isolated from aged mice (Wei *et al.*, 2000). The contention that IFN-γ is involved in an inflammatory response is supported by observations from disease paradigms. For example, IFN-γ mediated microglial activation in Alzheimer's disease and a synergistic relationship between Aβ and IFN-γ exists as evidenced by microglial release of nitric oxide following co-exposure (Goodwin *et al.*, 1995). The progression of EAE in mice was facilitated by IFN-γ (Popko and Baerwald, 1999; Wensky *et al.*, 2005). As with IL-1β, IFN-γ negatively impacted on synaptic plasticity in hippocampal slices (Vikman *et al.*, 2001) while behavioural changes following i.c.v. injection have been reported in rats

(Peng *et al.*, 1994). Although not explicitly shown, it may be postulated that the age-related impairment in LTP observed in this study may be due to the increase in IFN-γ, which, it is proposed triggers the increased IL-1β in the aged hippocampus.

Treatment with either dexamethasone or vitamin D_3 alone reduced the increase in IFN-γ concentration associated with age in the hippocampus; however the greatest reduction was elicited by treatment with a combination of both. Although no previous results exist showing the effect of dexamethasone and vitamin D_3 on IFN-γ concentration in the hippocampus, it has been reported that dexamethasone alone can inhibit IFN-γ concentration in T cells (Hodge *et al.*, 1999; Yamaki *et al.*, 2005), whole blood (Simmons *et al.*, 2005) and NK cells (Hodge *et al.*, 1999). Similarly, vitamin D_3 has been shown to target IFN-γ at the molecular and protein levels (Jirapongsananuruk *et al.*, 2000; Lemire *et al.*, 1992). Co-administration of dexamethasone and vitamin D_3 has been reported to inhibit IFN-γ release from lymphocytes in peripheral blood samples (Jirapongsananuruk *et al.*, 2000).

From these findings, it is proposed that IFN-γ is responsible for the activation of microglial cells, as evidenced by MHCII expression, in the aged hippocampus, with the consequent age-related increase in IL-1β. In the normal brain, glial activation exerts a protective effect by attacking pathogens, clearing debris and enhancing tissue repair; however in the stressed brain, cells become excessively activated and thus become destructive. It is evident that normal cell functioning is disrupted in the aged brain and as a consequence, cytokine secretion and signalling becomes unbalanced, with the pro-inflammatory responses escalating and the opposite occurring with respect to the anti-inflammatory responses. Thus, homeostasis is eliminated and functional and behavioural changes occur as exemplified by the age-related impairment in LTP. However, restoration of homeostasis is achieved by dexamethasone and vitamin D_3 and also IL-10, which successfully ameliorate several of the inflammatory mediators.

In an effort to elucidate source of IFN-γ, glial cell cultures were prepared and were treated with LPS, which can stimulate pro-inflammatory cytokine release. However, no

detectable IFN-γ was found in the supernatants. A similar result was found following assessment of supernatants obtained from cultured neurons (A. Lyons, personal communication). This is consistent with work from this laboratory which fails to establish any detectable IFN-γ release following treatment with other stimuli including LPS, Aβ and IL-1β. In contrast, there are reports of IFN-γ release from neurons treated with a parasite Trypanosome brucei brucei (Peng *et al.*, 1994) and from glial cells (Hirsch *et al.*, 2003). Because of the finding that IFN-γ is not released from resident cells of the CNS, the possibility that peripheral immune cells may be present in the aged brain was investigated. $CD4^+$ T cells and NK cells are well known to produce and release IFN-γ and therefore, their presence in the hippocampus was assessed. The hypothesis that the source of IFN-γ is peripheral is supported by the observation that in aged mice, IFN-γ immunoreactivity was found in endothelial cells at the choroid plexus (Wei *et al.*, 2000), an area where infiltrating cells are thought to gain access to the CNS (Ransohoff *et al.*, 2003).

The presence of $CD4^+$ T cells was detected in the hippocampus from young and aged rats using western immunoblotting and immunohistochemistry, with evidence of a slight increase in number in the aged brain. Similar results have been reported in CNS disease and stress. For example, it is well-documented that MS and EAE, the animal model of MS are exacerbated by T cell infiltration (see Ching *et al.*, 2005; Bo *et al.*, 2003; Trebst *et al.*, 2003). T cells are present in the CNS of patients and animals with cerebrovascular diseases (Wang *et al.*, 1992), leukencephalopathy (Tomimoto *et al.*, 1993), tumours (Paine *et al.*, 1986), cerebral meningitis, traumatic brain injury (see Ching *et al.*, 2005) and amyloid β angiopathies (Yamada *et al.*, 1996). These reports illustrate that lymphocytes enter the inflamed CNS and therefore perhaps it is not surprising that ageing is associated with T cell infiltration.

As a result of the finding that the cervical lymphatics facilitate communication between the CNS and the immune system, the idea that the brain was an immune-privileged organ has been quashed. In fact, in the normal brain, physiologically activated T cells constantly transverse the BBB and survey the CNS for antigens (Brown, 2001; Hofer *et*

al., 2004; see Merrill and Beneviste, 1996). In the absence of an antigen, the T cells will return to the peripheral circulation (Brown, 2001) or undergo apoptosis (Hofer *et al.*, 2004). The idea that the BBB must be permeable in order to allow infiltration, as occurs in MS, has been made redundant. Perry and colleagues (1997) illustrated that infiltration occurs where the BBB is intact and the diapedesis is the process by which infiltration occurs, as opposed to passive diffusion. Lymphocytes are stimulated to cross the BBB by chemotactic signals from the CNS such as those provided by chemokines. Chemokines facilitate the extravasation of lymphocytes (Vulcano *et al.*, 2003; Ching *et al.*, 2005) and the expression and secretion of these molecules are regulated by inflammatory cytokines such as IFN-γ (Vulcano *et al.*, 2003) and IL-1β, both of which are increased in the aged brain. Consistent with its increase in the aged hippocampus, the IL-1RI was found to be necessary for recruitment of leukocytes (Ching *et al.*, 2005). The hypothesis presented here is that once the T cells enter the CNS, they stimulate the activation of microglia, via the secretion of IFN-γ. This produces more IL-1β, along with other pro-inflammatory cytokines and amplifies the inflammatory process. In the aged brain, this inflammation spirals out of control, resulting in overactivation of glial cells and subsequent cell deterioration. The exacerbation of inflammation by T cell infiltration of the CNS is supported by Merrill and Beneviste (1996) and Biernack and colleagues (2004).

Treatment with dexamethasone and vitamin D_3 did not elicit any effect on the number of $CD4^+$ T cells in the aged hippocampus. However, vitamin D_3 alone (Jirapongsananuruk *et al.*, 2000), and in combination with dexamethasone (Barrat *et al.*, 2002) can inhibit Th1 cell development and favour the production of IL-10-producing T cells. In our study, we have not distinguished between cells with a Th1 or Th2 phenotype and therefore it is possible that although the number of $CD4^+$ T cells is unchanged following treatment, the phenotype may be shifted in favour of Th2 cells. Although we cannot explicitly determine whether treatment influences T cell polarisation in the aged brain, it has been shown in this study that Th1 cell cytokines are suppressed by dexamethasone and vitamin D_3.

A significant finding of this study is the detection of the presence of NK cells in the aged

hippocampus, using western immunoblotting and immunohistochemistry. To my knowledge, this is the first indication of infiltration of this type of peripheral cell into the CNS. NK cells have been found to release IFN-γ upon stimulation (Bluman *et al.*, 1996) and therefore present as the source of IFN-γ in the aged brain. NK cell biology is preserved with age (Solana *et al.*, 1999; Krishnaraj, 1997) and in fact, activation increases with age in the periphery (Borrego *et al.*, 1999). The data indicate that there is an enhanced number of $CD161^+$ cells in the hippocampus and increased activity as evidenced by the increase in IFN-γ concentration in aged rats. Immunohistochemistry directed against $CD161^+$ cells detected their presence around blood vessels, consistent with the contention of infiltration occurring in the aged brain. A study by Hasegawa and colleagues (2004) established a relationship between hippocampal neurons and activated NK cells *in vitro*; adherence of NK cells to the cultured neurons occurred which led to altered dendritic morphology. The idea that NK cells infiltrate the brain is supported by further work from this laboratory which has resulted in the isolation of $CD161^+$ cells from the whole rat brain using Dynabeads (R. Clarke, unpublished results).

NK cell activity and proliferation is regulated by interleukins, most notably, IL-2 and IL-12 (Masera *et al.*, 1999). Correlations between IL-2 concentration, NK cell activity and cytotoxicity and IFN-γ release have been drawn on numerous occasions, using various experimental paradigms (see Bodner *et al.*, 1998; Naume *et al.*, 1993). IL-2 stimulated NK cells to release IFN-γ in glioma patients (Urbani *et al.*, 1995), in patients with lymphohistiocytic syndrome (Yang *et al.*, 1994), in rats stimulated with acupuncture (Yu et al., 1997) and in patients with myelodyspastic syndromes (Cortelezzi *et al.*, 1996). *In vitro* results from the current study support these findings; IL-2 stimulation is shown to augment IFN-γ release from cultured NK cells. In addition, Aβ in combination with IL-2 elicited a similar effect (A. Lyons, unpublished results). Indeed, $CD161^+$ cells isolated from the whole brain exhibited increased IFN-γ release following IL-2 stimulation, *in vitro* (R. Clarke, unpublished results). To explore the possibility that an age-related increase in IL-2 might be responsible for the increase in IFN-γ, the concentration of IL-2 in the aged hippocampus was assessed; no change in the concentration was observed in tissue prepared from young and aged rats or following treatment with dexamethasone and

vitamin D_3. This is at odds with the finding that vitamin D_3 can inhibit IL-2 production in T cells (Jirapongsananuruk *et al.*, 2000). However, this may be explained by the fact that NK cells must be activated before their transport through the lymph nodes is permitted (Seaman, 2000) and thus, the IL-2 concentration in serum, rather than the hippocampus, may correlate with the enhanced NK cell activity observed in the aged brain.

Previous studies have shown that IFN-γ can be released by T cells and NK cells; however following stimulation with Staphylococcus aureus, IFN-γ release occurred specifically from NK cells, not T cells (Yoshihara *et al.*, 1993). It is suggested that the role played by T cells is in production of IL-2, which is then responsible for NK cell activation. Similarly, ligand-expanded or mature DCs enhanced NK cell activity (Yu *et al.*, 2001) by priming T cell development into a Th1 phenotype (Marcenaro *et al.*, 2005). As a result, it may be suggested that the $CD4^+$ cell infiltration seen in this study is responsible for activation of NK cells.

The classical role of NK cells are in mediating anti-tumour activities and, in keeping with this, dexamethasone decreased NK cell activity in a human tumour model (Nielsen et al., 2000). Similarly, NK cell cytokine production is regulated in a dose-dependent manner by glucocorticoids (Hodge *et al.*, 1999). Dexamethasone, in combination with cyclophosphamide, vinarisirne and doxorubin, attenuates advance blastic NK cell lymphoma/leukaemia (Shapiro *et al.*, 2003). In combination with vitamin D_3, dexamethasone can decrease the number of $CD161^+$ cells *in vivo* and also their activity, as measured by IFN-γ release *in vitro*. Dexamethasone alone has also been found to suppress NK cell function *in vitro* (Parrillo and Falici, 1978) while correlations between cortisol levels (Pedersen *et al.*, 1988), ACTH levels (Masera *et al.*, 1999; Hseuh *et al.*, 1994) and NK cell activity exist. As observed in this study, dexamethasone, in combination with vitamin D_3, attenuated the age-related increase in corticosterone levels in serum. Taken together with findings regarding cortisol and ACTH, it can be suggested that the mechanism by which dexamethasone inhibits NK cell activity may involve its inhibitory effect on the HPA axis.

Vitamin D_3 alone inhibited NK cell proliferation (Lemire *et al.*, 1992), activity (Merino *et al.*, 1989) and cytotoxicity (Leung, 1989). It also antagonised the mediators of NK cell activity, such as T cells (Kaneno *et al.*, 2002), IL-2 mRNA (Kaneno *et al.*, 2002; Lemire, 1992) and IL-2 production by T cells (Merino *et al.*, 1989) and therefore resulted in the inhibition of IL-2 stimulated activation of NK cells (Leung, 1989). The efficacy of vitamin D_3-induced inhibition is highlighted by the increased NK cell activity and subsequent IFN-γ release in rats with experimentally induced rickets (Kaneno *et al.*, 2002) and in human patients with vitamin D_3 deficiency (Sonnenfeld *et al.*, 1992). Further work is warranted to explicitly elucidate the mechanism by which dexamethasone and vitamin D_3 mediate the abrogation in NK cell infiltration and activity.

Although it is suggested that peripheral immunological capability decreases with age (Miller, 1996), central immune responses progress with age (Terao *et al.*, 2002). Findings from the current study suggest that increases in pro-inflammatory cytokines occur with age and are accompanied by enhanced downstream signalling. Signalling through IL-1β and its downstream mediators, JNK and caspase-3, result in cell deterioration as evidenced by PARP cleavage and an inability to sustain LTP. The age-related augmentation in serum corticosterone concentration may also contribute to this functional decline as treatment with dexamethasone and vitamin D_3 attenuated both the LTP impairment and corticosterone concentration. Restoration of LTP may be due to the amelioration of the age-induced increase in IL-1β concentration, another factor identified as a target for dexamethasone and vitamin D_3.

As it is thought that a balance exists between the production of pro- and anti-inflammatory cytokines, IL-10 was found to be decreased in the aged hippocampus. Compelling evidence from this laboratory shows that injection of IL-10 or administration of a treatment which increases IL-10 concentration can abrogate changes induced by inflammation and thus it is reasonable that dexamethasone and vitamin D_3 restore LTP via resolution of the balance between IL-1β and IL-10 signalling in the aged brain. Activation of microglia, as a result of increased IFN-γ concentration with age, may be the key component in the process which tips the balance in favour of IL-1β. The objectives of this study to establish the cellular signalling associated with age have been reached and

the evidence suggests that treatment with dexamethasone and vitamin D_3 successfully attenuated the age-related changes in the hippocampus, highlighting the potential benefits of anti-inflammatory treatment strategies in the aged brain. Although this study contributes to the understanding of cellular events in the aged brain, some questions still remain.

1. If IFN-γ is accepted as being the initiator of IL-1β signalling by stimulating activation of microglia, what is the stimulus for the age-related increase in IFN-γ concentration?

2. If it is agreed that peripheral cells are the source of IFN-γ in the hippocampus, what is the identity of the stimulus or antigen necessary for activation and thus facilitating infiltration?

3. The exact mechanisms of the neuroprotective effects of dexamethasone and vitamin D_3 are unknown. This study shows that they promote an anti-inflammatory response while suppressing a pro-inflammatory one, however how they achieve this is unclear.

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Appendix

Appendix I. Mean Data

(A) Pro-inflammatory Signalling *in vivo*

Parameter	Young				Aged				Units
	Con	Dex	Vit D_3	Dex & VitD_3	Con	Dex	Vit D_3	Dex & VitD_3	
IL-1β	77.39 ± 8.45	75.232 ± 0.23	41.22 ± 11	51.3 ±9.10	127.89 ±18.79	60.56 ±15.49	57.11 ±15	34.53 ± 10.44	pg/mg
IL-1 mRNA	0.51 ± 0.20			0.706 ± .11	1.24 ± 0.21			1.75 ± 0.18	Arbitrary values
IL-1RI mRNA	0.26 ± 0.07			0.06 ± 0.02	1.18 ±0.19			0.23 ± 0.10	Arbitrary values
pJNK	0.57 ± 0.12	0.63 ± 0.15	0.89 ± 0.16	0.69 ± 0.10	0.94 ± 0.05	0.76 ± 0.08	0.89 ± 0.11	0.77 ± 0.13	Arbitrary values
Caspase-3	0.1 ± 0.03	0.03 ± 0.01	0.06 ± 0.02	0.04 ± 0.01	0.008 ± 0.003	0.04 ± 0.01	0.06 ± 0.03	0.04 ± 0.01	Arbitrary values
PARP	50.57 ± 7.60	50.58 ± 7.3	59.69 ± 4.41	46.60 ± 16.02	91.7 ± 5.25	61.84 ± 2.57	56.67 ± 9.59	44.49 ± 12.58	Arbitrary values
% EPSP slope 0-2mins	128.656 ± 1.51			146.59 ± 1.8	118.33 ± 2.48			136.82 ± 2.38	%EPSP
% EPSP slope 35-40mins	128.14 ± 0.41			134.43 ± 1.56	92.66 ± 0.84			117.77 ± 2.08	%EPSP
Corticosterone	102.19 ± 31.48			29.7 ± 4.63	248.05 ± 59.0			39.7 ± 7.22	ng/ml

B) **Anti-inflammatory Signalling *in vivo***

Parameter	Young				Aged				Units
	Con	Dex	Vit D_3	Dex & VitD_3	Con	Dex	Vit D_3	Dex & VitD_3	
IL-10	164.03 ± 10.67	119.60 ± 10.76	190.29 ± 16.26	80.05 ± 37.28	105.81 ± 18.11	91.48 ± 24.22	50.86 ± 16.64	244.67 ± 13.55	pg/mg
IL-10 mRNA	0.42 ± 0.3			0.21 ± 0.01	0.31 ± 0.02			0.41 ± 0.06	Arbitrary values
pJAK1	2.21 ± 0.33	1.83 ± 0.29	1.77 ± 0.32	1.97 ± 0.37	0.98 ± 0.008	1.46 0.13	1.37 ± 0.08	1.57 ± 0.26	Arbitrary values
pSTAT-3	0.48 ± 0.04	0.53 ± 0.05	0.65 ± 0.1	0.77 ± 0.14	0.38 ± 0.02	0.41 ± 0.04	0.56 ± 0.1	0.45 ± 0.06	Arbitrary values

Effect of dexamethasone and vitamin D_3 and IL-10 *in vitro*

Parameter	Control-Treated			IL-1β-Treated			Units
	Control	Dex & Vit D_3	IL-10	Control	Dex & Vit D_3	IL-10	
IL-10	72.41 ± 22.11	87.21 ± 13.9		84.90 ± 10.90	133.00 ± 16.50		pg/ml
pJNK	24.54 ± 2.67	24.66 ± 2.84	23.95 ± 1.16	42.80 ± 1.87	26.29 ± 2.47	29.00 ± 0.84	%positive cells
Caspase-3	18.25 ± 1.32	18.33 ± 1.49	17.90 ± 0.81	42.25 ± 2.30	22.75 ± 0.88	24.79 ± 1.05	% positive cells

D) Ageing is associated with an increase in IFN-γ concentration and microglial activation

Parameter	Young				Aged				Units
	Con	Dex	Vit D_3	Dex & VitD_3	Con	Dex	Vit D_3	Dex & VitD_3	
IFN-γ	983.98 ± 232.95	1141.81 ± 345.05	1607.46 ± 388.58	1108.85 ± 87.99	1791.87± 24.32	1484.90 ± 162.41	1620.96 ± 206.57	1312.08 ± 259.01	pg/mg
OX-6 mRNA	0.0086 ± 0.005			0.002 ± 0.0004	0.15 ± 0.04			0.06 ± 0.02	Arbitrary values

E) Effect of LPS and IFN-γ on neurons and glia *in vitro*

Parameter	Neurons			Glia			Units
	Control	LPS	IFN-γ	Control	LPS	IFN-γ	
OX-6 mRNA				0.025 ± 0.02		0.25 ± 0.06	Arbitrary Values
IL-1β	11.57 ± 4.32	66.72 ± 17.24	37.71 ± 3.85	42.74 ± 3.24	120.84 ± 18.82	217.35 ± 42.38	pg/ml

F) IL-10 attenuates the IFN-γ-induced increase in IL-1β release from glia

Parameter	Control		IFN-γ		Units
	Control	IL-10	Control	IL-10	
OX-6 mRNA	42.74 ± 3.24	62.27 ± 11.85	217.35 ± 42.38	60.42 ± 6.90	Arbitrary Units

Ageing is associated with the presence of $CD4^+$ and $CD161^+$ cells and IL-2 in the brain

Parameter	Young		Aged		Units
	Control	Dex & Vit D_3	Control	Dex & Vit D_3	
$CD4^+$	0.29 ± 0.06	0.61 ± 0.22	0.40 ± 0.06	0.40 ± 0.08	Arbitrary Values
$CD161^+$	0.89 ± 0.10	0.75 ± 0.15	1.43 ± 0.15	0.78 ± 0.11	Arbitrary Values
IL-2	286.53 ± 13.82	376.18 ± 36.35	305.74 ± 41.98	257.91 ± 13.10	pg/mg

Effect of dexamethasone and vitamin D_3 on IFN-γ release from NK cells

Parameter	IL-2	IL-2 + Dex &Vit D_3 (6.8×10^{-11}mol/l)	IL-2 + Dex &Vit D_3 (1.36×10^{-10}mol/l)	IL-2 + Dex &Vit D_3 (6.8×10^{-10}mol/l)	Units
IFN-γ	100 ± 2.27	96.6 ± 3.41	88 ± 0.924	80 ± 2.96	%release

) IL-2 induces IFN-γ release from NK cells in vitro; abrogated by dexamethasone and vitamin D_3

rameter	Control			IL-2			Units
	Control	Dex & Vit D_3	IL-10	Control	Dex & Vit D_3	IL-10	
FN-γ	138.32 ± 5.66	108.6 ± 7.83	155.46 ± 9.6	820.17 ± 28.26	649.95 ± 9.66	819.96 ± 20.66	pg/ml

Appendix II. Addresses

Alexis	Alexis Corporation (UK) Ltd., P.O. Box 6757, Bingham, Nottingham NG13 8LS, United Kingdom.
Amersham	Amersham Biosciences Ltd., Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, United Kingdom.
Bio-Rad	Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire HP2 7TD, United Kingdom.
Biosource	Biosource International, 542 Flynn Road, Camarillo, CA 93012, USA.
Calbiochem	Calbiochem-Novabiochem Corp., 10394 Pacifica Centre Court, San Diego, CA 92121, USA.
Chemicon	Chemicon International Inc., 28820 Single Oak Drive, Temecula, CA 92590, USA.

Cruinn	Cruinn Diagnostics Ltd., Unit 5b, 6b, Hume Centre, Park West Industrial Estate, Dublin 12, Ireland.
DAKO	DakoCytomation California Inc., 6392 Via Real, Carpintera, CA 93013, USA.
Gibco	Gibco Ltd., 3 Fountain Drive, Inchinnan Drive, Paisley PA4 9RF, Scotland, United Kingdom.
IDS	Immunodiagnostic Systems Ltd., 10 Didcot Way, Boldon Business Park, Boldon, Tyne and Wear, NE35 9PD, United Kingdom.
Invitrogen	Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, United Kingdom.
Jencons	Jencons (Scientific) Ltd., Cherrycourt Way, Stanbridge Road, Leighton Buzzard, Bedfordshire, LU7 4UA, United Kingdom.

Lennox	Lennox Laboratory Supplies, John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.
MWG	MWG Biotech, Anzingerstr. 7a, 85560 Ebersberg, Germany.
Pall Gelman	Pall Gelman Sciences Inc., 2200 Northern Boulevard. East Hills, New York 11548, USA.
Pierce	Pierce Biotechnologies 3747 N. Meridian Road, P.O. Box 117, Rockford, IL 61105, USA.
Promega	Promega, 2800 Woods Hollow Road, Madison, WI 53711, USA.
R&D Systems	R&D Systems, 614 McKinley Place NE, Minneapolis, MN 55413, USA.
Santa Cruz	Santa Cruz Biotechnologies, 2161 Delaware avenue, Santa Cruz, CA 95060, USA.

Sarsdedt	Sarstedt Ltd., Sinnottstown Lane, Drinagh, Wexford, Ireland.
Serotec	Serotec Ltd., 22 Bankside, Station Approach, Kidlington, Oxford, OX5 1JE, United Kingdom
Sigma	Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset BH12 4QH, United Kingdom.
Vector	Vector Laboratories Inc., 30 Ingold Road, Burlingame, CA 94010, USA.
Whatman	Whatman plc., Whatman House, St.Leonard's Road, 20/20 Maidstone, Kent ME16 0LS, United Kingdom.

Appendix III. Solutions

The following solutions were used:

Electrode running buffer

Tris base, 25mM

Glycine, 192mM

SDS, 0.1% (w/v)

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

Phosphate buffered saline (PBS), pH 7.4

Na_2HPO_4, 80mM

NaH_2PO_4, 20mM

NaCl, 100mM

Phosphate buffered saline (PBS), pH 7.3 for ELISA

NaCl, 137mM

KCl, 207mM

Na_2HPO_4, 8.1mM

KH_2PO_4, 1.5mM

Sample Buffer

Tris-HCl, 0.05M, pH6.8

Glycerol 20% (v/v)

SDS 2% (w/v)

β-Mercaptoethanol 5% (v/v)

Bromophenol blue 0.05% (w/v)

Stacking gel (4%), pH6.8

Acrylamide/bis acrylamide (30% stock), 13%(v/v)

dH_2O, 60% (v/v)

Tris-HCl, 0.05M, pH6.8, 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 (12%), pH 8.8

Acrylamide/bis acrylamide (30% stock), 40% (v/v)

dH_2O, 33% (v/v)

Tris-HCl, 0.05M, pH6.8, 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, pH6.8, 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)

Transfer buffer, pH8.3

Tris base, 25mM

Glycine, 192mM

MeOH, 20%

SDS, 0.05% (w/v)

Tris-buffered saline (TBS), pH7.4

Tris-HCl, 20mM

NaCl, 150nM

X. Publications

Moore M. and Lynch M.A. (2003). Does anti-inflammatory treatment prevent the age-related changes in the hippocampus? *International Cytokine Society Abstract.*

Moore M. and Lynch M.A. (2004). Does treatment with dexamethasone prevent the age-related changes in the hippocampus? *Society for Neuroscience Abstracts.*

Moore M., Nolan Y. and Lynch M.A. (2005). Treatment of rats with dexamethasone and vitamin D_3 restores LTP; evidence of a role for IL-10. *British Neuroscience Association Abstracts.*

Moore M. and Lynch M.A. (2005).The age-related changes in the hippocampal concentration of interleukin-1β and interleukin-1β -induced signalling are attenuated by the synthetic corticoid, dexamethasone. *Biochemical Society Abstracts.*

Piazza A., Moore M. and Lynch M.A. (2005). The neuro-inflammatory changes induced by age and LPS are suppressed by treatment with dexamethasone and vitamin D_3. *British Neuroscience Association Abstracts.*

Piazza A., Moore M. and Lynch M.A. (2005). Dexamethasone and vitamin D_3 attenuate the LPS-induced microglial activation in an IL-10-dependent manner. *Biochemical Society Abstracts.*

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Among the reported effects of ageing is an increase in concentration of the pro-inflammatory cytokine, interleukin-1β (IL-1β). Here we investigate the role of the putative anti-inflammatory combination of dexamethasone and vitamin D_3 on age-related changes in rat hippocampus. Dexamethasone (1μg/ml) and vitamin D_3 (0.1μg/ml) was administered in drinking water to male Wistar rats, aged 3 and 22 months old (n=6 for all groups), for 2 weeks. Hippocampal concentrations of IL-1β and IL-10 were assessed by ELISA on homogenates prepared from these treatment groups. IL-1β concentration was significantly increased in hippocampus of aged, compared with young, rats as previously shown. The present data indicate that treatment with dexamethasone and vitamin D_3 did not alter IL-1β concentration in hippocampus of young rats but it reduced the increase observed in aged rats. The concentration of the anti-inflammatory cytokine, interleukin-10 (IL-10), was significantly decreased in aged, compared with young rats and treatment with dexamethasone and vitamin D_3 resulted in a reversal of the age-related change. The data indicate that inflammatory changes observed in hippocampus of aged rats are abrogated by dexamethasone and vitamin D_3.

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Moore M. and Lynch M.A. (2004). Does treatment with dexamethasone prevent the age-related changes in the hippocampus? *Society for Neuroscience Abstracts.*

Inflammation is thought to contribute to the detrimental effects on cellular function and signaling in the ageing brain. Here we investigate the role of a putative anti-inflammatory agent, the synthetic glucocorticoid hormone, dexamethasone on the age-related changes in the hippocampus. Dexamethasone (1μg/ml) was administered for 2 weeks in the drinking water of male Wistar rats, aged 3 and 22 months old. Activated microglia have been reported to be associated with the neurodegeneration observed in the aged brain and are a source of the pro-inflammatory cytokine, interleukin-1β (IL-1β). In this study, microglial activation, assessed by immunohistochemistry (IHC) was increased in the hippocampus of aged, compared with young, rats; treatment with dexamethasone attenuates this age-related change. The concentration of IL-1β, assessed by ELISA was significantly increased in hippocampal homogenate prepared from the aged, compared with the young, rats. Treatment with dexamethasone abrogated this change paralleling its effect on microglial activation. Phosphorylation of the downstream mediator of IL-1β signaling, c-Jun N-terminal kinase (JNK) was assessed by Western Immunoblotting. We demonstrated an age-related increase in JNK phosphorylation, which was attenuated by dexamethasone. Similarly, caspase-3 activation, assessed by IHC, was markedly increased in the hippocampus of aged, compared with young, rats and this was coupled with a significant increase in the cleavage of poly-(ADP)ribose polymerase (PARP). Dexamethasone reversed the age-related increase in both caspase-3 activation and PARP cleavage. These findings demonstrate that dexamethasone can prevent the inflammatory changes observed in the hippocampus of the aged rat.

This work is supported by the Higher Education Authority.

Moore M., Nolan Y. and Lynch M.A. (2005). Treatment of rats with dexamethasone and vitamin D_3 restores LTP; evidence of a role for IL-10. *British Neuroscience Association Abstracts.*

It is well documented that synaptic plasticity is imapired in the aged hippocampus. Evidence illustrates a decline in long-term potentiation (LTP) which may be caused partly by a decrease in the hippocampal concentration of the anti-inflammatory interleukin-10 (IL-10). In this study, male Wistar rats, aged 3 and 22 months old, received dexamethasone (30[micro]g/rat/day) and vitamin D_3 (3[micro]g/rat/day) in their drinking water for 2 weeks. Aged rats did not sustain LTP when compared with young rats; however aged rats which received dexamethasone and vitamin D_3 displayed LTP comparable to young rats. Consistent with our hypothesis, IL-10 concentration in the hippocampus was decreased in the aged, compared with young rats; dexamethasone and vitamin D_3 treatment restored the IL-10 concentration to levels seen in young rats. Immunostaining for the IL-10 receptor revealed functional receptors on the granule cells of the dentate gyrus. To investigate the effect of age on IL-10 signalling, phosphorylation of JAK1 and STAT3 were assessed. In the aged brain, a decrease in phosphorylation of both was evident, however treatment with dexamethasone and vitamin D_3 abrogated these changes. These findings suggest that the anti-infammatory effects of dexamethasone and vitmain D_3 are mediated by IL-10.

Moore M. and Lynch M.A. (2005).The age-related changes in the hippocampal concentration of interleukin-1β and interleukin-1β -induced signalling are attenuated by the synthetic corticoid, dexamethasone. *Biochemical Society Abstracts.*

Cellular dysfunction in the ageing brain is mediated in part by an increase in interleukin-1β (IL-1β) concentration and its downstream signalling. Microglia, whose activation is amplified in the aged hippocampus, are thought to be the source of this increased IL-1β. In this study, we examined the actions of dexamethasone, a synthetic glucocorticoid which has anti-inflammatory effects, in the hippocampus of aged and young rats. Dexamethasone (1μg/ml) was administered for 2 weeks in the drinking water of male Wistar rats, aged 3 and 22 months old. Assessment of microglial activation by immunohistochemistry showed an increase in CD11b-positive staining in the aged hippocampus compared to the young rats; treatment with dexamethasone attenuated this age-related change. IL-1β concentration, assessed by ELISA, was augmented in the hippocampus of aged, compared with young rats. Downstream mediators of IL-1β signalling such as c-Jun N terminal kinase, caspase-3 and poly (ADP)-ribose polymerase were increased in the aged compared with young, rats. The effect of dexamethasone treatment on this signalling paralleled its effect on microglial activation thereby demonstrating its anti-inflammatory effect in the aged hippocampus.

Piazza A., Moore M. and Lynch M.A. (2005). The neuro-inflammatory changes induced by age and LPS are suppressed by treatment with dexamethasone and vitamin D_3. *British Neuroscience Association Abstracts.*

Evidence indicates that IL-1β concentration is increased in the hippocampus of aged rats suggesting that there may be an age-related increase in microglial activation. Since it has been shown that dexamethasone and vitamin D_3 exert anti-inflammatory properties, we assessed the effect of treating aged rats for 2 weeks on a combination of dexamethasone (25μg/rat/day) and vitamin D_3 (2.5μg/rat/day). Analysis of microglial activation, using MHCII expression as a marker (assessed by measurement of OX6 mRNA), revealed that there was a significant age-related increase in microglial activation and that this was not evident in tissue prepared from aged rats which received dexamethasone and vitamin D_3. Administration of the two agents also significantly reversed the age-related increase in hippocampal IL-1β concentration although IL-1β mRNA was unaffected.

Analysis of changes *in vitro* was also conduced using lipopolysaccharide (LPS) to induce neuro-inflammation. Treatment of cultured cortical glia with dexamethasone and vitamin D_3 reversed the LPS-induced increase in IL-1β concentration and significantly inhibited the LPS-induced increase in MHCII expression.

We report that microglial activation is a feature of the aged brain and that dexamethasone and vitamin D_3 act as anti-inflammatory agents in the brain reversing the neuro-inflammatory changes induced by age and LPS *in vivo* as *in vitro*.

ACKNOWLEDGEMENTS: AP is supported by Science Foundation Ireland. MM is supported by the Higher Education Authority Ireland (PRTLI).

Piazza A., Moore M. and Lynch M.A. (2005). Dexamethasone and vitamin D_3 attenuate the LPS-induced microglial activation in an IL-10-dependent manner. *Biochemical Society Abstracts.*

It has been shown that lipopolysaccharide (LPS) increases microglial activation resulting in increased production of inflammatory cytokines like IL-1β. Here we investigated the possibility that LPS-induced changes in cultured cortical glia might be attenuated by the synthetic glucocorticoid, dexamethasone, and vitamin D3.

The data show that LPS significantly increased expression of MHCII, as assessed by OX6 mRNA, and that this was accompanied by a significant increase in IL-1β protein, although IL-1β mRNA was unchanged. Treatment of cells with dexamethasone and vitamin D3 reversed the LPS-induced increases in MHCII expression and IL-1β protein. Previous studies have indicated that the action of dexamethasone and vitamin D3 may be mediated by IL-10 and therefore we examined IL-10 mRNA and protein in cells treated with these agents. Both IL-10 mRNA and protein were significantly increased by dexamethasone and vitamin D3. We also report that, like dexamethasone and vitamin D3, IL-10 inhibited the LPS-induced increase in IL-1β concentration.

The data are therefore consistent with the hypothesis that dexamethasone and vitamin D3 increase IL-10 production and that this is the key to the anti-inflammatory effect of these agents.

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Analysis of Interleukin-1β-induced Cell Signaling Activation in Rat Hippocampus following Exposure to Gamma Irradiation

PROTECTIVE EFFECT OF EICOSAPENTAENOIC ACID*

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Among the many reported effects of irradiation in cells is activation of the stress-activated protein kinase, c-Jun N-terminal kinase (JNK), which has been shown to result in apoptotic cell death. The trigger that leads to JNK activation has not been identified, although, in rat hippocampus at least, irradiation-induced apoptosis has been coupled with increased accumulation of reactive oxygen species (ROS). Significantly, irradiation-induced changes in hippocampus are abrogated by treatment of rats with the polyunsaturated fatty acid, eicosapentaenoic acid (EPA). A close coupling between ROS accumulation and concentration of the pro-inflammatory cytokine, interleukin-1β (IL-1β) in hippocampus has been reported, and the evidence suggests that IL-1β may be responsible for the enhanced ROS production. Here we set out to assess the possibility that whole body γ-irradiation increases IL-1β concentration in hippocampus and to investigate the consequences of such a change. We present evidence that reveals that the irradiation-induced increase in IL-1β concentration in hippocampus is accompanied by increased expression of IL-1 type I receptor and IL-1 accessory protein and increased activation of IL-1 receptor-activated kinase. These changes, which were coupled with increased activation of JNK and evidence of apoptotic cell death, were absent in hippocampus of rats that received EPA treatment. Significantly, EPA treatment enhanced hippocampal IL-10 concentration that was inversely correlated with IL-1β concentration. The data are consistent with the idea that EPA exerts anti-inflammatory and neuroprotective effects in the central nervous system.

Interleukin-1β (IL-1β)[1] exerts a wide range of effects in the CNS, most of which have been attributed to its interaction with IL-1 type I receptor (IL-1RI (1)), although recent evidence has revealed actions of IL-1β that are independent of interaction with IL-1RI (2). IL-1RI activation by IL-1β initiates formation of a complex that requires recruitment of IL-1 accessory protein (IL-1AcP), an adaptor protein, MyD88, and a kinase, IL-1 receptor-associated kinase (IRAK (1)). Phosphorylation of IRAK is a pivotal step in activating the cascade of events that lead to the IL-1β-induced response; events in this cascade include activation of the stress-activated protein kinase, c-Jun N-terminal kinase (JNK). Increased IL-1β concentration in hippocampus is associated with impairment in synaptic function, and activation of JNK significantly contributes to this; for example, deficits in synaptic transmission, and consequently in long-term potentiation (LTP), have been coupled with increased IL-1β concentration and increased JNK activation in aged rats (3, 4) and lipopolysaccharide-treated rats (5). Hippocampal and cortical cell death has also been reported to accompany these changes (4–6).

Radiotherapy treatment for brain tumors has been shown to result in deficits in hippocampal-dependent cognitive function (7, 8), and in animal studies, exposure to irradiation exerts profound effects on hippocampal function that have been coupled with apoptosis. For example, irradiation inhibits LTP in CA1 *in vitro* (9) and in dentate gyrus *in vivo* (10) and impairs memory/learning (11, 12). These changes have been shown to be accompanied by apoptotic cell death (10, 13), decreased neurogenesis (14, 15), and apoptosis of proliferating stem cells in dentate gyrus (16), the area of the hippocampus that has been shown to be particularly susceptible to irradiation (17). Although several changes have been observed following irradiation, the cell signaling events leading to irradiation-induced cell loss have not been explained. In a recent study, we have coupled irradiation-induced impairment in synaptic function with accumulation of reactive oxygen species (ROS) in hippocampus and with apoptotic changes (10). Significantly, treatment of rats with eicosapentaenoic acid (EPA) prevented these changes. Others have reported that inflammatory changes occur in brain following irradiation and increases in IL-1β and tumor necrosis factor α mRNA expression have been described previously (18). Interestingly, a close correlation between inflammation, as assessed by IL-1β concentration, and accumulation of ROS has been identified in hippocampus. Specifically, IL-1β increases activity of superoxide dismutase (but not catalase and glutathione peroxidase) resulting in increased ROS accumulation (3, 19).

Here we have investigated the possibility that exposure of rats to whole body irradiation induces an increase in hippocampal IL-1β concentration and IL-1β-induced signaling. The data suggest that the irradiation-induced apoptotic changes in hippocampus are a consequence of increased IL-1β concentration and the subsequent up-regulation of JNK activation. We report

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[1] The abbreviations used are: IL-1β, interleukin-1β; IL-1RI, IL-1 receptor type I; IL-1AcP, IL-1 accessory protein; IRAK, IL-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; LTP, long-term potentiation; ROS, reactive oxygen species; EPA, eicosapentaenoic acid; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; p-JNK, phosphorylated JNK; p-p38, phosphorylated p38; PARP, poly(ADP-ribose) polymerase; TUNEL, TdT-mediated dUTP nick-end labeling; ERK, extracellular signal-regulated kinase; Ac-YVAD-CMK, acetyl-tyrosinyl-valyl-alanyl-aspartyl-chloromethyl-ketone; ANOVA, analysis of variance.

that these changes are prevented by treatment with EPA, which increases hippocampal concentrations of the anti-inflammatory cytokine, IL-10.

EXPERIMENTAL PROCEDURES

Animals—Thirty-six adult (3 months) male Wistar rats were housed in groups of six in the BioResources Unit, Trinity College, Dublin and maintained under a 12-h light schedule (ambient temperature 22–23 °C) with veterinary supervision for the duration of the study. Rats were randomly assigned to one of six treatment groups. Food and water intake was measured daily for 2 weeks, and at the end of this period, 12 rats were fed on normal laboratory chow supplemented with ethyl eicosapentaenoic acid (EPA; 250 mg (1%), dissolved in corn oil; Laxdale, UK), 12 rats were fed on laboratory chow supplemented with 500 mg (2%) of EPA, and the remaining 12 rats received control diet (laboratory chow to which only corn oil was added). Sufficient diet was prepared for 2 or 3 days at a time, and rats were offered 100% of their average daily food intake so that the full daily allowance of EPA would be ingested; dietary supplementation continued for 4 weeks. Food and water intake, and weight gained did not vary between groups, and there was no significant difference in daily food and water intake before and after dietary modifications were made.

At the end of this 4-week period, six rats from each treatment groups were exposed to whole body irradiation (10 Gy at a rate of 10 Gy/min; Nordion Gammacell cesium^{137} irradiator). The remaining rats were sham-irradiated. Rats were monitored for 4 days following irradiation and were then killed by cervical dislocation and decapitation. Hippocampal tissue was dissected free, sliced (350 × 350 μm) using a McIlwain tissue chopper, and stored at −80 °C in Krebs solution containing 10% dimethyl sulfoxide as previously described (3) until required for analysis.

The experimental conditions were chosen on the basis of findings from preliminary experiments in which irradiation dose and changes in hippocampal IL-1β concentration at 1, 2, and 4 days following irradiation were assessed. The data from these experiments indicated that 10 Gy at 10 Gy/min was not associated with weight loss or gastrointestinal disturbance, which occurred at higher doses. The data also indicated that the increase in hippocampal IL-1β concentration was maximal 4 days after irradiation.

Analysis of Hippocampal Concentrations of IL-1β and IL-10—Hippocampal slices were thawed by agitation in a water bath at 37 °C for 1.5–2 min. Tissue was homogenized in Krebs solution containing 2 mM $CaCl_2$, and samples were analyzed for concentrations of IL-1β and IL-10 by ELISA (R&D Systems) as previously described (4). Briefly, in the case of IL-1β, antibody-coated (100 μl; 1.0 μg/ml final concentration, diluted in phosphate-buffered saline (PBS), pH 7.3; goat anti-rat IL-1β antibody) 96-well plates were incubated overnight at room temperature, washed several times with PBS containing 0.05% Tween 20, blocked for 1 h at room temperature with 300 μl of blocking buffer (PBS, pH 7.3, containing 5% sucrose, 1% bovine serum albumin (BSA), and 0.05% NaN_3), and washed. IL-1β standards (100 μl; 0–1000 pg/ml in PBS containing 1% BSA) or samples were added, and incubation proceeded for 2 h at room temperature. Secondary antibody (100 μl; biotinylated goat anti-rat IL-1β antibody; final concentration 350 ng/ml in PBS containing 1% BSA and 2% normal goat serum) was added, and the mixture was incubated for 2 h at room temperature. Wells were washed, detection agent (100 μl; horseradish peroxidase-conjugated streptavidin; 1:200 dilution in PBS containing 1% BSA) was added, and the mixture was incubated for 20 min at room temperature. Substrate solution (100 μl; 1:1 dilution of H_2O_2 and tetramethylbenzidine) was added, and the mixture was incubated at room temperature in the dark for 1 h after which time the reaction was stopped using 50 μl of 1 M H_2SO_4.

A similar ELISA procedure was used for analysis of IL-10; in this case the coating antibody was mouse anti-rat IL-10 (100 μl; 4 μg/ml final concentration). IL-10 standards ranged from 0 to 4000 pg/ml (100 μl), and the secondary antibody was biotinylated goat anti-rat IL-10 (100 μl; final concentration, 100 ng/ml). Unfortunately, due to a technical difficulty, samples prepared from rats treated with 2% EPA could not be analyzed. For both cytokines, absorbance was read at 450 nm, values were corrected for protein and expressed as picograms of IL-1β or IL-10/mg of protein, and the detection limit was 5 pg/mg.

In one set of *in vitro* experiments, IL-1β was measured from primary hippocampal neuronal cultures (see below for preparatory details). Hippocampal neurons were divided into two pretreatment groups: control (culture media) or IL-10 (100 ng/ml). 1 h later, half of each group was either sham- or γ-irradiated (20 Gy; $n = 6$ per group). Twenty-four hours later, the supernatant was harvested and the IL-1β concentration was measured by ELISA and expressed as picograms of IL-1β/ml of supernatant.

Analysis of IL-1RAcP Expression, PARP Cleavage, IRAK, JNK, p38, and ERK Phosphorylation—In all cases hippocampal samples were homogenized in Krebs solution (containing 2 mM $CaCl_2$), equalized for protein concentration (20), and diluted so that the same concentration of protein was loaded onto each lane (0.7 mg/ml). Aliquots (10 μl, 1 mg/ml) were added to sample buffer (5 μl; Tris-HCl, 0.5 mM, pH 6.8; glycerol, 10%; SDS, 10%; β-mercaptoethanol, 5%; bromphenol blue, 0.05% w/v), boiled for 5 min, and loaded onto gels (10% SDS for IL-1RAcP, PARP, IRAK, JNK, and ERK; 12% for p38). Proteins were separated by application of 30-mA constant current for 25–30 min, transferred onto nitrocellulose strips (225 mA for 75 min), blocked for 2 h at room temperature in 2% bovine serum albumin (BSA) in Tris-buffered saline (TBS), and immunoblotted overnight at 4 °C with the appropriate antibody diluted in 0.1% BSA in TBS-T (0.1% Tween 20).

IL-1RAcP expression was determined by immunoblotting with a rabbit anti-rat IL-1RAcP antibody (1:500; QED Biosciences Inc., San Diego, CA). PARP cleavage was detected by incubating nitrocellulose strips with an antibody to both the cleaved and uncleaved forms of PARP (1:1000; BIOSOURCE). To estimate IRAK activity, proteins were immunoblotted with a rabbit polyclonal anti IRAK-1 antibody (1:500; Stressgen Biotechnologies Corp., Canada). To assess ERK, JNK, or p38 activity, proteins were immunoblotted with antibodies that specifically target either phosphorylated ERK (1:500; mouse monoclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated JNK (G-7, 1:300; Santa Cruz Biotechnology), or phosphorylated p38 (1:1000; mouse monoclonal IgG, Cell Signaling Technology). These nitrocellulose blots were stripped and reprobed for ERK (1:500; mouse monoclonal IgG, Santa Cruz Biotechnology), JNK (F-3, 1:200, Santa Cruz Biotechnology), and p38 (A-12, 1:100; Santa Cruz Biotechnology) to assess expression of total ERK, JNK, and p38.

In all cases, nitrocellulose strips were washed in TBS-T and incubated for 2 h at room temperature with secondary antibody diluted in TBS-T containing 0.1% BSA (horseradish peroxidase-linked anti-rabbit antibody (Amersham Biosciences, UK) at 1:500, 1:1000, and 1:2000 dilution for IRAK, IL-1RAcP, and PARP, respectively). Nitrocellulose strips were incubated with a peroxidase-linked anti-mouse IgG (Sigma, UK) in the cases of anti-active p-ERK (1:1000), the unphosphorylated form of ERK (1:1000), anti-active p-JNK (1:300), the unphosphorylated form of JNK (1:400), phosphorylated p38 (1:2000), and p38 (1:800). Protein complexes were visualized with SuperSignal (Pierce), and nitrocellulose strips were exposed to film for 5 s to 5 min depending on the antibody and processed using a Fuji x-ray processor. Quantitation of protein bands was achieved by densitometric analysis using two software packages, Grab It (Grab It Annotating Grabber 2.04.7, Synotics, UVP Ltd.) and Gelworks (Gelworks ID, Version 2.51, UVP Ltd.) 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 six separate experiments.

Analysis of IL-1RI Expression and Cytochrome c Translocation—IL-1RI expression was assessed in the membrane fraction, whereas cytochrome c analysis was performed on the cytosolic fraction. Samples were prepared by homogenizing hippocampal slices in lysis buffer, followed by centrifugation (15,000 × *g* for 10 min 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, 0.5 mg/ml). Similarly the supernatant (*i.e.* cytosolic fraction) was equalized, sample buffer added, and the final concentration was 300 μg/ml. Both preparations were boiled for 5 min, and 10 μl was loaded onto either 10% (IL-1RI) or 12% (cytochrome *c*) gels. Proteins were separated, transferred onto nitrocellulose strips, and immunoblotted.

IL-1RI membrane expression was assessed by incubating the nitrocellulose strips in the primary antibody (1:600; rabbit anti-rat IL-1RI IgG, Santa Cruz Biotechnology) in 0.1% BSA in TBS-T, whereas cytochrome c translocation was determined by incubating the nitrocellulose strips in cytochrome *c* (1:1250 in PBS-T containing 2% nonfat dried milk, rabbit polyclonal IgG, Santa Cruz Biotechnology). Immunoreactive bands were detected using peroxidase-conjugated anti-rabbit antibody (1:1000; Sigma) and SuperSignal (Pierce) and quantified by densitometry as before.

TUNEL Staining in Acutely Dissociated Cells—Hippocampal slices (350 μm) were washed in oxygenated Krebs solution, incubated in PBS containing collagenase (0.025%) for 30 min at room temperature, and washed in PBS to terminate collagenase digestion. Samples were tri-

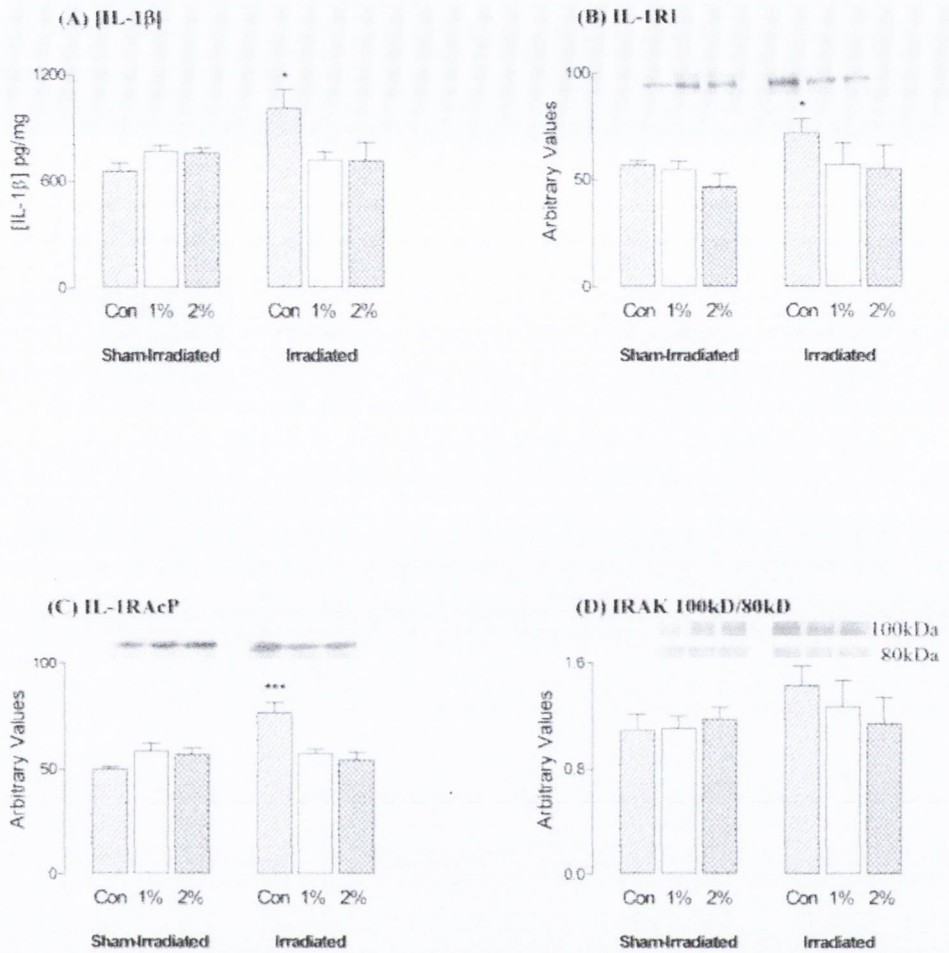


FIG. 1. **Irradiation up-regulates IL-1β concentration and signaling; this was prevented by EPA.** Mean IL-1β concentration (pg/mg; ± S.E.) was significantly increased in the hippocampus of irradiated rats that had received control diet (*A*; $p < 0.05$; ANOVA). This effect was prevented in irradiated rats that had received either 1% or 2% EPA diet, where the IL-1β concentration was similar to the sham-irradiated groups. There was a corresponding significant increase in IL-1RI (*B*; $p < 0.05$; ANOVA) and IL-1RAcP (*C*; $p < 0.001$; ANOVA) expression. A similar trend was evident when the ratio of phosphorylated (100 kDa) to unphosphorylated (80 kDa) IRAK was assessed, although significance was not reached (*D*). In *B–D* the *bar graphs* are represented by sample immunoblots. In the case of *D*, the sample immunoblots represent the 80-kDa unphosphorylated and the 100-kDa phosphorylated forms of IRAK.

turated using a fire-polished glass pipette and passed through a nylon mesh filter to remove tissue clumps. Dissociated cells were resuspended in PBS, and 100-μl aliquots were cytospun (600 rpm for 2 min) onto glass microslides (Shandon Cytospin III). Cells were fixed in methanol and stored at room temperature for later use. Apoptotic cell death was assessed using the DeadEnd colorimetric apoptosis detection system (Promega) according to the manufacturer's instructions. Staining was developed by addition of the chromogen 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 for 10 min. Apoptotic nuclei were stained dark brown, whereas negative cells were viewed by counter staining with 3% methyl green. Cells were dehydrated through alcohol to xylene and mounted, and 400 cells were counted per slide (~80 cells/field; 5 fields/slide) at ×40 magnification. TUNEL-positive cells were expressed as a percentage of the total cell number; six slides were assessed per experimental group.

Preparation of Primary Hippocampal Neuronal Cultures—Primary hippocampal neurons were established from 1-day postpartum Wistar rats and maintained in neurobasal medium (Invitrogen). Four experimental groups ($n = 6$/group) were assessed, which comprised sham-irradiated or γ-irradiated cells, with/without inhibitor (specific details below). Rats were decapitated, and the hippocampus was dissected free and incubated in sterile phosphate-buffered saline (PBS, Sigma) containing trypsin (0.25%; Sigma) for 20 min at 37 °C. The tissue was triturated five times in PBS containing trypsin inhibitor (0.1%; Sigma) and DNase (0.2 mg/ml; Sigma) and gently filtered through a sterile mesh filter (40 μm). Following centrifugation (2000 × *g* for 3 min) at 20 °C, the pellet was resuspended in neurobasal medium supplemented with heat-inactivated horse serum (10%; Sigma), penicillin (100 units/ml; Invitrogen), and glutamax (2 mM; Invitrogen). Suspended cells were plated out at a density of 1×10^5 cells on circular 10-mm diameter coverslips, coated with poly-L-lysine (40 μg/ml; Sigma), and incubated in a humidified atmosphere containing 5% CO_2:95% O_2 at 37 °C. After 48 h, cytosinearabinofuranoside (5 ng/ml; Sigma) was incubated in the culture medium to prevent proliferation of non-neuronal cells. Twenty-four hours later, preparations were randomly divided into one of four treatment groups: sham-irradiated or γ-irradiated hippocampal neurons incubated in the presence or absence of the caspase-1 inhibitor, Ac-YVAD-CMK. In the case of those to be treated with caspase-1 inhibitor, neurons were pretreated for 1 h with Ac-YVAD-CMK (100 nM; Calbiochem). Cells that were assigned to the irradiated group underwent γ-irradiation of 20 Gy at a rate of 10 Gy/min in a Nordion Gammacell cesium^{137} irradiator. The following day, cells were fixed with 4% paraformaldehyde in TBS and stored at 4 °C in TBS until required for immunocytochemistry. In a separate set of experiments, neurons were preincubated with the specific JNK inhibitor DJNKI1 (1 μM; Alexis) for 1 h prior to γ-irradiation and processed as above.

Immunocytochemistry and TUNEL Staining of Hippocampal Neurons—Sham- and γ-irradiated hippocampal neuronal cultures, which were pretreated with either Ac-YVAD-CMK (100 nM) or DJNKI1 (1 μM), were immunostained for either p-JNK or anti-active caspase-3. Cells were permeabilized in 0.1% Triton X-100 and 20 μg/ml proteinase K in TBS for 10 min and then washed in TBS. Non-reactive sites were blocked with TBS containing 10% normal goat serum (Vector, UK) and incubated for 2 h at room temperature. Cells were washed and incubated overnight at 4 °C in primary antibody; p-JNK (1:150; Santa Cruz Biotechnology) or anti-active caspase-3 antibody (1:150; Promega) di-

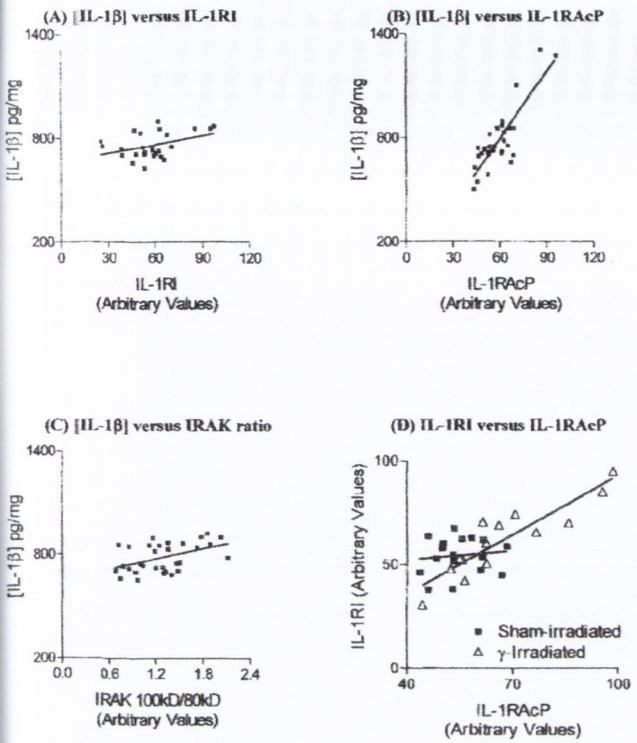


FIG. 2. **IL-1β concentration and elements of the signaling pathway are positively correlated.** There was a significant positive correlation between IL-1β concentration (pg/mg) and IL-1RI expression (*A*; $p < 0.04$; $r^2 = 0.16$), IL-1RAcP expression (*B*; $p < 0.0001$; $r^2 = 0.71$), and IRAK activity (*C*; $p < 0.01$; $r^2 = 0.21$). The γ-irradiation enhancement of IL-1RI and IL-1RAcP expression was also significantly correlated (*D*; $p < 0.001$; $r^2 = 0.81$, *open triangles*), although this was not the case when the sham-irradiated groups were assessed (*filled squares*).

luted in 2.5% normal goat serum in TBS. Endogenous peroxidases were blocked by immersion in 0.3% hydrogen peroxide for 10 min at room temperature, and cells were washed in TBS. Cells were incubated for 2 h at room temperature in secondary antibody; biotinylated anti-mouse IgG in the case of p-JNK (1:150; Vector Laboratories) and biotinylated anti-rabbit IgG (1:150; BIOSOURCE) for anti-active caspase-3. In all cases, cells were reacted with streptavidin-conjugated horseradish peroxidase (1:50 in TBS) for 30 min, and staining was developed by addition of the chromogen 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2 for 8 min. Cells were rinsed, counterstained with 3% methyl green, dehydrated, and mounted. Approximately 400 cells were counted per slide (30–50 cells/field; 8 fields/slide) at ×100 magnification, and six slides were analyzed per experimental condition. Positively immunostained cells were expressed as a percentage of the total number of cells counted.

In the case of p-JNK immunostaining a further distinction was made within the JNK-positive group. In some cells, JNK immunostained in a punctate manner and was localized throughout the cell. A second population of JNK-positive cells was also evident. These appeared smaller in morphology and were more densely stained, which was consistent with apoptotic changes (condensed nuclei and shrunken cell body).

Negative controls in which p-JNK and anti-active caspase-3 antibodies were heat-inactivated (*i.e.* boiling both antibodies for 10 min prior to primary antibody incubation) were conducted in parallel in each experiment. In addition, the primary antibody was omitted, and cells were incubated with TBS alone; this constituted another negative control.

Hippocampal neurons were also assessed for DNA fragmentation by TUNEL labeling. The protocol was identical to that described above, for acutely dissociated cells.

Statistical Analysis—Data are expressed as the mean ± S.E. A one-way analysis of variance (ANOVA) was performed to determine whether there were significant differences between conditions. When this analysis indicated significance (at the 0.05 level), *post hoc* Student Newman-Keuls test analysis was used to determine which conditions were significantly different from each other (Prism, GraphPad Software).

RESULTS

Fig. 1*A* shows that IL-1β concentration was similar in hippocampal tissue prepared from the three groups of sham-irradiated rats. Exposure to irradiation significantly enhanced IL-1β concentration in rats that received control diet by over 50% ($p < 0.05$; ANOVA, compared with sham-irradiated rats that received control diet). A preliminary time-course study demonstrated that, when the concentration of IL-1β was assessed 1, 2, and 4 days after γ-irradiation, the levels increased significantly with time.[2] Treatment with EPA prevented the irradiation-induced increase in IL-1β concentration. These findings were paralleled by changes in expression of membrane-associated IL-1RI (Fig. 1*B*). Thus receptor expression was similar in hippocampal tissue prepared from the three groups of sham-irradiated rats, and although exposure to irradiation significantly increased IL-1RI in hippocampus of rats that received control diet ($p < 0.05$; ANOVA), there was no significant change in irradiated rats that were treated with EPA. Consistent with the developing theme that irradiation up-regulated IL-1β-associated signaling is the finding that IL-1RAcP expression, which was similar in hippocampus of all sham-irradiated rats, was significantly enhanced in tissue prepared from irradiated rats that were maintained on the control diet (Fig. 1*C*; $p < 0.001$; ANOVA). Analysis of IRAK (80 and 100 kDa) revealed that irradiation enhanced the expression of the phosphorylated (100 kDa) form but did not alter expression of the unphosphorylated (80 kDa) form. Fig. 1*D* presents the data obtained from analysis of the ratio of 100- to 80-kDa IRAK. The data demonstrate that the mean ratio was enhanced in hippocampal tissue prepared from rats that were exposed to irradiation (although this did not reach statistical significance; $p < 0.11$) but that treatment with EPA attenuated this change.

That increased IL-1β concentration is positively correlated with components of the IL-1β signaling pathway is illustrated in Fig. 2. A significant correlation exists between IL-1β concentration and IL-1RI expression (*A*; $p < 0.04$; $r^2 = 0.16$), IL-1RAcP expression (*B*; $p < 0.0001$; $r^2 = 0.71$) and IRAK activity (*C*; $p < 0.01$; $r^2 = 0.21$). In addition, γ-irradiation enhanced the interaction between IL-1RI and IL-1RAcP, whereas the correlation between these measures was not statistically significant when data from sham-irradiated animals was assessed, there was a significant positive correlation in data obtained from irradiated rats (Fig. 2*D*; $p < 0.001$; $r^2 = 0.81$).

Previous reports have indicated that activation of JNK accompanies phosphorylation of IRAK and the data presented demonstrate parallel irradiation-induced responses in activity of both kinases. Fig. 3*A* shows that expression of phosphorylated JNK was similar in hippocampus of the 3 sham-irradiated groups but that it was significantly enhanced in tissue prepared from irradiated rats that were maintained on the control diet ($p < 0.001$; ANOVA). This irradiation-induced change was absent in tissue prepared from irradiated rats that were treated with EPA. Expression of total JNK was similar in all treatment groups (Fig. 3*B*). When JNK activity was assessed in a group of rats that received a higher irradiation dose (20 Gy), expression of p-JNK was increased in a dose-dependent manner (Fig. 3*C*; $p < 0.001$; ANOVA).

In contrast to the change in JNK phosphorylation, the evidence suggests that p38 phosphorylation was not significantly enhanced by irradiation (Fig. 3*D*). No effect of EPA was observed in either sham-irradiated or irradiated rats. Fig. 3*E* shows that total p38 expression was also similar in all treatment groups.

[2] A. M. Lynch, M. Moore, S. Craig, P. E. Lonergan, D. S. Martin, and M. A. Lynch, unpublished observation.

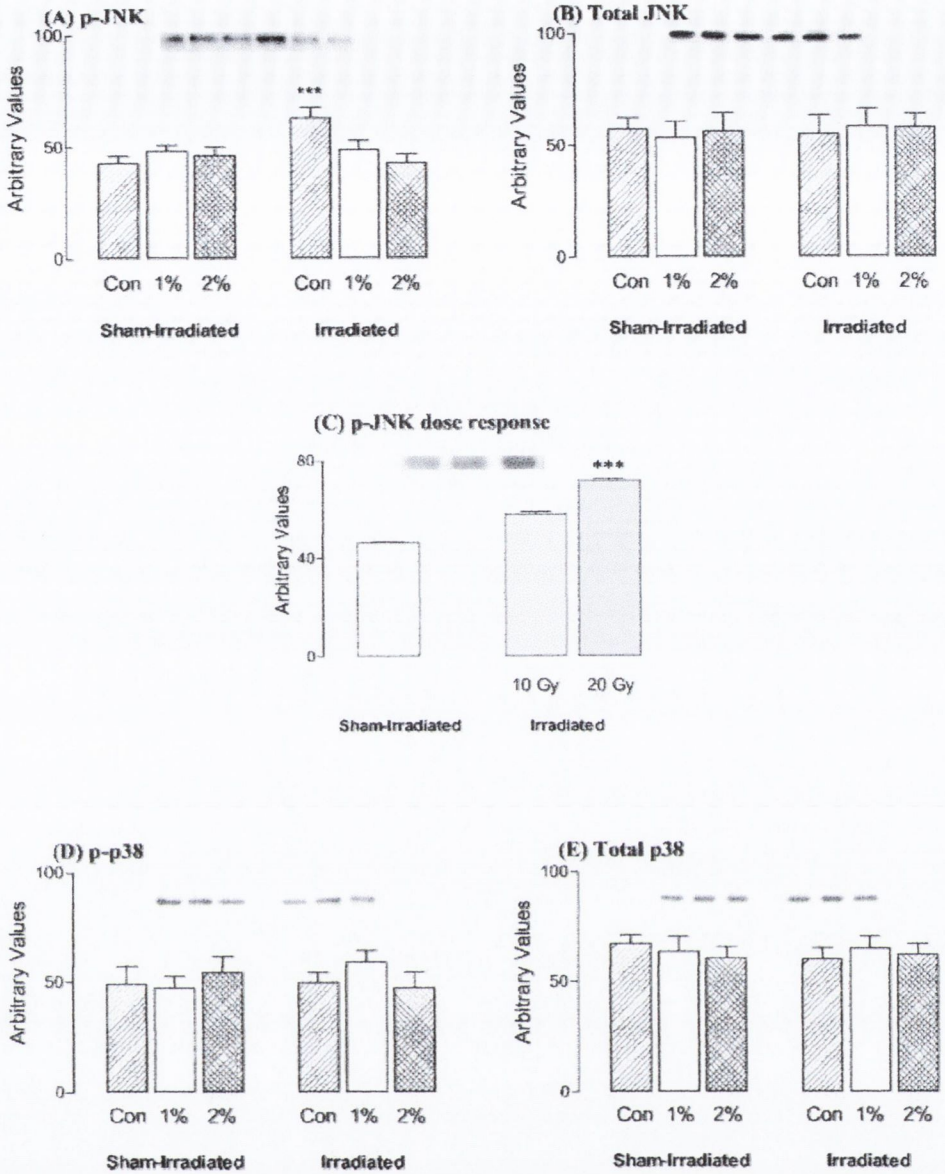


FIG. 3. **EPA abolishes the irradiation-induced increase in JNK activity.** p-JNK expression was significantly increased in hippocampus of irradiated rats on control diet (*A*; $p < 0.001$; ANOVA), but this activity was decreased to that of sham-irradiated levels when irradiated rats received EPA. However, there was no difference in total JNK levels across all groups (*B*). When p-JNK activity was assessed at different γ-irradiation doses, its expression was significantly increased in a dose-dependent manner (*C*; $p < 0.001$; ANOVA). In contrast, p38 activation was not altered by irradiation (*D*), and a similar pattern was evident for total p38 levels (*E*). The histograms represent the means (± S.E.) of six observations; the data were calculated by densitometric analysis and are expressed as arbitrary values. In *A–E*, sample immunoblots reflect the results obtained from statistical analyses.

Exposure to irradiation resulted in a significant decrease in ERK phosphorylation; thus the mean value in hippocampus prepared from irradiated rats that received the control diet was significantly reduced compared with the corresponding value in the sham-irradiated group (Fig. 4*A*; $p < 0.05$; ANOVA). Although EPA treatment failed to affect ERK phosphorylation in tissue prepared from sham-irradiated rats, it prevented the irradiation-induced effect. Although protein loading was similar in all experiments, the data indicate that total ERK expression was also decreased in tissue prepared from irradiated rats maintained on the control diet (Fig. 4*B*; $p < 0.05$; ANOVA), and in parallel with its effect on ERK phosphorylation, EPA treatment reversed the irradiation-associated decrease in total ERK expression.

That activation of JNK leads to mitochondria membrane perturbation has been previously demonstrated (10, 21), and the data presented here is consistent with this. Fig. 5*A* shows that cytochrome *c* translocation was significantly increased in tissue prepared from rats that were exposed to irradiation ($p < 0.05$; ANOVA). Cytochrome *c* has been shown to activate caspase-3 (12), which in turn leads to cleavage of PARP. Fig. 5*B* shows that in parallel with the irradiation-induced increase in cytochrome *c* translocation, PARP cleavage was significantly enhanced ($p < 0.001$; ANOVA). Treatment with EPA did not affect either measure in sham-irradiated rats, but it prevented the effects of irradiation. In parallel with upstream markers of cell death, there was also evidence of increased DNA fragmentation in the irradiated group as assessed by TUNEL labeling (Fig. 5*C*; $p < 0.01$; ANOVA), and this was prevented by the EPA diet.

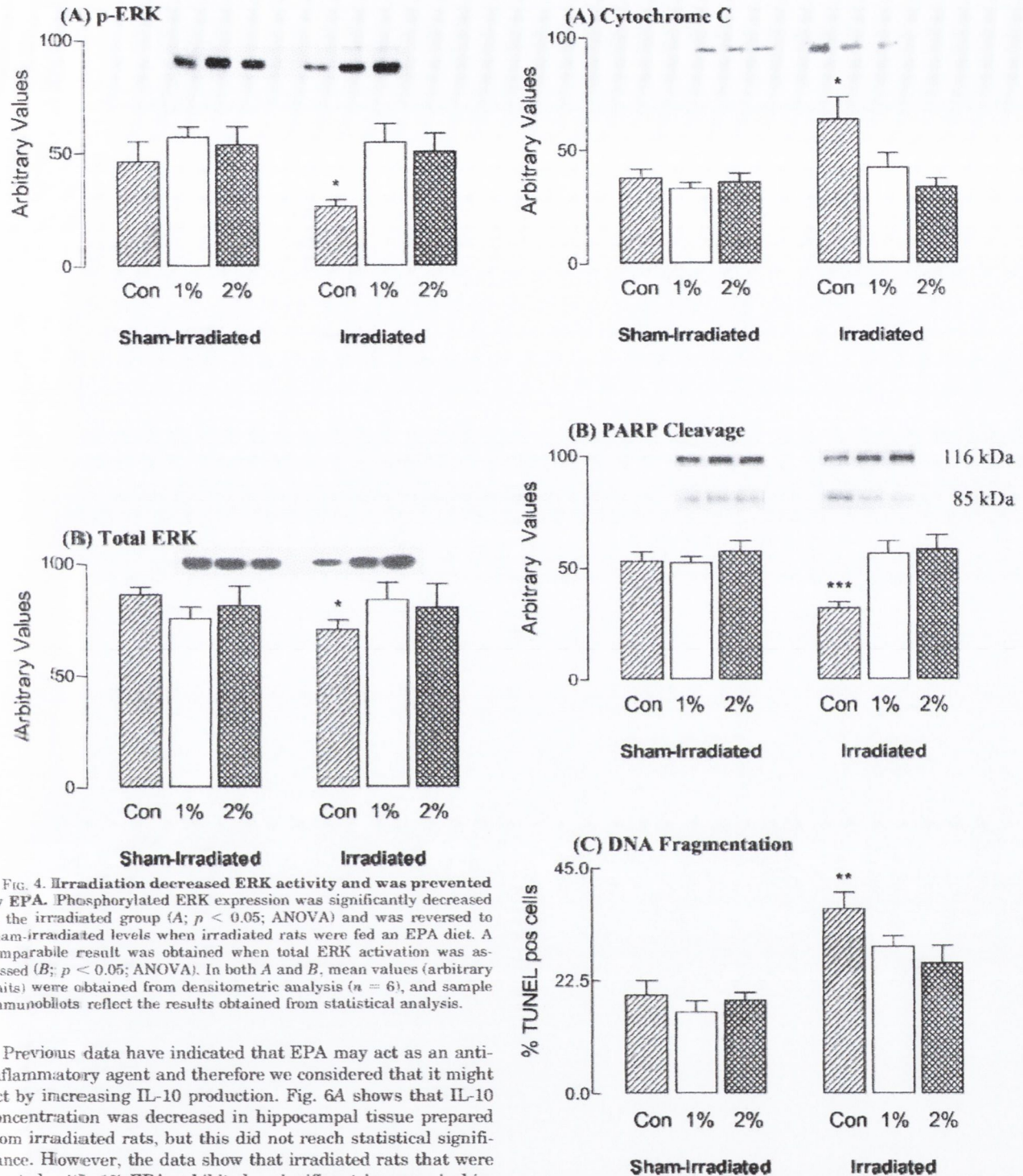


FIG. 4. **Irradiation decreased ERK activity and was prevented by EPA.** Phosphorylated ERK expression was significantly decreased in the irradiated group (*A*; $p < 0.05$; ANOVA) and was reversed to sham-irradiated levels when irradiated rats were fed an EPA diet. A comparable result was obtained when total ERK activation was assessed (*B*; $p < 0.05$; ANOVA). In both *A* and *B*, mean values (arbitrary units) were obtained from densitometric analysis ($n = 6$), and sample immunoblots reflect the results obtained from statistical analysis.

FIG. 5. **The irradiation-induced increase in markers of apoptosis is abolished by EPA.** Cytochrome *c* translocation (*A*; $p < 0.05$; ANOVA) and PARP cleavage (*B*; $p < 0.001$; ANOVA) were significantly increased in hippocampal tissue prepared from control-treated irradiated rats compared with sham-irradiated rats. In both cases, levels in EPA-treated irradiated rats were similar to that in the sham-irradiated groups, a result that is reflected in the sample immunoblots. Both the uncleaved (116 kDa) and cleaved (85 kDa) forms of PARP are represented by the immunoblots in *B*. Mean values (arbitrary units) were obtained from densitometric analysis ($n = 6$). In parallel, there was an irradiation-induced increase in fragmented DNA as assessed by TUNEL labeling (*C*; $p < 0.01$; ANOVA) and as with the other markers of apoptosis (*A* and *B*) dietary intervention with EPA significantly decreased the percentage of TUNEL-positive cells.

Previous data have indicated that EPA may act as an anti-inflammatory agent and therefore we considered that it might act by increasing IL-10 production. Fig. 6*A* shows that IL-10 concentration was decreased in hippocampal tissue prepared from irradiated rats, but this did not reach statistical significance. However, the data show that irradiated rats that were treated with 1% EPA exhibited a significant increase in hippocampal IL-10 concentration ($p < 0.01$; ANOVA). Fig. 6*B* demonstrates that irradiation significantly increases IL-1β release from cultured primary hippocampal neurons ($p < 0.001$; ANOVA), and this effect was prevented by pretreatment with IL-10 ($p < 0.01$). A significant inverse relationship is evident between the concentrations of IL-10 and IL-1β *in vivo* (Fig. 6*C*; $r^2 = 0.40$; $p < 0.01$).

To establish a sequential role for IL-1β and JNK in irradiation-induced apoptosis, γ-irradiated hippocampal neurons were assessed *in vitro* following pretreatment with either Ac-YVAD-CMK or D-JNKI1. Gamma-irradiation significantly increased

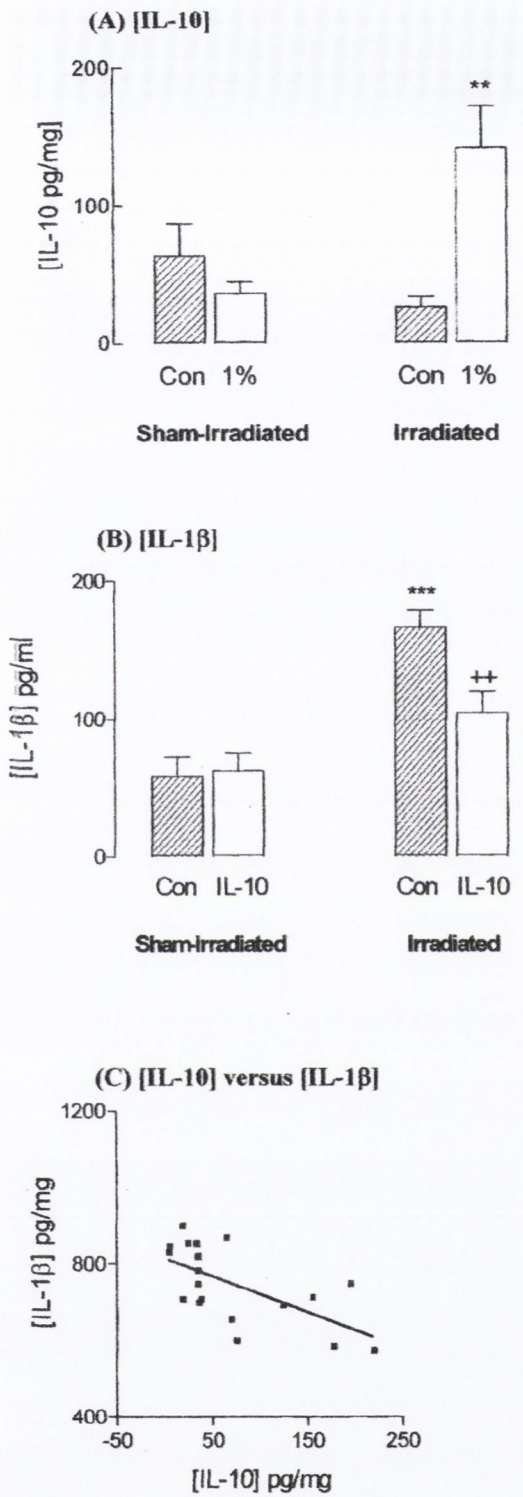


FIG. 6. **EPA induced a significant increase in IL-10 concentration.** Although IL-10 levels were decreased in the control-treated irradiated group, results did not reach statistical significance. However, in the 1% EPA-treated irradiated group, the concentration of IL-10 was significantly increased when compared with the sham-irradiated group (*A*; $p < 0.01$; ANOVA). The concentration of IL-1β released from primary hippocampal neurons was significantly increased following irradiation, when compared with the sham-irradiated group (*B*; ***, $p < 0.001$; ANOVA). Pretreatment with IL-10 (100 ng/ml) directly decreased the irradiation-induced increase in IL-1β (*B*; ++, $p < 0.01$; ANOVA). When *in vivo* concentrations of IL-1β and IL-10 were compared, a significant inverse correlation was revealed (*C*; $r^2 = 0.40$; $p < 0.01$).

the total number of JNK-positive cells (Fig. 7*A*; $p < 0.05$; ANOVA), and this increase was reversed by preincubation with the caspase-1 inhibitor Ac-YVAD-CMK indicating that IL-1β mediated the irradiation-induced increase in JNK immunostaining. Cells that were positive for p-JNK appeared to fall into two categories: those that were immunostained globally in a punctate manner and those that were smaller in morphology and more densely stained. The subpopulation of densely immunostained JNK-positive cells were also significantly increased in the irradiated group (Fig. 7*B*; $p < 0.01$; ANOVA), and the effect of irradiation was not observed in cells that were preincubated in the presence of Ac-YVAD-CMK. A micrograph represents both JNK-positive subtypes (Fig. 7*C*).

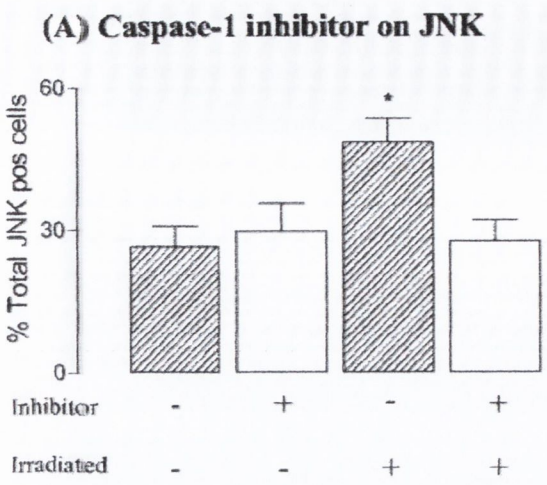
Two methods were used to evaluate apoptosis; neurons were assessed for anti-active caspase-3 immunostaining and by directly labeling fragmented DNA using TUNEL staining. Significantly, irradiation increased the numbers of caspase-3- and TUNEL-positive hippocampal cells (Figs. 8, *A–D*). Both indicators of apoptosis were significantly decreased in neurons that were incubated in the presence of Ac-YVAD-CMK, the caspase-1 inhibitor (Fig. 8*A*; $p < 0.001$, ANOVA and Fig. 8*C*; $p < 0.01$; ANOVA). Similar results were obtained in the irradiated neurons that were preincubated with DJNKI1 (Fig. 8*B*; $p < 0.01$, ANOVA and Fig. 8*D*; $p < 0.05$; ANOVA). Anti-active caspase-3 immunostaining is represented in Fig. 8*E* and TUNEL labeling in Fig. 8*F*.

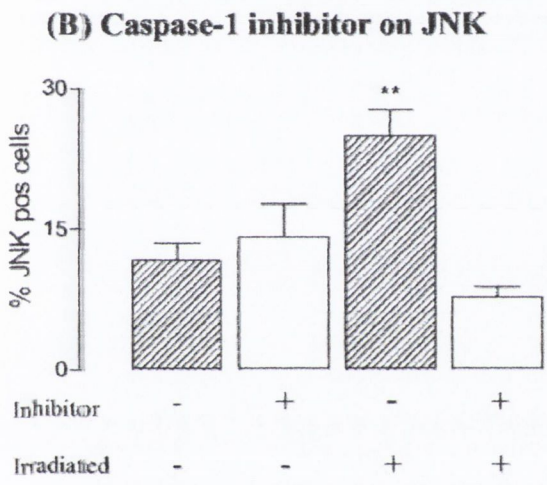
DISCUSSION

Here we demonstrate that exposure of rats to γ-irradiation resulted in an increase in IL-1β concentration and IL-1β-associated signaling events in hippocampus and that these changes were coupled with evidence of cell death. Significantly, the data show that treatment of rats with EPA completely abrogated the irradiation-induced increase in IL-1β and prevented cell death. EPA increased IL-10 concentration in hippocampus, and, because we have shown that the irradiation-induced increase in IL-1β is prevented by IL-10, we propose that this may be the key to the protective effect of EPA.

IL-1β concentration was significantly increased in hippocampal tissue prepared from irradiated, compared with sham-irradiated, rats; this is consistent with an earlier observation that indicated that irradiation increased expression of tumor necrosis factor α and IL-1β mRNA in brain tissue (18). A similar irradiation-induced increase in IL-1β concentration has been observed in a number of cells and tissues (22, 23), and increased circulating IL-1β concentration has been reported in patients following radiotherapy for brain tumors (24). In this study, EPA treatment prevented the irradiation-associated increase in IL-1β concentration and in other studies, EPA has been shown to have anti-inflammatory properties (25, 26). Specifically, it has been reported that EPA inhibits production of IL-1β by monocytes (27) and IL-6 by macrophages (28). In a recent study from this laboratory, we found that EPA treatment for 8 weeks attenuated the age-related increase in IL-1β concentration in hippocampus (6), whereas the lipopolysaccharide-induced increase in IL-1β concentration in hippocampus was inhibited by 4 weeks treatment with EPA (10). The mechanism by which this action occurs requires further elucidation, but it has been suggested that substitution of EPA for arachidonic acid, which would prevent cyclooxygenase-dependent production of prostaglandin E_2, may be a key element in blocking IL-1β formation (25).

There is a clear-cut correlation between IL-1β and elements of the IL-1β-induced signaling pathway as well as the fact that the interaction between IL-1RI and IL-1RAcP was potentiated by γ-irradiation. Data from three separate laboratories highlight an interesting parallel in keratinocytes: exposure of these





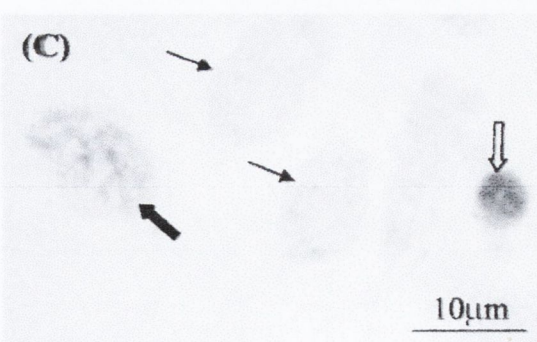


FIG. 7. **Inhibition of irradiation-induced IL-1β production decreased JNK activity *in vitro*.** Irradiation increased the total number of JNK-positive cells (*A*; $p < 0.05$; ANOVA) and when subdivided into two populations, those that were densely immunostained and apoptotic in morphology were also significantly increased in number (*B*; $p < 0.01$; ANOVA). When irradiated hippocampal cells were pretreated with Ac-YWAD-CMK (the caspase-1 inhibitor), the total number of JNK-positive cells was decreased (*A*) as well as the more densely stained population (*B*). At least 400 cells were counted at 100× magnification in each experimental group and there were $n = 6$ per group. A micrograph (*C*) represents the various cell populations analyzed and comprise negatively stained cells (*narrow arrows*), a globally immunostained JNK-positive cell (*filled arrow*), and a small densely stained JNK-positive cell (*open arrow*).

cells to UV irradiation has been reported to increase IL-1β concentration (22), IL-1RI expression (29), and IRAK phosphorylation (30). The likely outcome of these changes is up-regulation of IL-1β-induced signaling predicting increases in phosphorylation of JNK and p38 as previously described (3, 5). We found that activation of JNK, but not p38, was increased in tissue prepared from irradiated rats, which is consistent with the *in vitro* evidence presented here, as well as previous findings indicating that JNK phosphorylation in cultured cells was stimulated by exposure to irradiation (31). Significantly, the present data indicate that treatment with EPA attenuated these irradiation-induced increases.

Here we report that in addition to preventing the irradiation-induced increases in IL-1β signaling, EPA increases hippocampal IL-10 concentration; indeed there is an inverse correlation between hippocampal concentrations of IL-10 and IL-1β. The finding that EPA increases IL-10 has been confirmed in a recent study in which we observed that EPA abrogated the inhibitory effect of lipopolysaccharide on LTP and that this was accompanied by an EPA-induced increase in IL-10.[3] Significantly, we also found that IL-10 mimicked the effect of EPA by blocking the lipopolysaccharide-induced deficit in LTP (32). These data support previous observations indicating that supplementation of infant formula with docosahexaneoic acid, of which EPA is precursor, resulted in increased the proportion of CD4-positive T cells and, concomitantly increased IL-10 production (33). Similarly it has been shown that the beneficial effects of EPA in experimental acute pancreatitis are coupled with enhanced production of IL-10 (34).

The question of the mechanism of action of IL-10 arises, and here we show that it prevents the irradiation-induced increase in IL-1β in hippocampal neurons. Interestingly, it has also been shown that IL-10 down-regulates IL-1 mRNA in splenocytes (35) and IL-1 production in synovial tissue cells (36). That IL-10 prevents IL-1β-induced cell signaling events is probably as a consequence of its ability to decrease membrane expression of IL-1RI, as previously reported (37).

JNK activation triggers apoptosis in a variety of cultured cells (38); it has been proposed that phosphorylation of the mitochondrial membrane protein bcl-2 by JNK may be a significant event in induction of apoptosis. Bcl-2 phosphorylation results in its inactivation (39, 40), which leads to loss of mitochondrial membrane integrity and translocation of mitochondrial enzymes like cytochrome *c* to the cytosol (41). Here, we show both *in vivo* and *in vitro* that JNK activity was increased by irradiation. This was associated with increased cytochrome *c* translocation *in vivo* and increased activation of caspase-3 here, as well as in several studies (42, 43). The irradiation-induced increase in cytochrome *c* translocation, together with increased cleavage of the caspase-3 substrate, PARP, and increased TUNEL-labeling *in vivo* and *in vitro* are considered to be reliable indicators of apoptosis (44, 45). Taken together the data suggest that exposure of rats to γ-irradiation leads to apoptosis in hippocampus, providing an explanation for the marked cell loss observed in hippocampus following irradiation (46). Among the important findings of this study is the observation that the irradiation-induced increase in JNK activation is dependent on IL-1β, because it is blocked by inhibition of

[3] T. Kavanagh and M. A. Lynch, unpublished observation.

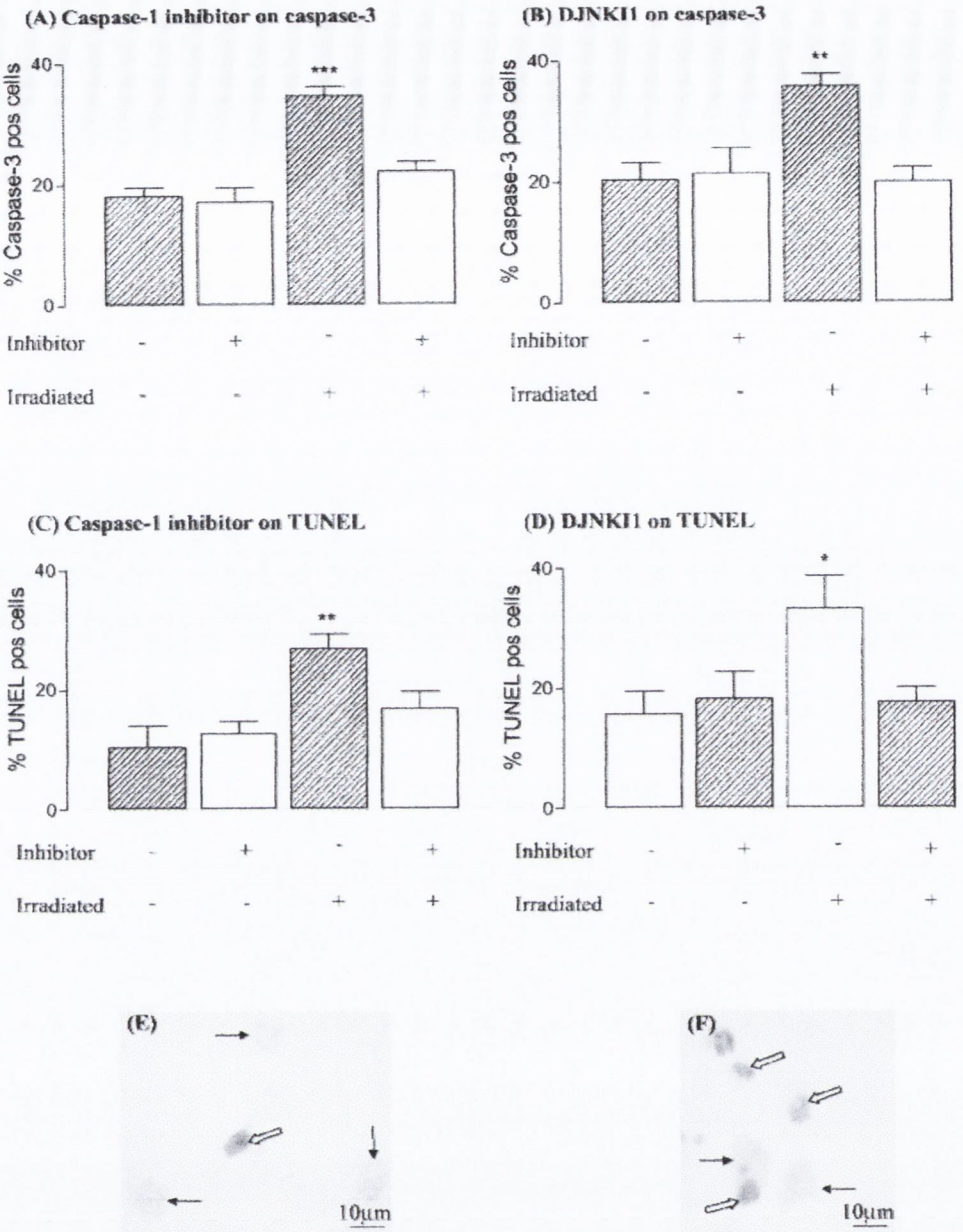


FIG. 8. **Inhibition of both IL-1β production and JNK activity decreased the irradiation-induced increase in markers of apoptosis.** The number of caspase-3- and TUNEL-positive cells was significantly increased in hippocampal cells, which were irradiated (*A*, $p < 0.001$; *B* and *C*, $p < 0.01$; *D*, $p < 0.05$; ANOVA). Irradiation-induced caspase-3 immunostaining was decreased in either the presence of the caspase-1 inhibitor, Ac-YVAD)-CMK (*A*) or DJNKI1 (*B*) the specific JNK inhibitor. Similarly, both Ac-YVAD-CMK and DJNKI1 significantly reduced the number of irradiation-induced TUNEL-positive cells (*C* and *D*, respectively). *Bar graphs* represent the counts obtained from at least 400 cells that were counted at ×100 magnification ($n = 6$/group). *Panel E* illustrates one caspase-3-positive cell (*open arrow*) surrounded by three negatively stained cells (*narrow arrow*). TUNEL-positive cells (*open arrows*) are represented in *panel F*; negatively stained cells are also shown (*narrow arrows*).

casapse-1, and that irradiation-induced apoptosis is mediated by IL-1β and JNK activation. EPA treatment inhibited these irradiation-induced changes, and the significance of this finding is 2-fold: first it suggests an interdependence of these factors and, second, it identifies a neuroprotective effect of EPA.

Irradiation decreased activation of ERK, which is generally considered to be a survival factor (47), and it is therefore possible that this contributed to the irradiation-induced apoptosis. It is of interest that EPA treatment reversed the irradiation-induced decrease in ERK activation. These observations in hippocampus therefore mirror the changes observed in Jurkat T cells in which apoptotic changes were linked with activation of JNK, but not activation of either p42 mitogen-activated protein kinase or p38 (48).

The question of the mechanism underlying the protective effect of EPA remains to be addressed. The present data indicate that EPA enhanced IL-10 concentration in hippocampus of rats that were exposed to irradiation. IL-10 is a potent anti-inflammatory cytokine, which we have shown abrogates irradiation-induced IL-1β release in hippocampal neurons. Additionally, IL-10 has been shown to inhibit the IL-1β-induced increase in activation of IRAK and JNK (37), and it suppresses the effects of IL-1β on LTP (37), behavior (49), and fever (50). We propose that the ability of EPA to

increase IL-10 expression may significantly contribute to the protective effect of EPA.

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Evidence that vitamin D_3 reverses age-related inflammatory changes in the rat hippocampus

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Abstract

One of the major challenges in neuroscience is to identify the changes which accompany aging and which contribute to the well-documented age-related deterioration in cognitive function. This is a particular challenge in the light of the vast array of reported changes, which include morphological changes like synaptic and perhaps cell loss, alteration in membrane composition and the resultant changes in function of membrane proteins, modulation of the hypothalamo–pituitary axis, impaired calcium homoeostatic mechanisms, alteration in enzyme function and decreased neurotransmitter release. In the past few years, evidence suggesting that an aged brain exhibits signs of oxidative stress and inflammatory stress has been accumulating, and recent evidence using microarray analysis has added support to this view. In this paper, we provide evidence to suggest that vitamin D_3 acts as an anti-inflammatory agent and reverses the age-related increase in microglial activation and the accompanying increase in IL-1β (interleukin-1β) concentration.

Increased IL-1β (interleukin-1β) concentration in hippocampus is associated with deficits in synaptic function

Cognitive deficits in aged rats, particularly deficits in spatial information processing, have been consistently reported by several groups for many years; thus it has been demonstrated that spatial learning, for example using the Morris Water maze, is reduced in aged rats [1]. Correlated with deficits in performance in spatial learning is a deficit in LTP (long-term potentiation) in hippocampus, a form of synaptic plasticity which is considered to be a biological substrate of learning and/or memory [2]. Among the changes which correlate with the age-related impairment in LTP is an increase in accumulation of ROS (reactive oxygen species) in hippocampus [3,4] which is indicative of oxidative stress, and a great deal of evidence accumulated in the past few years has coupled this with changes which are indicative of inflammation. These are characterized by an increase in hippocampal concentration of the pro-inflammatory cytokine IL-1β [5], which has been shown to be inversely correlated with the ability of rats to sustain LTP [6]. Significantly, an increase in hippocampal IL-1β concentration has been reported in rats treated with LPS (lipopolysaccharide) or Aβ (amyloid β) protein, and in rats exposed to irradiation as well as aged rats [7–10]. In each of these experimental conditions, LTP is decreased and IL-1β-induced cell signalling is up-regulated. Evidence indicates that IL-1β-induced activation of JNK (c-Jun N-terminal kinase) significantly contributes to the LPS- and Aβ-associated deficit in LTP since treatment of rats with the peptide inhibitor of JNK, D-JNKI1, suppresses the inhibitory effects of both on LTP [7,11]. This is supported by the findings of a number of studies which have revealed that when the age-related increase in IL-1β concentration is suppressed, and consequently when LTP is restored, the accompanying increase in activation of JNK is reversed. For example, treatment of rats with the polyunsaturated fatty acid, eicosapentaenoic acid which possesses anti-inflammatory properties, has been shown to abrogate the age-related, irradiation-induced and LPS-induced changes in IL-1β concentration and LTP and the associated change in JNK activation [8,10,12]. Similarly, treatment of rats with phosphatidylserine-bearing liposomes, which suppressed the LPS-induced increase in IL-1β concentration, also blocked the associated increase in JNK activation [13].

What is the cell source of IL-1β?

It is generally accepted that the primary cell source of IL-1β is activated microglia [7,14,15] and therefore, it might be predicted that microglial activation will accompany the age-related increase in hippocampal IL-1β concentration. However, although several studies have reported increased microglial activation in neurodegenerative diseases like AD (Alzheimer's disease) [16] and in animal models of AD [17], the evidence indicating an increase in activated microglia in the aged brain is equivocal. Whereas an increase has been reported in primates [18], there are conflicting reports relating to changes in aged rodents, with increased microglial activation in rat hippocampus being reported by some groups [19] but not others [20].

Key words: age, hippocampus, inflammation, interleukin-10 (IL-10), IL-1β, microglial activation.
Abbreviations used: AD, Alzheimer's disease; IFNγ, interferon-γ; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LTP, long-term potentiation; MHCII, major histocompatability complex II; PARP, poly(ADP-ribose) polymerase.
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IL-10 concentration is decreased in the hippocampus of the aged rat

Associated with the age-related increase in hippocampal concentration of IL-1β is an age-related decrease in the concentration of the anti-inflammatory cytokine, IL-10. IL-10, like IL-1β, is a pleiotropic cytokine that regulates a variety of functions of haemapoietic and non-haemapoietic cells [21], and its receptor is expressed on a wide range of cells including neurons [22]. Significant expression of the receptor has been observed in several brain areas [23], which explains the effect of IL-10 on central functions; for example, IL-10 has been shown to reverse the IL-1-induced fever which follows LPS injection [24], and to antagonize the effect of IL-1β on sleep [25]. At the level of the hippocampus, where receptors have also been identified, IL-10 has been shown to attenuate the effects of traumatic brain injury which have been attributed to an increase in IL-1β, and this was associated with a decreased concentration of IL-1 in the hippocampus [26]. The evidence indicates that IL-10 inhibits the actions of IL-1β, at least in some cases by inhibiting IL-1β production and/or release (see [27]), and this effect has been observed in the hippocampus, where it also decreases IL-1RI expression and IL-1β-induced cell signalling [28]. Whereas LPS has been shown to increase hippocampal concentration of IL-1β mRNA and protein, IL-10 antagonizes these effects, and the inhibition of LTP induced by treatment of rats with IL-1β or LPS has been shown to be abrogated by IL-10 [28]. In some cells, IL-10 may exert its actions by increasing the expression of IL-1 receptor antagonist production, for example in LPS-stimulated human polymorphonuclear leucocytes [29], but a similar effect in hippocampus has not been observed [28]. The findings of some studies have suggested that a significant component of the anti-inflammatory effect of IL-10 may be linked with its ability to modulate microglial function and to inhibit the subsequent cytokine production by microglia [30,31]. Consistently, IFNγ (interferon-γ)-induced activation of microglia has been shown to be down-regulated by IL-10 [30].

Evidence that vitamin D_3 possesses anti-inflammatory properties

It has been established that the role of vitamin D_3 extends beyond its traditional role in bone metabolism [32] and is capable of modulating immune function by acting on its receptor, the vitamin D receptor [32,33], which is expressed on several cell types, especially cells of the immune system [34]. Vitamin D_3 has been shown to reduce the inflammatory changes associated with delayed hypersensitivity, inflammatory bowel disease and experimental encephalomyelitis, and this has been attributed to the fact that the anti-inflammatory cytokines, transforming growth factor and IL-4, were increased by vitamin D_3, while the pro-inflammatory cytokines, IFNγ and tumour necrosis factor-α, were reduced [33,34]. On the basis of these findings it might be predicted that the anti-inflammatory effects of vitamin D_3 may extend to the hippocampus and therefore that some of the inflammatory changes induced by age in the hippocampus might be attenuated by treatment of aged rats with vitamin D_3. To assess this, young and aged rats were subdivided into two groups, an experimental group which received vitamin D_3 in their diet for 2 weeks and a control group which received standard laboratory chow. At the end of the 2 week treatment period, hippocampal tissue was analysed for evidence of microglial activation using phenotypic and functional markers. For phenotypic markers, tissue was assessed for expression of two markers of microglial activation, CD11b and MHCII (major histocompatability complex II), and IL-1β concentration was assessed as a functional marker of microglial activation. We also assessed hippocampal concentration of IL-10 in an effort to establish whether vitamin D_3 might exert its putative anti-inflammatory effect by increasing IL-10 concentration.

Evidence of age-related increase in microglial activation: effect of vitamin D_3

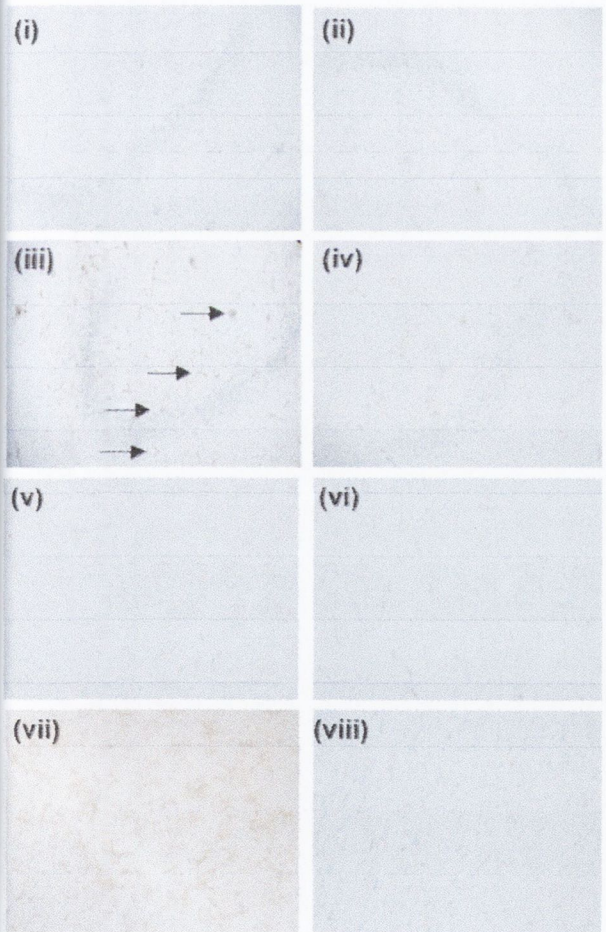
The data presented in Figure 1 indicate that the number of cells expressing MHCII was markedly increased in sections prepared from aged (iii) compared with young (i) control-treated rats and that, while vitamin D_3 treatment exerted no significant effect in young rats (ii), it reduced the age-related increase in the number of cells that expressed MHCII (iv). In a striking parallel, the data also indicate that CD11b expression was increased in aged (vii), compared with young (v), control-treated rats, whereas CD11b expression was decreased in aged rats that received vitamin D_3 (viii); no marked effect of treatment was observed in young rats (vi). These data indicate that, on the basis of phenotype, there is an age-related increase in microglial activation, and that vitamin D_3 treatment abrogated the age-related change.

Effect of vitamin D_3 treatment on the age-related increase in IL-1β concentration

One functional marker of increased activation of microglia is the consequent increase in IL-1β, and in an effort to confirm the phenotypic data, the hippocampal concentration of IL-1β was assessed in tissue prepared from aged and young control- and vitamin D_3-treated rats. The data indicate that mean hippocampal IL-1β concentration was significantly increased in tissue prepared from aged, compared with young, rats ($P < 0.05$; ANOVA: Figure 2(a), while the concentration of IL-1β was similar in tissue prepared from young rats and aged vitamin D_3-treated rats, and significantly lower in tissue prepared from aged vitamin D_3-treated, compared with aged control-treated rats ($P < 0.01$; ANOVA). It has been shown that increased IL-1β concentration in hippocampus is accompanied by a number of markers of deterioration in cell function. One of these markers is an increase in cleavage of DNA repair enzyme, PARP [poly(ADP-ribose) polymerase]. Here, we assessed expression of the cleaved (85 kDa) form of PARP using Western immunoblotting and show that there was a significant age-related increase in expression of 85 kDa PARP ($P < 0.01$; ANOVA; Figure 2b),

Figure 1 | Vitamin D_3 attenuates the age-related increase in microglial activation in hippocampus

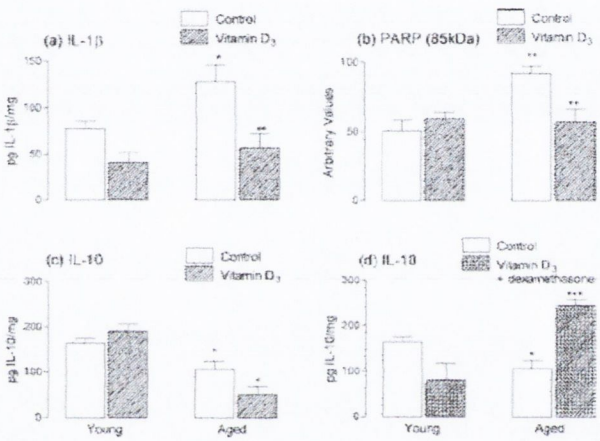
Young and aged rats were randomly divided into control and experimental groups; control animals were given laboratory chow and plain water while experimental animals were given vitamin D_3 (0.1 μg/ml) (in some cases in combination with 1 μg/ml dexamethasone) in their drinking water for 2 weeks. Rats were then killed by decapitation and cryostat sections were prepared for analysis of expression of CD11b or MHCII (as a measure of microglial activation) by fixing sections in ice-cold 100% ethanol, washing with PBS, blocking with 10% (v/v) normal goat serum/4% (w/v) BSA in PBS and treating overnight in a humidified chamber at 4°C with anti-CD11b antibody (1:100; OX-42, Serotec, Kidlington, Oxford, U.K.) or with OX-6 antibody, which assesses MHCII (1:100 in TBS containing 2% BSA; Serotec). Sections were washed, incubated with biotinylated anti-mouse IgG (1:200; Vector Laboratories, Peterborough, U.K.) and then incubated with 0.3% H_2O_2 in PBS to block endogenous peroxidases [13]. Hippocampal sections prepared from aged control-treated rats displayed increased OX-6 positive staining (iii) and CD11b positive staining (vii) compared with control-treated (i) and (v) or vitamin D_3-treated (ii) and (vi) young rats; treatment with vitamin D_3 blocked the age-related effect (iv) and (viii).



which was completely abrogated in tissue prepared from vitamin D_3-treated rats. Thus expression in this tissue was similar to that in tissue prepared from young rats and it

Figure 2 | Vitamin D_3 attenuates age-related changes in hippocampal tissue

The concentrations of IL-1β and IL-10, and PARP cleavage were assessed in hippocampal homogenate by ELISA and Western immunoblotting respectively as previously described [28]. Mean IL-1β concentration (**a**) and 85 kDa PARP expression (**b**) were significantly increased in hippocampal tissue prepared from control-treated aged, compared with young, rats ($^{*}P < 0.05$, $^{**}P < 0.01$; ANOVA); treatment with vitamin D_3 (0.1 μg/ml) attenuated this age-related change. Mean IL-10 concentration was significantly decreased in tissue prepared from control-treated aged rats compared with young rats ($^{*}P < 0.05$; ANOVA; **c** and **d**); treatment with vitamin D_3 alone did not induce any change (**c**) but, in combination with dexamethasone (1 μg/ml in drinking water; **d**) it completely reversed the change so that mean IL-10 concentration in tissue prepared from treated aged rats was significantly increased compared with that in control-treated aged rats ($^{***}P < 0.001$; ANOVA). Data are expressed as means ± S.E.M for six estimations.



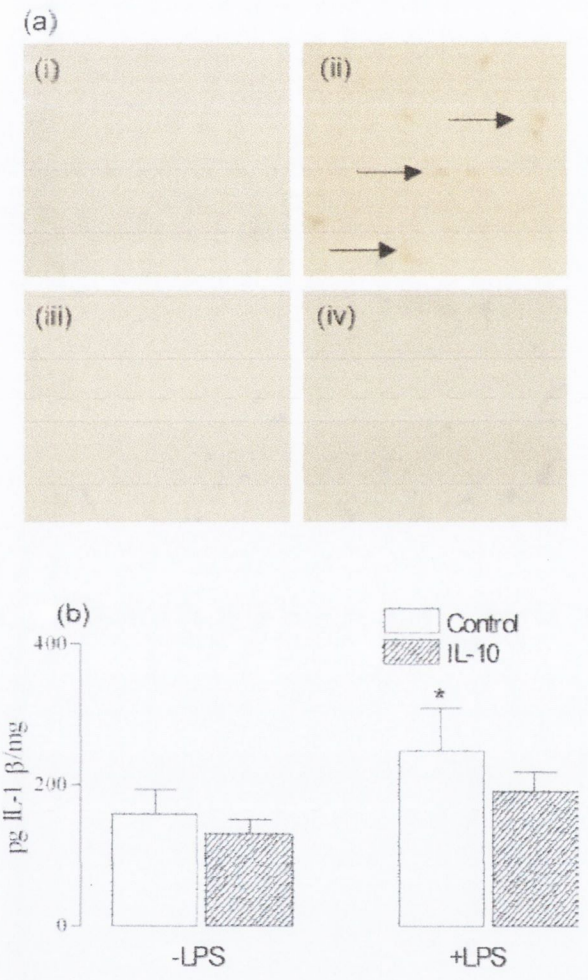
was significantly reduced compared with that in tissue prepared from control-treated aged rats ($P < 0.01$; ANOVA).

Does the anti-inflammatory effect of vitamin D_3 extend beyond its effect on IL-1β?

These data suggest that vitamin D_3 exerts anti-inflammatory effects and in an effort to establish whether this is a consequence of increased hippocampal concentration of IL-10, cytokine concentration was assessed in aliquots of the same tissue in which IL-1β concentration was assessed. Figure 2(c) indicates that mean IL-10 concentration was decreased in hippocampal tissue prepared from aged, compared with young, rats ($P < 0.05$; ANOVA), but that treatment of rats with vitamin D_3 failed to exert any significant effect in aged or young rats, therefore, the significant age-related decrease persisted in this treatment group ($P < 0.05$; ANOVA). In contrast with this, treatment of aged rats with a combination of vitamin D_3 and the synthetic glucocorticoid, dexamethasone, which has been shown to increase IL-10 concentration in peripheral tissues [35], reversed the age-related decrease in IL-10 concentration restoring it to that observed

Figure 3 | The LPS-induced increases in microglial activation and IL-1β are blocked by IL-10

Cultured cortical glia were incubated in the absence or presence of LPS (100 ng/ml in neurobasal medium) with or without a 1 h pre-treatment with IL-10 (100 ng/ml in neurobasal medium). Supernatant was removed and used for analysis of IL-1β concentration and cells were fixed and analysed for expression of MHCII using OX-6 staining; details of the methodology have been published [13]. (**a**) shows that LPS markedly increased the number of OX-6-positive cells [compare (ii) (LPS-treated cells) with (i) (control-treated cells)] and IL-10, which exerted no effect (iii), abrogated the LPS-induced change (iv). (**b**) Similarly, the LPS-induced increase in IL-1β ($P < 0.05$; ANOVA) was suppressed by treating cells with IL-10.



in hippocampal tissue prepared from young rats. These findings are similar to those which have been reported in peripheral tissues. While vitamin D_3 has been shown to inhibit pro-inflammatory cytokine production [36], the combination of dexamethasone and vitamin D_3 can induce naive T-cells to differentiate into IL-10-producing T regulatory cells [35,37] and to induce maturation of dendritic cells which in turn secrete IL-10 [38].

We argued that, if the increase in production of IL-1β is dependent on the presence of activated microglia then parallel changes in microglial activation and increased IL-1β concentration must be predicted. To examine this prediction and to further explore the potential of IL-10 to modulate microglial activation, we assessed the effect of LPS, in the presence and absence of IL-10, on MHCII expression and IL-1β concentration in cultured cortical glial cells. Cells were prepared from 1-day old Wistar rats and treated with LPS in the presence or absence of IL-10; supernatant was collected for analysis of IL-1β and cells were stained for evidence of MHCII expression. Figure 3(a) indicates that the number of cells which stained positively for MHCII was markedly increased in LPS-treated (ii) cultures compared with control (i), and IL-10 abrogated the LPS-induced upregulation of MHCII expression (iv), while IL-10 alone exerted no marked effect (iii). These data provide evidence based on phenotype, that microglial activation was increased and suggest that IL-10 abrogates the LPS-induced change. In parallel with this, and consistent with the idea that IL-10 modulates microglial activation, is the finding that the significant LPS-induced increase in IL-1β ($P < 0.05$; ANOVA; Figure 3b) was also inhibited by treatment of cells with IL-10. The data presented here indicate that IL-10 exerts a neuroprotective effect, which is consistent with our earlier observations which demonstrated that IL-10 antagonized the LPS-induced up-regulation of IL-1RI, IL-1β mRNA and protein and IL-1β-stimulated cell signalling [9,28]. The present findings suggest that these effects may be secondary to the modulatory effect of IL-10 on microglial function.

An anti-inflammatory role for vitamin D_3 has been recognized for some time and it has been specifically identified as exerting protection in conditions which are associated with polarization of T-cell responses to the Th1 phenotype [33,34]. In this paper, we have highlighted the fact that the modulatory effects of vitamin D_3 extend to the hippocampus and specifically that treatment of aged rats with vitamin D_3 attenuates the age-related up-regulation of inflammatory cytokines coupled with down-regulation of anti-inflammatory cytokines, which we have consistently shown to be associated with impairment in cognitive function.

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