Does IL-1F5 have anti-inflammatory actions in the brain?

By

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University of Dublin, Trinity College.
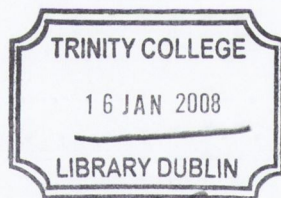
I.   Declaration

I declare that this thesis is entirely my own work apart from an experiment, indicated in the text, which was conducted by Melanie Watson. This work has not been previously submitted for a degree to this or any other university. I give my permission to the library to lend or copy this thesis.

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II.   Summary

Similarity in structure and sequence homology has led to the identification of additional members of the IL-1 ligand and receptor superfamilies. Because of the importance of IL-1 in immune and inflammatory reactions, elucidation of the respective roles of these recently described members is a priority. Thus far identification of a biological role for IL-1F5 has remained elusive. In this thesis investigation into a possible role for IL-1F5 in neuronal tissue was carried out and the findings of this study suggest that IL-1F5 abrogates IL-1β-induced inhibition of Long term potentiation (LTP), and associated increase in activation of c-Jun N terminal kinase (JNK). The data also show that IL-1F5 antagonizes the inhibitory effect of lipopolysaccharide (LPS) on LTP in rat dentate gyrus. The data indicate that this is associated with its ability to inhibit the LPS-induced activation of microglial cells and, as a consequence inhibit the increase in IL-1β and JNK.

The data show that IL-1F5 increases IL-4 mRNA and protein in glia, and the study investigated whether the anti-inflammatory effects of IL-1F5 are mediated through its ability to upregulate IL-4. These data show that the inflammatory effects of IL-1F5 are absent in mice deficient in IL-4 (IL-4$^{-/-}$ mice). The interaction of IL-1F5 with the novel orphan receptor SIGIRR was examined and IL-1F5 was shown to be unable to abrogate LPS-induced changes in SIGIRR$^{-/-}$ mice. Furthermore, IL-1F5 was unable to initiate an increase in IL-4 concentration in SIGIRR$^{-/-}$ mice. SIGIRR was found to be expressed in glial cells generated from rats and SIGIRR mRNA was upregulated in response to IL-1F5 in the hippocampus of wild-type C57BL/6 mice. Downstream IL-1F5 signalling was investigated and IL-1F5 increased phosphorylation of JAK1, STAT6, ERK1

and ERK2. In addition, IL-1F5 increased PPARγ activation. In the presence of the PPARγ antagonist GW9662, the inhibitory effect of IL-1F5 on LPS-induced increase in IL-1β was blocked.

The most significant finding of this study is that IL-1F5 exhibits anti-inflammatory activity and has the ability to abrogate changes induced by inflammatory mediators such as LPS and IL-1β. Furthermore the study has indicated that this anti-inflammatory ability is mediated via activation of the endogenous inhibitor IL-4. These data indicate an anti-inflammatory role for orphan receptor SIGIRR and indicate that the anti-inflammatory actions of IL-1F5 are reliant on SIGIRR interaction.

III.   Acknowledgements

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V. List of Abbreviations

The following abbreviations are used:

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
CNS	Central nervous system
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EGTA	Ethylene glycol bis (β-aminoethylether) N,N tetraacteic acid
ELISA	Enyzme linked immunosorbent assay
EPSP	Excitatory postsynaptic potential
ERK	Extracellular signal-regulated kinase
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HFS	High frequency stimulation
HRP	Horseradish peroxidase
ICE	Interleukin-1β converting enzyme
ICV	Intracerebroventricular
IL-1β	Interleukin-1β
IL-1R1	Interleukin-1 type 1 receptor
IL-1R2	Interleukin-1 type 2 receptor
IL-1ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-1RAcPb	Interleukin-1 receptor accessory protein B
IL-10	Interleukin-10
IL-6	Interleukin-6
IL-4	Interleukin-4
IL-1F5	Interleukin-1 Family member 5
IL-1F6	Interleukin-1 Family member 6

IL-1F7	Interleukin-1 Family member 7
IL-1F8	Interleukin-1 Family member 8
IL-1F9	Interleukin-1 Family member 9
IL-1F10	Interleukin-1 Family member 10
IFNγ	Interferon-γ
IP	Intraperitoneal
IRAK	Interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mGluR	metabotropic Glutamate receptor
mRNA	messenger Ribonucleic acid
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly- (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TBS-T	Tris buffered saline-tween

Introduction

1.1 The Hippocampus

1.1.1 Anatomy of the hippocampal formation

The hippocampal formation, located on the medial aspect of each hemisphere beneath the cortical structures, comprises the dentate gyrus, the hippocampus proper and the subiculum, and is part of the limbic system. All 3 are composed of temporal lobe allocortex, tucked into an S-shaped scroll along the floor of the lateral ventricle. The largest afferent connection of the hippocampal formation is the perforant path, which projects from layers II and III of the entorhinal cortex in the temporal lobe. There are three major pathways in hippocampus, the perforant fiber pathway from entorhinal cortex forms excitatory connections with the granule cells of dentate gyrus. The granule cells give rise to axons that form the mossy fiber pathway, which synapses with the pyramidal cells in area CA3. The pyramidal cells of area CA3 project to the pyramidal cells in CA1 by means of the Schaffer collateral pathway. (See Figure 1.1). This is known as the trisynaptic circuit.

It is now widely accepted that the hippocampus plays a central role in the storage of memory (Kandel & Squire, 2000). Studies involving lesions of the medial temporal lobe of monkeys demonstrated that these animals exhibited severe memory impairment (Mishkin, 1978). More recently, evidence for a role for the hippocampus in memory was demonstrated using magnetic resonance imaging and positron emission topography. These techniques assessed blood flow and oxygen consumption in the hippocampus and identified that these parameters fluctuated during learning tasks (Squire, 1992).

1.2   Long term potentiation

1.2.1 LTP

Synaptic plasticity is a term used to describe a change in synaptic function or efficacy in response to some stimulus, and can be defined as a change or modulation in synaptic transmission at a particular synapse. In 1973 Bliss and Lomo reported that trains of high frequency stimulation applied the to perforant path in anaesthetized rabbits increased amplitude of the excitatory postsynaptic potentials (EPSPs) in the target hippocampal neurons. This sustained increase in synaptic efficacy was termed long-term potentiation (LTP). The sustained increase in synaptic efficacy can be represented by assessing the effect of tetanic stimulation on EPSP. Low frequency stimulation (LFS; e.g. 0.33Hz) of the afferent input for extended periods of time elicits a synaptic response (EPSP) that remains stable in terms of slope and amplitude. High frequency stimulation (HFS; 250Hz) for a short period of time (often 3 or 4 trains of stimuli in one second) induces a short-lived tetanus. On return to LFS, an increase in the amplitude and slope of EPSP shows that the synaptic response has been strengthened, i.e. that LTP has been induced. LTP can be induced by a number of stimuli including high frequency stimulation/tetanic stimulation (Bliss & Lomo, 1973) or by inducing an increase in intracellular calcium (Malenka *et al.*, 1988). LTP was first characterised and found to be sustained at the synapses formed between the perforant path and the granule cells in the dentate gyrus of the hippocampus of anaesthetised rabbits (Bliss & Lomo, 1973). Since then LTP has been shown to occur in a wide variety of brain regions (e.g. amygdala, subiculum, cortex and striatum), and also in the other two main afferent pathways of the hippocampus; the mossy fibres projection from the dentate gyrus to the CA3 region, and the Schaffer collateral tract from CA3 to CA1 region.

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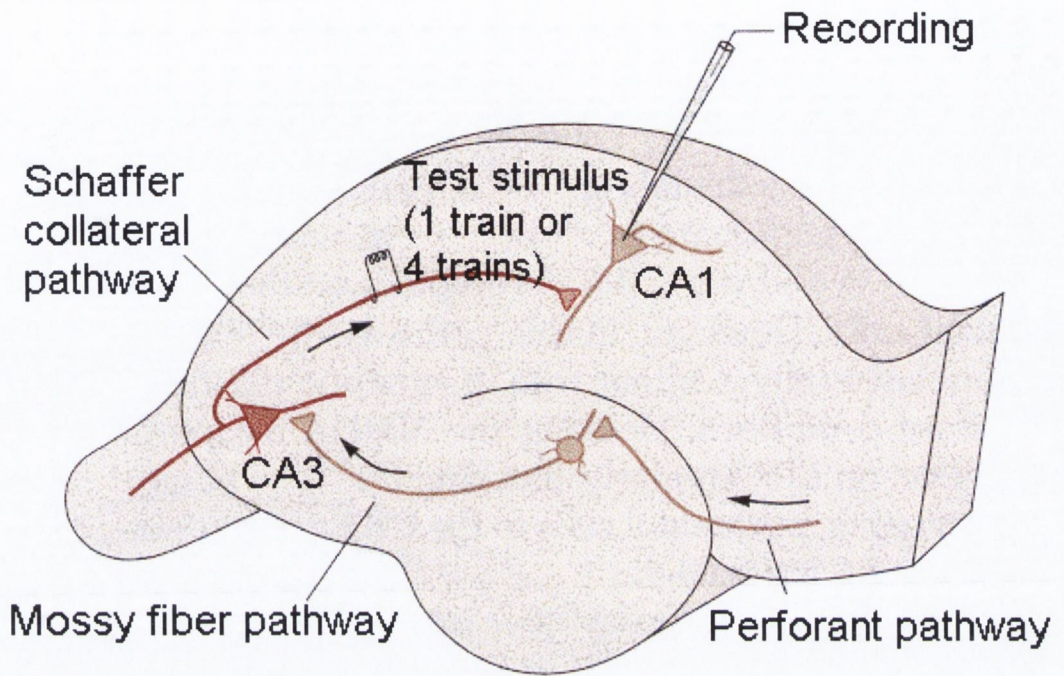
Figure 1.1: Anatomy of the Hippocampus

A transverse section through the hippocampus of the rat. Inputs reach the hippocampus from the entorhinal cortex through the perforant path, which makes synapses with the dendrites of the granule cells of the dentate gyrus and also with the CA3 pyramidal cells. The dentate cells project via the mossy fibres to the CA3 pyramidal cells, which in turn project via the Schaffer collaterals to the CA1 area. CA1 contains pyramidal cells which send axons to the subiculum. Long term Potentiation: Recordings are made intracellularly from CA1 neurons of the hippocampus while stimulation is applied to the Schaffer collaterals of CA3 neurons .

1.2.2 LTP and memory

There is evidence that similar mechanisms underlie LTP and memory. These include the requirement for activation of N-methyl-D aspartic acid (NMDA) receptors (Morris *et al.*, 1986; Tsien *et al.*, 1996; Kentros *et al.*, 1998), an increase in glutamate release, and increase in PIP_2 hydrolysis and an increase in aspartate in the CA1, CA3 and dentate gyrus of the hippocampus (Laroche *et al.*, 1990; Lynch *et al.*, 1990; Richter-Levin *et al.*, 1995). Also, involvement of Calcium-calmodulin- dependent kinase II (CaMKII), extracellular relared kinase (ERK) and protein kinase C (PKC) are associated with both LTP and memory (Lynch *et al.*, 2004). Temporal aspects of both memory and LTP are comparable; for instance, short term memory last for several hours and is independent of protein synthesis, whereas long-term memory lasts for days and longer, but is reliant on gene expression, protein synthesis and a myriad of downstream effectors to initiate new synaptic associations (Grecksch & Matthies, 1980; Quevedo *et al.*, 2004).

Hippocampal neurons have both non-NMDA (AMPA and kainate) and NMDA receptors. Non NMDA receptors participate in the early phase of the EPSP and gate ion channels with relatively low conductance that are permeable to both Na^+ and K^+ but usually not to Ca^{2+}. NMDA receptor-associated channels generate the late phase of the EPSP and have a higher conductance, being permeable to both Na^+ and K^+ but particularly to Ca^{2+}. Functioning of NMDA receptors depends on glycine being present in the extracellular fluid in sufficient quantities to bind to the NMDA-Receptor associated glycine binding site. The NMDA receptor-associated receptor differs further in that its channel is dual regulated, being not only ligand-gated but also voltage-gated. The presence of extracellular Mg^{2+} in the pore of the channel means that membrane depolarization must occur in order to allow ion influx through the channel. Membrane depolarization causes electrostatic repulsion of Mg^{2+} from its binding

site in NMDA receptor-associated channel (Collingridge *et al.*, 1983). Following production of a single action potential, the late phase of EPSP is usually quite short, as Mg^{2+} returns to the pore. However as neurons fire repeatedly, the late phase becomes longer as Mg^{2+} takes longer to be replaced into the pore. These events help to explain some of the main features of LTP.

1.2.3 Properties of LTP

The basic properties of LTP involve strengthening of synaptic efficacy through the coordinated firing of pre- and postsynaptic cells. There are three basic properties of LTP (Bliss & Collingridge, 1993). Firstly, when using high frequency stimulation to induce LTP, a critical number of fibers must be activated. Increasing the frequency of stimulation decreases the stimulation strength required to induce LTP – a property termed 'cooperativity' (Bliss & Collingridge, 1993). Secondly, when LTP is elicited at one set of synapses on a postsynaptic cell, adjacent synapses that were not activated do not undergo LTP – a property termed 'input specificity' (Andersen, 1977; Lynch *et al.*, 1977). Thirdly, LTP can be induced in a set of synapses undergoing subthreshold low frequency stimulation if their activation is concurrent with an LTP-inducing stimulus at another set of synapses on the same cell – a property termed 'associativity' (McNaughton *et al.*, 1978). This last property makes LTP an attractive mechanism for associating information carried by two different sets of afferents that synapse on the same postsynaptic cell.

1.2.4 Role of calcium in LTP

Several studies have identified that LTP can be produced in an NMDA-independent manner and that Ca^{2+} influx can act as a trigger for LTP. Lynch and colleagues (1983) demonstrated that intracellular injection of the Ca^{2+} chelator, EGTA, prevented the induction of LTP. Additionally, increasing concentrations of

postsynaptic Ca^{2+} can mimic LTP. Currently it is believed that a short-lasting (1-3sec) threshold level of Ca^{2+} must be reached in order to trigger LTP (Bliss & Collingridge, 1993). Thapsigargin, a drug that depletes intracellular stores of Ca^{2+}, has been shown to decrease the NMDA receptor-associated transient increase in Ca^{2+}. Furthermore, thapsigargin has also been shown to inhibit induction of LTP (Bortolotto & Collingridge, 1998). However it is not understood if Ca^{2+} can influence LTP induction independent of other factors and evidence suggests a family of G protein-coupled receptors known as metabotropic glutamate receptors (mGluR) may play a role in the induction for LTP. Upon Ca^{2+} influx into the postsynaptic cell a number of events must occur to translate the Ca^{2+} signal into a long-lasting increase in synaptic efficacy. Postsynaptic microinjection of CaMKII inhibitors have been shown to block the induction of LTP (Ito *et al.*, 1991). Furthermore it has been shown that by increasing concentrations of constitutively active CaMKII in CA1 cells, synaptic transmission was enhanced and sensitivity to tetanic stimulation suppressed (Pettit *et al.*, 1994).

1.3 Inflammation in the central nervous system (CNS)

1.3.1 Immunity in the CNS

The tissue and organs of the immune system, which defend the body against foreign and abnormal cells, rely on a complex and elaborate communication network. The system is controlled by hormonal and neural signals, including cytokines (McEwen, 1997). Pleuripotent haematopoetic stem cells, migrate to the liver, spleen and bone marrow, where they undergo differentiation and development into granulocytes, erythrocytes, lymphocytes or monocytes. Pathogen invasion presents the host with several challenges. Firstly, it must sense the pathogen, and secondly, deliver an appropriate

response. Protection against infection hinges on the close interplay between the innate and the adaptive immune systems. The innate and 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. Adaptive immunity is mediated by B and T-cells that proliferate clonally in response to a specific pathogen or antigen. These B and T-cells, which have differentiated from prelymphocytes and undergone maturation in the bone marrow, remain in the lymphocyte for their recruitment in the immune response. Innate immunity is a rapid response and, therefore, represents the first line of defence to an invading pathogen or foreign antigen (Medzhitov & Janeway, 1997).

Inflammation is a form of innate immunity 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 are manifest in the cardinal signs of inflammation – redness, heat, swelling, pain and tissue damage. Monocytes mature into macrophages in the peripheral nervous system (PNS) and microglia in the CNS and act to phagocytose foreign material and cell debris. This causes the release of hydrolytic and proteolytic enzymes which eliminate and destroy living organisms, ultimately leading to resolution of normal tissue structure and function.

1.3.2 The Blood brain barrier

In the past, the CNS was regarded as an 'immune-privileged site'. However, recently this view has been revised; now the CNS is regarded as an immunologically specialized site and, consistent with this view, the resident cells of the CNS, namely neurons and glia, have been found to be responsible for mediating immunoreactivity. The blood brain barrier (BBB), which is formed by the endothelial cells that line cerebral microvessels, has an important role in

maintaining a precisely regulated microenvironment for neuronal signalling. It acts as a 'physical barrier' because complex tight junctions between adjacent endothelial cells force most molecular traffic to take a transcellular route across the BBB, rather than moving paracellularly, as in most endothelial cells (Abbott *et al.*, 2006). Small gaseous molecules, such as O_2 and CO_2 can diffuse freely through the lipid membranes and this is also a route of entry for small lipophilic agents, including drugs such as barbiturates and ethanol. A selective 'transport barrier', permitting or excluding potentially harmful components and a 'metabolic barrier' consisting of enzymes which can metabolize potentially harmful peptides,-can inactivate and prohibit many neuroactive and toxic compounds (Campion *et al.*, 1996). However, it is now apparent that the BBB can be breached by immuno-active cells and the resident cells of the CNS, namely glia and neurons, are active in BBB functioning.

1.3.3 Resident cells of the CNS

Early histological studies show that brain capillaries are surrounded by, or closely associated with several cell types, including astrocytes, glia, pericytes, microglia and neuronal processes. These close cell-cell associations, particularly of astrocytes and brain capillaries, suggests that these interactions could be responsible for mediate the special features of the BBB, as well as mediating central inflammatory responses.

Until recently neurons were thought to play a passive role in inflammation. It now appears that neurons have an immune function; evidence demonstrates that neurons can produce a range of cytokines including interleukin-1β (IL-1β) (Minogue *et al.*, 2003) and also express the IFNγ, and major histocompatability complex (MHC) II gene (Neumann *et al.*, 1997). It appears that compromised nerve cells may have the ability to change their local environment, through interaction with surrounding glial cells.

The principal function of oligodendrocytes is to provide support to axons and to produce the fatty protein myelin, which forms an insulating sheath around the axons, promoting faster transmission of nerve signals.

Among glial cells of the CNS, oligodendrocytes and microglia have been recognized as having unique, specialized functions. For decades astrocytes have been considered to be non-excitable support cells of the brain. However, recent studies have recognized astrocyte's active communication properties, through which astrocytes can deliver specific messages to neighbouring cells in a calcium-mediated process known as gliotransmission. Calcium elevation in astrocytes results from release of Ca^{2+} from intracellular stores, activated by elevation of the second messenger inositol-1, 4, 5-triphosphate (IP_3). This messenger is generated as a consequence of the activity of phospholipase C, which in turn is activated by certain G-coupled receptors. Calcium waves were first thought to spread as a result of gap junction-mediated metabolic coupling between astrocytes, in the form of IP_3. ATP release appears to be an important component of long range Ca^{2+} signalling whereas shorter range signalling may be mediated by metabolic coupling through gap junctions. There seems to be a tightly coordinated regulation of the gap junction and ATP-mediated signalling pathways by molecules such as endothelin, glutamate, anandamide, and IL-1β (Rouach *et al.*, 2000; Enkvist & McCarthy, 1994). Because astrocytes are intimately associated with the synapse, enwrapping many pre- and post-synaptic terminals, and are in close contact with the capillaries, they are in a position to shuffle nutrients and metabolites between the blood supply and the active neuron. They therefore play an active metabolic role in the CNS. Astrocytes seem to play an important regulatory role in the processes of microglial differentiation and deactivation. Several factors released by astrocytes - TNFβ, macrophage colony-stimulating factor, and granulocyte/macrophage colony stimulating factor – can induce ramification and up-regulation of delayed rectifier outward K+ currents in microglia. Astrocytes are capable of suppressing production of the pro-inflammatory cytokine IL-12 (Hansson & Ronnback, 2003)

and can intefere with microglial phagocytosis. Microglia have a decreased ability to phagocytose senile plaques when cocultured with astrocytes.There are a number of suggestions as to how this process occurs. Astrocytes could down-regulate microglial scavenger receptors or alter levels of phagocytosis related trophic factors. Alternatively, astrocytes may induce ramification in microglial cells, thereby inhibiting microglial activation neccisary fro phagocytosis (DeWitt *et al.*, 1998).

In the injured CNS, reactive astrocytes form dense scar tissue, and this serves to compact inflammatory cells and re-seal the BBB after it has been broken down through insult or injury. Studies indicate that astrocytes may play an important role in the regeneration and recovery process following formation of this scar tissue. Recent work suggests that in chemically-induced, acute epilepsy models astrocytes contribute to the generation of synchronized epileptiform activity (Tian *et al.*, 2005). Astrocytes are thought to function via STAT3 signalling, a downstream effector molecule of several cytokines, involved in wound healing and cellular migration (Okada *et al.*, 2006) Astrocytes also control non-neuronal brain cells. For example, they attract cells to their territory through the release of chemokines, which are chemotactic agents that activate receptors on other cells. In this way astrocytes attract microglia and lymphocytes during inflammatory reactions (Volterra & Meldolesi, 2005). Recent work has revealed that astrocytes play an important protective role in Alzheimers disease (AD), where they are capable of binding to and degrading amyloid-β (Aβ), the protein that accumulates in the plaques of Alzheimers patients (Hartlage-Rubsamen *et al.*, 2003). Furthermore, astrocytes have been shown to express mRNA for the anti-inflammatory cytokine IL-4 (Brodie *et al.*, 1998) .

Microglia are the smallest of the glial cells. They serve as the representative cells of the immune system in the brain. Microglia protect the brain from invading micro-organsims and are thought to be similar in nature to macrophages in the periphery. Microglia are extremely sensitive to changes in

their microenvironment and are activated in all conditions that disrupt normal functioning (Kreutzberg, 1996). Microglia exist in two states; a resting state when they are not phagocytic and an active state when they have a distinctive amoeboid appearance and express cell surface markers associated with their phagocytic ability, such as MHC II. In recent years the advent of monoclonal antibodies to define cell surface determinants has led to the identification of microglia and derivatives as part of a group of cells related to monocytes and macrophages, that can release potential neurotoxins like tumor necrosis factor α (TNFα) and other cytokines, which may potentiate damage to nervous system cells (Gonzalez-Scarano & Baltuch, 1999). The macrophage antigens, CD11a, CD11b and CD11c have been identified on microglial cells and the increased expression of these antigens is associated with an induction of cytokines such as IL-1β and IL-6 (Griffin *et al.*, 1995). At the end stage of activation, microglia are phagocytic and act as the scavenger cells of the CNS. The neurotoxic potential of microglial cells is evidenced by their detection in post mortem analysis of brains of AD and Parkinsons Diseased patient brains in areas most effected by neurodegeneration (Rogers *et al.*, 1988), as well as their association with the pathogenesis of multiple sclerosis, amyotrophic lateral sclerosis and other autoimmune disorders. Microglial activation can be limited by a number of immunosuppressive agents, such as transforming growth factor-β (TGFβ), 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). IL-10 can inhibit MHC II expression in activated microglia and also the production of cytokines and chemokines which are essential for antigen presentation and immune defence (Ledeboer *et al.*, 2002).

1.3.4 Peripheral contribution to CNS inflammation

Peripheral cytokines have been shown to affect central behaviour and biochemical responses in brain; however the exact mechanism of this action is unclear. Delivered peripherally, cytokines induce fever, lethargy, reduced mobility, decreased learning, anorexia and decreased sleep (Rothwell, 1991). Peripheral immune cells, such as macrophages, are a source of cytokines, and are stimulated by injury, insult or infection, Macrophages are potently activated by binding of lipopolysaccharide (LPS). These peripherally-produced cytokines are thought to gain access to the CNS and exert their inflammatory effects. However, as cytokines are rather large molecules it is unlikely that they can simply pass through the tight junctions of the BBB. There are a number of theories as to how they infiltrate the CNS. Firstly, it has been documented that active T-cells can enter into the CNS (Wong *et al.*, 1997); this is significant as T-cells are a source of and also modulate cytokine production. Secondly, it has been demonstrated that passage can occur at circumventricular regions, where the BBB is less stringent (Licinio & Wong, 1997). Thirdly, cytokines or cytokine producing cells could gain access when the BBB is compromised. It has been suggested that cytokines can affect BBB permeability - binding of cytokines to receptors on endothelial cells of brain vasculature causes release of mediators such as nitric oxide (NO), chemokines and prostaglandins, which in turn can lead to an impairment of BBB integrity (Wong *et al.*, 1997). This increased permeability may therefore allow access of activated microglia or cytokines into the CNS. In addition, active transport mechanism for IL-1 and TNFα have been described (Dunn & Chuluyan, 1992; Gutierrez *et al.*, 1993).

1.3.5 T-cells and cytokines

T-cells are specialised white blood cells, which direct an ongoing immune response through the secretion of cytokines. T-cells recognize fragmented,

linear antigenic peptides when these are bound to proteins encoded by MHC genes. The T-cell receptor on an immature T cell binds to the MHC II molecule on the antigen presenting cell. Antigen presenting cells include dendritic cells, tissue macrophages and B-cells. Lymphocytes play a central role in controlling the acquired immune response and furthermore serve as crucial effector cells through antigen specific cytotoxic activity and the production of soluble cytokines, which then act as growth and differentiation factors for themselves and other cell types. The T helper cells are critical for the appropriate immune response and can be classified into Th1 and Th2 and Th3 subsets. The differentiation of T-cells into Th1 and Th2 determines whether humoral or cell-mediated immunity will predominate. Th1 cells lead to cell-mediated immunity which involves destruction of infected cells by cytotoxic T-cells or destruction of intracellular pathogens by activated macrophages. Th2 cells provide humoral immunity which activates B cells to make neutralizing antibodies. Th3 cells (also known as suppressor T cells) are a specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self. Experimental evidence from mouse models demonstrates that the immunosuppressive potential of these cells can be harnessed therapeutically to treat autoimmune diseases and facilitate transplantation tolerance (Shevach *et al.*, 2002). In addition new studies that link the cytokines IL-23 and IL-17 to immune pathogenesis previously attributed to the Th1 lineage have led to the delineation of a new effector Th cell — referred to as Th17. Release of IL-17 from Th-17 cells is associated with the production of IL-1β and TNF-α by human macrophage cells (Pelletier *et al.*, 1998). Thus, effective host response against intracellular pathogens requires selective production of Th1, Th3 and Th17 cells, while extracellular pathogens can be removed by humoral immunity with Th2 as the effector cells. In many infections both cell-mediated and humoral immunity play a role. The Th1 response is often viewed as pro-inflammatory; conversely Th2 is viewed as anti-inflammatory. The factors which influence the differentiation of naïve T cells to Th1,Th2 or Th3 include the type of antigen

presenting cell, the concentration of the antigen and the local cytokine environment (Szabo *et al.*, 2003). IL-4 predominantly induces Th2 differentiation, while IFNγ, IL-12 and IL-18 induce Th1 differentiationand IL-17 induces Th17 production (Zheng *et al.*, 2007)

1.4 Cytokines

Originally discovered in the immune system, cytokines are secreted proteins now known to be expressed by both neurons and glia. Cytokines are active during neural development (Mehler & Kessler, 1999) as well as in the adult nervous system during both normal function and pathological conditions (Rothwell, 1999). Different cytokines can exert similar effects on a single cell type - a phenomenon that allows one factor to compensate for another (Foster, 2001). Additionally most cytokines induce multiple effects in multiple cell types – making it difficult to determine the role of specific cytokines in the regulation of normal physiology (Foster, 2001). Interaction of cytokines with specific cell surface membrane receptors is required for induction of biological activity. Cytokine binding induces a conformational change which allows the intracellular domain of the receptor to interact with accessory molecules. Receptor binding results in the activation of downstream effectors and activation of protein kinases which phosphorylate protein substrates (Foster, 2001). There are two major pathways of signal transduction involved in cytokine activation – the first pathway, used by mitogenic cytokines utilizes tyrosine kinases as the signal transducers, either directly or indirectly linked to the intracellular domain of the receptor. The second pathway involves activation of phospholipases that produce small mediators that activate serine-threonine kinases.

Cytokines have been divided into "pro-inflammatory" and "anti-inflammatory" depending on their effects. As indicated by their name, pro-inflammatory cytokines promote inflammation and anti-inflammatory cytokines suppress the activity of pro-inflammatory cytokines; they suppress

genes for pro-inflammatory cytokines such as IL-1 and IFNγ. While IFNγ possesses anti-viral activity, its ability to augment pro-inflammatory cytokine activity and induce NO have lead to its classification as a pro-inflammatory cytokine (Dinarello, 2000). The most thoroughly investigated of the pro-inflammatory cytokines are IL-1 and TNFα while the most studied of the anti-inflammatory cytokines are IL-4 and IL-10.

1.5   Classical IL-1 Family

1.5.1   Classical IL-1 receptors; IL-1R1 and IL-1R2

The effects of IL-1 are mediated by the an 80kDa glycoprotein IL-1 type 1 receptor (IL-1R1), which acts in concert with IL-1RAcP to form a complex that initiates signal transduction (Sims *et al.*, 1993). The activation of this complex by IL-1 increases the expression of a large number of genes characteristic of inflammation. Concurrent with the idea of IL-1β as a neuromodulator is the finding that IL-1 receptors have been shown to be present in many brain regions with concentrationsin the hippocampus and hypothalamus (Wong & Licinio, 1994). IL-1R1 mRNA expression has been demonstrated in perivascular cells and some neuronal cell groups (Ericsson *et al.*, 1995). Binding of IL-1 to the IL-1R1 causes structural changes which allows recruitment of the IL-1RAcP which forms the active signalling complex. IL-1R2 acts as a negative regulator of IL-1R1 and is known as a decoy receptor. IL-1R2 is unable to bind either IL-1α or IL-1ra but has high affinity for IL-1β. IL-1R2 lacks a Toll-IL-1 receptor (TIR) domain, rendering it incapable of transducing a signal. It has the ability to recruit the IL-1RAcP thereby forming non-functioning complexes which regulate IL-1R1 signalling by sequestering available accessory protein (Lang *et al.*, 1998).

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1.5.2 IL-1α and -1β

IL-1 is a cytokine that is produced by and acts on many different cell types (Dinarello, 1996). IL-1 has the ability to upregulate the expression of many genes important in the initiation and regulation of inflammatory conditions (Dinarello, 1996). As such, IL-1 is critical in the management of host defence, however the potency of this cytokine means that imbalance in IL-1 levels can participate in the development of many diseases, including rheumatoid arthritis, AD and Parkinson's' disease. The classical members of the IL-1 family consist of IL-1α, IL-1β, IL-1receptor antagonist (IL-1ra) and IL-18. IL-1α and IL-1β were first isolated in the 1980s. Both IL-1α and IL-1β have similar properties in that they can activate the same signals and they both bind to the type 1 IL-1 receptor (IL-1R1; (Sims & Dower, 1994). However, differences in receptor affinities, cellular localization and sites of regulation and expression allows for a divergence of IL-1α and IL-1β functions *in vivo*. Furthermore, studies on knock out mice have shown that IL-1β but not IL-1α is required for T-cell dependent antibody production and IL-1β, but not IL-1α, is required for fever development (Alcami & Smith, 1992; Horai *et al.*, 1998). Both IL-1α and IL-1β are synthesized as precursor models of a 31 kDa form, which are cleaved enzymatically to release the active mature 17kDa form . Pro-IL-1α is biologically active but is anchored to the membrane via a lectin-like interaction, with little release in the circulation (Brody *et al.,* 1989; Mosley *et al.*, 1987). In contrast IL-1β is readily and rapidly exported from the cell. The method of release is thought to involve the packaging of IL-1β into vesicles and subsequent ATP-driven exocytosis (Andrei *et al.*, 1999). Pro IL-1β is cleaved by caspase-1 to form mature, biologically active IL-1β (Black *et al.*, 1988).

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1.5.3 IL-1 in the CNS

The cytokine IL-1 has diverse actions in the brain. It mediates host defence responses to local and systemic disease and injury and to neuroinflammation and cell death in neurodegenerative conditions. IL-1β is constitutively expressed at low levels in healthy adult brain by a variety of cell types. In response to local brain injury or insult, IL-1 is over-expressed by the CNS macrophage equivalent cell, the microglia (Hetier *et al.*, 1988). These are cells which are thought to be one of the principal producers of IL-1β (Mrak & Griffin, 2001).IL-1β mediates many actions in the CNS, , including modulation of EEG and neuronal activity, induction of NGF, self induction (of IL-1β), astrogliosis, increased metabolic rate, peripheral IL-6 release and a number of effects that can be collectively referred to a sickness behaviour; fever, lethargy, increased sleep, reduced social activity, reduced mobility, decreased learning, anorexia and decreased libido (Rothwell, 1991). Maximal changes in body temperature in response to IL-1β occur at lower doses after injection into the brain than when injected peripherally; in conscious rats, peripheral injection of 1-2μg recombinant IL-1β elicits maximal increases in body temperature and metabolic rate, but doses of only 5-10ng are required if injected centrally (Rothwell, 1991). IL-1β seems to be the main agonist induced in the CNS in response to systemic injury (e.g. infection, peripheral administration of LPS), or local (e.g. head injury or stroke) insults (Rothwell, 1999). IL-1β can elicit an array of responses which can potentially inhibit, exacerbate or induce neuronal damage and death. These include induction of fever and oedema, damage to the cerebral vasculature, activation of microglia, induction of neurotrophins, growth factors, adhesion molecules and release of NO and free radicals (Rothwell, 1999). Some of these responses serve to enable tissue repair to take place in an efficient manner, while some cause more tissue damage. However, it should be stressed that infusion of IL-1β into the brain of normal rats or

application to neuronal cultures does not cause overt damage or death to cells (Rothwell, 1999). It appears that IL-1β exacerbates damage to compromised neuronal cells, and that it may depend on other factors present in the damaged brain (Rothwell, 1999; Viviani et al., 2004). Although the etiology of CNS disorders vary, IL-1β does seem to be a common factor in processes leading to neurodegeneration.

1.5.4   IL-1receptor antagonist (IL-1ra)

IL-1ra is the third member of the IL-1 family. It is structurally similar to IL-1α and IL-1β and can bind to IL-1R1 with near equal affinity (Dripps et al., 1991), but is incapable of inducing intracellular responses (Hannum et al., 1990). The cytokine network is self-regulating through the action of opposing cytokines, the release of soluble cytokine receptors, and the production of antagonists of cytokine binding to receptors. IL-1ra is the first described naturally occurring specific receptor antagonist of any cytokine or hormone-like molecule. IL-1ra was originally described as an IL-1 inhibitory activity in the urine of patients with fever, and in the supernatants of monocytes cultured on adherent IgG (Arend, 1993). IL-1ra binds to the IL-1 receptors with avidity nearly equal to the two agonists (IL-1α and IL-1β), yet fails to activate cells.

Both type 1 and type 2 IL-1R are members of the IL-1 superfamily and possess three Ig-like domains in the extracellular positions. The extracellular domains are enzymatically cleaved and are present as soluble receptors both in the pericellular environment and in the circulation (Arend et al., 1998). Soluble IL-1R2 binds IL-1β much more avidly than IL-1ra, and may function as an inhibitor of IL-1β action in vivo. In contrast, soluble IL-1R1 binds IL-1ra almost selectively. Thus soluble IL-1R1 may bind exogenous IL-1ra in vivo and block or neutralise its natural anti-inflammatory actions (Arend et al., 1998). The results of early studies indicated that IL-1β and IL-1ra possess an identical β–pleated

sheet structure and that residues in the common open-faced surface of both molecules bind to the same site on IL-1R1. However, IL-1β interacted with two additional sites on the receptor, a β-bulge between strands 4 and 5 and a region around aspartic acid at residue 145. These interactions are though to be responsible for the difference in biological activity between IL-1β and IL-1ra. The detailed crystal structures of complexes of soluble IL-1R1 and IL-1β and IL-1ra were recently described. Both ligands bind to the soluble receptor with 1:1 stiochiometry, and the three Ig domains of the receptor were wrapped around both ligands.

The identification and characterisation of a second subunit of the IL-1 receptor complex, the IL-1 receptor accessory protein (IL-1RAcP), has further clarified the biochemistry and biology of the IL-1 system. The IL-1RAcP is a 570 amino acid protein of the IL-1 superfamily and possess limited homology to both IL-1R1 and IL-1R2. The accessory protein formed a complex with either IL-1α or IL-1β and IL-1R1, but IL-1ra failed to form this complex. Cell lines that do not express IL-1RAcP failed to elicit responses to IL-1β; these responses were restored by transfection of the cDNA for the accessory protein (Korherr *et al.*, 1997). Thus, the conformational changes induced in the ligand-receptor complex, by tight binding of the IL-1 agonist to domain 3 of the receptor, allow a secondary interaction of this complex with the IL-1RAcP, an effect absent in IL-1ra signalling. The most potent IL-1ra inducing substances *in vitro* are adherent IgG, LPS and IL-4, indicating that this cytokine may be readily produced *in vivo* in numerous chronic inflammatory and infectious diseases. Both IL-4 and IL-10 inhibited IL-1β production, while enhancing LPS-induced IL-1ra production by monocytes. In contrast fibrin enhanced IL-1β and suppressed IL-1ra production in human monocytes (Arenberg *et al.*, 1995). In an effort to investigate the physiological role of endogenous IL-1ra, mice were generated that lacked production of either isoform of IL-1ra or that over-produced sIL-1ra. Intraperitoneal injection of LPS in the IL-1ra knockout

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mice caused a greater number of fatalities in the knockout mice compared with normal mice, consistent with the known beneficial effects of IL-1ra in animal models of septic shock. In contrast, the mice lacking endogenous IL-1ra were less susceptible to infection with *Listeria* monocytogenes than were the normal mice, indicating the importance of IL-1β in resistance to infection with intracellular organisms. These initial studies established that the *in vivo* balance between endogenous IL-1 and IL-1ra is important in influencing the host response to infection.

IL-1ra is distributed widely throughout the body. IL-1ra mRNA was localized in the rat brain by *in situ* hybridisation in the hypothalamus, hippocampus and cerebellum (Arend *et al.*, 1998). Chronic intracerebroventricular infusion of IL-1β in rats induced expression of mRNAs for all three ligands of the IL-1 system in the same areas, particularly in cells in close proximity to the microvasculature. IL-1ra is also capable of crossing the BBB, thus protein measured in the brain may be of peripheral or local origin.

1.5.5 IL-1ra as an anti-inflammatory agent

The beneficial effects of administration of recombinant human IL-1ra in many experimental animal models of disease have been studied. Delivery of exogenous IL-1ra, usually intravenously (iv), led to at least partial prevention or treatment of disease. For example, iv injection of IL-1ra has been shown to be useful in the treatment of ischemic brain injury in rats, experimental allergic encephalomyelitis (EAE) in rats, osteoclast formation and bone reabsorption in ovariectomized mice and rats, collagen induced arthritis in mice and septic schock in mice and rats. A severe limitation to the therapeutic benefit of IL-1ra protein is the necessity to administer large amounts. Up to 100- to 1000-fold amounts of IL-1ra greater than the IL-1 agonists were necessary to inhibit biological activities. This may be required because target cells are sensitive to binding of only a few molecules of IL-1 per cell, and excess receptors are

present. In addition, the presence of the biologically inactive IL-1R2, either on the cell surface or as a soluble form in the cell microenvironment, may compete for the available IL-1ra. A mutant IL-1ra molecule that bound more avidly to IL-1R1 was a more potent antagonist of IL-1 effects *in vivo*. The delivery of IL-1ra by gene therapy has been recently examined in experimental animal models of arthritis. This approach led to a high concentration of IL-1ra in the synovium without the pre-systemic inhibition of IL-1 effects seen with the use of recombinant protein. Clinical trials of recombinant IL-1ra have been carried out in patients with sepsis syndrome. In an initial trial, three different doses of IL-1ra were infused intravenously over 3 days into patients with sepsis syndrome or septic shock. A dose dependent 28-day survival benefit was associated with IL-1ra treatment in this early clinical trial. Similarly, recombinant IL-1ra appeared to demonstrate some efficacy in patients with rheumatoid arthritis. In an initial clinical trial, patients with rheumatoid arthritis were treated with three different doses of IL-1ra protein by subcutaneous injection daily or three times a week for 3 weeks, followed by a 4-week maintenance phase of once weekly injections. The treatment was well tolerated, and patients receiving the daily dosage appeared to have significant clinical improvement (Campion *et al.*, 1996).

1.6 Novel IL-1 Family Members

The recent expansion of the IL-1 family to include six new ligands and new IL-1/Toll receptors has opened up areas with potential for further elucidating the pathways of IL-1 actions in the CNS. The six new members of the IL-1 family have been identified primarily through the use of DNA database searches for homologues to IL-1. A nomenclature has been adopted which names these new proteins IL-1F5 to IL-F10 and are classified as IL-1 family members based on amino acid sequence similarity, identity of gene structure, and predicted or known three-dimensional structure (Sims *et al.*, 2001). The sequence identity in

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the family ranges from 57% at its highest to 13% at its lowest (Nicklin *et al.*, 2002). The genes for IL-1F5 to IL-1F10 are located between the IL-1β and the IL-1ra loci, suggesting that they arose from a common ancestral gene that later became duplicated (Dunn *et al.*, 2001; Nicklin *et al.*, 2002; Taylor *et al.*, 2002). Little is known about the receptors, signalling or function of these new IL-1 family members.

1.6.1 IL-1F5

IL-1F5, formerly IL-1δ, is most closely related to IL-1ra, sharing a 44% sequence homology. IL-1F5 expression is much more restricted than that of the classical IL-1 family members. There is strong expression in foetal skin (Mulero *et al.*, 1999) and adult skin (Barton *et al.*, 2000) as well as abundance in psoriatic skin lesions (Debets *et al.*, 2001). Expression is also detected in immune cells such as monocytes (Barton *et al.*, 2000; Smith *et al.*, 2000), B cells and dendritic cells (Smith *et al.*, 2000) as well as LPS stimulated macrophages (Mulero *et al.*, 1999). Using Fc fusion proteins to overexpress receptors it has been shown that IL-1F5 is unable to bind IL-1R1, IL-1RAcP, IL-18R, IL-18RAcP, IL-1Rrp2, T1/ST2 (Smith *et al.*, 2000) TIGIRR or IL-1RAPL (Born *et al.*, 2000b). A study involving transfection of Jurkat cells with a plasmid encoding IL-1Rrp2, indicates that IL-1F5 has the ability to inhibit the activation of an NFκB luciferase reporter plasmid by another novel IL-1; IL-1F9 (Debets *et al.*, 2001). However, thus far, identification of a biological role for IL-1F5 has remained elusive.

1.6.2 IL-1F6

IL-1F6 has the highest sequence identity with IL-1F9 (Lin *et al.*, 2001). IL-1F6 is highly expressed in tonsil and fetal brain (Smith *et al.*, 2000). IL-1F6 is also expressed in immune cells, in LPS-stimulated monocytes and in B cells

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(Smith *et al.*, 2000). IL-1F6 was initial shown to lack a receptor, with Fc fusion studies indicating its inability to bind IL-1R1, IL-1RAcP, IL-18R, IL-18RAcP, IL-1Rrp2, TI/ST2 (Smith *et al.*, 2000), TIGIRR or IL-1RAPL (Born *et al.*, 2000b). However a recent study has identified IL-1F6 as having the ability to activate mitogen activated protein (MAP) kinase, JNK and ERK in addition to activation of the pathway leading to NFκB, via its interaction with the novel IL-1/Toll receptor IL-1Rrp2. Furthermore IL-1F6 binding with IL-1Rrp2 induced secretion of cytokines including IL-6 and IL-8 (Towne *et al.*, 2004).

1.6.3 IL-1F7

IL-1F7 is the only member of the novel IL-1 superfamily that has a pro-domain like that of the classical IL-1 members. Expression of IL-1F7 has been detected in fetal lung, colon, testes and B cells (Kumar *et al.*, 2000). IL-1F7 shares significant sequence homology with IL-18. The hallmark for IL-18 activity is its ability to induce IFNγ in T cells or natural killer cells in the presence of IL-2, IL-12 or IL-15 as co-stimulants (Bufler *et al.*, 2002). This effect is reliant on the interaction of IL-18 with a complex called the IL-18α chain. IL-1F7 has been shown to inhibit IL-18 activity by blocking recruitment of the IL-18α chain (Bufler *et al.*, 2002).

1.6.4 IL-1F8

IL-1F8 is most similar to IL-1F6 and is expressed in lung, testes, fetal brain as well as in LPS-stimulated monocytes and B cells (Smith *et al.*, 2000). Experiments using Fc fusion proteins have been shown IL-1F8 is unable to bind IL-1R1, IL-1RacP, IL-18R, IL-1Rrp2, T1/ST2, (Smith *et al.*, 2000), TIGIRR or IL-1RAPL (Born *et al.*, 2000). IL-1F8 is closely related to IL-1F6. IL-1F8 has been reported to trigger JNK and NFκB signalling in mammalian cell lines via a mechanism that requires IL-1Rrp2 and IL-1RAcP (Towne *et al.*, 2004). Recently

IL-1F8 mRNA expression has been shown in cultured microglia, O_2A cells, neurons and to a lesser extent in astrocytes in the CNS (Wang *et al.*, 2005). However, IL-1F8 was unresponsive to LPS, indicating that although IL-1F8 is expressed in the CNS it fails to induce the IL-1 classical signalling pathway in brain cells (Wang *et al.*, 2005). Recent studies have also indicated that IL-1F8 levels are not elevated in response to inflammatory arthritis synovial biopsies (Magne *et al.*, 2006).

1.6.5 IL-1F9

IL-1F9, formerly IL-1ε (Debets *et al.*, 2001) is expressed in the lung, TNFα and IFNγ-stimulated keratinocytes and epithelial cells, as well as oesophageal squamous cells and macrophages (Kumar *et al.*, 2000). Debets *et al* (2001) reported that IL-1F9 can induce NFκB activation in IL-1Rrp2 transfected Jurkat cells (Debets *et al.*, 2001). However, recent studies demonstrated that despite constitutive expression of IL-1Rrp2 mRNA in glial cells, IL-1F9 failed to activate classical IL-1 signalling pathways, including NFκB in these cells (Berglof *et al.*, 2003). Experiments have shown that LPS strongly reduces expression of IL-1Rrp2 mRNA in both mixed glia and microglia, which may suggest that during inflammatory situations, possible IL-1F9 actions might be suppressed by decreased expression of IL-1Rrp2 mRNA. This points to the possibility that IL-1F9 does not participate in brain inflammation, but may have trophic actions and contribute to the development of the brain or to physiological responses, which might occur via activation of signalling pathways distinct from those of IL-1β. A recent significant finding reported that IL-1F9 expression was significantly increased in bronchial epithelial cells in response to an inflammatory stimulus (Vos *et al.*, 2005).

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1.6.6 IL-1F10

IL-1F10 is the final member of the IL-1 superfamily and is closest in resemblance to IL-1ra and IL-1F5. Immunohistochemical data shows that IL-1F10 is released from activated B cells of the tonsil (Smith *et al.*, 2000). It has also been shown to bind a form of IL-1R, although its affinity is considerably less than that of the classical IL-1 family members. The implications of this binding are, as of yet, unclear.

1.7   The TLR superfamily

There are a growing number of mammalian homologues of IL-1R1 that contain a highly conserved region in their cytosolic domains (O'Neill & Dinarello, 2000). The homologous regions are also found in a receptor-like protein of the Drosophila fruit fly called Toll. This has resulted in the defining of a receptor superfamily – the IL-1R/toll-like receptor (TLR) superfamily. The IL-1/TLR superfamily consists of two sub-families, the IL-1R-related and the Toll-related molecules. The extracellular part of the IL-1R family is characterised by three Ig immunoglobulin domains. To date, six IL-1/TLR are known, but only ligands for IL-1R1, IL-18, TLR-2 and TLR-4 have been identified. The members of the TLR superfamily are structurally and functionally related. Signalling activity is reliant upon the conservation of the motifs of the cytoplasmic region of the receptors. Studies have shown that any alteration in these cytoplasmic domains results in a loss of signalling activity. Site directed mutation of the cytoplasmic domain of the IL-1R resulted in inactivation of IL-1R-mediated signal transduction (Heguy *et al.*, 1992). Overall the superfamily is quite diverse, but given the degree of homology in the TIR domain and the cross-species nature of the superfamily, it can be concluded that the superfamily represents an ancient signalling system involved in the host response to injury and infection.

Studies have identified orphan members of the IL-1/TLR superfamily; T1/ST2, IL-1RAPL, TIGIRR, IL-1Rrp2 and SIGIRR (O'Neill & Dinarello, 2000). These receptors all share a common overall structure with an intracellular Toll-IL-1 receptor (TIR) domain and /or three extracellular immunoglobulin (Ig)-like domains. The family members include IL-1R1, which binds IL-1 (Thomassen *et al.*, 1998), IL-1R1I (McMahon & Briggs, 1991), the IL-1 receptor accessory protein (IL-1RAcP), which plays a role in IL-1 signalling, the IL-18R (Thompson *et al.*, 1991), the IL-18 receptor accessory protein (IL-18RAcP) (Born *et al.*, 2000a), IL-1Rrp2 (Lovenberg *et al.*, 1996), which is reported to be responsive to IL-1F5 and IL-1F9 (Debets *et al.*, 2001). The IL-1R family also contains the orphan receptors T1/ST2, IL-1RAPL and TIGIRR/IL-1RAPL2/1L-1R9 (Born *et al.*, 2000b; Jin *et al.*, 2000; Sana *et al.*, 2000; Ferrante *et al.*, 2001). The single immunoglobulin domain containing IL-1 receptor related (SIGIRR) is an IL-1R family member with only one extra cellular Ig domain (Born *et al.*, 2000b).

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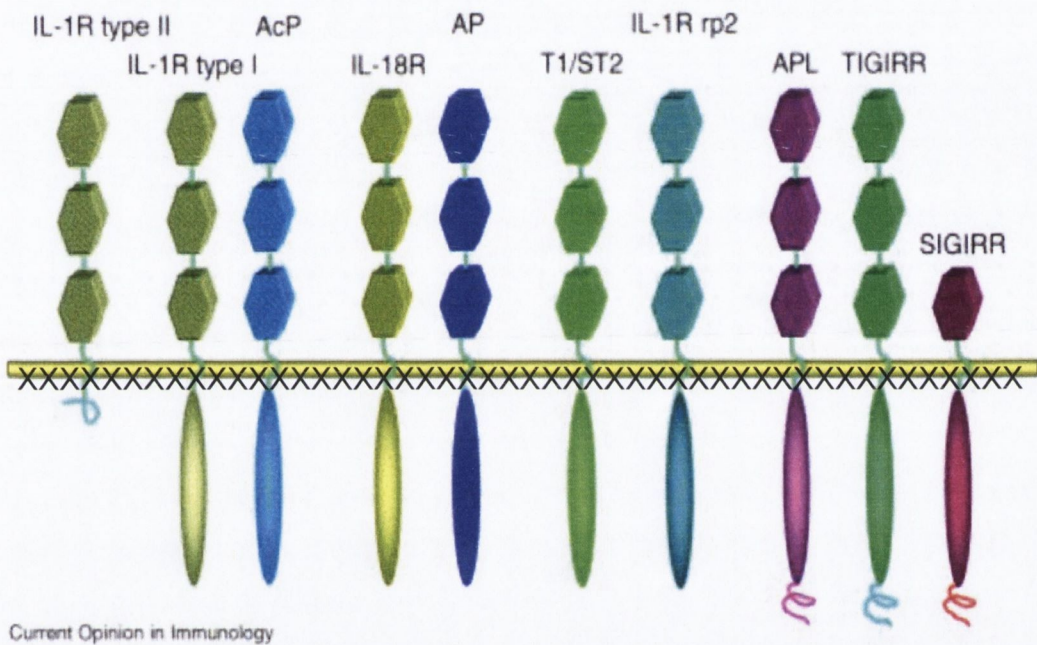
Figure 1.2: Novel members of the IL-1 receptor family

The IL-1 receptor family has ten members which share a common overall structure, with an intracellular Toll-IL-1 receptor domain and/or three extracellular Ig domains

(Adapted from Sims. (2002) IL-1 and IL-18 receptors, and their extended family. *Current Opinions in Immunology* **14**(1):117-122)

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1.7.1 T1/ST2

T1/ST2 is an orphan receptor of IL-1R with no known ligand. It has been shown to be unable to bind IL-1. T1/ST2 was originally identified in fibroblasts and was subsequently found to be expressed by mast cells. The receptor is expressed in Th2 cells, and therefore implicated in the development of these cells and their effects (Lohning et al., 1998). T1/ST2 is unable to bind IL-1α, IL-1β or IL-1ra . It is an orphan receptor with no known functional ligand. In the mouse, directed mRNA processing within the st2 gene leads to the production of both a membrane bound form of ST2 expressed primarily by haemopoietic cells and a soluble form of T1/ST2 predominantly expressed by fibroblasts (Gachter et al., 1996). Studies using either anti-T1/ST2 antibodies or a recombinant soluble form of T1/ST2 have demonstrated important roles for this molecule in regulating Th1/Th2 associated immune responses in vitro and in experimental inflammatory disease models in vivo (Xu et al., 1998; Coyle et al., 1999). Apart from regulation of disease outcome through modulation of Th1/Th2 bias, there is no direct evidence to suggest that T1/ST2 may also be involved in inflammatory responses. A recent study described a novel antiarthritic effect of soluble T1/ST2 (Gayle et al., 1996; Mitcham et al., 1996). This soluble form of T1/ST2 effectively suppressed murine collagen-induced arthritis even when administered after clinically evident onset of disease via specific suppression of the pathogenic pro-inflammatory responses. The clinical relevance of these observations is illustrated by parallel studies in experiments involving human cells, whereby soluble T1/ST2 protein suppressed cytokine release by macrophages following cell contact-dependent interaction with activated T cells (Leung et al., 2004). Previous studies have also indicated that T1/ST2 is unable to activate NFκB but is able to activate JNK and p38 MAP kinase (Brint et al., 2002).

1.7.2 IL-1RAPL and TIGIRR

IL-1RAPL was identified from patients suffering from non-specific X-linked mental retardation (MRX)(Carrie *et al.*, 1999). Non-overlapping deletions as well as nonsense mutation in the intracellular domain of this receptor are found in MRX patients. The exact function of this receptor is not known. It is highly expressed in postnatal brain structures that are important for learning and memory (Khan *et al.*, 2004). IL-1RAPL was characterised from homology searches and was found to share 72% amino acid sequence with another IL-1R/TLR superfamily member, TIGIRR. (Born *et al.*, 2000b). Like the other IL-1Rs, these two receptors contain a TIR domain in their intracellular region. However they also contain a 130-residue segment C-terminal to the TIR domain, which is absent in most of the other TLRs and IL-1Rs. Recent studies suggest that residues in the C terminal segment of IL-1RAPL may interact with neuronal calcium sensor-1 (NCS-1), and IL-1RAPL may have a role in regulating exocytosis of secretory and neurotransmitter substances NCS-1 is up-regulate in schizophrenic and bi-polar patients (Bahi *et al.*, 2003). Recently, crystallography studies have determined the structure of IL-1RAPL and have identified that it is unable to activate NFκB (Khan *et al.*, 2004). Mutations in the IL-1RAPL gene have been linked with the development of mental retardation, and due to the similarity of the receptors it is thought that TIGIRR may also be involved (Jin *et al.*, 2000). Chimeras produced, consisting of the extracellular and transmembrane domain of the IL-1R and cytoplasmic domain of TIGIRR or IL-1RAPL were non-responsive to IL-1 (Born *et al.*, 2000b). TIGIRR expression was predominantly in skin and liver, although overall expression levels are quite low, similarly IL-1RAPL and TIGIRR showed no involvement in either IL-1β or IL-18 cytokine signalling (Born *et al.*, 2000b).

1.7.3 IL-1Rrp2

Recently IL-1Rrp2 has been identified as the receptor for the orphan ligands IL-1F6 and IL-1F8 (Towne *et al.*, 2004). These novel cytokines, along with the cytokine IL-1F9 activate similar signalling molecules with a similar timecourse as IL-1β including the transcription factor, NFκB, and the JNK and ERK. IL-1Rrp2 is expressed in tissues such as skin and airway, where inflammatory mediators can serve as first line of defence against pathogens (Towne *et al.*, 2004). Previous studies have identified this signalling mechanism of IL-1F9 through IL-1Rrp2 and have indicated that IL-1F5 can antagonize IL-1F9 pro-inflammatory signalling via IL-1Rrp2 (Debets *et al.*, 2001).

1.7.4 SIGIRR

Sequencing of SIGIRR revealed that peptide sequences of mouse and human SIGIRR are 82% identical and show a 23% overall identity homology to mouse or human IL-1R1. Similarly the important cytoplasmic portion of SIGIRR contains the conserved motifs of the IL-1R1 superfamily. The SIGIRR sequence has a short extracellular region of only 118aa that corresponds to a single Ig domain of the IL-1R. Similar to IL-1Rrp2 two positions in the conserved domain of SIGIRR have been replaced, compared with IL-1R1 and the other members of the superfamily. The Ser and Tyr residues are replaced with a Cys and Leu residue. The importance of this is that Ser and Tyr have been shown to be essential for the signalling of IL-1R1. Investigation has shown that SIGIRR is unable to bind either IL-1β or IL-1α, despite have an overall homology of 42% with IL-1R1 (Lovenberg *et al.*, 1996). Chromosomal mapping revealed that although SIGIRR and IL-1R1 genes evolved from a common ancestor, SIGIRR is not located in proximity to the cluster of IL-1R family and superfamily members on the chromosome 2 (Sims *et al.*, 2001). SIGIRR contains a long cytoplasmic tail region, which extends beyond the last conserved region by

98aa. Previous experiments have shown that this region in Drosophila Toll may contribute to inhibitory activity (Norris & Manley, 1995). SIGIRR is the most widely expressed of all the IL-1/TLR superfamily. Analysis of human SIGIRR mRNA shows it is widely expressed in the oesophagus, stomach, duodenum, jejunum and ileum, colon, rectum, spleen, thymus and peripheral blood lymphocytes (Thomassen *et al.*, 1999). Similarly SIGIRR is expressed in primary and transformed cell lines derived from connective and lymphoid tissue (Thomassen *et al.*, 1999). Using EST database human SIGIRR was also found to be present in the adult brain (Thomassen *et al.*, 1999). However a further study reported almost no SIGIRR mRNA expression in the murine brain, heart, testes and skeletal muscle (Polentarutti *et al.*, 2003). There is a high expression of SIGIRR in epithelial cell lines, this high expression of SIGIRR suggests that SIGIRR may serve to moderate the immune response in organs which are constantly exposed to microorganisms, such as the colon, and the lung epithelial cells (Wald *et al.*, 2003).

Following induction of inflammation, the expression pattern of many genes involved in immunity is altered. Following injection with LPS there was a down-regulation in SIGIRR expression, at 6 hours and at 12 hours (Wald *et al.*, 2003). Similarly, the expression of SIGIRR was partially reduced in many murine organs following LPS stimulation, including brain, heart, lung and kifney tissue (Polentarutti *et al.*, 2003). One of the most important signalling cascades induced by IL-1R1 activation is the NFκ-B pathway. Replacement of the intracellular domain of mouse IL-1R1 with the SIGIRR intracellular domain resulted in the loss of normal IL-1R1 associated signalling. Stimulation with IL-1α failed to elucidate any IL-8 production (Thomassen *et al.*, 1999). One of the reasons for this inability to bind IL-1 could be that the single Ig domain of SIGIRR is simply too short to embrace the IL-1 ligand in the manner of IL-1R (Vigers *et al.*, 1997). It has been suggested that SIGIRR may function as an accessory protein for one of the IL-1R family members, however studies have shown that replacing the extracellular and transmembrane domains of SIGIRR

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with those of the IL-1RAcP failed to elicit any signal response after IL-1α stimulation (Thomassen *et al.*, 1999). *In vivo* challenges using SIGIRR-deficient mice show that these mice revealed greater induction of acute phase C-reactive protein (CRP) and chemokine genes in response to an intraperotoneal injection of the TLR4 agonist LPS. SIGIRR-deficient mice also showed hyperresponsiveness to IL-1, indicating that SIGIRR can function *in vivo* as a negative regulator of IL-1 and LPS signalling (Wald *et al.*, 2003). SIGIRR exerts a negative role in IL-1 induced NF-κB activation (Polentarutti *et al.*, 2003). Overexpression of cells with SIGIRR and NF-κB constructs failed to activate NF-κB expression, nor did it enhance IL-1R1-induced NF-κB activation. In contrast, treatment of these cells with IL-1 resulted in inhibition of IL-1R1 induced NF-κB activation (Polentarutti *et al.*, 2003). As a member of the IL-1 family, which plays a critical role in inflammation and immune response elucidation of SIGGIRs role is of paramount importance.

1.8   IL-1/Toll like receptor signalling

1.8.1 IL-1/TLR signalling

The presence of a conserved domain in a family of receptors suggest common signalling pathways. This appears to be the case for the IL-1/TLR superfamily. Common pathways include activation of the transcription factor NFκB, p38 and JNK.

1.8.2.1   NFκB activation

NFκB is a critical transcription factor for the immune and inflammatory responses because a large number of genes are NFκB regulated. Stimulation of

every member of the IL-1R/TLR superfamily leads to activation of NFκB transcription factor.. The key event is the phosphorylation and degradation of the inhibitory protein IκB. This leads to the release of NFκB, which translocates to the nucleus, binds to its consensus sequence on target genes, and up-regulates their expression. Two kinases responsible for IκB phopshorylation have been identified and named IκB kinase (IKK)1 and 2. Evidence has been presented that both are involved in NFκB activation by IL-1β (DiDonato *et al.*, 1997). In addition an upstream regulator of IKKs has been identified and termed NFκB-inducing kinase (NIK). NIK is also required for NFκB activation by IL-1 and is likely that NIK and IKKs are part of a large complex of proteins, which regulate NFκB activation in cells (O'Neill & Greene, 1998). The TNF receptor-associated factor (TRAF) family member, TRAF6, is necessary for NFκB activation. TRAF6 associates with IL-1R1 via the protein kinase IRAK. This complex then associates with NIK to activate the IKK complex and cause NFκB activation.

1.8.3 Mitogen activated protein kinases

The mitogen-activated protein kinases (MAP kinases) are the second major targets of TLR signalling. IL-1 has been shown to activate p38 MAP kinase and JNK (Derijard *et al.*, 1995; Raingeaud *et al.*, 1995; Lin *et al.*, 2001) and, in certain cell types, p42/p44 MAP kinase (Bird *et al.*, 1991; Guy *et al.*, 1991). Upon dimerisation the IL-1R/IL-1RAcP complex recruits the adapter molecule MyD88 (Burns *et al.*, 1998). MyD88 was the first adaptor to be described and is recruited following a dimerisation of two TIR domains. MyD88 has two binding domains, a Toll-IL-1 receptor (TIR) and a death domain (DD). MyD88 can interact with IL-1RacP through its TIR domain and also interact with IL-1 receptor associated kinase-4 (IRAK) through its DD domain (Wesche *et al.*, 1999). IRAK4 becomes activated and phosphorylates IRAK1. IRAK is recruited

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to the complex, a necessary step in activation of both NFκB and MAPK pathways as this complex leads to the activation of TRAF6. TRAF6 undergoes translocation from the membrane to the cytosol where it interacts with the TAK1/TAB complex, leading to phosphorylation of the downstream targets MAP Kinase kinase (MKK3/6) and finally activation of p38 (O'Neill & Greene, 1998).

1.8.4 c-Jun N-terminal Kinase (JNK)

JNKs are a family of kinases of the MAPK group. JNKs are also termed stress activated protein kinases (SAPK), as they were first described as being activated in response to various environmental stresses e.g. deprivation of trophic factors, or exposure to free radicals or LPS, as well as ischemia, heat shock or UV irradiation (Mielke & Herdegen, 2000). As a rule MAP kinase activation is mediated by MAPK kinase (MAPKK/MKK), which itself is activated by phosphorylation by a MAPKK kinase (MAPKKK/MEKK), creating a kinase-signalling cascade, these pathways, however, may be stimulated at any point along the cascade (Yang *et al.*, 1997; Tournier *et al.*, 1999). JNK is so called because it binds the NH$_2$ terminal activation domain of the transcription factor c-Jun, and phosphorylates it on Ser63 and Ser73. However, JNK has a wide variety of substrates, both transcription factors and non-nuclear proteins (Mielke & Herdegen, 2000). JNK is encoded by three genes, jnk1, jnk2 and jnk3. While jnk1 and jnk2 are ubiquitously expressed, jnk3 appears to be restricted to brain, testes and heart (Gupta *et al.*, 1996). Indeed, in the brain all three isoforms of JNK are active with JNK activity being 15-30 fold higher in the brain than in other tissues (Borsello & Bonny, 2004). This suggests that JNK has an important physiological role in the nervous system.

JNK plays a role in activation of cell death pathways and the inflammatory response. Activation of JNK leads to the modification of many substrates. Cytoplasmic targets include BCL-2, tau; phophorylation of which is a hallmark of neurodegenerative disease (Reynolds *et al.*, 1997). In addition JNK can regulate

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p53 by stabilising it, resulting in an increase in Fas and BAX expression and an increase in ROS, all of which have been linked to cell death.

1.9   Apoptosis

In a mature human, millions of cells die every minute, and we remain the same size only because cell division exactly balances cell death. Based on the characteristic way cells look when they die in different circumstances it was proposed in 1972 that normal cell deaths, as well as some pathological ones, can be identified as programmed cell death (suicide)(Kerr *et al.*, 1972). That is, that cells activate an intracellular death programme and kill themselves in a controlled way; - this process is now known as apoptosis. Early studies in C. elegans identified two genes, *ced-3* and *ced-4* required for apoptosis. The protein encoded by the *ced-3* gene was found to be very similar to a human protein called IL-1 converting enzyme (ICE; Miura *et al.*, 1993). ICE is an intracellular protein cleavage enzyme, or protease, that splices IL-1, the signalling protein that induces inflammation, from a larger precursor protein pro-IL-1β (Sabbatini *et al.*, 1997). The similarity between *ced-3* and ICE proteins was the first indication that the death programme depends on protein cleavage. Many more proteases have been identified and they all have the amino acid cysteine in their active site, and cleave their target proteins at specific aspartic acids residues, and are so called caspases. Each caspase is composed of a large inactive precursor, which itself is activated by cleavage at aspartic acids, usually by another caspase (Sabbatini *et al.*, 1997). In apoptosis, caspases are thought to be activated in an amplifying proteolytic cascade, cleaving one another in sequence. Once activated, caspases cleave other specific proteins in the cell to help kill the cell quickly and neatly (Alnemri *et al.*, 1996). They cleave proteins supporting the nuclear membrane, thereby helping to dismantle the nucleus; they cleave a protein that normally holds DNases in an inactive form, thereby freeing the DNases to cut up the cells nucleus; they cleave protein

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constituents of the cells skeleton and other proteins involved in the attachment of cells to their neighbours, thereby helping the dying cell to detach and making it easier to digest (Raff, 1998). Some caspases, such as ICE, appear not to be directly involved in apoptosis, but instead generate inflammatory signals such as IL-1β, which can initiate apoptosis via the JNK signalling pathway. When the CNS becomes infected with a virus some lymphocytes become activated and induce infected cells to kill themselves, thereby preventing the virus from multiplying and spreading to other cells. Killer lymphocytes have a second method of inducing cells to kill themselves. The lymphocytes produce a protein (Fas ligand) that binds to receptors (Fas) on the surface of the target cells, causing receptors to aggregate (Nagata & Golstein, 1995). The aggregated receptors recruit adaptor proteins from the cytoplasm, which in turn, recruit pro-caspase-8; the clustered pro-caspase molecules cleave and activate each other to begin the suicide sequence (Ashkenazi & Dixit, 1998). Remarkably, stressed cells can sometimes insert Fas and Fas ligand into their surface membrane, and use them to activate pro-caspases and kill themselves.

Pro-caspases can also become activated without a stimulating signal at the cell surface, especially if a cell is stressed or damaged. When mitochondria are damaged, for instance by a toxic drug, they can release a protein cytochrome c into the cytoplasm. This protein normally functions in the electron transport processes in mitochondria that generate most of the cells' ATP, the main energy carrier in cells. Once it is in the cytoplasm, however, cytochrome c has an entirely different function. It binds to an adaptor molecule, leading to the activation of procaspase-9, which in turn leads to apoptosis (Li *et al.*, 1997). One mechanism that triggers apoptosis when DNA is damaged is the cancer-suppressing protein p53, which accumulates and becomes activated in response to DNA damage; by various routes, the p53 can either stop cell division or induce apoptosis (Raff, 1998). There are many disorders where cells die prematurely; heart cells die in a heart attack, for example, and brain cells in a stroke. In these acute conditions, many cells die by necrosis. But some less

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badly damaged cells die by apoptosis, and the search is on for drugs that block the process in the hope that some of the apoptosis can be prevented.

1.10   Inflammation and cognitive decline

It might be predicted that these degenerative effects of sustainedinflammation would culminate in some functional effect on actual cognition. Indeed, this is the case, as observed in various models of inflammation; IL-1β has been shown to disrupt the acquisition of spatial learning information in the Morris water maze (Oitzl *et al.*, 1993).   Another pro-inflammatory cytokine, IL-6 was shown to be associated with deficits in avoidance learning (Heyser *et al.*, 1997) while a decline in cognitive function with age, including memory related functions, is well documented (Lynch, 1998). Normal cognitive function is reliant on synaptic efficacy. One way to assess synaptic efficacy is by analyzing the ability of neurons to sustain LTP (Bliss & Gardner-Medwin, 1973; Lynch, 2004).

1.11   Lipopolysaccharide (LPS)

1.11.1 LPS

One of the best known activators of the innate immune system is LPS from Gram-negative bacteria (Takeda & Akira, 2003). LPS plays a role in inducing Gram-negative disease states, including the potentially fatal condition of toxic shock syndrome/septic shock. LPS is one of the most potent microbial initiators of inflammation (Cohen, 2002) and functions to activate the innate immune response, the purpose of which is to coordinate a defensive response to a detected pathogen. This involves the activation of pro-inflammatory cytokines as well as other mediators (Cohen, 2002) designed to efficiently control growth of pathogens, and to destroy pathogens. Therefore,

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administration of LPS upregulates inflammatory mediators; TNFα, IL-1β (Vereker *et al.*, 2000a), NFκB (Zhang & Ghosh, 2000) and others, resulting in an activation of microglia and other immunoreactive cells. As a result of its potent and widespread effects that mimic an inflammatory reaction, LPS treatment is often used as a model of inflammation. Using this model, both LTP and cognitive function are impaired; acute LPS administration results in inhibition of LTP in the dentate gyrus (Vereker *et al.*, 2000b) inhibition of spatial learning (Shaw *et al.*, 2001), diminished performance in a passive avoidance task and the elevated maze (Jain *et al.*, 2002), and impairment of memory consolidation in contextual fear conditioning (Pugh *et al.*, 1998). The LPS molecule is composed of three regions; Lipid A, the core region and an O antigenic side chain. Lipid A consists of disaccharide, phosphoryl groups and fatty acid. The core region consists of an outer core and an inner core containing heptose and 2-keto-3-deoxyoctonic acid (KDO). Lipid A and KDO region are essential for LPS signalling and cytokine production. In animal studies LPS has been shown to induce negative systemic effects. Studies report a decrease in feeding, activity and social behaviour (Lacosta *et al.*, 1999). LPS has also shown to be associated with apoptosis in a variety of cell types such as mouse lymphocytes and hippocampal neurons (Norimatsu *et al.*, 1995; Vereker *et al.*, 2000b). It is clear that LPS can induce a myriad of effects in the CNS.

1.11.2 LPS and inflammation

Elucidation of the LPS signalling pathway has made a major contribution to understanding the immune response. LPS binding protein (LBP) was identified as a plasma protein that binds to the lipid moiety of LPS (Tobias *et al.*, 1986). The generation of LBP deficient mice revealed a non-redundant role for LBP in the response to LPS (Jack *et al.*, 1997). The formation of an LPS and LBP complex was shown to trigger the association of this complex with another LPS-binding molecule, CD14 (Tobias & Ulevitch, 1993). The first described

mammalian homologue of Drosophila Toll was found to work downstream from CD14 and is principally responsible for delivering the LPS signal (Medzhitov *et al.*, 1997). The importance of CD14 in the response to LPS has been demonstrated in CD14 deficient mice, these mice exhibited a reduced response to LPS (Haziot *et al.*, 1996; Moore *et al.*, 2000). The LPS receptor, TLR4, is a member of the IL-1R/TLR superfamily and is involved in the recognition of host-derived products. During inflammation or tissue injury, extracellular matrix components, such as fibronectin or collagen, are degraded by proteases. Degraded products then trigger inflammatory responses. Degraded products have been shown to induce their inflammatory responses via TLR4 (Smiley *et al.*, 2001).. Fibrinogen, a fragment of fibronectin has been shown to stimulate chemokine production from macrophages in a TLR4-dependent manner (Smiley *et al.*, 2001). Activation of TLR4 by microbial components leads to an induction of numerous genes that function in inflammatory and immune responses. These include inflammatory cytokines such as TNF-α, IL-1β and IL-6 and chemokines, such as MIP-1α, MIP-2. Activation of TLR4 also leads to an upregulation in MHC and co-stimulatory molecule expression on the cell surface (Medzhitov & Janeway, 1997; Akira, 2001; Medzhitov, 2001). Association of the LPS complex with TLR4 activates downstream inflammatory signalling pathways in a similar manner to other IL-1R/TLR superfamily members. MyD88 can interact with the TIR domain of TLR4. Upon stimulation, MyD88 recruits a death-like domain containing serine/threonine kinase, the IRAK1. Activation of IRAK1 by phosphorylation facilitates the interaction and activation of tumour necrosis factor receptor-associated factor 6 (TRAF6)(Cao *et al.*, 1996). TRAF-6 in turn associates and activates TGF-β activated kinase 1 (TAK1). TRAF-6-activated TAK1 phosphorylates IKKβ, which leads to the phosphorylation of the NF-κB pathway (Wang & Richmond, 2001)(see section 1.9).

1.11.3 LPS and IL-1β

One of the main cytokines produced in response to LPS is IL-1β and much evidence suggests that many LPS-induced effects are mediated by IL-1β. Consistently, IL-1β antagonists and IL-1ra attenuated the LPS-induced decrease in feeding (Swiergiel *et al.*, 1997) and impairment in learning and memory (Pugh *et al.*, 1998). These results suggest that IL-1β and/or IL-1α stimulation of their receptors is necessary for LPS to have its effects and recent evidence indicates that IL-1β and LPS function to activate the same inflammatory transcription factors. It is well documented that peripheral administration of LPS results in an increase in IL-1β concentration in the brain (Nolan *et al.*, 2004). There is also evidence that peripheral administration of LPS increase IL-1β mRNA expression and protein levels in the spleen, pituitary and the brain of mice and also in rat microglia (Goujon *et al.*, 1996). In addition to activating IL-1β centrally which can activate JNK and p38 signalling pathways (Vereker *et al.*, 2000b), LPS itself can directly activate JNK. LPS has been shown to activate MKK4 (O'Neill & Greene, 1998) and numerous studies have reported an LPS-associated increase in JNK activity (Hambleton *et al.*, 1996; Pyo *et al.*, 1998; Loscher *et al.*, 2000)

1.12 Anti-Inflammatory cytokines

The regulation of immune responses is accomplished by a network of soluble molecules, including cytokines that can function in a synergistic or antagonistic manner. Th cells secrete distinct subsets of cytokines (Mosmann *et al.*, 1986). Th1 cells produce IL-2, IFNγ, and TNFβ, which activate macrophages to stimulate cellular immunity and inflammation. Th1 cells promote IL-3 and GM-CSF secretion to stimulate the bone marrow to produce more leukocytes. Th2 cells promote IL-4, IL-5, IL-6, and IL-10 secretion, which stimulates antibody production by B cells. Th2 derived cytokines promote humoral

immunity and oppose Th1-dependent activities. Anti-inflammatory cytokines activate a number of signalling pathways that influence gene expression and can result in the suppression of pro-inflammatory cytokines, chemokines and certain cell surface molecules. Anti-inflammatory cytokines interact with their respective receptors in a similar manner to pro-inflammatory cytokines and the Janus activated protein kinase (JAK) and signal transducers and activators of transcription (STAT) signalling pathways are also employed to induce downstream effects (Keegan et al., 1994).

1.12.1 Interleukin-10 (IL-10)

IL-10 has potent effects in numerous cell populations. IL-10 was first described as a cytokine synthesis inhibiting factor (CSIF)(Fiorentino et al., 1989). Although IL-10 exerts potent anti-inflammatory effects it is referred to as an immunoregulatory cytokine. IL-10 promotes the development of a Th2 cytokine pattern by inhibiting the IFN-γ production by T-lymphocytes. IL-10 suppresses pro-inflammatory cytokine production by monocytes and macrophages (Fiorentino et al., 1991; Romagnani, 1995b, 1995a). It was suggested that IL-10 may exert its anti-inflammatory effect by blocking nuclear translocation of the NFκ-B P65/P50 heterdimer in macrophages (Wang et al., 1995; Clarke et al., 1998).

1.12.2 Interleukin-4 (IL-4)

IL-4 was originally described as a B-cell stimulating factor (Howard & Paul, 1982) inducing B-cell activation and proliferation. Since then the number of functions attributed to IL-4 has increased and includes modulation of macrophage functions and T-cell differentiation. IL-4 activates at least four distinct signalling pathways to influence gene expression – phosphatidylinositol-3 kinase (Hirasawa et al., 2000), phosphorylation of insulin

receptor substrates by IL-4Rα chain (Zamorano *et al.*, 1996) activation of Ras/MAP kinases including ERK (David *et al.*, 2001) and activation of the JAK/STAT pathway (Keegan *et al.*,1994). Binding of IL-4 to its receptor results in the translocation of STAT-6 to the nucleus (Nelms *et al.*, 1999). In the nucleus, STAT-6 binds to STAT-binding elements to activate gene transcription (Decker & Majchrzak, 1992). Genes induced by IL-4 include IL-4Rα, IL-1ra, IL-4 and MHC II (Paludan, 1998). IL-4 also inhibits activation of genes associated with inflammation including IL-1β, IL-12, TNFα and iNOS which generates NO (Paludan, 1998). While the mode of action of IL-4 is not entirely clear many of these activities are STAT-6 dependent. Ultimately, IL-4 binding results in the suppression of macrophage activity and the differentiation of Th cells towards Th2 cell type further promoting the bias towards anti-inflammatory cytokines (Paludan, 1998). Among the factors that regulate Th2 differentiation, IL-4 is the major known determinant. IL-4 is expressed as a 15-19 kDa protein and exists as a dimer (Yokota *et al.*, 1998). IL-4 is produced by T cells, mast cells and basophils. IL-4 and the closely related cytokine, IL-13, signal through a shared surface receptor IL-4α, which activates the transcription factor STAT-6 (Takeda & Akira, 2000; Jung *et al.*, 2002). STAT-6 deficient mice exhibit a similar phenotype to IL-4 deficient mice (Barnes, 2002).

1.13 Study Objectives:

The overall aim of this study was to assess the possibility that IL-1F5 might exert an anti-inflammatory effect in brain. Specifically, the objective was to investigate the modulating effect of IL-1F5 on IL-1β and especially LPS-induced changes in the hippocampus

Materials and Methods

2.1 Materials

Acrylamide Sigma

Actin antibody   Santa Cruz

Amonium persulfate   Sigma

Anti-goat IgG HRP Vector

Anti-mouse FITC conjugated antibody Vector

Anti-mouse IgG HRP Sigma

Bio-Rad dye reagent concentrate Bio-Rad

Bis-acrylamide Sigma

Bovine serum albumin Sigma

Bromophenol blue Sigma

B-27 supplement Gibco

Calcium chloride Lennox

CD11b antibody Serotec

Diethylpyrocarbonate

Dimethyl sulphoxide Sigma

Dithiothreitol Sigma

DNase Sigma

Dulbecco's modified Eagle media Gibco

EDTA Sigma

ERK antibody Santa Cruz

EGTA Sigma

Ethanol Lennox

EtOH Sigma

Fluorescent mounting medium Vector

Foetal bovine serum Gibco

Glucose Lennox

Glutamax Gibco

Glycine Sigma

Horse serum (heat-inactivated) Gibco

HEPES Sigma

Hydrochloric acid Lennox

Hyperfilm Amersham

Recombinant rat IL-1β R&D Systems

IL-1β ELISA DuoSet R&D Systems

IL-4 ELISA DuoSet R&D Systems

JAK1 antibody Santa Cruz

JNK1 antibody Santa Cruz

Lipopolysacharide Sigma

Magnesium sulphate Sigma

Magnesium chloride Sigma

β-Mercaptoethanol Sigma

Methanol Lennox

Neurobasal media Gibco

Nitrocellulose membrane Sigma

Normal goat serum Vector

Penicillin/Streptomycin Gibco

Phenylmethylsulphonyl fluoride Sigma

Sterile phosphate-buffered saline (10X) Sigma

Phospho-JNK antibody Santa Cruz

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

ReBlot Plus strong antibody stripping solution Chemicon

SIGGIR antiboby R&D Systems

Sodium azide Sigma

Sodium carbonate Sigma

Sodium chloride Sigma

Sodium dodecylsulphate Sigma

Sodium hydrogen carbonate Lennox

Sodium hydroxide Lennox

Sodium phosphate (monobasic) Sigma

Sodium phosphate (dibasic) Sigma

Standard grade No. 3 filter paper Whatman

Stat6 antibody Santa Cruz

Sucrose Lennox

Sulphuric acid Lennox

SuperSignal West Dura extended duration substrate Pierce

Tris-base Sigma

Tris-HCl Sigma

Trypsin Sigma

Trypsin Inhibitor Sigma

Tween-20 Lennox

Urethane Sigma

Vectastain ABC kit Vector Laboratories

IL-1F5 standard was kindly donated by Luke O Neill, Biochemistry Department, Trinity College Dublin

2.2 Animals

2.2.1 Housing of animals

An inbred strain of male Wistar rat was used for experiments, aged between 2 and 4 months, supplied by the Bioresources unit, Trinity College Dublin. The animals weighed between 200 and 350g. were maintained under a 24hour light dark cycle in the Bioresources unit. Food, consisting of normal Laboratory chow and water was available *ad libitum*. Ambient temperature was controlled between 22 and 23°C.

A C57 mouse strain was used as control for all experiments involving knockout animals. All animals were aged between 4 – 6 weeks and supplied by the Bioresources unit, Trinity College Dublin. The animals weighed between 20 and 40g. Animals were housed in groups of 5 and according to sex and maintained under a 24hour light dark cycle in an SPF environment in the Bioresources unit. Ambient temperature was controlled between 22 and 23°C.

An IL-4$^{-/-}$ mouse strain was used for some experiments. These animals were aged between 4-6 weeks and supplied by the Bioresources unit, Trinity College Dublin. The animals weighed between 20 and 40g. Animals were housed in groups of 5 and according to sex and maintained under a 24hour light dark cycle in an SPF free environment in the Bioresources unit. Alternatively breeding pairs were set up with one male and 2 female per cage. Ambient temperature was controlled between 22 and 23°C.

A SIGIRR$^{-/-}$ mouse strain was used for some experiments. These animals were kindly donated by Dr.A Mantovani, Department of Immunology and Cell Biology, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy. The animals weighed between 20 and 40g. Animals were housed in groups of 5 and according to sex and maintained under a 24hour light dark cycle in an SPF

environment in the Bioresources unit. Alternatively, breeding peirs were set up with one male/female pair per cage. Ambient temperature was controlled between 22 and 23°C.

Animal experimentation was performed under a license granted by the 0lnister for Health and Children (Ireland) under the Cruelty to Animals Act 1876 and the European Community Directive, 86/609/EC.

2.2.2 Lipopolysaacahride administration

In experimental treatment groups rats were anaesthetised by intraperitonael (ip) injection of urethane (1.5g/kg; 33% w/v). Depth of anaesthesia was determined by the absence of the pedal reflex and if needed a top up dose of urethane was used (maximum 2.5g/kg). Rats were then injected ip with sterile 0.9% w/v saline or lipopolysaacharide (LPS; 100μg/kg in sterile 0.9% w/v saline) from Escherichia coli serotype 0111:B4 (Sigma, Dorset, UK). Three hours following administration of saline/LPS, rats were assessed for their ability to maintain long-term potentiation (LTP).

In knockout experimental treatment groups mice were anaesthetised by ip injection of urethane (1.2g/kg; 33% w/v). Depth of anaesthesia was determined by the absence of the pedal reflex and if needed a top up dose of urethane was used (maximum 1.5g/kg). Mice were then injected ip with sterile 0.9% w/v saline or lipopolysaacharide (LPS; 200μg/kg in sterile 0.9% w/v saline). Three hours following administration of saline/LPS, mice were sacrificed.

2.2.3 IL-1β and IL-1F5 administration

In experimental treatment groups where rats were challenged with LPS, rats were given an intracerebroventricular (icv) injection. Fur on the scalp was clipped and the head was positioned in a head holder in a stereotaxic frame (ASI Instruments). A midline incision was made with a scalpel and the skin pulled back to reveal the skull. The peristeum was scraped clear and a Bregma

and Lamda were identified. A dental drill was used to drill a small hole in the skull 2.5mm posterior and 0.5mm lateral, to Bregma, through which agents could be injected. Rats were administered IL-1F5 (5µl of 30ng/ml) and 5mins later were administered saline or LPS by ip injection. Animals were assessed for their ability to sustain LTP 3 hours after injection. In some experiments rats were given IL-1β (5µl of 3.5ng/ml; R&D systems, UK) and IL-1F5 (3µg/ml) icv and in this case were assessed for their ability to maintain LTP after 30mins.

In experimental treatment groups where mice were challenged with LPS, mice were given an icv injection. Fur on the scalp was clipped and the head was positioned in a head holder in a stereotaxic frame (ASI Instruments). A midline incision was made with a scalpel and the skin pulled back to reveal the skull. The peristeum was scraped clear and a Bregma and Lamda were identified. A 0.3mm gauge needle was used to form a small hole in the skull 1mm posterior and 0.6mm lateral, to Bregma, though which agents could be injected. Mice were administered IL-1F5 and 5mins later were administered saline or LPS by ip injection

2.3   Induction of LTP *in vivo*

2.3.1 Preparation of animals

Following icv injection, animals were assessed for their ability to sustain LTP. A window of the skull was removed using a dental drill, allowing correct placement of electrodes. The dura mater was pierced and pulled back to expose the brain. The recording chamber, consisting of the stereotaxic frame was surrounded by a Faraday cage to inhibit outside interference. All instruments within the cage were grounded to eliminate 50Hz cycle noise.

2.3.2 Electrode implantation

Bipolar stimulating electrodes and unipolar recording electrodes (Clark Electromedical, UK) were used in this study. The stimulating electrode was placed on the surface of the brain, 4.4mm lateral to lambda. The recording

electrode was placed on the surface of the brain 2.5mm lateral and 3.9mm posterior to Bregma. The positions of the stimulating and recording electrodes were carefully monitored as they were lowered in increments through the cortical and hippocampal layers into the perforant path and granule cell layer of the dentate gyrus respectively, until the characteristic perforant path granule cell synapse response was observed. The depth of electrodes was finely adjusted so as to maximize the response. This was carried out by generating 0.1msec duration, 2msec delay, and 4V pulse through the stimulating electrode at a frequency of 0.1Hz. Evoked responses were picked up by the recording electrode and displayed on an Apple Macintosh computer (Performa 5200). The final depth of the recording electrode was between 2.5 and 3.5mm and for the stimulating electrode was between 2.5 and 3mm. Stimuli were then delivered at 30sec intervals.

2.3.3   EPSP recordings

The population field post-synaptic potential   (field EPSP) was used as a measure of excitatory synaptic transmission in the dentate gyrus. EPSP's were recorded by passing a single square wave of current at low frequency (0.033Hz, 0.1sec, 2msec delay) which was generated by a constant isolation unit (IsoFlex, UK), to the bipolar stimulating electrode. The evoked response was transmitted via a pre-amplifier (DAM 50; Differential Amplifier; gain 75, World Precision Instruments, USA) to an analogue digital converter (Maclab/2e, Analog Digital Instruments). This digitized system was then linked to an Apple Macintosh computer (Performa 5200) via a specifically written software package (Scope, Version 3.36).

The slope of EPSP was taken as an indicator of excitatory synaptic transmission. After a period of stabilization, test shocks at 1/30sec were recorded for a 10mins control period to establish baseline recordings. This was followed by a delivery of 3 trains of a stimuli (250Hz fro 200msec) at 30sec intervals. Recording at test shock frequency then resumed for 40mins.

2.4 Preparation of tissue

2.4.1 Dissection

Both rats and mice were killed by cervical dislocation and decapitation. The brains were rapidly removed. In some experiments involving rats one half of the brain was coated in OCT compound (Sakura Tissue-Tek, Netherlands), immersed in liquid N_2 and stored at -80°C until sections were prepared. From the remaining half of the brain the hippocampus and cortex were quickly dissected free on ice.

2.4.2 Preparation of slices for freezing

Freshly dissected tissue was sliced bidirectionally to a thickness of 350μm using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK). Tissue was placed in aliquots in Eppindorf tubes containing 1ml Krebs solution (composition in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_47H_2O$ 1.18, $NaHCO_3$ 16, glucose 10) with added $CaCl_2$ (2mM final concentration). Tissue slices were vortexed and allowed to settle, before being washed twice more in this Krebs solution. The slices were the rinsed with Krebs solution containing 2mM $CaCl_2$ containing 10% dimethyl sulphoxide (Haan and Bowen, 1981) and stored at –80°C until required for later analysis.

2.4.3 Protein Quantification

Protein quantification was assessed according to Bradford (1976). Samples were diluted 10μl in 150μl dH_2O in a 96-well plate (Starstedt microtest plate). Standards were prepared from a 200μg/ml stock solution of bovine serum albumin (BSA; Sigma Dorset, UK). A range of standards was prepared from this solution from 200μg/ml to 3.125μg/ml. Duplicate standards were added to the 96-well plate and Bio-Rad dye reagent concentrate (40μl; Bio-Rad, Hertfordshire, UK) was added to both standards and samples. Absorbance was

measured at 630nm using a 96-well plate reader (Labsystems Multiskan RC). A regression line was plotted (GraphPad Prism) and the concentration of protein was calculated and converted to mg protein/ml.

2.5 Experiments *in vitro*

2.5.1 Preparation of sterile coverslips

Glass coverslips (13mm diameter; Chance Propper, UK) were soaked in 70% ethanol for 1hr followed by overnight exposure to ultraviolet light. Coverslips were coated with poly-L-lysine (40µg/ml in sterile dH2O; Sigma, UK) for 1 hr at 37°C so as to provide a suitable surface for cells to adhere. Coated coverslips were air-dried, placed in 24-well plates (Greiner, Austria) and stored at 4°C until required.

2.5.2 Primary culture of hippocampal neurons from wistar rats

Primary hippocampal neurons were established from Wistar rats 1 day postpartum (Bioresources Unit, 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 resusupended in warm neurobasal medium (NBM; Gibco, UK), supplememted with penicillin (100U/ml; Gibco, UK), heat-inactivated horse serum (10% Gibco, UK), streptomycin (100U/ml; Gibco, UK), glutatmax (2mM; Gibco, UK) and B27 (1%; Gibco, UK). Resusupended neurons were plated onto each poly-L-lyseine coated glass coverslip at a density of 0.25×10^6 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.

2.5.3 Primary culture of cortical glia from wistar rats

Glia were isolated from cerebral cortices of waster rats 1 day postpartum (Bioresearches Unit, Trinity College, Dublin). Rats were decapitated and cortices dissected. Cortices were placed in 3ml DMEM supplemented with 10% foetal bovine serum (Gibco, UK), penicillin (100U/ml; Gibco, UK) and streptomycin (100U/ml; Gibco, UK). Tissue was triturated (x7), passed through a sterile nylon mesh filter and centrifuged (2500g for 3mins at 20°C). The pellet was resuspended in DMEM. Resuspended glia were placed on the centre of each coverslip and allowed to adhere to the glass coverslip for 2hrs in a humidified incubator containing 5% CO_2: 95% air at 37°C before 400µl of pre-warmed DMEM was added to each well. Cells were grown for 10 days prior to treatment and media replaced every 3 days.

2.5.4 Primary culture of mixed glia from mice

Glia were isolated from C57, IL-4$^{-/-}$ and SIGIRR $^{-/-}$ 1 day postpartum (BioResources Unit, Trinity College, Dublin). Mice were decapitated and the whole brain was dissected free. Whole brain samples from two animals were pooled and placed in 3ml DMEM supplemented with 10% foetal bovine serum (Gibco, UK), penicillin (100U/ml; Gibco, UK) and streptomycin (100U/ml; Gibco, UK). Tissue was triturated (x7), passed through a sterile nylon mesh filter and centrifuged (2500g for 3mins at 20°C). The pellet was resuspended in DMEM. Resuspended glia were placed on the centre of each coverslip and allowed to adhere to the glass coverslip for 2 hr in a humidified incubator containing 5%

CO_2: 95% air at 37°C before 400µl of pre-warmed DMEM was added to each well. Cells were grown for 10 days prior to treatment and media replaced every 3 days.

2.6 Cell Treatments

2.6.1 LPS

LPS from Escherichia coli serotype 0111:B4 (Sigma, Dorset, UK) was prepared as a stock solution of 1mg/ml in DMEM and used at a final concentration of 100ng/ml and 1µg/ml. Cells were treated with sterile filtered (0.2µm cellulose acetate membrane filter (Pall Gelman Sciences Inc., USA) LPS for 24 hrs.

2.6.2 IL-1F5

IL-1F5 (kindly donated by O Neill Lab, Trinity College Dublin) was diluted to a stock concentration of 300µg/ml in PBS and stored at -80°C. For cell treatment IL-1F5 was diluted to a final concentration of 3µg/ml in media. Cells were treated with sterile filtered (0.2µm cellulose acetate membrane filter (Pall Gelman Sciences Inc., USA) IL-1F5 for 24hrs.

2.6.3 IL-1β

Recombinant rat IL-1β (R&D Systems, USA) was prepared as a stock solution of 1µg/ml in sterile PBS and used at a final concentration of 5ng/ml in media. Cells were treated with sterile filtered (0.2µm cellulose acetate membrane filter (Pall Gelman Sciences Inc., USA) IL-1β for 24 hrs.

2.6.4 anti-SIGIRR

An antibody to the single immunoglobulin IL-1 related receptor (anti-mSIGIRR; R&D Systems, Minneapolis, USA). Anti-SIGIRR was diluted to a concentration of 100µg/ml in PBS. Working concentrations of 20µg/ml were prepared in media.

Cells were incubated in sterile filtered (0.2μm cellulose acetate membrane filter (Pall Gelman Sciences Inc., USA) anti-SIGIRR for a period of 4 hrs.

2.6.5 GW9662

An antagonist to Peroxisome proliferator-activated receptor-gamma (PPARγ) GW9662 (Sigma, UK) was diluted to a concentration of 10M in PBS. Working concentrations of 100nM were prepared in media. Cells were incubated in sterile filtered (0.2μm cellulose acetate membrane filter (Pall Gelman Sciences Inc., USA) GW9662 for a period of 1hr.

2.7 Analysis of Cytokines *ex vivo*

2.7.1 Preparation of samples

Hippocampal slices were thawed rapidly and washed 3 times in Krebs solution (composition in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10) containing 2mM $CaCl_2$. The slices were homogenized (x60 strokes) in Krebs solution (400μl) containing 2mM $CaCl_2$ using a 1ml glass homogeniser (Jencons, Bedfordshire, UK). Protein concentrations were assessed and equalized with Krebs solution containing 2mM $CaCl_2$. Samples were stored at -80°C until required.

2.7.2 Analysis of rat interleukin-1β concentration

Enzyme Linked ImmunoSorbent Assay (ELISA) method was used to determine the concentration of interleukin-1β (IL-1β). 96-well plates (Nunc-Immuno plate with MaxiSorp surface) were coated with capture antibody (100μl; 1μg/ml; goat anti-rat IL-1β in PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4 and 1.5mM KH_2PO_4 pH7.3; R&D systems, Minneapolis, USA) and incubated overnight at room temperature (RT). A wash buffer of PBS containing 0.05% tween-20, pH7.4 was used to wash the plate 3 times and a blocking buffer (300μl; PBS containing 1% BSA, 5% Sucrose and 0.05% NaN_3) was added to the wells and

the plates were incubated at RT for 1 hour. Standards were prepared from rat recombinant IL-1β (R&D systems Minneapolis, USA) diluted in PBS containing 1% BSA. Plates were washed 3 times in wash buffer and 100μl of standards and samples were incubated for 2 hours at RT. Following this incubation, plates were washed 3 times with wash buffer and streptavidin-horseradish peroxidase conjugate (100μl; 1:200 dilution in PBS containing 1% BSA; R&D systems, Minneapolis, USA) was added and incubated at RT for 20mins. The plates were washed 3 times with wash buffer and substrates solution (100μl; 1:1 dilution Reagent A (H_2O_2) and Reagent B (tetramethylbenzidine); R&D systems, Minneapolis, USA) was added to the well. The plates were incubated in the dark for 1 hour, creating a colour change to blue. A stop solution (1M H_2SO_4; 50μl) was added to each well. The plates were read at 450nm on a 96-well plate reader (Labsystems Multiskan RC). A standard curve was produced and results were expressed as pg IL-1β/mg tissue corrected for protein or as ρg/ml supernatant for *in vitro* experiments.

2.7.3 Analysis of mouse interleukin-1β concentration

Enzyme Linked ImmunoSorbent Assay (ELISA) method was used to determine the concentration of interleukin-1β (IL-1β). 96-well plates (Nunc-Immuno plate with MaxiSorp surface) were coated with capture antibody (100μl; 1μg/ml; anti-mouse IL-1β in PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4 and 1.5mM KH_2PO_4 pH7.3; R&D systems, Minneapolis, USA) and incubated overnight at RT. A wash buffer of PBS containing 0.05% tween-20, pH7.4 was used to wash the plate 3 times and a blocking buffer (300μl; PBS containing 1% BSA) was added to the wells and the plates were incubated at RT for 1 hour. Standards were prepared from mouse recombinant IL-1β (R&D systems Minneapolis, USA) diluted in PBS containing 1% BSA. Plates were washed 3 times in wash buffer and 100μl of standards and samples were incubated for 2 hours at RT. Following this incubation plates were washed 3 times with wash buffer and

streptavidin-horseradish peroxidase conjugate (100μl; 1:200 dilution in PBS containing 1% BSA; R&D systems, Minneapolis, USA) was added and incubated at RT for 20mins. The plates were washed 3 times with wash buffer and substrates solution (100μl; 1:1 dilution Reagent A (H_2O_2) and Reagent B (tetramethylbenzidine); R&D systems, Minneapolis, USA) was added to the well. The plates were incubated in the dark for 45mins, creating a colour change to blue. A stop solution (1M H_2SO_4; 50μl) was added to each well. The plates were read at 450nm on a 96-well plate reader (Labsystems Multiskan RC). A standard curve was produced and results were expressed as ρg IL-1β/mg tissue corrected for protein or as pg/ml supernatant for *in vitro* experiments.

2.7.4   Analysis of rat interleukin-4 concentration

ELISA method was used to determine the concentration of interleukin-4 (IL-4). 96-well plates (Nunc-Immuno plate with MaxiSorp surface) were coated with capture antibody (100μl; 2μg/ml; monoclonal mouse anti-rat IL-4 in PBS; R&D systems, Minneapolis, USA) and incubated overnight at RT. A wash buffer of PBS containing 0.05% tween-20, pH7.4 was used to wash the plate 3 times and a blocking buffer (300μl; PBS containing 1% BSA, 5% Sucrose and 0.05% NaN_3) was added to the wells and the plates were incubated at RT for 1 hour. Standards were prepared from rat recombinant IL-4 (R&D systems Minneapolis, USA) diluted in PBS containing 1% BSA. Plates were washed 3 times in wash buffer and 100μl of standards and samples were incubated for 2 hours at RT. Following this incubation plates were washed 3 times with wash buffer and detection antibody (100μl; 50ng/ml; biotinyltaed goat anti-rat IL-4 in PBS containing 1% BSA; R&D systems, Minneapolis, USA) was added and incubation continued for 2 hours at RT. The plates were washed 3 times in wash buffer and streptavidin horseradish peroxidase conjugate (100μl; 1:200 dilution in PBS containing 1% BSA; R&D systems, Minneapolis, USA) was added and incubation continued at RT for 1 hour. The plates were washed 3 times with

wash buffer and substrate solution (100µl; 1:1 dilution Reagent A (H_2O_2) and Reagent B (tetramethylbenzidine); R&D systems, Minneapolis, USA) was added to the well. The plates were incubated in the dark for 1 hour, creating a colour change to blue. A stop solution (1M H_2SO_4; 50µl) was added to each well. The plates were read at 450nm on a 96-well plate reader (Labsystems Multiskan RC). A standard curve was produced and results were expressed as ρg IL-4/mg tissue corrected for protein or as pg/ml supernatant for *in vitro* experiments.

2.7.5   Analysis of mouse interleukin-4 concentration

ELISA method was used to determine the concentration of interleukin-4 (IL-4). 96-well plates (Nunc-Immuno plate with MaxiSorp surface) were coated with capture antibody (100µl; 2µg/ml; goat anti-mouse IL-4 in PBS; R&D systems, Minneapolis, USA) and incubated overnight at RT. A wash buffer of PBS containing 0.05% tween-20, pH7.4 was used to wash the plate 3 times and a blocking buffer (300µl; PBS containing 1% BSA) was added to the wells and the plates were incubated at RT for 1 hour. Standards were prepared from mouse recombinant IL-4 (R&D systems Minneapolis, USA) diluted in PBS containing 1% BSA. Plates were washed 3 times in wash buffer and 100µl of standards and samples were incubated for 2 hours at RT. Following this incubation plates were washed 3 times with wash buffer and detection antibody (100µl; 50ng/ml; biotinyltaed goat anti-mouse IL-4 in PBS containing 1% BSA; R&D systems, Minneapolis, USA) was added and incubation continued for 2 hours at RT. The plates were washed 3 times in wash buffer and streptavidin horseradish peroxidase conjugate (100µl; 1:200 dilution in PBS containing 1% BSA; R&D systems, Minneapolis, USA) was added and incubation continued at RT for 1 hour. The plates were washed 3 times with wash buffer and substrate solution (100µl; 1:1 dilution Reagent A (H_2O_2) and Reagent B (tetramethylbenzidine); R&D systems, Minneapolis, USA) was added to the well. The plates were incubated in the dark for 45mins, creating a colour change to blue. A stop

solution (1M H_2SO_4; 50μl) was added to each well. The plates were read at 450nm on a 96-well plate reader (Labsystems Multiskan RC). A standard curve was produced and results were expressed as ρg IL-4/mg tissue corrected for protein or as pg/ml supernatant for *in vitro* experiments.

2.8 Fluorescent Immunocytochemistry

2.8.1 JNK phosphorylation

Following treatment of cultured neurons, coverslips were washed in TBS, and fixed with absolute alcohol. Cells were permeabilised with 0.1% Triton X-10 and non-reactive sites were blocked with goat serum (blocking buffer; 5% in PBS). To determine the intracellular distribution of phosphorylated JNK, cells were incubated overnight with an anti-active JNK antibody (100μl; 1:400 dilution in 10% blocking buffer; Santa Cruz, USA) purified from mouse serum. This antibody was raised against a peptide corresponding to a short amino acid sequence of JNK1 and JNK2 of human origin containing phosphorylated Threonine-183 and Tyrosine-185. Coverslips were washed 3 times in PBS and secondary antibody was added (100μl; 1:100 dilution; anti-mouse IgG conjugated to FITC; Sigma, UK) for 1hr at RT. Coverslips were washed several times in dH_2O, before being mounted onto microscope slides using a mounting medium for fluorescence (Vector, USA) and the perimeter of each coverslip was sealed using nail varnish. Mounted coverslips were viewed under x40 magnification by fluorescence microscopy (Leitz Orthoplan Microscope) using Improvision software (Improvision, UK). Cells were observed under excitation, 490nm; emission, 520nm for FITC labelled antibodies.

2.8.2 ERK Expression

Following treatment of cultured neurons, coverslips were washed in TBS, and fixed with absolute alcohol. Cells were permeabilised with 0.1% Triton X-10 and non-reactive sites were blocked with goat serum (blocking buffer; 5% in PBS).

To determine the intracellular distribution of phosphorylated ERK cells were incubated overnight with an anti-active ERK antibody (100μl; 1:400 dilution in 10% blocking buffer; Santa Cruz, USA) purified from mouse serum. This antibody was raised against a peptide corresponding to a short amino acid sequence of ERK1 and ERK2 corresponding to human ERK1 and ERK2 containing phosphorylated p44 and p42 sub-units. Coverslips were washed 3 times in PBS and secondary antibody was added (100μl; 1:100 dilution; anti-mouse IgG conjugated to FITC; Sigma, UK) for 1hr at RT. Coverslips were washed several times in dH_2O, before being mounted onto microscope slides using a mounting medium for fluorescence (Vector, USA) and the perimeter of each coverslip was sealed using nail varnish. Mounted coverslips were viewed under x40 magnification by fluorescence microscopy (Leitz Orthoplan Microscope) using Improvision software (Improvision, UK). Cells were observed under excitation, 490nm; emission, 520nm for FITC labelled antibodies.

2.8.3   SIGIRR Expression

Following treatment of cultured glia, coverslips were washed in TBS, and fixed with absolute alcohol. Cells were permeabilised with 0.1% Triton X-100 and non-reactive sites were blocked with goat serum (blocking buffer; 5% in PBS). To determine the intracellular distribution of SIGIRR, cells were incubated overnight with an anti-SIGIRR antibody (20μl; 1:500 dilution in 10% blocking buffer; R&D systems, UK) purified from goat serum. This antibody was raised against a peptide corresponding to a short amino acid sequence of mouse SIGIRR . Coverslips were washed 3 times in PBS and secondary antibody was added (100μl; 1:100 dilution; anti-goat IgG conjugated to FITC; Sigma, UK) for 1hr at RT. Coverslips were washed several times in dH_2O, before being mounted onto microscope slides using a mounting medium for fluorescence (Vector, USA) and the perimeter of each coverslip was sealed using nail varnish. Mounted coverslips were viewed under x40 magnification by fluorescence microscopy (Leitz Orthoplan Microscope) using Improvision software

(Improvision, UK). Cells were observed under excitation, 490nm; emission, 520nm for FITC labelled antibodies.

2.9 Immunostaining

2.9.1 Preparation of cryostat sections

Half brain preparations were maintained at -80°C. Using a tissue slicer (Leica CM1900), 20μM cryostat sections were prepared. The sections were mounted on gelatine coated slides and air-dried for 30mins. Sections were stored at −20°C until required for immunohistochemical analysis.

2.9.2 Staining sections for MHCII

Frozen cryostats sections were thawed at room temperature. Sections were fixed using ice-cold absolute ethanol for 10mins. Following this sections were washed using TBS (containing in mM: 137mM NaCl, 2.7mM KCL, 8.1mM Na_2HPO_4 and 1.5mM KH_2PO_4 pH7.3; R&D systems, Minneapolis, USA) and then blocked using as solution of 10% normal goat serum (NGS; Vector Laboartories, USA) containing 4% BSA in TBS . Sections were incubated overnight in a humidified chamber at 4°C   in the presence of OX-6 antibody (10μ1/ml; 1:100 in TBS; Serotec, UK). Sections were then washed in TBS and incubated in the presence of a secondary antibody, anti-mouse IgG (1:200 in TBS; Sigma, Dorset, UK). Washing in PBS was repeated and sections were placed in avidin-biotin-horseradish peroxidase solution, diluted in TBS for 1hr (Vectastain elite ABC kit, Vector Laboratories, USA) and then reacted with 3,3'-diaminobenzidine (DAKO Corporation, USA) and H_2O_2 for colour development. The reaction was terminated by washing the sections in double distilled H_2O, and positively stained cells were visualized under the light microscope at X4 and X40 magnification.

2.10   SDS-polyacrylamide gel electrophoresis

2.10.1   Preparation of hippocampal tissue samples

Hippocampal slices were thawed rapidly, washed 3 times in Krebs solution (composition in mM: NaCl 136, KCl 2.54, KH_2PO4 1.18, $MgSO_4$ 1.18, $NaHCO_3$ 16, glucose 10) containing 2mM $CaCl_2$ and homogenized using a 1ml glass homogeniser (Jencons, Bedfordshire, UK). Protein concentrations were assessed (see section 2.4.3) and equalized with Krebs containing 2mM $CaCl_2$. sample buffer (0.5M Tris-Hcl pH 6.8; 20% glycerol (w/v); 2% SDS (w/v); 5% ˇ-mercaptoethanol (v/v); 0.05% bromophenol blue (w/v)) was added to generate a final concentration of 1ng/ml and samples were boiled for 5mins.

2.10.2   Preparation of whole cell lysate

Following the treatment period , the 24-well plates were removed from the incuvator, placed directly on ice , the supernatent media was removed, and in some instances stored for cytokine analysis. The ocverslips were washed with phospahte buffered saline (PBS; 100mM NaCl,   80mM Na_2HPO_4, 20mM NaH_2PO_4 ; pH7.4)

To harvest celllsfor whole cell lysate, 100µl of Lysis Buffer (composition; 25mM HEPES, 5mM MgCl2, 5mM DTT, 5mM EDTA, 2mM PMSF, 10µg/ml leupeptin, 10µg/ml pepstatin A, pH7.4) was added to each well and rocked on ice for 15min. A 1ml syringe insert was used to scrape the cells from the coverslip and the lysis bufffer cell suspension was collected and frozen for further analysis.

2.10.3   Preparation of polyacrylamide gels

Polyacrylamide gels (1mm thick) were prepared with either a monomer concentration of 10% or 12% overlaid with 4% stacking gel between 10cm wide

glass plated, mounted on an electrophoresis unit (Sigma Techware, Dorset, UK Laemmli, 1970). Electrode running buffer (25mM Tris0base; 200mM glyceine; 17mM SDS) was added to the upper and lower reservoirs of the unit. Samples were loaded into the wells using a Hamilton MicroLiter syringe (10µl per well). A pre-stained molecular weight marker (5µl; Sigma, Dorset, UK or Santa Cruz, California, USA) was also loaded. A 32mA current was passed through the unit in order to separate proteins, according to their molecular weight. The current was switched off when the blue dye band was observed to have reached the bottom of the gel.

2.10.4   Semi-dry electrophoretic blotting of proteins

The gel was removed from the gel apparatus, placed on top of a sheet of nitrocellulose membrane (0.45úm pore size; Sigma, Dorset, UK), moistened in transfer buffer (25mM Tris-base; 192mM glyceine; 20%methanol (v/v); 0.05% SDS (w/v)) and cut to the size of the gel. A sandwich was produced by placing filter paper (Standard Grade no.3, Whatman, Kent, UK) was placed on top and beneath the nitrocellulose/gel. The sandwich was soaked in transfer buffer and placed on a titanium electrode (anode) of a semi dry blotter (Sigma, Dorset, UK). Air bubbles were removed. A constant current of 225mA was applied for 75mins.

2.10.5   Western Immunoblotting

The nitrocellulose membrane was blocked for non-specific binding and then probed with an antibody raised against the appropriate protein. The membrane was then washed and incubated with horseradish peroxidase (HRP) conjugated secondary antibody. A chemiluminescent detection agent was added and the membrane was exposed to 5 x 7 inch photographic film (Hyperfilm ECL, Amersham, Buckinghamshire, UK) and developed using a Fuji X-ray processor.

2.10.6   JNK phosphorylation

Non-specific binding was blocked by incubating nitrocellulose membranes overnight at 4°C in Tris-buffered saline (10ml; TBS; 20mM Tris-HCL; 150mM NaCl; pH7.6) containing 5% BSA. Mebranes were washed 3 times for 15mins in TBS-T (10ml). The primary antibody used was a mouse monoclonal IgG antibody raised against a peptide corresponding to a short sequence of JNK1 human origin (10ml; 1:300 dilution in TBS containing 0.1% BSA; Santa Cruz, California, USA). Membranes were incubated in the presence of the primary antibody for 2hrs at RT, and were washed 3 times for 15mins in TBS-T. The secondary antibody (10ml; 1:600 dilution; goat anti-mouse HRP in TBS containing 0.1% BSA; Sigma, Dorset, UK) was added and incubation proceeded for 1hr at RT. Membranes were washed 3 times for 15mins with TBS-T. Supersignal (Pierce, Illinois, USA) was added for 5 mins after which membranes were exposed to photographic film for 1sec in the dark, after which time the film was developed.

2.10.7   Phosphorylation of JAK1

Non-specific binding was blocked by incubating nitrocellulose membranes overnight at 4°C in Tris-buffered saline (10ml; TBS; 20mM Tris-HCL; 150mM NaCl; pH7.6) containing 5% BSA. Membranes were washed 3 times for 15mins with TBS-T (10ml). The primary antibody used was a rat monoclonal IgG antibody raised against a peptide corresponding to a short sequence of JAK1 of mouse origin (10ml; 200μg/ml; 1:200 dilution in TBS containing 0.1% BSA; Santa Cruz, California, USA).Membranes were incubated in the presence of the primary antibody for 2hrs at RT, and then washed 3 times for 15mins with TBS-T. The secondary antibody (10ml; 1:600 dilution; rat anti-goat HRP in TBS containing 0.1% BSA; Sigma, Dorset, UK) was added and incubation proceeded for 1hr at RT. Membranes were washed 3 times for 15mins with TBS-T. Supersignal (Pierce, Illinois, USA) was added for 5mins after which membranes were exposed to photographic film for 1sec in the dark, after which time the film was developed.

2.10.8   Phosphorylation of Stat6

Non-specific binding was blocked by incubating nitrocellulose membranes overnight at 4°C in Tris-buffered saline (10ml; TBS; 20mM Tris-HCl; 150mM NaCl; pH7.6) containing 5% BSA. Membranes were washed 3 times for 15mins with TBS-T. The primary antibody used was a rat monoclonal antibody raised against a peptide corresponding to a short sequence of Stat6 of (10ml; 200µg/ml; 1: 100 dilution in TBS containing 0.1% BSA; Santa Cruz, California, USA). Membranes were incubated in the presence of the primary antibody for 2hrs at RT, and then washed 3 times for 15mins with TBS-T. The secondary antibody (10ml; 1:600 dilution; goat anti-mouse HRP in TBS containing 0.1% BSA; Sigma, Dorset, UK) was added and incubation proceeded for 1hr at RT. Membranes were washed 3 times for 15mins with TBS-T. Supersignal (Pierce, Illinois, USA) was added for 5mins after which membranes were exposed to photographic film for 1sec in the dark, after which time the film was developed.

2.10.9   Phosphorylation of ERK

ERK phosphorylation was assessed in whole-cell lysate using a rat monoclonal IgG1 antibody raised against a peptide corresponding to a sequence of   short sequence of mouse ERK   (10 ml; 200µg/ml; 1:200 dilution in TBS-T containing 1% BSA; Santa Cruz, USA). Membranes were incubated in the presence of the primary antibody for 2 hours at RT, and then washed for 15 mins 3 times in TBS-T. The secondary antibody (10ml; 1:600 dilution; goat anti-mouse HRP in TBS containing 0.1% BSA; Sigma, Dorset, UK) was added and incubation proceeded for 1hr at RT. Membranes were washed 3 times for 15mins with TBS-T. Supersignal (Pierce, Illinois, USA) was added for 5mins after which membranes were exposed to photographic film for 1sec in the dark, after which time the film was developed.

2.10.10   Actin Expression

Following Western Immunoblotting for JNK, JAK1, Stat6 and ERK blots were stripped with an antibody stripping solution and reprobed for analysis of total actin expression to confirm equal loading of the protein. The primary antibody used was a mouse monoclonal IgG antibody corresponding to amino acid sequence mapping the terminus of actin (10ml; 1:200 dilution in TBS containing 0.1% BSA; Santa Cruz, California, USA). Membranes were incubated for 2hrs in the presence of the primary antibody and then washed 3 times for 15 mins with TBS-T. the secondary antibody (10ml; 1:500 dilution; goat anti-mouse IgG HRP in TBS containing 0.1% BSA; Sigma, Dorset, UK) was added and incubation resumed for 1hr at RT. Membranes were washed for 15mins, 3 times in TBS-T. Supersignal (Pierce, Illinois) was added for 5 mins and the membranes were exposed to film and developed.

2.10.11   Densitometry

In all cases quantification of protein bands was achieved by densitometric analysis using an Ultraviolet Transilluminator (GelDoc-It Imaging system, BioImaging Systems, UK). Values were expressed as arbitrary units.

2.11   Analysis of mRNA by reverse transcription PCR

2.11.1   Precautions

RNAses, which easily degrade RNA, are ubiquitous in the environment and therefore all solutions were treated before use with diethyl pyrocarbinate (DEPC; 0.1%(v/v) Sigma, UK), which inactivates RNAses. Solutions containing amines such as Tris were prepared with DEPC-treated H_2O because they cannot be treated directly with DEPC. Carrying out the procedures on ice prohibited degradation of RNA by endogenous RNAses.

2.11.2 RNA extraction from tissue

Hippocampal tissue, which was snap-frozen in liquid nitrogen upon dissection, was homogenized in Tri-reagent (Sigma, UK). To remove any insoluble tissue, the tubes were centrifuged for 10mins at 12,000 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 for 15mins at 12,000 x g 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 1ml Tri-reagent. Samples were incubated at RT for 10mins and then centrifuged for 10mins at 12,000 x g at 4°C. The supernatant was removed and the RNA pellet was was shed with 75% ethanol (Sigma, UK) and mixed well. Samples were centrifuged for 5mins at 7500 x g and then resuspended in DNase-RNase free H_2O (Sigma, UK). RNA samples were stored -80°C until required.

2.11.3 RNA extraction from cultured glia

Total RNA was extracted from cultured cotical glia using Tri-reagent (Sigma, Dorset, UK). Cultured glia were rinsed with sterile PBS and lysed directly on 24 well culture plates by adding 50μl reagent per well and scraping the cells using the rubber end of a 1ml syringe piston (B.Braun Medical Ltd, Melsungen, Germany). Cells were incubated at room temperature for 5mins to allow dissociation of nucleoprotein complexes. 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 for 15mins at 12,000 x g 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 1ml Tri reagent. Samples were incubated at

RT for 10mins and then centrifuged for 10mins at 12,000 x g at 4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol (Sigma, UK) and mixed well. Samples were centrifuged for 5mins at 7500 x g and then resuspended in DNase-RNase free H_2O (Sigma, UK). RNA samples were stored -80°C until required.

2.11.4 Analysis of RNA by gel Electrophoresis

Samples were run on a 1% (w/v) agarose gel to ensure RNA was intact and had not been degraded. Agarose gel was prepared in 1X tris borate EDTA (TBE) buffer (100ml; 0.08 M Tris; 0.04 boric acid; 1mM EDTA; pH 8.3). Ethidium Bromide (EtBr; Sigma, Dorset, UK) was added to give a final concentration of 0.5μg/ml. RNA samples were mixed with 3.5μl of H_2O and 1ml of X6 gel loading buffer (60% (w/v) glycerol, 0.4% (w/v) bromophenol blue) in preparation for gel electrophoresis. Samples were loaded onto gels and RNA was separated by application of a 90 V voltage to the gel apparatus. Migration of the bromophenol blue was monitored and the voltage was switched off when the blue dye band reached the bottom of the gel. The gel was visualized under UV light.

2.11.5 Reverse transcription

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

2.11.6 Polymerase chain reaction

A mastermix PCR mixture (final volume 25µl) containing 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 denaturing step of 95°C for 1mins, followed by 25-35 cycles consisting of a denaturing step of 95°C for 1min, and annealing step of 50-65°C (see table 2.1 for optimal; annealing temperatures) for 1 min and an extension step of 72°C for 1min 30sec. A final extension step of 72°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 visualized under a UV light and photographed using a UV transilluminator (Ultra Violet products Ltd., UK)

Target Gene	Primer sequence		Annealing Temperature (°C)	Band size	No. of cycles
β-actin	Sense	5'AGAAGAGCTATGAGCTGCCTGACG-3'	65		30
	Antisense	5'-CTTCTGCATCCTGTCAGCGATGC-3'		236	
IL-1β	Sense	5'-GCACCTTCTTTTCCTTCATC-3'	59		30
	Antisense	5'-CTGATGTACCAGTTGGGGAA-3'		447	
IL-4	Sense	5'-TCC ATG CAC CGA GAT GTT TGT ACC -3'	60		36
	Antisense	5'- CGT AGG ATG CTC CCT TTA TGA ACG -3'		299	
MHC II	Sense	5'-CAG TCA CAG AAG GCG TTT ATG-3'	58		32
	Antisense	5'-GAT CGC AGG CCT TGA ATG ATG-3'		245	
IL-4mse	Sense	5'-ACTTCTCAGAGATTGTGTCCA-3'	60		30
	Antisense	5'-CTCTAGGAAGAGTTTGTATC-3'		371	
18smse	Sense	5'-GGT TCG AAG ACG ATC AGA TAC -3'	60		30
	Antisense	5'-AAT GGG GTT CAA CGG GTT AC -3'		489	
MHCII mse	Sense	5'-CCAAGAAGAAAATGGCCACA	58		36
	Antisense	5'-TCCGCTGAGATGAACAACTGA		255	

2.11.7   Densitometry

In all cases quantification of mRNA bands was achieved by densitometric analysis using ZERO-Dscan image Analysis System (Scanalytics, USA). Values were expressed as arbitrary units.

2.12   Analysis of mRNA by QT-PCR

2.12.1   Precautions

RNAses which easily degrade RNA are ubiquitous in the environment and therefore all solutions were treated before use with diethyl pyrocarbinate (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. Furthermore, all working surface areas and all equipment (e.g. pipettes, racks. Beakers) were wiped with RNase Away and rinsed with DEPC-H$_2$O.

2.12.2   RNA extraction from tissue

Hippocampal tissue, which was snap-frozen in liquid nitrogen upon dissection, was transferred to RNase free 2ml tubes along with 353.5µl of Cell Lysis mastermix (cell lysis mastermix; 350µl RA1 buffer (from NucleoSpin® RNA II kit, Nagel, USA), 3.5µl β-mercaptoethanol molecular grade (Sigma, UK). Tissue was homogenized by two 5sec pulses with a polytron mixer (Nagel, USA). Samples were then placed in NucleoSpin® Filter units and centrifuged for 1 min at 11,000 x g. 350µl of 70% EtOH (sigma, UK) was added to the homogenized lysate and mixed by pipetting up and down (5 times). Lysate was loaded into a NucleoSpin® RNA II column and centrifuged for 30secs at 8,000 x g. 350µl of membrane desalting buffer was added and the lysate was centrifuged fro 1mins at 11,000 x g. DNA was digested by applying 95µl of DNase reaction mixture directly onto the centre of the silica membrane of the column and the sample was incubated at RT for 15mins. The silica membrane was washed and dried in a series of steps. 200µl of RA2 buffer (from NucleoSpin® RNA II kit, Nagel, USA) was added to the NucleoSpin® RNA II column and the column was centrifuged for 30secs at 8,000 x g. Following this step 600µl of RA3 buffer (from NucleoSpin® RNA II kit, Nagel, USA) was added to the NuceloSpin® RNA II column and centrifuged for 30secs at 8,000 x g. 250µl of RA3 buffer was added to the NuceloSpin® RNA II column and the column was

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centrifuged for 2mins at 11,000 x g to dry the membrane completely. The column was then placed into a prelabelled nucleasefree 1.5ml microcentrifuge tube and RNA was eluted in 60μl H_2O (RNase-free) and centrifuged for 1mins at 11,000 x g. This elution step was repeated in order to produce a high yield and concentration of RNA. RNA samples were then stored at -80°C.

2.12.3   RNA Quantification

Samples were run on a 1% (w/v) agarose gel to ensure RNA was intact and had not been degraded. The concentration of RNA can be measured due to its ability to absorb light at 260nm. A 1:200 dilution of RNA was prepared using DEPC-treated H_2O. Samples were loaded into a quartz cuvette and RNA concentration was measured using a spectrophotometer. An optical density (OD) reading of 1.0 at 260nm was equivalent to an RNA concentration of 40μg/ml. The purity of RNA was established by measuring the absorbance at 280nm. A ratio of OD_{260}/OD_{280} of 1.8-2.1 was indicative of pure RNA. RNA concentrations were recorded and the volume of RNA required to reverse transcribe 1μg of RNA was determined.

2.12.4   Reverse transcription

RNA samples were thawed on ice and the appropriate volume of RNA (=1μg) was loaded into PCR tubes along with 2 μl gDNA wipeout buffer (from Qiagen quantified® Reverse Transcription kit, Qiagen, USA) and the appropriate volume of RNase-free H_2O to make up to 12μl total volume. The samples were mixed, incubated at 42°C for 2mins and placed immediately on ice. RT mastermix (6μl; RT mastermix: 1μl Quantiscript Reverse TranscriptaseRA1, 5μl buffer mastermix; Qiagen, USA) was added and the samples were incubated at 42°C for 15mins, followed by 95°C for 3mins to inactivate reverse transcriptase. Newly synthesized cDNA was stored at -20°C.

2.12.5 Polymerase chain reaction

A mastermix PCR mixture (final volume 11μl) was produced contained 10μl of taqman MasterMix (Applied Biosysytems, USA) and 1μl of upstream and downstream primers (Applied Biosysytems, USA). cDNA sample was diluted 1:5 ratio or 1:3 ratio with RNase-free H_2O and 10μl was added to the mastermix. The samples were then loaded (21μl) in duplicate onto a MicroAmp® optical 96-well plate. PCR was run with an initial incubation of 50°C for 2mins to activate Uracil-N-Glycosylase (UNG), which destroys PCR products, followed by a denaturing step of 95°C for 10mins, followed by 40 cycles consisting of a denaturing step of 95°C for 15sec, and annealing step of 60°C for 1 mins to ensure complete extension of the PCR product (7300 real time PCR system, Applied Biosysems, USA).

2.12.6 PCR Quantification

Using QT-PCR software (7300 real-time PCR software) target genes in different samples were compared to a reference gene. These values were then normalized to an endogenous control and the relative difference between samples was expressed as a ratio. Values were expressed as relative quantities of specific genes.

2.13 Statistical Analysis

Data are expressed as means ± standard error of the means. A two-way Analysis of variance (2-way ANOVA) was performed where appropriate to determine whether significant differences existed between conditions. In some experiments a 3-way ANOVA was performed to determine whether significant differences existed between conditions. When an F ratio 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 (GB-STAT software). In some experiments, where only two conditions were examined, an unpaired students-t-test was used to investigate possible significant differences between conditions (Graph Pad PRISM). All graphs were plotted using Graph Pad Prism software.

Results

3.1 Analysis of IL-1β and LPS-induced effects on hippocampus of rats treated with IL-1F5

3.1.1 IL-1F5 abrogated the inhibitory effect of IL-1β on LTP

IL-1β is classically known as an inflammatory mediator of immune responses in the CNS. Evidence suggests a role for IL-1β as a modulator of memory function in addition to its pro-inflammatory properties and it has been shown to inhibit LTP. This finding is confirmed in the present study. LTP was recorded in the dentate gyrus of rats and percentage change is slope of the excitatory post synaptic potential (EPSP) was recorded. An increase in the mean percentage change in the slope of the EPSP in all groups was observed, following a delivery of high frequency train of stimuli (HFS) to the perforant path (at time 0). Figure 3.1 illustrates that while LTP was attenuated in IL-1β treated rats, IL-1F5 had the ability to abrogate this effect so that the change in EPSP slope induced by tetanic stimulation in control-treated rats was similar to that in rats treated with IL-1β in combination with IL-1F5.

Analysis of changes in the 2 minute period immediately following tetanic stimulation is represented in Figure 3.2 and shows that the mean percentage change in slope of EPSP was similar in IL-1β-treated rats and control-treated rats (121.32 ± 1.49 compared with 119.3 ± 2.46 respectively; n=6). There was no significant difference in percentage change in EPSP slope in IL-1F5-treated, compared with control-treated rats. The corresponding changes in the last 5 minutes of the experiment indicate that the change in EPSP slope induced by tetanic stimulation in IL-1β-treated rats was significantly lower than in IL-1β-treated rats (104.7 ± 0.52; n=6), compared with control-treated rats (118.5 ± 0.65; **p<0.01, 2-way ANOVA; n=6).

Similarly the mean percentage change in EPSP slope was significantly lower in IL-1β-treated rats compared with both IL-1F5-treated rats and rats which received both IL-1β and IL-1F5 (104.7 ± 0.52 compared with 121.4 ± 0.78 and

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115.5 ± 0.31 respectively; **p<0.01, 2-way ANOVA; n=6). There was no significant difference in percentage change in EPSP slope in IL-1F5-treated, compared with control-treated rats.

Figure 3.1   IL-1F5 attenuates the IL-1β-induced deficit in LTP

LTP was reduced in IL-1β-treated rats (▼; n=6) compared with saline-treated rats (■; n=6). LTP was similar in saline-treated and IL-1F5-treated (♦; n=6) and in IL-1F5-treated rats which received IL-1β(●; n=6). The mean slope of the population EPSP evoked by test stimuli delivered at 30 sec intervals before and after high frequency stimulation (hfs) is shown. Population EPSP slope is expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and values are expressed as means± SEM. SEMs are included for every 10th response.

(A)
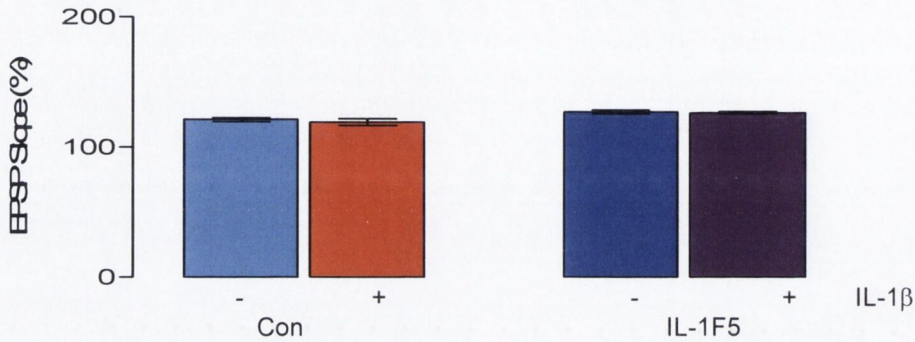
(B)
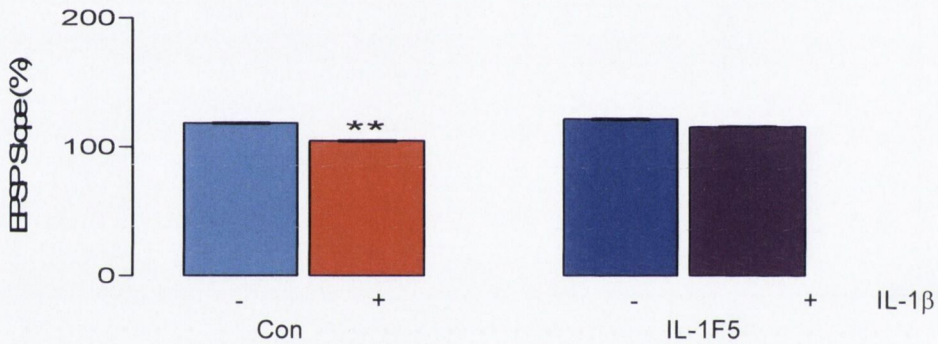
Figure 3.2 IL-1F5 attenuates the IL-1β-induced deficit in LTP

A. The mean percentage change in EPSP slope in the first 2 min post tetanic stimulation (compared with the 5 min period immediately proceeding tetanus) was similar in IL-1β-treated rats compared with saline-treated rats (n=6 in all groups). There was no significant difference in EPSP slope in saline-treated rats and IL-1β-treated rats which received IL-1F5

Interaction effect $F_{(1,15)}$ = 0.14, P=0.7151

B. The mean percentage change in EPSP slope in the last 5 min of recording (compared with the 5 min period immediately preceding tetanus) was significantly decreased in IL-1β-treated rats (**p<0.01; 2-way ANOVA; n=6 in all groups). There was no significant difference in EPSP slope in saline-treated rats and IL-1β-treated rats which received IL-1F5. There was no significant change in EPSP slope in IL-1F5-treated rats compared with saline-treated rats. Values are expressed as percentage change in EPSP slope and are means ± SEM.

Interaction effect $F_{(1,43)}$ = 108.56, P<0.0001

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3.1.2 IL-1F5 attenuated the IL-1β-induced JNK activation in hippocampal tissue

Several studies have reported that the IL-1β-induced impairment in LTP is accompanied by an increase in activation of c-jun N terminal kinase (JNK) and the data shown in this study provide further support for a correlation between these measures. To confirm the inhibitory effect of IL-1F5 on IL-1β induced changes, the interaction between these cytokines was assessed. Activation of JNK was assessed by gel electrophoresis and western immunoblotting using antibodies which specifically identify JNK phosphorylation on threonine 183 and tyrosine 185 and activation is expressed as a ratio of phosphorylated JNK (pJNK) to actin. Figure 3.3(A) shows a sample immunoblot indicating that pJNK expression was increased in hippocampal tissue prepared from IL-1β-treated (lane 2), compared with control-treated (lane 1) rats. Treatment with IL-1F5 appeared to abrogate the IL-1β-induced increase in pJNK (lane 4). IL-1F5 treatment alone, elicited similar levels of pJNK expression to control-treated rats (lane 3). Figure 3.3(B) indicates the mean data obtained from densitometric analysis. A significant increase in JNK activity, expressed as a ratio of pJNK to actin, was observed in hippocampal tissue prepared from IL-1β-treated ($2.25 \pm$ 0.17 arbitrary units; n=6), compared with control-treated (1.39 ± 0.1 arbitrary units; n=6) rats (*$p<0.05$; 2-way ANOVA; n=6). Figure 3.3(B) shows that the significant IL-1β-induced activation of JNK was attenuated in hippocampal tissue prepared from rats treated with IL-1F5 (2.25 arbitrary units $\pm$ 0.16 compared with 1.73 arbitrary units $\pm$ 0.18; +$p<0.05$; 2-way ANOVA; n=6). Importantly the data indicate that IL-1F5 alone exerted no effect (1.47 arbitrary units $\pm$ 0.11; n=6).

(A)

1   2   3   4

pJNK

Actin

(B)
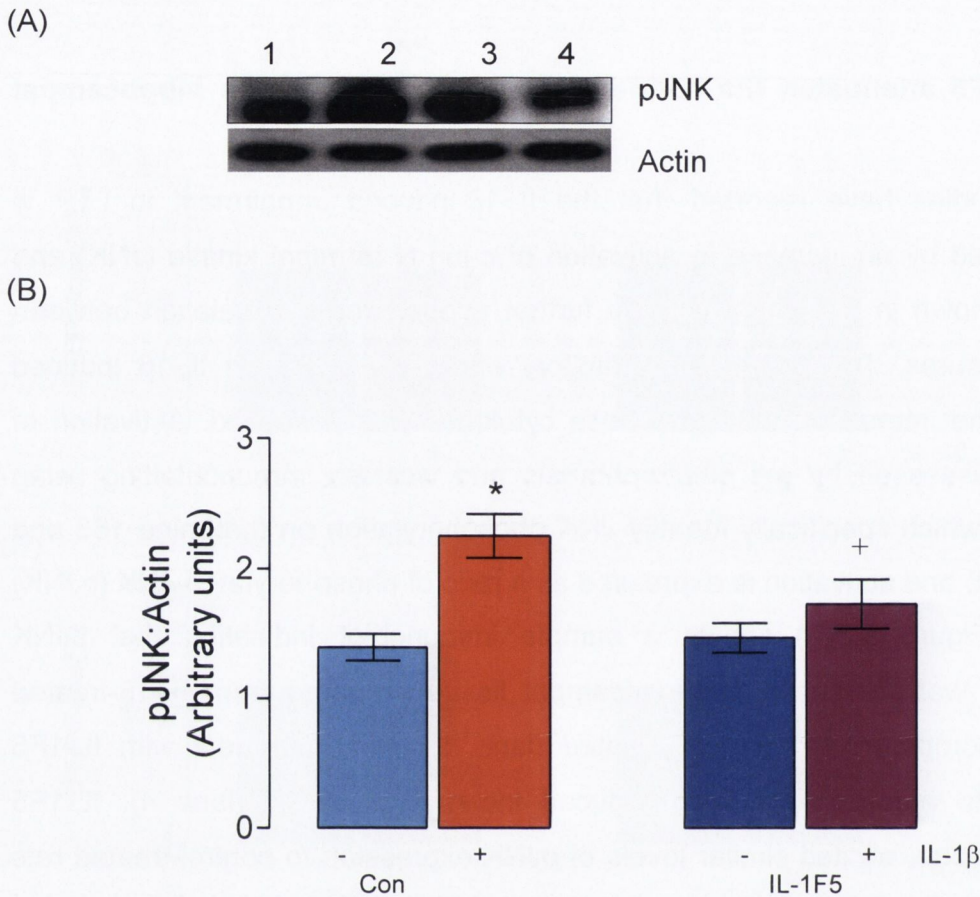
Figure 3.3 IL-1F5 attenuates the IL-1β-mediated increase in JNK in the hippocampus.

(A) A sample immunoblot shows expression of JNK as phosphorylation of the 46kDa isoform, in control-treated (lane 1), IL-1β-treated (lane 2), IL-1F5-treated (lane 3) and IL-1β and IL-1F5-treated (lane 4). A loading control of actin was used to check for equal protein loading.

(B) Expression of pJNK (46kDa) was significantly increased in hippocampal tissue prepared from IL-1β-treated rats (3.5ng/ml) compared with saline-treated rats ($^*p<0.05$; 2-way ANOVA; n=6). There was no significant difference in pJNK in hippocampal tissue prepared from saline-treated rats and hippocampal tissue prepared from IL-1β-treated rats which received IL-1F5 (lane 4; 30ng/ml IL-1F5; n=6). pJNK was significantly decreased in IL-1β-treated rats which received IL-1F5 compared with IL-1β-treated rats ($+p<0.05$; 2-way ANOVA; n=6). IL-1F5 exerted no significant effect (lane 3). Actin expression was used to check for equal protein loading and remained unchanged in all samples.

Interaction effect $F_{(1,16)} = 1.3$

3.1.3 IL-1F5 abrogated the inhibitory effect of LPS on LTP

It has been well documented that LPS administration increases the concentration of the pro-inflammatory cytokine IL-β in the rat hippocampus and that this effect plays a significant role in inhibiting LTP (Lynch *et al.*, 2004). In order to establish if IL-1F5 could affect this, LPS (100 μg/kg) was administered to Wistar rats and LTP was assessed. An increase in the mean percentage change in EPSP slope in all groups was observed following delivery of high frequency train of stimuli to the perforant path (at time 0). Figure 3.4 illustrates that while LTP was attenuated in LPS-treated rats, IL-1F5 had the ability to abrogate this effects so that the change in EPSP slope induced by tetanic stimulation in control-treated rats was similar to that in rats treated with LPS in combination with IL-1F5. Analysis of changes in the 2 minute period immediately following tetanic stimulation is represented on Figure 3.5 and shows that the mean percentage change in slope of EPSP was significantly lower in LPS-treated rats than in control-treated rats (121.32 ± 1.49 compared with 119.3±2.46 respectively; **$p<0.01$, 2-way ANOVA; n=6). IL-1F5 had the ability to abrogate this effect. Mean percentage change in EPSP slope was significantly lower in LPS-treated rats compared with IL-1F5 (127 ± 1.47; n=6) and rats treated with LPS and IL-1F5 (126.3 ± 0.85, **$p<0.01$, 2-way ANOVA; n=6). There was no significant difference in percentage change in EPSP slope in IL-1F5-treated, compared with control-treated rats. The corresponding changes in the last 5 minutes of the experiment indicate that the mean change in EPSP slope induced by tetanic stimulation in LPS-treated rats (97.35 ± 1.26) was significantly lower than in control-treated rats (133.0 ± 1.18; **$p<0.01$, 2-way ANOVA; n=6). Similarly the mean change in EPSP slope was significantly lower in IL-1β-treated rats compared with both IL-1F5-treated rats (125.3 ± 0.62; n=6) and rats which received both LPS and IL-1F5 (140.9 ± 0.56; **$p<0.05$, 2-way ANOVA; n=6). There was no significant difference in percentage change in EPSP slope in IL-1F5-treated, compared with control-treated rats.
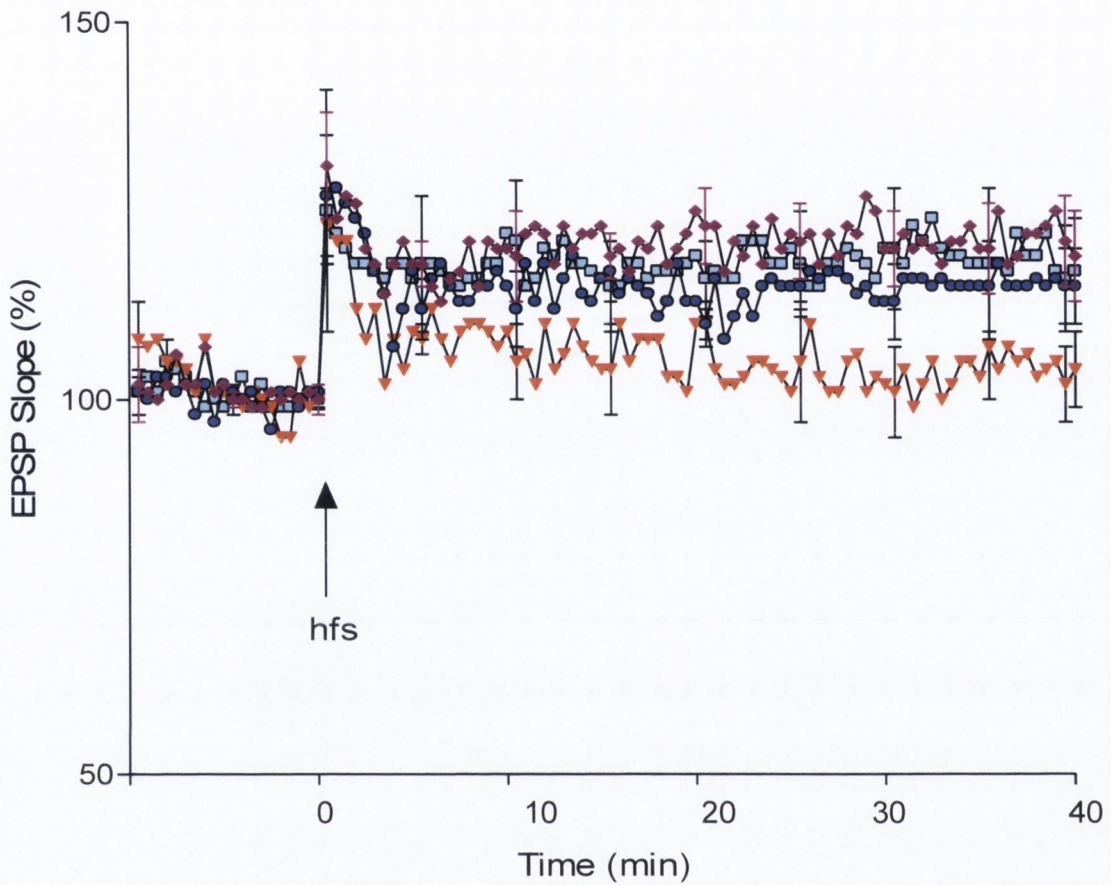
79
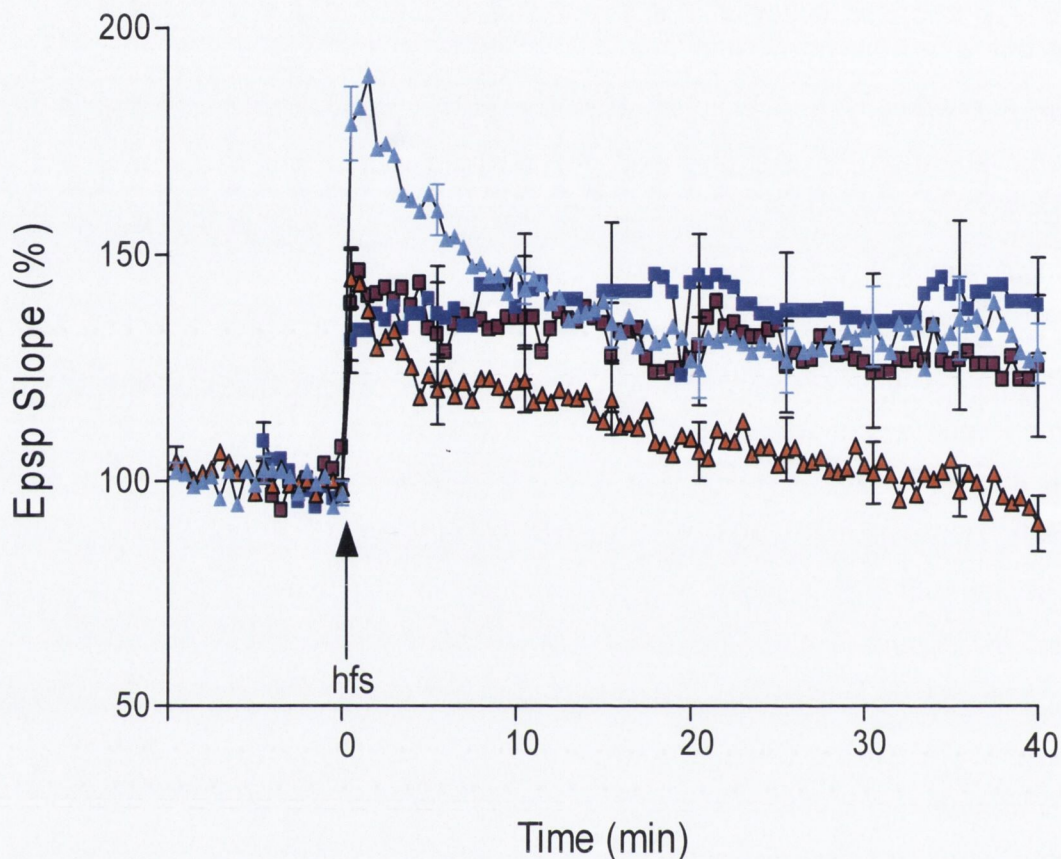
Figure 3.4 IL-1F5 attenuates LPS-induced deficit in LTP

LTP was impaired in LPS-treated rats (▲; n=6) compared with saline-treated rats (▲; n=6). LTP was similar in saline-treated and IL-1F5-treated (■; n=6)and in IL-1F5 treated rats which received LPS (■ ; n=6). The mean slope of the population EPSP evoked by test stimuli delivered at 30 sec intervals before and after high frequency stimulation (hfs) is shown. Population EPSP slope is expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and values are expressed as means± standard error of the mean of 6 observations. SEMs are included for every 10th response.
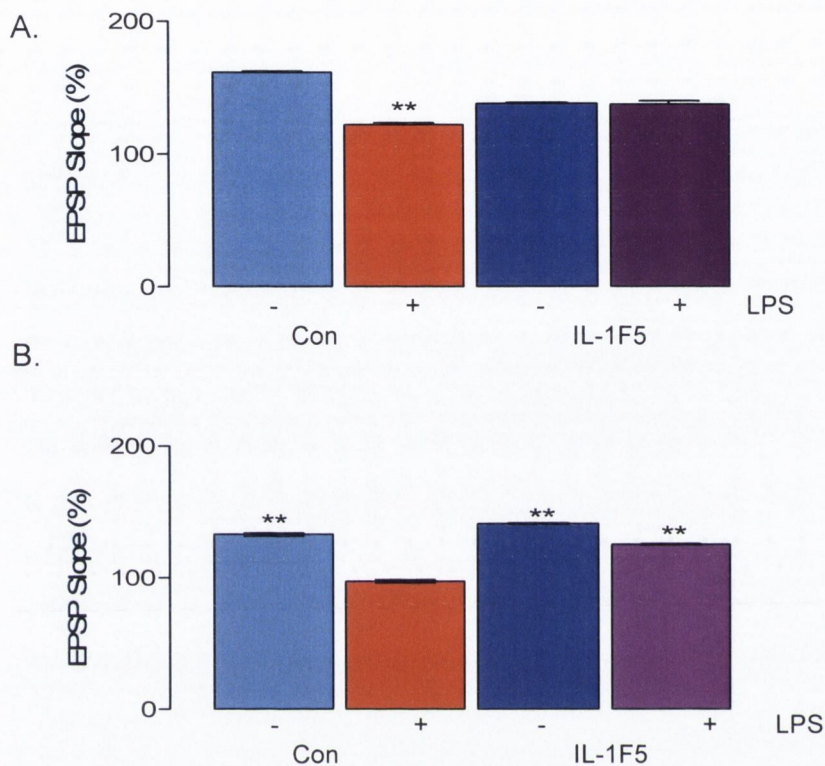
Figure 3.5 LPS administration affects percentage change in EPSP slope; reversal by treatment with IL-1F5

A. The mean percentage change in EPSP slope in the first 2 min post tetanic stimulation (compared with the 5 min period immediately proceeding tetanus) was significantly decreased in LPS-treated rats compared with saline-treated rats (*P<0.05; 2-way ANOVA; n=4 in all groups). There was no significant difference in EPSP slope in saline-treated rats and LPS-treated rats which received IL-1F5.

Interaction effect $F_{(1,12)}$ = 143.17 P<0.0001

B. The mean percentage change in EPSP slope in the last 5 min of recording (compared with the 5 min period immediately preceding tetanus) was significantly decreased in LPS-treated rats (*P<0.05; 2-way ANOVA; n=10 in all groups). There was no significant difference in EPSP slope in saline-treated rats and LPS-treated rats which received IL-1F5. There was no significant change in EPSP slope in IL-1F5-treated rats compared with saline-treated rats. Values are expressed as percentage change in EPSP slope and are means ± SEM.

Interaction effect $F_{(1,40)}$ = 13.52 P<0.001

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3.1.4 IL-1F5 attenuated LPS-induced microglial activation in hippocampal sections

When microglia receive an appropriate stimulus they become activated and release cytokines such as IL-1β. Activated microglia express MHC II proteins, which can be identified as increased expression of OX-6, and other cell surface markers such as CD11b. Microglial activation was assessed by immunohistological staining in cryostat sections prepared from rat brain. The first photomicrograph shown in Fig 3.6 is representative of what was observed in control-treated rats; it shows limited brown staining within the hippocampal formation. In contrast, a section prepared from an LPS-treated animal shows increased brown staining and an altered cell morphology, An example of a section prepared from an LPS and IL-1F5 treated rat demonstrates less intense staining than in LPS-treated rats. Treatment with IL-1F5 did not have any substantial effect in microglial activation. The pictures are representative of staining obtained in sections prepared from 6 animals for each treatment group.

Figure 3.6 IL-1F5 attenuates the LPS-induced increase in microglial activation in hippocampal sections

There was an observable increase in OX-6 in hippocampal sections prepared from LPS-treated rats (100μg/kg LPS), compared with control-treated rats. There was no observable difference in OX-6 staining in hippocampal sections prepared from LPS and IL-1F5 treated rats compared with control-treated rats. There was no significant difference in OX-6 staining in hippocampal sections prepared from IL-1F5-treated rats (30ng/ml), compared with control-treated rats. Scale bar represents 10μm

B) Two images of microglial cells in active state. Magnification x40

A
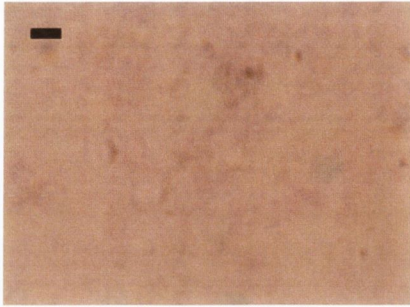
Con
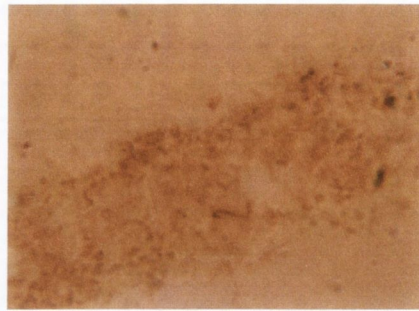
LPS

IL-1F5

LPS+IL-1F5

B

3.1.5 IL-1F5 attenuated the LPS-induced increase in IL-1β concentration

It has been reported that stimulated microglial cells provide the source of IL-1β (Tikka *et al.*, 2001). In order to establish whether increased microglial activation was associated with an increase the concentration of this cytokine, IL-1β was measured in hippocampal homogenate prepared from rats treated with LPS and IL-1F5 or the two in combination. Interleukin concentration was measured using an ELISA. Figure 3.7 shows that IL-1β concentration was increased significantly in hippocampal homogenate prepared from LPS-treated rats (94.25 ± 26.18 pg/mg; n=6) compared with tissue prepared from control-treated rats (36.53 ± 13.24 pg/ml; n=6; **p<0.01, 2-way ANOVA). Mean IL-1β concentration was decreased significantly in hippocampal tissue prepared from rats which were treated with LPS and IL-1F5 (47.84 ± 14.16 pg/mg) compared with LPS-treated rats (+p<0.05, 2-way ANOVA; n=6). IL-1F5 treatment did not elicit any significant effect in hippocampal tissue (22.26 ± 10.43 pg/mg; n=6).

3.1.6 IL-1F5 attenuated the LPS-induced JNK activation in hippocampal tissue

Stimulation of TLRs by specific ligands has been shown to induce activation of JNK (Robinson and Cobb, 1997), and evidence has indicated that the LPS-induced increase in IL-1β in the hippocampus is accompanied by an increase in JNK (Nolan et al., 2004). Activation of JNK was assessed by gel electrophoresis and western immunoblotting and activation is expressed as a ratio of pJNK to total JNK. Figure 3.8(A) shows a sample immunoblot indicating that pJNK expression was increased in hippocampal tissue prepared from LPS-treated (lane 2) compared with control-treated (lane 1) rats. Treatment with IL-1F5 appeared to abrogate the LPS-induced increase in pJNK (lane 4) and IL-1F5 treatment alone, elicited similar levels of pJNK expression to control-treated rats (lane 3). Figure 3.8(B) indicates the mean data obtained from

densitometric analysis. A significant increase in JNK activity, expressed as a ratio of pJNK to total JNK, was observed in hippocampal tissue prepared from LPS-treated (3.36 ± 0.32 arbitrary units; n=6) compared with control-treated (2.15 ± 0.24 arbitrary units; n=6) rats (*p<0.05; 2-way ANOVA; n=6). Figure 3.8 shows that the significant LPS-induced activation of JNK was attenuated in hippocampal tissue prepared from rats treated with IL-1F5 (3.36 ± 0.33 arbitrary units compared with 1.68 ± 0.24 arbitrary units; +p<0.05; 2-way ANOVA; n=6). Importantly the data indicate that IL-1F5 alone exerted no significant effect (2.58 ± 0.47 arbitrary units; n=6) in hippocampal tissue.

(This experiment was carried out by Melanie Watson)
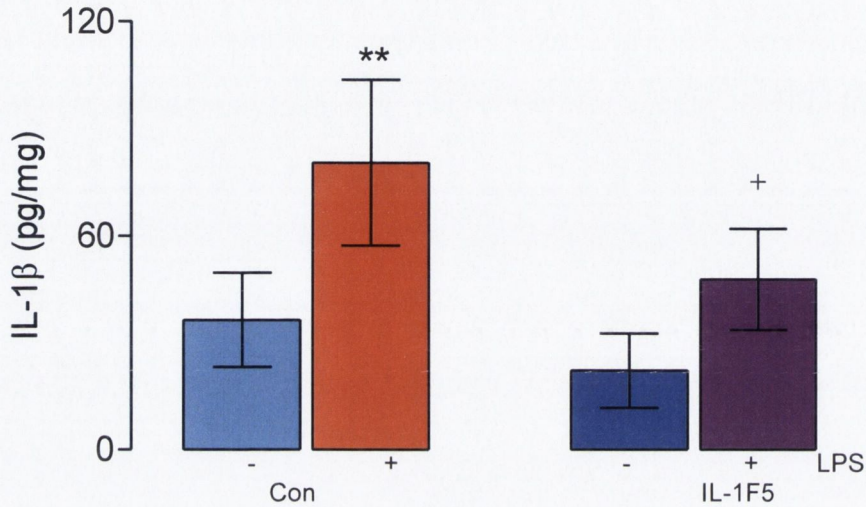
Figure 3.7 IL-1F5 attenuates the LPS-induced increase in IL-1β in the hippocampus.

IL-1β was significantly increased in hippocampal tissue prepared from LPS-treated rats compared with saline-treated rats (100μg/kg LPS; n=6; *P<0.05; 2-way ANOVA). There was no significant difference in IL-1β concentration in saline-treated rats and LPS- treated rats which received IL-1F5 (30ng/ml IL-1F5; n=6). IL-1β concentration was significantly decreased in hippocampal tissue prepared from LPS-treated rats which received IL-1F5 compared with LPS-treated rats ($^+$p<0.05; 2-way ANOVA; n=6). IL-1F5 exerted no significant effect. Values are expressed as pg per mg protein and are means ± SEM.
Interaction effect $F_{(}1,14) = 6.37$ P<0.05.
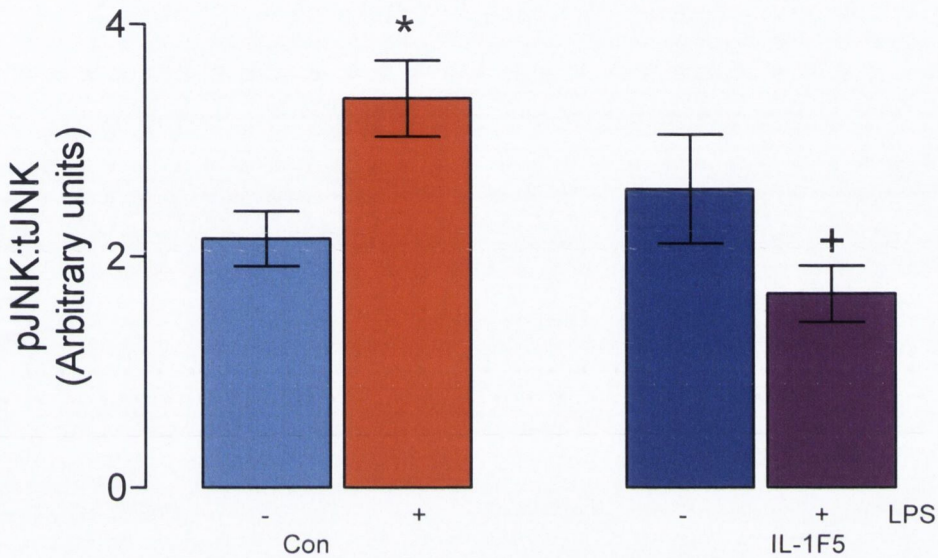
Figure 3.8 IL-1F5 attenuates the LPS-mediated increase in JNK in the hippocampus.

(A) A sample immunoblot shows expression of JNK as phosphorylation of the 46kDa isoform, in control-treated (lane 1), IL-1β-treated (lane 2), IL-1F5-treated (lane 3) and IL-1β and IL-1F5-treated (lane 4). A loading control of total JNK was used for this analysis.

(B) Expression of pJNK (46kDa) was significantly increased in hippocampal tissue prepared from LPS-treated rats (lane 2) compared with hippocampal tissue prepared from saline-treated rats (3.5ng/ml IL-1β; $^*p<0.05$; 2-way ANOVA; n=6). There was no significant difference in pJNK in hippocampal tissue prepared from saline-treated rats and LPS-treated rats which received IL-1F5 (30ng/ml IL-1F5; n=6). pJNK was significantly decreased in hippocampal tissue prepared from LPS-treated rats which received IL-1F5 compared with LPS-treated rats (+p<0.05; 2-way ANOVA; n=6). IL-1F5 exerted no significant effect. Total JNK expression was used to check for equal protein loading and remained unchanged in all samples.

Interaction effect $F_{(1,16)} = 2.5$

3.1.7 IL-1F5 induces an increase in IL-4 in the hippocampus

Data from several studies have identified that IL-4 can abrogate IL-1β induced changes and because IL-1F5 was shown to abrogate IL-1β-induced changes, it was considered that its action may be IL-4 dependent, therefore IL-4 concentration was measured in hippocamapal tissue using ELISA. Figure 3.9 demonstrates that the mean IL-4 concentration was significantly increased in hippocampal tissue prepared from IL-1F5-treated rats (57.89 ± 8.58 pg/mg; n=6) compared with control-treated rats (32.85 ± 4.98 pg/mg; *p<0.05, 2-way ANOVA; n=6). IL-4 concentration was similar in hippocampal tissue prepared from control-treated rats, IL-1β-treated rats (23.25 ± 3.71 pg/mg) and rats treated with IL-1β and IL-1F5 (32.59 ± 9.38 pg/mg).

It has also been shown that IL-4 can abrogate LPS effects (Maher et al., 2005) and therefore IL-4 was assessed in hippocampal tissue prepared from control-treated rats and rats treated with LPS, IL-1F5 or both. Figure 3.10 shows that IL-4 concentration was significantly increased in hippocampal tissue prepared from IL-1F5 treated rats (19.48 ± 1.92 pg/mg; *p<0.05, 2-way ANOVA), which confirms the data shown in Figure 3.9. IL-4 concentration was significantly increased in hippocampal tissue prepared rats which received LPS and IL-1F5 (17.37 ± 1.01 pg/mg) compared with LPS-treated rats (10.31 ± 1.5 pg/mg; + <0.05, 2-way ANOVA; n=6). LPS alone did not exert any effect, IL-4 concentration was similar in control-treated rats (14.15 ± 1.29pg/mg; n=6) and rats treated with LPS (10.31 ± 1.5 pg/mg).
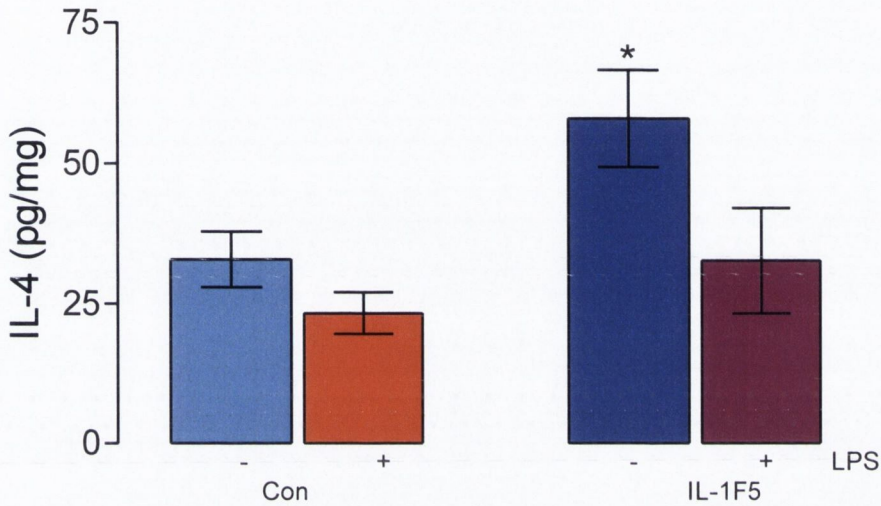
Figure 3.9 IL-4 concentration is increased in hippocampus following IL-1F5 treatment.

IL-4 concentration was significantly increased in hippocampal tissue prepared from IL-1F5-treated rats compared with saline-treated controls (30ng/ml IL-1F5; *P<0.05; 2-way ANOVA n=6). There was no significant difference in IL-4 concentration in hippocampal tissue prepared from saline-treated rats compared with IL-1β-treated (3.5ng/ml IL-β; n=6) which received IL-1F5. IL-4 concentration was significantly lower in hippocampal tissue prepared from IL-1β-treated rats compared with IL-1F5-treated rats (*P<0.05; 2-way ANOVA). Values are expressed as pg IL-4/mg tissue corrected for protein and are means±SEM.
Interaction effect $F_{(1,16)}$ =1.2
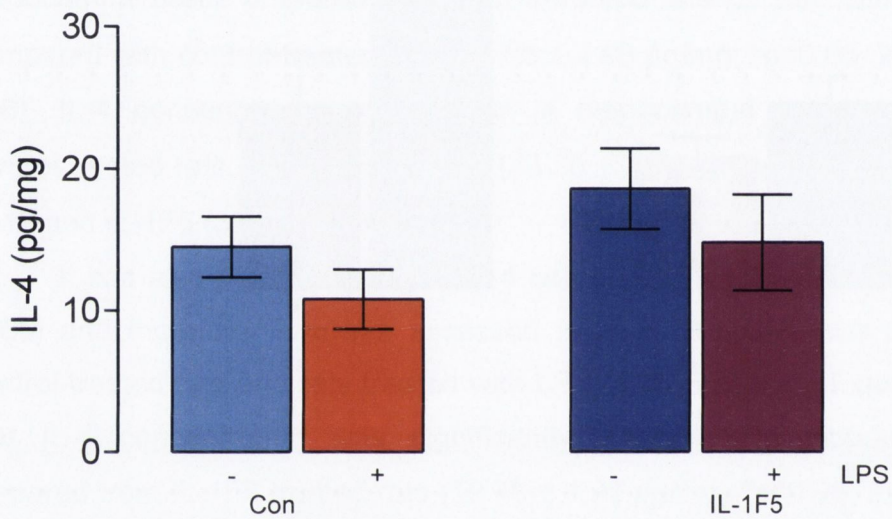
Figure 3.10 IL-4 concentration is increased in hippocampus following IL-1F5 treatment.

IL-4 concentration was slightly but not significantly increased in hippocampal tissue prepared from IL-1F5-treated rats compared with saline-treated controls (30ng/ml IL-1F5; n=6). IL-4 concentration was slightly, but not significantly lower in hippocampal tissue prepared from LPS-treated rats compared with IL-1F5-treated rats (100μg/kg LPS; n=6). There was no significant difference in IL-4 concentration in hippocampal tissue prepared from saline-treated rats compared with LPS-treated rats which received IL-1F5. Values are expressed as pg IL-4/mg tissue corrected for protein and are means±SEM.

Interaction effect F(1,32)=0.42 P= 0.84

3.2 Analysis of IL-1F5 effects in cultured mixed glial cells from rat

3.2.1 IL-1F5 induces an increase in IL-4 mRNA and protein in cultured glial cells

Past studies have indicated that IL-4 is produced by glia and to confirm this indication rat glial cells were prepared and treated with and without IL-1F5 and the cells and supernatants, were analyzed. Figure 3.11(A) shows a sample gel illustrating that IL-4mRNA expression was increased in IL-1F5-treated mixed glial cells, compared with control-treated mixed glial cells. Figure 3.11(B) represents the mean data obtained from densitometric analysis; this revealed a significant increase in IL-4 mRNA in IL-1F5-treated cells (5.14 ± 0.58 arbitrary units; n=7) compared with control-treated cells (3.19 ± 0.69 arbitrary units; **$p<0.01$, Student's t test for independent means; n=7).

The concentration of IL-4 was assessed in the supernatant prepared from glial cells. Figure 3.11(C) shows IL-4 protein concentration was significantly increased in IL-1F5-treated cells (51.31 ± 9.39 pg/ml) compared with control-treated cells (22.87 ± 5.19 pg/ml; **$p<0.01$, Student's t-test for independent means; n=8).

3.2.2 IL-1F5 did not effect IL-4 protein concentration when cells were co-treated with IL-1β

The concentration of IL-4 was assessed in supernatant prepared from rat cultured mixed glial cells, which had been challenged with IL-1F5 alone or with IL-1β. IL-4 concentration was increased in supernatent prepared from IL-1F5-treated cells (57.89 ± 9.38pg/ml; n=8; Figure 3.12) compared with control-treated cells (32.85 ± 4.98pg/ml; *$p<0.05$, 2-way ANOVA; n=8). IL-4 concentration in supernatent prepared from cells treated with IL-1β and IL-1F5 (28.45 ± 8.48 pg/ml; n=8) was similar to control-treated cells. There was no

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significant difference in IL-4 concentration in cells treated with IL-1β (23.25 ± 3.71pg/ml; n=8) compared with control-treated cells (32.85 ± 4.98pg/ml; n=8).

3.2.3 IL-1F5 attenuates the LPS-induced increase in IL-1β protein concentration in mixed glial cells

It has been shown that the LPS-induces an increase IL-1β concentration in cortical glial cells (Lynch *et al.*, 2006) and the data presented in Fig 3.13 support these findings. IL-1β concentration in supernatent prepared from LPS-treated (545.4 ± 39.17 pg/ml; n=6) cells was significantly greater than that in control-treated cells (81.54 ± 28.32 pg/ml; ***$p < 0.001$; 2-way ANOVA; n=6). This increase was not observed in LPS-treated cells co-treated with IL-1F5; in this case, mean IL-1β concentration was significantly lower than in LPS-treated glial cells (76.50 ± 29.04 pg/ml; +++$p < 0.001$, 2-way ANOVA; n=6). IL-1β concentration was not significantly affected in IL-1F5-treated cells (56.94 ± 14.68 pg/ml; n=6) compared with control-treated cells.

(A)

(B)   (C)
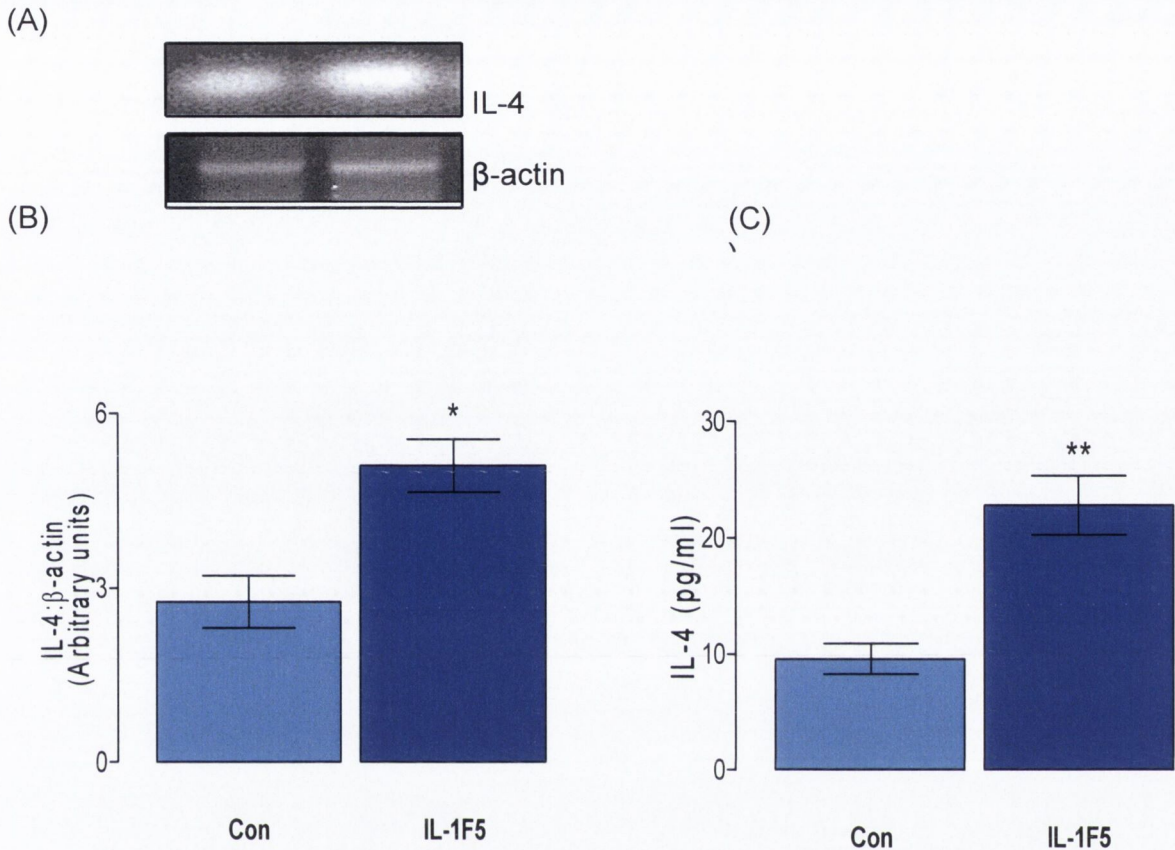
Figure 3.11 IL-4 mRNA and protein expression is increased in cultured mixed glial cells following IL-1F5 treatment

(A) A sample blot shows IL-4 mRNA expression, as shown by the appearance of bands at 299bp, in control-treated and IL-1F5-treated rats. A loading control of total β-actin was used for this analysis.

(B) IL-4 mRNA was increased in IL-1F5-treated cells compared with control treated cells (3μg/ml IL-1F5). Values are expressed as arbitrary units of IL-4:β-actin and are expressed as mean±SEM

(C) IL-4 concentration was significantly increased in IL-1F5-treated cells compared with control-treated cells (30μg/m; IL-1F5; *p<0.05; students unpaired t-test; n=6). Values are expressed as pg/ml supernatant and are means ± SEM.
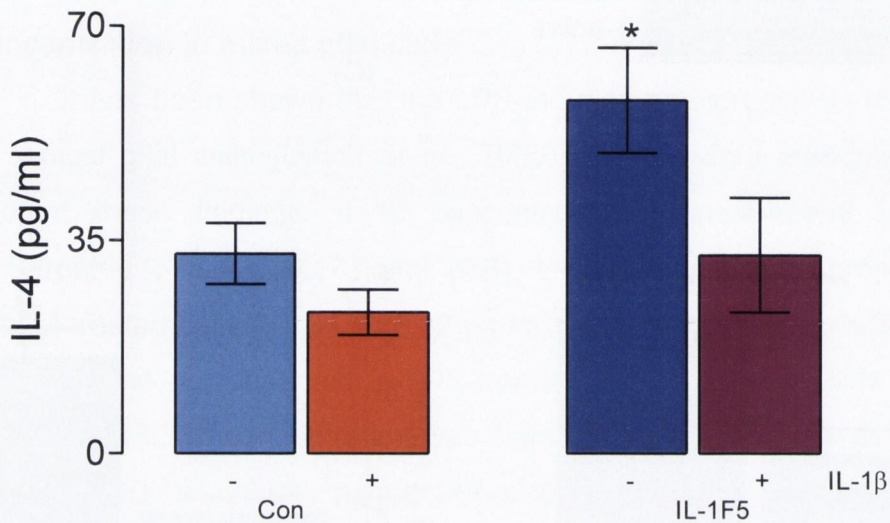
Figure 3.12 IL-4 concentration is increased in mixed glial culture following IL-1F5 treatment.

IL-4 concentration was significantly increased in IL-1F5-treated mixed glial cells compared with control-treated cells (3μg/ml IL-1F5; *P<0.05; 2-way ANOVA; n=6). There was no significant difference in IL-4 concentration in control-treated cells compared with IL-1β-treated cells which received IL-1F5. IL-4 concentration was significantly lower in IL-1β-treated cells compared with IL-1F5-treated cells (3.5ng/ml IL-1β; *P<0.05; 2-way ANOVA; n=6). Values are expressed as pg IL-4/ml supernatant and are means±SEM.

Interaction effect $F_{(1,27)}$ = 19.21, P=0.0002

94
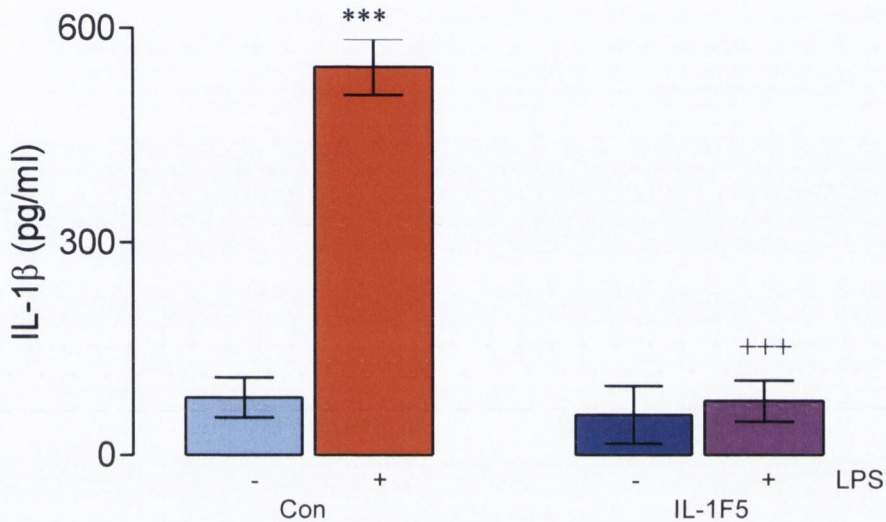
Figure 3.13   IL-1F5 attenuates the LPS-induced increase in IL-1β in mixed glial cells.

IL-1β concentration was significantly increased in supernatant prepared from LPS-treated cells (1μg/ml LPS; 2-way ANOVA; n=6) compared with supernatant prepared from control-treated cells (2-way ANOVA; n=6). There was no significant difference in IL-1β concentration control-treated and LPS- treated glial cells which received IL-1F5 (3μg/ml IL-1F5; n=6). IL-1β concentration was significantly decreased in LPS-treated glial cells which received IL-1F5 compared with LPS-treated cells (2-way ANOVA; n=6). IL-1F5 exerted no significant effect.

Values are expressed as pg/ml supernatant and are mean ± SEM

Interaction effect F(1,14) = 42.40 P<0.0001

3.3   Analysis of LPS-induced effects on hippocampus of wild-type C57BL/6 and IL-4$^{-/-}$ mice treated with IL-1F5

3.3.1 IL-1F5 did not affect MHCII expression in the hippocampus of wild-type C57BL/6 or IL-4$^{-/-}$ mice.

MHCII mRNA expression was analysed as an indication of microglial activation. MHCII mRNA was assessed in hippocampal tissue prepared from wild-type C57BL/6 and IL-4$^{-/-}$ mice. Figure 3.14(A) depicts a sample gel indicating MHCII expression in wild-type C57BL/6 (lane 1-4) and IL-4$^{-/-}$ (lane 5-8) mice. There appeared to be no difference in MHC II expression in LPS-treated (lane 2), compared with control-treated (lane 1), wild-type C57BL/6 mice. MHC II expression was similar in control-treated, IL-1F5-treated (lane 3) and LPS and IL-1F5-treated (lane 4) wild-type C57BL/6 mice. Similarly, there appeared to be no difference in MHC II expression in LPS-treated (lane 6), compared with control-treated (lane 5), IL-4$^{-/-}$ mice. MHC II expression was similar in control-treated, IL-1F5-treated (lane 7) and LPS and IL-1F5-treated (lane 8) IL-4$^{-/-}$ mice.

Figure 3.14(B) represents the mean data obtained from densitometric analysis. Treatment did not induce any significant difference in MHCII expression either in wild-type C57BL/6 or IL-4$^{-/-}$ mice. Densitometric analysis revealed that MHCII mRNA expression was significantly greater in hippocampal tissue prepared from control-treated IL-4$^{-/-}$ mice (0.13 ± 0.03 arbitrary units; n=6) compared with control-treated wild-type C57BL/6 mice (0.078 ± 0.03 arbitrary units; ##p<0.01, 3-way ANOVA; n=6). Similarly, MHCII mRNA expression was significantly greater in hippocampal tissue prepared from LPS-treated IL-4$^{-/-}$ mice (0.54 ± 0.06 arbitrary units; n=6) compared with LPS-treated wild-type C57BL/6 mice (0.17 ± 0.28 arbitrary units; **p<0.01, 3-way ANOVA; n=6). MHCII mRNA expression was significantly greater in IL-1F5-treated IL-4$^{-/-}$ mice (0.32 ± 0.04

arbitrary units; **p<0.01, 3-way ANOVA; n=6), compared with IL-1F5-treated wild-type C57BL/6 mice (0.16 ± 0.06 arbitrary units; **p<0.01, 3-way ANOVA; n=6). Similarly MHCII expression was significantly greater in IL-4$^{-/-}$ mice which were treated with LPS and IL-1F5 (0.74 ± 0.08 arbitrary units; n=6), compared with wild-type C57BL/6 mice which were treated with LPS and IL-1F5 (0.12 ± 0.03 arbitrary units; **p<0.05, 3-way ANOVA; n=6).

3.3.2 IL-1F5 did not affect IL-1β concentration in IL-4$^{-/-}$ hippocampal tissue.

It has been demonstrated that IL-4 can inhibit LPS induced IL-1β production. To address the possibility that IL-1F5 exerts its anti-inflammatory effects through IL-4, IL-1β protein concentration was assessed in tissue prepared from the hippocampus of wild-type C57BL/6 and IL-4 $^{-/-}$ mice. Figure 3.15 shows that IL-1β concentration was significantly increased in tissue prepared from the hippocampus of LPS-treated (236.30 ± 23.95 pg/mg; n=6), compared with control-treated wild-type C57BL/6 mice (98.71 ± 19.16 pg/mg; *p<0.05, 3-way ANOVA; n=6). IL-1β concentration was significantly decreased in tissue prepared from the hippocampus of LPS and IL-1F5 treated (159.5 ± 35.90pg/mg; n=6), compared with LPS-treated wild-type C57BL/6 mice (+p<0.05, 3-way ANOVA; n=6). There was no difference in IL-1β concentration in tissue prepared from IL-1F5-treated (126.80 ± 26.49pg/mg; n=6), compared with control-treated wild-type C57BL/6 mice.

In contrast, there was no significant difference in IL-1β concentration in tissue prepared from the hippocampus of LPS-treated (332.90 ± 59.50 pg/mg; n=6) compared with control-treated (258.9 ± 34.76pg/mg; n=6) IL-4$^{-/-}$ mice. Similarly there was no significant difference in IL-1β concentration in tissue prepared from the hippocampus of control-treated, compared with IL-1F5-treated (209.2 ± 26.92), or LPS and IL-1F5-treated (265.6 ± 32.45) IL-4$^{-/-}$ mice.

However, IL-1β concentration was higher in hippocampal tissue prepared from control-treated IL-4$^{-/-}$ mice, compared with the corresponding wild-type C57BL/6 groups (#p<0.05, 3-way ANOVA; n=6). This may account for the lack of

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effect of LPS in IL-4$^{-/-}$ mice and may be indicative of a 'ceiling' effect of LPS on IL-1β production, whereby there is 'exhaustion' of cytokine release. As in the case of MHC II mRNA, the mean values for IL-1β, in each treatment group were higher in IL-4$^{-/-}$, compared with wild-type C57BL/6 and the differences were significant in all cases except in the case of LPS treatment.

(A)

1   2   3   4   5   6   7   8

MHCII

(B)
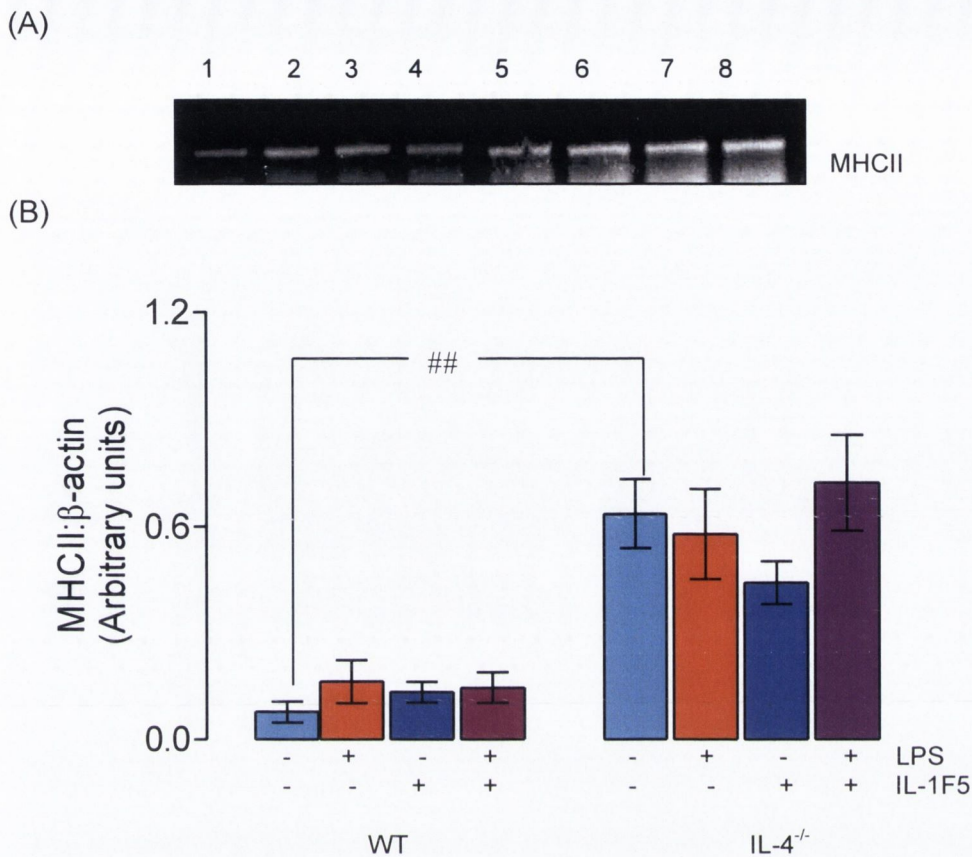
Figure 3.14 MHCII expression in the hippocampus of wild-type and IL-4$^{-/-}$ mice

(A) A sample agarose gel shows MHCII mRNA expression, as shown by the presence of the PCR bands of 250 base-pairs, in control-treated (lane 1), LPS-treated (lane 2), IL-1F5-treated (lane 3) and LPS and IL-1F5-treated wild-type mice (lane 4). Control-treated (lane 5), LPS-treated (lane 6), IL-1F5-treated (lane 7) and LPS and IL-15-treated (lane 8) SIGIRR$^{-/-}$ mice are also represented. A loading control of total β-actin was used for this analysis.

(B)There was no significant difference in MHCII expression in any treatment group in wild-type mice or IL-4$^{-/-}$ mice. There was a significant difference in MHC II mRNA expression in hippocampal tissue prepared from wild-type mice and IL-4$^{-/-}$ mice. MHC II mRNA expression was significantly increased in hippocampal tissue prepared from control-treated IL-4$^{-/-}$mice,compared with hippocampal tissue prepared from wild-type mice (##p<0.01. 3-way ANOVA; n=6). Values are expressed as arbitrary units of MHCII/β-actin and are means ± SEM)

Interaction effect F$_{(1,31)}$=2.7, P=0.0106
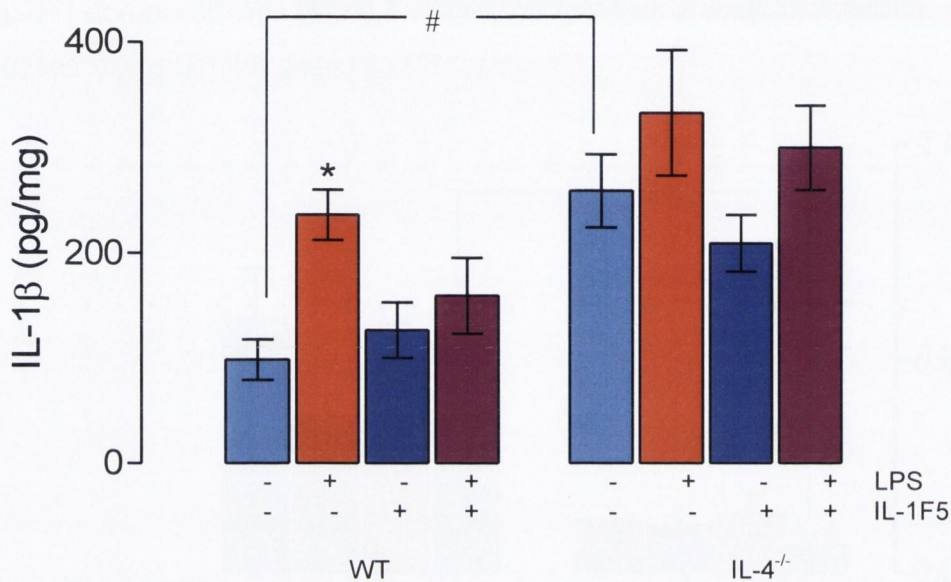
Figure 3.15   Effect of LPS and IL-1F5 on IL-1β in the hippocampus of wild-type and IL-4^{-/-} mice.

IL-1β was significantly increased in LPS-treated wild-type mice (200μg/kg LPS; 3-way ANOVA; n=6) compared with tissue prepared from saline-treated wild-type mice (3-way ANOVA; n=6). There was no significant difference in IL-1β concentration in tissue prepared from saline-treated and LPS- treated wild-type mice which received IL-1F5 (3μg/ml IL-1F5; n=6). IL-1β concentration was significantly decreased in hippocampal tissue prepared from LPS-treated wild-type mice which received IL-1F5 compared with LPS-treated wild-type mice (3-way ANOVA; n=6). IL-1F5 exerted no significant effect in wild-type mice.

Either LPS, IL-1F5 or the two in combination exerted any significant effect on IL-1β concentration in IL-4^{-/-} mice. IL-1β concentration was significantly increased in hippocampal tissue prepared from control-treated IL-4^{-/-}mice, compared with hippocampal tissue prepared from wild-type mice (#p<0.05, 3-way ANOVA; n=6)

Interaction effect $F_{(1,41)}$= 1.73, P<0.05

3.4 Analysis of LPS-induced effects on cultured mixed glial cells of wild type C57BL/6 and IL-4$^{-/-}$ mice treated with IL-1F5

3.4.1 IL-1F5 did not affect the LPS-induced increase in IL-1β concentration in IL-4$^{-/-}$ mixed glial cultures.

The data to this point suggest that IL-1F5 increases IL-4 and that the ability of IL-1F5 to abrogate LPS-induced changes in the hippocampus may be dependent on the modulatory action of IL-4. To further address the possibility that IL-1F5 exerts its anti-inflammatory effects via IL-4 in the brain, cultures of mixed glial cells were prepared from wild-type C57BL/6 and IL-4$^{-/-}$ mice and IL-1β concentrations were measured. Figure 3.16 shows that treatment of cells prepared from wild-type C57BL/6 mice with LPS significantly increased IL-1β concentration (38.58 ± 10.20 pg/ml; n=5), compared with control-treated (1.82 ± 0.71pg/ml; ***p<0.001, 3-way ANOVA; n=6). IL-1F5 had the ability to abrogate this effect. IL-1β concentration was significantly decreased in cells treated with LPS and IL-1F5-treated, compared with cells treated with LPS-treated alone (9.42 ± 2.47pg/ml; +++p<0.001, 3-way ANOVA; n=6). IL-1F5 alone exerted no significant effect, there was no significant difference in IL-1β concentration in cells treated with IL-1F5 (2.33 ± 0.82 pg/ml; n=8) and control-treated cells.

Treatment of cells prepared from IL-4$^{-/-}$ mice with LPS significantly increased IL-1β concentration (17.13 ± 1.23 pg/ml; n=8), compared with control-treated (1.59 ± 0.69 pg/ml; ***p<0.001, 3-way ANOVA; n=8). IL-1F5 was unable to abrogate this effect. IL-1β concentration was significantly greater in cells treated with LPS and IL-1F5 treated (15.17 ± 0.73 pg/ml; n=8), compared with control-treated cells generated from IL-4$^{-/-}$ mice (***p<0.001, 3-way ANOVA; n=8). Therefore there was no significant difference in IL-1β concentration in cells generated from IL-4$^{-/-}$ which were treated with LPS and IL-1F5 or LPS alone . IL-1F5 alone exerted no effect, IL-1F5-treatment did not significantly increase IL-1β concentration in cell generated from IL-4$^{-/-}$ mice (3.59 ± 0.78 pg/ml; n=8).

LPS-induced increase in IL-1β concentrations was significantly greater in cells prepared from wild-type mice, compared with cells prepared from IL-4$^{-/-}$ mice (###$p<0.001$, 3-way ANOVA; n=6)
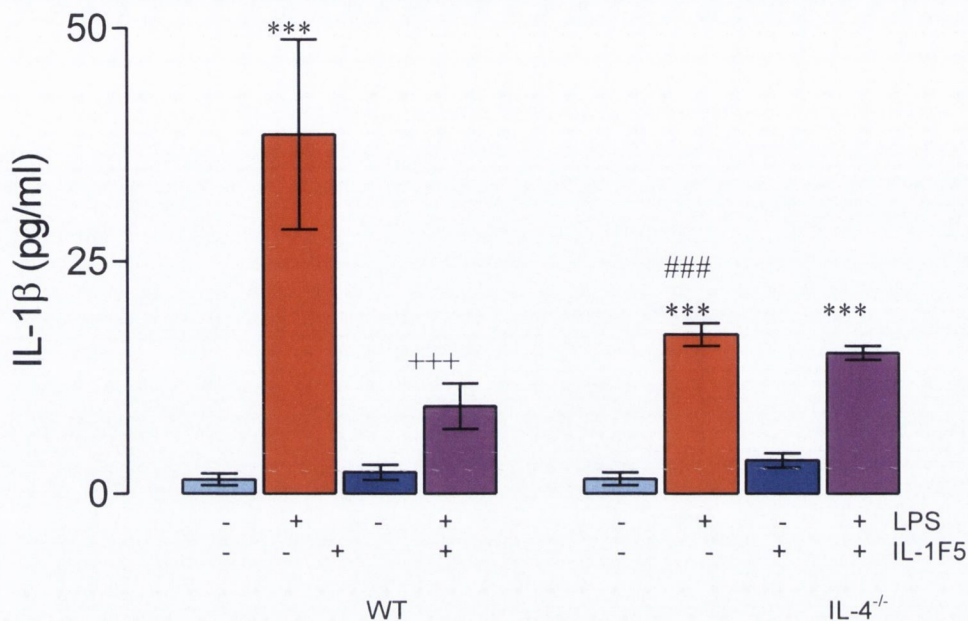
Figure 3.16   IL-1F5 attenuates the LPS-induced increase in IL-1β in wild-type but not IL-4$^{-/-}$ mixed glial cultures.

IL-1β was significantly increased in LPS-treated (200µg/kg LPS; 3-way ANOVA; n=6) compared control-treated cells generated from wild-type mice.(***p<0.001, 3-way ANOVA; n=6). There was no significant difference in IL-1β concentration in control-treated and LPS- treated cells which received IL-1F5 (3µg/ml IL-1F5; n=6). IL-1β concentration was significantly decreased in LPS-treated cells which received IL-1F5 compared with LPS-treated cells generated from wild-type mice (+++p<0.001, 3-way ANOVA; n=6). IL-1F5 exerted no significant effect in cells generated from wild-type mice.

IL-1β was significantly increased in LPS-treated cells generated from IL-4$^{-/-}$ mice (100ng/ml LPS; ***p<0.001, 3-way ANOVA; n=6) compared with control-treated cells generated from IL-4$^{-/-}$ cells .

IL-1β concentration was significantly increased in LPS-treated cells which received IL-1F5 compared with control-treated cells (3µg/ml IL-1F5; ***p<0.001, 3-way ANOVA; n=6). IL-1F5 exerted no significant effect in cells generated from IL-4$^{-/-}$ cells. The LPS-induced increase in IL-1β was significantly greater in cells prepared from wild-type mice, compared with cells prepared from IL-4$^{-/-}$ mice (###p<0.001, 3-way ANOVA; n=6)

Values are expressed as pg/ml supernatant and are mean ± SEM

Interaction effect: LPS/IL-1F5/IL-4$^{-/-}$ F$_{(1,46)}$ = 6.6, P=0.01

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3.5 Analysis of LPS-induced effects on cultured mixed glia treated with IL-1F5 and SIGIRR antibody

3.5.1 SIGIRR antibody treatment attenuated the IL-1F5-induced increase in IL-4 in mixed glial cells

The possibility that IL-1F5 might mediate its effects via an interaction with the orphan receptor SIGIRR was considered. SIGIRR has been confirmed to be expressed in the brain (Thomassen *et al.*, 2001). Primary cultures of mixed glial cells were prepared from Wistar rats and treated with IL-1F5 alone, or in combination with LPS and a SIGIRR antibody. IL-4 concentration was measured in the supernatent and Figure 3.17 shows that incubation of mixed glia in the presence of IL-1F5 caused a significant increase in IL-4 concentration (22.33 ± 2.87 pg/ml; n=24), compared with control-treated cells (13.12 ± 1.83 pg/ml; *p<0.05; 2-way ANOVA n=20). Incubation in the presence of an anti-SIGIRR antibody blocked this effect and therefore there no significant difference in IL-4 concentration was observed in IL-1F5-treated cells, which were pre-incubated for 4 hours in the presence of a SIGIRR antibody (10.92 ± 2.69 pg/ml; n=10), compared with control-treated cells. Treatment of cells with SIGIRR antibody alone also exerted no significant difference in IL-4 (15.98 ± 3.83 pg/ml; n=10), compared with control-treated cells.
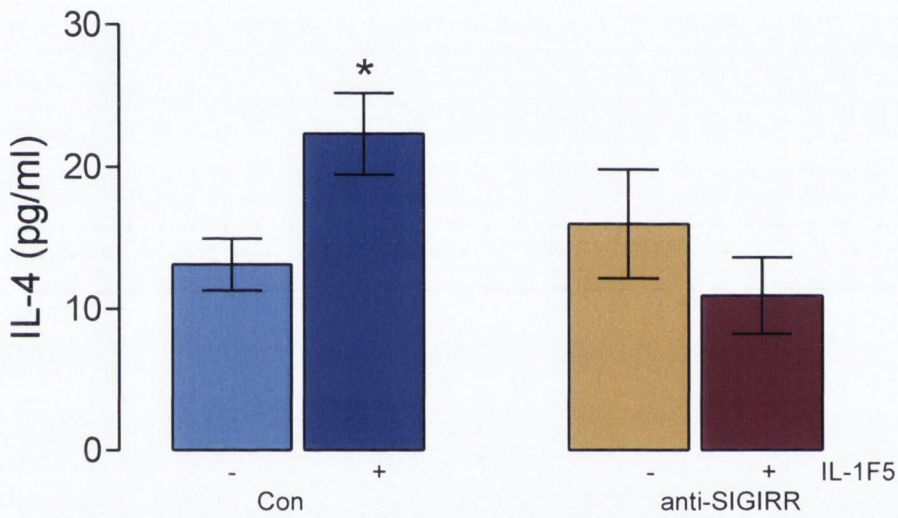
Figure 3.17 IL-1F5-induced increase in IL-4 is attenuated following pre-incubation with a SIGIRR antibody in mixedglia.

IL-4 concentration was significantly increased in mixed glial cells treated with IL-1F5 compared with control-treated cells (3μg/ml IL-1F5; [*]p<0.05; 2-way ANOVA; n=20). There was no significant difference in IL-4 in control-treated cells compared with IL-1F5-treated cells which underwent a 4 hour pre-incubation with a SIGIRR antibody (20μg/ml anti-SIGIRR). Cells treated with anti-SIGIRR alone exerted no effect (n=8).

Values are expressed as pg/ml supernatant and are mean ± SEM.

Interaction effect F(1,52) = 2.34 P=0.13

3.6 Analysis of LPS-induced effects in hippocampus of wild-type C57BL/6 and SIGIRR^{-/-} mice treated with IL-1F5

3.6.1 IL-1F5 did not affect MHCII mRNA expression in the hippocampus of wild-type C57BL/6 or SIGIRR^{-/-} mice.

To further investigate the role of SIGIRR in mediating the effects of IL-1F5 wild-type C57BL/6 and SIGIRR^{-/-} mice were challenged with LPS and IL-1F5. Tissue was prepared from the hippocampus of these animals and MHCII mRNA expression was assessed using quantitative-polymerase chain reaction (QT-PCR). MHCII expression levels were assessed relative to β-actin mRNA. Figure 3.18 shows MHCII mRNA expression was not significantly altered in response to any of the treatments in either hippocampal tissue prepared from wild-type C57BL/6 or SIGIRR^{-/-}. There was no difference in MHCII mRNA expression in hippocampal tissue prepared from LPS-treated (64.53 ± 18.23 arbitrary units; n=6), IL-1F5-treated (51.47 ± 17.10 arbitrary units), and LPS and IL-1F5-treated (95.43 ± 25.16; n=6), compared with control-treated wild-type C57BL/6 mice (37.12 ± 9.99 arbitrary units; n=6).

Similarly, there was no difference in MHCII mRNA expression in hippocampal tissue prepared from LPS-treated (68.95 ± 26.43 arbitrary units; n=6), IL-1F5-treated (11.47 ± 7.61 arbitrary units; n=6), LPS and IL-1F5-treated (128.50 ± 26.28 arbitrary units; n=6), compared with control-treated (13.78 ± 5.75 arbitrary units; n=6) SIGIRR^{-/-} mice. There was an increase in IL-1β concentration in LPS and IL-1F5-treated, compared with IL-1F5-treated SIGIRR^{-/-} cells (*$p < 0.05$, 3-way ANOVA; n=6).

MHC II expression levels were similar in hippocampal tissue prepared from wild-type mice and SIGIRR^{-/-} mice.

3.6.2 IL-1F5 did not affect the LPS-induced increase in IL-1β mRNA or protein concentration in SIGIRR$^{-/-}$ hippocampal tissue.

In parallel with MHC II mRNA IL-1β mRNA expression was investigated in hippocampal tissue prepared from wild-type C57BL/6 and SIGIRR$^{-/-}$ mice, using QT-PCR. Figure 3.19 shows that there was a significant increase in IL-1β mRNA expression in tissue prepared from the hippocampus of LPS-treated (322.14 ± 97.24 arbitrary units; n=5), compared with control-treated (75.14 ± 33.58 arbitrary units; *p<0.05, 3-way ANOVA; n=6) wild-type C57BL/6 mice. IL-1β mRNA was significantly decreased in tissue prepared from the hippocampus of LPS and IL-1F5-treated (140.71 ± 10.19 arbitrary units; n=6), compared with LPS-treated mice (+p<0.05, 3-way ANOVA; n=6). IL-1F5 alone exerted no effect, there was no significant difference in IL-1β mRNA expression in tissue prepared from the hippocampus of IL-1F5-treated (140.8 ± 14.37 arbitrary units; n=6), compared with control-treated wild-type C57BL/6 mice.

There was no significant difference in IL-1β mRNA expression in tissue prepared from the hippocampus of SIGIRR$^{-/-}$ mice, in response to any treatment. IL-1β mRNA expression was similar in control-treated (292.80 ± 88.21 arbitrary units; n=6), LPS-treated (242.00 ± 52.00 arbitrary units; n=6), IL-1F5-treated (272.90 ± 40.97 arbitrary units; n=6), and LPS and IL-1F5-treated (277.80 ± 77.93 arbitrary units; n=6) SIGIRR$^{-/-}$ mice.

IL-1β protein concentration was assessed in hippocampal tissue of wild-type C57BL/6 and SIGIRR$^{-/-}$ mice. In confirmation of previous data, IL-1β concentration was significantly increased in tissue prepared from the hippocampus of LPS-treated (155.60 ± 25.64 pg/mg; n=6), compared with control-treated (72.58 ± 25.22 pg/mg; *p<0.05, 3-way ANOVA; n=6) wild-type C57BL/6 mice. Figure 3.20 shows IL-1β concentration was significantly decreased in hippocampal tissue prepared from LPS and IL-1F5-treated (105.6 ± 12.42 pg/mg; n=6), compared with LPS-treated wild-type C57BL/6 mice (+p<0.05, 3-way ANOVA; n=6). IL-1F5 alone exerted no effect, therefore IL-1β concentration in tissue prepared from the hippocampus of IL-1F5-treated (81.96

± 23.75 pg/mg; n=6)mice was similar to hippocampal tissue prepared from control-treated wild-type C57BL/6 mice.

In contrast there was no significant difference in IL-1β concentration in tissue prepared from the hippocampus of SIGIRR$^{-/-}$ mice, in response to any treatment. IL-1β concentration was similar in control-treated (78.58 ± 17.44 pg/mg; n=6), LPS-treated (115.00 ± 23.33 pg/mg; n=6), IL-1F5-treated (99.85 ± 22.63 pg/mg; n=6), and LPS and IL-1F5-treated (113.4 ± 21.21 pg/mg; n=6) SIGIRR$^{-/-}$ mice.
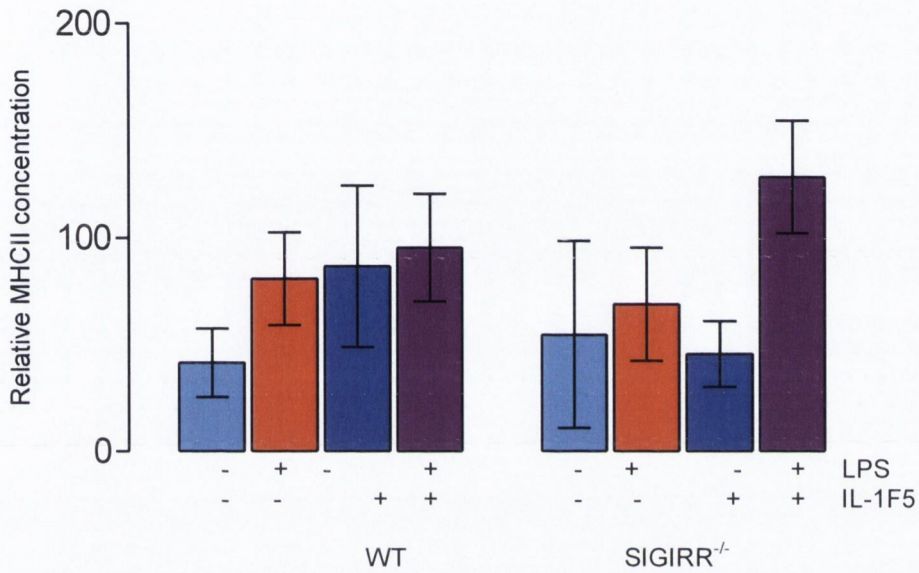
Figure 3.18 Effect of LPS and IL-1F5 on MHCII expression in wild-type and SIGIRR$^{-/-}$ mice

MHCII expression was not significantly altered in either wild-type or SIGIRR$^{-/-}$ mice in response to any treatment. There was no significant difference in MHC II mRNA expression in hippocampal tissue prepared from wild-type mice and SIGIRR$^{-/-}$ in response to any treatment.
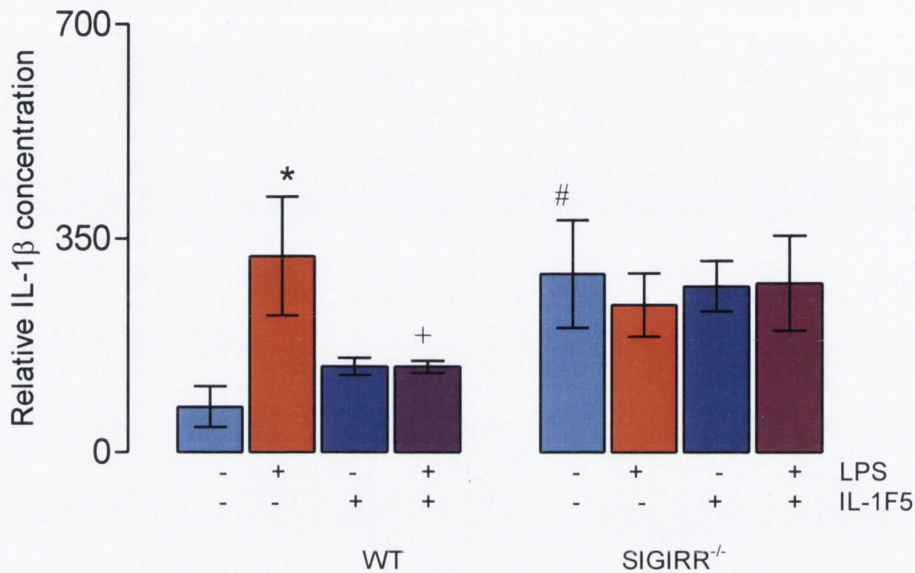
Figure 3.19 Effect of LPS and IL-1F5 on IL-1β mRNA expression in wild-type and SIGIRR^{-/-} mice

IL-1β mRNA expression was significantly increased in hippocampal tissue prepared from LPS-treated wild-type mice (200μg/kg LPS; *$p<0.05$, 3-way ANOVA; n=6) compared control-treated wild-type mice . There was no significant difference in IL-1β mRNA expression in hippocampal tissue prepared from control-treated and LPS- treated wild-type mice which received IL-1F5 (30ng/ml IL-1F5; n=6). IL-1β mRNA expression was significantly decreased in hippocampal tissue prepared from LPS-treated wild-type cells which received IL-1F5 compared with LPS-treated wild-type cells (+$p<0.05$, 3-way ANOVA; n=6). IL-1F5 exerted no significant effect in wild-type mice.

IL-1β mRNA expression was not significantly altered in a SIGIRR^{-/-} cells in response to any treatment. IL-1β mRNA was significantly increased inhippocampal tissue prepared from control-treated [SIGIRR-/-]mice, compared with wild-type mice ('$p<0.05$, 3-way ANOVA). β-actin was used a control gene in these experiments and remained unchanged between treatment groups in both wild-type and SIGIRR^{-/-}.

Interaction effect LPS/IL-1F5/SIGIRR-/- $F_{(1,31)} = 2.7$, $P=0.1106$

110
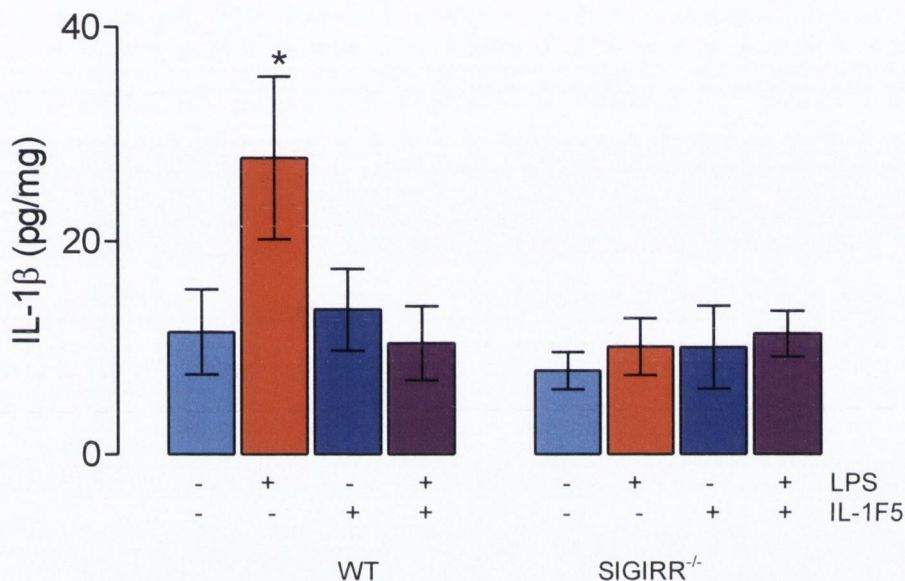
Figure 3.20 IL-1F5 attenuates the LPS-induced increase in IL-1β in the hippocampus of wild-type, but not SIGIRR^{-/-} mice

IL-1β was significantly increased in LPS-treated wild-type mice (200μg/kg LPS; 3-way ANOVA; n=6) compared control-treated wild-type mice (3-way ANOVA; n=6). There was no significant difference in IL-1β concentration in control-treated and LPS- treated wild-type mice which received IL-1F5 (30ng/ml IL-1F5; n=6). IL-1β concentration was significantly decreased in LPS-treated wild-type cells which received IL-1F5 compared with LPS-treated wild-type cells (3-way ANOVA; n=6). IL-1F5 exerted no significant effect in wild-type mice.
IL-1β concentration was not significantly altered in a SIGIRR^{-/-} cells in response to any treatment. Values are expressed as pg IL-1β/mg protein and are means±SEM

Interaction effect LPS/IL-1F5/SIGIRR^{-/-} $F_{(1,41)}$ = 0.5, P=0.48

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3.6.3 IL-1F5 did not induce an increase in IL-4 mRNA or protein concentration in SIGIRR$^{-/-}$ hippocampal tissue.

In an effort to determine the role SIGIRR plays in the anti-inflammatory effects of IL-1F5, the expression of IL-4 mRNA and concentration of IL-4 protein was assessed in hippocampal tissue prepared from wild-type C57BL/6 mice and SIGIRR$^{-/-}$ mice using QT-PCR. Figure 3.21 indicates IL-4 mRNA expression was significantly upregulated in hippocampal tissue prepared from IL-1F5-treated (20.22 ± 6.76 arbitrary units; n=6), compared with control-treated (6.16 ± 2.18 arbitrary units; *p<0.05, 3-way ANOVA; n=6), wild-type C57BL/6 mice. There was no significant difference in IL-4 mRNA expression in tissue prepared from the hippocampus of LPS-treated (6.11 ± 3.99 arbitrary units; n=6) compared with control-treated cells. Similarly, there was no significant difference in IL-4 mRNA expression in tissue prepared from the hippocampus wild-type C57BL/6 mice treated with LPS and IL-1F5 (8.79 ± 3.29 arbitrary units; n=6), compared with the control.

Mean IL-4 mRNA expression was similar in tissue prepared from the hippocampus of SIGIRR$^{-/-}$ mice, regardless of treatment group. There was no significant difference in IL-4 expression in control-treated (1.76 ± 1.44 arbitrary units; n=6), LPS-treated (3.41 ± 2.45 arbitrary units; n=6), IL-1F5-treated (3.34 ± 1.8 arbitrary units; n=6) and LPS and IL-1F5-treated (2.48 ± 1.9 arbitrary units; n=6) SIGIRR$^{-/-}$ mice.

There was a slight but insignificant difference IL-4 mRNA expression was in hippocampal tissue prepared from control-treated wild-type mice (6.16 ± 2.18 arbitrary units; n=6), compared with control-treated SIGIRR$^{-/-}$ mice (1.76 ± 1.44 arbitrary units; *p<0.05, 3-way ANOVA; n=6). This indicates that basal levels of IL-4 concentration were similar in the hippocampus of wild-type C57BL/6 and SIGIRR$^{-/-}$ mice.

The increase in IL-4 mRNA translated into an increase in IL-4 protein concentration in hippocampal tissue prepared from IL-1F5-treated wild-type

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C57BL/6 mice. Figure 3.22 shows that IL-4 protein concentration was significantly increased in tissue prepared from the hippocampus of IL-1F5-treated (27.32 ± 5.20 pg/mg; n=6), compared with control-treated (13.45 ± 4.2 4 pg/mg; *p<0.05, 3-way ANOVA; n=6) wild-type C57BL/6 mice. There was no significant difference in IL-4 concentration in the hippocampal tissue prepared from wild-type C57BL/6 mice treated with LPS and IL-1F5 (15.03 ± 3.30 pg/mg; n=7), compared with controls. LPS alone exerted no effect; thus IL-4 concentration was similar in hippocampal tissue prepared from LPS-treated (10.82 ± 1.67 pg/mg; n=7), compared with control-treated wild-type C57BL/6.

Mean IL-4 concentration was similar in all tissue prepared from the hippocampus of SIGIRR^{-/-} mice, regardless of treatment group. There was no significant difference in IL-4 concentration in the hippocampus of control-treated (19.35 ± 3.49 pg/mg; n=7), LPS-treated (14.17 ± 2.72 pg/mg; n=7), IL-1F5-treated (12.64 ± 2.86 pg/mg; n=7) and LPS and IL-1F5-treated (12.92 ± 2.28 pg/mg; n=7) SIGIRR^{-/-} mice.
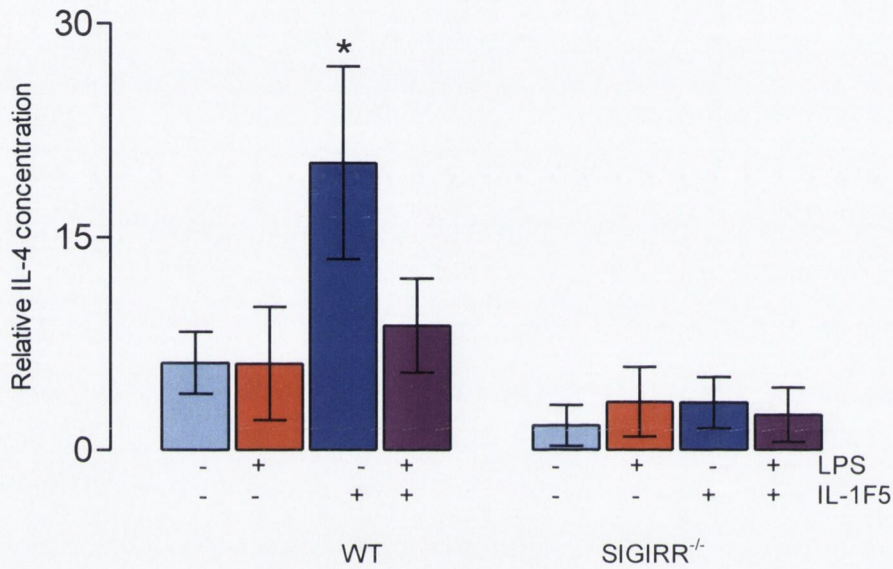
Figure 3.21 IL-1F5 induces an increase in IL-4 mRNA expression in the hippocampus of wild-type, but not SIGIRR$^{-/-}$ mice

IL-4 mRNA expression was significantly increased in hippocampal tissue prepared from IL-1F5-treated wild-type mice compared with control-treated mice (30ng/ml IL-1F5; *P<0.05; 2-way ANOVA; n=6). There was no significant difference in IL-4 mRNA expression in hippocampal tissue prepared from control-treated mice compared with LPS-treated mice which received IL-1F5. IL-4 mRNA expression was significantly lower in LPS-treated mice compared with IL-1F5-treated mice (200μg/kg LPS; *P<0.05; 2-way ANOVA; n=6). Values are a ratio of IL-4mRNA:β-actin and expressed as arbitrary units with means±SEM.

IL-4 mRNA expression was not significantly altered in SIGIRR$^{-/-}$ mice in response to any treatments.

Interaction effect F$_{(1,31)}$ = 10.98, P=0.0024

114
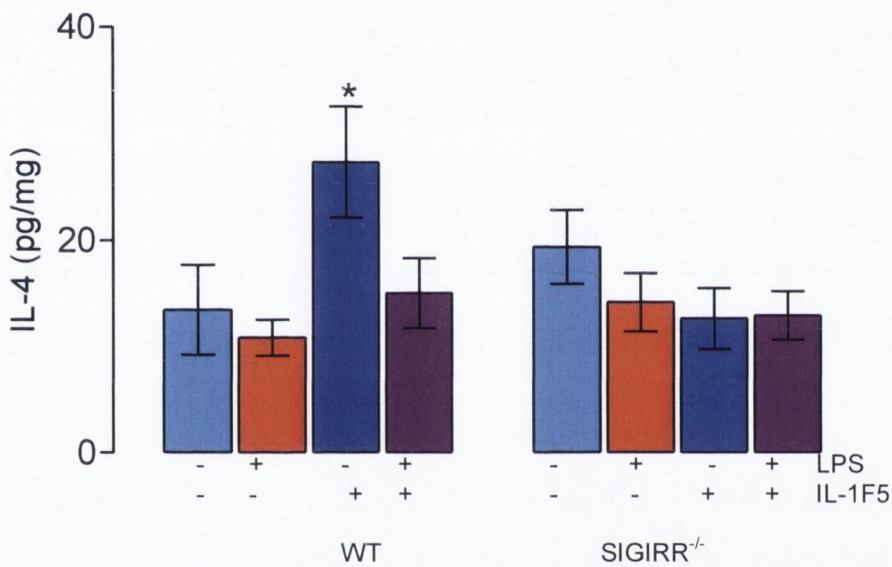
Figure 3.22 IL-1F5 increase IL-4 concentration in the hippocampus of wild-type but not SIGIRR$^{-/-}$ mice

IL-4 concentration was significantly increased in hippocampal tissue prepared from IL-1F5-treated wild-type mice compared with control-treated mice (30ng/ml IL-1F5; *P<0.05; 2-way ANOVA; n=6). There was no significant difference in IL-4 concentration in hippocampal tissue prepared from control-treated mice compared with LPS-treated cells which received IL-1F5. IL-4 concentration was significantly lower in LPS-treated cells compared with IL-1F5-treated cells (200µg/kg LPS; *P<0.05; 2-way ANOVA; n=6). Values are expressed as pg IL-4/mg protein and are means±SEM.

IL-4 concentration was not significantly altered in SIGIRR$^{-/-}$ mice in response to any treatments.

Interaction effect F$_{(1,41)}$ = 10.52, P=0.0023

3.7   Analysis of LPS-induced effects on cultures of mixed glial cells prepared from wild-type C57BL/6 and SIGIRR$^{-/-}$ mice treated with IL-1F5

3.7.1 IL-1F5 did not affect the LPS-induced increase in IL-1β mRNA or protein concentration in mixed glial cells prepared from SIGIRR$^{-/-}$ mice

To further investigate the possibility that IL-1F5 exerts its effects via an interaction with SIGIRR, mixed glial cultures were prepared from wild-type C57BL/6 and SIGIRR$^{-/-}$ mice and treated with LPS, IL-1F5 or the two in combination. IL-1β mRNA and protein concentration was assessed in all samples, as was IL-4 mRNA and protein concentration. IL-1β mRNA was assessed in cells prepared from wild-type C57BL/6 and SIGIRR$^{-/-}$ mice. Figure 3.23(A) depicts a sample gel indicating IL-1β mRNA expression in cells prepared from wild-type C57BL/6 (lane 1-4) and SIGIRR$^{-/-}$ (lane 5-8). IL-1β mRNA expression appeared to be increased in LPS-treated cells prepared from wild-type C57BL/6 mice (lane 2), compared with controls (lane 1). IL-1F5 appeared to abrogate this effect when administered with LPS (lane 4) with IL-1β mRNA expression levels similar to controls. IL-1F5 alone exerted no effect (lane 3). IL-1β mRNA expression levels appeared to be slightly increased in LPS-treated (lane 6) and LPS and IL-1F5-treated (lane 8) cell prepared from wild-type SIGIRR$^{-/-}$ mice, compared with controls (lane 5). Treatment with IL-1F5 alone exerted no effect on IL-1β mRNA expression in cells prepared from SIGIRR$^{-/-}$ mice (lane 7).

Figure 3.23(B) shows mean results obtained from densitometric analysis. Mean IL-1β mRNA expression was significantly increased in LPS-treated (6.71 $\pm$ 0.88 arbitrary units; n=6), compared with control-treated (2.34 $\pm$ 1.29 arbitrary units; *p<0.05, 3-way ANOVA; n=6) cells prepared from wild-type C57BL/6 mice. There was no significant difference in IL-1β mRNA expression in cells treated with both LPS and IL-1F5 (3.58 $\pm$ 0.78 arbitrary units; n=6), compared with

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controls. IL-1F5 alone exerted no effect, therefore IL-1β concentration was similar in cells treated with IL-1F5 (1.64 ± 1.11 arbitrary units; n=6), compared with controls.

IL-1β mRNA expression was increased in LPS-treated and LPS and IL-1F5-treated cells prepared from SIGIRR$^{-/-}$ mice, as depicted in the sample blot shown in Figure 3.23(A), but analysis of mean data indicated that there was no significant difference in IL-1β mRNA expression in LPS-treated (7.51 ± 0.85 arbitrary units; n=6), compared with control-treated (5.33 ± 0.46 arbitrary units; n=6) cells prepared from SIGIRR$^{-/-}$ mice. IL-1β mRNA expression was lower in IL-1F5-treated (2.18 ± 0.99 arbitrary units; n=6), compared with control-treated cells prepared from SIGIRR$^{-/-}$ mice. There was a significant increase in IL-1β mRNA in LPS and IL-1F5-treated (6.71 ± 0.71 arbitrary units; n=6), compared with IL-1F5-treated SIGIRR$^{-/-}$ cells (*$p < 0.05$, 3-way ANOVA; n=6).

Figure 3.24 shows IL-1β protein concentration was significantly increased in LPS-treated (24.15 ± 7.04 pg/ml; n=13), compared with control-treated cells prepared from wild-type C57BL/6 mice (4.2 ± 0.47 pg/ml; **$p < 0.01$, 3-way ANOVA; n=10). There was no significant difference in IL-1β concentration in LPS and IL-1F5-treated cells prepared from wild-type C57BL/6 mice (7.17 ± 1.65 pg/ml; n=11), compared with controls. IL-1F5 alone exerted no effect therefore IL-1β concentration was similar in IL-1F5-treated cells (4.25 ± 0.48 pg/ml; n=9) and control-treated cells.

IL-1β concentration was significantly increased in LPS-treated (26.24 ± 7.54 pg/ml; n=14), compared with control-treated (8.94 ± 1.92 pg/ml; *$p < 0.05$, 3-way ANOVA; n=14) cells prepared from SIGIRR$^{-/-}$ mice. There was a significant increase in IL-1β concentration in LPS and IL-1F5-treated (31.42 ± 5.13 pg/ml; n=11), compared with control-treated cells prepared from SIGIRR$^{-/-}$ mice (*$p < 0.05$, 3-way ANOVA; n=11). IL-1F5 alone exerted no effect therefore there was no difference in IL-1β concentration in IL-1F5-treated (11.19 ± 1.44 pg/ml; n=14), compared with control-treated cells prepared from SIGIRR$^{-/-}$ mice.

117
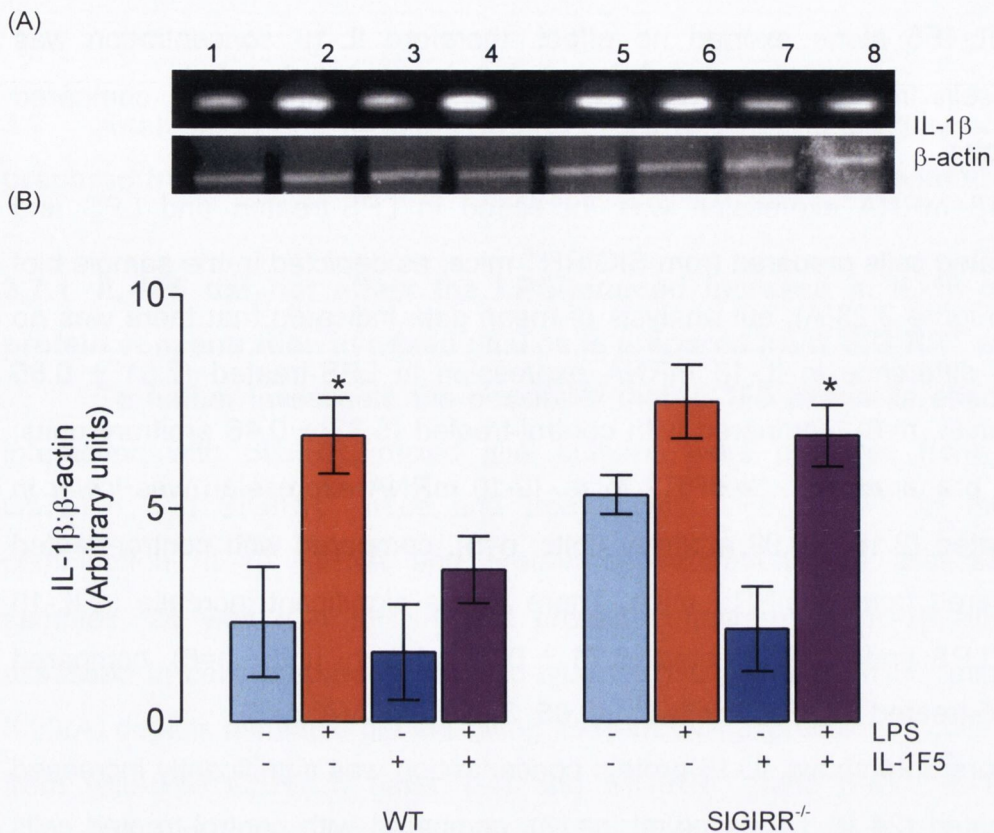
Figure 3.23 IL-1F5 attenuates the LPS-induced increase in IL-1β mRNA expression in wild-type but not SIGIRR-/- mixed glial cultures

(A) A sample agarose gel shows IL-1β mRNA expression, as shown by the presence of the PCR bands of 447 base-pairs, in control-treated (lane 1), LPS-treated (lane 2), IL-1F5-treated (lane 3) and LPS and IL-1F5-treated (lane 4) wild-type murine glial cells. Control-treated (lane 5), LPS-treated (lane 6), IL-1F5-treated (lane 7) and LPS and IL-1F5-treated (lane 8) mixed glial cells from SIGIRR$^{-/-}$ mice are also represented. A loading control of β-actin was used for this analysis.

(B) Treatment of cells prepared from wild-type mice with LPS significantly increased IL-1β mRNA expression, compared with control-treated cells (100ng/ml LPS; *p<0.05; 2-way ANOVA; n=6). There was no significant difference in IL-1β mRNA expression in tissue prepared from control-treated and LPS-treated cells which were treated with IL-1F5 (3µg/ml IL-1F5; n=6). IL-1F5 alone exerted no effect. IL-1β mRNA expression was not significantly altered in cells generated from SIGIRR$^{-/-}$ mice in response to any treatment. Values are expressed as arbitrary units of β-actin:IL-1β expression and are means ± SEM Values are expressed as arbitrary units of 18s:IL-4 expression and are means ± SEM.

Interaction effect $F_{(1,28)}$ = 3.10, P=0.091
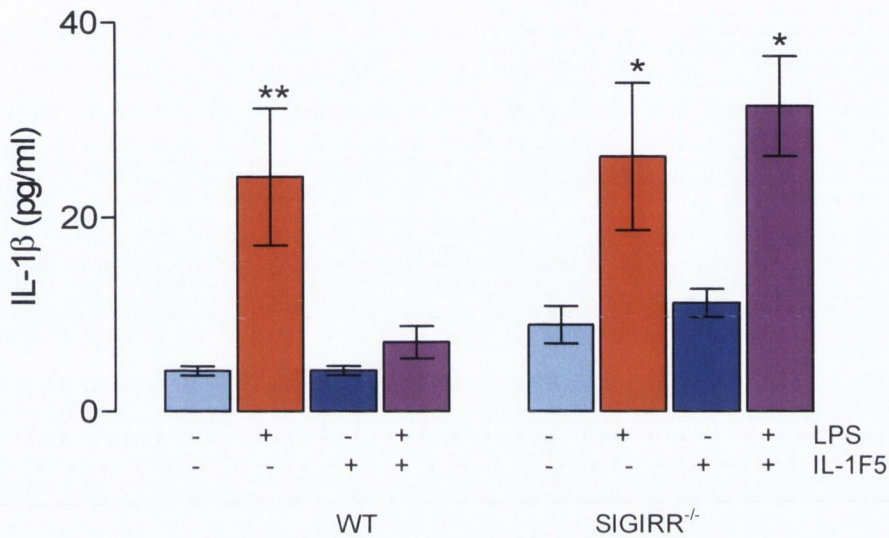
Figure 3.24 IL-1F5 attenuates the LPS-induced increase in IL-1β in wild-type but not SIGIRR$^{-/-}$ mixed glial cultures

Treatment of cells prepared from wild-type mice with LPS significantly increased IL-1β concentration compared control-treated wild-type cells (**p<0.01, 3-way ANOVA; n=6). There was no significant difference in IL-1β concentration in control-treated and LPS- treated cells which received IL-1F5 (3μg/ml IL-1F5; n=6). IL-1β concentration was significantly decreased in LPS-treated wild-type cells which received IL-1F5 compared with LPS-treated wild-type cells (3-way ANOVA; n=6). IL-1F5 exerted no significant effect in cells generated from wild-type mice. IL-1β was significantly increased in LPS-treated SIGIRR$^{-/-}$ cells (100ng/ml LPS;*p<0.05, 3-way ANOVA; n=6) compared with control-treated SIGIRR$^{-/-}$ cells . IL-1β concentration was significantly increased in LPS-treated cells which received IL-1F5 compared with control-treated cells (3μg/ml IL-1F5;*p<0.05, 3-way ANOVA; n=6). IL-1F5 exerted no significant effect in SIGIRR$^{-/-}$ cells Values are expressed as pg IL-1β/ml supernatant and are means±SEM.

Interaction effect F$_{(1,84)}$ = 2.73, P=0.10

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3.7.2 IL-1F5 did not induce an increase in IL-4 mRNA or protein concentration in mixed glial cultures prepared from SIGIRR$^{-/-}$ mice

The expression of IL-4 mRNA was assessed *in vitro* in mixed glial cultures prepared from wild-type C57BL/6 mice and SIGIRR$^{-/-}$ mice by RT-PCR. Figure 3.25(A) depicts IL-4 mRNA expression, measured as a ratio to 18S and illustrates greater expression of IL-4 mRNA expression in IL-1F5 (lane 3) and LPS and IL-1F5 (lane 4) samples, compared with control-treated (lane 1) and LPS-treated samples (lane 2).

Mean densitometric analysis revealed that IL-4 mRNA expression was significantly increased in IL-1F5-treated cells prepared from wild-type C57BL/6 mice (4.84 ± 0.41 arbitrary units; n=8), compared with control-treated cells prepared from wild-type C57BL/6 mice (2.22 ± 0.39 arbitrary units; *p<0.05, 3-way ANOVA; n=7). IL-4 mRNA expression was significantly increased in LPS and IL-1F5-treated (4.59 ± 1.1 arbitrary units; n=8), compared with control-treated cells prepared from wild-type C57BL/6 mice (2.33 ± 0.25 arbitrary units; *p<0.05, 3-way ANOVA; n=8). LPS alone exerted no affect, there was no difference in IL-4 mRNA expression in LPS-treated, compared with control-treated cells prepared from wild-type C57BL/6 mice. IL-4 mRNA expression was significantly greater in cells prepared from wild-type C57BL/6 compared with cells prepared from SIGIRR$^{-/-}$ mice (#p<0.05, 3-way ANOVA; n=6).

Figure 3.25(A) depicts IL-4 mRNA expression in mixed glial cells generated from SIGIRR$^{-/-}$ mice (lane 5-8). Figure 3.25(A) shows no detectable IL-4 mRNA expression in cells prepared from SIGIRR$^{-/-}$ mice. Mean densitometric analysis further verified that IL-4 mRNA was undetectable in cells prepared from SIGIRR$^{-/-}$ mice, regardless of treatment.

IL-4 protein concentration was assessed in the supernatant collected from cells prepared from wild-type C57BL/6 and SIGIRR$^{-/-}$ mice. Figure 3.26 shows

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that IL-4 concentration was significantly increased in IL-1F5-treated cells prepared from wild-type C57BL/6 mice (23.46 ± 5.32 pg/ml; n=6), compared with control-treated cells prepared from wild-type C57BL/6 mice (12.98 ± 2.48 pg/ml; *p<0.05, 3-way ANOVA; n=6). There was no significant difference in IL-4 concentration in LPS and IL-1F5-treated cells prepared from wild-type C57BL/6 mice (15.01 ± 4.89 pg/ml; n=6), compared with control-treated cells prepared from wild-type C57BL/6 mice. LPS alone exerted no effect, there was no difference in IL-4 concentration in LPS-treated cells (12.65 ± 1.66 pg/ml; n=6), compared with control-treated cells prepared from wild-type C57BL/6 mice.

There was no significant difference in IL-4 concentration in cells prepared from SIGIRR$^{-/-}$ mice, regardless of treatment. IL-4 concentration was similar in control-treated (9.25 ± 3.5 pg/ml; n=6), LPS-treated (10.13 ± 2.4 pg/ml; n=6), IL-1F5-treated (13.5 ± 1.76 pg/ml; n=6) and LPS and IL-1F5-treated cells prepared from SIGIRR$^{-/-}$ mice (14.40 ±3.17 pg/ml; n=6).

121
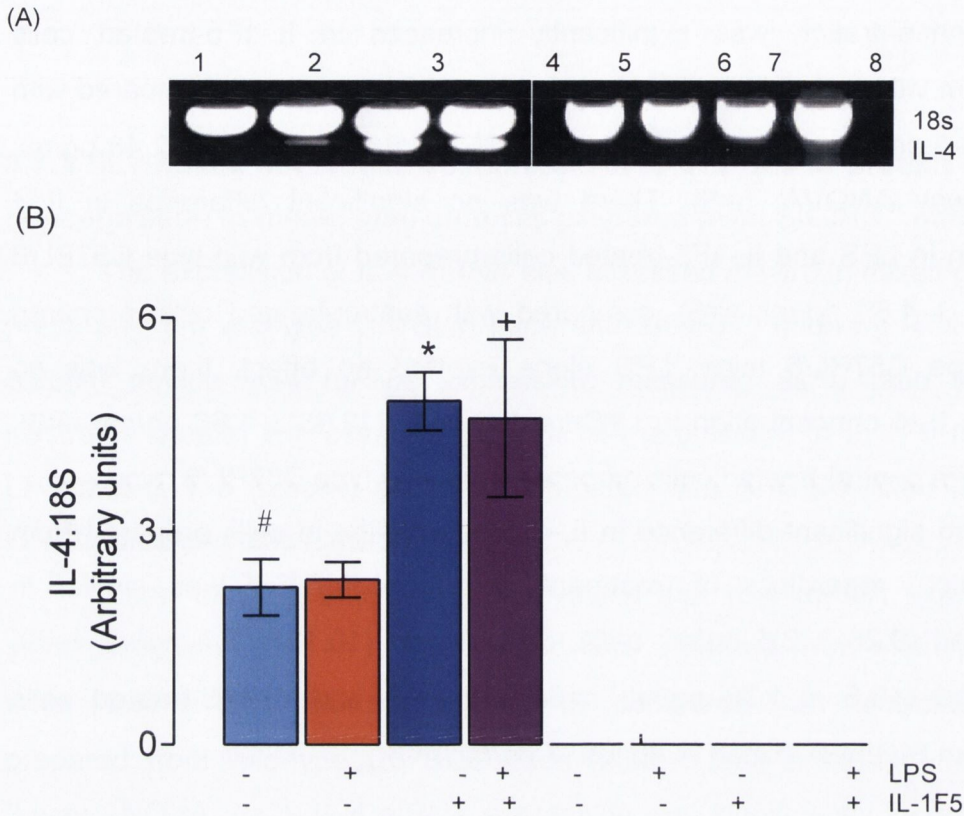
Figure 3.25 IL-4 mRNA is increased in wild-type mixed glial culture following treatment with IL-1F5

(A) A sample agarose gel shows IL-4 mRNA expression levels in control-treated (lane 1), LPS-treated (lane 2), IL-1F5-treated (lane 3) and LPS and IL-1F5-treated (lane 4) wild-type murine glial cells. Control-treated (lane 5), LPS-treated (lane 6), IL-1F5-treated (lane 7) and LPS and IL-1F5-treated (lane 8) mixed glial cells from SIGIRR^{-/-} mice are also represented.

(B) Treatment of cells prepared from wild-type mice with IL-1F5 significantly increased Il-4 mRNA expression compared with control-treated cells (3μg/ml IL-1F5; [*]$p<0.05$; 2-way ANOVA; n=6). IL-4 mRNA expression was significantly greater in LPS-treated cells which received IL-1F5 (100ng/ml LPS; [*]$p<0.05$; 2-way ANOVA; n=6) compared with control-treated cells. IL-4 mRNA expression was significantly lower in LPS-treated cells compared with IL-1F5-treated cells ([*]$p<0.05$; 2-way ANOVA; n=6). IL-4 mRNA was undetectable in mixed glial cells from SIGIRR^{-/-} mice. 18s expression in hippocampal tissue was similar in all treatment groups in both wild-type and SIGIRR^{-/-} mice (n=6). Il-4 mRNA was significantly greater in cells prepared from wild-type mice compared with cells prepared from SIGIRR^{-/-} mice (#$p<0.05$, 3-way ANOVA).Values are expressed as arbitrary units of 18s:IL-4 expression and are means ± SEM.

Interaction effect $F_{(1,35)}$ = 61.05, P=0.0026
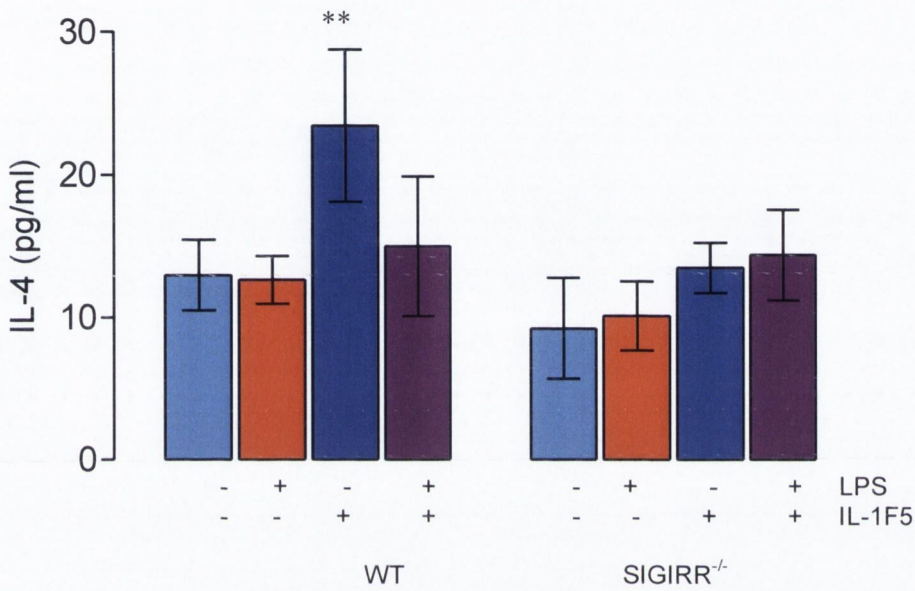
Figure 3.26 Effect of LPS and IL-1F5 on IL-4 in wild-type and SIGIRR$^{-/-}$ mixed glial cultures

IL-4 concentration was significantly increased in IL-1F5-treated compared with control-treated wild-type cells (3μg/ml IL-1F5; **p<0.01, 3-way ANOVA; n=6). There was no significant difference in IL-4 concentration in saline-treated cells compared with IL-1F5-treated cells generated from SIGIRR$^{-/-}$ mice. Values are expressed as pg IL-4/ml supernatant and are means±SEM.

Interaction effect: $F_{(1,20)}$ = 203.8, P=0.04

3.8 Analysis of SIGIRR expression *in vivo* and *in vitro*

3.8.1 IL-1F5 induces an increase in SIGIRR mRNA in wild-type C57BL/6 hippocampal tissue.

SIGIRR has been shown to be expressed in low levels in the brain (Thomassen *et al.*, 2001) and here SIGIRR mRNA expression was analyzed in hippocampal tissue prepared from wild-type C57BL/6 mice. Figure 3.27 shows the results obtained by QT-PCR. SIGIRR mRNA expression was increased in hippocampal tissue prepared from IL-1F5-treated (76.32 ± 20.58 arbitrary units; n=11), compared with control-treated wild-type C57BL/6 mice (23.84 ± 8.68 arbitrary units; **$p<0.01$, 2-way ANOVA; n=12). LPS exerted no effect on SIGIRR mRNA expression. SIGIRR mRNA expression was similar in the hippocampus of LPS-treated (16.61 ± 5.57 arbitrary units; n=8), LPS and IL-1F5-treated (16.15 ± 6.21 arbitrary units; n=9) and control-treated wild-type C57BL/6 mice.

3.8.2 IL-1F5 induces an increase in SIGIRR expression in mixed glial culture.

To investigate the cell type on which SIGIRR is expressed SIGIRR protein was assessed in cultured glial cells that had been treated with IL-1F5. Primary cultures of glia were prepared from Wistar rats. Figure 3.28 shows that SIGIRR expression was increased in glial cells that had been treated with IL-1F5, compared with control-treated cells.

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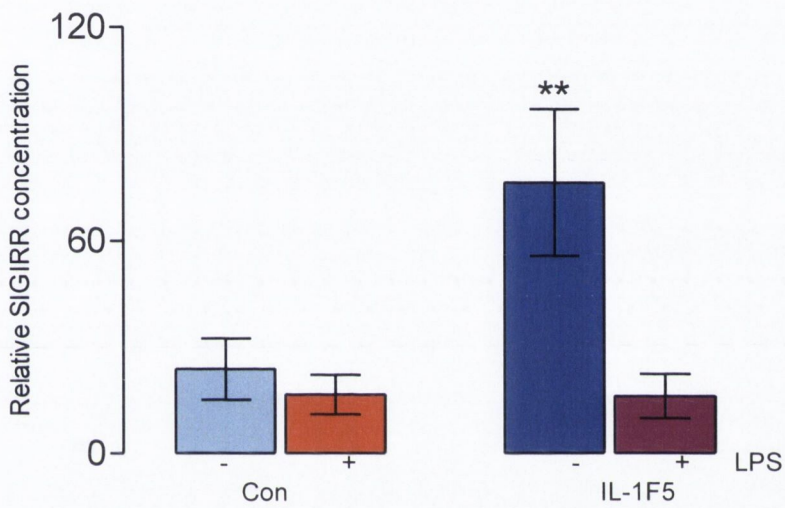
Figure 3.27 IL-1F5 induces an increase in SIGIRR mRNA expression in the hippocampus of wild-type mice

SIGIRR mRNA expression was significantly increased in IL-1F5-treated wild-type mice compared with control-treated mice (30ng/ml IL-1F5; **p<0.01; 2-way ANOVA; n=6). There was no significant difference in SIGIRR mRNA expression in control-treated mice compared with LPS-treated cells which received IL-1F5.

Interaction effect $F_{(1,20)}$=21.41, p=0.0027

SIGIRR PICTURE

Control
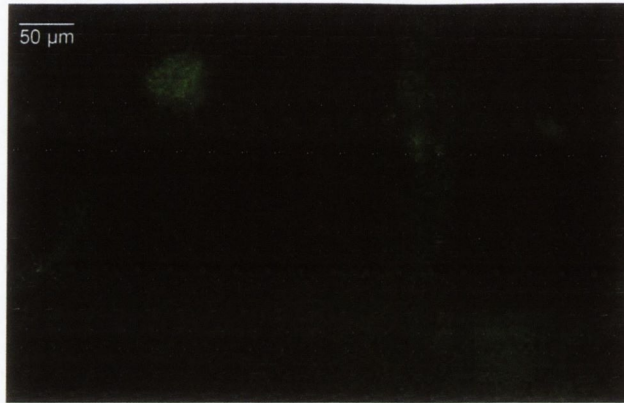
IL-1F5
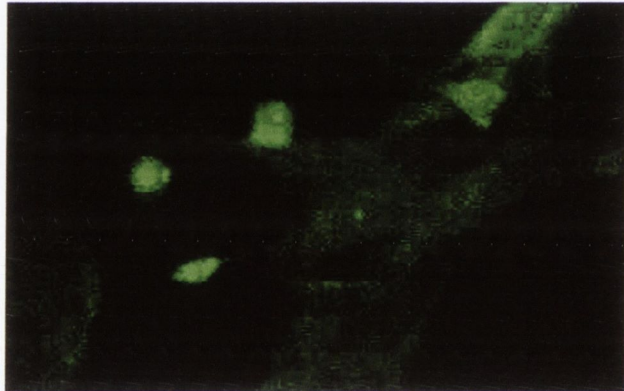
Figure 3.28 IL-1F5 increases SIGIRR expression in cultured mixed glial cells

Cultured mixed glial cells were incubated overnight with a fluorescent conjugated antibody for SIGIRR for visualization under fluorescent microscopy. Cells were examined at x40 magnification using an excitation wavelength of 497nm. IL-1F5-treated mixed glial cells exhibited increased fluorescent staining for SIGIRR compared with control-treated mixed glial cells

3.9 Analysis of IL-1F5 signaling *in vivo* and *in vitro*

3.9.1 JAK1 and STAT6 phosphorylation was increased in rat hippocampal tissue following treatment with IL-1F5.

To investigate the significant pathways induced by IL-1F5, rats were treated with IL-1F5 and hippocampal homogenate was prepared for gel electrophoresis and western blotting. In the first instance the effect of IL-1F5 on downstream modulators of IL-4 function - JAKs and STATs were examined. Binding of IL-4 to its receptor results in the phosphorylation of JAK and phosphorylation, and subsequent translocation of STAT-6 to the nucleus (Nelms *et al.*, 1999). In the nucleus, STAT-6 binds to STAT-binding elements to activate gene transcription (Decker *et al.*, 1997). Therefore expression of pJAK and pSTAT were assessed and Figure 3.29(A) depicts a sample immunoblot showing phosphorylated and total JAK in hippocampal tissue prepared from control-treated and IL-1F5-treated Wistar rats. It shows increased JAK phosphorylation in the IL-1F5-treated sample (lane 2), compared with a control-treated sample (lane 1). Mean data obtained from densitometric analysis indicated there was a significant increase in JAK1 phosphorylation in hippocampal tissue prepared from IL-1F5 treated rats (222.83 ± 61.51 arbitrary units; n=5), compared with control treated rats (106.45 ± 6.69 arbitrary units; **$p < 0.01$; Student's t test for independent means; n=5). This finding was coupled with a significant increase in downstream STAT6 phosphorylation. Figure 3.30(A) shows a sample immunoblot depicting STAT6 phosphorylation and actin expression in the hippocampus of control-treated and IL-1F5-treated rats . Mean data obtained from densitometric analysis indicates there was a significant increase in STAT6 phosphorylation in hippocampal tissue prepared from IL-1F5

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treated rats (163.14 ± 25.38 arbitrary units; n=5), compared with control-treated rats (95.40 ± 4.01; *p<0.05; Student's unpaired t test for independent means; n=5).

3.9.2 ERK1 and 2 phosphorylation were increased in rat hippocampal tissue following treatment with IL-1F5.

One of the consequences of JAK phosphorylation is activation of the ras-raf pathway and therefore, potentially, phosphorylation and activation of ERK. A sample immunoblot in Figure 3.31 depicts ERK phosphorylation and total ERK expression in hippocampal tissue prepared from the hippocampus of Wistar rats. The blot shows increased ERK1 and ERK2 phosphorylation in IL-1F5-treated (lane 2), compared with control-treated hippocampal tissue (lane 1). Mean data obtained from densitometric analysis indicate there was a significant increase in ERK1 phosphorylation in hippocampal tissue prepared from IL-1F5 treated (6.79 ± 2.45 arbitrary units; n=6), compared with control-treated rats (2.72 ± 0.68 arbitrary units; *p<0.05; Student's t test for independent means; n=5). This finding was coupled with a significant increase in ERK2 phosphorylation in hippocampal tissue prepared from IL-1F5-treated (9.49 ± 0.99 arbitrary units; n=5), compared with control-treated rats (4.00 ± 1.2 arbitrary units; *p<0.01, Student's unpaired t test for independent means; n=6).

3.9.3 ERK1/2 expression was increased in cultured rat neuronal cells following treatment with IL-1F5.

ERK expression was also investigated in cultured neuronal cells using a phosphorylated ERK1/2 antibody. Figure 3.32 shows immunflourescent staining of ERK in cultured primary hippocampal neurons, prepared from Wistar rats. The micrograph shows increased ERK staining in IL-1F5-treated neurons, compared with control-treated neurons.

(A)

pJAK1

tJAK

(B)
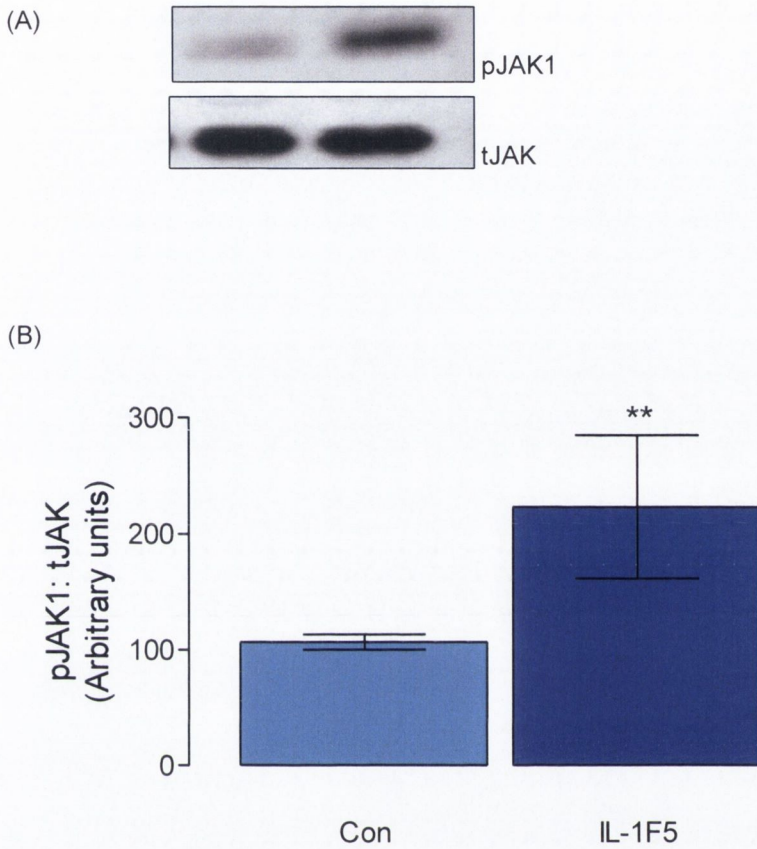
Figure 3.29 JAK1 phosphorylation is increased in hippocampus following IL-1F5 treatment

(A) A sample immunoblot shows JAK1 activation, as shown by the phosphorylation of the 130kDa form, in control-treated and IL-1F5-treated rats. A loading control of total JAK was used for this analysis.

(B) JAK1 phosphorylation was significantly increased in IL-1F5-treated rats compared with saline-treated rats (30ng/ml IL-1F5; * p<0.01; students unpaired t-test; n=6). Values are expressed as arbitrary units and are mean ± SEM.
Total JAK expression in hippocampal tissue was similar in saline-treated rats and IL-1F5-treated rats (n=6). Values are expressed as arbitrary units of total JAK:pJAK1 expression and are means ± SEM.

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(A)
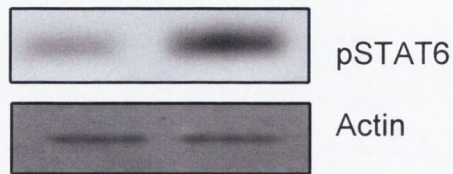
(B)
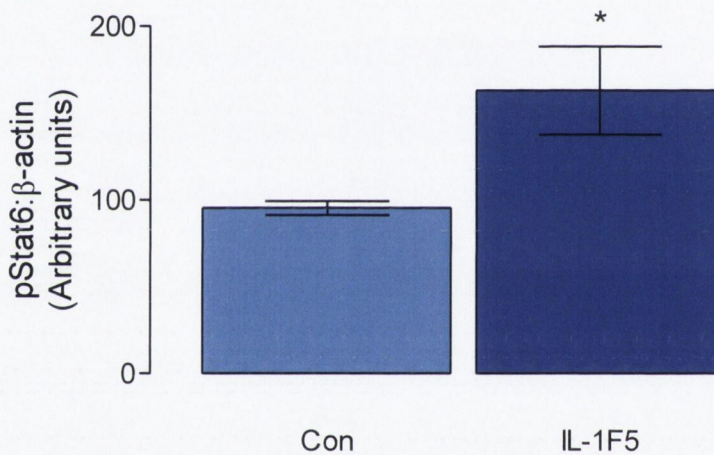
Figure 3.30 STAT6 phosphorylation is increased in hippocampus following IL-1F5 treatment

(A) A sample immunoblot shows STAT6 activation, as shown by the phosphorylation of the 100kDa form, in control-treated and IL-1F5-treated rats. A loading control of actin was used for this analysis.

(B) STAT6 phosphorylation was significantly increased in IL-1F5-treated rats compared with saline-treated rats (3μg/ml IL-1F5; *p<0.05; students unpaired t-test; n=6).

β-actin expression in hippocampal tissue was similar in saline-treated rats and IL-1F5-treated rats (n=6). Values are expressed as arbitrary units of actin:pSTAT6 expression and are means ± SEM.

(A)

pERK1

pERK1   pERK2

tERK

(B)

(C)
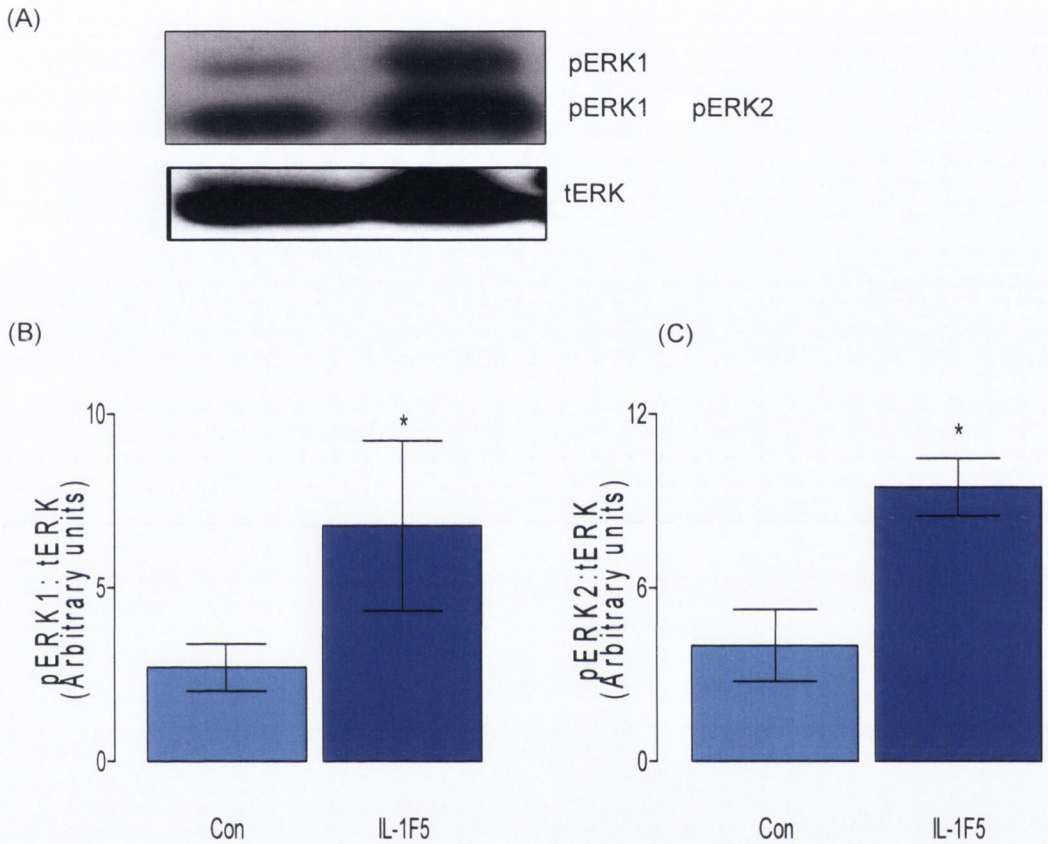
Figure 3.31 ERK1 and ERK2 phosphorylation is increased in hippocampus following IL-1F5 treatment

(A) A sample immunoblot shows ERK1 and ERK2 activation, as shown by the phosphorylation of the p44 and p42 subunits , in control-treated and IL-1F5-treated. A loading control of total ERK was used for this analysis.

(B) ERK1 phosphorylation was significantly increased in IL-1F5-treated rats compared with saline-treated rats (30ng/ml IL-1F5; *p<0.05; students unpaired t-test; n=6). Values are expressed as arbitrary units of pERK1:tERK expression and are means ± SEM.

(C) ERK2 phosphorylation was significantly increased in IL-1F5-treated rats compared saline-treated rats (30ng/ml IL-1F5; *p<0.05; students unpaired t test; n=6). Values are expressed as arbitrary units of pERK2:tERK expression and are means ± SEM.

Total ERK in hippocampal tissue was similar for tissue prepared from saline-treated rats and IL-1F5-treated rats (n=6).

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ERK PICTURE

Control
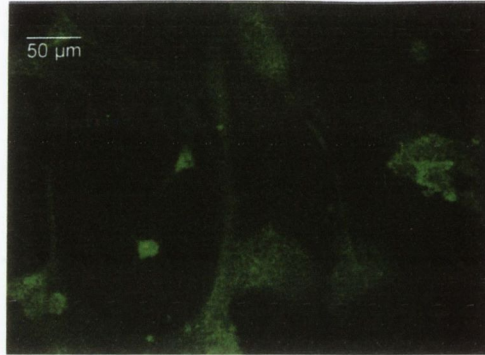
IL-1F5
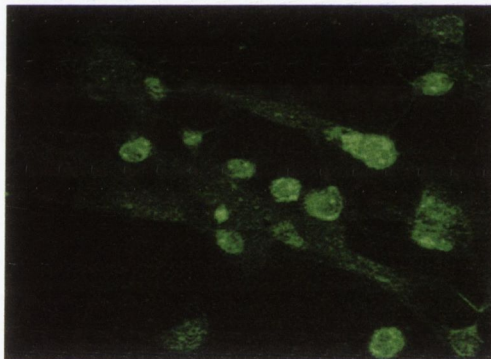
Figure 3.32 IL-1F5 increases ERK activation in cultured hippocampal neurons

Cultured hippocampal neurons were incubated overnight with a fluorescin conjugated antibody for phosphorylated ERK for visualization under fluorescent microscopy. Cells were examined at x40 magnification using an excitation wavelength of 497nm. IL-1F5-treated hippocampal neurons exhibited increased fluorescent staining for pERK compared with control-treated neurons.

3.9.4 PPARγ activation is increased in cultured mixed glial cells following treatment with IL-1F5.

IL-4 has been shown to induce activation of peroxisome proliferators-activated receptor gamma (PPARγ), which has been shown to mediate anti-inflammatory effects (Welch *et al.*, 2003). Therefore the effect of IL-1F5 on PPARγ activation was assessed. The sample immunoblot in Figure 3.33(A) depicts PPARγ activation and actin in glial cells prepared from Wistar rats. The blot shows increased expression of activated PPARγ in IL-1F5-treated (lane 2), compared with control-treated sample (lane 1). Mean data obtained from densitometric analysis indicate there was a significant increase in PPARγ expression in IL-1F5-treated (0.37 ± 0.05 arbitrary units; n=13), compared with control-treated glial cells (0.19 ± 0.01 arbitrary units; **$p<0.01$; Student's t test for independent means; n=11).

3.9.5 IL-1F5 did not abrogate the LPS-induced increase in IL-1β concentration in cultured mixed glial cells following treatment with GW9662

To further investigate the interaction between PPARγ and IL-1F5 and to determine if IL-1F5 might mediate its effects via PPARγ activation, a PPARγ inhibitor was used. Primary glial cultures were prepared from Wistar rats and incubated with or without IL-1F5, in the presence or absence of GW9662, and IL-1β concentration was evaluated. As shown in Figure 3.34 there was a significant increase in IL-1β concentration in LPS-treated (545.40 ± 39.17 pg/ml; n=6), compared to control-treated glial cells (105.90 ± 18.59 pg/ml; $p<0.05$, 3-way ANOVA; n=6). IL-1β concentration was significantly increased in LPS-treated, compared with LPS and IL-1F5-treated cells (104.10 ± 11.55 pg/ml; +$p<0.05$, 3-way ANOVA; n=6). IL-1F5 alone exerted no effect (86.19 ± 36.14 pg/ml; n=6), with IL-1β concentration similar to control-treated glial cells. IL-1β concentration was significantly increased in LPS and IL-1F5-treated cells which were incubated in the presence of GW9662 (237.20 ± 108.5 pg/ml; n=6),

compared with control-treated cells (*$p<0.05$, 3-way ANOVA; n=6). GW9662 alone exerted no effect, IL-1β concentration was similar in GW9662-treated (92.60 $\pm$ 35.44 pg/ml; n=6), compared with control-treated cells.

(A)

PPARγ

Actin
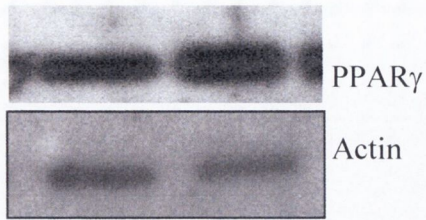
(B)
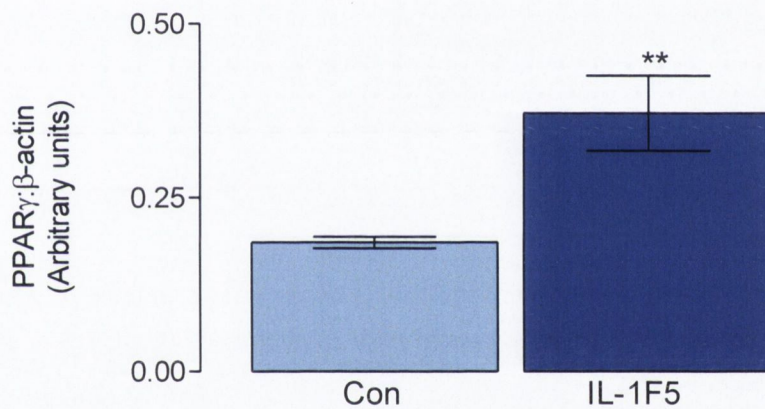
Figure 3.33 IL-1F5 –treatment is associated with an increase in activation of PPARγ in cultured cortical glia.

(A)) A sample immunoblot shows PPARγ activation, as shown by the presence of the 67kDa form, in control-treated and IL-1F5-treated. A loading control of actin was used for this analysis.

(B) PPARγ concentration was significantly increased in IL-1F5-treated cells compared with control-treated cells (3μg/ml IL-1F5; [**]p<0.01 students t-test; n=6).

Values are expressed as arbitrary units of actin:PPARγ activation and are means±SEM.

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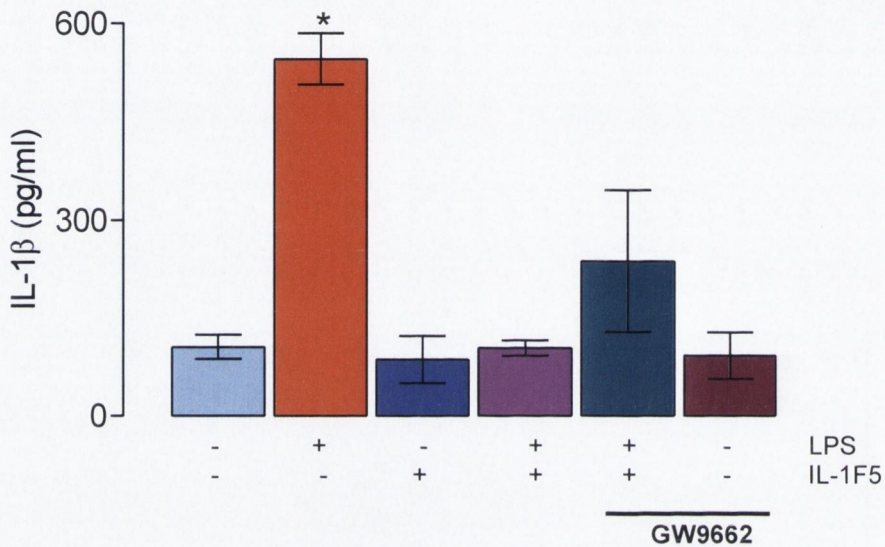
Figure 3.34 IL-1F5 attenuation of LPS-induced increase in IL-1β is blocked by pre-treatment with GW9662 in cultured cortical glia

IL-1β concentration was significantly increased in LPS-treated cells compared with control-treated cells (1μg/ml LPS; *p<0.05; 3-WAY ANOVA; n=6). There was no significant difference in IL-1β concentration in saline-treated cells compared with LPS-treated cells which received IL-1F5 (3μg/ml IL-1F5; n=5). IL-1β concentration was significantly increased in LPS-treated cells which received IL-1F5 and the antagonist GW9662 compared with control-treated cells (1μg/ml LPS; 3μg/ml IL-1F5; 20μM GW9662; *p<0.05; 3-WAY ANOVA; n=6). Similarly IL-1β concentration was significantly increased in LPS-treated cells which received IL-1F5 and GW9662, compared with IL-1F5-treated cells (3μg/ml IL-1F5; *p<0.05; 3-WAY ANOVA; n=6). There was no significant difference in IL-1β in control-treated cells compared with IL-1F5-treated cells. There was no significant difference in IL-1β in control-treated cells compared with GW9662 (n=6).

Values are expressed as pg/ml supernatant and are mean ± SEM

Interaction effect $F_{(1,39)}$ = 21.41, P=0.0057

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Discussion

This study set out to investigate the effects of IL-1F5 administration in the hippocampus with the emphasis on an analysis of IL-1F5 effects on LPS and IL-1β-induced inflammation and its potential modulatory effect in a number of animal models. The most significant finding of the study is that IL-1F5 had an anti-inflammatory effect, blocking several LPS and IL-1β induced changes in the hippocampus and also in glial cells. Importantly, the data showed that the effects of IL-1F5 are mediated through its induction of the anti-inflammatory cytokine IL-4. IL-1F5 shows high homology to IL-1ra, yet does not interact with any of the classical IL-1 receptors. An interesting finding of the study was that the novel orphan receptor, SIGIRR is present on glial cells, and is upregulated in response to IL-1F5. Furthermore, the data suggests that the effects of IL-1F5 may be mediated through its interaction with SIGIRR.

4.1 IL-1F5 inhibits IL-1β signalling in the brain

In the first instance evidence is presented which shows that intracerebroventricular injection of IL-1β induced inhibition of LTP in the dentate gyrus and a corresponding increase in JNK activation in the rat hippocampus. This supports previous evidence which indicates that IL-1β causes an inhibition of LTP in CA1 (Bellinger *et al.*, 1993), CA3 (Katsuki *et al.*, 1990) and dentate gyrus (Cunningham *et al.*, 1996; Murray and Lynch 1998).

Expression of IL-1β has been shown to be high in hippocampus and IL-1β-induced impairment in LTP has been consistently reported. Precisely how IL-1β exerts its deleterious effect on plasticity is unclear. IL-1β inhibits the release of acetylcholine (Rada *et al.*, 1991) and glutamate (Murray *et al.*, 1997) in hipppocampal synaptosomes. Furthermore, IL-1β negatively affects calcium influx in hippocampal synaptosomes and Ca^{2+} channel currents in hippocampal neurons (Murray *et al.*, 1997; Plata-Salaman and Ffrench-Mullen, 1994). Because LTP maintenance has been associated with increased glutamate release (Bliss and Collindridge, 1993), the inhibitory effect of IL-1β on glutamate release may be a factor in IL-1β induced inhibition of LTP. In addition, a huge number of molecules become activated in response to IL-1β. Recent evidence implicated the mitogen-activated protein (MAP) kinase family in the inhibitory effect of IL-1β. In particular the MAP

kinase, JNK is implicated. JNK is activated when it is phosphorylated. IL-1β binds to its cognate receptor, IL-1R1 and as a binary complex it recruits the accessory protein IL-1RAcP, to form an active signalling complex. The complex formed upon binding of IL-1β to IL-1R1 requires activation of TNF receptor-associated factor-6 (TRAF-6) to exert its downstream effects. Phosphorylation of this kinase has been associated with deficits in synaptic function; for instance, an increase in JNK activation has been shown to accompany age related impairments in the hippocampus (O Donnell *et al.*, 1998). The signalling pathways initiated by this complex activate and phosphorylate the MAP kinases, including JNK, culminating in the activation of the transcription factor NFκB (Neill *et al.*, 1998). JNK and its downstream activator c-jun are activated by environmental stress, including oxidative stress (Raingeaud *et al.*, 1995; Junger *et al.*, 1997) and activation leads to growth arrest or even cell death. JNK and p38, another stress activated protein kinase, are stimulated by IL-1β, which is consistent with the observation that IL-1β has been implicated in cell death, since both kinases have been shown to be activated in apoptosis (Rothwell *et al.*, 1995. Derijard *et al.*, 1994). Previous studies have shown that IL-1β induces an increase in reactive oxygen species, which in turn also increases activation of JNK.

The evidence shows that the IL-1 receptor antagonist IL-1ra inhibits the actions of IL-1β at IL-1R1, binding to the receptor but not activating any signalling pathways, acting as a highly selective competitive receptor antagonist (Eisenberg *et al.*, 1990). The neuroprotective effects of IL-1ra are evident in studies showing that IL-1ra reduced ischemic and excitotoxic brain damage and markedly inhibited the clinical symptoms of experimental allergic encephalomyelitis (EAE)(Aren *et al.*, 1998; Rothwell *et al.*, 1997). Previous studies at Trinity College Dublin have demonstrated that injection of IL-1ra abrogated IL-1β induced inhibition of LTP.. Furthermore IL-1ra administration was associated with an abrogation in the IL-1β-induced increase in JNK activation (Loscher *et al.*, 2003). However, in the same experiment administration of IL-1ra alone resulted in inhibition of LTP. In addition IL-1ra was associated with an increase in JNK activation and a decrease in glutamate release when administered alone. Interestingly, this effect was

maintained in the absence of IL-1R1 (Loscher et al., 2003). This result suggests that IL-1β may have the ability to abrogate IL-1ra, and that this activity occurs via interaction with a receptor distinct from IL-1R1. The crystal structure of both IL-1β and IL-1ra have been solved as free ligands and in complexes with IL-1R1. The two structures have the same overall β-trefoil fold but differ somewhat in loop length and structure. IL-1ra binds to IL-1R1 predominantly through interaction with domains 1 and 2 of the receptor, whereas IL-1β makes additional contacts with the membrane proximal domain 3, resulting in a rotation of this domain by about $20°$ relative to that of the IL-1ra bound receptor (Dunn et al., 2003). It is the interaction of IL-1β with domain 3 that is thought to bring about the conformation change required to recruit the IL-1RAcP and activate signalling (Dunn et al., 2003). It is therefore thought that this conformation change is critical for the recruitment of the accessory protein and for IL-1β signal transduction (Dunn et al., 2003).

A sequenced version of murine IL-1F5 was used in these studies to investigate its effect on IL-1β functioning. IL-1F5 shows 56% sequence identity to IL-1ra and although no IL-1ra like activity has been previously found for IL-1F5, activation of an NFκB reporter system by IL-1F9 has been shown to be inhibited by IL-1F5 in cells transfected with IL-1Rrp2 (Debets et al., 2001). The structure of IL-1F5 has been examined and compared with both IL-1β and IL-1ra. Within IL-1F5, the β-trefoil structure has been maintained, characteristic of IL-1 family membership (Debets et al., 2001) and studies report that IL-1F5 contains potential sites of receptor binding and accessory protein recruitment. However, similar to IL-1ra, IL-1F5 lacks the loop between the fourth and fifth β-strands, which is typical for IL-1 agonists (Debets et al., 2001). In addition, IL-1F5 modelling studies have been undertaken to assess wether or not IL-1F5 can bind to IL-1R1. These approaches revealed numerous clashes between side-chains in the loops and the corresponding binding sites in the receptor, which indicate that IL-1F5 does not bind IL-1R1 (Dunn et al., 2003).

The data presented here show that IL-1F5 abrogates IL-1β induced inhibition of LTP in the dentate gyrus and abrogates the IL-1β induced increase in JNK activation. While IL-1F5 had the ability to abrogate

IL-1β-induced changes in the hippocampus in a similar manner to IL-1ra, studies using Fc fusion proteins to overexpress receptors have indicated that IL-1F5 cannot bind IL-1R1; therefore, this ability is IL-1R1 independent.

4.2   IL-1F5 inhibits LPS signalling in the brain

LPS is a potent inducer of inflammatory mediators (Cohen, 2002), and can induce numerous changes centrally. Centrally mediated changes such as decreases in feeding, motor activity, and social behaviour (Bluthe *et al.*, 1992; Dantzer *et al.*, 1998; Lacosta *et al.*, 1999), as well as central changes in biochemical signalling (Loscher *et al.*, 2000) have been reported following intraperitoneal LPS administration. This suggests that peripherally administered LPS can induce a challenge in the brain, which can affect synaptic efficacy. The hypothalamo-pituitary-adrenal axis (HPA) may play a role as its activation has been shown to occur in response to various types of stress, and usually results in the production of corticosteroids from the adrenal cortex. This is of consequence as the hippocampus, effective functioning of which is particularly vulnerable to stress, has the highest concentration of glucocorticoid receptors in the brain (Yau *et al.*, 1995). It is known that HPA activation due to fear impairs memory (Diamond *et al.*, 1999), and administration of high concentrations of corticosteroids disrupt spatial learning (Luine *et al.*, 1994). IL-1β is also expressed in the brain in response to stress and LPS, and has been reported to activate the HPA. A number of studies at Trinity College Dublin have identified the fact that the LPS-induced inhibition of LTP is coupled with, and probably at least partially dependent on an increase in hippocampal concentration of IL-1β, since LPS-induced changes are abrogated by IL-1ra administration (Vereker *et al.*, 2000). Therefore, the LPS-induced inhibition of both the induction and the maintenance of LTP in dentate gyrus observed here is in accordance with previous findings (Vereker *et al.*, 2000; Nolan *et al.*, 2002) and is consistent with the observation that LPS has the ability to block LTP in other brain regions such as CA1 (Mizuno *et al.*, 2004).

LPS has been shown to activate peripheral modulators of inflammation, which in turn lead to inflammatory response in the CNS (Rothwell *et al.*, 1999). The CNS shows a well-organised innate immune reaction in response to systemic stressors (Lehnardt *et al.*, 2003). The CD14 and TLR4 receptors are constitutively expressed in the circumventricular organs, choroid plexus and leptomeninges. Circulating LPS is able to cause a rapid transcriptional activation of genes encoding CD14 and TLR4, as well as a wide variety of pro-inflammatory molecules in the circumventricular organs (Lehnardt *et al.*, 2003).

The results presented here indicate an observable increase in microglia activation in hippocampal sections taken from LPS-treated rats compared with control-treated rats and this is consistent with data showing that upregulation of pro-inflammatory mediators results in an increase in activation of the brain macrophage cells, microglia (Lehnardt *et al.*, 2003). Microglial cells monitor their environment, being able to respond to signs of homeostatic disturbance with a program of supportive and protective activities. Microglia express the LPS receptor, TLR4, therefore, the pro-inflammatory effect of LPS in the CNS is mediated through TLR4 binding on microglia and subsequent microglia activation (Lehnardt *et al.*, 2003).

Active microglia are the main sources of IL-1β in the brain (Giulian *et al.*, 1986; Van Dam *et al.*, 1995). In accordance with previous findings at Trinity College Dublin IL-1β concentration was significantly increased in LPS treated glial cells prepared from rat. Previous findings show a marked increase in IL-1β in glial cells in response to LPS (Nolan *et al.*, 2004). Once activated, microglial cells undergo a morphological change that renders them active. IL-1β is produced as an inactive 35 kDa precursor protein (pro-IL-1β), which is prototypically processed into 17 kDa mature IL-1β by an IL-1β converting enzyme (ICE) (Thornberry *et al.*, 1992). In the present study the LPS-induced increase in microglial activation corresponded with a significant increase in IL-1β in the hippocampus and this is consistent with the hypothesis that activated microglia are the source of IL-1β.

One of the most significant findings in this study is that IL-1F5 had the ability to abrogate LPS-induced changes. Thus the LPS-induced inhibition of

LTP was reversed by intracerebroventricular injection of IL-1F5, LPS-induced increase in microglial activation was suppressed and the increase in IL-1β and JNK associated with LPS was attenuated. These findings pose the question of how IL-1F5 might mediate its effects. One possibility is that the primary target for IL-1F5 is a microglial cell and that IL-1F5 may signal via an IL-1β independent pathway, or perhaps through TLR signalling.

4.3 Mechanism of action of IL-1F5 – A role for IL-4

The mechanisms underlying the inhibitory action of IL-1F5 on both LPS and IL-1β induced changes remained to be identified. It was considered that IL-1F5 could mediate its effects via activation of some endogenous inhibitor. There are a number of endogenous inhibitors of IL-1β, the most notable being IL-1ra, however it was previously shown that IL-1F5 acts independently of IL-1ra. Several anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and TGFβ have been identified, which can inhibit the release or actions of IL-1β (Rothwell, 1999) and in this study one of the anti-inflammatory cytokines was considered as a possible mediator of IL-1F5 effects.

Mechanisms that regulate the balance of Th1 ands Th2 cells, such as cytokines, are of great interest because they can determine the outcome of a disease. The anti-inflammatory cytokine IL-4 has been the subject of extensive research, as it emerges that it plays a pivotal role in modulating the inflammatory process. Many of the outcomes can be attributed to a downregulation of microglial production of cytokines, e.g., IL-1β and TNFα, or the attenuation of their secondary effects (Chao *et al.*, 1993; Dinarello, 1997; Kitamura *et al.*, 2000; Pahan *et al.*, 2000). IL-4 can also interfere with IL-1 bioactivity by enhancing IL-1ra synthesis (Dinarello, 1997). In addition, this cytokine can alter the microglial cell surface molecule expression (Chao *et al.*, 1993). Pre-treatment of microglial/neuronal cell co-cultures with murine IL-4 inhibited microglial-mediated neuronal cell injury (Chao *et al.*, 1993, Rothwell, 1999). Previous findings from the Trinity College Dublin Lab have reported the ability of IL-4 to reverse impairment of LTP (Nolan *et al.*, 2002, Barry *et al.*,

2005). IL-4 has been shown to   initiate increase in the release of anti-inflammatory agents such as TGFβ (Nelms *et al.*, 1999).

Activation of a number of signalling pathways leads to IL-4 influencing gene expression – phosphatidylinositol-3 kinase (Hirasawa *et al.*, 2000), phosphorylation of insulin receptor substrates by IL-4Rα chain (Zamorano *et al.*, 1996), activation of Ras/MAP kinases including ERK (David *et al.*, 2001), and activation of the JAK/STAT pathway (Keegan *et al.*,1994). Genes induced by IL-4 include IL-4 and IL-1ra (Paludan, 1998). Furthermore, IL-4 has the ability to inhibit activation of genes associated with inflammation including IL-1β, IL-12, TNFα and iNOS, which generate NO (Paludan, 1998). Ultimately, IL-4 binding to the IL-4R results in the suppression of macrophage activity and the differentiation of Th cells towards Th2 cell type further promoting certain types of anti-inflammatory activity (Paludan, 1998).

Previous studies at Trinity College Dublin have shown that aged rats, like LPS-treated rats exhibit a deficit in LTP and this is associated with an increased hippocampal concentration of IL-1β (Murray and Lynch,1998; Martin *et al.*, 2002), coupled with a decrease in the concentration of IL-4 (Nolan *et al.*, 2004). The same study also confirmed the importance of IL-4 in the maintenance of LTP by showing that IL-4 partially restores the age-related deficit and abrogates the LPS-induced and IL-β-induced deficit in LTP (Nolan *et al.*, 2004). In the present study it was considered that IL-1F5 might exert its anti-inflammatory effect by increasing IL-4 concentration in the hippocampus and the evidence presented is consistent with this.

In an effort to address this point and determine the effect of IL-1F5 on different cell types, a mixed glial culture was prepared. Previous studies have identified that functional IL-4 receptors are expressed in rat hippocampus (Nelm *et al.*, 1999), however, to date there has been little or no evidence indicating that resident brain cells are a source of IL-4. The data demonstrate two important findings. Firstly, that IL-4 mRNA expression is upregulated in glial cells in response to treatment with IL-1F5. Secondly, the data indicate that glial cells are capable of secreting IL-4 following IL-1F5 stimulation. It is proposed that secreted IL-4 activates IL-4 receptors located on neurons, thereby mediating the anti-inflammatory actions of IL-1F5 in the brain. This

finding agrees with previous work at Trinity College Dublin, indicating that glial cells can release IL-4, whereas neuronal cell lack this ability (Maher *et al*, 2005).

In CNS inflammation it has been shown that there may be a balance between microglia and astrocytes in regulating local immune reactions, including pro-inflammatory and anti-inflammatory responses. The cultures purified in this study contain a combination of glial cells, including astrocytes. Astrocytes have been shown to be involved in the anti-inflammatory response in the CNS, where they are involved in immunoprotective activities. Consequently, previous studies have indicated that a possible cell source for IL-4 is the astrocyte. Cultured mouse astrocytes have been shown to express IL-4 mRNA (Brodie *et al*., 1995), and in a STAT3 $^{-/-}$ mouse model, reactive astrocytes showed limited migration and resulted in a markedly widespread infiltration of inflammatory cells, neural disruption and demyelination with severe motor deficit after contusive spinal cord injury, indicating that astrocytes immunoprotective signalling is STAT3-dependent (Okada *et al*., 2006).

In accordance with previous findings at Trinity College Dublin IL-1β concentrations were significantly increased in LPS treated glial cells. A mixed glial culture was generated from neonatal rats and treated with LPS or IL-1F5 or the two in combination. Taken together with the *in vivo* study, the data show that IL-1F5 had the ability to abrogate the LPS-induced increase in IL-1β *in vitro*. This finding supports the hypothesis that IL-1F5 acts on glial cells to initiate IL-4 secretion, and raises the possibility that the anti-inflammatory effects of IL-1F5 may be mediated by IL-4.

4.4   IL-1F5 signalling in the absence of IL-4

In an effort to further test this hypothesis an IL-4 knockout animal was used. Mice homozygous for a mutation that inactivates the IL-4 gene were mated with C57BL/6 mice, and after 6 generations of mating withC57BL/6, more than 99% of the genetic background was C57BL/6. IL-4 deficient mice show normal T and B cell development, but strongly reduced serum levels of

IgG1 and IgE, resulting in a loss of IgG1 dominance in a T-cell-dependent immune response, and upon infection, a lack of detectable IgE (Kuhn *et al*, 1991). It had been documented that constitutive expression of IL-4, under the control of an MHC I promoter, leads to autoimmune type disorders, characterized by increased B cell-surface MHC II expression, elevated responsiveness of B cells to polyclonal *ex vivo* stimulation, and increased IgG1 and IgE serum levels in mice (Erb *et al.*, 1997). These mice showed increased production of autoantibodies, developed anaemia and manifested glomerulronephritis with immune disposition, eventually leading to end stage renal failure (Tepper *et al.*, 1990). Similar studies report that B cells from IL-4 deficient mice hyperexpress MHC II molecules (Kuhn *et al.*, 1991) and that these mice display increased susceptibility to MOG-induced experimental autoimmune encephalitis (EAE) (Willenborg *et al.*, 2001). These results indicate that some of the properties of IL-4 are critical for the physiology of the immune system.

The ability of IL-1F5 to downregulate MHCII mRNA expression was examined in the wild-type C57BL/6 and IL-4 knockout mice which were treated with LPS or IL-1F5 or the two in combination. The data indicate that MHC II mRNA expression was significantly greater in hippocampal tissue prepared from IL-4$^{-/-}$ mice, compared with wild-type C57BL/6 mice, in all treatment groups. However, treatment with either LPS or IL-1F5 failed to elicit any change in MHC II mRNA expression in hippocampal tissue prepared from wild-type C57BL/6 or IL-4$^{-/-}$ mice. This result was surprising and in conflict with other data presented here - as an immunohistochemical study observed an increase in MHC II expression in hippocampal sections prepared from LPS-treated rats. These results are inconsistent with previous findings that LPS initiates an increase in MHC II molecules in both in human and murine cells (Piani *et al.*, 2000). This result may indicate that changes which occurred in this experiment did not happen at a transcriptional level. Another factor which must be considered is that these experiments were undertaken in a mouse model, not a rat and the conditions were slightly different. It is accepted that in smaller rodents a larger dose of LPS is necessary to induce inflammatory changes. In experiments involving rats an LPS dose of 100 μg/kg was administered intraperitoneally and tissue was collected after three

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hours, whereas in experiments with mice, an LPS dose of 200 µg/kg dose was administered intraperitoneally and tissue was collected after three hours. It is possible that changes in MHC II induced by LPS may have needed longer to occur in mice, or perhaps the dose used was too low.

Subsequent to microglial activation, IL-1β concentration was determined and the data paralleled those observed in *in vivo* studies in the rat hippocampus; LPS increased IL-1β concentration and IL-1β concentration was attenuated following IL-1F5 treatment in hippocampal tissue prepared from wild-type C57BL/6 mice. IL-1β concentrations were higher in hippocampal tissue prepared from IL-4$^{-/-}$ mice than in hippocampal tissue prepared from wild-type C57BL/6 mice and this result is to be expected, considering that MHC II expression was increased in these animals. LPS failed to elicit an increase in IL-1β concentration in the hippocampus of IL-4$^{-/-}$ mice, therefore it is difficult to establish wether or not IL-1F5 had any effect in IL-4$^{-/-}$ mice. There are a number of reasons as to why LPS failed to elicit a response. As mentioned earlier, IL-4$^{-/-}$ mice are immunodeficient and have been shown to exhibit hyper reactivity to a number of inflammatory mediators, such a IFNγ (Kuhn *et al.*, 1991). IL-1β values were similar in hippocampal tissue prepared from LPS-treated wild-type C57BL/6 and control-treated IL-4$^{-/-}$ mice. Since immunity is concerned with maintaining a balance between pro-inflammatory and anti-inflammatory responses it is assumed that in the absence of IL-4 this balance is skewed in favour of a pro-inflammatory response, resulting in an increased level of IL-1β under normal conditions. Indeed, it can be further postulated that a 'threshold' may exist after which immune-compromised animals cannot elicit further response when challenged with an inflammatory stimulus. This phenomena has been explored in several studies, exhaustion of cytokine production has been studied in dendritic cells and can be induced by LPS and TNFα (Langenkamp *et al.*, 2000). A similar 'ceiling' effect has been demonstrated in the CNS, whereby a threshold level of upregulation of IL-1ra mRNA, in response to LPS treatment, was recorded (Gayle *et al.*, 1999).

To further investigate this effect, primary cultures of mixed glial cells were prepared from wild-type C57BL/6 and IL-4$^{-/-}$ neonatal mice. Similar to *in*

vitro studies in mixed glial cultures prepared from rats, the LPS-induced increase in IL-1β was abrogated following treatment with IL-1F5 in glial cells prepared from wild-type C57BL/6 mice. In comparison, IL-1F5 lacked the ability to abrogate the LPS-induced increase in IL-1β in cells prepared from IL-4$^{-/-}$ mice. This result indicates that IL-1F5 requires IL-4 activation to initiate its anti-inflammatory effects. Contrary to the *in vivo* data IL-1β concentrations were higher in cells prepared from wild-type C57BL/6 mice than cells prepared from IL-4$^{-/-}$ mice. Obviously a direct comparison between *in vivo* and *in vitro* work must be approached with reservation, not least because of the variation in conditions; animals received a 200 μg/kg dose of LPS and tissue was assessed 3h later, while mixed glial cultures were treated with a 100 ng/ml dose of LPS in media and incubated for 24 hours. The environment in brain *in situ* would comprise of neurons, glia and astrocytes as well as any infiltrating peripheral immune cells and circulating cells, while the *in vitro* environment is much more regulated, consisting of purely glial cells, with an absence of neuronal cells. While glial cells are thought to be the main producers of cytokines centrally, neuronal cells have been shown to initiate IL-1β release in response to LPS (Barry *et al.*, 2005). This could indicate that the increased basal level of IL-1β produced in the hippocampal tissue of IL-4$^{-/-}$ mice is induced by an increase in neuronal production of IL-1β. Furthermore, since IL-4 aids maintenance of BBB integrity (Poliani *et al.*, 2001), it is possible that IL-4 deficient animals have a compromised BBB, allowing the infiltration of immune cells and cytokines from the periphery.

4.5   Mechanism of action of IL-1F5 – A role for SIGIRR

It appears from these data that IL-1F5 has anti-inflammatory actions in the brain, which appear to be mediated, to some extent via activation of IL-4. However, the method of initiation of these signalling pathways remains unknown. As previously mentioned, IL-1F5 shares a high degree of homology with IL-1ra, However, due to its conformational shape, it is unable to bind IL-1R1. Further studies have indicated that IL-1F5 does not bind IL-1R1 but

may be a ligand for an orphan IL-1R (Dunn *et al.*, 2003). Recently, a number of novel IL-1R family members have been discovered, leading to the formation of the IL-1R/TLR superfamily. These receptors are characterised by having three Ig domains and sharing Toll-like signalling mechanisms, except in the case of one of the novel family members; the orphan receptor SIGIRR. SIGIRR contains only one extracellular Ig domain and previous data has indicated that SIGIRR may have anti-inflammatory functions. Within the IL-1R/TLR family, SIGIRR shows a higher similarity to the other members of the IL-1R subgroup. Sequencing of SIGIRR revealed that peptide sequences of mouse and human SIGIRR are 82% identical and show a 23% overall identity homology to mouse or human IL-1R1. Similarly, the important cytoplasmic portion of SIGIRR contains the conserved motifs of the IL-1R1 superfamily. The SIGIRR sequence has a short extracellular region of only 118aa that corresponds to a single Ig domain of the IL-1R. Similar to IL-1Rrp2, two positions in the conserved domain of SIGIRR have been replaced, compared with IL-1R1 and the other members of the superfamily. The Ser and Tyr residues are replaced with a Cys and Leu residue. The importance of these changes is that Ser and Tyr have been shown to be essential for the signalling of IL-1R1. Investigation has shown that IL-1Rrp2 is unable to bind either IL-1β or IL-1α, despite having an overall homology of 42% with IL-1R1 (Lovenberg *et al.*, 1996). Recent evidence suggests that SIGIRR may have an anti-inflammatory role. Analysis of differential expression of SIGIRR has indicated that it is highly expressed in the kidney; moderately expressed in the colon, small intestine, lung, spleen and liver; and weakly expressed in the brain (Wald *et al.*, 2003). Overexpression of SIGIRR in Jurkat and HepG2 cells led to a substantial reduction in IL-1 and IL-18 mediated activation of NFκB (Wald *et al.*, 2003). These results indicate that SIGIRR may function as a negative regulator of IL-1 and IL-18 signalling. Furthermore, it has been reported that SIGIRR deficient mice exhibit increased inflammatory responses to LPS injection and these findings led the authors to suggest that its activation may be anti-inflammatory (Garlanda *et al.*, 2004). Similarly, it was revealed that inflammation in response to LPS was enhanced in SIGIRR-deficient mice

(Wald *et al.*, 2003). The evidence suggested that SIGIRR binds to the TLR-IL-1R signalling components in a ligand-dependent manner, and consistently, cells from SIGIRR-deficient mice showed enhanced activation in response to IL-1 (Wald *et al.*, 2003). However, the mechanism by which SIGIRR negatively regulates IL-1 signalling remains unclear. As SIGIRR interacts with IL-1R1, IRAK and TRAF 6 after IL-1 treatment, SIGIRR may negatively regulate the IL-1 pathway through its interaction with the IL-1R complex. Studies have indicated that SIGIRR acts to negatively regulate IL-1R signalling via its interaction with the adaptor molecules, MyD88 and MAL. It has been suggested that SIGIRR acts by sequestering recruitment of these two downstream adaptor molecules by interacting with the extracellular Ig domain or by interacting with the intracellular TIR domain, thereby blocking recruitment of these molecules (Qin *et al.*, 1993).

The evidence presented here suggests that IL-1F5 acts on glial cells causing IL-4 release and that this effect relies on an interaction with SIGIRR, since the IL-1F5-induced increase in IL-4 is blocked in the presence of the SIGIRR antibody. These findings highlight two important issues. First, they identify a specific anti-inflammatory action of IL-1F5; second, they identify the possibility that IL-1F5 is a ligand for SIGIRR.

A transgenic SIGIRR deficient mouse population was developed (Garlanda *et al* 2004). Both wild-type C57BL/6 mice and SIGIRR$^{-/-}$ mice were treated with LPS or IL-1F5 or the two in combination. Results of a QT-PCR study indicate that MHC II mRNA expression was unchanged in any treatment group and was similar in the hippocampus of wild-type C57BL/6 and SIGIRR$^{-/-}$ mice. There was a trend toward LPS and IL-1F5 having an additive effect on MHC II mRNA expression in both animal models and LPS failed to elicit any significant increase in MHC II mRNA expression therefore, it is difficult to establish a role for IL-1F5 in the absence of an LPS-induced effect.

While LPS failed to affect the IL-1β mRNA expression levels in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice it is worth noting that basal IL-1β mRNA expression was significantly higher in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice compared with wild-type C57BL/6 mice. Consistent with previous findings, IL-1F5 abrogated the LPS-induced

increase in IL-1β in hippocampal tissue prepared from wild-type C57BL/6 mice, both at an mRNA and protein level. Similarly, LPS failed to elicit any significant increase in IL-1β concentration in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice. These results are in conflict with previous studies which report that, using *in vivo* challenges, SIGIRR-deficient mice show greater induction of acute phase C reactive protein (CRP) and chemokine genes in response to an intraperitoneal injection of LPS. SIGIRR deficient mice also showed hyperresponsiveness to IL-1, indicating that SIGIRR can function *in vivo* as a negative regulator of IL-1 and LPS signalling (Wald *et al.*, 2003). However, it should be noted that these studies analysed chemokine responses in the periphery and did not analyse CNS effects in SIGIRR deficient mice.

Since members of the IL-1R/TLR superfamily are involved in not only innate, but also pro-inflammatory/anti-inflammatory adaptive immune response after induction of inflammation, the ability of SIGIRR to modulate anti-inflammatory type immune responses was examined. Since the IL-1F5-induced increase in IL-4 is blocked in the presence of the SIGIRR antibody it was proposed that IL-1F5 might interact with SIGIRR to activate IL-4, therefore IL-4 mRNA and protein production were examined in the wild-type C57BL/6 and SIGIRR$^{-/-}$ mouse models. Consistent with findings in the rat model, IL-1F5 had the ability to significantly increase IL-4 mRNA expression and IL-4 protein concentration in hippocampal tissue prepared from wild-type C57BL/6 mice. In comparison, IL-1F5 failed to elicit any response in the absence of SIGIRR. Basal level of IL-4 mRNA expression was significantly lower in hippocampal tissue prepared from SIGIRR$^{-/-}$, compared with wild-type C57BL/6 mice. There was no difference in IL-4 mRNA expression response to any treatment in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice. These results suggest that SIGIRR plays a role in modulating inflammation, whereby in the absence of SIGIRR, IL-4 mRNA is downregulated. In parallel with this finding, IL-1F5 failed to elicit any increase in IL-4 protein concentration in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice.

To further investigate the actions of SIGIRR in the brain, mixed glial

cell cultures were prepared from wild-type C57BL/6 and SIGIRR^{-/-} neonatal mice and treated with LPS or IL-1F5 or the two in combination. Similar to the *in vivo* study, IL-1F5 abrogated the LPS induced increase in IL-1β mRNA expression and IL-1β concentration in cells prepared from wild-type C57BL/6 mice. In comparison, IL-1F5 lacked the ability to abrogate the LPS-induced increase in IL-1β mRNA expression in cells prepared from SIGIRR^{-/-} mice. This result suggests that IL-1F5 requires SIGIRR to initiate its anti-inflammatory effects. Consistent with this result, IL-1F5 also failed to abrogate the LPS-induced increase in IL-1β concentration in cells prepared from SIGIRR^{-/-} mice. Furthermore, basal levels of IL-1β were significantly higher in cells prepared from SIGIRR [-/-] mice, compared with cells prepared from wild-type C57BL/6 mice.

In addition to its inhibitory effect on LPS signalling, the effect of SIGIRR on anti-inflammatory responses in glial cells was also examined. Consistent with *in vivo* findings, IL-1F5 had the ability to significantly increase IL-4 mRNA expression and IL-4 protein concentration in cells prepared from wild-type C57BL/6 mice. In comparison, IL-1F5 failed to elicit any response in the absence of SIGIRR. IL-4 mRNA was undetectable in cells prepared from SIGIRR^{-/-} mice, regardless of treatment. In parallel with this finding, IL-1F5 failed to elicit any increase in IL-4 in cells prepared from SIGIRR^{-/-} mice. These data are in agreement with previous findings indicating that SIGIRR is required for host resistance against bacterial infection (Huang *et al.*, 2006), and that SIGIRR functions to negatively regulate proinflammatory cytokine production. However, some conflicting issues exist, for example it has been shown that in the absence of SIGIRR there is in an increase in pro-inflammatory cytokine production, but no difference in IL-4 cytokine production has been recorded (Huang *et al.*, 2006). The results presented here suggest that SIGIRR may participate in regulation and balance between proinflammatory and anti-inflammatory cytokines in the CNS.

SIGIRR mRNA expression was analysed in hippocampal tissue prepared from wild-type C57BL/6 mice and was shown to be upregulated in response to IL-1F5-treatment. In an effort to identify the cell type on which SIGIRR is expressed, mixed glial cells were prepared from neonatal rats and

SIGIRR expression was identified and shown to be increased in response to IL-1F5 treatment. The culture produced in the present study contains a mixture of glial cells, including astrocytes and microglia, and without a purified culture, it is not possible to unequivocally state which cells are expressing SIGIRR. However, previous studies have failed to detect SIGIRR mRNA in a microglial cell line (Dimcheff *et al.*, 2006). This could indicate that another cell type in the mixed glial culture could express SIGIRR.

4.6   IL-1F5 downstream signalling

The present study has established that IL-1F5 produces its anti-inflammatory effects through mediation of the anti-inflammatory cytokine IL-4, and this process is reliant on the presence of SIGIRR. In an effort to elucidate the exact mechanism by which IL-1F5 activates IL-4, some possible downstream signalling mechanisms were investigated; namely the interaction of IL-1F5 with the Janus kinase/signal transducers and activators of transcription factor (JAK/STAT). Three members JAK1, JAK2 and JAK3 have been demonstrated to be activated in response to IL-4R engagement and to be associated with components of the IL-4R complex. STAT6 is the primary STAT activated in response to IL-4 stimulation. It is critical in the activation or enhanced expression of many IL-4-responsive genes (Nelms *et al.*, 1999). IL-4R engagement results in the activation of JAK1 and JAK3 and phosphorylation of specific tyrosine residues in the receptor cytoplasmic region. STAT6 then binds to the phophorylated receptor through a highly conserved SH2 domain, enabling the activated kinases to phosphorylate STAT6 at a C-terminal tyrosine residue. Once phosphorylated, the STAT6 molecule disengages from the receptor and forms a homodimer through interaction of its SH2 domain with the C-terminal phosphotyrosine residue of a second STAT6 molecule. The dimerized STAT6 complex translocates to the nucleus where it binds to specific DNA motifs in the promotor of responsive genes (Nelms *et al.*, 199). The modulatory effect of IL-1F5 was confirmed in this study, both JAK1 and STAT6 protein expression was significantly increased in hippocampal tissue prepared from IL-1F5-treated rats.

Extracellular signal-regulated protein kinases (ERKs) are a family of

protein serine/threonine kinases that occupy a pivotal position in intracellular signalling pathways mediating mitogen/growth factor effects. The ERKs, and their signalling pathways, exhibit several novel characteristics, and the most studies are ERK1 and ERK2. Activation of ERK1 and ERK2 involved their phosphorylation on both a tyrosine and a threonine residue (Robbins et al., 1993). This novel form of activation appears to be catalysed specifically by an equally novel type of protein kinase; MEK. The wide range of extracellular signals capable of activating the ERKs belies the specificity of ERK phosphorylation by MEK. Downstream, the ERKs phosphorylate a relatively distinct set of substrates. In mammalian cells, nuclear transcription factors such as CREB are thought to be substrates for ERKs (Blenis, 1993). Activation of the JAK/STAT pathway is associated with activation of the Ras/MAP pathway in response to a number of cytokines, including IL-4 (Nelm et al., 1991). The Ras/MAPK pathway is initiated by the serine/threonine kinase Raf following its activation by Ras-GTP. Raf initiates a cascade of kinase activation events that ultimately result in the phosphorylation and activation of the mitogen activated protein kinases ERK1 and ERK2. Active ERK1/2 translocates to the nucleus and activates the expression of genes by phosphorylating transactivating factors, this can lead to cell growth and differentiation or to activation of genes such as c-fos,. IL-4 can dramatically activate this pathway. In this study both ERK1 and ERK2 were significantly increased in response to IL-1F5 treatment in tissue prepared from the hippocampus of rats.

ERKs are ubiquitously expressed in vertebrates, and the regulation of ERK phosphorylation and activity has been reported in numerous cell types from highly diverse origins. Similarly, in mammals ERKS are widely distributed throughout the body, however the highest levels of ERK mRNA are found in brain and spinal cord (Boulton et al., 1991), and the expression of ERK protein appears to parallel that of the mRNA. Studies have demonstrated directly that the activity of both ERK1 and ERK2 is increased in the hippocampus after seizure-producing electroconvulsive shock (Baraban et al., 1993). Also, in isolated hippocampal neurons, ERK phosphorylation is increased by epidermal growth factor treatment or glutamate receptor activation (Ortz et al., 1995). Assessment of ERK expression in this study

revealed increased ERK1/2 expression in IL-1F5-treated neuronal cells prepared from rats. The data here indicate a role for IL-1F5, in downstream signalling mechanisms associated with IL-4 and MAPK signalling.

4.7   IL-1F5 signalling – A Role for PPARγ

Until recently, the family of transcription factors termed peroxisome proliferators-activated receptors (PPARs) was believed to regulate genes predominantly associated with lipid and glucose metabolism. However, it is now clear that PPARs play a role in inflammation and immunity. Peroxisomes are subcellular organelles found in most plant and animal cells that perform diverse metabolic functions including H_2O_2-based respiration, β-oxidation of fatty acids, and cholesterol metabolism (Clark, 2002). In rodents, a large class of structurally diverse industrial and pharmaceutical chemicals, including herbicides, industrial solvents and hypolipidemic drugs leads to significant increases in the size and number of peroxisomes in liver, liver hypertrophy and transcription of genes encoding peroxisomal enzymes. The structurally diverse compounds that induce these effects are termed peroxisome proliferators. It is now known that PPARs are nuclear receptors which regulate numerous genes through ligand-dependent transcriptional activation and of the three members; PPARα, PPARδ and PPARγ, PPARγ has been shown to play a role in the differentiation and activation of monocytes and in the regulation of inflammatory activities (Clark, 2002). Studies have indicated an anti-inflammatory role for PPARγ; PPARγ ligands have been shown to induce apoptosis of activated human macrophages, decrease NOS activity and PPARγ ligands reverse ageing-related inflammatory cytokine production (Chinetti *et al.*, 1998, Colville-Nash *et al.*, 1998, Poynter *et al.*, 1998). In recent studies, which investigated the role of PPARγ in adaptive immunity, Huang *et al* (1999) investigated the effects of cytokines on macrophage PPARγ expression and function. They found that IL-4 strongly induced PPARγ expression and function in resident peritoneal macrophages and human peripheral blood monocytes. Furthermore it was found that IL-4 not only upregulated PPARγ expression in macrophages but also enhanced the

activation of PPARγ via the production of endogenous PPARγ ligands (Huang *et al.*, 1999). The relevance of PPARγ has been studied in several human autoimmune diseases and animal models of immune disease. Increased expression of PPARγ is associated with Alzheimer's disease, which is characterised by the extracellular deposition of β-amyloid fibrils within the brain, and activation of microglial cells associated with the amyloid plaque. The activated microglia subsequently secrete a diverse range of inflammatory products. Furthermore, treatment with PPARγ agonists, such as NSAIDs and thiazolidinediones, inhibited the β-amyloid-stimulated expression of genes for IL-6 and TNFα, and the expression of COX-2 (Combs *et al.*, 2000, Heneka *et al.*, 2000). The identification of endogenous ligands for PPARγ in different cell types is an important area of investigation.

In the present study the possibility that IL-1F5 may act as a PPARγ agonist was considered. Treatment of rat glial cells with IL-1F5 significantly increased PPARγ expression in glial cells prepared from rats.

The implications of removing PPARγ from the system have been investigated. CD36 functions in macrophages as a scavenger receptor and is involved in cholesterol breakdown. Recent studies have shown that PPARγ is required for the IL-4 induced increase in macrophage CD36 expression (Clark, 2002).

The data indicate a role for IL-1F5 as an endogenous PPARγ agonist. GW9662 is an irreversible PPAR antagonist first identified in a competition-binding assay against the human ligand-binding domain (Leesnitzer *et al.*, 2002). The antagonistic effect of GW9662 was measured by its ability to inhibit CD36 expression in peritoneal macrophages stimulated with IL-4 (Huang *et al.*, 1999). GW9662, at the concentration of ~1M, inhibited CD36 induction by IL-4 and antagonized PPARγ activation of the transfected (AOx)3-TK-Luc promoter gene by BRL49653 in a dose-dependent manner (Fu *et al.*, 2001). Recently, PPARγ has been implicated in the development and progression of atherosclerosis. PPARγ ligands, GW7845, ciglitazone and troglitazone, tested on human aortic smooth muscle cells (HASMC) inhibit osteoprotegerin (OPG) expression. GW9662 completely abolishes the effect of GW7845 and ciglitazone on OPG expression. In

addition, PPARγ activation inhibits OPG promoter activity (Fu *et al.*, 2001). GW9662 is a potent and irreversible antagonist of PPARγ, which does not lose its activity in cell cultures and is a valuable tool for determining specific PPARγ receptor-mediated functions in different biological systems. In the present study, GW9662 was used to assess the role of PPARγ in IL-1F5 inhibitory activity. Glial cells were pre-incubated with LPS and IL-1F5 in the presence or absence of GW9662. An LPS-induced increase in IL-1β was recorded in glial cells prepared from rats. IL-1F5 had the ability to abrogate this effect. However, the inhibitory effect of IL-1F5 on LPS-induced changes was absent in the presence of GW9662. The PPARγ antagonist blocked the inhibitory effect of IL-1F5.

This study established for the first time that IL-1F5 possesses anti-inflammatory actions in the brain, and that the anti-inflammatory effect is associated with an ability to antagonise IL-1β and stimulate production of IL-4. The study highlighted some significant roles for IL-1F5 that warrant further investigation. The data indicate that IL-1F5 has the ability to block the IL-1β-induced inhibition of LTP and the subsequent increase in JNK activation in the hippocampus of rats. This is the first study to report on any inhibitory effect of IL-1F5 on IL-1β-induced signalling. IL-1F5 also blocked the LPS-induced inhibition of LTP in the rat hippocampus. Furthermore, IL-1F5 abrogated the corresponding LPS-induced increase in microglial activation, IL-1β concentration and JNK activation. It was hypothesised that IL-1F5 mediates its inhibitory effects by induction of the endogenous anti-inflammatory cytokine IL-4 and IL-1F5 had no inhibitory effect in the absence of IL-4. The interaction between IL-1F5 and SIGIRR was examined and IL-1F5 had no inhibitory effect on LPS-induced changes in the absence of SIGIRR, highlighting a role for SIGIRR in IL-1F5 activity.

Future work:

This study set out to investigate a possible role for IL-1F5 as an antagonist. Although this study contributes to the understanding of IL-1F5 and SIGIRR in cellular events during inflammation, it also highlights some issues that should be addressed in further studies.

The data show that IL-1F5 has anti-inflammatory actions and also shows the ability to induce the anti-inflammatory cytokine, IL-4. This poses the question as to whether the anti-inflammatory actions of IL-1F5 are simply due to its ability to induce IL-4. Could it be that IL-1F5 alone exerts no anti-inflammatory activity? The study did aim to answer this question, using IL-4$^{-/-}$ mice, however the results presented here are difficult to interpret, due to a lack of LPS effect in some experiments. Therefore, further studies using IL-4$^{-/-}$ mice, must be undertaken to definitively answer this question. Another method for elucidating the actions of IL-1F5 could be the use of IL-1F5$^{-/-}$ animals. This model could also be used to investigate other inflammatory models of interest, for example Aβ or Age.

The study showed SIGIRR expression on glial cells generated from rats. The culture used in this study contained a variety of glial cell types, therefore it is not known on what particular cell SIGIRR is expressed. To address this, a pure microglia or astrocyte culture is necessary and staining could be repeated in these cultures. Another way of addressing the specific cell type on with SIGIRR is expressed is by using flow cytometry to identify specific cell populations within a culture and looking for co-localisation of SIGIRR.

The data presented here indicates that IL-1F5 has the ability to increase SIGIRR mRNA expression in the hippocampus. This phenomenon could be further explored by overexpressing IL-1F5, and investigating if SIGIRR is upregulated.

The data indicate that IL-1F5 activity is reliant on an interaction with SIGIRR. This poses the question, is IL-1F5 a ligand for SIGIRR? There are a number of ways in which this could be investigated. This could be addressed by staining both IL-1F5 and SIGIRR, and observing co-localisation using confocal microscopy. This could also be accomplished using flow cytometry techniques.

In addition, SIGIRR downstream signalling could be investigated. Using the SIGIRR$^{-/-}$ mice downstream effectors such as JAK and STAT could be investigated.

Recent studies, undertaken by John Simms, have added information to the novel IL-1 family member story. A novel IL-1 receptor accessory protein (IL-1RAcPb) has been discovered, which appears to act as an accessory protein in inhibitory signalling. Significantly, IL-1RAcPb is found only in brain and appears to be restricted to neuronal cells. It has been hypothesised that IL-1RAcPb could bind to a novel IL-1 receptor to form an alternative binding complex. Furthermore, signalling associated with this accessory protein appears to be anti-inflammatory; there is no induction of IL-1 genes with IL-1RAcPb, there is downregulation of IL-1-induced NFκB and there is no IL-6 production. IL-1F5 activity could be investigated in IL-1RAcPb$^{-/-}$ mice to establish if there is a relationship between IL-1F5 and IL-1RAcPb. In view of this study, the discovery of IL-1RAcP represents an exciting new mechanism by which IL-1F5 could be studied and could represent a specific method through which IL-1F5 mediates its anti-inflammatory activity.
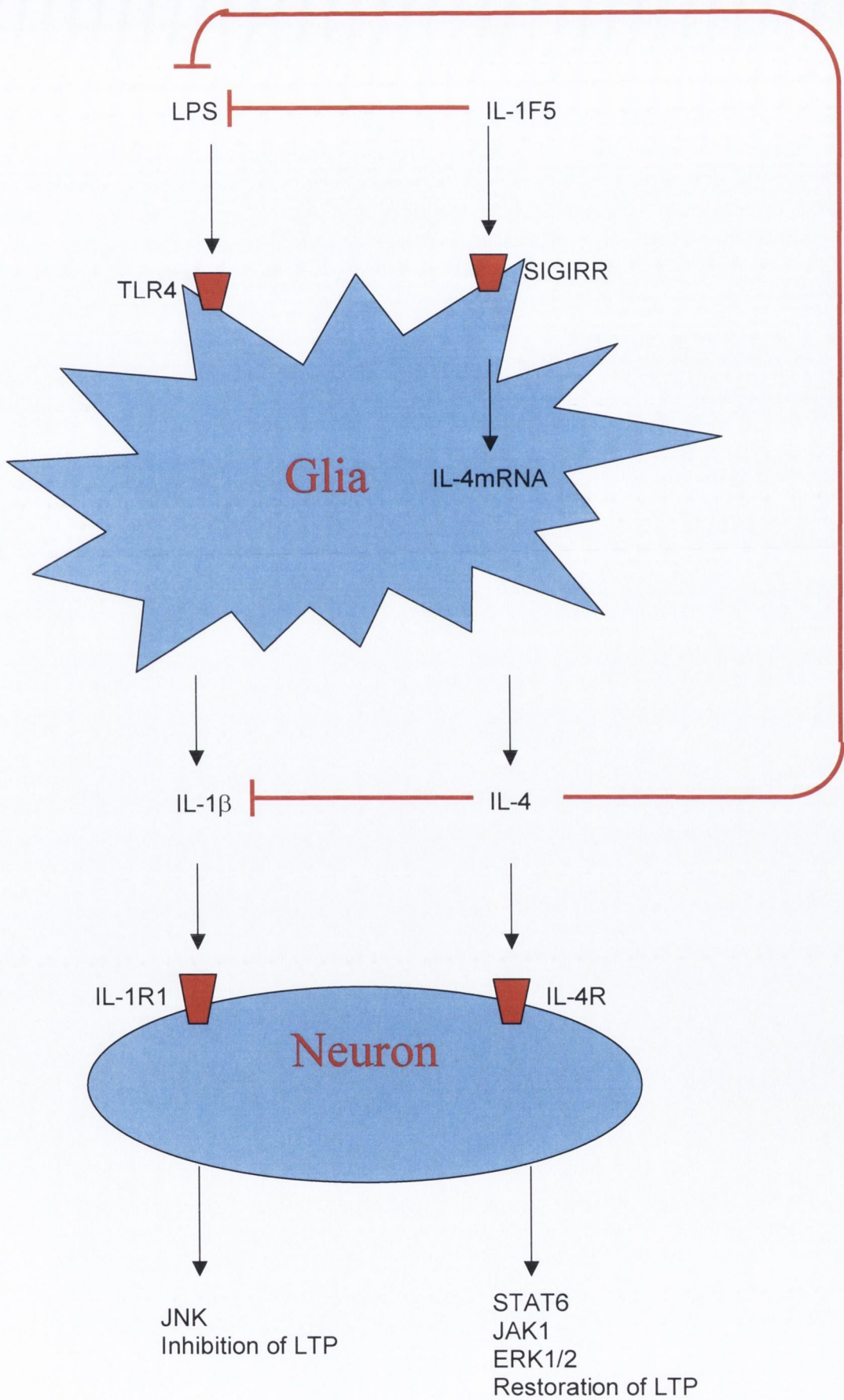
Figure 4.1 A Schematic showing IL-1F5 activation

IL-1F5 has the ability to inhibit IL-1β activity. IL-1F5 interacts microglial cell activation and with IL-1β functioning to abrogate IL-1β induced increase in JNK and p38. IL-1F5 induces an increase in IL-4 concentration from glial cells, which interacts with it IL-4R on neuronal cells and acts to induce JAK/STAT and ERK pathways. In the absence of IL-4 IL-1F5 loses its inhibitory abilities. IL-1F5 interacts with the orphan receptor SIGIRR, expressed on glial cells. In the absence of SIGIRR IL-1F5 fails to elicit an increase in IL-4 and loses its inhibitory abilities.

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VII. APPENDIX I

Raw data expressed as mean + standard error of mean.

% change in EPSP slope 0-2 mins pre-hfs

Control	IL-1β	IL-1F5	IL-1β+IL-1F5
119.3 ± 2.46	121..32 ± 1.49	127.6 ± 1.47	126.3 ± 0.85

% change in EPSP slope in last 5 of recording

Control	IL-1β	IL-1F5	IL-1β+IL-1F5
118.5 ± 0.65	104.7 ± 0.52	115.5 ± 0.31	121.4 ± 0.78

JNK phosphorylation in hippocampal tissue (Arbitrary units)

Control	IL-1β	IL-1F5	IL-1β+IL-1F5
1.39 ± 0.1	2.25 ± 0.17	1.47 ± 0.11	1.73 ± 0.18

% change in EPSP slope 0-2 mins pre-hfs

Control	LPS	IL-1F5	LPS+IL-1F5
121.32 ± 1.49	119.3 ± 2.46	127.0 ± 1.47	126.3 ±0.85

% change in EPSP slope in last 5 mins of recording

Control	LPS	IL-1F5	LPS+IL-1F5
133.0 ± 1.18	97.35 ± 1.26	125.3 ± 0.62	140.9 ± 0.56

IL-1β concentration in Hippocampal tissue (pg/mg)

Control	LPS	IL-1F5	LPS+IL-1F5
36.53 ± 13.24	94.25 ± 26.18	22.26 ± 10.43	36.53 ± 13.24

JNK activation in hippocampal tissue (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
2.15 ± 0.24	3.36 ± 0.32	2.58 ± 0.47	1.68 ± 0.24

IL-4 concentration in hippocampal tissue (pg/mg)

Control	LPS	IL-1F5	LPS+IL-1F5
32.85 ± 3.71	23.25 ± 3.71	57.89 ± 8.58	32.59 ± 9.38

IL-4 concentration cultured glial cells (pg/ml)

Control	IL-1F5
22.87 ± 5.19	51.31 ± 9.39

IL-4 mRNA in cultured glial cells (Arbitrary units)

Control	IL-1F5
3.19 ± 0.69	5.14 ± 0.58

IL-4 concentration in cultured glial cells (pg/ml)

Control	IL-1β	IL-1F5	IL-1β+IL-1F5
32.85 ± 4.98	23.25 ±3.71	57.89 ± 9.38	28.45 ± 8.48

IL-1β concentration in cultured glial cells (pg/ml)

Control	LPS	IL-1F5	LPS+IL-1F5
81.54 ± 28.32	545.4 ± 39.17	56.94 ± 14.68	76.50 ± 29.04

MHC II mRNA expression in hippocampal tissue from wild-type C57BL/6 mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
0.07 ± 0.03	0.17 ± 0.28	0.16 ± 0.06	0.12 ± 0.03

MHC II mRNA expression in hippocampal tissue from IL-4 $^{-/-}$ mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
0.13 ± 0.03	0.54 ± 0.06	0.32 ± 0.04	0.74 ± 0.08

IL-1β concentration in hippocampal tissue from C57BL/6 mice (pg/mg)

Control	LPS	IL-1F5	IL-1β+IL-1F5
98.71 ± 19.16	236.3 ± 23.95	126.80 ± 26.49	159.5 ± 35.90

IL-1β concentration in hippocampal tissue from IL-4$^{-/-}$ mice (pg/mg)

Control	LPS	IL-1F5	IL-1β+IL-1F5
258.9 ± 34.76	332.90 ± 59.50	209.2 ± 26.92	265.6 ± 32.45

IL-1β concentration in glial cells prepared from C57BL/6 mice (pg/ml)

Control	LPS	IL-1F5	LPS+IL-1F5
1.82 ± 0.71	38.58 ± 10.20	2.33 ± 0.82	9.42 ± 2.47

IL-1β concentration in glial cells prepared from IL-4$^{-/-}$ mice (pg/ml)

Control	LPS	IL-1F5	LPS+IL-1F5
1.59 ± 0.69	17.13 ± 1.23	3.59 ± 0.78	15.17 ± 0.73

IL-4 concentration in glial cells treated with anti-SIGIRR (pg/ml)

Control	Anti-SIGIRR	IL-1F5	Anti-SIGIRR+IL-1F5
13.12 ± 1.83	15.98 ± 3.83	22.23 ± 2.87	10.92 ± 2.69

MHC II mRNA expression in hippocampal tissue from C57BL/6 mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
37.12 ± 9.99	65.53 ± 18.23	51.47 ± 17.10	95.43 ± 25.16

MHC II mRNA expression in hippocampal tissue from SIGIRR$^{-/-}$ mice

Control	LPS	IL-1F5	LPS+IL-1F5
43.78 ± 5.75	68.95 ± 26.43	11.47 ± 7.61	128.50 ± 26.28

IL-1β mRNA in hippocampal tissue from C57BL/6 mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
75.14 ± 33.58	322.14 ± 97.24	140.8 ± 14.37	140.71 ± 10.19

IL-1β mRNA in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
292.80 ± 88.21	242.0 ± 52.0	272.90 ± 40.97	277.80 ± 77.93

IL-1β concentration in hippocampal tissue prepared from C57BL/6 mice (pg/mg)

Control	LPS	IL-1F5	LPS+IL-1F5
72.58 ± 25.22	155.60 ± 25.64	81.96 ± 23.75	105.6 ± 12.42

IL-1β concentration in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice (pg/mg)

Control	LPS	IL-1F5	LPS+IL-1F5
78.58 ± 17.44	115.0 ± 23.33	99.85 ± 22.63	113.4 ± 21.21

IL-4 mRNA in hippocampal tissue prepared from C57BL/6 mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
6.16 ± 2.18	6.11 ± 3.99	20.22 ± 6.76	8.79 ± 3.29

IL-4 mRNA in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
1.76 ± 1.44	3.41 ± 2.45	3.34 ± 1.8	2.48 ± 1.9

IL-4 concentration in hippocampal tissue prepared from C57BL/6 mice (pg/mg)

Control	LPS	IL-1F5	LPS+IL-1F5
13.45 ± 4.24	10.82 ± 1.67	27.32 ± 5.20	15.03 ± 3.30

IL-4 concentration in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice (pg/mg)

Control	LPS	IL-1F5	LPS+IL-1F5
19.35 ± 3.49	14.17 ± 2.72	12.64 ± 2.86	12.92 ± 2.28

IL-1β mRNA in glial cells prepared from C57BL/6 mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
2.34 ± 1.29	6.71 ± 0.88	272.90 ± 40.97	3.58 ± 0.78

IL-1β mRNA in glial cells prepared from SIGIRR$^{-/-}$ mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
5.33 ± 0.46	7.51 ± 0.85	2.18 ± 0.99	6.71 ± 0.71

IL-1β concentration in glial cells prepared from C57BL/6 mice (pg/ml)

Control	LPS	IL-1F5	LPS+IL-1F5
4.2 ± 0.47	24.15 ± 7.04	4.25 ± 0.48	7.17 ± 1.65

IL-1β concentration in glial cells prepared from SIGIRR$^{-/-}$ cells (pg/ml)

Control	LPS	IL-1F5	LPS+IL-1F5
8.94 ± 1.92	26.24 ± 7.54	11.19 ± 1.44	31.42 ± 5.13

IL-4 mRNA in glial cells prepared from C57BL/6 mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
2.22 ± 0.39	2.33 ± 0.25	4.84 ± 0.41	4.59 ± 1.1

IL-4 concentration in glial cells prepared from C57BL/6 mice (pg/ml)

Control	LPS	IL-1F5	LPS+IL-1F5
12.98 ± 2.48	12.65 ± 1.66	23.46 ± 5.32	15.01 ± 4.89

IL-4 concentration in glial cells prepared from SIGIRR$^{-/-}$ mice (pg/ml)

Control	LPS	IL-1F5	LPS+IL-1F5
9.25 ± 3.5	10.13 ± 2.4	13.5 ± 1.76	14.40 ± 3.17

SIGIRR mRNA in hippocampal tissue prepared from C57BL/6 mice (Arbitrary units)

Control	LPS	IL-1F5	LPS+IL-1F5
23.84 ± 8.68	16.61 ± 5.57	76.32 ± 20.58	16.15 ± 6.21

JAK1 in phosphorylation in hippopcampal tissue (Arbitrary units)

Control	IL-1F5
106±6.698	222.8±61.51

STAT6 phosphorylation in hippocampal tissue (Arbitrary units)

Control	IL-1F5
95.40±4.010	163.1±25.38

ERK1 phosphorylation in hippocampal tissue (Arbitrary units)

Control	IL-1F5
2.715±0.6816	6.791±2.45

ERK2 phosphorylation in hippocampal tissue (Arbitrary units)

Control	IL-1F5
4.001±1.244	9.485±0.9862

PPARγ expression in mixed glial cells prepared from rat (Arbitrary units)

Control	IL-1F5
0.19 ± 0.01	0.37 ± 0.054

IL-1β concentration in mixed glial cells treated with GW9662 prepared from rat (pg/ml)

Control	LPS	IL-1F5	LPS+IL-1F5	LPS+IL1F5 +GW9662	GW9662
105.90 ±18.59	545.40 ± 39.17	86.19 ± 36.14	104.10 ± 11.15	237.20± 108.5	92.60 ± 35.44

VIII. Appendix II Addresses

Alexis

        Alexis Corporation (UK) Ltd.,
        P.O. Box 6757,
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        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.

Cruin   Cruin 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.

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,
                            Scotland.

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

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2800 Woods Hollow Road,
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R&D Systems

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Santa Cruz

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Santa Cruz,
CA 95060,
USA.

Sarsdedt

Sarsdedt 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.

                        xxx

IX. 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, pH 6.8

Glycerol 20% (v/v)

SDS 2% (w/v)

β-Mercaptoethanol 5% (v/v)

Bromophenol blue 0.05% (w/v)

Stacking gel (4%), pH 6.8

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$_2$O, 33% (v/v)

Tris-HCl, 0.05M, pH 6.8, 25% (v/v)

SDS (10% w/v stock), 1% (v/v)

APS (10% w/v stock), 0.5% (v/v)

TEMED, 0.05% (v/v)

Separating gel (10%), pH 8.8

Acrylamide/bis acrlamide (30% stock), 33% (v/v)

dH$_2$O, 40% (v/v)

Tris-HCl, 0.05M, pH 6.8, 25% (v/v)

SDS (10% w/v stock), 1% (v/v)

APS (10% w/v stock), 0.5% (v/v)

TEMED, 0.05% (v/v)

Transfere Buffer, pH 8.3

Tris-base, 25mM

Glyceine, 102mM

MeOH, 20%

SDS, 0.05% (w/v)

Tris-buffered slaine (TBS), pH 7.4

Tris-HCl, 20mM

NaCl, 150nM

X. Publications

Costelloe, C.E., Lyons, A., and Lynch, M.A. (2005). Minocycline attenuates the inhibition of LTP induced by Amyloid-β. American Society for Neuroscience Abstract. U27: 910.9

Costelloe, C.E., Lyons, A. and Lynch, M.A. (2005). Role of microglial activation in the inhibition of LTP induced by Amyloid-β. Neurodegeneration Ireland. Abstract 060P

Costelloe, C.E. and Lynch, M.A. (2005). Strategies which increase hippocampal IL-4 concentration attenuate lipopolysaccahride and IL-1 induced inhibition of LTP. British Neuroscience Association National meeting. Abstract 37.10

Costelloe, C.E., Lyons, A., Callaghan, C., Minogue, A. and Lynhch, M.A (2004). The role of microglial activation in the inhibition of LTP. Royal Academy of Medicine Ireland. Abstract