The role of interleukin-1 receptors in brain cell signalling

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List of abbreviations

A2B5	Anti-GT3 ganglioside
AD	Alzheimer's disease
ANOVA	Analysis of variance
BBB	Blood brain barrier
BDNF	Brain derived nerve factor
BSA	Bovine serum albumin
CCL	CCL chemokine sub group
CO_2	Carbon dioxide
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
CXCL	CXCL chemokines sub group
CREB	cAMP response element binding protein
DAPI	4',6-diamidino-2-phenylindole, dihydrochoride
DIV	Day in vitro
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Immunosorbent assay
ERK1/2	Extracellular signal-regulated kinase p42/44
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FUDR	5'-fluoro-2-deoxyuridine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
HRP	Horseradish peroxidise

ICAM-1	Intercellular adhesion molecule-1
ICC	Immunocytochemistry
Icv	Intracerebroventricular
IL-10	Interleukin-10
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-5	Interleukin-5
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-13	Interleukin-13
icIL-1RA	Intracellular isoforms of IL-1RA
IL-1RA	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-1RAcPb	Interleukin-1 receptor accessory protein b
IL-1RN	IL-1RA gene
IL-1R1	Interleukin-1 type 1 receptor
IL-1R2	Interleukin-1 type 2 receptor
IRAK	Interleukin-1 receptor associated kinase
JNK	c-jun N-terminal kinase
kDa	KiloDalton
KO	Knock out
LPS	Lipopolysaccharide
MAP2	Microtubule-associated protein 2
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase
MCAo	Middle cerebral artery occlusion
MCP	Monocyte chemotactic proteins
MHC	Major histocompatibility complex

MIP	Macrophage inflammatory proteins
MMP9	Matrix metalloproteinase-9
mRNA	Messenger ribonucleic acid
MRF-1	Microglia response factor-1
MS	Multiple sclerosis
MyD88	Myeloid differentiation factor 88
NeuN	Marker of neuronal nuclei
NGF	Nerve growth factor
NF-κB	Nuclear factor-kappa B
NMDA	N-methyl-D-aspartic acid
nSMase	Neutral sphingomyelinase
PBS	Phosphate buffered saline
PTX3	Pentraxin-3
PD	Parkinson's disease
PDL	Poly-D-lysine
PDS	Plasma derived serum
PMSF	Phenyl-methansulfonyl-fluoride
PP2	4 -amino-5- (4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]-pyrimidine
RT-PCR	Reverse-transcriptase polymerase chain reaction
p38	Mitogen-activated protein kinase p38
PGE_2	Prostaglandin E2
cPLA2	Calcium dependent phospholipase A_2
SDS	Sodium dodecyl sulfate
sIL-1RAcP	Soluble interleukin-1 receptor accessory protein
sIL-1R1	Soluble interleukin-1 type 1 receptor
sIL-1R2	Soluble interleukin-1 type 2 receptor
Src kinase	A protein tyrosine kinase
TAE	Tris-acetic buffer and ethylene-diamine-tetra-acetic acid
TAK1	Transforming growth factor β activated kinase
TIR	Toll/ IL-1 receptor

TNFα	Tumour necrosis factor α
TRAF6	TNF receptor associated factor 6
UTP	Uridine triphosphate
VCAM-1	Vascular cell adhesion molecule-1
WT	Wild type

The role of interleukin receptors in brain cell signalling

The University of Mancester

Loan Nguyen Ph.D. 2010

Abstract

IL-1α and IL-1β are two IL-1 agonists which signals at the same receptor complex composed of IL-1R1/IL-1RAcP. However, IL-1α and IL-1β exert differential actions. A recent CNS-specific IL-1 receptor accessory protein, called IL-1RAcPb, has been characterised but its actions are unknown. In T cell line, over expression of IL-1RAcPb negatively regulate IL-1 action (Smith et al, 2009), but over-expression of IL-1RAcPb in HEK cell line induces IL-1 signaling (Lu et al, 2008). The role of IL-1RAcPb has not been studied in primary cells. The aim of this project was to investigate the role of IL-1RAcPb in IL-1-induced actions in neurones and glia, and to determine IL-1α and IL-1β differential actions in these two cell types. The role of IL-1RAcPb in IL-1-induced protein expression and IL-1α and IL-1β differential effects were investigated by treating WT and IL-1RAcPb$^{-/-}$ neurones and glia with IL-1α or IL-1β in the presence or absence of IL-1RA for 24 h followed by assessment of IL-6 induction by ELISA. The mechanism of IL-1RAcPb actions were studied by examining the effects of IL-1α or IL-1β on p38, ERK1/2 and Src kinase activation in neurones and glia by Western blot analysis. SB203580 (p38 inhibitor), UO126 (ERK1/2 inhibitor), and PP2 (Src kinase inhibitor) were used to determine the contribution of p38, ERK1/2 and Src kinase activation to IL-1-induced IL-6 synthesis in neuronal cultures.

In WT neurones, IL-1α and IL-1β were equipotent at inducing IL-6 synthesis and p38 activation, whilst both ligands failed to induce ERK1/2 or Src kinase activation. In IL-1RAcPb$^{-/-}$ neurones, IL-1α and IL-1β induced similar levels of IL-6, but IL-1β was more potent than IL-1α at inducing p38 activation. IL-1α-induced p38 activation was reduced in IL-1RAcPb$^{-/-}$ neurones compared to WT neurones. In contrast to WT neurones, ERK1/2 was activated in IL-1RAcPb$^{-/-}$ neurones in response to IL-1α, whilst Src kinase was not activated by IL-1α or IL-1β. IL-1-induced IL-6 synthesis was abolished by IL-1RA, SB203580, UO126 and PP2. Interestingly PP2, a specific Src kinase inhibitor also partially inhibited basal ERK1/2 activity.

In WT glial cells, IL-1α was more potent than IL-1β at inducing IL-6 synthesis but both cytokines induced ERK1/2 activation with equal potency. In IL-1RAcPb$^{-/-}$ glia, IL-1α and IL-1β were equally potent at inducing IL-6 synthesis and ERK1/2 activation. However, IL-α-induced-IL-6 synthesis was reduced in IL-1RAcPb$^{-/-}$ glia compared to WT glia. In both WT and IL-1RAcPb$^{-/-}$ glia, IL-1α and IL-1β induced p38 activation but not Src kinase activation .

In conclusion, this study showed that in neurones, IL-1RAcPb may contribute to IL-1α-induced p38 activation but negatively regulates IL-1-induced ERK1/2 activation, therefore IL-1RAcPb may have specific effects on different signalling pathways. The effect of IL-1RAcPb could also be cell specific, as IL-1RAcPb contributed to IL-1α-induced p38 signalling in neurones but IL-6 production in glia. The role of IL-1RAcPb remains largely unknown and more investigations are required to elucidate its role in IL-1 signalling in the brain.

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Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Loan Nguyen Date

Copyright Statement

Publications

Summers L, Kangwantas K, Nguyen L, Kielty C, Pinteaux E. Adhesion to the extracellular matrix is required for interleukin-1 beta actions leading to reactive phenotype in rat astrocytes. *Mol Cell Neurosci*, 2010 Jul;44(3):272-81.

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Posters

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Dedication

I would like to dedicate this thesis to my dear mother, for all her love and devotion. I hope that I have made her proud.

I would also like to dedicate this chapter of my life, like everything else in my life, to my daughter Nhu-Dinh who is 6 years old. Through tears and laughter we did it together.

1. Introduction

1.1. Inflammation in the CNS

Inflammation is a protective mechanism initiated by the host in response to noxious stimuli. It is typically characterised by pain, swelling, redness and increased temperature. Inflammation is a tightly orchestrated event involving many cell types and mediators. Macrophages and dendritic cells are amongst the first type of cells to be activated (for reviews see Lucas et al., 2006 and Perry et al., 1995). Activated cells release pro-inflammatory mediators including cytokines and chemokines to propagate inflammation. During inflammation, local blood vessels become compromised, resulting in leakage of plasma protein into the tissue, oedema and leukocyte invasion. Activated immune cells are central to inflammatory processes needed to remove pathogens, damaged cells, irritants or debris. Inflammation is an integral part of the healing process when regulated with timely precision, but it is not a feature of healthy tissue (for review see Allan and Rothwell, 2003).

1.1.1. Does inflammation occur in the CNS?

The brain was initially believed to be 'immune privileged' on three accounts. Firstly, it is isolated from the systemic immune system by the blood-brain barrier (BBB) and was thought to be deprived of influence from the systemic immune system. Secondly, it was thought not to possess antigen presenting cells such as dendritic cells, B cells and macrophages. Thirdly, it was thought that brain cells lack antigen-presenting molecules such as major histocompatibility complex (MHC) class I and II and therefore cannot be recognised by antigen-specific T lymphocytes (for review see Neumann 2001). However, these views are now challenged. In normal conditions, the central nervous system (CNS) is immunologically silent and an immune response is actively suppressed. The immune cells of the brain are glial cells, in particular microglia, which are actively kept quiescent via local interactions with intact neurones. However, in the presence of neurotoxins or trauma, neuronal activity is disturbed, glial cells are activated by inflammatory mediators and an inflammatory response is initiated (for review see Neumann, 2001). Therefore this belief of 'immune privilege' is now radically changed, and it is now accepted that

the brain, like any other organ in the body, is capable of developing an inflammatory response. Hallmarks of CNS inflammation include oedema, glial cell activation, invasion of circulating immune cells, production of pro-inflammatory mediators and a compromised BBB. But, unlike other organs within the body, the brain has limited capacity to regenerate. Consequently, CNS inflammation must be regulated with extra vigour as prolonged inflammation is harmful and can lead to chronic inflammatory CNS disease (for reviews see Allan and Rothwell, 2003; Neumann, 2001 and Rothwell et al., 1997). The role of inflammation in the CNS is unresolved. Despite overwhelming evidence for deleterious effects of inflammation, beneficial effects have also been described. Whether inflammation is friend or foe is a subject of intense research. It is postulated that inflammation could have a dual role in the CNS (for review see Hohlfeld et al., 2007), the fate of which could depend on the timing of activation.

1.1.2. Acute inflammatory response in the CNS

Acute brain injury refers to insults that result in a rapid loss of brain cells, often characteristic of conditions such as stroke, traumatic brain injuries and seizures. Ischaemic stroke is caused by a sudden occlusion of a cerebral artery, preventing blood flow and supply of oxygen and nutrients to a targeted brain area, leading to cerebral ischaemia. Cerebral ischaemia leads to a rapid depletion of energy stores, loss of ionic gradients, calcium influx, excitotoxic neurotransmitter release, and ultimately neuronal death and loss of associated neurological functions (for reviews see Muir et al., 2007; Graham and Hickey, 2002; Graham and Chen, 2001; Dirnagl et al., 1999 and Lee et al., 1999). In traumatic brain injury, brain damage is caused directly by a physical trauma, whilst in seizures it is caused by a transient wave of synchronous/excessive neuronal activity resulting in neuronal cell death. In all these cases, acute cell death is caused by necrosis, but delayed cell death is associated with the inflammatory response. Apoptotic cell death can also contribute to delayed brain injury, triggered or enhanced by neuroinflammatory processes.

1.1.3. Chronic inflammation of the CNS

Multiple sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease (PD) are progressive neurodegenerative disorders with unknown aetiology. The symptoms of these diseases are diverse and progressive, increasing in severity with disease progression. MS is characterised by demyelination of neurones, which can lead to motor and cognitive defects (for reviews see Steinman, 2008; Delegge and Smoke, 2008; Allan and Rothwell, 2003). AD is characterised by a progressive loss of memory and inability to form new memories. Beta-amyloid plaques and neurofibrilliary tangles are hallmarks of AD (for review see Shaftel et al., 2008). Tremor, muscle rigidity and bradykinesia are overt symptoms of PD. These symptoms are due to the loss of dopaminergic neurones in the *substantia nigra* (for review see Schulz and Falkenburger, 2004). It is clear that these diseases can affect different brain regions and different populations of CNS cells, but are commonly associated with chronic inflammation of the CNS. The hallmarks of inflammation, such as activated microglia, MHC up-regulation, pro-inflammatory cytokine production and invasion of leukocytes, are all present in these neurological disorders.

1.2. Cellular responses to CNS inflammation

The major brain cell types that respond to inflammation are the microglia, neurones, and astrocytes. The role of oligodendrocytes in inflammation is less clear. The specialised endothelial cells of the BBB also play a key role in regulating CNS inflammation.

1.2.1. Microglial response

Microglia are the resident monocytes/macrophages of the brain, derived from bone marrow precursor cells that migrate into the brain during early development (for review see John et al., 2003). In the adult brain the microglial population is constantly renewed by a specific population of monocytes from the circulation (Mildner et al, 2007). However, local renewal of microglia cells from CNS resident cells has also been suggested (Ajami et al, 2007).

Resting microglia are exquisitely sensitive cells that rapidly responds to any changes in their environment. Microglia respond to changes in brain homeostasis by altering their morphology and begin to proliferate (Denes et al., 2007 and for review see Perry, et al. 1995). Activated microglia assume phagocytic properties and antigen-presenting capacity through the expression of MHC molecules (for review see Kim and de Vellis, 2005). Consequently, invading leukocytes recognise antigenic material and trigger an immune response (for review see Neumann, 2001). Brain ischaemia, trauma or infection can all activate resting microglia to migrate to the site of injury or infection and produce pro-inflammatory cytokines, chemokines as well as mitogens (for review see Kim and de Vellis, 2005 and Kreutzberg, 1996). Nuclear transcription factor (NF-κB) and mitogen-activated protein kinase (MAPK) signalling are important in the activation and production of pro-inflammatory mediators in microglia (Kim et al,. 2004; for review see Kreutzberg, 1996). The role of activated microglia in CNS inflammation is to scavenge any debris or dying cells within the vicinity of the injured tissue. However, sustained microglial activation can lead to chronic inflammation and neuronal dysfunction and death (for review see Vilhardt, 2005).

Activated microglial cells also have beneficial properties as they can act as glutamate scavengers to remove excess glutamate from the extracellular vicinity to prevent excitotoxic cell death. Microglia can also produce neurotrophic factors including neurotrophins and growth factors (Imai, F. et al., 2007 and for review see Lai and Todd, 2006, and Kim and de Vellis, 2005) to support neurogenesis.

1.2.2. Astrocytic response

Astrocytes are the most abundant cell type in the human brain and have important roles in CNS development, energy metabolism, regulation of local cerebral blood flow, synaptic functions, maintenance of brain pH balance and establishment and maintenance of functional integrity of the BBB (for reviews see Sofroniew and Vinters, 2010 and Dong and Benveniste, 2001). Astrocytes are adaptive cells and become activated in response to any adverse changes in brain homeostasis. Activated astrocytes are hypertrophic, prolific and undergo altered gene expression, producing factors that can both contribute to or limit CNS injury

(for reviews see Liberto et al., 2004 and John et al., 2003). Mediated by NF-κB signalling, astrocytes are responsible for the production of a wide array of cytokines, chemokines and neurotrophic factors, as well as mediating the recruitment of leukocytes and neutrophils, contributing further to the inflammatory response (for reviews see Farina et al., 2007, Moynagh, 2005, and Dong and Benveniste, 2001). There are also suggestions that activated astrocytes may function as antigen-presenting cells that contribute to the resolution phase of inflammation (for review see Dong and Benveniste, 2001). Activated astrocytes also act as scavengers of excess glutamate and free radicals and produce growth factors to promote neurogenesis and restore CNS homeostasis (for reviews see Farina et al., 2007 and Liberto et al., 2004).

1.2.3. Neuronal response

Neurones are electrically excitable cells in the brain. These cells process and conduct information by sending electrical and chemical signals to neighbouring cells. Neurones are sensitive to infection and injury, but their sensitivity can vary in different brain regions, and different diseases can lead to selective and specific neuronal death. For example, PD affects neurones in the *substantia nigra* whilst Alzheimer's disease affects cortical and subcortical neurones.

Neuronal responses to inflammation are not fully characterised, however neurones in acute and chronic neuroinflammatory conditions display altered neurotrophin expression, neuronal calcium imbalance as well as protease activation and induction of many inflammatory mediators. The effects of these mediators on neuronal survival is unclear (for review see Neumar, 2000). NF-κB is activated in neurones after stroke, traumatic brain injury and epilepsy, and can influence the neurodegenerative process directly by altering neuronal gene expression. The role of NF-κB signalling in neuronal injury is complex, contributing prominently to neuronal survival but also neuronal death (for review see Mattson, 2005).

The specific contribution of microglia and astrocytes to CNS inflammation and their effects on neuronal survival is still not fully resolved. It is clear that mediators released by microglia can activate astrocytes, and mediators

released by astrocytes can activate microglial cells. This bidirectional level of activation can result in a rapidly escalated and uncontrolled inflammatory event and neuronal death (Figure 1.1). The regulatory mechanisms required to inhibit inflammation are poorly understood.

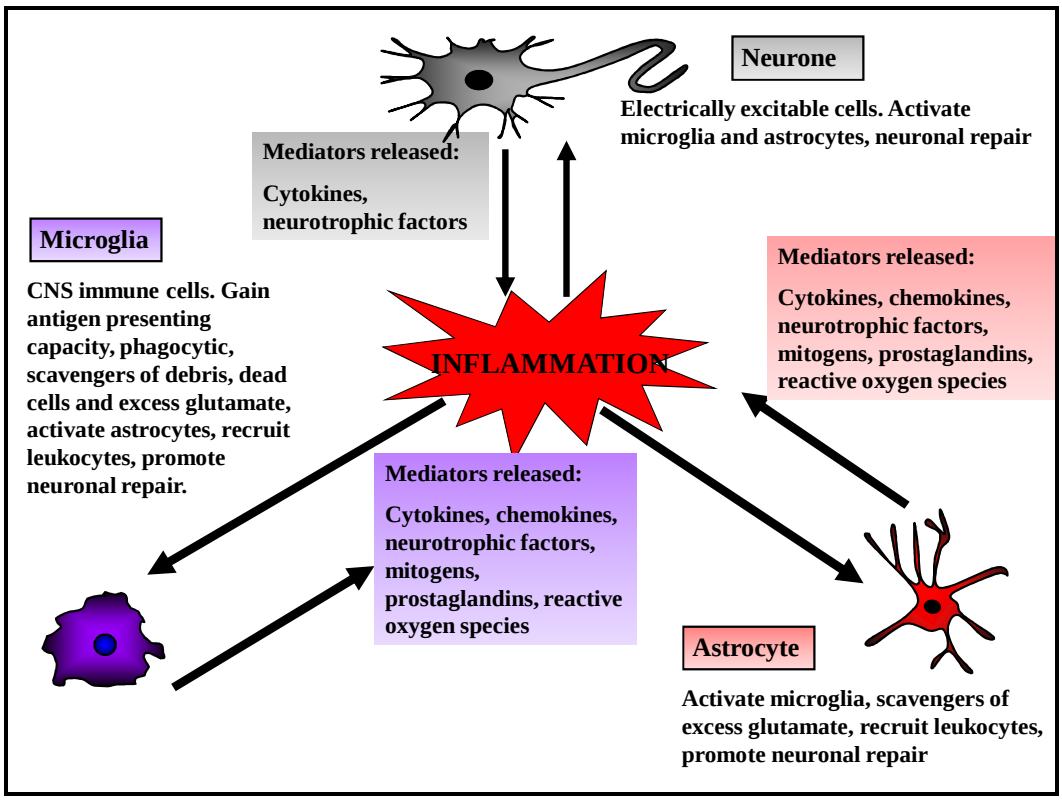


Figure 1.1. The effects of inflammation on different CNS cell types.

Inflammation activates microglial cells and astrocytes to release pro-inflammatory mediators as well as neurotrophic factors, which modulate inflammation itself. During inflammation, neurones release cytokines and neurotrophic factors to activate microglia and astrocytes to initiate an immune response.

1.2.4. Oligodendrocytes

Oligodendrocytes are myelin-forming glial cells that are abundant in the brain. However, the effect of inflammation on oligodendricytes is not as extensively reported when compared to other glial cell types such as microglia or astrocytes. Oligodendrocyte demise is associated with neuro-inflammatory conditions such as MS, AD and stroke (for reviews see Roth et al. 2005; Cannella and Raine 2004; Dewar et al. 2003), indicating its vulnerability to inflammatory damage. Indeed oligodendrocytes express both chemokine and cytokine receptors (Omari et al, 2005; Cannella and Raine, 2004) and have been shown to be compromised by endotoxins such as lipopolysaccharide (LPS) (Yao et al, 2010; Pang et al, 2003) and pro-inflammatory cytokines (Bhat et al, 1999; Robbins et al, 1987).

1.2.5. The blood brain barrier and brain endothelial cells

The blood brain barrier (BBB) is a functionally active barrier that regulates the migration of substances from the blood (circulation) to the brain to maintain brain homeostais. The BBB is a complex system of blood vessels formed by endothelial cells encapsulated by a layer of basal lamina, and supported by astrocytic cells. Endothelial cells in the BBB form tight junctions to regulate passage of substances across to the brain. During inflammation, the integrity of the BBB is compromised and becomes “leaky”, resulting in an influx of molecules that would have been otherwise restricted (for review see de Boer and Gaillard 2006 and de Vries et al. 1997). Endothelial cells of the BBB are sensitive to pro-inflammatory cytokines and chemokines, and respond to these proteins by the production of more chemokines, cytokines and adhesion molecules (Oynebraten et al, 2004; Stanimirovic et al, 1997). Adhesion proteins are key in the recruitment of leukocytes across the BBB contributing further to the inflammatory response (Van et al, 2003).

1.3. Mediators of CNS inflammation

During CNS inflammation, the integrity of neurones is compromised, and glial cells are activated. Activated microglia release an array of chemokines including macrophage inflammatory proteins (MIP), microglia response factor-1 (MRF-1), interleukin-8 (IL-8), monocyte chemotactic proteins (MCP) as well as prostaglandin E2 (PGE_2), reactive oxygen species and mitogenic factors (for reviews see Kim and de Vellis 2005; John et al., 2003; Allan and Rothwell, 2003; Kreutzberg, 1996). Activated microglia are also a source of inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) (Pinteaux et al, 2002; Lee et al, 1993). The primary cytokine involved in the activation of astrocytes at the early stages of inflammation is IL-1 (Lee et al, 1993). Activated astrocytes are also potent producers of chemokines including chemokines belonging to the CCL and CXCL family (CCL1, 2, 5 and CXCL10, 20, 12, 1, 2), IL-1, IL-6, TNF-α, (Lee et al, 1993) and matrix metalloproteinase-9 (pro-MMP9) (Thornton et al, 2007) (for reviews see Farina et al., 2007 and Dong and Benveniste, 2001). Neurones are the most vulnerable cells in the brain and their loss during neuroinflammation can lead to loss of function associated to that area of degeneration. Neurones are also producers of pro-inflammatory cytokines and neurotrophic factors (Tsakiri et al, 2008c; Isackson, 1995). The function of pro-inflammatory cytokines and chemokines is to activate neighbouring cells to amplify the local innate immune response as well as recruiting circulating immune cells to initiate the adaptive immune response. Cytokines can activate local microglia and astrocytes that produce chemokines, leading to leukocyte infiltration.

In addition to the expression of this array of pro-inflammatory mediators, inflammation can also induce the expression and release of mediators important for promoting cell growth and repair. These mediators include neurotrophins, brain-derived neurotrophic factors (BDNF) and nerve growth factors (NGF), all of which are expressed by microglia, astrocytes and neurones (for reviews see Farina et al., 2007; Kim and de Vellis 2005; Isackson, 1995). The neuroprotective actions of microglia can also be attributed to their ability to produce IL-1 receptor antagonist (IL-1RA), which can down-regulate IL-1 actions on astrocytes (Pinteaux et al, 2006) (Figure 1.2).

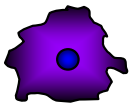
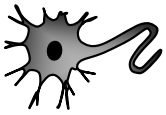
Cell type	Mediators released	References
 ***Microglia***	• Cytokines and chemokines (TNF, IL-8, IL-6, IL-1, IL-1RA, CCL family chemokines and CXCL family cytokines) • PGE_2 and mitogens factors • Reactive oxygen species • Neurotrophin, BDNF and NGF	• Lai and Todd, 2006 • Kim and de, 2005 • Vilhardt, 2005 • John et al, 2003 • Kreutzberg, 1996 • Perry, et al, 1995
 ***Astrocytes***	• Cytokines and chemokines (TNF, IL-6, IL-1, CCL1, 2, 5 and CXCL10, 20, 12, 1, 2, • PGE_2, pro-MMP9 and mitogens • Reactive oxygen species • Neurotrophin, BDNF and NGF	• Sofroniew and Vinters, 2010 • Farina et al, 2007 • Thornton et al 2007 • Moynagh, 2005 • Liberto et al, 2004 • John et al, 2003 • Dong and Benveniste, 2001
 ***Neurones***	• Neurotrophin, BDNF and NGF • TNF • IL-1 and IL-6	• Tsakiri et al, 2008 • Mattson, 2005 • Neumar, 2000 • Isackson, 1995

Figure 1.2. Mediators expressed by different brain cell types during inflammation.

Inflammation induces the activation of microglia and astrocytes to secrete pro-inflammatory cytokines, chemokines, neurotrophic factors and other mediators. Neurones also release neurotrophic factors and pro-inflammatory cytokines.Abbreviations: TNF, tumuor nercosis factor; IL-8, interleukin-8; IL-6, interleukin-6; IL-1, interleukin-1; IL-1RA, IL-1 receptor antagonist; PGE_2, prostaglandin E2; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; CCL1, 2, 5 and CXCL10, 20, 12, 1, 2, chemokines CCL and CXCL subclasses; MMP9, matrix metalloproteinase-9.

1.3.1. Cytokines and CNS inflammation

Cytokines have dual roles in CNS inflammation - detrimental and beneficial. The pro-inflammatory role of some cytokines is well recognised, but the same cytokine can also exert anti-inflammatory effects. TNF-α, IL-6 and IL-1 have been shown to exacerbate ischaemic damage and block neurogenesis, whilst inhibition of their expression significantly reduced brain damage and restored neurogenesis (Monje et al., 2003 and for reviews see Denes et al., 2009; Lai and Todd, 2006; Ekdahl et al., 2003; Kempermann and Neumann, 2003). The detrimental effects of IL-1 may be mediated in part by the induction of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by endothelial cells, leading to leukocyte recruitment into the brain (for review see Denes et al., 2009). Conversely, TNF-α, IL-6 and IL-1 is also neuroprotective in mouse neurones *in vitro* against N-methyl-D-aspartic acid (NMDA)-induced cell death (Carlson et al, 1999), whilst IL-6 is also neuroprotective *in vivo* after cerebral ischaemia in rats (Loddick et al, 1998). Additionally, IL-1 has been shown to enhance neurogenesis during differentiation of mesenchymal stem cells into neurones (Greco and Rameshwar, 2007).

In summary, regulated inflammation is a beneficial process to the host but deregulated inflammation is detrimental. Inflammation is a complex and dynamic mechanism involving many interacting cytokines, chemokines and signalling pathways. Amongst the different inflammatory mediators known to regulate neuroinflammation, IL-1 is the primary mediator. However its mechanism of action is not fully investigated. Therefore it is important to characterise the effects of IL-1 on different CNS cell types and investigate its mechanism of action to prevent the deleterious effects of IL-1 after injury.

1.4. The interleukin-1 system

There are currently eleven agonists in the IL-1 family including IL-1α, IL-1β, IL-1RA, IL-18, IL-33 and IL-1F5-10, and ten receptors including IL-1 type 1 receptor (IL-1R1), IL-1 type 2 receptor (IL-1R2), IL-1 receptor accessory protein (IL-1RAcP), IL-18Rα, IL-18Rβ, IL-1 receptor-like 1 (ST2), IL-1 receptor-like 2 (IL-1RL2), sinlge immunoglobulin IL-1 receptor-related moleculre (SIGIRR) and three immunoglobulin domain-containing IL-1 receptor-related 1 and 2 (TIGIRR1 and 2) in the IL-1 family, however, not all the functions of these receptors and agonists have been defined. Activation of these receoptors by their respective agonists is associated with activation of the MAPK and NF-κB signalling (see section 1.5.1). However, not all IL-1 receptors are signalling receptors. IL-1R2 is a non-signalling receptor (see section 1.4.2) and SIGIRR and TIGIRR are orphaned receptors. The presence of orphaned receptors within the IL-1 family suggests that our current understanding of IL-1 actions is incomplete (Figure 1.3) (for reviews see Sims and Smith, 2010; Allan 2005).

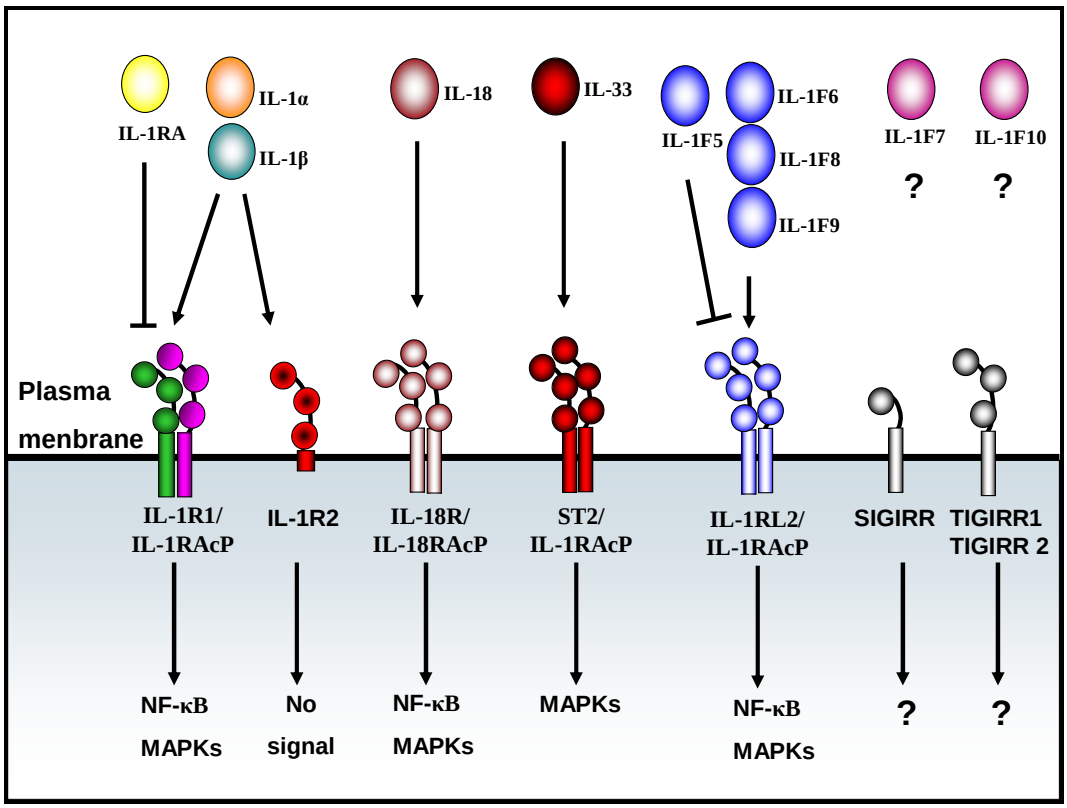


Figure 1.3. The IL-1 family members.

Schematic showing the association of different IL-1 ligands to their respective receptors. Of the eleven ligands, it is unknown which receptor(s) IL-1F7 and IL-1F10 are associated with. Actions of IL-1 agonists are regulated by IL-1RA, which blocks IL-1α and IL-1β, and IL-1F5, which regulates IL-1F6, IL-1F8 and IL-1F9. IL-1 actions are also regulated by IL-1R2 and SIGIRR. Abbreviations: IL-1R1, IL-1 type 1 receptor; IL-1R2, IL-1 type 2 receptor; IL-1RAcP, IL-1 receptor accessory protein; IL-18Rα, IL-18 receptor α; IL-18Rβ, IL-18 receptor β; ST2, IL-1 receptor-like 1, IL-1RL2, IL-1 receptor-like 2; SIGIRR, sinlge immunoglobulin IL-1 receptor-related moleculre and TIGIRR1 and 2, three immunoglobulin domain-containing IL-1 receptor-related 1 and 2. Modified from Sims and Smith, 2010 and Allan et al, 2005.

1.4.1. IL-1α, IL-1β and IL-1RA

The best characterised ligands of the IL-1 system are IL-1α, IL-1β and IL-1RA. IL-1α and IL-1β are agonists whereas IL-1RA is a competitive receptor antagonist. These ligands act by binding to IL-1R1 or IL-1R2. IL-1R1 is an active signalling receptor whilst IL-1R2 is a non-signalling decoy receptor. IL-1 signalling is involved in normal growth and development, as well as normal physiological functions including sleep (Fang et al, 1998), appetite (Matsuki et al, 2003), memory, and neuronal plasticity (Avital et al, 2003; Yirmiya et al, 2002). Disruption to IL-1 actions can result in an abnormal immune response. Arthritis (Pascual et al, 2005; Smeets et al, 2005), and atherosclerosis (Merhi-Soussi et al, 2005b; Moyer et al, 1991) are peripheral conditions associated with inflammation and over-active IL-1 signalling. Centrally, unregulated IL-1 signalling is reported in stroke pathology, traumatic brain injuries and seizures. IL-1 is also reported to be pro-convulsive (Balosso et al, 2008) and contributes to the detrimental effects of seizures (Patel et al, 2003). Collectively these studies indicate that IL-1 is an important element in the inflammatory cascade, both peripherally and centrally.

IL-1α and IL-1β are products of different genes, synthesised as large (31 kDa) precursor molecules before being cleaved to form mature 17 kDa proteins. Pro-IL-1α is the precursor of IL-1α, and is cleaved by proteases called calpains. Both pro-IL-1α and IL-1α are biologically active and remain mainly localised in the cytosol and the nucleus (for reviews see Sims and Smith, 2010; Arend et al., 2008; Laurincova, 2000; and Boraschi et al., 1996). Intracellularly, pro-IL-1α is reported to regulate cell migration (Merhi-Soussi et al, 2005a; McMahon et al, 1997), proliferation (Kawaguchi et al, 2004; Maier et al, 1994) and gene expression in various cell lines via intranuclear actions (Kawaguchi et al, 2004; Werman et al, 2004) (for review see Luheshi et al. 2009). The function of intracellular mature IL-1α is less well understood. Mature IL-1α, either intracellular or as result of endocytotic internalisation of secreted IL-1α, is transported to the nucleus where it is suggested to bind to DNA and exert intranuclear actions (Luheshi et al, 2009; Weitzmann and Savage, 1992; Grenfell et al, 1989). The specific intranuclear actions of mature IL-1α are however unknown.

In contrast, pro-IL-1β is biologically inactive and must be cleaved by the protease caspase-1 in order to become an active mature protein. Responses induced by IL-1β are from the secreted mature form of IL-1β (for reviews see Luheshi et al. 2009a; Allan et al. 2005; Dinarello 1997). Caspase-1 shares significant sequence homology with the death protein CED-3 found in the apoptotic pathway of *Caenorhabditis elegans* (Thornberry et al, 1992). This analogy has led to the suggestion that caspase-1 as well as IL-1 could be contributing factors in programmed cell death.

IL-1RA is a 17 kDa protein produced by the same cells that express IL-1α and IL-1β. There are three intracellular isoforms of IL-1RA (icIL-1RA 1, 2 and 3) and one secreted isoform (IL-1RA) (Palin et al., 2001**;** Muzio et al., 1999 and for reviews see Arend et al., 2008 and Arend and Guthridge, 2000). Both icIL-1RA and secreted IL-1RA are antagonists of IL-1-induced actions. Secreted IL-1RA is a competive inhibitor of secreted IL-1, acting at the IL-1R1 (Pinteaux et al, 2006), whereas icIL-1RA is antagonistic to pro-IL-1α actions (Merhi-Soussi et al, 2005a)

1.4.2. IL-1R1, IL-1R2 and IL-1RAcP

There are two well characterised IL-1 receptors: IL-1R1 (80 kDa) and IL-1R2 (68 kDa). Structurally, IL-1R1 and IL-1R2 are very similar, containing a membrane-spanning region and three immunoglobulin–like domains in the extracellular part of the protein (for reviews see Smith, 2010; Arend, 2008; Boutin et al., 2003). However, IL-1R1 has an intracellular signalling region called the Toll/IL-1R (TIR) domain which is essential for signalling, but is absent in IL-1R2 (McMahan et al, 1991). This explains why IL-1R1 is a signalling receptor, whereas IL-1R2 acts as a decoy receptor by scavenging excess IL-1 (Sims et al, 1993). IL-1R2 can also regulate IL-1 functions by preventing the formation of the IL-1 signalling complex (Malinowsky et al, 1998; Lang et al, 1998). IL-1R1 is internalised after activation (Brissoni et al, 2006; Solari et al, 1994) or as a part of the receptor's life cycle (Solari et al, 1994). Following endocytosis the receptor can be recycled (Solari et al, 1994), degraded or re-directed to other sites (Mizel et al, 1987). The half life of IL-1R1 is 11 h in the absence of IL-1 (Mizel et al, 1987). The life cycle of IL-1R2 is not known.

IL-1R1 and IL-1R2 also exists in soluble forms (for review see Boutin et al, 2003), and both are capable of binding to IL-1α and IL-1β (Arend et al, 1994) to inhibt IL-1 actions (for review see Allan et al, 2005). The enzymes responsible for the cleavage of IL-1 receptors belong to the metalloprotease family, but the specific protease involved in this process is unknown (Elzinga et al, 2009) (Figure 1.4). In addition to the receptor cleavage, soluble IL-1R2 can also be the result of alternative splicing of the IL-1R2 mRNA, making it an alternative splice variant of IL-1R2 (Liu et al, 1996). The intracellular domain of IL-1R1 and IL-1R2 can also be cleaved by γ-secretease into soluble intracellular fragments (Elzinga et al, 2009; Kuhn et al, 2007). These receptor fragments have been shown to mediate specific IL-1-induced actions in human embryonic kidney cell lines, but this could also be a mechanism of regulating IL-1 actions by preventing the coupling of IL-1R1 and the IL-1 receptor accessory protein (IL-1RAcP) TIR domain (Elzinga et al, 2009)

IL-1R1 alone cannot transduce signal, but requires association with the IL-1RAcP to recruit downstream adaptor molecules such as myeloid differentiation factor (88) (MyD88) and Toll-interacting protein (Tollip). This makes IL-1RAcP an essential secondary sub-unit crucial for IL-1 signalling (Cullinan et al, 1998; Wesche et al, 1997; Wesche et al, 1996). IL-1RAcP is 80 kDa in size when fully glycosylated, and has significant structural and sequence homology with IL-1R1, with three extracellular immunoglobulin-like domains, a transmembrane region and an intracellular TIR domain. Interaction of IL-1RAcP with IL-1R1 increases IL-1R1 affinity for its agonist (Cullinan et al, 1998; Greenfeder et al, 1995). There are four splice variants of IL-1RAcP; a membrane-bound IL-1RAcP, a soluble sIL-1RAcP (Jensen et al, 2000) a soluble sIL-1RAcPβ (Jensen and Whitehead, 2003), and a recently described splice variant called IL-1RAcPb (Smith et al, 2009; Lu et al, 2008) (See section 1.4.3 and Figure 1.5). The function of sIL-1RAcP is unclear but it has been proposed to be a competitive inhibitor of IL-1 actions by competing with membrane-bound IL-1RAcP for binding with IL-1R1, thus preventing the formation of the TIR domain heterodimer necessary for signalling (Jensen et al, 2000). sIL-1RAcP may also act as a powerful scavenger of released IL-1 (Smith et al, 2003). The opposing actions of IL-1RAcP and sIL-1RAcP on IL-1 signalling suggests that the

biological outcome of IL-1 actions may depend on which form of IL-1RAcP is predominantly expressed at the time of stimulation.

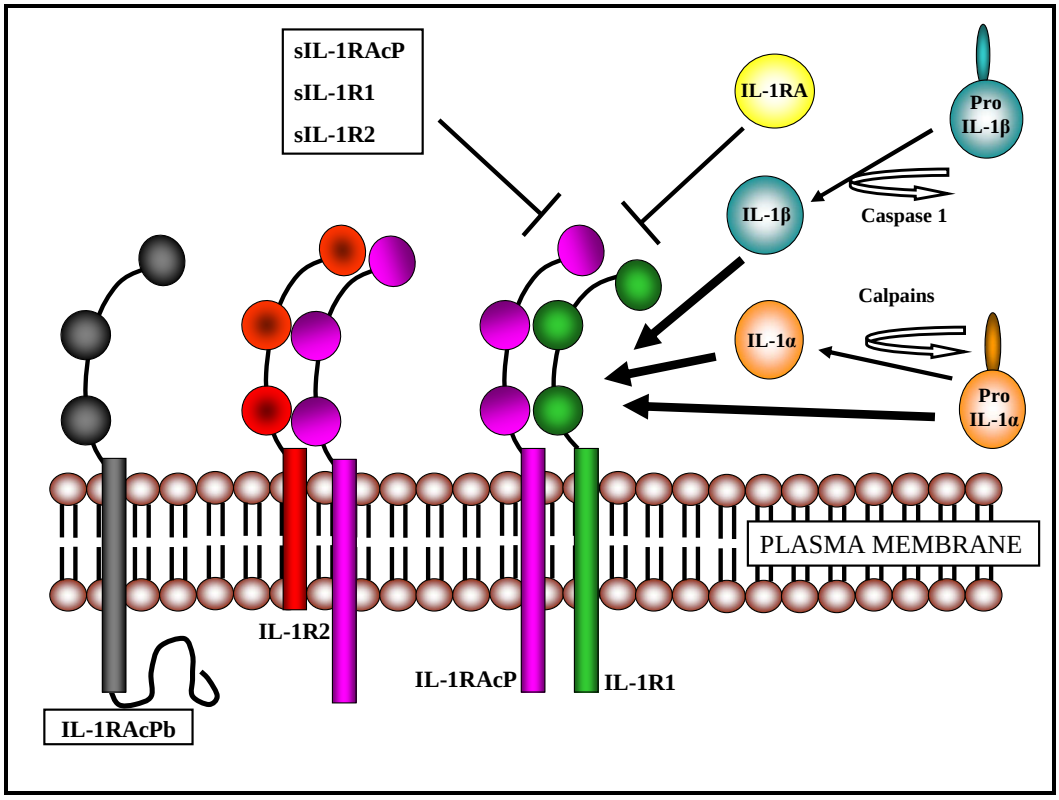


Figure 1.4. The IL-1 signalling system

IL-1α and IL-1β are produced as precursor molecules and cleaved by calpains and caspase-1 respectively to form mature IL-1 proteins. These agonists, like IL-1RA, bind to the signalling complex (IL-1R1/IL-1RAcP) to exert their effects, whilst IL-1RA inhibits IL-1α and IL-1β actions. IL-1R2 is a decoy receptor that negatively regulates IL-1 actions by scavenging excess IL-1 or associating with IL-1RAcP to prevent the formation of the signalling heterodimer. IL-1R1, IL-1R2 and IL-1RAcP also exist as soluble proteins. These soluble receptors function as modulators of IL-1 actions by scavenging excess IL-1 or recruiting IL-1RAcP to prevent the formation of the signalling complex. IL-1RAcPb is a recently described IL-1 receptor accessory protein. The function of this receptor is not fully characterised.

It is unclear how IL-1 interacts with the signalling complex, but previous reports have suggested that it is only IL-1R1 that binds to IL-1 (Wesche et al, 1998), upon which a conformational change occurs in the IL-1R1 structure, which then reveals a docking site for IL-1RAcP and the formation of the signalling heterodimer (Greenfeder et al, 1995). IL-1RAcP is suggested to stabilise the binding of IL-1 to IL-1R1 (Wesche et al, 1998). How IL-1RAcP interacts with IL-1 and IL-1R1 is still unclear. Binding of IL-1RA to IL-1R1 fails to induce the required binding conformation of IL-1R1, therefore docking of IL-1RAcP is prevented and no signal can be transduced. Hence IL-1RA acts as an antagonist (Casadio et al, 2001).

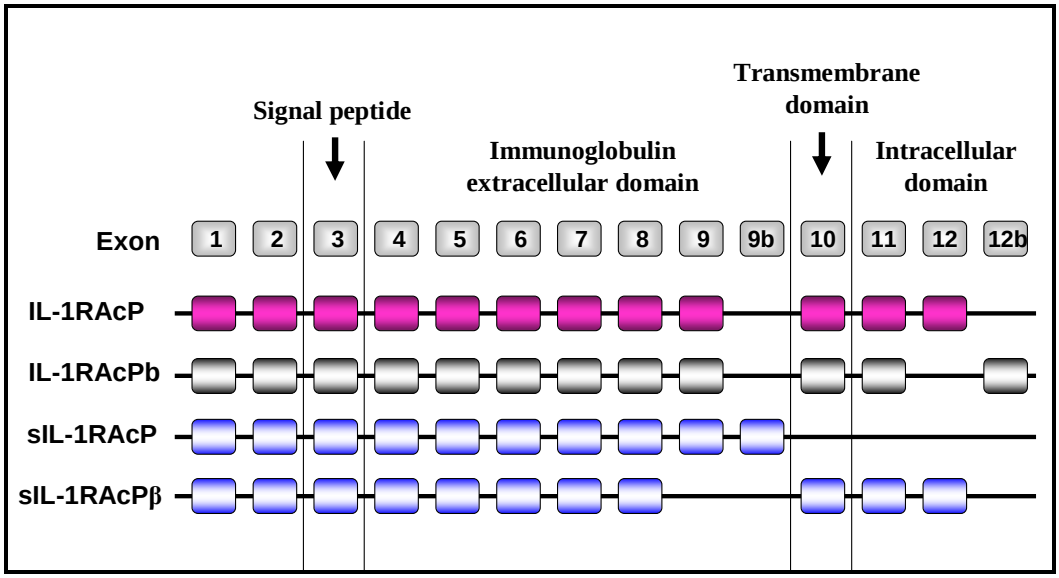


Figure 1.5. Expression of exons in different isoforms of IL-1RAcP.

Schematic showing the expression of different exons in different isoforms of IL-1RAcP. The signal peptide is encoded on exon 3 and the three extracellular immunoglobulins are encoded by exons 4-9/9b. The transmembrane region is encoded by exon 10 and the intracellular domain is encoded by exons 11-12/12b. Modified from Lu et al., 2008.

1.4.3. Interleukin-1 receptor accessory protein b (IL-1RAcPb)

Recently, two independent research groups (Smith et al, 2009; Lu et al, 2008) have identified an alternatively spliced isoform of IL-1RAcP in the human genome, named IL-1RAcP687 or IL-1RAcPb. As IL-1RAcPb will become the focus of this project, and since the two research groups presented opposing evidence for the function of IL-1RAcPb, this section will review both studies in some detail.

Genomic comparisons of IL-1RAcP and IL-1RAcPb revealed that these proteins are encoded by two different splice variants of the exon 12 of the *IL-1RAcP* gene (Figure 1.6). Like IL-1R1 and IL-1RAcP, IL-1RAcPb has three immunoglobulin-like domains, a transmembrane region and an intracellular TIR domain, but it also has an additional chain of 140 amino acid; forming a 'tail' at the carboxyl terminus (Smith et al, 2009).

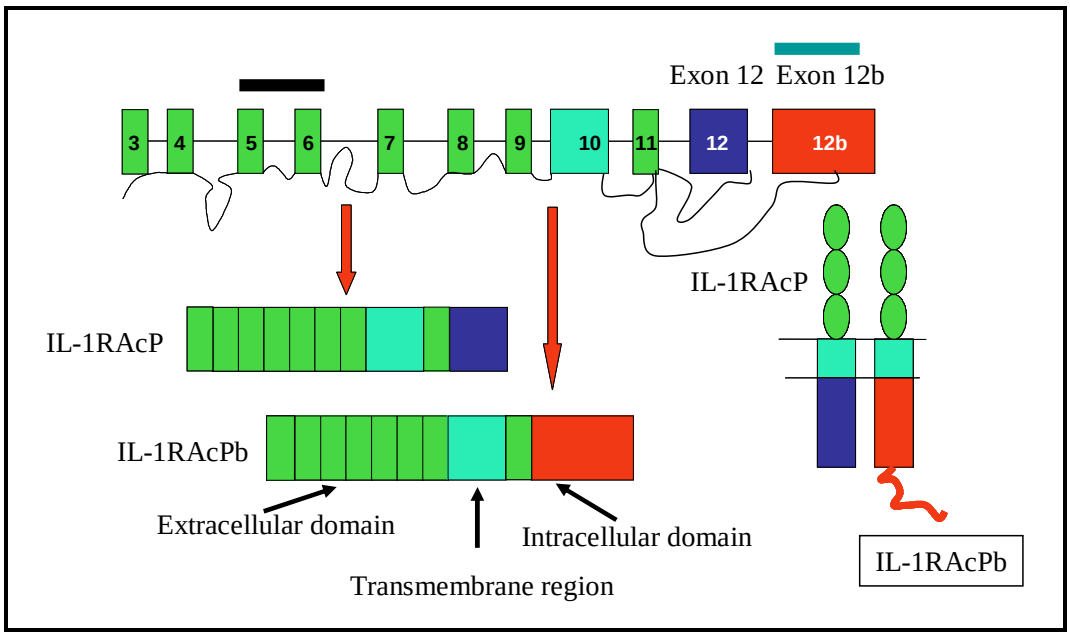


Figure 1.6. Schematic drawing of IL-1RAcP and IL-1RAcPb mRNA and their structural domains.

IL-1RAcP contains exon 12 but IL-1RAcPb contains exon 12b, which codes for an additional poly amino acid tail. The black horizontal bar indicates the disrupted section in IL-1RAcP knock-out ($^{-/-}$) modification and the green horizontal bar indicates the disrupted section in IL-1RAcPb$^{-/-}$ modification. Modified from Smith et al., 2009.

The search for the expression of IL-1RAcPb mRNA by real-time PCR in thirty one different human tissues, showed that the expression of IL-1RAcPb is restricted to the CNS, and was found predominantly in the whole brain, foetal brain, cerebellum and spinal cord (Smith et al, 2009). In the periphery, the only non-CNS tissue that expressed IL-1RAcPb mRNA are bronchial epithelial cells, however expression was only 3 % compared to whole brain IL-1RAcPb mRNA. It was also demonstrated that the pattern of expression of IL-1RAcPb in the brain followed the expression of neurone-specific microtubule-associated protein 2 (MAP2) mRNA, indicating that it is neuronal specific. The expression of IL-1RAcPb and IL-1RAcP however, does not coincide as IL-1RAcP expression co-localised with the expression of astrocyte-specific glial fibrillary acidic protein (GFAP) mRNA (Smith et al, 2009). In mouse hippocampus, the expression of IL-1RAcP is found in both neurones and astrocytes.

In EL4.16a cells (a T-cell sub cell line) it was shown that transfected IL-1RAcP and IL-1RAcPb co-immunoprecipitates with native IL-1R1 in the presence of IL-1β (Smith et al, 2009). Co-immunoprecipitation of transfected IL-1R1, IL-1RAcP, and IL-1RAcPb in human kidney HEK293T cells was also observed (Lu et al, 2008). Studies of the functional expression of IL-1RAcPb in EL4.16a indicate that IL-1RAcPb could be a negative modulator of IL-1 signalling in these cells. In cells transfected with *IL-1RAcP* gene, IL-1β induces the release of IL-2, IL-5 and IL-6. However, when cells are transfected to express IL-1RAcPb, these responses are abolished. It was demonstrated that IL-1RAcPb fails to recruit downstream signalling molecules of the IL-1 signalling cascade. IL-1RAcPb failed to activate MAPK such as p38, extracellular signal-regulated kinases (ERK1/2) and c-jun N-terminual kinases (JNK) when expressed in EL4.16a cells (Smith et al, 2009). This response was independent of the extra 140 amino acid C-terminus tail on the TIR domain. These results suggest that IL-1RAcPb may act similarly to that of the IL-1R2 decoy receptor. Despite having the intracellular TIR domain, IL-1RAcPb failed to transduce signal upon ligand binding.

In contrast, HEK293T cells transfected with IL-1RAcPb, recruited molecules of the IL-1 signalling cascade including Tollip and MyD88, as shown by immunoprecipitation. This process occurs independently of IL-1R1 and IL-1

action (Lu et al, 2008). Co-transfection of cells with IL-1R1 and IL-1RAcPb could also induce NF-κB binding to deoxyribonucleic acid (DNA) in HEK293T cells. Increasing NF-κB binding were detected with increasing expression of IL-1RAcPb (Lu et al, 2008). In a different set of experiments using human hepatoma HA22T/VGH cells, over-expression of IL-1RAcPb induced TNF-α and granulocyte macrophage-colony stimulating factor (GM-CSF) similarly to that of IL-1RAcP (Lu et al, 2008). Thus, in contrast to the findings of Smith et al. (2009), it was concluded that IL-1RAcPb is a mediator of IL-1 signalling.

To gain a better knowledge of the function of IL-1RAcPb, Smith et al. (2009) focused on the action of this receptor in primary mice neuronal-glial cell cultures. However, in order to evaluate the specific contribution of IL-1RAcP and IL-1RAcPb to IL-1 signalling, these authors cultured neuronal-glial cell cultures from IL-1RAcP$^{-/-}$ mice followed by transfection of target receptors (IL-1RAcP or IL-1RAcPb) by lentivirus. Microarray analysis of IL-1RAcP-transfected primary neuronal-glial population demonstrated substantial overlap in gene expression with wild-type (WT) C57/BL6 neuronal-glia cells when treated with IL-1 (Smith et al, 2009). This indirectly inferred that the absence of IL-1RAcPb did not affect affect cells' response to IL-1. However, when IL-1RAcP and IL-1RAcPb were co-transfected in IL-1RAcP$^{-/-}$ neuronal-glial cultures, the pattern of gene expression in response to IL-1 was different to that of IL-1RAcP transfection alone or WT cells, indicating that the presence of IL-1RAcPb may alter the the cells' response to IL-1. IL-1 treatment of co-transfected cells lead to an increase in specific genes expression, as well as a decrease in the expression of other classes of genes, whilst the expression of many classes of genes also remained unchanged. From these data, Smith et al. (2009) concluded that "IL-1RAcPb may down-modulate some but not all IL-1RAcP dependent IL-1 responses."

In vivo, IL-1RAcPb deficiency in mice does not exacerbate experimental autoimmune encephalomyelitis, and similar disease progression was observed in WT and IL-1RAcPb$^{-/-}$ animals (Smith et al, 2009). However, IL-1RAcPb did exert neuroprotective effects against LPS-induced neurotoxicity. LPS did not induce significant demyelination or neuronal cell loss in WT or IL-1RAcP$^{-/-}$ animals, but both were prominent in IL-1RAcPb$^{-/-}$ animals (Smith et al, 2009). However, this is a complex approach to show that IL-1RAcPb negatively regulates IL-1 actions.

There are many sequences of events between the infusion of LPS, the induction of endogenous IL-1 and the neuroprotection observed in IL-1RAcPb$^{-/-}$ animals. A more direct approach is needed to define the precise role of IL-1RAcPb *in vivo*.

These data provide the first insight into the structure and functions of IL-1RAcPb. However, these results must be interpreted with caution, as much of the data demonstrating negative/positive regulation of IL-1 action by IL-1RAcPb were derived from non-neuronal cell lines. Additionally, cultures used to investigate IL-1RAcPb activity in neurones contained 20-30% glial cells. The contribution of these cells to the microarray data could be significant and should be taken into account. The *in vivo* data from Smith et al. (2009) are inconclusive, which leaves open the question on the role of IL-1RAcPb in IL-1 signalling. Despite its expression being CNS-restricted and neuronal-specific, the functions of IL-1RAcPb in different CNS cells, including neurones, are unknown. Since the activity of IL-1R1 is dependent on the co-activation of IL-1RAcP, and that the expession of IL-1R1 and IL-1RAcP does not spatially coincide in different brain regions (see next section), it is important to characterise the function of IL-1RAcPb in these cells types to better understand the actions of IL-1 in the brain.

1.4.4. Expression of interleukin-1 receptors in the brain

There is controversy about the expression of IL-1R1, IL-1R2 and IL-1RAcP in different cell types and different brain regions, as the expression of these receptors does not always spatially coincide. The presence of IL-1RAcP in brain regions where IL-1R1 is absent may indicate independent function of IL-1RAcP or the presence of additional uncharacterised IL-1 receptors (for reviews see Boutin et al., 2003 and Touzani et al., 1999).

IL-1 receptors are expressed in both glia and neurones, as both cell types express mRNA for these proteins (Andre et al, 2005a) and both cell types are responsive to IL-1β treatment (Lee et al, 1993). Expression of IL-1 receptors in neurones has been reported in the developing nervous system of several species including mice, rats and frogs (Davis et al, 2006; Friedman, 2001; Jelaso et al, 1998), which suggests that IL-1R1 is expressed constitutively in neurones. However, the expression of IL-1R1 in neurones appears to be restricted to certain

brain regions, such as the hippocampus, the choroid plexus and ependymal epithelial cells lining the ventricles, as demonstrated in mice (French et al, 1999). Neurones cultured from mice cerebral cortex also express IL-1R1 and are responsive to IL-1α and IL-1β treatments (Tsakiri et al, 2008c). The cellular expression of IL-1 receptors is low, with only 50-200 receptors per cell; however this is sufficient for biological activity, as less than 10 occupied receptors are required for signal transduction to occur (for review see Dinarello, 1997).

Numerous studies have reported the presence of IL-1 receptors in astrocytes (Lin et al, 2006; Moynagh, 2005; Dunn et al, 2002; da Cunha A. et al, 1993), but the expression of IL-1R1 in microglia is controversial. There are opposing data on the expression of IL-1R1 in microglia. Studies on foetal human microglia have shown that IL-1R1 are present and that IL-1β treatment leads to further IL-1β, IL-6 and TNF-α production (Lee et al, 1993). Additionally, Andre et al. (2005), have shown IL-1R1 mRNA expression in mice microglia, and IL-1R1 is necessary for microglial activation in mice *in vivo* (Basu et al, 2002). IL-1α has also been shown to activate mouse microglia in culture by increasing the expression of receptors for antigens including CD4 and MCA-1 proteins (Yu et al, 1998). In contrast, there is also evidence indicating the absence of IL-1R1 in microglia. A radioligand binding assay on mouse primary glial cultures showed that IL-1 binds specifically to astrocytes but not microglia (Ban et al, 1993). This was later supported by a study reporting IL-1-induced astrocyte proliferation but not microglia proliferation in rat (Araujo and Cotman, 1995). More recently, Pinteaux et al. (2002) found very little evidence of constitutive expression of IL-1R1 mRNA in murine microglia, and microglial cells did not respond to IL-1β. However, there is evidence that stress induced by endotoxin (LPS) (Pinteaux et al, 2002) or penetrating brain injury (Friedman, 2001) induces IL-1R1 expression in these cells.

Literature on the expression of IL-1 receptors is biased towards IL-1R1 expression, and much less is known about the expression of IL-1R2 in different cell types. More attention should be given to this receptor because, as a decoy receptor, IL-1R2 possess anti-inflammatory actions. IL-1R2 mRNA expression has been reported to be most abundant in mouse microglia (Pinteaux et al, 2002) and absent from rat neonatal astrocytes (Juric and Carman-Krzan, 2001). In the

adult mouse brain, IL-1R2 is expressed in neurones of the hypothalamus in the absence of IL-1R1 (French et al, 1999). These reports provide only a small insight into the expression of IL-1R2. More research is needed to correlate the actions of IL-1R2 in pathology and to identify therapeutic targets.

1.4.5. Differences in IL-1α and IL-1β actions

Secreted IL-1α and IL-1β act by binding to IL-1R1 on the plasma membrane, which leads to the recruitment of IL-1RAcP and the formation of a signalling dimer complex. Structurally, IL-1α, IL-1β and IL-1RA share a common tertiary structure and all bind to IL-1R1. However; this similarity is not reflected in their affinity for IL-1R1 and IL-1R2. IL-1α, IL-1β and IL-1RA display different affinities for IL-1R1 and IL-1R2, with higher affinity of IL-1α and IL-1RA for IL-1R1, and IL-1β for IL-1R2 (Burger et al, 1995). In contrast, Juric and Carman-Krzan (2001), reported that IL-β has a 5-fold stronger affinity for IL-1R1 than that of IL-1RA and 6-fold stronger than that of IL-1α. Others have shown that IL-1α and IL-1β can bind to a common receptor with similar affinity (Bird and Saklatvala, 1986). Inconsistencies between these data could result from differences in IL-1 preparation or in cell types investigated, ranging from human dermal fibroblasts and synovial cells (Burger et al, 1995), to porcine connective tissue (Bird and Saklatvala, 1986) to rat astrocytes (Juric and Carman-Krzan, 2001). Additionally, there are multiple promoters regulating the expression of IL-1R1 (Ye et al, 1996) and these promoters display tissue-specific distribution (Chen et al, 2009). This observation suggests that the versatile nature of IL-1 could depend on which promoter is active; some promoters may constitutively be active, which may account for the basal level of IL-1R1 expression (Ye et al, 1993).

The differences between IL-1α and IL-1β are not limited to their differences in binding affinity for IL-1R1. It has been shown that recombinant murine IL-1α and recombinant human IL-1α can bind to human endothelial cells with equal affinity but induced differential responses (Thieme et al, 1987). In human endothelial cells, recombinant human IL-1α was more potent at inducing lymphocyte adhesion compared to recombinant murine IL-1α. Similarly, IL-1α and IL-1β could bind to IL-1R1 with high affinity, but only IL-1β, and not IL-1α,

induced NGF and IL-6 release from rodent glial cells (Andre et al, 2005b; Juric and Carman-Krzan, 2001). Also, IL-1β, but not IL-1α, is a potent inducer of IL-6 in neurones (Tsakiri et al, 2008c). In contrast, IL-1α is more effective than IL-1β in inducing substance P in cultured rat dorsal root ganglion (Skoff et al, 2009) and TNF-α in mouse epidermal cells (Beissert et al, 1998). IL-1α is also a potent inducer of prostaglandins in bovine endometrial endothelial and stromal cells, compared to IL-1β (Tanikawa et al, 2009). This indicates that it would be misleading to interpret the affinity of IL-1 for IL-1 receptors as potency, as strong ligand binding may not always translate to strong signal transduction.

These differences between IL-1α and IL-1β actions could be related to the interaction of IL-1RAcP with the IL-1/IL-1R1 complex. Anti-peptide targeting the immunoglobulin domain III of the IL-1RAcP protein showed that IL-1β-induced IL-6 in 3T3-L1 cells was inhibited by 70%, but that it affected IL-1α-induced responses only marginally (Yoon and Dinarello, 2007). IL-1 interaction with IL-1RAcP could be important in IL-1-induced responses, as this co-receptor is responsible for recruiting downstream molecules in the IL-1 signalling cascade (Huang et al, 1997; Volpe et al, 1997) (see section 1.4.2).

Current literature suggests that these differential effects of IL-1α and IL-1β may only occur in certain cell types. This indicates that there could be cell-specific expression of some components of the IL-1 signalling pathway (different expression of IL-1R1 or IL-1RAcP uncharacterised isoforms) or differential regulation of downstream signalling adaptor molecules recruitment by IL-1α and IL-1β. IL-1α and IL-1β administered by intraperitoneal injection in rats induced comparable effects on body temperature. However, when administered intra-cerebrally, IL-1β was much more potent than IL-1α at inducing fever. In contrast, IL-1α is more effective at reducing social behaviour than IL-1β when administered intra-cerebrally (Anforth et al, 1998). This study suggests that CNS cells are unequally sensitive to IL-1α and IL-1β, as intracerebral but not intraperitoneal injection of IL-1 induced differential effects. Indeed IL-1α and IL-1β have been shown to induce differential actions in mouse mixed glia (Andre et al, 2005b). These data suggest that the bioactivity of IL-1α and IL-1β could be cell type-dependent, tissue-dependent, species-dependent, and/or dependent on the end-point measured.

1.5. Interleukin-1-induced signalling

1.5.1. Mitogen activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) signalling pathways

IL-1R1 is a membrane-bound receptor that has been shown to be recruited to membrane stuctures called lipid rafts upon ligand binding (Oakley et al, 2009; Blanco et al, 2008). Binding of IL-1 to IL-1R1 induced rapid phosphorylation of IL-1R1 (Gallis et al, 1989), followed by the association of IL-1R1 with IL-1RAcP (Cullinan et al, 1998). It is suggested that after the initiation of IL-1β-induced signalling, the ectodomain of IL-1R1 is cleaved by metalloproteases to form soluble IL-1R1 leaving the TIR domain attatched to the lipid rafts to be internalised by endocytosis to form caveolar vesicles (Elzinga et al, 2009) (Figure 1.7). However in a different model of IL-1R1 signalling, IL-1β triggers rapid endocytosis of full-length IL-1R1 (Oakley et al, 2009; Blanco et al, 2008) (Figure 1.8).

The heterodimeric association of the TIR domains of IL-1R1 and IL-1RAcP inside the cell allows recruitment of the adaptor molecule MyD88 (Burns et al, 1998), IL-1 receptor associated kinases (IRAK-1, IRAK-2, and IRAK-4), and tollip (Elzinga et al, 2009). Tollip is important in the trafficking of IL-1R1 to endosomes and lysomes for degradation following activation (Brissoni et al, 2006). TNF receptor associated factor 6 (TRAF6) (Elzinga et al, 2009) and transforming growth factor β-activated kinase (TAK1) is also recruited to the caveolar signalling complex. It is reported that TRAF-6 induces ubiquitination of the IL-1R1 for subsequent proteolysis by γ-secretase (Twomey et al, 2009) that liberate soluble intracellular IL-1R1 fragments contributing to JNK phosphorylation and IL-6 production (Elzinga et al, 2009). TAK1 is important in the phosphorylation of NF-κB-inducing kinase to activate IκB kinase leading to NF-κB activation. IκBα is an inhibitory molecule that is bound to NF-κB in the cytoplasm, holding it in an inactive state. Phosphorylation of the IκBα releases NF-κB to migrate to the nucleus and initiate transcription of pro-inflammatory genes. TAK-1 can also activate the JNK, ERK1/2 and p38 signalling pathways (for reviews see Li and Qin 2005; Subramaniam et al., 2004; O'Neill and Greene, 1998) (Figure 1.9).

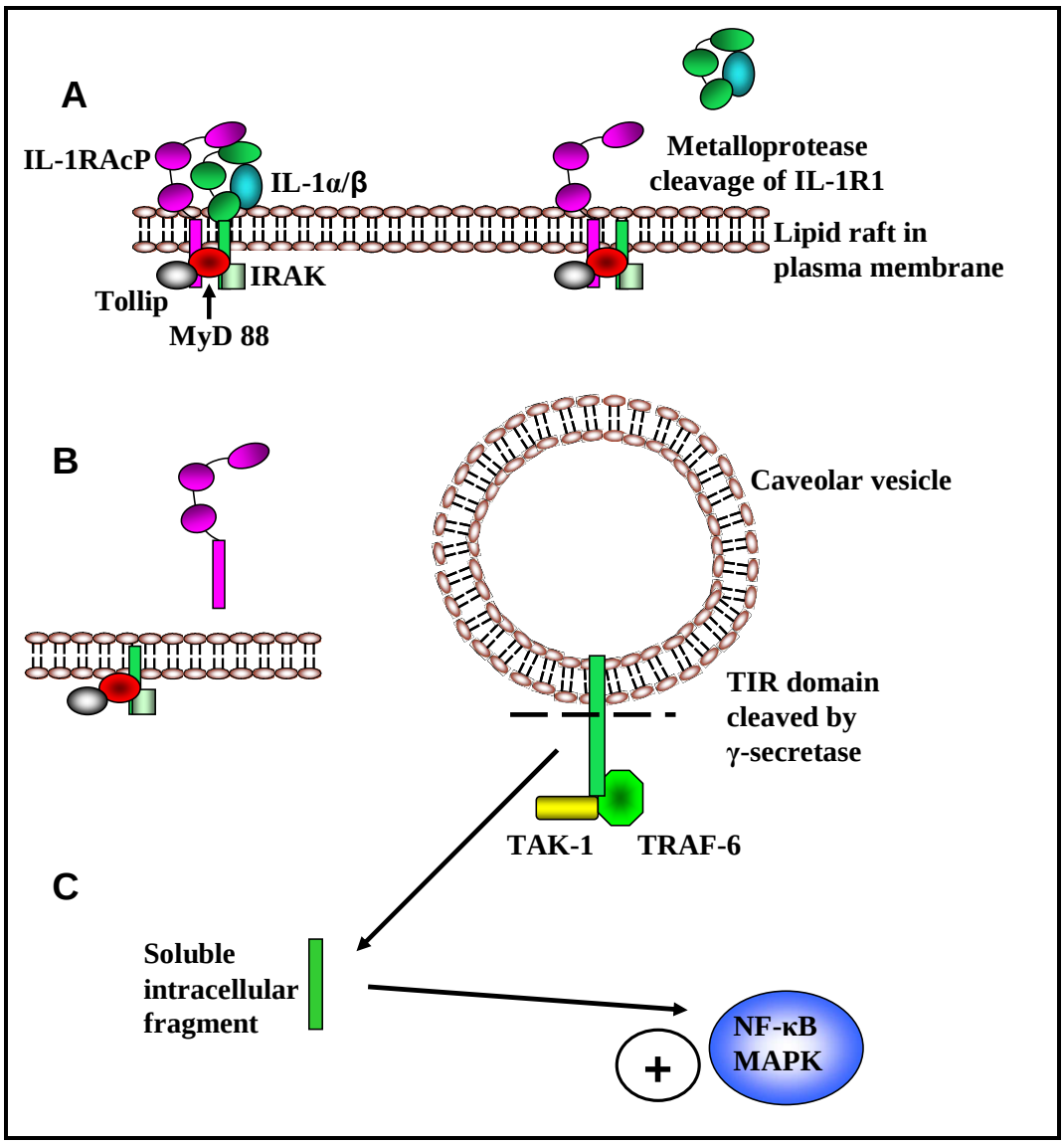


Figure 1.7. Schematic diagram showing endocytosis and proteolysis of IL-1R1

A. IL-1-bound IL-1R1 is translocated to lipid rafts in the plasma membrane and IL-1RAcP is recruited. Dimerisation of IL-1R1 and IL-1RAcP recruits adaptor molecules including MyD88, IRAK and tollip to the signalling complex. IL-1R1 is then cleaved by metalloproteases to liberate soluble IL-1R1. **B.** IL-1RAcP dissociates from the signalling complex and the IL-1R1 TIR domain is taken into the cell by endocytosis by caveolar vesicles. TRAF-6 and TAK-1 is recruited to the TIR domain for ubiquitination of IL-1R1 and subsequent cleavage of the TIR region by γ-secretase. **C.** The generated soluble intracellular fragment then activates MAPK and NK-κB signalling cascades. Modified from Elzinga et al. 2009.

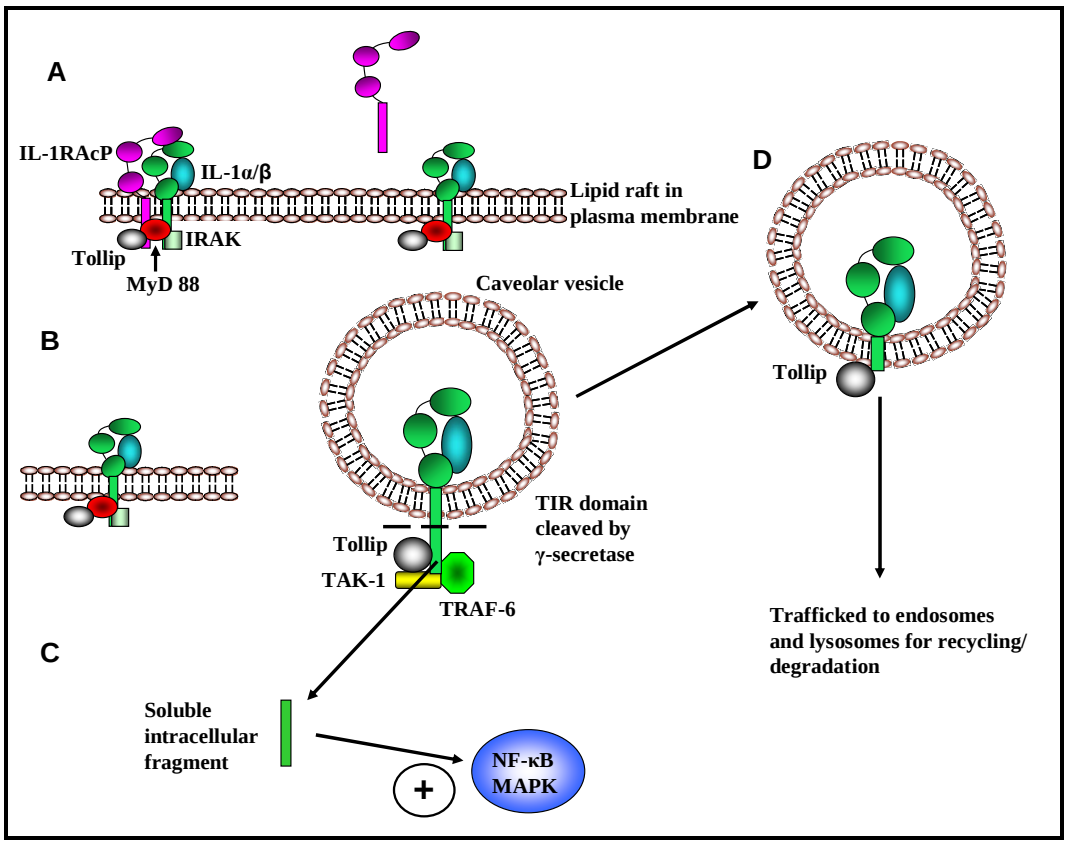


Figure 1.8. Schematic diagram showing endocytosis of IL-1R1 and intracellular cleavage of the TIR domain

A. IL-1-bound IL-1R1 is translocated to lipid rafts in the plasma membrane and IL-1RAcP is recruited. Dimerisation of IL-1R1 and IL-1RAcP recruits adaptor molecules including MyD88, IRAK and tollip to the signalling complex, after which IL-1RAcP dissociates. **B.** Full length IL-1R1 is taken into the cell by endocytosis in caveolar vesicles. TRAF-6 and TAK-1 is recruited IL-1R1 for ubiquitination and subsequent cleavage of the TIR region by γ-secretase. **C.** The generated soluble intracellular fragment then activates MAPK and NK-κB signalling cascades. **D.** IL-1R1 is trafficked to endosomes and lysomes for recycling or degradation. Modified from Elzinga et al. 2009; Brissoni et al. 2006.

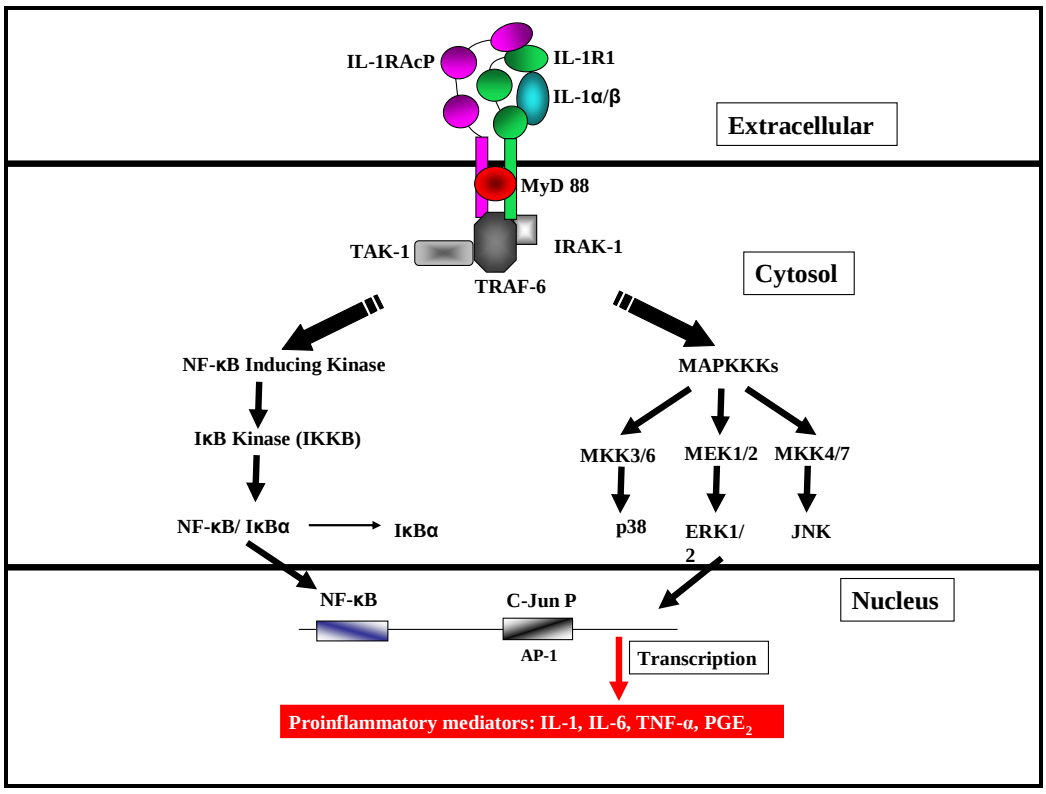


Figure 1.9. IL-1-induced MAPK and NF-κB signalling pathways.

Signalling pathways presented in this diagram are the best characterised pathways for IL-1 in different cell types. Binding of IL-1 to the IL-1R1/IL-1RAcP signalling complex recruits downstream signalling molecules including MyD88, IRAKs, TRAF-6 and TAK-1. TAK-1 phosphorylates NF-κB-inducing kinase, which in turns activates IκB kinase to phosphorylate IκBα and releases NF-κB to migrate into the nucleus to initiate gene transcription. TAK-1 can also activate MAPK kinase kinases (MAPKKK) leading to the activation of MAPK signalling pathways and production of pro-inflammatory mediators.

1.5.2. Sphingomyelinase and Src kinase signalling pathway

The NF-κB/MAPK kinase signalling pathway is insufficient to explain rapid changes in ionic currents induced by IL-1β found in neuronal cells (Diem et al, 2003). However, a second signalling pathway has been postulated, where Src kinase could be a key effector molecule. This signalling pathway is initiated with the activation of the IL-1 signalling complex (IL-1R1/IL-1RAcP) by IL-1. These events trigger the activation of neutral sphingomyelinase (nSMase) (Nalivaeva et al, 2000), leading to the hydrolysis of sphingomyelin to soluble ceramide (Davis et al, 2006). Ceramide then acts as a signalling molecule to phosphorylate a proto-oncogen tyrosine kinase (Src kinase). Activation of Src kinase leads to the activation of several ion channels including Na^+, K^+ and Ca^{++} channels (Tabarean et al, 2006; Desson and Ferguson, 2003; Ferri and Ferguson, 2003; Pita et al, 1999) and phosphorylation of NMDA receptor channels (Tsakiri et al, 2008a; Viviani et al, 2003). This pathway is proposed to account for the rapid response of IL-1β found in febrile responses (Sanchez-Alavez et al, 2006) (Figure 1.10).

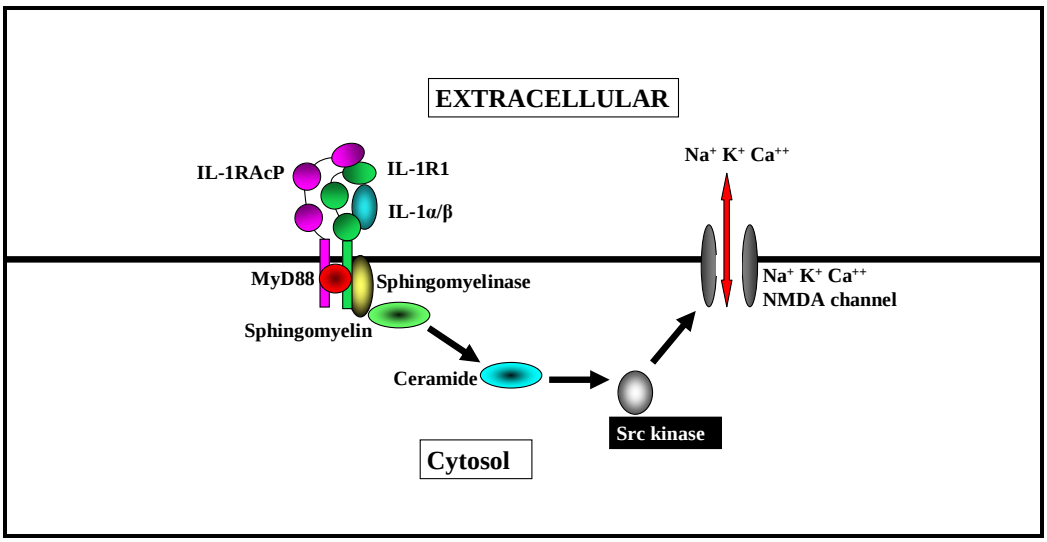


Figure 1.10. IL-1 and the Src kinase pathway.

IL-1 can initiate the Src kinase pathway by activating sphingomyelinase to degrade sphingomyelin to ceramide. Ceramide then phosphorylates Src kinase, which in turn phosphorylates NMDA channels or modulates ionic currents across ion channels. This pathway is believed to be involved in the rapid actions of IL-1 in neurones.

It is unclear how MAPK and Src kinase signalling pathways may contribute to IL-1-induced neuroinflammation. It is known that the IL-1-induced MAPK signalling pathway in glia leads to production of pro-inflammatory mediators (Andre et al, 2005a; Parker et al, 2002; Dunn et al, 2002). However, the role of IL-1-induced MAPK signalling in neurones is poorly understood. Src kinase activation in glial cells has been reported to be induced by cerebral ischaemia (Choi et al, 2005), but the role of IL-1 in Src kinase pathway activation in glial cells is unknown. Conversely, IL-1β has been shown to induce Src kinase activation in neurones, with subsequent IL-6 expression (Tsakiri et al, 2008a). However, the role of IL-1β-induced Src kinase in neuroinflammation is not clear. There are emerging data suggesting that Src kinase activation contributes to the detrimental effects of neuroinflammation (Hou et al, 2007; Lennmyr et al, 2004; Paul et al, 2001). The role of IL-1RAcPb in IL-1 signalling is unknown, and the contribution of IL-1RAcPb to CNS injuries also requires further investigation.

1.6. Interleukin-1 and ischaemic damage

1.6.1. IL-1R1-dependent damage

The contribution of IL-1 to various neurological diseases is too wide to review extensively. For the purpose of this thesis, the role of IL-1 in cerebral ischaemia will be the main focus. There is now extensive evidence indicating the contribution of IL-1 to ischaemic damage in rodents. Transient middle cerebral artery occlusion (MCAo) in mice induces a 323% increase in IL-1β mRNA expression in the cortex after 24 h (Boutin et al, 2001). Increased IL-1β mRNA expression in rats after permanent MCAo has been reported to peak at an earlier time-point of 12 h (Wang et al, 1997; Liu et al, 1993), whilst IL-1β protein is significantly increased in the cerebral cortex of rats after permanent MCAo at various time points (Skifter et al, 2002; Legos et al, 2000; Liu et al, 1993).

The role of IL-1α in cerebral ischaemia is poorly characterised. Cortical IL-1α mRNA expression in mice is unaffected after transient MCAo, but appears to be constitutively expressed (Boutin et al, 2001). This observation is in agreement with a previous study demonstrating that, in a model of permanent MCAo, expression of IL-1α protein does not change significantly compared to

sham-operated animals, but displays a trend towards an increase that reaches maximum level at day 3 post-surgery (Legos et al, 2000). Controversially, IL-1α mRNA expression is markedly increased in the cerebral cortex of mice subjected to hypoxia/ischaemia (Basu et al, 2005), or after LPS treatment of mouse primary microglia (Brough et al, 2002). These observations suggest that the induction of IL-1α expression could be dependent on the type of injury or stimulus.

It is difficult to asses the regional and cellular changes in IL-1 expression after ischaemia, since ischaemia-induced brain damage is dynamic and progressive. The ischaemic core is established soon after insult, however the penumbral region is difficult to determine as this area of damage is constantly evolving. A recent study showed that transient MCAo induced IL-1β protein expression at 1 h after reperfusion in astrocytes and microglia in the core of the ischaemic region, which then spread to astrocytes and microglia in the penumbra by 2 h after reperfusion. At 22 h, IL-1 protein expression in penumbral astrocytes were no longer detected but IL-1 expression in the penumbral microglia remained strong (Amantea et al, 2010).

Ischaemia-induced IL-1RA expression is delayed compared to IL-1β at both the mRNA and protein level. In rats, permanent MCAo induces an increase in IL-1RA mRNA expression that peaks at day 2 and expression is maintained until day 5 after permanent MCAo (Wang et al, 1997). In a different study, significant IL-1RA protein increase was detected at 12 h and peaked at day 5, compared to IL-1β, which peaked at day 3 (Legos et al, 2000).

The induction of IL-1 mRNA and proteins by MCAo is not indicative of its contribution to ischaemia-induced brain damage. The functional assessment of IL-1 in ischaemia is ascertained by the use of transgenic mice as well as IL-1RA. Despite the lack of induction by MCAo, the potential contribution of IL-1α to ischaemia is revealed by the use of IL-1$\alpha^{-/-}$ mice. IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice subjected to transient MCAo have similar brain damage compared to WT mice, whilst mice deficient in both cytokines showed reduced damage by 70%, suggesting a relationship between IL-1α and IL-1β in ischaemia-induced brain damage, and the possibility of compensatory mechanisms being triggered (Boutin et al, 2001). Deleting the *IL-1R1* gene in mice has also been shown to reduce total infarct volume induced by mild hypoxia/ischaemia by 60% (Basu et al, 2005) or

90 % (Lazovic et al, 2005), whereas deleting the IL-1RA gene (*IL-1RN)* in mice increases total infarct volume 3.6-fold following transient MCAo (Pinteaux et al, 2006). Furthermore, over-expression of IL-1RA in mouse brain was shown to be neuroprotective after transient MCAo (Yang et al, 1999) and *icv* injection of anti-rat IL-1RA antiserum into rat brain before permanent MCAo increases total infarct by 71% (Loddick et al, 1997). Additionally, exogenous IL-1RA has also been shown to be neuroprotective after MCAo in rodents (Clark et al, 2007; Stroemer and Rothwell, 1997), with improved functional outcome (Garcia et al, 1995). Importantly, IL-1RA is neuroprotective in rats when administered subcutaneously (Greenhalgh et al, 2010). This indicates that IL-1RA could have therapeutic potential in a clinical setting.

1.6.2. IL-1R1-independent damage

The neuroprotective effects of IL-1RA in ischaemia-induced brain damage indicate that actions of IL-1α and IL-1β in ischaemia are mediated by IL-1R1. However, there is evidence indicating that IL-1β can exacerbate ischaemic damage independently of IL-1R1 signalling (Touzani et al, 2002). In a study on mice, transient MCAo induced almost identical extent of ischaemia-induced damage in both WT and IL-1R1$^{-/-}$ mice, and *icv* injection of IL-1RA markedly reduced the ischaemic brain damage in WT mice but not in IL-1R1$^{-/-}$ mice. Additionally, *icv* injection of IL-1β worsened damage in WT and IL-1R1$^{-/-}$ mice, suggesting that IL-1β can act independently of IL-1R1 to exacerbate injury (Touzani et al, 2002). The authors suggested that there could be additional uncharacterised IL-1 receptors mediating IL-1β action, maybe a low affinity receptor, whose expression or response may be unmasked only in the absence of the high affinity IL-1R1. This hypothesis later gained support from *in vitro* experiments, showing that IL-1β changed the expression of over 400 genes in mice mixed glia cultured from IL-1R1$^{-/-}$ mice (Andre et al, 2005a).

A second hypothesis is that IL-1R2 could associate with an unknown accessory protein to induce signal transduction. It is unclear if IL-1RAcPb could serve as this co-receptor. Supporting this hypothesis, the receptor IL-13Rα$_2$ of the IL-13 system, which was once thought to be a decoy receptor, has been shown to be a functional signalling receptor (Fichtner-Feigl et al, 2006). Interestingly, the

IL-1 decoy receptor IL-1R2 has been suggested to be a critical component of the pro-IL-1α signalling complex in the nucleus (Kawaguchi et al, 2006). Additionally, it has been reported recently that IL-1RAcP could function as a co-receptor for IL-33 signalling (Lingel et al, 2009; Palmer et al, 2008), suggesting that it may posses the capacity to mediate signalling activity independently of IL-1R1. It is unclear which IL-1 receptor or co-receptor is involved in IL-1R1-independent signalling mechanisms, but it has been suggested to be independent of NF-κB and MAPKs pathways (Parker et al, 2002).

1.7. IL-1-induced actions in CNS cells

1.7.1. IL-1 actions on glia

The actions of IL-1 on microglial cells are controversial, as the expression of IL-1R1 on this cell type is unclear. There are reports that IL-1 induces IL-1, IL-6 and TNF whilst others have failed to show any IL-1-induced responses in microglia (Pinteaux et al, 2002; Lee et al, 1993). However, microglia are the main source of IL-1 in the CNS after injury (Lee et al, 1993). It was demonstrated that LPS induces IL-1β synthesis from human fetal migroglial cells in a concentration depedent manner. At their highest concentration of 10000 ng/ml, LPS induced 500pg/ml of IL-1β, but failed to induce the same response in astrocytes. In contrast, IL-1β is a key activator of astrocytes (Herx and Yong, 2001; Lee et al, 1993). Activated astrocytes produce a wide array of cytokines, chemokines and neurotrophic factors (see section 1.2.2 and 1.2.4) as well as cellular proliferation (da Cunha A. et al, 1993). It has been shown that IL-1-induced activation of astrocytes during inflammation can lead to neuronal cell death. Whilst IL-1 is not directly toxic to rat cortical neurones (Carlson et al, 1999), it can induce extensive neuronal death in the presence of astrocytes (Thornton et al, 2006). However, this effect may not always be true for different species. IL-1β failed to induce neuronal death in human foetal neuronal-glial cultures (Chao et al, 1995). The neurotoxic effect of IL-1β mediated by astrocytes is thought to be mediated by MMP9. Pro-MMP9 is released by astrocytes following IL-1β treatment (Thornton et al, 2007). Pro-MMP9 is non-toxic, but treatment with an MMP9 inhibitor reduces IL-1β-induced neurotoxicity by 93%

in vitro (Thornton et al, 2007). This suggests that pro-MMP9 released from astrocytes is cleaved to become active MMP9 before exerting its neurotoxic effects on neurones. IL-1β is also a potent inducer of MMP9 in human foetal neurones and contributes significantly to the brain damage seen in traumatic brain injury (Vecil et al, 2000).

Similarly, IL-1β alone was not toxic to mouse or rats oligodendrocytes (Zhang et al, 2005; Takahashi et al, 2003), but when cultured in the presence of microglial cells or astrocytes, IL-1β induces apoptotic cell death of oligodendrocytes, mediated by glutamate (Takahashi et al, 2003). Astrocyte- and microglial-mediated oligodendrocyte cell death was also demonstrated following LPS treatment (Pang et al, 2000). Oligodendrocytes expresses both IL-1β and IL-1R1 (Deng et al, 2008; Vela et al, 2002), however IL-1-induced responses in oligodendrocytes are not as extensively reported compared to IL-1-induced responses in astrocytes. In rat primary oligodendrocytes, IL-1 activates p38 signalling and inhibits growth factor-induced ERK1/2 phosphorylation as well as inhibiting growth factor-stimulated cellular proliferation (Vela et al, 2002). In oligodendrocyte cell lines, IL-1β treatment enhances the effect of TNF-γ-induced nitric oxide but the mechanism involved was not investigated (Bhat et al, 1999).

IL-1β induces the p38, JNK, ERK1/2 and NF-κB signalling pathways in mixed glial cultures and astrocytes (Parker et al, 2002; Pinteaux et al, 2002; Dunn et al, 2002) which leads to an increase in pro-inflammatory cytokines and neuronal death (Xie et al, 2004). These observations strongly suggest that IL-1β neurotoxicity is mediated by activation of the MAPK signalling cascade (including ERK1/2, JNK, p38 and NF-κB) in glia.

1.7.2. IL-1 actions on neurones

Evidence for the activation of NF-κB/MAPK signalling pathways in neurones by IL-1α or IL-1β is controversial. p38 activation is induced by IL-1β in rat hippocampal neurones (Srinivasan et al, 2004). However, in another study, IL-1β completely failed to activate ERK1/2, JNK or p38 in mouse cortical neurones (Tsakiri et al, 2008a). A further study reported activation of the neuronal-specific JNK-3 in neurones (Kim et al, 2007). This leaves open the possibility that IL-1 induces MAPK signalling in these cells.

IL-1β has been reported to have both hyperpolarising and depolarising effects on neurones (Motoki et al, 2008; Davis et al, 2006; Tabarean et al, 2006; Desson and Ferguson, 2003; Ferri and Ferguson, 2003) depending on the population of neurones being stimulated. The concentration of IL-1β is also an important factor in IL-1-induced responses in neurones. Low concentrations of IL-1 (pM range) inhibit, whilst higher concentrations (nM range) activate neurones in the *locus coerulus* of rats (Figure 1.11). The effects of IL-1RA on IL-1-induced responses on neurones are equally controversial. In one study, IL-1RA blocked IL-1β-induced neuronal hyperpolarisation in murine anterior hypothalamic neurones in culture (Davis et al, 2006), but was ineffective in rat retinal ganglion cells (Diem et al, 2003). In contrast, IL-1RA blocked IL-1β-induced neuronal depolarisation but not hyperpolarisation *in vitro* in rat subfornical organ neurones (Desson and Ferguson, 2003). Whilst both responses were abolished by IL-1RA (Borsody and Weiss, 2002). These inconsistencies suggest that the effects of IL-1RA on IL-1β-induced activity in neurones are not yet fully characterised or that some responses may be IL-1R1-independent.

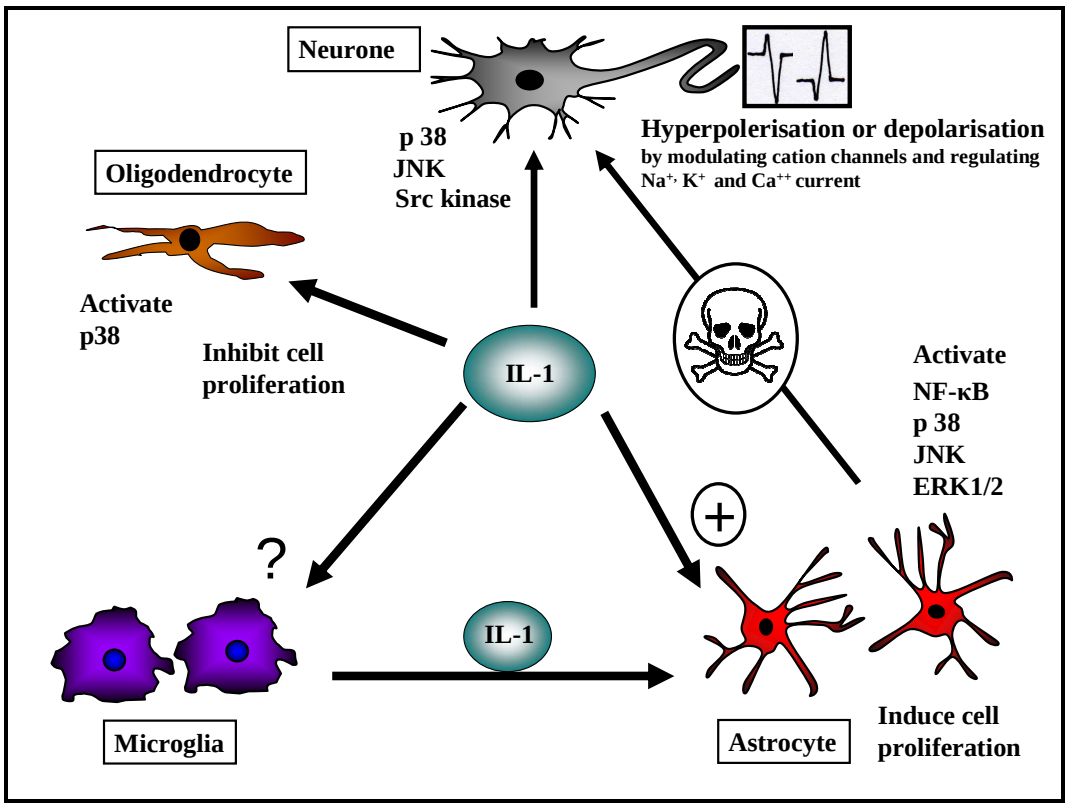


Figure 1.11. The effects of IL-1 on different cell types in the CNS.

The effects of IL-1 on microglial cells are controversial but these cells are recognised as major producers of IL-1. IL-1 is a potent activator of astrocytes resulting in NF-κB, p38, JNK and ERK1/2 activation, astrocyte proliferation and neuronal cell death. IL-1 activates p38 in oligodendrocytes but inhibits cell proliferation. IL-1 is not directly toxic to neurones but affects their electrical function, causing either hyperpolarisation or depolarisation. Abbreviations: NF-κB, Nuclear factor-kappa B; p38, Mitogen activated protein p38; JNK, c-Jun N-terminal kinase; ERK1/2, Extracellular signal-regulated kinase 1/2; Src kinase, Proto-oncogenic tyrosine kinase.

Critically, the signalling pathways mediating rapid responses to IL-1 are only partially characterised. The second signalling pathway involving the activation of nSMase/Src kinase (Davis et al, 2006; Nalivaeva et al, 2000) has been implicated in these fast IL-1-induced actions in neurones. This signalling pathway is activated in glial cells by ischaemia (Choi et al, 2005) but IL-1-induced Src kinase activation in glia has not been reported. The lack of knowledge on IL-1-induced Src kinase signalling in glia suggests that IL-1 may induce cell-specific signalling mechanisms in different cell types (Figure 1.12). Although there is evidence showing that Src kinase activation exacerbates ischaemic damage in rats and mice, and that inhibition of nSMase and Src kinase activity reduces infarct size (Ardizzone et al, 2007; Soeda et al, 2004; Paul et al, 2001), the specific contribution of IL-1-induced neuronal nSMase/Src kinase pathway to neurotoxicity and inflammation is unknown. The effects of nSMase/Src activation on neuronal electrical activity suggest that it could be involved in the normal physiological function of neurones.

It is difficult to assess the importance of each of these signalling pathways in physiological and pathophysiological states. What is clear is that NF-κB/MAPK and Src kinase signalling pathways are present in both glia and neurones, and although IL-1β may to activate cell-specific pathways, it is not clear which signalling pathways are activated in which cell type by IL-1. Whether these pathways are activated simultaneously and working in parallel, or whether they are co-ordinated during the inflammatory response also remains to be elucidated. The high potency of IL-1 and the many regulatory mechanisms involved to limit its activities suggest that these pathways are likely to be activated simultaneously and may act in parallel.

So far the NF-κB/MAPK and Src kinase signalling have only been discussed in the context of IL-1, however these signalling cascades are not exclusive to IL-1 and can be activated by other ligands such as LPS, SB100, glutamate, beta-amyloid and ceramide (Davis et al, 2006; Chaparro-Huerta et al, 2005; Kim et al, 2004; Xie et al, 2004). To investigate IL-1-induced cell-specific NF-κB/MAPK and Src kinase signalling, I have used IL-1RA to block any IL-1 specific signalling.

It is important to identify which signalling pathway is activated in which specific cell type as this may provide specific targets for new pharmacological interventions. It is unclear whether IL-1RAcPb mediates any of these signalling pathways, therefore it is necessary to study the involvement of IL-1RAcPb in both MAPK signalling pathway and Src kinase signalling pathway to understand how it may influence the progression of different pathological conditions of the CNS. Investigating the role of IL-1RAcPb may provide new insights into IL-1 signalling and also assist in the design of novel approaches for treatments of acute and chronic inflammatory diseases of the CNS.

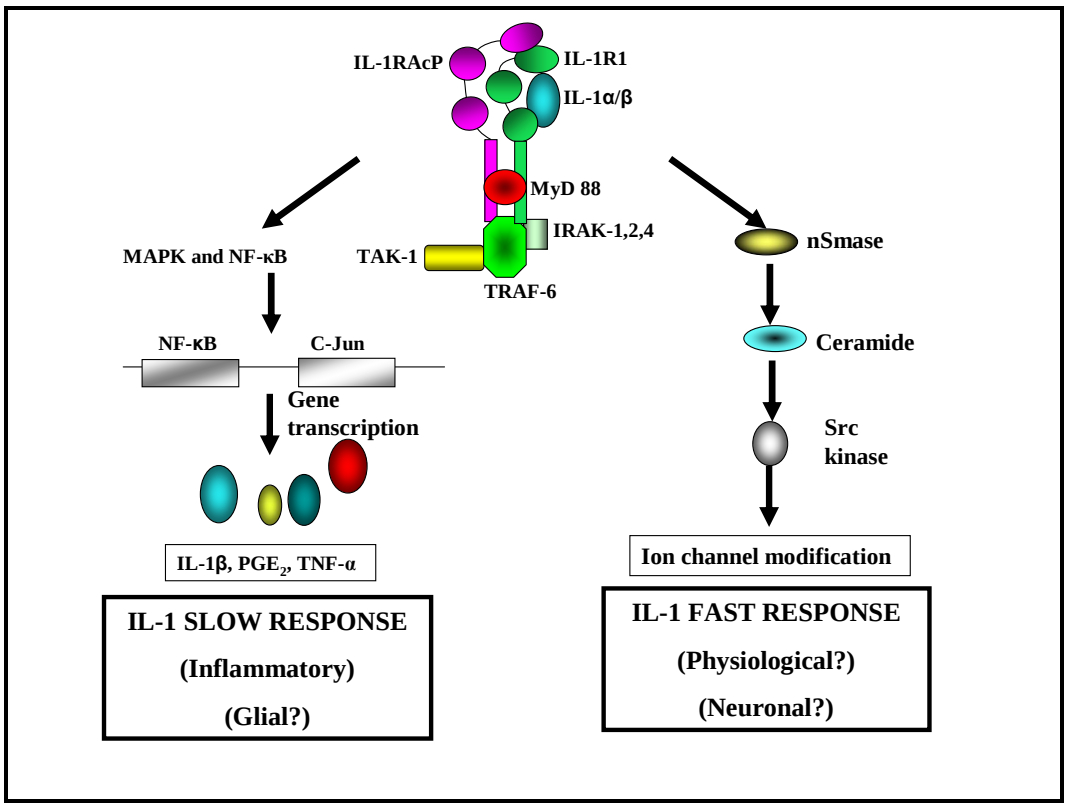


Figure 1.12. IL-1β-induced signalling pathways.

IL-1β-induced NF-κB/MAPK and Src kinase signalling pathways are present in the CNS. However, it is unclear whether the activation of these pathways is cell-specific. The contribution of these NF-κB/MAPK and Src kinase signalling to neuroinflammation is also unclear. Modified from Sanchez-Alavez et al. (2006).

1.8. Summary

Brain damage and neurological deficits associated with acute conditions such as stroke and traumatic brain injury, as well as chronic neurodegenerative disorders such as multiple sclerosis and Alzheimer's disease, are now recognised partly as a result of acute or chronic inflammation of the CNS. It is clear that glial cell activation and the subsequent release of pro-inflammatory cytokines are two key steps involved in the initiation and propagation of an inflammatory response in the CNS. IL-1 is a key cytokine contributing to the in inflammatory response, but it is unclear how IL-1 mediates the pathogenesis of many CNS diseases, acutely or chronically. IL-1 can induce an array of changes in a multitude of cell types, but the mechanism underlying IL-1-induced actions in different cell types remains to be fully characterised.

IL-1α and IL-1β are the best characterised agonists of the IL-1 system. These agonists signal by binding to the IL-1R1/IL-1RAcP signalling complex to initiate a signalling cascade. However, IL-1α and IL-1β display differential effects, indicating that IL-1α and IL-1β are capable of recruiting different signalling elements downstream of the IL-1R1/IL-1RAcP complex. IL-1 signalling pathways encompass NF-κB/MAPK activation and Src kinase activation. The IL-1 signalling system is indeed very complex. However, the identification of a new splice variant of IL-1RAcP called IL-1RAcPb may resolve some of the unanswered questions regarding IL-1 actions. This isoform may be specifically expressed in neurones, but it is unclear if this receptor could mediate the neuronal actions of IL-1. Further investigations are needed to elucidate the role of IL-1RAcPb in IL-1-induced MAPK signalling and IL-1-induced Src kinase activation. Clarifying the issues surrounding the function of IL-1RAcPb may provide novel targets for the therapeutic treatment of acute and chronic inflammatory diseases of the CNS.

1.9. Objectives

It is well known that IL-1R1 and IL-1RAcP are crucial components in the IL-1-induced signalling. However, the involvement of IL-1RAcPb in IL-1-induced MAPK signalling cascades or in the Src kinase pathway is completely unknown. The differential actions of IL-1α and IL-1β in neurones and glia are poorly understood. How IL-1RAcPb may contribute to these differences remains to be elucidated. The overall aim of this project was to investigate the role of IL-1RAcPb in IL-1α- and IL-1β-induced actions in neurones and glia. This was investigated by *in vitro* studies on the actions of IL-1α and IL-1β in glial and neuronal primary cell cultures from both WT and IL-1RAcPb$^{-/-}$ mice. There were three main objectives in this project:

1. To determine the role of IL-1RAcPb in IL-1-induced actions in neuronal and glial cultures at a signalling and functional level. The composition of primary neuronal and glial cultures were first characterised and IL-1-induced ERK1/2, p38 and Src kinase activation was assessed to determine the specific role for IL-1RAcPb. As IL-6 is induced by IL-1 in both neurones and glia, IL-6 was used as the end-point for the functional study of IL-1-induced actions in these cell types.
2. To investigate the possible differential actions of IL-1α and IL-1β in neurones and glia and how IL-1RAcPb may modulate these responses. For this study. neuronal and glial (prepared from WT and IL-1RAcPb$^{-/-}$ mice) cultures were treated with identical concentrations (in biological activity, International Units) of recombinant rat IL-1α or IL-1β in the presence or absence of IL-1RA.
3. To determine the contribution of MAPK and Src kinase signalling in IL-1-induced IL-6 release in neurones by pharmacological intervention. The ERK1/2 inhibitor UO126, MAPK p38 inhibitor SB 230508 and Src inhibitor PP2 (4 -amino-5- (4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]-pyrimidine) were used to investigate the effects of specific inhibition of these signalling pathways on neuronal IL-6 production.

2. Materials and methods

2.1. Introduction

IL-1 can induce numerous responses in many different cell types. In order to study the specific effects of IL-1 on neurones and glia, these cells were isolated and their responses to IL-1 were investigated *in vitro*.

2.2. Animals

This study used WT C57/BL6 mice, IL-1RAcP$^{-/-}$ and IL-1RAPb$^{-/-}$ all backcrossed onto C57/BL6JOlaHsd background for 10-12 generations. In IL-1RAcPb$^{-/-}$mice, only the exon 12b were deleted, however, in IL-1RAcP$^{-/-}$ mice, both exon 5 and 6 were disrupted in the *IL-1RAcP* gene therefore the function of IL-1RAcPb is also disrupted in IL-1RAcP$^{-/-}$ mice (Figure 1.6). Mice deficient in IL-1RAcP and IL-1RAcPb displayed normal growth and development, and fertility. There were no gross abnormalities in either mutant strains (Smith et al, 2009; Cullinan et al, 1998). In IL-1RAcPb$^{-/-}$ and WT mice, staining for myelin (with Weil) and neuronal cell bodies (using Thionine Nissl) showed that neurones were morphologically similar in both strains (Smith et al, 2009).

WT mice were supplied by Harlan Olac, UK, IL-1RAcP$^{-/-}$ and IL-1RAPb$^{-/-}$ were obtained from Dr John Sims (Amgen, USA). Animals were kept at 21ºC ± 1ºC, 55% ± 10% humidity and maintained in a 12 hour light-dark cycle with free access to food and water. All animals used in this study were sacrificed according to the Animals Scientific Procedures Act (UK) 1996.

2.3. Primary cortical neuronal cell cultures

Primary neuronal cell cultures were prepared from the brains of mouse embryos at 15-16 days of gestation. The pregnant mouse was euthanised by exposure to an increasing concentration of carbon dioxide (CO_2) and dislocation of the neck. Cell culture was performed under sterile conditions in an airflow hood. Briefly, the embryos were removed from their embryonic sacs and then decapitated, and the head pinned onto a wax petri dish. A sagittal incision was made from the brain stem to the nose. The skin was then peeled and pinned back, the hindbrain was discarded and cortices were lifted out and submerged in pre-

warmed (37ºC) Starve medium (Table 7.1, Appendix I). Cortices were then incubated in dissociation medium (Table 7.2, Appendix I) on a shaker for 30 min at 60 rpm and 37ºC (Stuart orbital incubator S150) to gently dissociate the cortices. The active components of the dissociation are trypsin and deoxyribonuclease. Trypsin dissociates the cortices by proteolysis, however during the process necrotic cell death is inevitable and consequently, the release of DNA into the medium. The presence of deoxynuclease digests these free-floating DNA to prevent cell clumping. The dissociation medium was then aspirated, and cortices were incubated in 2 ml ice-cold foetal calf serum (FCS) for 5 sec at room temperature to block trypsin activity. Cortices were then washed four times with a wash medium (Table 7.3, Appendix I) and re-suspended in a seeding medium (Table 7.4, Appendix I). Cells were then fully dissociated by triturating through different size stripettes, and passed through a nylon mesh of 80 μm pore size (John Stainer & Co, UK). Cells were then counted and seeded at a density of 6 x 10^5 cells/ml. Immediately before seeding, 20 μM of 5'-fluoro-2-deoxyuridine (FUDR) was added to inhibit the growth of glial cells. Cells were then seeded onto poly-D-lysine (PDL)-coated (20 μg/ml) 12-well tissue culture plates. The cultures were grown in an incubator at 37ºC, in a humidified atmosphere (5% CO_2, 95% air). After 4-5 days in vitro (DIV), the seeding medium was completely removed and replaced with a maintenance medium (Table 7.5, Appendix I), and 10 μM FUDR was added. At 8 DIV, half the medium was removed and replaced with fresh maintenance medium. The culture was used for experiments at 12 DIV.

2.4. Primary mixed glial cell cultures

Primary mixed glial cultures were prepared from the brains of 1-4 day old C57/BL6 mice. Cortices were removed in the same way as was done for neuronal cell cultures (see section 2.3) Cortices were collected and incubated in pre-warmed (37ºC) glial culture medium (Table 7.6, Appendix I). The meninges were removed by rolling the cortices and hind brain onto sterile filter papers. The brain tissue was then mechanically dissociated by triturating through different size stripettes. The cells were then centrifuged at 600g (Boeco, Germany, C-28) for 5 min, and the medium aspirated. Cells were then re-suspended into fresh glial

culture medium (9 ml per pup). The cultures were grown in an incubator at 37ºC, in a humidified atmosphere (5% CO_2, 95% air). After 4 DIV a complete medium change was carried out. Cells were maintained until reaching confluence with complete medium change every 4 DIV. Cells were used at approximately 16-21 DIV.

2.5. Immunocytochemistry

Neuronal cultures are highly susceptible to glial contamination therefore it is critical to determine the extent of this contamination. The level of glial contamination in pure neuronal cultures was assessed by immunocytochemistry (ICC). It is essential to this project to experiment on genetically modified animals; however, it is unclear how genetic modification may affect neuronal and glial growth and differentiation and, consequently, the composition of primary cultures. It was necessary to characterise neuronal and glial cultures from both WT and knock-out (KO) animals.

Cells used for ICC were cultured on PDL-coated glass coverslips. Cells were fixed with 4% paraformaldehyde and 4% sucrose for 15 min at room temperature, and permeabilised using 0.1% triton X-100 (prepared in phosphate buffered saline (PBS)) at room temperature for 10 min. Non-specific binding sites were blocked by incubating cultures in 10% FCS (diluted in PBS) at room temperature for 60 min. Cells were then washed six times with PBS and incubated with primary antibody as follows; Microglia and O_2A progenitor cells were detected by biotinylated tomato lectin (diluted 1: 200 in 1% normal donkey serum in PBS, room temperature, 60 min) and anti-GT3 ganglioside antibody (A_2B_5, diluted 1:10 in 1% normal donkey serum in PBS) at room temperature for 60 min, respectively. Neurones and astrocytes were detected using anti-neuronal nuclei antibody (NeuN, diluted 1:500 in 1% normal donkey serum in PBS) at room temperature for 60 min, and Cy3-conjugated anti-GFAP (diluted 1:500 in 1% normal donkey serum in PBS) at room temperature for 60 min respectively. After incubation, cells were washed six times with PBS

Cells incubated with NeuN and A_2B_5 antibodies were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary anti-mouse antibody. (diluted 1:500 in 1% normal donkey serum in PBS) at room temperature for 60

min (Table 7.7, Appendix I). All cells were mounted onto glass slides using DAPI (4',6-diamidino-2-phenylindole)-containing mounting medium (Vectashield, UK). Immunostaining was visualised using a fluorescent microscope and images were captured by a snapshot widefield confocal microscope (BX 51 Olympus fluorescence, Olympus, UK) and processed by the Meta Vue software (Nikon, UK). The proportion of each cell type was calculated by counting cells stained with specific markers and comparing it against the DAPI stained nuclei. This was repeated three times for each cell type.

2.6. Reverse-transcriptase polymerase chain reaction.

Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to assess any differences in gene expression between cultures. It was essential to determine the expression of genes within these cultures to ensure that expression of *IL-1RAcP* and *IL-RAcPb* genes was absent in cells from KO animals.

2.6.1. Ribonucleic acid extraction

Total ribonucleic acid (RNA) isolation was prepared from WT and KO neurones and glia using Trizol Reagent and purified by several steps of washing and centrifugation. Culture medium was aspirated and the cells lysed with 500 µl Trizol Reagent per 1 ml of medium. To increase yield of RNA extraction, the bottom of each well was carefully scraped before being transferred to RNAse-free Eppendorf tubes. Two hundred microlitres of chloroform was then added to each tube, and the tubes were shaken vigorously for 30 sec. Immediately after, the samples were centrifuged at 12000 g for 15 min at 4°C (Boeco, Germany, M-240R). The aqueous phase containing RNAs was carefully collected and transferred into fresh RNAse free tubes containing 250 µl of isopropanol and tubes were stored overnight at -20°C (to allow RNA precipitation). The following day, the RNAs were collected by centrifugation at 12000 g for 15 min. The supernatants were discarded and the RNA pellets were washed with 1 ml 70% ethanol, followed by a brief vortexing. After washing, the RNAs were centrifuged at 7500 g for 10 min at 4°C, and then washed in 1 ml 100% ethanol, after which RNA pellets were left to air dry at room temperature for 10 to15 min. RNAs were

finally dissolved into 20 µl RNA-free water. The RNA concentration and purity was quantified by measuring the light absorbency of the RNA between 260 nm and 280 nm using a nanodrop 1000 spectrophotometer (Thermo Scientific) and the software ND-1000 V3.7.0. RNA has its maximum absorbance at 260 nm and any detection at 280 nm would indicate contaimination. Any residues of DNA, proteins or derivatives from tissue/cells or lysis buffer are detected as contaminants. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the RNA preparation. An A260/A280 range of 1.8-2.0 was accepted as an indication of a pure RNA preparation.

2.6.2. Reverse transcriptase

For the reverse transcriptase step, it is required to have a minimum of 1 µg of RNA in a total volume of 10 µl. The RNA was first purified by treatment with DNase 1 amplification grade to digest any single or double stranded DNAs. This was done following the manufacturer's instructions. Briefly, 1 µg of RNA was incubated with a DNase mix (1 µl of 10 x DNase 1 reaction buffer, 1 µl DNase amplification grade 1 U/µl, and topped up to 10 µl with RNAse-free water) for 15 min at room temperature, after which the reaction was stopped by addition of 1 µl of 25 mM ethylenediaminetetraacetic acid (EDTA). RNAs were then incubated with 1µl of oligo dT at 70°C for 10 min, after which 9 µl of master mix (4 µl 5 x buffer, 2 µl dithiothreitol [DTT], 1 µl 2'-deoxynucleoside 5' triphosphate [dNTPs], 1 µl Moloney murine leukaemia virus reverse transcriptase [MMLV] and 1 µl recombinant ribonuclease inhibitor [RNase out]) was added to the sample and heated for 1 h at 37°C. The reaction was stopped by heating at 70°C for 15 min.

2.6.3. Polymerase chain reaction

For the polymerase chain reaction (PCR) step, 18 µl of PCR master mix (10 µl Biomix red, 0.4 µl forward primers [10 pM], 0.4 µl reverse primers [10 pM] and 8.2 µl pure water) was added to 2 µl of prepared cDNA sample (100 ng of cDNA) to make a total reaction volume of 20 µl necessary for PCR. Samples were loaded in duplicate and the housekeeping gene glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) was used as loading control. The primer sequence and PCR programmes used for IL-1RAcP, IL-1RAcPb and GAPDH mRNA amplification are listed in Table 7.9 (Appendix I) and Table 7.10 (Appendix I). PCR was performed using the Multi gene 2 thermal cycler (Labnet International Inc.).

The cDNA was visualised on a 1.5 % 1 x TAE (tris-acetic buffer and ethylene-diamine-tetra-acetic acid) agorose gel by electrophoresis with 5 µl ethidium bromide per 100 ml agarose. To each 20 µl of sample, 5 µl of 5 x loading buffer was added and 20 µl of sample was loaded into each well. The gel was electrophorised at 100 V for 60 min and the image captured using a camera (ImageQuant 350, GE healthcare U.K).

2.7. Cytokines

Cultured neurones and glia were treated with recombinant rat IL-1α, IL-1β and recombinant human IL-1 receptor antagonist (IL-1RA), all from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). The bioactivity of IL-1α and IL-1β is 127 IU/µg and 317 IU/µg respectively; both with an endotoxin content of 0.3 fg/10 ng. IL-1α and IL-1β were used at bioactivity range of 0.03-30 IU/ml, diluted in vehicle (0.9 % saline, 0.1% w/v low-endotoxine bovine serum albumin [BSA]). IL-1RA was used at a concentration that was 1000 times greater than that of IL-1α and IL-1β (in vehicle) to ensure that all IL-1 activity is effectively blocked.

2.8. Cell treatments

To investigate the effect of IL-1 on IL-6 expression in neurones and glia, neuronal and glial cultures were treated with vehicle, IL-1α or IL-1β (0.03–30 IU/ml) in the presence or absence of IL-1RA (5 min pre-treatment). The effects of the Src kinase inhibitor PP2 (Calbiochem, UK), ERK1/2 inhibitor UO126 (Calbiochem, UK) and p38 inhibitor SB203580 (Calbiochem, UK) on IL-6 expression were also studied in neurones. Cells were pre-incubated with the inhibitors (20 µM) for 15 min prior to the incubation with IL-1 (3 IU/ml). This concentration of UO126 and SB203580 is within the range routinely used by

different research groups on different CNS cells. Previous reports have used UO126 at 10 μM - 30 μM (Summers et al, 2010; Parker et al, 2002; Van Wagoner et al, 2000) and SB203580 at 10 μM - 50μM (Binshtok et al, 2008; Yan et al, 2007; Moolwaney and Igwe, 2005; Kim et al, 2004; Xie et al, 2004). PP2 was previously used at 10 μM (Tsakiri et al, 2008c; Sanchez-Alavez et al, 2006; Sanna et al, 2000), however, for consistency PP2 was used at 20 μM for this study, two times the concentration of what has been used. Cells were incubated with IL-1 for 24 h, after which medium was collected and cells lysed using 110 μl lysis buffer (

Table 7.11, Appendix I). Immediately before the addition of the lysis buffer to the cells, 100x protease inhibitor (Calbiochem, UK) and the protease inhibitor phenyl-methansulfonyl-fluoride (PMSF) were added to the lysis buffer, both at 10 µl/ml. Lysates were collected and samples were analysed by sandwich enzyme-linked immunosorbent assay (ELISA).

The effect of IL-1 on ERK1/2, p38 and Src kinase phosphorylation in neurones and glia was studied by treating neuronal and glial cultures with vehicle, IL-1α or IL-1β (0.03-30 IU/ml) in the presence or absence of IL-1RA for 15 min and 30 min respectively. The medium were aspirated and cells lysed using lysis buffer or ERK1/2 lysis buffer (Table 7.14, Appendix I). Lysates were collected and analysed by ELISA or Western blot. The effects of PP2, UO126 and SB203580 on their respective signalling cascades were also tested in neurones by pre-incubating the cells with the inhibitors (20 µM) for 15 min prior to incubation with IL-1 (3 IU/ml). The medium were aspirated and cells lysed with 110 µl of lysis buffer. Each well was carefully scraped with a pipette. Two times sample buffer (Table 7.12, Appendix I) was added (v/v) to each well, and lysates were collected and heated at 95^{o}C for 15 - 20 min, then the samples were analysed by Western blot.

2.9. Western Blot

Twenty microlitres of each sample was loaded and resolved (at 150 V) through a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel alongside precision blue molecular marker (Biorad, UK). Proteins were then transferred onto nitrocellulose membranes (Amersham, UK) by semi-dry transfer at 15 V for 60 min. Non-specific binding sites were blocked by incubation in blocking buffer (10% fat-free dry milk in 0.1% Tween-PBS) for 60 min. Membranes were then washed six times with 0.1% Tween-PBS, and incubated overnight at 4ºC with the following primary antibodies: total or phosphorylated ERK1/2 (diluted 1:1000 in 1% BSA in Tween-PBS), p38 (diluted 1:100 in 1% BSA in Tween-PBS), Src kinase (diluted 1:500 in 1% BSA in Tween-PBS), and MK2 (1:100 in 1% BSA in Tween-PBS) (all primary antibodies used for Western blotting were from New England Biolabs, UK). The membranes were then washed six times with Tween-PBS and incubated with a secondary horseradish peroxidase (HRP)-conjugated

anti-rabbit antibody (DAKO, Denmark) (diluted 1:500 in 10% milk in Tween-PBS) for 60 min at room temperature. Membranes that were probed with MK2 were also incubated with HRP-conjugated anti β-actin antibody. The membranes were washed again six times with Tween-PBS and incubated with chemiluminescent substrate (Amersham, UK). Detection of the secondary antibody was done by exposing the membrane to a camera (ImageQuant 350, GE healthcare U.K). Data were analysed semi-quantitatively by ImageQuant TL 7.0 image analysis software.

2.10. ELISA

IL-6 synthesis and release from neuronal and glial cells, and ERK1/2 activation in glial cells, were measured by ELISA (R&D Systems, UK). The basic steps involved for each of these ELISAs were the same. The initial step involved coating of 96-well microtitre plates (Nunc-immuno, Maxisorp, Denmark) with a capture antibody at the concentration recommended by the manufacturer. The plates were sealed with a sheet of parafilm and incubated overnight at room temperature. The following day, the plates were washed four times with ELISA wash buffer (0.05 % Tween- PBS) and blot dried. The plates were then incubated with 1% BSA in PBS for 1 h at room temperature to block non-specific binding sites. The plates were then washed and dried as described previously, and the samples and standards loaded (50 µl/well).

Samples used for IL-6 ELISA were loaded neat, whilst samples used for ERK1/2 ELISA were diluted 1:6 in diluent 8 (Table 7.14, Appendix I) before loading. The standards for both ELISAs were prepared according to manufacturer's instructions and samples and standards were loaded in duplicates. Blank wells were filled with lysis buffer, or the appropriate medium.

The plates were then incubated overnight at 4°C, after which they were washed four times with 0.05 % Tween-PBS and incubated with a biotinylated antibody (50 µl/well). The plates were incubated for 2 h at room temperature and washed four times as before. The plates were then incubated with HRP-conjugated streptavidin (diluted 1:200 in 1 % BSA in PBS) for 20 min at room temperature. The plates were then washed as described previously, and the substrate (Substrate Reagent A containing hydrogen peroxide and Substrate

Reagent B containing 3,3',5,5' tetramethylbenzidine, added v/v) (BD Biosciences, UK) was added and incubated at room temperature for another 20 min. The reaction was stopped by adding 26 µl of 1 M H_2SO_4. The optical density of each well was measured at 450 nm using a spectrocolorimeter (Synergy HT multi-detection microplate reader, Biotek instruments, UK). The amount of IL-6 produced and the level of ERK1/2 activation was calculated using the software Graphpad prism 5.0. The blanks were subtracted from the optical readings of all the samples and standards. A standard curve was generated and the quantity of IL-6 and phosphorylated ERK1/2 was calculated from the curve. The detection limit for IL-6 ELISA in neuronal lysates and supernatant was 10 pg/ml, and 11 pg/ml for glial supernatant. ERK1/2 data are expressed as fold increase compared to vehicle-treated cells as there was large variation in the basal expression of ERK1/2 in glial cells.

2.11. Statistical analysis.

Data are expressed as a mean ± standard deviation of at least three independent experiments. All data were analysed using Graphpad prism 5.0. All data were analysed using parametric tests as there is not an equivelant non-parametric test to the Two-way ANOVA. Comparisons between groups of data with one variable were analysed by one-way analysis of variance (ANOVA) followed by a Tukey multiple comparison test. When comparing data with two variables, two-way ANOVA and Bonferroni post hoc were used. Level of significance was accepted at $P<0.05$.

3. The role of IL-1RAcPb in IL-1-induced IL-6 expression in neurones and glia

3.1. Introduction

IL-6 is a cytokine which is expressed in a wide variety of cells. The ubiquitous nature of IL-6 is important for its role in cell growth and differentiation (for review see Tamm, 1989). In the CNS, IL-6 is expressed by the same cell types that express IL-1 (neurones, microglia and astrocytes) (for reviews see Van Wagoner and Benveniste, 1999; Gadient and Otten, 1997). IL-1β is a powerful inducer of IL-6 synthesis in glial cells (Andre et al, 2005b; Parker et al, 2002; Molina-Holgado et al, 2000b; Lee et al, 1993) and neurones (Tsakiri et al, 2008c), indicating that, in the CNS, IL-6 could be a mediator of IL-1 actions. The overall aim of this chapter was to determine the role of IL-1RAcPb in IL-1-induced actions in neurones and glia, one of which is the induction of IL-6 production (Tsakiri et al, 2008c; Parker et al, 2002). We investigated the role of IL-1RAcPb in IL-1-induced IL-6 expression using primary neuronal and glial cultures prepared from WT and IL-1RAcPb$^{-/-}$ mice. Genetically modified animals have become a powerful tool in science. However, the use of such models may have some limitations. Altering the genetic composition of an organism may lead to unexpected phenotype or compensatory mechanisms. Thus it is critical to characterise the effects of the altered gene expression. IL-1RAcPb is reported to be primarily expressed in neurones (Smith et al, 2009) but this observation needs to be confirmed. It is unclear if *IL-1RAcPb* gene deletion disrupts the expression of *IL-1RAcP* which could lead to a misinterpretation of IL-1RAcPb function.

The contribution of IL-1 to CNS diseases has been reviewed extensively, but the contribution of IL-6 in CNS disorders is controversial. Like IL-1, IL-6 expression is low in the healthy brain, but is dramatically increased during CNS disorders and central inflammation (for reviews see Juttler et al, 2002; Van Wagoner and Benveniste, 1999). However, the precise role of IL-6 in neuroinflammation is unclear. Specifically, the role of IL-1RAcPb in the production of IL-6 is not known. IL-1RAcPb has been shown to down-regulate the expression of some genes (such as the activating transcription factor 3 gene (ATF3), CCAAT/enhancer-binding protein delta gene (CEBPD) and ceruloplasmin gene (CP) – a copper binding glycoprotein) in primary neurones (Smith et al, 2009) and mediates NF-κB activation when over-expressed in HEK

cell lines (Lu et al, 2008), but its role in IL-1-induced IL-6 expression in neuronal and glial cells remains to be determined.

3.2. Aims

The aim of the first part of this study was to investigate the role of IL-1RAcPb in the cellular composition of neuronal and glial cell cultures, and to study the functional role of IL-1RAcPb on IL-1-induced IL-6. This aim is composed of two objectives:

1. To investigate the role of IL-1RAcPb in IL-1-induced IL-6 production in neurones and glial cultures.
2. To investigate the differential effects of IL-1α and IL-1β on IL-6 production in neurones and glial cultures.

3.3. Materials and methods

To characterise the cellular composition of neuronal and glial cultures, cells were stained by immunocytochemistry using specific markers for neurones (NeuN), astrocytes (GFAP), microglia (tomato lectin) and O_2A progenitor cells (A_2B_5) (see section 2.5 for full methods).

To investigate the functional role of IL-1RAcPb in IL-1-induced IL-6 production, WT and IL-1RAcPb$^{-/-}$ neurones and glial cultures were treated with vehicle, increasing concentrations of IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 24 h, in the presence or absence of IL-1RA. The concentration of IL-1 used for co-incubation of cells with IL-1RA was the optimal concentration for IL-1-induced IL-6, as reported previously for neurones (0.3 IU/ml) (Tsakiri et al, 2008a) and glial cells (3 IU/ml) (Parker et al, 2002). Conditioned medium and cell lysates were collected an assayed for IL-6 levels using a mouse-specific IL-6 sandwich ELISA (see sections 2.8 and 2.10 for full methods).

3.4. Results

3.4.1. Characterisation of neuronal cultures

Immunostaining showed that the cell somas of our cultured neurones were clustered together with long processes connecting each cluster. The neuronal cultures used in this project were composed of 98 ± 2 % neurones with 2 ± 2 % glial contamination. Glial cells found in neuronal cultures include the type 2 astrocytes and microglia. The type 2 astrocytes were large and fibrous and the spread of one astrocyte could stretch across many neuronal cell bodies (Figure 3.1A). The microglia found in neuronal cultures assumed their semi-activated phenotype typical of cultured microglia (Figure 3.1B). The microglial population in neuronal cultures was less than 1 %. The cellular compositions of WT and IL-1RAcPb$^{-/-}$ cultures were similar with no obvious differences.

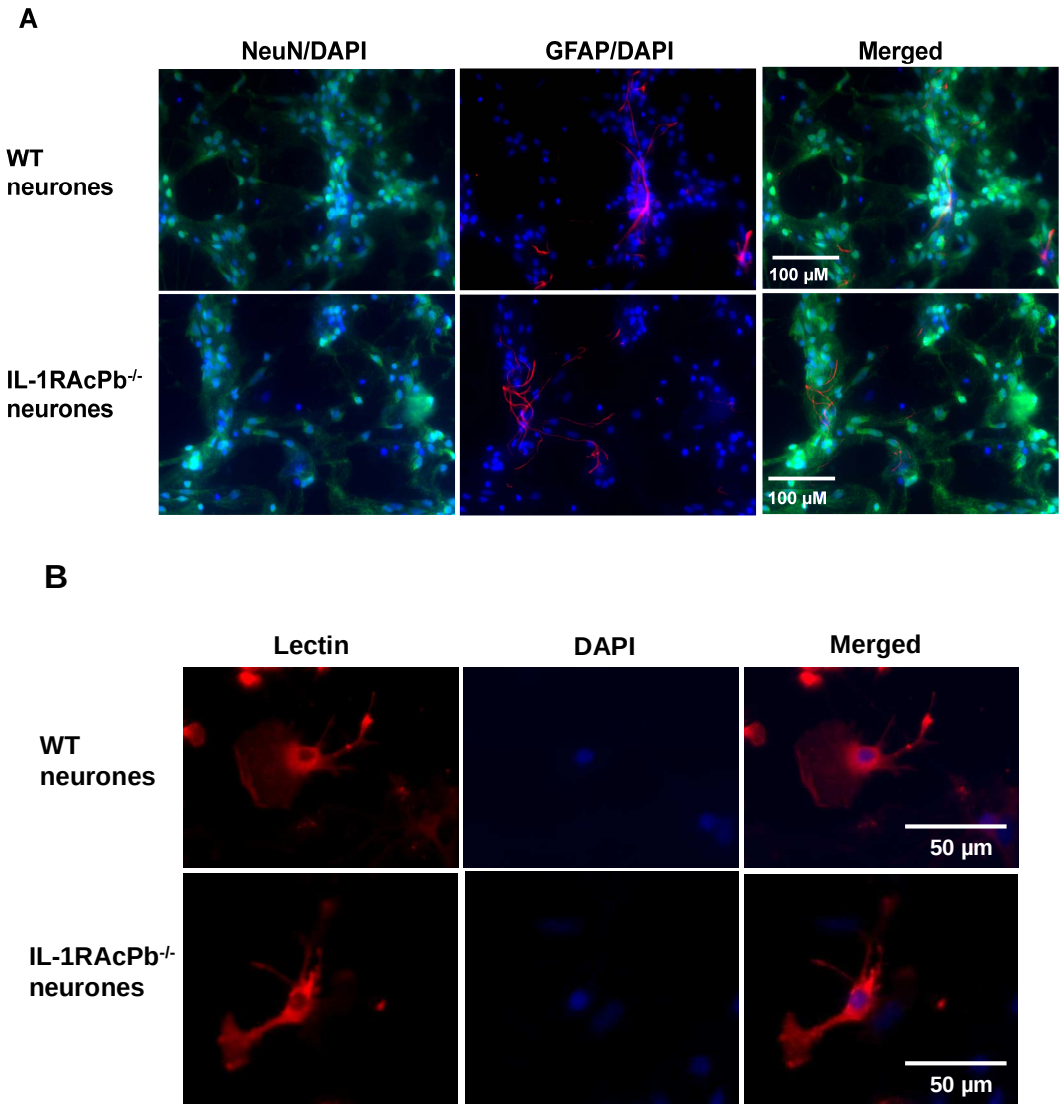


Figure 3.1. The cellular composition of WT and IL-1RAcPb$^{-/-}$ neuronal primary cultures.

Pure neuronal cultures from WT and IL-1RAcPb$^{-/-}$ mice at 12 days *in vitro* were double immunostained with NeuN, GFAP antibodies or tomato lectin, and were mounted using DAPI containing mounting medium. **A**. WT and IL-1RAcPb$^{-/-}$ neurones were identified as NeuN-positive cells (98 ± 2 % of the culture population) and astrocytes were identified by GFAP-positive cells (1-2 ± 2 % of the culture population). **B**. Single staining with tomato lectin showed that neuronal cultures from WT and IL-1RAcPb$^{-/-}$ mice also contained a small amount of microglial cells (less than 1 % of the culture population).

3.4.2. Characterisation of glial cultures

In primary mixed glial cultures (Figure 3.2A), astrocytes dominated the culture composition (82 ± 3 %). Astrocyte cell bodies were irregular in shape but were packed together to form a monolayer of cells at the bottom of the culture plate when the cells were confluent. The microglia grew on top of this monolayer. Microglial cells in culture were rounded and amoeboid shaped, indicating that they were in a partially activated state (Figure 3.2B). Microglial cells formed approximately 18 ± 3 % of the culture population. No O_2A progenitor cells were detected. The distribution of astrocytes and microglia in WT and IL-1RAcPb$^{-/-}$ cultures were similar, indicating that deleting the IL-1RAcPb gene had no effect on the composition of primary mixed glial cultures.

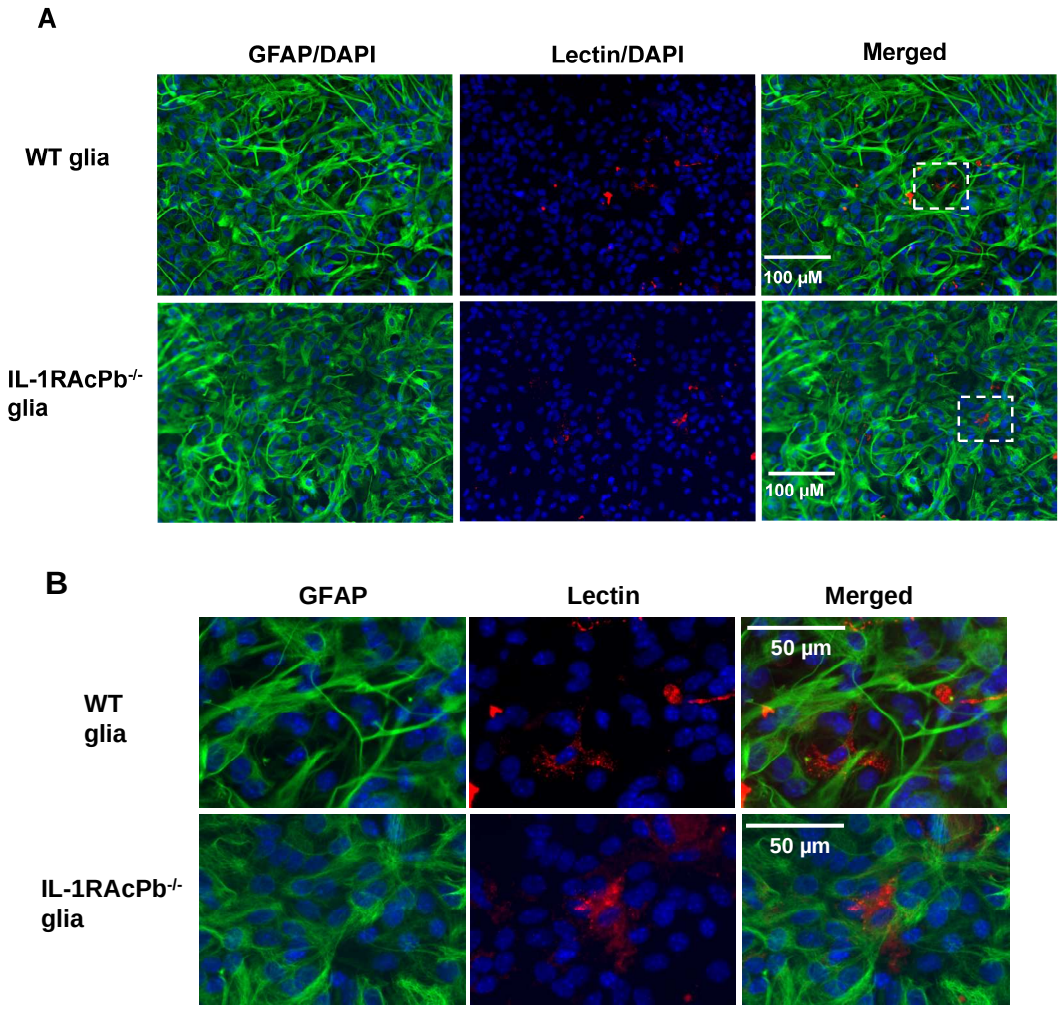


Figure 3.2. Cellular composition of WT and IL-1RAcPb$^{-/-}$ primary glial cultures

A. Mixed glial cultures were double immunostained with tomato lectin and GFAP antibody and mounted using DAPI containing mounting medium. Microglial cells were identified as lectin-positive cells (18 ± 3 % of the culture population) and astrocytes as GFAP-positive cells (82 ± 3 % of the culture population). **B.** Higher magnification of images shown in **A**.

RT-PCR showed that IL-1RAcPb mRNA was expressed strongly in WT neurones and detected faintly in WT glia (Figure 3.3A). IL-1RAcPb mRNA expression was absent in IL-1RAcPb$^{-/-}$ neurones and glia. The house keeping gene GAPDH was used as a loading control. Quantification of IL-1RAcP mRNA expression in WT and IL-1RAcPb$^{-/-}$ neurones and glia showed that the level of IL-1RAcP mRNA expression in WT and IL-1RAcPb$^{-/-}$ glia was 98 % identical and 100 % identical in neurones (Figure 3.3B), indicating that *IL-1RAcPb* deletion did not affect IL-1RAcP mRNA expression in IL-1RAcPb$^{-/-}$ cells.

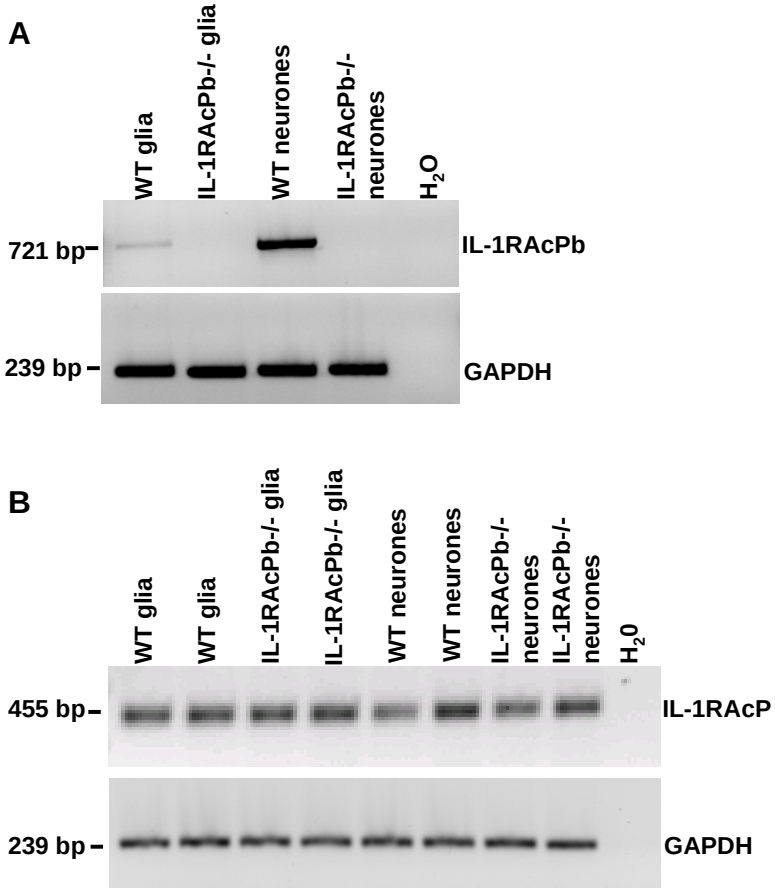


Figure 3.3. Expression of IL-1RAcPb and IL-1RAcP mRNAs in neurones and glia

WT and IL-1RAcPb$^{-/-}$ mouse cortical neuronal and glial cultures were analysed by RT-PCR for expression of IL-1RAcP, IL-1RAcPb and GAPDH mRNAs. **A.** IL-1RAcPb and GAPDH mRNA expression in neurones and glia. **B.** IL-1RAcP and GAPDH mRNA expression in neurones and glia.

3.4.3. IL-1-induced IL-6 expression in neurones

In WT neuronal cell lysates, IL-1α induced a minimum of 2-fold and a maximum of 6.5-fold increase in IL-6 expression compared to vehicle-treated cultures. IL-1-induced IL-6 expression in six independent cultures, but the magnitude was variable and statistical analysis revealed that only the highest concentration of IL-1α (30 IU/ml) induced a significant increase in IL-6 synthesis (46 ± 34 pg/ml) compared to vehicle treatment (10 ± 4 pg/ml) (Figure 3.4A). IL-1RA reduced IL-1α (0.3 IU/ml)-induced IL-6, however, as the level of IL-6 induced by IL-1α at 0.3 IU was non-significant, the inhibitory effect of IL-1RA at this concentration was also non-significant.

In contrast, IL-1β added at 0.3 IU/ml, 3 IU/ml or 30 IU/ml significantly induced IL-6 synthesis in neuronal cell lysates with a mean IL-6 expression of 32 ± 13 pg/ml, 35 ± 15 pg/ml and 34 ± 20 pg/ml respectively (Figure 3.4B). At the lowest concentration (0.03 IU/ml), IL-1β induced 25 ± 11 pg/ml of IL-6 expression but this was non-significant compared to vehicle treatment (10 ± 4 pg/ml). IL-1RA (1μg/ml) significantly inhibited IL-1β (0.3 IU/ml)-induced IL-6 expression. IL-1β-induced IL-6 in the presence of IL-1RA (1μg/ml), or vehicle treatment were at the detection limit level of the assay (10 pg/ml). IL-1RA alone had no effect on IL-6 induction in WT or IL-1RAcPb$^{-/-}$ neurones (Figure 7.1, Appendix II)

To quantify the differential effect of IL-1α and IL-1β on IL-6 expression in neurones, the level of IL-6 induced by these two cytokines was compared directly. In WT neuronal cell lysates, IL-1α and IL-1β induced IL-6 with similar potency. There was no significant difference between IL-1α- and IL-1β-induced IL-6 for any of the concentrations of IL-1 tested (Figure 3.5).

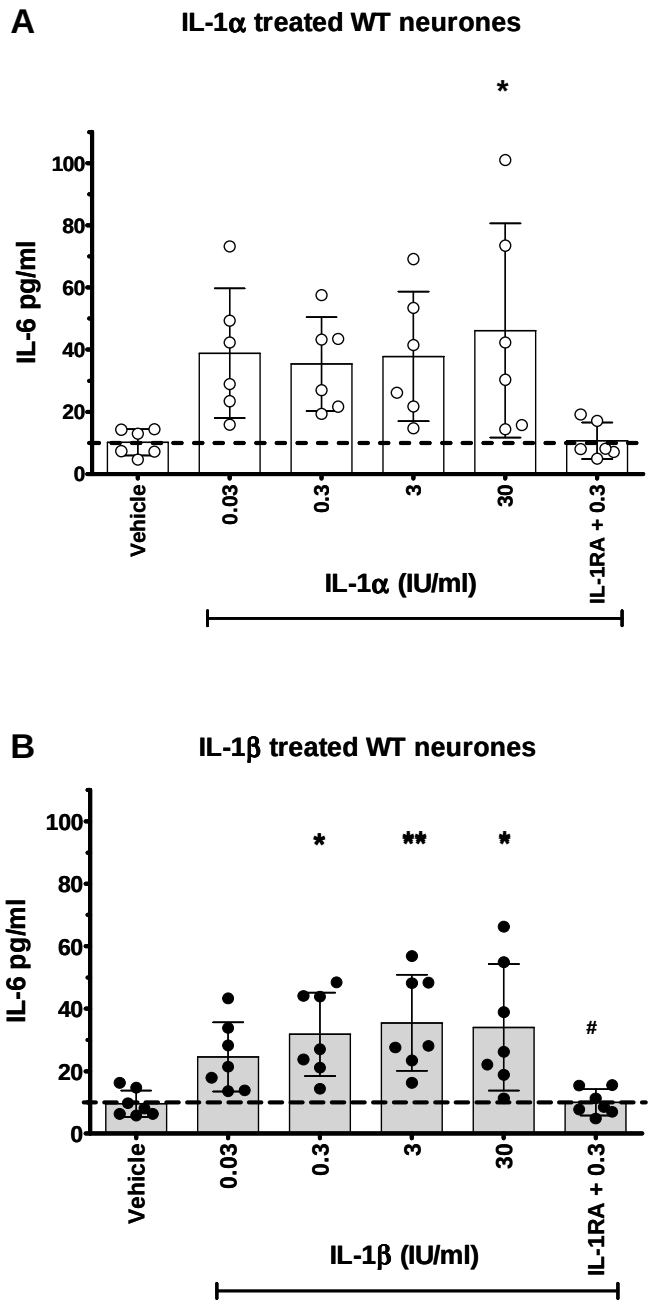


Figure 3.4. IL-1α- and IL-1β-induced IL-6 expression in WT neuronal cell lysates

WT neurones were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 24 h in the presence or absence of IL-1RA (1 μg/ml). Lysates were collected and analysed for IL-6 by ELISA. **A.** IL-1α induced significant levels of IL-6 only at 30 IU/ml: n=6. **B.** IL-1β significantly induced IL-6 at 0.3-30 IU/ml but at not the lowest concentration of 0.03 IU/ml: n=7. IL-1RA co-incubated with IL-1β (0.3 IU/ml) abolished IL-1β-induced IL-6 expression. One-way ANOVA and Tukey's multiple comparison tests.* $P< 0.05$, ** $P< 0.01$ IL-1 vs. vehicle. # $P< 0.05$ IL-1β (0.3 IU/ml) vs. IL-1RA co-incubation. Dashed line indicates the detection limit (10 pg/ml).

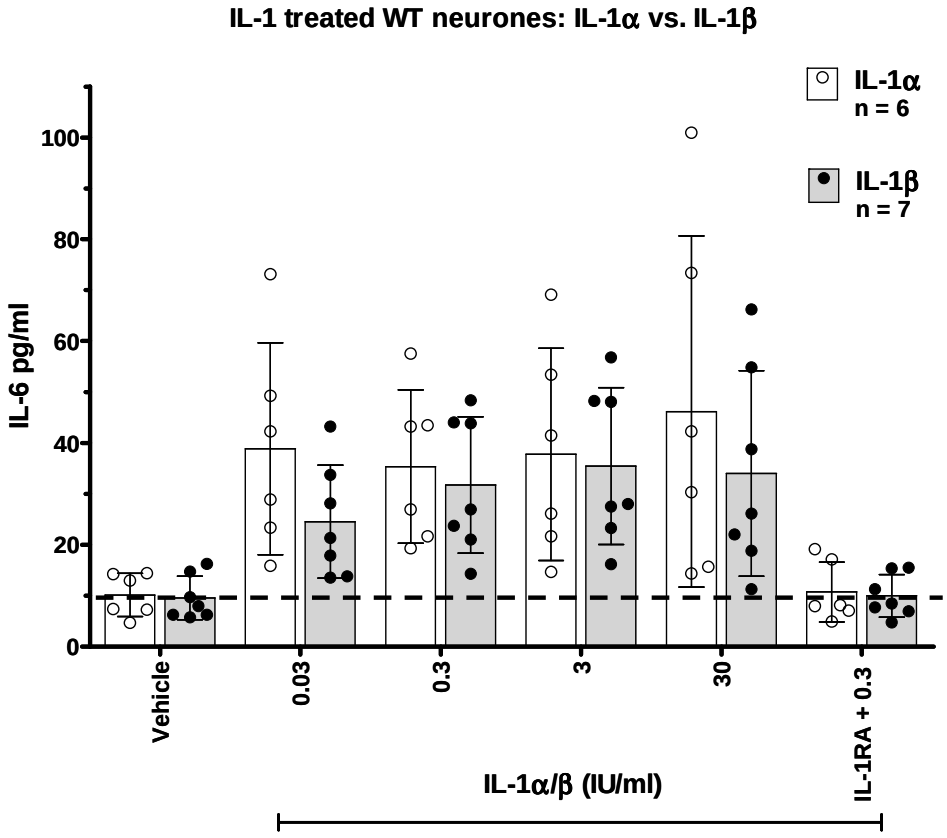


Figure 3.5. Comparison of IL-α- and IL-1β-induced IL-6 expression in WT neuronal cell lysates.

Comparison of the level of IL-6 induced by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), or IL-1RA (1 μg/ml) co-incubation showed that IL-1α and IL-1β induced similar levels of IL-6 expression in WT neurones. No significant difference between IL-1α- and IL-β-induced IL-6 was found for any concentrations of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (10 pg/ml).

To investigate the possible role of IL-1RAcPb in IL-1α- and IL-1β-induced IL-6, IL-1RAcPb$^{-/-}$ neurones were treated with IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) in the presence or absence of IL-1RA (1 μg/ml), and IL-6 expression was measured in cell lysates and culture supernatants.

In cell lysates of IL-1RAcPb$^{-/-}$ neurones, IL-1α (0.03-30 IU/ml) induced significant levels of IL-6 compared to vehicle treatment (Figure 3.6A). At 0.03 IU/ml, 0.3 IU/ml, 3 IU/ml, and 30 IU/ml, IL-1α induced 27 ± 7 pg/ml, 30 ± 16 pg/ml, 29 ± 12 pg/ml and 27 ± 8 pg/ml of IL-6 respectively. The lowest concentration of IL-1α (0.03 IU/ml) elicited maximum expression of IL-6. IL-1RA significantly blocked IL-1α (0.3 IU/ml)-induced IL-6. There was no significant difference in the levels of IL-6 expressed in the lysates of cells treated with increasing concentrations of IL-1α.

In IL-1RAcPb$^{-/-}$ neurones, IL-6 induction was significantly induced by 3 IU/ml of IL-1β (24 ± 9 pg/ml) and 30 IU/ml (25 ± 10 pg/ml) but not at the lower concentrations of 0.03 IU/ml (19 ± 11 pg/ml) or 0.3 IU/ml (20 ± 10 pg/ml) (Figure 3.6B). There was no significant difference in the potency of IL-1α and IL-1β at inducing IL-6 in IL-1RAcPb$^{-/-}$ neurones (Figure 3.7).

To assess the role of IL-1RAcPb in IL-1α and IL-1β activity, IL-1α induced IL-6 synthesis in WT and IL-1RAcPb$^{-/-}$ neurones was compared directly (Figure 3.8). The same comparison was repeated for IL-1β (Figure 3.9). There was no significant difference in IL-1-induced IL-6 expression in WT compared to IL-1RAcPb$^{-/-}$ neurones. IL-1α induced IL-6 expression with equal potency in WT and IL-1RAcPb$^{-/-}$ neurones. This was also the case for IL-1β-induced IL-6 in WT and IL-1RAcPb$^{-/-}$ neurones.

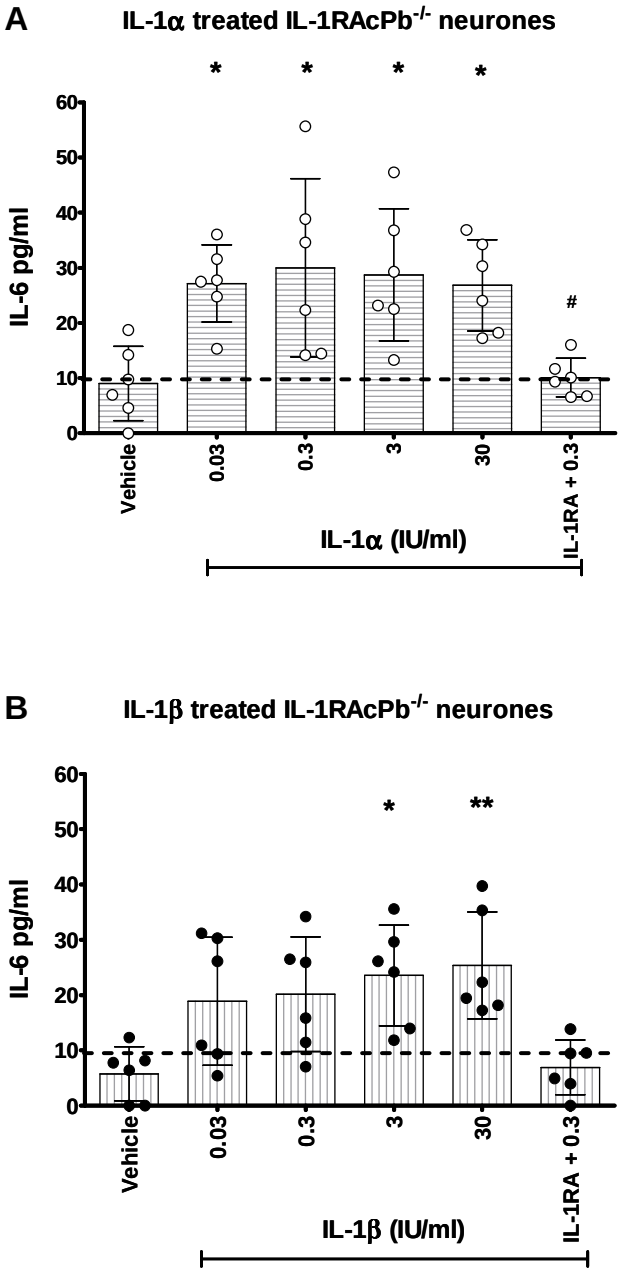


Figure 3.6. IL-1α- and IL-1β-induced IL-6 expression in IL-1RAcPb$^{-/-}$ neuronal cell lysates

IL-1RAcPb$^{-/-}$ neurones were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 24 h in the presence or absence of IL-1RA (1 μg/ml). Lysates were collected and analysed for IL-6 by ELISA. IL-1α (0.03-30 IU/ml: n=6) (**A**) and IL-1β (3 and 30 IU/ml: n=6) (**B**) induced significant levels of IL-6 expression but at 0.03 and 0.3 IU/ml, IL-1β-induced IL-6 was non-significant compared to vehicle. IL-1RA (1μg/ml) co-incubation abolished IL-1 (0.3 IU/ml)-induced IL-6 expression and vehicle induced minimal levels of IL-6. One-way ANOVA and Tukey's multiple comparison tests. * P< 0.05, ** P< 0.01 IL-1 vs. vehicle. # P< 0.05 IL-1α (0.3 IU/ml) vs. IL-1RA co-incubation. Dashed line indicates the detection limit (10 pg/ml).

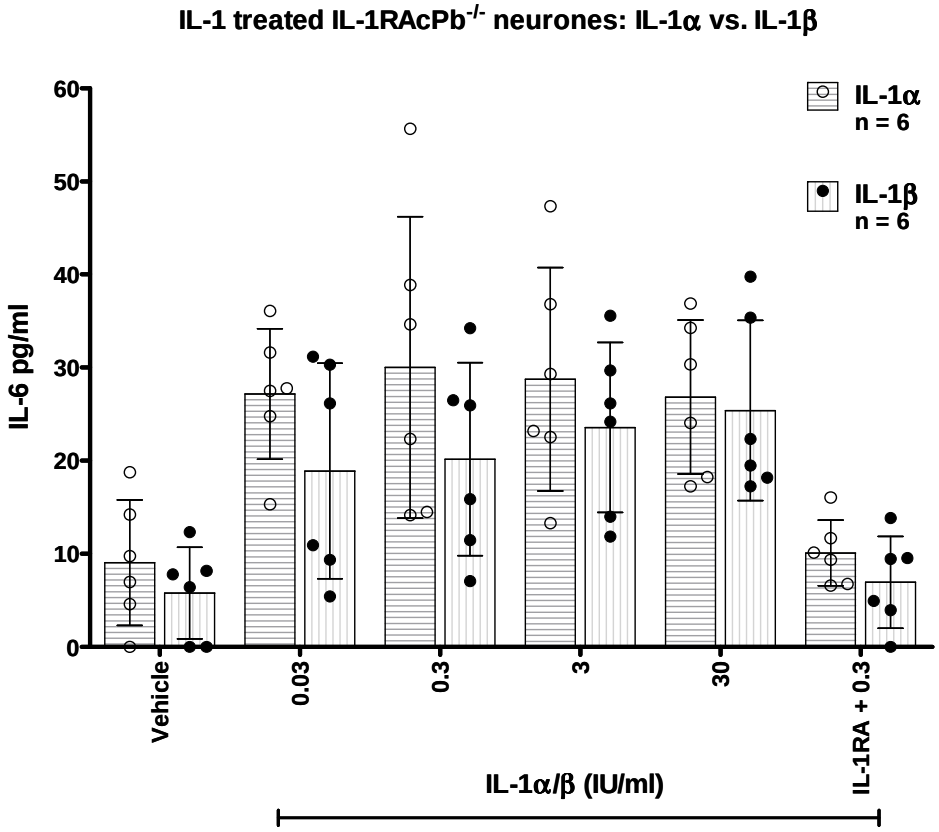


Figure 3.7. Comparison of IL-α- and IL-1β-induced IL-6 expression in IL-1RAcPb$^{-/-}$ neuronal cell lysates.

Comparison of the level of IL-6 induced by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), or IL-1RA (1 μg/ml) co-incubation showed that IL-1α and IL-1β induced similar levels of IL-6 expression in IL-1RAcPb$^{-/-}$ neurones. No significant difference between IL-1α- and IL-β-induced IL-6 was found for any concentration of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (10 pg/ml).

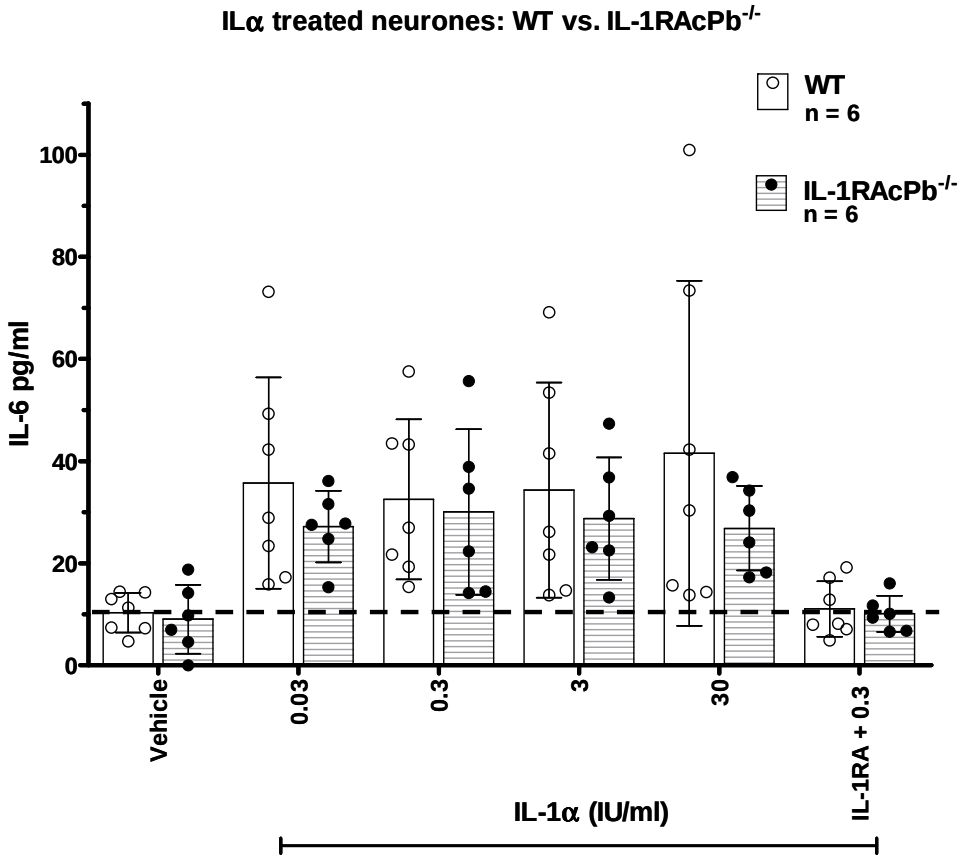


Figure 3.8. Comparison of IL-α-induced IL-6 expression in WT and IL-1RAcPb$^{-/-}$ neuronal cell lysates.

Comparison of the level of IL-6 induced by vehicle, IL-1α (0.03-30 IU/ml) or IL-1RA (1 μg/ml) co-incubation showed that IL-1α induced similar levels of IL-6 expression in WT and IL-1RAcPb$^{-/-}$ neurones. No significant difference was found between WT and IL-1RAcPb$^{-/-}$ neurones in IL-1α-induced IL-6 for any concentration of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (10 pg/ml).

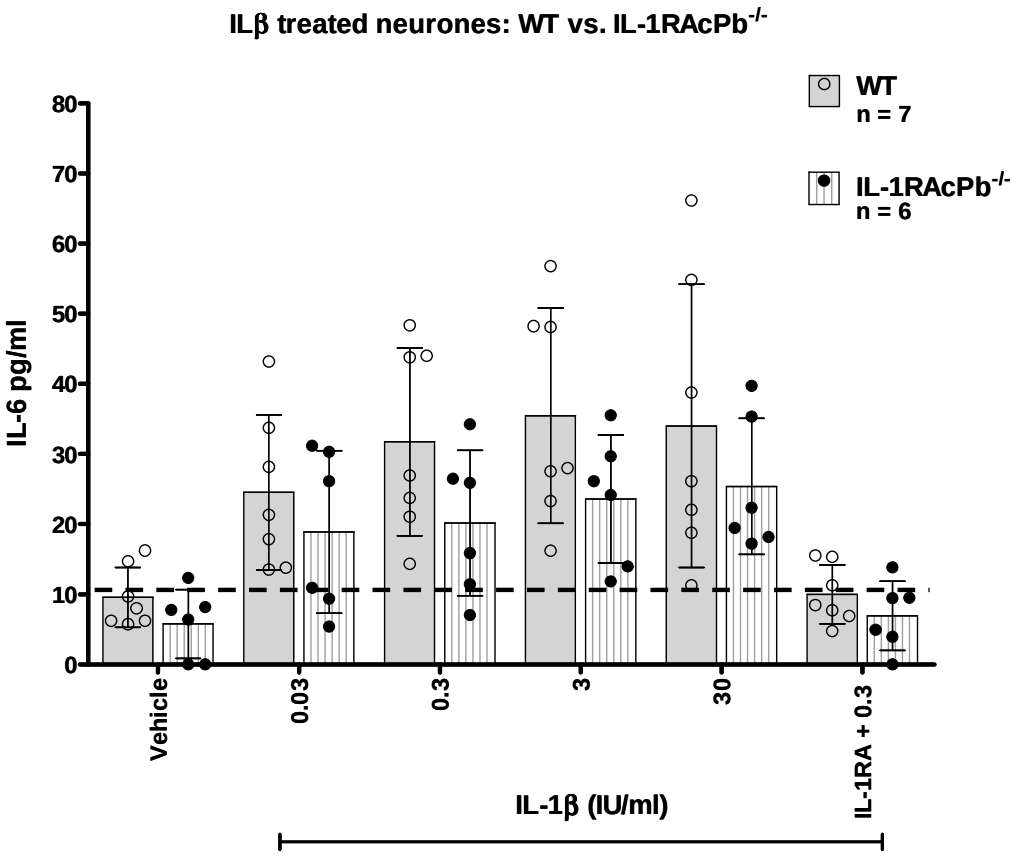


Figure 3.9. Comparison of IL-β-induced IL-6 expression in WT and IL-1RAcPb$^{-/-}$ neuronal cell lysates.

Comparison of the level of IL-6 induced by vehicle, IL-1β (0.03-30 IU/ml) or IL-1RA (1 μg/ml) co-incubation showed that IL-1β induced similar levels of IL-6 expression in WT and IL-1RAcPb$^{-/-}$ neurones. No significant difference was found between WT and IL-1RAcPb$^{-/-}$ neurones in IL-1β-induced IL-6 for any concentration of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (10 pg/ml).

3.4.4. IL-1-induced IL-6 release from neurones

In WT neurones, vehicle did not induce IL-6 release. In contrast IL-1α (0.03-30 IU/ml) strongly and significantly induced IL-6 release compared to vehicle treated cells (Figure 3.10A). The mean level of IL-6 release induced by 0.03 IU/ml, 0.3 IU/ml, 3 IU/ml and 30 IU/ml were 90 ± 36 pg/ml, 94 ± 27 pg/ml, 97 ± 42 pg/ml and 96 ± 36 pg/ml respectively. These data show that IL-1α-induced IL-6 release was not dependent over the concentrations tested. IL-1α (0.3 IU/ml)-induced IL-6 release was blocked in the presence of IL-1RA.

IL-1β also significantly induced IL-6 release at 0.3 IU/ml, 3 IU/ml and 30 IU/ml with a mean IL-6 release of 111 ± 53 pg/ml, 120 ± 60 pg/ml and 130 ± 89 pg/ml respectively (Figure 3.10B). At 0.03 IU/ml, IL-1β induced a mean of 90 ± 50 pg/ml of IL-6 release, but this was not significantly different to vehicle-treated cells. IL-1RA (1 μg/ml) significantly inhibited IL-1β (0.3 IU/ml)-induced IL-6 release. The expression of IL-6 induced by 0.3 IU/ml IL-1β in the presence of IL-1RA (1μg/ml), and vehicle-induced IL-6 release were below the detection limit.

Comparing the effects of IL-1α- and IL-1β-induced IL-6 release in WT neurones showed that IL-1α and IL-1β induced IL-6 release with similar potency for all of the concentrations tested (Figure 3.11).

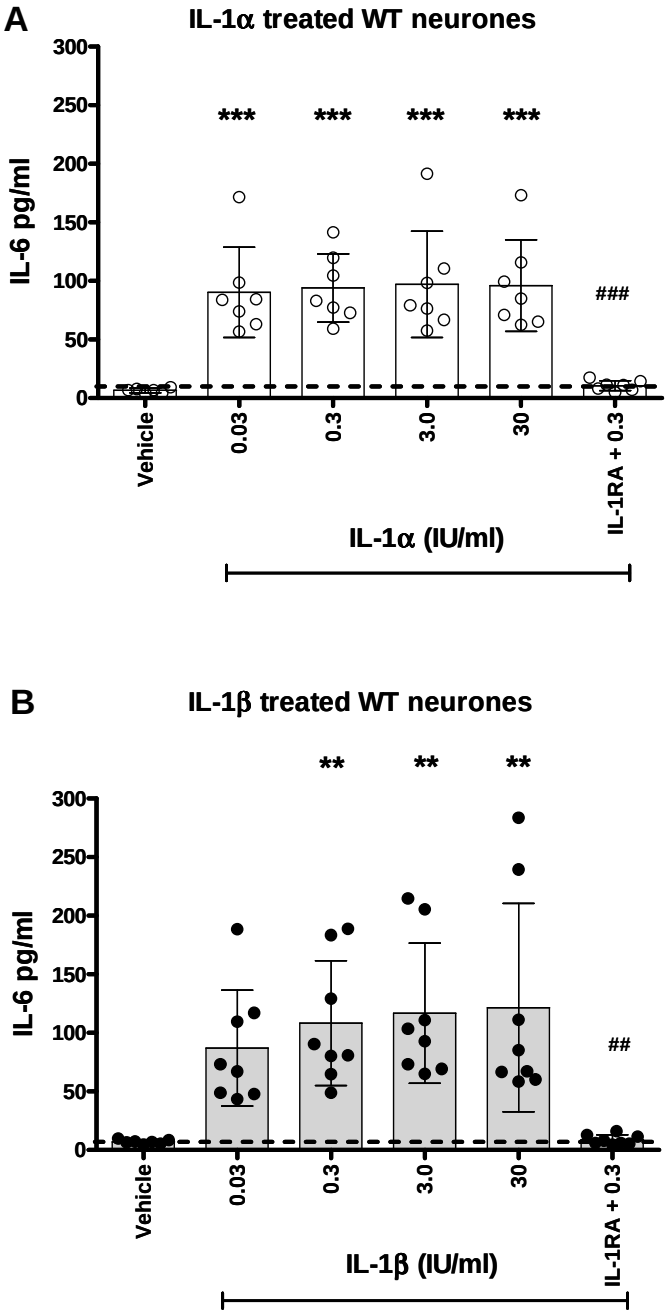


Figure 3.10. IL-1α- and IL-1β-induced IL-6 release in WT neuronal cultures

WT neurones were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 24 h in the presence or absence of IL-1RA (1 μg/ml). Supernatants were collected and analysed for IL-6 by ELISA. IL-1α (0.03-30 IU/ml: n=7) (**A**) and IL-1β (0.3-30 IU/ml: n=8) (**B**) significantly induced IL-6 release compared to vehicle. These responses were blocked by co-incubation with IL-1RA (1 μg/ml). One-way ANOVA and Tukey's multiple comparison tests. ** P< 0.01 and *** P< 0.001 IL-1 vs. vehicle. ## P< 0.01 and ### P< 0.001 IL-1 (0.3 IU/ml) vs. IL-1RA co-incubation. Dashed line indicates the detection limit (10 pg/ml).

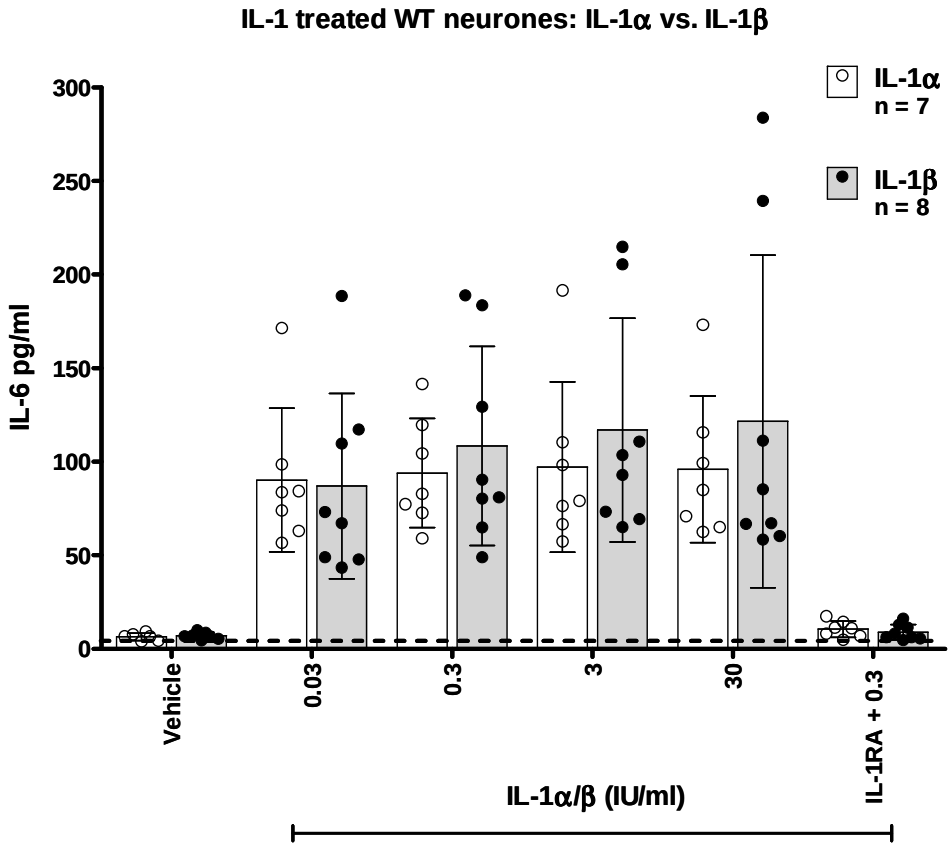


Figure 3.11. Comparison of IL-α- and IL-1β-induced IL-6 release in WT neuronal cultures.

Comparison of the level of IL-6 induced by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), or IL-1RA (1 μg/ml) co-incubation showed that IL-1α and IL-1β induced similar levels of IL-6 release in WT neurones. No significant difference between IL-1α- and IL-β-induced IL-6 release was found for any concentrations of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (10 pg/ml).

In IL-1RAcPb$^{-/-}$ neurones, vehicle treatment did not induce IL-6 release. IL-1α induced significant IL-6 release at 0.3 IU/ml (97 ± 56 pg/ml), 3 IU/ml (92 ± 48 pg/ml), and 30 IU/ml (96 ± 52 pg/ml), but not at the lower concentration of 0.03 IU/ml (78 ± 60 pg/ml). IL-1α did not induce IL-6 release from IL-1RAcPb$^{-/-}$ neurones in a concentration-dependent manner (Figure 3.12A). IL-1RA (1 μg/ml) completely blocked IL-1α (0.3 IU/ml)-induced IL-6 release.

All concentrations of IL-1β tested (0.03-30 IU/ml) caused significant IL-6 release. IL-1β at 0.03 IU/ml, 0.3 IU/ml, 3 IU/ml and 30 IU/ml induced 64 ± 26 pg/ml, 70 ± 35 pg/ml, 96 ± 28 pg/ml and 90 ± 38 pg/ml of IL-6 respectively (Figure 3.12B). IL-1RA (1 μg/ml) completely blocked IL-1β (0.3 IU/ml)-induced IL-6 release. There was no significant difference in the potency of IL-1α and IL-1β at inducing IL-6 release in IL-1RAcPb$^{-/-}$ neurones (Figure 3.13).

As in the cell lysates, there were no significant differences in IL-1-induced IL-6 in WT compared to IL-1RAcP$^{-/-}$ neurones (Figure 3.14 and Figure 3.15).

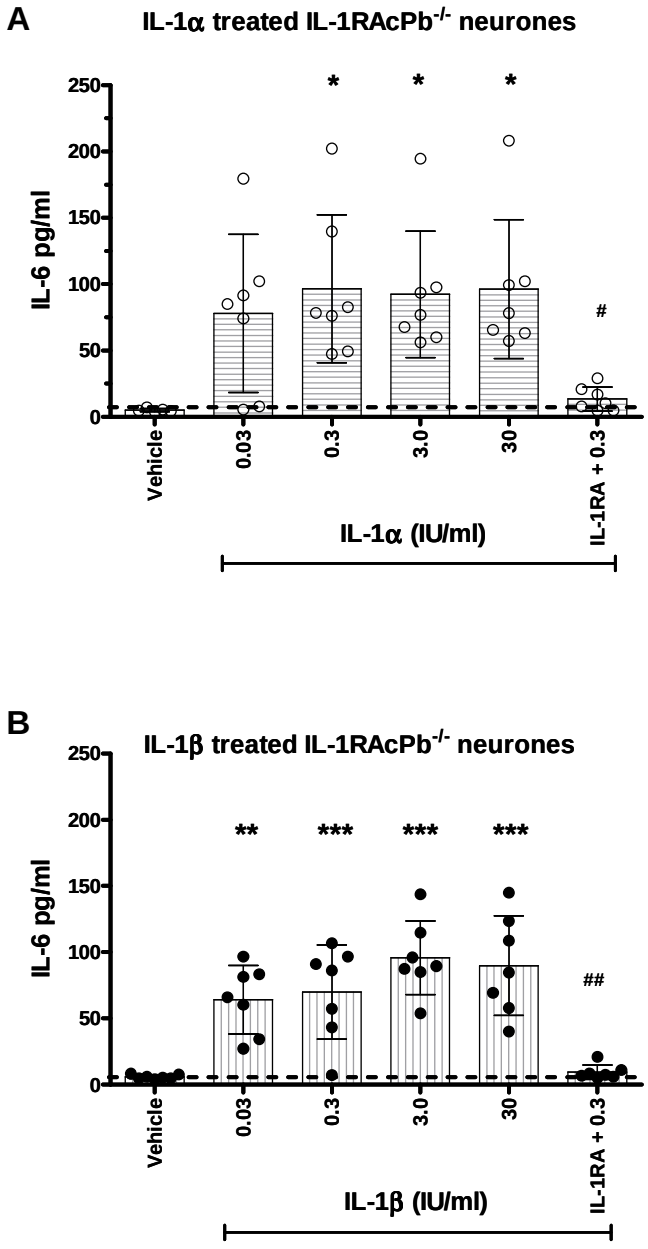


Figure 3.12. IL-1α- and IL-1β-induced IL-6 release in IL-1RAcPb$^{-/-}$ neuronal cultures

IL-1RAcPb$^{-/-}$ neurones were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 24 h in the presence or absence of IL-1RA (1 μg/ml). Supernatants were collected and analysed for IL-6 by ELISA. IL-1α (0.3-30 IU/ml: n=7) (**A**) and IL-1β (0.03-30 IU/ml: n=7) (**B**) induced significant release of IL-6. IL-1RA (1 μg/ml) completely blocked IL-1α- and IL-1β-induced IL-6 release. One-way ANOVA and Tukey's multiple comparison tests. * P< 0.05, ** P< 0.01 and *** P< 0.001 IL-1 vs. vehicle. # P< 0.05 and ## P< 0.01 IL-1 (0.3 IU/ml) vs. IL-1RA co-incubation. Dashed line indicates the detection limit (10 pg/ml).

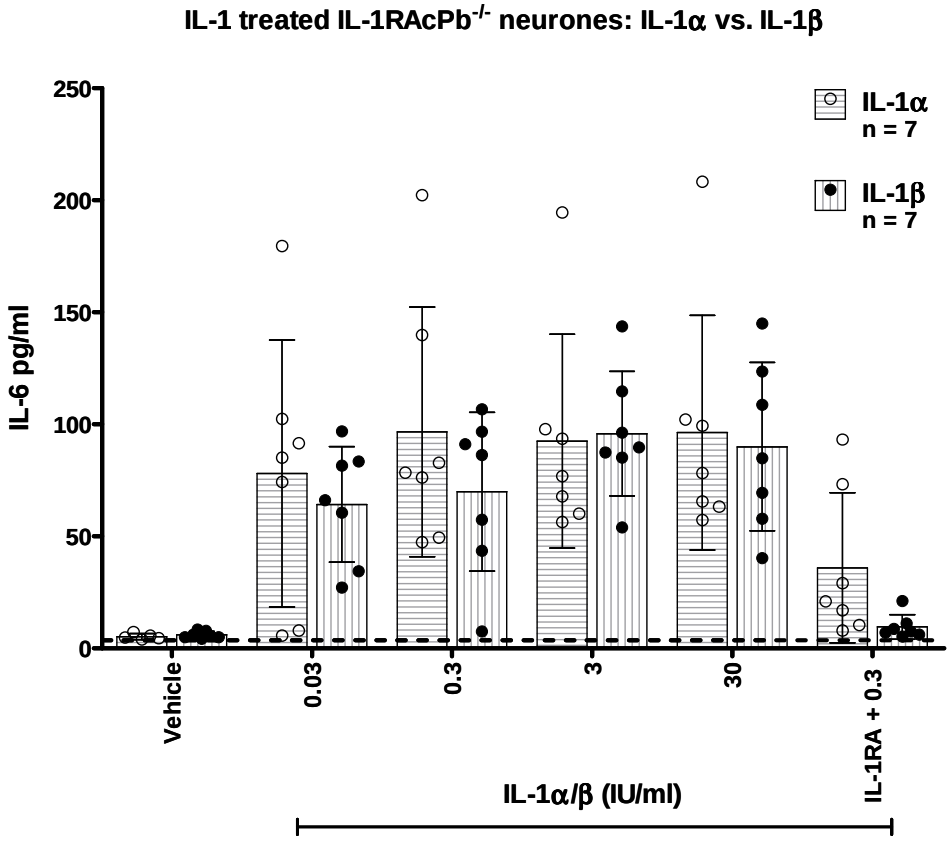


Figure 3.13. Comparison of IL-α- and IL-1β-induced IL-6 release in IL-1RAcPb$^{-/-}$ neuronal cultures.

Comparison of the level of IL-6 induced by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), or IL-1RA (1 μg/ml) co-incubation showed that IL-1α and IL-1β induced similar levels of IL-6 release in IL-1RAcPb$^{-/-}$ neurones. No significant difference between IL-1α- and IL-β-induced IL-6 release was found for any concentration of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (10 pg/ml).

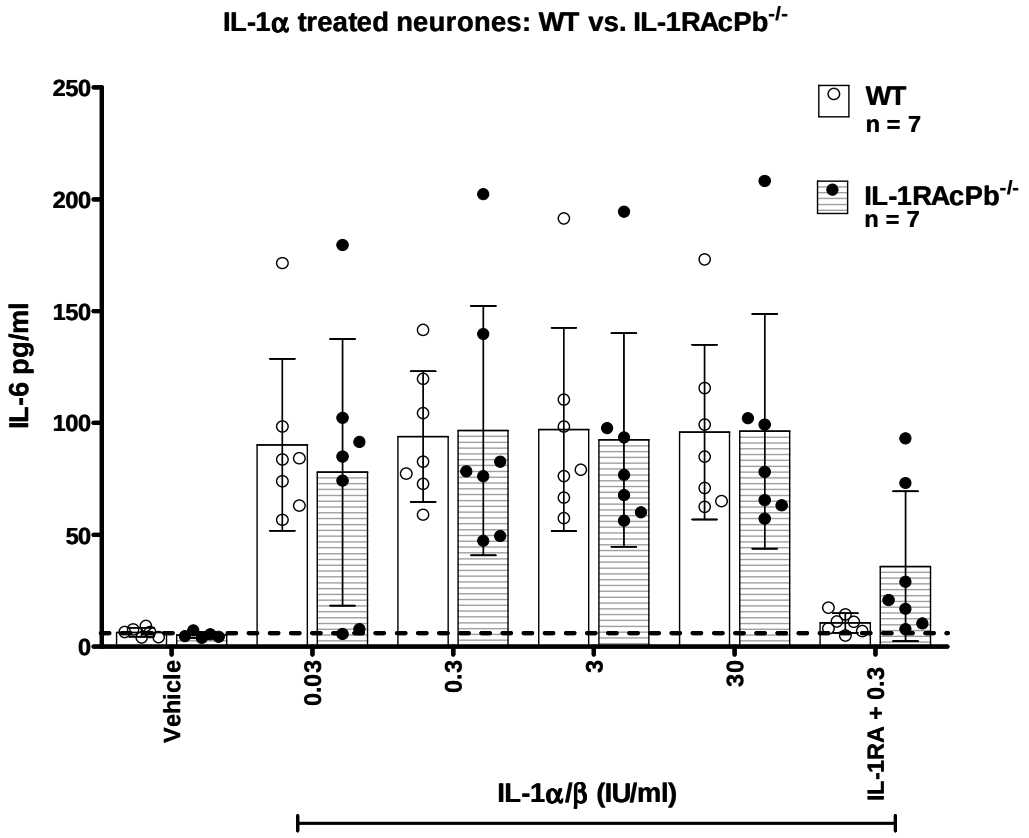


Figure 3.14. Comparison of IL-α- induced IL-6 release in WT and IL-1RAcPb$^{-/-}$ neuronal cultures

Comparison of the level of IL-6 induced by vehicle, IL-1α (0.03-30 IU/ml) or IL-1RA (1 μg/ml) co-incubation showed that IL-1α induced similar levels of IL-6 release in WT and IL-1RAcPb$^{-/-}$ neurones. No significant difference was found between WT and IL-1RAcPb$^{-/-}$ neurones in IL-1α-induced IL-6 release for any concentration of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (10 pg/ml).

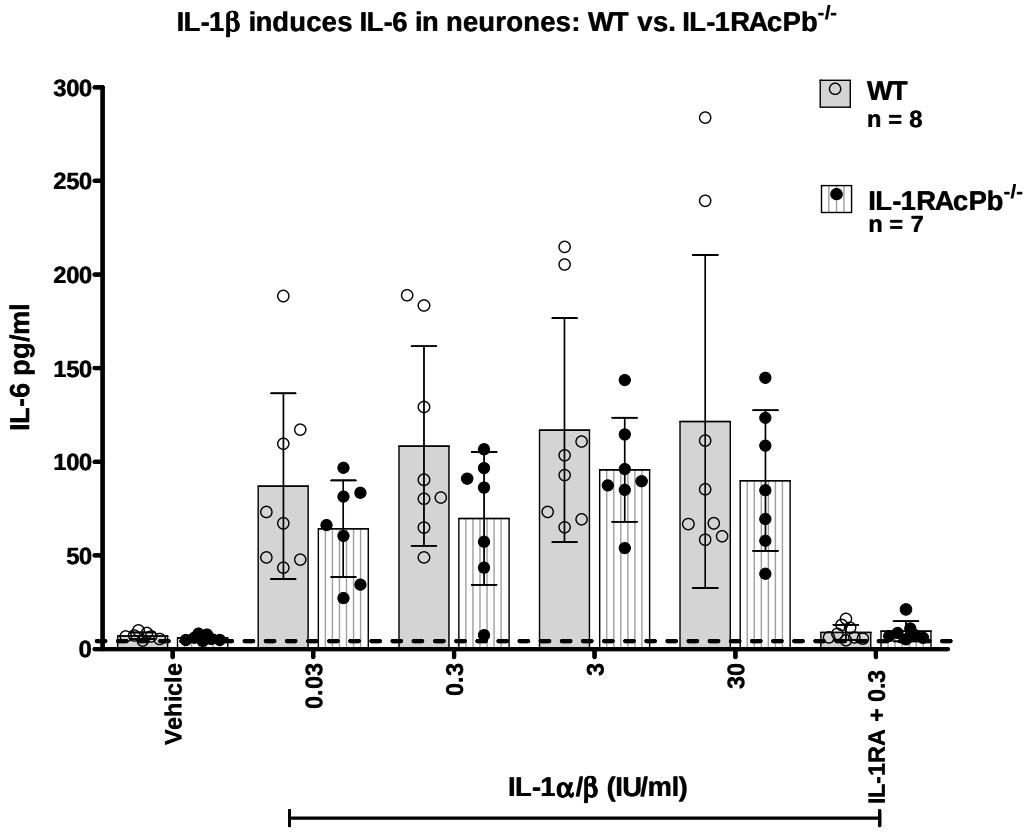


Figure 3.15. Comparison of IL-β-induced IL-6 release in WT and IL-1RAcPb$^{-/-}$ neuronal cultures

Comparison of the level of IL-6 induced by vehicle, IL-1β (0.03-30 IU/ml) or IL-1RA (1 μg/ml) co-incubation showed that IL-1β induced similar levels of IL-6 release in WT and IL-1RAcPb$^{-/-}$ neurones. No significant difference was found between WT and IL-1RAcPb$^{-/-}$ neurones in IL-1β-induced IL-6 release for any concentration of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (10 pg/ml).

3.4.5. IL-1-induced IL-6 release from glial cells

IL-1RAcPb expression in brain cells was found largely in neurones (Smith et al, 2009). However, glial cells in primary cultures also express this receptor, as detected by semi-quantitative RT-PCR (Figure 3.3). Although IL-1RAcPb mRNA expression in glial cells appear to be very low, its contribution to IL-1-induced actions in glial cells could be significant. To investigate the role of IL-1RAcPb in IL-1-induced actions in glial cells, primary glial cultures were treated with IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml) or LPS (1 μg/ml) for 24 h in the presence or absence of IL-1RA (10 μg/ml). Conditioned medium were collected and analysed for IL-6 by ELISA. LPS was used as a positive control for IL-6 induction in both WT and IL-1RAcPb$^{-/-}$ glial cultures. LPS (1 μg/ml) induced 1458 ± 381 pg/ml of IL-6 in WT glia and 1969 ± 278 pg/ml of IL-6 in IL-1RAcPb$^{-/-}$ glia.

In WT glial cultures, vehicle treatment induced no IL-6 release (13 ± 11 pg/ml). In contrast, IL-1α potently induced IL-6 release compared to vehicle (Figure 3.16A). The mean level of IL-6 release induced by 0.03 IU/ml, 0.3 IU/ml, 3 IU/ml and 30 IU/ml was 257 ± 149 pg/ml, 291 ± 83 pg/ml, 321 ± 23 pg/ml and 398 ± 110 pg/ml respectively. IL-1α (3 IU/ml)-induced IL-6 release was completely blocked by co-incubation with IL-1RA (10 μg/ml).

IL-1β potently induced IL-6 release from glial cultures. The level of IL-6 release induced by IL-1β (0.03-30 IU/ml) was significantly greater than that induced by vehicle. However, increasing concentrations of IL-1β did not induce a concentration-dependant release of IL-6. IL-1β at 0.03 IU/ml, 0.3 IU/ml, 3 IU/ml and 30 IU/ml induced IL-6 release from glial cultures of 108 ± 51 pg/ml, 179 ± 79 pg/ml, 194 ±72 pg/ml and 201 ± 76 pg/ml respectively (Figure 3.16B). Co-incubation with IL-1RA (10 μg/ml) completely blocked IL-1β (3 IU/ml)-induced IL-6 release in glia. The release of IL-6 induced by IL-1β (3 IU/ml) in the presence of IL-1RA (10μg/ml) was below the detection limit.

Comparing the effects of IL-1α- and IL-1β-induced IL-6 release from WT glial cultures showed that IL-1α induced IL-6 release with greater potency than IL-1β. This difference in IL-1 potency occurred at 0.03 IU/ml, 3 IU/ml and 30 IU/ml. At 0.3 IU/ml, IL-1α induced more IL-6 release than IL-1β, although this was not significant (Figure 3.17).

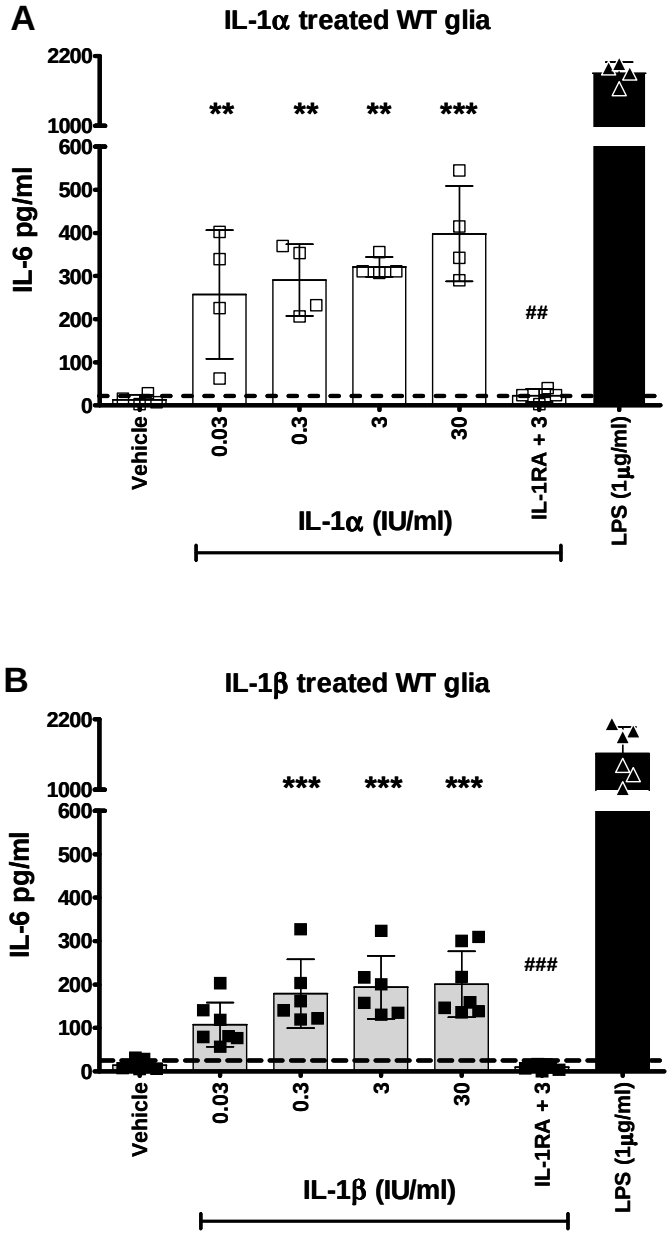


Figure 3.16. IL-1α- and IL-1β-induced IL-6 in WT glial cultures

WT glial cultures were treated with vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml) in the presence or absence of IL-1RA (10 μg/ml), or LPS (1μg/ml) for 24 h. Supernatants were collected and analysed for IL-6 release by ELISA. IL-6 detected in vehicle treated cells was low but IL-1α (0.3-30 IU/ml: n=5) (**A**) and IL-1β (0.3-30 IU/ml: n=6) (**B**) induced significant levels of IL-6 release compared to vehicle. Co-incubation with IL-1RA (10 μg/ml) blocked these responses. One-way ANOVA and Tukey's multiple comparison tests. * P< 0.05, ** P< 0.01 and *** P< 0.001 IL-1 vs. vehicle. # P< 0.05 and ### P< 0.001 IL-1 (0.3 IU/ml) vs. IL-1RA co-incubation. Dashed line indicates the detection limit (11 pg/ml).

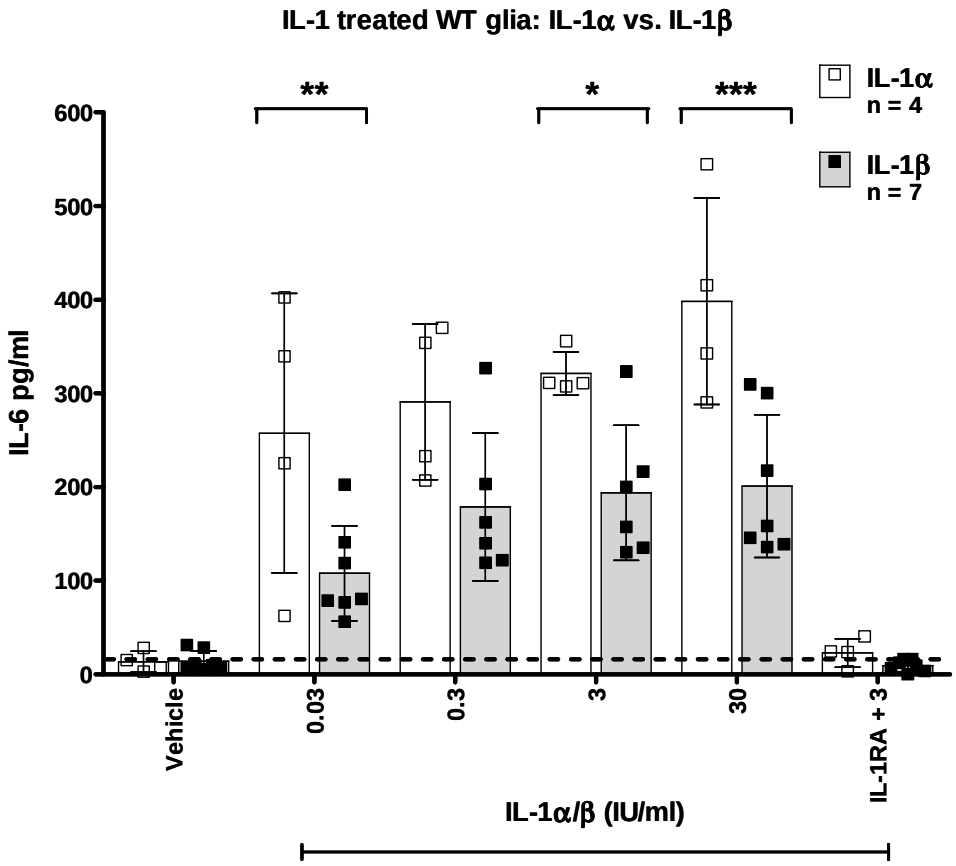


Figure 3.17. Comparison of IL-1α- and IL-β-induced IL-6 release in WT glial cultures

Comparison of the level of IL-6 induced by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), or IL-1RA (10 μg/ml) co-incubation showed that IL-1α induced IL-6 release with greater potency than IL-1β (at 0.03, 3 and 30 IU/ml). At 0.3 IU/ml, IL-1α and IL-1β induced IL-6 release with similar potency and no significant difference was detected between the two cytokines. Two-way ANOVA and Bonferroni post hoc test. * P< 0.05, ** P< 0.01 and *** P< 0.001 IL-1α vs. IL-1β. Dashed lines indicate the detection limit (11 pg/ml).

Vehicle treatment failed to release IL-6 from IL-1RAcPb$^{-/-}$ glial cultures, whilst IL-1α induced significant IL-6 release at the higher concentrations (0.3-30 IU/ml). At 0.03 IU/ml, 0.3 IU/ml, 3 IU/ml, and 30 IU/ml, IL-1α induced 155 ± 109 pg/ml, 209 ± 86 pg/ml, 199 ± 71 pg/ml and 221 ±100 pg/ml of IL-6 release respectively. Despite the high level of IL-6 release induced by IL-1α at 0.03 IU/ml (155 ± 109 pg/ml), this was not significantly different compared to vehicle treatment. (Figure 3.18A). IL-1RA (10 μg/ml) inhibited IL-1α (3 IU/ml)-induced IL-6, reducing it from 209 ± 86 pg/ml down to 15 ± 18 pg/ml.

IL-1β-induced IL-6 release in IL-1RAcPb$^{-/-}$ glial cultures displayed a similar trend to that of IL-1α-induced IL-6 release in IL-1RAcPb$^{-/-}$ glial cultures. IL-6 release was significantly high for the higher concentrations of IL-1β (0.3-30 IU/ml). The mean level of IL-6 release induced by increasing concentrations of IL-1β were 109 ± 40 pg/ml, 164 ± 56 pg/ml and 136 ± 52 pg/ml respectively (Figure 3.18B). Co-incubation with IL-1RA (10 μg/ml) blocked IL-1β (3 IU/ml)-induced IL-6 release. Comparing the level of IL-1α- and IL-1β-induced IL-6 release at each concentration (0.03-30 IU/ml) showed that the difference in potency between the two cytoknes, at the higher concentrations in WT glial cultures (Figure 3.17) were abrogated in IL-1RAcPb$^{-/-}$ glial cultures (Figure 3.19).

IL-1α (30 IU/ml)-induced IL-6 release in IL-1RAcPb$^{-/-}$ glia was significantly reduced compared to WT cultures (Figure 3.20), but IL-1β-induced IL-6 release was not significantly different in WT and IL-1RAcPb$^{-/-}$ glial cultures (Figure 3.21).

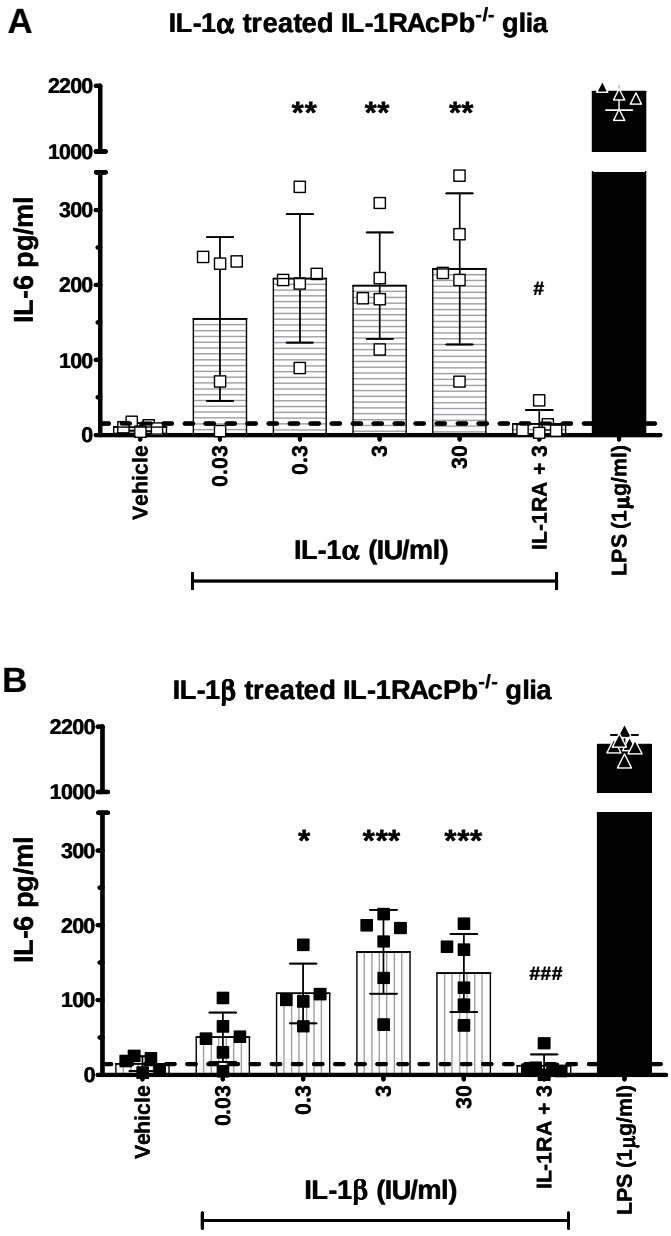


Figure 3.18. IL-1α- and IL-1β-induced IL-6 release in IL-1RAcPb$^{-/-}$ glial cultures

IL-1RAcPb$^{-/-}$ glial cultures were treated with vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml) in the presence or absence of IL-1RA (10 μg/ml) or LPS for 24 h. Supernatants were collected and analysed for IL-6 release by ELISA. Vehicle-induced IL-6 was low, however, IL-1α (0.3-30 IU/ml: n=5) (**A**) and IL-1β (0.3-30 IU/ml: n=6) (**B**) induced significant levels of IL-6 but not at the lower concentration of 0.03 IU/ml. IL-1RA (10 μg/ml) co-incubation significantly inhibited these responses. One-way ANOVA and Tukey's multiple comparison tests. * P< 0.05, ** P< 0.01 and *** P< 0.001 IL-1 vs. vehicle. # P< 0.05 and ### P< 0.001 IL-1 (0.3 IU/ml) vs. IL-1RA co-incubation. Dashed line indicates the detection limit (11 pg/ml)

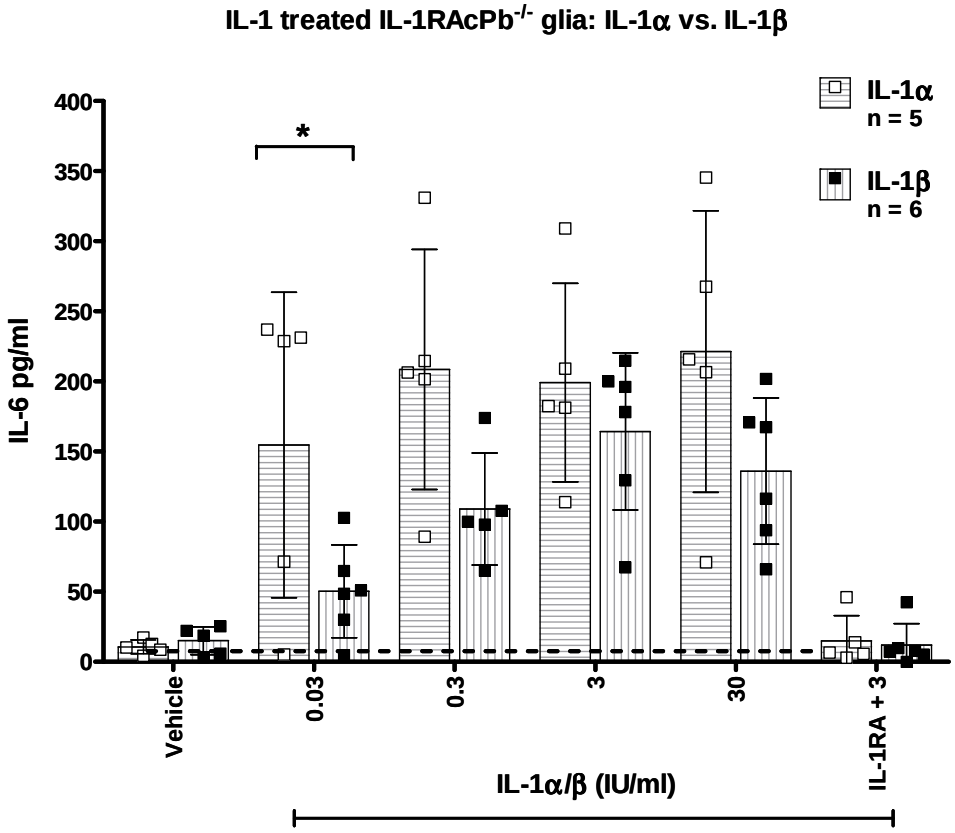


Figure 3.19. Comparison of IL-α- and IL-1β-induced IL-6 release in IL-1RAcPb$^{-/-}$ glial cultures.

Comparison of the level of IL-6 induced by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), or IL-1RA (10 μg/ml) co-incubation showed that IL-1α and IL-1β induced similar levels of IL-6 release at higher concentrations (0.3-30 IU/ml) in IL-1RAcPb$^{-/-}$ glia. At the lowest concentration (0.03 IU/ml), IL-1α was more potent than IL-1β. Two-way ANOVA and Bonferroni post hoc test.
* P< 0.05 IL-1α vs. IL-1β. Dashed line indicates detection limit (11 pg/ml).

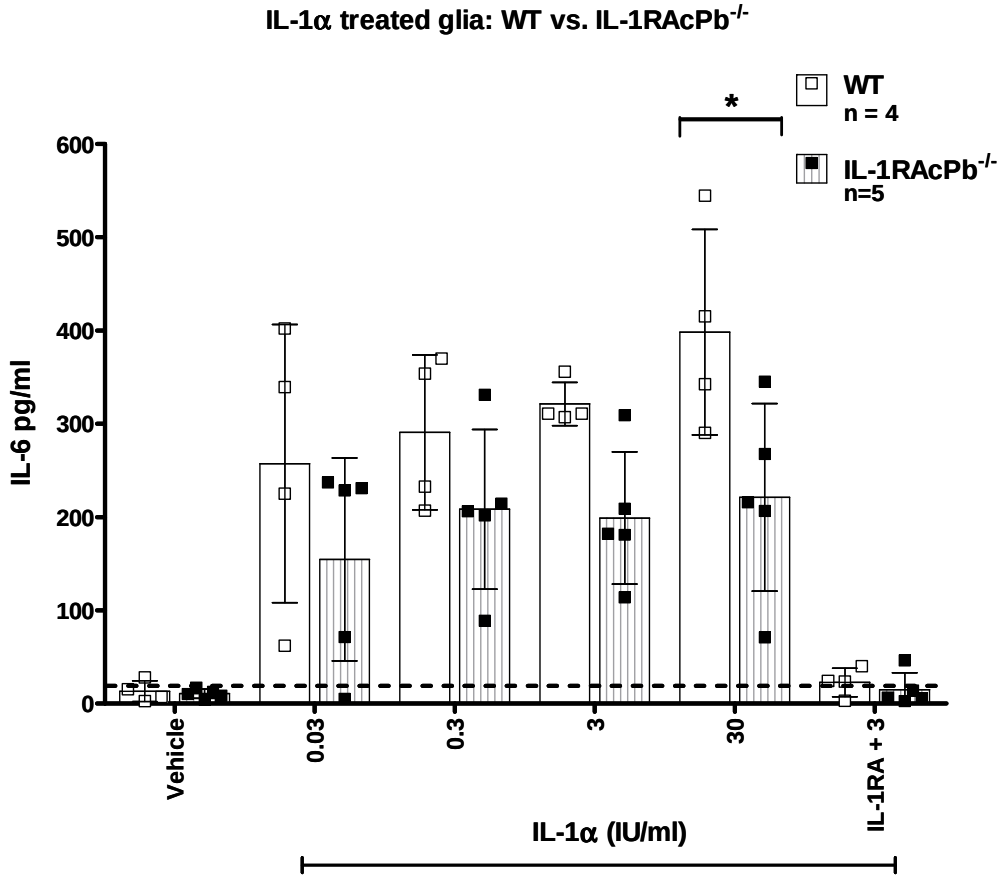


Figure 3.20. Comparison of IL-α-induced IL-6 release in WT and IL-1RAcPb$^{-/-}$ glial cultures

Comparison of the level of IL-6 induced by vehicle, IL-1α (0.03-30 IU/ml) or IL-1RA (10 μg/ml) co-incubation showed that IL-1α induced similar levels of IL-6 release at lower concentrations (0.03-3 IU/ml) in WT and IL-1RAcPb$^{-/-}$ glia. At the highest concentration (30 IU/ml), IL-1α was more potent in the WT than IL-1RAcPb$^{-/-}$ glia. Two-way ANOVA and Bonferroni post hoc test. * P< 0.05 WT vs. 1RAcPb$^{-/-}$. Dashed line indicates detection limit (11 pg/ml).

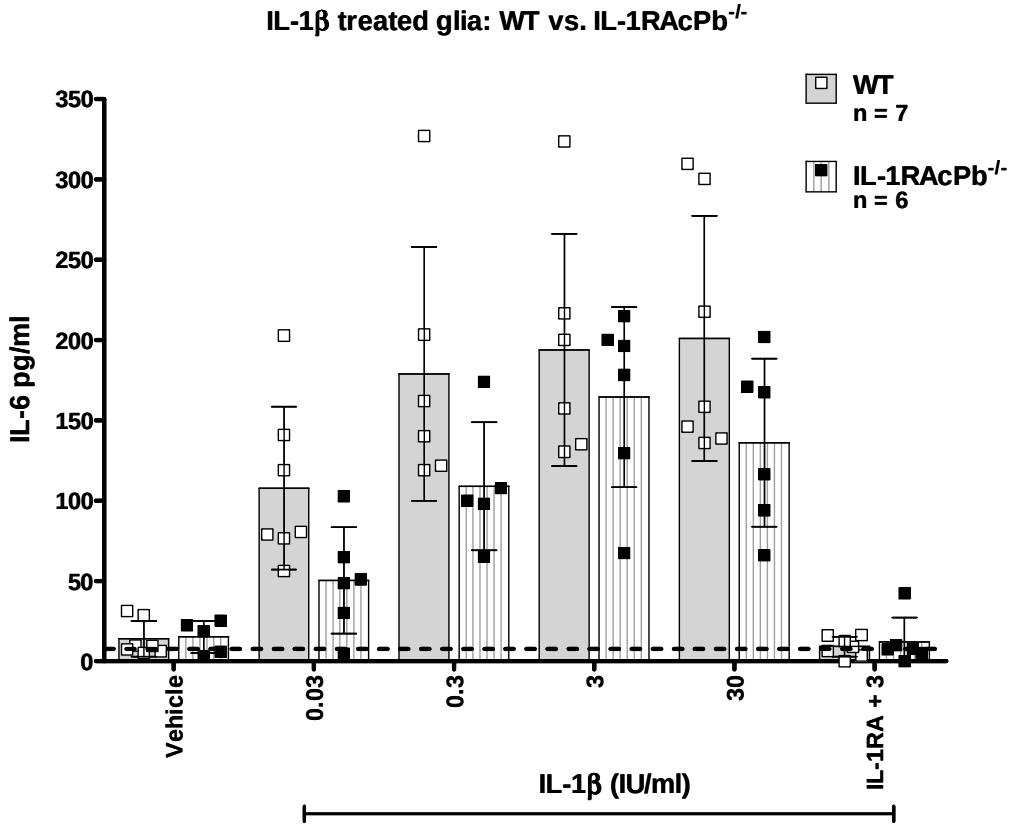


Figure 3.21. Comparison of IL-β-induced IL-6 in WT and IL-1RAcPb$^{-/-}$ glia

Comparison the level of IL-6 induced by vehicle, IL-1β (0.03-30 IU/ml) or IL-1RA (10 μg/ml) co-incubation showed that IL-1β induced similar levels of IL-6 release at all concentrations (0.03-3 IU/ml) in WT and IL-1RAcPb$^{-/-}$ glia.. No significant difference in IL-6 release was found between WT and IL-1RAcPb$^{-/-}$ glial cultures in response to IL-1β (0.03-30 IU/ml). Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (11 pg/ml).

3.5. Discussion

3.5.1. Cell culture characterisation

Recently, a new isoform of IL-1RAcP called IL-1RAcPb has been identified but the function of this receptor is controversial (Smith et al, 2009; Lu et al, 2008). The aim of this project was to determine the role of IL-1RAcPb in IL-1-induced actions in neurones and glia. To achieve this aim, we have tested the effect of IL-1RAcPb deletion on IL-1-induced IL-6 in these cell types by using genetically modified animals. Previous studies have demonstrated that genetic manipulation of the IL-1 family (with the exception of IL-1RA) does not adversely affect normal growth and development, or fertility (Cullinan et al, 1998; Horai et al, 1998; Glaccum et al, 1997). This could be due to the fact that IL-1 signalling is quiescent in the absence of infection or injury. Normal development in these transgenic animals suggests that IL-1 activity is not essential for development and can be compensated by other regulatory mechanisms. On contrary, IL-1RA$^{-/-}$ mice showed slow growth and smaller adult size (Horai et al, 1998), indicating IL-1RA may have an active role in normal growth and development and that overactivation of IL-1 signalling is not beneficial. Here we investigated whether deletion of IL-1RAcPb has any effect on the cellular composition of glial and neuronal cultures to be used.

Immunostaining of neuronal cultures from WT and IL-1RAcPb$^{-/-}$ animals showed that neurones from these two strains were morphologically similar. This is consistent with previous findings showing no significant difference in cellular morphology or composition between WT and IL-1R1$^{-/-}$ murine neurones (Tsakiri N, 2008). Phenotypically, both strains displayed clustering of neuronal cell bodies and a low level of glial contamination (2 ± 0.2 %) (Figure 3.1 and Figure 3.2). The formation of neuronal clusters is typical of neurones in culture supplemented with B27 (Chen et al, 2008), and the minor glial contamination is a feature of neuronal cultures supplemented with serum (Madarasz et al, 1991). Neuronal cultures grown in serum-free medium are reported to be pure and free from glial contamination (Tsakiri N, 2008).

Glial cells cultured from WT and IL-1RAcPb$^{-/-}$ mice were also morphologically similar. Immunostaining revealed that glial cells cultured from

WT and IL-1RAcPb$^{-/-}$ animals were similar in their composition of astrocytes and microglial cells. In mixed glial cultures, astrocytes were the most abundant cell type with a population of 82 ± 3% of the total cell population. Microglial cells formed the remaining part of the total population (18 ± 3 %) (Figure 3.2). It is unclear why O_2A progenitor cells, normally present in brain cell cultures, were absent from our neuronal and glial cultures. One possibility could be due to poor detection by the antibody, or to the fact that O_2A have differentiated into type-2 astrocytes (Noble, 1991) by 12 days *in vitro* and were detected as type 2 astrocytes in neuronal cultures and could not be distinguished from ordinary astrocytes by GFAP staining in mixed glial cultures. O_2A progenitor cells were probed using an A_2B_5 antibody previously proven to be effective by other members in our laboratory (Pinteaux et al, 2002) and other research groups (Brogi et al, 1997; Madarasz et al, 1991), indicating that the antibody is effective and specific.

Examining the cellular specificity of IL-1RAcPb expression in CNS cells revealed that IL-1RAcPb is expressed by neurones preferentially and expressed weakly in glial cells (Figure 3.3). This is consistent with the report of Smith et al (2009).

In summary, the cellular composition of neuronal and glial cell cultures from WT and IL-1RAcPb$^{-/-}$ animals were similar indicating that IL-1RAcPb does not contribute to the growth or development of neurones or glial cells *in vitro*. Our neuronal cultures were 98 % pure, and the cellular composition of our mixed glial cultures was 82 % astrocyte and 18 % microglia, making them valid models to investigate the effects of IL-1-induced actions in neurones and glial cells *in vitro*.

3.5.2. IL-1-induced IL-6 in neurones

In vivo, IL-6 protein expression is up-regulated in the cerebral cortex of rats after permanent MCAo (Legos et al, 2000). Transient MCAo in rats also induces up-regulation of cortical IL-6 mRNA expression (Ali et al, 2000) as well as IL-6 protein expression in microglia and neurones (Dihne et al, 2001; Block et al, 2000; Suzuki et al, 1999). IL-6 mRNA expression can also be detected in neurones of healthy rat brain by *in situ* hybridization (Gadient and Otten, 1994). *In vitro,* induction of IL-6 mRNA expression in rat primary cortical neurones was

stimulated by membrane depolarisation by forskolin (10 μM, 6 h) (Sallmann et al, 2000). IL-6 mRNA expression was also induced by NMDA (50 μM, 1 h and 3 h) in mouse cortical neurones (Ali et al, 2000). Additionally, IL-6 mRNA expression and protein release is induced by IL-1 from rat hippocampal neuronal cell lines, H19-7/IGF-IR cells (Bergamaschi et al, 2006) and mouse cortical neurones (Tsakiri et al, 2008c; Ringheim et al, 1995). Collectively, these studies indicate that IL-6 is an inflammatory cytokine that is expressed by neurones and can be induced by IL-1 (amongst others) *in vitro*.

To further characterise the role of IL-1RAcPb in IL-1α- and IL-1β-induced actions in neurones and glia, we investigated the effects of IL-1α and IL-β on IL-6 synthesis in WT and IL-1RAcPb$^{-/-}$ neuronal and glial cultures. Additionally, we also investigated the effects of IL-1α and IL-1β on pentraxin-3 (PTX3) production in neurones and glia to validate the IL-6 data. PTX3 is an acute phase protein which is induced in neurones and glial by seizure activities (Ravizza et al, 2001). IL-1β-induced PTX3 has been described previously in glial cells by Polentarutti et al (2000) (for review see Ortega-Hernandez et al, 2008). For this investigation, pure mouse cortical neuronal cultures were incubated with increasing concentration of IL-1α or IL-1β for 24 h, the suggested optimal time for IL-1-induced IL-6 in mouse cortical neurones (Ringheim et al, 1995) and IL-6 expression and release were measured.

This study demonstrated that IL-1β is a potent inducer of IL-6 in mouse cortical neurones as even the lowest concentration of IL-1 tested could induce significant IL-6 expression and release (Figure 3.4 and Figure 3.10). IL-1α also induced strong IL-6 production and release but large standard deviation within the lysates data prevented IL-1-induce IL-6 expression from reaching statistical significance (Figure 3.4) but IL-1-induced IL-6 release was potent (Figure 3.10), indicating IL-1α was also a potent inducer of IL-6 synthesis in neurones. A similar response was observed for PTX3 (Figure 7.2, Appendix III). Previous work by Tsakiri et al (2008c) also showed that low concentrations of IL-1β (0.03 IU/ml) could also induce significant level of IL-6 synthesis in mouse cortical neurones. It is unclear from these data whether IL-1-induced IL-6 is and all-or-nothing response as lower concentrations of IL-1 need to be tested. However, a previous study has shown that IL-1β induced IL-6 release from mouse cortical

neurones in a concentration-dependent manner ranging from 1 pM to 1 nM (Ringheim et al, 1995). Additionally, the present study demonstrated that IL-1-induced p38 phosphorylation (see next chapter) was also concentration-dependent, indicating that IL-1-induced IL-6 synthesis in neurones is graded.

In WT neuronal cell lysates, IL-1β induced significant amount of IL-6 except at the lowest concentration, but IL-1α induced significant level of IL-6 expression only at the highest concentration (Figure 3.4). This could be due to large variance in the data for IL-1α-induced IL-6. IL-1β-induced IL-6 synthesis in mouse cortical neurones was demonstrated previously by Tsakiri et al (2008c), however, the induced IL-6 was stored, until cellular depolarisation which then induces the release of IL-6 from neurones (Tsakiri et al, 2008b). In this study, IL-1-induced IL-6 was constitutively released into the medium (Figure 3.10). It is unclear why in our study IL-1-induced IL-6 in cortical neurones were contitutively released but IL-1-induced IL-6 in mouse cortical neurones studied by Tsakiri et al (2008b) were stored. The cause of this difference could be due to the difference in strain of mouse used in the two studies. In our study, we cultured neurones from C57/BL6 mice whereas Tsakiri et al (2008b) used C57/BL6 x 129sv mice, neurones from different strain may respond differently. Neurones used in our study could be partially depolarised in presence of IL-1 which could then lead to release of IL-1-induced IL-6. IL-1 is well documented to alter the neuronal electrical activities (Desson and Ferguson, 2003; Borsody and Weiss, 2002). Cultured neurones from C57/BL6 x 129sv could be resistant to the subtle effects of IL-1 on neuronal electrical activities at the concentrations tested. The 5 % plasma derived serum (PDS) in our culture medium compared to the serum free culture medium used by Tsakiri et al (2008b) may also contribute to this difference. The presence of serum introduced minor glial contamination which may alter the response of the co-habiting neurones.

IL-1-induced IL-6 release from neurones in our study is consistent with the dogma that IL-6 is a secreted protein (reviewed by Heinrich et al, 1990). Release of IL-6 in response to IL-1 has been reported previously in mouse cortical neurones (Ringheim et al, 1995) and hippocampal neuronal cell lines (Bergamaschi et al, 2006).

There is increasing evidence indicating that IL-1α and IL-1β have differential effects in specific cell types. Most relevant are reports on IL-1α and IL-1β differential actions in mouse cortical neurones. A previous study from our group demonstrated IL-1β, but not IL-1α, induced IL-6 production in mouse cortical neurones (Tsakiri et al, 2008c). This differential effect of IL-1α and IL-1β was not reproduced in the current study. I found that IL-1α and IL-1β induced IL-6 expression and release with equal potency (Figure 3.5 and Figure 3.11). This could be due to the large standard deviation in the data caused by outliers; however the level of IL-1α- and IL-1β-induced IL-6 did cluster closely together (Figure 3.11) suggesting IL-1α and IL-1β possesed similar bioactivity and that the responses observed were not artefacts of large standard deviations. Additionally IL-1α and IL-1β also equipotent at inducing PTX3 release from WT neurones (Figure 7.3, Appendix III). The difference between Tsakiri et al. (2008c) and the current study could again be the result of different strain of mouse used and/or the difference in composition of the culture medium.

3.5.3. The role IL-1RAcPb in IL-1-induced IL-6 in neurones

The IL-1 receptor accessory protein is critical for IL-1 signalling and deleting *IL-1RAcP* from neurones caused complete inhibition of IL-1α- and IL-1β-induced IL-6 expression and release (Figure 7.6, Appendix IV). IL-1RAcPb is highly expressed in neurones (Figure 3.3), but its contribution to neuronal function is completely unknown. In the present study, IL-1 induced constitutive release of IL-6 (Figure 3.10 and Figure 3.12), and IL-1α and IL-1β were equipotent at inducing IL-6 expression and release in WT and IL-1RAcPb$^{-/-}$ neurones (Figure 3.11 and Figure 3.13). This suggests that IL-1RAcPb is not essential in IL-1-induced IL-6 in neurones, however, the role of IL-1RAcPb in IL-1-induced changes in neuronal electrical activities is unclear.

The lack of effects of deleting *IL-1RAcPb* on IL-1-induced IL-6 in neurones is contrary to previous findings on the role of IL-1RAcPb when transfected in other cell types. In HEK293T cells, IL-1RAcPb was demonstrated to positively contribute to IL-1β signalling leading to the activation of NF-κB and induction of TNF-α and GM-CSF mRNA expression (Lu et al, 2008). However, IL-1RAcPb has also been suggested to be a negative modulator of IL-1β actions

in primary mouse cortical neurones. In IL-1RAcP$^{-/-}$ neurones, IL-1β treatment of neurones with IL-1RAcP reconstitution lead to expression of an array of gene that overlapped with WT neurones. However, incubating IL-1β with neurones which have had IL-1RAcP and IL-1RAcPb reconstitution (which should genetically resemble WT) resulted in down-regulation of certain gene expression (Smith et al, 2009). The authors concluded that IL-1RAcPb could down-regulate some but not all IL-1RAcP-dependent IL-1 responses. In this study, we did not observe any regulatory action of IL-1RAcPb on IL-6 expression in neurones. However, there are many differences in experimental procedures between our study and Smith et al. (2009). Although both studies used the same strain of mouse cortical neurones (C57/BL6), Smith et al. (2009) studied the role of IL-1RAcPb in IL-1RAcP$^{-/-}$ neurones by gene transfection, whereas in this study, the role of IL-1RAcPb was investigated by studying the effects of IL-*1RAcPb* gene deletion. The culturing protocols of primary neurones were also different between the two studies. In Smith et al. (2009), primary neurones were cultured in serum-free medium, in the absence of any glial proliferation inhibitor, and the level of glial contamination ranged from 20-30 %. Primary neurones used in this study were cultured in medium containing 5 % PDS and a minimum of 5 % glial proliferation inhibitor (see section 2.3). Glial contamination was 2 %. The cellular composition of neuronal cultures between Smith et al. (2009) and the current study is clearly very different, specifically in the population of glial cells. The contribution of 20-30 % glial population could significantly affect neuronal response to IL-1. Additionally, Smith et al. (2009) did not study the role of IL-1RAcPb in protein expression but explored its role in the expression of different protein by microarray analysis. It is important to interpret this microarray data with caution, as gene transcription does not indicate gene translation into a protein. Some genes are transcribed but are degraded before they are translated. Therefore it is difficult to compare data from Smith et al. (2009) and the current study as the cellular composition of the neuronal cultures, and the variable measured are different.

3.5.4. IL-1-induced IL-6 in glial cells

IL-1RAcPb was expressed at low level in glial cells (Figure 3.3), but the significance of this expression is unknown. IL-1 signalling is highly proficient and

activation of only a few receptors within a cell is sufficient to induce a maximum response (for review see Dinarello, 1997). Therefore, how low level of IL-1RAcPb expression may affect IL-1-induced actions in glia is not known.

This study demonstrated that IL-1α and IL-1β are potent activators of WT mouse mixed glia, leading to the production of IL-6 (Figure 3.16) and PTX3 (Figure 7.4, Appendix III). IL-1α was more potent than IL-1β at inducing IL-6 production from mixed glial cells at 0.03 IU/ml, 3 IU/ml) and 30 IU/ml (Figure 3.17) and PTX3 at 0.3 IU/ml (Figure 7.5, Appendix III). This observation is not in agreement with previous observations which showed that IL-1β is more potent than IL-1α at inducing IL-6 from mouse mixed glia (Andre et al, 2005b). However, IL-1α has been shown to be more effective than IL-1β at inducing responses in various conditions; IL-1α is more potent than IL-1β in activating mouse brain endothelial cells (Thornton et al, 2010), inducing substance P from rat dorsal root ganglion (Skoff et al, 2009), TNF-α release from mouse epidermal cells (Beissert et al, 1998) and prostaglandins release from bovine endothelial cells (Tanikawa et al, 2009).

3.5.5. The role of IL-1RAcPb in IL-1-induced IL-6 in glia

In IL-1RAcPb$^{-/-}$ glial cells, IL-1α and IL-1β potently induced IL-6 (Figure 3.18). Again IL-1α was more potent than IL-1β but only at 0.03 IU/ml (Figure 3.19). It appears that deleting IL-1RAcPb abrogated the differential effects between IL-1α and IL-1β seen in WT glial cultures treated with higher concentrations (3 IU/ml and 30 IU/ml) of the cytokines. In WT glia, IL-1α was more potent than IL-1β at inducing IL-6 suggesting that the presence of IL-1RAcPb may contribute to IL-1α-induced IL-6 in glia (Figure 3.20). This possible role of IL-1RAcPb in IL-1α signalling in glial cells is surprising as the expression of IL-1RAcPb mRNA in glia is marginal compared to IL-1RAcPb mRNA expression in neurones. However the large variance in our data could also introduce a false differential effect. What is clear is that IL-1RAcPb is not critical for IL-1-induced IL-6 in glial cells.

In summary, IL-1α and IL-1β induced significant release of IL-6 (and PTX3) from mouse cortical neuronal cultures and mixed glial cultures after 24 h treatment. IL-1α and IL-1β induced IL-6 with equal potency in WT and

IL-1RAcPb$^{-/-}$ neurones and, IL-1RAcPb did not alter IL-1-induced IL-6 in neurones. In glial cells, IL-1α was more potent than IL-1β at inducing IL-6 (and PTX3), and IL-1RAcPb may contribute IL-1α-induced IL-6 production in glial cells. However, these responses were measured after 24 h of IL-1 treatment which may not apply to other time points. There could be possible protein degradation of IL-6 after 24 h when compared to earlier time points such as 7 h investigated by Tsakiri et al. 2008c. However, the level of IL-6 protein detected in the two studies were similar (100 – 150 pg/ml), indicating that IL-6 degradation was low between 7 h and 24 h. A comparison between IL-1β-induced IL-6 in neurones at 7 h and 24 h was also undertaken which revealed that there was no difference in the amount of IL-1-induced IL-6 at 7 h and 24 h (data not shown). 24 h exposure to IL-1α and IL-1β may also modulate the expression of different components of the IL-1 signalling complex (IL-1R1, IL-1RAcP and IL-1RAcPb) that may affect IL-1-induced responses. Supporting this notion, IL-1R1 have been shown to be upregulated in the rat hippocampus neurones and astrocytes following 4 h IL-1β treatment (Friedman, 2001). It would be necessary to perform an analysis of expression of the different IL-1 receptors to confirm their contribution to IL-1-mediated responses.

IL-6 is a cytokine amongst many mediators which are induced by IL-1 in neurones and glial cells. The lack of effect of IL-1RAcPb on IL-1-induced IL-6 in neurones implies a redundant role for IL-1RAcPb in IL-1-induced IL-6 production in neurones. However, IL-1RAcPb may affect IL-1-induced actions in neurones at a signalling level. IL-1 has been shown to induce trophic factors as well as neurotransitters release (Skoff et al, 2009; Cho et al, 2005; Feleder et al, 1998; Isackson, 1995) and IL-1RAcPb may have a role in these responses. Thus the following chapters will investigate a specific role for IL-1RAcPb in IL-1-induced activation of key signalling pathways in neurones and glial cells.

4. The role of IL-1RAcPb in IL-1-induced signalling in neurones and glia

4.1. Introduction

4.1.1 Mitogen-activated protein kinase signalling

Mitogen-activated protein kinases (MAPK) and Src kinase are activated by phosphorylation of the enzyme to trigger distinct signalling pathways, both of which can be activated by IL-1. Extracellular signal-regulated kinases (ERK1 and ERK2) and p38 (p38α, p38β, p38γ and p38δ) are well characterised MAPK signalling elements. Activation of the MAPK signalling cascade follows a structured framework involving sequential activation of MAPK kinase kinase (MAPKKK), leading to MAPK kinase (MAPKK) activation and then activation of MAPK (ERK1/2, p38 and JNK) (Figure 1.9). The substrate for ERK1/2 and p38 are numerous and diverse to account for the many diverse responses attributed to MAPK signalling (for reviews see Roux and Blenis, 2004 and Johnson and Lapadat, 2002). Due to time limitations the effects of IL-1 on JNK signalling was not investigated in this study. However, IL-1-induced JNK phosphorylation has been reported in glial cells (Andre et al, 2005b; Parker et al, 2002) but not neurones (Tsakiri et al, 2008a; Srinivasan et al, 2004).

ERK1/2 and p38 are distributed in the cytoplasm in quiescent cells, whilst upon stimulation, they translocate into the nucleus to activate various transcription factors including cAMP response element-binding protein (CREB) c-Fos, c-jun and NF-κB. Activation of transcription factors can lead to enhanced stability of the transcription complex and activity (for reviews see Borders et al, 2008, Rubinfeld and Seger, 2005, Roux and Blenis, 2004 Harper and LoGrasso, 2001). p38α and p38β are the only two isoforms ubiquitously expressed in brain cells (for review see Harper and LoGrasso, 2001). Activation of p38 results in phosphorylation of MAPK activated protein kinase 2 and 3 (MK2 and MK3) and mitogen and stress-activated kinases (MSKs) well as transcription factors including ATF-1, Elk-1 and NF-κB. MK2 is an important element that promotes mRNA elongation and stabilisation. Therefore p38 activity could be important both for mRNA transcription as well as stability (for reviews see Borders et al, 2008, Rubinfeld and Seger, 2005 and Harper and LoGrasso, 2001).

IL-1β-induced ERK1/2 and p38 phosphorylation has been shown to both initiate and limit CNS inflammation. In neuroglioma and neuroblastoma cells,

IL-1β-induced PGE_2 release is mediated by ERK1/2 and p38 signal transduction. p38 also mediates IL-1β-induced cytosolic phospholipase A_2 (cPLA2) and cylcooxygenase-2 (COX-2) gene expression (Moolwaney and Igwe, 2005). However, p38 activation is also associated with anti-inflammatory functions, since it mediates the expression of the anti-inflammatory cytokine IL-10 (Kim et al, 2008).

It is unclear how IL-1RAcPb may contribute to these signalling pathways or CNS inflammation. Investigating the role of IL-1RAcPb in ERK1/2 and p38 signalling in brain cells may yield new insights into IL-1-induced actions. An improved understanding of IL-1 signalling could introduce greater flexibility when it comes to manipulating IL-1 signalling for therapeutic purposes.

4.1.2 Src kinase signalling

Src kinase is abundantly expressed in neural tissues (Cotton and Brugge, 1983) and plays an important role in neuronal differentiation and development (Cartwright et al, 1987 and for review see Salter, and Kalia, 2004). There are *in vitro* evidence to suggest that Src kinase enhances NMDA receptor activity in rat neurones (Yu et al, 1997; Chen and Leonard, 1996) by association with the NR2A subunit of the NMDA receptor (Liu et al, 2001; Kohr and Seeburg, 1996). As synaptic strength (activity-dependent neuronal changes) is partly regulated by NMDA receptor activities, modulation of NMDA receptors by Src kinase could contribute to physiological plasticity and brain damage (for review see Salter and Kalia, 2004).

Src kinase activation is induced by IL-1 and could also be involved in acute brain injury. Transient cerebral ischaemia in rats has been shown to induce Src kinase phosphorylation and NMDA receptor hyperphosphorylation (Takagi et al, 1999) and that NMDA receptor hyperactivity could be linked to IL-1β activity via Src kinase activation (Tsakiri et al, 2008a; Davis et al, 2006; Viviani et al, 2003). The role of IL-1RAcPb on IL-1-induced Src kinase activation in neurones and glia is unknown, but could be important as IL-1RAcPb is highly expressed in the CNS (Smith et al, 2009). If IL-1RAcPb is actively involved in IL-1-induced Src kinase signalling (whether regulatory or mediatory) its property could be harnessed to target inflammation.

4.2. Aims

To investigate the role of IL-1RAcPb in IL-1-induced signalling and the possible differential effects of IL-1α and IL-1β on signalling mechanisms. This part of the project is divided into two main objectives:

1. To determine the role of IL-1RAcPb in IL-1-induced signalling pathways activation in neurones and glia. The effects of IL-1 on MAPK and Src kinase will by examined in both glial and neuronal cells.
2. To investigate the differential effects of IL-1α- and IL-1β-induced MAPK and Src kinase activation in neurones and glia.

4.3. Materials and methods

For this study, WT and IL-1RAcPb$^{-/-}$ neuronal and glial cells were treated with vehicle or increasing concentrations of IL-1α or IL-1β (0.03-30 IU/ml) for 15 min (neurones) or 30 min (glia), in the absence or presence of IL-1RA. In neurones, 1 μg/ml of IL-1RA was co-incubated with 1 ng/ml (0.3 IU/ml) of IL-1α or IL-1β, and in glial cells, 10 μg/ml of IL-1RA was co-incubated with 10 ng/ml (3 IU/ml) of IL-1α or IL-1β. Cell lysates were collected for analysis of p38, ERK1/2 and Src kinase activity by Western blot analysis or specific ELISA (see sections 2.8 and 2.10).

4.4. Results

4.4.1. IL-1-induced p38 MAPK signalling in neurones

In WT neurones, vehicle induced weak or undetectable p38 activity (Figure 4.1A). Western blot analysis shows that IL-1α (0.03-30 IU/ml) induced a concentration-dependent activation of p38 (Figure 4.1B). At 0.03 IU/ml, IL-1α-induced p38 activation was non-significant compared to vehicle treatment. However, at higher concentrations (0.3-30 IU/ml), IL-1α-induced p38 was significantly greater than vehicle treatment. The response was maximal at 3 IU/ml of IL-1α. IL-1α at concentrations of 0.3 IU/ml, 3 IU/ml or 30 IU/ml induced a 4.3, 7.3 and 7.8 fold increase in p38 activity respectively. The presence of IL-1RA (1

μg/ml) decreased IL-1α-induced p38 activation but this was non-significant compared to IL-1α treatment (0.3 IU/ml) (Figure 4.1B).

IL-1β (0.03-30 IU/ml) also induced a concentration-dependent activation of p38 in WT neurones; the concentration of 3 IU/ml IL-1β was optimal for p38 activation. IL-1β at concentrations of 0.3 IU/ml, 3 IU/ml and 30 IU/ml, induced a 4.4, 5.2, and 5.3 fold increase in p38 activation respectively. IL-1RA (1μg/ml) significantly inhibited this response (Figure 4.2A and Figure 4.2B). Comparison of IL-α- and IL-1β-induced p38 activation in WT neurones showed that these two cytokines were equally potent for p38 activation (Figure 4.3).

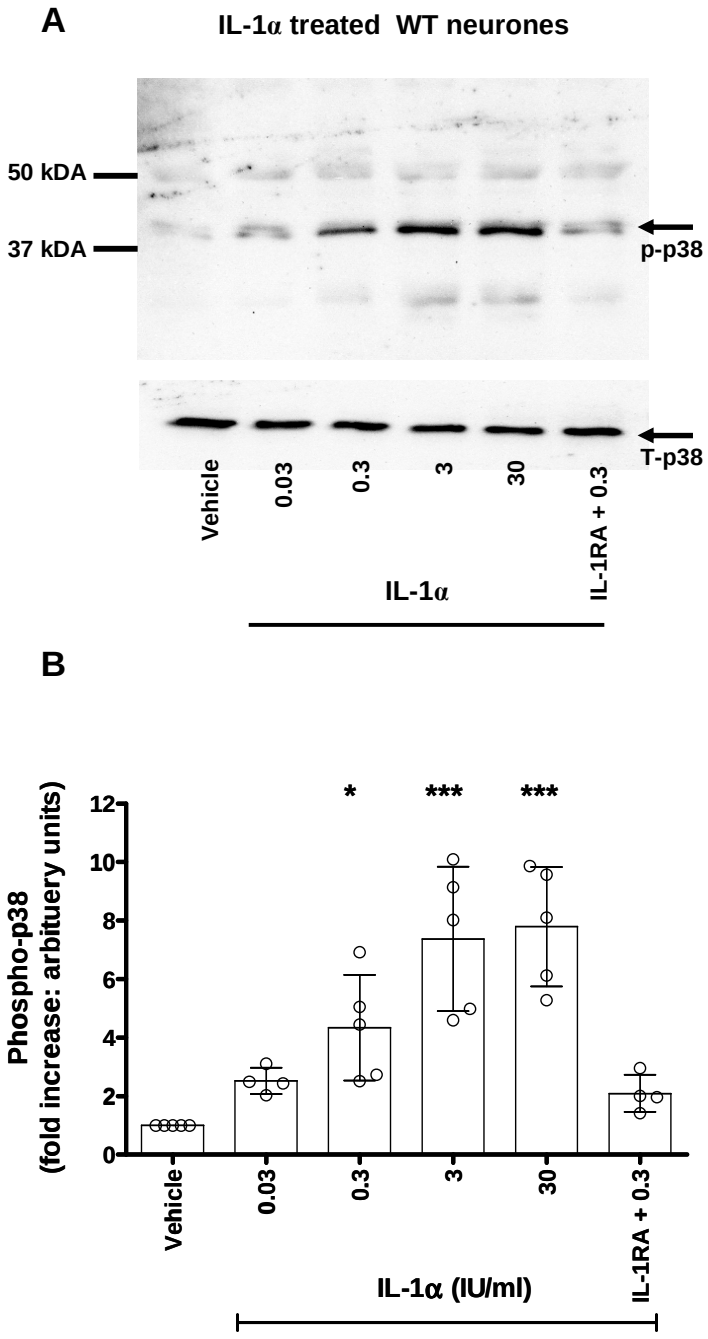


Figure 4.1. IL-1α-induced p38 activation in WT neurones

WT neuronal cultures were treated with vehicle or IL-1α (0.03-30 IU/ml) for 15 min in the presence or absence of IL-1RA (1 μg/ml). Lysates were collected and analysed for p38 activity by Western blot (**A**). **B**. ImageQuant analysis of blot showed that IL-1α induced p38 activation in a concentration-dependent manner (n=5). IL-1RA (1 μg/ml) co-incubation with IL-1α (0.3 IU/ml) reduced IL-1α-induced p38 activation. One-way ANOVA and Tukey's multiple comparison tests.* $P< 0.05$, *** $P< 0.001$ IL-1α vs. vehicle.

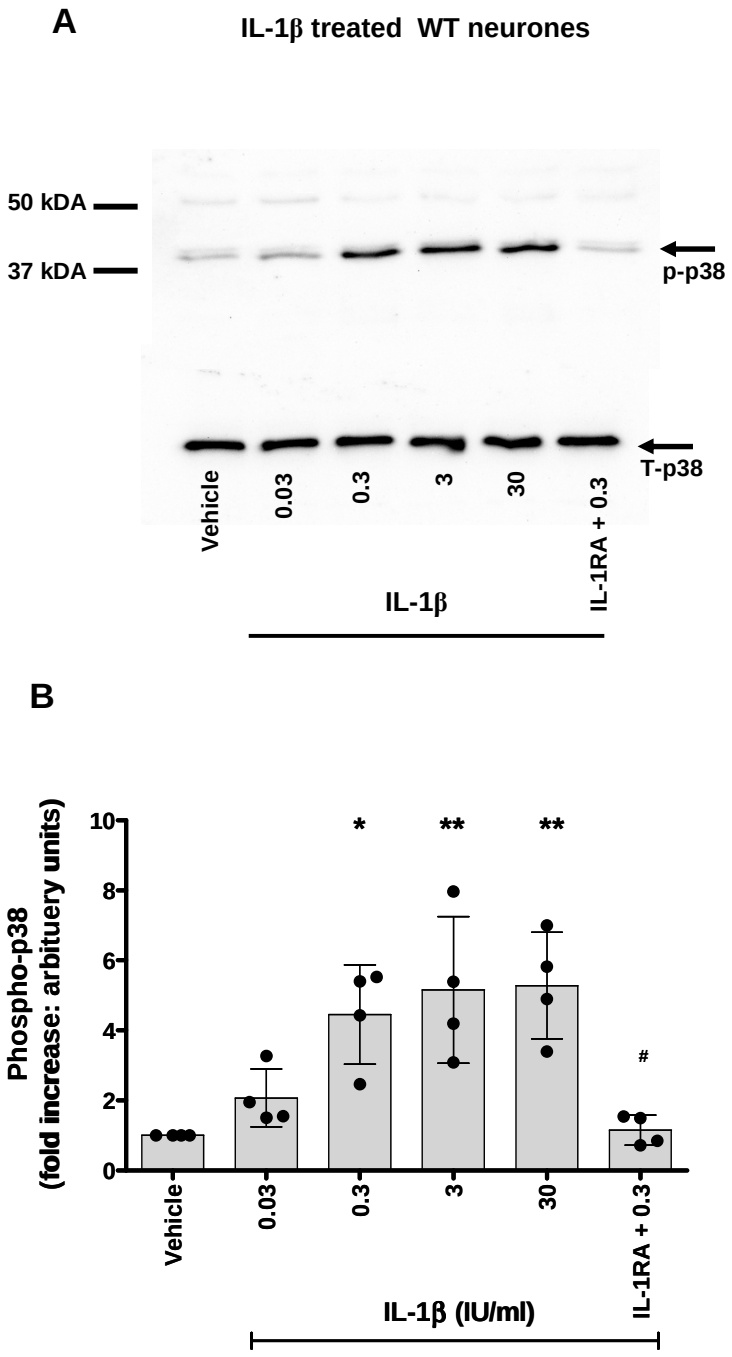


Figure 4.2. IL-1β-induced p38 activation in WT neurones

WT neuronal cultures were treated with vehicle or IL-1β (0.03-30 IU/ml) for 15 min in the presence or absence of IL-1RA (1 μg/ml). Lysates were collected and analysed for p38 activity by Western blot (**A**). **B**. ImageQuant analysis of blot showed that IL-1β induced p38 activation in a concentration-dependent manner (n=4). IL-1RA (1 μg/ml) co-incubation with IL-1β (0.3 IU/ml) significantly inhibited IL-1β-induced p38 activation. One-way ANOVA and Tukey's multiple comparison tests.* P< 0.05, ** P< 0.01 IL-1β vs. vehicle. # P< 0.05 IL-1β (0.3 IU/ml) vs. IL-1RA co-incubation.

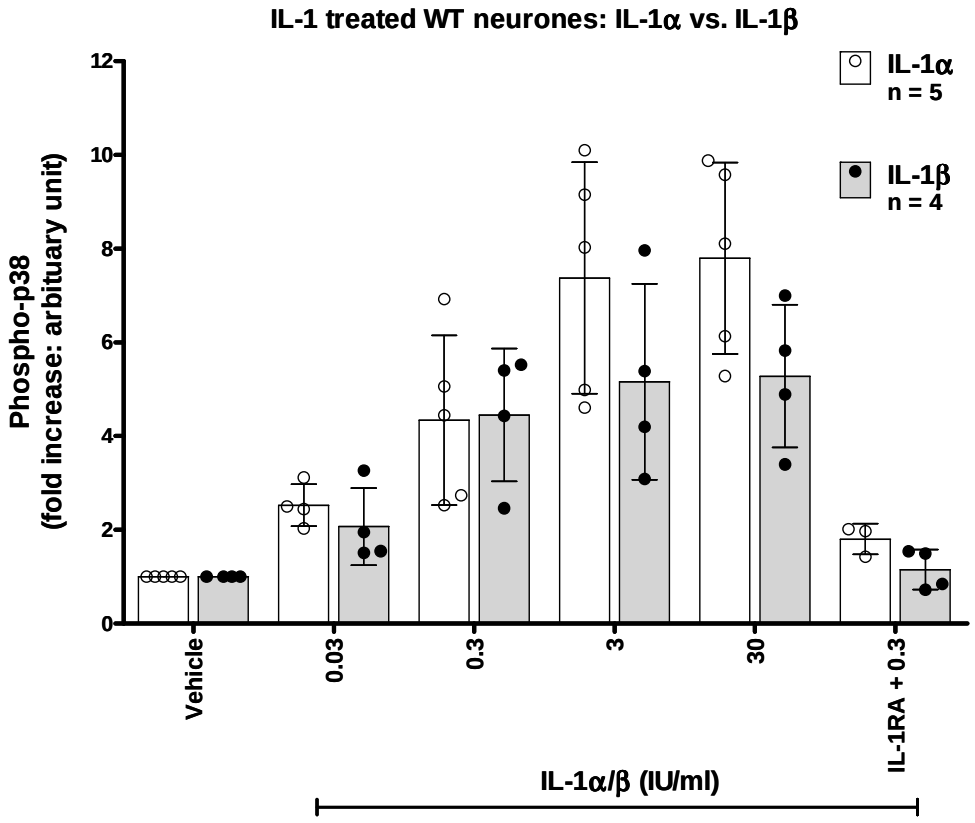


Figure 4.3. Comparison of IL-α- and IL-1β-induced p38 activation in WT neurones.

Comparison of the level of p38 activation induced by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), or IL-1RA (1 μg/ml) co-incubation showed that IL-1α and IL-1β induced similar levels of p38 activation in WT neurones. No significant difference between IL-1α- and IL-β-induced p38 activation was found for any concentrations of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test.

In IL-1RAcPb$^{-/-}$ neurones, vehicle-induced p38 activity was weakly detected (Figure 4.4A). IL-1α (0.03-30 IU/ml) induced a concentration-dependent p38 activation. At 0.03 IU/ml, IL-1α-induced p38 was not significant compared to vehicle treatment. However, at higher concentrations (0.3-30 IU/ml), IL-1α-induced p38 activation was significantly greater than vehicle treatment. The concentration response peaked at 3 IU/ml. 0.3 IU/ml, 3 IU/ml and 30 IU/ml of IL-1α induced an average of 3.7, 4.5 and 3.9 fold increase in p38 activation which was significantly blocked by the presence of IL-1RA (1 μg/ml) (Figure 4.4B).

IL-1β (0.03-30 IU/ml) also induced p38 activation in a concentration-dependent manner that reached saturation at 3 IU/ml (Figure 4.5A and B). At 0.3 IU/ml, 3 IU/ml and 30 IU/ml, IL-1β induced an average of 5.6, 7.5, and 7.8 fold increase in p38 activation respectively. IL-1RA (1μg/ml) significantly inhibited this response. A comparison of IL-α- and IL-1β-induced p38 activity showed that IL-1α-induced p38 activity was significantly lower compared to IL-1β treatment in IL-1RAcPb$^{-/-}$ neurones (Figure 4.6).

It is unclear if this difference in potency between IL-1α- and IL-1β-induced p38 activation in IL-1RAcPb$^{-/-}$ neurones is due to suppression of IL-1α activity or potentiation of IL-1β action. To investigate this phenomenon, we compared the activity of IL-1α-induced p38 activity in WT and IL-1RAcPb$^{-/-}$ neurones and repeated the same comparison for IL-1β. This analysis revealed that IL-1α-induced p38 activation was significantly decreased in IL-1RAcPb$^{-/-}$ neurones compared to WT for the higher concentrations of IL-1α (3-30 IU/ml) (Figure 4.7). IL-1RAcPb deletion did not affect IL-1β activity (Figure 4.8).

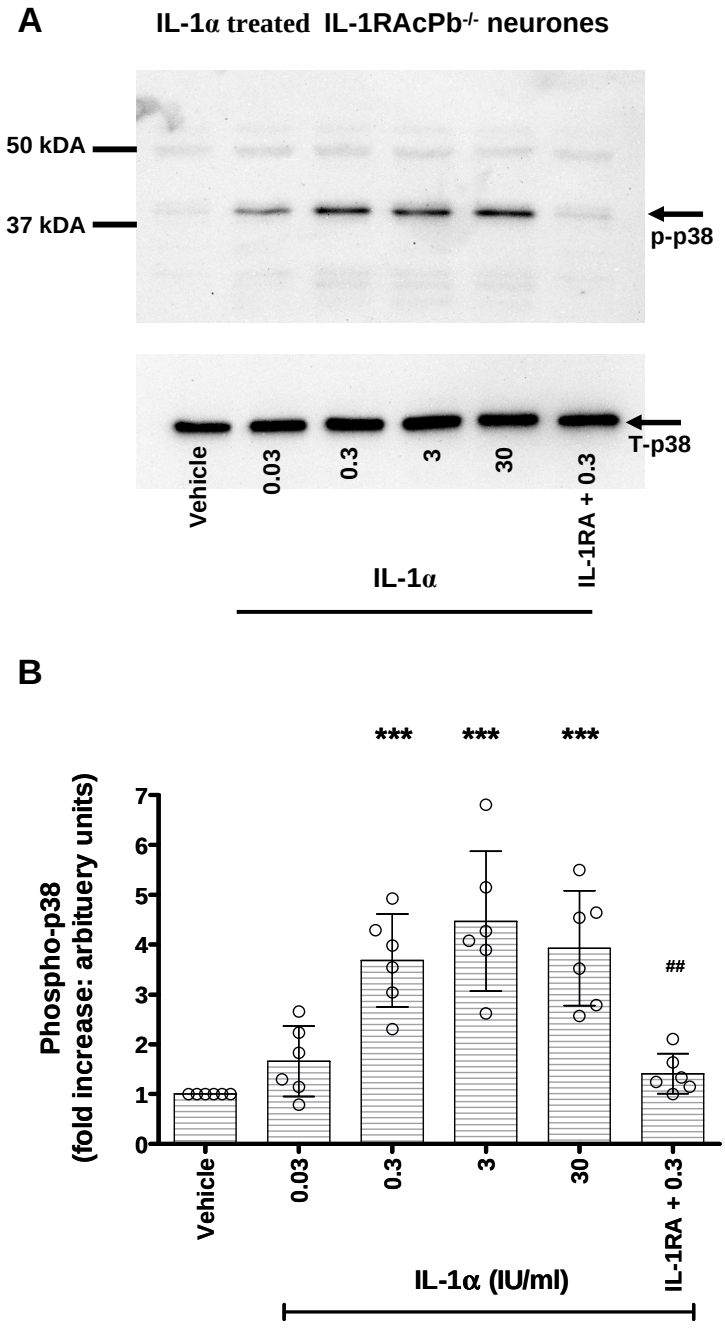


Figure 4.4. IL-1α-induced p38 activation in IL-1RAcPb$^{-/-}$ neurones

IL-1RAcPb$^{-/-}$ neuronal cultures were treated with vehicle or IL-1α (0.03-30 IU/ml) for 15 min, in the presence or absence of IL-1RA (1 μg/ml). Lysates were collected and analysed for p38 activity by Western blot (**A**). **B**. ImageQuant analysis of blot showed that IL-1α induced p38 activation in a concentration-dependent manner (n=6) IL-1RA (1 μg/ml) co-incubation with IL-1α (0.3 IU/ml) significantly reduced IL-1α-induced p38 activation. One-way ANOVA and Tukey's multiple comparison tests. *** P< 0.001 IL-1α vs. vehicle and ## P< 0.01 IL-1α (0.3 IU/ml) vs. IL-1RA co-incubation.

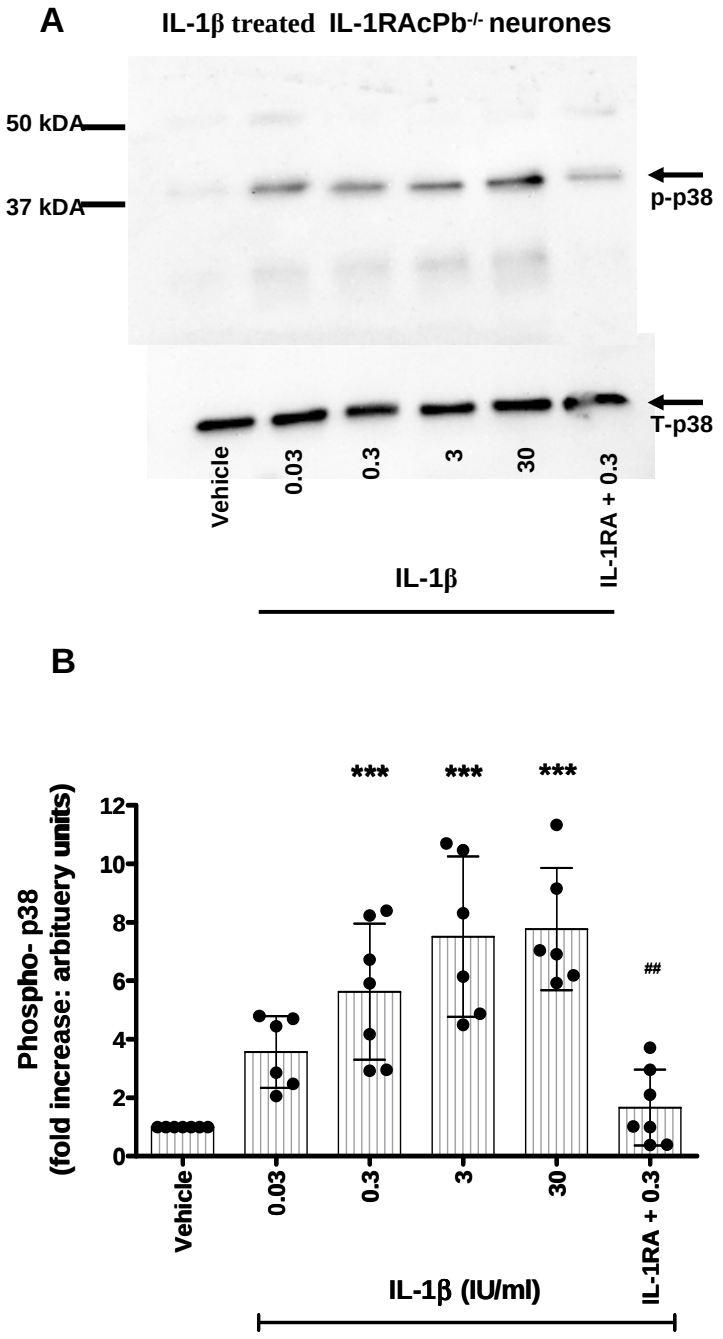


Figure 4.5. IL-1β-induced p38 activation in IL-1RAcPb$^{-/-}$ neurones

IL-1RAcPb$^{-/-}$ neuronal cultures were treated with vehicle or IL-1β (0.03-30 IU/ml) for 15 min, in the presence or absence of IL-1RA (1 μg/ml). Lysates were collected and analysed for p38 activity by Western blot (**A**). **B**. ImageQuant analysis of blot showed that IL-1β induced p38 activation in a concentration-dependent manner (n=7). IL-1RA (1 μg/ml) co-incubation with IL-1β (0.3 IU/ml) significantly inhibited IL-1β-induced p38 activation. One-way ANOVA and Tukey's multiple comparison tests. *** P< 0.001 IL-1β vs. vehicle. ## P< 0.01 IL-1β (0.3 IU/ml) vs. IL-1RA co-incubation.

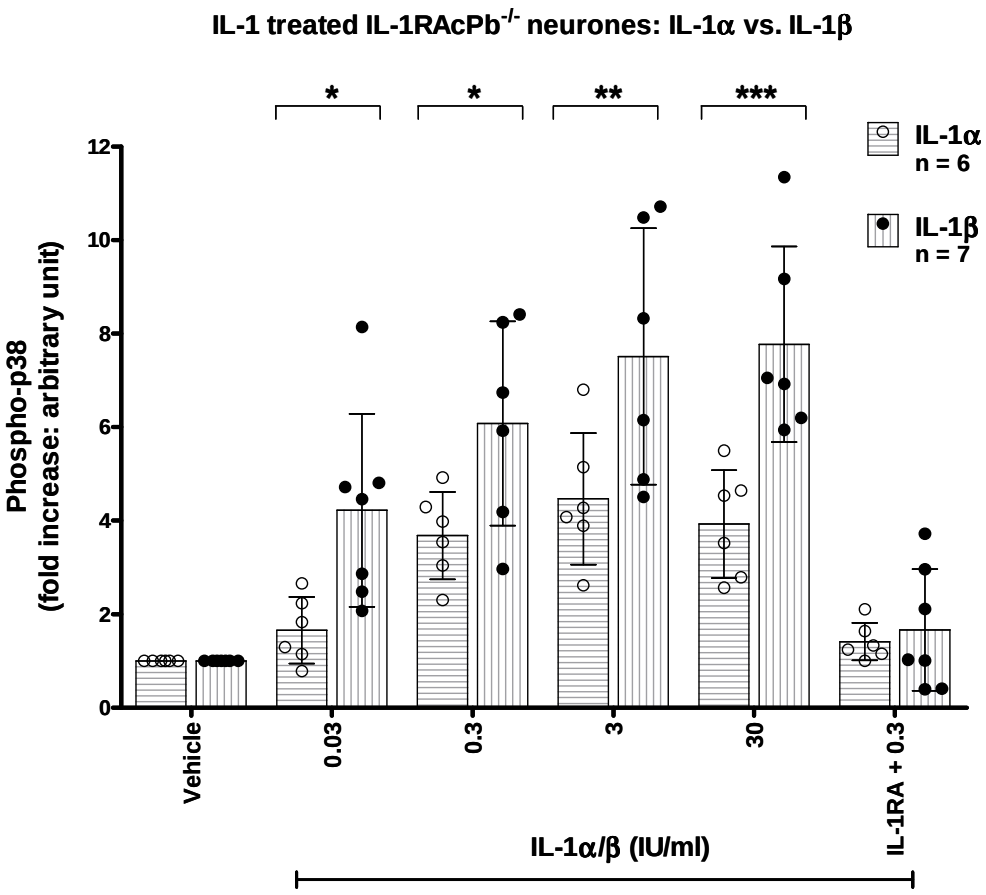


Figure 4.6. Comparison of IL-α- and IL-1β-induced p38 activation in IL-1RAcPb$^{-/-}$ neurones.

Comparison of the level of p38 activation by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), or IL-1RA (1 μg/ml) co-incubation showed that IL-1α-induced p38 activation was significantly less than IL-1β-induced p38 activation in IL-1RAcPb$^{-/-}$ neurones. This reduction in IL-1α-induced p38 activation was seen to all concentrations of IL-1 tested (0.03-30 IU/ml). * P< 0.05, ** P< 0.01 and *** P< 0.001 IL-1α vs. IL-1β. Two-way ANOVA and Bonferroni post hoc test.

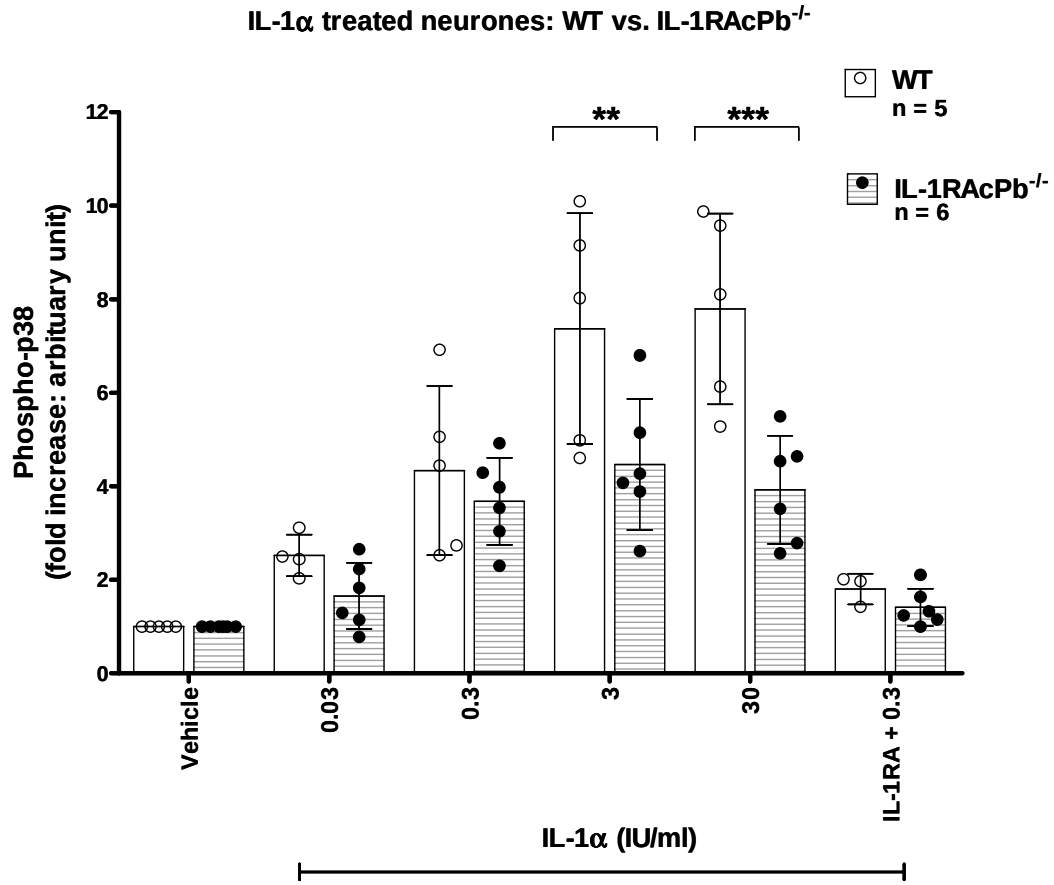


Figure 4.7. Comparison of IL-α-induced p38 activation in WT and IL-1RAcPb$^{-/-}$ neurones

Comparison of the level of p38 activation by vehicle, IL-1α (0.03-30 IU/ml) or IL-1RA (1 μg/ml) co-incubation showed that IL-1α-induced p38 activation was significantly less in IL-1RAcPb$^{-/-}$ neurones compared to WT neurones at higher concentrations (3-30 UI/ml). No significant difference was observed for the lower concentrations of IL-1α (0.03-0.3 IU/ml). * P< 0.05, ** P< 0.01 and *** P< 0.001 WT vs. IL-1RAcPb$^{-/-}$ neurones. Two-way ANOVA and Bonferroni post hoc test.

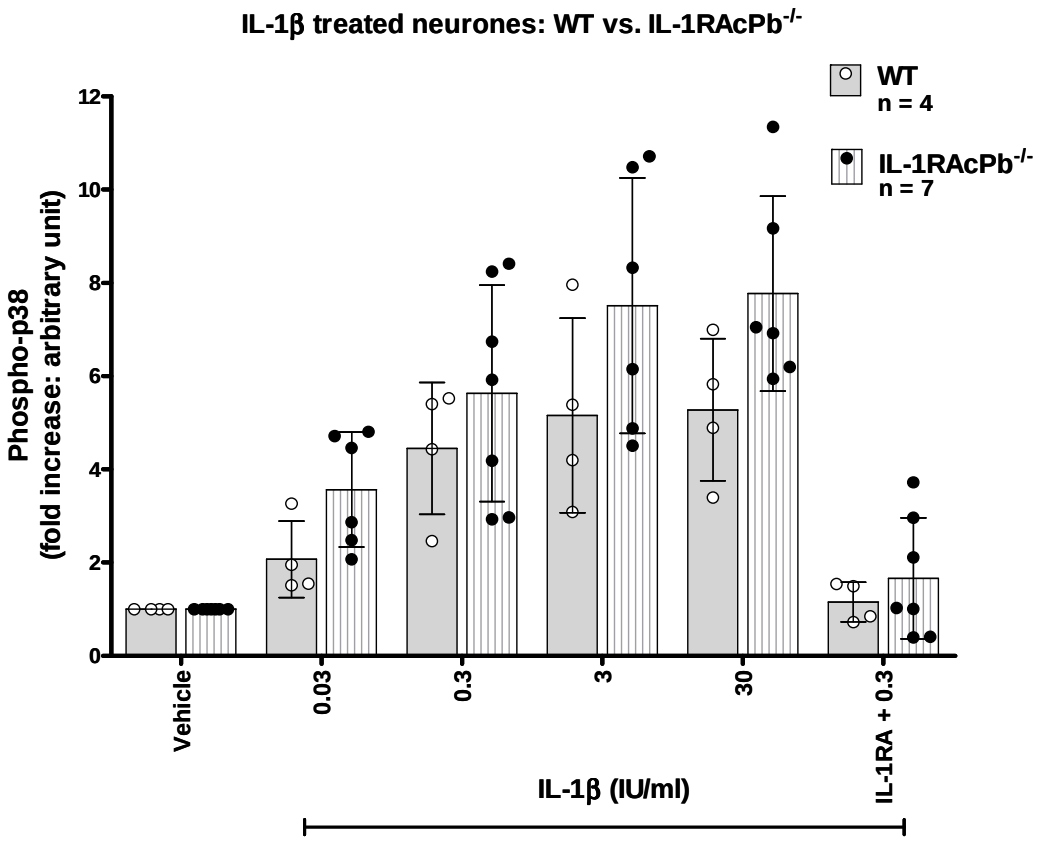


Figure 4.8. Comparison of IL-β-induced p38 activation in WT and IL-1RAcPb$^{-/-}$ neurones

Comparison of the level of p38 activation by vehicle, IL-1β (0.03-30 IU/ml) or IL-1RA (1 μg/ml) co-incubation showed that IL-1β-induced p38 activation in WT and IL-1RAcPb$^{-/-}$ neurones with equal potency. No significant difference was observed for the any of the concentrations of IL-1β tested. Two-way ANOVA and Bonferroni post hoc test.

4.4.2. The effects of IL-1 on ERK 1/2 signalling in neurones.

The effect of IL-1β on ERK1/2 signalling was tested in neurones. Vehicle treated cells showed a high basal level of ERK1/2 activity and IL-1β treatment did not further induce ERK1/2 activation even at the highest concentration (30 IU/ml) (Figure 4.9A). Phosphorylation of the two isoforms of ERK (p42 and p44) remained constant in the presence of IL-1β (0.03-30 IU/ml). Co-incubation of IL-1β (0.3 IU/ml) and IL-1RA (1 μg/ml) had no effect on ERK1/2 phosphorylation. Levels of total (non-phosphorylated and phosphorylated) ERK1/2 were identical in all treatment groups, indicating that sample loading was constant. Semi-quantitative analyses of the blots confirmed that IL-1β did not activate ERK1/2 in WT neurones (Figure 4.9B).

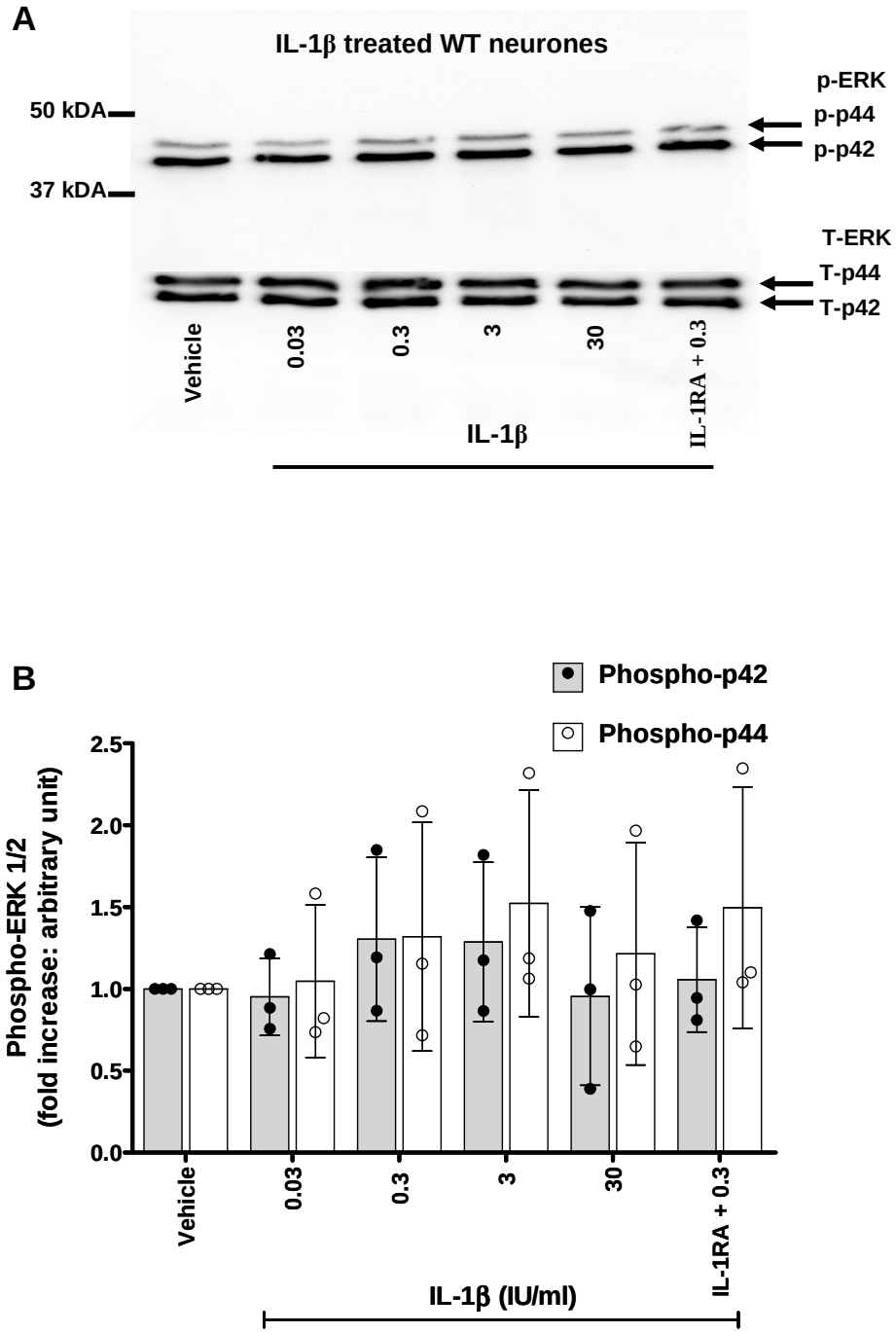


Figure 4.9. The effects of IL-1β on ERK1/2 activation in WT neurones.

WT neuronal cultures were treated with vehicle or IL-1β (0.03-30 IU/ml) for 15 min, in the presence or absence of IL-1RA (1 μg/ml). Lysates were collected and analysed for ERK1/2 activity by Western blot (**A**). Blot shown is representative of 3 independent experiments. **B**. ImageQuant analysis of blot showed that IL-1β did not activate ERK1/2 in WT neurones (n=3). IL-1RA (1 μg/ml) co-incubation with IL-1β (0.3 IU/ml) did not affect ERK1/2 activity. One-way ANOVA and Tukey's multiple comparison test was used to compare IL-1β treated group against vehicle treatment.

4.4.3. The effects of IL-1 on Src kinase signalling in neurones.

WT neurones treated with vehicle, IL-1α or IL-1β showed similar level of Src kinase activation (Figure 4.10). Co-incubation of IL-1RA (1 μg/ml) with IL-1 had no effect on Src kinase activation in neurones. .

Similarly, Src kinase was not activated in IL-1RAcPb$^{-/-}$ neurones by IL-1α or IL-1β treatments (Figure 4.11).

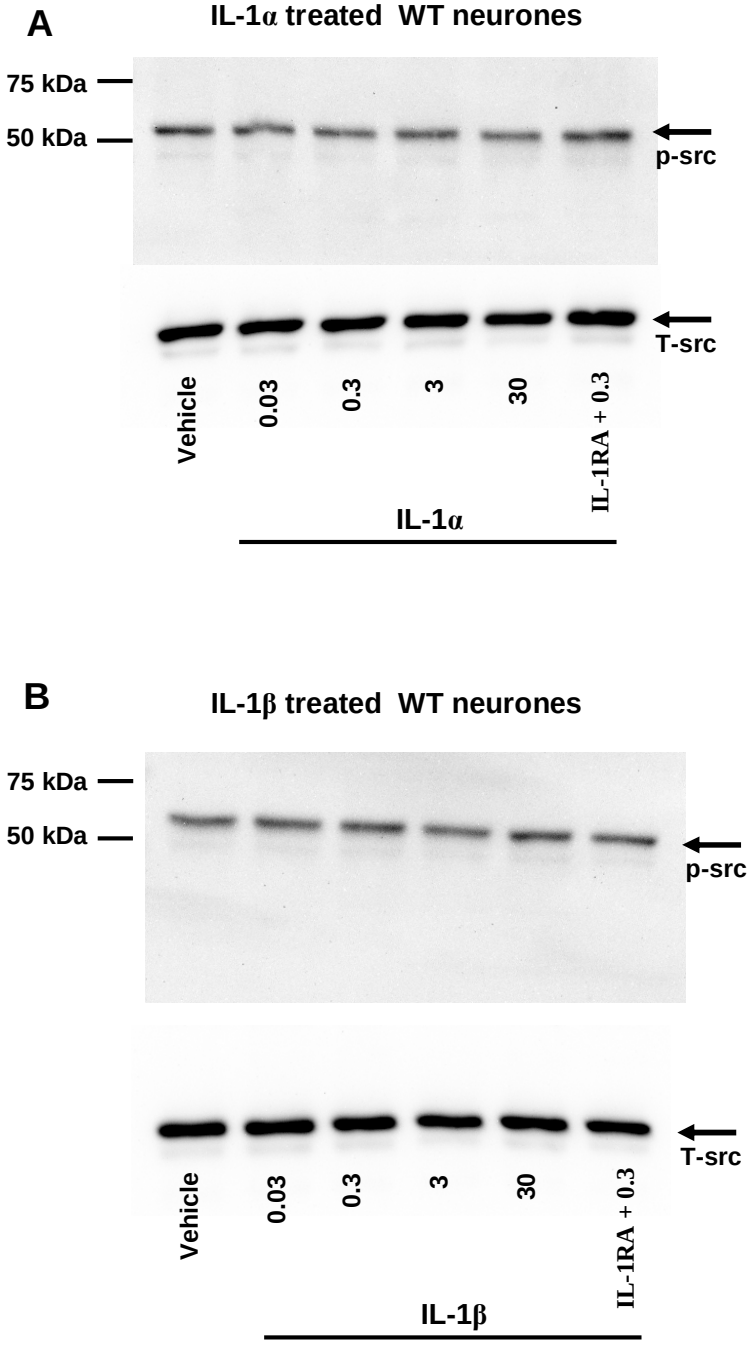


Figure 4.10. The effects of IL-1α and IL-1β in Src kinase activity in WT neurones

WT neuronal cultures were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 15 min, in the presence or absence of IL-1RA (1 μg/ml). Lysates were collected and analysed for Src kinase activity by Western blot. IL-1α (**A**) or IL-1β (**B**) treatments failed to induce Src kinase activity in WT neurones. IL-1RA (1 μg/ml) co-incubation with IL-1 (0.3 IU/ml) did not affect Src kinase activation. Blots shown are representative of 5 independent experiments. Blots were not analysed as no induction of Src kinase phosphorylation by IL-1 was observed.

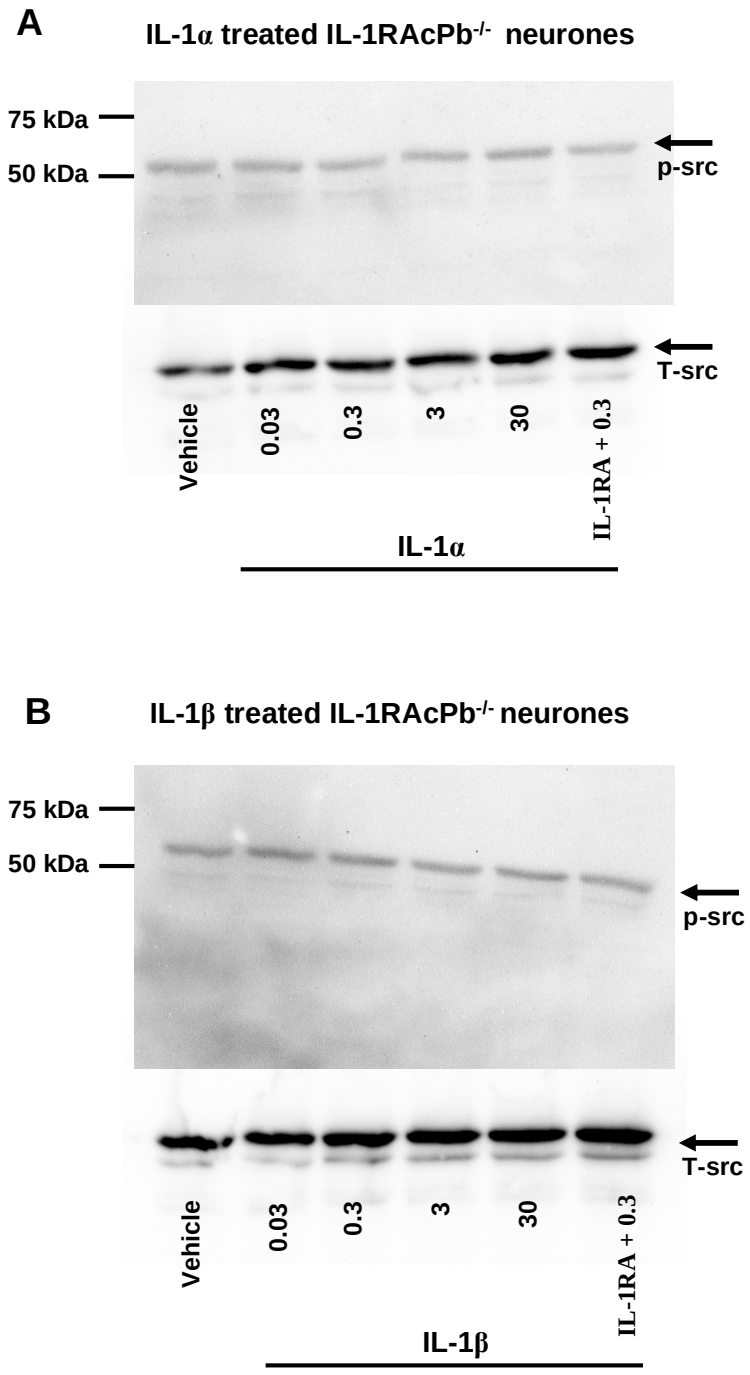


Figure 4.11. The effects of IL-1α and IL-1β in Src kinase activity in IL-1RAcPb$^{-/-}$ neurones

IL-1RAcPb$^{-/-}$ neuronal cultures were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 15 min in the presence or absence of IL-1RA (1 μg/ml). Lysates were collected and analysed for Src kinase activity by Western blot. IL-1α (**A**) or IL-1β (**B**) treatments failed to induce Src kinase activity in IL-1RAcPb$^{-/-}$ neurones. IL-1RA (1 μg/ml) co-incubation with IL-1 (0.3 IU/ml) did not affect Src kinase activation. Blots shown are representative of 3 independent experiments Blots were not analysed as no induction of Src kinase phosphorylation by IL-1 was observed.

4.4.4. IL-1-induced p38 MAPK signalling in glia

Western blot analysis showed that p38 was strongly induced by IL-1α and IL-1β in both WT (Figure 4.12) and IL-1RAcPb$^{-/-}$ glia (Figure 4.13). This response was specific to IL-1 as it was completely blocked by IL-1RA. However, due to undetected p38 activity in vehicle-treated cultures, it has not been possible to quantify the level of IL-α- or IL-1β-induced p38 in glia.

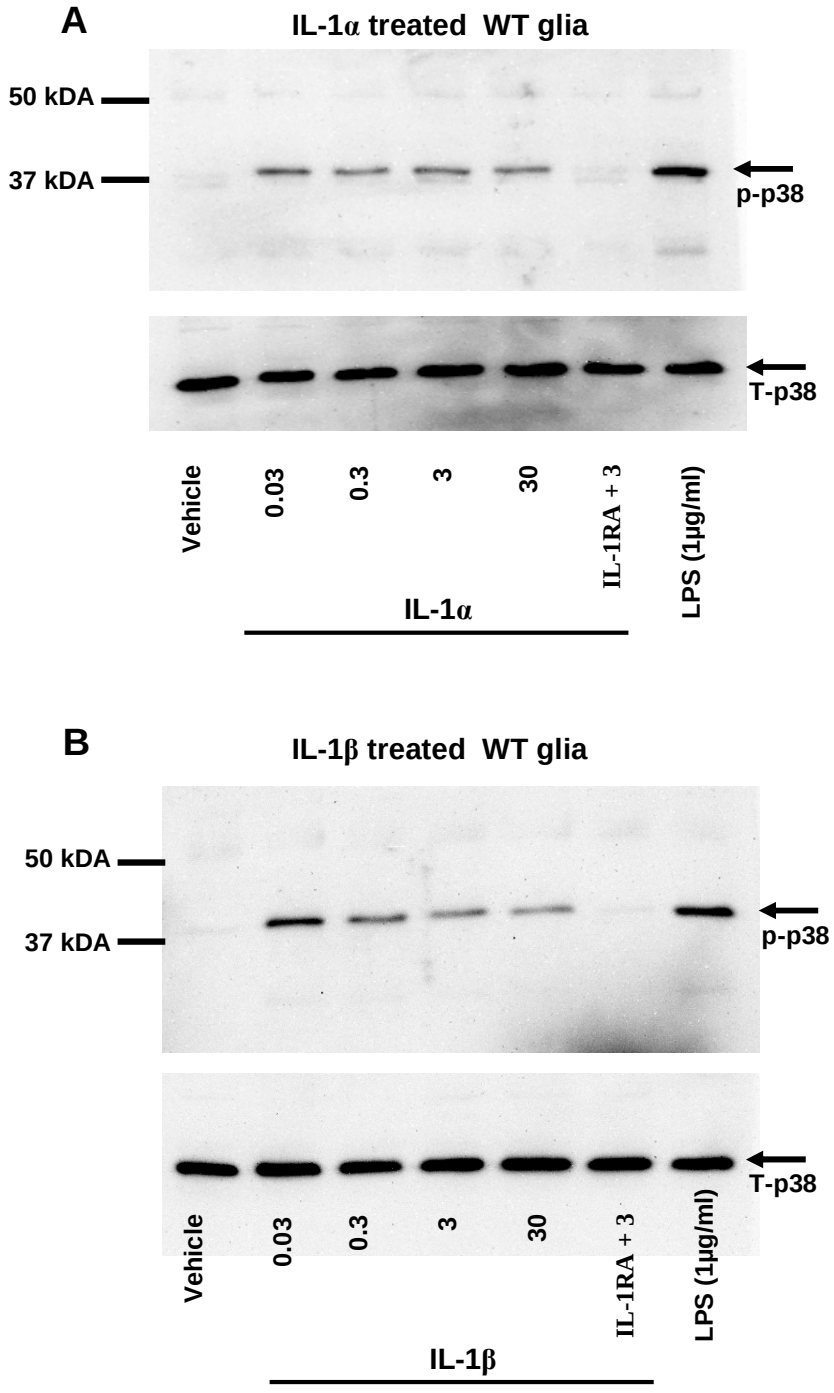


Figure 4.12. IL-1α- and IL-1β-induced p38 activation in WT glial cells

WT glial cultures were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 30 min, in the presence or absence of IL-1RA (10 µg/ml). Lysates were collected and analysed for p38 activation by Western blot. IL-1α (**A**) and IL-1β (**B**) induced p38 activation at all concentrations tested (0.03-30 IU/ml). IL-1RA (1 µg/ml) co-incubation with IL-1α (3 IU/ml) or IL-1β (3 IU/ml) inhibited IL-1-induced p38 activation in WT glial cultures. Blot shown are representative of four independent experiments.

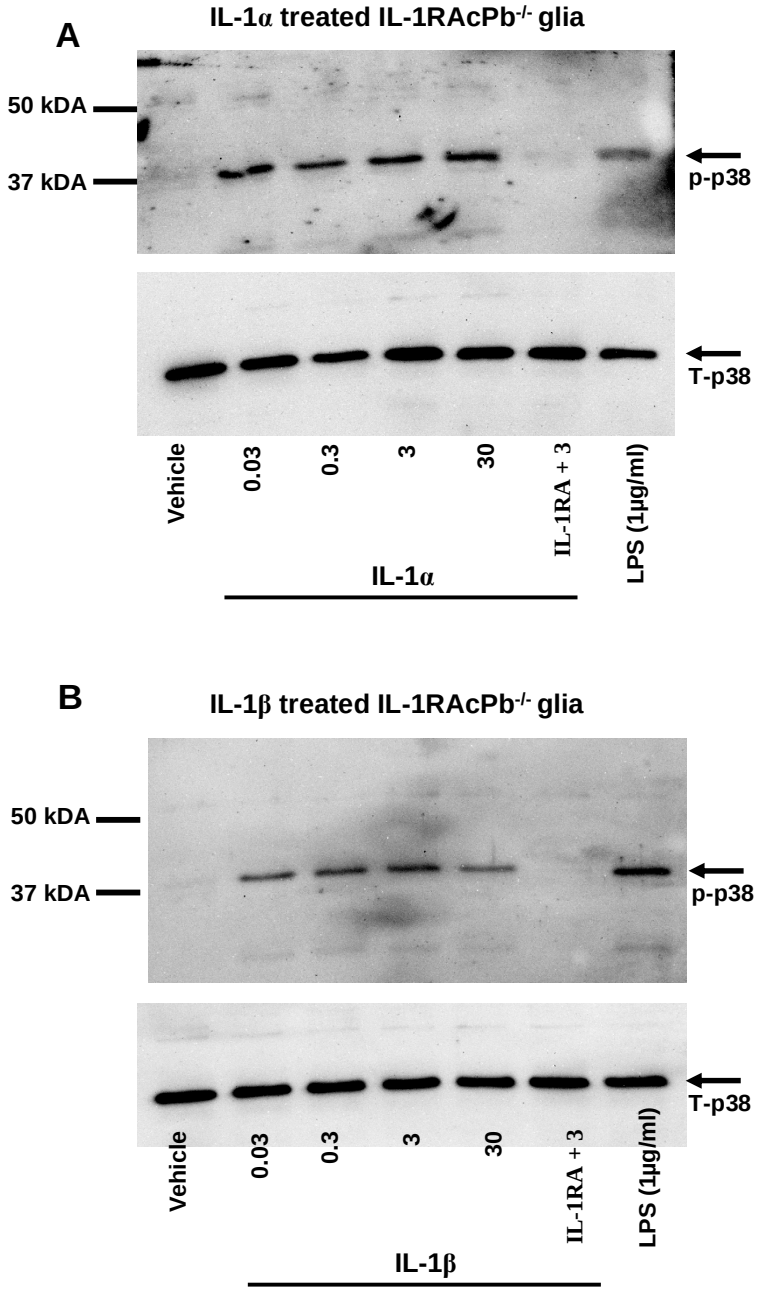


Figure 4.13 IL-1α- and IL-1β-induced p38 activation in IL-1RAcPb$^{-/-}$ glial cells

IL-1RAcPb$^{-/-}$ glial cultures were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 30 min, in the presence or absence of IL-1RA (10 µg/ml). Lysates were collected and analysed for p38 activation by Western blot. IL-1α (**A**) and IL-1β (**B**) induced p38 activation at all concentrations tested (0.03-30 IU/ml). IL-1RA (10 µg/ml) co-incubation with IL-1α or IL-1β inhibited IL-1-induced p38 activation in IL-1RAcPb$^{-/-}$ glia. Blots shown in (**A**) are representative of 3 independent experiments and blots shown in (**B**) are representative of 2 independent experiments. Blots were not quantified

4.4.5. IL-1-induced ERK1/2 signalling in glial cells

In WT glial cultures, IL-1α (0.03-30 IU/ml) induced significant ERK1/2 activation compared to vehicle treatment (Figure 4.14A). However, increasing concentrations of IL-1α did not induce increasing levels of ERK1/2 activation, and the lowest concentration of IL-1α (0.03 IU/ml) induced a near maximal response (two fold increase in ERK1/2 activation).

IL-1β (0.3-30 IU/ml) induced ERK1/2 activation in a concentration-dependent manner (Figure 4.14B). At 0.03 IU/ml of IL-1β, ERK1/2 activation was 1.5 fold higher compared to vehicle treatment, but this was not significant. At higher concentrations (0.3-30 IU/ml) IL-1β significantly induced ERK1/2 activation compared to vehicle treatment. 3 IU/ml IL-1β induced optimal ERK1/2 activation (2.3 fold increase compared to vehicle treatment) IL-1α- and IL-1β-induced ERK1/2 activation were abolished by co-incubation with IL-1RA (10 μg/ml).

IL-1α- and IL-1β-induced ERK1/2 activity in WT glial cultures were then compared to determine possible differential effects of these two cytokines on the activation of the ERK1/2 signalling pathway in glia. This comparison revealed that IL-1α and IL-1β induced ERK1/2 activation in WT glia with similar potency (Figure 4.15).

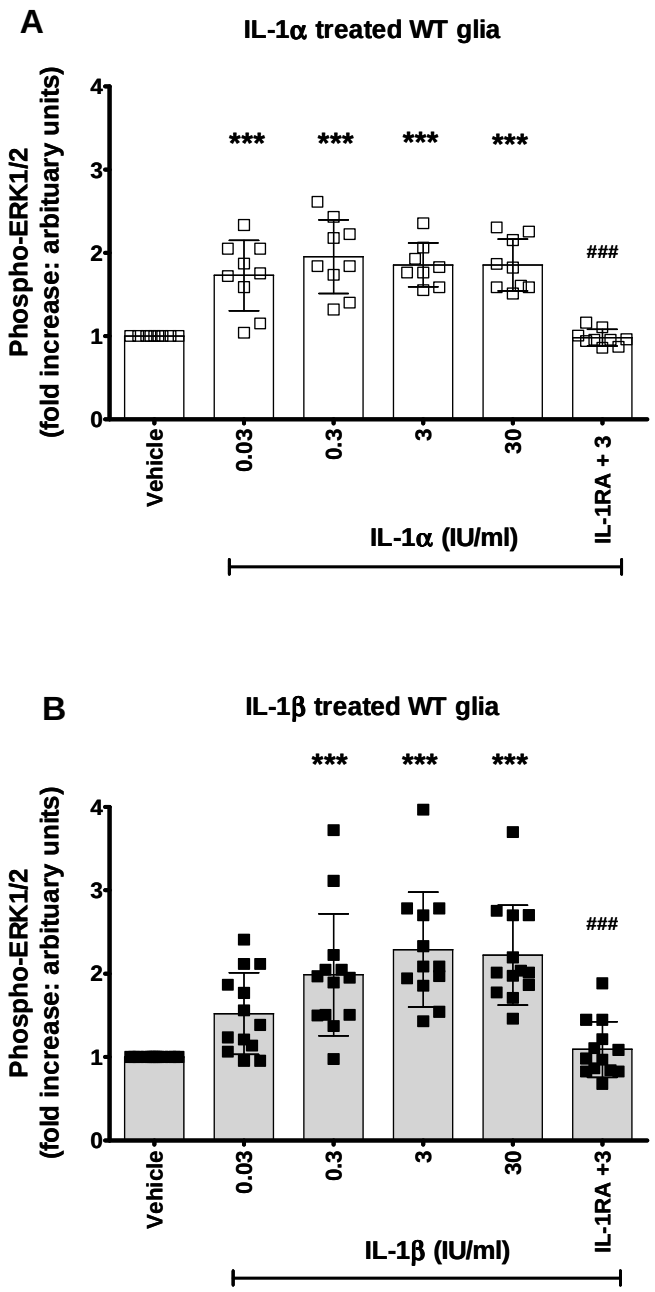


Figure 4.14. IL-1α- and IL-1β-induced ERK1/2 activation in WT glial cells

WT glial cultures were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 30 min in the presence or absence of IL-1RA (10 μg/ml). Cell lysates were collected and analysed for phosphorylated ERK1/2 by ELISA. IL-1α (**A**) and IL-1β (**B**) induced significant levels of phosphorylated ERK1/2 compared to vehicle treatment. IL-1α (n=9) induced ERK1/2 activation for all the concentrations of IL-1α tested (0.03-30 IU/ml). IL-1β (n=13) induced significant ERK1/2 activation at 0.3-30 IU/ml but not at the lower concentration of 0.03 IU/ml. These responses were blocked by IL-1RA (10 μg/ml). One-way ANOVA and Tukey's multiple comparison tests. *** P< 0.001 IL-1 vs. vehicle. ### P< 0.001 IL-1β (0.3 IU/ml) vs. IL-1RA co-incubation.

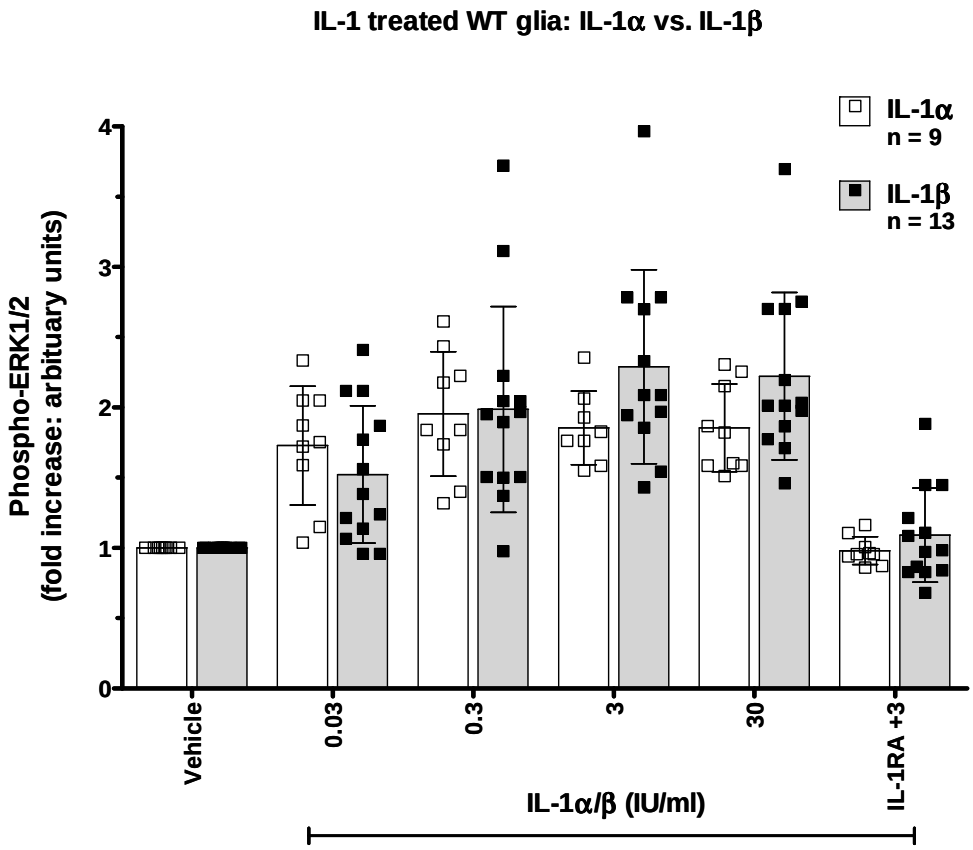


Figure 4.15. Comparison of IL-1α- and IL-1β-induced ERK1/2 activation in WT glial cultures.

Comparison of the level of ERK1/2 activation by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), or IL-1RA (10 μg/ml) co-incubation showed that IL-1α and IL-1β induced similar levels of phosphorylated ERK1/2 for all treatment groups in WT glia. No significant difference between IL-1α- and IL-1β-induced ERK1/2 activation was found for any concentrations of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test.

In IL-1RAcPb$^{-/-}$ glial cultures, IL-1α at 0.3 IU/ml and 30 IU/ml significantly induced ERK1/2 activation compared to vehicle treatment. However, at 0.03 IU/ml and 3 IU/ml, IL-1α induced ERK1/2 activation was not significantly different to vehicle-treated cultures. The concentration of IL-1α that induced maximal ERK1/2 activation in IL-1RAcPb$^{-/-}$ glial cultures was 0.3 IU/ml, which induced a 2.5 fold increase compared to vehicle treatment (Figure 4.16A).

IL-1β induced ERK1/2 activation in a concentration-dependent manner in IL-1RAcPb$^{-/-}$ glial cultures (Figure 4.16B). At the lowest concentration (0.03 IU/ml), IL-1β induced a slight but non-significant ERK1/2 activity compared to vehicle treatment. Increasing IL-1β concentrations induced increased ERK1/2 activation, with a peak at 3 IU/ml (2.3 fold increase compared to vehicle). This response was inhibited by IL-1RA (10 μg/ml). IL-1α and IL-1β induced ERK1/2 activation in IL-1RAcPb$^{-/-}$ glia with similar potency (Figure 4.17). IL-1α (Figure 4.18) and IL-1β (Figure 4.19) induced ERK1/2 activation to a similar level in both WT and IL-1RAcPb$^{-/-}$ glial cultures.

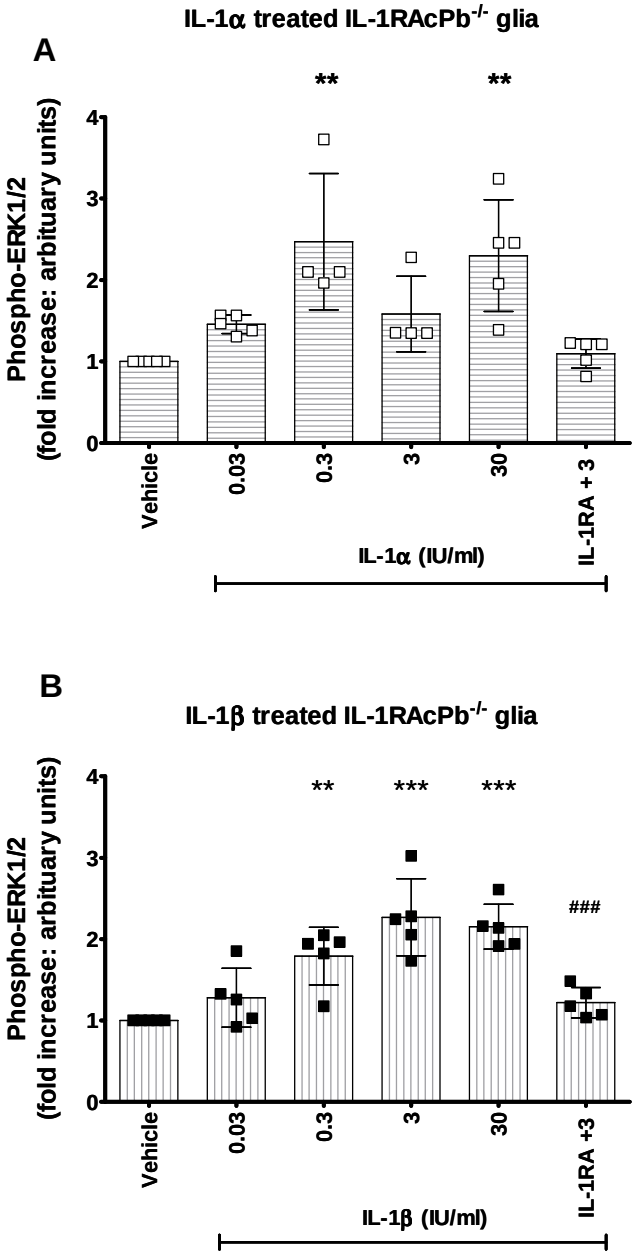


Figure 4.16. IL-1α- and IL-1β-induced ERK1/2 activation in IL-1RAcPb$^{-/-}$ glial cells

IL-1RAcPb$^{-/-}$ glial cultures were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 30 min in the presence or absence of IL-1RA (10 μg/ml). Cell lysates were collected and analysed for phosphorylated ERK1/2 by ELISA. IL-1α (**A**) and IL-1β (**B**) induced significant levels phosphorylated ERK1/2 compared to vehicle. IL-1α (n=5) induced ERK1/2 activation significantly at 0.3 and 30 IU/ml. IL-1β (n=5) induced significant level of phosphorylated ERK1/2 expression at higher concentrations (0.3-30 IU/ml) but not at the lower concentration of 0.03 IU/ml. IL-1β-induced ERK1/2 activation was blocked by IL-1RA (10 μg/ml). One-way ANOVA and Tukey's multiple comparison tests. ** P< 0.01 and *** P< 0.001 IL-1 vs. vehicle. ### P< 0.001 IL-1β (0.3 IU/ml) vs. IL-1RA co-incubation.

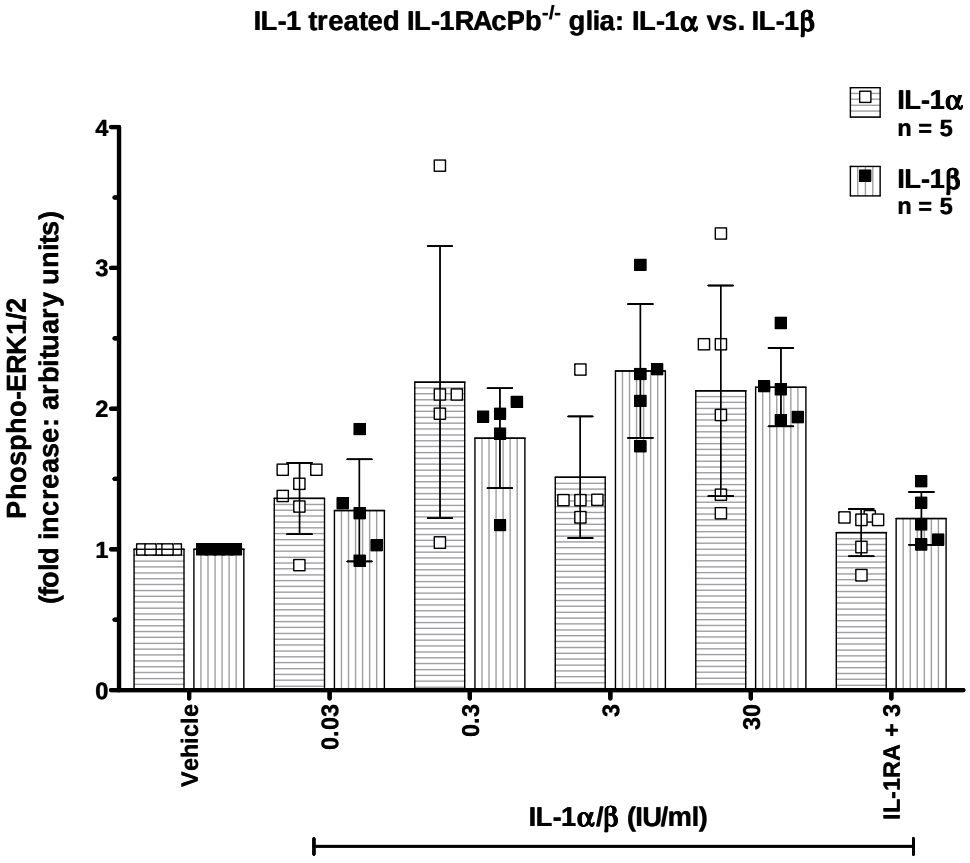


Figure 4.17. Comparison of IL-1α- and IL-1β-induced ERK1/2 activation in IL-1RAcPb$^{-/-}$ glial cells.

Comparison of the level of ERK1/2 activation by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml) or IL-1RA (10 μg/ml) co-incubation showed that IL-1α and IL-1β induced similar levels of phosphorylated ERK1/2 for all treatment groups in IL-1RAcPb$^{-/-}$ glia. No significant difference between IL-1α- and IL-1β-induced ERK1/2 activation was found for any concentrations of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test.

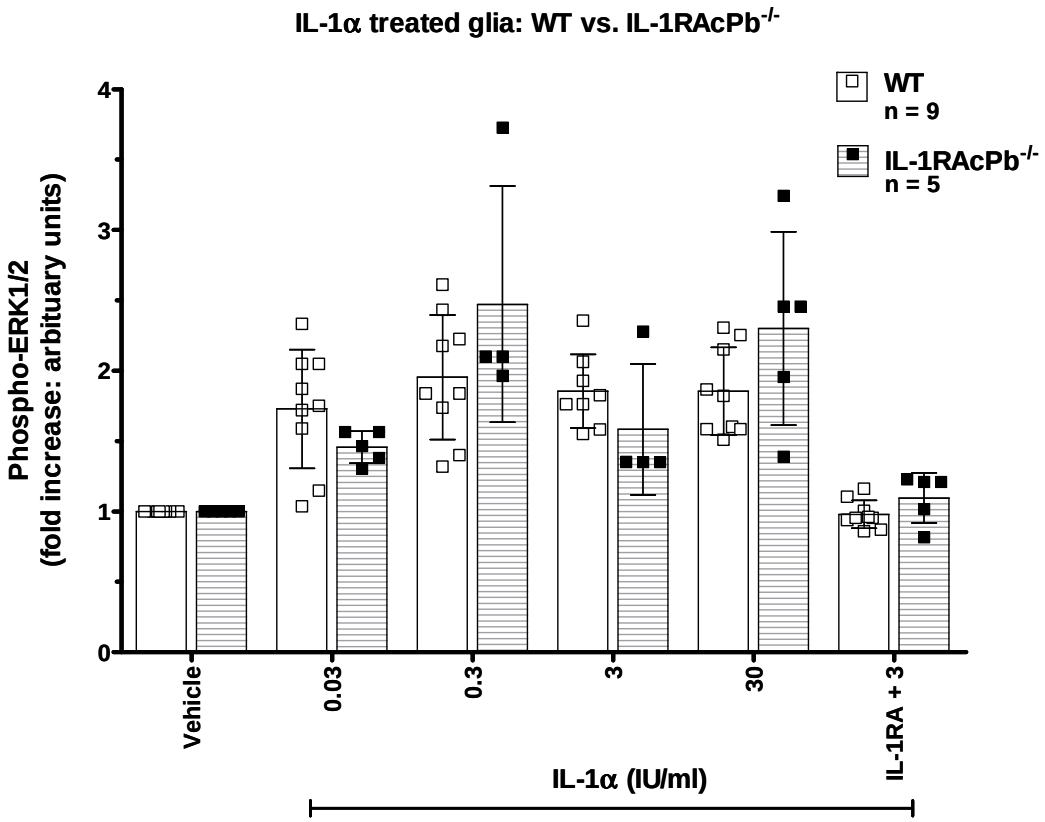


Figure 4.18. Comparison of IL-1α-induced ERK1/2 activation in WT and IL-1RAcPb$^{-/-}$ glial cells

Comparison of the level of ERK1/2 activation by vehicle, IL-1α (0.03-30 IU/ml) or IL-1RA (10 μg/ml) co-incubation showed that IL-1α-induced ERK1/2 activation in WT and IL-1RAcPb$^{-/-}$ glia with similar potency. No significant difference was observed for any of the concentrations of IL-1α tested. Two-way ANOVA and Bonferroni post hoc test.

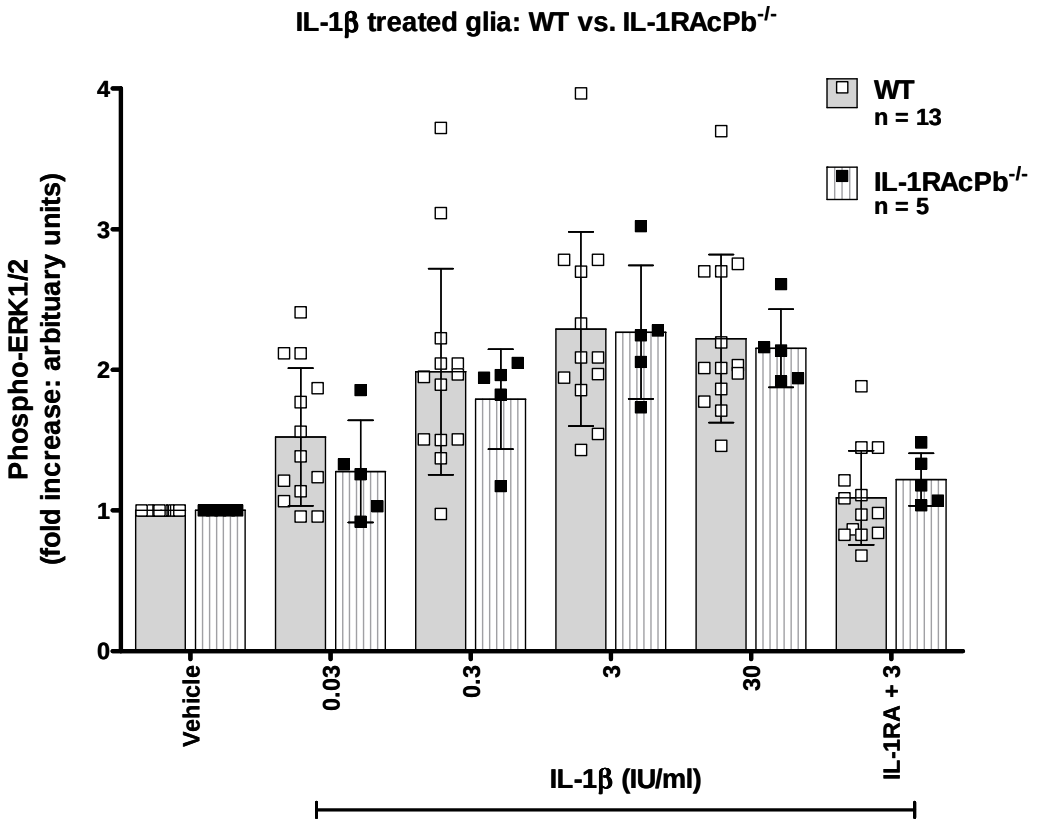


Figure 4.19. Comparison of IL-1β-induced ERK1/2 activation in WT and IL-1RAcPb$^{-/-}$ glial cultures

Comparison of the level of ERK1/2 activation by vehicle, IL-1β (0.03-30 IU/ml) or IL-1RA (10 μg/ml) co-incubation showed that IL-1β-induced ERK1/2 activation in WT and IL-1RAcPb$^{-/-}$ glia with similar potency. No significant difference was observed for any of the concentrations of IL-1β tested. Two-way ANOVA and Bonferroni post hoc test.

4.4.6. The effects of IL-1 on Src kinase signalling in glia.

In WT glia, vehicle treatment and IL-1 treatment induced similar levels of Src kinase phosphorylation (Figure 4.20). Co-incubation of IL-1RA (10 μg/ml) with IL-1 had no effect on Src kinase phosphorylation in glial cells. IL-1α and IL-1β also failed to induce Src kinase activation in IL-1RAcPb$^{-/-}$ glia (Figure 4.21).

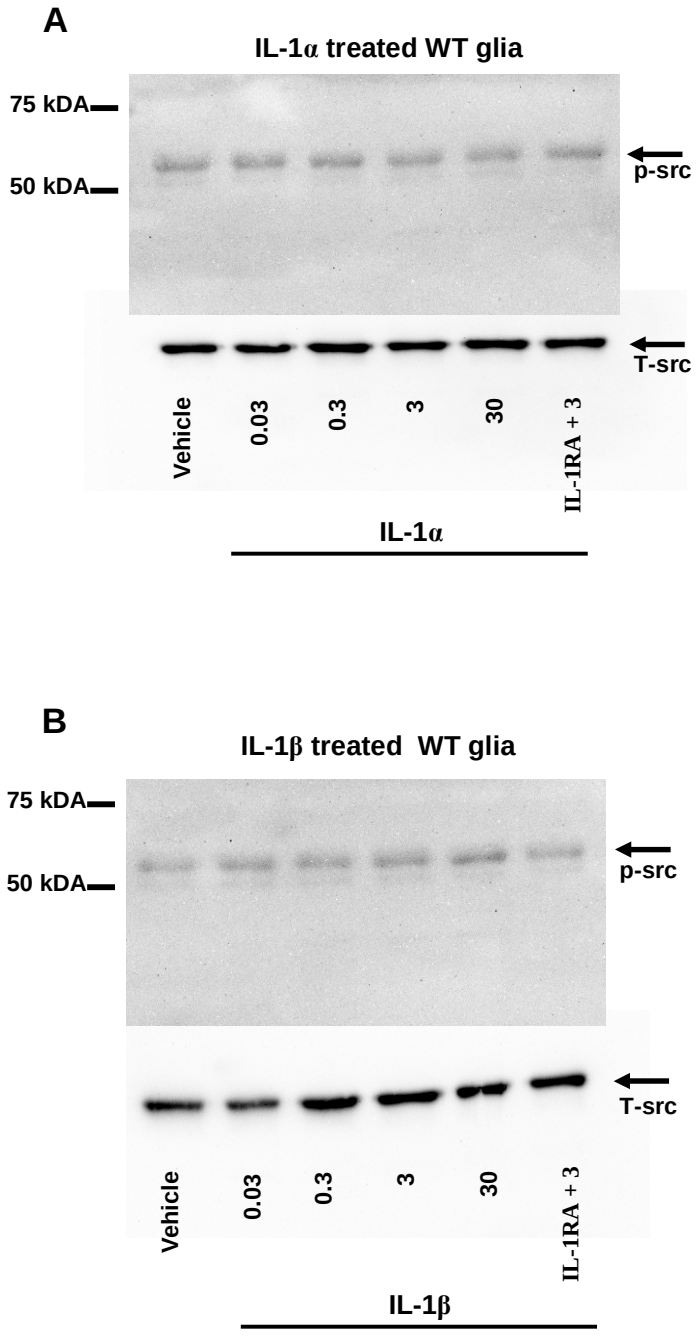


Figure 4.20. The effects of IL-1α and IL-1β on Src kinase activity in WT glial cells

WT glial cultures were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 30 min, in the presence or absence of IL-1RA (10 μg/ml). Lysates were collected and analysed for Src kinase activity by Western blot. IL-1α (**A**) and IL-1β (**B**) treatments failed to induce Src kinase activity in WT glia. IL-1RA (10 μg/ml) co-incubation with IL-1 (3 IU/ml) did not affect Src kinase activation. Blot shown is from one experiment.

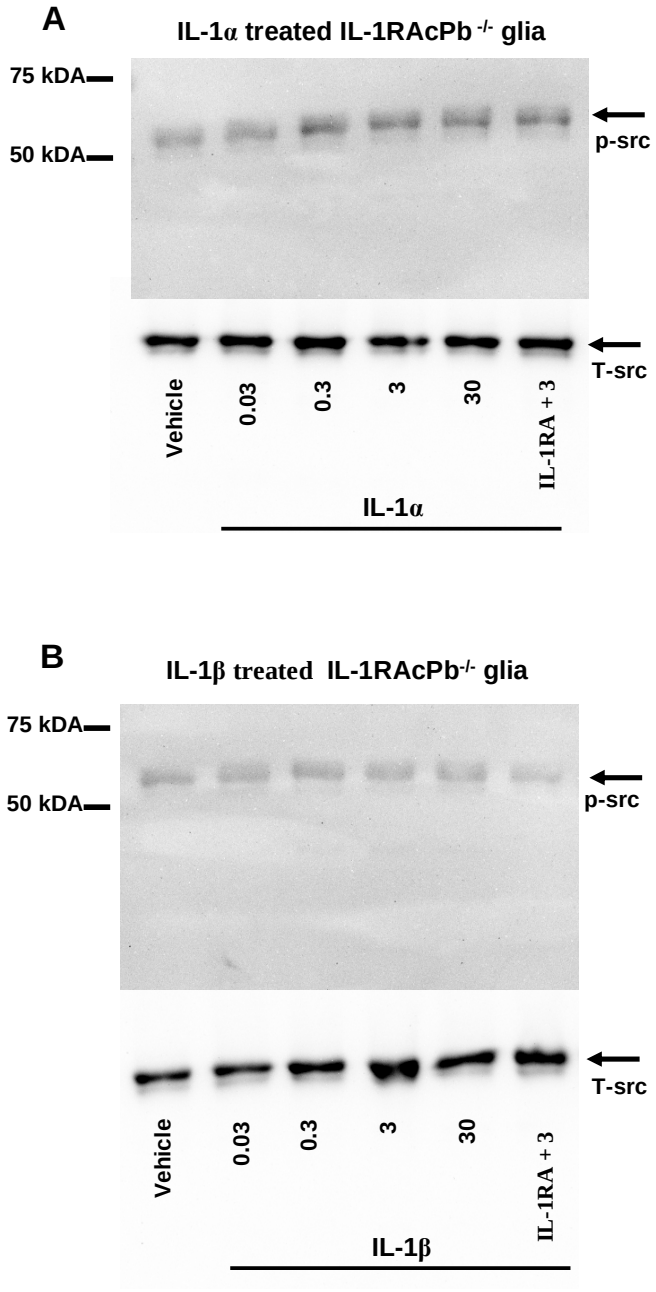


Figure 4.21. The effects of IL-1α and IL-1β on Src kinase activity in IL-1RAcPb$^{-/-}$ glial cells

IL-1RAcPb$^{-/-}$ glial cultures were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 30 min, in the presence or absence of IL-1RA (10 μg/ml). Lysates were collected and analysed for Src kinase activity by Western blot. IL-1α (**A**) and IL-1β (**B**) treatments failed to induce Src kinase activity in IL-1RAcPb$^{-/-}$ glia. IL-1RA (10 μg/ml) co-incubation with IL-1 (3 IU/ml) did not affect Src kinase activation. Blot shown is from one experiment.

4.5. Discussion

4.5.1. IL-1-induced p38 in neurones

IL-1α and IL-1β are potent inducers of IL-6 in both neuronal and glial cultures (Tsakiri et al, 2008c; Parker et al, 2002 and see Chapter 3). In glial cultures IL-1RAcPb may contribute to IL-1α induced IL-6 release; however, in neuronal cultures IL-1RAcPb deletion did not affect IL-1α- or IL-1β-induced IL-6 production (see Chapter 3). It is unclear how IL-1RAcPb may modulate signalling pathways leading to IL-6 synthesis in glial cells or if IL-1RAcPb affects specific signalling pathways in neurones. The aim of this chapter was to investigate the role of IL-1RAcPb in MAPK kinase and Src kinase signalling in neuronal and glial cells.

In this study I have shown that IL-1α and IL-1β are potent activators of p38 MAPK in neuronal cells. IL-1α and IL-1β induced p38 activation similarly in a concentration-dependent manner (Figure 4.1 and Figure 4.2). Published studies on IL-1-induced p38 signalling in neurones are controversial, since some have reported IL-1-induced p38 activation, whilst others failed to demonstrate a response. Indeed, p38 activation by IL-1β has been reported previously in cultured rat hippocampal neurones (Srinivasan et al, 2004) and rat dorsal root ganglion neurones (Binshtok et al, 2008), as well rat cortical neurones (Griffin et al, 2006; Li et al, 2003). Conversely, IL-1β did not induce p38 activation in cultured mouse cortical neurones (Tsakiri et al, 2008a). These studies indicate that IL-1-induced p38 activation in neurones could be species dependent, strain dependent, or brain region specific. Neurones used in this study and those previously by Tsakiri et al (2008a) were mouse cortical neurones, however with the same concentration of IL-1β and duration of treatment (15 min), we detected IL-1-induced p38 activation whilst Tsakiri et al (2008a) did not. It is unclear what is the cause of this discrepancy, but could be attributed to differences in mouse strain and culturing protocol. In this study C57/BL6 mice were used, and Tsakiri et al (2008a) used C57/BL6 x 129sv mice. The method in which cells were cultured in this study was distinctly different to Tsakiri et al. (2008a). The method used by Tsakiri et al. (2008a) involved trituration to homogenise the brain tissue, however, in our study, the brain tissue was first chemically dissociated by incubation in a

dissociation medium (Table 7.2, Appendix I) before being mechanically dissociated by trituration. Additionally, in our study, the neurones were maintained in medium that contained 5% PDS whereas Tsakiri et al (2008a) were grown in serum-free medium. These differences may affect the metabolic state of the neurones and their susceptibility to external stimulation.

Activation of p38 signalling pathway has been shown to result in the production of proinflammatory mediators such as IL-1β, TNF-α and IL-6 (Bergamaschi et al, 2006; Chaparro-Huerta et al, 2005; Kim et al, 2004), which may contribute to the damaging effects of inflammation. p38 is strongly activated after cerebral ischaemia in rats and is associated with neuronal death and functional deficit (Barone et al, 2001b). Therefore variations in p38 activation between species and brain tissue could account for the varied susceptibility of rodent species and brain regions to injury.

Differential effects of IL-1α and IL-1β on IL-6 induction and synthesis in mouse cortical neurones and glial cells have been reported previously (Tsakiri et al, 2008c; Andre et al, 2005b). However, it is unclear what mechanisms are responsible for this differential effect. IL-1α and IL-1β have been shown to activate identical signalling pathways (p38, ERK1/2 and JNK) in primary mouse mixed glial cells. This was demonstrated for all of the concentrations of IL-1 tested (0.3 IU/ml, 3 IU/ml, 30 IU/ml and150 IU/ml) and time points investigated (5 min, 15 min, 30 min and 60 min) (Andre et al, 2005b). In this study, IL-1α and IL-1β were equally potent at inducing p38 activation in WT mouse cortical neurones (Figure 4.3) at 15 min, which suggests that the differential action of IL-1α- and IL-1β-induced IL-6 in neurones could not be associated with differences in IL-1α- or IL-1β-induced p38 activation. Differences in IL-1α and IL-1β-induced p38 at other time points were not investigated and could potentially be of significant.

The role of IL-1RAcPb in IL-1-induced p38 activation is completely unknown. The data presented here show that IL-1α and IL-1β were potent inducers of p38 activation in IL-1RAcPb$^{-/-}$ neurones (Figure 4.4 and Figure 4.5), indicating that IL-1 signalling in neurones is not mediated by IL-1RAcPb. Additionally, IL-1α induced p38 activation was reduced in IL-1RAcPb$^{-/-}$ neurones compared to IL-1β (Figure 4.6), indicating a modulatory effect of IL-1RAcPb on

IL-1 signalling. Specifically, IL-1RAcPb was shown to contribute to IL-1α-induced p38 activation in neurones (Figure 4.7). IL-1RAcPb deletion had no effect on IL-1β-induced p38 activation in neurones (Figure 4.8). It is unclear how this differential effect of IL-1α- and IL-1β-induced p38 activation may affect the cellular response of neurones.

4.5.2. The effects of IL-1 on ERK1/2 signalling in neurones

ERK1/2 is activated by cerebral ischaemia and brain trauma in rats and is believed to be detrimental (Lu et al, 2005) but can also be neuroprotective (Irving et al, 2000; Kitagawa et al, 1999). It is unclear which mediators are involved in ERK1/2 activation in neurones in acute brain injury. Results from this study showed that in WT neurones, IL-1β failed to induce ERK1/2 activation (Figure 4.9). This is consistent with previous data from our laboratory and other studies in which IL-1β failed to induce ERK1/2 activation in mouse and rat cortical neurones (Soiampornkul et al, 2008; Tong et al, 2008; Tsakiri et al, 2008a) and rat hippocampal neurones (Srinivasan et al, 2004). Interestingly, IL-1β reduces ERK1/2 activation induced by neurotrophic factors such as neurotrophin-3 and BDNF in rat cortical neurones, and associated with that, a reduction in neuronal cell survival (Soiampornkul et al, 2008; Tong et al, 2008). This supports the hypothesis that IL-1 actions are detrimental during neuroinflammation. These studies suggest that neurones are capable of ERK1/2 signalling, but, *in vitro* studies have failed to detect any IL-1β-induced ERK1/2 in neurones. This indicates that IL-1 may induce cell specific signalling. The effects of IL-1α on ERK1/2 activation was not investaged in this study based on the fact that there were no differences between IL-1α and IL-1β-induced IL-6, p38 or Src kinase activation (see next section) in neurones. However, this is not to conclude that there is no difference in the effects of IL-1α and IL-1β on ERK1/2 phosphorylation in neurones.

4.5.3. The effects of IL-1 on Src kinase signalling in neurones

In the current study IL-1α and IL-1β failed to induce Src kinase activation in WT or IL-1RAcPb$^{-/-}$ neurones (Figure 4.10 and Figure 4.11). These

findings indicate that Src kinase does not mediate IL-1 actions on neurones. However, it was difficult to determine the effects of IL-1RAcPb on Src kinase activity in the absence of any Src kinase induction by IL-1α or IL-1β. In our study, basal Src kinase activity was high, as indicated by high Src kinase phosphorylation in the vehicle treated cells. In the presence of this high basal Src kinase activity, subtle IL-1-induced Src kinase phosphorylation maybe masked. Previous studies have demonstrated clear IL-1β-induced Src kinase activation (in the presence of high basal Src kinase phosphorylation) in mouse cortical and hypothalamic neurones (Tsakiri et al, 2008a; Davis et al, 2006) and rat hippocampal neurones (Viviani et al, 2003). IL-1-induced Src kinase activation in neurones occurs rapidly, as early a 2 min after IL-1β treatment and increases in a concentration-dependent manner (Davis et al, 2006). It is unclear what caused the discrepancy between these previous findings and the findings in the current study. One hypothesis is that these differences could be due to differences in species, strain of mouse, or population of neurones studied. In this study, we examined the effects of IL-1 on Src kinase activation in mouse cortical neurones. Tsakiri et al (2008a) also studied mouse cortical neurones, which eliminate differences in species and neuronal population as accountable for the differential results between the two studies. However the mouse strain used in this study was C57/BL6 and Tsakiri et al (2008a) used C57/BL7 x 129sv, which may account for the different results obtained between the two studies. The role of Src kinase activation in neuronal activity is unclear. Studies have shown that Src kinase activation contributes to IL-6 production in neurones (Tsakiri et al, 2008a) *in vitro* which may contribute to the inflammatory response *in vivo.*

4.5.4. IL-1 induced p38 MAPK signalling in glial cells

IL-1α and IL-1β strongly induced p38 activation in mouse mixed glia (Figure 4.12 and Figure 4.13). MAPK signalling is a recognised mechanism by which glial cells respond to external stimuli. Many previous studies have reported p38 activation in glial cells in both rats and mouse glial cultures by IL-1α and IL-1β (Andre et al, 2005b; Parker et al, 2002; Pinteaux et al, 2002; Dunn et al, 2002). In these studies astrocytes are the main cell type activated by IL-1α and IL-1β leading to p38 activation. Significant p38 activation occurred as early as 5

min after incubation with IL-1α (Dunn et al, 2002) but optimum p38 activation occurred 30 min after IL-1 incubation (Andre et al, 2005b; Parker et al, 2002). This time frame of optimum p38 activation is also similar in neurones which occurred at 20 min after IL-1β incubation (Srinivasan et al, 2004). This suggests that the mechanism of p38 activation in glial cells and neurones could be similar. A time course investigation is important when investigating the effects of an uncharacterised compound or the response of an uncharacterised system. However, a time course was not conducted in the present study as the incubation period was the same as that reported to be optimum for IL-1-induced MAPK activation in glial cells (Andre et al, 2005). It was difficult to determine the level of IL-1α- and IL-1β- induced p38 activation in glial cultures as there was no detectable p38 activation in the vehicle-treated group. We could not present the data in absolute value because each blot had different exposure time and could not be directly compared. As it has not been possible to quantify the level of p38 induced by IL-1, we could not determine the differential effects of IL-1α and IL-β or determine the role of IL-1RAcPb in IL-1-induced p38.

To clarify the role of IL-1RAcPb in IL-1-induced signalling in glial cells, we investigated the effects of IL-1-induced ERK1/2 activation in WT and IL-1RAcPb$^{-/-}$ glial cultures.

4.5.5. IL-1 induced ERK1/2 activation in glial cells

IL-1α and IL-1β were potent inducers of ERK1/2 activation in glial cells (Figure 4.14). In WT glia, IL-1α and IL-1β induced ERK1/2 activation with equal potency (Figure 4.15). These findings are consistent with previous findings (Andre et al 2005). IL-1α and IL-1β induced strong ERK1/2 activation in IL-1RAcPb$^{-/-}$ glia, with significant induction occurring at low concentration with both forms of IL-1 (0.3 IU/ml) (Figure 4.16). IL-1α and IL-1β induced ERK1/2 activation with similar potency in IL-1RAcPb$^{-/-}$ glia (Figure 4.17). There was also no significant difference between IL-1α-induced ERK1/2 activation in WT or IL-1RAcPb$^{-/-}$ glial cultures (Figure 4.18), or IL-1β-induced ERK1/2 activation in WT or IL-1RAcPb$^{-/-}$ glial cultures (Figure 4.19), which indicates that IL-1RAcPb does not regulate IL-1-induced ERK1/2 activation in glial cells. Data presented here suggest that the differential effect between WT and IL-1RAcPb$^{-/-}$ in IL-1α-

induced IL-6 in glial cells (see Chapter 3) was not due to differences in ERK1/2 activation. This suggests that the differential effect of IL-1α and IL-1β-induced IL-6 in glia could be due to other signalling mechanisms. I therefore investigated whether Src kinase signalling could contribute to the differential effect of IL-1α- and IL-1β-induced IL-6 synthesis in glial cultures.

4.5.6. The effects of IL-1 on Src kinase signalling in glial cultures

Src kinase is activated in hippocampal microglia by ischaemia (Choi et al, 2005) and spinal microglia after spinal nerve ligation (Katsura et al, 2006). However, the mediator which triggers Src kinase activation in glial cells is unclear. The results of the present study show that IL-1α and IL-1β failed to induce Src kinase activity in WT (Figure 4.20) or IL-1RAcPb$^{-/-}$ glial cells (Figure 4.21). This indicates that IL-1 may not be the mediator which activates Src kinase in glial cells following ischaemia. However, this is an *in vitro* study, which may not represent true events *in vivo*. Additionally, *in vivo* studies on injury-induced Src kinase activation reported Src kinase activation to be specific to microglial cells (Katsura et al, 2006; Choi et al, 2005), but the major cell type in our mixed glial cultures were mainly astrocytes with microglial cells forming the minor cell population. The method of observation and analysis may also contribute to the differences in our results and the *in vivo* data. Here Western blotting was used to detect Src kinase activation which is only a semi-quantitative method and may not detect subtle or cell-specific changes in Src kinase activation in the minor microglial population in our mixed glial cultures. Both previous papers (Katsura et al, 2006; Choi et al, 2005) used immunohistochemistry to detect Src kinase activation in microglial cells after injury. It is unclear which mediators are involved in the activation of Src kinase in glial cells *in vivo* but there are no *in vitro* findings to suggest that it is IL-1. This indicates that Src kinase signalling is not responsible for the differential effects of IL-1α- or IL-1β- induced IL-6 in glial cells.

4.6. Summary.

I investigated the effects of IL-1α and IL-1β-induced p38, ERK1/2 and Src kinase in neurones and glia, and my main findings are summarised in Table 4.1. IL-1α and IL-1β are potent activators of p38 in neurones and glial cells. IL-1α and IL-1β-induced p38 activation with similar potency in WT neurones. However, in IL-1RAcPb$^{-/-}$ neurones, IL-1α-induced p38 was suppressed compared to WT neurones. IL-1β did not induce ERK activation in neurones but IL-1α and IL-1β were potent inducers of ERK1/2 activation in glial cells, both in WT glial and IL-1RAcPb$^{-/-}$ glial cultures, acting with equal potency in both strains. This indicates that IL-1RAcPb is not a modulator of IL-1-induced ERK1/2 activation in glial cells. Finally, IL-1α and IL-1β failed to induce Src kinase activation in both strains of neurones and glial cultures.

Table 4.1. The effects of IL-1α and IL-1β on signalling mechanisms in neurones and glia.

Key: +++, strongly induced; ++ moderately induced; 0, not induced; NS, not studied.

	IL-1α			**IL-1β**		
	p38	**ERK1/2**	**Src kinase**	**p38**	**ERK1/2**	**Src kinase**
WT neurone	+++	NS	0	+++	0	0
IL-1RAcPb$^{-/-}$ neurones	++	NS	0	+++	0	0
WT glia	+++	+++	0	+++	+++	0
IL-1RAcPb$^{-/-}$ glia	+++	+++	0	+++	+++	0

These data indicate that IL-1 may have specific actions on different brain cells by activating specific signalling pathways. Our study also suggests that IL-1RAcPb may contribute to the action of IL-1α on p38 activation in neurones. However, the significance of this finding in terms of neuronal response to stress would require further investigation. Activation of these signalling pathways is associated with IL-6 production but the contribution of each specific signalling pathway to IL-6 expression is unclear and would require further studies. The following chapter will focus on the effects of pharmacological intervention on p38, ERK1/2 and Src kinase signalling pathways on the cellular response of neurones and glia. Understanding the specific mechanisms involved in a cellular response is necessary for pharmacological development targeting specific diseases.

5. Contribution of IL-1RAcPb and signalling pathways to IL-1-induced IL-6 production in neuronal cells

5.1. Introduction

It was demonstrated in the previous chapters that IL-1α and IL-β are potent inducers of IL-6 expression and p38 signalling activation, and that IL-1RAcPb may contribute to IL-1α-induced p38 and IL-6 synthesis activation in neurones and glia respectively. However, as neurones are the predominant cell type that express IL-1RAcPb, IL-1-induced neuronal responses was the focus of this chapter. IL-1-induced p38 activation in neurones is consistent with previous data, however, there is no direct evidence linking IL-1-induced p38 activation in neurones and IL-6 production (Srinivasan et al, 2004). A recent paper demonstrated that IL-6 production in mouse cortical neurones is mediated by Src kinase activation (Tsakiri et al, 2008a). However, IL-1-induced Src kinase activation was not observed in the current study. The aim of the this part of the study was to determine the contribution of MAPK and Src kinase signalling pathways to IL-1-induced IL-6 synthesis in WT and IL-1RAcPb$^{-/-}$ neurones. Since IL-1α and IL-1β were equipotent at inducing IL-6 in both WT and IL-1RAcPb$^{-/-}$ neurones, but IL-1α-induced p38 phosphorylation was reduced in IL-1RAcPb$^{-/-}$ compared to WT neurones, it is important to investigate if compensatory mechanisms have been triggered. Despite the lack of detected ERK1/2 and Src kinase activation after IL-1 treatment, subtle, undetected enzyme activity may still occur, and could be compensatory in IL-1RAcPb$^{-/-}$ neurones. To exclude the contribution of these signalling pathways in IL-1-induced IL-6 synthesis, ERK1/2 and Src kinase activity was blocked by pharmacological interventions. This chapter will also determine if identical signalling mechanisms are required for IL-1-induced IL-6 in WT and IL-1RAcPb$^{-/-}$ neurones.

MAPK including p38 and ERK1/2 signalling mechanisms, Src kinase signalling mechanism and IL-6 are all implicated in neuronal injury such as in response to cerebral ischaemia (see Chapter 1). However their specific contribution to ischaemia-induced brain damage is controversial; there is evidence suggesting that these mediators could be neuroprotective and/or neurotoxic after acute brain injury. IL-1 is induced by cerebral ischaemia (see Chapter 1), but the role of endogenous IL-1 during inflammation is unclear. Studies demonstrating the neuroprotective effect of IL-1RA indicate that ischaemia-induced endogenous IL-1 is detrimental and contributes to brain damage whereas inhibition of IL-1

signalling is protective (Vogt et al, 2008; Pinteaux et al, 2006; Loddick et al, 1997; Stroemer and Rothwell, 1997; Relton and Rothwell, 1992). Furthermore, IL-1RA has entered into phase II of clinical trial against stroke (Emsley et al, 2005). p38, ERK1/2 and Src kinase inhibitors are studied widely, targeting inflammation (Ardizzone et al, 2007; Hou et al, 2007; Lu et al, 2007; Katsura et al, 2006; Lu et al, 2005; Lennmyr et al, 2004; Wang et al, 2004; Legos et al, 2002; Legos et al, 2001; Barone et al, 2001b). There has been several clinical trials targeting ERK1/2 and p38 signalling for peripheral inflammation but as yet have not identified an effective or specific compound for human diseases (for reviews see Borders et al, 2008, Roux and Blenis 2004; Johnson and Lapadat. 2002).

In vitro studies showed that application of exogenous IL-1 in glial cultures induces p38 and ERK1/2 activation leading to the expression of IL-6 (Andre et al, 2005b; Parker et al, 2002). However, IL-1-induced neuronal responses are very different and are known to occur in the absence of MAPK signalling or IL-6 production (Davis et al, 2006; Desson and Ferguson, 2003; Borsody and Weiss, 2002). IL-1-induced IL-6 synthesis was demonstrated in neurones, but the effects of MAPK and Src kinase inhibitor on IL-1-induced IL-6 in neurones is not fully characterised. For this study, we have investigated the effects of SB203580 (p38 inhibitor), UO126 (ERK1/2 inhibitor) and 4 -amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]-pyrimidine (PP2, Src kinase inhibitor) on IL-1-induced IL-6 synthesis and release in neurones.

5.2. Aims

The aims of this part of the study were:

1. To determine the specific contribution of MAPK and Src kinase signalling in IL-1-induced IL-6 synthesis in neurones. The effect of ERK1/2 (UO126), p38 (SB203580) and Src kinase (PP2) inhibitors on IL-1-induced IL-6 in neurones was tested.
2. To determine whether the signalling mechanisms described above also mediate IL-1-induced IL-6 synthesis in IL-1RAcPb$^{-/-}$ neurones using MAPK and Src kinase inhibition.

5.3. Material and methods

WT and IL-1RAcPb$^{-/-}$ neurones were treated with vehicle, dimethyl sulfoxide (DMSO) (1 μl), IL-1α (3 IU/ml) or IL-1β (3 IU/ml) in the presence or absence of IL-1RA (10 μg/ml), UO126 (20 μM), SB203580 (20 μM), or PP2 (20 μM). Neurones were pre-treated with IL-1RA for 5 min; and MAPK inhibitors and PP2 for 15 min, before co-incubation with IL-1α or IL-1β for 15 min (for signalling) or 24 h (for IL-6). Cell lysates and supernatants were collected and analysed for IL-6 expression by Western blot or ELISA (see section 2.10). Cell lysates were also collected and analysed for MAPK/Src kinase activity by Western blot (see section 2.9) to determine the specificity of the inhibitors used.

5.4. Results

5.4.1 The effects of MAPK and Src kinase inhibitors on IL-6 production in neurones.

In the cell lysates IL-1α induced a slight but non-significant increase in IL-6 synthesis compared to DMSO treatment alone (Figure 5.1A).

The mean level of IL-6 release in culture supernatants induced by IL-1α was 139 ± 13 pg/ml (vs. 15 ± 3 pg/ml for DMSO), and this response was significantly reduced by co-incubation with the inhibitors. The percentages of IL-6 synthesis inhibition triggered by IL-1RA, UO126 and SB203580 were -80 %, -65 %, and -76 % respectively. The effect of PP2 on IL-1α-induced IL-6 was not statistically analysed because data was from a single experiment (Figure 5.1B).

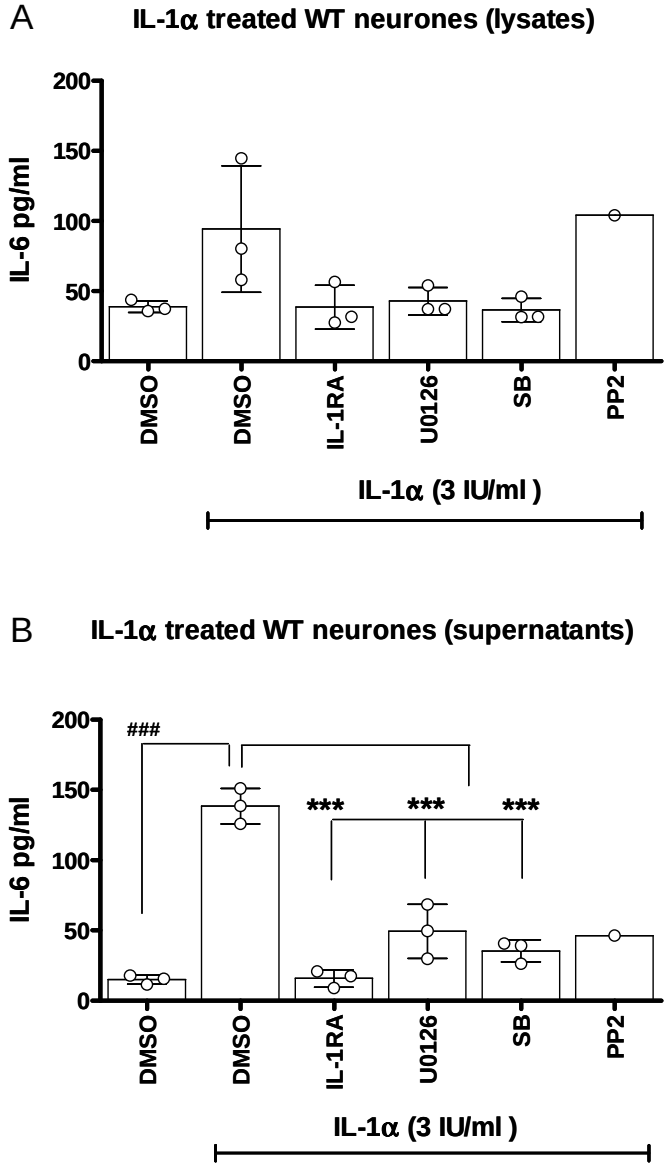


Figure 5.1. Effect of UO126, SB203580 and PP2 on IL-1α-induced IL-6 production in WT neuronal cells.

WT neuronal cultures were treated with DMSO, and/or IL-1α (3 IU/ml) for 24 h in the presence or absence of IL-1RA (10 μg/ml), UO126 (20 μM), SB203580 (20 μM) or PP2 (20 μM). Cell lysates (**A**) and supernatants (**B**) were collected and analysed for IL-6 expression by ELISA. IL-1α (n=3) induced significant levels of IL-6 release, and this response was markedly inhibited by co-incubation with IL-1RA, UO126 or SB203580. One way ANOVA and Tukey's multiple comparison test. *** P< 0.001 DMSO + IL-1α (3 IU/ml) vs. inhibitors. ### P< 0.01 DMSO + IL-1α (3 IU/ml) vs. DMSO alone.

IL-1β induced significant IL-6 expression (92 ± 33 pg/ml) compared to DMSO treatment (37 ± 4 pg/ml) (Figure 5.2A). IL-6 expression induced by IL-1β was significantly reduced in the presence of IL-1RA (-66 %) or SB203580 (-63 %). UO126 and PP2 reduced IL-1β-induced IL-6 expression but it was non-significant.

IL-1β induced significant IL-6 release (140 ± 21 pg/ml) in the culture supernatants compared to DMSO treatment alone (56 ± 11 pg/ml) (Figure 5.2B). IL-1β-induced IL-6 release was inhibited by IL-1RA (-93 %), UO126 (-79 %), SB203580 (-79 %) and PP2 (-61 %)

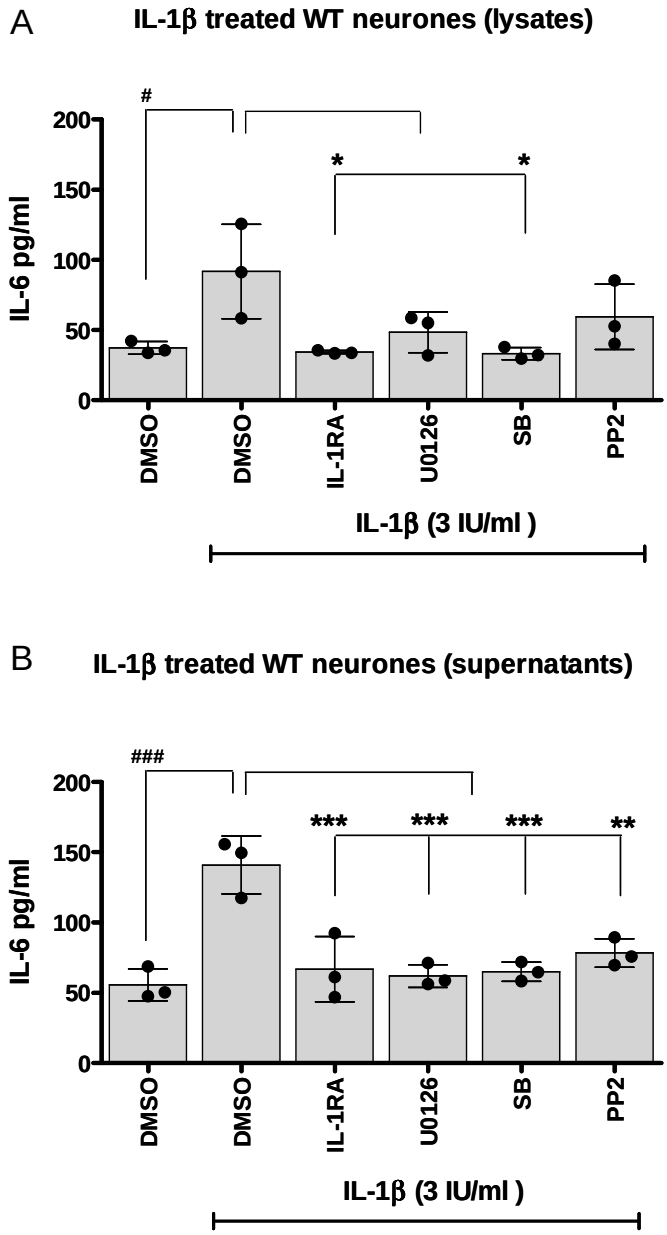


Figure 5.2. Effect of UO126, SB203580 and PP2 on IL-1β-induced IL-6 production from WT neuronal cells.

WT neuronal cultures were treated with DMSO and/or IL-1β (3 IU/ml) for 24 h in the presence or absence of IL-1RA (10 μg/ml), UO126 (20 μM), SB203580 (20 μM) or PP2 (20 μM). Cell lysates (**A**) and supernatants (**B**) were collected and analysed for IL-6 expression by ELISA. IL-1β (n=3) induced significant levels of IL-6 expression and release, and responses were abolished by co-incubation with IL-1RA, UO126, SB203580 or PP2. One way ANOVA and Tukey's multiple comparison test. * P<0.05, ** P< 0.01, *** P< 0.001 DMSO + IL-1α (3 IU/ml) vs. inhibitors. # P<0.05, ### P< 0.01 DMSO + IL-1α (3 IU/ml) vs. DMSO alone.

DMSO-treated IL-1RAcPb$^{-/-}$ neurones showed low IL-6 expression and release (Figure 5.3A). IL-1α-treated neurones showed significant IL-6 induction compared to DMSO treatment alone. IL-1α induced 50 ±18 pg/ml of IL-6, which was inhibited by IL-1RA, UO126 and SB203580 by -79 %, -48 %, and -57 % respectively. PP2 did not significantly inhibit IL-1α-induced IL-6 expression in IL-1RAcPb$^{-/-}$ neurones.

IL-1α induced an average of 97 ± 13 pg/ml of IL-6 release in the supernatants of IL-1RAcPb$^{-/-}$ neurones (Figure 5.3B). Co-incubation of IL-1α with IL-1RA, UO126, SB203580 or PP2 significantly inhibited IL-1 (3 IU/ml)-induced IL-6 by -79 %, -65 %, -76 % and -49 % respectively.

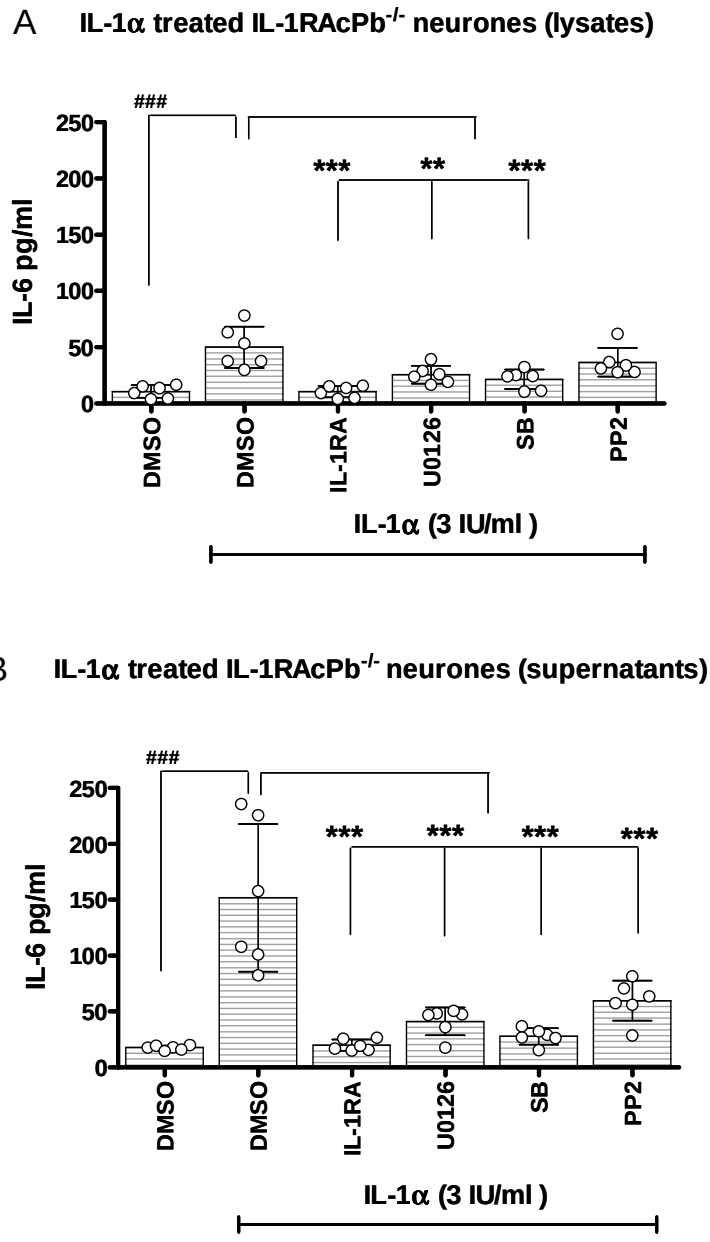


Figure 5.3. Effect of UO126, SB203580 and PP2 on IL-1α-induced IL-6 production from IL-1RAcPb$^{-/-}$ neuronal cells.

IL-1RAcPb$^{-/-}$ neuronal cultures were treated with DMSO and/or IL-1α (3 IU/ml) for 24 h in the presence or absence of IL-1RA (10 μg/ml), UO126 (20 μM), SB203580 (20 μM) or PP2 (20 μM). Cell lysates (**A**) and supernatants (**B**) were collected and analysed for IL-6 by ELISA. IL-1α (n=6) induced significant levels of IL-6 release and responses were inhibited by co-incubation with IL-1RA, UO126, SB203580 and PP2. One way ANOVA and Tukey's multiple comparison test. ** P< 0.01, *** P< 0.001 DMSO + IL-1α (3 IU/ml) vs. inhibitors. ### P< 0.01 DMSO + IL-1α (3 IU/ml) vs. DMSO alone.

IL-6 expression in IL-1β-treated IL-1RAcPb$^{-/-}$ neurones was significantly greater (74 ± 3 pg/ml) than that of DMSO-treated cells (26 ± 4 pg/ml) (Figure 5.4A). IL-1β-induced IL-6 synthesis in IL-1RAcPb$^{-/-}$ neurones was significantly reduced in the presence of IL-1RA (-70 %), UO126 (-55 %), SB203580 (-67 %) and PP2 (-54 %).

IL-1β treatment induced 135 ± 21 pg/ml IL-6 release in the culture supernatant compared to DMSO treatment alone (11 ± 3 pg/ml) (Figure 5.4B). This response was inhibited in the presence of IL-1RA, UO126, SB203580 and PP2 by -93 %, -79 %, -79 % and -61 % respectively.

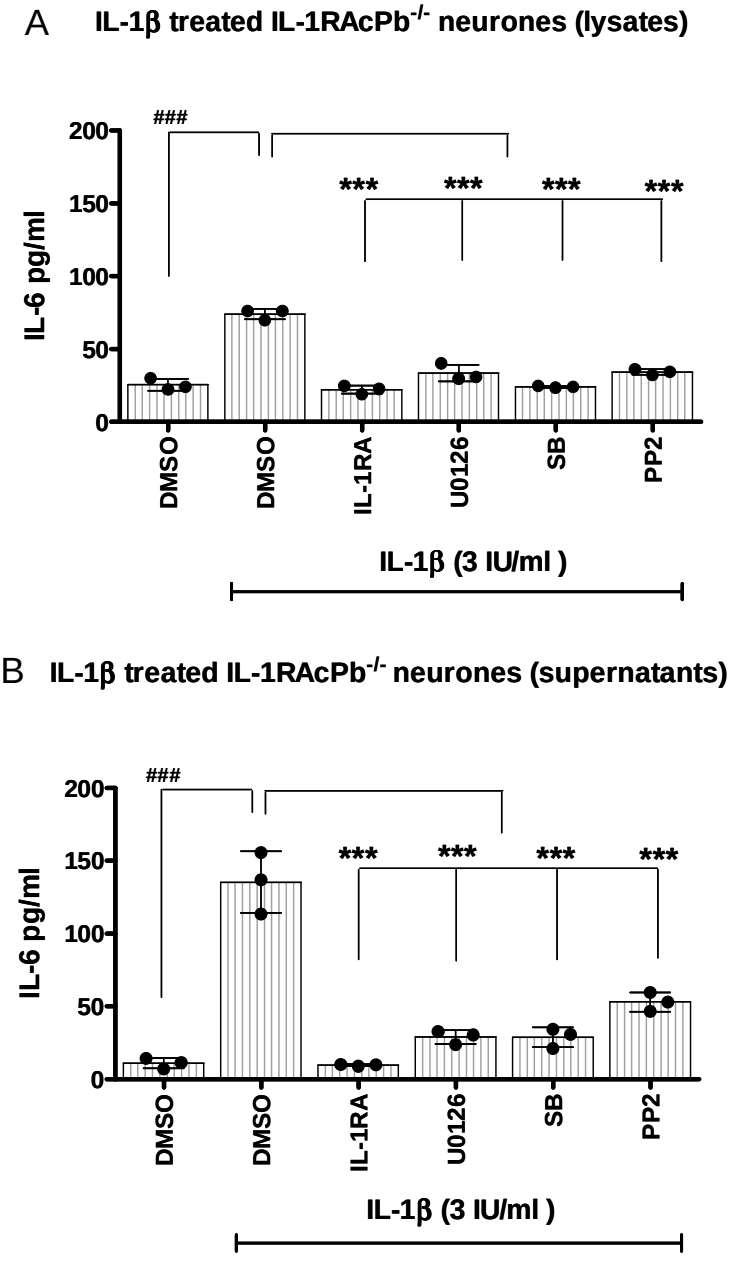


Figure 5.4. Effect of UO126, SB203580 and PP2 on IL-1β-induced IL-6 production from WT neuronal cells.

WT neuronal cultures were treated with DMSO or IL-1β (3 IU/ml) for 24 h in the presence or absence of IL-1RA (10 μg/ml), UO126 (20 μM), SB203580 (20 μM) or PP2 (20 μM). Cell lysates (**A**) and supernatants (**B**) were collected and analysed for IL-6 by ELISA. IL-1β (n=3) induced significant levels of IL-6 expression and release, and responses were inhibited by co-incubation with IL-1RA, UO126, SB203580 and PP2. One way ANOVA and Tukey's multiple comparison test. * P<0.05, ** P< 0.01, *** P< 0.001 DMSO + IL-1α (3 IU/ml) vs. inhibitors. # P<0.05, ### P< 0.01 DMSO + IL-1α (3 IU/ml) vs. DMSO alone.

5.4.2 Mechanism of UO126, SB203580 and PP2 inhibition

UO126, SB203580 and PP2 have all been shown to effectively inhibit IL-6 synthesis and release from neurones (Figure 5.1 to 5.4). However, the specificity of these inhibitors is unclear. ERK1/2 was not activated by IL-1β in WT neurones and Src kinase was not activated by IL-1α or IL-1β in WT or IL-1RAcPb$^{-/-}$ neurones (see Chapter 4). To investigate the mechanism of actions of the inhibitors used, cell lysates were collected from neurones treated with IL-1α, IL-1β with or without the inhibitors and assessed for MK2, Src kinase and ERK1/2 phosphorylation by Western blot. SB203580 is an inhibitor that blocks the phosphorylated form of p38 therefore to assess its specificity we measured the phosphorylation of MK2 a downstream signalling molecule activated by p38.

In vehicle- or DMSO-treated WT neurones, MK2, Src kinase or ERK1/2 were not induced. However, in the presence of IL-1α (Figure 5.5) or IL-1β (Figure 5.6), MK2 was strongly phosphorylated, indicative of upstream p38 activation. This response was inhibited by co-incubation with IL-1RA. UO126 or PP2 co-incubation with IL-1α or IL-1β did not affect p38 signalling, since IL-1α or IL-1β in the presence of UO126 or PP2 could still induce strong MK2 activation. SB203580 inhibited IL-1α- and IL-1β-induced p38 in neurones. Basal ERK1/2 activation was high in DMSO-treated cells and IL-1α or IL-1β did not induce ERK1/2 phosphorylation. Basal ERK1/2 signalling in untreated and neurones treated with IL-1α or IL-1β were completely inhibited by UO126 and partially inhibited by PP2. SB203580 did not affect ERK1/2 activity in neurones. Finally IL-1α or IL-1β did not to induce Src kinase activation and, UO126, SB203580 and PP2 had no effect on Src kinase phosphorylation.

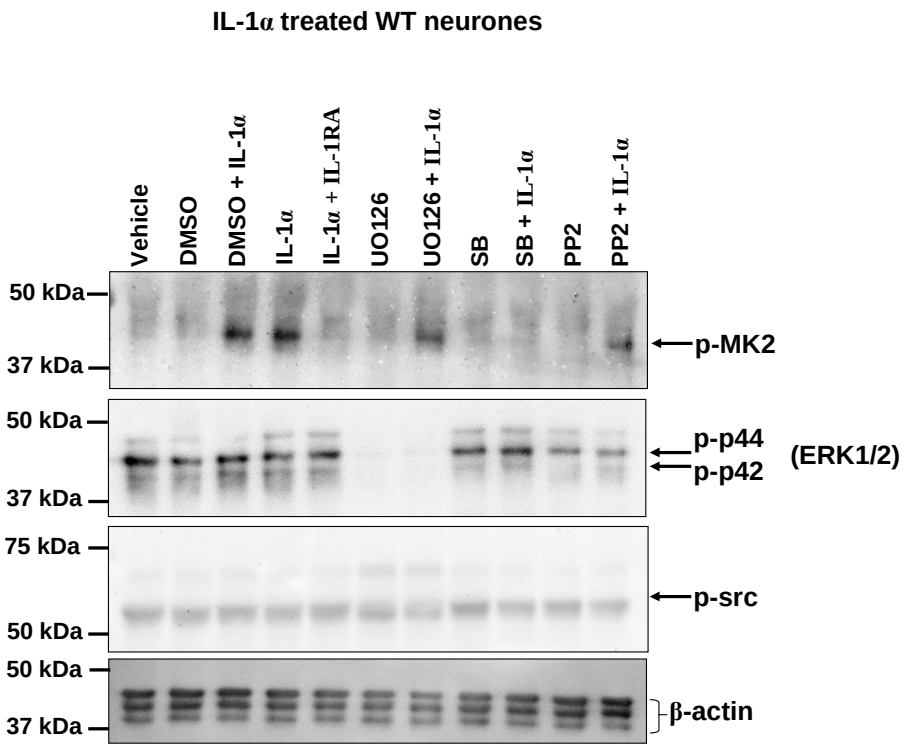


Figure 5.5. The effects of UO126, SB203580 and PP2 on IL-1α-induced signalling in WT neuronal cells.

WT neuronal cultures were treated with vehicle, DMSO and/or IL-1α (3 IU/ml) for 15 min in the presence or absence of IL-1RA (10 μg/ml), UO126 (20 μM), SB203580 (20 μM) or PP2 (20 μM). Cell lysates were collected and analysed for MK2, Src kinase and ERK1/2 phosphorylation by western blot. IL-1α induced MK2 phosphorylation but not Src kinase or ERK1/2 activation. SB203580 specifically inhibited p38 activity, and UO126 blocked basal ERK1/2 activity. PP2 also partially inhibited basal ERK1/2 activation in WT neurones. Image is from a single experiment.

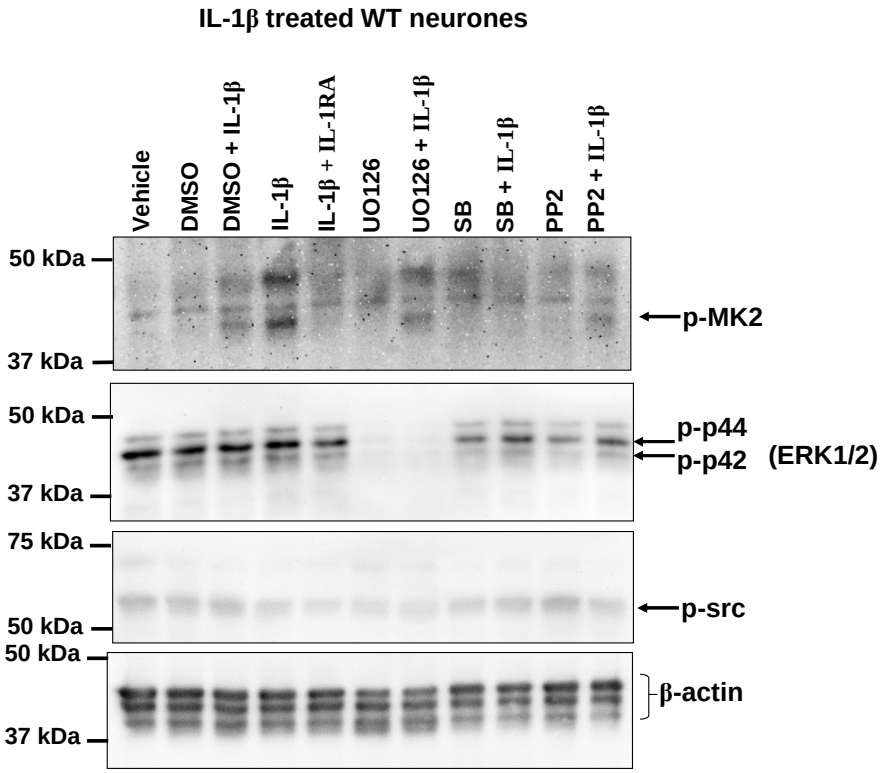


Figure 5.6. The effects of UO126, SB203580 and PP2 on IL-1β-induced signalling in WT neuronal cells.

WT neuronal cultures were treated with vehicle, DMSO and/or IL-1α (3 IU/ml) for 15 min in the presence or absence of IL-1RA (10 μg/ml), UO126 (20 μM), SB203580 (20 μM) or PP2 (20 μM). Cell lysates were collected and analysed for MK2, Src kinase and ERK1/2 phosphoryaltion by Western blot analysis. IL-1β induced MK2 phosphorylation but not Src kinase or ERK1/2 activation. SB203580 specifically inhibited p38 activity and UO126 blocked basal ERK1/2 activity WT neurones. Image is from a single experiment.

In IL-1RAcPb$^{-/-}$ neurones, vehicle or DMSO did not induce MK2, Src kinase or ERK1/2 phosphorylation (Figure 5.7), but IL-1α induced strong MK2 phosphorylation, and this response was abolished by co-incubation with IL-1RA. SB203580, but not UO126 or PP2 co-incubation reduced IL-α-induced p38 activation in IL-1RAcPb$^{-/-}$ neurones. IL-1α also induced ERK1/2 phosphorylation which was reduced by co-incubation with IL-1RA and completely abolished by UO126. SB203580 and PP2 did not inhibit IL-1α-induced ERK1/2 phosphorylation in IL-1RAcPb$^{-/-}$ neurones. IL-1α did not induce Src kinase activation in IL-1RAcPb$^{-/-}$ neurones, therefore the effect of UO126, SB203580 and PP2 on Src kinase phosphorylation could not be assessed.

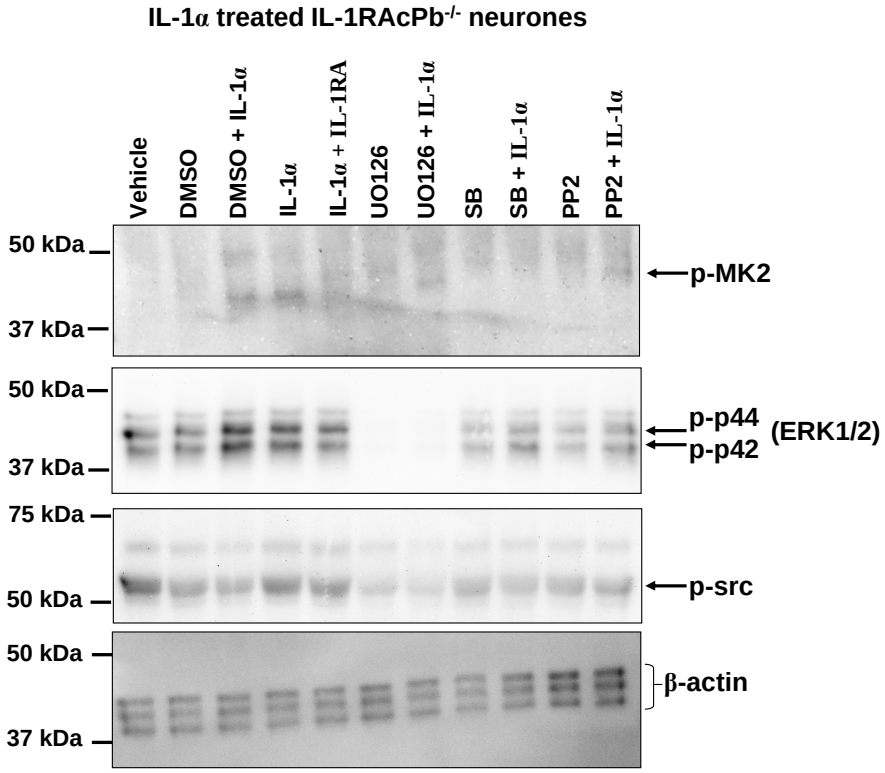


Figure 5.7. The effects of UO126, SB203580 and PP2 on IL-1α-induced signalling in IL-1RAcPb$^{-/-}$ neuronal cells.

IL-1RAcPb$^{-/-}$ neuronal cultures were treated with vehicle, DMSO and/or IL-1α (3 IU/ml) for 15 min in the presence or absence of IL-1RA (10 μg/ml), UO126 (20 μM), SB203580 (20 μM) or PP2 (20 μM). Cell lysates were collected and analysed for MK2, Src kinase and ERK1/2 phosphorylation by western blot. IL-1α induced MK2 and ERK1/2 phosphorylation but not Src activation. SB203580 specifically inhibited p38 activity and UO126 blocked basal ERK1/2 activity in IL-1RAcPb$^{-/-}$ neurones. Image is from a single experiment.

In IL-1RAcPb$^{-/-}$ neurones IL-1β also induced MK2 phosphorylation compared to vehicle- or DMSO-treated neurones (Figure 5.8). This response was blocked by co-incubation with IL-1RA. SB203580, but not UO126 or PP2 inhibited IL-β-induced p38 signalling in IL-1RAcPb$^{-/-}$ neurones. IL-1β induced strong ERK1/2 activation, but this response was not blocked by IL-1RA. Increased ERK1/2 activation was also detected in response to SB203580 alone or PP2 treatment alone, as well as IL-1β with SB203580 or PP2. Basal ERK1/2 signalling in IL-1RAcPb$^{-/-}$ neurones were completely inhibited by UO126. Finally, basal Src kinase phosphorylation was low but IL-1β failed to induce Src kinase activation in IL-1RAcPb$^{-/-}$ neurones, therefore the effect of UO126, SB203580 and PP2 on Src kinase phosphorylation could not be assessed.

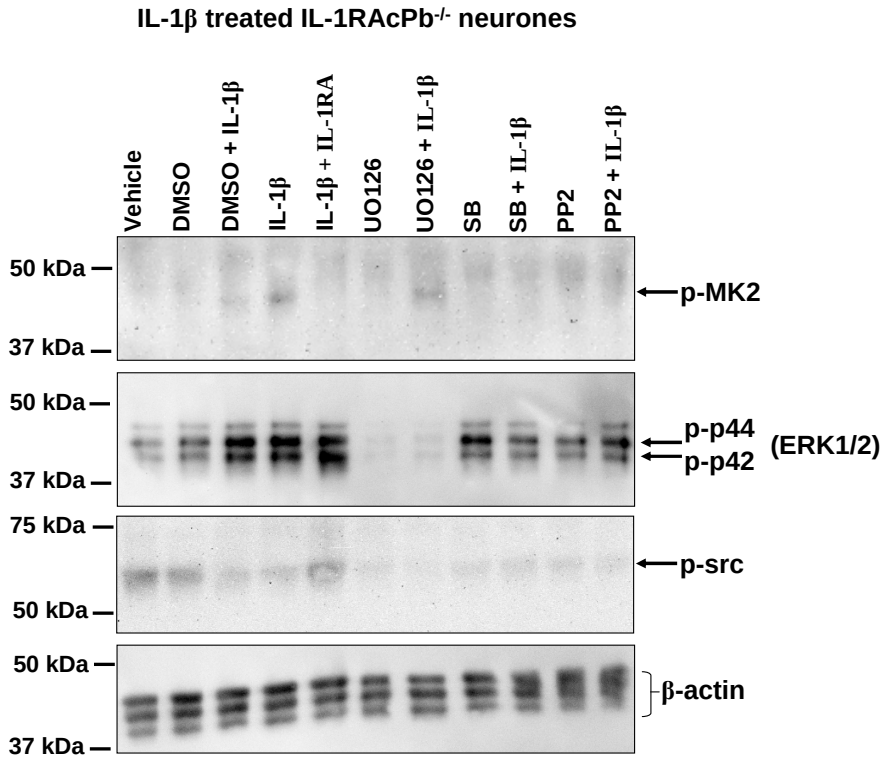


Figure 5.8. The effects of UO126, SB203580 and PP2 on IL-1β-induced signalling in IL-1RAcPb$^{-/-}$ neuronal cells.

IL-1RAcPb$^{-/-}$ neuronal cultures were treated with vehicle, DMSO and/or IL-1β (3 IU/ml) for 15 min in the presence or absence of IL-1RA (10 μg/ml), UO126 (20 μM), SB203580 (20 μM) or PP2 (20 μM). Cell lysates were collected and analysed for MK2, Src kinase and ERK1/2 phosphorylation by Western blot analysis. IL-1β induced specific MK2 phosphorylation. ERK1/2 phosphorylation was also increased in response to IL-1β but this was not blocked by IL-1RA. SB203580 specifically inhibited p38 activity and UO126 blocked basal ERK1/2 activity. Image is from a single experiment.

5.5. Discussion

In the previous chapter (see Chapter 4) it was shown that IL-1α and IL-1β were potent inducers of p38 activation in WT and IL-1RAcPb$^{-/-}$ neurones. In this chapter it was confirmed that IL-1-induced p38 activation in neurones is the mechanism responsible for IL-1-induced IL-6 synthesis in neurones (see Chapter 3).

IL-1α and IL-1β were potent activators of IL-6 production in WT and IL-1RAcPb$^{-/-}$ neurones. These responses were abolished in the presence of IL-1RA, SB203580, UO126 and PP2. The action of IL-1RA on IL-1-induced actions are well characterised, abrogating IL-1 signalling at the receptor level. However, the action of the other inhibitors is poorly understood. It is unclear how UO126 and PP2 could inhibit IL-1-induced IL-6 synthesis when IL-1 did not induce ERK1/2 or Src kinase activation (see Chapter 4). It was speculated that IL-1-induced ERK and Src activation could be too low to be detected by Western blot, or that activation of these signalling molecules could be very transient and was not detected at the time point investigated (15 min post IL-1 incubation)

SB203580 is a specific p38 inhibitor which has been shown to inhibit IL-1-induced activity in human bronchial epithelial cells (Newton et al, 2000), astrocytes (Dunn et al, 2002), as well as IL-1α-induced IL-6 synthesis in a neuronal cell line (Bergamaschi et al, 2006). IL-1-induced p38 activation in neuronal cell line suggests that p38 could be a functional mechanism in which IL-1 induces IL-6 in neurones. Here we have shown that SB203580 is an effective inhibitor of IL-1α- and IL-1β-induced IL-6 in primary neurones (Figure 5.1). SB203580 inhibited IL-1-induced IL-6 in cell lysates and supernatants suggesting that p38 signalling is likely an active pathway in which IL-1 induces IL-6 expression in neurones. In the present study SB203580 inhibited IL-1α- and IL-1β-induced p38 (as indicated by inhibition of MK2 phosphorylation), but did not affect ERK1/2 or Src kinase signalling (Figure 5.5 and Figure 5.6). This indicates that SB203580 is a p38 specific inhibitor. These data confirm that IL-1-induced p38 activation in neurones mediated IL-6 production.

IL-1β is not directly toxic to neurones, however, activation of p38 could be involved in the priming of neurones to be more susceptible to damage induced by other proinflammatory mediators. Direct application of exogenous IL-1, LPS

or interferon-γ (IFN-γ) onto pure neuronal cultures has been shown to be non-toxic to neurones *in vitro*. However, in the presence of glial cells, IL-1β (Thornton et al, 2006), LPS and IFN-γ could dose-dependently and and time-dependently induced neuronal death respectively (Xie et al, 2004). Pre-incubation of rat primary cortical neurones with IL-1β has also been shown to prime these cells to release soluble amyloid precursor protein-α (sAPPα) induced by nucleotide uridine3 triphosphate (UTP) (Kong et al, 2009). In the absence of IL-1β pre-treatment, UTP did not induce sAPPα release from neurones. Additionally IL-1β has been shown to enhance susceptibility to hypothermia-induced seizures in rats (Fukuda et al, 2009). These studies highlight the importance of IL-1 regulation in the CNS as even subtle changes in IL-1 level may prime neurones to become more sensitive to mediators/toxins in the event of an injury.

IL-1α- and IL-1β-induced IL-6 synthesis in IL-1RAcPb$^{-/-}$ neurones was not investigated previously by Lu et al (2008) or Smith et al (2009).Therefore the signalling mechanisms involved in IL-1-induced IL-6 in IL-1RAcPb$^{-/-}$ neurones completely unknown. In this study, SB203580 completely abolished IL-1α- and IL-1β-induced IL-6 synthesis in IL-1RAcPb$^{-/-}$ neurones (Figure 5.3 and Figure 5.4) and p38 activation (Figure 5.7 and Figure 5.8). This indicates that IL-1RAcPb is not required for the recruitment of downstream signalling molecules to activate p38 signalling to mediate IL-6 synthesis, and that IL-1RAcPb is not essential for IL-1-induced p38 signalling and IL-6 synthesis. This is in contrast to Smith et al (2009), who reported IL-1RAcPb as a negative regulator of IL-1-induced p38 activation in EL4 T-cell line. However, EL4 are T-cells which do not ordinary express IL-1RAcP, and over-expression of IL-1RAcP and IL-1RAcPb by transfection may alter cellular signalling mechanisms therefore biasing the result. In the current study, the role of IL-1RAcPb was studied in primary neurones that express IL-1R1, IL-1RAcP and IL-1RAcPb; therefore it is not surprising that these two studies produced opposing conclusions for the role of IL-1RAcPb in IL-1-induced signalling. Lu et al (2008) did not assess IL-1-induced p38 signalling, however they demonstrated that IL-1RAcPb could mediate NF-κB activity when over-expressed in HEK cells. NF-κB and p38 signalling are two independent signalling pathways (Srinivasan et al, 2004; Dunn et al, 2002). Data in this study suggests that IL-1RAcPb may potentiate IL-1α-induced p38 in

neurones (see Chapter 4), but the role of IL-1RAcPb in modulating IL-1-induced NF-κB signalling in neurones remains to be investigated.

IL-1-induced ERK1/2 activity has not been demonstrated previously in neurones, however it is an important mechanism for IL-1-induced actions in glial cells (Andre et al, 2005b; Parker et al, 2002; Molina-Holgado et al, 2000a). UO126 has been shown to be a specific ERK1/2 inhibitor in these cells. In this study UO126 was an effective inhibitor of IL-1-induced IL-6 synthesis in neurones suggesting that ERK1/2 signalling is involved in this response (Figure 5.1 and Figure 5.2). UO126 inhibited IL-1-induced IL-6 expression in both cell lysates and supernatants suggesting that ERK1/2 involvement in IL-6 production is at the level of protein expression. It is unclear how UO126 inhibits IL-1-induced IL-6 expression in neurones because ERK1/2 was not induced by IL-1β in WT neurones (see Chapter 4, Figure 4.9). Investigating the effects of UO126 on IL-1-induced ERK1/2 phosphorylation in neurones revealed that UO126 abolished all basal ERK1/2 phosphorylation in neurones (Figure 5.5 and Figure 5.6). The concentration of UO126 (20 μM) was higher than that used on astrocytes by Kim et al., (2004); Xie et al., (2004) and Parker et al., (2002) but the same as that used by Summers et al., (2010) and lower than that used by Van Wagoner et al., (2000) which indicates that the concentration of UO126 used in this study was in the accepted range for the specific inhibition of ERK1/2. The effect of different concentrations of UO126 on IL-1β-induced ERK1/2 in mixed glia was also performed and showed that there was no difference in activity between the 10 μM (the concentration most frequently used in previous literature) and 20 μM of UO126 (Figure 7.7, Appendix V). This finding indicates that although IL-1 did not induce ERK 1/2 activation in neurones, basal ERK1/2 activity may be essential for IL-1-induced IL-6 synthesis in neurones.

In IL-1RAcPb$^{-/-}$ neurones IL-1α and IL-1β induced ERK1/2 phosphorylation (Figure 5.7 and Figure 5.8). These responses were abolished by UO126. This is in contrast to the response in WT neurones (Figure 5.5 and Figure 5.6, and see section 4.4.2). These results suggest that IL-1RAcPb may suppress ERK1/2 activity in WT neurones and this inhibition was removed by the deletion of the *IL-1RAcPb* gene. This is consistent with previous findings by Smith et al (2009) who demonstrated that IL-1RAcPb is a negative regulator of IL-1β-

induced ERK1/2 activation when over-expressed in EL4 T-cell line. However, it is difficult to relate the function of IL-1RAcPb in EL4 cells to primary neurones as these are two completely different cell types. Additionally, our data are only preliminary, from a single experiment, and IL-1RA inhibited IL-1α-induced ERK1/2 but not IL-1β-induced ERK1/2 phosphorylation. Further studies are required to confirm these findings. Interestingly, work from our group has also shown, *in vivo,* that IL-1α exacerbated kainate-induced seizures in IL-1RAcPb$^{-/-}$ mice but not in WT C57/BL6 mice (unpublished data), although the mechanisms involved were not investigated. In that study, IL-1β (10 min pre-treatment) decreased seizure onset time, and increased seizure duration and the number of ictal seizure episodes induced by kainite in C57/BL6 mice, but IL-1α (10 min pre-treatment) had no effect. However, in IL-1RAcPb$^{-/-}$ mice, IL-1α (10 min pre-treatment) decreased seizure onset time, increased seizure duration and the number of ictal seizure episodes. Taken together, these studies indicate that IL-1RAcPb down-regulated IL-1α-induced ERK1/2 activity resulting in inhibition of seizure activities in WT mice.

In the present study, IL-1 did not induce Src kinase phosphorylation in neurones, however, a Src kinase specific inhibitor, PP2, blocked IL-1β-induced IL-6 production in neurones (Figure 5.2), which is consistent with previous findings from Tsakiri et al (2008a). Their study on mouse cortical neurones demonstrated that IL-1 induced strong Src kinase activation at an early time-point (5 min) and this response was blocked by IL-1RA and PP2. However, in this study, IL-1 did not induce Src kinase phosphorylation, and the relevance of IL-1-induced IL-6 inhibition by PP2 is unclear. Data presented here also showed that PP2 reduced basal ERK1/2 phosphorylation in neurones (Figure 5.5), suggesting that there could be some cross-talk mechanisms between Src kinase signalling pathway and ERK1/2 signalling pathway. This could be the mechanism by which PP2 inhibits IL-1-induced IL-6 synthesis in this study, which emphasises the importance of basal ERK1/2 actions in neuronal responses to IL-1. There is evidence suggesting that Src kinase signalling may converge at ERK1/2 signalling pathway and that actions of these two pathways may be orchestrated together for specific responses. Spinal nerve ligation in rats induces Src kinase and ERK1/2 activation in spinal microglial cells and both were inhibited by PP2 (Katsura et al,

2006). Additionally, in rat neural stem cells, carbachol induced the phosphorylation of Src kinase and ERK1/2 but both responses were abolished by PP1, another specific Src kinase inhibitor (Zhao et al, 2003).

PP2 also inhibited IL-1-induced IL-6 synthesis in IL-1RAcPb$^{-/-}$ neurones (Figure 5.3 and Figure 5.4). The role of IL-1RAcPb in IL-1-induced Src kinase signalling is not clear as IL-1 did not induce Src kinase activation in this study (see Chapter 4). Our investigation into the action of PP2 on IL-1-induced signalling in IL-1RAcPb$^{-/-}$ neurones is inconclusive, as PP2 did not affect IL-1-induced p38 or basal Src kinase activity. The action of PP2 on basal ERK1/2 activity is unclear because basal ERK1/2 phosphorylation, reduced by PP2 was detected only in for IL-1α and not IL-1β (Figure 5.7 and Figure 5.8). As there was no difference between the action of SB203580 and UO126 in WT and IL-1RAcPb$^{-/-}$ neurones, it can only be assumed that PP2 inhibited IL-1-induced IL-6 synthesis in IL-1RAcPb$^{-/-}$ neurones by reducing the basal activity of ERK1/2, as this was also observed in WT neurones. However, this hypothesis was drawn from a single experiment and further analyses are required to test the hypothesis.

In summary, IL-1α- and IL-1β-induced IL-6 synthesis in WT and IL-1RAcPb$^{-/-}$ neurones is mediated by p38 signalling and abolished by IL-1RA, SB203580, UO126 and PP2. These effects were observed in both cell lysates and supernatants, indicating that the signalling pathways are involved in the expression of IL-6. SB203580 inhibited IL-1-induced IL-6 synthesis by specifically blocking IL-1-induced p38 activation. UO126 inhibited IL-1-induced IL-6 synthesis by blocking basal ERK1/2 activity. Finally the inhibitory action of PP2 on IL-1-induced IL-6 synthesis may occur via decreased basal ERK1/2 activation. These data indicate that p38 and ERK1/2 signalling pathways are essential in IL-1-induced IL-6 expression in neurones, but the role of Src kinase signalling in IL-1-induced IL-6 expression is unclear. The complete inhibition of IL-1-induced IL-6 expression in both WT and IL-1RAcPb$^{-/-}$ neurones by these inhibitors suggests that identical signalling pathways are activated in WT and IL-1RAcPb$^{-/-}$ neurones and that IL-1RAcPb is not essential to IL-1 signalling.

6. General discussion and conclusion

6.1 Summary

The aim of this study was to determine the role of the newly characterised IL-1 accessory protein, called IL-1RAcPb, in the signalling mechanisms involved in IL-1 actions in neuronal and glial cells. IL-1RAcPb mRNA expression was strong in neuronal cultures when compared to glial cultures. *IL-1RAcPb* deletion did not alter the cellular composition of neuronal or glial cell cultures compared to WT cultures. This observation suggests that IL-1RAcPb does not affect neuronal or glial cell growth or differentiation. Additionally, IL-1RAcPb was not required for IL-1-induced IL-6 synthesis in neurones or glia and IL-1α and IL-1β were equipotent at inducing IL-6 in both WT and IL-1RAcPb$^{-/-}$ neurones. In WT glial cultures, IL-1α was more potent than IL-1β at inducing IL-6 synthesis, but both cytokines were equipotent in IL-1RAcPb$^{-/-}$ glia, which suggests that IL-1RAcPb may contribute to the action of IL-1α in WT glial cells, and thus to the differential actions of IL-1α and IL-1β in glial cells. Schematic diagrams summarising the affects of IL-1RAcPb on IL-1 actions on neuronal and glial cells are represented in Figure 6.1and Figure 6.2.

The contribution of IL-1RAcPb to IL-1-induced signalling pathways is unknown. Only p38 was activated by IL-1α and IL-1β in neurones, whilst ERK1/2 and Src kinase activation was not detected (Figure 6.1). IL-1α and IL-1β-induced p38 phosphorylation with equal potency in WT neurones, but IL-1β was more potent than IL-1α at activating p38 in IL-1RAcPb$^{-/-}$ neurones. These findings suggest that IL-1RAcPb may contribute to IL-1α-induced p38 activation in WT neurones. IL-1α and IL-1β induced p38 and ERK1/2 activation in glial cells, whilst Src kinase activation was not detected. IL-1RAcPb did not affect IL-1α- or IL-1β-induced ERK1/2 signalling in glial cells but its contribution to IL-1-induced p38 and Src kinase activation in glial cells remains unclear (Figure 6.2).

Although IL-1α and IL-1β did not induce ERK1/2 and Src kinase activation in neurones, the use of UO126 (ERK1/2 inhibitor) and PP2 (Src kinase inhibitor) as well as SB203580 (p38 inhibitor) showed that p38, ERK1/2 and Src kinase signalling pathways are central to IL-1-induced IL-6 synthesis in neurones (Figure 6.1).

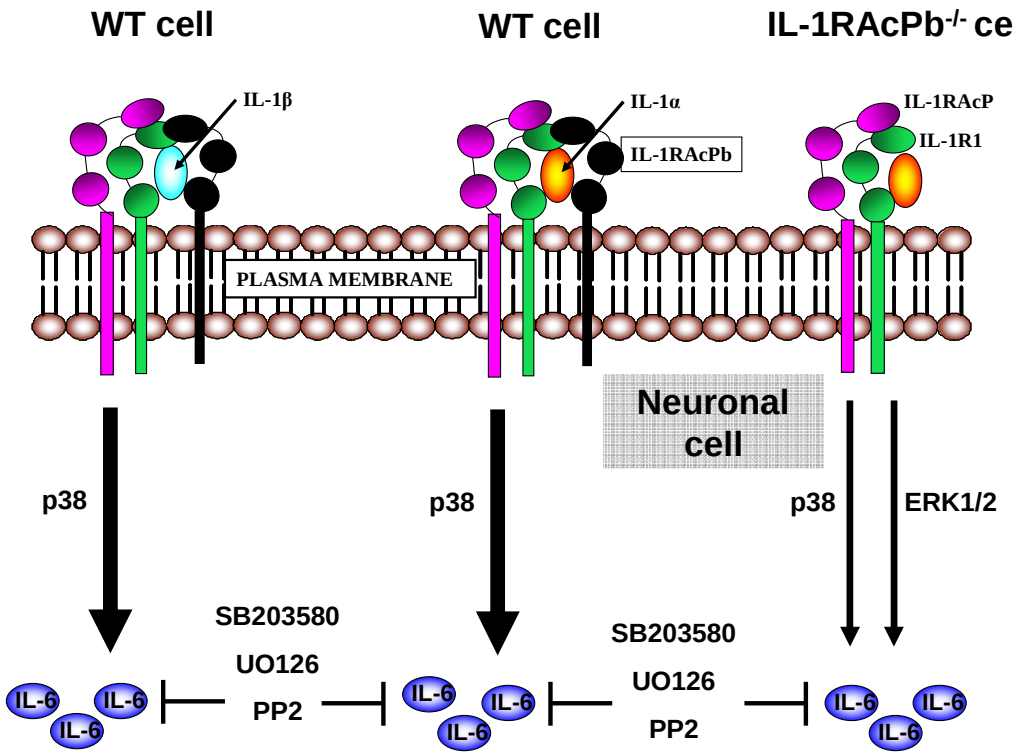


Figure 6.1. The role of IL-1RAcPb in IL-1-induced actions in neuronal cells

In WT neurones, IL-1α and IL-1β were equipotent at inducing IL-6 synthesis and p38 activation. However, in IL-1RAcPb$^{-/-}$ neurones, IL-1β was more potent than IL-1α at inducing p38 activation, this was caused by a diminished p38 activation induced by IL-1α in IL-1RAcPb$^{-/-}$ when compared to WT neurones. IL-1α and IL-1β-induced IL-6 in IL-1RAcPb$^{-/-}$ neurones were comparable. These results indicate IL-1RAcPb contributed specifically to IL-1α-induced p38 activation but not IL-6 synthesis. ERK1/2 was not activated by IL-1β in WT neurones but was induced by IL-1α in IL-1RAcPb$^{-/-}$ neurones indicating that IL-1RAcPb may negatively regulate IL-1α-induced ERK1/2 activation in WT neurones. Additionally, Src kinase was not activated by IL-1α or IL-1β in WT or IL-1RAcPb$^{-/-}$ neurones, but, PP2, a Src kinase specific inhibitor abolished IL-1α- and IL-1β-induced IL-6 in neurones, indicating that Src kinase activity is involved in IL-1-induced IL-6 synthesis. SB203580 and UO126 also inhibited IL-1α- and IL-1β-induced IL-6 in neurones indicating that p38 and ERK1/2 are important signaling pathways mediating IL-1-induced IL-6 in neurones. These data suggests that IL-1RAcPb may modulate multiple signalling pathways in neurones, but the specific action of IL-1RAcPb (whether positively or negatively regulating IL-1 action) may depend on the specific signaling pathway.

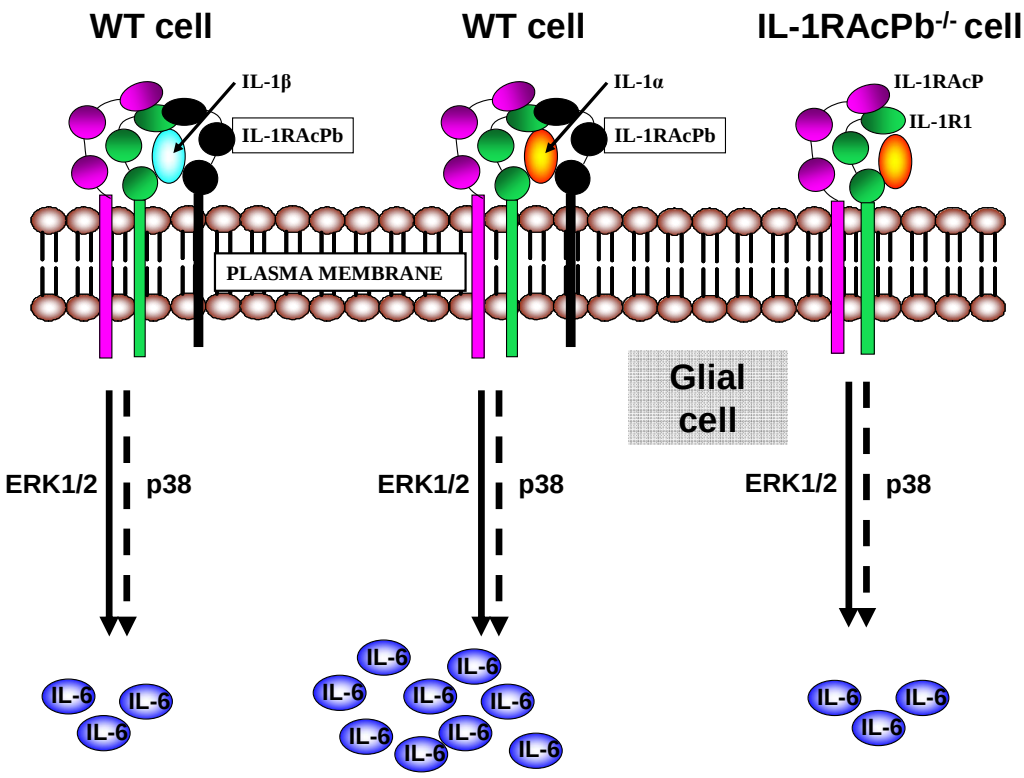


Figure 6.2. The role of IL-1RAcPb in IL-1-induced actions in glial cells

In WT glia, IL-1α was more potent than IL-1β at inducing IL-6 synthesis. However, in IL-1RAcPb$^{-/-}$ glia, the two cytokines were equipotent because IL-1α induced IL-6 was reduced in IL-1RAcPb$^{-/-}$ glia compared to WT glia. These results indicate that IL-1RAcPb contributed specifically to IL-1α-induced IL-6 synthesis in WT glia. The mechanism responsible for this differential effect is unknown because IL-1α and IL-1β induced ERK1/2 with similar strength in WT and IL-1RAcPb$^{-/-}$ glia. IL-1-induced p38 activation could not be quantified (indicated by broken line) and IL-1-induced Src kinase activation was not detected, therefore the contribution of these two signaling pathways in IL-1-induced IL-6 in glial cells is not clear.

6.2 Experimental approaches

The aim of an *in vitro* study is to investigate *in vivo* mechanisms in a restricted but more controlled environment. *In vivo* studies are often considered more valid since the mechanisms studied occurs in more physiological, spatial and temporal conditions that govern the response. However, it is difficult to study the contribution of a single cell type and/or specific molecular mechanisms *in vivo*. *In vitro* studies have the advantage that a specific response of interest can be studied in a single cell type population, in isolation, in the absence of other factors or contact with other cell types. By this method, the mechanisms, likely to be similar to those involved *in vivo*, can be identified *in vitro*. For example, *in vivo* studies have shown that cerebral ischaemia induces the activation of MAPK signalling (Skifter et al, 2002; Walton et al, 1998) and IL-1 expression (Boutin et al, 2001; Legos et al, 2000; Liu et al, 1993), and importantly, inhibition of these signalling pathways by IL-1RA (Emsley et al, 2005; Mulcahy et al, 2003; Yang et al, 1999; Yang et al, 1998; Stroemer and Rothwell, 1997; Betz et al, 1995; Relton and Rothwell, 1992), or specific inhibitors have been shown to be neuroprotective (Wang et al, 2004; Legos et al, 2001; Barone et al, 2001b). *In vitro*, MAPK activation in glial cells resulted in expression of proinflammatory cytokines such as IL-1, IL-6, PGE_2 (Andre et al, 2005b; Kim et al, 2004; Parker et al, 2002) and these responses were blocked by IL-1RA or specific inhibitors. These studies suggest that cerebral ischaemia induces MAPK activation in glial cells leading to an increase in proinflammatory mediator expression to induce neuronal cell death. Glial–mediated neuronal cell death has been demonstrated previously *in vitro* (Thornton et al, 2006; Xie et al, 2004). However, some responses could be abrogated in the absence of different cell types, therefore a lack of response such as that observed for the effect of IL-1 on Src kinase activation in the current study should be interpreted with caution.

Previous studies (Smith et al, 2009; Lu et al, 2008) have used cell lines to characterise the function of IL-1RAcPb. In this study the role of IL-1RAcPb in IL-1-induced actions was studied in primary neuronal and glial cultures, which is more physiologically relevant. The disadvantage of using a cell line is that the intracellular machinery in these cells is different to neurones and glia, therefore their responses to IL-1 could be modified. Additionally, both previous reports

investigated IL-1RAcPb action by gene transfection which could lead to many compensatory mechanisms being triggered, and over-expression of IL-1RAcPb could introduce bias into the IL-1 response. However using primary cell cultures also has its disadvantages. For instance, the influence of other cell types in the brain parenchyma on IL-1RAcPb-mediated IL-1 actions cannot be investigated, although neuronal and glial cells in cultures are morphologically and functionally similar to neurones and glia *in vivo*. Neurones in cultures are electrically excitable cells with the capacity to generate action potentials, form functional synapses and are capable of neurotransmitter expression such as substance P, and other proteins including L-1, IL-6 and NGF (Skoff et al, 2009; Tsakiri et al, 2008c; Carlson et al, 1999; Freidin et al, 1992; Kriegstein and Dichter, 1984; Dichter et al, 1983; Kriegstein and Dichter, 1983; Dichter, 1978). Glial cells in cultures are partially activated but the level of activation is considered minimum given that the level of signalling and proinflammatory mediators synthesised in control cells are very low. *In vivo,* following injuries such as cerebral ischaemia, p38 MAPK signalling is activated in astrocytes and microglial cells, and activation of these signalling pathways is also evident in glial cells *in vitro* (de Souza et al, 2008; Andre et al, 2005b). These studies indicate that neurones and glial cells in culture are valid systems to investigate the role of IL-1RAcPb in the mechanisms of IL-1 action *in vivo*.

In this study, RT-PCR, ELISA and Western blotting were used as methods of analysis. Whilst ELISA is a quantitative measure, RT-PCR and Western blotting are semi-quantitative measures which may not be sensitive enough to detect subtle but physiologically relevant changes. This may give rise to false negative results. In this study, RT-PCR data indicated that IL-1RAcPb deletion did not affect the expression of IL-1RAcP mRNA in neurones and glia, however, this needs to be confirmed by quantitative PCR as subtle changes in IL-1RAcP mRNA consequent to IL-1RAcPb deletion may not be detected by semi-quantitative RT-PCR. Similarly, Western blotting analysis indicated that IL-1 did not induced ERK1/2 and Src kinase activation in neurones (see Chapter 4), but this would need to be confirmed using quantitative analysis such as ELISA.

6.3 IL-1RAcPb expression in CNS cells.

The expression of IL-1RAcPb was previously shown to be most abundant in the CNS, with expression greater than IL-1RAcP in the brain (Smith et al, 2009). RT-PCR revealed that IL-1RAcPb is expressed in both neuronal and mixed glial cultures prepared from mice embryos and neonatal mice respectively. However, the expression of IL-1RAcPb was strong in neuronal cells but weak in glial cells, which is consistent with previous findings (Smith et al, 2009). Expression of IL-1RAcPb during early stages of development suggests that this receptor could be expressed constitutively in the brain. This is consistent with reports that IL-1R1 are expressed constitutively in rat hippocampus and *Xenopus laevis* (Friedman, 2001; Jelaso et al, 1998). Early expression of IL-1R1 in *Xenopus laevis* in the midbrain and hindbrain was suggested to be involved in promoting neuronal survival during CNS development which suggests that IL-1RAcPb could also have a similar role. However, IL-1RAcPb deletion did not affect neuronal or glial cell growth or differentiation *in vitro* suggesting that IL-1RAcPb is not essential for normal growth and development of these cells *in vitro*. The role of IL-1RAcPb in neurones and glial cells development *in vivo* remains to be investigated as the presence of other cell types in the brain parenchyma or the support of a functioning extracellular matrix, absent in our *in vitro* model, could contribute to the potential function of IL-1RAcPb on brain development.

The expression of IL-1RAcPb in specific glial sub-types is unclear. It is not known if the IL-1RAcPb mRNA expression in our mixed glial cultures (see section 3.4.3) was derived from the astrocytic population (82%), the microglial population (18%), or both. The expression of IL-1RAcPb in astrocytes may modulate IL-1 actions in these cells. However, the role of IL-1RAcPb expression in microglial cells is unknown since no IL-1 actions were detected in microglial cells (Pinteaux et al, 2002; Ban et al, 1993). To address this question, it would be necessary to study the expression of IL-1RAcPb in pure microglial or astrocytic cultures by PCR as there are currently no effective antibody against IL-1RAcPb to investigate this by double immunohistochemistry (IL-1RAcPb + GFAP, and IL-1RAcPb + tomato lectin) or Western blotting. The expression of IL-1RAcPb in specific cell types could be also determined by immunohistochemistry of

astrocytes or microglia combined with *in situ* hybridization for IL-1RAcPb. The expression of IL-1RAcPb in oligodendrocytes and O_2A progenitor cells was not determined in this study. Immuno-staining of cells in culture did not reveal any progenitor cells, however, this could be due to poor antibody and their presence could still be there, as has been demonstrated previously for mouse mixed glial cultures (Parker et al, 2002). The low level of IL-1RAcPb expression in our glial cultures could come from uncharacterised O_2A cells since these progenitor cells and neurones are derived from same neural precursor cell lineage. The expression of IL-1RAcPb by glial and progenitor cells may contribute to the acute and chronic inflammatory response of the CNS. Astrocytes and microglial are activated in response to acute brain damage (e.g. cerebral ischaemia) and in neurodegenerative diseases including Alzheimer's disease and multiple sclerosis. However, the role of IL-1RAcPb in these conditions is completely unknown.

6.4 The role of IL-1RAcPb in IL-1-induced IL-6 in neurones and glia

In a previous study (Tsakiri et al, 2008c), IL-1α and IL-1β were demonstrated to differentially induce IL-6 in mouse cortical neurones, however this was not observed in the current study. Differences in mouse strain and culturing protocol have been speculated as possible causes to this discrepancy (see Chapter 3). As we observed neither an increase nor a decrease in IL-1 response in IL-1RAcPb$^{-/-}$ neuronal culture when compared to WT, we can conclude that IL-1RAcPb did not contribute significantly to IL-1α- or IL-1β-induced IL-6 synthesis in neurones. However, neurones *in vitro* may lack cell-to-cell contacts or supporting factors from other cell types that could be essential for IL-1RAcPb functions. The effect of IL-1RAcPb on IL-1-induced neuronal IL-6 synthesis *in vivo* is not known. IL-6 was chosen as a read-out for IL-1 actions since it has been shown to be strongly induced by IL-1 in both neurones and glial cells (Tsakiri et al, 2008c; Parker et al, 2002). However, neurones also express other mediators including NGF, BDNF, substance P and neurotrophins (Skoff et al, 2009; Mattson, 2005; Isackson, 1995) which could be used as possible end-points to investigate the function of IL-1RAcPb.

IL-1-induced IL-6 is a mechanism involved in IL-1-induced fever (Chai et al, 1996; LeMay et al, 1990), however, this response involves the distal action of IL-1 and cross-talk between multiple brain regions (the hypothalamus and the pituitary which forms the hypothalamic-pituitary-adrenal axis), which could not be studied using cell culture. Distal action of IL-1 was demonstrated by the *icv* infusion of IL-1β and subsequent activation of NF-κB in subcortical brain regions such as the hypothalamus and amygdala (Konsman et al, 2000). It is unclear from the current study whether IL-1RAcPb could be involved in distal actions of IL-1 such as fever (Sanchez-Alavez et al, 2006; Konsman et al, 2000; Anforth et al, 1998). IL-6 is a potent pyrogen (Ulrich-Pur et al, 2000; Lenczowski et al, 1999; Sundgren-Andersson et al, 1998), IL-1RAcPb contributed to IL-1α-induced IL-6 in glial cells suggests that IL-1RAcPb may affect IL-1-induced distal actions such as fever.

IL-1RAcPb may contribute to IL-1α and IL-1β differential effects in glial cells by contributing preferentially to IL-1α-induced IL-6. Differential effects of IL-1α and IL-1β have been reported *in vitro* (Tsakiri et al, 2008c; Andre et al, 2005b; Juric and Carman-Krzan, 2001) and *in vivo* (Dube et al, 2001; Lemke et al, 1999; Beuscher et al, 1992). IL-1α and IL-1β are encoded by different genes, but act at the same complex receptor, IL-1R1/IL-1RAcP and contribute similarly to many biological responses, however, they display low sequence homology (27 %) (for review see Allan et al, 2005). The mechanism by which IL-1α or IL-1β interact with this signalling complex is not clear but the differential effects of IL-1α and IL-1β on IL-6 expression in glia suggests that IL-1α and IL-1β may bind to the signalling complex differently. Indeed, differential interaction between IL-1α and IL-1β to IL-1RAcP has been reported previously (Yoon and Dinarello, 2007), and binding of IL-1α to the signalling complex may lead to the recruitment of IL-1RAcPb and/or other adapter molecules to regulate IL-1α signalling mechanisms. The specific effects of IL-1RAcPb on IL-1α activity suggest that IL-1α may have a higher affinity for IL-1RAcPb than IL-1β. It would be necessary to do cell binding assay and ligand displacement studies on IL-1α and IL-1β in WT and IL-1RAcPb$^{-/-}$ cells to confirm this. It is well documented that IL-1α and IL-1β display different affinity for IL-1R1 and IL-1R2, and these affinities could be species dependent, or strain or cell specific (see Chapter 1).

These contributory effects of IL-1RAcPb on IL-1α action may help to explain some of the differences between IL-1α and IL-1β actions in the CNS.

6.5 The role of IL-1RAcPb in IL-1-induced signalling in neurones and glia

Studying the role of IL-1RAcPb on IL-1-induced IL-6 synthesis in neurones did not reveal an active role for IL-1RAcPb in this response. However, IL-1RAcPb appeared to contribute to IL-1α-induced p38 activation in neurones which indicates that IL-1RAcPb could affect other neuronal response mediated by p38 signalling. Indeed, IL-1-induced activation of the p38 signalling pathway has been shown to induce IL-6 in neuronal cell line (Bergamaschi et al, 2006), as well as expression of other proinflammatory genes including $cPLA_2$ and COX-2 gene in human neuroblastoma cells (Moolwaney and Igwe, 2005). *In vivo*, p38 is activated by cerebral ischaemia in rodents (Irving et al, 2000; Walton et al, 1998) and inhibiting p38 action is neuroprotective (Legos et al, 2001; Barone et al, 2001a; Barone et al, 2001b). The role of IL-1RAcPb in these experimental settings is unknown, but it is possible that IL-1RAcPb may contribute to ischaemia-induced brain damage driven by IL-1 (see Chapter 1) by contributing to IL-1α actions.

Conversely to p38 activation, IL-1β failed to induce ERK1/2 in WT neurones, but was activated by IL-1α in IL-1RAcPb$^{-/-}$ neurones. This suggests that IL-1RAcPb may have a negative regulatory role in IL-1α-induced ERK1/2 activity in neurones. The significance of IL-1α-induced ERK1/2 activation in IL-1RAcPb$^{-/-}$ neurones is unclear. Preliminary data from our laboratory have shown that IL-1α mediates kainate-induced seizures and subsequent brain damage in IL-1RAcPb$^{-/-}$, but not in WT mice (unpublished data), suggesting that IL-1RAcPb is effectively a negative modulator of IL-1α action. These data suggest that the mechanism of IL-1α-induced brain damage in kainate-induced seizures could be attributed to IL-1α-induced ERK1/2 signalling in IL-1RAcPb$^{-/-}$ neurones.

Although IL-1β did not induce ERK1/2 activation in WT neurones, basal ERK1/2 activity was essential for IL-1-induced IL-6 synthesis in neurones

because in the absence of basal ERK1/2 phosphorylation, IL-1 did not induce IL-6 (see Chapter 5). This indicates that IL-1-induced IL-6 expression requires activation of more than one signalling pathway. This is consistent with our understanding of the role of ERK1/2 and p38 in gene expression. Effectively, as activated ERK1/2 is involved in protein synthesis at a transcription level whereas MK2 is involved in protein synthesis at a translational level (see introduction to Chapter 4), inhibiting basal ERK1/2 activity could result in blockage of expression of downstream inflammatory mediators, and the activation of translational machinery is ineffective in the absence of transcription.

IL-1-induced protein expression is associated with IL-1 slow responses such as IL-1-induced IL-6 and PGE_2 during neuroinflammation. However, IL-1 is also associated with a rapid and transient response involving Src kinase activation and ion channel activation, resulting in changes in neuronal electrical properties (Tsakiri et al, 2008a; Davis et al, 2006; Sanchez-Alavez et al, 2006; Desson and Ferguson, 2003; Viviani et al, 2003; Borsody and Weiss, 2002), which is evident in seizures (Balosso et al, 2008). However, in this study, IL-α and IL-1β did not induce Src kinase activation in neurones or glia. The cause of this discrepancy could be related to the differences in species, strain of animals used, population of neurones studied and/or time-point investigated as well as limitations in the method of detection (see section 6.2). However, our chosen time-point was the suggested optimum time for IL-1-induced Src kinase in mouse cortical neurones (Tsakiri et al, 2008a). The role of IL-1RAcPb in IL-1-induced ionic channel activation was not investigated in this study, therefore remains a potential avenue for future investigations. Whole-cell patch-clamp recordings of WT and IL-1RAcPb$^{-/-}$ in response to IL-1 could be a potential technique to study the role of IL-1RAcPb in neuro-electrophysical responses.

In glial cells, IL-1RAcPb did not contribute to IL-1-induced ERK1/2 signalling, and its effect on p38 and Src kinase activation could not be determined. IL-1 has been demonstrated to induce NF-κB signalling in glial cells (Srinivasan et al, 2004) which could potentially be modulated by IL-1RAcPb. Indeed, knowing that activation of MAPK and NF-κB signalling pathways in glial cells leads to the production of proinflammatory mediators such as IL-1, IL-6, TNF and PGE_2 (Andre et al, 2005b; Kim et al, 2004; Parker et al, 2002; Dunn et

al, 2002), modulation of these signalling pathways by IL-1RAcPb could indicate a potential role for IL-1RAcPb in the inflammatory response of the CNS.

6.6 Conclusion

This study showed that IL-1RAcPb modulates selectively the action of IL-1α in neurones and glial cells *in vitro*. More specifically, IL-1RAcPb contributed to IL-1α-induced p38 activation but negatively regulated ERK1/2 activation in neurones. In glial cells, IL-1RAcPb contributed to IL-1α-induced IL-6 synthesis. These observations suggest that the action of IL-1RAcPb could affect multiple signalling cascades in parallel as well as being cell specific. IL-1RAcPb did not mediate IL-1β actions in neurones or glia, which suggests that IL-1RAcPb may modulate only some IL-1 responses (IL-1α-induced responses). However, further investigations are required to confirm these hypotheses.

6.7 Future directions

The role of IL-1RAcPb could be further studied by investigating the contribution to other IL-1 actions, for example, expression of other mediators such as TNF-α, PGE_2 or neurotrophic factors. IL-1-induced NF-κB signalling in neurones and glial could also be a potential avenue to further investigate the role of IL-1RAcPb in IL-1 actions. The effect of IL-1RAcPb on neuronal electrophysiological activity could also be investigated. IL-1 has been shown to affect different neuronal population differently including hyperpolarisation of mouse anterior hypothalamic neurones (Davis et al, 2006; Tabarean et al, 2006; Sanchez-Alavez et al, 2006), excitation of mouse cerebellar Purkinje cells (Motoki et al, 2008) and neurones in the paraventricular nucleus in rats (Ferri and Ferguson, 2003). Both IL-1β-induced hyperpolarisation and depolarisation were observed in rat subfornical organ neurones and *locus coeruleus* neurones (Desson and Ferguson, 2003; Borsody and Weiss, 2002). In mouse cortical neurones, IL-1-induced IL-6 and Src kinase activation (Tsakiri et al, 2008a), and p38 activation in rat hippocampal neurones (Srinivasan et al, 2004). Therefore, the next step in understanding the role of IL-1RAcPb would be to investigate the function of this receptor (in electrophysiology and IL-1-induced signalling pathways) in different neuronal populations.

In addition to the *in vitro* approach, the role of IL-1RAcPb in IL-1 signalling can also be studied *in vivo*. IL-1RAcPb has been shown to be a negative regulator of IL-1α action in kainite-induced seizures (unpublished data). This indicates that IL-1RAcPb may act as a negative modulator of IL-1α but not IL-1β actions in neurones. IL-1 is a key cytokine in acute and chronic neuroinflammation indicating that the role of IL-1RAcPb in the CNS could be wide ranging and unknown. The role of IL-1RAcPb in stroke, brain trauma, PD or AD are potential avenues for future investigations.

IL-1 actions independent of IL-1R1 has been shown both *in vitro* (Andre et al, 2006) and *in vivo* (Touzani et al, 2002), however, the role of IL-1RAcPb in these responses is unknown. One hypothesis is that IL-1RAcPb could be a co-receptor to an uncharacterised IL-1 receptor, which may contribute to IL-1-induced IL-1R1 independent actions. This thesis has so far only focused on the role of IL-1RAcPb in IL-1-induced actions, however IL-1RAcPb may also have a

role in IL-18- or IL-33-induced actions as both these ligands require the association of an accessory protein to initiate signalling. Investigating the role of IL-1RAcPb independent of IL-1R1 and IL-1 could be a new chapter for the characterisation of IL-1RAcPb actions.

7. Appendices

Appendix I. Recipes for medium and buffers and experimental reagents

Table 7.1. Starve medium

Reagents	Quantity	Source
Dulbecco's Modified Eagle's Medium (DMEM)	500 ml	Sigma
100 U/ml/ penicillin and 100 μg/ml streptomycin	5 ml	Sigma

Table 7.2. Dissociation medium

Reagents	Quantity	Source
Starve medium	2 ml	Table 7.1
10 x trypsin	200μl	Invitrogen
Deoxyribonuclease	375 U/ml	Sigma

Table 7.3. Wash medium

Reagents	Quantity	Source
Dulbecco's Modified Eagle's Medium (DMEM)	500 ml	Sigma
100 U/ml/ penicillin and 100 μg/ml streptomycin	5 ml	Sigma
Foetal calf serum (FCS)	50 ml	PAA Laboratories (UK)

Table 7.4. Seeding medium

Reagents	Quantity	Source
Neurobasal medium	500 ml	Invitrogen
B27 with antioxidants	10 ml	Invitrogen
Plasma derived serum (bovine)	25 ml	First Link (UK)
L-Glutamine 200mM (100x)	5 ml	Invitrogen
100 U/ml/ penicillin and 100 μg/ml streptomycin	5 ml	Sigma

Table 7.5. Maintenance medium

Reagents	Quantity	Source
Neurobasal medium	500 ml	Invitrogen
B27 without antioxidants	10 ml	Invitrogen
Plasma derived serum (bovine)	25 ml	First Link (UK)
L-Glutamine 200mM (100X)	5 ml	Invitrogen
100 U/ml/ penicillin and 100 μg/ml streptomycin	5 ml	Sigma

Table 7.6. Mixed glia culture medium

Reagents	Quantity	Source
Dulbecco's Modified Eagle's Medium (DMEM)	500ml	Sigma
100 U/ml/ penicillin and 100μg/ml streptomycin	5ml	Sigma
Foetal calf serum (FCS)	50 ml	PAA Laboratories (UK)

Table 7.7. Antibodies used to characterise neuronal and glial cultures

Reagents / antibody	Dilution	Source
A2B5 (anti-GT3 ganglioside)	1:10 (2% Donkey serum 0.5% BSA in primary diluent - 0.1% triton-X-100 in PBS with 1.5 mM sodium azide)	Gift from Christine Pigott and Peter Andrews (Sheffield University, UK)
Normal Donkey serum	2% (0.1% triton-X-100 in PBS with 1.5 mM sodium azide)	Jackson Immunoresearch
GFAP (anti-glial fibrillary acidic protein conjugated to Cy3)	1:500 (2% Donkey serum 0.5% BSA in primary diluent - 0.1% triton-X-100 in PBS with 1.5 mM sodium azide)	Sigma
Biotinylated tomatoe lectin	1:200 (2% Donkey serum 0.5% BSA in primary diluent - 0.1% triton-X-100 in PBS with 1.5 mM sodium azide)	Sigma
NeuN (Mouse anti-neuronal nuclei)	1:500 (2% Donkey serum 0.5% BSA in primary diluent - 0.1% triton-X-100 in PBS with 1.5 mM sodium azide)	Chemicon (Temecula, CA, USA)
DAPI (4',6-diamidino-2-phenylindole, dihydrochoride)		Vector Laboratories (Burlington, CA, USA).

Table 7.8. Reagents and their sources used for RT-PCR.

Reagents	Quantity	Source
Trizol reagent	500 µl/ml	Invitrogen
Chloroform	200 µl	Fisher scientific
Isopropanol	250 µl	Fisher scientific
Ethanol	1 ml	Fisher scientific
DNase 1 amplificatuion grade	1 µl	Invitrogen
Oligo (dT) primer	1 µl	Invitrogen
DTT	2 µl	Invitrogen
10 mM dNTPs	1 µl	Invitrogen
MMLV	1 µl	Invitrogen
RNase out	1 µl	Invitrogen
Biomix Red	1 µl	Bioline
Primers (10 pM)	0.4 µl	Eurofins MWG operon

Table 7.9. Primers used for genetic characterisation of culture

Primers	Sequence	
IL-1RAcP	Forward	5'-TTT CCT ATG ACC GAA ATG TGG-3'
IL-1RAcP	Reverse	5'-TCA TTG CTA GAC CAC CTG GG-3'
IL-1RAcPb	Forward	5'- TGT TTC CTA TGC AAG AAA TGT GGA AGA AGA GG-3'
IL-1RAcPb	Reverse	5'-TCA GAC TCT TGG AGA GGA GGC TTA CAG AGG-3'
GAPDH	Forward	5'-TGA ATG ACA TCA AGA AGG TGG TGG AG-3'
GAPDH	Reverse	5'-TCC TTG GAG GCC ATG TAG GCC AT-3'

Table 7.10. PCR programme for each gene

Primers	Initiation step	Denaturation step	Annealing step	Elongation step	Final elongation step	Cycles
IL-1RAcP	94 °C/ 5 min	94 °C 50 sec	**60 °C 50 sec**	72 °C 1 min,	72 °C 5min	**40**
IL-1RAcPb	94 °C/ 5 min	94 °C 50 sec	**58 °C 50 sec**	72 °C 1 min,	72 °C 5min	**40**
GAPDH	94 °C/ 5 min	94 °C 50 sec	**58 °C 30 sec**	72 °C 1 min,	72 °C 5min	**30**

Table 7.11. Lysis buffer

Reagents	Quantity	Source
50mM tris pH 7.5	1 ml	Sigma
NP40	100 µl	Sigma
0.5 M NaF	1 ml	Sigma
1M B-glycerophosphate	500 µl	Sigma
0.5 M Sodium orthovanadate	100 µl	Sigma
dH2O	7.2 ml	
100mM PMSF	10 µl/ml	Sigma

Table 7.12. 2 x Sample buffer (100ml)

Reagents	Quantity	Source
1M Tris pH 6.8	5 ml	Sigma
10% SDS	20 ml	Sigma
Bromophenol blue	100 mg	Sigma
glycerol	10 ml	Fisher Scientific.
Dithiothreitol	10 ml or 1.5 g	Sigma
dH2O	55 ml or 65 ml if using 1.5 g DTT	

Table 7.13. Diluent 8 pH 7.2 – 7.4 (100ml)

Reagents	Quantity	Source
EDTA	0.045 g in 100 ml PBS	Sigma
Triton-X 100	500 µl	Sigma
Sodium fluoride	0.021 g	Sigma

Table 7.14. Diluent 6 ERK1/2 lysis buffer pH 7.2 – 7.4 (100ml)

Reagents	Quantity	Source
EDTA	0.045 g in 100 ml PBS	Sigma
Triton-X 100	500 µl	Sigma
Sodium fluoride	0.021 g	Sigma
Urea (6 M)	36 g	Sigma
Sodium pyrophosphate (2.5 mM)	0.06 g	Sigma
Sodium orthovanadate (1 mM)	0.018 g	Sigma
100mM PMSF	10 µl/ml	Sigma

Appendix II. The effects of IL-1RA on neurones

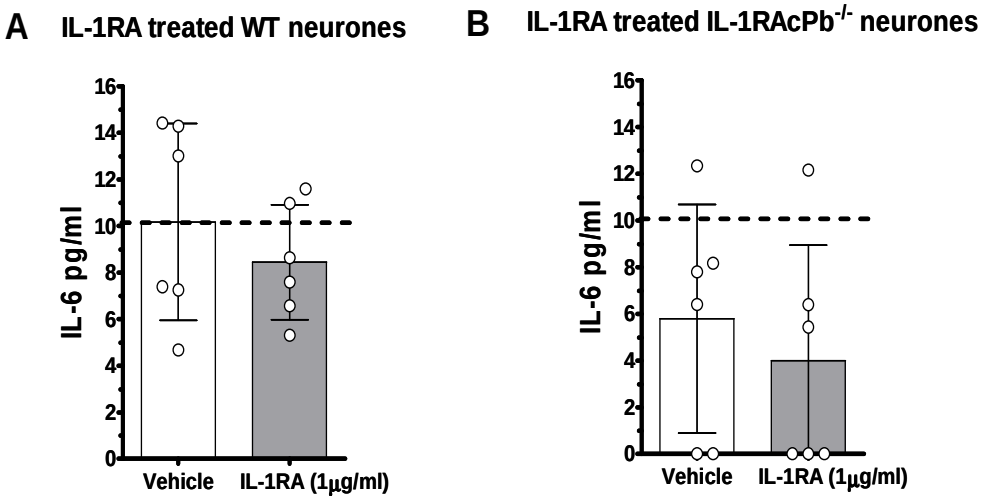


Figure 7.1. The effects of IL-1RA on neurones.

Neurones were treated with vehicle or IL-1RA (1µg/ml) for 24 h. Lysates were collected and analysed for IL-6 by ELISA. A. IL-1RA alone did not induce IL-6 in WT (n=6) or B. IL-1RAcPb$^{-/-}$ (n=6) neurones. One-way ANOVA and Tukey post-hoc test. Dashed line indicates detection limit (10 pg/ml).

Appendix III. IL-1-induced pentraxin-3 release in neurones and glia

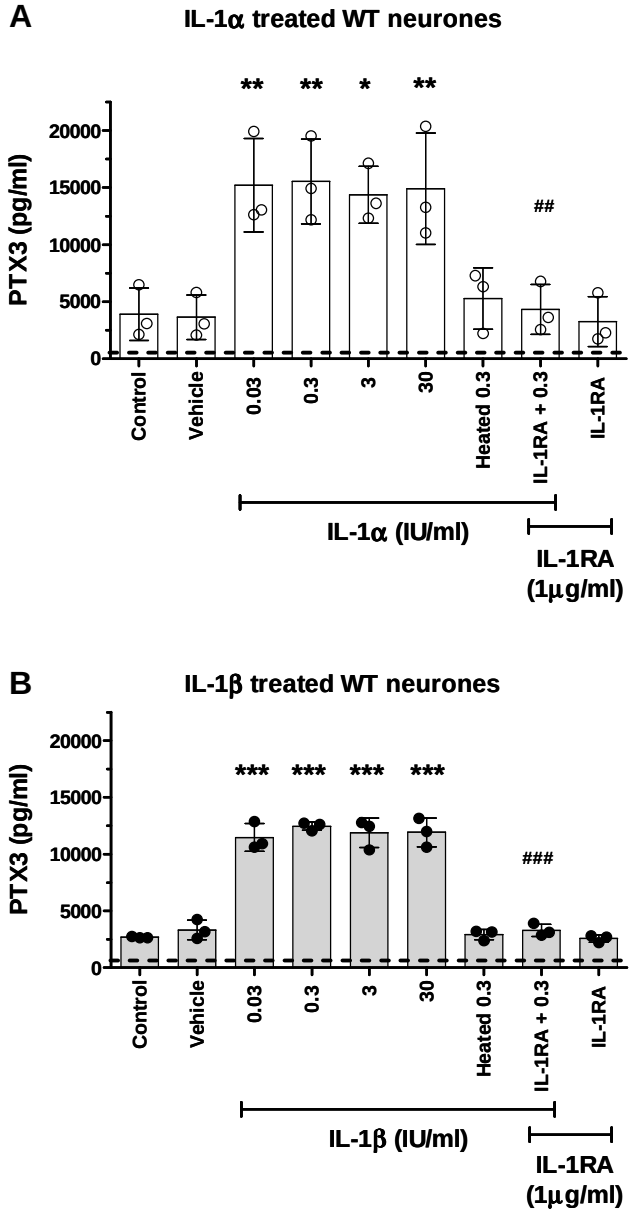


Figure 7.2. IL-1α- and IL-1β-induced PTX3 release in neuronal cultures.

WT neurones were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 24 h in the absence or presence of IL-1RA (1µg/ml), IL-1RA alone or heat-treated IL-1α/IL-1β. Supernatants were collected and analysed for PTX3 by ELISA. IL-1α (0.03-30 IU/ml: n=3) (A) and IL-1β (0.3-30 IU/ml: n=3) (B) significantly induced PTX3 release compared to vehicle. These responses were blocked by co-incubation with IL-1RA. * P< 0.05, ** P< 0.01, *** P<0.001 IL-1 vs. vehicle. $^{\#\#}$ P< 0.01, $^{\#\#\#}$ P<0.001 IL-1 (0.3 IU/ml) vs. ILRA co-incubation. One-way ANOVA and Tukey post-hoc test. Dashed line indicates detection limit (274 pg/ml).

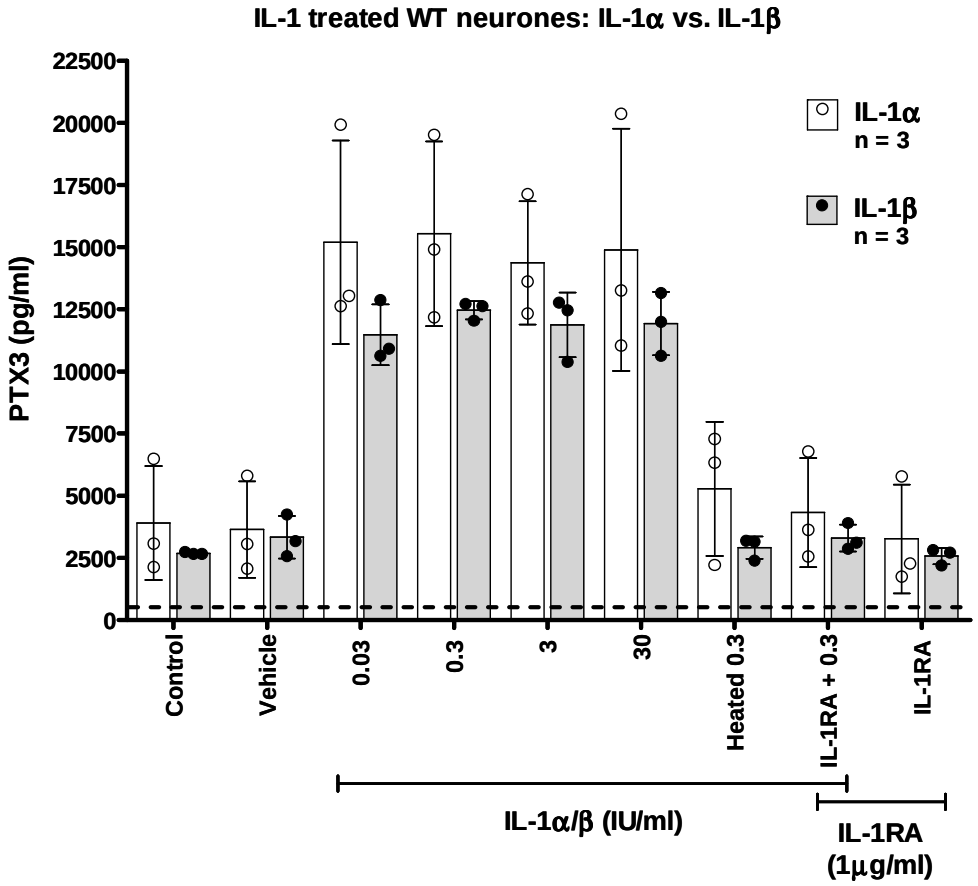


Figure 7.3. Comparison of IL-1α- and IL-1β-induced PTX3 release in neuronal cultures.

Comparison of the level of PTX3 induced by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), in the absence or presence of IL-1RA (1µg/ml), IL-1RA alone or heat-treated IL-1α/IL-1β showed that IL-1α and IL-1β induced similar levels of PTX3 release in WT neurones. No significant difference between IL-1α- and IL-β-induced PTX3 was found for any concentrations of IL-1 tested. Two-way ANOVA and Bonferroni post hoc test. Dashed line indicates detection limit (274 pg/ml).

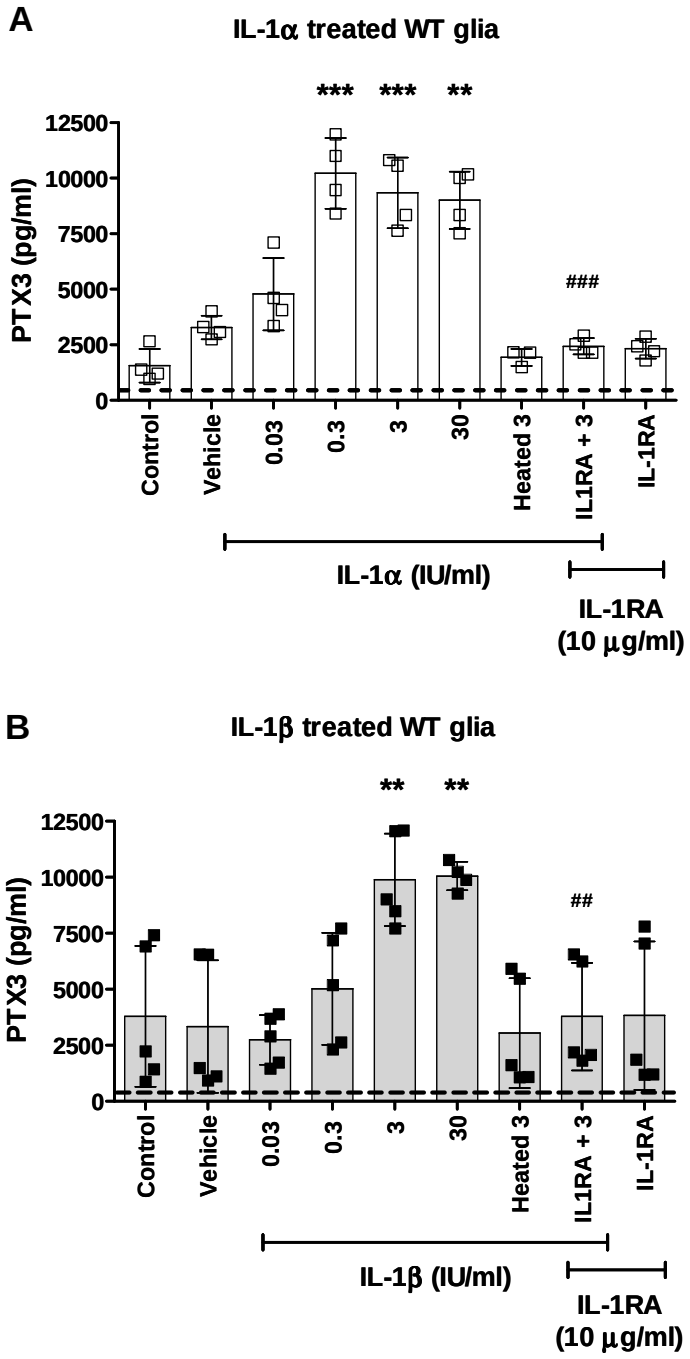


Figure 7.4. IL-1α- and IL-1β-induced PTX3 release in glial cultures.

WT glia were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 24 h in the absence or presence of IL-1RA (1µg/ml), IL-1RA alone or heat-treated IL-1α/IL-1β. Supernatants were collected and analysed for PTX3 by ELISA. IL-1α (0.3-30 IU/ml: n=4) (A) and IL-1β (3-30 IU/ml: n=5) (B) significantly induced PTX3 release compared to vehicle. These responses were blocked by co-incubation with IL-1RA. ** P< 0.01, *** P<0.001 IL-1 vs. vehicle. ## P< 0.01, ### P<0.001 IL-1 (0.3 IU/ml) vs. ILRA co-incubation. One-way ANOVA and Tukey post-hoc test. Dashed line indicates detection limit (90 pg/ml).

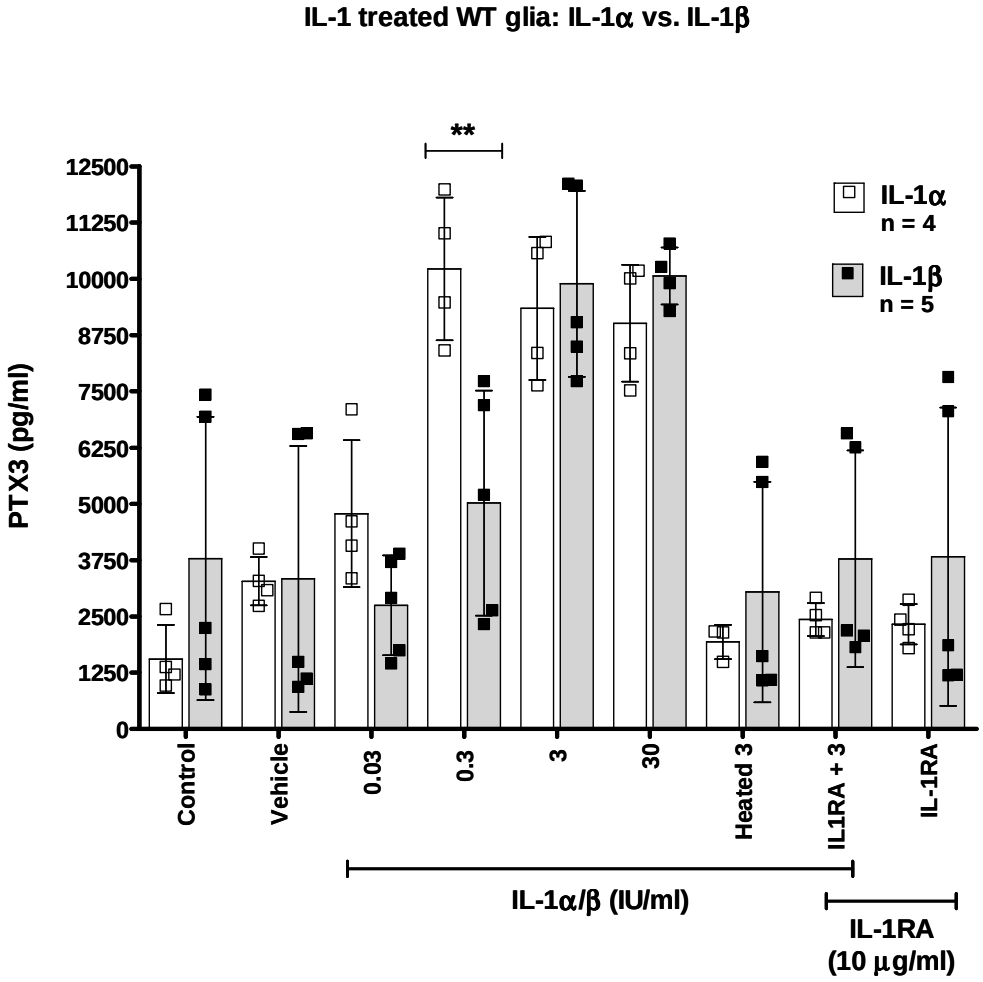


Figure 7.5. Comparison of IL-1α- and IL-1β-induced PTX3 release in glial cultures.

Comparison of the level of PTX3 induced by vehicle, IL-1α (0.03-30 IU/ml), IL-1β (0.03-30 IU/ml), in the absence or presence of IL-1RA (1µg/ml), IL-1RA alone or heat-treated IL-1α/IL-1β showed that IL-1α was significantly more potent than IL-1β at inducing PTX3 in glia at 0.3 IU/ml. Two-way ANOVA and Bonferroni post hoc test. . ** P<0.01 IL-1α vs. IL-β. Dashed line indicates detection limit (90 pg/ml).

Appendix IV. The effect of IL-1 on IL-6 expression in IL-1RAcP$^{-/-}$ neurones.

.

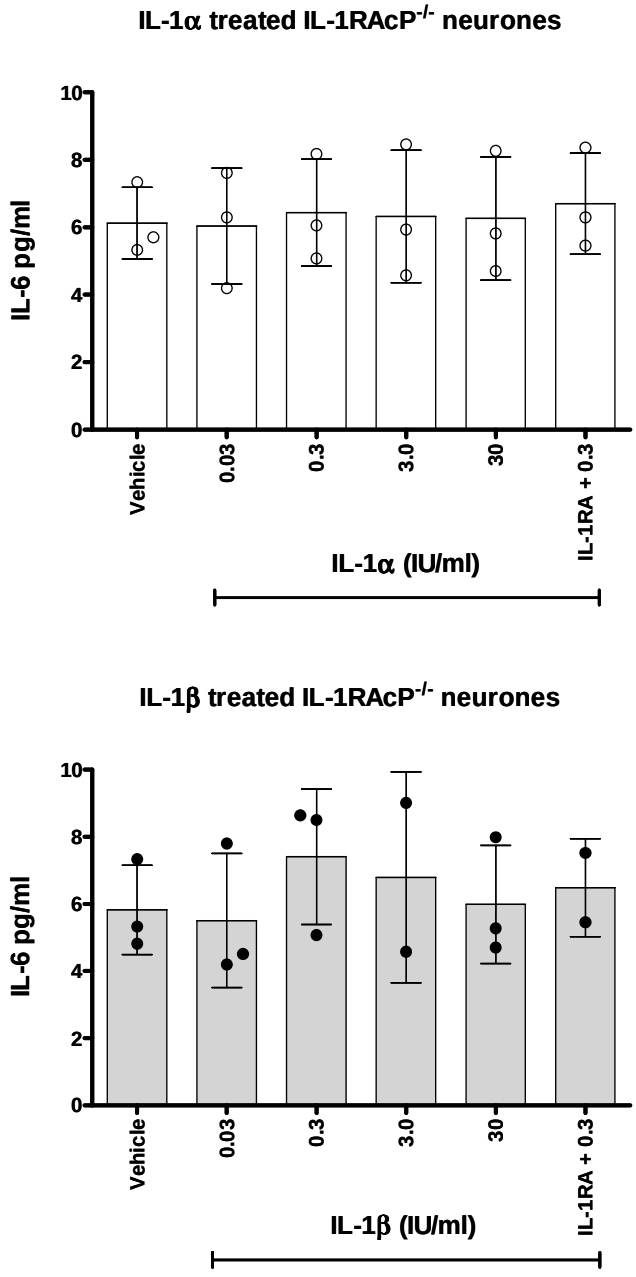


Figure 7.6. The effect of IL-1α- and IL-1β on IL-6 expression in IL-1RAcP$^{-/-}$ neuronal cultures

IL-1RAcP$^{-/-}$ neurones were treated with vehicle, IL-1α (0.03-30 IU/ml) or IL-1β (0.03-30 IU/ml) for 24 h in the presence or absence of IL-1RA (1 μg/ml). Supernatants were collected and analysed for IL-6 by ELISA. IL-1α (A) and IL-1β (B) failed to induce IL-6 at any concentration of cytokine tested. One-way ANOVA and Tukey's multiple comparison tests. Detection limit was 10 pg/ml.

Appendix IV. The effect of UO126 on IL-1β-induced ERK1/2 phosphorylarion in mixed glial cells

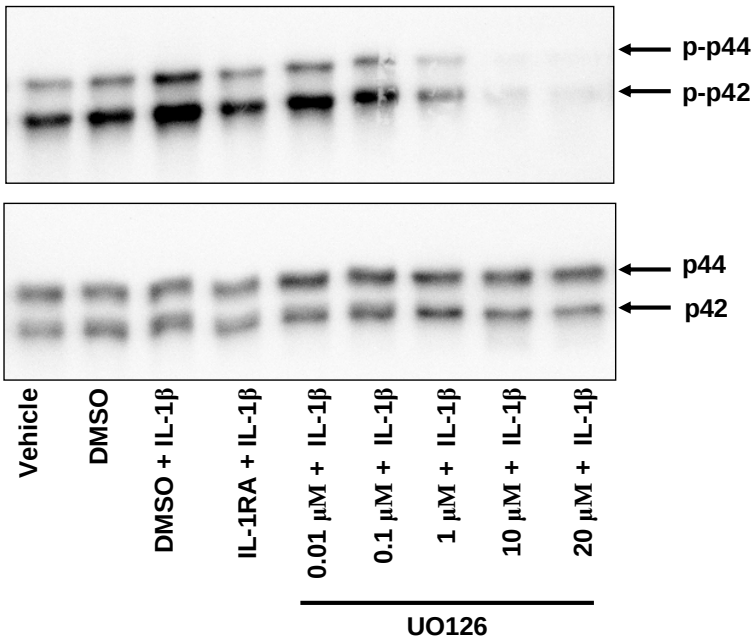


Figure 7.7. The effect of UO126 on IL-1β-induced ERK1/2 phosphorylarion in mixed glial cells

WT mixed glial cells were treated with vehicle, DMSO and/or IL-1β (3 IU/ml) for 15 min in the presence or absence of IL-1RA (10 μg/ml) or increasing concentration of UO126. Cell lysates were collected and analysed for ERK1/2 phosphorylation by Western blot analysis. IL-1β induced ERK1/2 activation that was blocked by IL-1RA and OU126 (1 μM-20 μM. UO126 inhibited both p42 and p44 isoform of ERK activation and at higher concentrations,10 μM and 20 μM, UO126 inhibited basal ERK1/2 activity.

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