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Noradrenergic modulation of the central nervous system immune response: A critical role for the β-adrenoceptor

by

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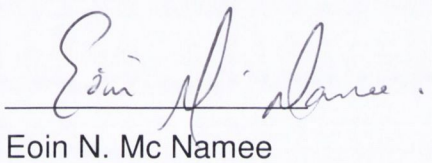

Eoin N. Mc Namee

II ABSTRACT

Evidence suggests that inflammation is a significant contributor to pathology in a number of neurodegenerative disease states. In this regard, the pro-inflammatory cytokine interleukin-1β (IL-1β) plays a key role in initiating an immune response within the central nervous system (CNS). However in addition to evidence indicating that IL-1β has detrimental effects for neuronal survival, other studies indicate that IL-1 can be neuroprotective possibly via induction of neurotrophic factors. The actions of IL-1β can be regulated by interleukin-1 receptor antagonist (IL-1ra), an endogenously produced antagonist that prevents IL-1β from acting on the IL-1 type I receptor [IL-1R(I)]. Consequently, the balance between IL-1ra/IL-1β is considered to be of pathological importance, and pharmacological strategies that tip the balance in favour of IL-1ra may be of therapeutic benefit in situations where IL-1β contributes to pathology. Evidence is emerging to suggest that the neurotransmitter noradrenaline elicits anti-inflammatory actions in the CNS, and consequently may play an endogenous neuroprotective role. Here we report that noradrenaline induces production of secreted IL-1ra from primary rat cortical mixed glial cells. This noradrenaline-induced increase in IL-1ra production is mediated via β-adrenoceptor activation, and downstream signaling via the cAMP-Protein Kinase A pathway and also requires activation of the MAP kinase ERK. In addition to increasing IL-1ra, noradrenaline increased expression of the IL-1type II receptor [IL-1R(II)]; a decoy receptor that serves to sequester IL-1β. The ability of noradrenaline to induce IL-1R(II) expression was also mediated via β-adrenoceptor activation and downstream signaling via Protein Kinase A and ERK. In parallel with its ability to increase IL-1ra and IL-1R(II) expression, noradrenaline prevented the neurotoxicity induced by conditioned medium from IL-1β-treated mixed glial cells. Considering the pivotal role played by IL-1β in neuroinflammation, the ability of noradrenaline to negatively regulate the IL-1 system and protect against IL-1β - induced neurotoxicity may be of therapeutic relevance in neurodegenerative disorders where inflammation contributes to pathology.

In this study we also examined the impact of increasing central noradrenergic tone *in vivo* on expression of components of the IL-1 system in rat brain. Specifically, *in vivo* studies conducted using pharmacological treatments that either enhance the central availability of noradrenaline (reboxetine + idazoxan combination), or directly stimulate central β_2-adrenoceptors were also shown to impact upon the central IL-1 system. In contrast to the *in vitro* situation where noradrenaline and β_2-adrenoceptor stimulation increased IL-1ra and IL-1R(II) without any alteration in IL-1β, increasing central noradrenergic tone or direct stimulation of central β_2-adrenoceptors *in vivo* induced IL-1ra and IL-1R(II), but also induced IL-1β. Based on a time-course study conducted with the centrally acting β_2-adrenoceptor agonist clenbuterol, IL-1β was produced first, and this was followed by prolonged expression of the negative regulators IL-1ra and IL-1R(II). Despite the increase in IL-1 induced by clenbuterol, there was no evidence of inflammatory signaling (assessed by IκBα expression) or apoptotic cell death (assessed by caspase3, BAX and Bcl-2 expression) in the hippocampus or cortex of clenbuterol treated rats.

In addition to the changes in the IL-1 system observed following administration of noradrenergic enhancers we also observed that the reboxetine + idazoxan combination induced mRNA expression of the broad-spectrum anti-inflammatory cytokine IL-10 in both cortex and hippocampus, and this was accompanied by an increase in IL-10 protein expression. In addition, reboxetine + idazoxan induced IL-10 signalling indicated by increased STAT3 phosphorylation and SOCS-3 mRNA expression. The ability of the drug combination to induce IL-10 production and signaling was mediated by β-adrenoceptor activation, as all of the aforementioned effects were blocked by the β-adrenoceptor antagonist propranolol. In addition, direct stimulation of central β_2-adrenoceptors with clenbuterol induced IL-10 production and signaling. In all, these data indicate that increasing central noradrenergic tone promotes an anti-inflammatory environment in the CNS, which may protect against neurodegeneration that occurs secondary to inflammation.

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V Abbreviations

Aβ	=	Amyloid-β
AD	=	Alzheimer's disease
APS	=	Ammonium persulfate
AP-1	=	Activator protein-1
ARA-C	=	Cytosine-arabinofuranoside
AR	=	Adrenoceptor
ATF2	=	Activating transcription factor 2
BCA	=	Bicinchoninic acid
BDNF	=	Brain derived neurotrophic factor
BSA	=	Bovine serum albumin
cAMP	=	Cyclic adenosine monophosphate
Ca^{2+}	=	Calcium
Cl^-	=	Cloride
CNS	=	Central nervous system
COX	=	Cyclooxygenase
C/EBP-β	=	CCAAT/enhancer binding protein-β
CREB	=	cAMP regulatory element binding protein
CSF	=	Cerebrospinal fluid
CT	=	PCR cycle number
DAG	=	Diacyl glycerol
DEPC	=	Diethyl Pyrocarbonate
DMEM	=	Dulbecco's modified eagles medium:F-12
DNA	=	Deoxyribonucleic acid
DTT	=	DL-Dithiothreitol
EAE	=	Experimental autoimmune encephalomyelitis
EDTA	=	Ethylenediaminetetra acetic acid
EtOH	=	Ethanol
ERK	=	Extracellular signal-regulated kinase
FCS	=	Foetal Calf Serum

FRET	=	fluorescence resonance energy transfer
GFAP	=	Glial fibrillary acidic protein
G_i	=	inhibitory G-protein
G_s	=	stimulatory G-protein
HCL	=	Hydrochloric acid
HSC	=	hematopoietic stem cells
HRP	=	Horse radish peroxidase
ICAM-1	=	Intra cellular adhesion molecule type-I
ICE	=	IL-1-converting enzyme
ICV	=	Intracerebroventricular
IDO	=	indoleamine-2,3-dioxygenase
Ig	=	Immunoglobulin
IKK	=	IκB kinase
IL-	=	Interleukin
IL-1ra	=	Interleukin-1 receptor antagonist
IL-1R(I)	=	Interleukin 1 type I receptor
IL-1R(II)	=	Interleukin 1 type II receptor
IL-1R(Acp)	=	Interleukin-1 receptor accessory protein
iNOS	=	Inducible nitric oxide synthase
I.P.	=	Intraperitoneal
IP3	=	Inositol tri-phosphate
IRAK	=	Interleukin-1 receptor-associated kinase
IFN	=	Interferon
IGF-1	=	Insulin like growth factor-1
H_2SO_4	=	Sulphuric acid
HIF	=	hypoxia-induced factors
Jak	=	tyrosine janus kinases
JNK	=	C-Jun N-terminal kinases
LDH	=	Lactate dehydrogenase
LC	=	Locus Ceruleus
LPS	=	Lipopolysaccaride

LTP	=	Long term potentiation
MAPK	=	Mitogen activated protein kinases
$MgCl_2$	=	Magnesium chloride
$MgSO_4$	=	Magnesium Sulphate
MHC	=	Major histocompatability complex
MPTP	=	1-methyl-4-phenyl-1,2,3,6-terahydropyridine
MS	=	Multiple sclerosis
NA	=	Noradrenaline
Na^+	=	Sodium
NET	=	Noradrenergic transporter
NFκB	=	Nuclear factor-kappa B
NK cell	=	Natural killer cell
NaH_2PO_4	=	Sodium phosphate monobasic monohydrate
Na_2HPO_4	=	di-Sodium hydrogen orthophosphate
NGF	=	Nerve growth factor
NMDA	=	N-methyl D-aspartate
NO	=	Nitric oxide
NaOH	=	Sodium hydroxide
OD	=	Optical density
PD	=	Parkinsons disease
P/S	=	Penicillin-streptomycin
PBS	=	Dulbecco's phosphate buffered saline
PGE_2	=	prostaglandin E_2
PKA	=	Protein kinase A
PKC	=	Protein kinase C
RNA	=	Ribonucleic acid
ROS	=	Reactive oxygen species
SDS-PAGE	=	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SOCS	=	Supressor of cytokine signaling
Stat	=	signal transducers and activators of the transcription
TBS	=	Tris buffered saline

TEMED	=	N,N,N',N'-Tetramethylethylene-diamine
TGF	=	Transforming growth factor
TMB	=	3,3',5,5',-Tetramethyl-benzidine
TNF	=	Tumour necrosis factor
TRAF	=	TNF receptor associated factor
VCAM	=	Vascular cell adhesion molecule
VMAT	=	Vesicular monoamine transporters

1. The Immune System

The immune system is a remarkably adaptive defence system that has evolved in humans to protect against invading pathogenic microorganisms (eg: bacterial, viral, fungal), tumors and to clear damaged cells. The immune system must be able to distinguish between pathogens or abnormal cells and healthy host cells so that it can direct its destructive powers towards their elimination. As a consequence of this dynamic complexity, the immune system is able to generate a tremendous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders, in addition to the recognition and destruction of abnormal cells. Humans have two types of immunity, innate (non-specific) and adaptive (specific). The activation of the innate immune response produces signals that stimulate and direct subsequent adaptive immune responses. Innate immunity comprises anatomical (eg: skin), physiologic (eg: fever response, stomach acid) and phagocytic (monocytes, neutrophils, tissue macrophages and dendritic cells) responses. In addition, inflammatory barriers have evolved to induce an immune reaction when required. For example, when there is tissue damage or infection, there is a subsequent induction of vascular permeability, the release of histamine, bradykinin, serotonin and prostaglandins into the sight of injury. This results in the activation of pain receptors, vasodilation of the blood vessels and subsequent influx of phagocytic cells to a sight of infection/damage (Stvrtinová, *et al.,* 1995).

All cells of both the innate and adaptive immune system are derived from hematopoietic stem cells (HSCs) in the bone marrow. HSC's are stem cells that give rise to all the blood cell types including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells). The mononuclear phagocytic cells consist of circulating monocytes in the blood and tissue macrophages (microglia in the brain). Monocytes can differentiate into tissue macrophages by complex processes

of cell enlargement, increases in hydrolytic enzyme production and the secretion of cytokines and chemokines. Although normally in a resting state, macrophages are activated by a variety of stimuli in the course of an immune response. There actions are further enhanced by cytokines secreted from Th and other immune cells. It is this ability to become activated that allows macrophages to phagocytose microbes and eliminate pathogens or infected/dieing cells. There ability to present antigens to lymphocytes is a critical step in the human immune system and bridges the gap between the innate and adaptive responses.

In contrast to non-specific innate responses, the adaptive immune response comprises specificity, diversity, memory and self/non-self recognition. An adaptive immune response requires the recognition of an antigen, which has been processed by a phagocytic innate cell, and the generation of a specific response to clear the threat by lymphocytes, T- and B-cells.

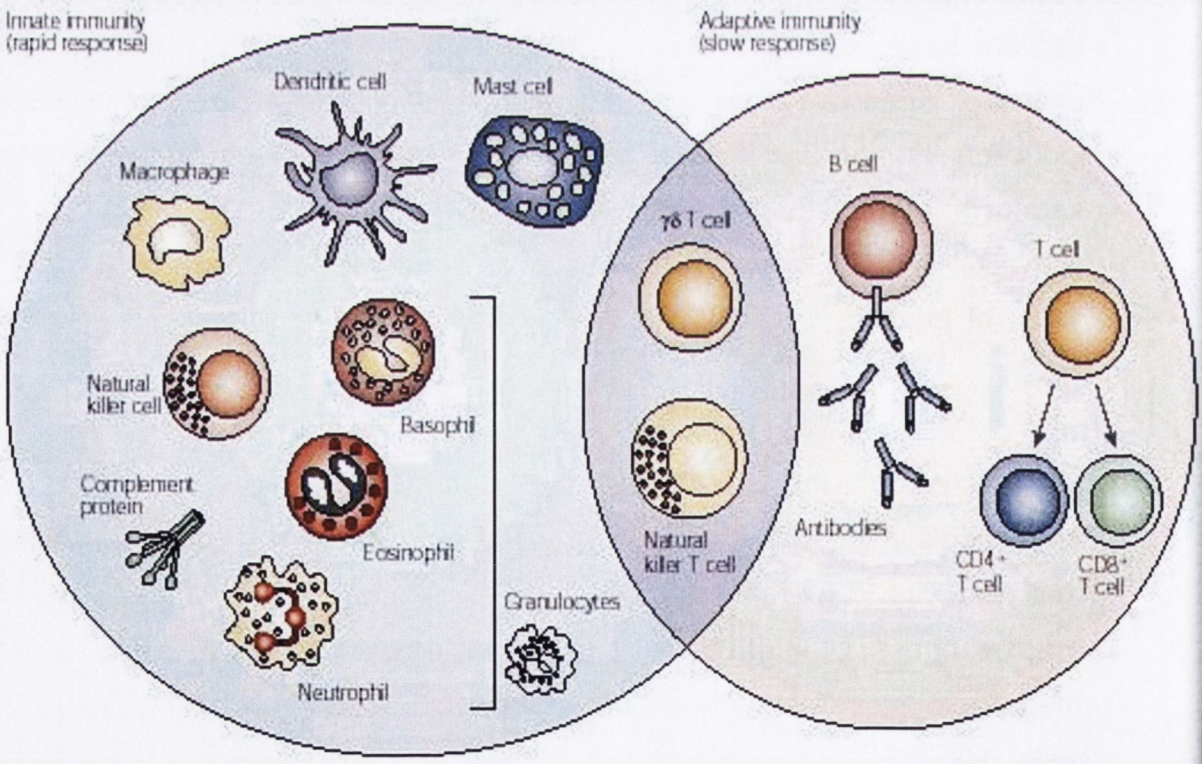


Figure 1.1: Cells of the immune system (Goldsby *et al.*, 2000)

T-cells mature predominantly in the thymus and there are two major subtypes: the cytotoxic T cell (CD8) and the helper T cell (CD4). Cytotoxic T cells are a sub-group of T cells that kill cells infected with viruses (and other pathogens), or are otherwise damaged or dysfunctional (Harty *et al.*, 2000). They are activated when their T cell receptor (TCR) binds to a specific antigen in a complex with the MHC Class I receptor of a phagocytic innate cell. Recognition of this MHC:antigen complex is aided by a co-receptor on the T cell, called CD8. The T cell then travels throughout the body in search of cells where the MHC I receptors bear this antigen. When an activated T cell contacts such cells, it releases cytotoxin's, such as perforin, which form pores in the target cell's plasma membrane, allowing ions, water and toxins to enter. The entry of another toxin called granulysin (a protease) induces the target cell to undergo apoptosis (Radoja et al., 2006). T cell killing of host cells is particularly important in preventing the replication of viruses. Helper T cells regulate both the innate and adaptive immune responses and help determine which types of immune responses the body will make to a particular pathogen (Abbas et al., 1996). Phagocytic innate cells present antigen on their Class II MHC molecules (MHC2). Helper T cells recognize these, with the help of their expression of CD4 co-receptor (CD4). The activation of a resting helper T cell causes it to release cytokines and other stimulatory signals that stimulate the activity of macrophages, killer T cells and B cells (Alberts *et al.*, 2002, Goldsby *et al.,* 2000)

B-cells mature in the bone marrow and contain antigen-binding receptors, allowing them to bind directly to an antigen and this antigen/antibody complex is taken up by the B cell and processed by proteolysis into peptides. The B cell then displays these antigenic peptides on its surface MHC class II molecules. This combination of MHC and antigen attracts matching helper T cells, which releases cytokines and activates the B cell (Kehry *et al.*, 1994). As the activated B cell then begins to divide, its offspring (plasma cells) secrete millions of copies of the antibodies that recognize this antigen. These antibodies circulate in blood plasma and lymph, bind to pathogens expressing

the antigen and mark them for destruction by complement activation or for uptake and destruction by phagocytes. Antibodies can also neutralize challenges directly, by binding to bacterial toxins or by interfering with the receptors that viruses and bacteria use to infect cells (Bowers, *et al.*, 2006, Sproul *et al.*, 2000).

1.1 Central Nervous System (CNS) immune response

There is now an extensive body of evidence to demonstrate that inflammatory events in the brain are major contributors to the pathogenesis of numerous neurological diseases, trauma and infection. Examples include Multiple Sclerosis, Alzheimer's disease, Parkinson's disease and human immunodeficiency virus (HIV) associated dementia (see Liu and Hong, 2003; Block and Hong, 2005; McGeer and McGeer, 1999; McGeer and McGeer, 2002 for reviews). Inflammatory mediators including cytokines, cell adhesion molecules and reactive oxygen species are detected in the CNS of patients suffering from these conditions, and also in animal models (see Allan *et al.*, 2001, Lucas *et al.,* 2006, Rothwell and Luheshi, 2000, for reviews). Interventions that inhibit or reduce the levels of expression of these agents would theoretically provide anti-inflammatory therapeutic benefits in many of these diseases. Recent progress in the use of anti-inflammatory therapies and immunization in animal models of CNS disorders indicates the importance of the immune response in resolving neurodegeneration (Allan *et al.*, 2001, Rothwell *et al.*, 1996).

1.1.1 *Glial cells*

In the human brain, neurons are out numbered ten-fold by supportive cells called glia (Greek: *glia*; glue) (see Stevens *et al.*, 2003 for review). Glia display diverse functions that include almost every aspect of nervous system function. Glia within the CNS can be assigned to one of three major classes based on there unique morphology and biochemistry, these include the oligodendrocytes, astrocytes and microglia. The myelinating glia – oligodendrocytes (including peripheral Schwann cells) provide insulating

layers of myelin membrane around neuronal axons, which allow neural impulses to propagate rapidly over long distances. Microglia and astrocytes are the glial subtypes most implicated in mediating inflammatory events in the brain, consequently these cell types are discussed in more detail below.

1.1.2 *Astrocytes*

These are large, star-shaped, glia which, have elongated processes tipped with end-feet. They are classed into two subgroups on the basis of their morphology, function and location. Protoplasmic astrocytes found in grey matter are closely associated with synapses, while fibrillary astrocytes in white matter contact the nodes of ranvier on neurons (Stevens *et al.,* 2003). The functions of astrocytes include homeostatic regulation of the extracellular K^+ concentration, removal of neurotransmitters from the synaptic cleft, regulating the supply of glucose to neurons, and generating the formation of a tight blood-brain barrier by inducing tight-junctions between endothelial cells (Jessen *et al.,* 2004). They also play an important role in inflammatory and disease states (Liberto *et al.,* 2004, Block *et al.,* 2005, Aloisi *et al.,* 1999). Astrocytes are a significant source of extracellular matrix proteins and factors and membrane bound cell adhesion molecules (Block *et al.*, 2005). Importantly, astrocytes are a major producer of neurotrophins, which are vital for the survival of neurons (Friedman *et al.*, 1993).

Glial fibrillary acidic protein (GFAP) is a 49.8 kDa non-soluble cytoplasmic filamentous protein that constitutes a portion of the cytoskeleton in astrocytes. GFAP belongs to the class III intermediate filamentous proteins and is though to be important in astrocyte motility and shape by providing structural stability to astrocytic processes (ENG *et al.,* 2000). Following CNS injury caused by trauma, genetic disorders or inflammation, astrocytes proliferate and display extensive hypertrophy of the cell body and processes and GFAP is markedly up-regulated. Alternatively, GFAP appears to be dramatically decreased during astrocytic malignancies. Astrocytes can also impair axon repair during CNS degeneration as they form a barrier through the formation of gliotic scars

around the site of injury. This gliosis is typified by astrocyte (and microglial) hypertrophy and proliferation and it can drastically alter the cell-cell signaling events required for normal glia-neuron function. For example, gliotic tissue may hault normal apoptotic signals as well as the ability to respond to insults like oxidative stress or infection, thus hindering axonal regeneration (Kielian *et al.*, 2004).

1.1.3 *Microglia*

Microglia are the resident 'immune cells' of the brain. They originate from the mesodermal hemopoietic precursor cells that enter the brain during embryonic development and are slowly turned over and replenished by proliferation in the adult CNS (Cuadros and Navascues, 1998, Vilhardt, *et al.* 2005). In the developing brain, microglia phagocytose surplus cells undergoing apoptosis and radial glia provide a temporary scaffold for the migration of newborn cortical and cerebral neurons (Barron *et al.*, 1995, Milligan *et al.,* 1991). Radial glia also provide important trophic support for migrating neurons (Stevens *et al.,* 2003). Recent studies have expanded on the role of glia in cellular homeostasis, in particular in the case of astrocytes, by proposing that glia might not only regulate neurogenesis but also themselves be neuronal progenitor cells (Nedergaard *et al.,* 2003).

In the healthy adult brain, microglia adopt a characteristic resting ramified morphology and serve a supportive role in immune surveillance and the clearing of cellular debris (Beyer *et al.*, 2000). However, the resting state of microglial is abandoned when they are presented with a pathological endogenous (eg: neuronal death, abnormal protein aggregation or immune cell interaction) or exogenous (infection) signal (see Vilhardt *et al.*, 2005, for review). Up-regulation of multiple phagocyte effector functions and expression of a range of molecular mediators allow activated microglia to respond specifically towards an insult by neurotrophic support, induction of an inflammation response and tissue repair. These functions can include cell proliferation, migration, phagocytosis, upregulation of antigen-presenting cell

receptors, secretion of pro-inflammatory cytokines (such as Interleukin (IL)-1 and Tumor necrosis factor (TNF)-α), chemokines, prostaglandins, proteases, and the generation of cytotoxic reactive oxygen and nitrogen intermediates (Aloisi *et al.*, 2001, Vilhardt *et al.*, 2005). Ongoing controversy exists regarding whether microglia are neuroprotective or neurotoxic when activated as in addition to producing toxic mediators, they also produce anti-inflammatory cytokines (such as IL-10 and transforming growth factor (TGF)-β) and neurotrophins, thus promoting neuronal survival (Liao *et al.*, 2005, Polazzi *et al.*, 2001). It is likely that microglia can serve multiple functions, depending on the progression of disease state and type of immune stimuli (Block *et al.* 2005).

In pathological conditions, microglia, have in particular received attention in a class of diseases characterized by CNS inflammation. These include Bacterial and Viral infections, Cerebral Ischemia, Multiple Sclerosis, Alzheimer's disease, Parkinson's disease, and HIV-associated dementia (see Block *et al.*, 2005, Aloisi *et al.,* 1999, Rothwell et al., 1995, Gonzalez-Scarano, 1995, for reviews). Microglia have been associated both with initiation and perpetuation of chronic inflammation in these conditions (Liu *et al,* 2003). For example, under basal conditions, microglia express low levels of the complement receptor 3 and major histocompatability complex I and II (MHC I / MHC II) receptors. However under stimulated conditions the expression of these cell surface receptors are up-regulated allowing for the association of microglia with invading encephalitogenic CD4 T lymphocytes (Salimi *et al.,* 2002). The rapid up-regulation of MHC antigens was amongst the first molecular changes described in multiple sclerosis as well as in different forms of the murine disease model, experimental autoimmune encephalomyelitis (EAE). It appeared that the up-regulation of MHC II was solely confined to activated microglia, macrophages and blood-borne leukocytes (Boyle *et al.,* 1990).

1.2 Cytokines and Neuroinflammation

Cytokines are low molecular weight regulatory proteins secreted by immune cells and various other cells in the body (eg: endothelial cells, keratinocytes and glia) in response to a number of different inflammatory stimuli. Cytokines collectively mediate the complex cell-cell interaction between lymphoid cells, inflammatory cells and the proliferation and differentiation of hematopoietic stem cells. They bind to specific receptors on the membrane of target cells, ultimately altering gene expression. Generally, a cytokines affinity for its specific receptor is very high, with dissociation constants ranging from 10^{-10} to 10^{-12}M, thus picomolar concentrations can exert an effect. Cytokines generally have a molecular weight of less than 30 kDa (Goldsby *et al.,* 2000).

The actions of cytokines may either promote or attenuate the inflammatory immune response and as such, they are generally classed as pro- or anti-inflammatory (Roitt *et al.*, 2001, Goldsby, *et al.*, 2000). A large body of evidence implicates diverse cytokine-secretion patterns to be responsible for orchestrating and influencing specific immune phenotypes. This is most evident in the cytokine secretion from subsets of T-helper cells (Th). Generally, a Th1 profile of expression drives cell-mediated responses including inflammation and tissue damage. Conversely, the Th2 subset of cytokines promotes allergic reactions and B-cell proliferation. Thus, cytokines are loosely classed as being either Th1-pro-inflammatory or Th2-anti-inflammatory (Goldsby *et al.*, 2000, O'Garra 1998). It is now widely accepted that both subsets of cytokines are expressed in the CNS and have a pivotal role to play in both neuroprotection and neurodegeneration (Rothwell *et al.,* 1995, Hopkins et al., 1995, Allan *et al.*, 2001, see Schiepers *et al.*, 2005 for review).

1.2.1 Cytokine expression in the CNS:

Although most cytokines are expressed at low or undetectable levels in the healthy adult brain, many are induced in response to injury or infection. Expression of interleukins (IL)-1, 2, 3, 4, 6, 8, and 12, Interferons (IFN's),

several chemokines, tumor necrosis factor-α (TNF-α) and numerous growth factors are induced rapidly by experimental and clinical insults to the CNS including systemic or brain infection and inflammation, stroke, excitotoxic brain damage, Alzheimer's disease, Parkinson's disease, Multiple Sclerosis, scrapie and Creuzfeldt-Jacob disease (see Hopkins *et al.*, 1995, Rothwell *et al.*, 1995, for review). Furthermore, the constitutive expression of cytokine receptors (for example, TNF-α, IL-1, 2, 6, 8, 10, 11, 12 and TGF-β) have been reported on most cell types throughout the brain (albeit, at low levels), and these often display rapid upregulation in response to injury or an inflammatory stimulus (see Allan *et al.*, 2001, Rothwell *et al*, 1995, Hopkins *et al.*, 1995, for review, Loddick *et al.*, 1998).

1.2.2 *Cytokine receptors and signaling*

Jak-Stat pathway

Most Interleukins, Interferons, and hemopoietins are structurally related and all induce homodimerization and activation of their cognate receptors, resulting in the activation of their downstream intracellular signalling pathways, predominantly receptor-associated tyrosine janus kinases (JAK's)-(JAK1, JAK2, JAK3 and Tyk2).

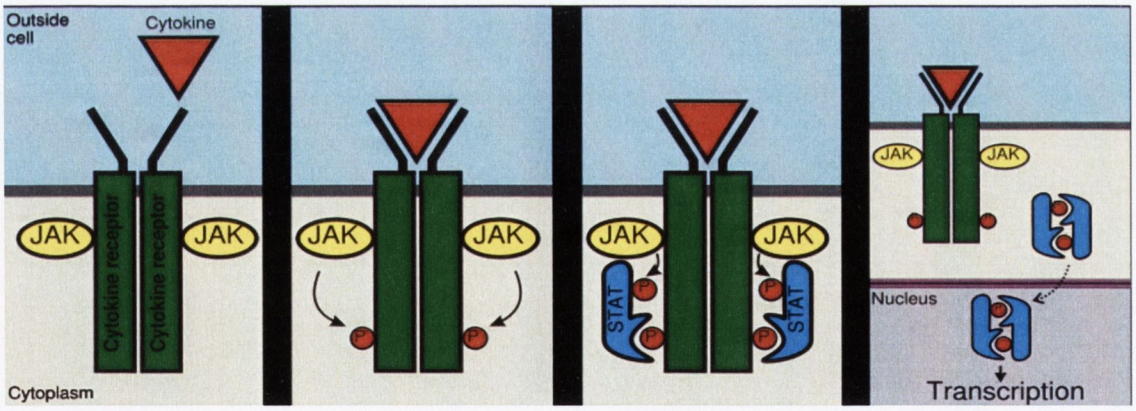


Figure 1.2: Jak-Stat signaling pathway
(http://en.wikipedia.org/wiki/Image:Jakstat_pathway.svg)

Induction of the JAK pathway by cytokines like IFN-γ and IL-10, results in activation of members of the signal transducers and activators of the

transcription family of proteins (STAT's). STAT's dimerize then translocate to the nucleus where they activate there target genes (see fig 1.2). Specificity of signaling is achieved by the use of different combinations of JAK's and STAT's. For example, IL-10 activates the STAT3 pathway and inhibits proliferation of macrophages (O'Farrell, *et al.*, 1998).

Mitogen Activated Protein Kinase pathway

The mitogen activated protein kinase's (MAPK) are a family of Serine/Threonine protein kinases widely conserved among eukaryotes and are involved in many cellular programs such as cell proliferation, cell differentiation, cell movement and cell death (see fig 1.3) (Pearson *et al.*, 2001).

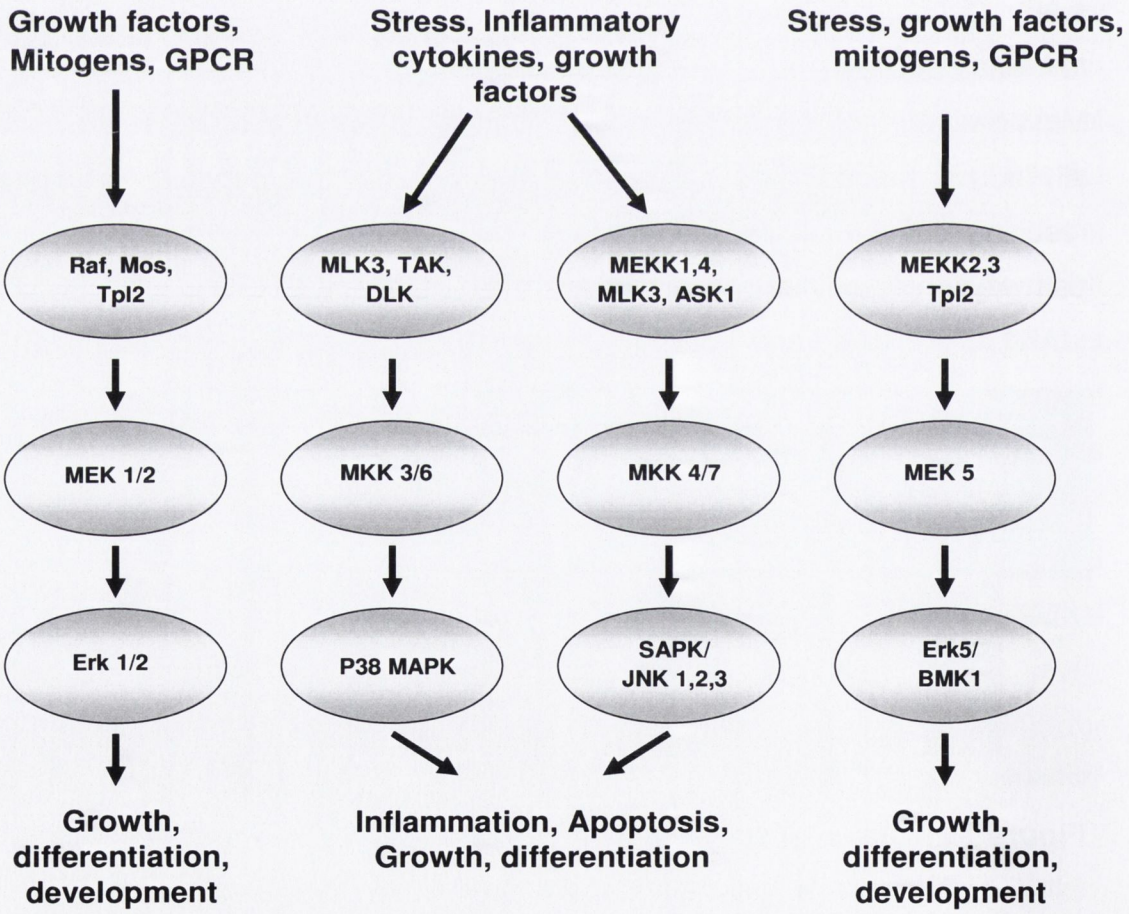


Figure 1.3: MAPK signaling pathway (adapted from Pearson *et al.*, 2001)

Extracellular signal-regulated kinases (ERK's) are widely expressed protein kinase intracellular signalling molecules which are involved in functions including the regulation of meiosis, mitosis, and post-mitotic functions in differentiated cells. Many different stimuli, including growth factors, cytokines, virus infection, ligands for heterotrimeric G protein-coupled receptors, transforming agents, and carcinogens, activate the ERK pathway (Kolch *et al.*, 2005).

In contrast, both the SAPK/JNK and p38 MAPK's are activated by a variety of cellular stresses including osmotic shock, inflammatory cytokines, bacterial endotoxin such as lipopolysaccaride (LPS), Ultraviolet light and growth factors (Roux *et al.*, 2004). In the CNS, It has been demonstrated that signaling events orchestrated by MAPK are vital for the development of hippocampal long term potentiation (LTP) and the consolidation of memory as well an inflammatory immune response, mediated by cytokines (Barry *et al.,* 2005, Minogue *et al.,* 2003).

NFκB pathway

The inflammatory cytokines such IL-1 and TNF-α, act on distinct membrane bound receptors but they can share some common signalling mechanisms including the mitogen activated protein kinases (MAPK) such as P38, c-Jun N-terminal kinase (JNK), extracellular response kinase (ERK) and also the NFκB pathway (see Allan *et al.,* 2001, for review, Vitkovic *et al.,* 2000). NFκB is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens (Gilmore *et al.*, 1999). NFκB plays a key role in regulating the immune response to infection. Consistent with this role, incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development.

While in an inactivated state, NFκB is located in the cytosol complexed with the inhibitory protein IκBα. A variety of extracellular signals can activate the

enzyme IκB kinase (IKK). IKK, in turn, phosphorylates the IκBα protein, which results in ubiquitination, dissociation of IκBα from NFκB, and eventual degradation of IκBα by the proteosome. The activated NFκB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements

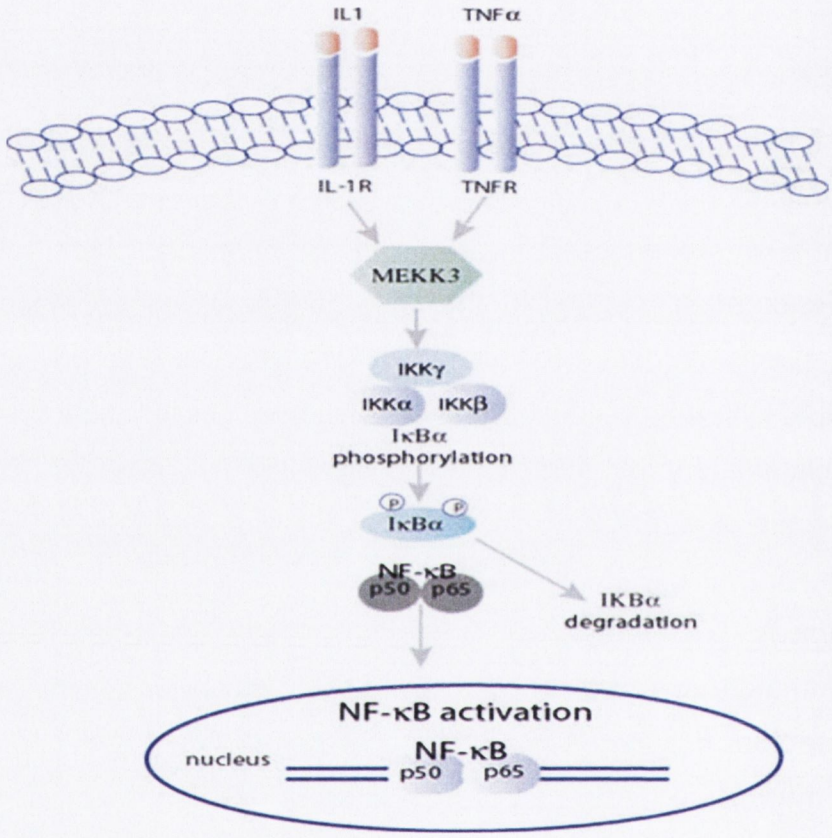


Figure 1.4: NFκB pathway (Panomics Inc., 2007)

NFκB plays a role in the regulation of neurotransmitter signaling within the brain. Low concentrations of the excitatory neurotransmitters glutamate, and its analogues N-methyl D-aspartate (NMDA) and kainite, can activate NFκB *in vitro* preparations of hippocampal neurons. Using *in vivo* mouse models of NFκB over-expression or suppression, NFκB appears to play a role in learning and behaviour (Meffert *et al.*, 2005)

Apoptotic signaling in the CNS

Neuronal cell death occurs by one of two processes, necrosis or apoptosis. Necrosis in the CNS generally follows an acute ischemic or traumatic injury to the brain (Kanduc *et al.,* 2002). Abrupt biochemical collapse in a specific area of the CNS leads to the generation of reactive oxygen species (ROS) and excitotoxins such as glutamate, calcium, and inflammatory cytokines. The hallmark histological features of necrotic cell death are mitochondrial and nuclear swelling and chromatin dissolution. This ultimately leads to cytoplasmic and nuclear membrane degeneration (Kerr *et al.,* 1972).

While neuronal apoptosis (programmed cell death) sculpts the developing brain it also has a potentially important role in neurodegenerative diseases. After an acute injury in the CNS, apoptosis often occurs in areas that are not directly affected by the injury. As such, it is a secondary cause of neuronal death in acute injuries such as stroke (MacManus *et al.,* 1993). Conversely, in chronic neurodegenerative conditions such as Alzheimer's disease (AD), apoptosis is the predominant form of cell death (Smale *et al.*, 1995). Histologically, the cytoplasm condenses, mitochondria and ribosome's aggregate, nucleus condenses and the chromatin aggregates. Intracellular acidification also occurs with the generation of ROS. The major proteins involved in the apoptotic process are proteases called caspase's (Alnemri *et al.*, 1996, Shi *et al.*, 2002). Along with members of the Bcl-2 family and mitochondrial cytochrome T, these proteins orchestrate the induction, or protect against, apoptosis, depending on the cellular signals and its microenvironment (see Yuan *et al.*, 2000, for review).

While it is difficult to assess the role of apoptosis directly in AD due to the chronic nature of disease progression, neurons in AD brains do show the histological hallmarks of apoptosis. Aggregated amyloid-β proteins induce an inflammatory environment with the generation of ROS and cytokines from glial cells; however, they can also bind directly to neuronal cell-surface

receptors. This can result in caspase activation, calcium influx and ROS release (Smale *et al.*, 1995).

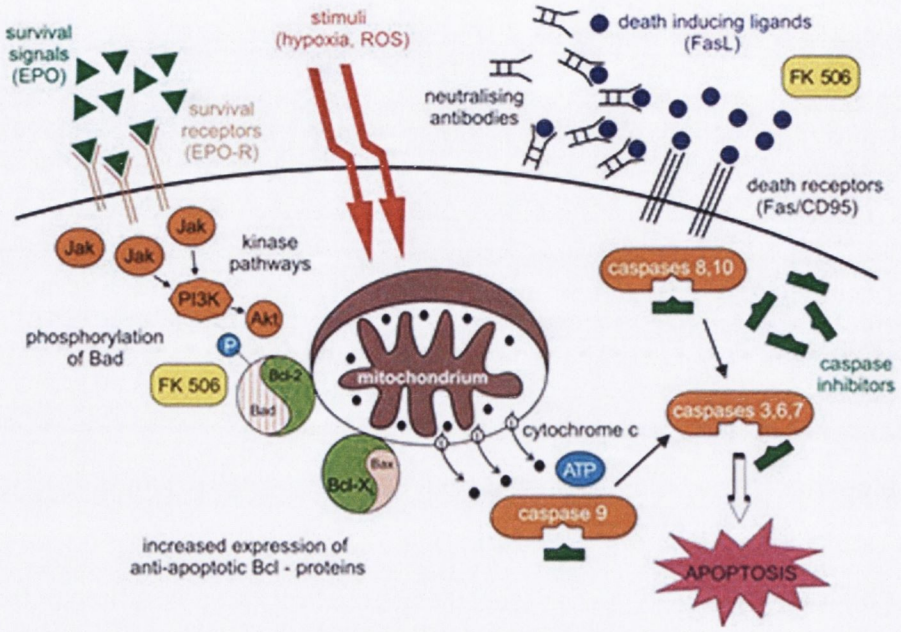


Figure 1.5: Neuronal apoptosis (Padosch, 2001)

1.3 Inflammation in Neurodegenerative disease

1.3.1 Acute neurodegeneration

The term acute neurodegeneration describes clinical conditions in which neurons are rapidly damaged and usually die in response to a sudden insult, such as stroke or acute bacterial infection (Barone *et al.,* 1999, Lopez-Cortes *et al.*, 1993). This type of aggressive insult is associated with local release of TNF-α and IL-1 (α and β) giving rise to leukocyte recruitment to the meninges and brain parenchyma. Cellular infiltration of peripheral innate inflammatory cells, particularly perivascular neutrophils and macrophages, into the brain in response to sudden injury or infection is also evident (Allan *et al.,* 2001, *Allan et al.*, 2005, Docagne *et al.*, 2005). Recent evidence indicates that influx of neurotrophils into the CNS is major contributor to the neurodegeneration that occurs in response to stroke (McColl *et al.*, 2007). Common pathways of neuronal cell death have been identified in response to a diverse range of inflammatory insults. These include early disruption of ion homeostasis,

excessive neuronal activation, seizures, massive release and impaired uptake of neurotransmitters such as glutamate, intracellular entry of Ca^{2+} as well as the release of inducible nitric oxide and free radicals (Allan *et al.*, 2001, Allan *et al.,* 2005, Block *et al.,* 2005). More recently, other factors have been identified, including activation of genes that initiate or induce apoptosis, influence glial and endothelial cells, extracellular matrix and invading peripheral immune cells. There is evidence to show that cytokines can activate some if not all of these factors (Allan *et al.*, 2001, 2005).

Stroke and ischaemic neurodegeneration

Acute neurodegenerative conditions such as cerebral ischaemia (e.g. stroke) and traumatic brain injury are characterized by rapid and usually severe insults to the brain that lead to substantial loss of nerve cell and associated functional deficits. In stroke, there is a cessation of blood supply to the brain as a result of a clot or haemorrhage, leading to increased glutamate release, oxidative stress and disrupted ion homeostasis (Allan *et al.,* 2003).

Whilst pro- and anti-inflammatory cytokine production is induced rapidly by experimental focal ischemia, neonatal hypoxic injury, excitotoxic, and brain trauma in response to epileptogenic agents (such as kainic acid) in rodents, the temporal and cellular profile of release varies with each model (Barone *et al.*, 1999, Allan *et al.*, 2001). For example, gene expression of the prototypical, pro-inflammatory cytokine interleukin-1β (IL-1β) is up-regulated in rodent disease models of stroke within fifteen to thirty minutes and protein levels within one hour after experimental brain damage (Touzani *et al.,* 1999). IL-1β can induce the production of prostaglandin E_2 (PGE_2) and reactive oxygen/nitrogen species such as nitric oxide (NO), which can further contribute to neurodegeneration (Gonzalez-Scarano *et al.*, 1999). As such, therapeutic intervention that inhibits the actions of IL-1β has shown to markedly reduce neuronal injury and glial activation. Thus, IL-1ra or small IL-1β receptor antagonists may prove beneficial as a treatment for acute neurodegenerative conditions (Allan *et al.*, 2001). In support of this,

administration of recombinant interleukin-1 receptor antagonist (IL-1ra) into the brain or periphery of rodents markedly inhibits brain damage that is caused by cerebral ischaemia or excitotoxins (Relton *et al.*, 1992, Dinarello *et al.,* 1991, Toulmond *et al.,* 1995). Similarly, over-expression of IL-1ra in the brain also inhibits ischaemic damage. Inhibition of the actions of endogenous IL-1ra by administration of neutralizing antibodies increases ischemic brain damage in rats, indicating that IL-1ra is a true functional endogenous inhibitor of neuronal damage (Allan *et al.*, 2001). Conversely, Injection of an anti-IL-1β antibody appears to be neuroprotective in ischaemic animal studies (Allan *et al.*, 2001).

In addition to IL-1β, previous literature has implicated the pro-inflammatory cytokine TNF-α with neurotoxicity in acute neurodegeneration. Inhibition of TNF-α, by treatment with either soluble TNF-α receptors, neutralizing antibodies or anti-sense oligonucleotides markedly reduce ischemic or traumatic brain damage in rodents (Barone *et al.*, 1997, Nawashiro *et al.*, 1996). It appears that all cell types in the CNS (Glia, neurons, endothelial cells) can express TNF-α and its receptors (Vitkovic *et al.,* 2000). TNF-α can activate apoptosis directly by activation of the FAS receptors that are known to be present in the CNS and *in vitro* studies have demonstrated that it induces apoptosis in a dose-dependent fashion in oligodendrocytes and neurons (Venters *et al.*, 1999).

1.3.2 Chronic neurodegeneration

In contrast to the aggressive acute tissue damage and inflammation observed with acute neurodegenerative conditions such as stroke, chronic neurodegenerative disorders such as AD and PD have a more complex aetiology and are generally multifactorial, with environmental, age and genetic background contributing to development and progression of the disease (see Campbell *et al.*, 2004 for review). Many of these factors lead to increase low-grade microglia activation and production of cytokines, superoxide radicals, iNOS and components of the complement system

(Lucas *et al.*, 2006). As such, chronic inflammation plays a role in the pathogenesis of AD, PD, and MS (Frederickson *et al.*, 1994, Mc Geer *et al*, 1998, Lucas *et al.*, 2006).

Multiple Sclerosis

Multiple sclerosis is a chronic disorder in which inflammation plays a clear role. Invasion of the CNS by T cells and macrophages leads to damage to the myelin sheaths surrounding axons, loss of neuronal function, and the formation of gliotic scar tissue (MS plaques). Since injury in the CNS varies widely between affected individuals, the clinical symptoms are heterogenous and can include fatige, muscle weakness, area's of numbness and paralysis and in over 85% of cases, include CSF antibodies for myelin proteins (Rudick *et al., 200,* Sospedra *et al.*, 2005). Characteristically, the disease progresses in cycles of relapse, often associated with systemic infection and inflammation, and remission

Recent microarray analysis has revealed that many genes related to inflammatory processes are up-regulated in the marginal zones of active demyelinating lesions (Mycko *et al.,* 2003). In animal models of the disease, experimental autoimmune encephalomyelitis (EAE), iNOS, COX2, complement, and the cytokines IL-1, IL-12 and TNF-α are increased, changes which are also observed in MS patients and correlate with disease progression (Raivich & Banati, 2004).

Alzheimer's disease

AD is a major cause of dementia, where neuronal damage begins in the temporal and parietal lobes of the cerebral cortex and progresses with time to the hippocampus and the amygdala (Braak and Braak, 1994). The disease is characterized by memory loss eventually leading to progressive and global dementia. The pathology of AD reveals the presence of insoluble structures or depositions in the cortical regions of the brain, classified primarily as β-

amyloid (Aβ)-containing extracellular plaques and intraneuronal neurofibrillary tangles.

AD was one of the first neurodegenerative diseases associated with neurotoxic microglia. Activated microglia can be found in large numbers in post-mortem AD brain tissue in and around the Aβ plaques (Mc Geer *et al*, 1988). A cytotoxic role for microglia in AD may be explained by the observation that Aβ will both recruit and activate microglia, and induce the production of neurotoxic mediators such as NO (Li *et al.*, 1996), TNF-α (Dheen *et al.*, 2005) and superoxide (Qin *et al.*, 2002). Activated microglia associated with Aβ also up-regulate a large cohort of pro-inflammatory cytokines. In an *in vitro* study, cultured human microglia exposed to $A\beta_{1-42}$ or $A\beta_{25-35}$ induced heightened gene expression of IL-β, IL-6, TNF-α, IL-8, IL-10, IL-12, MIP-1β and MCP-1 (Kim *et al.*, 2005). Furthermore *in vivo,* Intracerebroventricular (ICV) injections of Aβ(1-40) into rats was neurotoxic via the activation of the MAP kinase, JNK, and activation of the pro-apoptotic bax and cytochrome C. These effects were attenuated by pre-treatment with a caspase-1 inhibitor, implicating Aβ(1-40)-induced IL-1β as the neurotoxic mediator (Minogue *et al.*, 2003). Similarly, ICV administration of Aβ(1-42) into mice induces a time- and dose- dependent production of IL-1α, IL-1β, IL-6 and the chemokine, monocyte chemotactic protein-1 (MCP-1) in the hippocampus and cortex. Treatments with anti-inflammatory agents such as prednisolone, dexamethasone, and IL-10 inhibited production of these inflammatory cytokines and chemokines (Szczepanik *et al.,* 2001). A marked increase in microglial inflammation and release of the above-mentioned, inflammatory molecules is thought to be a major factor in disease amplification in AD (Sheng *et al.*, 1998).

A potentially important observation is that while inflammatory cytokines like IL-6, IL-1β and TNF-α can coordinate the majority of inflammatory changes in both glia and neurons observed in AD (predominantly by inducing expression of APP, complement and acute-phase response genes) a classic peripheral

immune response (involvement of peripheral T-cells or immunoglobulins) does not appear to occur as these components have not been detected in the AD brain to date (Donnelly et al., 1990, Potter et al., 1992, Gonzalez-Scarano and Baltuch, 1999).

Parkinson's disease

PD is the result of a specific and progressive neurodegeneration of pigmented nigrostriatal dopaminergic neurons (Whitton, 2007). The clinical symptoms of the disease include difficulty in initiating voluntary movement (akinesia), slowing and diminished range of movement (bradykinesia), a rhythmic involuntary tremor in patients at rest as well rigidity of the major muscle groups (Deumens *et al.*, 2002). The clinical symptoms of PD become apparent when loss of at least 50% of the dopaminergic neurons in the substantia nigra pars compacta occurs, leading to more than an 80% reduction in the levels of striatal dopamine (Deumens *et al.*, 2002, Lang and Lozano, 1998).

While the cause of the disease remains unclear, several theories implicate environmental toxins, genetic factors, mitochondrial dysfunction as well as free-radical-mediated neurotoxicity (Ben-Shachar *et al.*, 1995, Hoehn and Yhar, 1998, Rosenberg, 2002). A role of chronic CNS inflammation has also been strongly implicated in PD (Liu *et al.,* 2003, Mc Geer *et al.,* 2001, Whitton, 2007). Furthermore, activated amoeboid microglia are found close to degenerating substantia nigral neurons in patients with PD (Lucas *et al.,* 2005, Wahner *et al.,* 2007, Mc Geer *et al.,* 1998). This increase in microglial activation is accompanied by elevated levels of pro-inflammatory molecules such as TNF-α, IL-β, IFN-γ, IL-6, iNOS and cyclooxygenase-2 (COX-2) in the CSF and striatum in PD brains (Mogi *et al.,* 1995, 1996, 1999, Blum-Degen *et al.*, 1995, Knott *et al.*, 2000, Hunot *et al.*, 1996, Nagatsu *et al.*, 2000). These inflammatory mediators along with cellular debris released from dying dopaminergic neurons have the potential to amplify and sustain a

neuroinflammatory environment and exacerbate the loss of substania nigra neurons.

Inhibiting microglial activation with the opioid receptor antagonist, naloxone, or the tetracycline microglial inhibitor, minicycline, have proven beneficial in down-regulating microglial-induced cytokine production in animal models of PD (Lucas *et al.*, 2005, McGeer and McGeer, 2004). In addition, specific anti-inflammatory therapies appear to be beneficial, at least in animal models of PD, where COX-2 inhibitors reduce neuronal damage induced by the the selective dopaminergic neuronal neurotoxin, MPTP (1-methyl-4-phenyl-1,2,3,6-terahydropyridine) (McGeer and McGeer, 2004).

Depression

Depression is characterised by disorders in noradrenaline (NA) and 5-HT neurotransmission (Ressler & Nemeroff, 1999, 2000; Dursun *et al.,* 2001). It has been hypothesized that immune activation may be causally related to these signalling disorders, as pro-inflammatory cytokines have been implicated in alterations of NA and 5-HT turnover in brain regions assumed to be involved in major depression, including the hypothalamus, hippocampus, amygdala and prefrontal cortex (Dunn *et al.,* 1999). Synthesis of these neurotransmitters is largely dependent on the availability and metabolism of the 5-HT precursor, tryptophan. Cytokines including IL-1, 2, 6 and IFN-γ have been found to reduce the availability of the tryptophan precursor, predominantly by the activation of the tryptophan metabolizing enzyme, indoleamine-2,3-dioxygenase (IDO) (Heyes *et al.,* 1993, Stone & Darlington *et al.,* 2002).

Furthermore, there is increasing evidence that immunotherapy with IL-2 or IFN-α (in the treatment of viral infections) increases serum levels of IFN-γ, IL-6, 8 and 10 (Bonaccorso *et al., 2001;* Capuron *et al., 2001).* IFN-α-induced increases in IL-6 and IL-8 were found to be significantly correlated to symptoms of depression and anxiety (Bonaccorso *et al.*, 2001), suggesting

that the occurrence of depressive symptoms following IFN-α treatment may be secondary to the activation of cytokine production (Schiepers *et al.,* 2005). In accordance with this hypothesis, Capuron and colleagues have demonstrated that the symptoms of depression, anxiety and cognitive impairment associated with IFN-α immunotherapy was ameliorated by treatment with the antidepressant paroxetine (Capuron *et al.,* 2002).

Consistent with this finding, there is now a body of research to show that antidepressants have immunomodulatory properties. Hashioka and colleagues have shown that the selective noradrenaline reuptake inhibitor (NRI) Reboxetine, inhibits the production of IL-6 and NO from IFN-γ-treated mouse microglia *in vitro* (Hashioka *et al.*, 2007). Reboxetine also inhibited human T-cell motility and reduced the production of IFN-γ *in vivo* (Diamond *et al.,* 2005). In addition, Reboxetine, the NRI desipramine, and the selective serotonin reuptake inhibitors fluoxetine and clomipramine, inhibited the circulating levels of IFN-γ in LPS-stimulated rats (Diamond *et al.,* 2005).

1.4 Interleukin-1

The Interleukin (IL)-1 gene family consists of at least two agonists, IL-1α and IL-β, which require post-translational processing by the IL-1-converting enzyme (ICE) for biological activity (Li *et al.*, 1995) and a receptor antagonist (IL-1ra). All three share limited amino acid identity and bind to the same receptors on different cell types (Dinarello *et al.*, 1991). Following processing, IL-1α is expressed on the cell membrane, whereas IL-1β is secreted. IL-1β is considered a prototypical pro-inflammatory cytokine and has a pivotal role in both CNS and peripheral immunomodulation.

1.4.1 *Interleukin-1 receptors and intracellular signalling*

There are two major members of the IL-1 receptor gene family expressed in the CNS, the 80-kDa glycoprotein, IL-1 receptor type I [IL-1R(I)] and the 60-kDa type II receptor [IL-1R(II)]. The extracellular portions of both receptors are homologous (26-28%) and contain three Ig-like domains. Both receptors

are derived from a common receptor gene but with multiple promoters and cell-type-specific transcription events (Sims *et al.,* 1995). Both IL-1R(I) and IL-1R(II) contain a single trans-membrane domain. IL-1R(I) has a cytosolic domain of 213 amino-acids whereas the IL-1R(II) cytosolic domain is only 29 amino acids. While binding of IL-1R(I) induces receptor activation and a subsequent intracellular signalling cascade that culminates in the transcription of a host of inflammatory genes, binding of IL-1 to the IL-1R(II) induces no signalling within the cell. This has implicated the IL-1R(II) as an endogenous decoy receptor, and as such, increased expression of IL-1R(II) may be a mechanism to reduce biological activity of IL-1 (Dinarello *et al.,* 1994). Consistent with this theory is the fact that the IL-1R(II) receptor binds IL-1α and IL-1β with higher affinity that it binds sIL-1ra (Mc Mahan *et al.,* 1991).

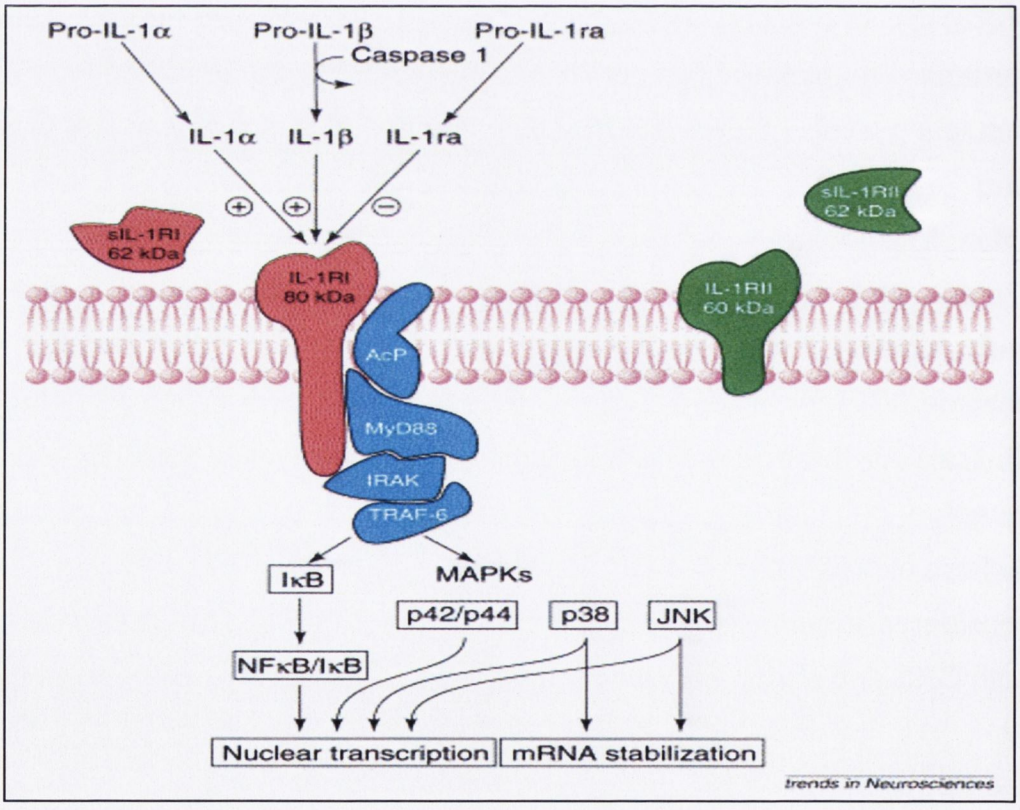


Figure 1.6: IL-1: Ligands, receptors and signalling

The IL-1R(I) protein requires IL-1 receptor accessory protein (IL-1RAcp) to transduce an intracellular signal when bound to IL-1. IL-1 binding causes activation of two kinases, IRAK-1 and IRAK-2, associated with the IL-1

receptor complex. IRAK-1 (IL-1 Receptor Associated Kinase) activates and recruits TRAF6 to the IL-1 receptor complex. TRAF6 activates two pathways, one leading to NFκB activation and another leading to mitogen activate protein (MAP) kinase activation. The TRAF associated protein ECSIT leads to c-Jun activation through the Map kinase/JNK signaling system. TRAF6 also signals through the TAB1/TAK1 kinases to trigger the degradation of IκB, and activation of NFκB (Allan *et al.*, 2001, Medzhitov *et al.,* 1998, Muzio *et al.*, 1997) (see fig. 1.6).

The IL-1 receptors can also be enzymatically cleaved from the membrane and function as soluble scavenging receptors. Thus, the soluble IL-1R(II) works in conjunction with sIL-1ra by blocking the ability of IL-1 to activate cells and induce inflammation. It has been demonstrated that the soluble IL-1R(II) binds IL-1 with the same affinity and to the same binding sites as the membrane bound receptor (Symons *et al.,* 1995). As there are fewer than 100 IL-1R(I)'s on most cell types, and of these, less than 5% need to be occupied for a biological response, IL-1 signal transduction must be highly efficient and greatly amplified (Dinarello *et al.,* 1994).

1.4.2 *Interleukin-1 receptor expression in the CNS*

Neurons, glia and endothelial cells as well as invading neutrophils, macrophages and T-cells, all express IL-1 receptors (Loddick *et a*l., 1998, Konsman *et al.*, 2000, Parnet *et al.*, 2002, Vitkovic *et al.,* 2000). Early autoradiography analysis in the rat has shown that the expression IL-1 receptors are diffusely localized throughout the brain, with the highest levels of expression in the granule cell layer of the cerebellum, dentate gyrus, hypothalamus and the pyramidal layer of the hippocampus (Farrar *et al.*, 1987). Later, mRNA analysis revealed that IL-1R(I) was localized on non-neuronal cells in the choroid plexus and the endothelial cells of the brain capillaries. Neuronal expression was most evident in the hippocampus, amygdala and hypothalamus (Ericsson et al., 1995, Parnet et al., 2002). In comparison to IL-1R(I), there is little literature concerning the expression

profile of the IL-1R(II) in rats, presumably due to the poor selectivity of available reagents for IL-1R(II) protein. Some reports have shown that IL-1R(II) mRNA is undetectable in the adult rat brain, but is dramatically up-regulated in the dentate gyrus, hippocampus and the basolateral amygdaloid nucleus in response to systemic administration with Kainic acid (Nishiyori et al., 1997). Docagne and colleagues have shown a variable expression profile of IL-1 receptors which was stimuli dependant in the rat brain. For example, microinjection of IL-1β into the striatum of rats showed and early increase in IL-1R(I) mRNA which dissipated after 24 hours. In contrast, a robust induction of IL-1R(II) was observed at 8 hours and expression remained 24 hours post treatment, whereas, only IL-1R(I) and IL-1β were induced by NMDA striatal microinjection (Docagne *et al.*, 2005).

In contrast to IL-1R(I) and IL-1R(II), the expression profile of IL-1R(Acp) mRNA appears to be ubiquitous throughout the rat brain in all regions tested such as the cortex, hippocampus, cerebellum and striatum.

1.4.3 *Interleukin-1 in CNS inflammation*

In the central nervous system (CNS), IL-1 has been implicated as an important mediator of tissue damage and destruction in a number of inflammatory diseases. It has been shown to be expressed in activated microglia, macrophages and endothelial cells in both animal models and human lesion in multiple sclerosis, and CNS tissue from Human Immunodeficiency Virus type 1 (HIV-1) encephalitis, Down syndrome, Alzheimer's disease and ischemia-induced excitotoxicity (Cannella *et al.*, 1995, Brosnan *et al.*, 1995, Hopkins *et al.*, 1995, Tyor *et al.*, 1992, Griffin *et al.,* 1989, Relton *et al.,* 1992). The promoter for the IL-1 gene has multiple *cis*-acting regulatory sites including those for hypoxia-induced factors (HIF), NFκB, Nuclear factor (NF)-IL-1βA-, Activator protein-1 (AP1) (cJun, cFos), Activating transcription factor 2 (ATF2), CCAAT/enhancer binding protein-β (C/EBP-β), and Elk1 that enables IL-1 mRNA to be transcribed in response to

a diverse range of stimuli. As such IL-1 is considered an early and dominant injury signal (Basu *et al.*, 2004, Auron *et al.*, 1998, Smith *et al.*, 1992).

Inappropriate expression of IL-1β in the CNS can facilitate CNS inflammation and subsequent dysfunction in a number of ways. IL-1β can activate the endothelium, up-regulating a number of inflammatory factors, including adhesion molecules, eicosanoids, and chemokines, inducing in blood-brain barrier permeability and recruitment of peripheral immune cells (Wong *et al.*, 1992, De Vries *et al.*, 1995, Liu *et al.*, 1996). In addition, IL-1β can potently induce the production of inducible nitric oxide synthase (iNOS) and subsequent nitric oxide production from both microglia and astrocytes. Nitric oxide is both a potent vasodilator and a neurotoxic agent (Liu *et al.*, 1996, Merrill *et al.*, 1993, Mitrovic *et al.*, 1994). IL-1β may also contribute to the establishment of chronic CNS inflammatory states through its induction of the pro-inflammatory cytokines TNF-α and IL-6 from human astrocytes and microglia as well as self regulating the induction of its own gene expression from microglia *in vitro* (Lee *et al.*, 1993). An important point to note is that while in a peripheral immune response, and *in vitro* data from CNS glia, IL-1β has been shown to regulate the expression of inflammatory cytokines such as TNF-α recent *in vivo* data has disputed this crosstalk in the brain. IL-1β injected into striatum failed to induce the expression of TNF-α. Likewise, TNF-α injection failed to induce IL-1 β expression. These results demonstrate an atypical cytokine expression pattern in the rodent brain (Blond *et al.,* 2002).

In acute MS, IL-1β appears to be expressed predominantly by microglial cells throughout the white matter in and around the gliotic lesions (Cannella *et al.,* 1995). In conjunction with other inflammatory mediators, IL-1β has an important role in orchestrating the inflammatory cell-cell interactions which can lead to oligodendrocyte cytotoxicity (Badovinac *et al.,* 1998). IL-1 immune reactivity can be demonstrated within the CNS in infiltrating macrophages and activate microglial cells during EAE in rats (Bauer *et al.,* 1993). In addition, IL-1β has been implicated in the activation of resting (both naïve and memory)

antigen-specific lymphocytes in EAE. Moreover, *in vitro* addition of sIL-1ra to activated anti-myelin T cells resulted in a decrease of their proliferation, encephalitogenic capacity and their expression of IL-2 receptor (Badovinac *et al.,* 1998).

Production of IL-1β is induced in vitro by both human monocytes and mice microglia in response to amyloid-β (Brugg *et al.*, 1995). Furthermore, IL-1β can regulate the expression of amyloid-β in both human endothelial cells and astrocytes *in vitro* (Brugg *et al.*, 1995, Goldgaber *et al.,* 1989). While IL-1β has a role in the inflammation observed in AD, as previously mentioned, animal models of AD have also shown that IL-1 has a deleterious affect on memory consolidation and long term potentiation (LTP) in the hippocampus (Lyons *et al.*, 2007, Minogue et al., 2003, see Pugh *et al.,* 1999, for review). Conversely, blockade of IL-1β signalling by CNS over-expression of IL-1ra, leads to facilitation of short and long-term memory in rats (Tanaka *et al.*, 2006).

1.4.4 *Combating the actions of IL-1β: IL-1 receptor antagonist and IL-1 receptor type II*

It is now well accepted that the cytokine network and subsequent inflammatory cascade of events is self-regulating through the action of opposing anti-inflammatory cytokines, the release of soluble cytokine receptors, and the production of antagonists which limit cytokine-receptor binding. With regard to the inflammation induced by IL-1β secretion, a number of mechanisms have evolved to limit and absorb the detrimental effects of IL-1β.

IL-1ra

IL-1β is biologically active at very low concentrations and only a few IL-1 receptors on each cell need to be occupied to elicit an intracellular signalling cascade (Dower *et al.*, 1986). Thus, the biological activity of IL-1 is generally kept under strict control *in vivo*. As previously mentioned, one of the products

of the IL-1 gene family is the endogenous receptor antagonist, IL-1ra. IL-1ra exists in three molecular forms: one secreted (sIL-1ra) and two intracellular forms (icIL-1ra type I and type II), which are encoded by a single gene (using two distinct promoters, Butcher *et al.,* 1994) and generated by alternative splicing of the first exon of each transcript. icIL-1ra type II is generated by alternative translation initiation of sIL-1ra mRNA (Malyak *et al.,* 1998a, Malyak *et al.,* 1998b). All three isoforms of this antagonist are differentially regulated and are stimuli and cell specific (Malyak *et al.*, 1998). Mature sIL-1ra is a 22kDa glycosylated cytokine generated from a precursor that includes an N-terminal leader sequence to permit classical secretion via the endoplasmic reticulum and golgi apparatus (Eisenberg *et al.,* 1990, Carter *et al.,* 1990). The human icIL-1ra protein sequence differs from sIL-1ra only at the N-terminus where the first 21-amino acids of the 25-amino acid leader sequence are replaced by four unique amino acids, and thus impairing secretion (Butcher *et al.,* 1994).

The secreted form of IL-1ra is an acute phase protein which acts as an endogenous antagonist, binding to the type I IL-1 receptor, without eliciting receptor activation (Lundkvist *et al.,* 1999, Gabay *et al.,* 1997). It has been proposed that the relative levels of IL-1β and IL-1ra may determine the extent of tissue injury in the CNS (Liu *et al.,* 1998). The imbalance between these two cytokines is indicated to be of pathological importance and has been implicated in many other inflammatory disease states, including Arthritis (Bresnihan *et al.,* 1998). The concentration of sIL-1ra in a given tissue is generally required to be 10-100 fold greater than the concentration of IL-1β to inhibit 50% of the biological activity of IL-1 (Arend *et al.,* 1990). In the synovial membrane of rheumatoid arthritis patients, the concentration of sIL-1ra is generally less than IL-1. A recombinant glycosylated form of human sIL-1ra has been developed specifically for the treatment of arthritis and is now commercially available. It has been shown to significantly limit the inflammatory disease state and improve patient mobility (Campion *et al.,* 1996, Breshnihan *et al.,* 1998, Jiang *et al.,* 2000).

IL-1β synthesis is also generated in the CNS during mechanical injury and IL-1β mimics some of the effects of injury, such as gliosis and neurovascularization (Relton et al., 1992). In a rodent model of focal cerebral ischemia, it was reported that injection with recombinant sIL-1ra significantly suppressed neuronal death from the ischemia-induced excitotoxicity implicating IL-1β as a potent mediator of excitotoxic brain damage (Relton *et al.,* 1992). The same group have also showed that mice lacking IL-1ra (IL-1ra knock-out) exhibited a dramatic increase in neuronal injury induced by transient cerebral ischemia compared to wild-type (WT) animals, giving further evidence to show that IL-1β is a mediator of excitotoxicity (Pinteaux *et al.,* 2006).

There is now a large body of research also emphasizing the anti-inflammatory and neuroprotective effects of IL-1ra *in vitro.* As previously mentioned, IL-1β can induce the production of pro-inflammatory cytokines TNF-α and IL-6 from human astrocytes and microglia as well as self regulating the induction of its own gene expression in microglia (Lee *et al.,* 1993). However, *in vitro*, sIL-1ra markedly suppresses production of TNF-α and iNOS in astrocytes induced by IL-1β (Liu *et al.,* 1998). In another *in vitro* study where combined treatment of mouse neuron-astrocyte co-cultures with IL-1β and IFN-γ induced significant neuronal toxicity, IL-1ra was the only anti-inflammatory cytokine to attenuate the neuronal degeneration. Surprisingly, anti-inflammatory cytokines such as TGF-β, IL-10 and IL-4 had no affect when co-incubated with the neuron-astrocyte co-cultures (Downen *et al.,* 1999). Treatment of organotypic-hippocampal slice cultures with N-methyl D-aspartate (NMDA) induced a significant increase in the number of activated microglia and degenerating neurons. Addition of recombinant IL-1β exacerbated this excitotoxic damage. Furthermore, treatment with sIL-1ra significantly reduced both the number of activated microglia and attenuated the NMDA/IL-1β-induced neuronal damage (Hailer *et al.,* 2005).

IL-1R(II)

It is clear that IL-1 is unique amoung cytokines as its actions in vivo are regulated both by an endogenous receptor antagonist, but also by membrane bound and soluble decoy receptor. The IL-1R(II) has been shown to bind to different epitopes on the IL-1β molecule than the IL-1R(I) and this variance has been implicated in the ability of IL-1R(II) to control the bioactivity of IL-1β at both the level of membrane bound receptor binding and peptide processing (Dinarello, *et al.,* 1991). Specifically, it has been shown *in vitro* using a human B-cell line, that the soluble IL-1R(II) can act as a carrier molecule that blocks the processing of the IL-1β precursor. This is presumably mediated by masking cleavage sites or altering the conformation of the pro-IL-1β molecule so that ICE or other proteolytic enzymes fail to recognize their cleavage sites (Symons *et al.,* 1995).

In conjunction with the sIL-1ra, the IL-1R(II) has potential therapeutic benefit as a mechanism to limit IL-1β biological activity during inflammatory states. For instance it was demonstrated in a rabbit antigen-induced T-cell mediated model of arthritis that sIL-1R type II significantly inhibited soft tissue swelling and joint necrosis (Dawson *et al.,* 1999), furthermore, Increased soluble IL-1R(II) correspond to reduced indices of tissue damage in chronic arthritis (Jouvenne et al., 1998). In light of these findings, a soluble recombinant IL-1R(II) has been proposed as a therapeutic for the treatment of rheumatoid arthritis. Furthermore, elevated soluble IL-1R(II) has been shown to regulate IL-1β production in human sepsis (Giri et al., 1994) and soluble IL-1R(II) gene transfer significantly attenuates an animal model of allograft rejection (Simeoni *et al.*, 2007). Over expression of IL-1R(II) mRNA was also described in the brain after systemic injections of Kainate (Nishiyori et al., 1997) or cerebral ischaemia (Wang et al., 1997).

It has been demonstrated *in vitro,* using a human B-cells that sIL-1ra binds to the IL-1R(II) with higher affinity when the receptor is membrane bound. However, upon its cleavage into the extra-cellular space, the binding affinity

of IL-1R(II) for sIL-1ra is reduced by up to 2000 fold, whereas its affinity for IL-1β remains high (Symons *et al.*, 1995). When bound to the cell membrane, the type I IL-1R increases its affinity for IL-1β (Symons *et al.,* 1995). If sIL-1ra and IL-1R(II) are to work in conjunction with each other to limit the biological actions of IL-1β then they cannot compete for binding *in vivo*.

1.5 Interleukin-10 and CNS Inflammation

1.5.1 *Interleukin-10 receptors and intracellular signaling*

Interleukin-10 (IL-10) is an 18 kDa non-glycosylated polypeptide secreted by monocytes/macrophages, B lymphocytes, keratinocytes and subclasses of the CD4+ T lymphocytes, whose physiological function is to inhibit inflammatory responses to Th type 1 cell-mediated immune responses (see Moore *et al.,* 2001, for review). IL-10 functions by binding to a multi-component receptor structure composed of at least 2 subunits: the IL-10R1 and the CRFB4/CRF2-4 molecule, now called IL-10R2, both members of the class II interferon receptor (IFNR)-like family (Liu *et al.*, 1994, Kotenko *et al.*, 1997, Spencer *et al.,* 1998). IL-10 binds a primary ligand-binding region of the IL-10R1 with high affinity to induce heterodimerisation with the IL-10R2 and subsequent intracellular signalling by recruiting Jak's (Kotenko *et al.*, 1997). IL-10R1 associates with Jak1 while IL-10R2 associates with Tyk2. This results in the phosphorylation of IL-10R1 on cytoplasmic tyrosine residues (Y446 and Y496), and subsequent STAT3 activation. STAT3 binds these sites via its SH2 (*Src* homology 2) domain, and is in turn, tyrosine–phosphorylated by the receptor associated Jaks (Weber-Nordt *et al.*, 1996).

STAT3 is subsequently homodimerised and translocated to the nucleus where it affiliates with high affinity to STAT-binding elements (SBE) in the promoters of IL-10-responsive genes (Wehinger *et al.*, 1996). STAT3 can also be phosphorylated in response to various cytokines and growth factors including IFN's, IL-5, IL-6 and bone morphogenic protein (BMP-2) (Hoey *et al.*, 1999, Levy *et al.*, 2006).

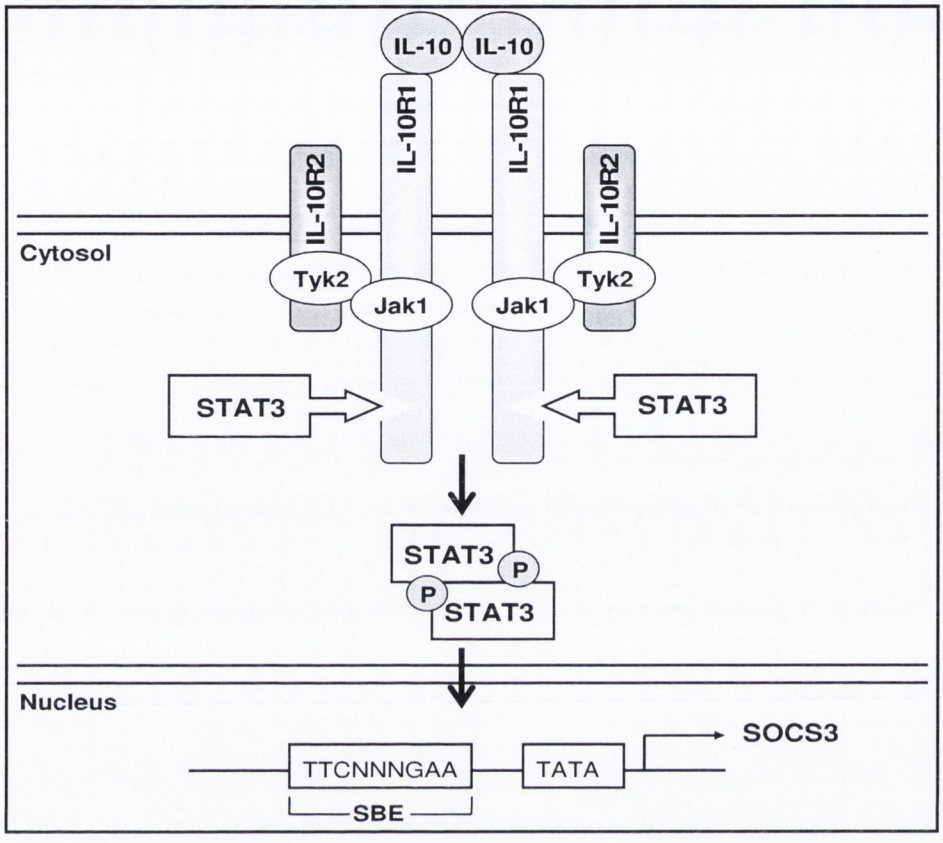


Figure 1.7: IL-10 signal transduction pathway
(Adapted from Donnelly *et al.*, 1999)

Recently, a family of STAT-inducible genes, suppressor of cytokine signalling (SOCS) have been identified and act in an anti-inflammatory feed-back loop. IL-10 rapidly induces SOCS-3 gene expression and protein production which functions by inhibiting Jak/STAT-dependent signalling of inflammatory cytokines including TNF-α and IFN-γ (Donnelly *et al.*, 1999, Starr *et al.,* 1997, Naka *et al.*, 1997).

1.5.2 *Interleukin-10 expression and action in the CNS*

The levels of both IL-10 and IL-10R1 appear to be constitutively expressed in the CNS. *In vitro* studies show that human microglial cells express mRNA for IL-10 and the IL-10R, whereas both microglia and astrocytes express IL-10 and IL-10R, mRNA and protein in mouse and rat cultures (Mizuno *et al.*, 1994, Ledeboer *et al.,* 2002). *In vivo* analysis has revealed that the IL-10 is

expressed in the human pituitary and hypothalamus amoung other regions (Rady *et al.,* 1995) and IL-10 receptor is constitutively expressed in the rat cortex, hippocampus, hypothalamus, cerebellum and pituitary (Ward *et al.*, 2001).

Although the actions of IL-10 are commonly viewed as 'anti-inflammatory', there is also evidence to suggest that it protects neurons and down-regulates glial responsiveness to disease in the absence of inflammation. Its actions counterbalance the activities of elevated IL-1β and TNF-α to maintain homeostasis. For example, microinjection of recombinant IL-10 into the rat hippocampus inhibits the inflammatory changes observed from IL-1β administration (Kelly et al., 2001). It has been suggested that the effects of IL-10 in the CNS are controlled by neurons, but they may be indirectly mediated by IL-10R on microglia and astrocytes (Vitkovic *et al.*, 2001, Laye, *et al.* 1999).

IL-10 is now implicated as an endogenous immunosuppressant which can contribute to recovery from CNS autoimmune disease, in particular experimental autoimmune encephalomyelitis (EAE) (Jander, S., *et al.*, 1998). The ability of regulatory T-cells ($CD25^{+}CD4^{+}$) to suppress EAE disease severity and improve clinical outcome has been attributed to their production of IL-10, independent of any change in the levels of IFN-γ or IL-2. IL-10 was detected within individual lesions during active disease of a primate model of EAE (Laman *et al.*, 1998). IL-10 mRNA has also been detected in the cerebrospinal fluid (CSF) of MS patients (Monteyne *et al.,* 1997) and appears to correlate with a favourable clinical response. Elevated CSF concentrations were detected during the stable rather than active phase of MS and therapeutic intervention with IFNβ increased the CSF levels of IL-10 mRNA and protein (Calabresi *et al.,* 1998, Rudick *et al.*, 1998).
IL-10 was reported to be elevated in the CSF of stroke patients with a peak in concentration 3 days after the initial injury (Tarkowski *et al.*, 1997). In animal models of ischemic neurotoxicity, recombinant IL-10 pre-treatment improves

clinical outcome by reducing infract size and pro-inflammatory cytokine production (Spera *et al.*, 1998, Knoblach *et al.*, 1998). The role of IL-10 as an anti-inflammatory molecule *in vivo* is supported by the phenotype of IL-10-deficient mice; these animals develop chronic colitis and subsequent colon cancer, which appears to be mediated by pro-inflammatory $CD4^+$ Th1 cells (Berg *at al.*, 1996, Kuhn *et al.,* 1993, Davidson *et al.*, 1996).

There is also a considerable body of research highlighting the specific anti-inflammatory properties of IL-10 *in vitro*. IL-10 has been shown to inhibit the LPS-induced production of the pro-inflammatory cytokines IL-6, TNF-α and IL-1β as well as nitric oxide and superoxide anion production from primary rat microglia cultures (Ledeboer *et al.*, 2000, Sawada *et al.*, 1999). IL-10 also down-regulates LPS- and cytokine-induced chemokine and adhesion molecule production (Shrikant, *et al.*, 1995, Hu *et al.*, 1999, Guo *et al.*, 1998). Another important finding is that IL-10 can potently down-regulate the microglia expression of antigen presentation molecules such as MHC class II (O'Keefe *et al.*, 1999, Frei *et al.*, 1994) and the co-stimulatory molecules B7-1 and B7-2. Cerebellar granular cells were protected from an *in vitro* model of excitotoxicity by IL-10 pre-treatment. It appears that IL-10 inhibited the glutamate induction of pro-apoptotic caspase-3 and NFκB-DNA binding (Bachis *et al.*, 2001). These findings indicate that IL-10 can both directly and indirectly exert multiple inhibitory effects on glial immune function in the CNS and demonstrates why there is now considerable promise for the use of agents that might increase the CNS expression of IL-10 as therapeutics.

1.6 Suppressor of Cytokine signalling-3 (SOCS-3)

The intracellular signaling of cytokines can be negatively regulated by a family of proteins called SOCS, containing 8 related members, SOCS 1-7, and cytokine-inducible Src homology 2 (SH2)-containing protein (Cis). All these proteins share a central SH2 domain, an N-terminal domain and a C-terminal conserved domain called a SOCS box (Yoshimura *et al.*, 2007, Campbell *et al.*, 2005). The tissue expression levels of SOCS proteins,

particularly SOCS1 and SOCS3, are not static and can be rapidly up-regulated in a number of cell types by various cytokines, including interleukins, interferons, and colony-stimulating factor families (Wang *et al.*, 2002). This may be explained by the fact that the SOCS3 promoter contains numerous potential regulatory elements including AP-1, C/EBPβ, NF-κB, Sp1 and Gas-like binding sites (Qin *et al.,* 2007). Previous literature has also shown that the promoters of many SOCS genes contain a STAT-responsive DNA binding site and their expression is dependent on Jak/STAT signaling (Naka, *et al.*, 1997, Starr *et al.*, 1997). Both SOCS1 and SOCS3 can inhibit JAK tyrosine kinase activity, as they have a kinase inhibitory sequence (KIR) in their amino terminal region, which acts as a pseudosubstrate. SOCS3 is believed to act via binding of its SH2 domain with the cytokine receptor (Yoshimura *et al.*, 2003).

SOCS3 expression can be induced by various cytokines including IL-6, IL-10 and IFN's (Anhuf *et al.*, 2000, Shen *et al.,* 2000). While there are some reports of a STAT3-independent mechanism of IL-10-induced SOCS3 increases in human neutrophils (Cassatella *et al.*, 1999), it is now well accepted that STAT3 phosphorylation following IL-10 receptor activation, is one of the major sources of SOCS3 induction in innate immune cells (Qin *et al.*, 2007, Berlato *et al.*, 2002). The up-regulation of SOCS3 by IL-10 has been implicated in its ability to inhibit the signaling of pro-inflammatory cytokines such as IL-6, IFN-γ and TNF-α. By mutating the SHP-2/SOCS3 binding sites of the IL-6 receptor gp130 subunit, mice develop a rheumatoid arthritis-like joint disease with increased production of Th1 cytokines and autoantibodies (Atsumi *et al.*, 2002).

There is limited literature available regarding the cellular expression pattern and function of SOCS3 in the CNS. It appears that SOCS3 is expressed ubiquitously, but at low levels under physiological conditions. Its CNS expression is dramatically up-regulated following peripheral administration with the bacterial endotoxin, lipopolysaccaride (LPS), IL-6, LIF and IL-1β, in

the hypothalamus and pituitary (Lebel *et al.*, 2000, Auernhammer *et al.*, 1998). The localization of SOCS3 to hypothalamic neurons and its marked up-regulation by different cytokines suggests that it plays an important role in cytokine-directed neuro-imuno-endocrine functions. The hypothalamic gene expression of SOCS3 is increased 3-fold following sub-cutaneous administration of complete freuds adjuvant in mice (Chesnokova *et al.*, 2000). SOCS3 expression is also increased in the hippocampal pyramidal and granule neurons, 24 hours after the induction of experimental seizures in rats (Rosell *et al.*, 2003).

SOCS3 expression may also regulate the non-immune functions of STAT proteins. Neuro-epithelial cells from mice that have a conditional SOCS3 knock-out, readily differentiate into GFAP-positive astrocytes when stimulated with LIF (Leukemia inhibitory factor) via a BMP-2 (bone morphogenic protein) and STAT-SMAD dependent mechanism. The authors postulate that SOCS3 acts in a positive regulatory loop to prevent the development and/or accumulation of reactive astrocytes at the site of inflammation (Fukuda *et al*, 2007). Consistent with this theory is the finding that injury to the adult spinal cord rapidly induces IL-6 production followed by SOCS3 up-regulation (Nesic *et al.*, 2002, Streit *et al.*, 1998). Okada et al. observed an increase in reactive astrocytes accumulation in the spinal cords of SOCS3-conditional knock-out mice (Okada *et al.*, 2006).

1.7 The noradrenergic system and its receptors

1.7.1 *Noradrenaline metabolism and mode of action*

The monoamine neurotransmitter noradrenaline (NA) is synthesized from L-tyrosine, an aromatic amino acid, to dihydroxyphenylanlanine (DOPA) by tyrosine hydroxylase (TH). DOPA is converted to dopamine by DOPA decarboxylase, and dopamine in converted to NA by the catalyst actions of the enzyme Dopamine-β-hydroxylase (DβH) in both noradrenergic neurons in the brain, as well as chromaffin neurons in the adrenal medulla (Rang *et al.*, 1999). NA is actively taken into the synaptic vesicles (by the vesicular

monoamine transporter) where it is stored, bound to a protein, chromogranin, and adenosine 5´-triphosphate. Both of these molecules are co-released with NA into synaptic vesicles. A small quantity of DβH is also released with NA and is not metabolized or subject to reuptake (Rang *et al.*, 1999).

Noradrenergic neurons are located mainly in the pons and the medulla, the largest group of neurons being the Locus Ceruleus (LC). Noradrenergic axons project via the medial forebrain bundle to most forebrain structures including the cortex, and hippocampus forming wide synapses allowing for considerable diffusion of the transmitter. High activity of the LC neurons results in increased expression of the tyrosine hydroxylase (TH) gene and *de novo* synthesis of the enzyme so that demand for NA synthesis can be met. The total number of estimated noradrenergic neurons, identified by tyrosine hydroxylase immunoreactivity and neuromelanin pigment content, in the LC of a normal young adult human brain ranges is from 45,000 to 60,000 (Cubells *et al.,* 1995., 1987, German *et al.,* 1988).

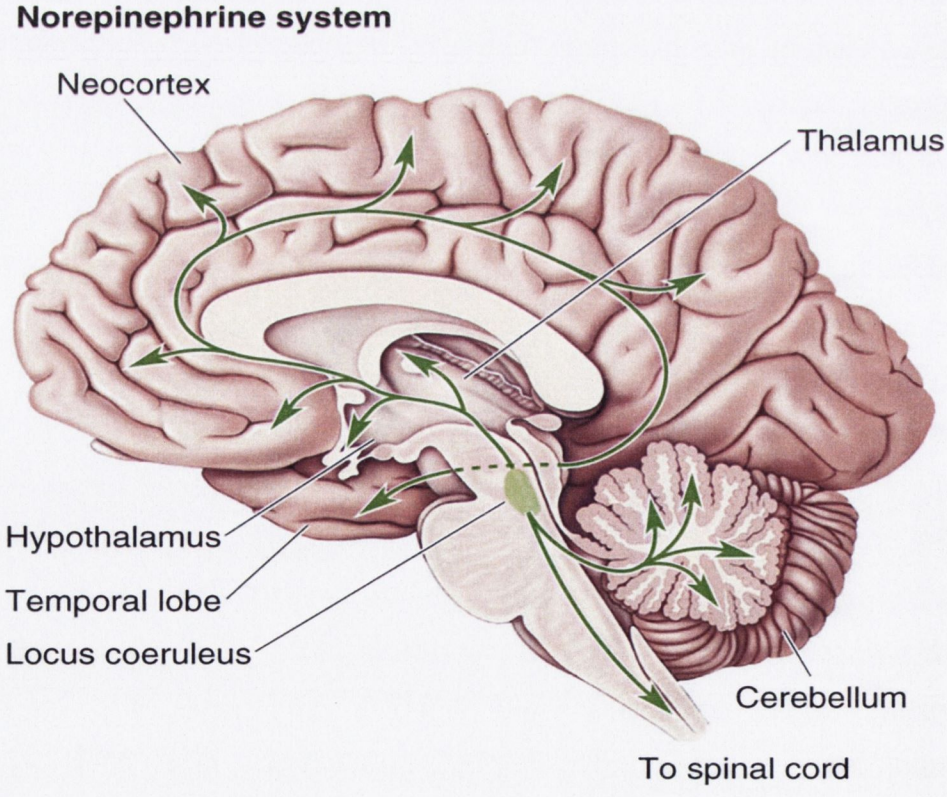


Figure 1.8: The CNS innervation of noradrenaline from the Locus Ceruleus

Two different types of LC axonal terminals have been characterized; conventional synaptic structures and varicosities that are believed to result in the extra-synaptic release of noradrenaline. As a result, NA may then diffuse over some distance before affecting adrenoceptors on neurons, glia and blood vessels (Parnaveles *et al.,* 1989, Marien *et al.,* 2004). As such, it has been suggested that noradrenaline is unique amoung neurotransmitters as it has the ability to acts as paracrine agent by diffusing away from the sight of its synaptic release (German *et al.,* 1992).

There are two enzymes that metabolise synaptic NA, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), and both are located in neurons and glia. NA is transported across the cell membrane by a sodium (Na^+) and chloride (Cl^-)-dependent transporter called the noradrenergic transporter (NET).

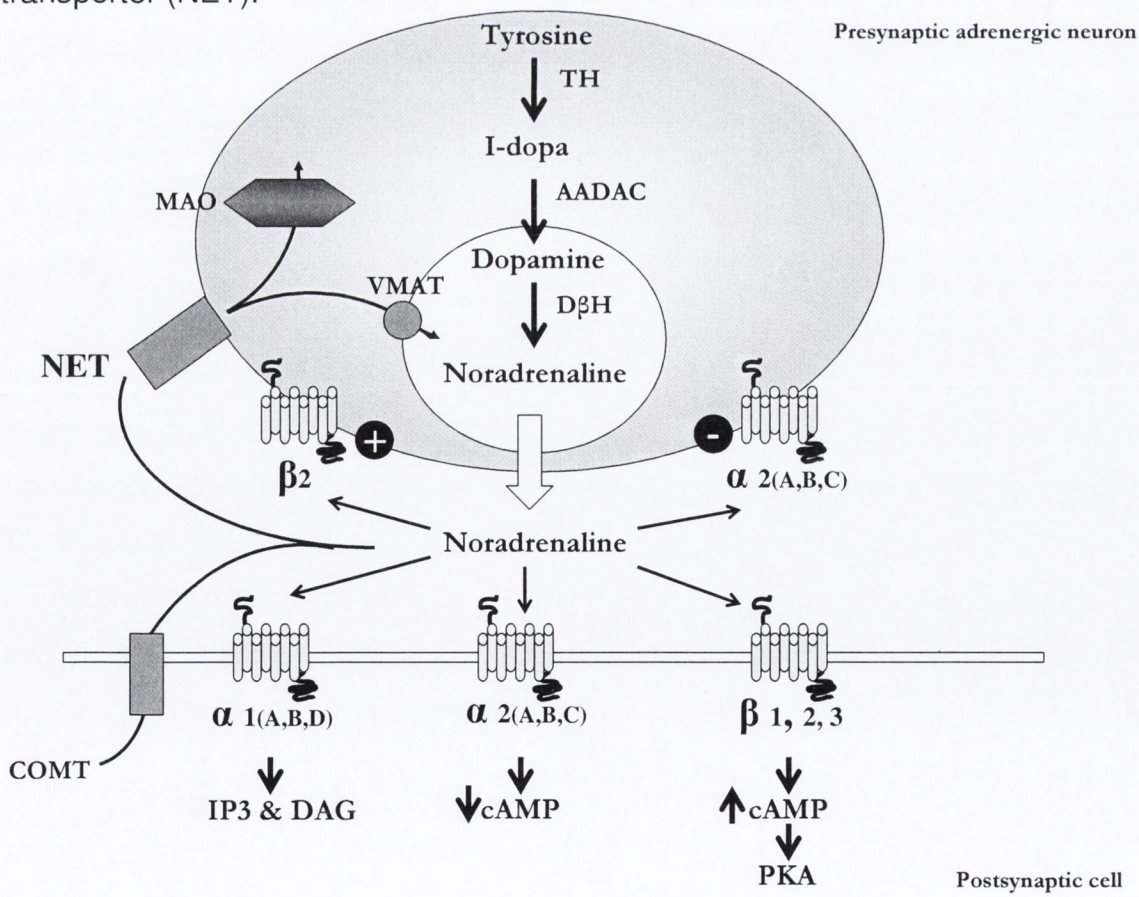


Figure 1.9: The biosynthesis of noradrenaline & adrenoceptor localisation (Adapted from Hein, 2006)

The NET is expressed presynaptically on neurons where it functions to clear released NA from the synaptic cleft. Approximately 70% of released NA is recycled rather than metabolised, by being sequestered by the intracellular vesicular monoamine transporters (VMAT) and stored in vesicles for re-release (see Eisenhofer, *et al.* 2001).

The NET is a high-affinity catecholamine transporter, which clears both DA and NA from the synaptic cleft and extracellular fluids, but it is a low capacity transporter that can become saturated in the presence of high NA concentrations (Eisenhofer *et al.,* 2001). As such, low concentrations of NA will be cleared efficiently from the synaptic cleft, however, larger amounts may saturate the NET and excess NA could then be available to diffuse to the interstitial fluid and affect neighbouring cells. The fact that the NET is primarily responsible for the termination of the adrenergic signal has made it the pharmacological target for disorders in which deficits in noradrenergic signalling are affected. The NET is also found on non-neuronal cells, including astrocytes and can be pharmacologically inhibited by selective NET inhibitors such as Reboxetine and Desipramine (Inazu *et al.*, 2003).
The post synaptic net effect of noradrenaline on cells of the cerebral or cerebellar cortex is to enhance the responsivity of neurons to other powerful excitatory (glutamatergic) and inhibitory (GABAergic) inputs while reducing spontaneous activity, thereby increasing the signal-to-noise ratio for trans-synaptic information transfer (Devilbiss *et al.,* 2000). Importantly, apart from the classical neurotransmitter signaling action, LC noradrenaline may fulfill additional capacities that influence the survival, maintenance and plasticity of CNS neurons, including regulation of endogenous neurotrophin systems, glial function, CNS energy utilization and extracellular homeostasis, sleep and anti-inflammatory effects (Marien *et al.,* 2004).

1.7.2 *Adrenoceptors*

Noradrenergic cell surface receptors, Adrenoceptors (AR's), are metabotrophic G-protein coupled and are activated by noradrenaline and

adrenaline binding. They are divided into two classes, the α- and β-AR's, in accordance with the type of G-protein they are coupled to. The α1 subtype is typically postsynaptic and coupled to the IP3/DAG second messenger system. The α2 subtype is presynaptic and act as autoregulatory inhibitory receptors by reducing cyclic AMP (cAMP) as they are coupled to an inhibitory G-protein (G_i). All of the β subtypes (1, 2, & 3) are coupled to stimulatory G-proteins (G_s) and as a result, increase the levels of intracellular cAMP. Recruitment of a G_s from the ligation of a β-adrenoceptors activates membrane bound adenylate cyclase and cytosolic adenosine tri-phosphate (ATP) to produce increased levels of intracellular cAMP, leading to a subsequent increase in cAMP dependent protein kinase A (PKA). PKA is a ubiquitous serine/threonine kinase that exists in an inactive state with its catalytic domains bound to regulatory domains. cAMP binds to the regulatory subunits of PKA, releasing the catalytic subunits in an enzymatically active form. The catalytic domains can further phosphorylate a broad spectrum of protein substrates, leading to changes in cell function and the expression. There appears to be cell and gene specific differences in the transcription factors that PKA can activate, although cAMP regulatory element binding protein (CREB) appears to be the most common. Others include NFκB, CREB-binding protein and CCAAT enhancer binding protein.

1.7.3 *Adrenoceptor localization in the brain*

The individual α-AR subtypes demonstrate unique patterns of mRNA distribution in the rat brain, with the α_1-AR more restricted in their distribution and the α_2-AR more widespread. The $\alpha_{1(A)}$-AR was primarily localized in the olfactory bulb, intermediate layers of the cortex, the hippocampus and the reticular nucleus of the thalamus. The $\alpha_{1(B)}$-AR was expressed in intermediate and deep layers of the cortex, thalamus, hippocampus, dorsal raphe and cerebellum. Although the $\alpha_{2(A)}$-AR message was relatively low in abundance, it was identified in the olfactory bulb, cortex, hippocampus, locus coeruleus, pons and cerebellum. The $\alpha_{2(C)}$-AR messenger RNA was localized in the cortex (particularly cingulate), hippocampus, caudoputamen, pons and

cerebellum. Multiple α-AR subtypes have significant sequence homology and similar pharmacologic properties; however, they each appear to possess unique patterns of messenger RNA distribution throughout the brain. The multiplicity of subtypes of alpha adrenergic receptors in specific brain regions may dictate the physiological and pharmacological responses to catecholamines (McCune *et al.,* 1993, Nicholas *et al.*, 1993).

Using autoradiography, the concentration of β-adrenoceptors in human postmortem brain tissue was highest in the hippocampus < cerebellum < thalamic nucleus < basal ganglia < midbrain < cortex with low concentrations in the white matter and hypothalamus (Reznikoff *et al.*, 1986). Analysis of rat brain tissue has revealed difference in the localization of adrenoceptors and also heterogeneous distribution of the different receptor subtypes compared to human brains (Rainbow *et al.*, 1984). High levels of β_1-AR's were observed in the cingulate cortex, layers I and II of the cerebral cortex, the hippocampus, the Islands of Calleja, and the gelatinosus, mediodorsal, and ventral nuclei of the thalamus. In contrast, high levels of β_2-AR's were found in the molecular layer of the cerebellum, over pia mater, and in the central, paraventricular, and caudal lateral posterior thalamic nuclei. Approximately equal levels of β_1- and β_2-AR's were localized to the substantia nigra, the olfactory tubercle, layer IV of the cerebral cortex, the medial preoptic nucleus, and all nuclei of the medulla. These pronounced differences in the ratio of β_1- and β_2-AR's among brain regions could suggest that the subtypes of β-AR's receptors may play different roles in neuronal function (Rainbow *et al.*, 1984). They may also be an indication of different glial subtypes and there regionally variable expression levels of β-AR's. These receptor distribution studies also point towards a lack of correlation between known regions of noradrenergic terminals and a high distribution of β-adrenoceptors.

As well as being localized on neurons, all three glial subtypes have been reported to express functional adrenoceptors. Rat astrocytes express α_1, α_2, β_1- and β_2-AR's (Ebersolt *et al.*, 1981, Salm and McCarthy, 1989, Hertz *et al.,*

2004) and rat microglia express α_1, α_2, β_1- and β_2-AR's, but not the β_3-AR (Tanaka *et al.*, 2002, Mori *et al.*, 2002). Recently, α_1-AR's have been localized on oligodendrocytes in the rat visual cortex (Nakadate *et al.,* 2006) and primary cultures of rat oligodendrocytes and oligodendrocyte progenitor cells express all three subtypes of the α_1-AR (α_1-A, B and D) and β-AR's (Khorchid *et al.*, 1999, 2002)

While both adrenaline and NA are endogenous ligands for AR's, the brain contains more NA than adrenaline. There are more NA neurons than adrenergic, and the density of noradrenergic fibers is far greater than that of adrenergic ones (Moore and Bloom, 1979). Pharmacological studies have revealed that adrenaline has a high affinity for β_1- and β_2-AR's and a moderate affinity for β_3-AR. Conversely, NA has a high affinity for β_1- and β_3-AR but a low affinity for the β_2-AR (Bylund et al., 1994).

1.7.4 *Physiological functions of adrenoceptors*

α_1-AR

As previously mentioned, upon activation of α_1-AR, a heterotrimeric G protein, Gq, activates phospholipase C (PLC), which causes an increase in IP3 and calcium. In the brain it is typically located post-synaptically. The general physiological affect of α_1-AR activation is the contraction of smooth muscle, particularly the vasoconstriction of blood vessels. They also serve a function in glycogenolysis from adipose tissue. Clinically, *α_1-AR* antagonists are used to treat hypertension (Rang *et al.*, 2003).

α_2-AR

α_2-AR have similar functions to the α_1-AR subtype in that they play a role in the vasoconstriction, however, they (both pre- and post-synaptically) are coupled to inhibitory G proteins, Gi, which renders adenylate cyclase inactive and thus reduce cellular levels of cAMP. This results in a decrease of synaptic release of noradrenaline and acetylcholine in the brain, as well as inhibition of lipolysis in adipose tissue and insulin in the pancreas (Rang *et*

al., 2003, Khan et al., 1999). Antagonist of the α_2-*AR* have a role in the treatment of depression as they increase the availability of synaptic noradrenaline.

β_1-*AR*

As previously mentioned, all of the β subtypes (1, 2, & 3) are coupled to stimulatory G-proteins (G_s) and as a result, increase the levels of intracellular cAMP. β_1-AR agonists increase heart rate and cardiac output by increasing cardiac muscle contractions. They are used clinically as a cardiac stimulant. Antagonists of β_1-AR's are used in hypertension and coronary heart disease's to decrease the cardiac output (Rang et al., 2003).

β_2-*AR*

β_2-AR activation results in the relaxation of smooth muscle. While β_2-AR agonists increase cardiac output by increasing heart rate and muscle contraction, the physiological response of this is minor in comparison to selective β_1-AR agonists. As such the main clinical use of β_2-AR agonist is in the vasodilation of bronchi and as such, selective β_2-AR agonists are use to treat asthma and chronic obstructive pulmonary disease (Rang et al., 2003, Frielle *et al.,* 1989, Taylor *et al,* 2002).

β_3-*AR*

The newly identified β_3-AR is mainly localized in adipose tissue and selective β_3-AR agonists mediate both the lipolysis of adipose tissue and aid in thermogenesis of skeletal muscle. As such, selective agonists have been studies as therapeutic treatment of obesity (Rang *et al.,* 2003, Emorine *et al.*, 1989, Krief *et al.,* 1993, Strosberg *et al.,* 1997).

1.8 Noradrenergic system degeneration in CNS disease

1.8.1 *Locus Coeruleus degeneration in Alzheimer's Disease*

As previously mentioned, the Locus Coeruleus (LC) is the main sub-cortical site for the synthesis of noradrenaline and its precursor enzymes. The Loss

of noradrenergic LC neurons, degeneration of noradrenergic projections and a decrease in cortical and hippocampal NA concentrations are well described features of various neurodegenerative diseases including Alzheimer's disease (AD) (Forno *et al.,* 1978, Wilcock et al., 1988, see Marien *et al.,* 2004, for review). Decreased LC neuronal counts are significantly correlated with the numbers of β-amyloid plaques, neurofibrillary tangles and the severity of dementia in AD (Bondareff *et al.*, 1987). Significantly, LC cell numbers are reduced by up to 60% at autopsy in Alzheimer's Disease, Parkinson's disease, and Down's syndrome, compared to normal aged-matched controls (German *et al.*, 1992).

This deficit in NA tone appears to contribute to the inflammation observed in animal models of Alzheimer's disease in vivo (Heneka *et al.,* 2002, Heneka et al., 2003, Kalinin et al., 2007) and *in vitro* (Hu et al., 1998). The lowered levels of NA in the AD brains may increase the β-amyloid-induced inflammation, including cytokine and NOS2 expression (Heneka *et al.*, 2002). As a result, the degeneration of LC neurons observed in AD may be a contributing factor for the increased inflammation and neuronal cell death observed.

1.8.2 *β_2-adrenoceptors in Multiple Sclerosis*

Immunohistochemical experiments have shown that astrocytes in human Multiple sclerosis (MS) tissue lack β_2-AR's. β_2-AR's could not be visualized on astrocytes in normal appearing white matter or in reactive astrocytes in chronic active and inactive plaques (De Keyser *et al.*, 1999, De Keyser *et al.*, 2004). Why β_2-AR's are lacking in MS tissue is not known, however because of their involvement in anti-inflammatory and supportive roles, their disappearance might have important pathophysiological consequences that are relevant for the pathogenesis of Multiple Sclerosis (De Keyser *et al.*, 2004).

When bound by noradrenaline under normal circumstances, *In vitro* studies using microglia, astrocytes and brain endothelial cells have demonstrated, β_2-AR's significantly down regulate inflammatory gene transcription via elevation of intracellular cAMP (Feinstein *et al.*, 2002). In the context of CNS inflammation, one of the most significant genes that NA can modulate is major histocompatability complex type II (MHC II). This membrane bound receptor is found on antigen presenting cells and with regard to MS, it functions by presenting myelin to auto-reactive T-cells. MHC II is predominantly found within the CNS on microglia (Zeinstra *et al.*, 2003, Ulvestad *et al.*, 1994) but by contrast, astrocytes in both normal and many inflammatory disease states, do not constitutively express MHC II. It has been shown that this is due to neurotransmitters like NA, which via activation of β2-AR's, down-regulate co-stimulatory molecules like MHC II, intra cellular adhesion molecule type-I (ICAM-I) and B7-1 (Zeinstra *et al.*, 2003, Frohman *et al.*, 1998, Lee *et al.*, 1999). The down-regulation of β2-AR's on astrocytes may possibly result in an increase of expression of adhesion molecules such as MHC II, which can aid the influx of T-cells, B-cells and macrophages into the CNS during inflammatory conditions (Zeinstra *et al.*, 2003, Frohman *et al.*, 1998, Lee *et al.*, 1999). Another important point to note is that the loss of β_2-AR's in MS tissue may impair the normal rates of astrocyte glycogenolysis and subsequent production of lactate. It is this secreted lactate that neuronal axons rely on to aerobically produce ATP and maintain regular ion gradients as well as sustain energy consuming functions (Brown *et al.*, 2003, De Keyser *et al.*, 2004). Thus, the β_2-AR's impairment on MS astrocytes may inadvertently lead to axonal degeneration, possibly via a breakdown of the normal ion gradients and Ca^{2+} influx into the mitochondria (De Keyser *et al.*, 2004).

1.9 Intrinsic CNS immunomodulation: A regulatory role for NA and the β-adrenoceptors

As mentioned previously, it is now well accepted that inflammatory processes in the CNS are a major contributing factor to the physiology and subsequent

pathology of neurological diseases, infection and trauma. The concept is emerging that endogenous mechanisms in the CNS could be an important factor for initiation, maintenance and lack of resolution of brain inflammation. Interestingly, the activation of intrinsic neurotransmitter pathways, particularly the NA system, could provide protection in neuroinflammatory conditions (Galea *et al.*, 2003).

Early studies revealed that NA attenuated IL-12 production from LPS-stimulated human whole blood cultures (Elenkov *et al.,* 1996) and also enhanced the synthesis of the anti-inflammatory cytokine, IL-10 in conjunction with it's suppression of TNF-α from LPS-stimulated human monocytes (Siegmund *et al.,* 1998). These were important observations considering the ability of IL-10 to inhibit the production of IL-1β, IL-6, IL-8 and TNF-α in monocytes and macrophages, as well as IFN-γ and TNF in natural killer cells and pointed towards a direct anti-inflammatory role of NA (Bogdan *et al.,* 1991). NA has also been shown to inhibit the LPS-induced production of IL-1 from human fibroblasts, endothelial cells and monocytes (Van der Poll *et al.,* 1997, Koff *et al.,* 1986) and IFN-γ production from human T- and NK cells (Sander *et al.,* 1997, Borger *et al.,* 1998).

In vitro studies of microglia, astrocytes and brain endothelial cells have also demonstrated that NA can infact suppress inflammatory gene expression (Feinstein *et al.*, 2002). In enriched cultures of rat microglia incubated with NA, NOS_2 activity was reduced by up to 70% (Galea *et al.*, 2003). In rat astrocyte cultures, incubation with NA significantly reduced the levels of LPS-induced NO release and NOS_2 mRNA expression. This affect was mediated by β- and not α-AR's as was evident from the use of selective antagonists (Feinstein *et al.*, 1998). A further anti-inflammatory role for β-AR's was demonstrated when the β-AR agonist salbutamol, inhibited circulating levels of IL-12 in humans (Panina-Bordignon *et al.,* 1997). In addition, salbutamol induces the ex vivo release of IL-4, IL-6 and IL-10 from concanavalin A-activated mice spleenocytes (Coqueret *et al.,* 1994). The selective β_2-AR

agonist clenbuterol, has also displayed anti-inflammatory properties as it reduces LPS-induced IL-6 and TNF-α production from human macrophages and *in vivo* using wistar rats (Izebond *et al.,* 1999). As such, It is the β_2-AR subtype that has been implicated in mediating the anti-inflammatory affects of NA on glia, as microglia have a higher expression of the β_2-AR than any other subtype (Mantyh *et al,* 1995, Mori *et al.,* 2002, Tanaka *et al.,* 2002).

In addition to its regulation of cytokine expression, NA has also demonstrated a negative regulation of cell adhesion molecules. *In vitro* studies using primary cultures of astrocytes have demonstrated that NA inhibits IFN-γ-induced MHC-II expression via β_2-adrenoceptor activation (Frohman *et al.*, 1988), a very significant observation considering the importance of MHC II in the presentation of antigens to infiltrating peripheral immune cells during chronic CNS inflammatory states. Brain endothelial cells treated with IL-1β induced a two-fold increase in the levels of the intracellular adhesion molecule (ICAM)-1 protein and *de novo* appearances of nitric oxide synthase-2 (NOS_2) activity. Stimulation of cAMP-dependent pathways with a cyclic AMP mimic or an adenylate cyclase activator decreases ICAM-1 expression (Balyasnikova *et al.*, 2000). It is important to note that as cAMP can modulate expression of pro-inflammatory molecules in brain endothelium, this may suggest that microvascular inflammatory processes may be regulated by NA (via β_2-adrenoceptor activation) *in vivo*.

Previous literature has revealed that stimulation of the β-AR and subsequent activation of intracellular cAMP can interfere with inflammatory intracellular signalling. For example, cAMP can interfere with the NFκB pathway by altering its phosphorylation state (Haraguchi *et al.,* 1996). The cAMP/PKA pathway can alsp compete with the NFκB (P50/P65) promoter binding site (Tsuruta *et al.,* 1995). Furthermore, cAMP also increases the levels of the inhibitory IκB proteins, which bind to and maintain NFκB in its inactive state in the cytosol (Neumann *et al.,* 1995). It has thus been suggested that the regulation and expression of members of the NFκB:IκBα signaling system

could contribute to the suppressive effects of NA in brain inflammation. In addition, NA suppressed NOS_2 gene transcription via the modification of transcription factors binding to a small region of the NOS2 promoter localized 58 bp upstream of a critical NFκB binding site (Gavrilyuk *et al.*, 2001). Furthermore, the signal transduction pathways used by IL-1β and TNF-α in astrocytes to induce ICAM-1/VCAM-1 expression are antagonized by cAMP-dependent PKA-mediated signaling pathways (Ballestas *et al.*, 1997). A generalized mechanism for NA transcriptional activation in the CNS may not exist but multiple affects may contribute to an overall anti-inflammatory phenotype (Feinstein *et al.*, 2002).

1.10 Pharmacological strategies for enhancing noradrenergic tone in the CNS

Despite the evidence that NA plays a tonic anti-inflammatory role in the CNS, little research has focused on the potential anti-inflammatory/neuroprotective actions of pharmacological strategies that increase central noradrenergic tone. Given previous literature indicating that NA has anti-inflammatory effects in the CNS, and evidence of NA deficits in a number of neurodegenerative disease states, strategies to augment NA availability in the CNS could have significant therapeutic effects. As NA cannot cross the blood-brain-barrier, inhibition of the transport mechanisms necessary for termination of adrenergic signalling could be a way by which to enhance its action in the CNS.

Noradrenaline re-uptake inhibitors:

NRI's are used clinically in the treatment of depression and other psychiatric disorders such as attention-deficit-hyperactivity disorder (ADHD) (Friedman *et al.*, 1996, Cipriani *et al.*, 2005, Nelson, 1999). These drugs increase noradrenaline availability by blocking the noradrenaline transporter (NET) thus, blocking re-uptake of the neurotransmitter.

In further support of the notion that an anti-inflammatory phenotype can be maintained following NRI treatment, it has been demonstrated that chronic treatment with the NRI desipramine, blocked the depressive-like behavioral symptoms and pro-inflammatory cytokine expression in rat cortex in response to an acute inflammatory challenge with LPS (Shen *et al.,* 1999). Desipramine has also demonstrated neuroprotective properties as it induced expression of bcl-2 and inhibited LPS-induced apoptosis of hippocampal neuronal stem cells (Huang *et al.,* 2007). Recently, Hashioka and colleagues have shown that the selective NRI reboxetine, inhibits the production of IL-6 and NO from IFN-γ-treated mouse microglia *in vitro* (Hashioka et al., 2007). Reboxetine also inhibited human T-cell motility and reduced the production of IFN-γ *in vivo* (Diamond et al., 2005).

α_2-AR antagonist:

α_2-AR antagonists are another class of drug that can potentiate the synaptic availability of NA *in vivo,* as pre-synaptically located α_2-AR negatively regulates NA release (van Veldhuizen *et al.*, 1993). Inhibition of this receptor subtype with antagonists such as idazoxan, has been shown to increase brain NA concentrations using *in vivo* microdialysis (Invernizzi and Garatti, 2004, van Veldhuizen *et al.*, 1993, Swanson *et al.*, 2006; Wortley *et al.*, 1999). α_2-AR antagonists have also displayed anti-inflammatory properties, for example, Idazoxan inhibits NOS-2 production from LPS-stimulated astrocytes (Feinstein et al., 1999) and F14413 inhibits Aβ-induced NOS-2 expression *in vivo* (Kalinin *et al.,* 2006). Furthermore, treatment with idazoxan and other α_2-AR antagonists such as yohimbine and dexefaroxan, have been shown to be neuroprotective in animal models of Huntington's disease (Martel *et al.,* 1998, Martel *et al.*, 2000), Parkinson's disease (Srinivasan *et al*, 2004) and brain ischemia (Craven, *et al.*, 1997, Gustafson *et al*, 1990).

Selective β-AR activation:

While β_2-AR agonists are best known for their clinical use as bronchodilators in asthma and chronic obstructive pulmonary disease, they also display

potent anti-inflammatory properties. For example, a range of β-AR agonists (procaterol, clenbuterol, fenoterol and terbutaline) inhibited LPS-induced TNF-α and IL-1β production from human peripheral blood mononuclear cells (Yoshimura *et al.*, 1997). Similarly, the β-AR agonist isoproterenol, inhibited LPS-induced TNF-α and IL-1β release from primary microglial cultures (Hetier *et al.*, 1991). The selective and short acting β_2-AR agonist, Salbutamol has been shown to inhibit the production of reactive oxygen species and TNF-α from alveolar macrophages *in vitro* (Gu *et al.,* 1996). While the highly selective and long lasting β_2-AR agonist salmeterol, inhibited LPS-induced TNF-α production *in vitro,* and circulating serum levels in the rat *in vivo* (Sekut *et al.*, 1995) as well as down-regulating release of the inflammatory mediators, histamine, leukotriene C4 and prostaglandin D2 from human lung tissue (Butchers *et al.*, 1991). Furthermore, in an *in vivo* study were healthy human subjects inhaled LPS, salmeterol treatment inhibited LPS-induced pulmonary neutrophils influx and degranulation, TNF-α release and HLA expression (Maris *et al.*, 2005).

Clenbuterol is a selective β-agonist with a relatively high affinity for the β_2-AR, developed as a long acting, orally active compound. Like most other β_2-AR's, the main clinical use is in asthma therapy where the compound produces relaxation of bronchial smooth muscle. As with many other β-AR agonist, there is now a body of research alluding to clenbuterol's anti-inflammatory properties. For example, Izeboud and colleagues, showed using both rats and a macrophage cell line, that clenbuterol attenuates LPS-induced IL-6 and TNF-α production *in vivo* and *in vitro* (Izeboud *et al.*, 1999). Clenbuterol is also lipophilic and as such, can pass the blood brain barrier, Previous research has showed a potentially neuroprotective role for clenbuterol, predominantly via the induction of astrocytic neurotrophins. Clenbuterol can induce astrocytic release of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and transforming growth factor-beta (TGF-β), all shown to be neuroprotective molecules (Hayes, *et al.*, 1995, Junker *et al.*, 2002, Zhu *et al.,* 2001). Clenbuterol-induced NGF protects hippocampal

neurons from excitotoxic cell death *in vitro* and ischaemia induced toxicity *in vivo* (Culmsee *et al.*, 1999a, Culmsee *et al.*, 1999b). The protective affects of clenbuterol pre-treatment on a mouse model of amyotrophic lateral sclerosis (ALS) have also been attributed to the it's induction of insulin-like growth factor (IGF-1) (Teng *et al.,* 2006). Furthermore, in an *in vivo* model of cerebral ischaemia, clenbuterol pre-treatment induced the anti-apoptotic bcl-2 and reduced the pro-apoptotic bax expression (Zhu *et al.*, 1999).

1.11 Objectives.

The objective of this project was to asses the ability of NA *in vitro*, and pharmacological strategies that enhance central availability of NA *in vivo,* to promote an anti-inflammatory phenotype in CNS glial cells. The role of the β-adrenoceptor in the anti-inflammatory actions of NA in glial cells was also studied. Specific experiments focused on:

1. The ability of NA to negatively regulate the IL-1 system in glial cells *in vitro,* and protect neurons against IL-1β-induced neurotoxicity.

2. The ability of pharmacological enhancement of central noradrenergic tone, or direct stimulation of central β_2-adrenoceptors to modify the IL-1 system *in vivo.*

3. The ability of pharmacological enhancement of central noradrenergic tone, or direct stimulation of central β_2-adrenoceptors to alter expression of other pro- and anti-inflammatory cytokines in the CNS *in vivo.* This study focused largely on the anti-inflammatory cytokine IL-10, as expression of this molecule was found to be profoundly altered by the pharmacological agents employed in this study.

Overall, this project has yielded significant insights into how NA and subsequent β-adrenoceptor activation exert an anti-inflammatory phenotype in the healthy CNS.

2.1 Materials

Animals

Wistar rats (1-2 days old)	Bioresources, TCD
Male Sprague Dawley rats (250-300g)	Harlan UK.
Laboratory rat diet	Red Mills

Cell Culture Materials

Acrodisc syringe filter (0.2μm)	Pall Corporation
Biocidal ZF ™	WAK-Chemie
B-27 growth factor	Invitrogen
Cell strainers (40μM)	BD Falcon
Coverslips (Plastic; 13 mm)	Sarstedt
Coverslips (glass; 13 mm)	Sarstedt
Cytotox 96-non-radioactive LDH assay kit	Promega
Disposable sterile scalpels	Swann-Morton
DNase	Sigma
Dulbecco's modified eagles medium:F-12 (DMEM)	Invitrogen
Dulbecco's phosphate buffered saline (PBS) (10X)	Sigma
Foetal Bovine Serum (FBS)	Invitrogen
Glutamax	Invitrogen
Haemocytometer	VWR international
Horse serum	Invitrogen
Neurobasal media	Invitrogen
Penicillin-streptomycin	Invitrogen
Plastic syringe (20ml and 1ml)	Becton Dickenson
Poly-L-lysine	Sigma
Polystyrene round bottomed tubes (5ml)	Becton Dickenson
Serological pipette (25ml)	Sarstedt
Sterile petri dishes	Sarstedt
Sterile transfer pipettes	Sarstedt
Sterile Combitips plus (2.5ml)	Eppendorf
Sterile Combitips plus (1ml)	Eppendorf
Sterile falcon tubes (50ml)	Sarstedt
Sterile falcon tubes (15ml)	Sarstedt

Sterile microtubes (2ml)	Sarstedt
Sterile 24 well plates	Sarstedt
Trypan Blue	Sigma
Trypsin	Sigma
Trypsin inhibitor	Sigma

ELISA: Plastics and kits

Maxisorp immunoplates for ELISA	Nunc
Rat Interleukin-1β (IL-1β) ELISA kit (Duoset)	R&D systems
Rat Interleukin-1ra (IL-1ra)	NIBSC, UK
Rat Interleukin-10 (IL-10) ELISA kit (Cytoset)	Biosource

Experimental Treatments

Clenbuterol	Tocris
Dibutyryl adenosine 3',5'-cyclic monophosphate (cAMP)	Sigma
Fomoterol hemisulphate	Tocris
Idaxozan hydrochloride	Tocris
KT5720	Calbiochem
(-)-Norepinephrine (+) bitartrate salt hydrate (99%)	Sigma
Phentolamine	Sigma
(±) Propranolol hydrochloride	Sigma
Reboxatine	Sigma
Salbutamol	Sigma
U0126	Promega

General Laboratory Chemicals

Acrylamide	Sigma
Ammonium persulfate (APS)	Sigma
Bicinchoninic acid (BCA) protein kit	Pierce
N' N' Bis Acrylamide	Sigma
Bovine serum albumin 96% (BSA)	Sigma
Bromophenol blue	Sigma
Chemiluminescence Hyperfilm	Amersham
CL-Xposure x-ray film	Pierce

Diethyl Pyrocarbonate (DEPC)	Sigma
di-Sodium hydrogen orthophosphate (Na_2HPO_4)	BDH
DL-Dithiothreitol (DTT)	Sigma
N-(1-naphthyl)-ethylenediamine dihydrochloride	Sigma
Ethylenediaminetetra acetic acid (EDTA)	Sigma
Glycerol	Sigma
Glycine	Sigma
Hepes	Sigma
Hydrochloric acid (HCL)	BDH
Industrial methylated spirits	Lennox
Normal goat serum	Vector
Magnesium chloride ($MgCl_2$)	Sigma
Magnesium Sulphate ($MgSO_4$)	Sigma
β-Mercaptoethanol	Sigma
Methanol (MeOH)	BDH
Phosphatase inhibitor cocktail I & II	Sigma
Potassium dihydrogen orthophosphate (KH_2PO_4)	BDH
Potassium chloride (KCL)	Merck
2-propanol	Sigma
Protease inhibitor cocktail	Sigma
Restore stripping solution	Pierce
Sodium carbonate (Na_2CO_3)	BDH
Sodium bicarbonate ($NaHCO_3$)	BDH
Sodium Chloride (NaCl)	BDH
Sodium dodecyl sulfate (SDS) 99%	Sigma
Sodium phosphate monobasic monohydrate (NaH_2PO_4)	Sigma
Sodium hydroxide (NaOH)	Sigma
Sodium nitrite ($NaNO_2$)	Sigma
Sucrose	Sigma
Sulphanilamide	Sigma
Sulphuric acid (H_2SO_4) 98%	BDH
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Sigma
3,3',5,5',-Tetramethyl-benzidine (TMB)	Dako
Tris Base	Sigma

Tris-HCl	Sigma
Tween 20	Sigma

General Laboratory Plastics

Microtest 96-well flat bottomed plates	Sarstedt
Pipette tips	Sarstedt
Microtubes (1.5ml)	Sarstedt
Microtubes (0.5ml)	Sarstedt
Plastic transfer pipettes	Sarstedt
Laboratory roll	Parafilm
Plastic syringe (1ml)	Becton Dickenson
50ml Yellow capped tubs	Sarstedt
Blood collection tubes 12ml	Sarstedt

Molecular Reagents

Absolute ethanol	Sigma
Agarose	Condra
Biosphere filter tips (1000, 200 and 100 µl)	Sarstedt
Diethyl pyrocarbonate	Sigma
Ethidium bromide	Sigma
High capacity cDNA archive kit	Applied Biosystems
Loading dye (6X)	Promega
Molecular grade water	Sigma
Optical adhesive covers	Applied Biosystems
PCR tubes	Sarstedt
RNA*later*™	Ambion
RNase away	Invitrogen
RNase-free 1.5ml and 2ml microfuge tubes	Ambion
RNAse Zap wipes	Ambion
Total RNA isolation kit	Macherney-Nagel
TaqMan gene expression assays	Applied Biosystems
TaqMan universal PCR master mix	Applied Biosystems
10X TBE buffer	Invitrogen
96-well optical reaction plates	Applied Biosystems

Western Blotting Reagents and Antibodies

Anti-mouse IgG	Sigma
Anti-Rabbit IgG	Amersham
Broad range molecular weight marker	Biorad
CHAPS	Sigma
CL-Xposure X-ray film	Pierce
Filter paper	Whatman
IL-1R(II) antibody	Santa cruz
Immobilin-P transfer membrane	Milliopre
Immobilin western chemiliuminescent HRP-substrate	Millipore
Monoclonal anti-β-actin antibody (mouse-anti rat)	Sigma
Nitrocellulose membrane	Sigma
Phospho-CREB antibody	Cell Signalling
Phosho-ERK antibody	Cell Signalling
Phospho-STAT3 antibody	Cell signalling
Restore™ Western Blot stripping buffer	Pierce
Supersignal® West Dura extended substrate solution	Pierce
Total-CREB antibody	Cell signalling
Total-ERK antibody	Cell signalling

Methods

2.2 In vitro cell culture

2.2.1 Aseptic technique

Aseptic techniques were utilised during all cell culture work and also in the preparation of cell culture reagents. This is necessary to maintain a sterile environment free from fungal, bacterial and viral infections that can alter normal cellular functions. Aseptic techniques utilised, include the use of sterile disposable plastics, and sterilisation of glassware, plastics and H_2O by autoclaving at 121°C for 30-60 minutes. Dissection equipment was baked for a minimum of two hours at 200°C to ensure sterility. All cell culture work was carried out in a laminar flow hood (Hera Safe, category 2). This allows only filtered air to come into contact with cells, thus preventing contamination with airborne pathogens. The interior of the hood was wiped down with 70% ethanol (EtOH) before and after use. The hood surface was also exposed to ultraviolet (UV) light for 15-30 minutes after use. Any items taken into the flow hood were lightly sprayed with 70% EtOH to prevent introduction of any pathogens to the hood work area. Disposable latex gloves were worn and sprayed with EtOH before use. Gloves were changed regularly during cell culture work. Cells were maintained in a sterile Nuaire incubator (95% air, 5% CO_2 at 37°C) and any items put in the incubator were lightly sprayed with EtOH to prevent contamination with any pathogens. Both the incubator and laminar flow hood were regularly cleaned with Biocidal ZF™ to maintain a sterile environment.

2.2.2 Preparation of tissue culture media & test compounds

Glial Culture Media: A working solution of complete dulbecco's modified eagle media (DMEM:F12) containing 10% (v/v) heat Inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin P/S (v/v) was prepared by filter sterilizing using a 0.2µm syringe filter, 50mls of FCS and 5mls of P/S into 500mls of DMEM:F12 (complete DMEM).

Neurobasal media: A solution of complete Neurobasal media (NBM) containing 10% (v/v) heat Inactivated horse serum (HS), 1% P/S (v/v) and

1% glutamax (v/v), was prepared by filter sterilizing using a 0.2µm syringe filter, 50mls of HS, 5mls of P/S and 5mls of glutamax into 500mls of neurobasal media. A working solution was prepared by adding 500µl of B-27 growth supplement to 50mls of complete-neurobasal media.

PBS: A working solution of 1x PBS (100mM NaCL, 80mM Na_2HPO_4, 20mM NaH_2PO_4) was prepared by adding 1ml of 10x sterile PBS to 9mls of d.d.H_2O.

Noradrenaline: Pilot dose-response studies were carried out with NA treatment using concentrations of 0.01-10µM. In the present study, A stock solution of noradrenaline (NA) was prepared by adding 0.337g of noradrenaline [Formula weight (F.W.) is 337] to 5ml of d.d.H_2O. The solution was filter sterilized as before by using a 0.2µm syringe filter. 200µl aliquots were prepared yielding a concentration of 100mM and stored at -20°C. Before use it was diluted to a 5µM working concentration in pre-warmed complete DMEM:F12.

Phentolamine: Pilot dose-response studies were carried out with phentolamine treatments using concentrations of 10µM or less. A concentration of 5µM was chosen as this dose did not induce any independent treatment effects, while still attenuating NA-induced gene and protein expression. A stock solution of phentolamine (non-selective α-adrenoceptor antagonist) was prepared by adding 50mg to 3.38ml of d.d.H_2O yielding a 50mM concentration. This solution was vortexed and filter sterilized as before by using a 0.2µm syringe filter. This stock solution was stored in 50µl aliquots at -20°C. A working concentration of 5µM was prepared using in pre-warmed complete DMEM:F12.

Propranolol: Pilot dose-response studies were carried out with propranolol as with phentolamine. In the present study, A stock solution of propranolol (non-selective β-adrenoceptor antagonist) was prepared by adding 50mg to 3.38ml of d.d.H_2O yielding a 50mM concentration. This solution was vortexed and filter sterilized as before by using a 0.2µm syringe filter. This

stock solution was stored in 50µl aliquots at -20°C. A working concentration of 5µM was prepared using in pre-warmed complete DMEM:F12.

Salbutamol: A 100mM stock solution was prepared by adding 0.866mls of d.d.H_2O to 25mg of salbutamol (selective β_2-adrenoceptor agonist). This solution was vortexed and filter sterilized as before by using a 0.2µm syringe filter. The stock solution was stored in 50µl aliquots at -20°C. A working concentration of 5µM was prepared using in pre-warmed complete DMEM:F12.

N^6, 2'-O-O-Dibutryladenosine; 3':5'-Cyclic monophosphate (db-cAMP): Pilot studies were carried out using a dose-response of db-cAMP with concentrations ranging from 1-100µM. In the present study, 1ml of d.d.H_20 was added to a 25mg vial of db-cAMP (cyclic AMP analogue) [with a F.W. of 491.4]. This yielded a stock solution of 51mM. This solution was vortexed and filter sterilized as before by using a 0.2µm syringe filter and stock aliquots were stored at -20°C. A working concentration of 100µM was prepared using in pre-warmed complete DMEM:F12.

KT5720: Pilot dose-response studies were carried out with KT5720 as with phentolamine. In the present study, A 932µM stock concentration of this Protein kinase A antagonist was prepared by adding 100µl of DMSO to 50µg of KT5720. This solution was vortexed and stock aliquots were stored at -20°C. A working concentration of 1µM was prepared using pre-warmed complete DMEM:F12. The media control treatments in all *in vitro* experiments using KT5720 were controlled for DMSO content.

U0126: Pilot dose-response studies were carried out with U0126 as with phentolamine. In the present study, A 10mM stock solution of the MEK 1/2 inhibitor U0126 was prepared by adding 234 µl of dimethyl sulphoxide (DMSO) to a 1mg vial of U0126. This solution was vortexed and filter sterilized as before by using a 0.2µm syringe filter. A working solution of 10µM was prepared with pre-warmed complete DMEM:F12. The media

control treatments in all *in vitro* experiments using U0126 controlled for DMSO content.

Recombinant rat IL-1β (rrIL-1β): Pilot studies were carried out using a dose-response of IL-1β treatment s at concentration of 0.1-10 ng/ml for 24, 48 and 72 hours. In the present study, a 20µg/ml stock solution of rrIL-1β was prepared by reconstituting 10µg of rrIL-1β with 500µl sterile PBS/1% (v/v) BSA. This solution was vortexed and stored in 10µl aliquots at -80°C. A working solution of 5ng/ml was prepared with pre-warmed complete DMEM:F12

2.2.3 Coating coverslips with poly-L-lysine

A 25mg stock of Poly-l-lysine was reconstituted by adding 3mls of autoclaved distilled H_2O and mixed using a vortex. When the solution was dissolved, 22mls of d.d. H_2O was added to bring the final volume to 25mls. 1ml aliquots (40µg/ml w/v) were prepared and one aliquot was used per glial/neuronal preparation. One day, prior to the preparation of the glial cells, a 1ml aliquot of Poly-L-lysine was added to 24mls of d.d. H_2O in a sterile 50ml falcon tube. The solution was vortexed and filter-sterilized through a 0.2µm syringe filter into a sterile Petri-dish.

Primary glial preparation: Sterile plastic 13mm coverslips were then added to the Petri-dish and gently pushed to the bottom. The petri-dish was placed in a 5% CO_2 incubator at 37°C for 1 hour.

Primary neuronal preparation: Pilot studies culturing primary neuronal cultures revealed a better growth rate and cell quality if they were grown on poly-l-lysine coated glass coverslips, rather than the plastic coverslips used in all glial culture experiments. As such, non-sterile 13mm glass coverslips were placed in a sterile 50ml falcon tube containing 50mls of ethanol for 24 hrs. The coverslips were then dried on sterile tissue paper inside the laminar hood and exposed to Ultra-violet light overnight. This protocol was employed to sterilize the coverslips. The glass coverslips

were then added to a Petri-dish containing the working solution of poly-L-lysine and incubated at 37°C for 1 hour in a 5% CO_2 incubator.

The coverslips where subsequently removed from the Poly-L-lysine working solution and indivually placed on sterile tissue paper which was previously soaked in 70% ethanol (EtOH) in the center of the laminar hood. When the coverslips were dry, they were individually placed into the wells of a 24×well tissue culture plate and stored in the hood to maintain their sterility.

2.2.4 Preparation of primary cortical mixed-glial cell cultures

This protocol was previously shown to yield primary mixed glial cultures containing astrocytes (70%) and microglia (30%) approximately, as seen by Ox-42 (microglial stain) and GFAP (astrocyte stain) immunocytochemistry (Nolan *et al.,* 2004). Six, one day old neonatal male wistar rats (PND2) were used per experiment. Each rat was taken into the hood, and was decapitated with a sharp scissors and the excess blood was soaked using a sterile paper towel that was previously sprayed with 70% EtOH. A small scissors was used to cut the skin from the base of the skull up to the nose. The skin was pulled away from the skull on each side and used to hold the skull in place. Each side of the skull was cut with a scissors along the sides at the level of the ears until the eyes were reached. At this point a curved forceps was used to pull back the skull, revealing the brain. The curved forceps was again used to carefully pinch out the cortices from both hemispheres. The cortices were then placed in a few drops of sterile1x PBS in a new Petri-dish. Any remaining meninges were carefully removes using a fine forceps. Cortices from the six rat pups were pooled. The pooled cortices were cross-chopped lightly using a new sterile scalpel in the Petri-dish. Using a sterile Pasteur pipette, the cortex homogenate was placed in 20mls of pre-warmed (37°C) complete DMEM in a 50ml falcon tube and was placed in a 37°C incubator for 20 min. The homogenate was subsequently removed from the incubator and in the hood, triturated with a sterile Pasteur pipette until all visible clumps had been removed. A nylon cell strainer (40μm) was placed on top of a sterile

50ml falcon tube and the 20mls of homogenate was carefully pipetted through it using the Pasteur pipette. The resulting solution was centrifuged for 3 min at 2000 x rpm at 20°C. Following centrifugation, the supernatant was carefully aspirated off and the cell pellet was re-suspended in 15ml of complete DMEM. The pellet was gently triturated with a fresh sterile Pasteur pipette until a homogenous cellular suspension was obtained. At this stage, a cell count was performed by adding 100µl of the cell suspension to 300µl of 1xPBS and 100µl of trypan-blue in a 2ml eppendorf microtube. This solution was vortexed and 50µl was placed under a microscope coverslip on a haemocytometer. The cell number was counted and calculated using a light microcope at the 40x magnification. The cell count was adjusted to 2 x 10^5 cells/ml.

Finally, 50µl of this adjusted cell suspension was placed into the center of each of the poly-L-lysine treated coverslips in the wells of the 24 x well plates. After one plate was coated, it was immediately placed in the incubator at 5% CO_2 and 37°C. The cells are left for two hours to allow them to adhere to the coverslips after which they are removed from the incubator and in the laminar flow hood, 400µl of complete DMEM is pipetted into each well. The plates were placed back into the 37°C incubator and the cells were grown for ten to eighteen days before treatments began. The media was changed every three days.

2.2.5 Preparation of primary cortical neuronal cell cultures

This protocol was previously shown to yield 97% pure cultures of primary neurons, as demonstrated by Neu-N immunocytochemistry (Fogarthy *et al.,* 2003). Cultures of primary rat cortical neurons were prepared from six, one day old neonatal male wistar rats (PND1) according to the same protocol as the primary mixed glia cultures with the following adaptations;

3 solutions of trypsin or trypsin inhibitor were prepared as follows:

Solution 1: Trypsin (0.3mg/ml)/sterile PBS.

Solution 2: Trypsin inhibitor (0.1mg/ml), DNase (0.2mg/ml) and 100µl of 1M $MgSO_4$.

Solution 3: 30% solution 2 in sterile PBS i.e. 3mls solution 2 to 7mls PBS.

When the meninges are removed from the cortices, they are placed in a Petri dish containing a few drops of a 3mg/ml Trypsin/sterile PBS (w/v) solution and mechanically dissociated. There are then placed in a 15ml falcon tube containing 2 mls of solution 1 and Incubated for 25 minutes at 37°C. Next, 4 mls of a solution 3 is added to inactivate the trypsin. The solution is flicked and the tissue allowed settle. The solution is the aspirated off the cortical tissue using a disposable pipette. 2 mls of solution 2 is added to the tissue and it is dissociated by trituration with a sterile disposable pipette. The solution is passed through a 40µm cell strainer into a sterile 50ml falcon tube and centrifuged at 2000g for 3 minutes at 20°C. The supernatant was aspirated off and the pellet re-suspended in a 15mls of pre-warmed NBM-B27. The pellet was gently triturated with a fresh sterile Pasteur pipette until a homogenous cellular suspension was obtained. At this stage, a cell count was performed by adding 100µl of the cell suspension to 300µl of 1xPBS and 100µl of trypan blue in a 2ml eppendorf microtube. This solution was vortexed and 50µl was placed under a microscope coverslip on a haemocytometer. The cell number was counted and calculated using a light microcope at the 40x magnification. The cell count was adjusted to 2×10^5 cells/ml.

Finally, 50µl of this adjusted cell suspension was placed into the center of each of the poly-L-lysine treated coverslips in the wells of the 24 x well plates. After one plate was coated, it was immediately placed in the incubator at 5% CO_2 and 37°C. The cells are left for two hours to allow them to adhere to the coverslips after which they are removed from the incubator and in the laminar flow hood, 400µl of complete B27-NBM was pipetted into each well. The plates were placed back into the 37°C incubator for 3 days. The media was then replaced with NBM containing 5µg/ml Cytosine-arabinofuranoside (ARA-C) to kill off glia and endothelial cells. This solution was incubated with the cells for 24 hours and the then replaced with complete NBM. The primary neuronal cultures were treated within 10 days of preparation.

2.2.6 Preparation of enriched primary microglia and astrocyte cell cultures

Primary glial cultures containing enriched astrocytes and microglia were prepared according to the same protocol as the primary mixed glia cultures, with the following adaptations;

A final single cell suspension of mixed glia was adjusted to 2 x 10^5 cells/ml according to the previous mixed glial protocol. However, instead of 24-well plates, 2ml of this solution was added to a T25 cm^2 tissue culture flask. The cells were allowed to adhere for up to 2 hours and the flasks were flooded 8mls of complete DMEM:F12 and cultured in the incubator at 5% CO_2 and 37°C for 24 hours. The media containing any cellular debris was aspirated off the monolayer of cells and fresh complete DMEM: F12 was added containing a working concentration of GM-CSF (20ng/ml) and M-CSF (5ng/ml). The cells were cultured for 7 days, after which the DMEM:F12 (+ M-CSF/GM-CSF) was replaced. On day 12-14 of culture, a layer of microglia will be loosely adherent to a monolayer a astrocytes on the surface of the culture flask. The flasks were sealed with parafilm and placed on an orbital shaker. They were agitated at 110 rpm for 2hr at room temperature to dissociate the microglia into solution. The flasks were gently tapped inside the laminar flow hood to dissociate any remaining microglia and the supernatant centrifuged in a new sterile 50ml falcon tube at 2,000 x rpm for 5 mins at 20°C. The media was aspirated off and the remaining cells pellet was re-suspended in 1ml of complete DMEM:F12. At this stage a cell count was performed and the cell number was adjusted to 5x10^4 cells/ml. The cells were then plated onto glass coverslips in 24-well culture plates, allowed to seed for 2 hours before the wells were flooded with 400µl complete DMEM:F12. These isolated microglia were used within 3 days of isolation.

The remaining astrocytes were washed with 5mls of sterile PBS. This was aspirated off and the astrocytes were incubated with 1ml trypsin-EDTA for 15min at 37°C to dissociate all cells from the culture flask. The trypsin

solution was inactivated by adding 5mls of complete DMEM:F12 and the solution collected into a new sterile 50ml falcon tube and centrifuged at 2000 x rpm for 3 minutes at 20°C. The media was aspirated off and the resulting pellet was re-suspend in 1ml complete DMEM:F12 and a cell count performed. The astrocytes were plated onto glass coverslips at a cell density of 1 x 10^5 cells/ml and treated within 3 days of isolation.

2.2.7 Cell culture treatments

For each experiment 24 well tissue culture plates were prepared with mixed glial, enriched microglia/astrocytes or neuronal cultures as described previously. The cells were grown until the adherent monolayer was between 70-100% confluency, after days 10-14. Each 24 well plate was divided into 4 or 6 treatment groups of which, either 4-6 wells were pooled to give 1 experimental sample. Each plate was considered as an n=1. On the day of treatment culture media was removed from the wells and treatments administered in a final volume of 300µl of pre-warmed DMEM:F12, with 10% FCS and 1% P/S. All pre-treatments were administered 30 minutes prior to the experimental treatment with the exception of U0126 which was for 20 minutes. Cell cultures were incubated at 37°C with 5% CO_2 with the various drug treatments for either 6 hours to assess gene expression or 48 hours to assess protein production, unless stated otherwise.

2.2.8 Harvesting cell-free supernatants from in vitro cell cultures

At the required time-points, the 24 well plates were individually placed on ice, and the supernatants from each well were quickly aspirated off and placed in 2ml microtubes. The corresponding wells of each treatment group were pooled in one microtube. The microtubes were stored, submerged in ice, and when one plate was finished, the supernatants were mixed and then centrifuged at 13,000 x rpm for 3minutes at 4°C to remove cellular debris. Following this, the supernatants were carefully aspirated out and pipetted into new microtubes, mixed, and pipetted out into 300µl aliquots, before being frozen at -80°C until cytokines were analysed by ELISA. This procedure was repeated for each plate individually.

2.3 In vivo studies

2.3.1 Animal husbandry

Male Sprague-Dawley rats (200-300g) were obtained from Harlan laboratories UK and housed in hard-bottomed polypropylene cages with wood shavings as bedding. Animals were housed four to a cage under standard laboratory conditions, with an ambient temperature of 20-24°Cand a 12 hour light:12 hour dark cycle (lights on 08.00 hours, lights off: 20.00 hours). Animals had free access to food and water and were fed a standard laboratory diet (Red Mills).

2.3.2 In vivo drug administration

To increase synaptic availability of NA, 4 hours prior to sacrifice, a treatment regime of acute intra-peritoneal (I.P.) drug delivery with the NA re-uptake inhibitor (NRI) reboxetine and the α2-adrenoceptor antagonist, idazoxan was used. This animal model has previously been shown to maximise the synaptic availability of NA in vivo, as measured by microdialysis (Sacchetti *et al.*, 1999).

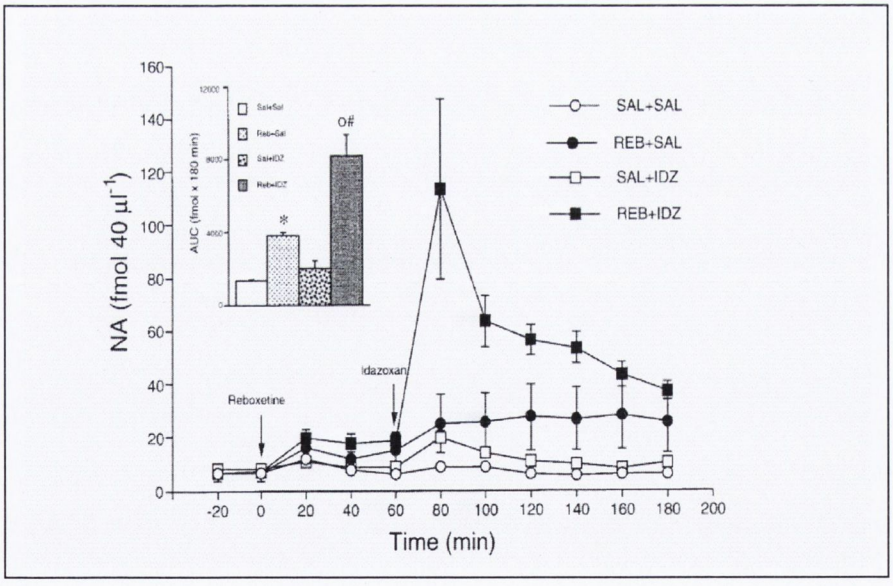


Figure 2.1: Idazoxan augments reboxetine-induced extracellular NA in the rat pre-frontal cortex

A microdialysis measure of extracellular concentrations of NA in the frontal cortex of rats given saline (Sal + Sal), 15 mg kg^{-1} reboxetine (Reb + Sal), saline + 1 mg

ml^{-1} idazoxan (Sal + IDZ) and reboxetine + idazoxan (Reb + IDZ) (adapted from Sacchetti *et al.*, 1999).

Reboxetine/Idazoxan study: Animals were acutely treated with intraperitoneal (I.P.) injections of reboxetine (15mg/kg), idazoxan (1 mg/kg) or both drugs in combination, 4 hours prior to sacrifice. A saline vehicle group was included to asses basal expression of CNS gene targets assessed.

Propranolol & Reboxetine/Idazoxan study: Animals were pre-treated with intraperitoneal (I.P.) injections of propranolol (10 mg/kg) or saline vehicle 60 minutes prior to acute treatment with reboxetine (15mg/kg) + idazoxan (1 mg/kg). A saline vehicle and propranolol (10 mg/kg) group were also included.

Clenbuterol time-course study: The concentrations of Clenbuterol used in this study were within a dose-range of *in vivo* literature (Culmsee *et al.,* 1999, Zhu *et al.,* 1999). An acute dose-response study was performed with intra-peritoneal (I.P.) injection of clenbuterol at 0.03, 0.1 and 0.5 mg/kg to asses the optimum dose range for assessing acute-gene expression of cytokines and cytokine receptors in the brain. In the present study, rats were treated with a single I.P. injection of either saline vehicle or Clenbuterol (0.5 mg/kg) 1, 4 or 8 hours prior to being sacrifice.

Propranolol & Clenbuterol study: Animals were pre-treated with intraperitoneal (I.P.) injection of propranolol (10 mg/kg) for 60 minutes prior to a 4 hour acute treatment with clenbuterol (0.5 mg/kg) prior to sacrifice. A saline vehicle and propranolol (10 mg/kg) group were also included.

Fomoterol study: Rats were acutely treated with intraperitoneal (I.P.) injections of either saline vehicle or fomoterol (0.5 mg/kg) 4 hours prior to sacrifice.

2.3.3 Harvesting brain tissue for post-mortem analysis

Following sacrifice the brain was quickly removed from the skull and dissected on ice. Tissue samples from various brain regions (cortex, hippocampus, hypothalamus and cerebellum) were transferred to 1.5ml microtubes and snap-frozen on dry ice and stored at –85°C until protein analysis. A portion of every brain region of interest from each subject was placed in RNase-free tubes containing RNA*later*™ and stored for up to seven days at 4°C. All samples were then removed form the RNA *later*™ solution, transferred to fresh RNAse-free tubes and frozen at –85°C until RNA extraction was performed. The cortex and hippocampus were used to analyse mRNA expression, and protein analysis was performed using the cortex samples.

2.4 Analysis of secreted cytokine concentration by ELISA

The sandwich Enzyme Linked Immunosorbent Assay (ELISA) method was used to determine the concentration of secreted cytokines.

Interleukin-1ra ELISA

IL-1ra standards and antibodies were obtained from Dr. Poole, NIBSC, Potters bar, UK. 96-well plates (NUNC, F96 MAXISORP-immuno plate) were coated with 100µl capture antibody (Rabbit anti-sIL-1ra; 2µg/ml, diluted in bicarbonate coating Buffer (0.1M $NaHCO_3$, 0.1M NaCL, pH 8.3) and incubated overnight at 4°C. The wells were washed four times with 400µl of wash buffer (0.5M NaCL, 2.5M $NaH_2PO_4.2H_2O$, 7.5M Na_2HPO_4, Tween-20 0.1%(v/v); pH 7.2) and blotted dry on a paper towel. The wells were blocked for one hour at room temperature with 100µl of bicarbonate coating buffer with 1% ovalbumin (w/v). The plate was again washed with 400µl of wash buffer and blotted dry on a paper towel. IL-1ra standards were prepared with serial dilutions containing 1000pg/ml to 0pg/ml using complete-DMEM (section 2.1.1) as a diluent. 100µl of the sample or standard in duplicate were pipetted into the wells and the plate was covered and incubated at 4°C overnight. Following this the wells were aspirated and washed as before, four times using 400µl of wash buffer and blotted dry on a paper towel. 100µl of detection antibody (1:500

dilution in wash buffer with 1% normal goat serum) was pipetted onto each well, the plates was covered and incubated at room temperature for 1hr. This was subsequently aspirated off and the wells were again washed with 400µl of wash buffer, before being blotted dry on a paper towel. 100µl of a working solution of avidin-HRP (1:5000 dilution in wash buffer) was added to each well, covered and incubated for 15 mins at room temperature, while avoiding direct light. The plate was again washed four times with wash buffer and blotted dry.

Finally, 100µl of substrate solution (TMB) was added to each well and plates were incubated for 10-30 minutes. 100µl of stop solution (1M H_2SO_4) was subsequently added to each well and the absorbance was measured at 450nm using a microtitre plate reader. A standard curve was constructed by plotting the standards against the absorbance. The results obtained are expressed as pg/ml.

Interleukin-1β ELISA

An IL-1β kit (Duo-set, R&D Systems) was used to perform these assays. 96-well plates (NUNC, F96 MAXISORP-immuno plate) were coated with 100µl capture antibody (0.297µg/ml, diluted in Phosphate Buffered Saline (PBS) 137mM NaCL, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.3) and incubated overnight at 4°C. The wells were washed four times with 400µl of PBS with 0.05% Tween-20 (v/v) and blotted dry on a paper towel. The wells were blocked for a minimum of one hour at room temperature with 300µl of blocking buffer (PBS, 1% BSA (w/v), 5% sucrose (w/v)). The plate was again washed with 400µl of PBS with 0.05% Tween-20 (v/v) and blotted dry on a paper towel. Recombinant IL-1β standards were prepared with serial dilutions containing 2000pg/ml to 0pg/ml using complete-DMEM:F12 (section 2.1.1) as a diluent. 100µl of the sample or standard (duplicated) were pipetted into the wells and the plate was covered and incubated at room temperature for 2 hours. Following this the contents of each well were aspirated and washed as before, four times using 400µl of PBS with 0.05% Tween-20 (v/v) and blotted dry on a paper towel. 100µl of detection antibody (IL-1β: 800ng/ml, diluted in PBS with 1% BSA (w/v) and

2% (v/v) normal goat serum were pipetted onto each well, the plates was covered and incubated at room temperature for 2hrs. This was subsequently aspirated off and the wells were again washed with 400µl of PBS and 0.05% Tween-20 (v/v), before being blotted dry on a paper towel. 100µl of a working solution of Horse radish peroxidase (HRP) conjugated streptavidin (1:200 dilution ; strepavidin-HRP with PBS and 1% BSA) was added to each well, covered and incubated for twenty minutes at room temperature, while avoiding direct light.

Finally, 100µl of substrate solution (TMB) was added to each well and plates were incubated for 10-30 minutes. 50µl of stop solution (1M H_2SO_4) was subsequently added to each well and the absorbance was measured at 450nm using a microtitre plate reader. A standard curve was constructed by plotting the standards against the absorbance. The results obtained are expressed as pg/ml.

Interleukin-10 ELISA

A rat IL-10 kit (Cytoset, Biosource) was used to perform this assay. 96-well plates (NUNC, F96 MAXISORP-immuno plate) were coated with 100µl capture antibody (1.25µg/ml, in Bicarbonate Coating Buffer; 51mM $NaHCO_3$, 50mM Na_2CO_3 pH 9.4) and incubated overnight at 4°C. The wells were washed four times with 300µl of wash buffer (PBS with 0.05% Tween-20) and excess wash buffer removed by blotting plate on a paper towel. Plates were blocked for a minimum of two hours at room temperature with 300µl of blocking buffer (PBS with 5% BSA). The plates were then washed as above.

100µl of recombinant IL-10 standard (0-1000pg/ml) or sample were added to plates and incubated at room temperature for 1.5 hrs. Plates were washed as previously and 100µl per well of biotinylated detection antibody (0.125µg/ml, in PBS containing 0.5% BSA, 0.1% Tween-20 and 5% fetal bovine serum) was added to the plates, which were then incubated for 1 hour at room temperature. Plates were washed as before and 100µl of the working solution streptavidin-horseradish peroxidase (HRP) conjugate

(1:2000 dilution in PBS with 0.5% BSA and 0.1% Tween-20) was added to each well and incubated for forty five minutes at room temperature, avoiding direct light. Following four washes, 100µl of substrate solution (TMB) was added to each well and plates were incubated for 10-30 minutes. 100µl of stop solution (1M H_2SO_4) was then added to each well and the absorbance measured at 450nm using a microtitre plate reader. A standard curve was constructed by plotting the standards against the absorbance and results obtained expressed as pg/ml of supernatant/serum or % control.

2.5 Lactacte dehydrogenase (LDH) assay

The CytoTox 96® (Promega) was used in this study to indirectly asses the levels of cytoplasmic LDH released into the cultured neuronal supernatants, after treatment with glial-conditioned media. LDH is a stable cytosolic enzyme that is released upon lysis. Therefore, measuring the levels of LDH in the supernatant inversely allows for a measurement of cell toxicity. Briefly, the supernatants of primary cell culture are aspirated off the cells and stored in 1.5 ml microtubes on ice, before centrifuging them at for 3 mins at 13, 000 x rpm to pellet any cellular debris. The adherent primary cells are exposed to 15µl of lysis solution (10X) (9% (v/v) Triton-x-100 in water) per 100 µl of culture medium, followed by incubation at 37ºC for 45-60 minutes. 50µl of the cell lysate is measured in conjunction with 50 µl of supernatant for every experimental treatment (in duplicate) by incubating with 50 µl of reconstituted substrate mix in a 96-well plate, for 30 mins at 37ºC. Following this, 50 µl of stop solution is added to arrest the reaction. Finally the absorbance is measured at 490nm. The data is interpreted by expressing % cytotoxicity as LDH absorbance in the supernatant (released) / LDH absorbance in the cell lysate (total cellular LDH).

2.6 Tissue preparation for western immunoblot

Cell cultures: Upon removal of cell supernatants, cells were washed twice with 300µl of ice-cold dulbeccos PBS to remove any remaining traces of FCS. A working solution of lysis buffer was prepared by adding DTT

(5mM) and 1% (v/v) of both protease / phosphatase inhibitors to a requisite amount of lysis buffer stock solution (Hepes (25mM), $MgCl_2$ (5mM), EDTA (5mM) in d.d.H_2O.). 75µl of lysis buffer was added to each coverslip of the different treatment groups. The plate was transferred to an orbital shaker, while on ice, an incubated for fifteen minutes. After this time the coverslips were scraped with the rubber-end of the insert in a 1ml syringe. The insert was either washed in d.d.H_2O or changed between each treatment group to avoid contamination. The lysis buffer/cell suspension was aspirated off each well into 1.5ml microtubes (The homogenate can be stored frozen at -80°C until immunoblotting experiments were performed).

Each microtube was subsequently homogenized by pipetteing the contents into a new 2ml flat-bottom microtube and using a polytron to shear the cells. The homogenate was centrifuged at 13,000 rpm at 4°C for 5 minutes to pellet any cellular debris. A 25µl sample was aspirated out of each microtube to perform a protein assay (section 2.4.1). Following the BCA-protein assay and sample equalization using lysis buffer stock, the samples were frozen at -20°C directly, with a 100µl of the equalized lysate added to 100µl of 1x Lamelli sample buffer (d.H2O, Tris-HCL (pH 6.8), Gycerol, 10% SDS, β-mercaptoethanol, 10X bromophenol blue). The samples were vortexed and then boiled for five minutes and placed in ice. The samples were vortexed again and then pipetted into 30µl aliquots until analysis by Western blotting (section 2.4.3).

Cellular membranes fraction to be analysed for membrane-bound proteins were incubated with 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) buffer (7M Urea, 2M thio Urea 2% (w/v) CHAPS, 65 mM DTT, d.d.H_20) and processed as above. As CHAPS buffer does not allow the use of a BCA protein assay for equalisation, a Biorad protein assay was performed instead.

Brain tissue from in vivo experiments (see section 2.3): Tissue samples from cortices were removed from the -80°C and the tissue was adjusted to

approximately 50mg. A 500 µl solution of PBS with 1% (v/v) protease and phosphotase inhibitors was added to each sample. The samples were mechanically homogenized with a polytron for 30 seconds in a 2ml flat-bottom microtube and centrifuged at 15,000 x rpm at 4°C for 30 minutes to pellet any cellular debris and non-soluble proteins. Aliquots of the resulting supernatant were equalized for protein content with a BCA protein assay and either used on the day of preparation for cytokine ELISA's or subsequently processed for Western immunoblotting as previously described. Cellular membranes pellet that were formed during centrification were used to analyse membrane-bound proteins by processing CHAPS buffer (7M Urea, 2M thio Urea 2% (w/v) CHAPS, 65 mM DTT, d.d.H_20) as above.

2.6.1 Protein quantification – BCA protein assay

The BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. The homogenized glial cells was quantified using this method. A working solution of 2000µg/ml of bovine serum albumin (BSA) was prepared by using the same lysis buffer that the samples were homogenized in as a diluent. A set of dilutions were prepared to give final protein concentrations as follows; 2000, 1500, 1000, 750, 500, 250, 125, 25, 0ug/ml BSA. The lysis buffer alone was used as a blank. Next 25µl of the dilutions and of the individual samples were pipetted in duplicate into the wells of a new 96 well plate. A BCA working solution was prepared by adding fifty parts of the BCA working reagent A to one part of B, to a sufficient volume. 200µl of this working solution was added to the wells containing the protein samples and the standard dilutions. The 96 well plate was covered and incubated at 37°C for 30 min. The plate was subsequently cooled to room temperature. The absorbance was obtained by reading the plate in a plate reader at 560nm.

The values obtained for the standards were used to plot a regression line. The concentration of protein in the samples was ascertained using this graph in a pg/ml concentration. The samples were equalized by adding the

required volume of lysis buffer as a diluent. They were subsequently processed for western blotting as in section 2.4.3

2.6.2 SDS-PAGE

Sodium dodecylsulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the equalized glial cell samples using a 'Biorad' SDS-PAGE rig as follows. Two glass plates were cleaned with 70% EtOH and a rubber gasket was placed around the round-bottomed plate. The two plates were arranged 1mm apart using two separators and they were held in place with a Biorad casting frame. 10% separating gels (2.425ml d.H20, 1.25 ml Bis/acrylamide bis, 1.25 ml Tris-HCL [pH 8.8], 50 µl SDS, 25 µl APS, 3 µl TEMED) were cast by gently pouring the solution between the two plates with a new Pasteur pipette. A temporary layer of isopropranol was placed on top of the gels to avoid evaporation. When the polyacrylamide gel solidifies, the isopropranol was aspirated off and the gel was washed twice with d.H_2O. A 4% stacking gel was prepared (1.525ml d.H20, 0.325 ml Bis/acrylamide bis, 0.625 ml Tris-HCL [pH 6.8], 25 µl SDS, 12.5 µl APS, 4 µl TEMED) and pipetted onto the separating gel. The well combs were carefully inserted into the gel and this was allowed to set for 30 min. The plates were arranged into the Biorad electrode assembly rig. The upper and lower reservoirs of the Biorad mini stand electrophoresis unit were filled with cold electrode running buffer (125mM Tris Base, 960mM glycine, 5% SDS (w/v)) and the well combs were carefully removed. The samples (20µl) were pipetted into the well along with 5µl of a broad–range molecular weight marker on the outside lanes only. A representative sample from each treatment group was added to each well of the gel. A 32mA current (Biometra-Standard power pack 25) was applied per individual gel for 40 min or before the protein bands ran off the gel.

2.6.3 Western immunoblot

The polyacryamide gels were removed from the gel rig and washed gently in transfer buffer (25mM Tris Base, 192mM Glycine, 20% MeOH (v/v), 0.05% SDS (w/v)). Pre-soaked (in transfer buffer) nitrocellulose paper and

filter paper were cut to the size of the gel and placed on a semi-dry blotter (Biometra), which was lightly moistened with transfer buffer. A 'sandwich' of filter paper, nitrocellulose, gel, and filter paper was placed on the graphite electrode (anode) of the semi-dry blotter. A Pasteur pipette was rolled over the 'sandwich' to remove any bubbles. The top electrode (cathode) was placed on the blotter and a current of 225mA was set for one and a half hours. The nitrocellulose paper was subsequently removed and placed in Tris-buffered saline (TBST-T; 20mM Tris-HCL, 150mM NaCL, containing 0.05 Tween-20 (v/v); pH: 7.6) as a wash step to remove the methanol of the transfer buffer. The membrane was the transferred to 10ml of blocking buffer consisting of TBS-T containing 5% (w/v) BSA, and blocked for two hours. The membrane was washed three times for 10 min each in TBS-T before being transferred to the primary antibody diluted in TBS-T with 1% (w/v) BSA. This was incubated overnight at 4°C before being washed again in 10ml of TBS-T for 3 x 10 min washes. The secondary antibody was incubated with the membrane at the required concentration, diluted in 1% BSA (w/v) TBS-T for 1 to 2 hours. The membrane was again washed in TBS-T, 3 x times, each for 10 min, and was subsequently exposed to 1ml of chemiluminescent solution. This was incubated for 5 min on the blot. The membrane was exposed to photographic film in a dark room and then developed and fixed using an automated developer. Following this step the membranes were immediately removed and washed 3 x times in TBS-T. 10mls of restore stripping solution was added to each blot for fifteen minutes. The blots were again washed for ten minutes 3 x times and re-blocked for two hours in 5% BSA (w/v) TBS-T at room temperature. The membranes were quickly washed and re-probed with a primary antibody overnight at 4°C and a secondary antibody for 2 hr at room temperature. Furthermore, the membranes were striped of there bound antibody as before and probed for β-actin at room temperature, diluted in 1% BSA (w/v) TBS-T for 1 to 2 hours. After the previously mentioned wash step the membrane was probed with secondary antibody, diluted in 1% BSA (w/v) TBS-T for 1 hour and exposed to photographic film as before. Densitometry was performed

on all blots obtained to semi-quantify the intensities of the bands and the values obtained were expressed as the optical densities.

1° Antibody	*band size & % gel*	*Dilution*	*Supplier*	*2° Antibody*	*Dilution*	*Supplier*
Mouse anti-**β-Actin** (monoclonal)	42 kDA / 10%	1/1000	Sigma	Goat-Anti mouse	1/3000	Sigma
Rabbit anti-**ERK**	42/44 kDA / 10%	1/1000	Cell Signalling	Donkey anti-rabbit	1/3000	Amersham
Rabbit anti-**P-ERK** (Thr202/204)	42/44 kDA / 10%	1/1000	Cell Signalling	Donkey anti-rabbit	1/3000	Amersham
Rabbit anti-**CREB**	44 kDA / 10%	1/1000	Cell Signalling	Donkey anti-rabbit	1/2500	Amersham
Rabbit anti-**P-CREB** (Ser133)	44 kDA / 10%	1/1000	Cell Signalling	Donkey anti-rabbit	1/2500	Amersham
Rabbit anti-**P-STAT3** (Tyr705)	86 kDA / 10%	1/1000	Cell Signalling	Donkey anti-rabbit	1/2000	Amersham

Table 2.1: Western immunoblot antibody specifications

2.7 Tissue preparation for real-time polymerase chain reaction (PCR)

2.7.1 RNA extraction

Purified RNA was extracted from cultured cells and CNS cortices and hippocampi using 350µl RA1 buffer per sample (total RNA isolation kit, Macherney-Nagel) containing 1% β-mercaptoethanol. The lysate from the treatment wells were pooled into an RNase-free microfuge tube and incubated for 5 minutes at room temperature to allow for complete dissociation of nucleo-protein complex, leading to the solubilisation of RNA and then disrupted using a polytron tissue disrupter (Kinetatica). Sample/cell homogenate were added to NucleoSpin® Filter units and

filtered by centrifugation at 13,000rpm for 1 minute. 350µl of 70% ethanol was added to each sample lysate and mixed by pipetting up and down approximately 5 times. Each sample mix was placed in NucleoSpin ® RNA II columns and centrifuged at 13,000rpm for 30 seconds to bind the RNA to the silica column. Following centrifugation the column was placed in a new collecting tube and 350µl of membrane desalting buffer (supplied) was added. The column was then centrifuged at 13,000rpm for 1 minute. DNA was digested using rDNase and DNase Reaction Buffer (supplied). rDNase was diluted 1:10 in DNase Reaction Buffer and 95µl of this solution was pipetted directly onto the centre of the silica column. Samples were incubated with DNase mix for 15 minutes at RT. 200µl buffer RA2 was added to the column and centrifuged at 13,000rpm for 30 seconds following which the column was placed in a new collecting tube. 600µl of RA3 buffer (50ml of ethanol added to 25ml of RA3 buffer concentrate) was added to each column and centrifuged at 13,000rpm for 30 seconds. The flow-through was discarded and the collecting tube re-used for the second RA3 wash. 250µl of RA3 buffer was added to each column and centrifuged at 13,000rpm for 2 minutes. Column was placed in a fresh RNAse-free microtube and RNA eluted by addition of 60µl of H_2O and centrifugation at 13,000rpm for 1 minute. Eluted RNA was then frozen and stored at -85 for qualification, quantification and reverse transcription.

2.7.2 RNA qualification & quantification

Assessment of RNA quality: RNA was separated on a 1% agarose gel to check integrity of extracted RNA samples. 2g of agarose was added to 130ml of TBE (1.0M Tris, 0.9M Boric acid, 0.01M EDTA) and fully dissolved by heating in microwave. The solution was allowed to cool such that container could be hand-held before 1.3µl of ethidium bromide was carefully added and swirled to mix. The agarose solution was poured into a sealed agarose gel tray containing a comb and allowed solidify for approximately 30 minutes. The tray was then transferred to gel tank (OWL model B2) and covered with 1X TBE running buffer. 3µl of RNA sample was mixed with 2µl DEPC H_2O and 1µl of loading dye and 4µl of this mixture loaded onto gel. RNA was separated using 90 volts for 30

minutes up to 1.5 hrs (or until yellow dye at front reached the end of the gel). The gel was then placed in a ultra violet (UV) transilluminator and the RNA visualized under UV light. Only extracted RNA that demonstrated visible 28S and 18S ribosomal RNA bands were used as this indicated that the RNA had not been degraded during the extraction process.

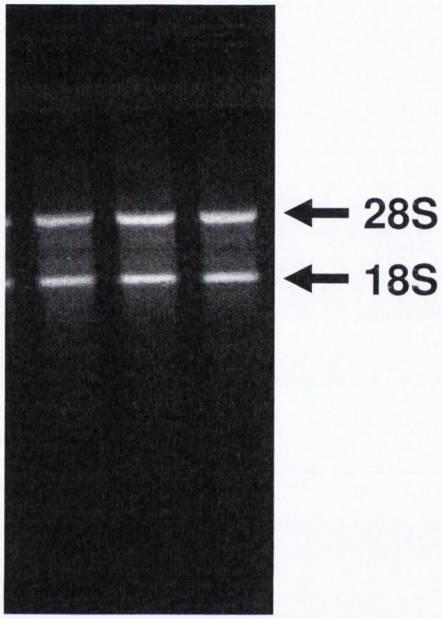


Figure 2.2: Representative picture of isolated cortical RNA

RNA quantification: The optical density (OD) of RNA was measured using a spectrophotometer (UV/vis Beckman Coulter Du730) to determine RNA concentration and purity. The concentration of RNA can be measured due to its ability to absorb light at 260nm. As an OD reading of 1.0 at 260nm is equivalent to an RNA concentration of 40µg/ml, sample RNA concentrations can be quantified using the following equation: RNA = OD_{260} X dilution factor X 40µg/ml. The purity of RNA may also be established by measuring absorbance at 280nm. A ratio of OD_{260}/OD_{280} of approx. 1.8-2.1 is indicative of pure RNA. All RNA samples used had ratios of >1.5. RNA concentrations were then equalised so that equal concentrations of RNA could be used as template for cDNA transcription. Samples were aliquoted in equal volumes until reverse- transcribed.

2.7.3 cDNA synthesis by Reverse Transcription

RNA samples were reverse transcribed into cDNA using a high capacity cDNA archive kit (Applied Biosystems) according to the manufactures

protocol. Briefly, 20-50µl of 0.5-2.5µg of RNA was mixed in a PCR mini-tube with an equal volume of 2X master mix that was made up as follows: 1:5 dilution of 10X Reverse Transcription Buffer, 1:12.5 dilution of 25X dNTPs, 1:5 dilution of Random Primers, 1:10 dilution of MultiScribe Reverse Transcriptase and 1:2.381 dilution of H_2O. Samples were then placed in thermal cycler and incubated at 25°C for 10 minutes followed by a 2 hour incubation at 37°C. Resultant cDNA was frozen at –20°C until ready for real time polymerase chain reaction (PCR) analysis.

2.7.4 Real-time PCR

Assesment of target genes was performed using Taqman gene expression assays containing specific target primers, and FAM-labelled MGB target probes (Applied biosystems). β-actin gene expression was used to normalize gene expression between samples, and was quantified using a β-actin endogenous control gene expression assay containing specific primers, and a VIC-labelled MGB probe for rat β-actin (order number 4352341E).

Amplification of a gene of interest and endogenous control (β-actin) was carried out for each cDNA sample. Briefly, cDNA was diluted 1:4 and 10µl of diluted cDNA was pipetted onto a PCR plate, to which 1.25µl of primer/probe, 1.25 of µl of β-actin/probe and 12.5µl of Taqman master mix was added. Samples were run in duplicate, and electronic pipettes (EDP3 20-200µl, 2-20µl and 10-100µl) were used to ensure pipetting accuracy. Samples were placed in the real-time PCR thermocycler (Applied Biosciences 7300) using the following programme; step 1: 95°C for 10 minutes, step 2: 95°C for15 seconds followed by 1minute at 60°C. Step two was repeated 40 times, and fluorescence read when the cycle was at 60°C for the duration of the programme.

During step two of the PCR reaction, the double stranded cDNA is denatured at 95°C for 15seconds. As the temperature begins to fall to 60°C (annealing and extension) the target probe is first to anneal to the single-stranded cDNA as it has a higher melting temperature than the target primers (Applied Biosystems). This probe contains a FAM/VIC dye

and a proprietary non-fluorescent quencher (NFQ) dye, this quencher prevents the dye from emitting a fluorescent signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems). At 60°C the primers anneal and the strand is extended by 5' nuclease activity of the Taq polymerase. This displaces the FAM/VIC-labeled probe causing the FRET between the dye and quencher to be broken, and the generation of a fluorescent signal. Due to the specificity of the probe and primers for the cDNA sequence, one fluorescent signal is generated for each new cDNA copy and measured during the annealing stage of the PCR cycle (60°C).

Target gene	Assay number
IL-1β	Rn00580432_m1
IL-1ra	Rn00573488_m1
IL-1R(I)	Rn00565482_m1
IL-IR(II)	Rn00588589_m1
IL-R(Acp)	Rn00492642_m1
IL-6	Rn00561420_m1
IL-10	Rn00563409_m1
TNF-α	Rn99999017_m1
SOCS3	Rn00585674_s1
Bcl-2	Rn99999125_m1
Bax	Rn02532082_g1
Caspase-3	Rn00563902_m1
IκBα	Rn01473658_g1
GFAP	Rn00566603_m1
Cd11b	Rn00709342_m1

Table 2.2: List of Taqman gene expression assays (Applied Biosystems)

2.7.5 Real-time PCR analysis

The ΔΔCT method (Applied Biosystems RQ software, Applied Biosystems, UK) was used to assess gene expression for all real-time PCR analysis (Livak & Schmittgen, 2001). This method is used to assess relative gene

expression by comparing gene expression of treated/experimental samples to a normal or untreated sample (control), rather than quantifying the exact copy number of the target gene. In this manner the fold-difference (increase or decrease) can be assessed between treated and control samples. The fold-difference is assessed using the cycle number (CT) difference between samples. Briefly, a threshold for fluorescence is set, against which CT is measured. To accurately assess differences between gene expression the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Thus, samples with low CT readings demonstrate high fluorescence, indicating greater amplification and hence, greater gene expression. When a PCR is 100% efficient a one-cycle difference between samples means a 2-fold difference in copy number (2^2), similarly a 5-cycle difference is a 32-fold difference (2^5). This method however assumes a linear response of experimental samples.

To measure this fold-difference relative to control, the CT of the endogenous control (β-actin) is subtracted from the CT of the target gene for each sample, thus accounting for any difference in cDNA quantity that may exist. This normalised CT value is called the (ΔCT). The CT difference (ΔCT) of the control is subtracted from itself to give 0, and subtracted from all other samples, this is the ΔΔCT value. The ΔΔCT (cycle difference corrected for β-actin) is then converted into a fold-difference. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, 2 to the power of the -ΔΔCT (difference in control and sample CT corrected for actin) gives the fold-difference in gene expression between the control and treated samples. The control sample always has a ΔΔCT value of 0, thus 0^{-2} gives a $2^{-\Delta\Delta CT}$ of 1, against which all other samples are referenced.

2.8 Statistical analysis

All data was analysed using the GB-STAT statistical software package (Dynamic Microsystems). Statistical comparisons were initially performed using a one or two-way analysis of variance (ANOVA) or student t-test with pooled variance as indicated in the experimental sections. If significant

changes were observed, the data was further analysed using Student Newman-Keuls *post hoc* test as appropriate. Data was deemed significant when $P<0.05$. Results are expressed as means and standard error of the mean (SEM).

3. Results

3.1 Modulation of the IL-1 ligands and receptors by NA *in vitro*

The following studies were carried out to asses to ability of NA to alter expression of the IL-1 ligands and receptors from both primary cortical mixed glial and neuronal cell cultures. DMEM:F12 Media vehicle or NA (5μM) was administered for 6 hours prior to the analysis of gene expression by real-time PCR. Vehicle or NA were also administered to cortical mixed glial cultures for 48 hours and the cell-free supernatants harvested for the assessment of cytokine production by ELISA.

Glial IL-1 ligand mRNA expression: NA (5μM) significantly induced IL-1ra ($P<0.05$) mRNA expression without altering the expression of IL-1β in primary cortical mixed glia cultures (figure 3.1.1a).

Glial IL-1 receptor mRNA expression: NA (5μM) significantly induced mRNA expression of IL-1R(II) ($P<0.01$) in primary mixed glia cultures. NA (5μM) failed to alter the expression of IL-1R(I) or IL-1R(acp) (figure 3.1.2.a).

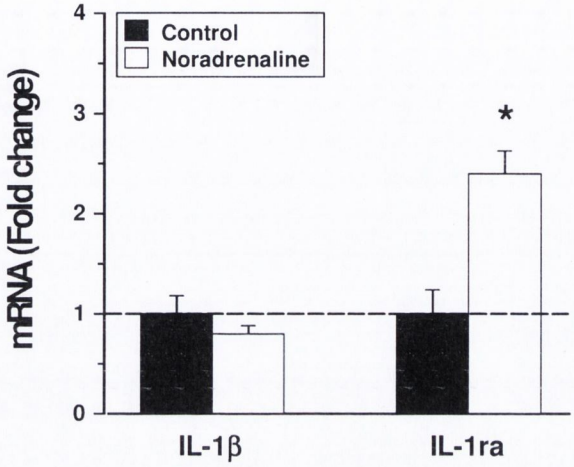
Neuronal IL-1 ligand mRNA expression: NA (5μM) treatment did not significantly alter the mRNA expression of either IL-1β or IL-1ra in primary cortical neuronal cultures (figure 3.1.1b).

Neuronal IL-1 receptor mRNA expression: NA (5μM) significantly induced mRNA expression of IL-1R(II) ($P<0.01$) in primary cortical neuronal cultures. NA (5μM) failed to alter the expression of IL-1R(I) or IL-1R(acp) (figure 3.1.2b).

Glial IL-1 and IL-1R(II) protein expression: NA significantly induced a dose-dependent increase in IL-1ra (1μM & 5μM: $P<0.05$, 10μM: $P<0.01$) production, independent of any increase in the production of IL-1β in primary cortical mixed glia cultures (figure 3.1.3a). NA (5μM) treatment did not induce a statistically significant increase in membrane-bound IL-1R(II) expression

after a 24 hour treatment. However, densitometric quantification of these western immunoblot bands was compromised by the poor quality and specificity of the commercially available antibody (figure 3.1.3b).

(a) Mixed glia



(b) Neurons

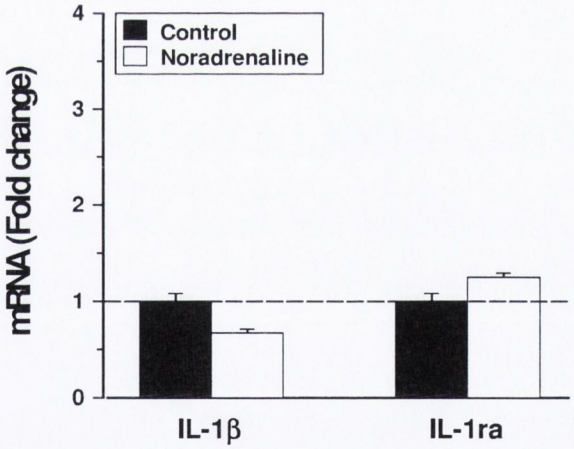
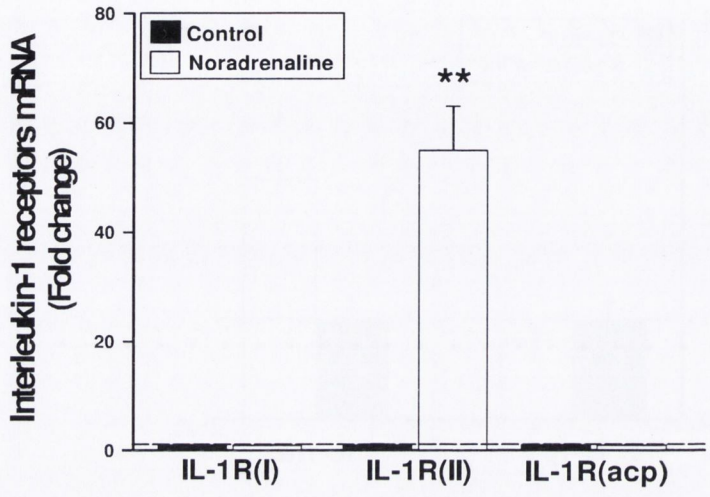


Figure 3.1.1: NA induced IL-1ra mRNA expression in primary cortical mixed glial cell cultures without altering IL-1β expression

NA (5μM) induces a significant increase in IL-1ra mRNA expression in primary mixed glial cells (a) after a 6 hour incubation and failed to alter expression of IL-1β (a). Neuronal IL-1ra or IL-1β mRNA expression was not altered by incubation with Noradrenaline (5μM) (b). Data are expressed as means + SEM (n=3-6). *P<0.05 vs. media control (Student t-test).

(a) Mixed glia



(b) Neurons

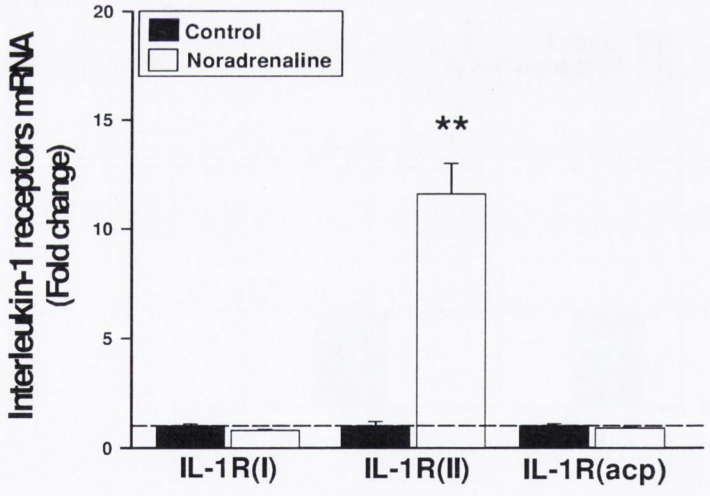


Figure 3.1.2: NA induced IL-1R(II) mRNA expression in primary cortical mixed glial and neuronal cell cultures

NA (5μM) significantly increased IL-1R(II) mRNA expression in primary cortical mixed glial (a) ($P<0.01$) and neuronal (b) ($P<0.01$) cultures after a 6 hour incubation. Treatment with NA (5μM) failed to alter IL-1R(I) or IL-1R(acp) expression in either cell type (a & b). Data are expressed as means + SEM (n=3-6). **$P<0.01$ vs. media control (Student t-test).

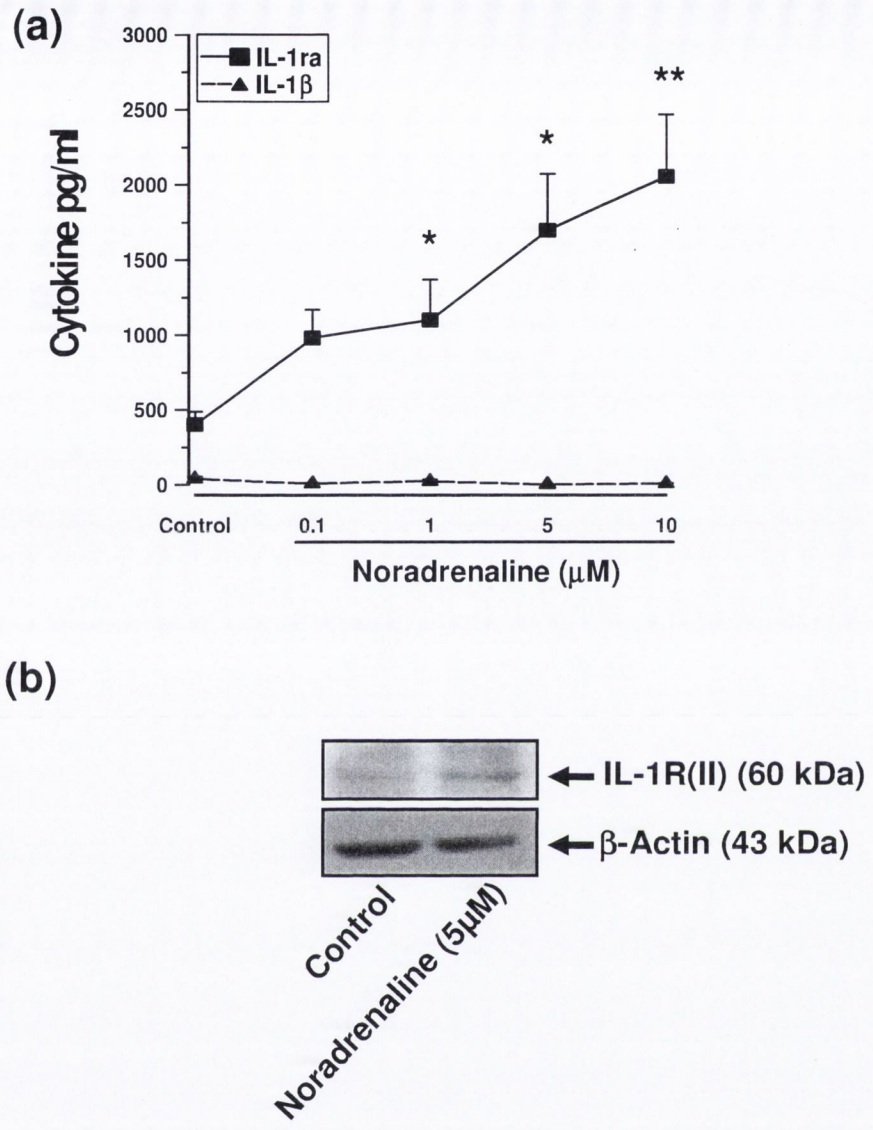


Figure 3.1.3: NA induced protein expression of IL-1ra and IL-1R(II) in primary cortical mixed glial cell cultures

NA induces a dose-dependent increase in IL-1ra protein production from primary mixed glial cells after a 48 hour incubation [One-way ANOVA: $F(5,36)=4.77$, $P< 0.01$] (a). This induction appears to be independent of IL-1β as NA failed to induce IL-1β protein production (a). NA (5μM) treatment also induced an increase in membrane-bound IL-1R(II) expression after a 24 hour treatment (b) (representative of 3 independent immunoblots-densitometry did not show significant treatment differences). Data expressed as means + SEM (n=7), *P<0.05, **P < 0.01, vs. media control (Newman-Keuls test).

3.2 Microglia mediate the ability of NA to induce IL-1ra and IL-1R(II) from cortical mixed glial cells *in vitro*

As the mixed preparation of primary cells used in these experiments best represents the glia cell ratio *in vivo*, it was proposed that this would be the optimum *in vitro* system to assay the basal expression of inflammatory cytokines and receptors, induced by noradrenaline. However, previous literature has identified microglia as being the predominant producer of IL-1 ligands and the sole producer of IL-1ra in the CNS (Judy *et al.*, 1998, Pinteaux *et al.*, 2006). To clarify the source of IL-1 ligands and receptors induced from mixed glia by NA, primary microglia and astrocytes were isolated in culture and assessed for their ability to express both IL-1ra and IL-1R(II) mRNA expression. DMEM:F12 media vehicle or NA (5μM) was administered for 6 hours prior to the analysis of gene expression by reverse transcriptase PCR.

NA induced IL-1ra and IL-1R(II) mRNA expression from enriched microglial cultures but not from astrocytes: NA (5μM) significantly induced mRNA expression of IL-1ra (a: $P<0.05$) and IL-1R(II) (c: $P<0.05$) in enriched microglia cell cultures following a 6 hour treatment. In contrast, NA (5μM) failed to alter the mRNA expression of IL-1ra and IL-1R(II) in astrocytes cultures (b & d) (figure 3.2).

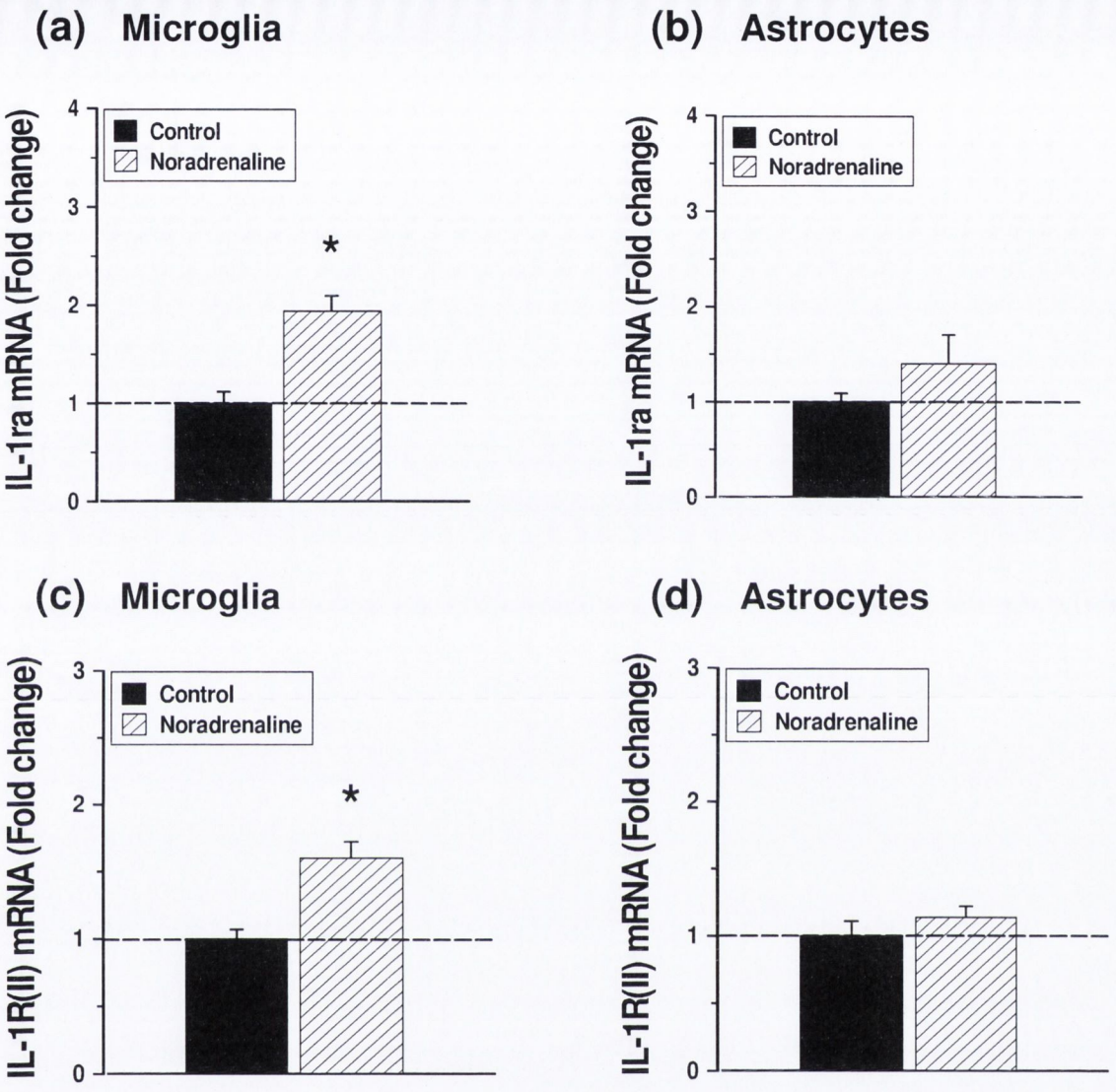


Figure 3.2: Noradrenaline increases IL-1ra and IL-1R(II) mRNA expression in primary microglia cells but not in Astrocytes

NA (5μM) increased IL-1ra (a) ($P<0.05$) and IL-1R(II) (c) ($P<0.05$) mRNA expression in primary microglial cells without significantly altering IL-1ra or IL-1R(II) mRNA expression in astrocytes (b & c). Data expressed as means ± SEM (n=6 mRNA). *$P < 0.05$ vs. media control (student-t test).

3.3 β-AR mediates the ability NA to induce IL-1ra and IL-1R(II) from cortical mixed glial cells *in vitro*: A critical role for PKA and ERK

Previous literature has identified the β_2-AR in mediating the anti-inflammatory properties of NA *in vitro* (Feinstein *et al.,* 1998, Feinstein *et al.*, 2002, Frohman *et al.*, 1988, Galea *et al.*, 2003). The following studies were carried out to determine whether β_2-AR activation and subsequent elevations in intracellular cAMP are responsible for the ability of NA to negatively regulate the Interleukin-1 system, by increasing glial expression of IL-1ra and IL-1R(II) *in vitro*.

Blockade of the β-AR inhibits NA-induced IL-1ra expression: To determine whether α- or β-AR activation was responsible for the induction of IL-1ra by NA, primary mixed glial cells were pre-treated with a non-selective α-AR antagonist phentolamine (5μM), or a non-selective β-AR antagonist propranolol (5μM), 30 minutes prior to a 6 hour treatment with NA (5μM). NA (5μM) induced a significant increase in IL-1ra ($P<0.01$) (figure 3.3.1a) mRNA expression and protein production ($P<0.01$) (figure 3.3.1b), and this was not attenuated by phentolamine pre-treatment (Figure 3.3.1a & b). In contrast, pre-treatment with propranolol (5μM) significantly attenuated NA (5μM)-induced IL-1ra mRNA expression ($P<0.01$) (figure 3.3.1c) and protein production ($P<0.01$) (figure 3.3.1d)

Blockade of the β-AR inhibits NA-induced IL-1R(II) expression: NA (5μM) induced a significant increase in IL-1R(II) mRNA expression ($P<0.01$) and this was not attenuated by a 30 minute pre-treatment with phentolamine (Figure 3.3.2a). In contrast, a 30 minute pre-treatment with propranolol (5μM) significantly attenuated NA-induced IL-1R(II) mRNA expression ($P<0.01$) (figure 3.3.2b).

The selective β_2-AR agonist, salbutamol mimics the ability of NA to induce IL-1ra and IL-1R(II) expression: The selective β_2-adrenoceptor agonist,

salbutamol (5μM), induces mRNA expression of IL-1ra ($P<0.01$) (figure 3.3.3a) and IL-1R(II) ($P<0.01$) (figure 3.3.3b).

The cAMP analogue, db-cAMP, mimics the ability of NA to induce IL-1ra and IL-1R(II) expression: The selective cAMP analogue, db-cAMP (100μM), induces gene expression of IL-1ra ($P<0.01$) (figure 3.3.4a) and IL-1R(II) ($P<0.01$) (figure 3.3.4b).

The selective PKA antagonist, KT5720, attenuates NA-induced IL-1ra and IL-1R(II) expression: NA (5μM) significantly induced both gene expression ($P<0.05$) (figure 3.3.5a) and protein production of IL-1ra ($P<0.05$) (figure 3.3.5b), and gene expression of IL-1R(II) ($P<0.01$) (figure 3.3.5c). These increases were attenuated by a 30 minute pre-treatment with the selective PKA antagonist, KT5720 (1μM) (a & 6: $P<0.05$, c: $P<0.01$).

NA increases ERK phosphorylation via the β-adrenoceptor: A 30 minute pre-treatment with the non-selective β–adrenoceptor antagonist, Propranolol (5μM), attenuated the NA-induced increase in ERK 1/2 phosphorylation, 15 minutes post-treatment as measuresd by western immunoblot ($P<0.05$) (figure 3.2.6).

The selective Mek 1/2 antagonist, U0126, attenuates NA-induced IL-1ra and IL-1R(II) gene expression: NA (5μM) significantly induced mRNA expression of IL-1ra ($P<0.01$) (figure 3.3.7a) and IL-1R(II) ($P<0.01$) (figure 3.3.7b). These increases were attenuated by a 20 minute pre-treatment with the selective Mek 1/2 antagonist, U0126 (10μM) (a: $P<0.05$, b: $P<0.01$). However, it is worth noting that the NA-induced induction of IL-1R(II) is still apparent in the propranolol treated group.

The selective Mek 1/2 antagonist, U0126, attenuates β_2-adrenoceptor-induced IL-1ra production: Both NA (5μM) and salbutamol (5μM) significantly induced protein production of IL-1ra ($P<0.01$). A 20 minute pre-treatment with

the selective Mek 1/2 antagonist, U0126 (10μM), significantly attenuated NA and Salbutamol-induced IL-1ra production ($P<0.01$) (figure 3.3.8).

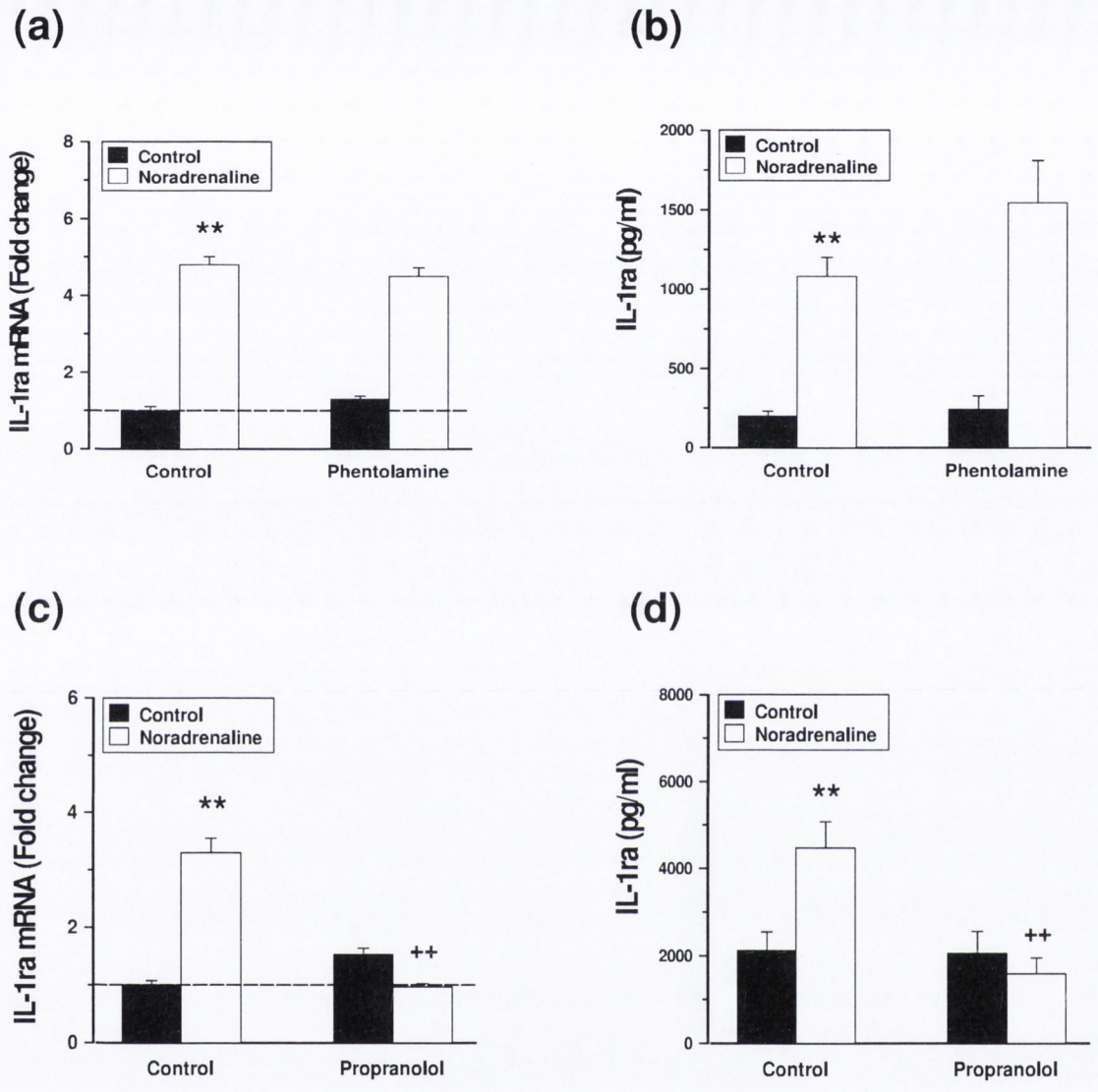
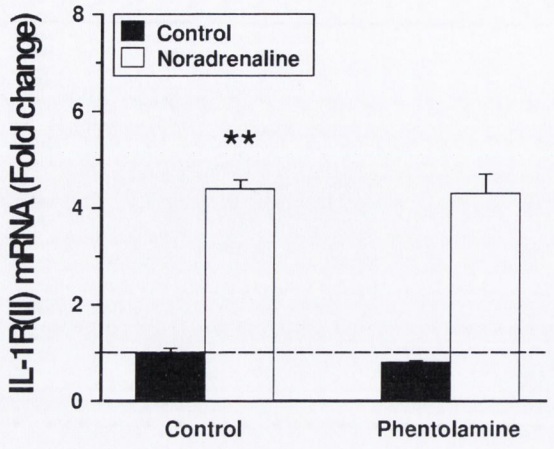


Figure 3.3.1: Blockade of the glial β-AR inhibits NA-induced IL-1ra expression

NA (5μM) induced a significant increase in IL-1ra gene expression (a & c) ($P<0.01$) and protein production (b & d) ($P<0.01$) from primary mixed glial cells. Phentolamine pre-treatment failed to inhibit NA-induced IL-1ra gene expression (a) or protein production (b). Two-way ANOVA demonstrated a significant interaction between propranolol (5μM) pre-treatment and NA-induced IL-1ra gene expression (c) [$F(3,16)=39.05$, $P<0.0001$] and protein production (d) [$F(3,28)=6.82$, $P<0.001$] Data expressed as means + SEM (n=6 mRNA, n=8 protein), **$P < 0.01$, vs. media control, ++$P<0.01$ vs. NA (5μM) (Newman-Keuls test).

(a)



(b)

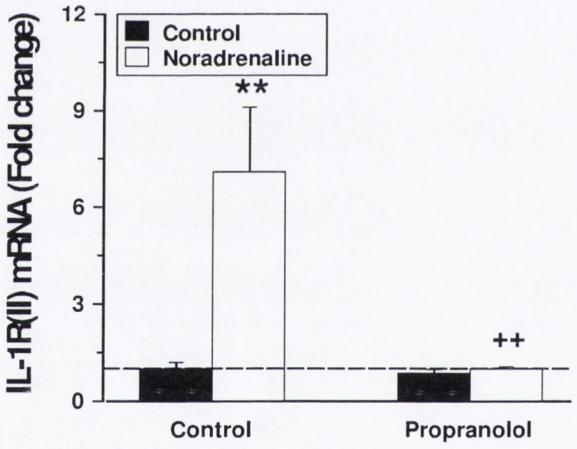
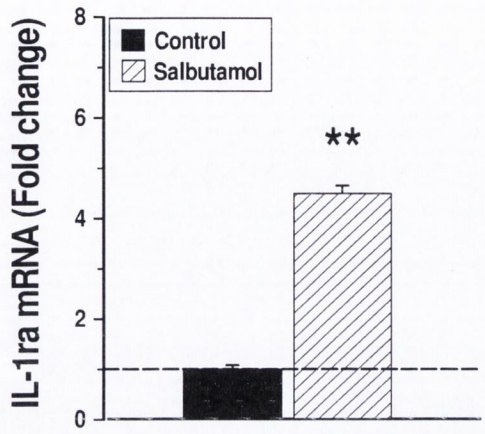


Figure 3.3.2: Blockade of the glial β-AR inhibits NA-induced IL-1R(II) expression

NA induced a significant increase in IL-1R(II) gene expression in primary mixed glial cells after a 6 hour incubation. Phentolamine pre-treatment failed to inhibit NA-induced IL-1R(II) production (a). Two-way ANOVA demonstrated a significant interaction between NA-induced IL-1R(II) mRNA expression and pre-treatment with propranolol (5μM) [$F(3,20)=7.15$, $P< 0.001$] (b). Data expressed as means + SEM (n=6), **$P < 0.01$, vs. media control, ++$P<0.01$ vs. Noradrenaline (5μM) (Newman-Keuls test).

(a)



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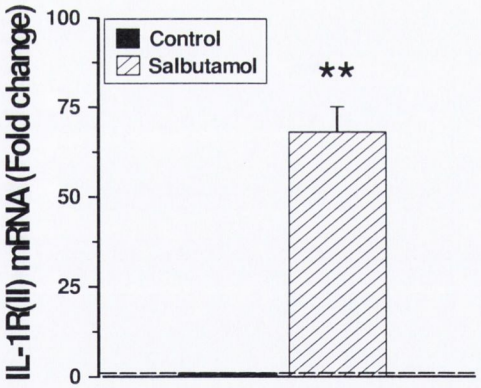
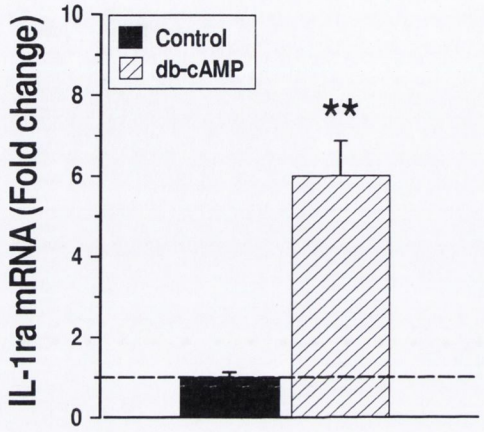


Figure 3.3.3: The selective β_2-AR agonist, Salbutamol, mimics the ability of NA to induced glial IL-1ra and IL-1R(II) expression

Salbutamol (5μM) induced a significant increase in IL-1ra gene expression (a) ($P<0.01$) and IL-1R(II) (b) ($P<0.01$) from primary mixed glial cells. Data is expressed as means + SEM (n=6). **$P<0.01$ vs. media control (Student t-test).

(a)



(b)

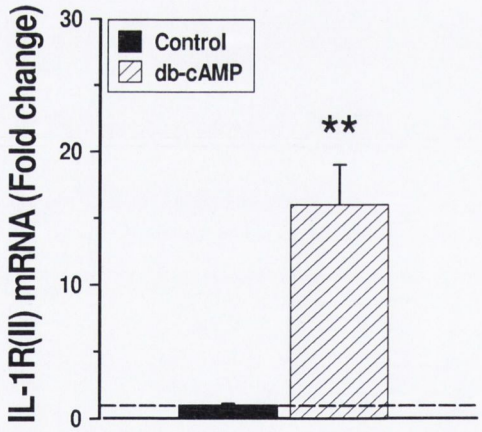


Figure 3.3.4: The selective cAMP analogue, db-cAMP mimics the ability of NA to induced glial IL-1ra and IL-1R(II) expression

db-cAMP (100μM) induced a significant increase in IL-1ra (a) ($P<0.01$) and IL-1R(II) (b) ($P<0.01$) gene expression from primary mixed glial cells. Data is expressed as means + SEM (n=6). **$P<0.01$ vs. media control (Student t-test).

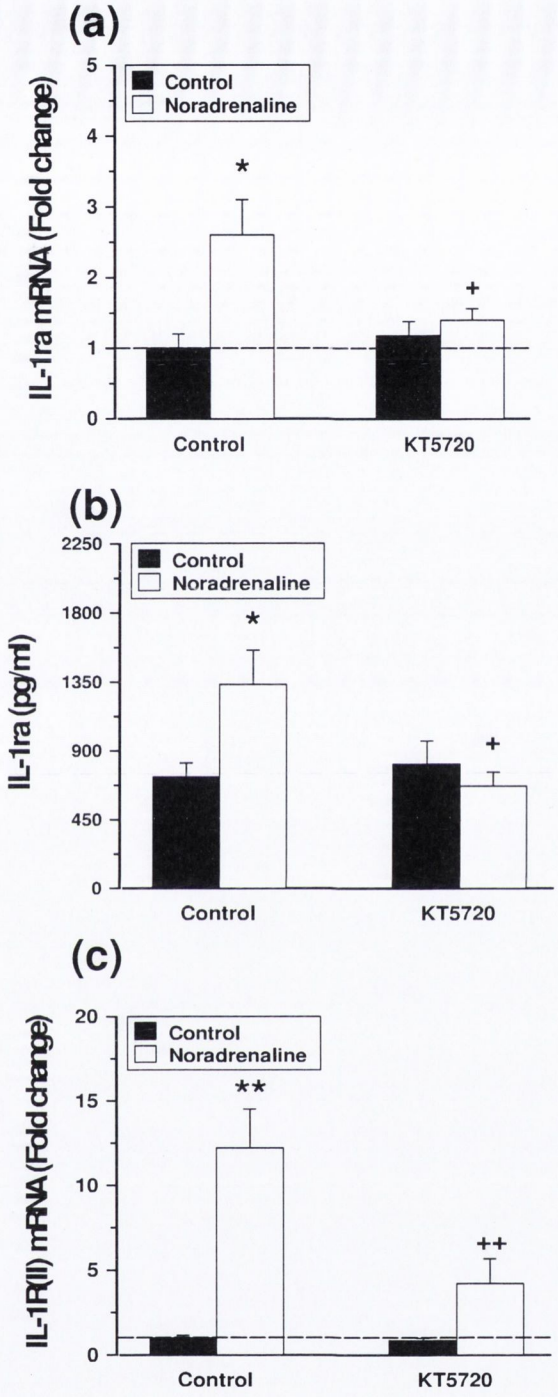


Figure 3.3.5: The selective PKA antagonist, KT5720, attenuates NA-induced IL-1ra and IL-1R(II) expression

NA induced a significant increase in both IL-1ra gene expression (a) ($P<0.05$) protein production (b) ($P<0.05$), and IL-1R(II) gene expression (c) ($P<0.01$) from primary mixed glial cells. Two-way ANOVA demonstrated a significant interaction between KT5720 (1μM) pre-treatment and NA-induced IL-1ra gene expression (a) [$F(3,19)=6.77$, $P=0.0027$] and protein production (c) [$F(3,18)=4.69$, $P<0.014$], and IL-1R(II) gene expression (c) [$F(3,16)=21.73$, $P<0.0001$]. Data expressed as means + SEM (n=3-6), *$P<0.01$, **$P < 0.01$, vs. media control, ++$P<0.01$ vs. NA (5μM) (Newman-Keuls test).

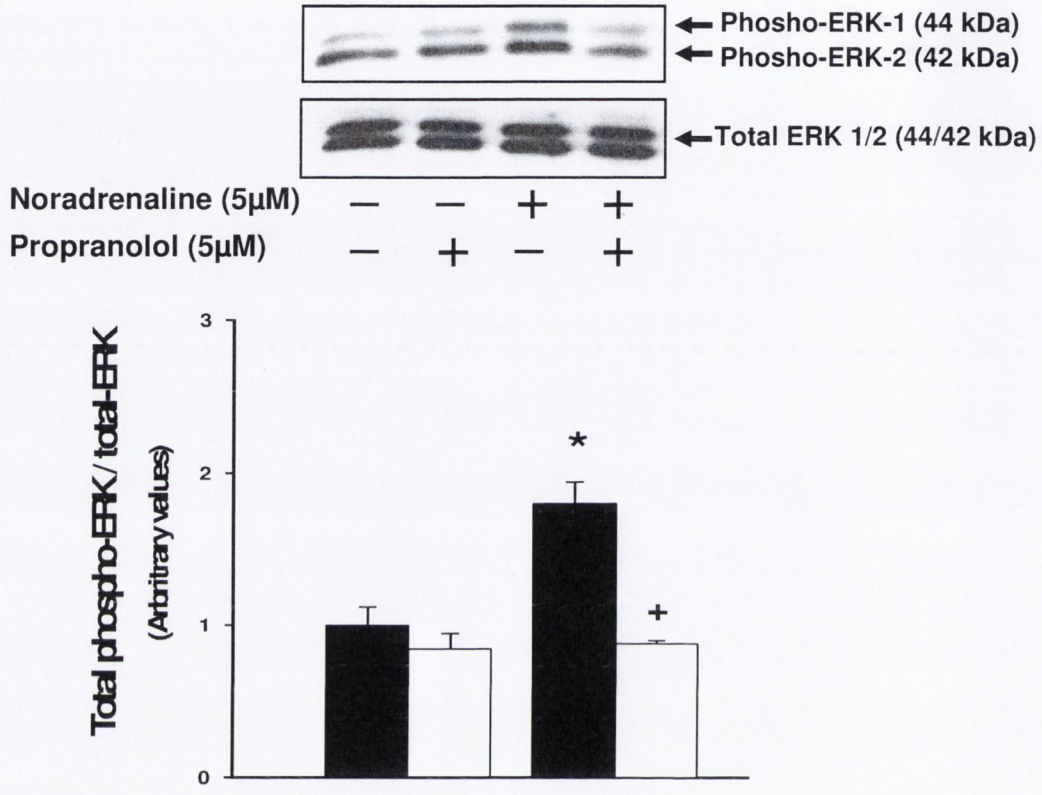
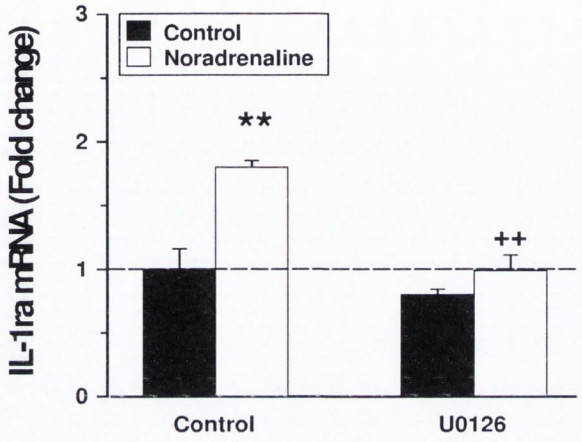


Figure 3.3.6: NA increases total-ERK phosphorylation via the β-AR

A 30 minute pre-treatment with the non-selective β–AR antagonist, Propranolol (5μM), attenuates the NA-induced increase in ERK 1/2 phosphorylation, 15 minutes post-treatment as measuresd by western immunoblot. Two-way ANOVA demonstrated a significant interaction between propranolol (5μM) pre-treatment and NA-induced P-ERK [$F(3,12)=6.12$, $P<0.005$] Data expressed as means + SEM (n=3). *P< 0.05 vs. media control, +P<0.05 vs. NA group (Newmans-keuls test).

(a)



(b)

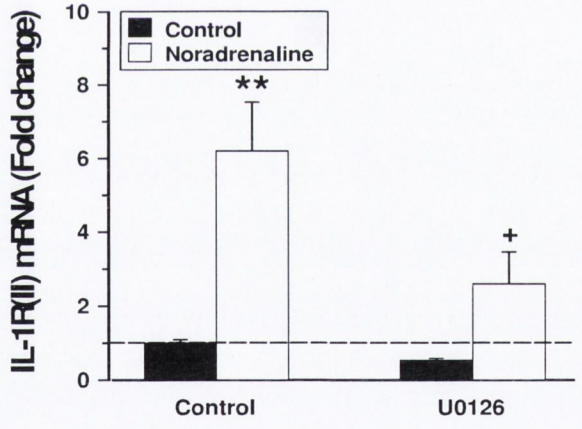


Figure 3.3.7: The selective Mek 1/2 antagonist, U0126, attenuates NA-induced IL-1ra and IL-1R(II) gene expression

NA-induced a significant increase in IL-1ra (a) ($P<0.01$) and IL-1R(II) gene expression (b) ($P<0.01$) from primary mixed glial cells. Two-way ANOVA demonstrated a significant interaction between U0126 (μM) pre-treatment and NA-induced IL-1ra (a) [$F(3,9)=21.27$, $P<0.0002$] and IL-1R(II) gene expression (b) [$F(3,7)=10.46$, $P<0.005$]. Data expressed as means + SEM (n=4), **$P < 0.01$, vs. media control, +$P<0.05$, ++$P<0.01$ vs. NA (5μM) (Newman-Keuls test).

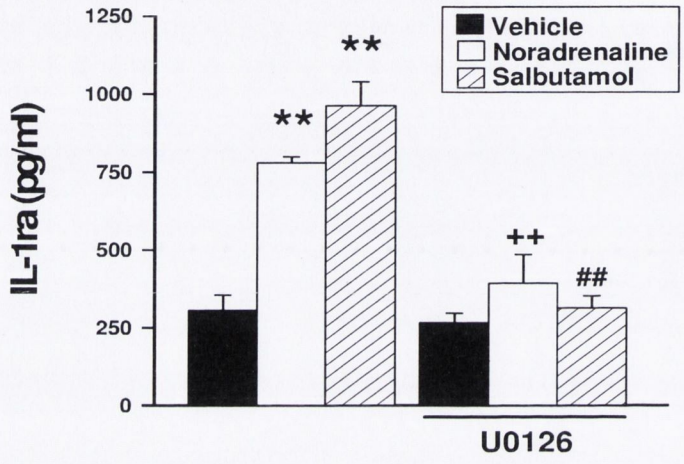


Figure 3.3.8: The selective Mek 1/2 antagonist, U0126, attenuates β2-AR-induced IL-1ra production

Both NA (5μM) and salbutamol (5μM) significantly induced protein production of IL-1ra ($P<0.01$) after a 48 hour treatment from primary mixed glial cells. A 20 minute pre-treatment with the selective Mek 1/2 antagonist, U0126 (10μM), significantly ($P<0.01$) attenuated both NA and Salbutamol-induced IL-1ra production. Two-way ANOVA demonstrated significant interaction between U0126 pre-treatment and both NA and Salbutamol ($P<0.01$). [$F(5,24)=26.37$, $P<0.0001$]. Data expressed as means + SEM (n=6), **$P < 0.01$, vs. media control, ++$P<0.01$ vs. NA (5μM), ## $P<0.01$ vs. salbutamol (5μM) (Newman-Keuls test).

3.4 NA inhibits IL-1β-induced neurotoxicity

NA inhibits IL-1β-induced neurotoxicity: Pilot studies were carried out using a dose-response of IL-1β (0.1-10 ng/ml) with time points of 24, 48 and 72 hours on primary cortical cultures of mixed glia and neurons to identify the optimal concentration of IL-1β. These studies revealed that concentrations of IL-1β up to 10ng/ml, had no direct toxic affect on primary neuronal cultures after a 48 hour treatment.

In the following study, primary cortical mixed glial cells were treated for 48 hours with either a media vehicle or NA (5μM) followed by a 24 hour treatment with recombinant IL-1β (5ng/ml). This glial-conditioned media (CM) was added to primary cortical neuronal cells for 48 hours. As a control, primary neurons cells were also treated with IL-1β (5ng/ml). IL-1β (5ng/ml) had no significant effect on neuronal viability (as measured by LDH release at a 48 hour time point). However, IL-1β (5ng/ml)-treated CM significantly induced neuronal cell death at a 48 hour time-point. This was attenuated by a 48 hour pre-treatment with NA (5μM) ($P<0.01$) (figure 3.4).

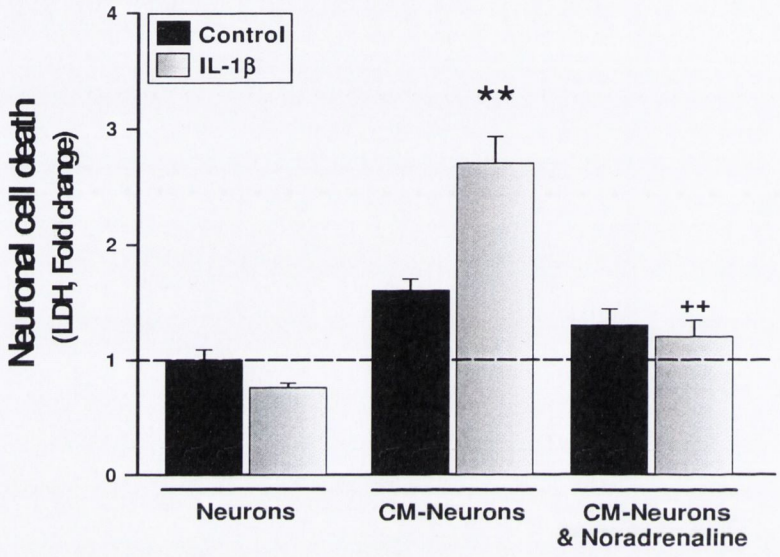


Figure 3.4: NA inhibits IL-1β-induced neurotoxicity

IL-1β (5ng/ml) had no significant direct affect on neuronal toxicity (as measured by LDH release). However, IL-1β (5ng/ml)-conditioned glial media (CM) significantly induced neuronal cell death at a 48 hour time-point (figure 3.3.1a). This was attenuated by pre-treatment with NA (5μM) ($P<0.01$).Two-way ANOVA showed a significant treatment interaction between IL-1β (5ng/ml)-CM-treated neurons and NA pre-treatment [$F(3,9)=14.21765$, $P<0.0009$)]. Data expressed as means + SEM (n=4). **$P<0.01$ vs. Neuronal media control, ++$P<0.01$ vs. IL-1β CM-treated neurons (Newmans-Keuls test)

3.5 Pharmacological enhancement of central NA tone *in vivo*; impact on the central interleukin-1 system

The previous studies have demonstrated that NA may act as an endogenous anti-inflammatory agent in the CNS, inducing an anti-inflammatory phenotype in the IL-1 system of glial cells *in vitro*. The aim of the following studies was to asses the impact of pharmacological intervention that increases the tone of noradrenaline in the CNS at regulating the interleukin-1 system in the rat CNS *in vivo*. To increase synaptic availability of NA, 4 hours prior to sacrifice, a treatment regime of acute intra-peritoneal (I.P.) drug delivery with the NA re-uptake inhibitor (NRI) reboxetine (15mg/kg), and the α2-adrenoceptor antagonist, idazoxan (1mg/kg) was used. This animal model has previously been shown to maximise the synaptic availability of NA in vivo, as measured by microdialysis (Sacchetti *et al.*, 1999).

Acute administration of the NRI, reboxetine and the α_2-adrenoceptor antagonist, idazoxan, modulate the gene expression of CNS interleukin-1 ligands in vivo: A 4 hour acute I.P. treatment with idazoxan (1mg/kg) failed to induce significant changes in any of the Interleukin-1 ligands (a-d). However, acute treatment with reboxetine (15mg/kg) induced a significant increase in IL-1β in both the cortex (a) ($P<0.05$) and the hippocampus (b) ($P<0.05$) as well as hippocampus Il-1ra (d) ($P<0.01$). Co-treatment with reboxetine and idazoxan induced a significant increase in both cortical (a) ($P<0.01$) and hippocampal (b) ($P<0.01$) IL-1β gene expression and cortical (a) ($P<0.01$) and hippocampal (b) ($P<0.01$) IL-1ra gene expression (figure 3.5.1).

Acute administration of the NRI, reboxetine and the α_2-adrenoceptor antagonist, idazoxan, modulate the gene expression of CNS interleukin-1 receptors in vivo: A 4 hour acute I.P. treatment with either reboxetine (15mg/kg) or idazoxan (1mg/kg) failed to induce significant changes in any of the Interleukin-1 receptors (a-f). Co-treatment with reboxetine and idazoxan

induced a significant increase in IL-1R(II) gene expression in both cortex (c) ($P<0.01$) and hippocampus (d) ($P<0.01$) (figure 3.5.2).

Acute treatment with reboxetine and idazoxan induce cortical CREB phosphorylation: A 4 hour treatment regime with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced an increase in β-adrenoceptor activation as measured by CREB phosphorylation which is indicative of increased cAMP signalling ($P<0.05$) (figure 3.5.3).

Acute treatment with reboxetine and idazoxan induced cortical GFAP mRNA expression: A 4 hour acute I.P. treatment with either reboxetine (15mg/kg) or idazoxan (1mg/kg) failed to induce significant changes either GFAP or cd11b mRNA expression. In contrast, a 4 hour co-treatment regime with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced a modest increase in cortical GFAP mRNA expression (figure 3.5.4).

The non-selective β-AR antagonist, propranolol, attenuates combined reboxetine and Idazoxan-induced changes in the CNS interleukin-1 system in vivo: A 4 hour acute drug treatment with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced significant increases in cortical IL-1β (a: $P<0.05$) and cortical (e: $P<0.01$) and hippocampal (f: $P<0.01$) IL-1R(II) mRNA expression. This induction was attenuated by a 30 minute pre-treatment with the β-AR antagonist, propranolol (10mg/kg). The combined treatment of reboxetine or idazoxan failed to induce a significant difference in the expression of either cortical or hippocampal IL-1ra (c & d) (figure 3.5.5).

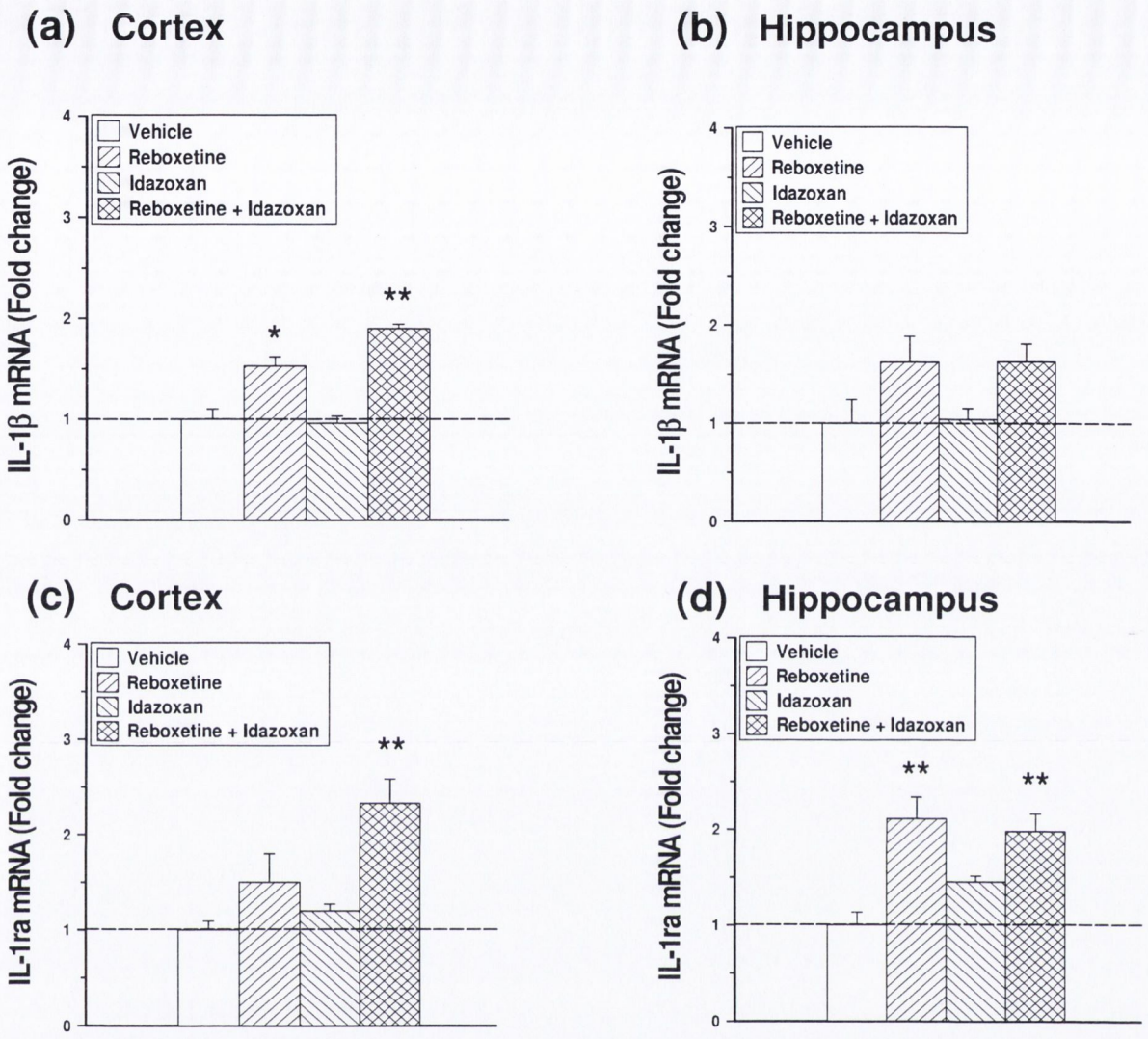


Figure 3.5.1: Modulation of CNS IL-1β and IL-1ra gene expression by acute treatment with reboxetine and idazoxan *in vivo*

A 4 hour acute treatment with idazoxan (1mg/kg) failed to induce significant changes in any of the Interleukin-1 ligands (a-d). In contrast, acute treatment with reboxetine (15mg/kg) induced a significant increase in cortical IL-1β (a; $P<0.05$) as well as hippocampal IL-1ra (d; $P<0.01$) mRNA expression. Co-treatment with reboxetine and idazoxan induced a significant increase in cortical [a; ONE-way ANOVA: $F(3,20)=25.09$, $P<0.0001$)] IL-1β and cortical [c; One-way ANOVA: $F(3,19)=3.97$, $P<0.023$)] and hippocampal [d;One-way ANOVA; $F(3,16)=7.72$, $P<0.002$)] IL-1ra gene expression. Data expressed as means + SEM (n=6). *$P<0.05$, **$P<0.01$ vs. Saline vehicle (Newmans-Keuls test).

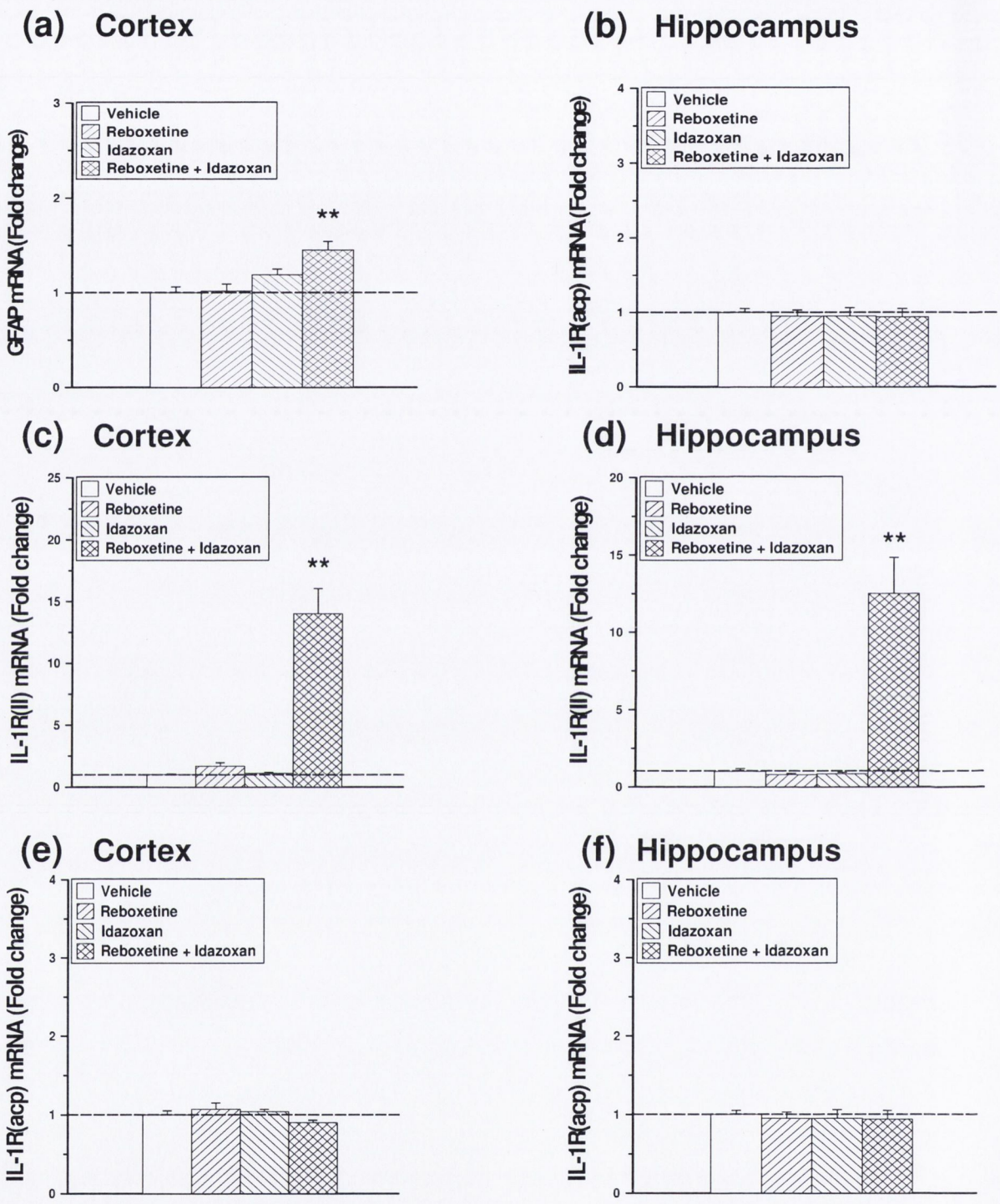


Figure 3.5.2: Induction of CNS IL-1R(II) gene expression by acute treatment wit reboxetine and idazoxan *in vivo*

A 4 hour acute I.P. treatment with either reboxetine (15mg/kg) or idazoxan (1mg/kg) failed to induc significant changes in any of the Interleukin-1 receptors (a-f). Co-treatment with reboxetine and idazoxa induced a significant increase in IL-1R(II) gene expression in both cortex [c; One-way ANOV $F(3,20)=24.52$, $P<0.0001$) and hippocampus [d; One-way ANOVA: $F(3,19)=18.32$, $P<0.001$)]. Da expressed as means + SEM (n=6). **P<0.01 vs. saline vehicle (Newmans-Keuls test).

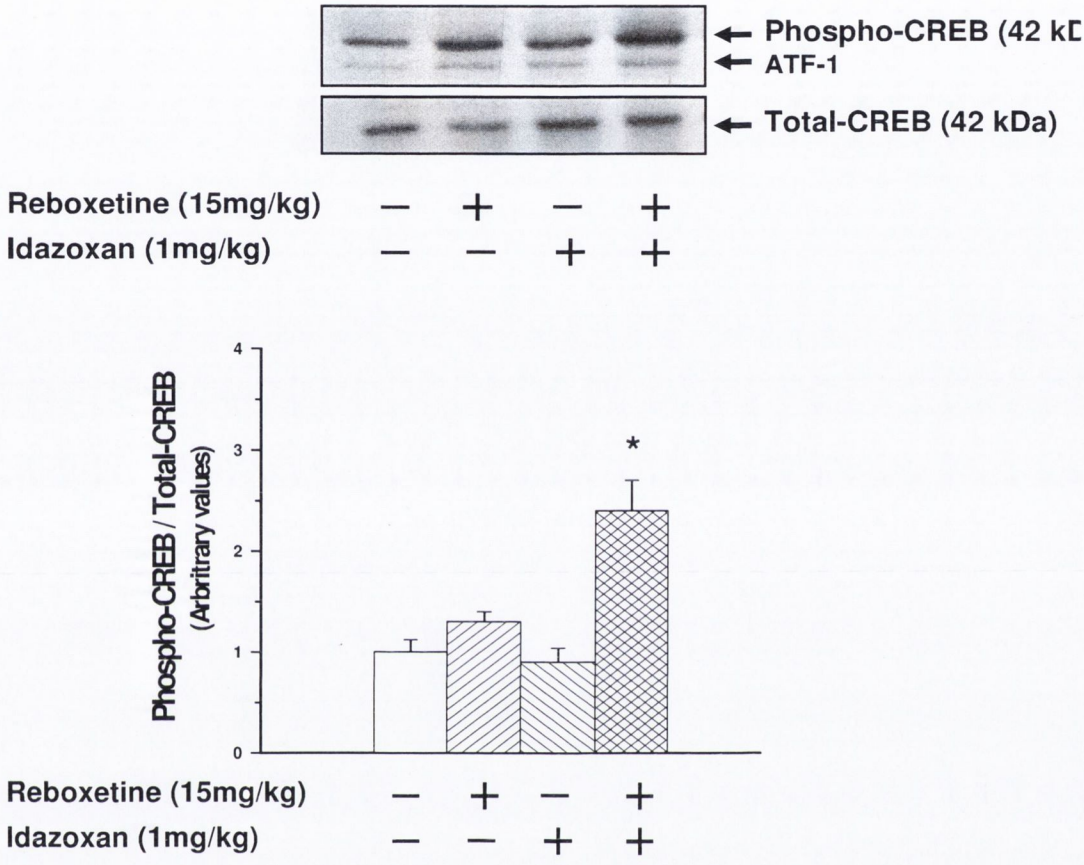


Figure 3.5.3: Acute treatment with reboxetine and idazoxan induce cortical CREB phosphorylation

While treatment with reboxetine or idazoxan alone failed to induce an induction of CREB phosphorylation, a 4 hour treatment regime with reboxetine (15mg/kg) and idazoxan (1mg/kg) in combination, induced a significant increase in β-adrenoceptor activation as measured by CREB phosphorylation [One-way ANOVA: $F(3,28)=6.82$, $P<0.01$]. ATF-1 (activating transcription factor-1 is a CREB-like transcription factor which is also immunodetected with this cell signalling (Ser133) antibody. Data expressed as means + SEM (n=4). *$P<0.05$ vs. saline vehicle (Newmans-keuls test).

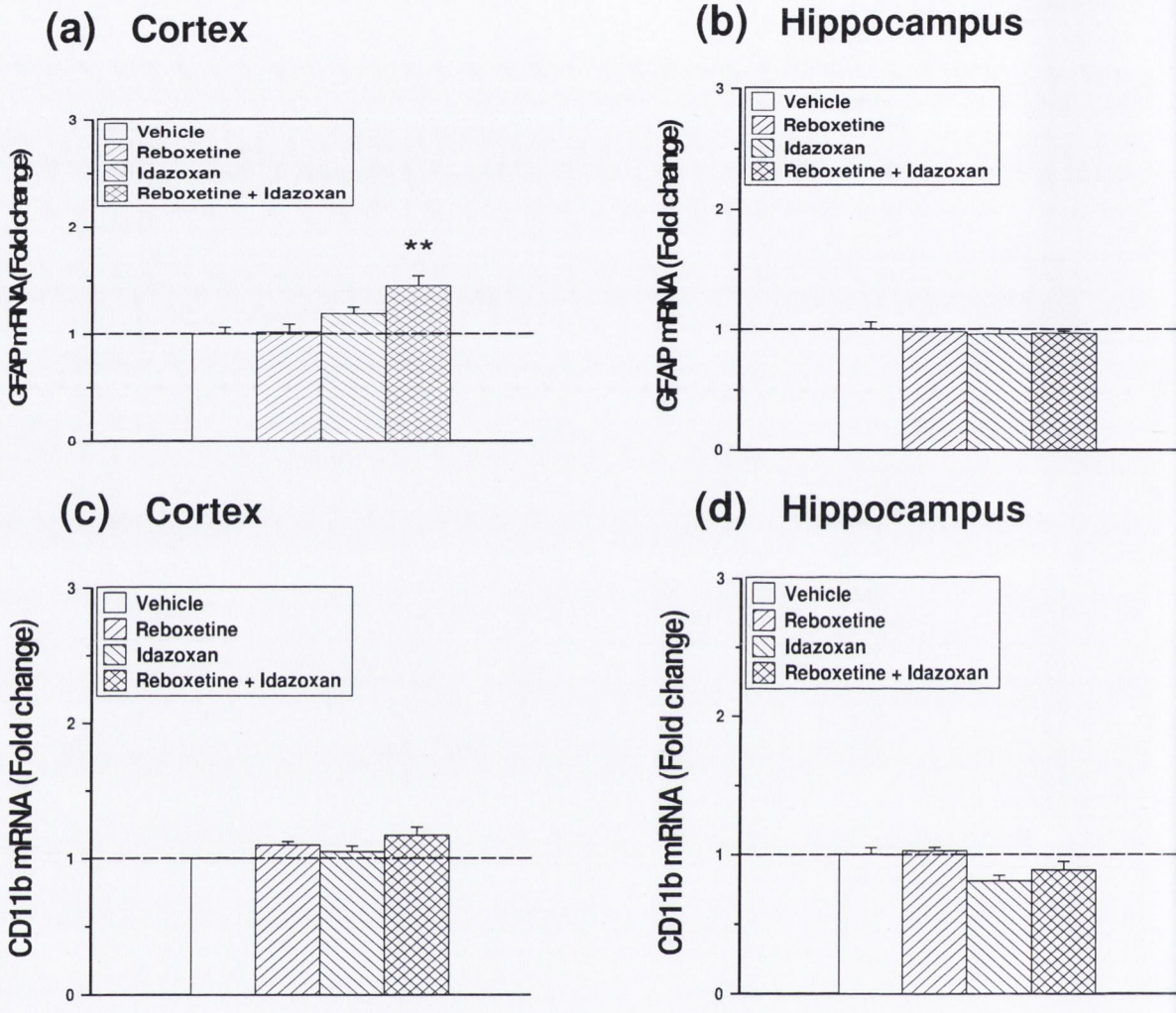


Figure 3.5.4: Acute treatment with reboxetine and idazoxan induced cortical GFAP mRNA expression

A 4 hour acute I.P. treatment with idazoxan (1mg/kg) failed to induce significant changes in either GFAP or cd11b mRNA expression (a-d). In contrast co-treatment with reboxetine (15 mg/kg) and idazoxan induced a significant increase in cortical [a; ONE-way ANOVA: $F(3,20)=6.075$, $P<0.0041$)] GFAP gene expression. Data expressed as means + SEM (n=6). *$P<0.05$ vs. Saline vehicle (Newmans-Keuls test).

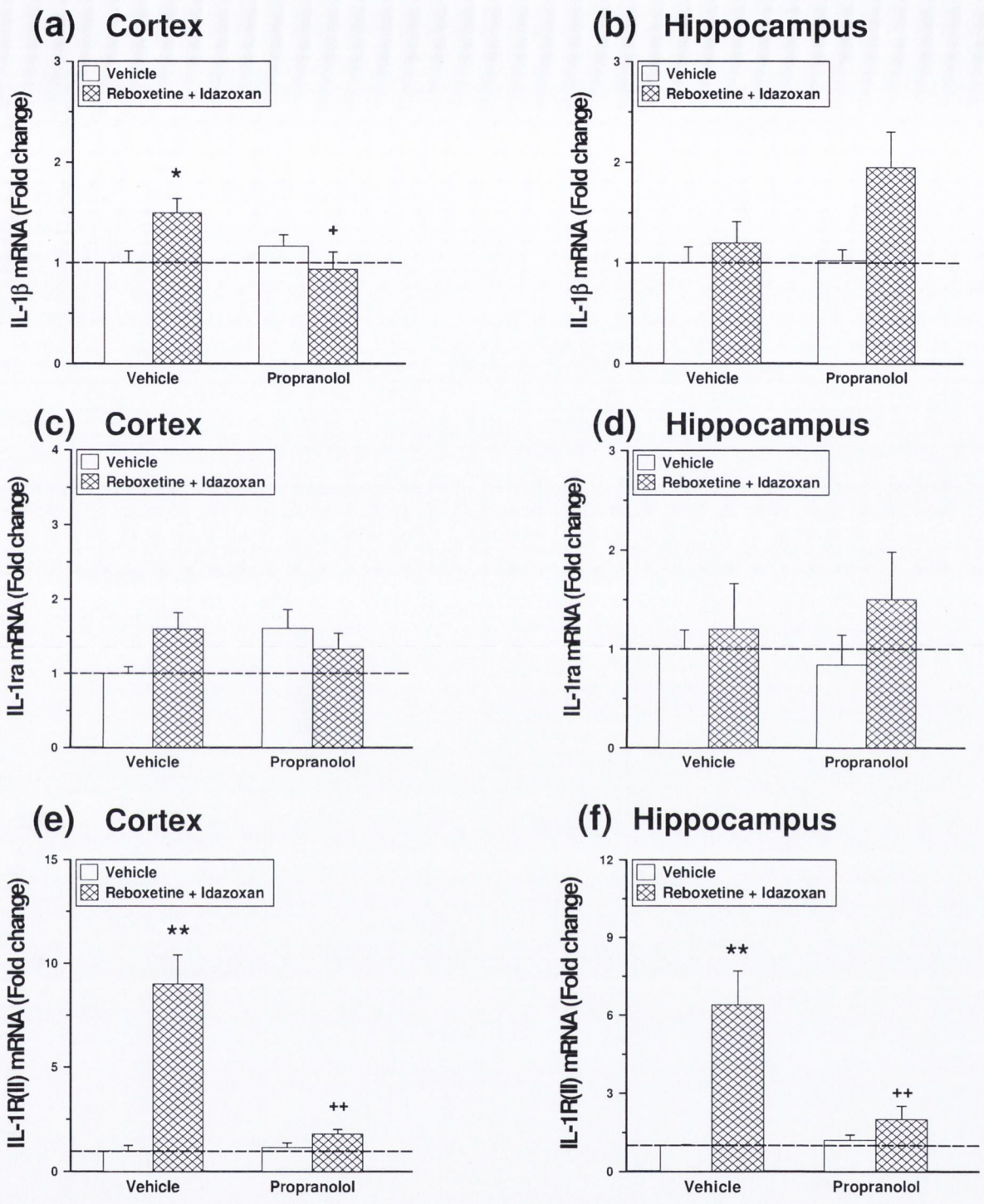


Figure 3.5.5: β-AR mediates the ability of combined reboxetine and idazoxan treatment to modulate the interleukin-1 system *in vivo*

A 4 hour acute drug treatment with reboxetine and idazoxan induced significant increases in cortical IL-1β (a) ($P<0.05$) and cortical (e) ($P<0.01$) and hippocampal (f) ($P<0.01$) IL-1R(II) mRNA expression. Two-way ANOVA showed a significant effect of treatment on cortical [a; $F(2,18)=5.87$, $P<0.005$)] IL-1β as well as cortical [e; $F(3,20)=24.52$, $P<0.0001$)] and hippocampal [f; $F(3,14)=42.89$, $P<0.0001$)] IL-1R(II) mRNA expression and a 30 minute propranolol (10mg/kg) pre-treatment. Data expressed as means + SEM (n=6). *$P<0.05$, **$P<0.01$ vs. saline vehicle, +$P<0.05$, ++$P<0.01$ vs. reboxetine & idazoxan (Newmans-Keuls test).

3.6 β-AR mediates the ability of NA to modulate the central rat interleukin-1 system *in vivo*

The selective β_2-AR agonist, clenbuterol, modulates the CNS gene expression of IL-1β and IL-1ra in vivo: Acute I.P. treatment with the selective β_2-AR agonist, clenbuterol (0.5 mg/kg) significantly induces cortical (a: $P<0.01$, 1 hour) and hippocampal (b: $P<0.05$, 1 & 4 hours) IL-1β mRNA expression and cortical (c: $P<0.01$, 1 & 4 hours, $P<0.05$, 8 hour) and hippocampal (d: $P<0.01$, 4 hour, $P<0.05$, 8 hour) IL-1ra expression (figure 3.6.1).

The selective β_2-AR agonist, clenbuterol, failed to modulate the CNS gene expression of IL-1R(I) and IL-1R(acp) in vivo: Acute I.P. treatment with the selective β_2-AR agonist, clenbuterol (0.5 mg/kg), did not alter the basal mRNA expression of IL-1R(I) or IL-1R(acp) in the rat cortex (a & b) or hippocampus (c & d) (figure 3.6.2).

The selective β_2-AR agonist, clenbuterol, modulates the CNS gene expression and protein production of IL-1R(II) in vivo: Acute I.P. treatment with the selective β_2-AR agonist, clenbuterol (0.5 mg/kg), significantly induces mRNA expression of IL-1R(II) in the rat cortex (a) ($P<0.01$) and hippocampus (b) ($P<0.01$). Clenbuterol (0.5 mg/kg) treatment also induced an increase in cortical membrane-bound IL-1R(II) expression after an 8 hour treatment (c) (figure 3.6.3).

The selective β_2-AR agonist, clenbuterol, induces cortical CREB phosphorylation: Clenbuterol (0.5 mg/kg) induced an increase in β-AR activation as measured by CREB phosphorylation at the 4 hour time-point which is indicative of β_2-adrenoceptor activation and increased cAMP signalling ($P<0.05$) (figure 3.6.4).

The selective β_2-AR agonist, clenbuterol, induces GFAP and cd11b mRNA expression in vivo: Acute I.P. treatment with the selective β_2-AR agonist,

clenbuterol (0.5 mg/kg), induced basal mRNA expression of GFAP and cd11b in the rat hippocampus (figure 3.6.5).

The β_2-AR agonist clenbuterol failed to induce gene expression for markers of apoptosis and inflammation in the rat hippocampus: Acute I.P. treatment regime with clenbuterol (0.5 mg/kg) failed to induce mRNA expression for caspase-3, Bcl-2 or Bax, which are all activated during apoptosis. Clenbuterol also failed to induce gene expression of IκBα, which is a negative regulator of the NFκB pathway, induced by IL-1β signaling (table 3.6.1).

The non-selective β-AR antagonist, propranolol, attenuates clenbuterol–induced changes in the CNS interleukin-1 system in vivo: A 4 hour acute I.P. treatment regime with the selective β_2-AR agonist, clenbuterol (0.5mg/kg), significantly induces mRNA expression of IL-1β (a & b; $P<0.01$), IL-1ra (c & d; $P<0.01$) and IL-1R(II) (e & f; $P<0.01$) in the rat cortex and hippocampus. This induction is attenuated by a 30 minute pre-treatment with the β-AR antagonist, propranolol (10mg/kg) (a-f; $P<0.01$) (figure 3.6.6).

The non-selective β-AR antagonist, propranolol, attenuates clenbuterol–induced increase in CREB phosphorylation the rat cortex: A 4 hour acute treatment with clenbuterol (0.5 mg/kg) induced an increase in phosphorylation of CREB in the rat cortex. This induction was attenuated by a 30 minute pre-treatment with the non-selective β-AR, propranolol (10mg/kg) (figure 3.6.7)

The selective β_2-AR agonist, fomoterol, mimics the affects of clenbuterol in modulating the CNS interleukin-1 system in vivo: A 4 hour acute I.P. treatment regime with the selective β_2-AR agonist, fomoterol (0.5mg/kg), significantly induces mRNA expression of IL-1β (a & b) ($P<0.01$), IL-1ra (c & d) ($P<0.01$) and IL-1R(II) (e & f) ($P<0.01$) in the rat cortex and hippocampus (figure 3.6.8).

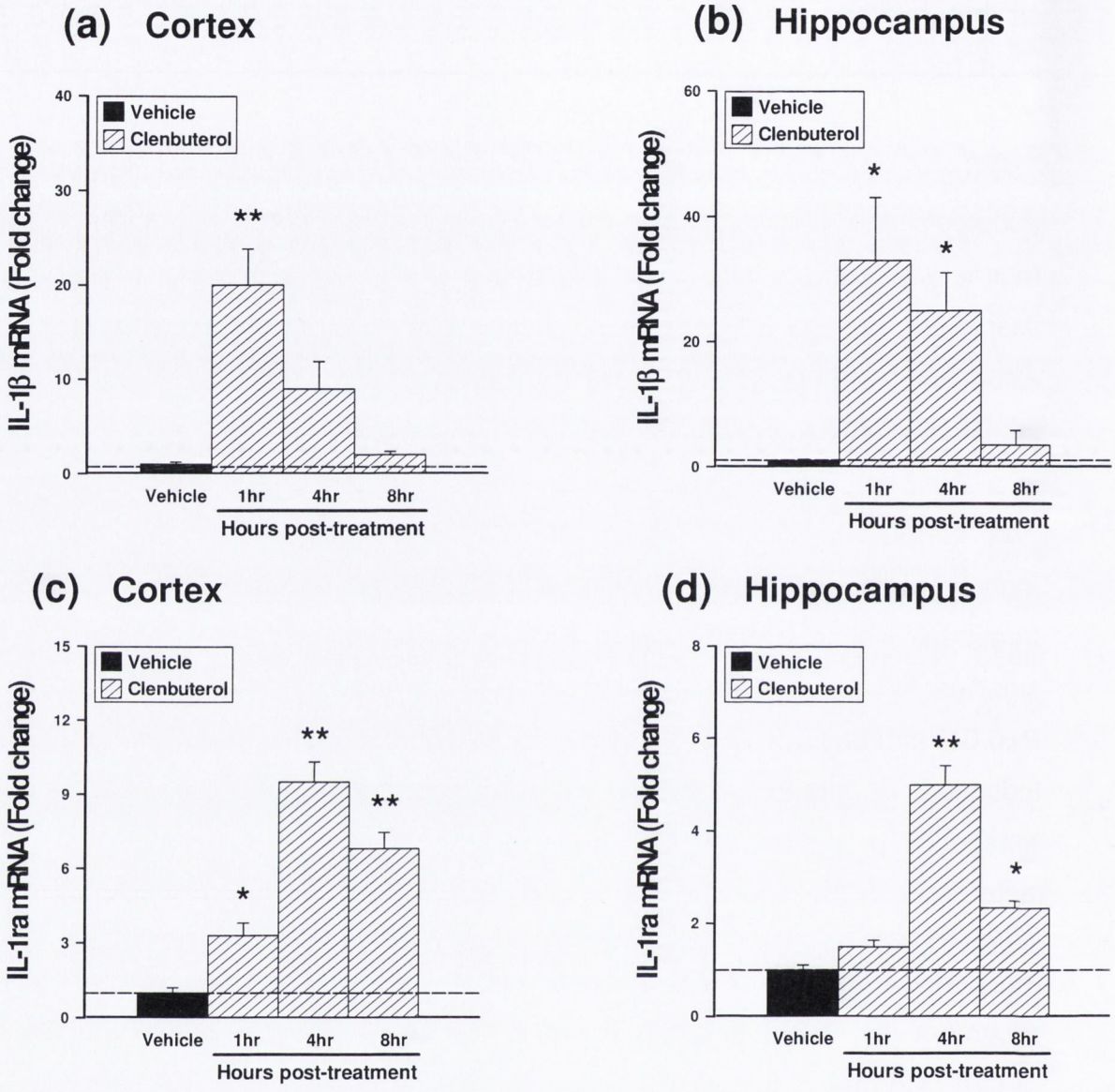


Figure 3.6.1: The selective β_2-AR agonist, clenbuterol, modulates the CNS gene expression of IL-1β and IL-1ra *in vivo*

Acute I.P. treatment with the selective β_2-AR agonist, clenbuterol, significantly induces mRNA expression of cortical [a: One-way ANOVA; $F(3,18)=10.75$, $P<0.0003$] and hippocampal [b: One-way ANOVA;$F(3,11)=8.26$, $P<0.003$] IL-1β and cortical [c: One-way ANOVA; $F(3,19)=27.45$, $P<0.0001$] and hippocampal [d: One-way ANOVA; $F(3,19)=41.74$, $P<0.0001$] IL-1ra. Data expressed as means + SEM (n=6). *P<0.05, **P<0.01 vs. saline vehicle (Newmans-Keuls test).

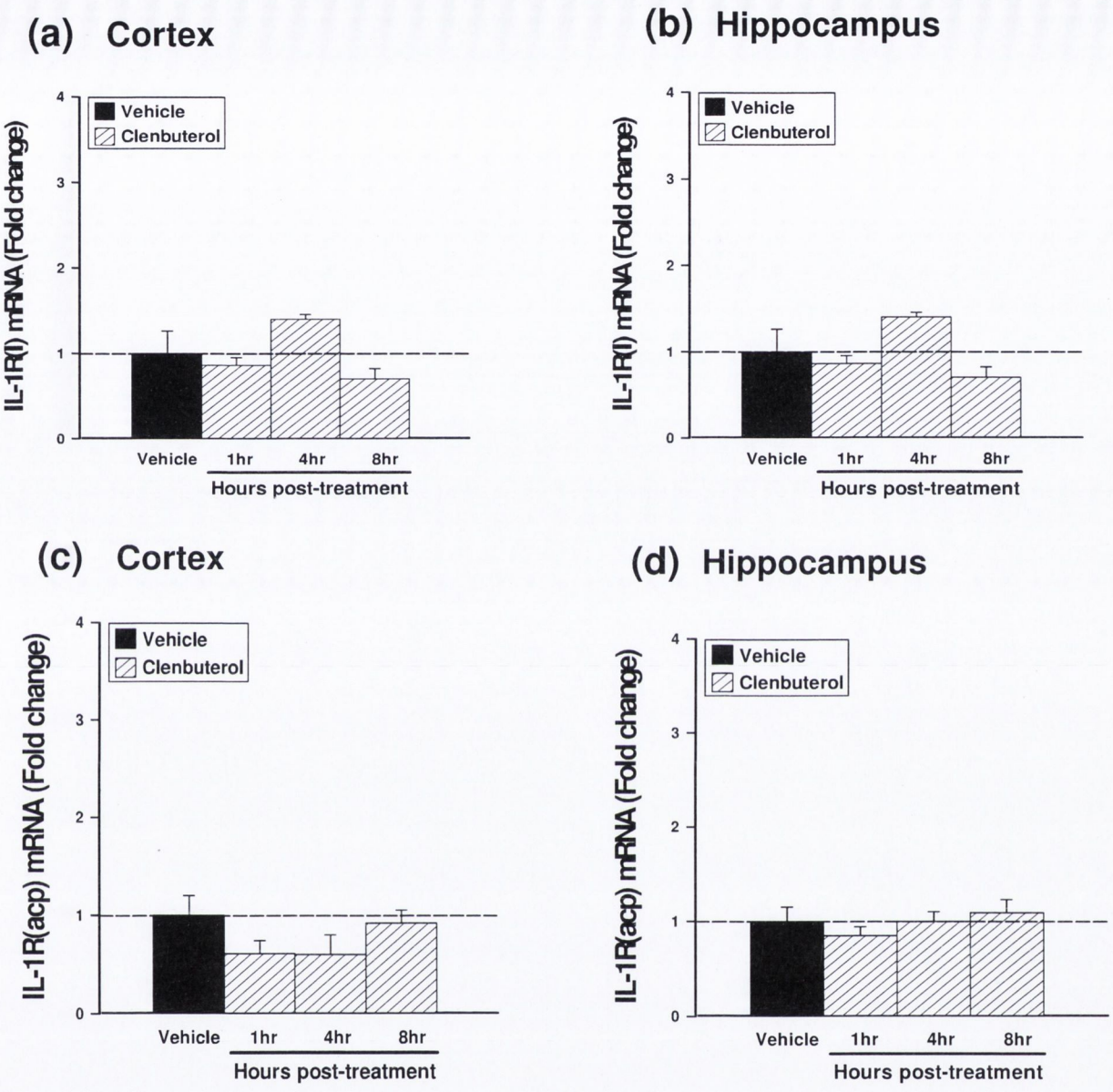


Figure 3.6.2: The selective β_2-AR agonist, clenbuterol, failed to modulate the CNS gene expression of IL-1R(I) and IL-1R(acp) *in vivo*

Acute I.P. treatment with the selective β_2-AR agonist, clenbuterol (0.5 mg/kg), did not alter the basal mRNA expression of IL-1R(I) or IL-1R(acp) in the rat cortex (a & b) or hippocampus (c & d). Data expressed as means + SEM (n=6).

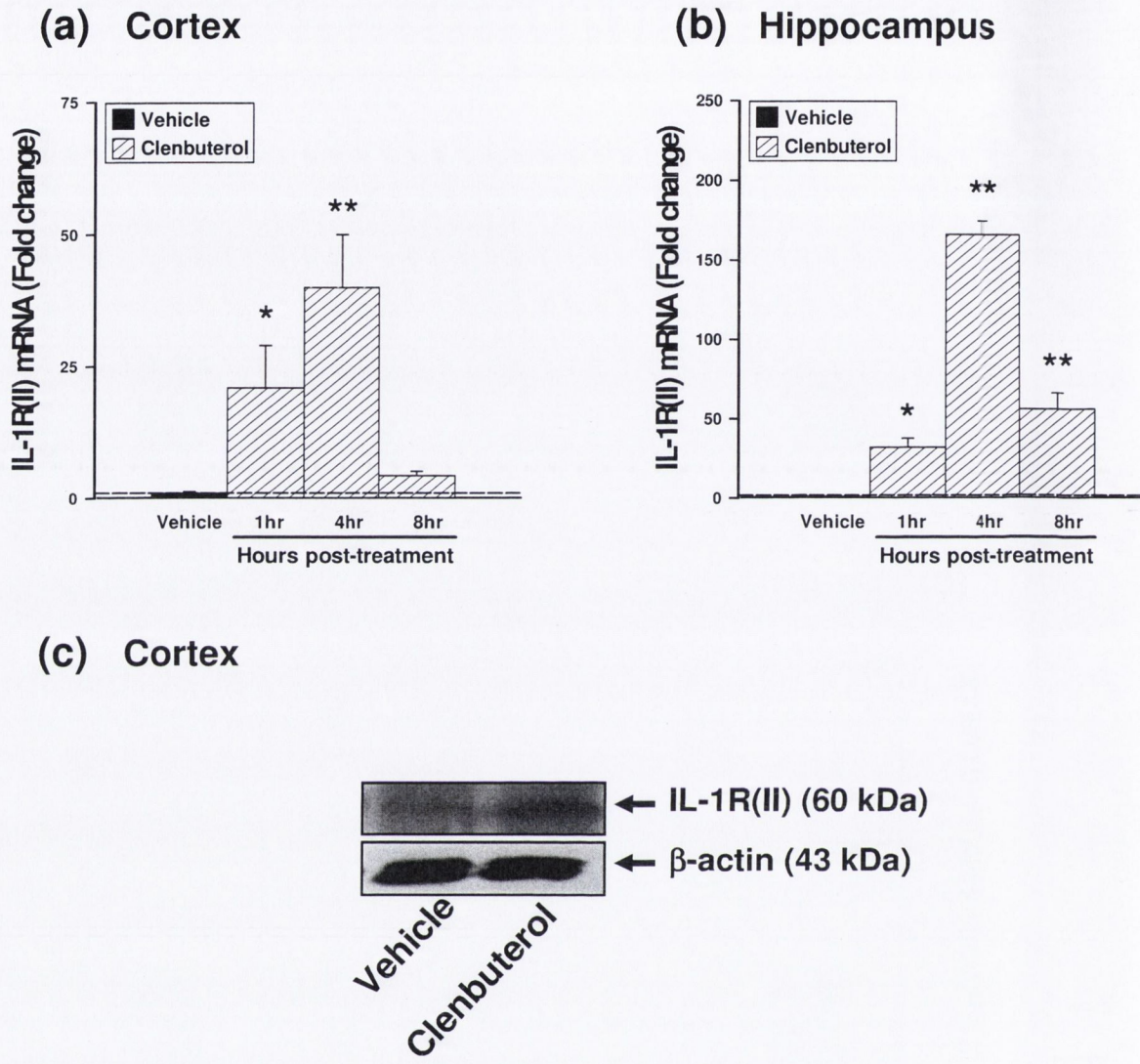


Figure 3.6.3: The selective β_2-AR agonist, clenbuterol, modulates the CNS gene expression and protein production of IL-1R(II) *in vivo*

Acute I.P. treatment with the selective β_2-AR agonist, clenbuterol, significantly induces mRNA expression of IL-1R(II) in the rat cortex [a; One-way ANova: $F(3,15)=18.99$, $P<0.0001$, 1 & 4 hours] and hippocampus [b; One-way ANOVA: $F(3,19)=97.43$, $P<0.0001$), 1, 4 & 8 hours). Clenbuterol (0.5 mg/kg) treatment also induced an increase in cortical membrane-bound, IL-1R(II) expression after an 8 hour treatment (c) (representative of 3 independent immunoblots). Data expressed as means + SEM (n=6). *P<0.05, **P<0.01 vs. saline vehicle (Newmans-Keuls test).

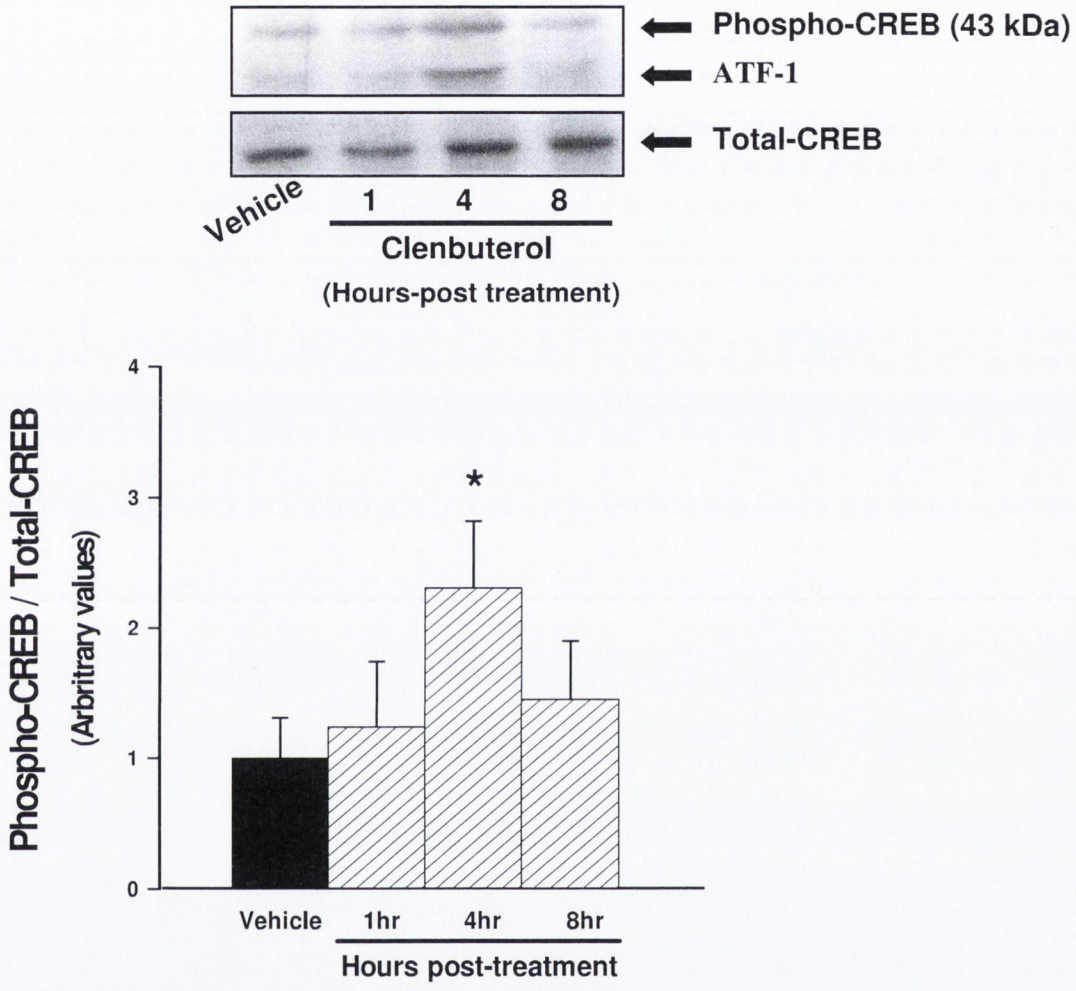


Figure 3.6.4: The selective β_2-AR agonist, clenbuterol, induces cortical CREB phosphorylation

Clenbuterol treatment induced and increase in β-AR activation as measured by CREB phosphorylation at the 4 hour time-point [One-Way ANOVA: $F(3,39)=3.59$, $P<0.02$]. Data expressed as means + SEM (n=4). *$P<0.05$ vs. saline vehicle (Newmans-Keuls test).

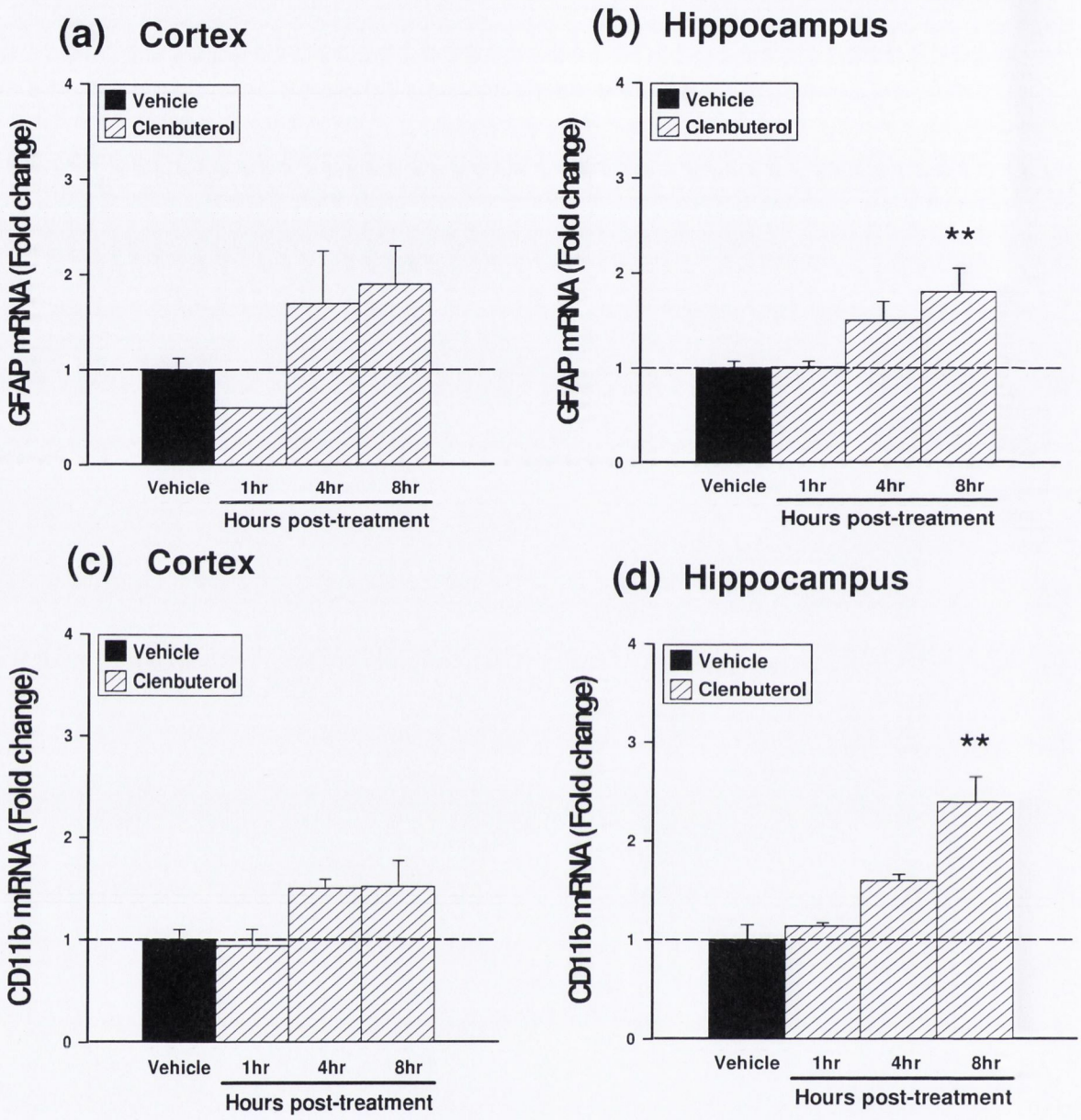


Figure 3.6.5: The selective β_2-AR agonist, clenbuterol, induces GFAP and cd11b mRNA expression *in vivo*

Acute I.P. treatment with the selective β_2-AR agonist, clenbuterol (0.5 mg/kg), induced basal mRNA expression of GFAP and cd11b in the rat hippocampus [b; One-way ANOVA: F(or hippocampus (c & d). Data expressed as means + SEM (n=6).

As outlined in the table below, the impact of selective β_2-AR activation on proxy indices of apoptosis and inflammatory signalling (as measured by induction of the NFκB pathway inhibitor, IκBα) were assessed. The immunomodulatory affects of clenbuterol are not due to unspecific drug toxicity as an acute I.P. treatment regime with clenbuterol (0.5 mg/kg) failed to induce mRNA expression for caspase-3, Bcl-2 or Bax, which are all activated during apoptosis. Clenbuterol also failed to induce gene expression of IκBα, which is a negative regulator of the NFκB pathway, induced by IL-1β signaling.

Table 3.6.1: The β_2-AR agonist clenbuterol fails to induce gene expression for markers of apoptosis and inflammation in the rat hippocampus

Cortex	**saline**	**1**	**4**	**8** (Hours-post treatment)
Caspase-3	1 ± 0.35	0.77 ± 0.02	1.14 ± 0.12	0.81 ± 0.03
Bcl-2	1 ± 0.02	1.07 ± 0.06	0.93 ± 0.04	1.16 ± 0.16
Bax	1 ± 0.15	1.15 ± 0.22	0.95 ± 0.28	1.14 ± 0.22
IκBα	1 ± 0.8	2 ± 0.42	1.4 ± 0.31	1 ± 0.1

Hippocampus	**saline**	**1**	**4**	**8** (Hours-post treatment)
Caspase-3	1 ± 0.2	1.07 ± 0.08	1.18 ± 0.19	0.9 ± 0.05
Bcl-2	1 ± 0.11	1.5 ± 0.18	1.4 ± 0.14	1.3 ± 0.1
Bax	1 ± 0.05	11 ± 0.08	1.17 ± 0.11	1.1 ± 0.04
IκBα	1 ± 0.1	06 ± 0.03	1.04 ± 0.04	1.08 ± 0.03

One-way analysis of variance revealed no significant effect of treatment on target gene expression. Data expressed as fold change of the mean clenbuterol (0.5 mg/kg) + SEM vs. Saline vehicle (n=6).

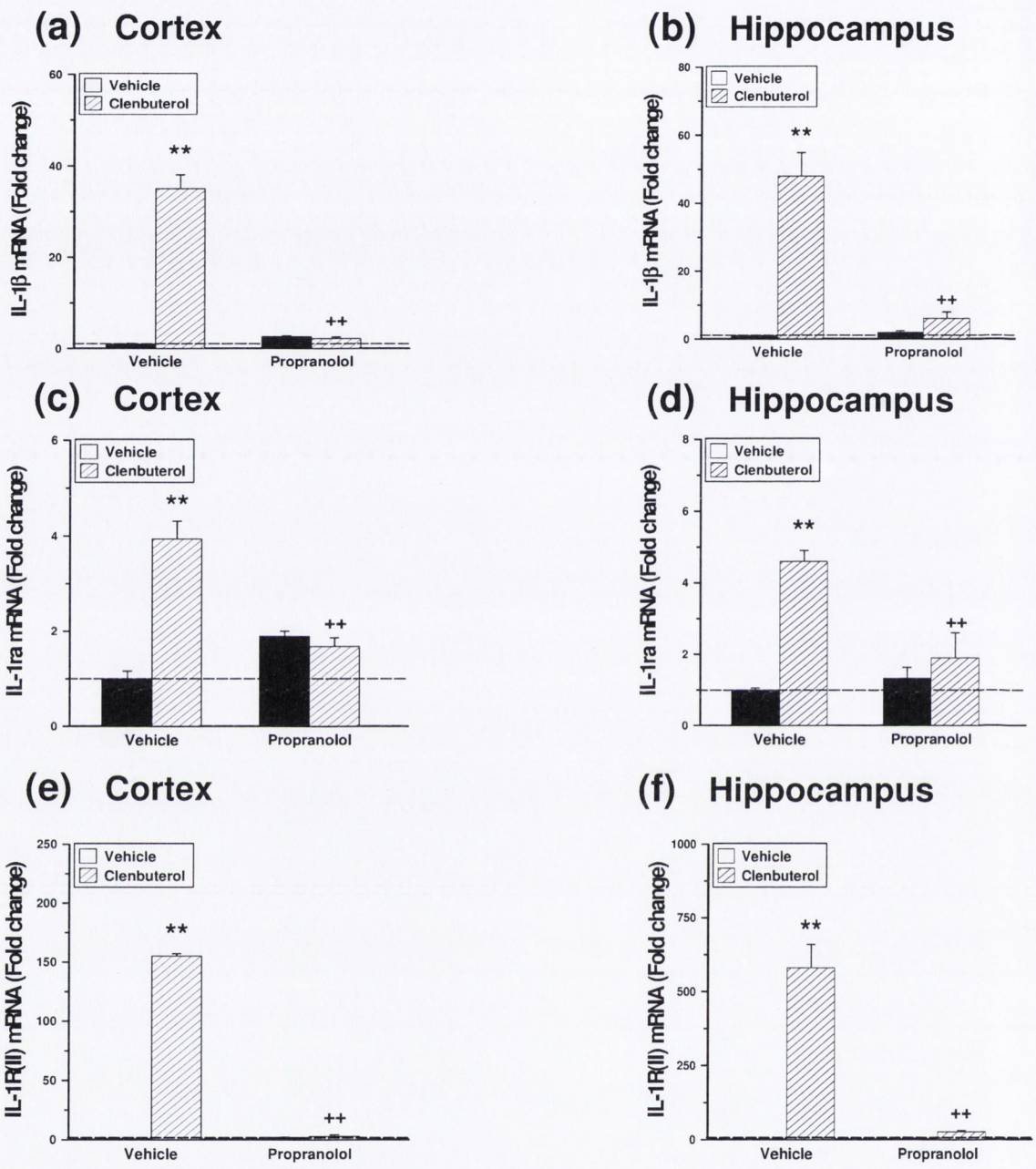


Figure 3.6.6: β-AR mediates the ability of acute clenbuterol treatment t modulate the interleukin-1 system *in vivo*

A 4 hour acute I.P. treatment with the selective β_2-AR agonist, clenbuterol (0.5mg/kg), significantl induces mRNA expression of IL-1β (a & b; P<0.01), IL-1ra (c & d; P<0.01) and IL-1R(II) (e & f; P<0.01 in the rat cortex and hippocampus. This induction is attenuated by a 30 minute pre-treatment with the β adrenoceptor antagonist, propranolol. Two-way ANOVA demonstrates a significant treatment interactio with clenbuterol and propranolol for cortical and hippocampal IL-1β [a; F(3,12)=15.28, P=0.0002)], [b F(2,14)=31.01, P<0.0001)], IL-1ra [c; F(3,13)=27.50, P<0.0001)] and [d; F(3,16)=34.49, P<0.001)], an IL-1R(II) [e; F(3,13)=39.57, P<0.0001)] and [f; F(3,16)=51.55, P<0.0001]. Data expressed as means SEM (n=6). **P<0.01 vs. saline vehicle, ++P<0.01 vs. clenbuterol (Newmans-Keuls test).

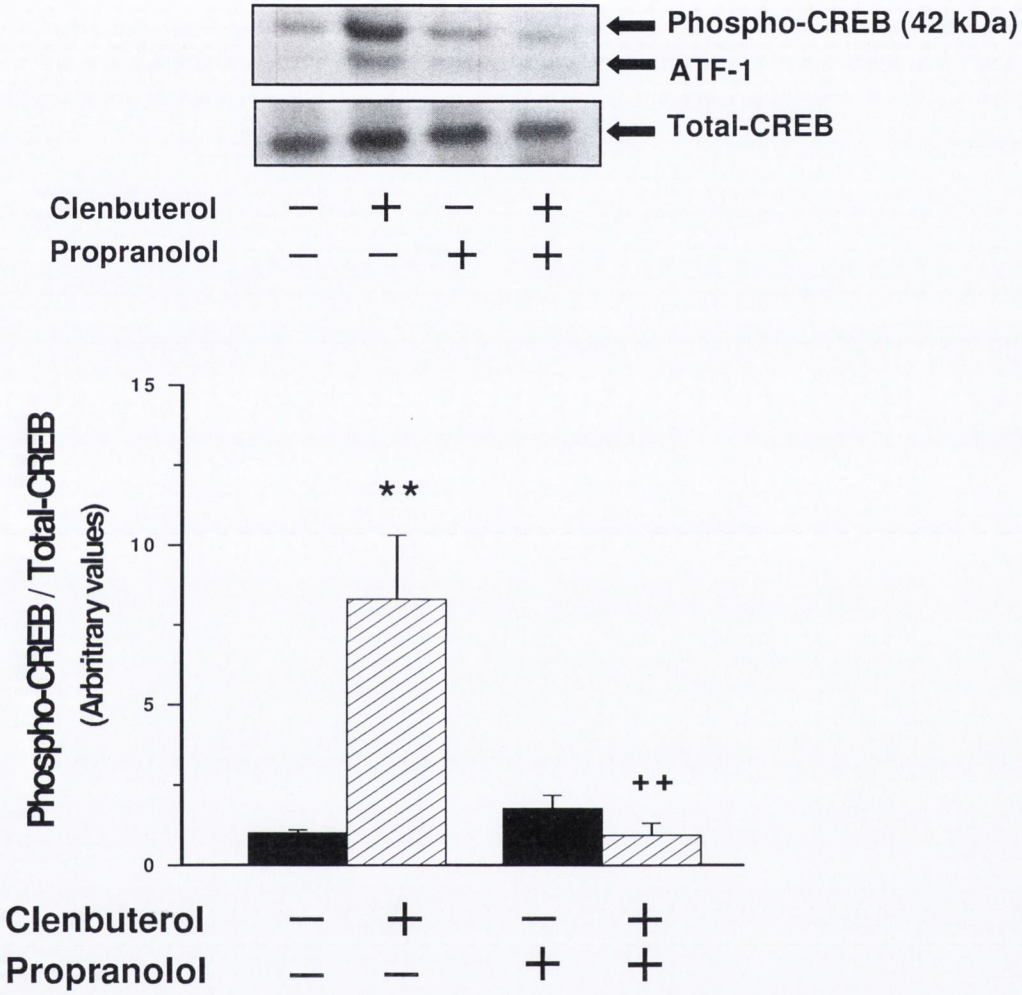


Figure 3.6.7: The non-selective β-AR antagonist, propranolol, attenuates clenbuterol–induced increase in CREB phosphorylation the rat cortex

A 4 hour acute treatment with clenbuterol (0.5 mg/kg) induced an increase in phosphorylation of CREB in the rat cortex ($P<0.01$). This induction was attenuated by a 30 minute pre-treatment with the non-selective β-AR antagonist, propranolol (10mg/kg). Two-way ANOVA showed a significant affect of treatment with propranolol and clenbuterol [$F(3,13)=73.97$, $P<0.004$]. Data expressed as means + SEM (n=6). **$P<0.05$ vs. saline vehicle, ++$P<0.01$ vs. clenbuterol treatment (Newmans-Keuls test).

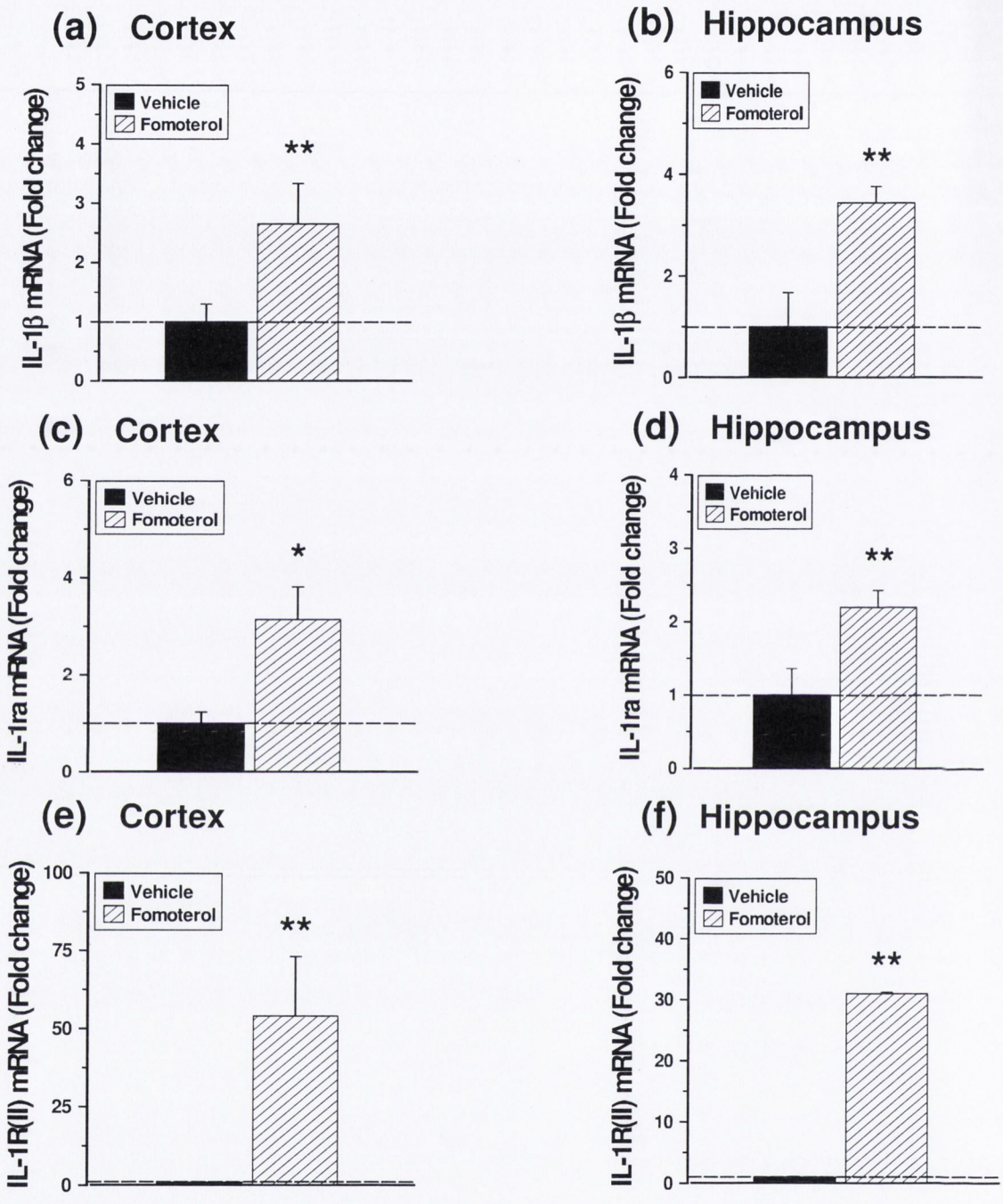


Figure 3.6.8: The selective β_2-AR agonist, fomoterol, mimics the affects of clenbuterol in modulating the CNS interleukin-1 system in vivo

A 4 hour acute I.P. treatment regime with the selective β_2-AR agonist, fomoterol (0.5mg/kg), significantly induces mRNA expression of IL-1β (a & b; $P<0.01$), IL-1ra (c; $P<0.05$, d; $P<0.01$) and IL-1R(II) (e & f; $P<0.01$) in the rat cortex and hippocampus. Data expressed as means + SEM (n=6). *$P<0.05$, **$P<0.01$ vs. saline vehicle (Student-t test).

3.7 Effects of increasing central noradrenergic tone or direct stimulation of the central β_2-AR, on expression of pro- and anti-inflammatory cytokines in the CNS

In this study, the impact of NRI and α_2-AR antagonist treatment, on CNS gene expression levels of inflammatory cytokines was assessed. A 4 hour acute I.P. treatment with reboxetine (15 mg/kg) induced an increase in IL-10 mRNA expression. This induction of IL-10 by reboxetine (15 mg/kg) was augmented by a combination with idazoxan (1 mg/kg). Neither drug treatment affected the expression levels of any of the other cytokines assessed.

Table 3.7.1: A summary of NRI and α_2-AR antagonist–induced levels of inflammatory gene expression in the rat cortex and hippocampus

Cortex	Saline	Reboxetine	Idazoxan	Reboxetine & Idazoxan
TNF-α	1 ± 0.02	0.96 ± 0.08	1.30 ± 0.08	1.6 ± 0.09*
IL-6	1 ± 0.04	1.01 ± 0.09	1.23 ± 0.12	1.34 ± 0.08
IL-10	1 ± 0.07	3.3 ± 0.5 *	0.8 ± 0.2	6.8 ± 1.4 **
IL-4	ND	ND	ND	ND
TGF-β	1 ± 0.09	0.62 ± 0.03	0.98 ± 0.13	0.63 ± 0.07

Hippocampus	Saline	Reboxetine	Idazoxan	Reboxetine & Idazoxan
TNF-α	1 ± 0.1	1.07 ± 0.08	1.34 ± 0.08	1.2 ± 0.13
IL-6	1 ± 0.	1.11 ± 0.15	0.94 ± 0.06	0.7 ± 0.12
IL-10	1 ± 0.07	1.8 ± 0.2 *	0.93 ± 0.12	5 ± 1.3 **
IL-4	ND	ND	ND	ND
TGF-β	1 ± 0.21	0.94 ± 0.19	0.9 ± 0.14	0.9 ± 0.14

One-way analysis of variance revealed significant effect of treatment on IL-10 gene expression with both reboxetine and combined reboxetine/idazoxan treatment [Cortex; $F(3,19)=14.70$, $P<0.0001$; hippocampus; $F(3,12)=4.48$, $P=0.024$]. Data expressed as fold change of the mean Reboxetine (15 mg/kg) or Idazoxan (1 mg/kg) + SEM vs. Saline vehicle (n=6).*$P<0.05$, **$P<0.01$ vs. saline vehicle. ND = not detected

The impact of the β_2-AR agonists, clenbuterol, on CNS gene expression levels of inflammatory cytokines was assessed. A 4 hour acute I.P. treatment with clenbuterol (15 mg/kg), up-regulated IL-10 mRNA expression *in vivo,* while reducing the relative mRNA expression of both TNF-α and IL-6.

Table 3.7.2: A summary of β_2-AR agonist–induced levels of inflammatory gene expression in the rat cortex and hippocampus

Cortex	**Saline**	**1**	**4**	**8** (Hours-post treatment)
TNF-α	1 ± 0.1	0.26 ± 0.05**	0.88 ± 0.006	1.3 ± 0.16
IL-6	1 ± 0.02	0.35 ± 0.07 *	0.99 ± 0.15	0.79 ± 0.14
IL-10	1 ± 0.03	10.5 ± 1.7 **	15 ± 4**	0.58 ± 0.4
IL-4	1 ± 0.12	0.6 ± 0.06	.49 ± 0.47	1.6 ± 0.45
TGF-β	1 ± 0.16	1.04 ± 0.09	1.12 ± 0.09	1.24 ± 0.14

Hippocampus	**Saline**	**1**	**4**	**8** (Hours-post treatment)
TNF-α	1 ± 0.13	0.35 ± 0.09 *	1.24 ± 0.13	1.51 ± 0.25
IL-6	1 ± 0.24	0.2 ± 0.02 *	1.5 ± 0.3	2 ± 0.38
IL-10	1 ± 0.12	4 ± 0.6	9 ± 1.6**	3.5 ± 0.3
IL-4	1 ± 0.15	0.5 ± .08	0.82 ± 0.24	1.16 ± 0.15
TGF-β	1 ± 0.31	1.15 ± 0.07	1.06 ± 0.11	1.35 ± 0.15

One-way analysis of variance revealed significant effect of treatment on target gene expression with clenbuterol (0.5mg/kg) treatment for IL-10 [Cortex; $F(3,18)=12.27$, $P<0.0001$; hippocampus; $F(3,19)=20.00$, $P<0.0001$], IL-6 [Cortex; $F(3,18)=4.35$, $P<0.018$; hippocampus; $F(3,13)=9.10$, $P=0.0016$] and TNF-α [Cortex; $F(3,19)=11.89$, $P<0.0001$; hippocampus; $F(3,19)=9.49$, $P=0.0005$]. Data expressed as fold change of the mean clenbuterol + SEM vs. Saline vehicle (n=6).*$P<0.05$, **$P<0.01$ vs. saline vehicle.

As the previous studies demonstrate an endogenous, anti-inflammatory role for cortical glial β-AR in vitro, by negatively regulating the IL-1 system, the following experiments were carried out to asses the impact of β_2-AR activation on the CNS gene expression of other pro- and anti-inflammatory cytokines. From the data in tables 3.7.1 and 3.7.2., it is evident that *in vivo* strategies which increase the tone of noradrenaline or activate the β-AR, induce IL-10 expression, while down-regulating the acute expression of TNF-α at 1 hour (there is still a subsequent modest increase in cortical TNF-α expression).

The anti-inflammatory cytokine Interleukin-10 (IL-10) has recently been shown to promote the survival of both neuronal and glial cells during models of excitotoxicity and inflammation *in vitro* (Molina-Holgado, *et al.,* 2001, Pahan, *et al.,* 2000, Zachary, *et al.*, 2003). However, little is known about the CNS modulation of IL-10 by noradrenaline and adrenoceptors.

Combined reboxetine and idazoxan treatment induces CNS IL-10 protein expression in vivo: A 4 hour acute I.P. co-treatment with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced a significant increase in cortical IL-10 protein production ($P<0.01$) (figure 3.7.1).

Combined reboxetine and idazoxan treatment induce CNS STAT3 phosphorylation in vivo: A 4 hour acute I.P. co-treatment with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced an increase in cortical IL-10 receptor signalling as measured by an induction of STAT3 phosphorylation (figure 3.7.2).

Induction of CNS SOC3 gene expression by acute treatment with reboxetine and idazoxan in vivo: A 4 hour acute I.P. treatment with either reboxetine (15mg/kg) or idazoxan (1 mg/kg) failed to induce a significant change in SOCS3 mRNA expression in the rat cortex (a). Co-treatment with reboxetine(15mg/kg) and idazoxan (1mg/kg) induced a significant increase in SOCS3 gene expression in the cortex (a; $P<0.01$) (figure 3.7.3).

β-AR mediates the ability of combined reboxetine and idazoxan treatment to induce CNS IL-10 and SOCS3 expression in vivo: A 4 hour acute I.P. co-treatment with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced a significant increase in IL-10 gene expression in both the cortex (a; $P<0.01$) and hippocampus (b; $P<0.01$) as well as SOCS3 gene expression in the hippocampus (b; $P<0.01$). A 30 minute pre-treatment with the β-adrenoceptor antagonist, propranolol (10 mg/kg) attenuated the treatments affects of reboxetine and idazoxan (figure 3.7.4).

The selective β_2-AR agonist, clenbuterol, modulates the CNS gene expression of IL-10 and SOCS3 in vivo: A 4 hour acute I.P. treatment with the selective β_2-adrenoceptor agonist, clenbuterol (0.5mg/kg), significantly induced cortical (a: $P<0.01$) and hippocampal ($P<0.01$) mRNA expression of IL-10 as well as cortical (a: $P<0.01$) and hippocampal ($P<0.01$) mRNA expression SOCS3 *in vivo* (figure 3.7.5).

The selective β_2-AR agonist, clenbuterol, induces cortical IL-10 protein expression in vivo: An acute I.P. treatment regime with the selective β_2-AR agonist, clenbuterol (0.5mg/kg), induced a significant increase in cortical IL-10 protein production ($P<0.01$) (figure 3.7.6).

The selective β_2-AR agonist, clenbuterol upregulates STAT3 phosphorylation in vivo:

An acute I.P. treatment regime with the selective β_2-AR agonist, clenbuterol (0.5mg/kg), induced a significant increase in IL-10 receptor signalling as measured by an induction of STAT3 phosphorylation ($P<0.05$) (figure 3.7.7).

The selective β-AR antagonist, propranolol, attenuates the ability of clenbuterol to induce CNS gene expression of IL-10 and SOCS3 in vivo: An acute I.P. treatment regime with the selective β_2-AR agonist, clenbuterol (0.5mg/kg), significantly induces mRNA expression of IL-10 (a; $P<0.01$) and SOCS3 (c; $P<0.01$) in the rat cortex and hippocampus (b; $P<0.01$, c; $P<0.01$)

respectively. This induction was attenuated by a 30 minute pre-treatment with the β-AR antagonist, propranolol (10 mg/kg) (a-d) ($P<0.01$) (figure 3.7.8).

The selective β_2-AR agonist, fomoterol, induces CNS IL-10 gene expression in vivo: A 4 hour acute I.P. treatment regime with the selective β_2-AR agonist, fomoterol (0.5mg/kg), mimics the ability of clenbuterol to significantly induces mRNA expression of IL-10 in the rat cortex (a; $P<0.01$) and hippocampus (b; $P<0.01$) (figure 3.7.9).

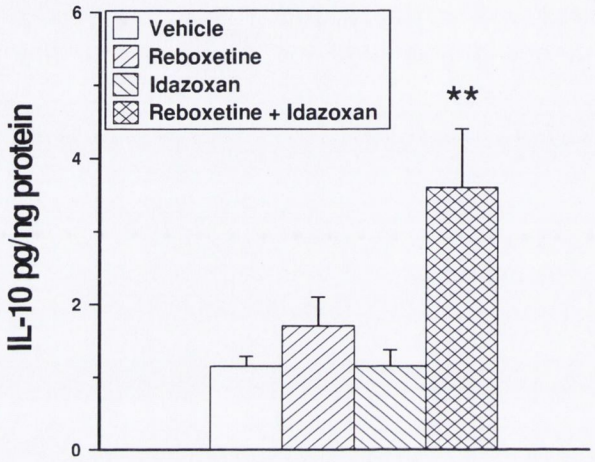


Figure 3.7.1: Combined reboxetine and idazoxan treatment induce CNS IL-10 protein expression *in vivo*

A 4 hour acute I.P. co-treatment with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced a significant increase in cortical IL-10 protein production [One-way ANOVA: $F(3,13)=6.29$, $P<0.007$)] in the rat cortex. Data expressed as means + SEM (n=6). **$P<0.01$ vs. saline vehicle (Newmans-Keuls test).

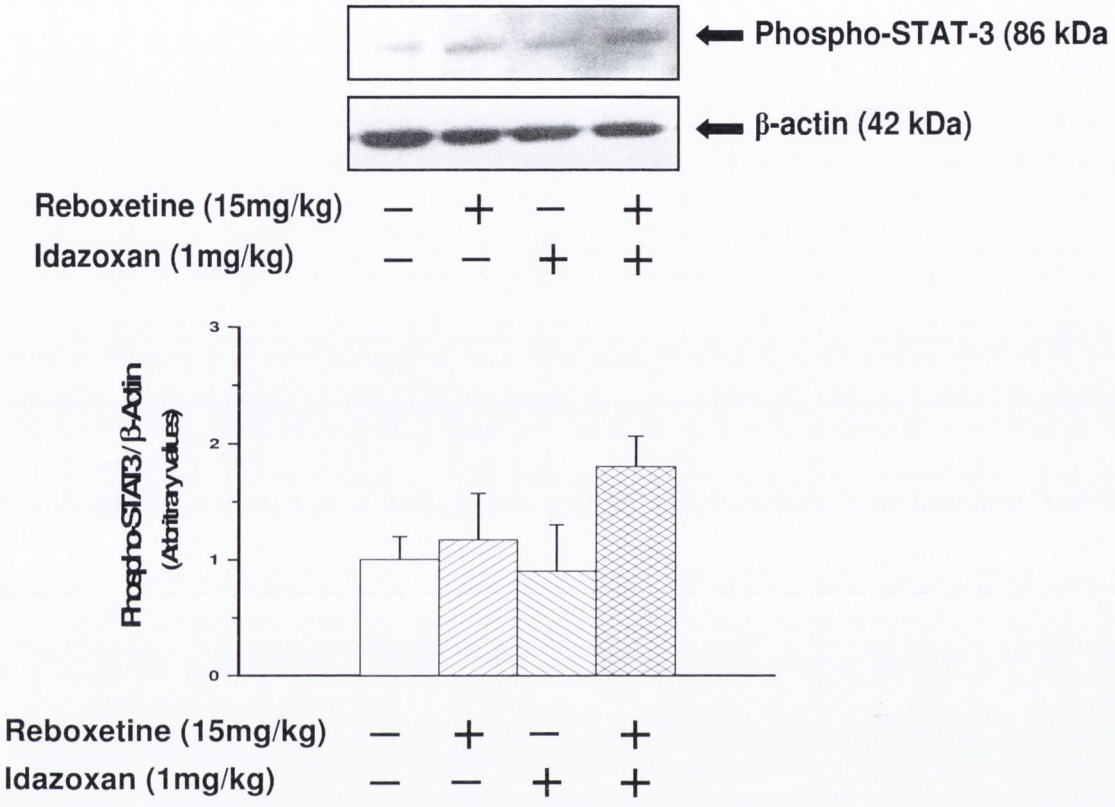


Figure 3.7.2: Combined reboxetine and idazoxan treatment induce CNS STAT3 phosphorylation *in vivo*

A 4 hour acute I.P. co-treatment with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced an increase in cortical IL-10 receptor signalling as measured by an induction of STAT3 phosphorylation. Data expressed as means + SEM (n=3).

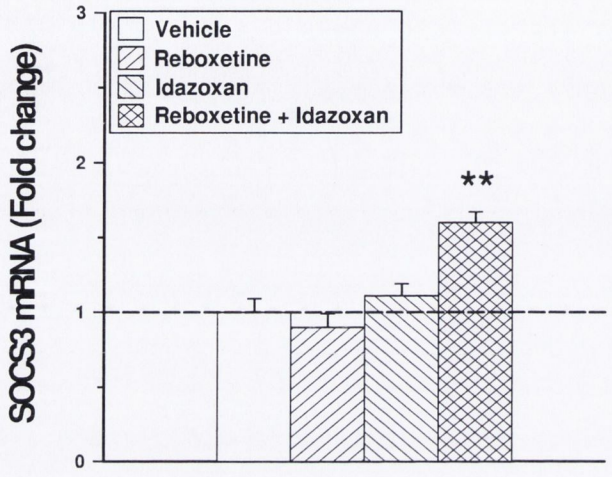


Figure 3.7.3: Induction of CNS SOC3 gene expression by acute treatment with reboxetine and idazoxan *in vivo*

A 4 hour acute I.P co-treatment with reboxetine(15mg/kg) and idazoxan (1mg/kg) induced a significant increase in SOCS3 gene expression in the rat cortex [One-way ANOVA: $F(3,20)=3.29$, $P<0.04$)]. Data expressed as means + SEM (n=6). **$P<0.01$ vs. saline vehicle (Newmans-Keuls test).

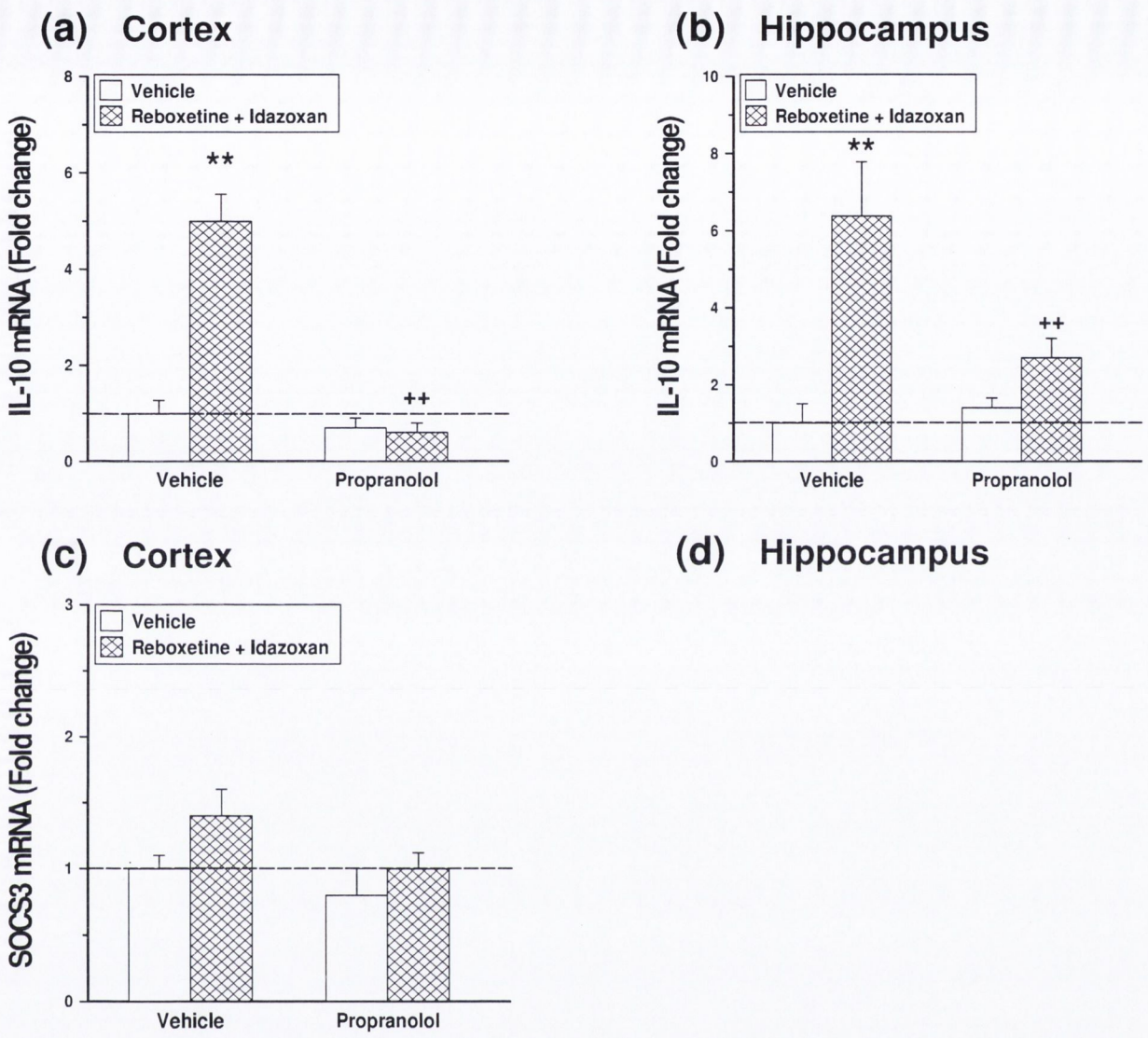


Figure 3.7.4: β-AR mediates the ability of combined reboxetine and idazoxan treatment to induce CNS IL-10 and SOCS3 expression *in vivo*

A 4 hour acute I.P. co-treatment with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced a significant increase in IL-10 gene expression in both cortex (a; $P<0.01$) and hippocampus (b; $P<0.01$) as well as SOCS3 gene expression in the hippocampus (b; $P<0.01$) respectively. Two-way ANOVA showed a significant effect of treatment on cortical [a; $F(3,19)=38.13$, $P<0.0001$)] and hippocampal [b; $F(3,11)=10.52$, $P<0.001$)] IL-10 as well as SOCS3 hippocampal [b; $F(3,19)=23.89$, $P<0.0001$)] mRNA expression and a 30 minute propranolol (10mg/kg) pre-treatment. Data expressed as means + SEM (n=6). **$P<0.01$ vs. saline vehicle, ++ $P<0.01$ vs. Reboxetine & Idazoxan (Newmans-Keuls test).

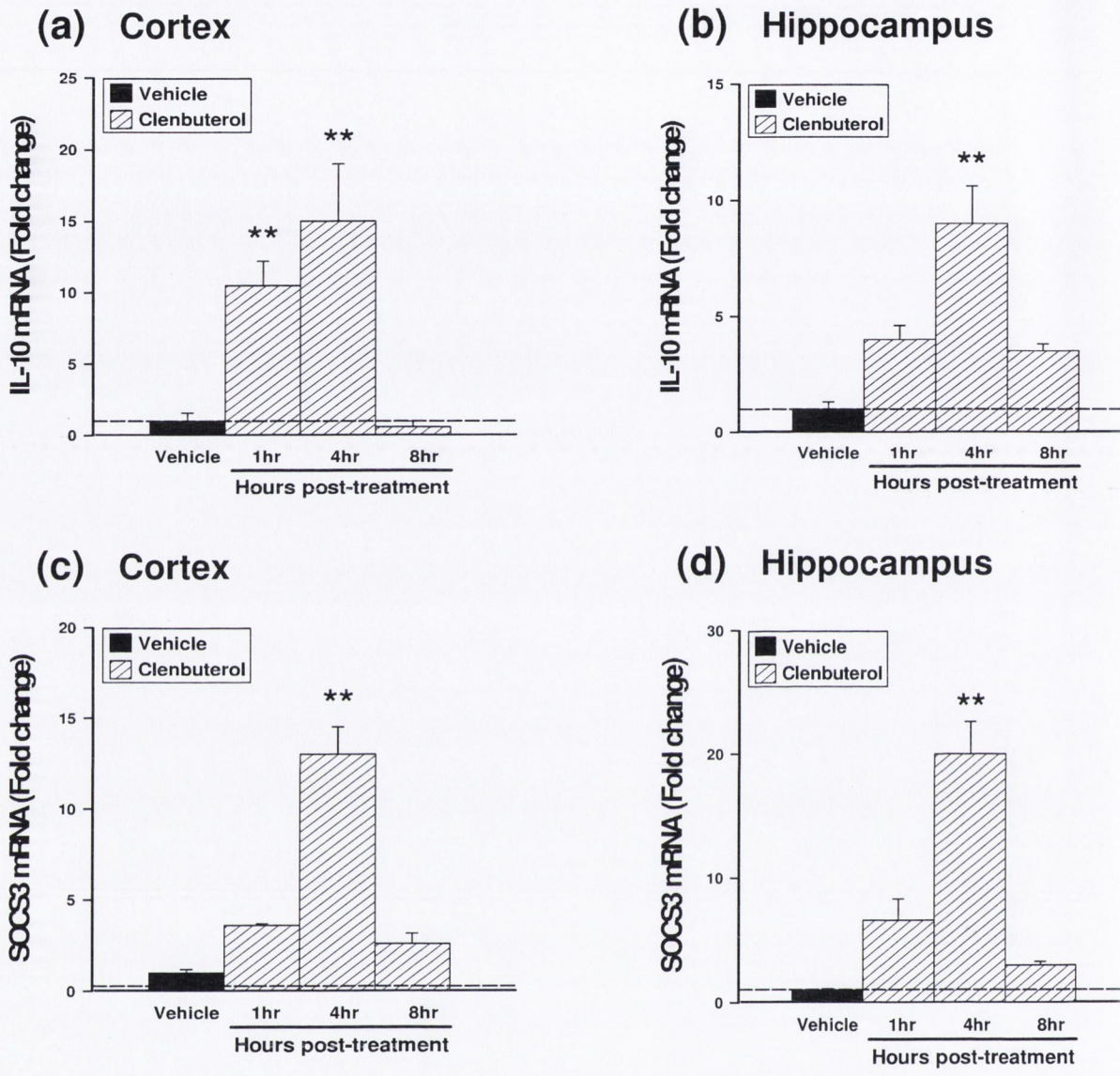


Figure 3.7.5: The selective β_2-AR agonist, clenbuterol, modulates the CNS gene expression of IL-10 and SOCS3 *in vivo*

An acute I.P. treatment regime with the selective β_2-AR agonist, clenbuterol (0.5mg/kg), significantly induces mRNA expression of cortical [a; One-way ANOVA: $F(3,18)=12.27$, $P<0.0001$), 1 & 4 hours] and hippocampal [b; One-way ANOVA: $F(3,13)=14.18$, $P<0.0002$), 4 hours] IL-10 as well as cortical [c; One-way ANOVA: $F(2,20)=6.80$, $P<0.002$), 4 hours] and hippocampal [d; One-way ANOVA: [$F(3,19)=20.01$, $P<0.0001$), 4 hours] SOCS3 expression respectively. Data expressed as means + SEM (n=6). **P<0.01 vs. saline vehicle (Newmans-Keuls test).

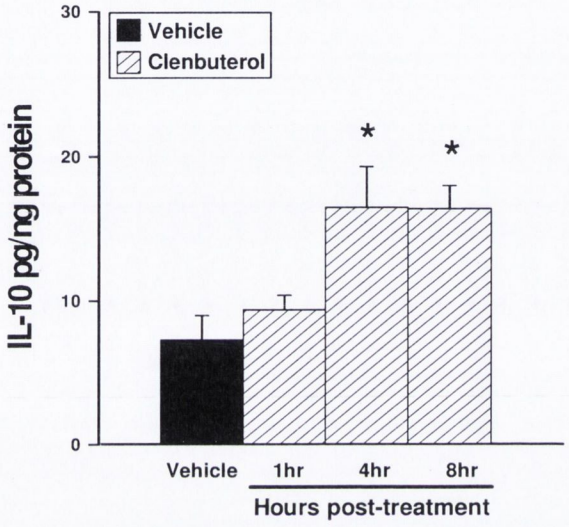


Figure 3.7.6: The selective β_2-AR agonist, clenbuterol, induces cortical IL-10 protein expression *in vivo*

An acute I.P. treatment regime with the selective β_2-AR agonist, clenbuterol (0.5mg/kg), induced a significant increase in cortical IL-10 protein production [a; One-way ANOVA: $F(3,14)=6.50$, $P=0.005$), 4 & 8 hours]. Data expressed as means + SEM (n=6). *$P<0.05$ vs. saline vehicle (Newmans-Keuls test).

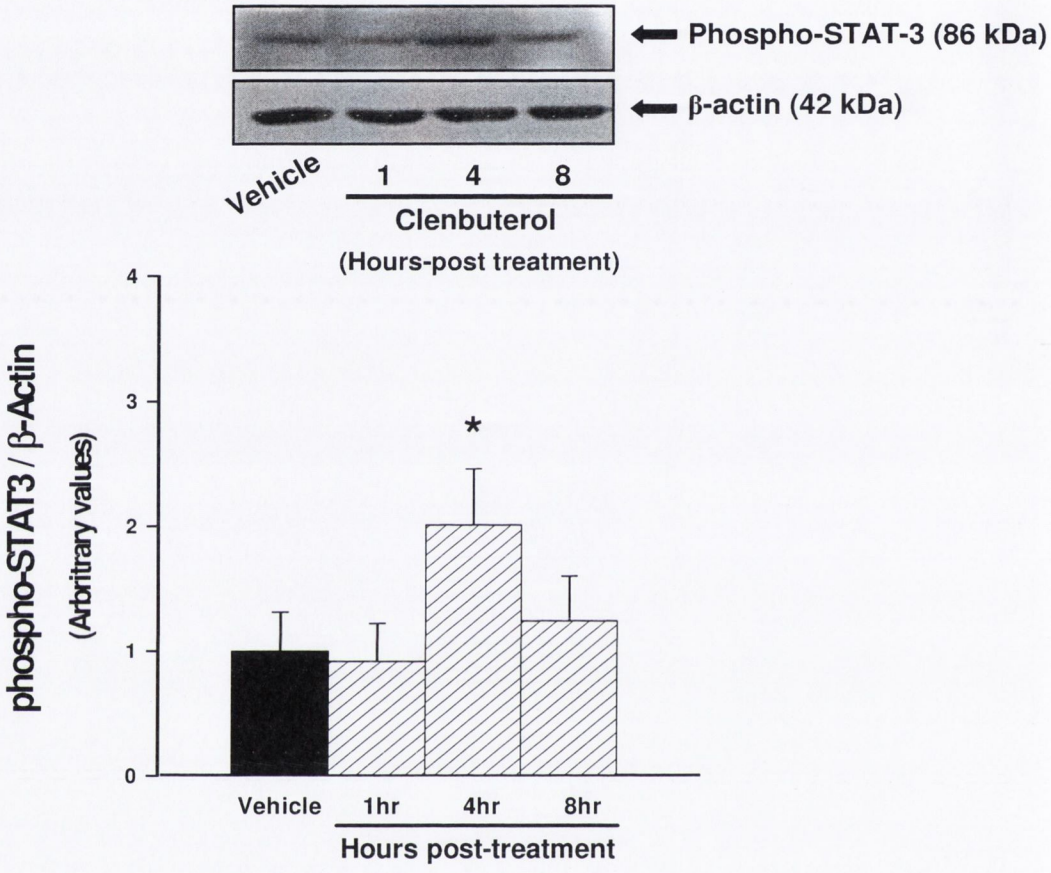


Figure 3.7.7: The selective β_2-AR agonist, clenbuterol up-regulates STAT3 phosphorylation in the rat cortex *in vivo*

An acute I.P. treatment regime with the selective β_2-AR agonist, clenbuterol (0.5mg/kg), induced a significant increase in IL-10 receptor signalling as measured by an induction of STAT3 phosphorylation [One-Way ANOVA: $F(3,18)=4.69$, $P<0.014$]. Data expressed as means + SEM (n=6). *$P<0.05$ vs. saline vehicle (Newmans-Keuls test).

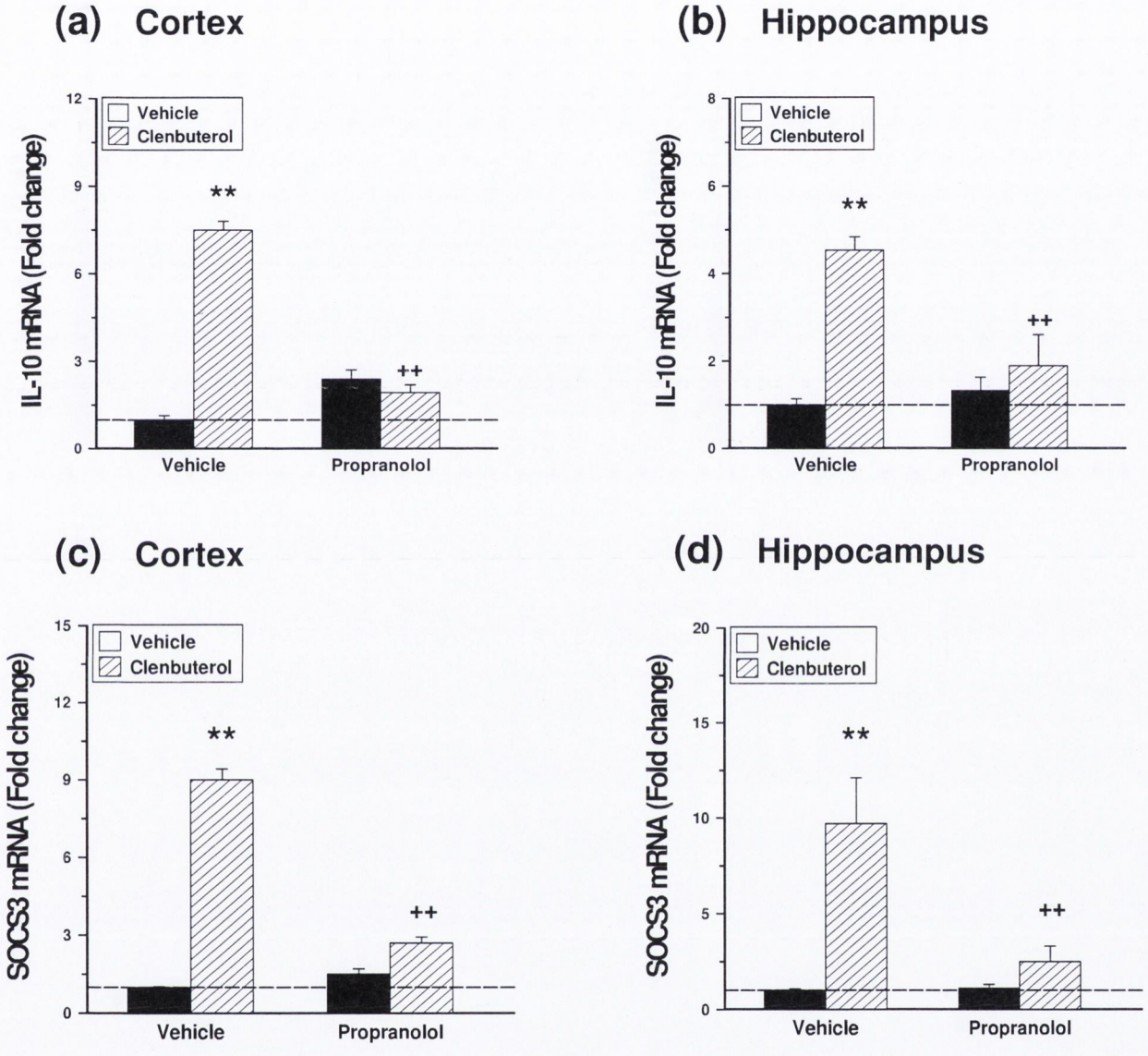
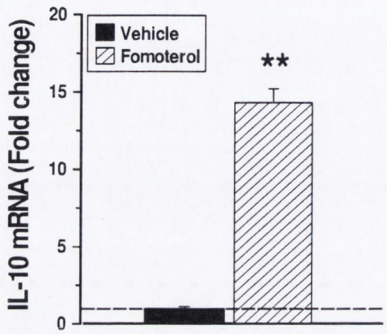


Figure 3.7.8: The selective β-AR antagonist, propranolol, attenuates the ability of clenbuterol to induce CNS gene expression of IL-10 and SOCS3 *in vivo*

An acute I.P. treatment regime with the selective β_2-AR agonist, clenbuterol (0.5mg/kg), significantly induces mRNA expression of IL-10 (a; $P<0.01$) and SOCS3 (c; $P<0.01$) in the rat cortex and hippocampus (b; $P<0.01$, c; $P<0.01$) respectively 4 hours post-treatment. Two-way ANOVA demonstrated a significant treatment interaction between clenbuterol and a 30 minute pre-treatment with propranolol (10 mg/kg) for cortical [a; $F(3,13)=4.05$, $P<0.03$)] and hippocampmal [b; $F(3,13)=10.73$, $P<0.0008$)] IL-10 and cortical [c; $F(3,15)= 13.17$, $P<0.0002$)] and hippocampal [d; $F(3,13)=73.97$, $P<0.0001$)] SOCS3 expression respectively. Data expressed as means + SEM (n=6). **$P<0.01$ vs. Saline vehicle, ++$P<0.01$ vs. Clenbuterol group (Newmans-Keuls test).

(a) Cortex



(b) Hippocampus

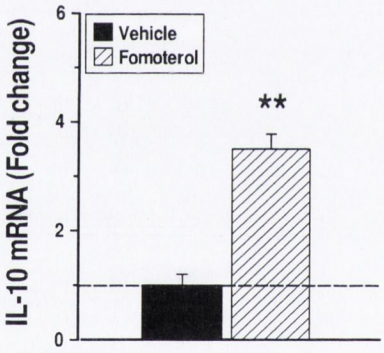


Figure 3.7.9: The selective β_2-AR agonist, fomoterol, induces CNS IL-10 gene expression *in vivo*

A 4 hour acute I.P. treatment regime with the selective β_2-AR agonist, fomoterol (0.5mg/kg), significantly induces mRNA expression of IL-10 in the rat cortex (a; $P<0.01$) and hippocampus (b; $P<0.01$). Data expressed as means + SEM (n=6). **$P<0.01$ vs. saline vehicle (Student-t test).

4. Discussion

The concept is emerging that activation of endogenous neuronal pathways can exert anti-inflammatory actions in the brain, and thereby provide protection in neuroinflammatory conditions (Galea *et al.,* 2003). Of particular interest is evidence indicating that the catecholamine neurotransmitter NA elicits anti-inflammatory actions within the CNS (Feinstein *et al.*, 1993, Galea *et al.*, 2003, Mori *et al.*, 2002, Elenkov *et al.*, 2000). In this regard, the purpose of this study was to examine the ability of NA, and activation of β_2-AR to promote an anti-inflammatory cytokine phenotype *in vitro* in primary mixed glial cells, and in rat brain *in vivo*.

The initial objective of the *in vitro* studies was to asses the ability of NA to modulate the IL-1 cytokine system (ligands and receptors) in mixed glial cells, and to determine the receptors and cellular signalling mechanisms employed by NA in modulating the IL-1 system in glial cells.

4.1 Negative regulation of the CNS IL-1 system by NA *in vitro*

A vast array of literature now implicates IL-1 as a major contributing factor in the initiation, persistence and lack of resolution of both acute and chronic inflammation in the central nervous system. IL-1 gene expression and production of IL-1 (α and β isoforms) protein is increased in a host of disease states including experimentally induced ischaemic, excitotoxic, and traumatic brain injury, Alzheimer's disease, multiple sclerosis, HIV-associated dementia and Parkinson's disease (see Allan et al., 2001, Rothwell & Luheshi, 2000, for reviews). While IL-1 is not directly neurotoxic when administered to otherwise healthy neurons *in vitro*, or when administered *in vivo* into the healthy brain, injection of low (picomolar) doses into the cerebral ventricles of rodents exposed to other insults markedly exacerbates inflammation and neuronal toxicity (Rothwell *et al.*, 2003). Thus antagonism of the actions of IL-1 has neuroprotective potential.

Here we demonstrate that *in vitro* exposure of mixed glial cells to noradrenaline up-regulates gene expression and protein levels of the endogenous antagonist of IL-1 receptors, IL-1ra, without altering expression of IL-1β. In contrast, NA

failed to induce IL-1ra expression in primary neuronal cultures, indicating that IL-1ra appears to be a glia cell specific phenomenon.

The importance of IL-1ra in regulating IL-1β has become apparent in recent years after it was shown that an endogenous imbalance between IL-1ra and IL-1β is of pathophysiological importance in many peripheral disease states such as inflammatory bowel disease and rheumatoid arthritis (Palin *et al.*, 2004). IL-1ra is generally found to be up-regulated in a dose-dependent manner by IL-1β in order to dampen down the inflammatory effects of IL-1β over-expression (Palin *et al.*, 2004). However in this study it is noteworthy that the increase in glial IL-1ra production induced by NA does not simply occur secondary to IL-1 production, as IL-1 production was not altered by NA. Thus it appears that NA induces a selective upregulation of IL-1ra; the anti-inflammatory ligand in the IL-1 system.

In addition to increasing IL-1ra expression, our results demonstrate that NA induces a robust increase in IL-1R(II) mRNA expression in mixed glial cells, as well as subsequent protein expression. This is significant, as IL-1R(II) is a decoy receptor that serves to sequester IL-1 and therefore like IL-1ra is a negative regulator of the IL-1 system. This increase in IL-1R(II) expression is also observed in primary cortical neurons following treatment with NA, however this NA-induced neuronal IL-1R(II) expression is of a lesser magnitude than what was observed in glial cells. NA-treatment of both mixed glia and neurons had no significant effect on the gene expression levels of either IL-1R(I) or IL-1R(Acp); molecules responsible for initiating IL-1 signalling. These data are in contrast to a previous study which reported that IL-1R(I) gene expression was down-regulated by low concentrations (nM) of β_2-AR agonists and db-cAMP in primary cultures of astrocytes, but not microglia (Tomozawa *et al.*, 1995).

While recombinant human IL-1ra is now a licensed subcutaneous treatment for rheumatoid arthritis (Cohen *et al.*, 2002, Bresnihan *et al.*, 1998) and is in a phase II clinical trials using intravenous infusions for stroke patients (Rothwell *et al.*, 2003), at 17 kDa, IL-1ra is a large protein and is unlikely to pass the blood brain barrier with ease. Thus, pharmacological intervention that would increase

the CNS expression levels of IL-1ra and/or IL-1R(II) may prove to be a beneficial alternative to peripheral administration of the recombinant protein. In the healthy CNS, the activation of intrinsic neuronal pathways that provide protection in neuroinflammatory conditions may be of therapeutic potential, considering the ability of glia to induce both IL-1ra and IL-1R(II) *in vitro* when activated by physiologically relevant doses of noradrenaline. Considering that IL-1ra is induced during brain insults, but usually slightly later than IL-1α and IL-1β, an endogenous "basal" increase in IL-1ra, in conjunction with IL-1R(II), may have the potential to pre-emptively block/dampen down the effects of IL-1β before it can cause a heightened state of inflammation.

Microglia mediate the ability of NA to negatively regulate the IL-1 system in vitro: In an effort to identify the cellular source of the NA-induced IL-1ra and IL-1R(II) expressed from mixed glial *in vitro*, enriched cultures of both microglia and astrocytes were treated with NA under the same conditions. Microglia appear to be the sole producer of IL-1ra and IL-1R(II), as astrocytes failed to respond to treatment (fig 3.2a-d). At least were IL-1ra is concerned, this is consistent with previous literature, which has identified microglia as being the predominant producer of IL-1 ligands and the sole producer of IL-1ra in the CNS (Pinteaux *et al.*, 2006). IL-1R(II) expression induced by NA in microglial cells is not of the same magnitude as that observed in mixed glial cultures. This may be due to experimental differences as enriched cultures of microglia utilize cytokines such as M-CSF and GM-CSF to induce heightened microglia differentiation. These may affect the functional ability of either glial subtype to respond maximally to NA, by altering the expression levels of the β-AR's or IL-1R(II) independently. Alternatively, the induction of IL-1R(II) by NA on microglial cells may require an, as yet unidentified, juxtacrine factor, present in mixed glial cultures. Oligodendrocytes present in the mixed glial culture system may also be the major IL-1R(II) expressing cell type, however as oligodendrocytes have limited influence on the CNS immune environment this would seem unlikely.

4.2 β-AR mediate the ability NA to induce IL-1ra and IL-1R(II) in cortical mixed glial cells *in vitro*: A critical role for PKA and ERK

A number of *in vitro* studies indicate that activation of β-AR on glial cells provide protection from inflammatory insults (Junker *et al.*, 2002), and decrease pro-inflammatory cytokine production (Mori *et al.*, 2002). Functional β_1-AR's have been demonstrated on glia, however, β_2-AR's are more highly expressed on microglia and produce greater amounts of cAMP upon stimulation than β_1-AR (Mori *et al.*, 2002). Thus, much of the anti-inflammatory effects of NA in the CNS have to date, been attributed to β_2-AR stimulation (Feinstein *et al.,* 1998, Feinstein *et al.*, 2002, Frohman *et al.*, 1988, Galea *et al.*, 2003, Elenkov *et al.,* 2000, for review).

To determine which adrenoceptor subtype was responsible for the NA-induced expression of IL-1ra and IL-1R(II) observed in the previously mentioned studies, primary mixed glial cells were pretreated with the non-selective β-AR antagonist, propranolol, and the non-selective α-AR antagonist, phentolamine, prior to the administration of NA. While phentolamine had no effect on NA-induced IL-1ra or IL-1R(II) expression, pretreatment with propranolol, significantly blocked NA induced IL-1ra gene expression and protein production, as well as IL-1R(II) gene expression, thus implicating β-adrenoceptors in mediating these effect of NA.

A role for glial β_2-AR-cAMP-PKA activation in the negative regulation of the CNS IL-1 system: Since the previous literature associates selective β_2-AR activation with the ability of NA to modulate immune function of CNS glia (see Elenkov et al., 2000 for review), here mixed glial cultures were incubated with the selective β_2-AR agonist salbutamol. Using mixed glial cultures under unstimulated conditions, salbutamol mimics the effects of NA as it induced mRNA of both IL-1ra and IL-1R(II).

Consistent with the ability of β_2-AR to activate adenylate cyclase and up-regulate intracellular cAMP, incubation of the primary mixed glial cells with the lipophilic cAMP analogue, db-cAMP, increased both IL-1ra and IL-1R(II) expression. Furthermore, as protein kinase-A (PKA) is the major intracellular

target for cAMP, it was not surprising to observe that pre-treatment with the selective PKA inhibitor, KT5720, blocked the NA-induced increase in IL-1ra mRNA and protein, as well as IL-1R(II) mRNA expression. Increases in intracellular levels of cAMP have been previously shown to be anti-inflammatory, and it is through that increased cAMP production that the β_2-AR is thought to mediate its immunosuppressive effects (Elenkov *et al.,* 2000). An increasing body of evidence suggests that activation of the β_2-AR-cAMP-PKA pathway, suppresses Th_1-pro-inflammatory cytokine production by promoting a Th_2-anti-inflammatory cytokine phenotype (Elenkov *et al.,* 2000). For example, NA suppresses IL-12 and TNF-α secretion from bacterial endotoxin-stimulated human monocytes and whole blood cultures (Severn *et al.*, 1992, Elenkov *et al.*, 1996), as well as mouse microglia (Hetier *et al.*, 1991) via an increase in cAMP. Conversely, NA and β_2-adrenoceptor agonists have been shown to boost bacterial-endotoxin-induced increases in IL-10 from human monocytes, mouse macrophages and rat serum (Elenkov *et al.*, 1996, van der Poll *et al.*, 1996, Siegmund et al., 1998, Connor *et al.*, 2005).

There is compelling evidence to show that activation of β_2-AR and elevated intracellular cAMP can negatively regulate glial immune function in the CNS. Pre-treatment with NA or cAMP-elevating agents blocked IL-1β/TNF-α-induced ICAM-1/VCAM-1 in astrocytes (Ballestas *et al.,* 1997), LPS-induced IL-1β and iNOS from microglia (Dello Russo *et al.*, 2004), glutamate-induced neurotoxicity (Junker *et al.,* 2002) and IFN-γ-induced MHC class II expression in astrocytes (Frohman *et al.,* 1988). One theory as to how the PKA/cAMP pathway can inhibit an inflammatory cytokine response is by impairing transcription, activation and subsequent nuclear translocation of the inflammatory transcription factor NFκB (Neumann *et al.*, 1995, Parry & Mackman., 1997, Elenkov *et al.*, 2000). This may be achieved by the fact that cAMP-induced CREB can compete for many of NFκB's target promoter binding sites (Neumann *et al.*, 1995, Parry & Mackman., 1997, Elenkov *et al.*, 2000). NA can also induce expression of the NFκB inhibitor, IκBα (Dello Russo *et al.*, 2004). Alternatively, NA treatment of mouse astrocytes and neurons induces the anti-inflammatory transcription factor, peroxisome proliferator activated receptor gamma (PPAR-γ) which has been shown to inhibit inflammatory cytokine

production by inducing both IκBα and heat shock protein-70 (HSP-70) expression (Klotz *et al.*, 2003, Heneka *et al.*, 2003).

The *in vitro* studies from this body of research are the first to highlight a mechanism by which NA-β_2-AR-cAMP act as an endogenous immunosuppressive mechanism by negatively regulating glial IL-1β expression in glial cells, independent of any immune response. These results may partially explain why the loss of NA *Locus Ceruleus* neurons, and subsequent central NA tone, contributes to inflammatory activation in chronic inflammatory states such as Alzheimer's disease (Heneka *et al.*, 2002).

Extracellular response kinase (ERK) is required for NA-induced IL-1ra and IL-1R(II) expression in glial cells in vitro: It has been suggested that G-protein coupled receptors, such as the β-adrenoceptor, activate ERK-1 and -2, either downstream of second messengers like cAMP and Ca^{2+} or, that the Gα- and/or Gβγ-subunits may directly or indirectly activate the Ras/Raf pathway upstream of ERK through adapter proteins, tyrosine kinases (Faure *et al.*, 1994, Kolch *et al.*, 2005, Lopez-Ilasaca *et al.*, 1998). Activation of ERK by either NA or cAMP-inducing agents has previously been shown to be neuroprotective by inducing brain-derived neurotrophic factor (BDNF) from hippocampal neurons, as well as protecting dopaminergic neurons from oxidative stress-induced toxicity (Chen *et al.*, 2007, Troadec *et al.*, 2002). In this study, the involvement of ERK in mediating NA-induced increases in IL-1ra and IL-1R(II) was assessed. Consistent with the previous literature, NA induced an increase in ERK-1 (P44) phosphorylation in primary mixed glial cultures via β-adrenoceptor activation, and pre-treatment with the β-AR antagonist, propranolol, inhibited this increase. As MEK 1/2 are directly upstream of ERK 1/2 signalling, pre-treatment of primary glial cells with the selective MEK inhibitor, U0126, inhibited both NA-induced IL-1ra and IL-1R(II) mRNA expression. These results are the first to show that β-adrenoceptor and subsequent ERK activation are responsible for the induction of glial IL-1ra and IL-1R(II) in the CNS.

NA inhibits IL-1β-induced neurotoxicity: Previous literature demonstrates that IL-1β is not directly neurotoxic to healthy neurons *in vitro* but rather, exacerbated

neuronal damage by increasing glial-derived agents such as reactive nitrogen species, free radicals, and inflammatory cytokines (Rothwell *et al.*, 2003, Ma *et al.*, 2002, Thornton *et al.*, 2006). The following study was carried out to asses the ability of NA to limit neuronal toxicity, induced by IL-1β treated, glial-conditioned media. Previously, culture media from microglia cells treated with bacterial endotoxin, which was added to primary cortical neurons, induced a dramatic increase in the expression of iNOS and neurotoxicity (as measured by LDH release). Pre-treatment with NA attenuated this toxic affect (Madrigal *et al.,* 2005). In the present study, IL-1β treatment of primary cortical neurons for 48 hours showed no toxic affects as measured by membrane instability and subsequent LDH release. However, addition of conditioned-media (CM) from mixed glial cells that were treated with IL-1β (for 24 hours), to neurons, induced almost a 3-fold increase in LDH release. Pre-treatment of glial cells with NA (for 48 hours), before the addition of IL-1β, attenuated the neurotoxic affects observed. These results enforce the concept that NA has the ability to act as an endogenous immunosuppressant within the CNS. The negative regulation of the glial IL-1 system by NA points towards a functional mechanism to block/dampen-down the effects of IL-1β in the CNS, before it can cause a heightened state of inflammation.

The exact mechanism employed by NA to inhibit toxicity, induced by CM from IL-1β-treated glia was not assessed in this study. While this work clearly identifies glial-derived soluble factors in the IL-1β-induced neurotoxicity, the use of an LDH assay is simply a measure of decreased membrane viability, and as such, could be as a result of toxicity, necrosis or end stage apoptosis. Measuring markers of apoptosis such as up-regulated Bcl and Bax family members as well as cleaved caspases-3 and -9, the cytosolic release of mitochondrial cytochrome C and the measurement of cleaved DNA by TUNEL staining would identify a direct link for IL-1β in the induction of apoptosis. Previous literature has already identified the activation of caspase-3 and free radical release in the neurotoxicity induced by IL-1β in primary rat neuron-astrocyte co-cultures (Thornton *et al.,* 2006).

4.3 Pharmacological enhancement of central noradrenergic tone *in vivo*; impact on the central interleukin-1 system

There is now a body of research indicating that loss of noradrenergic LC neurons plays a critical role in the progression of neuroinflammation and neurodegeneration (Heneka *et al.*, 2002, Grudzien *et al.*, 2007; Marien *et al.*, 2004). Whilst there is clear evidence that NA plays a tonic anti-inflammatory role in the CNS (Elenkov *et al.*, 2000), to date there has been a paucity of research focused on the potential anti-inflammatory/neuroprotective actions of pharmacological strategies that increase central noradrenergic tone. As anti-inflammatory cytokines are peptides in nature, the use of recombinant cytokine therapy in the clinical treatment of human CNS inflammation does not appear to be a plausible course of action, as these cytokines won't pass the blood brain barrier (see Allan *et al.*, 2001, for review). Despite the wealth of information and research into neurodegenerative disease, current pharmacological therapies only alleviate symptoms and slow disease progression. At present, there is no cure for Alzheimer's disease, Parkinson's disease, Multiple Sclerosis or any of the other neurodegenerative disorders. Therefore, the use of agents that would enhance the tone of NA within the CNS and thus, drive an anti-inflammatory cytokine phenotype may be a viable therapeutic treatment for neurodegenerative disorders.

In this study we examined the impact of increasing central noradrenergic tone on the IL-1 system *in vivo*. The NRI reboxetine and the α_2-AR antagonist idazoxan were administered to rats either alone, or in combination, in order to increase central noradrenaline availability.

Acute NRI treatment modulates the CNS IL-1 system in vivo: This present study investigated the ability of pharmacological blockade of central NA transporters as a means of increasing the endogenous synaptic availability of NA. In this study, an acute peripheral treatment with the NRI reboxetine induced a modest, yet significant, increase in cortical but not hippocampal IL-1β mRNA expression. Conversely, reboxetine increased hippocampal IL-1ra mRNA expression but failed to significantly increase cortical IL-1ra expression. While reboxetine treatment did not have a statistically significant influence on hippocampal IL-1β

or cortical IL-1ra, there was a trend towards increases. In this regard we postulate that addition of further experimental subjects to this experiment may have been required in order to increase the power and yield statistically significant results.

It has previously been shown that the blockade of NA transporters has the propensity to alter immune function by increasing the local concentrations of NA in the vicinity of peripheral immune cells. Chronic treatment with a range of antidepressants (all of which increase central noradrenergic tone) were shown to increase expression of the anti-apoptotic protein bcl-2 in a variety of brain structures (Kosten *et al.*, 2007). In a more recent study, Huang and colleagues, show that treatment of adult rat neural stem cells with desipramine, inhibited bacterial-endotoxin induced apoptosis by up-regulating bcl-2, and inhibiting production of IL-1β, IL-6 and TNF-α (Huang *et al.,* 2007).

The α_2-AR antagonist, idazoxan, augments the ability of reboxetine to modify the CNS IL-1 system in vivo: α_2-AR exert a tonic inhibitory control on adrenergic transmission and thereby modulating NA release (Ihalainen *et al.,* 2002, Trendelenburg *et al.,* 1999). Thus, pharmacological inhibition of α_2-AR may also represent a viable strategy to enhance NA concentrations in the brain by augmenting its actions (Invernizzi and Garatti, 2004). In this regard it is noteworthy that the α_2-AR antagonist idazoxan has previously been shown to produce considerable neuroprotective effects in animal models of Huntington's disease (Martel *et al.*, 1998), brain ischemia (Gustafson *et al.,* 1990, Craven *et al.*, 1997), and Parkinson's disease (Srinivasan *et al.,* 2004). As to whether such neuroprotective actions of idazoxan are related to the modulation of inflammatory processes is not clear.

In this study acute peripheral treatment of rats with idazoxan did not alter mRNA expression of either of the IL-1 ligands, IL-1β or IL-1ra, or any of the receptor subtypes. This result would indicate that the α_2-AR antagonist, idazoxan, was less efficacious at eliciting an immunomodulatory effect in the CNS compared to the NRI reboxetine, at least on the IL-1 system. It is possible that this is a result of the fact that α_2-AR antagonists at the dose employed in the present study (1mg/kg) were less efficacious than NRI treatment at increasing extracellular

NA in the CNS, as assessed using microdialysis (Geranton *et al.*, 2003, Wortley *et al.*, 1999). Previous literature shows that there was no significant increase in NA efflux in the frontal cortex of rats following administration of idazoxan (1mg/kg), but did observe a profound synergistic increase in NA efflux when this dose of idazoxan was combined with the selective NRI reboxetine (Sacchetti *et al.*, 1999). In line with that finding, the results of this study demonstrate that combined treatment with idazoxan and reboxetine induced a modest yet significant increase in mRNA expression of IL-1β in cortex, as well as cortical and hippocampal IL-1ra. In addition, combined treatment with idazoxan and reboxetine induced a profound increase in cortical and hippocampal IL-1R(II) expression. This is the first report of a pharmacological intervention that increases NA availability and subsequently modulates the IL-1 system in the CNS, independent of any immune stimulus.

In contrast to the profound increase in IL-1R(II) induced by the reboxetine/idazoxan combination, there was no significant changes observed in expression of either IL-1R(I) or IL-1R(Acp). These findings are consistent with other previous research stating that the low constitutive levels of IL-1R(I) expression in the brain don't appear to be easily altered, at least in animal models (Parnet *et al.*, 1994, Gayle *et al.*, 1999). In all these the data generated in this study indicate that a pharmacological intervention that increases central noradrenergic tone (reboxetine/Idazoxan combination) selectively increased expression of cortical IL-β and both cortical and hippocampal of the anti-inflammatory IL-1R(II).

β-AR's mediate ability of the reboxetine/idazoxan combination to modify the CNS IL-1 system in vivo: In the present study, acute treatment with both reboxetine and idazoxan induce cortical CREB phosphorylation, the major downstream transcription factor induced by β-AR signalling. Consistent with β-AR mediating the immunomodulatory affects of NA, the affects of combined reboxetine/idazoxan treatment on the IL-1 system were completely attenuated with a pre-treatment using the β-AR antagonist, propranolol. There are some previous reports of β-AR agonists inducing IL-1β from a macrophage cell line (Tan *et al.*, 2007), and β-AR being responsible for stress-induced

catecholamine increases in hypothalamic IL-1β (Blandino *et al.*, 2006, Johnson *et al.*, 2005).

There is now a general consensus from the previous literature that IL-1β can exert both adaptive and maladaptive responses in the brain, depending on the concentration and immune environment. For example, low concentrations of IL-1β protected cultured cortical neurons against an NMDA-induced *in vitro* model of excitotoxicity (Rothwell *et al.*, 1995) and enhanced survival of lesioned cultured dopaminergic neurons (Akaneya *et al.*, 1995). *In vivo*, infusion of human recombinant IL-1β into both the striatum and cortex failed to cause any overt neurotoxicity in its own right, but could significantly potentiate the damage caused by administration of excitotoxins (Lawrence *et al.,* 1998). Interestingly, while there is an exhaustive body of research outlining the inflammatory affects of IL-1β in CNS disease states (Allan *et al.*, 2001, Allan *et al,* 2005, Rothwell *et al.,* 2003, Basu *et al.,* 2004), IL-1β is expressed at high levels in the brain during prenatal and postnatal development (Giulian *et al.*, 1988, Mizuno *et al.,* 1994) and its expression declines to low constitutive levels in the normal adult brain (Vitkovic *et al.,* 2000). It has also been shown that remyelination is dramatically reduced in the absence of IL-1β (Mason *et al.*, 2001), and that IL-1β can induce oligodendrocyte proliferation and maturation (Vela *et al.*, 2002). Taken together, this previous research may indicate a role for IL-1β or the proteins it induces, in normal neurodevelopment.

To clarify the role of β-ARs in the regulation of the IL-1 system *in vivo*, the selective β_2-AR agonist, clenbuterol was used in this study. It appears that clenbuterol treatment, mimicked that of combined NRI/α_2-AR administration, in that it induced mRNA expression of both cortical and hippocampal IL-1β, IL-1ra and IL-R(II), however it does so to a much greater extent. As previously observed with selective β-AR treatment, there was no alteration in the expression levels of either IL-1R(I) or IL-1R(Acp). What is clear from this study is that the temporal expression profile of both ligands and IL-1R(II) is not the same. Maximal IL-1β expression is observed after 1 hour post-treatment, with expression reduced to near basal levels by 8 hours post-treatment. In contrast, IL-1ra and IL-1R(II) expression peaked after 4 hours with IL-1R(II) protein expression being observed at 8 hours. From the parameters of this study it is

unclear whether this observation has any functional consequences or whether the later peak in expression of IL-1ra and IL-1R(II) is a result of early IL-1β induction. It has previously been reported that cAMP analogues and PKA can induce the activation of both NF-κB and IL-1-inducible genes (Shirakawa *et al.*, 1989, Mizel *et al.*, 1990, Chedid and Mizel, 1990). The promoter for the IL-1β gene has multiple *cis*-acting regulatory sites including those for the transcription factors HIF, NFκβ, NFIL-1βA-, AP1 (cJun, cFos), ATF2, CEBPβ, and Elk1 that enables IL-1β mRNA to be transcribed in response to a diverse range of stimuli (Basu *et al.*, 2004, Auron *et al.*, 1998). The promoter for the IL-1ra gene also contains consensus sequences for possible NF-κB-, NFIL-1 beta A-, AP-1-, and CRE-binding sites (Smith *et al.*, 1992). As such, both IL-1β and IL-1ra can be induced by a range of cell-type and stimuli specific agents. Thus, as both IL-1β and IL-1ra promoters share many of the same transcription factor binding sites, further investigation will be required to ascertain whether selective β-AR agonists can preferentially induce expression of IL-1ra/IL-1R(II) over IL-1β.

To further clarify that the modulatory affects of clenbuterol in this study on the central IL-1 system were attributed to β-AR activation, pre-treatment with the β-AR antagonist, propranolol, attenuated clenbuterol induced increases in both cortical and hippocampal IL-1β, IL-1ra and IL-1R(II). Furthermore, clenbuterol induced CREB phosphorylation, as previously mentioned, a marker of β-AR-induced signalling, was abolished with propranolol pretreatment. The same treatment regime with a second selective β_2-AR agonist, fomoterol, mimicked clenbuterol's effects on the central IL-1 system (although, to a lesser degree of magnitude). Both β_2-AR agonists were used at the same dose (0.5 mg/kg) so the difference in the levels of magnitude of gene transcription might reflect a combination of differences in the lipophilicity and ability to cross the blood brain barrier, the pharmacodynamics and the metabolism of both agents. One may argue that as fomoterol also induces IL-1β mRNA expression as well as IL-1ra and IL-1R(II), this may rule out the possibility that the large clenbuterol-induced changes in the IL-1 ligands observed were due to any adverse side affects or toxicity. Consistent with this fact, acute clenbuterol treatment failed to alter the cortical expression of the apoptotic markers, bcl-2, bax or caspase-3, at any of the time points assessed (1-8 hours). These results are in agreement with

previous literature which showed that clenbuterol treatment (at the same dose used in this study; 0.5 mg/kg) failed to alter hippocampal expression of either bcl-2 or bax. However, in experimentally induced ischemia, pre-treatment with clenbuterol induced anti-apoptotic bcl-2 and reduced pro-apoptotic bax expression (Zhu *et al.*, 1999). The expression of the anti-inflammatory NFκB inhibitor, IκBα, was also assessed in the present study as an indication of possible inflammatory IL-1β signalling. However, cortical and hippocampal IκBα mRNA expression was unchanged by acute treatment with clenbuterol. While fomoterol induced a similar magnitude of RNA expression for IL-1ra and IL-1R(II) as clenbuterol, it only induced a modest 2-3 fold increase in IL-1β expression compared to clenbuterol's 50-fold induction. This may potentially reflect unwanted anabolic side affects associated with clenbuterol's high lipophilicity, however, the exact differences in the pharmacokinetics and their possible role in this discrepancy was not addressed in this body of work.

This study also showed an increase in markers of both microglial and astrocytic activation by clenbuterol (at 8 hours post-treatment). As clenbuterol has previously been shown to be neuroprotective via its ability to induce the production of neurotrophins from astrocytes, astrocyte activation (as measured by an increase in the astrocyte cytoskeletal protein, GFAP) was not surprising. The ability of selective β2-ARs such as clenbuterol to induce neuroprotection has been attributed to there ability to induce nerve growth factor (NGF) (Culmsee *et al.,* 1999). In fact, clenbuterol-induced NGF protects hippocampal neurons from excitotoxic cell death *in vitro* and ischaemia induced toxicity *in vivo* (Semkova *et al.*, 1999, Culmsee *et al.*, 1999a, Culmsee *et al.*, 1999b). Clenbuterol has also been reported to induce astrocytic release of NGF, brain derived neurotrophic factor (BDNF), and transforming growth factor-beta (TGF-β), all shown to be neuroprotective molecules (Hayes, *et al.*, 1995, Junker *et al.*, 2002, Zhu *et al.,* 2001). The protective affects of clenbuterol pretreatment on a mouse model of amyotrophic lateral sclerosis (ALS) have also been attributed to the its induction of insulin-like growth factor (IGF-1) (Teng *et al.,* 2006).

It was previously mentioned that IL-1β can have neuroprotective effects under certain conditions and it has been proposed that the neurotrophic actions of IL-1

are mediated via the induction of various neurotrophic factors, including NGF. Consistent with this, the ability of IL-1β to inhibit NMDA-induced neuronal toxicity *in vitro* was mediated via NGF (Strijbos *et al.*, 1995). In a similar study, IL-1β (10-50 ng/ml) conferred protection to NMDA-treated cortical neurons and pre-treatment with an anti-NGF antibody abolished this affect (Carlson *et al.*, 1999). The previously mentioned inability of IL-1β-knockout mice to remyelinate effectively was attributed to a lack of IL-β-induced IGF-1 production from glia. Thus, IL-1β-neurotrophin 'cross-talk' now appears to have important actions on neuronal survival. In this regard, IL-1 released locally from activated astrocytes following CNS injury can stimulate gene transcription of NGF and stabilization of NGF mRNA (Lindholm *et al.*, 1987), however, this may be secondary to its inflammatory effects.

Considering these findings, and the previously mentioned literature showing a neuroprotective role for clenbuterol, it would seem plausible that the β_2-AR-induced expression of IL-1β in the rat CNS observed in this study may not confer any detrimental affects. Especially considering the fact that the peak IL-1β expression is at 1 hour-post treatment, and its expression dissipates by 8 hours, followed by induction of IL-1ra and Il-1R(II). However, further studies are required to asses all of the IL-1β-induced gene profile including MMP-9, iNOS, COX2 and neurotrophins. The impact of Long term chronic treatment with β_2-AR's will also highlight any adverse effects of inflammatory gene transcription, particularly IL-1β.

4.4 Effects of increasing central noradrenergic tone or direct stimulation of the central β_2-ARs on expression of other pro- and anti-inflammatory cytokines in the CNS

As has been previously stated, there is now a body of literature to demonstrate that pharmacological agents such as antidepressants or β_2-AR agonists, display an array of anti-inflammatory and neuroprotective properties within the CNS. However, the majority of this research focuses on the impact of experimentally-induced inflammation or animal models of disease. There is little understanding as to the cellular mechanisms of how increased central NA tone and subsequent β_2-AR activation, influence the brains immune system during 'healthy'/basal conditions, and thus, how those mechanisms transform during

immune dysregulation observed in disease states. In conjunction with the assessment of antidepressants and selective β_2-AR agonists to alter the expression profile of the IL-1 system members within the CNS, this study also assessed the effect of central β_2-AR activation on other pro- and anti-inflammatory cytokines associated with neurological diseases. Of particular interest, was the observation that both the reboxetine/idazoxan combination and selective β_2-AR agonist clenbuterol-induced expression of the broad-spectrum anti-inflammatory cytokine IL-10 in both cortex and hippocampus. While the physiological role of NA in influencing IL-10 in glial cells has not been previously documented, a potentially important observation in the literature is that NA can enhance the synthesis of IL-10 in human mononuclear cells, in parallel with its ability to suppress production of pro-inflammatory cytokines under LPS stimulated conditions (Siegmund *et al.*, 1998).

CNS β_2-AR activation induces IL-10, while reducing TNF-α expression in the rat cortex and hippocampus: In this study, acute treatment with the NRI reboxetine, failed to modify expression of the pro-inflammatory cytokines IL-6 and TNF-α, or the anti-inflammatory cytokines IL-4 and TGF-β, in either the cortex or hippocampus. However, reboxetine induced a significant increase in the cortical and hippocampal mRNA expression levels of IL-10. This was augmented by co-treatment with the α_2-AR antagonist idazoxan. Moreover, this increase in IL-10 mRNA expression was accompanied by an increase in the levels of cortical IL-10 protein secretion after reboxetine/idazoxan co-treatment.

As the previous studies have shown that the use of centrally acting selective β_2-AR agonists, induce a more potent effect of treatment than combined reboxetine/idazoxan co-treatment, at least on the IL-1 system, the impact of clenbuterol on these cytokines was also assessed. Clenbuterol markedly reduced mRNA expression of the pro-inflammatory cytokines IL-6 and TNF-α 1hour post-treatment, however a significant rebounding increase in the expression levels of TNF-α was observed in the cortex 8 hours post-treatment. Whilst clenbuterol failed to alter the expression of the anti-inflammatory cytokines IL-4 or TGF-β, it induced a robust increase in IL-10 expression after 4 hours. As such, the modest increase in cortical TNF-α expression may be a result of the earlier increase in IL-10, as both of these cytokines have been

shown to regulate each other *in vivo.* The increase in IL-10 gene expression was also accompanied by a significant increase in cortical IL-10 protein expression, which was observed up to 8 hours post-treatment. These data give a further insight into the multiple cytokine expression profiles that the catecholamine-influenced brain can affect. The ability of β-AR activation within the CNS, to negatively regulate the pro-inflammatory cytokines TNF-α and IL-6 while conversely, enhancing the expression of the anti-inflammatory IL-10, independent of an immune stimulus, adds to the hypothesis that NA/β-AR provides an endogenous immunomodulatory mechanism within the healthy brain to self-regulate inflammation. However, the β-AR-induced increase in IL-β and the late increase in TNF-α observed in these studies points towards a more complex modulation of cytokine expression than simply being broadly anti-inflammatory.

IL-10 has recently been shown to promote the survival of both neuronal and glial cells during models of excitotoxicity, traumatic brain injury and inflammation *in vitro* (Molina-Holgado *et al.*, 2001, Pahan *et al.,* 2000, Bachis *et al.*, 2001) and in vivo (Kelly *et al.,* 2001, lynch *et al.,* 2004, Spera *at al.*, 1998, Knoblach *et al.,* 1998). Infact, IL-10 has been reported to have protective properties in a host of CNS inflammatory conditions. IL-10 also down-regulates microglial expression of MHC II (O'Keefe *et al.*, 1999) and microglial expression of the inflammatory chemokine RANTES (Hu *et al.,* 1999), it reduces bacterial endotoxin and Aβ(1-42)-induced CNS inflammation (Szczepanik *et al.,* 2001), reduces the expression of IL-6 and IL-2 ligands and receptors on microglia (Sawada et al., 1999), and can inhibit the course of EAE progression (Bettelli et al., 1998). The importance of IL-10 in regulating the immune response is most evident from experiments using the IL-10 knock-out mouse [IL-10($^{-/-}$)]. These mice develop enterocolitis and subsequent colon cancer, following an un-checked Th1-pro-inflammatory cytokine response (Berg *et al.*, 1996, Davidson *et al.*, 1996, Kuhn *et al.,* 1993). It had been previously postulated that the effects of IL-10 in the CNS was controlled by neurons (Vitkovic *et al.*, 2001), and the results of this study confirm a direct mechanism and immunoregulatory role for neuronal NA in IL-10 expression. As increased central NA and selective β-AR activation also induced a robust increase in IL-1β also, future studies

using IL-1R(I) KO mice would rule out any involvement of IL-1β with the induction of IL-10.

β_2-AR's mediate the induction of CNS IL-10 expression and signalling: Consistent with the previous results of this study showing that β-AR's are responsible for the immunomodulatory affects of NA within the CNS, the ability of both combined reboxetine/idazoxan treatment and activation of the β-AR with clenbuterol, to induce IL-10 expression, were completely blocked by a pre-treatment with propranolol. Acute treatment with a second selective β_2-AR agonist, fomoterol, mimicked clenbuterol's induction of IL-10 mRNA expression.

Moreover, as it is now well accepted that STAT3 phosphorylation following IL-10 receptor activation, is one of the major sources of SOCS3 induction in innate immune cells (Qin *et al.*, 2007, Berlato *et al.*, 2002), combined reboxetine/idazoxan as well as clenbuterol treatment, independently induced an increase in cortical STAT3 phosphorylation. Interestingly, both of the previously mentioned treatment regimes (reboxetine/idazoxan and clenbuterol), up-regulated the cortical and hippocampal mRNA expression of SOCS3. The induction of SOCS3 by IL-10 has been implicated in IL-10's ability to inhibit the signaling of pro-inflammatory cytokines such as IL-6, IFN-γ and TNF-α. By mutating the SHP-2/SOCS3 binding sites of the IL-6 receptor gp130 subunit, mice develop a rheumatoid arthritis-like joint disease with increased production of Th_1-pro-inflammatory cytokines and auto-antibodies (Atsumi *et al.,* 2002). The CNS expression of SOCS3 is dramatically up-regulated following peripheral administration with the bacterial endotoxin, IL-6, LIF and IL-1β, in the hypothalamus and pituitary (Lebel *et al.,* 2000, Auernhammer *et al.*, 1998). As previously mentioned, the localization of SOCS3 to hypothalamic neurons and its marked up-regulation by different cytokines suggests that it plays an important role in cytokine-directed neuro-imuno-endocrine functions. In the present study, it appears that SOCS3 may be induced by selective β_2-AR activation, independent of extracellular IL-10 concentrations and STAT3 signalling. This is evident from the fact that IL-10 protein was increased at 4-8 hours post-treatment with clenbuterol, in conjunction with a maximal phosphorylation of STAT3 at 4 hours. Whereas, the temporal profile of SOCS3 expressions appeared to mirror that of IL-10. This might indicate the same

cAMP-driven mechanism for induction of both IL-10 and SOCS3 in the CNS. The fact that pre-treatment with propranolol attenuated reboxetine/Idazoxan- and clenbuterol-induced SOCS3 expression further points towards a cAMP-dependent mechanism. These results are in agreement with two recent findings, that show the SOCS3 promoter has cAMP responsive elements and cAMP-inducing neuropeptides in the pituitary can mediate gene expression of SOCS3 in a PKA-dependent manner (Bousquet *et al.*, 2001). In addition, it was shown that exchange protein activated by cAMP (Epac), induced SOCS3 expression in vascular endothelial cells (Sands *et al.*, 2006). As IL-6 can also induce STAT3 phosphorylation, future studies could asses the expression levels of the IL-6 family members in the brains of reboxetine/Idazoxan and clenbuterol treated rats, to further asses the role of IL-10 and β-AR's in the induction of SOCS3.

4.5 Concluding remarks

There is now well documented evidence of the pro-inflammatory and neurotoxic affects of IL-1β expression in CNS diseases such as experimentally induced ischaemic, excitotoxic, and traumatic brain injury, Alzheimer's disease, Multiple Sclerosis, HIV associated dementia and Parkinson's disease (see Allan & Rothwell *et al.*, 2001, Rothwell & Luheshi, 2000, for reviews). Overall the results of this body of research shows that *in vitro* exposure of glial cells to NA negatively regulates the IL-1 system by inducing expression of IL-1ra and IL-R(II), independent of any increase in IL-1β expression. The data also clearly demonstrate that these anti-inflammatory action are mediated by β-AR activation and activation of PKA and ERK signaling. Moreover, negative regulation of the IL-1 system by NA has functional consequences for neurodegeneration, as NA also inhibited IL-1β induced neurotoxicity.

Furthermore, *in vivo* studies conducted using pharmacological treatments that either enhance the central availability of NA, or directly stimulate central β_2-ARs were shown to impact upon the IL-1 system in the CNS. In contrast to the *in vitro* situation where NA and β_2-AR stimulation increased IL-1ra and IL-1R(II) without any alteration in IL-1β production, increasing central noradrenergic tone or direct stimulation of central β_2-ARs *in vivo* induced IL-1ra and IL-1RII, but

also induced IL-1β. Based on the time course study conducted with the centrally acting β_2-AR agonist clenbuterol, IL-β was produced first, and this was followed by prolonged expression of the negative regulators IL-1ra and IL-1R(II). Previous literature has shown that IL-1β may also be protective to neurons under certain conditions by inducing the release of astrocytic neurotrophins. Functional evidence for this hypothesis stems from the ability of IL-β to inhibit NMDA-induced neuronal toxicity *in vitro*, mediated via NGF (Strijbos *et al.*, 1995). In addition, IL-1β conferred protection to NMDA-treated cortical neurons and pre-treatment with an anti-NGF antibody abolished this affect (Carlson *et al.,* 1999). In this regard, clenbuterol has demonstrated multiple neuroprotective and anti-inflammatory affects in experimental models of CNS inflammation and disease (Semkova *et al.*, 1999, Culmsee *et al.*, 1999a, Culmsee *et al.*, 1999b, Hayes, *et al.*, 1995, Junker *et al.*, 2002, Zhu *et al.,* 2001, Teng *et al.,* 2006) so it would seem unlikely that this early induction of IL-1β after 1 hour post-treatment would confer detrimental effects, it is more likely that prolonged or chronic expression of IL-1 would have detrimental effects for neuronal survival.

In addition to having actions on the IL-1 system, combined NRI/α_2-AR treatment or β_2-AR agonist treatment induced a profound induction of IL-10 expression and signalling in the CNS. The selective β_2-AR agonist, clenbuterol, also inhibited expression of the pro-inflammatory cytokines TNF-α and IL-6. As mentioned previously, the general understanding is that IL-10 activates STAT3 signalling which subsequently induces SOCS3 expression. However, this study demonstrated that the β_2-AR-induced increase in SOCS3 preceded the increase in IL-10 protein expression, therefore suggesting that the increase in SOCS-3 induced by β_2-AR stimulation occurs independent of IL-10. Most likely this is due to direct induction of SOCS-3 by β_2-AR-induced increases in cAMP that precede IL-10 production, in this regard it is well established that SOCS3 expression can be induced by cAMP (Bousquet *et al.*, 2001).

Taken together, the results of this study demonstrate that acute increases in central NA tone and β_2-AR activation, has the propensity to regulate the immune phenotype within the CNS under basal conditions and thus, may act as

an endogenous neuroprotective mechanism in conditions were inflammation contributes to disease progression. In accordance with this, the clinical use of agents such as NRI's and α_2-AR antagonists may prove useful in modulating the endogenous immunomodulatory potential of catecholamines like NA during conditions associated with CNS inflammation.

5. Future Directions

The research presented in this thesis has yielded a number of important leads for future research as outlined below.

1. Future research should focus on determining the efficacy of chronic treatment with NRIs at promoting an anti-inflammatory cytokine phenotype in the brain. In this regard, the acute reboxetine + idazoxan drug combination used in the present investigation is thought to mimic the extent of noradrenergic enhancement that occurs following chronic treatment with NRIs such as reboxetine. Chronic *in vivo* studies will be necessary to determine if the acute immunomodulatory effects observed in this thesis persist following long term treatment with these drugs. In parallel with assessing inflammatory measures, the impact of chronic NRI treatment on β_2-AR expression in the CNS should be assessed, as alterations in β_2-AR receptor density could impact upon the ability of NRIs to elicit anti-inflammatory actions following repeated treatment. Similarly, it needs to be determined if chronic treatment with β_2-AR agonists such as clenbuterol to elicit anti-inflammatory and neuroprotective effects following chronic treatment.

2. Future research should examine the efficacy of NRIs in neurodegenerative disease models such as experimental stroke, Alzheimer's disease models and EAE, all of which have a significant neuroinflammatory component that contributes to disease pathology.

3. The impact of clenbuterol administration in IL-1R1 knockout mice should be examined in order to determine the role of IL-1 in the neuroprotective effects of clenbuterol. For instance, could IL-1-induced NGF production underlie the neuroprotective effects of noradrenaline?

4. It is known that IL-10 can induce IL-1ra and IL-1RII in various cellular systems. To determine if IL-10 plays a role in the induction of central IL-1ra and IL-1R(II) expression that occurs in response to clenbuterol, these experiments should be repeated in IL-10 knockout mice.

5. Based on the results of the clenbuterol time-course experiment presented in this thesis it appears that the β_2-AR-induced increase in SOCS-3 occurs independent of IL-10. This finding should be confirmed using IL-10 knock out mice. In addition, the ability of clenbuterol to alter expression of other SOCS family members in the CNS should be examined.

6. Novel IL-10-family cytokines and their corresponding receptors have recently been identified, but their levels of expression (if any) and function in the CNS has not been examined to date. As activation of β_2-ARs can induce IL-10 expression in the CNS, the impact of β_2-AR to modify expression of novel IL-10 family members such as IL-19, IL-20, IL-22, IL-24 and IL-26 should be examined.

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