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Noradrenergic control of inflammatory processes in the central nervous system

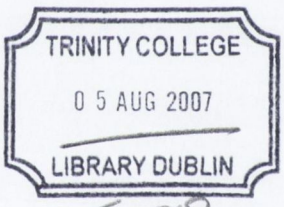
by

Joan B. O'Sullivan

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

Thesis submitted May 2007

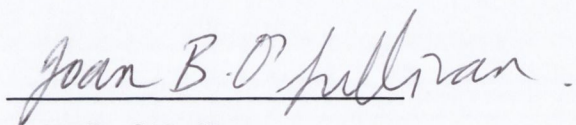
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II Summary

Evidence suggests that the monoamine neurotransmitter noradrenaline (NA) elicits anti-inflammatory actions in the central nervous system (CNS), and consequently may play an endogenous neuroprotective role in CNS disorders where inflammatory events contribute to pathology. In line with this hypothesis, we demonstrate that *in vitro* exposure of primary cortical mixed glial cells to NA suppresses expression of the pro-inflammatory cytokines IL-1β and TNF-α, suppresses induction of iNOS and nitric oxide production, and also suppresses mRNA expression of the chemokines RANTES, IP-10 and MIP-1α in response to the inflammagen lipopolysaccharide (LPS). Interestingly, NA also decreased production of the anti-inflammatory cytokine IL-10, but failed to alter production of the related anti-inflammatory cytokine TGF-β. As previous studies indicate that the noradrenaline reuptake inhibitor (NRI) desipramine (DMI) has anti-inflammatory properties, we examined the ability of DMI, and also the highly selective NRI atomoxetine (ATX), to alter pro-inflammatory cytokine production in mixed glial cells. In addition, as glial cells carry the noradrenaline transporter we hypothesized that NRIs may increase the anti-inflammatory actions of NA by inhibiting its reuptake from the culture medium. However, treatment of mixed glial cells with NRIs largely failed to alter inflammatory events induced by LPS, and also failed to enhance the anti-inflammatory action of NA. In contrast to the *in vitro* situation, acute *in vivo* treatment of rats with NRIs elicited an anti-inflammatory phenotype in rat cortex, and to a lesser extent in hypothalamus following systemic LPS administration, as indicated by reduced mRNA expression of the pro-inflammatory cytokines IL-1β and TNF-α, the enzymes iNOS and COX II, the inflammatory chemokines IP-10, RANTES and CINC-1, and the co-stimulatory molecule and glial activation marker CD40. DMI and ATX show similar efficacy at reducing inflammatory processes in the CNS, however, ATX had less of a tendency to induce an anti-inflammatory effect in the periphery, indicated by the results of inflammatory gene expression studies in spleen tissue. The α_2-adrenoceptor (α_2-AR) antagonists idazoxan (IDA), and RX 821002 (RX), were also examined in terms of anti-inflammatory potential. Whilst the results indicate that these agents were less efficacious than NRIs at inhibiting neuroinflammation, both compounds suppressed mRNA expression of CD40, IP-10 and CINC-1 in cortex and hypothalamus. None of the *in vivo* anti-inflammatory effects elicited by NRIs or α_2-AR antagonists could be mimicked by *in vitro* incubation of glial cells with these compounds, arguing against a direct modulatory effect on the inflammatory response in glial cells. Consequently it is suggested that the anti-inflammatory effects observed *in vivo* occur secondary to increased NA availability. Overall, this project has yielded significant insights into the ability of NA augmentation strategies to limit neuroinflammation.

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IV Abbreviations

Aβ	Amyloid beta
AD	Alzheimers disease
ADHD	Attention deficit hyperactive disorder
APP	Amyloid precursor protein
APC	Antigen presenting cell
AR	Adrenoceptor
ATX	Atomoxetine
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
cAMP	3'5'-cyclic adenosine monophosphate
CBP	CREB-binding protein
CINC-1	Cytokine-induced neutrophil chemotaxis
Cl^-	Chloride
CNS	Central nervous system
COMT	catechol-O-methyltransferase
COX-2	Cyclooxygenase-2
CRE	cAMP response element
CREB	CRE binding protein
CSF	Cerebrospinal fluid
CVO	Circumventricular organ
DA	Dopamine
DAG	1,2-diacylglycerol
D□H	Dopamine-β-hydroxylase
DOPA	Dihydroxyphenylanlanine
DHPG	Dihydrophenylglycol
DMI	Desipramine
DSP-4	N-(2-chloroethyl)-N-ethyl-2 bromobenzlamine
EAE	Experimental autoimmune encephalomyelitis
EM	Electron microscopy
EMT	Extraneuronal monoamine trasnporter

EPA	Eicosapentaenoic acid
ERK	Extracellular signal regulated kinase
FCS	Foetal calf serum
FDA	Federal drugs administration
FRET	Fluorescence resonance energy transfer
HRP	Horse radish peroxidase
ICAM	Intracellular cell adhesion molecule
IDA	Idazoxan
IFN	Interferon
IκB	Inhibitory kappa B
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP-10	IFN-inducible protein-10
IP_3	1,4,5-triphosphate
IRAK 1	IL-1 receptor associated kinase
IRF3	Interferon regulatory factor 3
JAK	Janus kinase
LBP	LPS-binding protein
LC	Locus coeruleus
LPS	Lipopolysaccharide
LT	Lymphotoxin
MAO	Monoamine oxidase
MAP	Mitogen activated protein
MAPK	MAP kinase
ME	Metanephrine
MHC class II	Major histocompatibility complex class 2
MIP	Macrophage inhibitory protein
MPP	1-methyl-4phenylpyridinium
RNA	Ribonucleic acid
MS	Multiple sclerosis
MyD88	Myeloid differentiation factor 88
MAL	MyD88-adaptor like
NA	Noradrenaline
Na+	Sodium
NET	Noradrenaline transporter

NGF	Nerve growth factor
NO	Nitric oxide
NFκB	Nuclear factor kappa B
NME	Normetanephrine
NRI	Noradrenline reuptake inhibitor
$ONOO^-$	Peroxynitrite
OVLT	Median eminence and vascular organ of lamina terminalis
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinsons disease
PKAs	cAMP-dependent kinases
PGE_2	Prostaglandin E_2
PNMT	Phenylethanolamine N-methyl transferase
PPAR	Peroxisome proliferator-activated receptor
RBCs	Red blood cells
ROS	Reactive oxygen species
SDS	Sodium dodecylsulphte
SERT	Serotonin transporter
SH2	Src-homology 2
SOCS	Suppressor of cytokine signalling
SSRI	Selective serotonin reuptake inhibitor
STATs	Signal Transducers and Activators of Transcription
TCA	Tricyclic antidepressant
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
TNF	Tumour necrosis factor
TLR	Toll like receptor
TMB	Tetramethyl-benzidine
TIR	Toll / IL-1 receptor
TRADD	TNFR-associated death domain
TRAF6	Tumour necrosis factor receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor-inducing IFNβ

TZD	Thiazolidinediones
VCAM	Vascular cell adhesion molecule
VMAT	Vesicular monoamine transporter

CHAPTER 1

Introduction

1. Introduction

Inflammation is part of a local non-selective immune response that occurs as a result of foreign matter invasion or tissue damage. The inflammatory response consists of increased blood flow, increased capillary permeability and an increase in leucocyte migration to the affected tissue. This normally results in swelling, redness, heat, and pain. While the effects of inflammation are unpleasant, it limits the spread of infection while phagocytic leukocytes clear pathogens and cellular debris. These cells are attracted to the site of injury or infection by chemotactic agents, and invade the tissue via cell adhesion molecules and increased permeability of capillary tissue.

The outcome of an inflammatory response depends on whether the invading pathogen or dead tissue is cleared, and when successful, the response is self-limiting. When the inflammatory response does not succeed, however, a chronic inflammatory reaction can develop.

1.1. Inflammation and the Brain

In general, the inflammatory response is similar in many tissues, with the exception of the brain, which was once considered an immune-privileged organ. It is now accepted that the inflammatory threshold of the brain is higher than that of peripheral tissues, and that brain inflammation does occur, but only when this upper limit is achieved. This suggests an immunosuppressive environment within the brain, rather than the absence of immunoreactivity.

The question then arises as to why an immunosuppressive environment should exist in the brain. Brain cells are secluded in their microenvironment by a skull that prevents much of the direct trauma incurred by other tissue, and the presence of the blood brain barrier (BBB). The BBB consists of a continuous layer of endothelial cells that are surrounded by tightly packed astrocytes. This barrier is strictly regulated and prevents infiltration of large molecules, such as leukocytes into the brain. When the BBB is compromised leukocyte infiltration can occur and can result in brain inflammation. In addition to the role of infiltrating peripheral immune cells in neuroinflammation it is noteworthy that the brain also has resident immune cells called microglia, and

when activated these cells can initiate inflammation in the CNS. It has also been demonstrated that some neurotransmitters have anti-inflammatory effects, namely the catelcholamines noradrenaline (NA) and dopamine (DA) (see Galea *et al.*, 2003 for review; Facchinetti *et al.*, 2004) and acetylcholine (see Gallowitsch-Puerta and Tracey, 2005; Czura *et al.*, 2003). Given the abundance of these neurotransmitters in the central nervous system (CNS) it is probable that they also contribute to an immunosuppressive environment in the brain. Moreover, the direct cell-cell interactions between neurons and microglia can exert a quiescent action on resident immune cells. Such direct communication can occur via the CD200 ligand (CD200L) expressed on neurons with the CD200 receptor expressed on microglia (see Neuman, 2001). Soluble factors from non-neuronal brain cells, astrocytes have also been demonstrated to prevent microglial activation *in vitro* (Wirjatijasa *et al.*, 2002).

CNS inflammation may not readily occur, but when it does it can be slow to resolve. This is due in part, to the BBB, and also to the actions of astrocytes that form scar tissue surrounding damaged areas (gliosis), that interferes with transmission of the neuronal signal. When inflammation fails to resolve a chronic inflammatory state can occur and this results in cell damage and eventual loss of neurons.

1.3. Non-neuronal cells of the CNS: Glia

Glia have numerous functions within the CNS, and comprise the majority of non-neuronal cells in the brain, outnumbering neurons in the ratio of 10:1 (Stevens, 2003.). Within the CNS they comprise the oligodendrocytes, astrocytes and microglia, and have a diverse range of functions in the development and normal functioning of the CNS. The response to injury or infection is primarily mounted by astrocytes and microglia, and as a result much attention has been focused on these cells in particular.

1.3.1. Microglia

Microglia are the resident immune cells in the CNS and are often termed "the macrophages of the brain" as they are believed to derive from blood monocytes that infiltrate the brain during development. Given this lineage, they are similar to macrophages in a number of respects, such as phagocytosis of foreign material and dead cells and the production of cytokines and soluble factors that can exert neuroprotective or neurotoxic effects (see Liu and Hong, 2003). Thus, these cells are considered the innate immune system of the brain, and form the first line of defence in brain infection (see Streit *et al.*, 2004 for review). In the healthy adult brain these cells proliferate slowly, adopt a ramified appearance, and maintain active immune surveillance (see Liu and Hong, 2003, and Vilhardt, 2005 for reviews). They are also extremely important in brain development where they phagocytose apoptotic cells.

Microglia are the first cells to respond to a CNS insult and when thus activated their morphology changes from a ramified state to a reactive semi-amoeboid state and then to an active amoeboid state. Concurrent with the change from ramified to amoeboid, microglia increase their capacity to phagocytose cellular debris and bacteria, present antigen to T-cells and secrete pro-inflammatory mediators.

In a similar manner to peripheral immune cells, when microglia become activated they upregulate a number of cell-surface receptors that enable them to co-ordinate the immune response, including the major histocompatibility complex class two (MHC II) and CD40. MHC II upregulation allows microglia and other antigen presenting cells (APCs) present phagocytosed antigen fragments to CD4+ T cells, termed T-helper cells or Th cells (Roitt *et al.*, 2001). Presentation of antigen will only result in T-cell activation in the presence of co-stimulatory molecules, of which CD40 is one. This cell surface marker is up-regulated on activated microglia and astrocytes (Abdel-Hak *et al.*, 1999; see also Dong and Beneviste, 2001 for review), thus if MHC II is also present both these cell types will be able to present antigen. Upon activation by its cognate ligand called CD40 ligand (CD40L) that is expressed on T-cells, CD40 can promote the release of pro-inflammatory cytokines. CD40 and CD40L interaction on T and B-cells can also result in antibody production (Roitt *et al.*,

2001). Both MHC II and CD40 are used as markers of microglial activation in investigative studies on CNS inflammation.

When microglia are activated by an immune stimulus they release a number of soluble factors that co-ordinate the inflammatory response in the brain. These include the release of cytokines and chemokines that allow the infiltration of peripheral immune cells (such as T-cells, macrophages, dendritic cells and neutrophils) into the brain and direct them to the site of injury. Chemokines and cytokines allow the extravasion of peripheral cells into the CNS by induction of cell adhesion molecules (CAM) such as intracellular CAM (ICAM) and vascular CAM (VCAM) that alter the permeability of the BBB (Moynagh *et al.*, 1994). Infiltration of the CNS parenchyma by peripheral blood cells allows for the secretion of cytokines not only by microglia but also by Th cells and monocytes, thereby contributing to the inflammatory response.

Not all soluble factors released from microglia are harmful, in fact some such as glia-derived neurotrophic factor and anti-inflammatory cytokines can promote cell survival. Many of the soluble factors released by microglia, however, can promote neurotoxicity such as pro-inflammatory cytokines and chemokines, matrix metalloproteinases, lymphotoxin (LT), nitric oxide (NO) and reactive oxygen species (ROS), (for reviews see Liu and Hong, 2003; Liberto *et al.*, 2004).

Inflammation in the CNS, as elsewhere in the body, results from the sequential release of pro-inflammatory chemokines and cytokines, and its resolution results from the production of anti-inflammatory cytokines and the accumulation of negative intracellular factors. This process initially involves the release of pro-inflammatory chemokines and cytokines that initiate the production of other cytokines, both pro- and anti-inflammatory, in addition to the soluble factors mentioned above.

1.3.2. Astrocytes

Astrocytes are the most numerous glia within the CNS, and have many functions integral to the maintenance of brain homeostasis. They surround blood vessels and neurons where they provide structural support, maintain the

integrity of the blood-brain barrier and regulate ion and neurotransmitter concentrations, respectively (Jessen, 2004; Stevens 2003). Such placement is advantageous in monitoring brain homeostasis and communicating with neurons and other glia via chemokine, cytokine and nerve growth factor (NGF) release. Astrocytes also contain active transport mechanisms by which they can remove neurotransmitters and other substances from extracellular fluid. The removal of glutamate from the extracellular space is an important example of their role in the maintenance of homeostasis as it can prevent the consequences of excitotoxicity resultant from excessive glutamate release. Activated astrocytes increase their capacity to clear glutamate via upregulation of the glutamate transporter and glutamine synthase (Liberto *et al.*, 2004), which is neuroprotective post-trauma. These cells also contain isoforms of the cytochrome P450 enzymes, which can metabolise eicosanoid and oestrogen among other substances (Abdulla and Renton 2005).

An important astrocytic function is the quiescent effects they elicit on neighbouring microglia. Neurons have also been demonstrated to exert a calming effect on microglia and these effects are mediated via the production of soluble factors such as TGFβ_1, and non-soluble cell-cell interactions (Vincent *et al.*, 1997; Zietlow *et al.*, 1999). Under inflammatory conditions astrocytes can also adopt immunological functions, one of which is antigen presentation. In this regard, astrocytic MHC II and CD40 can be up-regulated by inflammatory cytokines (Zeinstra *et al.*, 2000; Abdel-Haq *et al.*, 1999; see Dong and Benveniste, 2001 for review). Moreover, activation of astrocytic CD40 by CD40L has also been demonstrated to result in pro-inflammatory cytokine release by these cells *in vitro* (Abdel-Hak *et al.*, 1999).

1.4. Pathogen recognition

The following studies used lipopolysaccharide (LPS) to induce an inflammatory reaction *in vitro* and *in vivo*. LPS is a component of the cell wall of gram-negative bacteria and is recognised by the innate immune system as a foreign antigen via its pathogen-associated molecular pattern (PAMP). The first process in LPS recognition by the immune system is the binding of LPS by circulating soluble LPS-binding protein (LBP). Once bound by LBP, LPS can

then associate with CD14, a protein necessary for toll-like receptor (TLR) activation by LPS. CD14 exists in two forms, a membrane bound form expressed by a number of cell types, predominantly on those of the innate immune system, and a soluble form that allows cells that do not express CD14 to initiate TLR signalling (see Palsson-McDermott and O'Neill,2004). This complex can then bind to and activate the toll-like receptor 4 (TLR4) to cause downstream intracellular signalling (Janeway *et al.*, 2005). TLR4 has been demonstrated on astrocytes and microglia (Jack *et al.*, 2005).

To date there are 6 identified mammalian TLRs of which TLR4 is the one involved in recognition and response to gram negative bacteria (Bowie and O'Neill, 2000). TLRs are single transmembrane receptors that are part of the TIR (Toll / IL-1 receptor) superfamily. This superfamily of receptors all signal in a similar manner, due to the presence of an cytosolic TIR domain, that can associate with TIR domain-containing adaptor proteins, including myeloid differentiation factor 88 (MyD88) and MyD88-adaptor like (MAL) protein (see Liew *et al.*, 2005; Bowie and O'Neill, 2000, for reviews). As such, activation of TIRs and TLRs result in similar intracellular signalling, and much research has focussed on activation of the NFκB pathway and involvement of the mitogen activated protein (MAP) kinases following ligand binding to TLRs.

Activation of TLR4 by LPS binding results in dimerisation of the receptor and recruitment of either the MyD88 adaptor protein or the TIR domain-containing adaptor-inducing IFNβ (TRIF) adaptor protein. This occurs via the TIR domain on both receptor and adaptor protein. TLR activation can thus result in one of two main signalling pathways, called the MyD88-dependent or independent pathway as described below and depicted in figure 1.4.1.

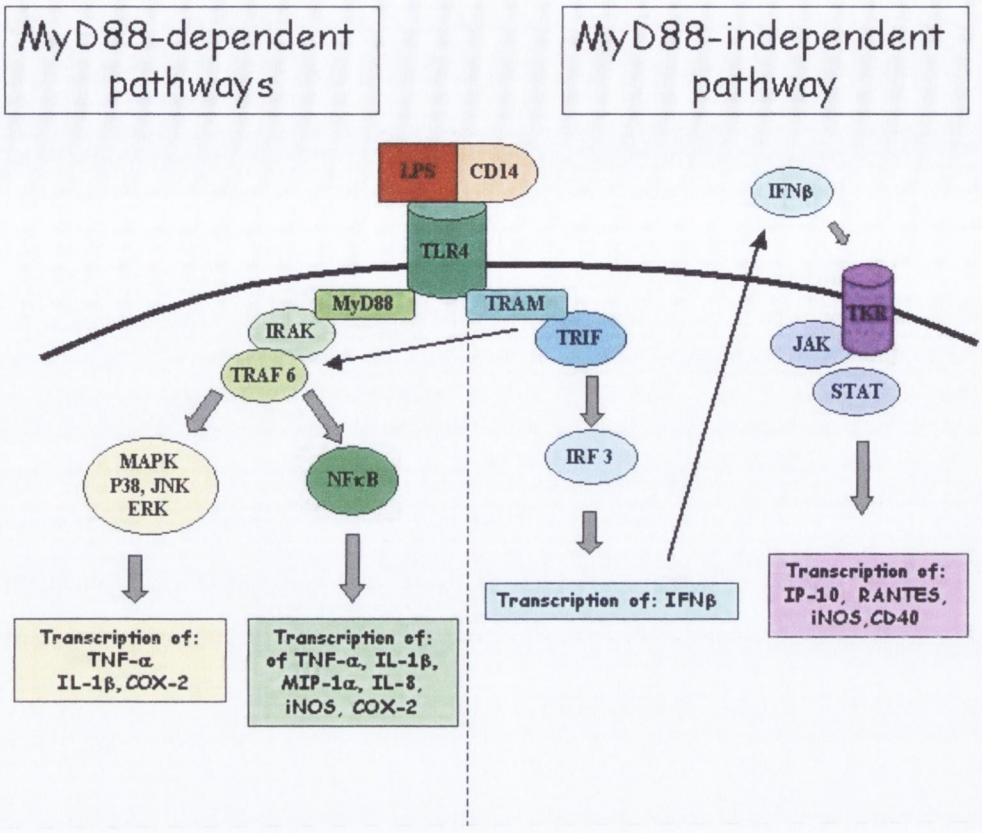


Figure 1.4.1: TLR4 signalling pathways

MyD88-dependent signalling: MyD88 associates with IL-1 receptor associated kinase (IRAK) 1 causing IRAK1 and IRAK 4 phosphorylation. IRAK1 dissociates from MyD88 and activates tumour necrosis factor receptor-associated factor 6 (TRAF6). This activates IκB kinases that phosphorylate inhibitory kappa B (IκB). Phosphorylated IκB is degraded by proteasomes and NFkB translocates to the nucleus to initiate gene transcription of pro-inflammatory cytokines and chemokines. TRAF6 activation can also activate the mitogen-activated protein kinases (MAPKs) resulting in inflammatory gene transcription.

MyD88-independent signalling: The adaptor protein TRIF-related adaptor molecule (TRAM) interacts with TLR4 to recruit TRIF. TRIF then interacts with TRAF6 causing phosphorylation of interferon regulatory factor (IRF) 3. IRF3 translocates to the nucleus and intiates gene transcription of type I interferons (IFN) such as IFNα/β. IFNβ activates its receptor in a paracrine or autocrine fashion resulting in activation of janus kinases (JAKs) that phosphorylate the Signal Transducers and Activators of Transcription (STATs) and initiate transcription of another set of inflammatory genes. TRAF6 activation via TRIF can also result in NFkB or MAPK activation. See also Kawai *et al.*, 2001; Moynagh, 2005; Jung *et al.*, 2005; Liew *et al.*, 2000.

Myd88-dependent signalling: Recruitment of MyD88 causes recruitment and association with the IL-1 receptor associated kinase (IRAK) 1 that leads to phosphorylation of IRAK1 and IRAK 4 and causes dissociation of IRAK1 from MyD88 allowing it to activate tumour necrosis factor receptor-associated factor 6 (TRAF6) (see Liew *et al.,* 2005 and Moynagh, 2005 for reviews). Interaction of IRAK with TRAF6 leads to activation of IκB kinases that phosphorylate inhibitory kappa B (IκB). The IκB protein is responsible for sequestering the transcription factor nuclear factor kappa B (NFκB) in the cytosol. Phosphorylation of IκB leads to its degradation by proteasomes allowing the translocation of NFkB to the nucleus where it binds with the 3',5'-cyclic adenosine monophosphate cAMP (cAMP) responding element (CRE)-binding protein (CREB)-binding protein (CBP) / p300. This allows the initiation of inflammatory gene transcription, including that of the pro-inflammatory cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α, IL-12, the chemokines macrophage inflammatory protein 1- α (MIP-1α, also called CCL3) and cytokine-induced neutrophil chemotaxis (CINC-1 the rat analogue of IL-8 [also called CXCL8]) and the inducible isoform of nitric oxide synthase (iNOS) (Jung *et al.*, 2005; see Beutler *et al.*, 2003; Kaibori *et al.*, 1999).

MyD88-independent signalling: TLR4 has also recently been demonstrated to signal via a MyD88-independent pathway (Hoebe *et al.*, 2003). This involves the recruitment of the adaptor protein named TRIF (see Hoebe *et al.,* 2003 and Jung *et al.*, 2005). TRIF requires the adaptor protein TRIF-related adaptor molecule (TRAM) to+ interact with TLR4. Upon recruitment TRIF can interact with TRAF6 to induce NFkB signalling (see Moynagh 2005, for review), or it can also cause phosphorylation of interferon regulatory factor (IRF) 3. Upon phosphorylation IRF3 translocates to the nucleus, where it binds to the transcription co-activator CBP/p300 to cause gene transcription of type I interferons (IFN) such as IFNα/β (see Moynagh, 2005; Hoebe and Beutler, 2004). Upon release IFNβ can activate its receptor in a paracrine or autocrine fashion. Binding of IFNβ to its receptor results in activation of janus kinases (JAKs) that phosphorylate the Signal Transducers and Activators of Transcription (STATs) to cause gene transcription of a different set of inflammatory genes to that induced by NFκB (see Moynagh, 2005, and Beutler

et al., 2003, for reviews). Genes transcription induced by TRIF-dependent TLR and subsequent IFNβ signalling include the chemokines IFN-inducible protein-10 (IP-10, also called CXCL10), Regulated upon Activation, normal T cells Expressed and Secreted (RANTES or CCL5) the enzyme iNOS, and the co-stimulatory molecule CD40 (see Beutler *et al.*, 2003; Hoebe and Beutler, 2004; Hoebe *et al.*, 2003).

Both these pathways can operate in a synergistic manner, and activation of both is necessary for activation-induced apoptosis in microglia (Jung *et al.*, 2005). There also appears to be cross-talk between the two pathways as MyD88 deletion still results in NFκB signalling, although it is somewhat delayed (see Beutler *et al.*, 2003; Kawai *et al.*, 1999).

Mitogen-activated protein kinases (MAPKs): Both pathways can also lead to MAP kinase signalling via TRAF6 that can activate a number of different kinases, LPS binding has been demonstrated to cause phosphorylation and activation of the kinases p38, c-Jun N-terminal protein kinase (JNK) or extracellular signal-regulated protein kinase (ERK) (see Beutler *et al.*, 2003; Huang *et al.*, 2004). Phosphoryated JNK can then associate with and phosphorylate the N-terminal domain of the transcription factor c-Jun leading to gene transcription. There is also evidence that JNK can phosphorylate a number of other transcription factors and non-nuclear genes such as those on mitochrondria, including Bcl2 family that can lead to Bax-dependent apoptosis (see Bogoyevitch, 2004). Thus, activation of the JNK MAPK pathway can lead to mitochrondrial-induced apoptosis or gene transcription. It appears that the p38 and JNK MAPKs are also important for pro-inflammatory signalling, indeed, it has been reported that inhibition of p38 can inhibit both TNF-α, IL-1β and IL-10 signalling (Foey *et al.*, 1998; also see also Saklatvala, 2004 for review). ERK activation on the other hand does not appear to be necessary for TNF-α or IL-1β induction (Uesugi *et al.*, 2006) although it can exert a synergistic action on mRNA stability induced by p38 (Brook *et al.*, 2006). Both ERK and p38 activation are believed to be necessary for IL-10 induction following LPS administration (Maloney *et al.*, 2005; Loscher *et al.*, 2005).

Activation of TLR4 by LPS can thus result in NFκB or IRF signalling, or activation of the MAPK pathways. Release of IFNβ resultant from IRF activation can also lead to JAK/STAT signalling, and it is via the multiplicity of signalling pathways that immune cells can invoke a repertoire of responses that ultimately serve to protect the host from infection. Deleterious effects of inflammation often result from sustained inflammation and recruitment of circulating lymphocytes into the brain can precipitate specific (such as MS) and non-specific neuronal damage (cell death).

1.5. Mediators of inflammation

Chemokines, cytokines and cell products such as NO and prostaglandins play a pivotal role the inflammatory response. Chemokines are responsible for attracting immune cells to an area of inflammation and can also have direct actions on target cells. Cytokines mediate their effects by activating specific receptors that initiate intracellular signalling that results in gene transcription (Roitt *et al.*, 2001).

1.5.1. Chemokines

Chemokines are very small (8-14 kDa) chemotactic proteins that regulate leukocyte migration under normal and pathogical conditions. During development both chemokines and cytokines are responsible for cell migration, proliferation and survival (Mehler and Kessler, 1997; Aroujo and Cotman, 1993). Chemokines can be directly induced by inflammatory molecules such as LPS or by pro-inflammatory cytokines. They are released by a number of cell types, including endothelial cells, macrophages, monocytes, microglia and astrocytes (Mennicken *et al.*, 1999).

There are four families of chemokines, so delineated on the basis of their cysteine residue position. Of these four branches the α- and β-chemokine comprise the largest members. The α-chemokines are also termed the CXC chemokines as there is one amino acid separating the cysteine residues and these chemokines can only signal via CXC receptors. The β-chemokines are also called the CC chemokines as their first two cysteine residues are adjacent to one another (see Luster, 1998; Charo and Ransohoff, 2006; Ubogu *et al.*,

2006 for reviews). Similarly to the CXC chemokines, the CC chemokines can only signal via CC receptors. The other two chemokine families are the CX3C and C chemokines, called the δ- and γ- chemokines, respectively.

Upon release by microglia and astrocytes chemokines attract leukocytes and lymphocytes to the site of inflammation / injury in a concentration-dependent manner (see Ubogu *et al.*, 2006). In addition to their chemotactic effects, they can also exert direct actions on cells. In this regard, it has been demonstrated that monocyte chemoattractant protein (MCP)-1 can directly alter integrity of endothelial tight junctions to allow infiltration of peripheral immune cells (Song and Pachter, 2004).

Chemokines of note in CNS inflammation and pathology include, but are not restricted to: IL-8, and IP-10 (members of the CXC family and termed CXCL8 and CXCL10, respectively) and MIP-1α and RANTES, (members of the CC chemokine family and termed CCL3 and CCL5, respectively, where L denotes ligand).

Interleukin 8 (CINC-1): IL-8 is not produced in rat or mouse, however rat CINC-1 shares sequence homology with IL-8 and appears to carry out similar functions to this human chemokine by binding to the IL-8 CXC receptor (CXCR)-2 (Ramos *et al.*, 2003; Shibata *et al.*, 2000). This chemokine is chemotactic for neutrophils and monocytes and has been shown to be up-regulated in the cerebrospinal fluid (CSF) but not sera of AD patients (Galimberti *et al.*, 2003). It is released predominantly by microglia *in vivo*, and has been demonstrated to be upregulated in animal models of ischaemic injury resulting from invasion of neutrophils and mononuclear cells (see Ambrosini and Aloisi, 2004). Pharmacological inhibition of IL-8 receptors has recently been demonstrated to limit leukocyte invasion and attenuate tissue damage in a rat reperfusion injury model (Garau *et al.*, 2005). This chemokine can be induced by the pro-inflammatory cytokine IL-1β, concurrent administration of intra-striatal IL-1β and anti-CINC-1 antibody was demonstrated to prevent neutrophil accumulation in the CNS (Anthony *et al.*, 1998). In addition to its

chemotactic properties IL-8 has been shown to induce IL-1β, IL-6 and COX-2 and TNF-α gene expression in human foetal microglia (Franciosi *et al.*, (2005).

Interferon-inducible protein-10: IP-10 (CXCL10) is a potent T-cell and monocyte chemoattractant and has been implicated in a number of CNS pathologies including MS and AD. IP-10 has been demonstrated in the CSF of MS patients, and is expressed by astrocytes in demyelinated MS lesions (Sorenson *et al.*, 2002). Indeed levels of IP-10 have been demonstrated to correlate with disease severity in experimental autoimmune encephalomyelitis (EAE), an animal model of MS and neutralisation of this chemokine ameliorated disease severity in mice (Fife *et al.*, 2001). Increased IP-10 concentrations have also been demonstrated in the CSF but not sera of AD patients (Galimberti *et al.*, 2003).

Regulated upon Activation, normal T cells Expressed and Secreted: RANTES (CCL5) is also chemotactic for T-cells and monocytes and akin to IP-10 is implicated in both MS and EAE (Boven *et al.*, 2000). In a similar manner to IP-10 this chemokine is predominantly expressed by astrocytes (Boven *et al.*, 2000). A greater number of CCR5 (receptor for RANTES and MIP-1α) positive T-cells are evident in the blood of MS patients T-cells while CCR5 positive cells are also evident in MS lesions (Balashov *et al.*, 1999).

Macrophage inflammatory protein-α: MIP-1α (CCL3) is predominantly a macrophage and monocyte chemoattractant, however it can also attract T-cells (Luster, 1998). MIP-1α is proposed to play a role in a number of CNS disorders, including MS and ischaemia (see Ambrosini and Aloisi, 2003). In contrast to both IP-10 and RANTES, MIP-1α is localised to microglia and macrophages in MS (Boven *et al.*, 2000; Balashov *et al.*, 1999). One of the receptors used by this chemokines is also upregulated in MS (Balashov *et al.*, 1999). MIP-1α has also been demonstrated on activated microglia in animal models of stroke (Kim *et al.*, 1995; Takami *et al.*, 1997).

Chemokine receptors are 7-transmembrane G-coupled receptors expressed on subsets of leukocytes. Receptors are believed to be G_i-linked and ligand

binding causes an increase in inositol triphosphate and subsequent protein kinase C activation. Activation of Ras and Rho family proteins also signal for cell motility events (see Luster, 1998). Chemokines can activate more than one receptor and there appears to be a lot of redundancy in the chemokine network. Chemokine receptors are found on a number of immune cells such as eosinophils, mast cells, basophils, microglia, T-cells, B-cells, natural killer cells (NKC), neutrophils and non-immune cells such as astrocytes, neurons and endothelial cells (see Ubogu *et al.*, 2006). The studies detailed herein focus on the role of two α-chemokines IP-10 and CINC-1 and that of the β-chemokines MIP-1α and RANTES. These cytokines have different roles in cellular chemotaxis and are induced by different signalling pathways, (see Table 1.5.1. below).

Table 1.5.1: Summary of chemokine, induction pathways and cellular receptor location

Chemokine	Induction pathway	Receptor	Receptor localisation
CXCL10 / IP-10	IRF3 NFκB*, p38+	CXCR3	Monocytes, T-cells, NKCs
CINC-1 (IL-8 / CXCL8 homologue)	NFκB p38	CXCR1 CXCR2	Neutrophils, monocytes, endothelial cells
CCL5 / RANTES	IRF3 JNK#	CCR1 CCR3 CCR5	Monocytes, T-cells eosinophils
CCL3 / MIP-1α	NFκB	CCR5 CCR8	T-cells, monocytes, basophils, eosinophils

Adapted from Luster, 1998; Charo and Ransohoff 2006; Karpus, 2001

*Kawai *et al.*, (2001); + Shen *et al.*, (2006); # Miyabayishi *et al.*, (2006)

1.5.2. Cytokines

Cytokines comprise a set of structurally diverse small proteins (8-80 kDa) that including the interleukins (IL), interferons (IFN) and colony-stimulating factors (Janeway *et al.*, 2005; Roitt *et al.*, 2001). The actions of these proteins may promote or attenuate the inflammatory process and as such, are generally

classed as pro- or anti-inflammatory. They are produced by immune cells such as macrophages, T-cells, monocytes, dendritic cells, microglia and kupffer cells as well as a number non-immune cell types, including astrocytes, endothelial and neuronal cells. In addition to their immunological functions cytokines are produced under normal physiological conditions and have a number of functions including cell growth and differentiation and cell-cell communication (Roitt *et al.*, 2001).

When immune cells come into contact with a bacterial, fungal or viral immune stimulus or a cell stressor such as ultraviolet light gene transcription of cytokines and chemokines occurs. The type of cytokines and chemokines induced will depend upon the stimulus. For instance, a gram-negative bacterial stimulus will promote gene transcription of the interleukins and tumour necrosis factor downstream of TLR4 signal transduction, whereas a double stranded RNA viral stimulus will predominantly induce IFN gene transcription following activation of TLR3 (Liew *et al.*, 2005).

Pro-inflammatory cytokines

The pro-inflammatory cytokines interleukin-1β & TNF-α are among the first pro-inflammatory mediators to be released in response to microglial activation (Liberto *et al.*, 2004), and they induce the production of other pro-inflammatory cytokines (termed the "cytokine cascade") and production of the neurotoxic product. As such, they are considered to be the primary mediators of the inflammatory response within the CNS. Other pro-inflammatory cytokines that are produced by infiltrating leucocytes within the brain parenchyma include IL-12, IFN-γ and IL-18.

Tumor necrosis factor-α: Much research has focused on TNF-α as it is the first cytokine to appear in systemic circulation following a bacterial stimulus and can induce production of a number of other cytokines in addition to pro-inflammatory mediators such as iNOS. This cytokine can have proliferative or toxic effects that can lead to cell survival or apoptosis, and this can depend upon the receptor activated, and concentrations of TNF-α present. TNFRs are divided into two main classes, those that contain a death domain and can

mediate apoptosis (cell death) and those that do not (see Aggarwal, 2003). TNFR1 (also called p55) and TNFR2 (also called p75) have distinct and common signalling pathways. TNFR1 contains a death domain and has been demonstrated on a variety of cell types including endothelial and tissue-resident cells such as astroytes, neurons and oligodendrocytes in addition to those of the immune system (Kuno *et al.*, 2006; Ohtori *et al.*, 2004; Buntinx *et al.*, 2004). TNFR2 does not contain a death domain and is predominantly expressed on immune cells, however it has also been demonstrated on astrocytes (Kuno *et al.*, 2006; Aggarwal, 2003).

Ligand activation of TNFR1 can induce apoptosis by association with TNFR-associated death domain (TRADD) protein that leads to recruitment of caspase 8 and activation of caspase 3. TRADD can also associate with TRAF2 to activate the protein kinases p38 and/ or JNK. TRAF2 can also lead to phosphorylation of IκB and subsequent NFκB signalling, as previously described for LPS. Binding of TNF-α to the TNFR2 does not induce apoptosis directly as this receptor does not contain a death domain. Instead it associates with TRAF2 to initiate either MAP kinase (p38) or NFκB signalling that can then induce the production of other pro- and anti-inflammatory cytokines and pro-inflammatory mediators such as iNOS, (Akama *et al.*, 2000). In a manner similar to LPS, this cytokine can elicit a number of different effects depending on the signalling pathway utilised, however, NFκB is the one most focused upon in the literature.

The response to TNF-α will depend on the cell to which it signals and the receptors found there. Much will also depend on extracellular concentrations of TNF-α as high concentrations of this cytokine results in an overwhelming inflammatory response that can end in endotoxic shock and death. This can occur when large amounts of NO are released and cause vascular relaxation and a drop in blood pressure following iNOS induction. TNF-α is a potent modulator of the immune response, causing the induction of adhesion molecules, cytokines, chemokines metallaproteases and inflammatory enzymes such as iNOS and cyclooxygenase –2 (COX-2) (Zhang *et al.*, 1998; Aggarwal, 2003). Together with IFN-γ, TNF-α has shown potent cytotoxic

activity towards tumour cells (Nakashima *et al*, 2005). This cytokine can also cause direct cell death of non-tumour cells, including neurons, by TNFR1-induced apoptosis (Yang *et al.*, 2002). This cytokine has been implicated in a variety of neurodegenerative disorders including AD, PD, MS and stroke (see Lucas *et al.*, 2006).

Interleukin-1β: IL-1β is another important cytokine involved in the innate inflammatory response, and can be induced by LPS and TNF. This cytokine binds with IL-1 receptors that belong to the TIR superfamily and thus signal in a similar manner to TLR4. There are two IL-1 receptors (IL-1R), IL-1RI and IL-1RII. The effects of IL-1β are mediated via activation of IL-1R1 and its accessory protein (IL-1RacP) that is necessary for signal transduction. Binding of IL-1β to IL-1R1 causes MyD88 recruitment and downstream phosphorylation of IRAKs and activation of TRAF6 as previously described for TLR signalling. This can lead to NFkB and/or MAP kinase signalling (see Fitzgerald and O'Neill 2000).

IL-1RII is very similar to IL-1R1, however, it has a shorter cytosolic tail that does not allow association with MyD88 and subsequent signal transduction (Fitzgerald and O'Neill 2000). IL-1R1 thus acts as a decoy receptor to bind circulating IL-1β without causing signal transduction.

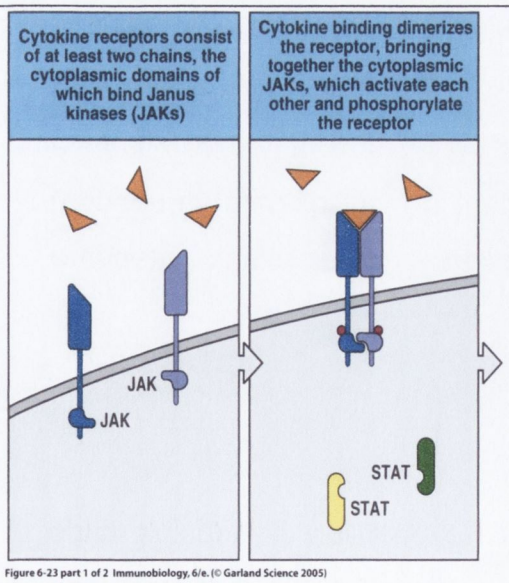
Administration of IL-1β into brain parenchyma results in IL-1β induction and parenchymal neutrophil recruitment (Blond *et al.*, 2002). This cytokine is also responsible for induction of COX-2, chemokines and iNOS expression (Akama *et al.*, 2000; Laflamme *et al.*, 1999; Fujishima *et al.*, 1993). This cytokine is implicated in a variety of neurodegenerative disorders including AD, MS and stroke (see Lucas *et al.*, 2006; Allan *et al.*, 2005).

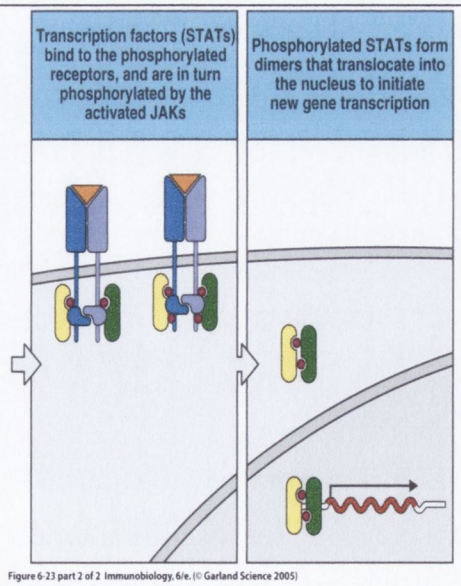
Anti-inflammatory cytokines

Interleukin-10: IL-10 is induced by inflammatory stimuli such as cytokines, LPS and viruses. Its temporal release follows that of pro-inflammatory cytokines and it serves to negatively regulate inflammation by inhibiting pro-inflammatory cytokine release. TNF-α has been demonstrated to induce IL-10 *in vitro*

(Sheng *et al.*, 1995) while IL-1β has also been postulated to play a role in IL-10 induction *in vivo* (Souza *et al.*, 2003). IL-10 has an auto-regulatory action on both IL-1β and TNF-α, and helps in the resolution of the inflammatory response (Ledeboer *et al.*, 2002; Denys *et al.*, 2002). IL-10 can also induce microglial ramification and reduce ICAM-1 expression *in vitro* (Wirjatijasa *et al.*, 2002). Ledeboer and colleagues also demonstrated an auto-regulatory role for IL-10 as it can down-regulate its own production from astrocytes.

IL-10 signals through a kinase receptor called IL-10R. Binding of IL-10 causes dimerisation of the receptor, bringing the cytosolic janus kinases (JAKs) into contact and subsequent phosphorylation of the receptor. This creates sites for Src-homology (SH2) domain-containing signalling proteins and allows association of STATs that are then phosphorylated by the activated JAKs. Phosphorylated STAT dimers then translocate to the cell nucleus where they activate the transcription of a variety of genes (see figure 1.4.2.). Examples of other cytokines that signal via JAK-STAT pathways are the interleukins IL-12, and the IFNs. The longevity of the cytokine signals tranduced by the JAK/STAT pathway is regulated by a family of JAK inhibitor proteins called Suppressors of Cytokine Signaling (SOCS), (Yoshimura *et al.*, 2003). SOCS proteins can also be induced by STATs and thus act in an autoregulatory fashion, (see Baetz *et al.*, 2004).





Reproduced from Immunobiology 2005.

Figure 1.4.2: JAK/STAT signalling pathway

Transforming growth factor β: TGFβ$_1$ is an anti-inflammatory cytokine produced by astrocytes, microglia, and immune cells. This cytokine inhibits production of pro-inflammatory cytokines and NO from macrophages (Xiao *et al.*, 2006), and also prevents microglial activation (Suzumura *et al.*, 1993). Some resports show up-regulation of this cytokine post-LPS and ischaemic damage in animal models (Cunningham *et al*, 2002; Zhu *et al.*, 2001). Divergent reports of LPS-induced changes in this cytokine *in vitro* have been reported, Xiao *et al.*, (2006) demonstrated an increase in macrophage TGFβ, while Vincent *et al.*, (1997) did not observe increases in this cytokine in glia following LPS administration.

IL-1 receptor antagonist: IL-1ra is an anti-inflammatory cytokine that shares structural homology to IL-1β and can bind to IL-1 receptors but does not induce IL-1 signalling. As such it functions as an endogenous competitive antagonist to block the actions of IL-1β. Wirjatijasa *et al.*, (2002) demonstrated that exogenous administration of this cytokine induced microglial ramification in isolated cultured microglia and reduced LPS-induced ICAM expression in rat microglial cultures.

As outlined above there are three main pathways via which cytokine signalling occurs; activation of Janus kinases (JAKS), activation of mitogen activated protein (MAP) kinases or NFκB signalling pathways, all of which can result in gene transcription. The inflammatory cytokines TNF-α and IL-1β are known to activate a number of signalling pathways and these pathways can influence one another to produce different cell-dependent and cytokine(s)-dependent effects. This provides us with a number of potential targets to alter or attenuate inflammatory signalling.

1.5.3. Other inflammatory mediators

LPS and pro-inflammatory cytokines can induce expression of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase II (COX-2), the enzymes responsible for the production of nitric oxide and prostaglandins respectively, two important mediators of inflammation.

iNOS and nitric oxide: iNOS is induced by a number of intracellular signalling molecules following TLR-4 signalling, however, it can also be induced by TNF-α and IL-1β (Akama *et al.*, 2000). Induction of iNOS results in a large release of NO that can cause a variety of effects including vasodilation that can alter BBB integrity, inhibition of mitochondrial function and production of reactive nitrogen species that can result in cell damage and death (see Pannu and Singh, 2006). iNOS is a calcium-independent isoform of NOS and its induction requires new protein synthesis and occurs in response to IL-1β, IFN-γ and TNF-α within 3-5 hours of exposure (Moncada *et al.*, 1991; Dawson and Snyder, 1994). iNOS utilises L-arginine to produce nitric oxide (NO) and NO functions as both an intercellular messenger and also as a cytotoxic molecule (Bernard *et al.*, 2000; Pannu and Singh, 2006). NO can inhibit the action of mitochondrial enzymes involved in electron transport (Pannu and Singh, 2006). NO also reacts with the superoxide O_2^- to form the potent oxidant peroxynitrite ($ONOO^-$) and it is this molecule that can result in cellular damage (see Pannu and Singh, 2006). In this manner NO can result in cell death of invading pathogens and also resident brain cells. This molecule has been implicated in a number of neurodegenerative disorders including AD, PD, and MS (see Pannu and Singh 2006).

COX-2 and prostaglandins: COX-2 is an inducible enzyme that is constitutively expressed in the brain (Phillis *et al.*, 2006). This enzyme is inducible following exposure of immune and non-immune cells including astrocytes, neurons and endothelial cells to an inflammatory stimulus and it is the rate-limiting enzyme in prostaglandin and thromboxine synthesis from arachadonic acid (see Tsatsanis *et al.*, 2006 for review). Although it is an NFκB-inducible gene mRNA stability and successful translation of this enzyme is also dependent on MAPK p38 activation (see Tsatsanis *et al.*, 2006; Saklatvala *et al.*, 2004). The catalytic activity of this enzyme can also be regulated by iNOS activity as peroxynitrites (metabolites of NO) increase activity of this enzyme (Landino *et al.*, 1996). This enzyme synthesises prostaglandin from arachadonic acid and the end product prostaglandins are involved in pain perception, vasodilation, platelet aggregation, oedema and fever (Vidensky *et al.*, 2003; also see Miller, 2006 and Phillis *et al.*, 2006 for review). Prostaglandin E_2 (PGE_2) derived from

COX-2 in the brain is primarily involved in fever and pain (see Miller, 2006, and Phillis *et al*,. 2006 for reviews). COX activation can also result in neuronal death resulting from the 'arachadonic acid cascade' whereby regulation of prostaglandin synthesis is uncontrolled. This can result in free radical and reactive oxygen species formation and resultant cellular damage (Phillis *et al.*, 2006). Prostaglandins can also exert some neurotrophic effects as receptor binding results in an increase in intracellular cAMP.

1.5.4. Cell surface co-stimulatory molecules

Effective functioning of the immune response relies on the ability of antigen presenting cells (APC's) such as microglia and infiltrating macrophages to present antigen associated with an MHC II molecule to the T-cell receptor (TCR) on naïve T helper cells. Following antigen presentation T cells become activated and clonal expansion of antigen specific T helper cells occurs followed by secretion of cytokines such as IFN-γ and IL-2 from Th1 cells, IL-4, IL-5 and IL-6 from Th2 cells and IL-10 and TGF-β from Th3/Treg cells (Mills and McGuirk, 2004).

In addition to the specific MHC class II/antigen – TCR interaction, other cell-cell interactions are necessary for effective T cell activation following antigen presentation. The first of these is the association of B7 molecules (B7.1 or B7.2) on the APC with CD28 or CTLA-4 on the T cell. B7 binding to CD28 or CTLA4 delivers an essential second signal to the T cell without which the T cell does not become activated (Delgado *et al.*, 1999). The second interaction necessary for T-cell activation is between CD40 present on APC's and CD40 ligand (L) which is present on T-cells (Mackey *et al.* 1998). Activation of APC's via CD40 is essential for their ability to effectively present antigen to T-cells. In this regard, triggering of CD40 on APC's leads to a significant upregulation of B7.1 and B7.2 thereby enhancing their ability to facilitate T-cell activation and expansion. Concomitant expression of CD40L on T cells is critical for their priming, expansion and maturation into effector cells capable of cytokine production.

CD40: CD40 is a member of the TNF-α receptor family and upon activation by its ligand CD40L can result in a number of important immunological effects, including immune cell activation, upregulation of co-stimulatory molecules and pro-inflammatory cytokine induction via NFκB and MAPKs (see Chen *et al.*, 2006). Thus CD40 / CD40L interaction can amplify the inflammatory response. As aforementioned, interaction of CD40 on APCs with CD40L on T-cells allows successful antigen presentation to occur, while CD40 / CD40L interaction between T- and B-cells allows successful activation of B-cell differentiation and antibody production (Roitt *et al.*, 2001). In this regard CD40 is considered an important inflammatory molecule that has been implicated in a number of neurodegenerative diseases, including AD, HIV-dementia, and MS to name a few (D'Aversa *et al.*, 2002; for reviews see Town *et al.*, 2001 and Chen *et al.*, 2006). Constitutive expression of this molecule is relatively low on microglia, however it is potently upregulated by LPS and pro-inflammatory cytokines such as IFNβ and IFNγ. NFkB binding sites have been identified in the CD40 promoter regions, and it has previously been reported that both TNF-α and IL-1β can augment IFN-induction of this molecule (Nguyen *et al.*, 1998).

Pro-inflammatory cytokines and chemokines, cytotoxic soluble mediators and cell surface co-stimulatory molecules as described above, are produced in large amounts by activated microglia (Liu and Hong, 2003; Liberto *et al.*, 2004; Qin *et al.*, 2002). Clearly the ability to dampen microglial activation or halt uncontrolled inflammation by regulation of either pro- or anti-inflammatory cytokines could be beneficial in preventing neuronal cell death. Much *in vivo* and *in vitro* evidence exists that NA exerts an anti-inflammatory role on glial cells, thus it has been proposed that loss of this neurotransmitter, or alterations in adrenergic signalling could result in sustained inflammation (Galea *et al.*, 2003).

1.6. Inflammation and neurodegenerative disorders

Many neurodegenerative disorders are accompanied by inflammation and it has been suggested that inflammation itself may be an important factor in the initiation and maintenance of many neurodegenerative disorders such as Alzheimers disease (AD), Parkinsons disease (PD), Multiple sclerosis (MS)

and AIDs-related dementia among others (see Liu and Hong, 2003; Block and Hong, 2005; McGeer and McGeer, 2002; McGeer and McGeer, 1999 for reviews). For instance, activated astrocytes and microglia have been demonstrated to surround amyloid plaques in AD and Lewy bodies in PD (McGeer *et al.*, 1987; McGeer *et al.*, 1988) and glial-derived cytokines and nitric oxide (NO) have been reported to be involved in the neurodegeneration that occurs in both AD and PD (Katsuse *et al.*, 2003). NA has been reported to have numerous anti-inflammatory effects within the CNS, and deficits in noradrenergic signalling have been reported in AD, MS and PD (Bondareff *et al.*, 1987, Mann *et al.*, 1982; Mann *et al.*, 1985; De Keyser *et al.*, 1999; Zarow *et al.*, 2003). Whether these deficits are causal to or a consequence of the disease state, however, remains a subject of debate. Given the anti-inflammatory effects of NA described in the literature, (see Feinstein *et al.*, 2002; Galea *et al.*, 2003 for reviews), it is probable that deficits in its transmission, whatever the cause, could have deleterious inflammatory results. To date, however, there are no established strategies that utilise the anti-inflammatory effects of this transmitter. As there is no known cure for any of the neurodegenerative diseases outlined above, interventions that limit or indeed prevent their initiation and/or progression could prove of great therapeutic benefit.

1.7. Peripheral and Central Models of neuroinflammation

There are a number if ways by which inflammation can be induced in a laboratory setting. A commonly used method is the *in vivo* or *in vitro* administration of lipopolysaccharide (LPS). *In vitro* or central administration of amyloid beta (Aβ) can also be used be induce an inflammatory reaction as this peptide causes microglia to become reactive, thus simulating the inflammatory reaction that occurs around amyloid plaques in AD (McGreer *et al.*, 1987). Other models include the induction of experimental stroke, induction of experimental autoimmune encephalomyelitis (EAE) which is an animal model of MS, and the use of transgenic animals, such as those expressing human amyloid precursor protein (APP), that mimic aspects of AD pathology (see Yang *et al.*, 2006).

1.7.1. In vitro models of neuroinflammation

Research into the functions of glial cells *in vivo* remains difficult due to the close structural relationship they maintain with other brain cells. As such, much of our knowledge of glial cell properties and functions is derived from *in vitro* cell culture studies. *In vitro*, LPS can be directly administered to cultured cells and this approach can be useful when characterisation of the inflammatory response of one or two cell types is the goal.

The mixed glial sytem used in the following studies involved the co-culture of the two major glial cells involved in inflammation, astrocytes and microglia. Although both cell subtypes can be cultured individually, a mixed glial system allows for interaction of these cell types to occur and also approximates the ratio of astrocyte:microglia cells as occurs *in vivo*. Isolated cultured microglia exhibit an amoeboid morphology and appear to be more reactive than those in a mixed culture, thus, co-culture with astrocytes may more accurately represent the *in vivo* physiological state of this cell types (Zietlow *et al.*, 1999; Tanaka and Maeda 1996). Although mixed glial primary cultures may contain a small number of oligodendrocytes and fibroblasts, cultures of this type typically contain approximately 70% astrocyte and 25-30% microglial cells. Primary glial cells are derived from neonatal rat pups and cultured for approximately 10 to 14 days, or until confluent, before being exposed to any treatments. The extent to which neonatal pup cells represent cells of the adult brain remains a matter of debate, however, they yield a higher cell density and are easier to culture than adult cells.

1.7.2. In vivo models of neuroinflammation

In vivo studies are invaluable when cellular, tissue and organ interactions are to be assessed. Given the complexity of both the immune and central nervous systems such studies are necessary to understand how these systems communicate and affect one another. This is of particular importance in the study of CNS inflammation as it has been well documented that peripheral inflammatory events can lead to CNS inflammation (Breder *et al.*, 1994; Kaneko *et al.*, 2005), and systemic infection/inflammation can accelerate neurodegenerative processes in the CNS (Perry *et al.*, 2003).

Induction of CNS inflammation *in vivo* involves the central or peripheral administration of an inflammatory stimulus such as LPS or Aβ or the induction of experimental stroke. The former is often used when the effects of peripheral mediators such as infiltrating leucocytes on brain function are unwanted. The administration of any substance into the brain requires the use of surgery, anaesthesia and recovery time, making it a time-consuming and expensive method. Where non-recoverable surgery experiments are performed the time-course of inflammation is restricted and any effects of anaesthesia on inflammatory measures must first be assessed. This method is particularly useful, however, in chronic studies where small amounts of an inflammatory stimulus are released into the brain over a period of time. This mimics the inflammatory state and subsequent neurodegeneration that occurs in human CNS disorders with an inflammatory component (Hauss-Wegrzyniak *et al.*, 1998). Any survival surgery obviously requires sterile conditions as infection arising from surgery or cannulae inplants would confound results.

Induction of neuroinflammation following systemic LPS administration: Systemic administration of an inflammatory stimulus is commonly used to induce brain inflammation as it is rapid, does not require surgery and provides the investigator with information on the initial stages of brain inflammation. This is an acute model of CNS inflammation and its effects on brain tissue are usually short-lived. One advantage of this method is that peripheral immune measures may also be assessed. This method may prove less useful, however, where peripheral immune measurements may affect CNS variables of interest. Upon systemic administration of LPS a large amount of TNF-α is released into the circulation, detectable at 30 minutes in serum and peaking at 90 minutes (Givalois *et al.*, 1994). IL-1β and IL-6 are also released in large quantities, peaking between 2 and 5 hours (Givalois *et al.*, 1994) while the pro-inflammatory cytokines interleukin-12 (IL-12-p70) and interferon gamma (IFNγ levels are elevated 6 hours post-LPS (Chakravarty *et al.*, 2005). The anti-inflammatory cytokine IL-10 has been found to peak in serum 90 minutes after challenge, returning to baseline between 3 and 6 hours (Matsumoto *et al.*, 2004). It is a generally held belief that LPS induces central inflammation via peripheral cytokine induction, which, via a number of mechanisms is believed

to induce cytokine expression in the brain. It was first discovered that systemic LPS invoked TNF-α in the brain over a decade ago when Breder *et al.*, (1994) demonstrated TNF-α mRNA in the circumventricular organs and nuclei of the hypothalamus and solitary tract using *in situ* hybridisation. Since then TNF-α and IL-1β mRNA has been demonstrated in murine locus coeruleus (LC) (Kaneko *et al.*, 2005) and increases in brain IL-1β have been shown in a variety of brain regions, including the cortex and hippocampus following systemic injection of LPS (Kavanagh *et al.*, 2004). Peripheral administration of LPS has also been shown to negatively affect hippocampal IL-10 concentrations (Kavanagh *et al.*, 2004). The inflammatory mediators iNOS, COX II and chemokines are also induced in the brain by peripheral administration of LPS and subsequent cytokine release (Elmquist *et al.*, 1997; McCann *et al.*, 1998). Indeed prostaglandins can readily cross the BBB to initiate microglial activation while chemokines are responsible for trafficking of leucocytes into the brain. Indeed it could be argued that systemic administration of an inflammatory stimulus better reflects the human situation where systemic infections may better reflect the tendency toward relapse in MS and delerium in AD (see Perry *et al.*, 2003).

As both LPS and peripherally produced cytokines are relatively large (8-80 kDa) hydrophilic proteins, direct access to the brain is denied by the BBB. Peripherally produced cytokines are thought to affect the brain by acting at the circumventricular organs that lack a functional BBB. Elmquist *et al.*, (1997) showed that intravenous injection of LPS induced COX-2 in perivascular microglia and meningeal macrophages. As COX is the rate-limiting enzyme in prostaglandin synthesis they hypothesised that COX-2 induction could result in the production of prostaglandins that may induce cytokine production within the brain. Production of pro-inflammatory cytokines such as TNF-α, IL-1β and IFN-γ following a systemic inflammatory insult can also alter the integrity of the BBB by inducing the expression of cell adhesion molecules (CAM) on endothelial cells, allowing for the preferential entry of CD4 positive cells into the CNS which can then differentiate into cytokine-secreting effecter cells (see Munoz-Fernandez *et al.*, 1998 for review). There is also evidence that peripherally released cytokines can influence the brain via a neurally mediated

mechanism. Specifically, stimulation of the peripheral vagus sensory nerve by circulating cytokines is believed to be at least partly responsible for some of the effects of systemically administered LPS and cytokines (Roth and de Souza, 2001; Wieczorek *et al.*, 2005). For instance, it has been demonstrated that vagotomy attenuates the ability of systemically administered LPS to induce IL-1β mRNA expression in the hippocampus and hypothalamus (Laye *et al.*, 1995). Similarly, the ability of systemically administered IL-1β to induce IL-1β mRNA expression in the CNS is attenuated by vagotomy (Hansen *et al.*, 1998). It is noteworthy that the ability of vagotomy to block IL-1β mRNA expression following systemic IL-1β administration is less profound in the hypothalamus that in other brain regions examined (Hansen *et al.*, 1998). This is most likely due to the fact that systemic inflammatory mediators have increased access to the hypothalamus via the circumventricular organs.

1.8. Noradrenaline and its receptors

Noradrenaline (NA) is a biogenic amine derived from the dietary amino acid tyrosine, that contains a benzene ring and an amine-containing side chain (Rang *et al.*, 1999; Leonard, 1997),(see Figure 1.8.1).

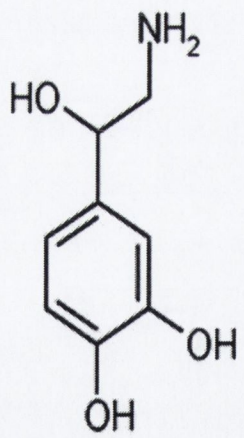


Figure1.8.1: Structure of Noradrenaline

NA has numerous roles both peripherally and centrally, including memory formation, REM sleep, arousal, attention, anxiety and immunomodulation (Ouyang 2004; Leonard, 1997; Alaniz *et al.*, 1999; for reviews see Aston-

Jones 1999 and Sara *et al.*, 1994). NA is catalysed from L-tyrosine, an aromatic amino acid, to dihydroxyphenylanlanine (DOPA) by tyrosine hydroxylase (TH). TH is thus the first and only rate-limiting step in the synthesis of dopamine (DA), NA and also adrenaline (Rang *et al.*, 1999).

DOPA is converted to dopamine by DOPA decarboxylase, and dopamine-β-hydroxylase (DβH) converts dopamine to NA. DβH is only found in cells capable of catecholamine synthesis. The NA is then catalysed to adrenaline by the enzyme phenylethanolamine N-methyl transferase (PNMT) (see Figure 1.8.2).

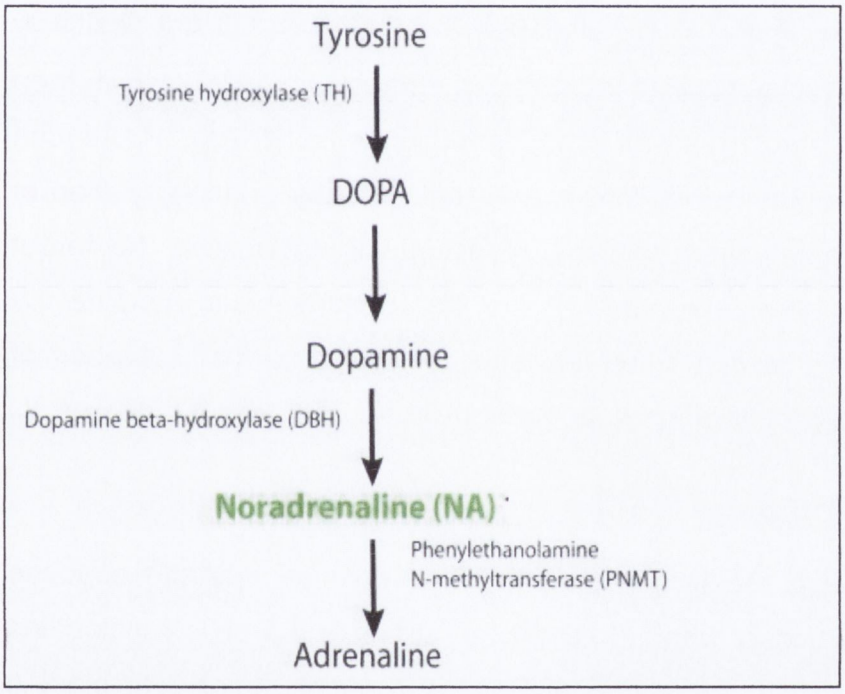


Figure 1.8.2: Schematic of noradrenaline synthesis

Adapted from the website: http://anatomy.ucsf.edu/ohara/noradrenaline_and_pain.htm

In neurons, NA is stored in high concentrations in synaptic vesicles that are maintained by active transport systems (Rang *et al.*, 1999). Nerve terminals also contain ATP and chromogranin A, both of which are released along with NA upon neuronal stimulation. Small amounts of soluble DβH are also released (Rang *et al.*, 1999).

There are a number of different noradrenergic receptors, all of which are seven transmembrane G-protein coupled. There are two basic types of adrenergic

receptors (AR), the alpha (α) and beta (β)-ARs, so delineated on the basis of their affinities and actions. There are two major classes of α-ARs, α_1 and α_2. α_1-ARs are coupled to the G-protein G_q, which stimulates phospholipase C, to activate inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) as second messengers (Rang *et al.*, 1999; Lodish *et al.*, 2000). α_2-AR are coupled to the G_i protein that inhibits the effector adenylyl cyclase thereby preventing the formation of the second messenger 3',5'-cyclic adenosine monophosphate cAMP (cAMP) and inhibiting calcium channels (Rang *et al.*, 1999; Lodish *et al.*,1999).

The β-ARs, (β_{1-3}), are all positively coupled to the G-protein G_s that positively stimulates adenylyl cyclase to increase levels of intracellular cAMP, which then mediates its effects through cAMP-dependent kinases (PKAs). PKAs can modulate the activities of various intracellular enzymes by phosphorylation of serine and threonine residues, thereby increasing or decreasing the target enzyme activity (Lodish *et al.*, 1999). More than one PKA can mediate the action of cAMP and this can differ between cell types so that elevation of cAMP can exert differential effects dependent on the cell type (Lodish *et al.*, 1999).

PKA's can also induce expression of genes that encode a cAMP-response element (CRE). This occurs when the catalytic subunit of the PKA translocates to the nucleus where it can phosphorylate the transcription factor CRE-binding (CREB) protein which interacts with a co-activator CBP/300 to initiate gene transcription of CRE-encoding genes (Lodish *et al.*, 2000) (see Figure 1.8.3)

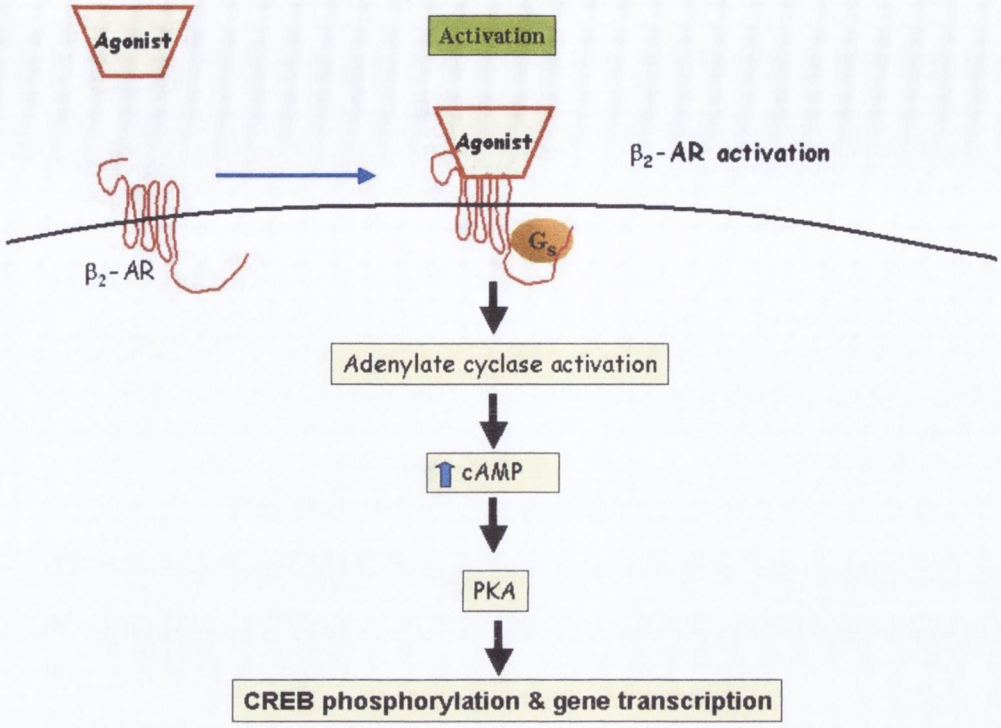


Figure 1.8.3: β_2-adrenoceptor signaling

Upon binding of an agonist the b2-AR the Gs protein activates adenlylate cyclase to increase cAMP. This activates PKAs that can exert a diverse range of intracellular effects and can also lead to CREB phosphorylation and gene transcription.

Both adrenaline and NA are capable of activating α- and β-ARs that are located throughout the body and exert tissue-specific responses upon stimulation. α-ARs demonstrate a higher affinity for NA than adrenaline whereas β-ARs display a greater affinity for adrenaline over NA (Rang *et al.*, 1999). As many cells co-express both adrenoceptor type, this means that differential receptor activation could occur depending on the concentrations of NA present. Low levels of NA, insufficient for β-AR stimulation, may be adequate to stimulate α-AR activation. Activation of β-ARs on glia is believed to mediate many of the anti-inflammatory actions of NA (Junker *et al.*, 2002; Mori *et al.*, 2002) and β_2-ARs are more highly expressed on microglia and cells of the immune system and produce greater amounts of cAMP upon stimulation than β_1-AR (Mori *et al.*, 2002).

Electron microscopy (EM) studies of the visual cortex by Aoiki (1992) and Aoiki and Pickel (1992) suggest that NA released from nerve terminals is not

confined to the synaptic cleft, meaning NA may be able to diffuse to neighbouring glia. These authors also visualised β-AR on astrocytes that were distant from the NA synapse. Functional α- and β-AR have been found on both astrocytes and microglia (Mori *et al.*, 2002; Aoki 1992; Aoki and Pickel, 1992) and the noradrenaline transporter (NET) has also been demonstrated on glial cells (Inazu *et al.*, 2003b). These studies indicate that NA is not only available to glia but may also affect their function via receptor activation, and can also be removed from the extracellular space by the astrocytic NET.

1.9. Noradrenaline release, reuptake and metabolism

The enzymes that metabolise NA, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), are located intracellularly in neurons and glia. As NA is a highly polar chemical, however, it does not easily cross the cell membrane and must be taken up into the cell for termination of the signal. This occurs via active transport across the cell membrane by a sodium (Na^+) and chloride (Cl^-)-dependent transporter called the noradrenergic transporter (NET). The NET, also called $uptake_1$ is expressed presynaptically on neurons where it functions to clear released NA from the synaptic cleft. NA taken up in this manner by neurons is either metabolised by MAOa to 3,4-dihydroxyphenylglycol (DHPG) or 3,4-dihydroxymandelic acid or it is sequestered by the intracellular vesicular monoamine trasporter (VMAT) to be stored in vesicles for re-release (see Eisenhofer, 2001 for review). As the VMAT has a higher affinity for NA than MAO, over 70% of released NA is recycled rather than metabolised (Eisenhofer *et al.*, 1988). In extraneuronal tissues COMT catalyzes conversion of norepinephrine to normetanephrine (NME) and DHPG to 3-methoxy-4-hydroxy-phenylglycol (Siegel *et al.*, 1999; Eisenhofer, 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, 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 indicated. Many selective and non-selective inhibitors of this transporter exist and are used in the treatment of both depression and attention deficit hyperactive disorders (ADHD) where deficits in noradrenergic signalling are proposed. To this end it has been demonstrated using microdialysis techniques that inhibition of the NET by selective noradrenaline reuptake inhibitors (NRIs) can increase NA concentrations over two-fold in distinct brain regions (see Sacchetti *et al.*, 1999). The NET is also found on non-neuronal cells, including the chromaffin cells of the kidney, capillary endothelial cells of the lung and cells of the placenta (see Eisenhofer, 2001 for review). Within the CNS functional NETs have been demonstrated on cultured astrocytes and can be pharmacologically inhibited by selective NET inhibitors such as desipramine (DMI), nisoxetine, and imipramine (Inazu *et al.*, 2003b).

Catecholamines can also be transported into cells via other non-neuronal transporters, namely the organic cation transporter 3 (OCT3), also termed the extraneuronal monoamine transporter (EMT) in humans and uptake$_2$ in rat and mouse (Grundemann 1998; Kekuda *et al.*, 1998; Wu *et al.*, 1998). This transporter is found on non-neuronal cells including human and rat astrocytes (Russ *et al.*, 1996; Inazu *et al.*, 2003ab), and has been demonstrated in human liver, heart and brain cortex, (Grundemann *et al.*, 1998). Although this transporter has a low affinity for NA, it is a high-capacity transporter that is Na^+ and Cl^--independent (Grundemann *et al.*, 1998). Uptake$_2$ is involved in the re-uptake of NA, adrenaline, DA, the neurotoxin 1-methyl-4phenylpyridinium (MPP+), histamine and cimetidine (Grundemann *et al.*, 1999). Grundemann and colleagues (1998) demonstrated that uptake$_2$ has a higher affinity for adrenaline, which was roughly 3-fold that of NA. This is not surprising given that adrenaline is a neurohormone that is secreted directly into the bloodstream and exerts its effects distal to the adrenal glands. Eisenhofer *et al.*, (1996) demonstrated that blockade of the EMT significantly increased plasma catecholamine levels, however, the extent to which uptake$_2$ is involved in clearance of NA in the CNS has not yet been clarified.

Noradrenergic innervation within the CNS: There are two main sources of noradrenergic innervation in the brain, termed the ventral and dorsal bundle. The dorsal bundle arises from cell bodies in the locus coerelus (LC) that project to the spinal cord and cerebellum, and via the median forebrain bundle to the entire cortex, hippocampus and indeed other regions such as the olfactory bulbs, and amygdala (see Figure 1.7.1). The ventrally located cell bodies (lateral tegmental nuclei) are more diffusely localised and project fibres, termed the ventral bundle to innervate the brainstem and hypothalamus.

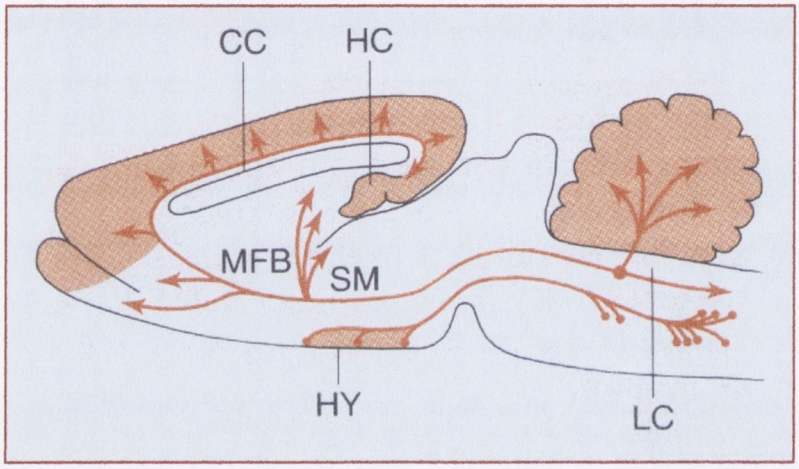


Figure 1.9.1: Noradrenaline pathways in the rat brain

From Basic Neurochemistry by Siegel *et al.*, (1999) 6th Ed. (pg 252)

1.10. The spleen and its regulation by noradrenaline

The spleen is a secondary lymphoid organ that is located at the upper left quadrant of the abdominal cavity behind the stomach. The secondary lymphoid organs have two major functions, the capture and presentation of antigens, and induction of antigen-specific T-lymphocytes. Thus, not only does this organ play an active role in immune surveillance but it is also a major site of antibody production.

The nature of spleen tissue is such that it can efficiently trap both pathogens and worn out red blood cells (RBCs) in its small capillary vessels (Roitt *et al.*, 2001). Additional functions of the spleen include the temporary storage of fresh RBCs, granulocytes and platelets and destruction of those that are worn out by macrophage phagocytosis (Roitt *et al.*, 2001). The spleen is innervated by the splenic nerve that is predominantly noradrenergic. Regulation of the spleen is

under autonomic control and receives input from the sympathetic nervous system (SNS) originating in the brain stem. Activation of the splenic nerve results in NA release that has diverse effects on circulating and resident splenic immune cells and dilation of the vasculature. Activation of the splenic nerve also results in the release of neuropeptide Y (see Elenkov *et al.*, 2000 for review). Mostly NA fibres are found in the white pulp area of the spleen, thus diffusible NA is available to T-cells, macrophages and resident B cells but not immature B-cells (Elenkov *et al.*, 2000).

Upon release in the spleen NA is available to act on a variety of immune cells, predominantly on those in the white pulp, macrophages, dendritic cells, and T-cells. Akin to noradrenergic neurons in the brain, NA fibres in the spleen demonstrate the capacity to store, release and re-uptake NA, indicating that this organ will be subject to the NA-enhancing effects of NRIs (see Elenkov *et al.*, 2000 for review). NA release in the speen is also subject to autoregulation by α_2-autoreceptors located on axon terminals, in this regard, a number of studies demonstrated increased NA concentrations following α_2-AR antagonist treatment (Elenkov and Vizi, 1995; Elenkov and Vizi, 1991)

1.11 Anti-inflammatory effects of NA in the CNS

Many *in vivo* studies provide evidence for the anti-inflammatory effects of NA using a number of different inflammatory models, while *in vitro* studies of isolated cell cultures have attempted to determine the pathways through which these effects are mediated. Peripheral anti-inflammatory effects on macrophages and Th1 cells are primarily mediated via the β_2-AR receptor (Verhoeckx *et al.*, 2005; Borger *et al.*, 1998). There is also evidence that NA can inhibit inflammatory processes in CNS via its actions on microglia and also on astrocytes (see Feinstein *et al.*, 2002; Galea *et al.*, 2003 for reviews)

Evidence from in vitro studies: NA administration and the subsequent increase in cAMP signalling has also been demonstrated to inhibit the expression of cytokine-induced adhesion molecules by astrocytes (Ballestas and Benveniste, 1997). The induction of adhesion molecules promotes leukocyte adhesion and trafficking across the BBB, leukocyte infiltration may then potentiate the

inflammatory status within the CNS. A number of studies have demonstrated that NA can reduce the production of pro-inflammatory cytokines in astrocytes and microglia (Nakamura *et al.*, 1998; Mori *et al.,* 2002; Dello Russo *et al.*, 2004) an effect mediated via β_2-AR activation and subsequent increases in cAMP signalling. Isoproterenol a β-AR agonist has been shown to decrease LPS- induced TNF-α and IL-6 in astrocytes (Nakamura *et al.*, 1998) while NA has been demonstrated to potently inhibit iNOS expression and NO production and IL-1β production in microglia (Dello Russo *et al.*, 2004). The reduction in iNOS expression in this study was not mediated by NA-induced decreases in IL-1β as inhibition of IL-1β did not affect NO production. Thus, NA may have a direct effect on iNOS expression and NO production. Mori *et al.*, (2002) further demonstrated that NA and β_1- and β_2-AR agonists suppressed mRNA expression of TNF-α and IL-6 in microglia. As expression of the β_1-AR was lower than that of β_2, and induced 10 times less cAMP upon stimulation, the authors attributed the down-regulation of pro-inflammatory cytokine expression primarily to β_2-AR activation. Clenbuterol, a β_2-AR agonist has been demonstrated to exert neuroprotective effects *in vitro* that are proposed to result from TGFβ induction of both brain-derived nerve factor (BDNF) and neural growth factor (NGF) (Zafra *et al.*, 1992; Culmsee *et al.*, 1999; Lindholm *et al.*, 1990).

Astrocyte expression of factors that promote growth and survival of neurones and oligodendrocytes, are increased following treatment with the β-AR agonists. Zafra *et al.*, (1992) demonstrated increased brain derived neurotrophic factor (BDNF) mRNA in astrocytes after NA treatment, while Culmsee *et al.*, (1999) demonstrated increases in NGF after clenbuterol treatment.

NA has also been demonstrated to increase the inhibitory protein IκBα in cultured astrocytes, levels of which are decreased when endogenous NA is depleted by DSP-4 *in vivo* (Gavrilyuk *et al.*, 2002). By binding to NFκB in the cytosol, IκB prevents the translocation of NFκB to the nucleus and thereby prevents NFκB signaling and induction of pro-inflammatory mediators. Activation of β-ARs by NA has been shown to suppress IFN-γ -induced MHC II

expression in astrocytes, thereby preventing them from acting as antigen-presenting cells (APCs) (Frohman *et al.*, 1988).

NA has been shown to induce expression of peroxisome proliferators-activated receptor gamma peroxisome proliferator-activated receptor-γ (PPAR-γ) (Klotz *et al.*, 2003). This receptor belongs to a family of nuclear hormone receptors that can bind PPAR elements to activate transcription (for review see Berger and Moller 2002). Endogenous ligands include 15-deoxy-delta-12,14-prostaglandin J2 (15d-PGJ_2) and polyunsaturated fatty acids, other agonists include non-steroidal anti-inflammatory drugs such as ibuprofen and the anti-diabetic drugs thiazolidinediones (TZDs). PPAR-γ is involved in the regulation of inflammatory responses, and is upregulated by NA, cAMP analogs, β-AR agonists and the natural ligand 15d-PGJ_2 (Klotz *et al.*, 2003; Bernardo *et al.*, 2000). Studies by Dello Russo *et al.,* (2003) show that PPAR-γ agonists increase glucose metabolism in astrocytes. Astrocytes can maintain glucose stores, metabolism of which provides lactate for neighbouring neurons. Activation of PPARγ on astrocytes may, therefore, be important in maintaining energy supply to CNS cells. Stimulation of β-AR also induces glycogenolysis in astrocytes, however, whether this is independent of PPARγ is not yet clear. Bernardo *et al.*, (2000) demonstrated that LPS down-regulates PPARγ expression in microglia.

No work to date appears to have examined the effect of NA on brain chemokines, however β-AR agonists have been demonstrated to inhibit RANTES release in airway endothelium and smooth muscle (Miyabayashi *et al.*, 2006; Ammit *et al.*, 2002; Hallsworth *et al.*, 2001). Moreover, adrenaline and β-AR agonists have been shown to inhibit MIP-1α and IL-8 production and MIP-1α mRNA expression in monocytes and macrophages, an action that was shown to be β_2-AR mediated (Li *et al.*, 2003a; Li *et al.*,2003b; Hasko *et al.*, 1998).

Evidence from in vivo studies: It is proposed that the absence of β-AR expression on astrocytes in MS, could allow these cells to function as antigen presenting cells to promote an inflammatory phenotype and contribute to the

production of neurodegenerative mediators such as cytokines and ROS in this disease (De Keyser *et al.*, 2004).

Using an animal model of stroke Zhu and colleagues (2001) demonstrated that pre-treatment with clenbuterol, a β_2-AR agonist up-regulated the anti-inflammatory cytokine TGFβ in rat hippocampus following transient forebrain ischaemia. Clenbuterol-induced increases in TGFβ concentrations in this study were associated with an increase in NGF and neuronal survival. NGF, BDNF and the trophic factor neuroregulin are all produced by astrocytes and promote growth and survival of neurones and oligodendrocytes. The neuroprotection afforded by clenbuterol could result from alterations in bcl-2, Bcl-xl and Bax proteins as demonstrated by Zhu *et al.*, (1999) in earlier studies. These studies demonstrated that clenbuterol increased the anti-apoptotic proteins Bcl-xl and Bcl-2 and down-regulated expression of the pro-apoptotic Bax protein following transient global ischaemia in rats. Thus, it is possible that NA exerts anti-inflammatory effects via a number of different mechanisms.

Heneka *et al.*, (2002) found that depletion of endogenous NA using N-(2-chloroethyl)-N-ethyl-2 bromobenzylamine (DSP-4) enhanced Aβ-induced cortical inflammation compared to animals with an intact noradrenergic system. DSP-4 selectively destroyed noradrenergic cells of the LC thereby disrupting noradrenergic projections to other parts of the CNS. In this study lack of endogenous NA was demonstrated to potentiate IL-1β, IL-6 concentrations and iNOS expression in neurons and glia (Heneka *et al.*, 2002). NA-deficient rats had an enhanced and sustained inflammatory response to Aβ with iNOS expression observed in neurons and microglia, an effect only observed in the microglia of NA-intact animals. In this study co-injection of NA or a β-AR agonist with Aβ attenuated the effects of DSP-4 on cortical inflammation. The authors proposed, therefore, that LC dysfunction early in AD could potentiate inflammation in response to Aβ that could cause retrograde degeneration of the LC, further perpetuating the cycle of inflammation and neural degeneration.

Despite the paucity of evidence regarding a potential role for NA in chemokine brain regulation, Hasko and colleagues (1998) demonstrated a suppression of

the monocyte and macrophage chemoattractant MIP-1α after *in vivo* administration of isoproterenol and LPS that was blocked by propranolol pre-treatment.

Overall it is clear that there is abundant evidence indicating that NA and β-AR agonists can exert anti-inflammatory effects in the CNS. Dysfunction of NA signalling could, therefore, allow the initiation of inflammatory processes within the CNS.

1.12. Noradrenergic loss in neurodegenerative disease states: A role in neuroinflammation?

Post-mortem studies have shown significant cell loss in the noradrenergic cell body the locus coeruleus (LC) in both Alzheimers (AD) and Parkinsons disease (PD) (Zarow *et al.*, 2003; Bondareff *et al.*, 1987). Alterations in adrenergic signalling have also been proposed to be involved in the pathology of MS (De Keyser *et al.*, 1999). As it has been proposed that NA tonically inhibits inflammatory processes in the CNS (see Feinstein *et al.*, 2002; Galea *et al.*, 2003 for reviews), when noradrenergic signalling is impaired, the threshold for inflammation to occur may then be lowered, allowing the brain to respond inappropriately to inflammatory stimuli that would not otherwise cause a reaction. Considering the large body of evidence suggesting that inflammation is a significant contributor to pathology in a range of neurodegenerative disorders (Mann *et al.*, 1982; Zarow *et al.*, 2003; Katsuse *et al.*, 2003; Imamura *et al.*, 2003; De Keyser *et al.*, 2004), it is suggested that noradrenergic loss or deficits in noradrenergic signalling may well contribute to neurodegeneration by promoting neuroinflammation.

Alzheimer's disease: There are gross abnormalities in many neurotransmitter systems in AD, deficits in the cholinergic system being a major hallmark of the disease. Disruptions in glutamatergic and serotonergic signalling also occur, while deficits in noradrenergic signalling are proposed to contribute to many of the behavioural manifestations of AD, including aggression, alterations in sleeping patterns and cognitive decline (Matthews *et al.*, 2002; Wu *et al.*, 2003). Clinical evidence of the contribution of NA in AD is often difficult to

interpret and often contradictory. For example, Palmer *et al.*, (1987) found no correlation between clinical or histological indices of noradrenergic markers and severity of AD, which is in contrast to Zarow *et al.*, (2003) who demonstrated LC cell loss that correlated with disease severity. Mann *et al.*, (1982) also reported LC cell loss in patients with Alzhemier's type dementia but not those with multi-infarct dementia. An upregulation of β-ARs and a comcomitant decrease in α2-AR localisation in AD brain has been observed and is believed to be a compensatory mechanism to combat NA loss in this disease (Kalaria *et al.*, 1989; Meana *et al.*, 1992; Pascual *et al.*, 1992). The variance in the literature regarding involvement of noradrenergic signalling in these diseases may be due to factors such as diagnosis, treatment, length and severity of disease, medication and cause of death.

Parkinsons disease: In PD the major neurotransmitter deficits are observed with DA in the substantia nigra, leading to progressive movement disability. Deficits in noradrenergic signalling and inflammatory processes have also been proposed to contribute to disease progression in PD. Zarow *et al.*, (2003) report greater neuronal loss in LC than nucleus basalis and substantia nigra in both AD and PD, while activated glia, pro-inflammatory cytokines and iNOS have been demonstrated in PD and AD brains post-mortem (Katsuse *et al.*, 2003; Imamura *et al.*, 2003).

Multiple sclerosis: De Keyser *et al.*, (1999) showed that MS brains lacked astrocytic β_2-AR, which were present as normal on neurones. Whether this is a symptom of, or one of the causal factors in this disease remains unknown, however, the neuroprotective and anti-inflammatory effects mediated by this receptor on astrocytes suggest the absence of this receptor subtype may be a contributory factor in the pathology of the disease (for review see De Keyser *et al.*, 2004). Clinical evidence of the contribution of NA in the above diseases is often difficult to interpret and sometimes contradictory.

The above neurodegenerative disorders are associated not only with alterations in noradrenergic signalling and/or NA deficiencies, but also comprise an inflammatory component. As such, it is possible that these two

factors are inter-related and may be contributory to disease occurrence or progression.

1.13. Pharmacological strategies to limit neuroinflammation

A number of epidemiological studies suggest that long-term anti-inflammatory therapy such as non-steriodal anti-inflammatory drugs may be helpful in preventing AD (for review see McGeer and McGeer, 1998). Similarly there is observational evidence that statin therapy, (used to lower cholesterol), may slow cognitive decline in AD patients (Masse *et al.*, 2005). Other epidemiological studies suggest that a diet rich in fish oils may help maintain brain function, and to this end, animal studies have demonstrated anti-inflammatory effects of the polyunsaturated fatty acid, eicosapentaenoic acid (EPA) (Kavanagh *et al.*, 2003; Lonergan *et al.*, 2004). Despite the wealth of information and research into neurodegenerative disease, current pharmacological therapies only alleviate symptoms and attempt to slow disease progression. At present there is no cure for AD, PD, MS or any other neurodegenerative disorders.

Given the abundance of evidence indicating that NA has anti-inflammatory effects in the CNS, and evidence of NA deficits in a number of neurodegenerative disease states it is suggested that strategies to augment NA availability in the CNS could have significant therapeutic effects. As NA cannot cross the BBB, inhibition of the transport mechanisms necessary for termination of adrenergic signalling could be a way to enhance its action in the CNS. There are a number of existing drugs that can inhibit the NET including desipramine (DMI) and atomoxetine (ATX). Alternatively, inhibition of α_2-ARs, autoreceptors that regulate NA release may also represent a viable strategy to enhance NA concentrations in the brain (Invernizzi and Garatti, 2004).

Noradrenaline reuptake inhibitors: DMI and ATX are used in the treatment of depression and attention-deficit-hyperactivity disorder (ADHD), respectively. DMI is an older generation tricyclic (TCA) antidepressant that has been demonstrated to have a number of anti-inflammatory effects, both *in vivo* and *in vitro* (Connor *et al.*, 2000; Diamond *et al.*, 2005; Shen *et al.*, 1999).

A functional NET has been demonstrated on astrocytes (Inazu *et al.*, 2003b; Takeda *et al.*, 2002) and inhibition of astroglial NET by a related TCA compound amitriptyline, resulted in suppression of LPS-induced TNF-α and IL-1β protein from both microglial and mixed glial cultures (Obuchowicz *et al.*, 2006). The anti-inflammatory profile of this drug has been far more extensively evaluated using *in vivo* animal models where it has been shown to inhibit serum TNF-α and IL-1β, and increase IL-10 release (Connor *et al.*, 2000; Shen *et al.*, 1999). To date, however, the potential anti-inflammatory action of this compound on brain inflammation has not been examined.

DMI can also inhibit the serotonin transporter (SERT), although to a much lesser extent than the NET (Leonard, 1997) and has a number of non-noradrenergic transporter-specific effects such as antagonism of α-ARs histamine, muscarinic and NMDA receptors (Rang *et al.*, 1999). In addition, DMI can also elicit cardiotoxic effects in overdose by inhibition of sodium and calcium channels (Deffois and Carter, 1996). All of the above result in negative side-effect and toxicity profiles for drugs of this class. It is for these reasons, and the advent of newer therapeutic agents with better safety profiles, that DMI is not generally used as a first line therapy for depression although it remains in clinical use. However in terms of modulating inflammation, DMI is the most widely studied TCA.

ATX is a more selective NRI than DMI that demonstrates minimal selectivity for the SERT or neuronal receptors (Bymaster *et al.*, 2002). It was initially developed as an anti-depressant more than twenty years ago, but was dropped from clinical trials when the selective serotonin reuptake inhibitor (SSRI), fluoxetine (Eli Lilly, [see Garland and Kirkpatrick, 2004]) showed more promise. In 2002, however, it received approval from the Federal drug administration (FDA) in the United States for treatment of ADHD (Garland and Kirkpatrick, 2004). To date it is the only non-stimulant drug approved for this condition and is indicated for both childhood and adult ADHD conditions. Bymaster *et al.*, (2002) demonstrated an increase in extracellular NA concentrations in rat frontal cortex within 30 minutes of administration that persisted for four hours. Despite the selectivity of this drug for the NET, to our

knowledge, no research has been carried out to test the anti-inflammatory potential of this drug.

Uptake$_2$ inhibitors: The non-neuronal transporter uptake$_2$ is not subject to inhibition by the NRIs described above, displaying as it does, a completely different pharmacological profile. This transporter is subject to inhibition by corticosterone and products of O-methylation of NA, namely normetanephrine (NME) and metanephrine (ME) (Eisenhofer, 2001). Although inhibition of uptake$_2$ increases plasma catecholamine levels, the extent to which glial uptake$_2$ blockade may increase brain NA concentrations or side-effects resultant from inhibition in glia are unknown (Eisenhofer *et al.*, 1996). Both ATX and DMI, however, are attractive therapeutic candidates as they are already in clinical use, are relatively safe, cross the BBB and one, at least demonstrates an anti-inflammatory profile.

α_2-adrenoceptor antagonists: Upon release NA exerts its actions via α- and β-ARs located post-synaptically and also α_2-AR that are located pre-synaptically. This α-AR subtype negatively regulates NA release. Inhibition of this receptor subtype has been demonstrated to increase brain NA concentrations using *in vivo* microdialysis (see Invernizzi and Garatti, 2004). Idazoxan (IDA) and RX 821002 (RX) were used in the following studies to examine the effect of α_2-AR blockade on inflammatory measures in the brain as both these compounds were shown to increase NA using microdialysis in rat studies (Swanson *et al.*, 2006; Wortley *et al.*, 1999). As IDA also blocks imidazoline receptors that are involved in blood pressure regulation, the α_2-AR blocker RX 821002 (RX) that has little/no affinity for imidazoline receptors was also used in the following studies. Not only have α_2-AR antagonists been shown to increase NA in their own right, they have also been demonstrated to synergistically increase NA concentrations when administered with NRIs (see Invernizzi and Garattini [2004] for review).

1.14. Objectives of this thesis

The overall objective of this project was to determine the ability of the monoamine neurotransmitter noradrenaline, and pharmacological strategies that enhance the action of noradrenaline to limit neuroinflammation.

Glial activation was induced *in vitro* and *in vivo* using bacterial lipopolysaccharide, and specific experiments focused on:

1. Examining the effect of noradrenaline on the pro-/anti-inflammatory cytokine balance in mixed glial cells.

2. The ability of noradrenaline reuptake inhibitors to augment the anti-inflammatory actions of noradrenaline in mixed glial cells

3. The ability of *in vivo* administration of NRI's and α_2-adrenoceptor antagonists alone and in combination to attenuate neuroinflammation induced by a systemic LPS challenge in rats

Overall, this project has yielded significant insights into the ability of noradrenaline augmentation strategies to limit inflammatory processes in the CNS

CHAPTER 2

Materials and methods

2.1 Materials

Animals

Wistar rats (1-3 days old)	Bioresources, TCD
Sprague Dawley rats	Harlan UK.
Laboratory rat diet	Red Mills

Cell Culture Materials

Acrodisc syringe filter (0.2μm)	Pall Corporation
Biocidal ZF ™	WAK-Chemie
Cell strainers (40μM)	BD Falcon
Disposable sterile scalpels	Swann-Morton
Dulbecco's modified eagles medium:F-12 (DMEM)	Invitrogen
Dulbecco's phosphate buffered saline (PBS) (10X)	Sigma
Foetal Bovine Serum (FBS)	Invitrogen
Haemocytometer	VWR international
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

ELISA: Plastics and kits

Maxisorp immunoplates for ELISA	Nunc
Rat Interleukin-1β (IL-1β) ELISA kit (Duoset)	R&D systems

Rat Interleukin-10 (IL-10) ELISA kit (Cytoset)	Biosource
Rat Interleukin-TNF-α ELISA kit (Opt EIA set)	BD Biosciences
Rat TGFβ$_1$E$_{max}$ ImmunoAssay System	Promega

Experimental Treatments

Anti-IL-1β neutralizing antibody	R&D systems
Anti-TNF-α neutralizing antibody	R&D systems
Atomoxetine	Eli Lilly
Desipramine hydrochloride	Sigma
Dibutyryl adenosine 3′,5′-cyclic monophosphate (cAMP)	Sigma
DL-Normetanephrine hydrochloride	Sigma
Escherichia coli Lipopolysaccharide (LPS) serotype 0111:B4	Sigma
Idaxozan hydrochloride	Tocris
(-)-Norepinephrine (+) bitartrate salt hydrate (99%)	Sigma
Normal goat IgG	R&D systems
(±) Propranolol hydrochloride	Sigma
Salbutamol	Sigma

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
Diethyl Pyrocarbonate (DEPC)	Sigma
di-Sodium hydrogen orthophosphate (Na_2HPO_4)	BDH
DL-Dithiothreitol (DTT)	Sigma
N-(1-naphthyl)- ethylenediamine dihydrochloride	Sigma
Ethylenediaminetetraacetic 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
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
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
Urethane	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 (see table 2.4.1)	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
Filter paper	Whatman
Monoclonal anti-iNOS antibody	Transduction Laboratories
Monoclonal anti-β-actin antibody	Sigma

Nitrocellulose membrane	Sigma
Restore™ Western Blot stripping buffer	Pierce
Supersignal® West Dura extended substrate solution	Pierce

METHODS

2.2. *In vivo* studies

2.2.1. 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°C and 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).

Physiological assessments

A record of body-weight was maintained as a general indication of the health and well-being of each animal throughout the study.

2.2.2. In vivo drug administration

Animals were handled daily for 4 days prior to experiment to reduce any possible stress caused by experimental injecting and handling.

NRI dose-response study: On day five the animals were administered an intraperitoneal (i.p.) injection of either vehicle (d. H_2O), DMI (3.2, 7.5 or 15mg/kg) or ATX (2.5, 5 or 10mg/kg) before being immediately challenged with LPS (250μg/ml). A vehicle control group was also included to give an estimate of basal expression of inflammatory genes in the brain.

IDA and RX study: On day five the animals were administered an injection (i.p.) of either vehicle (d. H_2O), IDA (1mg/kg) or RX (1mg/kg), before being challenged with LPS (250μg/kg). A vehicle control group was also included to give an estimate of basal expression of inflammatory genes in the brain

ATX/IDA interaction study: On day five the animals were administered an injection (i.p.) of either vehicle (d. H_2O), ATX (5mg/kg), IDA (1mg/kg) or ATX (5mg/kg) + IDA (1mg/kg) immediately before being challenged with LPS (250μg/kg).

2.2.3. *Harvesting tissue for mRNA expression*

A portion of cortex, the whole hypothalamus and a portion of spleen from each rat were placed in RNase-free tubes containing RNA*later*™ and stored for seven days at 4°C. All samples were then removed from the RNA*later*™ solution, transferred to fresh RNAse-free tubes and frozen at –85°C until RNA extraction was performed.

2.3. *In vitro* cell culture studies

2.3.1. *Preparatory work for cell culture*

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 sterilized with 70% ethanol (EtOH) (30% d.d.H_2O and 70% EtOH v/v) 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.

Preparation of culture plates: 25mg of Poly-L-lysine was reconstituted with 3mls of autoclaved distilled water (d.d.H_2O). The solution was mixed using a vortex, and when fully dissolved a further 22mls of d.d.H_2O was added, to give a final concentration of 1mg/ml. This solution was then filter-sterilised using a 0.2μm syringe filter. To prepare 24 well plates for culture, 1ml of Poly-L-lysine (1mg/ml)

was diluted 1:25 by the addition of 24ml of d.d.H_2O and 100µl (40µg/ml) used to coat each well of a 24 well culture plate. Poly-L-lysine was removed from the plates after 30 minutes, and plates left in the laminar flow hood overnight to ensure they were completely dry before use.

2.3.2. Preparation of culture media and test compounds

Culture media: Dulbecco's modified eagle's medium:F12 (DMEM) containing 10% foetal bovine serum (FCS) and 1% penicillin-streptomycin (complete DMEM) was used for all glial cell cultures. Briefly, 50ml of FCS and 5ml of penicillin-streptomycin were sterile filtered using a 0.2µm syringe filter and added to a 500ml bottle of DMEM:F12.

Phosphate buffered saline (PBS): A working 1X solution of PBS was prepared by adding 1ml of Dulbecco's sterile 10X PBS (100mM NaCL, 80mM Na_2HPO_4, 20mM NaH_2PO_4) to 9mls of d.d. H_2O.

LPS: 25mg of *Escherichia coli* Lipopolysaccharide (LPS) (serotype 0111:B4) was reconstituted in 1ml of d.d.H_2O. The solution was mixed using a vortex, and when fully dissolved 500µl (25mg/ml) was added to 62ml of complete DMEM (1:125 dilution) to give a 200µg/ml stock solution which was then sterile filtered using a 0.2µm syringe filter. Stock solution was frozen at –20°C in 1ml aliquots for future use. Before use it was diluted to a working concentration in pre-warmed DMEM.

Noradrenaline: A stock solution of noradrenaline (NA) [Formula weight (F.W.): 337] was prepared by adding 5ml of d.d.H_2O to 0.337g of NA to give a stock solution of 200mM that was then filter-sterilised using a 0.2µm syringe filter. This stock solution was frozen at –20°C in 200µl aliquots for future use. Before use it was diluted to a working concentration in pre-warmed DMEM.

Salbutamol: 1.732 ml of d.d.H_2O was added to 25mg of salbutamol with a F.W. of 288.7, to give a stock solution of 50µM. This stock solution was frozen at –20°C in 50ml aliquots. Before use it was filter-sterilised using a 0.2µm syringe filter and diluted to a working concentration in pre-warmed DMEM.

Di-butryl cAMP: 1ml of d.d.H_2O was added to 25mg of di-butyryl adenosine 3',5'-cyclic monophosphate (di-butyryl cAMP) with a F.W. of 491.4. This gave a stock solution of 51mM, which was aliquoted into smaller volumes and frozen at –20°C. Before use it was filter-sterilised using a 0.2μm syringe filter and diluted to a working concentration in pre-warmed DMEM.

Atomoxetine: 3ml of d.d.H_2O was added to 52.5mg of atomoxetine with a F.W. of 291.82. This gave a stock solution of 60mM, which was filter-sterilised using a 0.2μm syringe filter and aliquoted into smaller volumes and frozen at –20°C. Before use it was diluted to a working concentration in pre-warmed DMEM

Desipramine: 5ml of d.d.H_2O was added to 302.8mg of desipramine with a F.W. of 302.8. This gave a stock solution of 200mM, which was filter-sterilised using a 0.2μm syringe filter and aliquoted into smaller volumes and frozen at –20°C. Before use it was diluted to a working concentration in pre-warmed DMEM

Normetanephrine : 5ml of d.d.H_2O was added to 55mg of normetanephrine with a F.W. of 219.67. This gave a stock solution of 50mM, which was filter-sterilised using a 0.2μm syringe filter and aliquoted into smaller volumes and frozen at –20°C. Before use it was diluted to a working concentration in pre-warmed DMEM.

Idazoxan: 41.5ml of d.d.H_2O was added to 10mg of idazoxan with a F.W. of 240.69. This gave a stock solution of 1mM, which was filter-sterilised using a 0.2μm syringe filter and aliquoted into smaller volumes and frozen at –20°C. Before use it was diluted to a working concentration in pre-warmed DMEM

RX 821002: 36.94ml of d.d.H_2O was added to 10mg of RX 821002 with a F.W. of 270.72. This gave a stock solution of 1mM, which was filter-sterilised using a 0.2μm syringe filter and aliquoted into smaller volumes and frozen at –20°C. Before use it was diluted to a working concentration in pre-warmed DMEM

Anti-TNF-α neutralising antibody: 100μg of anti-TNF-α antibody was reconstituted with 1ml of sterile PBS to give a 0.1mg/ml stock solution, which was aliquoted into smaller volumes and frozen at –20°C. Before use it was diluted to a working concentration of 2μg/ml in pre-warmed DMEM.

Anti-IL-1β neutralising antibody: 100μg of anti-IL-1β antibody was reconstituted with 1ml of sterile PBS to give a 0.1mg/ml stock solution, which was aliquoted into smaller volumes and frozen at –20°C. Before use it was diluted to a working concentration of 0.25μg/ml in pre-warmed DMEM.

Normal Goat IgG: 1mg of normal goat antibody was reconstituted with 1ml of sterile PBS to give a 1mg/ml stock solution, which was aliquoted into smaller volumes and frozen at –20°C. Before use it was diluted to a working concentration in pre-warmed DMEM.

2.3.3. Preparation of primary glial cells

The culturing of primary glial cells is an *in-vitro* technique that involves the dissection of the brain, removal of the area(s) of interest and dissociation of the tissue to obtain a population of glia. Cells are then allowed to mature over a fourteen-day period before being used in experimental procedures. Glial cultures obtained in this way typically contain astrocytes (70%) and microglial (30%), which simulates the approximate ratio of glia in the normal brain (unpublished observations). Primary cultures such as these also represent unaltered phenotypes that have not been transformed and thus, were used in all studies.

Briefly, neonatal wistar rat pups (postnatal day 1-3) were taken into the laminar flow hood where they were decapitated using a large scissors and the skin cut down the midline to reveal the skull. Using a smaller scissors the skull was carefully cut on each side, at the level of the ears. A curved forceps was then used to gently pull back the skull and remove the exposed brain, which was placed on a petri dish. Here the hindbrain was removed using a scalpel. The forebrain was then placed in another petri dish containing 1X PBS where the meninges and adherent blood vessels were removed using a straight fine forceps. The remaining tissue was then cross-chopped using a scalpel and placed into a pre-labelled 15ml flacon tube containing 6ml of pre-warmed complete DMEM. This was placed in a humidified incubator (95% air, 5% CO_2, 37°C) until any further dissections were complete. Unless stated otherwise glial cultures were prepared from eight animals to give an n=8 for all experiments. When dissections were completed the tissue prepared first was returned to the laminar flow hood where it was gently triturated

using a transfer pipette until all visible clumps were removed. This was then passed through a cell strainer into a 50ml pre-labelled falcon tube. Procedure was repeated for all tissue samples. Samples were then centrifuged at 2,000rpm for 3 minutes at 20°C before being returned to the incubator. In the flow hood the supernatant from the first sample was removed and the pellet re-suspended in 6ml of preheated complete DMEM. This was then gently triturated using a plastic transfer pipette until a homogenous cellular suspension was obtained. Procedure repeated for all samples. At this point, a cell count was performed by adding 100μl of the cell suspension to 300μl of 1xPBS and 100μl of trypan blue in a 2ml microtube. This solution was mixed using a vortex 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. Cell counts achieved usually averaged 2×10^5 cells/ml.

50μl of this cell suspension was placed in the centre of each well of a 24 well plates, which were then placed in the incubator (humidified, 95% air, 5% CO_2). This procedure was repeated for all samples. Cells were allowed to adhere to the plates for a minimum of two hours before being flooded with 300μl of complete DMEM. Media was changed every 3-4 days and cells were treated on days 10-14, or when confluent.

2.3.4. Harvesting cell-free supernatants for cytokine ELISAs

After the requisite incubation time, the 24 well plates were removed from the incubator and supernatant removed and stored in 1.5ml microtubes according to the treatment plan (wells that received the same treatment pooled into one 1.5ml microtube). All samples were kept on ice before being centrifuged at 13,000rpm for 3 minutes at 4°C to remove any cell debris. Samples were then aliquoted (2-3 aliquots per sample) into new tubes and frozen at –85°C until cytokine analysis. This procedure was repeated for each plate respectively.

2.3.5. Harvesting glial cells for Western Immunoblotting

Upon removal of cell supernatants, wells were washed twice with Dulbeccos PBS (200μl) to remove all remaining supernatant. If not harvested immediately, plates were frozen at -85°C for harvesting at a later date.

On the day of harvest, DTT (5mM) and protease and phosphatase inhibitors (1% v/v) added to the requisite volume of lysis buffer stock solution (25mM Hepes, 5mM $MgCL_2$, 5mM EDTA in $d.H_2O$).

If necessary, plates were defrosted on ice, otherwise 50μl of lysis buffer was added to each well. The plate was transferred to a rock 'n' roller, and incubated on ice for fifteen minutes. Following this incubation period, the bottom of each well was scraped with the rubber-end of a 1ml syringe insert to remove cells from the bottom of the well. The lysis buffer was then aspirated into a 1.5ml microtube on ice. Cells from different wells that received the same treatment were pooled into one tube. The insert was washed in $d.H_2O$ between each treatment group to avoid cross-contamination.

Protein analysis was carried out on sonicated (10 microns for 2 seconds) samples, using a BCA protein assay. Protein concentrations were equalised using lysis buffer before being diluted 1:2 with western blotting sample buffer (1.1ml dH_2O, 0.5ml Tris-HCl (pH6.8), 0.8ml Glycerol, 0.8ml 10% SDS, 0.2ml β-mercaptoethanol). Samples were boiled for 5 minutes before being aliquoted into separate tubes and frozen at -85°C for western immunoblotting.

2.3.6. Harvesting glial cells for mRNA analysis

Supernatants were removed and 50μl of RA1 lysis buffer (total RNA isolation kit, Macherney-Nagel) containing 1% β-mercaptoethanol was pipetted directly onto wells of culture plates. The lysed cells and buffer were removed from the wells and placed in microtubes and disrupted using a polytron tissue disrupter (Kinetatica). mRNA extraction was then performed as described in section 2.4.5.

2.4. Analytical methods

2.4.1. Analysis of cytokine concentrations by ELISA

The sandwich enzyme-linked immunosorbent assay (ELISA) method was used to determine cytokine concentrations in serum, mixed-glial supernatants or supernatants prepared from brain tissue homogenates.

Interleukin (IL)-1β

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 of capture antibody [0.8μg/ml in Phosphate Buffered Saline (PBS): 137mM NaCL, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, 2.7mM KCL, pH 7.4] and incubated overnight at 4°C. The plates were washed four times with 300μl of wash buffer per well (PBS with 0.05% Tween-20) and excess wash buffer removed by blotting plate on a paper towel. The plates were then blocked with 300μl per well of blocking buffer (PBS with 1% BSA and 5% sucrose) for a minimum of one hour at room temperature. Following four washes, 100μl of samples or recombinant IL-1β standards (0-2000pg/ml in DMEM:F12) were added to the plates which were incubated for two hours at room temperature. Plates were washed as before, and 100μl of detection antibody (63μg/ml, diluted to a working concentration of 350ng/ml in PBS with 1% BSA and 2% normal goat serum) was added to each well and the plates incubated at room temperature for 2hrs. Plates were washed as before and 100μl of a working solution of Horse radish peroxidase (HRP)-conjugated streptavidin (1:200 dilution streptavidin-HRP in PBS and 1% BSA) was added to each well, covered and incubated for twenty minutes at room temperature, avoiding direct light.
Plates were washed and 100μl of substrate solution 3,3',5,5',-Tetramethyl-benzidine (TMB) added to each well.

This was incubated for approximately 20 minutes or prior to the colorometric reaction reaching saturation. 50μl of stop solution (1M H_2SO_4) was then added to each well and the absorbance measured at 450nm using a microtitre plate reader (Elx 800 Bio-Tek instruments Inc.). A standard curve was constructed by plotting the standards against the absorbance and results obtained expressed as pg/ml of supernatant/serum or % control

Interleukin (IL)-10

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 TMB substrate solution was added to each well. This was incubated for 20 minutes or prior to the colorometric reaction reaching saturation. 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.

Tumor Necrosis Factor-α

A TNF-α kit (Opt EIA set, BD Biosciences) was used to perform this assay. 96-well plates (NUNC, F96 MAXISORP-immuno plate) were coated with 100µl capture antibody, monoclonal anti-rat TNF-α antibody (1:250 dilution in bicarbonate coating Buffer: 3.36mM Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.5) and incubated overnight at 4°C. Plates were washed four times with 300µl of PBS containing 0.05% Tween-20 and excess wash buffer removed by blotting plate on a paper towel. Plates were blocked for one hour at room temperature with 200µl of assay diluent

(PBS, containing 10% fetal bovine serum) per well. Plates were washed as before and 100µl of TNF-α standard (0-2000pg/ml) or sample added to each well and incubated at room temperature for 2 hrs. Following this incubation time, plates were washed and 100µl of detection antibody, (biotinylated anti-rat TNF-α, 1:250 dilution in assay diluent) added to each well and incubated at room temperature for 1 hr. Plates were washed as described and 100µl of a working solution of Horse radish peroxidase (HRP) conjugated streptavidin (1:250 dilution in assay diluent) added to each well and incubated for 30 min at room temperature, avoiding direct light.

Following four washes, 100µl of TMB substrate solution was added to each well. This was incubated for 20 minutes or prior to the colorometric reaction reaching saturation. 50µl 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 or % control.

Transforming Growth Factor-β

A TGF-β_1 E_{max}® ImmunoAssay system (Promega) was used to perform this assay. 96-well plates (NUNC, F96 MAXISORP-immuno plate) were coated with 100µl capture antibody, monoclonal anti-rat TGFβ antibody (1:1000 dilution in bicarbonate coating Buffer: 25mM Na_2CO_3, 25mM $NaHCO_3$, pH 9.5) and incubated overnight at 4°C. Plates were allowed warm to room temperature (RT) before contents were flicked out and the excess removed by blotting the plate on a paper towel. 270µl of 1X block buffer (1:5 dilution of 5X Block buffer supplied in kit) was added to each well and the plate incubated at 37°C for 35 minutes. Plates were washed once with 400µl of Tris-buffered saline (TBS), (20mM Tris, 150mM NaCl) containing 0.05% Tween-20 (TBS-T) and excess wash buffer removed by blotting plate on a paper towel. To measure bioactive form of $TGF\beta_1$, 100µl of sample or $TGF\beta_1$ standard (recombinant human $TGF\beta_1$, 0-1000pg/ml) were added to each well and incubated at room temperature for 1 hour and thirty minutes with shaking (400-600rpm) or at 37°C without shaking. Following this incubation time, plates were washed 5 times with TBS-T, and 100µl of primary antibody, (1:1000 dilution of primary antibody diluted in 1X Sample buffer. Sample buffer diluted 1:10

from 10X sample buffer supplied in kit) added to each well. Plates were then incubated for 2 hours at RT with shaking. Plates were washed five times and 100μl of a TGFβ$_1$ Horse radish peroxidase (HRP) (1:100 dilution in 1X sample buffer) added to each well and incubated for two hours at RT with shaking. Plates were washed five times, as before and 100μl of TMB One solution (supplied with kit) added to each well and incubated for 15 minutes or prior to the colorometric reaction reaching saturation. 100μl of 1N HCl was then added to each well to stop the colormetric reaction 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 or % control.

2.4.2. Analysis of nitrite concentrations

A greiss assay was used to determine nitrite concentrations in mixed-glial supernatants. The formation of nitric oxide in the cell cannot be measured directly because it is broken down into two stable and non-volatile substrates – nitrite and nitrate. For this assay, standards of sodium nitrite are made up of known concentrations to make a standard curve (0-180μM nitrite). 100μl of sample and standard were loaded onto a 96 well plate. 50μl of Griess Reagent I containing 1% sulphanilamide in 5% orthophosphoric acid was added to the plate and incubated at room temperature for 10 minutes. 50μL of Griess Reagent II which contained N-1 naphthylenediamine dihydrochloride (0.1%) was then added to the plate and incubated at room temperature for a further 10 minutes. The absorbance was read at 450nm using a spectrometer and nitrite concentration was determined using the standard curve. Data expressed as μM/ml of supernatant.

2.4.3. Protein quantification-BCA assay

The BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. Protein content was quantified using this assay. A working solution of 2000μg/ml of bovine serum albumin (BSA) was prepared using lysis buffer stock as diluent. A set of dilutions were prepared to give final protein concentrations of: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0μg/ml BSA protein. Next 25μl of the dilutions and samples were pipetted in duplicate into the wells of a new 96 well

plate. A BCA working solution was prepared by adding one part of the BCA working reagent B to 50 parts of reagent A. 200μl of this working solution was added to each sample/standard. The 96 well plate was then covered and incubated at 37°C for 30 minutes. The plate was subsequently cooled to room temperature and the absorbance read at 560nm using a microtitre plate reader. A standard curve was constructed by plotting the standards against the absorbance and results obtained expressed as μg/ml of protein.

2.4.4. SDS-PAGE & Western Immunoblotting

SDS-PAGE: Sodium dodecylsulphte – 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 placed in the Biorad casting frame which was then secured in the casting stand. 7.5% separating gels (2.425ml d.H_20, 1.25ml Bis/acrylamide/Bis, 1.25ml Tris-HCl [pH 8.8], 50μl SDS, 25μl APS, 3μl TEMED) were cast by gently pipetting the freshly-made solution between the two plates (plastic or glass) using with a Pasteur pipette. A temporary layer of isopropranol was placed on top of the gels to avoid evaporation. When the polyacrylamide gel was set, the isopropranol or water was drained off and the top of the gel rinsed with d.H_2O. A freshly-prepared 4% stacking gel (1.525ml d.H_20, 0.325ml Bis/acrylamide/Bis, 0.625ml Tris-HCl [pH 6.8], 25μl SDS,12.5μl APS, 4μl TEMED) was then pipetted onto the separating gel. The well combs were carefully place into the stacking gel, which was allowed to set for ~30 min. The plates containing the gel (gel cassette) were removed from the casting frame and placed in the Biorad Electrode Assembly, this assembly was then inserted into the Biorad clamping frame which was lowered into the inner chamber of the Biorad mini stand. The inner chamber was then filled with cold electrode running buffer [125mM Tris Base, 960mM glycine, 5% SDS (w/v)] and the well combs carefully removed. 10 or 20μl of sample was pipetted into each well and 5μl of a broad–range molecular weight marker added to the outside gels lanes. A 32mA current (Biometra-Standard power pack 25) was applied per individual gel for 30-40 minutes or until the marker reached the end of the gel.

Western Immunobloting: 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)]. Nitrocellulose paper and filter paper (pre-soaked in transfer buffer) 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 filterpaper, gel, nitrocellulose, 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 applied 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) to remove the methanol of the transfer buffer. The membrane was then transferred to 10ml of blocking buffer consisting of TBS-T containing 5% (w/v) BSA, and blocked for two hours. The membrane was then washed four times for 10-15 minutes in TBS-T before being incubated (overnight at 4°C or for 2 hours at room temperature) with the primary antibody diluted to the requisite concentration in TBS-T with 2% BSA (w/v). Following incubation with the primary antibody the membrane was washed as described previously. The membrane was then incubated with secondary antibody diluted to the requisite concentration in 2% BSA (w/v) TBS-T for 1 hour. The membrane was washed as before and exposed to 1ml of chemiluminescent solution (Supersignal®) for one minute. Following exposure of the membrane to photographic film in a dark room the film was developed and fixed using an automated developer. Then membrane was then washed as before and re-blocked with 5% BSA (w/v) in TBS-T for 2 hours at room temperature, or overnight at 4°C. (Depending on the size of the next protein to be immunoblotted, the membrane could be stripped by incubating the membrane with 20mls of Restore™ stripping solution for ten minutes prior to blocking). Following blocking the membranes were washed and re-probed with primary antibody as before.

2.4.5. Preparation of samples for real-time polymerase chain reaction (PCR)

RNA extraction procedure: Total RNA isolation kit

A total RNA isolation kit (Macherney-Nagel) was used to extract RNA. Briefly, samples were removed from freezer, put in 350μl of RA1 buffer and 3.5μl of β-mercaptoethanol and homogenised using a polytron tissue disrupter (Kinetatica). To harvest cultured cells, 50μl of RA1 lysis buffer containing 1% β-mercaptoethanol was pipetted directly onto wells of culture plates. The lysed cells

and buffer were removed from the wells and placed in microtubes disrupted using a polytron tissue disrupter (Kinetatica).

Sample/cell homogenate was 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 30seconds. 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.

Assessment of RNA quality

RNA was separated on a 1% agarose gel to check integrity of extracted RNA samples. 1.3g 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. 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). At this point power supply disconnected from gel rig and gel taken to UV transilluminator and RNA visualised. 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.

RNA quantification

Optical density (OD) of RNA was measured using a spectrophotometer (UV/vis Beckman Coulter Du730) to determine RNA concentration and purity. 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.

Reverse Transcription of RNA

A high capacity cDNA archive kit (Applied Biosystems) was used to reverse transcribe samples extracted using the Total RNA isolation kit (Macherney-Nagel). 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.

2.4.6. Real-time PCR

Gene expression of inflammatory targets (see Table 2.1 for list) was assessed using off the shelf Taqman gene expression assays containing specific target primers, and FAM-labelled MGB target probes. 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).

Table 2.4.1: List of Taqman gene expression assays used

Gene name	Assay number	*NCBI Gene reference
TNF-α	Rn99999017_m1	NM_012675.2
IL-1β	Rn00580432_m1	NM_031512.1
iNOS	Rn00561646_m1	NM_012611.2
COX II	Rn00568225_m1	NM_017232.2
CD40	Rn01423583_m1	NM_134360.1
IL-10	Rn00563409_m1	NM_012854.1
RANTES	Rn00579590_m1	NM_031116.2
IP-10	Rn00594648_m1	NM_139089.1
CINC-1	Rn00578225_m1	NM_030845.1
MIP-1α	Rn00564660_m1	NM_013025.2

*Gene reference as listed on the National centre for biotechnology information (NCBI) Entrez-Nucleotide website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide

Plate set-up for single target (singleplex) QPCR: Briefly, cDNA was diluted 1:5 and 10μl of diluted cDNA was pipetted onto a PCR plate, to which 1μl of target or endogenous primer/probe and 10μl of Taqman master mix was added (21μl reaction volume). Samples were run in duplicate, and electronic pipettes (EDP3 20-200μl, 2-20μl and 10-100μl) were used to ensure pipetting accuracy.

Plate set-up for multi-target (multiplex) QPCR: Briefly, cDNA was diluted 1:4 and 10μl of diluted cDNA was pipetted onto a PCR plate, to which 1.25μl of target primer/probe and 1.25μl β-actin primer/probe and 12.5μl of Taqman master mix was added (25μl reaction volume). 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 Biosystems 7300) using the following programme; step 1: 95°C for 10 minutes, step 2: 95°C for 15 seconds followed by 1 minute at 60°C. Step two was repeated 40 times, and fluorescence read during the annealing and extension phase (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-labelled 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).

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. 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-fold difference is a 32-fold difference (2^5).

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 2^{-0} gives a $2^{\Delta\Delta CT}$ of 1, against which all other samples are referenced, as outlined in Table 2.2 below.

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Table 2.2: Example of the ΔΔCT method of analysis

	Target CT	β-actin CT	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$ Fold difference
Control	20	16	20 -16 = 4	4 - 4 = 0	$2^{-0} = 1$
Sample 1	21	16	21 – 16 = 5	5 - 4 = 1	$2^{-1} = 0.5$
Sample 2	22	16	22 – 16 = 6	6 - 4 = 2	$2^{-2} = 0.25$
Sample 3	19	16	19 – 16 = 3	3 - 4 = -1	$2^{-(-1)} = 2$
Sample 4	18	16	18 – 16 = 2	2 – 4 = -2	$2^{-(-2)} = 4$
Sample 5	17	16	17 – 16 = 1	1 – 4 = -3	$2^{-(-3)} = 8$

2.5. Statistical Analysis of Results

All data was analysed using a GB-STAT routine. Statistical comparisons were initially performed using a one, two, or three-way analysis of variance (ANOVA), with or without repeated measures, as indicated in the experimental sections. If significant changes were observed, the data was further analysed using Fishers LSD or Dunnetts *post hoc* test as appropriate. A p value less than 0.05 was considered statistically significant. Results are expressed as means and standard error of the mean (SEM) or mean percentage control and SEM. For real-time PCR analysis the fold difference was measured using the ΔΔCT as described. The fold difference in gene expression for each sample was expressed as a percentage of the LPS-treated group and statistical analysis was carried out as above.

CHAPTER 3

Results

3.1. Noradrenaline pre-treatment attenuates LPS-induced pro- and anti-inflammatory cytokine and iNOS production from cultured cortical glial cells

The following study was carried out to characterise the effects of NA on LPS-induced pro-inflammatory cytokine and NO production from cortical mixed glia over a 24-hour period. As anti-inflammatory cytokines negatively regulate pro-inflammatory cytokine production, the effects of NA on LPS-induced IL-10 and TGFβ production in mixed cortical glia were also assessed in this study. Vehicle or NA (10μM) was administered 2 hours prior to stimulation with LPS (1μg/ml) and cells co-incubated with NA for 6, 12 or 24 hours. DMEM was administered to unstimulated cells as a control treatment. Supernatants were removed and assessed for cytokine production by ELISA. Cells were harvested for Western Immunoblotting for iNOS expression, and concentrations of NO_2 concentrations were assessed in supernatants using the Greiss assay as a proxy measure of nitric oxide production.

Glial TNF-α production: LPS significantly induced TNF-α production 6, 12 and 24 hours post-treatment ($P<0.01$), and NA (10μM) significantly decreased LPS-induced TNF-α production ($P<0.01$) at all three timepoints (Figure 3.1.1a.).

Glial IL-1β production: LPS significantly induced IL-1β concentrations ($P<0.01$) 12 and 24 hours post-treatment. NA pre-treatment suppressed this LPS-induced increase in IL-1β production (10μM) at both timepoints ($P<0.01$) (Figure 3.1.1b)

Glial nitrite production and iNOS expression: LPS significantly induced nitrite production ($P<0.01$) 24 hours post-treatment, and this increase was significantly attenuated by pre-treatment with NA (10μM) (Figure 3.1.2a). NA pre-treatment also prevented LPS -induced iNOS expression 24 hours post-treatment (Figure 3.1.2b).

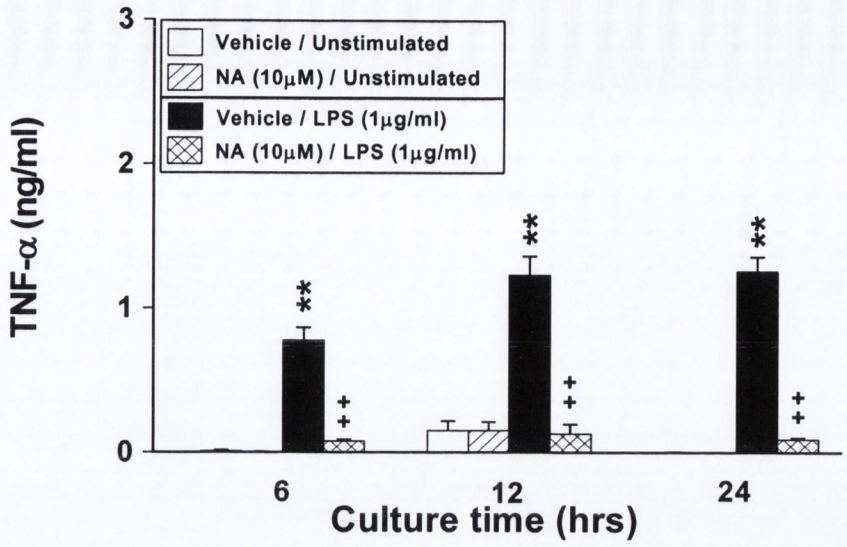
Glial IL-10 production: LPS significantly induced IL-10 production 6, 12 and 24 hours post-treatment ($P<0.01$). NA (10μM) pre-treatment significantly attenuated

this LPS-induced increase in IL-10 at 12 and 24 post-LPS treatment ($P<0.01$, Figure 3.1.3a).

Glial TGF-β production: Neither NA pre-treatment nor LPS treatment altered TGF-β concentrations in mixed glial cultures over the 24-hour period assessed (see Figure 3.1.3b).

Correlation between pro- and anti-inflammatory cytokines: A significant correlation was found to exist between TNF-α and IL-10 concentrations (Pearson $r = 0.69$, $P<0.001$, degrees of freedom = 94) and between IL-1β and IL-10 concentrations (Pearson $r=0.73$, $P<0.001$, degrees of freedom = 94) across all three timepoints.

(a)



(b)

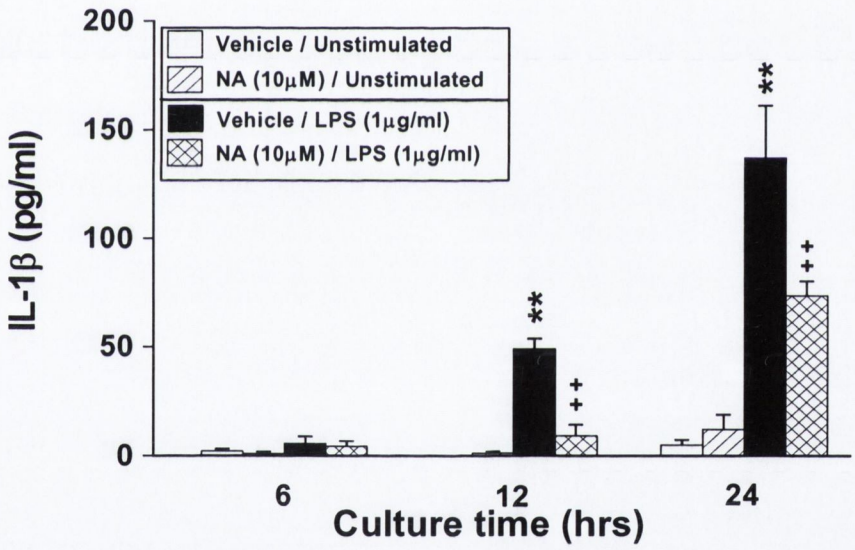


Figure 3.1.1: NA attenuates LPS-induced TNF-α and IL-1β production in mixed glial cultures

Vehicle / NA (10μM) were administered to cells 2 hours prior to vehicle/LPS (1μg/ml). (a) NA significantly attenuated LPS-induced TNF-α production at 6, 12 and 24 hours. (b) NA significantly attenuated IL-1β production at 12 and 24 hours post-treatment in mixed glial cultures.

Repeated 3-way ANOVA revealed a significant interaction between NA pre-treatment, LPS treatment and time on TNF-α [$F(2,56)=5.88$, $P<0.01$] and IL-1β [$F(2,56)=5.73$, $P<0.01$] production.

Data expressed as means and standard error of the mean (n=8). **P<0.01 versus Vehicle/Unstimulated counterparts, ++P<0.01 versus LPS-stimulated counterparts (Fishers LSD test).

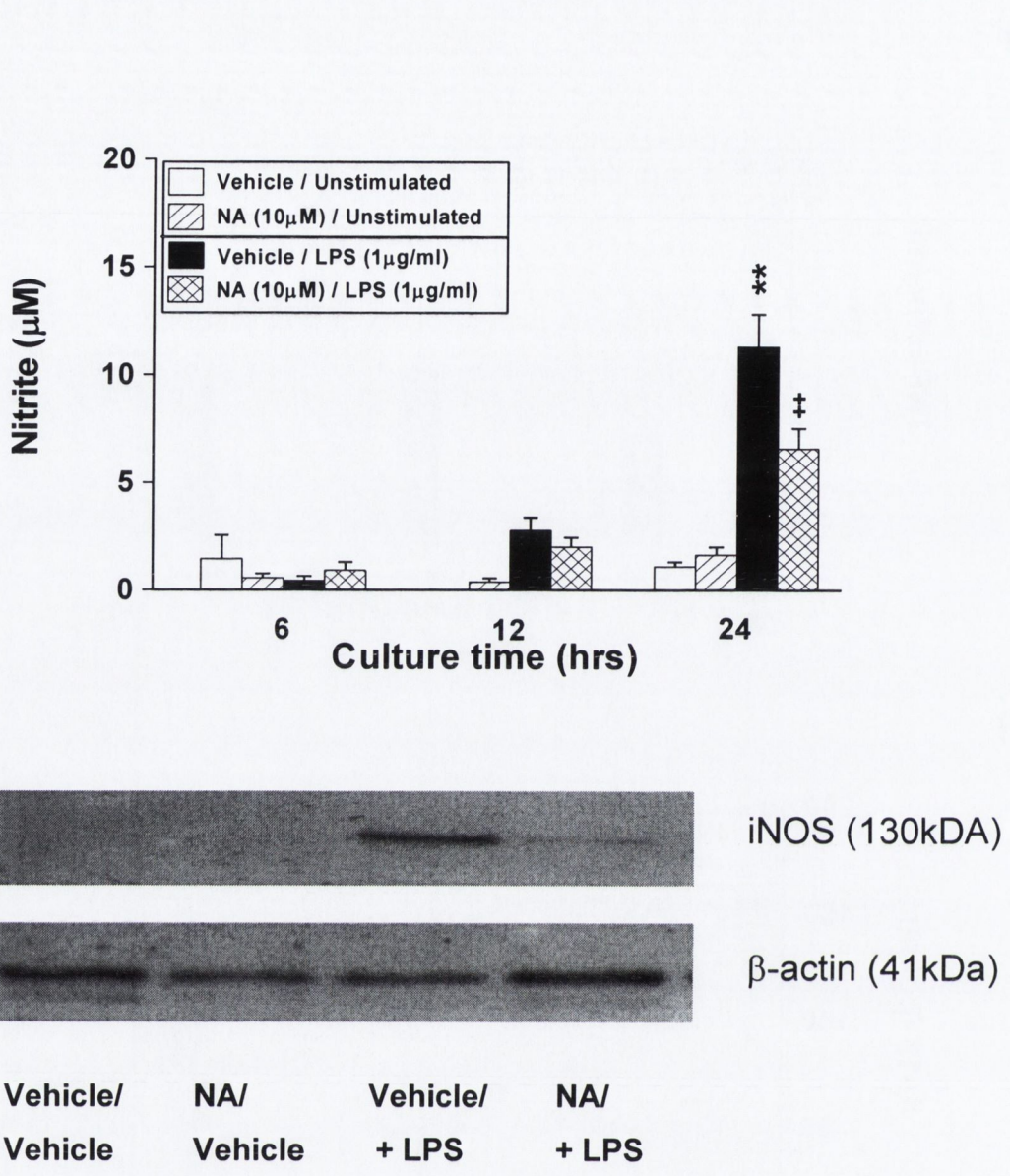


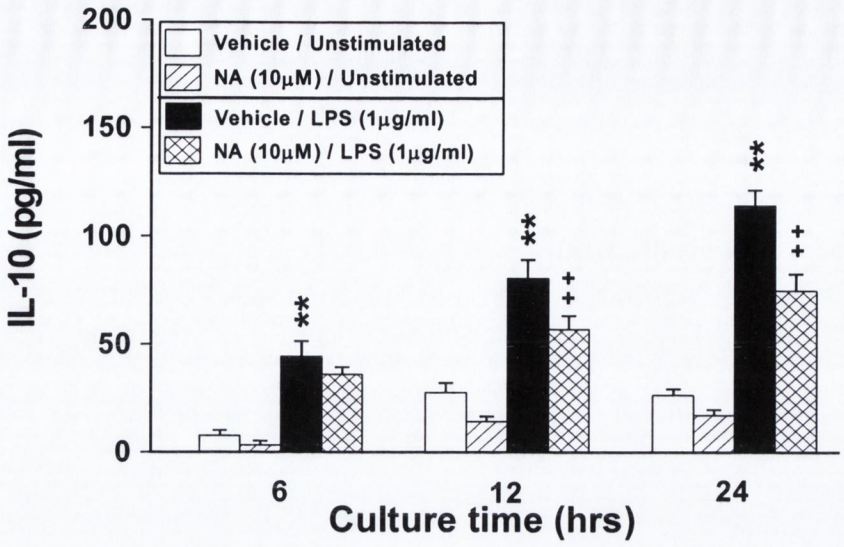
Figure 3.1.2: NA attenuates LPS-induced nitrite production and iNOS expression in mixed glial cultures

Vehicle / NA (10μM) were administered to cells 2 hours prior to vehicle/LPS (1μg/ml). (a) NA significantly attenuated LPS-induced nitrite production at 24 hours. (b) Sample immunoblot depicting the suppressive effect of NA (10μM) on LPS (1μg/ml)-induced iNOS expression 24 hours post stimulation (representative of 5 independent immunoblots).

Repeated 3-way ANOVA revealed a significant interaction between NA pre-treatment, LPS treatment and time, on nitrite [$F(2,56)=6.37$, $P<0.01$] production.

Data expressed as means and standard error of the mean (n=8). **P<0.01 versus Vehicle/Unstimulated counterparts, ++P<0.01 versus LPS-stimulated counterparts (Fishers LSD test).

(a)



(b)

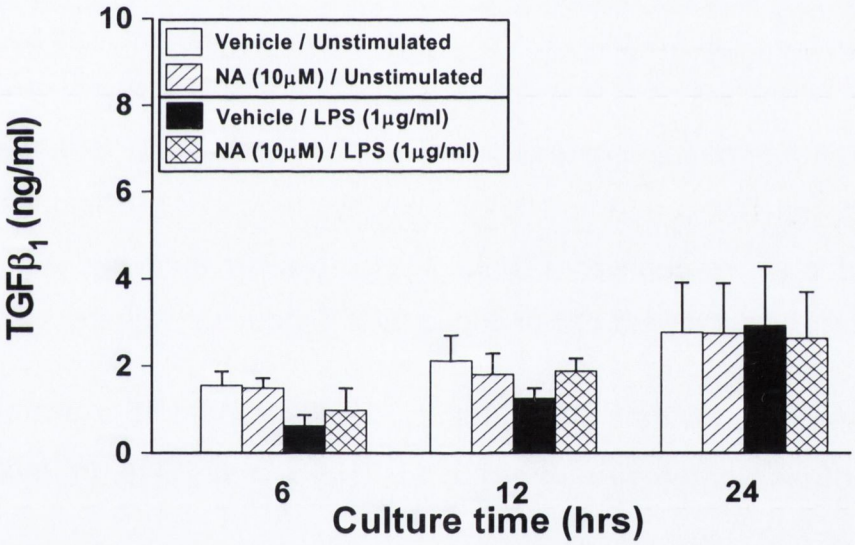


Figure 3.1.3: NA attenuates LPS-induced IL-10 but does not alter TGF-β production in mixed glial cultures

Vehicle / NA (10μM) were administered to cells 2 hours prior to vehicle/LPS (1μg/ml). (a) NA significantly attenuated LPS-induced IL-10 production at 12 and 24 hours. (b) NA did not alter TGF-β production at any timepoint in mixed glial cultures.

Repeated 3-way ANOVA revealed a significant interaction between NA pre-treatment, LPS treatment and time, on IL-10 [$F(2,56)=3.6$, $P<0.05$] but not TGFβ [$F(2,56)=0.16$, $P=0.85$] production. Data expressed as means and standard error of the mean (n=8). **P<0.01 versus Vehicle/Unstimulated counterparts, ++P<0.01 versus LPS-stimulated counterparts (Fishers LSD test).

3.2. Neutralisation of TNF-α, and/or IL-1β does not mimic the suppressive effect of noradrenaline on IL-10 production in mixed glial cells

In contrast to the literature, the previous studies demonstrate that LPS-induced IL-10 protein concentrations and mRNA are attenuated by NA pre-treatment in mixed glial cultures. As it has been reported that TNF-α and IL-1β are necessary for IL-10 induction in fetal microglia and macrophages, respectively (Sheng *et al.*, 1995; Souza *et al.*, 2003), it was hypothesised that NA may exert a suppressive effect on IL-10 by reducing production of TNF-α and IL-1β. In the following study TNF-α and IL-1β were neutralised, alone and in combination to determine whether TNF-α and/or IL-1β is required for LPS-induced IL-10 production in mixed glia. Control IgG, anti-TNF-α (2μg/ml), anti-IL-1β (0.25μg/ml) or anti-TNF-α (2μg/ml) + anti-IL-1β (0.25μg/ml) antibodies were co-administered with vehicle/LPS (0.1μg/ml). 24 hours post-treatment supernatants were collected for cytokine analysis by ELISA.

Neutralisation of the pro-inflammatory cytokines does not alter LPS-induced IL-10: Treatment with anti-TNF-α and anti-IL-1β antibody abrogated LPS-induced TNF-α and IL-1β production respectively (Table 3.3.1.). Neither antibody, administered alone or in combination altered LPS-induced IL-10 production (Figure 3.3.1).

Table 3.2.1: Verification that the concentrations of anti-TNF-α antibody and anti-IL-1β antibody employed neutralised TNF-α and IL-1β in mixed glial cultures

Cytokine (pg/ml)	Unstimulated Control	Control IgG + LPS	Anti-TNF-α + LPS	Anti-IL-1β + LPS	Anti-TNF-α/IL-1β + LPS
TNF-α	79 ± 15	1121 ± 157	17 ± 10 **	1404 ± 184	39 ± 36 **
IL-1β	0.2 ± 0.2	214 ± 80	233 ± 76	9 ± 9 **	0.2 ± 0.2 **

Control IgG, anti-TNF-α (2μg/ml), anti-IL-1β (0.25μg/ml) or anti-TNF-α + anti-IL-1β antibodies were co-administered with vehicle/LPS (0.1μg/ml). Treatment with anti-TNF-α and anti-IL-1β antibody abrogated LPS-induced TNF-α ($P<0.01$) and IL-1β ($P<0.01$) production.

One-way ANOVA revealed a significant effect of treatment on TNF-α [$F(4,28)=38.27$, $P<0.0001$] and IL-1β [$F(4,28)=7$, $P<0.001$] production. Data expressed as means and standard error of the mean (n=6-7). **$P<0.01$ versus control IgG (Fishers LSD test).

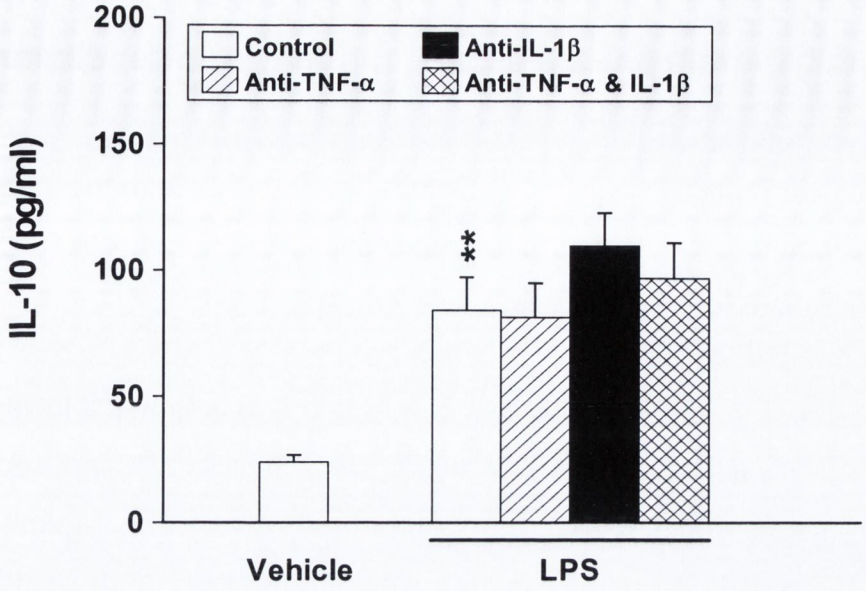


Figure 3.2.1: Neutralisation of TNF-α and/or IL-1β does not alter LPS-induced IL-10 production in mixed glial cultures.

Control IgG, anti-TNF-α (2mg/ml), anti-IL-1β (0.25μg/ml) or anti-TNF-α + anti-IL-1β antibodies were co-administered with vehicle/LPS (0.1μg/ml). Neutralisation of pro-inflammatory cytokines alone, or in combination, did not alter LPS-induced IL-10 concentrations.

One-way ANOVA revealed a significant effect of treatment on IL-10 [$F(4,28)=8.07$, $P<0.001$] production. Data expressed as means and standard error of the mean (n=6-7). *$P<0.05$, **$P<0.01$ versus control IgG (Fishers LSD test).

3.3. The suppressive effect of NA on LPS-induced IL-10 results from β_2-AR activation and subsequent elevations in intracellular cAMP

The following studies were carried out to determine whether β_2-adrenoceptor activation and subsequent elevations in intracellular cAMP mediate the suppressive action of NA IL-10 concentrations.

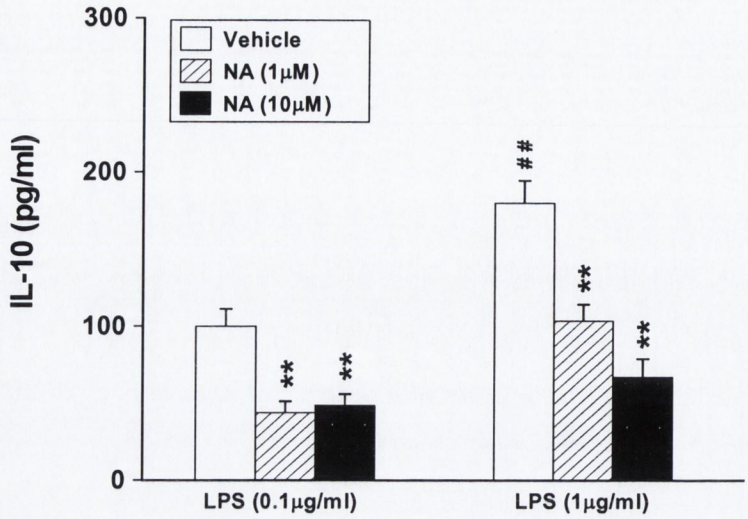
NA attenuates IL-10 protein and gene expression: To determine whether a lower concentration of NA (1μM) would exert a similar attenuating effect on LPS-induced IL-10, NA (1 and 10μM) was administered 2 hours prior to LPS (0.1 and 1μg/ml). 24 hours post-LPS treatment supernatants were collected and IL-10 concentrations assessed by ELISA. NA at both concentrations significantly attenuated LPS-induced IL-10 (see Figure 3.3.1a). To determine whether NA attenuated LPS-induced IL-10 at a transcriptional level, gene expression of this cytokine was examined using real-time PCR. NA (1 and 10μM) was administered 30 minutes prior to LPS and cells harvested for 6 hours later to examine mRNA expression. A vehicle control group was included to give an estimate of basal expression of IL-10 in mixed cortical glia under non-inflammatory conditions. LPS (1μg/ml) induced a 6-fold increase in glial IL-10 mRNA expression relative to control ($P<0.01$). NA (10μM) significantly attenuated LPS-induced IL-10 gene expression (Figure 3.3.1b).

The β_2-adrenoceptor agonist salbutamol exerts similar actions to NA and the β-AR antagonist propranolol blocks the suppressive action of NA on LPS-induced IL-10: To determine whether the β_2-adrenoceptor agonist salbutamol could mimic the action of NA in primary glial cells salbutamol (1 and 10μM) was administered 2 hours prior to LPS (0.1 and 1μg/ml). 24 hours post-LPS treatment supernatants were collected and IL-10 concentrations assessed by ELISA. 2 hour pre-treatment with NA (1 and 10μM) or salbutamol (1 and 10μM) prior to LPS (0.1 or 1μg/ml) treatment significantly decreased IL-10 production in glial cells ($P<0.01$, Figures 3.3.2a). To determine whether the β-AR antagonist could block the suppressive effects of NA on LPS-induced IL-10, propranolol (5μM) was administered 15 minutes prior to NA (10μM) and LPS (1μg/ml) administered 15 minutes post-NA

treatment. Propranolol (5μM) pre-treatment significantly attenuated the effects of NA on LPS-induced IL-10 ($P<0.01$) production (Figure 3.3.2b).

The cAMP analog di-butryl cAMP mimics the actions of noradrenaline and salbutamol on IL-10 production: As β_2-adrenoceptor activation increases intracellular cAMP concentrations, here we determined if the cAMP analog di-butryl cAMP could mimic the effects of NA and salbutamol on LPS-induced IL-10 production. Di-butryl cAMP (1-50μM) was administered 2 hours prior to LPS (1μg/ml). Di-butryl cAMP (50μM) significantly decreased LPS-induced IL-10 concentrations ($P<0.01$) in mixed glial cultures (Figure 3.3.3).

(a)



(b)

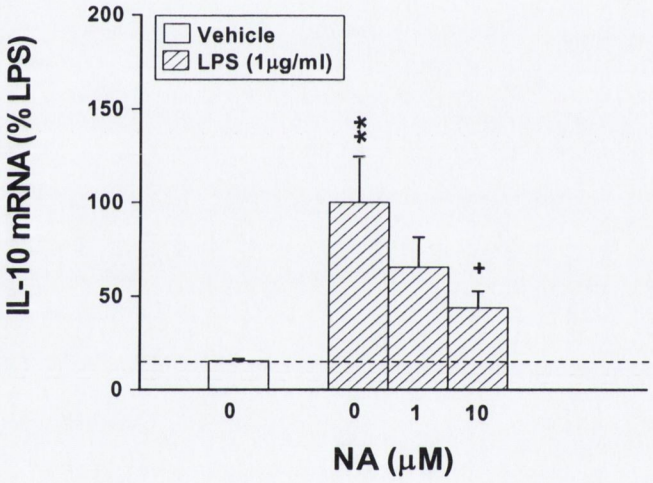


Figure 3.3.1: NA attenuates LPS-induced IL-10 protein and gene expression in mixed glial cultures

(a) Vehicle or NA (1 or 10μM) were administered to cells 2 hours prior to LPS (0.1or 1μg/ml). NA (1 and 10μM) significantly attenuated LPS (0.1 and 1μg/ml) -induced IL-10 production 24 hours post-treatment in mixed glial cultures. Two-way ANOVA revealed a significant interaction effect between NA pre-treatment and LPS treatment on IL-10 protein production [$F(2,36)=4.19$, $P<0.05$]. Data expressed as means and standard error of the mean (n=7-8). (b) NA (1 and 10μM) or vehicle were administered to cells 30 minutes prior to LPS (1μg/ml) and cells co-incubated with these treatments for 6 hrs. NA did not alter LPS-induced iNOS mRNA expression mixed glial cultures 6 hours post-treatment. One way ANOVA revealed a significant effect of treatment on IL-10 [$F(3,19)=5.69$, $P<0.01$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05 versus Vehicle/LPS group, # # P<0.01 versus LPS-stimulated counterparts (Fishers LSD test).

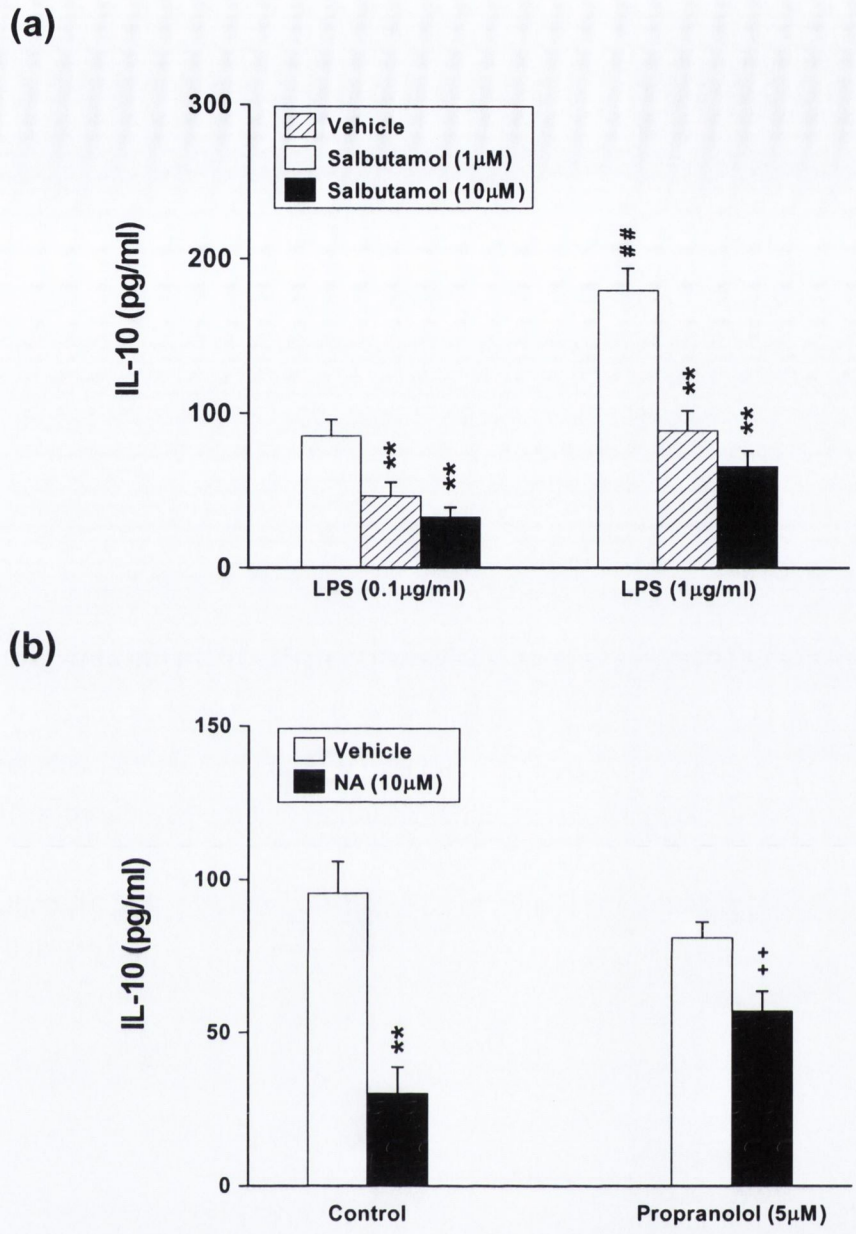


Figure 3.3.2: Salbutamol mimics and propranolol attenuates the NA-induced suppression of IL-10 in mixed glial cultures

(a) Vehicle, NA or salbutamol (1 or 10μM) were administered to cells 2 hours prior to LPS (0.1or 1μg/ml). (a) NA and (b) salbutamol (1 and 10μM) significantly attenuated LPS (0.1 and 1μg/ml) - induced IL-10 production 24 hours post-treatment in mixed glial cultures. Data expressed as means and standard error of the mean (n=7-8). (b) Vehicle or propranolol (5μM) were administered 15 minutes prior to NA (10μM), LPS (1μg/ml) was administered 15 minutes post-NA treatment. Propranolol attenuated NA-induced suppression of IL-10, 24 hours post-LPS treatment in mixed glial cultures. Data expressed as means and standard error of the mean (n=4).

Two-way ANOVA revealed a significant interaction effect between salbutamol pre-treatment and LPS treatment [$F(2,42)=4.62$, $P<0.05$] and a significant interaction effect between propranolol pre-treatment and NA treatment [$F(1,12)=6.9$, $P<0.05$] on IL-10 production. **$P<0.01$ versus respective control, ++$P<0.01$ versus NA-treated counterpart # # $P<0.01$ versus LPS-stimulated counterparts (Fishers LSD test).

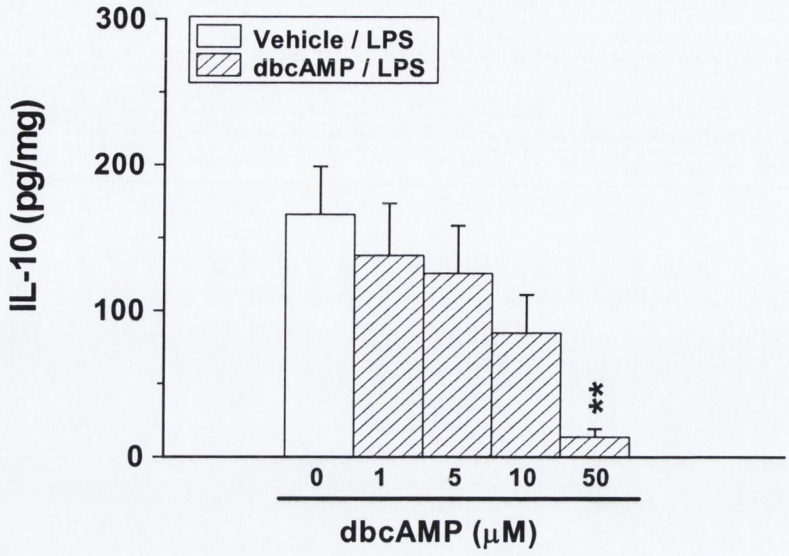


Figure 3.3.3: Di-butyryl cAMP suppresses LPS-induced IL-10 in mixed glial cultures

Di-butyryl cAMP (1-50μM) was administered 2 hours prior to LPS (1μg/ml) and cells co-incubated with these treatments for 24 hours. dbcAMP (50μM) significantly decreased LPS-induced IL-10 production in mixed glial cultures.

One-way ANOVA revealed a significant effect of dbcAMP -treatment [$F(4,30)=4.24$, $P<0.01$] on IL-10 concentrations. Data expressed as means and standard error of the mean (n=7)**$P<0.01$ versus Vehicle/LPS (Fishers LSD test).

3.4. Inhibition of the glial NA transporter does not alter LPS-induced pro-inflammatory cytokine production in cortical mixed glia

The following studies assessed the ability of the NA reuptake inhibitors (NRIs), DMI and ATX to alter LPS-induced TNF-α and IL-1β cytokine production in cortical glial cultures in the presence and absence of NA. To this end, vehicle (DMEM), DMI (1, 5 or 10μM) or ATX (1, 5 or 10μM) were administered 24 hours prior to LPS (1μg/ml) and cell supernatants removed for cytokine analysis 24 hours post-LPS administration.

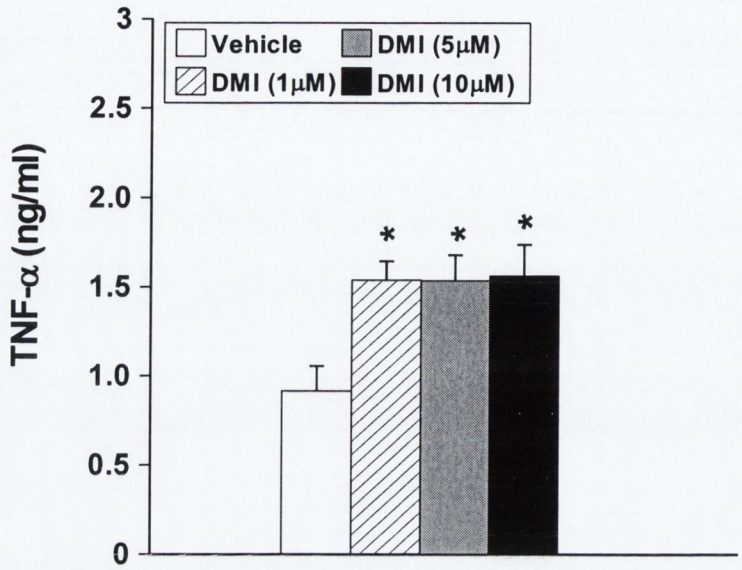
Effect of NRI treatment on glial TNF-α and IL-1β production in the absence of exogenous NA: DMI (1, 5 and 10μM) significantly augmented ($P<0.05$), while ATX did not alter LPS-induced TNF-α production (Figure 3.5.1a and b). ATX (5μM) significantly attenuated ($P<0.05$), while DMI did not alter LPS-induced IL-1β production (Figure 3.4.2a and b).

From the above study DMI (10μM) and ATX (10μM) were chosen to pre-treat cells 30 minutes prior to NA (0.1, 0.5, 1, 5 and 10μM) administration. Cells were co-incubated for 24 hours prior to LPS treatment. Supernatants were removed for cytokine analysis 24 hours post-LPS administration. The aim of this study was to determine if pre-treatment with NRI's could enhance the anti-inflammatory actions of exogenous noradrenaline in mixed glial cells.

Effect of NRI treatment on glial TNF-α production in the presence of exogenous NA: DMI (10μM) significantly attenuated ($P<0.05$) the suppressive effect of NA (1μM), on TNF-α concentrations, whereas ATX did not alter the suppressive effect of NA on LPS-induced TNF-α production (Figure 3.4.3a and b).

Effect of NRI treatment on glial IL-1β production in the presence of exogenous NA: ATX (10μM) significantly attenuated ($P<0.01$) the suppressive effect of NA (0.5 and 1μM), on IL-1β concentrations, whereas DMI did not alter the suppressive effect of NA on LPS-induced IL-1β production (Figure 3.4.4a and b).

(a)



(b)

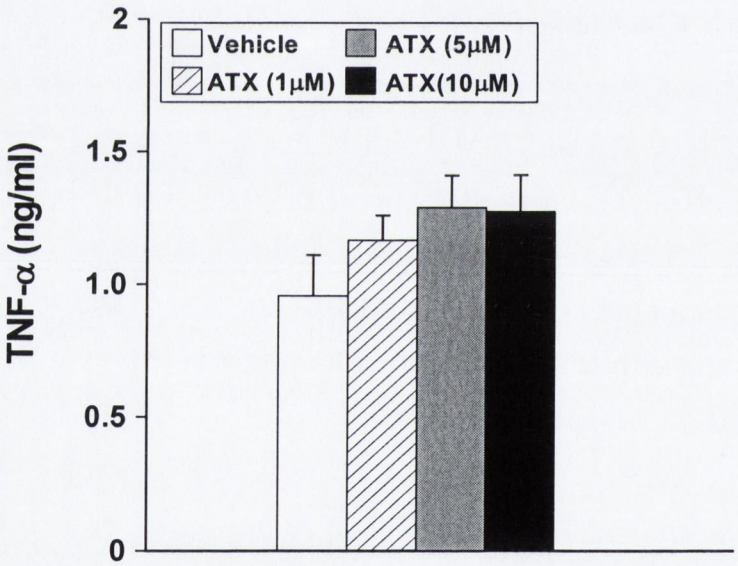


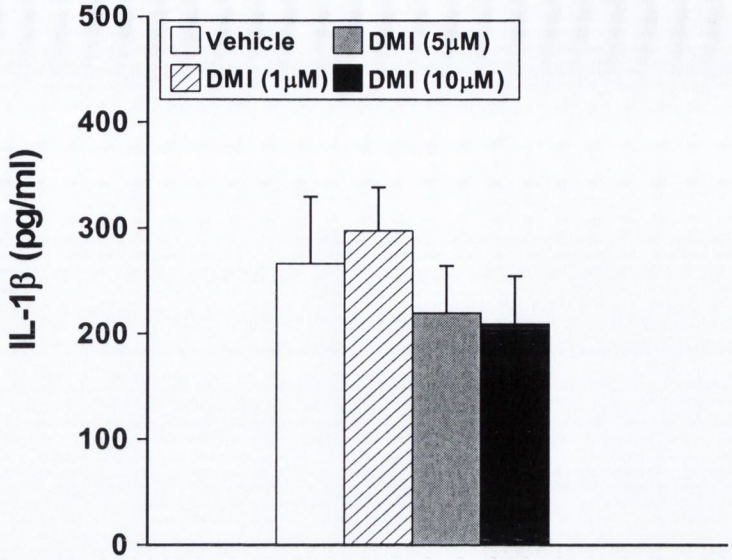
Figure 3.4.1: DMI augments while ATX does not alter LPS-induced TNF-α

DMI or ATX (1, 5 or 10μM) were administered 24 hours prior to LPS (1μg/ml) and cells co-incubated with these treatments for 24 hours. (a) DMI significantly augments LPS-induced TNF-α production in mixed glial culture. (b) ATX does not alter LPS-induced TNF-α production in mixed glial culture.

One-way ANOVA revealed a significant effect of DMI treatment [$F(3,16)=4.5$, $P<0.05$] but no significant effect of ATX treatment [$F(3,20)=1.4$, $P=0.27$] on TNF-α concentrations.

Data expressed as means and standard error of the mean (n=5-6). *$P<0.05$ versus Vehicle / LPS (Dunnetts test).

(a)



(b)

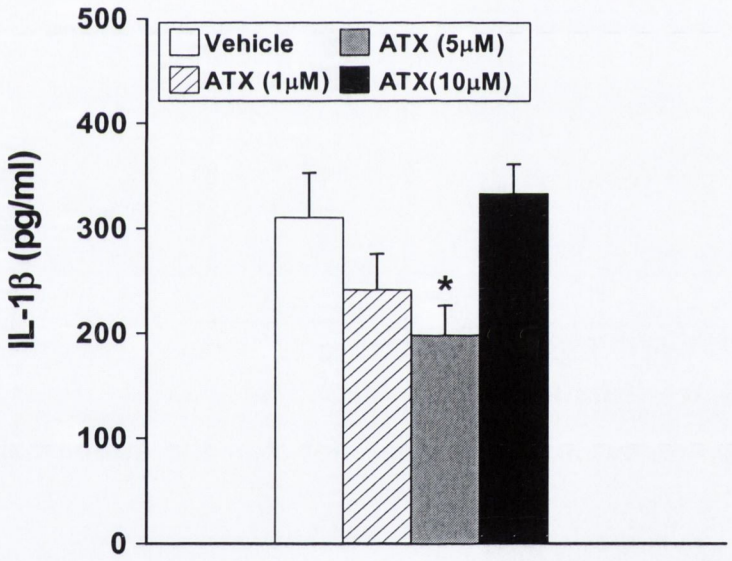


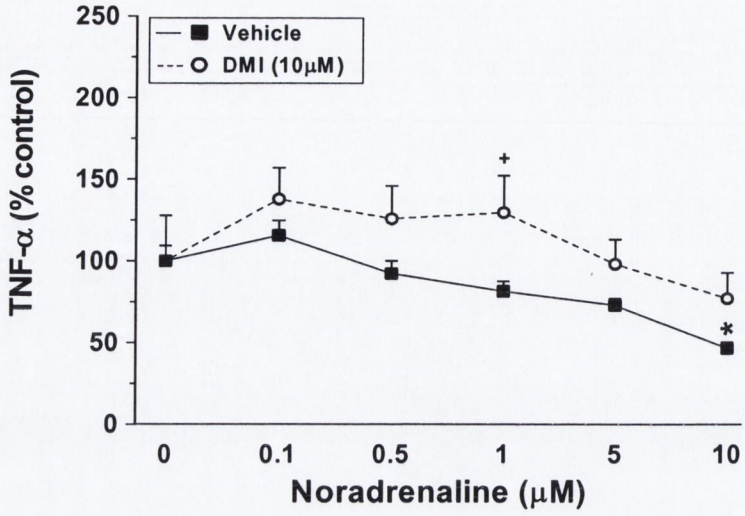
Figure 3.4.2: ATX (5μM) attenuates while DMI does not alter LPS-induced IL-1β

DMI or ATX (1, 5 or 10μM) were administered 24 hours prior to LPS (1μg/ml) and cells co-incubated with these treatments for 24 hours. (a) DMI does not alter LPS-induced IL-1β production in mixed glial culture. (b) ATX (5μM) significantly attenuates LPS-induced IL-1β production in mixed glial culture.

One-way ANOVA revealed no effect of DMI treatment [$F(3,19)=0.75$, $P=0.5$] and a significant effect of ATX treatment [$F(3,18)=3.37$, $P<0.05$] on IL-1β concentrations.

Data expressed as means and standard error of the mean (n=5-6). *$P<0.05$ versus Vehicle/LPS (Dunnetts test).

(a)



(b)

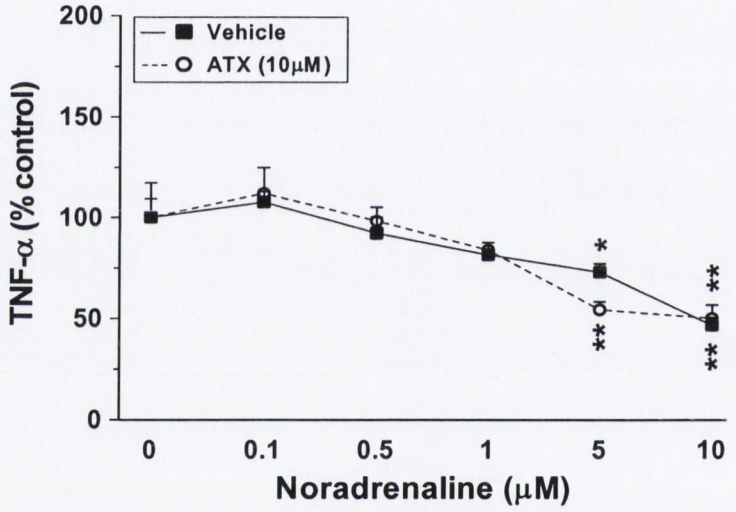
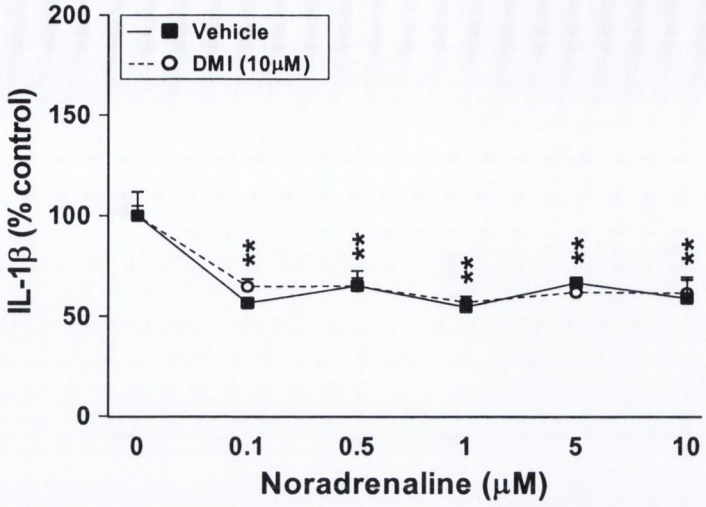


Figure 3.4.3: DMI attenuates, and ATX does not alter the suppressive effect of NA on LPS-induced TNF-α

Vehicle, DMI or ATX (10μM) were administered 30 minutes prior to NA (0, 0.1, 0.5, 5 and 10μM) and cells co-incubated with these treatments for 24 hours before the addition of LPS (1μg/ml), and samples were harvested 24hours later for cytokine analysis. (a) DMI (10μM) attenuates the suppressive effect of NA (1μM) on LPS-induced TNF-α production in mixed glial culture. (b) ATX (10μM) does not alter the suppressive effect of NA on LPS-induced TNF-α production in mixed glial culture. Two-way ANOVA revealed a significant effect of NA treatment [$F(5,82)=15.4$, $P<0.0001$], but no interactive effect of NA with DMI on TNF-a concentrations. Similarly a significant effect of NA [$F(5,84)=3.3$, $P<0.01$] but no interaction between ATX and NA on TNF–α concentrations was observed. Data expressed as mean (% control) and standard error of the mean (n=7-8). *P<0.05, **P<0.01 versus Vehicle/LPS, +P<0.05 versus Vehicle / NA same dose (Fishers LSD test).

(a)



(b)

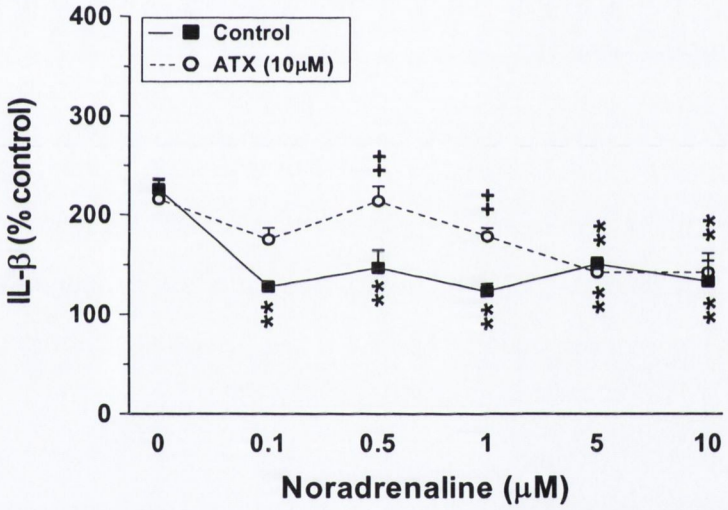


Figure 3.4.4: ATX attenuates, and DMI does not alter the suppressive effect of NA on LPS-induced IL-1β

Vehicle, DMI or ATX (10μM) were administered 30 minutes prior to NA (0, 0.1, 0.5, 5 and 10μM) and cells co-incubated with these treatments for 24 hours before the addition of LPS (1μg/ml) and samples were harvested 24hrs later for cytokine analysis. (a) DMI (10μM) does not alter the suppressive effect of NA on LPS-induced IL-1β production in mixed glial culture. (b) ATX (10μM) attenuates the suppressive effect of NA (0.5 and 1μM) on LPS-induced IL-1β production in mixed glial culture. Include ANOVA for sig effects of NA as opposed to the non-significant interactions Two-way ANOVA revealed a significant effect of NA treatment [$F(5,84)=3$, $P<0.05$] and DMI treatment [$F(1,84)=18.8$, $P<0.0001$] but no interactive effect of NA with DMI on IL-1β concentrations. A significant effect of NA [$F(5,84)=10.3$, $P<0.0001$]and ATX [$1,84)=28$,$P<0.0001$] but no interactive effect of NA with ATX [$F(5,84)=3.04$, $P=0.05$] on IL-1β concentrations was observed. Data expressed as mean (% control) and standard error of the mean (n=7-8). **P<0.01 versus Vehicle/LPS, ++P<0.01 versus Vehicle / NA same dose (Fishers LSD test).

Inhibition of the non-neuronal transporter uptake$_2$

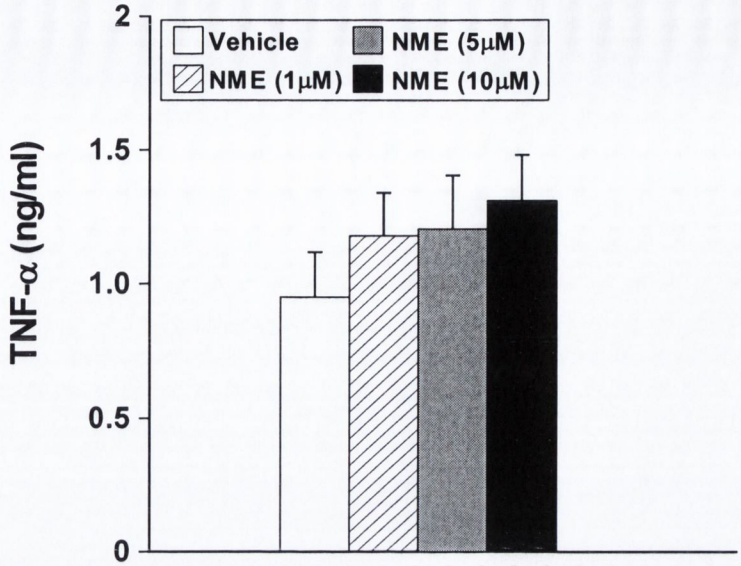
As neither DMI nor ATX augmented the anti-inflammatory effects of NA on pro-inflammatory cytokines, inhibition of the non-neuronal NA transporter (Uptake$_2$) was investigated. To this end the uptake$_2$ inhibitor normetanephrine (NME) was used to treat mixed glia, as previously described for DMI and ATX.

Effect of NME treatment on glial TNF-α and IL-1β production in the absence of exogenous NA: NME pre-treatment did not alter LPS-induced TNF-α or IL-1β production (see Figure 3.4.5a and b).

From the above study NME (10μM) was chosen to pre-treat cells 30 minutes prior to NA (0.1, 0.5, 1, 5 or 10μM) treatment. Cells were co-incubated for 24 hours prior to LPS treatment, and supernatants were removed for cytokine analysis 24 hours post-LPS administration.

Effect of NME treatment on glial TNF-α and IL-1β production in the presence of exogenous NA: NME (10μM) significantly augmented ($P<0.05$) LPS-induced TNF-α in the presence of NA (0.1μM), but failed to alter the suppressive effects of higher doses of NA on TNF-α production. NME did not alter the suppressive effect of NA on LPS-induced IL-1β concentrations (see Figure 3.4.6a and b).

(a)



(b)

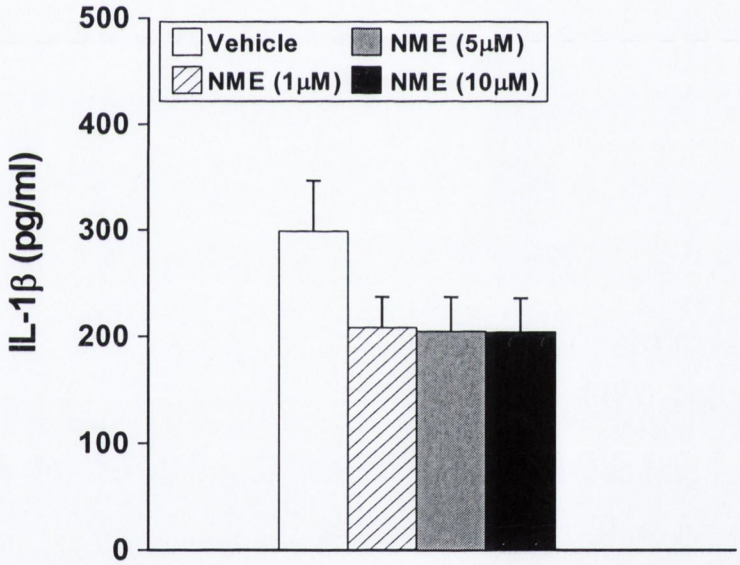
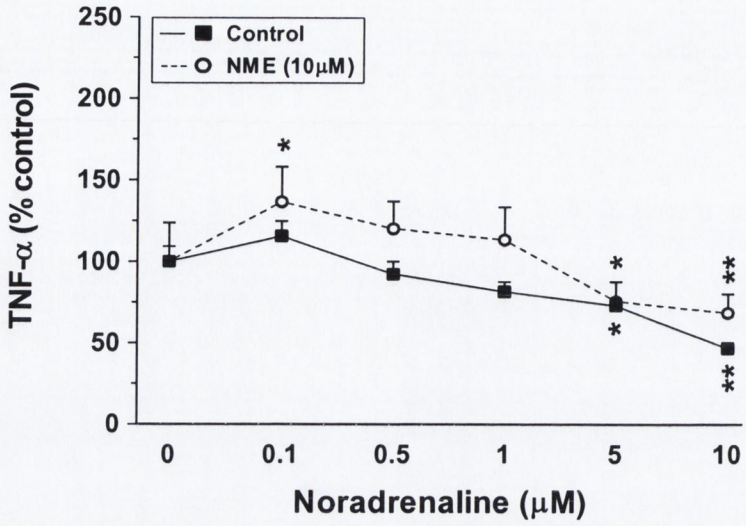


Figure 3.4.5: NME does not alter LPS-induced TNF-α or IL-1β

Vehicle or NME (1, 5 or 10μM) was administered 24 hours prior to LPS (1μg/ml) treatment and cells co-incubated with these treatments for 24 hours. (a) NME does not significantly alter LPS-induced TNF-α production in mixed glial culture. (b) NME does not alter LPS-induced IL-1β production in mixed glial culture.

One-way ANOVA revealed no effect of NME treatment on either TNF-α [$F(3,20)=0.74$, $P=0.54$] or IL-1β [$F(3,19)=1.62$, $P<0.22$] concentrations. Data expressed as mean (% control) and standard error of the mean (n=5-6).

(a)



(b)

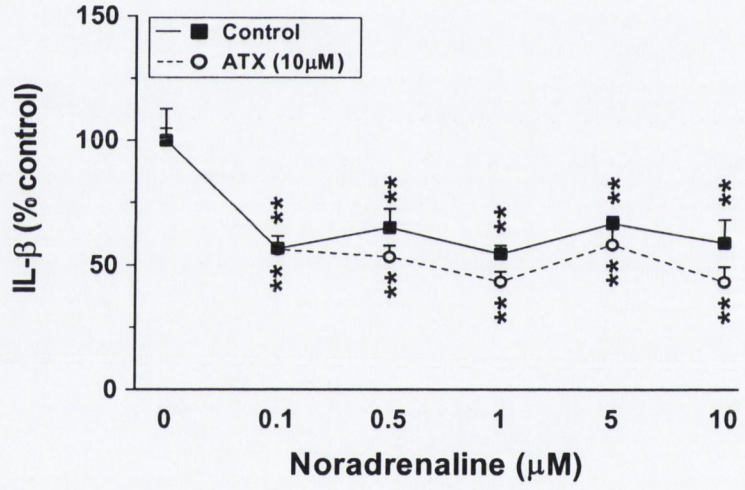


Figure 3.4.6: NME augments LPS-induced TNF-α but does not alter the suppressive effect of NA on IL-1β

Vehicle or NME (10μM) were administered 30 minutes prior to NA (0; 0.1; 0.5; 5 and 10μM) treatment and cells co-incubated with these treatments for 24 hours before the addition of LPS (1μg/ml) and a further 24-hour incubation. (a) NME (10μM) slightly augments LPS-induced TNF-α in the presence of NA (0.1μM). (b) NME does not alter the suppressive effect of NA on LPS-induced IL-1β production in mixed glial culture. Two-way ANOVA revealed a significant effect of NA treatment on TNF-α [$F(5,71)=15.51$, $P<0.0001$] and IL-1β [$F(5,84)=15.63$, $P<0.0001$] no interactive effect of NA with NME on concentrations of either cytokine. Data expressed as mean (% control) and standard error of the mean (n=6-8). *P<0.05, **P<0.01 versus Vehicle / LPS, +P<0.05 versus Vehicle / NA same dose (Fishers LSD test).

3.5. Assessment of the ability of acute treatment with noradrenaline-reuptake inhibitors to alter measures of brain inflammation: an *in vivo* analysis

As NA demonstrates anti-inflammatory actions on cortical rat primary glial cells the effects of noradrenaline reuptake inhibitors (NRIs) on brain indices of inflammation in both cortex and hypothalamus were assessed using an *in vivo* rat model. NRIs have been demonstrated to increase rat cortical concentrations of NA within 80 minutes of systemic administration, an effect that persists for up to 4 hours, as assessed by *in vivo* microdialysis (Sacchetti *et al.*, 1999). To determine whether increased availability of NA to glial cells would limit brain inflammation *in vivo*, the NRI's DMI (3.2, 7.5 or 15mg/kg;i.p.) and ATX (2.5, 5 or 10mg/kg;i.p.) were administered immediately prior to systemic challenge with LPS (250μg/kg;i.p.). A vehicle control group was included to give an estimate of basal expression of inflammatory genes in the brain. Four hours post-treatment animals were sacrificed and cortex, hypothalamus and spleen collected for inflammatory analyses. Inflammatory gene expression in spleen was conducted an indicator of the peripheral inflammatory response. mRNA expression of the pro-inflammatory cytokines TNF-α and IL-1β, the anti-inflammatory cytokine IL-10, the enzymes iNOS and COX-2, and the phenotypic marker of glial cell activation CD40 was examined using real-time PCR.

As outlined in Table 3.5.1. below, the magnitude of IL-1β, iNOS, COX-2 and IL-10 gene induction by LPS was greater in the hypothalamus than in the cortex. LPS-induced TNF-α gene expression was the same in both regions while CD40 induction was slightly greater in cortical than hypothalamic tissue. With the exception of IL-1β and CD40, the magnitude of LPS-induced gene expression was greater in splenic than in brain tissue.

Table 3.5.1: A summary of the magnitude of inflammatory gene expression induced by LPS in cortex, hypothalamus and spleen tissue, and the ability of acute NRI treatment to alter LPS-induced inflammatory responses

Target	Cortex			Hypothalamus			Spleen		
	Vehicle# + LPS	DMI + LPS	ATX + LPS	Vehicle# + LPS	DMI + LPS	ATX + LPS	Vehicle# + LPS	DMI + LPS	ATX + LPS
TNF-α	7-fold **	++	+	7-fold **	++	++	9-fold**	++	++
IL-1β	5-fold**	++	+	11-fold**	-	-	4-fold**	++	-
iNOS	25-fold**	++	+	73-fold**	-	-	88-fold**	++	-
COX-2	1.5-fold**	++	-	5-fold**	-	-	12-fold**	+	+
CD40	5-fold**	++	++	3-fold**	++	++	4-fold**	++	-
IL-10	1.4-fold	# #	-	22-fold**	-	-	27-fold**	-	-

= Fold increase in gene expression from non-LPS treated tissue.

One way analysis of variance revealed significant treatment effects on target gene expression. **P<0.01 fold increase versus Vehicle / Vehicle, +P<0.05, ++P<0.01 decrease (at any dose) versus Vehicle / LPS, # #P<0.01 increase versus Vehicle / LPS (Fishers LSD test).

In vivo indices of cortical inflammation: effects of NRI treatment

Magnitude of basal gene expression (in animals not treated with LPS) as demonstrated by lower CT values (Table 3.5.2.) were as follows: COX-2 > CD40 > TNF-α > IL-1β > iNOS > IL-10.

Magnitude of target gene induction by LPS: iNOS > TNF-α > CD40 > IL-1β > COX-2 > IL-10.

DMI and ATX elicited qualitatively similar effects, both drugs dose-dependently decreased mRNA expression for TNF-α and IL-1β, CD40 and the enzymes iNOS and COX-2. A differential effect of DMI and ATX was apparent on IL-10 and COX-2 mRNA expression in the cortex. DMI increased mRNA expression of the anti-inflammatory cytokine IL-10 and decreased that of COX-2I, while ATX was without effect on both these inflammatory parameters.

Table 3.5.2: A summary of basal and LPS-induced levels of inflammatory gene expression in cortical tissue.

Target	Ct for target Gene		CT for β-Actin		LPS-induced change	
	Control	LPS	Control	LPS	ΔCT (Target-Actin)	Fold increase
TNF-α	29.01	26.08	16.58	16.44	2.79	6.93
IL-1β	30.34	28.55	17.32	17.37	1.84	3.58
iNOS	33.0	28.68	16.74	17.07	4.66	25.23
COX-2	23.16	22.60	17.29	17.45	0.72	1.65
CD40	27.61	25.15	17.69	17.52	2.29	4.89
IL-10	35.97	35.43	16.94	16.89	0.49	1.4

CT = Cycle number

ΔCT = Difference in CT between Control and LPS treated animals corrected for β-actin

QPCR was carried out on cDNA reverse transcribed from 1.28μg of cortical RNA using a High Capacity cDNA archive kit (Applied Biosystems), as described previously.

Data represents the mean of 4-6 samples

TNF-α mRNA expression in rat cortex: LPS (250μg/kg) induced a 7-fold increase in TNF-α mRNA expression relative to control ($P<0.01$). This increase was significantly attenuated by DMI (15mg/kg, $P<0.05$) and ATX (10mg/kg, $P<0.05$) (Figure 3.5.1a).

IL-1β mRNA expression in rat cortex: LPS (250μg/kg) induced a 4-fold increase in IL-1β cortical mRNA expression relative to control ($P<0.01$). DMI (7.5 and 15mgkg) significantly attenuated LPS-induced IL-1β mRNA expression ($P<0.01$ and $P<0.05$). Similarly, ATX (5 and 10mg/kg) significantly attenuated IL-1β mRNA expression ($P<0.05$) in rat cortex, 4 hours post-treatment (Figure 3.5.1b).

iNOS mRNA expression in rat cortex: LPS (250μg/kg) induced a 23-fold increase in iNOS cortical mRNA expression relative to control ($P<0.01$). DMI dose-dependently suppressed the induction of iNOS mRNA expression by LPS,

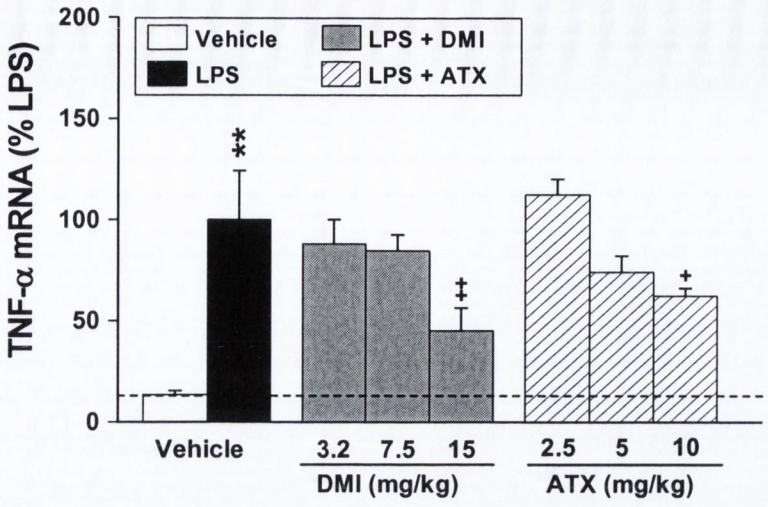
15mg/kg eliciting a significant effect ($P<0.01$). ATX (5mg/kg) also significantly decreased LPS-induced iNOS mRNA expression ($P<0.05$) in rat cortex, while ATX (10mg/kg) elicited a non-significant reduction in expression of this enzyme (Figure 3.5.2a).

COX-2 mRNA expression in rat cortex: LPS (250μg/kg) induced a 1.5-fold increase in COX-2 mRNA expression relative to control ($P<0.01$). DMI (15mgkg) significantly decreased LPS-induced COX-2 mRNA expression ($P<0.01$). ATX (2.5-10mg/kg) failed to alter mRNA expression of this enzyme (Figure 3.5.2b).

CD40 mRNA expression in rat cortex: LPS (250μg/kg) induced a 5-fold increase in CD40 mRNA expression relative to control ($P<0.01$). DMI (7.5 and 15mgkg) significantly attenuated LPS-induced increases in CD40 mRNA expression ($P<0.01$). Similarly, ATX (5 and 10mg/kg) significantly attenuated CD40 mRNA expression ($P<0.01$), 4 hours post-treatment (Figure 3.5.3a).

IL-10 mRNA expression in rat cortex: LPS did not significantly induce IL-10 mRNA expression in rat cortex 4 hours post-challenge. DMI (3.2mg/kg) and ATX (5mg/kg) induced a 2.5-fold increase in cortical IL-10 expression relative to control ($P<0.05$). In particular, DMI (15mg/kg) induced a 4-fold increase relative to control and a 2.5-fold increase of IL-10 mRNA expression relative to the LPS-treated group ($P<0.01$, Figure 3.5.3b).

(a)



(b)

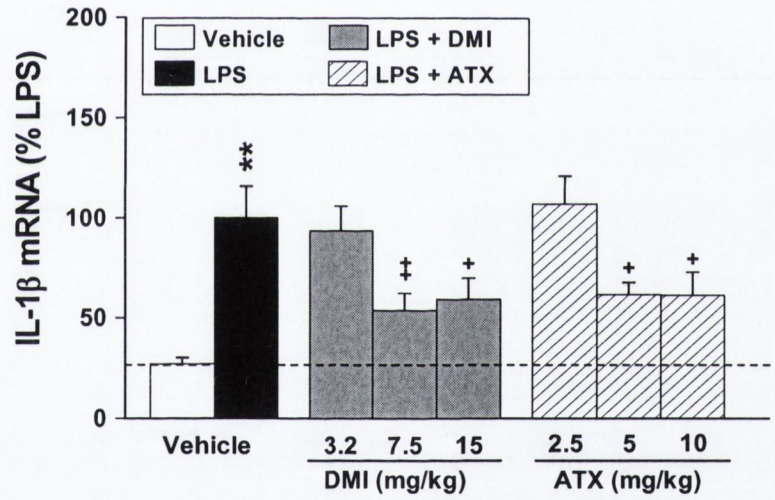
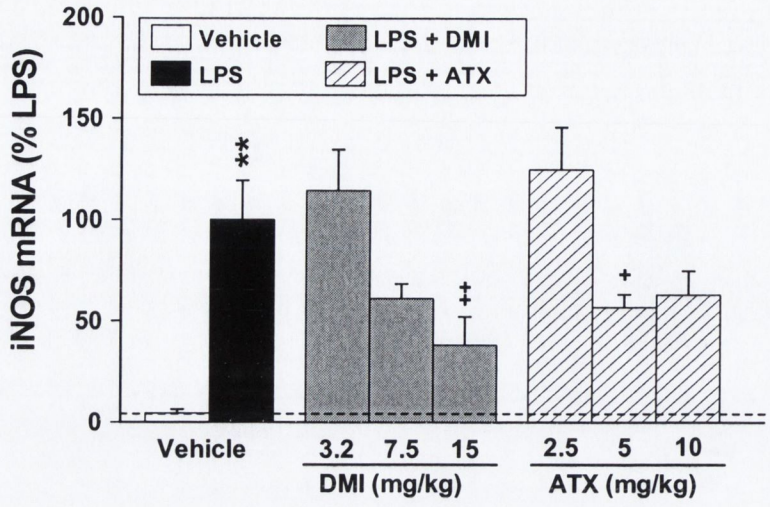


Figure 3.5.1: DMI and ATX attenuate LPS-induced TNF-α and IL-1β mRNA expression in rat cortex.

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (15mg/kg) and ATX (10mg/kg) significantly attenuated LPS-induced TNF-α mRNA expression. (b) DMI (7.5 and 15mg/kg) and ATX (5 and 10mg/kg) significantly attenuated LPS-induced IL-1β mRNA in rat cortex 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on TNF-α [$F(7,38)=7.31$, $P<0.001$] and IL-1β [$F(7,38)=6.27$, $P<0.0001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc* test).

(a)



(b)

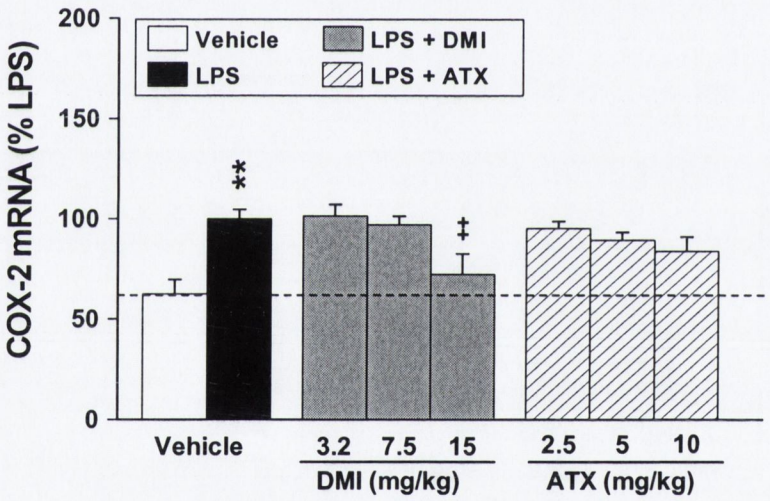


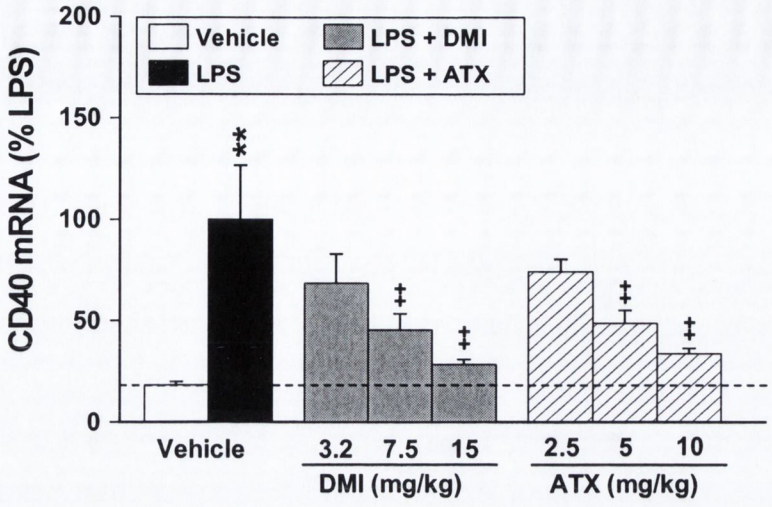
Figure 3.5.2: DMI and ATX suppress LPS-induced iNOS and COX-2 mRNA expression in rat cortex.

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (15mg/kg) and ATX (5mg/kg) significantly attenuated LPS-induced induction of iNOS mRNA expression in rat cortex 4 hours post- treatment. (b). DMI (15mg/kg) significantly attenuated, while ATX was without effect on LPS-induced induction of COX-2 mRNA in rat cortex 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on iNOS [$F(7,38)=7.85$, $P<0.0001$] and COX-2 [$F(7,39)=5.46$, $P<0.001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers *post hoc* test).

(a)



(b)

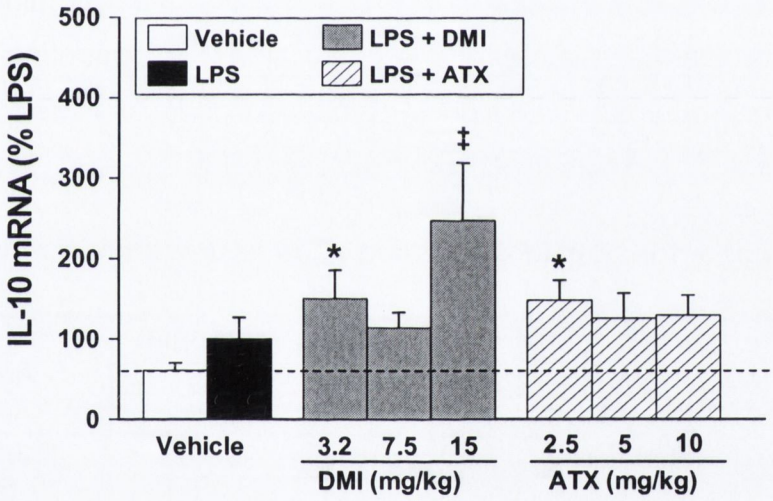


Figure 3.5.3: DMI and ATX dose-dependently attenuate LPS-induced CD40, while DMI induces IL-10 mRNA expression in rat cortex.

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (7.5 and 15mg/kg) and ATX (5 and 10mg/kg) significantly attenuated LPS-induced induction of CD40 mRNA in rat cortex 4 hours post- treatment. (b) DMI (3.2mg/kg) and ATX (2.5mg/kg) significantly induced IL-10 mRNA expression relative to control. DMI (15mg/kg) significantly induced IL-10 relative to both control and LPS groups in rat cortex 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on CD40 [$F(7,38)=7.31$, $P<0.001$] and IL-10 [$F(7,38)=7.31$, $P<0.001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

Acute *in vitro* treatment with noradrenaline reuptake inhibitors does not alter inflammatory gene expression in mixed cortical glia

To determine whether the anti-inflammatory actions of NRIs on LPS-induced inflammatory gene expression in rat cortex resulted from increased NA availability, or a direct effect of the drugs on glia, the effect of DMI and ATX on glial cells was examined *in vitro*. DMI or ATX (0.1, 1 or 10μM) were administered 30 minutes prior to LPS (1μgml) and cells incubated with these treatments for 6 hours. Six hours post-treatment cellular RNA was harvested and mRNA expression of the pro-inflammatory cytokines TNF-α and IL-1β, the anti-inflammatory cytokine IL-10, the enzymes iNOS and COX-2, and the phenotypic marker of glial cell activation CD40 was examined using real-time PCR. Only those markers that were altered *in vivo* were examined in mixed glia *in vitro*.

As demonstrated in Table 3.5.3 and Table 3.5.4. below, direct administration of DMI or ATX did not alter inflammatory gene expression, demonstrating that these drugs do not have any direct action on LPS-induced gene expression in mixed cortical glia.

Table 3.5.3: DMI does not alter LPS-induced gene expression in mixed cortical glia

Target	Vehicle + LPS	DMI (0.1μM) + LPS	DMI (1μM) + LPS	DMI (10μM) + LPS	ANOVA
TNF-α	100 ± 9	80 ± 9	108 ± 21	93 ± 8	F(3,18)=0.9, P=0.5
IL-1β	100 ± 17	73 ± 11	81 ± 12	89 ± 19	F(3,18)=0.7, P=0.6
iNOS	100 ± 25	60 ± 14	65 ± 17	89 ± 30	F(3,18)=0.8, P=0.5
COX-2	100 ± 7	91 ± 7	86 ± 5	102 ± 10	F(3,18)=1, P=0.4
CD40	100 ± 18	72 ± 12	83 ± 7	96 ± 21	F(3,18)=1.3, P=0.3
IL-10	100 ± 20	77 ± 19	68 ± 11	72 ± 17	F(3,18)=0.7, P=0.6

Data expressed as mean (% LPS control) with standard error of the mean (n=4-6). One-way analysis of variance revealed no significant effect of treatment on target gene expression.

Table 3.5.4: ATX does not alter LPS-induced gene expression in mixed cortical glia

Target	Vehicle + LPS	ATX (0.1μM) + LPS	ATX (1μM) + LPS	ATX (10μM) + LPS	ANOVA
TNF-α	100 ± 9	83 ± 5	118 ± 13	118 ± 23	F(3,18)=2, P=0.16
IL-1β	100 ± 17	86 ± 11	112 ± 10	107 ± 15	F(3,18)=0.5, P=0.7
iNOS	100 ± 25	77 ± 23	96 ± 25	86 ± 21	F(3,18)=0.2, P=0.9
CD40	100 ± 18	123 ± 22	100 ± 19	90 ± 14	F(3,18)=0.5, P=0.7

Data expressed as mean (% LPS control) with standard error of the mean (n=4-6). One-way analysis of variance revealed no significant effect of treatment on target gene expression.

In vivo indices of hypothalamic inflammation: effect of NRI treatment

The hypothalamus, via the hypothalamic-pituitary-adrenal (HPA) axis is responsible for co-ordinating the brain-immune defense mechanisms to inflammatory stimuli such as LPS. As such the effect of NRIs on the mRNA expression of the pro-inflammatory cytokines TNF-α and IL-1β, the anti-inflammatory cytokine IL-10, the enzymes iNOS and COX-2, and the phenotypic marker of glial cell activation CD40 was examined using real-time PCR.

Magnitude of basal gene expression (in animals not treated with LPS) were as follows: COX-2 > CD40 > TNF-α > IL-1β > iNOS > IL-10.

Magnitude of target gene induction by LPS was iNOS > IL-1β > TNF-α > COX-2 > CD40> IL-10.

Table 3.5.5: A summary of basal and LPS-induced levels of inflammatory gene expression in hypothalamic tissue.

Target	Ct for target Gene		CT for β-Actin		LPS-induced change	
	Control	LPS	Control	LPS	ΔCT (Target-Actin)	Fold increase
TNF-α	29.03	26.66	17.20	17.53	2.7	6.5
IL-1β	29.33	26.31	17.33	17.71	3.39	10.51
iNOS	34.84	28.85	17.33	17.54	6.2	73.39
COX-2	25.65	23.38	17.33	17.48	2.42	5.36
CD40	26.5	25.2	17.08	17.32	1.59	3.01
IL-10	37.27	32.97	17.11	17.29	4.48	22.32

CT = Cycle number

ΔCT = Difference in CT between Control and LPS-treated animals corrected for β-actin

QPCR was carried out on cDNA reverse transcribed from 1.11μg of cellular RNA using a High Capacity cDNA archive kit (Applied Biosystems), as described previously.

Data represents the mean of 4-6 samples

TNF-α mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 7-fold increase in TNF-α expression relative to control (P<0.01). This increase was significantly attenuated by DMI at all doses (P<0.05 and P<0 01). Similarly, ATX (2.5-10mg/kg) significantly attenuated LPS-induced mRNA expression of this cytokine (P<0.05 and P<0.01, Figure 3.5.4a).

IL-1β mRNA expression in rat hypothalamus: LPS (250μg/kg) induced an 11-fold increase in IL-1β mRNA expression relative to control (P<0.01). DMI did not significantly alter LPS-induced IL-1β mRNA expression although a non-significant augmentation was observed. ATX (5-10mg/kg) was without effect on IL-1β mRNA expression, expression levels remaining similar to LPS-treated animals (Figure 3.5.4b).

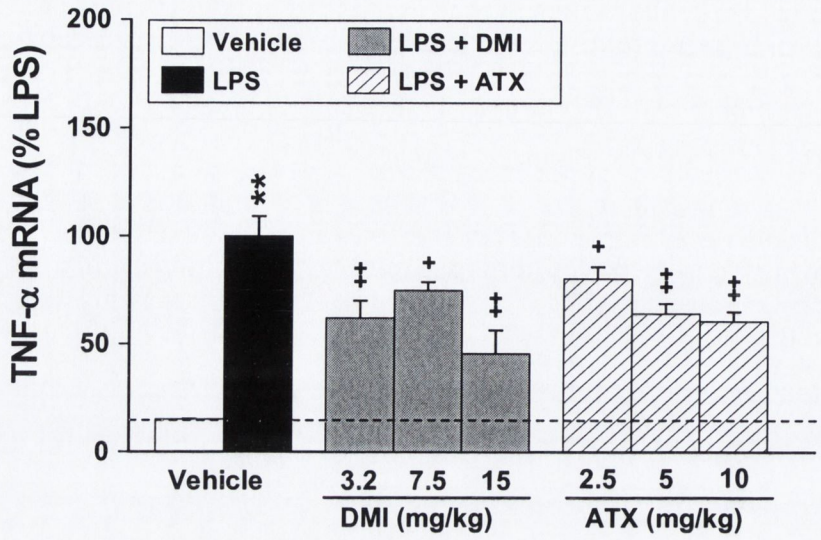
iNOS mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 50-fold increase in iNOS mRNA expression relative to control (P<0.01). DMI (all doses) and ATX (5 and 10mg/kg) non-significantly attenuated the induction of iNOS mRNA expression (50% reduction from LPS levels), although levels remained significantly greater than control (Figure 3.5.5a).

COX-2 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 5-fold increase in COX-2 mRNA expression relative to control (P<0.01). Neither DMI nor ATX significantly altered LPS-induced COX-2 mRNA expression, levels of this enzyme remaining similar to those of LPS-treated animals (Figure 3.5.5b).

CD40 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 3-fold increase in CD40 mRNA expression relative to control (P<0.01). DMI (7 and 15mgkg) significantly attenuated LPS-induced CD40 mRNA expression (P<0.05 and P<0.01, respectively). Similarly, ATX (5 and 10mg/kg) significantly decreased CD40 mRNA expression (P<0.05 and P<0.01, respectively, Figure 3.5.6a).

IL-10 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 22-fold increase in IL-10 mRNA expression relative to control (P<0.01). Neither DMI (3.2-15mgkg) nor ATX (2.5-10mg/kg) significantly altered LPS-induced IL-10 mRNA expression (Figure 3.5.6b).

(a)



(b)

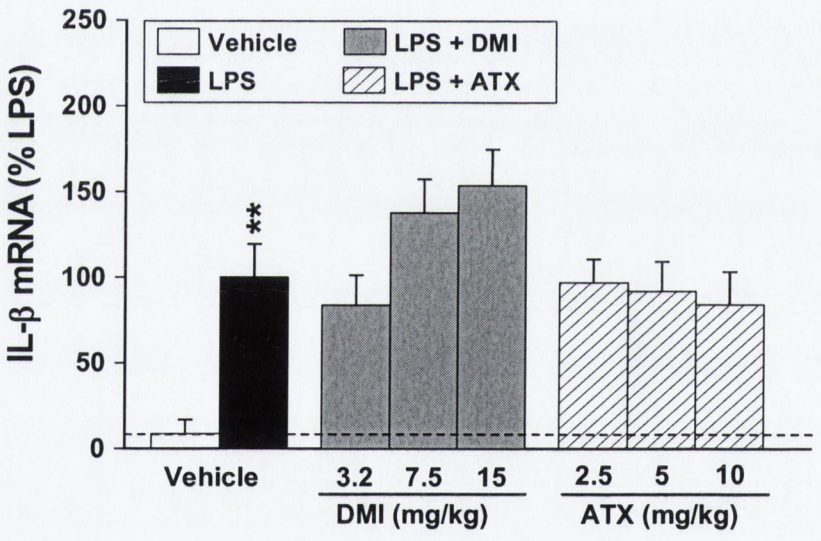


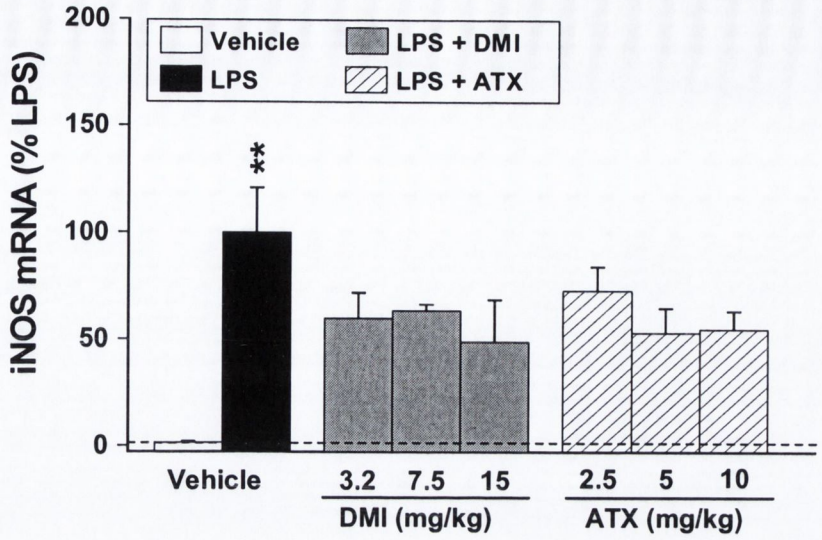
Figure 3.5.4: DMI and ATX attenuate LPS-induced TNF-α but not IL-1β mRNA expression in rat hypothalamus

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (3.2-15mg/kg) and ATX (2.5-10mg/kg) significantly attenuated LPS-induced induction of TNF-α mRNA expression. (b) Neither DMI nor ATX significantly altered LPS-induced induction of IL-1β mRNA expression in rat hypothalamus 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on TNF-α [$F(7,39)=$, $P<0.0001$] and IL-1β [$F(7,3)=6.49$, $P<0.001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

(a)



(b)

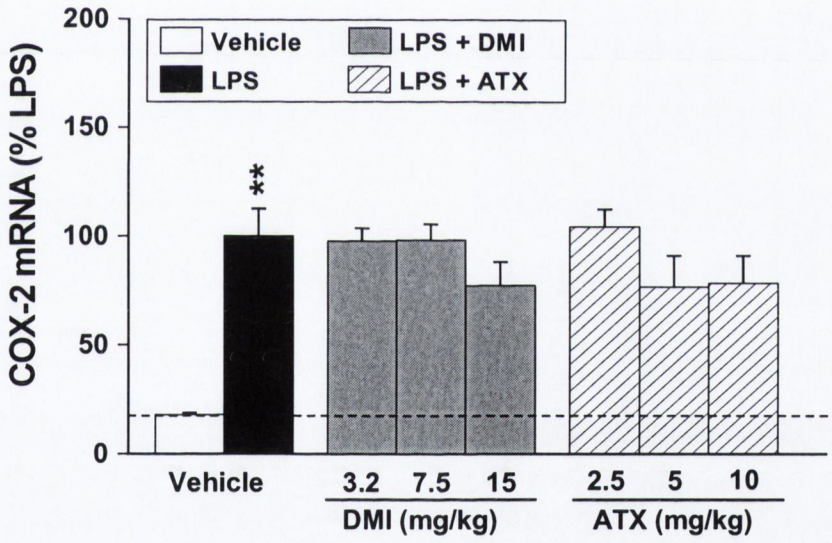
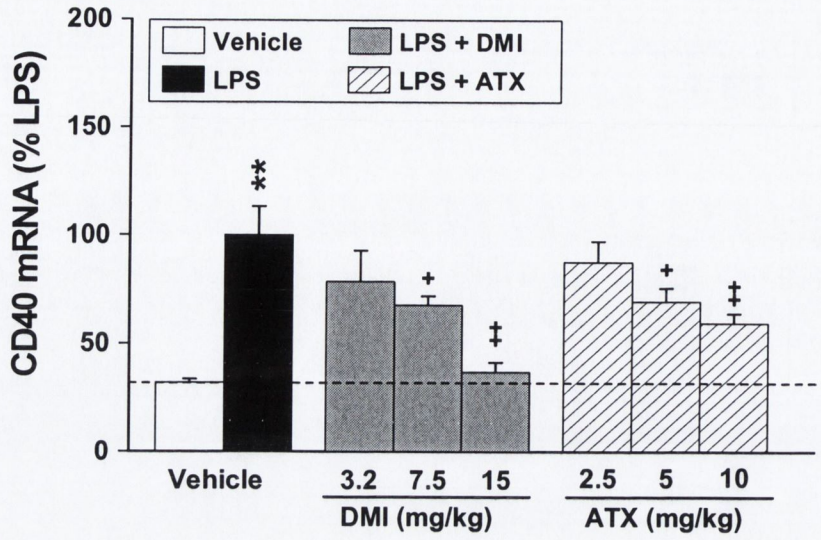


Figure 3.5.5: Neither DMI nor ATX alter LPS-induced iNOS or COX-2 mRNA expression in rat hypothalamus

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) Neither DMI (3.2-15mg/kg) nor ATX (2.5-10mg/kg) significantly altered LPS-induced induction of iNOS mRNA expression. (b) Neither DMI (3.2-15mg/kg) nor ATX (2.5-10mg/kg) significantly altered LPS-induced induction of COX-2 mRNA expression in rat hypothalamus 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on iNOS [$F(7,37)=4.59$, $P<0.001$] and COX-2 [$F(7,39)=8.46$, $P<0.0001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle (Fishers LSD *post hoc test*).

(a)



(b)

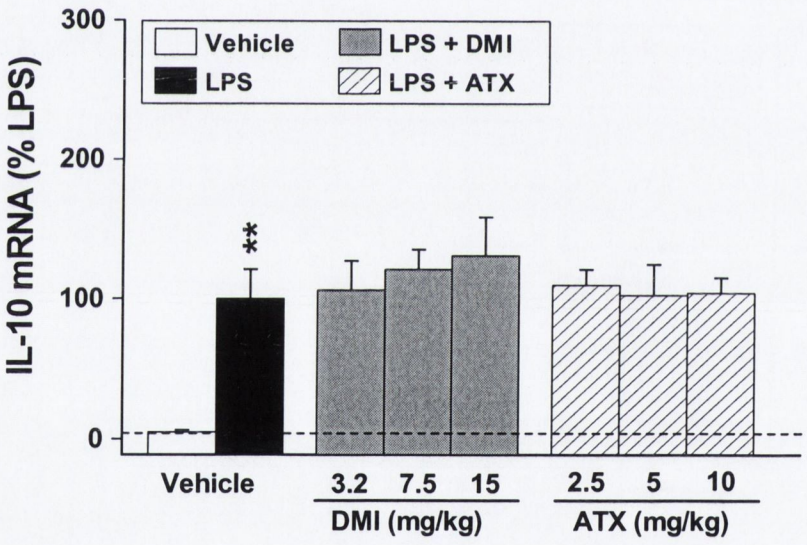


Figure 3.5.6: DMI and ATX dose-dependently suppress LPS-induced CD40 but do not alter IL-10 gene expression in rat hypothalamus

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (A) DMI (7.5 and 15mg/kg) and ATX (5 and 10mg/kg) significantly attenuated LPS-induced induction of CD40 mRNA. (B) Neither DMI nor ATX significantly alter IL-10 in rat hypothalamus 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on CD40 [$F(7,39)=7.74$, $P<0.0001$] and on IL-10 [$F(7,37)=5.22$, $P<0.001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

***In vivo* indices of splenic inflammation: effect of NRI treatment**

The spleen is a lymphoid organ that receives rich noradrenergic innervation from the sympathetic nervous system, and immune function in the spleen is under the control of noradrenergic innervation (see Straub, 2004 for review). As NRIs exert their effects both peripherally and centrally mRNA analysis of splenic tissue was carried out to determine if a similar profile of anti-inflammatory effects was evident in lymphoid tissue, or whether the anti-inflammatory actons of NRI's were confined to the central nervous system. A differential effect of NRIs on splenic and brain tissue could be attributable to the different cell types being acted upon by NA, i.e. glial cells in the brain versus peripheral lymphocytes. As such the effect of NRIs on the mRNA expression of the pro-inflammatory cytokines TNF-α and IL-1β, the anti-inflammatory cytokine IL-10, the enzymes iNOS and COX-2, and the phenotypic marker of immune cell activation CD40 was examined in the spleen using real-time PCR.

Magnitude of basal gene expression (in animals not treated with LPS) were as follows: IL-1β > CD40 > TNF-α > iNOS > IL-10 > COX-2.

Magnitude of target gene induction by LPS:

iNOS > IL-10> COX-2 >TNF-α >IL-1β > CD40.

Table 3.5.6: A summary of basal and LPS-induced levels of inflammatory gene expression in splenic tissue.

Target	Ct for target Gene		CT for β-Actin		LPS-induced change	
	Control	LPS	Control	LPS	ΔCT (Target-Actin)	Fold increase
TNF-α	24.58	20.31	15.97	14.93	3.23	9.40
IL-1β	21.91	18.60	15.30	14.14	2.15	4.44
iNOS	27.16	19.34	15.56	14.21	6.47	88.55
COX-2	30.02	24.34	16.64	14.54	3.59	12.02
CD40	22.21	19.10	15.77	14.61	1.94	3.85
IL-10	29.08	22.43	16.17	14.30	4.77	27.36

CT = Cycle number

ΔCT = Difference in CT between Control and LPS treated animals, corrected for β-actin

QPCR was carried out on cDNA reverse transcribed from 2.73μg of splenic RNA using a High Capacity cDNA archive kit (Applied Biosystems), as described previously.

Data represents the mean of 4-6 samples

TNF-α mRNA expression in rat spleen: LPS (250μg/kg) induced a 14-fold increase in TNF-α mRNA expression relative to control ($P<0.01$). Both DMI and ATX dose-dependently attenuated LPS-induced mRNA expression of this cytokine ($P<0.05$ and $P<0.01$, figure 3.5.7a).

IL-1β mRNA expression in rat spleen: LPS (250μg/kg) induced a 5-fold increase in splenic IL-1β mRNA expression relative to control ($P<0.01$). DMI (7.5 and 15mg/kg) significantly attenuated LPS-induced IL-1β mRNA expression ($P<0.05$ and $P<0.01$, respectively). ATX (2.5-10mg/kg) was without effect on IL-1β mRNA expression (figure 3.5.7b).

iNOS mRNA expression in rat spleen: LPS (250μg/kg) induced a 70-fold increase in splenic iNOS mRNA expression relative to control ($P<0.01$). DMI (15mg/kg)

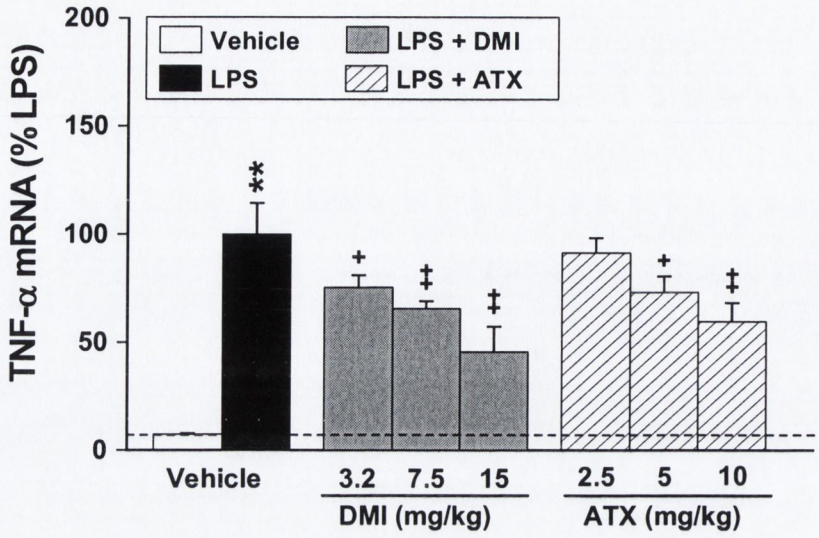
significantly attenuated LPS-induced iNOS expression, while ATX was without effect on LPS induction of iNOS mRNA (figure 3.5.8a).

COX-2 mRNA expression in rat spleen: LPS (250μg/kg) induced a 12-fold increase in splenic COX-2 mRNA expression relative to control ($P<0.01$). DMI (7.5 and 15mg/kg) and ATX (2.5 and 5mg/kg) significantly attenuated LPS-induced COX-2 mRNA expression (figure 3.5.8b).

CD40 mRNA expression in rat spleen: LPS (250μg/kg) induced a 4-fold increase in splenic CD40 mRNA expression relative to control ($P<0.01$). DMI (15mgkg) significantly attenuated LPS-induced CD40 mRNA expression ($P<0.01$). ATX (2.5-10mg/kg) was without effect on CD40 mRNA expression, expression levels remaining similar to LPS-treated animals (figure 3.5.9a)

Relative IL-10 mRNA expression in the spleen: LPS (250μg/kg) induced a 27-fold increase in splenic IL-10 mRNA expression relative to control ($P<0.01$). Neither DMI nor ATX significantly altered LPS-induced IL-10 mRNA expression (3.5.9b).

(a)



(b)

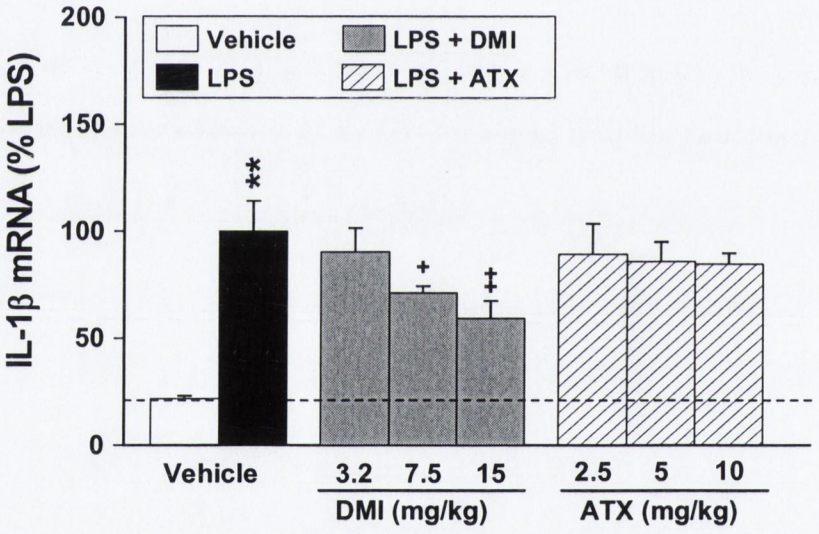
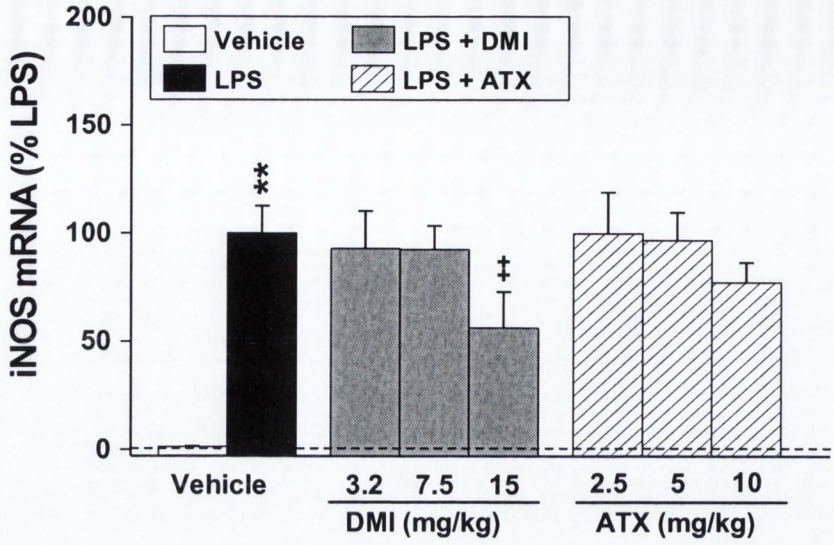


Figure 3.5.7: Both DMI and ATX suppress LPS-induced TNF-α mRNA, but only DMI suppresses IL-1β gene expression in rat spleen

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI and ATX dose-dependently attenuated LPS-induced of TNF-α mRNA expression. (b) DMI (7.5 and 15mg/kg) significantly attenuated and ATX (2.5-10mg/kg) did not alter LPS-induced IL-1β mRNA expression in rat spleen 4 hours post- treatment

One way ANOVA revealed a significant effect of treatment on TNF-α [$F(7,40)=11.73$, $P<0.0001$] and IL-1β [$F(7,38)=7.5$, $P<0.0001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

(a)



(b)

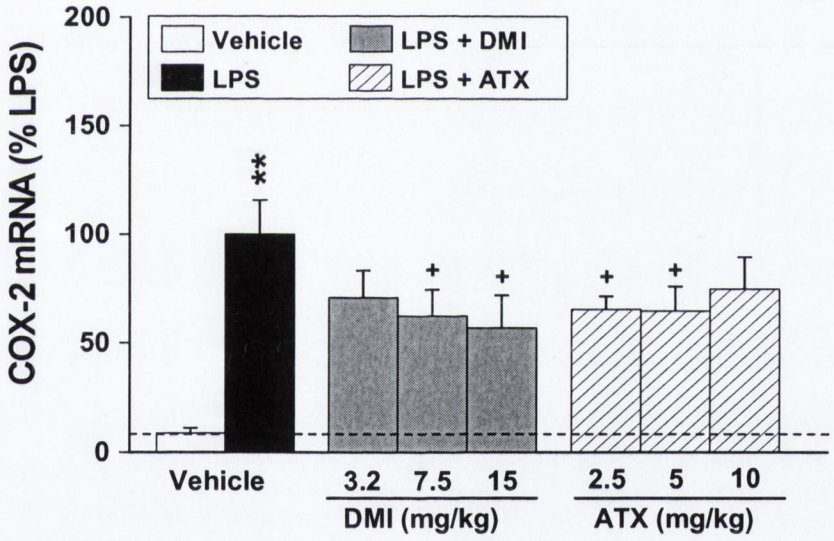
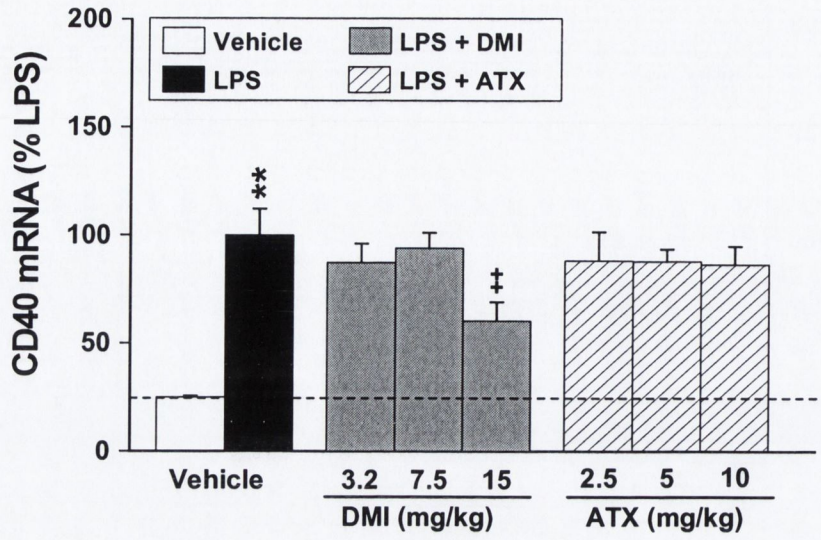


Figure 3.5.8: DMI attenuates LPS-induced iNOS and COX-2, ATX attenuates LPS-induced COX-2 gene expression only, in rat spleen

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (15mg/kg) significantly attenuated, while ATX did not alter LPS-induced of iNOS mRNA expression. (b) DMI (7.5 and 15mg/kg) and ATX (2.5 and 5mg/kg) attenuated LPS-induced COX-2 mRNA expression in the spleen 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on iNOS [$F(7,39)=6.93$, $P<0.0001$] and COX-2 [$F(7,40)=4.52$, $P<0.001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

(a)



(b)

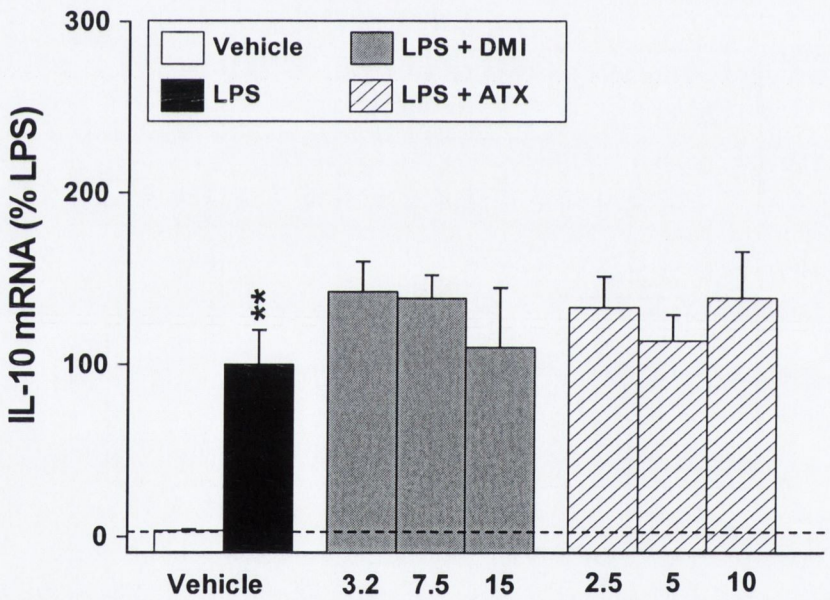


Figure 3.5.9: DMI attenuates LPS-induced CD40 mRNA expression, but neither DMI nor ATX alter IL-10 mRNA expression in the spleen.

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (15mg/kg) significantly attenuated, while ATX did not alter LPS-induced induction of CD40 mRNA expression. (b) Neither DMI nor ATX alter LPS-induced IL-10 mRNA in rat spleen, 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on CD40 [$F(7,39)=7.5$, $P<0.0001$] and IL-10 [$F(7,40)=4.93$, $P<0.001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

3.6. Assessment of the ability of α_2-adrenoceptor antagonists to alter measures of brain inflammation: an *in vivo* analysis

The following study assessed the ability of α_2-AR antagonists to suppress indices of brain inflammation *in vivo*. To this end, α_2-AR antagonists have been demonstrated to increase central concentrations of NA in various rat brain regions within 1-2 hours following systemic administration, as assessed by *in vivo* microdialysis (Wortley *et al.*, 1999; Swanson *et al.*, 2006; see Invernizzi *et al.*, 2004 for review). To determine whether α_2-AR blockade and subsequent increases in the availability of NA to glial cells would limit brain inflammation *in vivo*, the α_2-AR antagonists IDA (1mg/kg) and RX (1mg/kg) were administered immediately prior to challenge with either vehicle or LPS (250μg/kg;i.p.). Four hours post-treatment animals were sacrificed and cortex, hypothalamus and spleen collected for analysis of inflammatory markers. mRNA expression of the pro-inflammatory cytokines TNF-α and IL-1β, the anti-inflammatory cytokine IL-10, the enzymes iNOS and COX-2, and the phenotypic marker of glial cell activation CD40 was examined using real-time PCR.

As observed in the NRI dose-response study and outlined in Table 3.6.1. below, the magnitude of IL-1β, iNOS, COX-2 and IL-10 gene induction by LPS was greater in the hypothalamus than in the cortex. Magnitude of TNF-α gene expression was the same in both regions while CD40 expression was slightly greater in cortical tissue. With the exception of IL-1β and CD40, the magnitude of LPS-induced gene expression was greater in splenic than in brain tissue. LPS-induced iNOS gene expression was unaltered by α_2-AR antagonism in either brain or spleen tissue.

Table 3.6.1: Effect of α_2-adrenoceptor antagonists on inflammatory gene expression in cortex, hypothalamus and spleen tissue

Target	Cortex			Hypothalamus			Spleen		
	Vehicle# + LPS	IDA + LPS	RX + LPS	Vehicle# + LPS	IDA + LPS	RX + LPS	Vehicle# + LPS	IDA + LPS	RX + LPS
TNF-α	7-fold**	-	-	7-fold**	++	++	13-fold**	-	++
IL-1β	4-fold**	-	+	9-fold**	+	-	5-fold**	++	++
iNOS	23-fold**	-	-	55-fold**	-	-	89-fold**	-	-
COX-2	2-fold**	-	-	6-fold**	-	-	12-fold**	-	++
CD40	5-fold**	+	++	3-fold**	++	++	4-fold**	-	-
IL-10	1.4-fold	-	-	22-fold**	-	-	27-fold**	-	#

= Fold increase in gene expression from non-LPS treated animals

One-way analysis of variance revealed significant treatment effects on target gene expression. **P<0.01 fold increase versus Vehicle/Vehicle, +P<0.05, ++P<0.01 decrease (at any dose) versus Vehicle/LPS, #P<0.05 increase versus Vehicle/LPS (Fishers LSD test)

In vivo indices of cortical inflammation: effect of α_2-AR antagonism

RX (1mg/kg) attenuated LPS-induced IL-1β (P<0.05) and CD40 (P<0.01) mRNA expression in rat cortex. IDA (1mg/kg) also attenuated CD40 mRNA expression (P<0.05) but did not alter any other index of cortical inflammation in rat cortex.

TNF-α mRNA expression in rat cortex: LPS (250μg/kg) induced a 7-fold increase in TNF-α mRNA expression relative to control (P<0.01). This increase was unaffected by either IDA (1mg/kg) or RX (1mg/kg) treatment (Figure 3.6.1a).

IL-1β mRNA expression in rat cortex: LPS (250μg/kg) induced a 4-fold increase in IL-1β cortical mRNA expression relative to control (P<0.01). RX (1mg/kg) significantly decreased LPS-induced IL-1β mRNA expression (P<0.05) while IDA (1mg/kg) did not alter mRNA expression of this cytokine (Figure 3.6.1b).

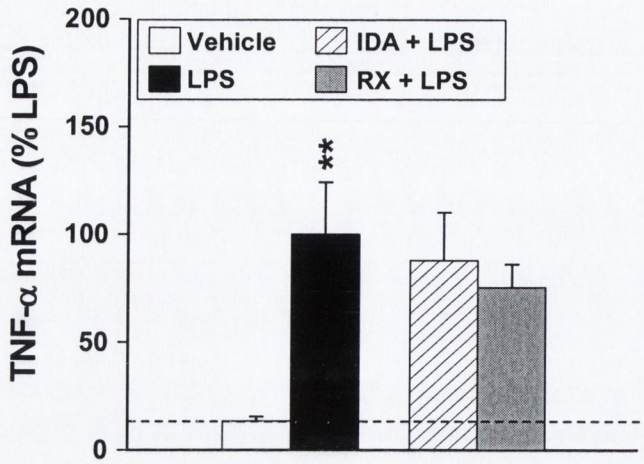
iNOS mRNA expression in rat cortex: LPS (250μg/kg) induced a 25-fold increase in iNOS mRNA expression relative to control ($P<0.01$). Neither IDA (1mg/kg) nor RX (1mg/kg) altered mRNA expression iNOS (Figure 3.6.2a).

COX-2 mRNA expression in rat cortex: LPS (250μg/kg) induced a 1.7-fold increase COX-2 mRNA expression relative to control ($P<0.01$).) Neither IDA (1mg/kg) nor RX (1mg/kg) altered mRNA expression of this enzyme (Figure 3.6.2b).

CD40 mRNA expression in rat cortex: LPS (250μg/kg) induced a 5-fold increase in CD40 mRNA expression relative to control ($P<0.01$). IDA and RX significantly decreased LPS-induced CD40 mRNA expression ($P<0.05$ and $P<0.01$, respectively, Figure 3.6.3a).

IL-10 mRNA expression in rat cortex: Neither LPS, IDA nor RX significantly altered IL-10 mRNA expression 4 hours post-treatment, although both LPS and α_2-AR antagonists non-significantly increased mRNA expression of this cytokine above control levels (Figure 3.6.3b).

(a)



(b)

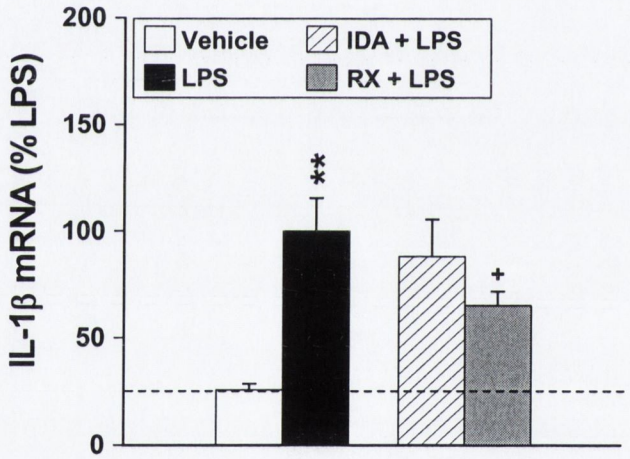


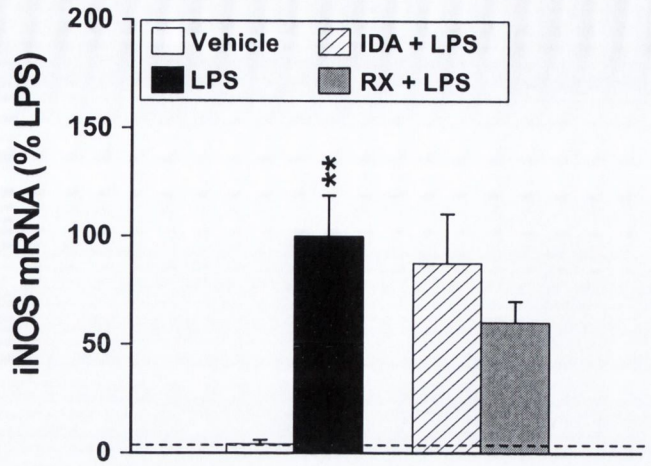
Figure 3.6.1: RX attenuates LPS-induced IL-1β mRNA expression in rat cortex

IDA (1mg/kg), RX (1mg/kg) or vehicle were administered to rats immediately prior to a challenge with vehicle or LPS (250μg/kg). (a) IDA and RX did not alter LPS-induced TNF-α mRNA expression. (b) RX significantly attenuated while IDA did not alter LPS-induced IL-1β mRNA expression in rat cortex 4 hours post -treatment ($P<0.05$).

One way ANOVA revealed a significant effect of treatment on TNF-a [$F(3,18)=8.63$, $P<0.01$] and IL-1b [$F(3,20)=7.24$, $P<0.01$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=4-6). **$P<0.01$ versus Vehicle/Vehicle, +$P<0.05$ versus Vehicle/LPS group (Fishers LSD *post hoc test*).

(a)



(b)

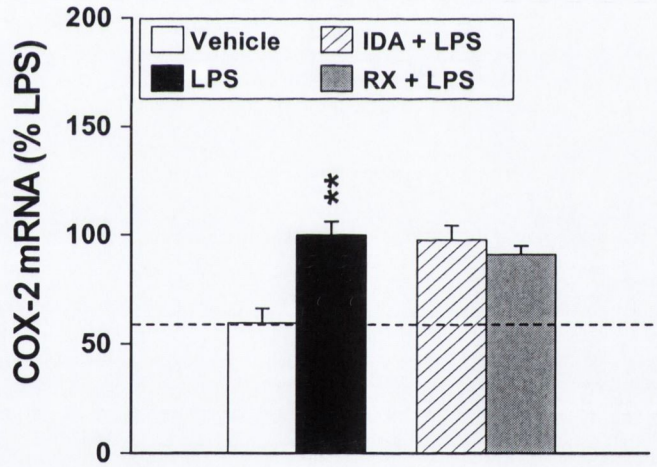


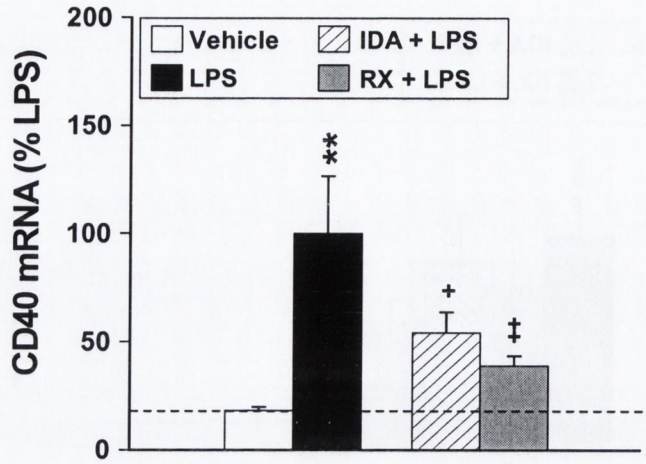
Figure 3.6.2. Neither IDA nor RX alter LPS-induced iNOS or COX-2 gene expression in rat cortex.

IDA (1mg/kg), RX (1mg/kg) or vehicle were administered to rats immediately prior to a challenge with vehicle or LPS (250μg/kg). (a) Neither IDA nor (b) RX altered LPS-induced iNOS or COX-2 mRNA expression in rat cortex 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on iNOS [$F(3,19)=10.83$, $P<0.001$] and COX-2 [$F(3,20)=9.77$, $P<0.001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle (Fishers *post hoc*).

(a)



(b)

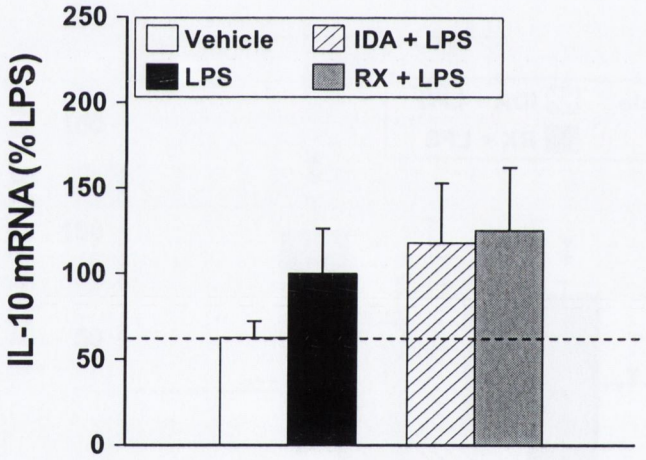


Figure 3.6.3. IDA and RX attenuate LPS-induced CD40, and non-significantly increase IL-10 gene expression in rat cortex

IDA (1mg/kg), RX (1mg/kg) or vehicle were administered to rats immediately prior to a challenge with vehicle or LPS (250μg/kg). (A) IDA and RX significantly attenuated LPS-induced CD40 mRNA in rat cortex. (B) LPS, IDA and RX non-significantly induce IL-10 mRNA expression treatment in rat cortex 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on CD40 [$F(3,18)=8.62$, $P<0.001$] and IL-10 [$F(3,20)=0.86$, $P=0.48$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers *post hoc*).

Acute treatment with α_2-AR antagonists do not alter IL-1β or CD40 gene expression in mixed cortical glia

To determine whether the anti-inflammatory actions of IDA on CD40 and RX on CD40 and IL-1β cortical gene expression resulted from increased NA availability or a direct effect of the drugs on glia, the effect of IDA and RX on glial cells was examined *in vitro*. IDA (0.1, 1 and 10μM) or RX (1μM) were administered 30 minutes prior to LPS (1μgml) and cells incubated with these treatments for 6 hours. A vehicle control group was included to give an estimate of basal expression of inflammatory genes in mixed cortical glia. Six hours post-treatment cellular RNA was harvested and mRNA expression of the pro-inflammatory cytokine IL-1β and the phenotypic marker of glial cell activation CD40 was examined using real-time PCR.

As demonstrated in Table 3.6.2 below, direct administration of RX and IDA did not alter IL-1β or CD40 gene expression, demonstrating no direct action of these drugs on LPS-induced gene expression in mixed cortical glia.

Table 3.6.2: IDA and RX do not alter LPS-induced gene expression in mixed cortical glia

Target	Vehicle + LPS	IDA (0.1μM) + LPS	IDA (1μM) + LPS	IDA (10μM) + LPS	RX (1μM) + LPS	ANOVA
IL-1β	100 ± 17	95 ± 24	86 ± 13	91 ± 15	86 ± 13	F(4,25)=0.1, P=0.97
CD40	100 ± 18	93 ± 21	99 ± 20	93 ± 15	85 ± 13	F(4,25)=0.1, P=0.98

Data expressed as mean (% LPS control) with standard error of the mean (n=4-6). One-way analysis of variance revealed no significant effect of treatment on target gene expression.

In vivo indices of hypothalamic inflammation: effect of α_2-AR blockade

The magnitude of IL-1β, iNOS, COX-2 and IL-10 gene induction by LPS was greater in the hypothalamus than in the cortex. As in the cortex, IDA and RX significantly attenuate LPS-induced CD40 gene expression in the hypothalamus. In addition, LPS-induced TNF-α mRNA expression was significantly attenuated by both α_2-AR antagonists in the hypothalamus, and IL-1β mRNA expression was significantly attenuated by IDA.

TNF-α mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 7-fold increase in TNF-α mRNA expression relative to control ($P<0.01$). This increase was significantly ($P<0.01$) attenuated by IDA and RX treatment (Figure 3.6.4a).

IL-1β mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 10-fold increase in IL-1β mRNA expression relative to control ($P<0.01$). IDA significantly attenuated IL-1β mRNA expression ($P<0.05$), while RX did not significantly alter LPS-induced IL-1β mRNA expression (Figure 3.6.4b).

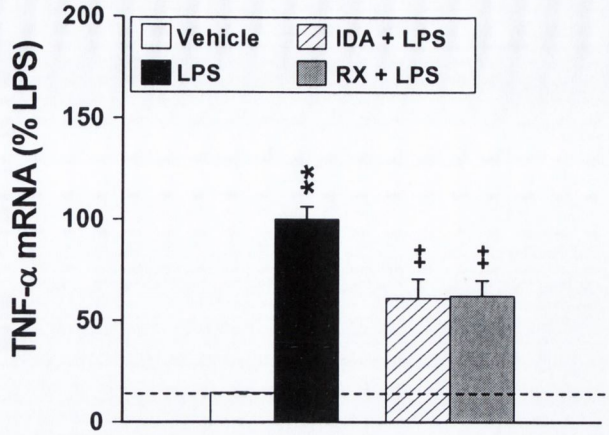
iNOS mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 50-fold increase in iNOS mRNA expression relative to control ($P<0.01$). Neither IDA nor RX altered LPS-induced iNOS gene expression (Figure 3.6.5a).

COX-2 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 6-fold increase in COX-2 mRNA expression relative to control ($P<0.01$). Neither IDA nor RX altered LPS-induced COX-2 gene expression (Figure 3.6.5b).

CD40 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 3-fold increase in hypothalamic CD40 mRNA expression relative to control ($P<0.01$). Both IDA and RX attenuated LPS-induced CD40 mRNA expression ($P<0.01$) (Figure 3.6.6a).

IL-10 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 22-fold increase in IL-10 mRNA expression relative to control ($P<0.01$). Neither IDA nor RX altered LPS-induced IL-10 mRNA expression (figure 3.6.6b).

(a)



(b)

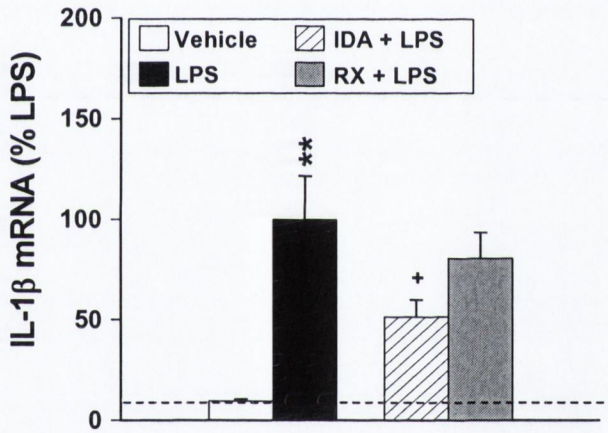


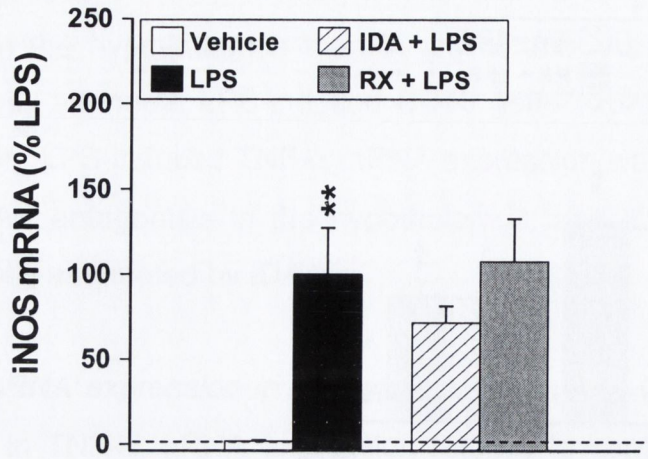
Figure 3.6.4: Both IDA and RX attenuate LPS-induced TNF-α mRNA expression, and IDA attenuates LPS-induced IL-1β mRNA expression in rat hypothalamus

IDA (1mg/kg), RX (1mg/kg) or vehicle were administered to rats immediately prior to a challenge with vehicle or LPS (250μg/kg). (a) IDA and RX significantly attenuated LPS-induced TNF-α mRNA. (b) IDA but not RX attenuated LPS-induced IL-1β mRNA expression in rat hypothalamus 4 hours post-treatment ($P<0.01$)

One way ANOVA revealed a significant effect of treatment on TNF-α [$F(3,20)=25.4$, $P<0.0001$] and IL-1β [$F(3,20)=8.92$, $P<0.001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **$P<0.01$ versus Vehicle/Vehicle; ++$P<0.01$ versus Vehicle/LPS group (Fishers *post hoc*).

(a)



(b)

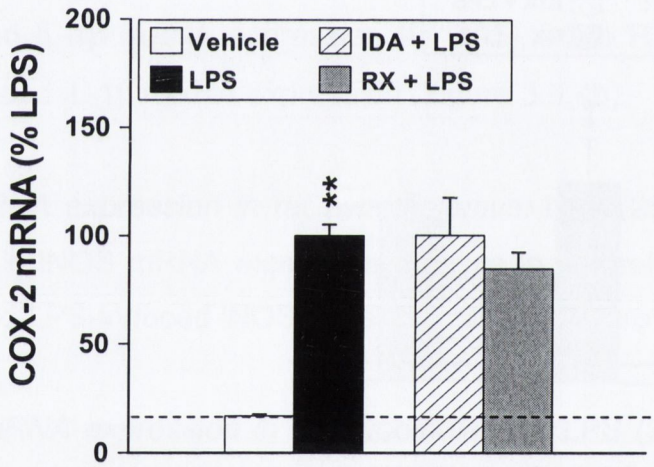


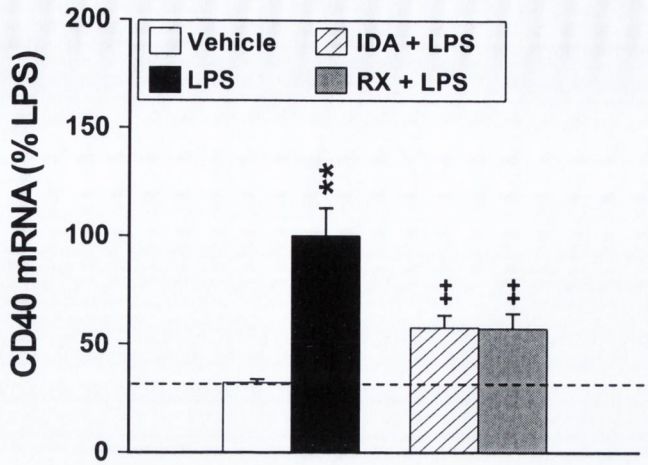
Figure 3.6.5: Neither IDA nor RX alter LPS-induced iNOS or COX-2 mRNA expression in rat hypothalamus

IDA (1mg/kg), RX (1mg/kg) or vehicle were administered to rats immediately prior to a challenge with vehicle or LPS (250μg/kg). (a) Neither IDA nor (b) RX significantly alter LPS-induced iNOS or COX-2 mRNA expression in rat hypothalamus 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on iNOS [$F(3,18)=6.56$, $P<0.01$] and COX-2 [$F(3,20)=15.93$, $P<0.0001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle (Fishers LSD *post hoc test*).

(a)



(b)

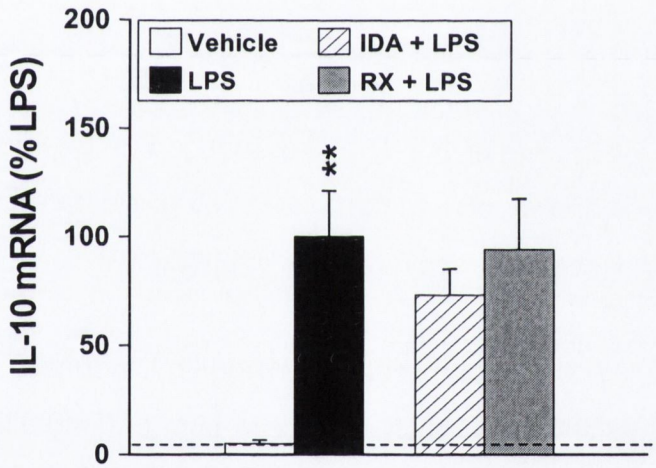


Figure 3.6.6: IDA and RX suppress LPS-induced CD40, but do not alter LPS-induced IL-10 gene expression in rat hypothalamus

IDA (1mg/kg), RX (1mg/kg) or vehicle were administered to rats immediately prior to a challenge with vehicle or LPS (250μg/kg). (a) Both IDA and RX significantly attenuated LPS-induced CD40 mRNA. (b) expression in rat hypothalamus 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on CD40 [$F(3,20)=12.71$, $P<0.0001$] and IL-10 [$F(3,19)=6.56$, $P<0.01$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS (Fishers LSD *post hoc test*).

In vivo indices of splenic inflammation: effect of α_2-AR blockade

RX attenuated TNF-α, IL-1β and COX-2 ($P<0.01$) gene expression, and augmented that of the anti-inflammatory cytokine IL-10 ($P<0.05$) in the spleen. IDA attenuated only LPS-induced IL-1β in splenic tissue, 4 hours post-treatment. LPS-induced CD40 is unaltered by either α_2-AR antagonist in spleen tissue.

TNF-α mRNA expression in rat spleen: LPS (250μg/kg) induced a 13-fold increase in TNF-α mRNA expression relative to control ($P<0.01$). RX significantly attenuated ($P<0.01$) LPS-induced TNF-α, while IDA did not alter gene expression of this cytokine (Figure 3.6.7a).

IL-1β mRNA expression in rat spleen: LPS (250μg/kg) induced a 5-fold increase in IL-1β mRNA expression relative to control ($P<0.01$). IDA significantly attenuated IL-1β mRNA expression ($P<0.01$), while RX did not alter gene expression of this cytokine (Figure 3.6.7b).

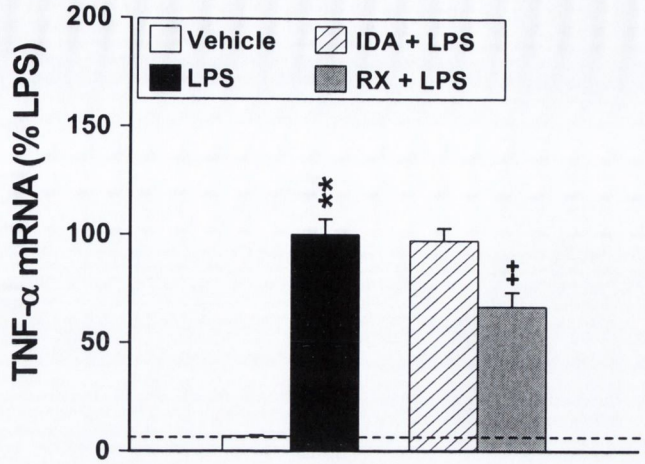
iNOS mRNA expression in rat spleen: LPS (250μg/kg) induced an 89-fold increase in splenic iNOS mRNA expression relative to control ($P<0.01$). Neither IDA nor RX altered LPS-induced iNOS mRNA expression (Figure 3.6.8a).

COX-2 mRNA expression in rat spleen: LPS (250μg/kg) induced a 12-fold increase in splenic COX-2 mRNA expression relative to control ($P<0.01$). RX, but not IDA significantly attenuated LPS-induced COX-2 expression (Figure 3.6.8b).

CD40 mRNA expression in rat spleen: LPS (250μg/kg) induced a 4-fold increase in CD40 mRNA expression relative to control ($P<0.01$). Neither IDA nor RX altered LPS-induced CD40 mRNA expression (Figure 3.6.9a).

IL-10 mRNA expression in rat spleen: LPS (250μg/kg) induced a 27-fold increase in IL-10 mRNA expression relative to control ($P<0.01$). RX, but not IDA significantly augmented LPS-induced IL-10 mRNA expression ($P<0.05$) (Figure 3.6.9b).

(a)



(b)

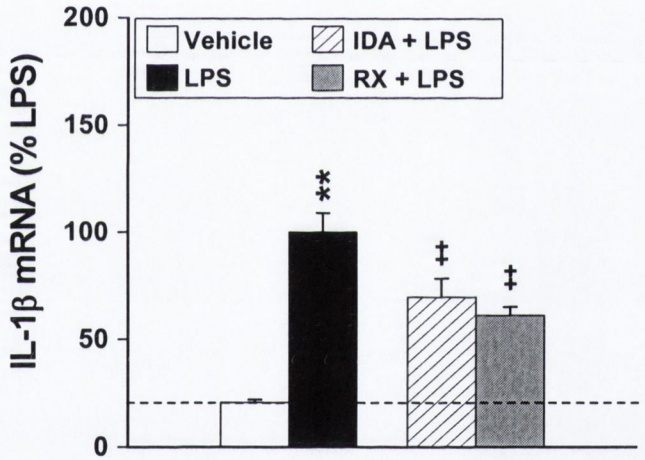


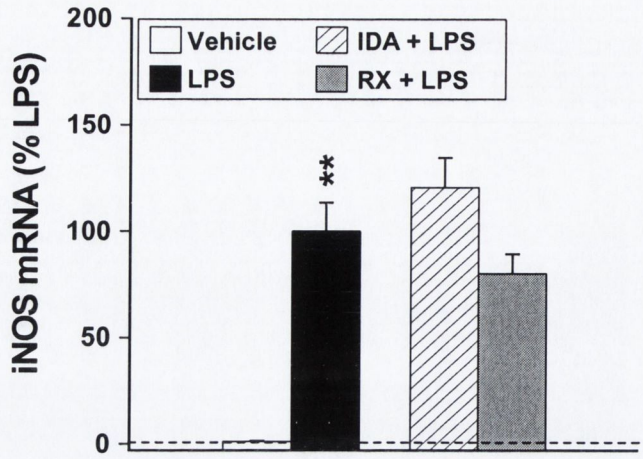
Figure 3.6.7: RX attenuates LPS-induced TNF-α and IL-1β mRNA expression, and IDA attenuates IL-1β mRNA expression in rat spleen

IDA (1mg/kg), RX (1mg/kg) or vehicle were administered to rats immediately prior to a challenge with vehicle or LPS (250μg/kg). (a) RX but not IDA significantly attenuated LPS-induced TNF-α mRNA expression in rat spleen. (b) Both IDA and RX significantly attenuated LPS-induced IL-1β in spleen 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on TNF-α [$F(3,19)=58.85$, $P<0.0001$] and IL1β mRNA expression [$F(3,20)=8.92$, $P<0.001$].

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers *post hoc*).

(a)



(b)

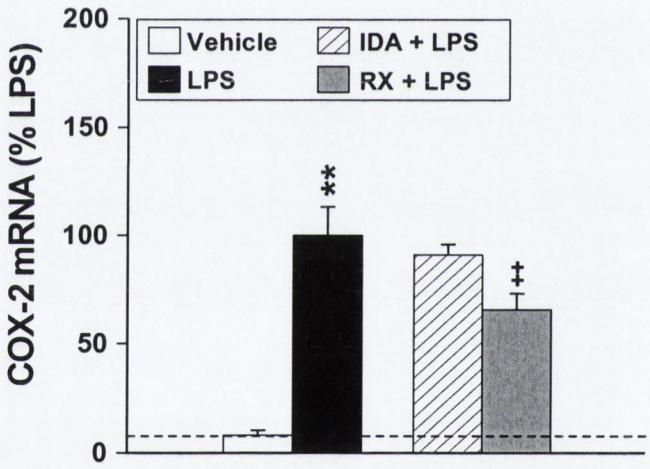


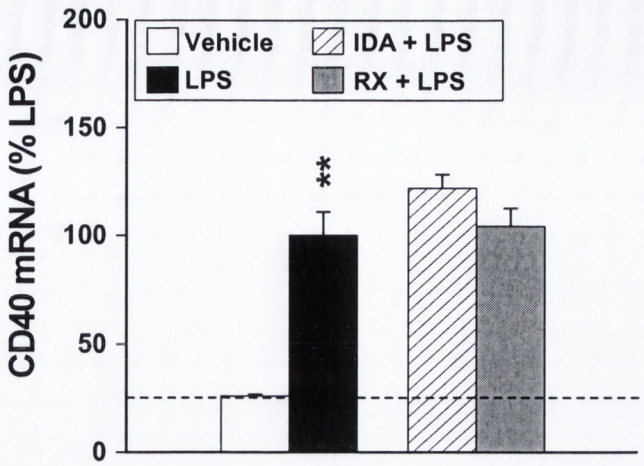
Figure 3.6.8: RX attenuates LPS-induced COX-2 mRNA expression in rat spleen

IDA (1mg/kg), RX (1mg/kg) or vehicle were administered to rats immediately prior to a challenge with vehicle or LPS (250μg/kg). (A) Neither IDA nor RX significantly alter LPS-induced iNOS mRNA expression in rat spleen. (B) HOW do they affect COX-2 in rat spleen 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on iNOS [$F(3,19)=24.62$, $P<0.01$] and COX-2 [$F(3,19)=25.71$, $P<0.0001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle (Fishers *post hoc*).

(a)



(b)

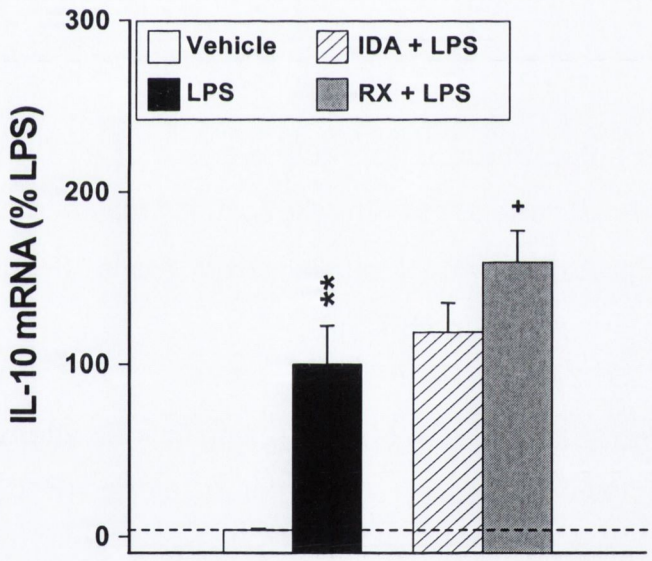


Figure 3.6.9: Neither IDA nor RX alter LPS-induced CD40 mRNA expression, but RX augments LPS-induced IL-10 mRNA expression in rat spleen

Vehicle, IDA (1mg/kg) or RX (1mg/kg) were administered to rats immediately prior to a challenge with vehicle or LPS (250μg/kg). (a) Neither IDA nor RX significantly alter LPS-induced CD40 mRNA expression. (b) RX significantly augments while IDA does not alter IL-10 gene expression in rat spleen 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on CD40 [$F(3,19)=30.6$, $P<0.0001$] and IL-10 [$F(3,19)=15.9$, $P<0.0001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05 versus Vehicle/LPS (Fishers *post hoc*).

3.7. Assessment of the ability of acute treatment with ATX together with IDA to attenuate inflammation in rat brain and spleen: an *in vivo* analysis

The following study assessed the ability of ATX in combination with the α_2-AR antagonist IDA to suppress indices of brain inflammation *in vivo*. To this end, Ida (1mg/kg) has been demonstrated to augment the effect of NRIs on extracellular NA concentrations *in vivo* (Wortley *et al.*, 1999; Swanson *et al.*, 2006; see Invernizzi *et al.*, 2004 for review). To determine whether IDA and ATX would exert a synergistic anti-inflammatory action on neuroinflammation, ATX (5mg/kg) or IDA (1mg/kg) were administered alone or in combination administered immediately prior to challenge with LPS (250μg/kg;i.p.). Four hours post-treatment animals were sacrificed and cortex, hypothalamus and spleen collected for inflammatory analyses. mRNA expression of the pro-inflammatory cytokines TNF-α, the enzyme iNOS and the phenotypic marker of glial cell activation CD40 were examined using real-time PCR.

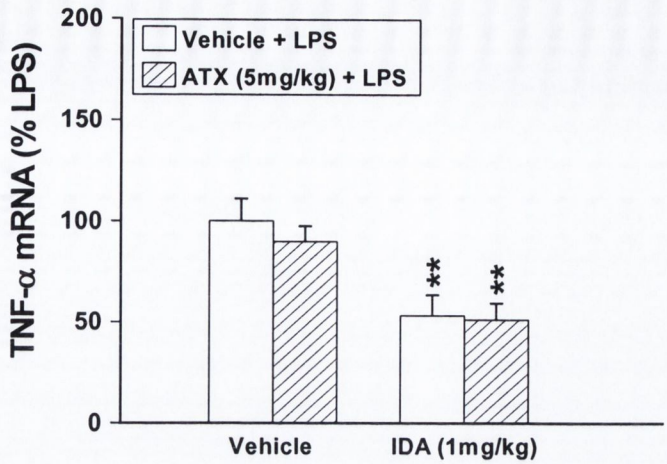
In vivo indices of cortical inflammation: interactive effect of ATX and IDA

TNF-α mRNA expression in rat cortex: IDA (1mg/kg) significantly attenuated LPS-induced TNF-α gene expression, when administered alone ($P<0.01$) or in combination with ATX ($P<0.01$, see figure 3.7.1a)

iNOS mRNA expression in rat cortex: IDA (1mg/kg) significantly attenuated LPS-induced iNOS gene expression, when administered alone ($P<0.01$) or in combination with ATX (5mg/kg), ($P<0.01$, see figure 3.7.1b).

CD40 mRNA expression in rat cortex: IDA (1mg/kg) significantly attenuated LPS-induced CD40 gene expression, when administered alone ($P<0.01$) or in combination with ATX (5mg/kg) ($P<0.01$, see figure 3.7.2a)

(a)



(b)

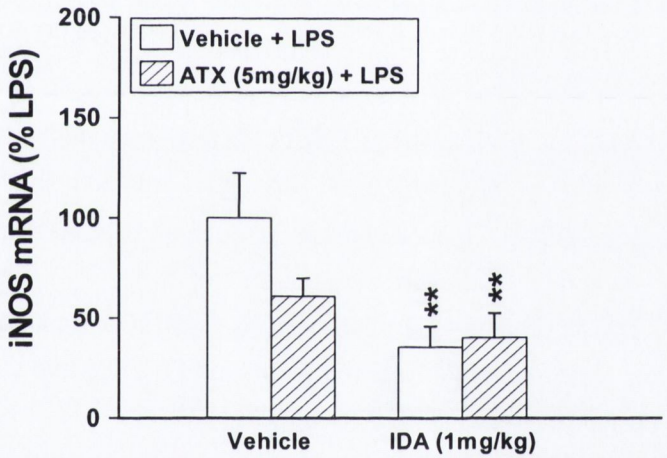


Figure 3.7.1. The α_2-AR antagonist IDA does not have a synergistic anti-inflammatory effect with the NRI ATX on LPS-induced TNF-α or iNOS gene expression in rat cortex

Vehicle, ATX (5mg/kg), IDA (1mg/kg), or ATX (5mg/kg) + IDA (1mg/kg) were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a, b) IDA alone, or in combination with ATX significantly attenuated LPS-induced TNF-α and iNOS gene expression. Treatment with ATX alone did not alter either TNF-α or iNOS mRNA expression in rat cortex 4 hours post-treatment.

Two-way ANOVA revealed no significant interaction between ATX and IDA on TNF-α [$F(1,20)=0.165$, $P=0.69$] or iNOS mRNA expression [$F(1,23)=2.26$, $P=0.146$]. Data expressed as means (% LPS) and standard error of the mean (n=5-7). **$P<0.01$ versus Vehicle/LPS (Fishers *post hoc*).

(a)

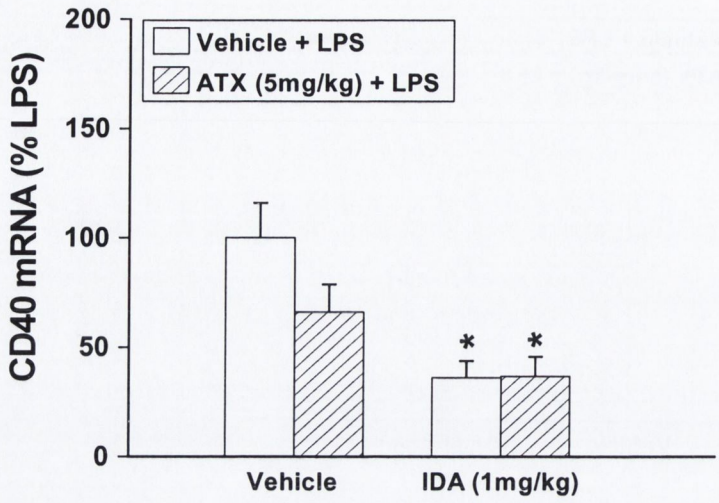


Figure 3.7.2. The α_2-AR antagonist IDA does not have a synergistic anti-inflammatory effect with the NRI ATX on LPS-induced CD40 gene expression in rat cortex

Vehicle, ATX (5mg/kg), IDA (1mg/kg), or ATX (5mg/kg) + IDA (1mg/kg) were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a, b) IDA alone, or in combination with ATX significantly attenuated LPS-induced CD40 gene expression. Treatment with ATX alone did not alter CD40 mRNA expression in rat cortex 4 hours post-treatment.

One way ANOVA revealed no significant interaction between ATX and IDA treatment on CD40 [$F(1,22)=0.01$, $P=0.92$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=6-7). **$P<0.01$ versus Vehicle/LPS (Fishers *post hoc*).

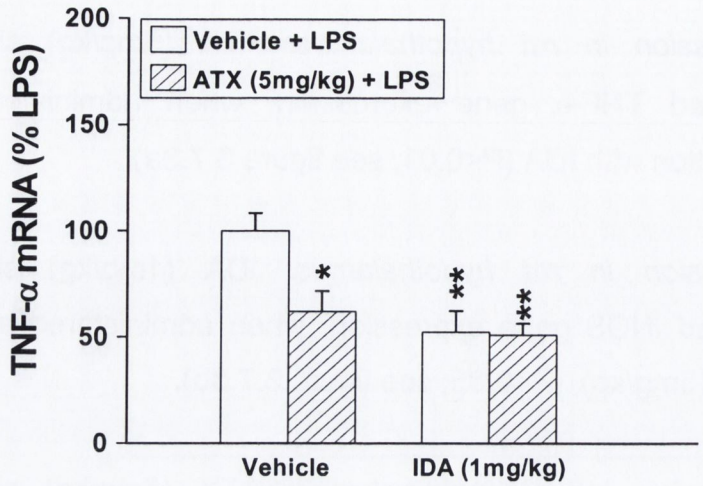
In vivo indices of hypothalamic inflammation: interactive effect of ATX and IDA

TNF-α mRNA expression in rat hypothalamus: ATX (5mg/kg) significantly attenuated LPS-induced TNF-α gene expression, when administered alone ($P<0.05$) or in combination with IDA ($P<0.01$, see figure 3.7.3a)

iNOS mRNA expression in rat hypothalamus: IDA (1mg/kg) significantly attenuated LPS-induced iNOS gene expression, when administered alone or in combination with ATX (5mg/kg), ($P<0.05$, see figure 3.7.3b).

CD40 mRNA expression in rat hypothalamus: ATX (5mg/kg) significantly attenuated LPS-induced CD40 gene expression, when administered alone ($P<0.05$) or in combination with IDA ($P<0.01$, see figure 3.7.4a)

(a)



(b)

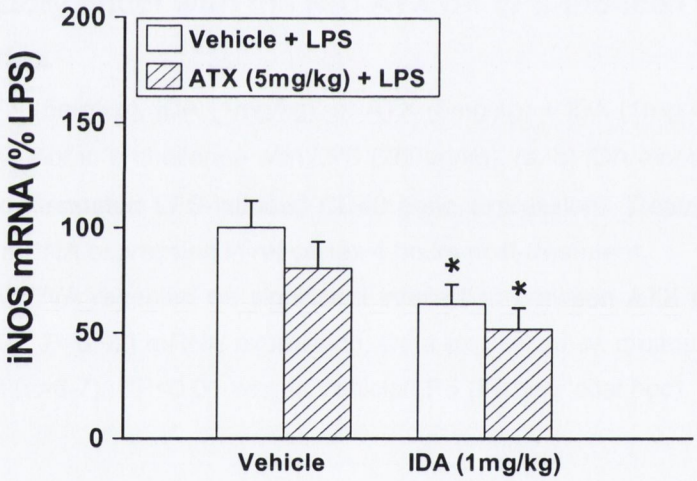


Figure 3.7.3. The α_2-AR antagonist IDA does not have a synergistic anti-inflammatory effect with the NRI ATX on LPS-induced TNF-α or iNOS gene expression in rat hypothalamus

Vehicle, ATX (5mg/kg), IDA (1mg/kg), or ATX (5mg/kg) + IDA (1mg/kg) were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) ATX, IDA and ATX + IDA significantly attenuated LPS-induced TNF-α gene expression. (b) IDA and ATX + IDA significantly attenuated iNOS mRNA expression in rat hypothalamus 4 hours post-treatment.

Two-way ANOVA revealed no significant interaction between ATX and IDA treatment on TNF-α [$F(1,23)=2.63$, $P=0.12$] and iNOS [$F(1,19)=0.1$, $P=0.76$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=5-7). **P<0.01 versus Vehicle/LPS (Fishers *post hoc*).

(a)

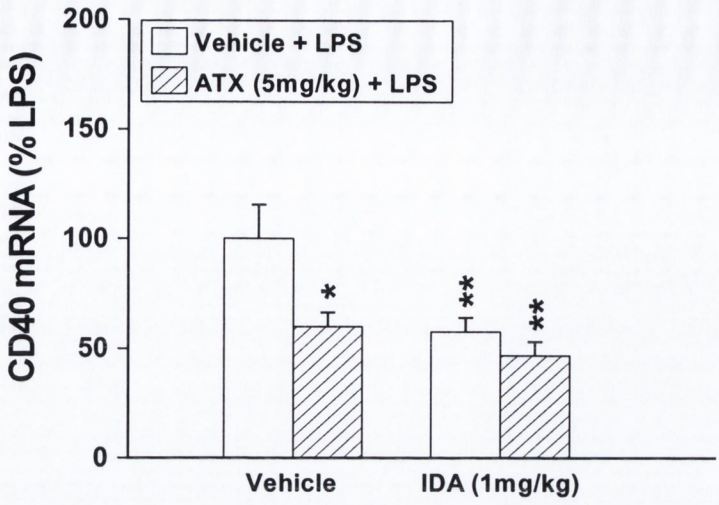


Figure 3.7.4. The α_2-AR antagonist IDA does not have a synergistic anti-inflammatory effect with NRI ATX on LPS-induced CD40 gene expression in rat hypothalamus

Vehicle, ATX (5mg/kg), IDA (1mg/kg), or ATX (5mg/kg) + IDA (1mg/kg) were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) ATX, IDA and ATX + IDA significantly attenuated LPS-induced TNF-α gene expression in rat hypothalamus 4 hours post-treatment. Two-way ANOVA revealed no significant interaction between ATX and IDA CD40 [$F(1,23)=1.58$, $P=0.22$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=6-7). **P<0.01 versus Vehicle/LPS (Fishers *post hoc*).

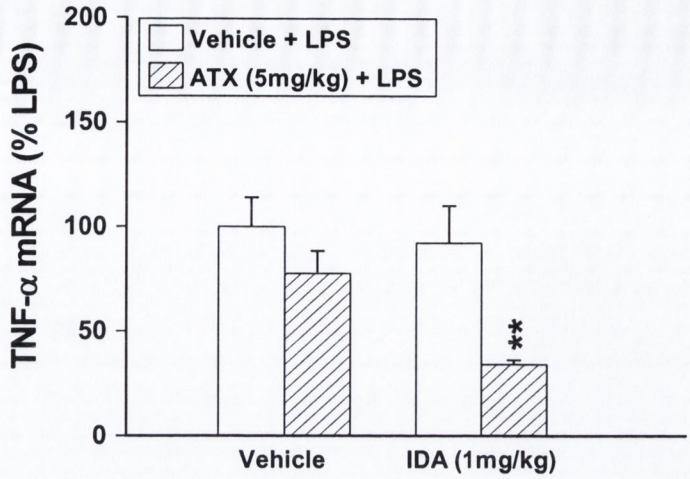
In vivo indices of splenic inflammation: interactive effect of ATX and IDA

TNF-α mRNA expression in rat spleen: Neither ATX (5mg/kg) nor IDA significantly altered LPS-induced TNF-α gene expression, when administered alone, however, when administered in combination they significantly attenuated TNF-α induced by LPS ($P<0.01$, see figure 3.7.5a)

iNOS mRNA expression in rat spleen: ATX (5mg/kg) and IDA (1mg/kg) significantly attenuated LPS-induced iNOS gene expression when administered alone ($P<0.05$, $P<0.01$ respectively) or in combination, ($P<0.01$, see figure 3.7.5b).

CD40 mRNA expression in rat spleen: ATX (5mg/kg) significantly attenuated LPS-induced CD40 gene expression, when administered alone ($P<0.01$) or in combination with IDA ($P<0.01$, see figure 3.7.6a). IDA (1mg/kg) did not alter LPS-induced CD40 when administered alone.

(a)



(b)

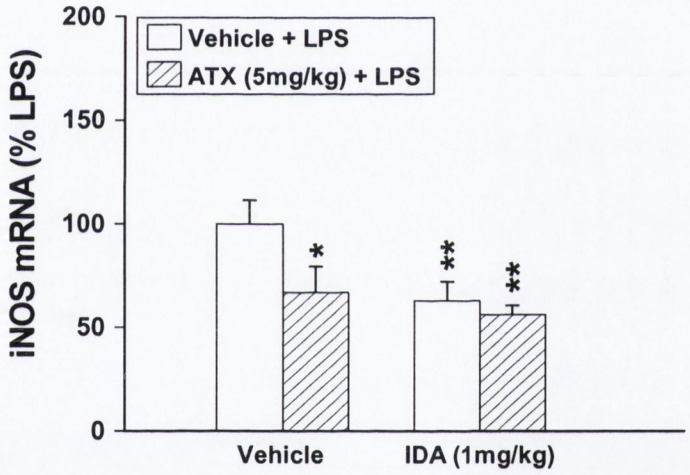


Figure 3.7.5. The α_2-AR antagonist IDA has synergistic anti-inflammatory effect with the NRI ATX on LPS-induced TNF-α, but not iNOS gene expression in rat spleen

Vehicle, ATX (5mg/kg), IDA (1mg/kg), or ATX (5mg/kg) + IDA (1mg/kg) were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) ATX + IDA significantly attenuated LPS-induced TNF-α gene expression. (b) ATX, IDA and ATX + IDA significantly attenuated LPS-induced iNOS gene expression in rat spleen four hours post-treatment.

Two-way ANOVA revealed no significant interaction between ATX and IDA on TNF-α [$F(1,23)=2.82$, $P=0.11$] or iNOS [$F(1,21)=1.9$, $P=0.18$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=6-7). **$P<0.01$ versus Vehicle/LPS (Fishers *post hoc*).

(a)

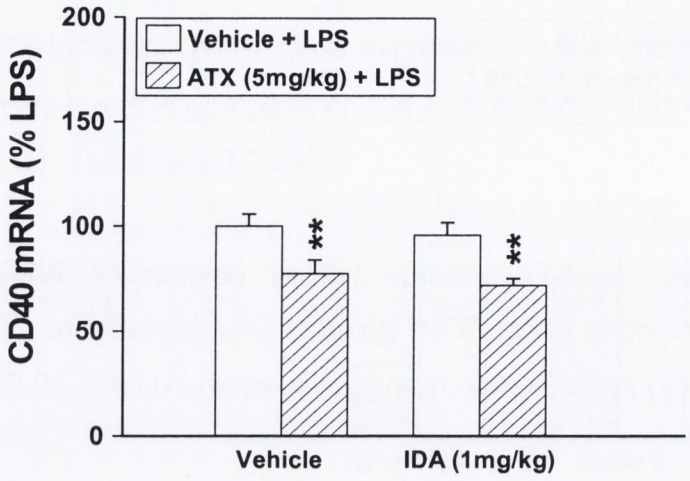


Figure 3.7.6. The α_2-AR antagonist IDA does not have a synergistic anti-inflammatory effect with the NRI ATX on LPS-induced CD40 gene expression in rat spleen

Vehicle, ATX (5mg/kg), IDA (1mg/kg), or ATX (5mg/kg) + IDA (1mg/kg) were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) ATX and ATX + IDA significantly attenuated LPS-induced CD40 gene expression.

Two-way ANOVA revealed a significant effect of treatment on CD40 [F(1,23)=0.15, P=0.9] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=6-7). **P<0.01 versus Vehicle/LPS (Fishers *post hoc*).

3.8. Assessment of the ability of noradrenaline to alter chemokine mRNA expression in rat cortical glia: an *in vitro* analysis

NA demonstrates anti-inflammatory actions, as demonstrated by the attenuation of LPS-induced cytokines, cell activation markers and inflammatory markers both *in vitro* and *in vivo*. Despite this, the effect of NA on expression of pro-inflammatory chemokines has not been assessed to date. Chemokines play a crucial role in co-ordinating the inflammatory response by directing leukocytes to sites of injury and inflammation, this is particularly important in allowing infiltration of leuckocytes across the BBB during the course of neuroinflammation.

To determine if NA alters LPS-induced chemokine expression in cortical glial cells *in vitro*, NA (1 or 10μM) was administered 30 minutes prior to LPS (1μg/ml). A vehicle control group was included to give an estimate of basal expression of inflammatory chemokines in glial cells. Six hours post-treatment cells were harvested for mRNA, and expression of the pro-inflammatory chemokines RANTES, IP-10, CINC-1 and MIP-1α was examined using real-time PCR.

Table 3.8.1. A summary of basal and LPS-induced levels of chemokine gene expression in cultured cortical glia

	Ct for target Gene		CT for β-Actin		LPS-induced change	
Target	Control	LPS	Control	LPS	ΔCT (Target-Actin)	Fold increase
RANTES	28.96	23.91	17.26	16.72	4.66	25.25
IP-10	24.02	19.22	17.05	16.50	4.37	20.67
CINC-1	23.91	20.90	17.16	17.1	2.97	7.83
MIP-1α	25.38	22.33	17.76	17.25	2.59	6.02

CT = Cycle number

ΔCT = Difference in CT between Control and LPS stimulated cells corrected for β-actin

QPCR was carried out on cDNA reverse transcribed from 0.84μg of cellular RNA using a High Capacity cDNA archive kit (Applied Biosystems), as described previously.

Data represents the mean of 5-6 samples

Basal gene expression (non-LPS stimulated) of target chemokines did not differ greatly in cultured glia, as assessed using QPCR. Magnitude of target gene induction by LPS was RANTES > IP-10 > CINC-1 > MIP-1α. NA significantly suppressed LPS-induced chemokine gene expression, with the exception of CINC-1, which was unaltered by NA treatment.

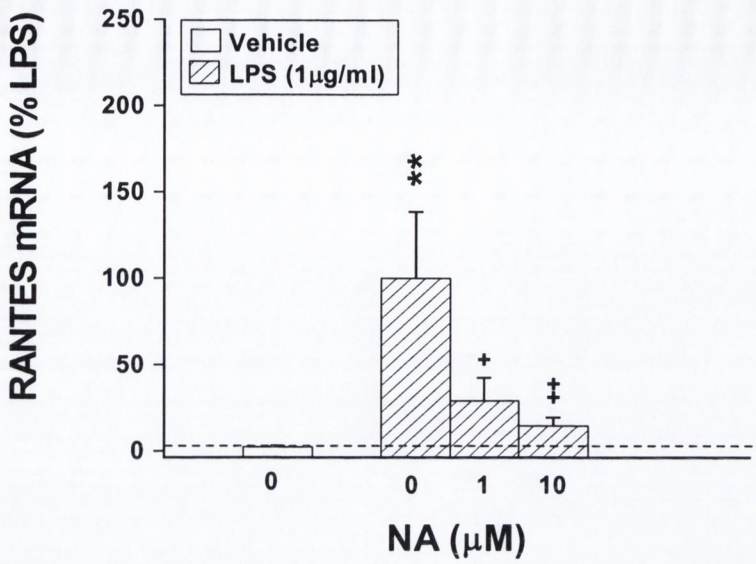
RANTES mRNA expression in rat mixed cortical glia: LPS (1μg/ml) induced a 25-fold increase in RANTES gene expression relative to control ($P<0.01$). NA (1 and 10μM) significantly attenuated LPS-induced RANTES mRNA expression ($P<0.01$) (Figure 3.8.1a).

IP-10 mRNA expression in rat mixed cortical glia: LPS (1μg/ml) induced a 21-fold increase in IP-10 gene expression relative to control ($P<0.01$). NA (10μM) significantly attenuated LPS-induced IP-10 mRNA expression ($P<0.05$) (Figure 3.8.1b).

CINC-1 mRNA expression in rat mixed cortical glia: LPS (1μg/ml) induced an 8-fold increase in CINC-1 gene expression relative to control ($P<0.01$). NA (1 and 10μM) did not alter LPS-induced CINC-1 mRNA expression (Figure 3.8.2a).

MIP-1α mRNA expression in rat mixed cortical glia: LPS (1μg/ml) induced a 6-fold increase in MIP-1α gene expression relative to control ($P<0.01$). NA (1 and 10μM) significantly attenuated LPS-induced MIP-1α mRNA expression in mixed glia, 6 hours post-treatment (Figure 3.8.2b).

(a)



(b)

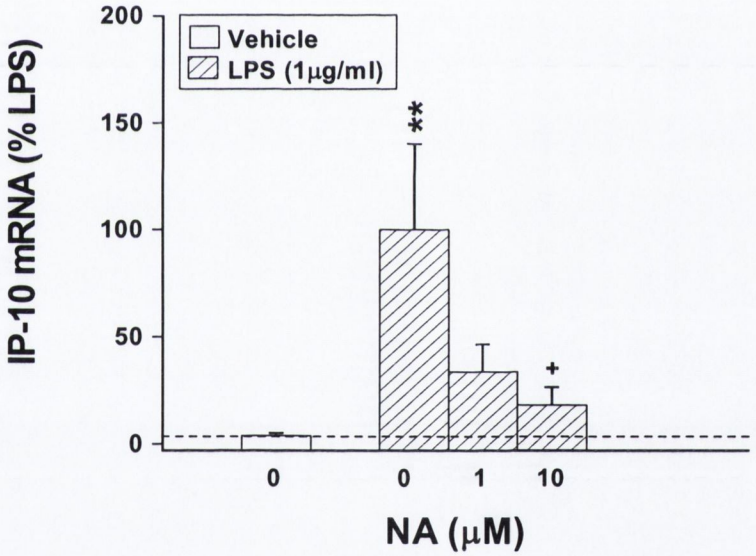
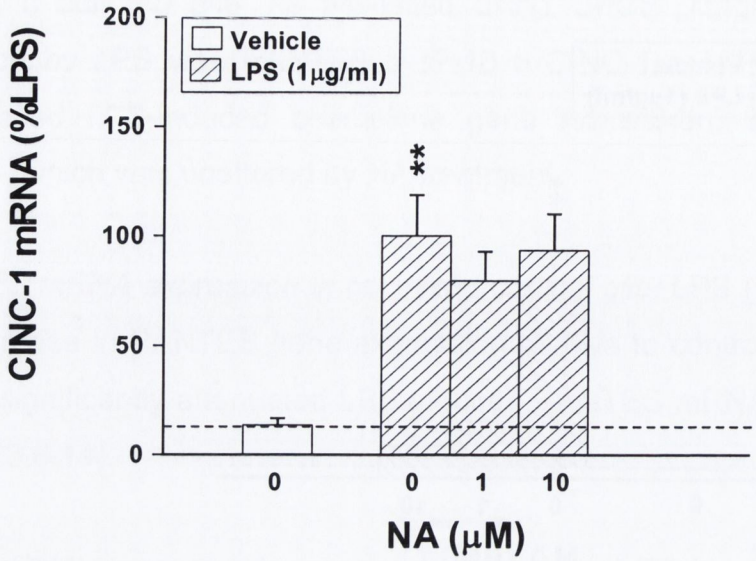


Figure 3.8.1: NA attenuates LPS-induced RANTES and IP-10 gene expression in mixed cortical glia.

NA or vehicle were administered to cells 30 minutes prior to LPS (1μg/ml) administration. (a) NA (1 and 10μM) significantly attenuated LPS-induced RANTES mRNA expression. (b) NA (1 and 10μM) significantly attenuated LPS-induced IP-10 mRNA in cultured cortical glia 6 hours post-treatment. Repeated one way ANOVA revealed a significant effect of treatment on RANTES [$F(3,19)=5.34$, $P<0.01$] and IP-10 [$(3,19)=3.94$, $P<0.05$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

(a)



(b)

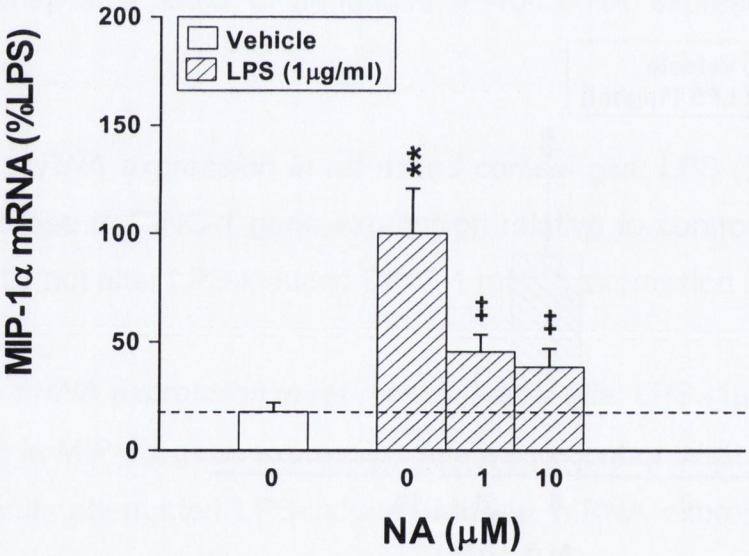


Figure 3.8.2: NA attenuates LPS-induced MIP-1α but does not alter CINC-1 gene expression in mixed cortical glia

NA or vehicle were administered to cells 30 minutes prior to LPS (1μg/ml) administration. (a) NA did not alter LPS-induced CINC-1 mRNA expression. (b) NA (1 and 10μM) significantly attenuated LPS-induced MIP-1α mRNA in cultured cortical glia 6 hours post-treatment.

Repeated one way ANOVA revealed a significant effect of treatment on CINC [$F(3,20=7.8$, $P<0.01$] and MIP-1α [$F(3,20)=8.57$, $P<0.001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc* test).

3.9. Assessment of the ability of acute treatment with noradrenaline-reuptake inhibitors to alter chemokine mRNA expression in rat brain and spleen: an *in vivo* analysis

As NA demonstrates anti-inflammatory actions on pro-inflammatory chemokines in cortical rat primary glial cells, the effects of noradrenaline reuptake inhibitors (NRIs) on cortical and hypothalamic chemokines were assessed using an *in vivo* rat model. To out knowledge no other studies have examined the effects of NA or NRIs on brain chemokines to date.

To determine whether increased availability of NA to glial cells would suppress LPS-induced chemokines *in vivo*, the NRI's DMI (3.2, 7.5 or 15mg/kg;i.p.) and ATX (2.5, 5 or 10mg/kg;i.p.) were administered immediately prior to systemic challenge with LPS (250μg/kg;i.p.), as previously described. A vehicle control group was included to give an estimate of basal expression of inflammatory chemokines in the brain. Four hours post-treatment animals were sacrificed and cortex, hypothalamus and spleen collected for inflammatory analyses. Inflammatory chemokine expression in spleen was assessed as an indicator of the peripheral inflammatory response. mRNA expression of the pro-inflammatory chemokines RANTES, IP-10, CINC-1 and MIP-1α was examined using real-time PCR.

As outlined in Table 3.9.1. below, the magnitude of chemokine gene induction by LPS was greater in the brain than in spleen, with the exception of MIP-1α, RANTES gene expression was only significantly induced in the cortex by LPS, although high basal levels of RANTES were observed in both brain and spleen (see Tables 3.9.2, 3.9.3 and 3.9.4 for relevant CT values).

Table 3.9.1: A summary of the magnitude of chemokine gene expression induced by LPS in cortex, hypothalamus and spleen tissue, and the ability of acute NRI treatment to alter LPS-induced chemokine expression

Target	Cortex			Hypothalamus			Spleen		
	Vehicle# + LPS	DMI + LPS	ATX + LPS	Vehicle# + LPS	DMI + LPS	ATX + LPS	Vehicle# + LPS	DMI + LPS	ATX + LPS
RANTES	4-fold**	++	++	1.5-fold	-	-	-	-	-
IP-10	69-fold**	++	++	180-fold**	++	++	37-fold**	++	-
CINC-1	45-fold**	++	++	142-fold**	+	-	198-fold**	+	-
MIP-1α	3-fold**	-	-	5-fold*	#	-	17-fold**	++	+

= Fold increase in gene expression from non-LPS treated animals

One-way analysis of variance revealed significant treatment effects on target gene expression. **P<0.01 fold increase versus Vehicle/Vehicle, +P<0.05, ++P<0.01 decrease (at any dose) versus Vehicle/LPS, # P<0.05 increase? versus Vehicle/LPS (Fishers LSD test).

***In vivo* chemokine mRNA expression in the cortex: effect of NRI treatment**

Magnitude of cortical basal gene expression (in animals not treated with LPS) were as follows: IP-10 > > RANTES > MIP-1α > CINC-1

Magnitude of target gene induction by LPS: IP-10 > CINC-1 > RANTES > MIP-1α (Table 3.9.2).

DMI and ATX elicited qualitatively similar effects on cortical chemokines, both drugs dose-dependently decreased mRNA expression of RANTES, IP-10 and CINC-1, while MIP-1α was unaffected by either NRI treatment.

Table 3.9.2: A summary of basal and LPS-induced chemokine mRNA expression in cortical tissue

	Ct for target Gene		CT for β-Actin		LPS-induced change	
Target	Control	LPS	Control	LPS	ΔCT (Target-Actin)	Fold increase
RANTES	28.87	27.1	16.61	16.78	1.93	3.82
IP-10	25.48	19.29	16.95	16.87	6.11	69.17
CINC-1	32.02	26.37	17.56	17.39	5.49	44.50
MIP-1α	31.30	29.8	17.65	17.52	1.37	2.59

CT = Cycle number

ΔCT = Difference in CT between Control and LPS treated animals, corrected for β-actin

QPCR was carried out on cDNA reverse transcribed from 1.28μg of cortical RNA using a High Capacity cDNA archive kit (Applied Biosystems), as described previously.

Data represents the mean of 4-6 samples

RANTES mRNA expression in rat cortex: LPS (250μg/kg) induced a 4-fold increase in RANTES mRNA expression relative to control ($P<0.01$). DMI (7.5 and15mg/kg) and ATX (5 and 10mg/kg) significantly attenuated LPS-induced RANTES mRNA expression (Figure 3.9.1a).

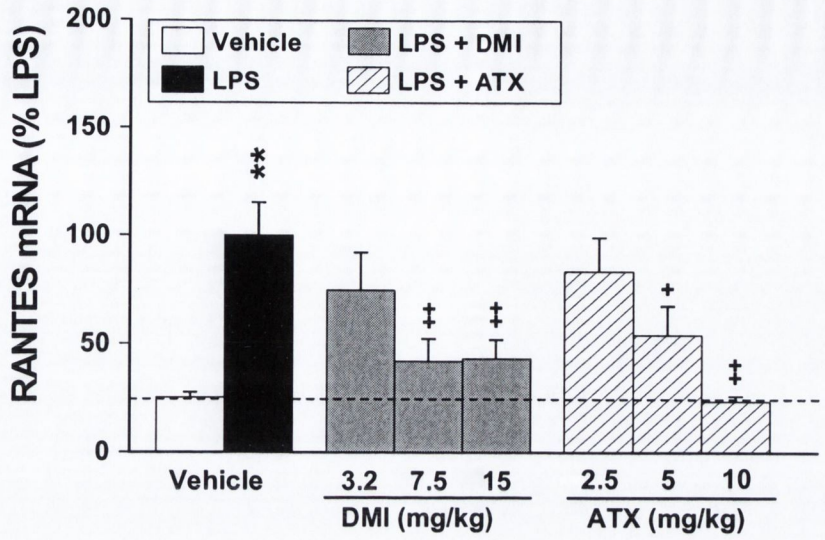
IP-10 mRNA expression in rat cortex: LPS (250μg/kg) induced a 70-fold increase in IP-10 mRNA expression relative to control ($P<0.01$). DMI (7.5 and 15mgkg) and

ATX (5 and 10mg/kg) significantly decreased LPS-induced IP-10 mRNA expression ($P<0.01$, Figure 3.9.1b).

CINC-1 mRNA expression in rat cortex: LPS (250μg/kg) induced a 45-fold increase in CINC-1 mRNA expression relative to control ($P<0.01$). DMI (7.5 and 15mgkg) and ATX (5 and 10mg/kg) significantly decreased LPS-induced CINC-1 mRNA expression ($P<0.01$, Figure 3.9.2a).

MIP-1α mRNA expression in rat cortex: LPS (250μg/kg) induced a 2.6-fold increase in MIP-1α mRNA expression relative to control ($P<0.01$). Neither DMI nor ATX significantly altered LPS-induced MIP-1α mRNA expression (Figure 3.9.2b).

(a)



(b)

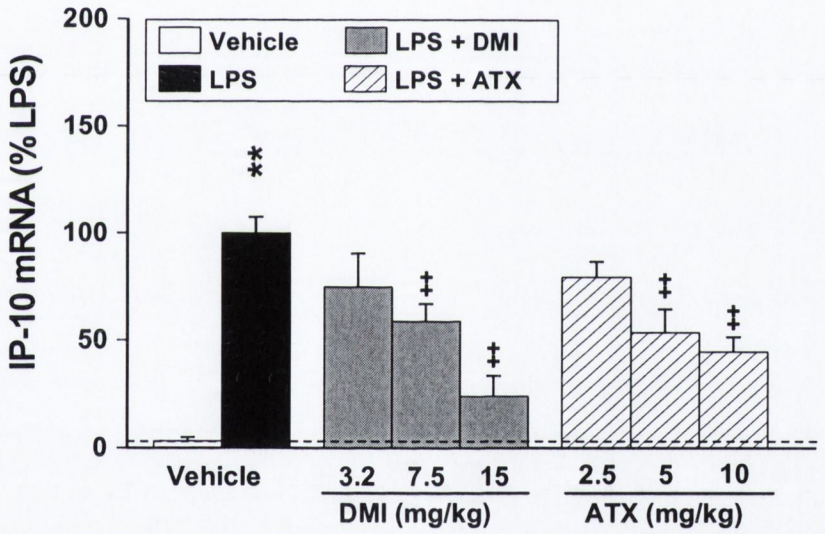


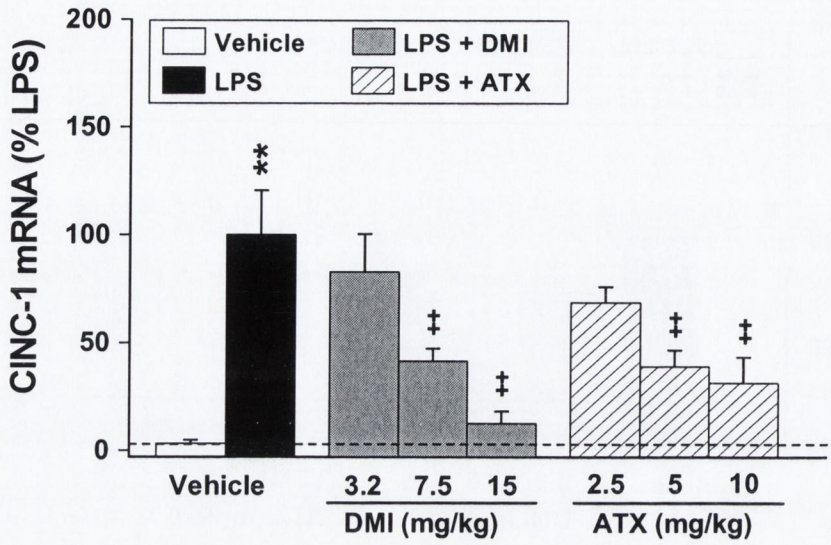
Figure 3.9.1: DMI and ATX attenuate LPS-induced RANTES and IP-10 gene expression in rat cortex.

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (7.5 and 15mg/kg) and ATX (5 and 10mg/kg) significantly attenuated LPS-induced RANTES mRNA expression. (b) DMI (7.5 and 15mg/kg) and ATX (5 and 10mg/kg) significantly attenuated LPS-induced IP-10 mRNA in rat cortex 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on RANTES [$F(7,40)=5.34$, $P<0.001$] and IP-10 [$(7,40)=11.31$, $P<0.0001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

(a)



(b)

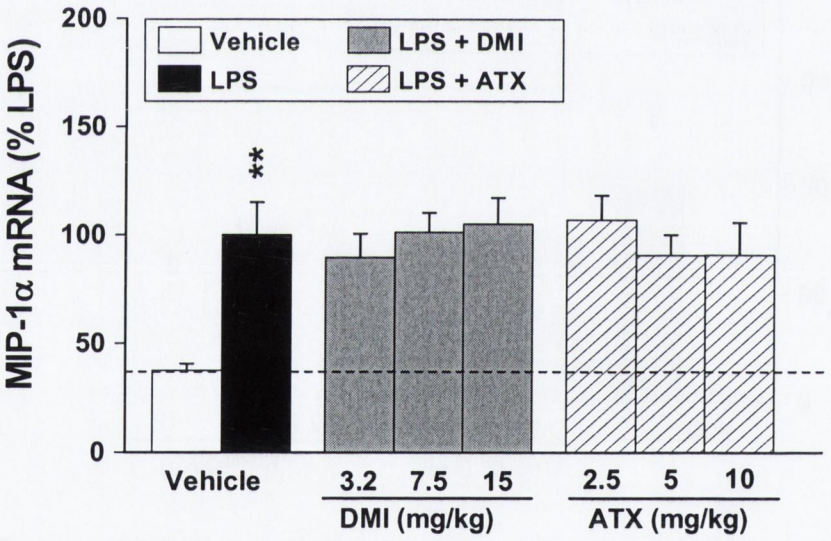


Figure 3.9.2: DMI and ATX attenuate LPS-induced CINC-1 gene expression but do not alter MIP-1α mRNA expression in rat cortex.

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (7.5 and 15mg/kg) and ATX (2.5-10mg/kg) significantly attenuated LPS-induced CINC-1 mRNA expression. (b) Neither DMI nor ATX altered LPS-induced MIP-1α mRNA in rat cortex 4 hours post- treatment. CINC-1 [$F(7,36)=8.09$, $P<0.0001$] and MIP-1α [$(7,40)=3.95$ $P<0.01$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

Acute treatment with noradrenaline-reuptake inhibitors does not alter chemokine gene expression in mixed cortical glia

To determine whether the anti-inflammatory actions of NRIs on chemokine mRNA expression in rat cortex resulted from increased NA availability, or a direct effect of the drugs on glia, the effect of DMI and ATX on glial cells was examined *in vitro*. DMI or ATX (0.1, 1 or 10μM) were administered 30 minutes prior to LPS (1μgml). 6 hours post-treatment cellular RNA was harvested and mRNA expression of the pro-inflammatory chemokines RANTES, IP-10 and CINC-1 was examined using real-time PCR. MIP-1α mRNA expression was not examined following *in vitro* exposure to ATX or DMI, as neither drug altered expression of this chemokine *in vivo*.

As demonstrated in Table 3.9.3 and 3.9.4, below, direct administration of DMI or ATX did not alter chemokine gene expression, demonstrating the lack of a direct action of these drugs on LPS-induced chemokine expression in mixed cortical glia.

Table 3.9.3: DMI does not alter LPS-induced chemokine gene expression in mixed cortical glia

Target	Veh. + LPS	DMI (0.1μM) + LPS	DMI (1μM) + LPS	DMI (10μM) + LPS	ANOVA
RANTES	100 ± 24	68 ± 14	84 ± 19	100 ± 20	F(3,18)=0.6, P=0.6
IP-10	100 ± 12	72 ± 8	89 ± 13	112 ± 20	F(3,18)=1.7, P=0.2
CINC-1	100 ± 8	91 ± 9	99 ± 7	116 ± 11	F(3,18)=1, P=0.3

Data expressed as mean (% LPS control) with standard error of the mean (n=4-6). One way analysis of variance revealed no significant effect of treatment on target gene expression.

Table 3.9.4: ATX does not alter LPS-induced chemokine gene expression in mixed cortical glia

Target	Veh. + LPS	ATX (0.1μM) + LPS	ATX (1μM) + LPS	ATX (10μM) + LPS	ANOVA
RANTES	100 ± 24	89 ± 21	108 ± 27	112 ± 17	F(3,18)=0.2, P=0.9
IP-10	100 ± 12	94 ± 18	102 ± 19	99 ± 6	F(3,18)=0.04, P=0.99
CINC-1	100 ± 8	134 ± 18	101 ± 13	107 ± 8	F(3,18)=1.6, P=0.2

Data expressed as mean (% LPS control) with standard error of the mean (n=4-6). One-way analysis of variance revealed no significant effect of treatment on target gene expression.

***In vivo* chemokines in the hypothalamus: effect of NRI treatment**

Magnitude of basal gene expression (in animals not treated with LPS) were as follows: RANTES > IP-10 > CINC >MIP-1α.

Magnitude of target gene induction by LPS: IP-10 > CINC-1 > MIP-1α > RANTES (Table 3.9.5)

Neither LPS, DMI nor ATX significantly altered mRNA expression for RANTES in the hypothalamus. Induction of hypothalamic CINC-1 mRNA was attenuated by DMI but not ATX, while DMI augmented and ATX did not alter LPS-induced MIP-1α in hypothalamus. In contrast, both NRIs attenuated LPS-induced IP-10 gene expression in hypothalamus.

Table 3.9.5: A summary of basal and LPS-induced levels chemokine gene expression in hypothalamic tissue

	Ct for target Gene		CT for β-Actin		LPS-induced change	
Target	Control	LPS	Control	LPS	ΔCT (Target-Actin)	Fold increase
RANTES	26.76	26.47	17.14	17.40	0.55	1.47
IP-10	26.93	19.62	16.65	16.84	7.5	180.54
CINC-1	32.32	25.30	17.66	17.78	7.15	141.93
MIP-1α	32.05	29.94	17.71	17.93	2.33	5.02

CT = Cycle number

ΔCT = Difference in CT between Control and LPS treated animals, corrected for β-actin

QPCR was carried out on cDNA reverse transcribed from 1.11μg of hypothalamic RNA using a High Capacity cDNA archive kit (Applied Biosystems), as described previously.

Data represents the mean of 5-6 samples

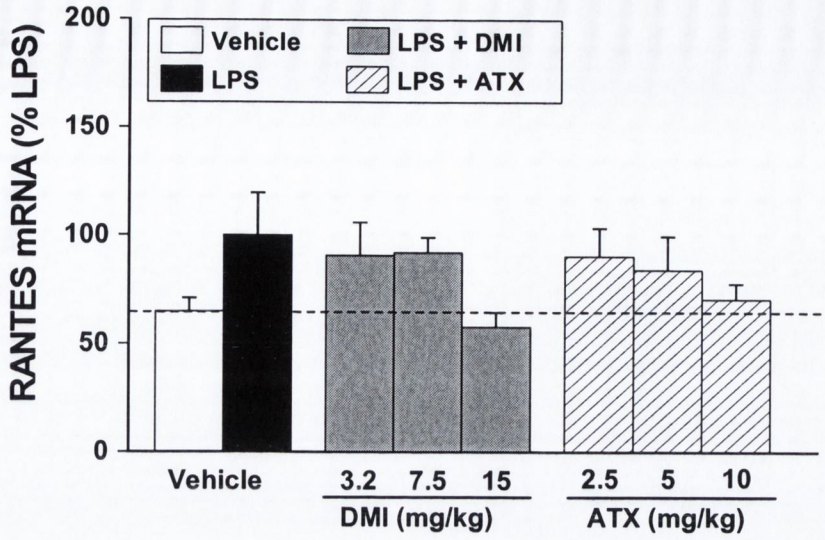
RANTES mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a non-significant increase (1.5-fold) in RANTES, and NRI treatment failed to significantly altered RANTES mRNA expression (Figure 3.9.3a).

IP-10 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 180-fold increase in IP-10 mRNA expression relative to control ($P<0.01$). DMI (15mg/kg) and ATX (5 and 10mg/kg) significantly decreased LPS-induced IP-10 mRNA expression ($P<0.01$, Figure 3.9.3b).

CINC-1 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 142-fold increase in CINC-1 gene expression relative to control ($P<0.01$). DMI (15mgkg), but not ATX significantly attenuated LPS-induced CINC-1 mRNA expression ($P<0.05$, Figure 3.9.4a).

MIP-1α mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 5-fold increase in MIP-1α gene expression relative to control ($P<0.05$). DMI (7.5mg/kg) significantly augmented ($P<0.05$) while ATX did not alter LPS-induced MIP-1α expression (Figure 3.9.4b).

(a)



(b)

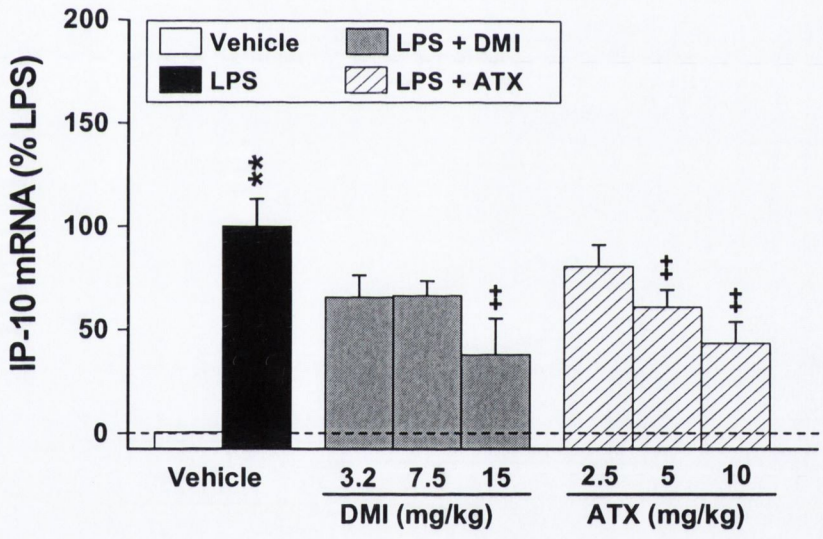


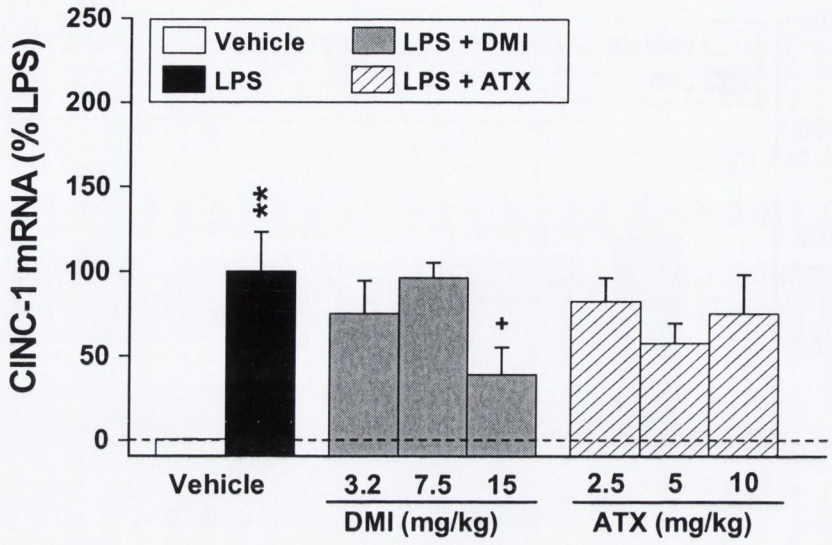
Figure 3.9.3: DMI and ATX attenuate LPS-induced RANTES and IP-10 gene expression in rat hypothalamus

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) LPS, DMI and ATX did not significantly alter RANTES gene expression in hypothalamus 4 hours post-treatment. (b) DMI (15mg/kg) and ATX (5 and 10mg/kg) significantly attenuated LPS-induced IP-10 mRNA in rat hypothalamus 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on RANTES [$F(7,39)=1.56$, $P=0.18$] and IP-10 [$F(7,39)=7.77$, $P<0.0001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

(a)



(b)

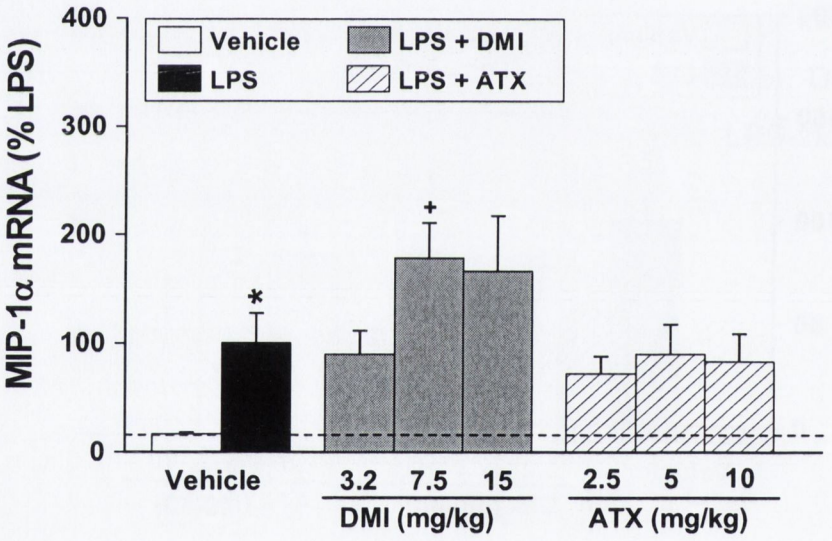


Figure 3.9.4: DMI attenuates LPS-induced CINC-1 but augments MIP-1α gene expression in rat hypothalamus

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (15mg/kg) significantly attenuated, while ATX did not alter CINC-1 gene expression in hypothalamus 4 hours post-treatment. (b) DMI (7.5mg/kg) augmented, while ATX did not alter LPS-induced MIP-1α mRNA in rat hypothalamus 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on CINC-1 [$F(7,38)=4.08$, $P<0.01$] and MIP-1α [$F(7,37)=3.65$, $P<0.01$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05 versus Vehicle/LPS group (Fishers LSD *post hoc test*).

***In vivo* chemokine mRNA expression in spleen: effect of NRI treatment**

Basal expression of IP-10 and RANTES was higher than the other chemokines, as demonstrated by lower CT values (Table 3.9.4). LPS induced the greatest gene expression of IP-10 and MIP-1a in spleen, with CINC-1 induced to a lesser extent. In contrast, LPS failed to induce expression of RANTES in spleen tissue. With the exception of MIP-1α, the magnitude of chemokine gene induction by LPS was less than that observed in the brain 4 hours post-treatment.

Table 3.9.6: A summary of basal and LPS-induced levels of chemokine gene expression in spleen tissue

	Ct for target Gene		CT for β-Actin		LPS-induced change	
Target	Control	LPS	Control	LPS	ΔCT (Target-Actin)	Fold increase
RANTES	19.85	18.87	15.45	13.99	0.48	0.72
IP-10	21.07	14.41	15.72	14.28	5.21	37.09
CINC-1	28.79	19.33	16.54	14.38	7.95	246.62
MIP-1α	27.11	21.63	16.05	14.66	4.09	17.08

CT = Cycle number

ΔCT = Difference in CT between Control and LPS treated animals, corrected for β-actin

QPCR was carried out on cDNA reverse transcribed from 2.73μg of splenic RNA using a High Capacity cDNA archive kit (Applied Biosystems), as described previously.

Data represents the mean of 5-6 samples

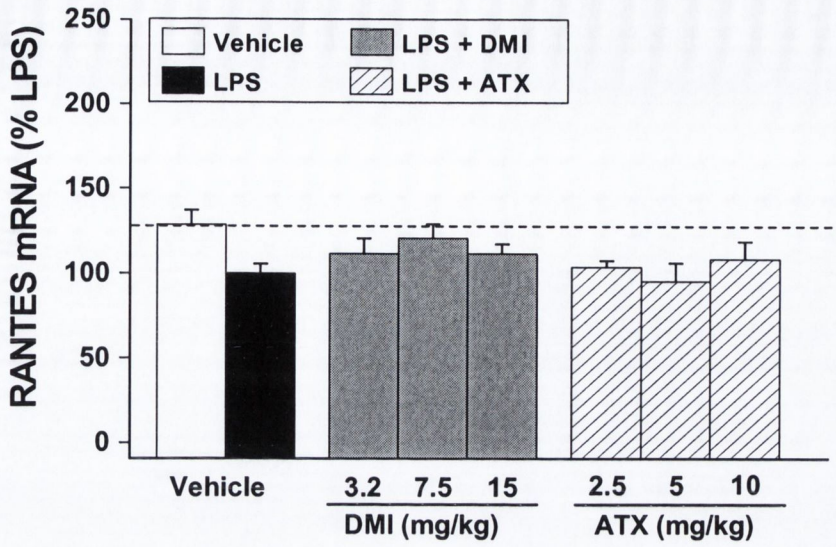
RANTES mRNA expression in rat spleen: Neither LPS (250μg/kg), nor NRI treatment significantly altered RANTES mRNA expression (Figure 3.9.5a).

IP-10 mRNA expression in rat spleen: LPS (250μg/kg) induced a 37-fold increase in IP-10 mRNA expression relative to control ($P<0.01$). DMI (15mg/kg) significantly attenuated ($P<0.01$), while ATX did not alter LPS-induced IP-10 mRNA expression in rat spleen 4 hours post-treatment (Figure 3.9.5b).

CINC-1 mRNA expression in rat spleen: LPS (250μg/kg) induced a 246-fold increase in CINC-1 mRNA expression relative to control ($P<0.01$). DMI (15mg/kg), but not ATX significantly attenuated LPS-induced CINC-1 mRNA expression ($P<0.05$, Figure 3.9.6a).

MIP-1α mRNA expression in rat spleen: LPS (250μg/kg) induced a 17-fold increase in MIP-1α mRNA expression relative to control ($P<0.01$). DMI (7.5 and 15mg/kg) and ATX (5 and 10mg/kg) significantly attenuated ($P<0.01$ and $P<0.05$, respectively) LPS-induced MIP-1α expression (Figure 3.9.6b).

(a)



(b)

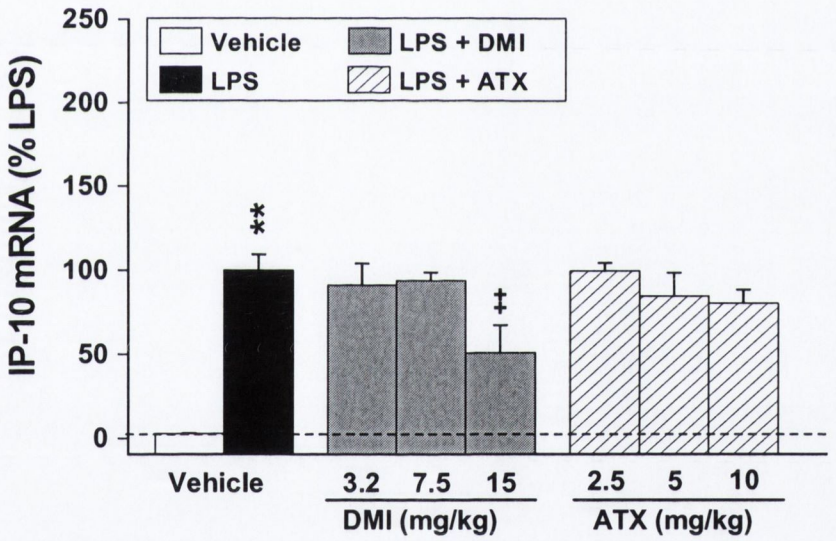


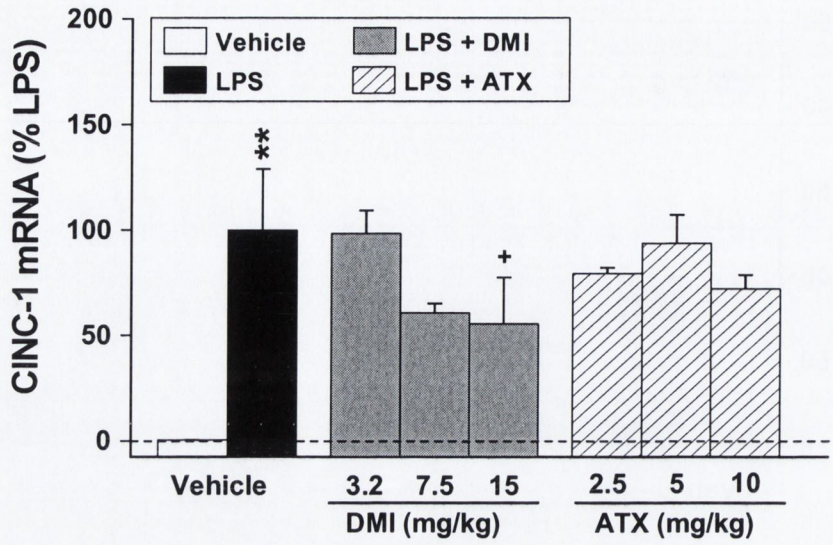
Figure 3.9.5: LPS, DMI and ATX are without effect on RANTES, while DMI attenuates LPS-induced IP-10 gene expression in rat spleen

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) LPS, DMI and ATX treatment were without effect on RANTES mRNA expression in spleen. (b) DMI (15mg/kg) significantly attenuated, while ATX did not alter IP-10 gene expression in spleen, 4 hours post-treatment.

One way ANOVA revealed no significant effect of treatment on RANTES [$F(7,39)=1.7$, $P=0.14$] and a significant treatment effect on IP-10 [$F(7,40)=10.63$, $P<0.0001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01versus Vehicle/LPS group (Fishers LSD *post hoc test*).

(a)



(b)

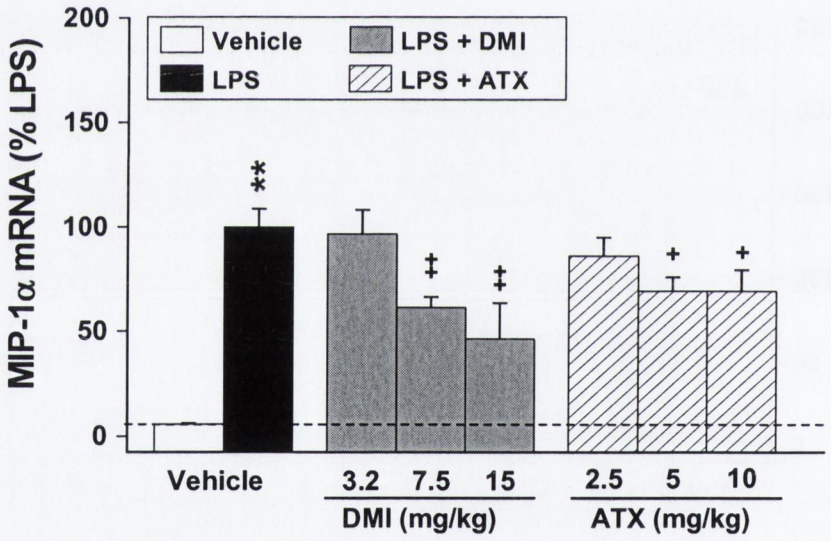


Figure 3.9.6: DMI but not ATX attenuates LPS-induced CINC-1, while both DMI and ATX attenuate LPS-induced MIP-1α gene expression in rat spleen

DMI, ATX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) DMI (15mg/kg) significantly attenuated, while ATX did not alter LPS-induced CINC-1 mRNA expression. (b) DMI (7.5 and 15mg/kg) and ATX (5 and 10mg/kg) significantly attenuated LPS-induced MIP-1α gene expression in spleen, 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on [$F(7,38)=4.67$, $P<0.001$] and MIP-1α [$F(7,39)=10.99$, $P<0.0001$] mRNA expression.

Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01versus Vehicle/LPS group (Fishers LSD *post hoc test*).

3.10 Assessment of the ability of α_2-adrenoceptor antagonists to alter brain chemokine expression: an *in vivo* analysis

As NRIs attenuated LPS-induced chemokines in the brain, the ability of the α_2-AR antagonists IDA and RX to effect the same result was assessed. Vehicle or the α_2-AR antagonists IDA (1mg/kg) and RX (1mg/kg) were administered immediately prior to challenge with either vehicle or LPS (250μg/kg;i.p.). Four hours post-treatment animals were sacrificed and cortex and hypothalamus removed for inflammatory analyses. Inflammatory gene expression in the spleen was conducted as an indicator of the peripheral inflammatory response. mRNA expression of the pro-inflammatory chemokines RANTES, IP-10 and CINC-1 was assessed using real-time PCR.

As outlined in table 3.10.1 below, the magnitude of chemokine gene induction by LPS was greatest in the brain. Moreover, suppression of chemokine mRNA displays a different profile in the brain and spleen, with greatest inhibition of chemokine mRNA by α_2-AR antagonists observed in the cortex.

Table 3.10.1. A summary of the magnitude of inflammatory gene expression induced by LPS in cortex, hypothalamus and spleen tissue, and the ability of acute NRI treatment to alter LPS-induced inflammatory responses

Target	Cortex			Hypothalamus			Spleen		
	Vehicle# + LPS	IDA + LPS	RX + LPS	Vehicle# + LPS	IDA + LPS	RX + LPS	Vehicle# + LPS	IDA + LPS	RX + LPS
RANTES	4-fold**	++	++	1.3-fold	-	-	0.75$	-	-
IP-10	72-fold**	+	++	196-fold**	++	++	39-fold**	-	-
CINC-1	51-fold**	++	++	85-fold**	++	+	242-fold**	+	++
MIP-1α	3-fold**	-	-	3.5-fold**	-	#	17-fold**	-	++

= Fold increase in gene expression from non-LPS treated animals

One-way analysis of variance revealed significant treatment effects on target gene expression.

**P<0.01 fold increase versus Vehicle / Vehicle, +P<0.05, ++P<0.01 decrease (at any dose) versus Vehicle / LPS, # P<0.05 increase versus Vehicle / LPS, $ P<0.05 decrease versus Vehicle / Vehicle (Fishers LSD test).

***In vivo* chemokines in the cortex: effect of α_2-AR antagonism**

IDA and RX elicited qualitatively similar effects on cortical chemokines, both drugs decreased mRNA expression of RANTES, IP-10 and CINC-1 induced by LPS, but were without effect of MIP-1α induction.

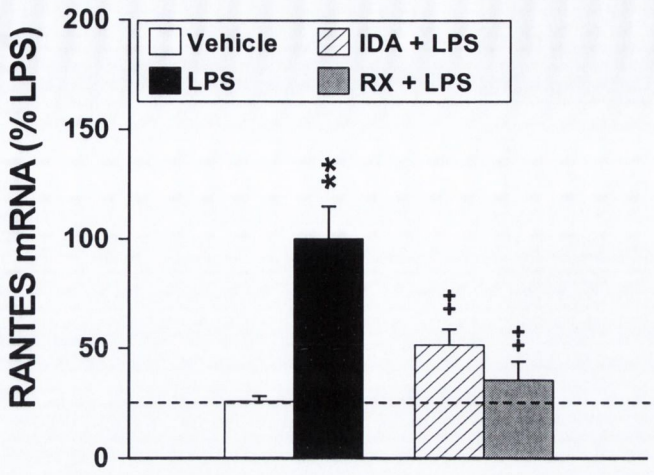
RANTES mRNA expression in rat cortex: LPS (250μg/kg) induced a 4-fold increase in RANTES gene expression relative to control ($P<0.01$). IDA and RX significantly attenuated this increase ($P<0.01$, figure 3.10.1a).

IP-10 mRNA expression in rat cortex: LPS (250μg/kg) induced a 70-fold increase in IP-10 gene expression relative to control ($P<0.01$). IDA and RX significantly attenuated this increase ($P<0.05$ and $P<0.01$, respectively), RX achieving a slightly greater attenuation (3.10.1b).

CINC-1 mRNA expression in rat cortex: LPS (250μg/kg) induced a 45-fold increase in CINC-1 gene expression relative to control ($P<0.01$). IDA and RX significantly attenuated LPS-induced CINC-1 mRNA expression ($P<0.01$) in rat cortex, 4 hours post-treatment (3.11.2a).

MIP-1α mRNA expression in rat cortex: LPS (250μg/kg) induced a 2.7-fold increase in MIP-1α gene expression relative to control ($P<0.01$). Neither IDA nor RX altered LPS-induced MIP-1α mRNA expression rat cortex, 4 hours post-treatment (3.10.2a).

(a)



(b)

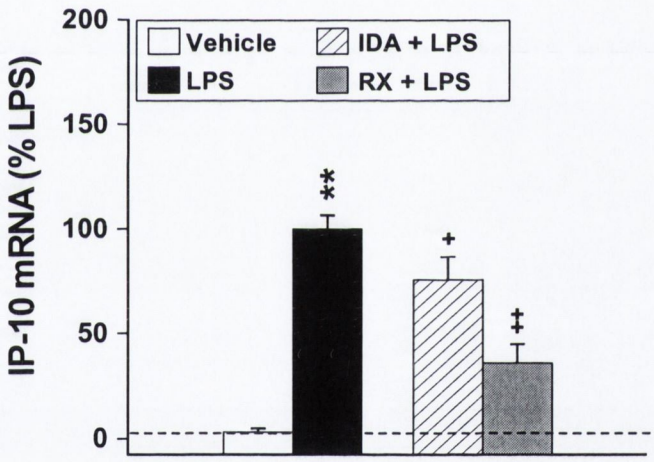
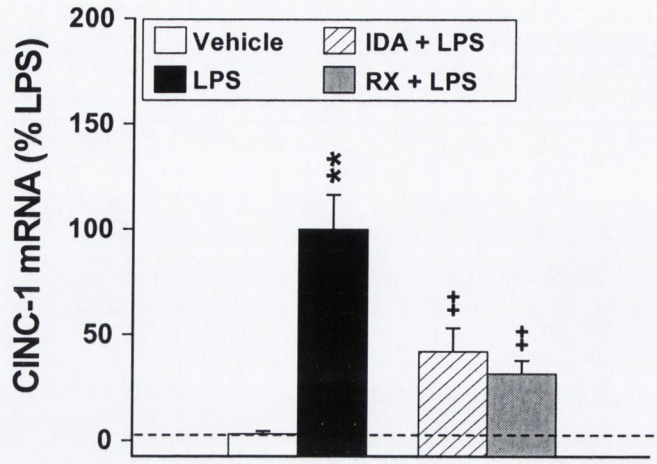


Figure 3.10.1: IDA and RX attenuate LPS-induced RANTES and IP-10 gene expression in rat cortex.

IDA, RX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) IDA and (b) RX significantly attenuated LPS-induced RANTES and IP-10 mRNA expression in rat cortex 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on RANTES [$F(3,20)=12.5$, $P<0.001$] and IP-10 [$(3,19)=33$, $P<0.0001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=5-6). **P<0.01 versus Vehicle/Vehicle; +P<0.05, ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc* test).

(a)



(b)

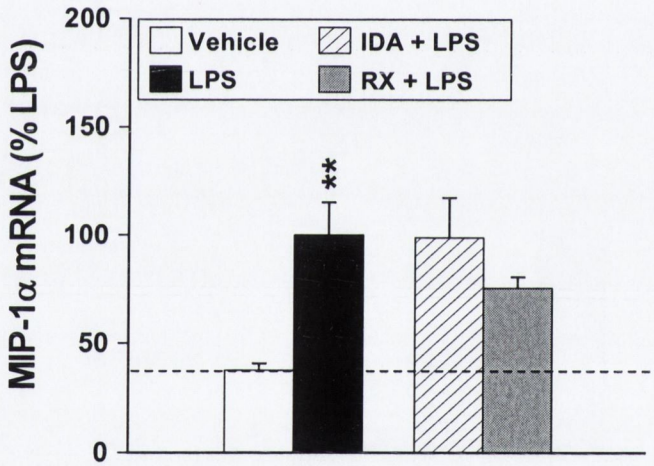


Figure 3.10.2: IDA and RX attenuate LPS-induced CINC-1, but do not alter MIP-1α gene expression in rat cortex.

IDA, RX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) IDA and RX significantly attenuated LPS-induced CINC-1 mRNA expression in rat cortex. (b) Neither IDA nor RX altered LPS-induced MIP-1α in rat cortex4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on CINC-1 [$F(3,18)=18.65$, $P<0.0001$] and MIP-1α [$F(3,20)=5.63$, $P<0.01$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc* test).

Acute treatment with α_2-AR antagonists do not alter chemokine gene expression in mixed cortical glia

To determine whether the anti-inflammatory actions of IDA on CD40 and RX on CD40 and IL-1β cortical gene expression resulted from increased NA availability or a direct effect of the drugs on glia, the effect of IDA and RX on glial cells was examined *in vitro*. IDA (0.1; 1; 10μM) or RX (1μM) were administered 30 minutes prior to LPS (1μgml) and cells incubated with these treatments for 6 hours. A vehicle control group was included to give an estimate of basal expression of inflammatory genes in mixed cortical glia. Six hours post-treatment cellular RNA was harvested and mRNA expression of the pro-inflammatory cytokine and the phenotypic marker of glial cell activation CD40 was examined using real-time PCR.

As demonstrated in table 3.11.2 below, direct administration of RX and IDA did not alter IL-1β or CD40 gene expression, demonstrating no direct action of these drugs on LPS-induced gene expression in mixed cortical glia.

Table 3.10.2. IDA and RX do not alter LPS-induced gene expression in mixed cortical glia

Target	Veh. + LPS	IDA (0.1μM) + LPS	IDA (1μM) + LPS	IDA (10μM) + LPS	RX (1mM) + LPS	ANOVA
RANTES	100 ± 24	98 ± 26	82 ± 15	90 ± 20	98 ± 23	F(4,25)=0.1, P=0.97
IP-10	100 ± 12	94 ± 16	89 ± 10	100 ±15	97 ± 12	F(4,25)=0.1, P=0.97
CINC-1	100 ± 8	111 ± 11	104 ± 8	107 ± 13	92 ± 8	F(4,25)=0.6, P=0.7

Data expressed as mean (% LPS control) with standard error of the mean (n=4-6). One-way analysis of variance revealed no significant effect of treatment on target gene expression.

In vivo chemokines in the hypothalamus: effect of α_2-AR blockade

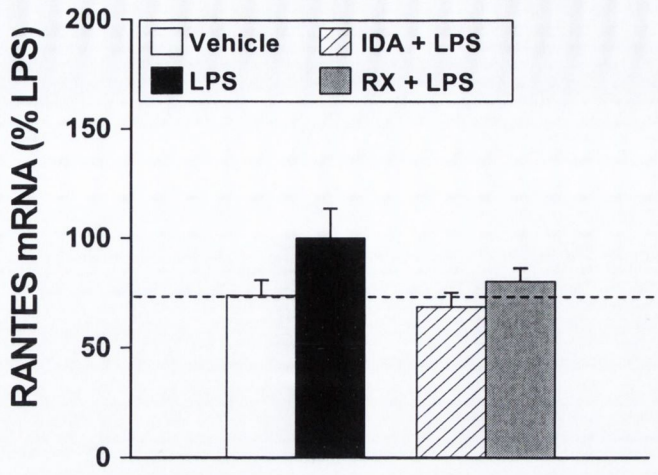
RANTES mRNA expression in rat hypothalamus: Neither LPS (250μg/kg), nor α_2-AR antagonist treatment significantly altered RANTES gene expression in rat spleen 4 hours post-treatment (figure 3.11.3a).

IP-10 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 196-fold increase in IP-10 gene expression relative to control ($P<0.01$). IDA (1mgkg) and RX (1mg/kg) significantly attenuated ($P<0.01$) LPS-induced IP-10 mRNA expression (Figure 3.10.3b).

CINC-1 mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 85-fold increase in CINC-1 gene expression relative to control ($P<0.01$). IDA ($P<0.01$) and RX ($P<005$) significantly attenuated LPS-induced CINC-1 mRNA expression (Figure 3.10.4a).

MIP-1α mRNA expression in rat hypothalamus: LPS (250μg/kg) induced a 17-fold increase in MIP-1α gene expression relative to control ($P<0.01$). IDA did not alter, while RX significantly augmented ($P<0.05$) LPS-induced MIP-1α expression (Figure 3.10.4b).

(a)



(b)

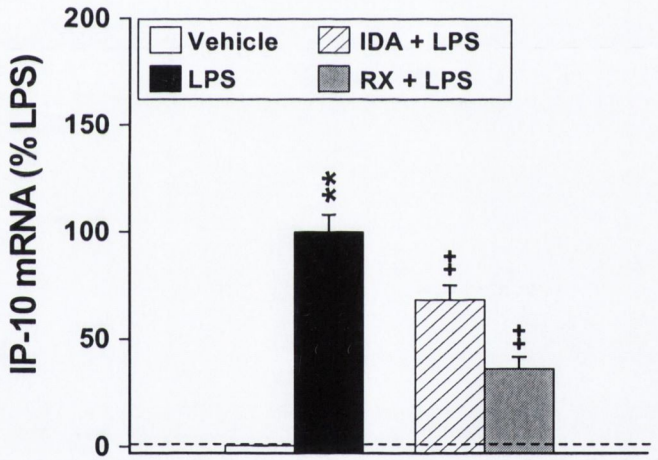
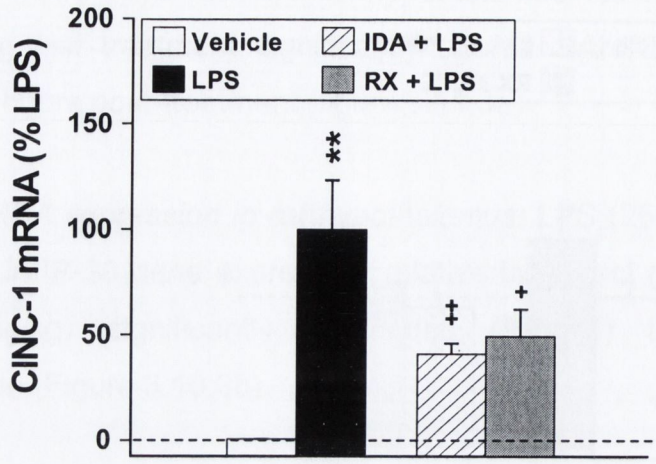


Figure 3.10.3. IDA and RX do not alter RANTES, but attenuate LPS-induced IP-10 gene expression in rat hypothalamus

IDA, RX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) LPS, IDA and RX did not alter RANTES mRNA expression. (b) IDA and RX significantly attenuated LPS-induced IP-10 in rat hypothalamus 4 hours post- treatment.

One way ANOVA revealed no significant effect of treatment on RANTES [$F(3,20)=2.43$, $P=0.095$] and a significant effect of treatment on IP-10 [$F(3,19)=53.62$, $P<0.0001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc* test).

(a)



(b)

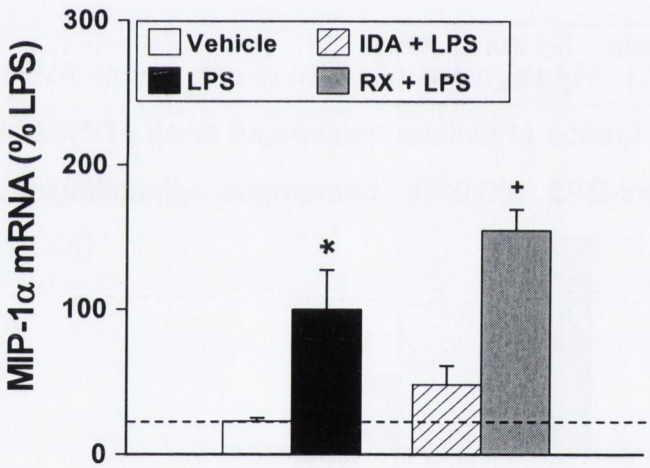


Figure 3.10.4. IDA and RX attenuate LPS-induced CINC-1and RX augments MIP-1α gene expression in rat hypothalamus

IDA, RX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) IDA and RX significantly attenuated LPS-induced CINC-1 mRNA expression in rat hypothalamus. (b) RX augmented LPS-induced MIP-1α in rat hypothalamus 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on CINC-1 [$F(3,19)=8.74$, $P<0.001$] and MIP-1α [$F(3,19)=11.86$, $P<0.001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc* test).

***In vivo* chemokines in the spleen: effect of α_2-AR blockade**

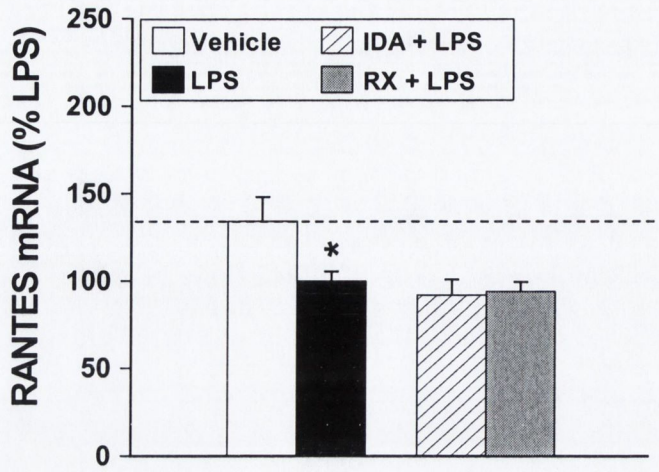
RANTES mRNA expression in rat spleen: LPS (250μg/kg) significantly attenuated while α_2-AR did not alter RANTES gene expression in rat spleen 4 hours post-treatment (Figure 3.10.5a).

IP-10 mRNA expression in rat spleen: LPS (250μg/kg) induced a 39-fold increase in IP-10 gene expression relative to control ($P<0.01$). Neither IDA nor RX significantly attenuated LPS-induced IP-10 mRNA expression in rat spleen 4 hours post-treatment (Figure 3.10.5b).

CINC-1 mRNA expression in rat spleen: LPS (250μg/kg) induced a 242-fold increase in CINC-1 gene expression relative to control ($P<0.01$). IDA ($P<0.05$) and RX ($P<0.01$) significantly attenuated LPS-induced CINC-1 mRNA expression (Figure 3.10.6a).

MIP-1α mRNA expression in rat spleen: LPS (250μg/kg) induced a 17-fold increase in MIP-1α gene expression relative to control ($P<0.01$). IDA did not alter, while RX ($P<0.01$) significantly attenuated LPS-induced MIP-1α expression (Figure 3.10.6b).

(a)



(b)

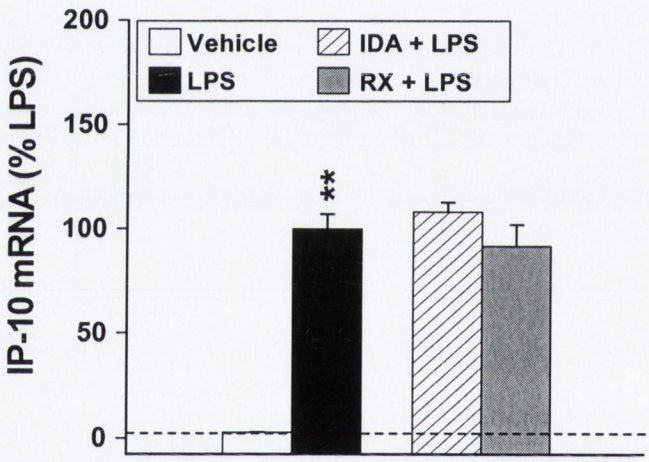
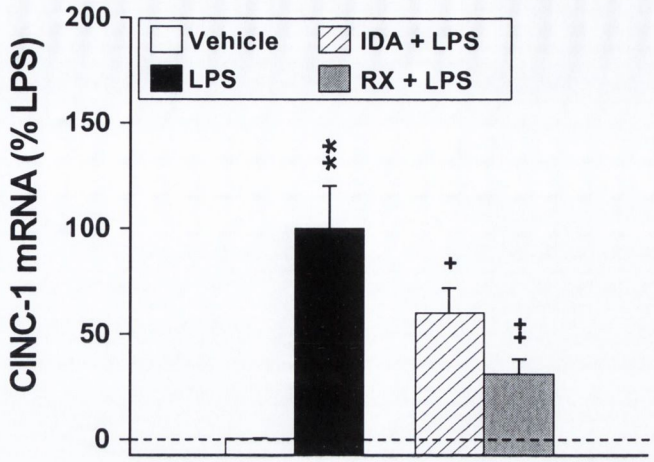


Figure 3.10.5: IDA and RX do not alter RANTES or LPS-induced IP-10 gene expression in rat spleen

IDA, RX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) LPS significantly attenuates basal RANTES, IDA and RX do not alter LPS induced suppression of RANTES mRNA expression in spleen. (b) Neither IDA nor RX altered LPS-induced IP-10 in rat spleen, 4 hours post- treatment.

One way ANOVA revealed a significant effect of treatment on RANTES [$F(3,20)=4.78$, $P<0.05$] and IP-10 [$F(3,19)=52.58$, $P<0.0001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc* test).

(a)



(b)

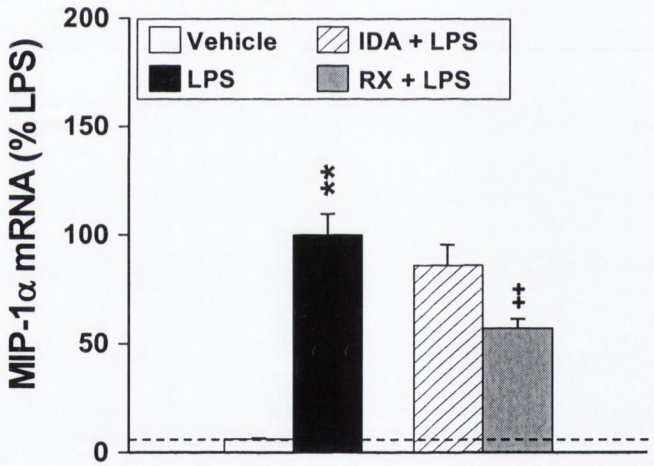


Figure 3.10.6. IDA and RX attenuate LPS-induced CINC-1, and RX attenuates MIP-1α gene expression in rat spleen.

IDA, RX or vehicle were administered to rats immediately prior to a challenge with LPS (250μg/kg). (a) IDA and RX significantly attenuated LPS-induced CINC-1 mRNA expression in rat spleen. (b) RX significantly attenuated LPS-induced MIP-1α mRNA expression in rat spleen 4 hours post-treatment.

One way ANOVA revealed a significant effect of treatment on CINC-1 [$F(3,18)=10.95$, $P<0.001$] and MIP-1α [$F(3,19)=3.51$, $P<0.05$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean (n=4-6). **P<0.01 versus Vehicle/Vehicle; ++P<0.01 versus Vehicle/LPS group (Fishers LSD *post hoc* test).

CHAPTER 4

Discussion

4. Discussion

4.1 NA exerts an anti-inflammatory action on glial cells

Evidence suggests that the monoamine neurotransmitter NA elicits anti-inflammatory actions in brain microglia and astrocytes (Mori *et al.*, 2002; Akama *et al.*, 2000; Feinstein *et al.*, 1993), and consequently plays an endogenous neuroprotective role in brain disorders where inflammatory events contribute to pathology. The first objective of the *in vitro* studies conducted here was to characterise the anti-inflammatory effects of NA in mixed glial cultures following stimulation with the inflammagen LPS, and to determine if increased production of the anti-inflammatory cytokines IL-10 or TGF-β play a role in the anti-inflammatory actions of NA.

Pro- and anti-inflammatory cytokines and iNOS: In line with previous reports, in this study it was demonstrated that NA significantly attenuated the ability of glial cells to produce the pro-inflammatory cytokines TNF-α and IL-1β, and also suppressed iNOS expression and NO production following stimulation with LPS (Mori *et al.*, 2002; Akama *et al.*, 2000; Feinstein *et al.*, 1993). IL-1β and TNF-α are both potent inducers of neuroinflammation and their expression in the CNS is implicated in the neuroinflammation that accompanies AD, PD, MS and cerebral ischaemia (Griffin, 2006; Hunot and Hirsche 2003; Carson 2002; Lucas *et al.*, 2006). Moreover, evidence from animal models also indicates distinct roles for these cytokines in neurodegenerative disorders. In this regard, IL-1β is implicated in AD, MS and MS-type pathology (Lucas *et al.*, 2006; Mulcahy *et al.*, 2003; see Griffin, 2006) while TNF-α is indicated as a major player in PD pathology (see Hunot and Hirsche, 2003). Indeed the roles of these cytokines in neuroinflammation are multifaceted, they are responsible for cytokine, chemokine, metalloprotease and cell adhesion molecule induction (Zhang *et al.*, 1998; Aggarwal, 2003). TNF-α can also cause neuronal apoptosis directly by interacting TNFR1, the death domain-containing receptor for TNF-α (Aggarwal, 2003).

NA treatment was also efficacious in suppressing iNOS expression and NO production following stimulation with LPS as observed elsewhere (Akama *et al.*, 2000; Feinstein *et al.*, 1993). iNOS is induced by a number of inflammatory insults and is also induced by both TNF-α and IL-1β (Akama *et al.*, 2000; Kozuka *et al.*,

2006). The release of large quantities of NO in the brain following iNOS induction by an inflammatory stimulus can exert a number of cytotoxic effects, resulting in altered protein functions (due to nitrosylation by NO), oxidative stress, cell damage and death (see Hunot and Hirsche, 2003). Indeed, both clinical and preclinical studies indicate an important role for NO in mediating neurotoxicity. In this regard iNOS deficient mice are partially protected against neurotoxity in an animal model of PD (Dehmer *et al.*, 2000) while clinical studies by Qureshi *et al*, (1995) and Iravani *et al.*, (2002) have observed increased nitrite concentrations in the CSF of PD patients and increased expression of iNOS by glia from PD brains.

TNF-α was the first cytokine to be induced by LPS in glial (6hrs), followed by a subsequent increase in IL-1β (12hrs), and the temporal induction of these cytokines concurs with that observed in the literature. Nitrite was not increased until 24 hours following LPS administration and this reflects the inducible nature of the enzyme iNOS that requires *de novo* protein synthesis (see Moncada *et al.*, 1991). Pre-treatment with NA (10μM) suppressed TNF-α and IL-1β release at the timepoints at which there were increased by LPS. Similarly NA attenuated both nitrite and iNOS protein expression twenty-four hours post-LPS administration.

In addition to producing pro-inflammatory cytokines, astrocytes, and to a lesser extent, microglia, produce anti-inflammatory cytokines such as IL-10 and TGFβ (Ledeboer *et al.*, 2002; Vincent *et al.*, 1997), and these agents inhibit pro-inflammatory cytokine production in the CNS (Bogdan *et al.*, 1992; Gerard *et al.*, 1993). Whilst studies have demonstrated that noradrenaline, β-AR agonists and cAMP can increase production of these anti-inflammatory cytokines in peripheral blood cells and macrophages (Agarwal and Marshall, 2000; Suberville *et al.*, 1996; Woo *et al.*, 2003) the ability of NA to alter IL-10 or TGF-β production from mixed glial cells has not been examined to date.

Consequently, in this study we examined the impact of noradrenaline on LPS-induced IL-10 and TGF-β production in primary mixed glial cultures. This investigation was conducted with a view to examining the role these anti-inflammatory cytokines may play in mediating the suppressive effect of noradrenaline on production of pro-inflammatory cytokines such as IL-1β and TNF-

α in the CNS. No effect of LPS on the anti-inflammatory cytokine TGF-β was observed in this study. Although significant increases in this cytokine have been observed in LPS-stimulated macrophages (Xiao *et al.*, 2006), our results are in agreement with those of Vincent and colleagues (1997) who reported no observable increase in glial TGFβ concentrations in response to LPS. Previous *in vivo* research has demonstrated an increase in TGF-β in the brain, however, this was observed after an intra-hippocampal injection of LPS (Cunningham *et al.*, 2002). Noradrenaline also failed to alter TGF-β production in the *in vitro* system presented here. This is in contrast to *in vivo* findings where the β_2-AR agonist clenbuterol was found to induce TGF-β production in an animal model of stroke (Zhu *et al.*, 2001). Based on the studies here, it can be concluded that TGF-β does not play a role in the anti-inflammatory actions of NA observed in mixed glial cultures.

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 the anti-inflammatory cytokine 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). Surprisingly, in the studies presented here, a suppression of IL-10 following NA pre-treatment was observed. This suppression of IL-10 was consistent across studies, and appeared to be regulated by NA at the level of transcription. Lowering the concentration of LPS used did not alter the suppressive effect of NA on IL-10, indicating that maximal induction by LPS was not preventing augmentation of this cytokine by NA. Suppression of this cytokine was also observed at lower NA concentrations, indicating that the attenuation of IL-10 by NA was unlikely to result from any toxic effect of NA metabolites on the cells (Khorchid *et al.*, 2002) and hence, IL-10 production.

As NA consistently attenuated LPS-induced IL-10 in mixed glia, an attempt was made to elucidate the mechanism by which this occurs. To this end TNF-α and/or IL-1β were neutralised to determine if the absence of these cytokines could mimic the suppressive effect of noradrenaline on LPS-induced IL-10 as a significant correlation was observed between the suppression of these pro-inflammatory

cytokines and suppression of IL-10 by NA. A dose-dependent induction of IL-10 protein and mRNA expression by TNF-α has previously been observed in cultured human foetal microglia (Sheng *et al.*, 1995), while TNF-α has also been found to induce IL-10 mRNA expression in human monocytes (Wanidworanun and Strober, 1993). Foey and colleagues (1998) also surmised that IL-10 synthesis in human monocytes was dependant upon either IL-1β and/or TNF-α production, while an independent role for IL-1β in IL-10 induction *in vivo* was postulated by Souza *et al.*, (2003), who found IL-1β concentrations to be positively correlated with both serum and intestinal IL-10 concentration following reperfusion injury in the rat gut.

Although neutralisation of TNF-α and IL-1β resulted in greater than 90% reduction of these cytokines, no effect on IL-10 concentrations were observed. Thus, while these cytokines may indeed be relevant to IL-10 induction in certain circumstances, the studies presented here demonstrate an independent role for LPS in IL-10 induction in glial cells.

IL-10 has been demonstrated to autoregulate its own production in rat glia and also human monocytes (Ledeboer *et al.*, 2002; de Waal Malefyt *et al.*, 1999). Based on these findings it is tempting to postulate that NA may increase IL-10 concentrations prior to the 6 hour time-point measured here, resulting in a later negative auto-regulation, however, examination of the literature shows that no other study has demonstrated LPS-stimulated induction of IL-10 prior to 6 hours in either peripheral or brain immune cells. In fact, IL-10 induction by LPS has not been demonstrated until 6 hours post-treatment in rat glial (Ledeboer *et al.*, 2002) and human microglial (Sheng *et al.*, 1995) and monocyte studies (de Waal Malefyt *et al.*, 1991).

β_2-ARs have been previously shown to mediate many of the immunological effects of NA (Verhoeckx *et al.*, 2005; Borger *et al.*, 1998). To determine whether β-AR activation was involved in the NA-induced suppression of IL-10, the effect of the β-AR blocker propranolol and the β_2 agonist salbutamol on LPS-induced cytokine production was examined in mixed glial cultures. It was observed that β-AR blockade by propranolol attenuated, while the β_2-AR agonist salbutamol mimicked the suppressive effects of NA on LPS-induced IL-10. As β_2-ARs are positively

coupled to adenlylate cyclase, activation of which results in cAMP production and activation of protein kinase A and cyclic-AMP response element binding protein (CREB). While a suppressive effect of intracellular cAMP on pro-inflammatory cytokines has previously been observed in microglia and astrocytes, (Nakamura *et al.*, 1999; Mori *et al.*, 2002) to date, the impact of cAMP increasing agents such as forskolin or di-butyryl cAMP on IL-10 production have not previously been examined in mixed primary glial cells. Shames *et al.*, (2001) documented that elevation of cAMP by either forskolin or cAMP increased IL-10 and suppressed TNF-α following LPS treatment in human monocytes. Similarly, Woo *et al.*, (2003) demonstrated increases in IL-10 following di-butyryl cAMP administration in an LPS-treated murine BV2 microglial cell line 24 hours post-treatment. Later studies by this group demonstrated an increase in both protein and mRNA following either forskolin or dbcAMP in this BV2 cell line (Woo *et al.*, 2004). Not all studies have consistently demonstrated increased IL-10 concentrations following β-AR agonists or cAMP-enhancing agents, however.

To this end, research on peripheral mucosal tissue (lungs and small intestine) has shown that activation of β_2–adrenoceptors by the specific agonist, salbutamol, decreases LPS-induced IL-10 production (Eijkelkamp, *et al.*, 2004). Using Kupffer cells, Dahle *et al.*, (2005) demonstrated a decrease in LPS-induced IL-10 mRNA expression and protein production following forskolin treatment, while observing a concurrent decrease in TNF-α protein and mRNA expression. The authors also demonstrated no change in IL-10 following treatment with the cell-permeable cAMP analogue 8-CPT-cAMP, and a non-significant increase following treatment with prostaglandin E_2, another agent demonstrated to increase intracellular levels of cAMP (Dahle *et al.*, 2005).

The question remains then, as to how β–adrenoceptor and cAMP enhancing agents increase LPS-induced IL-10 production in some cells, but decrease LPS-induced IL-10 production in mixed glia. EijKelkamp and colleagues postulated that tissue specific alterations in IL-10 production results from a complexity of intracellular signaling. IL-10 production is dependent on the regulation of several transcription factors such as Ap-1, SP-1 and NFκB (Kim *et al.*, 1992, Tone *et al.*, 2000), the activity of which can be down regulated by cAMP inducing agents, including β–adrenoceptor agonists (Farmer and Pugin 2000; Tone *et al.*, 2000).

Thus, it is possible that the level of IL-10 production is determined by the interplay of multiple transcription factors whose relative contribution may be tissue-specific.

Overall, it is clear from data collected here that noradrenaline attenuates LPS-induced IL-10 protein and mRNA expression in mixed glial cultures, and this occurs independently of TNF-α and IL-1β concentrations. The suppressive action exerted on IL-10 by NA results from activation of glial β_2-ARs and appears to be mediated by increased intracellular cAMP concentrations. As there are a number of both clinically-approved and illicit drugs that manipulate the central noradrenergic system, the suppression of glial IL-10 by NA could have important clinical consequences and therefore warrants further investigation.

Inflammatory chemokines: There is overwhelming evidence that chemokines can contribute to neuroinflammation by trafficking immune cells into the brain parenchyma (see Karpus, 2001; Ambrosini and Aloisi, 2004 for review). Despite the plethora of reports demonstrating a role for NA in peripheral inflammation regulation, no studies appear have investigated the potential of NA to regulate chemokines in the brain. Thus, the second objective of the *in vitro* studies conducted here was to determine if NA altered expression of a number of chemokines commonly implicated in CNS disease, following stimulation with the inflammagen LPS.

Here we observed that NA suppressed gene expression of a number of chemokines induced by LPS, namely the T-cell/monocyte chemoattractants RANTES, IP-10 and MIP-1α. Both RANTES and IP-10 are strongly implicated in MS, and in EAE; which is the most commonly used animal model of MS (Fife *et al.*, 2001; Sorenson *et al.*, 2002; Boven *et al.*, 2000). The role of chemokines in MS and EAE is not surprising given the requirement for T-cell recruitment into the CNS for pathology to occur. MIP-1α is also proposed to play a role in MS and neuroinflammatory processes accompanying cerebral ischaemia (see Ambrosini and Aloisi, 2003; Kim *et al.*, 1995; Takami *et al.*, 1997).

Although no investigations into the possible regulation of glial chemokines by NA have been undertaken, some evidence points toward a role for NA in chemokine

regulation in peripheral cells. Many of the studies reported in the literature focus on the potential of β–AR agonists to suppress MIP-1α and RANTES release, particularly in the context of asthma. Both these chemokines are important in this disease for their eosinophil-chemoattractant properties. In this regard, Miyabayashi and colleagues (2006) demonstrated a β-AR mediated suppression of RANTES from lung epithelium following a pro-inflammatory cytokine stimulus. Similar results were obtained by Hallsworth *et al.*, (2001) using airway smooth muscle cells. Suppression of both protein and expression of MIP-1α has been demonstrated by β-AR agonists and adrenaline in monocytes and macrophages (Hasko *et al.*, 1998; Li *et al.*, 2003).

The suppression of these chemokines by NA may result from either a direct effect of NA-induced intracellular signalling, or indirectly, by the reduction of pro-inflammatory cytokines. Hallsworth and colleagues (2001) showed that the suppression of RANTES from airway smooth muscle resulted from β-AR activation and was mimicked by cAMP-enhancing agents. It is also possible that a suppression of IL-1β and TNF-α may have prevented the induction of these chemokines, as previous studies demonstrate that IL-1β and TNF-α can induce MIP-1α, RANTES and IL-8 (CINC homologue) in human airway, foetal glial cells and intact rat brain (Hallsworth *et al.*, 2001; Hua and Lee, 2000; Anthony *et al.*, 1998). It is noteworthy, however, that only modest increases in the IFNβ-inducible chemokines RANTES and IP-10 were observed following TNF-α and / IL-1β administration to cultured glia (Hua and Lee, 2000). Given the dependence of RANTES and IP-10 on LPS and IFN-β signalling (see Beutler *et al.*, 2003; Hoebe and Beutler, 2004; Hoebe *et al.*, 2003) it is unlikely that a suppression of either TNF-α or IL-1β by NA was responsible for the profound suppression of these cytokines observed here. As microglial MIP-1α is induced by IL-1β and TNF-α *in vitro*, it is more likely that the suppression of MIP-1α induced by NA resulted from a suppression of these cytokines, or could also have resulted from increased cAMP signalling as previously reported by Hallsworth *et al.*, (2001) in airway smooth muscle cells. Given the demonstrated role of these chemokines in CNS pathology their potent suppression by NA could prove of future therapeutic benefit. However further studies are required to elucidate the precise mechanisms by which NA alters chemokine production in glial cells.

In contrast to the suppressive action of NA on RANTES, IP-10 and MIP-1α, NA failed to alter expression of the neutrophil chemoattractant CINC-1. This chemokine is induced by IL-1β and TNF-α in cultured human glia (Hua and Lee, 2000) while Anthony *et al.,* (1998) demonstrated that intracerebral administration of IL-1β potently induced CINC-1 and neutrophil accumulation in intact rat brain. While NA suppressed IL-1β and TNF-α, CINC-1 remained unaltered indicating that other mediators can obviously compensate to maintain CINC-1 in the absence of IL-1β. The time course of IL-1β production also suggests that this cytokine is not a critical mediator of CINC induction in mixed glial cells, in that CINC-1 mRNA is induced prior to the induction of IL-1β protein. In concordance with the inability of NA to alter CINC-1 expression observed here, it as been demonstrated that neither β-AR agonists nor cAMP-enhancing agents altered IL-8 production induced by IL-1β and TNF-α (Hallsworth *et al.*, 2001) in airway smooth muscle cells.

The precise intracellular mechanisms by which NA elicits an anti-inflammatory action on primary glia are unknown at present, however, a number of reports point toward a role for NA in the suppression of NFκB in certain cell types (Farmer and Pugin, 2000; Parry and Mackman, 1997). It is also possible that NA exerts its anti-inflammatory actions by the inhibition of one of the MAPK pathways, as suggested by Miyabayashi *et al.*, (2006). Indeed the studies presented here may yield some insights into the possible manipulation of LPS signalling by NA as both the IRF3/IFNβ-inducible chemokines (IP-10 and RANTES) were profoundly suppressed by NA treatment *in vitro*. However, future studies on the three main LPS signalling pathways, NFκB, MAPKs and IRF3 are necessary to determine possible targets NA in the suppression of both chemokine and cytokine production.

Despite the ability of NA to reduce production of the anti-inflammatory cytokine IL-10 from primary glial cells, the overall effect of NA was anti-inflammatory in nature, characterised by reduced production of pro-inflammatory cytokines, nitric oxide and inflammatory chemokines. Furthermore, it has been recently demonstrated that NA prevents micoglial activation, and consequential neurotoxicity in a microglial/neuron co-culture model (Madrigal *et al.*, 2005).

4.2. Inhibition of astrocyte neuronal or non-neuronal transporters does not exert anti-inflammatory actions *in vitro*

Given the potent anti-inflammatory actions of NA observed in glial cultures the third objective of the *in vitro* studies was to investigate the potential of NA-enhancing agents to augment these actions *in vitro*. A functional NET has been demonstrated on rat astrocytes (Inazu *et al.*, 2003b; Takeda *et al.*, 2002) suggesting that glia may be involved in termination of the noradrenergic signal *in vivo*. Initially, the ability of the NRIs DMI and ATX were assessed to determine if they had any direct actions on LPS-induced cytokine production or gene expression and chemokine gene expression in mixed glial cultures. Subsequently a dose of NRI that was without effect, or exerted minimal effect on pro-inflammatory cytokine production was administered with NA to determine if inhibition of the astrocytic NET could augment the anti-inflammatory actions of NA *in vitro*.

Direct administration of DMI augmented LPS-induced TNF-α but did not alter IL-1β production, or gene expression of these cytokines, nor did this compound alter LPS-induced chemokine mRNA expression *in vitro*. However it is noteworthy that, Obuchowicz *et al.*, (2006) demonstrated inhibition of LPS-induced IL-1β and TNF-α release in mixed glia with the related antidepressant compound amitriptyline and its metabolite nortriptyline, in the absence of exogenously administered NA. Obuchowicz and colleagues (2006) speculated that the inhibition of cytokine release observed in this study could result from non-transporter specific effects such as inhibition of adrenoceptors or direct effects of sodium or calcium influx as TCAs can inhibit these channels with varying degrees of affinity (Deffois *et al.*, 1996). However as DMI is also known to share the non-noradrenergic transporter-specific effects of amitriptyline it is not clear why the effects of this compound on glial pro-inflammatory cytokine production was at variance from those reported by Obuchowicz *et al.*, (2006). These authors incubated their mixed glial cultures for only 6 hours before determining TNF-α concentrations. Thus, it is possible that the longer DMI incubation period used in the present study (24hr) resulted in cell stress arising from non-noradrenergic effects of this compound that resulted in MAPK induction of TNF-α. In support of this theory, gene expression of both TNF-α and IL-1β was unaffected 6 hours following treatment with DMI.

As neither DMI nor ATX (10μM) exerted an anti-inflammatory action on cytokine production directly, the ability of a 10μM concentration of both drugs to augment the anti-inflammatory actions of NA on LPS-induced pro-inflammatory cytokine production was then examined. Whilst DMI has previously been demonstrated to inhibit TNF-α and IL-1β production *in vivo* (Connor *et al.*, 2000; Shen *et al.*, 1999), it failed to augment the suppressive action of NA on either TNF-α or IL-1β, and actually slightly attenuated the effect of NA on TNF-α. This concurs with a recent *in vitro* study by Diamond *et al.*, (2005) where DMI was observed to have no effect on LPS-stimulated pro-inflammatory cytokine production in human blood.

Although ATX exhibits greater selectivity for the NET, this drug was also ineffective in augmenting the suppressive effects of NA on LPS-induced TNF-α or IL-1β. Interestingly, although ATX (5μM) significantly attenuated LPS-induced IL-1β in the absence of NA, a higher dose of 10μM exerted an opposite effect, causing an augmentation of LPS-induced IL-1β. This was not observed in the presence of higher NA concentrations, however. There are no reports on the potential anti-inflammatory profile of ATX to date, however, reboxetine, a highly selective NRI was shown not to alter LPS-stimulated pro-inflammatory cytokine production in human blood (Diamond *et al.*, 2005). In fact the results reported by Diamond *et al.* (2005) suggest that a number of antidepressant compounds including the NRIs DMI and reboxetine have little effect on LPS-induced IL-1β and TNF-α production, and that the major anti-inflammatory action of these compounds results from their ability to suppress IFN-γ production from T-cells.

Neither DMI nor ATX demonstrated a direct effect on LPS-induced gene expression of the enzymes iNOS or COX-2, the anti-inflammatory cytokine IL-10, nor the chemokines RANTES, IP-10, CINC-1 and MIP-1α in mixed glial cultures. This indicates that the anti-inflammatory actions of NRIs observed *in vivo* (Shen *et al.*, 1999; Connor *et al.*, 2000; Section 3.3, this thesis) most likely result from increased NA availability at inflammatory cells, such as microglia/macrogphages, rather than a direct non-noradrenergic action on these cells.

Functional expression of the non-neuronal transporter, uptake$_2$ has been demonstrated on both human and rat astrocyte cells (Inazu *et al.*, 2003a; Russ *et*

al., 1996; Inazu *et al.*, 1999; Takeda *et al.*, 2002). Although this transporter demonstrates a low affinity for NA, it is a high capacity, sodium-independent transporter that is likely to play an important role in NA removal *in vitro,* because unlike the NET it should not become saturated in the presence of high NA concentrations (Grundemann *et al.*, 1998). Consequently the ability of the non-neuronal transporter inhibitor NME to augment the suppressive effect of NA on LPS-induced TNF-α and IL-1β production was also assessed in this study. However the results clearly demonstrate that inhibition of this transporter did not alter LPS-induced pro-inflammatory cytokine production in mixed glial cultures, nor did it augment the anti-inflammatory actions of NA on these cytokines.

In all, the results presented here demonstate that the NRIs DMI and ATX and the uptake$_2$ inhibitor NME largely fail to alter inflammatory processes in primary glial cells induced by LPS, and also fail to augment the anti-inflammatory actions of NA in glial cells. These data argue against any significant functional role of glial NET and uptake $_2$ transporters in noradrenaline uptake.

4.3 Ability of pharmacological strategies that enhance NA availability *in vivo* to elicit an anti-inflammatory action in the CNS

Although cell culture studies are a useful means to determine the basic response of one or two cell types they cannot provide the complexity of signalling and cell interactions of any tissue. Thus, it was decided to carry out *in vivo* studies to assess the anti-inflammatory potential of the NRIs DMI and ATX. Administration of NRIs *in vivo* results in inhibition of neuronal NET, the principle transporter responsible for regulating NA concentrations in the intact CNS. Pharmacological inhibition of the NET can result in a 2-4 fold increase in extracellular NA concentrations, as assessed using microdialysis techniques, depending on the inhibitor, dose administered, and brain region under investigation (see Invernizzi and Garattini, 2004). Normetanephrine was not used in these studies as it affects only non-neuronal NA transport present on CNS glial cells, and was observed to be ineffective in altering pro-inflammatory cytokines in glial cultures. In addition, peripheral inhibition of organic cation transport would most likely result in numerous side-effects unrelated to manipulation of NA availability and therefore do not represent a viable therapeutic target for inhibiting inflammatory processes in the CNS. The impact of the α_2-AR antagonists IDA and RX on inflammatory measures in the brain *in vivo* was assessed, as these compounds increase NA concentrations by a different mechanism to NRIs, and finally the ability of α_2-AR blockade to potentiate the anti-inflammatory effect of the NRI, ATX was examined. This augmentation approach was taken as it has been previously demonstrated that administration of an α_2-AR blocker in conjunction with a NRI results in a greater increase in NA extracellular concentrations than either compound administered alone (Wortley *et al.*, 1999; Swanson *et al.*, 2006; see Invernizzi and Garattini, 2004).

Induction of inflammatory gene expression in brain and spleen by systemic LPS administration: It is well documented that systemic administration of an inflammatory stimulus can induce cytokine production in the CNS. The inflammatory response of the cortex was examined as this region is involved in higher cognitive processing that is adversely affected in a number of neurodegenerative diseases. This region also receives rich noradrenergic innervation from the LC (Siegel *et al.*, 1999). Inflammatory measures in the

hypothalamus were also examined as this region is considered to be at the interface between brain and immune systems. This region also receives noradrenergic innervation, predominantly from the ventral noradrenergic bundle, a more diffuse noradrenergic pathway than the dorsal bundle innervating the cortex (Siegel *et al.*, 1999).

Inflammation in spleen tissue was also assessed, as the spleen is an immune organ richly innervated by the sympathetic nervous system, and immunological function in this organ is under sympathetic control (see Straub, 2004). The inflammatory response of this organ was assessed to determine if the anti-inflammatory effects of NA-enhancing agents were brain-specific. Similar effects of treatment in both brain and spleen may indicate that an action on glial cells is not the primary means by which enhanced noradrenergic tone exerts anti-inflammatory actions, and that reduced inflammation in the CNS may occur secondary to a reduction in inflammation in the periphery.

The primary caveat in examining inflammatory markers in non-perfused brains is the inability to differential between the products of circulating macrophages and monocytes etc., and those of the brain parenchyma. By examining a secondary immune organ such as the spleen, that is also subject to modulation by the NA-enhancing agents used here (Elenkov and Vizi, 1995; Vizi *et al.*, 1991; see Elenkov *et al.*, 2000 for review), it is possible to differentiate to some degree the peripheral and central effects of both LPS and NRI / α_2-AR antagonist treatment.

The magnitude of inflammatory-product induction by LPS was observed to be considerably less in the brain than in the spleen, underscoring the lack of inflammatory reactivity of CNS tissue. One notable exception was the greater induction of the chemokines IP-10 and RANTES (monocyte and T-cell attractants) in brain than in spleen. Peripheral administration of LPS elicited a greater inflammatory reaction in the hypothalamus than cortex, and this result corresponds with the physiological function of the hypothalamus. Specifically, the hypothalamus is considered to be the brain-immune system interface and the presence of circumventricular organs (CVOs) (median eminence and vascular organ of lamina terminalis [OVLT]) that lack a functional BBB (Fitzgerald and

Folan–Curran, 2002) allows the hypothalamus to act as a sensor and initiate appropriate response to inflammatory and indeed other peripheral stimuli.

In this regard the magnitude of induction of all inflammatory measures was greater in the hypothalamus than in the cortex, with the exception of TNF-α and CD40 whose induction by LPS was relatively similar in cortex, hypothalamus and spleen. In fact, the magnitude of inflammatory gene induction in the hypothalamus, with the notable exception of the chemokines, more closely resembled that of the spleen than the cortex. Akin to the results presented here, a greater induction of IL-1β expression has been observed in hypothalamus relative to the hippocampus following peripheral administration of IL-1β (Hansen *et al.*, 1998). Vagotomy studies have demonstrated only partial blockade of IL-1β expression in hypothalamus, indicating that this region can respond to inflammatory signals in the circulation, and is perhaps more susceptible to peripheral influence than other brain areas. Greater inflammatory gene induction in the hypothalamus is likely to result from direct cytokine entry in the hypothalamus at the CVOs. In this regard Konsman *et al.*, (1999) demonstrated IL-1β immunoreactivity in the CVOs, including the median eminance and OVLT, that was not discernible in other brain areas 4 hours following peripheral administration of LPS. These authors also demonstrated a similar pattern of iNOS induction (using *in situ* hybridization) as observed for IL-1β. Furthermore, TNF-α mRNA has been observed in the CVO's and nuclei of the hypothalamus and solitary tract (Breder *et al.*, 1994).

Clear dissimilarities in chemokine induction between the brain and spleen were also evident. Induction of RANTES and IP-10, the chemokines responsible for monocyte and T-cell trafficking, was greatest in the brain, whereas induction of the neutrophil and macrophage chemoattractants CINC-1 and MIP-1α was greatest in the spleen. It is noteworthy that RANTES was not significantly induced in the hypothalamus, and was actually decreased in the spleen following LPS treatment, while IP-10 induction was more than two-fold greater in hypothalamus than cortex These results perhaps point toward a role for RANTES and IP-10 in CNS inflammation which is distinct from their functions in the periphery. As both these chemokines are strongly implicated in the pathogensis of MS and its animal counterpart, EAE (Balashov *et al.*, 1999; Boven *et al.*, 2000) further investigation

into the induction profile of these chemokines in other brain regions is clearly warranted. Indeed the induction of these T-cell chemoattractants concomitant with CD40; a co-stimulatroy molecule that assists in successful antigen presentation (Mackey *et al.,* 1998), could have grave implications for systemic infections in patients suffering from MS.

Anti-inflammatory action of NRIs: The NRIs DMI and ATX were chosen to determine whether an acute increase in NA availability would exert anti-inflammatory actions in the brain following a systemic challenge. The potential of NRIs to suppress inflammatory measures in the brain has not previously been evaluated, thus a range of DMI and ATX doses were evaluated in this study. Previous investigations have ascertained that chronic and sub-chronic treatment with DMI (7.5mg/kg) can suppress circulating concentrations of the pro-inflammatory cytokines TNF-α and IL-1β, and increase circulating concentrations of the anti-inflammatory cytokine IL-10 following a systemic challenge with LPS (Shen *et al.*, 1999; Connor *et al.*, 2000). *In vivo* administration of a similar dose of DMI has been demonstrated to elicit a 2-3.5-fold increase in extracellular NA (see Invernizzi and Garattini, 2004 for review). Systemic injection of ATX (3mg/kg) has also been shown to elicit a 2.5-3 fold increase in frontal and prefrontal cortex (Swanson *et al.*, 2006; Bymaster *et al.*, 2002). Thus the doses chosen for this study were based around doses of DMI and ATX that have been previously shown to increase extracellular NA in the CNS.

Administration of DMI and ATX elicited qualitatively similar effects in the cortex, both drugs suppressed gene expression of TNF-α and IL-1β, CD40 and the enzymes iNOS and COX-2 in a dose-dependent manner. ATX showed a tendency to increase IL-10 gene expression, while DMI significantly increased IL-10 gene expression and decreased that of COX-2. The suppression of COX-2 by DMI in the brain was not of great magnitude relative to the potent suppression of iNOS, CD40 and pro-inflammatory cytokines observed with this compound. Administration of DMI has previously been shown to increase serum IL-10 after LPS challenge (Shen *et al.*, 1999), however, no studies have examined the potential of this or related compounds to increase central IL-10 concentrations. In this regard the present study is the first to demonstrate an increase in cortical IL-

10 gene expression following NRI treatment, this is an interesting observation considering our earlier finding that NA suppresses LPS-induced IL-10 production in glial cells.

The magnitude of inflammatory product suppression by NRI's was observed to be less in the hypothalamus than the cortex, with the exception of the chemokines. It is possible that the greater inflammatory reactivity of the hypothalamus relative to cortex may have rendered it less susceptible to noradrenergic suppression. In this regard innervation of the hypothalamus from the lateral tegmental nuclei is of a more diffuse nature than the LC innervation of the cortex, and this may impact negatively on potential enhancement of NA concentrations following NRI treatment.

Differential effects of NRI treatment in the cortex and hypothalamus may also result from different β_2-AR localisation in these areas, as it is via this receptor that NA is purported to exert anti-inflammatory effects on glia (Nakamura *et al.*, 1998; Mori *et al.*, 2002), however there is no published evidence to support such differential β_2-AR expression on glia in different brain regions. It is also possible that the discrepancy between NRI effects in hypothalamus and cortex may arise from NET localization. In this regard, Geranton *et al.*, (2003) demonstrated a difference in NET uptake between these areas, where uptake of NA was greater in frontal cortex than hypothalamus. Moreover, expression of this transporter is less in brainstem nuclei innervating the hypothalamus than in the cortex (Schroeter *et al.*, 2000; Jin *et al.*, 2004). The neurotoxin DSP-4 selectively destroys neurons originating from the LC but leaves those arising from the ventral bundle relatively intact. In this regard the 'sparing' effect of DSP4 treatment on this area is believed to result from differences in NET expression, as DSP-4 is believed to enter the nerve terminal via this transporter to cause its neurotoxic effect (Fritschy and Grzanna, 1991). Thus, it is possible that NRIs cannot elicit NA efflux in the hypothalamus to the same extent as cortex due to NET localisation in projections to this area, and this may explain the greater anti-inflammatory actions of NRI treatment in the cortex than hypothalamus.

Desipramine exerted similar actions in the cortex and spleen while ATX exerted a more potent anti-inflammatory action in the cortex than peripherally. In this regard, certain inflammatory measures appeared to be more susceptible to NRI treatment in different regions. iNOS suppression in the spleen was less than that observed in the brain following NRI treatment, however, this may relate to the greater induction of iNOS in the spleen. Induction of IL-10 in the spleen is also greater than that observed in the brain following systemic LPS treatment, in fact the greater IL-10 gene expression in this area may prevent further induction of IL-10 by NRI treatment. In contrast COX-2, whose magnitude is greater in the spleen than the brain following LPS treatment, is also more susceptible to suppression by both DMI and ATX in this organ. Interestingly, despite a similar induction of CD40 and IL-1β in brain and spleen, the magnitude of suppression by NRI treatment was greater in the brain than the spleen.

Overall, acute DMI treatment exerted a greater anti-inflammatory action than ATX in both brain and spleen following LPS challenge. This concurs with previous reports on the anti-inflammatory profile of this drug, however, given the similar fold-increases in extracellular NA concentrations reported following *in vivo* administration of these compounds, it is possible that some of DMIs actions are unrelated to its ability to inhibit the NET.

There are no reports to date on the potential of NA or NA-enhancing agents to elicit anti-inflammatory actions on glial-derived chemokines, either *in vivo* or *in vitro*. Interestingly the effect of DMI and ATX treatment on chemokine gene expression following LPS treatment were qualitatively and quantitatively similar. A greater suppression of the monocyte and T-cell chemoattractants IP-10 and RANTES, and the neutrophil attractant CINC-1 was observed in brain compared to spleen, with DMI and ATX eliciting a potent suppression of IP-10, RANTES and CINC-1 chemokines in the cortex. A similar effect was observed in the hypothalamus, with the exception of RANTES, which was not significantly increased in this region following LPS treatment. Given the importance of monocyte and T-cell invasion in the brain in disorders such as MS and AD, the potent suppression of these chemokines by NRIs could prove of future therapeutic importance.

Opposite effects of NRIs and NA on CINC-1 mRNA expression in the cortex and cortical glial cells was also evident in these studies. Chemokines have diverse roles in development (Rezaie *et al.*, 2002) and it is possible that their induction in primary glial cultures may reflect attendant developmental rather than inflammatory roles. In this regard, differences in BBB permeability and neutrophil recruitment to IL-1β has been shown to alter with age (Anthony *et al.*, 1997), and juvenile rats injected with either IL-1β or CINC demonstrated greater brain neutrophil recruitment and BBB permeability than adults (Anthony *et al.*, 1998). Induction of CINC-1 by LPS was greater in hypothalamic than cortical tissue. CINC is the rat homolog of IL-8, and it is noteworthy that IL-8 has been implicated in neuroprotection, and has been shown to induce both cell proliferation and myelin synthesis in an oligodendrocyte precursor cell line (Kadi *et al.*, 2006). As MIP-1α is also constitutively expressed in human foetal microglia, a potential role for this chemokine in brain development has been proposed (Rezaie *et al.*, 2002). Thus, the ability of NA to suppress MIP-1α but not CINC-1 in cultured neonatal cells *in vitro,* and the ability of NA-enhancing agents such as NRIs to attenuate CINC-1 but not MIP-1α in the adult brain *in vivo* may relate to the different roles these chemokines play in post-natal and adult brain.

While LPS-induced MIP-1α mRNA expression was significantly attenuated by NA treatment in cultured cortical glia, it was unaltered by either DMI or ATX in the cortex following systemic treatment with LPS, and was actually increased in the hypothalamus following DMI but not ATX treatment. It is possible that the augmentation of MIP-1α in the hypothalamus observed following treatment with DMI relates to the non-adrenergic effects of this drug as no augmentation was observed with the more selective NRI, ATX in this region. Previous studies by Hasko *et al.*, (1998) showed that systemic administration of the β-AR agonist isoproterenol, in addition to the α_2-AR antagonist CH-38083 attenuated LPS-induced MIP-1α serum concentrations in mice, indicating a role for endogenous catecholamines in chemokine regulation. In line with the findings of Hasko *et al.*, (1998) both DMI and ATX attenuated MIP-1α gene expression in the spleen, expression of which was far greater than in the brain following systemic LPS challenge. It is suggested that the differential induction and suppression of cytokines in spleen and brain may be due to their relative importance in these

areas or indeed their expression by certain cell types. In this regard both IP-10 and RANTES are predominantly expressed in astrocytes in MS, where MIP-1α is localised to microglia in this disease state (Boven *et al.*, 2000; Balashov *et al.*, 1999). Thus, induction of IP-10 and RANTES may occur more readily in the brain and also be subject to greater suppression by pharmacological agents such as NRIs than MIP-1α.

The suppression of cytokines, chemokines, iNOS and CD40 by NRIs highlights a unique strategy for pharmacological intervention in neuroinflammatory states. Indeed the contribution of IL-1β and TNF-α in initiating an inflammatory cascade in the brain, in addition to their potential for causing cell death via the activation of caspases and activation of death domain–containing receptors respectively make them primary targets for intervention. Similarly the ability to prevent T-cell and monocyte trafficking into the brain by suppression of IP-10 and RANTES could also provide neuroprotection from products released by such cells in the brain parenchyma. A concomitant decrease in iNOS and CD40 could prevent the production of cytotoxic products and also prevent antigen presentation in the brain.

Anti-inflammatory action of α_2-AR antagonists: The anti-inflammatory potential of the α_2-AR antagonists IDA and RX were also assessed in these studies as the mechanism by which these compounds increase extracellular NA concentrations differs from that of NRIs. IDA has been used in a number of studies to examine the effect of α_2-AR blockade on extracellular NA concentrations in the brain, and has proven efficacious in this regard (see Invernizzi and Garattini, 2004). As this compound also has some affinity for imidazoline receptors (that are involved in blood pressure control) the more selective α_2-AR blocker RX was also used. Previous investigations by Fessler and colleagues (1996) demonstrated a protective effect of α_2-AR blockade in rats treated with lethal and sublethal doses of LPS. To date, however, no investigations have evaluated the potential of α_2-AR blockade to inhibit inflammatory processes in the CNS.

Interestingly, treatment with α_2-ARs elicited qualitatively similar effects to NRIs on the chemokines IP-10 and CINC-1 in both the cortex and hypothalamus. An increase in MIP-1α expression, similar to that observed following DMI treatment

was seen in the hypothalamus following RX but not IDA treatment. The reason for this discrepancy between the action of the two drugs on hypothalamic MIP-1α expression is unknown at present. In general, the α_2-AR antagonists were much less efficacious at eliciting an anti-inflammatory effect in the CNS compared to the NRIs. This is likely to result from the fact that the α_2-AR antagonists at the dose employed in the present investigation (1mg/kg) were less efficacious than NRIs at increasing extracellular NA in the CNS, as assessed using microdialysis (Wortley *et al.*, 1999; Invernizzi and Garattini, 2004). Indeed Sacchetti *et al.*, (1999) did not observe any significant increase in NA efflux in frontal cortex following IDA (1mg/kg), but did observe a profound synergistic increase in NA efflux when this dose of IDA was combined with the selective NRI reboxetine. However it is interesting that the α_2-AR blockers were more efficacious at reducing inflammation in the hypothalamus than cortex, a situation opposite to what was observed in the case of the NRI's. In this regard, Heal *et al.*, (1993) proposed that α_2-AR localization was greater in hypothalamus than cortex, and a number of studies have demonstrated alterations in NA release between hypothalamus and cortex that are hypothesized to stem from differential localization of α_2 autoreceptors (Wortley *et al.*, 1999; Geranton *et al.*, 2003a).

There is overwhelming evidence that pro-inflammatory cytokines, chemokines and inflammatory mediators can contribute to inflammatory pathology in a number of disease states, including AD, PD, MS and cerebral ischaemia (see Liu and Hong, 2003; Town *et al.*, 2001; Chen *et al.*, 2006; Pannu and Singh 2006; Karpus, 2001). Thus, it is suggested that the ability of NRI's and α_2-AR's to inhibit IL-1β, TNF-α, iNOS, IP-10, RANTES, CINC-1 and CD40 expression in the CNS could be of therapeutic benefit in preventing/treating CNS disorders with an inflammatory component.

Do NRI's and α_2-AR antagonists synergise to elicit an enhanced anti-inflammatory response in the CNS?: ATX is a more selective NRI that unlike DMI, does not exhibit affinity for the SERT or neurotransmitter receptors. As this compound also exhibited less of an anti-inflammatory action *in vivo*, it was decided to combine this NRI with the α_2-AR antagonist IDA to determine if a synergistic anti-inflammatory effect would be observed. In this regard, synergism between NRIs and α_2-AR

blockers on extracellular NA concentrations has been demonstrated in rat cortex and hypothalamus, as assessed by microdialysis studies (Sacchetti *et al.*, 1999; Geranton *et al.*, 2003; Wortley *et al.*, 1999). Three inflammatory markers that were susceptible to modulation by NA-enhancing agents namely, TNF-α, iNOS and CD40 were chosen to examine the impact of NRI treatment in combination with α_2-AR blockade. No synergistic effect of treatment was observed on any of these measures in either cortex or hypothalamus, however a synergistic effect on TNF-α mRNA expression was observed in the spleen. As no synergism on brain inflammation was observed it is possible that the dose of IDA used was too high, and evoked too large a response to further augment by co-treatment with ATX. In this regard, 1mg/kg of IDA significantly attenuated all inflammatory measures studied in the cortex and hypothalamus, alone or in combination with ATX. In the previous studies IDA only attenuated LPS-induced TNF-α and CD40 in the brain, and in both studies exerted minimal effects on splenic tissue. Previous microdialysis studies by Sacchetti *et al.*, (1999) demonstrated no effect of IDA at this dose on NA concentrations in the frontal cortex, while Wortley *et al.*, (1999) only demonstrated a two-fold increase in extracellular NA concentrations following a 3 mg/kg dose of the more selective α_2-AR antagonist RX, in both frontal cortex and hypothalamus. To date no other studies have examined the effect of α_2-AR blockade and increased NA concentrations on inflammatory measures in the brain. It is possible however, that a lower dose of IDA in combination with ATX (5mg/kg) may well have potentiated the effects of ATX on inflammatory measures in the CNS. Comprehensive dose-response investigations into the potential anti-inflammatory actions of α_2-ARs are required in order to gain a better insight into the potential of augmentation strategies with these drugs.

Possible intracellular mechanisms by which NA and NA-enhancing agents exert anti-inflammatory actions: The signalling pathways affected by NA were not examined in these studies and further studies into the mechanism by which NA exerts its effects are clearly warranted. Binding of LPS to TLR4 activates three main pathways, NFkB, MAPKs and IRF3 (Kawai *et al.*, 2001; Moynagh; 2005; Jung *et al.*, 2005; Liew *et al.*, 2000). Thus, the relative sensitivity of genes induced by these signalling pathways to NA regulation may provide valuable clues into the mechanism whereby NA exerts anti-inflammatory actions.

In this regard, previous investigations in the ability of NA to exert anti-inflammatory actions proposed NFκB signalling as a target for NA in certain cell types (Farmer and Pugin, 2000; Neumann *et al.*, 1995). Parry and Mackman (1997) proposed that the inhibitory action of cAMP-elevating agents on NFκB signalling occur due to competition for the co-activator of transcription CREB-binding protein utilised by both PKA and NFκB to initiate gene transcription. There is also evidence to suggest that inhibition of one of the MAPK pathways is responsible for the action of NA and β-AR agonists on both chemokine and cytokine production. Specifically, previous studies by Miyabayashi and colleagues (2006) showed that isoproterenol inhibited RANTES release from lung epithelium partly by inhibition of MAPK JNK signalling.

The pattern of inhibition of the IFNβ-inducible chemokines IP-10, RANTES and CD40 signalling following LPS treatment perhaps provide the best clues into possible mechanisms whereby NA may alter inflammatory signalling. Thus, the consistent and potent inhibition of IP-10, RANTES and CD40, suggests that IRF3 and/or JAK/STAT signalling following IFNβ release may be more susceptible to intervention by NA and NA-enhancing agents (see Hoebe and Butler, 2004). Future studies should attempt to dissect the pathways whereby NA exerts its anti-inflammatory actions.

4.4 Concluding remarks

Overall *in vitro* exposure of glial cells to NA, and *in vivo* administration of NA-enhancing agents suppresses expression of the pro-inflammatory cytokines TNF-α and IL-1β, the T-cell/moncyte chemokines RANTES and IP-10, and the co-stimulatory molecule/glial activation marker CD40 induced by the experimental inflammagen LPS.

Considering the evidence that pro-inflammatory cytokines and NO contribute to pathology in a number neurodegenerative disorders such as AD, PD, MS (Lucas *et al.*, 20065; Mrak and Griffin, 2005; Hunat and Hirsch, 2003), it is suggested that the ability of NRI's and α_2-AR's to inhibit IL-1β, TNF-α and iNOS expression in the CNS could by of therapeutic benefit in preventing/treating CNS disorders with an inflammatory component.

In this regard both production of IP-10 and RANTES by glial cells in the CNS will facilitate monocyte and T-cell trafficking into the brain, while CD40 expression on glia cells will facilitate successful activation of infiltrating T-cells following antigen presentation. Induction of both these chemokines and CD40 by an inflammatory stimulus presents a unique opportunity whereby not only are T-cells directed to the brain, but when in the brain parenchyma can be successfully presented with antigen by activated microglia. The concurrent up-regulation of cytokines, cytotoxic molecules, vasodilatory factors and cell adhesion molecules can disrupt the integrity of the BBB allowing the entry of immune cells into the brain. Such an environment may not only precipitate neuronal damage but potentiate neuronal degeneration in AD, PD or MS (Mrak and Griffin, 2005; Perry *et al.*, 2003; Hunot and Hirsch, 2003). Indeed in terms of demyelinating diseases, such an environment may present a unique opportunity for self-antigen presentation within the CNS parenchyma.

The results of the present investigation clearly demonstrate an acute action of NA *in vitro*, and NA enhancing agents *in vivo* to inhibit inflammatory processes in glial cells and intact brain respectively. However, further research is required in order to determine if such anti-inflammatory actions are maintained following chronic *in vivo* treatment with NA-enhancing drugs. This is an important consideration as it

is well established that chronic treatment with NRI's downregulate β-AR expression in the CNS (Harkin *et al.,* 2000), although from the studies conducted to date it is not possible to say whether NRI's down regulate expression of the β_2-AR subtype present on glia, which is the receptor thought to mediate the anti-inflammatory actions of NA in the CNS. It is also noteworthy that chronic treatment with NRI's downregulate expression of autoinhibitory α_2-AR's (see Leonard, 1997), an effect that may be of relevance for the anti-inflammatory potential of these compounds following long-term administration.

Assuming that the anti-inflammatory actions of acute NRI treatment observed in the current investigation persists following long term or chronic treatment, it is suggested that NRIs could represent a viable strategy to combat the development of neuroinflammation. In this regard, long-term treatment with NRI's such as reboxetine and atomoxetine could represent a clinically feasible neuroprotective strategy, as existing clinical data demonstrates that these agents are well tolerated and safe when taken for prolonged periods (Zhou, 2004). In contrast, whilst DMI is a useful experimental tool that inhibits neuroinflammation, it is an unlikely candidate to therapeutic use considering its marked cardiotoxic effects in humans (see Leonard, 1997). Overall α_2-AR antagonists showed less potential in combating neuroinflammation, and combined with their potential to elicit significant effects on blood pressure renders them an unlikely choice as potential anti-inflammatory agents.

CHAPTER 5

Future directions

5. Future Directions

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

1. The ability of NA-enhancing agents to alter inflammatory indices in hippocampus and cerebellum should be assessed. In this regard, the hippocampus contains a high density of cytokine receptors and is negatively affected in AD. Localization of the β_2-AR is greatest in the cerebellum (unpublished observations), and as such it would be interesting to ascertain if NA-enhancing agents elicit a greater anti-inflammatory response in this region.

2. The ability of NRI's to inhibit cell death induced by LPS *in vivo* should be assessed. Markers of apoptotic cell death include caspase 3, PARP cleavage and Tunel staining, and can be assessed in *in vivo* tissue.

3. Future research should focus on determining the therapeutic efficacy of NRI's over long periods of time. Chronic *in vivo* studies will be necessary to determine if the acute anti-inflammatory 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 NRI's to elicit anti-inflammatory actions following repeated treatment.

4. Future research should also be devoted to assessing the action of NA and NA-enhancing agents on LPS-induced signal transduction. Specifically, the ability of NA and NRIs to alter LPS-induced MyD88-dependent signalling (NFκB and MAPK) and MyD88-independent signalling (IFNβ/IRF-3) should be assessed.

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

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Presentations and publications

Presentations and publications

J.B. O'Sullivan, A. Harkin, T.J. Connor (2006). The noradrenaline re-uptake inhibitor desipramine promotes an anti-inflammatory phenotype in rat cortex.

Poster presentation: Irish Society for Immunology meeting, Belfast, September 2006.

J.B. O'Sullivan, T.J. Connor (2006). The noradrenaline re-uptake inhibitor desipramine limits pro-inflammatory cytokine production in rat cortex *in vivo*. *Brain Behav. Immun.* (2006) 20 (Suppl. 1): 53.

Poster presentation: Psychoneuroimmunology Research Society meeting, Miami, USA, May 2006.

J.B. O'Sullivan, E.N. McNamee, N.M. Curtin, T.J. Connor (2005) Noradrenaline suppresses IL-1β and TNF-α in primary glial cells: a role for cAMP signaling or anti-inflammatory cytokines?

Poster presentation: Irish Society for Immunology meeting, Dublin, October 2005.

E.N. McNamee, **J.B. O'Sullivan**, T.J. Connor (2005) Noradrenaline suppresses pro-inflammatory cytokine production and to a lesser extent IL-10 production from LPS-stimulated primary mixed glial cultures: A role for β-adrenoceptor activation? *Ir. J. Med. Sci.* (2005) 174 (1, e2): 24.

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J.B. O'Sullivan, T.J. Connor (2005) Inhibition of the noradrenaline transporter or uptake-2 fails to augment the suppressive effect of noradrenaline on IL-1β and TNF-α production in primary glial cells. *Ir. J. Med. Sci.* (2005) 174 (1, e2): 22.

Poster presentation: Royal Academy of Medicine in Ireland meeting, Dublin, January 2005.