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β_2-adrenoceptors as Modulators of Glial Immune Function

Karen M. Ryan

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

Thesis submitted October 2009

Department of Physiology,
Trinity College Institute of Neuroscience,
Trinity College,
Dublin 2.

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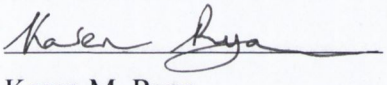

Karen M. Ryan

II Abstract

The endogenous anti-inflammatory and neurotrophic effects of noradrenaline are mediated by β_2-adrenoceptors on astrocytes and microglia in the central nervous system (CNS). Notably, clinical studies demonstrate that β_2-adrenoceptors are absent from astrocytes of patients with Multiple Sclerosis (MS) and loss of glial β_2-adrenoceptors is likely to contribute to the chronic inflammation observed in this disorder. Additionally it has been suggested that a loss of central noradrenergic tone may contribute to the development of Alzheimer's disease by facilitating the chronic neuroinflammation and amyloid-β deposition which is characteristic of this disease. The biological basis for the loss of β_2-adrenoceptors in MS is not known, therefore the possibility that exposure to an inflammatory stimulus could down-regulate glial β_2-adrenoceptor expression and responsiveness was examined here. The results demonstrate that *in vitro* exposure of primary mixed glial cultures to LPS+IFN-γ down-regulates β_2-adrenoceptor mRNA and cell surface expression (~50%) and reduces the accumulation of intracellular cAMP in response to the β_2-adrenoceptor agonist salbutamol, an effect found to be specific to β_2-adrenoceptor and not due to an effect on intracellular signaling events. Significantly, pre-treatment with the glucocorticoid dexamethasone prevented the suppression of β_2-adrenoceptor expression induced by LPS+IFN-γ and interestingly, dexamethasone also increased β_2-adrenoceptor expression in its own right in the absence of inflammation, an effect which may contribute to its therapeutic efficacy in MS. In contrast, *in vivo* a systemic LPS challenge did not impact on the mRNA expression or function of central β_2-adrenoceptors, and it is suggested that the glucocorticoid surge that occurs in response to systemic treatment with LPS could prevent β_2-adrenoceptor downregulation in vivo. Clenbuterol was shown to induce a dose-dependent activation of the IL-1 and IL-10 cytokine systems and it was shown that these effects are mediated by actions at central β_2-adrenoceptors, since they could be blocked by treatment with the selective β_2-adrenoceptor antagonist ICI 118,551. Notably, the clenbuterol-induced increase in IL-1 expression was found to occur in the absence of a classical immune response since clenbuterol did not impact on TNF-α, IL-6, iNOS, COX-II or IκBα expression. In addition, it was shown that the LPS-induced production of inflammatory mediators could be reduced by concurrent clenbuterol administration. Interestingly, chronic treatment with clenbuterol resulted in astrocytic activation and significantly reduced amyloid-β concentrations, an effect suggested to occur due to increased degradation of amyloid-β as opposed to inhibition of synthesis. In this regard, clenbuterol induced expression of MMP-2; an enzyme that is known to degrade amyloid-β. In contrast, clenbuterol did not influence expression of enzymes involved in amyloid-β synthesis. Clenbuterol also significantly reduced expression of RAGE; a receptor implicated in mediating amyloid-β-induced neurotoxicity. However, in spite of its beneficial central effects, clenbuterol was also found to induce negative peripheral effects such as a reduction in food and water intake, body-weight and locomotor activity of rats, effects which most likely occur due to its profound impact on heart rate. While it still remains to be determined if the peripheral effects of clenbuterol can be dissociated from its central effects, overall the data presented here indicate that activation of central β_2-adrenoceptors may represent a new target for pharmacological intervention in the treatment of neuroinflammatory diseases.

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VII Abbreviations

α-MSH	Alpha-melanocyte-stimulating hormone
AC	Adenylate cyclase
AD	Alzheimer's Disease
ADAM	A disintegrin and metalloproteinase
AICD	Amyloid precursor protein intracellular domain
ANOVA	Analysis of variance
APC	Antigen presenting cell
APP	Amyloid precursor protein
ATF	Activating transcription factor
ATP	Adenosine triphosphate
Aβ	Amyloid-β
β_2-AR	β_2-adrenoceptor
β-ARB	Beta adrenergic receptor binding protein
β-ARK	Beta-adrenergic receptor kinase
BBB	Blood brain barrier
BCA	Bicinchonic acid
BDNF	Brain derived neurotrophic factor
bFGF-2	Basic fibroblast growth factor
Bid	Bi-daily
BPM	Beats per minute
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
cAMP	cyclic adenosine 5' 3' monophosphate
CBP	Cyclic adenosine 5' 3' monophosphate response element binding protein
CD40L	CD40 ligand
cDMEM	Complete Dulbecco's modified Eagles medium
CNS	Central nervous system
CO_2	Carbon dioxide
COMT	Catechol-O-methyltransferase

con	Control
COX	Cyclooxygenase
CREB	cAMP response element binding
CSF	Cerebrospinal fluid
CSIF	Cytokine synthesis inhibitory factor
CT	Cycle time
CTF	C-terminal fragment
CVO	Circumventricular organ
db-cAMP	Dibutyryl cyclic adenosine 5'3' monophosphate
ddH_2O	Double-distilled water
DEPC	Diethyl pyrocarbonate
Dex	Dexamethasone
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
DTT	Dithiothreitol
DβH	Dopamine beta hydroxylase
EAE	Experimental allergic encephalomyelitis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra acetic acid
EIA	Enzymeimmunoassay
ELISA	Enzyme linked immunosorbancy assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal regulating kinase
EtOH	Ethanol
FCS	Fetal calf serum
fmol	Femtomole
FW	Formula weight
g	Gravity
GABA	Gamma amino-butyric acid

GDNF	Glial derived neurotrophic factor
GDP	Guanosine diphosphate
GFAP	Glial fibrillary associated protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G-protein coupled receptor
GPI	Glycosylphosphophatidylinositol
GRE	Glucocorticoid response element
GRK	G-protein receptor kinase
GTP	Guanosine triphosphate
HASM	Human airway smooth muscle
HPA	Hypothalamic-pituitary adrenal
hr	Hour
Hsp	Heat shock protein
IBMX	3-butyryl-1-methylxanthine
ICAM	Intracellular adhesion molecule
ICE	IL-1 converting enzyme
IDE	Insulin degrading enzyme
IFN	Interferon
IFN-GR	Interferon gamma receptor
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IGIF	Interferon-gamma inducing factor
IkB	Inhibitor of kappa B
IκBKK	Inhibitor of kappa B kinase kinase
IKK	Inhibitor of nuclear factor kappa B kinase
IL	Interleukin
IL-1Acp	Interleukin-1 accessory protein
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1beta
IL-1RAPL1	IL-1-receptor accessory-protein-like 1
IL-1ra	IL-1 receptor antagonist

IL-1R	Interleukin-1 receptor
IL-1RI	Interleukin-1 Type I receptor
IL-1RII	Interleukin-1 Type II receptor
IL-1RRP2	IL-1 receptor related protein 2
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-2	Interleukin-2
IL-6R	Interleukin-6 receptor
iNOS	Inducible nitric oxide synthase
ip	Intraperitoneally
IP-10	Interferon-inducible protein 10
IRAK	Interleukin-1receptor associated kinase
IRF	Interferon response factors
ISGF3	Interferon-stimulating gene factor 3
Jak	Janus tyrosine kinase
JNK	c-Jun amino-terminal kinase
kDa	Kilodalton
KIR	Kinase inhibitory domain
LBP	Lipopolysaccharide binding protein
LC	Locus coeruleus
LFA	Leukocyte function-associated molecule
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTP	Long-term potentiation
mA	Milliamp
MAG	Myelin associated glycoprotein
MAO	Monoamine oxidase
MAP	Mitogen-activated protein
MAPK	Mitogen-activate protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase

MBP	Myelin basic protein
MCP-1	Monocyte chemotactic protein-1
M-CSF	Mononuclear phagocyte colony stimulating factor
MEK	MAPK/ERK kinase
MHC	Major histocompatibility complex
min	Minute
MIP-1α	Macrophage inflammatory protein
ml	Millilitre
mM	Millimolar
MMP	Matrix metalloproteinase
MS	Multiple Sclerosis
MyD88	Myeloid differentiation factor 88
NADH	Nicotinamide adenine dinucleotide
Nep	Neprilysin
NET	Noradrenaline transporter
NFκB	Nuclear factor kappa B
ng	Nanogram
NGF	Neural growth factor
NHERF	Na^+/H^+ exchanger regulatory factor
NIK	Nuclear factor kappa B inducing kinase
NK	Natural killer
Nm	Nanometres
NMDA	N-methyl-D-aspartic acid
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NP-40	([Octy]phenoxy)polyethoxyethanol
NSB	Non-specific binding
O_2	Oxygen
OVLT	Organum vasculosum of lamina terminalis
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-tween 20
PCR	Polymerase chain reaction
PD	Parkinson's Disease
PDE4	Phosphodiesterase Type 4
PGE2	Prostaglandin E_2
PGH2	Prostaglandin H_2
PKA	Protein kinase A
PKC	Protein kinase C
PLA2	Phospholipase A_2
PLC	Phospholipase C
PNS	Peripheral nervous system
PRR	Pathogen recognition receptor
PVDF	Polyvinylidine
RAGE	Receptor for advanced glycation endproducts
RANTES	Regulated upon activation, normal T-cells expressed and secreted
ROS	Reactive oxygen species
rpm	Rotations per minute
RT-PCR	Real-time polymerase chain reaction
SAPK	Stress activated protein kinase
sAPP	Soluble amyloid precursor protein
sc	Subcutaneous
SDS-PAGE	Sodium dodecyl polyacrylamide electrophoresis
sec	Second
SEM	Standard error of the mean
SH	*Src* homology
SIGIRR	Single immunoglobulin IL-1-receptor-related molecule
siRNA	Small interfering ribonucleic acid
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TBS	Tris-buffered saline

TBS-T	Tris buffered saline-tween 20
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
Th	T-helper
TICAM1	TIR-containing adapter molecule
TIGIRR	Three-immunoglobulin-domain-containing IL-1-receptor-related molecule
TIMP-1	Tissue inhibitor of metalloproteinases
TIR	Toll-like receptor/Interlekin-1 receptor
TLR	Toll-like receptor
TMB	N,N,N,'N'-Tetramethylethylene-benzidine
TMEV	Theiler's murine encephalitis virus
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNF-α	Tumour necrosis factor alpha
TRAF	Tumour necrosis factor receptor associated factor
TRIF	TIR domain containing adapter-inducing IFN-β
Tyk-2	Tyrosine kinase 2
μM	Micromolar
UV	Ultraviolet
v/v	Volume per volume
VCAM	Vascular cell adhesion molecule
VIP	Vasoactive intestinal peptide
VMAT	Vesicular monoamine transporter
w/v	Weight per volume
WHO	World Health Organisation

Chapter 1

Introduction

1.1 Introduction

Neuroinflammation is known to play a significant role in the pathogenesis of many neurological diseases such as stroke, Multiple Sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease (PD) (Aloisi *et al.,* 2000; Liu & Hong, 2003; Chavarria & Alcocer-Varela, 2004). Whilst it was once thought that the brain was incapable of mounting an immune response it is now accepted that the blood brain barrier (BBB) provides the brain with "relative" rather than "absolute" protection from invading pathogens (Carson *et al.,* 2006; Galea *et al.,* 2007). Hence, it is now recognised that the brain has its own intrinsic protective mechanisms which act to dampen the inflammatory response. Much research has focused on the role of neurotransmitters in the regulation of neuroinflammation, one such neurotransmitter being noradrenaline (Galea *et al.,* 2003). Consequently, it has been found that within the central nervous system (CNS) noradrenaline exerts its anti-inflammatory and neurotrophic effects primarily through actions at β_2-adrenoceptors expressed on glial cells, the resident immune cells of the brain (Mori *et al.,* 2002). However, the noradrenergic system has been shown to be defective in many neurological diseases. For instance, in MS it has been found that β_2-adrenoceptors are absent from astrocytes in the white matter of patients suffering from the disease (De Keyser *et al.,* 1999; Zeinstra *et al.,* 2000). In addition, defects in the noradrenergic system have been shown to play a substantial role in the pathogenesis of other neurodegenerative disorders including Alzheimer's disease and Parkinson's disease (Marien *et al.,* 2004). Such defects in noradrenergic signalling pathways may inhibit the beneficial effects of endogenous noradrenaline in combating neuroinflammation. Thus, in the first instance, since β_2-adrenoceptors are absent on astrocytes in the MS brain, a neuroinflammatory disorder, the initial studies presented here examine the possible link between an inflammation and altered expression and responsiveness of glial β_2-adrenoceptors both *in vitro* (Chapter 3) and *in vivo* (Chapter 4). In addition, the work presented in this thesis also examines the potential of using pharmacological compounds to stimulate central glial β_2-adrenoceptors as a strategy for regulating the innate immune response of the brain. Since previous studies from this laboratory have shown that acute treatment with clenbuterol, a long-acting selective β_2-adrenoceptor agonist, can activate astrocytes and induce the expression of anti-inflammatory cytokines in the brain under basal conditions (McNamee, 2008) the work presented here further examines the ability of clenbuterol to regulate the immune response

within the CNS following both acute (Chapter 5) and chronic treatment (Chapter 7). Furthermore the ability of acute treatment with clenbuterol to impact on LPS-induced inflammation (Chapter 5) and the peripheral effects of clenbuterol (Chapter 6) were investigated in a bid to further examine its potential as a suitable therapeutic intervention for the treatment of neuroinflammation. Thus, the work presented here provides significant insight into the potential of regulating neuroinflammation by selectively targeting central β_2-adrenoceptors.

1.2 The innate immune response

The immune response plays a critical role in protecting the body from injury and invading pathogens and is often referred to as the body's "sixth sense". This response can be divided into two primary components: 1) an immediate innate immune response and 2) a slower adaptive immune response involving the activation of B- and T-cells resulting in immunological memory. The innate immune response is the body's first line of defence and plays a crucial role in the early recognition of invading pathogens. It is a non-specific response which triggers an inflammatory reaction owing to recognition of the highly evolutionarily conserved structures on pathogens termed pathogen-associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs) on cells of the immune system. Upon PAMP recognition, the PRRs trigger a number of intracellular signalling cascades to evoke an inflammatory response, the cardinal signs of which are heat, pain, swelling, redness and loss of function. Inflammation is known to involve the release of pro-inflammatory mediators such as cytokines, chemokines and cell adhesion molecules (CAMs), amongst others (Tracey, 2002; Andersson, 2005; Mogensen, 2009). However, while inflammation acts to protect the host against infection or injury it can have a dichotomous effect. In this regard, while inflammation plays an important repairing role the magnitude of this response is crucial since an insufficient response can result in immunodeficiency while an exaggerated response can lead to morbidity and mortality (Chavarria & Alcocer-Varela, 2004; Tracey, 2002).

1.2.1 Inflammation and the brain

It is a well known fact that the brain is less susceptible to inflammation than any other organ and as such has long been regarded as an immune privileged site owing to the presence of the

BBB. The BBB acts to prevent the access of foreign matter into the brain and is comprised of a complex network of pericytes, astrocytes and specialized cerebrovascular endothelial cells which are held together by complex tight occluding junctions (Carson *et al.*, 2006). However, as already mentioned, opinions regarding the immune privilege of the brain are beginning to change and it is now clear that this "privilege" is more "relative" than "absolute" and cannot be wholly explained by the presence of the BBB (Carson *et al.*, 2006; Galea *et al.*, 2007). Accordingly, it is now acknowledged that there are structures in the CNS that are devoid of BBB which provide entry routes for foreign particles. Collectively these are known as circumventricular organs (CVOs) of which seven have been described, namely these are the subfornical organ, the organum vasculosum of lamina terminalis (OVLT), median eminence, posterior pituitary, subcommissural organ, the pineal gland and the area postrema. Furthermore, it is now recognized that T-cells can enter the CNS from the periphery and while naive T-cells cannot be activated within the CNS, T-cells which have been primed in the periphery can cross the BBB and become re-stimulated upon antigen presentation within the CNS (Carson, 2002). As such it has been demonstrated that $CD4^+$ T-cells specific for myelin antigens form part of the normal T-cell repertoire and are found in the CNS of both MS patients and healthy individuals, with the incidence or function of these cells being no different between such individuals (De Keyser *et al.*, 2004b). Incidentally, the brain is also found to contain its own resident immune cells, the so called glial cells, which are said to play an important role in the inflammatory response of the CNS owing to their ability to release cytokines and act as antigen presenting cells (APCs). Thus, it is now widely accepted that the CNS can mount a well-organized response to infection or injury and that, rather than the absence of an immune response within the CNS, this process is under strict regulation (Galea *et al.*, 2003; Rivest, 2003). The activation of regulatory neuronal pathways, such as that of the cholinergic and noradrenergic pathways, is suggested to be one such mechanism and it has been suggested that activation of such pathways imparts protection to the vulnerable CNS under neuroinflammatory conditions (Aloisi *et al.*, 2000, Galea *et al.*, 2003, Tracey, 2002). In spite of this, the prevailing theory on many of the major neurodegenerative diseases, including MS, Alzheimer's disease and Parkinson's disease, suggests that an inappropriate inflammatory response contributes to their pathology leading to widespread neuronal death and impaired CNS function (Aloisi *et al.*, 2000; Liu & Hong 2003, Chavarria & Alcocer-Varela 2004).

However, it is now also recognized that inflammation can be both neurotoxic and neuroprotective (Carson *et al.*, 2006).

1.2.2 Physiological neuroinflammation

As previously mentioned, inflammation forms part of the innate immune response and is a method by which the body protects itself from invading pathogens. Likewise in the brain, inflammation acts to protect the vulnerable CNS from infiltrating toxins and pathogens by activating the resident immune cells of the CNS, the glia, and by inducing the release of various inflammatory mediators including cytokines, chemokines, nitric oxide (NO) and prostaglandins. Thus, while the BBB acts to prevent foreign matter from entering the CNS, it is now accepted that the CNS is itself capable of mounting an immune response in the absence of a breach of the BBB (Becher *et al.,* 2000; Carson *et al.,* 2006). Thus, under physiological conditions there are a number of mechanisms by which the CNS can induce an innate immune response, namely 1) by regulating the presentation of antigen to T-cells, 2) by influencing the polarization of T-cell cytokine responses and 3) through the activation of its own endogenous immune cells, the glia (Becher *et al.,* 2000). This acute physiological response and the inflammatory molecules it produces are suggested to play a significant role in the repair and recovery from injury and disease (Becher *et al.,* 2000; Piehl & Lidman, 2001). Thus, it has been found that microglia can initiate an immune response towards dying or damaged cells. In this regard, microglia have been shown to respond to the flipping of phosphatidylserine from the inner cell membrane to the outer cell membrane, an event which occurs during apoptosis (Becher *et al.,* 2000).

Activation of microglia induces the expression of pro-inflammatory cytokines and chemokines during the initial stages of the immune response allowing for permeabilization of the BBB and recruitment of peripheral immune cells into the CNS. As such, the expression of pro-inflammatory cytokines contributes further to the inflammatory response since these play a role in the activation and polarization of T-cells (Becher *et al.,* 2000; Lucin & Wyss-Coray, 2009). Astrocytes also play a role in the immune response of the CNS. Thus, upon insult to the CNS astrocytes are generally found to migrate towards the site of injury and form a glial scar which acts to insulate the damaged area. As such the activation of astrocytes appears to restrict inflammatory processes within the CNS since these cells play a role in the induction of T-cell

apoptosis (Piehl & Lidman, 2001). Neurons are also found to play a role in the immune response of the CNS and have been found to express major histocompatability complex (MHC) class I and to produce cytokines such as interferon-gamma (IFN-γ). In addition, they have also been shown to induce apoptosis of T-cells (Piehl & Lidman, 2001). While the inflammatory response is generally rapidly resolved, how a given insult is interpreted within the CNS can mean the difference between a beneficial or detrimental outcome. Thus, dysregulation of the inflammatory response can have detrimental consequences for the CNS if the response is either too passive or too aggressive as discussed below.

1.2.3 Pathophysiological neuroinflammation: inflammation in the aged brain

Inflammation is found to play a significant role within the aged brain. Thus it is known that in the aged brain there is a shift in the homeostatic balance and as such an imbalance exists between pro- and anti-inflammatory states. Essentially this shift results in a marked increase in steady-state levels of pro-inflammatory cytokines and a decrease in steady-state levels of anti-inflammatory cytokines. In this regard several pro-inflammatory cytokines, such as interleukin-6 (IL-6) have been shown to be increased during the normal aging process with a concomitant decrease being seen in anti-inflammatory cytokines such as interleukin-10 (IL-10) (Sparkman & Johnson, 2008). This imbalance in cytokine expression may predispose the CNS to the increased susceptibility to inflammation and neuronal damage commonly seen in aging. Furthermore, the aged brain has been shown to have an increased number of activated and primed microglia compared to that seen in the young brain (Sparkman & Johnson, 2008). In addition, it has been found that upon secondary stimulation these primed microglia can release excessive levels of pro-inflammatory cytokines leading to a greater severity of illness (Dilger & Johnson, 2008). It has been suggested that the increased expression of IFN-γ observed in the aged brain may be responsible for microglial priming observed in this state (Dilger & Johnson, 2008). Furthermore, it has been found that there is an increased expression of MHC class II molecules, complement receptors, toll-like receptor (TLR)-2, TLR-4 and CD14 molecules in the aged brain (Dilger & Johnson, 2008). Thus, it appears that the aged brain may be sensitised to produce an exaggerated immune response following infection or exposure to a stressor (Sparkman & Johnson, 2008). This sensitised state has also been found to result in impaired cognitive function following an inflammatory stimulus. Moreover, aged animals show a prolonged sickness behavioural response following an inflammatory challenge

in comparison to younger animals (Huang *et al.,* 2008). Thus, while the behavioural response to sickness is normally adaptive and reversible, the overproduction of pro-inflammatory cytokines in the aged brain may lead to pathological disease states and a maladaptive behavioural response (Dilger & Johnson, 2008). However, an alternative notion regarding the role of microglia in aging also exists. As such it has been proposed that microglia become dysfunctional and enter a senescent state in the aged brain, thus secreting diminished levels of neurotrophins and down-regulating their phagocytic function. This coupled with the increased expression of inflammatory mediators has been suggested to lead neuronal loss and the inefficient clearance of toxic proteins seen in neurodegenerative disease (Lucin & Wyss-Coray, 2009).

1.2.4 Pathological neuroinflammation: inflammation in neurodegenerative disease states

While the inflammatory response of the CNS is generally acute in nature and acts primarily to protect this delicate system, if unresolved the inflammatory response can turn from friend to foe. As such, chronic activation of the immune response of the CNS can lead to pathological neurodegenerative states such as that seen in MS and Alzheimer's disease (Becher *et al.,* 2000). However, whether neuroinflammation contributes directly to neurodegeneration or is a secondary response to neurodegenerative processes remains a matter for debate.

In contrast to the physiological immune response the response seen in neurodegeneration is chronic and low-grade. Furthermore, it appears that inflammation in neurodegenerative disease states occurs in the absence of significant compromise of the BBB or infiltration of peripheral immune cells (Carson *et al.,* 2006; Wyss-Coray & Mucke, 2002; Lucin & Wyss-Coray, 2009). As such it has been suggested that the chronic activation of glia and the concomitant production of inflammatory molecules witnessed in neurodegenerative states may represent a response to distress signals from dying neurons which survive for prolonged periods prior to their clearance by phagocytosis (Wyss-Coray & Mucke, 2002). This response to neurodegeneration may then trigger deleterious events such as oxidative stress and cytokine-receptor-mediated apoptosis (Hirsch & Hunot, 2009) Furthermore, it has also been proposed that microglial activation in neurodegeneration occurs due to the release of adenosine triphosphate (ATP), neurotransmitters, blood-clotting factors and changes in ion concentrations. In addition, the loss of inhibitory molecules on neurons that act to keep glia in

a quiescent state, such as CD200 & fractalkine, has been observed in many neurodegenerative diseases (Lucin & Wyss-Coray, 2009). Thus it seems likely that dysfunctional neurons or glia may induce neurodegenerative processes. Notably, a similar state of microglial priming to that witnessed in the aged brain has also been reported in several neurodegenerative disease states including Alzheimer's Disease, MS and an ME7 model of prion disease. The proposed mechanism for microglial priming in these conditions suggests that the expression of abnormal proteins, specific to each disease, acts as a priming factor for the microglia. For example, PRRs have been shown to play a role in initiating an immune response towards disease-associated molecules such as that of amyloid-β in Alzheimer's disease. Thus, while amyloid-β is endogenously expressed at physiological concentrations, changes in its abundance or location may result in glial activation, in particular microglial activation (Becher *et al.,* 2000). Furthermore, the presence of amyloid-β is found to induce microglial phagocytosis in a dose and time-dependent manner which is suggested to be an attempt to remove amyloid-β plaques (Tuppo & Arias, 2005), however, disease progression and the ongoing accumulation of amyloid plaques observed in Alzheimer's disease may occur owing to a dysfunction in this clearance system.

While a disruption in the BBB is one of the earliest signs in MS, leaky regions in the BBB are not always found to be associated with autoimmunity (Carson *et al.,* 2006). Thus in the animal model of MS, experimental allergic encephalomyelitis (EAE), it has been demonstrated that brain endothelial cells express MHC class II molecules prior to the infiltration of peripheral immune cells into the CNS. As such it appears that events within the CNS itself also play a significant role in the development of this neurodegenerative disease (Becher *et al.,* 2000). Furthermore, it has been found that endothelial cells *in vitro,* in the presence of IFN-γ, express B7 molecules suggesting that in the presence of an inflammatory stimulus endothelial cells may have the capacity to present antigen to T-cells and thus induce their activation. However, studies conducted to date have failed to provide conclusive evidence for this (Becher *et al.,* 2000). Perivascular macrophages are also found to express high levels of MHC class II constitutively and have been shown to influence the rate of proliferation of $CD4^+$ T-cells reactive to MBP. In this regard, it appears that perivascular macrophages may be required for the "homing" of T-cells to the CNS (Becher *et al.,* 2000). Notably, depletion of perivascular macrophages was shown to inhibit the onset of EAE since T-cells were found to accumulate in

the perivascular area but were incapable of infiltrating the parenchyma. In addition, in the Theiler's murine encephalitis virus (TMEV) model of MS, the CNS mounts a response against the virus which is thought to induce destruction of tissue thus releasing antigens that can be recognized by auto-reactive T-cells within the CNS or in lymph nodes. In turn these T-cells may be recruited to the target tissue and drive the inflammatory process further, a phenomenon termed epitope spreading. A second mechanism by which the CNS may mount an immune response to virus is by a process termed molecular mimicry. The suggested mechanism in this case involves the expression of antigens by invading viruses that mimic host proteins and this in turn may activate an immune response against neuroantigens which have homology with the viral protein (Becher *et al.,* 2000).

1.3 Glia: more than just 'glue' for the brain

The nervous system comprises two major cell types, namely neurons and the lesser known neuroglial cells. Neuroglia or more simply "glia" are the most numerous cells in the brain and constitute approximately 90% of the cells of the CNS (He & Sun 2007). The name glia originates from the Greek for 'glue' and although once thought to function simply in holding the nervous system together the notion is now coming to the fore that glia play a much more prominent role in the brain. There are three major types of glial cells found in the CNS, namely oligodendrocytes, microglia and astrocytes. While oligodendrocytes serve to myelinate neurons and provide the insulation necessary for neuronal impulse conduction, microglia and astrocytes are the cells primarily implicated in the immune response of the CNS.

1.3.1 Microglia

Microglia are the smallest of the glial cells and constitute approximately 20% of the glial population found within the CNS (Gehrmann *et al.,* 1995). They are derived from mesodermal haemopoietic precursor cells which infiltrate into the CNS early in development, hence microglia are physiologically and embryologically unrelated to any other cells within the CNS. Instead, microglia have much in common with the macrophages of the peripheral immune system since they are part of the mononuclear phagocyte lineage and primarily function as scavenger cells (He & Sun 2007). In support of this, all antibodies developed so

far against microglia are found to cross-react with macrophages (Gehrmann *et al.,* 1995, Vilhardt, 2005). The microglial population of the CNS consists of two major pools namely the parenchymal microglia which show slow turnover and the perivacular macrophages which are located within the BBB and show rapid turnover from a pool of bone marrow derived cells (Becher *et al.,* 2000; Piehl & Lidman, 2001). Perivascular microglia/macrophages are found located between the endothelium and brain parenchyma, a space which is known as the Virchow-Robin space in humans (Carson *et al.,* 2006).

In the normal CNS microglia are maintained in a quiescent state by a number of mechanisms. One such mechanism is the receptor-ligand interactions between neurons and microglia, for example the interaction of neuronal CD200 receptor with the microglial CD200 ligand, neuronal fractalkine with microglial CXCR1, neuronal CD22 with microglial CD45, to name but a few. Furthermore, microglia are also shown to be maintained in a quiescent state by soluble factors released from astrocytes and neurons, for example prostaglandins and the cytokine transforming growth factor 1 ($TGF\beta_1$). Additionally neuropeptides, such as vasoactive intestinal peptide (VIP) and α-melanocyte-stimulating hormone (α-MSH), and neurotransmitters, such as noradrenaline, are also found to influence the phenotype and function of microglia (Galea *et al.*, 2007; Gonzalez-Rey *et al.*, 2007). Under resting conditions microglia display a dendritic morphology with highly ramified processes and are shown to express low levels of the co-stimulatory molecules CD14, CD45 and MHC class II in the absence of CNS pathology (Vilhardt, 2005; Jack *et al.,* 2005a). However, it is known that microglia are the first cells in the CNS to respond to injury or infection and when exposed to insult they undergo a rapid change in morphology. As such, activation results in the transformation of microglia first from a ramified morphology to a rod-like shape and finally to an amoeboid macrophage-like shape. In addition to changes in morphology of the cells, activation also provokes changes in their gene expression profile (Vilhardt 2005). Two types of activated microglia have been identified: 1) the hypertrophic microglia, which express increased levels of marker molecules such as MHC antigens but which are not phagocytic, and 2) the fully phagocytic microglia (Gehrmann *et al.,* 1995). A rapid upregulation of MHC class II expression is observed following microglial activation in addition to increased expression of CAMs, such as intracellular adhesion molecule (ICAM)-1 and leukocyte function-associated molecule 1 (LFA-1). Appearance of such molecules is the earliest sign of microglial activation

(Aloisi *et al.,* 2000). CD11b can also be used as a marker of microglial activation since its expression is found to be increased by serum constituents following disruption of the BBB (Ransohoff & Perry, 2009). Activated microglia are also shown to express the co-stimulatory molecules CD-40, CD-80 (B7-1) and CD-86 (B7-2), all of which are necessary for T-cell activation. As a result microglia are said to act as APCs and to promote activation of T-cells within the CNS (Aloisi *et al.,* 2000).

In the developing brain activated microglia play an important role in the phagocytosis of surplus cells undergoing apoptosis. In addition, they are found to play a role in determining neuronal fate, promoting neuronal migration and inducing axonal growth (Vilhardt, 2005). However, activated microglia are also responsible for the secretion of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β), chemokines, prostaglandins and they are also implicated in the generation of reactive oxygen species (ROS) and nitrogen intermediates (Vilhardt, 2005). Notably, when microglia phagocytose cells which have been programmed to die their APC capacity becomes down-regulated, a mechanism which is suggested to be an attempt to limit the inflammatory response (Jack *et al.,* 2005a). Nevertheless, the chronic activation of these cells, such as that proposed to occur in many neuroinflammatory disorders, can have detrimental consequences for the CNS.

1.3.2 Astrocytes

Astrocytes are the most abundant of the glial cells and their development within the CNS stems from neuroectodermal cells. Astrocytes have an irregular "star-shaped" cell body and broad end-feet with which they can interact with neurons and help to form the BBB. In addition, astrocytes also play an important role in maintaining homeostasis within the CNS. In this regard, they function in maintaining ionic homeostasis, buffering excess neurotransmitters and secreting neurotrophic factors (Block & Hong 2005). Furthermore, astrocytes play an important role in synaptogenesis. In this respect, neuronal synapses are found to develop at the same time that astrocytes appear in the developing brain. In addition, astrocytes are also found to possess receptors for many neurotransmitters including those for glutamate, gamma amino-butyric acid (GABA), noradrenaline and acetylcholine (Tritsch & Bergles 2007). Incidentally, it has been found that can prevent glutamate-induced neurotoxicity by the removal of excess

synaptic glutamate following its release from the pre-synaptic terminal. Moreover, astrocytes are also believed to control levels of potassium in the extracellular space, which if left to accumulate would have disastrous consequences due to its role in neuronal excitability (Jessen 2004).

However, in spite of the beneficial effects of astrocytes these cells are also known to play a role in the immune response of the CNS, although they have a much slower reaction to insult than microglia. Upon activation astrocytes undergo hypertrophy which is characterized by a rapid increase in the expression of glial fibrillary acidic protein (GFAP), an intermediate filament found in mature astrocytes thought to be important for maintaining structural stability and modulating motility (Eng *et al.*, 2000). As such activated astrocytes act to encase areas of damage and can potentially mediate T-cell apoptosis due to the release of the anti-inflammatory cytokine $TGF\beta_1$, thus downregulating T-cell mediated injury within the CNS (Gehrmann *et al.,* 1995). In EAE, an animal model of MS, astrocytes are shown to respond at the late recovery stage and act to encase the inflammatory lesion preventing access of microglia thus potentially inducing downregulation of microglial activity. However, hypertrophy of astrocytes also results in the formation of glial scars and since astrocytes have been also been shown to express factors such as Nogo-A and myelin associated glycoprotein (MAG), which block axonal re-growth, this hypertrophy may contribute to CNS damage. Furthermore, it has also been observed that astrocytes are capable of acting as APCs (Nikcevich *et al.,* 1997; Soos *et al.,* 1998) and thus may contribute to CNS damage through the activation of T-cells, however, the extent to which astrocytes function as APCs remains a matter for debate. In this regard, astrocytes kept in IFN-γ deprived conditions *in vitro* show poor APC capacity, possibly due to a lack of co-stimulatory signals from other cells (Gehrman *et al.,* 1995), yet in MS lesions astrocytes have been found to express CD1 molecules which aid in the presentation of lipid antigens to specific clusters of T-cells (Aloisi *et al.,* 2000).

The activation of glial cells is an important host defence mechanism that occurs in a bid to protect the CNS during infection or environmental stress. While microglia have been shown to produce the chemokine macrophage inflammatory protein 1α (MIP-1α), attracting T-helper (Th)-1 cells to the CNS which are generally regarded as pro-inflammatory in nature, astrocytes produce the monocyte chemotactic protein-1 (MCP-1) which attracts both Th_1 and

Th_2 cells but primarily exerts a Th_2 anti-inflammatory response (Aloisi *et al.*, 2000). Along with cytokine and chemokine production activated glia can also produce the CAMs, VCAM-1 and intracellular adhesion molecule (ICAM)-1 which allow activated lymphocytes to diapedese across the BBB from the periphery (Steinman 1996, Chavarria & Alcocer-Varela 2003). The crucial role that glia play in the inflammatory response of the CNS is revealed by the finding that the infiltration of lymphocytes is not always necessary for neurotoxicity (Block & Hong, 2005). Bearing all of this in mind, chronic glial activation may have detrimental consequences for the CNS due to overproduction or sustained production of potentially neurotoxic molecules such as NO, ROS and the pro-inflammatory cytokines TNF-α and IL-1β (Xie *et al.*, 2004).

1.4 Mediators of CNS inflammation

1.4.1 Pro-inflammatory Cytokines

1.4.1.1 Tumour Necrosis Factor-α (TNF-α)

TNF-α has a molecular weight of 17 kilodalton (kDa) and is regarded as a trimeric protein which is pro-inflammatory in nature (Aschner, 1998; Tizard, 2008). *In situ* TNF-α forms part of the primary inflammatory response and is produced by activated macrophages, mast cells, T-cells, endothelial cells, B-cells, fibroblasts and glia in response to injury or invading pathogens (Chung & Benveniste, 1990; Tracey, 2002; Tizard, 2008). TNF-α transduces its signal through two distinct receptors (see Fig. 1.1), tumour necrosis factor receptor (TNFR) 1 which is expressed on virtually all cell types and TNFR2 which is expressed on immune and endothelial cells. Binding of TNF-α to its receptors can activate mitogen-activated protein kinase (MAPK) or nuclear factor kappa B (NFκB) signalling pathways (Bertazza & Mocellin 2008). While TNF-α is generally present as a secreted protein, a membrane-anchored form has also been identified and has been shown to play a role in intercellular signalling (Benveniste *et al.*, 1995). In the CNS it is reported that microglia express TNFR1 and TNFR2 while astrocytes predominantly express TNFR1 (Patrizio, 2004).

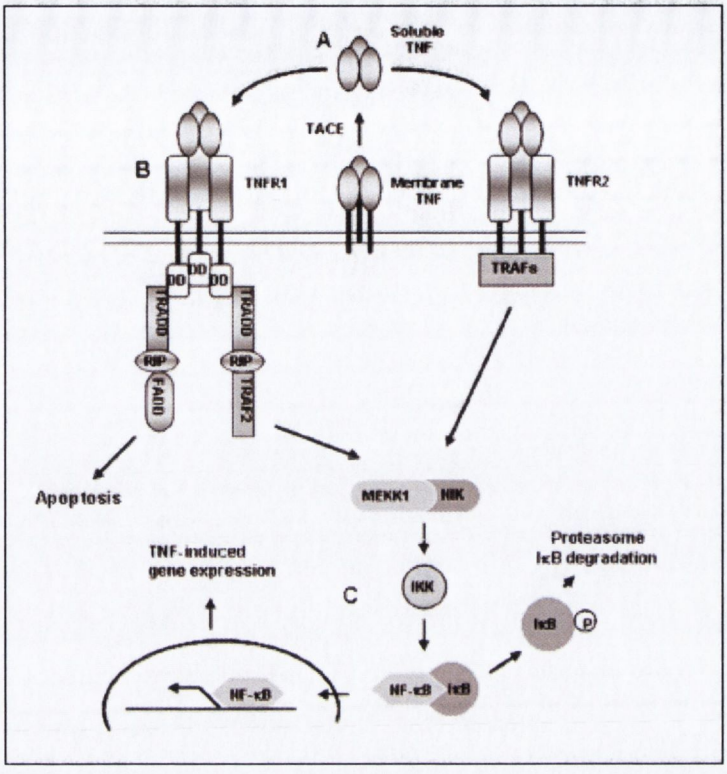


Figure 1.1: TNF-α Signalling

(From: www.ncbi.nlm.nih.gov/bookshelf/picrender.fcgi)

TNF-α is one of the primary mediators of the host response to invading gram-negative bacteria and is regarded as the master pro-inflammatory cytokine since it is generally the first cytokine to appear following injury. In line with this, TNF-α is essential for a complete inflammatory response since it stimulates the production of IL-1, NO via iNOS, prostaglandins via cyclooxygenase (COX)-II and ROS from other cells. In addition TNF-α induces the release of chemokines and CAMs to promote the attraction and activation of leukocytes. All of these effects lead to an amplified and prolonged inflammatory response. Furthermore, TNF-α appears to facilitate the switch from an innate to an adaptive immune response (see Tracey, 2002; Tizard, 2008). Within the CNS TNF-α is said to increase the permeability of endothelial cells in the BBB and to enhance the expression of CAMs thereby facilitating the recruitment of neutrophils, monocytes and lymphocytes to the CNS (Benveniste *et al.*, 1995). Furthermore, the presence of TNF-α has also been shown to induce the expression of other pro-inflammatory cytokines such as IL-1β and IL-6 and it has also been shown to induce its own expression. In addition, TNF-α has been found to induce the expression of ICAM-1 and

MHC class II in astrocytes, thereby promoting their ability to function as APCs. Notably, TNF-α alone does not increase the expression of MHC class II on astrocytes, rather it enhances that induced by IFN-γ or viruses (Benveniste *et al.*, 1995; Aschner, 1998). Increased TNF-α expression in the CNS has been observed following traumatic injury, ischemia and in AD, PD and MS. (Hofman *et al.*, 1989; Patrizio, 2004). Notably, TNF-α has been shown *in vitro* to induce damage to myelin and oligodendrocytes and thus may contribute to the myelin damage observed in MS (Benveniste *et al.*, 1995).

1.4.1.2 Interleukin-1 (IL-1)

The IL-1 superfamily is a pleiotropic cytokine family which can exert many different functions in the immune system. However, in general the term IL-1 refers to a 17kDa polypeptide of which there are two isoforms, namely interleukin-1alpha (IL-1α) and IL-1β. While IL-1α and IL-1β are encoded by separate genes they share sequence homology and are found to elicit similar biological actions. Both IL-1α and IL-1β are formed as precursors with a molecular weight of approximately 31kDa. Pro-IL-1α is synthesized in association with microtubules and can be cleaved by calpains which are calcium-dependent membrane associated cysteine proteases or other extracellular proteases to form mature IL-1α. In the main the expression of IL-1α remains intracellular, however, a small portion (~5%) is also found expressed on the surface of a number of cells. Both pro-IL-1α and mature IL-1α are biologically active and their actions are found to be primarily due to the regulation of intracellular events. In contrast, pro-IL-1β has no biological activity and requires cleavage by the protease caspase-1/IL-1β converting enzyme (ICE) in order to become active. ICE is initially synthesized as a 45kDa precursor protein which is cleaved to form an enzymatically active heterodimer consisting of 10 and 20 kDa chains. Two molecules of this ICE heterodimer can form a tetramer with two molecules of pro-IL-1β to initiate cleavage of pro-IL-1β and release mature IL-1β. While ICE is found to be constitutively expressed in most cell types, not all cells have the capacity to process pro-IL-1β. Activation of ICE occurs in response to activation of purinergic $P2X_7$ receptors by extracellular ATP (Rothwell, 2003; Mingam *et al.*, 2008). Following synthesis pro-IL-1β is found primarily in the cytosol, however, upon cleavage IL-1β is transported out of the cell. In general IL-1β is found to be constitutively expressed at low levels, however its expression becomes rapidly upregulated in response to injury. In the normal CNS microglia are the only cells shown to constitutively

express ICE, however, its expression in astrocytes, oligodendrocytes and neurons has been observed following experimental damage (Dinarello, 1996; Rothwell & Luheshi, 2000).

There are two receptors for IL-1, an 80kDa type I receptor and a 68kDa type II receptor. The type I receptor (IL-1RI), with a molecular weight of 80kDa, is a member of the IL-1 receptor/Toll-like receptor superfamily and is the major receptor responsible for IL-1β-mediated responses (Dinarello, 1996; Bowie & O'Neill, 2000). Its expression has been demonstrated on glial cells, endothelial cells, smooth muscle cells, epithelial cells, hepatocytes, fibroblasts and T-cells, amongst others (Dinarello, 1996; Lee *et al.*, 2002). IL-1RI is comprised of a single transmembrane domain and a cytoplasmic domain. Signalling through IL-1RI by its ligands requires the additional binding of the IL-1 receptor accessory protein (IL-1Acp). IL-1 binds to IL-1RI with a low affinity and this is suggested to cause a conformational change in the receptor enabling IL-1Acp to dock to the complex the consequence of which is higher affinity binding of IL-1. Signalling through IL-1RI primarily induces activation of the NFκB signalling pathway and extracellular signal-related kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) MAPK pathways (see Fig. 1.2). In contrast, the IL-1 type II receptor (IL-1RII) lacks an intracellular domain and appears to act as a decoy receptor that competes for the binding of IL-1, thus inhibiting IL-1-mediated responses. Its expression is found throughout the brain, mainly localized to neuronal soma. In addition, IL-1 signalling can also be inhibited by the highly selective endogenous antagonist IL-1 receptor antagonist (IL-1ra) of which there are three known isoforms, a 17kDa secreted form and two intracellular forms. Like IL-1α and IL-1β, IL-1ra is also initially synthesized as a precursor protein. IL-1ra is structurally homologous to the IL-1 polypeptides and hence is found to bind the same receptors. However, IL-1ra fails to trigger an interaction between IL-1RI and IL-1Acp and thus is biologically inactive. For this reason, IL-1ra may be considered an endogenous negative regulator of IL-1 signalling. However, while IL-1 and IL-1ra bind to IL-1RI with near equal affinity, a 10-100-fold molar excess of IL-1ra is often required to inhibit signalling of IL-1 through IL-1RI (Dinarello, 1996; Rothwell & Luheshi, 2000). While it has been shown that the expression of IL-1ra can be neuroprotective in neuroinflammatory states, its overexpression has been shown to lead to atrophy of the brain (Loddick *et al.*, 1997; Oprica *et al.*, 2007).

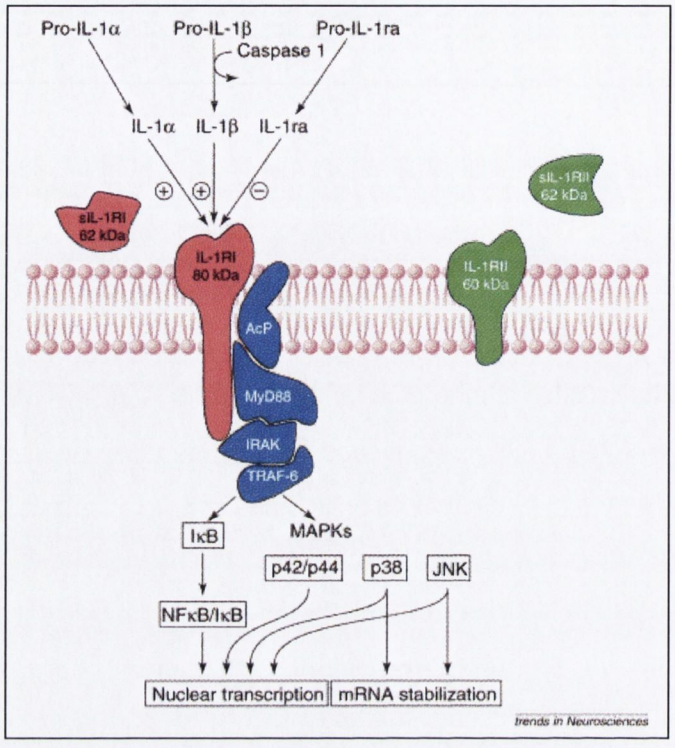


Figure 1.2: IL-1 Signalling
(From: Rothwell & Luheshi, 2000)

Another member of the IL-1 family which is found to share sequence homology with IL-1 is IL-18, formerly known as interferon-γ inducing factor (IGIF). Like IL-1, IL-18 is initially formed as a precursor and requires cleavage by ICE to release mature IL-18 which then binds to and elicits its effects through the IL-18 receptor. Both IL-18 and its receptor are found to be constitutively expressed in the CNS and IL-18 is found to be produced by both astrocytes and microglia (Rothwell & Luheshi, 2000).

IL-33 (IL-1F11) is another member of the IL-1 family which exhibits high expression within the CNS (Hudson *et al.,* 2008). Peripherally IL-33 exerts its effects on mast cells and Th2 polarized CD4+ T-cells inducing the expression of the type II cytokines IL-6 and IL-13 in a MyD88/NFκB-dependent manner. Within the CNS IL-33 is primarily localized within astrocytic nuclei and is found to be a potent inducer of type II innate immunity as it has been found to induce the expression of type II cytokines such as IL-4, IL-5 and IL-13. In association with IL-1RAcp, IL-33 signals through the ST2L receptor whose expression has been found predominantly on Th_2 cells (Bowie & O'Neill, 2000; Hudson *et al.,* 2008). Within the nucleus IL-33 is found bound to chromatin and so it has been shown to induce

transcriptional repressor activity and it has also been suggested that it acts additionally as a transcription factor (Luheshi *et al.*, 2009).

Through sequencing of the human genome a number of other novel members of the IL-1 superfamily have been identified to share sequence homology with IL-1α, IL-1β or IL-1ra. These new ligands include IL-1F5, IL-1F6, IL-1F7, IL-1F8, IL-1F9 and IL-F10 (see Table 1.1). With the exception of IL-1F7 which appears to signal through the IL-18R, these ligands signal primarily through the IL-1 receptor related protein 2 (IL-1RRP2) and activate downstream signalling pathways such as that of NFκB and the MAPKs. Sequencing also revealed the identity of three orphan receptors, namely IL-1-receptor accessory-protein-like 1 (IL-1RAPL1), single immunoglobulin IL-1-receptor-related molecule (SIGIRR) and three-immunoglobulin-domain-containing IL-1-receptor-related molecule (TIGIRR) (see Allan *et al.*, 2005). While some of these new members of the IL-1 family are found to be expressed within the CNS their exact physiological role remains a matter for current research.

Family Member	Alternative Name	Receptor	Signalling Pathways	Classification
IL-1α/IL-1F1	-	IL-1RI + IL-1Acp	NFκB, MAPK	Pro-inflammatory cytokine
		IL-1RII	No signal	
IL-1β/IL-1F2	-	IL-1RI + IL-1Acp	NFκB, MAPK	Pro-inflammatory cytokine
		IL-1RII	No signal	
IL-1ra/IL-1F3	-	IL-1RI	No signal	Antagonist of IL-1 signalling
		IL-1RII	No signal	
IL-18/IL-1F4	IL-1γ/IGIF	IL-18R + IL-18Acp	NFκB, MAPK	Pro-inflammatory cytokine
IL-1F5	IL-1δ/ IL-1HY/IL-1H3/IL-1RP3/IL-1L1/FIL-1δ	IL-1RRP2 + IL-1Acp	NFκB, MAPK	Anti-inflammatory cytokine
IL-1F6	IL-1ε	IL-1RRP2 + IL-1Acp	NFκB, MAPK	
IL-1F7	IL-1ζ/IL-1H/IL-1H4/IL-1RP1	IL-18R + IL-18Acp	NFκB, MAPK	
IL-1F8	IL-1η/IL-1H2	IL-1RRP2 + IL-1Acp	NFκB, MAPK	
IL-1F9	IL-1H1/IL-1RP2	IL-1RRP2 + IL-1Acp	NFκB, MAPK	
IL-1F10	IL-1HY2/FKSG75	-	-	
IL-33/IL-1F11	-	ST2L + IL-1Acp	NFκB	Anti-inflammatory cytokine

Table 1.1: Members of the IL-1 Family: Receptors & Signalling Pathways
(see Allan *et al.*, 2005; Costello *et al.*, 2008; Luheshi *et al.*, 2009)

IL-1 has is known to have diverse physiological effects. As such it has been implicated in slow-wave sleep, synaptic plasticity and appetite and is also known to induce fever, astrogliosis and neovascularisation within the CNS. The involvement of IL-1 in learning and memory has been found to follow an inverted U-shaped pattern (Goshen *et al.,* 2007). In this regard, it has been shown that physiological concentrations of IL-1 are necessary for hippocampal-dependent memory formation, and a slight increase in IL-1 concentrations has been shown to improve memory consolidation (Goshen *et al.,* 2007). However, any deviation from the physiological range can result in impairment in memory. In this regard, both excessive concentrations of IL-1, such as that seen in the aged brain, and the blockade of IL-1 signalling have both been found to impair long-term potentiation (LTP) and memory consolidation (Murray & Lynch, 1998; Avital *et al.,* 2003; Goshen *et al.,* 2007).

In the normal CNS IL-1 is found to be expressed by neurons, astrocytes and microglia (Tomozawa *et al.*, 1995; Rothwell & Luheshi, 2000). However, IL-1 can promote the expression of other pro-inflammatory cytokines such as TNF-α, IL-6 and granulocyte macrophage colony stimulating factor (GM-CSF). Furthermore, it has been found to induce the expression of CAMs and has also been implicated in antigen presentation and T-cell activation. Another potentially devastating role for IL-1β is the production of NO. While NO production is important for host defence it has also been implicated in neuronal damage in the context of the CNS (Aschner, 1998). Moreover, IL-1β has been shown to stimulate the growth, differentiation and maturation of astrocytes *in vitro* and its injection into the CNS leads to astrogliosis. Notably, IL-1β is found to be elevated in the cerebrospinal fluid (CSF) of guineas pigs with EAE and its expression is found to be increased in the brain and CSF of patients with MS and PD (Chung & Benveniste, 1990, Basu *et al.,* 2004). Nonetheless, while the expression of IL-1β can have potentially deleterious consequences, it has also been shown to promote nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 expression potentially leading to protection of neurons in early phases of brain injury (Tomozawa *et al.*, 1995; Tanabe *et al.*, 2009). Moreover, IL-1 has been found to promote remyelination (Mason *et al.*, 2001) and has also recently been demonstrated to be involved in the clearance of amyloid-β (Shaftel *et al.*, 2007).

1.4.1.3 Interferon-γ (IFN-γ)

IFN-γ is a 17-kDa polypeptide which is known to have anti-microbial and anti-tumour effects and is found to be released predominantly from activated T-cells. IFN-γ also acts to potentiate the immune response through the orchestration of leukocyte attraction and the direction, maturation and differentiation of immune cells such as T and B-cells. In addition, IFN-γ can induce the expression of MHC class I and II. Two types of IFNs are known, namely Type I and Type II IFNs. IFN-γ is currently the only Type II IFN known (Schroder *et al.,* 2004). The IFN-γ receptor consists of two ligand binding interferon-gamma receptor (IFN-GR)-1 chains and two IFN-GR2 chains. Signal transduction is mediated by means of the signalling machinery associated with the IFN-GR chains (see Fig. 1.3). Notably, The IFN-GR2 chains lack intrinsic kinase/phosphatase activity thus their association with signalling machinery is crucial. The IFNGR1 chains contain Janus tyrosine kinase (Jak)-1 binding motifs and a latent cytosolic factor signal transducer and activator of signalling (STAT)-1, required for receptor phosphorylation and signal transduction. IFN-GR2 has a Jak-2 binding motif and is also known to take part in signal transduction since, upon binding of ligand, Jak-2 undergoes autophosphorylation and activation. Activated Jak-2 transphosphorylates Jak-1, and Jak-1 in turn phosphorylates the IFN-GR1 forming two docking sites for the *Src* Homology (SH)-2 domain of STAT-1. Tyrosine phosphorylation induces the dissociation of a STAT-1 homodimer which then translocates to the nucleus and binds to promoter elements in order to initiate or suppress gene transcription. Signalling by STAT-1 can induce the expression of other transcription factors, for example interferon regulatory factor (IRF)-3, which act to further regulate gene transcription. Dissociation of the STAT-1 homodimer occurs within 1 minute of IFN-γ binding and its effects on transcription are found to occur after approximately 15-30 minutes. STAT-1 signalling is shown to cease 1 hour post-treatment, even in the presence of additional IFN-γ. IFN-γ signalling can be negatively regulated by the expression of suppressor of cytokine signalling (SOCS)-1 (Schroder *et al.,* 2004).

Notably, while the brain is normally devoid of IFN-γ, when the BBB is breached this cytokine becomes detectable. However, while IFN-γ is primarily produced in the periphery it has been proposed that astrocytes and microglia in some autoimmune diseases may locally secrete IFN-γ (Aschner 1998). In light of this, IFN-γ is the primary pro-inflammatory cytokine found in MS lesions (Hall *et al.,* 1997).

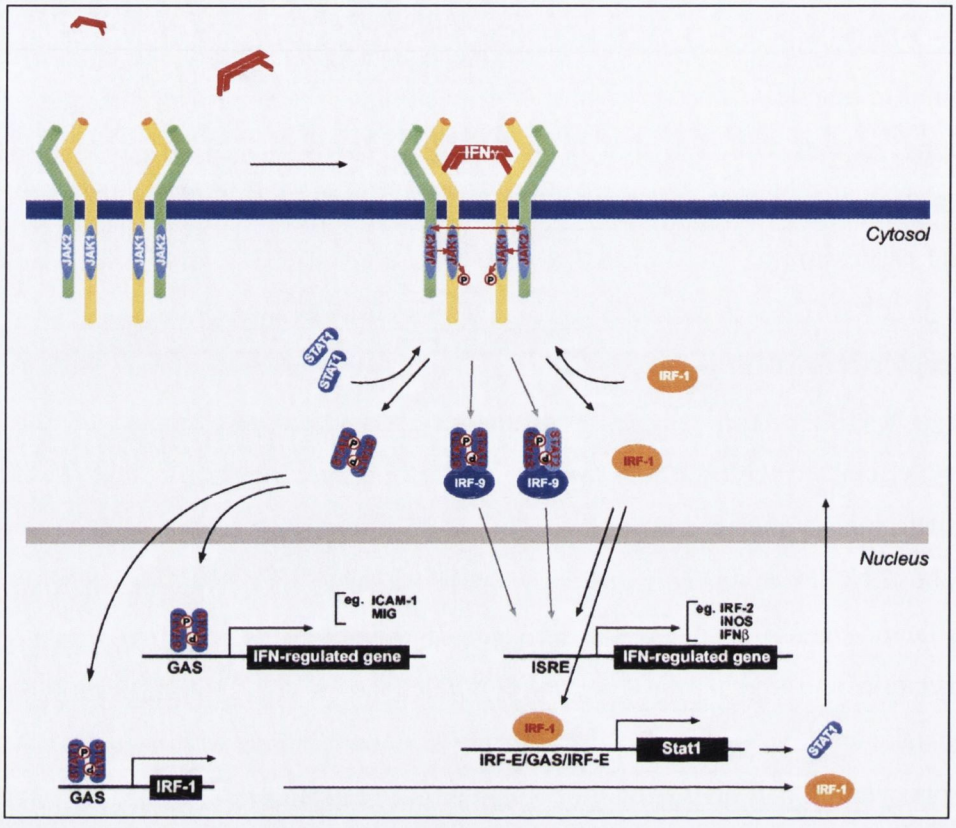


Figure 1.3: IFN-γ Signalling

(From: Schroder *et al.*, 2004)

1.4.1.4 Interleukin-6 (IL-6)

IL-6 is a member of the four-helical cytokine family and has a molecular weight of approximately 20-30kDa (Scheller & Rose-John, 2006; Tizard, 2008). IL-6 is found to be secreted by fibroblasts, monocytes, endothelial cells, B-cells, T-cells, astrocytes and microglia (Aschner, 1998) and its production can be stimulated by endotoxin, IL-1 and TNF-α (Tizard, 2008). In addition, it appears that the TNF-α-induced production of IL-6 may act to negatively regulate TNF-α expression (Aschner, 1998). IL-6 binds to a receptor complex comprised of a hexamer of two IL-6 receptors (IL-6R) and two molecules of the signal transducing protein gp130 (see Fig. 1.4) (Scheller & Rose-John, 2006). While expression of the IL-6 receptor is found on hepatocytes, leukocytes and microglia its expression is somewhat limited. In contrast, gp130 is expressed on most cell types and it has been shown that gp130 has the capacity to bind complexes of IL-6 and soluble IL-6R to initiate signalling in cells which do

not possess IL-6R, a mechanism referred to as transsignalling (Lee *et al.*, 2002; Scheller & Rose-John, 2006).

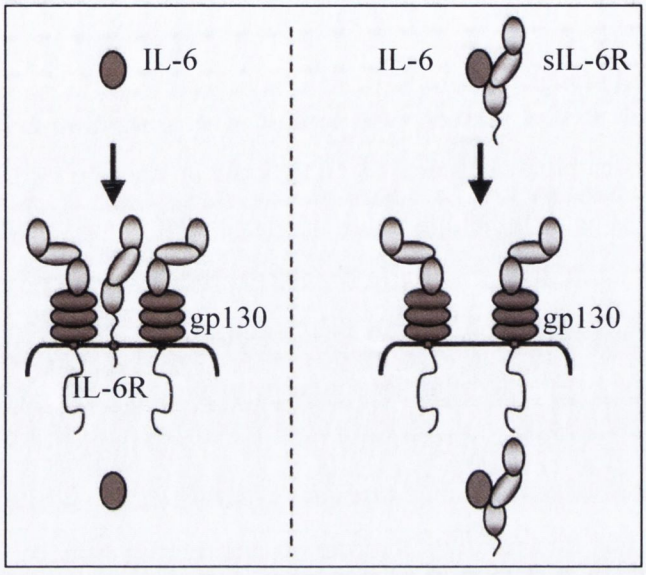


Figure 1.4: IL-6 Signalling
(adapted from Scheller & Rose-John, 2006)

IL-6 has been implicated in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, Crohn's disease, asthma, colon cancer and murine peritonitis (Scheller & Rose-John, 2006). In this regard, it has been shown that IL-6 plays a role in leukocyte recruitment, apoptosis, T-cell activation and it has also been demonstrated to have mitogenic actions on astrocytes and so may contribute to the induction of gliosis. In addition, it is thought that IL-6 can induce a switch in the inflammatory reaction changing it from a neutrophil-dominated process to a more macrophage-dominated process (Aschner, 1998; Scheller & Rose-John, 2006; Tizard, 2008). Furthermore, IL-6 is known to activate the hypothalamic-pituitary adrenal (HPA)-axis and thus is shown to play a role in the febrile response, however it is known to be far less potent than IL-1 (Dunn, 2006; Tizard, 2008). Of note is the suggestion that IL-6 can induce the secretion of NGF from astrocytes (Aschner, 1998).

1.4.2 Non-cytokine pro-inflammatory mediators

1.4.2.1 Nitric Oxide (NO)

Nitric oxide is synthesized by the enzyme nitric oxide synthase (NOS) through a series of sequential oxidation steps that converts the amino acid L-arginine to L-citrulline (Pannu & Singh, 2006). There are three subtypes of NOS known, namely endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed in the CNS while iNOS is not normally expressed in the post-natal brain, however its expression has been found in glia under inflammatory conditions (Pannu & Singh, 2006). During inflammation NO is predominantly found to be induced by iNOS (Galea & Feinstein, 1999). Furthermore, it has been demonstrated that the expression of iNOS can be induced by IL-1β, TNF-α and IFN-γ, which in many cases act synergistically. While the production of NO can be beneficial to the host due to its role in the elimination of infiltrating microbes, continued activation of iNOS and production of NO can result in tissue damage. In this regard, it has been found that NO can react with superoxide free radicals to produce peroxynitrite, an event which has been linked to cell death (Merrill *et al.,* 1993). In addition, the production of NO by microglia *in vitro* has been shown to be cytotoxic to oligodendrocytes (Merrill *et al.,* 1993). Upon its induction, iNOS continues to be expressed for 4-8 days and is shown to be 100-1000 more efficient in synthesizing NO than nNOS (Pannu & Singh, 2006). Consequently, there are generally important regulatory constraints in place which control the activity iNOS in order to ensure that its beneficial actions outweigh detrimental effects. Significantly, astrocytes and microglia are found to express iNOS in neuroinflammatory conditions such as demyelinating diseases, cerebral ischemia and Alzheimer's disease (Galea & Feinstein, 1999). Interestingly, the microglial expression of iNOS has been found to induce neuronal death however, co-treatment of glia with the neurotransmitter noradrenaline, a neurotransmitter which can dampen microglial activation, was shown to protect neurons (Madrigal *et al.,* 2005).

1.4.2.2 Cyclooxygenase (COX)-II and prostaglandins

Two isoforms of COX are known to exist in the body, namely COX-I and COX-II. COX-I is shown to be constitutively expressed while the expression of COX-II is low under normal conditions but can be rapidly induced in response to inflammation. COX-II is one of the three

enzymes found to be involved in the conversion of arachidonic acid to prostaglandin E_2 (PGE_2). The other two enzymes involved in this process include phospholipase A_2 (PLA_2) and PGE_2 isomerase (Quan *et al.*, 1998). The initial release of arachidonic acid from phospholipids of cell membranes requires the actions of PLA_2. Subsequently, COX-II converts arachidonic acid to prostaglandin H_2 (PGH_2), an unstable precursor of PGE_2. This is known to be the rate-limiting step in the production of PGE_2. Finally, PGH_2 is converted to PGE_2 by the actions of PGE_2 isomerase (see Fig. 1.5) (Cao *et al.*, 1995; Quan *et al.*, 1998). The mechanisms by which PGE_2 can elicit a response in the brain are disputed. Some suggest that PGE_2 is lipophilic and can cross the BBB to gain entry to the CNS, while others suggest that it activates sensory neurons which can then relay signals to the brain. However, it appears more likely that the expression of PGE_2 in the brain is mediated centrally (Blatteis *et al.*, 2005). PGE_2 signals through four different receptors, namely EP1-EP4, of which EP1 and EP2 are found to be expressed on microglia (Akundi *et al.*, 2005).

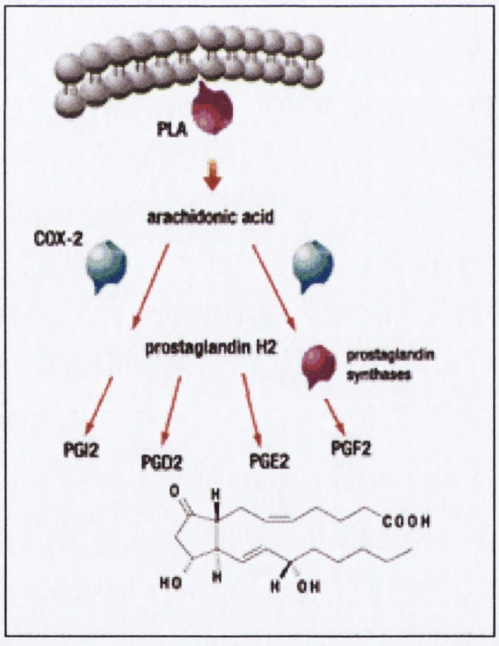


Figure 1.5: PGE2 production

(adapted from www.htrf.com/images/products/pge2_product.gif)

The expression of COX-II and prostaglandins in the CNS has been demonstrated to play a role in activation of the HPA-axis and the induction of fever. In addition, it has been found to stimulate the production of ROS by microglia and furthermore, increased expression is

observed in neuroinflammatory conditions such as hypoxia, ischemia, PD and AD (Cao *et al.*, 1995; Quan *et al.*, 1998; Akundi *et al.*, 2005). COX-II is shown to be constitutively expressed in the CNS in neurons of the cortex, hippocampus, dentate gyrus and amygdala (Quan *et al.*, 1998). Following an inflammatory challenge with lipopolysaccharide (LPS) it was demonstrated that the expression of COX-II increases by 50-80% above that of basal levels (Cao *et al.*, 1995). In addition it has been shown that, following an inflammatory challenge, COX-II is initially found expressed in the major blood vessels and meninges but later becomes evenly expressed throughout the brain in vascular and perivascular cells surrounding small blood vessels (Quan *et al.*, 1998).

1.4.2.3 Chemokines

Collectively chemokines are a family of small proteins which range in size from approximately 8-12kDa and are found expressed in both the peripheral nervous system (PNS) and CNS. There are four major classes of chemokines, namely C, CC, CXC and CX3C, which are designated according to the number of and distance between key cysteine residues. Chemokines signal through specific high and low-affinity G-protein coupled receptors (GPCR) which are 340-370 amino acids in length and, with a few exceptions, chemokines have the ability to act on more than one receptor. Pathways implicated in chemokine receptor signalling include those of cyclic adenosine 5'3' monophosphate (cAMP), phospholipase C (PLC), protein kinase C (PKC), MAPKs and Jak/STAT (Bajetto *et al.*, 2001; Adler *et al.*, 2005).

In the CNS, chemokines and their receptors are shown to be expressed on neural progenitor cells, neurons, astrocytes and microglia in regions such as the hypothalamus, limbic system, hippocampus, cortex, olfactory bulbs and cerebellum. Chemokines are known to play a major role in brain function and as such are associated with the trafficking of leukocytes and recruitment of inflammatory cells. In addition, they are found to be involved in neuronal development, neuronal apoptosis, the release of glutamate from astrocytes and the modulation of ion channel activity. A number of chemokines are found to have selective distribution within the CNS, among them are γ-interferon-inducible-protein 10 (IP-10/CXCL10) and regulated on activation normal T-cell expressed and secreted (RANTES/CCL5). Notably, the expression of chemokines and their receptors is found to be upregulated during inflammation.

In line with this, elevated levels of chemokines have been demonstrated in neuroinflammatory conditions such as MS, stroke, Alzheimer's disease and HIV encephalitis (Bajetto *et al.*, 2001; Adler *et al.*, 2005).

1.4.2.4 Cell adhesion molecules (CAMs)

CAMs are involved in the extravasation of leukocytes from the bloodstream and the trafficking of leukocytes to the site of inflammation during the inflammatory process. Furthermore, it is suggested that they can also act as co-stimulators for T-cell activation (Ballestas & Benveniste, 1997). Two primary CAMs are ICAM-1/CD54 and VCAM-1/CD106. ICAM-1 acts as a ligand for LFA-1 and Mac-1 (also known as CD11b/CD18), both of which are β2-integrins found expressed on granulocytes and lymphocytes. VCAM-1 acts as a ligand for the α2β1 and α4β7 integrins which are expressed on lymphocytes, monocytes, eosinophils and basophils. CAMs are suggested to be major players in the formation of synapses and also in the initiation of LTP. Furthermore, CAMs are also found to induce reorganization of the extracellular space and as such they may cause disturbances in the CNS resulting in disease (Jovanova-Nesic & Shoenfeld, 2006).

1.4.2.5 Co-stimulatory molecules

The CNS is no longer considered an immune privileged site and instead it is now recognized that T-cells can enter the CNS to carry out immune surveillance. The activation of T-cells is considered to be dependent on a two-signal process. First, APCs initiate an immune response by presenting processed antigens to $CD4^+$ T-cells. This event is dependent on the binding of MHC class II to T-cell receptors in association with the accessory molecule CD4 which results in the activation of downstream signalling pathways. In turn these signalling pathways can increase the expression of interleukin-2 (IL-2) and the IL-2 receptor and induce T-cell proliferation. In addition, the activation of downstream signalling pathways can increase the expression of CD40 on the surface of APCs which in turn can bind to the CD40 ligand (CD40L) on the T-cell surface resulting in the production of IFN-γ (O'Keefe *et al.*, 2002). CD40 is a member of the TNF receptor family and its expression has been found in numerous cell types including B-cells, monocytes, dendritic cells, vascular endothelial cells, smooth muscle cells and most importantly, astrocytes and microglia within the CNS. Under resting conditions the expression of CD40 on microglia is low but becomes markedly increased during inflammation and experimental autoimmune disease (Chen *et al.*, 2006a; Ponomarev *et*

al., 2006). The CD40/CD40L interaction can induce the expression of the B7 proteins, namely B7-1 (CD80) and B7-2 (CD86), on the surface of APCs. This in turn can bind to CD28 expressed on T-cells to initiate the second signal required for complete T-cell activation leading to T-cell clonal expansion (O'Keefe *et al.*, 2002). The CD40/CD40L interaction can also serve to amplify the inflammatory response through the induction of pro-inflammatory cytokines, nitric oxide, metalloproteinases and chemokines (Chen *et al.*, 2006a). However, when T-cells interact with antigen in the absence of co-stimulatory molecules they undergo anergy (O'Keefe *et al.*, 2002).

1.4.3 Anti-inflammatory Mediators

1.4.3.1 Interleukin-10 (IL-10)

IL-10 is an 18kDa protein which was first characterized as cytokine synthesis inhibitory factor (CSIF). IL-10 is a member of the class 2α-helical cytokine family which is comprised of IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29, interferons, and interferon-like molecules (Pestka *et al.*, 2004; Commins *et al.*, 2008; Mosser & Zhang, 2008). It has been shown that IL-10 can be released from Th_2 cells, regulatory T-cells (Tr_1, Th_1 & Th_{17}), $CD8^+$ T-cells, monocytes, macrophages, dendritic cells and glia, amongst others (Mosser & Zhang, 2008). All members of the IL-10 superfamily signal through a Jak/STAT pathway, however, the biological activities which they initiate differ according to the cells in which they are produced and the cells on which they act. IL-10 signalling occurs as a result of activation of the IL-10 two-receptor complex (see Fig. 1.6). This receptor complex is comprised of four transmembrane polypeptides composed of two IL-10R1 chains and two IL-10R2 chains. IL-10 binds to IL-10R1 with high affinity while its binding to IL-10R2 is of a lower affinity, however, engagement of IL-10R2 is essential for signalling to occur (Pestka *et al.*, 2004; Mosser & Zhang, 2008). The activation of Jak1 and tyrosine kinase 2 (Tyk2) occurs upon binding of IL-10 to the receptor complex, since Jak1 is constitutively bound to IL-10R1 while Tyk2 is constitutively bound to IL-10R2. Activation of Jak1 and Tyk2 is presumed to occur through cross-phosphorylation of two tyrosine residues (Y446 and Y496) on the intracellular domain of the receptor which in turn mediates the recruitment of STAT-3 to the receptor complex. Consequently, STAT-3 becomes phosphorylated allowing it to form homodimers and detach from the receptor. STAT-3 can then translocate to the nucleus where it induces transcription of STAT-3-responsive genes such as that of IL-10 itself and the SOCS

molecules, SOCS1 and SOCS3. Importantly the IL-10 receptor does not have SOCS-binding sites and so is not subject to regulation by SOCS proteins (Mosser & Zhang, 2008). STAT-3 is known to be important for limiting inflammation since its deletion has been found to result overactivation of the innate immune response and impaired antigen presentation (Qin *et al.*, 2006). A region in the C-terminal domain of the IL-10 receptor is found to mediate the anti-inflammatory activity of IL-10 but is not involved in STAT-3 phosphorylation. However, parallel activation of this region and STAT-3 phosphorylation is necessary for the anti-inflammatory activities of IL-10 to occur (Pestka *et al.*, 2004).

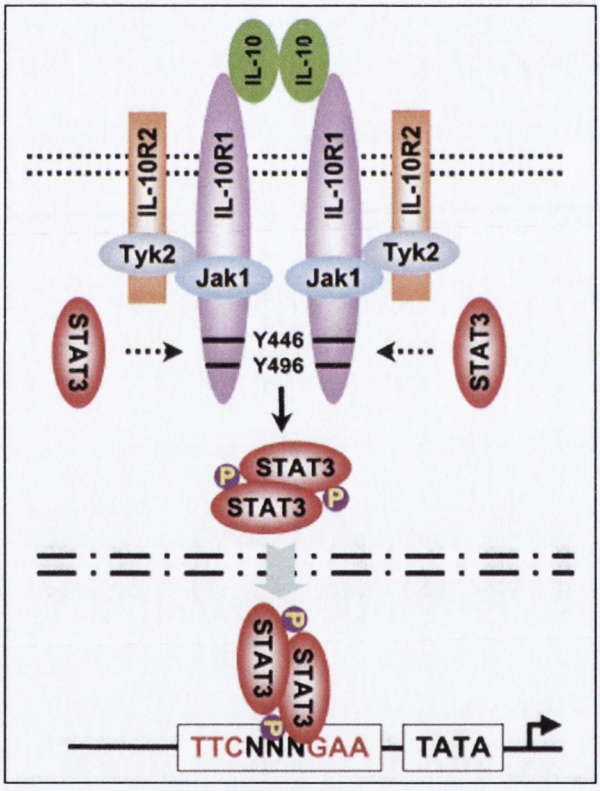


Figure 1.6: IL-10 Signalling

(From: Mosser & Zhang 2008)

IL-10 has been shown to promote the development of B1 cells, the proliferation of natural killer (NK) cells and to enhance the survival of B-cells, T-cells and tumour cells. While IL-10 signalling can initiate a wide variety of activities, its principle function is to limit the scale of the immune response. In this regard it has been shown that IL-10 blocks the production of a number of cytokines including IL-1, IL-4, IL-6, IL-8, IL-12 and TNF-α, amongst others. This

is suggested to occur owing to IL-10-induced inhibition NFκB activity, preventing the translocation of NFκB to the nucleus (Mosser & Zhang, 2008). IL-10 has also been shown to reduce the secretion of chemokines and the expression of the co-stimulatory molecules CD28, CD80, CD86 and MHC class II (Pestka *et al.*, 2004; Commins *et al.*, 2008; Mosser & Zhang, 2008). Notably, IL-10 knock-out mice die following what is normally considered to be a sublethal dose of LPS and further to this these mice also show exacerbated disease severity in models of experimental autoimmune diseases such as rheumatoid arthritis, EAE and inflammatory bowel disease (Mosser & Zhang, 2008). Significantly, the exogenous administration of IL-10 has been shown to attenuate inflammation in models of chronic renal disease, autoimmune encephalomyelitis, pneumococcal meningitis and following traumatic brain or spinal cord injury (Cua *et al.*, 2001; Kastin *et al.*, 2003; Mu *et al.*, 2005). While it has been reported that IL-10 can suppress the expression of pro-inflammatory cytokines induced by amyloid-β, it has also been reported that amyloid-β is incapable of inducing IL-10 expression. In light of this since IL-10 is a potent inhibitor of inflammation, the lack of IL-10 induction in response to amyloid-β may go some way to account for the chronic inflammation observed in Alzheimer's disease (Szczepanik *et al.*, 2001).

Notably, while IL-10 is stable in blood and brain, its ability to cross the BBB following peripheral administration is limited since it does not cross by a saturable transport system (Kastin *et al.*, 2003) therefore limiting its potential as a therapeutic treatment. Furthermore, the finding that IL-10R2 is expressed on most cell types may represent a problem for therapeutic administration of IL-10, since the administered dosage will be reduced by binding of IL-10 to non-target cells (Mosser & Zhang, 2008).

1.4.3.2 Suppressor of Cytokine Signalling 3 (SOCS3)

SOCS proteins were discovered as the first inducible feedback inhibitors of type I and type II cytokine receptors (Dalpke *et al.*, 2008). Eight members of the SOCS family are currently known to exist, namely SOCS-1 to -7 and cytokine-inducible SH2-containing protein (Qin *et al.*, 2007). The genes of SOCS proteins are small, containing only a few introns, and thus they bear resemblance to immediate early genes. While SOCS proteins are constitutively expressed, their transcription increases rapidly within minutes to hours upon inflammatory challenge (Dalpke *et al.*, 2008). All members of the SOCS family are known to contain a

central SH2 domain, an N-terminal domain, which is variable in length, and a C-terminal domain, more commonly known as the SOCS box (Qin *et al.*, 2007; Dalpke *et al.*, 2008; Mosser & Zhang, 2008). The SH2 domain acts to bind substrate while the SOCS box can complex with other molecules, such as elongins, to form an E3 ubiquitin ligase (Mosser & Zhang, 2008). Both SOCS1 and SOCS3 have an additional kinase inhibitory region (KIR) in their N-terminal domain which is said to be responsible for inhibition of Jak signalling (Qin *et al.*, 2007; Dalpke *et al.*, 2008). Negative regulation of cytokine signalling by SOCS proteins is thought to occur mainly at the level of the receptors through inhibition of Jak/STAT signalling (Kile & Alexander, 2001; Dalpke *et al.*, 2008). In line with this, the expression of SOCS3 is known to inhibit GM-CSF, IL-6, interferons, leptin, and leukemia inhibitory factor (LIF) by binding to their phosphorylated receptors (Kile & Alexander, 2001). Notably, SOCS3 has been shown to positively regulate ERK signalling. In contrast, SOCS3 has been shown to inhibit NFκB and cAMP signalling (Qin *et al.*, 2007).

SOCS3 expression can be induced by stimulation of Toll-like receptor 4 (TLR-4) by LPS or by activation of MAPKs (Dalpke *et al.*, 2008). As such, LPS is known to be a potent activator of SOCS3 expression in macrophages and microglia, an event which is primarily dependent on IL-10 and STAT-3 signalling since its expression was diminished in macrophages from IL-10-deficient mice and when STAT-3 was blocked by small interfering ribonucleic acid (siRNA) (Qin *et al.*, 2007). Furthermore, it has been found that LPS and IL-10 can work synergistically to increase the expression of SOCS3 (Qin *et al.*, 2006). However, SOCS3 expression following LPS administration is not solely dependent on IL-10 signalling at early timepoints, yet its continued expression is found to be IL-10-dependent (Qin *et al.*, 2007). The expression of SOCS3 is imperative since it has been shown that SOCS3 knock-out is embryonically lethal due to placental defects, thus conditional knock-out is required to study the functions of this protein (Dalpke *et al.*, 2008). Furthermore, impairment of SOCS3 has been found to lead to increased disease severity in a mouse model of IL-1-dependent inflammatory arthritis (Qin *et al.*, 2006).

1.5 Generation of an experimental inflammatory environment

The *in vitro* studies presented in this thesis employ a combined treatment of LPS + IFN-γ to instigate an inflammatory response in glial cells similar to that observed in MS. This treatment regimen results in cells being exposed to the T-cell (Th_1) cytokine IFN-γ, in addition to the pro-inflammatory cytokines IL-1β, TNF-α and members of the IL-12 superfamily (IL-12, IL-23, IL-27) which are induced by LPS (Pouly & Antel, 1999; Martino *et al.*, 2002). Systemic administration of LPS was employed for *in vivo* studies, since LPS is known to induce the expression of the pro-inflammatory mediators IL-1β, TNF-α, IFN-γ and iNOS in the CNS several hours after administration (Connor *et al.*, 2008; Konsman *et al.*, 2008; O'Sullivan *et al.*, 2009).

1.5.1 Lipopolysaccharide (LPS)

As previously discussed, the body detects the invasion of foreign microbes through the recognition of PAMPs by specific cells of the immune system resulting in the induction of a robust inflammatory response. One such PAMP is LPS, an endotoxin derived from the outer cell membrane of gram-negative bacteria which is known to affect the transcription of more than 1000 genes (Jerala, 2007). LPS signals through TLRs which are expressed on the surface of many cell types. TLRs are type I transmembrane receptors which are comprised of an extracellular domain consisting of multiple leucine rich repeats (LRRs) and a cytoplasmic domain which has an IL-1 receptor (IL-1R) homologous region known as a TLR/IL-1R (TIR) domain. Notably however, the extracellular immunoglobulin domain of IL-1R distinguishes it from TLRs. LPS signalling primarily occurs via activation of TLR-4 which is targeted against the lipid A moiety of gram-negative bacteria (Jerala, 2007). TLR-4 and the adapter protein CD14 are shown to be constitutively expressed in the CVOs and circulating LPS has been demonstrated to increase the expression of CD14 (Jerala, 2007). However, while it is known that TLR-4 is constitutively expressed in the CNS, the cell types on which it is found remains a matter for debate. In this regard, a recent study has shown TLR-4 to be present on microglia but not on astrocytes, oligodendrocytes or neurons (Akundi *et al.*, 2005). In contrast, other studies suggest that microglia express high levels of TLR-4 mRNA, while astrocytic expression is low (Jack *et al.*, 2005a).

LPS signalling through TLR-4 can go down one of two pathways (see Fig 1.7), namely a myeloid differentiation factor 88 (MyD88)-dependent or MyD88-independent pathway. The first step of LPS signalling involves the binding of LPS to the adapter protein LPS-binding protein (LBP), a 58-60kDa serum or cell-associated glycoprotein. The LPS-LBP complex can then bind with the glycosylphosphatidylinositol (GPI)-anchored adapter protein CD14, which can be expressed either anchored to the cell membrane or in a soluble form. CD14 transfers an LPS monomer to MD-2, a secreted adapter protein on the ectodomain of TLR-4, resulting in the formation of a trimeric LPS:MD-2:TLR-4 complex. MD-2 is crucial for the physiological response of TLR-4, since it is found that no signalling occurs in its absence. The final stage in LPS recognition is the rearrangement of the LPS:MD-2:TLR-4 complex to allow recruitment of adapter proteins to the intracellular TIR domain of TLR-4 (Jerala, 2007).

1.5.1.1 MyD88-dependent pathway

Upon ligand stimulation, the cytoplasmic tail of TLR-4 can associate with the adapter protein MyD88 (see Fig. 1.7). MyD88 is comprised of an N-terminal death domain and a C-terminal TIR domain. The death domain of MyD88 has been shown to associate with the adapter protein IL-1R associated kinase (IRAK), a serine kinase involved in the activation of another adapter protein identified as TNF receptor associated factor 6 (TRAF-6). Activated TRAF-6 goes on to activate another protein kinase, termed inhibitor of NFκB (IκB) kinase (IKK), possibly through the activation of NFκB inducing kinase (NIK) which can directly interact with IKKs (Takeuchi & Akira, 2001). IKK is composed of two catalytic subunits, namely α and β, and a regulatory subunit termed IKK-γ/NEMO (Nguyen *et al.*, 2002). The regulatory subunit is usually required for the activation of NFκB. NFκB is normally sequestered in an inactive state in the cytoplasm of almost all cell types and its activity is regulated by the presence of an inhibitory molecule, known as IκB, which is essential for the activation of NFκB in response to cytokines and PAMPs. IκB binds tightly to NFκB dimers and holds them in an inactive complex. Activation of IκB kinase kinase (IκBKK) phosphorylates IκB kinase and this in turn phosphorylates IκB at its serine and threonine residues. Following phosphorylation of IκB it undergoes polyubiquitination and is targeted to the proteasome for degradation and recycling. The phosphorylation of IκB activates NFκB which subsequently translocates to the nucleus where it can regulate the expression of approximately 60 genes involved in inflammation, for example those coding for cytokines (TNF-α, IL-1, IL-6),

chemokines, enzymes (COX-II and iNOS) and CAMs (Minagar *et al.*, 2002; Simi *et al.*, 2005). Thus, the MyD88 pathway is responsible for the fast activation of NFκB (Moynagh, 2005). Furthermore, the MyD88-dependent pathway is critical for the response to LPS since MyD88-deficient mice are shown to be resistant to LPS-induced shock, and macrophages derived from such mice do not secrete TNF-α, IL-6 or NO in response to LPS (Takeuchi & Akira, 2001).

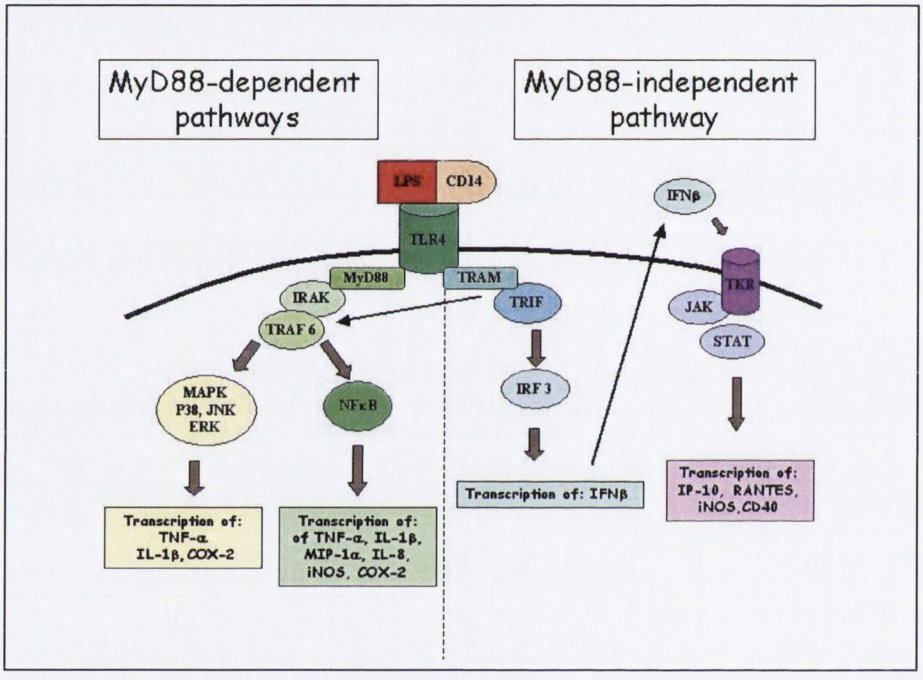


Figure 1.7: TLR signalling pathways: MyD88-dependent & MyD88-independent pathways (From: O'Sullivan 2007)

1.5.1.2 MyD88-independent pathway

In addition to the regular MyD88 pathway induced by LPS, a MyD88-independent pathway (see Fig. 1.7) has also been identified which is also found to involve CD14 and LBP (Jerala, 2007). Activation of the MyD88-independent pathway is responsible for dendritic cell (DC) maturation and the production of interferon-β (IFN-β). This pathway also activates NFκB, although much more slowly than that of the MyD88-dependent pathway. The MyD88-independent pathway was first identified in MyD88-deficient mice which were found to be unable to produce IL-1β, TNF-α or IL-6 in response to LPS but maintained the capacity to produce interferons. The key regulator of the MyD88-independent pathway is an adaptor

protein known as TRIF or TICAM-1 (TIR domain-containing adapter-inducing IFN-β or TIR-containing adapter molecule). TRIF is also found to activate NFκB signalling through TRAF-6. The activation of interferons occurs through the initial phosphorylation of IRFs, in particular IRF-3. IRF-3 is expressed at high constitutive levels in cells and is responsible for early IFN-β gene induction. Phosphorylation of IRF-3 occurs at serine and threonine residues in the C-terminal allowing a conformational change to occur whereby a DNA-binding domain becomes exposed along with an IRF-association domain. Dimerisation of IRFs consequently occurs and these dimers are then shuttled into the nucleus where they bind to motifs of relevant promoters. The activation of IRF also allows for its association with co-activators such as CBP (CREB binding protein) and p300. Furthermore, production of IFN-β results in the activation of a transcription factor complex known as IFN-stimulated gene factor 3 (ISGF3) which includes the transcription factors STAT-1 and STAT-2 and IRF-9. This complex is also found to induce the expression of IRF-7 which in turn can stimulate the transcription of IFN-α and IFN-β. Activation of this pathway can also lead to the expression of the IFN-inducible chemokines RANTES and IP-10 (Fitzgerald *et al.*, 2003; Moynagh, 2005).

1.5.1.3 MAPK Signalling Pathways

Signalling by TLRs can also lead to the activation of a number of MAP kinase cascades including that of p38 and JNK (see Fig. 1.8). p38 and JNK are classed as stress-activated protein kinases (SAPKs). Three members of the JNK family have been identified so far, namely JNK-1, JNK-2 and JNK-3. JNK-1 and JNK-2 are found to be ubiquitously expressed while JNK-3 is expressed primarily in the heart, brain and testis. Four members of the p38 family have been identified so far, namely p38α, p38β, p38γ and p38δ (Rincon & Davis, 2009). MAPK signalling occurs when the small GTPase Ras becomes activated by ligand binding and phosphorylates a MAPK kinase kinase (MAPKKK), most likely MEKK1, which in turn phosphorylates a MAPK kinase (MAPKK), either MKK4 or MKK7 for the JNK pathway or MKK3 or MKK6 for the p38 pathway. MEKK1 appears to regulate the activation of JNKs but fails to activate p38, for which the activating MAPKKK is as yet unidentified Activated MAPKK can subsequently stimulate the phosphorylation of either JNK or p38 (Kyriakis & Avruch, 1996; Herlaar & Brown, 1999; Rincon & Davis, 2009). JNK and p38 in turn go on to activate the transcription factors c-jun and activating transcription factor 2 (ATF-2). In addition, p38 can activate the transcription factor c-fos (see Fig. 1.8). These

transcription factors are known to control the recruitment of leukocytes to the site of inflammation and the transcription of inflammatory cytokines, in addition to playing an essential role in cell growth, cell differentiation, cell cycle and cell death (Ono & Han 2000). Both JNK and p38 are found to be activated during the innate and adaptive phases of the immune response. Notably, the resolution of the immune response appears to require downregulation of JNK and p38 signalling (Rincon & Davis, 2009).

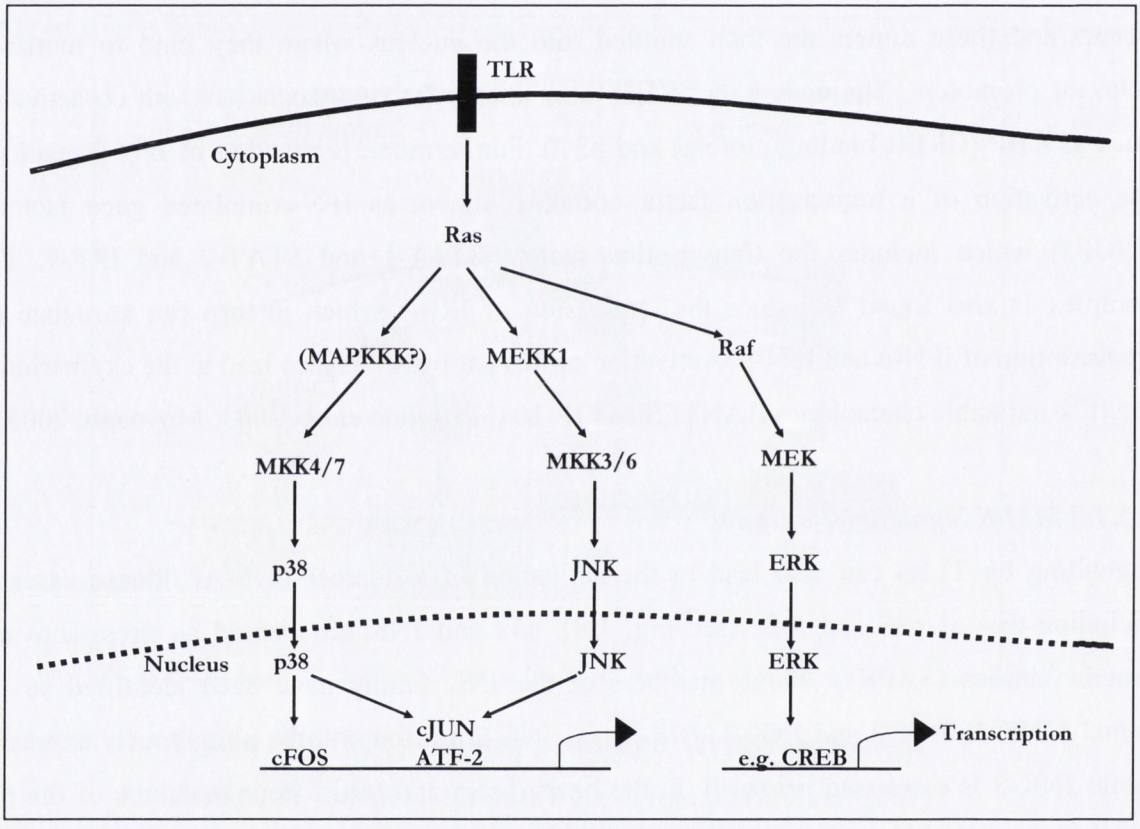


Figure 1.8: MAPK Signalling

ERK is another member of the MAPK family. ERK signalling is initiated by the activation of the small G-protein Ras which in turn phosphorylates the cytosolic protein kinase Raf. Raf in turn phosphorylates the protein kinase MAPK/ERK kinase (MEK) leading to the phosphorylation of ERK (see Fig. 1.8). ERK has been shown itself to phosphorylate a number of substrates resulting in alterations in gene expression, metabolism and morphology of cells. ERK signalling has been found to play a role in determining cell fate, since it is involved in cell proliferation, differentiation, senescence and survival. Notably, it has been shown that there is cross-talk between the ERK and cAMP signalling pathways and that ERK can

phosphorylate phosphodiesterase Type 4 (PDE4) inactivating the enzyme and leading to increased levels of cAMP. This cAMP can then feedback on PKA leading to an inhibition of ERK, suggested to be a negative feedback loop by which ERK can regulate its own expression. In addition, the reverse situation has also been found to occur whereby cAMP is capable of inducing the expression of ERK (Dumaz & Marais, 2005). Significantly, the activation of JNK and p38 has been demonstrated to differentiate Th_0 T-cells into Th_1 T-cells while activation of ERK in the absence of JNK and p38 differentiates Th_0 T-cells into Th_2 T-cells (Rincon & Davis, 2009).

1.6 Therapeutic anti-inflammatory agents – glucocorticoids

Glucocorticoids act as immunosuppressants and are commonly used in the treatment of many inflammatory and autoimmune diseases. The predominant effect of glucocorticoids is to switch off inflammatory genes encoding for cytokines, chemokines, adhesion molecules, enzymes, receptors and proteins (Barnes, 2006) and to switch on genes encoding anti-inflammatory molecules such as those of IL-10, IL-1ra and lipocortin-1 (Barnes, 1998). Glucocorticoids exert their effects by binding to glucocorticoid receptors located in the cytoplasm of cells. To date only one glucocorticoid receptor has been identified. Structurally the receptor has a binding domain located at the C-terminal end and two zinc fingers in the middle of the molecule which interact with deoxyribonucleic acid (DNA). The inactive receptor is found bound to two molecules of heat shock protein (hsp) 90, an immunophilin protein and a number of other inhibitory proteins which forms a complex of approximately 300kDa (Glezer & Rivest, 2004). The hsp90 molecules prevent the receptor from localizing to the nucleus in the absence of ligand and, as such, effectively act as "molecular chaperones". The receptor becomes activated upon binding of glucocorticoid at which point hsp90 dissociates, and the receptor moves into the nucleus where it binds DNA (Barnes, 1998). DNA binding occurs in what are termed glucocorticoid response elements (GREs) which are located in the 5'-upstream promoter region of genes responsive to glucocorticoids. Binding of glucocorticoid receptors to GREs can result in either the induction or repression of a gene. Induction of gene transcription occurs when glucocorticoids acetylate histone residues and thus cause unwinding of DNA coiled around the residues ultimately opening up the chromatin structure and allowing transcription factors to bind more easily. Repression is caused by the

opposite effect whereby histone residues are deacetylated and DNA winds more tightly around the histone residue resulting in dense chromatin structures which are not easily accessible by transcription factors.

Glucocorticoids inhibit the transcription of many cytokines (IL-1β, TNF-α, GM-CSF) chemokines (RANTES and IL-8), CAMs (ICAM-1) and are also shown to prevent the induction of iNOS and COX-II. Additionally, glucocorticoids have been shown to promote cell survival and decrease apoptosis. Glucocorticoids are also said to regulate the activity of NFκB through IκB. As such, there is evidence to suggest that glucocorticoids regulate the predominant form of IκB, IκB-α, in mononuclear cells and T-lymphocytes, although IκB-α does not express a GRE. Notably, glucocorticoid response elements have been found in the promoter region of β-adrenoceptors (Xu, 2001).

1.7 Noradrenaline: an endogenous immunomodulator

Noradrenaline is a catecholamine neurotransmitter synthesized from the amino acid tyrosine. Its structure comprises a benzene ring, two hydroxyl groups, an intermediate ethyl chain and a terminal amine group (see Fig. 1.9). Noradrenergic terminals are principally found in the Locus Coeruleus (LC), a noradrenergic nucleus located in the pontine tegmentum in the lateral wall

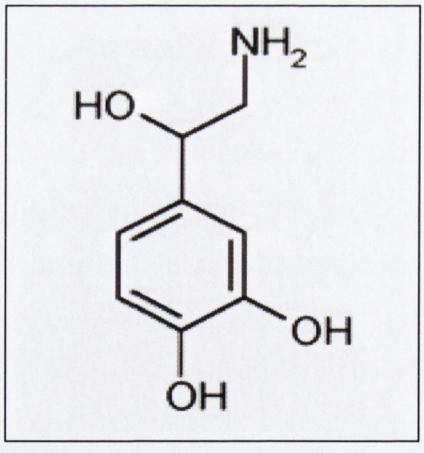


Figure 1.9: Structure of Noradrenaline

of the fourth ventricle of the brain, with terminals also found in the lateral tegmentum. Neurons from the lateral tegmentum innervate the dorsal nucleus of the vagus, the nucleus of

the tractus solitarius, the spinal cord, brainstem, hypothalamus and basal telencephalon, whilst neurons from the LC innervate the spinal cord, brainstem, cerebellum, thalamus, hypothalamus, basal telencephalon and the cerebral cortex (see Fig. 1.10) (Feinstein *et al.,* 2002; Loder *et al.,* 2002). Consequently, noradrenergic nerve terminals are found widely distributed throughout the CNS. Unlike other classical neurotransmitters, noradrenaline is not released merely from the synaptic terminal of the neuron where it is synthesized, but instead is released at varicosities along the entire length of the neuron and as such, noradrenaline is considered to act on neighbouring cells in a fashion similar to that of a paracrine agent (Feinstein *et al.,* 2002). Thus, since noradrenaline is released into the extracellular fluid it is available to bind not only to its neuronal receptors but also to adrenoceptors expressed on microglia and astrocytes (Mori *et al.,* 2002).

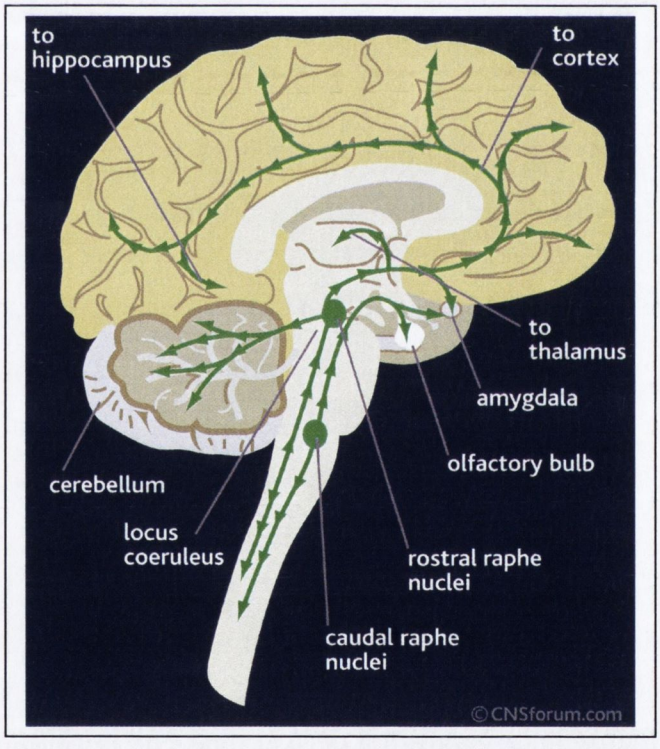


Figure 1.10: Locus Coeruleus Projection Pathways

(From: www.cnsforum.com)

Biosynthesis of noradrenaline begins with the conversion of sources of dietary phenylalanine to the amino acid tyrosine in the liver by the enzyme phenylalanine hydroxylase, although tyrosine can also be sourced directly from the diet. Within the adrenal medulla, tyrosine is

converted to dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase (TH), which requires the presence of the co-factor tetrahydropteridine. This is known to be the rate-limiting step in the biosynthesis of noradrenaline. DOPA is subsequently converted to dopamine by DOPA decarboxylase and dopamine is can then be converted to noradrenaline by the enzyme dopamine-β-hydroxylase (DβH) (Leonard, 1997). The biosynthetic pathway can go one step further with the conversion of noradrenaline to adrenaline by the enzyme phenethanolamine N-methyltransferase. Upon its release noradrenaline exerts its effects in the CNS through adrenoceptors located on neurons and glia which will be discussed in detail later.

Once released, noradrenaline must be quickly taken back up for degradation and recycling in order to cease activation of the neurotransmitter and thus, cease neuronal stimulation. The enzymes that metabolise noradrenaline, namely monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), are located intracellularly. Since noradrenaline is a highly polar molecule that cannot cross the cell membrane it is translocated across the plasma membrane by a pre-synaptic neuronal 80kDa transporter protein known as the noradrenaline transporter (NET). NET is a member of a family of 12 transmembrane Na^+- and Cl^- -dependent neurotransmitter transporters and its activity has been shown to be reduced or inhibited in low Na^+ conditions and low temperatures. In addition to being targeted for degradation, noradrenaline can also be taken up by the vesicular monoamine transporter (VMAT), which has a higher affinity for noradrenaline than MAO, and so noradrenaline can be sequestered into storage vesicles (~70%) for later re-release. Storage of noradrenaline allows for a decreased need for *de novo* synthesis (Eisenhofer, 2001). Astrocytes have been shown to express mRNA for NET and this has been identified to be the same NET as that expressed on neurons. Thus, glia may act to control the activity of noradrenaline by inactivating any transmitter that escapes neuronal re-uptake since the presence of NET on astrocytes is in accordance with their intracellular expression of MAO and COMT (Inazu *et al.,* 2003).

Noradrenaline functions primarily in the regulation of mood, attention, arousal, learning and memory (Mori *et al.,* 2002). Moreover, studies have shown that noradrenaline can suppress the expression of inflammatory genes such as those encoding iNOS, CAMs, MHC class II, IL-1β and TNF-α in microglia, astrocytes and brain endothelial cells *in vitro* and it is thought that in this way neuronal systems may be able to self-regulate inflammation (Feinstein *et al.*, 1993;

Feinstein *et al.*, 2002). Furthermore, noradrenaline has been found to suppress the proliferation of microglial cells and is also known to alter the function of macrophages and mononuclear phagocytes in the periphery (Mori *et al.*, 2002). However, it has been suggested that the predominant mechanism by which noradrenaline contributes to a lack of inflammatory response within the CNS is through the suppression of MHC class II expression on astrocytes, thus preventing them from functioning as APCs (Feinstein *et al.*, 2002).

1.8 Adrenoceptors

Noradrenaline is the endogenous agonist for α- and β-adrenoceptors, which are found expressed on many cell types within both the PNS and CNS. α-adrenoceptors are divided into two primary subtypes namely α_1- and α_2-adrenoceptors which can be further subdivided into $\alpha_{1A,B}$ and $\alpha_{2A,B,C,D}$ (Civantos Calzada & Aleixandre de Artinano, 2001). The β-adrenoceptors are divided into β_1-, β_2- and β_3-adrenoceptor subtypes. In the CNS, microglia are found to express mRNA encoding α_{1A}-, α_{2A}-, β_1- and β_2-adrenoceptors but are devoid of mRNA for the β_3-adrenoceptor, which is primarily shown to be expressed in adipose tissue. α_1-adrenoceptors are located post-synaptically and are found to be coupled via the Gq G-protein to PLC, the activation of which leads to an increase in the concentration of free Ca^{2+} in the cytosol (Giembycz & Newton, 2006). α_2-adrenoceptors are expressed pre-synaptically and are linked to the inhibitory G-protein, Gi. Thus, their activation is found to inhibit the action of adenylate cyclase and the release of noradrenaline. As such, α_2-adrenoceptors are considered to function as autoreceptors, regulating the release of noradrenaline (Ordway, 2007). All β-adrenoceptors are found to be positively coupled to adenylate cyclase and activation of these receptors results in increased production of the second messenger cAMP (Tanaka *et al.*, 2002).

It has been proposed that noradrenaline acts as an endogenous immunomodulator within the CNS through actions at β-adrenoceptors expressed on glial cells, eliciting anti-inflammatory effects and ultimately contributing to neuroprotection (Feinstein *et al.*, 2002). While noradrenaline is known to have a higher affinity for β_1-adrenoceptors, the expression of β_2-adrenoceptors is higher than β_1-adrenoceptors on microglia. Thus, the actions of noradrenaline at β_2-adrenoceptors is said to be of greater importance within the CNS (Mori *et al.*, 2002).

1.8.1 β-adrenoceptors

β-adrenoceptors are glycoproteins consisting of 402-470 amino-acid residues with an overall molecular weight of approximately 64kDa (Xu, 2001). Structurally β-adrenoceptors have an extracellular amino terminus, three interconnecting extracellular loops, three intracellular loops and an intracellular carboxyl terminus and for this reason β-adrenoceptors are classed as members of the GPCR class A superfamily (Liggett, 2002; Swaminath *et al.*, 2004). As already mentioned, there are three subtypes of β-adrenoceptor, namely β_1, β_2 and β_3, which are primarily activated by the catecholamine neurotransmitter noradrenaline (Swaminath *et al.*, 2004). In the periphery, β-adrenoceptors are found to be expressed on skeletal muscle, cardiomyocytes, adipocytes and lymphocytes (Xiang & Kobilka, 2003). Within the CNS β-adrenoceptors are shown to be expressed on neurons and glia (Mori *et al.*, 2002; Hertz *et al.*, 2004; Hein, 2006). Notably, changes in β-adrenoceptor expression and function have been found to occur in autoimmune diseases such as MS, rheumatoid arthritis and myasthenia gravis (De Keyser *et al.*, 1999).

While both the β_1- and β_3-adrenoceptor subtypes are expressed in the brain (Nakadate *et al.*, 2006), the anti-inflammatory effects of noradrenaline on glial cells appears to be primarily mediated through activation of the β_2-adrenoceptor (Mori *et al.*, 2002; De Keyser *et al.*, 2004). The β_2-adrenoceptor is positively coupled to the membrane bound enzyme adenylate cyclase through a trimeric G_s protein, which is comprised of G_α and $G_{\beta\gamma}$ subunits (Johnson, 2001). Upon binding of noradrenaline to the receptor it was originally believed that the receptor underwent a conformational change resulting in a catalytic exchange of the receptor bound guanosine triphosphate (GTP) for guanosine diphosphate (GDP), as was thought to be the case for all GPCRs. This conformational change was believed to result in dissociation of the α-subunit (G_α) from the βγ-subunit ($G_{\beta\gamma}$) of the G-protein and it was suggested that each of these subunits then went on to generate intracellular signalling cascades. However, it is now known that the β_2-adrenoceptor can exist in two states, an active and an inactive

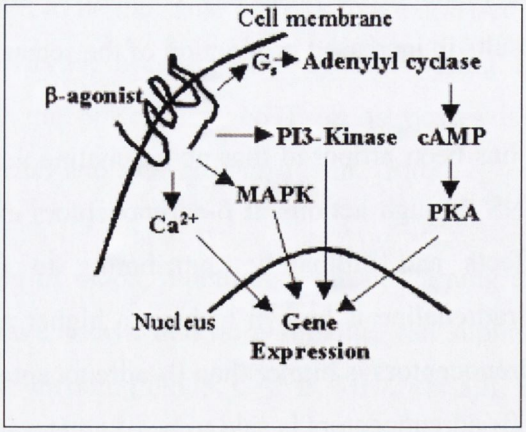


Figure 1.11: The β-adrenoceptor signalling pathway
(From: www.ans.iastate.edu)

state of which the active state may consist of a number of subspecies, with each favouring different intracellular signalling pathways (Johnson, 1998; Liggett, 2002). Under resting conditions, in the absence of agonist, it appears that the receptor can toggle between both the active and inactive state (Liggett, 2002). Agonists may therefore exert their effect not by causing a conformational change in the receptor, as was previously thought, but rather by temporarily stabilizing the receptor in its activated state where the G_α subunit of the G-protein is associated with GTP (Johnson, 1998). This idea is enhanced by the fact that when the receptor is in its inactive state, with GDP bound, β-agonists are unable to bind (Johnson, 1998).

The G_α subunit of the β_2-adrenoceptor has been shown to couple to the membrane bound enzyme adenylate cyclase and activate its enzymatic activity. Adenylate cyclase is the enzyme responsible for the conversion of ATP to cAMP, a second messenger which acts by phosphorylating protein kinase A (PKA) (see Fig. 1.11). PKA can then activate many other proteins and transcription factors, for instance cAMP-regulatory element binding protein (CREB), along with inducing phosphorylation of the receptor itself. In addition to coupling to G_s proteins and the activation of PKA, the β_2-adrenoceptor can also couple to G_i proteins and activate MAPK pathways through the actions of the $G_{\beta\gamma}$ subunit which may in turn also lead to downregulation of the receptor (Johnson, 1998; Kohm & Sanders, 2001; Liggett, 2002).

Signalling of GPCRs must be rapidly attenuated to avoid the overstimulation of cells. This attenuation is achieved by utilization of a mechanism known as homologous receptor desensitization and occurs owing to uncoupling of the receptor from its G-protein, thus preventing receptor signalling (Vroon *et al.*, 2005). Desensitization of the β_2-adrenoceptor is suggested to occur due to the activation of G-protein receptor kinases (GRKs). GRKs initially phosphorylate the agonist-occupied receptor at its intracellular C-terminal domain leading to recruitment of proteins known as arrestins to the receptor which can mediate receptor internalization, however, the receptor can also be phosphorylated on sites in its third intracellular loop by PKA or PKC (Millman *et al.*, 2004). The means by which the receptor is desensitized is dependent on the density of receptors bound by agonist since it has been observed that at low density the receptor is phosphorylated by PKA but at high density it is phosphorylated by GRKs (Rousseau *et al.*, 1997). The main GRK involved in the

internalization of the β_2-adrenoceptor is GRK2, also known as β-adrenergic receptor kinase 1 (β-ARK1), although there is also some evidence to suggest a role for GRK3/β-adrenergic receptor kinase 2 (β-ARK2) (Pitcher *et al.*, 1998; Fan *et al.*, 2001; Luttrell & Lefkowitz, 2002). GRK2 appears to function in increasing the affinity of the receptor for β-arrestin since *in vitro* it has been shown that the binding affinity of β-arrestin$_1$ to the β_2-adrenoceptor increases 10-30 fold following receptor phosphorylation by GRK2 (Luttrell & Lefkowitz, 2002). β-arrestins inhibit receptor signalling in one of two ways either 1) by sterically inhibiting the interaction of the receptor with its G-protein or 2) by functioning as adapter molecules, coupling GPCRs to the proteins involved in endocytosis and receptor internalization (Miller *et al.*, 2000). β-arrestins are essential for the downregulation of the β_2-adrenoceptor since downregulation does not occur in β-arrestin1/2 double knockout mouse embryos (Luttrell & Lefkowitz, 2002). Subsequently, internalized receptors are either taken into early endosomes, where they are dephosphorylated by phosphatase 2A and recycled back to the plasma membrane fully sensitised, or into lysosomes, where they are degraded (Miller *et al.*, 2000; Fan *et al.*, 2001; Millman *et al.*, 2004).

1.8.2 The β_2-adrenoceptor mediates the anti-inflammatory and neurotrophic properties of noradrenaline

The notion that noradrenaline can act as an endogenous immunomodulator eliciting anti-inflammatory effects and contributing to neuroprotection via stimulation of glial β_2-adrenoceptors is a viable one since, upon its release, noradrenaline is not confined to the vicinity of the synaptic cleft and so is available to bind to adrenoceptors expressed on proximal microglia and astrocytes (Galea *et al.*, 2003, Mori *et al.*, 2002). The intracellular cAMP generated by stimulation of β_2-adrenoceptors is known to have an inhibitory action on the transcription of a number of inflammatory genes. In this regard, activation of PKA and the subsequent phosphorylation of CREB induced by stimulation of microglial β_2-adrenoceptors with noradrenaline has been shown to inhibit the production of NO and superoxide anions, both of which contribute to neuronal damage (Mori *et al.*, 2002). Moreover, studies in astrocytes reveal that an increase in intracellular cAMP levels results in the suppression of iNOS and ICAM-1 (Feinstein *et al.*, 1993, Ballestas & Benveniste 1997). Furthermore, it has been found that astrocytes can express MHC class II *in vitro* upon exposure to the cytokine IFN-γ which enables them to act as APCs and present myelin-basic protein (MBP) to T-cells.

However, exposure to noradrenaline is found to reduce MHC class II expression on mouse astrocytes, an effect shown to be mediated by activation of β_2-adrenoceptors (Frohman *et al.*, 1988; Feinstein *et al.*, 2002; Constantinescu *et al.*, 2005).

In addition, β_2-adrenoceptor agonists have been shown to activate astrocytes and promote neuroprotection through the induction of neurotrophins. Neurotrophic factors are peptides which are essential for neuronal development, axonal growth and synaptogenesis, both during development and in the adult brain. However, dysregulation of neurotrophin expression has been implicated in the progression of many neurodegenerative diseases (Connor & Dragunow, 1998) since such alterations can result in necrosis, apoptosis and reduced synaptic plasticity in the CNS. Encouragingly, it has been reported that noradrenaline can induce BDNF expression in cultured astrocytes through activation of the cAMP pathway (Conderelli *et al.*, 1994) and the selective β_2-adrenoceptor agonist clenbuterol has been shown to increase NGF mRNA and protein levels by 200-300% (Culmsee *et al.*, 1999a). Furthermore, *in vivo* studies have also shown that clenbuterol can induce the expression of NGF, basic fibroblast growth factor-2 (bFGF-2) and TGF-β_1 mRNA in the CNS following ischemia (Culmsee *et al.*, 1999b).

1.9 The noradrenergic system and neuroinflammation

Owing to the fact that noradrenaline plays such an important role in regulating the neuroinflammatory response any deficits in this system would have disastrous consequences for the CNS. Indeed the noradrenergic system, or rather deficits in the noradrenergic system, has been linked to the aetiology of many neurodegenerative diseases. In this regard, changes in the LC-noradrenergic system, primarily involving significant reductions in LC cell numbers, have been observed in Parkinson's disease, Alzheimer's disease, Huntington's disease, HIV-related dementia and MS (Marien *et al.*, 2004). Many of these diseases are characterized by an unresolved inflammatory component involving chronic activation of microglia and astrocytes and the excessive production of pro-inflammatory cytokines and other inflammatory mediators in the CNS, an effect which ultimately results in neuronal degeneration.

1.9.1 The noradrenergic system and MS

It appears that noradrenaline, or rather loss of the anti-inflammatory effects of noradrenaline, might contribute to the development of MS since it has been shown that the symptoms of EAE, a rodent model of MS, are worsened by chemical sympathectomy, an experimental procedure that reduces endogenous noradrenaline and cAMP levels. In support of this, symptoms of EAE are shown to be suppressed by the administration of β_2-adrenoceptor agonists (Giorelli *et al.,* 2004). Interestingly, evidence is now coming to the fore which suggests that the demyelination associated with the progression of MS may be due to a loss of β_2-adrenoceptors from astrocytes. In this regard, quantitative radiography and immunohistochemistry studies on brain slices from MS patients revealed that β_2-adrenoceptors were absent from astrocytes in both normal appearing white matter and astrogliotic plaques in the white matter of patients suffering from MS but were present on those from controls, which included individuals suffering from other neuroinflammatory diseases (De Keyser *et al.,* 1999; Zeinstra *et al.,* 2000). Significantly, while the β_2-adrenoceptor was shown to be absent from astrocytes in the CNS of MS patients, its expression on neurons was unaffected (De Keyser *et al.,* 2004a). Notably, no association has been found between polymorphisms of the β_2-adrenoceptor gene and the incidence of MS (De Keyser *et al.,* 2004a).

Overall it has been suggested that the absence of astrocytic β_2-adrenoceptors in MS might result in an inability of endogenous noradrenaline to elicit its anti-inflammatory effects within the CNS and, as such, implies a means by which astrocytes might be converted to functional APCs. Furthermore, the absence of the receptor could contribute to neuronal damage for a number of reasons (see Fig. 1.12). Firstly, the transcription of trophic factors, such as neuregulin, BDNF and NGF in astrocytes is dependent on cAMP signalling (Zafra *et al.,* 1992). Hence, loss of the receptor, and the associated decrease in cAMP, would likely result in a loss of trophic support for neighbouring neurons which in turn could contribute to neuronal death and apoptosis of oligodendrocytes. Secondly, the production of NO along with other pro-inflammatory molecules, such as IL-1β and TNF-α, could become upregulated in the absence of noradrenergic actions at β_2-adrenoceptors leading to damage of neurons and oligodendrocytes. Thirdly, most of the brain's energy source is stored in astrocytes in the form of glycogen. Glycogenolysis is dependent on stimulation of astrocytic β_2-adrenoceptors and cAMP production, therefore decreased production of intracellular cAMP would lead to a

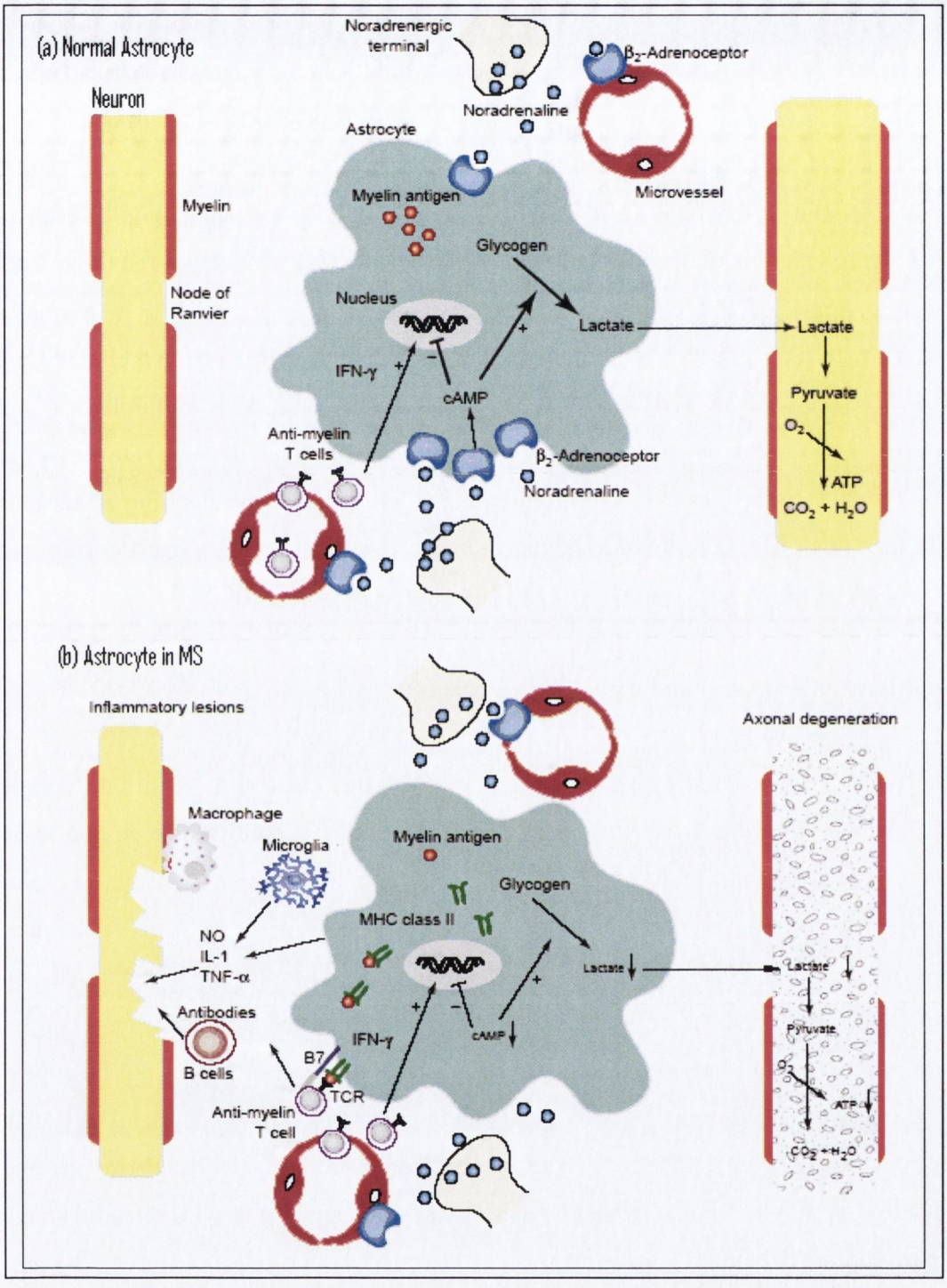


Figure 1.12: Astrocytes in healthy individuals and those affected by MS
(a) The effects of noradrenaline binding to the β_2-adrenergic receptor on astrocytes in healthy individuals (b) The loss of the β_2-adrenergic receptor from astrocytes in MS and its consequences (adapted from De Keyser *et al.*, 2004)

decline in the amount of glycogenolysis taking place, the eventual outcome of which being a reduction in energy supplies available to neurons. This would lead to progressive degeneration of axons, the longest of which would be affected first. Energy deprivation of neurons may also lead to injury caused by excessive levels of Ca^{2+} influx due to failure of the Na^{+}/K^{+} pump, since the Na^{+}/K^{+} pump relies on ATP to function. Failure of this mechanism could result in uncontrolled depolarization of the cell allowing for the opening of voltage-sensitive Ca^{2+} channels and reversal of the operation of the Na^{+}/Ca^{2+} exchanger, which is normally responsible for extruding excess Ca^{2+} from the cell. Ultimately, a rise in intracellular Ca^{2+} concentrations could lead to activation of degradative enzymes, such as proteases and phospholipases, which break down neurofilaments and induce neuronal damage (De Keyser *et al.,* 2004b). Damage to myelin and neurons can result in impaired conduction of nervous impulses. In turn this could contribute to latencies in the visual, auditory, somatosensory and motor systems, all of which are symptoms of MS (Compston & Coles, 2002).

1.9.2 The noradrenergic system and Alzheimer's disease

The World Health Organisation (WHO) reported in 2009 that there are 18 million people worldwide currently suffering from Alzheimer's disease and this is predicted to double to 34 million by the year 2025 (http://www.searo.who.int). As such, this disease represents a major debilitating disorder for an ever aging population. First described in 1911 by Alois Alzheimer, Alzheimer's disease is considered to be a multifactorial disease characterized by neuroinflammation, extracellular amyloid plaques and intraneuronal neurofibrillary tangles (Maccioni *et al.*, 2001; Maccioni *et al.*, 2009). The presence of amyloid-β does not be appear to be directly neurotoxic and under physiological conditions may even be beneficial as it may play a role in learning and memory (Garcia-Osta & Alberini, 2009). However, the overaccumulation of amyloid-β can trigger a cascade of events leading to widespread damage of the CNS as seen in Alzheimer's disease.

The role of neuroinflammation in Alzheimer's disease is not fully understood and the question remains as to whether neuroinflammation directly contributes to or is a secondary factor in the course of this disease. The current hypothesis appears to support the later as it has been shown that amyloid-β can attract and activate microglia leading to an accumulation of microglia

around sites of amyloid-β deposition in the brain. One proposed mechanism of microglial activation in response to amyloid-β is via binding of amyloid-β to the receptor for advanced glycation endproducts (RAGE) and the M-CSF receptor (Tuppo & Arias, 2005). Furthermore, studies suggest that the exposure of microglia, astrocytes and neurons to amyloid-β induces the production of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, and chemokines, such as IL-8, MCP-1 and MIP-1α. While this response may initially act as a neuroprotective mechanism, and an attempt to clear amyloid-β from the brain, the continued activation of these cells and the continued production of pro-inflammatory mediators may lead to damage of delicate neuronal populations. Furthermore, amyloid-β has also been found to recruit peripherally circulating macrophages across the BBB and so can further contribute to the inflammatory burden of the CNS in this manner (see Tuppo & Arias, 2005 and references therein).

Alzheimer's disease is generally associated with deficits in the cholinergic system. However, post-mortem results have revealed that there are also significant alterations in other neurotransmitter systems, such as the serotonergic and glutamatergic systems but most importantly the noradrenergic system. In light of this, studies have shown that there is a more significant correlation between the duration of illness and the degree of noradrenergic cell loss in the LC than with the degree of cell loss observed in the nucleus basalis (Marien *et al.*, 2004). The presence of amyloid-beta (Aβ) has been found to increase iNOS and IL-1β production in microglial cells, however, when the LC was ablated there was a 5-6 fold greater increase in response to amyloid-β and a more prolonged expression of these inflammatory mediators thus reinforcing the importance of the noradrenergic system in the regulation of the inflammatory response (Heneka *et al.*, 2002).

The accumulation of amyloid-β plaques in the CNS has been suggested to be a contributory factor in the development of Alzheimer's disease. The amyloid-β peptide is comprised of beta-sheets and has a molecular weight of approximately 4kDa. It is formed from proteolysis of amyloid precursor protein (APP), a type I glycoprotein localized on chromosome 21 which is found to be ubiquitously expressed. Proteolysis of APP occurs owing to actions of α, β or γ-secretases (see Fig. 1.13). The N-terminal domain of APP projects towards the extracellular region while the C-terminal domain is located within the cytoplasmic region. Cleavage of APP

by α-secretase directs APP processing down the non-amyloidogenic pathway to release soluble APPα (sAPPα) and a membrane bound C-terminal fragment (CTF), CTFα/C83. CTFα can then undergo further cleavage to yield a p3 fragment and an APP intracellular domain (AICD). In contrast, cleavage by β-secretase directs APP processing down the amyloidogenic pathway to release sAPPβ and membrane bound CTFβ/C99. CTFβ can be further cleaved by γ-secretase to yield amyloid-β and AICD (Bandyopadhyay et al., 2006; McLoughlin & Miller, 2008). The amyloid hypothesis of Alzheimer's disease suggests that cleavage generally occurs on the N-terminal side of APP to release amyloid-β peptides, most commonly amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ of which amyloid-$\beta_{1\text{-}42}$ has a higher capacity to aggregate than any other form (Maccioni *et al.*, 2001). The major genes implicated in Alzheimer's disease include those for APP, presenilin-1, presenilin-2 and 5-10% of Alzheimer's disease cases are found to be due to genetic mutation (Maccioni *et al.*, 2001). Presenilins form the active site of the multiprotein γ-secretase complex and are intramembrane aspartyl proteases whose protein chains span the cell membrane many times and are capable of cleaving proteins within the hydrophobic lipid bilayer at two or more bonds (Fortini, 2003; Selkoe & Wolfe, 2007). Presenilin-1 is known to be involved in normal processing of APP and so missense mutations can lead to a loss-of-function resulting in increased amyloid-β accumulation (Maccioni *et al.*, 2001; Selkoe & Wolfe, 2007). Furthermore, a lack of presenilin function has been shown result in impaired signalling by N-cadherin, a cell-surface protein associated with neuronal survival and neuronal plasticity and in addition it can lead to reduced activity of the CBP pathway which is important for long-term memory formation (Fortini, 2003). Significantly, it has been found that increasing concentrations of intracellular cAMP, through the use of PDE4 inhibitors, can ameliorate memory deficits and neurodegeneration in animal models (Beglopoulos & Shen, 2006).

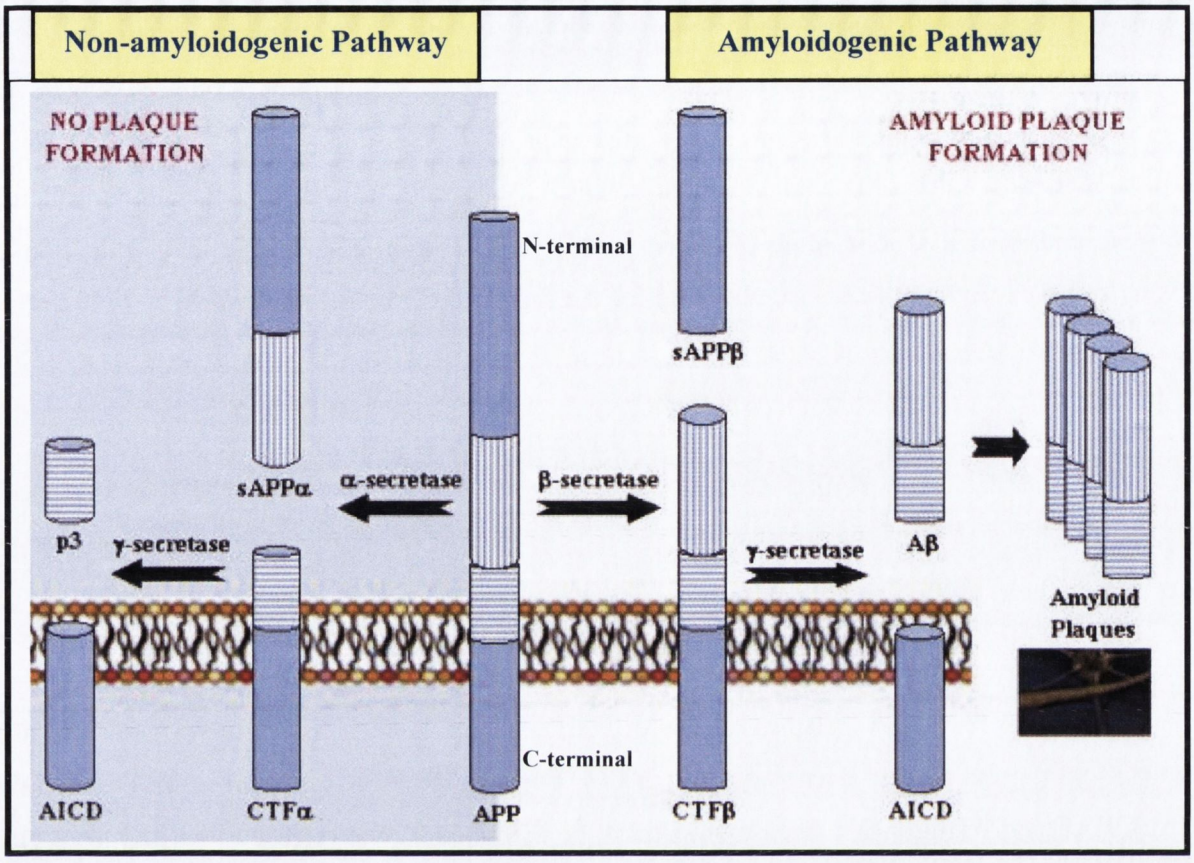


Figure 1.13: APP processing depicting non-amyloidogenic and amyloidogenic pathways
(adapted from: http://www.ebi.ac.uk/interpro/potm/2006_7/Page2_files/image004.jpg)

Amyloid-β is found to be continuously produced and its clearance is generally dependent on the activity of a number of scavenger receptors and degrading enzymes. The key amyloid-β degrading enzymes are the zinc metalloendopeptidases, primarily neprilysin (NEP) and insulin-degrading enzyme/insulysin (IDE), which are expressed neuronally. It has been found that levels of these enzymes decrease with age and moreover their expression is decreased in Alzheimer's disease (Yasojima *et al.,* 2001; Cook *et al.,* 2003; Caccamo *et al.,* 2005). The importance of these enzymes in the clearance of amyloid-β is revealed by the fact that endogenous levels of amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ are significantly elevated in the brains of neprilysin and insulysin knock-out mice. Moreover, it has been demonstrated that the chronic overexpression of both neprilysin and insulysin reduces the burden of amyloid plaques in mice (Eckman & Eckman, 2005). In addition, the concentrations of amyloid-β found in the brain are also dependent on the activity of amyloid-β scavenger receptors. For instance, it has been shown that amyloid-β can interact with a number of receptors expressed on the microglial

surface, for example scavenger receptor A, CD36, CD40, TLR-2 and RAGE (Chen *et al.*, 2006b; Farfara *et al.*, 2008). Thus, it is proposed that microglia may act as scavengers for amyloid-β in the brain, however, while microglia have been demonstrated to be capable of phagocytosing amyloid-β, their degradative capacity appears to be somewhat limited (Chung *et al.*, 1999). Thus, it is likely that in the case of Alzheimer's disease the production of amyloid-β exceeds its clearance allowing for the formation of amyloid plaques.

1.9.2.1 Matrix metalloproteinases (MMPs)

Other enzymes capable of degrading amyloid-β are the matrix metalloproteinases (MMPs). MMPs regulate the composition of the extra cellular-matrix (ECM) through proteolysis. MMPs can be classified into collagenases, gelatinases and stromelysins based on their substrate specificity (Medina *et al.*, 2003; Liuzzi *et al.*, 2004). They are found to play a role in embryonic development, morphogenesis, tissue resorption and remodelling. In addition they are highly expressed in inflammation and diseases such as arthritis, cardiovascular disease, distal colitis and cancer (Heneka *et al.*, 2006). Diseases of the CNS such as HIV-dementia, MS, EAE and Alzheimer's disease are said to be mediated by the release of MMPs from glia. Significantly, amelioration of the symptoms of EAE has been shown to occur upon administration of MMP inhibitors (Pagenstecher *et al.*, 1998; Liuzzi *et al.*, 2004).

The MMPs of interest for the purposes of the work presented here are the gelatinases, namely gelatinase A (MMP-2) and gelatinase B (MMP-9) whose main substrate is gelatine (Heneka *et al.*, 2006). The structure of MMPs comprises an N-terminal signal peptide domain, a C-terminal domain which is responsible for substrate binding and a catalytic domain which is found to contain a zinc binding site, a calcium ion and methionine. This serves to give structure, stability and enzymatic activity to the MMP. In addition, MMPs also have a pro-domain containing a cysteine residue which is found to bind to the catalytic zinc, maintaining the MMPs in a latent form until they undergo activation (Heneka *et al.*, 2006). In this regard, MMPs are initially secreted as zymogens and their activation requires proteolysis of the pro-peptide domain to induce a conformational change. Pro-MMP-2 is produced as a 72kDa pro-enzyme while pro-MMP-9 has a molecular weight of 92kDa (Medina *et al.*, 2003). In the CNS, both MMP-2 and MMP-9 are found to be constitutively expressed by both astrocytes and microglia (Gottschall *et al.*, 1995). However, while MMP-9 is expressed at low levels

under normal conditions its expression can be increased by factors such as cytokines (Heneka *et al.*, 2006; Yin *et al.*, 2006). Notably, it has been shown that the administration of LPS to cultures enhances the expression of MMP-2 in astrocytes but not microglia, whilst LPS is found to increase the expression of MMP-9 in both astrocytes and microglia (Liuzzi, *et al.*, 2004; Yin, *et al.*, 2006). Notably, it has been suggested that MMP-9 may be responsible for BBB breakdown allowing for the excessive invasion of immune cells into the CNS during inflammation (Lindberg *et al.*, 2001; Heneka *et al.*, 2006).

The expression of MMPs is under strict control at the levels of transcription, synthesis, activation, inhibition and glycosylation (Heneka *et al.*, 2006). In general, the selective inhibitor, tissue inhibitor of metalloproteinases (TIMP)-1, is secreted in combination with MMPs. Notably, TIMP-1 is found to be highly expressed in activated astrocytes (Pagenstecher *et al.*, 1998). TIMP-1 is one member of a family of four proteins, namely TIMP-1 to -4, which have the capacity to bind MMPs in a 1:1 ratio (Pagenstecher *et al.*, 1998). Thus, the activity of MMPs involves a delicate balance between activation of the enzyme and inhibition induced by TIMPs and as such any imbalance may lead to pathological events (Liuzzi *et al.*, 2004). In this regard, it is known that there is an imbalance in the expression of MMP/TIMP in rheumatoid arthritis and MS (Lindberg *et al.*, 2001). In addition, MMPs are suggested to play a role in Alzheimer's disease since levels of MMP-2 and MMP-9 have been shown to be elevated surrounding amyloid plaques in aged amyloid precursor protein/presenilin-1 mice. In further support of this, levels of amyloid-β are shown to be significantly increased in the brains of *mmp*-2 and *mmp*-9 knock-out mice. Moreover, it has been shown that MMP-2 is capable of degrading amyloid-β extracted from Alzheimer's disease brains and additionally it has been shown that the viral transfection of TIMP-1 into astrocytic cultures inhibits their ability to degrade amyloid-β (Roher *et al.*, 1994; Yin *et al.*, 2006). Thus, these reports all suggest a role for MMPs in the clearance of amyloid-β.

1.10 β_2-adrenoceptor agonists

The physiological activity of β-adrenoceptor agonists is known to be dependent on their structure and activity at the receptor. Their activity is also found to be dependent on the rate of absorption, metabolism, elimination and distribution of the drug to target tissues. In general, β-adrenoceptor agonists have a substituted six-membered aromatic ring, which is essential for

their biological activity, with hydroxyl groups such as halogens, amines, hydroxymethyl groups or a various combination of these attached. As such, β-adrenergic agonists fall into a number of categories dependent on their aromatic substitutions, namely phenols, resorcinols, catechols and saligenins, and it is these chemical substitutions which also determine the longevity of the compound (Smith, 1998). For instance, salbutamol is categorised as a saligenin. The therapeutic use of salbutamol is limited since it is rapidly deactivated by enzymes in the liver and intestine. In addition, due to its chemical substitutions, salbutamol is also known to have a low affinity for the β_2-adrenoceptor. β_2-adrenoceptor agonists can also be categorised into those which directly activate the receptor (salbutamol), those which are taken into a membrane depot (formoterol) and those which can bind to an auxiliary binding site that is receptor dependent (salmeterol) (Johnson, 2001). It is suggested that rather than inducing a conformational change in the receptor β-adrenoceptor agonists stabilise it in its active form with GTP bound. On the contrary, β-adrenoceptor antagonists are thought to bind with high affinity to a low-energy inactivated form of the receptor and thus shift the equilibrium away from the activated form. As such, it appears that β-adrenoceptor agonists and antagonists are not competitive in the classical sense of the meaning since they do not bind to the same region on the receptor.

The selective β_2-adrenoceptor agonist clenbuterol was specifically designed to resist rapid degradation and elimination by enzymes active toward the aromatic hydroxyl groups. The aromatic ring of clenbuterol is substituted with dichloroaniline (see Fig. 1.14) and it is for this reason that clenbuterol appears more lipophilic than other β-agonists since dichloroaniline is shown to have a high log P value. However, this has also been suggested to be the reason why clenbuterol passes readily into adipose tissue. Clenbuterol is a long-acting β-agonist with a reported half-life of approximately 30 hours in rats. Owing to its long half-life, it has been shown clinically that for continued therapy clenbuterol need only be administered twice daily whereas other β-agonists require administration up to 3-12 times daily (see Smith, 1998). Clenbuterol is metabolized by N-dealkylation, N-oxidation and sulphate conjugation. Its primary metabolite is 4-N-Hydroxylamine which is found primarily in the urine (Zalko *et al.*, 1998). In rats dosed with clenbuterol, 60% of the drug is found to be excreted in the urine after 8 days whilst the remaining 20% is excreted in the faeces (Zalko *et al.*, 1998).

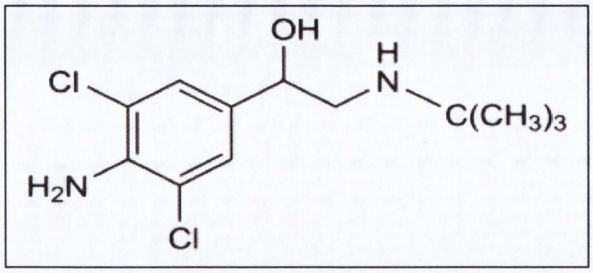


Figure 1.14: Structure of Clenbuterol

(From: www.medicinescomplete.com)

The administration of clenbuterol has been shown to be protective in many different disease states. In this regard, clenbuterol has been shown to be protective in models of myocardium ischemia/reperfusion injury, ischemia and amyotrophic lateral sclerosis (Semkova *et al.*, 1996; Culmsee *et al.*, 1999; Zhu *et al.*, 1999; Zhu *et al.*, 2001; Junker *et al.*, 2002; Teng *et al.*, 2006; Liu *et al.*, 2008). Furthermore, clenbuterol enhances production of the N-methyl-D-aspartate (NMDA) antagonist kynurenic acid *in vitro*; an endogenous compound known to have neuroprotective and anti-convulsant properties (Luchowska *et al.*, 2008). In addition, it has been shown that clenbuterol increases the expression of NGF, bFGF-2, TGF-β_1 following focal ishaemia (Culmsee *et al.*, 1999b). In light of this, since neurotrophins do not cross the BBB their induction by pharmacological stimulation of β_2-adrenoceptors might overcome the difficulty in using them as treatment for neurodegenerative diseases. Moreover, recent studies have also shown that clenbuterol can modulate central expression of members of the IL-1 and IL-10 families (McNamee, 2008) and thus may represent a new therapy for the treatment of neuroinflammatory disease. See Table 1.2 below for a list of β-adrenoceptor agonists and antagonists used in this thesis outlining their half-life and receptor affinity.

Name	Classification	Half-Life ($t_{½}$)	Kd (nM)	Reference
Salbutamol	**Agonist at β_2-adrenoceptor**	**1.5-2hr**	**320**	**Martin *et al.*, 1971; Liang & Mills, 2001**
Clenbuterol	**Agonist at β_2-adrenoceptor**	**30 hr**	**14**	**Smith, 1998; Liang & Mills, 2001**
Formoterol	**Agonist at β_2-adrenoceptor**	**1.7 hr**	**74**	**Sasaki *et al.*, 1982; Johnson, 1998**
Metoprolol	**Antagonist at β_1-adrenoceptor**	**37 min**	**55**	**Belpaire *et al.*, 1990; Baker, 2005**
ICI 118,551	**Antagonist at β_2-adrenoceptor**	**20min**	**0.66**	**O'Donnell & Wanstall, 1980 Moresco *et al.*, 2000**

Table 1.2: β-adrenoceptor agonists and antagonists

1.11 Objective

The overall objective of this project was to examine the therapeutic potential of targeting glial β_2-adrenoceptors for the treatment of neuroinflammation. As discussed earlier, β_2-adrenoceptors have been shown to be absent from astrocytes in the CNS of people suffering from MS (De Keyser *et al.*, 1999; Zeinstra *et al.*, 2000), a disease associated with neuroinflammation. Therefore, the ability of an inflammatory stimulus to alter the expression and function of β_2-adrenoceptors on glial cells *in vitro* was examined here in an attempt to examine the impact of inflammation on β_2-adrenoceptor expression and function (Chapter 3). An inflammatory stimulus in the form of LPS + IFN-γ was used for *in vitro* studies as it replicates, to some extent, the inflammatory environment seen in the MS brain. Furthermore, the impact of a systemic inflammatory challenge on central β_2-adrenoceptor expression and function was examined *in vivo* to determine if effects observed in *in vitro* studies translate to the *in vivo* situation where cells are in their natural environment (Chapter 4). In addition, the work presented here examined the ability of selective stimulation of central β_2-adrenoceptors to regulate an immune response of the CNS *in vivo* using the selective β_2-adrenoceptor agonist

clenbuterol under basal conditions using both acute (Chapter 5) and chronic (Chapter 7) treatments and in acute LPS-induced neuroinflammatory conditions (Chapter 5). In addition the peripheral effects of clenbuterol were examined (Chapter 6) in a bid to further elucidate its suitability as a therapeutic treatment. Thus, specific experiments focused on:

1) The impact of inflammation on the expression and function of glial β_2-adrenoceptors both *in vitro* and *in vivo*.

2) The ability of direct stimulation of central β_2-adrenoceptors with clenbuterol to alter the expression of pro- and anti-inflammatory cytokines *in vivo*.

3) Determining the lowest dose at which clenbuterol can induce an immune response within the CNS and examining the ability of this low-dose clenbuterol to impact on LPS-induced inflammation.

4) Determining the receptor subtype responsible for the clenbuterol-induced immune response within the CNS.

5) Assessing the neuroinflammatory profile and the peripheral effects induced by clenbuterol in comparison to LPS, specifically effects on locomotor activity, heart rate, body-temperature, body-weight and food-motivated behaviour.

6) Assessing the ability of chronic stimulation of central β_2-adrenoceptors with clenbuterol to continue to induce an immune response and to alter amyloid load in the CNS.

Chapter 2

Materials & Methods

2.1 Materials

Animals

Wistar rats (1-3 days old)	Bioresources, TCD
Sprague-Dawley Rats	Harlan, UK
Laboratory Rat Diet	Red Mills, Ireland

Cell Culture Materials

Acrodisc syringe filter (0.2μm)	Pall Corporation, Ireland
Biocidal ZF ™	WAK-Chemie Medical GmbH, Germany
25cm Cell scrapers	Sarstedt, Ireland
Cell strainers (40μM)	BD Falcon, UK
Coverslips (Plastic; 13 mm)	Sarstedt, Ireland
Disposable sterile scalpels	Swann-Morton Ltd., UK
Dulbecco's modified eagles medium:F-12 (DMEM)	Invitrogen, USA
Dulbecco's phosphate buffered saline (PBS) (10X)	Sigma-Aldrich, Ireland
Fetal Calf Serum (FCS)	Invitrogen, USA
Haemocytometer	VWR international, Ireland
Penicillin-streptomycin	Invitrogen, USA
Plastic syringe (1ml, 20ml, 50ml)	BD, UK
Poly-L-lysine	Sigma-Aldrich, Ireland
Serological pipette (25ml)	Sarstedt, Ireland
Sterile 24-well plates	Sarstedt, Ireland
Sterile Combitips plus (1ml)	Eppendorf, USA
Sterile Combitips plus (2.5ml)	Eppendorf, USA
Sterile falcon tubes (15ml)	Sarstedt, Ireland
Sterile falcon tubes (50ml)	Sarstedt, Ireland
Sterile microtubes (2ml)	Sarstedt, Ireland
Sterile petri dishes	Sarstedt, Ireland
Sterile transfer pipettes	Sarstedt, Ireland
T25cm^2 Flasks	Sarstedt, Ireland

T75cm^2 Flasks	Sarstedt, Ireland
Trypan Blue	Sigma-Aldrich, Ireland
Trypsin-EDTA	Sigma-Aldrich, Ireland

Assay Kits

Bicinchonic acid (BCA) protein assay	Pierce, USA
cAMP Biotrak Enzymeimmunoassay kit	Amersham Biosciences, UK
MSD® 96-well MULTI-SPOT® Human/Rodent (4G8) Abeta Triplex Ultra-Sensitive Assay	MesoScale, USA
Pinpoint Cell Surface Protein isolation kit	Pierce, USA
Rat Interleukin-1β (IL-1β) ELISA kit (Duoset)	R&D systems, UK
Rat TNF-α ELISA kit (Opt EIA set)	BD Biosciences, UK

Experimental Treatments

3-isobutyryl-1-methylxanthine	Sigma-Aldrich, Ireland
Clenbuterol	Sigma-Aldrich, Ireland
Dexamethasone Hydrochloride	Sigma-Aldrich, Ireland
Forskolin	Sigma-Aldrich, Ireland
Granulocyte macrophage colony stimulating-factor (GM-CSF)	R&D Systems, UK
ICI 118,551	Tocris Bioscience, UK
Interferon-γ (IFN-γ)	R&D Systems, UK
Lipopolysaccharide (LPS)	Sigma-Aldrich, Ireland
Macrophage colony-stimulating factor (M-CSF)	R&D Systems, UK
Metoprolol	Sigma-Aldrich, Ireland
Resazurin	Sigma-Aldrich, Ireland
Salbutamol	Sigma-Aldrich, Ireland

General Laboratory Chemicals

2-propanol	Sigma-Aldrich, Ireland
Acrylamide	Sigma-Aldrich, Ireland
Ammonium persulfate (APS)	Sigma-Aldrich, Ireland

β-Mercaptoethanol	Sigma-Aldrich, Ireland
Betadine	Medlock Medical Ltd., UK
Bicinchoninic acid (BCA) protein kit	Pierce, USA
Bovine serum albumin 96% (BSA)	Sigma-Aldrich, Ireland
Bromophenol blue	Sigma-Aldrich, Ireland
Chemiluminescence Hyperfilm	Amersham, UK
CL-Xposure x-ray film	Pierce, USA
Diethyl Pyrocarbonate (DEPC)	Sigma-Aldrich, Ireland
Dimethyl Sulfoxide	Sigma-Aldrich, Ireland
Disodium hydrogen orthophosphate (Na_2HPO_4)	VWR Int., Ireland
DL-Dithiothreitol (DTT)	Sigma-Aldrich, Ireland
Ethylenediaminetetra acetic acid (EDTA)	Sigma-Aldrich, Ireland
Glycerol	Sigma-Aldrich, Ireland
Glycine	Sigma-Aldrich, Ireland
Hydrochloric acid (HCl)	VWR Int., Ireland
Industrial methylated spirits	Lennox, Ireland
Ketamine	Bioresources, TCD
Methanol	Lennox, Ireland
N-(1-naphthyl)-ethylenediamine dihydrochloride	Sigma-Aldrich, Ireland
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Sigma-Aldrich, Ireland
3,3',5,5',-Tetramethyl-benzidine (TMB)	Dakocytomation, Ireland
N' N' Bis Acrylamide	Sigma-Aldrich, Ireland
Normal goat serum	Vector, UK
NP-40 ([Octy]phenoxy)polyethoxyethanol)	USB corporation, Germany
Orthophosphoric acid	VWR Int., Ireland
Phosphatase inhibitor cocktail I & II	Sigma-Aldrich, Ireland
Potassium chloride (KCl)	Merck, UK
Potassium dihydrogen orthophosphate (KH_2PO_4)	VWR Int., Ireland
Protease inhibitor cocktail	Sigma-Aldrich, Ireland
Restore stripping solution	Pierce, USA
Sodium bicarbonate ($NaHCO_3$)	VWR Int., Ireland

Sodium carbonate (Na_2CO_3)	VWR Int., Ireland
Sodium chloride (NaCl)	VWR Int., Ireland
Sodium dodecyl sulfate (SDS) 99%	Sigma-Aldrich, Ireland
Sodium hydrogen carbonate	VWR Int., Ireland
Sodium hydroxide (NaOH)	Sigma-Aldrich, Ireland
Sodium nitrite ($NaNO_2$)	Sigma-Aldrich, Ireland
Sodium phosphate dibasic ($NaHPO_4$)	Sigma-Aldrich, Ireland
Sodium phosphate monobasic monohydrate (NaH_2PO_4)	Sigma-Aldrich, Ireland
Sucrose	Sigma-Aldrich, Ireland
Sulphanilamide	Sigma-Aldrich, Ireland
Sulphuric acid (H_2SO_4) 98%	VWR Int., Ireland
Triton X-100	Sigma-Aldrich, Ireland
Trizma-Base	Sigma-Aldrich, Ireland
Tris-HCl	Sigma-Aldrich, Ireland
Tween 20	Sigma-Aldrich, Ireland
Xyzaline	Bioresources, TCD

General Laboratory Wares

50ml yellow capped tubs	Sarstedt, Ireland
F96 Maxisorp immunoplates for ELISA	Nunc, USA
Filter paper	Whatman, USA
Laboratory roll-parafilm	VWR Int., Ireland
Microtest 96-well flat bottomed plates	Sarstedt, Ireland
Microtubes (0.5ml)	Sarstedt, Ireland
Microtubes (1.5ml)	Sarstedt, Ireland
Pipette tips	Sarstedt, Ireland
Plastic syringe (1ml)	BD, UK
Plastic transfer pipettes	Sarstedt, Ireland

Molecular Reagents

10X TBE buffer	Invitrogen, USA
96-well optical reaction plates	Applied Biosystems, UK

Absolute ethanol	Sigma-Aldrich, Ireland
Agarose	Sigma-Aldrich, Ireland
Biosphere filter tips (1000, 200 and 100µl)	Sarstedt, Ireland
Diethyl pyrocarbonate	Sigma-Aldrich, Ireland
Ethidium bromide	Sigma-Aldrich, Ireland
High capacity cDNA archive kit	Applied Biosystems, UK
Loading dye (6X)	Promega, UK
Molecular grade water	Sigma-Aldrich, Ireland
Optical adhesive covers	Applied Biosystems, UK
PCR tubes	Sarstedt, Ireland
RNase away	Invitrogen, USA
RNA later	Ambion, UK
RNase Zap wipes	Ambion, UK
RNase-free 1.5ml and 2ml microfuge tubes	Ambion, UK
RNase-free H_2O	Sigma-Aldrich, Ireland
TaqMan gene expression assays	Applied Biosystems, UK
TaqMan universal PCR master mix	Applied Biosystems, UK
Total RNA isolation kit	Macherey-Nagel GmbH & Co., Germany

Western Blotting Reagents and Antibodies

Anti-mouse IgG	Sigma-Aldrich, Ireland
Anti-Rabbit IgG	Amersham, UK
anti-β-actin antibody	Santa-Cruz Inc., USA
β_2-adrenoceptor antibody	Santa Cruz Inc., USA
Broad range molecular weight marker	BioRad, USA
CL-Xposure X-ray film	Pierce, USA
GFAP antibody	Sigma-Aldrich, Ireland
JNK antibody	Cell Signalling, USA
Monoclonal anti-β-actin antibody	Sigma-Aldrich, Ireland
Nitrocellulose membrane	Sigma-Aldrich, Ireland
p38 antibody	Cell Signalling, USA

p42/44 (ERK) antibody	Cell Signalling, USA
Phospho-JNK antibody	Cell Signalling, USA
Phospho-p38 antibody	Cell Signalling, USA
Phospho-p42/44 (ERK) antibody	Cell Signalling, USA
Phospho-STAT-3 Antibody	Cell Signalling, USA
Polyvinylidene (PVDF)	Millipore, Ireland
Restore™ Western Blot stripping buffer	Pierce, USA
SOCS3 antibody	Santa Cruz, USA
STAT-3 Antibody	Cell Signalling, USA
Supersignal® West Dura extended substrate solution	Pierce, USA

Methods

2.2 *In vitro* studies

2.2.1 Aseptic technique

All cell culture work, including the preparation of cell culture reagents, was carried out in a laminar flow-hood (Hera safe, category 2) and aseptic technique was utilised throughout. The use of the laminar flow-hood ensures that cells only come in contact with filtered air and is necessary to ensure that cells do not become contaminated with air-borne pathogens from the normal atmosphere. To ensure that the flow-hood remained sterile the interior was sprayed with 70% ethanol (EtOH) (30% double distilled water (d.d.H_2O) + 70% EtOH v/v) both prior to and following use. Any items taken into the hood were lightly sprayed with 70% EtOH beforehand to prevent the introduction of any pathogens into the working area of the flow-hood. Other aseptic techniques included the use of sterile disposable plasticware and any glassware or d.d.H_2O used for culture work was sterilised prior to use by autoclaving at 121°C for 2 hours (hr). Dissection equipment was sterilised by soaking overnight in 70% EtOH followed by baking at 180°C for a minimum of 2 hr to remove any pathogens. The hood surface was also exposed to ultraviolet (UV) light for a minimum of 30 minutes (min) after use. Disposable powder-free latex gloves were worn throughout all culture work and changed frequently. Gloves were sprayed lightly with 70% EtOH before use in the flow-hood. Cells were maintained in a Nuaire incubator at 37°C, 95% oxygen (O_2), 5% carbon dioxide (CO_2) and any items placed in the incubator were lightly sprayed with 70% EtOH to prevent pathogenic contamination. Both the incubator and laminar flow-hood were cleaned regularly with Biocidal ZF^{TM} followed by 70% EtOH to maintain a sterile, pathogen-free environment. It is necessary to maintain a sterile environment for culture work and to utilise aseptic techniques throughout in order to maintain an environment free from fungi, bacteria or viruses which can interfere with normal cellular functions or cause cell death.

2.2.2 Preparation of culture media and test compounds

Culture Media: Using a 0.2 micrometre (μm) syringe filter and a 50 millilitre (ml) syringe 50ml (10%) of Fetal Calf Serum (FCS) and 5ml (1%) of Penicillin-streptomycin were filter-sterilised into a 500ml bottle of Dulbecco's modified eagle's medium:F12 (DMEM:F12) to give complete DMEM:F12 (cDMEM). This cDMEM was used for the preparation of all glial cultures.

Phosphate Buffered Saline (PBS): 5ml of Dulbecco's sterile 10x PBS (100 millimolar (mM) NaCl, 80mM Na_2HPO_4, 20mM NaH_2PO_4) was added to 45ml of d.d.H_2O to yield a 1x working concentration of PBS.

Macrophage-colony stimulating factor (M-CSF): 25 μg M-CSF was reconstituted by adding 1ml 0.1% bovine serum albumin (BSA) in PBS to yield a stock solution of 25μg/ml. The stock solution was aliquoted into 50μl aliquots and stored at -20°C. Prior to use the stock solution was diluted to a working concentration in pre-warmed cDMEM.

Granulocyte macrophage-colony stimulating factor (GM-CSF): 50μg/ml GM-CSF was reconstituted in 1ml 0.1% BSA in PBS to yield a stock solution of 50μg/ml. This stock solution was aliquoted into 20μl aliquots and stored at -20°C. Prior to use the stock solution was diluted to a working concentration in pre-warmed cDMEM.

Lipopolysaccharide (LPS): 25 mg LPS [*Escherichia coli* serotype 0111:B4] was dissolved in 1ml d.d.H_2O. The solution was mixed using a vortex and when fully dissolved 500μl of this 25mg/ml solution was added to 62ml of cDMEM (1:125 dilution) to yield a 200μg/ml stock solution which was then filter-sterilised using a 0.2μm syringe filter. This stock solution was aliquoted into 1ml aliquots and stored at –20°C. Prior to use the stock solution was diluted to a working concentration in pre-warmed cDMEM.

Interferon-gamma (IFN-γ): A stock solution of 100μg/ml was prepared by dissolving 100μg IFN-γ in 1ml PBS, 1mM dithiothreitol (DTT), 0.1% BSA. The solution was mixed using a vortex until fully dissolved. This stock solution was then aliquoted into 20μl aliquots and

stored at -20°C. Prior to use the stock solution was diluted to a working concentration in pre-warmed cDMEM.

Salbutamol: A 50 µM stock solution was prepared by dissolving 25mg (Formula weight (F.W.) 288.7g) salbutamol in 1.732ml d.d.H_20 and the solution was mixed using a vortex until fully dissolved. This stock solution was then aliquoted into 50µl aliquots and stored at -20°C. Prior to use the stock solution was diluted to a working concentration in pre-warmed cDMEM.

Forskolin: A 100mM stock solution was prepared by dissolving 0.041g (F.W. 410.5g) forskolin in 1ml Dimethyl Sulfoxide (DMSO) and the solution was mixed using a vortex until fully dissolved. This stock solution was then aliquoted into 25µl aliquots and stored at -20°C. Prior to use the stock solution was diluted to a working concentration in pre-warmed cDMEM.

Dexamethasone: A stock solution of 5.35 x 10^{-3}M concentration of dexamethasone was prepared (F.W. 392.47g) in cDMEM and the solution was mixed using a vortex until fully dissolved. This stock solution was then aliquoted into 1ml aliquots and stored at -20°C. Prior to use 1ml of the stock was added to 4.35ml cDMEM to yield a 1mM solution. This 1mM solution was then diluted to a working concentration in pre-warmed cDMEM.

3-Isobutyl-1-methylxanthine (IBMX): A 44.99µM stock solution of IBMX (F.W. 222.25g) was prepared by adding 10mg IBMX to 1ml d.d.H_2O with 0.2% DMSO and the stock solution was warmed, sonicated and vortexed until fully dissolved. This stock solution was then aliquoted into 10µl aliquots and stored at -20°C. Prior to use the stock solution was diluted to a working concentration in pre-warmed cDMEM.

2.2.3 Preparation of cell culture plates

25mg Poly-L-lysine was reconstituted in 5ml sterile d.d.H_2O. The solution was vortexed to ensure thorough mixing and then was subsequently added to a further 20ml d.d.H_2O to yield a final stock concentration of 1mg/ml. The stock solution was then filter-sterilised through a 0.2µm syringe filter using a 25ml syringe and aliquoted into 1ml aliquots and stored at -20°C until required. To prepare plates for cell culture a 1ml aliquot of Poly-L-lysine was added to

24ml d.d.H_2O. The solution was vortexed to ensure thorough mixing and the wells were coated by adding 75µl of solution to the centre of each of the wells of a 24-well plate. The plates were transferred to the incubator for 1 hr and were subsequently removed back into the laminar flow-hood where the Poly-L-lysine was removed. To ensure the plates were fully dry prior to use they were left in the laminar flow-hood overnight.

2.2.4 Preparation of primary mixed glial cultures

Primary mixed glial cultures were prepared from 1-3 day old neonatal male Wistar rats [Bioresources Unit, Trinity College]. As stated previously, all cell culture preparations were carried out utilising aseptic conditions. Briefly, the neonates were taken into the flow-hood and decapitated using a large, sharp scissors. Using a smaller scissors an incision was made into the skin down the midline and the skin was pulled back to reveal the skull. Using the smaller scissors incisions were made along each side of the skull at the level of the ears and the skull cap was removed to expose the brain. Using a straight forceps meninges were removed and the cortical tissue was subsequently removed by pinching it out using a curved forceps. The tissue was placed in a few drops of sterile 1x PBS in a Petri-dish. Any remaining meninges or adherent blood-vessels were subsequently removed. The tissue was cross-chopped using a sterile scalpel and then transferred to a 15ml Falcon tube containing 2ml of pre-warmed cDMEM per animal used. The tube was placed in a humidified incubator (95% O_2, 5% CO_2) and incubated at 37°C for 20 min. Subsequently, the homogenate was gently triturated several times using a pasteur pipette until all visible clumps were removed. The suspension was then passed through a 40µm nylon cell strainer into a sterile 50ml Falcon tube. This solution was centrifuged at 2,000 x rotations per minute (rpm) for 3 min at 20°C. The tube was returned to the flow-hood and the supernatant was gently aspirated. The cell pellet was re-suspended in 7.5ml cDMEM by trituration using a pasteur pipette. At this stage a cell count was performed by adding 10µl cell suspension to 30µl sterile 1x PBS and 10µl Trypan Blue in a 1ml microtube. The solution was vortexed and allowed to sit for 30 seconds (sec) and then 10µl was placed under a glass microscope coverslip on a haemocytometer. The cell number was counted and calculated using 40x magnification on a light microscope [Nikon Eclipse E200]. Cell counts usually averaged 2 x 10^5 cells/ml.

50µl of the cell suspension was placed into the centre of each well of a 24-well tissue culture plate which had previously been coated with Poly-L-lysine as described in 2.2.3. The plates were then placed in the incubator where cells were allowed to adhere to the wells for a minimum of 2 hr. Plates were subsequently removed back into the flow-hood where each well was flooded with 300µl cDMEM.

For the biotinylation assay in 2.2.8 cells were cultured in T75cm^2 flasks. Briefly, cells were prepared as described above and 800µl of the re-suspended cell solution was placed in the centre of the flasks at a plating density of 2.16 x 10^5 cells/flask. The flasks were swirled gently to spread the solution over the base and were immediately placed into the incubator where the cells were allowed to adhere for a minimum of 2 hr prior to flooding with 19.2ml cDMEM per flask.

Culture media was replaced every 3-4 days and cells were treated on days 10-14 or when confluent.

2.2.5 Preparation of enriched microglial and astrocytic cultures

Enriched cultures of primary microglia and astrocytes were prepared as in 2.2.4 above with the following adaptations:

The cell pellet was re-suspended in cDMEM to give a final cell count of 2 x 10^5 cells/ml and 2ml of this cell suspension was added per T25cm^2 flask. The flasks were placed in the incubator where the cells were allowed to adhere for a minimum of 2 hr prior to flooding with 8ml cDMEM. Flasks were incubated in a humidified incubator (95%O_2, 5%CO_2) and incubated at 37°C for 24 hr. Media was replaced 24 hr after the cells were plated to remove any cellular debris. 8ml cDMEM supplemented with the growth factors GM-CSF (20 nanogram (ng)/ml) and M-CSF (5ng/ml) was added to each of the flasks. Cells were cultured for 7 days after which time the media was once again replaced with cDMEM supplemented with GM-CSF and M-CSF. On day 9-10 a layer of microglia loosely adheres to a monolayer of astrocytes and at this point the cells were split. The necks of the flasks were wrapped in parafilm and the flasks were placed on an orbital shaker at 110 x rpm for 2 hr at room

temperature to dissociate the microglia into solution. The flasks were subsequently taken back into the flow-hood and tapped approximately 30 times to dissociate any remaining microglia. The supernatant was poured into a 50ml Falcon tube and centrifuged at 2000 x rpm for 5 min at 20°C. The supernatant was aspirated and the pellet was re-suspended in 1ml cDMEM. At this stage a cell count was performed as described in 2.2.4 and the cell suspension was adjusted to give a final cell density of 1.5 x 10^5 cells/ml. 50µl of the cell suspension was added to each well of a 24-well plate previously coated with Poly-L-lysine as described in 2.2.3. Plates were placed in the incubator where cells were allowed to adhere for a minimum of 2 hr prior to flooding with 300µl cDMEM. Microglia were maintained in an incubator for up to 3 days prior to treatment.

Astrocytes were isolated by adding 1ml Trypsin-ethylenediaminetetraacetic acid (EDTA) to the flasks which were then were placed back into the incubator for 15 min. The flasks were subsequently removed back into the flow-hood where they were gently tapped to remove any adherent astrocytes with continual viewing under 40x magnification using a light microscope until all cells had detached. The cell suspension was poured into a 50ml Falcon tube and the solution was centrifuged at 2000 x rpm for 3 min at 20°C. The supernatant was gently aspirated and the cell pellet was re-suspended in 1ml cDMEM. A cell count was carried out as described in 2.2.4 and the cell suspension was adjusted to yield a final cell density of 1.5 x 10^5 cells/ml. 50µl of the cell suspension was added to the centre of each of the wells of a 24-well tissue culture plate which had previously been coated with Poly-L-lysine as described in 2.2.3. Plates were placed back in the incubator where cells were allowed to adhere for a minimum of 2 hr prior to flooding with 300µl of cDMEM. Astrocytes were maintained in an incubator for up to 3 days prior to treatment.

2.2.6 Harvesting cell free supernatants for cytokine ELISAs

Following treatment and the requisite incubation time, 24-well plates were removed from the incubator and supernatants were harvested and pooled according to the treatment plan into 1.5ml microtube (i.e. supernatants from wells that received the same treatment were pooled into the same microtube). Microtubes were kept on ice prior to being centrifuged at 13,000 x rpm for 3 min at 4°C to pellet any cell debris. The supernatant was aliquoted into fresh

microtubes (2-3 aliquots per sample) and the aliquots were frozen and stored at -80°C until required for cytokine analysis.

2.2.7 Harvesting glial cells for cAMP assay

Following treatment and the requisite incubation time the supernatant was aspirated from the wells of a 24-well plate and 200µl/well of diluted lysis reagent 1B (0.25% solution of Dodecyltrimethylammonium Bromide in 0.05M Acetate buffer, pH 5.8) from the cAMP Biotrak Enzymeimmunoassay (EIA) System kit [Amersham Biosciences] was added. Plates were agitated on ice on a gyrorocker for 10 min. Wells were then scraped using a 1000µl pipette tip to detach adherent cells and the lysate was transferred to a 1.5ml microtube pooling wells which had received the same treatment. The pipette tip was changed between treatment groups to prevent cross-contamination of samples. Samples were placed on ice and analysed immediately for cAMP as described in 2.4.6.

2.2.8 Harvesting glial cells for biotinylation assay

Following treatment and the requisite incubation time the supernatant was aspirated from the T75cm^2 flasks and cells were washed twice with 8ml ice-cold PBS. A Pinpoint Cell Surface Protein Isolation kit [Pierce] was used for biotinylation of samples. Briefly, 10ml Sulfo-NHS-SS-Biotin (supplied) was added per flask and the cells were agitated at 4°C on a gyrorocker for 30 min. 500µl of quenching solution (supplied) was added per flask to stop the reaction and the flasks were gently tipped back and forth to ensure even coverage of the cells. The cells were gently scraped into solution using a 25cm cell scraper and transferred to a sterile 15ml Falcon tube. Each flask was rinsed with a 2.5ml volume of Tris-Buffered Saline (TBS) and the rinse volume was added to the 15ml tube. The tubes were centrifuged at 500 x g for 3 min at 4°C. The supernatant was discarded and 125µl of Lysis Buffer (supplied) containing a 1:100 dilution of protease inhibitors was added to the cell pellet and the cells were re-suspended into solution by pipetting. Biotinylation of samples was carried out as in 2.4.7.

2.2.9 Harvesting glial cells for mRNA analysis

Following treatment and the requisite incubation time the supernatant was aspirated from the wells and 100µl RA1 lysis buffer [supplied in Total RNA isolation kit, Machery Nagel GmbH & Co.] containing 1% β-mercaptoethanol was pipetted directly into each of the wells. Cells were scraped using a 1000µl filtered pipette tip to remove adherent cells. The lysate was transferred to an RNase-free 2ml microtube pooling wells which had received the same treatment. The pipette tip was changed between treatment groups to prevent cross contamination. RNA was then extracted from the samples as described in 2.4.10. If RNA extraction did not take place immediately after harvesting the samples were frozen at -80°C in RA1 lysis buffer containing 1% β-mercaptoethanol. Subsequently, samples were allowed to defrost slowly at room temperature prior to extraction.

2.3 *In vivo* studies

2.3.1 Animal husbandry

Male Sprague-Dawley rats were obtained from Harlan Laboratories, UK (weight on arrival: ~200g). Rats were housed three per cage in hard-bottomed polypropylene cages with wood shavings as bedding. Rats were maintained under standard laboratory conditions in an ambient temperature of 22 ± 2°C and lighting was controlled on a 12 hour light-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, Ireland]. The experimental procedures employed were in compliance with the European Council Directive (86/609/EEC).

2.3.2 *In vivo* drug administration

All animals were handled daily for at least four days prior to drug administration to alleviate any stress caused by experimental handling or injection.

Study 1: 4hr/24 hr LPS + 4hr Clenbuterol Study

Rats were administered an intraperitoneal (i.p.) injection of either a control of 0.89% (w/v) saline (0.045g NaCl/50ml d.d.H_2O) or LPS (250μg/ml). A number of rats were placed back in their home cage for 4 hr prior to sacrifice. 24 hr post-LPS administration rats were administered an i.p. injection of either 0.89% (w/v) saline or clenbuterol (0.5mg/kg) and were placed back in their home cage. Rats were then sacrificed 4 hr post-injection. (see Fig. 4.1, Chapter 4 for study design)

Study 2: Clenbuterol dose-response study

Rats were administered an i.p. injection of either a control of 0.89% (w/v) saline or clenbuterol (0.03, 0.1, 0.3mg/kg). Rats were placed in locomotor activity cages for 15 min 2 hr post-injection to monitor activity prior to being returned to their home cages. Rats were then sacrificed 4 hr post-injection.

Study 3: Clenbuterol low dose-response study

Rats were administered an i.p. injection of either a control of 0.89% (w/v) saline or clenbuterol (0.003, 0.01, 0.03mg/kg). Rats were placed in locomotor activity cages for 15 min 2 hr post-injection to monitor activity prior to being returned to their home-cages. Rats were then sacrificed 4 hr post-injection.

Study 4: LPS + low-dose clenbuterol study

Rats were administered an i.p. injection of either a control of 0.89% (w/v) saline or clenbuterol (0.03mg/kg). Rats were then immediately administered an i.p. injection of either control or LPS (250μg/kg). Rats were placed in locomotor activity cages for 15 min 2 hr post-injection to monitor activity prior to being returned to their home cages. Rats were sacrificed 4 hr post-injection.

Study 5: Clenbuterol + selective β_1-adrenoceptor antagonist metoprolol or selective β_2-adrenoceptor antagonist ICI 118,551

Rats were administered an i.p. injection of either a control of 0.89% (w/v) saline, the selective β_2-adrenoceptor antagonist ICI 118,551 (10mg/kg) or the selective β_1-adrenoceptor antagonist metoprolol (10mg/kg), all containing 0.2% (v/v) Tween-20, 30 min prior to the i.p.

administration of either 0.89% (w/v) saline or clenbuterol (0.05mg/kg). Rats were placed in locomotor activity cages for 15 min 2 hr post-injection to monitor activity prior to being returned to their home cages. Rats were sacrificed 4 hr post-injection.

Study 6: Telemetry study - comparison between LPS & clenbuterol

Rats which had previously been implanted with radiotelemetry devices (see 2.3.4.1) were administered a subcutaneous (s.c.) injection of either a control of 0.89% (w/v) saline, clenbuterol (0.05mg/kg) or LPS (250µg/kg). Heart rate, body temperature and locomotor activity of rats were monitored at 10 min intervals for 24 hr using PDT 4000HR E-mitters and the Windows PC-based acquisition system VitalView™ [Mini-Mitter, USA].

Study 7: Telemetry study - clenbuterol + selective β_1-adrenoceptor antagonist metoprolol or selective β_2-adrenoceptor antagonist ICI 118,551

Rats which had previously been implanted with radiotelemetry devices (see 2.3.4.1) were administered an s.c. injection of either a control of 0.89% (w/v) saline, the selective β_2-adrenoceptor antagonist ICI 118,551 (10mg/kg) or the selective β_1-adrenoceptor antagonist metoprolol (10mg/kg), all containing 0.2% (v/v) Tween-20, 30 min prior to the s.c. administration of either 0.89% (w/v) saline or clenbuterol (0.05mg/kg). Heart rate of rats was monitored at 10 min intervals for 24 hr using PDT 4000HR E-mitters and the Windows PC-based acquisition system VitalView™ [Mini-Mitter, USA].

Study 8: Telemetry study - clenbuterol + selective β_1-adrenoceptor antagonist metoprolol or selective β_2-adrenoceptor antagonist ICI 118,551

Rats which had previously been implanted with radiotelemetry devices (see 2.3.4.1 below) were administered an s.c. injection of either a control of 0.89% (w/v) saline or clenbuterol (0.003, 0.01, 0.03mg/kg). Heart rate of rats was monitored at 10 min intervals for 24 hr using PDT 4000HR E-mitters and the Windows PC-based acquisition system VitalView™ [Mini-Mitter, USA].

Study 9: Chronic clenbuterol study

Rats were administered an i.p. injection of either a control of 0.89% (w/v) saline or clenbuterol (0.03mg/kg) twice daily (morning & evening) for 14 days. The final injection was administered 4 hr prior to sacrifice.

2.3.3 Harvesting tissue for post-mortem analysis

Immediately following sacrifice of rats brains were removed from the skull and dissected by hand on a Petri-dish containing dry-ice. Portions of cortex for protein analysis were placed in a 1.5ml microtube and snap-frozen on dry ice prior to storage at -80°C. Portions of cortex, hippocampus and hypothalamus for mRNA analysis were transferred to an RNase/DNase-free 1.5ml microtube containing 500µl RNA later solution. Samples for RNA analysis were subsequently stored at 4°C for up to two weeks prior to being transferred from the RNA later solution to fresh RNase/DNase-free microtubes and stored at -80°C.

2.3.4 Telemetry

2.3.4.1 Surgical procedure

Rats (~250-300g) were anaesthetised with ketamine (100mg/ml) and xylazine (10mg/ml). Rats were secured to a heated sterile surgical surface with adhesive tape. The ventral surface of the abdomen and the thorax to the area of the axilla slightly cranial on the right side was shaved and the shaved area was swabbed with Betadine. A 2cm midline abdominal incision was made into the skin 1cm below the diaphragm. The abdomen was opened by making a 2cm incision along the linea abla. The body of the E-mitter was slipped into the abdominal cavity along the sagittal plane and dorsal to the digestive organs with both leads situated in the direction of the head of the animal. The abdominal cavity was gently massaged to allow internal organs to settle. The heart leads were then gently threaded through two small holes in the abdominal wall made to the right and left of the incision. The negative lead was attached using a trochar and sleeve and the lead was passed under the skin to an incision made near the clavicle and left to rest on the pectoralis superficialis. The positive lead was passed under the skin to an incision made to the left of the xiphoid process, cranial to the last rib and left to rest on the

posterior pectoralis superficialis. Steel ferrules were placed over the leads and subsequently crimped into tabs which detect the heart signal. Leads were secured by suturing into the chest muscles. The surgical procedure was finished by closing the abdominal opening. Animals were then returned to their home-cages and allowed to recover for 10-14 days.

2.3.4.2 Heart rate monitoring

Heart rate is monitored from voltage created by the R-wave of the QRS complex which triggers a pulse which can be detected by the ER-4000 energizer/receiver. The Vitalview™ system then computes heart rate from the pulses corresponding to the R-waves which is then reported by the system as beats per minute (BPM) value.

2.3.4.3 Temperature monitoring

Temperature is monitored from signals sent from the transponder to the receiver as a set of pulses whose frequency is dependent on temperature. The receiver converts this pulse frequency into a serial bit stream that can be read by the computer. Calibration values are provided by the manufacturer (normally over the range 32-42°C) which are entered into the software system prior to data collection. These calibration values are then used to convert transponder frequency values to °C which is reported by the system to an accuracy of 0.1°C.

2.3.4.4 Activity monitoring

Activity is monitored by the system as a measurement of changes in signal strength between the transponder and the receiver. The system interprets this as an indication that the transponder has moved and thus scores it as an activity count. The system is capable of recording one count per second. However, these counts can only be regarded as a qualitative measure of movement since they give no quantification of the magnitude or direction of movement of the animal. Behaviours measured primarily include exploratory behaviour and ambulation since the transponder is implanted in the peritoneal cavity and thus finer movements such as feeding and grooming will not be registered as activity.

2.3.5 Other measurements for *in vivo* studies

2.3.5.1 Body-weight measurements

Rats were weighed using a three-point balance prior to injection. Rats were then subsequently re-weighed 24 hr post-injection in order to calculate changes in body weight measured in grams.

2.3.5.2 Food intake

Rat chow pellets were weighed using a three-point balance prior to injection of rats. Rat chow pellets were subsequently re-weighed 24 hr post-injection in order to calculate food intake measured in grams.

2.3.5.3 Water intake

Water bottles were weighed using a three-point balance prior to injection of rats. Water bottles were subsequently re-weighed 24 hr post-injection in order to calculate water intake measured in grams which was then subsequently converted to ml (1g = 1ml).

2.3.5.4 Locomotor activity cages

2 hours post-injection, animals were removed from their home cage and placed individually into activity monitor cages (32cm x 20cm x 18 cm; length x width x height) which were connected to an AM1051 data logger [Benwick Electronics]. Each activity monitor cage is equipped with 2 sets of horizontal infrared beams, positioned at 3cm and 15cm above the base of the cage. Both sets of beams consist of a 12 beam by 7 beam matrix, forming a grid of 66 x 2.54cm^2 cells within the cage. Activity is recorded as the number of times a beam changes from unbroken to broken and is split into static or mobile activity. An animal is considered mobile if its central position changes by more than 2 grids (5.08cm^2). Rearing is recorded by the upper set of infrared beams. Active time is also logged, an animal is considered inactive if

no beam has been broken for 5 seconds. Locomotor activity measures were recorded at 1 min intervals for 15min, following which time the animals were returned to their home cage.

2.3.6 Preparation of tissue samples for western immunoblotting

Approximately 30mg cortex tissue was cross-chopped and placed in a 1.5ml microtube on ice. 10 volumes Lysis Buffer (50mM Tris-HCl, pH8; 150mM NaCl; 1% ([Octy]phenoxy)polyethoxyethanol (NP-40)) containing 1% protease and phosphatase inhibitors was added per sample. Samples were sonicated on ice at 5 microns for 5 sec using a Soniprep 150 [Sanyo]. Samples were then centrifuged at 15,000 x rpm at 4°C for 30 min. The supernatant was transferred to a fresh 1.5ml microtube and the pellet was stored at -80°C. A 1:5 dilution of the supernatant was prepared and a protein assay was carried out as in section 2.4.5. Protein concentrations of samples were adjusted to 4.5mg/ml. Samples were aliquoted into 25µl aliquots and stored at -80°C until use for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblotting as described in section 2.4.9.

2.3.7 Preparation of tissue samples for mRNA analysis

A polytron blade [Kinematica] and forceps were rinsed before use with RNase away followed by diethylpyrocarbonate (DEPC) H_2O to prevent RNA degradation. Approximately 25mg of tissue was cross-chopped using a sterile scalpel and added to 350µl RA1 buffer [supplied in Total RNA isolation kit, Machery Nagel GmbH & Co.] containing 1% β-mercaptoethanol in a round-bottomed microtube. Samples were homogenised for approximately 30 sec with the polytron. The scalpel and polytron blade were rinsed between samples using DEPC H_2O to prevent cross-contamination of samples. RNA isolation was carried out as in section 2.4.10.

2.3.8 Preparation of tissue for MSD® Multi-spot biomarker detection

Approximately 20mg of tissue was cross-chopped and homogenised in a 2ml round-bottomed microtube using a polytron [Kinematica] in 10 volumes (w/v) Lysis Buffer (50mM NaCl pH 10, 1%SDS) containing 1% protease and phosphatase inhibitors. Samples were spun at 21,500 x gravity (g) for 40 min at 4°C. The supernatant was transferred to a new 1.5ml microtube and

a protein assay was carried out as in 2.4.5. Protein was equalised to a concentration of 2.8mg/ml and 10% (v/v) of neutralising buffer (0.5M Tris-HCl, pH 6.8) was added to each sample. Samples were aliquoted into 20µl aliquots and stored at -80°C until required.

2.4 Analytical techniques

2.4.1 Cell viability assay - alamar blue assay

A 440µM solution of alamar blue dye was prepared by dissolving 25mg Resazurin (F.W. 251.17g) in 226.214ml d.d.H_2O. The dye was added to the wells of a 24-well culture plate at 10% v/v ratio with treatments. A set of wells containing media only (no cells) and another set containing cells but no treatments (control) were included in the experimental design. Following treatment plates were placed in the incubator for 24 hr after which time the supernatant was removed from the wells and placed directly into wells of a 96-well flat bottomed plate. The absorbance was then read at 600nm using a microtitre plate reader [Bio-Tek Instruments Inc.].

Percentage viability of cells was assessed using the following equation:

%Viability = ((Am - As) / (Am - Ac)) *100

where Am = average absorbance of wells containing media only,
Ac = average absorbance of wells containing cells without treatments
As = absorbance of a particular sample.

2.4.2 Analysis of nitrite concentrations (Greiss assay)

The production of nitrite by glial cultures was measured using a Greiss assay. Nitric oxide is an unstable compound which is rapidly broken down into two stable and non-volatile substrates, nitrite and nitrate, and so since nitric oxide cannot be measured directly the Greiss assay is a way of determining its production in cells in an indirect manner. A stock 18mM solution of sodium nitrite ($NaNO_2$) was prepared by dissolving 62mg of $NaNO_2$ in 50ml d.d.H_2O. Standards ranging from 0µM to 180µM were prepared by carrying out dilutions of

the $NaNO_2$ stock solution in cDMEM. Samples were centrifuged at 13,000 x rpm at 4°C for 1 min to pellet any cellular debris. 100µl of each of the standards and samples was plated out onto a 96-well plate in duplicate. 50µl of Greiss Reagent I (1% Sulphanilamide in 85% orthophosphoric acid) was added to each well and the plate was incubated at room temperature for 10 min. 50µl of Greiss Reagent II (0.1% N-(1-naphthyl-ethylenediamine dihydrochloride)) was then added to each well and the plate was incubated at room temperature for a further 10 min. The absorbance was read at 450 nanometres (nm) using a microtitre plate reader [Bio-Tek Instruments Inc.]. A standard curve was constructed and total nitrite concentration for each of the samples was calculated by comparison against the standard curve.

2.4.3 Analysis of TNF-α concentrations by ELISA

TNF-α w as measured using a TNF-α ELISA kit [BD Biosciences] according to the manufacturer's instructions. Briefly, a 96-well NUNC F96 MAXISORP-immuno plate was coated with 100µl of capture antibody (1:250 dilution of TNF-α coating antibody in 0.1M sodium carbonate, pH9.5) and incubated overnight at 4°C. The coating antibody was aspirated and wells were washed four times with 400µl wash buffer (PBS, pH 7.3, containing 0.05% Tween-20) using a plate washer [TECAN]. Any excess wash buffer remaining in the wells was removed by blotting the plate dry on a paper towel. Plates were blocked at room temperature for 1 hr using 200µl assay diluent (PBS containing 10% FCS) per well. Plates were washed as before and blotted dry. A 1:82.5 dilution of the stock standard of 165ng/ml was carried out in order to obtain a 2000pg/ml top standard. A series of 2-fold serial dilutions of the standards was carried out to yield an eight point standard curve ranging from 0pg/ml to 2000pg/ml. The limit of detection for this kit is 15pg/ml. 100µl of each of the samples and standards was loaded onto the plate in duplicate. The plate was covered and incubated at room temperature for 2 hr. The samples and standards were aspirated and the plate was washed as before. 100µl of detection antibody (1:250 dilution of biotinylated anti-rat TNF-α antibody in 0.1M sodium carbonate, pH 9.5) was added per well and the plate was covered and incubated at room temperature for 1 hr. The detection antibody was aspirated and the plate was washed as before. 100µl of Streptavidin-HRP Enzyme Reagent (1:250 dilution in 0.1M sodium carbonate, pH 9.5) was added to each of the wells and the plate was covered and incubated at

room temperature for 30 min. The Streptavidin-HRP enzyme reagent was aspirated and 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was carefully added to each well. The plate was covered and incubated at room temperature for approximately 30 min until the colorimetric reaction reached saturation at which time 50µl of 1M sulphuric acid (H_2SO_4) was carefully added to each well to stop the reaction. The plate was gently tapped to ensure thorough mixing and the absorbance was read at 450/630nm using a microtitre plate reader [Bio-Tek Instruments Inc.]. A standard curve was constructed by plotting the standards against the absorbance obtained and the unknown concentration of TNF-α in the samples was determined by comparison against the standard curve. Results obtained were expressed as pg/ml of supernatant.

2.4.4 Analysis of IL-1β concentrations by ELISA

IL-1β concentrations were measured using an IL-1β ELISA kit [DuoSet, RnD Systems] according to the manufacturer's instructions. Briefly, a 96-well NUNC F96 MAXISORP-immunoplate was coated with 100µl of capture antibody (144µg/ml stock solution of anti-rat IL-1β diluted to 0.8µg/ml working concentration in PBS) and incubated overnight at room temperature. The capture antibody was aspirated and wells were washed four times with 300µl wash buffer (PBS, pH7.3 containing 0.05% Tween-20). Any excess wash buffer remaining in the wells was removed by blotting the plate dry on a paper towel. The plate was blocked by adding 300µl block buffer (PBS containing 1% BSA, 5% sucrose) per well and then incubated at room temperature for 1 hr. The plate was washed as before and blotted dry. A top standard of 2000pg/ml was prepared from a stock standard of 100ng/ml. A series of 2-fold serial dilutions was carried out to obtain an eight point standard curve ranging from 0pg/ml to 2000pg/ml. 100µl of each of the samples and standards was loaded onto the plate in duplicate and the plate was covered and incubated at room temperature for 2 hr. The samples and standards were aspirated and the plate was washed as before. 100µl of detection antibody (63µg/ml biotinylated anti-rat IL-1β antibody diluted to a working concentration of 350ng/ml in PBS containing 1% BSA) was added per well and the plate was covered and incubated at room temperature for 2 hr. The plate was washed as before and 100µl of Streptavidin-HRP Enzyme Reagent (1:200 dilution in PBS containing 1% BSA) was added to each of the wells and the plate was covered and incubated at room temperature for 20 min. The Streptavidin-

HRP enzyme reagent was aspirated and the plate was washed as before. 100µl of TMB substrate solution was carefully added to each well. The plate was covered and incubated at room temperature for approximately 30 min until colorimetric saturation was reached at which point the reaction was stopped by the careful addition of 50µl 1M H_2SO_4 to each of the wells. The plate was gently tapped to ensure thorough mixing and the absorbance was read at 450/630nm using a microtitre plate reader [Bio-Tek Instruments Inc.]. A standard curve was constructed by plotting standards against absorbance and the unknown concentration of IL-1β in the samples was determined by comparison against the standard curve. Results were expressed as pg/ml of supernatant.

2.4.5 Bicinchonic acid (BCA) protein assay

Protein concentrations were determined using the BCA protein assay kit [Pierce] as per the manufacturer's instructions. Briefly, a stock solution of BSA of 20mg/10ml d.d.H_2O was prepared. A working solution of 2000µg/ml was prepared and a series of dilutions was carried out to give standards with a final BSA protein concentration of 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000µg/ml. 25µl of each of the standards and samples was pipetted into the wells of a 96-well plate in duplicate. A BCA working solution was prepared by adding 50 parts BCA Reagent A to 1 part BCA Reagent B (supplied). The solution was vortexed and 200µl of this working reagent was carefully added to each of the wells. The 96-well plate was covered and incubated at 37°C for 30 min. The absorbance was read at 595nm using a microtitre plate reader [Bio-Tek Instruments Inc.]. A standard curve was constructed by plotting the standards against the absorbance obtained and the unknown concentration of each of the samples was determined by comparison against the standard curve. Results were expressed as µg/ml.

2.4.6 cAMP Assay

Intracellular cAMP was measured using a cAMP Biotrak Enzymeimmunoassay system [Amersham Biosciences] immediately following the harvesting of cells as described in 2.2.7. Briefly, a standard curve ranging from 0 to 3200 femtomole (fmol) was constructed by carrying out serial 1:2 dilutions of the standards. 100µl of lysis reagent 1B (0.25%

Dodecyletrimethylammonium Bromide solution) and 100µl of lysis reagent 2B (supplied) were pipetted into the non-specific binding (NSB) well. 100µl of lysis reagent 1B was pipetted into the zero standard well. 100µl of each of the standards was plated out in duplicate and 100µl of each of the samples was plated out individually. 100µl of anti-cAMP serum was added to all wells except the blank and NSB wells. The plate was covered and incubated at 4°C for 2 hr. 50µl of cAMP-peroxidase conjugate (in 0.05M acetate buffer, pH5.8; 0.02% (w/v) BSA) was added to each of the wells. The plate was covered and incubated at 4°C for 1 hr. The solution was aspirated and wells were washed four times with 400µl assay wash buffer (0.01M Phosphate buffer, pH7.5; 0.05% (w/v) Tween-20) using a plate washer [TECAN] and the plate was blotted dry on a paper towel. 150µl TMB substrate was added to each well. The plate was covered and incubated at room temperature for 15 min after which time the colour reaction was stopped by addition of 50µl 1M H_2SO_4 stop solution. The absorbance was read at 450nm using a microtitre plate reader [Bio-Tek Instruments Inc.]. A standard curve was constructed and levels of cAMP expressed in the samples were determined by comparison against the standard curve and later plotted as fmol/µg protein.

2.4.7 Biotinylation assay

The biotinylation assay was carried out using a Pinpoint Cell Surface Protein isolation kit [Pierce] according to the manufacturer's instructions. Briefly, following harvesting of lysates as described in 2.2.8 the suspension was subsequently transferred to a 1.5ml microtube. Cells were disrupted by sonicating on ice using five 1 sec bursts at 5 microns using a Soniprep 150 [Sanyo]. The samples were incubated on ice for 30 min with samples being vortexed for 5 sec every 5 min. Additional sonications were carried out every 10 min to improve solubility. Samples were centrifuged at 10,000 x g for 2 min at 4°C. The supernatant was then transferred to a new 1.5ml microtube. 125µl of Immobilized NeutrAvidin Gel (supplied) was added to a spin column. Columns were centrifuged at 1,000 x g for 1 min at 4°C and the flow-through was discarded. 125µl of wash buffer was added to the gel. The column was centrifuged at 1,000 x g for 1 min at 4°C and the flow-through was discarded (repeated twice). The cell supernatant was added to the gel and columns were incubated at room temperature for 1 hr on a gyrorocker. Columns were centrifuged at 1,000 x g for 1 min, the flow-through was discarded and 125µl of wash buffer (containing 1% protease inhibitors) was added to the

column. The columns were centrifuged at 1,000 x g for 1 min and the flow-through was discarded (repeated three times). 100µl of sample buffer (62.5mM Tris-HCl, pH 6.8; 1% (w/v) SDS; 10% (v/v) glycerol) containing 1M DTT was added to the gel and columns were incubated at room temperature for 1 hr on a gyrorocker after which time the column was placed in a new collection tube. The column was centrifuged at 1,000 x g for 2 min. 15µl of 10x Bromophenol Blue was added to the eluate and the eluate was aliquoted into 50µl aliquots and stored at -20°C until required for analysis using SDS-PAGE and western immunoblotting as described in 2.4.9.

2.4.8 MSD® 96-well MULTI-SPOT® Human/Rodent (4G8) Abeta Triplex Ultra-Sensitive Assay

Amyloid-β concentrations were measured using an MSD® 96-well MULTI-SPOT® Human/Rodent (4G8) Abeta Triplex Ultra-Sensitive Assay kit [MesoScale Discovery] as per the manufacturer's instructions. Briefly, 150µl of Blocker A solution (supplied) was added to each well of the 96-well plate and the plate was covered and incubated at room temperature for 1 hr. Standards for amyloid-β_{1-38}, amyloid-β_{1-40}, amyloid-β_{1-42} were prepared immediately before use to prevent aggregation of peptides by adding the correct volume of DMSO as indicated on each of the vials to yield a 0.1mg/ml stock solution. The peptide solutions were gently vortexed to ensure they were fully dissolved. A 10µg/ml stock was prepared for each of the peptides by adding 10µl of the 0.1mg/ml stocks to 90µl of 1% Blocker A solution. A 0.1µg/ml stock of each peptide was prepared by adding 5µl of the 10µg/ml solution to 495µl of 1% Blocker A solution. The highest standard was prepared by adding 80µl of the 0.1µg/ml amyloid-β_{1-40} peptide solution and 24µl each of the 0.1µg/ml amyloid-β_{1-38} and amyloid-β_{1-42} peptide solutions to 672µl of 1% Blocker A solution. Subsequent standards were prepared by carrying out 3-fold serial dilutions to yield a standard curve with concentrations ranging from 0 to 3,000pg/ml for amyloid-β_{1-38} and amyloid-β_{1-42} and from 0 to 10,000pg/ml for amyloid-β_{1-40}. A 1:40 dilution of samples was carried out in Lysis Buffer containing 1% protease and phosphatase inhibitors (as in 2.3.8). The blocker solution was aspirated and the plate was washed x 4 using 400µl/well of 1x Tris wash buffer (supplied). 25µl of the detection antibody solution (3ml per plate: 60µl 50x SULFO-TAG 4G8 detection antibody, 30µl 100x Blocker G solution, 2910µl 1% Blocker A solution) was added to each well and 25µl of standards or

samples were immediately added to each well. The plate was covered and incubated at room temperature for 2 hr. The solution was aspirated and the plate was washed x 4 using 400µl/well 1x Tris wash buffer. Exactly 150µl/well 2x MSD read buffer T (supplied) was added to each well and the plate was read within 30 min on the sector imager. A BCA protein assay was again carried out on the diluted samples and amyloid-β concentrations were expressed as pg/mg of protein.

2.4.9 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Immunoblotting

2.4.9.1 SDS-PAGE

Samples to be analysed using western immunoblotting were added in a 1:1 v/v ratio to 5x Laemmli sample buffer (0.5M Tris-HCl, pH 6.8; 10% (w/v) sodium dodecyl sulphate; 10% (v/v) glycerol; 5% (v/v) β-mercaptoethanol; 0.05% (w/v) bromophenol blue) and were boiled for 10 min at 100°C prior to loading on gels for SDS-PAGE. Electrophoresis was carried out using SDS-polyacrylamide gels (7.5%, 10% or 15% separating gel: 4% stacking gel). Briefly, gels were prepared by pouring the separating gels between two glass plates held firmly together with a casting frame [BioRad]. A layer of isopropanol was poured onto the gel to prevent evaporation. The separating gel was allowed to set for a minimum of 30 min after which time the isopropanol was poured off and the gel was rinsed with d.d.H_20. Subsequently, the stacking gel was poured between the two glass plates and a 10-well comb was placed in the gel to allow wells to form. Once the gels were set they were placed in an electrode assembly and clamped in place. The assembly was then placed into a gel rig [BioRad Mini-PROTEAN 3]. The middle chamber and bottom of the gel rig were filled with 1x electrode running buffer (125mM Tris-base, pH 8.3; 960mM glycine; 0.5% SDS). The combs were removed from the gels and 10µl of sample was loaded per well with the exception of biotinylated samples where 30µl of sample was loaded per well. 10µl Broadrange Molecular Marker [BioRad] was loaded onto gels alongside samples. Proteins were separated by applying a constant current of 33 milliamps (mA) per gel for 20-60 min or until the blue dye of the sample buffer had run to the bottom of the gel.

2.4.9.2 Semi-dry transfer

Proteins were transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membranes using a semi-dry blotter at a constant current of 225mA for 90 min. Briefly, following electrophoresis gels were removed from the glass plates and placed on a layer of nitrocellulose or PVDF. PVDF requires activation in methanol for 15 sec followed by soaking in Milli-Q water [Millipore] prior to use. 2 pieces of filter paper were soaked in anode buffer I (0.3M Tris, 10% methanol), 1 piece of filter paper was soaked in anode buffer II (25mM Tris, 10% methanol) and 3 pieces of filter paper were soaked in cathode buffer (25mM Tris, 40mM glycine, 10% methanol) for 30 sec. A sandwich of the filter paper, nitrocellulose/PVDF membranes and gel was prepared as outlined in Figure 2.1. Briefly, filter paper soaked in anode buffer I was placed on the anode (+) plate of the semi-dry transfer unit and filter paper soaked in anode buffer II was placed on top. The nitrocellulose/PVDF membrane was placed on top of this layer of filter paper, followed by the gel. Finally, filter paper soaked in cathode buffer was placed on top and any bubbles which had formed between the layers were carefully eliminated by rolling a pasteur pipette over the sandwich. The cathode (-) plate was placed onto the unit. Proteins migrate from the cathode end of the unit to the anode end. In this way protein samples were transferred onto nitrocellulose/PVDF membranes for 1.5 hr at 55mA.

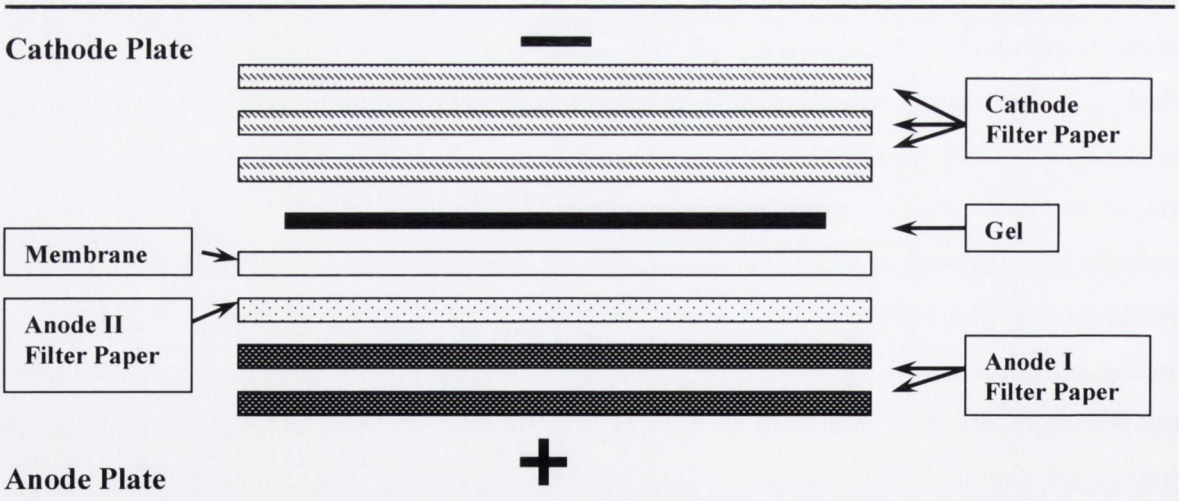


Figure 2.1: Diagram of sandwich preparation for semi-dry transfer

2.4.9.3 Western immunoblotting

Membranes were blocked using 20ml of 5% BSA in TBS-Tween20 (TBS-T) for 1 hr. The membrane was washed 4 x 10 min in 20ml PBS-T. The membrane was incubated with the primary antibody at the appropriate dilution (Table 2.1) in 10ml of 1% BSA in TBS-T overnight at 4°C. Membranes were washed 4 x 10 min in 20ml PBS-T. The secondary antibody was applied to the membrane at the appropriate dilution (Table 2.1) in 10ml 2% BSA in TBS-T and incubated for 1 hr at room temperature. The membrane was washed 4 x 10 min in 20ml PBS-T. Blots were exposed to Supersignal West Dura enhanced chemiluminescence [Pierce] for 1 min or Immobilon Western HRP substrate [Millipore] for 5 min and developed using an automated developer [Agfa CP1000]. Blots were stripped using Re-Store Western Blot Stripping Buffer [Pierce] prior to re-probing. Protein bands were quantified using an MCID analysis system [Imaging Research Inc.].

Target	Separating gel (%)	M.Wt. (kDa)	Primary Antibody Conc.	Secondary Antibody Conc.
β_2-adrenoceptor	10%	65	1:200 [Santa Cruz:SC-570]	1:500 Anti-Rabbit [Amersham:NA934]
GFAP	10%	50	1:1000 [Sigma: G3893]	1:10,000 Anti-Mouse [Sigma: A8924]
Phospho-STAT-3	7.5%	75	1:1000 [Cell Signalling: 9131s]	1:10,000 Anti-Rabbit [Amersham: NA934]
STAT-3	7.5%	75	1:1000 [Cell Signalling: 9132]	1:10,000 Anti-Rabbit [Amersham:NA934]
SOCS3	12.5%	30	1:1000 [Santa-Cruz:SC-9023]	1:10,000 Anti-Rabbit [Amersham: NA934]
Phospho-ERK	10%	42 & 44	1:1000 [Cell Signalling: 9101s]	1:10,000 Anti-Rabbit [Amersham:NA934]
ERK	10%	42 & 44	1:1000 [Cell Signalling: 9102]	1:10,000 Anti-Rabbit [Amersham:NA934]
Phospho-JNK	10%	46 & 54	1:1000 [Cell Signalling: 9251s]	1:10,000 Anti-Rabbit [Amersham:NA934]
JNK	10%	46 & 54	1:1000 [Cell Signalling: 9252]	1:10,000 Anti-Rabbit [Amersham:NA934]
Phospho-p38	10%	40	1:1000 [Cell Signalling]	1:10,000 Anti-Rabbit [Amersham:NA934]
p38	10%	40	1:1000 [Cell Signalling:9212]	1:10,000 Anti-Rabbit [Amersham:NA934]
β-actin	-	43	1:1000 [Sigma: A5441]	1:10,000 Anti-Mouse [Sigma: A8924]
β-actin	-	43	1:400 [Santa-Cruz: SC-8432]	1:500 Anti-Mouse [Sigma:A8924]

Table 2.1: Antibody dilutions for western immunoblotting

2.4.10 Analysis of mRNA expression

All surfaces were cleaned using RNase Zap wipes [Ambion Inc.] prior to carrying out RNA work to eliminate RNases and prevent RNA degradation. Biosphere Filter Tips [Sarstedt] were used for all RNA work.

2.4.10.1 RNA extraction

A Nucleospin RNA II Total RNA isolation kit [Machery-Nagel GmbH & Co.] was used to extract RNA as per the manufacturer's protocol. Cells or tissue were prepared for RNA extraction as desribed in 2.2.9 and 2.3.7 respectively. The sample was added to a Nucleospin Filter unit and filtered by centrifugation at 13,000 x rpm for 1 min. The Nucleospin Filter unit was discarded and 350µl 70% EtOH was added to the sample. The sample and EtOH were mixed by triturating ~8 times. Each sample was transferred to a Nucleospin RNA II column placed in a 2ml centrifuge tube. The RNA was bound to the membrane by centrifugation at 13000 x rpm for 30 sec. The column was placed in a new collecting tube and 350µl of membrane desalting buffer was added to the column which was subsequently centrifuged at 13000 x rpm for 1 min to dry the membrane. Any DNA present was digested by adding a preparation of rDNase and rDNase reaction buffer (supplied). Briefly, 10µl rDNase was added to 90µl DNase reaction buffer and 95µl of this reaction mixture was pipetted directly onto the centre of the silica membrane of the column. The columns were then incubated at room temperature for 15 min. To inactivate the rDNase 200µl of RA2 buffer was added to the column which was then centrifuged at 13000 x rpm for 30 sec. The column was place in a new collecting tube and 600µl of RA3 buffer was added to the column and the column was centrifuged at 13000 x rpm for 30 sec. The flow-through was discarded and 250µl RA3 buffer was added to the column which was centrifuged at 13000 x rpm for 2 min in order to completely dry the membrane. The column was placed in a 1.5ml nuclease-free microtube (supplied) and 60µl of RNase-free water was added to the column. The column was centrifuged at 13000 x rpm for 1 min to elute highly pure RNA. RNA was subsequently stored at -80°C for later qualification, quantification and reverse transcription.

2.4.10.2 Analysis of RNA integrity

The integrity of the RNA samples was evaluated by separating the RNA on a 1% agarose gel. 1.3g of agarose powder was dissolved fully in TBE (1.0M Tris, 0.9M Boric acid, 0.01M EDTA) by heating in a microwave for approximately 2 min in a glass vessel. The solution was allowed to cool to a temperature at which the container could be handled prior to the addition of 1.3µl ethidium bromide. The solution was gently swirled to ensure thorough mixing and poured onto a sealed agarose gel tray. A comb was added to allow for the formation of loading wells and any bubbles in the gel were removed using a pipette tip to ensure correct running of the samples. The gel was allowed to set for 30 min and the tray was transferred to a gel tank and covered with 1x TBE running buffer. 3µl of RNA was mixed with 2µl RNase-free H_2O and 1µl of loading dye. The samples were vortexed to ensure thorough mixing and 4µl of this solution was loaded to the gel. RNA was separated by running it at 90 volts for 1-1.5 hr or until the front dye had reached the end of the gel. The gel rig was disconnected from the power source and the gel was taken to a UV transilluminator for RNA visualisation. Only RNA which displayed both an 18s and 28s ribosomal band were used for PCR as these indicate that the RNA is completely intact and has not been degraded.

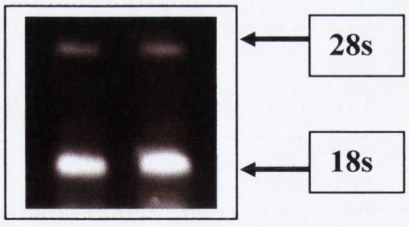


Figure 2.2: Representative picture of intact RNA displaying ribosomal 18s and 28s bands

2.4.10.3 Spectophotometric quantification of RNA

A NanoDrop was used to carry out spectrophotometric quantification of RNA. Briefly, 1µl of RNase-free H_2O was added to the pedestal of the NanoDrop to initialise it. This H_2O was wiped off using tissue paper and a further 1µl of H_2O was used to blank the instrument and this was again wiped away using tissue paper. Subsequently, the RNA samples were flicked gently to ensure thorough mixing and 1µl of each of the samples in turn was pipetted onto the

pedestal and the concentration was determined in ng/µl. The pedestal was cleaned between samples using tissue paper and a fresh 10µl filtered pipette tip was used for each sample to prevent cross-contamination of samples. The ratio between the A260 and A280 readings was used to determine the purity of the RNA, where a ratio of 2 shows pure RNA. The RNA was equalised using RNase-free H_2O as a diluent. RNA from cells was equalised to 10ng/µl and that from tissue was equalised to 60ng/µl. Equalised samples were then aliquoted into 20µl aliquots and stored at -20°C until required.

2.4.10.4 cDNA synthesis

A high-capacity cDNA archive kit [Applied Biosystems] was used to reverse transcribe RNA samples extracted using the total RNA isolation kit [Machery-Nagel GmbH & Co.]. Briefly, 10-20µl of RNA was mixed in a 1:1 ratio with a 2x Master Mix in a microtube. The Master Mix was prepared as follows: 1:5 dilution of 10x Reverse Transcription Buffer, 1:12.5 dilution of 25x dNTPs, 1:5 dilution of 10x Random Primers, 1:10 dilution of Multiscribe Reverse Transcriptase, 1:2.381 dilution of RNAse/DNase-free H_2O. The samples were vortexed and centrifuged using a mini-centrifuge and subsequently placed in a Thermocycler [PTC-200, MJ Research]. The samples were incubated at 25°C for 10 min followed by incubation at 37°C for 2 hr. Finally cDNA was stored at -20°C for further use in PCR analysis.

2.4.10.5 Muti-target (Multiplex) quantitative Real Time-PCR (RT-PCR)

Gene expression was assessed using Taqman gene expression assays containing specific target primers and FAM-labelled MGB target probes (Table 2.2) [Applied Biosystems]. β-actin gene expression was used to normalise 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 (Table 2.2) [Applied Biosystems].

Briefly, for cell samples a 1:5 dilution of cDNA was carried out in RNase/DNase-free water and for tissue samples a 1:4 dilution of cDNA was carried out in RNase/DNase-free water. Since each sample acts as its own control 10µl of each of the samples was plated out singly onto a 96-well optical reaction plate [Applied Biosystems]. A Taqman Master Mix containing

12.5µl Taqman Universal PCR Master Mix, 1.25µl of β-actin primer/probe and 1.25µl target primer/probe was added to each well. Electronic pipettes (EDP3 20-200µl, 2-20µl and 10-100µl) were used to ensure pipetting accuracy. The plate was covered with an optical adhesive cover [Applied Biosystems] and centrifuged at 800 x rpm for 20 sec. The reaction was run in an Applied Biosystems 7300 real-time PCR machine using the following programme: 90°C for 10 min, 90°C for 15 sec for 40 cycles and then 60°C for 1 min for 40 cycles. The data was analysed using Applied Biosystems relative quantification software.

Target	Primer Code
ADAM-10	Rn01530753_m1
ADAM-17	Rn00571880_m1
Adrb1 (Beta-adrenoceptor 1)	Rn00824536_s1
Adrb2 (Beta-adrenoceptor 2)	Rn00560650_s1
Ager (RAGE)	Rn00584249_m1
APP (amyloid precursor protein)	Rn00570673_m1
β-actin (VIC/MGB pl)	4352340E
BACE1	Rn00569988_m1
CD11b	Rn00709342_m1
CD40	Rn01423583_m1
CD68	Rn01495634_g1
CD86	Rn00571654_m1
COX-II	Rn00568225_m1
CSF-1 (M-CSF)	Rn00576849_m1
CSF-r1 (M-CSF receptor)	Rn01491957_m1
FGF-2	Rn00570809_m1
GDNF	Rn00755092_m1
GFAP	Rn00566603_m1
ICAM-1	Rn00564227_m1
IDE (Insulysin)	Rn00565839_m1
IFN-γ	Rn00594078_m1
IGF-1	Rn00710306_m1
IkBa	Rn01473658_g1
IL-1 receptor antagonist	Rn00573488_m1
IL-1 Type I Receptor	Rn00565482_m1
IL-1 Type II Receptor	Rn00588589_m1
IL-10	Rn00563409_m1
IL-18	RN00824548_m1
IL-1alpha	Rn00566700_m1
IL-1beta	Rn00580432_m1
IL-33	Rn01759837_m1
IL-6	Rn00561420_m1
IP-10	Rn00594648_m1
MME (Neprilysin)	Rn00561572_m1
MMP-2	Rn01538177_m1
MMP-9	Rn00579162_m1
NGF-β	Rn01533872_m1
NOS2 (iNOS)	Rn00561646_m1
PSEN1 (presenilin-1)	Rn00569763_m1
PSEN2 (presenilin-2)	Rn00579412_m1
RANTES	Rn00579590_m1
Scarb1	Rn00580588_m1
SOCS3	Rn00585674_s1
TGF-beta 1	Rn00572010_m1
TIMP-1	Rn00587558_m1
TLR-2	Rn02133647_s1
TNF-alpha	Rn93699017_m1

Table 2.2: List of primers/probes for PCR

2.4.10.6 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 time (CT) difference between samples. Briefly, a threshold for fluorescence is set against which CT is measured. To accurately assess differences between gene expression the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Thus, samples with low CT readings demonstrate high fluorescence, indicating greater amplification and hence, greater gene expression. When a PCR is 100% efficient a one-cycle difference between samples means a 2-fold difference in copy number (2^2), similarly a 5-cycle difference is a 32-fold difference (2^5).

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

2.5 Statistical analysis

All data was analysed using the statistical analysis package GB-STAT [Dynamic Microsystems Inc.]. Statistical comparisons were performed using either a Student's *t*-test or a one or two-way analysis of variance (ANOVA) as indicated in the experimental sections. If

significant changes were observed following ANOVA the data was further analysed using either a Dunnett's Procedure or Student Newman-Keuls *post hoc* test as appropriate. A p value less than 0.05 was deemed statistically significant. Results are expressed as means with standard error of the mean (SEM) or mean percentage control with SEM.

Chapter 3

Regulation of β_2-adrenoceptor expression & function on glial cells in vitro by an inflammatory stimulus

3.1 Introduction

Noradrenaline is widely regarded as an endogenous anti-inflammatory and neurotrophic neurotransmitter within the CNS and dysregulation of the noradrenergic system has been proposed to be a contributing factor in a number of neurodegenerative diseases including MS, Alzheimer's Disease, Parkinson's Disease, Huntington's Disease and HIV related dementia, amongst others (Feinstein *et al.*, 2002; Marien *et al.*, 2004). These diseases are generally characterized by an unresolved inflammatory response with chronic activation of microglia and astrocytes within the CNS (Marien *et al.*, 2004). Chronic glial activation may have detrimental consequences for neurons since it can result in the overproduction or sustained production of potentially neurotoxic molecules such as NO, ROS and the pro-inflammatory cytokines TNF-α and IL-1β (Xie *et al.*, 2004). For this reason noradrenergic regulation of neuroinflammation has been the subject of much research over the past number of years. As such, it is now widely accepted that the anti-inflammatory and neurotrophic effects of noradrenaline within the CNS are mediated primarily through its actions at β_2-adrenoceptors (De Keyser *et al.*, 1999; Mori *et al.*, 2002; Zeinstra *et al.*, 2002). In this regard, stimulation of β_2-adrenoceptors using selective β_2-adrenoceptor agonists has been shown to activate astrocytes and induce the expression of the neurotrophins NGF, bFGF-2 and TGF-β_1 (Nakamura *et al.*, 1998; Culmsee *et al.*, 1999 a,b; Zhu *et al.*, 2001). Furthermore, studies have also shown that noradrenaline can suppress the proliferation of microglial cells and alter the function of macrophages and mononuclear phagocytes and it has also been shown to suppress the expression of inflammatory genes such as those of iNOS, IL-1β and TNF-α in microglia, astrocytes and brain endothelial cells *in vitro* (Feinstein *et al.*, 2002; Mori *et al.*, 2002). Notably, in EAE, a rodent model of MS, chemical sympathectomy which reduces endogenous levels of noradrenaline and cAMP in the brain, is found to worsen disease symptoms whereas β_2-adrenoceptor agonists can suppress symptoms (Giorelli *et al.*, 2004).

Intriguingly, it has been observed that β_2-adrenoceptors are absent from astrocytes in both normal appearing white matter and astrogliotic plaques in the white matter of patients suffering from MS, but are present on tissue from control individuals suffering from other neuroinflammatory conditions (De Keyser *et al.*, 1999; Zeinstra *et al.*, 2000). Such a phenomenon suggests an inability for endogenous noradrenaline to elicit its anti-inflammatory

effects in the CNS thus leading to the neuroinflammation that is characteristically observed in MS. Previous studies have reported that pro-inflammatory cytokines can impact on the expression and function of β_2-adrenoceptors expressed on human airway smooth muscle (HASM) cells and rat lung membranes (Moore *et al.,* 1999; Mak *et al.,* 2002). With this in mind, the following studies were undertaken in an attempt to elucidate a link between inflammation and the absence of β_2-adrenoceptor expression on astrocytes observed in the MS brain. Furthermore, studies have shown that the synthetic glucocorticoid dexamethasone can influence the expression and function of the β_2-adrenceptor under both basal (Dangel *et al.,* 1996) and inflammatory conditions (Moore *et al.,* 1999; Mak *et al.,* 2002). Thus, since glucocorticoids have shown therapeutic efficacy in EAE (Tsutsui *et al.,* 2008) and as they are also commonly used in the treatment of MS (Brusaferri *et al.,* 2000) the impact of dexamethasone on β_2-adrenoceptor expression in mixed glia *in vitro* under both basal and inflammatory conditions was also examined here.

As such the objectives of the following studies were:

1) To determine the anti-inflammatory and neurotrophic actions of noradrenaline and selective β_2-adrenoceptor agonists (salbutamol and formoterol) in primary mixed glial cultures in the presence of the endotoxin LPS.

2) To determine whether incubation with a combined treatment of LPS + IFN-γ induces a pro-inflammatory response in primary mixed glial cultures and to determine the impact of this treatment regimen on cell viability.

3) To determine the impact of LPS + IFN-γ on intracellular cAMP accumulation in response to the selective β_2-adrenoceptor agonist salbutamol and its impact on the cell surface and mRNA expression of the β_2-adrenoceptor in primary mixed glial cultures.

4) To examine the ability of LPS and IFN-γ both alone and in combination to induce a pro-inflammatory response and to examine their impact on the expression and responsiveness of the β_2-adrenoceptor in primary mixed glial cultures.

5) To examine the ability of LPS + IFN-γ to induce a pro-inflammatory response in enriched cultures of astrocytes and microglia and to determine the impact on β_2-adrenoceptor mRNA expression in these cultures.

6) To determine whether pre-treatment with the glucocorticoid dexamethasone, a drug commonly used in the treatment of MS, can inhibit the attenuation of β_2-adrenoceptor expression induced by treatment with LPS + IFN-γ in primary mixed glial cultures.

3.2 Methods

3.2.1 Subjects and drug treatments

Primary mixed glial cultures and enriched cultures of microglia and astrocytes were prepared and maintained in cDMEM for 10-14 days as previously described in the methods section (Chapter 2). LPS (100ng/ml), IFN-γ (20ng/ml), salbutamol (0.1, 1 & 5μM), noradrenaline (5μM), formoterol (5μM), forskolin (10μM), IBMX (100μM) and dexamethasone (0.01 & 1μM) were dissolved and administered to glial cultures as described in the methods section (Section 2.2, Chapter 2).

3.2.2 Experimental Procedure

The design for this set of experiments was as set out in Figures 3.1 to 3.6 below. Treatment of glial cultures was carried out as previously outlined in the methods (Section 2.2, Chapter 2). Harvesting of supernatants and cell lysates for analyses and the techniques of RNA extraction, cDNA synthesis, RT-PCR, alamar blue assay, IL-1β & TNF-α ELISAs, Greiss assay, cAMP assay, biotinylation assay and western immunoblotting used in these studies were outlined previously in the methods section (Section 2.2 & 2.4, Chapter 2)..

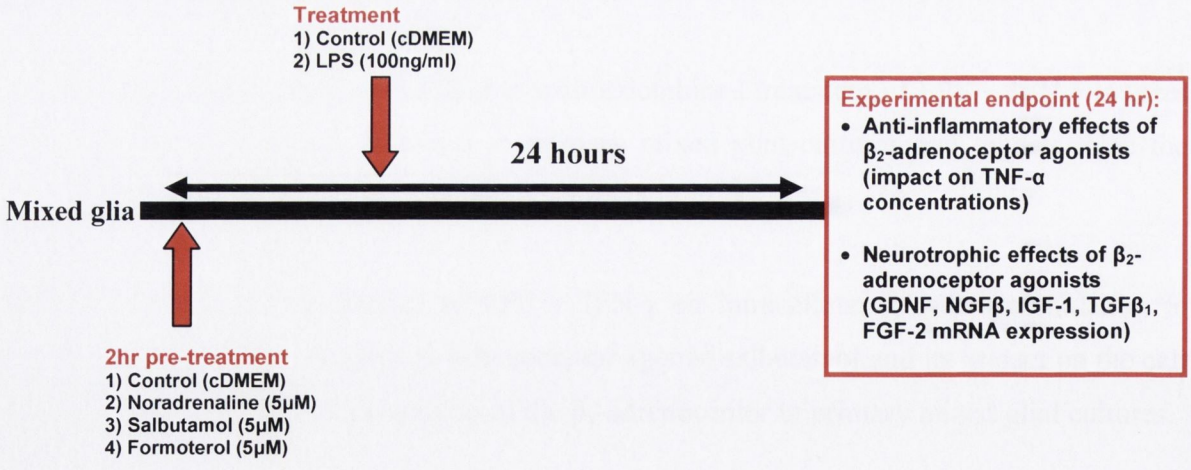


Figure 3.1: Experimental design for study examining the ability of noradrenaline and selective β_2-adrenoceptor agonists to exert anti-inflammatory and neurotrophic effects in glia

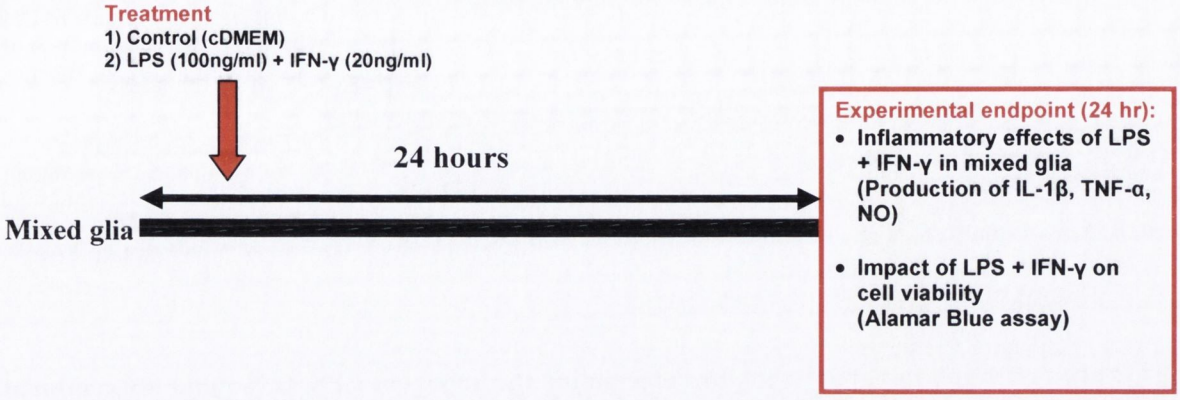


Figure 3.2: Experimental design for determining the ability of LPS + IFN-γ to induce a pro-inflammatory response in glia and to examine its impact on cell viability

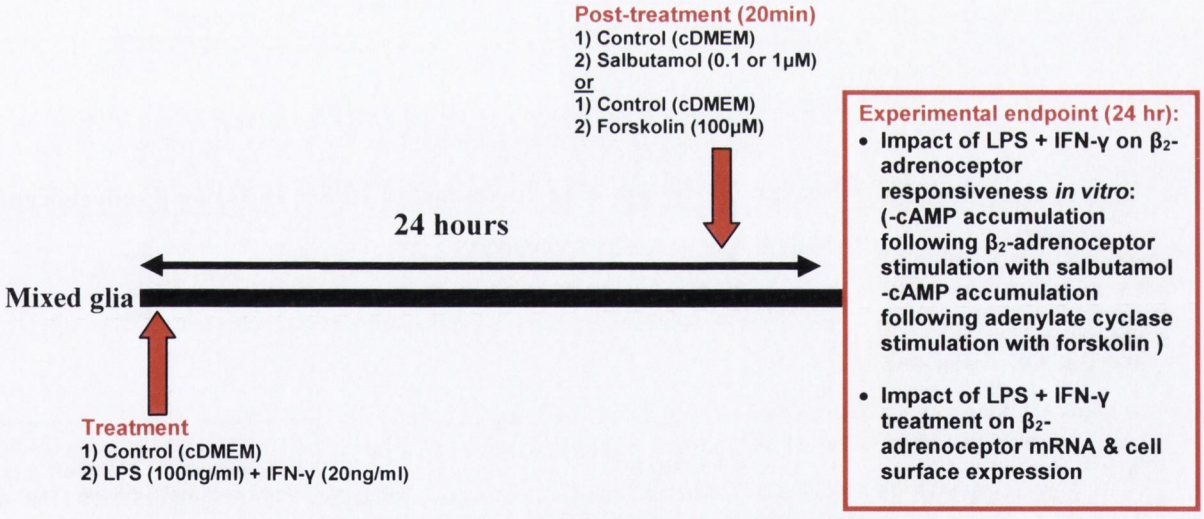


Figure 3.3: Experimental design for determining the impact of LPS + IFN-γ on β_2-adrenoceptor expression and responsiveness in glia

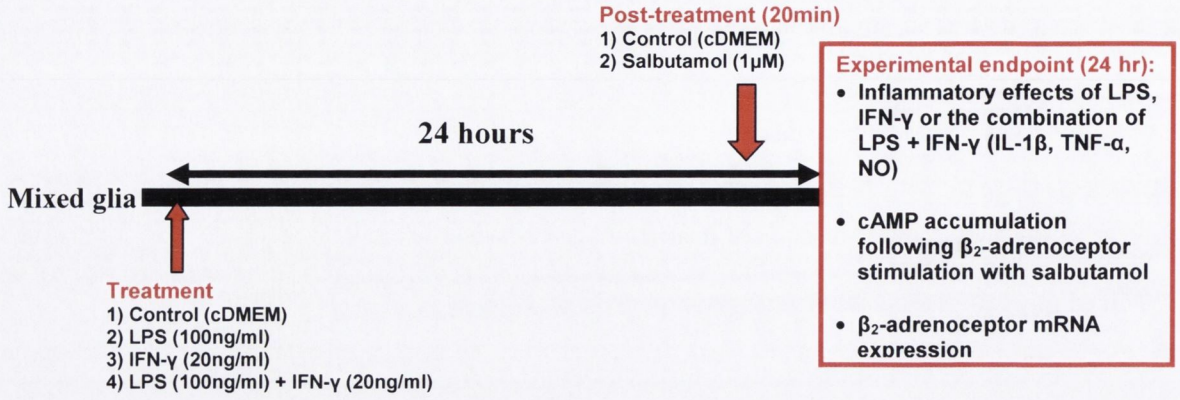


Figure 3.4: Experimental design for determining the impact of LPS, IFN-γ and the combination of LPS + IFN-γ on β_2-adrenoceptor expression and responsiveness in glia

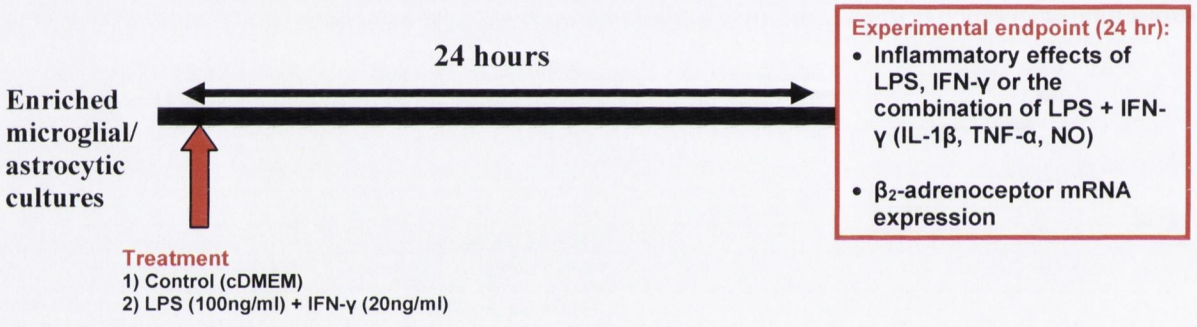


Figure 3.5: Experimental design for determining the impact of LPS + IFN-γ on β_2-adrenoceptor expression in enriched microglial and astrocytic cultures

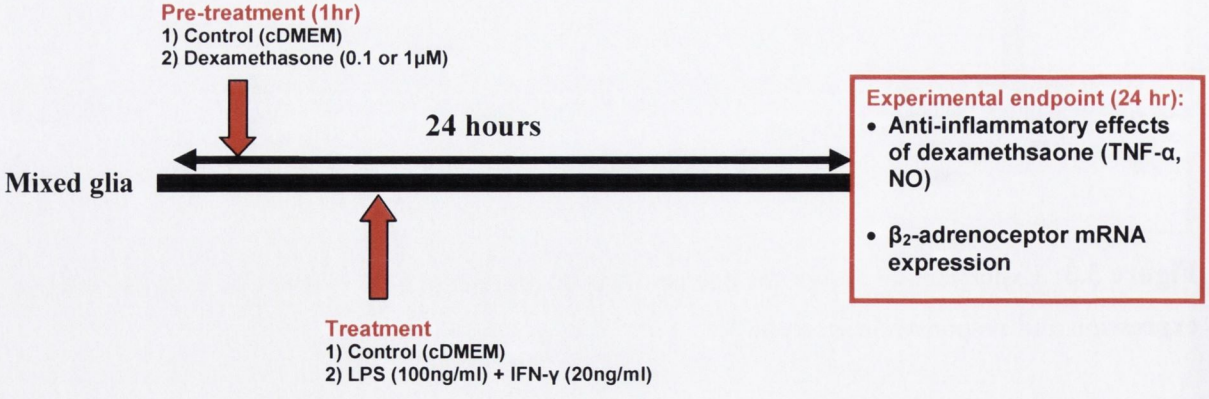


Figure 3.6: Experimental design for determining the ability of pre-treatment with dexamethasone to attenuate the effect of LPS + IFN-γ on β_2-adrenoceptor expression in glia

3.3 Treatment of primary mixed glial cultures with noradrenaline or the β_2-adrenoceptor agonists salbutamol and formoterol significantly attenuates production of TNF-α and increases expression of the neurotrophin GDNF in response to LPS

As previously mentioned a vast array of literature has suggested that noradrenaline elicits its anti-inflammatory and neurotrophic effects via stimulation of the β_2-adrenoceptor on glial cells within the CNS. Therefore, the purpose of the following study was to determine if pre-treatment of primary mixed glial cultures with noradrenaline or the selective β_2-adrenoceptor agonists salbutamol or formoterol could impact on the production of the pro-inflammatory cytokine TNF-α or on expression of the neurotrophins GDNF, NGF-β, TGF-β_1, FGF-2 or insulin-like growth factor (IGF-1).

Noradrenaline, salbutamol and formoterol reduce the LPS-induced production of TNF-α: Treatment of primary mixed glial cultures with LPS (100ng/ml) significantly increased the production of TNF-α ($P<0.01$) compared to unstimulated counterparts over a 24 hr period as assessed by ELISA. Pre-treatment of cultures for 2 hr with noradrenaline (5μM), salbutamol (5μM) or formoterol (5μM) prior to exposure to LPS significantly reduced the production of TNF-α compared to control counterparts ($P<0.01$). [Figure 3.3.1 a, Newman-Keuls, n=5-6].

Salbutamol and formoterol augment the LPS-induced expression of GDNF: Treatment of primary mixed glial cultures with LPS (100ng/ml) did not alter the mRNA expression of GDNF compared to unstimulated counterparts over a 24 hr period as assessed by RT-PCR. However, pre-treatment of cultures for 2 hr with salbutamol (5μM) or formoterol (5μM) prior to exposure to LPS significantly increased the mRNA expression of GDNF compared to control counterparts ($P<0.01$). Whilst, pre-treatment of cultures with noradrenaline (5μM) was also shown to increase LPS-induced GDNF expression this did not reach statistical significance. [Figure 3.3.1 b, Newman-Keuls, n=5-6].

Formoterol augments LPS-induced expression of NGFβ: Treatment of primary mixed glial cultures with LPS (100ng/ml) significantly increased the mRNA expression of NGFβ ($P<0.01$) compared to unstimulated counterparts over a 24 hr period as assessed by RT-PCR. Pre-treatment of cultures for 2 hr with formoterol (5μM) prior to exposure to LPS augmented the

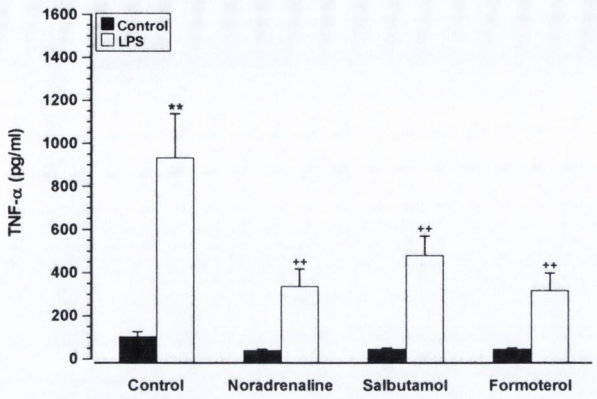
LPS-induced expression of NGFβ compared to control counterparts ($P<0.01$). However, pre-treatment of cultures with noradrenaline (5μM) or salbutamol (5μM) had no significant effect on NGFβ expression. [Figure 3.3.1 c, Newman-Keuls, n=5-6].

Neither noradrenaline, salbutamol nor formoterol altered LPS-induced reduction of IGF-1: Treatment of primary mixed glial cultures with LPS (100ng/ml) significantly reduced the mRNA expression of IGF-1 ($P<0.01$) compared to unstimulated counterparts over a 24 hr period as assessed by RT-PCR. Pre-treatment of cultures for 2 hr with noradrenaline (5μM), salbutamol (5μM) or formoterol (5μM) had no effect on the LPS-induced reduction of IGF-1 expression. [Figure 3.3.2 a, Newman-Keuls, n=5-6].

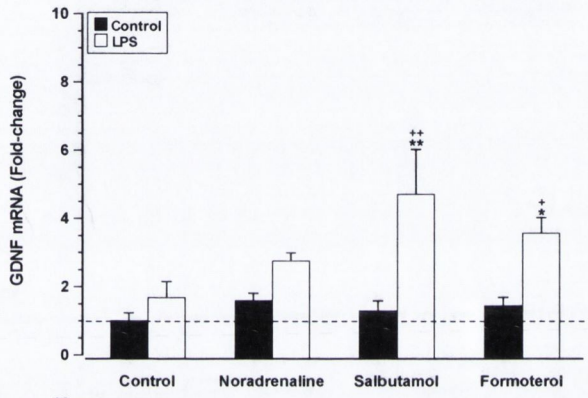
Neither noradrenaline, salbutamol nor formoterol altered expression of TGF-β_1: Treatment of primary mixed glial cultures with LPS (100ng/ml) did not alter the mRNA expression of TGF-β_1 compared to unstimulated counterparts over a 24 hr period as assessed by RT-PCR. Pre-treatment of cultures for 2 hr with noradrenaline (5μM), salbutamol (5μM) or formoterol (5μM) had no effect on TGF-β_1 expression. [Figure 3.3.2 b, Newman-Keuls, n=5-6].

Neither noradrenaline, salbutamol nor formoterol altered expression of FGF-2: Treatment of primary mixed glial cultures with LPS (100ng/ml) did not alter the mRNA expression of FGF-2 compared to unstimulated counterparts over a 24 hr period as assessed by RT-PCR. Pre-treatment of cultures for 2 hr with noradrenaline (5μM), salbutamol (5μM) or formoterol (5μM) had no effect on FGF-2 expression. [Figure 3.3.2 c, Newman-Keuls, n=5-6].

a) TNF-α



b) GDNF



c) NGFβ

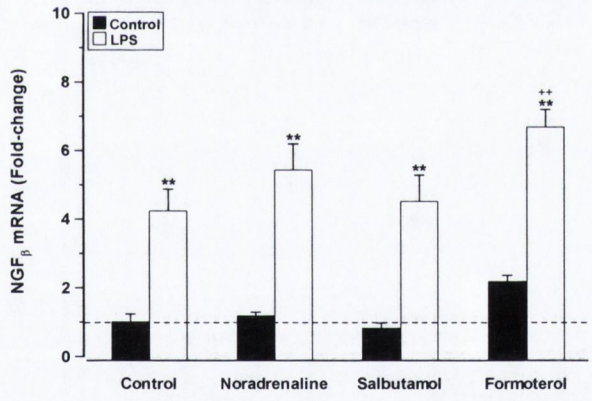
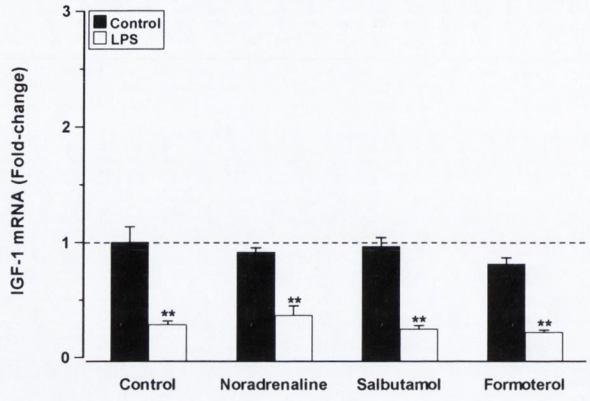


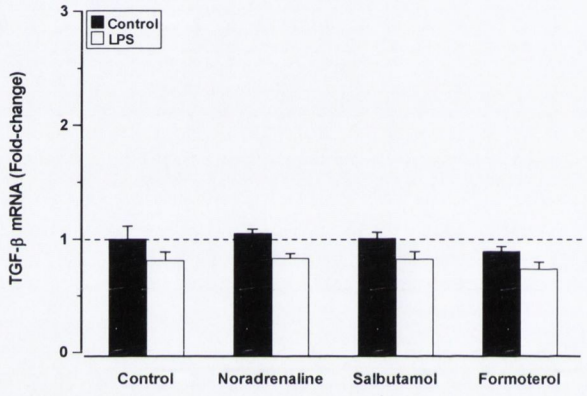
Figure 3.3.1: Noradrenaline and β_2-adrenoceptor agonists decrease TNF-α production and selectively increase GDNF and NGF-β expression

Primary mixed glial cultures were treated for 2 hr with either control (cDMEM), noradrenaline (5μM), salbutamol (5μM) or formoterol (5μM) followed by treatment with either control or LPS (100ng/ml) and were subsequently incubated for 24 hr. (a) A two-way ANOVA revealed a significant interactive effect of treatment on the production of TNF-α [$F_{(3,40)}$=4.07, P=0.0129]. (b) A two-way ANOVA revealed a significant interactive effect of treatment on GDNF mRNA expression [$F_{(3,38)}$=4.69, P=0.007]. (c) A two-way ANOVA revealed a significant effect of treatment with β_2-agonists [$F_{(3,40)}$=5.83, P=0.0021] and LPS treatment [$F_{(1,40)}$=125.81, P<0.0001] on NGFβ mRNA expression. Data are expressed as means with standard error of the mean (n=5-6). *P<0.05, **P<0.01 versus control/unstimulated counterparts, +P<0.05, ++P<0.01 versus LPS control counterpart (Newman-Keuls).

a) IGF-1



b) TGF-β_1



c) FGF-2

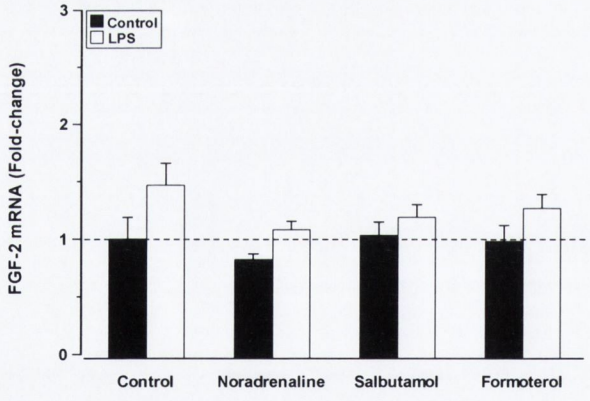


Figure 3.3.2: Noradrenaline and β_2-adrenoceptor agonists have no effect on IGF-1, TGF-β_1, FGF-2 or expression

Primary mixed glial cultures were treated for 2 hr with either control (cDMEM), noradrenaline (5μM), salbutamol (5μM) or formoterol (5μM) followed by treatment with either control or LPS (100ng/ml) and were subsequently incubated for 24 hr. (a) A two-way ANOVA revealed a significant effect of LPS on IGF-1 mRNA expression [$F_{(1,40)}$=177.88, P<0.0001]. (b) A two-way ANOVA revealed a significant effect of LPS on TGFβ_1 mRNA expression [$F_{(1,40)}$=15.94, P=0.0003]. (c) A two-way ANOVA revealed a significant effect of LPS on FGF-2 mRNA expression [$F_{(1,40)}$=9.86, P=0.0032]. Data are expressed as means with standard error of the mean (*n*=6). **P<0.01 versus control/unstimulated counterparts (Newman-Keuls).

3.4 Treatment with LPS + IFN-γ increases production of nitric oxide and the pro-inflammatory cytokines IL-1β and TNF-α in primary mixed glial cultures without affecting cell viability

The following studies were carried out to verify that a combined treatment of LPS + IFN-γ induces the production of pro-inflammatory cytokines, namely TNF-α and IL-1β, and nitrite, as an indirect measure of NO production, in primary mixed glial cultures. Additionally, the viability of glial cultures following stimulation with LPS + IFN-γ was assessed to ensure that this treatment regimen did not instigate a toxic effect on cells.

LPS + IFN-γ induces production of TNF-α: A combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) significantly increased TNF-α concentrations ($P<0.0001$) in primary mixed glial cultures compared to unstimulated counterparts after 24 hr as assessed by ELISA. [Figure 3.4.1 a, Student's *t*-test, $n=8$].

LPS + IFN-γ induces production of IL-1β: A combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) significantly increased IL-1β concentrations ($P<0.0001$) in primary mixed glial cultures compared to unstimulated counterparts after 24 hr as assessed by ELISA. [Figure 3.4.1 b, Student's *t*-test, $n=8$].

LPS + IFN-γ induces the production of nitrite: A combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) significantly increased nitrite concentrations ($P<0.0001$) in primary mixed glial cultures compared to unstimulated counterparts after 24 hr as assessed by Greiss assay. [Figure 3.4.1 c, Student's *t*-test, $n=8$].

LPS + IFN-γ has no effect on cell viability: A combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) had no effect on the viability of cells in primary mixed glial cultures compared to unstimulated counterparts after 24 hr as assessed by alamar blue assay. [Figure 3.4.2, Student's *t*-test, $n=12$].

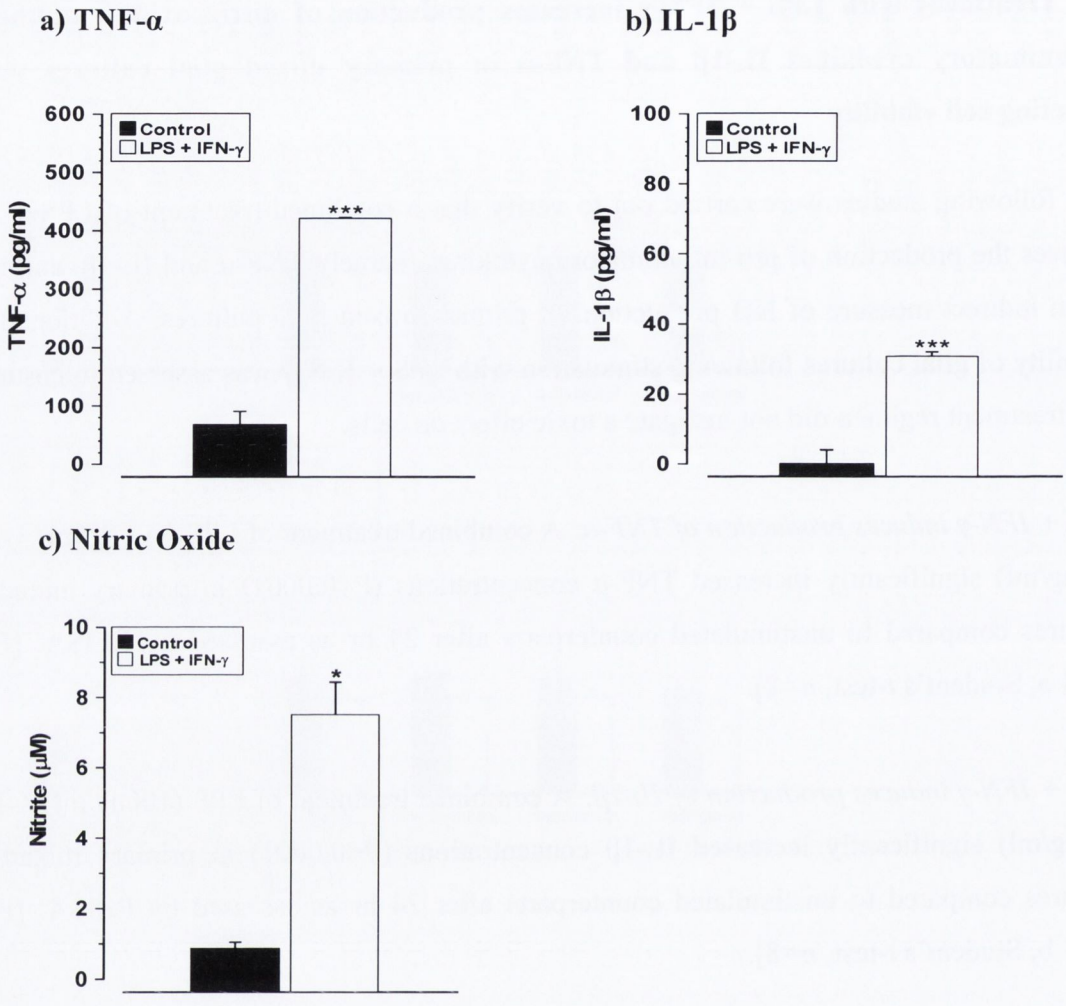


Figure 3.4.1: Treatment with LPS + IFN-γ significantly increases the production of TNF-α, IL-1β and nitric oxide in primary mixed glial cultures after 24 hr

Primary mixed glial cultures were incubated with either control (cDMEM) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr. (a) A Student's *t*-test revealed a significant effect of LPS + IFN-γ on the production of TNF-α ($P<0.001$) compared to control/unstimulated counterparts. (b) A Student's *t*-test revealed a significant effect of LPS + IFN-γ on the production of IL-1β ($P<0.001$) compared to control/unstimulated counterparts. (c) A Student's *t*-test revealed a significant effect of LPS + IFN-γ on the production of nitrite ($P<0.05$) compared to control/unstimulated counterparts. Data are expressed as means with standard error of the mean ($n=8$). *$P<0.05$, ***$P<0.001$ versus control/unstimulated counterparts (Student's *t*-test).

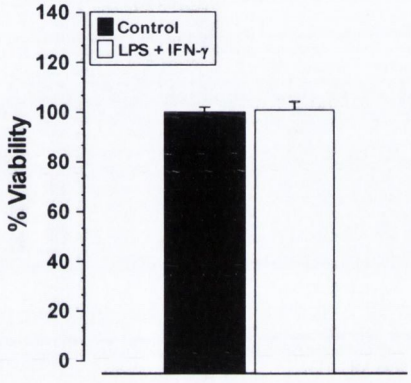


Figure 3.4.2: Treatment with LPS + IFN-γ does not affect viability of primary mixed glial cultures after 24 hr

Primary mixed glial cultures were incubated with either control (cDMEM) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) in combination with 10% (v/v) resazurin dye for 24 hr. A Student's *t*-test revealed no significant effect of LPS + IFN-γ on the viability of primary mixed glial cultures compared to control/unstimulated counterparts after 24 hr. Data are expressed as means with standard error of the mean (n=12).

3.5 Treatment with LPS + IFN-γ suppresses accumulation of intracellular cAMP in primary mixed glial cultures in response to the selective β_2-adrenoceptor agonist salbutamol

The β_2-adrenoceptor has been shown to be absent from astrocytes in MS brain, a disease associated with neuroinflammation and glial activation (De Keyser *et al.*, 1999). It has been well established that stimulation of β_2-adrenoceptors leads to an increase in the concentrations of intracellular cAMP (Rosenberg & Li, 1995; Mak *et al.*, 2000). Thus, the following studies were carried out to assess the impact of treatment with an inflammatory stimulus of LPS + IFN-γ on the accumulation of intracellular cAMP in response to the selective β_2-adrenoceptor agonist salbutamol in order to determine if this treatment regimen can impact on the responsiveness of the β_2-adrenoceptor. In addition, the specificity of any effect induced by LPS + IFN-γ on the β_2-adrenoceptor was assessed in this study by measuring cAMP accumulation in response to forskolin, a direct activator of adenylate cyclase. All cAMP analyses were carried out in the presence of 3-isobutyl-1-methylxanthine (IBMX), a non-specific phosphodiesterase inhibitor, in order to prevent cAMP degradation following drug treatments.

Treatment with LPS + IFN-γ reduces accumulation of intracellular cAMP in response to stimulation with the β_2-adrenoceptor agonist salbutamol: Treatment of primary mixed glial cultures with the β_2-adrenoceptor agonist salbutamol (0.1 or 1μM) for 20 min, in the presence of the phosphodiesterase inhibitor IBMX (100μM), significantly increased the accumulation of intracellular cAMP in a dose-dependent manner ($P<0.01$) compared to unstimulated control counterparts. In contrast, when cultures were exposed to LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr prior to treatment with salbutamol, in the presence of IBMX, there was a blunted increase in the accumulation of intracellular cAMP in response to salbutamol ($P<0.01$). [Figure 3.5.1, Newman-Keuls, $n=7$].

Treatment with LPS + IFN-γ did not alter accumulation of intracellular cAMP in response to stimulation of adenylate cyclase with forskolin: Treatment of primary mixed glial cultures with the adenylate cyclase activator forskolin (10μM) for 20 min, in the presence of the phosphodiesterase inhibitor IBMX (100μM), significantly increased the accumulation of

intracellular cAMP ($P<0.01$) compared to unstimulated control counterparts. Notably, when cultures were exposed to LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr prior to treatment with forskolin, in the presence of IBMX, there was no effect on the accumulation of intracellular cAMP in response to forskolin compared to control counterparts. [Figure 3.5.2, Newman-Keuls, $n=6$].

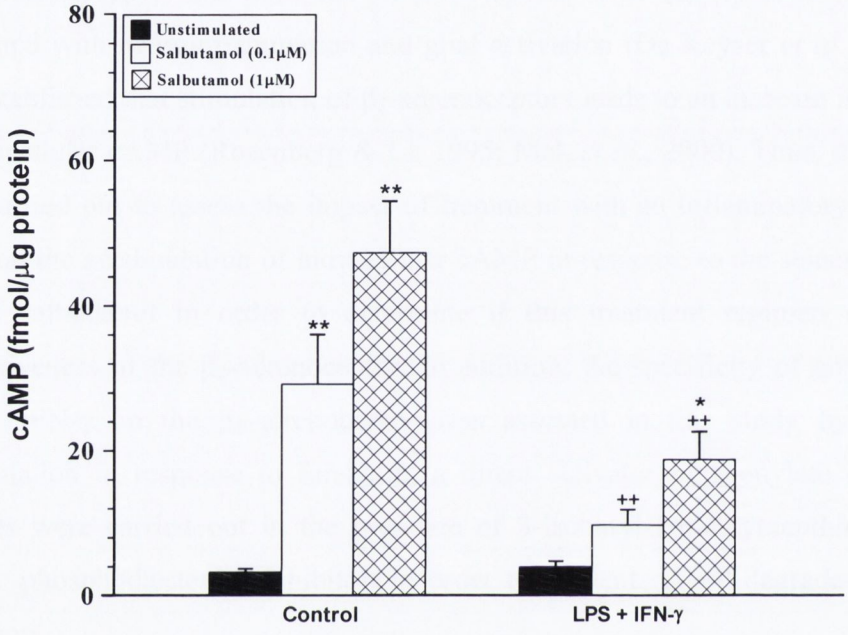


Figure 3.5.1: Treatment with LPS + IFN-γ suppresses accumulation of intracellular cAMP in response to stimulation of the β_2-adrenoceptor with the β_2-adrenoceptor agonist salbutamol

Primary mixed glial cultures were incubated with either control (cDMEM) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr followed by subsequent incubation with either control or salbutamol (0.1μM or 1μM) in the presence of IBMX (100μM) for 20 min. A two-way ANOVA revealed a significant interactive effect of LPS + IFN-γ and salbutamol on the accumulation of intracellular cAMP [$F_{(2,36)}$=5.73, P=0.0069]. Data are expressed as means with standard error of the mean (n=7). **P<0.01, *P<0.05 versus control/unstimulated counterparts, ++P<0.01 versus control counterparts (Newman-Keuls).

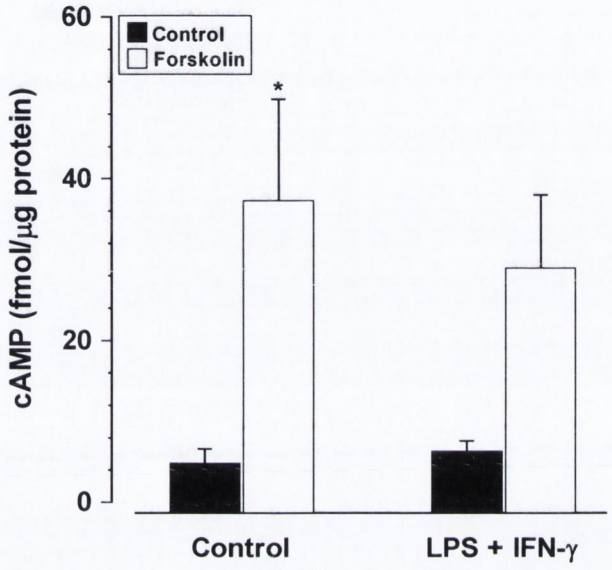


Figure 3.5.2: Treatment with LPS + IFN-γ does not affect accumulation of intracellular cAMP in response to the adenylate cyclase activator forskolin

Primary mixed glial cultures were incubated with either control (cDMEM) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr followed by subsequent incubation with either control or forskolin (10μM) in the presence of IBMX (100μM) for 20 min. A two-way ANOVA revealed a significant effect of treatment with forskolin on the accumulation of intracellular cAMP [$F_{(1,20)}$=12.4, P=0.0021]. Data are expressed as means with standard error of the mean (n=6). *P<0.05 versus control/unstimulated counterparts (Newman-Keuls).

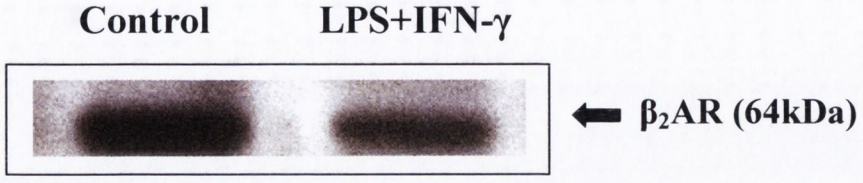
3.6 Treatment with LPS + IFN-γ decreases cell surface and mRNA expression of the β_2-adrenoceptor

The results of the previous study reveal that there is impaired responsiveness of the β_2-adrenoceptor to the β_2-adrenoceptor agonist salbutamol following treatment of mixed glial cultures with LPS + IFN-γ. This effect is demonstrated to be due to a direct impact on the β_2-adrenoceptor itself since there was no effect on intracellular cAMP accumulation in response to direct stimulation of adenylate cyclase. It has previously been shown that impaired accumulation of intracellular cAMP following exposure to IL-1β is associated with a reduction in the density of β_2-adrenoceptors in lung membranes (Mak *et al.*, 2002). Therefore it was hypothesized that reduced expression of the β_2-adrenoceptor at the cell surface might account for the impaired accumulation of intracellular cAMP observed in primary mixed glial cells following exposure to LPS + IFN-γ in this study. It was also hypothesised that there may also be a reduction in the expression of β_2-adrenoceptor at the mRNA level which could in turn lead to reduced receptor density at the cell surface. Therefore, the cell surface and mRNA expression of the β_2-adrenoceptor was examined here in response to LPS + IFN-γ.

Treatment with LPS + IFN-γ reduces the cell surface expression of the β_2-adrenoceptor: Treatment of primary mixed glial cultures with LPS + IFN-γ significantly reduced ($P<0.05$) the expression of the β_2-adrenoceptor at the level of the cell surface compared to control counterparts 24 hr post-treatment as determined by biotinylation assay. [Figure 3.6.1 a,b, Student's *t*-test, $n=3$].

Treatment with LPS + IFN-γ reduces the mRNA expression of the β_2-adrenoceptor: Treatment of primary mixed glial cultures with LPS + IFN-γ significantly reduced ($P<0.01$) the mRNA expression of the β_2-adrenoceptor as compared to control counterparts 24 hr post-treatment as assessed using RT-PCR. [Figure 3.6.2, Student's *t*-test, $n=8$].

a) Sample Immunoblot



b) Optical Density

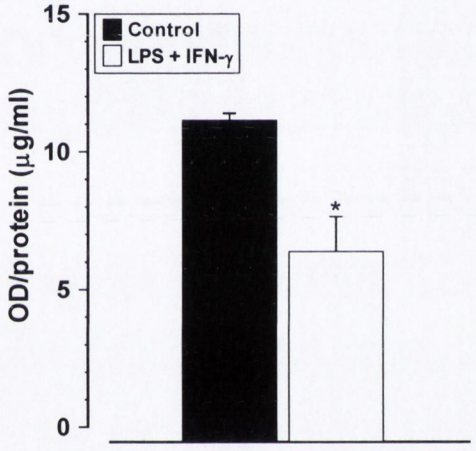


Figure 3.6.1: Treatment with LPS + IFN-γ reduces cell surface expression of the β_2-adrenoceptor

Primary mixed glial cultures were incubated with either control (cDMEM) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr. (a) Sample immunoblot representative of three independent experiments depicting decreased β_2-adrenoceptor expression on the cell surface of primary mixed glial cultures following 24 hr incubation with LPS + IFN-γ. (b) Graph representing optical density of immunoblot bands corrected for protein content of cell lysates. A Student's *t*-test revealed a significant effect of LPS + IFN-γ on the cell surface expression of the β_2-adrenoceptor. Data are expressed as means with standard error of the mean (n=3). *$P<0.05$ versus control/unstimulated counterparts (Student's *t*-test).

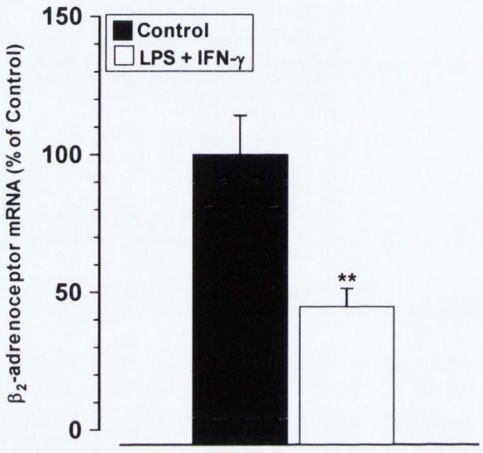


Figure 3.6.2: Treatment with LPS + IFN-γ reduces mRNA expression of the β_2-adrenoceptor

Primary mixed glial cultures were incubated with either control (cDMEM) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr. A Student's *t*-test revealed a significant effect of LPS + IFN-γ on β_2-adrenoceptor mRNA expression ($P<0.01$) compared to unstimulated control counterparts. Data are expressed as mean percentage control with standard error of the mean ($n=8$). **$P<0.01$ versus control/unstimulated counterpart (Student's *t*-test).

3.7 Both LPS and IFN-γ alone decrease expression and responsiveness of the β_2-adrenoceptor but it is the combination of LPS + IFN-γ that has the most profound effect

The results from the previous studies show that treatment of primary mixed glial cultures with LPS + IFN-γ reduces the responsiveness of the β_2-adrenoceptor to stimulation with salbutamol and decreases both the cell surface and mRNA expression of the receptor. Thus, the following studies were undertaken to determine whether it is LPS or IFN-γ that is the primary mediator of these effects. In addition, the production of the pro-inflammatory mediators IL-1β, TNF-α and nitric oxide in mixed glial cultures was determined in response to LPS and IFN-γ both alone and in combination.

TNF-α production is increased by LPS and the combination of LPS + IFN-γ: Treatment of primary mixed glial cultures with LPS (100ng/ml) alone significantly increased TNF-α production ($P<0.01$) while treatment with IFN-γ (20ng/ml) alone did not alter TNF-α production compared to unstimulated control counterparts as assessed using ELISA. However, when cultures were exposed to a combined treatment of LPS + IFN-γ the presence of IFN-γ augmented the LPS-induced increase in TNF-α production ($P<0.01$). [Figure 3.7.1 a, Newman-Keuls, $n=6$].

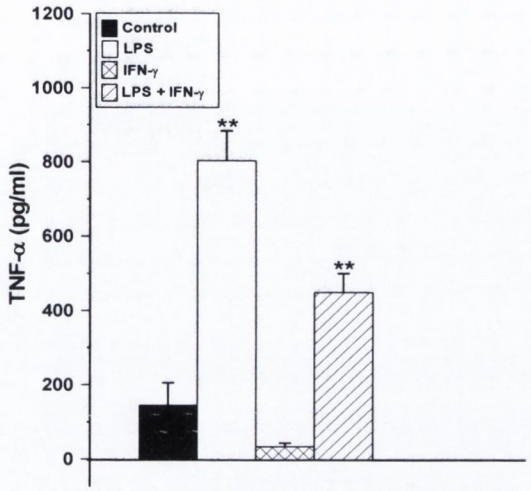
IL-1β production is increased by LPS: Treatment of primary mixed glial cultures with IFN-γ (20ng/ml) alone or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) failed to alter IL-1β production compared to unstimulated control counterparts as assessed using ELISA. However, treatment of cultures with LPS alone significantly increased IL-1β production ($P<0.01$). [Figure 3.7.1 b, Newman-Keuls, $n=6$].

Nitric oxide production is increased by LPS and the combination of LPS + IFN-γ: Treatment of primary mixed glial cultures with LPS (100ng/ml) alone significantly increased the production of nitrite ($P<0.01$) while IFN-γ (20ng/ml) alone failed to alter nitrite production compared to unstimulated control counterparts as assessed by Greiss assay. However, when glial cultures were exposed to a combined treatment of LPS + IFN-γ the presence of IFN-γ augmented the LPS-induced production of nitrite ($P<0.01$). [Figure 3.7.1 c, Newman-Keuls, $n=6$].

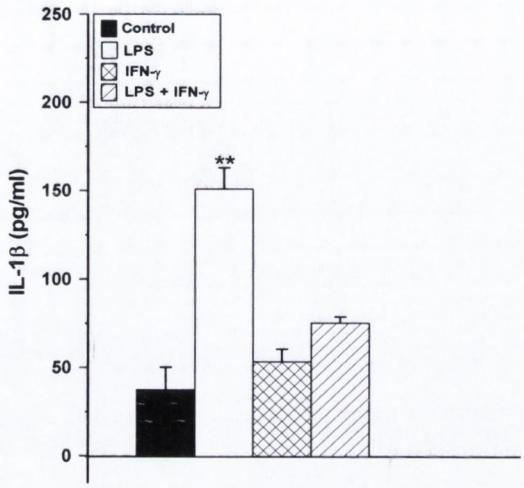
The combination of LPS + IFN-γ has the most profound effect on β_2-adrenoceptor mRNA expression: Treatment of primary mixed glial cultures with both LPS and IFN-γ alone significantly decreased the mRNA expression of the β_2-adrenoceptor ($P<0.01$), however, it was the combined treatment of LPS + IFN-γ that had the most profound effect on β_2-adrenoceptor mRNA expression ($P<0.01$) compared to unstimulated control counterparts as assessed using RT-PCR. [Figure 3.7.2 a, Newman-Keuls, $n=8$].

The combination of LPS + IFN-γ has the most profound effect on β_2-adrenoceptor responsiveness: Stimulation of β_2-adrenoceptors with the β_2-adrenoceptor agonist salbutamol (1μM) for 20 min significantly increased the accumulation of intracellular cAMP ($P<0.01$) in primary mixed glial cultures. Exposure of glial cultures to LPS (100ng/ml) and IFN-γ (20ng/ml) alone 24 hr prior to stimulation with salbutamol reduced the responsiveness of the β_2-adrenoceptor, although these failed to attain significance. However, exposure of glial cultures to a combined treatment of LPS + IFN-γ 24 hr prior to stimulation with salbutamol significantly reduced the accumulation of intracellular cAMP ($P<0.05$). [Figure 3.7.2 b, Newman-Keuls, $n=4$].

a) TNF-α



b) IL-1β



c) Nitric Oxide

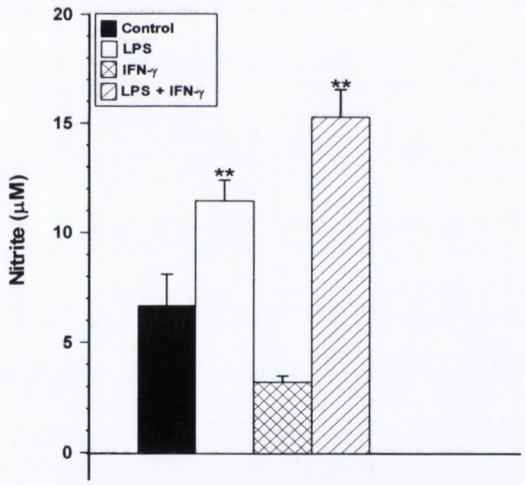
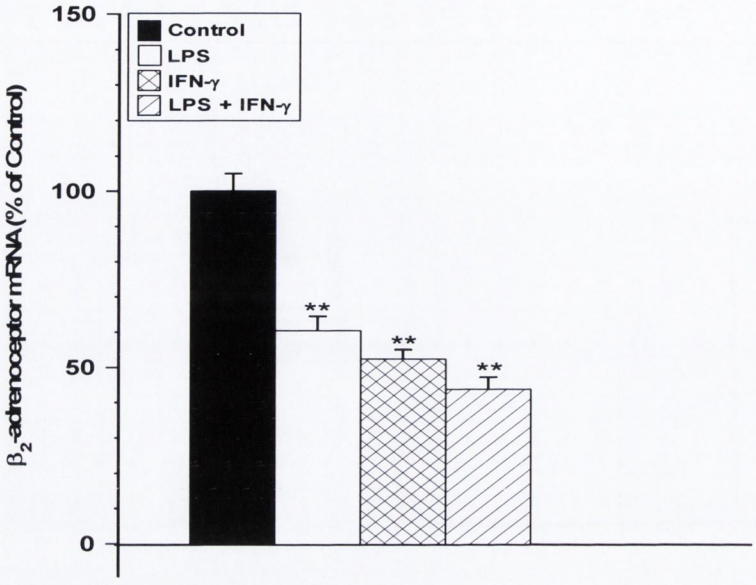


Figure 3.7.1: Effect of LPS and IFN-γ alone and in combination on TNF-α, IL-1β and nitric oxide production

Primary mixed glial cultures were incubated with either control (cDMEM), LPS (100ng/ml), IFN-γ (20ng/ml) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr. a) A one-way ANOVA revealed a significant effect of treatment on TNF-α production [$F_{(3,18)}$=38.65, P<0.0001] compared to unstimulated control counterparts. (b) A one-way ANOVA revealed a significant effect of treatment on IL-1β production [$F_{(3,18)}$=24.61, P<0.0001] compared to unstimulated control counterparts. (c) A one-way ANOVA revealed a significant effect of treatment on nitrite production [$F_{(3,20)}$=24.02, P<0.0001] compared to unstimulated control counterparts. Data are expressed as means with standard error of the mean (n=6). **P<0.01 versus control/unstimulated counterparts (Newman-Keuls).

a) mRNA



b) cAMP

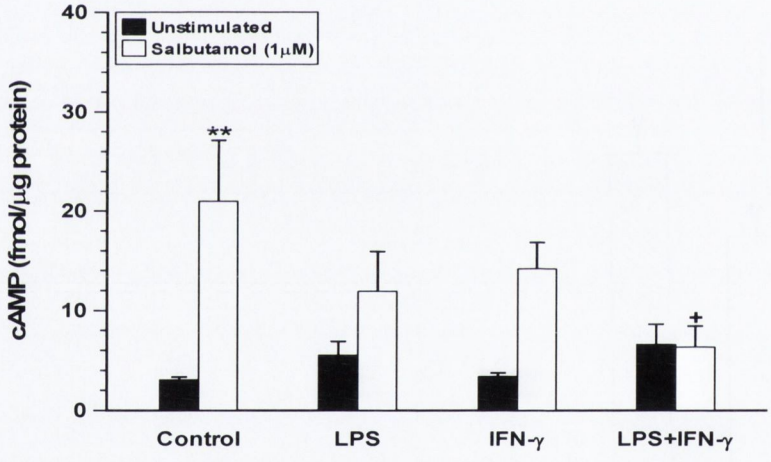


Figure 3.7.2: The combination of LPS + IFN-γ has the most profound effect on β_2-adrenoceptor mRNA expression and responsiveness

Primary mixed glial cultures were incubated with either control (cDMEM), LPS (100ng/ml), IFN-γ (20ng/ml) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr. (a) A one-way ANOVA revealed a significant effect of treatment on β_2-adrenoceptor mRNA expression [$F_{(3,31)}$=40.65, P<0.0001] compared to unstimulated control counterparts. Data are expressed as means % control with standard error of the mean (n=8). b) Following treatment with inflammatory stimuli for 24 hr primary mixed glial cultures were subsequently incubated with either control or salbutamol (1μM) in the presence of IBMX for 20 min. A two-way ANOVA revealed a significant effect of treatment with inflammatory stimuli and salbutamol on the accumulation of intracellular cAMP [$F_{(3,24)}$=3.29, P=0.0381]. Data are expressed as means with standard error of the mean (n=4). **P<0.01,*P<0.05 versus control/unstimulated counterparts, +P<0.05 versus salbutamol control (Newman-Keuls).

3.8 Treatment with LPS + IFN-γ reduces mRNA expression of the β_2-adrenoceptor in enriched cultures of both microglia and astrocytes

The previous studies examined the effect of an inflammatory stimulus on the β_2-adrenoceptor in primary mixed glial cultures, however the β_2-adrenoceptor has been shown to be missing merely from astrocytes in MS brain (De Keyser *et al.,* 1999, Zeinstra *et al.,* 2002). Thus, the following studies were undertaken to examine the impact of LPS + IFN-γ on β_2-adrenoceptor mRNA expression in enriched cultures of both microglia and astrocytes to determine the primary cell type effected in these studies. In addition, the primary cell type responsible for the production of the inflammatory mediators TNF-α, IL-1β and nitric oxide was also examined.

Treatment with LPS + IFN-γ induces the production of TNF-α in both microglia and astrocytes: Treatment of enriched cultures of microglia and astrocytes with LPS + IFN-γ significantly increased the production of TNF-α in both microglia ($P<0.01$) and astrocytes ($P<0.01$) compared to unstimulated control counterparts as assessed by ELISA. [Figure 3.8.1 a, Student's *t*-test, $n=6$].

Treatment with LPS + IFN-γ induces the production of IL-1β in microglia but not astrocytes: Treatment of enriched cultures of microglia and astrocytes with LPS + IFN-γ significantly increased the production of IL-1β in microglia ($P<0.05$) but the presence of IL-1β was undetectable in astrocytes either under basal conditions or following treatment with LPS + IFN-γ compared to unstimulated control counterparts as assessed by ELISA. [Figure 3.8.1 b, Student's *t*-test, $n=6$].

Treatment with LPS + IFN-γ induces the production of nitric oxide in both microglia and astrocytes: Treatment of enriched cultures of microglia and astrocytes with LPS + IFN-γ significantly increased the production of nitrite both microglia ($P<0.001$) and astrocytes ($P<0.001$) compared to unstimulated control counterparts as assessed by Greiss assay. [Figure 3.8.1 c, Student's *t*-test, $n=6$].

Treatment with LPS + IFN-γ reduced β_2-adrenoceptor mRNA expression in both microglia and astrocytes: Treatment of enriched cultures of microglia and astrocytes with LPS + IFN-γ significantly reduced β_2-adrenoceptor mRNA expression in both microglia ($P<0.01$) and

astrocytes ($P<0.01$) compared to unstimulated control counterparts as assessed by RT-PCR. [Figure 3.8.2, Student's *t*-test, $n=6$].

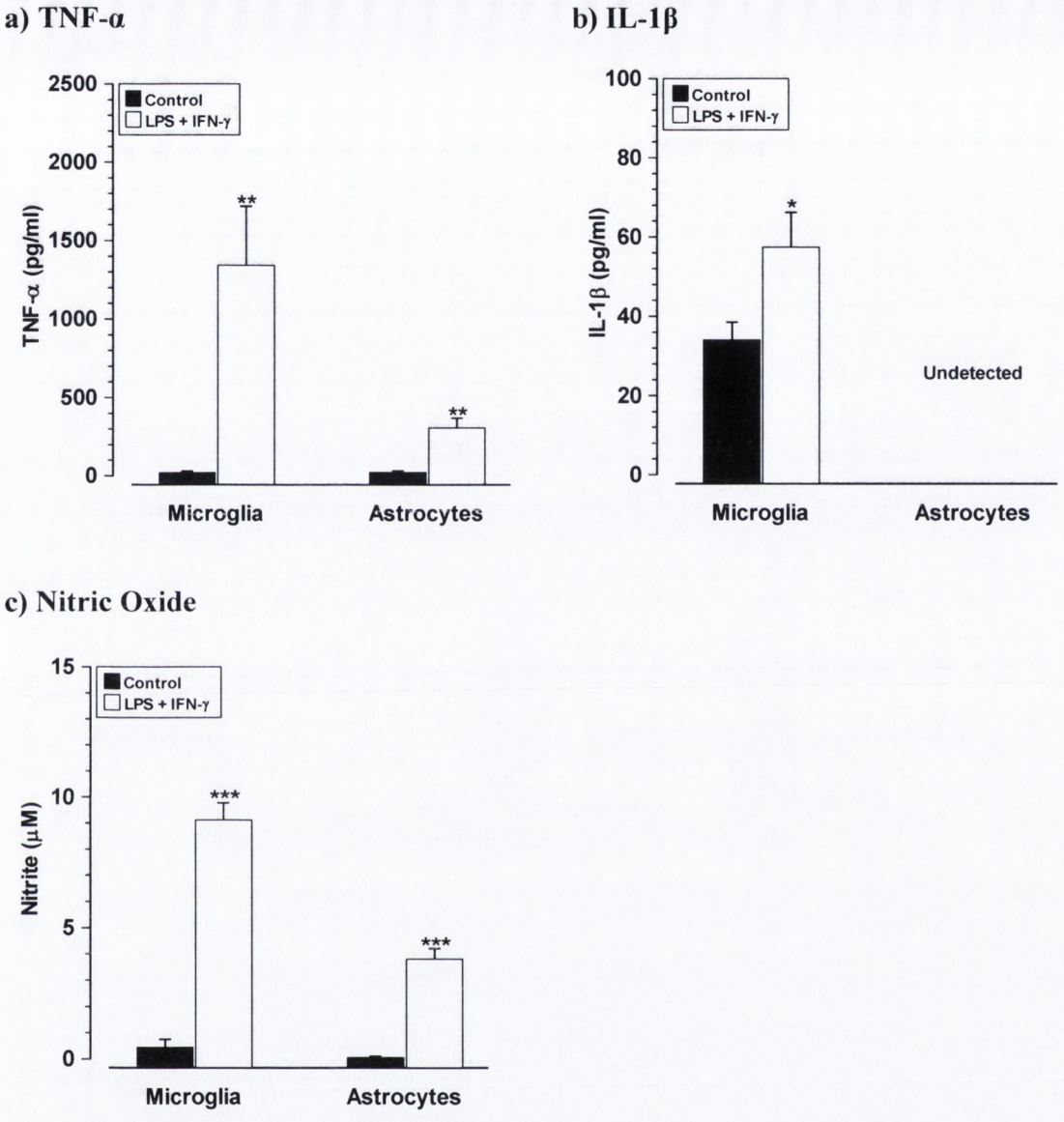


Figure 3.8.1: LPS + IFN-γ induces the production of IL-1β in enriched microglial cultures and the production of TNF-α and nitrite in enriched cultures of both astrocytes and microglia

Enriched cultures of astrocytes or microglia were incubated with either control (cDMEM) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr. (a) A Student's *t*-test revealed a significant effect of LPS + IFN-γ on TNF-α production in both microglia ($P<0.01$) and astrocytes ($P<0.01$) compared to unstimulated control counterparts. (b) A Student's *t*-test revealed a significant effect of LPS + IFN-γ on IL-1β production in microglia ($P<0.05$) but not astrocytes compared to unstimulated control counterparts. (c) A Student's *t*-test revealed a significant effect of LPS + IFN-γ on the nitrite production in both microglia ($P<0.001$) and astrocytes ($P<0.001$) compared to unstimulated control counterparts. Data are expressed as means with standard error of the mean ($n=6$). ***$P<0.001$, **$P<0.01$, *$P<0.05$ versus control counterpart (Student's *t*-test).

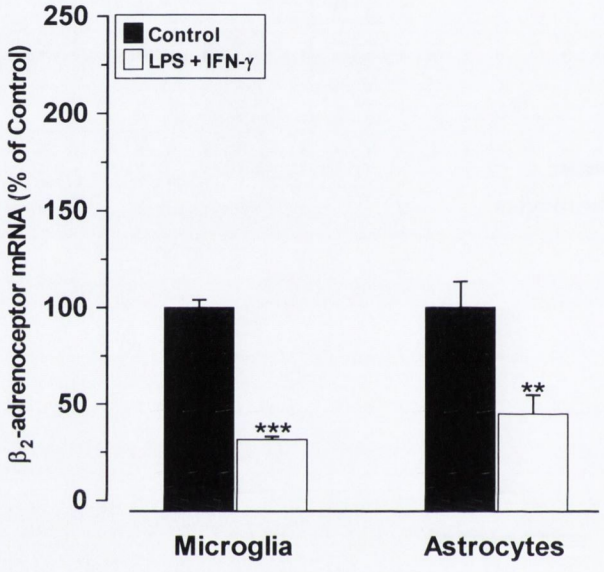


Figure 3.8.2: LPS + IFN-γ attenuates β_2-adrenoceptor mRNA expression in enriched cultures of both microglia and astrocytes

Enriched cultures of astrocytes or microglia were incubated with either control (cDMEM) or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr. A Student's *t*-test revealed a significant effect of LPS + IFN-γ on β_2-adrenoceptor mRNA expression in both microglia ($P<0.001$) and astrocytes ($P<0.01$) compared to unstimulated control counterparts. Data are expressed as mean percentage control with standard error of the mean ($n=6$). ***$P<0.001$, **$P<0.01$ versus control counterparts (Student's *t*-test).

3.9 Dexamethasone dose dependently inhibits the attenuation of β_2-adrenoceptor mRNA expression induced by treatment with LPS + IFN-γ in primary mixed glial cultures

Glucocorticoids are commonly used in the treatment of inflammatory diseases since one of their predominant mechanisms of action is to switch off genes encoding pro-inflammatory cytokines, chemokines and adhesion molecules (Barnes, 2006). In addition, previous studies have shown that the synthetic glucocorticoid dexamethasone (Dex) is effective in the treatment of both EAE and MS (Brusaferri *et al.,* 2000; Tsutsui *et al.,* 2008). Furthermore, it has been shown that dexamethasone can influence the expression and function of the β_2-adrenoceptor under both basal (Dangel *et al.,* 1996) and inflammatory conditions (Moore *et al.,* 1999; Mak *et al.,* 2002). Therefore, the purpose of this study was to determine if the attenuation of β_2-adrenoceptor mRNA expression induced by LPS + IFN-γ could be inhibited by pre-treatment with dexamethasone.

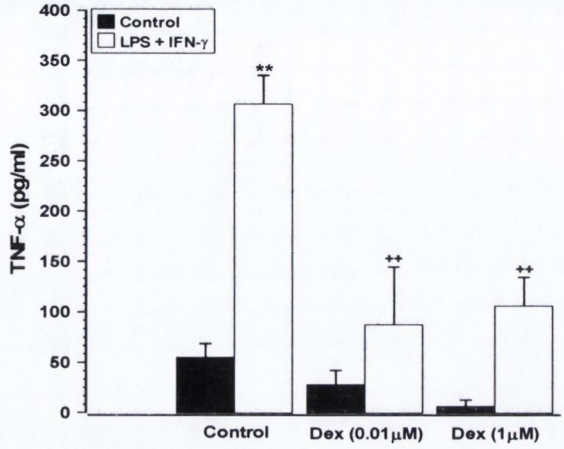
Dexamethasone attenuates the LPS + IFN-γ-induced production of TNF-α: Treatment of primary mixed glial cultures with LPS + IFN-γ significantly increased the production of TNF-α ($P<0.01$) compared to unstimulated control counterparts after 24 hr as assessed by ELISA. Pre-treatment with dexamethasone (0.01, 1μM) significantly attenuated the LPS + IFN-γ-induced production of TNF-α ($P<0.01$). [Figure 3.9.1 a, Newman-Keuls, $n=6$].

Dexamethasone attenuates the LPS + IFN-γ-induced production of nitric oxide: Treatment of primary mixed glial cultures with LPS + IFN-γ significantly increased nitrite concentrations ($P<0.01$) compared to unstimulated control counterparts after 24 hr as assessed by Greiss assay. Pre-treatment with dexamethasone (0.01, 1μM) significantly attenuated the production of LPS + IFN-γ-induced nitrite ($P<0.01$). In addition, 1μM dexamethasone significantly reduced basal concentrations of nitrite ($P<0.05$). [Figure 3.9.1 b, Newman-Keuls, $n=6$].

Dexamethasone dose-dependently inhibits LPS + IFN-γ-induced attenuation of β_2-adrenoceptor mRNA expression: Treatment of primary mixed glial cultures with LPS + IFN-γ significantly reduced β_2-adrenoceptor mRNA expression ($P<0.05$) compared to unstimulated control counterparts as assessed by RT-PCR. However, pre-treatment of cultures with dexamethasone (0.01, 1μM) dose-dependently inhibited the LPS + IFN-γ-induced attenuation of β_2-adrenoceptor mRNA expression ($P<0.01$). Furthermore, treatment with dexamethasone

significantly increased β_2-adrenoceptor mRNA in its own right ($P<0.01$). [Figure 3.9.2, Newman-Keuls, $n=6$].

a) TNF-α



b) Nitric Oxide

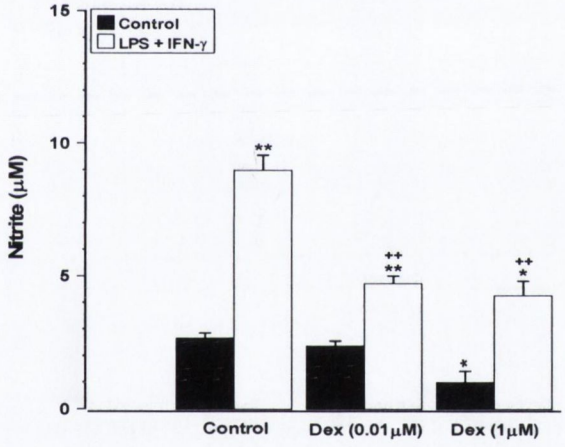


Figure 3.9.1: The glucocorticoid dexamethasone suppresses LPS + IFN-γ-induced production of TNF-α and nitric oxide

Primary mixed glial cultures were incubated with either control (cDMEM) or dexamethasone (0.01μM or 1μM) for 1 hr followed by subsequent incubation with either control or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr. (a) A two-way ANOVA revealed a significant interactive effect of dexamethasone and LPS + IFN-γ treatment on the production of TNF-α [$F_{(2,30)}$=13.62, P<0.0001]. (b) A two-way ANOVA revealed a significant interactive effect of dexamethasone and LPS + IFN-γ treatment on the production of nitrite [$F_{(2,30)}$=5.77, P=0.0076]. Data are expressed as means with standard error of the mean (*n*=6). **P<0.01, *P<0.05 versus control/unstimulated counterpart, ++P<0.01 versus control counterpart (Newman-Keuls).

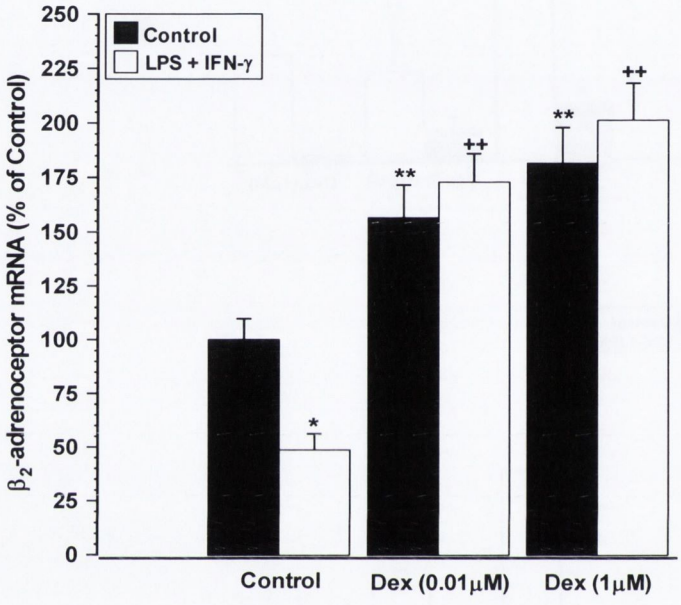


Figure 3.9.2: The glucocorticoid dexamethasone prevents LPS + IFN-γ-induced attenuation of β_2-adrenoceptor mRNA expression

Primary mixed glial cultures were incubated with either control (cDMEM) or dexamethasone (0.01μM or 1μM) for 1 hr followed by subsequent incubation with either control or a combined treatment of LPS (100ng/ml) + IFN-γ (20ng/ml) for 24 hr. A two-way ANOVA revealed a significant interactive effect of dexamethasone and LPS +IFN-γ treatment on β_2-adrenoceptor mRNA expression [$F_{(2,29)}$=4.32, P=0.0228]. In addition, a two-way ANOVA revealed a significant effect of dexamethasone treatment alone on β_2-adrenoceptor mRNA expression [$F_{(2,29)}$=40.31, P<0.0001]. Data are expressed as mean percentage control with standard error of the mean (n=6). **P<0.01, *P<0.05 versus control/unstimulated counterpart, ++P<0.01 versus control counterpart (Newman-Keuls).

3.10 Discussion

There is compelling evidence to suggest that the neurotransmitter noradrenaline acts as an endogenous immunomodulatory agent exerting its anti-inflammatory and neurotrophic effects through activation of β_2-adrenoceptors which are found predominantly on astrocytes and microglia within the CNS (De Keyser *et al.,* 1999, Zeinstra *et al.,* 2002). However, since it has been observed that β_2-adrenoceptors are absent from astrocytes in the white matter of patients suffering from MS, an autoimmune disease characterized by glial activation, neuroinflammation and neurodegeneration, the primary objective of this study was to determine whether exposure of primary mixed glial cultures *in vitro* to an inflammatory stimulus of LPS + IFN-γ can impact upon the responsiveness or expression of glial β_2-adrenoceptors. For the purposes of these studies primary mixed glial cultures were employed following a protocol which yields cultures consisting of approximately 70% astrocytes:30% microglia (O'Sullivan *et al.*, 2009), a ratio similar to that found *in vivo*. In addition, the inflammatory stimulus of a combined treatment of LPS + IFN-γ results in cells being exposed to the T-cell (Th1) cytokine IFN-γ in addition to the LPS-induced production of the pro-inflammatory cytokines IL-1β, TNF-α and members of the IL-12 superfamily (IL-12, IL-23, IL-27), all of which have all been found to be present in lesions in the MS brain (Pouly & Antel 1999; Martino *et al.,* 2002).

3.10.1 Selective β_2-adrenoceptor agonists exerts anti-inflammatory and neurotrophic actions in glial cells following exposure to the endotoxin LPS

The results presented here demonstrate that pre-treatment of primary mixed glial cultures with noradrenaline or the selective β_2-adrenoceptor agonists salbutamol or formoterol significantly attenuates the LPS-induced production of TNF-α. This is in line with the literature since it has previously been shown that stimulation of β-adrenoceptors with the non-selective β-adrenoceptor agonist isoproterenol can reduce the LPS-induced increase in TNF-α expression *in vivo* (Elenkov *et al.*, 1995). Furthermore, isoproterenol has been demonstrated to inhibit the LPS-induced activity of the TNF-α promoter when administered to astrocytic cultures, an effect shown to be specific to the β_2-adrenoceptor since the selective β_2-adrenoceptor antagonist ICI 118,551 blocked the effects of isoproterenol while the selective β_1-adrenoceptor antagonist atenolol had no impact (Nakamura *et al.*, 1998).

The results presented here also show that administration of LPS to primary mixed glial cultures has no effect on the expression of the neurotrophin GDNF, however, when cultures were pre-treated with either salbutamol or formoterol, both selective β_2-adrenoceptor agonists, there was a synergistic effect between LPS and the agonist resulting in an upregulation of GDNF expression. The literature shows that GDNF plays an important role in development and its expression is essential for neuronal survival in the LC-hippocampal pathway, since GDNF knock-out animals have noradrenergic neurons which are reduced in size and number compared to their wild-type counterparts (Quintero *et al.*, 2004). In contrast to the observations on GDNF, administration of LPS to primary mixed glial cultures induced a robust increase in the expression of the NGFβ, an effect that was significantly augmented by pre-treatment of cells with the selective β_2-adrenoceptor agonist formoterol. In contrast pre-treatment of cells with noradrenaline had no effect on either GDNF or NGFβ expression. A previous report from Zafra *et al.,* (1992) demonstrated increased expression of NGFβ in astrocytes *in vitro* in response to the pro-inflammatory cytokine IL-1β, however, while exposure of astrocytes to noradrenaline was found to induce NGF expression, this was found to be dependent on culture conditions (Zafra *et al.*, 1992). Notably, reports in the literature have shown that salbutamol is incapable of inducing the expression of NGF mRNA in astrocytes. It is suggested that this is due to the lack of a catechol ring in its structure thus implying that the expression of NGF may be due to a mechanism other than stimulation of adrenoceptors (Furukawa *et al.*, 1989).

While the induction of the neurotrophin $TGF\beta_1$ by LPS has also been shown occur *in vivo,* an effect found to be protective against the excitotoxic injury induced by kainic acid (Boche *et al.*, 2003), the results presented here demonstrate that administration of LPS to primary mixed glial cells failed to induce expression of the $TGF\beta_1$. Furthermore, pre-treatment of cultures with noradrenaline, salbutamol or formoterol did not impact on the expression of $TGF\beta_1$. This is noteworthy since in addition to the important roles $TGF\beta_1$ plays in tissue repair, inflammation, cell adhesion, growth and differentiation (Boche *et al.*, 2003) it has also been shown to modulate the density and function of β_2-adrenoceptors and can additionally cause alterations in the activity of adenylate cyclase (Mak *et al.*, 2000). The results presented here also demonstrate that administration of LPS to primary mixed glial cultures failed to induce the expression of FGF-2. Pre-treatment with noreadrenaline, salbutamol or formoterol did not impact on the expression of FGF-2. This is in contrast to *in vivo* studies which have found that

stimulation of β_2-adrenoceptors induces the expression of FGF-2, a neurotrophin known to play an important role in neuronal maintenance and repair (Follesa & Mocchetti, 1993; Riva *et al.*, 1998). Surprisingly, the results from the current study also demonstrate that LPS decreases the expression of IGF-1 in mixed glial cultures. IGF-1 is known to be essential for proper development of the CNS and is an important stimulator of myelin synthesis (Chesik *et al.*, 2007). However, while activation of the β_2-adrenoceptor plays an important role in regulation of the IGF system and can suppress the proliferation of astrocytes induced by IGF-1, IGF-1 can in turn induce phosphorylation of the β_2-adrenoceptor thereby blocking its activation (Chesik *et al.*, 2007). Nonetheless, the results presented here show that pre-treatment of cultures with noradrenaline, salbutamol or formoterol fails to impact on the LPS-induced reduction in IGF-1 expression. Ultimately, the anti-inflammatory and neurotrophic effects of β_2-adrenoceptor stimulation demonstrated in the current study appears to occur due to attenuation of LPS-induced TNF-α production and selective upregulation of GDNF and NGF-β expression.

3.10.2 Induction of inflammatory mediators in glia

In order to verify that a treatment regimen of LPS + IFN-γ induces an inflammatory response in mixed glial cultures *in vitro* the production of the pro-inflammatory mediators TNF-α, IL-1β and nitric oxide were assessed. Evidently the concentrations of LPS + IFN-γ (100ng/ml and 20ng/ml respectively) employed in this study were found to evoke a vigorous inflammatory response. In this regard, LPS + IFN-γ generated a significant increase in concentrations of the pro-inflammatory cytokine TNF-α. *In situ* TNF-α forms part of the primary inflammatory response and is known to be produced by activated macrophages in response to injury or invading pathogens. The production of TNF-α is essential for the initiation of a complete inflammatory response as its production triggers the release of IL-1, NO and ROS by other cells, all of which play an important role in inflammation (Tracey, 2002). Previous studies have shown that *in vitro* astrocytes do not produce TNF-α, however, upon stimulation with LPS astrocytes produce TNF-α in a dose-dependent manner (Chung & Benveniste, 1990). The results presented here also demonstrate a sizeable increase in concentrations of IL-1β in mixed glial cultures in response to LPS + IFN-γ. This is in line with previous reports since it has been

demonstrated that exposure of microglia to the same dose of 100ng/ml LPS *in vitro* induces the expression of IL-1β and iNOS after 24 hr (Xie *et al.,* 2004). In line with previous reports which have shown that stimulation of isolated microglia with LPS results in increased production of nitrite in comparison to unstimulated cells (Mori *et al.* 2002), the results presented here demonstrate that LPS + IFN-γ induces a significant increase in nitric oxide production in mixed glial cultures. It is well established that, when activated, microglia produce the free radical NO, synthesized by iNOS from the conversion of L-arginine to L-citrulline (Dello Russo *et al.*, 2004; Pannu & Singh, 2006). Thus, NO production gives a good indication of glial activation in response to an inflammatory stimulus.

Significantly the concentrations of LPS + IFN-γ employed in this study were found to have no effect on glial viability. This was assessed using an alamar blue assay which is based on the principal that viable cells convert resazurin to resorufin resulting in a colorometric change. This finding is critical since any effects on the β_2-adrenoceptor induced by treatment with LPS + IFN-γ cannot therefore by attributed to a reduction in cell number or viability in these cultures.

3.10.3 Regulation of β_2-adrenoceptor responsiveness by an inflammatory stimulus

Given that *in vitro* glial cells were capable of generating an inflammatory response to treatment with LPS + IFN-γ and the fact that inflammation is said to play a critical role in the pathology of many neurodegenerative disorders the primary goal of this study was to determine the effect of LPS + IFN-γ on glial β_2-adrenoceptor expression and function. Stimulation of β_2-adrenoceptors with the endogenous agonist noradrenaline is known to lead to an increase in the production of intracellular cAMP in co-cultures of astrocytes and neurons (Rosenberg & Li, 1995). In addition, previous work has shown that incubation of rabbit tracheal smooth muscle segments with IL-1β and TNF-α results in hyporesponsiveness β_2-adrenoceptors to stimulation with the β-agonist isoproterenol (Hakonarson *et al.*, 1996). Impaired responsiveness to β-agonists has also been found following exposure of HASM cells to IL-1β (Moore *et al.*, 1999 a). In line with this, the results presented here demonstrate that exposure to salbutamol, a selective β_2-adrenoceptor agonist, significantly increases the production of intracellular cAMP in mixed glial cultures in a dose-dependent manner. However, when glia were previously exposed to LPS + IFN-γ the ability of salbutamol to

increase intracellular cAMP was greatly diminished compared to control counterparts. It is important to note that the reduction in intracellular cAMP cannot be attributed to accelerated degradation of cAMP in these cultures since cAMP accumulation was measured in the presence of the phosphodiesterase inhibitor IBMX in a bid to slow cAMP degradation and thus eliminate any such possibility.

LPS + IFN-γ failed to have any effect on the accumulation of intracellular cAMP in response to forskolin, a direct activator of adenylate cyclase. Thus, it can be said that the reduction in cAMP accumulation in response to salbutamol induced by LPS + IFN-γ is an event specific to the β_2-adrenoceptor itself and not due to an effect on intracellular signalling events. This is in line with previous work in HASM cells where the accumulation of cAMP following treatment with forskolin was no different in cells pre-treated with an inflammatory stimulus of IL-1β compared to controls showing that inflammatory regulation of the β_2-adrenoceptor is independent of effects on the adenylate cyclase system (Moore *et al.*, 1999 a). Thus, the decrease in intracellular cAMP accumulation observed in this study following LPS + IFN-γ treatment is suggestive of receptor internalization or decreased transcription of receptor protein.

3.10.4 Treatment with LPS + IFN-γ reduces the expression of the β_2-adrenoceptor at both the cell surface and mRNA levels

In view of the fact that treatment with LPS + IFN-γ decreases β_2-adrenoceptor responsiveness in mixed glial cultures it was important to determine if decreased receptor responsiveness was due to internalization of the receptor and a reduction in receptor density at the level of the cell surface. Notably, it was found that there is a significant decrease (~50%) in the density of β_2-adrenoceptors present at the cell surface level following treatment with LPS + IFN-γ. This may explain the decrease in intracellular cAMP accumulation since stimulation of the receptor and uncoupling of the Gα subunit from its G-protein is required for cleavage of ATP by the enzyme adenylate cyclase in order to produce cAMP (Johnson, 1998). As such a decrease in receptor number at the cell surface would result in fewer binding sites for the agonist and therefore reduce the ability of the agonist to generate a suitable response. These findings reflect the observations of Mak *et al.* (2002) who showed that following the treatment of rats

with IL-1β the density of β_2-adrenoceptor expressed in lung membranes was reduced and this was found to be associated with impaired cAMP accumulation following stimulation with the β-agonist isoproterenol. Decreased cell surface expression of the β_2-adrenoceptor following exposure to LPS + IFN-γ may be due to recruitment of GRKs and β-arrestins to the receptor. GRKs are phosphoproteins responsible for the phosphorylation of GPCRs at their serine and threonine residues when the receptor is agonist-bound (Pitcher *et al.,* 1999) while β-arrestins are versatile adapter proteins essential for the internalization and desensitization of the β_2-adrenoceptor (Luttrell & Lefkowitz, 2002). However, attempts to co-immunoprecipitate the β_2-adrenoceptor in the current study failed (data not shown) therefore the events mediating reduced β_2-adrenoceptor expression at the cell surface still warrants further investigation.

Investigating β_2-adrenoceptor hyporesponsiveness and the reduction in β_2-adrenoceptor cell surface expression further it was deemed necessary to examine whether these events were merely attributable to a reduction in receptor expression at the protein level or whether these results could additionally be attributed to a decrease in the expression of the receptor at the mRNA level. Using quantitative RT-PCR it was shown that there is approximately a 50% reduction in β_2-adrenoceptor mRNA expression following the incubation of cultures with LPS + IFN-γ versus those levels seen in unstimulated cultures. This effect on the mRNA expression of the receptor is noteworthy since a decline in transcription may lead to decreased translation of β_2-adrenoceptor protein, an effect which would in turn lead to a reduction in the expression of the receptor at the cell surface. Previous reports have suggested that down-regulation of β_2-adrenoceptor mRNA expression occurs due to the up-regulation of a 35kDa protein, the so-called β-adrenergic receptor mRNA binding protein (β-ARB), which binds to AU-rich sequences and ultimately destabilizes β_2-adrenoceptor mRNA (Port *et al.*, 1992). However, the mechanisms involved in down-regulation of β_2-adrenoceptor mRNA expression induced by treatment with LPS + IFN-γ were beyond the scope of the current study.

3.10.5 Combination of LPS + IFN-γ has most profound effect on β_2-adrenoceptor expression and responsiveness

It has previously been demonstrated that administration of LPS *in vitro* induces the expression of IL-1β and iNOS in microglia and NO in J774 murine macrophages. However, when IFN-γ

was given in combination with LPS a synergistic effect between LPS and IFN-γ was found which resulted in a more robust response (Korhonen, 2002; Xie, 2004). In addition, it has also been found that astrocytes express TNF-α in response to treatment with LPS but do not express TNF-α in response to IFN-γ. However, treatment with LPS and IFN-γ in combination induces a synergistic effect on the production of TNF-α owing to a priming effect of IFN-γ on astrocytes (Chung *et al.*, 1991). Therefore, the induction of inflammatory mediators in response to LPS, IFN-γ and the combined treatment of LPS + IFN-γ was examined in the current study in primary mixed glial cultures. In line with the previous reports, treatment of glial cultures with LPS induced a robust increase in the production of TNF-α, IL-1β and nitric oxide, while treatment with IFN-γ failed to stimulate the production of any of these. However, when IFN-γ was administered to cultures in combination with LPS it enhanced the LPS mediated production of nitric oxide but not TNF-α or IL-1β. The priming effect of IFN-γ on the induction of pro-inflammatory mediators is a well known phenomenon. While its mechanisms are still under investigation a number of possibilities have been proposed. In this regard, it has been shown that the administration of IFN-γ alone has no effect on the production of IL-6 or TNF-α in human gingival fibroblasts, however, administration of IFN-γ augments the LPS-induced expression of these cytokines, an effect found to occur due to an IFN-γ-induced increase in CD14 and MyD88 expression (Mochizuki *et al.*, 2004). In addition, it has also been suggested that the priming effect of IFN-γ on monocytes occurs due to a mechanism at the mRNA level since primed monocytes transcribe TNF-α mRNA at a faster rate than their unprimed counterparts, an event found to correlate to an increase in NFκB activation (Hayes *et al.*, 1995).

Given that the earlier studies illustrate that treatment of glial cultures with LPS + IFN-γ induces hyporesponsivenness of the β_2-adrenoceptor to stimulation with salbutamol and reduces the cell surface and mRNA expression by approximately 50% it was deemed necessary to establish whether LPS or IFN-γ was the primary mediator in these events. As such it was found that incubation of cultures with either LPS or IFN-γ alone significantly reduced the mRNA expression of the β_2-adrenoceptor, however, treatment with LPS + IFN-γ in combination had the most profound effect. Furthermore, such effects of inflammatory mediators on mRNA expression were found to translate to the reduced responsiveness of the receptor to stimulation with salbutamol. In this regard, while both LPS and IFN-γ alone were

shown to suppress intracellular cAMP accumulation in their own right, it was the combined treatment that has most powerful effect. For this reason, the remaining studies utilized the combined treatment of LPS + IFN-γ.

3.10.6 Treatment with LPS + IFN-γ attenuates β_2-adrenoceptor expression in enriched cultures of astrocytes and microglia

In view of the fact that the β_2-adrenoceptor was found to be absent merely from astrocytes in the CNS of MS patients (De Keyser *et al.*, 1999; Zeinstra *et al.*, 2002) and since for the previous studies mixed glial preparations were used it was deemed necessary to determine if treatment with the inflammatory regimen employed in this study was regulating the expression of the β_2-adrenoceptor purely in astrocytes or if this phenomenon might occur in microglia as well. Furthermore, the primary cell type involved in the production of inflammatory mediators induced by the treatment regimen of LPS + IFN-γ *in vitro* was also examined here.

Accordingly, the results presented here demonstrate that both microglia and astrocytes produce nitric oxide in response to treatment with LPS + IFN-γ, however, microglia by far had the most vigorous response. Microglia were shown to express basal levels of IL-1β and this was shown to be enhanced following treatment with LPS + IFN-γ. In contrast, IL-1β was undetectable in astrocytes either under basal conditions or following incubation with LPS + IFN-γ. There is much controversy about the production of IL-1β by astrocytes, however, the results observed in this study might be accounted for by a report by Pearson *et al.,* (1999) who showed that IL-1β is predominantly expressed by microglia *in vivo* and enhanced production occurs rapidly and within 1-4 hours of glutamate-induced excitotoxicity. However, astrocytes were found to have a more delayed response which was shown to occur 2-7 days post-lesion, an event which might implicate microglia in the induction of IL-1β production by astrocytes. In the present study TNF-α was found to be produced by both microglia and astrocytes, albeit to a lesser extent in astrocytes. *In vitro* investigations have shown that astrocytes produce TNF-α in response to LPS + IFN-γ (Chung & Benveniste, 1990; Chung *et al.*, 1991), however, since the concentration obtained in the current study from the astrocytes is lower than that from the microglia this may also implicate the necessity for the presence of microglia for the induction of astrocytic TNF-α.

Surprisingly, when enriched cultures of astrocytes and microglia were utilised a reduction in the expression of β_2-adrenoceptor mRNA was observed in both cell types. Furthermore, while β_2-adrenoceptor expression was reduced by approximately 50% in astrocytes, similar to that seen in mixed glial cultures, expression in microglia was reduced to a slightly greater degree of approximately 60%. Although the β_2-adrenoceptor was demonstrated to be absent from astrocytes in CNS of MS patients its expression was found to be unaffected on neurons in lesions examined (De Keyser *et al.*, 2004a). However, expression of β_2-adrenoceptors on microglia has not been examined since microglia are not found to be present within the lesions in MS (Zeinstra *et al.*, 2002) and so loss of the receptor on these cells could also influence inflammation within the CNS.

There has been much controversy over the expression of TLR-4 on glial cells. Since LPS binds to and elicits its effects through TLR-4, which was recently shown by to be present foremost on microglia within the CNS with only low levels expressed on astrocytes (Akundi *et al.*, 2005; Jack *et al.*, 2005a), it was deemed necessary to employ mixed glial cultures for further studies in order to elicit the full inflammatory effects of LPS + IFN-γ treatment on astrocytes. Moreover, since β_2-adrenoceptor downregulation was shown to occur in both microglia and astrocytes in response to LPS + IFN-γ it was additionally resolved to continue to utilize mixed glial cultures since this culture condition best reflects the *in vivo* environment of these cells.

3.10.7 Dexamethasone inhibits the LPS + IFN-γ induced suppression of β_2-adrenoceptor mRNA expression

The results presented here reveal that pre-treatment of glial cultures with the synthetic glucocorticoid dexamethasone inhibits the LPS + IFN-γ induced production of nitric oxide and TNF-α from mixed glial cultures in a dose-dependent fashion. This is in contrast to the work of Korhonen *et al.,* (2002) who showed that a one hour pre-treatment of murine J774 macrophages with dexamethasone inhibited the LPS-induced production of iNOS and NO also in a dose dependent fashion (0.1-10μM) but had no effect when cells were treated with a combination of LPS + IFN-γ. These authors suggested that iNOS mRNA was more stable

when cells were treated with combination of LPS + IFN-γ and that iNOS induction by LPS + IFN-γ was not a GRE mediated event and thus, could not be regulated by dexamethasone. In spite of this, dexamethasone has been shown to destabilize iNOS mRNA in LPS treated cells through a mechanism requiring *de novo* protein synthesis. Thus, the differences between the results of this study and that of Korhonen *et al.,* could possibly be accounted for by differences in cell type used. The iNOS promoter contains a number of transcription factor binding sites and as such differences in its regulation may be observed depending on species, stimulus and cell type examined (Kozuka *et al.,* 2007). In line with the inhibition of TNF-α production induced by pre-treatment with dexamethasone observed here, previous reports have shown that the LPS-induced expression of TNF-α in human fetal astrocytes was inhibited by co-treatment with dexamethasone with similar findings having additionally been reported in monocytes/macrophages (Velasco *et al.,* 1991).

Significantly, the results presented here show that pre-treatment of primary glial cultures with dexamethasone prevents the LPS + IFN-γ-induced downregulation of β_2-adrenoceptor mRNA expression in a dose dependent manner. Interestingly, treatment with dexamethasone was also shown to augment basal levels of β_2-adrenoceptor mRNA expression. GREs are present in the promoter regions of the β-adrenoceptor and as such glucocorticoids have been demonstrated to up-regulate β-adrenergic gene expression with the greatest effect on the β_2-adrenoceptor since this has been shown to have the most GRE in its promoter region (Xu, 2001). In line with this, a GRE at position -379 to -365 in the 5' flanking region of the rat β_2-adrenoceptor has been found to mediate the gene transcription induced by glucocorticoids in HepG2 cells (Cornett *et al.*, 1998). In addition, the glucocorticoid dexamethasone has previously been shown to increase the expression of the β_2-adrenoceptor various cells and tissues types including that of the human lung, the H9c2 rat heart cell-line, hamster smooth muscle cells and DDT1 MF-2 hamster vas deferens cells (Collins *et al.*, 1988; Hadcock *et al.*, 1989; Mak *et al.*, 1995; Dangel *et al.*, 1996). Furthermore, in line with the results presented here, previous reports have also shown that treatment with dexamethasone prevents the IL-1β-induced decrease in β_2-adrenoceptor responsiveness in HASM cells (Moore *et al.*, 1999a). The process by which glucocorticoids regulate β_2-adrenoceptors is known as heterologous regulation, a process whereby hormones or drugs which are not specific ligands for adrenergic receptors can regulate receptor responsiveness (Collins *et al.*, 1988)

While pre-treatment with dexamethasone was shown to inhibit the LPS + IFN-γ-induced decrease in β_2-adrenoceptor mRNA expression it remains to be determined if this effect translates to restoration of receptor function in response to the β_2-adrenoceptor agonist salbutamol.

3.10.8 Summary

Overall, the results from the current study demonstrate that stimulation of the β_2-adrenoceptor on glial cells in the presence of an inflammatory stimulus (LPS) suppresses production of pro-inflammatory cytokines and induces the expression of neurotrophins *in vitro*, thus reinforcing previous reports on the neuroprotective properties of activation of glial β_2-adrenoceptors. However, the results from the current study also demonstrate that exposure of glial cells *in vitro* to an inflammatory stimulus of LPS + IFN-γ significantly reduces the expression and responsiveness of the β_2-adrenoceptor. Thus, were such an event to occur *in vivo* it would significantly impede the ability of endogenous noradrenaline or exogenously administered β_2-adrenoceptor agonists to elicit anti-inflammatory and neurotrophic effects. Significantly, pre-treatment of glial cultures with the glucocorticoid dexamethasone inhibited this LPS + IFN-γ-induced reduction in β_2-adrenoceptor expression, however, it remains to be determined whether this effect also translates to the restoration of responsiveness of the β_2-adrenoceptor. Surprisingly, treatment of glial cultures with dexamethasone was also found to increase the expression of the β_2-adrenoceptor under basal conditions, an event which might account for its therapeutic efficacy in the treatment of neuroinflammatory disease states. Ultimately, these results point to the β_2-adrenoceptor as a promising therapeutic target for the treatment of neuroinflammatory and neurodegenerative diseases.

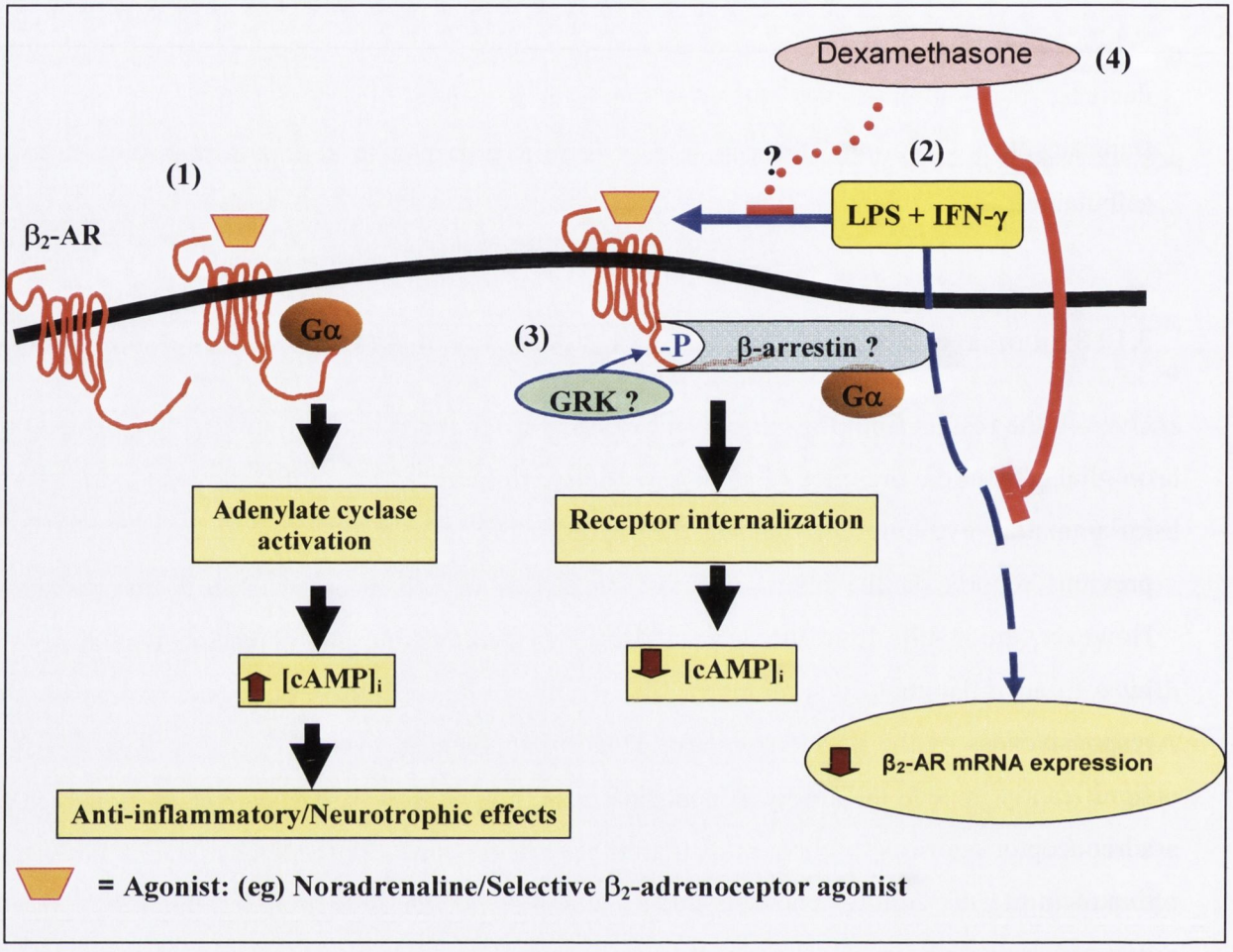


Figure 3.7: Schematic representation of *in vitro* results

Stimulation of β_2-adrenoceptors with either noradrenaline or selective β_2-adrenoceptor agonists activates adenylate cyclase and induces the accumulation of intracellular cAMP resulting in anti-inflammatory and neurotrophic effects in primary mixed glial cultures (1). Exposure to an inflammatory stimulus of LPS + IFN-γ downregulates the cell surface and mRNA expression of the β_2-adrenoceptor in glia (2). In addition, exposure to LPS + IFN-γ impairs the accumulation of intracellular cAMP following stimulation of the β_2-adrenoceptor. Downregulation of β_2-adrenoceptor expression at the cell surface level may be due to the recruitment of GRK and β-arrestin to the receptor (3). Pre-treatment with the synthetic glucocorticoid dexamethasone attenuates the impact of LPS + IFN-γ on β_2-adrenoceptor mRNA expression (4). The ability of dexamethasone to restore cell surface expression and responsiveness of the β_2-adrenoceptor following exposure to LPS + IFN-γ remains to be established.

Chapter 4

Examination of the expression and responsiveness of the β_2-adrenoceptor in rats in vivo following a systemic LPS challenge

4.1 Introduction

As discussed in the previous chapter deficiencies in the noradrenergic system have been implicated in the progression of many neurodegenerative disorders including MS, Alzheimer's Disease, Huntington's Disease and Parkinson's Disease, to name but a few (Feinstein *et al.*, 2002; Hertz *et al.*, 2004; Marien *et al.*, 2004). The findings from the preceding chapter show that exposure of primary mixed glial cultures *in vitro* to an inflammatory stimulus of LPS + IFN-γ downregulates the expression and responsiveness of the β_2-adrenoceptor, an event which could hinder the neuroprotective effects of endogenous noradrenaline within the CNS. The use of an *in vitro* system is convenient for examining the molecular mechanisms involved in such a phenomenon. However, it is also necessary to extrapolate these results to an *in vivo* model whereby the effect of inflammation on glial β_2-adrenoceptor expression and function can be examined where the cells are in their natural environment under the influence of factors released from the other cells that constitute the CNS, namely neurons and oligodendrocytes. Therefore, the following studies were undertaken to examine the expression and function of central β_2-adrenoceptors in rats *in vivo* following a systemic inflammatory challenge with LPS.

LPS has been reported to provoke an acute phase response and induce activation of the HPA-axis cauing a robust increase in the expression of pro-inflammatory mediators within the CNS (Beishuizen & Thijs, 2003). A number of pathways have been described by which peripherally administered LPS might bring about such a response within the CNS. The stimulation of primary afferent nerves such as the vagus nerve which can directly relay signals to the CNS is one such pathway, the so called neural pathway. Another is the humoral pathway whereby LPS can bind to TLRs on cells in the CVOs and choroid plexus instigating an immune response within the CNS due to the peripheral release of pro-inflammatory cytokines which can then enter the brain by volume diffusion. Another suggestion is that cytokines can enter the CNS from the periphery by binding to saturable cytokine transporters at the BBB. In addition it has also been proposed that peripherally circulating IL-1 elicits a response in the brain by binding to IL-1 receptors on perivascular macrophages and endothelial cells on brain venules inducing the local production of PGE_2 within the CNS (Dantzer *et al.*, 2008; Tizard, 2008). Regardless of the pathway involved, systemic administration of LPS has been previously demonstrated to induce IL-1β expression in the brain in a temporal and spatial

manner, an effect which subsides after 24 hr (Quan *et al.*, 1998a). Furthermore, the dose of LPS employed in the present study (250μg/kg;i.p.) has previously been established to induce sickness behaviour in rats and trigger a robust increase in the expression of the pro-inflammatory mediators IL-1β, TNF-α, IFN-γ and iNOS in the CNS several hours after administration (Konsman *et al.*, 1999; Connor *et al.*, 2008; O'Sullivan *et al.*, 2009). Thus, according to these previous reports the cytokine profile induced in the CNS by systemic LPS administration is reflective of that induced by the treatment regimen of LPS + IFN-γ employed in the previous *in vitro* model.

Central β_2-adrenoceptors represent a promising therapeutic target for the treatment of neuroinflammatory disease. In this regard, previous work from this laboratory has shown that stimulation of central β_2-adrenoceptors by peripheral administration of the brain penetrable β_2-adrenoceptor agonist clenbuterol activates glia and upregulates the expression of the anti-inflammatory mediators IL-10 and SOCS3 and in addition increases the expression of IL-1ra and the IL-1 Type II receptor, negative regulators of the IL-1 signalling system, under non-inflammatory conditions. Such effects were shown to occur in a temporal manner and all were due to actions on β-adrenoceptors since they could be blocked by administration of the non-selective β-adrenoceptor antagonist propranolol (McNamee, 2008). Thus, *in vivo* stimulation of central β_2-adenoceptors with clenbuterol 24 hr post-LPS challenge was examined to assess the impact of LPS on central β_2-adrenoceptors responsiveness in this study.

Therefore, the aims of the present study were as follows:

1) To confirm the ability of LPS (250μg/kg;i.p.) to increase expression of the pro-inflammatory mediators IL-1β, TNF-α, iNOS and IFN-γ in rat CNS 4 hr post-challenge.

2) To examine the impact of systemic LPS administration on the body-weight of rats over a 24 hr period as a marker of the response of the animal to LPS.

3) To determine the impact of systemic LPS administration on the mRNA expression of the β_2-adrenoceptor in rat CNS 4 hr and 1 day post-challenge.

4) To determine the impact of systemic LPS administration on the responsiveness of the β_2-adrenoceptor in rat CNS 1 day post-challenge by assessing responsiveness to the brain penetrable β_2-adrenoceptor agonist clenbuterol.

4.2 Methods

4.2.1 Subjects and drug treatments

Male Sprague-Dawley rats (320-400g) were housed 4 per cage under a 12 hr light-dark cycle (lights on 08:00) in a temperature-controlled room (22 ± 2 °C) with food and water available *ad libitum* for the duration of the experiment as outlined in the methods (Section 2.3, Chapter 2). LPS and clenbuterol were dissolved in 0.89% (w/v) saline and were administered i.p. at doses of 250μg/kg and 0.5mg/kg respectively. Control animals were administered an i.p. injection of 0.89% (w/v) saline alone.

4.2.2 Experimental Procedure

The design for this set of experiments was as set out in Figure 4.1 below. Briefly, rats were injected on day 1 with either control (0.89% (w/v) saline) or LPS (250μg/kg) i.p. and rats were sacrificed 4 hr (day 1) or 28 hr (day 2) post-injection. On day 2 rats were injected with either control or clenbuterol (0.5mg/kg) i.p. 24 hr post-LPS injection and rats were sacrificed 4 hr post-clenbuterol injection.

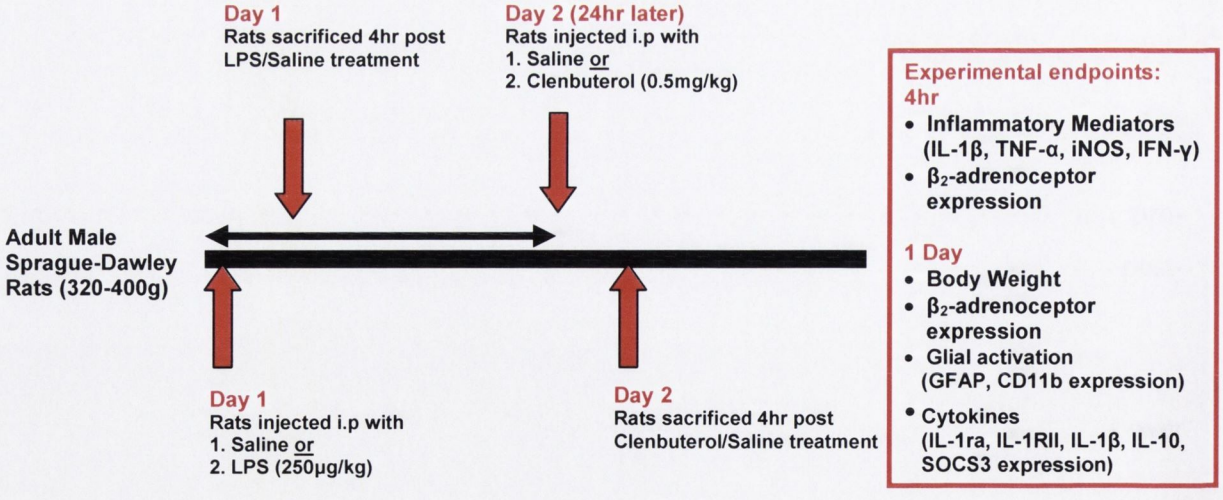


Figure 4.1: Experimental design for examination of the impact of a systemic LPS challenge on the expression and responsiveness of central β_2-adrenoceptors

Ultimately this study yields 6 treatment groups as outlined in table 4.1 below. Cortical and hippocampal brain regions were analyzed for the mRNA expression of the pro-inflammatory mediators IL-1β, TNF-α, iNOS and IFN-γ from tissue harvested 4 hr post-LPS challenge. β_2-adrenoceptor mRNA expression was analyzed in cortex and hippocampus 4 and 28 hr post-LPS challenge. In addition, to determine responsiveness of central β_2-adrenoceptors to clenbuterol 1 day post-LPS challenge mRNA expression of CD11b, GFAP, IL-1β, IL-1ra, IL-1 Type II receptor, IL-10 and SOCS3 was assessed. Preparation of tissue and RT-PCR were carried out as outlined in the methods section (Section 2.3 & 2.4, Chapter 2).

Group	Treatment	Timepoint for sacrifice
1	Control	4 hr post-challenge
2	LPS	4 hr post-challenge
3	Control + Control	28 hr post-challenge
4	Control + Clenbuterol	28 hr post-challenge
5	LPS + Control	28 hr post-challenge
6	LPS + Clenbuterol	28 hr post-challenge

Table 4.1: Treatment groups for study examining the impact of a systemic LPS challenge on the expression and responsiveness of central β_2-adrenoceptors

4.3 Systemic LPS administration induces the production of pro-inflammatory mediators in rat CNS 4 hr post-challenge and reduces body-weight of rats 24 hr post-challenge

As previously discussed systemic administration of LPS is known to induce the expression of pro-inflammatory cytokines in the rat CNS. Therefore, the purpose of the following study was to confirm the ability of systemic LPS administration, at this dose (250μg/kg) and route of administration (i.p.), to elicit a similar inflammatory profile in the CNS to that used in the mixed glial cultures in the previous chapter. The expression of cytokines in the CNS was measured at a time-point of 4 hr post-LPS administration since previous reports from this laboratory have demonstrated a significant increase in the expression of the pro-inflammatory mediators iNOS, IL-1β, IFN-γ and TNF-α in the CNS at this time-point (Connor *et al.*, 2008; O'Sullivan *et al.*, 2009). In addition this time-point was chosen since, as already mentioned, the effect of systemic LPS on the expression of pro-inflammatory cytokines in the CNS is shown to dissipate by 24 hr (Quan *et al.*, 1998a; Connor *et al.*, 2008). Furthermore, systemically administered LPS is known to cause a reduction in appetite and body-weight of animals (Kent et al., 1996; Layé *et al.*, 2000). Therefore, body-weights of rats were measured before and 24 hr after systemic LPS administration in this study as a measure of the response of the animal to LPS.

LPS induces TNF-α expression: Systemic administration of LPS (250μg/kg;i.p) significantly increased TNF-α mRNA expression in rat cortex ($P<0.05$) and hippocampus ($P<0.01$) 4 hr post-injection compared to saline controls. [Figure 4.3.1 a, Student's *t*-test, n=3-7].

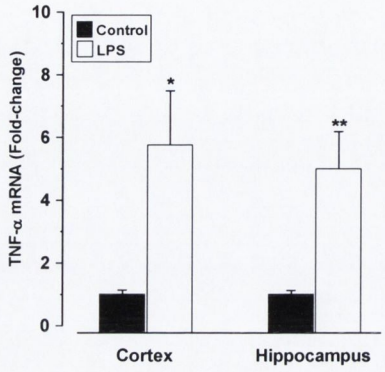
LPS induces IL-1β expression: Systemic administration of LPS (250μg/kg;i.p) significantly increased IL-1β mRNA expression in rat cortex ($P<0.01$) and hippocampus ($P<0.001$) 4 hr post-injection compared to saline controls. [Figure 4.3.1 b, Student's *t*-test, n= 3-6].

LPS induces IFN-γ expression: Systemic administration of LPS (250μg/kg;i.p) significantly increased IFN-γ mRNA expression in rat cortex ($P<0.01$) but not hippocampus 4 hr post-injection compared to saline controls. [Figure 4.3.1 c, Student's *t*-test, n=4-5].

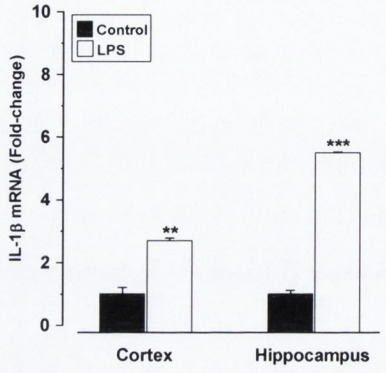
LPS induces iNOS mRNA expression: Systemic administration of LPS (250μg/kg;i.p) significantly increased iNOS mRNA expression in rat cortex ($P<0.001$) and hippocampus ($P<0.001$) 4 hr post-injection compared to saline controls. [Figure 4.3.1 d, Student's *t*-test, $n=3$-6].

Body-weight: Systemic administration of LPS (250μg/kg;i.p.) caused a robust reduction in the body-weight of rats ($P<0.0001$) 24 hr post-injection compared to saline controls, indicative of a response to endotoxin. [Figure 4.3.2, Student's *t*-test, $n=10$-12].

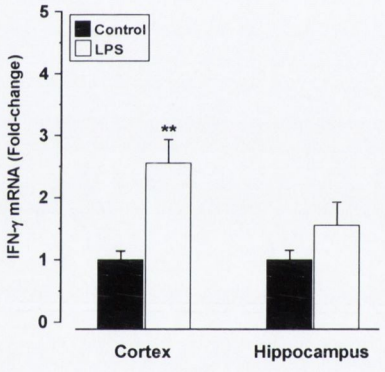
a) TNF-α mRNA expression



b) IL-1β mRNA expression



c) IFN-γ mRNA expression



d) iNOS mRNA expression

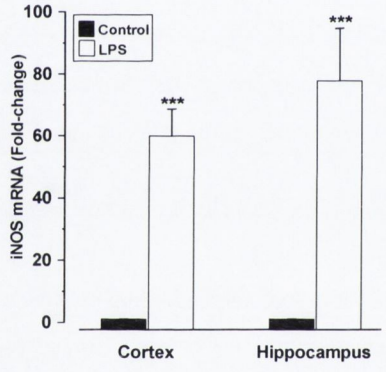


Figure 4.3.1: Systemic LPS administration induces a pro-inflammatory response in rat cortex and hippocampus 4 hr post-challenge

LPS (250μg/kg) or control (0.89% (w/v) saline) were administered i.p. and rats were sacrificed 4 hr post-challenge. a) A Student's *t*-test revealed a significant effect of LPS on TNF-α mRNA expression in rat cortex ($P<0.05$) and hippocampus ($P<0.01$) compared to saline controls. (b) A Student's *t*-test revealed a significant effect of LPS on IL-1β mRNA expression in rat cortex ($P<0.01$) and hippocampus ($P<0.001$) compared to saline controls. (c) A Student's *t*-test revealed a significant effect of LPS on IFN-γ mRNA expression in rat cortex ($P<0.01$) but not hippocampus compared to saline controls. (d) A Student's *t*-test revealed a significant effect of LPS on iNOS mRNA expression in rat cortex ($P<0.001$) and hippocampus ($P<0.001$) compared to saline controls. Data are expressed as means with standard error of the mean (n=3-7). *** $P<0.001$, **$P<0.01$, *$P<0.05$ versus saline control counterparts (Student's *t*-test).

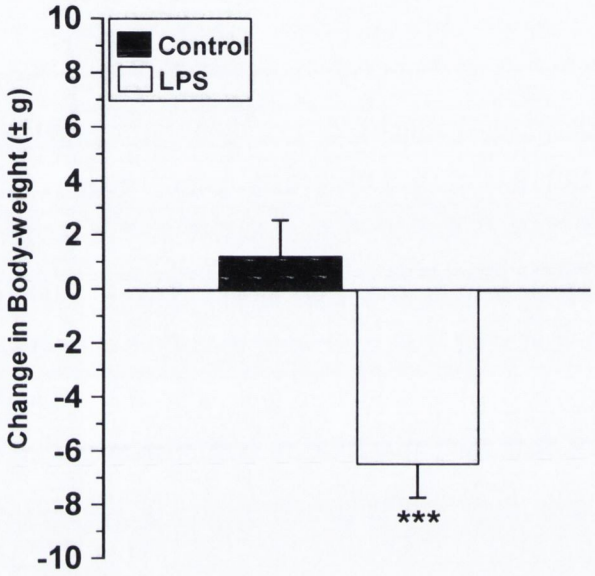


Figure 4.3.2: Systemic LPS administration reduces body-weight of rats over a 24 hr period

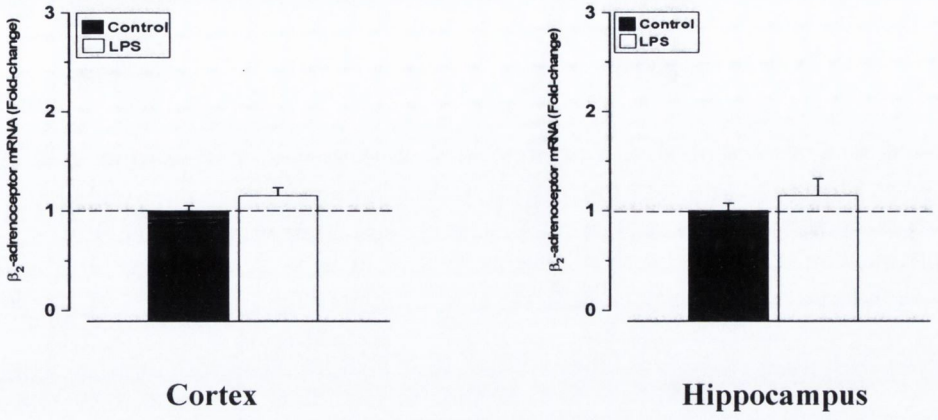
LPS (250μg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats and body-weights were measured prior to and 24 hr post-challenge. A Student's *t*-test revealed a significant effect of LPS on body-weight of rats ($P<0.001$) compared to saline controls. Data are expressed as means with standard error of the mean (n=10-12). ***$P<0.001$ versus saline control counterpart (Student's *t*-test).

4.4 Systemic LPS administration does not impact on β_2-adrenoceptor mRNA expression in rat CNS

The results from the previous chapter show that treatment of glial cells *in vitro* with LPS + IFN-γ results in reduced β_2-adrenoceptor expression and responsiveness after 24 hr. It has been well established that β_2-adrenoceptors are expressed in the rat brain under normal conditions (Salm & McCarthy, 1989). In addition, previous studies have shown that β_2-adrenoceptors are primarily expressed on glia, principally astrocytes, in the rat CNS (Salm & McCarthy, 1989; Mantyh *et al.*, 1995) and that their expression becomes significantly increased following experimentally induced neuronal injury (Mantyh *et al.*, 1995). Since β_2-adrenoceptors have been shown to be absent from astrocytes in an *in vivo* situation in the CNS of patients suffering from MS (De Keyser *et al.*, 1999), the purpose of the current study was to determine if a peripheral inflammatory challenge, similar to that induced by a single bacterial infection in humans, could impact upon the expression or responsiveness of central β_2-adrenoceptors *in vivo* where glia are in their natural environment. For the purposes of this study β_2-adrenoceptor mRNA expression was measured at timepoint of 4 hr, since expression of the pro-inflammatory mediators IL-1β, IFN-γ, TNF-α and iNOS was found to be increased in the CNS at this timepoint following LPS challenge. Expression of the receptor was also examined 1 day post-LPS challenge to keep in line with the timepoint used in the *in vitro* studies in chapter 3.

β_2-adrenoceptor mRNA expression: Systemic administration of LPS (250μg/kg;i.p) had no effect on β_2-adrenoceptor mRNA expression in rat cortex or hippocampus 4 hr or 1 day post-challenge compared to saline controls. [Figure 4.4.1 a,b, Student's *t*-test, n=4-9].

a) 4 hours



b) 1 day

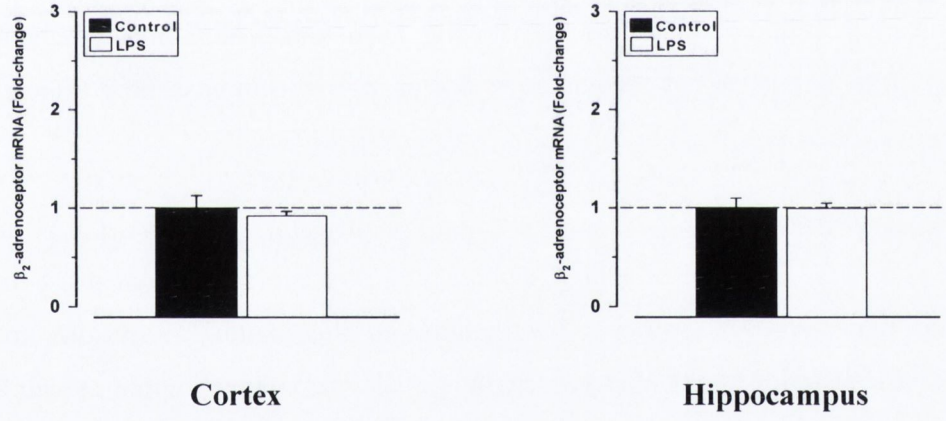


Figure 4.4.1: Systemic LPS administration does not alter β_2-adrenoceptor mRNA expression in rat cortex or hippocampus 4 hr or 1 day post-challenge

LPS (250μg/kg) or control (0.89% (w/v) saline) were administered i.p. and rats were sacrificed 4 hr or 28 hr post-challenge. (a) A Student's *t*-test revealed no significant effect of LPS on β_2-adrenoceptor mRNA expression in rat cortex or hippocampus 4 hr post-challenge. (b) A Student's *t*-test revealed no effect of LPS on β_2-adrenoceptor mRNA expression in rat cortex or hippocampus 1 day post-challenge. Data are expressed as means with standard error of the mean (n=4-9).

4.5 Impact of systemic LPS administration on β_2-adrenoceptor function in rat CNS in response to the brain penetrable selective β_2-adrenoceptor agonist clenbuterol

In contrast to the *in vitro* studies in the previous chapter, the results presented here demonstrate that systemic LPS administration does not impact on β_2-adrenoceptor mRNA expression in the rat CNS *in vivo*. However, this does not rule out the possibility that systemic LPS administration could impact on the β_2-adrenoceptor at a functional level. In the *in vitro* experiments β_2-adrenoceptor function was assessed by examining intracellular cAMP accumulation in response to stimulation with the β_2-adrenoceptor agonist salbutamol following LPS + IFN-γ treatment. Thus, in a similar manner responsiveness of central β_2-adrenoceptors following systemic LPS administration was assessed *in vivo* using the long-acting brain penetrable β_2-adrenoceptor agonist clenbuterol. The activation of astrocytes and expression of anti-inflammatory mediators in the CNS in response to clenbuterol was chosen as the experimental endpoint since, as previously discussed, stimulation of central β_2-adrenoceptors with clenbuterol has already been demonstrated to induce these effects under non-inflammatory conditions at a 4 hr timepoint (McNamee, 2008).

GFAP mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased GFAP mRNA expression in cortex ($P<0.05$) but not hippocampus compared to saline controls 4 hr post-administration, indicative of astrocytic activation in the cortex. Exposure to LPS (250µg/kg;i.p.) 24 hr prior to clenbuterol challenge did not impact upon the ability of clenbuterol to increase GFAP expression in cortex ($P<0.01$) or hippocampus ($P<0.05$). [Figure 4.5.1 a, Newman-Keuls, n=4-6].

CD11b mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased CD11b mRNA expression in hippocampus ($P<0.05$) but not cortex compared to saline controls 4 hr post-administration, indicative of microglial activation in the hippocampus. Exposure to LPS (250µg/kg;i.p.) 24 hr prior to clenbuterol challenge did not impact on CD11b expression in the cortex, neither did it impact on the ability of clenbuterol to increase CD11b expression in hippocampus ($P<0.05$). [Figure 4.5.1 b, Newman-Keuls, n=4-6].

IL-10 mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased IL-10 mRNA expression in cortex ($P<0.05$) but not hippocampus compared to saline controls 4 hr post-

administration. Exposure to LPS (250μg/kg;i.p.) 24 hr prior to clenbuterol challenge did not impact upon the ability of clenbuterol to increase IL-10 expression in cortex ($P<0.05$) or hippocampus ($P<0.05$). [Figure 4.5.2 a, Newman-Keuls, n=4-6].

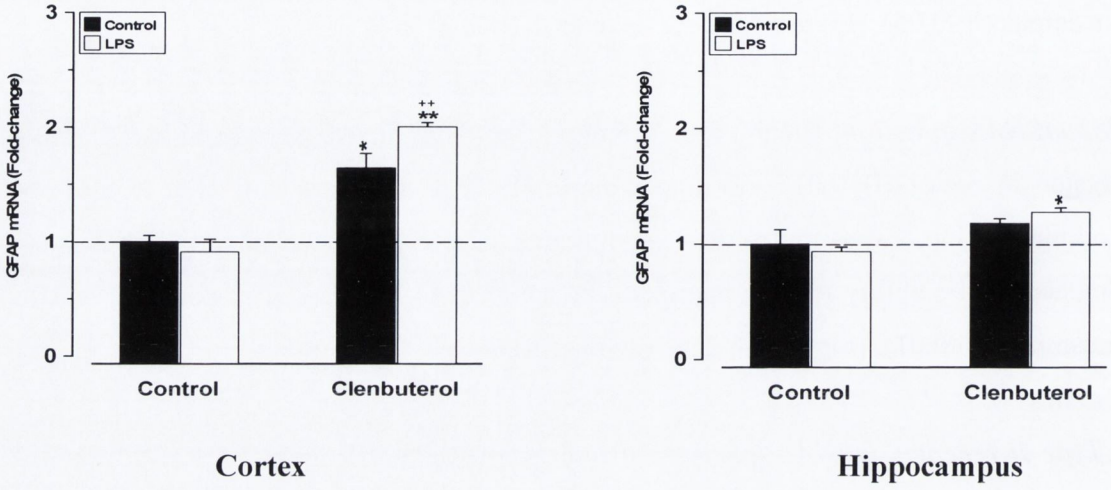
SOCS3 mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased SOCS3 mRNA expression in cortex ($P<0.01$) and hippocampus ($P<0.01$) compared to saline controls 4 hr post-administration. Exposure to LPS (250μg/kg:i.p.) 24 hr prior to clenbuterol challenge did not impact on the ability of clenbuterol to induce SOCS3 expression in cortex ($P<0.01$) or hippocampus ($P<0.01$). [Figure 4.5.2 b, Newman-Keuls, n=4-6].

IL-1 Type II receptor mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased IL-1 Type II receptor mRNA expression in cortex ($P<0.01$) and hippocampus ($P<0.01$) compared to saline controls 4 hr post-administration. Exposure to LPS (250μg/kg;i.p.) 24 hr prior to clenbuterol challenge did not impact on the ability of clenbuterol to induce IL-1 Type II receptor expression in cortex ($P<0.01$) or hippocampus ($P<0.01$). [Figure 4.5.3 a, Newman-Keuls, n=4-6].

IL-1ra mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased IL-1ra mRNA expression in cortex ($P<0.01$) and hippocampus ($P<0.01$) compared to saline controls 4 hr post-administration. Exposure to LPS (250μg/kg;i.p.) 24 hr prior to clenbuterol challenge did not impact on the ability of clenbuterol to induce IL-1ra expression in cortex ($P<0.01$) or hippocampus ($P<0.01$). [Figure 4.5.3 b, Newman-Keuls, n=4-6].

IL-1β mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased IL-1β mRNA expression in cortex ($P<0.05$) and hippocampus ($P<0.01$) compared to saline controls 4 hr post-administration. Exposure to LPS (250μg/kg;i.p.) 24 hr prior to clenbuterol challenge augmented the clenbuterol-induced increase in IL-1β expression in cortex ($P<0.01$) and hippocampus ($P<0.01$). [Figure 4.5.3, Newman-Keuls, n=4-6].

a) GFAP mRNA expression



b) CD11b mRNA expression

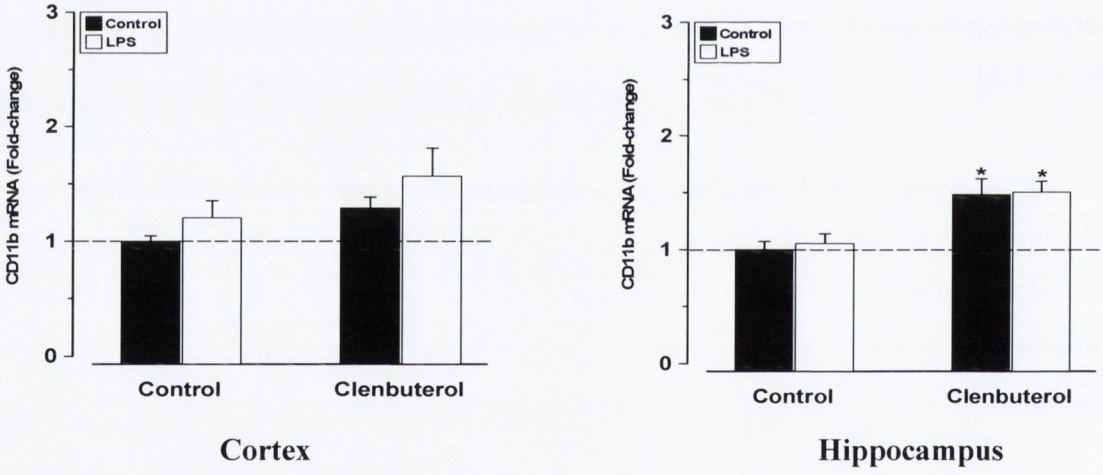
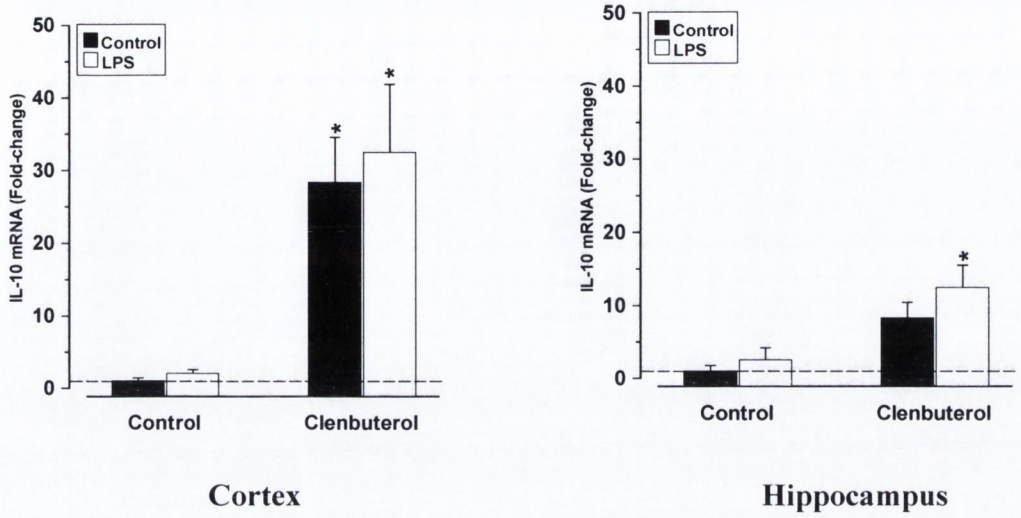


Figure 4.5.1: Systemic LPS administration does not impact on β_2-adrenoceptor responsiveness to clenbuterol as indicated by increased GFAP and CD11b mRNA expression

LPS (250μg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats followed 24 hr later by administration of either clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and rats were sacrificed 4 hr post-clenbuterol challenge. a) A two-way ANOVA revealed a significant effect of clenbuterol on GFAP mRNA expression in cortex [$F_{(1,18)}$=33.14, P<0.0001] and hippocampus [$F_{(1,18)}$=20.68, P=0.0002]. b) A two-way ANOVA revealed a significant effect of clenbuterol on CD11b mRNA expression in hippocampus [$F_{(1,18)}$=18.76, P=0.0004] but not cortex. Data are expressed as means with standard error of the mean (n=4-6). **P<0.01, *P<0.05 versus saline control, ++P<0.01 versus clenbuterol control (Newman-Keuls).

a) IL-10 mRNA expression



b) SOCS3 mRNA expression

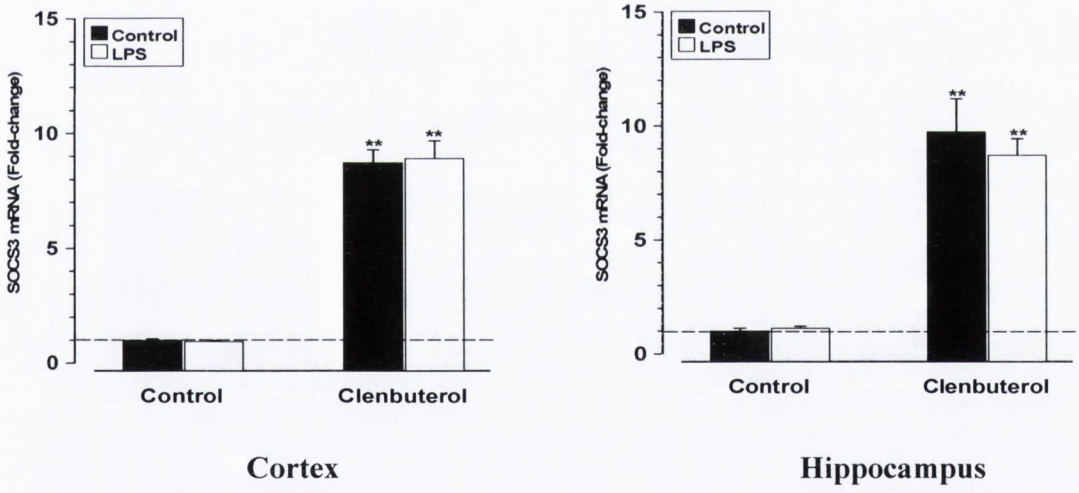
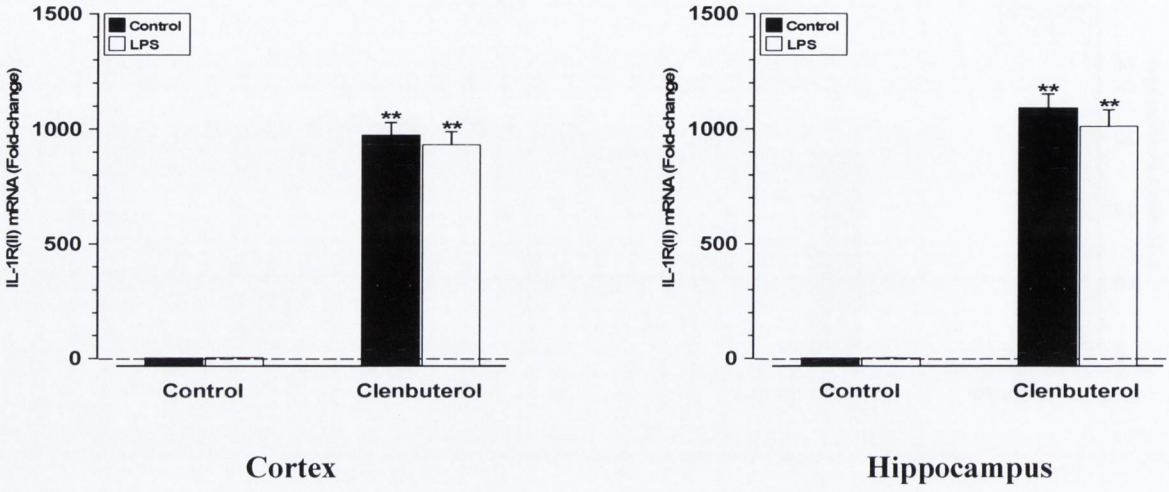


Figure 4.5.2: Systemic LPS administration does not impact on β_2-adrenoceptor responsiveness to clenbuterol as indicated by increased IL-10 and SOCS3 mRNA expression

LPS (250μg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats followed 24 hr later with administration of either clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and rats were sacrificed 4 hr post-clenbuterol challenge. a) A two-way ANOVA revealed a significant effect of clenbuterol on IL-10 mRNA expression in cortex [$F_{(1,18)}$=20.8, P<0.0002] and hippocampus [$F_{(1,17)}$=14.16, P=0.0016]. b) A two-way ANOVA revealed a significant effect of clenbuterol on SOCS3 mRNA expression in cortex [$F_{(1,18)}$=215.61, P<0.0001] and hippocampus [$F_{(1,18)}$=80.89, P<0.0001]. Data are expressed as means with standard error of the mean (n=4-6). **P<0.01, *P<0.05 versus saline control (Newman-Keuls).

a) IL-1 Type II mRNA expression



b) IL-1ra mRNA expression

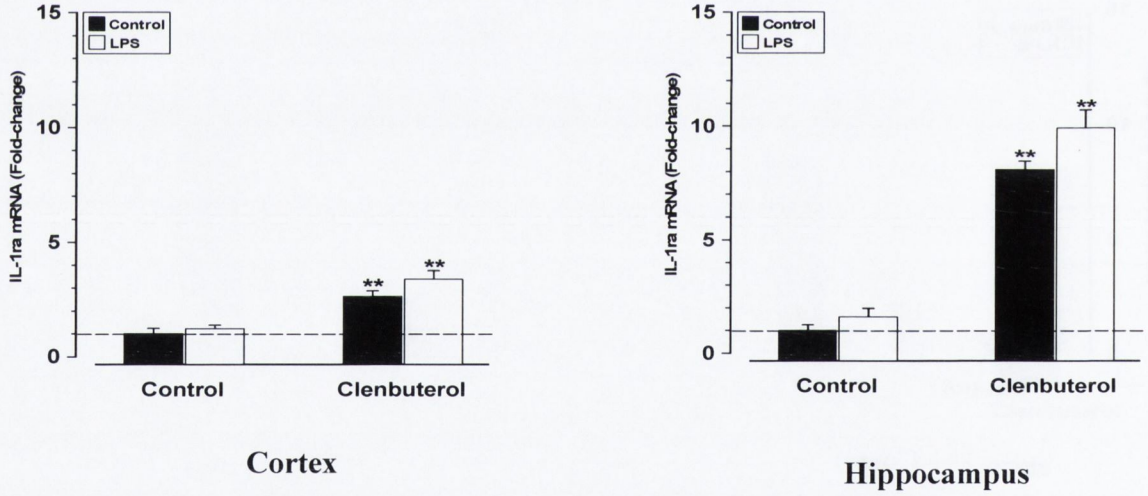


Figure 4.5.3: Systemic LPS administration does not impact on β_2-adrenoceptor responsiveness to clenbuterol as indicated by increased IL-1R(II) and IL-1ra mRNA expression

LPS (250μg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats followed 24 hr later with administration of either clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and rats were sacrificed 4 hr post-clenbuterol challenge. a) A two-way ANOVA revealed a significant effect of clenbuterol on IL-1 Type II receptor mRNA expression in cortex [$F_{(1,18)}$=466.22, P<0.0001] and hippocampus [$F_{(1,18)}$=403.31, P<0.0001]. b) A two-way ANOVA revealed a significant effect of clenbuterol on IL-1ra mRNA expression in cortex [$F_{(1,18)}$=46.93, P<0.0001] and hippocampus [$F_{(1,18)}$=116.22, P<0.0001]. Data are expressed as means with standard error of the mean (n=4-6). **P<0.01 versus saline control (Newman-Keuls).

IL-1β mRNA expression

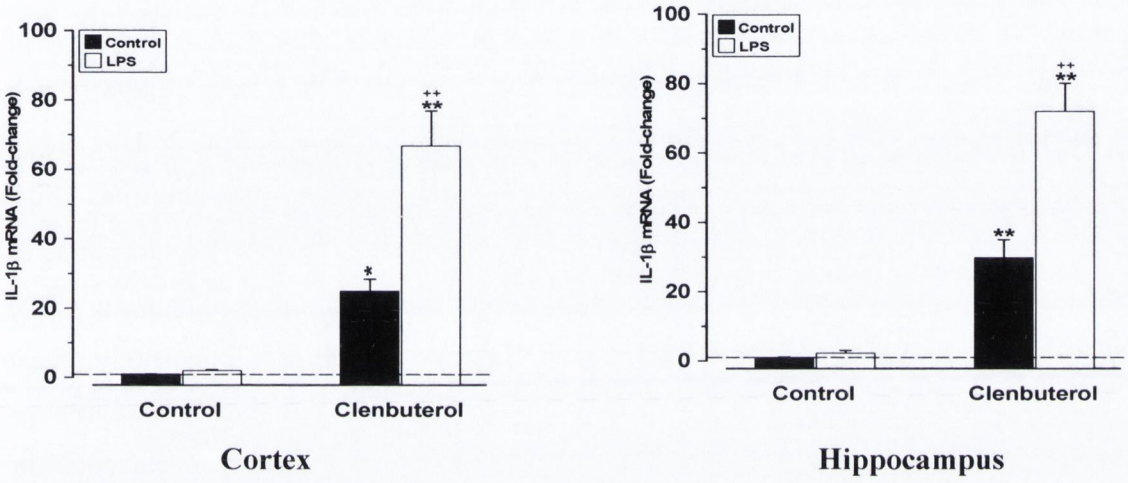


Figure 4.5.4: Systemic LPS administration enhanced clenbuterol-induced IL-1β expression

LPS (250μg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats followed 24 hr later with administration of either clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and rats were sacrificed 4 hr post-clenbuterol challenge. A two-way ANOVA revealed a significant interactive effect between LPS and clenbuterol treatment on IL-1β mRNA expression in cortex [$F_{(1,18)}$=11.89, P=0.0029] and hippocampus [$F_{(1,18)}$=14.63, P=0.0012]. Data are expressed as means with standard error of the mean (n=4-6). **P<0.01, *P<0.05 versus saline control, ++P<0.01 versus clenbuterol control (Newman-Keuls).

4.6 Discussion

4.6.1 Systemic LPS administration induces body-weight loss in rats and a pro-inflammatory profile in the CNS

It is well established that administration of endotoxin in the form of LPS perturbs the homeostasis of animals owing to the activation of the HPA-axis inducing fever, increased sleep, anorexia and a reduction in body-weight (Dantzer *et al.*, 2008; Tizard, 2008). Such events are suggested to be mediated primarily by the release of the major pro-inflammatory cytokines IL-1 and TNF-α from activated macrophages in the periphery (Beishuizen & Thijs, 2003). In this regard, it is well established that IL-1β and TNF-α act on the hypothalamus to provoke alterations in the normal homeostatic functions of the animal, termed an acute phase response (Beishuizen & Thijs, 2003; Tizard, 2008). The results presented in this study reveal that a single systemic injection of LPS induces a robust increase in the expression of the pro-inflammatory mediators IL-1β, TNF-α, iNOS and IFN-γ in rat cortex and hippocampus 4 hr post-injection. This is in line with previous reports from this laboratory which show that a single systemic injection of LPS at the same dose of 250μg/kg;i.p. induces a robust increase in TNF-α, IL-1β, IFN-γ and iNOS in rat cortex 4 hr post-administration (Connor *et al.*, 2008; O'Sullivan *et al.*, 2009). In addition, the pro-inflammatory mediators induced by systemic LPS administration in this study *in vivo* is qualitatively similar to that used to stimulate glial cells *in vitro* in the previous chapter. Since a systemic challenge with LPS induced the expression of IFN-γ in the brain it was not necessary to co-administer IFN-γ with LPS as for the *in vitro* study.

The results observed here show that in line with the increase in cortical and hippocampal cytokine expression, systemic LPS administration induced a significant reduction in body-weight of rats over a 24 hr period. Previous reports have indicated that peripheral or central administration of IL-1β induces anorexia in rats, an event suggested to be mediated by the actions of IL-1β in the ventromedial and lateral hypothalamus (Kent *et al.*, 1996). Furthermore, this dose of LPS (250μg/kg;i.p.) has already been shown by other groups to induce a similar decrease in the body-weight of rats 6 hr post-injection, an effect proposed to occur due to an increase in the mobilization of energy stocks which is necessary to mount a

febrile response in a bid to return normal homeostatic function (Konsman *et al.*, 2008). Thus, the results presented here show that the route of administration and dose used in the current study induces a robust LPS effect in the animals.

4.6.2 Systemic LPS administration does not impact on β_2-adrenoceptor mRNA expression in CNS

Despite the fact that a robust increase in expression of the pro-inflammatory mediators IL-1β, TNF-α, IFN-γ and iNOS was observed following systemic LPS administration, no change in β_2-adrenoceptor mRNA expression was observed 4 hr or 1 day post-LPS challenge in either cortex or hippocampus. This is in contrast to the reduced β_2-adrenoceptor mRNA expression observed in glial cultures in response to a similar inflammatory profile in the previous chapter (Chapter 3). However, while this study demonstrates no change in the mRNA expression of the β_2-adrenoceptor it does not rule out the possibility that LPS may be impacting on the protein expression of the receptor nor does it rule out the possibility that LPS may affect downstream accumulation of cAMP in response to stimulation of central β_2-adrenoceptors as was observed in the *in vitro* study. Thus, further studies are required to address these issues in order to fully assess the impact of LPS on β_2-adrenoceptor expression and function *in vivo*. In addition, while this study revealed no change in total β_2-adrenoceptor mRNA expression in rat CNS the impact of LPS on receptor expression in individual cell types, principally astrocytes and microglia, has not been examined. Thus, this is another issue which warrants further investigation. Ultimately, these results demonstrate that while *in vitro* cultures of primary mixed glia are useful for determining the molecular mechanisms by which an inflammatory challenge can regulate β_2-adrenoceptor expression and function the results obtained from *in vitro* models cannot always be extrapolated to the *in vivo* situation where glia are under the influence of other cells of the CNS such as neurons and oligodendrocytes. Furthermore, the LPS-induced activation of the HPA-axis acts to initiate a negative feed-back system through the release of glucocorticoids (Beishuizen & Thijs, 2003). Thus, by binding to GREs on β_2-adrenoceptors glucocorticoids produced in response to LPS could prevent the down-regulation of β_2-adrenoceptor expression in a similar manner to that of the synthetic glucocorticoid dexamethasone in the *in vitro* model. It has been suggested previously that a hyporeactive HPA-axis may play a role in inflammatory diseases (Beishuizen & Thijs, 2003). Significantly,

the progression of EAE has been shown to be associated with desensitization of the HPA-axis (Stefferl *et al.*, 1999) and Lewis rats, which express low basal levels of corticosterone, are found to be more susceptible to EAE than Brown Norway or Dark Agouti rats, which express higher basal levels of corticosterone (Stefferl *et al.*, 1999). In addition, repeated administration of LPS causes desensitization of the HPA-axis due to inhibition by glucocorticoids at the pituitary level (Stefferl *et al.*, 1999; Beishuizen & Thijs, 2003). Therefore, the role of the HPA-axis in the regulation of β_2-adrenoceptor expression is a matter which warrants further investigation.

4.6.3 Systemic LPS administration does not impact on responsiveness of central β_2-adrenoceptors to the selective β_2-adrenoceptor agonist clenbuterol

Since a systemic inflammatory challenge with LPS was found to have no effect on the expression of the β_2-adrenoceptor in either cortex or hippocampus it was deemed necessary to examine the possibility that inflammation may reduce β_2-adrenoceptor responsiveness *in vivo*. As previously described, this study was carried out in a similar manner to the *in vitro* studies whereby responsiveness of central β_2-adrenoceptors was examined by stimulation with the brain penetrable selective β_2-adrenoceptor agonist clenbuterol. It has previously been shown that clenbuterol increases concentrations of intracellular cAMP in a dose-dependent fashion in cortical slices from rat brain, an effect shown to be specific to stimulation of the β_2-adrenoceptor since the accumulation of intracellular cAMP was significantly blunted in the presence of the β_2-adrenceptor antagonist ICI 118,551 but was unaffected by the β_1-adrenoceptor antagonist ICI 89,406 (Ordway *et al.*, 1987). It is well established that stimulation of central β_2-adrenoceptors with clenbuterol is neuroprotective (Semkova *et al.*, 1996; Culmsee *et al.*, 1999b; Zhu *et al.*, 1999; Zhu *et al.*, 2001; Junker *et al.*, 2002; Teng *et al.*, 2006). In this regard, clenbuterol has previously been shown to upregulate the expression of the growth factor TGF-β_1 and provide protection to neurons in a model of transient forebrain ischemia (Zhu *et al.*, 2001). In addition, clenbuterol is shown to be protective in a murine model of amyotrophic lateral sclerosis by inducing a delay in disease onset (Teng *et al.*, 2006). Furthermore, clenbuterol has been shown to be neuroprotective in a model of focal cerebral ischemia owing to stimulation of β_2-adrenoceptors and the activation of astrocytes (Junker *et al.*, 2002).

In light of this, the results presented here demonstrate that peripheral administration of clenbuterol (0.5mg/kg;i.p.) induced a significant increase in GFAP mRNA expression in rat cortex 4 hr post-injection, indicative of astrocytic activation. This induction was shown to be unimpeded by prior exposure to LPS. Significantly, prior exposure to LPS was found to augment the clenbuterol-induced increase in GFAP expression in the cortex. In contrast, clenbuterol did not induce GFAP expression in rat hippocampus, however, when clenbuterol was administered to rats which had been previously exposed to LPS a significant increase in GFAP expression was observed. It is already recognized that β-adrenoceptors are responsible for astrocytic activation. In this regard, treatment with L-propranolol, a non-selective β-adrenoceptor antagonist, in a model of motor neuron degeneration was shown to reduce astrocytic hypertrophy and decrease the expression of GFAP (Sutin & Griffith, 1993). Furthermore, it is has also been shown that incubation of astrocytes *in vitro* with the β-adrenergic agonist (+)-isoproterenol induces rapid changes in astrocytic morphology, an event mediated by increased accumulation of intracellular cAMP (Shain *et al.*, 1987). Moreover, the clenbuterol-induced activation of astrocytes observed here is significant since it has previously been shown that clenbuterol conveys neuroprotection following permanent occlusion of the middle cerebral artery, an effect mediated by the global activation of astrocytes and the induction of the neurotrophins NGF and bFGF (Culmsee *et al.*, 1999a,b). The difference in astrocytic activation found in cortex and hippocampus in this study might be accounted for by a recent study by Guo *et al.*, (2007) who showed that almost all of the β_2-adrenoceptors expressed in rat hippocampus are located on neurons with very little expression on glia staining positive for GFAP. However, while GFAP was found to be significantly increased at the mRNA level it is not known if this is due to an increase in the total number of astrocytes in the brain or simply due to a change in the activation state and morphology of already existing astrocytes. This is important to note since previous studies have shown that stimulation of β-adrenoceptors results in a change in the morphology of astrocytes from an epithelial-like to a stellate morphology, most likely due to cAMP-dependent phosphorylation of GFAP (Shain *et al.*, 1987). Therefore, further investigations will be necessary in order to determine the impact of clenbuterol on astrocytes fully, for example though the use of immunohistochemistry.

The results of the present study demonstrate that stimulation of central β_2-adrenoceptors with clenbuterol has no effect on the expression of CD11b, a marker of microglial activation, in rat

cortex. In contrast, clenbuterol significantly increased the expression of CD11b in the hippocampus of both naïve rats and rats that were previously exposed to LPS. It has been established that microglia express the β_2-adrenceptor *in vitro* and these receptors have been shown to be functional since stimulation with the selective β_2-adrenoceptor agonist terbutaline results in a significant increase in concentrations of intracellular cAMP (Mori *et al.*, 2002). However, the results presented here are in contrast to those of Fujita *et al.,* (1998) who showed that stimulation of microglial β_2-adrenoceptors *in vitro* with β-agonists increases intracellular cAMP concentrations resulting in suppression of microglial proliferation. Then again, less attention has been paid to the activation of microglia by β_2-adrenoceptor agonists in the hippocampus *in vivo* and as such the data presented here may represent heterogeneity amongst microglia and the expression of neurotransmitter receptors on these cells within the CNS.

Previous work from this laboratory has shown that peripheral administration of clenbuterol (0.5mg/kg) induces an anti-inflammatory cytokine profile in rat cortex and hippocampus. As such, clenbuterol has been demonstrated to upregulate the expression of the anti-inflammatory cytokine IL-10 and its downstream signalling molecule SOCS3 in a temporal manner in rat CNS under non-inflammatory conditions (McNamee, 2008). In line with these observations the results shown here demonstrate that administration of clenbuterol to naïve rats induces the mRNA expression of IL-10 and SOCS3 in rat cortex and hippocampus. Reassuringly, exposure to LPS 24 hr prior to clenbuterol administration had no impact on the ability of clenbuterol to induce the expression of these molecules. This suggests that prior exposure to systemic LPS did not impede responsiveness of the β_2-adrenoceptor to stimulation with clenbuterol. These results are significant since previous reports have indicated that IL-10 can have neuroprotective effects. *In vitro* administration of IL-10 suppresses expression of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α induced by amyloid-β and LPS in a time and dose-dependent manner (Szczepanik *et al.*, 2001). *In vivo,* peripheral administration of IL-10 has been shown to exert anti-inflammatory effects and thus improve conditions such as autoimmune encephalomyelitis, pneumococcal mengitis and LPS-induced inflammation (Kastin *et al.*, 2003). In addition, IL-10 has been shown to improve neurological recovery and significantly decrease levels of TNF-α and IL-1β following traumatic brain injury in rats (Knoblach *et al.*, 1999). Moreover, in a rat model of EAE, intrathecal administration of IL-10 gene therapy, in the form of a plasmid construct coding for rat IL-10 (pDNA-IL-10^{F129S}) has

been shown to reverse the behavioural symptoms and histopathological changes associated with the disease (Sloane *et al.*, 2009). IL-10 is known to be constitutively expressed in the normal human brain (Szczepanik *et al.*, 2001), however, while IL-10 is found to be relatively stable in blood and brain and is capable of crossing the BBB intact it does not cross the BBB by a saturable transport system and therefore its entry into the CNS is slow and limited (Kastin *et al.*, 2003). Thus, whilst IL-10 can elicit anti-inflammatory effects, high circulating levels of IL-10 need to be induced in order for systemic administration of IL-10 to have effects in the CNS (Cua *et al.*, 2001). Therefore, pharmacological enhancement of IL-10 in the brain through stimulation of β_2-adrenoceptors with agonists such as clenbuterol may offer a new therapeutic target for the treatment of neuroinflammatory diseases.

Cytokine signalling is generally transient in nature. Accordingly SOCS proteins are known to control cytokine signalling and as such are involved in limiting the innate immune response. SOCS proteins were the first known inducible feedback inhibitors of cytokine signalling and have been shown to inhibit JAK activity and MAPK activation. While SOCS proteins are expressed at low constitutive levels in cells they can be rapidly upregulated upon cytokine stimulation since they bear the properties of immediate early genes and are mainly regulated at the transcriptional level (Dalpke *et al.*, 2008). Previous studies from this laboratory have shown that SOCS3 expression is increased following stimulation of central β_2-adrenoceptors with clenbuterol (McNamee, 2008). In line with this, the results presented here show that SOCS3 expression is induced by clenbuterol in the cortex and hippocampus of naïve animals 4 hr post-injection. Significantly, prior exposure to LPS 24 hr does not impede the ability of clenbuterol to upregulate the expression of this molecule. SOCS3 expression is critical since its deletion has been found to be lethal to the developing embryo due to defects in placental development (Dalpke *et al.*, 2008). Furthermore, knock-out of SOCS3 in a model of acute inflammatory arthritis resulted in exacerbated inflammation, splenomegaly and increased numbers of neutrophils in the synovium, blood, spleen and bone-marrow. Knock-out of SOCS3 was also found to result in hyperactivation of T lymphocytes and increased antigen presentation suggesting that SOCS3 is a negative regulator of CD4+ T lymphocyte activation (Wong *et al.*, 2006). Therefore, upregulation of SOCS3 expression in the CNS by therapeutic targeting of the β_2-adrenoceptor could have important consequences for the treatment of inflammatory diseases.

In addition to the increased expression of IL-10 and SOCS3 clenbuterol has previously been shown to regulate the IL-1 system under non-inflammatory conditions through the induction of IL-1β and the upregulation of negative regulators of the IL-1 signalling system, IL-1ra and the IL-1 Type II receptor. More importantly, clenbuterol has been demonstrated to have no effect on the expression of IL-1 Type I receptor or the IL-1 accessory protein, both of which are essential for IL-1 signalling (McNamee, 2008). The results presented here demonstrate that stimulation of central β_2-adrenoceptors increases the mRNA expression of IL-1ra and IL-1 Type II receptor in rat cortex and hippocampus 4 hr post-administration. Yet again, prior exposure to LPS does not impact on the expression of these molecules in response to clenbuterol. IL-1ra, a naturally occurring inhibitor of the IL-1 signalling system, is known to compete with IL-1 for binding sites on IL-1 receptors, however, IL-1ra is incapable of initiating a signalling cascade since it is unable to link the subunits of the receptor necessary for signalling (Cannon, 2000). The increased expression of IL-1ra shown here is noteworthy since the literature shows that IL-1ra is neuroprotective since it reduces glial activation, inhibits neutrophil infiltration into the CNS, reduces ischaemic brain injury, improves neurological function following injury and can inhibit symptoms of EAE (Martin & Near, 1995; Rothwell & Luheshi, 2000). The IL-1 type II receptor is another known negative regulator of IL-1 signalling. Found expressed on the cell surface, the IL-1 Type II receptor acts as a decoy receptor capable of binding IL-1 thus preventing access of the ligand to the functional IL-1 Type I receptor (Allan, 2000). However, in contrast to the IL-1 Type I receptor the IL-1 Type II receptor lacks the capacity to induce intracellular signalling (Docagne *et al.*, 2005). While the IL-1 Type II receptor is not constitutively expressed in the CNS, microinjection of IL-1β into the rat brain has been shown to induce the expression of IL-1 Type II receptor in the microvasculature and infiltrating neutrophils (Docagne *et al.*, 2005). Thus, the upregulation of IL-1ra and IL-1 Type II receptor expression by clenbuterol may have beneficial consequences for neuroinflammatory conditions.

Again in line with previous studies from this laboratory, clenbuterol was found to upregulate the expression of IL-1β in naive rats, a cytokine typically considered to be pro-inflammatory in nature. IL-1β is known to induce a pro-inflammatory phenotype when it engages with the IL-1 Type I receptor on the surface of cells, triggering the activation of downstream MAPKs such as p38, ERK1/2 and JNK (Rothwell & Luheshi, 2000) and transcription factors such as

NFκB (Bowie & O'Neill, 2000). IL-1 expression has been found to be rapidly induced in rats under ischaemic, hypoxic or excitotoxic conditions leading to neuronal loss, however, injection of IL-1 into the brain of naive rats under non-inflammatory conditions does not contribute to neuronal injury (Rothwell & Luheshi, 2000). *In vitro* studies have previously shown that stimulation of microglia but not astrocytes with the β-agonist isoproterenol or with the cAMP analogue dibutyryl cAMP (db-cAMP) induces a robust increase in the mRNA expression of IL-1β, an effect dependent on β-adrenoceptor signalling since it was prevented by pre-treatment with the non-selective β-adrenoceptor antagonist propranolol (Tomozawa *et al.*, 1995). In spite of this, the principal cell type responsible for the induction of IL-1β by clenbuterol has not been elucidated in the present study. However, previous studies have indicated that clenbuterol-induced IL-1 expression co-localises with astrocytes as opposed to microglia (Tanvaneer, 2008). Notably, while clenbuterol has been shown to increase the expression of IL-1β in the rat brain and induce activation of ERK1/2 and NFκB it fails to activate the pro-apoptotic MAPKs, JNK or p38 (Heffernan, 2009). Therefore, the increased expression of IL-1β in the CNS could have beneficial consequences. Significantly, the results presented here demonstrate that when rats were exposed to LPS prior to administration of clenbuterol it augmented the clenbuterol-induced increase in GFAP and IL-1β expression. Thus, in line with previous reports (Sparkman & Johnson, 2008; Sloane *et al.*, 2009), it appears that exposure to LPS may prime the CNS resulting in a more robust immune response upon clenbuterol administration. However, in contrast to previous reports on priming of microglia, the results presented here suggest that exposure to LPS induces a more robust immune response to clenbuterol through priming effects on astrocytes.

4.6.4 Summary

The results presented in this chapter reveal that while a single systemic administration of LPS reduces body-weight of rats and induces a robust increase in the expression of the pro-inflammatory mediators IL-1β, TNF-α, iNOS and IFN-γ in the CNS it does not impact on the expression or responsiveness of central β_2-adrenoceptors. However, the current study employed only a single dose of LPS. It is possible that the glucocorticoid surge which occurs following systemic LPS administration may lead to an inability of LPS to impact on β_2-adrenoceptor expression in a similar manner to that shown with LPS + IFN-γ in the presence

of dexamethasone in glial cells *in vitro*. Therefore, administration of higher doses of LPS or repeated administration of LPS should also be investigated to determine if they have any effect on the expression or function of the β_2-adrenoceptor *in vivo* since repeated administration of LPS can lead to desensitization of the HPA-axis and reduced glucocorticoid production. In spite of this, the results presented here demonstrate that *in vitro* findings do not always extrapolate to the *in vivo* situation and therefore it is necessary to employ both experimental methods for studies involving physiological responses. Overall however, the current results point to the β_2-adrenoceptor as a promising therapeutic target for the treatment of neuroinflammation since its stimulation induces the expression of the anti-inflammatory mediators IL-10, SOCS3, IL-1ra and the IL-1 Type II receptor both under basal conditions and following systemic LPS administration. However, clenbuterol was also shown to induce elevated expression of CD11b and GFAP, markers of glial activation, and furthermore increased the expression of the pro-inflammatory cytokine IL-1β. While the activation of glia can be advantageous since these cells are involved in the clearance of debris and restoring homeostasis, it can also lead to gliosis and may even contribute to neurodegeneration. Furthermore, the beneficial versus harmful effects of IL-1β are concentration dependent. Therefore, the impact of clenbuterol-induced elevations in IL-1β concentrations on the CNS, in particular in neuroinflammatory situations, warrants further investigation. Thus, while clenbuterol induces a profound anti-inflammatory profile further evaluation is necessary to determine if the increased expression of glial activation markers and IL-1β leads to a beneficial effect or whether these effects are a potentially detrimental side-effect of the drug.

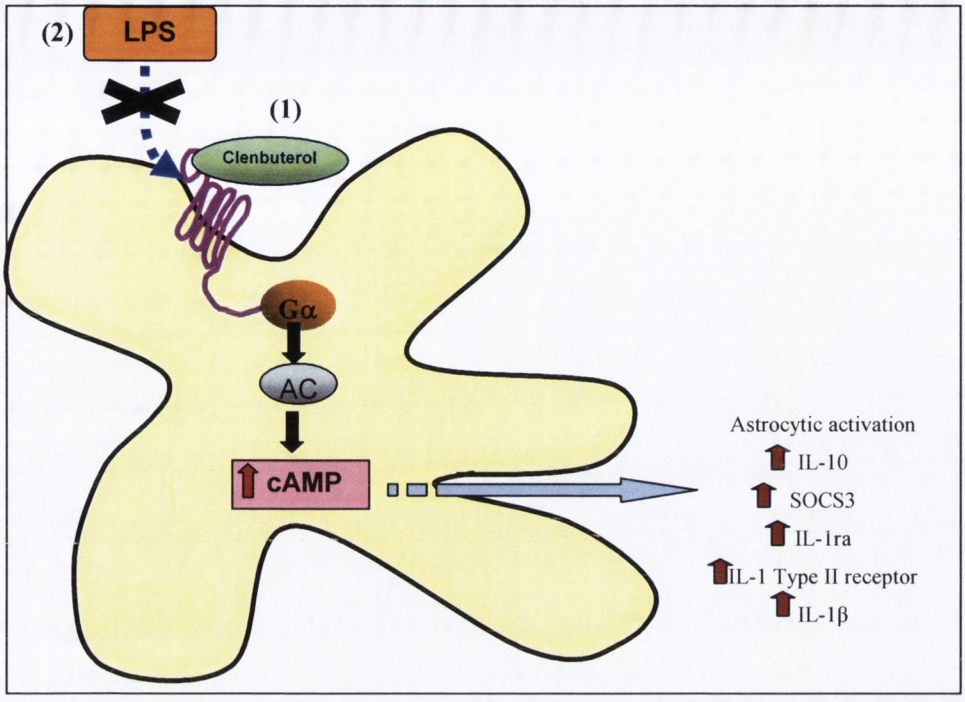


Figure 4.2: Schematic representation of results from *in vivo* study

Stimulation of central β_2-adrenoceptors with the selective β_2-adrenoceptor agonist clenbuterol *in vivo* induces astrocytic activation and the expression of IL-10, SOCS3, IL-1ra, IL-1 Type II receptor and IL-1β in rat cortex and hippocampus (1). Exposure to a systemic inflammatory challenge of LPS does not impact on the expression of central β_2-adrenoceptors nor does it impact on responsiveness of β_2-adrenoceptors since it does not impede the ability of clenbuterol to induce astrocytic activation or the expression of IL-10, IL-1ra, IL-1 Type II receptor or IL-1β (2).

4.7 General overview of *in vitro* and *in vivo* studies: inflammation impacts on β_2-adrenoceptor expression and function *in vitro* but not *in vivo*

The results presented in Chapter 3 demonstrate that an inflammatory challenge with LPS + IFN-γ down-regulates both the expression and function of β_2-adrenoceptors on glial cells *in vitro*. In this regard, it was found that an inflammatory challenge down-regulates the expression of the β_2-adrenoceptor at both the cell surface (protein) and mRNA levels. Furthermore, the accumulation of intracellular cAMP in response to stimulation of glial β_2-adrenoceptors with a β_2-adrenoceptor agonist, i.e. salbutamol, is blunted following exposure of glia to an inflammatory challenge of LPS + IFN-γ. Significantly, the impact of LPS + IFN-γ on the mRNA expression of the β_2-adrenoceptor was shown to be reversible and could be attenuated by prior exposure of glia to the synthetic glucocorticoid dexamethasone.

In contrast, the results presented in the current chapter demonstrate that when a systemic inflammatory challenge of LPS is administered to rats *in vivo* it induces a pro-inflammatory profile in the CNS which is qualitatively similar to that used in the *in vitro* model but it does not impact on the mRNA expression of central β_2-adrenoceptors. Furthermore, LPS did not impact on the responsiveness of central β_2-adrenoceptors to stimulation with the selective β_2-adrenoceptor agonist clenbuterol since, in line with previous reports (McNamee, 2008) clenbuterol induced the expression of GFAP, IL-1ra, IL-1 Type II receptor, IL-10 and SOCS3 both under basal conditions and following prior exposure of rats to LPS. Furthermore, it appears that a prior inflammatory challenge might even prime the CNS to yield a more robust increase in astrocytic activation and IL-1β expression, whether this is a beneficial or detrimental effect remains to be determined. In contrast to the *in vitro* studies however, the results from the *in vivo* study did not examine the expression of the β_2-adrenoceptor on individual cell types nor did it examine downstream signalling or the production of cAMP in response to stimulation of β_2-adrenoceptors with clenbuterol. Thus, it is possible that LPS may still impact on either the protein expression of the β_2-adrenoceptor or its downstream signalling pathways.

The synthetic glucocorticoid dexamethasone was shown to attenuate the impact of LPS + IFN-γ on glial β_2-adrenoceptor expression *in vitro*. Since no impact of LPS on glial β_2-adrenoceptors was observed *in vivo* it is likely that the release of endogenous glucocorticoids induced by systemic LPS administration may prevent the down-regulation of the β_2-adrenoceptor in a similar manner to that witnessed with dexamethasone *in vitro*. Since it has been reported that the HPA-axis is dysfunctional in many neuroinflammatory disease states including MS (Stefferl *et al.,* 1999; Beishuizen & Thijs, 2003; Heesen *et al.,* 2007) dysregulation of endogenous glucocorticoid actions might account for the absence of β_2-adrenceptors observed on astrocytes in the MS brain. Furthermore, the ability of synthetic glucocorticoids to regulate the expression of β_2-adrenoceptors both under basal and inflammatory conditions may account for their therapeutic efficacy in such conditions.

Overall, the results presented here demonstrate that stimulation of central β_2-adrenoceptors may represent a suitable therapeutic target for the treatment of neuroinflammation since neither their expression nor responsiveness appears to be affected by an inflammatory

challenge *in vivo*. Notably, the results presented here also demonstrate that findings from *in vitro* studies do not always translate to a similar finding *in vivo*. Therefore, caution must be warranted when drawing conclusions from work carried out *in vitro* and attempts should be made to replicate any significant findings in *in vivo* models also.

Chapter 5

Dose-dependent effect of clenbuterol on the IL-1 system and behaviour - a role for β_2-adrenoceptors?

5.1 Introduction

The previous chapter investigated the impact of systemic inflammation on β_2-adrenoceptor expression and function *in vivo* and thus the possibility of exploiting the β_2-adrenoceptor as a therapeutic target for the treatment of neuroinflammatory disease. Since the results presented in the earlier chapter show that a systemic inflammatory challenge has no apparent impact on the β_2-adrenoceptor *in vivo* the following studies were undertaken to elucidate the ability of an acute treatment with the brain penetrable selective β_2-adrenoceptor agonist clenbuterol to regulate glial activation and the induction of pro- and anti-inflammatory mediators under both basal and inflammatory conditions.

A vast amount of literature indicates that therapeutic stimulation of central β_2-adrenoceptors can elicit neuroprotection following insult to the CNS. In this regard, the β_2-adrenoceptor agonist clenbuterol has previously been shown to reduce the infarct volume and increase neuronal viability following middle cerebral artery occlusion and focal cerebral ischaemia (Junker *et al.*, 2002). Additionally, clenbuterol induces the expression of the neurotrophins NGF, bFGF and TGFβ_1 and astrocytic activation following ischaemia (Semkova *et al.*, 1996; Culmsee *et al.*, 1999). *In vitro* studies have also demonstrated the neuroprotective potential of clenbuterol since it has been shown to enhance the production of the endogenous NMDA receptor antagonist kynurenic acid in glial cultures (Luchowska *et al.*, 2008). Furthermore, administration of clenbuterol can delay the onset of disease in a mouse model of amyotrophic lateral sclerosis (Teng *et al.*, 2006). In line with these reports, the results from the previous chapter (Chapter 4) and those from previous studies carried out in this laboratory have shown that a dose of 0.5mg/kg clenbuterol can activate astrocytes and increase the expression of the anti-inflammatory mediators IL-10, SOCS3, IL-1 Type II receptor and IL-1ra in the CNS (McNamee, 2008). Surprisingly, this dose of clenbuterol was also found to induce the expression of the prototypical pro-inflammatory cytokine IL-1β (Chapter 4 this thesis; McNamee, 2008). It has previously been suggested that stimulation of facilitatory β_2-adrenoceptors on noradrenergic neurons can induce the release of noradrenaline (Murugaiah & O'Donnell, 1995) which may then subsequently act on post-synaptic β_1-adrenergic receptors. Since previous reports have also demonstrated that administration of the β-adrenergic agonist isoproterenol to microglia *in vitro* induces the expression of IL-1β (Tomozawa *et al.*, 1995) it was speculated that a dose of clenbuterol such as that employed previously might be acting to

increase IL-1β expression through stimulation of β_1-adrenoceptors on microglia in addition to effects on the β_2-adrenoceptor. Further to this, previous reports have also suggested that higher doses of clenbuterol may suppress its neuroprotective effects due to a reduction in mean arterial blood pressure and increased levels of glucose in the blood stream which ultimately lead to decreased tissue pH (Culmsee *et al.*, 1999). Additionally, the dose of clenbuterol shown to induce an acute immune response in the CNS previously (Chapter 4 this thesis; McNamee, 2008) was much higher (~17 times) than that used in humans whereby doses of 20-40μg are therapeutically efficacious in the treatment of asthma and are generally administered over prolonged periods up to twice or three times daily (Kamburoff & Prime 1977; Wheatley, 1982; Hida *et al.,* 1985; Sorau *et al.,* 2007). Therefore, the main purpose of the following studies was to elucidate the lowest dose at which clenbuterol can induce an immune response in the CNS following an acute challenge in rats. Furthermore, the ability of clenbuterol to elicit protection against the inflammation induced by co-administration of LPS given systemically was also examined.

Clenbuterol has previously been shown to increase IL-1β expression in the CNS (Chapter 4 this thesis; McNamee, 2008), a pro-inflammatory cytokine known to play a role in the reduced locomotor activity and social exploration associated with classical endotoxin-induced sickness behaviour (Bluthe *et al.*, 2000; Wieczorek & Dunn, 2006). Thus, the impact of acute clenbuterol administration on locomotor activity of rats was also examined here.

Therefore, the aims of these studies were as follows:

1) To determine the impact of peripheral clenbuterol administration on the locomotor activity of rats 2 hr post-injection using doses ranging from 0.003-0.3mg/kg.

2) To determine the lowest dose at which clenbuterol induces an immune response in the rat CNS following peripheral challenge 4 hr post-injection using doses ranging from 0.003-0.3mg/kg.

3) To determine if co-administration of a low dose of clenbuterol can impact upon the inflammatory profile induced in rat CNS by systemic LPS administration.

4) To determine whether the β_1- or β_2-adrenoceptor subtype is responsible for the clenbuterol-induced changes in immune mediators in the rat CNS and locomotor activity by pre-treatment of rats with the selective β_1-adrenoceptor antagonist metoprolol or the selective β_2-adrenoceptor antagonist ICI 118,551.

5.2 Methods

5.2.1 Subjects and drug treatments

Male Sprague-Dawley rats (280-310g) were housed 3 per cage under a 12 hr light-dark cycle (lights on 08:00) in a temperature-controlled room (22 ± 2 °C) with food and water available *ad libitum* for the duration of the experiment. Clenbuterol and LPS were dissolved in 0.89% (w/v) saline as described in the methods (Section 2.3, Chapter 2) and were administered i.p. at doses of 0.003, 0.01, 0.03, 0.1 or 0.3mg/kg and 250μg/kg respectively. Metoprolol and ICI 118,551 were dissolved in 0.89% (w/v) saline with 0.2% (v/v) Tween-20 and were both administered i.p. at a dose of 10mg/kg. Control rats were administered an i.p. injection of 0.89% (w/v) saline alone.

5.2.2 Experimental Procedure

The design for this set of experiments was as set out in Figures 5.1 to 5.3 below. Locomotor activity was monitored by placing animals in locomotor activity cages for 15 min 2 hr post-injection prior to returning the animal to its home-cage for a further 2 hr before sacrifice at 4 hr post-injection as previously outlined in the methods section (Section 2.3, Chapter 2). Preparation of tissue for cDNA synthesis and RT-PCR were carried out as outlined in the methods section (Section 2.3 & 2.4, Chapter 2).

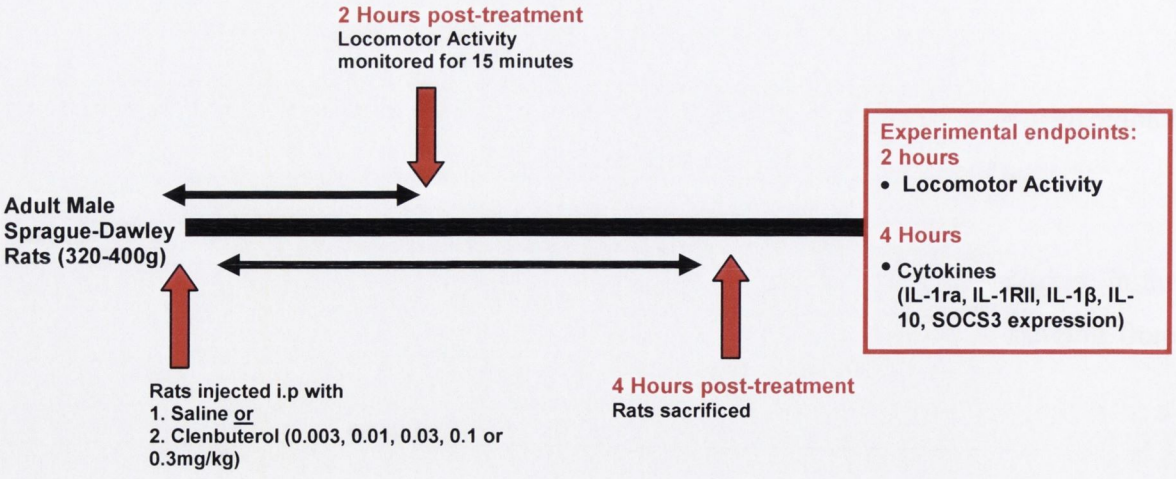


Figure 5.1: Experimental design to determine dose-dependent effects of clenbuterol

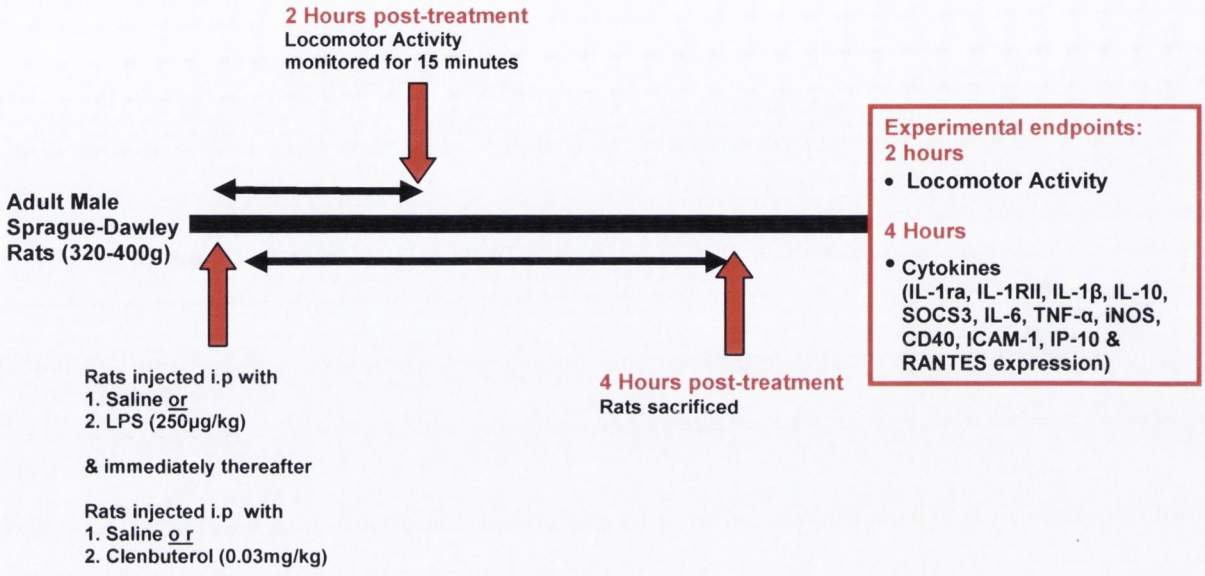


Figure 5.2: Experimental design to determine impact of low-dose clenbuterol on LPS-induced neuroinflammation

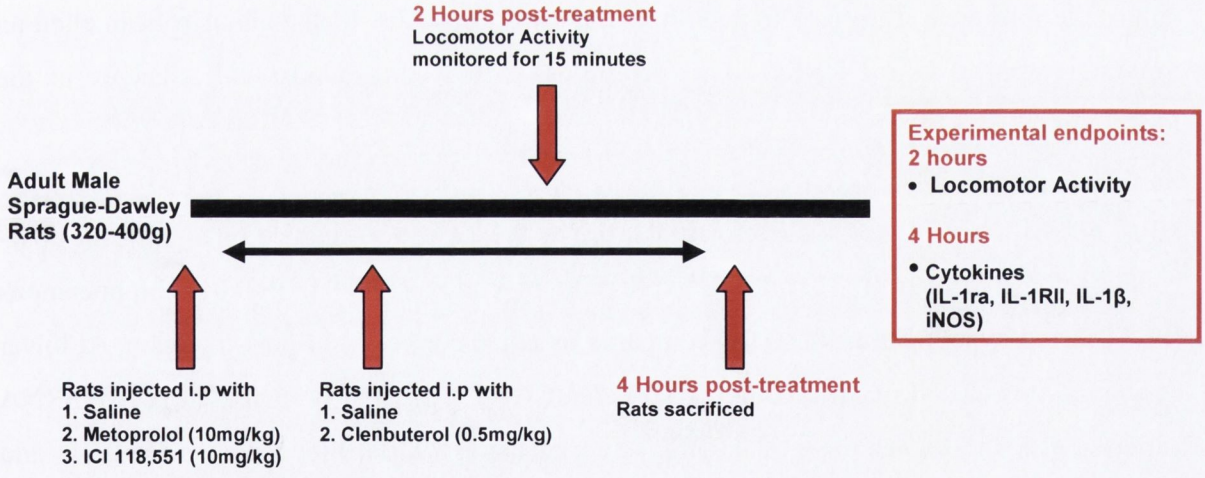


Figure 5.3: Experimental design to determine β-adrenoceptor subtype responsible for inducing central effects of clenbuterol

5.3 Effect of varying doses of the β_2-adrenoceptor agonist clenbuterol on cytokine expression in rat CNS

As previously discussed a vast range of doses at which clenbuterol can elicit neuroprotective effects in the CNS have been described in the literature (Du Yan *et al.*, 1997; Culmsee *et al.*, 1999; Zhu *et al.*, 1999; Zhu *et al.*, 2001; Junker *et al.*, 2002). Furthermore, the results from the previous chapter and those from previous studies from this laboratory demonstrate that in addition to the activation of glia and induction of anti-inflammatory cytokines, the high dose of clenbuterol (0.5mg/kg;i.p.) employed also increases expression of the pro-inflammatory cytokine IL-1β in rat CNS (Chapter 4 this thesis; McNamee, 2008). Thus, the aim of the following study was to determine if increased expression of anti-inflammatory mediators and the increase in IL-1β expression induced by peripheral administration of clenbuterol is dose-dependent and whether the increase in IL-1β and anti-inflammatory cytokine expression could be dissociated. In addition, since clenbuterol was found to increase the expression of IL-1β in the CNS, a cytokine associated with the reduced locomotor activity and social exploration associated with classical sickness behaviour (Bluthe *et al.*, 2000; Wieczorek & Dunn, 2006), the impact of clenbuterol on the locomotor activity of rats was examined. Thus, the overall aim of the following study was to determine the lowest dose at which clenbuterol can elicit an immune response in rat CNS and to determine if this corresponds with changes in the locomotor activity of rats.

IL-1β mRNA expression: Clenbuterol (0.03, 0.1 or 0.3mg/kg;i.p.) induced a significant dose-dependent increase in IL-1β mRNA expression in cortex ($P<0.05$, $P<0.01$), hippocampus ($P<0.01$) and hypothalamus ($P<0.01$) compared to saline controls 4 hr post-injection. At lower concentrations (0.003, 0.01, 0.03mg/kg;i.p.) clenbuterol significantly increased IL-1β mRNA expression at the higher dose of 0.03mg/kg in cortex ($P<0.01$), hippocampus ($P<0.01$) and hypothalamus ($P<0.01$) compared to saline controls. However, clenbuterol had no effect on IL-1β expression at the lower doses of 0.003mg/kg and 0.01mg/kg. [Figure 5.3.1 a,b,c, Dunnett's Procedure, n=4-9].

IL-1 Type II mRNA expression: Clenbuterol (0.03, 0.1 or 0.3mg/kg;i.p.) induced a significant dose-dependent increase in IL-1 Type II receptor mRNA expression in cortex ($P<0.05$,

P<0.01), hippocampus (P<0.01) and hypothalamus (P<0.01) compared to saline controls 4 hr post-injection. At lower concentrations (0.003, 0.01, 0.03mg/kg;i.p.) clenbuterol significantly increased IL-1 Type II receptor mRNA expression at the higher dose of 0.03mg/kg in cortex (P<0.01), hippocampus (P<0.01) and hypothalamus (P<0.01) compared to saline controls. However, clenbuterol had no effect on IL-1 Type II receptor expression at the lower doses of 0.003mg/kg and 0.01mg/kg. [Figure 5.3.2 a,b,c, Dunnett's Procedure, n=4-9].

IL-1ra mRNA expression: Clenbuterol (0.03, 0.1 or 0.3mg/kg;i.p.) induced a significant dose-dependent increase in IL-1ra mRNA expression in cortex (P<0.01), hippocampus (P<0.01) and hypothalamus (P<0.01) compared to saline controls 4 hr post-injection. At lower concentrations (0.003, 0.01, 0.03mg/kg;i.p.) clenbuterol significantly increased IL-1ra mRNA expression at the higher dose of 0.03mg/kg in cortex (P<0.05) while doses of 0.01mg/kg and 0.03mg/kg induced a significant increase in IL-1ra mRNA expression in hippocampus (P<0.01) and hypothalamus (P<0.05, P<0.01 respectively) compared to saline controls. However, clenbuterol had no effect on IL-1ra expression at the lower dose of 0.003mg/kg. [Figure 5.3.3 a,b,c, Dunnett's Procedure, n=4-9].

iNOS mRNA expression: Clenbuterol (0.03, 0.1 or 0.3mg/kg;i.p.) induced a significant dose-dependent increase in iNOS mRNA expression in cortex (P<0.01) and hypothalamus (P<0.01) compared to saline controls 4 hr post-injection. While a similar trend was shown in the hippocampus for this dose-range these results did not reach significance. At lower concentrations (0.003, 0.01, 0.03mg/kg;i.p.) clenbuterol significantly increased iNOS mRNA expression at doses of 0.01 and 0.03mg/kg in cortex (P<0.05, P<0.01 respectively) and in hippocampus (P<0.05) and hypothalamus (P<0.05) at a dose of 0.03mg/kg compared to saline controls. However, clenbuterol had no effect on iNOS expression at the lower dose of 0.003mg/kg [Figure 5.3.4 a,b,c, Dunnett's Procedure, n=4-9].

Locomotor Activity: Clenbuterol (0.03, 0.1 or 0.3mg/kg;i.p.) induced a significant dose-dependent decrease in the total time rats were active in the locomotor activity cages (P<0.01) compared to saline controls 2 hr post-injection. In addition, clenbuterol induced a significant dose-dependent decrease in the rearing totals (P<0.01), mobile totals (P<0.01) and static totals (P<0.05, P<0.01) of rats 2 hr post-administration. At a dose of 0.03mg/kg clenbuterol caused a significant decrease (P<0.01) in the time rats were active in the locomotor activity cages

compared to saline controls and, in addition, caused a significant decrease in the rearing totals ($P<0.01$), mobile totals ($P<0.01$) and static totals ($P<0.01$) of rats. In contrast, the lower doses of 0.003mg/kg and 0.01mg/kg had no effect on any of the parameters of locomotor activity measured. [Figure 5.3.5 a,b,c,d, Dunnett's Procedure, $n=6$].

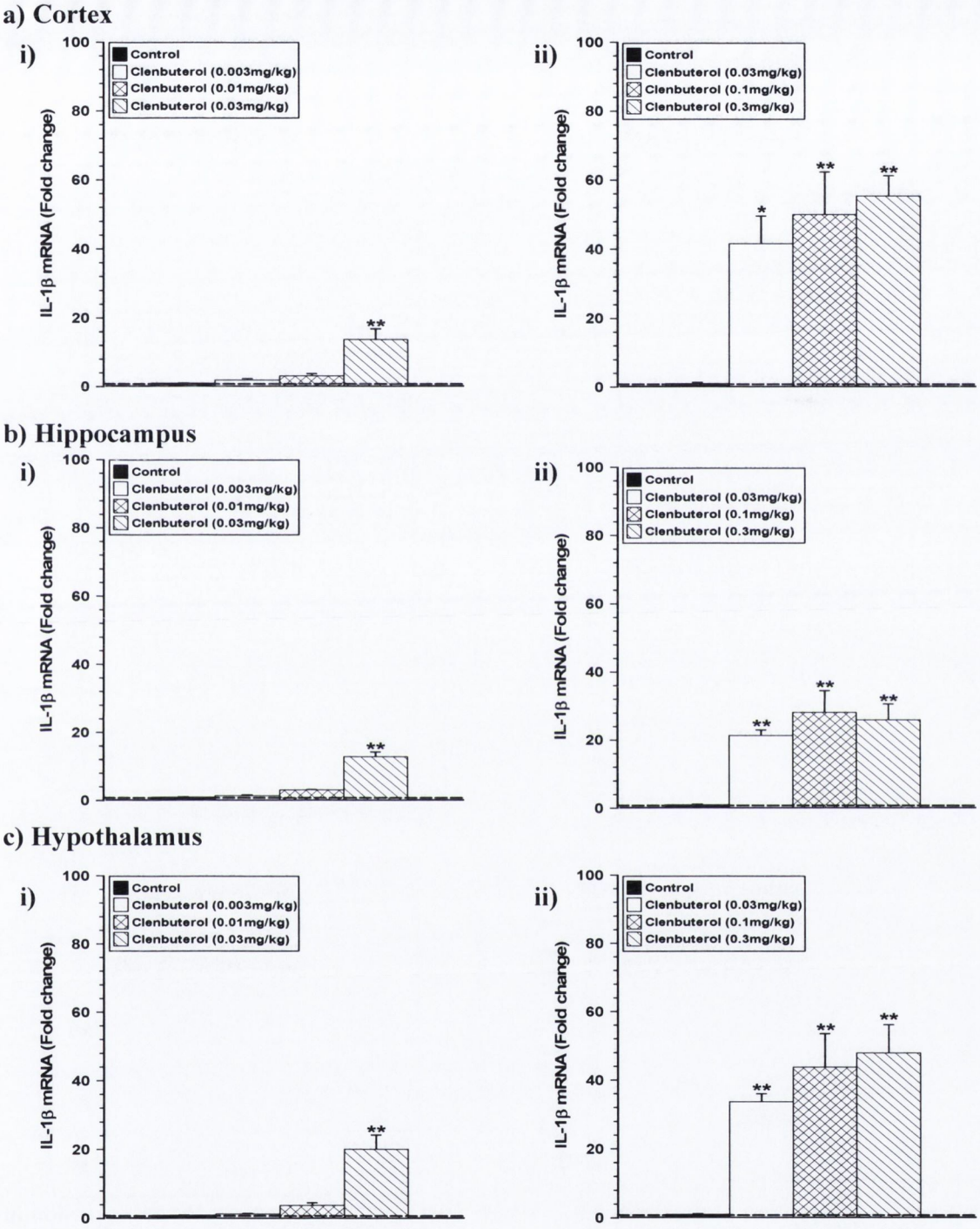


Figure 5.3.1: Clenbuterol dose-dependently increases IL-1β mRNA expression in rat CNS

Clenbuterol (0.003, 0.01, 0.03, 0.1 or 0.3 mg/kg) or control (0.89% (w/v) saline) were administered i.p. and rats were sacrificed 4 hr post-injection. A one-way ANOVA revealed a significant effect of clenbuterol treatment on IL-1β mRNA expression in a) cortex i) [$F_{(3,18)}$=12.43, P=0.0001], ii) [$F_{(3,19)}$=8.11, P=0.0011], b) hippocampus i) [$F_{(3,18)}$=53.62, P<0.0001], ii) [$F_{(3,20)}$=9.42, P=0.0004] and c) hypothalamus i) [$F_{(3,19)}$=18.53, P<0.0001], ii) [$F_{(3,20)}$=12.83, P<0.0001]. Data are expressed as means with standard error of the mean (n=4-9). **P<0.01, *P<0.05 versus saline control (Dunnett's Procedure).

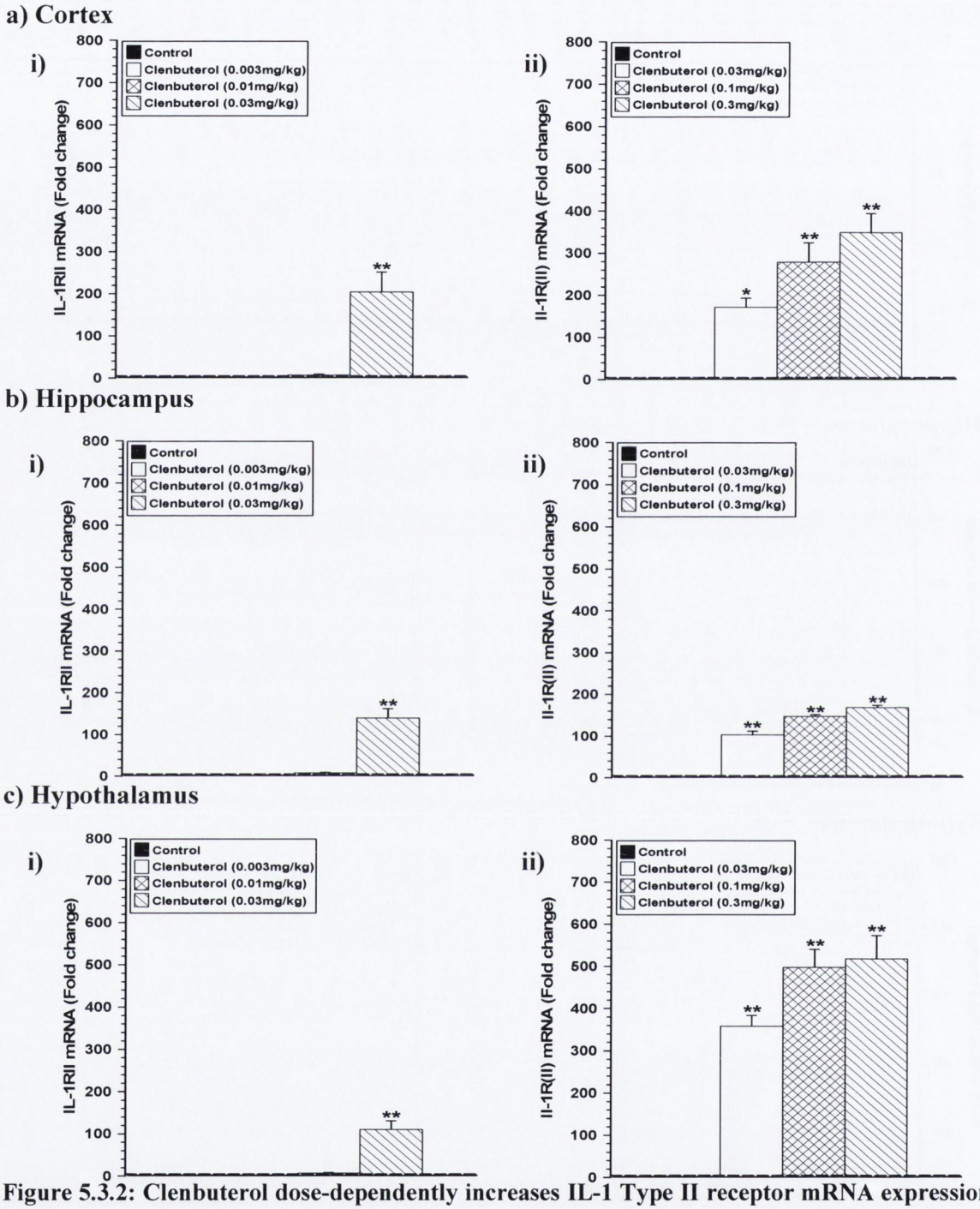


Figure 5.3.2: Clenbuterol dose-dependently increases IL-1 Type II receptor mRNA expression in rat CNS

Clenbuterol (0.003, 0.01, 0.03, 0.1 or 0.3 mg/kg) or control (0.89% (w/v) saline) were administered i.p. and rats were sacrificed 4 hr post-injection. A one-way ANOVA revealed a significant effect of clenbuterol treatment on IL-1 Type II receptor mRNA expression in a) cortex i) [$F_{(3,19)}$=17.17, P<0.0001], ii) [$F_{(3,19)}$=17.03, P<0.0001], b) hippocampus i) [$F_{(3,19)}$=34.96, P<0.0001], ii) [$F_{(3,18)}$=123.37, P<0.0001] and c) hypothalamus i) [$F_{(3,17)}$=28.68, P<0.0001], ii) [$F_{(3,20)}$=40.65, P<0.0001]. Data are expressed as means with standard error of the mean (n=4-9). **P<0.01, *P<0.05 versus saline control (Dunnett's Procedure).

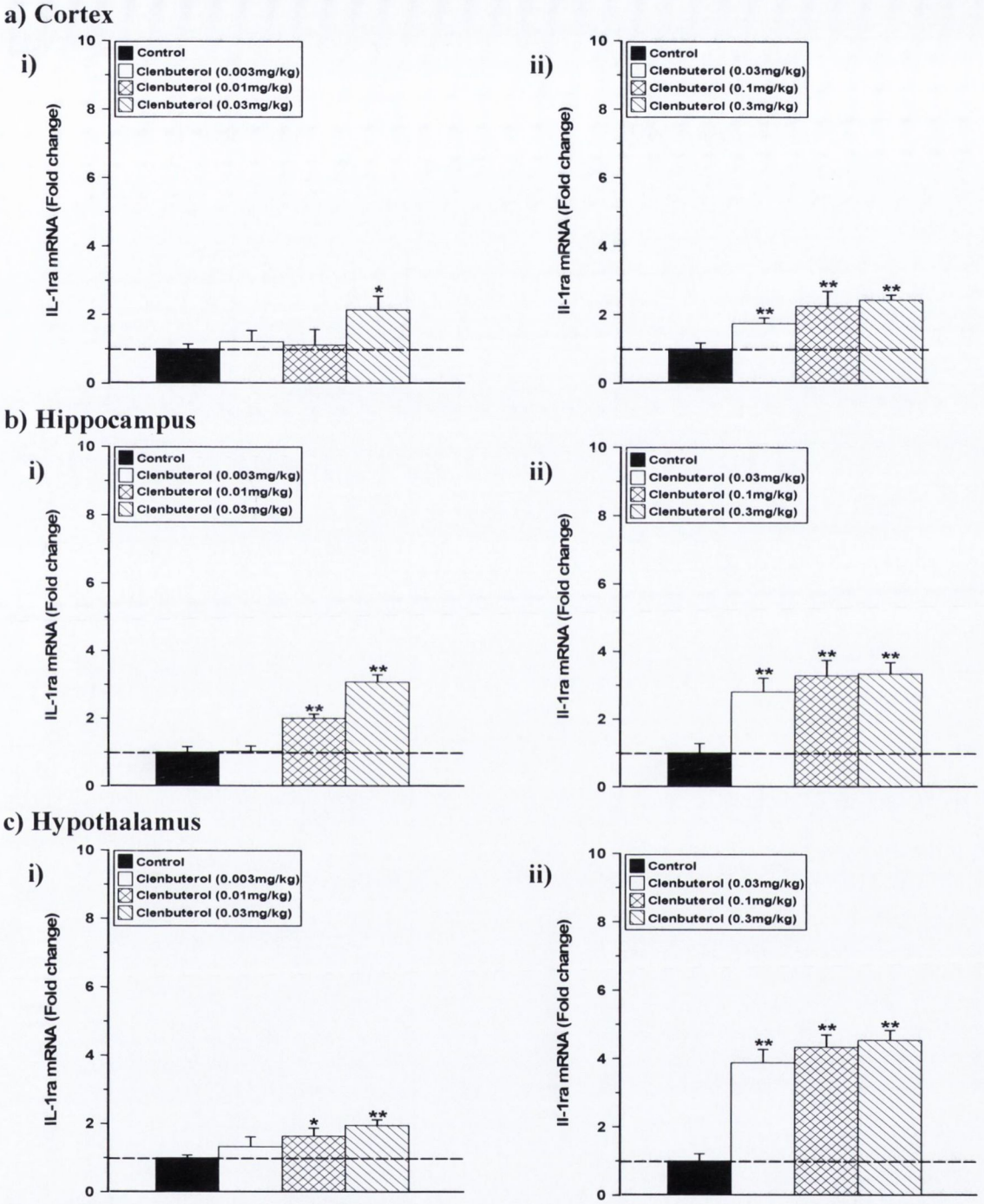


Figure 5.3.3: Clenbuterol dose-dependently increases IL-1ra mRNA expression in rat CNS

Clenbuterol (0.003, 0.01, 0.03, 0.1 or 0.3 mg/kg) or control (0.89% (w/v) saline) were administered i.p. and rats were sacrificed 4 hr post-injection. A one-way ANOVA revealed a significant effect of clenbuterol treatment on IL-1ra mRNA expression in a) cortex i) [$F_{(3,19)}$=3.27 P=0.044], ii) [$F_{(3,20)}$=6.47, P=0.0031], b) hippocampus i) [$F_{(3,18)}$=31.47, P<0.0001], ii) [$F_{(3,20)}$=8.35, P=0.0008] and c) hypothalamus i) [$F_{(3,19)}$=7.38, P=0.0018], ii) [$F_{(3,20)}$=24.87, P<0.0001]. Data are expressed as means with standard error of the mean (n=4-9). **P<0.01 saline control (Dunnett's Procedure).

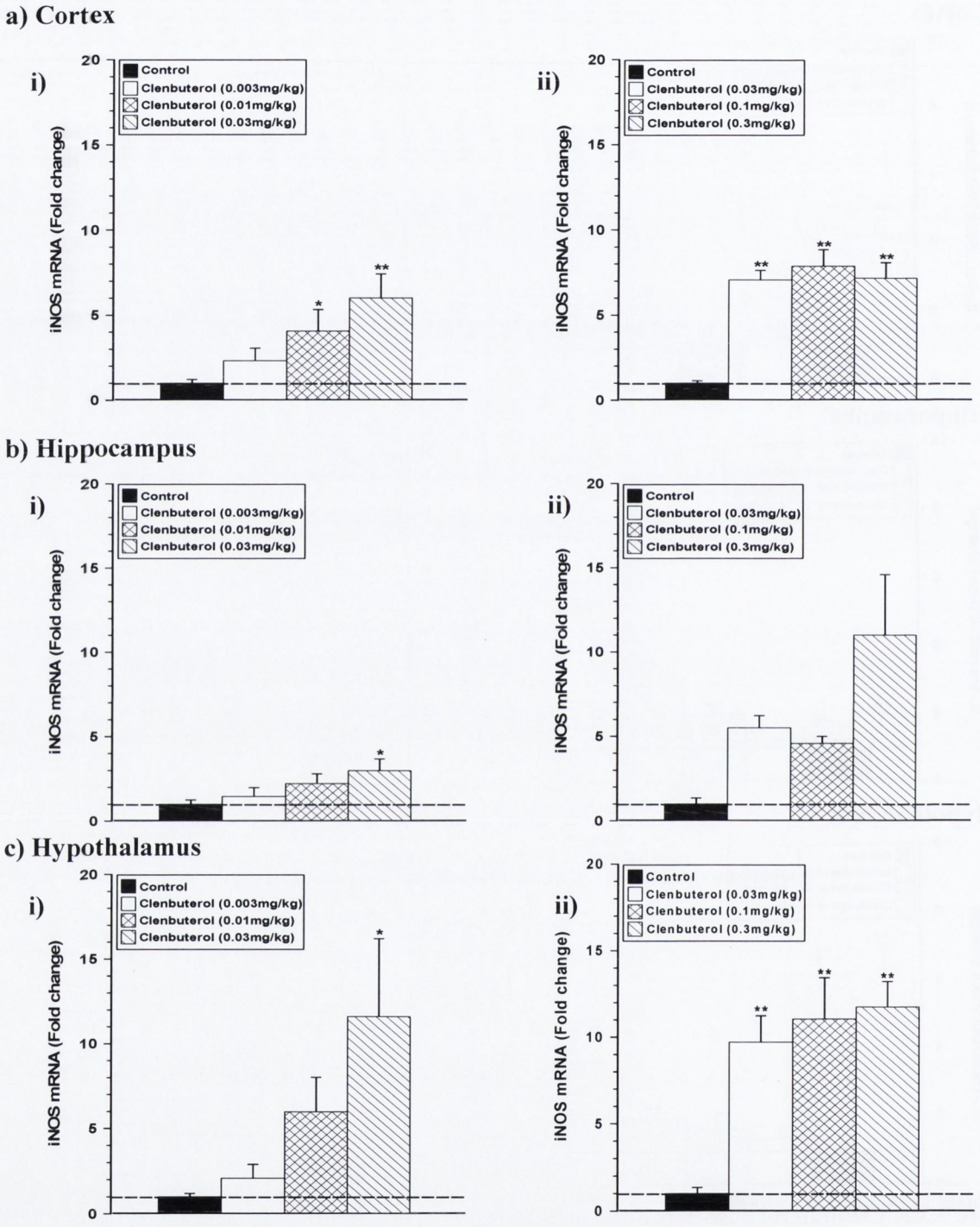


Figure 5.3.4: Clenbuterol dose-dependently increases iNOS mRNA expression in rat CNS

Clenbuterol (0.03, 0.1 or 0.3 mg/kg) or control (0.89% (w/v) saline) were administered i.p. and rats were sacrificed 4 hr post-injection. A one-way ANOVA revealed a significant effect of clenbuterol treatment on iNOS mRNA expression in a) cortex i) [$F_{(3,18)}$=7.78, P=0.0015], ii) [$F_{(3,20)}$=19.75, P<0.0001], b) hippocampus i) [$F_{(3,19)}$=3.69, P=0.03], ii) [$F_{(3,14)}$=2.96, P=0.07] and c) hypothalamus i) [$F_{(3,16)}$=3.84, P=0.03], ii) [$F_{(3,20)}$=10.34, P=0.0003]. Data are expressed as means with standard error of the mean (n=5-7). **P<0.01, *P<0.05 versus saline control (Dunnett's Procedure).

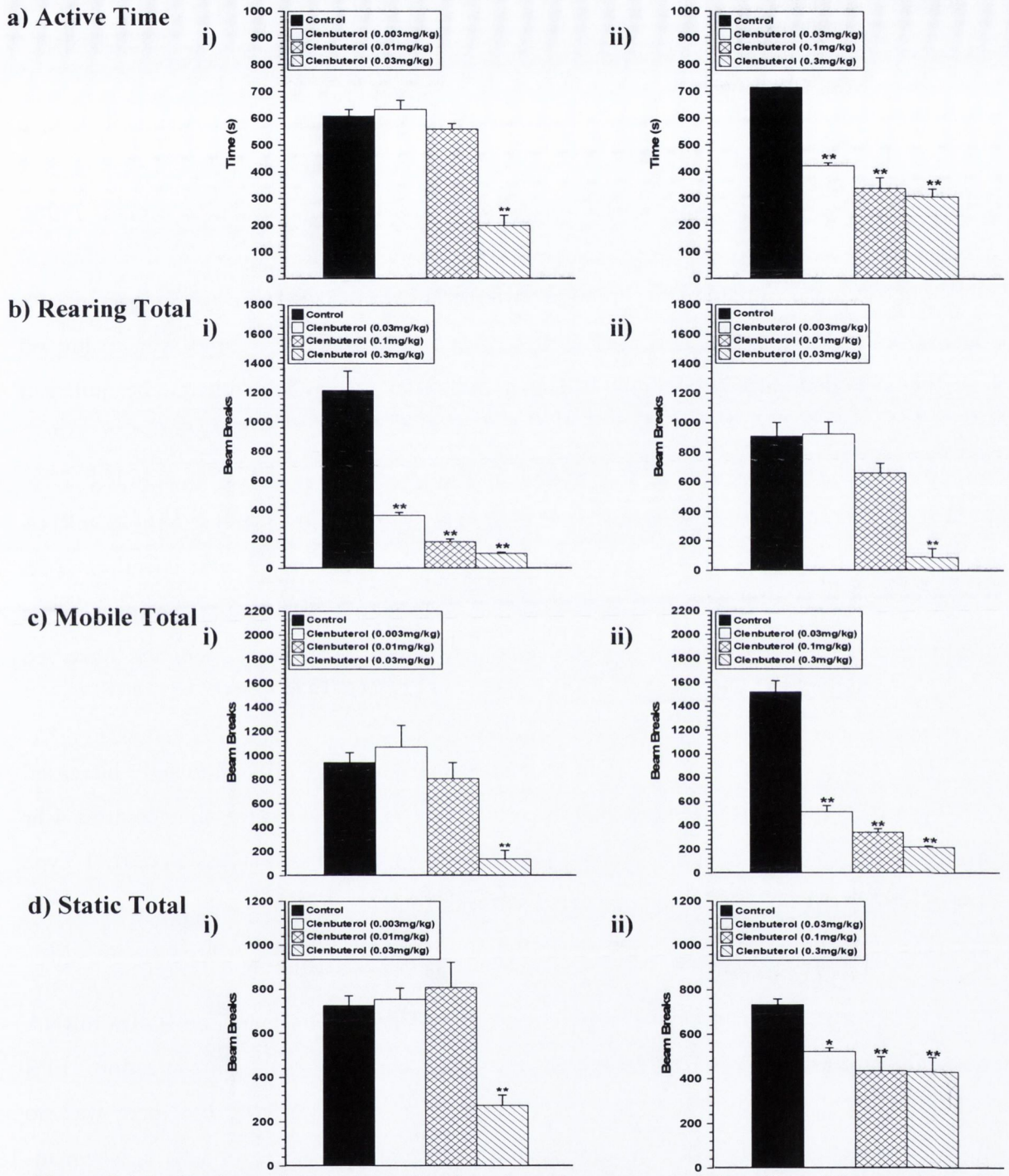


Figure 5.3.5: Clenbuterol dose-dependently reduces locomotor activity of rats 2 hr post-injection

Clenbuterol (0.003, 0.01, 0.03, 0.1 or 0.3 mg/kg) or control (0.89% (w/v) saline) were administered i.p. and the locomotor activity of rats was monitored for 15 min 2 hr post-injection. A one-way ANOVA revealed a significant effect of clenbuterol treatment on a) active time i) [$F_{(3,19)}$=44.55, P<0.0001], ii) [$F_{(3,20)}$=53.71, P<0.0001], b) rearing total i) [$F_{(3,19)}$= 20.09, P<0.0001], ii) [$F_{(3,20)}$=57.22, P<0.0001], c) mobile total i) [$F_{(3,19)}$=15.1, P<0.0001], ii) [$F_{(3,20)}$=115.82, P<0.0001] and d) static total i) [$F_{(3,19)}$=16.46, P<0.0001], ii) [$F_{(3,20)}$=9.02, P=0.0006]. Data are expressed as means with standard error of the mean (n=6). **P<0.01, *P<0.05 versus saline control (Dunnett's Procedure).

5.4 Effect of low-dose clenbuterol on LPS-induced inflammatory mediators in rat CNS

The results from the previous study revealed that the lowest dose at which clenbuterol elicits a full immune response in rat CNS was 0.03mg/kg. This dose is therapeutically relevant since a dose of 10-20μg (0.01-0.02mg) is used orally in humans for bronchodilation (Smith, 1998). Therefore, the aim of the following study was to determine if this low-dose of clenbuterol could impact on the inflammation induced in the CNS in response to co-administered LPS given systemically. For the purposes of these studies only effects in the cortex were examined since the response induced in both the cortex and hippocampus has been found to be similar in the previous studies.

IL-1β mRNA expression: Clenbuterol (0.03mg/kg;i.p.) significantly increased IL-1β mRNA expression in cortex ($P<0.01$) compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) did not significantly impact on the expression of IL-1β alone, however, when co-administered with clenbuterol a significant increase in IL-1β expression was observed ($P<0.01$). [Figure 5.4.1 a, Newman-Keuls, n=6-8].

IL-1 Type II receptor mRNA expression: Clenbuterol (0.03mg/kg;i.p.) significantly increased IL-1 Type II receptor mRNA expression in cortex ($P<0.01$) compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) did not significantly impact on the expression of IL-1 Type II receptor alone but when co-administered with clenbuterol a significant increase in IL-1 Type II receptor expression was observed ($P<0.01$). [Figure 5.4.1 b, Newman-Keuls, n=6-8].

IL-1ra mRNA expression: Clenbuterol (0.03mg/kg;i.p.) significantly increased IL-1ra mRNA expression in cortex ($P<0.05$) compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) significantly increased IL-1ra expression alone ($P<0.05$) but there was no significant effect observed when co-administered with clenbuterol. [Figure 5.4.1 c, Newman-Keuls, n=6-8].

IL-6 mRNA expression: Clenbuterol (0.03mg/kg;i.p.) had no significant effect on IL-6 mRNA expression in rat cortex compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) significantly increased IL-6 expression ($P<0.01$) and when co-administered with clenbuterol a

significant attenuation of LPS-induced IL-6 expression was observed ($P<0.05$). [Figure 5.4.2 a, Newman-Keuls, n=6-8].

TNF-α mRNA expression: Clenbuterol (0.03mg/kg;i.p.) had no significant effect on TNF-α mRNA expression in cortex compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) significantly increased TNF-α expression ($P<0.01$) and when co-administered with clenbuterol TNF-α was no longer increased relative to the clenbuterol control group. [Figure 5.4.2 b, Newman-Keuls, n=6-8].

iNOS mRNA expression: Clenbuterol (0.03mg/kg;i.p.) had no significant effect on iNOS mRNA expression in cortex compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) significantly increased iNOS expression ($P<0.01$) and when co-administered with clenbuterol a significant attenuation of LPS-induced iNOS expression was observed ($P<0.05$). [Figure 5.4.2 c, Newman-Keuls, n=6-8].

IL-10 mRNA expression: Clenbuterol (0.03mg/kg;i.p.) significantly increased IL-10 mRNA expression in cortex ($P<0.05$) compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) did not significantly impact on the expression of IL-10 either alone or when co-administered with clenbuterol. [Figure 5.4.3 a, Newman-Keuls, n=6-9].

SOCS3 mRNA expression: Clenbuterol (0.03mg/kg;i.p.) significantly increased SOCS3 mRNA expression in cortex ($P<0.01$) compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) significantly increased the expression of SOCS3 both alone ($P<0.05$) and when co-administered with clenbuterol ($P<0.01$). [Figure 5.4.3 b, Newman-Keuls, n=6-8].

CD40 mRNA expression: Clenbuterol (0.03mg/kg;i.p.) had no significant effect on CD40 mRNA expression in cortex compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) significantly increased CD40 expression ($P<0.01$) and when co-administered with clenbuterol a significant attenuation of LPS-induced CD40 expression was observed ($P<0.01$). [Figure 5.4.4 a, Newman-Keuls, n=6-8].

ICAM-1 mRNA expression: Clenbuterol (0.03mg/kg;i.p.) had no significant effect on ICAM-1 mRNA expression in cortex compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) significantly increased ICAM-1 expression ($P<0.01$) and when co-

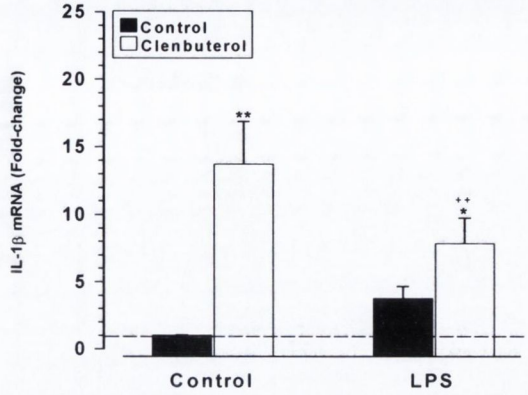
administered with clenbuterol a significant attenuation of LPS-induced ICAM-1 expression was observed ($P<0.05$). [Figure 5.4.4 b, Newman-Keuls, n=6-8].

IP-10 mRNA expression: Clenbuterol (0.03mg/kg;i.p.) had no significant effect on IP-10 mRNA expression in cortex compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) significantly increased IP-10 expression ($P<0.05$) and when co-administered with clenbuterol a significant attenuation of LPS-induced IP-10 expression was observed ($P<0.05$). [Figure 5.4.4 c, Newman-Keuls, n=6-8].

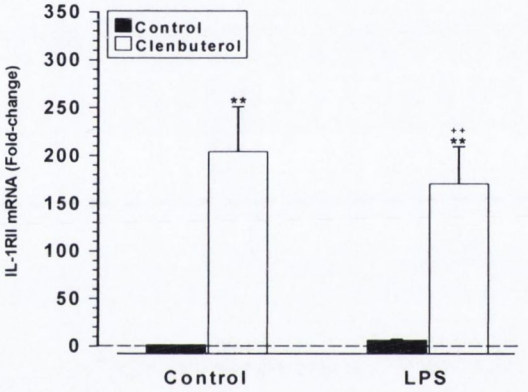
RANTES mRNA expression: Clenbuterol (0.03mg/kg;i.p.) had no significant effect on RANTES mRNA expression in cortex compared to saline controls 4 hr post-injection. LPS (250μg/kg;i.p.) significantly increased RANTES expression ($P<0.05$) and when co-administered with clenbuterol RANTES was no longer increased relative to the clenbuterol control group. [Figure 5.4.4 d, Newman-Keuls, n=6-8].

Locomotor activity: Clenbuterol (0.03mg/kg;i.p.) or LPS (250μg/kg;i.p.) either alone or in combination significantly decreased the time rats were active in the locomotor activity cages ($P<0.01$) compared to saline controls measured 2 hr post-injection. In addition, clenbuterol and LPS both alone and in combination significantly decreased the rearing totals ($P<0.01$), mobile totals ($P<0.01$) and static totals ($P<0.01$) of rats. [Figure 5.4.5 a,b,c,d Dunnett's Procedure, n=6].

a) IL-1β expression



b) IL-1 Type II receptor expression



c) IL-1ra expression

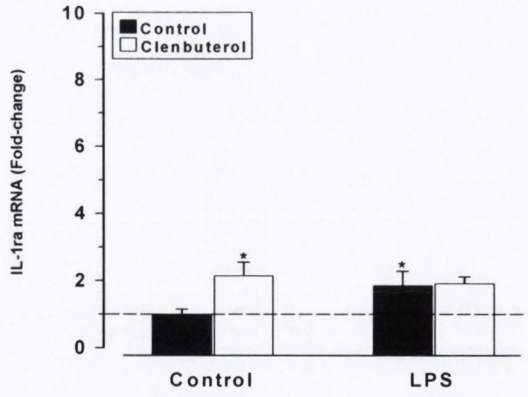


Figure 5.4.1: Low dose clenbuterol increases IL-1β and IL-1 Type II receptor mRNA expression under basal conditions and following systemic LPS administration in rat cortex

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats followed by concurrent i.p. administration of LPS (250μg/kg) or control (0.89% (w/v) saline). a) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on IL-1β mRNA expression [$F_{(1,22)}$=6.1, P=0.02]. b) A two-way ANOVA revealed a significant effect of clenbuterol treatment on IL-1 Type II receptor mRNA expression [$F_{(1,23)}$=45.26, P<0.0001]. c) A two-way ANOVA revealed no significant effect of clenbuterol treatment on IL-1ra mRNA expression. Data are expressed as means with standard error of the mean (n=6-9). **P<0.01, *P<0.05 versus saline control, ++P<0.01 versus LPS control counterpart (Newman-Keuls).

a) IL-6 expression

b) TNF-α expression

c) iNOS expression

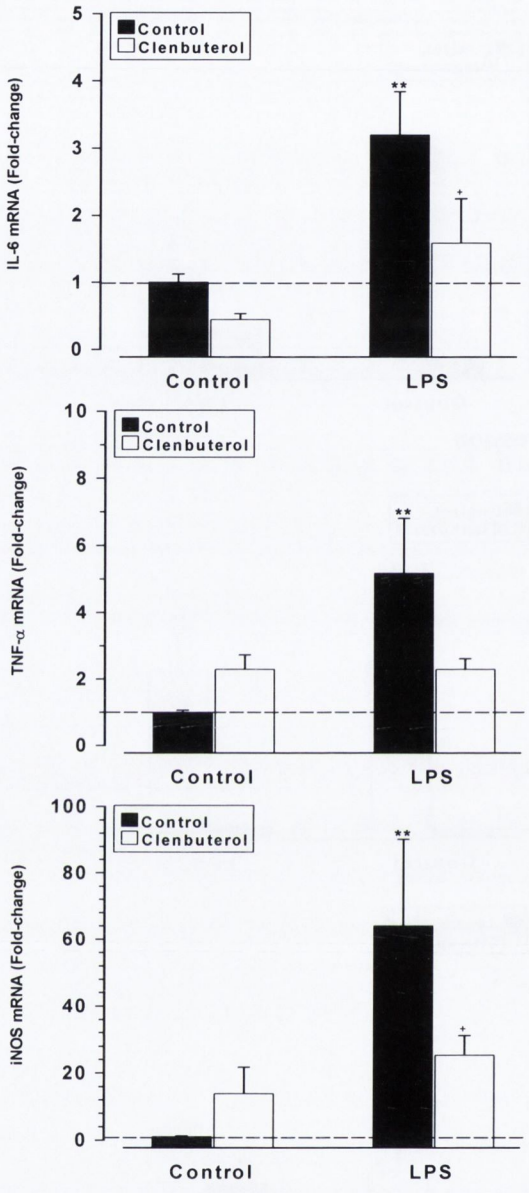
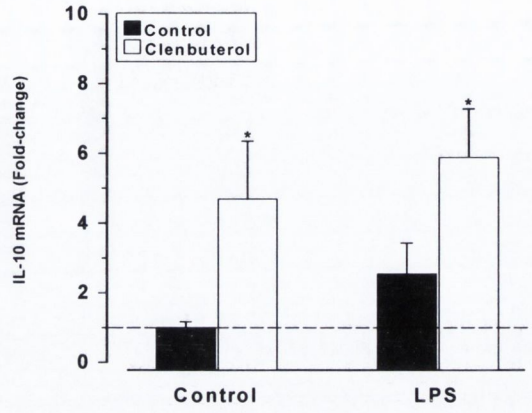


Figure 5.4.2: Low dose clenbuterol suppresses IL-6, TNF-α and iNOS mRNA expression induced by systemic LPS administration in rat cortex

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats followed by concurrent i.p. administration of LPS (250μg/kg) or control (0.89% (w/v) saline). a) A two-way ANOVA revealed a significant effect of clenbuterol and LPS treatment on IL-6 mRNA expression [$F_{(1,21)}$=6.4, P=0.02 & $F_{(1,21)}$=15.13, P=0.0008 respectively]. b) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on TNF-α mRNA expression [$F_{(1,23)}$=7.26, P=0.01]. c) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on iNOS mRNA expression [$F_{(1,23)}$=4.25, P=0.05]. Data are expressed as means with standard error of the mean (*n*=6-9). **P<0.01 versus saline control, ++P<0.01 versus LPS control counterpart (Newman-Keuls).

a) IL-10 expression



b) SOCS3 expression

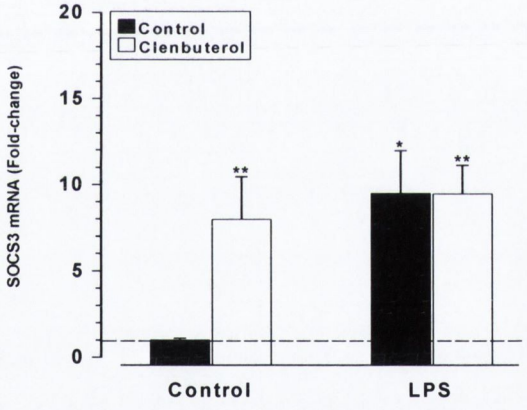
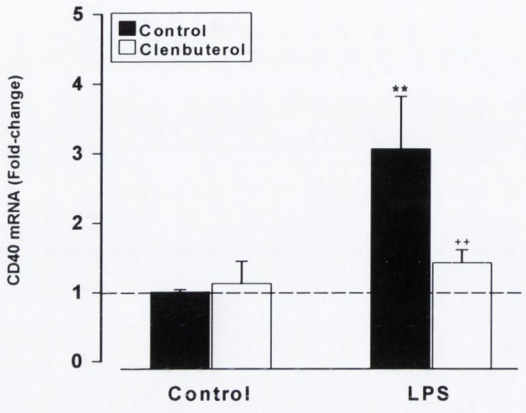


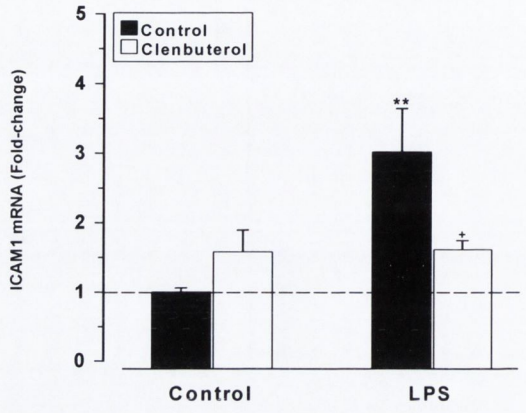
Figure 5.4.3: Low dose clenbuterol increases IL-10 and SOCS3 mRNA expression under basal conditions and following systemic LPS administration in rat cortex

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats followed by concurrent i.p. administration of LPS (250μg/kg) or control (0.89% (w/v) saline). a) A two-way ANOVA revealed a significant effect of clenbuterol treatment on IL-10 mRNA expression [$F_{(1,23)}$=11.18, P=0.003]. b) A two-way ANOVA revealed a significant effect of LPS treatment on SOCS3 mRNA expression [$F_{(1,23)}$=8, P<0.01]. Data are expressed as means with standard error of the mean (*n*=6-8). **P<0.01, *P<0.05 versus saline control (Newman-Keuls).

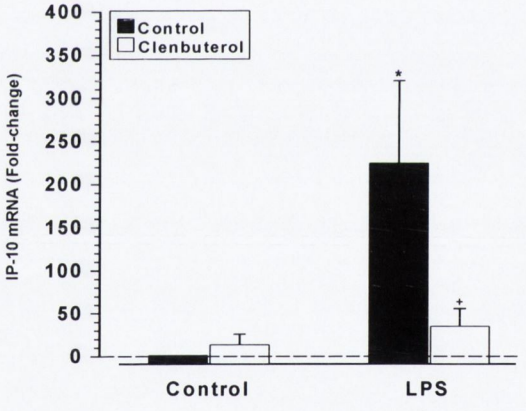
a) CD40 expression



b) ICAM-1 expression



c) IP-10 expression



d) RANTES expression

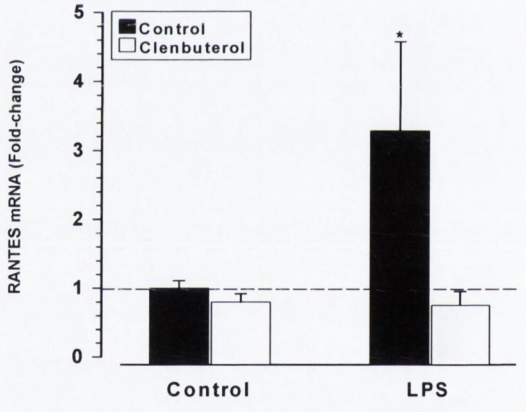


Figure 5.4.4: Low dose clenbuterol suppresses CD40, ICAM-1, IP-10 and RANTES mRNA expression induced by systemic LPS administration in rat cortex

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats followed by concurrent i.p. administration of LPS (250μg/kg) or control (0.89% (w/v) saline). a) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on CD40 mRNA expression [$F_{(1,22)}$=5.1, P=0.03]. b) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on ICAM-1 mRNA expression [$F_{(1,20)}$=7.75, P=0.01]. c) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on IP-10 mRNA expression [$F_{(1,17)}$=4.56, P=0.05]. d) A two-way ANOVA revealed a significant effect of clenbuterol treatment on RANTES mRNA expression [$F_{(1,17)}$=4.68, P=0.05]. Data are expressed as means with standard error of the mean (n=6-8). **P<0.01, *P<0.05 versus saline control, ++P<0.01, +P<0.05 versus LPS control counterpart (Newman-Keuls).

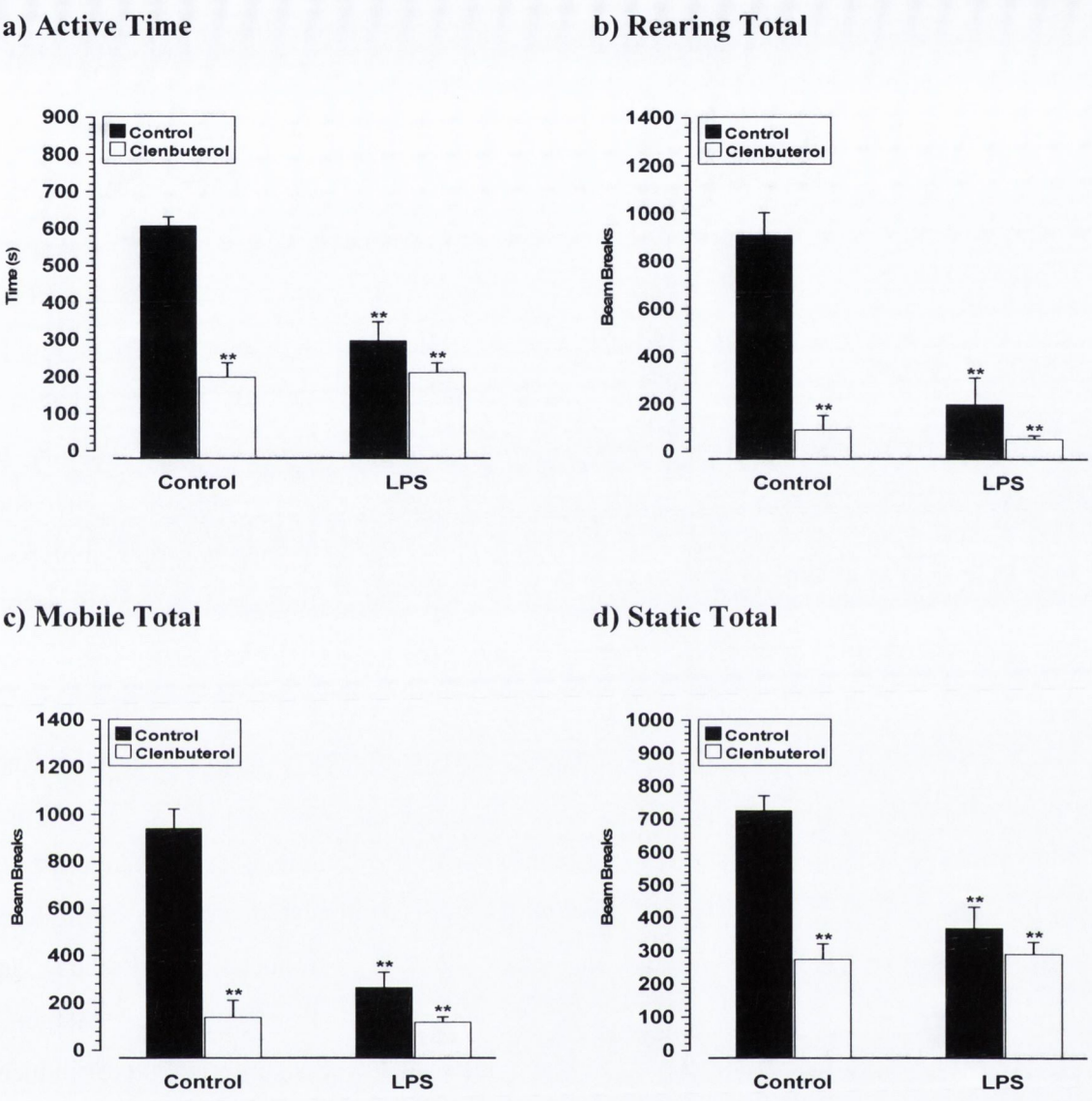


Figure 5.4.5: Both low-dose clenbuterol and LPS reduce the locomotor activity of rats alone and in combination 2 hr post-injection

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats followed by concurrent i.p. administration of LPS (250μg/kg) or control (0.89% (w/v) saline). a) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on active time [$F_{(1,23)}$=21.2, P=0.0001]. b) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on rearing total [$F_{(1,23)}$=15.14, P=0.0007]. c) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on mobile total [$F_{(1,23)}$=20.7, P=0.0001]. d) A two-way ANOVA revealed a significant effect of clenbuterol + LPS treatment on static total [$F_{(1,23)}$=14.13, P=0.001]. Data are expressed as means with standard error of the mean (n=6-9). **P<0.01 versus saline control (Newman-Keuls).

5.5 An investigation into the β-adrenoceptor subtype responsible for mediating the effects of clenbuterol in rat CNS

Studies have shown that *in vitro* the neuroprotective effects of clenbuterol can be achieved through stimulation of either β_1- or β_2-adrenoceptors, however, *in vivo* the effects have been found to be mediated through stimulation of β_2-adrenoceptors (Junker *et al.*, 2002). Additionally, reports have also implied that stimulation of facilitatory pre-synaptic β_2-adrenoceptors can induce a positive-feedback mechanism resulting in the release of noradrenaline from noradrenergic terminals which can then act on post-synaptic β_1-adrenoceptors within the CNS (Murugaiah & O'Donnell, 1995; Zhang *et al.*, 2005). Therefore, the aim of the following study was to determine if the β_1- or β_2-adrenoceptor subtype is responsible for the induction of GFAP, IL-1β, IL-1ra, IL-1 Type II receptor and iNOS mRNA expression by clenbuterol in rat CNS.

GFAP mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased GFAP mRNA expression in cortex ($P<0.01$), hippocampus ($P<0.01$) and hypothalamus ($P<0.01$) compared to saline controls 4 hr post-injection. Pre-treatment with the β_2-adrenoceptor antagonist ICI 118,551 (10mg/kg;i.p.) 30 min prior to clenbuterol significantly attenuated the clenbuterol-induced increase in GFAP expression in cortex ($P<0.01$), hippocampus ($P<0.01$) and hypothalamus ($P<0.05$). In contrast, pre-treatment with the β_1-adrenoceptor antagonist metoprolol (10mg/kg;i.p.) 30 min prior to clenbuterol had no effect on clenbuterol-induced GFAP expression. [Figure 5.5.1 a, b, c, Newman-Keuls, n=5-6].

IL-1β mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased IL-1β mRNA expression in cortex ($P<0.01$), hippocampus ($P<0.01$) and hypothalamus ($P<0.01$) compared to saline controls 4 hr post-injection. Pre-treatment with the β_2-adrenoceptor antagonist ICI 118,551 (10mg/kg;i.p.) 30 min prior to clenbuterol had no significant effect on the clenbuterol-induced increase in IL-1β expression in cortex but significantly attenuated IL-1β expression in hippocampus ($P<0.01$) and hypothalamus ($P<0.05$). In contrast, pre-treatment with the β_1-adrenoceptor antagonist metoprolol (10mg/kg;i.p.) 30 min prior to clenbuterol had no effect on the clenbuterol-induced increase in IL-1β expression. [Figure 5.5.2 a, b, c, Newman-Keuls, n=5-6].

IL-1 Type II receptor mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased IL-1 Type II receptor mRNA expression in cortex ($P<0.01$), hippocampus ($P<0.01$) and hypothalamus ($P<0.01$) compared to saline controls 4 hr post-injection. Pre-treatment with the β_2-adrenoceptor antagonist ICI 118,551 (10mg/kg;i.p.) 30 min prior to clenbuterol significantly attenuated the clenbuterol-induced increase in IL-1 Type II receptor expression in cortex ($P<0.01$), hippocampus ($P<0.01$) and hypothalamus ($P<0.01$). In contrast, pre-treatment with the β_1-adrenoceptor antagonist metoprolol (10mg/kg;i.p.) 30 min prior to clenbuterol had no effect on the clenbuterol-induced increase in IL-1 Type II receptor expression. [Figure 5.5.3 a, b, c, Newman-Keuls, n=5-6].

IL-1ra mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased IL-1ra mRNA expression in cortex ($P<0.05$), hippocampus ($P<0.01$) and hypothalamus ($P<0.01$) compared to saline controls 4 hr post-injection. Pre-treatment with the β_2-adrenoceptor antagonist ICI 118,551 (10mg/kg;i.p.) 30 min prior to clenbuterol attenuated the clenbuterol-induced increase in IL-1ra expression in cortex ($P<0.05$), hippocampus ($P<0.01$) and hypothalamus ($P<0.01$). In contrast, pre-treatment with the β_1-adrenoceptor antagonist metoprolol (10mg/kg;i.p.) 30 min prior to clenbuterol had no effect on the clenbuterol-induced increase in IL-1ra expression in hippocampus or hypothalamus but induced a significant increase in IL-1ra expression in cortex ($P<0.05$) compared to clenbuterol controls. [Figure 5.5.4 a, b, c, Newman-Keuls, n=5-6].

iNOS mRNA expression: Clenbuterol (0.5mg/kg;i.p.) significantly increased iNOS mRNA expression in cortex ($P<0.01$), hippocampus ($P<0.01$) and hypothalamus ($P<0.05$) compared to saline controls 4 hr post-injection. Pre-treatment with the β_2-adrenoceptor antagonist ICI 118,551 (10mg/kg;i.p.) 30 min prior to clenbuterol significantly attenuated the clenbuterol-induced increase in iNOS expression in cortex ($P<0.01$), hippocampus ($P<0.01$) and hypothalamus ($P<0.01$). In contrast, pre-treatment with the β_1-adrenoceptor antagonist metoprolol (10mg/kg;i.p.) 30 min prior to clenbuterol had no effect on the clenbuterol-induced increase in iNOS expression. [Figure 5.5.5 a, b, c, Newman-Keuls, n=5-6].

Locomotor activity: Clenbuterol (0.5mg/kg;i.p.) significantly decreased the time rats were active in the locomotor activity cages ($P<0.01$) compared to saline controls measured 2 hr

post-injection. In addition, clenbuterol significantly decreased the rearing totals ($P<0.01$), mobile totals ($P<0.01$) and static totals ($P<0.01$) of rats. Pre-treatment with the β_2-adrenoceptor antagonist ICI 118,551 (10mg/kg;i.p.) 30 min prior to clenbuterol significantly inhibited the clenbuterol-induced decrease in active time ($P<0.01$), rearing totals ($P<0.01$), mobile totals ($P<0.01$) and static totals ($P<0.01$). In contrast, pre-treatment with the β_1-adrenoceptor antagonist metoprolol (10mg/kg;i.p.) 30 min prior to clenbuterol had no effect on the clenbuterol-induced decrease in active time, rearing totals, mobile totals or static totals. [Figure 5.5.6 a,b,c,d Dunnett's Procedure, n=5-6].

a) Cortex

b) Hippocampus

c) Hypothalamus

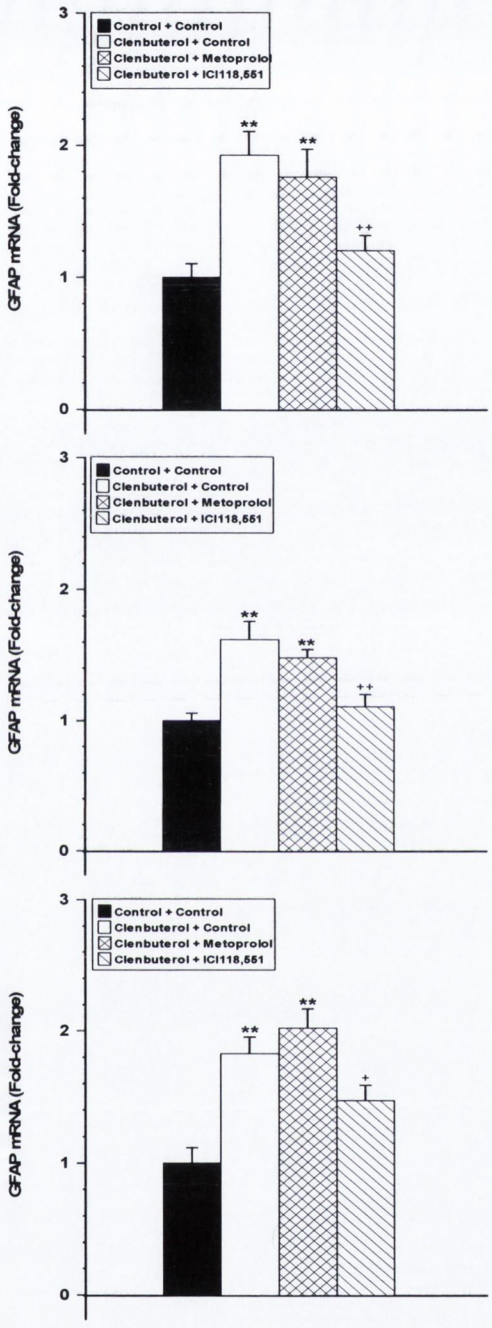


Figure 5.5.1: Clenbuterol-induced increase in GFAP mRNA expression is mediated by β_2-adrenoceptors in rat CNS

Metoprolol (10mg/kg), ICI 118, 551 (10mg/kg) or control (0.89% (w/v) saline) were administered i.p. 30 min prior to i.p. administration of clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and rats were sacrificed 4 hr post-clenbuterol injection. A one-way ANOVA revealed a significant effect of treatment on GFAP mRNA expression in a) cortex [$F_{(3,19)}$=9.93, P=0.0004], b) hippocampus [$F_{(3,19)}$=12.95, P<0.0001] and c) hypothalamus [$F_{(3,19)}$ =10.51 P=0.0003]. Data are expressed as means with standard error of the mean (n=5-6). **P<0.01 versus saline control, ++P<0.01versus clenbuterol control counterparts (Newman-Keuls).

a) Cortex

b) Hippocampus

c) Hypothalamus

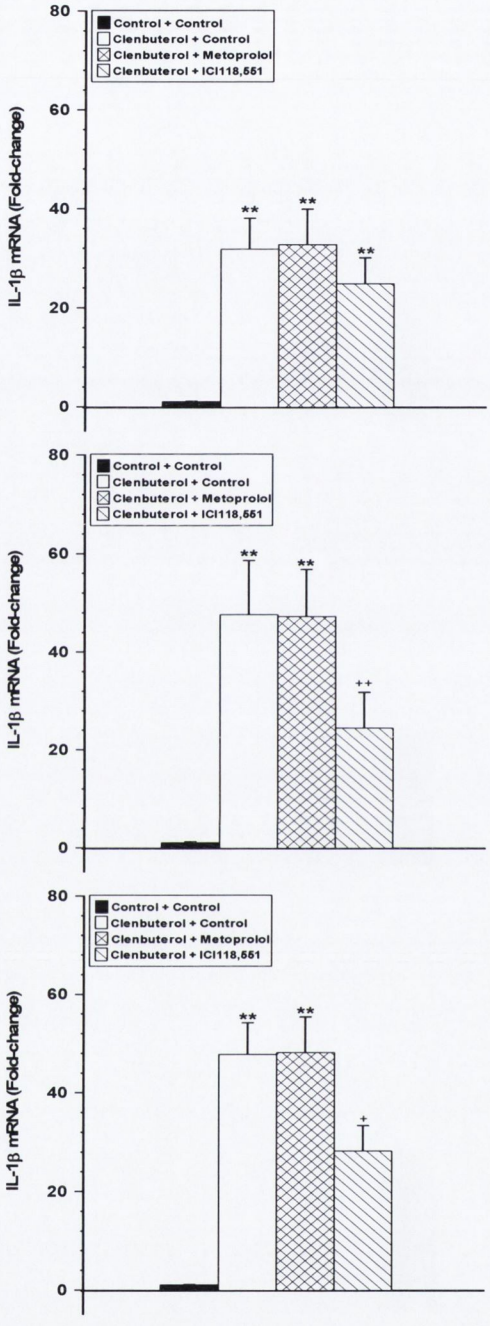


Figure 5.5.2: Clenbuterol-induced increase in IL-1β mRNA expression is partially mediated by β_2-adrenoceptors in rat CNS

Metoprolol (10mg/kg), ICI 118, 551 (10mg/kg) or control (0.89% (w/v) saline) were administered i.p. 30 min prior to i.p. administration of clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and rats were sacrificed 4 hr post-clenbuterol injection. A one-way ANOVA revealed a significant effect of treatment on IL-1β mRNA expression in a) cortex [$F_{(3,19)}$=13.63, P<0.0001], b) hippocampus [$F_{(3,19)}$=13.52, P<0.0001] and c) hypothalamus [$F_{(3,19)}$=29.18, P<0.0001]. Data are expressed as means with standard error of the mean (n=5-6). **P<0.01 versus saline control, ++P<0.01, +P<0.05 versus clenbuterol control counterparts (Newman-Keuls).

a) Cortex

b) Hippocampus

c) Hypothalamus

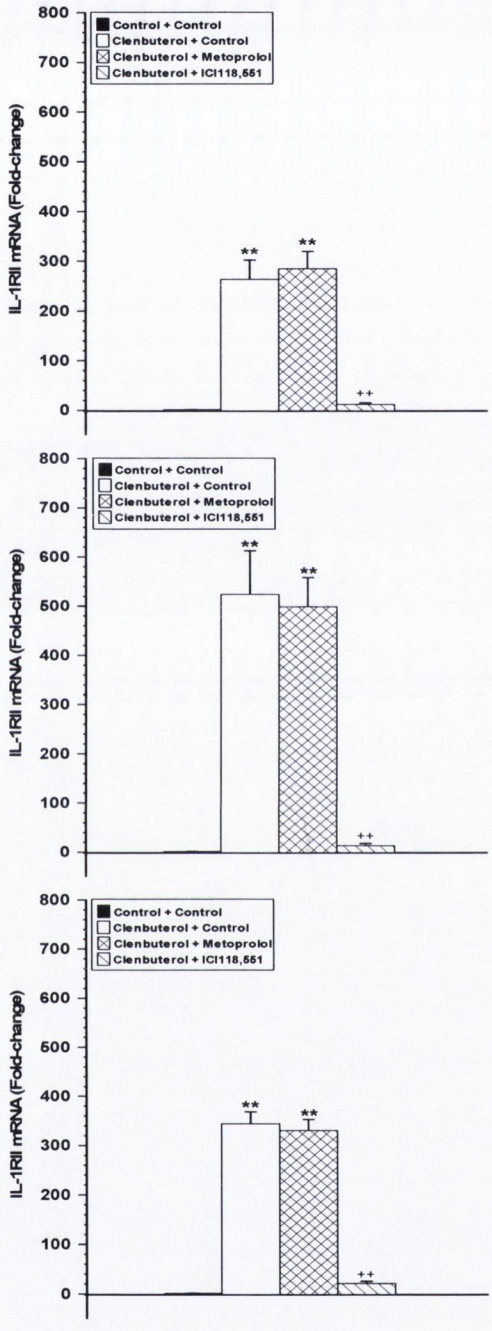


Figure 5.5.3: Clenbuterol-induced increase in IL-1 Type II mRNA expression is mediated by β_2-adrenoceptors in rat CNS

Metoprolol (10mg/kg), ICI 118, 551 (10mg/kg) or control (0.89% (w/v) saline) were administered i.p. 30 min prior to i.p. administration of clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and rats were sacrificed 4 hr post-clenbuterol injection. A one-way ANOVA revealed a significant effect of treatment on IL-1 Type II receptor mRNA expression in a) cortex [$F_{(3,19)}$=40.96, P<0.0001], b) hippocampus [$F_{(3,19)}$=35.76, P<0.0001] and c) hypothalamus [$F_{(3,19)}$=148.53, P<0.0001]. Data are expressed as means with standard error of the mean (n=5-6). **P<0.01 versus saline control, ++P<0.01 versus clenbuterol control counterparts (Newman-Keuls).

a) Cortex

b) Hippocampus

c) Hypothalamus

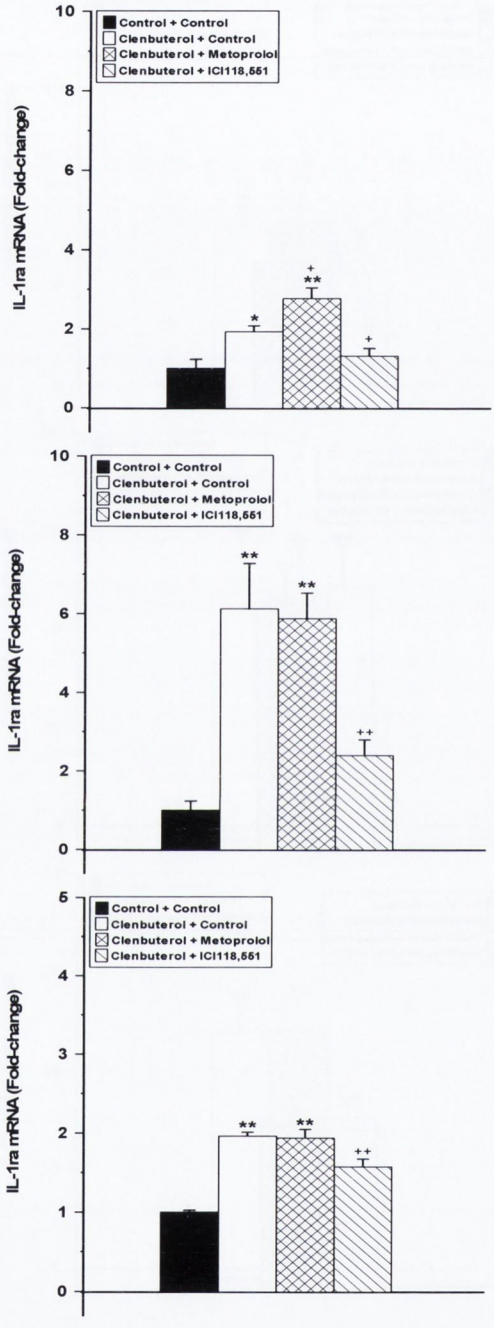
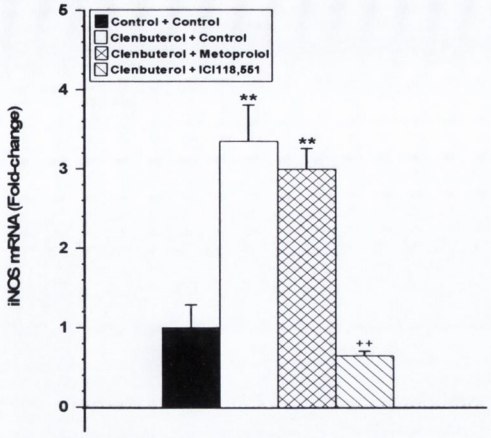


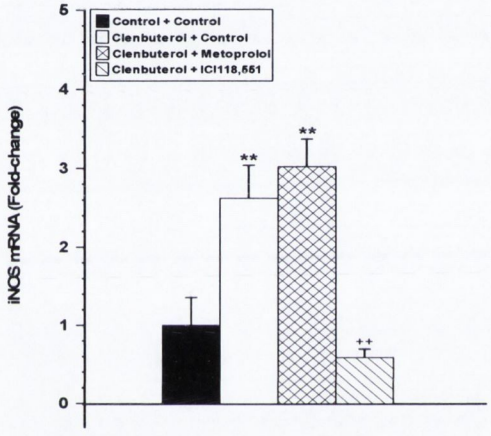
Figure 5.5.4: Clenbuterol-induced increase in IL-1ra mRNA expression is mediated by β_2-adrenoceptors in rat CNS

Metoprolol (10mg/kg), ICI 118, 551 (10mg/kg) or control (0.89% (w/v) saline) were administered i.p. 30 min prior to i.p. administration of clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and rats were sacrificed 4 hr post-clenbuterol injection. A one-way ANOVA revealed a significant effect of treatment on IL-1ra mRNA expression in a) cortex [$F_{(3,19)}$=13.7, P<0.0001], b) hippocampus [$F_{(3,19)}$=12.65, P<0.0001] and c) hypothalamus [$F_{(3,19)}$=44.55, P<0.0001]. Data are expressed as means with standard error of the mean (n=5-6). **P<0.01 versus saline control, ++P<0.01, +P<0.05 versus clenbuterol control counterparts (Newman-Keuls).

a) Cortex



b) Hippocampus



c) Hypothalamus

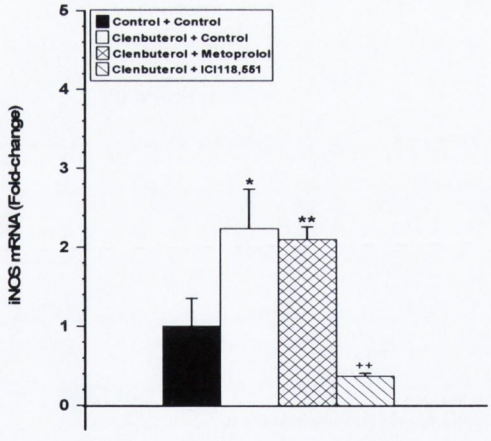


Figure 5.5.5: Clenbuterol-induced increase in iNOS mRNA expression is mediated by β_2-adrenoceptors in rat CNS

Metoprolol (10mg/kg), ICI 118, 551 (10mg/kg) or control (0.89% (w/v) saline) were administered i.p. 30 min prior to i.p. administration of clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and rats were sacrificed 4 hr post-clenbuterol injection. A one-way ANOVA revealed a significant effect of treatment on iNOS mRNA expression in a) cortex [$F_{(3,19)}$=16.81, P<0.0001], b) hippocampus [$F_{(3,19)}$=10, P=0.0004] and c) hypothalamus [$F_{(3,19)}$=7.65, P=0.0015]. Data are expressed as means with standard error of the mean (n=5-6). **P<0.01, *P<0.05 versus saline control, ++P<0.01 versus clenbuterol control counterparts (Newman-Keuls).

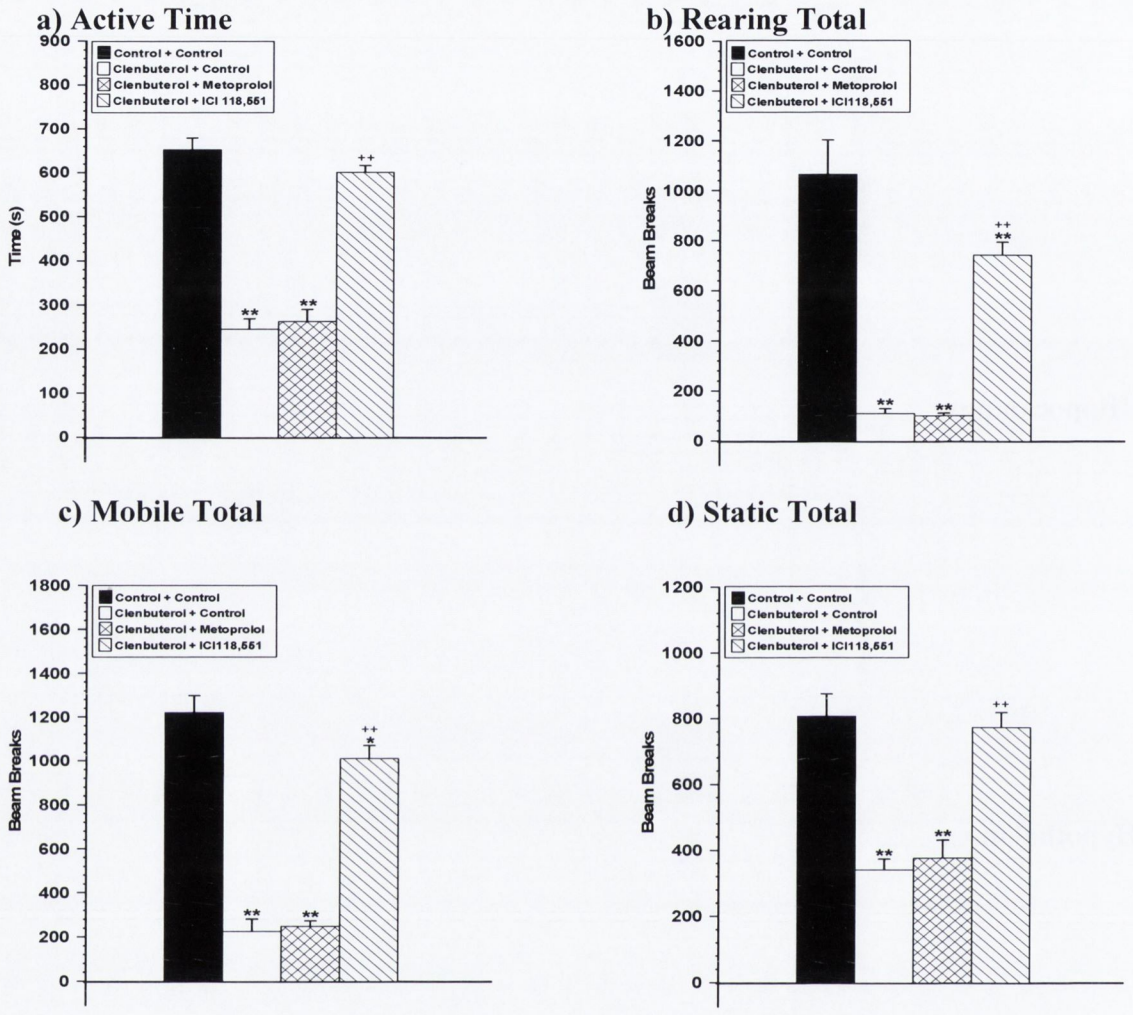


Figure 5.5.6: Clenbuterol-induced reduction in locomotor activity in rats is mediated by β_2-adrenoceptors

Metoprolol (10mg/kg), ICI 118, 551 (10mg/kg) or control (0.89% (w/v) saline) were administered i.p. 30 min prior to i.p. administration of clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline) and locomotor activity of rats was monitored for 15 min 2 hr post-clenbuterol injection. A one-way ANOVA revealed a significant effect of treatment on a) active time [$F_{(3,20)}$=68.12, P<0.0001], b) rearing total [$F_{(3,20)}$=41.27, P<0.0001], c) mobile total [$F_{(3,20)}$=78.87, P<0.0001] and d) static total [$F_{(3,20)}$=23.16, P<0.0001]. Data are expressed as means with standard error of the mean (n=6). **P<0.01, *P<0.05 versus saline control, ++P<0.01 versus clenbuterol control counterparts (Newman-Keuls).

5.6 Discussion

5.6.1 Effects of clenbuterol in the CNS are dose-dependent

Clenbuterol has previously been shown to have a high affinity for β-adrenoceptors both *in vitro* and *in vivo* since it induces a significant displacement of the radioactively labeled β-adrenergic antagonist (-)-[^{125}I]iodopindolol. In addition, clenbuterol is known to be highly lipophilic and thus when administered peripherally it been shown to be capable of binding to central β_2-adrenoceptors with a high affinity (Conway *et al.*, 1987). Thus, peripherally administered clenbuterol has the capacity to provoke a response within the CNS through activation of central β-adrenoceptors. In line with this, the results from the previous chapter (Chapter 4) and those from previous studies in this laboratory (McNamee, 2008) demonstrate that clenbuterol can activate astrocytes and invoke actions on the immune processes of the CNS.

Previous studies report that peripheral administration of the selective β_2-adrenoceptor agonists clenbuterol and formoterol increase the expression of IL-1β, IL-1ra and the IL-1 Type II receptor in rat cortex and hippocampus (McNamee, 2008). Furthermore, the results in the previous chapter demonstrate that clenbuterol can increase IL-1β, IL-1ra and IL-1 Type II receptor expression under basal conditions and following systemic LPS administration. Thus, in keeping with this, the current study demonstrates that acute clenbuterol administration dose-dependently regulates members of the IL-1 family. Clenbuterol is found to significantly increase the mRNA expression of IL-1β, IL-1ra and IL-1 Type II receptor in rat cortex, hippocampus and hypothalamus in a dose-dependent manner. As discussed previously the increased expression of the IL-1 Type II receptor and IL-1ra are noteworthy since both are negative regulators of the IL-1 signalling system. Notably, the two lower doses of 0.003mg/kg and 0.01mg/kg clenbuterol did not impact on the expression of IL-1β or IL-1 Type II receptor in rat CNS while the very lowest dose of 0.003mg/kg showed no effect on IL-1ra expression. It is hypothesized that these lower doses of clenbuterol do not cross the BBB at concentrations capable of inducing an immune response within the CNS but instead are rapidly metabolized by the liver and excreted as previously described by Zalko *et al.*, (1998).

Since previous studies have shown that acute administration of clenbuterol has no effect on the expression of the IL-1 Type I receptor or IL-1 accessory protein in rat CNS, both of which are

imperative for IL-1 signalling (McNamee, 2008), the expression of iNOS was evaluated as a down-stream measure of IL-1 signalling. Clenbuterol induced a dose-dependent increase in the expression of iNOS in rat cortex, hippocampus and hypothalamus following an acute challenge with clenbuterol. The lowest dose of 0.003mg/kg once again showed no effect. This increase in iNOS expression is to some extent in line with previous reports which have suggested that catecholamines can dose-dependently enhance the expression of iNOS in LPS-stimulated macrophages, an effect shown to be mediated by activation of the β_2-adrenoceptor (Chi *et al.*, 2003; Lin *et al.*, 2005). However, there are conflicting reports in the literature about the effects of β-adrenergic stimulation on the activity of iNOS since it has also been demonstrated that the administration of noradrenaline or β-adrenergic agonists can suppress the LPS-induced increase in iNOS and the production of nitrite (Mori *et al.*, 2002; O'Sullivan *et al.*, 2009). Thus iNOS, and ultimately NO production, appears to have a dichotomous role in the CNS. As such, iNOS activity results in the expression of NO which on the one hand can have beneficial effects for the host since it helps to eliminate bacteria, viruses and tumour cells. On the other hand, when the activity of iNOS is sustained over long periods of time it becomes deleterious to the host resulting in the pathogenesis of diseases such as rheumatoid arthritis, septic shock and within the CNS it can induce damage to oligodendrocytes, demyelination and axonal damage (Galea & Feinstein, 1999; De Keyser *et al.*, 2004).

A vast array of literature has shown that the central expression of IL-1β, in particular that in the hypothalamus, is associated with reduced locomotor activity and social exploration in animals (Wieczorak & Dunn, 2006; Bluthé *et al.,* 2000). As already discussed, the results presented here demonstrate that an acute clenbuterol challenge provokes a dose-dependent increase in the expression of IL-1β in all brain regions examined at the higher doses employed (0.03-0.3mg/kg). In addition, an acute challenge with clenbuterol was found to cause a dose-dependent decrease in the total activity of the rats when placed in a novel locomotor cage. Additionally, the other parameters of locomotor activity, namely static, mobile and rearing totals, were all significantly reduced at doses ranging from 0.03mg/kg to 0.3mg/kg 2 hr post-clenbuterol administration. Notably, the two lowest doses of 0.003mg/kg & 0.01mg/kg clenbuterol did not impact on the locomotor activity of rats. Thus it might be hypothesized that the increase in IL-1β expression and is responsible for the reduction in locomotor activity since the lowest doses of clenbuterol (0.003 & 0.01mg/kg) did not induce IL-1β expression

nor did they impact on locomotor activity. However, the relationship between the clenbuterol-induced increase in IL-1β expression and reduction in locomotor activity remains to be determined by further studies in IL-1 Type I receptor knock-out mice.

5.6.2 LPS-induced inflammation can be attenuated by co-administration of low-dose clenbuterol in rat CNS

The results discussed above demonstrate that the lowest dose at which clenbuterol induces a consistent immune response in the CNS is 0.03mg/kg. As previously mentioned, this dose is similar to that used therapeutically in humans (10-20μg) for bronchodilation (Smith, 1998). Thus, since the effects of clenbuterol were evaluated in naive rats until this point it was deemed necessary to examine the impact of this dose of clenbuterol on the neuroinflammation induced by systemic LPS administration. As discussed in the previous chapter, systemic LPS administration induces a robust increase in the expression of pro-inflammatory cytokines and chemokines in rat CNS (Konsman *et al.*, 1999; O'Sullivan *et al.*, 2009).

Clenbuterol was found to significantly increase the expression of IL-10 and SOCS3 both in the presence and absence of LPS. Moreover, in line with the previous studies, low-dose clenbuterol significantly increased the expression of IL-1β, IL-1 Type II receptor and IL-1ra under basal conditions. In the presence of LPS clenbuterol was still found to notably increase the expression of IL-1β and IL-1 Type II receptor, however, IL-1ra expression did not significantly increase above basal levels. With regard to the expression of IL-1β the results presented here are somewhat in contrast to those presented in Chapter 4 where clenbuterol was administered 24 hr post-LPS treatment. From that study it appears that prior exposure to LPS may prime the CNS to produce a more robust increase in clenbuterol-induced IL-1β expression. However, the results from the present study suggest that when both agents are co-administered the presence of LPS may impede the ability of clenbuterol to induce IL-1β expression.

Promisingly, the current study reveals that low-dose clenbuterol can attenuate the pro-inflammatory profile induced by systemic LPS administration when both drugs were given concurrently. In this regard, clenbuterol significantly reduced the LPS-induced mRNA

expression of the pro-inflammatory mediators IL-6 and iNOS and reduced the mRNA expression of the pro-inflammatory cytokine TNF-α. The impact of clenbuterol on LPS-induced TNF-α expression is in line with the result presented in the *in vitro* studies (Chapter 3) where it was found that pre-treatment of primary mixed glial cultures with noradrenaline or the selective β_2-adrenergic agonists salbutamol or formoterol suppressed the expression of LPS-induced TNF-α. The attenuation of LPS-induced TNF-α expression by administration of selective β-adrenergic agonists has been widely reported by other groups. As such, previous studies have demonstrated that treatment with β-adrenergic agonists attenuates the LPS-induced increase in TNF-α, an effect shown to be mediated primarily by β_2-adrenoceptor stimulation (Elenkov *et al.*, 1995; Kuroki *et al.*, 2004). In addition one group also reported that increasing the amount of noradrenaline available in the synapse, through the use of selective α_2-adrenergic agonists, blunted the expression of TNF-α elicited by LPS administration (Elenkov *et al.*, 1995). Furthermore, the effect of isoproterenol on LPS-induced TNF-α and IL-6 expression *in vitro* was shown to be due to β-adrenergic regulation of the promoter activity of TNF-α and IL-6 (Nakamura *et al.*, 1998).

Notably co-administration of clenbuterol with LPS was found to reduce the LPS-induced expression of the co-stimulatory molecules CD40, the integrin ICAM-1 and the chemokines IP-10 and RANTES in rat cortex. This is noteworthy since integrins are known to play a crucial role in leukocyte extravasation and the activation and proliferation of T-cells in the CNS and not surprisingly they are found to be overexpressed in inflammatory diseases of the CNS such as MS and AIDS-related dementia and EAE (Ballestas & Benveniste, 1997; Campbell *et al.*, 2002). It has previously been shown that administration of db-cAMP, noradrenaline or forskolin do not induce the expression of ICAM-1 in astrocytes *in vitro*, however, when these agents were administered to cells which were subsequently exposed to TNF-α or IL-1β a significant increase in ICAM-1 was observed at both the mRNA and protein levels, an event mediated by the induction of intracellular cAMP (Ballestas & Benveniste, 1997). Additionally, β_2-adrenoceptor agonists, salbutamol and terbutaline, have been shown to inhibit the expression of the co-stimulatory molecules ICAM-1 and CD40 in monocytes, an effect again found to be mediated by the β_2-adrenoceptor (Kuroki *et al.*, 2004). Significantly, reports have also suggested that enhanced intracellular cAMP accumulation and the increased expression of IL-10 which ensues suppresses ICAM-1 expression in astrocytes (Ballestas &

Benveniste, 1997). Encouragingly the impact of clenbuterol on LPS-induced neuroinflammation in rat CNS reflects that of previous work from this laboratory which demonstrated that *in vitro* administration of noradrenaline reduces the LPS-induced expression of chemokines in glia and increasing the availability of noradrenaline in the synapse *in vivo* through the use of noradrenaline reuptake inhibitors or α_2-adrenoceptor antagonists suppresses the LPS-induced expression of chemokines and co-stimulatory molecules in the CNS (O'Sullivan, 2007).

The results of this study also demonstrate that both clenbuterol and LPS reduce locomotor activity of rats to a similar extent. In this regard both clenbuterol and LPS reduced the total activity of rats in all parameters measured to approximately the same degree when rats were placed in novel locomotor activity cages. Notably, no synergistic effect was observed on locomotor activity when LPS and clenbuterol were co-administered.

5.6.3 Effects of clenbuterol are mediated via stimulation of β_2-adrenoceptors in rat CNS

The results presented so far have shown that peripheral administration of clenbuterol elicits an immune response in a dose-dependent manner in the rat CNS and when co-administered with LPS clenbuterol has the ability to reduce the LPS-induced expression of pro-inflammatory mediators. The effects of clenbuterol on astrocytic activation and cytokine expression have previously been shown to occur due to stimulation of β-adrenergic receptors since the effects could be blocked by administration of the non-selective β-adrenergic antagonist propranolol (McNamee, 2008). However, this previous study did not delineate between effects induced by the varying subtypes of β-adrenoceptor present in the brain, specifically whether the effects of clenbuterol were elicited by the β_1- or β_2-adrenoceptor subtypes. Therefore, the present study aimed to determine whether the β_1- or β_2-adrenoceptor subtype is primarily responsible for the effects of clenbuterol through the use of the selective β_1-adrenoceptor antagonist metoprolol and the selective β_2-adrenoceptor antagonists ICI 118,551.

The results presented here reveal that the increase in GFAP mRNA expression and hence astrocytic activation observed following peripheral clenbuterol administration is mediated by stimulation β_2-adrenoceptors since the β_2-adrenoceptor antagonist ICI 118,551 impeded the

expression of GFAP in rat cortex, hippocampus and hypothalamus. In contrast, the β_1-adrenoceptor antagonist metoprolol did not impact on GFAP expression in any of the regions examined. These results are in line with previous *in vitro* studies which report that clenbuterol induces changes in astrocytic morphology through a β_2-adrenoceptor mediated pathway (Junker *et al.*, 2002).

In addition, the results presented here show that the clenbuterol-induced increase in IL-1β, IL-1ra and the IL-1 Type II receptor expression is mediated by β_2-adrenoceptors since expression could be blocked by ICI 118,551 but not metoprolol. However, clenbuterol-induced IL-1β expression was not found to be significantly altered by ICI 118,551 or metoprolol in the cortex. Since previous studies have shown that propranolol can block clenbuterol-induced IL-1β expression (McNamee, 2008) it might be suggested that an interplay between actions both β-adrenoceptor subtypes is responsible for the clenbuterol-induced increase in IL-1β expression in the cortex. Surprisingly, blockade of β_1-adrenoceptors with metoprolol augmented the clenbuterol-induced increase in IL-1ra in the cortex. Furthermore, the results show that the clenbuterol-induced increase in iNOS expression is mediated by the β_2-adrenoceptor since administration of ICI 118,551 but not metoprolol blocked its expression.

In addition to the role in mediating the effects of clenbuterol on immune processes in the CNS the results presented here imply that activation of β_2-adrenoceptors is responsible for the clenbuterol-induced reduction in locomotor activity of rats. In line with the earlier studies peripheral administration of clenbuterol induced a significant reduction in all parameters of locomotor activity measured. However, pre-treatment of rats with β_2-adrenoceptor antagonist ICI 118,551 prior to clenbuterol blocked the effects on locomotor activity and thus the activity of these rats was shown to be similar to that of the controls. However, pre-treatment with the β_1-adrenoceptor antagonist metoprolol was found to have no impact on the clenbuterol-induced reduction in locomotor activity.

Overall, these results are not surprising since β_2-adrenoceptors are known to be responsible for the increase in intracellular cAMP accumulation observed in the rat CNS in response to clenbuterol (Ordway *et al.*, 1987). Furthermore, the neuroprotective effects of clenbuterol

have already been shown in the literature to be mediated by the β_2-adrenoceptor (Junker *et al.*, 2002; Culmsee *et al.*, 2007).

5.6.4 Summary

In conclusion, the results presented here show that peripheral administration of clenbuterol induces a dose-dependent increase in the expression of cytokines in rat CNS with similar effects being observed in all three brain regions examined, namely the cortex, hippocampus and hypothalamus. Not surprisingly, in line with previous reports, these effects were found to be largely mediated via stimulation of central β_2-adrenoceptors and not β_1-adrenoceptors. Furthermore, the lowest dose at which clenbuterol was shown to induce a consistent immune response in the CNS was a dose of 0.03mg/kg;i.p., a dose which is similar to that used therapeutically in humans. In addition administration of this low-dose of clenbuterol was demonstrated to elicit anti-inflammatory effects in rat cortex following systemic administration of the endotoxin LPS. In spite of the grand anti-inflammatory profile induced by clenbuterol it was also shown to induce a significant increase in IL-1β expression in the CNS, an effect which corresponds with reduced locomotor activity of rats. Thus, for this reason the peripheral effects of clenbuterol warrant further investigation as shall be discussed in the following chapter (Chapter 6).

Chapter 6

Examination of the peripheral effects of clenbuterol in rats; comparison with LPS

6.1 Introduction

The results from the previous chapter demonstrate that acute administration of clenbuterol increases the expression of IL-1β in the rat CNS, an effect found to correspond with a decrease in locomotor activity. IL-1 is a potent neuromodulator which has been consistently shown to play a role in the behavioural response of animals to injury or infection, a response which has come to be known as sickness behaviour. First classified by Benjamin Hart in 1988 sickness behaviour is the stereotypical response to injury or infection and occurs due to alterations in the normal homeostasis of an animal. This highly adaptive motivational state is classically associated with fever, hypophagia, hypersomnia, cachexia, hyperalgesia and anhedonia; behavioural alterations which are found to be associated with the increased actions of cytokines on the brain, in particular that of IL-1β, TNF-α and IL-6 (see Hart, 1988; Dantzer, 2001; Dantzer *et al.,* 2008). In line with this, the systemic administration of the endotoxin LPS is known to initiate an acute phase response triggering the release of IL-1, TNF-α and IL-6 from peripheral macrophages, an event which culminates in the induction of behavioural depression which aims to restore homeostasis in the animal (Beishuizen & Thijs, 2003). The aforementioned cytokines (IL-1β, TNF-α & IL-6) are known to be pyrogenic in nature and have been suggested to play a role in the febrile response through their actions at receptors in the CNS, in particular those within the hypothalamus, resulting in a decreased firing-rate of warm-sensitive neurons and a concomitant increase in the firing rate of cold-sensitive neurons (Rothwell & Luheshi, 2000; Tizard, 2008). Furthermore, the LPS-induced expression of IL-1β, TNF-α and IL-6 in the hypothalamus has been shown to be associated with a reduction in food-motivated behaviour (Layé *et al.,* 2000).

The increased expression and release of prostaglandins, in particular prostaglandin E_2, has also been found to play a role in the behavioural response to endotoxin. Prostaglandins are produced from the actions of enzymes known as prostaglandin synthases. In this regard, the prostaglandin synthase COX-II converts arachidonic acid to prostaglandin E_2. While COX-II is found to be constitutively expressed in the CNS its expression is rapidly upregulated in response to LPS administration (Cao *et al.*, 1995; Quan *et al.*, 1998). Moreover, COX-II has also been shown to mediate in part the febrile response since the rise in core body-temperature observed following peripheral administration of LPS or IL-1 can be blocked by the

administration of selective COX-II inhibitors (Cao *et al.*, 1995; Wieczorek & Dunn, 2006). Thus the following studies were undertaken in order to determine if administration of clenbuterol induces a neuroinflammatory response similar to that induced by systemic administration of the endotoxin LPS and to assess further its impact on the behaviour of rats.

β-adrenoceptors are found to be widely expressed in the periphery. The β_1-adrenoceptor subtype is known to predominate in the cardiac system and outnumbers the β_2-adrenoceptor by approximately 4:1 in the ventricles and 2:1 in the atria (Wallukat, 2002; Brodde *et al.*, 2006). Stimulation of cardiac β-adrenoceptors regulates heart rate by increasing the contractility and accelerating the relaxation of the heart, an effect found to be dependent on G_i coupling (Xiang & Kobilka, 2003; Zheng *et al.*, 2004). As clenbuterol is known to act on β_2-adrenoceptors, the capacity of clenbuterol to alter heart rate was examined in the following studies, since it is possible that a non-specific stress response induced by clenbuterol may account for its ability to reduce locomotor activity. LPS was used as a comparator since its administration has been previously demonstrated to increase heart rate, an effect brought about by increases in plasma concentrations of noradrenaline and adrenaline (Zhou & Jones, 1993).

The technique employed for the purposes of these studies was that of telemetry which utilizes PDT 4000HR E-Mitters (Mini Mitter, OR, USA) in order to acquire data for heart rate, core body-temperature and locomotor activity. This system utilizes battery-free radiotelemetry devices which are implanted into the peritoneal cavity of the rat. The technique allows for measurement of behavioural and physiological responses in rats and curtails a need for human contact therefore minimizing stress to the animal. In addition, this method also allows for concurrent measurement of both physiological and behavioural responses in the animal without distortion to any of the parameters under investigation (Harkin *et al.*, 2002).

Thus, the objectives of the following studies were to:

1) Compare the effect of LPS and clenbuterol on the mRNA expression of pro-inflammatory cytokines and COX-II in rat hypothalamus.

2) Compare the effect of LPS and clenbuterol on food intake, water consumption, body-weight, locomotor activity, body-temperature and heart rate in rats.

3) Determine the ability of the β_1-adrenoceptor antagonist metoprolol and the β_2-adrenceptor antagonist ICI 118,551 to block the effects of clenbuterol on food intake, water consumption and heart rate in rats.

4) Determine the impact of a low dose of clenbuterol (0.03mg/kg) on heart rate in rats in a bid to dissociate the negative effect of clenbuterol on heart rate from the beneficial effects induced by clenbuterol observed in the CNS.

6.2 Methods

6.2.1 Subjects and drug treatments

Male Sprague-Dawley rats (340-400g) were singly housed under a 12 hr light-dark cycle (lights on 08:00) in a temperature-controlled room (22 ± 2 °C) with food and water available *ad libitum* for the duration of the experiment. Rats were implanted with HR E-Mitters as outlined in the methods section (Section 2.3, Chapter 2). Clenbuterol and LPS were dissolved in 0.89% (w/v) saline and were administered i.p. or s.c. at doses of 0.003, 0.01, 0.03 or 0.5mg/kg and 250µg/kg respectively. Metoprolol and ICI 118,551 were dissolved in 0.89% (w/v) saline with 0.2% (v/v) Tween-20 and were administered s.c. at a dose of 10mg/kg. Control rats received an injection of 0.89% (w/v) saline alone.

6.2.2 Experimental Procedure

The design for this set of experiments was as outlined in Figures 6.1 to 6.3 below. Food, water and body-weights were measured as outlined in the methods section (Section 2.3, Chapter 2). Heart rate, core body-temperature and locomotor activity were measured and analysed using the VitalView™ system as outlined under the telemetry section of the methods section (Section 2.3, Chapter 2). Rats were allowed at least one week of wash-out prior to additional challenge. Preparation of tissue for cDNA synthesis and RT-PCR was carried out as outlined in the methods section (Section 2.3 & 2.4, Chapter 2).

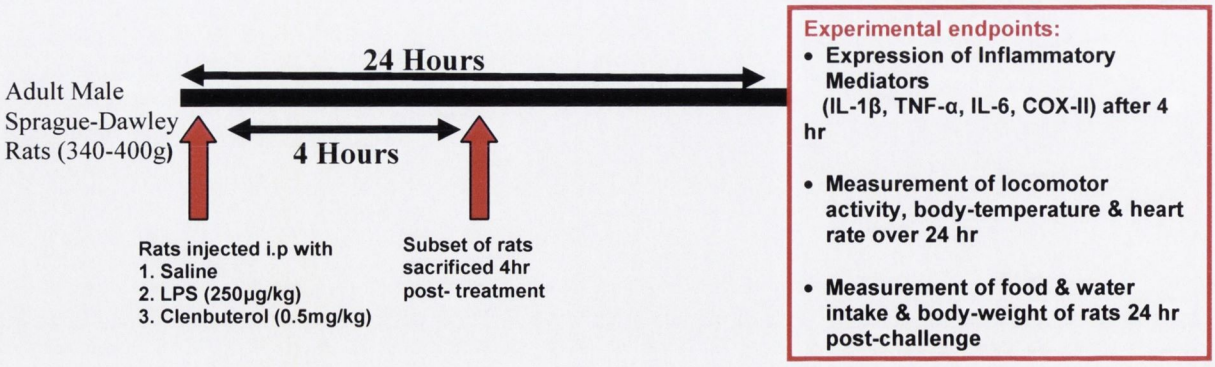


Figure 6.1: Experimental design for comparing the neuroinflammatory & peripheral effects of clenbuterol to those induced by LPS

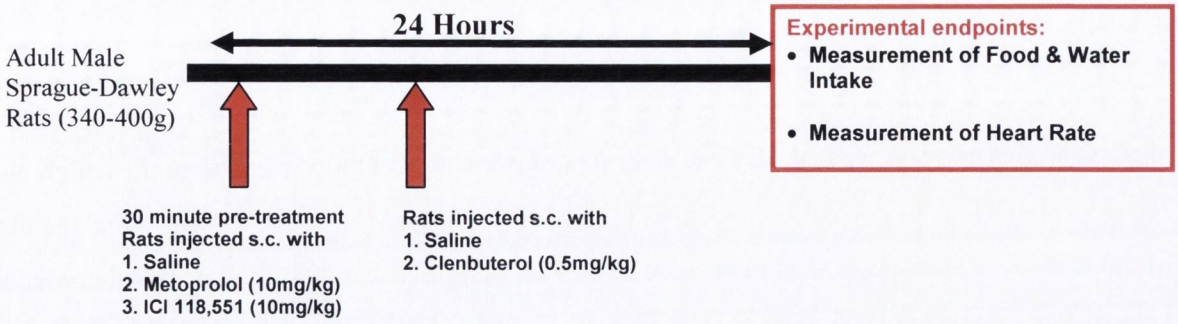


Figure 6.2: Experimental design for determining β-adrenoceptor subtype responsible for inducing the effect of clenbuterol on food-motivated behaviour and heart rate

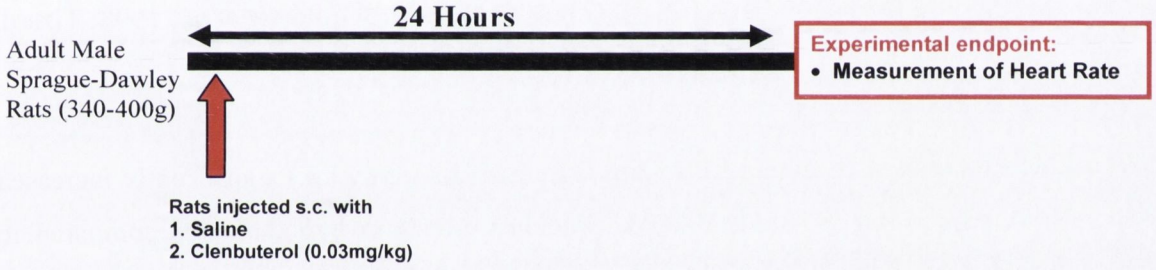


Figure 6.3: Experimental design for determining the impact of low-dose clenbuterol on heart rate

6.3 Effect of Clenbuterol and LPS on the induction of a pro-inflammatory response in rat hypothalamus 4 hr post-administration

As already discussed, "classical sickness behaviour" is associated with the actions of the cytokines IL-1β, TNF-α, IL-6 and the prostaglandin synthase COX-II in the brain which can provoke alterations in normal homeostasis (Dantzer, 2001; Dantzer *et al.,* 2008). Thus, the aim of the following study was to compare the ability of clenbuterol and the classical inflammagen LPS to induce the expression of IL-1β, TNF-α, IL-6 and COX-II in the rat hypothalamus. In addition, since the results of the previous chapter revealed that both LPS and clenbuterol reduce the locomotor activity of rats, the physiological mechanisms responsible for this, namely effects on feeding, body-weight, cardiac function and body-temperature, were examined in greater detail in this study, as these are all known to be implicated in the behavioural response to endotoxin and IL-1β (Zhou & Jones, 1993; Porter *et al.*, 1998; Tizard, 2008)

IL-1β mRNA expression: Systemic LPS administration (250μg/kg;i.p.) significantly increased IL-1β mRNA expression (~9-fold; $P<0.05$, Student's *t*-test) in hypothalamus compared to saline controls 4 hr post-injection. However, administration of clenbuterol (0.5mg/kg;i.p.) induced a more robust increase in IL-1β mRNA expression (~25-fold; $P<0.01$) than LPS. [Figure 6.3.1 a, Newman-Keuls, n=6-7].

TNF-α mRNA expression: Systemic LPS administration (250μg/kg;i.p.) induced a robust increase in TNF-α mRNA expression (~4-fold; $P<0.01$) in hypothalamus compared to saline controls 4 hr post-injection. In contrast, clenbuterol (0.5mg/kg;i.p.) failed to alter TNF-α mRNA expression. [Figure 6.3.1 b, Newman-Keuls, n=6-7].

IL-6 mRNA expression: Systemic LPS administration (250μg/kg;i.p.) induced a robust increase in IL-6 mRNA expression (~6-fold; $P<0.01$) in hypothalamus compared to saline controls 4 hr post-injection. In contrast, clenbuterol (0.5mg/kg;i.p.) failed to alter IL-6 mRNA expression. [Figure 6.3.1 c, Newman-Keuls, n=6-7].

COX-II mRNA expression: Systemic LPS administration of LPS (250μg/kg:i.p.) induced a robust increase in COX-II mRNA expression (~6-fold; $P<0.01$) in hypothalamus compared to saline controls 4 hr post-injection. In contrast, clenbuterol (0.5mg/kg;i.p.) failed to alter COX-II mRNA expression. [Figure 6.3.1 d, Newman-Keuls, n=6-7].

6.3.2 Comparison of the impact of clenbuterol and LPS on heart rate, body temperature and behaviour in rats

Food intake: Systemic LPS administration (250μg/kg;s.c.) significantly reduced food intake ($P<0.01$) of rats compared to saline controls over a 24 hr period. Comparably, clenbuterol (0.5mg/kg;s.c.) also significantly reduced food intake of rats compared to saline controls ($P<0.01$) and compared to the LPS group ($P<0.01$). [Figure 6.3.2 a, Newman-Keuls, n=6].

Water intake: Systemic LPS administration (250μg/kg;s.c.) significantly reduced water intake ($P<0.05$) of rats compared to saline controls over a 24 hr period. Comparably, clenbuterol (0.5mg/kg;s.c.) also significantly reduced water intake ($P<0.01$) of rats compared to saline controls. [Figure 6.3.2 b, Newman-Keuls, n=6].

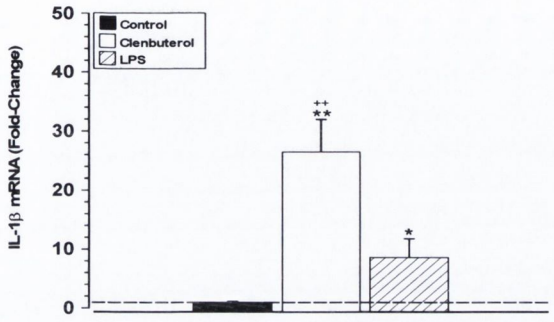
Body-weight: Systemic LPS administration (250μg/kg;s.c.) significantly reduced body-weight ($P<0.01$) of rats compared to saline controls over a 24 hr period. In contrast, clenbuterol (0.5mg/kg;s.c.) did not significantly alter the body-weight of rats compared to saline controls. [Figure 6.3.2 c, Newman-Keuls, n=6].

Locomotor activity: Systemic LPS administration (250μg/kg;s.c.) reduced the locomotor activity of rats, an effect which persisted for approximately 12 hr post-injection. When locomotor activity was pooled for the 12 hr lights-out period LPS significantly reduced locomotor activity ($P<0.01$) compared to saline controls. Similarly, administration of clenbuterol (0.5mg/kg;s.c.) reduced locomotor activity, an effect which persisted for approximately 12 hr post-injection. When locomotor activity was pooled for the 12 hr lights-out period clenbuterol significantly reduced locomotor activity ($P<0.01$) compared to saline controls. [Figure 6.3.3 a, b, Newman-Keuls, n=6].

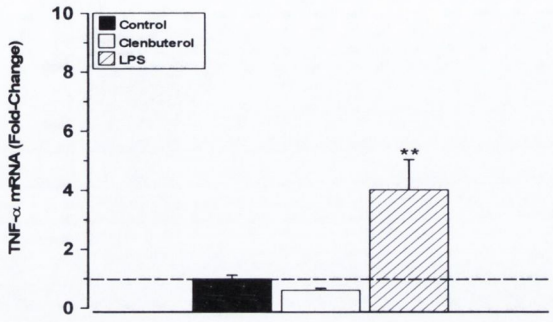
Body-temperature: Systemic LPS administration (250µg/kg;s.c.) induced a febrile response in rats which persisted for approximately 10 hr post-injection. When temperature data was pooled for the 12 hr lights-out period, LPS was shown to significantly increase body-temperature ($P<0.01$) compared to saline controls. In contrast, administration of clenbuterol (0.5mg/kg;s.c.) reduced body-temperature which persisted for approximately 11 hr post-injection. When temperature data was pooled for the 12 hr lights-out period, clenbuterol significantly decreased body-temperature ($P<0.01$) compared to saline controls. [Figure 6.3.4 a, b, Newman-Keuls, $n=6$].

Heart rate: Systemic LPS administration (250µg/kg;s.c.) increased the heart rate of rats, an effect which persisted for approximately 24 hr post-injection. When the heart rate data was pooled for the 12 hr lights-out period LPS significantly increased heart rate ($P<0.01$) compared to saline controls. Similarly, administration of clenbuterol (0.5mg/kg;s.c.) also increased the heart rate, an effect which again persisted for approximately 24 hr post-injection, however this was found to be of a much greater magnitude than that observed for LPS. When the heart rate data was pooled for the 12 hr lights-out period clenbuterol significantly increased heart rate compared to saline controls ($P<0.01$) and also compared to the LPS group ($P<0.01$). When the heart rate data was pooled for the 12 hr lights-on period clenbuterol significantly increased heart rate compared to saline controls ($P<0.01$) and also compared to the LPS group ($P<0.01$). [Figure 6.3.5 a, b, Newman-Keuls, $n=6$].

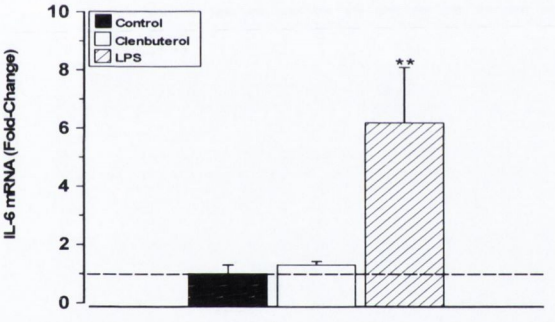
a) IL-1β mRNA expression



b) TNF-α mRNA expression



c) IL-6 mRNA expression



d) COX-II mRNA expression

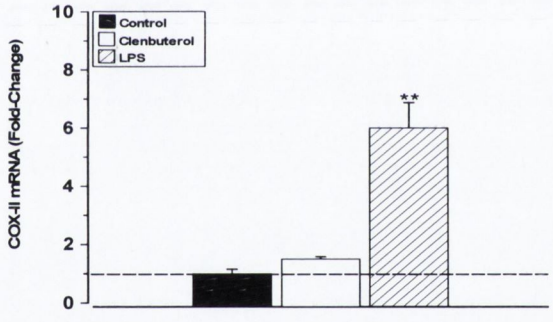
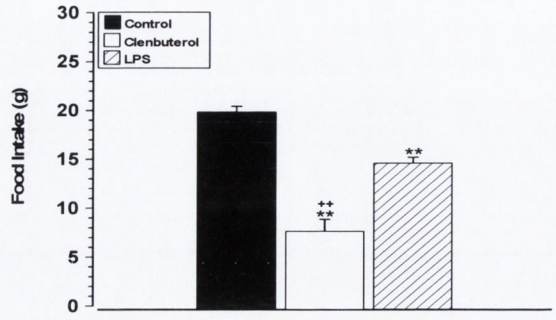


Figure 6.3.1: Systemic LPS administration induces IL-1β, TNF-α, IL-6 and COX-II mRNA expression while clenbuterol selectively induces IL-1β mRNA expression in rat hypothalamus

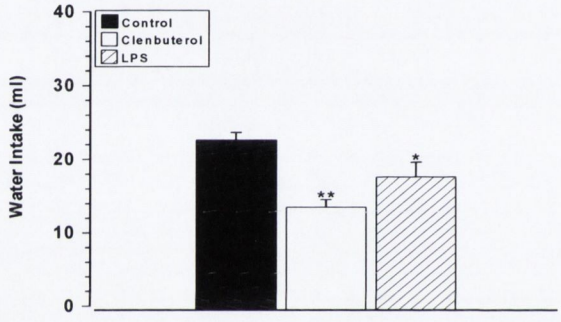
Clenbuterol (0.5mg/kg), LPS (250μg/kg) or control (0.89% (w/v) saline) were administered i.p. and rats were subsequently sacrificed 4 hr post-injection. a) A one-way ANOVA revealed a significant effect of treatment on IL-1β mRNA expression [$F_{(2,17)}$=12.79, P<0.0004]. b) A one-way ANOVA revealed a significant effect of treatment on TNF-α mRNA expression [$F_{(2,17)}$=11.54, P<0.0007]. c) A one-way ANOVA revealed a significant effect of treatment on IL-6 mRNA expression [$F_{(2,17)}$=7.95, P<0.0036]. d) A one-way ANOVA revealed a significant effect of treatment on COX-II mRNA expression [$F_{(2,17)}$=33.72, P<0.0001]. Data are expressed as means with standard error of the mean (n=6-7). **P<0.01 versus saline control, ++P<0.01 versus LPS group (Newman-Keuls).

In order to examine the induction of IL-1β expression by LPS, the control and LPS groups were isolated and analysed using a Student's t-test. This was necessary since the robust clenbuterol-induced increase in IL-1β expression precluded the detection of differences between the control and LPS groups.

a) Food Intake



b) Water Intake



c) Body-Weight

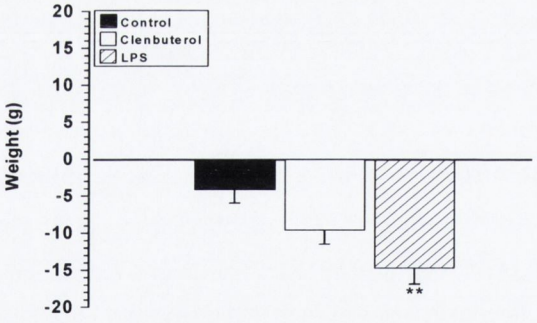
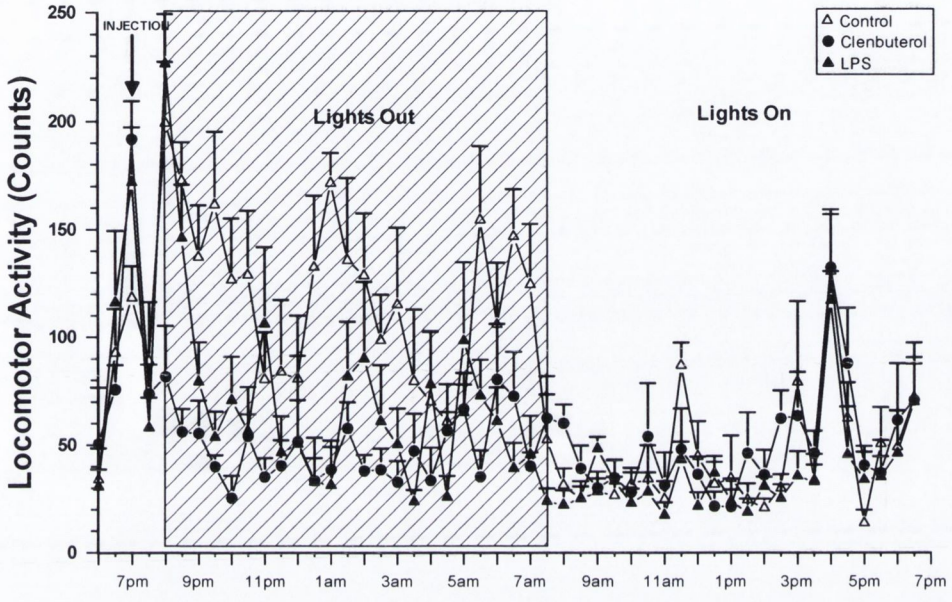


Figure 6.3.2: Systemic administration of LPS or clenbuterol reduces food and water intake and induces weight-loss in rats

Clenbuterol (0.5mg/kg), LPS (250µg/kg) or control (0.89% (w/v) saline) were administered s.c. to rats and food intake, water intake and body-weight were measured over a 24 hr period. a) A one-way ANOVA revealed a significant effect of treatment on food intake [$F_{(2,15)}$=51.36, P<0.0001]. b) A one-way ANOVA revealed a significant effect of treatment on water intake [$F_{(2,15)}$=9.79, P=0.0019]. c) A one-way ANOVA revealed a significant effect of treatment on body-weight [$F_{(2,15)}$=7.58, P=0.0053]. Data are expressed as means with standard error of the mean (n=6). **P<0.01, *P<0.05 versus saline control, ++P<0.01 versus LPS group (Newman-Keuls).

a)



b)

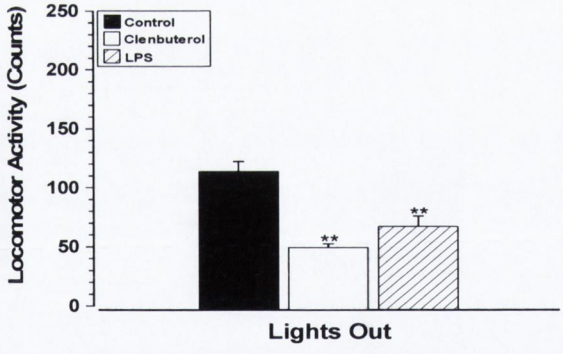
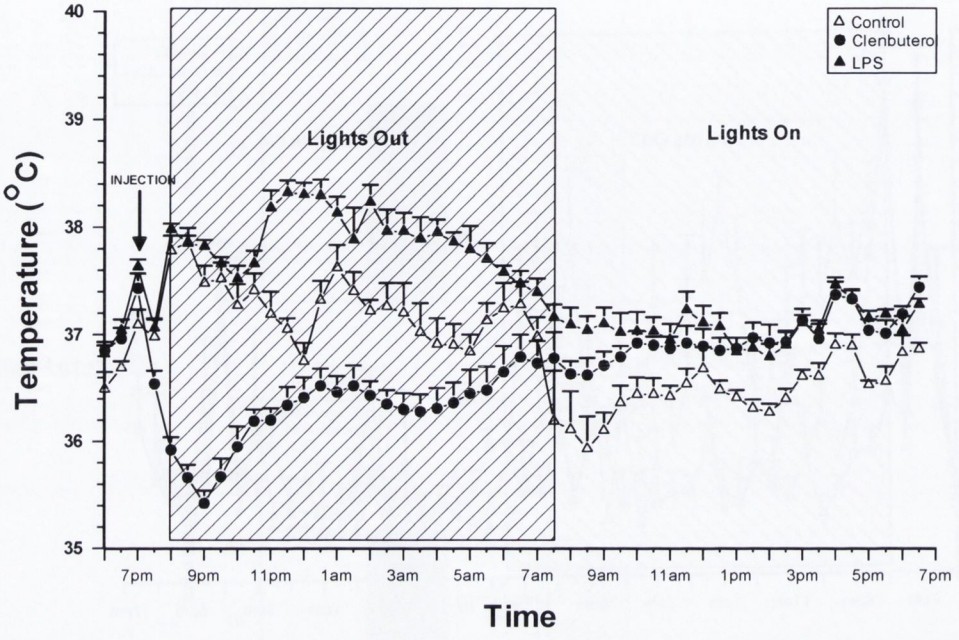


Figure 6.3.3: Systemic administration of LPS or clenbuterol reduces the locomotor activity of rats

Clenbuterol (0.5mg/kg), LPS (250μg/kg) or control (0.89% (w/v) saline) were administered s.c. to rats. a) Both clenbuterol and LPS reduce locomotor activity over a 24 hr period. b) A one-way ANOVA revealed a significant effect of treatment on locomotor activity [$F_{(2,72)}$=20.56, P<0.0001] during the 12 hr lights-out period. Data are expressed as means with standard error of the mean (n=6). **P<0.01 versus saline control (Newman-Keuls).

a)



b)

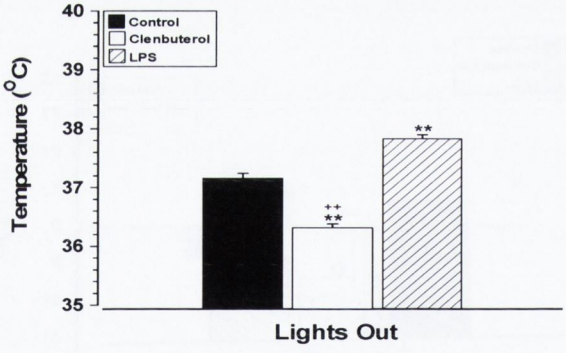
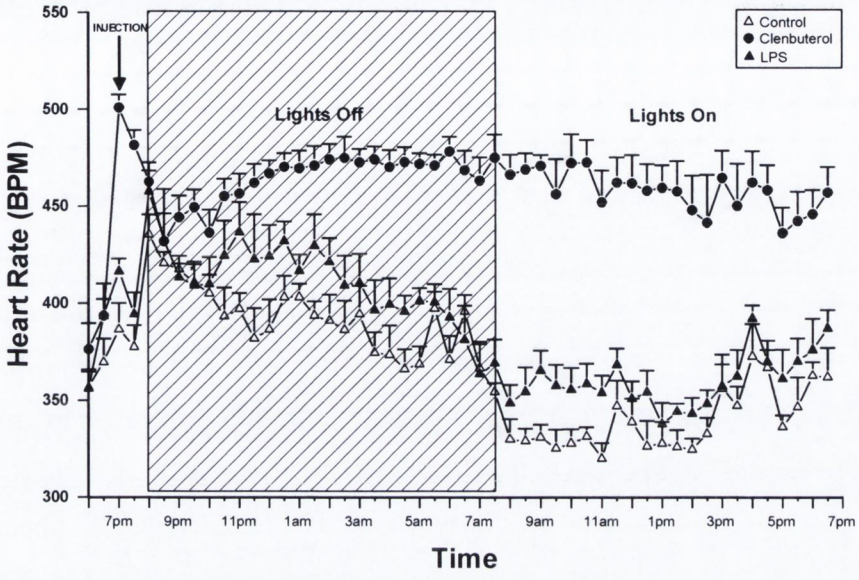


Figure 6.3.4: Systemic administration of LPS increases body-temperature of rats while clenbuterol reduces body-temperature of rats

Clenbuterol (0.5mg/kg), LPS (250μg/kg) or control (0.89% (w/v) saline) were administered s.c. to rats. a) LPS increases body-temperature while clenbuterol decreases body-temperature over a 24 hr period b) A one-way ANOVA revealed a significant effect of treatment on body-temperature [$F_{(2,72)}$=107.03, P<0.0001] during the 12 hr lights-out period. Data are expressed as means with standard error of the mean (n=6). **P<0.01 versus saline control, ++P<0.01 versus LPS group (Newman-Keuls).

a)



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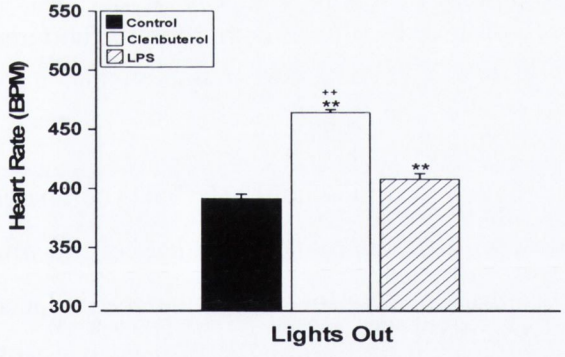

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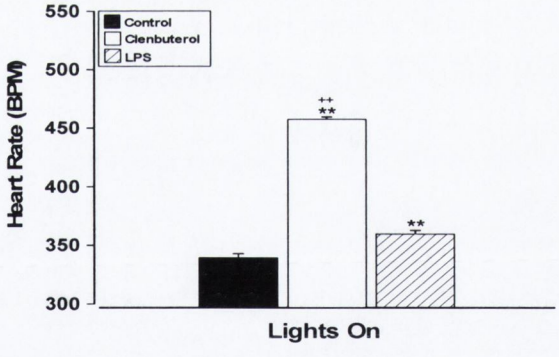


Figure 6.3.5: Systemic administration of LPS or clenbuterol increases heart rate of rats

Clenbuterol (0.5mg/kg), LPS (250μg/kg) or control (0.89% (w/v) saline) were administered s.c. to rats. a) Both clenbuterol and LPS increase heart rate over a 24 hr period. b) A one-way ANOVA revealed a significant effect of treatment on heart rate [$F_{(2,72)}$=100.61, P<0.0001] during the 12 hr lights-out period. c) A one-way ANOVA revealed a significant effect of treatment on heart rate [$F_{(2,63)}$=492.12, P<0.0001]. Data are expressed as means with standard error of the mean (n=6). **P<0.01 versus saline control, ++P<0.01 versus LPS group (Newman-Keuls).

6.4 Examination of the ability of the selective β_1-antagonist metoprolol and β_2-antagonist ICI 118,551 to block the peripheral effects of clenbuterol

The results from the previous study demonstrate that clenbuterol induces a profound increase in heart rate in comparison to that induced by LPS. Therefore, since the β_1-adrenoceptor is the primary subtype responsible for cardiac function it was suspected that the clenbuterol-induced tachycardia could be due to activation of β_1-adrenoceptors secondary to the release of noradrenaline induced by stimulation of facilitatory pre-synaptic β_2-adrenoceptors (Murugaiah & O'Donnell, 1995). Therefore, the following study was undertaken in order to determine whether the clenbuterol-induced increase in heart rate was mediated via actions at β_1- or β_2-adrenoceptors. In order to dissociate between such actions the selective β_1-adrenoceptor antagonist metoprolol or the selective β_2-adrenoceptor antagonist ICI 118,551 were administered to rats 30 min prior to clenbuterol and the impact on food and water intake and heart rate was measured over a 24 hr period. Since clenbuterol did not significantly alter body-weight in the previous study it was not analysed here.

6.4.1 Impact of metoprolol and ICI 118,551 on clenbuterol-induced reduction in food and water intake and increase in heart rate

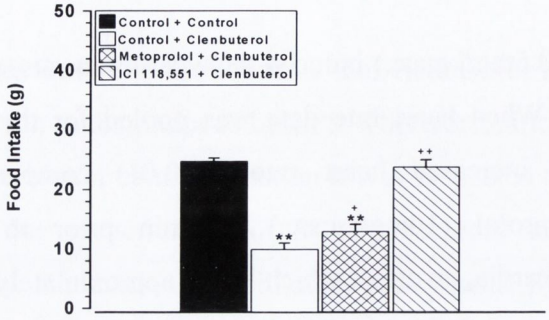
Food Intake: Clenbuterol (0.5mg/kg;s.c.) significantly reduced food intake ($P<0.01$) compared to saline controls over a 24 hr period. Administration of metoprolol (10mg/kg;s.c.) 30 min prior to clenbuterol induced a modest yet significant attenuation of the clenbuterol-induced decrease in food intake ($P<0.05$). However, a significant reduction in food intake was still shown in comparison to saline controls ($P<0.01$). In contrast, administration of ICI 118,551 (10mg/kg;s.c.) 30 min prior to clenbuterol completely blocked the clenbuterol-induced reduction in food intake ($P<0.01$). [Figure 6.4.1 a, Newman-Keuls, $n=6$].

Water Intake: Clenbuterol (0.5mg/kg;s.c.) significantly reduced water consumption ($P<0.05$) compared to saline controls over a 24 hr period. Administration of metoprolol (10mg/kg;s.c.) 30 min prior to clenbuterol did not significantly alter the clenbuterol-induced reduction in water consumption. In contrast, administration of ICI 118,551 (10mg/kg;s.c.) 30 min prior to

clenbuterol significantly blocked the clenbuterol-induced reduction in water consumption ($P<0.01$). [Figure 6.4.1 b, Newman-Keuls, $n=6$].

Heart rate: Clenbuterol (0.5mg/kg;s.c.) induced tachycardia in rats, an effect which persisted for 24 hr post-injection. When heart rate data was pooled for the 12 hr lights-out period clenbuterol significantly increased heart rate ($P<0.01$) compared to saline controls. Administration of metoprolol (10mg/kg;s.c.) 30 min prior to clenbuterol attenuated clenbuterol-induced tachycardia, an effect which lasted approximately 5 hr before wearing off. When heart rate data was pooled for the 12 hr lights-out period metoprolol induced a modest yet significant reduction in clenbuterol-induced tachycardia ($P<0.01$). Comparably, administration of ICI 118,551 (10mg/kg;s.c.) 30 min prior to clenbuterol attenuated clenbuterol-induced tachycardia, an effect which persisted for 24 hr. When heart rate data was pooled for the 12 hr lights-out period ICI 118,551 significantly reduced clenbuterol-induced tachycardia ($P<0.01$). Notably, during the 12 hr lights-on period ICI 118,551 induced a profound reduction in the clenbuterol-induced tachycardia ($P<0.01$). [Figure 6.4.2 a, b, c, Newman-Keuls, $n=6$].

a) Food Intake



b) Water Intake

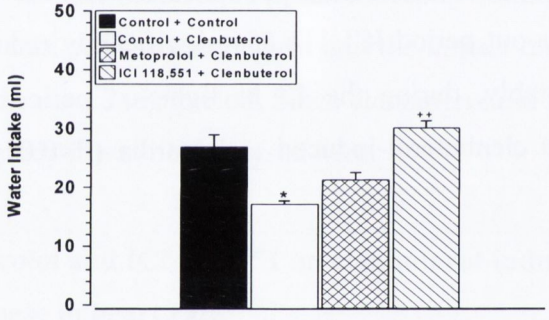


Figure 6.4.1: Administration of the β_1-adrenoceptor antagonist metoprolol attenuates the clenbuterol-induced reduction in food intake while the β_2-adrenoceptor antagonist ICI 118, 551 completely blocks the clenbuterol-induced reduction in food and water intake

Metoprolol (10mg/kg), ICI 118,551 (10mg/kg) or control (0.89% (w/v) saline) were administered s.c. to rats 30 min prior to s.c administration of clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline). a) A one-way ANOVA revealed a significant effect of treatment on food intake [$F_{(3,20)}$=7.31, P<0.0017]. b) A one-way ANOVA revealed a significant effect of treatment on water intake [$F_{(3,20)}$=57.47, P<0.0001]. Data are expressed as means with standard error of the mean (n=6). **P<0.01, *P<0.01 versus saline control, ++P<0.01, +P<0.05 versus clenbuterol control (Newman-Keuls).

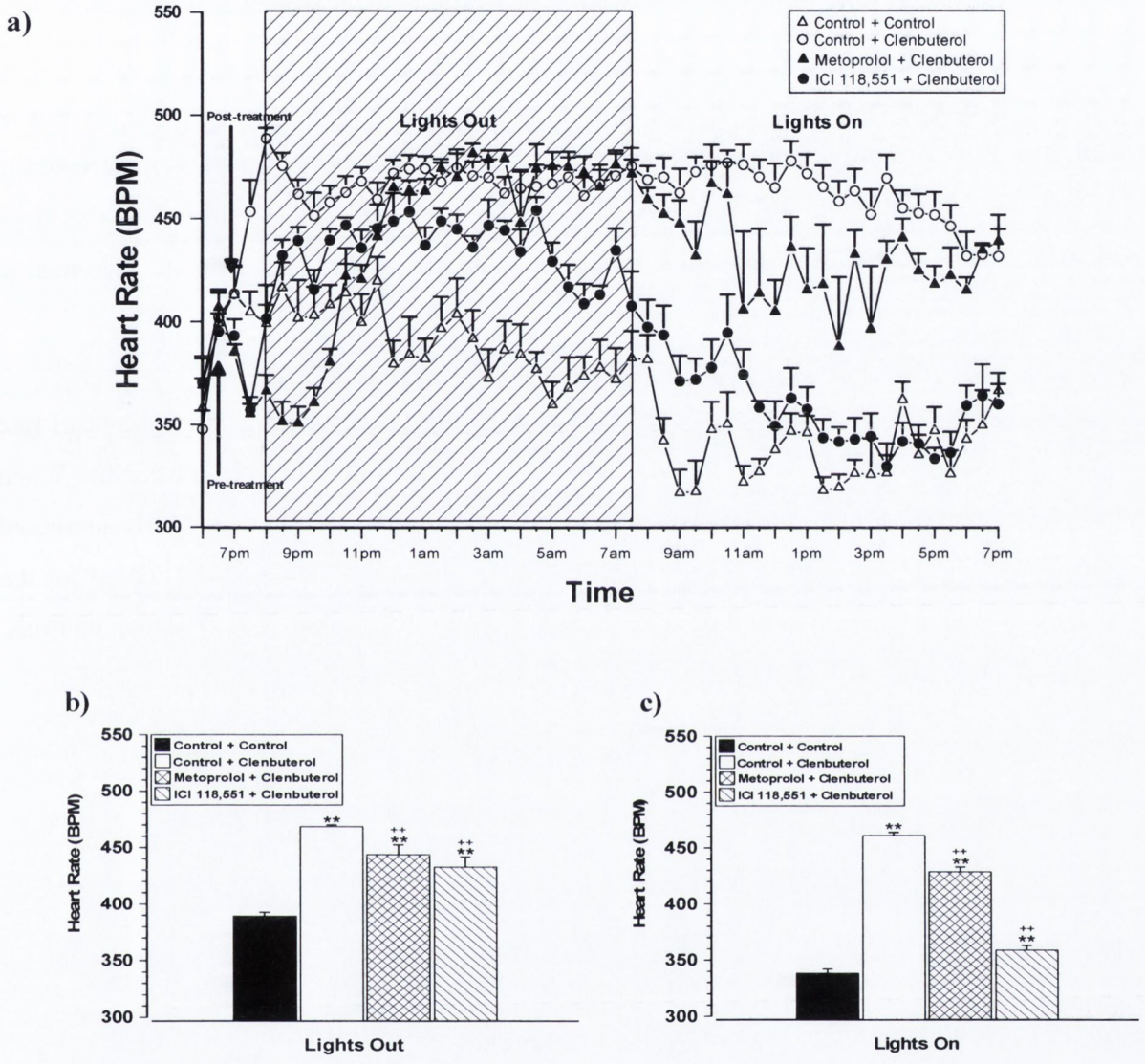


Figure 6.4.2: Administration of the β_1-adrenoceptor antagonist metoprolol or the β_2-adrenoceptor antagonist ICI 118,551 attenuates the clenbuterol-induced tachycardia

Metoprolol (10mg/kg), ICI 118,551 (10mg/kg) or control (0.89% (w/v) saline) were administered s.c. to rats 30 min prior to s.c. administration of clenbuterol (0.5mg/kg) or control (0.89% (w/v) saline). a) Clenbuterol increased heart rate over a 24 hr period. Pre-treatment with metoprolol or ICI 118,551 attenuated the clenbuterol-induced increase in heart rate. b) A one-way ANOVA revealed a significant effect of treatment on heart rate [$F_{(3,96)}$=41.6, P<0.0001] during the 12 hr lights-out period. c) A one-way ANOVA revealed a significant effect of treatment on heart rate [$F_{(3,88)}$=230.98, P<0.0001] during the 12 hr lights-on period. Data are expressed as means with standard error of the mean (n=6). **P<0.01 versus saline control, ++P<0.01 versus clenbuterol control (Newman-Keuls).

6.5 Examination of the impact of a low dose of clenbuterol on heart rate of rats

Results from the previous chapter demonstrated that 0.03mg/kg clenbuterol is the lowest dose capable of impacting on inflammatory processes in the rat CNS in the presence and absence of LPS. Thus, in light of the results reported here so far, the following study was undertaken to examine the impact of this low dose of clenbuterol (0.03mg/kg) on the heart rate of rats since this was the dose chosen to be used for a chronic study, the results for which are presented in the following chapter (Chapter 7).

Heart rate: Administration of low dose clenbuterol (0.03mg/kg; s.c.) increased the heart rate of rats, an effect which persisted for approximately 10 hr before returning to baseline. When heart rate data was pooled for the 12 hr lights-out period clenbuterol significantly increased heart rate ($P<0.01$) compared to saline controls. When the heart rate data was pooled for the 12 hr lights-on period, clenbuterol had no significant effect compared to saline controls. [Figure 6.5.1 a, b, Student's *t*-test, n=5-6].

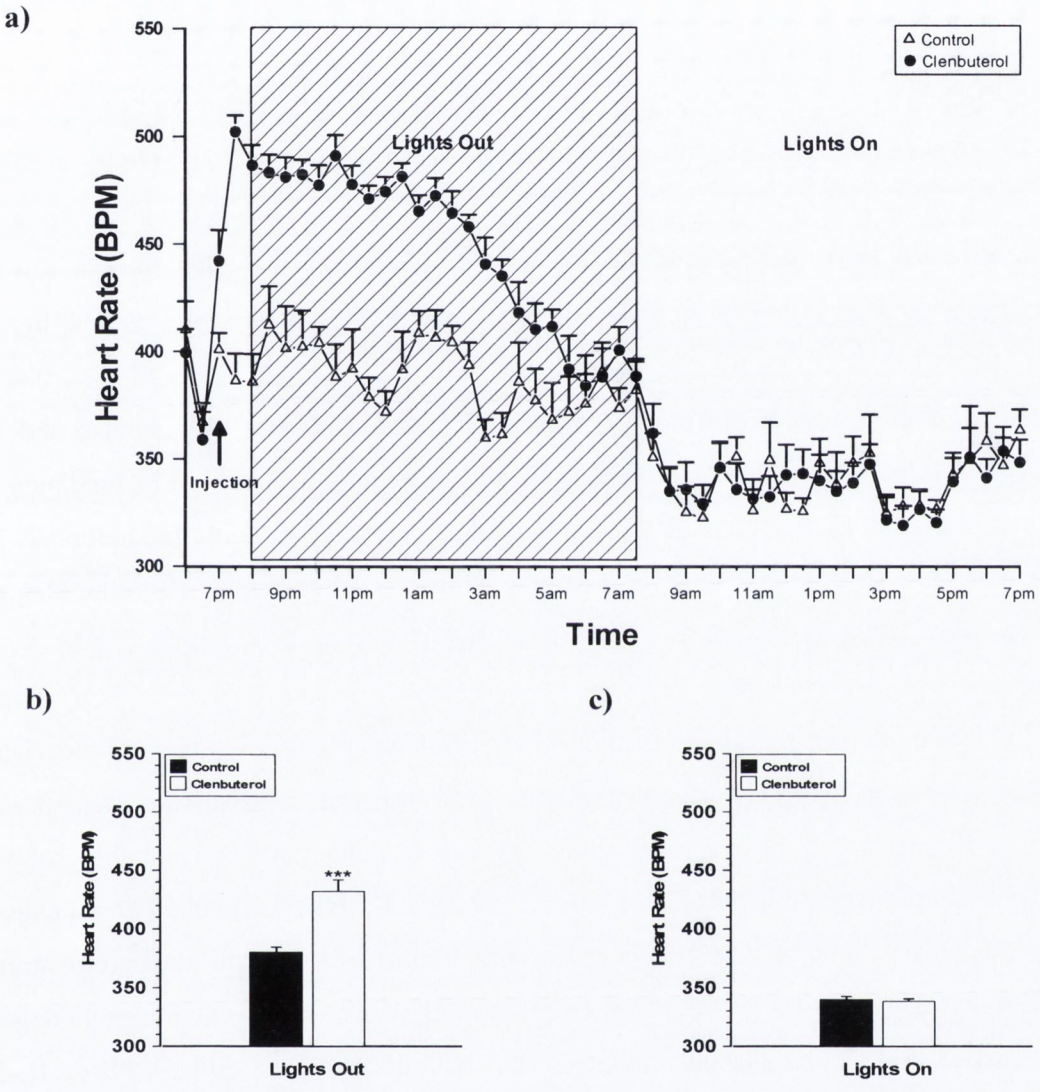


Figure 6.5.1: Administration of a low-dose of clenbuterol induces a transient increase in the heart rate of rats

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered s.c. to rats. a) Low dose clenbuterol (0.03mg/kg) transiently increased the heart rate over a 24 hr period. b) A Student's *t*-test revealed a significant effect of treatment on heart rate ($P<0.0001$) during the 12 hr lights-out period. c) A Student's t-test revealed no significant effect of treatment on heart rate. Data are expressed as means with standard error of the mean ($n=6$). ***$P<0.01$ versus saline control (Student's *t*-test).

6.6 Discussion

6.6.1 Clenbuterol selectively induces the expression of IL-1β in the hypothalamus and does not induce classical sickness behaviour in rats

The results presented here demonstrate that systemic LPS administration induces a robust increase in the mRNA expression of TNF-α, IL-6, COX-II and to a lesser extent IL-1β in rat hypothalamus. In contrast, clenbuterol was found to selectively increase the expression of IL-1β and did not impact on TNF-α, IL-6 or COX-II expression. Notably, the ability of clenbuterol to induce IL-1β expression was shown to be much more powerful than that of LPS. These findings are noteworthy since a classical neuroinflammatory response and the concomitant behavioural response associated with it are generally accepted to be mediated by the induction and combined actions of the pro-inflammatory and pyrogenic mediators IL-1β, TNF-α, IL-6 and COX-II in the CNS (Beishuizen & Thijs, 2003; Blatteis *et al.*, 2005; Dantzer *et al.*, 2008; Tizard, 2008).

Both LPS and clenbuterol were demonstrated to significantly impact on the food-motviated behaviour of rats since the food and water intake of rats was reduced over the 24 hr period post-injection. Significantly, the decrease in food and water intake induced by clenbuterol was shown to be much more profound than that induced by LPS. Both LPS and clenbuterol caused a reduction in body-weight of rats. However, in spite of the fact that the clenbuterol group were found to eat and drink far less than the LPS group, they did not display the same degree of weight-loss. It is well established that peripheral administration of either LPS or IL-1β induces hypophagia and a reduction in body-weight in rats, an effect suggested to be mediated through humoral pathways since neither vagal nor non-vagal afferent nerves from the upper gut have been shown to be involved (Porter *et al.*, 1998; Wieczorek & Dunn, 2006). The reduction in body-weight induced by clenbuterol is in contrast to previous reports which demonstrated that clenbuterol increases body-weight, however, increased body-weight is generally reported following chronic administration of clenbuterol (Huang *et al.*, 2000; Patiyal & Sharma, 2007). Notably, one study did report that while chronic administration of clenbuterol did not effect total body-weight of animals it did appear to induce a mild stress response since the thymus of the clenbuterol group was significantly reduced in size compared

to control animals (Shirato *et al.*, 2007). Thus, it is possible that a stress response may be responsible for the reduction in body-weight occurring here.

Further to this, in line with the results presented in the previous chapter, both clenbuterol and LPS significantly reduced the locomotor activity of rats over a 24 hr period. When locomotor activity was pooled for the lights-out period, the time at which rats are most active, the activity of the rats which received either clenbuterol or LPS was significantly less than that of the control group. Notably, clenbuterol was found to have a more profound impact on locomotor activity than LPS. It has previously been shown that peripheral injection of IL-1β results in prolonged increases in brain noradrenaline, an effect demonstrated to reduce the locomotor activity of rats (Wieczorek & Dunn, 2006). Thus, it seems plausible that the impact of clenbuterol on locomotor activity may be linked to the increased expression of IL-1β in the hypothalamus. However, it must also be noted that previous studies have shown that the clenbuterol-induced reduction in locomotor activity can be attenuated by administration of hydrophilic non-selective β-adrenoceptor antagonists, which are incapable of crossing the BBB. This indicates that the effects of clenbuterol on locomotor activity may be mediated by peripheral and not central mechanisms (Geyer & Frampton, 1988; O'Donnell, 1993). Therefore, the mechanisms mediating the clenbuterol-induced reduction in locomotor activity observed in the present study is a matter which warrants further investigation.

Systemic LPS administration was shown in the current study to induce a febrile response in rats, an event which a classically linked to sickness behaviour. The "central thermostat" regulates body-temperature by means of neurons located in the medial and lateral parts of the pre-optic nucleus, the anterior hypothalamus and regions of the septum and acts to maintain core body-temperature over a narrow range regardless of fluctuations in the ambient temperature (Tizard, 2008). However, it is known that cytokines and prostaglandins have the capacity to induce fever by acting on receptors in these areas causing a reduction in the firing rate of warm-sensitive neurons with the effect of increasing heat production and reducing heat loss. Thus, it is suggested that sick animals seek warmer environments and display reduced activity in order to minimize heat loss (Tizard, 2008). Furthermore, the febrile response plays an important role in sickness behaviour since it lowers the threshold required for the production of inflammatory mediators, thus aiding the elimination of pathogens (Tizard,

2008). In light of this, the LPS-induced reduction in body-weight of rats in the current study might be accounted for by the necessity for increased mobilization of energy stores required for the initiation of a febrile response (Konsman *et al.*, 2008). However, in contrast to LPS-induced fever, clenbuterol induced a mild hypothermic reaction in rats. It is known that clenbuterol can cause widespread vasodilation in both animals and humans (Daubert *et al.*, 2007). Thus, given that the predominant subtype of receptor present in the vasculature is the β_2-adrenoceptor, whose role is to induce relaxation of smooth muscle through increased concentrations of intracellular cAMP (Werstiuk & Lee, 2000), widespread vasodilation might account for the reduction in body-temperature observed in this study.

The results presented here show that LPS significantly increases the heart rate of rats, an effect which was found to persist for up to 18 hr. This corresponds with reports from the literature since it has been demonstrated that the administration of endotoxin increases plasma noradrenaline and adrenaline concentrations which in turn bring about a decrease in mean blood pressure and an increase in heart rate (Zhou & Jones, 1993). Comparably, clenbuterol also increased the heart rate of rats, however, this increase was of a much greater magnitude than that induced by LPS. As already discussed in section 6.1, the β_1-adrenoceptor is the predominant subtype expressed in the heart, where it outnumbers the β_2-adrenoceptor by approximately 70%:30% in the atria and 80%:20% in the ventricles (Wallukat, 2002; Brodde *et al.*, 2006). It has previously been reported that clenbuterol can induce changes in heart rate both *in vitro* and *in vivo* (Hoey *et al.*, 1995; Mazzanti *et al.*, 2007) and while it has been found that clenbuterol acts principally on β_2-adrenoceptors it has been suggested that the clenbuterol-induced increase in heart rate occurs due to an interplay between both β_1- and β_2-adrenoceptor subtypes (Mazzanti *et al.*, 2007). Thus, it might be hypothesized that the profound tachycardia induced by clenbuterol occurs owing to hypotension evoked by stimulation of vascular β_2-adrenoceptors. This in turn could result in a baroreceptor reflex response which could provoke the release of noradrenaline via facilitatory β_2-adrenoceptors which can then act on cardiac β_1-adrenoceptors to influence heart rate.

6.6.2 Impact of metoprolol and ICI 118,551 on clenbuterol-induced reduction in food and water intake and increase in heart rate

In order to determine if stimulation of β_1- or β_2-adrenoceptors was responsible for the profound tachycardia induced by clenbuterol, rats were administered either the β_1-adrenoceptor antagonist metoprolol or the β_2-adrenoceptor antagonist ICI 118,551 prior to clenbuterol. The results demonstrate that metoprolol caused a slight attenuation in clenbuterol-induced tachycardia, however, its effects were found to be short-lived, wearing off after approximately 5 hr. In contrast, ICI 118,551 induced a much greater and persistent attenuation of clenbuterol-induced tachycardia. These findings reflect previous *in vitro* studies in which clenbuterol was found to induce a dose-dependent increase in heart rate in isolated guinea-pig atria, an effect which could be antagonized by ICI 118,551 but to a significantly lesser extent by the β_1-adrenoceptor antagonist atenolol (Mazzanti *et al.*, 2007), thus implying that the β_2-adrenoceptor is responsible for the clenbuterol-induced increase in heart rate. Similarly, a study by Hoey *et al.*, (1995) showed that the effect of clenbuterol on heart rate *in vivo* could be completely reversed by administration of ICI 118,551 but was only reduced by approximately 50% when the β_1-adrenceptor antagonist CYP20712A was administered. Notably, when the results from the present study were pooled for the 12 hr lights-out period, it was found that ICI 118, 551 did not fully block the clenbuterol-induced tachycardia and surprisingly the magnitude of attenuation induced by both ICI 118, 551 and metoprolol were found to be similar. However, ICI 118,551 had a much more profound effect than metoprolol during the lights-on period. Thus, it seems likely that an interplay between actions at both β_1- and β_2-adrenoceptors is responsible for the clenbuterol-induced tachycardia observed here.

The clenbuterol-induced reduction in food and water intake was found to be mediated by the activation of β_2-adrenoceptors since the effects were completely blocked when rats were pre-treated with ICI 118,551 while pre-treatment with metoprolol had only a modest effect. The effect of metoprolol is unlikely biologically relevant since these rats were still shown to eat significantly less than either the control or ICI 118,551 + clenbuterol groups. Additionally, metoprolol did not impact on the clenbuterol-induced reduction in water consumption. Thus, it is likely that the clenbuterol-induced increase in heart rate may provoke a non-specific stress

response, resulting in reduced food and water intake, since blocking the profound clenbuterol-induced tachycardia with ICI 118,551 also reverses effects on feeding.

6.6.3 Examination of the impact of low dose of clenbuterol on heart rate of rats

Since the lowest dose of clenbuterol capable of impacting on immune processes in the CNS was 0.03mg/kg, this dose was chosen to be used for a chronic study to determine if such effects persist over time. However, in light of the results presented in this chapter it was deemed necessary to determine the impact of this dose on heart rate, since this dose was shown to induce an increase in IL-1 expression in the CNS and a decrease in locomotor activity in the previous chapter (Chapter 5). While the results presented here demonstrate that this low-dose of clenbuterol increases heart rate, its effects are transient and persist for approximately 10 hr before returning to baseline. This is significant since clenbuterol toxicity in humans is known to lead to symptoms including headache, dizziness, nervousness, peripheral vasodilation, hypothermia and tachycardia. However, this dose of 0.03mg/kg is found to be in line with that already used therapeutically in humans (Daubert *et al.*, 2007) and therefore, since its peripheral effects are minimal, might represent a promising therapy for the treatment of neuroinflammatory conditions. Notably, it has been reported that clenbuterol causes an increase in blood flow and heart rate during the initial days of treatment however, these effects are found to subside with continued treatment (Hoey *et al.*, 1995).

6.6.4 Summary

Te results presented here demonstrate that LPS causes classical neuroinflammatory and behavioural response characterized by an increase in the expression of IL-1β, TNF-α, IL-6 and COX-II in the hypothalamus which is accompanied by fever, increased heart rate, hypophagia, cachexia, and reduced locomotor activity. In contrast, clenbuterol selectively induces the expression of IL-1β in the hypothalamus. Furthermore, it appears that the impact of clenbuterol on behaviour might be accounted for by the induction of a stress response caused by its profound effects on heart rate, since the reduction in food and water intake were found to be reversed when the clenbuterol-induced tachycardia was attenuated with ICI 181,551. In light of this, in order for clenbuterol to be used therapeutically for the treatment of

neuroinflammatory disease further studies will be necessary to attempt to dissociate the beneficial effects of activation of central β_2-adrenoceptors from the negative effects peripheral effects.

Chapter 7

Chronic treatment with low-dose clenbuterol activates astrocytes & the IL-1 system & reduces amyloid-β concentrations in rat CNS

7.1 Introduction

The results from the previous chapters demonstrate that acute treatment with clenbuterol activates central β_2-adrenoceptors and elicits anti-inflammatory actions under both basal and inflammatory conditions in the CNS. However, since neuroinflammatory disease states are chronic in nature and require ongoing administration of therapeutics, the following study was undertaken to determine if the impact of clenbuterol on immune processes in the CNS would persist over time, in this instance for the duration of a chronic study 14 day study. A dose of 0.03mg/kg was chosen for the purposes of this study since this was found to be the lowest dose capable of impacting on immune processes in the CNS (Chapter 5) and was also found to have minimum peripheral effects (Chapter 6). Notably, it has previously been shown that chronic administration of low-dose clenbuterol does not impact on the density of β_2-adrenoceptors in cortex or hippocampus (Dooley *et al.,* 1983). In addition, as previously mentioned, this dose is also comparable to that already used therapeutically in humans. Furthermore, doses in this range have been shown to be well tolerated in humans over roughly the same period of time (Kamburoff & Prime, 1977) and additionally over a more prolonged periods in a clinical trial for the treatment of amyotrophic lateral sclerosis (Sorau *et al.,* 2007).

An interesting finding from the acute studies was the selective increase in the expression of IL-1β induced by clenbuterol in the CNS. This was surprising since IL-1 is, in the main, considered to be the prototypic pro-inflammatory cytokine and is known to play a major role in the inflammatory response. While low levels of IL-1 are constitutively expressed in the brain, its expression can be rapidly increased in response to injury (Rothwell & Luheshi, 2000; Shaftel *et al.*, 2008). Furthermore, elevated IL-1 expression has been detected in the brains of patients with neuroinflammatory diseases such as MS, Alzheimer's disease and HIV dementia (Shaftel *et al.*, 2008) and for this reason, increased IL-1 has been classically considered to induce detrimental effects in the CNS. In spite of this, IL-1 is also found to elicit beneficial effects in the brain since it has been shown to play an important role in memory consolidation (Shaftel *et al.*, 2008) and sleep (Rothwell & Luheshi, 2000). IL-1 itself is not directly neurotoxic since administration of IL-1 *in vitro* does not affect neuronal viability and injection of IL-1 into the brain is shown to have no effect under basal conditions, however, IL-1 is known to exacerbate the damage caused to the CNS by other means (Grundy *et al.*, 1999;

Rothwell & Luheshi, 2000; Shaftel *et al.*, 2008). Significantly, new reports are coming to the fore which challenge ideas about the role this prototypical pro-inflammatory cytokine plays in disease. Notably, with respect to Alzheimer's disease recent studies have proposed that both IL-1α and IL-1β can decrease concentrations of amyloid-β (Bandyopadhyay *et al.*, 2006; Tachida *et al.*, 2008). In light of this, since clenbuterol is found to induce a robust increase in the expression of IL-1β in rat CNS, the impact of chronic clenbuterol treatment on amyloid-β concentrations was examined in the present study. Concentrations of amyloid-β in the normal brain are generally found to be in the range of 200-1000pM with amyloid-β_{1-42} concentrations being closer to the lower end of the range while amyloid-β_{1-40} concentrations are found at the upper end of the range (Best *et al.*, 2005; Garcia-Osta & Alberini, 2009). While these concentrations are low in comparison to those found in transgenic rat models of Alzheimer's disease where concentrations of amyloid-β are generally found to be in the nanomolar range (Flood *et al.*, 2009), studies have evaluated the possibility of detecting changes in endogenous levels of amyloid-β following treatment (Best *et al.*, 2005).

Thus, the aims of this study were as follows:

1) To determine if chronic clenbuterol treatment alters the expression of the β-adrenergic receptors.

2) To determine the impact that chronic clenbuterol treatment has on glial activation.

3) To determine the ability of chronic clenbuterol treatment to activate the IL-1 system and induce expression of the anti-inflammatory cytokine IL-10.

4) To determine the signalling pathways activated by chronic clenbuterol treatment.

5) To determine whether chronic clenbuterol treatment can impact on levels of amyloid-β_{1-40} and amyloid-β_{1-42}.

6) To determine the impact of chronic clenbuterol on APP processing pathways and its impact on the expression of molecules involved in amyloid-β phagocytosis and degradation.

7.2 Methods

7.2.1 Subjects & Drug Treatments

Male Sprague-Dawley rats (200-250g) were housed 3 per cage under a 12 hr light-dark cycle (lights on 08:00) in a temperature-controlled room (22 ± 2 °C) with food and water available *ad libitum* for the duration of the experiment as described in the methods section (Section 2.3, Chapter 2). Clenbuterol was dissolved in 0.89% (w/v) saline and was administered i.p. at a dose of 0.03mg/kg twice daily. Control rats received an i.p. injection of 0.89% (w/v) saline alone.

7.2.2 Experimental Procedure

The design for this experiment was as set out in Figure 7.1 below. RT-PCR, western immunoblotting and MSD® Multi-spot biomarker detection procedures were carried out as outlined in the methods section (Section 2.3 & 2.4, Chapter 2).

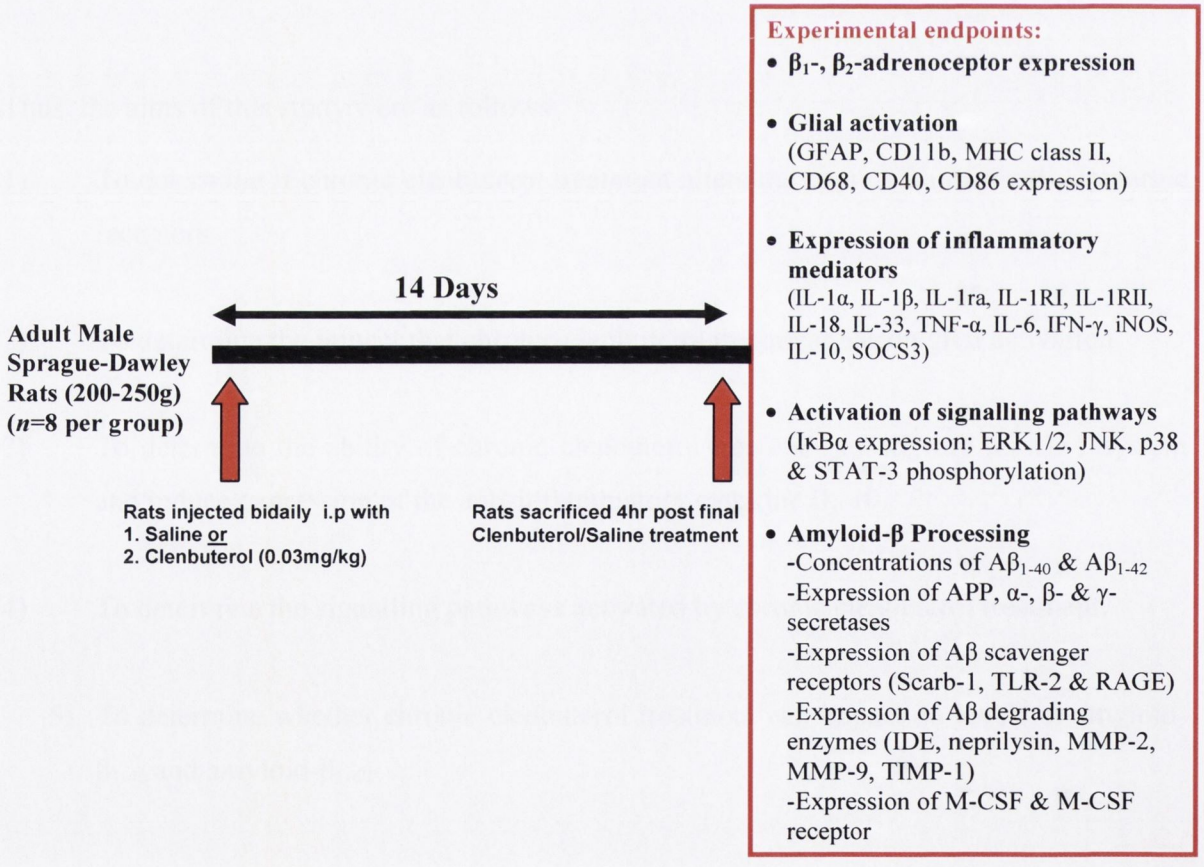


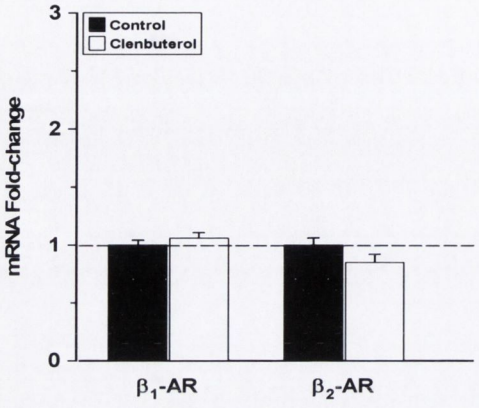
Figure 7.1: Experimental design for assessing impact of chronic clenbuterol treatment on immune processes and amyloid-β processing within the CNS

7.3 Chronic clenbuterol treatment does not influence the mRNA expression of either the β_1- or β_2-adrenoceptor in the CNS

Previous reports have shown that chronic administration of clenbuterol has minimal effects on the density of β_2-adrenoceptors in cortex and hippocampus (Dooley *et al.,* 1983). Thus, the aim of this study was to verify that chronic administration of a dose of clenbuterol of 0.03mg/kg;bid over a 14 day period does not impact on the expression of central β_1- or β_2-adrenoceptors.

β-adrenoceptors: Chronic administration of clenbuterol (0.03mg/kg;i.p.;bid) did not alter the mRNA expression of either the β_1-adrenoceptor or the β_2-adrenoceptor in cortex or hippocampus compared to saline controls [Figure 7.3.1 a, b, n=8]

a) Cortex



b) Hippocampus

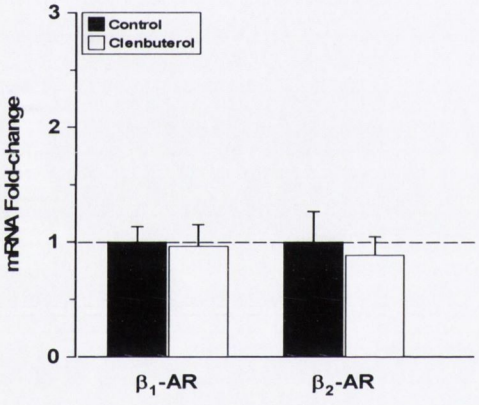


Figure 7.3.1: Chronic clenbuterol treatment has no effect on the mRNA expression of either the β_1-adrenoceptor or the β_2-adrenoceptor in cortex or hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two weeks. A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of either the β_1-adrenoceptor or β_2-adrenoceptor in cortex or hippocampus. Data are expressed as means with standard error of the mean (n=8).

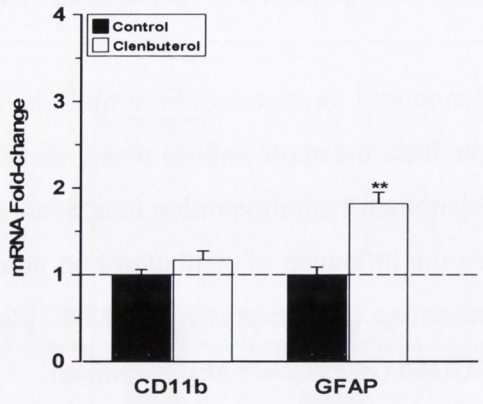
7.4 Chronic clenbuterol treatment induces glial activation in the CNS

Owing to the fact that chronic clenbuterol treatment did not alter the expression of central β-adrenoceptors and since the results from the acute studies reveal that clenbuterol induces glial activation, the ability of chronic clenbuterol administration to activate glia was examined here. In addition, to further characterize the influence of clenbuterol on glial activation and as such their capacity to act as antigen presenting cells, expression of MHC class II, CD68 and the co-stimulatory molecules CD40 and CD86 (B7-2) were also examined.

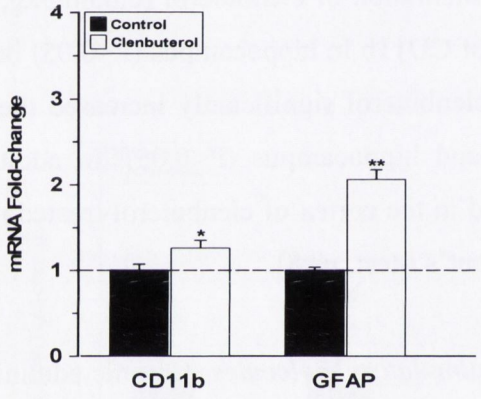
GFAP and CD11b: Chronic administration of clenbuterol (0.03mg/kg; i.p.; bid) significantly increased the mRNA expression of CD11b in hippocampus ($P<0.05$) but not cortex compared to saline controls. Furthermore, clenbuterol significantly increased the mRNA expression of GFAP in both cortex ($P<0.05$) and hippocampus ($P<0.05$). In addition, increased protein expression of GFAP was observed in the cortex of clenbuterol-treated rats compared to saline controls. [Figure 7.4.2 a,b,c, Student's *t*-test, $n=8$].

MHC class II, CD68 and the co-stimulatory molecules: Chronic administration of clenbuterol (0.03mg/kg; i.p.; bid) significantly increased the mRNA expression of MHC class II in cortex ($P<0.001$) and hippocampus ($P<0.01$) compared to saline controls. In addition clenbuterol significantly increased the mRNA expression of CD86 in cortex ($P<0.001$) and hippocampus ($P<0.05$). In contrast, clenbuterol did not alter the mRNA expression of CD40 or CD68 in either cortex or hippocampus. [Figure 7.4.3 a,b, $n=7$-8].

a) Cortex



b) Hippocampus



c) Cortical GFAP protein

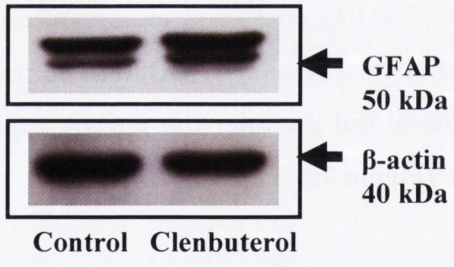
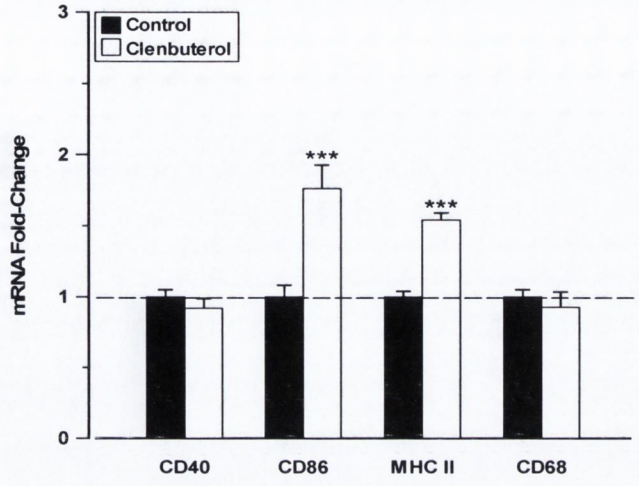


Figure 7.4.2: Chronic clenbuterol treatment increases GFAP mRNA expression in cortex and hippocampus, GFAP protein expression in cortex and CD11b mRNA expression in hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two weeks. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of GFAP in cortex (a: $P<0.01$) and hippocampus (b: $P<0.01$). A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of CD11b in hippocampus (b: $P<0.05$) but not cortex. c) Sample immunoblot depicting increased cortical GFAP protein expression induced by clenbuterol representative of 8 independent experiments. Data are expressed as means with standard error of the mean (n=7-8). **$P<0.01$, *$P<0.05$ versus saline control (Student's *t*-test).

a) Cortex



b) Hippocampus

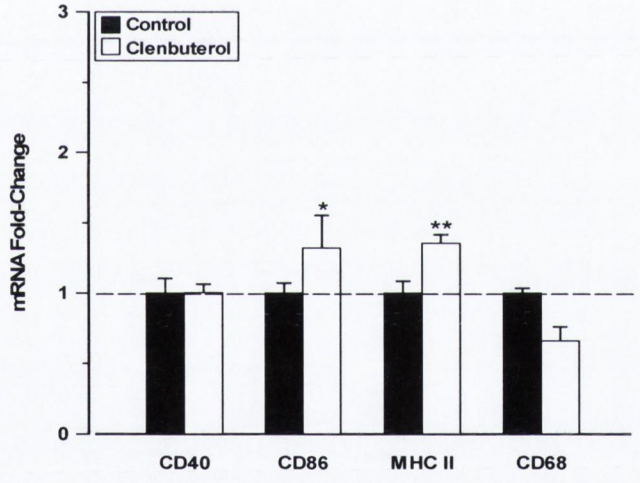


Figure 7.4.3: Chronic clenbuterol treatment increases CD86 and MHC class II mRNA expression but does not alter CD40 or CD68 expression in cortex and hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two weeks. A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of CD40 or CD68 in cortex or hippocampus. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of CD86 mRNA expression in cortex (a: $P<0.001$) and hippocampus (b: $P<0.05$). A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of MHC class II in cortex (a: $P<0.001$) and hippocampus (b: $P<0.01$). Data are expressed as means with standard error of the mean (n=7-8). ***$P<0.001$, **$P<0.01$, *$P<0.05$ versus saline control (Student's *t*-test).

7.5 Chronic clenbuterol treatment selectively activates the IL-1 system in cortex and hippocampus

The results from the previous chapters demonstrate that acute administration of clenbuterol increases the mRNA expression of the anti-inflammatory mediators IL-1ra and IL-1 Type II receptor, both negative regulators of the IL-1 signalling system, in addition to the anti-inflammatory cytokine IL-10 and its downstream signalling molecule SOCS3. Surprisingly, acute administration of clenbuterol was also found to selectively increase the expression of the cytokine IL-1β in the absence of an effect on the expression of other pro-inflammatory mediators. Therefore, the aim of the following study was to determine if the acute effects of clenbuterol on the IL-1 and IL-10 systems persist for the duration of a chronic 14 day treatment regimen. Additionally, the impact of chronic clenbuterol treatment on the expression of the pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ was also examined. The expression of IκBα was assessed as a marker of NFκB activation since acute studies have shown that IκBα is transiently expressed in response to clenbuterol (Heffernan, 2009). Moreover, since acute clenbuterol treatment has been demonstrated to induce phosphorylation of ERK but not JNK or p38 (Heffernan, 2009), the signalling pathways activated by chronic clenbuterol treatment were investigated here.

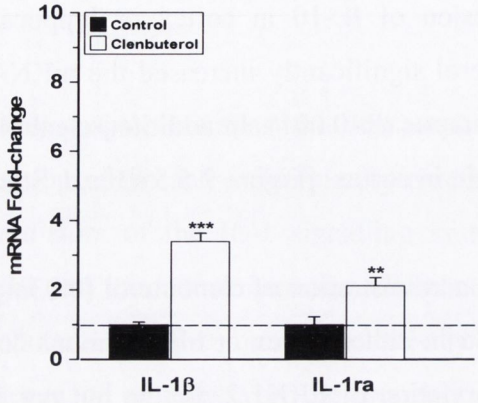
IL-1 system: Chronic administration of clenbuterol (0.03mg/kg; i.p., bid) significantly increased the mRNA expression of IL-1β and IL-1 Type II receptor in cortex ($P<0.001$) and hippocampus ($P<0.001$) compared to saline controls. In addition, clenbuterol significantly increased the expression of the IL-1ra in both cortex ($P<0.01$) and hippocampus ($P<0.001$). Furthermore, clenbuterol significantly increased the expression of IL-1α and IL-18 in cortex ($P<0.01$) and hippocampus ($P<0.05$, $P<0.01$ respectively). In contrast, clenbuterol did not alter on the expression of the IL-1 Type I receptor or IL-33. [Figures 7.5.1, 7.5.2, 7.5.3, Student's *t*-test, $n=7$-8].

Other pro-inflammatory mediators: Chronic administration of clenbuterol (0.03mg/kg; i.p., bid) significantly increased the mRNA expression of TNF-α in hippocampus ($P<0.05$) but not cortex compared to saline controls. In contrast, clenbuterol did not alter the expression of IL-6, IFN-γ or iNOS in either cortex or hippocampus. [Figure 7.5.4 a,b, Student's *t*-test, $n=8$].

IL-10 system: Chronic administration of clenbuterol (0.03mg/kg; i.p., bid) had no significant effect on the mRNA expression of IL-10 in cortex or hippocampus compared to saline controls. In contrast, clenbuterol significantly increased the mRNA expression of SOCS3 in cortex ($P<0.001$) and hippocampus ($P<0.001$). In addition, clenbuterol increased SOCS3 and phosphorylated STAT-3 protein in cortex. [Figure 7.5.5 a,b,c,d, Student's *t*-test, n=5-8].

Signalling pathways: Chronic administration of clenbuterol (0.03mg/kg; i.p., bid) did not alter the mRNA expression of IκBα in either cortex or hippocampus compared to saline controls. Clenbuterol induced phosphorylation of ERK1/2 protein but not JNK or p38. [Figure 7.5.6 a,b,c,d, n=4-8].

a) Cortex



b) Hippocampus

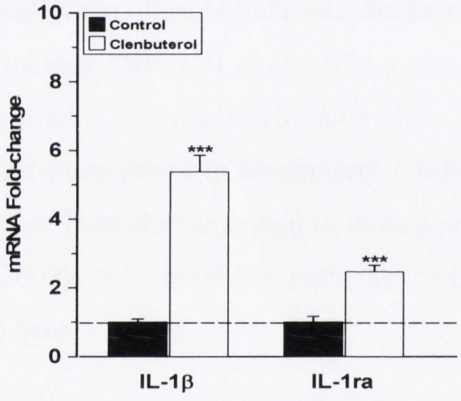
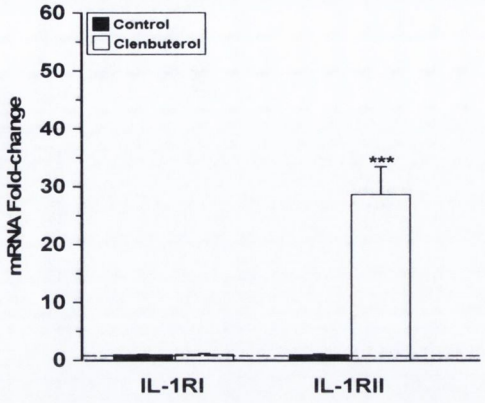


Figure 7.5.1: Chronic clenbuterol treatment increases IL-1β and IL-1ra mRNA expression in cortex and hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of IL-1β in cortex (a: $P<0.001$) and hippocampus (b: $P<0.001$). A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of IL-1ra in cortex (a: $P<0.01$) and hippocampus (b: $P<0.001$). Data are expressed as means with standard error of the mean (n=7-8). ***$P<0.001$, **$P<0.01$ versus saline control (Student's *t*-test).

a) Cortex



b) Hippocampus

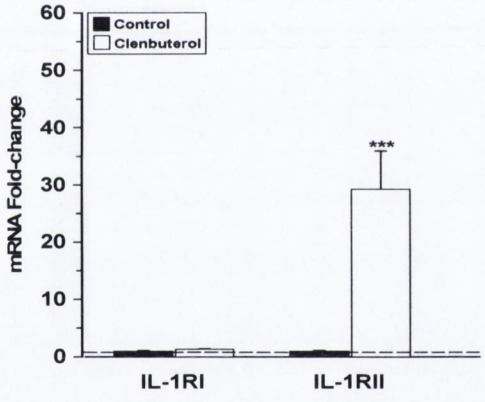
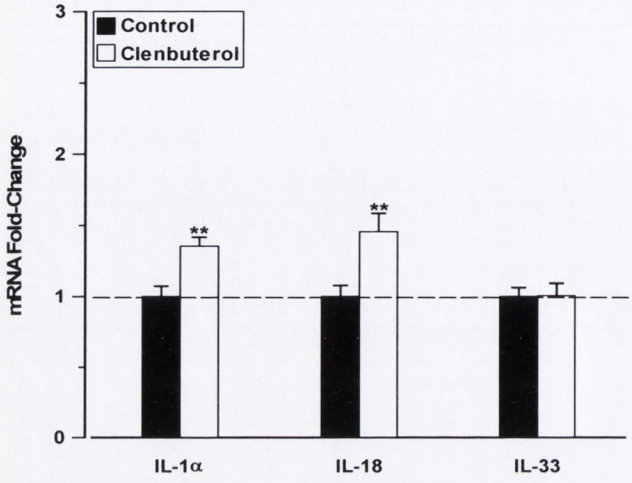


Figure 7.5.2: Chronic clenbuterol treatment increases IL-1 Type II receptor but not IL-1 Type I receptor mRNA expression in cortex and hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of IL-1 Type I receptor in cortex or hippocampus. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of IL-1 Type II receptor in cortex (a: $P<0.001$) and hippocampus (b: $P<0.001$) compared to saline controls. Data are expressed as means with standard error of the mean (n=7-8). ***$P<0.001$ versus saline control (Student's *t*-test).

a) Cortex



b) Hippocampus

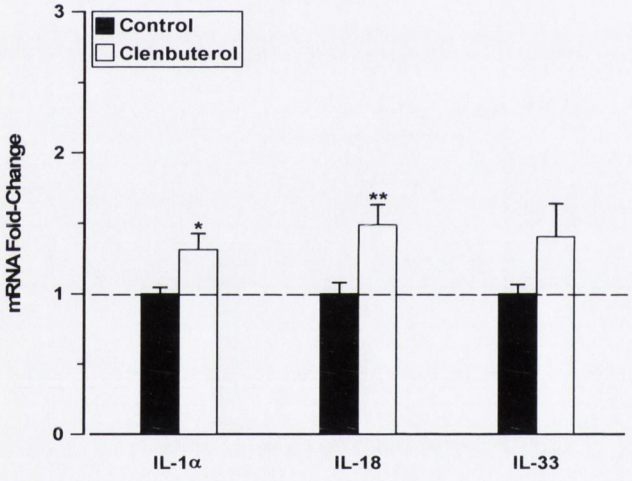
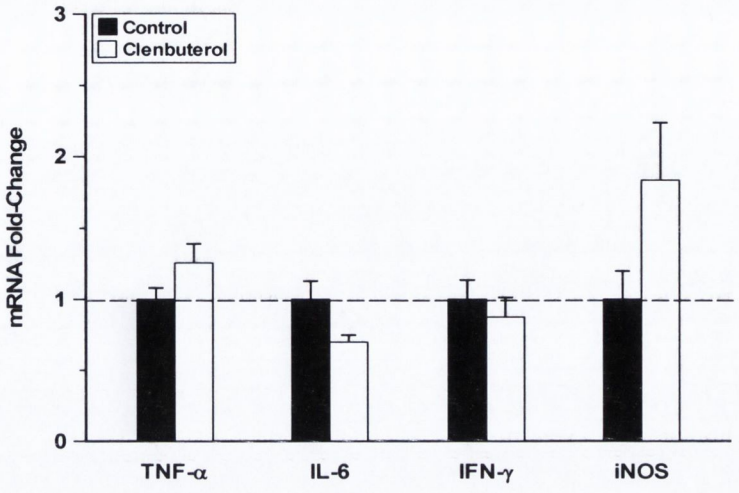


Figure 7.5.3: Chronic clenbuterol treatment increases IL-1α and IL-18 but not IL-33 mRNA expression in cortex and hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of IL-1α in cortex (a: $P<0.01$) and hippocampus (b: $P<0.05$). A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of IL-18 in cortex (a: $P<0.01$) and hippocampus (b: $P<0.01$). A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of IL-33 in cortex or hippocampus. Data are expressed as means with standard error of the mean (n=7-8). **$P<0.01$, *$P<0.05$ versus saline control (Student's *t*-test).

a) Cortex



b) Hippocampus

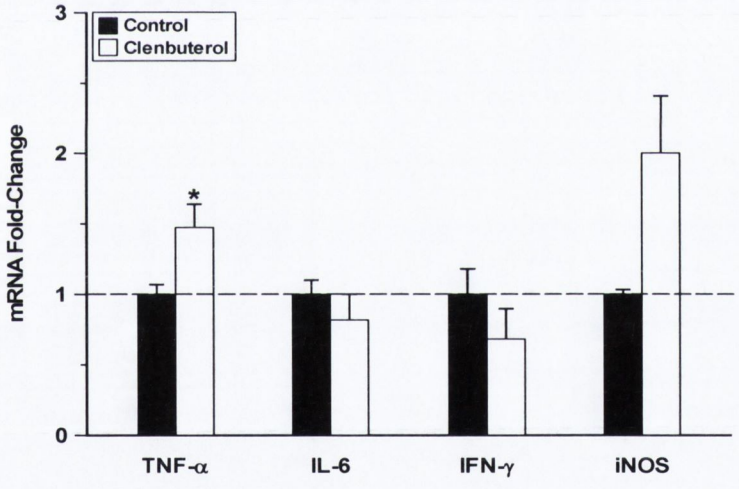


Figure 7.5.4: Chronic clenbuterol treatment does not induce a classical inflammatory response in cortex or hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of TNF-α in hippocampus (b: $P<0.05$) but not cortex. A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of IL-6, IFN-γ or iNOS in cortex or hippocampus. Data are expressed as means with standard error of the mean ($n=8$). *$P<0.05$ versus saline control (Student's *t*-test).

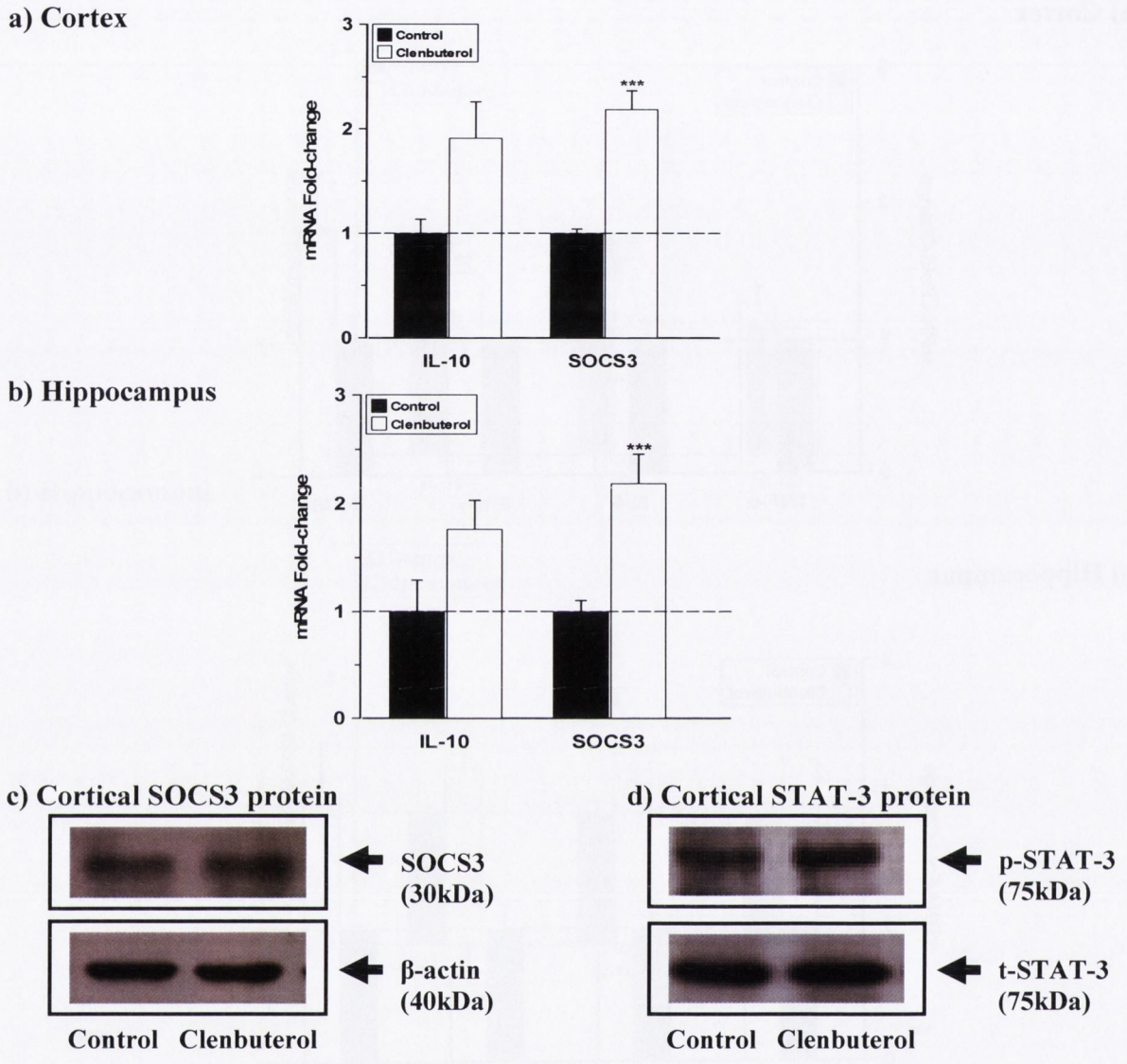


Figure 7.5.5: Chronic clenbuterol treatment increases IL-10 and SOCS3 mRNA expression in cortex and hippocampus and increases SOCS3 and phospho-STAT-3 protein in the cortex

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of IL-10 in cortex or hippocampus. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of SOCS3 in cortex (a: $P<0.001$) and hippocampus (b: $P<0.001$). c) Sample immunoblot depicting increased cortical SOCS3 protein induced by clenbuterol representative of 4 independent experiments. d) Sample immunoblot depicting increased cortical STAT-3 phosphorylation induced by clenbuterol representative of 7 independent experiments. Data are expressed as means with standard error of the mean (n=5-8). ***$P<0.001$ versus saline control (Student's *t*-test).

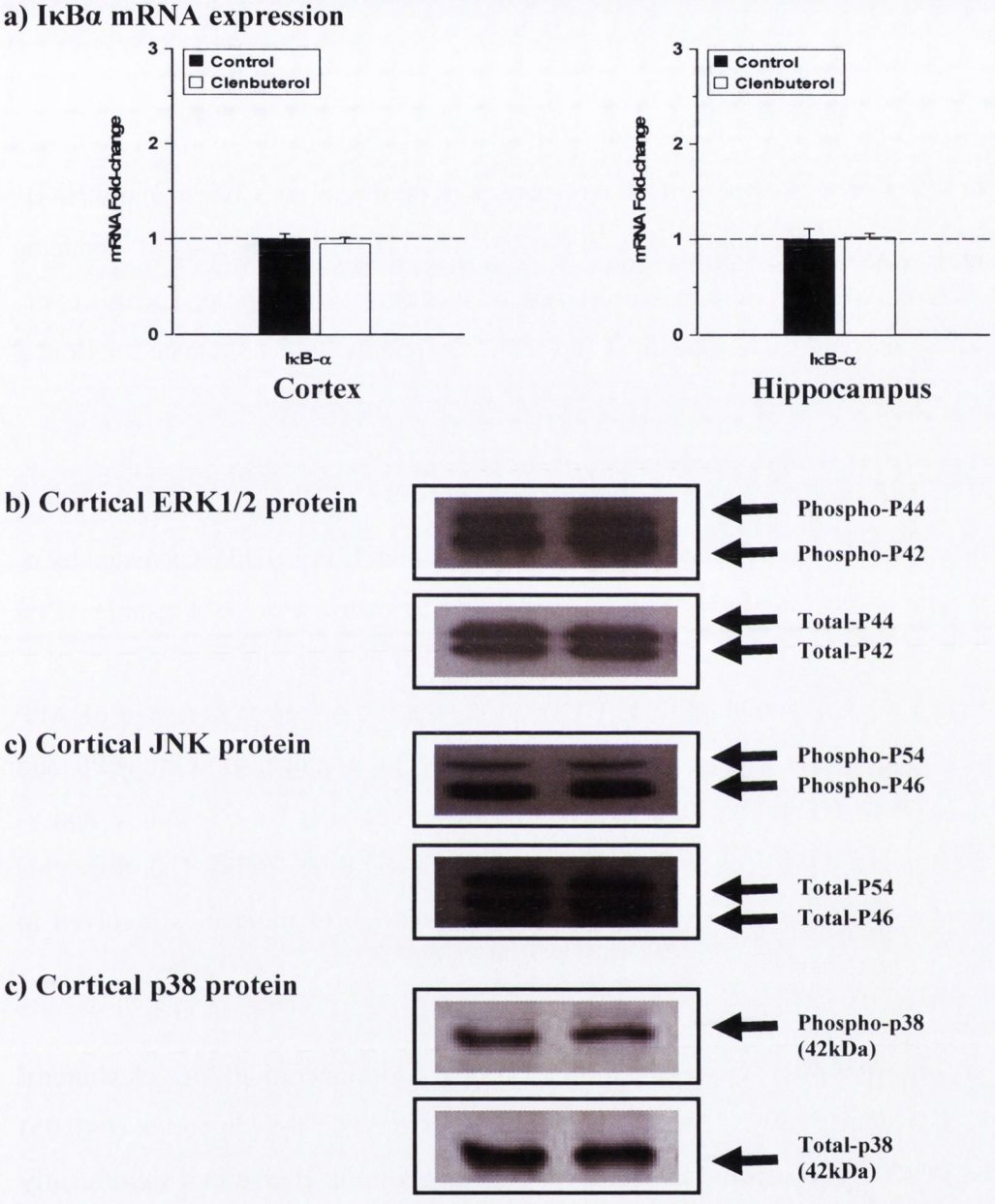


Figure 7.5.6: Chronic treatment with clenbuterol increases the phosphorylation of ERK1/2 but not JNK or p38 in cortex

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. a) A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of IκBα in cortex or hippocampus compared to saline controls. b) Sample immunoblot depicting chronic clenbuterol-induced phosphorylation of ERK1/2 in cortex representative of 4 independent experiments. c) Sample immunoblot demonstrating that chronic clenbuterol treatment does not induce phosphorylation of JNK in cortex representative of 6 independent experiments. d) Sample immunoblot demonstrating that chronic clenbuterol treatment does not induce phosphorylation of p38 in cortex representative of 8 independent experiments. Data are expressed as means with standard error of the mean (n=8).

7.6 Chronic clenbuterol treatment reduces amyloid-β concentrations in cortex and hippocampus

The results presented so far have shown that, similar to the acute studies, chronic treatment with clenbuterol selectively increases members of the IL-1 family in the CNS, namely IL-1β, IL-1α and IL-18. Previous studies have indicated that IL-1 is capable of reducing concentrations of amyloid-β_{1-40} and amyloid-β_{1-42} (Bandyopadhyay *et al.*, 2006; Tachida *et al.*, 2008) therefore, the current study examined the ability of clenbuterol to reduce amyloid-β concentrations in the naive rat brain.

APP is a Type I membrane-spanning protein which is subject to sequential cleavage by either α-secretase or β-secretase on its N-terminal domain (see Chapter 1, Fig. 1.13). Cleavage by α-secretase directs APP processing down the non-amyloidogenic pathway to yield soluble APPα and a membrane bound CTF-α. CTFα can then undergo further cleavage by ε-secretase and γ-secretase to produce a p3 fragment and an AICD. In contrast, β-secretase cleavage of APP directs APP processing down the amyloidogenic pathway to produce soluble APPβ and membrane bound CTFβ. CTFβ can then undergo further processing by ε-secretase and γ-secretase to yield amyloid-β peptides and AICD (McLoughlin & Miller, 2008). Therefore, this study also examined the impact of clenbuterol on the expression of molecules involved in amyloid-β synthesis and clearance.

Amyloid-β_{1-40} & amyloid-β_{1-42} concentrations: Chronic administration of clenbuterol (0.03mg/kg; i.p., bid) significantly reduced concentrations of amyloid-β_{1-40} in cortex ($P<0.05$) and hippocampus ($P<0.001$) compared to saline controls. In addition, clenbuterol significantly reduced concentrations of amyloid-β_{1-42} in cortex ($P<0.05$) and hippocampus ($P<0.05$). [Figure 7.6.1 a,b, Student's *t*-test, $n=8$].

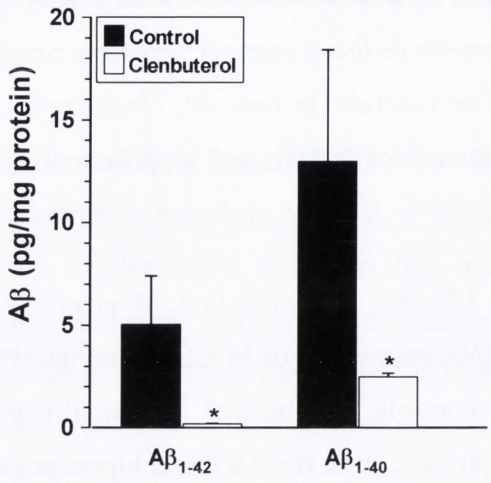
APP & secretases: Chronic administration of clenbuterol (0.03mg/kg; i.p., bid) did not alter the mRNA expression of APP, ADAM-10, ADAM-17 or presenilin-1 in cortex or hippocampus compared to saline controls. Clenbuterol significantly increased the mRNA expression of BACE-1 ($P<0.01$) and presenilin-2 ($P<0.01$) in hippocampus but not cortex. [Figure 7.6.2 a,b, $n=6$-8].

Amyloid-β scavenger receptors: Chronic administration of clenbuterol (0.03mg/kg; i.p., bid) did not alter the mRNA expression of the scavenger receptors Scarb-1 or TLR-2 in cortex or hippocampus compared to saline controls. In contrast, clenbuterol significantly decreased the mRNA expression of RAGE in cortex ($P<0.01$) and hippocampus ($P<0.05$). [Figure 7.6.3 a,b, n=6-8].

M-CSF & M-CSF receptor: Chronic administration of clenbuterol (0.03mg/kg; i.p., bid) significantly increased the mRNA expression of M-CSF in cortex ($P<0.001$) and hippocampus ($P<0.05$) compared to saline controls. Clenbuterol also significantly increased the mRNA expression of the M-CSF receptor in cortex ($P<0.05$) and hippocampus ($P<0.05$). [Figure 7.6.4 a,b, Student's t-test, n=6-8].

Amyloid-β degrading Enzymes: Chronic administration of clenbuterol (0.03mg/kg; i.p., bid) did not alter the mRNA expression of IDE or MMP-9 in cortex or hippocampus compared to saline controls. Clenbuterol significantly increased the mRNA expression of neprilysin in cortex ($P<0.05$) but significantly decreased the expression of neprilysin in hippocampus ($P<0.01$). In contrast, clenbuterol significantly increased the mRNA expression of MMP-2 in cortex ($P<0.001$) and hippocampus ($P<0.05$) and TIMP-1 in cortex ($P<0.01$) and hippocampus ($P<0.01$). [Figure 7.6.5 a,b, n=6-8].

a) Cortex



b) Hippocampus

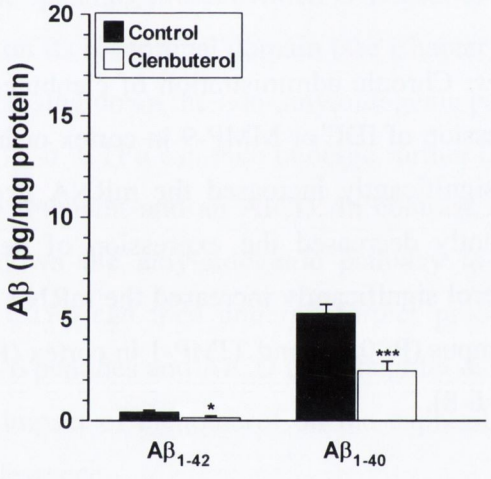
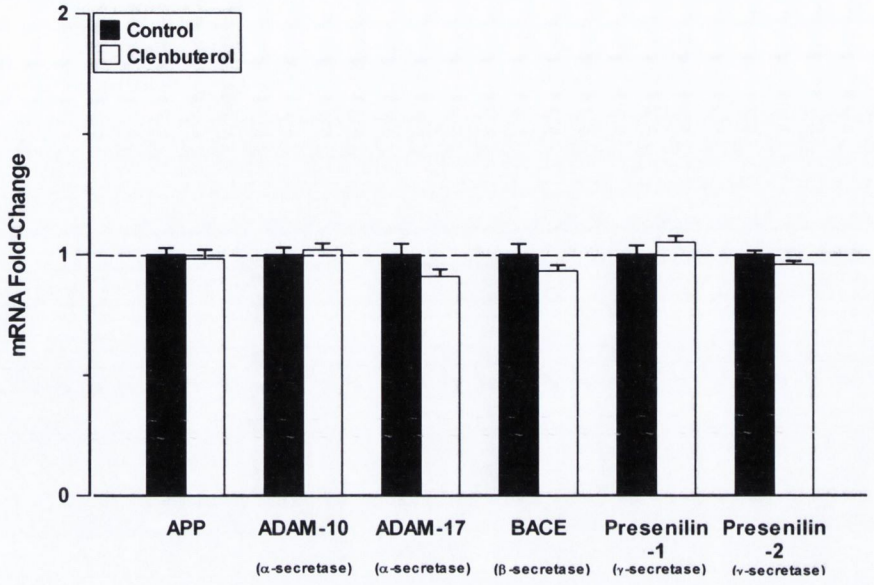


Figure 7.6.1: Chronic clenbuterol treatment reduces amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ concentrations in cortex and hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed a significant effect of treatment on amyloid-$\beta_{1\text{-}40}$ concentrations in cortex (a: $P<0.05$) and hippocampus (b: $P<0.001$). A Student's *t*-test revealed a significant effect of treatment on amyloid-$\beta_{1\text{-}42}$ concentrations in cortex (a: $P<0.05$) and hippocampus (b: $P<0.05$). Data are expressed as means with standard error of the mean ($n=8$). ***$P<0.001$, *$P<0.05$ versus saline control (Student's *t*-test).

a) Cortex



b) Hippocampus

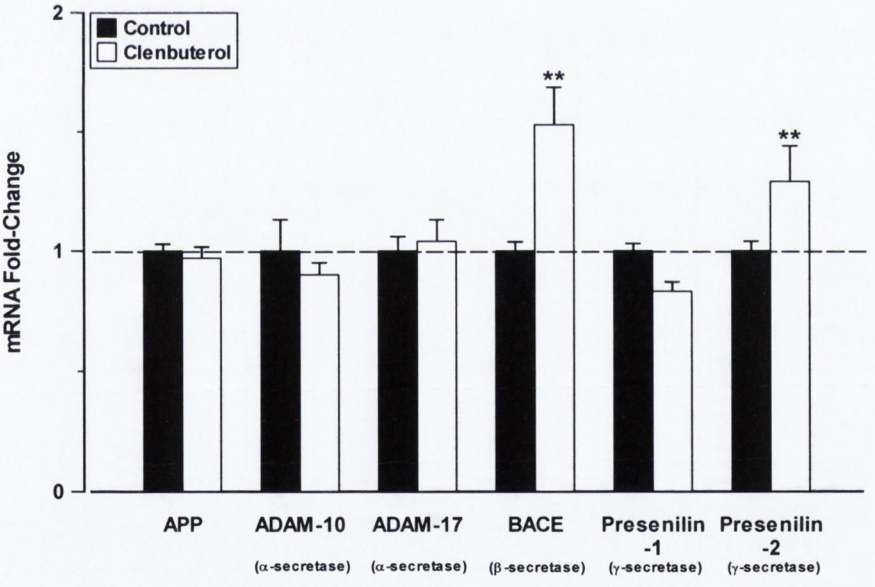
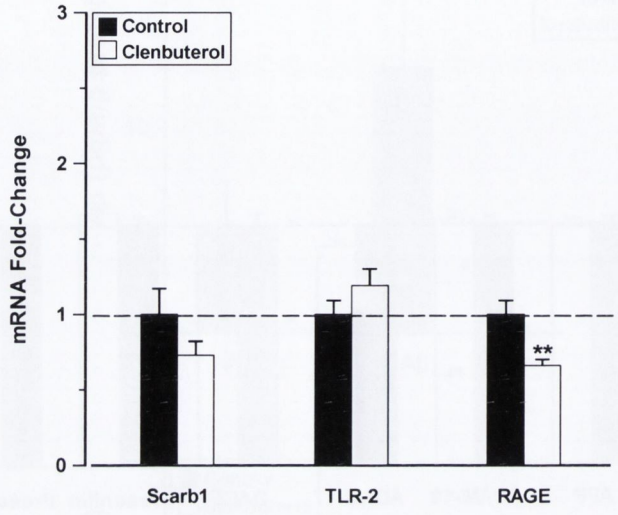


Figure 7.6.2: Chronic clenbuterol treatment largely fails to alter the expression of molecules involved in amyloid-β synthesis in cortex and hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of BACE (b: $P<0.01$) and Presenilin-2 (b: $P<0.01$) in hippocampus but not cortex. A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of APP, ADAM-10, ADAM-17 or Presenilin-1 in cortex or hippocampus. Data are expressed as means with standard error of the mean (n=6-8). **$P<0.01$ versus saline control (Student's *t*-test).

a) Cortex



b) Hippocampus

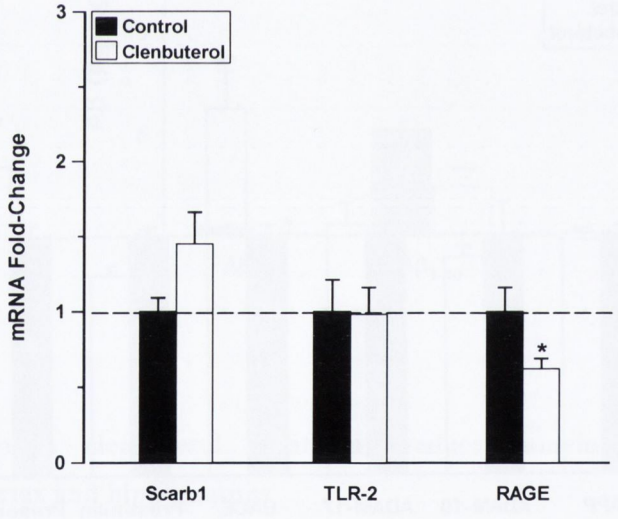
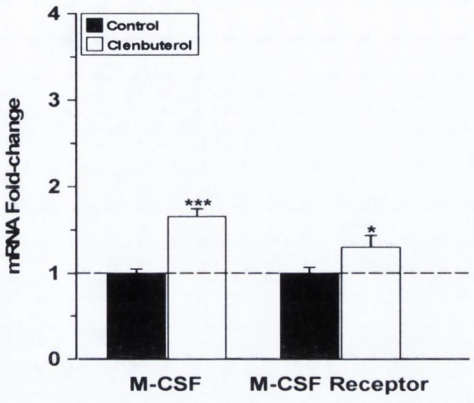


Figure 7.6.3: Chronic clenbuterol treatment reduces RAGE mRNA expression but fails to alter the expression of other amyloid-β scavenger receptors in cortex and hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of Scarb-1 or TLR-2 in cortex or hippocampus. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of RAGE in cortex (a: $P<0.01$) and hippocampus (b: $P<0.05$). Data are expressed as means with standard error of the mean (n=6-8). **$P<0.01$, *$P<0.05$ versus saline control (Student's *t*-test).

a) Cortex



b) Hippocampus

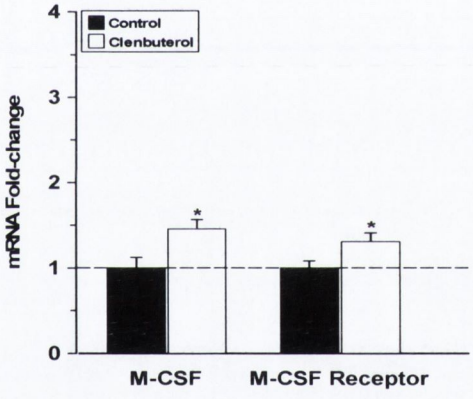
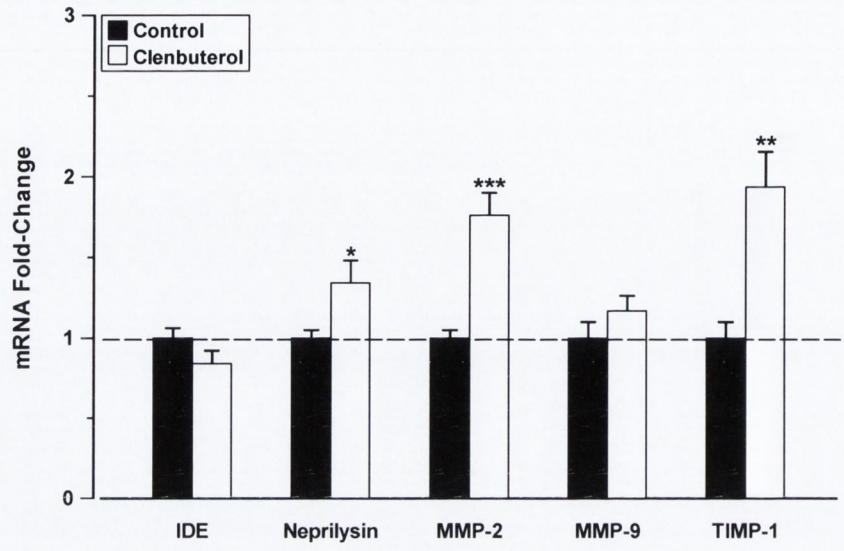


Figure 7.6.4: Chronic clenbuterol treatment increases M-CSF and M-CSF receptor mRNA expression in cortex and hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of M-CSF in cortex (a: $P<0.001$) and hippocampus (b: $P<0.05$). A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of M-CSF receptor in cortex (a: $P<0.05$) and hippocampus (b: $P<0.05$). Data are expressed as means with standard error of the mean (n=6-8). ***$P<0.001$, *$P<0.05$ versus saline control (Student's *t*-test).

a) Cortex



b) Hippocampus

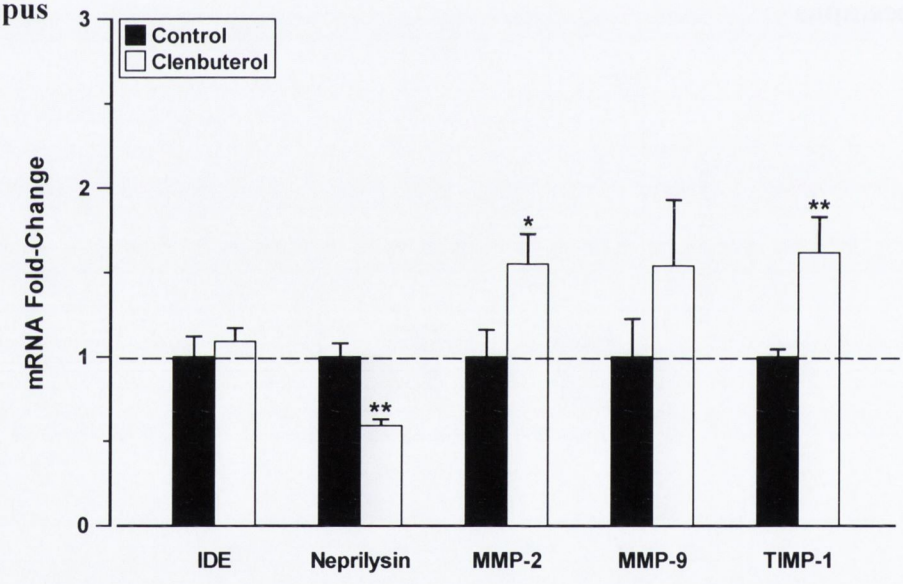


Figure 7.6.5: Chronic clenbuterol treatment increases MMP-2 & TIMP-1 mRNA expression but largely fails to alter the expression of other amyloid-β degrading enzymes in cortex and hippocampus

Clenbuterol (0.03mg/kg) or control (0.89% (w/v) saline) were administered i.p. to rats bi-daily for two-weeks. A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of MMP-2 in cortex (a: $P<0.001$) and hippocampus (b: $P<0.05$) and TIMP-1 in cortex (a: $P<0.01$) and hippocampus (b: $P<0.01$). A Student's *t*-test revealed a significant effect of treatment on the mRNA expression of neprilysin in cortex (a: $P<0.05$) and hippocampus (b: $P<0.01$) compared to saline controls. A Student's *t*-test revealed no significant effect of treatment on the mRNA expression of IDE or MMP-9 in cortex or hippocampus. Data are expressed as means with standard error of the mean (n=6-8). ***$P<0.001$, **$P<0.01$, *$P<0.05$ versus saline control (Student's *t*-test).

7.7 Discussion

7.7.1 Chronic treatment with clenbuterol has no impact on the expression of β-adrenoceptors in cortex or hippocampus

The results presented here demonstrate that repeated administration of low-dose clenbuterol (0.03mg/kg; i.p.) for 14 days does not impact on the mRNA expression of either the β_1- or β_2-adrenoceptor in cortex or hippocampus. Previous studies have shown that repeated administration of clenbuterol can down-regulate the density of β_2-adrenoceptors in the cerebellum (Ordway *et al.*, 1987; Vos *et al.*, 1987). Significantly however, it has also been shown that the repeated administration of low-dose clenbuterol has little impact on the density of β_2-adrenoceptors in cortex or hippocampus (Dooley *et al.,* 1983; O'Donnell & Frazer, 1985). Thus, since chronic administration of clenbuterol was shown here to have no effect on the mRNA expression of β-adrenoceptors in the CNS its ability to induce glial activation was examined.

7.7.2 Chronic clenbuterol treatment induces glial activation in cortex and hippocampus

In line with the acute studies, the results presented here demonstrate that chronic treatment with clenbuterol induces a robust increase in astrocytic activation in both cortex and hippocampus, as indicated by increased GFAP expression. In contrast, clenbuterol was found to cause a modest yet significant increase in the expression of CD11b, a marker of microglial activation, only in the hippocampus. This is notable since astrocytic activation is said to convey neuroprotection whilst microglial activation may have detrimental consequences for the CNS. Furthermore, as previously discussed, these results imply heterogeneity between populations of microglial cells. Since both microglia and astrocytes are capable of acting as antigen-presenting cells within the CNS markers for this were also examined, namely MHC class II and the co-stimulatory molecules CD40 and CD86. While microglia constitutively express MHC class II and CD86 *in vivo* the astrocytic expression of these molecules is limited (De Keyser *et al.*, 2004b). MHC class II is known to engage with T-cell receptors to initiate their activation, however, the expression and engagement of the co-stimulatory molecules CD40, CD80 and CD86 is required in order to provoke a full T-cell response (O'Keefe *et al.*, 2002). Surprisingly, the results presented here show that chronic administration of clenbuterol

increases the expression of MHC class II and CD86 in both cortex and hippocampus but does not alter the expression of CD40. These results are of note since previous studies have shown that microglia which express increased MHC class II and CD86 display the ramified morphology of resting microglia but are capable of phagocytosing myelin without destructive consequences and as such have been proposed to convey a protective effect within the CNS (Bechmann *et al.*, 2001). However, the expression of CD68, a marker of microglial lysosomal activity (Ulvestad *et al.*, 1994; Malm *et al.*, 2008), was found to be unchanged here.

7.7.2 Chronic treatment with clenbuterol activates the IL-1 system and induces phosphorylation of ERK1/2 in rat CNS

As discussed in the previous chapters, stimulation of central β_2-adrenoceptors following acute treatment with clenbuterol increases the mRNA expression of IL-1β, IL-1ra, IL-1 Type II receptor in the CNS. Significantly these effects were found to persist with chronic clenbuterol administration. In contrast, clenbuterol did not alter the expression of IL-33 which is a novel member of the IL-1 family known to be in involved in type 2 immunity owing to its induction of IL-6 and IL-13 expression and actions on mast cells and Th_2-polarized $CD4^+$ T-cells (Hudson *et al.*, 2008). Furthermore, chronic clenbuterol administration did not alter the expression of the IL-1 Type I receptor which is in line with previous reports from acute studies with clenbuterol (McNamee, 2008). Remarkably, increased expression of two other members of the IL-1 family, namely IL-1α and IL-18, was also observed in the current study. Previous reports have shown that IL-18 expression is upregulated in peripheral blood mononuclear cells (PBMCs) in response to stimulation of β_2-adrenoceptors, however, this effect was found to occur in addition to increases in TNF-α and IFN-γ expression (Takahashi *et al.*, 2004). In contrast, the effects of clenbuterol on members of the IL-1 family were shown here to occur in the absence of a classical inflammatory response since, with the exception of increased TNF-α expression in the hippocampus, clenbuterol did not impact on the expression of the pro-inflammatory mediators IL-6, TNF-α, IFN-γ or iNOS. Notably these findings are reflective of previous work which demonstrated that stimulation of microglial β-adrenoceptors increases the mRNA expression of IL-1β but not IL-6 or TNF-α *in vitro* (Tanaka *et al.*, 2002). It has also been reported that noradrenaline and the β_2-adrenoceptor agonist terbutaline can increase intracellular cAMP concentrations in microglia which in turn is shown to suppress the

mRNA expression of IL-6 and TNF-α, however, the authors did not report on effects on IL-1β (Mori *et al.*, 2002).

β-adrenergic agonists are suggested to exert their anti-inflammatory effects by suppressing the activation and subsequent translocation of NFκB to the nucleus (Farmer & Pugin, 2000). In this regard, it has been found that noradrenaline increases levels of IκBα, an inhibitor of NFκB, in neurons which correlated with suppression of iNOS expression (Madrigal *et al.*, 2006). In line with this previous studies have reported that the expression of IκBα, is increased in response to stimulation of central β_2-adrenoceptors following acute clenbuterol administration, however this effect was shown to be transient in nature (Heffernan, 2009). However, the results presented here demonstrate that chronic treatment with clenbuterol does not impact on the expression of IκBα. It has also previously been demonstrated that acute clenbuterol treatment induces phosphorylation of ERK1/2 but not p38 or JNK (Heffernan, 2009). Similarly, chronic clenbuterol treatment induced the phosphorylation of ERK1/2 in the cortex but did not induce p38 or JNK. This is noteworthy since it has previously been shown that noradrenaline is capable of inducing ERK1/2 phosphorylation *in vitro*, an event which was found to be mediated through actions at β-adrenoceptors and was shown to result in the increased expression of IL-1ra and IL-1 Type II receptor (McNamee *et al.*, 2009). This finding is also notable since the induction of ERK1/2 is found to be involved the production of neurotrophins and the survival of certain cell types, for example dopaminergic neurons (Troadec *et al.*, 2002). Furthermore, previous reports have also suggested that IL-1β is capable of stimulating the phosphorylation of ERK1/2 in human pulmonary epithelial cells (Lin *et al.*, 2005a), which is notable given the clenbuterol-induced increase in IL-1β expression observed here. In contrast however, phosphorylation of JNK and NFκB was also reported in that study (Lin *et al.*, 2005a). As already mentioned, clenbuterol does not induce the phosphorylation of either JNK or p38 which is significant since p38 and JNK are known to control the recruitment of leukocytes and the transcription of pro-inflammatory cytokines during the inflammatory process as well as playing an essential role in apoptosis (Ono & Han, 2000; Lin *et al.*, 2005a). Notably, neuronal death has been found to occur due to glia-induced activation of p38 and JNK pathways but not ERK1/2 (Xie *et al.*, 2004).

7.7.3 Chronic treatment with clenbuterol activates IL-10 signalling and its downstream mediator SOCS3

The results from this study show that chronic administration of clenbuterol induces a modest increase in the mRNA expression of the anti-inflammatory cytokine IL-10. Notably, clenbuterol was found to induce the phosphorylation of the IL-10 signalling molecule STAT-3 and the mRNA and protein expression of the downstream mediator of IL-10 signalling SOCS3. This finding is significant since it has been demonstrated that IL-10 is capable of suppressing pro-inflammatory cytokine expression in response to amyloid-β and LPS *in vitro* (Szczepanik *et al.*, 2001). Moreover, these findings also support reports which demonstrate that IL-10 induces phosphorylation of STAT-3, an event which was shown to be critical for the induction of SOCS3 (Qin *et al.*, 2006). Notably, it has been demonstrated that SOCS3 can positively regulate the ERK signalling pathway and inhibit NFκB activation (Qin *et al.*, 2007). The results presented here are in line with previous studies which reported that stimulation of β_2-adrenoceptors either with selective β_2-adrenoceptor agonists or by enhancing noradrenergic tone results in increased expression of IL-10 and its downstream mediator SOCS3 in the CNS of rats under basal conditions. However, it has been suggested that the increase in SOCS3 expression induced by β_2-adrenoceptor activation may be independent of IL-10 signalling since STAT-3 phosphorylation was maximal at 4 hr but IL-10 and SOCS3 display a similar temporal pattern with maximal increases at 4-8 hr (McNamee, 2008). In this regard, it has also been reported that STAT-3 phosphorylation can occur in response to IL-1β expression, an event shown to be mediated by an interplay between p38 and ERK1/2 signalling pathways (Ng *et al.*, 2001; Tanabe *et al.*, 2009). This is noteworthy given the increase in IL-1β expression and STAT-3 phosphorylation found following chronic clenbuterol treatment in this study. Thus, the increase in STAT-3 phosphorylation and SOCS3 expression observed here may be dependent on IL-1 as opposed to IL-10 expression. On the whole, pharmacological increases in IL-10 and SOCS3 by means of stimulation of central β_2-adrenoceptors may have beneficial consequences for the treatment of neuroinflammatory disease.

7.7.4 Chronic treatment with clenbuterol reduces amyloid-β concentrations in rat CNS

The results presented here demonstrate that selective stimulation of central β_2-adrenoceptors with clenbuterol over a 14 day period reduces concentrations of amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ in both cortex and hippocampus. This is significant since dysregulation of the noradrenergic system is a well known feature of Alzheimer's disease (Heneka *et al.*, 2002; Marien *et al.*, 2004). In this regard, previous studies have shown that selective destruction of Locus Coeruleus neurons with the neurotoxin N-(2-chloroethyl)-N-ethyl-2 (DSP-4) potentiates the amyloid-β-induced expression of iNOS, IL-1β, and IL-6; effects which were shown to be attenuated when animals were administered noradrenaline or the non-selective β-adrenoceptor agonist isoproterenol (Heneka *et al.*, 2002). However, in contrast to the results presented here are the findings from a recent study which implied that stimulation of central β_2-adrenoceptors with clenbuterol results in internalization of the receptor and translocation of γ-secretase to late endosomes in the CNS of both naive rats and APPswe/PS1ΔE9 double transgenic mice; an effect which resulted in increased processing of APP and increased amyloid-β accumulation in the transgenic mice. However, such effects were shown to be independent of β_2-adrenoceptor signalling since neither forskolin nor db-cAMP were found to impact on γ-secretase activity and mutations in the β_2-adrenoceptor did not to abolish the effects of agonist stimulation (Ni *et al.*, 2006). Notably, the dose of clenbuterol used in that study was 0.5mg/kg, a dose roughly 17 times higher than that employed here and a much higher dose than could be used therapeutically in humans. Thus, while those authors suggested that γ-secretase associates with the β_2-adrenoceptor during endocytosis, the use of low-dose clenbuterol appears to avoid downregulation of the β_2-adrenoceptor in this study. Thus clenbuterol is demonstrated to yield beneficial effects in the present study owing to effects on β_2-adrenoceptor signalling pathways. However, the therapeutic use of clenbuterol in humans still warrants caution since a recent study has shown that two polymorphisms in the gene for the β_2-adrenoceptor (Gly16Arg & Gln27Glu) increase the risk for Late Onset Alzheimer's disease, due to increased receptor responsiveness to endogenous noradrenaline resulting in enhanced γ-secretase activity and increased amyloid accumulation (Yu *et al.*, 2008).

It was hypothesized that the reduction in amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ concentrations demonstrated here might occur as a result of the selective upregulation of members of the IL-1

family by clenbuterol, since this effect is shown to occur in the absence of a "classical immune response". Incidentally, it has previously been shown that IL-1β can increase the α-secretase mediated cleavage of APP leading to increased concentrations of soluble APPα and C83 and decreased APPβ, amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ concentrations owing to reduced β-secretase cleavage of APP. This effect was suggested to occur due to the selective upregulation of a disintegrin and metalloproteinase (ADAM)-17 (TACE) expression by IL-1β since no effects on ADAM-9, ADAM-10 or BACE1 were observed (Tachida *et al.*, 2008). In addition, IL-1α has also been found to reduce concentrations of amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ *in vitro* in a U373 astrocytoma cell line both intra- and extracellularly. This effect was linked to an increase in the mRNA and protein levels of ADAM-10 and ADAM-17, with a corresponding increase in α-secretase and soluble APPα. Notably, the actions of IL-1α was found to involve activation of ERK, p38 and PI3K signalling pathways but not JNK (Bandyopadhyay *et al.*, 2006). In contrast, while chronic clenbuterol treatment is shown to reduce concentrations of amyloid-β in the current study it did not influence the expression of either ADAM-10 or ADAM-17 at the mRNA level. However, this does not completely rule out any effects of clenbuterol on the enzymatic activity of either ADAM-10 or ADAM-17 since this is a matter which still warrants further investigation. Overall however, it seems unlikely that clenbuterol reduces concentrations of amyloid-β due to an effect on APP processing since it was found that clenbuterol did not alter the expression of APP itself or presenilin-1, presenilin-2 or β-secretase in the cortex, however, clenbuterol was found to increase expression of β-secretase and presenilin-2 in the hippocampus. Notably, previous studies have demonstrated that the γ-secretase cleavage of APP is dependent on activation of a JNK-dependent MAPK pathway (Liao *et al.*, 2004; Lee & Das, 2008). However, it was found that clenbuterol did not alter levels of JNK in the current study.

Another hypothesis regarding the development of Alzheimer's disease suggests that it may result from downregulation of amyloid-β scavenger receptors and degrading enzymes in the CNS. In this regard, microglia isolated from the brains of aged PS1-APP transgenic mice are found to have a two to fivefold decrease in the expression of the scavenger receptors SRA and RAGE. A decrease in the expression of the degrading enzymes insulysin, neprilysin and MMP-9 was also found (Hickman *et al.*, 2008). Incidentally, a deficiency in TLR-2 expression results in the increased accumulation of amyloid-$\beta_{1\text{-}42}$, an effect found to correlate with

deficiencies in spatial and contextual memory (Richard *et al.*, 2008). In spite of this, the results presented here demonstrate that clenbuterol largely fails to alter the expression of the amyloid-β scavenger receptors Scarb-1 or TLR-2. Surprisingly, clenbuterol did significantly reduce the mRNA expression of RAGE. RAGE is a multiligand cell surface receptor whose engagement can result in sustained cellular activation through the induction of pro-inflammatory signalling pathways via NFκB activation and the stimulation of nicotinamide adenine dinucleotide (NADH) oxidase activity, ultimately leading to tissue destruction due to the production of free radicals and oxidative stress (Maccioni *et al.*, 2001; Bierhaus *et al.*, 2005). Furthermore, the results presented here are noteworthy since binding of amyloid-β to RAGE on the surface of neurons has been shown to be neurotoxic (Du Yan *et al.*, 1997). In addition, it has been demonstrated that RAGE is capable of transporting amyloid-β into the brain at concentrations known to play a role in the pathophysiology of Alzheimer's disease (Maccioni *et al.*, 2001).

Reports from the literature demonstrate that microglia are capable of phagocytosing fibrillar and soluble amyloid-β *in vitro*, an event that is homogenous over the entire population of microglia. However, while phagocytosis of amyloid-β does not impact on microglial viability, they are found to slowly re-release intact fibrillar amyloid-β. Thus, while internalization of soluble amyloid-β is known to be non-saturable it appears that microglia are incapable of degrading amyloid-β. This is in contrast to reports on macrophages which are found to be capable of phagocytosing and degrading amyloid-β *in vitro* (Chung *et al.*, 1999; Majumdar *et al.*, 2007). The inability of microglia to degrade amyloid-β is surprising since microglia are derived from macrophage lineage and therefore it would be expected that these cell types would play a similar role in the clearance of extracellular debris. Significantly, it has been found that when microglial lysosomes are acidified, by treatment with M-CSF or through the activation of PKA by forskolin, the ability of microglia to degrade fibrillar amyloid-β is recovered (Majumdar *et al.*, 2007). A study by Kawata *et al.*, (2005) showed that animals with the osteopetrosis mutation (op/op) have a reduced number of macrophages, possess no systemic M-CSF and have reduced numbers of microglia in their cortices. Furthermore, these animals display an increased number of amyloid-β plaques in their cerebral cortices. Notably, injection of M-CSF was found to reduce levels of amyloid-β deposits. Furthermore, microglia were found to have enhanced survival rates in the presence of toxic levels of amyloid-β when

exposed to M-CSF (Du Yan *et al.*, 1997). Notably, the results presented here demonstrate that chronic clenbuterol treatment increases the expression of M-CSF and the M-CSF receptor, an effect which might be speculated to account for the reduced concentrations of amyloid-β observed herein. However, as discussed earlier the expression of CD68, a marker of microglial lysosomal activity, was unaltered by treatment with clenbuterol.

The findings from this study also show that treatment with clenbuterol increases the mRNA expression of MMP-2 in rat cortex and hippocampus. This is a noteworthy finding since it has previously been shown that MMP-2 (gelatinase A), an extracellular Zn-metalloproteinase, is capable of cleaving amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ *in vitro* by hydrolysis at the Lys16-Leu17, Leu34-Met35 and Met35-Val36 peptide bonds (Roher *et al.*, 1994; Filiz *et al.*, 2008). In addition, MMP-2 has also been found to be capable of degrading insoluble amyloid-β 40-42 residues long purified from Alzheimer's disease brains, an event again shown to occur through cleavage at Lys16-Leu17, Leu34-Met35 and Met35-Val36 peptide bonds (Roher *et al.*, 1994). In contrast, MMP-9 (gelatinase B), which has also been found to cleave amyloid-β at Lys16-Leu17, Ala30-Ile31, Leu34-Met35 and Gly 37-Gly38 peptide bonds (Filiz *et al.*, 2008), was not induced by clenbuterol. However, it appears that, while MMP-2 is capable of enzymatically degrading soluble monomers of amyloid-β, it is incapable of degrading the aggregated peptide (Roher *et al.*, 1994). Thus, the ability of clenbuterol-induced MMP-2 to clear amyloid-β warrants further investigation, since the results shown here only reveal increased expression of this molecule at the mRNA level and did not investigate if such MMP-2 was functional. This will be important to evaluate since the expression of the endogenous inhibitor of MMPs TIMP-1 was also found to be significantly increased in response to clenbuterol. However, this increased TIMP-1 expression might be beneficial as it might act to prevent excessive activation of MMP-2. Furthermore it has been noted that TIMPs also have the capacity to act as growth factors (Pagenstecher *et al.*, 1998).

7.7.5 Summary

Overall the results presented here show that chronic administration of low-dose clenbuterol does not impact on the mRNA expression of either the β_1- or β_2-adrenoceptor in rat CNS. In addition, chronic treatment with clenbuterol was shown to induce glial activation and

selectively increase the expression of the IL-1 system, in the absence of a classical inflammatory response. Furthermore, clenbuterol was demonstrated to induce activation of the ERK1/2 signalling pathway, while having no impact on NFκB activation or that of the pro-apoptotic MAPKs p38 or JNK. In addition, clenbuterol increased the expression of IL-10 and induced activation of its signalling molecule STAT-3 and down-stream mediator SOCS3. However, the activation of STAT-3 might also be accounted for by increased IL-1 expression since the literature demonstrates that IL-1 is capable of inducing STAT-3 phosphorylation. Significantly, chronic administration of clenbuterol reduced concentrations of amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ in rat cortex and hippocampus. This effect is suggested to occur owing to increased degradation of amyloid-β since no effect on APP processing was observed. In this regard, the reduction in amyloid-β concentrations was found to correspond with a significant increase in the mRNA expression of the amyloid-β degrading enzyme MMP-2. In addition, clenbuterol was found to increase the expression of the cytokine M-CSF and its receptor, which has the capacity to acidify lysosomes thus inducing the phagocytosis and degradation of amyloid-β. Notably, clenbuterol was also shown to decrease the expression of RAGE, a scavenger receptor which implicated in amyloid-β neurotoxicity. Overall, the findings from this study suggest that activation of central β_2-adrenoceptors could represent a new therapeutic target for the treatment of Alzheimer's disease and other neuroinflammatory conditions.

Chapter 8

Concluding Remarks

8.1 An inflammatory challenge downregulates β_2-adrenoceptor expression and function *in vitro* but not *in vivo*

The results reported in this thesis have revealed a number of significant findings regarding the regulation and function of β_2-adrenoceptors on glial cells. The findings from the *in vitro* studies demonstrate that stimulation of β_2-adrenoceptors on glial cells, either with noradrenaline or selective β_2-adrenoceptor agonists, can elicit anti-inflammatory and neurotrophic actions in response to the inflammagen LPS. However, it was also found that an inflammatory stimulus of LPS + IFN-γ can down-regulate expression of the β_2-adrenoceptor at the both the mRNA and cell surface levels. In addition, it was found that LPS + IFN-γ reduces the accumulation of intracellular cAMP in response to stimulation of the β_2-adrenoceptor with the β_2-agonist salbutamol. This was found to be mediated by direct actions on the receptor itself and was not due to any effects on adenylate cyclase, since no effect was found in response to direct activation of adenylate cyclase by forskolin. These findings are noteworthy since the β_2-adrenoceptor has been shown to be absent from astrocytes in the brains of people from MS (De Keyser *et al.*, 2004), an effect which could impede the neuroprotective effects of endogenous noradrenaline by disrupting the anti-inflammatory and neurotrophic actions of this neurotransmitter. Moreover, MS is characterized by an unresolved inflammatory response, thus the findings presented here suggest that neuroinflammation may contribute to the absence of the β_2-adrenoceptor observed in MS. Notably, while the β_2-adrenoceptor has been found to be absent from astrocytes in MS brain, its expression on microglia has not been examined. However, the results presented here demonstrate that an inflammatory challenge can reduce β_2-adrenoceptor expression on both astrocytes and microglia. Significantly, the findings from this study also demonstrate that the synthetic glucocorticoid dexamethasone inhibits the LPS + IFN-γ-induced downregulation of β_2-adrenoceptor expression. This is noteworthy since glucocorticoids are widely used for the treatment of neuroinflammatory disease (Barnes, 2006). Furthermore, it was demonstrated that dexamethasone can also increase basal levels of β_2-adrenoceptor expression in its own right, an effect which might account for its therapeutic efficacy in MS. However, it remains to be determined whether the dexamethasone-induced inhibition of β_2-adrenoceptor downregulation translates to restoration of receptor responsiveness to agonist stimulation.

In contrast, when LPS was given systemically to rats it was found to have no impact on the expression of central β_2-adrenoceptors in an *in vivo* situation. Furthermore, LPS did not impact on the function of central β_2-adrenoceptors since, in line with previous reports (McNamee, 2008), the expression of the anti-inflammatory mediators IL-1ra, IL-1 Type II receptor, IL-10 and SOCS3 were all induced in response to stimulation of β_2-adrenoceptors with clenbuterol and these effects were not impeded by prior exposure to LPS. Thus, these studies demonstrate that while *in vitro* models are useful for examining molecular mechanisms involved in physiological processes in detail, the results do not always extrapolate to an *in vivo* model, where cells are in their normal environment and are under the influence of other factors. It is possible that in the *in vivo* model used here, the systemic administration of LPS induces a glucocorticoid surge owing its well established action on the HPA-axis and that, in a similar manner to the effect of dexamethasone on β_2-adrenoceptors *in vitro,* this inhibits the ability of LPS to downregulate the β_2-adrenoceptor *in vivo*. It is reported that the HPA-axis is dysfunctional in many neuroinflammatory disease states (Stefferl *et al.*, 1999; Beishuizen & Thijs, 2003; Heesen *et al.*, 2007), thus this may account for the absence of β_2-adrenoceptors observed on astrocytes in MS brain. Furthermore, the glial cultures used for the *in vitro* studies presented here were prepared from neonatal rat brain, however the *in vivo* results were obtained from studies carried out on adult rats. The purpose of preparing cells cultures from neonatal rats is owed to the fact that they are easier to culture and maintain than adult cells. However, it is possible that the β_2-adrenoceptor is more susceptible to downregulation by an inflammatory stimulus at early stages of development. More likely is the matter that glia grown *in vitro,* in the absence of neurons, display altered receptor expression, potentially rendering the β_2-adrenoceptor more susceptible to downregulation *in vitro* (Hertz *et al.*, 2004).

Surprisingly it appears that *in vivo,* prior exposure to LPS may increase the sensitivity of central β_2-adrenoceptors to subsequent stimulation with clenbuterol. As such, the results presented here demonstrate that clenbuterol induces the expression of IL-1β under basal conditions, however, when animals were exposed to LPS prior to clenbuterol administration, there was a synergistic effect between LPS and clenbuterol on IL-1β. In a similar manner, it was shown that clenbuterol induces astrocytic activation under basal conditions, as indicated by increased GFAP expression, and again when animals were exposed to LPS prior to clenbuterol a synergistic effect on astrocytic activation was observed. Thus, it appears that

exposure to LPS may "prime" cells of the CNS, primarily astrocytes, resulting in an exaggerated response to later immune stimulation. "Priming" is a phenomenon well documented in the literature and most commonly associated with microglia. Recent literature has suggested that the aged brain is in a constant state of inflammation and that a subset of microglia are in a persistent "primed" state. Exposure of such "primed" microglia to a subsequent immune stimulus has been found to result in an exaggerated inflammatory response (Dilger & Johnson, 2008; Sparkman & Johnson, 2008). In contrast, the "priming" effect that is demonstrated here is shown to be specific to effects on astrocytes and the IL-1 system, as it is not seen with any of the other molecules examined. In support of this, it has previously been shown that clenbuterol-induced IL-1 co-localises with astrocytes (Tanveneer, 2008). Whether this effect is beneficial or detrimental remains to be fully determined. Overall, while inflammation is normally a beneficial response for the host, where prior exposure to inflammation occurs it can have detrimental consequences for the brain.

8.2 Clenbuterol stimulates central β_2-adrenoceptors and elicits anti-inflammatory effects in the CNS

The findings presented here reveal that peripheral administration of the β_2-adrenoceptor agonist clenbuterol can elicit anti-inflammatory effects in the CNS. As such, the results indicate that clenbuterol induces the expression of the anti-inflammatory cytokine IL-10 and its downstream signalling molecule SOCS3, an event likely mediated through the phosphorylation and translocation of STAT-3 to the nucleus. These findings are significant since IL-10 is found to have beneficial actions in neuroinflammatory disease states (see Kastin *et al.*, 2003; Sloane *et al.*, 2009). Moreover, IL-10 is known to have neuroprotective effects since it has been shown that blockade of IL-10 results in exacerbated neuronal cell death in response to LPS (Park *et al.*, 2007). In addition, studies have also shown that IL-10 can prevent glutamate induced cell-death and can regulate apoptosis (Bachis *et al.*, 2001; Boyd *et al.*, 2003). However, in spite of its beneficial effects in the CNS, peripherally administered IL-10 is limited in its ability to cross the BBB. Thus, pharmacological enhancement of its expression by the stimulation of central β_2-adrenoceptors, such as through the peripheral administration of clenbuterol, may represent a new therapeutic target for the treatment of neuroinflammatory and neurodegenerative disease. In further support of this, low-dose

clenbuterol was shown to elicit anti-inflammatory actions in the CNS by suppressing the LPS-induced expression of pro-inflammatory cytokines, chemokines and adhesion molecules, reinforcing the idea that clenbuterol may be beneficial for the treatment of neuroinflammation.

Significantly the results presented here demonstrate that peripheral administration of clenbuterol can regulate the central IL-1 system. In this regard, increased expression of the endogenous antagonist of the IL-1 system, IL-1ra, and the decoy receptor for IL-1 signalling, IL-1 Type II receptor, were both found to be increased by clenbuterol in a dose-dependent manner. Surprisingly, clenbuterol also selectively increased the expression of IL-1β, IL-1α and IL-18, in the absence of an effect on other pro-inflammatory mediators. This regulation of the IL-1 family was found to be dependent on actions of clenbuterol at β_2-adrenoceptors, as opposed to β_1-adrenoceptors, since the increase in expression could be blocked by ICI 118,551 but not metoprolol. These findings are noteworthy since it is known that while IL-1 is generally regarded as the prototypical pro-inflammatory cytokine, its expression in the absence of other inflammatory mediators does not contribute to neurotoxicity (Rothwell & Luheshi, 2000). Furthermore, it has been shown that the expression of IL-1 is essential for the induction of certain growth factors and that IL-1-deficient mice display impaired remyelination (Nguyen *et al.*, 2002). In addition, the importance of IL-1 expression in the CNS is reinforced by the finding that chronic blockade of IL-1 signalling by overexpression of IL-1ra results in atrophy of the brain (Oprica *et al.*, 2007). Recent reports have also suggested that IL-1 can clear amyloid-β through actions on APP processing mechanisms (Tachida *et al.*, 2008; Bandyopadhyay *et al.*, 2006). Thus, the selective upregulation of IL-1 expression by clenbuterol may have beneficial consequences for the CNS, but this remains to be determined.

The findings from these studies also demonstrate that clenbuterol induces a profound decrease in the locomotor activity of rats, an effect which was initially suspected to be a consequence of the increased expression of IL-1β in the brain. However, when the peripheral effects of clenbuterol were examined in closer detail it was found that clenbuterol does not induce classical sickness behaviour when compared with the endotoxin LPS. Instead, it appears more likely that clenbuterol induces a stress response, owing to its profound effects on heart rate, and that this is responsible for the clenbuterol-induced reduction in food and water consumption and locomotor activity. While ICI 118,551 was found to block the negative

peripheral effects of clenbuterol, it also blocks the effects of clenbuterol on immune processes in the CNS. Thus, further studies will be necessary to attempt to dissociate the peripheral and central effects of clenbuterol in order for it to be used therapeutically for the treatment of neuroinflammation. Encouragingly reports have already suggested that the peripheral effects of clenbuterol subside with repeated administration (Hoey *et al.*, 1995).

8.3 Chronic stimulation of central β_2-adrenoceptors reduces amyloid load in the CNS

Results from this thesis also demonstrate that chronic administration of clenbuterol activates astrocytes and reduces concentrations of amyloid-$\beta_{1\text{-}40}$ and amyloid-$\beta_{1\text{-}42}$ in the CNS. This reduction in amyloid was initially hypothesized to occur due to the selective upregulation of IL-1 by clenbuterol since previous reports have shown that IL-1 is capable of directing amyloid processing down the non-amyloidogenic pathway leading to reduced concentrations of amyloid-β (Bandyopadhyay *et al.*, 2006; Tachida *et al.*, 2008). However, it appears more likely that the reduction in amyloid induced by clenbuterol occurs due to enhanced degradation of amyloid-β as opposed to any effects on APP processing since clenbuterol largely failed to alter the expression of the secretases responsible for amyloid production. Instead it appears that the reduction in amyloid-β occurs due to astrocytic activation induced by clenbuterol and the enhanced expression of the amyloid-β degrading enzyme MMP-2. In this regard, the ability of MMP-2 to degrade amyloid-β has been well established in the literature (Roher *et al.*, 1994; Yin *et al.*, 2006). In further support of this idea is a recent report which found that reduced levels of MMP-2 in the CSF of Alzheimer's patients correlates with low levels of amyloid-β concentrations in the CSF, an effect which may increase the accumulation of amyloid plaques in the CNS (Mlekusch *et al.,* 2009). In addition, the results presented here demonstrate that clenbuterol increases the expression of the cytokine M-CSF, which might act to acidify glial lysosomes and thus aid to increase degradation of phagocytosed amyloid-β as has previously been alluded to in the literature (Kawata *et al.*, 2005; Majumdar *et al.*, 2007; Filiz *et al.*, 2008). Significantly, clenbuterol was also found to reduce the expression of RAGE, a receptor which has been implicated in mediating amyloid-induced neurotoxicity and transporting amyloid into the brain at concentrations known to play a role in the pathophysiology of Alzheimer's disease (Du Yan *et al.*, 1997; Maccioni *et al.*, 2001; Bierhaus *et al.*, 2005).

Alzheimer's disease is currently the main cause of dementia in the world and is said to be the fourth major cause of death following cancer, heart disease and stroke (Maccioni *et al.*, 2009). The WHO report for 2009 indicates that there are 18 million people currently suffering from Alzheimer's disease worldwide and this is predicted to double to 34 million by the year 2025 (http://www.searo.who.int). All in all Alzheimer's disease is a devastatingly debilitating disease which still evades medical treatment. While the results of the present study are in contrast to those of Ni *et al.*, (2006) who showed that administration of clenbuterol can increase amyloid load in the CNS due to endocytosis of γ-secretase complex with the β_2-adrenoceptor, the dose employed in that study was approximately 17 times that used in the present study, and a much higher dose than could be used therapeutically in humans. In addition, the effects of clenbuterol reported in that study were also shown to occur in the absence of β_2-adrenoceptor signalling, thus stimulation of β_2-adrenoceptors and their downstream signalling pathways may hold potential in aiding clearance of amyloid-β and thus be beneficial for the treatment of Alzheimer's disease. However, caution must be taken with this since, while clenbuterol is found to elicit beneficial effects in the CNS, the profound peripheral effects induced by the drug still warrant further investigation in order to determine the mechanisms by which they occur and by which they can be overcome.

8.4 Conclusion

Overall the findings presented here are consistent with the literature and demonstrate that stimulation of β_2-adrenoceptors on glial cells elicits anti-inflammatory and neurotrophic effects both *in vitro* and *in vivo*. While anti-inflammatory cytokines and neurotrophins can have beneficial actions in the CNS, their therapeutic use is limited owing to the restricted ability of such molecules to cross the BBB. Significantly, despite the fact that inflammation was found to reduce the expression and responsiveness of glial β_2-adrenoceptors *in vitro,* no such effect was found *in vivo*. Therefore, stimulation of central glial β_2-adrenoceptors and activation of their downstream signalling pathways holds promise as a new therapeutic strategy for the treatment of neuroinflammatory disease.

Chapter 9

Future Directions

9.1 Future Directions

The findings presented in this thesis have yielded a number of important leads for future research as outlined below:

9.1.1 In Vitro Studies

1) First and foremost it remains to be determined if the effects of dexamethasone also translate to restoration of β_2-adrenoceptor protein expression at the cell surface level and, most importantly, restoration of receptor responsiveness to stimulation with agonists.

2) The signalling mechanisms involved in the down-regulation of β_2-adrenoceptor should be examined, in particular effects of NFκB, p38, JNK, ERK1/2 and STAT-1 inhibitors either alone or in combination should be examined since all of these pathways may be activated by treatment with LPS + IFN-γ. Furthermore, the impact of COX-II inhibitors on the expression of the β_2-adenoceptor should also be examined since it has been demonstrated in the literature that the COX-II inhibitors indomethacin and NS-398 can inhibit IL-1β-induced β_2-adrenoceptor downregulation in HASM cells.

3) The mechanisms by which β_2-adrenoceptor downregulation occurs should be examined further. Specifically, co-immunoprecipitation studies should be carried out to examine the involvement of GRKs and β-arrestins. In addition, the involvement of β-ARB should be examined since an increase in β-ARB expression has been shown in the literature to correlate with downregulation of β_2-adrenoceptor expression. In addition, it has also been shown that β-ARB expression can be reduced by dexamethasone resulting in increased β_2-adrenoceptor expression.

9.1.2 In Vivo Studies

1) It was demonstrated here that systemic LPS administration does not impact on the expression or function of central β_2-adrenoceptors *in vivo*, however, future studies should examine the expression and function of central β_2-adrenoceptors in chronic inflammatory conditions, for example in an EAE model.

2) Owing to the discrepancy between the *in vitro* and *in vivo* data it is plausible that the glucocorticoid surge which occurs following administration of LPS *in vivo* could prevent the reduction in β_2-adrenoceptor expression in a similar manner to that observed with dexamethasone *in vitro*. Thus, the effects of blocking glucocorticoid release *in vivo* using selective glucocorticoid antagonists should be investigated to determine if this would induce β_2-adrenoceptor downregulation in response to LPS treatment.

3) The role of IL-1 in clenbuterol-induced neuroprotection should be investigated further in IL-1RI knock-out mice. In particular, the literature suggests that the increases in IL-10 may be dependent on IL-1-induced STAT-3 phosphorylation, therefore this warrants investigation.

4) The potential neuroprotective effects of stimulation of the β_2-adrenoceptor with the β_2-agonist clenbuterol *in vivo* should be investigated further. In this regard, the impact of clenbuterol on chronic neuroinflammation should be examined, such as in EAE and models of Alzheimer's disease. In addition, the involvement of clenbuterol-induced MMP-2 in the clearance of amyloid-β should be investigated further both *in vitro* and additionally *in vivo* in MMP-2 knock-out mice.

5) Further studies will also be necessary to determine if the negative peripheral effects of clenbuterol can be dissociated from the beneficial effects observed in the CNS. In this regard, the use of hydrophilic β-adrenoceptor antagonists which are incapable of crossing the BBB should be investigated. In addition, to determine if the clenbuterol-induced decrease in locomotor activity is mediated by the increased expression of IL-1 in the brain the behavioral effects of clenbuterol should be examined in IL-1RI knock-out mice. Further studies should also investigate the peripheral effects of chronic clenbuterol treatment to determine if the negative effects observed in the acute studies subside with time, and in order to establish whether clenbuterol represents a potential therapy for the treatment of neuroinflammatory disease.

References

Adler MW, Geller EB, Chen X & Rogers TJ. (2005). Viewing chemokines as a third major system of communication in the brain. *AAPS J* **7,** E865-870.

Aksoy MO, Mardini IA, Yang Y, Bin W, Zhou S & Kelsen SG. (2002). Glucocorticoid effects on the β-adrenergic receptor-adenylyl cyclase system of human airway epithelium. *J Allergy Clin Immunol* **109**, 491-497.

Akundi RS, Candelario-Jalil E, Hess S, Hull M, Lieb K, Gebicke-Haerter PJ & Fiebich BL. (2005). Signal transduction pathways regulating cyclooxygenase-2 in lipopolysaccharide-activated primary rat microglia. *Glia* **51,** 199-208.

Allan SM, Tyrrell PJ, Rothwell NJ. (2005). Interleukin-1 and neuronal injury. *Nat Rev Immunol.* **5,** 629-640.

Allan SM. (2000). The role of pro- and anti-inflammatory cytokines in neurodegeneration. *Ann N Y Acad Sci* **917,** 84-93.

Aloisi F, Ria F & Adorini L. (2000). Regulation of T-cell responses by CNS antigen-presenting cells: different roles for microglia and astrocytes. *Immunol Today* **21,** 141-147.

Andersson J. (2005). The inflammatory reflex--introduction. *J Intern Med* **257,** 122-125.

Aoki C. (1992). Beta-adrenergic receptors: astrocytic localization in the adult visual cortex and their relation to catecholamine axon terminals as revealed by electron microscopic immunocytochemistry. *J Neurosci* **12,** 781-792.

Aschner M. (1998). Immune and inflammatory responses in the CNS: modulation by astrocytes. *Toxicol Lett* **102-103,** 283-287.

References

Avital A, Goshen I, Kamsler A, Segal M, Iverfeldt K, Richter-Levin G, Yirmiya R. (2003). Impaired interleukin-1 signaling is associated with deficits in hippocampal memory processes and neural plasticity. *Hippocampus* **13**, 826-834.

Bachis A, Colangelo AM, Vicini S, Doe PP, De Bernardi MA, Brooker G & Mocchetti I. (2001). Interleukin-10 prevents glutamate-mediated cerebellar granule cell death by blocking caspase-3-like activity. *J Neurosci* **21,** 3104-3112.

Bajetto A, Bonavia R, Barbero S, Florio T & Schettini G. (2001). Chemokines and their receptors in the central nervous system. *Front Neuroendocrinol* **22,** 147-184.

Baker JG. (2005). The selectivity of beta-adrenoceptor antagonists at the human beta1, beta2 and beta3 adrenoceptors. *Br J Pharmacol.* **144,** 317-322.

Ballestas ME & Benveniste EN. (1997). Elevation of cyclic AMP levels in astrocytes antagonizes cytokine-induced adhesion molecule expression. *J Neurochem* **69,** 1438-1448.

Bandyopadhyay S, Hartley DM, Cahill CM, Lahiri DK, Chattopadhyay N & Rogers JT. (2006). Interleukin-1alpha stimulates non-amyloidogenic pathway by alpha-secretase (ADAM-10 and ADAM-17) cleavage of APP in human astrocytic cells involving p38 MAP kinase. *J Neurosci Res* **84,** 106-118.

Barnes PJ. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci (Lond)* **94,** 557-572.

Barnes PJ. (2006). Corticosteroids: the drugs to beat. *Eur J Pharmacol* **533,** 2-14.

Basu A, Krady JK & Levison SW. (2004). Interleukin-1: a master regulator of neuroinflammation. *J Neurosci Res* **78,** 151-156.

Becher B, Prat A, Antel JP. (2000). Brain-immune connection: immuno-regulatory properties of CNS-resident cells. *Glia.* **29,** 293-304.

Bechmann I, Peter S, Beyer M, Gimsa U & Nitsch R. (2001). Presence of B7--2 (CD86) and lack of B7--1 (CD(80) on myelin phagocytosing MHC-II-positive rat microglia is associated with nondestructive immunity in vivo. *FASEB J* **15,** 1086-1088.

Beglopoulos V & Shen J. (2006). Regulation of CRE-dependent transcription by presenilins: prospects for therapy of Alzheimer's disease. *Trends Pharmacol Sci* **27,** 33-40.

Beishuizen A & Thijs LG. (2003). Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *J Endotoxin Res* **9,** 3-24.

Belpaire FM, De Smet F, Vynckier LJ, Vermeulen AM, M. T. Rosseel MT, Bogaert MG & Chauvelot-Moachon L. (1990). Effect of aging on the pharmacokinetics of atenolol, metoprolol and propranolol in the rat. *J Pharmacol Exp Ther.* **254**, 116-122.

Benveniste EN, Huneycutt BS, Shrikant P & Ballestas ME. (1995). Second messenger systems in the regulation of cytokines and adhesion molecules in the central nervous system. *Brain Behav Immun* **9,** 304-314.

Bertazza L & Mocellin S. (2008). Tumor necrosis factor (TNF) biology and cell death. *Front Biosci* **13,** 2736-2743.

Best JD, Jay MT, Otu F, Ma J, Nadin A, Ellis S, Lewis HD, Pattison C, Reilly M, Harrison T, Shearman MS, Williamson TL, Atack JR. (2005). Quantitative measurement of changes in amyloid-beta(40) in the rat brain and cerebrospinal fluid following treatment with the gamma-secretase inhibitor LY-411575 [N2-[(2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N1-[(7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl]-L-alaninamide]. *J Pharmacol Exp Ther.* **313,** 902-8.

Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, Stern DM & Nawroth PP. (2005). Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med* **83,** 876-886.

Blatteis CM, Li S, Li Z, Feleder C & Perlik V. (2005). Cytokines, PGE2 and endotoxic fever: a re-assessment. *Prostaglandins Other Lipid Mediat* **76,** 1-18.

Block ML & Hong JS. (2005). Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Prog Neurobiol* **76,** 77-98.

Bluthe RM, Laye S, Michaud B, Combe C, Dantzer R & Parnet P. (2000). Role of interleukin-1beta and tumour necrosis factor-alpha in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type I receptor-deficient mice. *Eur J Neurosci* **12,** 4447-4456.

Boche D, Cunningham C, Gauldie J & Perry VH. (2003). Transforming growth factor-beta 1-mediated neuroprotection against excitotoxic injury in vivo. *J Cereb Blood Flow Metab* **23,** 1174-1182.

Boyd ZS, Kriatchko A, Yang J, Agarwal N, Wax MB & Patil RV. (2003). Interleukin-10 receptor signaling through STAT-3 regulates the apoptosis of retinal ganglion cells in response to stress. *Invest Ophthalmol Vis Sci* **44,** 5206-5211.

Bowie A & O'Neill LA. (2000). The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *J Leukoc Biol* **67,** 508-514.

Brodde OE, Bruck H & Leineweber K. (2006). Cardiac adrenoceptors: physiological and pathophysiological relevance. *J Pharmacol Sci* **100,** 323-337.

Brusaferri F & Candelise L. (2000). Steroids for multiple sclerosis and optic neuritis: a meta-analysis of randomized controlled clinical trials. *J Neurol* **247**, 435-442.

Caccamo A, Oddo S, Sugarman MC, Akbari Y, LaFerla FM. (2005) Age- and region-dependent alterations in Abeta-degrading enzymes: implications for Abeta-induced disorders. *Neurobiol Aging.* **6,** 645-654.

Campbell SJ, Wilcockson DC, Butchart AG, Perry VH & Anthony DC. (2002). Altered chemokine expression in the spinal cord and brain contributes to differential interleukin-1beta-induced neutrophil recruitment. *J Neurochem* **83,** 432-441.

Cannon JG. (2000). Inflammatory Cytokines in Nonpathological States. *News Physiol Sci* **15,** 298-303.

Cao C, Matsumura K, Yamagata K & Watanabe Y. (1995). Induction by lipopolysaccharide of cyclooxygenase-2 mRNA in rat brain; its possible role in the febrile response. *Brain Res* **697,** 187-196.

Carson MJ. (2002). Microglia as liaisons between the immune and central nervous systems: functional implications for multiple sclerosis. *Glia* **40,** 218-231.

Carson MJ, Doose JM, Melchior B, Schmid CD & Ploix CC. (2006). CNS immune privilege: hiding in plain sight. *Immunol Rev* **213,** 48-65.

Chavarria A & Alcocer-Varela J. (2004). Is damage in central nervous system due to inflammation? *Autoimmun Rev* **3,** 251-260.

Chen K, Huang J, Gong W, Zhang L, Yu P & Wang JM. (2006a). CD40/CD40L dyad in the inflammatory and immune responses in the central nervous system. *Cell Mol Immunol* **3,** 163-169.

Chen K, Iribarren P, Hu J, Chen J, Gong W, Cho EH, Lockett S, Dunlop NM & Wang JM. (2006b). Activation of Toll-like receptor 2 on microglia promotes cell uptake of Alzheimer disease-associated amyloid beta peptide. *J Biol Chem* **281,** 3651-3659.

Chesik D, Glazenburg L, De Keyser J & Wilczak N. (2007). Enhanced proliferation of astrocytes from beta(2)-adrenergic receptor knockout mice is influenced by the IGF system. *J Neurochem* **100,** 1555-1564.

Chi DS, Qui M, Krishnaswamy G, Li C & Stone W. (2003). Regulation of nitric oxide production from macrophages by lipopolysaccharide and catecholamines. *Nitric Oxide* **8,** 127-132.

Chung H, Brazil MI, Soe TT & Maxfield FR. (1999). Uptake, degradation, and release of fibrillar and soluble forms of Alzheimer's amyloid beta-peptide by microglial cells. *J Biol Chem* **274,** 32301-32308.

Chung IY & Benveniste EN. (1990). Tumor necrosis factor-alpha production by astrocytes. Induction by lipopolysaccharide, IFN-gamma, and IL-1 beta. *J Immunol* **144,** 2999-3007.

Chung IY, Norris JG & Benveniste EN. (1991). Differential tumor necrosis factor alpha expression by astrocytes from experimental allergic encephalomyelitis-susceptible and -resistant rat strains. *J Exp Med* **173,** 801-811.

Civantos Calzada B & Aleixandre de Artinano A. (2001). Alpha-adrenoceptor subtypes. *Pharmacol Res* **44,** 195-208.

Collins S, Caron MG & Lefkowitz RJ. (1988). Beta-adrenergic receptors in hamster smooth muscle cells are transcriptionally regulated by glucocorticoids. *J Biol Chem* **263,** 9067-9070.

Commins S, Steinke JW & Borish L. (2008). The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. *J Allergy Clin Immunol* **121,** 1108-1111.

Compston A & Coles A. (2002). Multiple sclerosis. *Lancet* **359,** 1221-1231.

Condorelli DF, Dell'Albani P, Mudo G, Timmusk T & Belluardo N. (1994). Expression of neurotrophins and their receptors in primary astroglial cultures: induction by cyclic AMP-elevating agents. *J Neurochem* **63,** 509-516.

Connor B & Dragunow M. (1998). The role of neuronal growth factors in neurodegenerative disorders of the human brain. *Brain Res Rev* **27,** 1-39.

Connor TJ, Starr N, O'Sullivan JB & Harkin A. (2008). Induction of indolamine 2,3-dioxygenase and kynurenine 3-monooxygenase in rat brain following a systemic inflammatory challenge: a role for IFN-gamma? *Neurosci Lett* **441,** 29-34.

Constantinescu CS, Tani M, Ransohoff RM, Wysocka M, Hilliard B, Fujioka T, Murphy S, Tighe PJ, Sarma JD, Trinchieri G & Rostami A. (2005). Astrocytes as antigen-presenting cells: expression of IL-12/IL-23. *J Neurochem* **95,** 331-340.

Conway PG, Tejani-Butt S & Brunswick DJ. (1987). Interaction of beta adrenergic agonists and antagonists with brain beta adrenergic receptors in vivo. *J Pharmacol Exp Ther* **241,** 755-762.

Cook DG, Leverenz JB, McMillan PJ, Kulstad JJ, Ericksen S, Roth RA, Schellenberg GD, Jin LW, Kovacina KS, Craft S. (2003) Reduced hippocampal insulin-degrading enzyme in late-ons*et al*zheimer's disease is associated with the apolipoproteinE-epsilon4 allele. *Am J Pathol* **162**, 313–319

Cornett LE, Hiller FC, Jacobi SE, Cao W & McGraw DW. (1998). Identification of a glucocorticoid response element in the rat beta2-adrenergic receptor gene. *Mol Pharmacol* **54,** 1016-1023.

Costelloe C, Watson M, Murphy A, McQuillan K, Loscher C, Armstrong ME, Garlanda C, Mantovani A, O'Neill LA, Mills KH, Lynch MA. (2008). IL-1F5 mediates anti-inflammatory activity in the brain through induction of IL-4 following interaction with SIGIRR/TIR8. *J Neurochem* **105,** 1960-1969.

Cua DJ, Hutchins B, LaFace DM, Stohlman SA & Coffman RL. (2001). Central nervous system expression of IL-10 inhibits autoimmune encephalomyelitis. *J Immunol* **166,** 602-608.

Culmsee C, Junker V, Thal S, Kremers W, Maier S, Schneider HJ, Plesnila N & Krieglstein J. (2007). Enantio-selective effects of clenbuterol in cultured neurons and astrocytes, and in a mouse model of cerebral ischemia. *Eur J Pharmacol* **575,** 57-65.

Culmsee C, Semkova I & Krieglstein J. (1999a). NGF mediates the neuroprotective effect of the beta2-adrenoceptor agonist clenbuterol in vitro and in vivo: evidence from an NGF-antisense study. *Neurochem Int* **35,** 47-57.

Culmsee C, Stumm RK, Schafer MK, Weihe E & Krieglstein J. (1999b). Clenbuterol induces growth factor mRNA, activates astrocytes, and protects rat brain tissue against ischemic damage. *Eur J Pharmacol* **379,** 33-45.

Dalpke A, Heeg K, Bartz H & Baetz A. (2008). Regulation of innate immunity by suppressor of cytokine signaling (SOCS) proteins. *Immunobiology* **213,** 225-235.

Dangel V, Giray J, Ratge D & Wisser H. (1996). Regulation of beta-adrenoceptor density and mRNA levels in the rat heart cell-line H9c2. *Biochem J* **317 (Pt 3),** 925-931.

Dantzer R, O'Connor JC, Freund GG, Johnson RW & Kelley KW. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* **9,** 46-56.

Dantzer R. (2001). Cytokine-induced sickness behaviour: Mechanisms and implications. *Ann NY Acad Sci.* **933**, 222-234.

Daubert GP, Mabasa VH, Leung VW & Aaron C. (2007). Acute clenbuterol overdose resulting in supraventricular tachycardia and atrial fibrillation. *J Med Toxicol* **3,** 56-60.

De Keyser J, Wilczak N, Leta R & Streetland C. (1999). Astrocytes in multiple sclerosis lack beta-2 adrenergic receptors. *Neurology* **53,** 1628-1633.

De Keyser J, Zeinstra E & Wilczak N. (2004a). Astrocytic beta2-adrenergic receptors and multiple sclerosis. *Neurobiol Dis* **15,** 331-339.

De Keyser J, Zeinstra E, Mostert J & Wilczak N. (2004b). Beta 2-adrenoceptor involvement in inflammatory demyelination and axonal degeneration in multiple sclerosis. *Trends Pharmacol Sci* **25,** 67-71.

Dello Russo C, Boullerne AI, Gavrilyuk V & Feinstein DL. (2004). Inhibition of microglial inflammatory responses by norepinephrine: effects on nitric oxide and interleukin-1beta production. *J Neuroinflammation* **1,** 9.

Dilger RN & Johnson RW. (2008). Aging, microglial cell priming, and the discordant central inflammatory response to signals from the peripheral immune system. *J Leukoc Biol* **84,** 932-939.

Dinarello CA. (1996). Biologic basis for interleukin-1 in disease. *Blood* **87,** 2095-2147.

Docagne F, Campbell SJ, Bristow AF, Poole S, Vigues S, Guaza C, Perry VH & Anthony DC. (2005). Differential regulation of type I and type II interleukin-1 receptors in focal brain inflammation. *Eur J Neurosci* **21,** 1205-1214.

Dooley DJ HKBH. (1983). Differential decrease of the central beta-adrenergic receptor in the rat after subchronic infusion of desipramine and clenbuterol *Neurochem Int* **5,** 333-338.

Du Yan S, Zhu H, Fu J, Yan SF, Roher A, Tourtellotte WW, Rajavashisth T, Chen X, Godman GC, Stern D & Schmidt AM. (1997). Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. *Proc Natl Acad Sci U S A* **94,** 5296-5301.

Dumaz N & Marais R. (2005). Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on the anniversary prize of the Gesellschaft fur Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. *FEBS J* **272,** 3491-3504.

Dunn AJ. (2006). Effects of cytokines and infections on brain neurochemistry. *Clin Neurosci Res* **6,** 52-68.

Eckman EA & Eckman CB. (2005). Abeta-degrading enzymes: modulators of Alzheimer's disease pathogenesis and targets for therapeutic intervention. *Biochem Soc Trans* **33,** 1101-1105.

Eisenhofer G. (2001). The role of neuronal and extraneuronal plasma membrane transporters in the inactivation of peripheral catecholamines. *Pharmacol Ther* **91,** 35-62.

Elenkov IJ, Hasko G, Kovacs KJ & Vizi ES. (1995). Modulation of lipopolysaccharide-induced tumor necrosis factor-alpha production by selective alpha- and beta-adrenergic drugs in mice. *J Neuroimmunol* **61,** 123-131.

Eng LF, Ghirnikar RS & Lee YL. (2000). Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). *Neurochem Res* **25,** 1439-1451.

Fan G, Shumay E, Malbon CC & Wang H. (2001). c-Src tyrosine kinase binds the beta 2-adrenergic receptor via phospho-Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization. *J Biol Chem* **276,** 13240-13247.

Farfara D, Lifshitz V & Frenkel D. (2008). Neuroprotective and neurotoxic properties of glial cells in the pathogenesis of Alzheimer's disease. *J Cell Mol Med* **12,** 762-780.

Farmer P & Pugin J. (2000). beta-adrenergic agonists exert their "anti-inflammatory" effects in monocytic cells through the IkappaB/NF-kappaB pathway. *Am J Physiol Lung Cell Mol Physiol* **279,** L675-682.

Feinstein DL, Galea E & Reis DJ. (1993). Norepinephrine suppresses inducible nitric oxide synthase activity in rat astroglial cultures. *J Neurochem* **60,** 1945-1948.

Feinstein DL, Heneka MT, Gavrilyuk V, Dello Russo C, Weinberg G & Galea E. (2002). Noradrenergic regulation of inflammatory gene expression in brain. *Neurochem Int* **41,** 357-365.

Filiz G, Price KA, Caragounis A, Du T, Crouch PJ & White AR. (2008). The role of metals in modulating metalloprotease activity in the AD brain. *Eur Biophys J* **37,** 315-321.

Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM & Golenbock DT. (2003). LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* **198,** 1043-1055.

Flood DG, Lin YG, Lang DM, Trusko SP, Hirsch JD, Savage MJ, Scott RW & Howland DS. (2009). A transgenic rat model of Alzheimer's disease with extracellular Aβ deposition. *Neurobiol Aging* **30,** 1078–1090

Follesa P & Mocchetti I. (1993). Regulation of basic fibroblast growth factor and nerve growth factor mRNA by beta-adrenergic receptor activation and adrenal steroids in rat central nervous system. *Mol Pharmacol* **43,** 132-138.

Fortini ME. (2003). Neurobiology: double trouble for neurons. *Nature* **425,** 565-566.

References

Fujita H, Tanaka J, Maeda N & Sakanaka M. (1998). Adrenergic agonists suppress the proliferation of microglia through beta 2-adrenergic receptor. *Neurosci Lett* **242,** 37-40.

Furukawa Y, Tomioka N, Sato W, Satoyoshi E, Hayashi K & Furukawa S. (1989). Catecholamines increase nerve growth factor mRNA content in both mouse astroglial cells and fibroblast cells. *FEBS Lett* **247,** 463-467.

Galea E & Feinstein DL. (1999). Regulation of the expression of the inflammatory nitric oxide synthase (NOS2) by cyclic AMP. *FASEB J* **13,** 2125-2137.

Galea E, Heneka MT, Dello Russo C & Feinstein DL. (2003). Intrinsic regulation of brain inflammatory responses. *Cell Mol Neurobiol* **23,** 625-635.

Galea I, Bechmann I & Perry VH. (2007). What is immune privilege (not)? *Trends Immunol* **28,** 12-18.

Garcia-Osta A & Alberini CM. (2009). Amyloid beta mediates memory formation. *Learn Mem* **16,** 267-272.

Gehrmann J, Matsumoto Y & Kreutzberg GW. (1995). Microglia: intrinsic immuneffector cell of the brain. *Brain Res Brain Res Rev* **20,** 269-287.

Geyer MA & Frampton SF. (1988). Peripheral mediation of effects of clenbuterol on locomotor and investigatory behavior in rats. *Pharmacol Biochem Behav* **30,** 417-420.

Giembycz MA & Newton R. (2006). Beyond the dogma: novel beta2-adrenoceptor signalling in the airways. *Eur Respir J* **27,** 1286-1306.

Giorelli M, Livrea P & Trojano M. (2004). Post-receptorial mechanisms underlie functional disregulation of beta2-adrenergic receptors in lymphocytes from Multiple Sclerosis patients. *J Neuroimmunol* **155,** 143-149.

Glezer I & Rivest S. (2004). Glucocorticoids: protectors of the brain during innate immune responses. *Neuroscientist* **10,** 538-552.

Gonzalez-Rey E, Chorny A & Delgado M. (2007). Regulation of immune tolerance by anti-inflammatory neuropeptides. *Nat Rev Immunol* **7,** 52-63.

Goshen I, Kreisel T, Ounallah-Saad H, Renbaum P, Zalzstein Y, Ben-Hur T, Levy-Lahad E, Yirmiya R. (2007). A dual role for interleukin-1 in hippocampal-dependent memory processes. *Psychoneuroendocrinology.* **32(8-10),** 1106-15.

Gottschall PE, Yu X & Bing B. (1995). Increased production of gelatinase B (matrix metalloproteinase-9) and interleukin-6 by activated rat microglia in culture. *J Neurosci Res* **42,** 335-342.

Grundy RI, Rothwell NJ & Allan SM. (1999). Dissociation between the effects of interleukin-1 on excitotoxic brain damage and body temperature in the rat. *Brain Res* **830,** 32-37.

Guo M, Pascual RM, Wang S, Fontana MF, Valancius CA, Panettieri RA, Jr., Tilley SL & Penn RB. (2005). Cytokines regulate beta-2-adrenergic receptor responsiveness in airway smooth muscle via multiple PKA- and EP2 receptor-dependent mechanisms. *Biochemistry* **44,** 13771-13782.

Guo NN & Li BM. (2007). Cellular and subcellular distributions of beta1- and beta2-adrenoceptors in the CA1 and CA3 regions of the rat hippocampus. *Neuroscience* **146,** 298-305.

Hadcock JR, Wang HY & Malbon CC. (1989). Agonist-induced destabilization of beta-adrenergic receptor mRNA. Attenuation of glucocorticoid-induced up-regulation of beta-adrenergic receptors. *J Biol Chem* **264,** 19928-19933.

Hakonarson H, Herrick DJ, Serrano PG & Grunstein MM. (1996). Mechanism of cytokine-induced modulation of beta-adrenoceptor responsiveness in airway smooth muscle. *J Clin Invest* **97,** 2593-2600.

Hall GL, Wing MG, Compston DA & Scolding NJ. (1997). beta-Interferon regulates the immunomodulatory activity of neonatal rodent microglia. *J Neuroimmunol* **72,** 11-19.

Harkin A, O'Donnell JM & Kelly JP. (2002). A study of VitalView for behavioural and physiological monitoring in laboratory rats. *Physiol Behav* **77,** 65-77.

Hart BJ. (1988). Biological basis of the behaviour of sick animals. *Neurosci Biobehav Rev* **12,** 123-137.

Hayes MP, Freeman SL & Donnelly RP. (1995). IFN-gamma priming of monocytes enhances LPS-induced TNF production by augmenting both transcription and MRNA stability. *Cytokine* **7,** 427-435.

He F & Sun YE. (2007). Glial cells more than support cells? *Int J Biochem Cell Biol* **39,** 661-665.

Heesen C, Gold SM, Huitinga I & Reul JM. (2007). Stress and hypothalamic-pituitary-adrenal axis function in experimental autoimmune encephalomyelitis and multiple sclerosis - a review. *Psychoneuroendocrinology* **32,** 604-618.

Hein L. (2006). Adrenoceptors and signal transduction in neurons. *Cell Tissue Res* **326,** 541-551.

Heffernan S (2009). The ability of the β2-Adrenoceptor to regulate the Interleukin 1 family: Neuronal, Immunological and Behavioural implications. *Unpublished thesis* (M.Sc.), University of Dublin Trinity College.

Heneka MT, Galea E, Gavriluyk V, Dumitrescu-Ozimek L, Daeschner J, O'Banion MK, Weinberg G, Klockgether T & Feinstein DL. (2002). Noradrenergic depletion potentiates beta -amyloid-induced cortical inflammation: implications for Alzheimer's disease. *J Neurosci* **22,** 2434-2442.

Heneka MT, Ramanathan M, Jacobs AH, Dumitrescu-Ozimek L, Bilkei-Gorzo A, Debeir T, Sastre M, Galldiks N, Zimmer A, Hoehn M, Heiss WD, Klockgether T & Staufenbiel M. (2006). Locus ceruleus degeneration promotes Alzheimer pathogenesis in amyloid precursor protein 23 transgenic mice. *J Neurosci* **26,** 1343-1354.

Herlaar E & Brown Z. (1999). p38 MAPK signalling cascades in inflammatory disease. *Mol Med Today* **5,** 439-447.

Hertz L, Chen Y, Gibbs ME, Zang P & Peng L. (2004). Astrocytic adrenoceptors: a major drug target in neurological and psychiatric disorders? *Curr Drug Targets CNS Neurol Disord* **3,** 239-267.

Hickman SE, Allison EK & El Khoury J. (2008). Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *J Neurosci* **28,** 8354-8360.

Hida W, Sakurai M, Ichinose M, Shindoh C, Chonan T, Kikuchi Y, Inoue H, Talcishima T (1985). Effect of clenbuterol on peripheral airway obstruction in bronchial asthma Curr. Med. Res. Opin.. 9,616.

Hirsch EC, Hunot S. (2009). Neuroinflammation in Parkinson's disease: a target for neuroprotection? *Lancet Neurol.* **8(4),** 382-97.

Hoey AJ, Matthews ML, Badran TW, Pegg GG & Sillence MN. (1995). Cardiovascular effects of clenbuterol are beta 2-adrenoceptor-mediated in steers. *J Anim Sci* **73,** 1754-1765.

Hofman FM, Hinton DR, Johnson K & Merrill JE. (1989). Tumor necrosis factor identified in multiple sclerosis brain. *J Exp Med* **170,** 607-612.

Huang Y, Henry CJ, Dantzer R, Johnson RW, Godbout JP (2008). Exaggerated sickness behavior and brain proinflammatory cytokine expression in aged mice in response to intracerebroventricular lipopolysaccharide. *Neurobiol Aging.* **29(11),** 1744-1753

Huang H, Gazzola C, Pegg GG & Sillence MN. (2000). Differential effects of dexamethasone and clenbuterol on rat growth and on beta2-adrenoceptors in lung and skeletal muscle. *J Anim Sci* **78,** 604-608.

Hudson CA, Christophi GP, Gruber RC, Wilmore JR, Lawrence DA & Massa PT. (2008). Induction of IL-33 expression and activity in central nervous system glia. *J Leukoc Biol* **84,** 631-643.

Inazu M, Takeda H & Matsumiya T. (2003). Functional expression of the norepinephrine transporter in cultured rat astrocytes. *J Neurochem* **84,** 136-144.

Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E, Shapiro A & Antel JP. (2005a). TLR signaling tailors innate immune responses in human microglia and astrocytes. *J Immunol* **175,** 4320-4330.

Jack C, Ruffini F, Bar-Or A & Antel JP. (2005b). Microglia and multiple sclerosis. *J Neurosci Res* **81,** 363-373.

Jerala R. (2007). Structural biology of the LPS recognition. *Int J Med Microbiol* **297,** 353-363.

Jessen KR. (2004). Glial cells. *Int J Biochem Cell Biol* **36,** 1861-1867.

Johnson M. (1998). The beta-adrenoceptor. *Am J Respir Crit Care Med* **158,** S146-153.

Johnson M. (2001). Beta2-adrenoceptors: mechanisms of action of beta2-agonists. *Paediatr Respir Rev* **2,** 57-62.

Jovanova-Nesic K & Shoenfeld Y. (2006). MMP-2, VCAM-1 and NCAM-1 expression in the brain of rats with experimental autoimmune encephalomyelitis as a trigger mechanism for synaptic plasticity and pathology. *J Neuroimmunol* **181,** 112-121.

Junker V, Becker A, Huhne R, Zembatov M, Ravati A, Culmsee C & Krieglstein J. (2002). Stimulation of beta-adrenoceptors activates astrocytes and provides neuroprotection. *Eur J Pharmacol* **446,** 25-36.

Kamburoff P.L. & Prime F.J. (1977). The bronchodilator effect of NAB365. Br. J. clin. Pharmac. **4**, 67-71.

Kastin AJ, Akerstrom V & Pan W. (2003). Interleukin-10 as a CNS therapeutic: the obstacle of the blood-brain/blood-spinal cord barrier. *Brain Res Mol Brain Res* **114,** 168-171.

Kawata T, Tsutsui K, Kohno S, Kaku M, Fujita T, Tenjou K, Ohtani J, Motokawa M, Shigekawa M, Tohma Y & Tanne K. (2005). Amyloid beta protein deposition in osteopetrotic (op/op) mice is reduced by injections of macrophage colony stimulating factor. *J Int Med Res* **33,** 654-660.

Kent S, Bret-Dibat JL, Kelley KW & Dantzer R. (1996). Mechanisms of sickness-induced decreases in food-motivated behavior. *Neurosci Biobehav Rev* **20,** 171-175.

Kile BT & Alexander WS. (2001). The suppressors of cytokine signalling (SOCS). *Cell Mol Life Sci* **58,** 1627-1635.

Knoblach SM, Fan L & Faden AI. (1999). Early neuronal expression of tumor necrosis factor-alpha after experimental brain injury contributes to neurological impairment. *J Neuroimmunol* **95,** 115-125.

Kohm AP & Sanders VM. (2001). Norepinephrine and beta 2-adrenergic receptor stimulation regulate CD4+ T and B lymphocyte function in vitro and in vivo. *Pharmacol Rev* **53,** 487-525.

Konsman JP, Kelley K & Dantzer R. (1999). Temporal and spatial relationships between lipopolysaccharide-induced expression of Fos, interleukin-1beta and inducible nitric oxide synthase in rat brain. *Neuroscience* **89,** 535-548.

Konsman JP, Veeneman J, Combe C, Poole S, Luheshi GN & Dantzer R. (2008). Central nervous action of interleukin-1 mediates activation of limbic structures and behavioural depression in response to peripheral administration of bacterial lipopolysaccharide. *Eur J Neurosci* **28,** 2499-2510.

Korhonen R, Lahti A, Hamalainen M, Kankaanranta H & Moilanen E. (2002). Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing mRNA in lipopolysaccharide-treated macrophages. *Mol Pharmacol* **62,** 698-704.

Kozuka N, Kudo Y & Morita M. (2007). Multiple inhibitory pathways for lipopolysaccharide- and pro-inflammatory cytokine-induced nitric oxide production in cultured astrocytes. *Neuroscience* **144,** 911-919.

Kuroki K, Takahashi HK, Iwagaki H, Murakami T, Kuinose M, Hamanaka S, Minami K, Nishibori M, Tanaka N & Tanemoto K. (2004). beta2-adrenergic receptor stimulation-induced immunosuppressive effects possibly through down-regulation of co-stimulatory molecules, ICAM-1, CD40 and CD14 on monocytes. *J Int Med Res* **32,** 465-483.

Kyriakis JM & Avruch J. (1996). Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J Biol Chem* **271,** 24313-24316.

Layé S, Gheusi G, Cremona S, Combe C, Kelley K, Dantzer R, Parnet P. (2000). Endogenous brain IL-1 mediates LPS-induced anorexia and hypothalamic cytokine expression. *Am J Physiol.: Regul Integr Comp Physiol.* **279**, R93-R98.

Lee S & Das HK. (2008). Inhibition of basal activity of c-jun-NH2-terminal kinase (JNK) represses the expression of presenilin-1 by a p53-dependent mechanism. *Brain Res* **1207,** 19-31.

Lee YB, Nagai A & Kim SU. (2002). Cytokines, chemokines, and cytokine receptors in human microglia. *J Neurosci Res* **69,** 94-103.

Leonard BE (1997). Fundamentals of Psychopharmacology. John Wiley and Sons Ltd., Chicester

Liang W & Mills S. (2001). Profile of ligand binding to the porcine beta2-adrenergic receptor. *J Anim Sci* 79, 877-883.

Liao YF, Wang BJ, Cheng HT, Kuo LH & Wolfe MS. (2004). Tumor necrosis factor-alpha, interleukin-1beta, and interferon-gamma stimulate gamma-secretase-mediated cleavage of amyloid precursor protein through a JNK-dependent MAPK pathway. *J Biol Chem* **279,** 49523-49532.

Liggett SB. (2002). Update on current concepts of the molecular basis of beta2-adrenergic receptor signaling. *J Allergy Clin Immunol* **110,** S223-227.

Lin FS, Lin CC, Chien CS, Luo SF & Yang CM. (2005). Involvement of p42/p44 MAPK, JNK, and NF-kappaB in IL-1beta-induced ICAM-1 expression in human pulmonary epithelial cells. *J Cell Physiol* **202,** 464-473.

Lin WC, Tsai PS & Huang CJ. (2005). Catecholamines' enhancement of inducible nitric oxide synthase-induced nitric oxide biosynthesis involves CAT-1 and CAT-2A. *Anesth Analg* **101,** 226-232.

Lindberg RL, De Groot CJ, Montagne L, Freitag P, van der Valk P, Kappos L & Leppert D. (2001). The expression profile of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in lesions and normal appearing white matter of multiple sclerosis. *Brain* **124,** 1743-1753.

Liu B & Hong JS. (2003). Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J Pharmacol Exp Ther* **304,** 1-7.

Liu P, Xiang JZ, Zhao L, Yang L, Hu BR & Fu Q. (2008). Effect of beta2-adrenergic agonist clenbuterol on ischemia/reperfusion injury in isolated rat hearts and cardiomyocyte apoptosis induced by hydrogen peroxide. *Acta Pharmacol Sin* **29,** 661-669.

Liuzzi GM, Mastroianni CM, Latronico T, Mengoni F, Fasano A, Lichtner M, Vullo V & Riccio P. (2004). Anti-HIV drugs decrease the expression of matrix metalloproteinases in astrocytes and microglia. *Brain* **127,** 398-407.

Loddick SA, Wong ML, Bongiorno PB, Gold PW, Licinio J & Rothwell NJ. (1997). Endogenous interleukin-1 receptor antagonist is neuroprotective. *Biochem Biophys Res Commun* **234,** 211-215.

Loder C, Allawi J & Horrobin DF. (2002). Treatment of multiple sclerosis with lofepramine, L-phenylalanine and vitamin B(12): mechanism of action and clinical importance: roles of the locus coeruleus and central noradrenergic systems. *Med Hypotheses* **59,** 594-602.

Luchowska E, Kloc R, Wnuk S, Olajossy B, Wielosz M & Urbanska EM. (2008). Clenbuterol enhances the production of kynurenic acid in brain cortical slices and glial cultures. *Pharmacol Rep* **60,** 574-577.

Lucin KM & Wyss-Coray T. (2009). Immune activation in brain aging and neurodegeneration: too much or too little? *Neuron* **64,** 110-122.

Luheshi NM, Rothwell NJ, Brough D. (2009). Dual functionality of interleukin-1 family cytokines: implications for anti-interleukin-1 therapy. *Br J Pharmacol.* **157(8),** 1318-1329.

Luttrell LM & Lefkowitz RJ. (2002). The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* **115,** 455-465.

Maccioni RB, Munoz JP & Barbeito L. (2001). The molecular bases of Alzheimer's disease and other neurodegenerative disorders. *Arch Med Res* **32,** 367-381.

Maccioni RB, Rojo LE, Fernandez JA & Kuljis RO. (2009). The role of neuroimmunomodulation in Alzheimer's disease. *Ann N Y Acad Sci* **1153,** 240-246.

Madrigal JL, Dello Russo C, Gavrilyuk V & Feinstein DL. (2006). Effects of noradrenaline on neuronal NOS2 expression and viability. *Antioxid Redox Signal* **8,** 885-892.

Madrigal JL, Leza JC, Polak P, Kalinin S & Feinstein DL. (2009). Astrocyte-derived MCP-1 mediates neuroprotective effects of noradrenaline. *J Neurosci* **29,** 263-267.

Majumdar A, Cruz D, Asamoah N, Buxbaum A, Sohar I, Lobel P & Maxfield FR. (2007). Activation of microglia acidifies lysosomes and leads to degradation of Alzheimer amyloid fibrils. *Mol Biol Cell* **18,** 1490-1496.

Mak JC, Nishikawa M, Shirasaki H, Miyayasu K & Barnes PJ. (1995). Protective effects of a glucocorticoid on downregulation of pulmonary beta 2-adrenergic receptors in vivo. *J Clin Invest* **96,** 99-106.

Mak JC, Rousell J, Haddad EB & Barnes PJ. (2000). Transforming growth factor-beta1 inhibits beta2-adrenoceptor gene transcription. *Naunyn Schmiedebergs Arch Pharmacol* **362,** 520-525.

Malm TM, Magga J, Kuh GF, Vatanen T, Koistinaho M & Koistinaho J. (2008). Minocycline reduces engraftment and activation of bone marrow-derived cells but sustains their phagocytic activity in a mouse model of Alzheimer's disease. *Glia* **56,** 1767-1779.

Mantyh PW, Rogers SD, Allen CJ, Catton MD, Ghilardi JR, Levin LA, Maggio JE & Vigna SR. (1995). Beta 2-adrenergic receptors are expressed by glia in vivo in the normal and injured central nervous system in the rat, rabbit, and human. *J Neurosci* **15,** 152-164.

Marien MR, Colpaert FC & Rosenquist AC. (2004). Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain Res Brain Res Rev* **45,** 38-78.

Martin D & Near SL. (1995). Protective effect of the interleukin-1 receptor antagonist (IL-1ra) on experimental allergic encephalomyelitis in rats. *J Neuroimmunol* **61,** 241-245.

Martin LE, Hobson JC, Page J A & Harrison C. (1971). Metabolic studies of salbutamol-3H: A new bronchodilator, in rat, rabbit, dog and man. Eur. J. Pharmac. **14**, 183-199.

Martino G, Adorini L, Rieckmann P, Hillert J, Kallmann B, Comi G & Filippi M. (2002). Inflammation in multiple sclerosis: the good, the bad, and the complex. *Lancet Neurol* **1,** 499-509.

Mason JL, Suzuki K, Chaplin DD & Matsushima GK. (2001). Interleukin-1beta promotes repair of the CNS. *J Neurosci* **21,** 7046-7052.

Mazzanti G, Di Sotto A, Daniele C, Battinelli L, Brambilla G, Fiori M, Loizzo S & Loizzo A. (2007). A pharmacodynamic study on clenbuterol-induced toxicity: beta1- and beta2-adrenoceptors involvement in guinea-pig tachycardia in an in vitro model. *Food Chem Toxicol* **45,** 1694-1699.

McLoughlin DM & Miller CC. (2008). The FE65 proteins and Alzheimer's disease. *J Neurosci Res* **86,** 744-754.

McNamee EN (2008). Noradrenergic modulation of the central nervous system immune response-a critical role for the β_2-adrenoceptor. *Unpublished thesis* (Ph.D), University of Dublin Trinity College.

Medina C, Videla S, Radomski A, Radomski MW, Antolin M, Guarner F, Vilaseca J, Salas A & Malagelada JR. (2003). Increased activity and expression of matrix metalloproteinase-9 in a rat model of distal colitis. *Am J Physiol Gastrointest Liver Physiol* **284,** G116-122.

Merrill JE, Ignarro LJ, Sherman MP, Melinek J & Lane TE. (1993). Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J Immunol* **151,** 2132-2141.

Miller WE, Maudsley S, Ahn S, Khan KD, Luttrell LM & Lefkowitz RJ. (2000). beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis. *J Biol Chem* **275,** 11312-11319.

Millman EE, Rosenfeld JL, Vaughan DJ, Nguyen J, Dai W, Alpizar-Foster E, Clark RB, Knoll BJ & Moore RH. (2004). Endosome sorting of beta 2-adrenoceptors is GRK5 independent. *Br J Pharmacol* **141,** 277-284.

Minagar A, Shapshak P, Fujimura R, Ownby R, Heyes M & Eisdorfer C. (2002). The role of macrophage/microglia and astrocytes in the pathogenesis of three neurologic disorders: HIV-associated dementia, Alzheimer disease, and multiple sclerosis. *J Neurol Sci* **202,** 13-23.

Miners JS, Baig S, Palmer J, Palmer LE, Kehoe PG & Love S. (2008). Abeta-degrading enzymes in Alzheimer's disease. *Brain Pathol* **18,** 240-252.

Miners JS, Baig S, Palmer J, Palmer LE, Kehoe PG & Love S. (2008). Abeta-degrading enzymes in Alzheimer's disease. *Brain Pathol* **18,** 240-252.

Mingam R, De Smedt V, Amédée T, Bluthé RM, Kelley KW, Dantzer R, Layé S. (2008). In vitro and in vivo evidence for a role of the P2X7 receptor in the release of IL-1 beta in the murine brain. *Brain Behav Immun.* **22(2),** 234-44.

Mochizuki S, Kobayashi M, Suzuki T, Oikawa A, Koseki T, Nishihara T & Hasegawa K. (2004). Gamma-interferon enhances expression of CD14/MyD88 and subsequent responsiveness to lipopolysaccharide from Actinobacillus actinomycetemcomitans in human gingival fibroblasts. *J Periodontal Res* **39,** 333-343.

Mogensen TH. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* **22,** 240-273

Moore PE, Laporte JD, Gonzalez S, Moller W, Heyder J, Panettieri RA, Jr. & Shore SA. (1999a). Glucocorticoids ablate IL-1beta-induced beta-adrenergic hyporesponsiveness in human airway smooth muscle cells. *Am J Physiol* **277,** L932-942.

Moore RH, Hall HS, Rosenfeld JL, Dai W & Knoll BJ. (1999b). Specific changes in beta2-adrenoceptor trafficking kinetics and intracellular sorting during downregulation. *Eur J Pharmacol* **369,** 113-123.

Moresco RM, Matarrese M, Soloviev D, Simonelli P, Rigamonti M, Gobbo C, Todde S, Carpinelli A, Kienle MG, Fazio F. (2000). Synthesis and in vivo evaluation of [11C]ICI 118551 as a putative subtype selective beta2-adrenergic radioligand. *Int J Pharm.* **204,** 101-109.

Mori K, Ozaki E, Zhang B, Yang L, Yokoyama A, Takeda I, Maeda N, Sakanaka M & Tanaka J. (2002). Effects of norepinephrine on rat cultured microglial cells that express alpha1, alpha2, beta1 and beta2 adrenergic receptors. *Neuropharmacology* **43,** 1026-1034.

Mosser DM & Zhang X. (2008). Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* **226,** 205-218.

Moynagh PN. (2005). TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway. *Trends Immunol* **26,** 469-476.

Mu W, Ouyang X, Agarwal A, Zhang L, Long DA, Cruz PE, Roncal CA, Glushakova OY, Chiodo VA, Atkinson MA, Hauswirth WW, Flotte TR, Rodriguez-Iturbe B & Johnson RJ. (2005). IL-10 suppresses chemokines, inflammation, and fibrosis in a model of chronic renal disease. *J Am Soc Nephrol* **16,** 3651-3660.

Murugaiah KD & O'Donnell JM. (1995). Facilitation of norepinephrine release from cerebral cortex is mediated by beta 2-adrenergic receptors. *Life Sci* **57,** PL327-332.

Nakadate K, Matsukawa M & Okado N. (2006). Identification of adrenoceptor subtype-mediated changes in the density of synapses in the rat visual cortex. *Neuroscience* **138,** 37-46.

Nakamura A, Johns EJ, Imaizumi A, Abe T & Kohsaka T. (1998). Regulation of tumour necrosis factor and interleukin-6 gene transcription by beta2-adrenoceptor in the rat astrocytes. *J Neuroimmunol* **88,** 144-153.

Ng DC, Long CS & Bogoyevitch MA. (2001). A role for the extracellular signal-regulated kinase and p38 mitogen-activated protein kinases in interleukin-1 beta-stimulated delayed signal tranducer and activator of transcription 3 activation, atrial natriuretic factor expression, and cardiac myocyte morphology. *J Biol Chem* **276,** 29490-29498.

Nguyen MD, Julien JP & Rivest S. (2002). Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nat Rev Neurosci* **3,** 216-227.

Ni Y, Zhao X, Bao G, Zou L, Teng L, Wang Z, Song M, Xiong J, Bai Y & Pei G. (2006). Activation of beta2-adrenergic receptor stimulates gamma-secretase activity and accelerates amyloid plaque formation. *Nat Med* **12,** 1390-1396.

Nikcevich KM, Gordon KB, Tan L, Hurst SD, Kroepfl JF, Gardinier M, Barrett TA, Miller SD. (1997) IFN-gamma-activated primary murine astrocytes express B7 costimulatory molecules and prime naive antigen-specific T cells. *J Immunol.* 158(2), 614-21.

O'Donnell JM. (1993). Reduced locomotor activity of rats mediated by peripheral beta adrenergic receptors. *Res Commun Chem Pathol Pharmacol* **82,** 375-378.

O'Donnell JM & Frazer A. (1985). Effects of clenbuterol and antidepressant drugs on beta adrenergic receptor/N-protein coupling in the cerebral cortex of the rat. *J Pharmacol Exp Ther* **234,** 30-36.

O'Donnell SR & Wanstall JC. (1980). Evidence that ICl 118,551 is a potent, highly beta2-selective adrenoceptor antagonist and can be used to characterize beta-adrenoceptor populations in tissues. Life Sciences **12**, 671-677.

O'Keefe GM, Nguyen VT & Benveniste EN. (2002). Regulation and function of class II major histocompatibility complex, CD40, and B7 expression in macrophages and microglia: Implications in neurological diseases. *J Neurovirol* **8,** 496-512.

Ono K & Han J. (2000). The p38 signal transduction pathway: activation and function. *Cell Signal* **12,** 1-13.

Oprica M, Hjorth E, Spulber S, Popescu BO, Ankarcrona M, Winblad B & Schultzberg M. (2007). Studies on brain volume, Alzheimer-related proteins and cytokines in mice with chronic overexpression of IL-1 receptor antagonist. *J Cell Mol Med* **11,** 810-825.

Ordway GA, Schwartz MA, Frazer A (2007) Brain Norepinephrine:Neurobiology and therapeutics. Cambridge University Press.

Ordway GA, O'Donnell JM & Frazer A. (1987). Effects of clenbuterol on central beta-1 and beta-2 adrenergic receptors of the rat. *J Pharmacol Exp Ther* **241,** 187-195.

O'Sullivan JB (2007). Noradrenergic control of inflammatory processes in the central nervous system. *Unpublished thesis* (Ph.D.), University of Dublin Trinity College.

O'Sullivan JB, Ryan KM, Curtin NM, Harkin A & Connor TJ. (2009). Noradrenaline reuptake inhibitors limit neuroinflammation in rat cortex following a systemic inflammatory challenge: implications for depression and neurodegeneration. *Int J Neuropsychopharmacol* **12,** 687-699.

Pagenstecher A, Stalder AK, Kincaid CL, Shapiro SD & Campbell IL. (1998). Differential expression of matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase genes in the mouse central nervous system in normal and inflammatory states. *Am J Pathol* **152,** 729-741.

Pannu R & Singh I. (2006). Pharmacological strategies for the regulation of inducible nitric oxide synthase: neurodegenerative versus neuroprotective mechanisms. *Neurochem Int* **49,** 170-182.

Park KW, Lee HG, Jin BK & Lee YB. (2007). Interleukin-10 endogenously expressed in microglia prevents lipopolysaccharide-induced neurodegeneration in the rat cerebral cortex in vivo. *Exp Mol Med* **39,** 812-819.

Patiyal SN & Sharma S. (2007). Chronic oral administration of beta-adrenoceptor agonist clenbuterol affects myosin heavy chain (MHC) expression in adult mouse heart. *Physiol Res* **56,** 275-283.

Patrizio M. (2004). Tumor necrosis factor reduces cAMP production in rat microglia. *Glia* **48,** 241-249.

Pearson VL, Rothwell NJ & Toulmond S. (1999). Excitotoxic brain damage in the rat induces interleukin-1beta protein in microglia and astrocytes: correlation with the progression of cell death. *Glia* **25,** 311-323.

References

Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y & Fisher PB. (2004). Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol* **22,** 929-979.

Pitcher JA, Freedman NJ & Lefkowitz RJ. (1998). G protein-coupled receptor kinases. *Annu Rev Biochem* **67,** 653-692.

Pitcher JA, Tesmer JJ, Freeman JL, Capel WD, Stone WC & Lefkowitz RJ. (1999). Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases. *J Biol Chem* **274,** 34531-34534.

Ponomarev ED, Shriver LP & Dittel BN. (2006). CD40 expression by microglial cells is required for their completion of a two-step activation process during central nervous system autoimmune inflammation. *J Immunol* **176,** 1402-1410.

Port JD, Huang LY & Malbon CC. (1992). Beta-adrenergic agonists that down-regulate receptor mRNA up-regulate a M(r) 35,000 protein(s) that selectively binds to beta-adrenergic receptor mRNAs. *J Biol Chem* **267,** 24103-24108.

Porter MH, Hrupka BJ, Langhans W & Schwartz GJ. (1998). Vagal and splanchnic afferents are not necessary for the anorexia produced by peripheral IL-1beta, LPS, and MDP. *Am J Physiol* **275,** R384-389.

Pouly S & Antel JP. (1999). Multiple sclerosis and central nervous system demyelination. *J Autoimmun* **13,** 297-306.

Qin H, Roberts KL, Niyongere SA, Cong Y, Elson CO & Benveniste EN. (2007). Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia. *J Immunol* **179,** 5966-5976.

Qin H, Wilson CA, Roberts KL, Baker BJ, Zhao X & Benveniste EN. (2006). IL-10 inhibits lipopolysaccharide-induced CD40 gene expression through induction of suppressor of cytokine signaling-3. *J Immunol* **177,** 7761-7771.

Quan N, Whiteside M & Herkenham M. (1998a). Time course and localization patterns of interleukin-1beta messenger RNA expression in brain and pituitary after peripheral administration of lipopolysaccharide. *Neuroscience* **83,** 281-293.

Quan N, Whiteside M & Herkenham M. (1998b). Cyclooxygenase 2 mRNA expression in rat brain after peripheral injection of lipopolysaccharide. *Brain Res* **802,** 189-197.

Quintero EM, Willis LM, Zaman V, Lee J, Boger HA, Tomac A, Hoffer BJ, Stromberg I & Granholm AC. (2004). Glial cell line-derived neurotrophic factor is essential for neuronal survival in the locus coeruleus-hippocampal noradrenergic pathway. *Neuroscience* **124,** 137-146.

Ransohoff RM & Perry VH. (2009). Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol* **27,** 119-145.

Richard KL, Filali M, Prefontaine P & Rivest S. (2008). Toll-like receptor 2 acts as a natural innate immune receptor to clear amyloid beta 1-42 and delay the cognitive decline in a mouse model of Alzheimer's disease. *J Neurosci* **28,** 5784-5793.

Rincon M & Davis RJ. (2009). Regulation of the immune response by stress-activated protein kinases. *Immunol Rev* **228,** 212-224.

Riva MA, Molteni R & Racagni G. (1998). Differential regulation of FGF-2 and FGFR-1 in rat cortical astrocytes by dexamethasone and isoproterenol. *Brain Res Mol Brain Res* **57,** 38-45.

Rivest S. (2003). Molecular insights on the cerebral innate immune system. *Brain Behav Immun* **17,** 13-19.

Roher AE, Kasunic TC, Woods AS, Cotter RJ, Ball MJ & Fridman R. (1994). Proteolysis of A beta peptide from Alzheimer disease brain by gelatinase A. *Biochem Biophys Res Commun* **205,** 1755-1761.

References

Rosenberg PA & Li Y. (1995). Adenylyl cyclase activation underlies intracellular cyclic AMP accumulation, cyclic AMP transport, and extracellular adenosine accumulation evoked by beta-adrenergic receptor stimulation in mixed cultures of neurons and astrocytes derived from rat cerebral cortex. *Brain Res* **692,** 227-232.

Rothwell N. (2003). Interleukin-1 and neuronal injury: mechanisms, modification, and therapeutic potential. *Brain Behav Immun.* **17(3),** 152-157.

Rothwell NJ & Luheshi GN. (2000). Interleukin 1 in the brain: biology, pathology and therapeutic target. *Trends Neurosci* **23,** 618-625.

Rousseau G, Guilbault N, Da Silva A, Mouillac B, Chidiac P & Bouvier M. (1997). Influence of receptor density on the patterns of beta2-adrenocepter desensitization. *Eur J Pharmacol* **326,** 75-84.

Salm AK & McCarthy KD. (1989). Expression of beta-adrenergic receptors by astrocytes isolated from adult rat cortex. *Glia* **2,** 346-352.

Sasaki H, Kamimura H, Shiobara Y, Esumi Y, Takaichi M & Yokoshima T. (1982). Disposition and metabolism of formoterol fumarate, a new bronchodilator, in rats and dogs. *Xenobiotica.* **12,** 803-812

Scheller J & Rose-John S. (2006). Interleukin-6 and its receptor: from bench to bedside. *Med Microbiol Immunol* **195,** 173-183.

Schroder K, Hertzog PJ, Ravasi T & Hume DA. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75,** 163-189.

Selkoe DJ & Wolfe MS. (2007). Presenilin: running with scissors in the membrane. *Cell* **131,** 215-221.

Semkova I, Schilling M, Henrich-Noack P, Rami A & Krieglstein J. (1996). Clenbuterol protects mouse cerebral cortex and rat hippocampus from ischemic damage and attenuates glutamate neurotoxicity in cultured hippocampal neurons by induction of NGF. *Brain Res* **717,** 44-54.

Shaftel SS, Griffin WS & O'Banion MK. (2008). The role of interleukin-1 in neuroinflammation and Alzheimer disease: an evolving perspective. *J Neuroinflammation* **5,** 7.

Shaftel SS, Kyrkanides S, Olschowka JA, Miller JN, Johnson RE & O'Banion MK. (2007). Sustained hippocampal IL-1 beta overexpression mediates chronic neuroinflammation and ameliorates Alzheimer plaque pathology. *J Clin Invest* **117,** 1595-1604.

Shain W, Forman DS, Madelian V & Turner JN. (1987). Morphology of astroglial cells is controlled by beta-adrenergic receptors. *J Cell Biol* **105,** 2307-2314.

Shirato K, Tanihata J, Motohashi N, Tachiyashiki K, Tomoda A & Imaizumi K. (2007). Beta2-agonist clenbuterol induced changes in the distribution of white blood cells in rats. *J Pharmacol Sci* **104,** 146-152.

Simi A, Edling Y, Ingelman-Sundberg M & Tindberg N. (2005). Activation of c-fos by lipopolysaccharide in glial cells via p38 mitogen-activated protein kinase-dependent activation of serum or cyclic AMP/calcium response element. *J Neurochem* **92,** 915-924.

Sloane E, Ledeboer A, Seibert W, Coats B, van Strien M, Maier SF, Johnson KW, Chavez R, Watkins LR, Leinwand L, Milligan ED & Van Dam AM. (2009). Anti-inflammatory cytokine gene therapy decreases sensory and motor dysfunction in experimental Multiple Sclerosis: MOG-EAE behavioral and anatomical symptom treatment with cytokine gene therapy. *Brain Behav Immun* **23,** 92-100.

Smith DJ. (1998). The pharmacokinetics, metabolism, and tissue residues of beta-adrenergic agonists in livestock. *J Anim Sci* **76,** 173-194.

Soos JM, Morrow J, Ashley TA, Szente BE, Bikoff EK, Zamvil SS. (1998) Astrocytes express elements of the class II endocytic pathway and process central nervous system autoantigen for presentation to encephalitogenic T cells. *J Immunol.* **161,** 5959-5966.

Soraru G, Pegoraro E, Spinella P, Turra S, D'Ascenzo C, Baggio L, Mantovan MC, Vergani L & Angelini C. (2006). A pilot trial with clenbuterol in amyotrophic lateral sclerosis. *Amyotroph Lateral Scler.* 7, 252–254

Sparkman NL & Johnson RW. (2008). Neuroinflammation associated with aging sensitizes the brain to the effects of infection or stress. *Neuroimmunomodulation* **15,** 323-330.

Stefferl A, Linington C, Holsboer F & Reul JM. (1999). Susceptibility and resistance to experimental allergic encephalomyelitis: relationship with hypothalamic-pituitary-adrenocortical axis responsiveness in the rat. *Endocrinology* **140,** 4932-4938.

Steinman L. (1996). Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* **85,** 299-302.

Street JA & Walsh A. (1984). Inhibition of synaptosomal [3H]Noradrenalie uptake by β-adrenoceptor blocking drugs: influence of lipophilicity. *Eur J Pharmacol* **102**, 315-324.

Sutin J & Griffith R. (1993). Beta-adrenergic receptor blockade suppresses glial scar formation. *Exp Neurol* **120,** 214-222.

Swaminath G, Xiang Y, Lee TW, Steenhuis J, Parnot C & Kobilka BK. (2004). Sequential binding of agonists to the beta2 adrenoceptor. Kinetic evidence for intermediate conformational states. *J Biol Chem* **279,** 686-691.

Szczepanik AM, Funes S, Petko W & Ringheim GE. (2001). IL-4, IL-10 and IL-13 modulate A beta(1--42)-induced cytokine and chemokine production in primary murine microglia and a human monocyte cell line. *J Neuroimmunol* **113,** 49-62.

Tachida Y, Nakagawa K, Saito T, Saido TC, Honda T, Saito Y, Murayama S, Endo T, Sakaguchi G, Kato A, Kitazume S & Hashimoto Y. (2008). Interleukin-1 beta up-regulates TACE to enhance alpha-cleavage of APP in neurons: resulting decrease in Abeta production. *J Neurochem* **104,** 1387-1393.

Takahashi HK, Iwagaki H, Mori S, Yoshino T, Tanaka N & Nishibori M. (2004). Beta 2-adrenergic receptor agonist induces IL-18 production without IL-12 production. *J Neuroimmunol* **151,** 137-147.

Takahashi HK, Morichika T, Iwagaki H, Yoshino T, Tamura R, Saito S, Mori S, Akagi T, Tanaka N & Nishibori M. (2003). Effect of beta 2-adrenergic receptor stimulation on interleukin-18-induced intercellular adhesion molecule-1 expression and cytokine production. *J Pharmacol Exp Ther* **304,** 634-642.

Takeuchi O & Akira S. (2001). Toll-like receptors; their physiological role and signal transduction system. *Int Immunopharmacol* **1,** 625-635.

Tanabe K, Nishimura K, Dohi S & Kozawa O. (2009). Mechanisms of interleukin-1beta-induced GDNF release from rat glioma cells. *Brain Res* **1274,** 11-20.

Tanaka KF, Kashima H, Suzuki H, Ono K & Sawada M. (2002). Existence of functional beta1- and beta2-adrenergic receptors on microglia. *J Neurosci Res* **70,** 232-237.

Tanvaneer, R (2008). The effect of acute treatment with selective β_2 adrenoceptor agonist clenbuterol on IL-1 system in the rat cortex. *Unpublished thesis* (M.Sc.), University of Dublin Trinity College.

References

Teng YD, Choi H, Huang W, Onario RC, Frontera WR, Snyder EY & Sabharwal S. (2006). Therapeutic effects of clenbuterol in a murine model of amyotrophic lateral sclerosis. *Neurosci Lett* **397,** 155-158.

Tizard I. (2008). Sickness behavior, its mechanisms and significance. *Anim Health Res Rev* **9,** 87-99.

Tomozawa Y, Yabuuchi K, Inoue T & Satoh M. (1995). Participation of cAMP and cAMP-dependent protein kinase in beta-adrenoceptor-mediated interleukin-1 beta mRNA induction in cultured microglia. *Neurosci Res* **22,** 399-409.

Tracey KJ. (2002). The inflammatory reflex. *Nature* **420,** 853-859.

Tritsch NX & Bergles DE. (2007). Defining the role of astrocytes in neuromodulation. *Neuron* **54,** 497-500.

Troadec JD, Marien M, Mourlevat S, Debeir T, Ruberg M, Colpaert F & Michel PP. (2002). Activation of the mitogen-activated protein kinase (ERK(1/2)) signaling pathway by cyclic AMP potentiates the neuroprotective effect of the neurotransmitter noradrenaline on dopaminergic neurons. *Mol Pharmacol* **62,** 1043-1052.

Tsutsui S, Vergote D, Shariat N, Warren K, Ferguson SSG, Power C. (2008). Glucocorticoids regulate innate immunity in a model of multiple sclerosis: reciprocal interactions between the A1 adenosine receptor and β-arrestin-1 in monocytoid cells. *FASEB J* **22,** 786-796.

Tuppo EE & Arias HR. (2005). The role of inflammation in Alzheimer's disease. *Int J Biochem Cell Biol.* **37**, 289-305.

Ulvestad E, Williams K, Bjerkvig R, Tiekotter K, Antel J & Matre R. (1994). Human microglial cells have phenotypic and functional characteristics in common with both macrophages and dendritic antigen-presenting cells. *J Leukoc Biol* **56,** 732-740.

Velasco S, Tarlow M, Olsen K, Shay JW, McCracken GH, Jr. & Nisen PD. (1991). Temperature-dependent modulation of lipopolysaccharide-induced interleukin-1 beta and tumor necrosis factor alpha expression in cultured human astroglial cells by dexamethasone and indomethacin. *J Clin Invest* **87,** 1674-1680.

Vilhardt F. (2005). Microglia: phagocyte and glia cell. *Int J Biochem Cell Biol* **37,** 17-21.

Vos P, Davenport PA, Artymyshyn RP, Frazer A & Wolfe BB. (1987). Selective regulation of beta-2 adrenergic receptors by the chronic administration of the lipophilic beta adrenergic receptor agonist clenbuterol: an autoradiographic study. *J Pharmacol Exp Ther* **242,** 707-712.

Vroon A, Kavelaars A, Limmroth V, Lombardi MS, Goebel MU, Van Dam AM, Caron MG, Schedlowski M & Heijnen CJ. (2005). G protein-coupled receptor kinase 2 in multiple sclerosis and experimental autoimmune encephalomyelitis. *J Immunol* **174,** 4400-4406.

Wallukat G. (2002). The beta-adrenergic receptors. *Herz* **27,** 683-690.

Wheatley D. (1982). Clenbuterol ('Spiropent'):a longacting bronchodilator. *Curr. Med. Res. Opin.* **8**, 113.

Werstiuk ES & Lee RM. (2000). Vascular beta-adrenoceptor function in hypertension and in ageing. *Can J Physiol Pharmacol* **78,** 433-452.

Wieczorek M & Dunn AJ. (2006). Relationships among the behavioral, noradrenergic, and pituitary-adrenal responses to interleukin-1 and the effects of indomethacin. *Brain Behav Immun* **20,** 477-487.

Wong PK, Egan PJ, Croker BA, O'Donnell K, Sims NA, Drake S, Kiu H, McManus EJ, Alexander WS, Roberts AW & Wicks IP. (2006). SOCS-3 negatively regulates innate and adaptive immune mechanisms in acute IL-1-dependent inflammatory arthritis. *J Clin Invest* **116,** 1571-1581.

Wyss-Coray T & Mucke L (2002). Inflammation in neurodegenerative disease – a double-edged sword. *Neuron* **35,** 419-432.

Xiang Y & Kobilka B. (2003). The PDZ-binding motif of the beta2-adrenoceptor is essential for physiologic signaling and trafficking in cardiac myocytes. *Proc Natl Acad Sci U S A* **100,** 10776-10781.

Xie Z, Smith CJ & Van Eldik LJ. (2004). Activated glia induce neuron death via MAP kinase signaling pathways involving JNK and p38. *Glia* **45,** 170-179.

Xu B. (2001). The importance of beta-adrenergic receptors in immune regulation: a link between neuroendocrine and immune system. *Med Hypotheses* **56,** 273-276.

Yasojima K, Akiyama H, McGeer EG, McGeer PL. (2001). Reduced neprilysin in high plaque areas of Alzheimer brain: a possible relationship to deficient degradation of beta-amyloid peptide. *Neurosci Lett.* **297,** 97-100.

Yin KJ, Cirrito JR, Yan P, Hu X, Xiao Q, Pan X, Bateman R, Song H, Hsu FF, Turk J, Xu J, Hsu CY, Mills JC, Holtzman DM & Lee JM. (2006). Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-beta peptide catabolism. *J Neurosci* **26,** 10939-10948.

Yoshimura A, Naka T & Kubo M. (2007). SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol* **7,** 454-465.

Yu JT, Tan L, Ou JR, Zhu JX, Liu K, Song JH & Sun YP. (2008). Polymorphisms at the beta2-adrenergic receptor gene influence Alzheimer's disease susceptibility. *Brain Res* **1210,** 216-222.

Zafra F, Lindholm D, Castren E, Hartikka J & Thoenen H. (1992). Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J Neurosci* **12,** 4793-4799.

Zalko D, Debrauwer L, Bories G & Tulliez J. (1998). Metabolism of clenbuterol in rats. *Drug Metab Dispos* **26,** 891-899.

Zeinstra E, te Riele P, Langlois X, Wilczak N, Leysen J & de Keyser J. (2002). Aminergic receptors in astrogliotic plaques from patients with multiple sclerosis. *Neurosci Lett* **331,** 87-90.

Zeinstra E, Wilczak N & De Keyser J. (2000). [3H]dihydroalprenolol binding to beta adrenergic receptors in multiple sclerosis brain. *Neurosci Lett* **289,** 75-77.

Zhang HT, Huang Y, Mishler K, Roerig SC & O'Donnell JM. (2005). Interaction between the antidepressant-like behavioral effects of beta adrenergic agonists and the cyclic AMP PDE inhibitor rolipram in rats. *Psychopharmacology (Berl)* **182,** 104-115.

Zheng M, Han QD & Xiao RP. (2004). Distinct beta-adrenergic receptor subtype signaling in the heart and their pathophysiological relevance. *Sheng Li Xue Bao* **56,** 1-15.

Zhou ZZ & Jones SB. (1993). Involvement of central vs. peripheral mechanisms in mediating sympathoadrenal activation in endotoxic rats. *Am J Physiol* **265,** R683-688.

Zhu Y, Culmsee C, Roth-Eichhorn S & Krieglstein J. (2001). Beta(2)-adrenoceptor stimulation enhances latent transforming growth factor-beta-binding protein-1 and transforming growth factor-beta1 expression in rat hippocampus after transient forebrain ischemia. *Neuroscience* **107,** 593-602.

Zhu Y, Prehn JH, Culmsee C & Krieglstein J. (1999). The beta2-adrenoceptor agonist clenbuterol modulates Bcl-2, Bcl-xl and Bax protein expression following transient forebrain ischemia. *Neuroscience* **90,** 1255-1263.

Websites:

WHO organisation, Alzheimer's Disease Facts and Figures, accessed 29 September 2009
http://www.searo.who.int/en/Section1174/Section1199/Section1567/Section1823_8066.htm

Publications

E.N. McNamee, E.W. Griffin, K.M. Ryan, K.J. Ryan, S. Heffernan, A. Harkin, T.J. Connor. (2010). Noradrenaline acting at beta-adrenoceptors induces expression of IL-1beta and its negative regulators IL-1ra and IL-1RII, and drives an overall anti-inflammatory phenotype in rat cortex. *Neuropharmacology*.59(1-2):37-48.

K.M. Ryan, O.K. Mahfoud, R.S. Jones, A. Harkin, T.J. Connor (2009) The β_2-adrenoceptor agonist clenbuterol activates astrocytes and reduces amyloid-β concentrations and RAGE expression in rat cortex and hippocampus. *Glia* (Vol. 57, s106)

K.M. Ryan, A. Harkin, T.J. Connor (2009). A systemic LPS challenge does not alter central $\beta_2$adrenoceptor expression or reponsiveness. *Brain Behav Immun* (Vol. 23, Issue 6, Supp.2, s54-s55)

K.M. Ryan, E.N. McNamee, T.J. Connor (2008). The glucocorticoid dexamethasone prevents inflammation-induced suppression of glial β_2-adrenoceptor expression: Implications for Multiple Sclerosis. *Brain Behav Immun* (Vol 22, Issue 4, Supp 1, p26-27)

K.M. Ryan, E.N. McNamee, T.J. Connor (2008). Inflammation down-regulates β_2-adrenoceptor expression on primary glial cells: reversal by the glucocorticoid dexamethasone. *European Neuropsychopharmacology* 18 (p.s25-26)

K. Ryan, E.N. McNamee, T.J. Connor (2006) Exposure of primary glial cells to an inflammatory stimulus results in reduced β_2-adrenoceptor expression and responsiveness. International Journal of Neuropsychopharmacology 9(s1): s203.

K. Ryan, E.N. McNamee, T.J. Connor (2006) Exposure of primary glial cells to an inflammatory stimulus results in reduced β_2 -adrenoceptor expression and responsiveness. Irish Journal of Medical Science 175(3) es1: 92.