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Effect of the β_2 -adrenoceptor Agonist Clenbuterol on Inflammatory Signalling in the CNS and Behaviour

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

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Kathryn (Katie) Ryan

II Summary

This thesis explored the anti-inflammatory and neuroprotective properties elicited by stimulation of the noradrenergic receptor, the β_2 -adrenoceptor, in the rat brain. Neuroinflammation is a feature of many chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and multiple sclerosis and is driven mainly by activated microglia. The release of inflammatory mediators such as cytokines, chemokines, cell adhesion molecules and free radicals by microglia leads to slow and progressive destruction of neurons and axons in the CNS. Impairment of noradrenergic function is also suspected to contribute to the pathogenesis of neurodegenerative diseases. For example, in Alzheimer's disease and Parkinson's disease a profound loss of noradrenergic neurons occurs in the locus coeruleus. In addition, the noradrenergic system is reported to play an important role in the recovery from brain injury. There is growing interest in the central β_2 -adrenoceptors as a neuroprotective target due to the fact that stimulation of β_2 -adrenoceptors on glial cells induces expression of neurotrophic factors and promotes an anti-inflammatory phenotype. Thus, mechanisms that increase central noradrenergic tone may be beneficial in treating inflammation-related neurodegeneration.

Here we examined the impact of the brain penetrant β_2 -adrenoceptor agonist clenbuterol on the activity of the inflammatory mediator NF κ B in the rat brain. Specifically, *in vivo* studies whereby rats received an intraperitoneal (i.p.) injection of clenbuterol showed that clenbuterol suppressed central NF κ B activity and this was accompanied by an increase in the inhibitory I κ B α molecule. A time-course study conducted with clenbuterol showed that the increase in I κ B α protein expression coincided with the suppression of NF κ B activity, this suggested that the increased I κ B α mediates the clenbuterol-induced suppression of NF κ B activity. These effects are not limited to clenbuterol as two other β_2 -adrenoceptor agonists, formoterol and salbutamol, also suppressed NF κ B activity in rat brain. The findings confirmed, through the use of selective and non-selective antagonists for β_1 - and β_2 -adrenoceptor subtypes, that the effect of clenbuterol on NF κ B activity and I κ B α expression was mediated predominantly by the β_2 -adrenoceptor. Importantly, an experiment whereby rats were pre-treated with an i.p. injection of clenbuterol prior to intracerebroventricular injection of LPS showed that clenbuterol was also capable of attenuating LPS-induced NF κ B activity and this appeared to be mediated by an increase in I κ B α . Clenbuterol also successfully attenuated LPS-

induced expression of the inflammatory mediators TNF- α , iNOS and ICAM-1, and it increased expression of the broad-spectrum anti-inflammatory cytokine IL-10 and its downstream signalling molecules STAT-3 and SOCS-3. While clenbuterol also independently increased IL-1 β expression, this was accompanied by increased expression of interleukin-1 receptor antagonist (IL-1ra), an endogenously produced antagonist that prevents IL-1 β from acting on the IL-1type I receptor, and IL-1type I receptor (IL-1RII), a “decoy” receptor that binds IL-1 β without initiating the signalling cascade. Thus, IL-1ra and IL-1RII may serve to limit IL-1 β activity. Further *in vivo* work showed that pre-treatment with the glucocorticoid dexamethasone prior to the β_2 -adrenoceptor agonist clenbuterol elicited complimentary anti-inflammatory actions in the rat brain, more so than either agent alone. Dexamethasone blocked the clenbuterol-induced increase in IL-1 β while maintaining the beneficial effects of clenbuterol such as increased expression of IL-1ra, IL-1RII, IL-10 and SOCS-3. In addition, dexamethasone potentiated the clenbuterol-induced increase in I κ B α and combined treatment with the two agents suppressed NF κ B activity. Finally, central IL-1 β expression induced by clenbuterol was not involved in the suppressive effects of clenbuterol on locomotor activity or feeding. In addition, chronic administration of clenbuterol did not induce an anxious or depressive like phenotype in rats even in the presence of increased central IL-1 β .

Thus, these data indicate that administration of the β_2 -adrenoceptor agonist clenbuterol generates an anti-inflammatory cytokine profile and can attenuate LPS-induced inflammation, in the rat brain. In addition, these results present persuasive evidence that β_2 -adrenoceptors in combination with glucocorticoids induce complimentary anti-inflammatory actions in the rat CNS. Overall, while further research is needed to determine the mechanisms involved in the effect of clenbuterol on behaviour and ways in which these behavioural effects may be overcome, β_2 -adrenoceptor agonists may be a promising new therapeutic strategy for combating neurodegeneration that occurs secondary to inflammation.

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VII Abbreviations

AD	Alzheimer's disease
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCA	Bichinchonic acid
BDNF	Brain-derived neurotrophic factor
b.i.d.	Twice per day
BLA	Basolateral amygdala
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
COMT	Catechol O-methyltransferase
COPD	Chronic obstructive pulmonary disease
COX-II	Cyclooxygenase-2
Ct	Cycle time
Cx	Neocortex
DC	Dendritic cell
Dex	Dexamethasone
EMT	Extracellular monoamine transporter
ERK	Extracellular signal regulated kinase
FCx	Frontal cortex
g	Gram
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element

GTP	Guanosine triphosphate
Hipp	Hippocampus
hr	Hour
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
ICAM-1	Intracellular adhesion molecule-1
IFN- γ	Interferon gamma
IKK	Inhibitory kappa-B kinase
IL-10	Interleukin-10
IL-10RI	Interleukin-10 receptor-1
IL-10RII	Interleukin-10 receptor-2
IL-1ra	Interleukin-1 receptor antagonist
IL-1RAcp	Interleukin-1 receptor accessory protein
IL-1RI	Interleukin-1 type 1 receptor
IL-1RII	Interleukin-1 type 2 receptor
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K _d	Dissociation constant
kg	Kilogram
LABA	Long acting beta-adrenoceptor agonist
LC	Locus coeruleus
LPS	Lipopolysaccharide
MAD-3	Mitotic arrest deficient-3
MAL	MyD88 adaptor-like
MAO	Monoamine oxidase
MAPK	Mitogen activated protein kinase

mg	Milligram
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
<i>n</i>	Number of subjects in each treatment group
NA	Noradrenaline
NEMO	NF κ B essential modulator
NET	Noradrenaline transporter
NF κ B	Nuclear factor- κ B
NGF	Nerve growth factor
NK cells	Natural killer cells
nM	nanomolar
NO	Nitric oxide
OB	Olfactory bulb
<i>p</i>	Probability
PAMPS	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PCx	Piriform cortex
PD	Parkinson's disease
P-I κ B α	Phosphorylated I κ B α
PKA	Protein kinase A
PKC	Protein kinase C
PLA2	Type-II phospholipase A2
PRR	Pattern recognition receptor
P-STAT-3	Phosphorylated STAT-3
PVDF	Polyvinylidene fluoride
RLU	Relative light units
s	Second
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM	Standard error of mean
SNS	Sympathetic nervous system
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
T _(1/2)	Half-life
TAB2	TAK1-binding protein-2
TAK-1	Transforming growth factor- β -activated kinase
TGF- β	Transforming growth factor beta
TIR	Toll/Interleukin-1 like receptor
Thal	Thalamus
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor alpha
Tollip	Toll-interacting protein
TRAF-6	Tumour necrosis factor receptor-associated factor-6
TRAM	TRIF-related adaptor molecule
TRIF	TIR adaptor Toll/IL-1R domain containing adaptor-inducing IFN β
μ g	microgram
v/v	volume per volume
VCAM	Vascular cell adhesion molecule
VMAT	Vesicular monoamine transporter
w/v	weight per volume

Chapter 1

Introduction

1.1 The immune system

Vertebrates possess two types of immunity: innate and adaptive. The innate immune response represents the first line of defence during host infection and therefore plays a crucial role in the early response to invading pathogens. It is a non-specific response mediated by phagocytic cells and antigen presenting cells (APCs) which are genetically programmed to detect pathogenic microbes. The innate immune response employs both physical and chemical barriers to infection, as well as various cells which detect invading pathogens thus activating an immune response (Medzhitov & Janeway, 2000). Physical and chemical defence mechanisms include epithelium and secretions from mucosal surfaces, among others. Cellular components include antigen-presenting dendritic cells (DCs), phagocytic macrophages, and cytotoxic natural killer (NK) cells (Basset *et al.*, 2003). The adaptive immune system is responsible for eliminating pathogens in the late phase of infection. In contrast to innate immune cells, adaptive immune cells, B and T lymphocytes, employ antigen receptors that are not genetically encoded but are created *de novo* in each organism. Thus, the adaptive immune response is highly specific and is also responsible for generating immunological memory in the late phase of infection.

1.1.1 The central nervous system – an immune privileged site?

The central nervous system (CNS) is comprised of the brain and spinal cord. For decades, the CNS was believed to be immunologically separate from the peripheral immune system (Medawar, 1948; Barker & Billingham, 1977). However, despite Sir Peter Medawar's observation in the mid-20th century that the brain is an "immune privileged" site, it is now known that both innate and adaptive immune responses do take place in the CNS. This is partly facilitated by the absence of a blood-brain barrier (BBB) in certain brain areas such as the circumventricular organs (CVO) (Blatteis, 1992), where leakage of circulating molecules from the bloodstream into the CVO and possibly into adjacent CNS tissue, may occur (Konsman & Dantzer, 2001). It is important to note however that CNS immune responses are tightly regulated, due to the presence of a BBB in most other areas of the brain which largely restricts the exchange of immune cells. Also, the fact that the brain is encased in rigid bone makes it intolerant to the increase in extracellular fluid volume that accompanies many inflammatory reactions and because CNS neurons are mostly non-regenerative and vulnerable to damage from inflammatory

molecules, it seems that the CNS has limited capacity for immune reactivity (Hendriks *et al.*, 2005). In support of this, it has been widely demonstrated that it is much more difficult to elicit a typical leukocyte response in the CNS compared to other internal organs (Medawar, 1948; Barker & Billingham, 1977). For example, implantation of tumours in the CNS initiates a slower reaction compared to the skin (Head & Griffin, 1985; Stevenson *et al.*, 2002). More recently, inoculation of the influenza virus into the brain parenchyma showed slow and inefficient clearance (Stevenson *et al.*, 2002). Taken together, this data suggests that it is much more difficult to elicit an immune response against antigens in the CNS compared to other internal organs.

1.1.2 Resident immune cells of the CNS – microglia and astrocytes

Despite the idea that the CNS is an immune privileged site, it is clear that immune reactions do occur in the CNS (Bechmann, 2005; Wekerle, 2006) and it is probably more accurate to refer to it as a site of selective and modified immune reactivity (Ransohoff *et al.*, 2003). Immune reactions in the CNS are governed by glial cells including microglia and astrocytes. While one role of glial cells is to surround and support neurons they also play an important role in maintaining the immunosuppressive environment of the brain.

For example, microglia are constantly surveilling the CNS environment and phagocytosing cell debris. They have been described as the major resident immunocompetent cells in the brain (Benveniste, 1997; Lynch, 2009) and play a key role in mediating the inflammatory response through the release of inflammatory cytokines (Kato *et al.*, 1996). Much controversy exists over the neuroprotective versus neurodegenerative roles of activated microglia (Minton, 2001). On the one hand, activated microglia serve functions which maintain neuronal survival, by releasing anti-inflammatory cytokines such as transforming growth factor-beta (TGF- β) (Pratt & McPherson, 1997) and interleukin-10 (IL-10) (Ledeboer *et al.*, 2002). They also provide trophic support to neurons and clear up toxic cellular debris (Morgan *et al.*, 2004; Liao *et al.*, 2005). However, activated microglia can also damage or induce apoptosis of neurons through the release of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF- α) (Lokensgard *et al.*, 2001) and free radicals. There is also an abundance of literature which incriminates activated microglia as participants in neurodegenerative disorders such as multiple sclerosis,

Alzheimer's disease, and Parkinson's disease (see Minagar *et al.*, 2002; Nelson *et al.*, 2002; Streit, 2002). Thus, current evidence suggests that microglia act as a “double edged sword” with both neuroprotective and neurodegenerative properties.

Astrocytes are star-shaped cells that lack axons and dendrites. Their numerous processes extend to and surround neurons and blood vessels. They are usually identified in the CNS by the expression of glial fibrillary acidic protein (GFAP), a major component of astrocytic intermediate fibrils or glial fibrils (Eng *et al.*, 1971). Astrocytes possess a variety of receptors such as G-protein coupled receptors, receptors for chemokines and growth factors and receptors involved in the immune response such as Toll-like receptors (TLRs) (Kimelberg, 1995; Owens, 2005; Liu & Neufeld, 2007). Thus, astrocytes are well equipped to carry out various housekeeping roles as well as providing support to neurons under normal and inflammatory conditions, partly through release of growth factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Rudge *et al.*, 1992).

It is clear that activation of microglia and astrocytes is an important host defence mechanism during periods of stress in the CNS. While chronic activation of microglia has been implicated in many neurodegenerative diseases with detrimental effects in the CNS, astrocytic activation is thought to have more of a neuroprotective effect. In this regard, in Alzheimer's disease, activated microglia are found in close proximity to senile plaques where they produce various pro-inflammatory cytokines such as TNF- α and IL-1 β as well as neurotoxic free radicals that contribute to disease progression (Block *et al.*, 2007). On the other hand, astrocytes can release neurotrophic factors and may reduce the damaging effects of microglia activation (Rudge *et al.*, 1992; Vincent *et al.*, 1996; Aloisi *et al.*, 1997). However, there is still much debate as to whether glial cells are involved in the triggering of disease states or whether they are activated in response to the diseased state. A better understanding of the functions of glial cells has great therapeutic potential in treating the diseased or injured brain.

1.2 Neuroinflammation

Inflammation in the CNS, or neuroinflammation, describes a process involving activation of glial cells, cytokine and chemokine imbalance, neuronal damage, and neurodegeneration (Downer, 2011), and is a pathogenic feature of many neurodegenerative diseases such as multiple sclerosis, Parkinson's disease, Alzheimer's disease and stroke. Due to its complex nature, it is difficult to construct a linear sequence of events which describe the process of neuroinflammation. The idea that neuroinflammation is detrimental (Griffin *et al.*, 1989) implies that it precedes neurodegeneration. However, conflicting reports from animal studies show that glial activation may occur secondary to neuronal damage (Streit *et al.*, 2004). Thus, it appears that depending on the type of injury and the microenvironmental conditions, glial activation may have both harmful and beneficial functions during neuroinflammation. For example, while activated microglia are capable of inducing cell death and are the primary sources of pro-inflammatory cytokines in the brain (Lokensgard *et al.*, 2001; Tahraoui *et al.*, 2001), they are also known to enable neuronal survival (Ledeboer *et al.*, 2000). Similarly, while activated astrocytes can release neurotrophic factors and may reduce the damaging effects of microglia activation (Rudge *et al.*, 1992; Vincent *et al.*, 1996; Aloisi *et al.*, 1997), the scars that are often formed due to reactive astrogliosis may impede functional recovery after CNS injury (Hamby & Sofroniew, 2010). Thus, neuroinflammation must involve a complex series of feedback loops between neurons and glial cells making it extremely difficult to define a single mechanism.

Neuroinflammation may be acute or chronic. Before the term "neuroinflammation" became widely used, the acute CNS tissue response to injury was referred to as "reactive gliosis" whereby activated astrocytes and microglia rapidly responded to injury through the release of inflammatory factors. These days, the term "neuroinflammation" is mostly used in reference to chronic inflammation caused by the cumulative effect of activated astrocytes and microglia and the subsequent worsening of the initial neuro-destructive incident. The term is also most often used in reference to CNS disease rather than CNS injury. Multiple sclerosis is one example of CNS disease whereby axon degeneration occurs as a result of chronic inflammation (Hampton *et al.*, 2008). Inflammatory molecules generated by CNS immune cells serve to regulate various cellular processes. Infection or tissue damage within the CNS activates signalling cascades which eventually

lead to immune cell activation and proliferation with the aim of restoring the tissue to its normal state. However, if the inflammatory response continues unchecked a chronic inflammatory state may develop with detrimental consequences such as the development of neurodegenerative disease.

1.2.1 Immune signalling during neuroinflammation

As mentioned above, glial cells play an active role in the CNS immune response. Along with dendritic cells and natural killer cells, glial cells possess microbial sensors called pattern recognition receptors (PRRs); these receptors recognise invariant molecular structures on pathogens called pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). Several families of PRRs have been discovered, of which the family of TLRs has been studied most extensively (Medzhitov & Janeway, 2000; Akira *et al.*, 2006). In mammals, TLRs are either expressed on the plasma membrane or in endosomal/lysosomal organelles (Takeda & Akira, 2005). Specifically, it is the cell surface TLR-4 that recognises the microbial pattern on lipopolysaccharide (LPS) of Gram-negative bacteria (Iwasaki & Medzhitov, 2010). Upon PAMP recognition, PRRs send an inflammatory signal to the host and initiate proinflammatory responses by activation of a cascade of signalling pathways. Activation of these signalling pathways results in gene expression and synthesis of a multitude of molecules such as cytokines, chemokines, cell adhesion molecules (CAMs) and immunoreceptors (Akira *et al.*, 2006), which are all involved in the early response to infection. These responses ultimately lead to clearance of infection. However, in certain situations PRRs may recognise host factors as “danger” which may lead to inflammatory diseases or autoimmunity (Beg, 2002; Matzinger, 2002).

1.3 Mediators of the CNS immune response

Of particular importance in CNS inflammation are immunomodulators known as cytokines. These small proteins are called “cytokines” because almost all nucleated cells are capable of synthesising, releasing and, thus, responding to them (Dinarello, 2000). Some cytokines promote inflammation and are called *pro-inflammatory* cytokines; others suppress genes for pro-inflammatory cytokines and are known as *anti-inflammatory* cytokines. It is important to note that, although cytokines are listed as either pro- or anti-

inflammatory, they may function differently depending on the biological process (Dinarello, 2000). The present section provides a description of some of the key mediators involved in the immune response.

1.3.1 Interleukin-1 (IL-1)

The pro-inflammatory cytokine interleukin-1 (IL-1) is a 17kD protein consisting of two closely related isoforms: IL-1 α and IL-1 β . Both isoforms are synthesised in the cytoplasm as precursors. The IL-1 α precursor is biologically active (Mosley *et al.*, 1987), in other words it is free to bind to receptors and activate cells, and mostly resides intracellularly; it may also be cleaved by calpain and released from the cell. In contrast, the IL-1 β precursor is inactive and must be cleaved by the enzyme caspase-1 (formerly known as IL-1 β -converting enzyme or ICE) before it is fully activated and transported out of the cytoplasm (Dinarello, 1998).

There are two receptors for IL-1, IL-1 type I receptor (IL-1RI) which mediates IL-1 signalling and IL-1 type II receptor (IL-1RII) which does not initiate the signalling cascade. Thus, IL-1RII is thought to be a “decoy” receptor which inhibits IL-1 signalling (Colotta *et al.*, 1994). The IL-1RI requires the additional binding of IL-1 receptor accessory protein (IL-1RAcP) for signal transduction (Greenfeder *et al.*, 1995). IL-1RI also binds IL-1 receptor antagonist (IL-1ra) a competitive antagonist of IL-1RI which blocks all actions of IL-1 (Dinarello, 1998). Even though IL-1ra binds to the IL-1RI with the same affinity as IL-1 α and IL-1 β it fails to trigger a response (Granowitz *et al.*, 1992) and thus is a useful tool for blocking IL-1 actions and limiting neuronal damage.

The actions of IL-1 are diverse. For example, IL-1 can cause fever, “suppression of appetite and loss of weight, modulation of sleep, alterations in endocrine, immune, and nervous system functions, changes in behaviour as well as influences on synaptic plasticity, neuronal transmission, epilepsy,” and neuronal damage (Rothwell, 2003). There is also some evidence that IL-1 expression may have beneficial consequences and these will be discussed shortly. IL-1 is constitutively expressed in the human and rodent brain, albeit at low levels and mostly in the form of IL-1 β (Touzani *et al.*, 1999; Rothwell, 2003). Most cells in the brain are capable of expressing IL-1 including neurons, astrocytes, microglia, oligodendrocytes and endothelial cells (Touzani *et al.*,

1999). Microglia are the key cells involved in its early expression, and synthesis of IL-1 occurs rapidly following CNS insult; mRNA is expressed within 15 minutes and protein within 60 minutes (Vitkovic *et al.*, 2000; Rothwell, 2003).

1.3.1.1 The role of IL-1 in neuroinflammation

IL-1 α and IL-1 β are rapidly upregulated following neuronal injury. Their involvement in acute inflammation, following stroke and traumatic head injury, has been well documented, but it is also known to be present in the brain and cerebrospinal fluid of patients with chronic neuroinflammatory disorders, such as multiple sclerosis and Parkinson's disease (Chung *et al.*, 1991; Basu *et al.*, 2004). Although most cells of the brain can release IL-1, microglial cells are the early releasers during neuroinflammation (Giulian *et al.*, 1986; Pearson *et al.*, 1999). IL-1 is thought to play an essential role in neuronal injury and this has been demonstrated repeatedly by studies showing that administration of IL-1ra provides protection against neuronal injury caused by cerebral ischaemia, excitotoxins and trauma (Relton & Rothwell, 1992; Stroemer & Rothwell, 1997). This is supported by other data that shows that deletion of the IL-1 gene reduces ischaemic brain damage in mice (Boutin *et al.*, 2001). IL-1 is thought to contribute to brain inflammation by binding to the IL-1R1 on neurons, astrocytes and microglia. Activation of astrocytes by IL-1 leads to increased cell proliferation (astrogliosis) and increased expression of genes encoding cytokines, chemokines, and cell adhesion molecules, some of which have the potential to be neurotoxic but others may be beneficial and promote neuronal survival. Conversely, activation of microglial cells by IL-1 appears to mediate predominantly neurotoxic effects. The precise mechanisms by which IL-1 mediates neuronal injury are very difficult to pin down due to the array of roles that IL-1 plays in different systems. However, it is known that IL-1 signalling predominantly leads to activation of the transcription factor nuclear factor- κ B (NF κ B) which ultimately leads to transcription of many pro-inflammatory genes, as described in the next section.

1.3.1.2 IL-1 signalling via the NF κ B pathway

It is widely accepted that IL-1 is a member of the IL-1 receptor and TLR superfamily that signal through the NF κ B pathway (Chao *et al.*, 1997; Parker *et al.*, 2002). Activation of

NF κ B ultimately results in expression of genes encoding chemokines, cytokines such as TNF- α and IL-6, and adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1); all of which are involved in driving the immune response (Subramaniam *et al.*, 2004).

The following cascade occurs upon binding of IL-1 to its receptor: Firstly, a molecule known as myeloid differentiation factor 88 (MyD88) is recruited. This is a critical protein for IL-1 signalling and is composed of a Toll/IL-1R (TIR) domain and a death domain, and it acts as an adaptor to recruit IL-1 receptor-associated kinase (IRAK) (Wesche *et al.*, 1997; Takeuchi *et al.*, 2000) with the help of Toll-interacting protein (Tollip) (Burns *et al.*, 2000). IRAK is phosphorylated at the receptor complex (Cao *et al.*, 1996a) and subsequently dissociates to interact with TNF receptor-associated factor 6 (TRAF-6) (Cao *et al.*, 1996b). Interaction of IRAK with TRAF-6 leads to activation of the kinase TAK-1 (transforming growth factor- β -activated kinase) (Ninomiya-Tsuji *et al.*, 1999). TAK-1 with the help of TAK1-binding protein-2 (TAB-2) activates the inhibitory κ B (I κ B) kinase (IKK) complex. TRAF-6 can also activate other mitogen activated protein kinases (MAPK) that stimulate the IKK complex, these are described elsewhere by Moynagh (2005). The IKK complex is composed of two catalytic subunits, IKK α and IKK β , and a scaffolding subunit, IKK γ /NEMO (NF κ B essential modulator). This scaffolding subunit is thought to be a critical component of the NF κ B pathway as a mutation of NEMO in mice is lethal due to massive hepatic apoptosis (Rudolph *et al.*, 2000) (**Figure 1.1**).

In the resting cell, NF κ B resides in the cytoplasm where it is bound to an inhibitory molecule I κ B, the most common forms being I κ B α and I κ B β (Ghosh *et al.*, 1998). This interaction sequesters NF κ B in the cytoplasm and inhibits its DNA-binding activity. Activation of the IKK complex by TRAF-6 and other molecules leads to phosphorylation and degradation of I κ B which releases NF κ B allowing it to translocate to the nucleus (Karin & Ben-Neriah, 2000) where it regulates the expression of numerous genes involved in inflammation such as pro-inflammatory cytokines (IL-1, TNF- α and IL-6), chemokines, enzymes such as cyclooxygenase-2 (COX-II) and inducible nitric oxide synthase (iNOS), and CAMs (Minagar *et al.*, 2002; Simi *et al.*, 2005). Notably, I κ B α is rapidly re-synthesised following NF κ B activation due to the presence of a transcriptional binding site for I κ B on the NF κ B promoter. This is a critical inhibitory step. Newly

synthesised cytoplasmic I κ B α translocates to the nucleus where it terminates the activity of NF κ B by transporting it back to the cytoplasm (Arenzana-Seisdedos *et al.*, 1995; and 1997) (**Figure 1.1**).

Dysregulation of NF κ B could play a role in the pathogenesis of inflammatory and autoimmune disorders (Yamamoto & Gaynor, 2001). However, although NF κ B may be an attractive therapeutic target in inflammatory disease, it is important to note that it also plays an important role in normal cell function, such as the mounting of an immune response. Furthermore, it is involved in the embryonic formation of limbs and bones. Thus, total inhibition of NF κ B may lead to detrimental side effects. In order to minimise negative effects, new therapies should aim to target specific components of the NF κ B pathway that are involved in the particular disease (Li & Verma, 2002).

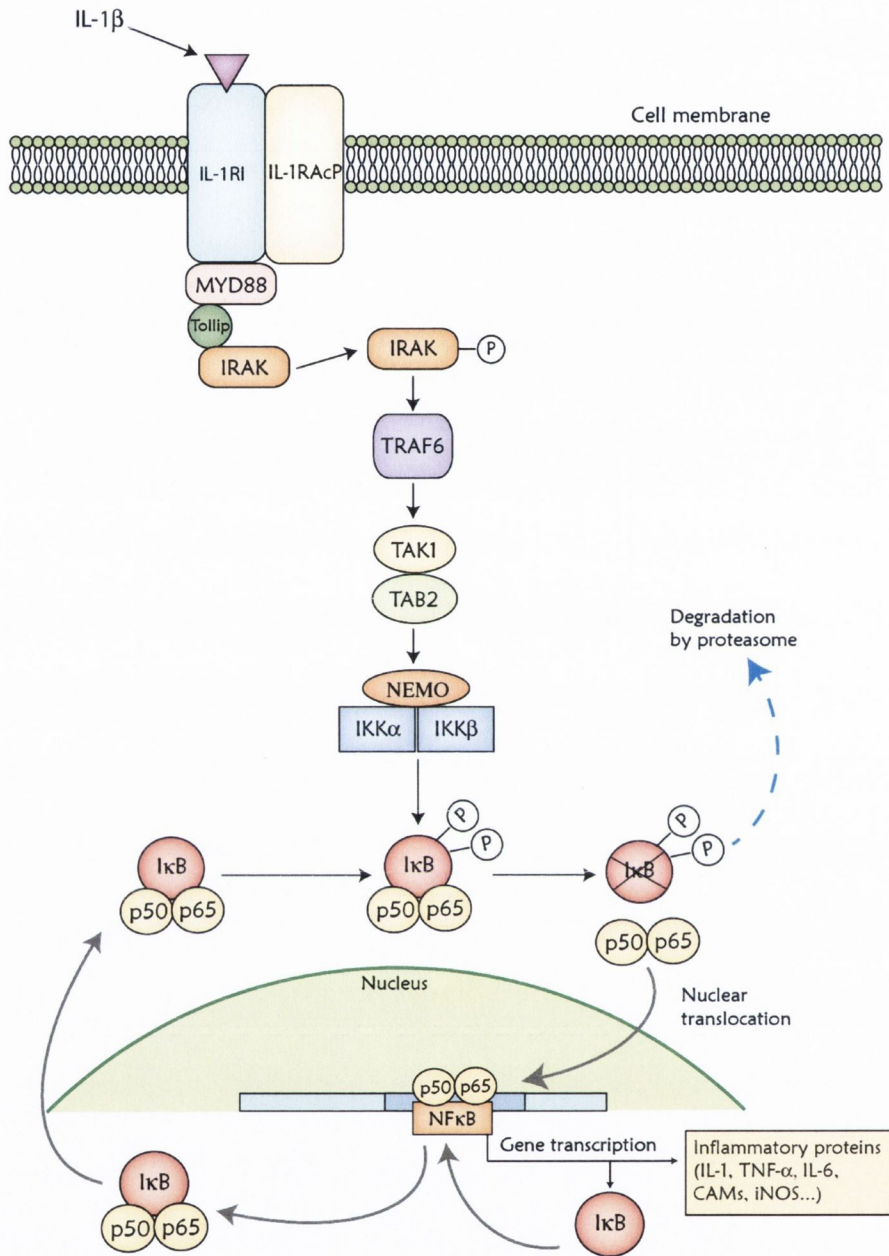


Figure 1.1. IL-1 signalling via IL-1R1 activates the NF κ B pathway. Binding of IL-1 β to the IL-1R1 leads to recruitment of MyD88. With the help of tollip, MyD88 recruits IRAK which is subsequently phosphorylated and dissociates to interact with TRAF-6. This leads to activation of TAK-1 and with the help of TAB-2 the IKK complex (IKK α and IKK β , and a scaffolding subunit, IKK γ /NEMO) is activated. In the resting cell, the two NF κ B subunits (p50 and p65) reside in the cytoplasm bound to I κ B. Activation of the IKK complex leads to phosphorylation and degradation of I κ B which releases NF κ B allowing it to translocate to the nucleus where it regulates the expression of numerous genes involved in inflammation as well as I κ B α .

1.3.1.3 IL-1 and sickness behaviour

IL-1 expression is induced during CNS inflammation and it not only influences the local brain microenvironment but also influences systemic functions that the host carries out in response to inflammation. For example, the release of IL-1 and other cytokines and inflammatory mediators during inflammation triggers a response known as “sickness behaviour” (Kent *et al.*, 1992). It is part of the body’s natural reaction to fight infection (Hart, 1988) and includes development of fever, reduced food intake and body mass, fatigue, as well as reduced social exploration (Dantzer, 2009). Central IL-1 is thought to be an important mediator of sickness behaviour because injection of IL-1 directly into the rat brain produces sickness behaviours such as reduced locomotor activity, food intake and body mass (Anforth *et al.*, 1998; Nadjar *et al.*, 2005). This is supported by studies that show that administration of IL-1ra prevents certain behavioural effects of LPS such as reduced feeding and social exploration (Bluthe *et al.*, 1992; Laye *et al.*, 2000; Konsman *et al.*, 2008).

There is still much debate as to whether central or peripheral IL-1 mediates the reduction in food intake that accompanies sickness behaviour. For instance, the latency to depressed feeding is shorter following intraperitoneal (i.p.) administration (1 hour) compared to intracerebroventricular (i.c.v.) administration (2-4 hours) (Kent *et al.*, 1996) which suggests that it is mediated by peripheral IL-1 β . In contrast, when IL-1 β is administered i.p., a much greater dose (100 fold) is required to suppress feeding compared to the dose required when injected i.c.v. (Eisenberg *et al.*, 1990) which suggests that central IL-1 β is the main mediator. In addition, caspase-1 knockout mice, which lack the enzyme responsible for activating IL-1 β , are less sensitive to the depressing effects of i.c.v. LPS but not i.p. LPS on food intake (Burgess *et al.*, 1998), thus supporting a role for central IL-1 β . The mechanisms regarding the depressive effect of LPS on feeding therefore remain unclear, it is possible however that both central and peripheral IL-1 β can play a part (Kent *et al.*, 1996).

Notably, sickness behaviours are not solely mediated by IL-1. Fever is a rise in body temperature above normal, and body temperature is regulated by temperature sensitive neurons in the hypothalamus, thus cytokines must act centrally to induce fever. IL-1 β is a cytokine with known pyrogenic properties and is known to act in the CNS to induce fever

(Berkenbosch *et al.*, 1987; Ericsson *et al.*, 1994). However, it appears that IL-1 is not the only mediator of fever because LPS-induced fever is not blocked by i.c.v. administration of IL-1ra (Konsman *et al.*, 2008). Rather than acting independently, IL-1 most likely acts in synergy with other cytokines to induce sickness behaviours. For example, IL-1 and IL-6 are thought to act synergistically to induce fever and reduce appetite (Lenczowski *et al.*, 1999; Campbell *et al.*, 2010).

Thus, while IL-1 β is thought to be the predominant mediator of sickness behaviour; other cytokines such as TNF- α and IL-6 also play a part. This is further evidenced by the fact that IL-1RI knockout mice, while being non-responsive to IL-1, still display depressed behaviour in response to LPS (Bluthe *et al.*, 2000). However, when TNF- α is blocked in these mice the behaviourally depressing effects of LPS are reduced, supporting a role for TNF- α . While the signalling pathways that mediate the depressive effect of IL-1 on behaviour have not been fully elucidated, it is likely that NF κ B signalling is involved because blocking of NF κ B signalling is known to reduce IL-1-induced sickness behaviours (Kubota *et al.*, 2000).

1.3.1.4 Can IL-1 expression be beneficial?

While IL-1 is known predominantly for its pro-inflammatory characteristics, low levels of expression may be beneficial in the brain (see **Figure 1.2**). For example, IL-1 can stimulate the synthesis of anti-inflammatory cytokines such as IL-1ra, IL-4, IL-10 and transforming growth factor- β (TGF- β), which may exert a negative feedback effect on the inflammatory cascade (Touzani *et al.*, 1999). IL-1 is also known to stimulate the release of NGF from astrocytes (Carman-Krzan *et al.*, 1991), and this has been shown to be neuroprotective in cultured primary cortical neurons (Strijbos & Rothwell, 1995). In addition, administration of exogenous IL-1 to the healthy rat brain does not lead to neuronal damage, however administration of IL-1 to the injured brain, or when co-administered with other cytokines, significantly enhances neuronal toxicity (Stroemer & Rothwell, 1997). Thus, it appears that IL-1 expression on its own does not cause neuronal damage, but in the presence of other cytokines a synergistic effect on neuronal damage is observed.

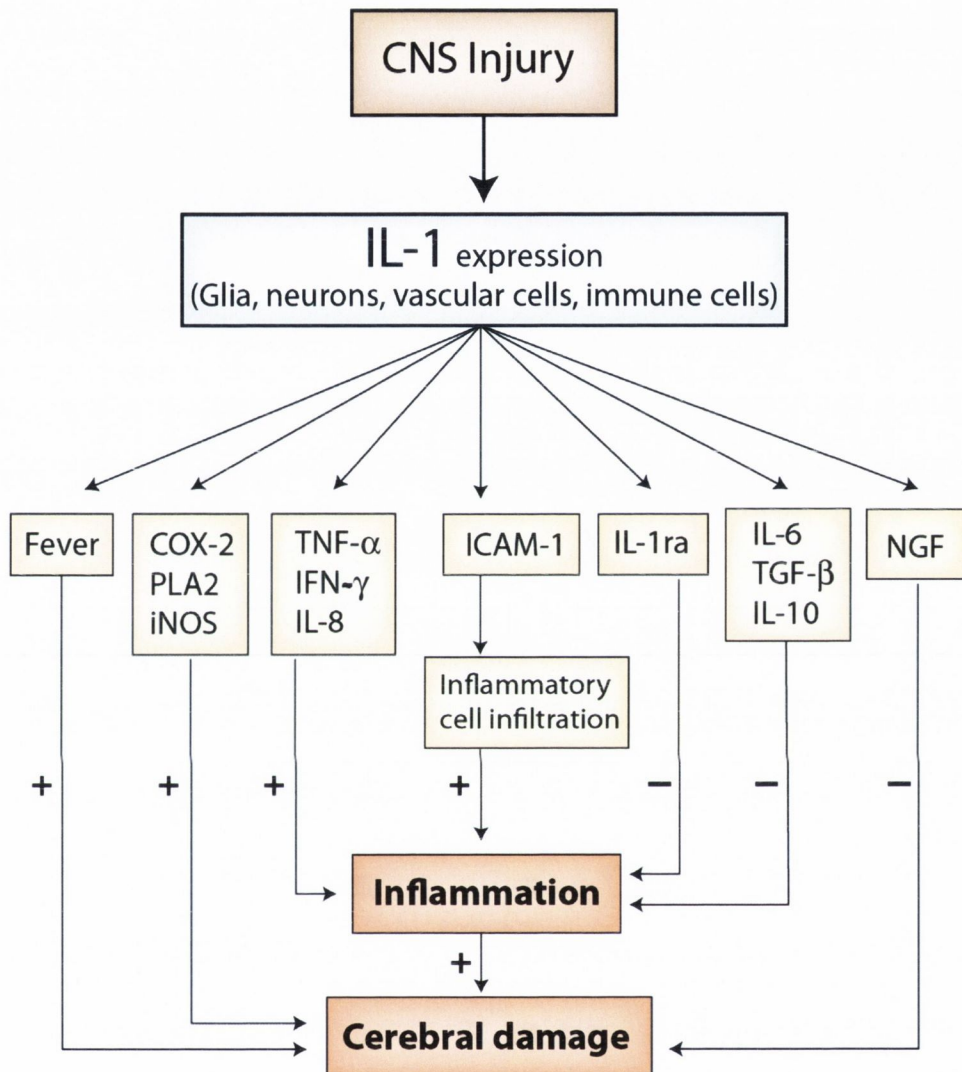


Figure 1.2. Summary of the proposed effects of interleukin-1 β following brain injury. IL-1 can be expressed by numerous cell types after brain injury. It can have many actions on aspects of central nervous system function that might contribute to or limit subsequent neuronal damage [modified from (Touzani *et al.*, 1999)].

1.3.2 Tumour Necrosis Factor- α (TNF- α)

TNF- α is a 212-amino acid protein that localises to the cell surface (Pennica *et al.*, 1984) and is generated in response to a variety of CNS insults. In addition to its ability to trigger apoptosis of tumour cells, TNF- α is one of the main mediators of the inflammatory response (Chen & Goeddel, 2002). This cytokine signals through two receptors, TNF-RI and TNF-RII which are expressed throughout the CNS on microglia, astrocytes and neurons (Kinouchi *et al.*, 1991). Binding of TNF- α to its receptor ultimately leads to activation of two major transcription factors, NF κ B and JNK (c-Jun N-terminal kinase). Transcription factor activation up-regulates pro-inflammatory genes such as type-II phospholipase (PL) A₂, COX-II, and iNOS, all of which increase the synthesis of platelet-activating factor and leukotrienes, and NO. Chemokines are also produced which attract and facilitate movement of infiltrating leukocytes (Dinarello, 2000). Through this signalling network TNF- α regulates diverse biological processes such as neuronal development, cell survival, and synaptic transmission (Schneider-Brachert *et al.*, 2004). Notably, TNF- α acts synergistically with another pro-inflammatory cytokine IL-1, which both act through the NF κ B pathway and trigger similar inflammatory mediators such as PLA₂ and COX-II.

1.3.3 Interleukin-6 (IL-6)

The cytokine IL-6 is present in the brain at low levels under normal conditions and becomes elevated following brain injury or inflammation. The physiological roles of IL-6 are complex and not fully understood. Evidence suggests that IL-6 can be both neuroprotective and neurodestructive in the CNS (see Van Wagoner & Benveniste, 1999). In the brain, IL-6 is secreted by endothelial cells, neurons and in particular, astrocytes (Juttler *et al.*, 2002; De Keyser *et al.*, 2008). IL-6 signals through a receptor complex composed of the IL-6 receptor and the signal transducing protein gp130. Binding of IL-6 and its receptor leads to activation of various signalling pathways such as Janus kinase (JAK)/signal transducer and activator of transcription (STAT), MAPK, and NF κ B (Heinrich *et al.*, 1998).

While *in vitro* and *in vivo* studies demonstrate that IL-6 is involved in neuronal survival, protection, and differentiation (Gadient & Otten, 1997; Loddick *et al.*, 1998), IL-6

overexpression also contributes to the pathophysiology of many diseases. This is supported by results from a murine model in which IL-6 is highly expressed. In this model, the mice develop neurodegeneration, blood brain barrier destruction, angiogenesis and impaired learning (Heyser *et al.*, 1997) and this may occur due to increased central production of inflammatory cytokines following IL-6 overproduction (Di Santo *et al.*, 1996). Thus, it is imperative that IL-6 levels are tightly regulated in order to preserve its beneficial functions and prevent its potentially destructive effects. IL-6 levels can be regulated by other cytokines such as IL-1 β and TNF- α . In addition, LPS-induced IL-6 expression can be regulated by the β -adrenoceptor agonist isoproterenol (Nakamura *et al.*, 1998) and the synthetic glucocorticoid dexamethasone (Nakamura *et al.*, 1998).

1.3.4 Inducible Nitric Oxide Synthase (iNOS)

Inducible nitric oxide synthase is one of three isoforms that generate NO. It is expressed in various cell types including microglia and astrocytes in the CNS following neurotoxic or inflammatory damage (Galea *et al.*, 1992). The other two forms are constitutively expressed in neurons (nNOS) and endothelial cells (eNOS) (Jaffrey & Snyder, 1995). Nitric oxide is synthesised by the various isoforms of NOS through the conversion of the amino acid L-arginine to L-citrulline (Stuehr, 1999). NO is a free-radical which has both neuroprotective and neurotoxic effects. At low concentrations NO is involved in neurotransmission and vasodilation, however it is potently neurotoxic at high levels. The excessive levels of NO that are produced as a result of iNOS expression in the CNS is thought to contribute to neuronal death during neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Dawson & Dawson, 1998; Iravani *et al.*, 2002). In addition, it is hypothesised that NO contributes to oligodendrocyte degeneration in demyelinating diseases such as multiple sclerosis (Liu *et al.*, 2002). iNOS expression is promoted by the bacterial endotoxin LPS and also by various proinflammatory cytokines such as IL-1 β , interferon- γ (IFN- γ) and TNF- α (Simmons & Murphy, 1992). It is also thought that the I κ B/NF κ B transcription pathway is essential for iNOS gene transcription and initiation of a proinflammatory response (Pannu & Singh, 2006). Thus, in the design of iNOS inhibitors for the treatment of NO-mediated neurodegenerative diseases, the design of NF κ B inhibitors may be useful seeing as NF κ B is a regulatory step for iNOS expression.

1.3.5 Interleukin-10 (IL-10) and suppressor of cytokine signalling-3 (SOCS-3)

IL-10 has long been recognised as a potent anti-inflammatory cytokine and is a member of a group of cytokines including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29 (Commins *et al.*, 2008). The members of this superfamily are genetically related and signal through similar receptors and signalling cascades, however, their functions are quite diverse and include immune suppression and anti-tumour activity (Commins *et al.*, 2008). The most common sources of IL-10 are regulatory T lymphocytes, monocytes and B cells (Del Prete *et al.*, 1993). IL-10 signals through a two-receptor complex consisting of two copies each of the IL-10 receptor-1 (IL-10R1) and IL-10 receptor-2 (IL-10R2). IL-10 binds to IL-10R1 with high affinity and to IL-10R2 to a lesser extent. However, association of IL-10R2 with IL-10R1 is essential for the receptor complex to function. Binding of IL-10 to the receptor complex results in activation of the JAK/STAT pathway (Mosser & Zhang, 2008). Firstly, activation of JAK1 and Tyk2, which are associated with IL-10R1 and IL-10R2 respectively, occurs. This results in phosphorylation of the cytoplasmic end of the receptor complex and recruitment of STAT-3 to IL-10R1 (Donnelly *et al.*, 2004). Subsequently, the recruited STAT-3 molecules form a homodimer which translocates to the nucleus and binds to the promoter regions of various genes (Donnelly *et al.*, 2004). A number of genes are activated by STAT-3 binding; including IL-10 itself and SOCS-3 which can feed back to control STAT-3 activation. SOCS-3 also exerts inhibitory effects on various cytokine genes.

The main biological effects of IL-10 are mediated through dendritic cells and macrophages where inhibition of MHC class II molecule expression and co-stimulatory molecules, CD80 and CD86, occurs. IL-10 also inhibits the production of inflammatory cytokines such as IL-1, IL-6, TNF- α , chemokines, and macrophage matrix metalloproteases (MMPs) (Mosser & Zhang, 2008). In addition, IL-10 increases the release of IL-1ra by macrophages, and as discussed previously, IL-1ra is a negative regulator of the inflammatory cytokine IL-1. The therapeutic benefits of IL-10 have been investigated for the last decade. The importance of IL-10 in limiting autoimmune pathologies can be seen in IL-10-deficient mice studies. Almost every autoimmune state including experimental autoimmune encephalitis, rheumatoid arthritis, and inflammatory bowel disease, is exacerbated in mice lacking IL-10 (Mosser & Zhang, 2008). Similarly, some bacterial infections that are normally tolerated by the body are often lethal to mice

lacking IL-10 (Gazzinelli *et al.*, 1996; Hunter *et al.*, 1997). However, despite the potential benefits of increasing IL-10, the use of this cytokine as a therapeutic agent has only been marginally successful to date. The main reason for this is that IL-10 overexpression can also result in immunosuppression in some cases (Mosser & Zhang, 2008).

1.4 Generation of an experimental inflammatory environment using LPS

Neuroinflammation is thought to contribute to the pathophysiology of various acute and chronic neurodegenerative diseases (Eikelenboom *et al.*, 1994; McGeer & McGeer, 1995; Hauss-Wegrzyniak *et al.*, 1998b; Griffin & Mrak, 2002; Allan *et al.*, 2005). The gram-negative bacterial cell wall component LPS is a potent pro-inflammatory agent (Rietschel *et al.*, 1994) which is often used to study the inflammatory response in the brain (Montero-Menei *et al.*, 1996; Szczepanik *et al.*, 1996). LPS can be administered peripherally or centrally. The present thesis utilises central i.c.v. administration of LPS to the rat brain and this is known to create an environment that is typical of a normal inflammatory response, i.e. one which includes activation of glial cells and increased pro-inflammatory cytokine release such as IL-1 β and TNF- α (Hauss-Wegrzyniak *et al.*, 1998a).

LPS is an endotoxin that is derived from the outer cell membrane of gram-negative bacteria (Rietschel *et al.*, 1994). As described earlier in this chapter, host immune cells possess pattern recognition receptors (PRRs) which recognise microbial patterns called pathogen-associated molecular patterns (PAMPs). One major class of PRR are the Toll-like receptors (TLRs). The TLR-4 subtype recognises LPS and is located on the surface of many cell types in the CNS including microglia and astrocytes (Akundi *et al.*, 2005; Jack *et al.*, 2005). Binding of LPS to TLR-4 induces the formation of a symmetric TLR4-MD2-LPS multimer which, along with the recruitment of the adaptor protein CD14, initiates signalling (Hennessy *et al.*, 2010). TLR-4 has an intracellular Toll/IL-1R (TIR) domain which engages adaptor proteins and triggers activation of two signalling cascades: namely the myeloid differentiation factor 88 (MyD88)-dependent or MyD88-independent pathway.

The MyD88-dependant pathway requires the bridging adaptor protein MyD88 adaptor-like (MAL) which associates with MyD88. MyD88 then activates members of the serine/threonine kinase interleukin-1 receptor-associated kinase (IRAK) family which triggers downstream signalling cascades that lead to activation of NF κ B and subsequent induction of pro-inflammatory cytokines. The MyD88-independant pathway requires the bridging adaptor TRIF-related adaptor molecule (TRAM) to associate with TRIF (TIR adaptor Toll/IL-1R domain-containing adaptor-inducing IFN- β). This leads to activation

of the transcription factor IRF-3 and the induction of genes such as those encoding Type I interferons (Hennessy *et al.*, 2010) (**Figure 1.3**).

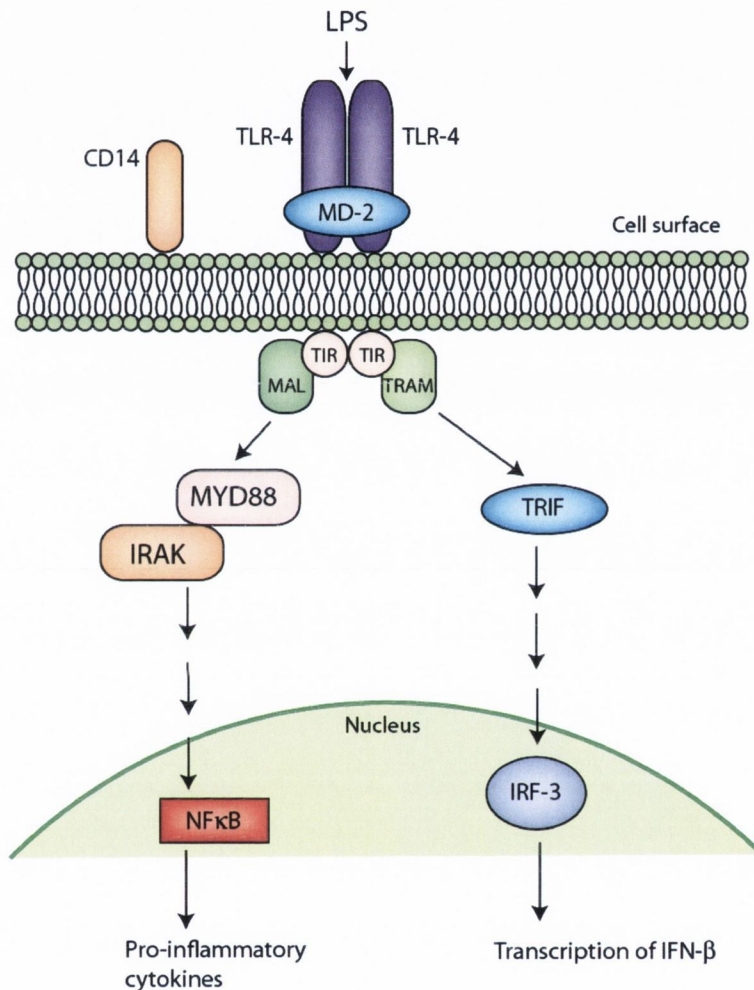


Figure 1.3. TLR-4 signalling through MyD88-dependant and MyD88-independent pathways. Binding of LPS to the TLR-4 receptor complex can induce two separate signalling pathways; MyD88-dependant and MyD88-independent. The MyD88-dependant pathway is initiated when the intracellular TIR domain engages the adaptor protein MAL which then associates with MyD88. Subsequent activation of the IRAK family triggers a signalling cascade that leads to activation of NFκB and subsequent induction of pro-inflammatory cytokines. The MyD88-independent pathway requires engagement of the TIR domain to TRAM which then associates with TRIF ultimately leading to activation of IRF-3 and transcription of IFN-β.

1.5 Modulation of cytokine release by the sympathetic nervous system

1.5.1 Sympathetic nervous system overview

The sympathetic nervous system (SNS) is a major component of the autonomic nervous system and provides a communicative link between the brain and the immune system via direct neural influences (Elenkov *et al.*, 2000). Sympathetic nerve fibres originate in the brain stem and give rise to pre-ganglionic fibres which leave the CNS via the thoracic and lumbar spinal nerves. They connect with post-ganglionic fibres located in ganglia located on either side of the spinal column, and from here the post-ganglionic fibres extend to innervate various tissues. Following activation by stressors the SNS predominantly releases the catecholamine neurotransmitter noradrenaline (NA) from nerves, though adrenaline is also released to a lesser extent (Von Euler, 1946). Centrally, neurons that release NA (noradrenergic neurons) project from a region called the locus coeruleus (LC) and supply NA to almost all parts of the CNS including the hippocampus, frontal cortex and entorhinal cortex (Feinstein *et al.*, 2002) which are involved in cognitive function, learning, and memory (**Figure 1.4**). In addition, a substantial body of noradrenergic neurons are found in the caudal brainstem and these neurons project to the paraventricular nucleus of the hypothalamus (Sawchenko & Swanson, 1982). Due to the well known involvement of the hypothalamus in mediating sickness behaviours, these caudal brainstem projections are likely to be involved in the initiation of sickness behaviours (Gaykema & Goehler, 2011). Thus, activation of the NA system by stress leads to release of central NA throughout the brain and an increase in arousal and vigilance and, simultaneously, NA release in the periphery controls fuel metabolism, heart rate, blood vessel tone, and thermogenesis (Elenkov *et al.*, 2000).

1.5.2 Noradrenaline

As mentioned above, NA is a catecholamine neurotransmitter in the brain, the major source of which are noradrenergic neurons located in the LC. The LC is a noradrenergic nucleus located on the lateral aspect of the fourth ventricle and sends branches to almost all forebrain structures (Aston-Jones *et al.*, 2000). Depletion of LC neurons and their noradrenergic projections and a decrease in cortical noradrenaline are prominent features of various neurodegenerative diseases, including Alzheimer's disease and Parkinson's

disease, which will be discussed in more detail later. It is important Following release into the synapse, NA is free to interact with adrenergic receptors (adrenoceptors) on the pre- and post-synaptic membranes. Importantly, NA is not confined to the synaptic cleft and can reach proximal astrocytes and microglia (Aoki, 1992). In fact, astrocytes (Aoki, 1992; Milner *et al.*, 2000) and microglia (Mori *et al.*, 2002) are known to express adrenoceptors. Thus, NA could be referred to as a paracrine signalling molecule.

Noradrenaline does not remain in the synapse for very long. Excess NA must be cleared from the synapse to prevent overstimulation of cells. Excess NA is transported back into the pre-synaptic neuron via the high affinity noradrenaline transporter (NET) located in the pre-synaptic membrane, or into neighbouring cells by the extracellular monoamine transporter (EMT). Neuronal metabolism of NA occurs by oxidative deamination catalysed by the enzymes monoamine oxidase (MAO) and catechol O-methyltransferase (COMT) (Eisenhofer, 2001). However, only 30% of re-captured NA is deaminated; the other 70% is sequestered into storage vesicles through the vesicular monoamine transporter (VMAT) (Eisenhofer *et al.*, 1987; Eisenhofer *et al.*, 1988). In this way NA stores are maintained.

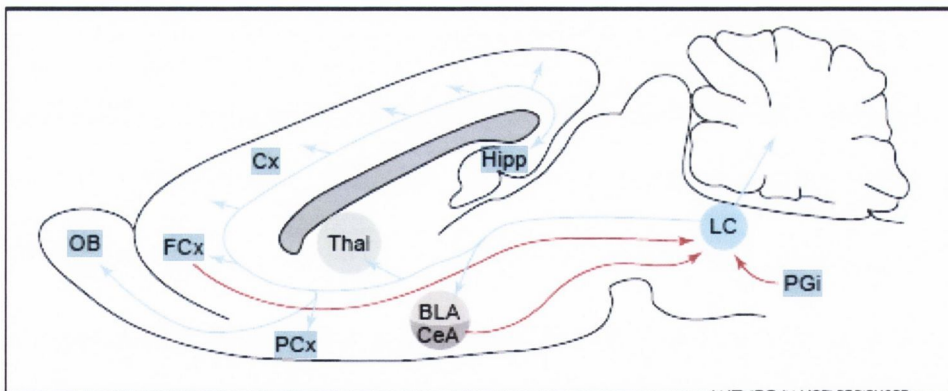


Figure 1.4. Distribution of noradrenergic projections from the locus coeruleus in the rat brain. The locus coeruleus (LC) sends branches to almost all forebrain structures including the basolateral nucleus of the amygdala (BLA), the thalamus (Thal), the olfactory system (olfactory bulb [OB] and piriform cortex [PCx]), the neocortex (Cx), including the frontal cortex (FCx), and the hippocampus (Hipp). Red arrows indicate the projections that the LC receives from other areas of the brain [this is not discussed in present section]. From Bouret & Sara (2005).

1.5.3 The noradrenergic receptor (adrenoceptor)

Adrenoceptors belong to the superfamily of G-protein-coupled receptors (GPCRs) and can be broadly divided into two different classes, α -adrenoceptors and β -adrenoceptors (Pierce *et al.*, 2002). They are located in the plasma membrane of neuronal and non-neuronal target cells (Hein, 2006). The endogenous catecholamines adrenaline and noradrenaline transmit their signals across the plasma membrane via these adrenoceptors. It was originally suggested that the excitatory action of catecholamines was mediated via the α -adrenoceptors, whereas inhibitory actions were mediated via the β -adrenoceptors (Ahlquist, 1948). Recently, 9 adrenoceptor subtypes have been identified: three α_1 -adrenoceptors (α_{1A} , α_{1B} , α_{1C}), three α_2 -subtypes (α_{2A} , α_{2B} , α_{2C}), and three β -adrenoceptors (β_1 , β_2 , β_3) (Bylund *et al.*, 1994). Studies of knockout mice carrying deletions in the genes encoding individual adrenoceptor subtypes have shed an enormous amount of light on the specific functions of these receptors (Brede *et al.*, 2004; Philipp & Hein, 2004). As the focus of this thesis is on β -adrenoceptors, the next sections will focus solely on this adrenoceptor subtype.

1.5.4 β -adrenoceptor structure and distribution

The β -adrenoceptor is composed of 413 amino acid residues of approximately 46,500 daltons (Henderson *et al.*, 1990). The following β -adrenoceptor subdivisions: β_1 , β_2 , and β_3 , are classically identified in cardiac, airway smooth muscle, and adipose tissue, respectively (Johnson, 2006). However, the brain is also densely innervated with adrenergic and noradrenergic neurons most of which originate in the LC (Foote *et al.*, 1983). In rat brain, β_1 -adrenoceptors have been identified in cerebral cortex, thalamus, pineal gland, and sympathetic ganglia, while β_2 -adrenoceptors are expressed in the olfactory bulb, cerebral cortex, hippocampus, hypothalamus, pineal gland, and spinal cord (Nicholas *et al.*, 1996). β_3 -adrenoceptors are expressed mainly in adipose tissue and not in the brain (Tanaka *et al.*, 2002), and thus will not be discussed further here. While both β_1 - and β_2 -adrenoceptor subtypes are expressed in the brain (Nakadate *et al.*, 2006) it appears that the expression level of β_1 -adrenoceptors is lower than that of β_2 -adrenoceptors in microglial cells (Mori *et al.*, 2002). With this in mind, and given the evidence that stimulation of central β_2 -adrenoceptors elicits anti-inflammatory effects in the CNS (Feinstein *et al.*, 2002; McNamee *et al.*, 2010c), it is likely that the anti-

inflammatory actions of NA may be mediated by the β_2 -adrenoceptors to a greater extent (Mori *et al.*, 2002).

1.5.5 β_2 -adrenoceptor signalling through cAMP

It is now known that the β_2 -adrenoceptor oscillates between inactive and active states (Liggett, 2002). The β_2 -adrenoceptor is coupled to adenylylase through a heterotrimeric G-protein (Rodbell *et al.*, 1971). This protein, G_s , consists of α , β and γ subunits; the α subunit binds guanosine triphosphate (GTP) and guanosine diphosphate (GDP) whereas the β and γ subunits form a tight $\beta\gamma$ dimer (Gilman, 1987). After agonist binding, the β_2 -adrenoceptor couples to the α -subunit of the G_s -protein together with a molecule of GTP. The activated heterotrimer dissociates into an α subunit and the $\beta\gamma$ dimer (Gilman, 1987). Hydrolysis of GTP to GDP leads to reassociation of the heterotrimer and the adrenoceptor reverts to the inactivated state (Ross & Wilkie, 2000). Upon β_2 -adrenoceptor activation the α -subunit of the G_s protein couples to and activates the enzyme adenylylase which in turn converts adenosine triphosphate (ATP) into cyclic AMP (cAMP). The resultant increase in cAMP activates protein kinase A (PKA) which phosphorylates many different substrates including other kinases and transcription factors (**Figure 1.5**).

A rapid downregulation of receptor signalling must occur in order to prevent over-excitation of cells. There are three possible methods of desensitisation depending on the extent of the β_2 -adrenoceptor response: (1) uncoupling of the receptors from adenylylase, (2) internalisation of uncoupled receptors, and (3) phosphorylation of internalised receptors (Johnson, 2006). The most common method of desensitisation results from phosphorylation of the receptor by PKA and protein kinase C (PKC) (Pitcher *et al.*, 1992). This phosphorylation results in uncoupling of the receptor from adenylylase and binding of the receptor to β -arrestin. Specifically, PKA-mediated receptor phosphorylation of the β_2 -adrenoceptor uncouples the receptor from the G_s protein and couples it to the G_i protein (which inhibits adenylylase) thus acting as a switching mechanism and limiting receptor function (Daaka *et al.*, 1997; Zamah *et al.*, 2002). Coupling of the receptor to the G_i protein initiates further signalling pathways such as the extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK) pathway. After a longer period of exposure to the agonist, internalisation of the uncoupled receptor occurs (Chuang & Costa, 1979). After hours of agonist exposure, the

internalised receptors are degraded by a process that is regulated by β -arrestin and involves ubiquitination (Shenoy *et al.*, 2001; Pierce *et al.*, 2002; Johnson, 2006).

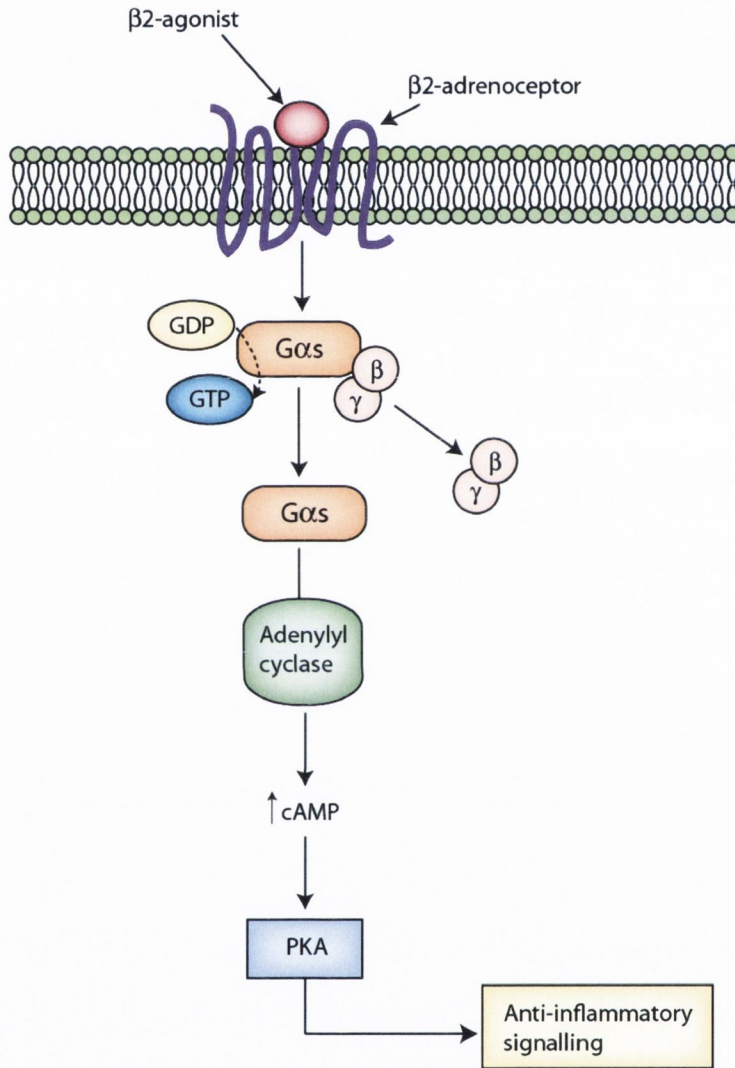


Figure 1.5. β_2 -adrenoceptor signalling through cAMP. The seven-transmembrane β_2 -adrenoceptor is coupled to adenylate cyclase through a heterotrimeric G-protein. After agonist binding, the β_2 -adrenoceptor couples to the α -subunit of the G_s -protein together with a molecule of GTP. The activated G-protein dissociates into an α subunit and the $\beta\gamma$ dimer. The α -subunit of the G_s protein couples to and activates the enzyme adenylate cyclase which in turn converts ATP into cAMP. The resultant increase in cAMP activates PKA which phosphorylates many different substrates including other kinases and transcription factors [Modified from (Pierce *et al.*, 2002)].

1.5.6 Noradrenaline modulates inflammatory signalling in the brain

Evidence indicates that NA suppresses inflammatory gene expression in the brain (Feinstein *et al.*, 2002; Heneka *et al.*, 2002; Marien *et al.*, 2004). Multiple studies indicate that activation of β -adrenoceptors on microglia reduces the LPS-induced inflammatory cytokines, TNF- α , IL-6 and NO (Chang & Liu, 2000; Farber *et al.*, 2005). Anti-inflammatory effects of β -adrenoceptor stimulation have also been observed in animal models of Alzheimer's disease, Parkinson's disease, and excitotoxicity (Galea *et al.*, 2003; Gleeson *et al.*, 2010). Recent data from our laboratory demonstrated that noradrenaline reuptake inhibitors, which increase the synaptic availability of NA, reduce inflammation following a systemic inflammatory challenge (O'Sullivan *et al.*, 2009; O'Sullivan *et al.*, 2010). Despite the known anti-inflammatory effects of NA, some studies show that NA release is associated with increased IL-1 β in the CNS in the absence of inflammatory stimuli, and this is thought to be mediated by microglia (Tomozawa *et al.*, 1995; Johnson *et al.*, 2005; Blandino *et al.*, 2006). However, the NA-induced increase in IL-1 β is known to be accompanied by its negative regulators IL-1ra and IL-1RII which may serve to limit any pro-inflammatory effects of IL-1 β expression (McNamee *et al.*, 2010c). It is difficult to describe one particular mechanism by which NA exerts its anti-inflammatory actions in the brain as knowledge of the molecular mechanisms underlying the effect of NA in cells is quite limited (Feinstein *et al.*, 2002); however, the induction of anti-inflammatory cytokines appears to be related to the level of cAMP (Kambayashi *et al.*, 1995), which is mediated by β -adrenoceptor activation. Thus it most likely involves binding of noradrenaline to the β -adrenoceptor and activation of cAMP signalling pathways.

1.5.7 Impairment of the noradrenergic system and its impact on neurodegeneration

Impairment of noradrenergic function is suspected to contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) where a profound loss of noradrenergic neurons occurs in the LC. For example, a reduction in LC neurons of approximately 83.2% in AD and 67.9% in PD has been shown at autopsy, and this is more than the percentage neuron loss in the two main areas associated with AD and PD, the nucleus basalis and substantia nigra respectively (German *et al.*, 1992; Zarow *et al.*, 2003). Noradrenergic depletion in the LC can be

experimentally induced using the selective noradrenergic neurotoxin DSP-4 (Fritschy & Grzanna, 1991). In light of the immunosuppressive properties of NA in the CNS mentioned above, evidence suggests that LC ablation potentiates A β -induced inflammation in rat cortex (Heneka *et al.*, 2002). This finding suggests that the characteristic LC loss that occurs in AD patients, coupled with a decrease in NA, may render the brain more vulnerable to inflammatory processes and contribute to the genesis of neuroinflammatory disease (Feinstein *et al.*, 2002).

The noradrenergic system is reported to play an important role in the recovery from brain injury. For instance, focal cortical injury resulting from stroke leads to a reduction in NA and other monoamines in the brain and this can persist for up to 40 days in the rat (Robinson *et al.*, 1975). Literature suggests that agents capable of enhancing NA in the brain promote functional recovery in these rats. For example, rats with unilateral injury to the sensorimotor cortex develop transient hemiplegia and have difficulty traversing a narrow elevated beam, and it has been shown that i.c.v. administration of NA promotes recovery from hemiplegia (Boyeson & Feeney, 1990). In support of this, depletion of NA using the noradrenergic neurotoxin DSP-4 slows down recovery from hemiplegia (Boyeson *et al.*, 1992; Goldstein & Bullman, 1997). In addition, clinical studies whereby human stroke patients were treated with amphetamine, a monoamine agonist that increases NA levels, in conjunction with a motor training programme showed improved recovery compared to placebo-treated controls (Walker-Batson *et al.*, 1995). Thus, extensive evidence suggests that impairment of the LC-noradrenergic system may contribute to the pathogenesis of neurological diseases that have an inflammatory component. In this regard, methods which preserve NA levels in the brain could be of benefit in the treatment of AD and other neurodegenerative diseases (Feinstein *et al.*, 2002; Marien *et al.*, 2004).

1.5.8 Therapeutic benefit of increasing central noradrenergic tone using β_2 -adrenoceptor agonists

Despite the evidence that NA and β_2 -adrenoceptor stimulation elicit anti-inflammatory actions in the brain (Feinstein *et al.*, 2002; Heneka *et al.*, 2002; Mori *et al.*, 2002; Kalinin *et al.*, 2007; McNamee *et al.*, 2010a), the anti-inflammatory effects of agents that directly stimulate the β_2 -adrenoceptor in the CNS have not been extensively studied to date. The

use of both long-acting and short-acting β -adrenoceptor agonists are useful tools for studying the functional role of the β -adrenoceptor. Several of these agonists, such as salmeterol and formoterol, are currently in use therapeutically for the treatment of asthma and chronic obstructive pulmonary disease (COPD) (Tashkin & Cooper, 2004; McKeage & Keam, 2009).

There is growing interest in the central β_2 -adrenoceptors as a neuroprotective target due to the fact that stimulation of β_2 -adrenoceptors on glial cells induces expression of neurotrophic factors and also promotes an anti-inflammatory phenotype (Culmsee *et al.*, 1999a; Culmsee *et al.*, 1999b; Hertz *et al.*, 2004; Counts & Mufson, 2010; McNamee *et al.*, 2010a; McNamee *et al.*, 2010b; McNamee *et al.*, 2010c). In addition, NA plays an important role in limiting neuroinflammation *in vivo* (Feinstein *et al.*, 2002; Heneka *et al.*, 2002; Kalinin *et al.*, 2007). Agonists most likely exert their actions by binding to and stabilising the β_2 -adrenoceptor in its active state rather than inducing a conformational change in the receptor (Onaran *et al.*, 1993). A useful tool for determining a specific role for the β_2 -adrenoceptor in promoting anti-inflammatory gene expression and limiting neuroinflammation, thereby excluding the possibility of a role for other β -adrenoceptor subtypes, is through the use of selective and non-selective agonists and antagonists for β_1 - and β_2 -adrenoceptor subtypes (**Table 1.1**).

One such agonist is clenbuterol, a brain-penetrant β_2 -adrenoceptor agonist used in the treatment of respiratory disorders including asthma and COPD (Baronti *et al.*, 1980; Tondo *et al.*, 1985; Papiris *et al.*, 1986; Boner *et al.*, 1988) and more recently it has been shown to have neuroprotective properties both *in vivo* and *in vitro* (Culmsee *et al.*, 1999a; Culmsee *et al.*, 1999b). Specifically, clenbuterol has neuroprotective actions in rodent models of cerebral ischaemia (Semkova *et al.*, 1996; Zhu *et al.*, 1998; Culmsee *et al.*, 1999b; Junker *et al.*, 2002) and in both *in vitro* and *in vivo* models of excitotoxicity (Semkova *et al.*, 1996; Gleeson *et al.*, 2010). It has recently been demonstrated that clenbuterol induces expression of the pro-inflammatory cytokine IL-1 β and its negative regulators IL-1ra and IL-1RII in rat brain (McNamee *et al.*, 2010c). In addition, clenbuterol induces expression of the broad spectrum anti-inflammatory cytokine IL-10 and its downstream signalling molecule SOCS-3 in rat brain (McNamee *et al.*, 2010b) and thus may be effective in the treatment of neuroinflammatory disease.

Name	Classification	Half-life ($t_{1/2}$)	K_d (nM)	References
Clenbuterol	Agonist at β_2 -adrenoceptor (brain penetrant)	26-30 hr	14	(Kopitar & Zimmer, 1976; Liang & Mills, 2001)
Formoterol		1.7 hr	74	(Sasaki <i>et al.</i> , 1982; Johnson, 1998)
Salbutamol		1.5-2 hr	320	(Liang & Mills, 2001)
Metoprolol	Antagonist at β_1 -adrenoceptor	37 min	55	(Belpaire <i>et al.</i> , 1990; Baker, 2005)
ICI 118,551	Antagonist at β_2 -adrenoceptor	20 min	0.66	(Liang & Mills, 2001)
Propranolol	Antagonist at β_1 - and β_2 -adrenoceptor (brain penetrant)	40 min	0.54	(Shand <i>et al.</i> , 1972; Liang & Mills, 2001)
Nadolol	Antagonist at β_2 -adrenoceptor (non brain penetrant: peripherally acting)	15.2-15.8 hr*	-	(Schiff & Saxey, 1984)

Table 1.1. List of β -adrenoceptor agonists and antagonists used in this thesis. Half-life cited here refers to that of a single dose in rats. * refers to the value in humans [table modified with permission (Ryan, 2010)].

1.5.9 Secondary physiological effects of clenbuterol treatment

Our laboratory and others have consistently shown that clenbuterol induces behavioural effects in rodents such as a reduction in locomotor activity and reduced food intake (Goldschmidt *et al.*, 1984; Geyer & Frampton, 1988; O'Donnell, 1993; Ryan, 2010). However, the underlying mechanisms behind the behavioural actions of clenbuterol are unclear. Notably, these behavioural effects are paralleled by an increase in central IL-1 β (Ryan, 2010) and as mentioned earlier in this chapter, when administered either peripherally or directly into the rodent brain IL-1 β causes a variety of sickness behaviours including fever, anorexia, fatigue, as well as reduced social exploration (Plata-Salaman *et al.*, 1988; Dantzer *et al.*, 1998; Kluger *et al.*, 1998; Konsman *et al.*, 2008). Thus, it is possible that the clenbuterol-induced increase in central IL-1 β may be

involved in mediating the suppressive effects of clenbuterol on behaviour, however this remains to be elucidated and is therefore one of the objectives of this thesis.

In addition to inducing sickness behaviours, central IL-1 β is known to precipitate symptoms of depression and anxiety (Dantzer, 2001). From a therapeutic point of view, depression and anxiety are undesirable drug side-effects. Therefore, if β_2 -adrenoceptor agonists such as clenbuterol were to be considered for clinical use it would be imperative to investigate the long-term effects of β -agonist drugs, such as clenbuterol, on IL-1 β and on depression and anxiety. To the best of our knowledge the long-term behavioural effects of clenbuterol have not been investigated and so this will be another focus of the present thesis.

1.6 Anti-inflammatory effects of glucocorticoids in the brain

Glucocorticoids act by binding to the glucocorticoid receptor (GR), a cytoplasmic receptor which translocates to the nucleus and modulates the expression of a number of genes (Hollenberg *et al.*, 1985). This receptor consists of two heat shock protein molecules plus a number of other proteins (Beato *et al.*, 1996). Entry of glucocorticoids into the cell and subsequent binding to the GR leads to a conformational change in the receptor (Bledsoe *et al.*, 2002). The multi-protein complex dissociates allowing translocation of the GR to the nucleus where it binds DNA sequences known as glucocorticoid response elements (GREs) to activate gene transcription (Lieberman *et al.*, 2007). Genes that are known to be targeted include a number of inflammatory cytokines (e.g. IL-6, IL-1 β , and TNF- α), enzymes (e.g. iNOS, COX-2, and MMPs), and adhesion molecules (e.g. ICAM-1, VCAM, and E-selectin) (See De Bosscher *et al.*, 2003; Lieberman *et al.*, 2007).

A number of synthetic glucocorticoids have been developed which effectively reduce the inflammation present in inflammatory diseases such as asthma and rheumatoid arthritis. The success of synthetic analogues such as dexamethasone is largely due to their ability to mimic natural corticosteroids and reduce the expression of inflammatory cytokines. Dexamethasone is known to inhibit transcription of NF κ B (Auphan *et al.*, 1995; Scheinman *et al.*, 1995). This occurs by upregulation of I κ B α and retention of NF κ B heterodimers in the cytoplasm. As NF κ B critically regulates a number of cytokines, its inhibition would block cytokine secretion; this may partly explain the immunosuppressive actions of glucocorticoids. Notably, β_2 -adrenoceptors (Collins *et al.*, 1988; Mak *et al.*, 1995) and IL-1 type II receptor (Colotta *et al.*, 1993) are also upregulated by glucocorticoids in the long term (24-48 hour period).

Regarding the actions of glucocorticoids in the brain, dexamethasone is known to inhibit cytokine production from glial cells (Nishida *et al.*, 1989; Velasco *et al.*, 1991; Chao *et al.*, 1992; Kimberlin *et al.*, 1995). While the synergistic anti-inflammatory effects of combined treatment with glucocorticoids and β -adrenoceptor agonists have been demonstrated in the periphery (Greening *et al.*, 1994; Shrewsbury *et al.*, 2000), evidence of glucocorticoid-adrenoceptor interactions in the brain is limited to studies regarding memory formation rather than inflammation. In this regard, glucocorticoids are known to

increase synthesis and release of noradrenaline, possibly by blocking the catecholamine transporter and preventing re-uptake of noradrenaline into the pre-synaptic terminal (Markey *et al.*, 1982; Grundemann *et al.*, 1998). These synergistic interactions are speculated to cause enhanced memory consolidation through interaction with the β -adrenoceptor-cAMP/PKA system (Roosendaal *et al.*, 2002). One of the aims of this thesis is to explore the synergistic anti-inflammatory effects of combined treatment with a glucocorticoid and a β_2 -adrenoceptor agonist in the brain.

1.7 Objective

The overall objective of this project was to examine the potential for central β_2 -adrenoceptors to be exploited as a therapeutic target for the treatment of neuroinflammatory disease. The specific aims were:

- 1) To examine the anti-inflammatory effects of the β_2 -adrenoceptor agonist clenbuterol in the rat brain, with particular focus on the impact of clenbuterol on NF κ B activity.
- 2) To investigate the anti-inflammatory effect of combined administration of a β_2 -adrenoceptor agonist and a glucocorticoid in rat brain.
- 3) To determine whether central expression of IL-1 β mediates the suppression of locomotor activity and feeding induced by clenbuterol treatment.
- 4) To examine whether chronic treatment with a low dose of clenbuterol induces depressive/anxious behaviour in tandem with its ability to activate the central IL-1 system.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Animal husbandry

Male Sprague-Dawley rats

Harlan, UK

Harlan Teklad Irradiated Diet

Harlan, Wisconsin

2.1.2 Surgical equipment

BD Plastipak luer 1 ml syringe

Becton, Dickinson and Co.

BD Microlance 3 25G × 3/8" needle

Becton, Dickinson and Co.

MSS - 3 Isofluorane Vaporiser

MSS International Ltd.

Royal™ - 35W Single Use Skin Stapler

Tyco Healthcare UK Ltd.

Gilson's MINIPULS 3 peristaltic pump

Gilson, Inc.

2.1.3 Assay Kits

NE-PER Nuclear and Cytoplasmic Extraction Reagents

ThermoFisher Scientific

NFκB p65 Transcription Factor Kit

ThermoFisher Scientific

Pierce BCA Protein Assay Kit

ThermoFisher Scientific

2.1.4 General Laboratory Chemicals

2-propanol

Sigma-Aldrich, Inc.

Acrylamide

Sigma-Aldrich, Inc.

Ammonium persulfate (APS)

Sigma-Aldrich, Inc.

Betadine

Medlock Medical, Ltd.

Bovine serum albumin

Sigma-Aldrich, Inc.

Bromophenol blue

Sigma-Aldrich, Inc.

Disodium hydrogen orthophosphate (Na₂HPO₄)

Sigma-Aldrich, Inc.

Glycerol

Sigma-Aldrich, Inc.

Glycine

Sigma-Aldrich, Inc.

Hydrochloric acid (HCl)

VWR Int., Ireland

Isopentane

Sigma-Aldrich, Inc.

Methanol

Sigma-Aldrich, Inc.

N,N,N',N'-Tetramethylethylene-diamine (TEMED)

Sigma-Aldrich, Inc.

N' N' Bis Acrylamide

Sigma-Aldrich, Inc.

Phosphatase inhibitor cocktail I & II

Sigma-Aldrich, Inc.

Potassium chloride (KCl)	Merck, UK
Protease inhibitor cocktail	Sigma-Aldrich, Inc.
Saccharin	Sigma-Aldrich, Inc.
Sodium chloride (NaCl)	Sigma-Aldrich, Inc.
Sodium dodecyl sulfate (SDS) 99%	Sigma-Aldrich, Inc.
Sodium hydroxide (NaOH)	Sigma-Aldrich, Inc.
Sodium phosphate dibasic (Na ₂ HPO ₄)	Sigma-Aldrich, Inc.
Sodium phosphate monobasic monohydrate (NaH ₂ PO ₄)	Sigma-Aldrich, Inc.
Tris-HCl	Sigma-Aldrich, Inc.
Triton X-100	Sigma-Aldrich, Inc.
Trizma-Base	Sigma-Aldrich, Inc.
Tween-20	Sigma-Aldrich, Inc.
β-Mercaptoethanol	Sigma-Aldrich, Inc.
Urethane	Sigma-Aldrich, Inc.

2.1.5 Pharmacological Agents

Clenbuterol	Sigma-Aldrich, Inc.
Dexamethasone hydrochloride	Sigma-Aldrich, Inc.
Formoterol hemifumerate	Tocris Bioscience, UK
ICI 118,551 hydrochloride	Tocris Bioscience, UK
Lipopolysaccharide (LPS)	Sigma-Aldrich, Inc.
Metoprolol	Sigma-Aldrich, Inc.
Nadolol	Sigma-Aldrich, Inc.
Propranolol hydrochloride	Sigma-Aldrich, Inc.
Salbutamol hemisulfate	Tocris Bioscience, UK
IsoFlo® 100% Isoflurane	Abbott Laboratories Ltd.

2.1.6 Western Blotting

Anti-mouse IgG antibody (whole molecule) (#8924)	Sigma-Aldrich, Inc.
Anti-rabbit IgG, HRP-linked whole antibody	GE Healthcare, Ltd.
Immobilon Western Chemiluminescent HRP Substrate	Millipore
IκBα (L35A5) mouse mAb antibody (#4814)	Cell Signaling Technology
p38 MAPK antibody (#9212)	Cell Signaling Technology
Phospho-IκB-α (Ser32) (14D4) antibody (#2859)	Cell Signaling Technology

Phospho-p38 MAPK (Thr180/Tyr182) antibody (#9211)	Cell Signaling Technology
Phospho-SAPK/JNK (Thr183/Tyr185) antibody (#9251)	Cell Signaling Technology
Phospho-Stat3 (Tyr705) antibody (#9131)	Cell Signaling Technology
Polyvinylidene fluoride (PVDF) membrane	Millipore
Re-blot plus strong solution (×10)	Millipore
SAPK/JNK antibody (#9252)	Cell Signaling Technology
Stat3 antibody (#9132)	Cell Signaling Technology
Mini PROTEAN-3 system	Bio-Rad Laboratories Inc.
Precision Plus Protein™ dual colour standards	Bio-Rad Laboratories Inc.

2.1.7 PCR Reagents

High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Pty Ltd.
Nuclease-free H ₂ O	Sigma-Aldrich, Inc.
Specific target primers/probes (see Table 2.2)	Applied Biosystems, Pty Ltd.
TaqMan® Gene Expression Assay Kit	Applied Biosystems, Pty Ltd.
TaqMan® Universal PCR Master Mix	Applied Biosystems, Pty Ltd.
Total RNA Isolation Kit	Macherey-Nagel GmbH&Co.

2.1.8 General Laboratory Machines

Applied Biosystems 7300 Real-Time PCR System	Applied Biosystems
Cordless polytron (#Z359971-1EA)	Sigma-Aldrich, Inc.
FujiFilm LAS3000 Intelligent Darkbox	FujiFilm Corporation
MJ Research PTC-200 Thermo Cycler	MJ Research, Inc.
NanoDrop spectrophotometer	Mason Technology Ltd.
ELX800 Universal Microplate Reader	Biotek Instruments Inc.

2.1.9 General Laboratory Ware

Filter paper	Whatman, USA
Microtest 96 well plate (flat bottom)	Sarstedt, Inc.
Microtubes (0.5ml)	Sarstedt, Inc.
Microtubes (1.5ml)	Sarstedt, Inc.
Optical 96-well reaction plate	Applied Biosystems, Pty Ltd.
Optical adhesive cover for PCR	Applied Biosystems, Pty Ltd.
Pipette tips	Sarstedt, Inc.

2.1.10 Computer Software

Adobe Illustrator CS5 (figures)

AM1051 data logger (locomotor activity monitor)

Applied Biosystems RQ Software. Version 1.3.1

EthoVision

Fig. P Version 2.98

GB-STAT™

ImageJ 1.44p

Adobe Systems Inc.

Benwick Electronics

Applied Biosystems, Pty Ltd.

Noldus Information

Technology

Fig. P Corporation

Dynamic Microsystems Inc.

Wayne Rasband, NIH, USA

2.2 Methods

2.2.1 Animal husbandry

Male Sprague-Dawley rats (200-300g) were obtained from Harlan Laboratories, UK. Rats were housed in hard-bottomed polypropylene cages with stainless steel wire tops and allowed to habituate in the housing conditions for at least seven days before experimental treatment. Wood shavings were used as bedding. Animals were housed in an air-conditioned room with a 12:12-h light/dark cycle (lights on at 8am). Food and water were available *ad libitum*. All rats living in a common cage received the same drug treatment. Rats were housed 4 per cage.

2.2.2 *In vivo* experimental design

All animals were handled daily for at least seven days prior to drug administration. All pharmacological agents were prepared by dissolving in 0.89% (w/v) saline with the exception of dexamethasone which was dissolved in 0.89 % (w/v) saline containing 0.2% (v/v) Tween-20. Control animals received a 0.89% (w/v) saline vehicle. Controls in the dexamethasone study received a 0.89% (w/v) saline vehicle containing 0.2% (v/v) Tween-20. All experimental protocols were in compliance with the European Communities Council directive (86/609/EEC).

Study 1: β_2 -adrenceptor agonist clenbuterol: a time course study

Rats received an intraperitoneal (i.p) injection of either clenbuterol (0.5 mg/kg) or a saline vehicle (0.89% [w/v] saline) and were sacrificed at 3 time points: 1 hour, 4 hours or 8 hours post-injection. This study yielded the following four treatment groups: (1) saline vehicle, (2) clenbuterol 1 hour, (3) clenbuterol 4 hour and (4) clenbuterol 8 hour. $n = 5$ per group.

Study 2: β_2 -adrenceptor agonist clenbuterol: a dose-response study

Rats received an intraperitoneal (i.p) injection of clenbuterol at the following three doses: 0.03 mg/kg, 0.1 mg/kg and 0.3 mg/kg. Control animals received a saline vehicle (0.89% [w/v] saline; i.p). Rats were sacrificed 4 hours post-injection. This study yielded the

following four treatment groups: (1) saline vehicle, (2) clenbuterol 0.03 mg/kg, (3) clenbuterol 0.1 mg/kg and (4) clenbuterol 0.3 mg/kg. $n = 6$ per group.

Study 3: β_2 -adrenoceptor agonist clenbuterol + the non-selective β -adrenoceptor antagonist propranolol, the selective β_1 -adrenoceptor antagonist metoprolol or the selective β_2 -adrenoceptor antagonist ICI-118551

Rats received an i.p. injection of the non-selective β -adrenoceptor antagonist propranolol (10 mg/kg), the selective β_1 -adrenoceptor antagonist metoprolol (10 mg/kg) or the selective β_2 -adrenoceptor antagonist ICI-118551 (10 mg/kg) 30 minutes prior to i.p. administration of clenbuterol (0.5 mg/kg). Control animals received a saline vehicle (0.89% [w/v] saline; i.p). Rats were sacrificed 4 hours post-clenbuterol injection. This study yielded the following five treatment groups: (1) saline vehicle, (2) clenbuterol, (3) propranolol + clenbuterol, (4) metoprolol + clenbuterol, (5) ICI-118551 + clenbuterol. $n = 6$ per group.

Study 4: β_2 -adrenoceptor agonists Salbutamol + Formoterol

Rats received an i.p. injection of the β_2 -adrenoceptor agonist salbutamol (0.5 mg/kg) or formoterol (0.5 mg/kg). Control animals received a saline vehicle (0.89% [w/v] saline; i.p). Rats were sacrificed 4 hours post-injection. This study yielded the following three treatment groups: (1) saline vehicle, (2) salbutamol, (3) formoterol. $n = 6$ per group.

Study 5: Intracerebroventricular LPS time course

Rats received an intracerebroventricular (i.c.v) injection of LPS (1 μ g/5 μ L) into the lateral ventricle (see section 2.2.5 for full surgery details). Control animals received a saline vehicle (0.89% [w/v] saline; i.c.v). Rats were perfused with heparinised saline at two time points post-i.c.v. injection; 1 hour or 2 hours. This study yielded the following three treatment groups: (1) saline vehicle, (2) LPS 1 hour, (3) LPS 2 hours. $n = 3$ per group.

Study 6: β_2 -adrenoceptor agonist clenbuterol + intracerebroventricular LPS

Rats received an i.p. injection of clenbuterol (0.5 mg/kg). Control animals received a saline vehicle (0.89% [w/v] saline; i.p). One hour later rats received an i.c.v. injection of LPS (1 μ g/5 μ L) into the lateral ventricle. Control animals received a saline vehicle (0.89% [w/v] saline; i.c.v). Rats were perfused with heparinised saline 2 hours post-LPS

administration. This study yielded the following four treatment groups: (1) saline vehicle, (2) clenbuterol alone, (3) LPS alone, (4) clenbuterol + LPS. $n = 6$ per group.

Studies 7-9: Each study has a part A and a part B. Part A assessed the effects of drug treatment on locomotor activity and brain IL-1 β levels. Part B assessed the effects of drug treatment on food intake only.

Study 7a: Non-selective brain permeable β -adrenoceptor antagonist propranolol + clenbuterol (for locomotor activity and brain tissue)

Rats were pre-treated with the non-selective brain permeable β -adrenoceptor antagonist propranolol (10 mg/kg; i.p) 30 minutes prior to clenbuterol (0.5 mg/kg; i.p). Control animals received a saline vehicle (0.89% [w/v] saline; i.p). Locomotor activity was recorded 2 hours post-clenbuterol administration for a 15 minute period after which they were returned to their home cage. Rats were sacrificed 4 hours post-clenbuterol injection. This study yielded four treatment groups: (1) saline vehicle, (2) clenbuterol, (3) propranolol, (4) propranolol + clenbuterol. $n = 6$ per group.

Study 7b: Non-selective brain permeable β -adrenoceptor antagonist propranolol + clenbuterol (for food intake)

Rats were pre-treated with propranolol (10 mg/kg) 30 minutes prior to clenbuterol (0.5 mg/kg), all via i.p. injection. Control animals received a 0.89% (w/v) saline vehicle. Rats were singly housed and food intake was measured for 24 hours following drug treatment. $n = 5$ per group.

Study 8a: Glucocorticoid dexamethasone + β_2 -adrenoceptor agonist clenbuterol (for locomotor activity and brain tissue)

Rats were pre-treated with dexamethasone (1 mg/kg; i.p) 30 minutes prior to clenbuterol (0.5 mg/kg; i.p). Control animals received a saline vehicle (0.89% [w/v] saline; i.p). Locomotor activity was recorded 2 hours post-clenbuterol administration for a 15 minute period after which they were returned to their home cage. Rats were sacrificed 4 hours post-clenbuterol injection. This study yielded four treatment groups: (1) saline vehicle, (2) clenbuterol, (3) dexamethasone, (4) dexamethasone + clenbuterol. $n = 6$ per group.

Study 8b: Glucocorticoid dexamethasone + β_2 -adrenoreceptor agonist clenbuterol (for food intake)

Rats were pre-treated with dexamethasone (1 mg/kg) 30 minutes prior to clenbuterol (0.5 mg/kg) via i.p. injection. Control animals received a saline vehicle (0.89% [w/v] saline; i.p). Rats were singly housed and food intake was measured for 24 hours following drug treatment. $n = 5$ per group.

Study 9a: Selective peripheral β_2 -adrenoreceptor agonist nadolol + clenbuterol (for locomotor activity and brain tissue)

Rats were pre-treated with the peripheral β_2 -adrenoceptor antagonist nadolol (5 mg/kg; i.p) 30 minutes prior to clenbuterol (0.5 mg/kg; i.p). Control animals received a saline vehicle (0.89% [w/v] saline; i.p). Locomotor activity was recorded 2 hours post-clenbuterol administration for a 15 minute period after which they were returned to their home cage. Rats were sacrificed 4 hours post-clenbuterol injection. This study yielded four treatment groups: (1) saline vehicle, (2) clenbuterol, (3) nadolol, (4) nadolol + clenbuterol. $n = 6$ per group.

Study 9b: Selective peripheral β_2 -adrenoreceptor agonist nadolol + clenbuterol (for food intake)

Rats were pre-treated with nadolol (5 mg/kg) 30 minutes prior to clenbuterol (0.5 mg/kg) via i.p. injection. Control animals received a saline vehicle (0.89% [w/v] saline; i.p). Rats were singly housed and food intake was measured for 24 hours following drug treatment. $n = 5$ per group.

Study 10: Chronic administration of the β_2 -adrenoceptor agonist clenbuterol

Clenbuterol (0.03 mg/kg; i.p) was administered to rats twice daily for 20 days. On day 21 rats received one clenbuterol injection and were sacrificed 4 hours later. Control animals received a saline vehicle (0.89% [w/v] saline; i.p). A two-bottle saccharin preference test was performed throughout the study and elevated plus maze and open field tasks were performed on days 9 and 13 respectively. This study yielded two treatment groups: (1) saline vehicle, (2) clenbuterol. $n = 8$ per group.

2.2.3 Harvesting of tissue for post-mortem analysis

Immediately following sacrifice, rats brains were removed from the skull and dissected by hand on an ice-cold Petri-dish. Portions of tissue for analysis were placed in 1.5ml micro tubes and snap-frozen on dry ice prior to storage at -80°C.

2.2.4 Behavioural Analysis

2.2.4.1 Locomotor activity

Two hours post-injection, animals were removed from their home cage and placed individually into activity cages (32 cm × 20 cm × 18 cm; length × width × height) connected to an AM1051 data logger. Each activity cage was equipped with two sets of horizontal infrared beams to monitor rearing, mobile and static activities, as well as the active and mobile times. The two beams were positioned 3 cm and 15 cm above the base of the cage and consisted of a 12 beam by 7 beam matrix, forming a grid of 66×2.54 cm² cells within the cage. Activity was recorded as the number of times a beam changed from unbroken to broken. An animal was considered mobile if its central position changed by more than 2 grids (5.08 cm²). Rearing was recorded by the upper set of infrared beams. Active time was also logged, an animal was considered inactive if no beam had been broken for 5 s. Locomotor activity was recorded at 1 min intervals for 15 min; following this, the animals were returned to their home cage. Locomotor activity cages were thoroughly cleaned between each animal.

2.2.4.2 Open field

The open field task took place under dim light conditions in a windowless room. A video camera mounted at ceiling height directly above the open field arena recorded activity during the experimental period. The extra-maze cues (furniture, light source and position of experimenter) were kept constant throughout the study. The open field consisted of a circular arena enclosed by a wall. Two zones were defined in the arena: a “safe” (peripheral) zone and an “unsafe” (central) zone. The animal was placed in the centre of the arena and left to explore for a period of 5 min. The following parameters were determined using specialised computer software from EthoVision: (1) in the whole arena: distance moved; (2) in the central zone: total time spent in the zone, number of entries into the zone, distance moved; (3) in the peripheral zone: total time spent in the zone, distance moved. The open field arena was thoroughly cleaned between each animal.

2.2.4.3 Elevated plus maze

The elevated plus maze task took place under dim light conditions in a windowless room. A video camera mounted at ceiling height directly above the plus maze recorded activity during the experimental period. The apparatus consisted of two opposing open and closed arms (closed arms were enclosed by walls forming a cross with a square centre area). Each rat was placed into the centre of the plus maze facing a closed arm. Each animal was left to explore for a period of 5 min. The following parameters were calculated: (1) in the open arms: time spent, number of entries; (2) in the closed arms: time spent, number of entries. In addition, the ratio (percentage) of open arm entries relative to the total was calculated. An arm entry was defined as the rat exiting the central platform and entering the arm with front paws. The elevated plus maze area was thoroughly cleaned between each animal.

2.2.4.4 Saccharin preference test

A 2-bottle test was conducted in the animals' home cage. One bottle contained water alone and the other bottle contained a 0.01% (w/v) saccharin solution prepared with tap water. Daily water and saccharin intake was measured by weighing the drinking bottles on an electronic balance. The left–right position of the saccharin and water bottles were alternated daily in this test.

2.2.5 Surgical techniques

2.2.5.1 Intracerebroventricular (ICV) surgery

Anaesthesia was induced using 5% gaseous isoflurane. Rats were then placed in a stereotaxic frame and were maintained on 2.5 - 3% gaseous isoflurane. After shaving and sterilising the top of the head a midline sagittal incision of approximately 1.5 cm in length was made between the ears. A burr hole was drilled through the skull over the left lateral ventricle (stereotaxic coordinates: 0.09 cm posterior to bregma, 0.14 cm left lateral to midline, 0.36 cm ventral from skull surface) through which a glass micropipette (with a < 50 µm tip diameter) delivered 5 µl of the compound of interest at a rate of 1 µl/min. The micropipette was withdrawn slowly and the incision was closed up using surgical staples. The rat was removed from the stereotaxic frame and singly housed while recovering from anaesthesia.

2.2.5.2 Transcardial perfusion

Rats were given a lethal dose of 30% urethane. A thoracotomy was performed to expose the beating heart and an incision was made in the right ventricle from which a blood sample was taken for later analysis. The left ventricle was pierced with a needle which was attached to the tubing of the perfusion pump and clamped in place. The rat was perfused with heparinised saline for 20 minutes. Once perfusion was complete, animals were decapitated and the brain was removed from the skull and dissected on an ice-cold Petri dish. Brain tissue was placed in 1.5 ml micro tubes and snap-frozen on dry ice prior to storage at -80°C.

2.2.6 Other measurements for *in vivo* studies

2.2.6.1 Bodyweight

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

2.2.6.2 Food intake

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

2.2.6.3 Water intake

Water bottles were weighed using a three-point balance prior to injection/surgery. Water bottles were subsequently re-weighed 24 hr post-injection in order to calculate water intake. Water weight in grams was subsequently converted to millilitres (1g = 1ml).

2.2.7 Molecular Techniques

2.2.7.1 Analysis of mRNA expression

Real-time polymerase chain reaction (PCR) is a powerful technique used for a broad range of applications including quantifying gene expression in cells and tissues. Frequently, it is used in conjunction with reverse transcription in order to quantify messenger RNA (mRNA). Analysis of mRNA expression from cells and tissue was performed in 3 steps: (1) RNA extraction from tissue; (2) synthesis of complimentary DNA (cDNA); (3) mRNA amplification using real-time PCR. Filtered pipette tips were used throughout the process to minimize contamination by aerosols.

Step 1a: RNA extraction

RNA was isolated using a NucleoSpin® RNA II Total RNA isolation kit according to the manufacturer's instructions. Cells or tissue were disrupted in 350 µl RA1 lysis buffer (supplied with kit) and 3.5 µl β-mercaptoethanol using a polytron for ~30 s or until a homogeneous solution was obtained. To reduce viscosity, the lysate was filtered through a NucleoSpin® filter for 1 min at 11,000 × g. Following this, 350 µl 70% ethanol was added to the lysate, mixed by pipetting up and down (8 times) and loaded to a NucleoSpin® RNA II column. The column was centrifuged for 30 s at 11,000 × g to bind the RNA to the column. The column was desalted by the addition of 350 µl membrane desalting buffer and centrifuged for 1 min at 11,000 × g. Digestion of rDNase was performed by preparing a DNase reaction mixture (briefly, 10 µl reconstituted rDNase plus 95 µl reaction buffer for rDNase, per sample) and applying 95 µl of this DNase reaction mixture directly onto the centre of each column. The column was incubated at room temperature for 15 min and was then washed 3 times, as follows: (1) 200 µl RA2 buffer (supplied with kit) to inactivate the rDNase, centrifuge for 30 s at 11,000 × g; (2) 600 µl RA3 buffer (supplied with kit), centrifuge for 30 s at 11,000 × g; (3) 250 µl RA3 buffer, centrifuge for 2 min at 11,000 × g. Finally, highly pure RNA was eluted by addition of 60 µl RNase-free H₂O to the column and centrifuging at 11,000 × g for 1 min. The RNA was subsequently stored at -80°C.

Step 1b: RNA quantification

Quantification of RNA was carried out using a NanoDrop spectrophotometer. The NanoDrop was initialised by placing 1 μl RNase-free H_2O onto the reading platform/pedestal. This H_2O was removed using some tissue paper and a further 1 μl RNase-free H_2O was placed onto the pedestal to create a blank reading. RNA was defrosted on and maintained on ice throughout this process. The RNA-containing tube was flicked gently to ensure thorough mixing of the contents. 1 μl RNA was placed onto the pedestal and a concentration reading was obtained in $\text{ng}/\mu\text{l}$. The pedestal was wiped down with tissue paper between each sample. The purity of the RNA was demonstrated by the ratio between the A260 and A280, where a ratio of 2 indicated pure RNA. The RNA was normalised to 60 $\text{ng}/\mu\text{l}$ using RNase-free H_2O as a diluent.

Step 2: cDNA synthesis

A High Capacity cDNA Reverse Transcription Kit was used for reverse transcription of total RNA to single stranded cDNA. The kit components were thawed on ice and a 2 \times RT master mix was prepared on ice according to Table 2.1. A 1:1 ratio of RNA and 2 \times RT master mix (20 μl + 20 μl) were mixed together by vortexing. This mixture was then placed in a thermo cycler with the following thermal cycle profile: 25 $^\circ\text{C}$ for 10 min, 37 $^\circ\text{C}$ for 2 hr. The resultant cDNA was stored at -20 $^\circ\text{C}$.

Component	Volume per reaction (μl)
10 \times RT Buffer	4
25 \times dNTP Mix (100 mM)	1.6
10 \times RT Random Primers	4
Multiscribe Reverse Transcriptase (50 U/ μl)	2
Nuclease-free H_2O	8.4

Table 2.1. 2 \times RT Master Mix components

Step 3a: Assessment of gene expression by multiplex real-time PCR

Real-time PCR was performed using TaqMan[®] Gene Expression Assays which contain forward and reverse primers, and a FAM[®] (fluorescein amidite)-labelled MGB (minor groove binder) TaqMan probe for each gene of interest. The assay ID's for the genes examined are listed in Table 2.2. 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.

A 1 in 4 dilution of cDNA was prepared using nuclease-free H₂O. A 25 μ l volume was added to each well of the 96-well optical reaction plate as follows: 10 μ l cDNA, 12.5 μ l TaqMan[®] Universal PCR Master Mix, 1.25 μ l β -actin primer/probe, and 1.25 μ l target primer/probe. The plate was covered with an optical adhesive cover and centrifuged at 800 \times rpm for 20 s. 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 (denaturation) and then 60°C for 1 min for 40 cycles (transcription). Gene expression was calculated using the $\Delta\Delta$ Ct method (where Ct is the threshold cycle) with Applied Biosystems Sequence Detection Software as outlined in the next section.

Step 3b: Real-time PCR analysis

The $\Delta\Delta$ Ct method [Applied Biosystems RQ software] was used to assess gene expression for all real-time PCR analysis. This method assesses relative gene expression by comparing gene expression in treated/experimental samples to normal or untreated samples (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 expressions 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 (2^1), 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 Δ Ct. The Δ Ct value for the control sample is subtracted from itself, to give 0, and also subtracted from all other samples to correct for β -actin. This is the $\Delta\Delta$ CT value which 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 $-\Delta\Delta$ Ct (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 $\Delta\Delta$ Ct value of 0, thus $0-2$ gives a $2^{-\Delta\Delta$ Ct of 1, against which all other samples are referenced.

Target	Primer code
β -actin	(VIC/MGB pl) 4352340E
CD11b	Rn00709342_m1
GFAP	Rn00566603_m1
I κ B- α	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-1 β	Rn00580432_m1
NOS-2 (iNOS)	Rn00561646_m1
SOCS-3	Rn00585674_s1
TNF- α	Rn93699017_m1

Table 2.2. List of primers/probes for PCR

2.2.7.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting

Tissue preparation

Cytoplasmic extracts were prepared using the Nuclear and Cytoplasmic Extraction Reagents Kit (see section 2.2.7.4). The protein concentration of cytoplasmic extracts was determined and samples were equalised to 4000 µg/ml protein using CER1 buffer from the extraction kit mentioned above. Each sample was combined with 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) in a 1:1 ratio. Samples were heated at 65°C for 5 min prior to being loaded on the polyacrylamide gel.

SDS-PAGE

Electrophoresis was carried out using SDS-polyacrylamide gels (10% separating gel; 4% stacking gel. **Table 2.3**). The separating gel was prepared and poured between two glass plates which were held firmly together using the Bio-Rad casting frame. A layer of isopropanol was poured over the top of the separating gel (to prevent evaporation). The gel was allowed to set for at least 30 min after which the isopropanol was rinsed off using dH₂O. The stacking gel was poured between the glass plates and a 10-well comb was inserted to create the wells. The gel was allowed to set for at least 30 min. The glass plates (containing the gel) were placed in a gel holder cassette which was then lowered into a Bio-Rad mini-PROTEAN gel rig. A 1× electrode running buffer (125 mM Tris-base, pH 8.3; 960 mM glycine; 0.5% SDS) was poured into the middle chamber and the bottom of the gel rig. The combs were removed from the gel and 10 µl of sample and 5 µl of molecular weight marker were loaded per well. The samples were separated by applying a constant current of 40 mA until the blue dye of the sample buffer had run as far as the bottom of the gel.

	Volume for 1 gel	
	4 %	10 %
d.H₂O	1.525 ml	2.01 ml
Acrylamide/Bis	0.325 ml	1.665 ml
Tris-HCl (pH 8.8)	0.625 ml	1.25 ml
SDS	25 μ l	50 μ l
APS	12.5 μ l	25 μ l
TEMED	4 μ l	3 μ l

Table 2.3. SDS-polyacrylamide gel components

Semi-dry transfer

Proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane via a semi-dry transfer method. Firstly, one piece of PVDF membrane and six pieces of transfer paper per gel were cut to match the size of the gel. The PVDF membrane was activated by immersion in methanol for 30 s and was then placed in distilled H₂O for 2 min. one piece of filter paper was soaked in anode buffer-1 (0.3M Tris-HCL, 10% methanol), two pieces soaked in anode buffer-2 (25 mM Tris-HCL, 10% methanol), and three pieces soaked in cathode buffer (25 mM Tris-HCL, 40 mM Glycine, 10% methanol). The “sandwich” of filter paper was placed on the anode plate of a semi-dry transfer unit as seen in Figure 2.1. A Pasteur pipette was rolled over the sandwich to remove any bubbles and the cathode plate was placed on the unit. Proteins were transferred to the PVDF membrane at 100 mA per gel sandwich for 50 min.

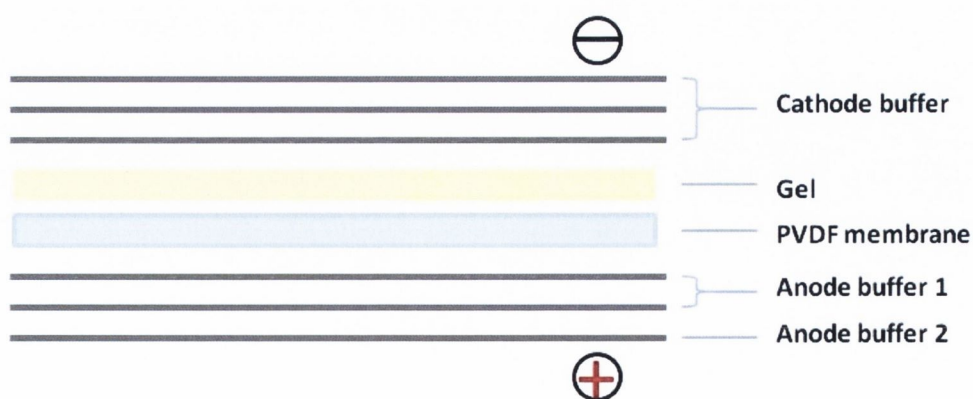


Figure 2.1: Sandwich preparation for semi-dry transfer

Western immunoblotting

The PVDF membrane was removed from the semi-dry transfer unit and placed in 10 ml of blocking buffer (TBS-T containing 5% marvel non-fat dried milk) for one hour at room temperature. The membrane was washed 3×10 min in TBS-T and incubated with the appropriate concentration of primary antibody (see **Table 2.5**), diluted in TBS/Tween (0.1% v/v) containing 5% BSA, overnight at 4°C with gentle agitation. The membrane was washed 3×10 min in TBS-T and incubated with the appropriate concentration of secondary antibody, diluted in TBS/Tween (0.1% v/v) containing 1% BSA, for one hour at room temperature. The membrane was washed 3×10 min and incubated in chemiluminescent HRP substrate solution for 5 min. Chemiluminescence was measured using a CCD camera system (FujiFilm LAS3000 Intelligent Darkbox). Densitometry was performed on the resulting images using Image-J software.

Target	Molecular Weight (kDa)	1°Antibody concentration	2°Antibody concentration
IκBα	39	1:1000	Anti-mouse 1:5000
Phospho-IκBα	39	1:1000	Anti-rabbit 1:5000
β-Actin	42	1:1000	Anti-mouse 1:2000
STAT3	79, 86	1:1000	Anti-rabbit 1:5000
Phospho-STAT3	79, 86	1:1000	Anti-rabbit 1:5000
JNK	46, 54	1:1000	Anti-rabbit 1:5000
Phospho-JNK	46, 54	1:1000	Anti-rabbit 1:5000
p38	43	1:1000	Anti-rabbit 1:5000
Phospho-p38	43	1:1000	Anti-rabbit 1:5000

Table 2.4: Western immunoblotting antibody dilutions

2.2.7.3 Bicinchoninic acid (BCA) protein assay

The Pierce BCA Protein Assay Kit is a detergent-compatible formulation based on bicinchoninic acid for the colorimetric detection and quantification of total protein. Firstly, a stock solution of bovine serum albumin (BSA) of 20 mg/10ml d.H₂O was prepared. A series of dilutions of the BSA stock solution were prepared to give the following final protein concentrations: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 µg/ml. 25 µl of each of the standards and the samples were loaded to each well of a Microtest 96 well plate (flat bottom). A working reagent (WR) was prepared by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B (supplied). 200 µl of WR was added to each well on top of the standard or sample. The 96-well plate was covered and incubated at 37°C for 30 min after which absorbance was measured at 595 nm. 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.

2.2.7.4 Nuclear and cytoplasmic extraction

Nuclear and cytoplasmic fractions were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology) according to the manufacturer's instructions. Briefly, 20 mg of tissue was washed in 200 µl PBS and centrifuged $500 \times g$ for 5 min at 4°C. The PBS was removed and tissue was homogenised in CERI and CERII buffers to disrupt the cell membrane and release cell contents. The homogenate was centrifuged at $16,000 \times g$ for 5 min at 4°C. The supernatant (cytoplasmic extract) was transferred to a clean pre-chilled tube and stored at -80°C. The pellet fraction was resuspended in NER buffer and vortexed for 15 s every 10 min, for a total of 40 min. The tube was centrifuged at $16,000 \times g$ for 10 min at 4°C. The supernatant (nuclear extract) was transferred to a clean pre-chilled tube and stored at -80°C.

Equalisation of extracts

Prior to performing the NFκB p65 assay (section 2.2.7.5), the protein concentration of each sample was determined using the BCA protein assay kit and equalised using NER

buffer (supplied with extraction kit). After equalisation one final BCA protein assay was performed and the values were taken into account when calculating NFκB p65 activity.

2.2.7.5 NFκB p65 detection using the Thermo Scientific Pierce NFκB p65 Transcription Factor Kit

NFκB p65 activation was measured using the Thermo Scientific Pierce NFκB p65 Transcription Factor Kit. The kit contains a streptavidin-coated 96-well plate with the bound biotinylated-consensus sequence for NFκB p65 which specifically binds only the active forms of NFκB p65. This chemiluminescent ELISA-based assay provides greater sensitivity than traditional methods for measuring active NFκB p65 including gel-shift or electrophoretic mobility shift assays (EMSA) and colorimetric ELISA-based assays.

The assay was performed according to manufacturer's instructions. Briefly, a working binding buffer (supplied with kit) was prepared according to the manufacturer's recommendations and 50 µl was added to each well along with 10 µl of the nuclear extract which was prepared from the NE-PER nuclear and cytoplasmic extraction kit (see section 2.2.7.4). The plate was incubated for 1 hour at RT with mild agitation after which the well contents were discarded and the plate was tapped on a stack of paper-towels to remove all liquid. The plate was washed three times with 200 µl of wash buffer. 100 µl of the diluted primary antibody (1:1000) was added to each well and incubated for 1 hour at RT without agitation. The plate was washed three times with 200 µl of wash buffer. 100µl of diluted secondary antibody (1:10,000) was added to each well and incubated for 1 hour at RT without agitation. Finally, the plate was washed four times with 200 µl of wash buffer and 100 µl of the chemiluminescent solution was added to each well. Chemiluminescence was measured using a luminometer set to 200 ms and a sensitivity value of 135. Data was expressed as relative light units (RLU)/µg protein (protein concentration after equalisation as determined by BCA assay).

2.2.8 Statistical analysis

All data was analysed using the statistical analysis package GB-STAT. Statistical comparisons were performed using either a Student's *t*-test or analysis of variance (ANOVA) as indicated in the individual results chapters. If significant changes were observed following ANOVA the data was further analysed using a Student Newman-Keuls or Dunnet's *post hoc* test as appropriate. A value of $p < 0.05$ was deemed statistically significant. Results are expressed as mean with standard error of the mean (SEM) or mean percentage control with SEM.

Chapter 3

β_2 -adrenoceptor stimulation suppresses NF κ B activity and promotes an overall anti-inflammatory phenotype following LPS-induced inflammation in rat brain

3.1 Introduction

The locus coeruleus (LC) is the main site of noradrenaline synthesis in the CNS. It has been well documented that the loss of LC neurons and decreased cortical noradrenaline concentrations are features of chronic neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Feinstein *et al.*, 2002; Marien *et al.*, 2004). There is growing interest in stimulation of noradrenergic receptors, in particular the central β_2 -adrenoceptors, due to the fact that when stimulated they promote an anti-inflammatory phenotype in glial cells (Culmsee *et al.*, 1999a; Culmsee *et al.*, 1999b; Hertz *et al.*, 2004; Counts & Mufson, 2010; McNamee *et al.*, 2010a). They are also known to have neuroprotective actions in rodent models of cerebral ischaemia (Semkova *et al.*, 1996; Zhu *et al.*, 1998; Culmsee *et al.*, 1999b; Junker *et al.*, 2002) and in both *in vitro* and *in vivo* models of excitotoxicity (Semkova *et al.*, 1996; Gleeson *et al.*, 2010).

Our laboratory has recently demonstrated that the β_2 -adrenoceptor agonist clenbuterol increases expression of the pro-inflammatory cytokine IL-1 β , its negative regulators IL-1ra and the interleukin-1 type II decoy receptor (IL-1RII) (McNamee *et al.*, 2010a; 2010c) and also induces expression of the broad spectrum anti-inflammatory cytokine IL-10 and its downstream signalling molecule SOCS-3 in the CNS (McNamee *et al.*, 2010b). The actions of IL-1 β can be regulated by IL-1ra, which prevents IL-1 β from acting on the IL-1RI (Carter *et al.*, 1990) and by the IL-1RII; a decoy receptor that serves to sequester IL-1 (Colotta *et al.*, 1994). Increased expression of these two molecules prevents prolonged activation of the IL-1 system and thus may be beneficial in combating the deleterious effects of IL-1 β on brain function.

Despite a largely anti-inflammatory profile, clenbuterol induces IL-1 β . It was important to investigate whether any downstream pro-inflammatory signalling pathways were being activated by IL-1 β . One major neurotoxic signalling pathway activated by IL-1 β is the NF κ B pathway. It controls gene expression in immunity and inflammation (Kopp & Ghosh, 1995) and has been implicated in various chronic inflammatory diseases such as multiple sclerosis (Gveric *et al.*, 1998; Bonetti *et al.*, 1999) and Alzheimer's disease (Boissiere *et al.*, 1997; Lukiw & Bazan, 1998). Literature suggests that IL-1 β is capable of inducing persistent NF κ B activation in human astrocytes (Aloisi *et al.*, 1992; Moynagh *et al.*, 1994; Griffin & Moynagh, 2006). Thus, it was imperative to investigate

whether clenbuterol-induced IL-1 β activated the NF κ B pathway. Previous work by our group demonstrated that I κ B α mRNA was induced following clenbuterol treatment (McNamee *et al.*, 2010c). Given the inhibitory effect of I κ B α on NF κ B activation it was plausible that clenbuterol could in fact suppress NF κ B activation. However, I κ B α mRNA alone was not enough to determine the end effect on NF κ B activity. Further investigation of I κ B α protein and NF κ B DNA binding activity were required.

A number of studies were carried out to investigate the effects of the β_2 -adrenoceptor agonist clenbuterol on NF κ B activation and I κ B α expression in the rat brain. Of particular interest were the cortical and hippocampal regions. Both regions receive rich noradrenergic innervation from the LC and the cortex in particular is involved in higher cognitive processing that is adversely affected in many neurodegenerative diseases (Burton *et al.*, 2004; Whitwell *et al.*, 2007). In addition, they are regions of high metabolic activity known to highly express NF κ B compared to other tissues (Kaltschmidt *et al.*, 1994).

The objectives of the present chapter are:

1. To determine the effects of the β_2 -adrenoceptor agonist clenbuterol on NF κ B activity in the rat brain and to investigate a role for I κ B.
2. To confirm a role for β_2 -adrenoceptor activation in mediating the effects of clenbuterol on the NF κ B system.
3. To determine the effects of the β_2 -adrenoceptor agonist clenbuterol on NF κ B activation in response to an inflammatory challenge.

3.2 Methods

3.2.1 Animal handling

Male Sprague-Dawley rats (250-300g) were obtained from Harlan Laboratories, UK. Rats were housed 4 per cage in an air-conditioned room with a 12:12-h light/dark cycle (lights on at 8am). Food and water were available *ad libitum*. Animals were handled daily for at least seven days prior to drug administration.

3.2.2 Experimental procedures

All pharmacological agents were dissolved in 0.89% (w/v) saline. Clenbuterol was administered via intraperitoneal (i.p.) injection at doses of 0.03, 0.1, 0.3 or 0.5 mg/kg. Formoterol and salbutamol were administered i.p. at a dose of 0.5 mg/kg. LPS was administered via intracerebroventricular (i.c.v.) injection at a dose of 1 μ g/5 μ L. Control animals were administered a 0.89% (w/v) saline vehicle. Tissue was prepared for Western blot, RT-PCR and NF κ B binding assay. A detailed description of experimental procedures and molecular techniques can be found in Chapter 2.

3.3. Effect of the β_2 -adrenoceptor agonist clenbuterol on NF κ B activity and I κ B expression in rat brain

As previously mentioned, literature published by this group and others has demonstrated that targeting of noradrenergic β -adrenoceptors in the rat brain elicits neuroprotective effects in rodent models of cerebral ischaemia (Semkova *et al.*, 1996; Zhu *et al.*, 1998; Culmsee *et al.*, 1999b; Junker *et al.*, 2002) and in both *in vitro* and *in vivo* models of excitotoxicity (Semkova *et al.*, 1996; Gleeson *et al.*, 2010). Our laboratory demonstrated that the β_2 -adrenoceptor agonist clenbuterol suppresses expression of the pro-inflammatory cytokines IL-6 and TNF- α , promotes expression of the anti-inflammatory cytokines such as IL-10, IL-1RII and IL-1ra, but paradoxically, also increases expression of IL-1 β in rat brain, albeit in a controlled manner (McNamee *et al.*, 2010a; 2010b; 2010c). As IL-1 β is a major inducer of NF κ B, a key transcription factor involved in the expression of inflammatory mediators, the present chapter aimed to examine NF κ B activity in clenbuterol-treated animals. NF κ B activation is controlled by inhibitory I κ B proteins that sequester it in an inactive state in the cytoplasm (see Chapter 1). In the present section, the impact of clenbuterol on NF κ B activity and I κ B expression are examined in rat brain. Part A outlines the results of a time course study whereby rats were administered a 0.5 mg/kg dose of clenbuterol and sacrificed at 1, 4 and 8 hours time points. Part B outlines the results of a dose-response study whereby rats were administered clenbuterol at doses of 0.03, 0.1 and 0.3 mg/kg and sacrificed 4 hours later.

Part A: A time course study. Effect of the β_2 -adrenoceptor agonist clenbuterol on NF κ B activity and I κ B expression in rat brain, 1, 4 and 8 hours post-administration

Figure 3.3.1. The β_2 -adrenoceptor agonist clenbuterol induced a time-dependent suppression of NF κ B activity in rat brain

ANOVA revealed a significant effect of clenbuterol (0.5 mg/kg; i.p) on NF κ B p65 binding (NF κ B activity) in nuclear fractions of both cortex [$F_{(3, 17)} = 5.25, p=0.0095$] and hippocampus [$F_{(3, 17)} = 15.72, p<0.0001$]. *Post hoc* analysis showed a time-dependent suppression of NF κ B activity in cortex 4 hours ($p<0.01$) and 8 hours ($p<0.01$) post-clenbuterol treatment compared to saline vehicle. A time-dependent suppression of NF κ B activity was also observed in hippocampus 1 hour ($p<0.05$), 4 hours ($p<0.01$) and 8 hours ($p<0.01$) post-treatment, compared to saline vehicle [One-way ANOVA followed by *post hoc* Dunnett's Procedure; $n=5-6$].

Figure 3.3.2 A, B. The β_2 -adrenoceptor agonist clenbuterol induced a time-dependent increase in I κ B α mRNA expression in rat brain

ANOVA revealed a significant effect of clenbuterol (0.5 mg/kg; i.p) on I κ B α mRNA expression in both cortex [$F_{(3, 14)} = 3.72, p=0.0372$] and hippocampus [$F_{(3, 16)} = 4.32, p=0.0207$]. *Post hoc* analysis showed an increase in I κ B α mRNA expression 1 hour post-clenbuterol treatment in both cortex ($p<0.01$) and hippocampus ($p<0.01$), compared to saline vehicle [One-way ANOVA followed by *post hoc* Dunnett's Procedure; $n=5-6$].

Figure 3.3.2 C, D. The β_2 -adrenoceptor agonist clenbuterol induced a time-dependent increase in I κ B α protein expression in rat brain

ANOVA revealed a significant effect of clenbuterol (0.5 mg/kg; i.p) on cytosolic I κ B α protein expression in both cortex [$F_{(3, 10)} = 10.38, p=0.0021$] and hippocampus [$F_{(3, 12)} = 4.81, p<0.05$]. *Post hoc* analysis showed a time-dependent increase in I κ B α protein expression 4 hours and 8 hours post-clenbuterol treatment in both cortex ($p<0.01$) and hippocampus ($p<0.05$) compared to saline vehicle [One-way ANOVA followed by *post hoc* Dunnett's Procedure; $n=3-4$].

Figure 3.3.3. The β_2 -adrenoceptor agonist clenbuterol induced a time-dependent increase in I κ B β mRNA expression in rat brain

ANOVA revealed a significant effect of clenbuterol (0.5 mg/kg; i.p) on I κ B β mRNA expression in cortex [$F_{(3, 15)} = 4.63, p=0.0174$], and a trend in towards significance in hippocampus [$F_{(3, 15)} = 2.71, p=0.0819$]. *Post hoc* analysis showed an increase in I κ B β mRNA expression 1 hour post-clenbuterol treatment in both cortex ($p<0.01$) and hippocampus ($p<0.05$), compared to saline vehicle [One-way ANOVA followed by *post hoc* Dunnett's Procedure; $n=5-6$].

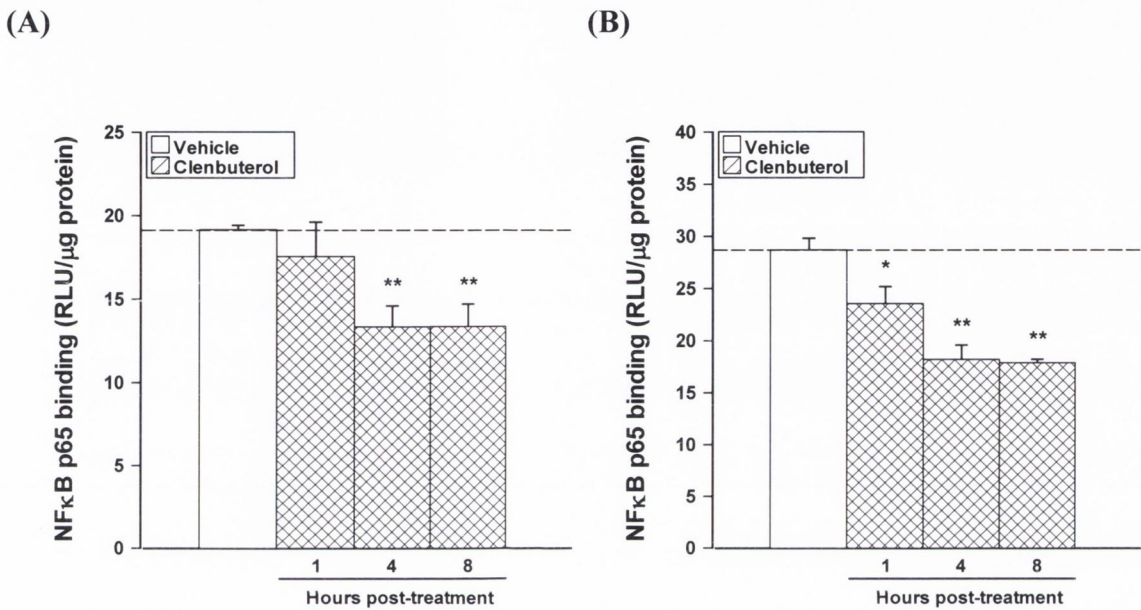


Figure 3.3.1. The β_2 -adrenoceptor agonist clenbuterol induced a time-dependent suppression of NF κ B activity in rat brain. Clenbuterol (0.5 mg/kg) or vehicle (0.89% w/v saline) was administered i.p. and rats were sacrificed at 1, 4 and 8 hour time points post-injection. Clenbuterol induced a time-dependent suppression of NF κ B p65 binding (NF κ B activity) in rat cortex (A) and hippocampus (B). Data expressed as mean + SEM ($n=5-6$). * $p<0.05$, ** $p<0.01$ vs. saline vehicle (One-way ANOVA followed by *post hoc* Dunnett's Procedure).

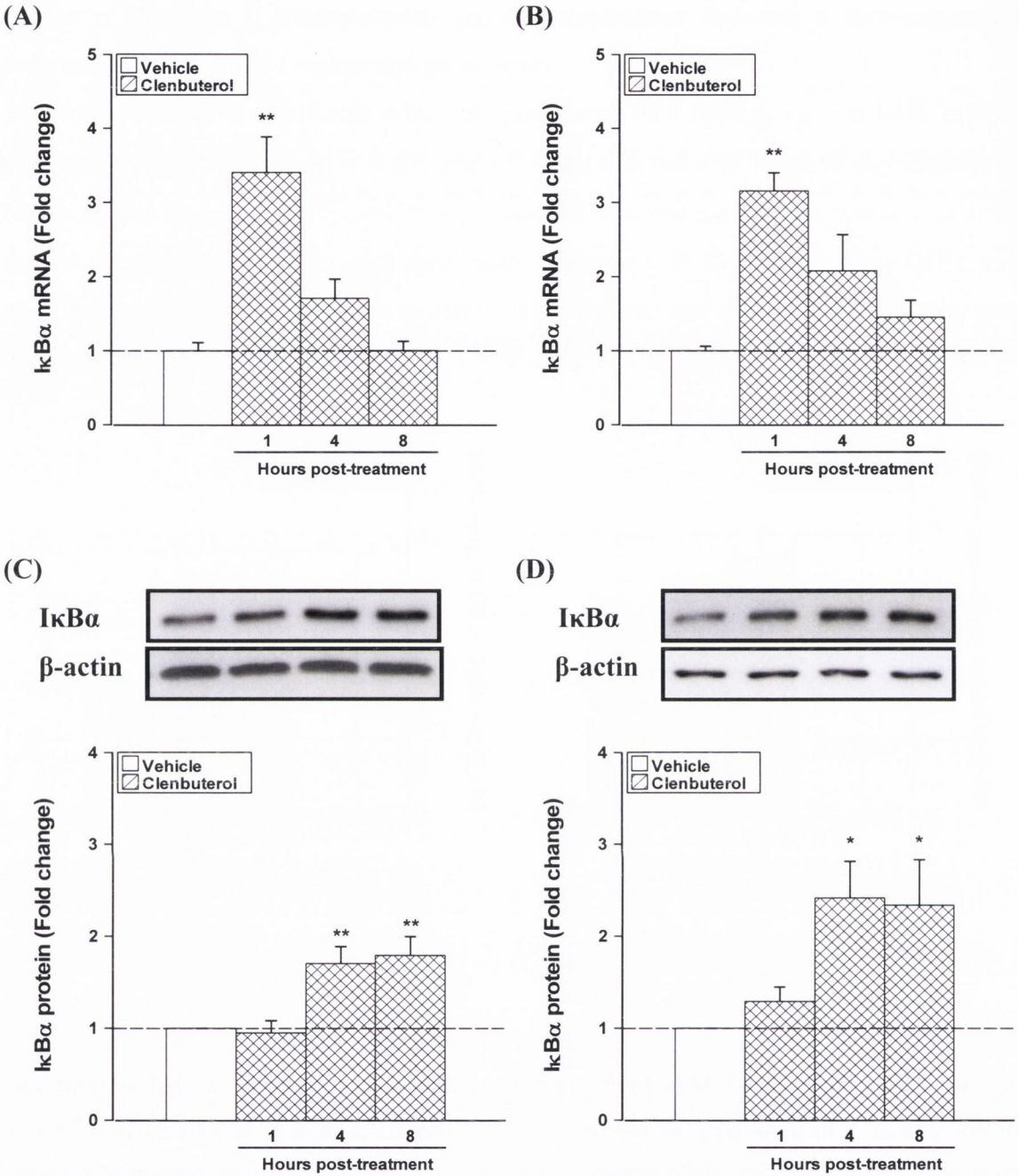


Figure 3.3.2. The β_2 -adrenoceptor agonist clenbuterol induced a time-dependent increase in IκBα mRNA and protein in rat brain. Clenbuterol (0.5 mg/kg) or vehicle (0.89% w/v saline) was administered i.p. and rats were sacrificed at 1, 4 and 8 hour time points post-injection. Clenbuterol induced a time-dependent increase in IκBα mRNA expression in cortex (A) and hippocampus (B) and an increase in IκBα protein expression in cortex (C) and hippocampus (D). Data expressed as mean + SEM ($n=5-6$). mRNA data expressed as fold-change vs. saline vehicle. * $p<0.05$, ** $p<0.01$ vs. saline vehicle (One-way ANOVA followed by *post hoc* Dunnett's Procedure).

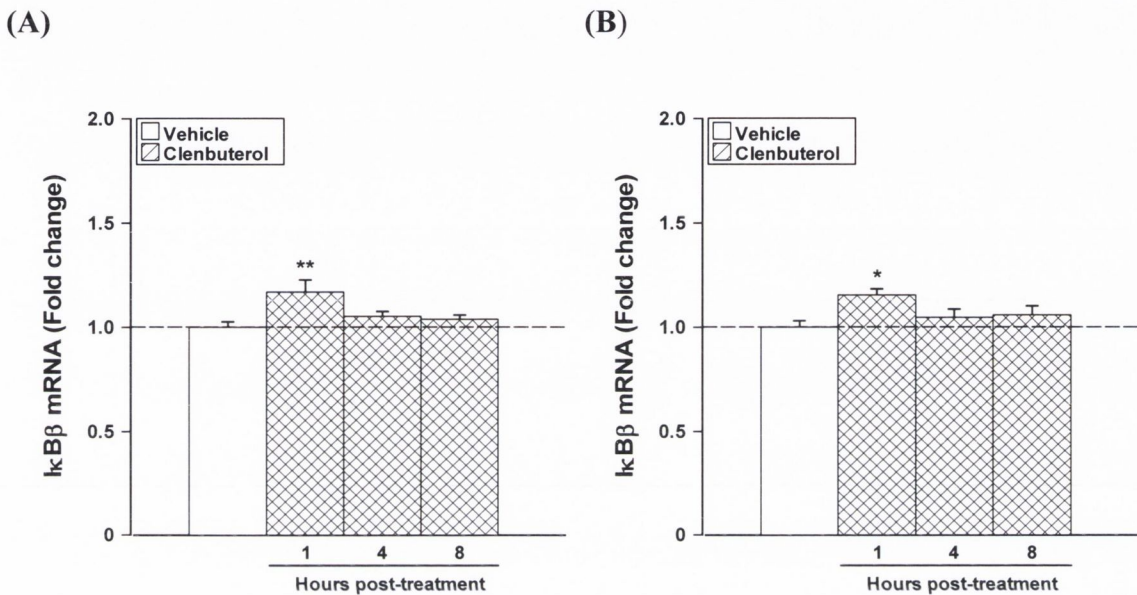


Figure 3.3.3. The β_2 -adrenoceptor agonist clenbuterol induced a time-dependent increase in I κ B β mRNA expression in rat brain. Clenbuterol (0.5 mg/kg) or vehicle (0.89% w/v saline) was administered i.p. and rats were sacrificed at 1, 4 and 8 hour time points post-injection. Clenbuterol induced a time-dependent increase in I κ B β mRNA expression in rat cortex (**A**) and hippocampus (**B**). Data expressed as mean + SEM ($n=3-4$). mRNA data expressed as fold-change vs. saline vehicle. * $p<0.05$, ** $p<0.01$ vs. saline vehicle (One-way ANOVA followed by *post hoc* Dunnett's Procedure).

Part B: A dose-response study. Effect of β_2 -adrenoceptor agonist clenbuterol on NF κ B activity and I κ B α expression in rat brain at three different doses (0.03, 0.1, and 0.3 mg/kg)

Figure 3.3.4. The β_2 -adrenoceptor agonist clenbuterol induced a dose-dependent suppression of NF κ B activity in rat brain

ANOVA revealed a significant effect of clenbuterol on NF κ B p65 binding (NF κ B activation) in nuclear fractions of both cortex [$F_{(3, 17)} = 8.19, p=0.0014$] and hippocampus [$F_{(3, 17)} = 6.31, p=0.0045$]. *Post hoc* analysis showed that NF κ B activity was suppressed at all three doses ($p<0.05$ - $p<0.01$) in both cortex and hippocampus, compared to saline vehicle [One-way ANOVA followed by *post hoc* Dunnett's Procedure; $n=5-6$].

Figure 3.3.5. The β_2 -adrenoceptor agonist clenbuterol induced a dose-dependent increase in I κ B α mRNA expression in rat brain

ANOVA revealed a significant effect of clenbuterol on I κ B α mRNA expression in both cortex [$F_{(3, 17)} = 13.66, p<0.0001$] and hippocampus [$F_{(3, 17)} = 5.62, p=0.0072$]. *Post hoc* analysis showed that I κ B α mRNA was increased by the 0.1 mg/kg ($p<0.05$) and 0.3 mg/kg ($p<0.01$) doses in cortex and by the 0.3 mg/kg dose ($p<0.01$) alone in hippocampus, compared to saline vehicle. The lowest dose of 0.03 mg/kg failed to induce I κ B α mRNA expression [**Figure C, D**; One-way ANOVA followed by *post hoc* Dunnett's Procedure; $n=4-6$].

Figure 3.3.6. The β_2 -adrenoceptor agonist clenbuterol induced a dose-dependent increase in I κ B α protein expression in rat brain

ANOVA revealed a significant effect of clenbuterol on cytosolic I κ B α protein expression in both cortex [$F_{(3, 15)} = 3.54, p=0.0406$], and a trend towards significance in hippocampus [$F_{(3, 15)} = 2.92, p=0.0684$]. *Post hoc* analysis showed that I κ B α protein was increased by the 0.3 mg/kg dose in cortex ($p<0.01$) and by both the 0.1 mg/kg and 0.3 mg/kg dose in hippocampus ($p<0.05$), compared to saline vehicle [One-way ANOVA followed by *post hoc* Dunnett's Procedure; $n=4-5$].

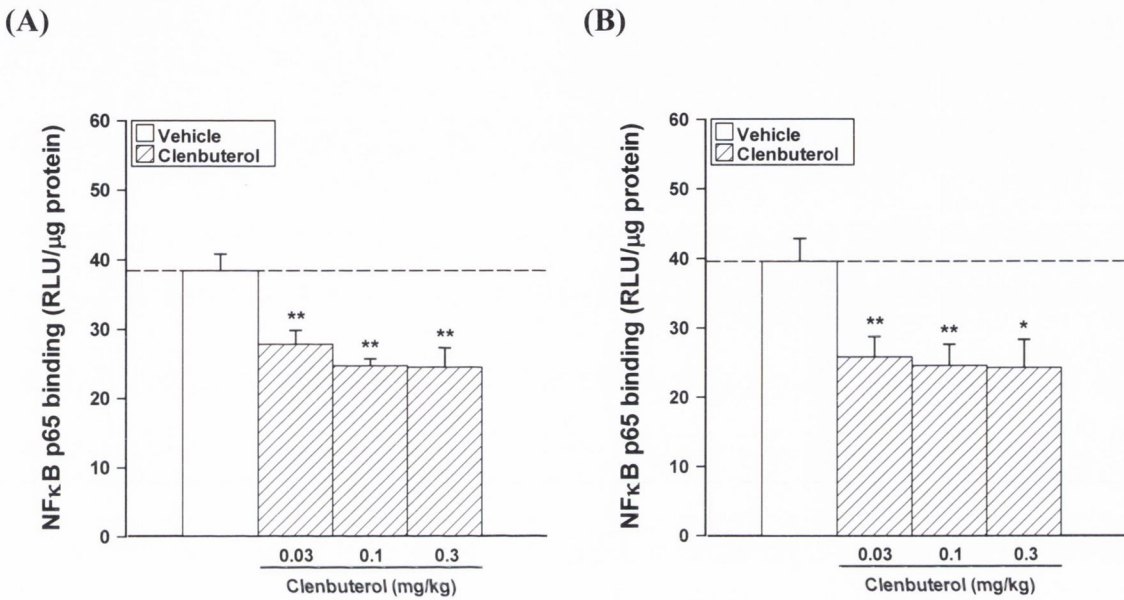


Figure 3.3.4. The β_2 -adrenoceptor agonist clenbuterol induced a dose-dependent suppression of NF κ B activity in rat brain. Clenbuterol (0.03, 0.1 or 0.3 mg/kg) or vehicle (0.89% w/v saline) were administered i.p. and rats were sacrificed 4 hours post-injection. Clenbuterol suppressed NF κ B p65 binding (NF κ B activity) in rat cortex (A) and hippocampus (B). Data expressed as mean + SEM ($n=5-6$). * $p<0.05$, ** $p<0.01$ vs. saline vehicle (One-way ANOVA followed by *post hoc* Dunnett's Procedure).

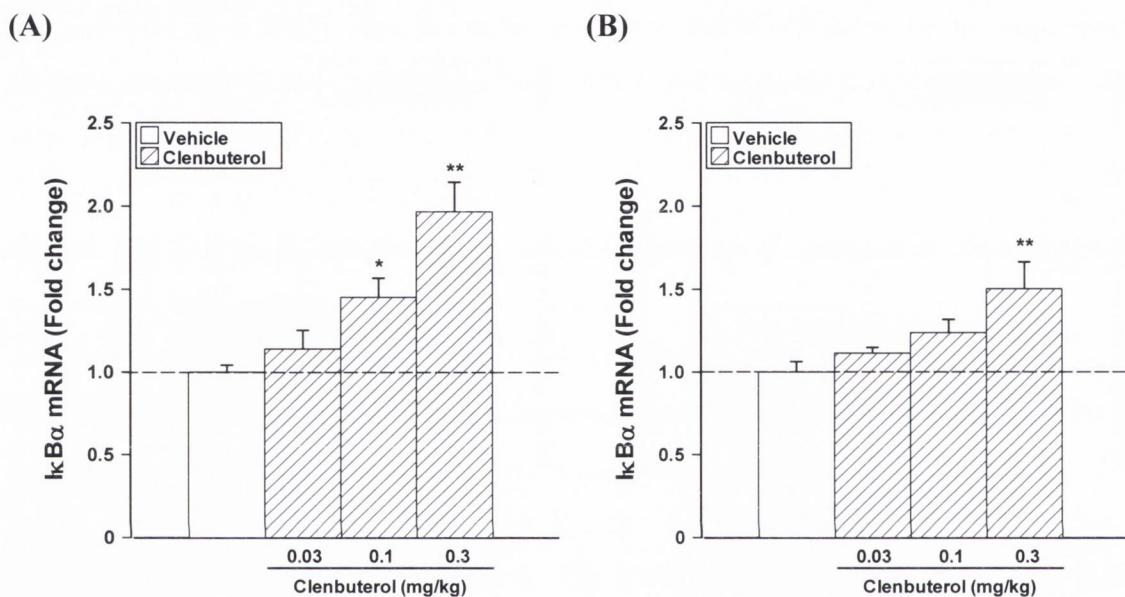


Figure 3.3.5. The β_2 -adrenoceptor agonist clenbuterol induced a dose-dependent increase in IκBα mRNA expression in rat brain. Clenbuterol (0.03, 0.1 or 0.3 mg/kg) or vehicle (0.89% w/v saline) were administered i.p. and rats were sacrificed 4 hours post-injection. Clenbuterol induced a dose-dependent increase in IκBα mRNA expression in rat cortex (A) and hippocampus (B). Data expressed as mean + SEM ($n=4-6$). Data expressed as fold-change vs. saline vehicle. * $p<0.05$, ** $p<0.01$ vs. saline vehicle (One-way ANOVA followed by *post hoc* Dunnett's Procedure).

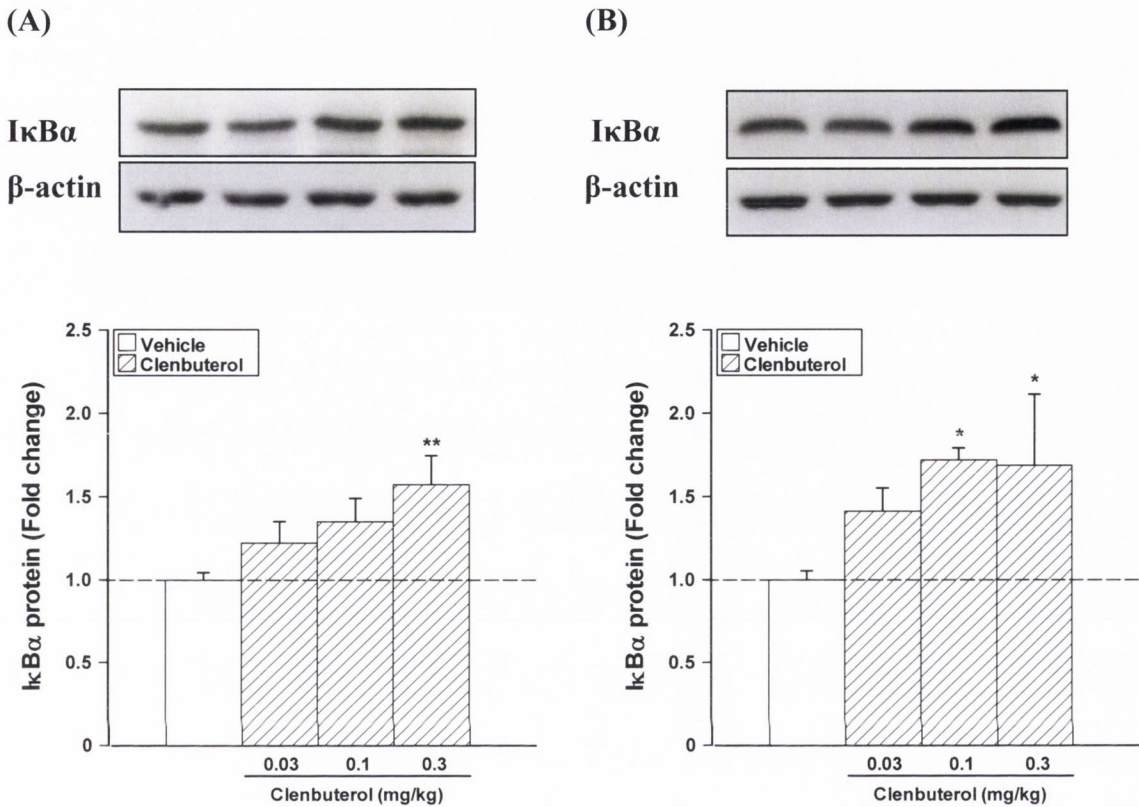


Figure 3.3.6. The β_2 -adrenoceptor agonist clenbuterol induced a dose-dependent increase in IκBα protein expression in rat brain. Clenbuterol (0.03, 0.1 or 0.3 mg/kg) or vehicle (0.89% w/v saline) were administered i.p. and rats were sacrificed 4 hours post-injection. Clenbuterol induced a dose-dependent increase in IκBα protein expression in rat cortex (A) and hippocampus (B). Data expressed as mean + SEM ($n=4-5$). Data expressed as fold-change vs. saline vehicle. Results presented in upper panels are representative images of Western blots. * $p<0.05$, ** $p<0.01$ vs. saline vehicle (One-way ANOVA followed by *post hoc* Dunnett's Procedure).

3.4 Is the clenbuterol-mediated suppression of NFκB activity mediated specifically by the β₂-adrenoceptor in rat brain?

The previous section demonstrated that the β₂-adrenoceptor agonist clenbuterol suppressed NFκB activity and induced the inhibitory IκBα molecule. Of the three subtypes of β-adrenoceptor (β₁, β₂, and β₃) literature suggests that neuroprotective properties are mediated mainly via the β₁ and β₂ subtypes *in vitro* (Semkova *et al.*, 1996; Junker *et al.*, 2002), and in particular via the β₂-adrenoceptors *in vivo* (Culmsee *et al.*, 1999b). Our group recently demonstrated that clenbuterol-induced activation of the IL-1 system is mediated via the β₂-adrenoceptor (Ryan, 2010). However, the role of the β₂-adrenoceptor in clenbuterol-mediated suppression of NFκB activity had not been explored. Therefore, the aim of the following study was to determine whether the clenbuterol-induced suppression of NFκB was mediated specifically by the β₂-adrenoceptor in an attempt to reinforce the argument for the β₂-adrenoceptor as a neuroprotective target. In the present study rats were pre-treated with the selective β₁-adrenoceptor antagonist metoprolol, the non-selective β-adrenoceptor antagonist propranolol or the selective β₂-adrenoceptor antagonist ICI-118551 prior to clenbuterol treatment.

Figure 3.4.1 A, B. Clenbuterol-induced suppression of NFκB activity is mediated specifically by the β₂-adrenoceptor in rat brain

ANOVA revealed a significant effect of clenbuterol (0.5 mg/kg; i.p) on NFκB p65 binding (NFκB activity) in nuclear fractions of both cortex [$F_{(4, 21)} = 3.94, p=0.0154$] and hippocampus [$F_{(4, 21)} = 5.53, p=0.0033$]. *Post hoc* analysis showed, as seen before, that clenbuterol treatment alone suppressed NFκB activity in cortex ($p<0.05$) and hippocampus ($p<0.01$), compared to saline vehicle. Pre-treatment with the selective β₁-adrenoceptor antagonist metoprolol (10 mg/kg; i.p) had no effect on clenbuterol-induced suppression of NFκB activity, thus NFκB activity remained significantly reduced in cortex ($p<0.05$) and hippocampus ($p<0.01$), compared to saline vehicle. Pre-treatment with the non-selective β-adrenoceptor antagonist propranolol (10 mg/kg; i.p) and the selective β₂-adrenoceptor antagonist ICI-118551 (10 mg/kg; i.p) blocked the clenbuterol-induced suppression of NFκB activity [One-way ANOVA followed by *post hoc* Newman Keuls; $n=5-6$].

Figure 3.4.1 C, D. Clenbuterol-induced increase in I κ B α mRNA expression is mediated specifically by the β_2 -adrenoceptor in rat brain

ANOVA revealed a significant effect of clenbuterol (0.5 mg/kg; i.p) on I κ B α mRNA expression in cortex [$F_{(4, 23)} = 17.97$, $p < 0.0001$] and hippocampus [$F_{(4, 23)} = 8.31$, $p = 0.0003$]. *Post hoc* analysis showed, as seen before, that clenbuterol significantly increased I κ B α mRNA expression in cortex ($p < 0.01$) and hippocampus ($p < 0.01$), compared to saline vehicle. Pre-treatment with the selective β_1 -adrenoceptor antagonist metoprolol had no effect on clenbuterol-induced I κ B α mRNA expression, thus I κ B α mRNA expression remained significantly increased in cortex ($p < 0.01$) and hippocampus ($p < 0.01$), compared to saline vehicle. The ability of clenbuterol to induce I κ B α mRNA expression was blocked by pre-treatment with the non-selective β -adrenoceptor antagonist propranolol and the selective β_2 -adrenoceptor antagonist ICI-118551 in cortex ($p < 0.01$) and hippocampus ($p < 0.01$) [One-way ANOVA followed by *post hoc* Newman Keuls; $n = 5-6$].

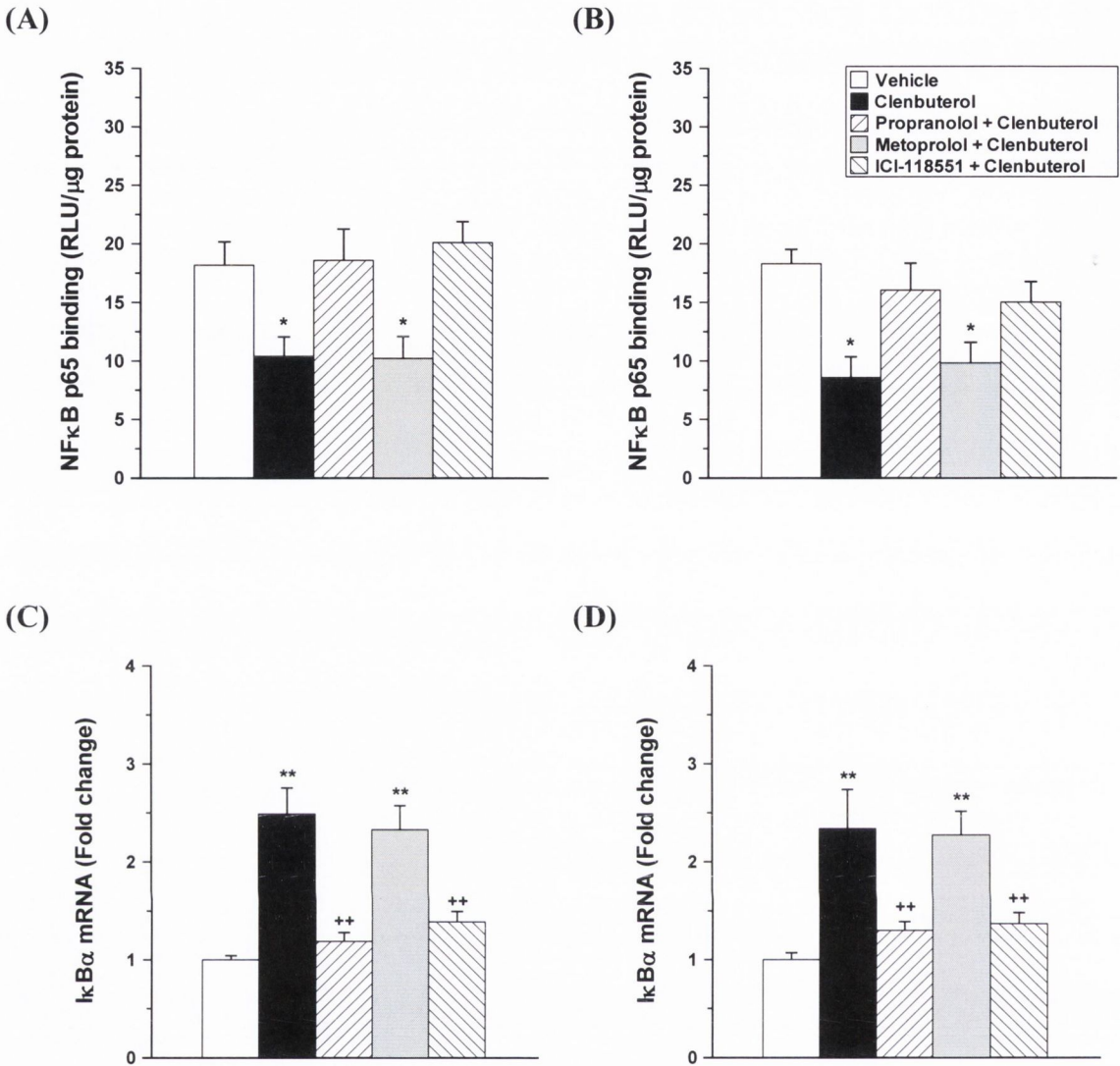


Figure 3.4.1. Clenbuterol-induced suppression of NFκB activity and induction of IκBα mRNA expression is mediated specifically by the β₂-adrenoceptor in rat brain. Rats were pre-treated with the non-selective β-adrenoceptor antagonist propranolol (10 mg/kg; i.p), selective β₁-adrenoceptor antagonist metoprolol (10 mg/kg; i.p) or the selective β₂-adrenoceptor antagonist ICI-118551 (10 mg/kg; i.p) 30 min prior to clenbuterol (0.5 mg/kg; i.p). Rats were sacrificed 4 hour post clenbuterol injection. The ability of clenbuterol to suppress NFκB activity and increase IκBα mRNA expression was prevented by pre-treatment with propranolol and ICI-118551, but not metoprolol in cortex (A + C) and hippocampus (B + D). Data expressed as mean + SEM (n=5-6). mRNA data expressed as fold-change vs. saline vehicle. **p*<0.05, ***p*<0.01, vs. saline vehicle; ++ *p*<0.01, vs. clenbuterol vehicle (One-way ANOVA followed by *post hoc* Newman Keuls).

3.5 Can other β_2 -adrenoceptor agonists, namely formoterol and salbutamol, mimic the effects of clenbuterol on NF κ B and I κ B α ?

Formoterol and salbutamol are β_2 -adrenoceptor agonists that are used as bronchodilators in the treatment of asthma and chronic obstructive pulmonary disease (Baronti *et al.*, 1980; Boner *et al.*, 1988; Tashkin & Cooper, 2004). Our group and others have demonstrated that formoterol and salbutamol are capable of suppressing LPS-induced TNF- α in the brain *in vivo* (Elenkov *et al.*, 1995; McNamee *et al.*, 2010c). While clenbuterol is a useful drug for the investigation of the anti-inflammatory effects of central β -adrenoceptor stimulation, concerns have arisen regarding its safety due to undesirable effects on skeletal and cardiac muscle such as tremor and tachycardia, and due to its off-target effects on ion channel function (Desaphy *et al.*, 2003). Due to the fact that formoterol and salbutamol are more readily used and have a better safety record, the aim of this study was to investigate whether these agonists could elicit similar effects to clenbuterol on NF κ B and I κ B α in rat brain.

Figure 3.5.1. The β_2 -adrenoceptor agonists salbutamol and formoterol suppressed NF κ B activity in rat brain

ANOVA revealed a significant effect of treatment on NF κ B activity in both cortex [$F_{(2, 15)} = 17.99$, $p < 0.0001$] and hippocampus [$F_{(2, 15)} = 22.04$, $p < 0.0001$]. Consistent with this finding, *post hoc* analysis showed that both salbutamol and formoterol suppressed NF κ B activity in cortex and hippocampus ($p < 0.01$), compared to saline vehicle [One-way ANOVA followed by *post hoc* Newman Keuls; $n=6$].

Figure 3.5.2. I κ B α mRNA expression is increased by the β_2 -adrenoceptor agonist formoterol but not by salbutamol

ANOVA revealed a significant effect of treatment on I κ B α mRNA expression in both cortex [$F_{(2, 15)} = 11.26$, $p = 0.001$] and hippocampus [$F_{(2, 15)} = 12.99$, $p = 0.0005$]. *Post hoc* analysis showed that formoterol increased I κ B α mRNA expression in cortex and hippocampus ($p < 0.01$), compared to saline vehicle. In contrast, salbutamol had no effect on I κ B α mRNA expression [One-way ANOVA followed by *post hoc* Newman Keuls; $n=6$].

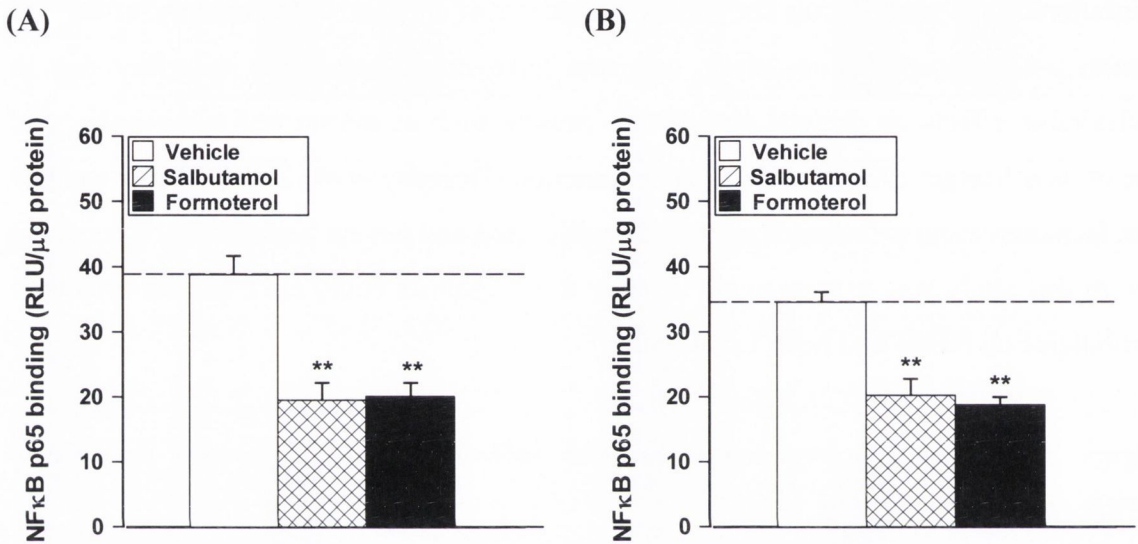


Figure 3.5.1. The β_2 -adrenoceptor agonists salbutamol and formoterol suppressed NFκB activity. Salbutamol (0.5 mg/kg; i.p), formoterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered and rats were sacrificed 4 hours post-injection. Both salbutamol and formoterol suppressed NFκB activity in cortex (A) and hippocampus (B). Data expressed as mean + SEM ($n=6$). ** $p<0.01$ vs. saline vehicle [One-way ANOVA followed by *post hoc* Newman Keuls].

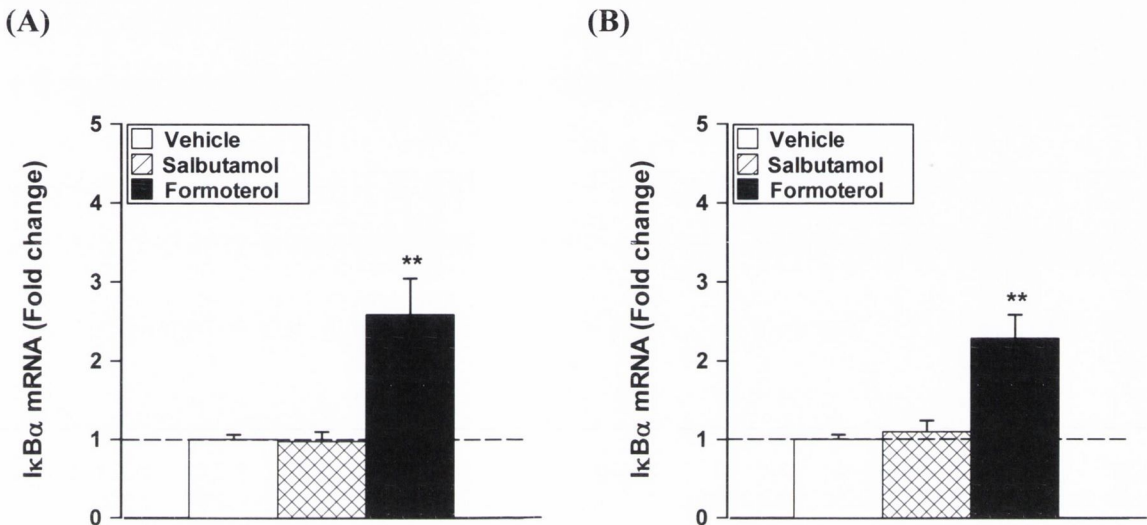


Figure 3.5.2. IκBα expression is increased by the β-adrenoceptor agonist formoterol but not by salbutamol. Salbutamol (0.5 mg/kg; i.p), formoterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered and rats were sacrificed 4 hours post-injection. IκBα mRNA expression was induced by formoterol in both cortex (A) and hippocampus (B). Salbutamol had no effect on IκBα mRNA expression. Data expressed as mean + SEM ($n=6$). Data calculated as fold change compared to saline vehicle. ** $p<0.01$ vs. saline vehicle [One-way ANOVA followed by *post hoc* Newman Keuls].

3.6 Can LPS-induced NFκB be attenuated by pre-treatment with the β₂-adrenoceptor agonist clenbuterol in rat brain?

The results presented in the previous sections demonstrate that clenbuterol alone is capable of suppressing NFκB activity in the rat brain. Dysregulated NFκB signalling is a known feature of a number of inflammatory disorders. For example, in Alzheimer's disease NFκB is known to be activated around early plaque stages (Kaltschmidt *et al.*, 1997). Thus, methods which modulate or inhibit NFκB activity may be a useful clinical tool. The β₂-adrenoceptor agonist clenbuterol has previously been shown to be neuroprotective in animal models of excitotoxicity (Gleeson *et al.*, 2010) and against IL-1β-induced neurotoxicity (McNamee *et al.*, 2010b). The aim of the present study was to investigate the ability of β₂-adrenoceptor stimulation to suppress NFκB activity induced by a central i.c.v. LPS injection.

Due to the fact that this was the first time that i.c.v. injection of LPS had been performed in our lab, a small pilot study was carried out to ensure that this technique could induce NFκB activity in rat brain and to determine the optimum time point for detection of NFκB activity post-injection (Part A). Following this a second study was carried out whereby animals were pre-treated with a peripheral clenbuterol injection followed by a central i.c.v LPS injection (Part B).

Part A: A pilot study to determine whether i.c.v. injection of LPS can induce NFκB activity and to determine the optimum time point for detection of NFκB activity post-injection

Figure 3.6.1 A, B. LPS increased NFκB activation two hours post-i.c.v injection.

ANOVA revealed a significant effect of LPS (1μg/5μL) on NFκB activation in nuclear fractions of cortex [$F_{(2, 6)} = 6.90, p=0.0278$], and a trend towards significance in hippocampus [$F_{(2, 5)} = 4.11, p=0.0879$]. *Post hoc* analysis showed that LPS significantly increased NFκB activity in cortex 2 hours post-injection ($p<0.01$), compared to saline vehicle. *Post hoc* analysis showed that LPS significantly increased NFκB activity in hippocampus 2 hours post-injection ($p<0.05$), compared to saline vehicle [One-way ANOVA followed by *post hoc* Dunnett's Procedure; $n=2-3$].

Figure 3.6.1 C, D. LPS had a tendency to reduce IκBa protein two hours post-i.c.v injection.

Due to the small sample size here ($n=2$ per group) it is not possible to perform statistical analysis. However, a trend towards decreased IκBa protein expression was observed two hours after LPS injection in cortex and hippocampus, compared to saline vehicle.

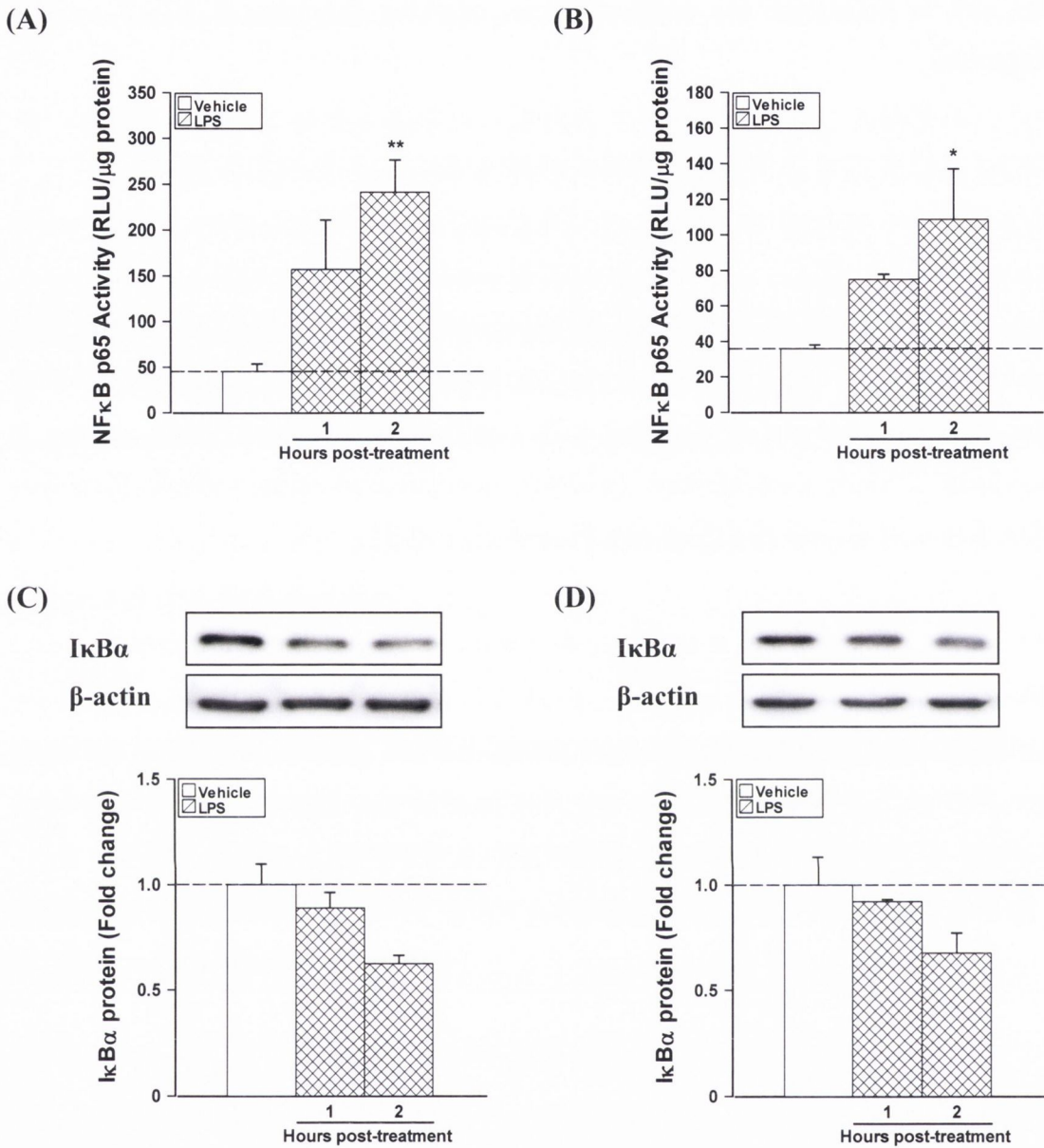


Figure 3.6.1. LPS increased NFκB activation and reduced IκBα protein expression two hours post-i.c.v injection. Rats were administered LPS (1μg/5μL; i.c.v. to lateral ventricle) and were sacrificed 1 and 2 hours post LPS treatment. LPS induced a time-dependent increase in NFκB activity in cortex (A) and hippocampus (B). LPS induced a significant time-dependent decrease in IκBα protein expression in cortex (C) and hippocampus (D). Results presented in the upper panels are representative images of Western blots. Data expressed as mean + SEM ($n=2-3$). * $p<0.05$, ** $p<0.01$ vs. saline vehicle (One-way ANOVA followed by *post hoc* Dunnett's Procedure).

Part B: Can pre-treatment with the β_2 -adrenoceptor agonist clenbuterol suppress LPS-induced NF κ B activity in rat brain?

The previous section revealed that i.c.v. injection of LPS (1 μ g/5 μ L) successfully induced NF κ B activity at the 2 hour time point. In the following study rats were pre-treated with the β_2 -adrenoceptor agonist clenbuterol one hour prior to i.c.v. injection of LPS and NF κ B activity and I κ B α expression were measured 2 hours post-LPS injection.

Figure 3.6.2. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol suppressed LPS-induced NF κ B activation.

ANOVA revealed a significant clenbuterol x LPS interaction in both cortex [$F_{(1,18)} = 19.14, p=0.0004$] and hippocampus [$F_{(1,18)} = 5.74, p=0.0277$]. Consistent with this, *post hoc* analysis showed that clenbuterol pre-treatment significantly attenuated the LPS-induced NF κ B p65 binding (NF κ B activity) in both cortex ($p<0.01$) and hippocampus ($p<0.01$) [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n=5-6$].

Figure 3.6.3. The β_2 -adrenoceptor agonist clenbuterol had no effect on the LPS-induced increase in I κ B α mRNA.

ANOVA revealed a significant LPS effect in cortex [$F_{(1,18)} = 176.92, p<0.0001$] and hippocampus [$F_{(1,18)} = 29.5639, p<0.0001$]. *Post hoc* analysis showed that LPS increased I κ B α mRNA expression in cortex ($p<0.01$) and hippocampus ($p<0.01$), compared to saline vehicle. *Post hoc* analysis also showed that clenbuterol pre-treatment had no effect on LPS-induced I κ B α mRNA expression, thus I κ B α mRNA remained significantly induced compared to control ($p<0.01$) [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n=5-6$].

Figure 3.6.4. The β_2 -adrenoceptor agonist clenbuterol blocked the LPS-induced suppression of I κ B α protein.

ANOVA revealed a significant effect of clenbuterol in I κ B α protein expression in cortex [$F_{(1,18)} = 27.13, p=0.0003$] and hippocampus [$F_{(1,8)} = 6.95, p=0.0299$]. *Post hoc* analysis revealed that pre-treatment with clenbuterol blocked the LPS-induced reduction in I κ B α protein expression in cortex ($p<0.01$) and hippocampus ($p<0.05$) [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n=3-4$].

Figure 3.6.5. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol reduced LPS-induced phosphorylation of I κ B α protein.

ANOVA revealed a significant effect of LPS in cortex [$F_{(1,12)} = 16.19$, $p=0.0017$] and hippocampus [$F_{(1,12)} = 11.06$, $p=0.006$]. *Post hoc* analysis showed that clenbuterol pre-treatment significantly attenuated LPS-induced phosphorylation of I κ B α protein in cortex ($p<0.05$) and hippocampus ($p<0.05$) [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n=4$].

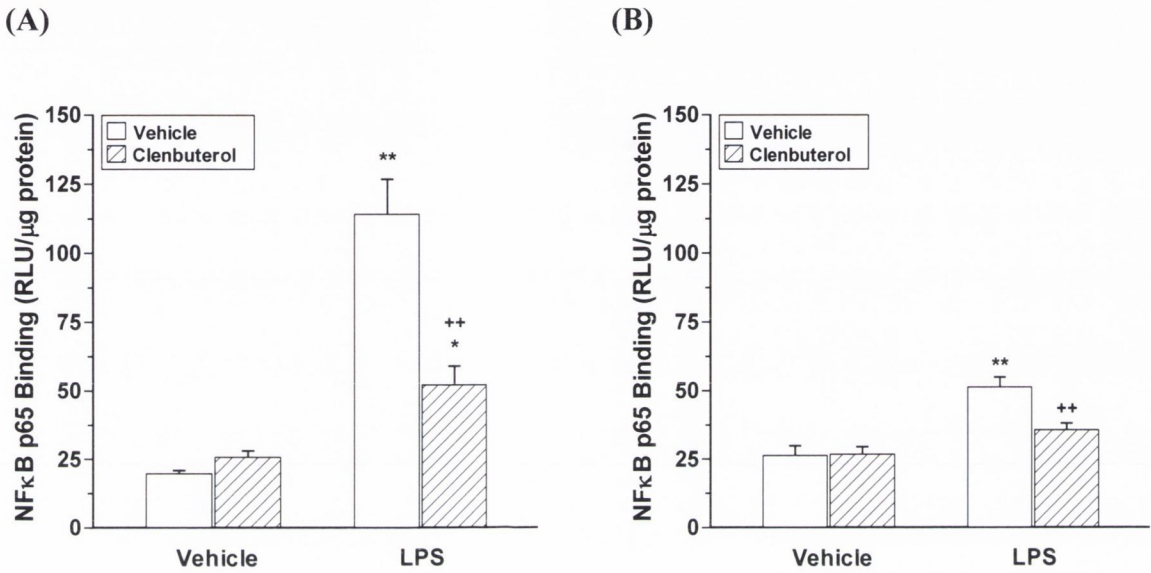


Figure 3.6.2. The β_2 -adrenoceptor agonist clenbuterol suppressed LPS-induced NFκB activation. Clenbuterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1μg/μL; i.c.v) or vehicle (0.89% w/v saline; i.c.v). Rats were sacrificed 2 hours post LPS injection. Clenbuterol treatment significantly attenuated LPS-induced NFκB activity in cortex (A) and hippocampus (B). Data expressed as mean + SEM ($n=5-6$). * $p<0.05$, ** $p<0.01$ vs. saline vehicle; ++ $p<0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

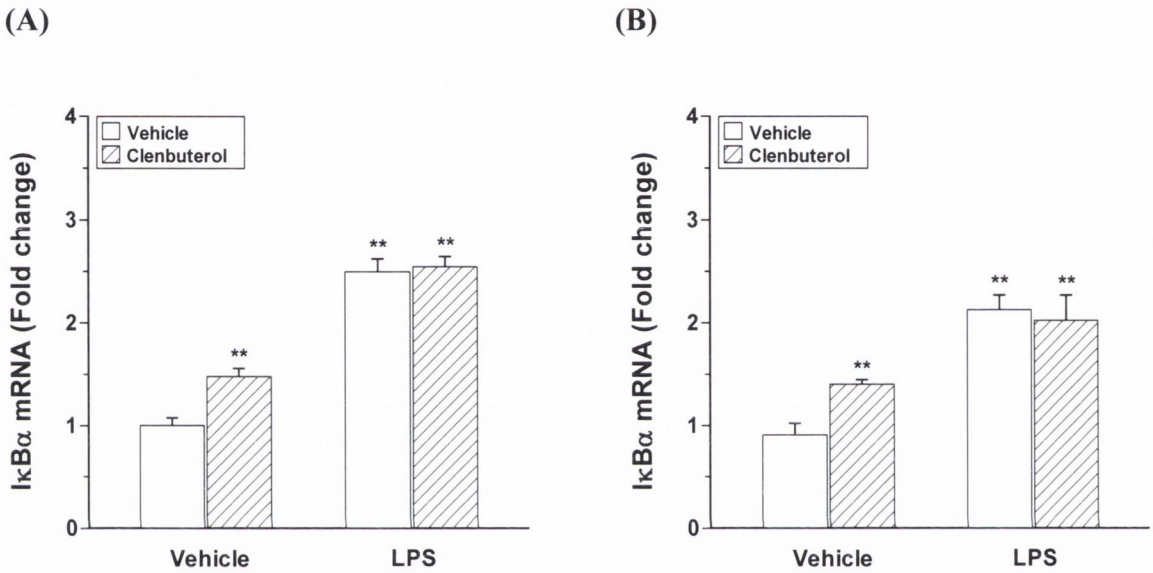


Figure 3.6.3. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol had no effect on the LPS-induced increase in IκBα mRNA. Clenbuterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1μg/μL; i.c.v) or vehicle (0.89% w/v saline; i.c.v). Rats were sacrificed 2 hours post LPS injection. IκBα mRNA expression was increased in response to both clenbuterol and LPS in cortex (A) and hippocampus (B). Data expressed as mean + SEM. mRNA data expressed as fold change compared to control ($n=5-6$). ** $p < 0.01$ vs. saline vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

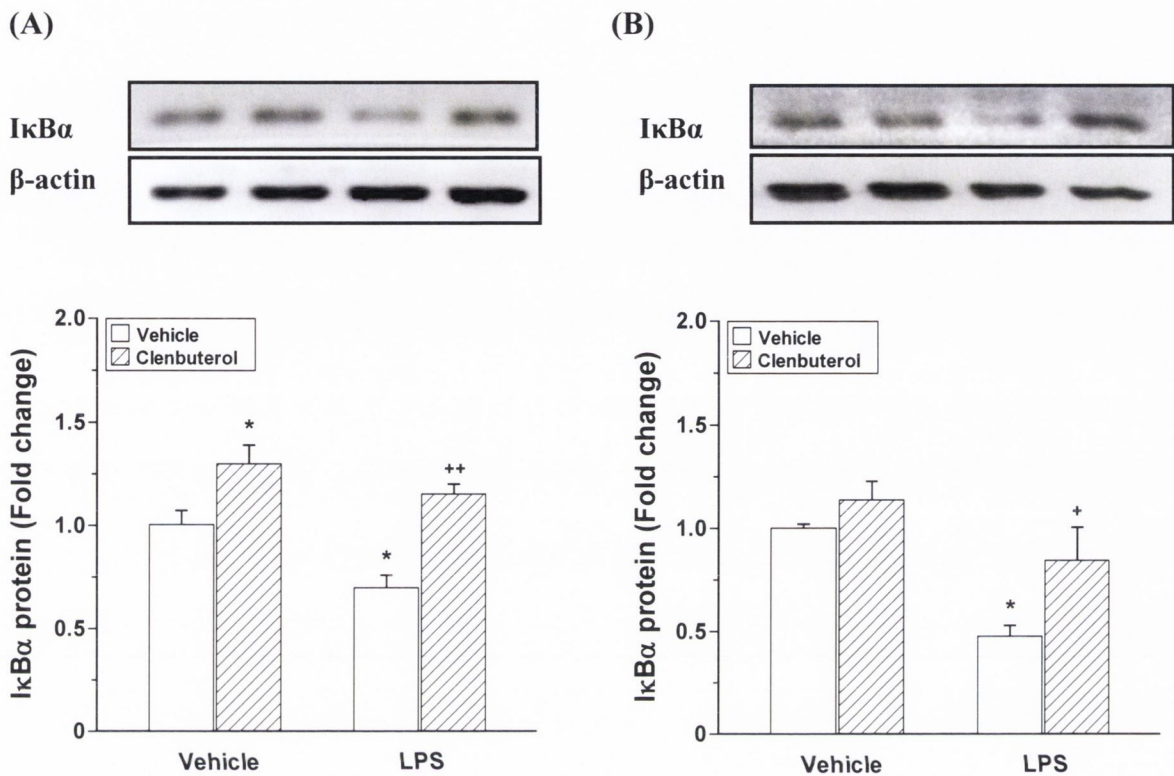


Figure 3.6.4. The β_2 -adrenoceptor agonist clenbuterol blocked the LPS-induced suppression of I κ B α protein. Clenbuterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1 μ g/ μ L; i.c.v) or vehicle (0.89% w/v saline; i.c.v). Rats were sacrificed 2 hours post LPS injection. The LPS induced reduction in total I κ B α protein was blocked by clenbuterol pre-treatment in cortex (A) and hippocampus (B). Data expressed as mean + SEM and as fold change compared to control ($n=3-4$). Results presented in the upper panels are representative images of Western blots. * $p < 0.05$ vs. saline vehicle; + $p < 0.05$, ++ $p < 0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

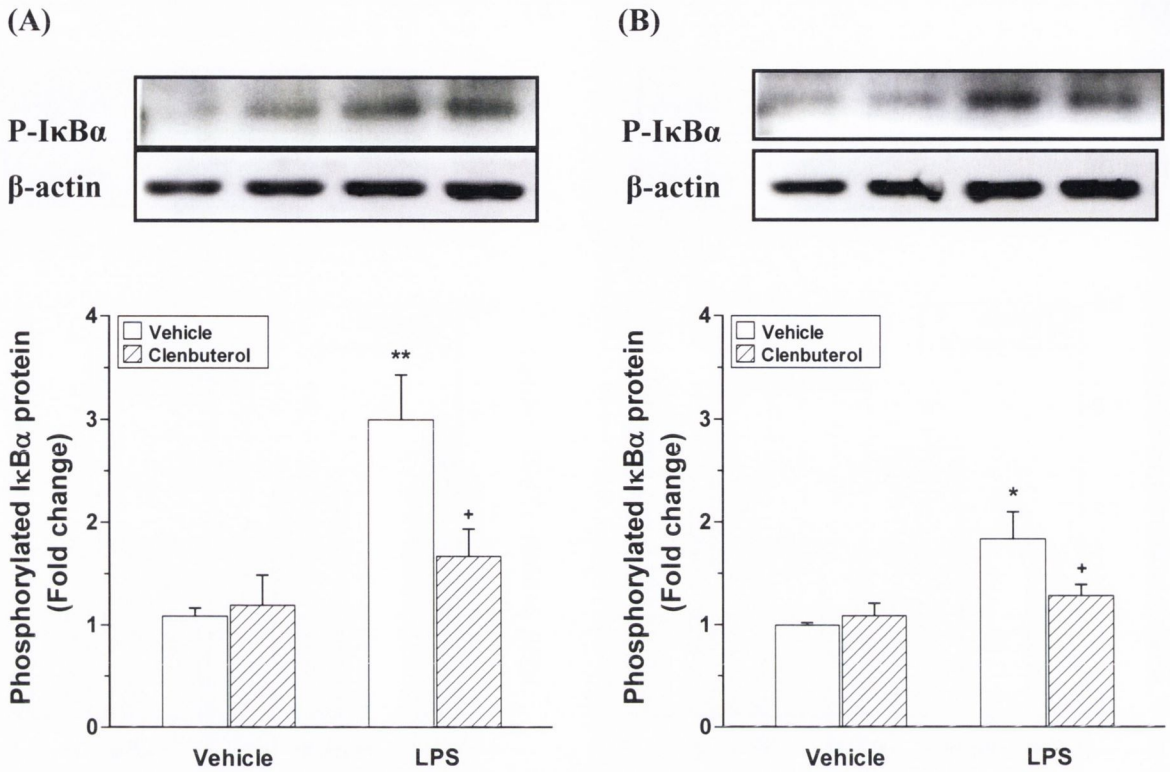


Figure 3.6.5. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol attenuated LPS-induced phosphorylation of IκBα protein. Clenbuterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1 μ g/ μ L; i.c.v) or vehicle (0.89% w/v saline; i.c.v). Rats were sacrificed 2 hours post LPS injection. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol significantly attenuated the LPS-induced phosphorylation of IκBα protein in both cortex (A) and hippocampus (B). Data expressed as mean + SEM and as fold change compared to control ($n=4$). Results presented in the upper panels are representative images of Western blots. * $p<0.05$, ** $p<0.01$ vs. saline vehicle; + $p<0.05$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

3.7 Can β_2 -adrenoceptor stimulation modulate other NF κ B-inducible genes in rat brain?

Activation of NF κ B in glial cells mainly results in inflammatory signalling through induction of many pro-inflammatory genes such as TNF- α , IL-1 β , and IL-6 and adhesion molecules such as ICAM-1 and VCAM-1. The present section examines whether clenbuterol-induced suppression of NF κ B leads to downstream suppression of the NF κ B inducible genes TNF- α and ICAM-1.

Figure 3.7.1. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol attenuated LPS-induced TNF- α expression in rat brain.

ANOVA revealed a significant clenbuterol x LPS interaction in both cortex [$F_{(1,18)} = 13.88, p=0.0015$] and hippocampus [$F_{(1,18)} = 17.97, p=0.0005$]. Consistent with these findings, *post hoc* analysis showed that pre-treatment with clenbuterol blocked the LPS-induced increase in TNF- α mRNA expression in both cortex ($p<0.01$) and hippocampus ($p<0.01$), compared to LPS treatment alone [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n=5-6$].

Figure 3.7.2. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol attenuated LPS-induced ICAM-1 expression in rat brain.

ANOVA revealed a significant clenbuterol x LPS interaction in both cortex [$F_{(1,18)} = 17.88, p=0.0005$] and hippocampus [$F_{(1,18)} = 6.29, p=0.022$]. Consistent with these findings, *post hoc* analysis showed that pre-treatment with clenbuterol blocked the LPS-induced increase in ICAM-1 mRNA expression in both cortex ($p<0.01$) and hippocampus ($p<0.01$), compared to LPS treatment alone [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n=5-6$].

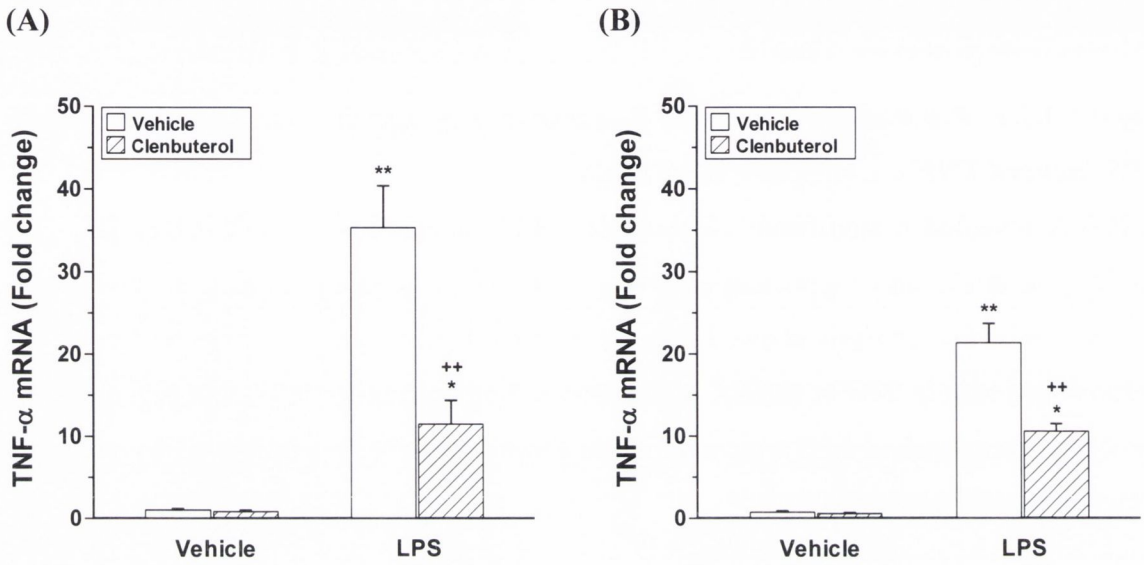


Figure 3.7.1. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol attenuated LPS-induced TNF- α expression in rat brain. Clenbuterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1 μ g/ μ L; i.c.v) or vehicle (0.89% w/v saline; i.c.v). Rats were sacrificed 2 hours post LPS injection. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol significantly attenuated the LPS-induced increase in TNF- α mRNA expression in both cortex (A) and hippocampus (B). Data expressed as mean + SEM and as fold change compared to control ($n=5-6$). * $p<0.05$, ** $p<0.01$ vs. saline vehicle; ++ $p<0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

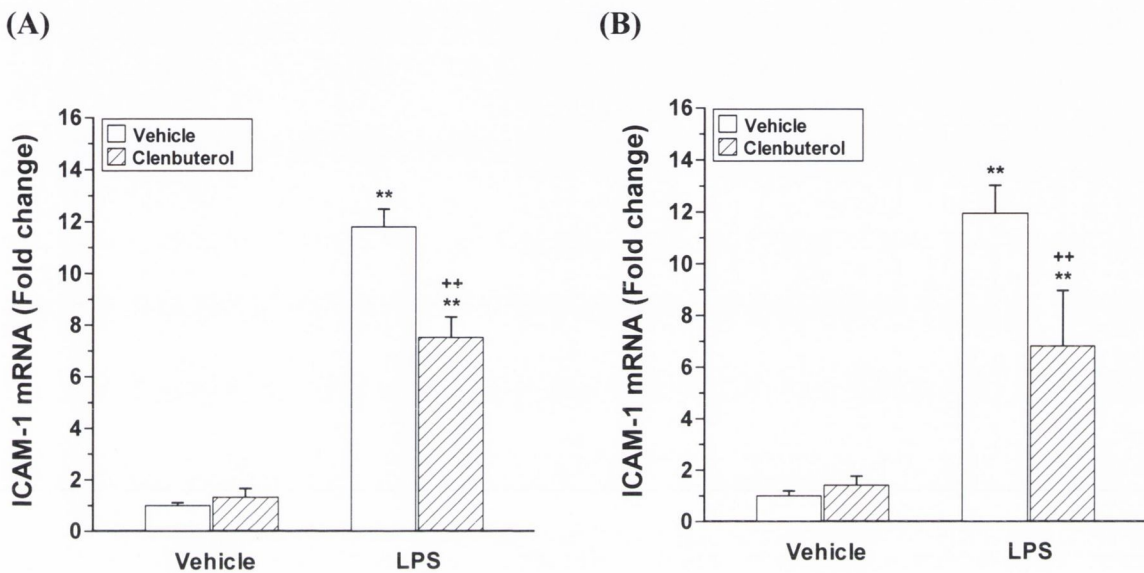


Figure 3.7.2. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol attenuated LPS-induced ICAM-1 expression in rat brain. Clenbuterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1 μ g/ μ L; i.c.v) or vehicle (0.89% w/v saline; i.c.v). Rats were sacrificed 2 hours post LPS injection. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol significantly attenuated the LPS-induced increase in ICAM-1 mRNA expression in both cortex (A) and hippocampus (B). Data expressed as mean + SEM and as fold change compared to control ($n=5-6$). ** $p<0.01$ vs. saline vehicle; ++ $p<0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

3.8 Along with the suppression of the pro-inflammatory mediator NFκB, can pre-treatment with clenbuterol increase anti-inflammatory mediators following an inflammatory LPS challenge in rat brain?

As clenbuterol is known to induce the broad spectrum anti-inflammatory cytokine IL-10 and its downstream signalling molecule SOCS-3 in the CNS (McNamee *et al.*, 2010b) expression of these two molecules as well as another important signalling molecule in the cascade, phosphorylated STAT-3 (P-STAT-3), were examined. Another member of the SOCS family, SOCS-1, has been implicated in the negative regulation of NFκB and so the ability of clenbuterol to induce SOCS-1 expression was also investigated. Finally, IL-10 is known to negatively regulate the cytokine IL-6 and furthermore, LPS-induced IL-6 expression can be regulated by the β-adrenoceptor agonist isoproterenol (Nakamura *et al.*, 1998) thus, the present study also looked at the ability of clenbuterol to regulate LPS-induced IL-6 expression.

Figure 3.8.1 A, B. Pre-treatment with the β₂-adrenoceptor agonist clenbuterol significantly enhanced IL-10 mRNA expression following an LPS challenge.

ANOVA revealed a significant clenbuterol x LPS interaction in both cortex [$F_{(1,19)} = 86.13, p < 0.0001$] and hippocampus [$F_{(1,19)} = 8.04, p = 0.0106$]. *Post hoc* analysis showed that pre-treatment with clenbuterol prior to LPS significantly enhanced IL-10 mRNA expression in cortex ($p < 0.01$) and hippocampus ($p < 0.01$), compared to LPS alone [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n = 5-6$].

Figure 3.8.1 C, D. Pre-treatment with the β₂-adrenoceptor agonist clenbuterol significantly enhanced phosphorylated STAT-3 (P-STAT-3) expression following an LPS challenge.

ANOVA revealed a significant effect of clenbuterol and a significant effect of LPS on P-STAT-3 in cortex [clenbuterol: $F_{(1,19)} = 48.54, p < 0.0001$; LPS: $F_{(1,19)} = 9.87, p < 0.0054$] and hippocampus [clenbuterol $F_{(1,19)} = 38.97, p < 0.0001$; LPS: $F_{(1,19)} = 7.37, p = 0.014$]. Consistent with these findings, *post hoc* analysis showed that clenbuterol alone significantly increased P-STAT-3 in cortex ($p < 0.01$) and hippocampus ($p < 0.01$), compared to saline vehicle. In addition, *post hoc* analysis showed that clenbuterol pre-treatment prior to LPS treatment further enhanced P-STAT-3 expression in cortex

($p < 0.01$) and hippocampus ($p < 0.01$) compared to LPS alone [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n = 5-6$].

Figure 3.8.2. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol significantly enhanced SOCS-3 mRNA expression following an LPS challenge.

ANOVA revealed a significant effect of clenbuterol and a significant effect of LPS on SOCS-3 mRNA expression in cortex [clenbuterol: $F_{(1,20)} = 47.91$, $p < 0.0001$; LPS: $F_{(1,20)} = 57.52$, $p < 0.0001$] and hippocampus [clenbuterol: $F_{(1,20)} = 53.73$, $p < 0.001$; LPS: $F_{(1,20)} = 24.95$, $p < 0.001$]. Consistent with these findings, *post hoc* analysis showed that both clenbuterol and LPS independently increased SOCS-3 mRNA expression in cortex ($p < 0.01$) and hippocampus ($p < 0.01$; $p < 0.05$, respectively), compared to saline vehicle. Furthermore, LPS-induced SOCS-3 mRNA expression was significantly enhanced by clenbuterol pre-treatment in cortex ($p < 0.01$) and hippocampus ($p < 0.01$), compared to LPS alone [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n = 6$].

Figure 3.8.3. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol significantly enhanced IL-6 expression following an LPS challenge.

ANOVA revealed a significant clenbuterol x LPS interaction in cortex [$F_{(1,20)} = 11.12$, $p = 0.0033$]. Consistent with this finding, *post hoc* analysis showed that pre-treatment with clenbuterol significantly enhanced the LPS-induced increase in IL-6 mRNA in cortex ($p < 0.01$). ANOVA revealed a significant effect of clenbuterol in hippocampus [$F_{(1,20)} = 5.74$, $p = 0.0265$]. *Post hoc* analysis showed that pre-treatment with clenbuterol significantly enhanced the LPS-induced increase in IL-6 mRNA in hippocampus ($p < 0.05$) [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n = 6$].

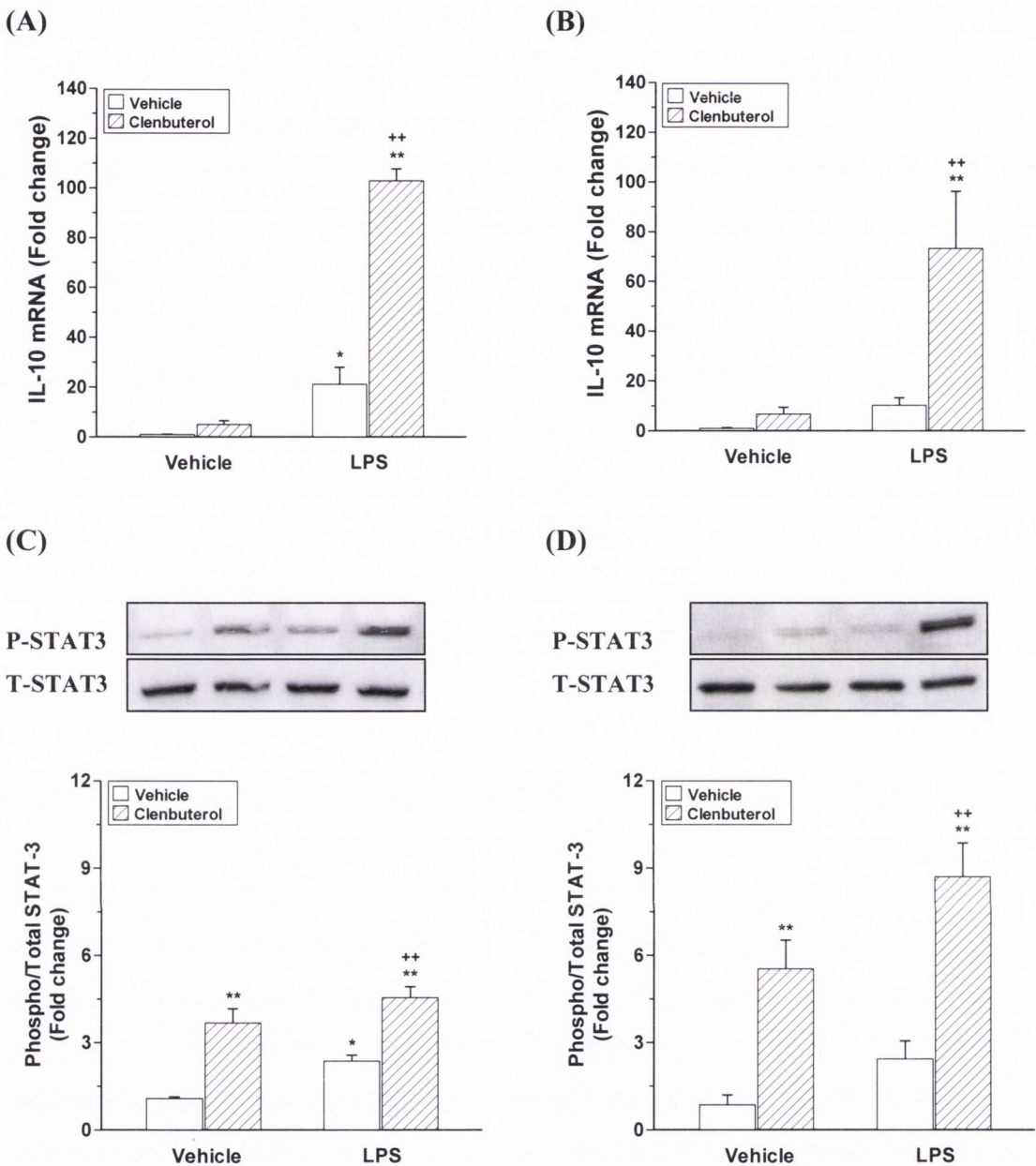


Figure 3.8.1. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol significantly enhanced IL-10 and P-STAT-3 following an LPS challenge. Clenbuterol (0.5 mg/kg; i.p) or saline vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1 μ g/5 μ L; i.c.v) or saline vehicle. Rats were sacrificed 2 hours post LPS injection. Clenbuterol pre-treatment significantly enhanced IL-10 mRNA expression in cortex (A) and hippocampus (B) and P-STAT-3 expression in cortex (C) and hippocampus (D) of LPS treated animals, compared to LPS alone. Data expressed as mean + SEM ($n=5-6$). mRNA data expressed as fold-change vs. saline vehicle. Results presented in the upper panels are representative images of Western blots. * $p<0.05$, ** $p<0.01$ vs. saline vehicle; ++ $p<0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

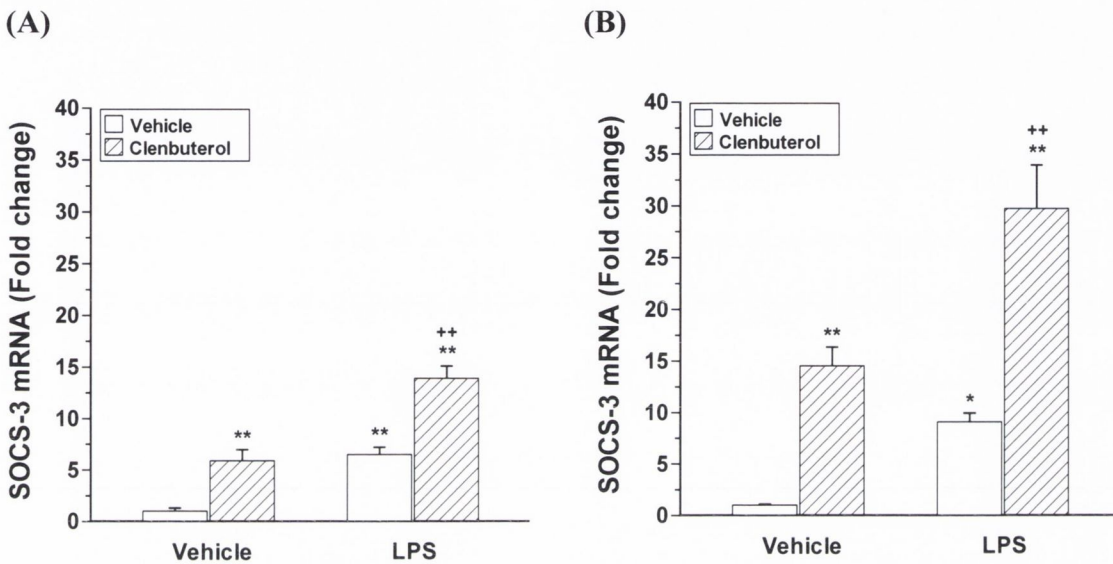


Figure 3.8.2. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol significantly enhanced SOCS-3 expression following an LPS challenge. Clenbuterol (0.5 mg/kg; i.p) or saline vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1 μ g/5 μ L; i.c.v) or saline vehicle. Rats were sacrificed 2 hours post LPS injection. Clenbuterol pre-treatment significantly enhanced SOCS-3 mRNA expression in cortex (A) and hippocampus (B) of LPS treated animals compared to LPS alone. Data expressed as mean + SEM ($n=6$). mRNA data expressed as fold-change vs. saline vehicle. * $p<0.05$, ** $p<0.01$ vs. saline vehicle; ++ $p<0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

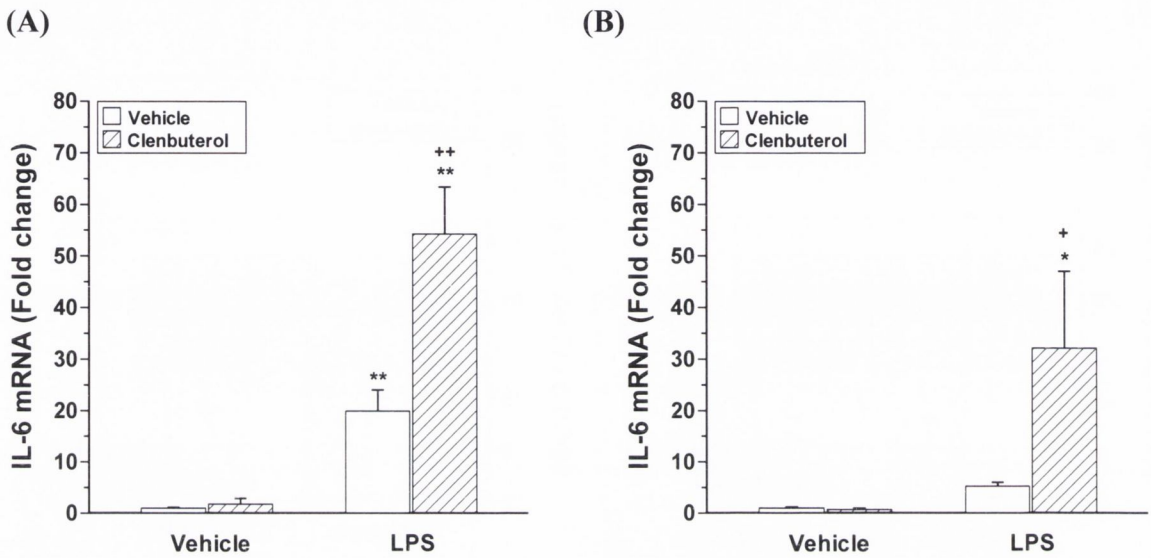


Figure 3.8.3. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol significantly enhanced IL-6 expression following an LPS challenge. Clenbuterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1 μ g/5 μ L; i.c.v) or vehicle (0.89% w/v saline; i.c.v). Rats were sacrificed 2 hours post LPS injection. While treatment with clenbuterol alone had no effect on IL-6 mRNA expression, pre-treatment with clenbuterol prior to LPS significantly enhanced IL-6 mRNA expression in cortex (A) and hippocampus (B), compared to LPS alone. Data expressed as mean + SEM ($n=6$). mRNA data expressed as fold-change vs. saline vehicle. * $p<0.05$, ** $p<0.01$ vs. saline vehicle; + $p<0.05$, ++ $p<0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

3.9 Effect of clenbuterol pre-treatment on LPS-induced activation of the IL-1 system.

Clenbuterol is known to induce IL-1 β and its negative regulators IL-1ra, IL-1RII when administered alone and following a peripheral LPS challenge. This section is novel in that it reports the effects on clenbuterol pre-treatment on the IL-1 system following a central LPS challenge.

Figure 3.9.1. The β_2 -adrenoceptor agonist clenbuterol has no effect on IL-1 β expression in rat brain.

ANOVA revealed a significant effect of LPS on IL-1 β mRNA expression in cortex [$F_{(1,18)} = 114.63, p < 0.0001$] and hippocampus [$F_{(1,18)} = 70.57, p < 0.0001$]. ANOVA revealed no effect of clenbuterol on IL-1 β mRNA in cortex [$F_{(1,18)} = 1.80, p = 0.1968$] or hippocampus [$F_{(1,18)} = 2.66, p = 0.1199$]. Consistent with these findings, *post hoc* analysis showed that clenbuterol pre-treatment had no effect on LPS-induced IL-1 β mRNA expression in cortex or hippocampus compared to LPS alone [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n = 5-6$].

Figure 3.9.2 A, B. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol increased IL-1ra mRNA expression, a negative regulator of IL-1 β , in rat brain.

ANOVA revealed a significant effect of clenbuterol and a significant effect of LPS on IL-1ra mRNA expression in cortex [clenbuterol: $F_{(1,19)} = 9.85, p = 0.0054$; LPS: $F_{(1,19)} = 26.9, p < 0.0001$] and hippocampus [clenbuterol: $F_{(1,19)} = 7.04, p = 0.0157$; LPS: $F_{(1,19)} = 12.79, p = 0.002$]. Consistent with these findings, *post hoc* analysis showed that clenbuterol pre-treatment significantly enhanced the LPS-induced IL-1ra mRNA expression in both cortex ($p < 0.05$) and hippocampus ($p < 0.05$), compared to LPS alone [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n = 5-6$].

Figure 3.9.2 C, D. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol increased IL-1RII mRNA expression, the decoy receptor for IL-1 β , in rat brain.

ANOVA revealed a significant effect of clenbuterol on IL-1RII mRNA expression in cortex [$F_{(1,19)} = 186.39, p < 0.0001$] and hippocampus [$F_{(1,19)} = 357.83, p < 0.0001$]. Consistent with these findings, *post hoc* analysis showed that clenbuterol alone caused a profound increase in IL-1RII mRNA in cortex ($p < 0.01$) and hippocampus ($p < 0.01$),

compared to saline vehicle. In addition, clenbuterol pre-treatment caused an equally profound increase in IL-1RII mRNA expression in LPS-treated animals in cortex ($p<0.01$) and hippocampus ($p<0.01$), compared to LPS alone [Two-way ANOVA followed by *post hoc* Newman-Keuls test; $n=5-6$].

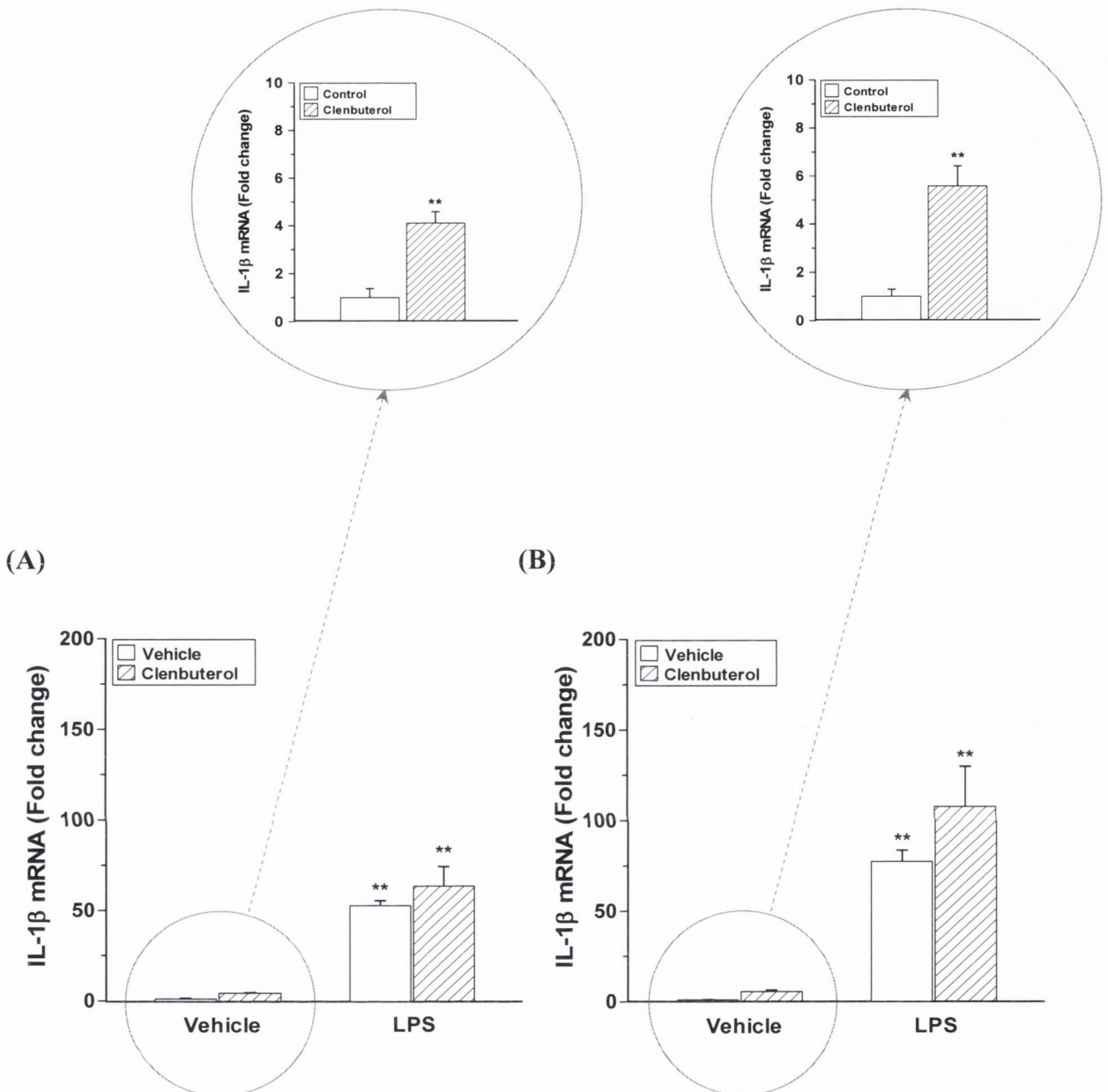


Figure 3.9.1. The β_2 -adrenoceptor agonist clenbuterol has no effect on IL-1 β expression in rat brain. Clenbuterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1 μ g/5 μ L; i.c.v) or vehicle (0.89% w/v saline; i.c.v). Rats were sacrificed 2 hours post LPS injection. Clenbuterol pre-treatment had no effect on LPS-induced IL-1 β mRNA in cortex (A) or hippocampus (B). However, clenbuterol alone induced IL-1 β mRNA expression compared to saline vehicle (shown in magnified upper panels). Data expressed as mean + SEM ($n=5-6$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test/Student's *t*-test for upper panels).

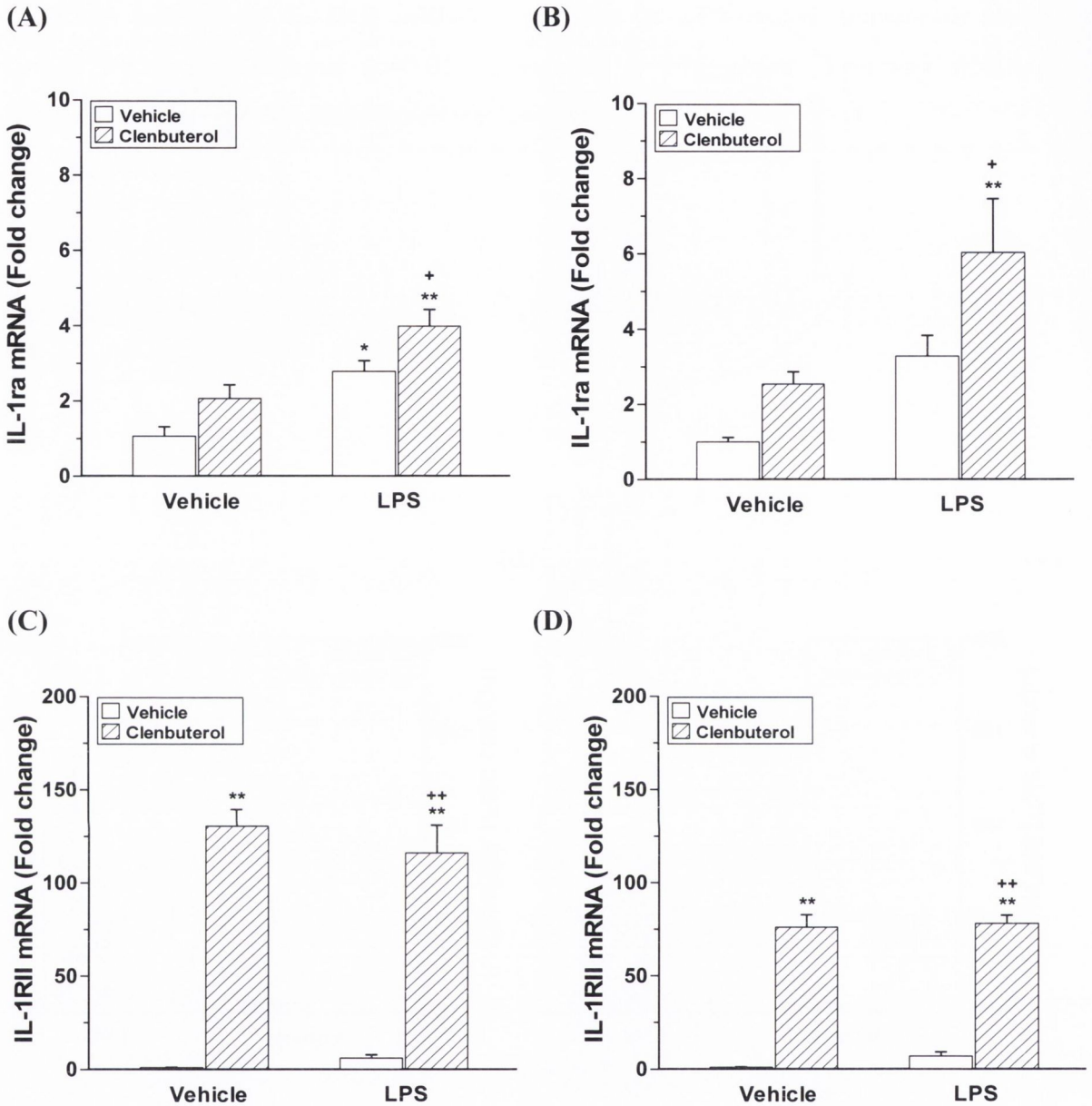


Figure 3.9.2. Pre-treatment with the β_2 -adrenoceptor agonist clenbuterol increased IL-1ra and IL-1RII, two negative regulators of IL-1 β , in rat brain. Clenbuterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered 1 hour prior to LPS (1 μ g/5 μ L; i.c.v) or vehicle (0.89% w/v saline; i.c.v). Rats were sacrificed 2 hours post LPS injection. Clenbuterol pre-treatment increased the LPS-induced expression of IL-1ra mRNA in cortex (A) and hippocampus (B) and IL-1RII mRNA in cortex (C) and hippocampus (D). Data expressed as mean + SEM ($n=5-6$). mRNA data expressed as fold-change vs. saline vehicle. * $p<0.05$, ** $p<0.01$ vs. saline vehicle; + $p<0.05$, ++ $p<0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman-Keuls test).

3.10 Discussion

3.10.1 The effects of clenbuterol on NF κ B and I κ B α in rat brain are time-dependent and dose-dependent

Due to its lipophilic properties, peripherally administered clenbuterol is capable of crossing the blood brain barrier and entering the CNS where it targets β_2 -adrenoceptors on glial cells and neurons. An increasing volume of literature supports the theory that central β -adrenoceptor stimulation leads to neuroprotective properties in animal models of neuroinflammation (see Chapter 1). A major pathway that is activated during inflammation as part of the host defence is the NF κ B pathway. NF κ B is a transcription factor that regulates key proteins involved in inflammation. As well as being present in the periphery NF κ B is also found in glial cells and neurons of the CNS (Sparacio *et al.*, 1992; Moynagh *et al.*, 1993; Ward & Hagg, 2000). In resting cells most NF κ B molecules reside in a latent form bound to the inhibitory I κ B proteins. When induced by signals such as TNF- α or LPS, I κ B becomes phosphorylated and subsequently degraded leaving NF κ B free to translocate to the nucleus where it activates numerous inflammatory genes. Our laboratory recently found that peripheral administration of the β_2 -adrenoceptor agonist clenbuterol resulted in increased I κ B α mRNA (McNamee *et al.*, 2010c) which is an indirect measure of NF κ B activation. It was unclear whether the clenbuterol-induced I κ B α mRNA was a result of NF κ B activation. Thus, the objective of the present study was to investigate the time course of NF κ B activity and I κ B α expression following clenbuterol administration.

Rat cortex and hippocampus were examined for NF κ B and I κ B expression 1, 4 and 8 hours following peripheral clenbuterol administration. In keeping with findings by McNamee *et al.*, (2010c) the present study found a robust and transient increase in inhibitory I κ B α mRNA and to a lesser extent I κ B β mRNA 1 hour post-clenbuterol injection. This was followed by a time-dependent increase in I κ B α protein expression at 4 hours which was mirrored by a time-dependent decrease in NF κ B activity at 4 hours. Thus, the increase in I κ B α protein expression coincided with the suppression of NF κ B activity, which suggests that the increased I κ B α mediates the clenbuterol-induced suppression of NF κ B activity. This was a significant finding because I κ B α mRNA can also be induced in response to NF κ B activation. The I κ B α promoter contains multiple

NF κ B binding sites which functionally induce I κ B α gene expression in response to NF κ B activation (Verma *et al.*, 1995) which is important for re-establishing the cytoplasmic pools of NF κ B/I κ B α complexes (Baldwin, 1996). Thus, increased I κ B α mRNA can also be an indicator of previous NF κ B activation. Importantly, the time course in the present study ruled out the possibility that clenbuterol induced NF κ B activity. In fact, the time course of events suggests that clenbuterol actually suppressed NF κ B activity.

Evidence in the literature shows that a range of doses of clenbuterol are capable of eliciting neuroprotective effects in the brain (Zhu *et al.*, 1999; Culmsee *et al.*, 1999a, b; Zhu *et al.*, 2001; Junker *et al.*, 2002; McNamee *et al.*, 2010b), but there is no evidence, to our knowledge, regarding the effect of varying doses on NF κ B activity. At higher doses clenbuterol is known to induce behavioural effects such as reduced locomotor activity and feeding (Goldschmidt *et al.*, 1984; Geyer & Frampton, 1988; O'Donnell, 1993). Thus the aim of this study was to examine whether a lower dose of clenbuterol could elicit the same effects on NF κ B:I κ B α signalling. The following three doses were chosen: 0.03, 0.1 and 0.3 mg/kg. The results demonstrated that clenbuterol acted in a dose-dependent manner. The 0.1 and 0.3 mg/kg doses effectively suppressed NF κ B activity and increased I κ B α expression. However, while the lowest dose of 0.03 mg/kg significantly suppressed NF κ B activity, it had no effect on I κ B α signalling 4 hours post-administration.

3.10.2 The β_2 -adrenoceptor plays a key role in mediating the effects of clenbuterol on NF κ B:I κ B α signalling in rat brain

Results from the previous studies showed that clenbuterol affected NF κ B:I κ B α signalling in a time- and dose-dependent manner. Our group have recently shown that the effects of clenbuterol on cytokine expression are mediated via the β -adrenoceptor and with the use of selective agonists and antagonists of β_1 - and β_2 -adrenoceptor subtypes the effects were shown to be specifically mediated by the β_2 -adrenoceptor subtype (Ryan, 2010; McNamee *et al.*, 2010a). Evidence in the literature also suggests that the neuroprotective effect of clenbuterol in astrocytes and neurons are mediated via the β_2 -adrenoceptor (Junker *et al.*, 2002; Culmsee *et al.*, 2007). However, clenbuterol is known to have some off-target effects such as modulation of ion channel function (Desaphy *et al.*, 2003). The aim of the present study was to determine whether the effects of clenbuterol on NF κ B:I κ B α signalling were also mediated specifically via the β_2 -subtype by using the

selective β_1 -adrenoceptor antagonist metoprolol, the non-selective β -adrenoceptor antagonist propranolol and the selective β_2 -adrenoceptor antagonist ICI-118,551.

The results from the present study show that the effects of clenbuterol on NF κ B activity and I κ B α expression in rat brain are mediated exclusively by the β_2 -adrenoceptor. In hippocampus and cortex, pre-treatment with the selective β_2 -adrenoceptor antagonist ICI-118,551 and the non-selective β -adrenoceptor antagonist propranolol prevented the clenbuterol-induced suppression of NF κ B activity. In contrast, pre-treatment with the selective β_1 -adrenoceptor antagonist metoprolol failed to reverse the clenbuterol-induced suppression of NF κ B activity, thereby supporting a specific role for the β_2 -adrenoceptor. Regarding I κ B α expression; pre-treatment with the selective β_2 -adrenoceptor antagonist ICI-118,551 and the non-selective β -adrenoceptor antagonist propranolol prevented the clenbuterol-induced increase in I κ B α expression while the selective β_1 -adrenoceptor antagonist metoprolol failed to reverse clenbuterol-induced I κ B α expression. Thus, the β_2 -subtype was found to mediate the effects of clenbuterol on I κ B α also.

These results highlight a role for the β_2 -adrenoceptor in the clenbuterol-induced suppression of NF κ B activity. We hypothesise that this is mediated by an increase in inhibitory I κ B α expression. While we found that β_2 -adrenoceptor stimulation by clenbuterol alone is capable of inducing I κ B α expression, others found that I κ B α was only induced when the β_1 - and β_2 -adrenoceptor agonist isoproterenol was used in combination with LPS and not when used alone (Farmer & Pugin, 2000). This is surprising because β -adrenoceptor stimulation results in increased cAMP (Ordway *et al.*, 1987) and this is thought to be the main signalling pathway by which the anti-inflammatory effects are mediated. Due to the discrepancy between our results and that of Farmer and Pugin (2000) a subsequent study was carried out to determine whether other β_2 -adrenoceptor agonists, namely formoterol and salbutamol, could elicit similar NF κ B:I κ B α effects.

3.10.3 Effect of the selective β_2 -adrenoceptor agonists formoterol and salbutamol on NF κ B activity and I κ B α expression

The results discussed above demonstrate that stimulation of the β_2 -adrenoceptor using clenbuterol suppresses NF κ B and this is accompanied by an increase in I κ B α . While

clenbuterol is a useful drug for the investigation of the anti-inflammatory effects of β -adrenoceptor stimulation, concerns have arisen regarding its safety due to undesirable effects on skeletal and cardiac muscle, and off-target effects on ion channel function (Desaphy *et al.*, 2003). The aim of the present study was to investigate whether two other β_2 -adrenoceptor agonists that have been used for the treatment of respiratory disorders could elicit similar effects to clenbuterol regarding NF κ B and I κ B α . Salbutamol is a short acting β_2 -adrenoceptor agonist while formoterol is similar to clenbuterol in that it is long acting.

The results from the present study demonstrate that both salbutamol and formoterol suppress NF κ B activity in rat cortex and hippocampus 4 hours post-administration. Interestingly, I κ B α was only increased by formoterol and not by salbutamol. Salbutamol is known to increase cAMP, albeit in cells of the periphery (Yang *et al.*, 2010) and cAMP is a known inducer of I κ B α (Neumann *et al.*, 1995). Therefore, it is surprising that salbutamol does not increase I κ B α here. One explanation could be due to salbutamol being a short-acting β_2 -agonist whereas formoterol is a long-acting β_2 -agonist (LABA). The clenbuterol time course study demonstrated that the clenbuterol-induced increase in I κ B α is transient, so perhaps the salbutamol-induced increase in I κ B α was missed due to the single 4 hour time point used here. Alternatively, salbutamol may suppress NF κ B via another mechanism. For example, SOCS-1 can bind directly to the p65 subunit of NF κ B and facilitate its degradation (Ryo *et al.*, 2003). Therefore, further study is warranted to investigate the effects of salbutamol on SOCS-1 expression.

The formoterol results complement other data in the literature. The anti-inflammatory effects of formoterol have previously been demonstrated both in the periphery (Donnelly *et al.*, 2010) as well as in the CNS (McNamee *et al.*, 2010a and 2010c). The results of the present study support earlier findings from our group which demonstrate a formoterol-induced increase in I κ B α mRNA (McNamee *et al.*, 2010c). Furthermore, the present study also found that I κ B α protein was increased. This supports the hypothesis that increased I κ B α is likely to be responsible for the formoterol-induced NF κ B suppression.

3.10.4 LPS-induced inflammation can be attenuated by pre-treatment with clenbuterol in the rat brain

The effects of clenbuterol have so far been evaluated in naive rats. Thus, the objective of the present study was to examine the effects of clenbuterol in a neuroinflammatory environment. Inflammation in the brain involves activation of microglia and production of cytokines and reactive oxygen species (Haus-Wegrzyniak *et al.*, 1998). Most of the inflammatory processes in the brain can be mimicked by treatment with LPS, a method which is widely used in rodents to investigate CNS inflammatory processes. In the present study, LPS was administered via intracerebroventricular (i.c.v.) injection into the lateral ventricle due to its ability to induce widespread inflammation in various regions of the rat brain (Renton *et al.*, 1999).

Initially, a small pilot study was carried out whereby rats received an i.c.v. injection of LPS and were sacrificed at two time points. This study demonstrated that the i.c.v. technique employed was capable of inducing NF κ B activity in the brain regions of interest, namely cortex and hippocampus. In particular, it demonstrated that NF κ B activity was significantly increased 2 hours post LPS injection. This 2 hour time point was used in the subsequent experiment to determine the effect of clenbuterol pre-treatment on LPS-induced NF κ B activity, whereby rats were pre-treated with clenbuterol prior to central LPS administration and sacrificed 2 hours post-LPS administration. Results demonstrate that clenbuterol successfully blocked LPS-induced NF κ B activity in both cortex and hippocampus. Clenbuterol also blocked the NF κ B-inducible genes TNF- α and ICAM-1. This effect is likely to be due to reduced activation of NF κ B. A similar result was found by Farmer and Pugin (2000) using the β -adrenoceptor agonist isoproterenol in human monocytic cells. However, to the best of our knowledge, this is the first time this has been demonstrated in an *in vivo* rat model of brain inflammation.

Western blotting demonstrated that LPS increased phosphorylation of I κ B α protein thereby depleting total-I κ B α protein in cortex and hippocampus. Clenbuterol pre-treatment prevented the LPS-induced phosphorylation of I κ B α and prevented the depletion of total-I κ B α protein. In contrast, LPS caused an increase in I κ B α mRNA both independently and with clenbuterol pre-treatment. However, the effects of LPS on I κ B α mRNA and protein are not surprising. It has been well documented that LPS treatment

leads to phosphorylation and degradation of I κ B α protein, thus freeing NF κ B and allowing it to translocate to the nucleus where it induces transcription of pro-inflammatory genes (Karin & Ben-Neriah, 2000). In addition, LPS-induced NF κ B activation increases I κ B α mRNA due to the presence of a transcriptional binding site for I κ B on the NF κ B promoter. This is a critical inhibitory step. Newly synthesised cytoplasmic I κ B α translocates to the nucleus where it terminates the activity of NF κ B by transporting it back to the cytoplasm (Arenzana-Seisdedos *et al.*, 1997 and 1997). Therefore, the proposed hypothesis is that clenbuterol increases newly synthesised I κ B α mRNA, which translocates to the nucleus thereby blocking LPS-induced NF κ B activation. This is a plausible explanation as even a small (2-fold) increase in cytoplasmic I κ B α is known to substantially inhibit NF κ B activation (Miyamoto *et al.*, 1994).

We propose that clenbuterol increases I κ B α by the following mechanisms: Clenbuterol can activate cAMP via the β_2 -adrenoceptor (Ordway *et al.*, 1987) and cAMP activity has been shown to suppress the immune response (Rappaport & Dodge, 1982; Hasler *et al.*, 1983). A downstream signalling molecule of cAMP called protein kinase-A (PKA) inhibits nuclear translocation of NF κ B p65 by stabilisation of I κ B α and blocking its phosphorylation in T-cells (Neumann *et al.*, 1995). Thus, it is likely that clenbuterol suppresses LPS-induced NF κ B activity by preventing phosphorylation of I κ B α via PKA. However, the present study also demonstrated *de novo* synthesis of I κ B α mRNA. Previously published data suggests that β_2 -agonists may activate the intracellular glucocorticoid receptor which is known for eliciting anti-inflammatory properties and is capable of inducing the I κ B α gene (Auphan *et al.*, 1995; Scheinman *et al.*, 1995; Eickelberg *et al.*, 1999). Thus, clenbuterol may increase I κ B α via two mechanisms: stabilisation of I κ B α by inhibiting its phosphorylation and *de novo* synthesis of I κ B α through induction of gene transcription (**Figure 3.1**). To our knowledge, this is the first time that the β_2 -adrenoceptor clenbuterol has been shown to suppress LPS-induced NF κ B activity *in vivo*.

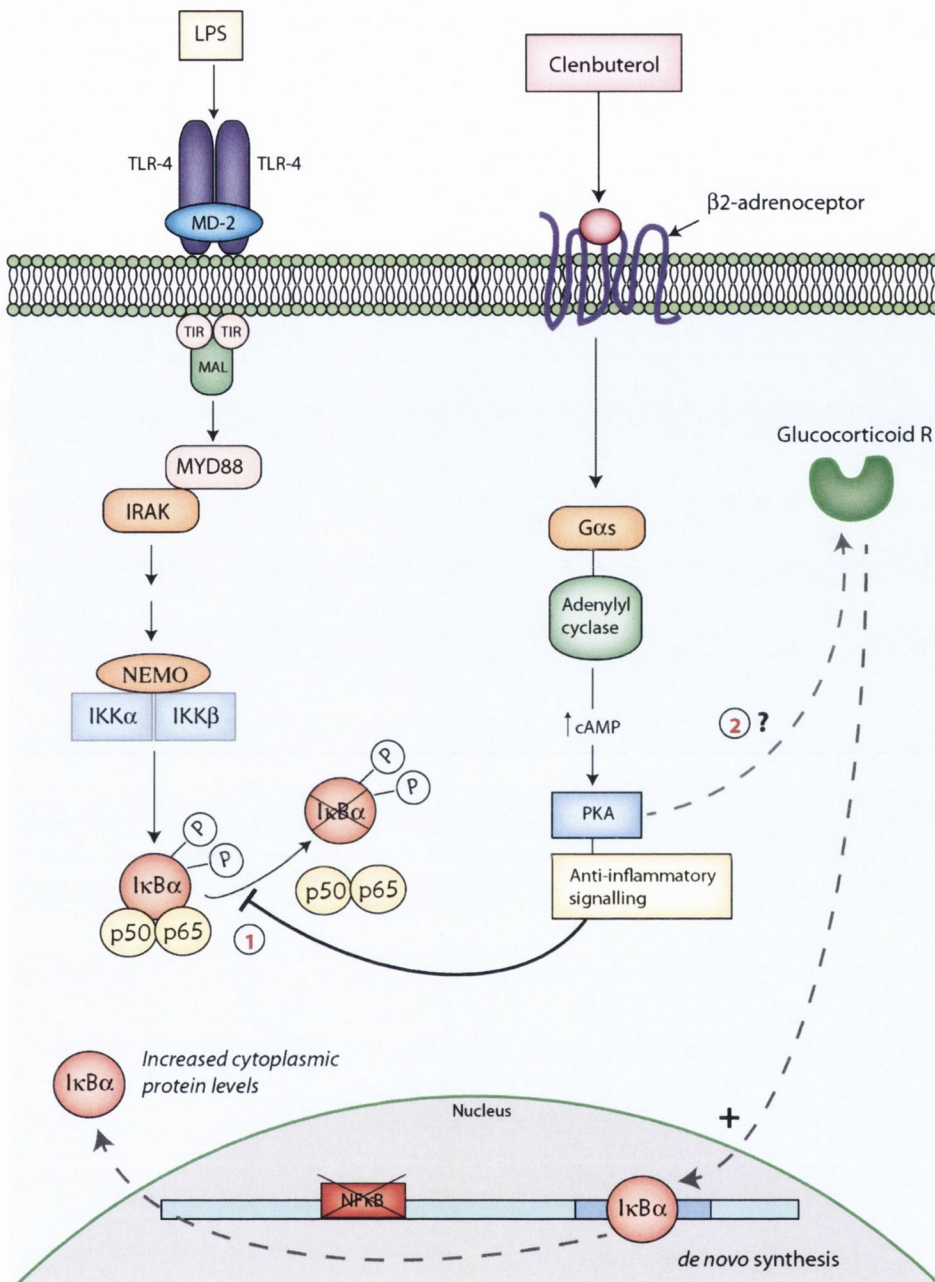


Figure 3.1: Proposed mechanism by which clenbuterol suppresses LPS-induced NFκB p65 activity. 1) Clenbuterol-induced activation of PKA may block phosphorylation of IκBα thereby preventing translocation of NFκB to the nucleus and its activation. 2) PKA-induced activation of the glucocorticoid receptor may be partly responsible for the clenbuterol-induced increase in IκBα mRNA.

3.10.5 Clenbuterol-induced IL-10 signalling – a possible inhibitor of NFκB activity?

Both noradrenaline and β -adrenoceptor agonists have been shown to induce expression of the broad-spectrum anti-inflammatory cytokine IL-10 and its downstream signalling molecule SOCS-3 (Szabo *et al.*, 1997; Curtin *et al.*, 2009; McNamee *et al.*, 2010a). Recently work in our group demonstrated that clenbuterol induces IL-10 and SOCS-3 mRNA in rat cortex at basal levels and following peripheral LPS administration (Ryan, 2010). Results from the present study demonstrate similar results whereby IL-10 and SOCS-3 expression are significantly enhanced when clenbuterol is administered prior to central LPS. In addition, the present study shows that STAT-3 phosphorylation is induced by clenbuterol alone and to an even greater extent following pre-treatment prior to LPS. STAT-3 phosphorylation is essential for the anti-inflammatory actions of IL-10 and for induction of SOCS-3, and several groups have reported that IL-10 is capable of reducing NFκB activity through prevention of IκBα phosphorylation and by preventing the binding of NFκB subunits to its binding motif (Shames *et al.*, 1998; Schottelius *et al.*, 1999). Schottelius *et al.* (1999) show that IL-10 acts by blocking the IKK molecule upstream of IκBα (see Chapter 1 for NFκB signalling pathway). To further support the inhibitory role of IL-10, a study showed that pre-treatment of dendritic cells with IL-10 prevented LPS-induced NFκB activity (Bhattacharyya *et al.*, 2004). As previously described, clenbuterol prevents IκBα phosphorylation in rat brain following LPS exposure and it is possible that IL-10 is a key mediator of this process. However, the literature above describes *in vitro* studies based on cells of the periphery. Further work using selective IL-10 inhibitors is certainly warranted, in order to verify the role of clenbuterol-induced IL-10 in mediating the suppression of NFκB activity in the CNS (**Figure 3.2**).

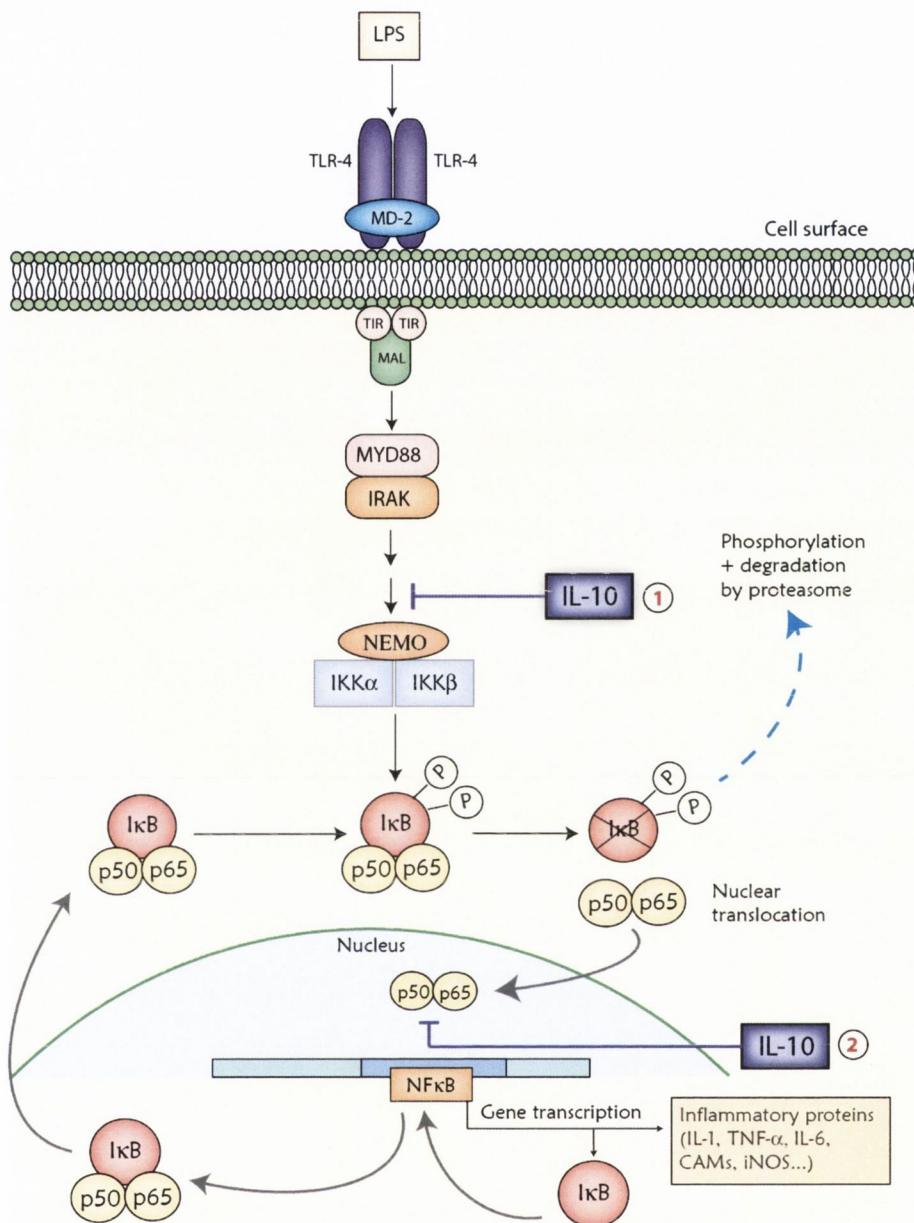


Figure 3.2: IL-10-induced inhibition of NFκB activity. IL-10 may inhibit the NFκB pathway at at least two levels: 1) inhibition of IKK activation leading to reduced phosphorylation of IκBα and 2) preventing the binding of NFκB subunits to their binding motif. Both of which may serve to inhibit NFκB activity [amended from (Williams *et al.*, 2004)].

3.10.6 Clenbuterol-induced IL-6 signalling – a beneficial effect?

Along with TNF- α and IL-1 β , IL-6 is one of the early cytokines released in response to injury and is a double-edged sword with regard the role it plays in the brain. As well as its reported proinflammatory characteristics there is a growing body of literature demonstrating that IL-6 can actually provide neuroprotection, stimulate neurotrophic factor release and promote neuronal survival (Gadient & Otten, 1997; Loddick *et al.*, 1998). Both the IL-10 receptor (IL-10R) and IL-6 receptor (IL-6R) can activate STAT-3 and SOCS-3 however, the IL-10R is thought to induce anti-inflammatory genes while IL-6R induces pro-inflammatory genes. Unfortunately, it is very difficult to distinguish between IL-10R-activated and IL-6R-activated STAT-3 (Murray, 2007). Both IL-10 and IL-6 are increased following LPS and further-enhanced with clenbuterol pre-treatment, so how can we be certain the observed increase in STAT-3 is induced by IL-10 or by IL-6 in the present study. We hypothesise that it is IL-10-mediated due to the fact that it has been shown repeatedly in our laboratory that clenbuterol and other β_2 -adrenoceptor agonists increase IL-10 expression (Ryan, 2010; McNamee *et al.*, 2010a) and have no effect on IL-6, in fact in some cases basal levels of IL-6 are reduced by clenbuterol.

Therefore, the source of increased IL-6 in clenbuterol plus LPS-treated animals is unknown and requires further investigation. The mechanism certainly involves LPS as clenbuterol alone has no effect on IL-6. It is important to note that the increased IL-6 could actually have beneficial effects in the brain. IL-6 has been found to be neuroprotective in neurons exposed to glutamate neurotoxicity (Ali *et al.*, 2000). It can reduce the infarct volume in a focal cerebral ischemia model (Herrmann *et al.*, 2003) and it can enhance the differentiation and survival of primary neurons (Horton *et al.*, 1998). Moreover, current *in vitro* data from our group demonstrates that noradrenaline-induced IL-6 from glial cells increases the number and length of neuritic branches from primary cortical neurons (Day *et al.*, 2011 unpublished). Thus, the increase in IL-6 seen in the present study may contribute to the beneficial and neuroprotective properties of β_2 -adrenoceptor stimulation.

3.10.7 Clenbuterol pre-treatment activates negative regulators of IL-1 β during inflammation

In line with previous studies (McNamee *et al.*, 2010a), clenbuterol independently increased the expression of IL-1ra and IL-1RII in rats. However, this is the first demonstration that clenbuterol pre-treatment increases IL-1ra following a central administration of LPS. Despite the ability of clenbuterol to induce the anti-inflammatory molecules IL-1ra, IL-1RII and IL-10, clenbuterol also independently increased IL-1 β while having no effect on LPS-induced IL-1 β expression. While IL-1 β is typically known to be a pro-inflammatory cytokine (Rothwell & Luheshi, 2000), there is a growing body of evidence suggesting that its expression could also have beneficial consequences. For example, IL-1 can induce synthesis of growth factors like NGF, and other cytokines such as IL-10, IL-4, IL-1ra and TGF- β which are anti-inflammatory in nature (Touzani *et al.*, 1999). IL-1ra and TGF- β in particular have been demonstrated to exert neuroprotective effects against ischaemic insult (Relton & Rothwell, 1992; Buisson *et al.*, 1998). Notably, while clenbuterol has been shown to increase the expression of IL-1 β in the rat brain and induce activation of ERK1/2 it fails to activate the pro-apoptotic MAPKs, JNK or p38 (Heffernan, 2009 unpublished) and there is no evidence of expression of other inflammatory cytokines like TNF- α , IFN- γ and IL-6 (Ryan, 2010; McNamee *et al.*, 2010c). In addition, increased expression of IL-1ra and IL-1RII prevents prolonged activation of the IL-1 system and thus may be beneficial in combating any deleterious effects of IL-1 β on brain function. Taking all of this evidence in to account, we suggest that clenbuterol-induced IL-1 β does not play a detrimental role in the brain.

3.10.8 Summary

The results presented here demonstrate that peripheral administration of clenbuterol reduces NF κ B activity in rat cortex and hippocampus and this occurs in a time- and dose-dependent manner. In tandem with this, clenbuterol increases the inhibitor of NF κ B, I κ B α . These effects are mediated, unsurprisingly, by the β_2 -adrenoceptor rather than the β_1 -adrenoceptor. Importantly, these effects are not limited to clenbuterol as two other β_2 -adrenoceptor agonists, formoterol and salbutamol, also suppress NF κ B; although effects of salbutamol appear to be independent of I κ B α . Clenbuterol is also capable of suppressing NF κ B in an LPS-induced inflammatory environment. We hypothesise that

the suppression of NF κ B is mediated by increased I κ B α caused by reduced I κ B α phosphorylation. Clenbuterol also increases expression of the broad spectrum anti-inflammatory cytokine IL-10 and its downstream signalling molecules STAT-3 and SOCS-3. As IL-10 is known to reduce I κ B α phosphorylation, clenbuterol-induced IL-10 is a possible mediator of reduced I κ B α phosphorylation. While clenbuterol had no effect on LPS-induced IL-1 β it induced the negative regulator of IL-1 β , IL-1ra. Given the ability of glucocorticoids to suppress IL-1 β expression in the CNS the next chapter examines the effect of the glucocorticoid dexamethasone on clenbuterol-induced IL-1 β expression and also investigates the ability of the two agents to elicit synergistic anti-inflammatory effects in the rat brain.

Chapter 4

*Combined treatment with the
 β_2 -adrenoceptor agonist clenbuterol
and the glucocorticoid dexamethasone
induces a synergistic anti-inflammatory
cytokine phenotype in rat brain*

4.1 Introduction

The pro-inflammatory cytokine interleukin-1 β (IL-1 β) has been implicated as a mediator of neuroinflammation (see Allan *et al.*, 2005) and excessive production of IL-1 β , particularly in the context of microglial activation, has been implicated in the pathophysiology of neurodegenerative states such as Alzheimer's disease, Parkinson's disease and ischaemic stroke (Allan *et al.*, 2005; Block *et al.*, 2007; Heneka & O'Banion, 2007). IL-1 β signals by binding to the IL-1 type I receptor (IL-1RI) and association of the IL-1RI with its accessory protein, and this signalling system is present in the CNS (Ericsson *et al.*, 1995). The actions of IL-1 β can be regulated by interleukin-1 receptor antagonist (IL-1ra), which prevents IL-1 β from acting on the IL-1 type I receptor (IL-1RI) (Carter *et al.*, 1990), and by the IL-1 type II receptor (IL-1RII); a decoy receptor that serves to sequester IL-1 (Colotta *et al.*, 1994). These molecules prevent excessive or prolonged activation of the IL-1 system (Bessis *et al.*, 2000; Arend, 2002), thus increased expression of these two molecules may serve to combat the deleterious effects of IL-1 β on brain function.

IL-10 is a broad spectrum anti-inflammatory cytokine and has neuroprotective properties in animal models of neurodegenerative disease (Bethea *et al.*, 1999; Arimoto *et al.*, 2007; de Bilbao *et al.*, 2009; Zhou *et al.*, 2009). IL-10 inhibits expression of a range of inflammatory molecules in glial cells both *in vitro* and *in vivo* (Ledeboer *et al.*, 2000; Molina-Holgado *et al.*, 2001; Qin *et al.*, 2006; Park *et al.*, 2007), thereby preventing the development of neuroinflammation which can drive the neurodegenerative process. Independent of its anti-inflammatory actions IL-10 has direct trophic actions on neurons and inhibits glutamate-induced neurotoxicity (Bachis *et al.*, 2001; Boyd *et al.*, 2003; Zhou *et al.*, 2009). IL-10 receptor activation results in downstream induction of suppressor of cytokine signalling-3 (SOCS-3); a molecule that acts to inhibit Jak/STAT-dependent signalling of inflammatory cytokines including TNF- α , IL-6 and IFN- γ (Starr *et al.*, 1997; Donnelly *et al.*, 1999). Functional studies demonstrate that SOCS-3 mediates some of the anti-inflammatory actions of IL-10 in glial cells (see Baker *et al.*, 2009) and plays a protective role in animal models of multiple sclerosis and cerebral ischaemia (Raghavendra Rao *et al.*, 2002; Li *et al.*, 2006).

There is a growing interest in central β_2 -adrenoceptors as a neuroprotective target due to the fact that stimulation of β_2 -adrenoceptors promotes an anti-inflammatory phenotype in glial cells (Culmsee *et al.*, 1999a, b; Hertz *et al.*, 2004; McNamee *et al.*, 2010a, b). Clenbuterol is a brain penetrant β_2 -adrenoceptor agonist used in the treatment of respiratory disorders including asthma and chronic obstructive pulmonary disease (Baronti *et al.*, 1980; Papiris *et al.*, 1986; Boner *et al.*, 1988), and has neuroprotective properties both *in vitro* and *in vivo* (Culmsee *et al.*, 1999a, b). Specifically, clenbuterol has neuroprotective actions in rodent models of cerebral ischaemia (Semkova *et al.*, 1996; Zhu *et al.*, 1998; Culmsee *et al.*, 1999b; Junker *et al.*, 2002) and in both *in vitro* and *in vivo* models of excitotoxicity (Semkova *et al.*, 1996; Gleeson *et al.*, 2010). We recently demonstrated that clenbuterol induces expression of IL-1 β and its negative regulators IL-1ra and IL-1RII in rat brain, and suppresses expression of other inflammatory cytokines including TNF- α and IL-6 (McNamee *et al.*, 2010a). Clenbuterol also induces expression of IL-10 and its downstream signalling molecule SOCS-3 in rat brain (McNamee *et al.*, 2010b). Moreover, clenbuterol suppresses the CNS inflammatory response following administration of the excitotoxin kainic acid (Gleeson *et al.*, 2010) and following a systemic challenge with bacterial lipopolysaccharide (McNamee *et al.*, 2010a). Considering this evidence we conclude that clenbuterol drives an anti-inflammatory phenotype in rat brain.

As the anti-inflammatory agent dexamethasone can inhibit cytokine production from glial cells (Nishida *et al.*, 1989; Velasco *et al.*, 1991; Chao *et al.*, 1992; Kimberlin *et al.*, 1995), we determined if dexamethasone could inhibit activation of the IL-1 system induced by β_2 -adrenoceptor activation in rat brain. Specifically, we examined the impact of dexamethasone pre-treatment on clenbuterol-induced IL-1 β , IL-1ra and IL-1RII expression, and on activation of NF κ B and induction of the IL-1 β -inducible gene inducible nitric oxide synthase (iNOS). In addition, as evidence indicates that glucocorticoids can increase IL-10 production (Mozo *et al.*, 2004; Xia *et al.*, 2005) we considered the possibility that dexamethasone may further enhance the clenbuterol-induced increase in central IL-10 and SOCS-3 expression. Thus the overall objective of this study was to determine if a combined treatment with the β_2 -adrenoceptor agonist clenbuterol and the glucocorticoid dexamethasone could elicit complementary anti-inflammatory actions in rat brain.

4.2 Methods

4.2.1 Animal handling

Male Sprague-Dawley rats (250-300g) were obtained from Harlan Laboratories, UK. Rats were housed 4 per cage in an air-conditioned room with a 12:12-h light/dark cycle (lights on at 8am). Food and water were available *ad libitum*. Animals were handled daily for at least seven days prior to drug administration.

4.2.2 Experimental procedures

Clenbuterol was dissolved in 0.89% (w/v) saline while dexamethasone (dex) was dissolved in 0.89 % (w/v) saline containing 0.2% (v/v) Tween-20. Drugs were administered i.p. at the following doses: clenbuterol 0.5 mg/kg, dexamethasone 1 mg/kg. Rats were sacrificed 4 hours post-clenbuterol injection. Tissue was prepared for RT-PCR and nuclear extracts were prepared for examination of NF κ B activity. A detailed description of experimental procedures and molecular techniques can be found in Chapter 2.

4.3 Dexamethasone blocks the clenbuterol-induced increase in IL-1 β and iNOS expression, without altering induction of IL-1ra and IL-1RII

As previously discussed, systemic administration of the β_2 -adrenoceptor agonist clenbuterol is known to induce expression of IL-1 β and its negative regulators IL-1ra and IL-1RII in rat brain. As the anti-inflammatory agent dexamethasone can inhibit cytokine production from glial cells the purpose of this study was to determine if dexamethasone could inhibit activation of the IL-1 system induced by β_2 -adrenoceptor activation.

Figure 4.3.1. Dexamethasone blocks the ability of clenbuterol to induce expression of IL-1 β in rat brain.

ANOVA revealed a significant dexamethasone \times clenbuterol interaction for IL-1 β mRNA expression in cortex [$F_{(1,16)} = 18.88, p=0.0005$], hippocampus [$F_{(1,15)} = 26.44, p=0.0001$] and striatum [$F_{(1,15)} = 28.02, p<0.0001$]. Consistent with these findings, the clenbuterol-induced increase in IL-1 β mRNA expression was significantly attenuated by pre-treatment with dexamethasone in cortex ($p<0.01$), hippocampus ($p<0.01$) and striatum ($p<0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=4-5$].

Figure 4.3.2. Dexamethasone blocks the ability of clenbuterol to induce expression of iNOS in rat brain.

ANOVA revealed a significant dexamethasone \times clenbuterol interaction for iNOS mRNA expression in cortex [$F_{(1,16)} = 26.96, p<0.0001$], hippocampus [$F_{(1,15)} = 16.43, p=0.001$] and striatum [$F_{(1,15)} = 108.62, p<0.0001$]. Consistent with these findings, the clenbuterol-induced increase in iNOS mRNA expression was significantly attenuated by pre-treatment with dexamethasone in cortex ($p<0.01$), hippocampus ($p<0.01$) and striatum ($p<0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=4-5$].

Figure 4.3.3. Dexamethasone does not alter the ability of clenbuterol to induce IL-1ra expression in rat brain.

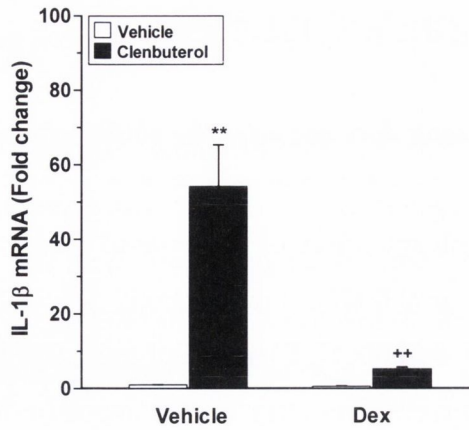
ANOVA revealed a significant effect of clenbuterol on IL-1ra mRNA expression in cortex [$F_{(1,16)} = 17.08, p=0.0008$], hippocampus [$F_{(1,15)} = 83.27, p<0.0001$] and striatum [$F_{(1,15)} = 45.68, p<0.0001$]. Clenbuterol treatment independently increased IL-1ra mRNA expression in cortex ($p<0.05$), hippocampus ($p<0.01$) and striatum ($p<0.01$), compared to saline vehicle. Pre-treatment with dexamethasone failed to alter the ability of clenbuterol

to induce IL-1ra expression in cortex, hippocampus or striatum [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=4-5$].

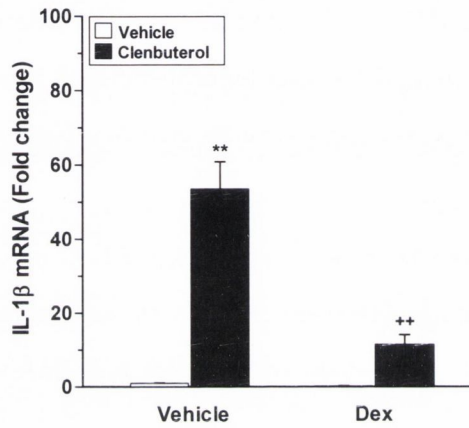
Figure 4.3.4. Dexamethasone does not alter the ability of clenbuterol to induce IL-1RII expression in rat brain.

ANOVA revealed a significant effect of clenbuterol on IL-1RII mRNA expression in cortex [$F_{(1,16)} = 246.16$, $p < 0.0001$], hippocampus [$F_{(1,15)} = 154.38$, $p < 0.0001$] and striatum [$F_{(1,15)} = 1013.80$, $p < 0.0001$]. Clenbuterol treatment independently increased IL-1RII mRNA expression in cortex ($p < 0.01$), hippocampus ($p < 0.01$) and striatum ($p < 0.01$), compared to saline vehicle. Pre-treatment with dexamethasone failed to alter the ability of clenbuterol to induce IL-1RII expression in cortex or striatum. In fact, dexamethasone pre-treatment further augmented the clenbuterol-induced increase in IL-1RII mRNA in hippocampus [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=4-5$].

(A) Cortex



(B) Hippocampus



(C) Striatum

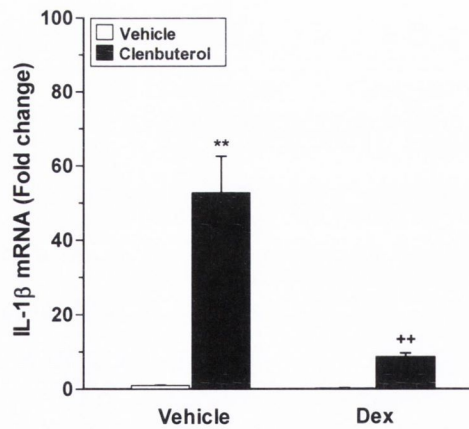
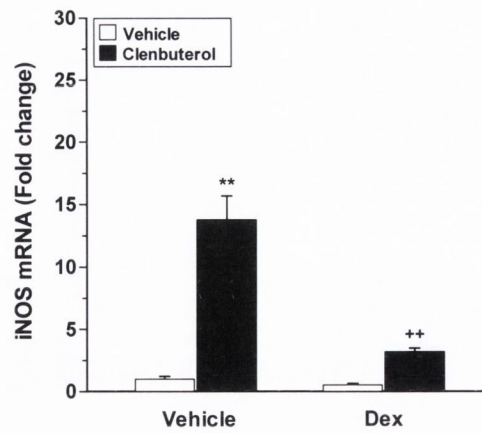
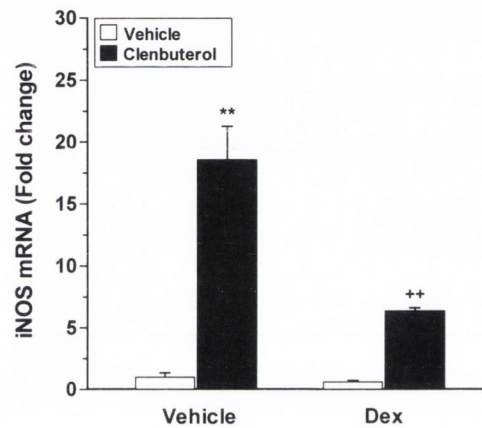


Figure 4.3.1. Dexamethasone blocks the ability of clenbuterol to induce expression of IL-1 β in rat brain. Rats were pre-treated with dexamethasone (1 mg/kg; i.p) 1 hour prior to clenbuterol (0.5 mg/kg; i.p) treatment. Control animals received a saline vehicle. Rats were sacrificed 4 hours post clenbuterol injection. Dexamethasone attenuated the clenbuterol-induced increase in IL-1 β mRNA expression in cortex (A), hippocampus (B) and striatum (C). Data expressed as mean + SEM ($n=4-5$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle, ++ $p<0.01$ vs. clenbuterol (two-way ANOVA followed by *post-hoc* Newman-Keuls test).

(A) Cortex



(B) Hippocampus



(C) Striatum

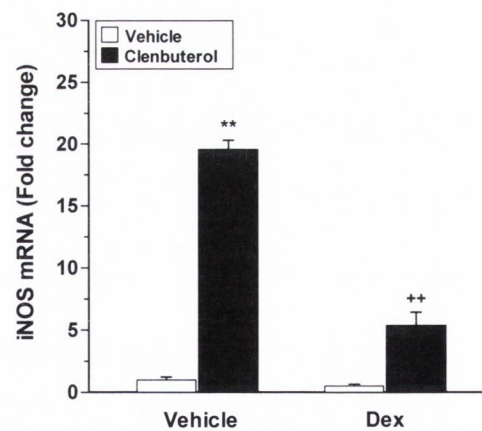
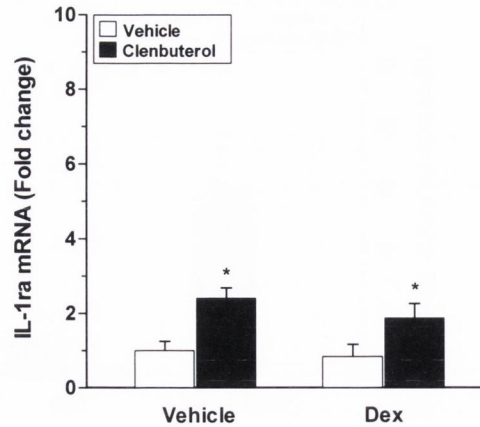
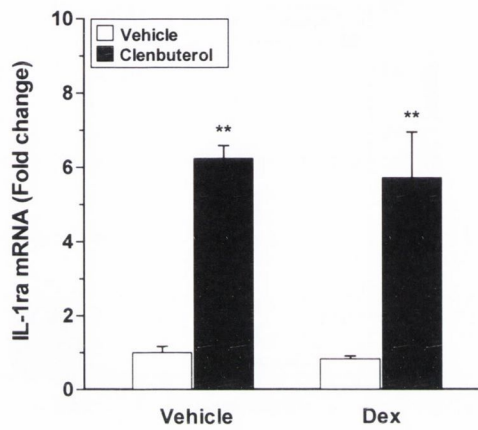


Figure 4.3.2. Dexamethasone blocks the ability of clenbuterol to induce expression of iNOS in rat brain. Rats were pre-treated with dexamethasone (1 mg/kg; i.p) 1 hour prior to clenbuterol (0.5 mg/kg; i.p) treatment. Control animals received a saline vehicle. Rats were sacrificed 4 hours post clenbuterol injection. Dexamethasone attenuated the clenbuterol-induced increase in iNOS mRNA expression in cortex (A), hippocampus (B) and striatum (C). Data expressed as mean + SEM ($n=4-5$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle, ++ $p<0.01$ vs. clenbuterol (two-way ANOVA followed by *post-hoc* Newman-Keuls test).

(A) Cortex



(B) Hippocampus



(C) Striatum

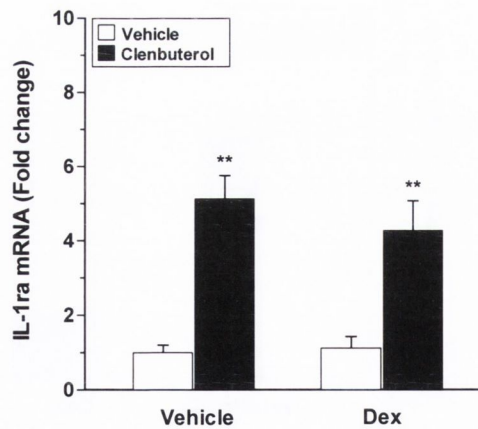
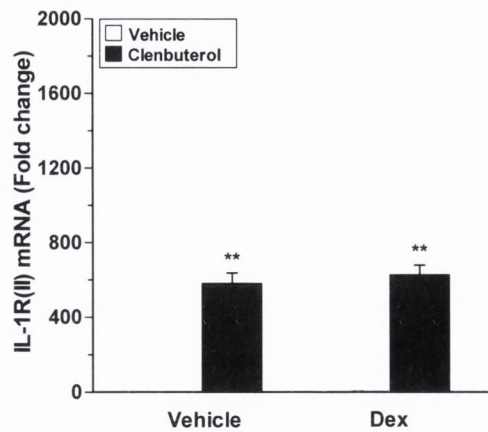
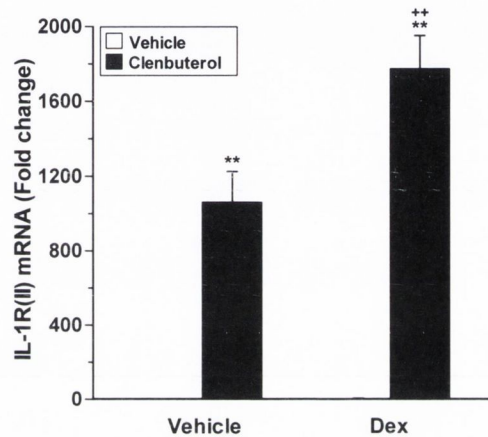


Figure 4.3.3. Dexamethasone does not alter the ability of clenbuterol to induce IL-1ra expression. Rats were pre-treated with dexamethasone (1 mg/kg; i.p) 1 hour prior to clenbuterol (0.5 mg/kg; i.p) treatment. Control animals received a saline vehicle. Rats were sacrificed 4 hours post clenbuterol injection. Dexamethasone failed to alter the ability of clenbuterol to induce IL-1ra mRNA expression in cortex (A), hippocampus (B) or striatum (C). Data expressed as mean + SEM ($n=4-5$). mRNA data expressed as fold-change vs. saline vehicle. * $p<0.05$, ** $p<0.01$ vs. saline vehicle (two-way ANOVA followed by *post-hoc* Newman-Keuls test).

(A) Cortex



(B) Hippocampus



(C) Striatum

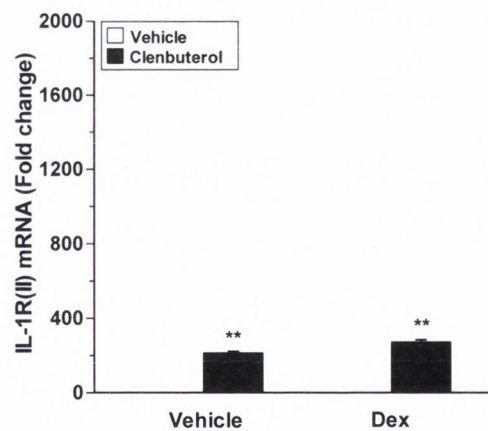


Figure 4.3.4. Dexamethasone largely does not alter the ability of clenbuterol to induce IL-1RII expression in rat brain. Rats were pre-treated with dexamethasone (1 mg/kg; i.p) 1 hour prior to clenbuterol (0.5 mg/kg; i.p) treatment. Control animals received a saline vehicle. Rats were sacrificed 4 hours post clenbuterol injection. Dexamethasone had no effect on the ability of clenbuterol to induce IL-1RII mRNA in cortex (A) or striatum (C) and in fact further augmented the increase in IL-1RII in hippocampus (B). Data expressed as mean + SEM ($n=4-5$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle; ++ $p<0.01$ vs. clenbuterol (two-way ANOVA followed by *post-hoc* Newman-Keuls test).

4.4 Effect of pre-treatment with the glucocorticoid dexamethasone on clenbuterol-induced suppression of NFκB p65 activity and induction of IκBα expression

Figure 4.4 A-C. Dexamethasone and clenbuterol induce a synergistic increase in IκBα expression in rat brain.

ANOVA revealed a significant effect of dexamethasone (dex) and of clenbuterol (clen) on IκBα mRNA expression in all three brain regions: cortex [dex: $F_{(1,16)} = 56.41$, $p < 0.001$; clen: $F_{(1,16)} = 24.16$, $p < 0.001$]; hippocampus [dex: $F_{(1,15)} = 75.6$, $p < 0.001$; clen: $F_{(1,15)} = 19.72$, $p < 0.001$]; striatum [dex: $F_{(1,15)} = 25.43$, $p < 0.001$; clen: $F_{(1,15)} = 8.76$, $p < 0.01$]. Consistent with these results, both clenbuterol and dexamethasone independently increased IκBα mRNA expression in all three regions compared to saline vehicle ($p < 0.05$ - $p < 0.01$). Notably, dexamethasone pre-treatment markedly enhanced the clenbuterol-induced IκBα mRNA expression in all three regions ($p < 0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=4-5$].

Figure 4.4 D-F. While both dexamethasone and clenbuterol suppress NFκB activity, treatment with these two agents together does not result in further suppression of NFκB activity in rat brain.

ANOVA revealed a significant dexamethasone × clenbuterol interaction for NFκB p65 DNA binding (NFκB activity) in cortex [$F_{(1,16)} = 4.43$, $p = 0.05$], hippocampus [$F_{(1,15)} = 26.63$, $p < 0.001$] and striatum [$F_{(1,16)} = 29.81$, $p < 0.001$]. Consistent with these results, both clenbuterol and dexamethasone independently suppressed NFκB activity in all three regions compared to saline vehicle, as did combined treatment with the two agents ($p < 0.05$ - $p < 0.01$). However, combined treatment did not result in a further suppression of NFκB activity compared to clenbuterol alone [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=4-5$].

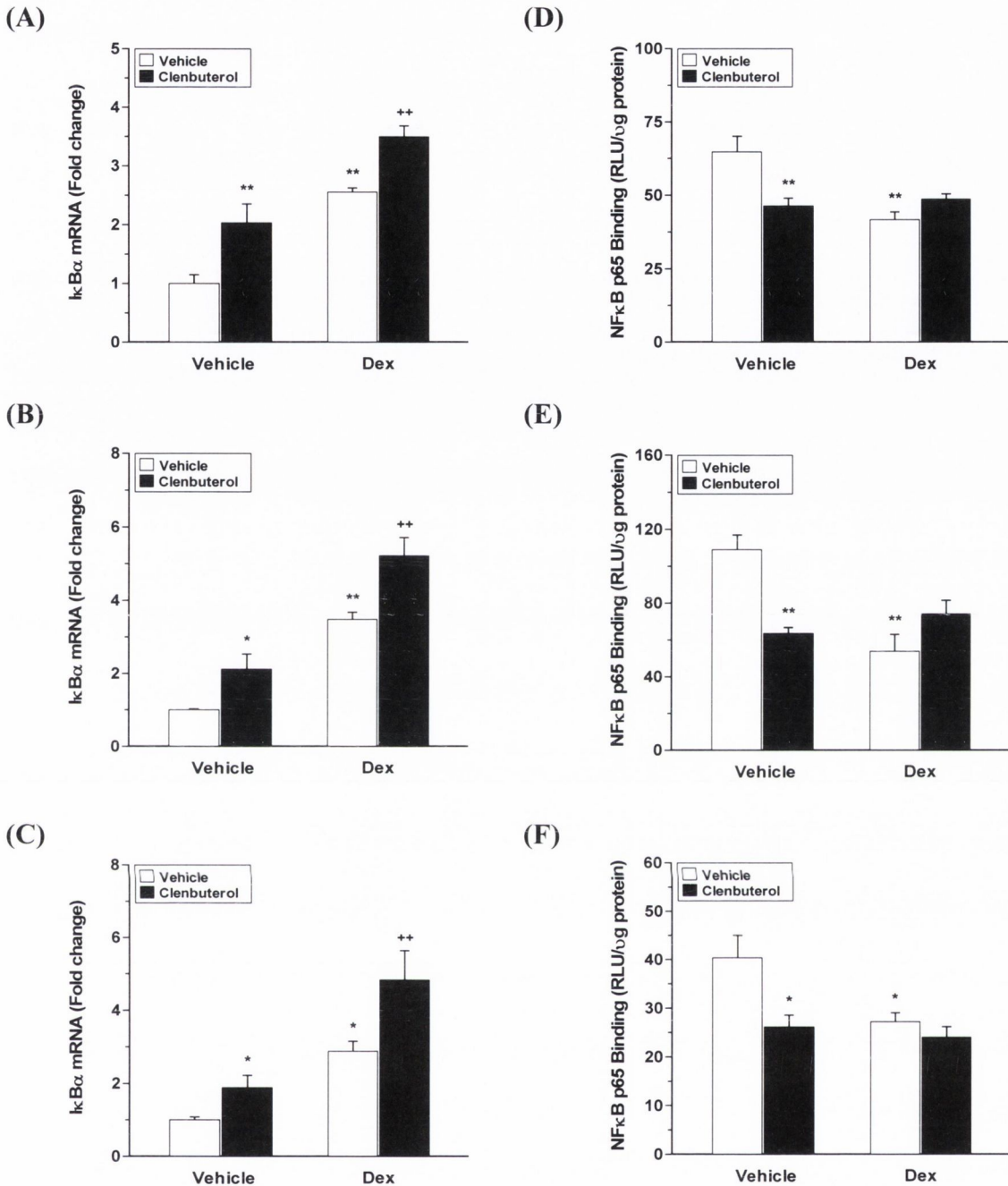


Figure 4.4. Dexamethasone and clenbuterol induce a synergistic increase in IκBα which is not accompanied by further decrease in NFκB activation in rat brain. Rats were pre-treated with dexamethasone (1 mg/kg; i.p) 1 hour prior to clenbuterol (0.5 mg/kg; i.p) treatment. Control animals received a saline vehicle. Rats were sacrificed 4 hours post clenbuterol injection. Clenbuterol and to a greater extent dexamethasone induces expression of IκBα mRNA in rat cortex (A), hippocampus (B) and striatum (C). Clenbuterol and dexamethasone both inhibit NFκB activity in cortex (A), hippocampus (B) and striatum (C). Data expressed as mean + SEM ($n=4-5$). mRNA data expressed as fold-change vs. saline vehicle. * $p<0.05$, ** $p<0.01$ vs. saline vehicle, ++ $p<0.01$ vs. clenbuterol (two-way ANOVA followed by *post-hoc* Newman–Keuls test).

4.5 Effect of dexamethasone on clenbuterol-induced IL-10 and SOCS-3 expression

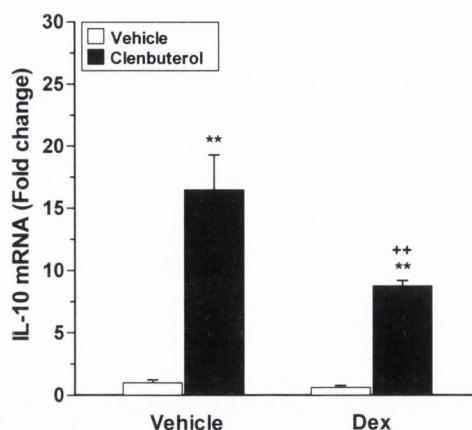
Figure 4.5.1. Clenbuterol is still effective at increasing IL-10 expression in rat brain following dexamethasone treatment.

ANOVA revealed a significant effect of clenbuterol on IL-10 mRNA expression in cortex [$F_{(1,16)} = 68.30, p < 0.0001$], hippocampus [$F_{(1,13)} = 18.12, p = 0.0009$] and striatum [$F_{(1,14)} = 59.67, p < 0.0001$]. Pre-treatment with dexamethasone had no effect on the ability of clenbuterol to induce IL-10 mRNA expression in hippocampus and striatum. In cortex however, whilst combined treatment with clenbuterol and dexamethasone significantly increased IL-10 mRNA in cortex compared to control, this increase was attenuated when compared to the induction of IL-10 induced by clenbuterol alone ($p < 0.01$). Consistent with this finding, a two-way ANOVA revealed a significant dexamethasone \times clenbuterol interaction for IL-10 mRNA in cortex [$F_{(1,16)} = 6.62, p < 0.05$] [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=4-5$].

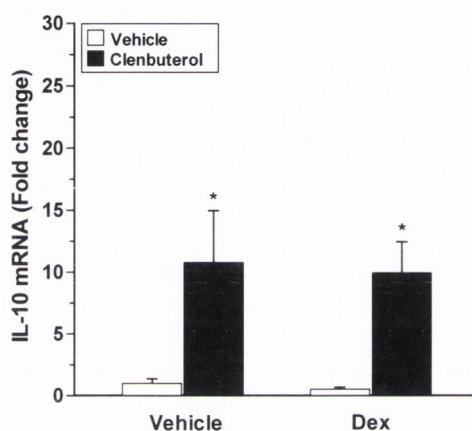
Figure 4.5.2. Clenbuterol is still effective at increasing SOCS-3 expression in rat brain following dexamethasone treatment.

ANOVA revealed a significant effect of clenbuterol on SOCS-3 mRNA expression in cortex [$F_{(1,16)} = 125.95, p < 0.0001$], hippocampus [$F_{(1,15)} = 97.52, p < 0.0001$] and striatum [$F_{(1,15)} = 239.7, p < 0.0001$]. ANOVA revealed a significant dexamethasone \times clenbuterol interaction for SOCS-3 in the cortex [$F_{(1,16)} = 14.74, p < 0.01$] and striatum [$F_{(1,15)} = 11.79, p < 0.01$] but not hippocampus. Consistent with these results, combined treatment with clenbuterol and dexamethasone significantly increased SOCS-3 mRNA expression in all three regions compared to saline vehicle ($p < 0.01$). While the ability of clenbuterol to induce SOCS-3 expression was maintained in hippocampus, and was reduced by approximately 50% in the cortex and 30% in the striatum following combined treatment with dexamethasone [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=4-5$].

(A) Cortex



(B) Hippocampus



(C) Striatum

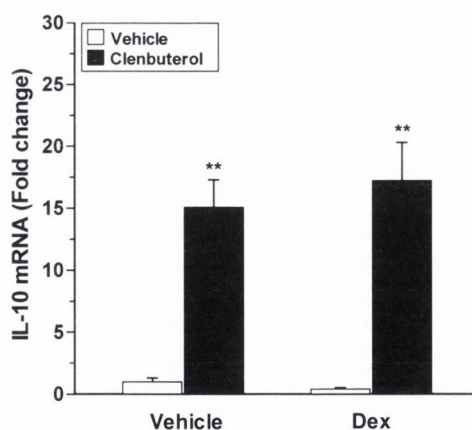


Figure 4.5.1. Clenbuterol is still effective at increasing IL-10 expression in rat brain following dexamethasone treatment. Rats were pre-treated with dexamethasone (1 mg/kg; i.p) 1 hour prior to clenbuterol (0.5 mg/kg; i.p) treatment. Control animals received a saline vehicle. Rats were sacrificed 4 hours post clenbuterol injection. Dexamethasone failed to alter the ability of clenbuterol to induce IL-10 mRNA expression in both hippocampus (B) and striatum (C), but not cortex (A). Data expressed as mean + SEM ($n=4-5$). mRNA data expressed as fold-change vs. saline vehicle. * $p<0.05$, ** $p<0.01$ vs. saline vehicle, ++ $p<0.01$ vs. clenbuterol (two-way ANOVA followed by *post-hoc* Newman-Keuls test).

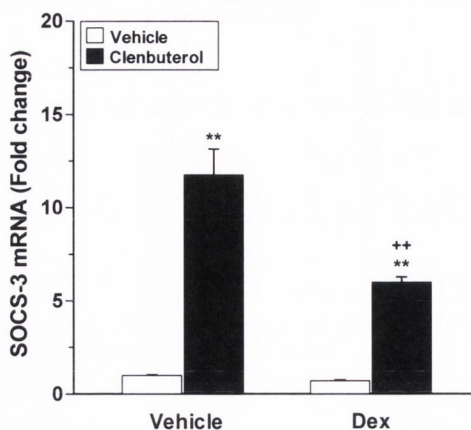
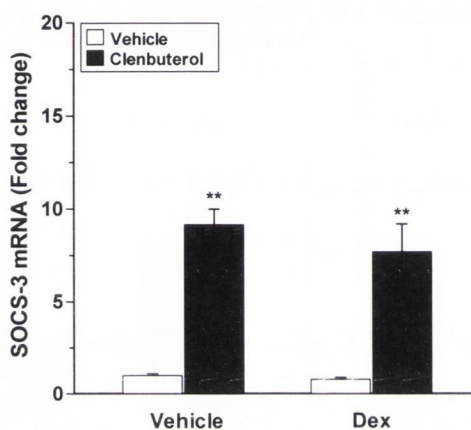
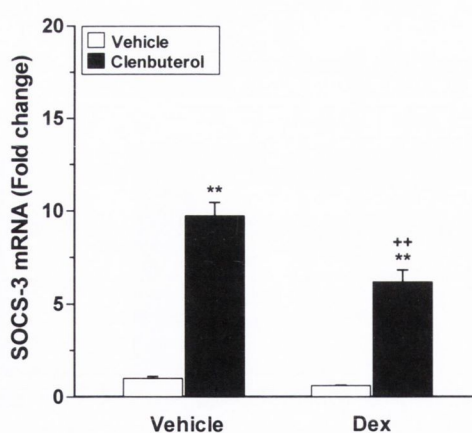
(A) Cortex**(B) Hippocampus****(C) Striatum**

Figure 4.5.2. Clenbuterol is still effective at increasing SOCS-3 expression in rat brain following dexamethasone treatment. Rats were pre-treated with dexamethasone (1 mg/kg; i.p) 1 hour prior to clenbuterol (0.5 mg/kg; i.p) treatment. Control animals received a saline vehicle. Rats were sacrificed 4 hours post clenbuterol injection. Dexamethasone failed to alter the ability of clenbuterol to induce SOCS-3 mRNA expression in hippocampus (B), but not cortex or striatum (A, C). Data expressed as mean + SEM ($n=4-5$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle, ++ $p<0.01$ vs. clenbuterol (two-way ANOVA followed by *post-hoc* Newman-Keuls test).

4.6 Discussion

4.6.1 Combined treatment with clenbuterol and dexamethasone blocks the clenbuterol-induced increase in IL-1 β and simultaneously increases negative regulators of IL-1 β

Consistent with previous work in our laboratory, clenbuterol treatment alone increased expression of IL-1 β , and its negative regulators IL-1ra and IL-1RII (McNamee *et al.*, 2010a), and in this study we report that these changes are also evident in hippocampus and striatum. Furthermore, this study shows that the anti-inflammatory glucocorticoid dexamethasone completely blocks the clenbuterol-induced increase in IL-1 β while maintaining levels of IL-1ra and IL-1RII. In fact, IL-1RII expression was further augmented by dexamethasone in hippocampus. These data show that IL-1 β can be expressed independent of IL-1ra and IL-1RII in the CNS highlighting a mechanistic dissociation between IL-1 β and its negative regulators and this is in agreement with other studies both in cultured glial cells, and in the intact brain (Docagne *et al.*, 2005; McNamee *et al.*, 2010c). The ability of dexamethasone to inhibit clenbuterol-induced IL-1 β mRNA expression is consistent with data indicating that dexamethasone inhibits IL-1 β transcription (Knudsen *et al.*, 1987). Dexamethasone also inhibited expression of the IL-1 β -inducible gene iNOS in the CNS. It is well established that iNOS expression and the subsequent production of nitric oxide contributes to the neurodegenerative process (Brown & Neher, 2010) thus the ability of dexamethasone to inhibit the clenbuterol-induced increase in iNOS is likely to be of benefit in combating neurodegeneration.

4.6.2 Clenbuterol and dexamethasone act synergistically to increase I κ B α expression

The robust induction of I κ B α gene expression observed following dexamethasone treatment is in agreement with another report that demonstrates widespread activation of I κ B α mRNA following peripheral dexamethasone treatment (Quan *et al.*, 2000). This is also consistent with the well-established role of I κ B α up-regulation in inhibiting NF κ B activation and in the anti-inflammatory actions of dexamethasone (Auphan *et al.*, 1995; Scheinman *et al.*, 1995). Interestingly, combined treatment with clenbuterol and dexamethasone further increased I κ B α expression compared to either agent alone. It is possible that dexamethasone and clenbuterol increase I κ B α expression via two separate

mechanisms. It was originally thought that dexamethasone blocks NF κ B through a physical interaction between the glucocorticoid receptor (GR) and NF κ B (Ray & Prefontaine, 1994). However, it is more likely the case that dexamethasone acts by inducing the I κ B α gene MAD-3 (Mitotic arrest deficient-3), leading to increased synthesis of I κ B α . The newly synthesised I κ B α then translocates to the nucleus where it sequesters NF κ B (Auphan *et al.*, 1995). NF κ B plays an important role in the induction of many immunoregulatory genes including pro-inflammatory cytokines such as IL-1, IL-6 and TNF- α . Thus, inhibition of NF κ B accounts for the anti-inflammatory effects of glucocorticoids and this inhibition of NF κ B is a possible mechanism by which dexamethasone blocks IL-1 β in this study.

The fact that clenbuterol alone can increase I κ B α is consistent with results in Chapter 3 and this is not surprising given that β_2 -agonists are known to inhibit NF κ B through stabilisation and accumulation of I κ B possibly through the activation of a transcription factor such as CREB which co-operates with NF κ B to increase I κ B α transcription (Farmer & Pugin, 2000). As mentioned above, combined treatment with dexamethasone and clenbuterol further increased I κ B α expression compared to either agent alone, suggesting that dexamethasone does not block the ability of clenbuterol to inhibit NF κ B.

4.6.3 Dexamethasone does not alter the ability of clenbuterol to induce IL-10 and SOCS-3

In line with previous results from our group this study found that IL-10 and its downstream signalling molecule SOCS-3 were increased in response to clenbuterol treatment in both cortex and hippocampus (McNamee *et al.*, 2010b); additionally, this study reports for the first time an increase in both molecules in the striatum. Interestingly, dexamethasone did not alter the ability of clenbuterol to induce IL-10 and SOCS-3 in the hippocampus and striatum. However, whilst clenbuterol still induced a robust induction of IL-10 and SOCS-3 in the cortex when administered in combination with dexamethasone, this increase was approximately 50% less than that induced by treatment with clenbuterol alone. The observed induction of these two molecules with combined clenbuterol and dexamethasone treatment is likely to be of benefit in combating neurodegeneration as induction of IL-10 and SOCS-3 elicit a broad spectrum of anti-inflammatory actions in the brain including inhibition of inflammatory cytokine and

chemokine production, inhibition of MHC class II expression and co-stimulatory molecule expression (Berlato *et al.*, 2002; Qin *et al.*, 2006). IL-10 also has direct trophic actions on neurons and inhibits glutamate-induced neurotoxicity (Bachis *et al.*, 2001; Boyd *et al.*, 2003; Zhou *et al.*, 2009) effects that occur independently of its anti-inflammatory actions.

4.6.4 Possible mechanisms involved in the synergistic anti-inflammatory effects of combined administration of a glucocorticoid and a β_2 -adrenoceptor agonist in the brain

The present study is the first to demonstrate the synergistic effects of glucocorticoids and β -agonists on cytokine expression in the brain. The mechanism by which glucocorticoids and β -agonists modulate these cytokines in the brain has yet to be explored. However, mechanistic links between these two agents have been explored in the periphery. For instance, it is known that β_2 -adrenoceptor agonists can activate the GR in human lung fibroblasts even in the absence of a glucocorticoid, and this appears to involve cAMP (Eickelberg *et al.*, 1999). It is possible that both clenbuterol and dexamethasone alone both activate the GR and when administered together result in an additive effect on GR activation. This could explain the additive effect of combined treatment on I κ B α expression. Further investigation of the ability of clenbuterol to independently activate the GR would be needed to confirm this as a mechanism in the brain. In addition, glucocorticoids are known to increase transcription of β_2 -adrenoceptors in both lung and nasal tissue and in glial cells of the brain (Mak *et al.*, 1995; Baraniuk *et al.*, 1997; Ryan, 2010). Thus, in a complementary way, each class of drug enhances the others receptor.

Evidence of glucocorticoid-noradrenergic interactions in the brain is limited to studies regarding memory formation rather than inflammation. In this regard, glucocorticoids are known to increase synthesis and release of noradrenaline (Markey *et al.*, 1982). Noradrenaline availability can also be increased by corticosterone which blocks the catecholamine transporter and prevents re-uptake of noradrenaline into the pre-synaptic terminal (Grundemann *et al.*, 1998). These synergistic interactions are speculated to cause enhanced memory consolidation through interaction with the β -adrenoceptor-cAMP/PKA system (Rooszendaal *et al.*, 2002). Perhaps these same mechanisms play a role in the regulation of cytokines seen in the present study. Perhaps dexamethasone

increases noradrenaline levels and through increased cAMP/PKA signalling leads to increased anti-inflammatory signalling in synergy with clenbuterol-induced cAMP/PKA signalling. However, the exact mechanism remains unknown. In this regard, the results from this chapter were recently published and this appears to be the only literature available regarding the synergistic anti-inflammatory effects of these agents in the brain (Ryan *et al.*, 2010).

4.6.5 Summary

Based on these data, it appears that combined treatment with dexamethasone and a β_2 -adrenoceptor agonist may be more beneficial than treatment with either agent alone in treating neuroinflammation and combating inflammation-related neurodegeneration. Due to the fact that clenbuterol induces IL-1 β expression and as IL-1 β is known to induce sickness behaviours including the suppression of locomotor activity and feeding, the next chapter will examine the role of central IL-1 β expression in the suppressive effect of clenbuterol on locomotor activity and food consumption in rats.

Chapter 5

*A role for central IL-1 β in the
suppression of locomotor activity and
feeding induced by the β_2 -adrenoceptor
agonist clenbuterol?*

5.1 Introduction

Earlier results both from this thesis and others in our group demonstrate that β_2 -adrenoceptor agonist clenbuterol increases expression of IL-1 β in the CNS (cortex, hippocampus, striatum and hypothalamus) (Ryan *et al.*, 2010; McNamee *et al.*, 2010b), albeit in a controlled manner. For instance, it is accompanied by an increase in its negative regulators IL-1 receptor antagonist (IL-1ra) and IL-1 receptor type-II (IL-1RII), without activation of downstream pro-inflammatory cytokines such as TNF- α , IL-6, and as demonstrated in the previous chapter, without NF κ B activation (in fact, NF κ B is suppressed). In parallel with its ability to induce central IL-1 β , clenbuterol induces behavioural effects in rodents such as a reduction in locomotor activity and feeding (Goldschmidt *et al.*, 1984; Geyer & Frampton, 1988; O'Donnell, 1993). However, the underlying mechanisms of the behavioural actions of clenbuterol are unclear and conflicting reports exist regarding the role of peripheral versus central β -adrenoceptors (Goldschmidt *et al.*, 1984; Geyer & Frampton, 1988; O'Donnell, 1993).

The increase in central IL-1 β parallels the reduction in locomotor activity and food consumption induced by clenbuterol (Ryan, 2010). When administered either peripherally or directly into the rodent brain IL-1 β causes a variety of sickness behaviours including fever, anorexia and depressed behaviour (Plata-Salaman *et al.*, 1988; Dantzer *et al.*, 1998; Kluger *et al.*, 1998; Konsman *et al.*, 2008). We hypothesise that the induction of central IL-1 β by clenbuterol could mediate the suppressive effect of clenbuterol on locomotor activity and feeding in rats.

The objective of this study was to determine the role of central IL-1 β expression in the suppressive effect of clenbuterol on locomotor activity. This study examined the ability of three pharmacological agents to block the clenbuterol-induced increase in hypothalamic IL-1 β expression and reduction in locomotor activity. The experimental aims were as follows:

- 1) To determine if administration of the anti-inflammatory glucocorticoid dexamethasone could block the clenbuterol-induced increase in IL-1 β and suppression of locomotor activity and feeding.

- 2) To determine if treatment with either centrally or peripherally acting β_2 -adrenoceptor antagonists could block the clenbuterol-induced increase in IL-1 β and suppression of locomotor activity and feeding.

5.2 Methods

5.2.1 Animal handling

Male Sprague-Dawley rats (250-300g) were obtained from Harlan Laboratories, UK. Rats were housed 4 per cage in an air-conditioned room with a 12:12-h light/dark cycle (lights on at 8am). Food and water were available *ad libitum*. Animals were handled daily for at least seven days prior to drug administration.

5.2.2 Experimental procedures

All pharmacological agents were dissolved in 0.89% (w/v) saline with the exception of dexamethasone which was dissolved in 0.89% (w/v) saline containing 0.2% (v/v) Tween-20.

Two separate experiments were carried out with the aim of:

1) Examining the effect of clenbuterol-induced central IL-1 β on locomotor activity:

Rats were pre-treated with dexamethasone (1 mg/kg), nadolol (5 mg/kg), or propranolol (10 mg/kg) 30 minutes prior to clenbuterol (0.5 mg/kg), all via i.p. injection. Control animals received a 0.89% (w/v) saline vehicle. Locomotor activity was recorded 2 hours post-clenbuterol administration for a 15 minute period. Rats were sacrificed 4 hours post-clenbuterol injection and tissue was prepared for RT-PCR.

2) Examining the effect of clenbuterol-induced central IL-1 β on food intake:

Rats were pre-treated with dexamethasone (1 mg/kg), nadolol (5 mg/kg), or propranolol (10 mg/kg) 30 minutes prior to clenbuterol (0.5 mg/kg), all via i.p. injection. Control animals received a 0.89% (w/v) saline vehicle. Food intake was measured for 24 hours following drug treatment. No brain tissue was taken in this study.

A detailed description of the experimental procedures and molecular techniques can be found in Chapter 2.

5.3 Effect of dexamethasone pre-treatment on clenbuterol-induced changes in hypothalamic IL-1 β mRNA expression, food intake and locomotor activity

The glucocorticoid dexamethasone is known to have anti-inflammatory properties and can reduce IL-1 β expression in the brain (Velasco *et al.*, 1991; Kimberlin *et al.*, 1995; Moore *et al.*, 2007). The aim of this study was to examine the effects of pre-treatment with dexamethasone on the clenbuterol-induced increase in central IL-1 β expression and reduction in locomotor activity.

Figure 5.3 A. Dexamethasone blocks the ability of clenbuterol to induce IL-1 β mRNA expression

ANOVA revealed a significant dexamethasone \times clenbuterol interaction for IL-1 β mRNA expression in hypothalamus [$F_{(1,16)} = 31.15$, $p < 0.0001$]. Consistent with this finding, *post hoc* analysis showed that the clenbuterol-induced increase in IL-1 β was significantly attenuated by pre-treatment with dexamethasone ($p < 0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=5$].

Figure 5.3 B. Dexamethasone fails to reverse the suppressive action of clenbuterol on food intake

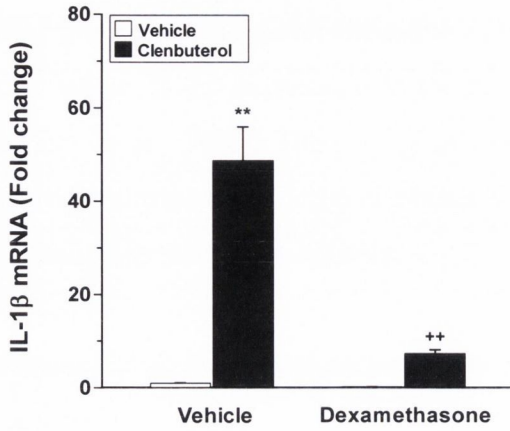
ANOVA revealed a significant dexamethasone \times clenbuterol interaction for food intake [$F_{(1,16)} = 11.73$, $p = 0.0035$]. *Post hoc* analysis showed that clenbuterol significantly reduced food intake ($p < 0.01$). However, pre-treatment with dexamethasone failed to reverse the clenbuterol-induced reduction in food intake, in fact food intake was further suppressed ($p < 0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=5$].

Figure 5.3 C, D. Dexamethasone fails to alter the suppressive action of clenbuterol on locomotor activity

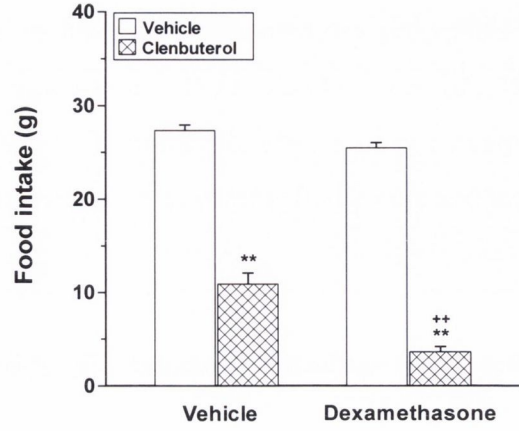
ANOVA revealed a significant effect of clenbuterol on active time [$F_{(1,19)} = 427.76$, $p < 0.0001$] and mobility [$F_{(1,19)} = 54.58$, $p < 0.0001$] but no effect of dexamethasone on active time [$F_{(1,19)} = 0.00185$, $p = 0.9663$] or mobility [$F_{(1,19)} = 0.00052$, $p = 0.9821$]. Consistent with these findings, *post hoc* analysis showed that clenbuterol significantly reduced both active time ($p < 0.01$) and mobility ($p < 0.01$). However, pre-treatment with

dexamethasone failed to alter the clenbuterol-induced reduction in active time or mobility [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=5$].

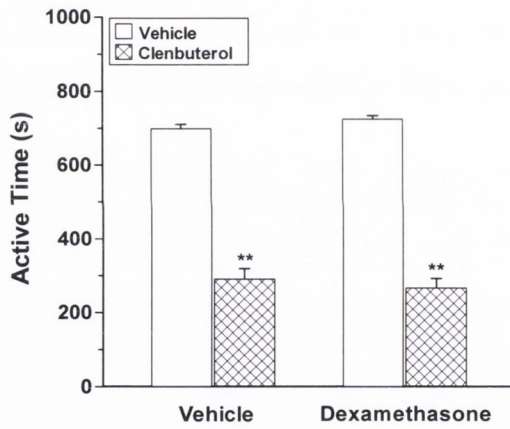
(A) Hypothalamic IL-1 β



(B) Food intake



(C) Locomotor activity



(D) Locomotor activity

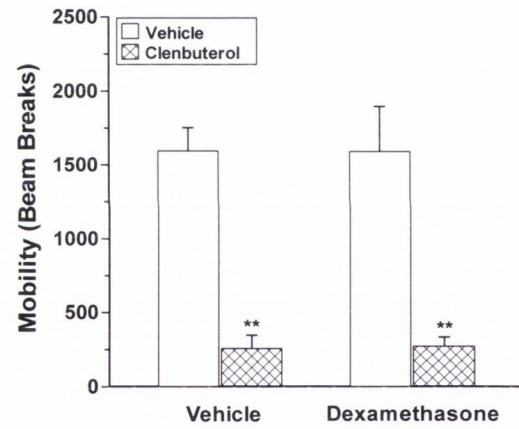


Figure 5.3: Dexamethasone blocks the ability of clenbuterol to induce IL-1 β expression but fails to alter the suppressive action of clenbuterol on food intake and locomotor activity.

Rats were pre-treated with dexamethasone (1mg/kg; i.p) 1 hour prior to clenbuterol treatment (0.5mg/kg; i.p). Control animals received a saline vehicle. Locomotor activity was monitored 2 hours post clenbuterol injection. Rats were sacrificed 4 hours post clenbuterol injection. Dexamethasone attenuated the clenbuterol-induced increase in hypothalamic IL-1 β mRNA expression (A). Dexamethasone pre-treatment failed to reverse the clenbuterol-induced suppression of food intake, in fact, food intake was suppressed to a slightly greater extent than clenbuterol alone (B). Dexamethasone pre-treatment had no effect on the clenbuterol-induced suppression of active time (C) or mobility (D). Data expressed as mean + SEM ($n=5$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle, ++ $p<0.01$ vs. clenbuterol (two-way ANOVA followed by *post-hoc* Newman-Keuls test).

5.4 Effect of nadolol pre-treatment on clenbuterol-induced changes in hypothalamic IL-1 β mRNA expression, food intake and locomotor activity

The present study examined the role of peripheral β -adrenoceptors on clenbuterol-induced reduction in locomotor activity. Nadolol is a peripherally acting β -adrenoceptor antagonist that does not cross the blood brain barrier.

Figure 5.4 A. Nadolol fails to inhibit central clenbuterol-induced IL-1 β expression

ANOVA revealed a significant nadolol \times clenbuterol interaction for IL-1 β mRNA expression in hypothalamus [$F_{(1,23)} = 14.85, p=0.001$]. *Post hoc* analysis showed that clenbuterol significantly increased IL-1 β mRNA expression ($p<0.01$), compared to saline vehicle. This was not attenuated by pre-treatment with nadolol. In fact, treatment with nadolol in combination with clenbuterol increased IL-1 β mRNA expression compared to clenbuterol treatment alone ($p<0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=6$].

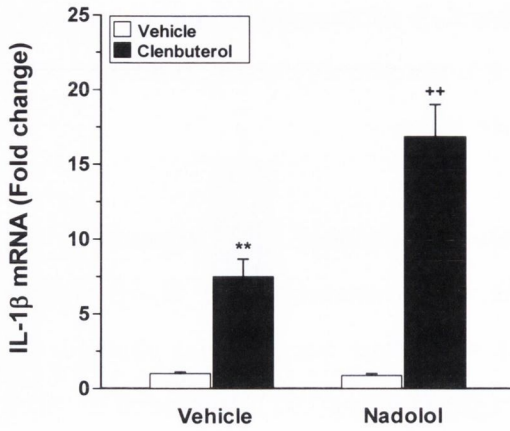
Figure 5.4 B. Nadolol effectively blocks the suppressive effect of clenbuterol on food intake

ANOVA revealed a significant nadolol \times clenbuterol interaction for food intake [$F_{(1,16)} = 57.34, p<0.0001$]. *Post hoc* analysis showed that pre-treatment with nadolol blocked the clenbuterol-induced reduction in food intake ($p<0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=5$].

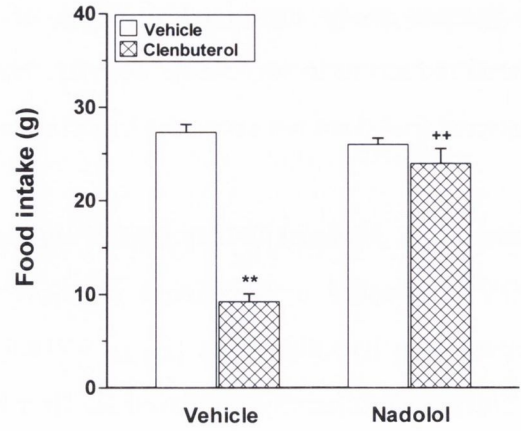
Figure 5.4 C, D. Nadolol effectively blocks the suppressive effect of clenbuterol on locomotor activity

ANOVA revealed a significant nadolol \times clenbuterol interaction for both active time [$F_{(1,23)} = 26.13, p<0.0001$] and mobility [$F_{(1,22)} = 14.54, p=0.0012$]. *Post hoc* analysis showed that pre-treatment with nadolol blocked the clenbuterol-induced reduction in both active time ($p<0.01$) and mobility ($p<0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=6$].

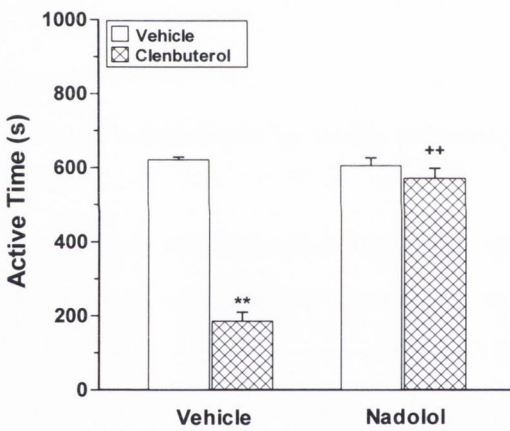
(A) Hypothalamic IL-1 β



(B) Food intake



(C) Locomotor activity



(D) Locomotor activity

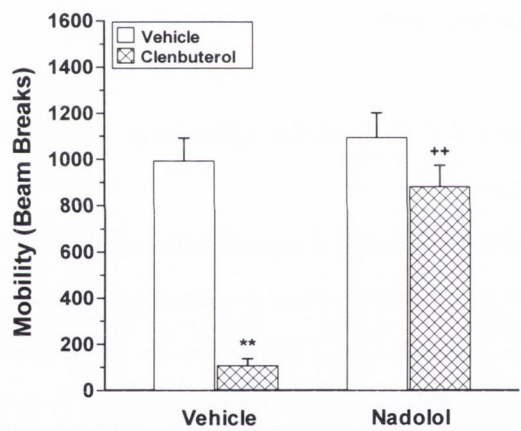


Figure 5.4: Nadolol fails to inhibit central clenbuterol-induced IL-1 β expression and effectively blocks the suppressive effect of clenbuterol on feeding and locomotor activity. Rats were pre-treated with nadolol (5mg/kg; i.p) 30 min prior to clenbuterol (0.5mg/kg; i.p) treatment. Control animals received a saline vehicle. Locomotor activity was monitored 2 hours post clenbuterol injection. Rats were sacrificed 4 hours post clenbuterol injection. Nadolol failed to attenuate the clenbuterol-induced increase in hypothalamic IL-1 β mRNA expression (A) but completely blocked the clenbuterol-induced reduction in feeding (B) active time (C) and mobility (D). Data expressed as mean + SEM ($n=6$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle, ++ $p<0.01$ vs. clenbuterol (two-way ANOVA followed by *post-hoc* Newman-Keuls test).

5.5 Effect of propranolol pre-treatment on clenbuterol-induced changes in hypothalamic IL-1 β mRNA expression, feeding and locomotor activity

The results from the previous section demonstrate that the peripherally acting β -adrenoceptor antagonist nadolol failed to inhibit clenbuterol-induced central IL-1 β expression while it completely blocked the suppressive effect of clenbuterol on locomotor activity. This supports a role for peripheral β -adrenoceptors in the clenbuterol-induced reduction in locomotor activity. To further investigate the role of the peripheral β -adrenoceptor rats received propranolol, a non-selective brain permeable β -adrenoceptor antagonist propranolol, prior to clenbuterol.

Figure 5.5 A. Propranolol completely blocks central clenbuterol-induced IL-1 β expression

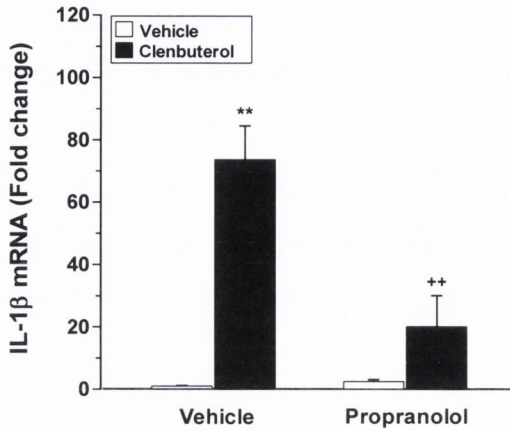
ANOVA revealed a significant propranolol \times clenbuterol interaction for IL-1 β mRNA expression in hypothalamus [$F_{(1,19)} = 13.92, p=0.0018$]. *Post hoc* analysis showed that the clenbuterol-induced increase in IL-1 β mRNA was significantly attenuated by pre-treatment with propranolol ($p<0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=5$].

Figure 5.5 B. Propranolol effectively reverses the suppressive effect of clenbuterol on food intake

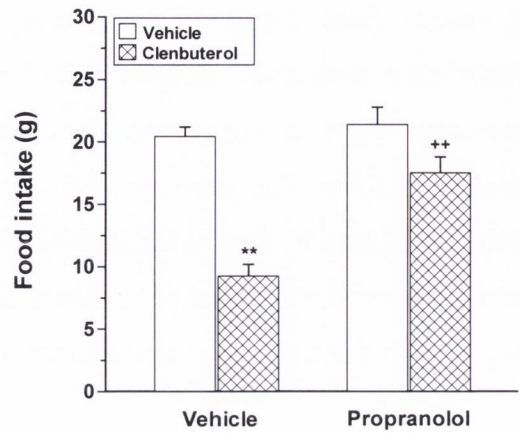
ANOVA revealed a significant propranolol \times clenbuterol interaction for both active time [$F_{(1,16)} = 10.80, p=0.0046$]. Consistent with these findings, *post hoc* analysis showed that pre-treatment with propranolol reversed the clenbuterol-induced reduction food intake ($p<0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=5$].

Figure 5.5 C, D. Propranolol effectively reverses the suppressive effect of clenbuterol on locomotor activity

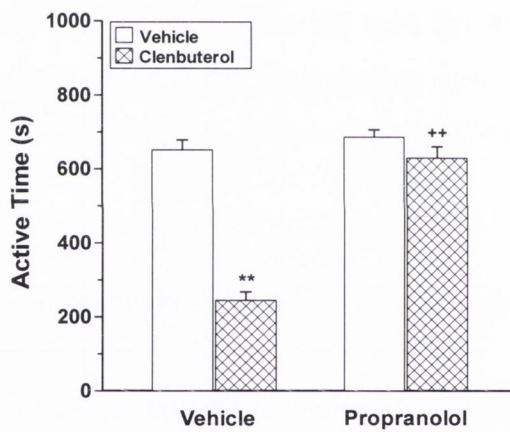
ANOVA revealed a significant propranolol \times clenbuterol interaction for both active time [$F_{(1,23)} = 47.38, p<0.0001$] and mobility [$F_{(1,23)} = 57.07, p<0.0001$]. Consistent with these findings, *post hoc* analysis showed that pre-treatment with propranolol reversed the clenbuterol-induced reduction in both active time ($p<0.01$) and mobility ($p<0.01$) [Two-way ANOVA followed by *post-hoc* Newman-Keuls test; $n=5$].

(A) Hypothalamic IL-1 β 

(B) Food intake



(C) Locomotor activity



(D) Locomotor activity

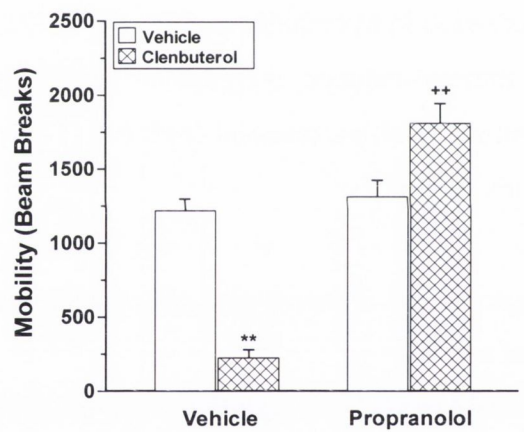


Figure 5.5: Propranolol completely blocks central clenbuterol-induced IL-1 β expression and effectively reverses the suppressive effect of clenbuterol on food intake and locomotor activity. Rats were pre-treated with propranolol (10 mg/kg; i.p) 1 hour prior to clenbuterol (0.5 mg/kg; i.p) treatment. Control animals received a saline vehicle. Locomotor activity was monitored 2 hours post clenbuterol injection. Rats were sacrificed 4 hours post clenbuterol injection. Propranolol attenuated the clenbuterol-induced increase in hypothalamic IL-1 β mRNA expression (A) and completely blocked the clenbuterol-induced reduction in food intake (B) active time (C) and mobility (D). Data expressed as mean + SEM ($n=5$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle, ++ $p<0.01$ vs. clenbuterol (two-way ANOVA followed by *post-hoc* Newman–Keuls test).

5.6 Discussion

5.6.1 The clenbuterol-induced reduction in locomotor activity is not caused by central IL-1 β expression and appears to be mediated by peripheral β -adrenoceptors.

Administration of the glucocorticoid dexamethasone blocked induction of hypothalamic IL-1 β by clenbuterol. This observation supports findings that glucocorticoids exert potent anti-inflammatory actions through the inhibition of pro-inflammatory gene expression (Barnes & Karin, 1997; Karin, 1998) thereby providing protection from a harmful over-activation of the immune system (Besedovsky *et al.*, 1986; De Bosscher & Haegeman, 2009). However, in spite of reducing IL-1 β in the brain, dexamethasone failed to alter the suppressive action of clenbuterol on locomotor activity. This result was surprising given the substantial evidence that exogenously administered IL-1 β leads to suppression of locomotor activity (Plata-Salaman *et al.*, 1988; Kent *et al.*, 1994; Swiergiel & Dunn, 2007; Harden *et al.*, 2008). Nevertheless, it is clear that central IL-1 β does not mediate the clenbuterol-induced reduction in locomotor activity.

In support of this, the peripherally acting β -adrenoceptor antagonist nadolol failed to inhibit central clenbuterol-induced IL-1 β expression, however, it effectively blocked the suppressive effect of clenbuterol on feeding and locomotor activity. In addition, the brain penetrant β -adrenoceptor antagonist propranolol, which completely blocked the central clenbuterol-induced induction of IL-1 β , effectively blocked the suppressive effect of clenbuterol on feeding and locomotor activity. These results support the idea that clenbuterol-induced suppression of feeding and locomotor activity occurs independently of its ability to induce central IL-1 β expression. Furthermore, it indicates that peripheral β -adrenoceptors mediate the suppression of feeding and locomotor activity induced by clenbuterol. However, this is not surprising as previous studies indicate that peripheral β -adrenoceptors mediate the suppressive effect of clenbuterol on behaviour (Geyer & Frampton, 1988; O'Donnell, 1993).

Given the fact that the clenbuterol-induced suppression of locomotor activity is paralleled by an induction of IL-1 β in the rat brain, and IL-1 β is known to induce sickness-like behaviours such as reduced locomotor activity, it was surprising that central IL-1 β did

not play a role in the behavioural effects elicited by this drug. However, this may be explained by the fact that our group has demonstrated that clenbuterol induces expression of IL-1ra and the interleukin-1 type II decoy receptor (IL-1RII) in a range of brain regions including the hypothalamus (Ryan, 2010; McNamee *et al.*, 2010a), both of which prevent excessive or prolonged activation of the IL-1 system (Colotta *et al.*, 1994; Bessis *et al.*, 2000; Arend, 2002). In addition, clenbuterol induces expression of the broad spectrum anti-inflammatory cytokine IL-10 in the rat brain, including the hypothalamus (Ryan, 2010; McNamee *et al.*, 2010b), and IL-10 has been shown to inhibit LPS-induced suppression of locomotor activity and food consumption (Hollis *et al.*; Bluthe *et al.*, 1999). Thus increased expression of the anti-inflammatory molecules IL-1ra, IL-1RI and IL-10 may serve to limit the effects of clenbuterol-induced IL-1 β on brain function and behaviour.

It is possible that a non-specific stress response may be the cause of the clenbuterol-induced reduction in locomotor activity. Clenbuterol is known to stimulate β_2 -adrenoceptors of the heart which induces cardiovascular changes such as increased heart rate and blood flow, and these effects have been shown to subside with continued treatment (Brockway *et al.*, 1987; Hoey *et al.*, 1995). In support of this, our laboratory has shown that i.p. administration of clenbuterol to rats results in a transient tachycardic response which subsides after approximately 10 hours (Ryan, 2010). It is possible that nadolol and propranolol block the suppressive effect of clenbuterol on locomotor activity through a depressive action on peripheral cardiac β -adrenoceptors, thereby maintaining a normal heart rate. While heart rate was not monitored in this particular set of experiments, previous work in our laboratory supports this theory whereby treatment with the β_2 -adrenoceptor antagonist ICI 118,551 blocked the effects of clenbuterol on heart rate in rats (Ryan, 2010). The same study showed that clenbuterol treatment reduced water and food intake and this was reversed when the clenbuterol-induced tachycardia was attenuated by ICI 118,551. For this reason, it appears that clenbuterol induces a non-specific stress response rather than classical sickness behaviour.

5.6.2 Summary

The underlying mechanisms of the behavioural actions of clenbuterol are unclear and conflicting reports exist regarding the role of peripheral versus central β -adrenoceptors.

The results presented here demonstrate that the clenbuterol-induced reduction in feeding and locomotor activity is mediated specifically by peripheral β_2 -adrenoceptors. In addition, the suppression of locomotor activity occurs independently of the ability of clenbuterol to induce central IL-1 β expression. Thus, it appears that the clenbuterol-induced reduction in feeding and locomotor activity is mediated by a non-specific stress response, possibly due to the effect of clenbuterol on cardiac β_2 -adrenoceptors, rather than being mediated by central IL-1 β . This further reinforces the idea that clenbuterol induces central IL-1 β in a controlled manner and without negative consequences downstream such as increased pro-inflammatory signalling or sickness-like behavioural effects. With this in mind, the final chapter examines the effects of long term β_2 -adrenoceptor agonist administration on cytokine expression and behaviour in rodents.

Chapter 6

*Chronic treatment with the
 β_2 -adrenoceptor agonist clenbuterol
activates the central interleukin-1
system without provoking depressive or
anxiety-like behaviour in rats*

6.1 Introduction

It has recently been demonstrated that the brain permeable β_2 -adrenoceptor agonist clenbuterol selectively activates the central IL-1 system without inducing expression of other inflammatory cytokines including TNF- α , IL-6 and IFN- γ (Ryan, 2010). Moreover, our laboratory has shown that this induction of IL-1 β is short-lived and accompanied by increased expression of IL-1ra and IL-1RII; two negative regulators of the IL-1 system. Double immunohistochemical staining showed that clenbuterol-induced IL-1 β expression is localised to astrocytes as opposed to microglia, and this is supported by the fact that clenbuterol treatment consistently induces expression of the astrocytic activation marker GFAP, whilst having little effect on expression of the microglial activation marker CD11b (Tanvaneer, 2008). Excessive production of IL-1 β , particularly in the context of microglial activation, has been implicated in the neurodegenerative process (see Allan *et al.*, 2005 for review; Patel *et al.*, 2006) and also in the pathogenesis of psychiatric disorders such as depression and anxiety (Maes *et al.*, 1997; Huang *et al.*, 2004; Howren *et al.*, 2009).

IL-1 β expression in the CNS is known to induce sickness-like behaviours such as reduced locomotor activity, feeding, and anxiety-like behaviours (Dantzer, 2001). Considering the evidence that IL-1 β can precipitate symptoms of depression and anxiety the aim of this study was to investigate the ability of chronic treatment with clenbuterol (30 μ g/kg; b.i.d; 20 days) to activate the central IL-1 system and to provoke anxiety and depression-like behaviour (anhedonia) in rats. Two well known tests for anxiety-like behaviour in rodents, the open field test and the elevated plus maze were used. In addition, the saccharin-preference test was performed to assess whether clenbuterol caused a depressive-like phenotype. Acute administration of clenbuterol is known to induce a reduction in locomotor activity as demonstrated in Chapter 5. Unpublished results from our group demonstrate that this is accompanied by reduced feeding (Ryan, 2010). Thus, rodent feeding and body weight were also monitored throughout the present study to determine if these acute effects subsided over the course of continued administration. In addition, in order to determine the cellular source of the IL-1 β produced by clenbuterol the expression of the astrocyte and microglial activation markers were examined.

The specific aims of this study were:

- 1) To examine the effect of chronic clenbuterol treatment on the expression of IL-1 β and other cytokines in the CNS.
- 2) To examine the impact of chronic clenbuterol treatment on anxiety behaviour and anhedonia using the elevated plus maze, open field, and saccharin preference tests.
- 4) To examine the effect of chronic clenbuterol treatment on food and water intake.

6.2 Methods

6.2.1 Animal handling

Male Sprague-Dawley rats (250-300g) were obtained from Harlan Laboratories, UK. Rats were housed 2 per cage in an air-conditioned room with a 12:12-h light/dark cycle (lights on at 8am). Food and water were available *ad libitum*. Animals were handled daily for at least seven days prior to drug administration.

6.2.2 Experimental procedures

Chronic administration of low-dose clenbuterol

Clenbuterol was dissolved in 0.89% (w/v) saline and was administered i.p. at a dose of 0.03 mg/kg twice daily for 20 days. Control animals received an i.p. injection of 0.89% (w/v) saline twice daily for 20 days. A two-bottle saccharin preference test was performed for the duration of the study whereby rats had free access to both water and saccharin. During week 2 of 3, rats performed an elevated plus maze task on day 9 and an open field task on day 14 to assess whether clenbuterol caused anxiety-like behaviour. On day 21 rats received one final injection of clenbuterol or vehicle. Rats were sacrificed 4 hours post-injection. This study yielded two treatment groups: (1) saline vehicle, (2) clenbuterol 0.03 mg/kg. $n=8$ per group.

6.3 Effect of chronic administration of the β_2 -adrenoceptor agonist clenbuterol on cytokine expression in rat CNS

Cytokine expression was assessed in the cortex hypothalamus and hippocampus. The hippocampus was of particular interest due to the important role that it plays in modulating fear and anxiety (Goosens, 2011).

Figure 6.3.1. Chronic clenbuterol administration induces expression of IL-1 β in rat brain

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) induced a significant increase in IL-1 β mRNA in cortex ($t(14) = -4.22, p=0.0009$), hippocampus ($t(14) = -9.02, p<0.0001$), and hypothalamus ($t(14) = 0.19, p=0.0002$) compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=8$].

Figure 6.3.2. Chronic clenbuterol administration does not induces expression of iNOS in rat brain

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) had no effect on iNOS mRNA expression in cortex ($t(11) = 0.55, p=0.8547$), hippocampus ($t(10) = 1.02, p=0.3298$), or hypothalamus ($t(11) = 0.21, p=0.9363$) compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=6-8$].

Figure 6.3.3. Chronic clenbuterol treatment induces IL-1ra expression in rat brain

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) induced a significant increase in IL-1ra mRNA in cortex ($t(12) = 3.70, p=0.0029$), hippocampus ($t(14) = -4.01, p=0.0013$), but not hypothalamus ($t(14) = -0.47, p=0.6398$) compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=7-8$].

Figure 6.3.4. Chronic clenbuterol treatment induces IL-1RII expression in rat brain

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) induced a significant increase in IL-1RII mRNA in cortex ($t(13) = 6.40, p=0.0003$), hippocampus ($t(14) = -5.35, p=0.0001$), and hypothalamus ($t(14) = -3.87, p=0.0017$) compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=7-8$].

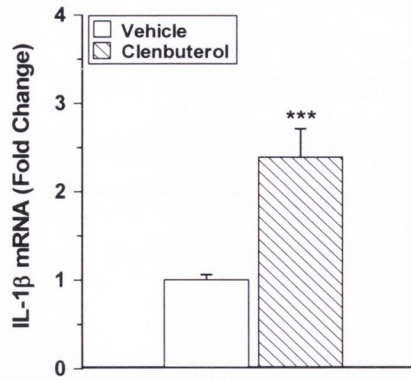
Figure 6.3.5. Chronic clenbuterol treatment did not induce IL-10 expression in rat brain

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) failed to induce a significant increase in IL-10 mRNA in cortex ($t(14) = 1.38, p=0.1903$), hippocampus ($t(10) = 1.31, p=0.2198$), or hypothalamus ($t(14) = 0.70, p=0.4928$) compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=6-8$].

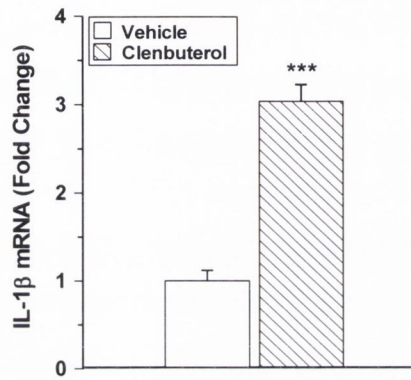
Figure 6.3.6. Chronic clenbuterol treatment did not induce expression of the inflammatory markers TNF- α or IL-6 in rat brain

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) failed to induce a significant increase in TNF- α mRNA in cortex ($t(12) = 1.55, p=0.1540$), hippocampus ($t(14) = 1.94, p=0.0722$), or hypothalamus ($t(13) = 1.88, p=0.0822$), compared to saline vehicle. Clenbuterol also failed to induce a significant increase in IL-6 mRNA in cortex ($t(12) = 1.20, p=0.9060$), or hypothalamus ($t(14) = 1.30, p=0.2162$), compared to saline vehicle. In fact, clenbuterol managed to suppress IL-6 mRNA in the hippocampus ($t(13) = 2.99, p=0.0105$) [Student's *t*-test (Pooled Variances) $n=6-8$].

(A) Cortex



(B) Hippocampus



(C) Hypothalamus

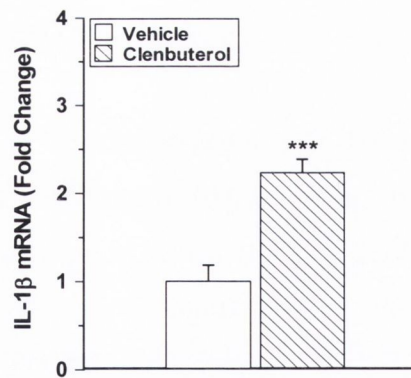


Figure 6.3.1. Chronic clenbuterol administration induces expression of IL-1β in rat brain.

Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol increased expression of IL-1β mRNA in cortex (A), hippocampus (B) and hypothalamus (C). Data expressed as mean + SEM ($n=8$). mRNA data expressed as fold-change vs. saline vehicle. *** $p<0.001$ vs. saline vehicle (Student's t -test).

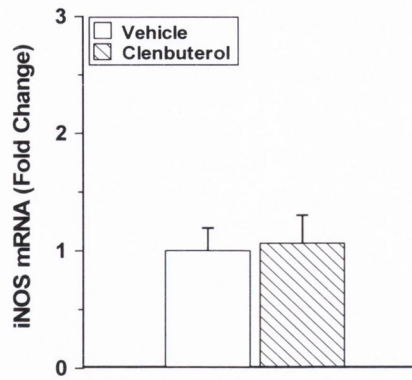
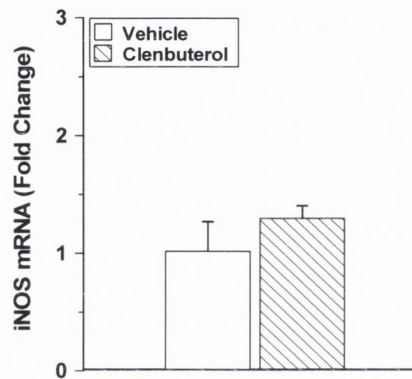
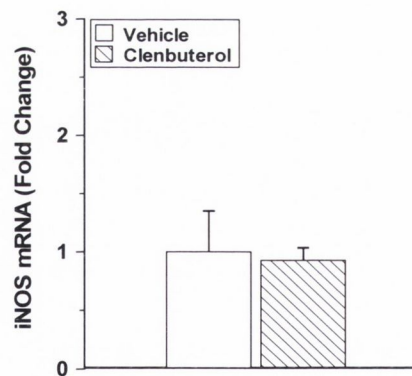
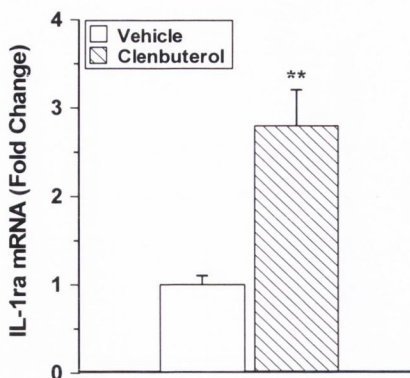
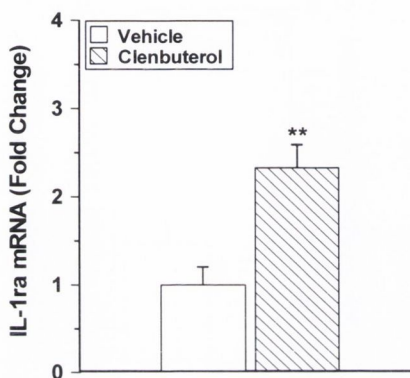
(A) Cortex**(B) Hippocampus****(C) Hypothalamus**

Figure 6.3.2. Chronic clenbuterol does not induce iNOS in rat brain. Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol failed to induce iNOS in cortex (A), hippocampus (B) or hypothalamus (C). Data expressed as mean + SEM ($n=6-8$). mRNA data expressed as fold-change vs. saline vehicle (Student's t -test).

(A) Cortex



(B) Hippocampus



(C) Hypothalamus

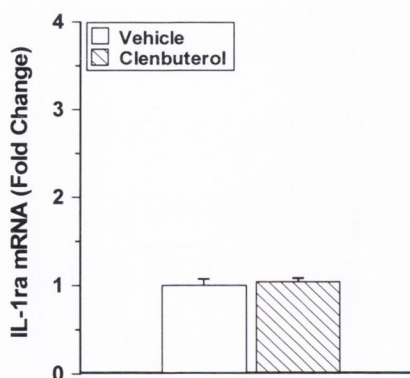


Figure 6.3.3. Chronic clenbuterol administration largely induces expression of IL-1ra in rat brain. Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol increased expression of IL-1ra mRNA in cortex (A), hippocampus (B) and hypothalamus (C). Data expressed as mean + SEM ($n=7-8$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle (Student's t -test).

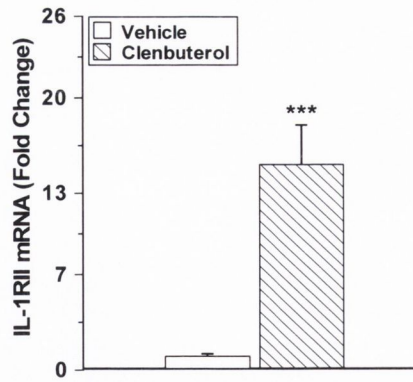
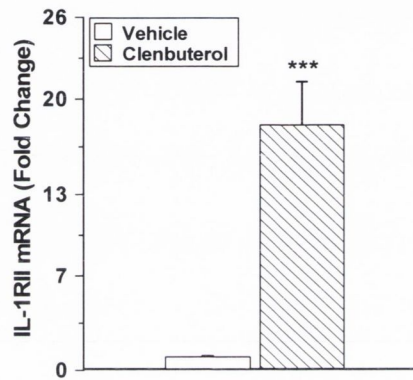
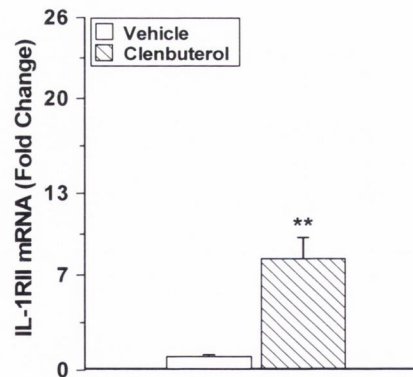
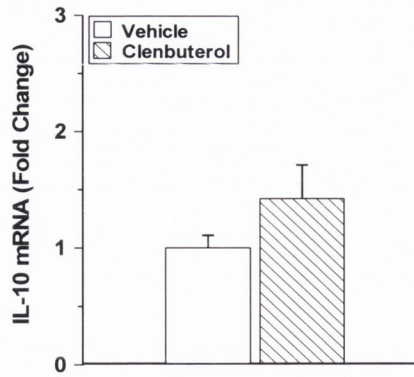
(A) Cortex**(B) Hippocampus****(C) Hypothalamus**

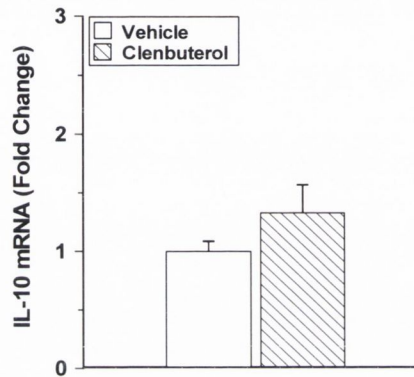
Figure 6.3.4. Chronic clenbuterol administration induces expression of IL-1RII in rat brain.

Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol increased expression of IL-1RII mRNA in cortex (A), hippocampus (B) and hypothalamus (C). Data expressed as mean + SEM ($n=7-8$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$, *** $p<0.001$ vs. saline vehicle (Student's t -test).

(A) Cortex



(B) Hippocampus



(C) Hypothalamus

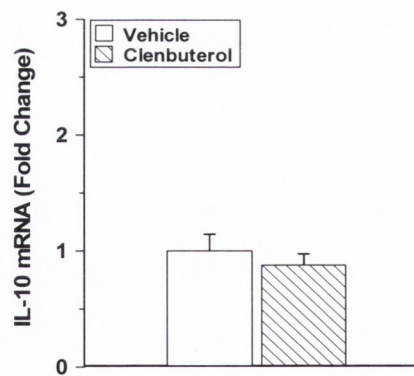


Figure 6.3.5. Chronic clenbuterol treatment was ineffective at inducing IL-10 in rat brain. Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol failed to induce IL-10 mRNA in cortex (A), hippocampus (B) or hypothalamus (C). Data expressed as mean + SEM ($n=7-8$). mRNA data expressed as fold-change vs. saline vehicle (Student's *t*-test).

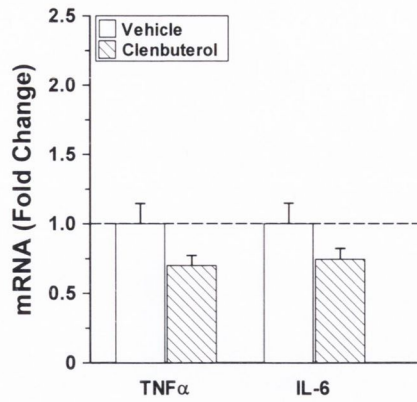
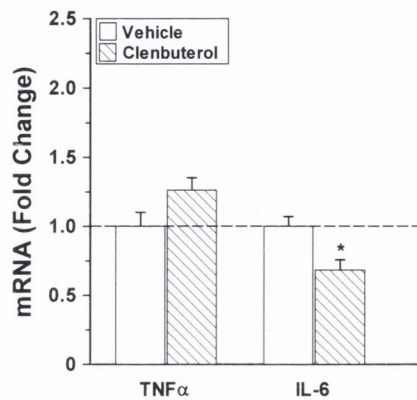
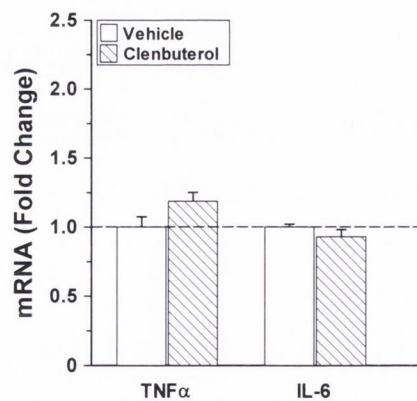
(A) Cortex**(B) Hippocampus****(C) Hypothalamus**

Figure 6.3.6. Chronic clenbuterol treatment did not induce expression of the inflammatory markers TNF- α or IL-6 in rat brain. Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol failed to induce either TNF- α or IL-6 mRNA expression in cortex (A), hippocampus (B) or hypothalamus (C). In fact, clenbuterol suppressed IL-6 in hippocampus (B). Data expressed as mean + SEM ($n=6-8$). mRNA data expressed as fold-change vs. saline vehicle. * $p<0.05$ vs. saline vehicle (Student's t -test).

6.4 Impact of chronic clenbuterol administration on astrocytic and microglial markers

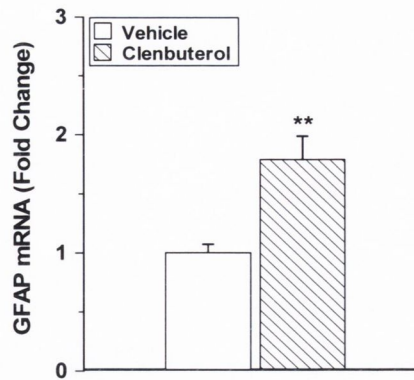
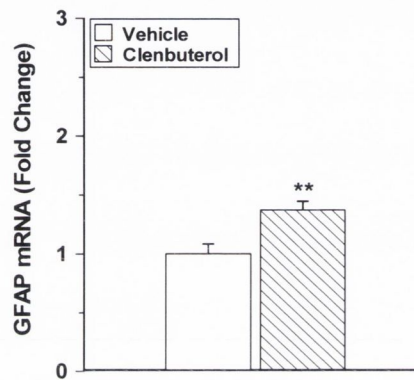
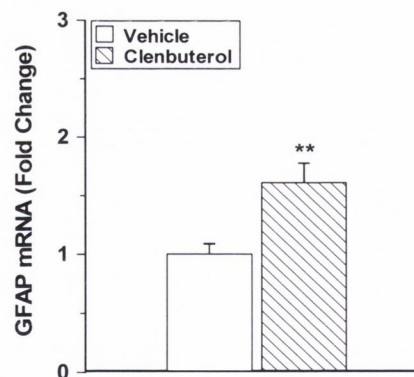
The ability of clenbuterol to activate glial cells in the brain was assessed here. Glial fibrillary acidic protein (GFAP) was used as a marker of astrocytic activation and CD11b was used as a marker of microglial activation in cortex and hypothalamus.

Figure 6.4.1. Chronic clenbuterol treatment induced expression of GFAP in rat brain ***GFAP mRNA expression***

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) induced a significant increase in GFAP mRNA in cortex ($t(13) = -3.90, p=0.0018$), hippocampus ($t(14) = -3.85, p=0.0018$), and hypothalamus ($t(14) = -3.32, p=0.005$) compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=7-8$].

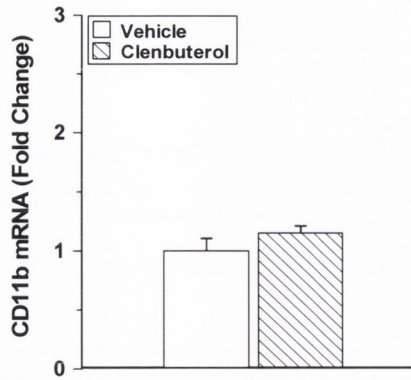
Figure 6.4.2. Chronic clenbuterol treatment was largely ineffective at inducing CD11b in rat brain

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) failed to induce CD11b mRNA expression in cortex ($t(13) = -1.20, p=0.2518$) and hippocampus ($t(14) = -1.63, p=0.1261$). However, clenbuterol induced a significant increase in CD11b mRNA in hypothalamus ($t(13) = -3.10, p=0.0078$) [Student's *t*-test (Pooled Variances) $n=7-8$].

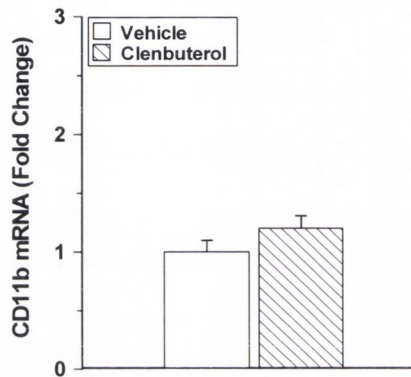
(A) Cortex**(B) Hippocampus****(C) Hypothalamus****Figure 6.4.1. Chronic clenbuterol treatment induced expression of GFAP in rat brain.**

Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol induced GFAP mRNA expression in cortex (A), hippocampus (B) and hypothalamus (C). Data expressed as mean + SEM ($n=7-8$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle (Student's t -test).

(A) Cortex



(B) Hippocampus



(C) Hypothalamus

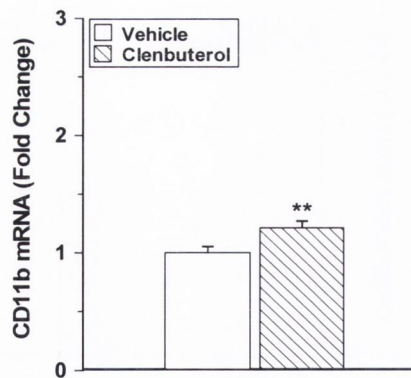


Figure 6.4.2. Chronic clenbuterol treatment was largely ineffective at inducing CD11b in rat brain. Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol failed to induce CD11b in cortex (A) and hippocampus (B) but induced CD11b in hypothalamus (C). Data expressed as mean + SEM ($n=8$). mRNA data expressed as fold-change vs. saline vehicle. ** $p<0.01$ vs. saline vehicle (Student's t -test).

6.5 Impact of chronic clenbuterol administration on anxiety-like behaviour using the elevated plus maze and open field tests

As shown in Figure 6.3.1, chronic clenbuterol administration induced IL-1 β mRNA expression. As IL-1 β expression in the CNS is known to evoke sickness-like behaviours in the rat such as anxiety, this study assessed anxiety-like behaviour in rats using the elevated plus maze and open field tests. Rats that spend less time exploring the open arms of the elevated plus maze and prefer to reside in the enclosed arms are considered to display an anxious phenotype. Thus, time spent in open arms and the number of entries each rat made into the open arms was measured. In the open field test, anxious rats display thigmotaxis (i.e. they stay close to the outer walls of the open field and don't venture into the open space in the centre of the field). Thus, as a measure of anxiety, the number of visits to the outer zone, time spent in outer zone and distance travelled in the outer zone was measured.

Figure 6.5.1 A. Chronic clenbuterol treatment does not alter time spent in open arms

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) did not alter the time spent in open arms ($t(14) = 0.79, p=0.4409$) of the elevated plus maze compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=8$].

Figure 6.5.1 B. Chronic clenbuterol treatment does not alter number of entries into open arms

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) did not increase number of entries into open arms ($t(14) = 0.06, p=0.9551$) of the elevated plus maze compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=8$].

Figure 6.5.2 A. Chronic clenbuterol treatment does not alter number of visits to outer zone

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) did not alter the number of visits to the outer zone ($t(14) = 0.31, p=0.7631$) of the open field compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=8$].

Figure 6.5.2 B. Chronic clenbuterol treatment does not alter time spent in outer zone

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) did not alter the time spent in the outer zone ($t(14) = 0.91, p=0.3761$) of the open field compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=8$].

Figure 6.5.2 C. Chronic clenbuterol treatment does not alter distance travelled in outer zone

Student's *t*-test revealed that clenbuterol (0.03 mg/kg; b.i.d; 21 days) did not alter the distance travelled in the outer zone ($t(14) = 0.77, p=0.4571$) of the open field compared to saline vehicle [Student's *t*-test (Pooled Variances) $n=8$].

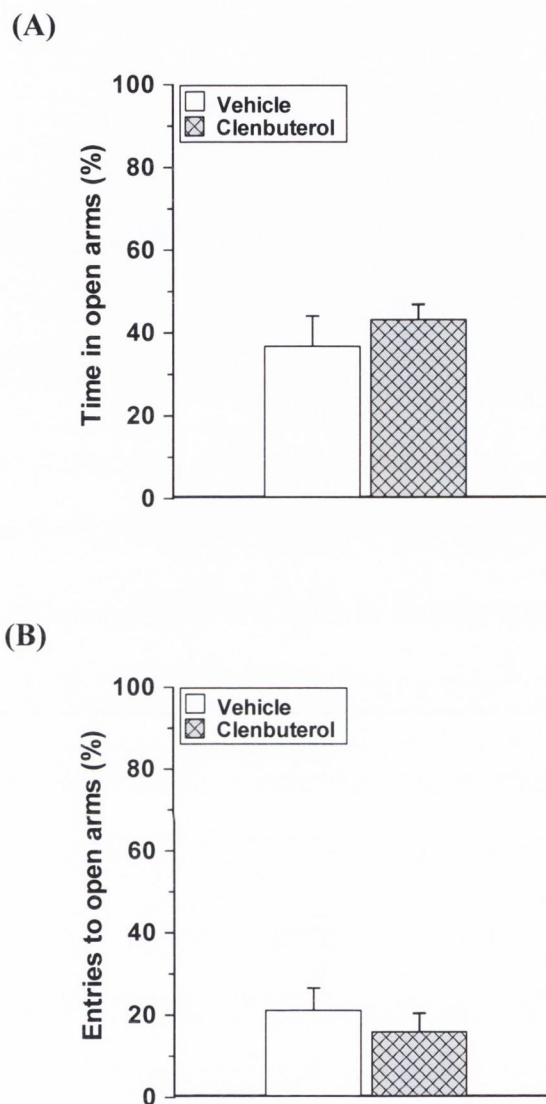
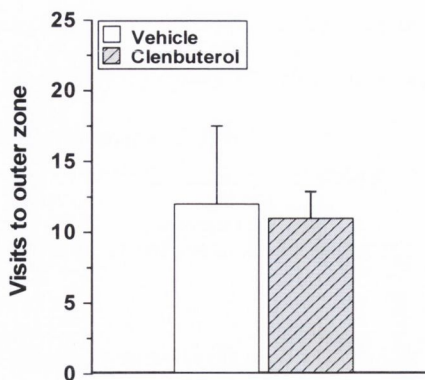
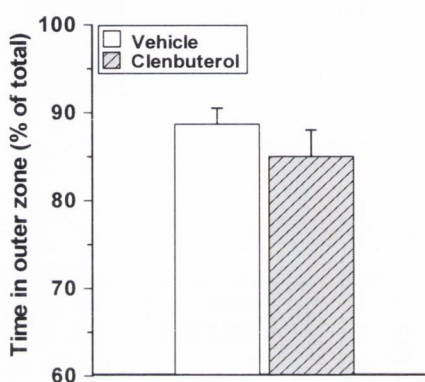


Figure 6.5.1. Chronic clenbuterol treatment does not induce anxiety-like behaviour in the elevated plus maze test. Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol failed to alter time spent in open arms (A) or number of entries into open arms (B). Data expressed as mean + SEM ($n=8$; Student's t -test).

(A)



(B)



(C)

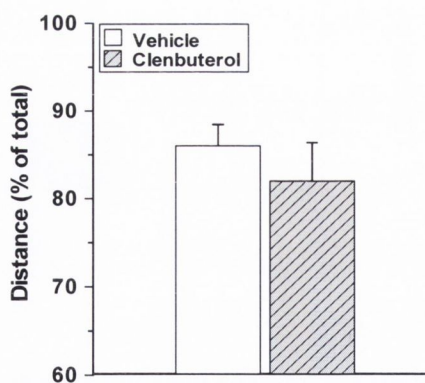


Figure 6.5.2. Chronic clenbuterol treatment does not induce anxiety-like behaviour in the open field test. Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Rats were sacrificed 4 hours after the final clenbuterol injection. Clenbuterol failed to alter visits to the outer zone (A), time spent in outer zone (B), or distance travelled in outer zone (C). Data expressed as mean + SEM ($n=8$; Student's *t*-test).

6.6 Impact of chronic clenbuterol administration on anhedonia using the saccharin preference test

As shown in Figure 6.3.1, chronic clenbuterol administration induced IL-1 β mRNA expression. As IL-1 β expression in the CNS is thought to evoke sickness-like behaviours in the rat, this study assessed anhedonic (depressed) behaviour in rats following clenbuterol administration using the saccharin preference test. An anhedonic rat will cease to gain pleasure from drinking sweetened water and as a result will show less preference for sweetened water. **Note:** There were 8 rats per treatment group in this study and rats were housed 2 per cage. Cage-mates were in the same treatment group. For saccharin preference measurements the total drinking volumes for each cage of 2 animals was recorded, thus there are 4 readouts per treatment group here rather than 8, i.e. $n=4$.

Figure 6.6. Chronic clenbuterol treatment does not alter saccharin preference in the rat ANOVA revealed no significant clenbuterol x time interaction for saccharin preference [$F_{(7, 42)} = 0.3090, p=0.9459$]. Consistent with this finding, *post hoc* analysis revealed that clenbuterol did not affect saccharin preference at any time point compared to saline vehicle [Two-way repeated-measures ANOVA with Bonferroni *post hoc* test; $n=4$].

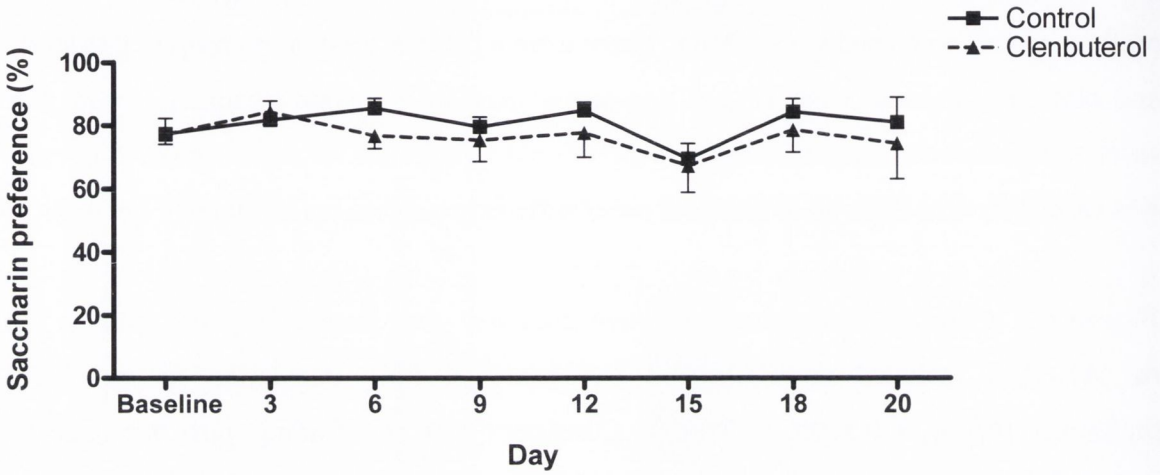


Figure 6.6. Chronic clenbuterol treatment does not induce anhedonia in the saccharin preference test. Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Animals were given free access to saccharin solution and tap water in their home cage for three days prior to the start of the experiment (baseline) and for the duration of the experiment. Two-way repeated-measures ANOVA revealed no significant effect of clenbuterol on saccharin preference at any time point. Data expressed as mean + SEM (Bonferroni *post hoc* test; $n=4$).

6.7 Impact of chronic clenbuterol treatment of body weight and food intake

Previous unpublished results from our group demonstrate that clenbuterol treatment results in reduced feeding in rodents (Ryan, 2010). Thus, body weight and feeding measurements were recorded throughout the present study to determine if the acute effects of clenbuterol subsided over the course of continued administration.

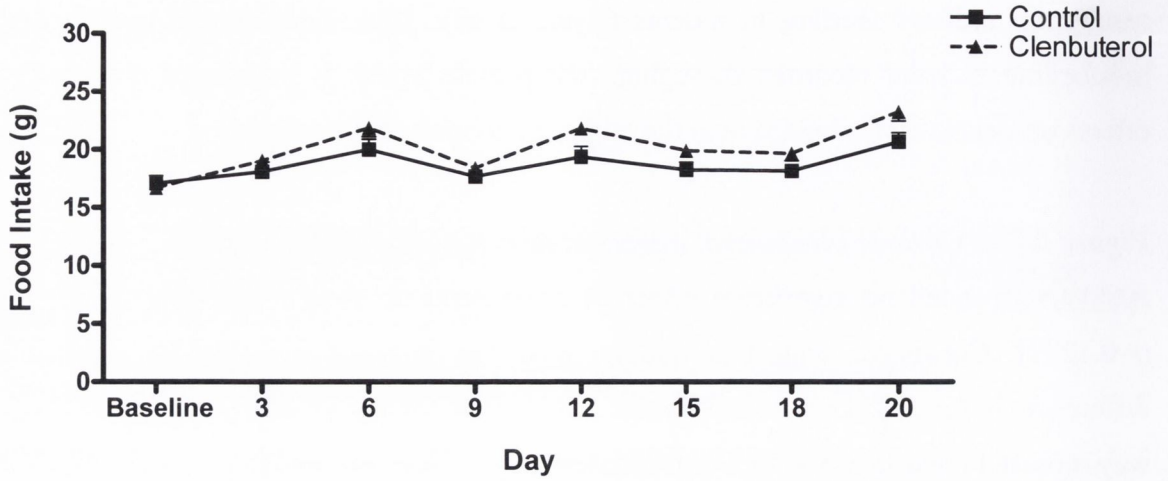
Figure 6.7 A. Chronic clenbuterol treatment does not alter rat food intake

ANOVA revealed no significant effect of clenbuterol on food intake [$F_{(7, 42)} = 3.12$, $p=0.1279$]. Consistent with this finding, *post hoc* analysis revealed no significant difference in food intake between control and clenbuterol groups at any time point [Two-way repeated-measures ANOVA with Bonferroni *post hoc* test; $n=4$].

Figure 6.7 B. Chronic clenbuterol treatment does not alter rat body weight

ANOVA revealed no significant effect of clenbuterol on body weight [$F_{(7, 98)} = 3.35$, $p=0.0885$]. Consistent with this finding, *post hoc* analysis revealed no significant difference in body weight between control and clenbuterol groups at any time point [Two-way repeated-measures ANOVA with Bonferroni *post hoc* test; $n=4$].

(A)



(B)

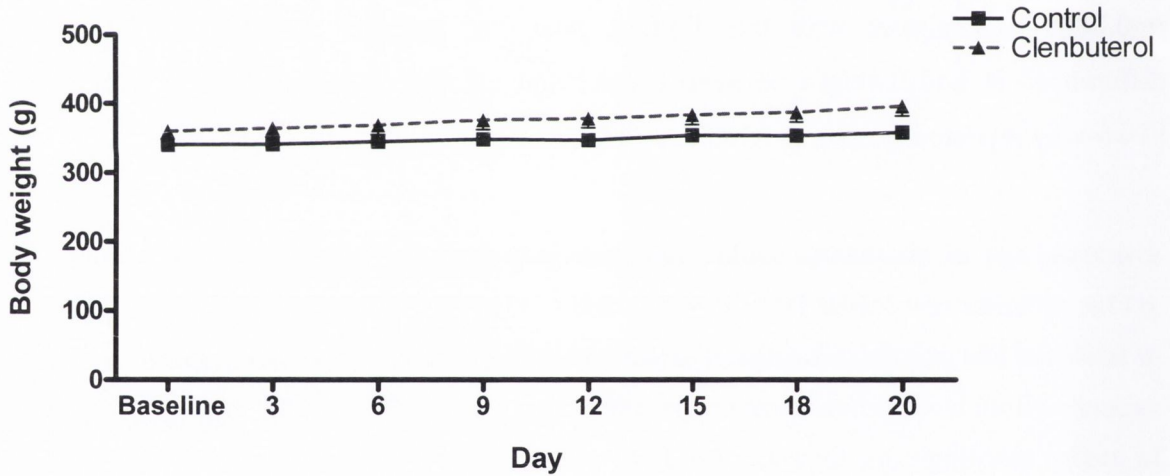


Figure 6.7. Chronic clenbuterol treatment does alter food intake or body weight in the rat. Clenbuterol (0.03 mg/kg) or vehicle (0.89% [w/v] saline) was administered i.p. to rats b.i.d. for 21 days. Food intake and body weight measurements were taken throughout the experiment. Two-way repeated-measures ANOVA revealed no significant effect of clenbuterol on food intake (A) or body weight (B) at any time point. Data expressed as mean + SEM (Bonferroni *post hoc* test; food intake $n=4$; body weight $n=8$).

6.8 Discussion

6.8.1 Chronic clenbuterol treatment activates the IL-1 system without inducing expression of other inflammatory cytokines in rat CNS

The results presented here show that chronic treatment with low-dose clenbuterol (0.03 mg/kg, twice daily for 21 days) induces a 2.5 fold increase in IL-1 β mRNA expression compared to a 15-20 fold increase observed by others following acute administration of the same dose (Ryan, 2010). Thus, IL-1 β expression subsides following continued clenbuterol administration in cortex and hypothalamus. In addition, chronic clenbuterol treatment has no effect on the IL-1 β -inducible gene iNOS. This is important as iNOS expression and the subsequent production of nitric oxide is known to be a contributing factor in the neurodegenerative process (Brown & Neher, 2010). Importantly, chronic clenbuterol administration induces the expression of IL-1ra and IL-1RII, two negative regulators of IL-1 β . As the inflammatory cytokine IL-1 β has been implicated as a key mediator of inflammation (see Allan *et al.*, 2005) the presence of its negative regulators IL-1ra and IL-RII may serve to combat the deleterious effects of IL-1 β on brain function (Bessis *et al.*, 2000; Arend, 2002). Clenbuterol selectively activates IL-1 β as it had no effect on the pro-inflammatory mediators TNF- α and IL-6 which are involved in the classical inflammatory response. This has previously been shown in our laboratory following clenbuterol administration *in vivo* (Ryan, 2010) and is supported by literature demonstrating that stimulation of microglial β -adrenoceptors increases the mRNA expression of IL-1 β but not TNF- α or IL-6 *in vitro* (Tanaka *et al.*, 2002). In addition, the β -adrenoceptor-induced increase in cAMP is known to suppress TNF- α and IL-6 mRNA levels in microglia, however the authors did not report on effects on IL-1 β (Mori *et al.*, 2002).

6.8.2 Chronic clenbuterol treatment does not induce the broad spectrum anti-inflammatory molecule IL-10

Chronic treatment with a low dose of the β_2 -adrenoceptor agonist clenbuterol (0.03 mg/kg; b.i.d; 21 days) did not induce IL-10 mRNA expression in the present study. This is in contrast to other studies which clearly demonstrate that β_2 -adrenoceptor agonists can induce IL-10 expression at both mRNA and protein levels as well as IL-10 signalling

(McNamee *et al.*, 2010b). In addition, noradrenaline and the use of noradrenaline reuptake inhibitors are known to increase IL-10 expression (Szabo *et al.*, 1997; Zhou *et al.*, 2005; Curtin *et al.*, 2009; McNamee *et al.*, 2010b). Thus, the results from the present study are unexpected. However, they may be explained by the low dose of clenbuterol that was used because the 0.03 mg/kg dose has previously been shown to be ineffective at inducing IL-10 mRNA in the rat brain (McNamee *et al.*, 2010b). In this same paper the authors find that a 0.3 mg/kg dose is in fact the lowest dose of clenbuterol that is capable of inducing IL-10 in cortex and hippocampus and this is supported by findings in Chapter 3 of this thesis whereby a higher dose of clenbuterol is capable of inducing IL-10 mRNA. Thus, the most likely explanation for the lack of induction of IL-10 in the present study is that the dose of clenbuterol used was too low. In order to induce IL-10 and its beneficial anti-inflammatory properties, such as inhibition of inflammatory cytokines, chemokines and MHC class II and co-stimulatory molecule expression in glial cells both *in vitro* and *in vivo* (Frei *et al.*, 1994; Shrikant *et al.*, 1995; Ledebuer *et al.*, 2000; Molina-Holgado *et al.*, 2001; Szczepanik *et al.*, 2001; Lynch *et al.*, 2004; Qin *et al.*, 2006; Park *et al.*, 2007) a more potent stimulation of the β_2 -adrenoceptor would be required. This could thereby help to prevent the development of neuroinflammation which can drive the neurodegenerative process.

6.8.3 Chronic clenbuterol treatment activates astrocytes but not microglia

The results of the present study support previous findings which suggest that clenbuterol is an activator of astrocytes (Junker *et al.*, 2002; Ryan, 2010) as evidenced by expression of the astrocytic marker glial fibrillary acidic protein (GFAP). Clenbuterol induced a modest increase in the microglial marker CD11b in the hypothalamus, however, in line with previous results, clenbuterol did not induce CD11b in the cortex (Ryan, 2010). These findings are notable as astrocytic activation is known to have neuroprotective properties (Rudge *et al.*, 1992; Vincent *et al.*, 1996; Aloisi *et al.*, 1997) while chronic activation of microglia has been implicated in many neurodegenerative diseases with detrimental effects in the CNS (Block *et al.*, 2007). Indeed clenbuterol has previously been shown to induce neuroprotection via activation of the β_2 -adrenoceptor and subsequent induction of nerve growth factor (NGF) expression (Culmsee *et al.*, 1999a). In addition, clenbuterol-induced NGF protects against excitotoxic cell death *in vitro* and

ischaemia-induced toxicity *in vivo* in hippocampal neurons (Semkova *et al.*, 1996; Culmsee *et al.*, 1999b).

6.8.4 Chronic clenbuterol treatment does not cause an anxious or depressive phenotype in the rat

As central IL-1 β expression has been previously been implicated in precipitating anxiety and depressive-like behaviours, this study examined the impact of chronic treatment with clenbuterol on anxiety behaviour using the elevated plus maze and open field tests, and examined depressive-like behaviour (anhedonia) using the saccharin preference test. Clenbuterol-treated rats showed no evidence of anxious behaviour in the elevated plus maze. Due to an innate fear of open spaces an anxious rat avoids the open arms and spends the majority of the time in the closed arms. The present results show no difference between groups with regard to time spent in the open arms or the number of entries in to open arms. Similarly, clenbuterol-treated rats did not display anxious behaviour in the open field test. When placed into the open field arena a healthy rat will explore the novel environment i.e. both inner and outer zones of the open field. Anxiety in the rat manifests as thigmotaxis or a tendency to stay near the perimeter (outer zone) of the open field. Clenbuterol did not affect the number of entries to the outer zone or the time spent in the outer zone. Reduced exploration in the open field is also used as an indicator of anxiety-like behaviour. Clenbuterol treatment did not affect exploration in this study as indicated by the fact that both control and clenbuterol-treated rats travelled the same distance during the open field test. These results indicate that chronic clenbuterol treatment does not precipitate an anxious phenotype in rats.

In this study, clenbuterol also had no effect on saccharin preference over a 21 day period indicating that clenbuterol treatment did not induce depressive-like behaviour in the rat. The saccharin preference test is often used to measure depressive-like behaviour in rats. Depression is a complex disorder and is associated with a variety of behavioural abnormalities. Some of the behavioural features that are mimicked by rats include anhedonia (inability to experience pleasurable emotions from normally pleasurable things such as eating), appetite/weight change, and sleep disturbances among others (Overstreet *et al.*, 2007). Anhedonia is a prominent sign of depression which is evidenced in the rat by decreased sweet intake in the saccharin preference test and it is clear from this study

that clenbuterol fails to induce an anhedonic response. Chronic clenbuterol administration had no effect on body weight or food intake which suggests that the reduction in feeding seen after acute clenbuterol administration is transient and the animals regain their appetite and maintain a healthy weight with continued treatment.

There is now a general consensus that inflammatory mediators including IL-1 β can exert both adaptive and maladaptive responses in the brain, depending on the concentration, the duration of exposure and the overall immune environment (Pinteaux *et al.*, 2006; Goshen *et al.*, 2007; Spulber *et al.*, 2009). The fact that clenbuterol-induced IL-1 β does not induce an anxious or depressed phenotype could be partly due to the low concentration of IL-1 β expressed during long-term administration of clenbuterol. In addition as mentioned above, clenbuterol induces expression of IL-1ra and the IL-1RII in a range of brain regions including the hypothalamus, both of which prevent excessive or prolonged activation of the IL-1 system (Bessis *et al.*, 2000; Arend, 2002). Thus, perhaps increased expression of the anti-inflammatory molecules IL-1ra and IL-1RII may serve to limit the effects of clenbuterol-induced IL-1 β on brain function and behaviour. In this regard, whilst both clenbuterol and noradrenaline reuptake inhibitors have neuroprotective (Zhu *et al.*, 1999; Culmsee *et al.*, 1999a; Gleeson *et al.*, 2010) and memory enhancing properties (Clinton *et al.*, 2006; Ramos *et al.*, 2008; Seu *et al.*, 2009), any role that IL-1 system components may have in such neuroprotective or memory enhancing actions of these drugs remains to be elucidated.

6.8.5 Summary

The results presented here demonstrate that clenbuterol is well tolerated following continued administration. Despite the small induction of IL-1 β there was no anxiety or depressive-like behaviours observed and there were no changes in rat food intake or body weight. In addition, a similar cytokine profile was observed compared to acute administration of clenbuterol whereby the IL-1 system was activated in a controlled manner whereby the negative regulators of IL-1 β , IL-1ra and IL-1RII were also induced. In addition, IL-1 β was selectively induced as shown by the lack of induction of other mediators involved in the typical inflammatory response, i.e. TNF- α and IL-6. All of these long-term effects appear to be mediated by astrocytes rather than microglia which is notable as astrocytic activation is known to have neuroprotective properties while chronic

activation of microglia has been implicated in many neurodegenerative diseases with detrimental effects in the CNS. Thus overall, β_2 -adrenoceptor stimulation by clenbuterol appears to suppress the innate inflammatory response in the brain following long term administration and this is not accompanied by any behavioural changes, despite the induction of IL-1 β .

Chapter 7

Concluding Remarks

7.1 β_2 -adrenoceptor stimulation suppresses NF κ B activity and promotes an anti-inflammatory phenotype in LPS-induced inflammation in rat brain

The results presented here demonstrate that the β_2 -adrenoceptor agonist clenbuterol has a profound ability to suppress NF κ B activity in the rat brain under normal non-inflamed conditions and at very low doses. It appears that clenbuterol suppresses NF κ B activity by increasing the inhibitory I κ B proteins, in particular the I κ B α subtype. The findings also confirm, through the use of selective and non-selective antagonists for β_1 - and β_2 -adrenoceptor subtypes, that the effect of clenbuterol on NF κ B activity and I κ B α expression is mediated predominantly by the β_2 -adrenoceptor. Initially, it was proposed that the suppressive actions of β_2 -adrenoceptor stimulation on NF κ B were mediated largely by increased I κ B α , due to the ability of both clenbuterol and formoterol to increase I κ B α expression. However further investigation demonstrated that another β_2 -agonist, salbutamol, suppressed NF κ B without increasing I κ B α . Thus, it is clear that I κ B α is not the sole mediator of β_2 -adrenoceptor agonist-induced suppression of NF κ B activity and other mechanisms remain to be determined.

The results discussed thus far were investigated under normal conditions in the rat brain. No previous description of the effects of β_2 -adrenoceptor agonists on LPS-induced NF κ B activity in the rat brain could be found in the literature. The data presented here clearly show that clenbuterol suppressed the LPS-induced activation of NF κ B and this was accompanied by an increase in inhibitory I κ B α expression. Specifically, it appears that clenbuterol blocked the LPS-induced phosphorylation of I κ B α , thus sequestering NF κ B in the cytoplasm and preventing transcription of inflammatory genes. Literature from *in vitro* experiments based on cells of the periphery suggests that IL-10 can inhibit I κ B α phosphorylation. There is no evidence of this in the CNS, however results from the present thesis and from others in our group demonstrate that clenbuterol increases IL-10 expression and signalling through STAT-3 (McNamee *et al.*, 2010b). Thus, it is possible that the inhibition of I κ B α phosphorylation by clenbuterol could be mediated by the clenbuterol-induced increase in IL-10. However, this remains to be determined. As a result of the suppression of NF κ B activity, downstream signalling events were also suppressed such as TNF- α and ICAM-1. The results of the present study support other work which implicates the regulation of NF κ B activity in the anti-inflammatory or immunosuppressive effects of β_2 -adrenoceptor agonists (Farmer & Pugin, 2000).

Over-activation of the NF κ B pathway is a pathogenic feature of neuroinflammatory disorders such as Alzheimer's disease. In this regard, it is thought that activation of NF κ B plays a role in the early phases of Alzheimer's disease when initiation of neuritic plaques and neuronal apoptosis occurs (Kaltschmidt *et al.*, 1999). Thus, methods which inhibit NF κ B may be a useful therapeutic tool in acute and chronic diseases where the inflammatory response becomes dysregulated. However, it is important to note that NF κ B plays an essential role in regulating the host immune response and maintaining host defences. For this reason, suppression of NF κ B may not be beneficial in the long term. Others have suggested that blocking specific points in the NF κ B signalling cascade, such as IKK activity, might reduce the potential side effects of long-term NF κ B suppression (Yamamoto & Gaynor, 2001).

Consistent with previous reports from our laboratory, the present results demonstrate that clenbuterol independently induced IL-1 β expression and had no effect on LPS-induced IL-1 β (Ryan, 2010; McNamee *et al.*, 2010c). Simultaneously, clenbuterol increased expression of two negative regulators of IL-1 β , the IL-1 receptor antagonist (IL-1ra) and the IL-1 type II decoy receptor (IL-1RII), both independently and in the presence of LPS, again replicating earlier findings from our group (McNamee *et al.*, 2010c). Due to the fact that expression of IL-1ra and IL-1RII prevents prolonged activation of the IL-1 system (Carter *et al.*, 1990; Colotta *et al.*, 1994), it was hypothesised that they may be beneficial in combating any deleterious effects of clenbuterol-induced IL-1 β on brain function. In this regard, the results presented here show that clenbuterol-induced IL-1 β fails to induce the classical IL-1-mediated signalling pathway including activation of NF κ B. In fact, NF κ B is suppressed by clenbuterol. This data supports previous work showing that clenbuterol selectively activates the IL-1 system without activating other pro-inflammatory mediators such as TNF- α , IL-6, IFN- γ and MAPK, or the IL-1 signalling pathways JNK or p38 (Heffernan, 2009; Ryan, 2010; McNamee *et al.*, 2010c). Thus, the results presented here reinforce the evidence that clenbuterol elicits anti-inflammatory and immunosuppressive actions in the rat brain and suggest that if β_2 -adrenoceptor agonists were to be used therapeutically it is unlikely that a subsequent increase in IL-1 β would contribute to inflammation or cause injury in the brain.

7.2 Co-administration of β_2 -adrenoceptor agonist and glucocorticoid elicits complimentary anti-inflammatory actions in the rat brain

The results reported in this thesis demonstrate for the first time the synergistic anti-inflammatory actions of combined treatment with the β_2 -adrenoceptor agonist clenbuterol and the synthetic glucocorticoid dexamethasone in the rat brain. There is little to no evidence in the literature regarding the complimentary effects of combined treatment with these two agents on inflammatory mediators in the CNS. The vast majority of research has focused on inflammatory disorders of the periphery such as asthma and COPD where the combination of long-acting β_2 -adrenoceptor agonists (LABAs) and inhaled glucocorticoids provide better control of asthma than either agent alone and are now the “gold standard” of therapy (Greening *et al.*, 1994; Shrewsbury *et al.*, 2000). The complimentary effects of combined treatment in the periphery are thought to be mediated by two actions: 1) the ability of glucocorticoids to increase transcription of the β_2 -adrenoceptor gene; 2) the ability of β_2 -agonists to activate the glucocorticoid receptor (GR) via increased cAMP and PKA (Barnes, 2002). Thus, in a complementary way, each class of drug enhances the others receptor. Both clenbuterol and dexamethasone independently induce anti-inflammatory actions in the brain. For example, dexamethasone is known to inhibit cytokine production from glial cells (Nishida *et al.*, 1989; Velasco *et al.*, 1991; Chao *et al.*, 1992; Kimberlin *et al.*, 1995) and clenbuterol has neuroprotective actions in rodent models of cerebral ischaemia (Semkova *et al.*, 1996; Zhu *et al.*, 1998; Culmsee *et al.*, 1999b; Junker *et al.*, 2002) and in both *in vitro* and *in vivo* models of excitotoxicity (Semkova *et al.*, 1996; Gleeson *et al.*, 2010). So in theory, co-administration of these two agents should be beneficial in the brain. In the present experiment, pre-treatment with dexamethasone prior to clenbuterol blocked the clenbuterol-induced increase in IL-1 β and iNOS in rat brain. In addition, dexamethasone had no effect on clenbuterol-induced expression of the anti-inflammatory mediators IL-1ra, IL-1RII, IL-10 and SOCS-3. Furthermore, dexamethasone pre-treatment induced an additive (and perhaps synergistic) increase in the NF κ B-inhibitory molecule I κ B α (without an additive effect on NF κ B suppression). The mechanism by which the present effects occur has yet to be investigated. However, our laboratory has previously shown that dexamethasone is capable of independently increasing expression of β_2 -adrenoceptors in glial cells of the brain (Ryan, 2010). In addition, given the fact that β -adrenergic signalling in the brain activates the downstream effectors cAMP and PKA

(Hertz *et al.*, 2004; Marien *et al.*, 2004) and these are known to be responsible for β_2 -agonist-mediated activation of the GR in the periphery, it is possible that clenbuterol activates the GR in the brain in a similar manner (**Figure 7.1**). In this regard, the impact of clenbuterol on GR activation in the CNS warrants further investigation.

In summary, administration of these two agents elicited complimentary anti-inflammatory actions in the brain. It is still unclear whether pre-treatment with dexamethasone prior to clenbuterol results in an additive anti-inflammatory effect, because of the two agents affecting a common mechanism, or whether there is true synergy. Some studies based on cells of the periphery have shown true synergy between the two classes of drugs, however this remains to be elucidated in the CNS.

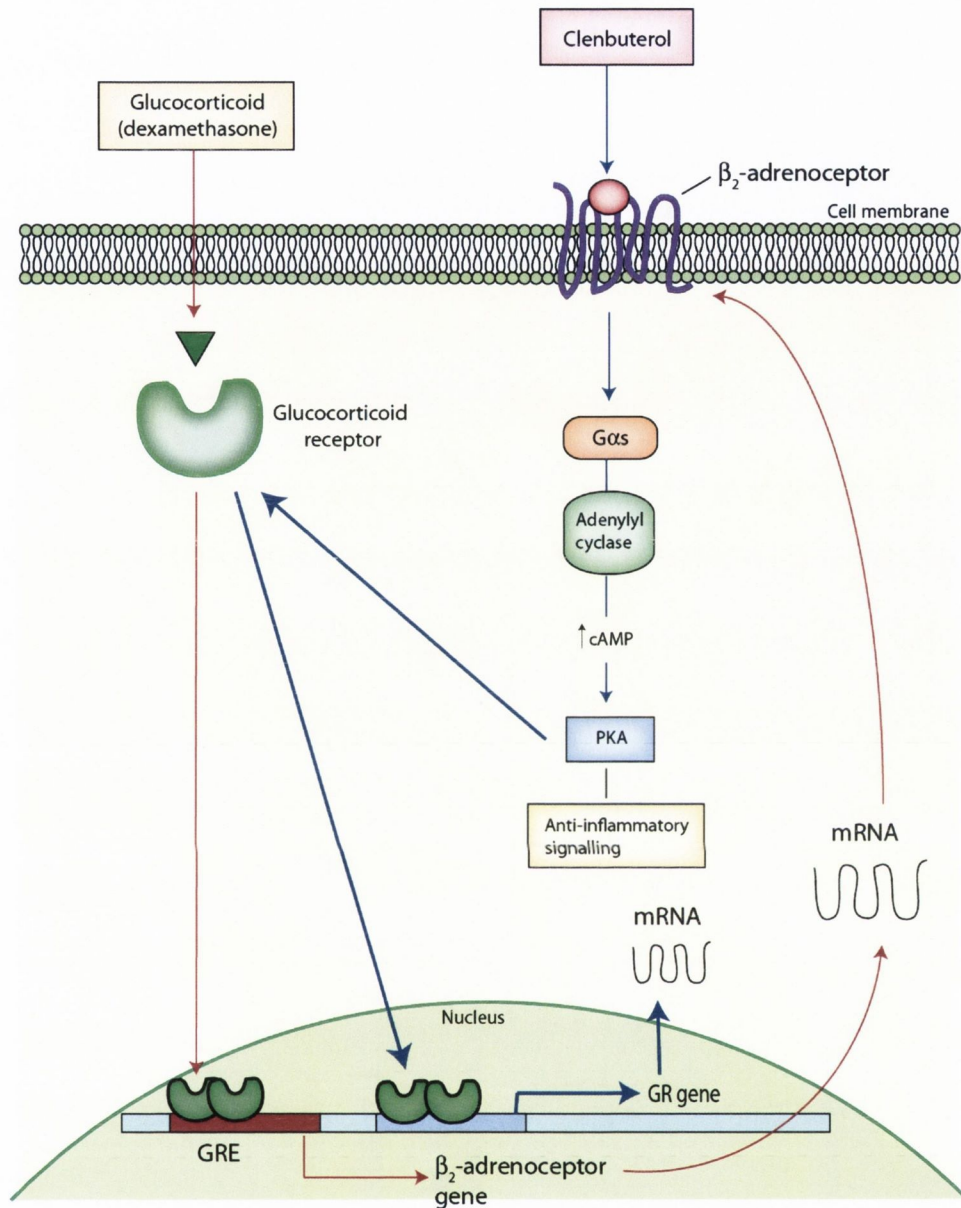


Figure 7.1: Proposed complimentary actions of the β_2 -adrenoceptor agonist clenbuterol and the glucocorticoid dexamethasone in the CNS. In a complementary way, each class of drug enhances the others receptor. Dexamethasone enters the cell and binds to the intracellular glucocorticoid receptor (GR) which translocates to the nucleus and binds to the glucocorticoid response elements (GRE) in the promoter region of the β_2 -adrenoceptor gene resulting in increased synthesis of β_2 -adrenoceptors. The β_2 -adrenoceptor agonist clenbuterol activates PKA which may have a direct effect on the nuclear translocation of the GR and increase synthesis of the GR. mRNA: messenger ribonucleic acid [modified from (Barnes, 2002)].

7.3 Central expression of IL-1 β induced by clenbuterol has no adverse effects on behaviour in rats

A role for central IL-1 β in the profound decrease in locomotor activity and food intake induced by clenbuterol was investigated. As central IL-1 β expression is known to mediate sickness-like behaviours in rodents, such as reduced locomotor activity and reduced food intake (Plata-Salaman *et al.*, 1988; Dantzer *et al.*, 1998; Konsman *et al.*, 2008), it was proposed that the depressed behaviour induced by clenbuterol was a consequence of the parallel increase in central IL-1 β . Reassuringly however, the results showed that the reduction in locomotor activity and food intake occurred regardless of whether IL-1 β was expressed in the brain or not. Thus, the fact that IL-1 β has no effects on sickness behaviour further supports the argument put forward in this thesis that central IL-1 β induced by clenbuterol is unlikely to elicit any negative downstream effects. Previous unpublished results from our laboratory had already shown that there is a difference between the sickness behaviour elicited by clenbuterol compared to the classical sickness behaviour elicited by LPS (Ryan, 2010). This work by Karen Ryan proposed that the depressed behaviour caused by clenbuterol was more of a stress response, caused by its profound ability to transiently increase heart rate, because blocking the clenbuterol-induced tachycardia reversed the clenbuterol-induced reduction in feeding. Furthermore, Karen Ryan also showed that the selective β_2 -adrenoceptor antagonist ICI-118,551 (which blocks both central and peripheral β_2 -adrenoceptors) blocked the behavioural effects, suggesting that β_2 -adrenoceptor activation was responsible for the depressed behaviour. While this did not determine whether the behaviour was mediated by central or by peripheral receptors, the results from the present thesis aimed to dissociate between the two. Data presented here show that the clenbuterol-induced behavioural effects were in fact mediated by peripheral β_2 -adrenoceptors because the peripherally acting β -adrenoceptor antagonist nadolol effectively blocked the suppressive effect of clenbuterol on feeding and locomotor activity as did the brain penetrant β -adrenoceptor antagonist propranolol which blocked both central and peripheral receptors. Thus, if β_2 -adrenoceptor agonists were to be used therapeutically for the treatment of neuroinflammation their effects on cardiac β_2 -adrenoceptors would have to be addressed. Encouragingly, reports have already suggested that the peripheral effects of clenbuterol subside with repeated administration (Brockway *et al.*, 1987; Hoey *et al.*, 1995).

Importantly, results from this thesis also demonstrate that long-term administration of clenbuterol even in the presence of central IL-1 β expression does not provoke anxious or depressive-like behaviour in rats. In this regard, rats did not display anxious behaviour in the open field or elevated plus maze tests. In addition, they did not display depressive-like behaviours as evidenced by no change in the saccharin preference test. This was reassuring because IL-1 β is thought to be involved in precipitating these behaviours (Dantzer, 2001). The clenbuterol-induced expression of the anti-inflammatory molecules IL-1ra and IL-1RII was maintained following chronic administration, thus perhaps increased expression of these molecules serves to limit the effects of clenbuterol-induced IL-1 β on brain function and behaviour. In support of the idea that IL-1 β is not involved in any detrimental effects in the brain, no increase in other inflammatory mediators that usually accompany IL-1 β activity (such as TNF- α , iNOS and IL-6) was observed. Overall, it appears that β_2 -adrenoceptor stimulation by clenbuterol suppresses the innate inflammatory response in the brain following long term administration and this is not accompanied by any behavioural changes, despite the induction of IL-1 β .

7.4 Clinical implications

Overall, given the ability of such low concentrations of β_2 -adrenoceptor agonists to generate an anti-inflammatory cytokine profile in the brain and the ability of clenbuterol in particular to reduce LPS-induced inflammation, β_2 -adrenoceptor agonists appear to have significant potential in regulating CNS inflammation. While some β_2 -adrenoceptor agonists, like formoterol and salmeterol, are currently used therapeutically to treat chronic pulmonary diseases, like COPD, the results presented here indicate that this class of compounds could also be beneficial in combating neurodegenerative diseases that have an inflammatory component. In particular, these results present persuasive evidence that β_2 -adrenoceptors in combination with glucocorticoids interact in a beneficial way in the CNS. Potential benefits of combined treatment include the ability of glucocorticoids to prevent the downregulation of the β_2 -adrenoceptor that can occur with chronic use of β -agonists, also β_2 -agonists may potentiate the local anti-inflammatory actions of glucocorticoids (Barnes, 2002). In addition, the present results show that the glucocorticoid dexamethasone prevents the increase in IL-1 β that occurs with some types of β_2 -adrenoceptor agonists such as clenbuterol, further supporting the capacity of glucocorticoids to prevent any potentially adverse consequences of chronic β_2 -agonist

therapy. While further research is needed to determine the mechanisms involved in the effect of clenbuterol on behaviour and ways in which these behavioural effects may be overcome, β_2 -adrenoceptor agonists may be a promising new therapeutic strategy for combating inflammation-related neurodegeneration.

Chapter 8

Future Directions

8.1 Future Directions

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

1) Results presented here propose that the β_2 -adrenoceptor agonist clenbuterol suppresses NF κ B activity via the stabilisation of I κ B α . Evidence suggests that IL-10 is capable of stabilising I κ B α by preventing I κ B α phosphorylation. It would be interesting to inhibit clenbuterol-induced IL-10 in the brain to determine whether it was involved in the stabilisation of I κ B α and the subsequent suppression of NF κ B activity.

2) The stabilisation of I κ B α may not be the only mechanism by which clenbuterol suppresses NF κ B activity because the β_2 -adrenoceptor agonist salbutamol was capable of suppressing NF κ B activity in the absence of I κ B α expression. Due to the ability of β_2 -agonists to activate SOCS family members and the ability of SOCS-1 in particular to suppress NF κ B activity, it would be interesting to see if SOCS-1 mRNA expression was increased in the brains of salbutamol-treated animals.

3) Further studies will be necessary to determine the cellular source and site of action of IL-1 β , IL-10, and I κ B α . To determine whether these molecules are released from microglia or astrocytes, rats should be pre-treated with minocycline (microglial inhibitor) and arundic acid (astrocytic inhibitor) prior to clenbuterol treatment. In addition, double immunostaining of clenbuterol-treated rat brains should be performed.

4) The mechanisms underlying the complimentary anti-inflammatory effects of combined treatment with the β_2 -adrenoceptor agonist clenbuterol and the glucocorticoid dexamethasone warrants further investigation. Given the anti-inflammatory properties of glucocorticoid receptor (GR) activation and the ability of β_2 -adrenoceptor agonists to independently activate the GR it would be interesting to measure GR activation to determine whether combined treatment with clenbuterol and dexamethasone has an additive effect on GR activation compared to either agent alone.

5) Cytokine expression following chronic clenbuterol administration is reduced compared to acute administration. β_2 -adrenoceptor desensitisation is known to occur

following continued stimulation of β_2 -adrenoreceptor agonists and this may be induced by IL-1 β . The effects of continued clenbuterol treatment on β_2 -adrenoceptor stability should be explored.

6) The role of IL-1 in clenbuterol-induced neuroprotection should be investigated further in IL-1RI knockout 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.

7) 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.

Publications

Publications

Ryan KJ, Griffin ÉW & Connor TJ. (2010). Complementary anti-inflammatory actions of the beta-adrenoceptor agonist clenbuterol and the glucocorticoid dexamethasone in rat brain. *Journal of Neuroimmunology* **232**, 209-216.

Peer-reviewed journal article

Ryan KJ, Griffin ÉW, Yssel JD, Ryan KM, Harkin A, Connor TJ. (2011). Stimulation of central β_2 -adrenoceptors suppresses NF- κ B activity in hippocampus and cortex: A role for I κ B. *Brain, Behavior, and Immunity*, **25** (Suppl 2): S222.

Abstract for poster presentation at the annual Psychoneuroimmunology Research Society conference, Chicago, Illinois

Ryan KJ, Griffin ÉW, Yssel JD, Ryan KM, Harkin A, Connor TJ. (2011). Stimulation of central β_2 -adrenoceptors suppresses activation of NF κ B in rat brain. *Irish Journal of Medical Science*. **180** (Suppl 9):S290–S291.

Abstract for oral presentation at the annual Royal Academy of Medicine Ireland meeting, Dublin, Ireland

Ryan KJ, Ryan KM, Griffin ÉW, McNamee EN, Harkin A, Connor TJ. (2010). A role for central IL-1beta in the suppression of locomotor activity induced by the beta₂-adrenoceptor agonist clenbuterol? *Brain, Behavior, and Immunity*, **24** (Suppl 1): S2-S2.

Abstract for poster presentation at the annual Psychoneuroimmunology Research Society conference, Dublin, Ireland

Ryan KJ, Griffin ÉW, Connor TJ. (2010). Combined treatment with the β_2 -adrenoceptor agonist clenbuterol and the glucocorticoid dexamethasone induces a synergistic anti-inflammatory cytokine phenotype in rat brain. *Irish Journal of Medical Science*. **179** (Suppl 7):S300.

Abstract for poster presentation at the annual Neuroscience Ireland conference, Trinity College Dublin, Ireland [Awarded 1st prize for poster presentation]

Ryan KJ, Ryan KM, Griffin ÉW, Heffernan S, McNamee EN, Harkin A, Connor TJ. (2010). A role for central IL-1 β in the suppression of locomotor activity induced by the β_2 -adrenoceptor agonist clenbuterol? *FENS Forum Abstracts*. **Volume 5**, 164.34.

Abstract for poster presentation at the annual Forum of European Neuroscience (FENS), Amsterdam

Ryan KJ, Griffin ÉW, Connor TJ. (2009). Treatment with the β_2 -adrenoceptor agonist clenbuterol activates the central interleukin-1 system without provoking depressive or anxiety-like behaviour in rats. *Irish Journal of Medical Science*. **179** (Suppl 3):S138.

Abstract for poster presentation at the annual Neuroscience Ireland conference, Trinity College Dublin, Ireland

Other poster presentations (unpublished)

Ryan KJ, Griffin ÉW, Yssel JD, Ryan KM, Harkin A, Connor TJ. (2011). Stimulation of central β_2 -adrenoceptors suppresses activation of NF κ B in rat brain.

Poster presentation at the annual Block Multiple Sclerosis Symposium, Dublin, Ireland

Ryan KJ, Griffin ÉW, Yssel JD, Ryan KM, Harkin A, Connor TJ. (2011). Stimulation of central β_2 -adrenoceptors suppresses NF- κ B activity in hippocampus and cortex: A role for I κ B.

Poster presentation at the Cytokines Action in the Brain Symposium, Dublin, Ireland

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