REGULATION OF SUSCEPTIBILITY AND RESISTANCE TO EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS BY NEUROENDOCRINE AND IMMUNE FACTORS

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science, University of London.

By

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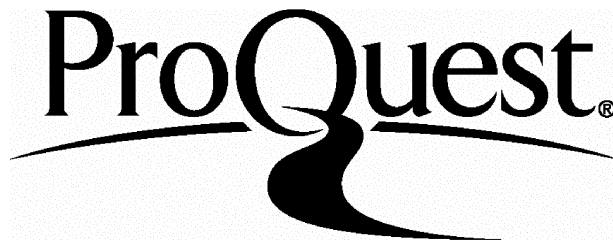Department of Neurochemistry,
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ABSTRACT

Susceptibility to the induction of the T-cell mediated autoimmune disease experimental allergic encephalomyelitis (EAE) in Lewis rats is attributed to the decreased production of corticosterone compared to the histocompatible EAE-resistant Fischer rat. This hypo-responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis appears to be caused by a defect in hypothalamic corticotrophin-releasing factor-41 (CRF) secretion, possibly a consequence of abnormal signal transduction in the CRF neuron.

Activation of the HPA axis with either bacterial endotoxin (lipopolysaccharide, LPS), interleukin (IL)-1β or tumour necrosis factor (TNF)–α increased plasma corticosterone levels up to 10-fold greater in Fischer rats than in Lewis rats. However, second messenger and neurotransmitter production in the paraventricular nucleus of the hypothalamus in response to LPS was greater in the Lewis rat suggesting that the Lewis CRF neuron requires greater stimulation and/or must increase production of second messengers to precipitate a steroid response which is substantially smaller than that observed in the Fischer rat. In contrast, changes in hypothalamic cyclic adenosine monophosphate, prostaglandin E_2 and noradrenaline following administration of IL-1β were similar in both strains and were not as great as those seen following LPS. It is possible that cytokines produced following LPS administration (e.g. IL-1β, TNF-α) may act synergistically to activate the systems measured in this study or that IL-1β stimulates alternative pathways which lead to adrenocortical activation.

Recovery from transfer EAE in intact Lewis rats was associated with increased numbers of apoptotic T lymphocytes (32%, 7 days post transfer) in the spinal cord at the time of the expected increase in plasma corticosterone levels; intact Fischer rats

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displayed reduced T cell numbers in the spinal cord, increased T cell apoptosis (44%, 7 d.p.t.) and milder disease. Adrenalectomised Lewis and Fischer rats developed severe and often fatal EAE and had low numbers of apoptotic T cells (max. 8.5 %) in the spinal cord. Glucocorticoid mediated apoptosis of T cells, the initiator cell type for the induction of EAE, appears to be an effective and important mechanism for recovery from EAE and perhaps contributes to disease resistance.

Induction of EAE is dependent on secretion of T helper 1 (Th1) cytokines while recovery is associated with a switch to a Th2 cytokine profile which may underlie the subsequent refractoriness to re-induction of disease. Increased corticosterone secretion in Fischer rats may suppress the development of a Th1 response and may be a contributory factor in conferring resistance. The incidence of EAE but not the severity was increased in Fischer rats following administration of IL-12 whereas Lewis rats developed severe EAE with rapid onset, the likely result of increased Th1 cytokine production and macrophage activation. Peripheral administration of IL-12 to Lewis rats up to one week after full recovery from paralysis resulted in clinical relapse when EAE was induced by active immunisation, whilst co-administration of myelin basic protein with IL-12 was required to elicit relapse following recovery from transfer EAE, suggesting IL-12 (re)-activates antigen specific T-cells peripherally, triggering a secondary wave of cellular infiltration and disease relapse. Disease exacerbation and re-induction was accompanied by extensive up-regulation of inducible nitric oxide synthase immunoreactivity, microglial activation and a predominance of macrophages in CNS lesions.

These studies highlight the importance of CNS control of the HPA axis in determining resistance and recovery from EAE through corticosterone-mediated

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mechanism(s) and also suggest a role for the Th1-cell-inducing cytokine, IL-12, in mediating disease relapse by promoting macrophage-mediated pathology.

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Abbreviations

ACTH	adrenocorticotrophic hormone		IFN	interferon
ADX	adrenalectomised		IL	interleukin
ANOVA	analysis of variance		iNOS	inducible nitric oxide synthase
AVP	arginine vasopressin		INT	intact
BBB	blood brain barrier		i.p.	intraperitoneal
cAMP	cyclic adenosine monophosphate		ir	immunoreactive
			ISNT	*in situ* nick translation
CD	cluster of differentiation		i.v.	intravenous
CFA	complete Freund's adjuvant		LEW	Lewis (rat)
CNS	central nervous system		LPS	lipopolysaccharide
COX	cyclooxygenase		M	molar
CRF	corticotropin releasing factor-41		MBP	myelin basic protein
			MHC	major histocompatibility complex
CS	corticosterone			
(a)CSF	(artificial) cerebrospinal fluid		MOG	myelin oligodendrocyte glycoprotein
DEX	dexamethasone			
DNA	deoxyribonucleic acid		mr	murine recombinant
d.p.i.	days post immunisation		mRNA	messenger ribonucleic acid
d.p.t.	days post transfer		MS	multiple sclerosis
EAE	experimental allergic encephalomyelitis		NA	noradrenaline
			NO	nitric oxide
EBSS	Earle's balanced salt solution		PBS	phosphate buffered saline
EIA	enzyme immunoassay		PPD	purified protein derivative
F344	Fischer (rat)		PGE$_2$	prostaglandin E$_2$
FCS	foetal calf serum		PLP	proteolipid protein
GC	glucocorticoids		PVN	paraventricular nucleus
GM-CSF	granulocyte-macrophage colony stimulating factor		RIA	radioimmunoassay
			rr	rat recombinant
h	hour		TBS	Tris buffered saline
HPA	hypothalamic-pituitary-adrenal		TGF	transforming growth factor
HPLC	high performance liquid chromatography		Th	T helper
			TNF	tumour necrosis factor
i.c.v.	intracerebroventricular			

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CHAPTER I

Pathogenic Mechanisms of Inflammatory Demyelination in the Central Nervous System

1.1   Introduction

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS).   Although the aetiology of MS is unknown, evidence from animal models |indicate that it is an organ-specific autoimmune disease, mediated by autoreactive T lymphocytes and/or autoantibodies directed against myelin antigen(s) resulting in the characteristic pathology of inflammatory cell infiltration and localised myelin loss. The occurrence of "neuroparalytic accidents" associated with inflammatory demyelination in the CNS was observed in some patients following immunisation with Pasteur's rabies vaccine (a mixture of attenuated rabies virus and rabbit CNS tissue) in the 1880's and led to experiments to elucidate the paralysis-causing factor in the vaccine. Neurological signs and CNS lesions were induced in rabbits and monkeys following repeated injections of CNS tissue indicating the nervous tissue and not the virus was responsible for the observed paralysis (Rivers, Sprunt & Berry, 1933; Rivers & Schwentker, 1935). The addition of Freund's mycobacterial adjuvant enhanced the encephalitogenicity of the CNS tissue to the extent that a single injection could induce disease in experimental animals (Freund, Stern & Pisani, 1947; Morgan, 1947). Experimental allergic encephalomyelitis (EAE) is mediated by T cells directed against myelin antigens in the CNS and is established as a reproducible animal model for studying human inflammatory demyelinating diseases such as MS.

1.1.1 Immunopathogenesis of Multiple Sclerosis

Evidence for an immunological component in the development of MS can be clearly demonstrated by histological analysis of post-mortem CNS tissue of MS patients. In actively demyelinating lesions (or plaques) numerous perivenous cuffs are present which are composed of infiltrating T- and B-lymphocytes, plasma cells and macrophages (Mφ), while the hypercellular interface between normal and degenerating myelin is comprised mainly of phagocytic cells such as macrophages and activated microglia (Cuzner *et al.*, 1988). In actively demyelinating lesions phagocytic cell bodies or processes are observed either between the axon and the myelin sheath or between individual myelin lamellae and contain myelin fragments (Lassmann, 1983) although whether this represents myelin being directly targeted by macrophages or merely phagocytosis of previously degenerated myelin has not been resolved. The detection in normal appearing MS white matter of focal areas of macrophage-like cells, expressing major histocompatibility complex (MHC) class II molecules (DR and DQ), with readily detectable myelin basic protein (MBP) immunoreactivity but no obvious myelin loss around the cells would suggest that processing of myelin by these cells may precede the inflammatory lymphocytic reaction (Li *et al.*, 1993). Release of MBP or other myelin protein peptides from these sites could then lead to activation of specific immune responses.

The presence of lymphocytes and macrophages in the demyelinating MS lesion suggests that the disease is the result of a cell mediated autoimmune response against a myelin protein or the myelin producing oligodendrocytes (McFarlin & McFarland, 1982). As yet, no specific autoantigen has been identified nor has a conclusive association with a viral or bacterial infection leading to the development of MS been

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demonstrated. T cell reactivity to MBP, proteolipid protein (PLP) and other myelin proteins such as myelin associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG) can be demonstrated from peripheral blood and cerebrospinal fluid (CSF) of MS patients (Kerlero de Rosbo, 1993), demonstrating that autoreactive T cells to CNS white matter antigens are produced by MS patients. However, similar reactivity is reported for lymphocytes isolated from healthy control subjects (Kerlero de Rosbo, 1993) suggesting that the autoreactive cells, *per se*, may not be disease-causing but that inappropriate regulation of these cells leads to autoimmune disease. It is possible that a bacterial or viral infection initiates events that lead to focal demyelination; although the processes by which this might occur are not fully understood, possible mechanisms include molecular mimicry (Wucherpfennig & Strominger, 1995) or the changing of host cell antigen(s) expression such that it is no longer recognised as "self" protein by the immune system and thus attacked. These remain prime areas of research since a number of animal viruses such as Theiler's murine encephalomyelitis virus (Lipton, 1975), mouse hepatitis virus (Herndon *et al.*, 1975) and canine distemper virus (Krakowka *et al.*, 1973) can lead to inflammation and demyelination in the CNS.

In order to elucidate the possible cause and underlying immunopathogenesis of MS a number of animal models have been developed, with EAE in Lewis rats representing one extensively characterised model.

1.1.2 Experimental Allergic Encephalomyelitis: An Animal Model of Multiple Sclerosis

EAE is most commonly studied in susceptible strains of mice and rats although it can be induced in a number of species such as guinea-pigs, rabbits and monkeys

(Paterson, 1966, 1976) by the subcutaneous injection of an emulsion containing spinal cord homogenate, purified myelin or myelin proteins such as MBP or PLP mixed with Freund's complete adjuvant (CFA) and heat killed *Mycobacterium tuberculosis* in a process known as active immunisation. Spleen or lymph node cells taken from these animals 10 to 12 days post immunisation can transfer EAE to normal syngenic recipients (Paterson, 1960), the inducer cells having been identified to be CD4^{+} T cells (Pettinelli & McFarlin, 1981). Stimulation of the cells *in vitro* with concanavalin A or the priming antigen (e.g. MBP) markedly enhances the ability of these cells to transfer disease (Panitch & McFarlin, 1977; Richert *et al.*, 1979; Panitch, 1980). EAE can also be induced by injection of T cell lines directed against the myelin proteins MBP and PLP (Ben-Nun, Wekerle & Cohen, 1981; van der Veen *et al.*, 1990).

In the Lewis rat, an acute monophasic clinical disease episode is seen. Neurological symptoms, preceded by weight loss, present with loss of tonus in the tail and progress to paresis then complete paralysis of the hind limbs about 12-14 days post active immunisation and 5-7 days post transfer of neuroantigen specific T cells. Clinical signs resolve spontaneously and the animals are fully recovered approximately 5-7 days after onset. The disease is characterised pathologically by perivascular infiltration of T lymphocytes and macrophages with limited demyelination (Lassmann *et al.*, 1986; Pender, 1987) The neurological deficits are thought to be the result of impaired conduction of nerve impulses due to demyelination in the CNS (Pender, 1987, 1988) and/or oedema associated with the inflammatory response (Simmons *et al.*, 1982; Kerlero de Rosbo *et al.*, 1985). Pronounced demyelination has been described in MBP-induced EAE following the administration of a monoclonal antibody to MOG and in EAE induced by the N-terminal domain (amino acids 1-125) of MOG although these

models have an acute and severe clinical course MOG (Linington *et al.*, 1988; Adelmann *et al.*, 1995). Chronic relapsing EAE models which more closely resemble MS both clinically and histopathologically (Lassmann, 1983) can be induced in mice by the adoptive transfer of MBP- or PLP-reactive lymphocytes (Mokhtarian *et al.*, 1984; van der Veen *et al.*, 1989), in juvenile strain 13 or Hartley guinea-pigs by inoculation of syngenic CNS tissue homogenate emulsified in CFA (Raine & Stone, 1977; Wisniewski and Keith, 1977), in MBP-immunised Lewis rats following low dose cyclosporin treatment (Polman *et al.*, 1988) and Lewis rats sensitised with purified MOG or a MOG-derived peptide (amino acids 35-55; Johns *et al.*, 1995).

1.1.3 Generation of a T Cell Mediated Autoimmune Response in Experimental Allergic Encephalomyelitis

Following active immunisation, antigen (e.g. MBP) is taken up by professional antigen presenting cells (APC; e.g. dendritic cells, macrophages or B lymphocytes) which migrate to the lymph nodes draining the site of injection where the antigen is processed and the resultant peptide presented in association with major histocompatibility complex (MHC) class II molecules to T helper (Th) cells of the CD4 subtype. CD4$^+$ Th cells differentiate into two distinct subpopulations following antigenic stimulation, depending on the cytokine milieu and activation by costimulatory signals, which are characterised by the profile of cytokines secreted and the effector functions mediated (Mosmann & Coffman, 1989; Seder & Paul, 1994). Type 1 Th cells (Th1) secrete interferon (IFN)–γ, interleukin (IL)-2 and tumour necrosis factor (TNF)–β promoting delayed-type hypersensitivity responses, cell-mediated immunity and macrophage activation. Type 2 Th cells (Th2) enhance humoral immunity, stimulating B

cell, mast cell and eosinophil proliferation, immunoglobulin (Ig) production (IgG1 and IgE) through the secretion of IL-4, IL-5, IL-6 and IL-10 (Powrie & Coffman, 1993). It has been demonstrated that $CD4^+$ Th cells specific for an encephalitogenic peptide of MBP must be able to secrete inflammatory cytokines such as IFN-γ and TNF-α and/or b to induce EAE whilst Th clones which recognise the same encephalitogenic peptide but secrete IL-4 or IL-10 and no IFN-γ or TNF are unable to induce EAE (Powell *et al.*, 1990; Baron *et al.*, 1993).

While the engagement of the T cell receptor (TCR) with the antigen-MHC ligand provides specificity to the immune response, further signals are necessary for full activation and phenotypic differentiation of T cells. An important costimulatory signal is provided by the B7 antigen, a member of the Ig gene superfamily, which is expressed on APC (Munro *et al.*, 1994). At least two molecules are involved in the B7 costimulatory pathway, B7-1 (CD80) and B7-2 (CD86) which bind to receptors, CD28 and CTLA4, on T cells (Linsley *et al.*, 1991; Freeman *et al.*, 1991, 1993a, b). Activation of precursor T cells from MBP-specific T cell receptor (TCR) transgenic mouse lines by incubation with the MBP Ac(1-11) peptide in the presence of monoclonal antibodies to either B7-1 or B7-2 resulted in dramatic skewing of the Th phenotype: anti-B7-1 increased the production of IL-4, whereas anti-B7-2 increased the production of IFN-γ (Kuchroo *et al.*, 1995). APC also produce IL-12 which induces the production of several cytokines from T and natural killer (NK) cells, in particular IFN-γ, and promotes Th1-specific immune responses (Chan *et al.*, 1991; Manetti *et al.*, 1993). Stimulation of naïve TCR transgenic T cells *in vitro* with antigen (ovalbumin) in the presence of IL-12 drives the differentiation of the cells towards the Th1 phenotype (Hsieh *et al.*, 1993) characterised by enhanced IFN-γ production, whereas addition of IL-4 or IL-10 resulted in the

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generation of a Th2 phenotype (Hsieh *et al.*, 1992; Seder *et al.*, 1992). The interaction

between CD28 on T cells with the B7 ligand (or stimulation of T cells with anti-CD28

antibodies) effectively synergises with IL-12 in inducing IFN–γ production from mouse

and human lymphocytes; blocking the B7/CD28 interaction with the fusion protein,

CTLA4-Ig, significantly inhibits the induction of IFN–γ by IL-12 (Murphy *et al.*, 1994;

Kubin, Kamoun & Trinchieri, 1994). Furthermore, IFN–γ production from Th1 clones

stimulated by antigen and spleen APC is efficiently blocked if both CTLA4-Ig and

neutralising IL-12 antibodies are added (Murphy *et al.*, 1994). The cytokines IL-1β, IL-

2 and TNF–α also augment the IL-12-induced synthesis of IFN–γ (D'Andrea *et al.*,

1993; Wu *et al.*, 1993) and neutralisation of either TNF–α or IL-1β in IL-12-stimulated

peripheral blood lymphocyte cultures significantly inhibits IFN–γ production (D'Andrea

et al., 1993). As well as enhanced IFN–γ synthesis, activation of human T cells with IL-

12 and anti-CD28 antibodies resulted in the synthesis of granulocyte-macrophage

colony-stimulating factor (GM-CSF) and TNF–α indicating that IL-12 can affect the

production of several cytokines (Kubin, Kamoun & Trinchieri, 1994).

The importance of Th1/Th2 development through costimulatory signals in

autoimmune disease has been demonstrated *in vivo* using EAE. Thus, blockade of B7-1

and -2 by CTLA4-Ig or selective blockade of B7-1 reduced the incidence and severity of

EAE and was associated with an inhibition of Th1 cytokines and an increase in Th2

cytokines (Khoury *et al.*, 1995; Kuchroo *et al.*, 1995). In a mild form of EAE, a

worsening of clinical signs was observed in animals treated with B7-2 antibodies,

consistent with an increase in Th1 cytokines (Kuchroo *et al.*, 1995).

1.1.4   Entry of Inflammatory Cells and Antigen Presentation in the Central Nervous System

In normal animals, very few lymphocytes are detectable in the CNS (Wekerle *et al.*, 1986) suggesting that the CNS is not subject to the same degree of immunological surveillance as non-CNS tissue. However, from studies of animal models of CNS inflammation it is apparent that the number of lymphocytes entering the CNS can be increased dramatically, a process primarily dependent on the activation state of the lymphocytes. Activated T cells enter the CNS whilst those in a resting state are excluded (Hickey, Hsu & Kimura, 1991). The ability of T cells to enter is not influenced by phenotype, MHC class II restriction or antigen specificity, although only cells capable of reacting with a CNS antigen remain in the tissue or recycle to it and initiate inflammation while those directed against non-CNS antigens exit within 1 or 2 days (Hickey, Hsu & Kimura, 1991). Once in the CNS, continued cytokine production by infiltrated leukocytes may enhance the expression of adhesion molecules and MHC antigens thus recruiting further T cells, monocytes/macrophages, facilitating the presentation of antigen to the newly recruited T cells and propagating the inflammatory response.

Cell adhesion and migration are central to the entry of lymphocytes into any tissue. Circulating cells are attracted to inflammatory foci through the generation of chemotactic gradients by chemokines, members of a family of readily inducible proinflammatory cytokines (Oppenheim *et al.*, 1991). Chemokines such as monocyte chemotactic protein (MCP)-1, RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted) and macrophage inflammatory protein (MIP)-1–α and –β primarily attract monocytes and T cells (Schall *et al.*, 1990, 1993; Carr *et al.*, 1994).

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Chemokine mRNA expression is induced by Th1 cytokines, IFN–γ and TNF–α (Ohmori et al., 1993; Schrum et al., 1996) and elevated levels of MCP-1, RANTES and MIP-1α mRNA and/or protein are found in the CNS of animals with EAE (Hulkower et al., 1993; Glabinski et al., 1995; Karpus et al., 1995). Further evidence of a critical role for chemokines in the pathogenesis of EAE is suggested by the ability of anti-MIP-1α antibody to treat and prevent transfer EAE, probably as a result of inhibiting leukocyte recruitment rather than proliferation or effector function (Karpus et al., 1995). MIP-1β induces adhesion of T cells to vascular cell adhesion molecule (VCAM)-1, an endothelial ligand for the integrin very late activation antigen (VLA)-4 on leukocytes (Tanaka et al., 1993) indicating that chemokines might promote the migration of selected cell types by activating integrin-mediated adhesion of leukocytes to the endothelium (Springer, 1994).

Expression of intercellular adhesion molecule (ICAM)-1 and VCAM-1 on CNS vascular endothelial cells, microglia and astrocytes increases dramatically prior to the onset of clinical signs of EAE, coinciding with the immigration of leukocytes into the CNS, remains elevated during the acute phase of the disease and diminishes during the recovery phase (Canella, Cross & Raine, 1991; Dopp, Breneman & Olschowka, 1994). The increased expression of adhesion molecules observed prior to the onset and during the clinical phase of EAE is probably due to the action of cytokines such as IFN–γ, TNF-α/β and IL-1-α/β. Indeed, each of these cytokines has been shown to increase gene or protein expression of ICAM-1 and VCAM-1 on cerebrovascular endothelial cells and astrocytes in vitro (Frohman et al., 1989; McCarron et al., 1993; Shrikant et al., 1994). The interaction of VCAM-1 with the ligand VLA-4 on leukocytes is of utmost importance for both cell entry and pathogenicity since MBP-reactive CD4$^+$ T cell

clones with low level surface expression of VLA-4 bind weakly to endothelial cells, fail to enter CNS parenchyma and do not induce EAE whereas clones expressing high levels of VLA-4 enter the brain parenchyma and are encephalitogenic (Baron *et al.*, 1993). Furthermore, antibodies to a4 integrin (VLA-4) or its ligand, VCAM-1, delayed the onset and suppress clinical signs of EAE and reduce leukocyte accumulation in the CNS (Yednock *et al.*, 1992; Baron *et al.*, 1993; Kent *et al.*, 1995).

Microglia constitutively express low levels of MHC class II molecules in the normal CNS (Hayes, Woodroofe & Cuzner, 1988; Gehrmann, Banati & Kreutzberg, 1993) and this rapidly increases in response to both inflammatory, e.g. EAE (Hickey, Osborn & Kirby, 1985; Matsumoto *et al.*, 1986; Vass *et al.*, 1986) and non-inflammatory, e.g. neuronal damage (Streit, Graeber & Kreutzberg, 1989) conditions. Of two populations recovered from adult rat normal CNS it is the bone marrow-derived, blood-borne transitional macrophage, not the resident microglia, that is the effective APC for EAE-inducing MBP-reactive CD4$^+$ T cells (Ford, *et al.*, 1995).

1.1.5   Progression of Inflammation and Tissue Damage in EAE

The presence of T cells in the CNS secreting cytokines such as IFN–γ can activate infiltrating macrophages and resident microglia to produce inflammatory mediators including nitric oxide (NO), reactive oxygen species (ROS; e.g. superoxide, hydrogen peroxide, hydroxyl radicals), proteases and cytokines which contribute to tissue damage. The expression of cytokines in the CNS during the course of active or transfer EAE in mice and rats has been studied using the reverse transcriptase/polymerase chain reaction technique to detect cytokine mRNA or with immunocytochemistry to detect the actual cytokines. Increased expression of IL-12,

TNF–α and β, IL-1α and β, IL-2 and IFN–γ is observed prior to disease onset and during the clinical phase (Merrill *et al.*, 1992; Bauer *et al.*, 1993; Issazadeh *et al.*, 1995a, b; Renno *et al.*, 1994) supporting the concept of a Th1 cytokine response initiating and maintaining the disease process. Functionally, IL-2 is necessary for the survival of T cells activated by antigen in association with MHC within the CNS (Duke & Cohen, 1986). IFN–γ enhances both the antigen presenting (MHC class II) and phagocytic (Fc-receptor) capacity of microglia and macrophages whilst IL-1 can act in an autocrine and paracrine fashion to modulate Fc-receptor numbers on CNS phagocytic cells (Loughlin *et al.*, 1992). TNF–α and –β have been shown to induce myelin degradation and oligodendrocyte apoptosis respectively *in vitro* (Selmaj & Raine, 1988; Selmaj *et al.*, 1991) and overexpression of TNF–α by neurons or astrocytes in transgenic mice triggers a chronic inflammatory demyelinating response in the CNS (Probert *et al.*, 1995). However, IL-1α and IFN–γ have been shown to reduce the MBP content of myelinated cultures suggesting other cytokines are capable of mediating demyelination (Loughlin *et al.*, 1994). Administration of TNF–α or IL-1α, augments the severity of EAE (Kuroda & Shimamoto, 1991; Jacobs *et al.*, 1991) whilst neutralisation of either cytokine by administration of antibodies, soluble receptors or receptor antagonists abrogates EAE (Selmaj, Raine & Cross, 1991; Selmaj *et al.*, 1995; Jacobs *et al.*, 1991; Martin *et al.*, 1995).

Several lines of evidence point to an important role of macrophages as effector cells in the pathogenesis of EAE. Whilst MBP- or PLP-specific T cells induce paralytic disease following transfer, T cells specific for MOG or S100b (an astrocyte-derived calcium binding protein) fail to induce a gross neurological deficit despite a severe inflammatory response in the CNS. The inability of MOG- and S100b-specific T cells to

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elicit severe disease is associated with a decrease in the extent of parenchymal (as opposed to perivascular) inflammation and a selective decrease in the migration of activated (ED1$^+$) macrophages into the CNS in comparison with severe EAE induced by MBP-specific T cells when macrophages constitute approximately 50% of the infiltrating cells in the CNS (Linington et al., 1993; Kojima et al., 1994). Depletion of peripheral macrophages has been shown to suppress clinical EAE despite the continued presence of inflammatory infiltrates in the CNS (Brosnan, Bornstein & Bloom, 1981; Huitinga et al., 1990, 1995).

Activated T cells synthesise, whereas macrophages constitutively express, matrix metalloproteinases (MMP; Hibbs et al., 1985; Montgomery et al., 1993). MMPs have the potential to further exacerbate CNS damage by degrading the constituents of the basement membrane of the BBB, enhancing the ability of inflammatory cells to enter the CNS (Rosenberg et al., 1992). Furthermore, gelatinase B is able to cleave MBP into encephalitogenic peptides which react with T cells from MS patients (Proost et al., 1993). Proteolytic activity has been demonstrated in the CSF and CNS tissue of MS patients and during EAE (Gijbels et al., 1992, 1993; Cuzner et al., 1976). In this context, perhaps it is not surprising that inhibition of various types of proteases suppress the development of EAE (Brosnan et al., 1980; Gijbels et al., 1994; Hewson et al., 1995).

Activation of phagocytic cells leads to a marked increase in oxygen consumption and increased activity of the NADPH oxidase complex which can lead to the production of superoxide (O_2^-) and subsequently hydrogen peroxide. These oxygen species are highly reactive and can damage cells, such as oligodendrocytes (Konat & Wiggins, 1985; Griot et al., 1990; Kim & Kim, 1991) by peroxidation of membrane lipids. Nitric

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oxide (NO) is produced by neurons, endothelial cells and phagocytes and is synthesised

from L-arginine in a reaction catalysed by nitric oxide synthase (NOS). Macrophages

and microglia express an inducible form of NOS (iNOS) following stimulation with IL-

1, TNF-α and IFN-γ either alone or in combination (Sheffler et al., 1995; Murphy et

al., 1993). Interaction of NO with reactive oxygen species gives rise to peroxynitrite,

which may underlie the mechanism of microglial cytotoxicity towards oligodendrocytes

(Merrill et al., 1993; Mitrovic et al., 1994). Mononuclear and polymorphonuclear

leukocytes isolated from the CNS of rats with hyperacute EAE secrete increased

amounts of oxygen- and nitrogen-derived free radicals (MacMicking et al., 1992) and

NO has been demonstrated in the spinal cords of mice with EAE by electron

paramagnetic resonance spectroscopy (Lin et al., 1993). Furthermore, CNS iNOS

expression parallels the course of EAE (Okuda et al., 1995) and inhibition of iNOS

ameliorates the disease (Cross et al., 1994) suggesting a causal role in the pathogenesis

of EAE.

1.1.6 Downregulation of Inflammation and Recovery from EAE

Spontaneous recovery of Lewis rats from actively and passively induced EAE is

dependent on an increase in endogenous corticosterone levels which occurs at the peak

of clinical signs (Levine, Sowinski & Steinetz, 1980; MacPhee, Antoni & Mason, 1989;

MacKenzie, Leonard & Cuzner, 1989). The mechanisms by which corticosterone

assists in recovery from EAE probably operate on many levels. Thus, glucocorticoid-

induced inhibition of IL-2 and IL-2 receptor synthesis suppresses the proliferation of T

lymphocytes (Gillis, Crabtree & Smith, 1979; Grabstein et al., 1986); access of

leukocytes to the target antigen is reduced by modulating BBB permeability (Paul &

Bolton, 1995), down regulation of cell adhesion molecules and MHC class II expression (Cronstein *et al.*, 1992; Loughlin, Woodroofe & Cuzner, 1993; Snyder & Unane, 1982) and chemokine production (Mukaida *et al.*, 1991). In addition to inhibiting the production of "pro-inflammatory" cytokines such as IL-1 and TNF–α glucocorticoids can up-regulate production of IL-1 receptor antagonist and soluble receptors for IL-1 and TNF–α which antagonise the relevant cytokine (Snyder & Unane, 1982; Guyre *et al.*, 1988; Colotta & Mantovani, 1994; Gabay *et al.*, 1997; Wilckens & De Rijk, 1997).

The role of specific suppressor or regulatory cells in the recovery from disease is unclear, with both CD4^{+} and CD8^{+} T cells being implicated. CD4^{+} suppressor T cells isolated from the spleens of rats following recovery from EAE inhibit the production of IFN–γ, but not IL-2, by EAE effector cells stimulated *in vitro* with MBP through a mechanism involving secretion of TGF–β (Karpus & Swanborg, 1989, 1991). Co-culture of suppressor T cell lines with EAE effector cells prior to transfer prevents onset of EAE (Ellerman, Powers & Brostoff, 1988) and administration of "recovery-associated suppressor cells" after the appearance of clinical signs arrests the course of ongoing disease (Varriale *et al.*, 1994). The secretion of IL-4 by CD4^{+} suppressor T cells in response to EAE effector cells (Karpus, Gould & Swanborg, 1992) and expression of IL-10 mRNA in the CNS during recovery from EAE (Kennedy *et al.*, 1992) suggest a switch from a Th1- to a Th2-mediated response is associated with resolution of clinical EAE. Indeed, administration of Th2 cytokines (IL-4, IL-10) or TGF–β prevent onset or improve the clinical course of ongoing EAE (Rott, Fleischer & Cash, 1994; Racke *et al.*, 1991; Johns *et al.*, 1991) whereas neutralisation of these cytokines leads to worsening of disease (Racke *et al.*, 1992; Johns & Sriram, 1993). Furthermore, increased IL-4 secretion by lymphocytes, enhanced expression of IL-4, IL-

10 and IL-13 mRNA and reduced IFN–γ and TNF–α production has been reported following both *in vitro* and *in vivo* exposure to glucocorticoids (Daynes & Araneo, 1989; Daynes *et al.*, 1990; Ramírez *et al.*, 1996).

CD8^{+} suppressor T cells have been isolated from spleens of Lewis rats following recovery from EAE induced by transfer of an MBP-specific CD4^{+} T cell line and from rats rendered resistant to the passive transfer of EAE by pre-treatment with injections of attenuated encephalitogenic line T cells (Sun, Ben-Nun & Wekerle, 1988a; Sun *et al.*, 1988b). In a study of EAE in B10.PL mice immunised with a peptide of murine MBP, depletion of CD8^{+} T cells had no effect on disease severity or recovery and no spontaneous relapses occurred. However, whilst control mice were resistant to reinduction of EAE, CD8^{+} T cell depleted mice developed a second episode of disease when re-challenged with the same MBP-peptide (Jiang, Zhang & Pernis, 1992). In contrast, CD8^{+} T cell depletion in Lewis rats had no effect on the course of actively or passively induced EAE and disease could not be reinduced following recovery (Sedgwick, 1988).

Recovery from acute EAE in Lewis rat is associated with a reduction in the number of T cells in the CNS (McCombe, de Jersey & Pender, 1994; Zeine & Owens, 1993) which might reflect emigration from or death within the CNS of these cells. The occurrence of apoptotic cells in CNS of LEW rats during EAE was first described by Pender *et al.*, (1991) and were identified as both oligodendrocytes, postulating that death of these cells may cause myelin loss seen in EAE and MS, and lymphocytes, suggesting that death of these cells might underlie recovery from EAE. Subsequent studies using immunolabelling techniques to identify apoptotic cell types have demonstrated apoptotic T cells in the CNS during the course of EAE and that the

maximum proportion of apoptotic T cells is associated with recovery (Pender *et al.*, 1992; Schmied *et al.*, 1993; Tabi, McCombe & Pender, 1994). As glucocorticoids can induce apoptosis in mature T cells (Zubiaga, Munoz & Huber, 1992), the endogenous CS release that occurs at peak clinical signs of EAE in the Lewis rat may contribute to the T cell apoptosis in the CNS (Pender *et al.*, 1992; Schmied *et al.*, 1993).

1.1.7 Triggers of Relapse in Acute EAE

Following recovery from acute EAE induced by active immunisation with MBP in CFA (MBP-EAE), rats become resistant to active reinduction of EAE (Willenborg, 1979; Hinrichs, Roberts & Waxman, 1981), although there appears to be a period immediately following recovery (between days 21-30 post immunisation) when a second episode of paralysis can be induced in some animals by reimmunisation with MBP in CFA or soluble MBP (MacPhee & Mason, 1990). In contrast, rats are susceptible to the induction of EAE by the transfer of MBP-specific lymphocytes following recovery from MBP-EAE (Willenborg, 1979; Hinrichs, Roberts & Waxman, 1981) although the severity may be reduced (MacPhee & Mason, 1990). Rats that have recovered from transfer EAE display partial (Welch, Holda & Swanborg, 1980; Ben-Nun & Cohen, 1981) or complete (Hinrichs, Roberts & Waxman, 1981) susceptibility to reinduction of MBP-EAE and are fully susceptible to reinduction of EAE by transfer of MBP-specific T cells (Hinrichs, Roberts & Waxman, 1981; Ben-Nun & Cohen, 1981).

Certain immunosuppressive drugs can precipitate relapse of EAE in Lewis rats depending on the treatment regime. Following recovery from active EAE a single injection of cyclophosphamide induced a relapse of clinical signs which may be attributable to the selective elimination of suppressor cells by this drug (Minagawa *et al.*,

1987). While administration of a high dose of cyclosporin A (25 mg kg^{-1}) prevents the development of EAE (Bolton, Allsopp & Cuzner, 1982), low-dose cyclosporin A therapy (2-4 mg kg^{-1}) converts acute EAE into a chronic relapsing disease (Polman *et al.*, 1988). A chronic relapsing form of EAE is also seen after oral administration of FK506 for 12 days following induction of active EAE (Deguchi *et al.*, 1991) whilst disease is prevented when given intramuscularly (Inamura *et al.*, 1988).

The bacterial superantigens, staphylococcal enterotoxin A and B (SEA and SEB), have been shown to precipitate relapses in mice when administered following recovery from the initial episode of paralysis and induce attacks in those with subclinical disease (Schiffenbauer *et al.*, 1993; Brocke *et al.*, 1993; Crisi *et al.*, 1995). Tumour necrosis factor may play a role in mediating these effects since administration of an antibody to TNF–α delayed the onset of initial signs of disease triggered by SEB while intraperitoneal injection of TNF–α produced relapses of similar frequency and severity to those induced by SEB (Crisi *et al.*, 1995).

1.2 Neuroendocrine and Immune System Interactions

The fundamental task of the immune system is to distinguish "self" from "non-self" and to respond accordingly to any encounters with these antigens. It is now accepted that the immune system does not function independently in coping with the potentially vast array of "foreign" and "self" stimuli but interacts with neuroendocrine and autonomic nervous systems through a bi-directional flow of cytokines, hormones, neuropeptides and neurotransmitters. The balance between the activities of these systems is of critical importance since over- or under-activity of either arm could be detrimental to the health of the organism. The over-production of glucocorticoids might

lead to a compromised immune system and leave the host vulnerable to infection. Conversely, reduced glucocorticoid production might allow uncontrolled activation of immune cells and excessive production of cytokines and other immune mediators possibly resulting in inflammatory tissue damage and autoimmune disease.

1.2.1 *The Hypothalamic-Pituitary-Adrenal Axis*

Activation of the hypothalamic-pituitary-adrenal (HPA) axis represents a mechanism by which the body maintains homeostasis in a stressful environment. Under basal conditions the system exhibits a circadian rhythm which is closely related to the sleep/wake cycle. Thus, in humans plasma corticosteroid levels are at their peak in the morning and at their nadir in the evening whereas this pattern is reversed in nocturnal animals such as rats. Activation of the pituitary-adrenocortical system is orchestrated *via* the hypothalamus which receives a diversity of information and subsequently affects many endocrine functions through its close association with the pituitary gland. Somatic and psychogenic stressors, circadian drive and humoral signals initiate the cascade by releasing hypothalamic adrenocorticotrophic hormone (ACTH) secretagogues into the hypophyseal-portal circulation (Antoni, 1986; Plotsky, 1987). The major ACTH secretagogue was identified in 1981 as a 41 amino acid peptide and named corticotrophin releasing factor-41 (CRF; Vale *et al.* 1981). Arginine vasopressin (AVP) has also been demonstrated to posses corticotrophin releasing activity and, although not as potent as CRF, AVP acts synergistically with CRF both *in vitro* and *in vivo* to potentiate ACTH release from the anterior pituitary gland (Gillies *et al.*, 1982; Buckingham, 1985; Rivier & Vale, 1983). Binding of ACTH to receptors on cells of the adrenal cortex results in the synthesis of glucocorticoids (principally cortisol in humans

and corticosterone in rats) and their subsequent secretion into the circulation. Regulation of the HPA axis is completed by a negative feedback loop exerted by glucocorticoids on the secretion of ACTH and its hypothalamic releasing factors (Dallman *et al.*, 1987).

1.2.2   Immune-Mediated Activation of the HPA Axis

Activation of the immune system by, for example, injection of bacterial endotoxin (lipopolysaccharide, LPS) or virus (e.g. Newcastle Disease Virus) into animals results in maximally elevated plasma levels of ACTH and corticosterone within two hours (Tilders *et al.*, 1984; Besedovsky & Del Rey, 1989). Increased levels of TNF–α, IL-1β and IL-6 are found in the circulation (Givalois *et al.*, 1994) and in the brain (Hillhouse & Mosley, 1993; Gatti & Bartfai, 1993; Schöbitz *et al.*, 1993) following LPS administration. Macrophages and neutrophils are a potential sources of peripheral cytokine production since both cell types express a receptor for endotoxin (CD14) and secrete TNF–α in response to LPS (Schumann *et al.*, 1990; Wright *et al.*, 1990; Haziot, Tsuberi & Goyert, 1993). Furthermore, depletion of macrophages prevented HPA axis activation and reduced plasma IL-1 concentration in response to a subpyrogenic, but not a pyrogenic, dose of LPS (Derijk *et al.*, 1991). CNS glial cells including astrocytes (Fontana *et al.*, 1982) and ameboid microglia (Giulian *et al.*, 1986) produce IL-1 *in vitro* whereas only microglia express IL-1 *in vivo* following LPS administration and also during EAE (Van Dam *et al.*, 1992; Bauer *et al.*, 1993). In addition to IL-1, a plethora of pro-inflammatory cytokines expressed both peripherally and centrally during EAE (including TNF–α, IFN–γ and IL-6) activate the HPA axis and may thereby regulate the disease course. The mode and site of action of HPA axis

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activation is outlined below for the major cytokines implicated in EAE.

Intravenous (Uehara *et al.*, 1987), intraperitoneal (Berkenbosch *et al.*, 1987) or intracerebral (Barbanel *et al.*, 1990; Matta *et al.*, 1990) administration of IL-1β stimulates the release of ACTH and glucocorticoids. Interleukin-1α appears to have a similar effect although it is considerably less potent (Rivier *et al.*, 1989; Matta *et al.*, 1993). The mechanism by which IL-1 affects the HPA axis is controversial since it is generally considered that IL-1 does not cross the BBB in significant amounts (Banks *et al.*, 1991). A direct action of IL-1 on ACTH secretion has been demonstrated in primary cultures of rat anterior pituitary cells *in vitro* (Bernton *et al.*, 1987; Cambronero *et al.*, 1992b) although the stimulatory effect of IL-1 *in vivo* can be inhibited by administration of a neutralising antibody to CRF suggesting that the main site of action of IL-1 is the hypothalamus (Sapolsky *et al.*, 1987). Furthermore, IL-1 causes a decrease in CRF immunoreactivity in the median eminence indicating enhanced release (Berkenbosch *et al.*, 1987; Whitnall *et al.*, 1992), an increase in the concentration of CRF in the hypophyseal portal circulation (Sapolsky *et al.*, 1987) and elevates AVP and CRF concentrations in the median eminence and paraventricular nucleus of the hypothalamus measured by push-pull cannula (Watanobe & Takebe, 1993, 1994). *In vitro*, IL-1 stimulates CRF release from rat hypothalami (Cambronero *et al.*, 1992a; Loxley *et al.*, 1993) and enhances CRF mRNA expression (Suda *et al.*, 1990). Like IL-1, IL-6 and TNF–α cause rapid increases in plasma ACTH levels in rats when given intravenously and this effect can be reversed by the administration of CRF antibodies providing evidence for a hypothalamic site of action of both these cytokines (Naito *et al.*, 1988; Sharp *et al.* 1989; Bernardini *et al.*, 1990; Spinedi *et al.* 1992). Unlike IL-1, TNF–α appears to have a relatively weak stimulatory effect (Bernardini *et al.*, 1990)

whilst IL-6 has a delayed secretory effect on ACTH release from rat anterior pituitary cells (Lyson & McCann, 1991; Matta *et al.*, 1992). A role for IFN–α should not be overlooked as hypothalamic CRF secretion is increased following exposure to the cytokine, at least *in vitro* (Gisslinger *et al.*, 1993).

Two receptors for IL-1 have been identified to date. The type I receptor binds IL-1α and IL-1β with similar affinity (Dower *et al.*, 1986) while the type II receptor exhibits a much higher affinity for IL-1β (Dinarello, 1991). In the rat brain, fairly widespread binding of ^{125}I-labelled IL-1α (Farrar *et al.*, 1987) and IL-1β (Katsuura, Gottschall & Arimura, 1988) has been described, with a significant signal in the hypothalamus. Recently mRNA for type I receptors, but not type II IL-1 receptors, has been demonstrated in rat cerebrovascular endothelial cells (CVEC) and incubation of these cells with recombinant rat IL-1β resulted in dose-dependent increases in IL-6, PGE_2 and prostacyclin which may act to translate the action of peripherally secreted IL-1 to the CNS (Van Dam *et al.*, 1996).

Demonstration of high affinity IL-6 binding sites in the hypothalamus (Cornfield & Sills. 1991) and expression of both IL-6 and IL-6 receptor mRNA in discrete areas of the brain such as the hippocampus and hypothalamus is consistent with an important neuroendocrine role of IL-6 (Schöbitz *et al.* 1993; Gadient & Otten 1993)

An alternative or additional pathway of cytokine to brain communication is provided by stimulation of subdiaphragmatic vagal afferents by local increases in tissue levels of cytokines. This route of activation is not restricted to IL-1 but also holds for TNF–α- and LPS-induced responses. Thus, subdiaphragmatic vagotomy blocks hypothalamic noradrenaline depletion and elevation of CS (Fleshner *et al.*, 1995), induction of IL-1β mRNA in mouse hypothalamus and hippocampus (Layé *et al.*, 1995)

and hyperalgesia (Watkins *et al.*, 1995) induced by IL-1β, TNF–α or LPS.

1.2.3 Effect of HPA axis on Immune-Mediated Inflammation

The final products of HPA axis activation, glucocorticoids (GC), are crucial

mediators of the neuroendocrine-immune interaction exerting both anti-inflammatory

and immunosuppressive actions in order to modulate the immune response and return

the body to a state of homeostasis. The importance of GC in preventing excessive

amplification of immune and inflammatory cell activation is clearly demonstrated by (i)

the increased lethality of LPS injection in adrenalectomised mice due to the unrestrained

production of TNF and IL-1 (Bertini, Bianci & Ghezzi, 1988), (ii) the development of

autoimmune diseases in Lewis rats and obese strain chickens which display "blunted"

corticosteroid responses to immune stimuli (Sternberg *et al.*, 1989a; Wick, Hu &

Gruber, 1992) and (iii) the ability to induce autoimmune disease in normally resistant

animals following adrenalectomy or antagonism of glucocorticoid receptors (Mason,

MacPhee & Antoni, 1990; Sternberg *et al.*, 1989a).

The anti-inflammatory effects of GC's are mediated through the suppression of

production of a number of pro-inflammatory molecules including cytokines, chemokines,

adhesion molecules and products of arachadonic acid metabolism (Barnes & Adcock,

1993; section 1.1.6 above). Arachadonic acid, once liberated from cell membranes by

phospholipase A_2, is metabolised to leukotrienes (chemotactic and vasoactive) by the

action of lipoxygenase or to prostaglandins and thromboxanes (vasoactive) by

cyclooxygenase. Glucocorticoids induce the production of a protein, lipocortin 1, which

inhibits phospholipase A_2 (Flower, 1988) thus suppressing the production of the pro-

inflammatory eicosanoids. The mechanism of action for the inhibition of cytokine

production is less well characterised but is thought to be through the induction of a

nuclear transcription factor, IkB, which inhibits the activity of NFkB, a key activator of

many immune effector genes (Scheinman *et al.*, 1995; Auphan *et al.*, 1995).

1.3   Neuroendocrine Factors in Experimental Autoimmune Diseases

Lewis (LEW) rats are commonly used to study the fundamental mechanisms

involved in the aetiology of autoimmune diseases. In contrast to the histocompatible

Fischer (F344) strain, Lewis rats are susceptible to a broad array of experimental

autoimmune diseases, particularly those which are primarily T cell-mediated. Thus,

LEW rats develop adjuvant arthritis (AA) induced by an intradermal immunisation of

heat-killed mycobacteria suspended in mineral oil (Battisto *et al.* 1982) or

intraperitoneal injection of streptococcal cell wall (SCW)-peptidoglycan polysaccharide

(Sternberg *et al.* 1989a). F344 rats become susceptible to AA by using the more

powerful adjuvant paraffin oil instead of mineral oil to suspend the mycobacteria

although the disease is less severe than in LEW rats (Langerijt *et al.*, 1993).

Furthermore, whilst LEW rats develop EAE in response to immunisation with myelin

proteins or following the transfer of T-cells reactive to myelin components, F344 rats do

not.

The increased susceptibility of LEW rats to experimentally induced autoimmune

diseases has been attributed to defective functioning of the HPA axis. Compromised

HPA axis activity is manifest in a reduction in basal levels of plasma corticosteroids with

blunted circadian variation (Griffin & Whitacre. 1991). A diminished HPA axis

response to a variety of stimuli such as SCW, IL-1, CRF or restraint stress is seen in

LEW rats whereas F344 rats show a robust increase in plasma ACTH and

corticosterone levels (Sternberg *et al.* 1989a). This defect in the LEW rat is thought to originate at the level of the hypothalamic paraventricular nucleus where both CRF synthesis and secretion are reduced compared with F344 rats, possibly as a consequence of a disrupted signal transduction mechanism in the CRF neuron (Sternberg *et al.* 1989b; Calogero *et al.* 1992). These neuroendocrine responses appear to play a critical role in determining susceptibility and resistance to disease in LEW and F344 rats as treatment of LEW rats with the synthetic glucocorticoid dexamethasone, beginning at the time of SCW challenge, is associated with marked attenuation of the disease. Conversely, arthritis and severe inflammation can be induced in otherwise SCW arthritis-resistant F344 rats by inhibiting the action of corticosterone with the glucocorticoid receptor antagonist RU 486 (Sternberg *et al.* 1989a).

During the course of EAE in LEW rats induced either by immunisation with MBP or following the transfer of MBP-sensitised leukocytes, plasma corticosterone levels are markedly elevated in animals with signs of the disease (MacPhee *et al.*, 1989; MacKenzie *et al.*, 1989). The increase in glucocorticoid levels occurs just prior to the onset of paralysis with the peak at the time of maximum clinical disease. This HPA response appears to explain the spontaneous recovery of LEW rats from EAE and its importance is emphasised by the fact that removal of the adrenal glands results in a more rapid disease onset which invariably results in death. The lethal effect of adrenalectomy is not altered by replacement of basal corticosterone levels but can be overcome by corticosterone replacement therapy which mimics the endogenous production of corticosterone in intact animals immunised with MBP (MacPhee *et al.* 1989). Moreover, EAE resistant rats such as the Piebald-Viral-Glaxo (PVG) and F344 strains can be rendered susceptible by adrenalectomy (fatal disease course seen in both strains)

and this effect can be reversed by suitable replacement corticosterone therapy as in LEW rats (Mason *et al.* 1990).

A number of factors, including control of Th cell differentiation, cytokine production and HPA axis activity which might influence the development of autoimmune diseases, may be controlled by genes within and outside MHC loci (Murray *et al.*, 1989, 1992; Mustafa *et al.*, 1993, 1994; Scott *et al.*, 1994; Happ *et al.*, 1988). Thus, rats with different MHC haplotypes but on a constant LEW background genome displayed differential susceptibility to EAE induction with MBP peptide 63-88: MHC haplotypes with susceptibility to EAE (RT1^a, RT1^c and RT1^l) showed a Th1-biased response associated with IFN–γ production, while MHC haplotypes which conferred resistance (RT1^d and RT1^u) displayed a Th2 response with production of IL-4 in response to antigen (Mustafa *et al.*, 1993, 1994). PVG and AO strain rats with RT1^u MHC haplotype remain resistant to EAE following adrenalectomy whereas congenic PVG rats with the same background genes as PVG but with RT1^c or RT1^l MHC genes become susceptible following removal of the adrenal glands (Mason *et al.*, 1990). Similarly, F344 rats which have the same MHC haplotype as LEW rats (RT1^l) become susceptible to SCW-induced arthritis following blockade of glucocorticoid receptors (Sternberg *et al.*, 1989a) indicating that corticosterone-mediated resistance is determined by genes outside the MHC. In addition, germ free (GF) F344 rats develop AA similar to LEW rats even though GF F344 rats respond to i.p. IL-1α with an increase in plasma corticosterone levels which are identical to conventional F344 rats (Langerijt *et al.* 1993). Using different strains of rats that vary in their stress response, raised in GF or conventional environments, Langerijt *et al.*, (1994) suggested that the diversity of the intestinal bacterial flora was of the greater significance in determining

disease susceptibility, while the HPA axis response modulated the severity of the disease.

1.4   Aims of Thesis

The aetiology of autoimmune disease is clearly multi-factorial, possibly involving several genes controlling immune activation, modulation by hormonal systems and exposure to environmental factor(s).

Two strains of rat have been used in the experiments described in this thesis. Lewis rats are susceptible to the induction of EAE whilst Fischer rats are resistant. As the strains used share the same MHC background genes (RT1^l) the differences in susceptibility must be determined by factors controlled by genes outside this locus. The activity and sensitivity of the hypothalamic-pituitary-adrenal axis under basal physiological conditions and following immune activation is greater in the resistant F344 rat and it is the increased production of corticosterone that is believed to confer resistance in this strain. Differences in the production of CRF by the hypothalamus have been demonstrated between the two strains which are thought to be due to disruption of signal transduction pathways in the CRF neurons. Central noradrenergic pathways which innervate the hypothalamic PVN may be important in activating the HPA axis in response to an immune stimulus. Thus, hypothalamic second messenger production and noradrenaline release in the hypothalamus have been investigated following immune activation to determine if differences in the central control of HPA axis activity may underlie susceptibility to autoimmune disease.

Glucocorticoids have a variety of immunosuppressive effects and are clearly important in determining resistance to EAE in F344 rats and contributing to recovery in

LEW rats, since adrenalectomy results in severe and fatal EAE in both strains. One possible mechanism by which GC's limit inflammation is through induction of apoptosis in active immune cells, particularly those that are autoreactive. To investigate the role of corticosterone in mediating apoptosis of immune effector cells (T cells and macrophages) in the target organ of EAE (CNS), transfer EAE was induced in intact and adrenalectomised Lewis and Fischer rats and apoptotic cells quantified in paraffin embedded sections of lumbar spinal cord using both morphological criteria and *in situ* nick translation in combination with immunocytochemistry (in a collaborative study with Prof. H. Lassmann and Dr. M. Schmied) at various time points during the disease.

Cytokines play an important role in the initiation of EAE and subsequent resolution of disease. The development of EAE is dependent on the ability of T cells to secrete cytokines of the Th1 type (IFN–γ, IL-2, TNF–β). Glucocorticoids can suppress the production of Th1 cytokines and induce Th2 cytokine production suggesting another level of control for corticosteroids on the activity of the immune system. The ability of a single cytokine, interleukin-12, known to be a potent inducer of Th1 cytokine production to affect the development of EAE in LEW and F344 rats was investigated. Since recovery is associated with a switch to a Th2 response (IL-4, IL-10, TGF–β), the ability of IL-12 to induce relapse in LEW rats was also studied.

CHAPTER II

Second Messenger and Neurotransmitter Changes in the Paraventricular Nucleus of Lewis and Fischer Rats Following Immune-Mediated Activation of the Hypothalamic-Pituitary-Adrenal Axis Measured by *in vivo* **Microdialysis.**

2.1   Introduction

2.1.1   Hypothalamic-Pituitary-Adrenal Axis Function in Lewis and Fischer Rats

Compromised HPA axis activity in the EAE-susceptible LEW rat is manifest in reduced basal and inflammatory mediator-stimulated ACTH and CS secretion compared with F344 rats (Sternberg *et al.*, 1989a) which is thought to be a consequence of a defect at the level of the hypothalamic CRF neuron. Following stimuli such as SCW, IL-1α, behavioural stressors and neurotransmitters, CRF synthesis and secretion is significantly enhanced compared to the LEW response to the same stimuli (Sternberg *et al.*, 1989b, 1992; Calogero *et al.*, 1992). The pituitary corticotrope represents another possible site contributing to HPA axis dysfunction in the LEW rat. Fewer corticotropes are present in the anterior pituitary of LEW compared to F344 rats resulting in reduced expression of proopiomelanocortin mRNA and decreased content and secretion of ACTH from anterior pituitary cells cultured under basal conditions (Zelazowski *et al.*, 1992). The ACTH response to CRF is blunted in LEW rats compared to F344 rats both *in vitro* (Bernadini *et al.*, 1996) and *in vivo* (Sternberg *et al.*, 1989a) which may be due to defective adenylate cyclase signalling following CRF receptor activation (Bernadini *et al.*, 1996).

2.1.2   Hypothalamic Mediators of Pituitary-Adrenocortical Activation

A strong inverse correlation between hypothalamic noradrenaline (NA) content

and plasma CS levels at peak clinical signs of EAE has been demonstrated (Leonard *et al.*, 1991), indicating a role for central noradrenergic pathways in the regulation of the HPA axis during the course of EAE. Central and peripheral administration of LPS or IL-1α/β stimulates noradrenaline release in the hypothalamus which parallels the increase in plasma ACTH and corticosterone concentrations (Dunn, 1988, 1992; Kabiersch *et al.*, 1988). Depletion of NA in the PVN, either by direct injection of 6-hydroxydopamine (6-OHDA) either into the PVN or the ventral noradrenergic ascending bundle, the main source of noradrenergic innervation of the PVN (Sawchenko & Swanson, 1982), reduced the corticosterone response to i.p. injection of IL-1α (Chuluyan *et al.*, 1992). In addition, the IL-1-induced increase in NA metabolism in the hypothalamus can be prevented by pre-treatment with either the cyclo-oxygenase inhibitor, indomethacin, or anti-CRF antibody (Mefford & Heyes, 1990; Terao, Oikawa & Saito, 1993) suggesting a role for eicosanoids (e.g. PGE$_2$) and CRF in mediating the hypothalamic NA response to peripheral immune activation.

Prostaglandins, particularly PGE$_2$, have been implicated in the stimulatory action of LPS and cytokines on HPA axis activity. Administration of indomethacin or a PGE antagonist suppressed or abolished the increase of plasma ACTH levels induced by IL-1α/β (Katsuura *et al.*, 1988, 1990; Watanabe *et al.*, 1990). Microinjection of PGE$_2$, but not PGD$_2$, into the preoptic area increased plasma ACTH levels, probably *via* stimulation of hypothalamic CRF secretion, as systemic pre-treatment with anti-CRF antibody significantly suppressed this response (Katsuura *et al.*, 1990; Watanabe *et al.*, 1990). Furthermore, increased PGE$_2$ production, as determined by immunocytochemistry (Van Dam *et al.*, 1993), push-pull perfusion (Watanobe & Takebe, 1994) and microdialysis (Komaki, Arimura & Koves, 1992) was detected in

several brain areas including the PVN, median eminence (ME), medial preoptic area (MPOA) and the organum vasculosum of the lamina terminalis (OVLT) following peripheral LPS or IL-1β administration indicating a potential role for the eicosanoid in neuroendocrine responses to endotoxin or IL-1 challenge.

Several lines of evidence suggest that cAMP may act as a second messenger in mediating both CRF and AVP secretion following stimulation of the hypothalamus. Thus, forskolin (which increase cAMP accumulation through activation of adenylate cyclase) stimulated the release of CRF and AVP from cultured hypothalamic cells (Hu *et al.*, 1992b) while CRF release induced by IL-1β, NA or 5-hydroxytryptamine was blocked by IP-20, a selective inhibitor of cAMP-dependent protein kinase A (Hu *et al.*, 1992a, c, d). Inhibition of type 4 phosphodiesterase (PDE-4, the enzyme responsible for the hydrolysis of cAMP) elicited CRF secretion from rat isolated hypothalami and increased serum CS concentration in rats following i.p. administration (Hadley *et al.*, 1996; Kumari *et al.*, 1997). In addition, release of immunoreactive CRF from human cultured placental cells was increased by IL-1β and was associated with increased intracellular cyclic nucleotide concentrations (Petraglia *et al.*, 1990).

2.2.3   Rationale and Aims

One possible defect in the CRF neurons of LEW rats could be a disrupted signal transduction mechanism linking immune or psychological stimuli to the production of CRF. By using the techniques of microdialysis or push-pull perfusion, temporal changes in levels of neurotransmitters and second messengers can be measured in discrete areas of the brain in conscious, freely moving animals thus enabling the mechanisms and

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mediators involved in various experimental paradigms to be elucidated. These techniques could be used to examine second messengers, hormones, neurotransmitters, etc. involved in HPA axis activation during EAE. However, problems arise when using microdialysis during EAE since surgical stress can activate the HPA axis (Udelsman & Chrousos, 1988) resulting in mild or no clinical signs of EAE (own observations); if probes are implanted earlier to allow animals to overcome the surgery-induced activation of the HPA axis, there is an increased possibility of the probe becoming blocked due to gliosis (Benveniste & Diemer, 1987). To overcome these problems the present study used bacterial endotoxin (lipopolysaccharide, LPS) and IL-1β which are widely used to study the mechanisms by which acute activation of the immune system stimulates the HPA axis (Rivier, Chizzonite & Vale, 1989; Derijk *et al.*, 1991; Tilders *et al.*, 1994). Endotoxin stimulates the secretion of a number of cytokines, including IL-1, TNF and IFN–γ from activated macrophages and lymphocytes (Dinarello, 1984; Morrison & Ryan, 1987). Whilst the increased production of these cytokines probably contributes to the pathogenesis of EAE, their ability to stimulate the HPA axis suggests a possible role in resolving the disease. Endotoxin/cytokine-stimulated HPA axis activation is thought to be indirect, involving a range of CNS nuclei, neurotransmitters and second messenger systems which impinge on the PVN as outlined above.

The present study aimed to measure hypothalamic mediators implicated in HPA axis activation (i.e. PGE_2, cAMP and NA) following peripheral immune stimuli (LPS and IL-1β) to establish whether differential signalling pathway responsiveness underlies the characteristic HPA axis profiles of LEW and F344 rats.

2.2   Materials and Methods

2.2.1   Animals

Adult female LEW and F344 rats (Charles River, U.K.) weighing 170 - 210g were housed in pairs under a 12 hour light-dark cycle (lights on 06.00h - 18.00h) at 21°C. Rats were handled and weighed daily for at least one week before experimentation. Rat diet (Labsure, Poole, Dorset, U.K.) and tap water were available *ad libitum*.

2.2.2   Dose- and Time-Course Responses of Corticosterone to Bacterial Endotoxin (Lipopolysaccharide) and Recombinant Cytokines

All experiments were performed in LEW and F344 rats without microdialysis probes except the dose-response study of LPS. LPS and recombinant cytokines were dissolved in sterile saline (0.9% w/v sodium chloride, NHNN pharmacy) and injected intraperitoneally (i.p.) between 09:00 and 11:00 h in a volume of 0.2-0.3 ml. Animals in control groups were administered an equal volume of sterile saline. Basal CS levels were determined in plasma samples from rats killed at 09:00 h.

(i)   Lipopolysaccharide: Previous work in this laboratory examining the effect of two doses of LPS (20 or 200 μg kg^{-1}; LPS from *E.coli*, serotype 055:B5; Sigma, U.K.) on hypothalamic cAMP production and plasma CS secretion in LEW rats showed greater stimulation of both parameters by the 200 μg kg^{-1} dose and was therefore used in experiments described in this chapter. In the time course experiment, plasma CS was determined 0.5, 1, 2, 3 and 4 hours after injection of saline or 200 μg kg^{-1} LPS (n=4/time-point for each strain).

(ii)   Murine Recombinant Interleukin-1β: Murine recombinant (mr) IL-1β was purchased from R&D Systems, Oxford, U.K. Plasma CS concentration was determined two hours after injection of saline or increasing doses of mrIL-1β to groups of LEW (0.25-2.0 μg/rat) and F344 (0.05-1.0 μg/rat) rats (n=5/group for each dose). In the time course experiment, plasma CS was measured 0.5, 1 and 2 h following injection of saline or mrIL-1β (0.5 μg/rat; n=4/time-point for each strain).

(iii)   Murine Recombinant Tumour Necrosis Factor-α: Murine rTNF-α (amino terminal truncated form) was obtained from R&D Systems, Oxford, U.K. Rat rTNF-α was obtained from the National Institute of Biological Standards and Controls, Potters Bar, U.K. through the BIOMED Concerted Action "Cytokines in the Brain". Increasing doses of mrTNF-α were administered to groups of LEW (0.25-2.0 μg/rat) and F344 (0.05-1.0 μg/rat) rats while rat rTNF-α was administered at 10 μg/rat (n=5/group for each dose); plasma CS concentration was measured after 2 h.

(iv)   Rat Recombinant Interferon-γ: Rat rIFN-γ was obtained from Gibco Life Technologies, Paisley, U.K. and administered to groups of LEW and F344 rats at 0.1-10 μg/rat (n=5/group for each dose); plasma CS was determined after 2 h.

At the appropriate time-point animals were removed to a separate room and killed by decapitation (the process taking 20-30 seconds) for collection of trunk blood into ice-chilled lithium heparin blood collection tubes (Becton Dickinson, H. M & S Ltd., U.K.). Blood was centrifuged (1500 g for 15 min at 4°C) and duplicate plasma samples (200 μl) stored in microcentrifuge tubes (BDH, Dorset, U.K.) at -80°C until analysis for corticosterone content by radioimmunoassay (RIA; Immunodiagnostic Systems Ltd., Tyne & Wear, U.K.).

2.2.3 Microdialysis Probe Construction

Concentric microdialysis probes were constructed essentially as described by Hutson *et al.* (1985). Pre-cut lengths of 24 gauge steel tubing (length 15mm; Coopers Needle Works, Birmingham, U.K.) were fixed onto Blu-tack placed on metric graph paper. All steps were performed under a Meiji EM2-Z stereo microscope (x20 - x40 magnification; Micro Instruments (Oxford) Ltd., U.K.) lit by a dual fibre optic illuminator (Flexilux 150 HL Universal, Micro Instruments (Oxford) Ltd.). Two 30 mm lengths of vitreous silica (VS 150-075, SGE, Milton Keynes, U.K.) were inserted into the steel tubing such that one piece protruded by 10 mm and 5 mm from each end whilst the other protruded by 20 mm from one end only and were held in place using epoxy resin. Using the underlying graph paper the 10 mm length of silica was then cut to 3 or 4 mm and a 2 cm length of Cuprophan- (Enka AG, Germany) or Filtral 12-dialysis membrane (Hospal, Rugby, U.K.) was placed over the end. The dialysis membrane was cut to approx. 3.5 mm or 4.5 mm and sealed with epoxy resin around the 24 gauge tubing and at the remaining open end leaving a 3 mm (IL-1 experiments) or 4 mm (LPS experiments) length of membrane free for the diffusion of molecules into the artificial cerebrospinal fluid (aCSF). The inlet (5 mm protruding) and outlet (20 mm protruding) ends of the silica tubing were connected to polyethylene tubing (internal diameter (i.d.) 0.28 mm, length 6 cm; Portex Ltd., Kent, U.K.) using 27 gauge steel tubing (length 8 mm, Coopers Needle Works, U.K.) and sealed with epoxy glue, dental acrylic and hot melt adhesive (Figure 2.1). Immediately prior to implantation a 1 ml syringe was attached to the inlet tube and aCSF gently perfused through the probe to check for minimal resistance to infusion and leaks. Any observed to leak or with high resistance were discarded.

Figure 2.1   Schematic Diagram of *in vivo* Microdialysis Probe

Infusion of aCSF from constant flow pump

Hot-melt adhesive

Dental acrylic

Epoxy resin

Collection of dialysate for analysis

Polyethylene tubing (i.d. 0.28 mm)

27 guage steel tubing (length 8 mm)

Vitreous silica

24 guage steel tubing (length 15 mm)

Dialysis membrane (Mol. Wt. cut off approx. 10 kDa)

aCSF

3 or 4 mm

0.2 mm
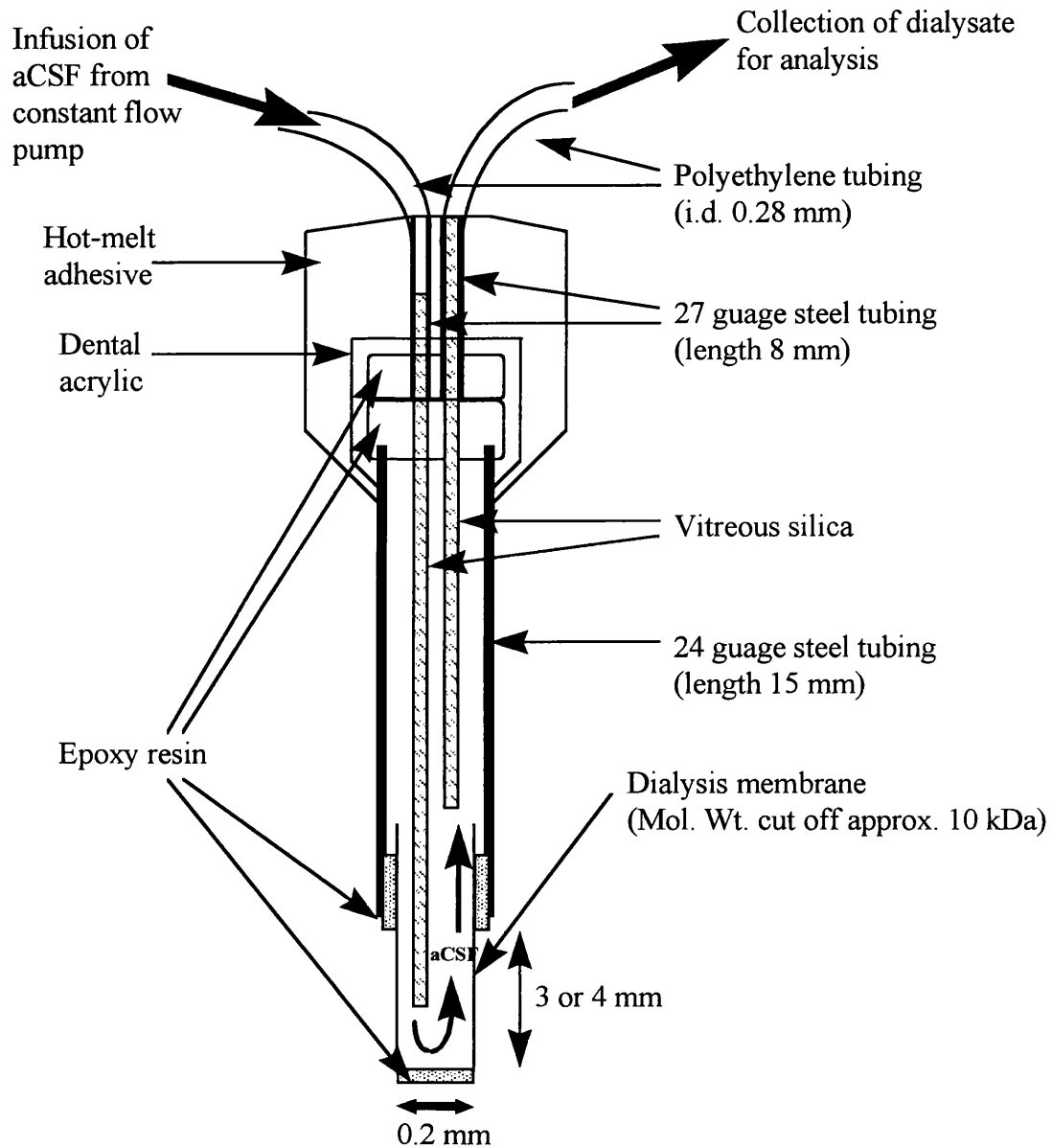
Construction of the microdialysis probe used for *in vivo* collection of prostaglandin E_2, cyclic adenosine monophosphate and noradrenaline from the paraventricular nucleus of the hypothalamus. aCSF = artificial cerebrospinal fluid.

In vitro recoveries for probes with 4 mm long, cuprophan dialysis membranes were determined by placing probes in small beakers of aCSF containing either cAMP (16 nM) or PGE_2 (500 and 2000 pg ml^{-1}) and perfused with aCSF (+ 1mM theophylline for cAMP) at 1.1 μl min^{-1}. *In vitro* recovery of cAMP/PGE_2 in the dialysate was expressed as a percentage of the concentration of cAMP/PGE_2 in the beaker. Under these conditions the *in vitro* recoveries were (mean ± SEM) 8.2±2.9% (cAMP, n=6), 18.5±1.7% (PGE_2 500 pg ml^{-1}, n=4) and 17±1.2% (PGE_2 2000 pg ml^{-1}, n=4); *in vivo* values were not corrected for *in vitro* recovery.

2.2.4 Microdialysis Probe Implantation

Anaesthesia was induced with halothane (2%, May & Baker, U.K.) in nitrous oxide:oxygen (1:1, 0.9 L min^{-1}). Animals were then placed in a stereotaxic frame (David Kopf Instruments, USA), which received a continuous infusion of halothane (1 to 1.5% in $N_2O:O_2$), with the incisor bar set 3.3 mm below the interaural line to achieve a flat skull position. Loss of reflex removal of the hind foot in response to being squeezed between thumb and finger indicated the required depth of anaesthesia had been attained. A strip of fur in the centre of the head was shaved, an incision made in the skin (approx. 1.5 cm long) and the skull exposed. A 3mm hole was drilled through the skull using co-ordinates according to Paxinos and Watson (1982) as follows: 1.8 mm posterior and 0.7 mm lateral to bregma (Figure 2.2A) and an incision of the dura was made with the tip of a 27 gauge hypodermic needle. The probe was positioned in the centre of the drill hole and gradually lowered to the required depth (8.5 mm ventral to the dural surface), taking 2-3 minutes; the dialysis membrane was kept rigid during implantation by slowly passing perfusion fluid through it. However, it was found that on a number of occasions

(7%) the probe was positioned posterior to the PVN in LEW rats and forward of the PVN in F344 rats. Also, in F344 rats, it appeared that the probe was being implanted too deeply. Thus, the co-ordinates were altered as follows: for F344 rats, 2.0 mm posterior, 0.55 mm lateral to bregma and 7.5 mm ventral to the dural surface; for LEW rats, 1.2 mm posterior, 0.55 mm lateral to bregma and 8.5 mm ventral to the dural surface. Probes were secured with dental cement (Dentsply Ltd., Surrey, U.K.) to two screws placed in the skull. Following surgery, animals were housed individually in clear perspex cages (l x w x h: 30 x 30 x 20 cm) with a hole in the lid to allow the inlet/outlet lines to be attached to the microdialysis probe.

On the day of the experiment the polyethylene inlet tubing was connected to a 50 cm length of identical tubing which was then attached to a gas-tight 1.0 ml Hamilton syringe held in a microinfusion pump (Harvard Apparatus infusion pump 22, Harvard Apparatus Ltd., Greenford, U.K.). The outlet line was connected to a 20 cm length of polyethylene tubing (i.d. 0.2 mm) and the dialysate sample collected into Eppendorf tubes (BDH, U.K.).

2.2.5   Experimental Protocol for Cerebral Dialysis

Due to space limitations in the room where the dialysis was carried out, probes were implanted into 5 animals at a time and i.p. treatment randomly assigned. Perfusion experiments were started on individually housed freely-moving animals 16 - 20 h after probe implantation (i.e. between 09.30 and 10.00 h) by passing aCSF of composition (mM): NaCl 125, KCl 2.5, $MgCl_2$ 1.18 and $CaCl_2$ 1.26, through the probe. For experiments in which cAMP was to be measured, the aCSF was supplemented with 1mM theophylline (Sigma, Dorset, U.K.) to prevent breakdown of cAMP in the

samples. After a washout period of 30 minutes, samples were collected every 20 minutes (IL-1β) or 30 minutes (LPS) at a flow rate of 1.1 μl min^{-1} into 500 μl microcentrifuge tubes, frozen on solid CO_2 and stored at -80°C until analysis of relevant constituents. For studies in which noradrenaline (NA) was to be measured, a flow rate of 0.5 μl min^{-1} was used and the dialysate was collected into 250 μl microcentrifuge tubes containing 5μl of 0.01% perchloric acid to prevent oxidation of the monoamine.

Sterile saline (0.9% w/v), LPS (200 μg kg^{-1}), LPS and indomethacin (Sigma, U.K.; 200 μg kg^{-1} and 50 mg kg^{-1} respectively) or mrIL-1β (0.5 μg/rat) dissolved in 0.9% saline were injected intraperitoneally following the collection of 3-4 basal samples; dialysate samples were collected for two hours (IL-1β) or four hours (LPS) post-injection.

At the end of each perfusion period, rats were removed to a separate room and killed by decapitation (the process taking 20-30s). Trunk blood was collected into lithium heparin vacutainer tubes as described above and their brains rapidly removed, frozen on solid CO_2 and stored at -80°C. Sections of frozen brain (25 μm) were cut on a cryostat (Slee, U.K.), stained with haematoxylin and eosin and probe position verified microscopically (Figure 2.2.B). Data from animals with incorrect probe position were excluded from the study.

2.2.6   Analysis of Dialysate Samples

Determination of dialysate levels of PGE_2 and cAMP was performed without purification or extraction procedures, using commercially available radio- and enzyme-immunoassay (RIA/EIA) kits (Amersham Life Science, Amersham, UK). The assays are

Figure 2.2   Placement of Microdialysis Probe in the Paraventricular Nucleus (PVN) of the Hypothalamus
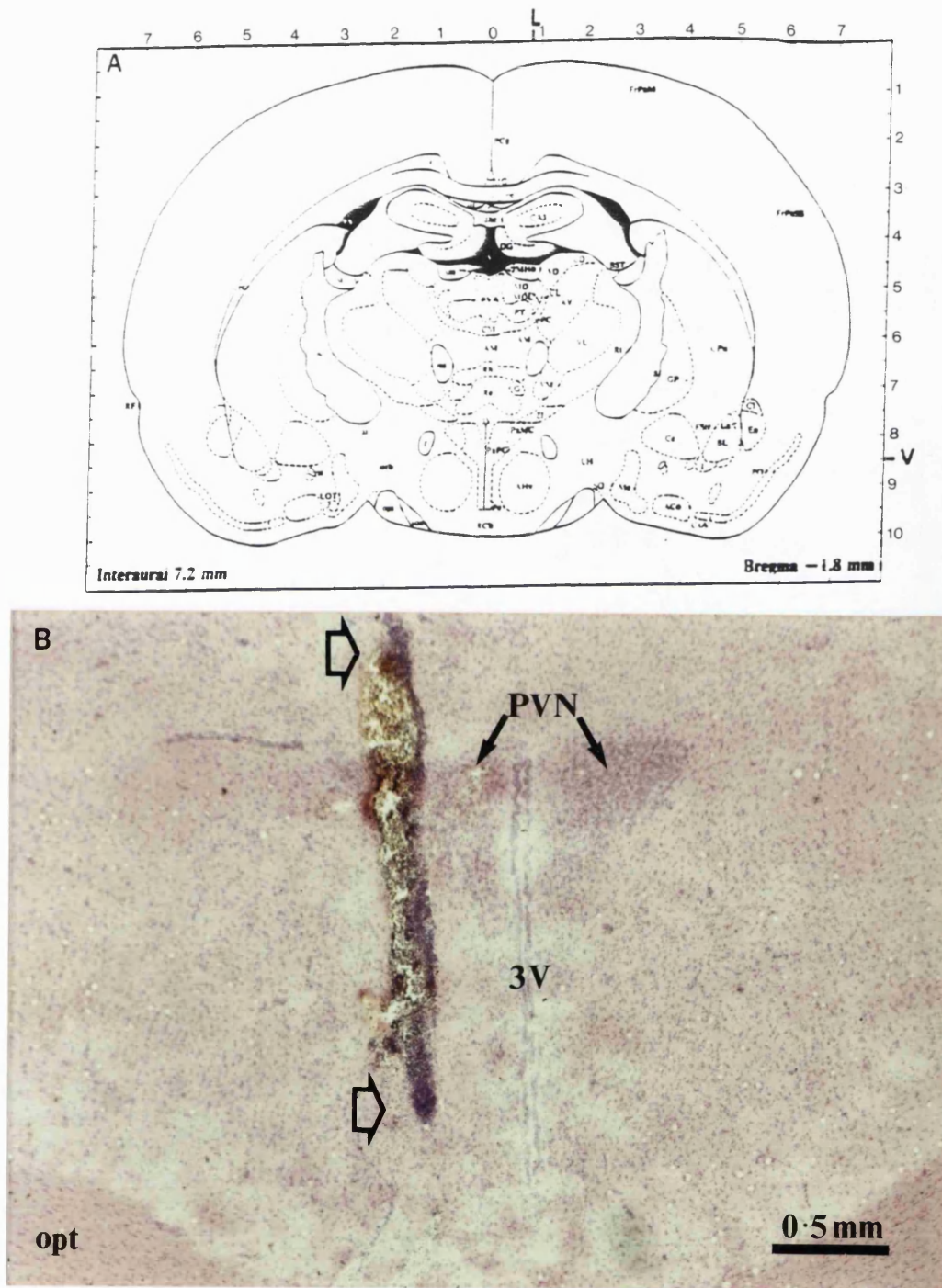
(A) Co-ordinates for implantation of microdialysis probe into the PVN of the hypothalamus: 1.8mm posterior, 0.7mm lateral (L) to bregma and 8.5mm ventral (V) from the skull surface. Taken from Paxinos and Watson (1982).

(B) Photomicrograph of a coronal section through the hypothalamus stained with haematoxylin & eosin. The track made by the tip of the probe can clearly be seen (between open arrows). 3V = third ventricle; opt = optic tract.

based on the competition between unlabelled (i.e. standard or unknown) cAMP or PGE_2 and a known quantity of radio- or enzyme-labelled cAMP or PGE_2 for the binding sites of a fixed amount of cAMP- or PGE_2-specific antibody. In the EIA the cAMP or PGE_2 antibody binds to a secondary antibody immobilised onto the wells of a 96-well polystyrene microtitre plate. Thus, any unbound ligand can be removed by washing each well thoroughly. In the RIA, separation of the antibody-bound PGE_2 from unbound ligand is achieved by addition of a polymer-bound secondary antibody directed toward the anti-PGE_2 antibody which remains in the test tube as a pellet following centrifugation and decantation of the supernatant. The amount of cAMP- or PGE_2-immunoreactivity in each sample can be obtained from a standard curve by interpolation, where the amount of labelled ligand bound by the antibody is inversely proportional to the concentration of added unlabelled cAMP or PGE_2 in the sample. The enzyme label was horseradish peroxidase (HRP) with 3,3',5,5'-tetra-methylbenzidine/hydrogen peroxide used as substrate. The reaction was stopped by the addition of 1.0 M sulphuric acid and the resultant colour read at 450nm in a microtitre plate spectrophotometer (Anthos HT II, Anthos Labtec Instruments, Salzburg). The radiolabel was ^{125}I and radioactivity in each pellet was measured for 1 minute in a gamma scintillation counter (MiniGamma 1275, LKB-Walac, Finland).

Dialysate samples were stored at -80°C for up to four weeks prior to assay. Cyclic AMP and PGE_2 EIA kits were purchased in batches (with the same batch number) and reagents pooled. Dialysates were assigned to plates such that samples from saline- and LPS- or IL-1β-injected Lewis and Fischer rats were present on each plate. Kit reagents supplied lyophilised were reconstituted using deionised water or assay buffer as described in the manufacturers instruction leaflet.

(i)   Prostaglandin E_2 Radio-Immunoassay

Dialysates from initial microdialysis experiments in which LEW rats were injected with saline, LPS or LPS and indomethacin were analysed for PGE_2 content by RIA. Dialysates were collected for 30 minute intervals, four pre-injection and 8 post-injection. Two consecutive samples were pooled into 1 hour samples (some basal samples were assayed singly), the volumes determined and made up to 100 μl using assay buffer (0.05M Tris-HCl buffer, pH 7.4 containing 0.9% sodium chloride, 0.01% Triton X-100 and 0.0057% thimerosal) provided with the kit. One hundred μl of a solution containing methoxyamine hydorchloride and sodium acetate in water:ethanol (9:1 v/v), pH 5.6 was added to each sample and mixed thoroughly. The resulting solution was heated to 60°C for 1h to convert PGE_2 in the samples to the methyl oximate derivative. After heating, the samples were diluted to a final volume of 500 μl with assay buffer and 100 μl aliquots added to appropriately labelled polypropylene tubes (12 x 75mm) in duplicate. One hundred μl of each standard (supplied as methyl oximate derivative and diluted with assay buffer) were added to appropriate tubes in duplicate and 100 μl of $[^{125}I]$-PGE_2 (supplied as methyl oximate derivative) added to all tubes. One hundred μl of PGE_2 antiserum (raised in rabbit against methyl oximate derivative of PGE_2) was pipetted into all tubes except the non-specific binding (NSB) and total counts (TC) tubes. Two hundred μl of assay buffer was added to the NSB and TC tubes and 100 μl pipetted into the zero standard (B_0) tubes. All tubes were vortex mixed and incubated for 2 h at 25°C in a water bath. The second antibody (250 μl; donkey anti-rabbit serum coated onto magnetizable polymer particles) was added to all tubes except TC and incubated at room temp for 15 minutes. The antibody bound

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fraction was then separated by centrifugation (4°C, 10 min, 1500g), the supernatant poured off and the tubes inverted on a pad of absorbent tissues to drain for 5 minutes before counting the radioactivity for 1 minute in a gamma scintillation counter. The sensitivity of the assay was 8 pg ml^{-1}. The concentration of PGE$_2$ in basal samples of single dialysates compared to those that were pooled were very similar when expressed as pg ml^{-1}.

(ii)   Prostaglandin E$_2$ Enzyme-Immunoassay

The volume of each dialysis sample was determined and made up to 50 μl using assay buffer (0.1M phosphate buffer, pH 7.5 containing 0.9% sodium chloride, 0.1% bovine serum albumin and 0.01% thimerosal) provided with the kit. One hundred μl of assay buffer was added to the NSB wells, 50 μl assay buffer to the B$_0$ wells and 50 μl of each standard or unknown were added to the appropriate wells with the standards run in duplicate and the samples singly. Fifty μl of PGE$_2$-antiserum was added to all wells except the blank and NSB wells, plates were gently shaken and incubated at 4°C for 3 hours. HRP-labelled PGE$_2$ (50 μl) was then added to all wells except the blank and incubated at 4°C for 1 hour. Wells were then aspirated, by flicking the inverted plate, and washed four times with 400 μl of wash buffer (0.01M phosphate buffer, pH 7.5 containing 0.05% Tween 20), ensuring any residual volume was removed by tapping the inverted plate on tissue paper. Enzyme substrate (150 μl) was then added to all wells and mixed at room temperature for 30 minutes while a blue colour developed. The addition of 100 μl 1M sulphuric acid to all wells resulted in the development of a yellow reaction product which was read immediately in a microtitre plate spectrophotometer at

450 nm, using the blank wells (only enzyme substrate added) to zero the plate-reader.

PGE$_2$ concentration of aCSF was below the limit of detection of the assay and the 8 pg ml^{-1} standard made up in aCSF gave values of 7.9 and 7.8 pg ml^{-1} (duplicates from single assay). Aliquots of PGE$_2$ standards (2 pg, 4 pg, 8 pg and 16 pg) were stored at -20°C and used in subsequent assays of different kit batches to monitor inter-assay variability, giving concentrations of (mean value of duplicates): 2.1 pg (2 pg standard); 5.4, 5.1, 2.9 pg (4 pg standard); 7.9 pg (8 pg standard); 16.3 pg (16 pg standard). Intra- and inter-assay coefficients of variation were 7.7% and 10.6% respectively and the sensitivity of the assay was 16 pg ml^{-1} (manufacturer's data).

(iii)   Cyclic Adenosine Monophosphate Enzyme-Immunoassay

The volume of each dialysis sample was determined and made up to 50 μl using assay buffer (0.05M sodium acetate buffer, pH 5.8 containing 0.02% bovine serum albumin and 0.005% thimerosal) provided with the kit. Standards, diluted in assay buffer, and samples were acetylated by the addition of 25 μl of a mixture of acetic anhydride:triethylamine (ratio 1:2) to increase the sensitivity of the assay (2 fmol/well ≡ 40 fmol ml^{-1}). One hundred μl of cAMP-antiserum was added to all wells except the blank and NSB wells followed by 50 μl aliquots from all acetylation tubes to the appropriate wells, with the standards run in duplicate and the samples singly. One hundred and fifty μl of assay buffer was added to the NSB wells. Plates were gently shaken and incubated at 4°C for 2 hours. HRP-labelled cAMP (150 μl) was then added to all wells except the blank and incubated at 4°C for 1 hour. Wells were then aspirated, by flicking the inverted plate, and washed four times with 400 μl of wash buffer (0.01M

phosphate buffer, pH 7.5 containing 0.05% Tween 20), ensuring any residual volume was removed by tapping the inverted plate on tissue paper. Enzyme substrate (150 μl) was then added to all wells and mixed at room temperature for 30 minutes while a blue colour developed. The addition of 100 μl 1M sulphuric acid to all wells resulted in the development of a yellow reaction product which was read immediately in a microtitre plate spectrophotometer at 450 nm, using the blank wells (only enzyme substrate added) to zero the plate-reader.

Theophylline did not appear to interfere with the concentration of cAMP determined in dialysates as aCSF containing theophylline were below the detection limit of the assay (2 fmol/well); the 32 fmol/well standard made up in aCSF containing theophylline gave concentrations of 24.7 and 31.9 fmol/well (duplicates of single assay). Aliquots of cAMP standards (2 fmol, 4 fmol and 16 fmol) were stored at -20°C and used in subsequent assays of different kit batches to monitor inter-assay variability, giving concentrations of (mean value of duplicates): 1.2 fmol (2 fmol standard); 4.8, 5, 3.5 fmol (4 fmol standard); 16.9, 17.5 fmol (16 fmol standard). The intra- and inter-assay coefficients of variation were 4.7% and 4.3%, respectively (manufacturer's data).

(iv)   Noradrenaline

Dialysate NA concentration was determined using high performance liquid chromatography (HPLC) with electrochemical detection by David Forster at Department of Physiology and Pharmacology, Queen's Medical Centre, University of Nottingham, UK. Briefly, the sample volume was determined and injected directly onto the HPLC without purification. The HPLC column (i.d. 2.1 mm x 10 cm long) was packed with 3 μm Hypersil ODS (Technicol Ltd., Manchester, U.K.). The mobile phase

consisted of 110 mM sodium acetate, 1 mM disodium EDTA, 100 mg L^{-1} sodium lauryl

sulphate and 20% methanol in double distilled, deionised water and adjusted to pH 5.1

with glacial acetic acid. The mobile phase was filtered using a Millipore 0.45 μm filter,

degassed under vacuum and sonicated for 5 min. before use. The pump flow rate was

set to 0.2 ml min^{-1}. Both electrodes were connected in series and the potential set at

+0.85 V against a Ag/AgCl reference electrode and the voltage output from the

amperometric detector was measured on a Spectra Physics SP4290 integrator.

A set of 1 x 10^{-8} M standard NA solutions were run at the beginning, middle and

end of each analytical run. A difference in the heights of the standard peaks of less than

6% was considered to be acceptable variation.

2.2.7 Determination of Plasma Corticosterone Concentration

Plasma CS levels were measured, without purification/extraction procedures,

using a commercially available RIA (Immunodiagnostic Systems, Tyne & Wear, U.K.).

The assay is based on the ability of a limited quantity of antibody to bind a fixed amount

of radiolabelled (^{125}I) antigen. The percentage of bound [^{125}I]-CS decreases as a

function of the increasing concentration of unlabelled CS in the test sample. Separation

of the bound and free [^{125}I]-CS is accomplished by the addition of a second antibody

directed toward the immunoglobulin present in the original antiserum, producing a

precipitate. The quantity of corticosterone in the test sample is determined by

comparing the radioactivity of the precipitate, after centrifugation and aspiration, with

values established using known standards in the same assay system.

Plasma samples were stored at -80°C for up to four weeks and RIA kits

purchased in batches (with the same lot number) allowing reagents to be pooled. All

plasma samples from LEW rats and saline-treated F344 rats were diluted 1:10 while samples from LPS-, IL-1β-, IFN–γ- and TNF–α- treated F344 rats were diluted 1:20 with assay buffer (phosphate buffered saline) supplied with the kit. Comparisons of 1:10 and 1:20 dilutions of the same sample were performed to monitor the linearity of the dilutions within the assay. The sensitivity of the assay was 0.39 ng ml^{-1}.

The assay was carried out in 12 x 75 mm polypropylene tubes. Two hundred μl of assay buffer was added to the NSB tubes, 100 μl assay buffer to the B_0 tubes and 100 μl of each standard or sample, in duplicate, to the appropriate tubes. [^{125}I]-Corticosterone (100 μl) was added to all tubes followed by 100 μl of corticosterone antiserum (rabbit anti-corticosterone) to all tubes except NSB and total counts (TC) tubes. All tubes were vortex mixed and incubated overnight (16-24 hours) at 4°C. One hundred μl of precipitating antibody (goat anti-rabbit gamma globulin) was added to all tubes except TC, vortex mixed and incubated at room temperature for 1 hour. Following the addition of 1.0 ml of 0.9% saline to all tubes except TC, bound and free [^{125}I]-corticosterone was separated by centrifugation (1500-1600 g, 15 min, 4°C) and the supernatant aspirated using a fine glass pipette attached to a vacuum pump, taking care not to dislodge the pellet. Radioactivity (counts per minute) in each pellet was measured for one minute using a gamma counter (MiniGamma 1275, LKB-Walac, Finland).

Aliquots of corticosterone standards (1.9 and 15.6 ng ml^{-1}) were stored at -20°C and used in subsequent assays of different kit batches to monitor inter-assay variability, giving concentrations of (mean value of duplicates): 2.7, 2.7, 1.3, 1.3 ng ml^{-1} (1.9ng ml^{-1} standard); 13.3, 16.9, 12.5 ng ml^{-1} (15.6 ng ml^{-1} standard). The intra- and inter-assay coefficients of variation were 7% and 15% respectively (manufacturer's data).

2.2.8 Data Presentation and Statistical Analysis

All results are expressed as means ± SEM. Data is presented in one of three ways: (1) as absolute amounts (i.e. CS in ng ml^{-1}; cAMP in fmol 30μl^{-1}; PGE$_2$ in pg ml^{-1}; NA in fmol 10μl^{-1}); N.B. values obtained for CS, cAMP and PGE$_2$ represent immunoreactive (ir) rather than biologically active levels of the substance; (2) as a percentage of respective basal values (i.e. the average of 2-4 samples collected before administration of saline, LPS or IL-1β). Transformation of the data accounts for the inter-animal variation of dialysate levels to give a clearer picture of the temporal profile of cAMP, PGE$_2$ or NA production; (3) as the mean difference from basal value % (MDBV %), calculated as follows: mean post injection value [%] - mean pre injection value [100%]).

Statistical analysis was performed using SPSS for Windows version 7.0. One-way analysis of variance (ANOVA) was used to test for overall differences between more than two experimental groups. Tukey's honestly significant difference (HSD) test was used as a post hoc test for comparisons between individual group means at a significance level of p=0.05. Student's t-test for unpaired data was used to compare differences between two experimental groups, with p<0.05 indicating a significant difference. Two-way ANOVA was used to assess whether the presence of a microdialysis probe had any effect on the CS response to i.p. injection of saline LPS or IL-1β.

Statistical analyses of changes in dialysate concentrations of cAMP, PGE$_2$ and NA were performed on a summary measure of the data (Matthews *et al.*, 1990). This method avoids comparisons of groups over a series of time points which are not likely to be independent of one another. The question to be answered was: is the overall value of

the outcome variable the same in different groups? To answer this question the summary measure chosen was the overall mean production of cAMP, PGE_2 or NA post injection. Student's t-test was used to compare the effects of treatment with LPS or IL-1β on second messenger/neurotransmitter production in LEW and F344 rats.

2.3 Results

2.3.1 Basal Plasma Corticosterone Concentration

Basal plasma CS concentration was determined in plasma samples obtained at 09:00 h from LEW (n=7) and F344 (n=8) rats without microdialysis probes and was significantly greater in F344 rats (LEW 51±7; F344 83±3 ng ml^{-1}; p<0.05, Student's t-test).

2.3.2 Time-Course Response to LPS

Similar temporal changes in plasma CS concentration were observed in LEW and F344 rats following 200 μg kg^{-1} LPS although the magnitude of the response was considerably greater in the F344 rat (Figure 2.3.1). No change in plasma CS levels was seen at any time-point following injection of saline compared to non-injected controls; thus, these data were pooled and are shown as baseline CS concentration. In LEW rats, there were no detectable changes in plasma CS levels of rats killed 30 min and 60 min after LPS injection. However, plasma CS was significantly elevated above baseline CS concentration by 120 min post LPS injection (p<0.05, one-way ANOVA plus Tukey's HSD test), peaked at 180 min and remained significantly above baseline 240 min post injection. In F344 rats a marked, but non-significant, increase in plasma CS levels was seen in rats killed 60 min post LPS injection. At 120 min post LPS injection plasma CS

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concentration was significantly greater than baseline levels (p<0.01, ANOVA plus Tukey's HSD test) and 6-fold greater than that of LEW rats. Plasma CS remained significantly greater than baseline at 180 and 240 min following LPS injection and was approximately 10-fold greater than LPS-injected LEW rats at the same time-points.

2.3.3 Dose- and Time-Course Responses to Interleukin-1β

Interleukin-1β caused a dose- and time-dependent increase in plasma CS levels in both LEW and F344 rats and, similarly to LPS-treated rats, the magnitude of the response was considerably greater in the F344 rat (Figure 2.3.2 A, B).

The dose-response relationship of plasma corticosterone concentration in LEW and F344 rats measured 2 h after i.p. administration of mrIL-1β is shown in figure 2.3.2 A. In F344 rats a significant increase in plasma CS compared to baseline levels was seen following a dose of 0.25 μg/rat IL-1β (p<0.01, ANOVA plus Tukey's HSD test) whereas a dose of 0.5 μg/rat was required to produce a significant increase above baseline steroid levels in LEW rats (p<0.05). The maximal CS response was seen following a dose of 0.5 μg/rat in F344 rats and 1.0 μg/rat in LEW rats.

Figure 2.3.2 B shows the time-course response of plasma CS in LEW and F344 rats to a dose of 0.5 μg/rat mrIL-1β. No change in plasma CS levels was seen at any time-point following injection of saline compared to non-injected controls in either strain; thus, these data were pooled and are shown as baseline CS concentration. Plasma CS levels were rapidly (at 30 min post injection) and significantly increased above baseline levels following IL-1β administration in both strains (p<0.01, ANOVA plus Tukey's HSD test) and remained significantly elevated up to 120 min post injection.

2.3.4 Corticosterone Production Following Administration of Tumour Necrosis Factor-α

The experiments using mrTNF-α and rrTNF-α were performed on different days; separate saline control groups for each strain were included on each day and were used to make relevant statistical comparisons although the data are pooled and shown as one saline control group in figure 2.3.3. One-way ANOVA revealed no significant change in plasma CS concentration in either strain measured two hours post i.p. injection of mrTNF-α (0.05-2.0 µg/rat) compared to saline injected controls. However, i.p. administration of rrTNF-α (10 µg/rat) elicited a significant increase in plasma CS levels in both rat strains compared to saline-treated controls ($p < 0.05$, Student's t-test).

2.3.5 Corticosterone Production Following Administration of Interferon-γ

Intraperitoneal injection of rat recombinant IFN-γ (0.1-10.0 µg/rat) did not significantly alter plasma CS concentration of either LEW or F344 rats, compared to saline injected control animals, measured 2 h post injection (Figure 2.3.4).

Figure 2.3.1   Time Course Response of Plasma Corticosterone Concentration to Intraperitoneal Injection of Lipopolysaccharide in Lewis and Fischer Rats
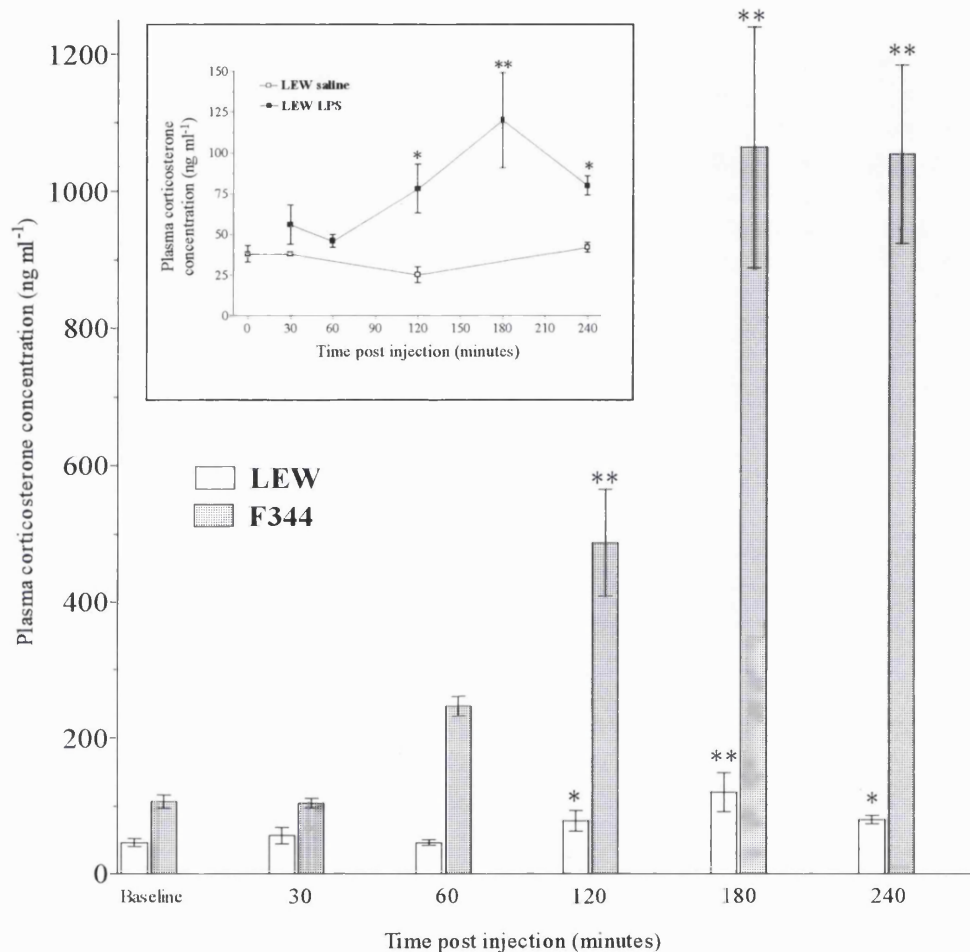
Lewis (LEW) and Fischer (F344) rats were injected intraperitoneally with either 0.9% sterile saline or lipopolysaccharide (LPS; 200 μg kg^{-1}) and animals killed at the times indicated for collection of trunk blood. Plasma corticosterone concentration (ng ml^{-1}) was determined by radioimmunoassay and the mean ± SE of values from 4 animals at each time-point is plotted; baseline represents pooled values obtained from non-injected and saline injected controls. Inset shows time-course response of LEW rats plotted on a different scale to demonstrate the stimulatory effect of LPS more clearly. *p<0.05 and **p<0.01 vs. respective baseline (ANOVA plus Tukey's HSD test).

Figure 2.3.2   Dose- and Time-Course Responses of Plasma Corticosterone
Concentration to Intraperitoneal Administration of Interleukin-1β in
Lewis and Fischer Rats
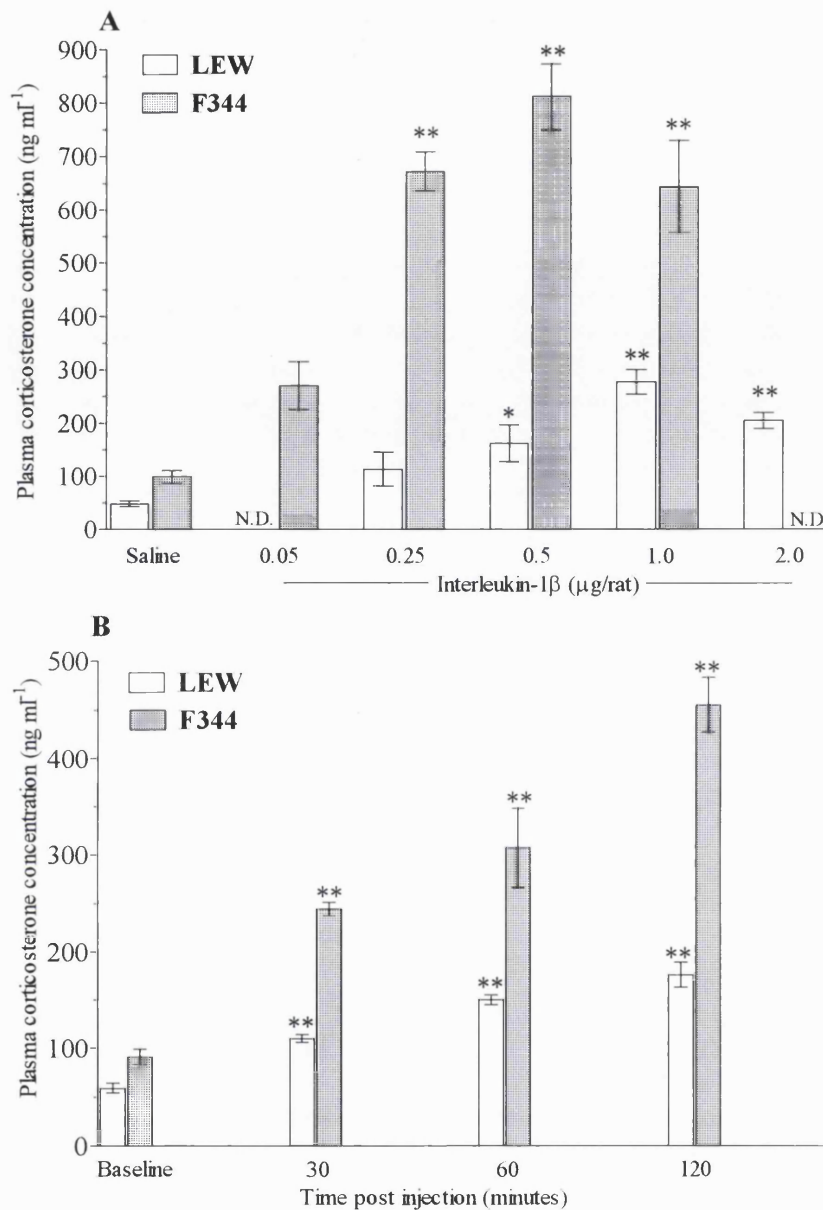
(A) Dose dependent increase in plasma corticosterone concentration in both Lewis
(LEW) and Fischer (F344) rats measured 2 hours following intraperitoneal injection of
sterile saline or murine recombinant interleukin (IL)-1β (0.05 - 2.0 μg/rat). Each bar
represents the mean ± SE of values from 5 rats. (B) Time course response of plasma
corticosterone concentration measured up to 2 hours following i.p. injection of
mrIL-1β (0.5 μg/rat). Each time point represents the mean ± SE of values from 4 rats;
baseline represents pooled data obtained from non-injected and saline injected controls.
N.D. = not done. *p < 0.05 and **p < 0.01 vs. respective saline or baseline (ANOVA
plus Tukey's HSD test).

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Figure 2.3.3   Effect of Tumor Necrosis Factor-α on Plasma Corticosterone
                      Concentration in Lewis and Fischer Rats
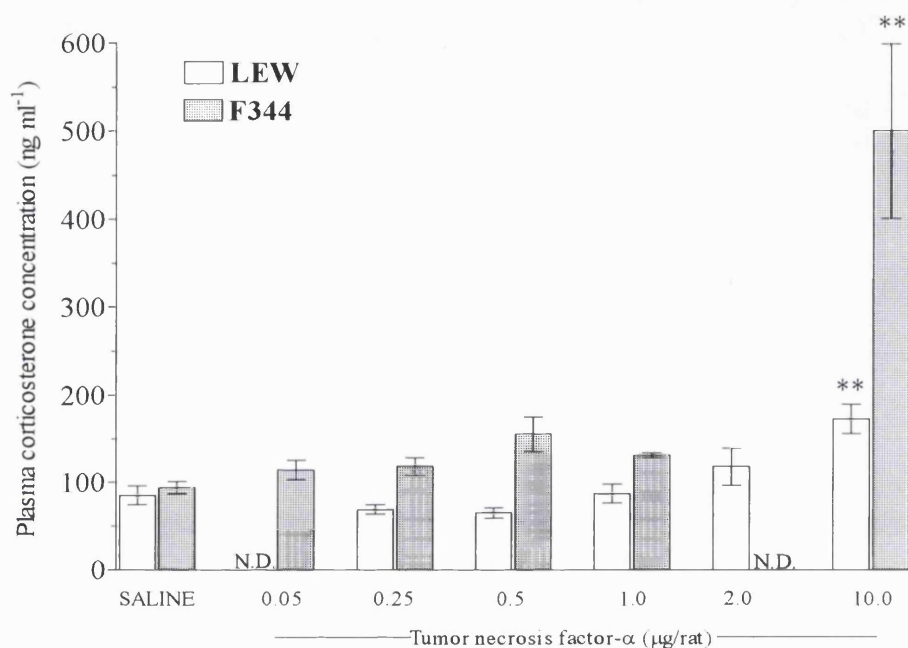
Lewis (LEW) and Fischer (F344) rats were injected i.p. with either saline, murine recombinant TNF-α (0.05 - 2.0μg/rat) or rat recombinant TNF-α (10μg/rat) and killed after 2 hours for collection of trunk blood. Plasma corticosterone concentration (ng ml^{-1}) was determined by radioimmunoassay and the mean ± SE of values from 5 animals at each dose is shown. N.D. = not done.

The experiments using murine and rat TNF-α were performed on different days with separate saline control groups (n = 5) on each day which have been pooled in the figure. One-way ANOVA showed no effect of mrTNF-α on plasma corticosterone concentration in either strain. **p < 0.05 vs. respective saline, Student's t-test.

Figure 2.3.4   Effect of Interferon-γ on Plasma Corticosterone Secretion in
Lewis and Fischer Rats
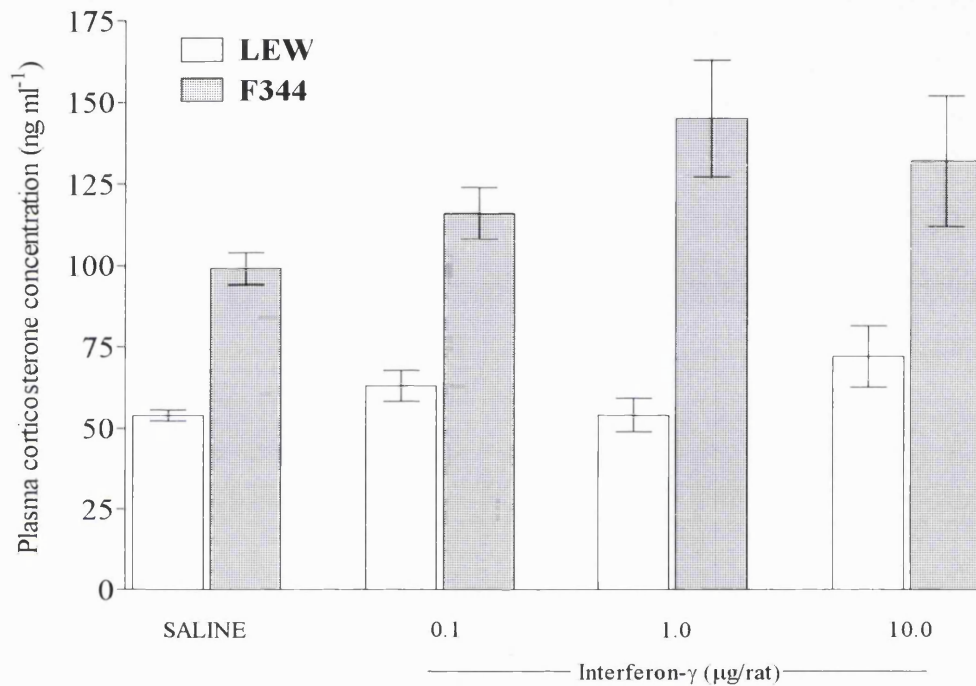
Lewis (LEW) and Fischer (F344) rats were injected intraperitoneally with either sterile
saline or rat recombinant interferon-γ (0.1 - 10.0 μg /rat) and killed after two hours for
collection of trunk blood. Plasma corticosterone concentration (ng ml^{-1}) was
determined by radioimmunoassay and the mean ± SE of values from 5 rats at each dose
is shown.
One-way ANOVA showed no effect of rrIFN-γ on plasma corticosterone concentration
for either strain.

2.3.6   Effect of Microdialysis Probe on Plasma Corticosterone Responses

Table 2.1 shows the plasma corticosterone response of LEW and F344 rats with and without microdialysis probes to LPS and IL-1β. Plasma CS concentration in both LEW and F344 rats, with or without microdialysis probes, measured 240 min after injection of LPS was significantly greater than saline injected controls ($p<0.001$, two-way ANOVA). In LEW rats no significant effect of the microdialysis probe was seen whilst in F344 rats plasma CS was significantly reduced in rats with microdialysis probes ($p<0.001$, two-way ANOVA; Table 2.1 A).

Administration of IL-1β resulted in significantly higher plasma CS concentration in both LEW and F344 rats, with or without microdialysis probes, measured 2 h post injection compared to saline ($p<0.001$, two-way ANOVA; Table 2.1 B). In LEW rats with microdialysis probes, plasma CS was significantly higher following both saline and IL-1β injection compared to rats without probes although there was no significant interaction between treatment and the presence of a microdialysis probe ($p=0.234$; Table 2.1 B).

2.3.7   Basal Production of cAMP, PGE₂ and Noradrenaline in LEW and F344 Rats

Figure 2.3.5 shows basal production of cAMP (fmol $30\mu l^{-1}$), PGE$_2$ (pg ml^{-1}) and noradrenaline (fmol $10\mu l^{-1}$) collected in 2-4 dialysate fractions from LEW and F344 rats following a 30 minute washout period and prior to administration of saline or LPS (LPS experiments) and saline or IL-1β (IL-1 experiments).

The mean basal dialysate cAMP concentration of F344 rats used in LPS and IL-1 experiments and LEW rats used in IL-1 experiments was very similar. In LPS

Table 2.1 Plasma Corticosterone Concentration in Lewis and Fischer Rats With and Without Microdialysis Probes Following Intraperitoneal Administration of Either Saline, LPS or IL-1β

A

Plasma Corticosterone Concentration (ng ml⁻¹)								
Lewis (240 min)				Fischer (240 min)				Statistical Differences
Saline - probe (n = 8)	Saline + probe (n = 19)	LPS - probe (n = 8)	LPS + probe (n = 22)	Saline - probe (n = 7)	Saline + probe (n = 26)	LPS - probe (n = 8)	LPS + probe (n = 31)	Main effects: a vs. b and c vs. d, p < 0.001 Interactions:
62 ± 8^a	91 ± 7^a	188 ± 42^b	197 ± 21^b	136 ± 12^a	127 ± 16^a	$1329\pm151^{b,c}$	$667\pm53^{b,d}$	LEW, none p=0.654; F344, p<0.001

B

Lewis (120 min)				Fischer (120 min)				Statistical Differences
Saline - probe (n = 9)	Saline + probe (n = 24)	IL-1β - probe (n = 9)	IL-1β + probe (n = 25)	Saline - probe (n = 9)	Saline + probe (n = 21)	IL-1β - probe (n = 8).	IL-1β + probe (n = 30)	Main effects: a vs. b and c vs. d, p < 0.001 Interactions:
$47\pm3^{a,c}$	$89\pm7^{a,d}$	$168\pm19^{b,c}$	$234\pm8^{b,d}$	78 ± 6^a	150 ± 13^a	589 ± 98^b	773 ± 59^b	LEW, none p=0.234; F344, none p=0.401

Plasma was collected from animals 240 min (A) or 120 min (B) after i.p. administration of sterile saline, LPS (A; 200 µg kg⁻¹) or IL-1β (B; 0.5 µg/rat). Plasma corticosterone concentrations (ng ml⁻¹) are presented as mean ± SEM. Significant differences, derived by two-way ANOVA, are encoded as superscripts and the p value for main effects (a vs. b = effect of LPS/IL-1β; c vs. d = effect of probe) and 2-way interactions are listed.

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experiments, basal cAMP concentration was significantly greater in LEW rats compared to F344 rats (p<0.01, Student's t-test; Figure 2.3.5 A). It should be noted that mean basal production of cAMP in LEW rats subsequently injected with saline was similar to that of F344 rats and was increased in LEW rats subsequently injected with LPS (Figure 2.3.6 A) although this was not significant (p=0.07, Student's t-test).

The basal production of PGE_2 in the dialysates collected from LEW rats used in LPS and IL-1 experiments was similar. However, PGE_2 production in LEW rats was 3.5-fold greater compared to F344 rats used in LPS experiments (p<0.001, Student's t-test) whilst no difference was observed between LEW and F344 rats used in IL-1 experiments (Figure 2.3.5 B).

Basal dialysate NA concentration was very similar for both LEW and F344 rats used in LPS and IL-1 experiments (Figure 2.3.5 C).

Figure 2.3.5 Basal Concentration of Cyclic AMP, Prostaglandin E_2 and Noradrenaline in Hypothalamic Dialysates From Lewis and Fischer Rats
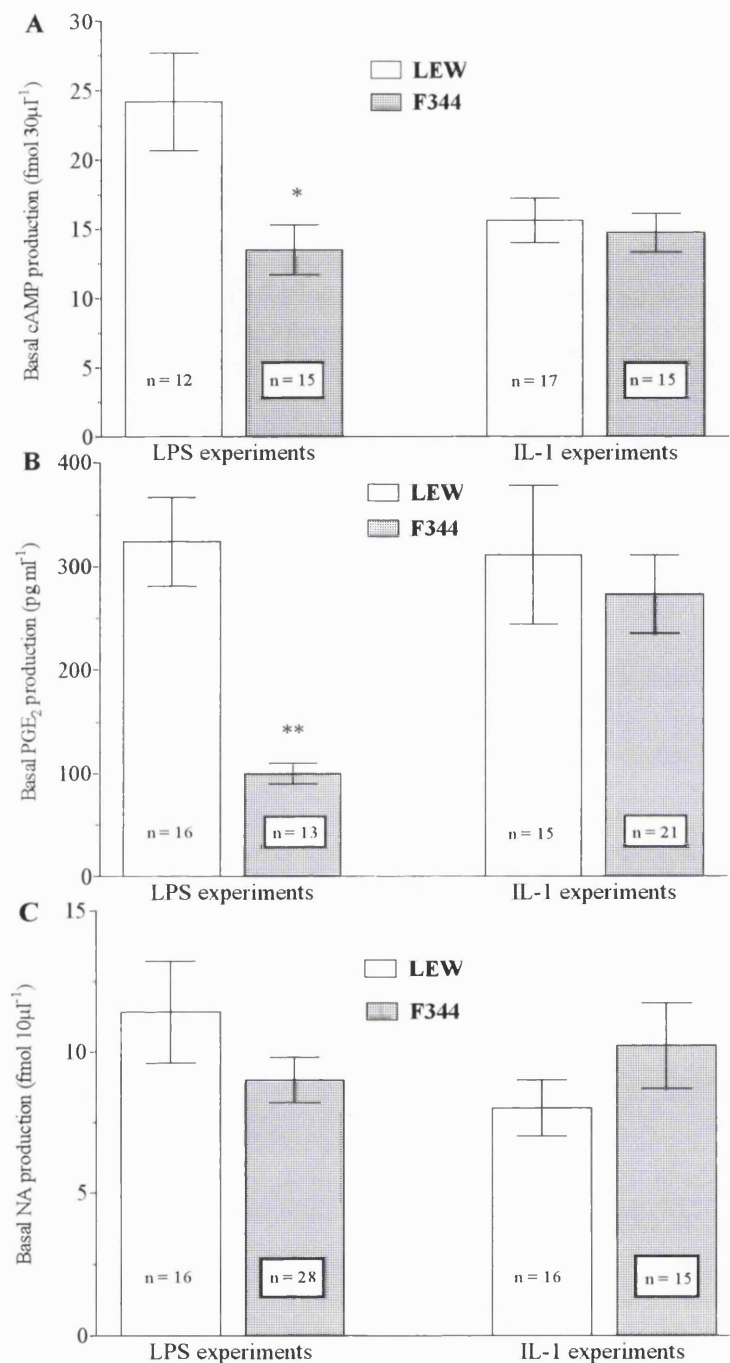
Cyclic AMP (cAMP), prostaglandin (PG) E_2 and noradrenaline (NA) content was measured in 2 - 4 dialysate fractions collected prior to administration of saline or endotoxin (LPS experiments) or saline or interleukin (IL)-1β (IL-1 experiments) to Lewis (LEW) and Fischer (F344) rats. The mean basal concentration for each individual animal was taken and the mean and SE for each group is represented by the columns and bars; the number of animals in each group is given within the columns. *p<0.01 and **p<0.001 vs. LEW, Student's t-test.

In the following sections (2.3.8 to 2.3.13) plasma corticosterone concentration, measured at the end of the dialysis collection period, is presented in the figures showing temporal changes of cAMP, PGE$_2$ or NA in response to LPS or IL-1β. In all cases, LPS or IL-1β administration induced significant increases in plasma CS levels compared to saline injection in both strains of rat and the LPS- or IL-1β-induced CS response was significantly greater in F344 rats compared to LEW rats.

2.3.8 Effect of LPS on Hypothalamic cAMP Production

Figure 2.3.6 shows the temporal profile of cAMP concentration measured in dialysate fractions collected from the anterior hypothalamus of LEW and F344 rats for 90 to 120 minutes prior to and 240 minutes following i.p. injection of saline or LPS expressed as absolute concentration (A, B) or as a percentage of the basal cAMP production (C, D). No changes in dialysate cAMP concentration were observed over the collection period in either LEW or F344 rats following saline administration. In LEW rats the cAMP concentration began to increase at 30 minutes post LPS injection and remained elevated throughout the collection period with a peak at 120 mins (Figure 2.3.6 A, C). In F344 rats, the concentration of cAMP in dialysates was lower than that detected in samples from LEW rats (Figure 2.3.6 B) and although elevated above saline controls with a peak after 60 minutes (141% of basal production), cAMP levels decreased and were not elevated above saline treated controls by 150 min post LPS injection (Figure 2.3.6 D).

The mean production of cAMP post injection (Figure 2.3.14 A) demonstrates both the significant increase of cAMP production in LEW rats following LPS compared to saline (p<0.005, Student's t-test) and the significantly greater production of cAMP in

LEW rats following LPS injection compared to F344 rats ($p<0.01$, Student's t-test). The mean production of cAMP following LPS administration in F344 rats was not significantly different from saline controls.

Co-administration of indomethacin (50 mg kg^{-1}) with LPS (200 μg kg^{-1}) to LEW rats prevented the increase in hypothalamic cAMP production and plasma corticosterone concentration measured up to 140 minutes post injection (Figure 2.3.7 A). Statistical analysis of the mean difference from basal values (MDBV) of cAMP following each treatment showed a significant increase following LPS administration compared to both saline and LPS + indomethacin ($p<0.01$, one-way ANOVA plus Tukey's HSD test) and is presented as a percentage of the MDBV in figure 2.3.7B.

2.3.9   Effect of LPS on Hypothalamic PGE₂ Production

The temporal profile of PGE_2 production measured in dialysate fractions collected from the anterior hypothalamus of LEW and F344 rats for up to 90 minutes prior to and 240 minutes following i.p. injection of saline or LPS is shown in figure 2.3.8, expressed as absolute concentration (A, B) or as a percentage of the basal production (C, D). No changes in dialysate PGE_2 concentration were observed over the collection period in either LEW or F344 rats following saline administration. In LEW rats the PGE_2 response was similar in profile to that of cAMP, rising by 60 min, reaching a peak at 120 min and declining thereafter but remaining elevated above saline treated controls up to 240 min (Figure 2.3.8 A, C). PGE_2 production was increased after 60 min in F344 rats and maintained at a steady level until declining at 240 minutes although the concentration of PGE_2 in dialysates of F344 rats was lower than that of LEW rats (Figure 2.3.8 B). The relative increase in PGE_2 production following LPS was similar in

both strains when expressed as a % of basal production (Figure 2.3.8 C, D).

The mean concentration of PGE_2 in dialysates collected following injection of LPS was significantly greater than following saline injection in both LEW ($p<0.05$, Student's t-test) and F344 ($p<0.005$) rats (Figure 2.3.14 B). In addition, mean dialysate PGE_2 concentration following LPS was significantly greater in LEW rats compared to F344 rats ($p<0.01$; Figure 2.3.14 B).

Concomitant administration of indomethacin with LPS to LEW rats prevented the increase in hypothalamic PGE_2 seen in animals injected with LPS alone, although CS concentration at the end of the experiment (i.e. 240 min post injection) was the same as LPS injected animals (Figure 2.3.9 A). Administration of indomethacin alone to LEW rats without microdialysis probes had no effect on corticosterone production measured 240 minutes post injection (ng ml^{-1}, n=4): saline 86±5; indomethacin 82±6. The MDBV of dialysate PGE_2 in LEW rats following LPS was significantly greater than in LEW rats injected with saline ($p<0.01$, one-way ANOVA plus Tukey's HSD test) or LPS + indomethacin ($p<0.05$), and is expressed graphically as MDBV (%) in figure 2.3.9 B.

2.3.10 Effect of LPS on Hypothalamic Noradrenaline Production

The overall mean production of NA following LPS administration was not significantly different from saline injected controls in both LEW and F344 rats (Figure 2.3.14 C). Whilst individual rats of both strains showed increases in dialysate NA concentration following LPS injection, the response was pulsatile, variable in magnitude and not synchronous. This is reflected in the temporal profiles of NA accumulation in dialysate samples which show that apart from peaks at 90 and 210-240 minutes after LPS, NA production was similar to saline injected controls (Figure 2.3.10).

The highest dialysate concentration of NA was measured in a sample from a LEW rat 90 min after LPS administration (74.5 fmol $10\mu l^{-1}$) whilst the highest dialysate NA concentration in a sample from a F344 rat was 37 fmol $10\mu l^{-1}$ (also obtained 90 min after LPS administration). The mean maximum NA concentration obtained at any time-point post-injection of LPS or saline in each strain was calculated (fmol $10\mu l^{-1}$): LEW saline 17.4 ± 3; LEW LPS 36.8 ± 10; F344 saline 11.2 ± 2; F344 LPS 19.5 ± 2. Statistical analysis revealed no significant difference between LEW rats injected with saline or LPS (p=0.09, Student's t-test) although a statistically significant difference was apparent in F344 rats (p=0.005). In addition, statistical analysis revealed no significant difference between the maximum NA response to LPS of LEW and F344 rats (p=0.119, Student's t-test).

Figure 2.3.6 Cyclic AMP Production in the Anterior Hypothalamus of Lewis and Fischer Rats Following Administration of LPS
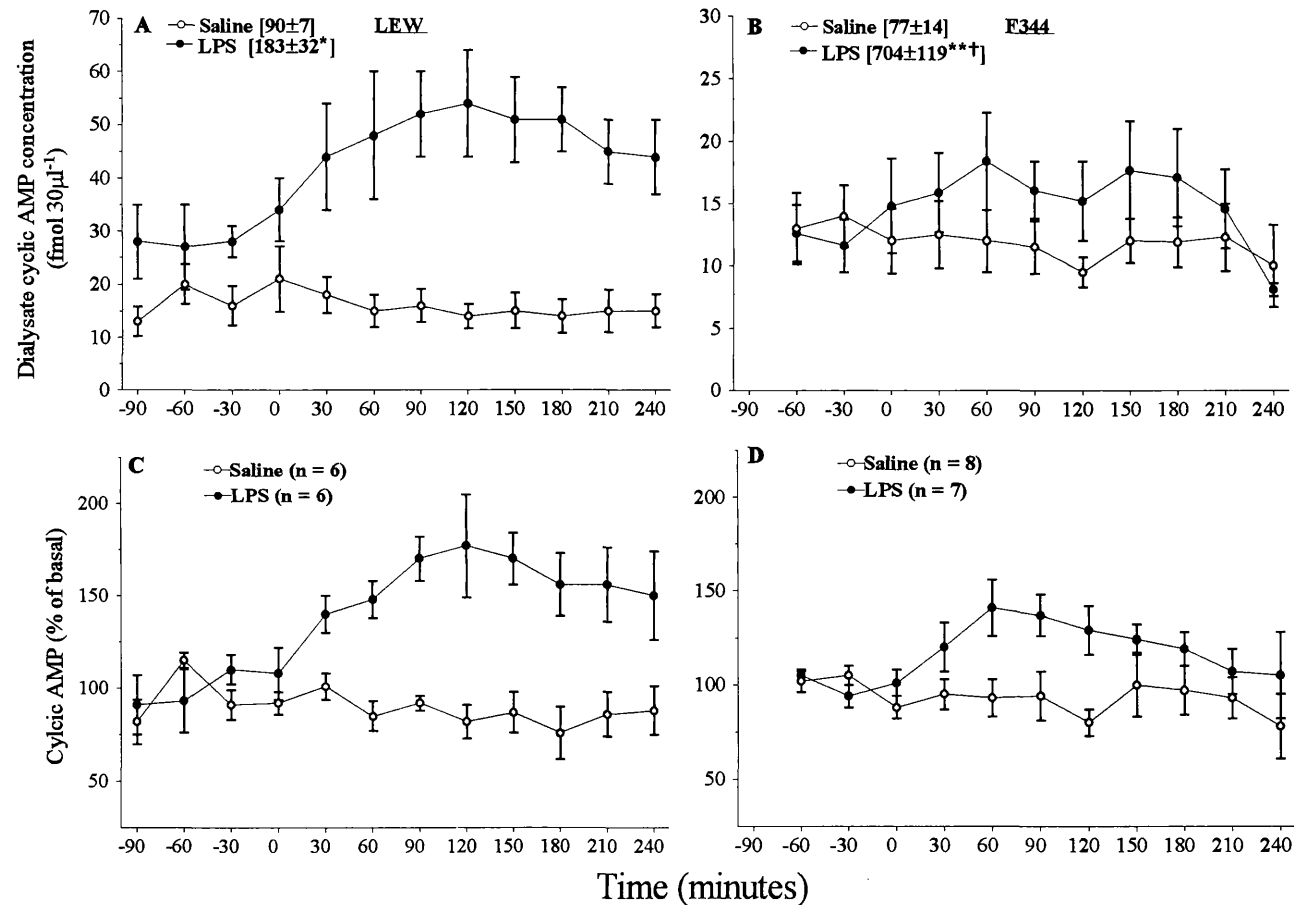
The effect of i.p. saline or LPS (200 μg kg⁻¹) administered following collection of the 0 minute dialysis sample on the concentration of cyclic AMP (fmol 30μl⁻¹) recovered from the anterior hypothalamus of (A) Lewis and (B) Fischer rats. Cyclic AMP production expressed as the percentage of the respective basal cAMP level is presented for Lewis (C) and Fischer (D) rats. Each time point represents the mean ± SE for 6-8 animals per group (n values in brackets C, D). Mean plasma corticosterone concentration (ng ml⁻¹) measured 240 min post injection is given in brackets (A, B). *p < 0.05 and **p < 0.01 vs saline; †p < 0.01vs. LEW LPS, Student's t-test.

Figure 2.3.7   Effect of Indomethacin on LPS-Induced Hypothalamic Cyclic AMP
Production in Lewis Rats
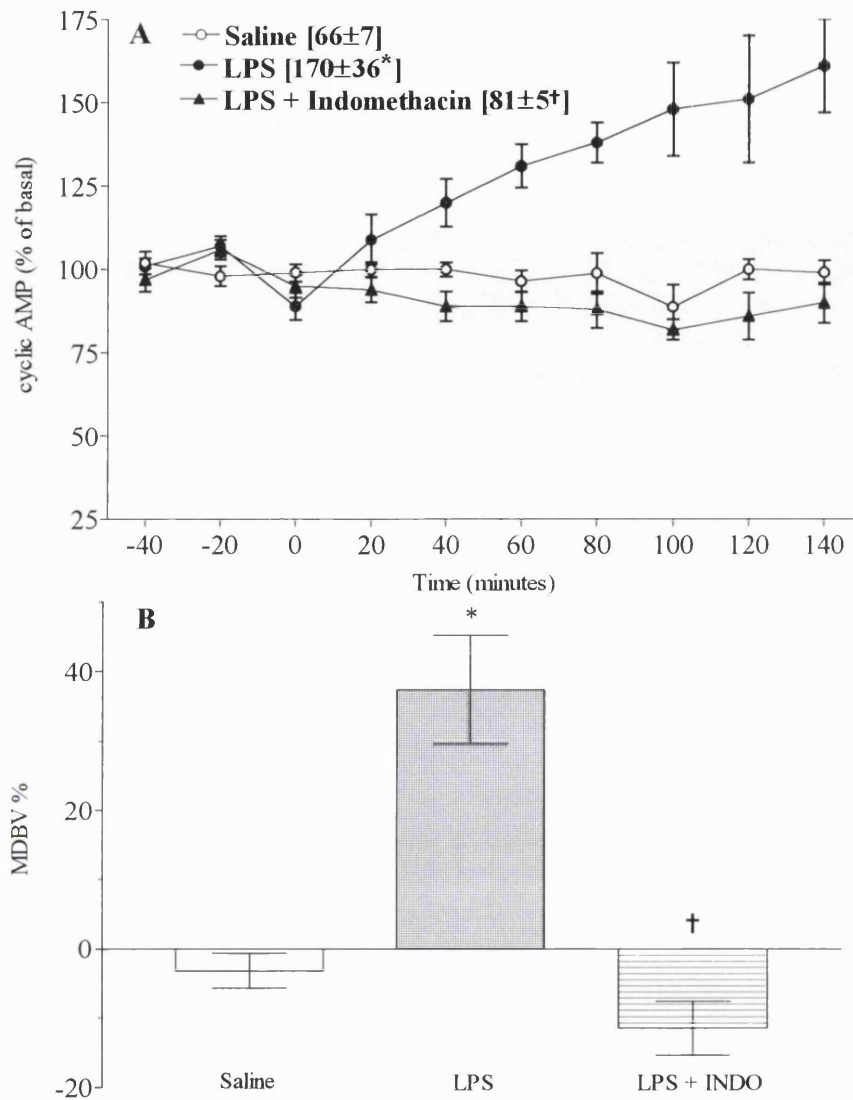
(A) The effect of saline (n = 6), LPS ($200\mu g$ kg^{-1}; n = 5) or LPS plus indomethacin
(INDO; $200\mu g$ kg^{-1} and 50mg kg^{-1} respectively; n = 4), administered following collection
of the 0 min sample, on cyclic AMP concentration in dialysates recovered from the
anterior hypothalamus of LEW rats. Each time point, mean ± SE, is expressed as the
percentage of the respective basal cAMP level (basal values (fmol $30\mu l^{-1}$): saline 16.3 ±
2.4; LPS 18.0 ± 2.5; LPS + INDO 20.2 ± 0.8). Values in brackets represent the mean
plasma corticosterone concentration (ng ml^{-1}) collected 140 min following each
treatment. (B) Mean difference from basal value (MDBV, %) following each i.p.
treatment. *$p < 0.01$ vs saline and †$p \leq 0.01$ vs. LPS; one-way ANOVA followed by
Tukey's HSD test.

Figure 2.3.8 Prostaglandin E_2 Production in the Anterior Hypothalamus of Lewis and Fischer Rats Following Administration of LPS
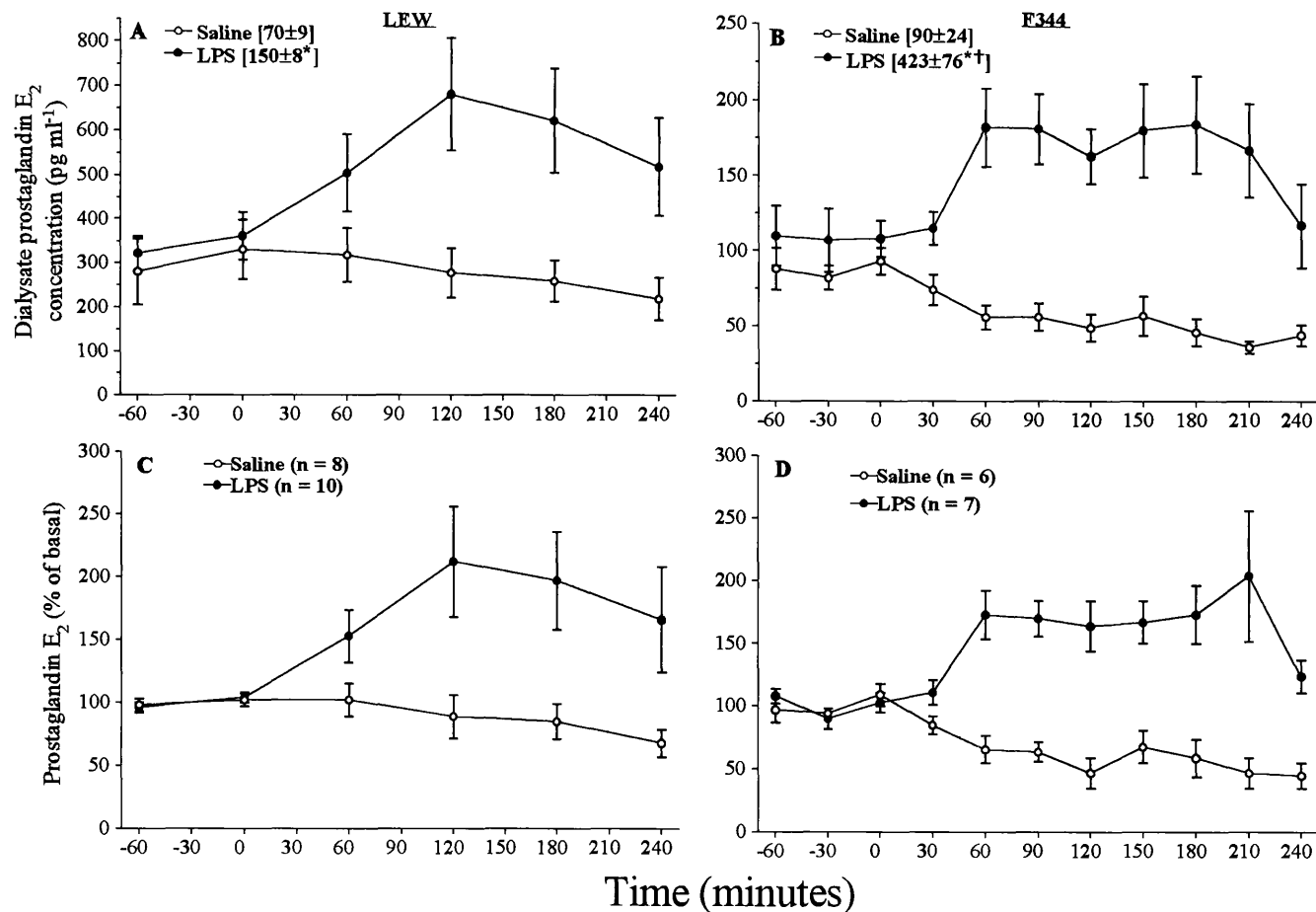
The effect of i.p. saline or LPS (200 µg kg⁻¹), administered following collection of the 0 min dialysis sample, on the concentration of PGE_2 (pg ml⁻¹) recovered from the anterior hypothalamus of (A) Lewis and (B) Fischer rats. PGE_2 production expressed as the percentage of the respective basal PGE_2 level is presented for Lewis (C) and Fischer (D) rats. Each time point represents the mean ± SE for 6-9 animals per group (n values in brackets C, D). Mean plasma corticosterone concentration (ng ml⁻¹) measured 240 min post injection is given in brackets (A, B). *p < 0.01 vs. saline; †p = 0.01 vs. LEW LPS, Student's t-test.

Figure 2.3.9   Effect of Indomethacin on LPS-induced Hypothalamic
Prostaglandin E_2 production in Lewis Rats
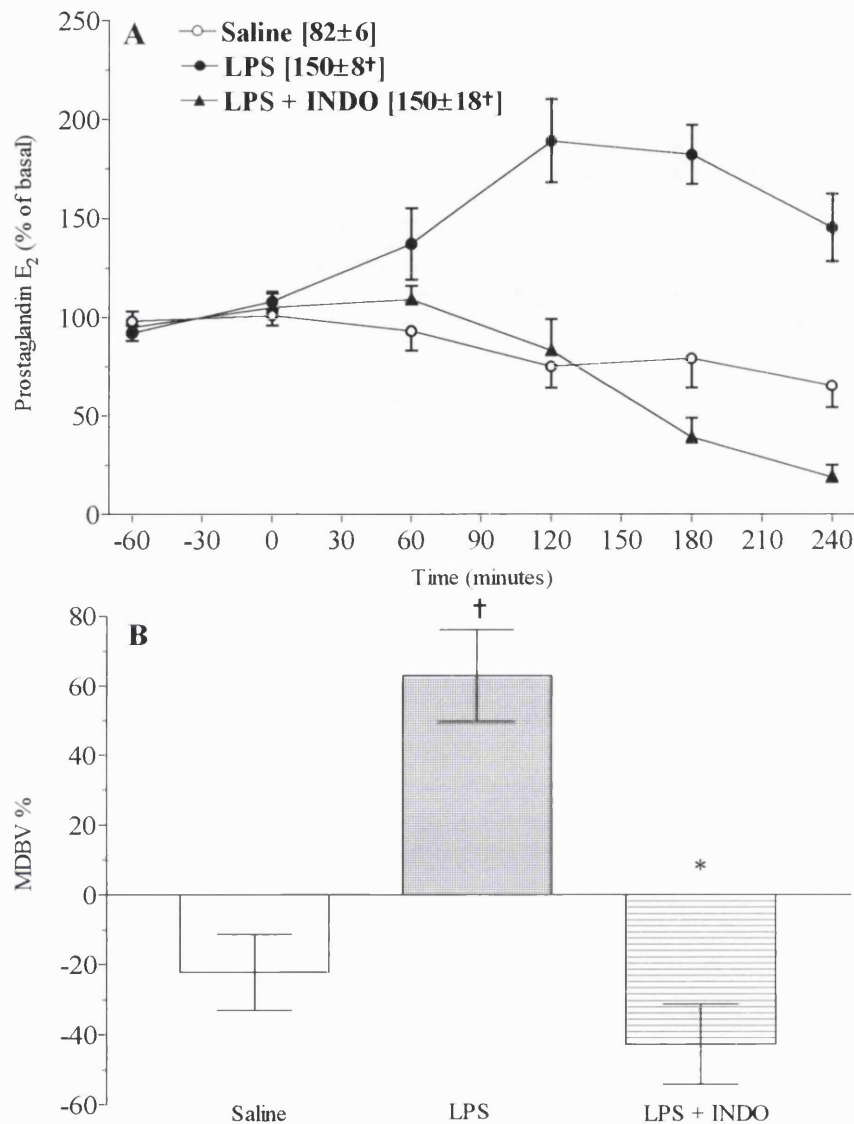
(A) The effect of saline (n = 7), LPS (200μg kg^{-1}; n = 10) or LPS plus indomethacin
(INDO; 200μg kg^{-1} and 50mg kg^{-1} respectively; n = 4) on prostaglandin E_2 concentration in
dialysates recovered from the anterior hypothalamus of LEW rats. Each time point, mean
± SE, is expressed as the percentage of the respective basal PGE_2 level (basal values, pg
ml^{-1}): saline 340± 82; LPS 346± 54; LPS + INDO 173± 44). Values in brackets represent
the mean plasma corticosterone concentration (ng ml^{-1}) collected 240 minutes following
each treatment. (B) Mean difference from basal value (MDBV, %) following each i.p.
treatment. *p < 0.05 vs. LPS and †p < 0.01 vs. saline, one-way ANOVA followed by
Tukey's HSD test.

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Figure 2.3.10 Noradrenaline Production in the Anterior Hypothalamus of Lewis and Fischer Rats Following Administration of LPS

Time (minutes)
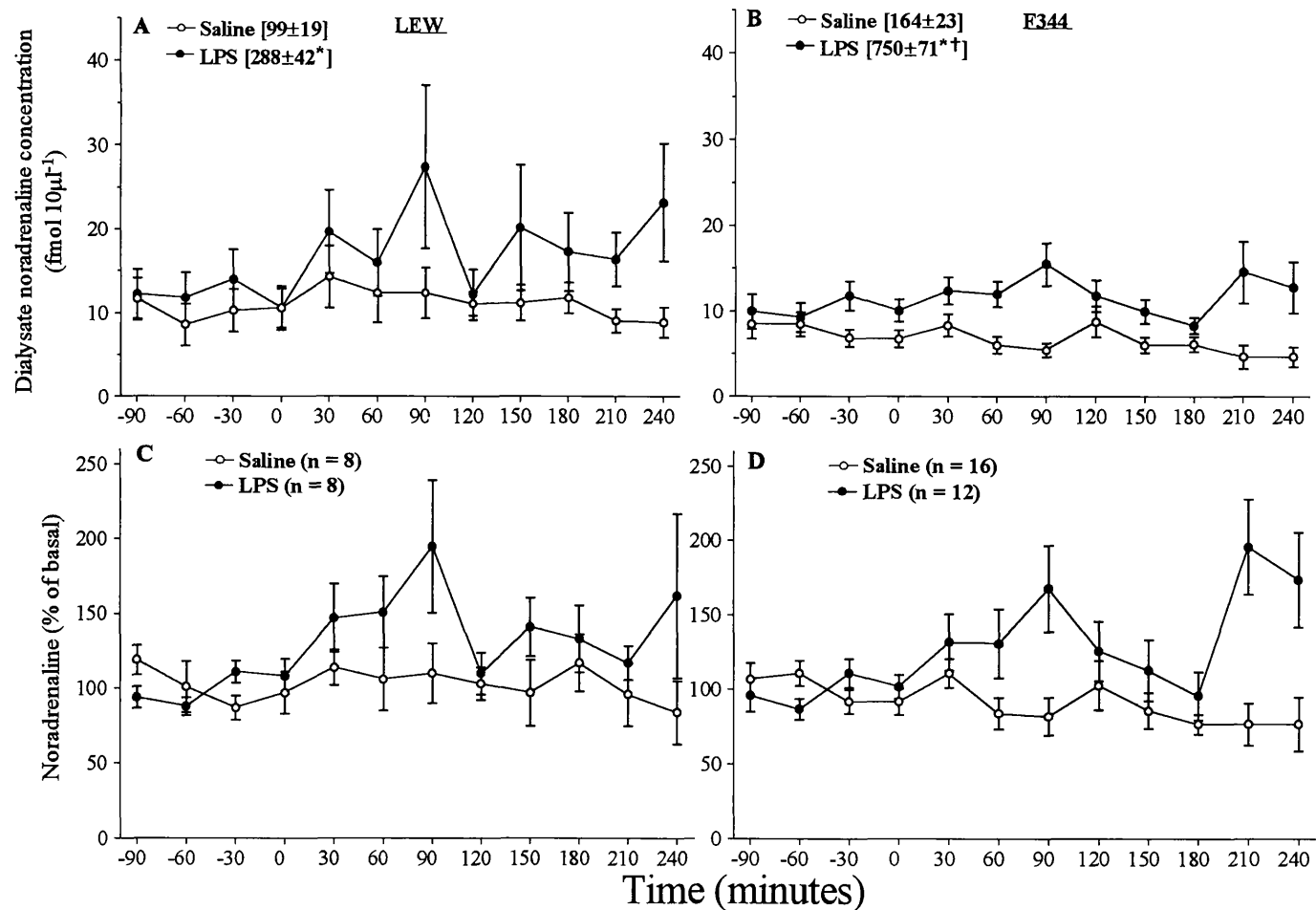
The effect of i.p. saline or LPS (200 μg kg⁻¹), adminstered following collection of the 0 min dialysis sample, on the concentration of noradrenaline (fmol 10μl⁻¹) recovered from the anterior hypothalamus of (A) Lewis and (B) Fischer rats. NA production expressed as the percentage of the respective basal NA level is presented for Lewis (C) and Fischer (D) rats. Each time point represents the mean ± SE for 8-16 animals per group (n value in brackets C, D). Mean plasma corticosterone concentration (ng ml⁻¹) measured 240 min post injection is given in brackets (A, B). $^*p < 0.01$ vs. saline; $^\dagger p < 0.01$ vs. LEW LPS, Student's t-test.

2.3.11 Effect of IL-1β on Hypothalamic cAMP Production

A rapid increase in hypothalamic cAMP production was apparent in both strains after IL-1β administration with dialysate cAMP concentration being elevated above saline injected controls within 20 minutes (Figure 2.3.11). In LEW rats, cAMP concentration gradually declined over the next 100 minutes (Figure 2.3.11A, C) whereas dialysate cAMP concentration in F344 rats remained elevated throughout the collection period (Figure 2.3.11 B, D). Thus, although the magnitude of the cAMP response to IL-1β was very similar in both strains, with an approximately 2-fold increase in the mean dialysate cAMP concentration following IL-1β compared to saline, this was statistically significant for F344 rats (p=0.02, Student's t-test) but just failed to reach statistical significance for LEW rats (p=0.06; Figure 2.3.14 D).

2.3.12 Effect of IL-1β on Hypothalamic PGE₂ Production

In LEW rats, injection of IL-1β resulted in a slight increase in dialysate PGE_2 concentration compared to basal levels whereas no increase was seen following administration of saline (Figure 2.3.12 A). A rapid (within 20 minutes) increase in dialysate PGE_2 levels was observed in F344 rats following IL-1β administration which declined over the next 100 minutes (Figure 2.3.12 B). Whilst the absolute concentration of PGE_2 in dialysates collected from both strains of rat were similar following injection of either saline or IL-1β, an increase was suggested following IL-1β injection when expressed as a percentage of basal production, with a peak at 40 minutes (147 % of basal) in LEW rats and 20 minutes (200 % of basal) in F344 rats (Figure 2.3.12 C, D). However, the mean post-injection concentration of PGE_2 following administration of

IL-1β was not significantly different from saline injected controls in both strains of rat and no difference in dialysate PGE_2 concentration was observed between the strains (Figure 2.3.14 E).

2.3.13 *Effect of IL-1β on Hypothalamic NA Production*

In LEW rats no change in dialysate NA concentration was seen following i.p. injection of either saline or IL-1β (Figure 2.3.13 A, C). In F344 rats, the absolute concentration of NA in dialysate samples appeared greater in the saline control group compared to the IL-1β group (Figure 2.3.13 B). However, whilst there was no change in dialysate NA concentration following injection of saline, a slight increase in NA concentration was detected 40 and 60 minutes following IL-1β administration compared to basal levels (168 % of basal at 60 minutes) which declined to basal levels by 80 minutes post IL-1β (Figure 2.3.13 D). Overall production of NA following IL-1β administration was not different from that following saline in both strains of rat (Figure 2.3.14 F).

Figure 2.3.11 Cyclic AMP Production in the Anterior Hypothalamus of Lewis and Fischer Rats Following Administration of Interleukin-1β
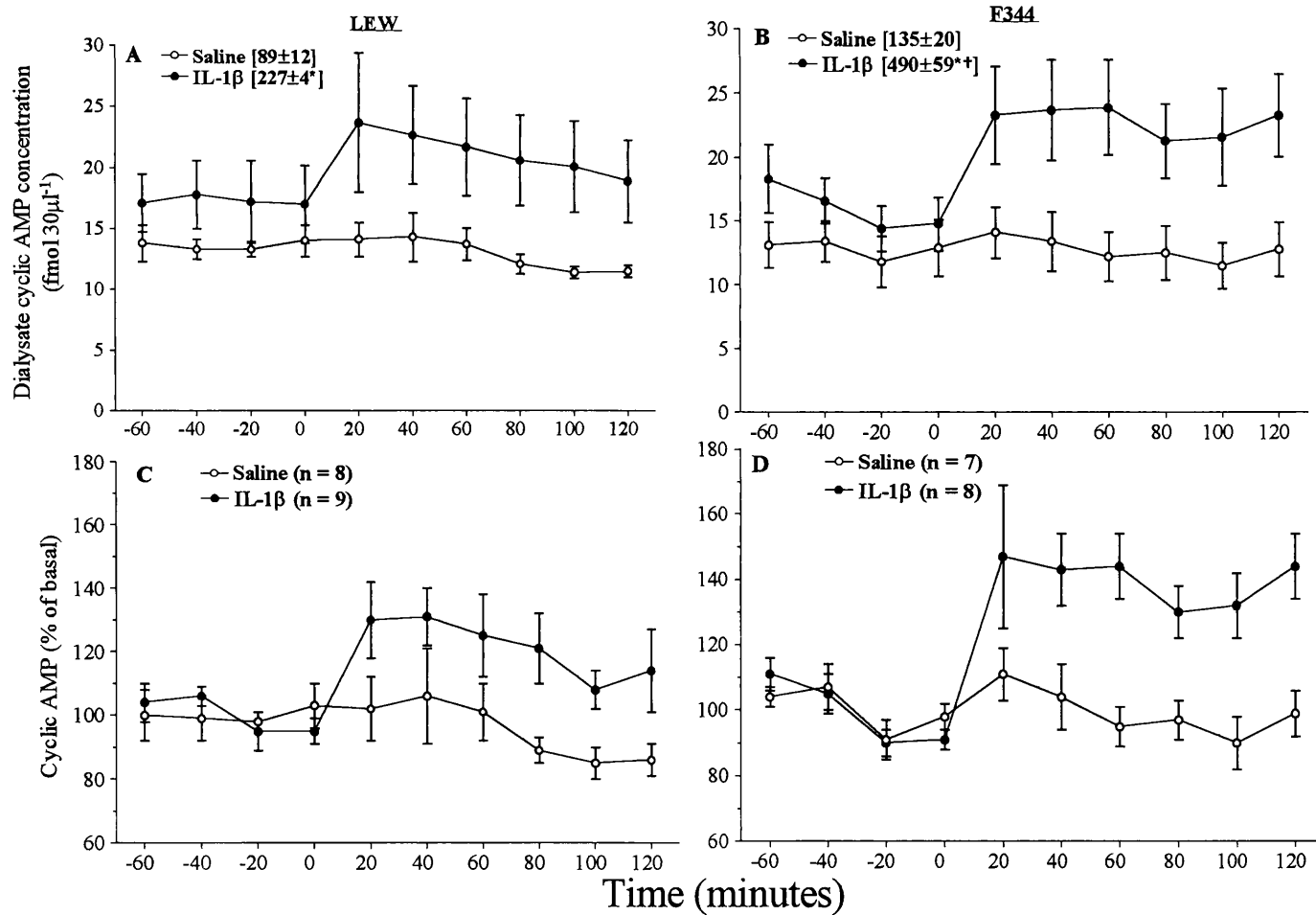
The effect of i.p. saline or IL-1β (0.5 µg/rat), administered following collection of the 0 min dialysis sample, on the concentration of cAMP (fmol 30µl^{-1}) recovered from the anterior hypothalamus of (A) Lewis and (B) Fischer rats. Cyclic AMP production expressed as the percentage of the respective basal level is presented for Lewis (C) and Fischer (D) rats. Each time point represents the mean ± SE for 8 or 9 animals per group (n value in brackets C, D). Mean plasma corticosterone concentration (ng ml^{-1}) measured 120 min post injection is given in brackets (A, B). *p < 0.01 vs. saline; †p < 0.01 vs. LEW IL-1β, Student's t-test.

Figure 2.3.12 Prostaglandin E₂ Production in the Anterior Hypothalamus of Lewis and Fischer Rats Following Administration of Interleukin-1β

A —○— Saline [68±7] LEW
   —●— IL-1β [256±21*]

B —○— Saline [122±19] F344
   —●— IL-1β [968±71**†]

C —○— Saline (n = 8)
   —●— IL-1β (n = 7)

D —○— Saline (n = 7)
   —●— IL-1β (n = 14)

Dialysate prostaglandin E₂ concentration (pg ml⁻¹)

Prostaglandin E₂ (% of basal)

Time (minutes)
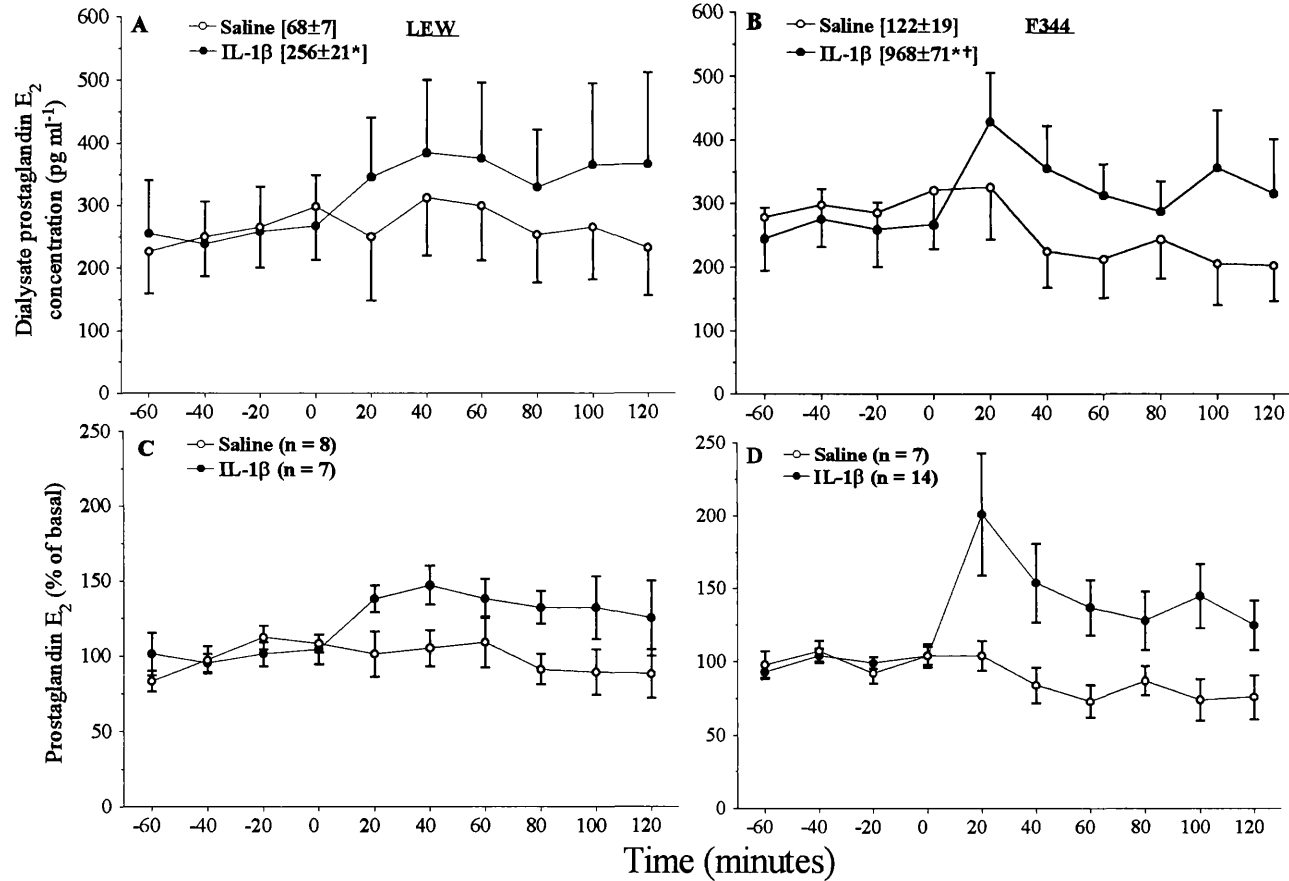
The effect of i.p. saline or IL-1β (0.5 µg/rat), administered following collection of the 0 min dialysis sample, on the concentration of PGE₂ (pg ml⁻¹) recovered from the anterior hypothalamus of (A) Lewis and (B) Fischer rats. PGE₂ production expressed as the percentage of the respective basal level is presented for Lewis (C) and Fischer (D) rats. Each time point represents the mean ± SE for 7 - 14 animals per group (n values in brackets C, D). Mean plasma corticosterone concentration (ng ml⁻¹) measured 120 minutes post injection is given in brackets (A, B). *p < 0.01 vs. saline; †p < 0.01 vs. LEW IL-1β, Student's t-test.

Figure 2.3.13 Noradrenaline Production in the Anterior Hypothalamus of Lewis and Fischer Rats Following Administration of Interleukin-1β
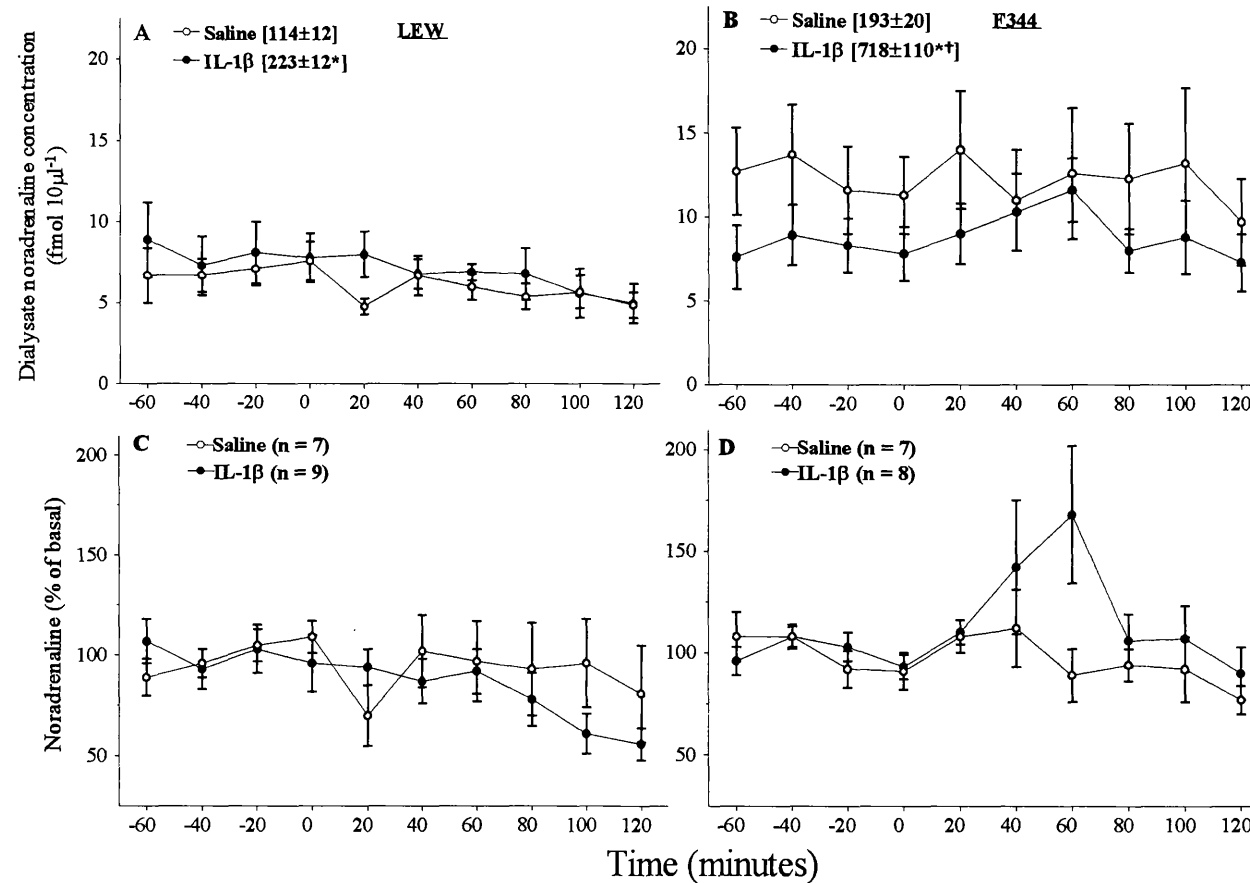
Effect of i.p. saline or IL-1β (0.5 μg/rat), administered following collection of the 0 min dialysis sample, on the concentration of noradrenaline (fmol 10μl⁻¹) recovered from the anterior hypothalamus of (A) Lewis and (B) Fischer rats. NA production expressed as the percentage of the respective basal level is presented for Lewis (C) and Fischer (D) rats. Each time point represents the mean ± SE for 7-9 animals per group (n values in brackets, C, D). Mean plasma corticosterone concentration (ng ml⁻¹) measured 120 min post injection is given in brackets (A, B). *p < 0.01 vs. saline; †p < 0.01 vs. LEW IL-1β, Student's t-test.

Figure 2.3.14   Mean Production of Cyclic AMP, Prostaglandin E_2 and Noradrenaline Following Injection of Saline, LPS or IL-1β in Lewis and Fischer Rats
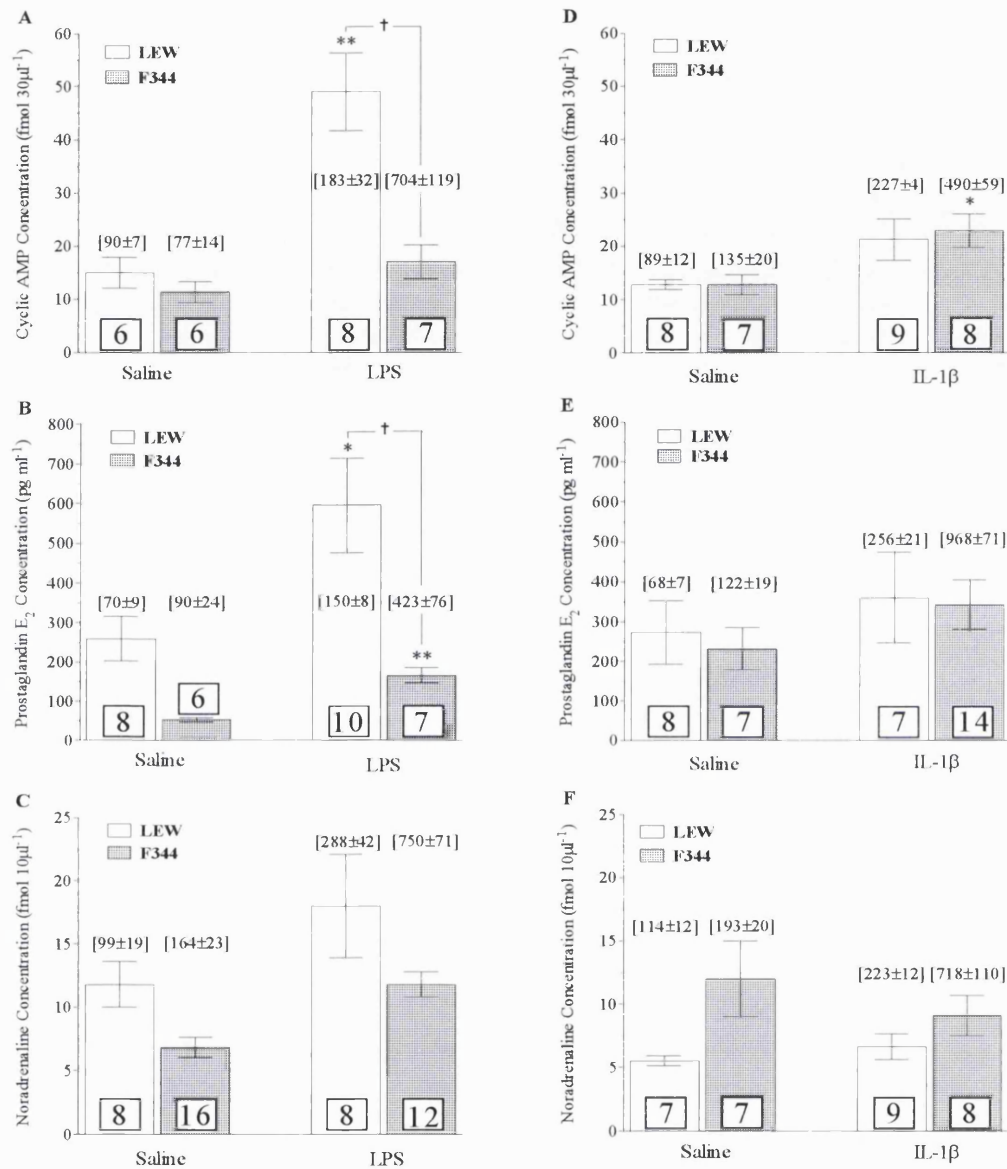
Mean post-injection concentration of cAMP, PGE_2 and NA in dialysate samples from the anterior hypothalamus of Lewis (LEW) and Fischer (F344) rats. The total concentration of each substance collected from individual rats following injection of LPS (A-C; 4 hours), IL-1β (D-F; 2 hours) or saline was divided by the number of samples collected during the relevant time period and the mean ± SE is presented for each group (n values within columns). Corticosterone concentration ± SE (ng ml-1) is shown in brackets. Statistically significant differences refer to second messenger data: *$p < 0.05$ and **$p < 0.005$ vs. respective saline; †$p < 0.01$ as indicated on the figure, Student's t-test.

2.4 Discussion

The results of this chapter show that i.p. injection of LPS, mrIL-1β or rrTNF-α to LEW and F344 rats activates the HPA axis, indexed by increased plasma corticosterone concentration, with up to 10-fold greater production in F344 rats. However, hypothalamic PGE_2 and cAMP production was significantly greater in LEW rats compared to F344 rats in response to LPS administration despite the reduced CS response, whilst IL-1β induced similar changes in PGE_2 and cAMP production in both strains and were not as great as seen following LPS administration. Indomethacin inhibited hypothalamic PGE_2 and cAMP production and prevented the early (2h) but not later (4h) increase in plasma CS secretion induced by LPS administration in LEW rats implicating a role for prostaglandins in mediating LPS-induced cAMP production and acute activation of the HPA axis. Noradrenaline does not appear to mediate the effect of IL-1β on CS production whilst distinct temporal increases in dialysate NA levels of both LEW and F344 rats in response to LPS suggest a role for this neurotransmitter in the sustained activation of the HPA axis.

2.4.1 Considerations For Data Interpretation of the Microdialysis Study

Insertion of a microdialysis probe results in some degree of trauma to surrounding tissue as indicated by a localised increase in glucose metabolism and decreased blood flow 3 h after implantation of the probe which normalise after 24 h (Benveniste *et al.*, 1987). Detection of substances which normally do not cross the BBB in dialysate samples following implantation of the dialysis probe demonstrate that the BBB is disturbed around the dialysis probe. This is believed to be a short lived phenomenon, with the BBB re-established 30 to 120 minutes after insertion of the probe

(Blasberg, Fenstermacher & Patlak, 1987; Tossman & Ungerstedt, 1986). However, the passage of inulin from blood to dialysate as well as the presence of serum albumin (detected by Evan's blue and immunohistochemistry) around the probe and surrounding brain tissue measured 24 h after probe implantation suggest that prolonged disruption of the BBB may occur following probe implantation (Westergren *et al.*, 1995). The amount of tissue trauma and BBB disturbance associated with microdialysis probe implantation may be determined by several factors including the rate at which the probe is inserted into the brain tissue and the stability of the probe within the tissue once implanted (Benveniste, 1989; Westergren *et al.*, 1995). For the experiments described in this chapter, the microdialysis probe was inserted into the brain over a 2-3 minute period and was firmly held in place by means of dental cement anchored by two screws placed into the skull, hopefully minimising tissue trauma and BBB damage. However, it cannot be stated unequivocally that any of the substances used in this study, all of which were administered peripherally, did not have access to the brain *via* the area surrounding the probe.

The cellular source and the site of production of cAMP and PGE$_2$ recovered from the anterior hypothalamus (AH) cannot be stated with certainty. The microdialysis probe was inserted into the AH such that the dialysis membrane was situated in the PVN, the site of CRF-containing neurons implicit to the HPA axis response to stimuli, thus enabling the release of locally active mediators to be monitored. However, the dialysis membrane extended both above and below the PVN and so any cAMP, PGE$_2$ or NA produced in these regions may have contributed to the amount in the dialysate. *In vitro*, LPS and IL-1 stimulated the release of PGE$_2$ from microglia and astrocytes but not neurons (Katsuura *et al.*, 1989; Bauer *et al.*, 1997) whilst cAMP in dialysates from

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rat cortex stimulated with NA was probably of neuronal or glial origin (Stone & John, 1990). The finding that the concentration of PGE_2 in dialysates recovered from the PVN of rats given i.v. IL-1β was only slightly reduced by perfusion of the probe with indomethacin suggested that the eicosanoid was not synthesised there but may have diffused into the PVN from other areas (Komaki, Arimura & Koves, 1992). Whether extracellular cAMP measured in dialysates is the result of overflow of intracellular cAMP, perhaps through cell membranes disrupted by implantation of the microdialysis probe, or represents a secreted species is not clear although dialysate levels of cAMP are closely correlated with the intracellular cAMP response (Egawa, Hoebel & Stone, 1988). Measurement of neurotransmitter levels using microdialysis does not reflect 'synaptic release' but rather the overflow or diffusion of released transmitter which has been subjected to the normal mechanisms that control the synaptic levels, i.e. re-uptake and metabolism in the case of noradrenaline (Marsden, 1990). The physiological relevance of NA levels determined by microdialysis is limited by the recovery of the transmitter across the dialysis membrane and the sensitivity of the assay. The 20-30 minute collection period required to accumulate detectable levels of NA may mask short bursts of release in response to a physiological/pathological stimulus.

Basal levels of PGE_2 in dialysates obtained from the PVN of Sprague Dawley (SD) rats were markedly lower (19 ± 0.26 pg ml^{-1}; Komaki, Arimura & Koves, 1992) than those presented in this chapter. Whilst some methodological similarities exist, such as beginning the perfusion 20-24 h after probe implantation and a flow rate of 1.2 μl min^{-1}, differences in the length (2 mm) and type of dialysis membrane, the lower *in vitro* recovery (5.4 %), the strain and sex of rat used, the anaesthetic and the radioimmunoassay kit used may account for the observed differences between the two

studies. Recently, dialysate PGE_2 levels that were very similar to those found in this chapter were reported where the microdialysis membrane was situated at the posterior limit of the PVN and the anterior limit of the ventromedial hypothalamus (Gerozissis *et al.*, 1997). Basal hypothalamic dialysate levels of cAMP in LEW and F344 rats were closely comparable with those described in the cortex and striatum of SD rats (Stone & John, 1990; Hutson & Suman-Chauhan, 1990). Basal extracellular NA levels were comparable to those obtained using concentric dialysis probes placed in the hippocampus and hypothalamus of SD rats (Kalen *et al.*, 1988; Routledge & Marsden, 1987) and were very similar in both LEW and F344 rats.

The significantly lower basal levels of PGE_2 and cAMP in F344 rats compared to LEW rats used in LPS experiments is postulated to be related to the glucocorticoid milieu in each strain or to increased corticosterone secretion induced by surgery (Udelsman & Chrousos, 1988; Udelsman *et al.*, 1987) which may have had a dampening effect on PGE_2 and cAMP production; corticosteroids are known to suppress the production of PG's in a variety of *in vitro* and *in vivo* preparations (Snyder & Ueanue, 1982; Flower, 1988) and corticosterone administration prior to probe implantation reduced both basal and isoprotorenol-stimulated cAMP levels (Stone, Egawa & Colbjornsen, 1989). However, basal PGE_2 production was only slightly lower in F344 rats than LEW rats used in IL-1 experiments. From figure 2.3.6A it can be seen that basal production of cAMP was elevated in only one group of animals, LEW rats subsequently injected with LPS, suggesting that "biological variability" between batches of animals used rather than endogenous glucocorticoid levels account for the differences observed between LEW and F344 rats. Whilst the presence of a microdialysis probe appeared to have some effect on plasma CS responses to LPS/IL-1β, the effect was not

consistent. Thus, the CS response (at the end of dialysis experiments) of F344 rats with probes following LPS was reduced whilst in LEW rats injected with either saline or IL-1β the CS response was greater than in probe-free animals. The possibility that differences in corticosterone secretion produced by surgery or the stress of having a microdialysis probe in the brain may have affected subsequent basal and stimulated production of the substances under investigation cannot be excluded. Plasma CS was not measured in animals 20 h following probe implantation so it is not known if CS levels had returned to basal levels following the surgical manipulation. This could be addressed by inserting a jugular vein cannula while the animals are anaesthetised for probe implantation, allowing subsequent blood samples to be taken from awake animals with minimal stress to measure plasma CS levels prior to the start of microdialysis and at the same time points as dialysate fractions are collected to determine if corticosterone levels correlate with hypothalamic second messenger production. The interpretation of the findings from the present study is difficult but it should be noted that the history (and possibly source) of experimental animals may influence biological variables: a number of physical and psychological stresses (Dugue *et al.*, 1993; Zhou *et al.*, 1993) increase endogenous cytokine levels which may alter both the response of hypothalamic mediators and the HPA axis to subsequent acute challenges with cytokines, endotoxin or other stressors (Rivier, 1995). One disadvantage of using awake animals for microdialysis is that the animal is susceptible to all kinds of influences ranging from the 'restraint' stress of tubing to reactions in response to a new environment. In these experiments animals were taken from their home cage, where they were in pairs, and placed into individual dialysis cages to which they were not accustomed, as soon as they awoke from the anaesthetic. On some occasions during dialysis, animals would try to

pull on the inlet/outlet lines attached to the probe suggesting they were agitated by their presence. Thus, it is conceivable that these factors may have influenced neurotransmitter/second messenger release in this study.

2.4.2 Activation of the HPA Axis

It is evident from the results of this study that the corticosterone response to i.p. administration of LPS is markedly greater in F344 rats compared to LEW rats confirming previous studies in the same rat strains treated i.p. with either SCW (chemically related to LPS) or LPS (Sternberg *et al.*, 1989a; Grota *et al.*, 1997). Endotoxin-induced activation of the HPA axis is thought to be mediated by cytokines, particularly IL-1 and TNF (Shalaby *et al.*, 1989; Zanetti *et al.*, 1992), released from peripheral macrophages (Derijk *et al.*, 1991; Tilders *et al.*, 1994); indeed, administration of either IL-1 receptor antagonist, antibody to IL-1 receptors or antiserum to TNF–α has been shown to inhibit LPS-induced ACTH release (Schotanus, Tilders & Berkenbosch, 1993; Perlstein *et al.*, 1993; Ebisui *et al.*, 1994) and both plasma ACTH and CS concentrations are increased following injection of IL-1α/β and TNF–α (Berkenbosch *et al.*, 1987; Van der Meer *et al.*, 1996). The data in this chapter support a stimulatory role for both mrIL-1β and rrTNF–α on CS secretion in both rat strains. The lack of a stimulatory effect of mrTNF–α on corticosterone production in the present study compared to similar doses of mrIL-1β may reflect the greater potency of IL-1β reported by others (Butler *et al.*, 1989; Van der Meer *et al.*, 1996) or may be due to species differences since rat rTNF–α resulted in a marked increase in plasma corticosterone concentration in both strains of rat; the effect of lower doses of rrTNF–α

on plasma corticosterone production should be part of future work. No change in corticosterone production was seen in either strain of rat in this study following administration of rrIFN–γ. Whilst administration of hrIFN–γ has been shown to increase cortisol levels in both healthy individuals and cancer patients (Holsboer *et al.*, 1988; Späth-Schwalbe *et al.*, 1989), no effect was observed in mice injected with IFN–γ (Besedovsky *et al.*, 1986) and is therefore probably not a key cytokine in the mediation of LPS-induced HPA axis activation, at least in LEW and F344 rats.

Variations in the kinetics of appearance of IL-1 and TNF after LPS challenge have been observed, with TNF levels reaching a peak after 1 hour and declining thereafter while IL-1 attained maximum levels within 2-4 h and were sustained longer (Shalaby *et al.*, 1989; Butler *et al.*, 1989; Zanetti *et al.*, 1992), suggesting that these cytokines may play different roles at different times. The temporal relationship between cytokine concentrations in blood and the HPA axis response to acute immune stimuli remains poorly understood, however, as i.v. endotoxin administration markedly elevates plasma ACTH concentrations before a rise in plasma levels of TNF–α, IL-1 or IL-6 is detected (Givalois *et al.*, 1994). It has been suggested that the dose of LPS may be critical in determining the participation of certain cytokines: low doses of endotoxin may rely on TNF–α alone to activate the HPA axis, while the response to larger doses (> 10 mg kg^{-1}), as used in this study, may involve both TNF–α and IL-1β (Ebisui *et al.*, 1994).

Immunoneutralisation of CRF or lesions of the PVN inhibited IL-1-induced ACTH secretion suggesting that cytokines act at the level of the hypothalamus to cause CRF release (Sapolsky *et al.*, 1987; Rivest & Rivier, 1991); however, the entry of cytokines into the brain is restricted by the BBB and while transport mechanisms across the BBB have been described for IL-1 and TNF it appears that only small amounts

(<1%) of injected recombinant cytokines actually reach the brain tissue (Banks *et al.*, 1991; Gutierrez, Banks & Kastin, 1993). However, it seems likely that significant accumulation of blood-derived cytokines within the brain would occur following prolonged and marked elevations of their blood concentrations accompanied by disruption of the BBB as might occur during EAE (Pan *et al.*, 1996). Increased protein and mRNA expression of IL-1, TNF–α and IL-6 in the brain were reported following systemic LPS administration (Ban, Haour & Lenstra, 1992; Gatti & Bartfai, 1993; Quan, Sundar & Weiss, 1994; Layé *et al.*, 1994) supporting the concept that local synthesis of cytokines in certain brain structures may convey peripheral immune stimuli to the CNS. Thus, sustained stimulation of the HPA axis following LPS injection depends largely on the differential temporal expression of, at least, IL-1 and TNF in both the periphery and the CNS.

2.4.3 *Mechanisms of HPA Axis Activation by LPS/Cytokines*

LPS- and IL-1-induced adrenocortical activation is thought to be mediated through stimulation of hypothalamic intermediaries, including PGE_2 (Katsuura *et al.*, 1990; Komaki, Arimura & Koves, 1992; Van Dam *et al.*, 1993), NA (Dunn, 1988, 1992; Kabiersch *et al.*, 1988) and cAMP (Hu, Tannahill & Lightman, 1992), resulting in CRF secretion. In this study the technique of *in vivo* microdialysis was used to measure temporal changes in hypothalamic noradrenaline, PGE_2 and cAMP production which accompany endotoxin- or IL-1β-induced HPA axis activation in adult LEW and F344 rats.

The temporal changes in PGE_2 production in both LEW and F344 rats and the duration of the response in this study were in close agreement with those described in

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the anterior hypothalamus following administration of endotoxin (Sirko, Bishai & Coceani, 1989) and IL-1β (Komaki, Arimura & Koves, 1992; Watanobe & Takebe, 1994). The response to LPS was slower in onset (60 minutes) compared to that induced by IL-1β (20 minutes) reflecting, as discussed above, the requirement for LPS to stimulate cytokine production. In absolute terms, LPS-stimulated PGE_2 concentrations were approximately 3-fold greater in LEW rats compared to F344 rats whereas PGE_2 levels following IL-1β were very similar in both strains and were not as great as following LPS. It is possible that greater levels of IL-1β are produced following LPS injection than occur following administration of the cytokine alone or, alternatively, that LPS stimulates the production of several cytokines (e.g. IL-1β, TNF-α, IL-6) which act synergistically to stimulate PGE_2 production. Another possible explanation of the differences between the magnitude of the PGE_2 response to LPS compared to IL-1β may be due to differences in cyclooxygenase-2 (COX-2) upregulation. COX-2, an inducible isoform of COX which is stimulated by LPS or cytokines and inhibited by dexamethasone, plays a major role in the rapid production of prostaglandins during inflammation (Goppelt-Struebe, 1995; O'Banion et al., 1996). Increased COX-2 mRNA was detected throughout the brain in non-neuronal cells associated with blood vessels, the meninges and the choroid plexus 45-60 minutes following LPS administration (Cao et al., 1995, Breder & Saper, 1996) and COX-2-like immunoreactivity was seen in perivascular microglia and meningeal macrophages at 2 and 6 h after LPS administration (Elmquist et al., 1997). Following administration of a high dose of IL-1β (30 μg kg^{-1}, i.p.), only a slight increase in COX-2 mRNA was seen in rat brain after 1.5 hours with a peak after 4 h (Cao et al., 1996). Thus, the increased production of PGE_2 in the

hypothalamic PVN following LPS compared to IL-1β in LEW rats may be due to the rapid and sustained up-regulation of COX-2.

Interleukin-1β may bypass the need for increased PGE_2 production in the anterior hypothalamus by stimulating, either directly or through intermediaries, nerve terminals in the median eminence (ME) causing CRF release and hence ACTH and CS secretion. The ME contains a large number of peptidergic and monoaminergic nerves terminating close to the hypophysial portal vessels (Negro-Vilar, 1982); in this region the BBB is poorly developed and represents a point at which circulating cytokines can cross to the brain. The rapid increases in plasma ACTH and corticosterone concentration (approx. 30 min) following systemic injection of IL-1β occur before changes in hypothalamic neuronal activity as indicated by expression of the neuronal activation marker, c-fos. Thus, increased c-fos mRNA expression was observed 1 h after IL-1β administration and increased c-fos immunoreactivity after 3 h (Brady *et al.*, 1994; Ericsson, Kovács & Sawchenko, 1994) in contrast to the rapid (30 min) increase in c-fos immunoreactive neurons of the PVN following systemic LPS injection (Wan *et al.*, 1993). Direct administration of IL-1α or b into the ME rapidly stimulates ACTH secretion in the rat (Matta *et al.*, 1990, 1993). As increased secretion of CRF appears to be a necessary step in elevating ACTH/CS secretion following systemically administered cytokines (Lee & Rivier, 1994), it seems likely that the CRF nerve terminals in the ME represent the primary, but not only, target of blood-borne IL-1β. In any extension of this study it would be important to determine the effect of TNF-α on the production of hypothalamic mediators of HPA axis activation already measured following LPS and IL-1β administration, particularly in light of the finding that human

rTNF-α mimics the CS changes and increased c-fos-ir neurons in the PVN produced by LPS administration (Tolchard et al., 1996).

In F344 rats the absolute response of PGE_2 to LPS was smaller than that of LEW rats although the relative increase compared to basal production was very similar. Accordingly, one might speculate that if the basal production of PGE_2 in F344 rats had been the same as in LEW rats (as observed in IL-1 experiments) then PGE_2 production in response to LPS would have been similar in both strains of rat. Alternatively, the higher basal and LPS-stimulated CS levels in F344 rats may have suppressed COX-2 up-regulation/activity resulting in the smaller PGE_2 response. It would be of great interest to examine COX-2 expression in neuroendocrine structures and investigate the regulation of this enzyme in relation to HPA axis activation.

Several lines of evidence suggest that PG's act as the final common pathway for immune mediators in HPA axis activation. Thus, the cyclooxygenase inhibitor indomethacin blocked the early but not the late phase of cytokine- and LPS-induced ACTH and CS secretion in vivo (Katsuura et al., 1988, 1990; Dunn & Chuluyan, 1992) and also inhibited IL-1-stimulated CRF release in vitro (Navarra et al., 1991). Moreover, hypothalamic elevations in PGE_2 in response to endotoxin were reversed following systemic indomethacin administration (Sirko, Bishai & Coceani, 1989). The data in this chapter are in accord with the above findings since indomethacin blocked the LPS-induced increase in hypothalamic PGE_2 production up to 240 min post injection whilst the plasma CS response was attenuated at 140 min but not 240 min in LEW rats suggesting that hypothalamic PGE_2 is an important mediator of the acute (up to 2 h) response of the HPA axis to LPS but is not crucial for sustained activation of this axis.

The results in this chapter represent the first demonstration of an in vivo

hypothalamic cAMP response to LPS and IL-1β administration. Hypothalamic cAMP production was rapidly increased following LPS administration although the response was greater in magnitude and duration in LEW rats compared to F344 rats. Maximum cAMP levels in LEW rats following LPS were similar to those observed in the striatum and cortex of SD rats following forskolin or NA stimulation (Hutson & Suman-Chauhan, 1990; Stone & John, 1990). In contrast, IL-1β administration produced a cAMP response of similar magnitude in both strains although the duration was greater in F344 rats. As the LPS-induced cAMP response in LEW rats was completely abolished by systemic administration of indomethacin it seems that hypothalamic prostaglandin production may determine cAMP levels. This may not be directly via stimulation of PG receptors however, as indomethacin also prevented the IL-1-induced increase in hypothalamic NA metabolism (Mefford & Heyes, 1990) and this neurotransmitter is known to bind to receptors which activate adenylate cyclase (Summers & McMartin, 1993). Further experiments are required to investigate the mechanism(s) by which hypothalamic cAMP production is increased following LPS or cytokines, with the effects of peripheral and central administration of PG and NA receptor antagonists being of particular interest. Regardless of the exact mechanism, hypothalamic cAMP production appears to be dependent on PG production, differences in the latter between LEW and F344 rats and LPS- and IL-1β-induced responses having been discussed above.

Whether hypothalamic cAMP production is critical for activation of the HPA axis is not known. Interestingly, it has recently been shown that systemic administration of PDE-4 inhibitors increase plasma ACTH and serum CS concentrations and evoke release of ACTH and CRF from pituitary and hypothalamic tissue respectively (Hadley

et al., 1996; Kumari *et al.,* 1997). It would be interesting to measure hypothalamic cAMP production *in vivo,* using microdialysis, following administration of these compounds. Moreover, given the appropriate pharmacological tools, it would be pertinent to investigate further the role of hypothalamic cAMP in immune-mediated activation of the HPA axis by targeted inhibition of hypothalamic cAMP production or blocking cAMP-dependent signalling pathways.

Various workers have demonstrated the ability of IL-1 and LPS to stimulate noradrenaline metabolism in the hypothalamus of mice and rats as indexed by decreased NA content and the concomitant increase in the concentration of the NA catabolite 3-methoxy,4-hydroxyphenylethyleneglycol (MHPG) in homogenised tissue samples (Kabiersch *et al.,* 1988; Dunn, 1988, 1992). The peak MHPG:NA ratio occurred approximately 2 h post injection of LPS and remained elevated at 4 h (Dunn, 1992) whereas a more rapid increase (40 min) in hypothalamic MHPG was observed after i.p. administration of IL-1 (Dunn & Chuluyan, 1992).

In contrast to the data presented in this chapter where the mean dialysate NA concentration was only increased 90 and 210-240 minutes after LPS administration, the NA response to LPS in the medial hypothalamus of Sprague-Dawley rats appeared to be temporally synchronised, with increased dialysate NA levels beginning 80 minutes post injection, reaching a peak at 120 minutes and declining to basal levels by 240 minutes (Lavicky & Dunn, 1995). These results provide support for the concept that peripheral administration of LPS stimulates cerebral noradrenergic neurons although whether this contributes to activation of the HPA axis is not clear.

Although a percentage increase above basal dialysate NA levels was suggested in F344 rats (i.e. 142-168 % of basal) 40 and 60 minutes after IL-1β injection, the absolute

concentration of NA in the samples was similar to those of saline treated controls. Furthermore, no difference in dialysate NA levels was seen in LEW rats injected with either saline or IL-1β. The failure of IL-1β to stimulate NA release above basal values may be due to the dose of IL-1β being too low. Using SD rats, MohanKumar & Quadri (1993) demonstrated that a dose of 1 μg/rat (350-400 g rats used) increased NA levels only 8% above pre-treatment values whereas 5 μg/rat increased NA release by 44% above pre-treatment levels demonstrating a dose-dependent effect. However, the dose used in this study (0.5 μg/rat, approx. 200g rats used) was effective in stimulating CS secretion suggesting that hypothalamic NA release is not essential for IL-1β-induced HPA axis activation in LEW and F344 rats.

Depletion of NA in the PVN using 6-OHDA has been shown to abolish the ACTH and CS response to neural stimuli (Feldman & Weidenfield, 1993), ether stress (Gaillet et al., 1993), intracerebral (Weidenfield, Abramsky & Ovadia, 1989) and i.p. (Chuluyan et al., 1992) administration of IL-1 but did not alter the response to 20 min of restraint stress (Chuluyan et al., 1992). Furthermore, in LEW rats with an 80% reduction in hypothalamic NA content following i.c.v. 6-OHDA administration, plasma corticosterone levels were as high as sham-treated controls at the peak of clinical signs of EAE (Leonard et al., 1991). These observations highlight the complexity of the role of NA in mediating the activity of the HPA axis.

Whilst it is recognised that smaller adrenal size, fewer pituitary corticotrophs and a defective hypothalamic CRF-secreting neuron are all contributory factors to the differences in the HPA response of LEW rats compared to F344 rats (Sternberg et al., 1989a; Calogero et al., 1992; Zelazowski et al., 1992), the hyposecretion of CS in LEW rats following sustained immune activation (e.g. following LPS injection or induction of

EAE) may be the result of a defective hypothalamic signalling pathway which ultimately renders LEW rats susceptible to autoimmune disease.

It is evident from the data presented in this chapter that increased hypothalamic second messenger (PGE_2 and cAMP) production is associated with the corticosterone response to immune stimuli in both LEW and F344 rats and that the corticosteroid response is markedly greater in the latter. Interleukin-1β induced PGE_2 and cAMP responses of similar magnitude in both strains of rat whereas LPS enhanced production of both mediators in LEW rats. The fact that LEW rats can mount a strong PGE_2 and cAMP response following an immunological stimulus, which precipitates a steroid response substantially smaller than that observed in F344 rats, suggests that signalling components downstream of these mediators may be defective in LEW rats.

CHAPTER III

Apoptosis of T-Cells During Experimental Allergic Encephalomyelitis: A Mechanism for Resistance and Recovery?

3.1 Introduction

3.1.1 Apoptosis and Glucocorticoids

It is now clear that cell death is a key physiological, as well as pathological process which proceeds via two different mechanisms, namely necrosis and apoptosis. Necrosis occurs following tissue damage and results in the rapid collapse of the internal homeostasis of the cell after lysis of the plasma membrane and degradation of cytoplasmic organelles resulting in the release of cellular contents into the surrounding area, contributing to the development of inflammation associated with this type of cell death. In contrast, apoptosis is an active, non-inflammatory, physiological process whereby a reactive cell undergoes a series of characteristic changes, including loss of cell volume and DNA degradation, observed as the condensation and clumping of chromatin in the nucleus as a result of endonuclease activation (Wyllie, 1987). Finally, cell fragments are pinched off with cellular contents sealed within and these "apoptotic bodies" are rapidly phagocytosed and degraded, thus preventing the release of proinflammatory cytoplasmic contents into the surrounding tissue.

An important characteristic of apoptosis is that it is induced in response to or following withdrawal of cell specific stimuli and can be inhibited by the action of cell specific hormones, cytokines, growth factors and mitogens (Wyllie, Kerr & Currie, 1980). T cell receptor-mediated apoptosis occurs during the elimination of autoreactive, immature T-cells in the thymus (Smith *et al.*, 1989) and therefore, a defective apoptotic mechanism could lead to the development of autoimmunity. During the foetal and

neonatal stage of development corticosteroids are synthesised in thymic epithelial cells where they antagonise T-cell receptor-mediated apoptotic death (Vacchia, Papadopoulos & Ashwell, 1993; Iseki, Mukai & Iwata, 1991) however adrenal derived glucocorticoids are potent mediators of thymocyte apoptosis (Wyllie, 1980). Thus whilst the glucocorticoid milieu may prevent the development of "self" T-cells in the mature animal this depends on the maturation state of the thymocyte and other costimulatory molecules. Mature T-cells are resistant to glucocorticoid-induced apoptosis (Nieto et al., 1992) until the cells have been activated (Tuosto et al., 1994), whereupon the sensitivity to apoptosis increases with repeated stimulation (Salmon et al., 1994). Elevation of circulating levels of corticosterone either by immune stimuli (Gruber et al., 1994) or steroid implants (Garvy et al., 1993) induces apoptosis in vivo, whilst severe thymolysis was reported during EAE, concomitant with the rise in endogenous corticosterone (Levine, Sowinski & Steinetz, 1980).

3.1.2 Apoptosis in Experimental Allergic Encephalomyelitis

The adrenocortical response is central to recovery from and resistance to experimental allergic encephalomyelitis (EAE) in the Lewis (LEW) and Fischer (F344) rat strains respectively. EAE can be induced in the LEW rat by transfer of myelin basic protein (MBP)-sensitised T-cells which subsequently infiltrate the CNS causing inflammation (Richert et al., 1981). The clinical phase of the disease is manifest in a reversible hind limb paralysis which is accompanied by changes in circulating corticosterone (MacPhee, Antoni & Mason, 1989). EAE-susceptible Lewis rats exhibit reduced basal and stress-stimulated corticosterone release relative to disease resistant F344 and Piebald-Viral-Glaxo (PVG) rats (Sternberg et al., 1992; Smith et al., 1994;

Mason, MacPhee & Antoni, 1990). Following adrenalectomy, EAE resistant rats are rendered susceptible to the disease whilst in the Lewis rat disease onset is more rapid and severe; in each strain a fatal paralysis ensues (Mason, MacPhee & Antoni, 1990; Leonard *et al.*, 1991). Disease resistance or the normal disease course can be restored by administration of a suitable corticosterone regime (Mason, MacPhee & Antoni, 1990) indicating that susceptibility to and spontaneous remission from EAE is influenced by the corticosterone tonus. Whilst glucocorticoids exert a variety of immunosuppressive and anti-inflammatory effects, including regulation of effector cell entry to the target organ, they may confer protection in EAE through the induction of apoptosis of immune cells.

Apoptotic cells have been demonstrated in the CNS during EAE in LEW rats and were identified on the basis of location and morphology as oligodendrocytes and lymphocytes (Pender *et al.*, 1991). In both active and transfer EAE, up to 49% of T-cells displayed signs of apoptosis in the CNS of SD and LEW rats and this was associated with recovery from the disease (Schmied *et al.*, 1993). Both groups postulated the involvement of corticosterone in the process. Hence corticosterone-driven apoptosis of T-cells in EAE may have an important contributory role in recovery from the disease in the LEW rat and inhibit the development in resistant rat strains in early stages of the disease process.

3.1.3 Detection of Apoptotic Cells in situ

Apoptotic cell death is characterised by the cleavage of chromatin DNA into approximately 180-base pair fragments due to the activation of endogenous endonucleases (Wyllie, 1980). Originally, detection of apoptosis was performed by

extracting DNA which was then run on an agarose gel, with a characteristic "ladder" of DNA bands indicating apoptosis (Wyllie, 1980; Cohen & Duke, 1984) or by morphological criteria defined by electron microscopy (Cohen, 1991; Wyllie, Kerr & Currie, 1980). By virtue of the ability to obtain a quantitative read out, light or electron microscopical determination of nuclear morphological changes remain the 'gold standard' for detection of apoptotic cells *in situ*.

Recently, enzymatic methods were adapted to study apoptosis at the cellular level on tissue sections and in cell culture systems (Gavrieli, Sherman & Ben-Sasson, 1992; Gold *et al.*, 1993). These techniques take advantage of the ability of the enzymes terminal deoxynucleotidyl transferase (TdT) and DNA polymerase I to add labelled deoxynucleotides to the 3'-hydroxyl ends of double-stranded DNA breaks (Sambrook, Fritsch & Maniatis, 1989). *In situ* nick translation (ISNT) uses DNA polymerase I and is used to detect DNA fragmentation in tissue sections by the incorporation of digoxigenin-11-dUTP which is subsequently visualised by immunohistochemistry (Iseki, 1986; Gold *et al.*, 1993, 1994). In the early stages of apoptosis the cytoplasmic components and the composition of the cell membrane remain intact (Wyllie, Kerr & Currie, 1980) thus allowing identification of the cells undergoing apoptosis by immunocytochemistry (Gold *et al.*, 1993; Schmied *et al.*, 1993).

3.1.4 Aims

The work described in this chapter was undertaken to examine whether endogenously released corticosterone was an important factor in the induction of T cell apoptosis in the CNS and if this process contributed to the resistance to and recovery from EAE seen in F344 and LEW rats respectively. As methods for both morphological

characterisation of apoptotic cells and for combining ISNT with immunocytochemistry were established in Prof. Hans Lassmann's laboratory, a collaborative study was set up to quantitatively define the cells undergoing apoptosis in the spinal cord of intact (INT) and adrenalectomised (ADX) LEW and F344 rats following the transfer of MBP-sensitised splenocytes.

3.2   Materials and methods.

3.2.1   Preparation of Guinea Pig Myelin Basic Protein

Guinea pig MBP was prepared using the method of Dunkley and Carnegie, (1974). Adult Dunkin-Hartley guinea pigs were killed by cervical dislocation followed by exsanguination and the brain and spinal cord rapidly removed into ice-cold PBS. Tissue was defatted by homogenising in 900 ml of ice-cold chloroform:methanol (2:1) for 5 min and the suspension filtered under vacuum using a sintered glass funnel. The lipid-extracted tissue was washed with 500 ml methanol, homogenised for a further 5 min with ice-cold deionised water and the pH carefully decreased to 3 by dropwise addition of 1M HCl. This pH was maintained for 30 min by the further addition of HCl while stirring on ice and the suspension centrifuged (24, 000 x g, 30 min, 4°C). The residue was reextracted at pH 3 for a further 30 min by the same procedure and following centrifugation (24, 000 x g, 30 min, 4°C) the two supernatants were dialysed overnight (Visking 18/32 dialysis tubing, BDH, U.K.) at 4°C against deionised water and then freeze-dried. The crude MBP extracts were resuspended in PBS and stored at -20°C until the protein content was determined.

3.2.2   Measurement of Protein Concentration (Lowry Method)

Protein concentration of the crude MBP extract was determined using a

spectrophotometric assay based on the coloured product formed by the reduction of copper-treated protein by Folin & Ciocalteu's phenol reagent (Lowry *et al.*, 1951). All standards and samples were assayed in duplicate. A standard curve was prepared by using bovine serum albumin (Sigma, U.K.) diluted in deionised water giving a range of 0-30 μg protein (stock BSA 0.2 mg ml^{-1}; total volume 150 μl). Samples were diluted 1:10 with deionised water to give a final volume of 150 μl. Following the addition of 50 μl of 0.4N NaOH and 1 ml of solution X (see below) to all tubes and vortex mixing, the samples were incubated for 15 min at room temperature. Folin-Ciocalteu's phenol reagent (BDH, U.K.) was mixed 1:1 with water and 100 μl added to all tubes; after vortex mixing, samples were left in the dark for 15 minutes at room temperature and the absorbance measured at a wavelength of 750 nm (Ultrospec 2000 UV/visible spectrophotometer, Pharmacia Biotech, U.K.). The protein content of the crude MBP extract was determined by interpolation of the absorbances against the standards.

Solution X: 99 volumes 2% Na_2CO_3, 0.02% NaK tartrate in 0.1M NaOH
                1 volume 1% $CuSO_4.3H_2O$. Mix just before use.

3.2.3   *Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Separation of the MBP extracts was performed using SDS-PAGE in a Bio-Rad Mini-Protean II electrophoresis cell. Gels were poured with a 12% resolving gel (375 mM Tris/HCl, pH 8.8, 0.1% SDS, 12% acrylamide, 0.3% bis-acrylamide, 0.05% ammonium persulphate (APS) and 0.05% N,N,N',N'-tetramethylethylenediamine (TEMED)) and a 4% stacking gel (125 mM Tris/HCl, pH 6.6, 0.1% SDS, 4% acrylamide, 0.1% bis-acrylamide, 0.05% APS and 0.1% TEMED). An aliquot of crude extract was diluted with deionised water to give a protein concentration of 20 μg 10μl^{-1} which was then mixed with 10 μl of 2 x sample buffer to give final concentrations of 1% SDS, 125 mM Tris/HCl, pH 6.8, 5% glycerol, 0.05% bromophenol blue and 1% 2-

mercaptoethanol. Samples were heated at 95°C for 4 minutes, left to cool and then loaded onto the gel and electrophoresed at 180 V for 45-60 minutes. Gels were then stained with Coomassie blue (0.25% Coomassie blue in 50% methanol, 10% acetic acid) for at least one hour and destained overnight with 50% methanol, 10% acetic acid. Gels were then scanned on a flatbed scanner connected to a PC and the amount of MBP as a percentage of the total protein determined using image analysis software (ImageQuant, Molecular Dynamics Ltd., Bucks, U.K.).

3.2.4   Animals

Adult female Lewis (LEW; 180-220g) and Fischer (F344; 170-190g) were obtained from either Charles River, U. K. or Harlan, U. K. and housed in pairs in a room with controlled lighting (lights on 06:00h to 18:00h) and temperature (21-23°C) with free access to food and water.

3.2.5   Surgery and Drug Administration

Bilateral adrenalectomy was performed by the dorsal approach under halothane anaesthesia (1.5% in oxygen:nitrous oxide [1:1; 0.9 L min^{-1}]). The animals were subsequently maintained for 1-2 weeks in the conditions described above and given 0.9% w/v sodium chloride in tap water to drink. Adrenalectomy was verified *post mortem* and any rats with incomplete surgery were excluded from the study.

The type II steroid receptor antagonist RU 486 (mifepristone; Research Biochemicals International, Herts, U.K.) was administered to intact LEW rats to block the action of endogenously released corticosterone. Supplies of RU 486 were limited and two dosing and administration regimes were used based on previously published protocols which were effective at blocking corticosterone action in EAE and SCW-

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arthritis (Bolton & Flower, 1989; Sternberg *et al.*, 1989a): (1) rats were orally dosed twice daily (9 a.m. and 5 p.m.), on days 3-10 post transfer of 4×10^7 MBP-sensitised splenocytes with either 1% carboxymethylcellulose containing 0.05% Tween 80 (vehicle) or RU 486 in this vehicle at 20 mg kg^{-1} body weight; (2) RU 486 was dissolved in 250 µl of absolute ethanol and made up to 5 ml with 0.9% sterile saline (final concentration 12 mg ml^{-1}). Rats were injected i.p. once daily (10 a.m.), on days 3-7 post transfer of 4×10^7 MBP-sensitised splenocytes with either 0.25 ml of vehicle (5% ethanol in saline) or RU 486 at 15 mg kg^{-1} body weight.

3.2.6 Adoptive Transfer of EAE.

Adrenal intact Lewis rats were injected subcutaneously, under halothane anaesthesia, in each hind foot with 50 µl of an emulsion containing guinea pig MBP (final concentration 1 mg ml^{-1}) in complete Freund's adjuvant (CFA; Difco Laboratories, Surrey, U.K.). The emulsion was prepared by homogenising 10 ml of MBP (2 mg ml^{-1} in sterile saline) with 10 ml CFA supplemented with 100 mg heat killed *Mycobacterium tuberculosis* H37Ra (Difco, U.K.; final concentration 5 mg ml^{-1}). Animals were killed eleven or twelve days later, the spleens removed and placed immediately into ice-cold Earle's balanced salt solution (EBSS; Gibco Ltd., Paisley, U.K.). A cell suspension was prepared by pressing the spleens through a stainless steel gauze/mesh with the rubber end of a plunger from a sterile 20 ml syringe (Becton Dickinson, UK) into 50mls of EBSS and centrifuged at 1500 g for 10 minutes at 4°C. After removing the supernatant, the cell pellet was resuspended in 50 ml EBSS and filtered through two nylon meshes (placed on top of each other) with pore diameters of 132 and 80 µm to remove tissue fragments (see Woodroofe and Cuzner, 1995). Splenic lymphocytes were cultured at 2×10^6 cells ml^{-1} in RPMI 1640 with L-glutamine medium supplemented with 100 U ml^{-1}

penicillin, 100 μg ml^{-1} streptomycin (Gibco Ltd., Paisley, Scotland), 10% heat inactivated foetal calf serum (First Link UK Ltd), 2 μM 2-mercaptoethanol, 1mg ml^{-1} indomethacin (Sigma, Poole, U.K.) and 10 μg ml^{-1} MBP for 72 hrs at 37°C in an atmosphere of 5% CO_2:95% O_2. Harvested cells were washed three times in unsupplemented EBSS and 4x10^7 splenocytes were transferred i.p. to naïve INT and ADX LEW and F344 rats.

Due to the severe and often fatal clinical EAE with rapid onset observed in ADX rats given 4x10^7 cells a cell titration (4 x 10^7, 4 x 10^6, 1 x 10^6 and 5 x 10^5 cells/rat) was performed in LEW rats only; a dose of 1x10^6 cells/rat to ADX LEW rats was found to delay the onset and prolong the survival of some animals to 6 days post transfer (d.p.t.). This low cell dose was subsequently administered to INT and ADX LEW and F344 rats.

On days 0, 3, 4, 5, 6, 7 and 10 post transfer rats were lethally anaesthetised with pentobarbitone sodium (150 mg kg^{-1}; Sagatal™, Rhône Mérieux Ltd., Essex, U.K.) and perfused through the left ventricle with 50 ml of PBS (pH 7.35-7.45) followed by 50 ml of freshly prepared 4% paraformaldehyde in PBS. The spinal cord, spleen and mesenteric lymph nodes were removed, post-fixed for four hours in 4% paraformaldehyde at 4°C then stored in PBS for transportation to Austria where tissue was routinely embedded in paraffin.

3.2.7 Assessment of Clinical EAE.

Animals were monitored daily and clinical disease scored as follows: (0) no clinical signs; (1) flaccid tail and weight loss (2) hind limb hypotonia with further weight loss (3) complete hind limb paralysis (4) paralysis of the lower part of the body and weakness of the forelimbs; (5) death. In addition, intermediate score were assigned to animals which, for example showed a loss of tonicity in the distal half of the tail (score =

0.5) or paralysis of one hind limb (score = 2.5).

3.2.8 Determination of Plasma Corticosterone Concentration.

Plasma corticosterone was measured in LEW rats 0, 3, 4, 6, 7, and 10 d.p.t. of 4×10^7 or 1×10^6 cells but tissue from these animals was not used for the determination of apoptotic cells. Animals were rapidly removed to a separate room, killed by a blow to the back of the head followed by decapitation and trunk blood collected into ice-chilled lithium heparin vacutainer tubes (Beckton-Dickinson, U.K.). Following centrifugation (1500 g for 15 minutes at 4°C), plasma was stored in 200 µl aliquots at -70°C until assayed for corticosterone content using a commercially available radioimmunoassay kit as described in chapter II (section 2.2.9).

3.2.9 Detection and Identification of Apoptotic Cells.

All tissue processing, detection and quantification of apoptotic cells was performed by Dr. M. Schmied, Research Unit of Experimental Neuropathology, Vienna, Austria and confined to the lumbar spinal cord, mesenteric lymph nodes and the spleen. Slides of lumbar spinal cord sections were returned to England and quantification of T cell (W3/13$^+$) and macrophage (ED1$^+$) infiltration and detection of apoptotic cells by nuclear morphological changes and ISNT was carried out by A.K.H.

Tissue was coded so the evaluation of apoptotic cell numbers was carried out without knowledge of the cell dose administered, the day post transfer on which the tissue was taken or the adrenal state of the animal. Apoptotic cells were identified at the light microscopical level by characteristic morphological criteria and ISNT with cell type determined by immunocytochemistry.

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(i)   *In situ* nick translation

Five µm thick tissue sections were cut onto slides (coated with 2% v/v 3-aminopropyltriethoxysilane (Sigma, U.K.) in acetone), dried on a hot plate (50°C) and stored at room temperature until required. Sections were deparaffinised in xylene (2 x 10 min), rinsed twice in 96% ethanol for 3 minutes each, treated with chloroform for 5 minutes, passed through graded ethanol (96%, 70% and 50%, 2 min each) and washed in Tris-buffered saline (TBS, pH 7.5). The ISNT reaction mixture consisted of 5 µl of 10 x nick translation buffer (0.5M Tris-HCl, pH 7.2, 0.1M $MgSO_4$, 500 µg ml^{-1} bovine serum albumin; Sambrook, Fritsch & Maniatis, 1989), 1µl digoxigenin labelled nucleotides (Dig DNA labelling mixture, Boehringer Mannheim, East Sussex, U.K.), 1µl (5 units) of DNA polymerase 1 (Boehringer Mannheim) and 43µl distilled water. Tissue sections were incubated with 50µl of the reaction mixture, covered with a parafilm square and incubated for 1 hour at 37°C in a humidified chamber. The parafilm was removed by rinsing in TBS and the sections covered with 2% normal sheep serum in TBS (NSS/TBS) for 30 minutes. The sections were then exposed for 1 hour, at room temp, to an alkaline phosphatase-labelled anti-digoxigenin antibody (Boehringer Mannheim) at a dilution of 1:250 in 1% NSS/TBS followed by 3 five minute washes in TBS. Two hundred µl of freshly prepared substrate solution (0.45 mg ml^{-1} nitro-blue tetrazolium and 0.17 mg ml^{-1} 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim) in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) was applied to the sections and colour reaction stopped after 3-5 minutes by washing in TBS (3 x 5 min). Subsequently, cell phenotype was determined by immunocytochemistry. After blocking with 10% foetal calf serum in TBS (FCS/TBS) for 30 min sections were incubated overnight at 4°C with a monoclonal antibody against either T lymphocytes (W3/13, 1:50; Sera-lab, Sussex, U.K.) or macrophages (ED1, 1:1000; Serotec, Oxford,

U.K.) diluted in FCS/TBS. Following three 5 minute washes in TBS, sections were incubated for 60 min at room temp with a biotinylated anti-mouse IgG (1:200; Amersham International, Bucks, U.K.) in TBS containing 3% normal rat serum. Unbound antibody was removed by washing in TBS and peroxidase-labelled avidin solution (1:100; Sigma, U.K.) was applied to the sections for 60 min. After washing, peroxidase activity was detected by placing the sides in a solution of 0.5 mg ml^{-1} 3, 3'-diaminobenzidine (Sigma, U.K.) in phosphate-buffered saline (PBS) containing 0.03% v/v hydrogen peroxide (BDH, U.K.) for 5-10 minutes. Sections were washed in tap water, lightly counterstained in Mayer's haematoxylin (10 secs) and mounted in Aquamount (BDH, U.K.).

ii)   Morphological criteria

To confirm that ISNT labelling was indicative of apoptosis, morphological changes in immunocytochemically identified cells were assessed in serial sections to those used for ISNT. Tissue sections were processed as described above except the chloroform step was replaced by a 15 minute incubation in 0.2% v/v hydrogen peroxide in methanol (BDH, U.K.) solution. After rehydrating the tissue through graded alcohols and washing in distilled water, sections were incubated with FCS/PBS and immunocytochemistry carried out as described above using PBS in place of TBS and counterstaining in haematoxylin for 1-2 min. Apoptosis was identified by the presence of either crescentic masses of condensed chromatin lying against the nuclear envelope or rounded masses of uniformly dense chromatin. Comparing the number of apoptotic cells determined either by morphological criteria or by ISNT in consecutive sections has previously been shown to have a correlation of nearly 1:1 (r=0.935, Gold *et al.*, 1993).

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3.2.10 Quantification and Statistics

The total number of W3/13$^+$ T cells, ED1$^+$ macrophages and apoptotic cells was determined using a 100 point stereological grid placed in the microscope ocular lens (ocular x10, objective x100). Apoptotic cells were identified and quantified by morphological criteria and ISNT. In each case one complete cross section of the lumbar spinal cord was examined with a standardised area of 3.0-3.2 mm^2. The results were averaged for each time point and are presented as the number of cells/mm^2 ± standard error. Comparisons of data between and within treatment groups were conducted using the Mann-Whitney U-test for nonparametric data; significance was accepted if p<0.05. In the subsequent analysis, tissue from animals which died before culling was not processed.

3.3 Results.

3.3.1 Guinea Pig Myelin Basic Protein

The crude MBP extract was separated on a 12% SDS polyacrylamide gel and stained with Coomassie blue. The major band detected in both extracts was approximately 20 KDa, corresponding to MBP (Figure 3.3.1). Scanning revealed the MBP band to represent 53% and 43% of the total protein in the first and second extracts respectively (Figure 3.3.1, lane 1 and 2). The extracts were pooled and diluted to give a final MBP concentration of 10 mg ml^{-1}; 2 ml aliquots were stored at -70°C until required.

3.3.2 Detection and Characterisation of Apoptotic Cells in the CNS

Apoptotic cells were detected in the spinal cord of rats with EAE using both morphological criteria and ISNT (Figure 3.3.2) and were found mainly within the

Figure 3.3.1 <u>Polyacrylamide Gel of Crude Myelin Basic Protein (MBP)</u>
<u>Extract</u>
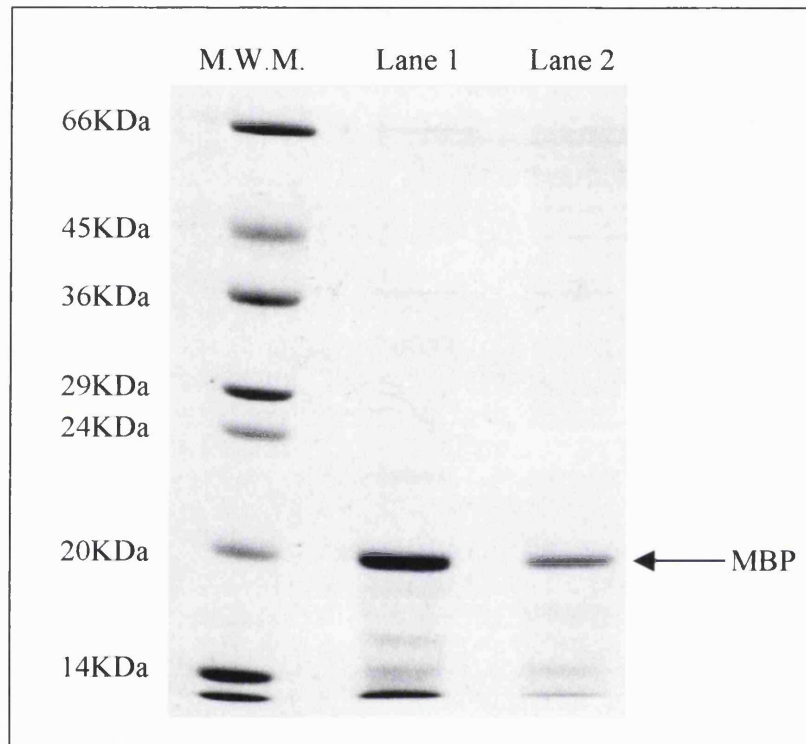
The first (lane 1) and second (lane 2) crude MBP extracts (20 μg protein)
were run on a 12% sodium dodecyl sulphate polyacrylamide gel and stained
with Coomassie blue. The major band in both extracts was approximately 20
KDa, corresponding to MBP. M.W.M. = molecular weight markers.

Figure 3.3.2 <u>Detection and Characterisation of Apoptotic T Cells in the CNS During EAE by *in situ* Nick Translation and Morphological</u> <u>Criteria</u>

(a) Spinal cord of an adrenalectomised rat 4 days post transfer (d.p.t.) of 1×10^6 MBP-sensitised splenocytes; only occasional apoptotic T cells could be detected by double labelling with ISNT and immunocytochemistry (ICC) for W3/13 (arrow; x120). (b) Increased numbers of apoptotic T cells in the spinal cord of an intact rat 4 d.p.t. of 4×10^7 cells, identified by ISNT and ICC (arrows, x120). (c) Apoptotic T cells identified by W3/13 ICC and morphological nuclear alterations (counterstained with haematoxylin, arrows) within the perivascular cuff (pc) and the parenchyma ; the open arrowheads denote uncharacterised apoptotic cells (x560). (d) and (e) Double staining of DNA fragmentation by ISNT (blue) and immunocytochemistry for T cells (brown); small arrows denote apoptotic T cells whilst open arrowheads indicate some cells that are labelled by ISNT but do not show membrane immunoreactivity for W3/13 (d: x200; e: x560).

Figure 3.3.2 <u>Detection and Characterisation of Apoptotic T Cells in the CNS During EAE by *in situ* Nick Translation and Morphological Criteria</u>
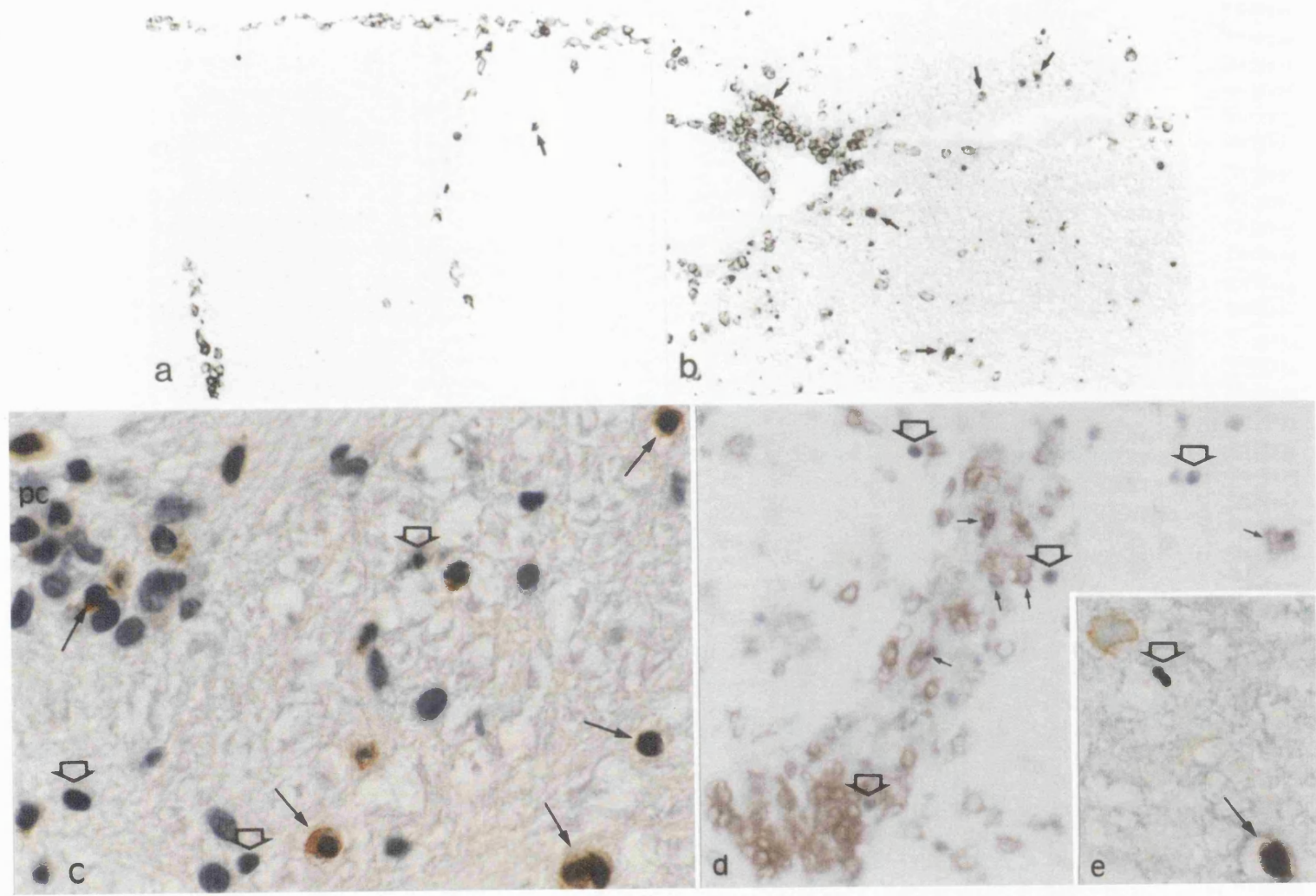
parenchyma associated with inflammatory cuffs around blood vessels. The majority of apoptotic cells in CNS lesions were T cells: of the apoptotic cells in INT rats administered 4×10^7 and 1×10^6 splenocytes, 58-93% and 55-93% respectively were T cells, and in ADX rats T cells represented 40-49% and 60-76% of the apoptotic cells following administration of 4×10^7 and 1×10^6 splenocytes respectively. Apoptotic macrophages represented less than 1% of the total macrophage number in the spinal cord of both INT and ADX rats injected with either cell dose. A number of unidentified cells were apparent (e.g. Figure 3.3.2 c) which may have been W3/13 positive cells at an advanced stage of apoptosis resulting in loss of membrane immunoreactivity.

3.3.3 Indices of Clinical Disease and Pathology in Intact and Adrenalectomised Rats Given 4×10^7 Cells

INT LEW rats administered 4×10^7 cells exhibited the typical disease course characterised by complete hind limb paralysis 6 d.p.t. and recovery by 10 d.p.t. (Figure 3.3.3 A). In contrast, INT F344 rats administered the same number of cells developed a mild disease (max. clinical score 1) and were fully recovered by day 7 (Figure 3.3.3 B). Administration of 4×10^7 cells to ADX rats of either strain resulted in earlier disease onset and rapid disease progression, often resulting in death by 4 d.p.t. (Figure 3.3.3 A, B).

The clinical data presented in figure 3.3.3 B relating to F344 rats was the result of one experiment with four INT and four ADX rats; the number of T cells and apoptotic T cells in the CNS of these animals is presented in table 3.3.1. Administration of either 4×10^7 or 1×10^6 cells to INT and ADX F344 rats in three subsequent experiments failed to induce any clinical or histological signs of EAE, whilst INT LEW rats injected at the same time with 4×10^7 cells exhibited both clinical signs and CNS

inflammation. Post mortem examination revealed no evidence of adrenal tissue in ADX F344 rats and plasma corticosterone levels were below the detection limit of the assay (0.8 ng ml^{-1}). Therefore it was not possible to perform a time profile of T cell entry and apoptosis in the CNS of F344 rats and the results in the following paragraphs refer solely to LEW rats.

T cells and macrophages were first detected in the CNS of both INT and ADX LEW rats on day 3 post transfer although a significantly greater number of T cells and macrophages were evident in tissue from ADX compared to INT rats ($p < 0.05$; Figure 3.3.4 A, C). By 4 d.p.t. there was no difference in T cell number between the two groups while macrophage numbers remained significantly elevated in the ADX animals ($p < 0.01$). In INT rats the profile of T cell and macrophage accumulation in inflammatory cuffs was similar, although whilst T cells began to decrease in number at 6 d.p.t., this was not apparent until 10 d.p.t. for macrophages.

Apoptotic T cells were first observed in INT animals 3 d.p.t., the greatest number being detected on day 6 (61 cells/mm^2; Table 3.3.2) which correlates with both the peak clinical symptoms and an increase in circulating corticosterone levels (Table 3.3.3). When expressed as a percent of the total number of T cells, the peak in percent T cell apoptosis (32%; Table 3.3.4) was observed 7 d.p.t. and associated with the beginning of recovery and elevated plasma corticosterone (Table 3.3.3). ADX rats administered 4x10^7 splenocytes exhibited few apoptotic T cells throughout the shortened disease course and numbers were lower than those observed in INT rats (Table 3.3.2). Furthermore, when the comparison was made at similar clinical scores (i.e. ADX, score 2.2 [4 d.p.t.] vs INT, score 2.4 [5 d.p.t.], Table 3.3.4) then a clear difference in percent apoptosis between the two groups was apparent (INT 18.9% vs ADX 3.8%; $p < 0.01$).

Figure 3.3.3 Clinical Course of Transfer EAE in Intact and Adrenalectomised Lewis and Fischer Rats
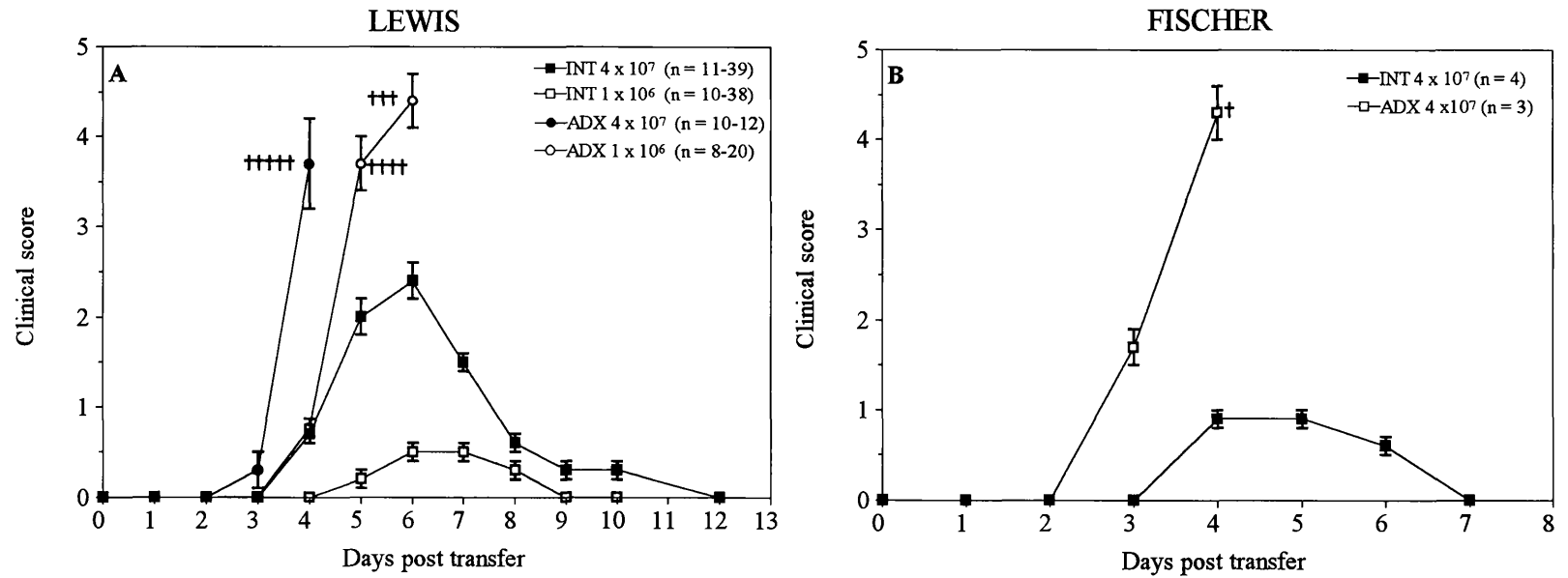
Clinical scores of intact (INT) and adrenalectomised (ADX) Lewis (A) and Fischer (B) rats following i.p. transfer of either 4×10^7 or 1×10^6 MBP-sensitised spleen cells. Each point represents the mean and standard error of individual daily scores from all animals used in up to seven separate experiments throughout the study.

† represents the number of rats which died as a result of EAE.

Figure 3.3.4 Total Number of T Cells and Macrophages in the Spinal Cord of Intact and Adrenalectomised Lewis Rats in Relation to Clinical Score
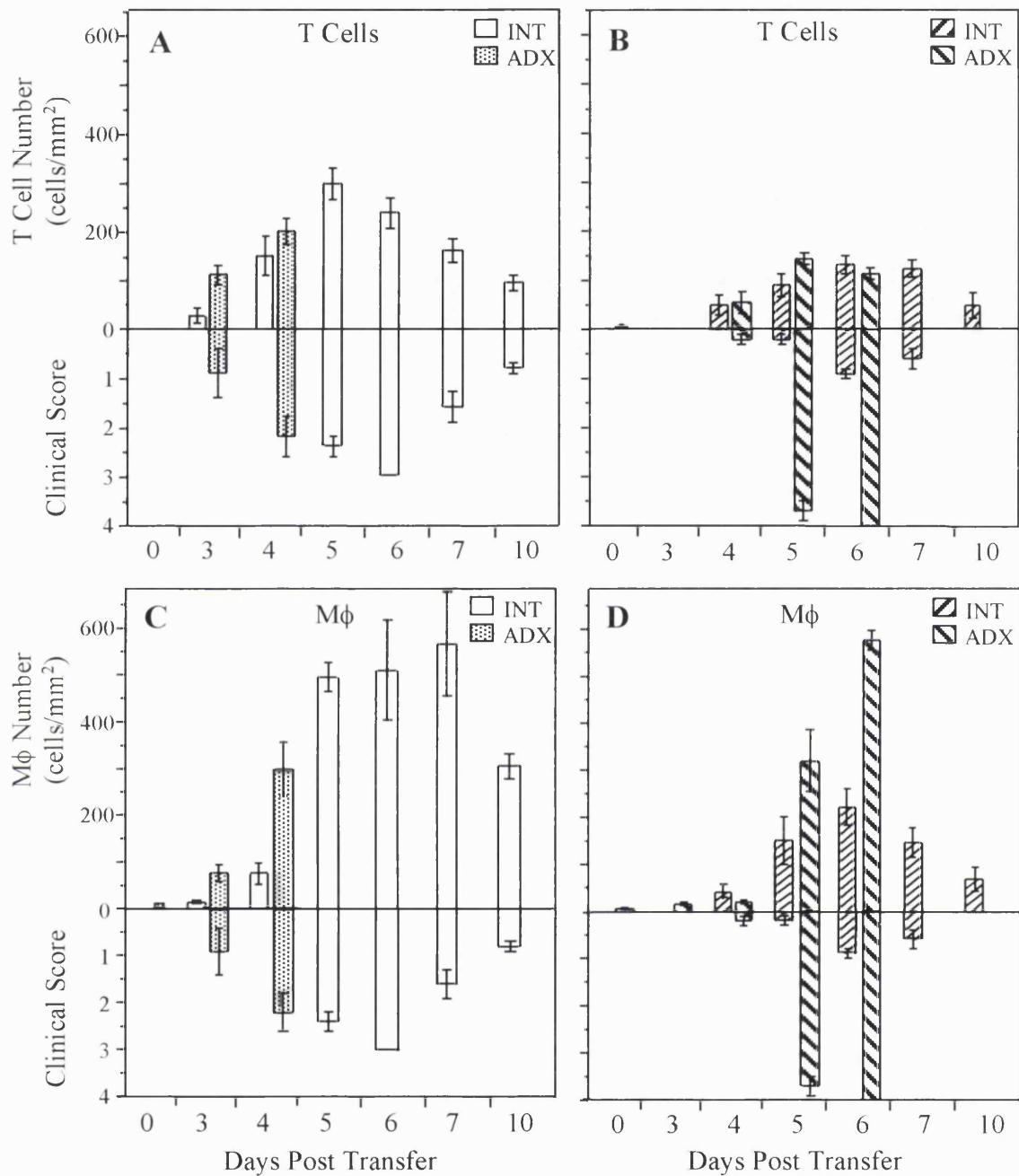
Total number of (A, B) T cells (W3/13 +ve) and (C, D) macrophages (Mϕ, ED1 +ve) and clinical score of intact (INT) and adrenalectomised (ADX) Lewis rats given either 4×10^7 (A, C) or 1×10^6 (B, D) MBP-sensitised spleen cells. Each column represents the mean and standard error of the total number of cells in transverse lumbar spinal cord sections expressed as cells/mm^2, where n = 4-10.

Table 3.3.1 <u>Total Number of Apoptotic T Cells in the CNS of Intact and Adrenalectomised Fischer Rats with EAE</u>

Treatment	Day Post Transfer	Clinical Score	Total W3/13 (cells/mm^2)	Apoptotic W3/13 (cells/mm^2)	% W3/13 apoptotic
INT F344	7	0r	59	14	24
INT F344	7	0r	9	3	33
INT F344	7	0r	62	35	56
INT F344	7	0r	42	27	64
Mean		**0**	**43**	**19.8**	**44.3**
ADX F344	4	ncs	-	-	-
ADXF344	4	5	-	-	-
ADX F344	4	4	104	7	7
ADX F344	4	4	76	3	4
Mean[a]		**4**	**90**	**5**	**5.5**

Adrenalectomised (ADX) or intact (INT) Fischer rats were injected intraperitoneally with 4 x 10^7 MBP-sensitised splenocytes. Animals were culled on day 4 or day 7 post transfer and total number of T cells (W3/13$^-$) and the number of apoptotic T cells in the lumbar spinal cord were determined. [a]For ADX animals, tissue from the animal that displayed no clinical signs (ncs) and the animal that died as a result of EAE (score 5) was not used for determination of T cell numbers. 0r denotes recovery from clinical signs of EAE at the time of culling (max. score = 1).

3.3.4 Clinical and Pathological Findings in Adrenalectomised Lewis Rats Given 1x10⁶ Cells

Given the severe nature of EAE in ADX rats injected with 4×10^7 cells, a cell titration was conducted in ADX rats in an attempt to reduce the severity and extend the course of the disease (data not shown). By decreasing the dose to 1×10^6 cells, disease onset was delayed to 4 d.p.t. (i.e. the same as in INT rats given 4×10^7 cells) and animals survived upto 6 d.p.t. although by this time the animals were very moribund (Figure 3.3.3 A). Figure 3.3.4 D shows the accumulation of macrophages in the CNS of ADX LEW rats injected with 1×10^6 was similar to that in INT LEW rats given 4×10^7 cells, although T cell numbers were consistently lower in the ADX group ($p < 0.05$, 4, 5 and 6 d.p.t. compared to INT 4×10^7; Figure 3.3.3 B). In addition, ADX rats injected with 1×10^6 cells displayed significantly lower T cell apoptosis at 4, 5 and 6 d.p.t. than INT rats given 4×10^7 cells whether expressed in absolute numbers or percentage terms (Table 3.3.2) and when compared at similar clinical scores (Table 3.3.4).

3.3.5 Mild EAE in Intact Lewis Rats Given 1x10⁶ Cells

As a control, INT animals were also administered to 1×10^6 cells. This dosing regime produced a mild form of EAE, the maximal signs being either partial or complete tail hypotonia, apparent 6-8 d.p.t. and "recovery" was attained 9 d.p.t. (Figure 3.3.3 A). In contrast to animals administered 4×10^7 cells, plasma corticosterone concentration did not differ from day 0 values during the mild disease course in rats administered 1×10^6 cells (Table 3.3.3). INT rats administered 1×10^6 cells had approximately 2 to 3-fold fewer CNS T cells and macrophages compared to INT rats given the 4×10^7 cell dose, with the exception of at 7 and 10 d.p.t. when no difference in T cell number between the two groups was evident (Figure 3.3.4). Moreover, INT rats receiving 1×10^6 cells

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displayed significantly lower T cell apoptosis at 4, 5, 6 and 10 d.p.t. when compared to INT animals administered 4×10^7 cells ($p < 0.05$; Table 3.3.2). Although ADX rats displayed increased apoptosis 5 d.p.t., there was no difference between INT and ADX rats given 1×10^6 cells on days 4 and 6 (Table 3.3.2).

3.3.6 Inflammatory Cell Number in Relation to Clinical Score in Intact and Adrenalectomised Lewis Rats

The T cell and macrophage number and coincident clinical score of INT and ADX rats given both cell doses are shown in figure 3.3.4. Comparison of INT and ADX rats given either cell dose revealed striking differences; for example, 4 d.p.t. of 4×10^7 cells, despite similar infiltrating T cell numbers, no clinical symptoms were apparent in the INT rat whereas 30% of the ADX animals were dead and the remainder severely affected (Figure 3.3.4 A). Equally severe clinical symptoms were displayed on day 6 by ADX rats given 1×10^6 cells, yet T cell numbers in the CNS were the same as for the matching INT rats which presented mild clinical symptoms (Figure 3.3.4 B). Macrophage numbers appeared more closely correlated with clinical score, however on days 5 and 6 similar macrophage numbers in INT and ADX rats given 4×10^7 and 1×10^6 cells respectively, were associated with more severe clinical disease in the latter group (Figure 3.3.4 C and D).

3.3.7 Effect of RU 486 on EAE and T Cell Apoptosis

Neither oral or intraperitoneal administration of the glucocorticoid receptor antagonist, RU 486, appeared to have any effect on the clinical disease course of EAE compared to vehicle treated controls. Furthermore, T cell numbers in the spinal cord were very similar in vehicle- and RU 486-treated animals (Tables 3.3.5 and 3.3.6) and

Table 3.3.2 Total Number of Apoptotic T Cells in the CNS of Intact and Adrenalectomised Lewis Rats During the Course of EAE

	T cell apoptosis (cells/mm^2)			
Days Post Transfer	INT 4×10^7	ADX 4×10^7	ADX 1×10^6	INT 1×10^6
0	0±0[0]	0±0[0]	0±0[0]	0±0[0]
3	0.4±0.2[1]	2.6±2.4[2]	0±0[0]	-
4	15.8±8.0[9]	6.8±2.0[4]	1.4±0.9*[1]	1.6±0.9*[2]
5	54.7±6.1[19]	-	12.5±2.4**[8]	4.1±3.1**[3]
6	60.8±14.7[24]	-	10.5±5.2**[9]	16.2±3.8*[11]
7	51.0±10.7[32]	-	. -	21.8±10.1-[16]
10	16.5±2.5[18]	-	-	5.3±3.1*[6]

Values represent the mean and standard error of apoptotic T cells (cells/mm^2) from individual intact (INT) and surviving adrenalectomised (ADX) Lewis rats at various time points following the transfer of either 4×10^7 or 1×10^6 MBP-sensitised splenocytes, where n = 4-10. Figures in parentheses show the percentage of the total T cell number that were apoptotic. *p<0.05 and **p<0.01 vs INT 4×10^7; -p=0.063 vs INT 4×10^7, Mann-Whitney U-test.

Table 3.3.3 Plasma Corticosterone Concentration During the Course of EAE in Lewis Rats Induced by the Transfer of 1 x 10^6 or 4 x 10^7 MBP-Sensitised Splenocytes

	Plasma corticosterone concentration (ng ml^{-1})					
Number of	Days Post Transfer					
cells transferred	0	3	4	6	7	10
1 x 10^6	84±18	-	82±15	83±16	72±8	75±11
4 x 10^7	71±12	86±18	-	110±26	277±59†	84±18

Values represent the mean ± SE of plasma corticosterone concentrations (ng ml^{-1}) measured by radioimmunoassay from intact Lewis rats at various time points following the transfer of either 1x10^6 or 4x10^7 MBP-sensitised splenocytes, where n=4-7. †p=0.01 vs. day 0, Student's t-test.

Table 3.3.4 <u>Percent T Cell Apoptosis in the CNS of Intact and Adrenalectomised Lewis Rats in Relation to EAE Clinical Score</u>

	Percent (%) T cell Apoptosis			
Clinical score	INT 4×10^7	ADX 4×10^7	ADX 1×10^6	INT 1×10^6
0-1	8.6 ± 2.9^4 $17.8 \pm 1.3(r)^{10}$	1.9 ± 1.8^3	$1.4 \pm 0.9^{4*}$	1.7 ± 0.8^4 2.6 ± 1.5^5 11.0 ± 2.0^6 16.0 ± 6.0^7 $5.8 \pm 3.3(r)^{10}$
1-2	$32.1 \pm 4.0(r)^7$	-	-	-
2-3	18.9 ± 1.6^5	$3.8 \pm 1.4^{4**}$	-	-
3-4	24.2 ± 4.4^6	-	$8.2 \pm 1.6^{5*}$ $8.5 \pm 3.8^{6\dagger}$	-

Values represent the mean and standard error of the percent of T cell apoptosis from individual intact (INT) and surviving adrenalectomised (ADX) Lewis rats administered either 1×10^6 or 4×10^7 MBP-sensitised splenocytes, where n = 4-10. (r) denotes recovering or recovered rats. Bold superscripted numbers represent the number of days post cell transfer. *p<0.05 and **p<0.01 vs INT 4×10^7; -p=0.064 vs INT 4×10^7, Mann-Whitney U-test.

Table 3.3.5 Effect of Orally Administered RU 486 on T Cell Apoptosis in the CNS of Lewis Rats with EAE

Treatment	Day post transfer	Clinical score	Total W3/13 (cells/mm^2)	Apoptotic W3/13 (cells/mm^2)	% W3/13 apoptotic
Vehicle	5	ncs	7	0	0
Vehicle	5	ncs	6	0	0
Vehicle	5	2.5	220	96	60
Vehicle	5	2.5	300	43	14
Mean[a]		**2.5**	**260**	**69.5**	**37**
Vehicle	10	ncs	2	0	0
Vehicle	10	0r	101	15	14
Vehicle	10	0r	96	24	25
Vehicle	10	0r	15	3	20
Mean[a]		**0**	**70.1**	**14**	**19.7**
RU 486	5	ncs	4	0	0
RU 486	5	3	233	55	24
RU 486	5	3	282	26	9
RU 486	5	3	130	14	25
Mean[a]		**3**	**215**	**31.7**	**19.3**
RU 486	10	ncs	1	0	0
RU 486	10	0r	78	10	13
RU 486	10	0r	182	9	5
RU 486	10	0r	71	9	13
Mean[a]		**0**	**110.3**	**9.3**	**10.3**

Lewis rats were orally dosed, twice daily, on days 3 to 10 post transfer of 4×10^7 MBP-sensitised splenocytes with either 1% carboxymethylcellulose containing 0.05% Tween 80 (vehicle) or RU 486 at 20 mg kg^{-1}. Animals were culled on day 5 or 10 post transfer and total number of T cells (W3/13$^+$) and the number of apoptotic T cells in the lumbar spinal cord were determined. [a]Rats which displayed no clinical signs of EAE (ncs) have been excluded from the calculation of mean values. 0r denotes recovered from clinical signs of EAE at the time of culling (max. clinical score = 3).

Table 3.3.6   Effect of Intraperitoneally Administered RU 486 on T Cell Apoptosis in the CNS of Lewis Rats with EAE

Treatment	Day post transfer	Clinical score	Total W3/13 (cells/mm²)	Apoptotic W3/13 (cells/mm²)	% W3/13 apoptotic
Vehicle	5	2	333	25	8
Vehicle	5	2.5	281	25	9
Vehicle	5	2.5	283	25	9
Vehicle	5	2.5	285	25	9
Vehicle	5	2	270	19	7
Mean		**2.3**	**290.4**	**23.8**	**8.4**
Vehicle	7	2r	121	12	10
Vehicle	7	2.5r	166	32	19
Vehicle	7	2r	139	20	14
Vehicle	7	1.5r	187	24	13
Vehicle	7	2.5r	213	22	10
Mean		**2.1**	**165.2**	**22**	**13.2**
RU 486	5	2.5	303	33	11
RU 486	5	1	200	20	10
RU 486	5	2.5	416	61	15
RU 486	5	1.5	219	10	5
RU 486	5	3.5	257	25	10
Mean		**2.2**	**279**	**29.8**	**10.2**
RU 486	7	0.5r	144	15	10
RU 486	7	1.5r	140	12	9
RU 486	7	1.5r	176	23	13
RU 486	7	3	131	10	8
RU 486	7	2	202	13	6
Mean		**1.7**	**158.6**	**14.6**	**9.2**

Lewis rats were injected i.p. once daily on days 2-7 post transfer of 4×10^7 MBP-sensitised splenocytes with either sterile saline containing 5% ethanol (vehicle) or RU 486 at 15 mg kg^{-1}. Animals were culled on day 5 or 7 post transfer and total number of T cells (W3/13$^+$) and the number of apoptotic T cells in the lumbar spinal cord were determined. r after a clinical score denotes animal recovering from EAE at the time of culling (max. clinical score = 3).

whilst there appears to be fewer apoptotic T cells in animals treated orally with RU 486 the small group numbers and the lack of disease development in several animals make interpretation difficult (Table 3.3.5); no difference in numbers of apoptotic T cells was apparent in animals administered RU 486 by i.p. injection (Table 3.3.6).

Post mortem examination of animals injected i.p. with RU 486 revealed the presence of white deposits on the liver, stomach and other tissue within the peritoneal cavity although no residue was seen in animals treated orally.

3.4   Discussion.

The data presented in this chapter indicate that administration of 4×10^7 MBP-sensitised splenocytes to intact Lewis rats resulted in normal disease course (score 3), an increase in plasma corticosterone concentration, high numbers of apoptotic T cells in the spinal cord and recovery from EAE. Intact Fischer rats, which are known to have greater corticosterone production, developed mild disease (score 1), had high T cell apoptosis and recovered from EAE. However, adrenalectomised rats of both strains given 4×10^7 cells rapidly developed severe disease (score 4-5), had low numbers of apoptotic T cells in the spinal cord and fatal disease. Disease onset was delayed following administration of a lower number of MBP-sensitised cells (1×10^6) to adrenalectomised Lewis rats although ultimately, severe disease developed (score 4-5), there was low T cell apoptosis and animals did not recover. Administration of the low cell dose (1×10^6) to intact Lewis rats resulted in mild clinical signs (score 0.5-1), no change in circulating corticosterone levels, low T cell apoptosis and 'recovery' from EAE. Where intact and adrenalectomised rats displayed comparable clinical scores, T cell apoptosis was significantly lower in adrenalectomised animals. Thus, corticosterone appears to play a crucial role in the resolution of clinical signs of EAE in Lewis rats and

in limiting disease onset in Fischer rats. Apoptotic elimination of autoreactive effector T cells represents one mechanism by which corticosterone may confer protection from disease onset and bring about recovery from EAE.

3.4.1   Resistance of Fischer Rats to Experimental Allergic Encephalomyelitis

This study further emphasises the absolute requirement of the adrenocortical response in recovery from EAE in the Lewis rat and whilst a critical role for corticosterone in mediating resistance of Fischer rats was evident from an initial experiment this was not substantiated in subsequent experiments. The resistance of ADX F344 rats was not the result of incomplete adrenalectomy as no residual tissue was observed on examination post mortem. In addition, corticosterone was not detected in plasma samples indicating no other source of corticosterone was responsible for the resistance. Resistance to EAE induction following ADX has been observed in PVG.RT1^{u} and AO rats (RT1^{u}),   and while perivascular inflammatory lesions were evident in the spinal cord of ADX Brown Norway (BN) rats, clinical signs developed only in ADX BN rats treated with RU 486. (Mason, MacPhee & Antoni, 1990; Peers et $al.$, 1995). However no data is available concerning EAE resistance in ADX F344 rats. The assumptions of corticosterone-mediated resistance to EAE in F344 rats were based on the ability of the glucocorticoid receptor antagonist, RU 486, to precipitate severe arthritis in F344 rats (Sternberg et $al.$, 1989a) and the fact that PVG rats with RT1^{l} MHC genes and increased corticosterone production compared to LEW rats become susceptible to EAE following ADX (Mason, MacPhee & Antoni, 1990). It is possible that a genetic difference, arising from mutation, contamination or residual heterozygosity could account for the observed resistance in these F344 rats. Although, data from the animal suppliers indicate that the LEW and F344 rats used were of the

RT1^{l} MHC haplotype it has been suggested that rats previously assigned the same MHC haplotype are not fully identical in the RT1 chromosomal segment because they have only been typed by serology and not by mixed lymphocyte culture and F1 tests (Hedrich, 1983). An example of non-MHC control over EAE susceptibility was reported in a closed colony of LEW rats which had been randomly bred for 5 years (Waxman *et al.*, 1981). Rats in the colony (termed Lewis resistant (Le-R)) were initially susceptible to EAE induction whilst after 5 years only 23% of Le-R rats developed disease even though they were histocompatible with EAE susceptible LEW rats. Thus, within the Le-R colony there appears to be two separate populations, one susceptible to EAE, the other resistant; this type of situation may have arisen within colonies of F344 rats used in these studies.

The results of the initial experiment suggest that fewer T cells are present in the CNS of INT F344 rats compared to INT LEW rats 7 d.p.t. of 4 x 10^{7} MBP-sensitised cells. This may account for the mild clinical disease in these rats, although INT LEW rats administered 1x10^{6} cells also presented with similarly mild clinical signs yet T cell numbers in the CNS on day 7 p.t. were 3-fold greater than in F344 rats. The fate of the adoptively transferred MBP-sensitised spleen cells in EAE-resistant rats requires further investigation, although given the resistance of ADX F344 rats it may be beneficial to use PVG.RT1^{l} rats. Whilst it is unknown whether greater numbers of T cells accumulated in the CNS of F344 rats at earlier time points following cell transfer, it is possible that high levels of corticosterone may suppress T cell entry to the CNS in F344 rats by a number of mechanisms such as down regulation of cell adhesion molecules and chemokine production (discussed in section 1.1.5). Another possibility is that in the higher corticosteroid environment of F344 rats, the transferred cells are depleted peripherally, perhaps by apoptosis in splenic germinal centres (Zheng *et al.*, 1996),

reducing the pool of activated T cells capable of entering the CNS and initiating disease. Nevertheless the limited data presented in this chapter indicate that a greater proportion of T cells in the CNS of INT F344 rats undergo apoptosis and very few apoptotic cells are seen in the CNS of ADX F344 rats supporting a role for corticosterone-mediated apoptosis of T cells within the CNS as a mechanism which limits the induction of EAE in this rat strain.

3.4.2   Cellular Infiltration of the CNS in Intact and Adrenalectomised Lewis Rats

Basal glucocorticoid tone does not appear to affect T cell surveillance of the CNS, as in the present study T cells were largely undetectable in the CNS of INT and ADX LEW rats prior to the induction of EAE. The profile of T cell and macrophage accumulation in the CNS of INT LEW rats was similar to that previously described (Schmied et al., 1993; Matsumoto & Fujiwara, 1987), with the initial wave of T cells being superseded by an influx of macrophages in numbers approximately 2-3 times that of T cells. This highlights the importance of T cells in initiating (Panitch & McFarlin, 1977) and macrophages as effectors of the disease process (Huitinga et al., 1990).

The observation that significantly more T cells and macrophages accumulated earlier in the CNS lesions of ADX animals indicates that the cells were indeed more rapidly recruited and the tissue more readily penetrated in the absence of corticosterone once the inflammatory condition was initiated. Moreover, in ADX rats not only does cell recruitment occur earlier than in INT animals but fewer cells are required to precipitate the clinical symptoms. This is probably a consequence of the lack of glucocorticoid-mediated immune suppression operating centrally during the clinical phase of EAE. The resulting escalation in inflammatory mediator production in the absence of corticosterone may recruit greater numbers of non-specific T cells and

macrophages, enabling unbridled exacerbation of the inflammatory foci. In addition, this may preclude the production of down-regulatory cytokines such as IL-4, IL-10 and TGF-β (Rott, Fleischer & Cash, 1994; Racke et al., 1991, 1994) and the shift to a suppressor/recovery T cell profile (Varriale et al., 1994), mechanisms suggested to be involved in recovery from EAE.

Surprisingly, in both INT and ADX rats only a 2 to 3-fold difference in T cell and macrophage numbers in CNS lesions was apparent between groups injected with 4×10^7 and 1×10^6 cells, despite a 40-fold difference in the number of splenocytes initially transferred. This suggests that the proportion of non-specific T cells in the CNS of rats given the lower cell dose is greater than rats receiving 4×10^7 cells, however it appears that relatively few MBP-specific T cells are required to enter the CNS initially to recruit further non-specific T cells and macrophages. Alternatively, T cell entry into the meninges, cuffs and parenchyma may reach saturation at relatively low transferred T cell levels.

3.4.3 Detection of Apoptotic Cells in the CNS During the Course of EAE

The identification of dying cells within tissue sections by morphological criteria alone may not wholly reflect the presence of apoptosis since DNA fragmentation can be found not only in histologically defined apoptotic cells, but also in morphologically intact cells going through the process of programmed cell death (Umansky, 1982; Motyka & Reynolds, 1991). Enzymatic labelling of DNA strand breaks may not only detect apoptosis but also the random fragmentation of DNA seen in necrosis (Gold et al., 1994). The combination of morphological criteria and ISNT labelling used in this study combined with immunocytochemistry allowed the unequivocal identification of dying cells and the type of cell death occurring in situ in pathological tissue.

3.4.4   Glucocorticoids and T Cell Apoptosis During EAE

The current findings imply that corticosterone-driven T cell apoptosis contributes to the reduction in CNS inflammation and recovery from EAE in the Lewis rat. Two significant issues were not addressed in the present study: (i) whether corticosterone replacement therapy in ADX rats would affect the clinical severity of EAE and the amount of T cell apoptosis; (ii) whether glucocorticoid treatment of INT LEW rats would increase T cell apoptosis and suppress clinical signs of EAE. Replacement of basal corticosterone levels in ADX rats failed to prevent the fatal outcome of EAE induced by transfer of $5x10^7$ MBP-sensitised spleen cells or immunisation with MBP-CFA; however replacement of corticosterone to levels which mimicked the endogenous production in INT animals with EAE resulted in remission of clinical signs (MacPhee, Antoni & Mason, 1989). Thus if corticosterone-mediated apoptosis of T cells is an important mechanism for resolution of EAE then increased numbers of apoptotic T cells would be expected in the CNS of ADX rats treated with a suitable dose of corticosterone. Administration of dexamethasone to INT LEW rats has been shown to suppress clinical signs of EAE and was associated with increased numbers of apoptotic T cells in the spinal cord (McCombe *et al.*, 1996; Nguyen, McCombe & Pender, 1997) suggesting that induction of T cell apoptosis may underlie, at least partly, the beneficial clinical effects of corticosteroids in EAE and possibly MS.

Pender *et al.* (1991) first described apoptosis of oligodendrocytes and T cells in the CNS of Lewis rats with EAE, subsequently reporting that between 5-10% of T cells in the spinal cord lesions were apoptotic (Pender *et al.*, 1992). In both active and transfer EAE, up to 49% of T cells in the CNS of SD and Lewis rats were apoptotic at the time of recovery from the disease (Schmied *et al.*, 1993). Both groups postulated the involvement of corticosterone in the process. T cell apoptosis also occurs in sciatic

nerve during experimental autoimmune neuritis (EAN), an inflammatory demyelinating disease of the peripheral nervous system, with the highest levels (~10%) also found during recovery which were augmented by i.v. prednisolone treatment (max. 42%; Zettl *et al.*, 1995). In the present study, the profile of T cell apoptosis in INT rats given 4×10^7 cells paralleled the expected changes in corticosterone secretion, with the absolute number of apoptotic T cells greatest on day 6. The percent T cell apoptosis, however, reached a maximum (32.1%) on day 7, concurrent with the beginning of disease remission, a finding also reported by Tabi *et al* (1994). Furthermore, the temporal profile of percent T cell apoptosis in this study and that of Tabi *et al.* (1994) is almost identical although the peak level in the latter (8-11%) was considerably lower, the disparity perhaps being due to the method of detecting apoptotic cells, namely flow cytometric analysis of DNA stained with propidium iodide from T cell enriched extracts of spinal cord. In addition, Tabi *et al.* (1994) reported the preferential deletion of $Vb8.2^+$ MBP-specific T cells via apoptosis, although these cells maximally constituted only 36% of all apoptotic T cells. Although not determined in the present study, it is possible that selective, apoptotic deletion of transferred MBP-specific T cells contributed to the total. However, as the proportion of MBP-specific T cells in CNS lesions during EAE is relatively low (less than 4%; Cross *et al.*, 1991), corticosterone-mediated "bystander" apoptosis of non-specific infiltrating T cells cannot be excluded.

3.4.5 Possible Mechanisms of T Cell Apoptosis in vivo

The importance of corticosterone in the apoptotic process is highlighted in the observation that adrenal INT rats given 4×10^7 cells consistently displayed significantly higher T cell apoptosis compared to ADX rats given either cell dose. As indicated previously, mature, peripheral blood T lymphocytes are resistant to glucocorticoid-

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induced apoptosis until the cells have been activated, whereupon the sensitivity to apoptosis increases with repeated stimulation. This also occurs with encephalitogenic (MBP-specific) T cells *in vitro* where apoptosis is enhanced by addition of dexamethasone or hydrocortisone after 72 h of antigen-specific stimulation but not at earlier time points (Gold *et al.*, 1996). In the present study, where spleen cells were transferred to naïve recipients following a 3 day re-stimulation period with MBP *in vitro*, T cells were thus primed to both respond to the stimulating antigen or activate the apoptotic pathway on encountering antigen and endogenous corticosteroid.

Glucocorticoids may initiate apoptosis in the inflammatory foci indirectly by down-regulating the production of cytokines such as IL-1 and IL-2 whilst increasing production of transforming growth factor (TGF)–β. Mature T lymphocytes undergo apoptosis when deprived of IL-2 (Duke & Cohen, 1986), whereas IL-2 primes $CD4^+$ and $CD8^+$ mouse T lymphocytes to undergo apoptosis when, subsequently, they are exposed to antigen which triggers the TCR (Lenardo, 1991; Migliorati *et al.*, 1993). Thus, in the ADX rat where the production of cytokines such as IL-1 and IL-2, which can rescue T-cells from apoptosis (McConkey *et al.*, 1990; Duke & Cohen, 1986), is probably enhanced then apoptosis may be inhibited and activation or proliferation potentiated, whereas in INT animals IL-2 primed T cells may be more prone to apoptosis following TCR activation due to the downregulation of IL-1 and IL-2 by increased corticosterone production. Expression of TGF–β mRNA in the CNS was found to increase at the height of clinical EAE, shortly preceding recovery (Issazadeh *et al.*, 1995b) and administration of this cytokine prevents onset or improves the clinical course of ongoing EAE suggesting an important role in downregulation of the immune response and recovery from EAE. In addition to inhibiting T cell proliferation (Kehrl *et al.*, 1986) and downregulating MHC class II expression (Schlüsener, 1990), TGF–β

induces apoptosis of antigen- and IL-2-activated T cells *in vitro* (Weller *et al.*, 1994) and may contribute to the induction of T cell apoptosis observed during EAE. Thus, whether or not T cells undergo apoptosis may be influenced by the local cytokine environment; conversely, the local cytokine environment may be modulated by the presence of apoptotic cells: the addition of apoptotic peripheral blood lymphocytes to LPS-activated peripheral blood mononuclear cells or purified monocytes resulted in increased secretion of the anti-inflammatory cytokine IL-10 and decreased secretion of the pro-inflammatory cytokines IL-1β, TNF–α and IL-12 suggesting that inflammation and cell mediated immunity may be inhibited in conditions associated with increased apoptosis (Voll *et al.*, 1997).

In the dermal sensitisation site of active EAE a large T cell infiltrate was apparent although no apoptotic T cells were seen (Schmied *et al.*, 1993). Furthermore, examination of spleen and draining lymph nodes during EAN and EAE in this and previous studies (Schmied *et al.*, 1993; Zettl *et al.*, 1995), revealed low numbers of apoptotic T cells which correlated with neither disease activity or adrenal state. Whilst corticosterone is essential for the degree of T cell apoptosis in the CNS associated with the recovery phase of EAE, the presence of low numbers of apoptotic T cells in ADX rats and animals with low circulating levels of the steroid (i.e. INT given 1x10^6 cells) suggests that several pathways to T cell apoptosis operate in the CNS. The apparent organ specificity of T cell apoptosis suggests that local resident cells may render T cells susceptible to apoptosis directly or through soluble factors, whilst the relative lack of stimulating antigen (i.e. MBP) in peripheral tissue may account for the low levels of T cell apoptosis observed; apoptosis of MBP-reactive T cells *in vitro* is increased in the presence of added MBP and is dependent on the type of APC used (Ford *et al.*, 1996; Klyushnenkova & Vanguri, 1997). In the CNS, astrocytes, resident microglia (MG) and

non-MG CNS macrophages might have a central role. Indeed, it has recently been shown that MBP-specific T cells underwent apoptosis following 72 h in culture with MBP when IFN–γ-treated astrocytes were used as APC but not when thymic APC were used (Gold *et al.*, 1996). Furthermore, the proportion of apoptotic T cells was increased by addition of either hydrocortisone or dexamethasone to the astrocyte-T cell cultures but not thymic APC-T cell cultures and the glucocorticoid mediated apoptosis was inhibited by RU 486 (Gold *et al.*, 1996). However, it is microglial cells rather than astrocytes that express MHC class II molecules during EAE (Matsumoto *et al.*, 1986) making it unlikely that this process occurs *in vivo*. CNS macrophages have been separated into two populations on the basis of the relative expression of CD45, with CD45^{low} phenotype identifying resident microglia and CD45^{high} defining the non-MG CNS (transitional) macrophage (Ford *et al.*, 1995). Interestingly, although activated CD45^{low} MG express MHC class II, MBP-reactive T cells failed to proliferate in response to added antigen which was in stark contrast to T cells cultured in the presence of CD45^{high} CNS-derived macrophages or lymph node APC (Ford *et al.*, 1996; Klyushnenkova & Vanguri, 1997). Furthermore, 20-44% of MBP-specific T cells stimulated with MBP in the presence of activated CD45^{low} MG underwent apoptosis whilst no apoptotic changes were observed in T cells cultured alone or in the presence of other APC (Ford *et al.*, 1996; Klyushnenkova & Vanguri, 1997).

In the EAE-resistant BN rat strain, which also have higher basal concentration of corticosterone than LEW rats (Villas, Dronsfield & Blankenhorn, 1991), constitutive- and IFN–γ-stimulated-expression of MHC class II molecules on resident microglia (CD45^{low}) was greater than that of Lewis rat MG and suggests that hyperinducibility of Ia on MG correlates with EAE resistance (Sedgwick *et al.*, 1993; Klyushnenkova & Vanguri, 1997). Furthermore, MG of both strains induced apoptosis of antigen-

activated (MBP) T cells suggesting that resident microglia, far from promoting inflammation in the CNS, actually participate in the downregulation of the pathological processes during EAE (Klyushnenkova & Vanguri, 1997).

It would be of great interest to examine the behaviour of MBP-reactive T cells from LEW, F344, and PVG (all RT1^l) rats in terms of proliferative response to antigen and the extent to which T cells from the different strains undergo apoptosis in the presence of different antigen presenting cell types (derived from peripheral and CNS tissue). Furthermore, the possibility that MBP-reactive T cells from EAE-resistant F344 and PVG rats may be more susceptible to apoptosis in the presence of corticosterone or dexamethasone should be investigated as this may shed more light on the importance of endogenous corticosteroid production and apoptosis of T cells as protective mechanisms in the induction of CNS autoimmune disease.

Glucocorticoid-induced apoptosis in rat thymocytes has been shown to be a steroid receptor-mediated process because it can be blocked by the glucocorticoid receptor antagonist RU 486 and cannot be induced in cells devoid of glucocorticoid receptors (Compton, Caron & Cidlowski, 1987; Wyllie *et al.*, 1984). Thus, administration of RU 486 during the course of EAE might have been expected to inhibit T cell apoptosis and lead to worsening of clinical signs. Given the finding of insoluble deposits within the peritoneal cavity of animals injected i.p. with this compound and no worsening of clinical signs following either oral or i.p. administration of RU 486 it appears likely that the apparent lack of inhibition of T cell apoptosis may have been due to RU 486 not accessing the circulation and thus not gaining access to glucocorticoid receptors.

3.4.6 Fate of Apoptotic Inflammatory Cells

The low levels of apoptotic macrophages (< 1%) in both INT and ADX animals suggest that corticosterone is not involved in the apoptotic process in these cells. This is supported by the finding that only 4% of macrophages/microglia (OX-42^{+}) were apoptotic in spinal cord sections from LEW rats injected with 4 mg kg^{-1} of dexamethasone at the onset of clinical signs (Nguyen, McCombe & Pender, 1997). Apoptosis, along with cell migration, may contribute to the mechanisms of clearance of inflammatory T cells from the CNS during recovery from EAE. It has been suggested that the majority of CD4^{+} T cells that accumulate within the CNS at the onset of disease are lost from the CNS within 48hr (Zeine & Owens, 1993). In the periphery, the apoptotic process occurs rapidly (4 - 6 hrs; Compton & Cidlowski, 1992); if similar kinetics are observed in the CNS then apoptosis may account for the loss of CD4^{+} T cells described in EAE. However, *in vivo*, at sites of inflammation and injury (Wyllie, Kerr & Currie, 1980), and *in vitro* (Fadok *et al.*, 1992) apoptotic lymphocytes are readily phagocytosed by macrophages, whilst in the present study it appeared that the number of apoptotic T cells in the CNS ingested by either macrophages or neural scavenger cells was negligible in both INT and ADX rats. Further studies are necessary to elucidate the fate of apoptotic T cells in the CNS. The paucity of apoptotic macrophages detected in this study and during peripheral inflammation (Bellingan *et al.*, 1996) suggests that migration rather than apoptosis is the major route to clearance of this cell type during the resolution of inflammation.

In summary, evidence of apoptotic T cells and macrophages in post mortem tissue from MS patients (Ozawa *et al.*, 1994; Dowling *et al.*, 1997) and the findings presented in this chapter suggest that this form of cell death may be an important mechanism to regulate the extent of inflammatory responses in CNS autoimmune

disease. Furthermore the importance of glucocorticoids in contributing to the death of infiltrating lymphocytes by apoptosis is highlighted and indicates that apoptosis may be an important mechanism by which high-dose glucocorticoid therapy contributes to the treatment of optic neuritis and acute relapses of MS.

CHAPTER IV

Exacerbation of Experimental Allergic Encephalomyelitis and Induction of Relapse in Lewis but not Fischer rats by Interleukin-12

4.1   Introduction

4.1.1   Interleukin-12

Interleukin (IL)-12 is a heterodimeric cytokine of 70 - 75 kDa (p70) formed by two disulphide-linked glycosylated polypeptide chains of approximately 40 (p40) and 35 (p35) kDa (Kobayashi *et al.*, 1989; Stern *et al.*, 1990). Bioactive p75 is produced upon stimulation by professional APC, such as dendritic cells, macrophages/microglia, neutrophils and B cells (Ma *et al.*, 1995; Aloisi *et al.*, 1997). Recently, IL-12 has been recognised as a key regulator of immune responses through its effects on T cells and natural killer (NK) cells. While IL-12 can induce production of TNF–α, granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-2 it is a particularly potent inducer of IFN–γ from T and NK cells and enhances proliferation and cytolytic activity of these cells (Trinchieri & Scott, 1995). In addition, IL-12 promotes the development of Th1-type cells both *in vitro* and *in vivo* (Manetti *et al.*, 1993; Hsieh *et al.*, 1993; Sypek *et al.*, 1993) and has been implicated in the induction of Th1-mediated organ specific autoimmune diseases (Trembleau *et al.*, 1995).

4.1.2   The Role of Interleukin-12 in Autoimmunity

Interleukin-12 has a pivotal role in the development of several Th1 CD4$^+$ T cell mediated autoimmune disease models, including collagen induced arthritis, insulin dependent diabetes mellitus and EAE (Germann *et al.*, 1995; Trembleau *et al.*, 1995; Leonard, Waldburger & Goldman, 1995).

In a murine model of EAE, PLP-sensitised lymph node cells (LNC) stimulated *in*

vitro in the presence of IL-12 and transferred to naive recipients resulted in severe disease with rapid onset compared to mice that received LNC stimulated with PLP alone (Leonard, Waldburger & Goldman, 1995; Waldburger *et al.*, 1996). Similarly, *in vivo* administration of IL-12 following transfer of PLP-sensitised LNC resulted in rapid onset of severe clinical signs and a protracted disease course. Stimulation of LNC *in vitro* with PLP and IL-12 resulted in a doubling of the number of IFN–γ producing cells and a ten-fold increase in IFN–γ secretion, whilst IL-4 producing cells were three-fold lower compared to LNC stimulated with PLP alone. Furthermore, neutralisation of endogenous IL-12 by an antibody administered every other day for 12 days from the time of cell transfer reduced the incidence and severity of EAE induced by transfer of PLP-sensitised lymph node cells to mice (Leonard, Waldburger & Goldman, 1995) indicating an important role for endogenous IL-12 in the development of the disease. In a chronic relapsing model of EAE in Biozzi mice, administration of either murine p40 homodimer alone or in combination with an anti-p70 monoclonal antibody significantly inhibited induction of disease and appeared to be effective when administered after secondary immunisation suggesting IL-12 may be important in the relapsing phase of the disease as well as induction (see Lamont & Adorini, 1996).

4.1.3 Rationale and Aims

The resistance of F344 rats to induction of EAE and other inflammatory autoimmune diseases compared to histocompatible, EAE-susceptible LEW rats is attributed to increased basal and stimulated secretion of corticosterone (Sternberg *et al.*, 1989a; Wilder, 1995). Since removal of the immunosuppressive influence of corticosterone, by antagonism of glucocorticoid receptors or adrenalectomy, results in development of SCW-induced arthritis (Sternberg *et al.*, 1989a) and EAE (chapter III)

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in F344 rats it is clear that resistance is not an inherent characteristic of F344 background genes. Glucocorticoids act at many levels of the immune system to modulate the immune response including inhibition of IFN–γ production and promotion of Th2 dependent immunity in favour of Th1 type responses. In ADX F344 rats these immunosuppressive influences are absent and following adoptive transfer of MBP-sensitised splenocytes severe EAE develops. Given the ability of IL-12 to skew the Th-cell population to the Th1 type it was hypothesised that *in vitro* stimulation of MBP-sensitised splenocytes with MBP and IL-12 prior to adoptive transfer or *in vivo* administration of IL-12 following induction of EAE would bypass the effect of endogenous CS and enhance the Th1 response, manifest as overt EAE in F344 rats and increased disease severity in LEW rats.

The mechanisms of recovery from acute monophasic EAE in LEW rats are not clearly understood but include a shift from a Th1- to a Th2-cytokine response (Kennedy *et al.*, 1992), induction of T cell apoptosis in the CNS (Schmied *et al.*, 1993; Chapter III) and increased plasma corticosterone levels (MacPhee, Antoni & Mason, 1989; MacKenzie, Leonard & Cuzner, 1989). Following recovery, LEW rats are refractory to disease reinduction which is attributed to active immunosuppression mediated by $CD4^+$ T cells (Killen & Swanborg, 1982; Karpus & Swanborg, 1991). Thus, the potential of IL-12 to overcome the presumed Th2-mediated recovery response and elicit a relapse of clinical signs of EAE in LEW rats by administration of the cytokine following recovery from either actively induced or passively transferred EAE was investigated.

4.2   Methods

4.2.1   Animals

Adult female LEW and F344 rats (190-220g) were purchased from Charles

River (Kent, U.K.) and housed in pairs under a 12 hour light-dark cycle (lights on 06.00h - 18.00h) at 21°C for at least one week before experimentation. Rat diet (Labsure, Poole, Dorset, U.K.) and tap-water were available *ad libitum*.

4.2.2 Murine Recombinant Interleukin-12

Murine recombinant (mr) IL-12 (gift from Dr. John Leonard, Genetics Institute, Cambridge, MA, USA) had a specific activity of 4.6 x 10^6 U/mg and endotoxin contamination < 0.641 EU/mg (*Limulus* amebocyte assay).

4.2.3 Induction of EAE

Active EAE was induced as described in chapter III (section 3.2.3) by subcutaneous injection of guinea-pig MBP in CFA (MBP-CFA). Control animals were injected with an emulsion of CFA and *M. tuberculosis* alone (CFA). For adoptive transfer of EAE (section 3.2.3), cells obtained from MBP-CFA-immunised animals were stimulated *in vitro* with MBP (10 mg ml^{-1}) whilst cells from CFA-immunised animals were stimulated with purified protein derivative (PPD; 10 mg ml^{-1}) from *M. tuberculosis* (NHNN pharmacy). Harvested cells were washed thoroughly in unsupplemented EBSS and 4x10^7 splenocytes were transferred i.p. to naive recipients.

4.2.4 In vivo Administration of IL-12 Following Induction of Active EAE

Murine rIL-12 (3 mg/rat, 0.2 ml i.p. in 0.9% w/v sterile saline) was administered to LEW and F344 rats following MBP-CFA or CFA immunisation. LEW rats received mrIL-12 or saline on days 5, 7 and 9 post immunisation (p.i.), whilst F344 rats were injected on days 3, 5, 7, 9 and 11 p.i.. Separate groups of LEW rats were immunised with MBP-CFA and allowed to recover from EAE; mrIL-12 was then administered

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immediately after recovery on days 17, 19 and 21 p.i. (when animals displayed scores of 0 or 1); one week after complete recovery on days 26, 28 and 30 p.i.; two weeks after complete recovery on days 33, 35 and 37 p.i.; or six weeks after complete recovery on days 57, 59 and 61 p.i.. Control animals received either an equivalent volume of sterile saline or no treatment. Animals were killed at various time-points by a blow to the back of the head followed by decapitation and trunk blood was collected into ice-chilled lithium-heparin tubes and, in some experiments, SST® gel and clot activator-vacutainer tubes (Becton Dickinson, UK), centrifuged (1500 g for 15 min at 4°C) and plasma and serum stored at -80°C until required for determination of (i) plasma corticosterone concentration by RIA (IDS Ltd.) and (ii) serum nitrate concentration using the Greiss reaction. After removing the brain and spinal cord the brainstem and cervical spinal cord were dissected, mounted in OCT compound (BDH, UK) on cork discs, rapidly frozen on solid CO_2 and stored at -80°C until required for histological and immunocytochemical analysis.

4.2.5 In vivo Administration of IL-12 After the Transfer of MBP- or PPD-Stimulated Spleen Cells

Murine rIL-12 (3 mg/rat, 0.2 ml i.p. in 0.9% sterile saline) or sterile saline (0.2 ml/rat i.p.) was administered to LEW rats on days 0, 1 and 2 post transfer (p.t.) of 4×10^7 MBP- or PPD-stimulated spleen cells. Separate groups of LEW rats that received 4×10^7 MBP-stimulated cells were allowed to recover from EAE and were then injected i.p. with either saline (0.2 ml/rat), MBP (10 mg/rat), mrIL-12 (3 mg/rat) or MBP (10 mg/rat) plus mrIL-12 (3 mg/rat) on days 9, 11 and 13 p.t.. Animals were killed at various time points for collection of blood and CNS tissue as described above.

4.2.6 In vitro Incubation of MBP-Stimulated Spleen Cells With IL-12 Before Cell Transfer

Splenocytes were cultured in the presence or absence of mrIL-12 (20 ng ml^{-1}). After 72 h the cells were washed 3 times in EBSS and 4 x 10^7 cells transferred i.p. to naive female LEW or F344 rats. Animals were killed on days 6 and 7 p.t. and plasma and CNS tissue collected and stored as described above.

4.2.7 Assessment of clinical EAE

Animals were weighed daily and observed for clinical signs of EAE as described in chapter III (section 3.2.4).

4.2.8 Immunocytochemistry and Histopathological Evaluation.

Rats were killed at various stages post immunisation and the brain and spinal cord were removed and rapidly frozen on solid CO_2. Longitudinal frozen sections (10 μm thick, 1-1.5 cm in length) of brain stem/cervical spinal cord were cut and thaw mounted onto poly-L-lysine- (1mg ml^{-1}; Sigma, U.K.) coated slides, dried in air for at least 30 minutes then stored frozen at -20°C until processed for immunocytochemistry. All incubations were carried out at room temperature in a humidified staining tray to prevent the sections drying out; phosphate buffered saline (PBS; pH 7.2 - 7.4) was used for all washing steps and to dilute all antibodies and the avidin-biotin peroxidase complex (ABC). The primary antibodies used to examine resident and infiltrating cells in the CNS are listed in table 4.1. Tissue was fixed in absolute ethanol (1 min, room temp.), washed in PBS and then incubated for 30 min with 2.5% normal horse serum (75 μl/ section). After tipping off serum, the primary antibody (70 μl/section) was applied for 1 hour. Following two five minute washes, 75 μl/section of a biotinylated

Table 4.1   Primary Antibodies Used in Immunocytochemical Analysis of CNS Tissue
During the Course of EAE

Antibody	Specificity	Dilution	Source
MRC OX34	CD2 (T cells)	1:500	Serotec, Oxford, UK
ED1	CD68(like) lysosomal membrane antigen on Mφ/MG	1:500	Serotec
MRC OX42	CD11b; complement receptor 3 on MG/Mφ	1:1000	Serotec
5.2E4	Glial fibrillary acidic protein (astrocytes)	1:1000	MS Laboratory.
Clone 6	inducible nitric oxide synthase	1:200	Transduction Laboratories (Affiniti Research Products, Exeter, UK)
MRC OX6	RT1B MHC II (Ia)	1:500	Serotec
1A29	ICAM-1 (CD54)	1:1000	Serotec
anti-IL-12	human IL-12	1:10 - 1:1000	Genetics Institute, Cambridge, MA, USA
DB.1	Interferon-γ	1:10 - 1:1000	Serotec
anti-TNF-α	Rat and mouse TNF-α	1:10 - 1:1000	Serotec

All antibodies were mouse anti-rat monoclonal antibodies except anti-IL-12 (mouse anti-human) and anti-TNF-α (rabbit polyclonal antibody which recognises rat and mouse TNF-α).

Abbreviations/symbols: Mφ, macrophages; MG, microglia; MHC, major histocompatibility complex; ICAM, intercellular adhesion molecule; TNF, tumour necrosis factor.

anti-mouse IgG (rat adsorbed; 1:200; Vector Laboratories, Peterborough, U.K.) was applied for 30 minutes. The ABC solution was prepared according to the manufacturer's instructions (Vector Laboratories, U.K.) at least 30 minutes before use to allow the complex to form. After washing off excess biotinylated antibody, 75 µl/ section of the ABC solution was applied and left for 45 minutes. Unbound ABC was removed by two 5 minute washes and peroxidase activity was detected by placing the slides in a solution of 0.5 mg/ml 3,3'-diaminobenzidine (Sigma, Poole, U.K.) in PBS containing 0.013% hydrogen peroxide for 5 min; in some instances, nickel (II) chloride (final conc. 0.04%) was included with the above solution to produce a black reaction product (Newcombe and Cuzner, 1988) where OX-42 antibody was used and sections were not counterstained. Rinsed sections were counterstained in Mayer's haematoxylin (30 sec; BDH, Poole, U.K.) followed by washing in running tap water, dehydrated through graded alcohols (70%, 100%, 100%, 45 seconds each), cleared in xylene (100%, 45 seconds; 100%, 5-10 minutes) and mounted in DPX (BDH, U.K.). Control sections with no primary antibody were included in each staining run.

For haematoxylin and eosin (HE) staining, tissue sections were fixed as for immunocytochemistry and then placed in Harris' haematoxylin (BDH, U.K.) for 2 minutes, rinsed in tap water and the staining differentiated by dipping the slides in 70% ethanol containing 1% HCl and washing in tap water for 5 min. Slides were then placed in eosin (1%; BDH, U.K.) for 30 seconds, washed in tap water and dehydrated and mounted as described above.

For all quantification, slides were coded so that scoring was performed without knowledge of the treatment (i.e. saline or mrIL-12) administered to the rat. Histological assessment of CNS inflammation for each animal was determined on two or three sections of brainstem taken at approximately 20 µm intervals and routinely stained with

HE or on sections that had been used for immunocytochemistry and counterstained with haematoxylin (no antibody control and ICAM-1). The number and size of CNS lesions was determined as follows: individual cuffs were scored as 1 = perivascular inflammation ≤ three cells thick; 2 = perivascular cuff > three cells thick and 3 = perivascular inflammation and parenchymal infiltration. The histopathological score was then calculated for each animal by adding the scores for all cuffs in a section and the mean of two or three sections taken.

The number of T cells (CD2$^+$) was determined in a randomly selected cuff and expressed as a percent of the total number of haematoxylin stained nuclei in the cuff. This was repeated on a serial section for macrophages (ED1$^+$) in the same cuff. The process was performed on three separate cuffs and the average taken to give the percent T cell/macrophage composition. The number of cuffs with iNOS immunoreactivity in one brainstem section from each animal were counted and expressed as a percentage of the total number of inflammatory cuffs in that section.

4.2.9 Measurement of Serum Nitrate (NO$_3^-$) Plus Nitrite (NO$_2^-$)

The method used was that of Giovannoni *et al* (1997b) which was adapted from the nitrate reductase and Griess reaction methods of Hevel and Marletta (1994). Freshly prepared PBS (pH 7.4), made with deionised water, was used for dilutions of serum samples and reagents. Serum samples were diluted 1:4 or 1:10 and 400 µl placed in Ultrafree-MC® centrifuge tubes (Millipore, Watford, UK) and filtered by centrifugation at 11,500 g for 30 minutes to remove proteins which may interfere with the assay. The assay was performed in flat-bottomed 96-well polystyrene microtitre plates (Flow Laboratories, U.K.). Fifty µl of standard, sodium nitrate (Sigma, UK; 0-50 µM), or serum filtrate sample were added to wells in duplicate. Fifty µl of PBS containing

nitrate reductase (final conc. 300 UL^{-1}; Boehringer Mannheim, East Sussex, UK) and β-

NADPH (final conc. 25 μM; Sigma, UK) was added to each well and left to incubate

for 3 hours at room temperature. Excess β-NADPH was then consumed by adding L-

glutamic dehydrogenase (final conc. 500 UL^{-1}; Sigma, UK), α-ketoglutaric acid (final

conc. 100 mM; Sigma, UK) and NH$_4$Cl (final conc. 4 mM; Sigma, UK) in 50 μl of PBS

and incubated at 37°C for 10 minutes. Having converted nitrate to nitrite, the Griess

reaction can be used to determine nitrite concentration: 50 μl of Griess reagent 1 (1%

sulphanilamide (Sigma, UK) in 5% concentrated phosphoric acid) and 50 μl of Griess

reagent 2 (0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma, UK) in water)

were added to each well and left for 10 minutes at room temperature for the colour

reaction to develop. The plates were then read using a plate reader (Titertek) with an

absorbance wavelength of 540 nm.

4.2.10 Effect of IL-12 on Plasma Corticosterone Secretion in Lewis and Fischer Rats

Murine rIL-12 (0.5 or 5 μg/rat; 0.2 ml) or sterile saline was administered i.p. to

LEW and F344 rats between 09:00 and 11:00 h. Animals were killed two hours later

and plasma prepared and stored as described above until assayed for corticosterone

content by RIA (IDS Ltd., U.K.).

4.2.11 Statistical Analysis

The Kruskal-Wallis test (a non-parametric ANOVA) was used to determine

overall differences between more than two experimental groups. Comparisons between

individual group means were determined using the non-parametric Mann-Whitney U-

test, with $p < 0.05$ accepted as a statistically significant difference.

4.3   Results

4.3.1   Effect of in vivo Administration of Interleukin-12 on the Development of Actively Induced EAE.

(i)   Pre-clinical mrIL-12 administration.

Following inoculation with MBP in CFA, F344 rats were injected with either saline (n = 8) or mrIL-12 (n = 8) on days 3, 5, 7, 9 and 11 p.i. No clinical signs of EAE had developed by day 15 p.i. and four animals from each group were culled for histopathological examination. Of the four remaining control animals, only one developed mild symptoms on days 18 - 20 p.i (maximum clinical score = 1; 15 g body weight loss over 6 days), whilst three of the four remaining mrIL-12 treated F344 rats displayed weight loss (7-20 g) over the same time period and developed clinical signs on day 16 p.i. (score 0.5-1 lasting 2-5 days).

LEW rats inoculated with MBP in CFA developed EAE with onset between 11 and 15 d.p.i. and severity of score 2-3. Injection of 3 µg mrIL-12 on days 5, 7, and 9 post inoculation resulted in earlier onset, prolonged duration and more severe disease relative to the control group (Table 4.3.1, Expt. 1). The moribund state observed in 5 out of 8 IL-12-treated rats necessitated that the animals were culled; the remaining 3 rats, which progressed to scores 2.5-3, recovered completely. LEW rats inoculated with CFA only and injected with saline or mrIL-12 on days 5, 7 and 9 p.i. did not display any clinical symptoms.

(ii)   Post-recovery mrIL-12 administration.

The ability of IL-12 to precipitate relapses following recovery from the initial bout of paralysis in EAE was tested up to 6 weeks after recovery. In an initial

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experiment 6 out of 11 rats which had completely recovered from active EAE (maximum clinical score=3) displayed signs of relapse ranging from weight loss and floppy tail (score 1) to complete hind limb paralysis (score 3) when injected on 3 alternate days with 3μg mrIL-12 immediately after recovery (days 17/19/21 p.i., score 0/1; Table 4.3.1, Expt. 1). Using the same treatment regime IL-12 induced relapses in 100% of a separate group of 8 rats, all of which displayed a second phase of partial or complete hind limb paralysis (Table 4.3.1, Expt. 2). The onset of the relapse was four days after recovery and disease duration (6 days) was similar to the primary bout of paralysis (Table 4.3.1). In one animal, a single injection of IL-12 was sufficient to produce a relapse of score 3. Control animals did not exhibit any signs of disease relapse. Interleukin-12 could induce a clinically apparent relapse up to one week post recovery, with 7/7 rats exhibiting clinical scores between 0.5 and 3 (Table 4.3.1, Expt. 3). By 2 weeks post recovery, cytokine administration resulted only in mild relapse in 2/9 rats (weight loss and clinical score of 0.5-1) whilst 6 weeks post recovery IL-12 administration was without effect.

4.3.2 Effect of in vivo Administration of Interleukin-12 on the Development of Clinical EAE in Lewis Rats Following Cell Transfer

(i)   Pre-clinical mrIL-12 administration.

In order to determine if IL-12 had a direct toxic effect on animals following the transfer of activated T-cells, two groups of Lewis rats were injected with 4×10^7 PPD-sensitised splenocytes and received either saline (n=7) or mrIL-12 (n=7) on days 0, 1 and 2 p.t.. Animals in both groups maintained normal weight gain and showed no sign of illness (i.e. alert, normal posture and gait and smooth fur) throughout the ten days

they were observed.

Following transfer of 4×10^7 MBP-sensitised splenocytes, Lewis rats were injected i.p. with either saline (n=8) or mrIL-12 (n=8) on days 0, 1 and 2 p.t.. Two rats from each group were killed prior to the onset of clinical signs, on day 3 p.t., for histological analysis of CNS tissue. All animals had lost weight by day 4 p.t. and clinical signs developed on day 5 p.t. although a marked increase in clinical scores of IL-12-treated rats was observed (individual scores, saline: 1, 1, 1, 1, 1.5, 2; IL-12: 2, 2.5, 2.5, 3, 4, 4). Indeed, the severity of EAE (score 4) necessitated that four IL-12-treated animals (two on day 5 p.t. and two on day 6 p.t.) were culled; tissue from saline-treated animals was obtained at the same time-points. Maximal clinical signs in the remaining two rats in both treatment groups were apparent on days 6 and 7 p.t. and animals made complete recovery by day 9 (saline-treated) or day 10 p.t. (IL-12-treated; Table 4.3.2).

(ii)   Post-recovery mrIL-12 administration.

Animals that were left to recover from EAE and then immediately treated with either saline, MBP or mrIL-12 on days 9, 11 and 13 p.t. did not re-develop clinical signs of EAE and rapidly regained weight lost during the initial phase of EAE, during the observation period of up to 10 days following the last IL-12 injection. However, 3/7 animals co-injected with MBP and mrIL-12 developed clinical signs (max. 1, 1.5 and 2) beginning 3-5 days following the last injection (Table 4.3.2). Four animals did not develop overt clinical signs but failed to regain weight at the same rate as animals in the other three treatment groups.

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Table 4.3.1   Effect of Pre-Clinical and Post-Recovery Administration of Interleukin-12 on Clinical Signs of Active EAE in Lewis Rats

Primary bout of disease					Relapse				
Treatment	Incidence	Onset[a]	Severity	Duration[c]	Treatment	Incidence	Onset[b]	Severity	Duration[c]
Experiment 1 EAE + saline (5, 7, 9 d.p.i.)	9/9	14 (11-15)	3 (2-3)	6.5 (6-7)	Experiment 1 -	-	-	-	-
EAE + IL-12 (5, 7, 9 d.p.i.)	11/11	12** (10-12)	3 (2.5-4)	9* (7-10)	-	-	-	-	-
EAE	11/11	13 (12-13)	3 (1.5-3)	6 (5-7)	recovery + IL-12 (17, 19, 21 d.p.i.)	6/11	5.5 (3-6)	1.5 (1-3)	5 (4-8)
Experiment 2 EAE	5/5	16 (14-18)	2.5 (2-3)	4 (3-7)	Experiment 2 recovery + saline (17, 19, 21 d.p.i.)	0/5	-	0	0
EAE	8/8	13 (12-13)	3	6 (3-6)	recovery + IL-12 (17, 19, 21 d.p.i.)	8/8	4 (3-5)	3** (2.5-3)	6** (5-7)
Experiment 3 EAE	3/3	17 (16-18)	2.5 (2-3)	3 (2-3)	Experiment 3 recovery + saline (26, 28, 30 d.p.i.)	0/3	-	0	0
EAE	7/7	14 (13-16)	3 (2.5-3)	5 (5-7)	recovery + IL-12 (26, 28, 30 d.p.i.)	7/7	7 (6-8)	2* (0.5-3)	5* (1-8)

Murine recombinant interleukin (IL)-12 (3 μg/rat) or saline (0.2 ml/rat) was administered intraperitoneally on various days post inoculation (d.p.i. as indicated) with MBP in CFA. Values represent the median (and range). *$p < 0.05$ and **$p < 0.01$ vs. saline control, Mann-Whitney U-test. [a]Onset - d.p.i.; [b]Onset - days after first IL-12 injection; [c]Duration - number of days clinical signs were observed.

Table 4.3.2   Clinical Data from Lewis Rats Administered Various Treatment Regimes Following Transfer of 4×10^7 MBP-Sensitised Splenocytes

	Primary bout of disease				**Relapse**				
Treatment (0, 1, 2 d.p.t.)	Incidence	Onset[a]	Severity	Duration[c]	Treatment (9,11,13 d.p.t.)	Incidence	Onset[b]	Severity	Duration[c]
EAE + saline	6/6	5	2.5 (1 - 3‡)	4		-	-	-	-
EAE + IL-12	6/6	5	4 (3 - 4)	5		-	-	-	-
EAE	9/10	5 (5 - 6)	2 (1.5 - 2.5)	3 (2 - 4)	recovery + saline	0/10	-	0	0
EAE	13/16	5	2 (1.5 - 3)	4 (2 - 5)	recovery + IL-12	0/16	-	0	0
EAE	7/8	5 (5 - 6)	2 (1.5 - 2)	3 (2 - 4)	recovery + MBP	0/8	-	0	0
EAE	7/8	5	2 (1 - 3)	3 (2 - 3)	recovery + IL-12 + MBP	3/8	8 (7 - 9)	1.5 (1 - 2)	†

Saline (0.2 ml/rat), murine recombinant interleukin (IL)-12 (3 µg/rat), myelin basic protein (MBP; 10 µg/rat) or mrIL-12 (3 µg/rat) plus MBP (10 µg/rat) were administered intraperitoneally on the indicated days post transfer (d.p.t.) of 4×10^7 MBP-sensitised splenocytes. Values represent median (and range). [a]Onset - d.p.t.; [b]Onset - days after first IL-12 injection; [c]Duration - number of days clinical signs were observed; ‡ two rats were culled on day 5 p.t. (for comparison of CNS tissue with IL-12-treated rats) with scores of 1 and 1.5; remaining animals developed scores of 2.5-3. † animals were culled when clinical signs were still present so exact duration is unknown.

4.3.3 Effect of in vitro Incubation of MBP-sensitised Splenocytes with Interleukin-12 on Development of Clinical EAE After Cell Transfer

Transfer of 4×10^7 MBP-reactive splenocytes to Fischer rats did not induce development of EAE. These animals, observed for up to 10 days post transfer, maintained normal weight gain and gait. However, of ten F344 rats administered cells that had been cultured in the presence of mrIL-12, six showed weight loss of between 5 and 15 grams on day 4 p.t. which was regained by day 5 p.t. (Figure 4.3.1 A); one animal that lost 15 grams did not regain weight and displayed a clinical score of 1 on days 5 and 6 pt.

LEW rats that received 4×10^7 MBP-sensitised splenocytes cultured in the presence or absence of mrIL-12 began to lose weight on day 4 p.t. which continued up to 7 d.p.t. (Figure 4.3.1 A). Clinical signs developed in all LEW rats administered MBP-sensitised splenocytes, beginning on day 5 p.t. and seven out of nine animals reached clinical scores ≥ 2 on day 6; by day 7 p.t. two out of seven animals had developed complete hind limb paralysis (score 3) and three out of seven had paralysis of one hind limb (score 2.5; Figure 4.3.1 B). Lewis rats that received 4×10^7 cells cultured with MBP and mrIL-12 developed severe EAE with earlier onset (3 out of 11 animals had developed clinical signs on day 4 p.t.; Figure 4.3.1 B). On day 5 p.t., 10 out of 11 animals had clinical scores ≥ 2 and this was significantly greater than the disease severity in control animals ($p < 0.01$, Mann-Whitney U-test). The increased severity of the disease was more apparent on day 6 and 7 p.t. when two animals on each day developed fatal paralysis (Figure 4.3.1 B).

Figure 4.3.1   Weight Changes and Clinical Scores of Lewis and Fischer Rats
Following Transfer of MBP-Sensitised Splenocytes Cultured *in
vitro* with Interleukin-12
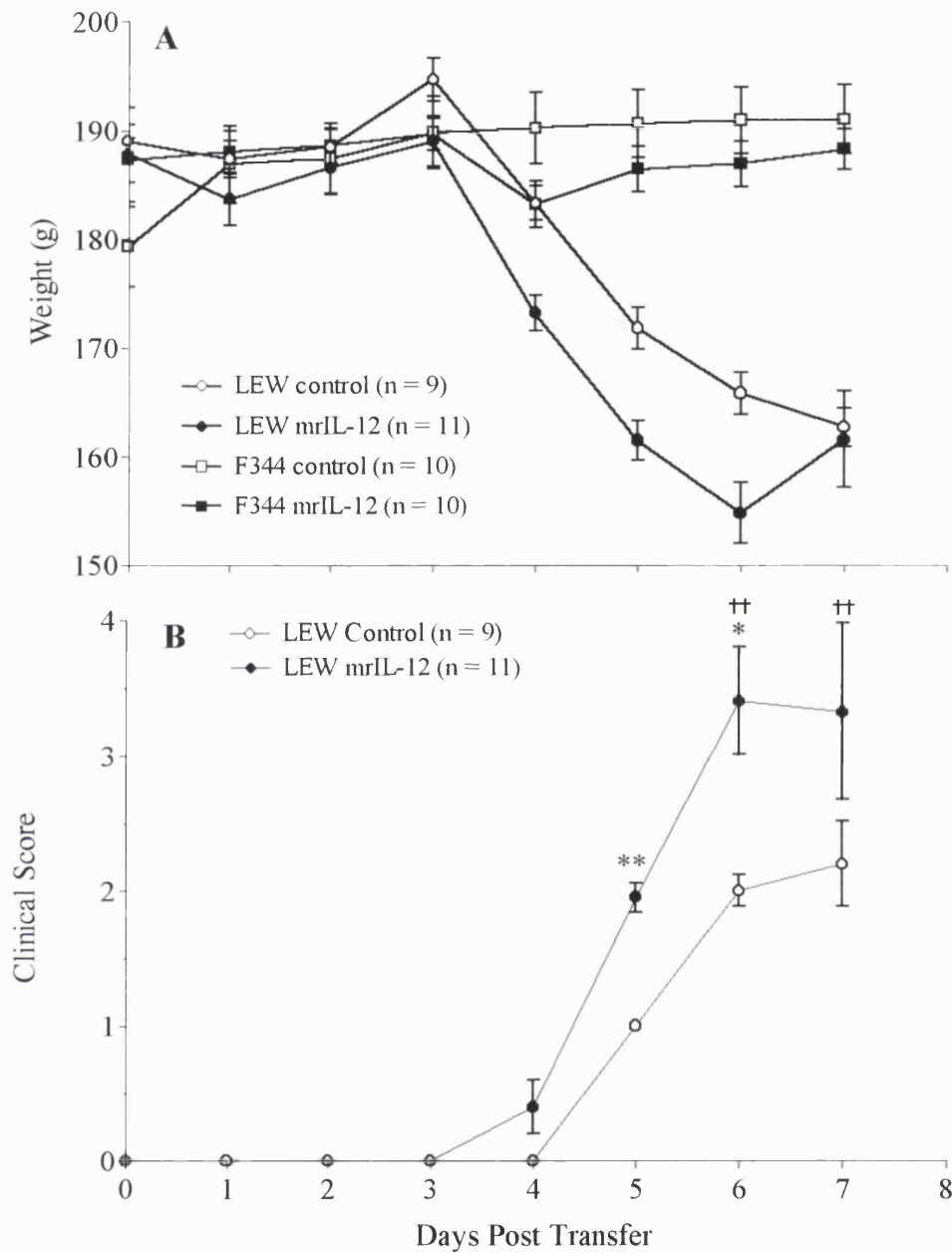
(A) Weight changes of Lewis (LEW) and Fischer (F344) rats and (B) clinical signs of
LEW rats following the transfer of 4×10^7 MBP-reactive splenocytes, cultured *in vitro*
in the absence (control) or presence of murine recombinant interleukin-12 (mrIL-12;
20 ng ml^{-1}). Values represent mean ± SE of 9-11 animals per group and are the result
of two separate experiments. *p<0.05; **p<0.01 vs. control. † indicates the number
of animals that died as a result of EAE.

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4.3.4   Immunopathological Changes in the Central Nervous System During EAE and the Effect of Interleukin-12.

Perivascular and parenchymal cellular infiltrates consisting of T cells (CD2$^+$) and macrophages (ED1$^+$) were observed during the course of EAE and persisted for at least two to three weeks post recovery from transfer and active EAE, respectively. Interleukin-12 treatment resulted in a marked accumulation of ED1$^+$ macrophages compared to T cells in perivascular cuffs and extensive activation of macrophages and parenchymal microglia as indicated by expression of inducible (type II) nitric oxide synthase (iNOS) immunoreactivity and morphological changes.   Control animals inoculated with CFA or injected with PPD-sensitised splenocytes and treated with either saline or IL-12 displayed no signs of CNS inflammation. Of the antibodies listed in table 4.1, immunoreactivity of some (anti-IL-12, anti-TNF–α, anti-IFN–γ) could not be detected in either CNS- or splenic-tissue sections taken from animals with peak clinical signs of EAE. Intercellular adhesion molecule (ICAM)-1 was expressed in CNS tissue sections but did not appear to be affected by mrIL-12 treatments and is therefore not discussed further in any detail.

(i)   <u>Administration of saline or mrIL-12 following active induction of EAE.</u>

A significant increase in histopathological score in brain stem sections from Lewis rats injected pre-clinically with mrIL-12 was observed, reflecting increased numbers of perivascular cuffs and increased parenchymal infiltration at pre- and peak-clinical symptoms relative to control animals (Table 4.3.3 A i, ii). This was also the case during the relapse induced by IL-12, administered either immediately on recovery from the primary bout of paralysis or beginning one week post recovery (Table 4.3.3 B, C).

Furthermore, the cellular composition of perivascular infiltrates at peak clinical signs of both primary disease and relapse was profoundly affected by IL-12 (Table 4.3.3). Thus, inflammatory cuffs in brainstem sections from animals injected with mrIL-12 had fewer T-lymphocytes ($CD2^+$) and increased numbers of macrophages ($ED1^+$) in contrast to control animals which had similar numbers of these cell types (Figure 4.3.2 A-D). Parenchymal microglia ($OX-42^+$) displayed an activated morphology, indicated by an enlarged, rounded cell body with thick, short processes in tissue from animals treated pre-clinically with IL-12 compared to the ramified morphology of microglia in EAE control animals (Figure 4.3.3 A, B). Inducible NOS immunoreactivity was rarely detected in tissue from any stage of EAE in control animals (Figure 4.3.3 C), however at peak clinical symptoms during both mrIL-12-exacerbated primary disease and mrIL-12-induced relapse many iNOS positive cells were present in 14-91% of perivascular infiltrates (Table 4.3.4 A, B, C). On the basis of morphological criteria, $iNOS^+$ cells were considered to be macrophages and microglia (Figure 4.3.3 D).

In tissue from animals with clinical relapse following treatment with IL-12 immediately post recovery, parenchymal microglia had a ramified morphology similar to control animals although large numbers of small round $OX-42^+$ cells (infiltrating macrophages) were observed in perivascular cuffs many of which were iNOS positive (Figure 4.3.6 K, L, O, P). Macrophage/microglial activation was less pronounced during relapse induced by IL-12 administered one week post recovery from initial disease, e.g. MHC class II (OX-6) staining was less intense and iNOS staining was more diffuse (not shown).

Whilst no inflammation was present in the control F344 rats culled on day 15 p.i., a few small lesions were observed in animals injected with mrIL-12

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Table 4.3.3   Effect of Interleukin-12 on CNS Pathology in Lewis Rats with Active EAE

(A) Saline or mrIL-12 administered on days 5, 7 and 9 post inoculation

Treatment	n	Hist. score	% T cells	% Mφ	% iNOS⁺
(i) preclinical (day 12 p.i.)					
EAE control	4	13 (0-28)	12 (0-48)	11 (0-42)	0
EAE + IL-12	3	69* (19-115)	27 (20-35)	68† (58-74)	32* (0-65)
(ii) clinical (day 12-14 p.i.)					
EAE control	3	102 (82-141)	52 (48-56)	43 (33-53)	0
EAE + IL-12	6	231* (172-291)	23* (17-27)	70*†† (59-72)	70* (28-91)

(B) Saline or mrIL-12 administered on days 17, 19 and 21 post inoculation

Treatment	n	Hist. score	% T cells	% Mφ	% iNOS⁺
(iii) relapse (day 23-24 p.i.)					
EAE control	5	117 (75-163)	54 (40-61)	· 52 (45-58)	0
EAE + IL-12	5	264* (132-355)	22* (18-38)	75*†† (69-81)	51** (14-75)

(C) Saline or mrIL-12 administered on days 26, 28 and 30 post inoculation

Treatment	n	Hist. score	% T cells	% Mφ	% iNOS⁺
(iv) relapse (day 34 p.i.)					
EAE control	3	79 (40-126)	52 (10-83)	29 (4-47)	0
EAE + IL-12	2	226 (203-251)	41 (22-52)	53 (32-76)	36 (29-42)

Lewis rats were immunised with MBP and injected i.p. with either saline (EAE control) or IL-12 (EAE + IL-12) either pre-clinically (A), immediately following recovery from the primary bout of disease (B) or one week post recovery (C). (i) - (iv) indicates the time at which the tissue was taken for immunocytochemical/histological analysis.

Histopathological (Hist.) score was calculated as described in section 4.2.8 and is the mean of three sections for each animal, stained with haematoxylin & eosin. The number of T cells (CD2⁺) in three inflammatory cuffs/animal was counted and expressed as a percentage of the total number of haematoxylin stained nuclei within each cuff (%T cells). The process was repeated in the same cuffs in a serial section stained for macrophages (Mφ, ED1⁺). The number of inflammatory cuffs with inducible nitric oxide synthase immunoreactive cells (iNOS⁺) in a single brainstem section is expressed as a percentage of the total number of cuffs in the section.

Values in the table represent the mean (and range) with n values for each group shown. *$p < 0.05$ and **$p < 0.01$ vs. EAE control; †$p < 0.05$ and ††$p < 0.01$ vs. T cells, Mann-Whitney U-test.

Figure 4.3.2 <u>Immunopathological Changes in the CNS of Lewis Rats Administered Interleukin-12 Following Induction of Active EAE</u>

CONTROL (Score 3)   +IL-12 *in vivo* (Score 3)
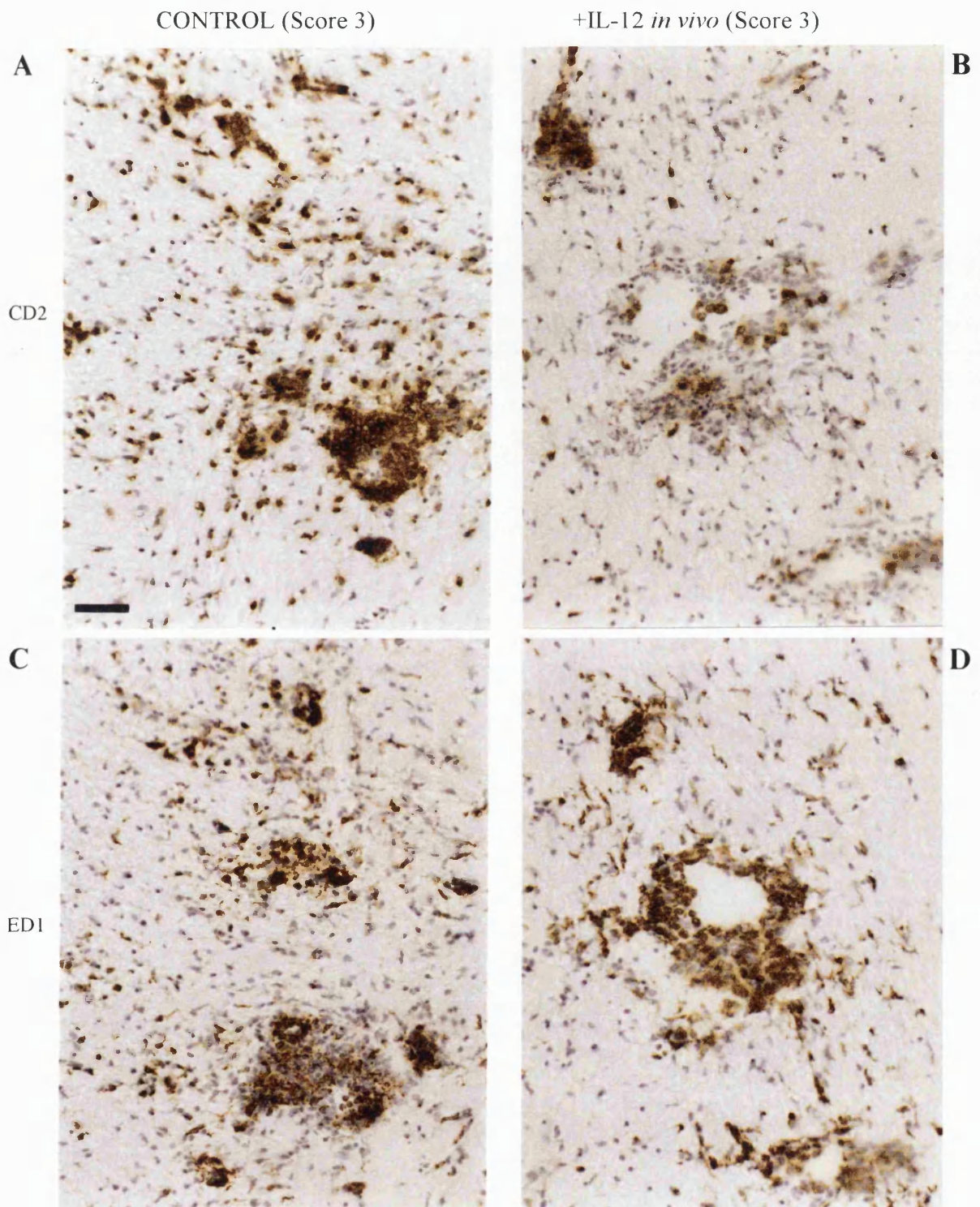
Brainstem sections from Lewis rats administered saline or mrIL-12 (3 µg/rat) on days 5, 7 and 9 post inoculation with MBP-CFA. At peak clinical signs of EAE, inflammatory cuffs of IL-12-treated animals contained few T cells (CD2; B) and many macrophages (ED1, Mϕ; D) whilst similar numbers of T cells and Mϕ were seen tissue from saline treated controls (A, C). Scale bar (A-D) = 50µm.

Figure 4.3.3 <u>Macrophage/Microglial Activation by IL-12 in Lewis Rats with Active EAE</u>

CONTROL   + IL-12 *in vivo*
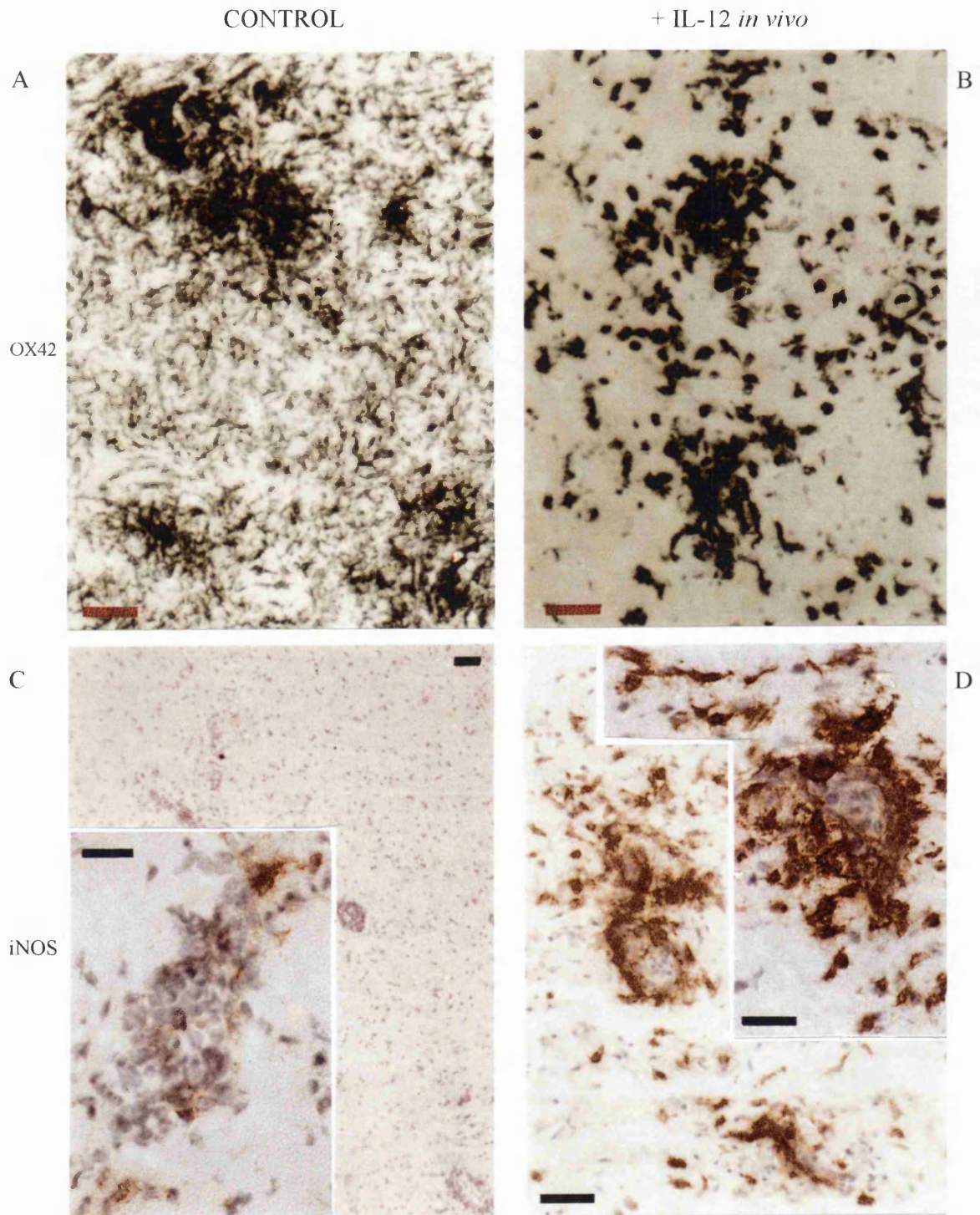
Tissue from animals described in Figure 4.3.2. Parenchymal OX-42$^+$ microglia (MG) in tissue from IL-12 treated animals (B) have an activated morphology of rounded cell bodies and retracted processes compared to saline treated animals with EAE (A). Widespread immunoreactivity for inducible nitric oxide synthase (iNOS) was detected in tissue from IL-12 treated animals (D) in cells identified morphologically as Mφ/MG (D inset) compared to the almost complete absence of iNOS staining in tissue from animals with EAE (C). Scale bar (A-D) = 50μm, (C, D insets) = 25μm.

(histopathological score 4-7) and this was associated with faint staining of MHC II on parenchymal microglia (not shown). Interestingly, inflammation was observed in all animals culled on day 21 p.i.; in saline treated animals a greater histopathological score was associated with the presence of clinical signs whilst in IL-12 treated rats a similar histopathological score was seen in two animals although clinical signs were only observed in one (Table 4.3.3). In contrast to LEW rats, T lymphocytes appear to dominate in perivascular lesions in both saline and mrIL-12 treated F344 rats (Table 4.3.4; Figure 4.3.4 A-D). MHC class II (OX-6) expression was similar in both groups and parenchymal microglia had a resting morphology (Figure 4.3.5 A-D).

(ii)   Administration of saline or mrIL-12 following transfer of MBP-sensitised splenocytes.

Evidence of immune activity within the CNS was apparent by day 3 p.t., before clinical signs had developed, in animals injected with mrIL-12 following transfer of MBP-sensitised cells: staining of MHC class II molecule was observed on (infiltrating) cells around some blood vessels and a few parenchymal microglia (not shown).

In contrast to *in vivo* mrIL-12 treatment during actively induced EAE the histopathological score at peak clinical signs was similar in animals injected with saline or mrIL-12 although the predominance of macrophages over T cells in perivascular cuffs (Table 4.3.5 A) and increased parenchymal macrophage/ microglia staining compared to saline treated animals was apparent (Figure 4.3.6 A, B, E, F). Expression of iNOS was confined to cells in one perivascular cuff at peak signs of EAE in saline treated animals whilst 62% of cuffs were iNOS$^+$ in mrIL-12 treated animals (Table 4.3.5 A; Figure 4.3.6 D, H).

Table 4.3.4   CNS Immunopathology Associated with Mild or No Clinical Signs of Active EAE in Fischer Rats

Treatment	Maximum clinical score	Histopathological score	% T cells	% Mφ
	0	6	54	14
Saline	0	8	63	28
	1	94	67	26
	0	5	68	33
	0.5	8	59	20
IL-12	0	35	62	26
	1	29	58	37
	1	7	49	38

Fischer rats were inoculated with MBP in CFA and injected intraperitoneally with mrIL-12 or saline 3, 5, 7, 9 and 11 days post inoculation (d.p.i.). Animals were culled 21 d.p.i. for analysis of inflammation in the brainstem. Histopathological score was calculated as described in section 4.2.8 and is the mean of two sections used for immunocytochemistry (no primary antibody and ICAM-1). %T cells and % Mφ (macrophages) is the mean of two or three cuffs in serial sections stained with CD2 (T cells) and ED1 (Mφ).

Figure 4.3.4 <u>CNS Immunopathology Associated with Mild Clinical Signs of EAE in</u>
<u>Fischer rats</u>

CONTROL   +IL-12 *in vivo*
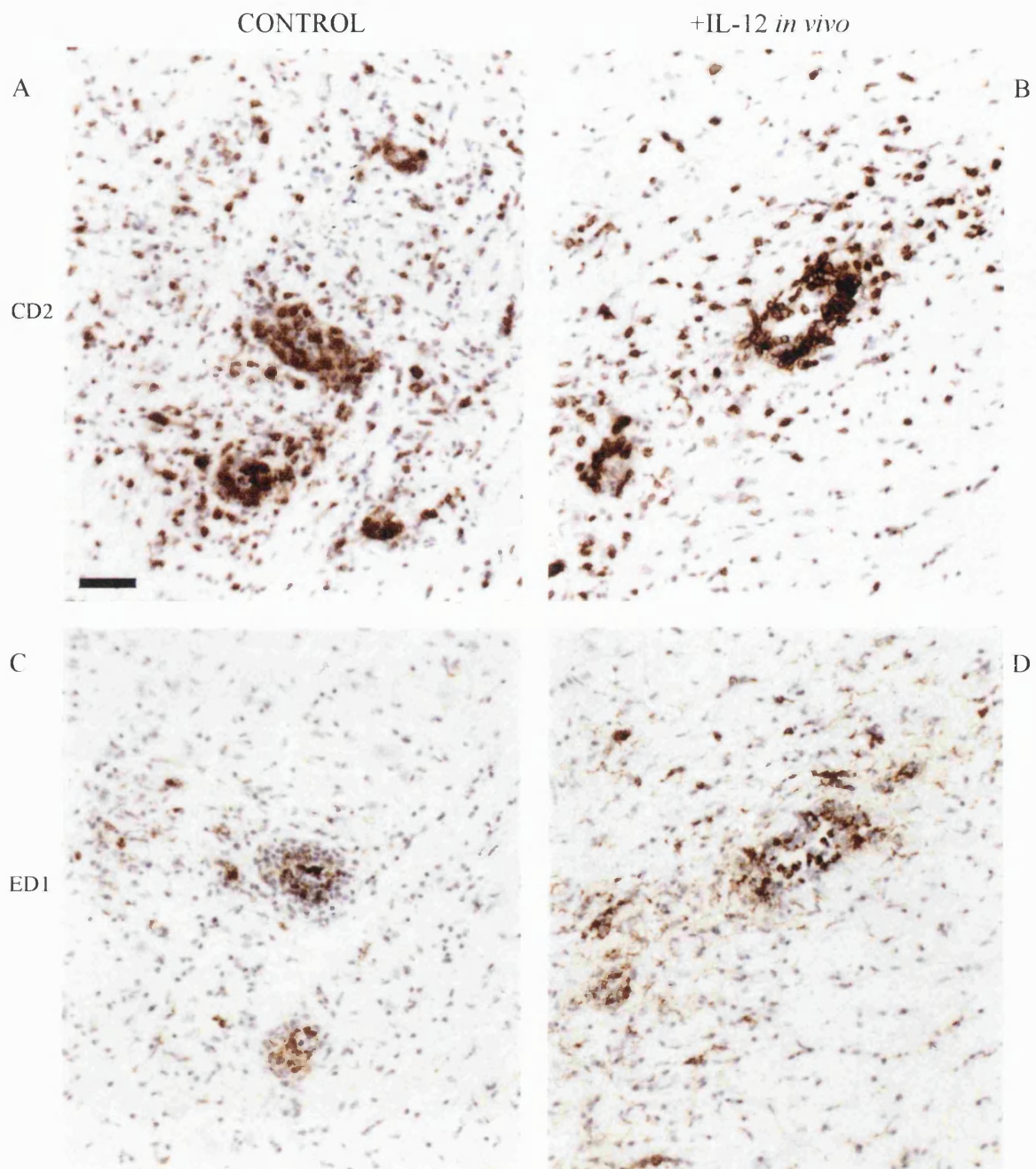
Brainstem sections from Fischer rats that developed clinical EAE (max. score of 1)
following inoculation with MBP-CFA (control) and administered mrIL-12 (3µg/rat) on
days 3, 5, 7, 9 and 11 post inoculation (p.i.; + IL-12 *in vivo*). Tissue taken on day 21 p.i.
when clinical signs were no longer apparent. T lymphocytes (CD2, A, B) are abundant
while few macrophages (ED1, C, D) are observed. Scale bar (A-D) = 50µm.

Figure 4.3.5 <u>CNS Immunopathology Associated with Mild Clinical Signs of EAE in Fischer rats</u>
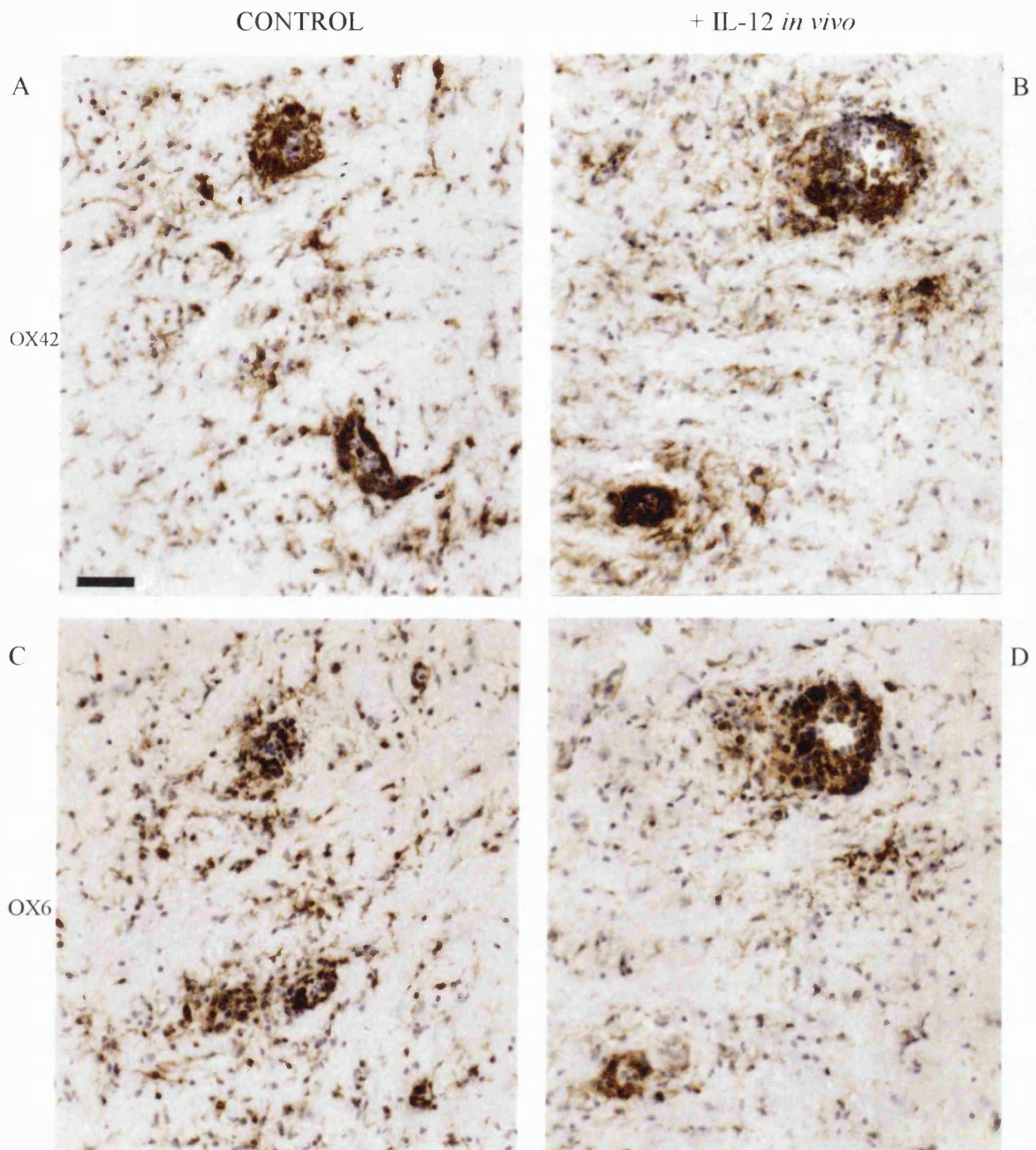
Tissue from same animals as described in Figure 4.3.4. Administration of mrIL-12 does not appear to have increased the activation of parenchymal microglia (OX-42; A, B) as both morphology and MHC class II expression (OX-6; C, D) are similar in control and mrIL-12 treated animals. Scale bar = 50μm.

Administration of either saline, mrIL-12 or MBP following recovery from transfer EAE did not induce relapse of clinical signs although immunocytochemical analysis of CNS tissue taken on day 18 or 19 p.t. revealed inflammatory lesions were still present consisting of varying numbers of T cells and macrophages (Table 4.3.5 B; Figure 4.3.6 I, J) and similar levels of MHC class II molecule expression in all treatment groups. However, animals in which clinical relapse was induced following co-administration of MBP and mrIL-12 had a higher histopathological score reflecting greater number of inflammatory foci (Table 4.3.5 B) containing large numbers of T cells and macrophages (Figure 4.3.6 I, J, M, N). Parenchymal OX-42$^+$ microglia did not show an activated morphology following IL-12 or IL-12 and MBP administration in contrast to changes seen following pre-clinical administration of IL-12. However, many round OX-42$^+$ cells were observed in perivascular cuffs of animals with relapse and iNOS immunoreactivity was associated with these cells (as was seen during IL-12-induced relapse following active induction of EAE).

(iii)   Transfer of MBP-sensitised splenocytes incubated ± mrIL-12 *in vitro.*

Immunocytochemical analysis was performed on brainstem sections obtained from LEW and F344 rats on day 6 or 7 post transfer (peak signs of disease in LEW rats). No infiltrating cells were apparent in tissue taken from F344 rats. In LEW rats, fewer and smaller inflammatory foci in animals given cells incubated in the presence of mrIL-12 were observed despite the increased severity of clinical disease in these animals (Table 4.3.5 C). The T cell and macrophage composition of brainstem perivascular infiltrates from LEW rats given cells incubated with mrIL-12 was the same as following *in vivo* IL-12 treatment, with few T cells and increased numbers of macrophages

Table 4.3.5 Effect of *in vitro* and *in vivo* IL-12 Treatment on CNS Pathology of Adoptively Transferred EAE in Lewis rats

(A) *In vivo* administration on days 0, 1 and 2 post transfer

Treatment	n	Hist. score	% T cells	% Mφ	% iNOS⁺
EAE + saline	4	59 (46-76)	65 (59-69)	41 (36-51)	0
EAE + IL-12	4	55 (47-59)	48* (35-62)	65**† (56-71)	62* (45-75)

(B) *In vivo* administration on days 9, 11 and 13 post transfer

Treatment	n	Hist. score	% T cells	% Mφ	% iNOS⁺
EAE + saline	3	25‡ (16-40)	56 (49-62)	55 (51-61)	0
EAE + IL-12	3	37‡ (9-63)	72 (61-83)	36 (22-60)	3 (0-6)
EAE + MBP	3	15‡ (9-23)	38 (22-56)	44 (37-50)	0
EAE + IL-12 and MBP	3	86‡ (79-100)	62 (31-58)	47 (24-66)	50* (43-54)

(C) Cells cultured ± mrIL-12 *in vitro*

Treatment	n	Hist. score	% T cells	% Mφ	% iNOS⁺
EAE control	6	63 (41-86)	69 (50-79)	45 (39-58)	0
EAE + IL-12	5	14 (3-25)	16** (6-22)	69**†† (63-74)	60* (36-69)

Interleukin-12 (3 μg/rat) was administered to Lewis rats following transfer of 4x10^7 MBP-sensitised splenocytes either pre-clinically (A) or immediately following recovery from the primary bout of disease (B). (C) Cells were cultured in the presence of MBP and mrIL-12 (20 ng ml^{-1}; EAE + IL-12) or absence of IL-12 (EAE control) prior to transfer.

Histopathological (Hist.) score was calculated as described in section 4.2.8 and is the mean of three sections for each animal, stained with haematoxylin & eosin. The number of T cells (CD2$^+$) in three inflammatory cuffs/animal was counted and expressed as a percentage of the total number of haematoxylin stained nuclei within each cuff (%T cells). The process was repeated in the same cuffs in a serial section stained for macrophages (Mφ, ED1$^+$). The number of inflammatory cuffs with inducible nitric oxide synthase immunoreactive cells (iNOS$^+$) in a single brainstem section is expressed as a percentage of the total number of cuffs in the section.

‡ no significant difference between groups (p=0.069, Kruskal-Wallis test). *p≤0.05 and **p<0.01 vs. respective control; †p<0.05 and ††p<0.01 vs. T cells (Mann-Whitney U-test).

Figure 4.3.6 <u>Summary of Immunopathological Changes in the CNS of Lewis Rats with EAE Following IL-12 Treatment</u>

Immunopathological changes in the CNS of Lewis rats following both active induction or adoptive transfer of EAE and either *in vitro* or *in vivo* treatment with IL-12 were very similar. Exacerbation of primary (1°) disease by IL-12 was associated with increased macrophage (Mφ; ED1) and decreased T cell (CD2) accumulation in perivascular cuffs (E, F) compared to controls (A, B), and Mφ/microglial (MG) activation indicated by morphological changes (C, G) and iNOS immunoreactivity (D, H). T cells and, to a lesser extent, Mφ persisted in the CNS of rats following recovery from EAE (I, J) and in animals with clinical relapse precipitated by IL-12 (plus MBP for transfer EAE; M, N). Microglial morphology was similar in animals with relapse and controls (K, O) although increased activation of Mφ/MG in animals with relapse was evident from the expression of iNOS immunoreactivity (L, P).

A, B, D, E, F, H: 4×10^7 MBP-sensitised splenocytes; saline or IL-12 injected on days 0, 1 and 2 post transfer.

I, J, M, N: 4×10^7 MBP-sensitised splenocytes; saline or IL-12 + MBP injected on days 9, 11 and 13 post transfer.

C, G: 4×10^7 MBP-sensitised splenocytes cultured +/- IL-12 in vitro.

K, L, O, P: Active EAE; saline or IL-12 injected on days 17, 19 and 21 post inoculation.

C, G: scale bar (shown in C) = 100 μm; all others: scale bar (shown in A) = 50 μm.

Figure 4.3.6   <u>Summary of Immunopathological Changes in the CNS of Lewis Rats with EAE Following IL-12 Treatment</u>

T cells (CD2+)   Mφ/MG (ED1+)   MG/Mφ (OX-42+)   iNOS

1° Disease

EAE control

EAE + IL-12

Relapse

EAE control (no relapse)

EAE + IL-12
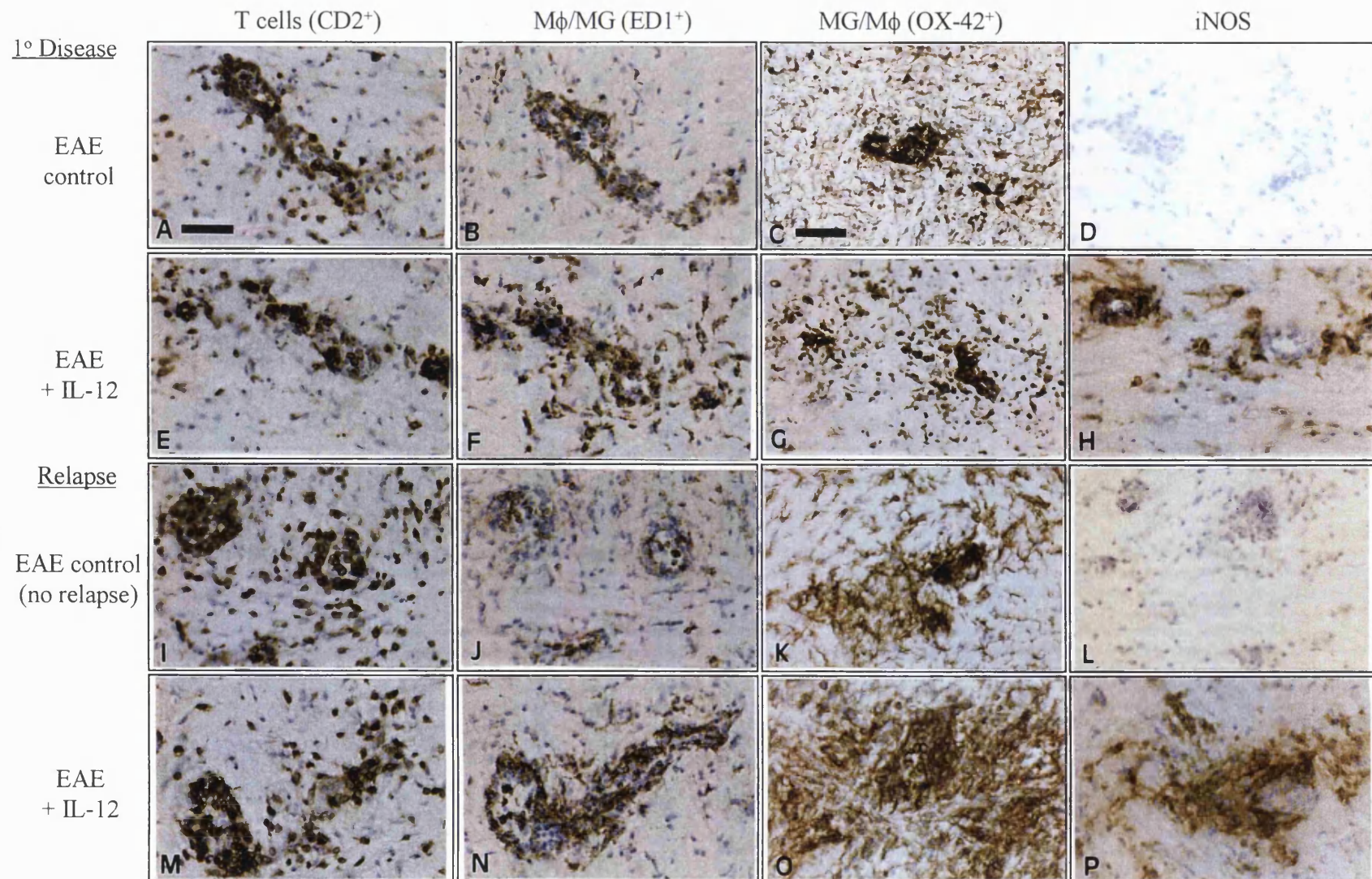
compared to controls (Table 4.3.3 A). Microglial cells (OX-42⁺) also displayed an activated morphology in sections from animals given mrIL-12-treated cells with more retracted processes and an enlarged, rounded cell body (Figure 4.3.6 C, G). Inducible NOS staining was not detected in sections from control animals at the peak of clinical EAE whereas 60% of cuffs in brainstem sections from animals given mrIL-12-treated cells were positive for iNOS immunoreactivity (Table 4.3.5 C).

4.3.5 Serum Nitrate (NO_3^-) Plus Nitrite (NO_2^-) and Plasma Corticosterone Concentrations During the Course of EAE.

Nitrite and nitrate represent end products of nitric oxide production and their concentrations in biological fluids are useful markers for the activity of nitric oxide synthases.

(i)   Active EAE ± mrIL-12

Serum levels of NO_3^- plus NO_2^- during the course of active EAE were not significantly different from non-injected control rats (p=0.419, Kruskal-Wallis test; Table 4.3.6 A). Administration of mrIL-12 on days 5, 7 and 9 following induction of active EAE in LEW rats resulted in a significant elevation of serum NO_3^- plus NO_2^- levels on days 12-13 compared to saline-treated animals with similar clinical scores (p<0.05, Mann-Whitney U test), which returned to control values following recovery (Table 4.3.6 A). Relapse induced by administration of mrIL-12 either immediately or one week following recovery from the initial disease episode was not associated with increased serum NO_3^- plus NO_2^- at the time of peak clinical scores (Table 4.3.6 B, C).

Plasma corticosterone concentration was significantly increased in both control and mrIL-12 treated LEW rats at peak clinical signs compared to non-injected control

rats (Table 4.3.6 A) and similar levels were found at peak clinical signs of relapse induced by IL-12 administered immediately post recovery (Table 4.3.6 B).

Following active induction of EAE in F344 rats and injection of either saline or mrIL-12 on days 3, 5, 7, 9 and 11 p.i., plasma was obtained prior to the onset of clinical signs (13 d.p.i.) and following recovery from mild clinical signs (or at the same time-point as recovered animals for those that did not develop clinical signs, 21 d.p.i.). No significant difference was observed in plasma CS concentration between the groups (ng ml^{-1}, n=4/group): (13 d.p.i.) saline 152±7; IL-12 160±7; (21 d.p.i.) saline 161±22; IL-12 224±44 (p=0.152, Kruskal-Wallis test).

(ii)   Transfer EAE ± mrIL-12 *in vivo*

Only two animals were taken at each time-point so no statistical analysis has been performed on the data. No variation in serum nitrate plus nitrite levels was observed in LEW rats administered saline following transfer of either MBP- or PPD-sensitised cells. Administration of mrIL-12 resulted in greater serum NO_3^- plus NO_2^- levels than saline controls on day 3 p.t. of MBP- or PPD-sensitised cells and on day 5 p.t. of MBP-sensitised cells (comparable with levels observed during active EAE + IL-12). By day 6 p.t. serum NO_3^- plus NO_2^- levels had fallen to basal levels even though animals had clinical scores of 4 (Table 4.3.7).

Similar plasma corticosterone levels were determined in samples obtained from saline and IL-12 treated animals injected with either PPD- or MBP-stimulated cells. Due to the small number of animals at each time point it is difficult to describe temporal changes in corticosterone production during the course of EAE; values are shown in table 4.3.7.

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(iii)   <u>Transfer EAE ± mrIL-12</u> *in vitro*

Serum was not obtained from these animals so NO_3^- plus NO_2^- levels could not be determined. Plasma corticosterone levels were only measured in surviving LEW rats at peak clinical signs of EAE (day 6 or 7 p.t.) and were found to be (ng ml^{-1}) 272±68 in controls (n=6) and 1184±156 in animals injected with IL-12 treated cells (n=3).

4.3.6   Acute Effect of Intraperitoneal Administration of mrIL-12 on Plasma Corticosterone Concentration

In contrast to a marked increase in plasma corticosterone secretion measured two hours following i.p. administration of mrIL-1β, no acute stimulatory effect of mrIL-12 on plasma corticosterone secretion was observed in either LEW or F344 rats (Figure 4.3.7).

Table 4.3.6   Serum Nitrate Plus Nitrite Concentration and Plasma Corticosterone Concentration in Lewis Rats Following Administration of Saline or IL-12 During the Course of Active EAE

(A) Saline or mrIL-12 administered on days 5, 7 and 9 post inoculation

Treatment	n	DPI	Clinical score	$NO_3^- + NO_2^-$ (μM)	CORT (ng ml^{-1})
normal controls	5	-	-	40 ± 6	72 ± 9
EAE + saline	4	12	0-1	57 ± 12	103 ± 11
	3	14	3	47 ± 2	188 ± 40[†]
	5	21-22	rec.	52 ± 5	100 ± 10
EAE + IL-12	3	12	0-1	194 ± 5*	90 ± 15
	6	12-13	3-4	188 ± 14*	159 ± 18[†]
	3	21-22	rec.	78 ± 12	108 ± 15
CFA + saline	3	16	-	87 ± 16	110 ± 7
CFA + IL-12	3	16	-	94 ± 9	75 ± 10

(B) Saline or mrIL-12 administered on days 17, 19 and 21 post inoculation

Treatment	n	DPI	1° clinical score	relapse score	score when culled	$NO_3^- + NO_2^-$ (μM)	CORT (ng ml^{-1})
EAE + saline	5	23-24	2-3	0	0	67 ± 8	82 ± 7
EAE + IL-12	5	23-24	3	2.5-3	2.5-3	85 ± 6	183 ± 39*
	8	28-29	3	1-3	0	82 ± 3	95 ± 23

(C) Saline or mrIL-12 administered on days 26, 28 and 30 post inoculation

Treatment	n	DPI	1° clinical score	relapse score	score when culled	$NO_3^- + NO_2^-$ (μM)	CORT (ng ml^{-1})
EAE + saline	2	34	2-3	0	0	87, 44	N.D.
EAE + IL-12	2	34	3	2-3	2-3	59, 70	N.D.
	3	36	2.5-3	2-3	1.5-2	68 ± 6	N.D.
	2	40	3	0.5	0	54, 55	N.D.

Lewis rats were immunised with MBP in complete Freund's adjuvant (CFA) or CFA alone and injected with either saline or mrIL-12 on various days post inoculation (DPI). Serum nitrate (NO_3^-) plus nitrite (NO_2^-) concentration (μM) and plasma corticosterone concentration (CORT, ng ml^{-1}) were determined following collection of trunk blood on various DPI during the primary (1°) disease episode (A) or during relapse induced by IL-12 administration beginning either immediately post recovery from 1° disease (B) or one week post recovery (C). rec. indicates rats recovered from EAE. N.D. means not done. $NO_3^- + NO_2^-$ and CORT concentrations are presented as mean ± SE for groups of n ≥ 3 or individual values where n = 2. *$p<0.05$ vs. respective saline control and [†]$p<0.05$ vs. normal control (Mann-Whitney U-test).

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Table 4.3.7   Serum Nitrate Plus Nitrite Concentration and Plasma Corticosterone Concentration in Lewis Rats Administered Either Saline or IL-12 Following Transfer of MBP- or PPD-Sensitised Splenocytes

| | Days Post Transfer | | | | | | | | | | | |
| | 3 | | | 5 | | | 6 | | | 10 | | |
Treatment	Clin. score	NO$_3^-$ + NO$_2^-$ (μM)	CORT (ng ml^{-1})	Clin. score	NO$_3^-$ + NO$_2^-$ (μM)	CORT (ng ml^{-1})	Clin. score	NO$_3^-$ + NO$_2^-$ (μM)	CORT (ng ml^{-1})	Clin. score	NO$_3^-$ + NO$_2^-$ (μM)	CORT (ng ml^{-1})
PPD + saline	ncs	63, 32	174, 166	ncs	33, 24	149, 212	N.D.	N.D.	N.D.	ncs	N.D.	103, 162
PPD + IL-12	ncs	126, 152	84, 121	ncs	8, 27	188, 208	N.D.	N.D.	N.D.	ncs	12, 44	134, 135
MBP + saline	0, 0	31, 36	144, 219	1, 1.5	37, 31	133, 61	3, 3	35, 24	175, 187	0r, 0r	35, 58	75, 112
MBP + IL-12	0, 0	193, 175	205, 246	4, 4	207, 201	198, 248	4, 4	54, 45	416, 221	0r, 0r	33, 22	176, 116

Lewis rats were injected with either saline or mrIL-12 on days 0, 1 and 2 following transfer of 4x10^7 MBP- or PPD-sensitised splenocytes. Serum nitrate (NO$_3^-$) plus nitrite (NO$_2^-$) concentration (μM) and plasma corticosterone concentration (CORT, ng ml^{-1}) were determined following collection of trunk blood on various days post transfer. EAE clinical scores (clin. score), NO$_3^-$ + NO$_2^-$ and CORT concentrations are presented as individual values where n = 2. 0r indicates rats recovered from EAE. N.D. means not done. Animals that received PPD-sensitised cells developed no clinical signs (ncs).

Figure 4.3.7 Acute Effect of Intraperitoneal Administration of mrIL-12 on Plasma Corticosterone Concentration in Lewis and Fischer Rats
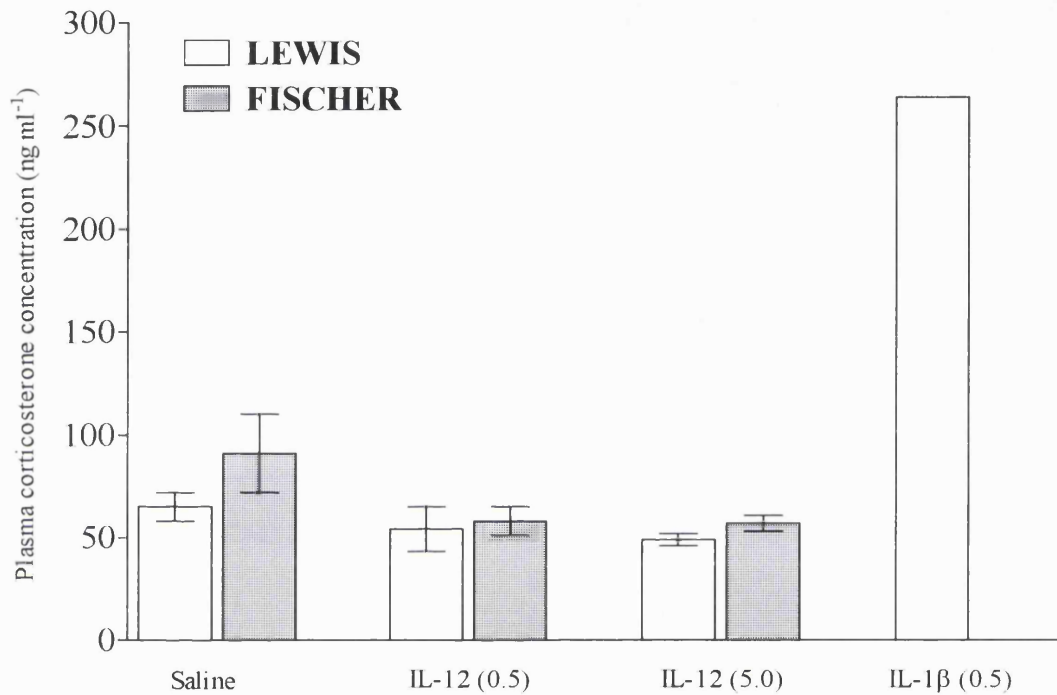
Plasma corticosterone concentration of Lewis and Fischer rats collected two hours post intraperitoneal (i.p.) injection of either saline or murine recombinant (mr) interleukin (IL)-12 (0.5 or 5 μg/rat). Bars represent mean ± SE of 5 animals per group. At the same time two Lewis rats were administered mrIL-1β (0.5 μg/rat), plasma collected two hours later and included in the same assay as plasma from mrIL-12 treated animals.

4.4 Discussion

The data presented in this chapter show that clinical signs of EAE in LEW rats were exacerbated following transfer of MBP-sensitised spleen cells exposed to mrIL-12 during *in vitro* stimulation with MBP and following *in vivo* administration of mrIL-12 after induction of active or adoptive transfer EAE, consistent with previously reported studies using adoptive transfer of PLP-sensitised LNC in SJL/J mice (Leonard, Waldburger & Goldman, 1995; Waldburger *et al.*, 1996). In F344 rats, the incidence but not the severity of actively induced EAE was increased following administration of IL-12. Clinical relapse could be induced in LEW rats by IL-12 administration, beginning up to 1 week post recovery from active EAE; co-administration of antigen (MBP) with IL-12 precipitated relapse in some LEW rats when administered immediately following recovery from transfer EAE. The enhanced severity of primary disease and the induction of relapse by IL-12 was associated with increased numbers of ED1$^+$ macrophages in perivascular cuffs, activation of parenchymal microglia and expression of inducible nitric oxide synthase immunoreactivity by these cells.

4.4.1 Resistance of Fischer Rats to Experimental Allergic Encephalomyelitis

In this study only one F344 rat developed EAE following active immunisation with MBP in CFA and disease severity was considerably reduced compared to LEW rats. This suggests that F344 rats are capable of mounting a Th1-type response but the T cells have compromised encephalitogenic capacity (e.g. decreased IFN–γ/TNF production) or that the corticosteroid response limits Th1 generation and prevents development of full blown disease. The former appears to be the case in B10.S mice since resistance to EAE is overcome following *in vitro* stimulation of syngenic MBP-

sensitised lymphocytes with IL-12 prior to transfer, resulting in enhanced IFN-γ production (Segal & Shevach, 1996). The increased incidence of mild disease following injection of mrIL-12 to MBP-immunised F344 rats indicates the Th1 initiator population can be expanded yet is unable to induce paralytic disease. The importance of an endogenous regulatory mechanism in F344 rats which prevents EAE expression is highlighted by the observation that IL-12-treated MBP-sensitised splenocytes transferred to LEW rats precipitate severe, often fatal, EAE whereas in F344 rats only mild clinical symptoms, if any, are apparent. The development of severe, fatal EAE in ADX F344 rats induced by adoptive transfer (chapter III), comparable to that seen in LEW rats after transfer of IL-12-stimulated MBP-sensitised T cells, strongly suggests that adrenal corticosterone is the key to resistance in the F344 rat.

Plasma was only obtained from F344 rats at two time-points (13 and 21 d.p.i.) following active induction of EAE and CS concentration was similar on each occasion in both saline- and IL-12-treated groups. The CS concentration in F344 rats was as high as that observed at the peak clinical signs of EAE in LEW rats and it would be very interesting to determine the temporal profile of circulating corticosteroid levels in F344 rats to see if larger increases occur following antigenic challenge or whether the levels observed in this study are sufficient to limit the induction of disease.

4.4.2   Central Nervous System Pathology and Clinical Symptoms of EAE

Histological evaluation of CNS tissue from LEW rats taken at peak clinical signs of transfer EAE revealed fewer or similar numbers of perivascular cuffs in the IL-12-treated groups compared to controls. In particular, LEW rats that received cells cultured *in vitro* with mrIL-12 had relatively few small perivascular cuffs whereas

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control animals had numerous cuffs, many with parenchymal infiltration, reflected in the large difference in histopathological score which takes account of both number and size of inflammatory cuffs. Given the earlier onset of disease in animals administered IL-12-treated cells it is possible that the transferred cells were targeted to the CNS more rapidly resulting in earlier development of CNS lesions which were beginning to resolve at the time the tissue was obtained; analysis of tissue taken at earlier time points of exacerbated disease is required to clarify this unexpected finding. Administration of mrIL-12 following induction of active EAE in LEW rats resulted in a significant increase in CNS lesion load both pre-clinically and at peak clinical signs of disease compared to control animals. However, the number and size of inflammatory lesions may not necessarily correlate with the severity of clinical symptoms, since more severe disease in rats treated with IL-12 *in vivo* following cell transfer was associated with similar histopathological scores to control animals and CNS inflammation was observed in F344 rats which displayed mild or no clinical signs. In saline treated F344 rats, the greatest histopathological score was observed in the animal which developed clinical EAE and this was comparable to inflammation seen in LEW rats. In addition, of two IL-12-treated F344 rats with similar histopathological scores clinical signs were only observed in one.

The cellular composition and 'activity' of the cells within CNS inflammatory foci may be of greater importance in determining clinical symptoms. Immunocytochemical analysis of brainstem sections from LEW rats at peak clinical signs of EAE showed a decrease in the proportion of T cells ($CD2^+$) and an increase in the proportion of macrophages ($ED1^+$) in perivascular cuffs and activated microglia in the parenchyma following *in vitro* and *in vivo* mrIL-12 treatments compared to controls. The proportion

of ED1$^+$ cells in perivascular cuffs and in the parenchyma was lower than that of T cells in both control and IL-12-treated F344 rats, in stark contrast to pathological changes seen in LEW rats. Thus, the mild clinical signs observed in F344 rats may be the result of reduced infiltration of macrophages into the CNS. Indeed, despite extensive CNS inflammation only mild clinical signs are observed in LEW rats following transfer of MOG- or S100β-specific T cells which is believed to be due to the reduced infiltration of macrophages (Linington et al., 1993; Kojima et al., 1994) and depletion of macrophages suppresses the development of EAE (Brosnan, Bornstein & Bloom, 1981; Huitinga et al., 1990) indicating a pivotal role of phagocytic cells in mediating pathophysiological events in EAE. Several explanations for the observed predominance of macrophages over T cells in perivascular cuffs following IL-12-induced exacerbation in LEW rats are possible: there may be fewer non-specific T cells attracted to the inflammatory site or more rapid loss of T cells from the CNS (either by migration or apoptosis) than is normally observed. Alternatively, macrophages may be preferentially recruited perhaps in response to altered adhesion molecule or chemokine profiles or proliferation of Mϕ/MG in the CNS may be enhanced; indeed IL-12 is known to increase the production of GM-CSF, a potent inducer of microglial proliferation (Lee et al., 1994); current and proposed work is aimed at examining these possibilities.

Microglia were inferred to be activated based on morphological features such as short, thick processes and a rounded cell body (Streit, Graeber & Kreutzberg, 1989) and expression of the antigen recognised by the monoclonal antibody ED1 (Damoiseaux et al., 1994), while both macrophages and microglia expressed iNOS. Increased numbers of macrophages in perivascular cuffs and coincident microglial activation would contribute to disease exacerbation since both cell types are a major source of

inflammatory mediators and proteases (Bauer *et al.*, 1996).

4.4.3 Exacerbation and Relapse of EAE Induced by Interleukin-12

Whilst the aggravation of clinical signs of EAE in LEW rats treated with IL-12 confirmed previous reports of enhanced clinical severity in a mouse model of EAE (Leonard, Waldburger & Goldman, 1995; Waldburger *et al.*, 1996), a significant finding of this study was the ability of IL-12 to induce clinical relapse in LEW rats up to one week following complete recovery from actively induced EAE. The rapid recurrence of clinical disease suggests that the cytokine may reactivate residual inflammatory T-cells and macrophages in the CNS; however in transfer EAE, in which CNS lesions and MHC class II expression also persist following recovery, IL-12 administration did not induce relapse. This suggests that in active EAE the peripheral depot of MBP may be necessary for IL-12 to initiate an encephalitogenic Th1 cell response from a pool of previously MBP-sensitised cells which subsequently trigger a secondary wave of cellular infiltration and disease relapse. The absence of IL-12-induced relapse following transfer EAE may be accounted for by the lack of antigen in the periphery, especially in view of the finding that mild relapse could be provoked if MBP was administered (i.p.) simultaneously with IL-12. As recovery from EAE is associated with the apoptotic deletion of T cells in the CNS (Schmied *et al.*, 1993; Chapter III) the lack of a residual cohort of encephalitogenic T cells may preclude the induction of relapse by IL-12 even if antigen presentation is occurring in the CNS. After the cessation of IL-12 treatment, the secondary bout of disease is probably regulated in much the same way as the primary disease, through increased circulating CS, production of Th2 cytokines and induction of apoptosis in the CNS (MacPhee, Antoni & Mason, 1989; Kennedy *et al.*, 1992; Schmied

et al., 1993). The ability of IL-12 to induce relapse up to 1 week but not 2-6 weeks after recovery from active EAE is in agreement with previous reports that describe an interval of up to 30 days after immunisation during which mild relapses may occur spontaneously or after reimmunisation with MBP-CFA or soluble MBP (McFarlin, Blank & Kibler, 1974; MacPhee & Mason, 1990). After this period, Lewis rats are refractory to disease reinduction (Willenborg, 1979; MacPhee & Mason, 1990) which may be attributed to active immunosuppression mediated by CD4$^+$ T cells (Killen & Swanborg, 1982; Karpus & Swanborg, 1991). Thus IL-12 was able to overcome the early but not established post-recovery suppressor cell response, perhaps as a result of the diminuation of priming antigen in the periphery.

Whilst IL-12 appeared to have no acute stimulatory effect on plasma corticosterone concentration in either LEW or F344 rats it is unlikely that the increased severity of EAE and relapse induced by IL-12 administration in LEW rats was the result of a suppressed corticosterone response. In fact, plasma CS levels at the peak of clinical signs following *in vivo* administration of IL-12 during both active and transfer EAE in LEW rats were similar to saline-treated rats. Furthermore, following transfer of cells that had been cultured with IL-12 *in vitro* to LEW rats, plasma CS levels were exceptionally high (the samples were re-assayed to confirm the initial results) suggesting that the IL-12-primed cells were secreting increased levels of cytokines capable of activating the HPA axis (e.g. TNF-α) although the increased severity of disease (and fatal paralysis in some) suggests that this was not sufficient to counteract the enhanced encephalitogenic capacity of the transferred cells.

Induction of relapses in a monophasic EAE model in mice has been achieved using the bacterial superantigen, staphylococcal enterotoxin B (SEB), and the cytokine

TNF–α (Brocke *et al.*, 1993; Crisi *et al.*, 1995). The incidence of relapses induced by SEB was reduced by administration of transforming growth factor (TGF)–β2 while administration of IL-10 prevented relapses induced by TNF–α (Crisi *et al.*, 1995) suggesting that, like the primary episode of EAE, relapse is induced by a Th1-mediated response. The mechanism of IL-12-induced exacerbation of initial disease and relapse has not been investigated but would appear to be similar in both paradigms as identical pathological changes were observed in the CNS during relapse as in exacerbation of EAE following *in vitro* and *in vivo* IL-12 treatment.

As IL-12 is a potent stimulator of IFN–γ production it might be predicted that the downstream effects of this cytokine are responsible for the exacerbation and relapse of EAE. However, although stimulation of LNC *in vitro* with PLP and mrIL-12 resulted in increased production of IFN–γ and TNF–α, addition to the culture medium of a neutralising antibody to either IFN–γ or TNF–α failed to prevent the exacerbation of clinical signs upon subsequent transfer (Leonard, Waldburger & Goldman, 1995) suggesting IL-12 may directly effect T cell encephalitogenicity independently of IFN–γ and TNF–α or that production of these cytokines in the absence of the antibody occurs following cell transfer. In addition, preliminary data obtained in Balb/c mice lacking the gene encoding IFN–γ suggest that EAE can be enhanced by in vivo administration of IL-12 (J. Leonard, personal communication) supporting an IFN-γ-independent mechanism of exacerbation of EAE by IL-12. Interestingly, LNC from C57BL/6 IFN-γ-knockout mice were found to produce greater amounts of GM-CSF in response to IL-12 compared with cells from both IL-12-treated wild-type mice and untreated IFN-γ-knockout controls (Leonard *et al.*, 1997) which might be responsible for the

exacerbation of disease both in the absence and presence of IFN-γ and may contribute to the increase in the number of activated macrophages and microglia in the inflammatory lesions observed during IL-12-induced exacerbation and relapse observed in this chapter.

Another possible mediator of the aggravation and relapse of clinical signs of EAE induced by IL-12 is NO, the production of which is stimulated by the Th1-type cytokines IFN-γ, TNF-α and IL-1 either alone or in combination (Murphy et al., 1993; Taub & Cox, 1995). Nitric oxide is rapidly oxidised to nitrite (NO_2^-) and nitrate (NO_3^-), both of which can be measured in several biological fluids as an indication of NO production (Johnson et al., 1995). While others have demonstrated iNOS immunoreactivity in the CNS and increased serum levels of NO_3^- plus NO_2^- during the course of active EAE in Lewis rats (Van Dam et al., 1995; S. Ruuls, PhD thesis) the present study has failed to confirm this. Serum nitrate/nitrite levels reported by Ruuls reached a peak of approximately 60 μM at the height of clinical signs on day 15 p.i. which is similar to the concentration determined in the experiments performed in this chapter. However, the basal (day 0 p.i.) and the early disease phase (score 1, 12 d.p.i.) concentrations were lower (25-30 μM) than in the present study (e.g. 57±12 μM, 12 d.p.i., score 0-1) and thus day 15 values were found to be significantly elevated. Whether these concentrations of nitrate plus nitrite are clinically relevant is unknown; similar concentrations were reported in serum from MS patients and were significantly greater than normal control subjects although this was not related to disability (Giovannoni et al., 1997a).

In rats injected with mrIL-12 following active induction or passive transfer of EAE serum NO_3^- plus NO_2^- concentration was significantly elevated prior to the onset of

clinical signs and at peak clinical signs levels. However, serum levels of NO_3^- plus NO_2^- do not appear to correlate with the disease activity, since rats given PPD-sensitised cells and injected with IL-12 also had raised serum nitrate/nitrite levels with no outward appearance of illness whilst 6 d.p.t. of MBP-sensitised cells serum nitrate/nitrite had returned to basal levels even though animals displayed clinical scores of 4. It is likely that the increased production of NO within the CNS is more important in determining disease severity and in this study iNOS immunoreactivity was only detected in CNS tissue from IL-12 treated LEW rats during both primary disease exacerbation and relapse. This was surprising given the findings from a number of other workers of iNOS immunoreactive cells in inflammatory infiltrates during EAE in Lewis rats (Van Dam *et al.*, 1995; Zhao *et al.*, 1996; S. Ruuls, PhD thesis). The ability of aminoguanidine (a selective inhibitor of iNOS) to ameliorate clinical signs of EAE (Cross *et al.*, 1994; Zhao *et al.*, 1996) suggests that NO contributes to the clinical signs of EAE. However, two other inhibitors of NOS (N^{ω}-nitro-L-arginine-methylester and N^{G}-monomethyl-L-arginine) augmented the clinical signs of EAE suggesting NO may play an immunoregulatory role in EAE (Ruuls *et al.*, 1996). Further experiments are proposed to determine the role of NO in the clinical exacerbation of EAE and relapse induced by IL-12 by treating animals with NOS inhibitors at various stages following induction of active EAE.

It is tempting to speculate that SEB-induced relapses might be mediated by the production of IL-12, given the ability of many infectious agents to induce IL-12 production and develop Th1-type immune responses (Gazzinelli, 1996; Hsieh *et al.*, 1993). There is much interest in attempting to link infectious agents/superantigens with autoimmunity; although there is little evidence to suggest that infectious agents cause

MS, relapses are frequently associated with viral infections (Sibley *et al.*, 1985). It is enticing to hypothesise that the triggering effect is mediated by IL-12 although other cytokines, notably IFN-γ which caused exacerbation of clinical signs and high relapse rate in MS patients (Panitch et al., 1987), cannot be excluded. In this context, it would be pertinent to examine the ability of known inducers of IL-12 (e.g. fixed *Staphylococcus aureus* or LPS) to aggravate EAE and elicit relapses in animals following recovery from EAE.

The finding of increased expression of mRNA for IL-12 in the CNS of Lewis rats prior to the onset of clinical signs and in acute MS plaques from cases of short duration suggest this cytokine may play a role in initiating the early events in MS and EAE, resulting in conditions which stimulate T cell activation and induction of Th1-type immune responses (Issazadeh *et al.*, 1995a; Windhagen *et al.*, 1995). Furthermore, increased production of IL-12 has been demonstrated in PBMC from patients with progressive MS suggesting this cytokine may also be involved in the elaboration of chronic disease (Balashov *et al.*, 1997).

The data in this chapter confirm that IL-12 administration exacerbates the primary clinical episode of EAE and demonstrate that a cytokine with the potential to induce IFN-γ production and promote Th1-type responses can induce a relapse of clinical signs of EAE in rats normally refractory to re-induction of disease. By conjecture, IL-12 may contribute to macrophage-mediated disease exacerbation and relapse in patients with MS and represents a novel therapeutic target for treatment of this inflammatory demyelinating disease.

Chapter V

General Conclusions

In multiple sclerosis the presence and persistence of immunopathology in the form of $CD4^+$ T cells and macrophages in CNS lesions and the intermittent reactivation of the immune system are suggestive of an imbalance between autoaggressive Th1-type and immunoregulatory Th2-type responses as demonstrated in EAE. Thus factors which affect the Th1:Th2 ratio could have an important bearing on the development and progression of MS. A growing body of evidence suggests that interactions between the neuroendocrine and immune systems are important in this respect (Smith & Hewson, 1998) and the data presented in this thesis has confirmed that the HPA axis plays a significant role in determining resistance to and recovery from EAE.

An increase in circulating corticosterone levels is evident during the clinical phase of EAE, associated with resolution of neurological symptoms. Activation of the HPA axis during EAE may well be due to the increased production of cytokines, such as IL-1 and TNF, both centrally and peripherally. Indeed, peripheral administration of IL-1β, TNF-α and LPS (a known inducer of both cytokines) resulted in marked increases in plasma corticosterone concentration which was much greater in EAE-resistant rats. The hypothalamus is probably the prime site of action for cytokines released following immune activation as elevated corticosterone levels were associated with increased PGE_2 and cAMP accumulation in dialysates from the hypothalamic paraventricular nucleus and inhibition of hypothalamic second messenger production prevented the adrenocortical response, at least acutely. Further investigation of the role of specific cytokines and second messenger pathways involved in stimulation of the HPA axis in Lewis and Fischer rats following chronic immune activation (e.g. EAE) may lead to a more thorough understanding of the mechanisms controlling the activity of the HPA axis with

implications for susceptibility to autoimmune diseases in humans.

The importance of the adrenocortical response in determining resistance to EAE induction and recovery was highlighted by the development of severe and often fatal EAE in adrenalectomised rats of both strains. Recovery from EAE was associated with increased numbers of apoptotic T cells in the CNS and increased plasma corticosterone levels whilst few apoptotic T cells were detected in the CNS of adrenalectomised rats. This suggests that corticosterone-driven apoptosis may represent one of the acute anti-inflammatory effects of glucocorticoids that contribute to disease resistance and recovery.

Administration of IL-12, a potent inducer of Th1-type immune responses, failed to induce any gross neurological deficit in Fischer rats, again highlighting the overriding regulatory influence of corticosterone in determining resistance to EAE in this rat strain. In contrast, IL-12 administration caused exacerbation of clinical signs of EAE in Lewis rats and overcame the post-recovery refractory state to precipitate full paralytic relapse. This was associated with an increased accumulation of macrophages in the CNS and activation of microglia, manifest as morphological changes and immunoreactivity for iNOS, implicating nitric oxide as a direct effector mechanism. Future studies are planned to investigate electrophysiological correlates of white matter damage with NO production during the course of IL-12-induced exacerbation and relapse of EAE.

The work presented in this thesis highlights the interactions between the HPA axis and the immune system, which have profound implications for the progression of clinical signs of EAE. Whilst animal models have limitations and some of the concepts applied may be a gross oversimplification of the situation in humans (e.g. Th1/Th2 paradigm; hypo-/hyper-responsive HPA axis), they offer a valuable means to unravelling the cellular mechanisms which underlie autoimmune disease.

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