Neural and Endocrine Regulation of in vivo Splenic Immune Function in the Rat

By Jonathan C. Meltzer

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree of

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Department of Human Anatomy and Cell Science

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Abstract

Stress is defined as any alteration in homeostasis. Although stress can significantly impact on health and disease, the mechanisms remain elusive. It is known that the efferent response to any stress is mediated primarily by the hypothalamus through the sympathetic nervous system (SNS) and the hypothalamic pituitary adrenal axis (HPAA), and that these systems primarily inhibit immune function. However few studies have addressed these complex interactions in vivo. The goals of this thesis are to determine the in vivo contribution of the HPAA and SNS in regulating splenic cytokine production in response to bacterial lipopolysaccharide (LPS); and to establish how this relationship is altered during psychological stress. Adult rats were left intact or given combinations of sham surgeries, splenic nerve cuts, and adrenalectomies (ADX) and allowed to recover for 7-10 days. At various intervals following i.v. LPS injections, cytokines were measured by Northern blotting and ELISAs. HPAA activity was indexed by plasma corticosterone levels and SNS activity was indexed by splenic and plasma catecholamine levels. Dose response studies established that maximum levels of splenic cytokines were induced in response to 1 μg of LPS while maximal changes in plasma corticosterone, plasma epinephrine and splenic norepinephrine were observed in response to 1, 10 and 100 μg of LPS respectively. Significantly, the 0.1μg dose of LPS-induced substantial cytokine levels without activating the above systems. In the second experiment, minimal changes in LPS-induced splenic cytokine levels were observed in

response to ADX, splenic nerve cut, or a combination of the two procedures. In the third experiments this paradigm was repeated in animals that were exposed to 15 minutes of 1.6 mA intermittent footshock directly following the injection of 0.1μg of LPS. Although footshock was immunosuppressive to most indices of cytokine production, neither splenic nerve cut nor ADX individually abrogated the effects of stress on splenic immune function. However the combination of these two manipulations significantly abrogated the immunosuppressive effects of stress on cytokine production. These results indicate that the effects of stress on immune function are mediated by the HPAA and the SNS.

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Abbreviations

6OHDA-	6-hydroxydopamine
A1/A2/A6-	noradrenergic cell groups in medulla
ACTH-	adrenocorticotropin releasing factor
ADX-	adrenalectomy
AH-	anterior hypothalamus
ANOVA-	analysis of variance
ANS-	autonomic nervous system
AP-	area postrema
AP-1-	transcription factor containing c-fos
BNST-	bed nucleus of the stria terminalis
C1/C2/C6-	adrenergic cell groups in medulla
CeA-	central nucleus of the amygdala
CGRP-	calcitonin gene related peptide
CMI-	cell-mediated immunity
CNS-	central nervous system
COX-	cyclooxygenase
CREB-	cAMP response element binding protein
CRF-	corticotrophin releasing factor
CVO-	circumventricular organ
DEPC-	diethylpyrocarbonate (RNase inhibitor)
DMH-	dorsomedial nucleus of the hypothalamus
DTH-	delayed type hypersensitivity
E-	epinephrine
ELISA-	enzyme linked immunosorbent assay
ENS-	enteric nervous system
EP1-6-	prostaglandin receptors
FADD-	fas associated death domain protein
GAPDH-	glyceraldehyde 3-phosphate dehydrogenase
GR-	glucocorticoid receptor type 2
HIV-	human immunodifficiency virus
HPAA-	hypothalamic pituitary adrenal axis
HYPOX-	hypophysectomy
i.a.-	intraarterial
i.c.v.-	intercerebroventricular
i.m.	intramuscular
i.p.-	interperitoneal
i.v.-	intravenous
ICE-	interleukin-1 beta converting enzyme

IFNγ-	interferon gamma
IKB-	I kappa beta (NFKB inhibitor)
IL-1	interleukin-1 beta
IL-1RA-	interleukin-1 receptor antagonist
IL-2-	interleukin-2
IL-6-	interleukin-6
IL-10-	interleukin-10
IL-12-	interleukin-12
IL-15-	interleukin-15
IML-	intermediolateral cell column of the spinal cord
iNOS-	inducible nitric oxide synthase
LH-	lateral hypothalamus
lps-	genetic locus for the LPS response
LPS-	lipopolysaccharide or endotoxin
LSA-	lateral septal area
MAPK-	mitogen activated protein kinase
ME-	median eminence
MPOA-	medial preoptic area
MR-	mineralocorticoid receptor or glucocorticoid receptor type 1
MSH-	alpha melanocyte stimulating hormone
NE-	norepinephrine
NFKB-	nuclear factor kappa beta (transcription factor)
NK-	natural killer (cell)
NOS-	nitric oxide synthase
NPY-	neuropeptide Y
NTS-	nucleus of the solitary tract
OVLT-	vascular organ of the lamina terminalis
OXY-	oxytocin
PFC-	plaque forming cell assay
PGE2-	prostaglandin E2
PNS-	parasympathetic nervous system
POA-	preoptic area
POMC-	proopiomelanocortin
PRL-	prolactin
PVN-	paraventricular nucleus of the hypothalamus
RIA-	radioimmunoassay
rRNA-	ribosomal RNA subunit
SCID-	severe combined immunodifficiency
SCO-	subcommisural organ
SFO-	subfornical organ

SIRS-	systemic inflammatory response syndrome
SNS-	sympathetic nervous system
SON-	supraoptic nucleus
SRBC-	sheep red blood cells
TACE-	TNF alpha converting enzyme
TNF-	tumor necrosis factor alpha
TRADD-	TNF receptor associated death domain protein
TRAF-	TNF receptor associated factor
VIP-	vasoactive intestinal peptide
VMH-	ventromedial nucleus of the hypothalamus
VP-	vasopressin

1. Review of the literature

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1.1. Introduction: organization of homeostatic processes

Homeostasis, as described by Walter J. Cannon in the 1930s is the ability of an organism to ensure survival by maintaining a consistent internal environment or steady state (reviewed in [1,2]). The response of an organism to alterations of its external or internal environments (or the perception thereof) is referred to as the stress response [3]. The stress response includes a coordination of metabolic, physiological and behavioral alterations designed to nullify the change(s) imposed by the stressor. These responses are necessary for survival and are coordinated by the hypothalamus [1]. Some examples of these responses include shivering for heat generation, thirst when water is required, fever and malaise in response to illness and an enhanced state of alertness when a predator is observed. The reaction to stress is beneficial to an organism (coping) when it serves to preserve survival of the organism. However when the reaction to stress far exceeds the initial insult, the stress response can be detrimental to the organism's survival [3]. Cannon realized that if the organism could not deal with the stress, secondary irrelevant effects would occur [2]. This is evident even today as individuals under chronic stress have the potential to suffer physiological and psychological health problems more often than non-stressed individuals [4-8] .

Cannon contended that the autonomic nervous system (ANS) regulated the

response to stress. The ANS is composed of sympathetic (SNS), parasympathetic (PNS) and enteric components (ENS) where the SNS and PNS were the primary systems responsible for maintaining homeostasis [1]. The SNS is primarily responsible for initiating "flight or fight responses" (such as fleeing from a predator) via the release of blood borne substances such as catecholamines (norepinephrine (NE) or epinephrine (E)) from the adrenal medulla and sympathetic nerve endings. These mediators immediately increase heart rate, dilate pupils, constrict cutaneous vascular beds (shunting blood to the internal organs and muscles) and increase blood sugar levels to ensure the greatest potential of fight or flight [9]. The antithesis of this system is the PNS which acts in "rest and digest" situations [1]. The balanced opposition between these two systems ensures the most efficient energy expenditure possible depending on the situation. For example digestion becomes a low survival priority when being chased by a predator as the systems required for fight of flight take precedence.

In 1936 Hans Selye described the General Adaptation Syndrome [10]. This syndrome demonstrated that irrespective of the type of stressor, a common set of responses would be elicited by an organism in order to maintain homeostasis. Sustained activation of this response resulted in peptic ulcers, thymic involution and eosinopenia [11]. The main effector of the General Adaptation syndrome was the release of glucocorticoids (corticosterone) from the adrenal cortex which was regulated by the hypothalamus via the pituitary gland (this system is referred to as the hypothalamic-pituitary-adrenal axis or HPAA). Later, in 1976, Selye modified his description of the

General Adaptation Syndrome by recognizing that the organism's response to stress was not identical in magnitude for all stressors, but similar systems would be engaged for different stressors [11]. Selye also observed that the same stressor may elicit different responses in different individual organisms probably due to genetic and environmental factors.

This concept of coordinated response to stress was recently extrapolated on by I.J Kopin, and colleagues who looked at corticosterone and catecholamine release in response to various stressors [2,12]. They found that these same output systems were activated in response to all stressors including cardiovascular stress (hemorrhage), inflammatory stress (formalin), temperature stress (cold exposure), psychological stress (immobilization) and metabolic stress (insulin injection). However the magnitude and relative proportions of the final mediators differed between stressors. For example cold stress-induced higher levels of NE relative to E, but the absolute levels of both molecules were lower in comparison with other stressors. Insulin injections, which produce hypoglycemia [13], induced more E relative to NE and more E relative to all other stressors. Therefore although there were common consequences of stress irrespective of the stressor, differences occurred based on the nature of the stressor.

Besides the physical nature of the stressor, intensity, controllability and perception may impact on the organism's response and ability to cope with the stressor [14-17]. For example a certain amount of exercise may be welcome by some individuals, while for others it is clearly a psychological stressor [18]. Although adaptation can occur

in response to chronic stresses, this adaptation may produce altered responses to other stressors [18-20] or cause dysregulation in other systems which may result in illness [5,21-27].

In support of Cannon, Selye, and Kopin, the literature supports a coordinated response of the SNS and adrenal cortex (via the hypothalamus) to various other stressors in addition to the above mentioned challenges. These include, but are not limited to physical stress such as acoustic stimuli [28], exercise [18] and ether [29], environmental stress such as cage switching [30] or handling [12], and inflammatory/immune stimuli such as bacterial endotoxin [31,32], cytokines [33,34] , turpentine [35] and viruses [33,34,36,37]. Whether these stresses are psychological, inflammatory, metabolic or physical, they all have the ability to signal the same output systems. However the pathways by which these challenges activate this system may differ with respect to anatomy and physiology [29,38-42] .

Based on recent literature to be reviewed below, the host's response to inflammatory stress activates the same central systems and peripheral compensatory responses as other stressors. Subsequently these compensatory responses modify (usually inhibit) the inflammatory reaction. These pathways are present in many animal models and are located in phylogenetically ancient brain systems suggesting these responses are an integral part of survival. The characterization of these pathways and relationships in response to an inflammatory stress is in its infancy.

The primary objective of this thesis is to characterize the role of the nervous

system in the in vivo response of an organism to inflammatory stress. Traditional immunology strictly delineates between inflammation and immunity where immunity is antigen specific and requires B and T cells and inflammation does not. However given the holistic perspective of neuroimmunologists, that many of the same cells are involved in both types of responses, and that the brain's reaction to both types of challenges are similar, such strict semantic delineation will not be made in this document. Therefore the immune system or immunity refers to any cell of immune origin involved in either a specific or innate response.

This thesis will summarize the current understanding of the neural and neuroendocrine interactions with the immune system and will describe experiments aimed at expanding our understanding of the neural-immune regulatory system in response to an inflammatory agent, lipopolysaccharide (LPS). As well, these experiments will test the effects of psychological stress on the modulation of the inflammatory response as an insight to how non immune stressors impact on health and disease. The importance of studying the neural modulation of inflammation is based on the fact that inflammation is an integral component of the host's response to injury and disease and is intimately linked with the adaptive immune response [43,44]. The inflammatory response is a critical first line of defence against pathogens, yet when the inflammatory reaction is uncontrolled, it can cause as much or more damage to the host than the initial stimulus [45,46]. Therefore the mechanisms by which the host regulates the response to an inflammatory stress gives critical insight as into the central mechanisms responsible for

the maintenance of homeostasis.

1.2. Evidence of brain-immune interactions; bidirectional communication between the nervous, endocrine and immune systems.

Empirically, it has been known for centuries that moods and stress can influence health. However it was not until the 20th century that morphological and physiological evidence for these observations were obtained. In the 1930s Cannon [1] and Selye [10] described the stress response in terms of efferent output from the SNS and adrenal cortex respectively. In the late 1960s G.F. Solomon proved experimentally that stress altered aspects of immune function, inferring that the efferent systems which were activated during stress, may also alter immune function [26,27,47]. However it wasn't until the 1970s when Hugo Besedovsky demonstrated that the SNS and the adrenal gland were directly capable of immune regulation [48]. This unambiguously illustrated that the same systems responsible for adaptation to physical and perceived stressors also mediated the response to immune-related stressors. Significantly, Besedovsky also proved that peripheral immune responses could alter brain catecholamine levels [49,50] in areas (the hypothalamus) that controlled the SNS and HPAA; showing that this relationship was bidirectional.

The ability of the brain and central nervous system to influence immune function has since been based on many lines of evidence. In the early 1980s T.L. Roszman's lab

[51-53] demonstrated that lesions in the central nervous system altered immune function. Secondly, many other investigators demonstrated that specific neuropeptides and neurotransmitters when injected into the brain, activated the SNS and HPAA and altered immune function [54]. Thirdly it was demonstrated that like other neurally-mediated responses, the immune system could be classically conditioned (reviewed in [55]). As previously mentioned, Solomon demonstrated that neurally mediated responses, such as the response to psychological stressors, could alter immune function [26,27]. Subsequently Keller and colleagues demonstrated that it was the HPAA axis and SNS that were directly responsible for this phenomenon [56-58]. Anatomical evidence linking the SNS and the immune system was first provided by D.L. Felten who discovered that all immune organs are innervated [59-64] while Nance and Burns demonstrated that the nerve supply to most immune organs was entirely sympathetic [65]. Subsequently it was found that immune cells had receptors for catecholamines as well as for glucocorticoids [66-69], the latter of which are used clinically as immune suppressants. Early evidence for the role of sensory nerves in the regulation of immune function comes from the determination that mediators present in small diameter C-fibers (which are involved in pain transmission) are released antidromically upon stimulation and are pro-inflammatory. J.D. Levine and his lab positively correlated the amount of substance P innervation with severity and susceptibility to adjuvant-induced arthritis in rats [70]. Based on the above-mentioned experimental data it is clear that through the nervous system and HPAA the brain can influence parameters of immune function.

Primary evidence that the immune system could signal the brain was that the SNS [71-77] and HPAA [78,79] were activated following immune challenges. Also, areas within the brain that were activated following immune challenge (as determined by multi-unit analysis [80,81], by nerve recordings [49], by measuring neurotransmitters [49,50,72] or by immunocytochemistry [32]) were the same areas implicated in the control the SNS and HPAA. It was also shown that cytokines could alter neurally-mediated events such as behavior [33,82] and fever [83-85] thus demonstrating functional effects of these observations. In 1987 Farrar et al. demonstrated that receptors for the cytokine IL-1 were located throughout the brain [86]. Following this, a feedback loop was demonstrated when injections of cytokines into the brain were found to subsequently down regulate peripheral immune function [87-89]. Sensory pathways of the nervous system were also shown to affect the brain-immune communications as cutting the vagus nerve abrogated activation of the CNS in response to certain immune stimuli [90]. Also, Levine et al. showed in their arthritis model that arthritis can be induced bilaterally (similar to humans) even though the adjuvant was injected unilaterally. This effect of bilateral induction could be abrogated by eliminating the afferent nerve fibers with capsaicin [70,91,92].

The basic principles of Besedovsky's model continue to be supported; however, our knowledge regarding the complexity and scope of this system has increased tremendously. The first section of this literature review will describe the brain's influence on the immune system (section 1.3.). Topics to be dealt with include the nature and

anatomy of both the SNS and HPAA, their roles in regulating immune function, and their interrelation. This review will next describe the immune system's influence on the brain (section 1.4.). Topics to be discussed include the nature of the inflammatory response, measures of neural activation, how immune signals reach the brain and the central mediators and pathways that are activated in response to peripheral immune challenges. Following this, the effects of psychological stress on immunity will be discussed (section 1.5.) as psychological stress activates the same central systems as inflammatory stress. The final section will describe our lab's contributions to this field, our working model (section 1.6.) and the objectives for this thesis (section 1.7.).

1.3. Neural regulation of immunity

1.3.1. Organization of the ANS

This section will deal with the organization of the ANS and includes a brief description of some of the areas in the brain that are involved in receiving, processing and reacting to environmental signals. This includes a brief description of sympathetic, parasympathetic and neuroendocrine systems, with emphasis on the areas involved in brain-immune interactions.

1.3.1.1. The Hypothalamus

They key structure in the ANS is the hypothalamus. It serves as the central

integration point for both afferent and efferent signals. Afferent signals can be acquired from the circulation, visceral organs and from senses such as smell, sight and taste [93]. The hypothalamus then integrates all the available information and initiates a coordinated response from various output systems (if required), including the PNS, SNS and HPAA.

Much of the information regarding the anatomical connections of the hypothalamus with other structures in the brain and nervous system has been described by A.D. Loewy, P.E. Sawchenko, L.W. Swanson and colleagues. Swanson authored one of the most comprehensive reviews on the hypothalamus to date [94]. In it he describes the role of the hypothalamus as the integrator of autonomic, endocrine and behavioral function resulting in the maintenance of homeostasis, survival and reproduction. This includes coordinating functions such as feeding, defence, maintaining body temperature and care for offspring. In a ventral-dorsal direction the hypothalamus extends from the bottom of the thalamus to the base of the brain. In the rostral to caudal direction it extends from the vascular organ of the lamina lateral terminalis (OVLT) in the third ventricle to the mamilliary body. Swanson described the hypothalamus as a 3 by 4 grid with three sections from medial to lateral (M-L) and four sections from anterior to posterior (A-P). Starting with the third ventricle as a midline, the sections from M-L are designated periventricular, medial and lateral. The four A-P sections starting at the OVLT are preoptic, anterior, tuberal and mamilliary. For the purposes of this review the hypothalamus will be described from medial to lateral.

The periventricular zone of the hypothalamus contains such structures as the

OVLT, periventricular nucleus, suprachiasmatic nucleus, arcuate nucleus and the paraventricular nucleus (PVN). The PVN is probably the most important nucleus in the hypothalamus in terms of autonomic regulation. The PVN is divided into three main areas from M-L starting at the third ventricle. The most medial portion is called the medial parvocellular region [94]. This region is comprised predominantly of corticotrophin releasing factor (CRF or CRH) positive cells that synapse on the hypophyseal-portal vessels in the median eminence (ME) of the pituitary gland [95]. CRF is released into these vessels, transported to the anterior pituitary (adenohypophysis) and causes the release of andrenocorticotropin releasing hormone (ACTH). ACTH travels in the circulation and causes the release of glucocorticoids from the adrenal cortex (this circuit will be dealt with in greater detail in the section on the HPA axis). Other releasing hormones from the hypothalamus function on the anterior pituitary in a similar manner. This region of the hypothalamus receives extensive innervation from the limbic system, brainstem, subfornical organ (SFO) as well as from other areas of the hypothalamus. The next anatomical region of the PVN is the magnocellular division and it is analogous in composition to the magnocellular region of the supraoptic nucleus (SON). Both magnocellular regions contain large cells that predominantly contain oxytocin (OXY) or VP. These cells project directly to the posterior pituitary (neurohypophysis) where they are released into the general circulation. Nerve supply to the magnocellular division is also from the brainstem, SFO and limbic system. The most lateral portion of the PVN is the lateral parvocellular division. These cells are positive for CRF, OXY, VP and other

substances, and synapse on brainstem neurons (some of which are vagal preganglionics) and sympathetic preganglonic cells in the spinal cord. Afferents to this cell group also include projections from the brainstem, limbic system, other hypothalamic cell groups and the SFO.

The medial zone of the hypothalamus is involved in the regulation of specifically motivated behaviors. This region contains the medial preoptic area (MPOA) which functions in body temperature regulation, the anterior hypothalamus (AH) which functions in visceral and gustatory sensation, the dorsomedial hypothalamus (DMH) and the ventromedial hypothalamus (VMH) which functions in feeding, rage and sexual behavior. This area also has reciprocal connections with other areas of the hypothalamus, the brainstem and the limbic system.

The lateral zone of the hypothalamus also has many reciprocal connections. This zone is primarily concerned with behaviors relevant to hunger, thirst and aggression and sends projections to other areas of the hypothalamus, the cortex and the spinal cord.

1.3.1.2. Connections to the hypothalamus

There are many structures that have reciprocal connections with the hypothalamus and specifically with the PVN [94]. The most relevant structures for the brain-immune axis include brainstem nuclei, the limbic structures and circumventricular organs (CVOs) because they relay information to the hypothalamus on the visceral environment, the external environment and emotional state of the organism, and blood

borne chemicals respectively.

The brainstem is composed of (from rostral to caudal) the midbrain, pons and medulla. Visceral information is relayed to the sensory nucleus of the vagus called the nucleus of the solitary tract (NTS) where it is then transmitted to other brain areas including the PVN. There are many transmitter systems throughout the brain that could potentially connect the brainstem to the hypothalamus. However it is the adrenergic and noradrenergic systems that have been definitively shown to transfer visceral information to the PVN from vagal afferents [94,96-98]. These structures include adrenergic cell groups (C1, C2 and C3) located in the medulla and noradrenergic cell groups located in the medulla and pons. This includes the A1 group in the caudal ventrolateral medulla, the A2 group located in the NTS (which is located in the dorsomedial medulla) and the A6 region in the pons which is also known as the locus ceruleus [94,99-104]. These brain regions, along with other areas, form an interconnected network that functions to process visceral information before transmitting it to higher centers like the PVN for integration. The resulting signal integration can then activate a combination of systems including the SNS, the HPAA or vagal preganglioinc motor neurons in the medulla. Functional and anatomical testing of catecholamine circuits from the brainstem to the hypothalamus has been conducted in our lab [105] and in the labs of A.J. Dunn [39], D. Saphier [106-108], P.E. Sawchenko [41,95,109] and others [110]. These aspects will be detailed in later sections as they are especially pertinent in the response to inflammatory stimuli.

The Limbic system consists of structures such as the hippocampus, Amygdala,

bed nucleus of the stria terminalis (BNST) and lateral septal area. These structures function collectively to influence and regulate cognitive function and emotions. For example the hippocampus is involved in learning and memory while the amygdala is involved in emotional responses [111]. It has been shown that cells from the amygdala and hypothalamus are functionally associated [112,113]. Cells from the amygdala also synapse on midbrain cells which have bidirectional communication with the hypothalamus [112,114]. The hippocampus is also functionally linked to the PVN, however the connection may be directed through other limbic structures such as the BNST and the Lateral septal area (LSA) [115,116]. These relationships have been functionally demonstrated by Saphier and others who showed that electrical stimulation of the amygdala increased activity in the BNST [117] and induced a corticosterone response [40]. The corticosterone response was blocked by lesions of the BNST.

Circumventricular organs (CVOs) are structures in the brain where the blood brain barrier (BBB) is leaky [118]. These structures include the posterior pituitary, choroid plexus, OVLT, area postrema (AP-beside the NTS), SFO, median eminence (ME) and subcommissural organ (SCO). The OVLT, SFO and AP have the ability to monitor the blood levels of specific chemicals and ions and send this information directly to other brain areas. The AP for example is located near in fourth ventricle beside the NTS to which it is also connected [118,119]. Catecholamine cell groups such as the A2/C2 regions are located within the NTS and project to many brain areas including the PVN. The SFO and OVLT project to the PVN as well [118].

Although afferent connections to the hypothalamus and in particular the PVN are complex and seem fairly ubiquitous, each region of the PVN has a unique afferent innervation from limbic regions, brainstem nuclei and CVOs [94]. For example the magnocellular division of the PVN receives most of its visceral afferent information via the A1 cell group, where as the parvocellular region receives it innervation mainly via the A2 group with smaller contributions from A1, C1 and A6 regions [94,120-122] . These differences demonstrate an anatomical basis for the differential activation of different afferent pathways for different stressors.

1.3.1.3. Projections from the brain to the spinal cord

The basic organization of the sympathetic nervous system is that neurons from the brain synapse on sympathetic preganglionic neurons in the spinal cord which synapse on sympathetic post ganglionic neurons outside the spinal cord which directly innervate the target organ. The sympathetic preganglionic neurons reside in the intermediolateral cell column (IML) of the thoracolumabr spinal cord. Many of the axons from preganglionic cells will travel in white rami communicants and synapse with postganglionic cells in the paravertebral sympathetic chains. The postganglionic axons will leave the ganglia in grey rami and will join mixed nerves that contain motor and sensory fibers. These nerves will directly innervate the target organs. Other preganglionic axons will travel through sympathetic chain and synapse with postganglionic cells in prevertebral ganglia located throughout the body before contributing to the target organ's innervation. Some examples

of these ganglia include the celiac ganglia and the superior mesenteric ganglia [1].

A.D. Loewy's studies formed the foundation for determining the structures in the brain that directly innervate the preganglionic SNS cells. In these studies, Loewy made use of pseudorabies virus which is a powerful tract tracing tool. When injected into an organ, this virus is transported from the axons to the cell bodies (known as retrograde transport) and continues to move transynaptically until the animal is killed. Therefore the longer the survival period, the more synaptic connections are identified [123]. Loewy injected this virus in various locations throughout the body such as the adrenal gland and the tail with the goal of identifying the brain areas responsible for afferent projections to the SNS preganglionic cells [123]. He found that many similar brain areas were identified, irrespective of the target organ [124,125]. The typical pattern would be for example that on day 4 just the IML would be virus positive after a visceral injection. On day 5 the ventrolateral medulla, ventromedial medulla , the A5 region in the medulla, the raphe nucleus in the medulla and the PVN would all be labelled. This was verified by Ding et al., after injection of retrograde tracer into the rabbit renal nerve [126]. On day 6 other brain areas were labelled, probably representing areas synapsing on the areas identified in day 5. These areas included the BNST, MPOA, DMH and VMH [123]. However there were some minor differences in labelling based on the locations of the ganglia or target organs. For example injections in the superior cervical ganglion also caused virus to show up in the lateral hypothalamus (LHA), suggesting that portions of the efferent SNS may be activated in a specific or selective manner [125].

Topographical organization and innervation is common are the CNS. In the spinal cord Weaver and colleagues found that similar spinal levels innervated a variety of organs. They stimulated one spinal level at a time from T3-L4 and observed responses in each of the splenic, renal and mesenteric nerves. Although they determined that spinal segments did not selectively innervate a specific set of post-ganglionic neurons, the intensity of response varied for each nerve depending on the segment activated [127]. In support of this, Pyner and Coote [128] and Appel and Elde [129] demonstrated that there was specificity of target innervation from the preganglionic cells to the postganglionic cells based on retrograde labelling of different target organs or ganglia. Appel and Elde [129] used retrograde transport of two different tracers in two different targets (the cervical sympathetic trunk or adrenal medulla) to determine the location of the preganglionic cells. They found that although there were areas of overlap, the same cell did not innervate both targets. As well, although both sets of preganglionics were innervated by somatostatin afferents, only the preganglionics for the cervical sympathetic chain were innervated by oxytocin positive fibers. This suggested that central oxytocin may be able to excite peripheral SNS nerves without causing the release of E from the adrenal gland. Pyner and Coote used retrograde transport with three different tracers to determine the location of preganglionic cells for the superior cervical ganglion, stellate ganglion and the adrenal medulla. Although there were areas of overlap with respect to the general anatomical region, again the preganglionc cells for each organ were located in anatomically distinct columns within the IML. Our lab has contributed to these anatomical observations by

functionally demonstrating that after an inflammatory challenge, the latency for activation in the splenic nerve is reduced, compared to the renal nerve, even though the preganglionics arise from similar spinal levels [76].

1.3.1.4. Neurotransmitters

There is the potential for many neurotransmitters to be involved in the regulation of SNS preganglionic neurons based on the regions of the brain that Loewy identified [125]. Some of these neurotransmitters include NE, E, OXY, VP, CRF, somatostatin, serotonin, substance P, and enkephalins [130-132]. It is not clear which of these transmitters are involved in the response to inflammatory stress, however our lab has recently identified that central oxytocin is crucial for LPS-induced splenic nerve activity [133].

The neurotransmitters involved in the regulation of target organs by the peripheral SNS are better characterized. In the SNS acetylcholine is the transmitter used between pre and postganglionic neurons (mainly via nicotinic receptors [134]) while NE is the major transmitter between the postganglionic cells and target organs. An exception is the adrenal medulla which releases NE and E into the bloodstream as opposed to releasing them in a paracrine manner similar to other postganglionic cells. Since the nature of catecholamine pharmacology has been known for decades, many tools exist for studying the interactions between the SNS and the immune system. These tools include surgical and chemical lesions of specific brain areas or peripheral nerves. For example there are adrenergic

agonists and antagonists which are specific for receptor subtypes and have known abilities to either cross or not cross the blood brain barrier. Also, there are substances that can inhibit catecholamine synthesis (alpha-methyl p-tyrosine), storage (reserpine), and release (guanethidine), substances that inhibit the preganglionic signals that cause catecholamine release (chlorisondamine- nicotinic receptor antagonist) [134] and substances that are neurotoxic to catecholamine synthesizing neurons such as 6-hydroxydopamine (6OHDA) [135]. Although there may be other substances in SNS postganglionic terminals such as neuropeptide Y (NPY) which may have the potential to regulate immune function [136-140], the majority of the reports implicate catecholamines as the primary peripheral neurotransmitter for immune regulation.

Catecholamine synthesis is initiated with the conversion of the amino acid tyrosine to Dopa by tyrosine hydroxylase (this enzyme is the rate limiting step in catecholamine synthesis). Dopa is then converted to dopamine by dopamine decarboxyalse. This compound is then converted to NE by dopamine beta hydroxylase. NE can then be converted to E by phenylethanolamine-N-methyltransferase [134]. Catecholamines are readily taken up by all cells and degraded by monamine oxidase and catechol-O-methyltransferase. Catecholamines exert their effects on all organs and body systems including the immune system [66] through alpha and beta adrenergic receptors [141]. It has been shown that catecholamines are good markers for sympathetic activity [142,143]. The majority of NE is released from the nerve terminals while E is released from the adrenal medulla [144,145]. The release of NE and E also occurs during an

inflammatory stress response [77,146-149] , and these mediators have the ability to regulate their own release via presynaptic adrenergic receptors [150].

1.3.2. The Effect of the SNS on immunity

Anatomically, the sympathetic nervous system has the potential to regulate immune function based on the fact that sympathetic nerves innervate immune organs and are in close contact with immune cells [59-65,151] which have adrenergic receptors [66-68,152,153]. As well, circulating catecholamines from the adrenal medulla can bind adrenergic receptors in circulating or tissue-bound immune cells. The ability of the peripheral SNS to functionally alter aspects of the immune system has been demonstrated both for innate and acquired immunity. Some such studies have investigated the change in the nature of the immune response in animals whose sympathetics have been modified chemically and/or surgically. Other studies have documented the change in immunity after activation of the SNS by chemical means or by psychological stimuli such as stress.

The response to a particular pathogen may require aspects of both natural and adaptive immunity [44]. Natural or innate immunity includes barriers such as skin, proteins of the complement system, phagocytic cells (macrophage, monocytes, PMN), and natural killer (NK) cells. This system is activated immediately upon the first exposure to the activating substance (immunogen). Although the innate response is rapid, there is little specificity and diversity. As well, some of the mediators responsible for host defence in this system can rapidly cause more damage than the original pathogen if

left unchecked. Adaptive immunity relies on T-cells and B-cells and has humoral (antibodies) and cell-mediated (CD8+ T-lymphocytes) components. Immunogens that are recognized by antibodies or T-cell receptors are referred to as antigens. If the adaptive system is exposed to an antigen for the first time, it may take a few days to mount a response. However since this system has memory, subsequent responses to the same antigen may get larger, be more specific and have a more rapid onset time [44]. The natural and adaptive systems interact heavily to combat pathogens. For example the inflammatory response initiates a series of signals to alert the adaptive immune system of a potential pathogen. These signals include soluble mediators such as cytokines and the expression of cell surface molecules for antigen presentation and cell to cell contact. The adaptive response can augment the innate response by stimulating its effectors (ie. the activation of macrophages in the delayed type hypersensitivity reaction (DTH)) or by making the innate system more efficient (ie. antibodies opsonizing targets for engulfment by phagocytic cells or for NK cell-mediated killing). Much of the communication between immune cells is controlled by cytokines.

Cytokines are small proteins that 1) are produced in the effector/ activation stage of immunity; 2) have brief, self-limited secretion; 3) are pleotrophic and are produced by many cell types; 4) have redundant functions; 5) regulate cell division; 6) influence the production of other cytokines; and that 7) convey their effects through receptors [45]. Certain cytokines may only be produced in certain situations or against certain pathogens. For example in the early stages of infection, the innate system produces tumor necrosis

factor (TNF), interleukin-1 (IL-1) and IL-6 in response to bacterial immunogens while type 1 interferons, IL-12 and IL-15 are produced in response to viral immunogens. Cytokines also have the ability to regulate the characteristics of the adaptive response. For example IL-4 and IL-10 promote humoral immunity (known as the TH2 response) while interferon gamma (IFNγ), IL-18 and IL-12 promote a cell-mediated response (TH1 response). The type of response required by the host depends on the nature of the antigen, therefore regulation of the TH1/TH2 balance is critical [45]. With respect to cytokine production, the preceding description is an over simplification as many cytokines will be produced to some or all of the above pathogens. For example IFNγ, IL-10 and IL-12 are expressed in ceratin innate responses as well as adaptive responses while TNF is expressed in adaptive responses as well as innate responses to viral and bacterial immunogens.

Chemokines are a subset of smaller cytokines that function to stimulate cell motility and act as a chemoattractants [45]. These proteins can attract many cell types including macrophages, T-cells and PMN.

1.3.2.1. sympathetic regulation of natural immunity

The influence of the SNS on natural immunity can be demonstrated by using immunological parameters such as the response to mitogens, NK cell function, the production of cytokines and inflammation. The basic premise is that SNS activation is inhibitory to the innate immune system [48,154].

Mitogens are molecules which non-specifically activate a large cell population such as macrophages or T-cells. Examples include endotoxin (also known as lipopolysaccharide or LPS) from gram negative bacteria and superantigens from gram positive bacteria. In some of the original reports on this subject, Roszman's lab [51,53] found that lesions in the anterior hypothalamus (AH) decreased the response to mitogens. These changes were not mediated by glucocorticoids, ruling out the HPAA in this situation. Saito et al., found that stimulation of VMH increased peripheral NE and E. [155] which decreased mitogenesis [156]. This effect was abrogated by propanolol, a beta adrenergic blocker, nerve section and chlorisondamine suggesting it was NE release from nerve terminals and not circulating E that was mediating this response. In vitro findings directly support the suppressive role of NE in the response to mitogens [154]. Similarly, when neuropeptides such as CRF [157] are exogenously administered into certain brain areas, they are able to activate the SNS and inhibit the response to mitogens [158]. The importance of the mitogen response is that these molecules are constituents of major pathogens that have been harmful to higher organisms for so long that specific receptors have evolved for their detection (ie. a specific receptor for LPS will be described in later sections). Although measuring the response to mitogens is one of the older dependent measures of immune function used in brain-immune studies, the pitfall is that this assay is in vitro and removes the cells from their microenvironment. Thus, studies utilizing the in vitro mitogen response may not directly prove that specific brain areas and neuropeptides are involved in the immune response to mitogens, but they do illustrate the potential for

neural regulation of immunity.

The neural regulation of NK cell function is similar to the regulation of the mitogen response. Like the mitogen assay, early studies of NK cell function utilized an in vitro assay system. Roszman's lab (Cross et al., [159]) found that lesions in the AH decreased splenic NK cell activity and Katafuchi et al. [160], found that lesioning of the MPO increased splenic nerve activity and decreased NK cell function. The decrease in NK cell function was blocked by splenic denervation. They also demonstrated that intercerebroventricular (i.c.v.) interferon alpha increased splenic nerve activity and decreased NK cell function. This effect was also blocked by cutting the splenic nerve or with naldolol (a beta blocker that does not cross the blood brain barrier)[161,162]. Irwin et al. [163] found that CRF in the lateral ventricle increased peripheral NE and decreased NK cell function. The inhibition of NK cell function was blocked by 6OHDA treatment. These studies again demonstrate that the central and peripheral pathways are able to regulate the immune system. An alternative perspective is to consider that in vitro studies may indicate that the SNS's influence on immunity is so robust that it continues to be present when the immune cells are transferred into a dish. The best evidence for SNS regulation of in vivo NK cells is from Shamgar Ben Eliyahu's lab who used an NK-sensitive tumor model to assess NK function [164]. They found that peripherally acting beta agonists increased tumor metastasis, which was reversed by beta blockers. Surgical stress, which activates the SNS did the same thing as the beta agonist. Thus catecholamines are inhibitory to NK function despite the fact that they promote the

movement of NK cells from tissues into circulation [164-166]. It may be E from the adrenals that contributes to this effect as both ADX and naldolol (a peripheral beta blocker) reduced the immune suppression of NK cells in response to i.c.v. IL-1, but 6OHDA treatment did not.

There are many reports of catecholamines being able to regulate cell movement and circulation [167-171]; however, the effects of catecholamines on cell trafficking are unclear. Some reports suggest that pre-treatment of catecholamines influence immune cells to accumulate in immune organs [167], where other reports suggest that catecholamines decrease lymphocyte binding to endothelium [170] and promote cell movement to the circulation [168,169]. Besedovsky's lab recently showed that cell movement was inversely proportional to flow resistance in an ex vivo spleen perfusion model and that catecholamines had the tendency to increase cellular flow despite being vasoconstrictors [172].

Many papers have reported the effects of adrenergic agonists and antagonists on cytokine production in humans, mice and rats in response to LPS. The vast majority of these reports have utilized in vitro assay systems. In terms of in vitro TNF production, increases in the intracellular cAMP levels that occur with activation of the beta adrenergic receptor [141] cause a decrease in TNF mRNA and secreted protein [173-188] . This is verified by the fact that phosphodiesterase inhibitors such as Rolipram, which block the degradation of intracellular cAMP, also decrease TNF production [189,190]. This is thought to occur primarily with activation of the B2 subtype receptor [184,187,188] ,

however some reports suggest a B1- receptor mediated mechanism may be responsible [191]. Hu et al., found that an alpha adrenergic agonist increased TNF production in rat peritoneal macrophages in response to LPS [192]. Spengler et al., confirmed these results with an alpha-2 agonist by showing that both TNF mRNA and protein were increased [193] in response to LPS combined with alpha-2 adrenergic receptor stimulation.

In terms of in vivo reports, the literature is less complete. It is known that exogenously administered E will decrease LPS-induced TNF in plasma and tissue [178,194] . Elenkov et al., [195] and Hasko et al., [196] demonstrated that TNF production in mice could be inhibited in vivo with an alpha-2 adrenergic antagonist. The alpha-2 antagonist was shown to inhibit presynaptic postganglionic A2 receptors, thus blocking negative feedback inhibition that NE has on its own release [150,197]. This effect was dependent on intact sympathetic transmission as chlorisondamine abrogated this initial inhibition. Propanolol, a beta adrenergic antagonist, reversed the effect of the A2 antagonist and increased TNF production in response to LPS. It is uncertain whether this reversal was due to modulation of immune cells directly or action at the nerve terminals [195]. Interestingly, blockade of sympathetic transmission with chlorisondamine did not affect LPS-induced TNF production [195], raising concerns regarding the physiological relevance of in vitro measures of immune function. However, recent reports out of this same lab contradict these finding by demonstrating that mice treated with reserpine, an alkaloid that depletes NE, dopamine and serotonin, increased LPS-induced TNF production in the circulation [198]. The reasons for this contradiction

were not discussed.

There is also evidence that catecholamines reduce IL-1 beta production after LPS treatment [185,199]; however, the evidence is not as convincing as it is for TNF. Some studies describe a greater effect of catecholamines on TNF production than on IL-1 production [185]. Others reported that there was no effect of catecholamines on IL-1 production although TNF production was significantly altered in the same cells [179].

The effects of catecholamines on IL-6 production are also not clear. It seems to be paradigm specific as some studies report that catecholamines increase IL-6 production [173,178,196,200-203] while other reports suggest that catecholamines are inhibitory to IL-6 production [174,176]. Evidence that supports SNS stimulation of IL-6 production includes the fact that IL-6 is increased by stress in the absence of LPS [204-207]. As well, central inflammatory agents can increase peripheral IL-6, and these effects are dependent on an intact SNS [208,209]. A likely explanation of the discrepancy was put forth by Straub et al., who demonstrated that in the presence of bacteria NE inhibited IL-6, but in the absence of bacteria NE increased IL-6 production [210].

The effects of catecholamines on the expression of other cytokines are even less well documented. Some studies have shown that catecholamines can increase IL-10 production [173,175,177] while others describe an inhibitory effect on IL-10 levels [211,212]. Similar to TNF, the effect of catecholamines on the production of IL-12 appears to be inhibitory [213,214]. Interestingly, it was found that cutting the splenic nerve decreases the chemokine production in the spleens of rats with *trypanasoma brucei*

brucei [215], an effect opposite to what might be expected in view of a possible anti inflammatory role for the SNS.

Besides catecholamines, their are other molecules in nerve fibers that can potentially regulate cytokine production. For example CGRP, NPY and VIP have also been shown to alter cytokine production [216] and substance P has been shown to increase LPS-induced TNF [217] and IL-12 production [218]. However the majority of reports concerned with the influence of the SNS on LPS-induced cytokine production have concentrated on the role of catecholamines and not these other mediators.

The peripheral and central pathways that activate the SNS in response to peripheral inflammation are not fully characterized. However central cytokines can activate the SNS as demonstrated by the fact that cytokines administered i.c.v. increase splenic nerve activity as well as peripheral catecholamine release and turnover [74,219-221] . In this proposed feedback loop, i.c.v. cytokines also downregulate immune function [89,161,222,223] which is partially or completely due to NE release from nerve terminals [89,223]. It has been shown that alpha MSH (MSH) is induced centrally by inflammatory agents [224] and is an important central mediator for the inhibitory SNS pathways [225-229]. MSH given i.c.v. reduces peripheral inflammation, and this effect is dependent on an intact SNS [228,230]. It is possible that pathogens like the HIV virus are able to subvert this regulatory pathway for their own purposes [222,231-233]. Firstly, it has been shown that elevated MSH is proportional to a reduction in HIV titer [234]. Secondly, the HIV coat protein, GP120, reduces MSH-induced NE turnover in the spleen

[231]. As expected, GP120 and HIV cause an increase in pro-inflammatory cytokines like TNF in rats [231,232] and humans [233] and decrease anti-inflammatory cytokines like IL-10 in humans [233]. Our lab has found a similar dysregulation with respect to increased TNF production in spinal cord injured rats [235].

J.D. Levine's lab showed that the SNS is involved in the pathogenesis of joint inflammation; a model for rheumatoid arthritis. They demonstrated that intact postganglionic sympathetic fibers and substance P afferents are critical in maintaining inflammation [70,91,92,236]. In agreement with Levine, Lorton et al., recently found that systemic treatment with 6OHDA reduced the severity of arthritis, but selective treatment of the draining lymph nodes with 6OHDA increased adjuvant induced arthritis as predicted by prior studies with the SNS and immune function [237]. The studies by Lorton and Levine suggest that although the SNS is primarily inhibitory to immune cells and immune function, the SNS has proinflammatory properties at least where the joints are concerned. The mechanism by which the SNS potentiates inflammation in the joints is unknown; however, Levine's lab proposed a model where presynaptic beta adrenergic receptors on sympathetic post ganglionic neurons caused the release of mediators like prostaglandins that worked in concert with mast cells and afferent nerve fibers to potentiate the severity of joint inflammation [91,238-240] .

1.3.2.2. sympathetic regulation of adaptive immunity

Antigens can also activate the SNS as determined by an increase in splenic nerve

activity after administration of a protein antigen [241] and the increase of both electrical activity [242] and transmitter release [50] in the brainstem and hypothalamus after inoculation with sheep red blood cells (SRBC). It is also known that lesions in certain brain areas will influence the humoral response to antigens [243,244]. Also, antigen administration can increase nerve fiber density in immune compartments [245]. In terms of cell-mediated immunity, the few papers that have investigated this response suggest that NE is inhibitory to this process [246,247]. Although the regulation of the humoral response by the SNS is well characterized, no consensus yet exists as to whether the SNS provides an inhibitory or facilitory influence on antibody production. It is known is that both the SNS and HPAA can inhibit cytokines involved in natural and cell-mediated immunity such as TNF and IL-12 [214]. Thus catecholamines and glucocorticoids tend to shift adaptive immunity to a TH2 response since the relationship between TH2 and TH1 is one of mutual inhibition [248]. In general, nerve fibers have been shown to be a more important mediator of humoral immune responses than either the adrenal medulla or adrenal cortex [48,249-251].

Evidence in favour of catecholamines providing a facilitory influence to the humoral response includes the 1977 report by Kasahara et al., (later verified by Hall et al., in 1982 [252]) which demonstrated that mice treated with 6OHDA have an impaired response to SRBC as assessed in vitro by the Plaque Forming Cell assay (PFC)[253]. In our lab, Zalcman et al., [254] demonstrated that cutting the splenic nerve did not alter the PFC response to SRBC. However, the IL-2 induced increased in the splenic PFC

response to SRBC was dependent on intact splenic innervation and was additionally shown to be mediated through beta adrenergic receptors. In vitro results verified this by demonstrating that NE increased LPS-induced antibody production in B cells (this effect was independent of T cells and macrophages) [255] and enhanced LPS-induced B-cell proliferation [256]. Recently it was shown that dopamine beta hydroxylase (DBH) $^{-/-}$ mice (DBH synthesizes NE from dopamine) are more susceptible to bacterial infection and have impaired T-cell function as compared to normal litter mates [257]. However it is unknown if these effects are due to the lack of NE or the substantial increase in dopamine which is inhibitory to B-cell function [255]. Perhaps the most compelling evidence that NE is facilitory to the humoral response comes from V.M. Sanders and colleagues. They demonstrated that NE can effect both T cells and B-cells and that NE given at the time of in vitro immunization increased the PFC response to SRBC [258]. This effect was blocked by propanolol, a beta adrenergic receptor antagonist and mimicked by terbutaline, a beta 2 adrenergic receptor agonist [259], proving this effect was mediated by the beta-2 adrenergic receptor. The next and perhaps most significant finding was that only TH1 cell lines (at rest and during stimulation) had B2 adrenergic receptors [152,153]. This was determined by binding studies and verified by the fact that terbutaline decreased IL-2 and IFNγ expression in TH1 clones but did not affect cytokine expression in TH2 clones. The clones were exposed to NE prior to reconstitution with B-cells, proving the effects of terbutaline were on T-cells. To extend these studies in vivo, they reconstituted SCID (severe combined immunodeficiency) mice with antigen specific T and B cells. Since the

T-cells were TH2 clones, only the B cells had adrenergic receptors [260]. Animals that were depleted with 6OHDA prior to reconstitution demonstrated a decreased primary IgM response and decreased primary and secondary IgG responses. The decreased antibody response in 6OHDA-treated mice was mimicked by a beta adrenergic receptor blocker and partially restored with a B2 adrenergic receptor agonist. They also demonstrated an increase in B7-2 (a T-cell co-stimulator molecule on B-cells) in NE-treated B cells. Therefore NE can effect B-cell function as well as T-cell function.

Evidence that catecholamines are inhibitory to antibody production was first proposed by Besedovsky in 1979 [48]. In this report he demonstrated that surgical denervation of the spleen or 6OHDA treatment increased the PFC response to sheep red blood cells in vitro. They also noticed that preceding the peak in the PFC response, splenic NE levels dropped. Interestingly, adrenalectomy (ADX) did not have an effect by itself, but it augmented the 6OHDA effect when both ADX and 6OHDA were combined. They also demonstrated that the levels of splenic NE inversely correlated with the magnitude of the antibody response [261]. Animals with a low splenic NE content had a higher antibody response to antigen and vice versa. Other groups found that the decrease in splenic NE in response to antigen was due to a comparable total amount of NE which was diluted by the larger spleen suggesting the increased response was due to the increased cellularity [262]. Similar to the mitogen response and NK cell function determinations, stimulation of the AH decreased IgG levels in normal animals [263]. The exact mechanism for the inhibitory action of catecholamines on the antibody response is

unknown; however, NE does have the potential to decrease MHC-2 expression [264,265]. Our lab has also provided evidence for the inhibition of the humoral response by NE. Green-Johnson et al., [266] demonstrated that epilepsy prone mice, which have a higher splenic NE content than epilepsy resistant control mice, also show a relative decrease in vivo IgG response to PFC. Supporting this, they found that a beta 2 adrenergic agonist, when given in the late stages of immunization, decreased the antibody response in normal animals. Interestingly, when the T cells were removed from the spleen they exhibited normal responses, demonstrating that the in vitro response do not always mimic the natural microenvironment for these cells. Supporting this, Green-Johnson et al., [267] demonstrated that mice with a greater cerebellar NE content (same splenic NE content) also had a lower PFC response. However, if B-cells were taken out of the body, they reacted normally to in vitro stimulation. Other reports have used 6OHDA treated animals and observed an increase in the antibody response as well [61,268] .

There are no obvious explanations as why some labs have found catecholamines to facilitate the humoral response and others found them to be inhibitory. Potential explanations include the timing of NE exposure. It is known that NE acting through B-adrenergic receptors will increase intracellular cAMP. However cAMP at the right time will increase antibody production and at the wrong time it may be inhibitory [269]. This may have to do with the cell cycle as NE has been shown to induce more B-cell precursors to differentiate, but did not have the same effect on IgG producing cells. Another possible explanation for these contradictory results is due to the use of 6OHDA.

6OHDA is a SNS toxin but it can activate the HPAA [270,271] as well as the adrenal medulla and central catecholamine circuits [272]. Also, upon injection of 6OHDA, there is a bolus of NE released from the nerve terminals. Thus despite being able to inhibit SNS function and the influence of the SNS on immunity, 6OHDA may activate alternate pathways that have a similar role to the SNS with respect to immune regulation. Another problem with the use of 6OHDA in neural immune studies is that is able to cross the blood brain barrier and eliminate central circuits in young animals but not older ones [61,273] . Therefore depending on the age of the animal the results of chemical sympathectomy on aspects of immune function will differ. Thus the unpredictable and contradictory results on this topic may be due in part to the unpredictable nature of 6OHDA's effects on the host.

1.3.3. The HPAA regulation of immunity

1.3.3.1. Organization of the HPAA

The HPAA is the other major efferent path by which the brain can regulate the immune system. The HPAA consists of peptides released from the medial parvocellular region of the hypothalamus into the hypophyseal portal vessels in the median eminence. These hormones cause the release of ACTH from the anterior pituitary which then cause the release of glucocorticoids from the adrenal cortex (primarily corticosterone in the rat and cortisol in humans). Glucocorticoids bind receptors on the target cells and mediate

their effects by translocating to the cell nucleus and affecting transcriptional processes and mRNA levels [69].

The hypothalamic peptides, CRF and VP, are the main mediators of ACTH release from the anterior pituitary [274-277]. They are both located in the medial parvocellular PVN, and often in the same cells [278,279]. Although they work in concert, CRF is primarily responsible for the initial portion of acute HPAA activation [275,277] while VP is more critical in the later stages of acute and chronic HPAA activation [275,280]. CRF is a 41 amino acid peptide found throughout the brain and is the principal transmitter of stress-related signals [275,277,281,282]. CRF receptor type 1 mediates most of the central effects of CRF. The type 2 CRF receptor (A and B forms) is located in the brain and the periphery and binds the CRF-like peptide urocortin 10-40X more strongly than CRF [281,283]. Chronic HPAA activation induced by chronic CRF causes weight loss and thymic involution [284]. This can be abrogated by adrenalectomy (ADX) [285]. Transgenic CRF mice have overactive HPAAs and reduced measures of immune function. This is also eliminated by ADX [284,285]. Studies with $CRF^{-/-}$ mice demonstrate that animals lacking CRF receptor type 1, lack the proper stress response and HPAA activation, the latter due to low levels of ACTH [286,287]. Besides CRF and CRF receptor synthesis, there is a CRF binding protein which can regulate the effects of CRF by preventing CRF from binding its receptor [288]. This and other evidence firmly establishes CRF as the main mediator of ACTH secretion.

VP is a 9 amino acid peptide that like CRF is expressed throughout the PVN and

causes the release of ACTH from the anterior pituitary [289]. There are at least three types of VP receptors; the V1a type in liver and smooth muscle, the V1b type in the anterior pituitary and the V2 in the kidney [290,291]. VP may be more important than CRF in stimulating ACTH release in the later stages of acute activation of the HPAA [275] and during the course of chronic HPAA activation [280,292].

The main pathway of HPAA counter regulation is by feedback inhibition from circulating glucocorticoids via central receptors. This has been demonstrated by measuring central and peripheral HPAA activity in response to glucocorticoid synthesis inhibitors, glucocorticoid receptor antagonists, ADX animals or exogenous glucocorticoid administration. In 1972 it was demonstrated by Dallman et al., that ADX increases plasma ACTH; an effect reversed by exogenous corticosterone [293]. P.E. Sawchenko and others established that central CRF and VP mRNA (not OXY) is increased in the medial parvocellular region of rats in response to ADX, chemical inhibition of glucocorticoid synthesis, specific antagonists or hypophysectomy (HYPOX) [294-299] . Many of these effects were reversed upon exogenous administration of corticosterone [295]. In addition to this, responses of the HPAA to stress or LPS can be exaggerated in ADX animals due to the lack of feedback inhibition [300,301].

Glucocorticoids mediate their effects through the two types of glucocorticoid receptors. The type 1, high affinity receptors are called mineralocorticoid receptors (MR) and they bind mineralocorticoids as well as glucocorticoids. The type 2 receptors are called glucocorticoid receptors (GR) and bind only glucocorticoids [69,302,303].

Although both receptors are distributed throughout the brain, MRs are mainly located in the areas of the limbic system, particularly the lateral septal area and hippocampus [303,304] while GRs have a more uniform distribution throughout the limbic system, brainstem, pituitary and hypothalamus. Corticosterone is released in a pattern or diurnal rhythm where the a.m. levels are lower than the p.m. levels. Findings by Bradbury et al., [305] which were extrapolated by Spencer et al. [304] suggest that both MR and GR are involved in glucocorticoid feedback. Essentially, the higher affinity MR receptor. is sufficient for monitoring and maintaining glucocorticoid levels during the low period in the morning. However, in the evening or during times of activation such as stress, both MR and GR are required. The functional receptor levels are also regulated by circulating glucocorticoid levels. Miller et al., found that ADX increased the maximal amount of MR and GR binding in the hippocampus and that stress (maximum physiological level of glucocorticoids) decreased the hippocampal binding of glucocorticoids [306]. Similar effects have been shown by others [307-309]. Lack of glucocorticoids have also been shown to increase GR on spleen cells, while high levels of glucocorticoids decrease splenic glucocorticoid binding [306,307]. The hippocampus was much more susceptible to regulation than other areas of the brain or periphery [306,307].

Glucocorticoid feedback on the HPAA has the potential to occur through many anatomical sites that express GR and/or MR. These include areas that are part of or connect to structures of the HPAA including the brainstem [303,310], hippocampus [303,306], lateral septal area [303], the pituitary [306] and the PVN [28,311]. The most

convincing evidence suggests that the hippocampus is the major site of glucocorticoid feedback. This region has the largest binding capacity for glucocorticoids and the receptors are the most sensitive to feedback [306]. Also, the hippocampus has been shown to tonically inhibit the HPAA as severing the fornix (which connects the hippocampus to the hypothalamus) increases VP and CRF mRNA in the parvocellular PVN [312-314].

1.3.3.2. Glucocorticoids and immune function

Rationale for HPAA involvement in immune regulation is firstly suggested by the fact that the HPAA is activated in response to inflammatory agents [10,78,315]. Other evidence comes from the study of histocompatible rat strains [316] (Lewis and Fischer) which have different disease susceptibilities that are directly attributed to HPAA function. Fischer rats have a hyperactive HPAA while Lewis rats have hypoactive HPAA and are more susceptible to autoimmune diseases [317]. When considering the effect of glucocorticoids on immunity it is critical to differentiate physiological effects from pharmacological effects. Just because exogenous glucocorticoids can down regulate many aspects of immune function, it does not mean they do so in the course of a natural response. For example, corticosterone binds MR preferentially to GR. However the synthetic glucocorticoid, dexamethasone, binds GR more readily and GR is the primary glucocorticoid receptor type on immune cells [305,306]. Therefore exogenous glucocorticoids, specifically dexamethasone, can alter aspects of immune function that are

not normally regulated by the HPAA [48,249-251]. Glucocorticoids levels are tightly regulated and are bound in plasma by albumin and corticosterone binding protein and subsequently bind receptors that translocate to the nucleus in order to exert effects [318]. Therefore measuring circulating levels from denatured samples is not always indicative of glucocorticoid action because the proportion of bound versus free hormone can be altered as can the glucocorticoid binding capacity [319,320].

Activation of the HPAA in response to inflammatory agents such as cytokines, LPS, turpentine and viruses can be fairly rapid (ie. glucocorticoid levels peak at around one hour post-LPS) [321-324]. Glucocorticoids, especially dexamethasone, have been shown to decrease TNF [325-331], IL-1 [325,328,331] and IL-6 [326,331-337] production in response to LPS injection. Conversely, surgical adrenalectomy or blockade of glucocorticoid receptors with RU486 (antagonizes both MR and GR), can alter the response to LPS in terms of increased cytokine production, increased behavioral effects and increased mortality [336,338-349]. The increased lethality to LPS seen in ADX animals is due to the lack of catecholamines as well as the lack of glucocorticoids. Both have major roles in regulating the cardiovascular and metabolic adaptations required in the stress response [13,344,350-352] . Some reports however have observed that the effects of chemical ADX were similar to surgical ADX in terms of the host's increased TNF response to LPS [339,343]. This suggests that glucocorticoids, not catecholamines are the primary inhibitors of cytokines in response to LPS: a finding supported by Molina and Abumrad [353]. It is unknown exactly how glucocorticoids mediate their effects, but since

they bind DNA, they have the potential to effect the regulation of both pro- and anti-inflammatory molecules. The anti-inflammatory properties of glucocorticoids may be mediated through the induction of inhibitory molecules such as IKB [354,355]. IKB is an endogenous inhibitory protein bound to NFKB which is a transcription factor involved in the expression of cytokines in response to LPS.

Although glucocorticoids are not significantly increased in a humoral response [48,249-251], stress-induced levels of glucocorticoids have been shown to alter some components of adaptive immunity [356]. Dhabhar et al., demonstrated that stress increased the delayed type hypersensitivity reaction (DTH), and this was probably due to a glucocorticoid mediated change in cell traffic out of the blood and into the tissues [357-359].

It has also been reported that components of the HPAA can alter immune function independent of the HPAA. For example CRF may be produced directly in immune tissues [360,361]while glucocorticoids can be released independent of increases in ACTH [362,363] suggesting alternative pathways may exist for adrenal cortex activation [364].

1.3.4. Communication Between the SNS and the HPAA

Although the SNS and the HPAA have been described separately, there are many aspects of these systems that are interconnected [365-367]. Central interactions can be demonstrated by the fact that the same central mediators (ie. CRF) activate SNS

postganglionic cells, the adrenal medulla and the adrenal cortex (as measured by circulating NE, E and glucocorticoids respectively) [157,163,368]. As well, feedback from one system may affect the others. For example, glucocorticoids can decrease stress-induced levels of peripheral NE and sympathetic nerve activity [366,369,370]. In response to stressful stimuli animals with adrenalectomy, adrenal medulectomy or removal of the adrenal cortex have shown increased peripheral NE secretion, while 6OHDA treated animals showed increases in peripheral E release as compared to controls [135,142,145,271,366,371-373] . It has also been shown that corticosterone is released and c-fos (a marker for neuronal activation) is expressed in the PVN in response to 6OHDA. However it is unknown if this represents a compensatory response to sympathectomy or is the normal response to nerve damage induced by 6OHDA [246,271,374-376] . Stress has also been shown to induce mRNA for catecholamine synthesizing enzymes in the adrenal medulla. Interestingly this effect is blocked by HYPOX and restored with ACTH [377], while denervation has no effect. Our lab has contributed to this literature by demonstrating that splenic nerve activity is increased in response to immune stimulation in acute ADX animals [378]. Aside from central feedback mechanisms, catecholamines and glucocorticoids may regulate each other locally within the adrenal gland. It is known that the adrenal cortex is innervated and that the innervation may be required for diurnal variation [367,379-381]. As well, mediators from nerves can effect glucocorticoid secretion [382-386] . Therefore in determining the effect of the HPAA and SNS on immunity, cooperative responses must be considered as the system

as a whole functions to preserve homeostasis.

1.3.5. Other endocrine mediators of immunity

There are other circulating factors which can regulate immune function and these include prolactin, growth hormone, nerve growth factor, estrogen, progesterone, testosterone and opioids [387-389]. Nagy and Berczi observed that antibody production against sheep red blood cells, the skin response to dinitrochlorobenzene and the development of adjuvant induced arthritis were all markedly suppressed in HYPOX animals [387]. They later described the role of lactogenic hormones in maintaining immunocompetence [388]. Interestingly, prolactin has been found to increase during the response to endotoxin, [390] and more recent reports have demonstrated prolactin's pro-inflammatory role with respect to cytokine production [391-394]. Therefore the effect of prolactin on immunity may be antagonistic to both the SNS and HPAA.

Sex steroids also play a role in immune regulation. In terms of cytokine production the consensus is that estrogen increases cytokine production [395-398] while testosterone, growth hormone and progesterone decrease cytokine production [398-400]. Ben Eliyahu's lab (Page et al., [401] and Shakhar et al., [402]) found that surgery increased NK cell-sensitive lung metastasis in female rats and that this effect was modulated during the menstrual cycle. Interestingly, Wetmore et al., found that kanic acid lesions in the lateral septal area deceased the antibody response to ovalbumin in female rats but not in male rats [403]. It is unknown if these observations are functionally related

to Ben Eliyahu's, however it is clear that sex hormones can alter immunity.

Opioid peptides have also been shown to alter immunity. Opioids are a class of peptides that are produced throughout the CNS, are cleaved from precursor proteins and bind three known types of opioid receptors; mu, kappa and delta. Enkephalins are cleaved from the pro-enkephalin molecule, dynorphins are cleaved from the pro-dynorphin molecule and beta endorphin is cleaved from the pro-opiomelanocortin precursor (POMC). Opioid peptides are involved in pain modulation pathways and can exert effects in the brain, spinal cord and periphery [404]. Opioids are also released into the circulation from the pituitary during stress [405,406] and can potentially bind opioid receptors located on primary afferents [407] and on immune cells [408]. It is known that opioids are immunosuppressive [409-412], but the mechanism(s) by which opioids alter immune function are unclear. The majority of evidence suggests that the immunosuppressive effects of opioids are mediated centrally. It is known that i.c.v. injection of beta endorphin increases circulating levels of NE, E, and ACTH [413,414] and the immunosuppressive effects of opioids can be abrogated with ganglionic blockers or adrenergic antagonists [411,415,416]. Also, opioid agonists such as morphine can activate central catecholamine systems [417,418]. Morphine injected into specific brain areas like the peri-aqueductal grey area reduced T cell proliferation, NK cell function and LPS-induced TNF production in macrophages [412]. One report described that stress is only immunosuppressive if immunocyte endorphin concentration is increased [419]. It is unknown if this relationship was causative or coincident. However MSH, another

hypothalamic peptide cleaved from POMC, has both central and peripheral anti-inflammatory properties [230,420], so the same may be true for opioids.

D. Befus, R. Mathison and colleagues described a critical role for the submandibular salivary gland in regulating lung inflammation [421,422]. They found that removal or de-afferentiation of the superior cervical ganglion reduced lung inflammation in response to the nematode Nippostrongylus brasiliensis [422]. This effect was reversed upon removal of the submandibular gland suggesting that sympathetic innervation tonically inhibited molecules that down-regulated lung inflammation. The submandibular salivary gland is known to have high levels of nerve growth factor and transforming growth factor beta, both of which can alter immune function [45,422-424] . In conclusion, although the SNS and HPAA are the major efferent paths by which the brain regulates immune function, other neuroendocrine molecules also contribute to this regulatory process.

1.4. Effects of the immune system on the CNS

It has been shown that LPS, antigens and other mitogens and inflammatory agents can all activate the brain in a similar fashion. LPS has been the agent of choice for many neural immune studies due to its ability to reliably activate the host's response and because it models a clinically relevant condition. As well, the response to LPS can be modulated by the dose administered. Lower doses of LPS will induce cytokines, fever, c-

fos expression and nerve activity without corresponding blood pressure changes, shock and/or death [425-429]. Therefore it is not necessary to administer doses that will induce shock and/or death in order to study how the immune system signals the brain.

The pathology of LPS is due to the cascade of events initiated by macrophage derived cytokines. Therefore the initial events induced by LPS are the most critical in determining the final outcome of the host's response. Thus the main goal of this section will be to describe the mechanisms by which LPS (via the immune system) signals the brain and elicits HPAA and SNS responses which subsequently function to negatively feedback on cytokine production. Specific topics to be discussed include the nature of LPS response, pathways by which the immune system signals the brain, the central mediators involved in activation of the CNS in response to immune stimuli and the functional effects of immune activation on neurally-mediated processes.

1.4.1. The Biology of LPS

LPS, also known as lipopolysaccharide or endotoxin, is a molecule on the outer portion of gram negative bacteria that is one of the most potent inflammatory agents known. The defense against LPS-containing bacteria is so critical to survival that many higher organisms have evolved a specific receptor-mediated system to mobilize the natural defences against these pathogens. LPS is comprised of an outer O-antigen (polymer of oligosaccharides), a sugar core and Lipid A embedded in the bacterial cell membrane [430,431]. The latter portion is responsible for most of the immunogenic activity of LPS

[432].

Although traditionally thought of as a B-cell mitogen, LPS can activate many cell types, especially macrophage and endothelial cells [433]. LPS can be given in small enough doses to specifically activate the immune system or in large enough doses to induce widespread effects such as systemic cytokine production, complement activation, hypoglycemia, fever, plasma extravasation, loss of blood pressure and eventually shock and death [434,435]. LPS induces a self-perpetuating sequence of events where a decrease in blood pressure will sometimes cause decreased organ perfusion resulting in ischemia [435]. As well, hemorrhage or shock may cause bacterial release from gut, introducing a new supply of LPS into the bloodstream [436]. Also, LPS causes a decreased response of the endothelium to vasoconstrictors like NE making clinical treatment more difficult [437-440]. Once in the bloodstream LPS ends up in many tissues including the kidneys, lungs, and spleen [430], however the liver is the main source of LPS clearance [441,442]. LPS is transported to the liver by plasma proteins and lipoproteins and is cleared by the gut via the biliary tract [432,443].

Similar to Selye's concept, systemic inflammatory response syndrome (SIRS) is the clinical term for the non-specific response to physiological insults [444,445]. To be classified with this condition, two or more of the following symptoms must be present: increased heart rate, increased respiratory rate and increased white blood cell count. The non-specific nature of this condition is evident in that a marathon runner is likely to have at least two of these symptoms. Sepsis however, is defined as SIRS with an identifiable

pathogen and pus in normally sterile locations. Sepsis can proceed to multisystem failure induced by the hypofusion of tissues, a condition also known as shock [434,444,445]. The mortality in sepsis is 33% but increases to 72% once the systemic effects are evident [443]. It was found that 50% of the cases of shock are due to gram negative bacteria [446]. The current treatment for shock consists of maintaining ABC- airways, breathing, circulation. This is achieved through blood transfusions, pressors (dopamine, dobutamine, NE), Vitamin K (for clotting), and bicarbonate [447]. Antibiotics are also administered despite the fact that one of the side effects is a potential release of more LPS from dead bacteria [430].

Gram negative sepsis is a persistent problem in health care in that there are up to 500,000 cases per year [444] It is the 13th leading cause of death in the U.S. costing 5-10 billion dollars annually [444,448]. Incidents of septic shock are increasing due to the increased average life span, increased age of surgical patients, immunosuppressive therapies, drug resistant strains of bacteria and an increase in invasive procedures [435]. It is critical that the study of the LPS response is continued as the vast majority of clinical trials have not produced significantly improved therapies [449,450]. It is unknown why there are often promising results in animal studies that fail in human trials. This may be due to the fact that in humans, sepsis is a complication of the disease process and not the disease itself [450]. As well, treatment is often given to animals at a much earlier point in the course of the disease than is often the case for humans. It is also important to remember that the reaction of the host to an LPS bolus is transient, while in human sepsis

there are live bacteria which cause chronic activation. Therefore blockade of components of the immune response such as cytokine production, at the wrong time may be detrimental to the elimination of a live infection. This is because cytokines are required to combat live bacteria but are an unnecessary side effect of LPS [450,451]. Also, different species have drastically different sensitivities to LPS which also may be a factor as to why a treatment works in some cases but not others. For example the LD 50 in rats is reported to be 2.5 mg/kg , while rabbits are much more sensitive (at least 5 times)[452]. Mice are similar to rats in their sensitivity to LPS [453,454] while humans are more like rabbits in that experimental studies only use between 0.4-0.8 ng/ml of LPS [455,456].

1.4.2. Molecular Events of LPS Signalling

There are many redundant and interconnected systems involved in the pathogenesis of the LPS response [434,451]. This includes induction or activation of macrophage [45,451,457], endothelial cells [458], the arachidonic acid pathway [459-463], reactive oxygen intermediates [434], nitric oxide production [464,465], the acute phase response from the liver [466,467], histamine [468,469], bradykinin [470] and the complement system [434,471,472]. This cascade of events is initiated primarily by macrophage derived mediators such as TNF [434,471]. TNF can induce many of the same symptoms as LPS including shock [473] and blockade of TNF can reduce the sequelae of LPS-induced inflammation [474]. Although TNF has a pivotal role in initiating the systemic response to LPS, it is not a good predictor of mortality suggesting that the

downstream events are more critical in determining survival [475]. IL-6, a cytokine that can be induced by both LPS and TNF, is a good predictor of mortality in endotoxin induced shock, however its direct effect on the pathogenesis of shock is unknown [476]. Given the fact that there are redundant interconnected pathways, it is important to learn how LPS activates individual cells and initiates its pathogenic cascade.

LPS activates cells through a receptor mediated processes where LPS binding protein (from the circulation) transfers LPS from the circulation to membrane bound CD14 or to CD11/18 (beta integrin) [430,477-479]. LPS activation through either of these membrane bound proteins results in cellular activation as determined by the nuclear location and DNA binding of the transcription factor NFKB [480,481]. However both of these receptors lack the internal machinery to effect the activation of NFKB [477]. The molecule(s) that transduce the signal from the CD14 receptor to the interior of the cell was unknown until recently and was discovered through the study of LPS resistant (C3H/HEJ) and LPS-sensitive (C3H/HEN) mouse strains [482,483]. A genetic locus was identified (designated *lps)* that when mutated, conferred resistance to LPS but increased susceptibility to bacteria [482,483]. An important discovery that helped to identify the molecule responsible for transducing the LPS signal was made in drosophila with the characterization of an IL-1 receptor homolog [484,485]. It was subsequently demonstrated in mammals that LPS activates NFKB via the mammalian counterpart of this IL-1 receptor homolog, *toll4*. It was also found that a mis-sense mutation in *toll*4 was able to confer resistance to LPS in normal mice, thus making it the likely candidate for the

lps locus [482,483,486,487]. Another potential candidate for the *lps* locus was the *toll*2 receptor. This molecule was found by some labs to be essential for LPS-induced NFKB activation. It was also found to require CD14 in order to activate NFKB and similar to the drosophila homolog, had much of the same signalling machinery [488-490]. It is currently unknown whether *toll*4 and *toll*2 are redundant, separate, or interlinked pathways. Takeuchi et al., suggested that both receptors may be involved in pathogen-induced cellular activation, depending on the nature of the bacteria. They found that *toll*4 may be more critical in signalling for gram negative bacteria while *toll*2 is more important for gram positive bacteria [491]. The actual intracellular pathways that confer the signals from either *toll*4 or *toll*2 to NFKB are not fully elucidated. However like the cytokine signalling cascades, many mediators and pathways have been implicated and this includes tyrosine kinase pathways [481,492-494] , mitogen activated protein kinase (MAPK) pathways [493-496] and protein kinase C [495,497].

Aside from genetic resistance to LPS, resistance to LPS can be induced by repeated exposure to either LPS or pro-inflammatory cytokines [498-500]. This phenomenon is known as LPS tolerance and has important implications with respect to surviving chronic infections. All aspects of the host response including HPAA activation and cytokine production are attenuated upon repeated exposure to LPS in many different species [501,502]. The mechanism behind LPS-induced tolerance is unknown; however, down regulation of intracellular signalling pathways is likely involved [496,503]. Others have proposed that tolerance is actually a reprogramming of function as suggested by the

reduction of LPS-induced IL-1 and TNF simultaneous with a potentiation of LPS-induced NO production [504].

1.4.3. Cytokine induction by LPS

LPS rapidly and transiently induces the production of cytokines which serve to stimulate natural immunity and protect the host from infection. However, overproduction of these molecules can damage the host if not properly controlled. This section will describe the biology and function of the main cytokines involved in the response to LPS.

TNF, IL-1 and IL-6 are produced in a cascade like fashion and can initiate most of the host's responses to LPS [45,79,505,506]. These cytokines are produced mainly in macrophage [507-513] but other cells such as endothelial cells and PMN can also produce these molecules [514-516]. The production of TNF, IL-1 and IL-6 are correlated [517] and are often produced in the same cell (as determined by confocal microscopy) [518]. The temporal pattern of expression for these molecules is similar in all mammals where TNF is induced first, then IL-1, and finally IL-6 [45]. TNF is usually present in the plasma by 30 minutes, peaks at an hour (hr) to 90 minutes and is down by 3 hr post-LPS injection [45,321,429,519-521] . IL-1 is present by 1 hour, peaks at 2 hr and is gone by 4 hr where as IL-6 is present at 2 hr, peaks by 3-4 hr and is gone by 5 hr post-LPS [45,321,519]. In vitro studies show a similar sequence of cytokine activation, but the temporal pattern is more spread out. For example in vivo TNF mRNA can be elevated at 15 minutes post-LPS and the protein elevated at one hour post-LPS. In vitro, the

corresponding times are 1 hr and 5 hr respectively [522]. These molecules also form a feedback loop where TNF induces both IL-1 and IL-6 [523-526], IL-1 induces IL-6 [203,527-529] but inhibits TNF [527,529] and IL-6 inhibits both TNF and IL-1 [517,530,531] . All three cytokines have shown the potential to activate the HPAA [532]. However some reports suggest that IL-6 only has a role in sustaining the HPAA response to LPS [322,533,534] while other reports suggest that IL-6 can't activate the HPAA [535]. The results of the latter report may be due to the fact that the use of human cytokines in rats does not induce as robust a response as using rat cytokines [536].

1.4.3.1. TNF

TNF is perhaps the single most important mediator in the response to LPS. TNF is produced mainly by macrophages but can also be produced by activated T cells, NK cells and mast cells [45]. TNF is produced as a membrane bound protein with an extracellular C-terminal that is rapidly cleaved by a zinc-dependent matrix metalloproteinase called TNF alpha converting enzyme (TACE) [537]. TACE mRNA is constitutively expressed in all tissues at a low level [538] and does not increase in response to LPS [539]. TNF is active as a trimer [540] and can bind to one of two receptors that are ubiquitously expressed on a wide variety of cells. TNF receptor type 1 (TNF R1 or p55) mediates most of the effects of LPS, while the type 2 receptor (TNF R2 or p75) serves as a permissive activator of TNF R1 and functions to bind membrane bound TNF [541-543]. Circulating receptors also exist as putative inhibitors of TNF

induced activation.

TNF was originally described in two different contexts: as a soluble mediator that killed tumor cells [544] and as a molecule that caused cachexia [545]. In 1985 Beutler et al. demonstrated that these two molecules were identical [545]. They also demonstrated that blocking TNF could prevent endotoxic shock in mice [474]. The role of TNF in the host response is in part demonstrated by TNF deficient and transgenic animals. Over expression of TNF in the CNS causes inflammation. $TNF^{-/-}$ mice are more resistant to LPS but are more susceptible to living pathogens like Listeria monocytogenes [546]. Similarly, TNF $R1^{-/-}$ mice are also more susceptible to bacterial infections [547]. TNF is also required for proper B-cell development as these $TNF^{-/-}$ mice lack follicles in immune organs [546]. The effects of TNF on the host response depend on the amount of TNF produced. Abbas et al. [45] suggest that lower amounts of TNF will activate endothelium, macrophages and PMN and induce the production of other cytokines. A moderate amount of TNF will also induce the production of other cytokines, and may alter body temperature, activate the acute phase response, induce cachexia and cause blood coagulation. High amounts of TNF will induce hypoglycemia, thrombosis, relaxation of vascular endothelium, decreased blood pressure and decreased organ perfusion; also known as shock [45].

Since the regulation of TNF is so critical to survival, there are many points at which it can be regulated [548-550]. This is evident as LPS can increase the baseline transcription rate of TNF by 3X, the mRNA levels by 50-100X and the amount of

protein by 1000-10,000X [551,552]. Transcriptional regulation is evident due to the rapid and transient induction of TNF mRNA and locations in the promoter region for constitutively expressed transcription factors such as NFKB [553,554]. TNF mRNA also is subject to post-transcriptional regulation. This includes differential splicing in certain cell types, a decreased mRNA stability induced by glucocorticoids and sequences within the 3' region of the mRNA that cause destabilization [327,552,555,556]. The potential for post-translational regulation includes inducible proteins such as heat shock protein 72 which can bind TNF and protect the host from endotoxemia [548.550.557] .

TNF exerts much of its effects on other cells through the activation of transcription factors like AP-1 and NFKB [558]. The activation of these transcription factors is mediated primarily through TNF R1, however TNF R2 can also activate some of these pathways [542,559]. Activation of TNF R1 can induce NFKB binding to DNA and subsequent cellular activation or induce programmed cell death (apoptosis) [401,542,543]. TNF R1 does not have the ability to confer intracellular signals on its own and relies on a group of accessory proteins for this function. The cascade of proteins associated with TNF R1 is initiated with the binding of the TRADD protein to TNF R1 [560]. TRADD is then bound by TRAF 2 which is a common intermediate for both TNF R1 and TNF R2 signalling. TRAF 2 activates a series of protein kinases which are responsible for the cellular effects of TNF including the activation of the transcription factors NFKB and AP-1 [543,561,562]. TRADD can also be bound by FADD, which induces the apoptosis pathway [543]. Other reports suggest that TNF-mediated events

are also induced through membrane-phospholipid signalling pathways [563].

1.4.3.2. IL-1

IL-1 has been described as a mediator of inflammation and as a costimulator for T cells. IL-1 has two forms: IL-1 alpha which is primarily membrane bound and IL-1 beta which is primarily in the circulation. These molecules come from separate genes but share a 30% homology and bind to the same receptors. It is the beta form of IL-1 that is most critical for the host response to LPS and thus is the only one described in the following sections. IL-1 can be produced by many cell types including macrophages, keratinocytes, epithelial cells and endothelial cells. Like TNF, there are two receptors where the type 1 receptor (IL-1R1) mediates the majority of effects [564]. The IL-1 family of molecules is unique in that it includes an endogenous antagonist, IL-1 receptor antagonist (IL-1RA). Like TNF, IL-1 is produced in a precursor form which must be cleaved by an enzyme (interleukin-1 beta converting enzyme or ICE) to become active [564].

IL-1 and TNF have many overlapping functions [565] and like TNF, the effects of IL-1 are dependent on the levels in the circulation [45,566]. The effects of IL-1 include the induction of slow wave sleep, cachexia, fever and the acute phase response [45,564,567]. IL-1 was also the first cytokine shown to activate the HPAA [79,506] . The difference between IL-1 and TNF is that IL-1 does not produce tissue injury and is not lethal when injected systemically [45]. The importance of IL-1 in the response to LPS is in part demonstrated by the fact that ICE -/- mice are resistant to endotoxic shock

where IL-1RA$^{-/-}$ mice are more susceptible to the effects of LPS than control mice [568]. However, redundancies in this system exist as molecules besides IL-1 are able to signal the brain in response to peripheral inflammation as revealed by the fact that both ICE and IL-1$^{-/-}$ mice have normal HPAA responses to LPS [569-571]. However IL-1R1$^{-/-}$ have a reduced response to turpentine, and are more susceptible to listeria monocytogenes suggesting that IL-1 is the primary mediator in response to these challenges [572]. The effects of IL-1 are also demonstrated by exogenous administration of IL-1RA. IL-1RA has been shown to block many IL-1 and LPS-induced effects [566,573-575] , but to be effective, the IL-1RA concentration must be 100 fold higher than the IL-1 concentration [576]. Others have shown that although IL-1RA is effective at inhibiting IL-1 induced responses, it is ineffective against the LPS-induced host response [577]. This corroborates the clinical situation where the use of IL-1RA did not fare well in multicenter trials for treatment of endotoxic shock [578,579].

IL-1 mediated activation is dependent on a protein kinase cascade comprised of many of the same molecules as the TNF and LPS-induced cascades [561,580]. However other systems may be involved depending on cell type [581,582]. These signals are activated primarily through IL-1R1 in conjunction with IL-1R accessory protein. MyD88 is then recruited to this complex which activates IL-1R associated kinases 1 and 2. These proteins, along with TRAF6 mediate the effects of IL-1 including NFKB activation and IL-6 production [561,580]. Also, like TNF, IL-1 is subject to regulation at multiple points in its expression [525,583,584].

1.4.3.3. IL-6

IL-6 is a cytokine produced mainly by macrophages but it can also be produced by other cell types such as fibroblasts, hepatocytes and endothelial cells [45]. Although IL-6 is a growth factor for activated B-cells, its more characterized role is with the regulation of the acute phase response. The acute phase response is a rapid adjustment of plasma proteins in response to trauma, stress, burns etc. [45,585]. Some of the proteins produced during the acute phase response include clotting factors, anti-proteinases and opsonins. Although IL-6 is not responsible for most of the deleterious effects of endotoxemia, it is a good predictor for outcome and severity [476,586-588] . Another role for IL-6 is in sustaining the final phase cytokine/LPS-induced HPAA cascade. For example, blockade of IL-6 reduces corticosterone production at later times such as 3 hr post LPS, but not at 1 hr post LPS [322,533]. In agreement with this, IL-$6^{-/-}$ mice show c-fos expression after LPS, but the expression is shorter lived [534]. At least in mice, IL-6 is the major factor in the local inflammatory response to i.m. turpentine. It was shown that IL-$6^{-/-}$ mice demonstrate a normal response to LPS, but are unable to mount an HPAA response to i.m. turpentine [589]. It is possible that the reason IL-$1^{-/-}$ mice also do not mount an HPAA response to i.m. turpentine is due to the lack of IL-6 [531].

1.4.4. Measures of Central Activation

There are many measures of neural activation utilized in brain-immune studies.

These include measuring peripheral HPAA and SNS activity (via glucocorticoids and catecholamines respectively), indexing changes in central mRNA for hypothalamic mediators and catecholamine synthesizing enzymes, measuring central neurotransmitter levels and/or turnover, recording central and peripheral nerve activity and assessing central c-fos expression. As well, more functional measures such as behavioral changes and body temperature have also been used. The rationale for measuring catecholamines, glucocorticoids and nerve activity has been described. This section describes the merits of using c-fos expression, behavioral changes and changes in body temperature as indicators of central activation.

C-fos is a proto-oncogene that interacts with c-jun to form the AP-1 transcription complex. C-fos has been extensively used as a marker for neuronal activation as its expression is transitory and independent of protein synthesis [590-594] . Wan et al., showed that LPS given i.c.v., i.p., or i.v. maximally induced c-fos expression in the PVN, SON, NTS and A2 regions of the brain 2-3 hours after injection [32,90]. Others have described c-fos expression in additional structures following LPS including the arcuate nucleus, ME, OVLT, MPOA, LC, Parabrachial nucleus, amygdala, AP, ventrolateral medulla and other central structures [31,595-599] , as well as in vagal afferents [600] and preganglionic sympathetic neurons [601]. Cytokines [505,602-605], CRF [606], psychological stress [90,606-608] and central PGE2 [105,609] all induce similar patterns of c-fos expression. Although c-fos is a good functional marker for neuronal activation, its biological function in response immune mediated central activation is unknown. One

possibility is that since it binds the promoter regions of catecholamine synthesizing enzymes [610-612], it may mediate the compensatory response to various stimuli. Hunt et al., found that after peripheral inflammation, c-fos expression was localized in the dorsal horn of the spinal cord, not the dorsal root ganglion. This suggests that cells expressing c-fos may be postsynaptic to the cell that is initially activated by the stimulus [613].

The hypothalamus is the major integration center for homeostasis [94], therefore behaviors which function to preserve homeostasis may be engaged by the hypothalamus. Sickness behavior in response to LPS or other challenges is a set of protective measures designed to preserve energy. These behaviors include hypomobility, hypophagia, increased sleep, decreased libido and decreased exploration [33,614]. The rationale behind energy conservation is that the body increases its core temperature in order to inhibit the pathogen replication cycle [33]. For every degree increase, the body requires an extra 10% in energy [85]. This increase in the set point of the body temperature is known as fever. Fever is a phylogenetically old response to infection that represents a coordinated physiological response with autonomic, neuroendocrine and behavioral components [84,85]. Fever is maintained in part by a decreased blood flow to cutaneous vascular beds and a decrease in sweat production. Fever is often accompanied by shivering, an increase in blood pressure, anorexia and malaise [83,84] . Fever is regulated by the hypothalamus and mainly by the POA, OVLT and AH regions [85,615-619]. Fever can be induced by central and peripheral LPS [620,621], turpentine [35,83], central and peripheral cytokines

[83,615,620,622], central PGE2 [618,619] and stress [621,623-626] in many species.

1.4.5. How immune signals reach the brain.

It is clear that the immune system activates the brain as demonstrated by the effects of an immune challenge on the SNS and HPAA [10,48,76]. Concurrently there is increased c-fos expression in the PVN and brainstem [32], there is increased catecholamine turnover in the hypothalamus [50], and there are changes in body temperature [85,627,628] and behavior [629]. The first reports of cytokines being able to activate the brain were in 1987 from Sapolsky et al., [506] and Berkenbosch et al., [79] who demonstrated that IL-1 could activate the HPAA. However, since increases in corticosterone and splenic nerve activity often precede detectable changes in plasma IL-1 [429,519,630,631], other mediators must play a role in brain activation, at least in the initial stages. This section will examine the potential pathways by which peripheral immune stimuli such as cytokines can activate the brain. The more contentious issue of central cytokine production and their roles as signalling intermediates in neural processes will be described in section 1.4.6.1. The exact pathways by which immune signals reach the brain remain unknown but candidate pathways include cytokines activating the brain through the CVOs, through disruption of the BBB, through direct activation of endothelial cells as well as through afferent nerves [70,90,632].

1.4.5.1. The role of circulating cytokines

There is evidence to suggest that peripheral cytokines can activate the brain via CVOs. CVOs are areas in the brain that have leaky blood brain barriers and are in contact with both the CSF and blood. CVOs monitor blood levels for specific peptides and electrolytes and send afferent projections to other brain areas such as the brainstem and hypothalamus [118,119]. Examples of CVOs include the OVLT, SFO and AP. It is currently unknown if CVOs function to actively signal the CNS regarding peripheral inflammatory agents, act as a barrier to prevent circulating cytokines from leaking into the brain and activating the CNS or produce cytokines and prostaglandins that are released on the brain side of the BBB and then diffuse to the central structures that are known to be activated during peripheral inflammation.

Ascending catecholaminergic fibers from the NTS (A2 region) are essential in transmitting signals from peripheral (i.v.) immune challenges to the brain [105,605,633] . The NTS is in direct communication with the AP [118,119,634] and the role for the AP and other CVOs in monitoring the blood for immune agents is suggested by the fact that CVOs express mRNA for TNF, IL-1 and LPS receptors [635-637]. Interestingly some studies found that CVOs expressed c-fos at lower doses than other brain areas [595,596]. In support of this, Lee et al., found that removal of the AP significantly attenuated ACTH and corticosterone release and c-fos mRNA expression in the NTS and PVN in response to i.v. IL-1 [603]. However, the NTS is often damaged upon removal of the AP so non-specific effects on the NTS cannot be entirely ruled out for this effect [118,603]. In contrast to this work, Ericsson et al., found that neither AP ablation nor

subdiaphragmatic vagotomy altered c-fos in the PVN and NTS after i.v. IL-1 [109]. The reasons for the differences in results between these two groups are not known; however, dose may be a factor as Ericsson used a much higher dose than Lee (1.87 μg/kg vs 0.5 μg/kg). Ericsson did determine that CVOs express c-fos in response to IL-1, but it took a 10-fold higher dose than was required to induce fos expression in the PVN [605]. Derijk et al., [638] also showed that alternative pathways of brain activation may exist depending on the dose of the stimulus. Therefore higher doses of cytokine may activate additional and redundant pathways.

The expression of c-fos, CD14 and IKB in CVOs and endothelial cells indicate that these cells may also produce cytokines and other intermediates in response to immune challenge [637,639,640]. These mediators would then be released inside of the blood brain barrier and cause central activation. This is suggested by the fact that CVOs and endothelial cells are the first areas in the brain that produce cytokines in response to LPS [636,641,642] and that cytokines can be found in the CSF and leptomeninges [641,642] despite not being able to cross the blood brain barrier [223,642-644] . PGE2 is a lipophilic mediator that can cross the BBB and definitively plays a role in signalling the brain in response to peripheral inflammation. Both Wan et al., [90] and Ericsson [109] found that blockade of PGE2 production attenuated c-fos expression in the PVN in response to an inflammatory stimulus (either i.p. or i.v.). Ericsson et al., [109] found that injection of PGE2 into the medulla mimicked i.v IL-1 in terms of c-fos induction [109] and MacNeil et al., found that central PGE2 injections mimicked the effect of systemic

LPS on the activation of splenic nerve activity [645]. Scammell et al. [646] found that PGE2 injected into the POA was involved in the induction of fever. Therefore it is possible that cytokines can activate perivascular cells in the brain to produce PGE2 (which can cross the BBB) which then activates neurons that innervate the hypothalamus. In support of this, both LPS and cytokine receptors are localized on brain endothelial cells and CVOs, and LPS, turpentine and cytokines induce c-fos, COX-2, IKB and cytokines in these same cells [636,637,647-654] .

Cytokine receptors are distributed throughout the brain [86,635,655-657] which suggests that cytokines can activate the brain directly; however as previously mentioned, cytokines can't cross the blood brain barrier [223,642-644] . Other proposed mechanisms of how peripheral cytokines can gain access to central cytokine receptors include LPS disrupting the BBB [658,659], immune cell migration into the brain [660-662], or cytokines being imported into the brain by active transport mechanisms [663,664]. In support of this, Katsuura et al., demonstrated that i.v. IL-1 induced corticosterone more rapidly if the OVLT was lesioned. They also showed that it took a longer time for IL-1 to induce corticosterone release if the POA was lesioned (the OVLT and POA are in extremely close proximity) [665]. This suggested that if the barrier was removed (the OVLT) the reaction was more rapid, but if the structure that relayed the signal to the HPAA was ablated (the POA) then the reaction was slower. However the doses of LPS that are high enough to disrupt the BBB are often high enough to induce shock and potentially death. Therefore it is unlikely that this signalling pathway mediates "normal"

inflammatory processes. As far as macrophage migration and the active transport process are concerned, it is likely that they are also not major signalling pathways as they work on limited scales. However cytokines like IL-1 are extremely potent biological signals, and as noted by Maier et al., it is unknown how much IL-1 is required to enter the brain in order to activate it [632].

1.4.5.2. The role of afferent nerves

Some of the first evidence to suggest that afferent nerves transmit immune-related signals to the brain were made by and Basbaum and Levine. They found that joints with a greater afferent innervation had more severe adjuvant-induced arthritis [70]. They also found that capscaisin, which destroys small afferents nerve fibers, attenuated inflammation in both the injected joint and its bilateral counterpart (in this model unilateral injection causes bilateral inflammation of the joints via the CNS) [92]. Wan et al., were the first to show that visceral innervation played an important role in the activation of the CNS in response to an inflammatory response [90]. They discovered that subdiaphragmatic vagotomy abrogated c-fos expression in the PVN in response to i.p. LPS. However c-fos expression in response to i.v. LPS was only mildly attenuated. Recently, Gaykema et al., extended these finding to include c-fos expression in the nodose ganglion [666]. Others labs found that cytokine expression in the brain was abrogated in vagotomized animals in response to i.p. LPS or i.p. IL-1 [667,668]. The mechanism by which this occurs is still unknown. However the labs of S.F. Maier and L.N. Watkins

have proposed that local cytokines can directly activate afferent nerve fibers. They showed that biotinylated IL-1RA binds glomus cells located in vagal paraganglia [669] and that IL-1 protein is found in the connective tissue of the vagus nerve [600]. This is supported by Ek et al., who showed IL-1R1 mRNA expression in the cell bodies of vagal sensory neurons, an increase in c-fos expression in these cells after i.v IL-1 and an increase in vagal nerve activity after i.v. IL-1 [670]. However, Ek et al., also demonstrated that prostaglandin receptor mRNA was present in the cell bodies of vagal afferents suggesting that visceral prostaglandins may also play a role. Some studies have observed the effect of vagotomy on response to i.v. immune challenges [632,671]; however, the reasons for these effects are unknown.

In summary, it is likely that many overlapping and redundant mechanisms and mediators exist to signal the brain in response to peripheral immune challenges. For example although Wan et al., found that subdiaphragmatic vagotomy significantly reduced c-fos expression in response to i.v. LPS, the magnitude of this change was nowhere near the effect observed for i.p. LPS and not given much emphasis. This effect was verified by Gaykema et al., looking at fos expression in the nodose ganglion [666]. The lack of a major vagotomy effect on c-fos expression in response to i.v. challenges may be due to the fact that LPS is able to activate vagal afferents above the diaphragm as well as activating brain endothelial cells and CVOs. This can't be proven directly as animals with cervical vagotomies do not survive long enough to complete the studies [632]. Vagotomy may also not work with all i.p. LPS challenges as serum cytokines are still increased [632]

and LPS is detectable systemically within 15 minutes [672]. Further evidence suggesting alternative mechanisms were proposed by Turnbull and Rivier who showed that the early corticosterone and ACTH response to i.m. turpentine was mediated by afferent nerves while the later peak was due to circulating cytokines, specifically IL-6 [274]. In terms of mediators, although IL-1 was the first cytokine shown to activate the brain, TNF and IL-6 can also activate the HPAA, and in some cases are more critical. For example increases in LPS-induced corticosterone (30 minutes [519,630]) and splenic nerve activity (22 minutes [645]) often precede increases in serum IL-1. In some reports increases in plasma TNF coincide temporally with increases in corticosterone levels and nerve activity [429]. Therefore since TNF can induce IL-1, it is likely that TNF is the major mediator in the host's response to LPS, at least in the initial stages [673]. Also likely is the fact that both LPS (via CD14 on endothelial cells) and cytokines activate the same systems and do so in a cascade-like fashion. This is suggested by the fact that blockade of IL-6 is only able to attenuate corticosterone and c-fos production at later time points [322,534] and that blockade of both TNF and IL-1 is not able to block the corticosterone response to LPS [577]. Also of consequence is that IL-1 or $ICE^{-/-}$ mice continue to show HPAA responses to LPS [569-571]. Therefore, like most critical regulatory processes, redundant systems exist.

1.4.6. Mediators of central activation.

It is known that viral infections, i.m. turpentine as well as i.v., i.p., and i.c.v. LPS

and cytokines can all activate the brain as determined by one or more of the following: increased HPAA activity [32,79,506,533,674-677], induction of fever [35,620,622,627,678-680], increased nerve activity and/or peripheral catecholamine release [73,75,681,682], increased central neurotransmitter turnover [36,535], behavioral changes [33,42,629,683,684] and c-fos expression in the PVN and brainstem [31,90,109,505,596,597,602,605]. Irrespective of how these signals arrive, the central signalling pathways must have similarities as ultimately common anatomical structures are involved in the responses to these diverse stimuli. Wan et al., [90] and Ericsson et al., [109] demonstrated that both LPS and IL-1 (respectively) induced c-fos in the brainstem and PVN. Also, these increases in c-fos expression, following both i.v. and i.p injections could be attenuated or abrogated by indomethacin. Indomethacin was also shown to abrogate the increase in splenic nerve activity observed after LPS [645] and abrogated some, but not all, of the behavioral effects of influenza virus [42,684]. Related to this, c-fos and other indicators of central activation were attenuated or abrogated upon elimination of the ascending catecholamine pathways that connect the brainstem to the hypothalamus [105,605,633] . However, the effects of psychological stress on HPAA activity and c-fos expression were not eliminated by either indomethacin or the depletion of ascending catecholamine pathways. This suggests that there are central pathways that are specifically activated in response to immune-related stressors [39,41,105].

The likely (although relatively simplistic) cascade of immune activated central mediators as proposed by Ericsson et al., [109], and verified by our lab [105] include: an

immune challenge which activates prostaglandins which activate ascending catecholamine pathways which activate CRF cells to cause HPAA and SNS activation.

The rest of this section will describe the role of central cytokines, prostaglandins, ascending catecholamine fibers and other mediators with respect to mediating the central signals involved with the peripheral immune response. Data presented on this cascade will amend, refute or clarify this pathway with respect to the different dependent measures being described including SNS activity, HPAA activity, fever, c-fos expression and sickness behavior.

1.4.6.1. Cytokines

It is important to distinguish between the role of central and peripheral cytokines. From the previous sections it is known that peripheral cytokines can activate the brain. Also, although local inflammation is different in the CNS as opposed to the periphery [660,685], i.c.v. LPS will induce cytokines and inflammation in the brain [661,662,675,686-689] . The question remains as to the role, if any, of central cytokines in the biochemical signalling pathways that activate the SNS, HPAA, fever and changes in behavior in response to peripheral immune challenges (reviewed in detail by Maier et al., [632]). The confusion regarding this topic is best summarized by Turnbull et al., who showed that the ACTH response to i.m. turpentine could be blocked by inhibiting central TNF protein [675]. This was in spite of the fact that no increase in circulating TNF was observed, and both bioassay an in situ hybridization failed to detect central TNF mRNA

or protein. RT-PCR detected low levels of TNF mRNA throughout the brain, however no difference was observed between experimental and control animals. Also, some of the areas in the brain that expressed cytokines, were areas like the cortex which are not likely to be involved in signalling the hypothalamus in response to peripheral immune challenges.

The role for central cytokines as part of the signalling cascade in response to peripheral stimuli is suggested by the fact that there are cytokine receptors in the brain [86,655-657,690,691], some groups have reported constitutive expression of cytokine mRNA and protein in the brain [642,643,692-696] and that i.v LPS and cytokines do not cross the BBB, yet induce HPAA and SNS activation [84,642-644,668,692,697] (although this can be partially explained by cytokine receptors on endothelial cells and CVOs.). Further evidence includes that central cytokines are increased during non immunological stimuli such as psychological stress [207,698,699], and that the blockade of central cytokines alters measures of central and peripheral activation [574,675,700-704].

However, further scrutiny of these data raises more questions. First, there are many reports that show central cytokines are not expressed basally and must therefore be induced. Many of these reports also show that induction occurs in a cascade-like manner. This means that the CVOs and meninges are the first areas to express cytokines, and usually by 1-3 hr, while the brain parenchyma is not usually positive for cytokines until at least 5 hr post-LPS [641,642,648,650,651,699]. This could suggest that the integrity of

the BBB is compromised and central cytokine induction is a function of diffusion of inflammatory mediators. Many of these latter studies rely on immunohistochemistry and in situ hybridization to localize cytokines while most of the studies that show basal expression utilize RT-PCR, bioassays and ELISAs; techniques which require homogenization of the tissue. Thus the anatomical specificity of the molecules in question is more difficult to determine as circulating cells and endothelial cells can also produce cytokines. Another consideration is that even if there is central induction of these cytokines, their late appearance would preclude their mediating the immediate neural and behavioral responses. Therefore it is perplexing how blockade of undetectable levels of central cytokines in the early stages of a response can have inhibitory effects on behavior. HPAA activation and immune function [632,675]. It is also possible, as suggested by Maier at al., these molecules may be so potent that undetectable levels can mediate systemic effects [632].

The dose of LPS is also an important consideration in these studies as doses that are able to induce cytokines in the CSF and brain in the same time frame as the spleen and plasma, can also disrupt the BBB [658,705]. It is also possible that macrophages can migrate through the BBB [660], but this does not usually occur until later in the inflammatory reaction and approximately at the same time that cental cytokines appear [661,662]. Pitossi et al., [697] disagreed with these contentions as they observed an increase in cytokines in the brain simultaneously with the spleen using a dose of LPS they claimed did not rupture the BBB (20μg i.p. per mouse) [697]. However the effects of

this dose of LPS to on the BBB were not tested directly but rather referred the work of Lustig et al., [659]. Using a model of neuroinvasion, Lustig et al., demonstrated that 100% of the animals (mice) showed a disrupted BBB after 100μg of i.v. LPS. However, 50% showed a disrupted BBB in response to 10μg of i.v. LPS. Although Pitossi used an i.p. challenge of 20μg, it is known that LPS is in the circulation within 15 minutes of an i.p. injection despite a first pass through the liver [672]. Thus the possibility of a disrupted BBB can't be ruled out. Another point against cytokines as central mediators of the peripheral immune response is that cytokines and cytokine receptors have been localized in brain areas that are not related to the signalling cascade in question. Unless there is local inflammation within the CNS, the relevance of cytokines and their receptors in areas like the cortex, striatum and cerebellum [86,643,655,697,706]is difficult to explain unless they are predominantly endothelial in origin. In the latter case, their relevance has been discussed in the previous sections.

1.4.6.2. Prostaglandins

Prostaglandins are synthesized from arachidonic acid by cyclooxygenase (COX) which has two isoforms: a constitutive form, COX-1, and an inducible form, COX-2. Although prostaglandins have many peripheral functions, including direct effects on the cardiovascular system and the immune system, this section is concerned with prostaglandins' role as central mediators during an inflammatory response.

Unlike cytokines, the role for prostaglandins as central mediators in the response

to peripheral immune challenges is firmly established. Central prostaglandins have been shown to be involved in LPS and cytokine induced fever [707-710], splenic and renal nerve activity [73-75,645,711,712], peripheral catecholamine release and/or turnover [72,713,714], central catecholamine turnover [72], c-fos induction [90,105,109,609,715-717], HPAA activity [665,718,719], behavioral alterations [42,684] and the regulation of peripheral immunity [717,720]. Although central PGE2 is the most thoroughly studied cyclooxygenase dependent prostaglandin in the response to peripheral LPS, i.c.v. injections of PGF2 and PGD2 also have the ability to alter the LPS-induced peripheral cytokine production [Nance et al., unpublished observations]. As well, i.c.v. PGF2 can increase plasma NE [721] and induce fever [722] while i.c.v. PGD2 can induce slow wave sleep [723] and increase hypothalamic NE turnover [72]. This suggests that more than one cyclooxygenase dependent prostaglandin may be involved in regulating the peripheral response to LPS.

The means by which PGE2 can affect centrally-mediated functions are unknown. This is because both peripheral (peripheral meaning outside the BBB) [652,670,724] and central prostaglandin [646,725,726] formation have the potential to regulate central processes. We will describe fever as an example as it has been known for many decades that central PGE2 can induce fever [85]. In 1983 Blatteis et al., showed that the anterior region of the third ventricle (which contains the POA and OVLT) was important in the febrile response to LPS [727]. This was later verified by other labs [646,707,710,728]. Also, it is known that i.v. LPS induces PGE2 in the POA and that these rising levels

correspond to the appearance of fever. Both fever and the levels of PGE2 in the POA were inhibited by indomethacin [707]. However there is a possibility that prostaglandins from many sites play a role in fever induction. In response to certain doses of LPS, a biphasic fever is produced in many species [729]. It was shown that the first phase is blocked by subcutaneous indomethacin while the second phase required i.c.v. indomethacin in order to be attenuated. The peripheral source of PGE2 is most likely from vascular endothelial and microglial cells as well as cells from the CVOs. These cells produce COX-2 in response to LPS and cytokines [639,652,724,730-738] as well as expressing c-fos and IKB [31,596,597,604,648,649,739] . LPS is known to induce NFKB which can transcribe COX-2 mRNA [740,741], while IKB expression (the inhibitory protein to NFKB) is likely a compensatory response to this. COX-2 is the likely isoform responsible for PGE2 production during fever as COX-$1^{-/-}$ mice still have fever in response to LPS, but COX-$2^{-/-}$ mice do not [742]. Also, specific COX-2 inhibitors reduce or attenuate fever in response to LPS and cytokines [733,735,743]. Related to this, glucocorticoids can inhibit stimulated, but not basal levels of PGE2 in ex vivo brain slices [744]. Endothelial PGE2 is an effective mediator because endothelial cells are located throughout the brain and PGE2 can readily diffuse across the BBB to exert physiological effects [654]. Ek et al., demonstrated that prostaglandin receptor mRNA was present in the cell bodies of vagal afferents suggesting that visceral prostaglandins may also play a role in signalling the brain in response to peripheral inflammation [670]. In terms of central PGE2, COX-2 can be found in neurons [653] and inhibition of PGE2 formation

directly in the POA has been shown to inhibit LPS-induced fever. However injections of PGE2 in the POA were more pyrogenic the closer the injections were to the OVLT. This contributes to the notion that diffusion from endothelial or CVO cells is the most likely source of PGE2 in the brain [728]. It has also been shown in vitro that IL-1 can induce prostaglandins synthesis from hypothalamic and hippocampal explants [725,726]. Unfortunately it is unknown if the source of the PGE2 is from neurons or endothelial cells.

In order to identify the locations in the brain where PGE2 is exerting its effects, studies have explored the effects of microinjections of PGE2 in various brain areas as well as looking at receptor distribution. It was first shown that i.c.v. PGE2 will induce many of the same effects as peripheral LPS or cytokines [105,639,645,715,717]. Injections of prostaglandins i.c.v. [645] and in both the medulla [109] and POA [728] mimic the effects of peripheral LPS or cytokines. The highest density of binding sites for PGE2 were found in the anterior wall of the third ventricle [745,746]. The NTS, PVN and LC also have PGE2 binding sites [746]. In terms of receptors there are at least six types of receptors that bind prostaglandins: EP1, EP2, EP3a, EP3b, EP3c and EP4 [747]. Sugimoto et al., [748] found EP3 receptor mRNA was widely distributed throughout the brain including areas such as the POA and the hypothalamus [748]. Zhang and Rivest found basal EP2 mRNA expression in the BNST, LSA, SFO, VMH, CeA, LC and AP, and basal EP4 mRNA expression in the POA, PVN, SON, PB, NTS, and VLM [749]. Functionally, an i.c.v. EP1 agonist, but not an i.c.v. EP2 agonist, was shown to activate splenic nerve

activity [711]. However much remains to be elucidated on the location and characterization of the functional EP receptors involved in mediating the central response to peripheral challenge.

In the most simple interpretation of the sequence in the signalling cascade, PGE2 is upstream from CRF. This is suggested by the fact that CRF induced splenic nerve activity is not blocked by indomethacin yet PGE2 induced nerve activity is blocked by alpha helical CRF (a CRF antagonist) [712]. In support of this Terao et al., showed that i.p IL-1, i.c.v. PGE2 and i.c.v. CRF all induced NE turnover in the spleen. Indomethacin blocked the IL-1 but not the CRF induced turnover of splenic NE [72]. Also, it was shown by Bernardini et al., that hypothalamic cells will secrete CRF in vitro if stimulated with ecosanoids [750]. MacNeil et al found that i.c.v. blockade of CRF slightly but significantly reduced splenic nerve activity in response to i.c.v. PGE2. Interestingly, this effect was more pronounced for PGE2 induced renal nerve activity. They also found that the renal nerve's increased activity was blocked by OXY and VP antagonists whereas the increase in activity of the splenic nerve was most potently inhibited by OXY antagonists [133]. This reinforces the view that CRF is not the only hypothalamic peptide involved in activating the SNS and HPAA. However the pathways that mediate fever induction and the other aspects of central activation may differ due to ubiquitous nature of prostaglandins and their receptors. For example, Rothwell found that alpha helical CRF blocked the fever in response to PGF2 but not PGE2 suggesting that although both CRF and PGE2 can induce fever, they may do so by different mechanisms [722].

1.4.6.3. Catecholamines and other central mediators

It has been shown that CRF and other hypothalamic peptides are important in activating the SNS and HPAA in response to immune challenges. However the question remains as to the upstream mediators that regulate the release of these peptides from the hypothalamus in response to these peripheral challenges. It is known that PGE2 is upstream of the hypothalamic peptides in terms of mediating responses to peripheral immune challenges [133,712,751]; however a role for central catecholamines has also been demonstrated. This is based on the fact that noradrenergic cell groups that are activated in response to peripheral inflammatory challenges contain mRNA for PGE2 receptors and innervate CRF cells in the PVN [94,95,102,104,106,108,120,310,752-754] . Secondly, NE release and turnover are increased in the hypothalamus in response to various stresses and immune stimuli [755-758] . Also, stimulation of the ascending catecholamine fibers increases CRF in the portal circulation [759] and CRF mRNA in the hypothalamus [760] (this effect can also be shown in vitro [761]). In support of this, removal of the noradrenergic ascending pathways decreases CRF in hypophyseal portal blood [762,763]. Functionally, ablation of the ascending catecholamine fibers from the brainstem to the hypothalamus can abrogate or attenuate HPAA activation and c-fos expression in the PVN in response to LPS or IL-1[39,41,105,764]. Interestingly, destruction of these pathways also abrogates some of the effects of ether stress [765-768], but not of footshock stress [39,41,105]. It is unknown which central adrenergic receptor is critical to

activating the central systems in response to a peripheral immune challenge, however some reports have suggested that the alpha-1 receptor is involved [769].

Although the evidence presented indicates that PGE2 activates NE positive cells which in turn activate hypothalamic peptides, this is a simplified explanation as other mediators may be involved depending on the nature of the initial challenge and the nature of the output measure studied. As well, these mediators can be a part of the same or separate pathways depending on the paradigm. For example, CRF is a major mediator of psychological stress [277] and is distributed in many brain areas that are involved in the stress response. There is a reciprocal relationship between NE and CRF in that CRF can induce NE release in certain paradigms, which is opposite in sequence as to what is described above. Nakamori et al., demonstrated that alpha helical CRF attenuated fever in response to cage switch but not in response to either i.c.v. PGE2 or i.p. IL-1 [623]. However alpha helical CRF did attenuate the fever in response to i.c.v. IL-1 [623]. Therefore there is not a single linear pathway that mediates all the centrally-controlled responses to immune challenge.

To further illustrate the complexity of these pathways, many investigators have shown that mediators other than cytokines, PGE2, catecholamines and CRF are involved in mediating the central response to immune challenge. These include glutamate, GABA, histamine, NO, serotonin and MSH (reviewed by Turnbull et al., [677]). Wan et al., found that PGE2, glutamate and histamine were involved in the LPS-induced expression of c-fos in the PVN and SON [90]. The role of histamine in the LPS-induced HPAA response to

endotoxin was confirmed by Givalois et al. [770], and Knigge et al [771]. Knigge et al., found that LPS-induced increases in ACTH and beta endorphin release were blocked by an i.p. or i.c.v. histamine receptor 1 antagonist. This is in agreement with Wan et al., who showed that c-fos expression was also abrogated by an histamine receptor 1 antagonist, but not a histamine receptor 2 antagonist.

A.T.K. Jackson showed that LPS-induced c-fos could be attenuated with the NOS blocker, L-NAME [105]. L-NAME could also attenuate PGE2-induced c-fos suggesting NOS was downstream of PGE2 and facilitatory to c-fos production. In support of this Lee et al., [772] and Harada et al., [773] found that LPS increased NOS mRNA in the PVN. Also, McCann et al., stated that NO neurons can stimulate the release of CRF and OXY [774]. Recent reports from Rivier and colleagues support Jackson's original observations [775] in that they found that i.c.v. injection of the NOS donor, SIN-1, increased plasma ACTH (via a CRF dependent mechanism), and increased the hnRNA for CRF and VP. However, they also found that NOS activation was not dependent on prostaglandin synthesis.

Serotonin is another possible central mediator in the response to peripheral inflammation as there are serotonin positive fibers in the PVN and SON [776]. In support of this it has been found that serotonin metabolism increases in response to peripheral LPS and cytokines[535,777]. Also, blockade of serotonin reduced corticosterone release and c-fos expression in response to LPS [769,778]. However others have shown no effect on corticosterone release or c-fos expression in serotonin depleted animals (as compared

to control) in response to LPS [779]. The reasons for the contradictory results between these groups are unknown.

As previously mentioned, MSH is induced centrally by inflammatory agents [224] and is an important central mediator for the inhibitory SNS pathways [225-229]. MSH given i.c.v. reduces peripheral inflammation, and this effect is dependent on an intact SNS [228,230].

In summary, further study is warranted to determine if PGE2, NE, histamine, NO, glutamate, serotonin, MSH and other mediators represent distinct or interrelated pathways that activate the HPAA and SNS in response to peripheral immune challenge.

1.5. The Effect Psychological Stressors on Brain-Immune Interactions

The relationship between emotion/mood and immunity has been observed empirically for generations. More recent studies have used statistically-based methods to verify that stress can suppress immunity in humans [4-7,22-25,780,781] . Conversely, depression has been associated with inflammation, demonstrating a potential reciprocal relationship [21]. Defining the stressor-specific pathways that mediate these relationships are the next challenge to this field. As predicted by Selye, different stressors whether they be psychological or immunological, will activate the same central systems [33,34,38,42,90], although by different mechanisms [39,41,105]. As well, different psychological stressors, such as exposure to ether or restraint may also work though

distinct pathways [782,783]. This section will briefly describe the activation of the SNS and HPAA in response to stress and summarize the possible central mediators involved in the activation of the stress response and the adaptation to stress. Following this, the effect of stress on immunity will be reviewed.

1.5.1. Stress-induced activation of the HPAA and SNS

Psychological stress will be broadly defined as being a stress with a predominantly interpretive component. In animal models psychological stressors include forced exercise, ether, immobilization/restraint, tailshock and footshock stress. Ether and shock stress have noxious physical components as well as psychological components, so they are not pure psychological stressors. Pan et al., [784] demonstrated that the noxious components of a stressor (hot water or electro acupuncture) will induce c-fos the brain in anesthetized animals. However, the increased heart rate observed with these stressors was eliminated with anesthetic, suggesting an interpretive component. The noxious stressors however could not signal the brain in capsaicin-treated animals (no pain afferents). Other stresses such as cold stress and insulin stress are primarily physiological stressors, but no doubt have psychological components as well.

The main indices of peripheral HPAA and SNS activation are elevated levels of peripheral catecholamines and corticosterone respectively. Although the central pathways and mediators are not definitively elucidated, CRF, VP and OXY release from the hypothalamus are all potentially involved for both the SNS and HPAA responses to

stress [1,157,275,785]. All three of these molecules innervate brain stem and spinal cord neurons and can cause the release of ACTH. The PVN is the main structure involved in relaying the central signals of stress to the SNS and HPAA as lesioning of the PVN will attenuate the ACTH response to stress [786] and abrogate the stress-induced immune suppression [787].

The response of the HPAA to stress is rapid. Activation is initiated with the release of neuropeptides from the hypothalamus (CRF, OXY, VP [788,789]) which causes the release of ACTH from the anterior pituitary which causes the release of glucocorticoids from the adrenal cortex. Corticosterone may be increased as quickly as 2 minutes after stress [790], peaks by 15-20 minutes [786,791] and is back to baseline by 1 hour. Glucocorticoids feedback onto the brain and decrease CRF release as well as NE turnover in the PVN in vitro [792]. Both CRF and VP have been shown to be essential in mediating the release of ACTH in response to stress and can be released from the same cells [793]. CRF has been considered the main central mediator of stress as CRF is widely expressed throughout the CNS, including areas that are involved in the stress response [18,788]. In support of this, within 5 minutes of i.c.v. CRF, rats show an increased heart rate, body temperature, and incidents of stress-related behaviors (grooming, digging and locomotion) [794]. Also, CRF type 1 receptor$^{-/-}$ mice have a reduced anxiety and a low HPAA response to stress [286,287] and blockade of central CRF can attenuate the ACTH response to ether stress by 75% [277]. VP may not be as critical in the initial response to acute stress, however VP plays an important role in the latent phase of the

acute stress response and in response to chronic stress [19,292,795,796]. The role of OXY in the HPAA response to stress is not as clear. OXY has been shown to be released in greater amounts than vasopressin in response to LPS, but the opposite holds true for stress [360]. However OXY is decreased in the hypothalamus and increased in spinal cord 1 minute after stress [797]. OXY magnocellular cells are activated after stress [93,607] and like VP and CRF, OXY is increased in the circulation 2 minutes after stress [789]. As well blockade of central OXY has been shown to reduce the corticosterone response to stress [798].

Stress can cause compensatory responses in the neurons that are activated in response to stimuli. This includes an increased rate of transcription for stress-induced mediators such as CRF and VP as measured by heteronuclear RNA (hnRNA) and mRNA. Protein and mRNA levels for the transcription factor c-fos can also be induced by stress and can be used as indices of activation. CRF hnRNA is increased within 5 minutes in the parvocellular PVN while CRF mRNA is increased by 30 minutes [282,300,799]. VP hnRNA in the parvocellular PVN is up by 30 minutes and down by 120 minutes after stress. VP mRNA is up by 90 minutes and down at 120 [800]. C-fos mRNA can be induced as early as 5 minutes after stress but does not peak until 30 minutes post-stress [300]. C-fos protein usually peaks at 2 hr post-stress [93]. C-fos is a ubiquitous transcription factor that is induced rapidly in response to stimuli . Therefore it is an excellent activational marker but its functions are unknown. It is also unknown which transcription factor(s) mediates the quick compensatory responses to stress, however it

must be constitutively present. CREB is a good candidate as it requires only phosphorylation to become activated and this occurs within the same time frame and same location within the PVN as the other markers of activation, and it can bind the c-fos promoter [799,801]. Activation of the SNS in response to psychological stress is also rapid. Peripheral catecholamines are increased within 5 minutes of stress [802] and peak by 20 minutes [803]. CRF may be a common central mediator between the HPAA and the SNS. It is known that i.c.v. CRF can increase SNS activity [157,804] and that this activity affects the immune system [805,806].

1.5.2. Activation of the Extrahypothalamic regions in response to stress

Although the hypothalamus is the main control center for the efferent response to stress, other areas mediate the afferent signalling of stress to the hypothalamus so that proper behavioral, physiological and locomotive responses can be initiated. The pathways and regions in the brain that have the potential to process stress-related signals are extensive. These includes central noradrenergic [757,758,807-814] , dopaminergic [38,807,809,813-816], histaminergic [817] and serotonergic [807,809,812,814] pathways connecting the brainstem, hypothalamus, cortex and limbic structures.

In order to determine the regions involved in a particular response, several indices of activation can be used such as neurotransmitter levels, neurotransmitter turnover and c-fos expression. Transmitter levels usually drop in an area that has been activated by stress while the metabolite levels are increased. Using these indicators, psychological

stress activates many of the same brain areas as immune challenge [90,818,819]. These areas include the PVN (mainly the parvocellular CRF cells but also magnocellular oxytocin cells and spinal cord projecting cells) as well as other areas of the hypothalamus, regions of the brainstem and limbic system [93,820]. For example the amygdala is an important area in integrating behavioral and physiological responses to stress [821]. The amygdala is involved in freezing behavior, tachycardia and the release of adrenaline, noradrenaline, prolactin and the corticosterone in response to footshock [809,821,822] . Stimulation of the amygdala induces many of the same responses as central CRF injections [823] suggesting that markers of activation are accurate in determining areas of functional significance.

As previously mentioned, each stressor will activate both unique and similar central systems. For example some reports suggest that ascending noradrenergic pathways (from the medulla to the PVN) are important mediators of stress. Evidence for this is that the HPAA and c-fos responses to LPS, IL-1 and ether were reduced upon destruction of these pathways [39,41,105,764,766-768,824,825]. However many of these same reports also showed that removal of these noradrenergic pathways did not alter HPAA activation or c-fos expression in response to footshock stress [39,41,105]. The difference may be that the response to footshock is mediated by higher integrative centers in the brain while the response to IL-1 and ether are mediated primarily by ascending brainstem pathways.

1.5.3. Adaptation to stress

The stress response is an adaptive process that is geared for survival. This is illustrated by the fact that exposure to acute stress can alter the levels of VP in the hypothalamus weeks after the stress occurs [19,826,827]. According to Anisman and Zacharko it is important to also consider the impact of chronic stressors as many of the stressors encountered by humans are chronic in nature [813]. Repeated exposure to the same stress may be able to down-regulate the stress response over time (also known as habituation) and increase the response to new stressors. This may serve to protect the organism from the secondary irrelevant effects as described by Cannon [1-3] .

The adaptation to stress and the response to chronic stress are mediated centrally and peripherally. As previously mentioned, the long term adaptations may be more dependent on hypothalamic VP than CRF. There are many reports of acute stressors (LPS, IL-1, surgery, footshock) selectively being able to increase the stores of AVP in the ME, but not of CRF [19,826,827]. This increase in VP augments the HPAA response to a second acute stressor 11 days later [827]. VP is also selectively increased in chronic stress paradigms [280,292,791]. Although chronic stress tends to habituate the activation of the HPAA in response to stress, basal levels of HPAA hormones may be increased [828].

Although it is common for an animal to habituate to a stressor, it is not a universally-observed effect for all animals or for all stress-activated systems [93]. For example it is known that gastric lesions in response to stress can be attenuated with

repeated exposures to that stress [829]. Also, many reports suggest that levels of hypothalamic NE can be habituated [810,830] in response to chronic stress. Several other reports suggest that although NE levels may be the same between control and chronically stressed animals, there are higher levels of NE metabolites in those brain areas which argue against habituation as the increased NE turnover suggests increased activity [3,831]. Reports also suggest that c-fos can be habituated to restraint stress [832] even in ADX rats suggesting that corticosterone feedback is not the only factor in habituation. Peripheral catecholamines can also be habituated to stress, but this is dependent on the stressor intensity [611,833,834]. Habituation of the corticosterone response may also be dependent on the intensity of the stressor [835]. For example the response to mild stresses such as handling will habituate to some extent [835-838] . Habituation is also common, but not universally observed for restraint stress [292,362,839] . which is less severe than footshock [840,841]. Another report showed habituation to a mild but not severe footshock paradigm [835]. Others using similar paradigms could not habituate the corticosterone response [842-844]. However footshocks administered over a 24 hr period (arguably the most intense paradigm) can lead to habituation of the corticosterone response [843] suggesting that the relationship between intensity and habituation is not linear and/or not the only factor predictive of a response. Also, different strains, species and individual animals will habituate to stressors to different degrees [845]. Animals that respond vigorously to the first exposure tend to habituate more readily [846]. It is also known that habituation is stressor specific. For example, if an animal is habituated to

restraint stress, it will either react normally or overreact to a novel stressor such as swim stress [847-851]. When determining habituation of the HPAA it is vital to measure corticosterone and not just ACTH as some reports demonstrate habituation of ACTH but not the corticosterone response [852-854]. As well, the HPAA and SNS may not be coordinated in terms of habituation [855].

1.5.4. The Effect of Psychological Stress on Natural Immunity.

Like inflammatory stress, psychological stress (also referred to as just "stress" in this thesis) activates the HPAA and SNS. Therefore the effects of stress should accentuate or augment the typical reaction of the SNS and HPAA to that particular immune challenge. For many challenges, these systems inhibit immune function, therefore it is expected that psychological stress is predominantly suppressive to immune function. This however depends on the immune outcome measured [856].

Some of the first studies describing the effects of stress on natural immunity were by Keller et al., who used the mitogen response as their main dependent measure [56-58] . In 1981, they first observed that stress was immunosuppressive to the mitogen response, and this was in proportion to the intensity of the stressor [56]. Secondly, they determined that the effects of stress were observed in ADX animals [57] suggesting that there are corticosteroid-independent pathways of stress-induced immune suppression. In 1988 they demonstrated that HYPOX animals still showed stress-induced immune suppression suggesting that the SNS and not pituitary factors was responsible for the

corticosteroid independent effects; but they never proved it directly [58]. In 1990 Rabin and colleagues verified that stress was potentially immunosuppressive to the mitogen response, but stress could also enhance immunity based on the intensity of the stress, the time the cells were challenged relative to stress, and the strain of rat used [857-859]. They showed that the immunosuppressive effects of stress were dependent on either beta adrenergic receptors or an intact adrenal gland depending on the anatomical origin of cells [859]. Not surprisingly from an anatomical standpoint, the adrenal gland was responsible for suppressing the mitogen response in peripheral circulating cells while naldolol blocked the suppression of splenic cells (presumably by blocking the release of NE from the splenic nerve). In 1993, our lab demonstrated that cutting the splenic nerve abrogated the immunosuppressive effects of stress on the splenic mitogen response [860]. This definitively showed that the SNS had the potential to mediate stress-induced immune suppression.

Stress, whether surgical [861] or psychological [862,863] can also decrease NK cell function. Irwin et al., found that stress-induced suppression of NK cell function was dependent on the SNS and central CRF [54,163,864,865]. Shimizu et al., verified that stress stimulated NE release which inhibited NK cell function, and that the original source of the NE was from the splenic nerve [144].

The effect of stress on LPS-induced TNF and IL-1 production is inconsistent between labs. Some reports suggest that stress inhibits the production of LPS-induced TNF and IL-1 [866,867] while in vitro studies with lung alveolar macrophages

demonstrate that stress increases LPS-induced TNF and IL-1 [868-870]. These later studies also show that the stress-induced potentiation of cytokines can be blocked by chlorisondamine [871]. It is unknown if these contradictory effects are the product of an in vitro environment or are due to the differences in the functional properties of macrophages from different locations [872]. The effects of stress on IL-6 production are also debatable. Some reports have used RT-PCR and determined that stress reduces LPS-induced IL-6 mRNA levels [867]. Others have shown an increase in circulating IL-6 levels after psychological stress [204-207,873] that was intensity- dependent [204]. This is in accordance with the information presented in section 1.3.2.1. which showed the ability of catecholamines to induce IL-6.

Evidence suggests that IL-1 is increased during stress in areas of the brain that mediate the stress response [207,698,699], and that blocking IL-1 abrogates the functional effects of stress [698,874,875]. For example, IL-1RA was found to decrease stress-induced increases in ACTH and hypothalamic catecholamines [698,874] . Given that IL-1 can induce the release of CRF [79,506], it is plausible that a central source of IL-1 induced by stress may mediate central CRF release in response to stress. Of interest is that in some reports IL-1RA had to be given no later than 5 minutes prior to stress, otherwise no effect was observed [698,874]. This suggests that the IL-1 was already synthesized and stored in preparation for rapid release. Nguyen et al., [699] demonstrated with ELISA assays that stress increased IL-1 in the hypothalamus by 2 hr, but only in ADX rats. However there were high basal levels present in all regions tested.

1.5.5. The Effect of Psychological Stress on Adaptive Immunity.

Both glucocorticoids and catecholamines have been shown to regulate aspects of cell-mediated and humoral immunity. In terms of cell-mediated immunity, J.F. Sheridan and colleagues have shown that restraint stress suppressed the cytotoxic T lymphocyte reaction to herpes simplex virus through adrenal dependent and independent mechanisms [863,876,877]. They also showed that stress reduced the cell-mediated immune response to influenza virus in mice [878] and that this was beneficial for survival depending on the strain of mouse [879]. They and others found that glucocorticoids decreased blood lymphocyte levels and caused the movement of cells into the tissues despite being immunosuppressive [56,168,880,881]. Related to this phenomenon, stress can modulate the delayed type hypersensitivity reaction (DTH). Dhabhar et al., demonstrated that acute stress increased DTH as well as lymphocyte redeployment [357]. As a survival mechanism the authors offered the explanation that in terms of fight or flight, the skin would be the most likely location for infection, and thus the redistribution of lymphocytes to this location serves a protective role. They also found that chronic stress reduced the DTH reaction, thus demonstrating a necessary adaptation to the high levels of glucocorticoids over a prolonged period. Thus the stress-induced migration of immune cells to the skin may help explain why stress can induce or exacerbate skin-mediated autoimmune conditions despite the overwhelming evidence that suggests that stress is immunosuppressive.

Although both glucocorticoids and catecholamines can mediate adaptive immunity, nerve fibers may be more important regulators of humoral responses than either the adrenal medulla or adrenal cortex [48,249-251,860]. The effects of stress on humoral immunity were first demonstrated by G.F. Solomon who found that certain types of stress were immunosuppressive to antibody production [26,27]. The suppressive effects of stress on humoral immunity have been verified by some labs [356,860] and refuted by others [882]. Similar to the mitogen response, our lab demonstrated that cutting the splenic nerve abrogated the immunosuppressive effects of stress on the spleen cell response to SRBC [860].

A more likely explanation is that stress can be immunosuppressive or immunoenhancing depending on the immune parameter measured [856]. It could be expected that psychological stress would augment the natural course of brain-immune interactions as the same systems are activated. Stress can vigorously activate both the HPAA and SNS, whereas during the course of some of the natural immune responses only one of these systems may be activated [48,249-251]. Also, the type of stress is critical, as not all paradigms are equally intense nor exert the same effects on immunity [26,808,862,883]. This includes the differential effects observed between chronic and acute stress [357]. The effects of stress on immunity are also dependent on the strain of animal used, as different strains can have different or even opposite responses to the same challenge [879,884]. Another reason that the effects of stress are not uniform between paradigms or labs studying similar models is the timing of stress relative to the immune

challenge. Zalcman et al., found that there was a critical period after inoculation of SRBC when stress (footshock) was immunosuppressive (72 hr after inoculation), otherwise stress had no effect on this immune response [885]. In a conditioning paradigm, they found that animals stressed 2 weeks prior to immunization and re-exposed to stressor related cues 72 hr after immunization were immune suppressed in terms of the response to SRBC [808,886]. In contrast, when the stressor was initially applied immediately after immunization, re-exposure to the stressor-related cues was found to provoke an immunoenhancement [808,883]. Similarly, Zalcman et al., found in both mice and rats that IL-2 increased the antibody response to SRBC only if it was given in close temporal proximity to inoculation [254].

It is unknown if stress-induced alteration of immunity is by design or is merely a byproduct from the activation of systems more relevant to short term survival. Dhabhar et al., showed that with the DTH reaction, stress caused a redistribution of cells to areas with a greater chance of being exposed to a pathogen in a life-threatening situation [357]. This is contrary to what might be expected since stress will increase the TH2 response at the expense of the TH1 response [248,887]; and the DTH reaction is a TH1 response. Experimentally, stress has been shown to be beneficial to an overactive immune system in that Kort et al., demonstrated that stress reduced kidney allograft rejection in rats [888].

1.6. The working model

The working model of the brain-immune regulatory system is primarily based on the data that our lab has contributed to the literature during the past 14 years. This includes data on how the immune system signals the brain, the central activation pathways within the brain and the mechanisms through which the HPAA and SNS regulate immunity. Our current working model is summarized in figures 1-1 and 1-2.

The first contributions from our lab were in the late 1980s when it was discovered that the innervation to the thymus and the spleen was exclusively sympathetic [65,889] . As well, lesions of extrahypothalamic brain structures such as the lateral septal area resulted in alterations of both natural and adaptive immunity [243,244,254]. One of the most pivotal studies in the field of brain-immune interactions was by Brown et al., in 1991 [89]. In this report he found that i.c.v. IL-1 activated the HPAA and suppressed IL-1 production in splenic macrophages stimulated in vitro with LPS. Subsequently, Vriend et al., demonstrated that i.c.v. injections of IL-1 also activated the SNS as indexed by increased turnover of NE in the spleen [219]. These results demonstrated the existence of a cytokine feedback loop that activates the CNS and which subsequently alters peripheral immune function. This regulatory feedback loop was also shown to be cytokine-specific as production of IL-1, but not transforming growth factor was affected. It was also demonstrated in this report that both ADX and splenic nerve cut individually abrogated the immunosuppressive effects of i.c.v. IL-1 on splenic macrophage IL-1 production. The

combination of splenic nerve section and ADX were additive and potentiated in vitro IL-1 production in macrophages from animals given i.c.v. IL-1 more than either ADX or nerve cut alone. This synergy between the SNS and ADX corroborated Besedovsky's original 1979 model [48]. In further support of this model, MacNeil et al., found that acute ADX increased LPS-induced splenic and renal nerve activity and reduced the latency of activation for the renal nerve [378]. These studies illustrate how the SNS and HPAA are related functionally as well as anatomically.

IL-1 is the most studied cytokine in terms of brain-immune interactions. We helped prove that IL-1 isn't the only cytokine responsible for signalling the brain as Zalcman et al., demonstrated that different cytokines (IL-1, IL-2 and IL-6) caused specific alterations in central neurotransmitters levels and behaviors [535,629]. In support of Zalcman, MacNeil et al., found that in response to LPS, mice unable to produce mature IL-1, had a normal corticosterone response and c-fos expression pattern [570].

In 1997 we collaborated with Dr. Catherine Rivier's group at the Salk Institute in determining a role for central cytokines in the inflammatory response to turpentine [675]. They found that i.m. turpentine increased ACTH as did i.c.v. TNF. They could inhibit the ACTH response to turpentine by an i.c.v. injection of TNF antagonist. However no TNF could be detected in the plasma and no TNF was detected by in situ hybridization or bioassay in the brain. Although RT-PCR detected low levels of TNF mRNA levels in the brain, the levels were not changed by i.m. turpentine. This study shows a functional role for cytokines in the brain despite the fact that they can't be localized.

C.A.Y. Vriend et al., demonstrated that pathogns such as the HIV virus have the potential to subvert bidirectional signalling between the brain and the immune system [231,232]. GP120, the HIV coat protein, did not by itself induce sympathetic nerve activity but inhibited MSH-induced sympathetic nerve activity. As previously described, MSH is a central mediator of peripheral anti-inflammatory circuits and in AIDS patients the circulating levels are inversely proportional to HIV levels in the blood [234]. Vriend found that central GP120 increased splenic TNF and IL-1 mRNA levels in vivo, which agreed with her earlier in vitro studies and recent studies by other investigators with human subjects [233]. A similar dysregulation was observed by MacNeil et al., who observed increases in splenic TNF production in spinal cord lesioned rats (as compared to controls) given LPS [235]. These results are in agreement with observations by Li Zhou who demonstrated that splenic macrophages from splenic nerve cut animals produced more cytokines after exposure to LPS [890]. Therefore the SNS has the ability to regulate cytokine production in vitro as well as in vivo. Zalcman et al., found that the splenic nerve has the potential to regulate humoral immunity. Cutting the splenic nerve did not alter the response to SRBC in vivo. However the SRBC response was increased if IL-2 was injected i.p. close to the time of inoculation [254]; and this potentiation was dependent on an intact splenic nerve. In extending Zalcman's findings to footshock stress, Wan et al., showed that the immunosuppressive effects of footshock stress on the SRBC response and the response to mitogen were mediated by the splenic nerve [860]. Green-Johnson et al., demonstrated that an increase in either peripheral or cental catecholamine

levels could inhibit the humoral response to SRBC [266,267]. However when the T or B lymphocytes were removed and stimulated in vitro, they functioned normally. This suggested that the in vivo microenvironment is critical in establishing immune function and in vitro assays may not necessarily reflect this physiological process.

To index the activation of the brain following LPS, Wan et al., used c-fos as a marker of activation. They found that LPS-induced c-fos expression whether it was injected i.v., i.p or i.c.v. [32,90]. Areas positive for c-fos expression following i.c.v. LPS were the PVN and the A2 region in the medulla. Higher doses of i.p. LPS-induced c-fos in the A1 region, the SON and the arcuate nucleus [32]. Corticosterone levels were increased and peaked at lower doses of i.p. LPS than c-fos expression further demonstrating differential sensitivity of these responses. Footshock stress induced a similar pattern of central activation to LPS; however, c-fos was expressed in additional brain areas such as the lateral septal area, amygdala, POA and the LH [90].

In an attempt to determine the chemical specificities of these afferent systems, various inhibitors were given i.p. in an attempt to inhibit c-fos production in the hypothalamus in response to i.v. LPS and footshock stress [90]. First it was found that both indomethacin, a COX inhibitor and MK801, a glutamate NMDA receptor antagonist, each inhibited c-fos expression following both i.p. and i.v. LPS. MK801 also blocked c-fos expression in response to footshock stress, but indomethacin did not. This report was also the first to demonstrate that vagotomy completely blocked the response to i.p. LPS but not to i.v. LPS. Only a small but significant attenuation was observed for

i.v. LPS. Vagotomy did not block the induction of c-fos in response to footshock. This shows that LPS can signal components of the same system as footshock albeit through different afferent signalling pathways. As well, LPS administered by different routes can activate the same systems by different mechanisms.

The next series of studies was initiated by A.T.K. Jackson. The purpose was to determine the chemical specificities of the cells in the PVN that were c-fos positive after i.v. LPS [105]. Jackson found that LPS-induced c-fos was colocalized with OXY, VP and nitric oxide synthase (NOS) activity. She then found that central injections of L-NAME, an NOS inhibitor, blocked LPS-induced c-fos in the hypothalamus. As an extension of the indomethacin studies, she found that PGE2 induced a similar pattern of c-fos in the brain as LPS, and that this response was blocked by central injections of L-NAME. This demonstrated that PGE2 activated NOS positive cells that were likely positive for VP or OXY, as well as c-fos. Interestingly, she also found that unilateral brainstem and hypothalamic cuts, which reduced noradrenergic brainstem inputs to the PVN, inhibited LPS, but not stress-induced c-fos on the same side of the brain. This reinforced the concept that c-fos in one area of the brain areas can be induced through multiple pathways and that adrenergic innervation, PGE2 and NOS were all involved in c-fos induction after i.v. LPS.

B.J. MacNeil et al., then tested which central mediators were relevant to SNS activity. Using nerve recordings, they found that increases in splenic nerve activity were induced in response to lower doses of i.v. LPS than it took to increase renal nerve

activity. Also, splenic nerve activity was increased with reduced latency in response to LPS as compared to the renal nerve [76]. This proved that the SNS could be activated in an anatomically and organ-specific manner [76]. Following up on previous studies, they also found that PGE2 activated the splenic nerve in a similar matter as LPS, but much more rapidly [645]. This suggested that central PGE2 is a likely mediator of LPS-induced splenic nerve activation. In support of this they blocked the effects LPS on sympathetic nerve activity with i.c.v. indomethacin. Indomethacin given i.v. also blocked this response, but a much higher dose was required than for i.c.v. administration indicating that central, not peripheral prostaglandin synthesis was the mediator of these effects. Central injections of VP and OXY, but not CRF, were found to increase nerve activity [[645] and unpublished observations]. However, the splenic nerve activity in response to PGE2 was blocked fully by an OXY antagonist, minimally by a CRF antagonist, and was unaffected by a VP antagonist. These results suggest that endogenous VP may not be related to the sympathetic regulation of the spleen [133]. To connect central mediators with immune function, Pan et al., investigated the effect of central PGE2 on the peripheral response to a low dose of LPS [717]. It was found that PGE2 injected i.c.v. reduced splenic TNF but not IL-1 beta mRNA and this effect was partially inhibited by splenic nerve cut. Therefore, given the appropriate central signal, it was established in vivo that the splenic nerve inhibited TNF mRNA in response to LPS.

My contribution to the preceding data was the development of in vivo measures of immune function for the rat. At the initiation of my project (as well as for other

projects in the lab) there were no reliable means to assay cytokine levels in the rat in vivo. This was important as cytokines play a pivotal role in all aspects of immunity. Also, the strength of our model has been the ability to assay immune measures in vivo in the rat, which is the model of choice for neuroscience. The manuscripts I have contributed to this field are not included as a part of this thesis, but are included in appendix 2. These are technical papers on immunohistochemistry [151], Northern blotting [891] and in situ hybridization [892]. These dependent measures have been used for the last few years in our lab to analyze further the in vivo study of brain-immune interactions [232,235,570,675,717] . These techniques, along with recently available, commercially produced ELISA kits will be used to answer the primary objectives of my thesis.

Figure 1-1: Schematic diagram of brain-immune communications.

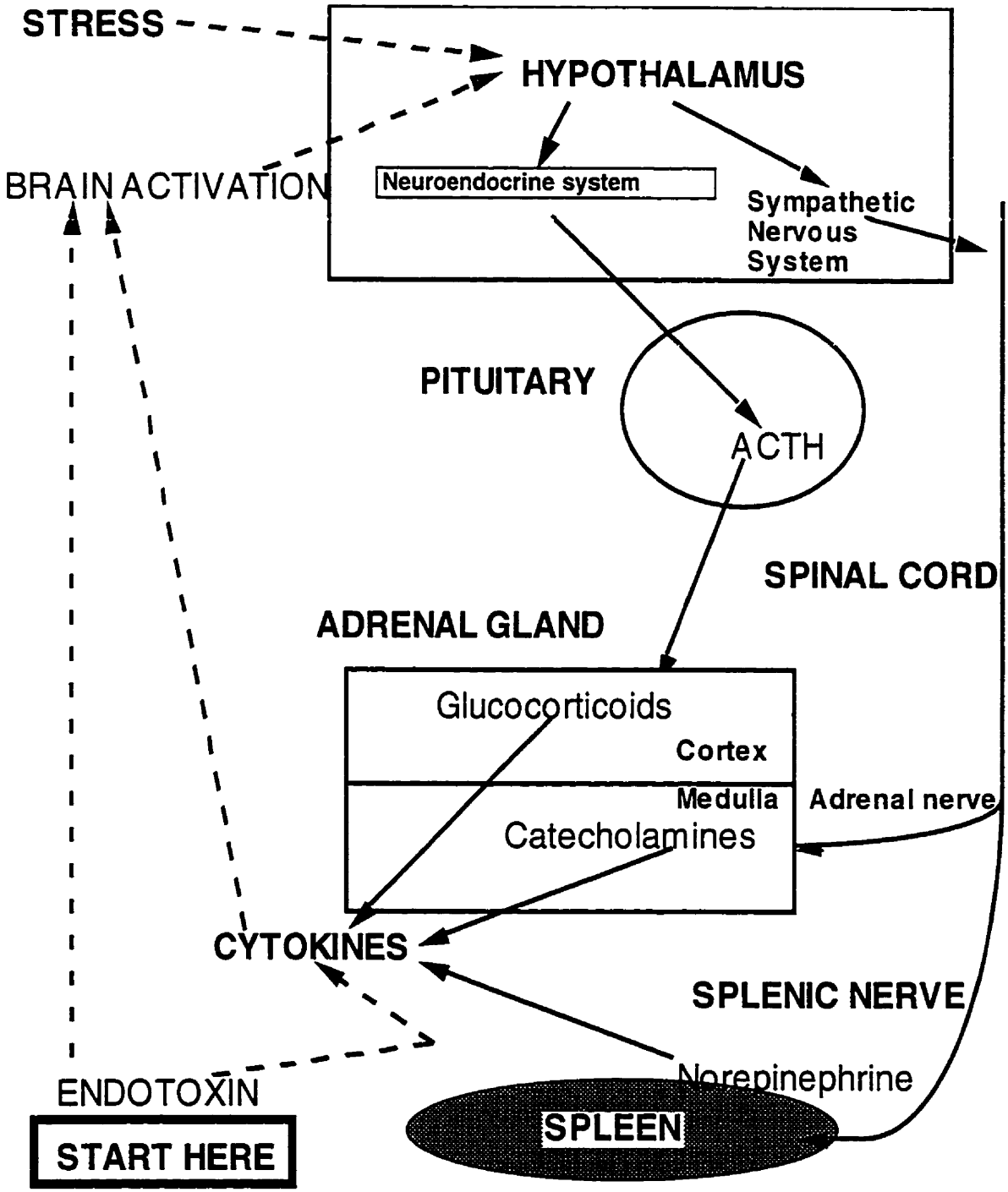
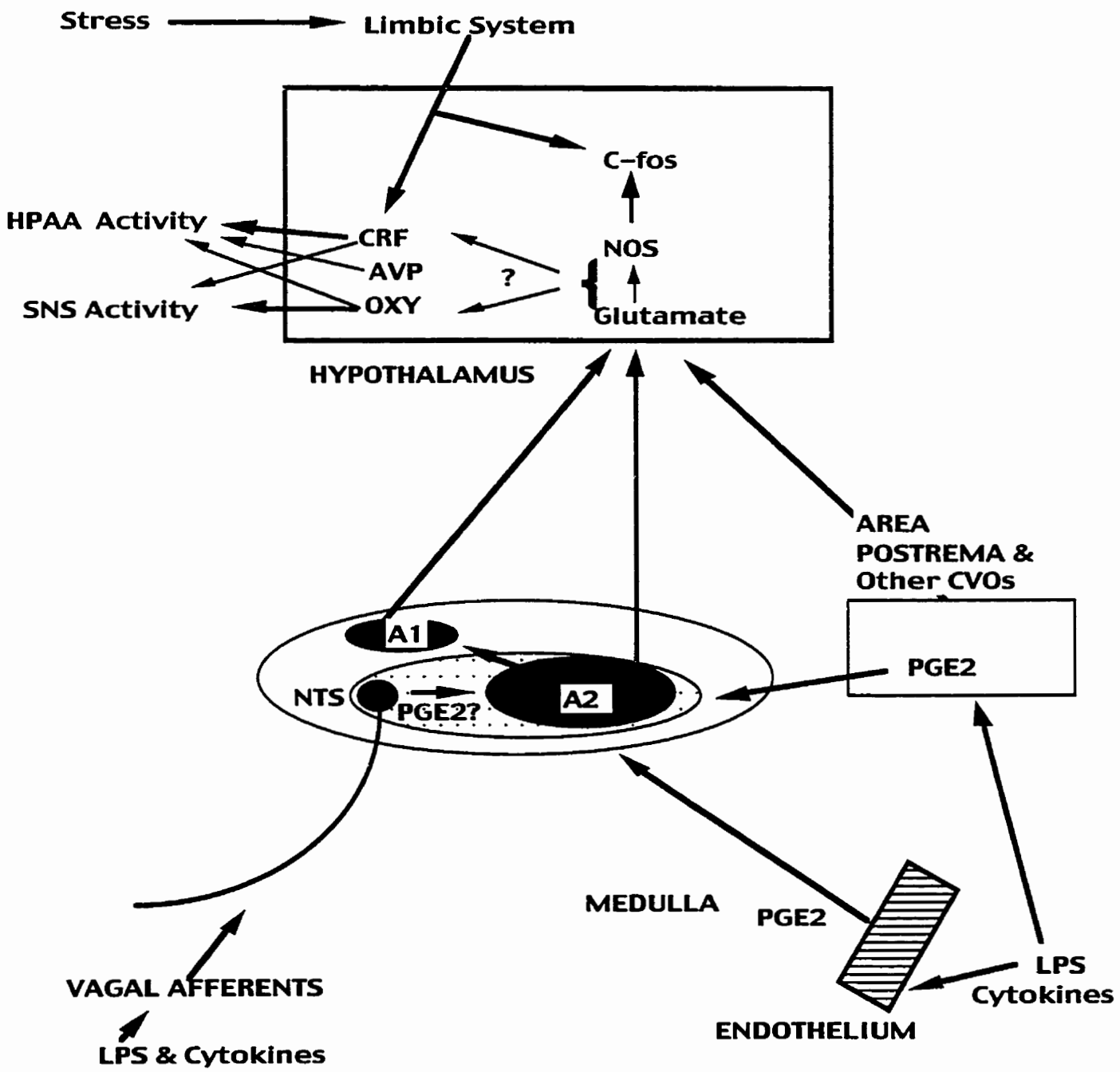


Figure 1-2: Proposed schematic representation of the central activation pathways in response to LPS and footshock stress.



1.7. Specific Objectives

1) To develop further an in vivo model system for analyzing brain-immune interactions, and to determine the dose response and time course for the in vivo production of cytokines and activation of the HPAA and SNS in response to i.v. LPS.

2) To determine if cutting the splenic nerve alters in vivo splenic cytokine production after i.v. LPS.

3) To determine if the splenic nerve mediates the effects of stress on in vivo splenic cytokine production.

2. Materials and Methods

2. Materials and methods

Some of the descriptions below are adapted from previously published manuscripts which are in appendix 2 of this thesis [151,891,892].

2.1. Animals

2.1.1. Background

All studies utilized adult male Sprague-Dawley rats (225-250 g) that were obtained from Charles River, (Dorval Quebec). The rats were kept on a 12-12 light/dark cycle and given food and water *ad libitum* (water for adrenalectomized (ADX) rats contained 0.9% saline). All animals were handled for 2 days then taken through the experimental procedure (weighing, mock injection etc.) for 3 days prior to the actual experiment. All procedures were approved by the animal ethics committee at the University of Manitoba and the CCAC.

2.1.2. Surgeries

Rats undergoing surgery were anesthetized i.p. with 60 mg/kg sodium pentobarbital or a 1.3 ml/kg of a 2:1 mixture of Ketamine (100 mg/ml) and Rhompun (Xylazine-20 mg/ml) containing 0.04 ml of atropine. The surgeries comprised combinations of: sham operations, splenic nerve cuts and bilateral adrenalectomies as previously described [65,89,860]. For both splenic nerve cuts and bilateral

adrenalectomies (ADX), incisions were made in the posterolateral abdominal wall and the procedures were carried out with the aid of a dissecting microscope. The splenic nerve was found by dissecting the splenic neurovascular bundle (via a posterior approach), distal to the bifurcation of splenic artery and vein into upper and lower splenic branches. All branches of the nerve (usually two per artery) were identified and stripped away from their respective artery with forceps. Splenic norepinephrine levels were measured to verify the effectiveness of the nerve cut. The adrenal glands were located by gross inspection, the artery to the adrenal gland was clamped and the glands were removed. These surgeries were verified by measuring plasma catecholamine and epinephrine levels. Sham surgeries were similar to both the splenic nerve cuts and ADX except that the respective structures were left intact. Following surgery, the incisions were sewn up, sutured and disinfected with iodine. Animals were then given i.m. injections of 0.1 ml Derapin and 2.5 mg/kg of Banamine. Animals were allowed to recover for 7-10 days, during which time they were singly housed and handled.

2.1.3. LPS injection and stress protocol

Prior to injection, animals were weighed and then put under a heat lamp for 3 minutes to dilate the tail veins. They were then put in a restrainer and injected i.v. via the tail vein with saline or various doses of lipopolysaccharide (LPS; E.Coli serotype 055:B5 lot L-2637 obtained from Sigma, Missussauga, ON) and were killed at numerous time intervals following LPS administration. The doses of LPS ranged from 0.0001 μg per rat to

1 mg per rat depending on the study. Following this, the animals were killed by decapitation or overdose with pentobarbital and both trunk blood and spleens were collected for various assays. Trunk blood was collected from animals in 15 ml polypropylene tubes containing 150µl of 0.5M EDTA. The tubes were kept on ice until the completion of the experiment, spun for 20 minutes at 4°C at 3000 RPM, and the plasma was collected and stored at -70°C until analysis. Immediately after decapitation, the spleen was removed from the animal, sectioned into 6 parts, frozen in liquid nitrogen and stored at -70°C. Any animals showing signs of illness or infection were removed from the study.

For the stress studies, animals were exposed for 15 minutes of 1.6 mA intermittent footshock. Each shock lasted 5s and was preceded by a 15s warning tone. The average interval between shocks was 3.5 minutes with a range from 2-5 minutes. Apparatus controls were exposed to the intermittent warning tone but not the shock while homecage controls remained in their cage as opposed to being exposed to either the apparatus or the footshock. Animals were injected with LPS either prior to or following stress. Following the completion of the experiment, animals were either killed by decapitation or overdosed with pentobarbitol and samples were collected as described above. Additional details of each experiment are provided for each study.

2.2 Northern blotting and RNA probe synthesis

2.2.1. Isolation of RNA

RNA was isolated from rat tissue with Trizol™ reagent (Life Technologies Burlington, ON) according to the manufacturer's instructions. Briefly, 1/6 of a rat spleen was homogenized in 2 ml of Trizol™ reagent and left to stand at room temperature for 5 minutes. Following this, 400 µl chloroform was added, the sample was vigorously shaken, incubated at room temperature for 5 minutes and centrifuged for 15 minutes at 12,000G at 4°C. After this, 1 ml of isopropanol was added to the aqueous phase to pellet the RNA. The pellet was washed in 2ml of 75% ethanol, air dried, and suspended in 200µl of diethylpyrocarbonate (DEPC)-treated water. Samples were either quantified right away or frozen at -70°C. To quantitate the amount of RNA in the samples, they were heated to 55°C for 10 minutes and measured by U.V. spectrophotometry at 260 and 280nm.

2.2.2. First strand cDNA synthesis and amplification

First strand synthesis was performed with the Superscript-2™ pre-amplification system (Life Technologies, Burlington, ON) or the Expand-RT™/ Expand™ Hi-Fidelity reverse transcription/ PCR systems (Roche Diagnostics, Laval, PQ) according to the manufacturer's instructions. To amplify the first strand according to Life Technologies, 0.2-2µl of first strand synthesis product was added to 0.5µm dNTP mix, 1.5mM $MgCl_2$, Taq DNA polymerase (2 units), and 20 pmol of each primer. PCR cycling parameters were as follows: 90°C (5 min.) then 35 cycles: 94°C (45s), 60 °C (45s), 72 °C (2 min.) and 7 min extension at 72°C. However the annealing temperature was varied empirically

depending on the primer pairs. PCR products were visualized by ethidium bromide on 2% agarose gels with a DNA mass ladder (Life Technologies) to estimate the yield.

2.2.3. Production of RNA probes and probe templates

An aliquot of first strand synthesis or first strand amplification product (0.1-1µl) was added to a PCR reaction tube containing a 3' (lower) primer with a T7 RNA polymerase promoter sequence as previously described in appendix 2 [891,892]. Primers were designed using Oligo™ 5.0 for the Macintosh (a list of primers used can be found in appendix 1). Cycling parameters were identical to those mentioned above. After the reaction, the PCR products were used as templates for both DNA and RNA probes. Probe templates for beta-Actin and TNF were sequenced by non-radioactive cycle sequencing (Silver Sequence DNA sequencing system (Promega, Madison, WI), while the 28S ribosomal RNA (rRNA), GAPDH and the IL-1 cDNA fragments were blunt-end cloned into a plasmid and sequenced with S^{35} dATP as previously described [892] (and found in appendix 2). Further verification of these and other recently made templates were made by fluorescently-detected cycle sequencing as performed by the Manitoba Institute of Cell Biology (MICB)'s core sequencing service. Prior to the reaction, PCR products were purified by phenol/chloroform extraction [892] and sequenced with T7 polymerase sequencing primer (see appendix 1). The reaction was carried out with 50 ng of PCR product and 3.2 pmol of primer as suggested by the MICB and Perkin Elmer.

Digoxigenin (Dig)-labelled RNA probes were produced and quantified using the

Dig nucleic acid production and detection kits (Roche, Laval, PQ). Briefly, 4-6μl of PCR product was in vitro transcribed using T7 RNA polymerase with Dig-UTP present. RNA probe yield was determined by comparing serial dilutions of probe to Dig-labelled control RNA standards, detection by sheep anti-Dig alkaline phosphatase-conjugated antibody (1/5000, Roche, Laval, PQ) and NBT/BCIP (0.46 mM NBT, 0.43 mM BCIP in 50 mM MgCl2/100 mM Tris in 100 mM NaCl, pH 9.3). ^{32}P-labelled DNA probes were made with Life Technology's random priming kit. All solutions were treated with diethylpyrocarbonate (DEPC) where necessary.

2.2.4. Northern blotting.

RNA was isolated from rat spleen with Trizol™ reagent as described above. The RNA (10 μg per sample) was denatured for 10 minutes at 65°C and loaded on a 1.2% agarose/ formaldehyde gel and run for 3 hours at 50V. Samples were transferred overnight to nylon membrane (Roche) by capillary action with SSC buffers. RNA was fixed on the membrane with a 2 hour incubation at 80°C and the integrity of the samples was verified by visualizing the ribosomal RNA subunits under UV light. The membranes were equilibrated in 2X SSC and placed in pre-hybridization solution (50% formamide, 5X SSC, 0.1% sodium laurosarcosine, 0.2% SDS, and 2% Roche blocking agent) at 42°C for 1 hour. Depending on the RNA sequence, membranes were then hybridized overnight at 60-68°C with 5-50 ng/ml of Dig-labelled RNA probe. Membranes were washed two times in 2X SSC/0.1% SDS for 15 min at room temperature and two times in 0.1X SSC/0.1%

SDS at 60-68°C for 25 minutes. Membranes were blocked for 1 hour in 1% Roche block/ maleic acid buffer, incubated with sheep anti-Dig peroxidase-conjugated antibody (1/5000, Roche) for one hour at room temperature, rinsed 3X in maleic acid buffer and visualized using the Roche chemiluminescence kit (Laval, PQ). The blots were exposed to film anywhere from 15 seconds to 30 minutes. The blots were then reprobed with a ^{32}P-labelled cDNA loading control probe overnight at 42°C. The following day the membranes were washed two times in 2X SSC/0.1% SDS for 15 min at room temperature and two times in 0.1X SSC/0.1% SDS at 42°C for 25 minutes. The membranes were then exposed to film anywhere from 15 minutes to 16 hr.

2.2.5. Quantitation and loading controls

The images were scanned using a CCD Camera and analyzed with MCID software (Guelph Ontario) with greyscale analysis. The optical density of the bands were determined by taking the area of the largest band and comparing the density and area of the remaining bands to this standard. Areas directly above or below the bands that were devoid of signal, were used to subtract background variations caused by uneven lighting, developing etc. The levels of cytokine mRNA were expressed as a ratio to loading control RNA. Statistics were analyzed on the Macintosh Version of Statsview 4.5.

To ensure that neither overexposure nor underexposure of the blots to the film impacted on our experimental results, the blots (both cytokines and loading controls) were routinely exposed to film for various time intervals. Also, each blot had one sample

(that was positive for the cytokine in question), loaded in amounts of 15, 10, 7.5 and 5μg of RNA per well to help determine if a film was over exposed or underexposed. Our preliminary studies found that only the most extreme overexposures and underexposures would significantly alter the experimental effects. This supports previous studies which show that the effective range of detection for differences in optical is quite broad [893,894] and belays the concerns associated with the lack of a linear relationship between optical density and pixel value [895].

Northern blotting is a powerful technique to determine the steady state level of specific mRNA species in vitro and in vivo. The limitations are that it does not give an index of either mRNA transcription or degredation. Upon careful review of the literature it was found that most manuscripts that utilized Northern blotting were only interested in "present or absent" scenarios. Also, many of the manuscripts that employed this technique for semi-quantitative measures overexposed the films for the loading controls and represented all the lanes as having equal amounts of RNA. The dilemma of a reliable loading control was best reviewed by Spanakis who suggested that all loading control/housekeeping genes are subject to regulation depending on the situation [896]. This was verified by others who demonstrated that actin [897] GAPDH [898-900] , and 28S rRNA [901,902] could all be regulated in specific paradigms. However, whether by probing [894,898,903] or staining the membrane with ethidium bromide [904,905], the 28S subunit of ribosomal RNA (28S rRNA) emerged as the best option for the majority of experimental scenarios. We corroborated this in numerous experiments by comparing

raw cytokine levels, raw loading control levels, ethidium bromide staining and ratios of cytokines to loading controls (data not shown). We found that a reliable loading control should not drastically alter the experimental effects observed with the probe(s) of interest (ie. cytokine probes) and the levels of the loading control should not be statistically different among experimental groups. The 28S rRNA probe met these criteria for all experiments. Although we found that both GAPDH and beta actin gave comparable results to 28S rRNA in most of the experiments in which they were directly compared (and thus could be used interchangeably), the 28S rRNA was selected as our primary loading control.

2.3. ELISA assays

Cytokine protein levels in rat spleens and plasma were determined by ELISA according to the procedure of Molina et al., [906] with a few modifications. Spleens were homogenized (100 mg/ml) at 4°C in PBS containing 1mM PMSF, 1μg/ml pepstatin, 1μg/ml aprotinin, 1μg/ml leupeptin, 0.5% Triton X-100 and 0.05% sodium azide. Samples were centrifuged at 20,000G and filtered through Nalgene 22μm syringe filters (Fisher, Nepean, ON). The samples were then aliqouted and frozen at -70°C until use. Tissue protein content was determined using the Bradford reagent from Bio-Rad (Mississauga, ON). The rest of the ELISA procedure was carried out according to the manufacturer's instructions (Biosource, Camarillo, CA). Preliminary assays were required

in order to determine the optimal protein concentrations used for each kit. All samples and standards were run in duplicate. The plates were read at 450nm and the unknown samples were compared against the standard curve with various software packages such as Assay Zap™ and Statsview™ 4.5 for Macintosh.

2.3.1. TNF

For all assays 5-20 (usually 10) μg of total splenic protein (in 50μl) was added to 50μl of assay buffer and 50μl of biotinylated anti TNF antibody. This mixture was incubated for 90 minutes at room temperature, after which the wells were aspirated and washed 4 times. After this, 100μl of streptavidin-HRP was added to each well and the plates sat at room temperature for 45 minutes. Again, the wells were aspirated, and the plates washed 4 times. To this, 100μl of stabilized chromogen was added and the reaction was stopped after 30 minutes. For serum TNF, the plasma was diluted 1/100 in 50μl of assay buffer which was then added to another 50μl of assay buffer. From this point, the procedure was carried out in exactly the same manner as described above.

2.3.2. IL-1

For all assays 5-20 (usually 20) μg of total splenic protein (in 50μl) was added to 50μl of assay buffer. The plate was then incubated for 3 hr at room temperature after which the wells were aspirated and rinsed 4 times. After this, 100μl of biotinylated anti IL-1 beta was added to each well and the plates sat for one hour at room temperature. The

wells were then aspirated and rinsed 4 times after which 100µl of streptavidin-HRP was added an the plates were incubated for 30 minutes at room temperature. After 4 rinses, the chromogen solution was added and the reaction incubated for 30 minutes at room temperature until addition of the stop solution. For serum IL-1, the 50µl of plasma was added to 50µl of assay buffer. From this point, the procedure was carried out in exactly the same manner as described above.

2.3.3. IL-6

For all assays 5-20 (usually 10) µg of total splenic protein (in 50µl) was added to 50µl of assay buffer. The plate was then incubated for 2 hr at 37°C after which the wells were aspirated and rinsed 4 times. After this, 100µl of biotinylated anti IL-6 was added to each well for one hour at room temperature. The wells were then aspirated and rinsed 4 times after which 100µl of streptavidin-HRP was added and the plate incubated for 30 minutes at room temperature. After 4 rinses, the chromogen solution was added and the samples were incubated for 30 minutes at room temperature until addition of the stop solution. For serum IL-6, the 50µl of plasma was added to 50µl of assay buffer. From this point, the procedure was carried out in exactly the same manner as described above.

2.4. Immunohistochemistry

This procedure was adapted from studies by Meltzer et al., [151] in appendix 2.

Sections of fresh-frozen rat spleen (approximately one-sixth) were post-fixed in Periodate Lysine Paraformaldehyde for 4 hr and then cryoprotected in 30% sucrose. Serial 50 μm sections of spleen were cut on a freezing microtome and incubated with proteinase K (1-2 μg/ml only for ED-1 and TNF staining) for 30 minutes at 37°C. Following 3 rinses in PBS, the sections were incubated overnight at room temperature in PBS with 1% normal goat serum, 2% BSA and 1% triton-X 100 containing one of rabbit anti-mouse TNF (neutralizing antibody 1/5000, formerly Genzyme now R and D systems Minneapolis, Minnesota), mouse anti-rat ED1 (1/2000, Cedarlane, Hornby, ON), mouse anti-rat Pan T (MRC clone OX-52, 1/500, Cedarlane) or mouse anti-rat pan B cell (MRC clone OX-33, Cedarlane). Sections were then rinsed 3 times for 10 minutes each with PBS and incubated for 2 hours in alkaline phosphatase-conjugated goat anti-rabbit (1/1000, formerly Cappel, now ICN) or alkaline phosphatase-conjugated goat anti-mouse (1/750, ICN) in PBS with 1% normal goat serum and 1% triton-X 100. The sections were rinsed three times in PBS, developed in NBT/BCIP and coverslipped in glycerol gel. The percentage area of positive staining was determined with a digital video microscope and the Macintosh version of NIH Image. Positive staining was expressed as a percentage of the total area of spleen in that optical field. Analyses for each animal was performed blinded on three separate tissue sections. Statistics were analyzed on the Macintosh Version of Statsview 4.5.

2.5. Radioimmune assays for corticosterone

Corticosterone standards were made by adding 29.55µl of 1000nM corticosterone standard to 4ml of assay buffer (PBS with 0.1% sodium azide and 0.1% BSA or gelatin) to generate a 2560 pg/ml standard. After vortexing, halving dilutions were done for our standard curve until a concentration of 20 pg/ml was reached. Plasma samples were thawed and diluted either 1/500 or 1/1000 in assay buffer depending on the experiment. Both samples and standards were heated for 45 minutes at 60°C in a water bath after which the tubes were gradually cooled to room temperature. The samples were then divided into three 500µl portions and processed as triplicates in glass test tubes. After this, 100µl of corticosterone tracer was added by repeater pipet (10,000 cpm/100µl-obtained from ICN 07-120026) followed by 100µl of antibody (at a 1/1200 dilution to make the final working dilution 1/8400; this was obtained from ICN, catalog number 07-120016 or 07-120017). These samples were incubated at 4°C overnight. The next day, charcoal dextran was prepared by adding 0.075g of dextran (T-70 from Pharmacia) to 100ml of assay buffer until it dissolved. After this, 0.15g of charcoal was added (Norit A from Fisher) and the solution was kept stirring on ice for 30 minutes. 0.5ml of this solution was then added to the each test tube, the samples were vortexed and incubated for 30 minutes at 4°C from the end of the dextran/charcoal addition. The samples were then centrifuged for 30 minutes at 2000 RPM (4°C) with no brake. 1ml of each sample was then pipetted into a plastic scintillation vials to which was added 3ml of scintillation cocktail (Ecolume-ICN 88247002). The samples were then mixed at a high speed for 1 hr on a shaker. Following this, the samples were placed in the counter and sat there

overnight before counting was commenced. The unknown samples were compared against the standard curve with Assay Zap™ or Statsview 4.5 for Mackintosh.

2.6. Catecholamine determinations

Frozen Spleens: Catecholamine levels were determined as previously described [89,219,860] . Frozen spleens were weighed and homogenized in 0.1 M perchloric acid containing 0.1 mM EDTA and 0.1 mM sodiummetabisulfite. Homogenates were centrifuged at 2500 rpm for 10 min and 1.0 ml of the supernatant was taken for alumina extraction using the ESA (Chelmsford, MA) catecholamine methodology with an internal standard. Samples were separated by HPLC using a Beckman Model 114M solvent delivery system with a 10cm C-18 (3 micron particle size) column (Higgins Analytical,Inc.). The assay was accomplished with an ESA Coulochem 5100A electrochemical detector with a model 5011 cell. Analysis was accomplished using a Beckman System Gold. Data were expressed as ng NE/mg frozen tissue weight. Statistics were analyzed on the Macintosh Version of Statsview 4.5.

Rat plasma: 0.5 ml of rat plasma was extracted with alumina using the ESA (Chelmsford, MA) plasma catecholamine methodology with an internal standard. Samples were separated by HPLC using a Beckman Model 114M solvent delivery system with a 10 cm C-18 (3 micron particle size) column (Higgins Analytical, Inc.). The assay was

accomplished with an ESA Coulochem 5100A electrochemical detector with a model 5011 cell. Analysis was accomplished using a Beckman System Gold. Data were expressed as pg NE or pg E/ml of plasma. Statistics were analyzed on the Macintosh Version of Statsview 4.5.

2.7. Statistics

One and two tailed T-tests, and one and two way ANOVAs were performed on the Macintosh versions of Statsview 4.5 and Statistica 4.1. The ANOVAs were analyzed using the Fisher LSD post-hoc test. The lowest standard for significance between groups for all tests was set at $p=0.05$. All error bars in the graphs represent standard error of the mean. Additional statistical details for each experiment are provided for each study.

3. Experiment 1

Characterization of the in vivo response to i.v. LPS in the rat.

3. Experiment 1: Characterization of the in vivo response to i.v. LPS in the rat.

3.1 Overview

Prior to testing the role of the splenic nerve and the adrenal glands in regulating splenic cytokine production, parametric data was collected with respect to our experimental model. Time course and dose response studies were conducted to assess splenic cytokines mRNA and protein and measures of HPAA and SNS activation in response to LPS. Time course studies have been previously reported in the literature, however no comprehensive dose response study has been conducted with respect to all the above parameters in the same animals. It was expected that all of these parameters would exhibit a dose-response relationship. We found that cytokine mRNA followed the pattern previously reported in the literature. The dose response for cytokines was steep in that there was only one dose (0.1μg) which induced cytokine mRNA and protein expression between baseline and maximal expression in a statistically significant manner. It was also found that the effect of LPS on plasma corticosterone, plasma epinephrine and splenic NE levels were maximal at increasing doses of LPS; 10μg 100μg and 1000 μg respectively.

3.2. Objectives

1. To characterize the time course for splenic TNF, IL-1 and IL-6 mRNA and protein expression in response to i.v. LPS in the rat.

2. To complete a dose response study of splenic cytokine production in response to i.v. LPS.

3. To measure plasma and brain cytokines levels in response to i.v. LPS.

4. To perform a dose response study on HPAA and SNS activation by measuring plasma corticosterone, E. and NE and splenic NE following i.v. LPS.

3.3. Hypotheses

1. That i.v. LPS will induce splenic cytokine expression in the rat in a similar time course as other species [45].

2. That splenic cytokine levels will be proportional to the dose of i.v. LPS.

3. That plasma cytokines will mirror splenic cytokines but may peak at a later time point.

4. Any changes in brain cytokine levels at the early stages of LPS activation will only be observed with doses of LPS that have the potential to disrupt the BBB.

5. That the SNS and HPAA will be activated by LPS in a dose dependent manner.

3.4. Rationale

LPS was chosen as an inflammatory agent because it is a pro-inflammatory constituent of a clinically relevant pathogen (gram negative bacteria) and mimics the host's response to bacteria except that the host's response to LPS is induced and resolved more rapidly [640,907,908]. This first set of experiments was designed to determine the response of the host to i.v. LPS in terms of cytokine production, HPAA activation and SNS activation. In addition to verifying that the host response to LPS in terms of SNS and HPAA activation is dose dependent [32,45,595,866], it was necessary to establish the optimal dose(s) of LPS for all subsequent experiments. First, a detailed time course study was conducted in order to establish optimal intervals for assessing cytokine production. Next, a dose response study was conducted at the time interval of maximal TNF and IL-1 expression in order to determine the optimal test dose of LPS. Too low a dose of LPS may preclude the assessment of an altered immune response as cytokines may not be detectable (basement effect) while too high a dose of LPS may over stimulate

the immune system such that any physiological manipulation could not alter cytokine production (ceiling effect).

The time course of cytokine production and HPAA and SNS activation in response to LPS have been previously documented, but typically not in the same experiments [45,146,429,519,566,630,866,909]. These reports suggest that all these mediators may be increased within the first hour after LPS administration consistent with their central roles in the initial response to inflammatory stimuli. However, the specific doses of LPS at which these systems are engaged are not well characterized and there are no studies in which the same animals have been used to measure all the pertinent parameters. Givalois et al., conducted the most comprehensive study to date on the time course of plasma cytokines and measures of HPAA activation in response to 3 doses of i.v. LPS (approximately 1.5μg, 7.5μg and 300μg per animal)[519]. Although they used the same LPS strain and rat strain that our lab utilizes, their dose response curve was not complete enough for our purposes. They did not provide information on the time course of cytokine mRNA or protein within the spleen, (the organ utilized in our experimental system) and no information on splenic or plasma catecholamines. Thus, the first goal was to complete the time course for splenic cytokine mRNA and protein production with an i.v. dose of LPS that was known to induce cytokines (based on preliminary studies) in order to determine the optimal point at which to assess the LPS dose response.

The spleen was chosen as the index of immune function in our experimental model for several reasons. Firstly, it is has a large macrophage population, the primary effector

in the early response to LPS [910] and is a site where the body will be exposed early to blood borne antigens [911-914]. Secondly, the size of the rat spleen allows multiple end measures to be made from the same animal. Thirdly, in order to test the effect of the SNS on cytokine production, we required an organ that could easily and selectively be denervated without affecting the innervation of other organs or the afferent nervous system. The spleen is an ideal organ for this purpose as its innervation is exclusively sympathetic and the nerve is accessible for surgical denervation [65]. This is an important consideration as the alternative, chemical denervation with 6OHDA precludes selective denervation of a particular organ. Also, there are many potential side effects of 6OHDA that may affect the host's immune function. A final reason for using the spleen as a model system is that splenic macrophages are in close contact to splenic nerves (as demonstrated by confocal microscopy) [151] and functionally, the splenic nerve is known to be activated in response to LPS [76].

In view of the fact that cytokines are subject to post-transcriptional and post-translational regulation, it was decided that both cytokine mRNA and protein would be measured. For example, if glucocorticoids affect in vivo cytokine mRNA stability, but not cytokine mRNA levels, the in vivo effect may only be detectable in the protein measurements. Although measures of all three cytokines were conducted, the emphasis was on TNF production as it is the first cytokine produced in the inflammatory cytokine cascade and mediates or initiates most of the physiological effects of LPS [45].

Cytokine levels were also measured in the plasma and brain to approach the issue

of circulating and central cytokine involvement in the central response to LPS. Measuring brain levels of cytokines in areas that are involved in the central response to LPS (such as the hypothalamus) and areas that are not involved (such as the cortex) may indicate if central cytokines are part of a signalling cascade or a part of a non-specific inflammatory response similar to other organs.

Plasma corticosterone was assayed as a measure of HPAA activation while plasma NE, E and splenic NE acted as indicies of the activity of the sympathetic nervous system. These results form the basis of the experiments to test if whether the HPAA or SNS is directly involved in the in vivo regulation of splenic cytokine production.

3.5. Experimental Design

All studies utilized adult male Sprague-Dawley rats (225-250 g) that were obtained from Charles River, (Dorval Quebec). The rats were kept on a 12-12 light/dark cycle and given food and water were given *ad libitum*. All animals were singly housed, handled for 2 days then taken through the experimental procedure (weighing, mock injection etc.) for 3 days before the actual experiment. Prior to injection, animals were weighed and then put under a heat lamp for 3 minutes to dilate their tail veins. They were then put in a restrainer and injected i.v. in the tail vein with saline or various doses of lipopolysaccharide (LPS; E.Coli serotype 055:B5 lot L-2637) and were killed at various time intervals following LPS. The doses of LPS ranged from 0.0001μg per rat to 1mg per

rat depending on the study. Animals were killed by decapitation or overdosed with pentobarbital depending on the experiment. Trunk blood, brains and spleens were collected for various assays. The brains were microdissected into hypothalamus, hippocampus and cortex in cold PBS prior to freezing in liquid nitrogen. Northern blots, ELISAs, immunhistochemistry, RIA for corticosterone and catecholamine determinations were performed as described in the materials and methods section. Experiments were analyzed by ANOVA, one and two tailed T-tests as described in the materials and methods. All procedures were approved by the animal ethics committee at the University of Manitoba and the CCAC.

3.6. Results

3.6.1. Time course for splenic cytokines

The time course for the cytokine mRNA and protein was determined in response to 100μg of i.v. LPS and indicated that the inflammatory cytokines increased in a sequential fashion. TNF was detected first followed by IL-1 and finally by IL-6. There was a significant overall effect of time on splenic TNF mRNA levels [$F(5,12)=21.27$, $p<0.0001$] (figure 3-1) where maximal levels were observed at both 30 minutes and 1 hr after LPS as compared to baseline ($p<0.0001$). These levels declined significantly by 2 hr ($p<0.0001$) and were at baseline by 6 hr post-injection. Time points prior to 30 minutes

were not tested in these experiments. The expression of splenic TNF protein levels also displayed a significant overall effect with respect to time [$F(5,12)=13.19$, $p=0.0002$] (figure 3-2). Splenic TNF protein was significantly increased (versus baseline) at 30 minutes post-injection ($p=0.019$), peaked at 1 hr (as compared to 30 minutes $p=0.004$), decreased by 2 hr (as compared to 1 hr $p=0.0295$) and returned to baseline by 4 hr ($p=0.0031$ as compared to 2 hr). Unpublished observations with immunohistochemistry for splenic TNF protein corroborated these results.

The time course for splenic IL-1 mRNA (figure 3-3) was similar to TNF mRNA in that there was and overall significant effect [$F(5,12)=72.82$, $p<0.0001$]. In terms of individual groups there was a significant increase at 30 minutes (compared to baseline, $p<0.0001$). IL-1 mRNA levels were further increased at 1 hr post-injection ($p=0.0002$). Levels were still high at 2 hr post-injection but lower than 1 hr ($p=0.0001$) and approaching baseline by 4 hr post-injection ($p=0.0001$ as compared to 2 hr). Interestingly, there was detectable baseline expression for splenic IL-1 protein (figure 3-4) and the overall effect of time remained significant [$F(5,12)=45.67$, $p<0.0001$]. In terms of individual groups, there was a trend for increased IL-1 protein levels at 30 minutes post-LPS versus baseline ($p=0.056$) which was further increased by 1 hr (versus 30 minutes $p=0.002$) and peaked at 2 hr post-LPS (versus 1 hr $p<0.0001$). IL-1 protein levels were still high at 4 hr, but lower than at 2 hr ($p=0.0017$) and were still elevated at 6 hr ppost-injection but were lower than the 4 hr post-injection levels ($p=0.0034$). We found that plasma TNF but not plasma IL-1 was detectable at 1 hr post-injection although both were

detected at 2 hr post-injection (data not shown).

Splenic IL-6 mRNA had a delayed time course in comparison with IL-1 mRNA (figure 3-5) but a significant overall effect was observed [$F(5,12)=4.45$, $p=0.016$]. No IL-6 mRNA expression was observed until 1 hr after LPS ($p=0.069$ as compared to baseline), with a peak at 2 hours ($p=0.071$ as compared to 1 hr and $p=0.0018$ as compared to either 0 hr or 30 minutes) and a decreased level of expression at 4 hr as compared to the 2 hr time interval ($p=0.0214$). The lack of significant effects among the various groups is due to the variability, which is more prominent for splenic IL-6 protein levels as there was no significant overall effect of time on splenic IL-6 protein levels [$F(5,12)=1.88$, $p=0.17$ ns] (figure 3-6). In terms of the individual time intervals, IL-6 protein peaked at 2 hr post-injection with significant differences between 2 hr and baseline ($p=0.02$) and 2 hr and 30 minutes ($p=0.03$).

3.6.2. Dose response for splenic cytokines

Based upon the results from the time course analysis, one hour post-injection was chosen as the point to test the dose response for i.v. LPS as this interval was the peak for splenic TNF production and the other cytokines were also detectable. There was a significant overall effect for the dose of LPS on TNF mRNA production [$F(6,28)=88.71$, $p<0.0001$] (figure 3-7). In terms of the response to the individual doses of LPS, TNF mRNA was barely detectable above baseline in response to 0.01μg of i.v. LPS. The level of TNF mRNA induced after 0.1μg of LPS was higher than the amount induced by the

0.01μg dose (p=0.0003) but lower than the levels induced by 1μg of LPS (p<0.0001). There were no differences between the effects of 1μg, 10μg, 100μg and 1000μg of i.v. LPS on splenic TNF mRNA levels. Therefore the 0.1μg dose emerged as the dose between baseline and maximum expression. A similar trend was observed for splenic TNF protein (figure 3-8) in terms of the 0.1μg dose and overall effects [F(6,28)=34.13, p<0.0001]. This was confirmed by immunohistochemistry (data not shown). The ELISA assay for splenic TNF protein detected a wider dose response from 1μg-1000μg than that observed for the mRNA. It was found that there was significantly more splenic TNF protein produced in response to 100μg of LPS as compared to the amount produced in response to 1μg of LPS (P=0.0009) (figure 3-8).

Similar to TNF, there was an overall effect of the dose of LPS on splenic IL-1 mRNA [F(6,28)= 26.76, p<0.0001] (figure 3-9). The levels of IL-1 mRNA expressed at the individual time intervals followed the same trend as the TNF mRNA in that the 0.01μg dose induced minimal expression, while the IL-1 mRNA levels peaked after 1μg of LPS. The 0.1 μg dose induced significantly more IL-1 mRNA than the 0.01μg dose (p=0.0012), and significantly less than the 1μg dose (p=0.0005). There was no difference in IL-1 mRNA levels in response to 1μg, 10μg, 100μg or 1000μg of i.v. LPS. Splenic IL-1 protein (figure 3-10) showed a similar overall effect of dose [F(6,28)=45.70, p<0.0001] and similar effects between groups as the mRNA for splenic IL-1. It was observed that the amount of IL-1 protein induced by the 0.1μg dose was higher than the amount induced by the 0.01μg dose (p=0.012) but less than the amount induced by the 1μg dose

($p<0.0001$). There was no difference in splenic IL-1 protein levels in response to 1μg, 10μg, 100μg or 1000μg of i.v. LPS. The difference between splenic mRNA and protein was that IL-1 protein production was not increased above baseline in response to 0.01μg dose of LPS.

There was also a significant overall effect of the dose of LPS on splenic IL-6 mRNA [$F(6,28)=5.07$, $p=0.0012$] (figure 3-11). However the response was not proportional to the dose of LPS in that expression was either present or absent. Doses lower than 1μg did not induce IL-6 mRNA, while doses of 1μg or higher did induce IL-6 mRNA. The response to 1μg was the peak response and equal to the response induced by the 10μg, 100μg, and 1000μg doses of LPS. The results for splenic IL-6 protein (figure 3-12) were similar to the mRNA in that there was a significant overall effect of the dose of LPS on protein production [$F(6,28)=3.42$, $p=0.016$] and the protein was either present or absent with no significant differences between groups.

3.6.3. Dose response for plasma and brain cytokines

TNF was the only plasma cytokine measured as plasma IL-1 was not detectable. The assay for IL-6 was not performed as it was not detectable until later time intervals than IL-1. The results for plasma TNF (figure 3-13) were similar to splenic TNF protein and mRNA levels in that there was a significant main effect of the dose of LPS on TNF protein expression [$F(6,28)=31.38$, $p<0.0001$]. In terms of the individual groups it was found that the 0.1μg dose induced higher levels of plasma TNF than baseline ($p=0.038$)

yet lower than the 1μg dose (p=0.003). Also, the 0.01μg dose did not induce TNF protein levels above baseline. The difference between the plasma levels and the splenic levels of TNF protein was that plasma levels showed more of a dose effect. This was determined by the fact that the 10μg dose of LPS-induced higher levels of plasma TNF than the 1μg dose (p=0.025) whereas in the spleen these doses induced equivalent levels of both TNF protein and mRNA. The 10μg dose however was no different from either the 100μg or 1000μg doses.

Based on the literature it was determined that if the levels of central cytokines could change in response to peripheral LPS at 1 hour post-injection, it would likely be in response to a dose of LPS that could disrupt the BBB. Therefore the preliminary study only assayed brains from animals treated with either saline or 1000μg of i.v. LPS (figures 3-14 and 3-15). The areas assayed included the hippocampus, hypothalamus and cortex. No difference between saline and LPS treatment was observed in any of the areas, nor were there differences in cytokine levels between the three anatomical locations.

3.6.4. Dose response for HPAA and SNS activation

There was an overall significant effect of the dose of LPS on plasma corticosterone levels [$F(6,28)=32.50$, $p<0.0001$]. The mean baseline plasma corticosterone level was 158 ng/ml and they were not different from levels induced in response to the 0.01μg or 0.1μg doses of i.v. LPS (figure 3-16). However, the 1μg dose induced a mean corticosterone level of 504 ng/ml, which was significantly different from

the 0.1μg dose ($p<0.0001$). These elevated levels however were no different from those induced by the 10μg, 100μg, or 1000μg doses. Therefore the observed levels of plasma corticosterone were either high or low and did not show a graded dose response.

The overall effect of the dose of LPS on plasma E (figure 3-17) was not significant [$F(6,28)=2.37$, $p=0.056$ ns]; however there was a trend for more E to be produced in response to doses of 10μg or higher as compared to the lower doses. For example 10μg of LPS induced more E than 0.01μg ($p=0.04$) while 1000μg of LPS induced more E than 1μg ($p=0.027$). These results showed a similar pattern to the effect of the dose of LPS on plasma corticosterone, except the effect was shifted one dose to the right. There were no significant effects of the dose of LPS on plasma NE [$F(6,28)=0.80$, $p=0.57$ ns] (figure 3-18), although a pattern for increased levels was indicated at the 1000μg dose. Similar to plasma E, there was no significant overall effect of the dose of LPS on splenic NE levels [$F(6,28)=1.07$, $p=0.411$ ns] (figure 3-19); however, levels of splenic NE appeared to decrease in response to the 100 and 1000μg doses of LPS.

3.7. Figures (* indicates that P≤0.05)

Figure 3-1: Time course for splenic TNF mRNA following 100μg of i.v. LPS. Rats were injected with 100μg of i.v. LPS and killed at various points post-injection. Splenic TNF mRNA [F(5,12)=21.27, p<0.0001, n=18] levels were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

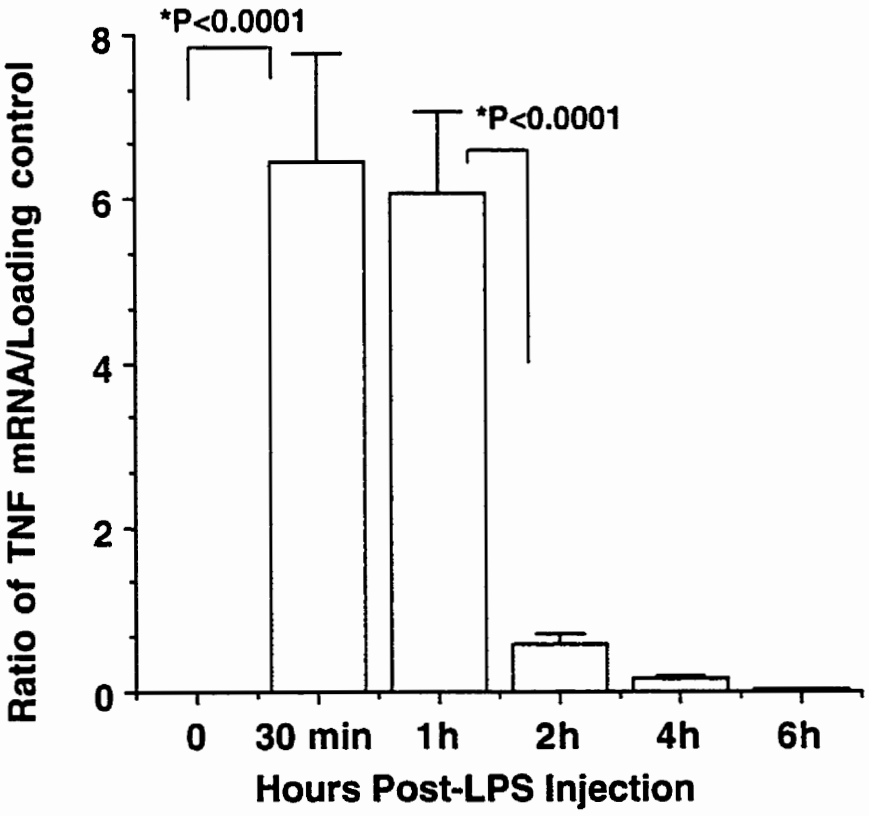


Figure 3-2: Time course for splenic TNF protein following 100μg of i.v. LPS. Rats were injected with 100μg of i.v. LPS and killed at various points post-injection. Splenic TNF protein levels [$F(5,12)=13.19$, $p<0.0001$, $n=18$] were measured by ELISA and expressed as pg/mg of splenic protein. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

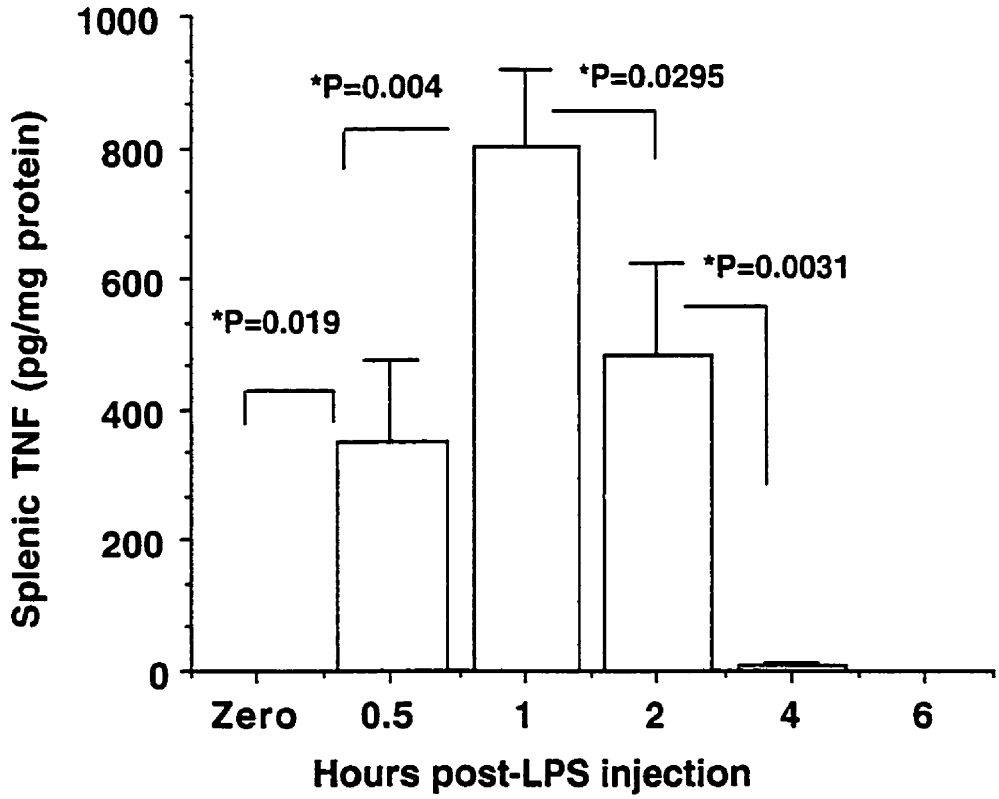


Figure 3-3: Time course for splenic IL-1 mRNA following 100μg of i.v. LPS. Rats were injected with 100μg of i.v. LPS and killed at various points post-injection. Splenic IL-1 mRNA levels [$F(5,12)=72.82$, $p<0.0001$, $n=18$] were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

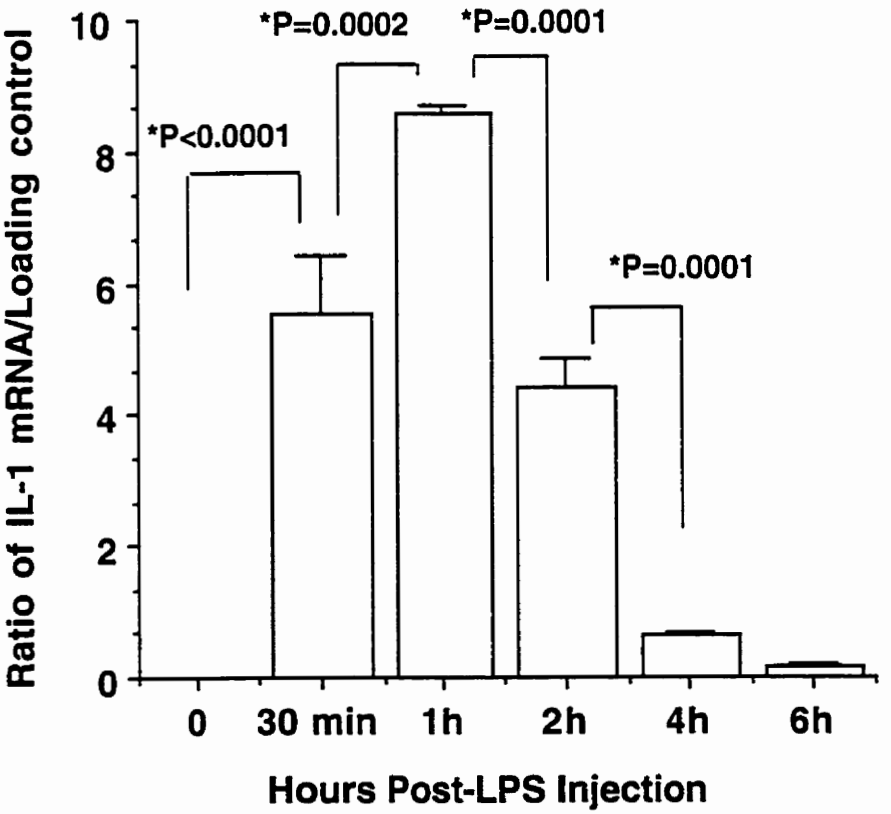


Figure 3-4: Time course for splenic IL-1 protein following 100μg of i.v. LPS. Rats were injected with 100μg of i.v. LPS and killed at various points post-injection. Splenic IL-1 protein levels [$F(5,12)=45.67$, $p<0.0001$, $n=18$] were measured by ELISA and expressed as pg/mg of splenic protein. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

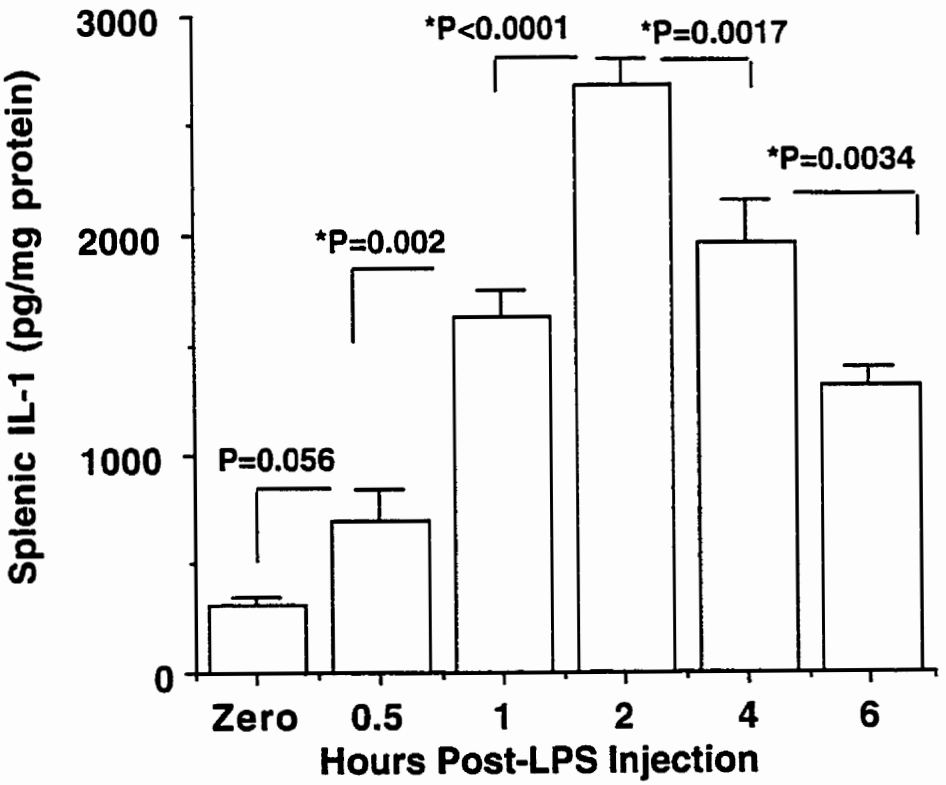


Figure 3-5: Time course for splenic IL-6 mRNA following 100μg of i.v. LPS. Rats were injected with 100μg of i.v. LPS and killed at various points post-injection. Splenic IL-6 mRNA levels [F(5,12)=4.45, p=0.016, n=18] were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

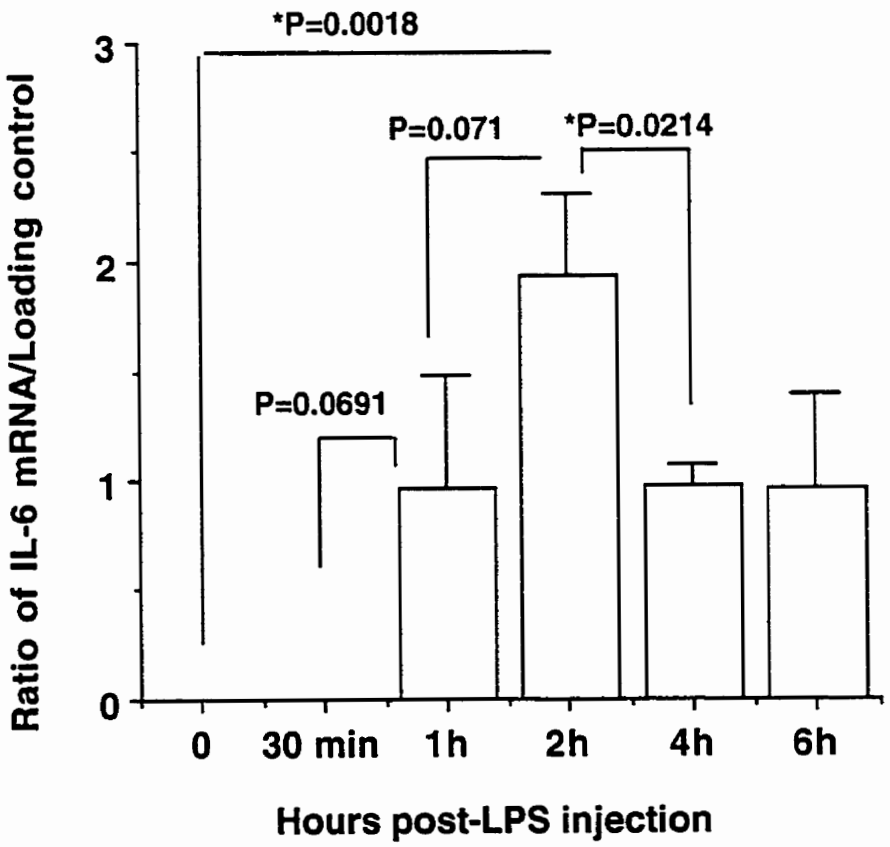


Figure 3-6: Time course for splenic IL-6 protein following 100μg of i.v. LPS. Rats were injected with 100μg of i.v. LPS and killed at various points post-injection. Splenic IL-6 protein levels [F(5,12)=1.88, p=0.17ns, n=18] were measured by ELISA and expressed as pg/mg of splenic protein. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

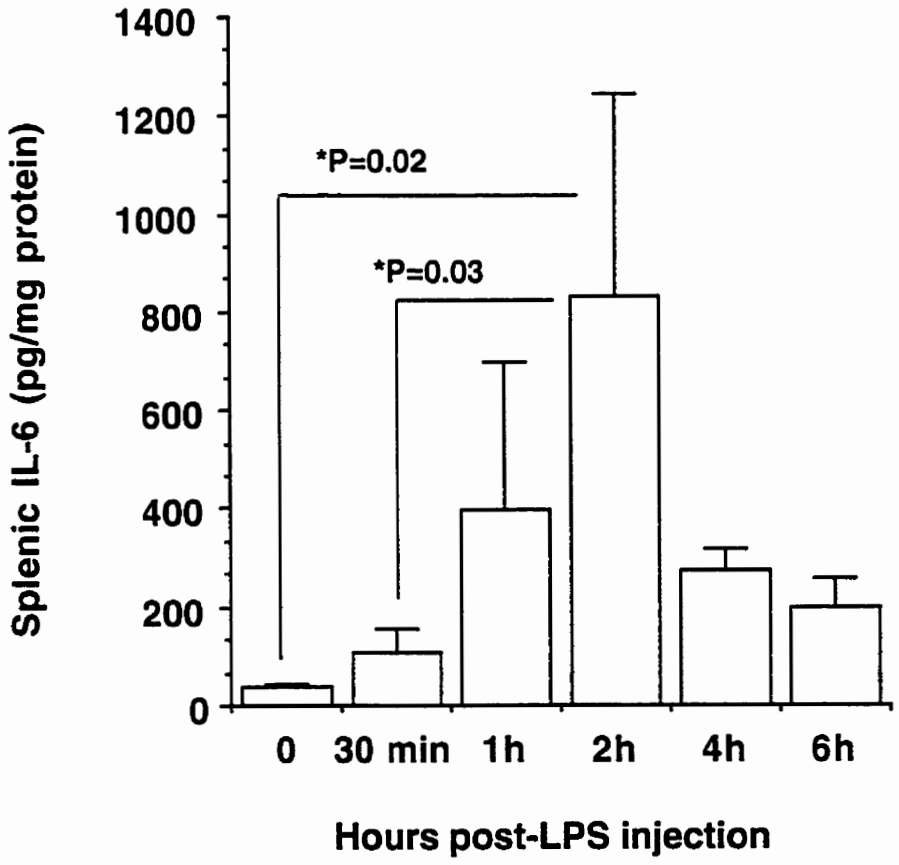


Figure 3-7: Dose response for splenic TNF mRNA one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic TNF mRNA levels [$F(6,28)=88.71$, $p<0.0001$, $n=35$] were analyzed by Northern blotting and expressed as a ratio relative the loading control. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

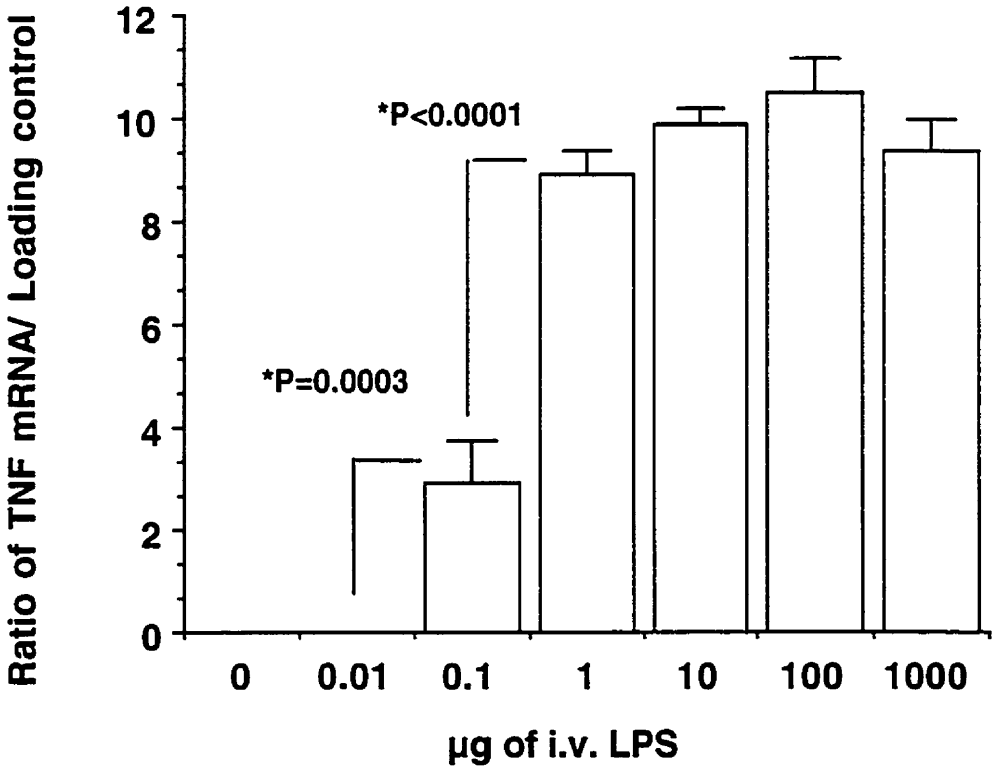


Figure 3-8: Dose response for splenic TNF protein one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic TNF protein levels [$F(6,28)=34.13$, $p<0.0001$, $n=35$] were measured by ELISA and expressed as pg/mg of splenic protein. The groups were compared by ANOVA and error bars represent +/-1 standard error of the mean.

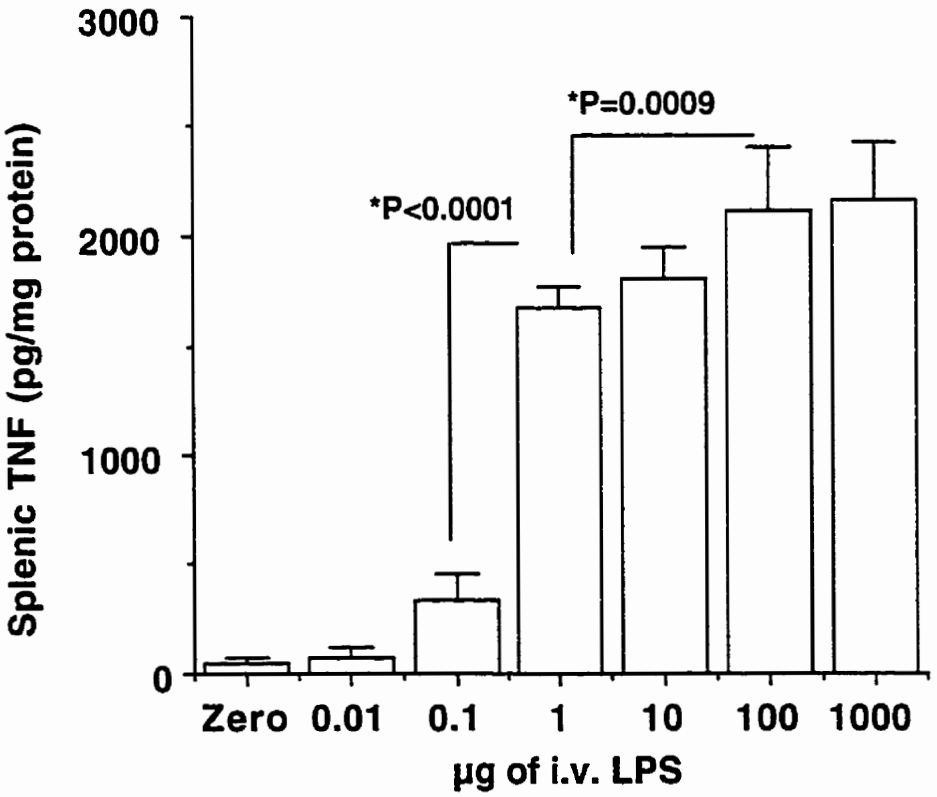


Figure 3-9: Dose response for splenic IL-1 mRNA one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic IL-1 mRNA levels [$F(6,28)=26.76$, $p<0.0001$, $n=35$] were analyzed by Northern blotting and expressed as a ratio relative the loading control. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

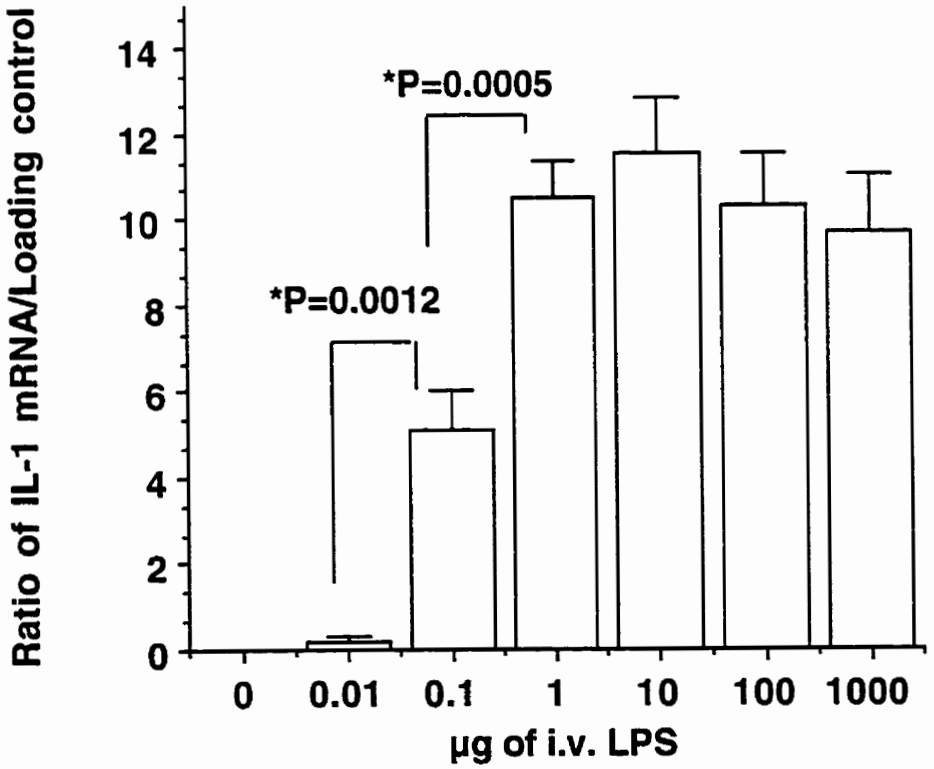


Figure 3-10: Dose response for splenic IL-1 protein one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic IL-1 protein levels [$F(6,28)=45.70$, $p<0.0001$, $n=35$]were measured by ELISA and expressed as pg/mg of splenic protein. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

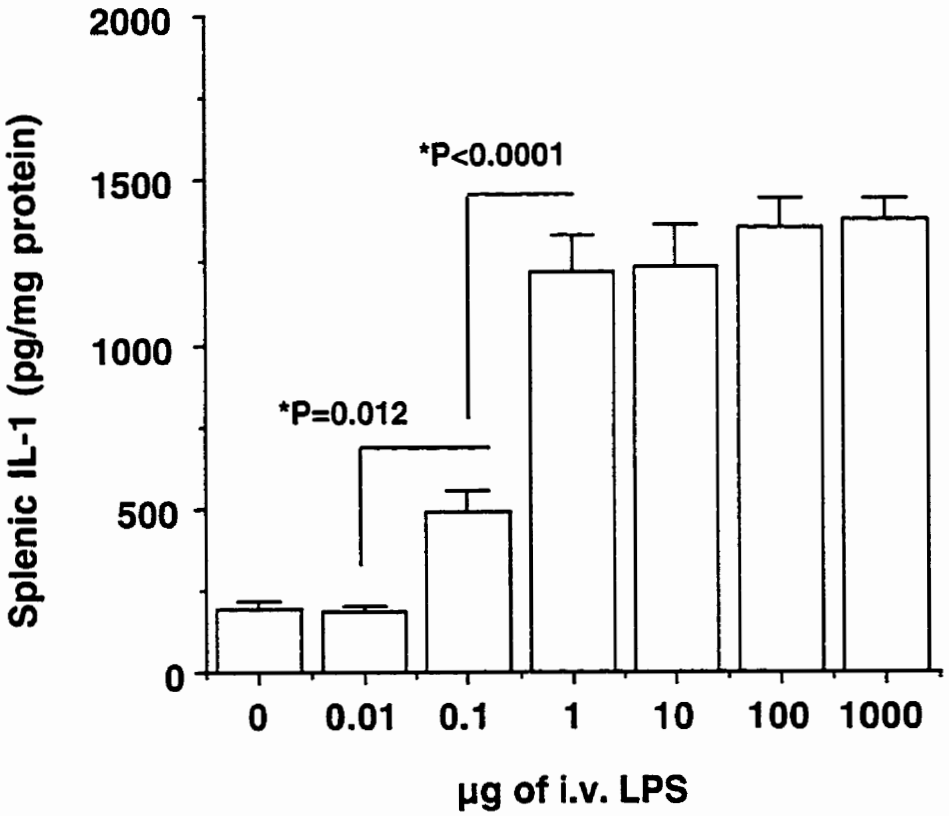


Figure 3-11: Dose response for splenic IL-6 mRNA one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic IL-6 mRNA levels [F(6,28)=5.07, p=0.0012, n=35] were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

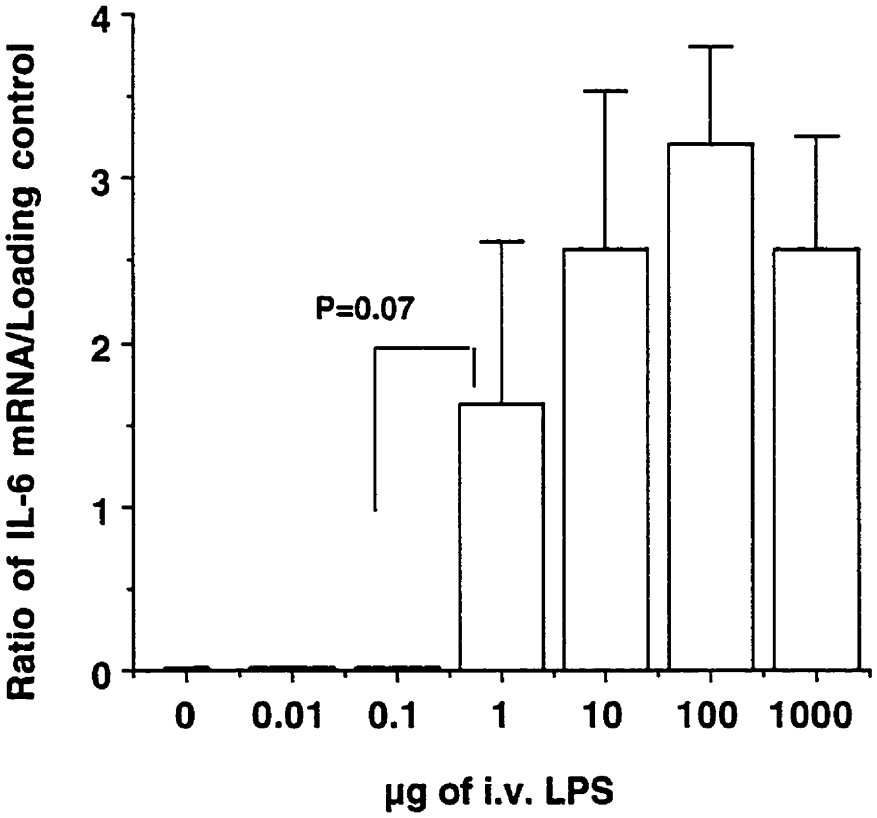


Figure 3-12: Dose response for splenic IL-6 protein one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic IL-6 protein levels [F(6,28)=3.42, p=0.016, n=35] were measured by ELISA and expressed as pg/mg of splenic protein. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

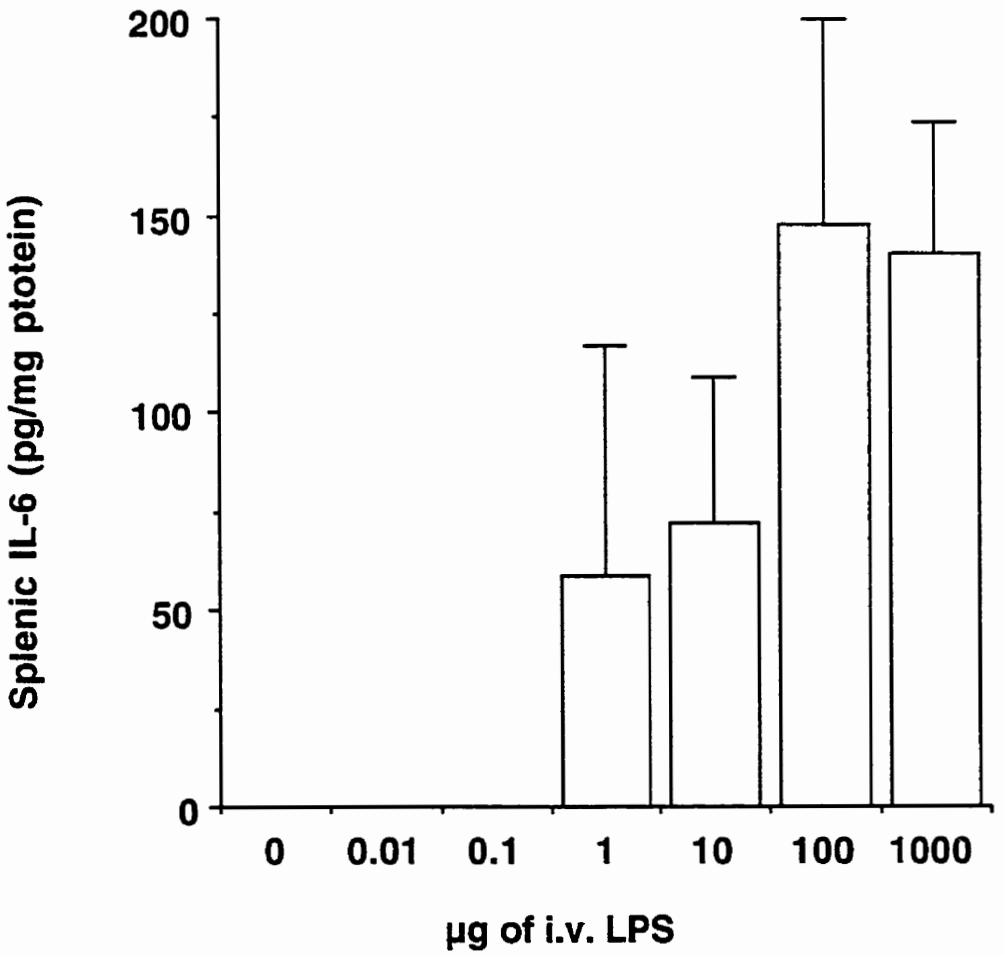


Figure 3-13: Dose response for plasma TNF protein one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Plasma TNF protein levels [$F(6,28)=31.38$, $p<0.0001$, $n=35$] were measured by ELISA and expressed as ng/ml. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

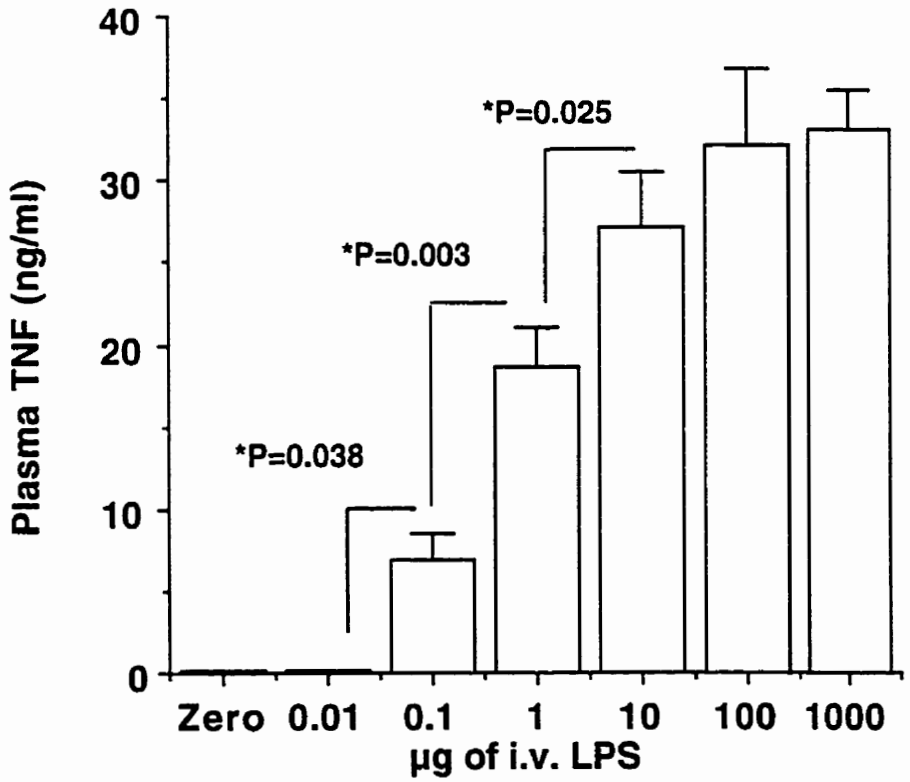


Figure 3-14: Measurement of TNF protein in several brain areas 1 hr after 1000μg of i.v. LPS. Rats were injected i.v. with either saline (SAL) or 1000μg of LPS and killed at 1 hour post-injection. Brain TNF protein levels were measured by ELISA and expressed as pg/mg of brain protein. HIP-hippocampus [T(8)=0.085, p=0.93ns, n=10], HYP-hypothalamus [T(8)=0.174, p=0.87ns, n=10] and COR-cortex [T(8)=0.82, p=0.43ns, n=10]. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

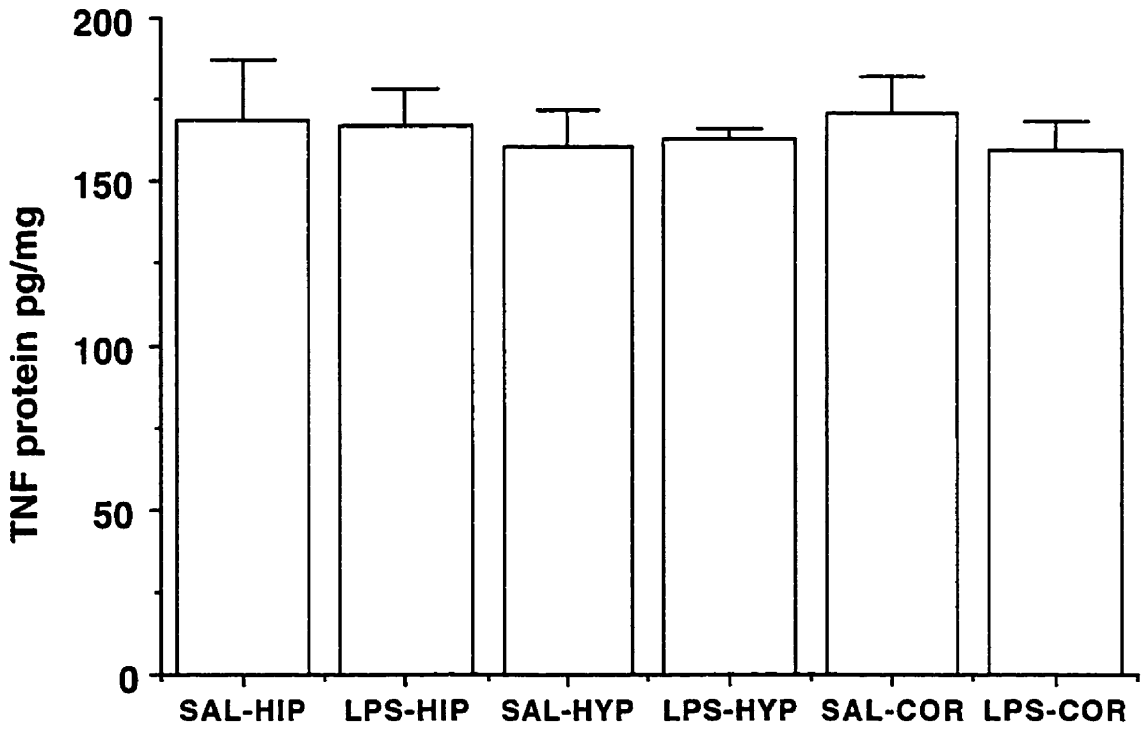


Figure 3-15: Measurement of IL-1 protein in several brain areas 1 hr after 1000μg of i.v. LPS. Rats were injected i.v. with either saline (SAL) or 1000μg of LPS and killed at 1 hour post-injection. Brain IL-1 protein levels were measured by ELISA and expressed as pg/mg of brain protein. HIP-hippocampus [T(8)=0.172, p=0.87ns, n=10], HYP-hypothalamus [T(8)=0.543, p=0.60ns, n=10] and COR-cortex [T(8)=0.40, p=0.70ns, n=10]. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

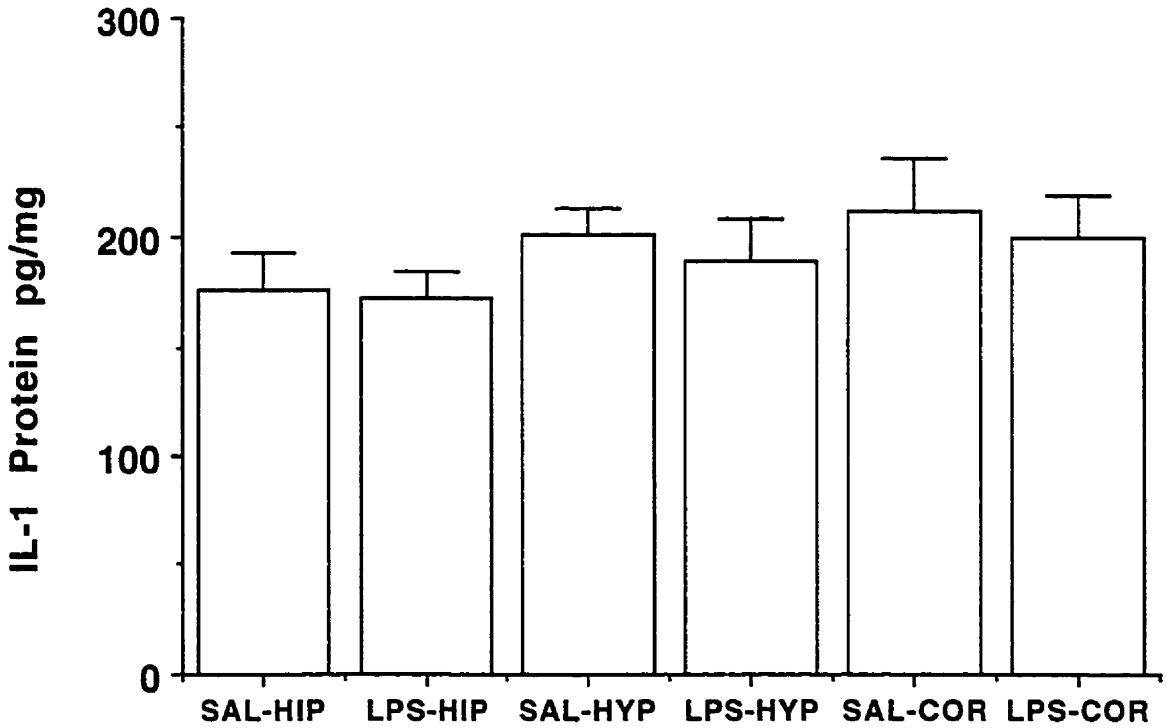


Figure 3-16: Dose response for plasma corticosterone one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Plasma corticosterone levels [$F(6,28)=32.50$, $p<0.0001$, $n=35$] were measured by RIA and expressed as ng/ml. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

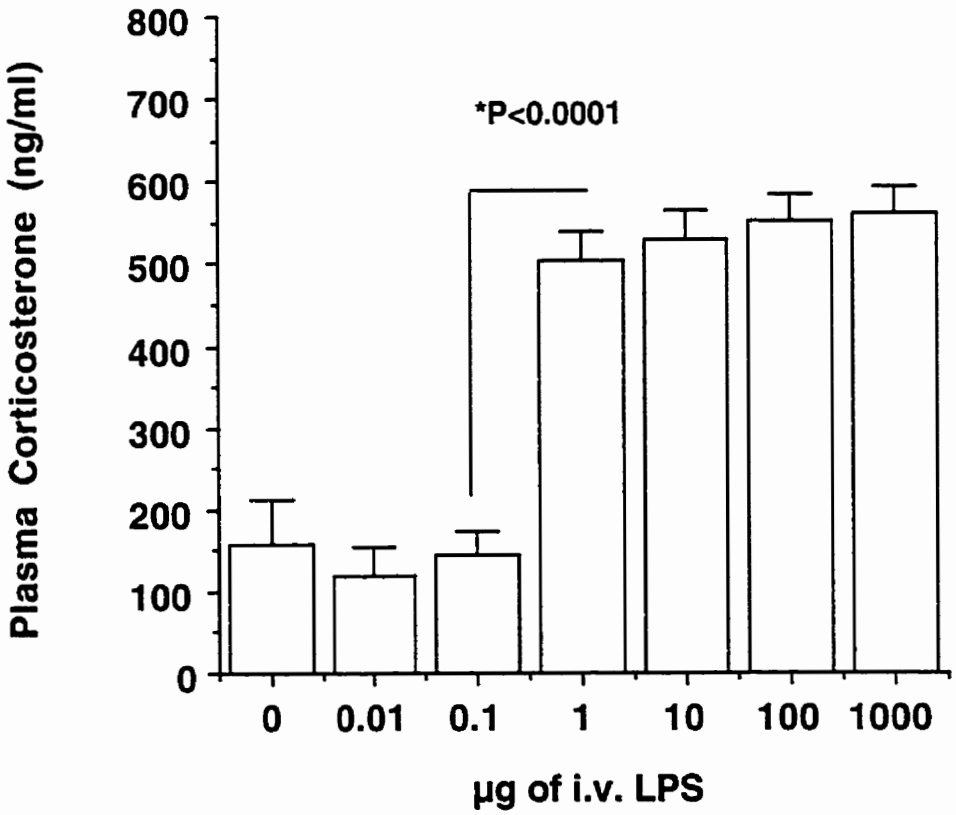


Figure 3-17: Dose response for plasma epinephrine one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Plasma epinephrine levels [F(6,28)=2.37, p=0.056, n=35] were measured by HPLC and expressed as pg/ml. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

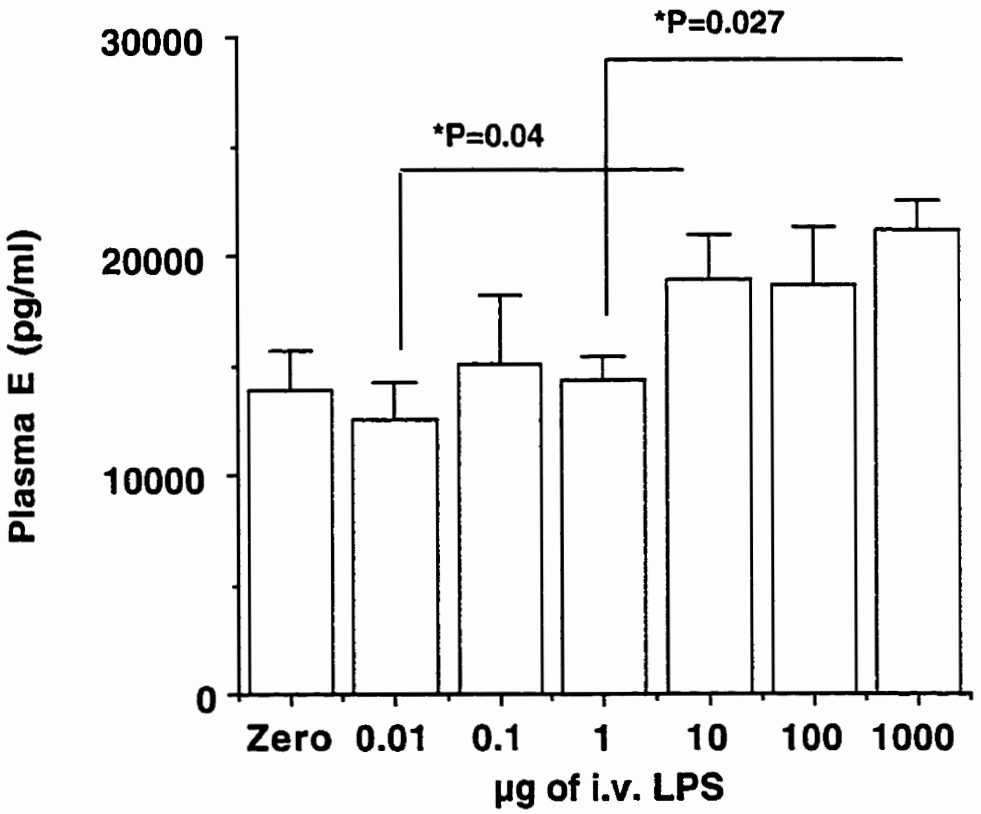


Figure 3-18: Dose response for plasma norepinephrine one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Plasma norepinephrine [F(6,28)=0.80, p=0.57ns, n=35] levels were measured by HPLC and expressed as pg/ml. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

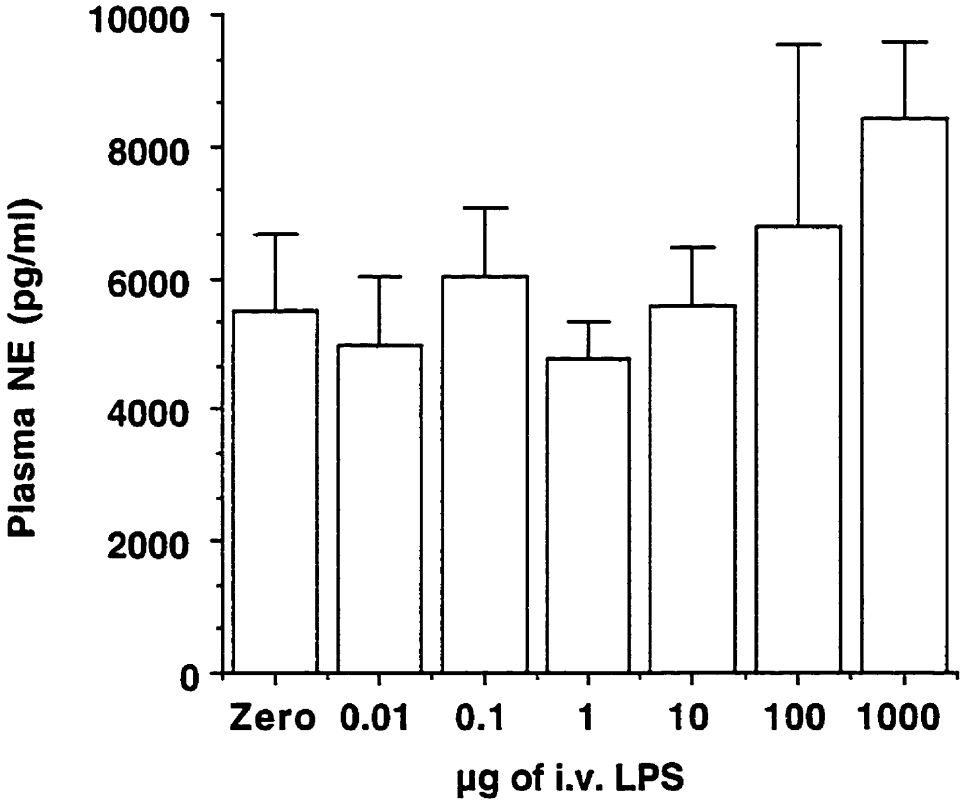
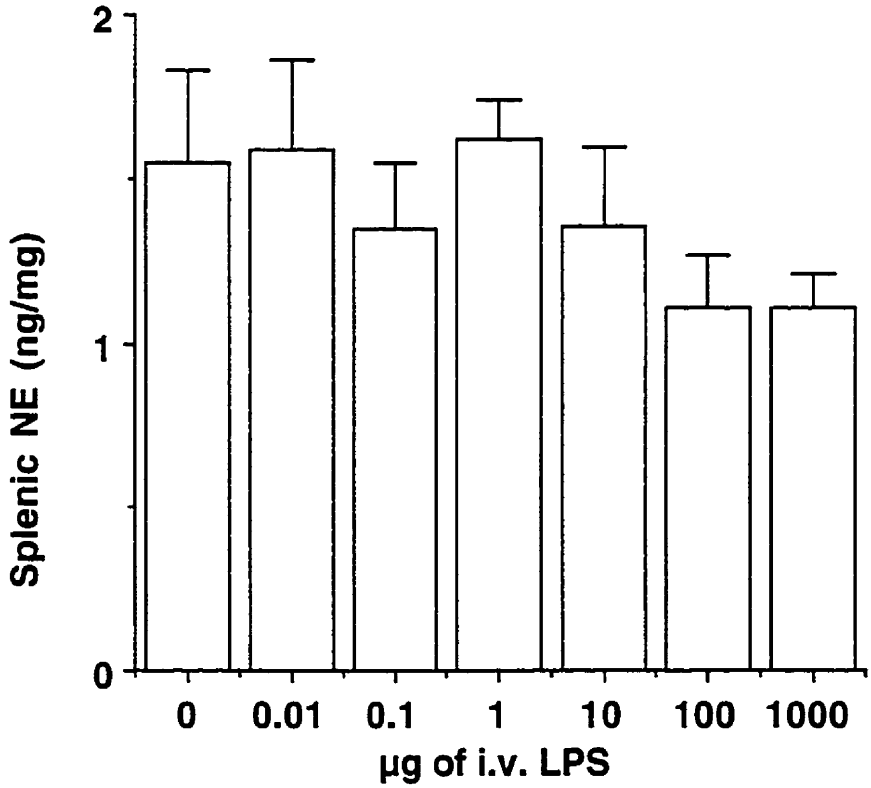


Figure 3-19: Dose response for splenic norepinephrine one hour following i.v. LPS. Rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Plasma norepinephrine levels [F(6,28)=1.07, p=0.41ns, n=35] were measured by HPLC and expressed as ng/mg of splenic protein. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.



3.8. Discussion

3.8.1. Technical considerations

At the initiation of our experiments it was decided that since cytokines mediate many of the host's response to LPS, they were the best in vivo indicators of immune function in response to an inflammatory stimulus. Therefore we required reliable in vivo means to measure cytokine expression as brain-immune interactions involve the interplay between many physiological systems and removing immune cells from their microenvironment can eliminate this relationship [266,267]. We thus decided that Northern blotting would be an ideal technique for this purpose in that it was sensitive, it could be used for semi-quantitative comparisons, and that we could generate the necessary probes ourselves in the absence of commercial reagents [891,892]. However cytokines are susceptible to regulation at various points in their production and increases in mRNA levels may not always be indicative of increased protein levels [551,552,583,584,915] . Therefore measuring the protein levels for these cytokines along with the mRNA was important as mediators like glucocorticoids can effect TNF mRNA by 50% but almost completely eliminate protein production [327]. Our early pilot experiments with bioassays suggested they were unsuitable for our purposes. Although we had an established immunohistochemistry protocol [151], this technique was too dependent on the quality of the primary antibody. This is evidenced by the fact that we had prominent and specific staining for TNF (which mirrored the results obtained with

our Northern blots) but we could not detect IL-1 despite trying upwards of 10 different commercial and privately produced anti IL-1 antibodies. Other reports suggested that although immunohistochemistry can index changes in the presence and location of a molecule, ELISAs and bioassays are better for quantification purposes [188,916]. Western blotting for cytokines was also considered as a possible end measure as it would give evidence of both translational levels of the protein (precursor form) and post-translational modification (secreted form). However in our hands, this technique lacked the required detection sensitivity. Thus recently developed commercial ELISA kits have been employed in our experimental model to assess cytokine protein levels.

3.8.2. The time course for splenic cytokines in response to i.v. LPS

The time course for splenic cytokines in the rat followed a similar pattern as described by Givalois et al [519], Perretti et al., [321] and Turnbull et al., [677] for plasma cytokines. The only difference was that our time course peaked and ended at earlier intervals probably due to the fact that increases in tissue cytokine levels precede increases in plasma cytokine levels. Consistent with the above reports, TNF peaked first, IL-1 second and IL-6 third. Splenic TNF mRNA peaked at 30-60 minutes and returned to baseline by 4 hr while splenic TNF protein was present at 30 minutes, peaked at 1 hr, and was gone by 4 hr. IL-1 mRNA levels were elevated by 30 minutes post-injection but IL-1 protein was not significantly increased until 1 hr post-injection and peaked at 2 hr post-injection. IL-6 mRNA was not present until 1 hr, peaked at 2 hr and was maintained

at an increased level from 4 to 6 hr post-injection. In our pilot studies, only TNF was detected in the plasma at 1 hour post-injection, however both TNF and IL-1 were detected at 2 hr post-injection. This indicates that although IL-1 has been described as the primary mediator of LPS-induced central activation, it is probably only responsible for a part of the response as it is produced and reaches the circulation too late to initiate centrally mediated host responses. This is corroborated by other time course studies [519] as well as by studies in IL-$1^{-/-}$, IL-$1R^{-/-}$ or $ICE^{-/-}$ mice which show a normal HPAA response to LPS [570,571,917].

The host's response to LPS and/or cytokines includes the release of catecholamines and corticosterone, and at higher doses can include induction of fever, behavioral changes and blood pressure changes. The time course for changes in plasma catecholamines and corticosterone in rats in response to i.v. LPS has been extensively described. Essentially, elevated levels of ACTH and corticosterone can be detected as early as 15 minutes after i.v. LPS and usually peak at 1-2 hr post-injection [321,427,519,630,631,677,918] . Therefore it is likely that ACTH is secreted immediately in response to LPS [630]. Similar to HPAA activation, c-fos expression can be increased in the PVN as early as 15 minutes in response to an intraarterial (i.a.) injection of LPS [919], but generally peaks at 2-3 hr post-injection [31,597-599]. The onset of SNS activation in response to i.v. LPS is also rapid, within 20-30 minutes [76,645]. This effect was measured in the splenic nerve and determined to be an immune-specific effect [76,645]. This is probably the best indicator of SNS activation as splenic

levels of NE may not reflect activity as well as turnover studies [219]. However higher doses of LPS (arguably doses that induce cardiovascular changes) can cause increases in plasma NE and E that can be detected as early as 20-30 minutes post-injection [77,147,920] .

It is not immediately clear which mediators represent the direct response to the LPS and which mediators represent the counter response. Functionally however it is known that glucocorticoids and catecholamines inhibit cytokine production, fever induction (through both central feedback and reduction in cytokine production [621.921]) and the behavior changes associated with the inflammatory response. However in a temporal context, corticosterone and catecholamines can appear at the same time interval as plasma cytokines or can even precede them [519]. So although cytokines can activate the HPAA , SNS [74], and mediate the other downstream effects of LPS such as fever, LPS itself must send the first signal to the brain to activate the HPAA and SNS. Proof of this includes the fact that blocking individual cytokines does not block the response to LPS [577]. LPS receptors (CD14) are located in the brain and thus may mediate the early central effects of LPS similar to the activation of immune cells [637]. Also, although peripheral cytokines can induce SNS and HPAA activity, they would have to be transcribed, translated and in the circulation as early as 5 minutes post-LPS to mediate the initial response to inflammation.

The only way cytokines could mediate the initial effects of LPS activation is if they were present centrally, in a basal state, and could activate the HPAA and SNS

immediately in response to LPS. Functional evidence for this exists as blockade of central cytokines can abrogate some of the physiological effects of peripheral inflammation [675,702,704] and central cytokines have been proposed as signalling intermediates for the stress response [207,698,874]. The problem (as described in the literature review) is that the anatomical evidence does not support the functional evidence. The basal expression of cytokines is not evident [675], and when cytokines are detected, it is often many hours after the initial activation of the HPAA and SNS [699]. Although ELISAs can detect basal levels of cytokines in the brain as determined by our results and the results of others [699], we found there are minimal differences between brain areas and no treatment effects. Therefore the relevance and specificity of the results are questionable.

3.8.3. The effect of dose on the host's response to LPS

The host's response to LPS is not an all or nothing event, therefore the dose administered will dictate which systems that will be activated in order to preserve homeostasis. Smaller doses may only cause local activation while higher doses can cause systemic responses such as shock. Although the time course can be moderately altered by the dose of LPS [519], this response pattern is quite consistent between species. Although the LPS dose response data for catecholamines, corticosterone and cytokine levels have been reported, these dependent measures have not been assessed in the same animals. This is important because not only do species have different sensitivities to LPS, but different strains of rats will react differently to the same stimulus

[205,317,630,845,922] . Taking these factors into account, our results are discussed in reference to other studies using i.v. doses of LPS in Sprague-Dawley rats whenever possible, to ensure the most meaningful comparisons.

3.8.3.1. Peripheral cytokines

Similar to our time course experiments, the dose response experiment was primarily designed to measure splenic levels of cytokines. It was first found that the dose response for splenic cytokines was relatively steep. Minimal induction (usually not statistically different from baseline) for TNF and IL-1 mRNA was seen in response to 0.01μg of LPS while maximal induction was present in response to 1μg of i.v. LPS. The levels of TNF and IL-1 mRNA induced by the 0.1μg dose were each significantly different from and intermediate to the levels induced by the 0.01μg and 1μg doses. Splenic IL-1 protein mimicked this pattern exactly. The response of splenic TNF protein was similar except that the levels were further increased in response to the 100μg and 1000μg doses of LPS in comparison to the 1μg and 10μg doses. It is unlikely that this small increase in TNF protein is responsible for the more severe host response at these higher LPS doses as this effect was not evident for plasma TNF. IL-6 mRNA was only detectable in response to the 1μg and higher doses of LPS. We did not determine whether lower doses of LPS would induce detectable IL-6 levels as peak IL-6 expression is 2 hr post-injection. Like the mRNA, splenic IL-6 protein was only detectable in response to a 1 μg or higher dose of LPS at 1 hr post-injection.

TNF was the only cytokine detectable in the plasma at 1 hr post-injection. Similar to splenic mRNA and protein studies, it was found that the 0.1μg of LPS-induced circulating TNF protein levels between baseline and maximum. However, the dose response for circulating TNF protein levels was less steep than observed for the spleen. Specifically, the 1μg dose of LPS-induced plasma TNF levels that were higher than baseline yet lower than the maximum levels observed in response to 10 μg of LPS. In contrast, there was no difference in splenic TNF levels in response to the 1μg and 10μg doses of LPS. One explanation for the wider dose response seen for circulating cytokine levels versus splenic cytokine levels in response to LPS is that increased TNF protein turnover occurs in response to the higher doses of LPS. Therefore although it may appear that splenic TNF levels are the same in response to two doses of LPS, it is possible that the higher dose of LPS induces more TNF production while simultaneously causing more TNF to be released into the plasma. Another possibility is that since the spleen is not the major contributor to circulating TNF levels, that the dose response for plasma TNF more closely mirrors the dose response from other organs such as the liver [923,924]. It has not been determined if the dose response for TNF production is different in the liver as compared to the spleen in response to i.v. LPS. Overall however, there was a strong relationship between the changes in cytokine mRNA and protein after i.v. LPS.

Givalois et al., [519] found that circulating TNF peaked after their lowest dose of LPS tested using the same rat strain and same strain of LPS (approximately 1.5μg of i.v. LPS/rat). They observed that the circulating IL-1 levels was less responsive to LPS than

TNF in that IL-1 was not detected in the plasma until the medium dose of LPS (approximately 7.5μg/rat), and not until 2 hours post-injection. These levels peaked in response to the high dose (approximately 90μg of i.v. LPS/rat). IL-6 appeared in response to the lowest dose, but not until 2 hr post-injection. Like IL-1, circulating IL-6 also peaked in response to the high dose of LPS. Therefore our results agree with those of Givolais et al., in that there is a differential sensitivity between TNF and IL-6. However we did not observe a differential sensitivity in response to LPS between IL-1 and TNF in terms of splenic mRNA and protein levels. Other reports have also confirmed that plasma cytokine levels peak at relatively low doses of LPS that do not engage the maximal host response [569].

3.8.3.2. Central cytokines

It is known that peripheral cytokines can activate the brain through a number of potential routes. However there is still no consensus as to the role of central cytokines in response to peripheral inflammation. Cytokines can be detected centrally after LPS but most studies have detected these molecules only at time intervals [650,651,925] and doses [659] that suggest a disrupted BBB. However, the fact that the blockade of central cytokines can disrupt the response to peripheral inflammation even in circumstances where central and circulating cytokines are not detectable [675] provides functional evidence for a regulatory role for cytokines within the brain. Interestingly, central cytokines have been implicated in the central signalling pathways in response to non-

immune stressors as well [207,698,699,874].

In our dose response study, we determined if changes in central cytokine levels could be detected in response to a dose of LPS that is known to disrupt the BBB, at a time period (1 hr post LPS) during which multiple regulatory systems are fully engaged. We conceded that any observed effect with this simple assay would not indicate the mechanism by which central cytokine levels were increased. This is due to the fact that increased levels of central cytokines could be due to the induction of cytokines directly by the brain parenchyma, through leakage from the periphery (via endothelial cells, CVOs or physical disruption of the BBB), or as an artifact from elevated plasma cytokine levels.

Basal levels of expression for both TNF and IL-1 protein were detected in all three tested areas of the brain (hippocampus, hypothalamus and cortex). These levels were equivalent to the unstimulated splenic levels of TNF and IL-1. This was somewhat unexpected. Although we anticipated some level of basal splenic cytokine levels in non-pathogen-free rats, we did not expect these same basal levels in the brain which is regarded as an immune-privileged site. Also unexpected was that all three brain areas had similar levels of cytokines. Therefore the hypothalamus which is involved in brain-immune signalling had the same basal levels as the cortex, which is presumably not involved in these pathways. Importantly we found no LPS effect on the levels of brain cytokines in any area 1 hr after 1000μg of i.v. LPS.

In order to block central cytokine production, animals must be cannulated. It is also possible that the regulatory role of central cytokines on peripheral immune function

is an artifact due to the trauma of the cannulation surgery. We know from our own unpublished observations that cytokine mRNA will be expressed at the site of the cannula. This is predicted by other studies which suggest that any trauma or inflammation has the potential to induce cytokine production [906]. Woiciechowsky et al., found that brain injury can cause sympathetic storm which activates inhibitory molecules like IL-10 [926,927]. Therefore it is possible that the central cytokines induced by the surgery are somehow contributing to the regulation of peripheral immune function and are not an inherent part of the central signalling pathway.

In summary our results did not clarify the contentious issue of the role or presence of central cytokines in response to peripheral inflammation. As well, we were not able to determine if high levels of cytokines truly exist in the non-infected brain or are artifacts of our detection method. We did however demonstrate, in contrast to Sacoccio et al. [705], that the dose response and time course between the brain and the spleen are different. Sacoccio et al. observed increased TNF levels in the hypothalamus 30 minutes following 7.5μg of i.v. LPS/rat with the same strain of LPS that we used. We did not corroborate these finding despite giving the rats a dose of LPS that could potentially disrupt the BBB [658,659]. We did not look at later time points (4-24 hrs post-injection) as it is well established that LPS at these intervals will induce central cytokines, and the question of cytokine production in response to central inflammation is not in dispute. Therefore the role of central cytokines as signalling intermediates in the regulatory response to inflammatory stress and other types of stress remains an important and

unresolved issue in this field.

3.8.3.3. Corticosterone

Similar to Gartner et al., [928] our baseline levels of corticosterone in Sprague-Dawley rats were approximately 150 ng/ml. These levels did not increase in response to either 0.01μg or 0.1μg of i.v. LPS. Plasma corticosterone levels peaked in response to 1μg of i.v. LPS (500 ng/ml) and saturated at this level being unchanged in response to 10μg, 100μg and 1000μg of i.v. LPS. These data concur with other reports that described maximal corticosterone responses following low doses of i.v. LPS [929]. Thus in our model, maximal corticosterone levels were achieved at the same dose of LPS that induced maximal splenic cytokine production, a 10 fold lower dose than required to produce detectable increases in plasma E, and a 100 fold lower dose than is required to produce detectable changes in splenic NE. As well, prior studies in our lab found that increases in corticosterone occurred at lower doses of LPS than c-fos expression [32]. Therefore the HPAA appears to be the most sensitive central counterregulatory mechanism in response to peripheral inflammation.

Other studies that investigated corticosterone levels in response to i.v. LPS reported lower baseline levels than we observed in our studies. For example, Feuerstein et al., [429], reported a baseline level of corticosterone at 40 ng/ml ,and peak levels of only 200 ng/ml. Other reports show baselines in the same range as Feuerstein (or as low as 5 ng/ml) but obtained peaks similar to ours [519,866,918]. These lower baselines

potentially allow for more sensitive assays. For example Feuerstein et al. [429] and Ebisui et al. [929], saw a corticosterone response in the range of 0.03μg of i.v. LPS/rat. Feuerstein even reported that corticosterone was increased in response to lower doses of LPS than that required to induce cytokines [429]. Therefore it is important to consider that our higher baseline levels of corticosterone may have precluded detecting increases in corticosterone in response to even lower doses of LPS.

Our higher baseline can be explained either by biological variability [930] or by differences in our injection procedure. In our protocol animals were extensively handled which functioned to habituate them to the mild stressors that they would be exposed to during the course of the experiment [837,838,842,931,932]. However the handling and tail vein injection procedure may cause some corticosterone release despite this habituation [842]. Habituation still may not explain the difference in our baseline as compared to the other groups as the corticosterone response to a mild stress usually returns to baseline by 1 hr, the interval at which we measured corticosterone [15,835,928,933-936] . The likely difference is that the reports which demonstrated low baseline levels of corticosterone administered the LPS and vehicle via a catheter, whereas we injected our animals manually via the tail vein. This is indirectly suggested by Beno and Kimura [866] who saw their corticosterone levels increase from 57 ng/ml to 150 ng/ml after tail vein injection. Yet Gartner et al., [928] had a baseline of 150 ng/ml with no treatments, so biological variability cannot be ruled out.

3.8.3.4. Catecholamines

Catecholamines are important in maintaining homeostasis. It is known that plasma epinephrine originates from the adrenal medulla while the majority of NE (90-95%) originates from nerve terminals (however, an increased percentage of NE may be released from the adrenals in response to the appropriate stimulation [142,143,145]). We found that plasma E was increased in the 10μg-1000μg groups as compared to the 0-1μg groups. Circulating NE, did not differ between any pair of groups, although there was a trend to increase at the 1000μg dose.

Plasma catecholamines are important in many aspects of homeostatic regulation, and can be released in response to many challenges. Therefore the effect of increased levels of plasma E on immune function may be a byproduct of another regulatory process. This is especially plausible for the response to LPS as higher doses, besides being an inflammatory challenge, can also affect blood pressure, body temperature and energy regulation. Lang et al. [426], found that metabolic changes and increases in body temperature were induced in Sprague-Dawley rats in response to LPS doses as low as 0.3 μg (i.v.) per rat, although these changes did not usually occur until 2 hours post-injection. However higher doses of LPS, such as 30μg/rat, could induce metabolic changes at 1 hr post-injection. Changes in cardiac output and blood pressure were also apparent at doses of 30μg/rat (and above), but not at doses of 3μg/rat. In support of this, S.B. Jones and colleagues also found that in Holtzman rats, doses of 30μg/rat and above, but not 3μg/rat, induced changes in blood pressure at 1 hr post-injection. They also found that both NE

and E were increased in the plasma at 1 hr post-injection in response to the doses that affected blood pressure [77,146,148]. Therefore it is unclear if plasma catecholamines can increase in response to doses of LPS that do not alter the host's blood pressure. The patterns of body temperature change are also subject to the dose of LPS [425,595] as lower doses may increase body temperature but doses that induce some measure of lethality may also decrease body temperature [425].

It is unknown if the 10μg dose of LPS used in our experiment altered blood pressure, body temperature or metabolism at 1 hr. However, it is possible that catecholamines are released in response to LPS in an immune-specific manner (independent of the above mentioned factors) as MacNeil et al., demonstrated that increases in splenic nerve activity occurred prior to increases in renal nerve activity in response to LPS [76]. Besides 10μg being the lowest dose at which we observed an increase in plasma E, it is the lowest dose at which we have obtained consistent c-fos protein induction in the PVN (unpublished observations). Interestingly, although maximal cytokine expression was observed in the spleen at doses well below 10μg, plasma TNF peaked at 10μg/rat. Therefore it is plausible that levels of E may be increased to keep a ceiling on circulating cytokine production. In summary, in response to lower doses of LPS, corticosterone and basal E and NE would appear sufficient to maintain homeostasis, however at higher doses, additional systems and regulatory processes are likely activated.

The results also showed that the 100μg and 1000μg doses of i.v. LPS caused NE levels in the spleen to be depleted as compared to all the other doses. It is known that

splenic NE originates from splenic nerves and thus NE levels are a potential marker for SNS activity in the spleen [144,937]. The difficulty with this measure of activity (as determined by Vriend et al. [219]) is that NE turnover in the spleen in response to an inflammatory stimulus can increase despite there being no change in splenic NE levels (E is not detectable in the spleen). Another way to measure SNS activity in the spleen is through nerve recordings [76]. The limitation of this technique is that the animals are anesthetized, this can potentially complicate interpretation due to decreased nerve activity [76]. MacNeil et al., [76] demonstrated that splenic nerve activity increases in response to LPS and that this increase precedes increases in renal nerve activity [76]. Therefore early activation of the splenic nerve in response to LPS may represent an immune-specific signal. In support of Vriend's observation, MacNeil [76] found that splenic nerve activity increases in 66% of the rats in response to LPS 0.4μg of LPS per rat, but doses of 10μg or higher were required for a response in 100% of the animals. Although anesthetized animals were used, MacNeil found that the dose of LPS required to increase splenic nerve activity in 66% of the animals was many fold less that the dose of LPS we required to deplete splenic NE levels. Therefore it is possible that in conscious animals this low dose of LPS, or even lower doses such as 0.1μg, could increase splenic nerve activity in 100% of the animals tested. So although our results can't definitively determine if the splenic nerve is activated in response to doses of LPS below 100μg, it is likely that the role of the nerve in response to LPS is most prominent in response to this dose as indicated by the depleted NE levels.

3.8.3.5. Perspectives

The purpose of these studies was to determine the time course and dose response of splenic mRNA and protein in response to LPS. The main findings are that splenic cytokine mRNA expression corresponded to cytokine protein levels. Also, peak corticosterone levels corresponded with peak levels of splenic cytokines. Increases in plasma E occurred at a 10 fold higher dose of LPS than was required to induce maximal splenic cytokines levels whereas detectable changes in splenic NE levels occurred at a 100 fold dose higher than required to produce maximal splenic cytokine levels. Therefore we have characterized doses of LPS that can potentially initiate the different components of the host's response to LPS, including doses capable of activating splenic cytokine production without activating the brain (the 0.1μg dose). It is not known from these results if the splenic cytokine levels peak from 1μg-1000μg because they have reached a biological ceiling or if the SNS and HPAA are functioning at these higher doses of LPS to restrain further production.

4. Experiment 2

Effect of the SNS and HPAA on splenic cytokines in response to LPS.

4. Experiment 2: Effect of the SNS and HPAA on splenic cytokines in response to LPS.

4.1. Overview

The main objective of this experiment was to test Besedovsky's model of neural-immune feedback. Besedovsky demonstrated that both the adrenal glands and the sympathetic nervous system can independently inhibit immune function, and the combination of these two systems had an additive influence on immune regulation. Our lab previously supported this model using an in vitro system of LPS induced cytokine production and predicted similar findings with respect to the in vivo model tested in this thesis. Experiments were designed to test the individual and combined effects of surgically denervating the splenic nerve and removing the adrenal glands on in vivo splenic cytokine production in response to i.v. LPS. It was found that neither manipulation, individually or combined, significantly alter either splenic TNF, IL-1 or IL-6, mRNA or protein, in response to the many dose/time points tested. In summary these experiments suggest that Besedovsky's model is not applicable to LPS induced splenic cytokine production in vivo in the rat in response to i.v. LPS.

4.2. Objectives

1. To determine if the splenic nerve regulates in vivo splenic cytokine production in response to i.v. LPS.

2. To assess the effects of ADX on in vivo splenic cytokine production in response to i.v. LPS.

3. To examine the interaction between the adrenal glands and the splenic nerve on in vivo splenic cytokine production.

4.3. Hypotheses

That cutting the splenic nerve will increase splenic cytokine production in response to i.v. LPS and this effect will be potentiated in ADX rats.

4.4. Rationale

Besedovsky found that both the SNS and HPAA are activated in response to immune stimuli and both can suppress immune function [48]. Therefore the overall objective of this experiment is to determine if Besedovsky's feedback model is relevant

with respect to LPS-induced cytokine production. The spleen is a model organ for testing the effects of the SNS on immunity as the innervation to the spleen is entirely sympathetic [65,938] and nerve fibers are in close proximity to immune cells [64,151,939-943] . The first evidence suggesting the involvement of the splenic nerve in splenic immune function was by Besedovsky et al., who demonstrated that cutting the splenic nerve increased the plaque forming cell (PFC) response to sheep red blood cells [48]. Our lab extended these findings in a report by Brown et al., who demonstrated that splenic macrophage from animals treated with i.c.v. IL-1 produced less IL-1 in vitro in response to LPS than macrophages from vehicle-injected controls; significantly, this effect was abrogated by cutting the splenic nerve [89]. Our lab also demonstrated that macrophages from untreated splenic nerve cut animals produced more cytokines than cells from sham animals when stimulated in vitro with LPS [890]. Consistent with this, we also showed that the splenic nerve can be selectively activated in response to LPS and that central PGE2 is an important mediator of this activation [76,105,645] . It is also known that i.c.v. PGE2 downregulates cytokine production in response to a low dose of peripheral LPS and that this effect can be partially abrogated by cutting the splenic nerve [717]. In vitro studies by other labs strongly support an inhibitory role for catecholamines with respect to LPS-induced TNF production [173-193] , a moderate role with respect to IL-1 production [179,185,199] and a moderate role with respect to IL-6 production [173,174,176,178,196,200-203] . Therefore the majority of the literature supports our hypothesis that cutting the splenic nerve will disinhibit in vivo cytokine

production in response to i.v. LPS.

Additional rationale for objective 1: The dose response and time course studies described in the last chapter were used to determine the optimal conditions in which to test the effects of the SNS on LPS-induced cytokine production. Examining every time/dose combination in this paradigm is logistically impossible, therefore we focused on the optimal time point for TNF expression as it is the first cytokine in the cascade of events that mediate the host response to LPS, and the literature suggests that TNF can be regulated by the SNS. We tested whether cutting the splenic nerve altered the baseline expression of splenic cytokines or increased the ceiling levels of TNF production. For this study we chose the 10μg dose of LPS as it induced maximal cytokine expression, was the lowest dose tested which induced 100% of the animals to show increased splenic nerve activity in response to LPS [76] and consistently induced central c-fos expression in the PVN. These factors suggested that 10μg of LPS was able to consistently activate the central systems which control the splenic nerve. In order to determine if cutting the splenic nerve prolonged the cytokine response to LPS, we examined cytokine production at 2 hr-post-injection with 10μg of i.v. LPS. In order to determine if cutting the splenic nerve would potentiate a submaximal immune response to LPS, we examined cytokine production at 1 hr post-injection in response to the 0.1μg dose of i.v. LPS. The cytokine response to this dose of LPS was consistently between baseline and maximal responses in previous studies and thus gave us the potential to detect both inhibitory and facilitory

responses. Although our parametric results indicated a close correspondence between cytokine mRNA and protein expression in response to LPS, it is possible that the SNS could affect cytokine production at the level of protein expression. Thus we assessed whether cutting the splenic nerve modified both cytokine mRNA and protein.

Additional rationale for objectives 2-3: The effects of the splenic nerve on splenic immune function as described by both Brown et al. [89], and Besedovsky et al. [48], were accentuated in ADX animals. Therefore it is predicted that in response to i.v. LPS the combination of cutting the splenic nerve and ADX will show a greater disinhibition of cytokine production than nerve cut or ADX alone. For this set of experiments, the optimal dose of LPS was reassessed as it has been shown that ADX animals are 100-1000X more sensitive to the effects of LPS than adrenal-intact animals [339,342-344,944,945] . Besides the effects of ADX on indices of cardiovascular function and mortality, Ramachandra et al. [339], demonstrated increased cytokine production (40-60X) in ADX mice in response to LPS. Therefore another dose response study was conducted in ADX rats in order to establish an optimal dose of LPS with which to determine the effects of nerve cuts. This was done on the premise that the response curve may be shifted 1-3 doses to the left in ADX rats as compared to the response in intact animals. Similar to objective 1, the doses of LPS we selected in the nerve cut/ADX studies represented a dose at which an increase or decrease in the response could be observed as well as a higher dose that is known to drive sympathetic activation. These experiments

were also designed to determine if ADX alone increased splenic cytokine production in response to i.v. LPS in our model.

4.5. Experimental Design

All studies utilized adult male Sprague-Dawley rats (225-250 g) that were obtained from Charles River, (Dorval Quebec). The rats were kept on a 12-12 light/dark cycle and given food and water *ad libitum* (water for ADX rats contained 0.9% saline). Animals underwent combinations of sham surgeries, ADX and splenic nerve cuts and were allowed to recover for 7-10 days. All animals were singly housed, handled for 2 days then taken through the experimental procedure (weighing, mock injection etc.) for 3 days before the actual experiment. Prior to injection, animals were weighed and then put under a heat lamp for 3 minutes to dilate the tail veins. They were then placed in a plastic restrainer and injected i.v. via the tail vein with saline or various doses of lipopolysaccharide (LPS; E.Coli serotype 055:B5 lot L-2637) and were killed at 1 or 2 hr following LPS injection. The doses of LPS ranged from 0.0001μg per rat to 1mg per rat depending on the study. The animals were killed by decapitation and blood and spleens were collected for various assays. Northern blots, ELISAs, immunhistochemistry, RIA for corticosterone and catecholamine determinations were performed as described in the materials and methods section (section 2). Plasma E, corticosterone and splenic NE were measured to verify the surgeries. Experiments were analyzed by ANOVA, one and two

tailed T-tests as described in section 2. The nerve cut/ADX studies were analyzed by ANOVA in a 2X2 design looking at the effects of ADX, splenic nerve cut and the combination of the two procedures on splenic cytokine production (the groups tested were sham/sham, sham/nerve cut, ADX/sham and ADX/nerve cut). All procedures were approved by the animal ethics committee at the University of Manitoba and the CCAC. All ADX surgeries were verified by measuring plasma corticosterone and epinephrine levels while splenic nerve cut surgeries were verified by measuring splenic NE.

4.6. Results

4.6.1. Objective 1: The effect of the splenic nerve cut on splenic cytokine production

For this series of experiments two-tailed T-tests were used because in the literature catecholamines have been shown to both potentiate and inhibit LPS-induced cytokine production [178,192,946]. The first experiment tested if there was a difference in cytokine production between intact and splenic nerve cut animals in response to saline injection. It was found that without LPS, cytokine levels were not detectable in either group (data not shown). The next experiment determined if peak cytokine levels could be increased in splenic nerve cut animals 1 hr after 10μg of i.v. LPS (figure 4-1). It was found that cutting the splenic nerve had no significant effect on TNF [T(8)=0.524, p=0.61 ns], IL-1 [T(10)=2.01, p=0.07] or IL-6 [T(14)=0.122, p=0.905 ns] mRNA levels in response to this challenge, however there was a trend for decreased IL-1 mRNA levels in splenic

nerve cut animals (p=0.07). The results for the splenic TNF and IL-6 protein levels were similar to the mRNA levels and the trend observed for decreased IL-1 mRNA production in the splenic nerve cut groups was not observed for IL-1 protein (data not shown). The same paradigm was repeated 2 hr after 10μg of i.v LPS in order to determine if the time course for splenic cytokines would be prolonged in splenic nerve cut animals. There was no difference in the splenic TNF [T(7)=0.95, p=0.375 ns], IL-1 [T(7)=1.09, p=0.31 ns] and IL-6 [T(8)=0.52, p=0.621 ns] mRNA levels between nerve cut and sham operated animals (figure 4-2). Not shown is that the results for the protein are similar to the mRNA. The fourth experiment investigated if the submaximal response to LPS-induced with 0.1μg dose could be increased in splenic nerve cut animals. Like the other two experiments, there was no effect on TNF [T(8)=0.808, p=0.44 ns] or IL-1 [T(8)=0.402, p=0.7 ns] mRNA (figure 4-3) or protein levels (data not shown). IL-6 was not detectable in response to 0.1μg of LPS at 1 hr post-injection in this experiment. Like Vriend et al., we found that cutting the splenic nerve eliminated >90% (usually 100%) of splenic NE [937] (not shown). Also, we did not observe any differences in plasma corticosterone or E levels in nerve cut rats as compared to the sham operated controls (data not shown).

4.6.2. Objective 2: The effect of the ADX and splenic nerve cut on splenic cytokine production

4.6.2.1. ADX dose response to i.v. LPS

A significant overall effect of the dose of LPS was observed with respect to TNF mRNA production in ADX rats [$F(6,21)=21.62$, $p<0.0001$] (figure 4-4). It was found that submaximal responses were elicited by 0.01μg and 0.1μg of LPS whereas maximal responses were induced by 1μg of LPS. The 0.01μg dose was significantly different from the 0.1μg dose ($p=0.0029$) which was different from the 1μg dose ($p<0.0001$). In a separate experiment, TNF mRNA levels in response to the 10μg dose of LPS were not significantly different from the response to the 100μg and 1000μg doses in ADX rats [$F(2,11)=1.03$, $p=0.4$ ns] (data not shown). Overall, the dose response for splenic TNF protein [$F(6,20)=127.3$, $P<0.0001$] (figure 4-5) was similar to the results of the mRNA dose response. Splenic TNF protein levels in response to the 0.1μg dose of LPS in ADX rats were higher than the levels induced in response to the 0.01μg dose of LPS ($p=0.0002$), yet below the levels induced by the 1μg dose ($p<0.0001$). One difference however was the fact that the 10μg dose induced more TNF protein than the 1μg dose ($p=0.0025$). This pattern was not observed in reference to splenic TNF mRNA. Splenic TNF protein production in response to the 10μg dose of LPS was not significantly different from the response to the 100μg and 1000μg doses in ADX rats [$F(2,11)=0.267$, $p=0.77$ ns] (data not shown).

There was also a significant overall effect of the dose of LPS on IL-1 mRNA production in ADX rats [$F(6,21)=28.22$, $p<0.0001$]. Interestingly, this dose response was shifted one dose to the left as compared to the response of TNF mRNA and protein (figure 4-6). The 0.01μg dose induced significantly more IL-1 mRNA than the 0.001μg

dose (p=0.0028), and significantly less that the 0.1μg dose (p=0.0006), which was the peak. No differences were found between the responses to 10μg, 100μg and 1000μg of i.v. LPS in ADX rats [F(2,11)=2.83, p=0.13 ns] (data not shown). Similar to the mRNA there was an overall effect of the dose of LPS on splenic IL-1 protein [F(6,20)=60.18, p<0.0001] (figure 4-7). Unlike the effect observed with IL-1 mRNA, it was found that the 0.01μg dose of LPS only showed a tendency to increase IL-1 protein above baseline. However consistent with other experiments, the 0.1μg dose induced more IL-1 protein than the 0.01μg dose (p=0.0077) and less than the 1μg dose (p<0.0001). Similar to the TNF protein, the 10μg dose of LPS-induced more splenic IL-1 protein than the 1μg dose in ADX rats (p=0.0062). However the response to the 10μg dose of LPS was not significantly different from the response to the 100μg and 1000μg doses in ADX rats [F(2,11)=0.62, p=0.94 ns] (data not shown).

Similar to what was observed in intact animals, there was an overall effect of the dose of LPS on splenic IL-6 mRNA [F(6,21)=5.77, p=0.001] and IL-6 was not induced by doses lower than 1μg of i.v. LPS (figure 4-8). However, in this experiment there was a significant difference in IL-6 mRNA levels between the 1μg dose and the 10μg dose in ADX rats (p=0.018). Similar to the other cytokines, no differences were found between the response to 10μg, 100μg and 1000μg of i.v. LPS in ADX rats [F(2,11)= 0.41, p= 0.67 ns] (data not shown). Determination of splenic IL-6 levels was not done for the lower doses, however it was found that IL-6 levels in response to the 10μg dose of LPS were not significantly different from the response to the 100μg and 1000μg doses in ADX rats

[F(2,11)=1.4, p=0.29 ns] (data not shown).

Also not shown was that ADX eliminated plasma E by >95% and reduced the detectable plasma corticosterone levels to <20 ng/ml. ADX did not affect splenic or plasma NE levels (data not shown).

4.6.2.2. Direct comparison of cytokine levels in sham and ADX rats following i.v. LPS

We next determined if ADX rats produced more cytokines in response to i.v. LPS than sham operated rats. In response to 0.01μg of i.v. LPS, ADX rats produced more splenic TNF mRNA than sham operated rats, however this effect was not statistically significant [T(6)=1.2, p=0.14, one-tailed, ns] (figure 4-9). This pattern was not present for splenic IL-1 mRNA levels [T(6)=0.5, p=0.52, one-tailed, ns] (figure 4-9). The same pattern was also observed for splenic TNF protein levels [T(6)=1.2, p=0.14, one-tailed, ns] (figure 4-10), however the differences were also not significant. Like the mRNA, no differences in splenic IL-1 protein levels were observed between ADX and control rats (figure 4-10) [T(6)=-0.21, p=0.57, one-tailed, ns]. IL-6 mRNA and protein were not detected in response to this dose of LPS. Similar to the 0.01μg dose, ADX rats produced more TNF mRNA in response 10μg of i.v. LPS than sham operated animals, but this difference was also not significant [T(9)=1.08, p=0.15, one-tailed, ns] (figure 4-11). A similar trend was observed for IL-1 mRNA [T(9)=1.46, p=0.089, one-tailed, ns] but not for IL-6 mRNA [T(9)=0.37, p=0.36, one-tailed, ns] (figure 4-11). There were no differences between ADX and control rats in terms of TNF [T(9)=-0.08, p=0.53, one-

tailed, ns], IL-1 [T(9)=-1.027, p=0.83, one-tailed, ns] and IL-6 [T(9)=0.83, p=0.21, one-tailed, ns] protein levels (data not shown). All analyses in this section were done with a one-tailed T-test as it was expected that ADX would increase cytokine production.

4.6.2.3. Effect of splenic nerve cut in ADX rats in response to i.v. LPS

The combination of splenic nerve cut and ADX on the cytokine response to i.v. LPS was analyzed with an ANOVA using a 2X2 design where the groups tested were sham/sham, sham/nerve cut, ADX/sham and ADX/nerve cut. In response to 0.01μg of i.v. LPS (figure 4-12) there were no significant overall differences in TNF mRNA expression [F(3,22)=1.14, p=0.36 ns]. In terms of main effects, there was no nerve cut effect, but there was a trend for an ADX effect [F(1)=2.95, p=0.10]. In terms of differences between individual groups, there was a pattern for LPS to induce higher levels of TNF mRNA in ADX/sham rats as compared to sham/sham rats, however this was not statistically significant (p=0.11). Splenic protein for TNF showed similar results to the mRNA in that there was no overall effect or main effect of splenic nerve cut, however there was a trend for a significant main effect of ADX [F(1)=3.61, p=0.08] (data not shown). The results for IL-1 mRNA and protein in response to 0.01μg of i.v. LPS were even less dramatic than for TNF in that there were no significant overall effects, no significant main effects and no differences between groups (data not shown). IL-6 was not detected in response to this dose of LPS.

This same paradigm was tested in response to 100μg of i.v. LPS. It was found

that the pattern of TNF mRNA expression was similar to the response observed to 0.01μg in that there was no significant overall effect, no main effects of ADX or nerve cut and no differences between groups (figure 4-13). However there was a tendency for ADX to increase TNF mRNA expression as compared to sham animals, but this was not statistically significant. The results were similar for splenic TNF protein and IL-1 and IL-6 mRNA and protein in response to 100μg of i.v. LPS, except no patterns were evident (data not shown).

4.6.2.4. Other observations

The effects of splenic nerve cut and ADX on splenic cytokine mRNA production was also determined for ICE, TACE, IL-18, MCP-1, MIP-1 beta, IFN gamma, IL-12 p40, and RANTES. For the vast majority of these molecules there were no overall effects, no main effects of either ADX or nerve cut and no differences between groups. The exceptions were that although there was no overall effect of treatment on IFN gamma mRNA [$F(3,32)=1.93$, $p=0.15$ ns], there was a significant main effect of ADX on IFN gamma mRNA levels which were decreased in ADX rats [$F(1)=4.29$, $p=0.047$] (data not shown). Although there was also no overall treatment effect on IL-12 p40 expression [$F(3,32)=1.62$, $p=0.20$ ns], there was a main effect of ADX on IL-12 p40 expression, where IL-12 p40 expression was increased in ADX rats [$F(1)=4.8$, $p=0.0364$]. No nerve cut effect or interactions between ADX and nerve cut were observed for either IFN gamma or IL-12 p40.

In these same experiments splenic weight was indexed as a ratio to body weight and analyzed by 2X2 ANOVA (figure 4-14). An overall treatment effect was observed [$F(3,31)=4.85$, $p=0.007$] as were main effects for both nerve cut [$F(1)=8.3$, $p=0.007$) and for ADX [$F(1)=6.54$, $p=0.016$], but there was no interaction between these effects. In terms of the differences between individual groups, the ADX/nerve cut group had a higher index of spleen weight to body weight than ADX/Sham ($p=0.023$), sham/nerve cut ($p=0.037$) and sham/sham ($p=0.0007$). To further investigate the potential reasons for the increase in splenic weight in response to splenic nerve cut, immunohistochemical analysis of pan B-cell (OX 33 positive), pan T-cell (OX-52 positive) and macrophage (ED-1 positive) cell markers was conducted on 50 μm spleen sections (figure 4-15). It was found that splenic nerve cut did not significantly affect the percent area of staining for either ED-1 [$T(22)=0.487$, $p=0.63$ ns] or OX-33 [$T(22)=1.38$, $p=0.18$ ns] positive cells, but the percent immunostaining for OX-52 [$T(22)=3.71$, $p=0.0012$] was significantly decreased in the spleens from nerve cut animals.

4.7. Figures (* indicates that P≤ 0.05)

Figure 4-1: Effect of splenic nerve cut on splenic cytokine mRNA 1 hr after 10μg of i.v. LPS. Control rats and rats with splenic nerve cuts were injected with 10μg of i.v. LPS and killed at 1 hr post-injection. Splenic TNF [T(8)=0.524, p=0.53ns, n=10], IL-1 [T(10)=0.2.01, p=0.07ns, n=12], and IL-6 [T(14)=0.122, p=0.91ns, n=16] mRNA levels were measured by Northern blotting and are expressed as a ratio relative to the loading control. Groups were compared by a two-tailed T-test and error bars represent +/-1 standard error of the mean.

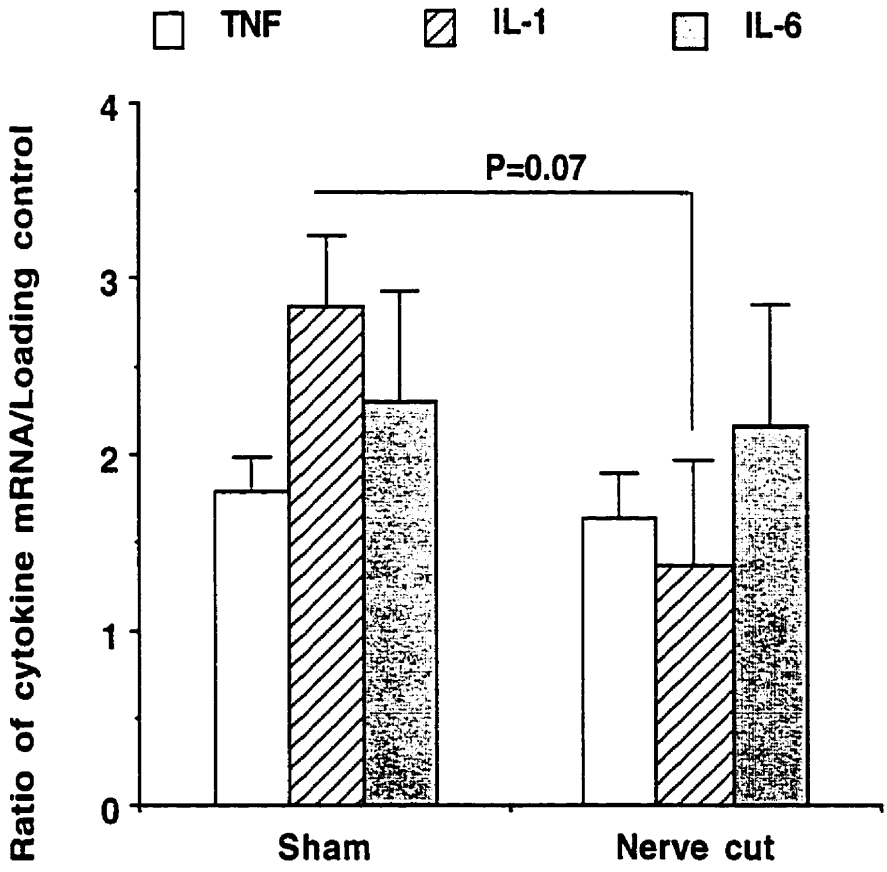


Figure 4-2: Effect of splenic nerve cut on splenic cytokine mRNA 2 hr after 10μg of i.v. LPS. Control rats and rats with splenic nerve cuts were injected with 10μg of i.v. LPS and killed at 2 hr post-injection. Splenic TNF [T(7)=0.95, p=0.38ns, n=9], IL-1 [T(7)=1.09, p=0.31ns, n=9], and IL-6 [T(8)=0.52, p=0.62ns, n=10] mRNA levels were measured by Northern blotting and are expressed as a ratio relative to the loading control. Groups were compared by a two-tailed T-test and error bars represent +/-1 standard error of the mean.

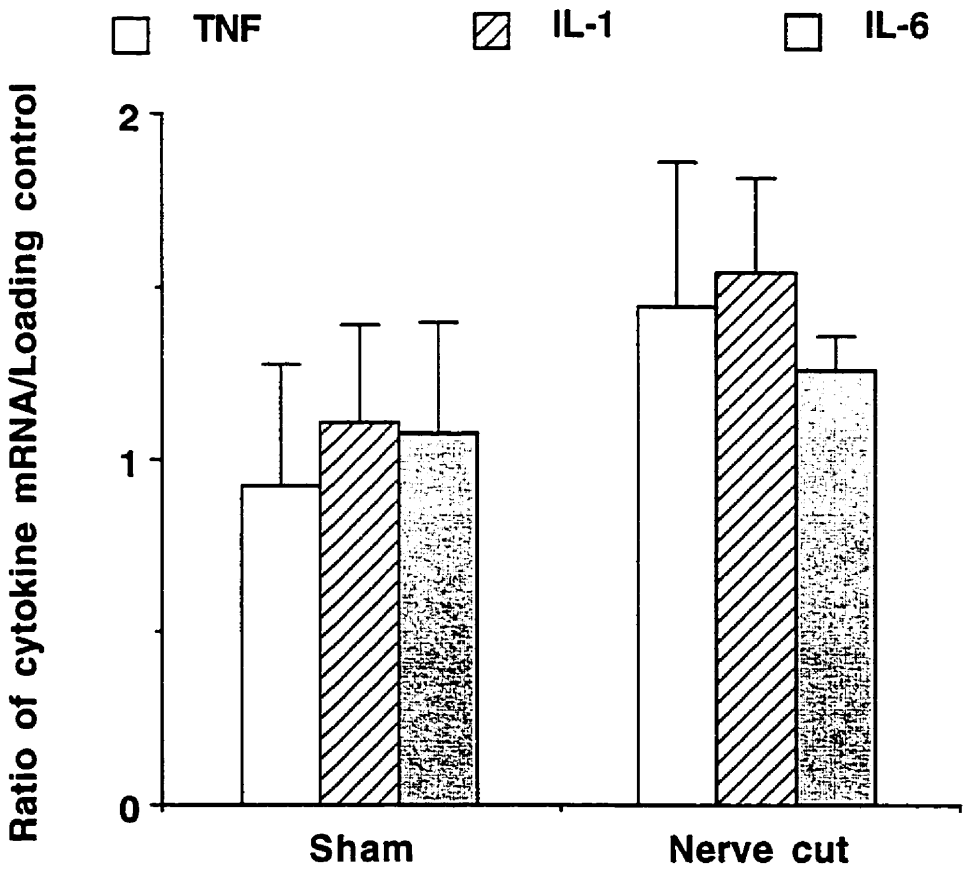


Figure 4-3: Effect of splenic nerve cut on splenic cytokine mRNA 1 hr after 0.1μg of i.v. LPS. Control rats and rats with splenic nerve cuts were injected with 0.1μg of i.v. LPS and killed at 1 hr post-injection. TNF [T(8)=0.81, p=0.44ns, n=10] and IL-1 [T(8)=0.4, p=0.7ns, n=10] mRNA levels were measured by Northern blotting and are expressed as a ratio relative to the loading control. Groups were compared by a two-tailed T-test and error bars represent +/-1 standard error of the mean.

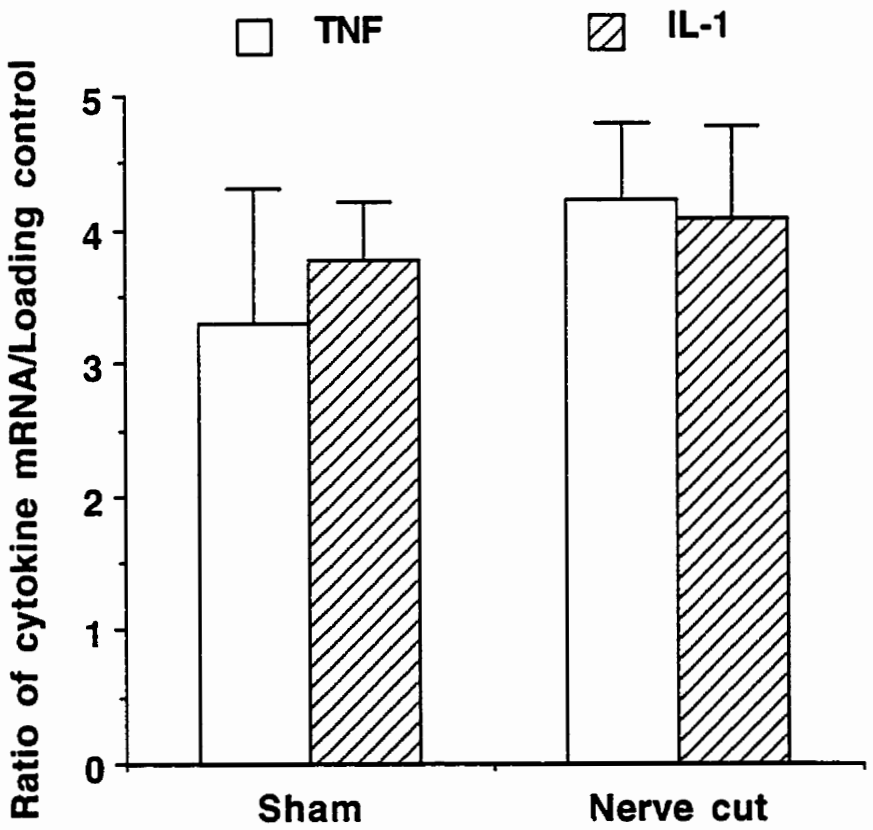


Figure 4-4: Dose response for splenic TNF mRNA one hour following i.v. LPS in ADX rats. ADX rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic TNF mRNA levels [$F(6,21)=21.62$, $p<0.0001$, $n=28$] were analyzed by Northern blotting and expressed as a ratio relative the loading control. Groups were compared by ANOVA and error bars represent +/-1 standard error of the mean.

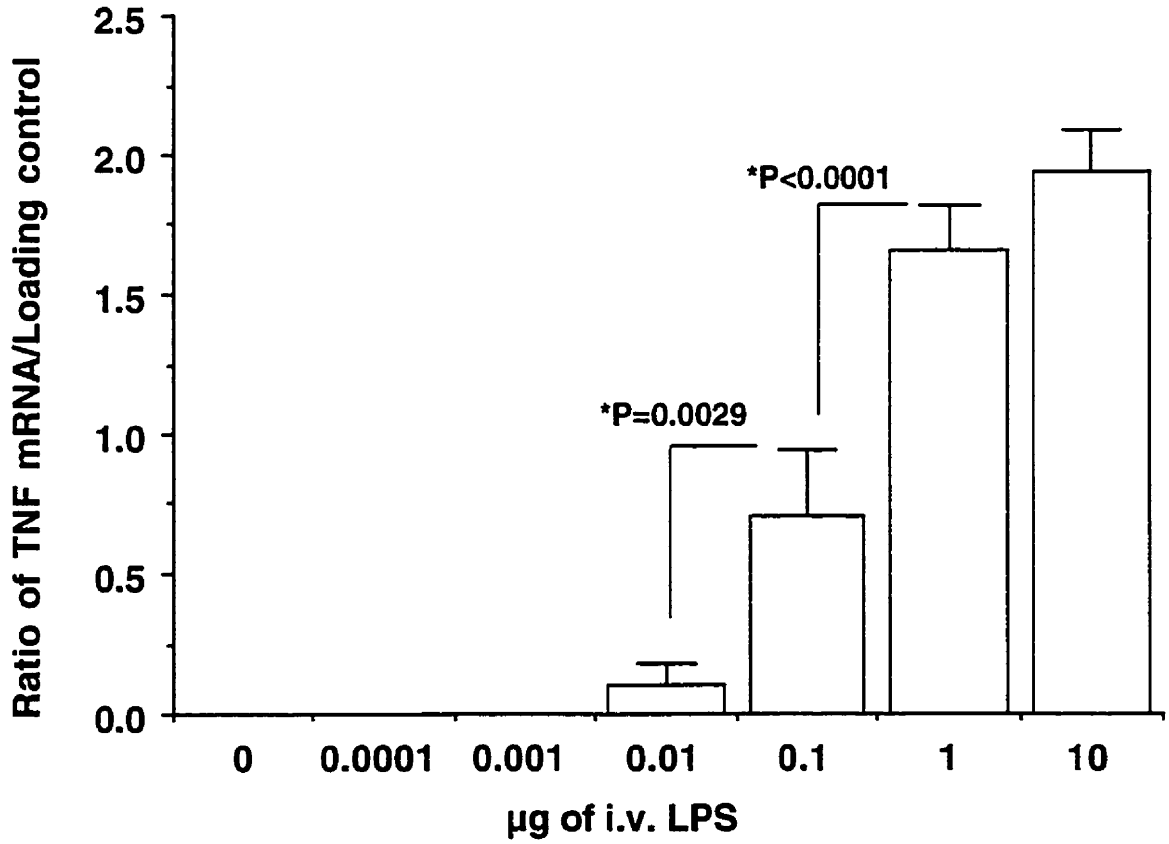


Figure 4-5: Dose response for splenic TNF protein one hour following i.v. LPS in ADX rats. ADX rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic TNF protein levels [$F(6,20)=127.3$, $p<0.0001$, $n=27$] were measured by ELISA and expressed as pg/mg of splenic protein. Groups were compared by ANOVA and error bars represent +/-1 standard error of the mean.

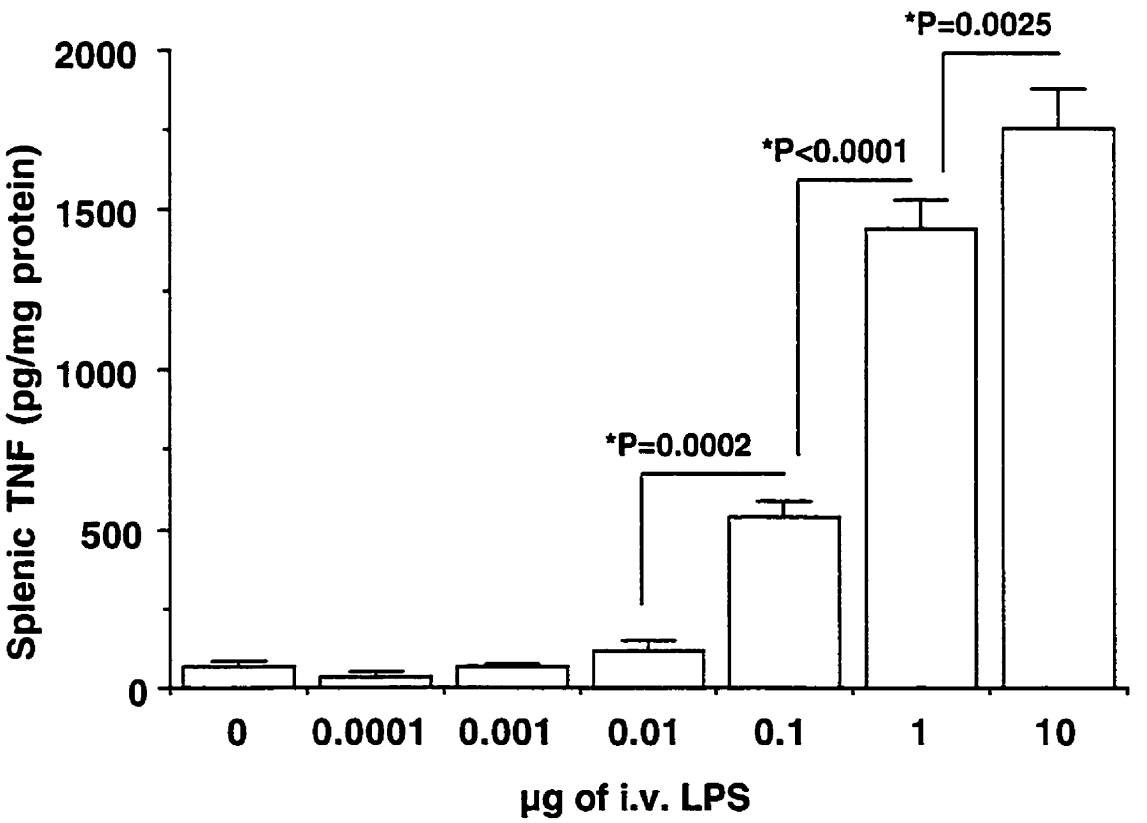


Figure 4-6: Dose response for splenic IL-1 mRNA one hour following i.v. LPS in ADX rats. ADX rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic IL-1 mRNA levels [$F(6,21)=28.22$, $p<0.0001$, $n=28$] were analyzed by Northern blotting and expressed as a ratio relative the loading control. Groups were compared by ANOVA and error bars represent +/-1 standard error of the mean.

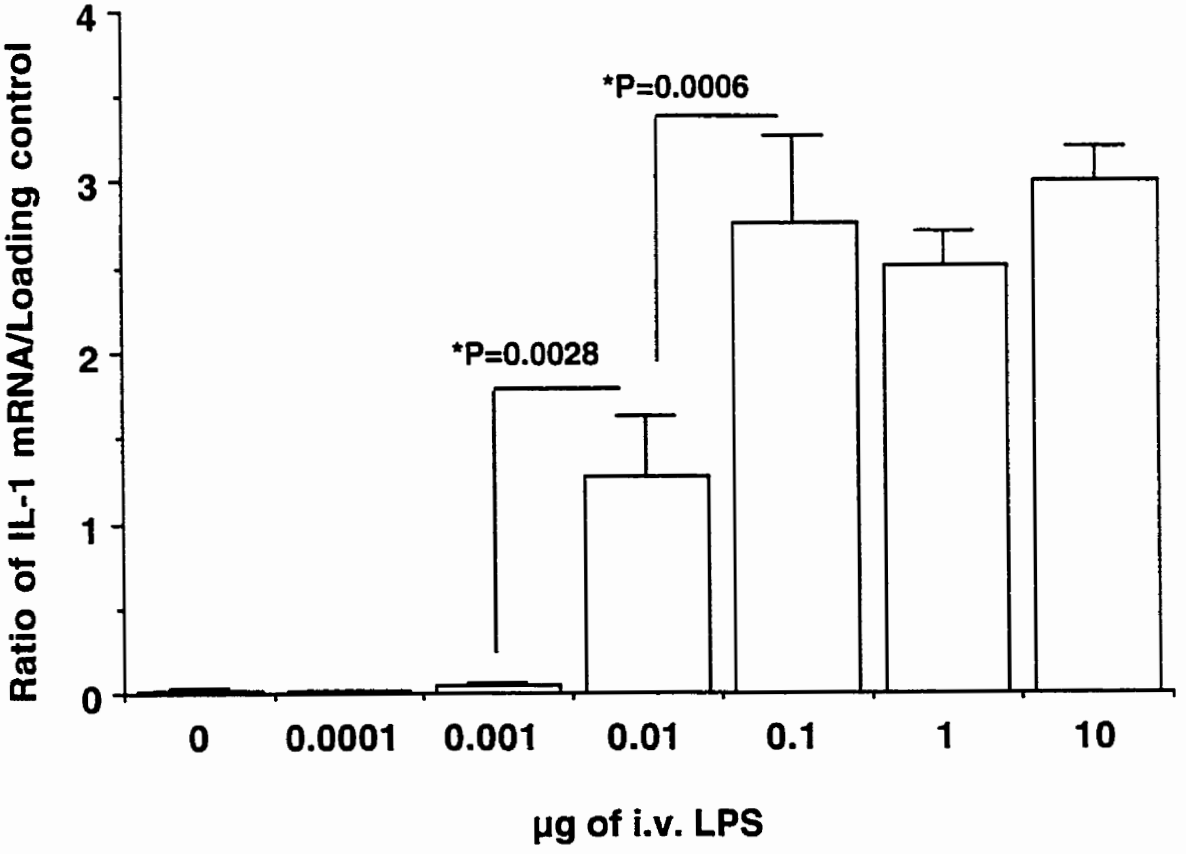


Figure 4-7: Dose response for splenic IL-1 protein one hour following i.v. LPS in ADX rats. ADX rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic IL-1 protein levels [$F(6,20)=60.18$, $p<0.0001$, $n=27$] were measured by ELISA and expressed as pg/mg of splenic protein. Groups were compared by ANOVA and error bars represent +/-1 standard error of the mean.

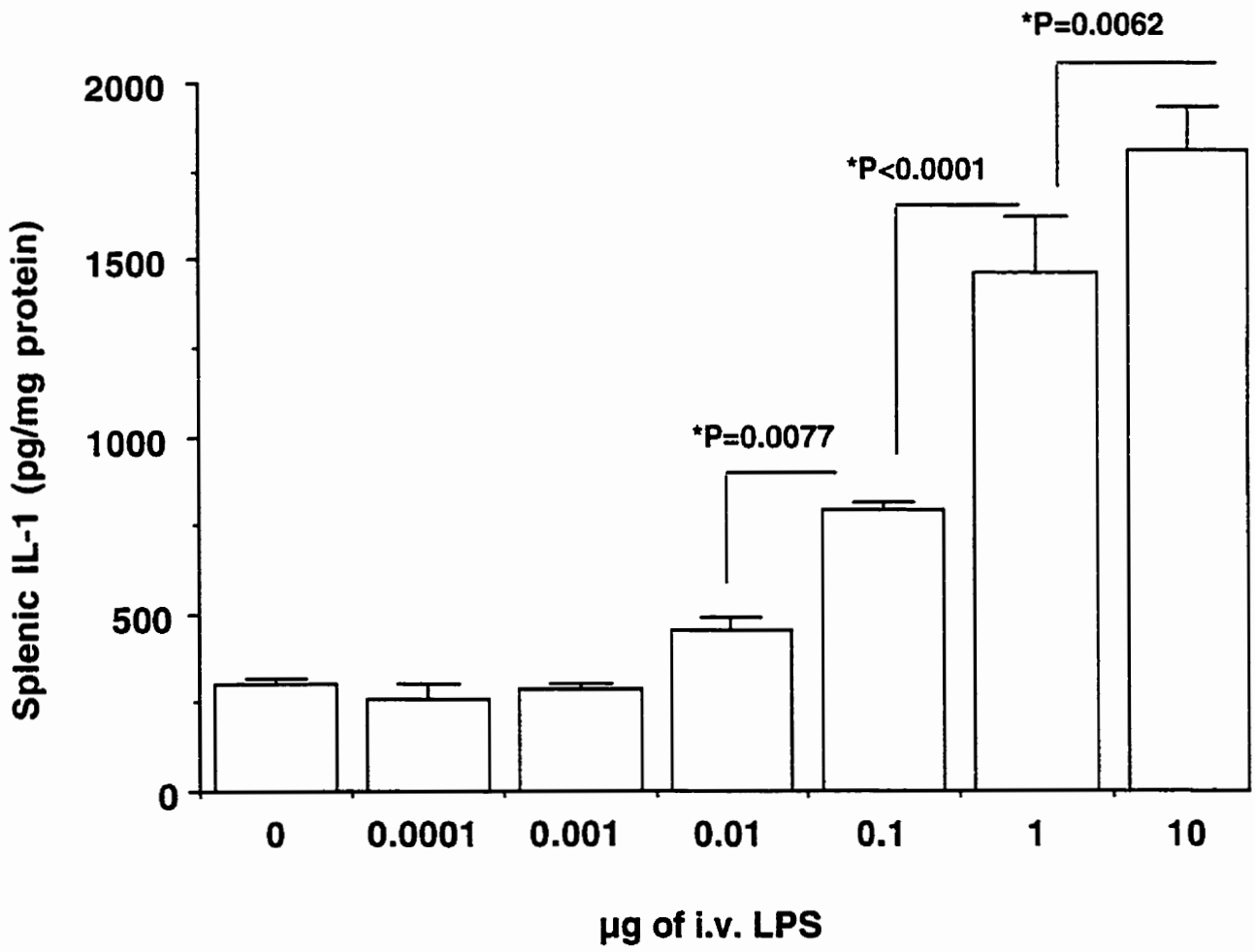


Figure 4-8: Dose response for splenic IL-6 mRNA one hour following i.v. LPS in ADX rats. ADX rats were injected with various doses of i.v. LPS and killed at 1 hour post-injection. Splenic IL-6 mRNA levels [F(6,21)=5.77, p=0.001, n=28] were analyzed by Northern blotting and expressed as a ratio relative the loading control. Groups were compared by ANOVA and error bars represent +/-1 standard error of the mean.

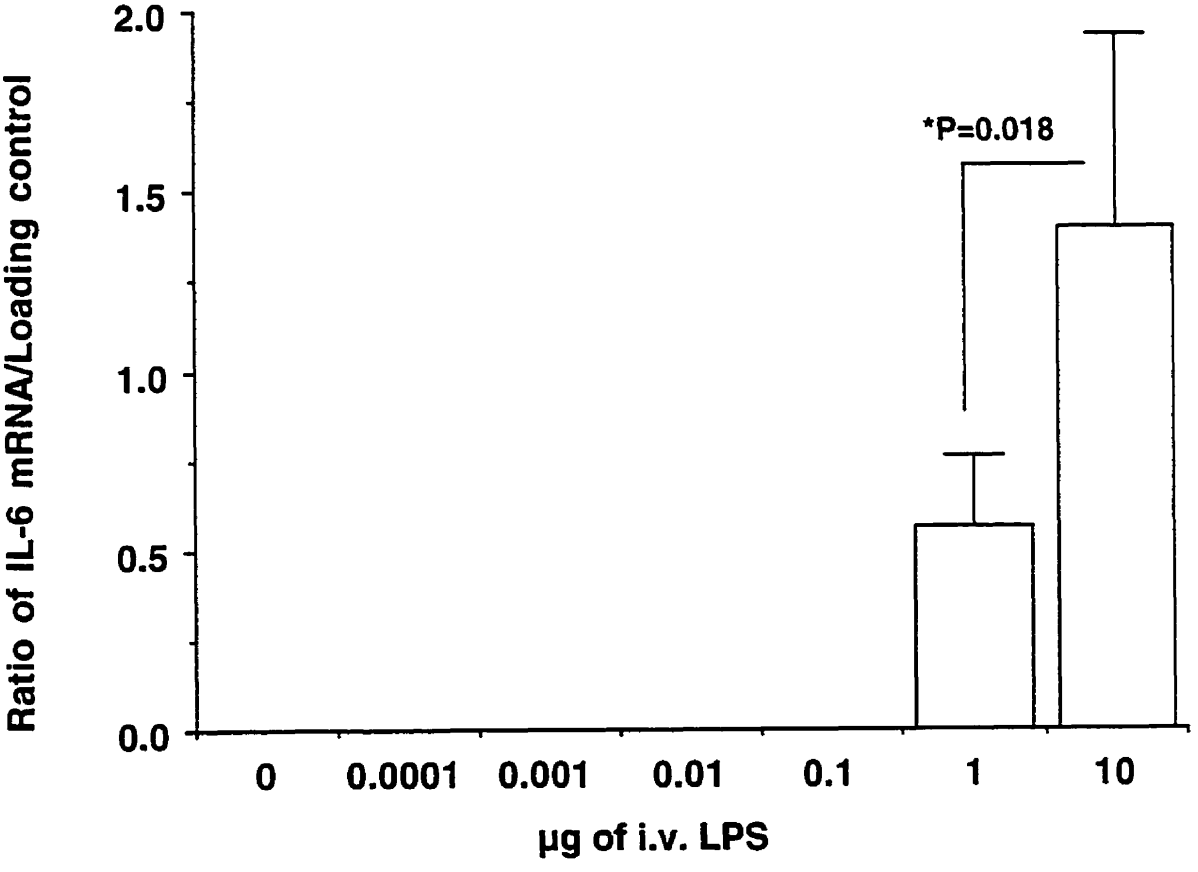


Figure 4-9: Effect of ADX on splenic cytokine mRNA one hour following 0.01µg of i.v. LPS. ADX and sham rats were injected with 0.01µg of i.v. LPS and killed at 1 hour post-injection. Splenic TNF [T(6)=1.2, p=0.14ns, n=8] and IL-1 [T(6)=0.5, p=0.5ns, n=8] mRNA levels were measured by Northern blotting and are expressed as a ratio relative to the loading control. The groups were compared with a one-tailed T-test and error bars represent +/-1 standard error of the mean.

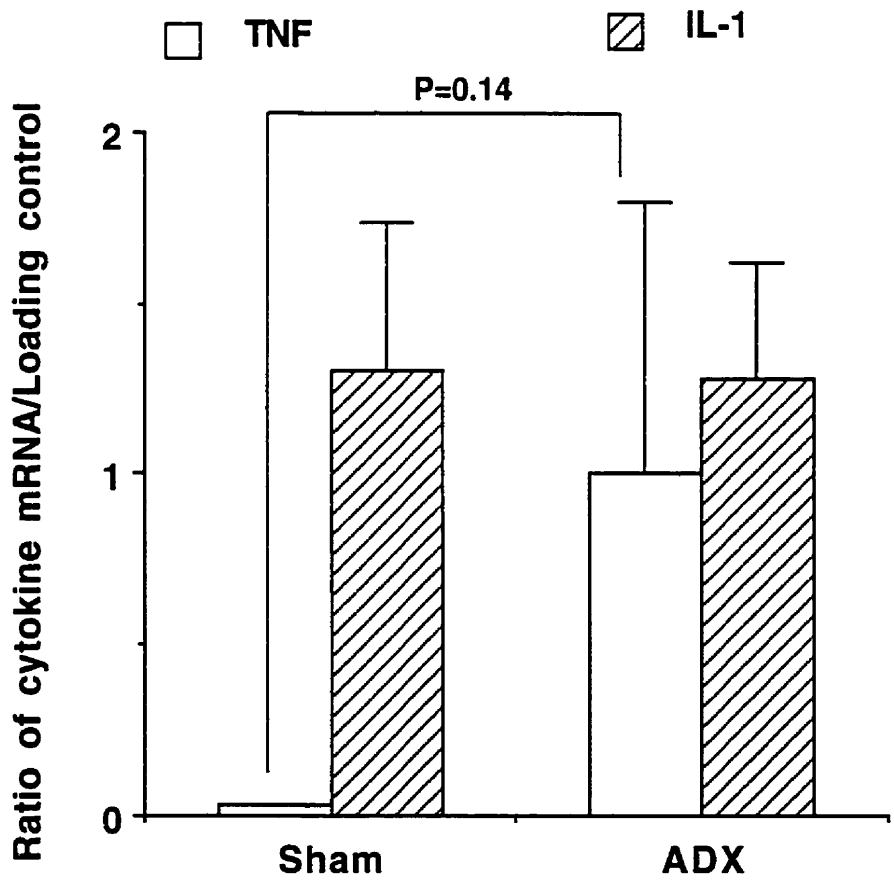


Figure 4-10: Effect of ADX on splenic cytokine protein one hour following 0.01μg of i.v. LPS. ADX and sham rats were injected with 0.01μg of i.v. LPS and killed at 1 hour post-injection. Splenic TNF [T(6)=1.2, p=0.14ns, n=8] and IL-1 [T(6)=-0.21, p=0.57ns, n=8] protein levels were measured by ELISA and expressed as pg/mg of splenic protein. The groups were compared with a one-tailed T-test and error bars represent +/-1 standard error of the mean.

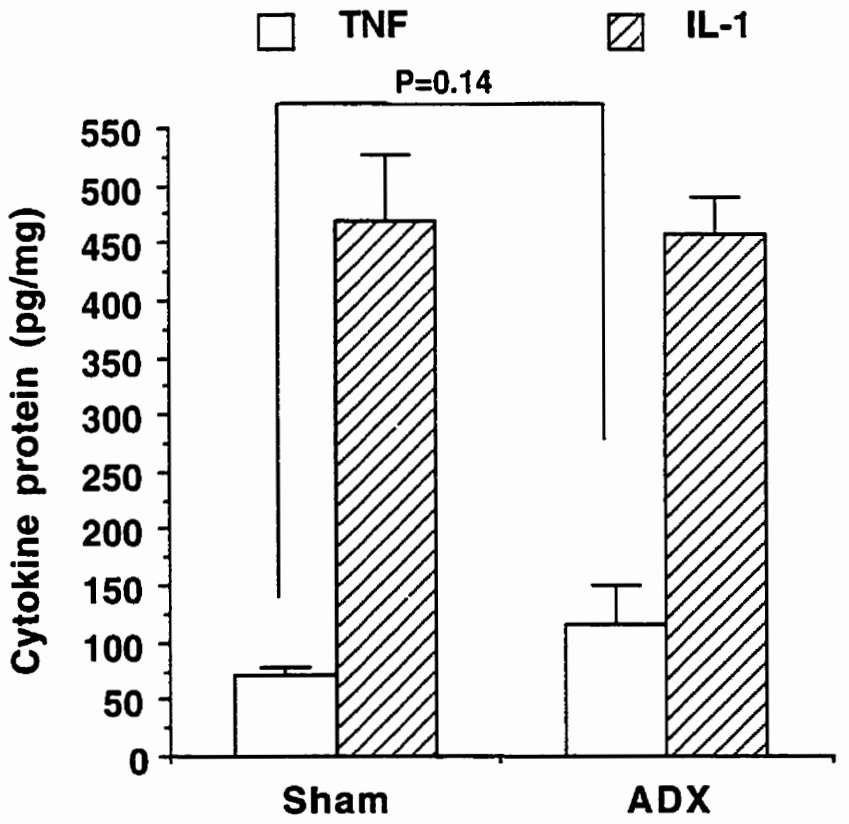


Figure 4-11: Effect of ADX on splenic cytokine mRNA one hour following 10μg of i.v. LPS. ADX and sham rats were injected with 10μg of i.v. LPS and killed at 1 hour post-injection. Splenic TNF [T(9)=1.08, p=0.15ns, n=11], IL-1 [T(9)=1.46, p=0.09ns, n=11] and IL-6 [T(9)=0.37, p=0.36ns, n=11] mRNA levels were measured by Northern blotting and are expressed as a ratio relative to the loading control. The groups were compared with a one-tailed T-test and error bars represent +/-1 standard error of the mean.

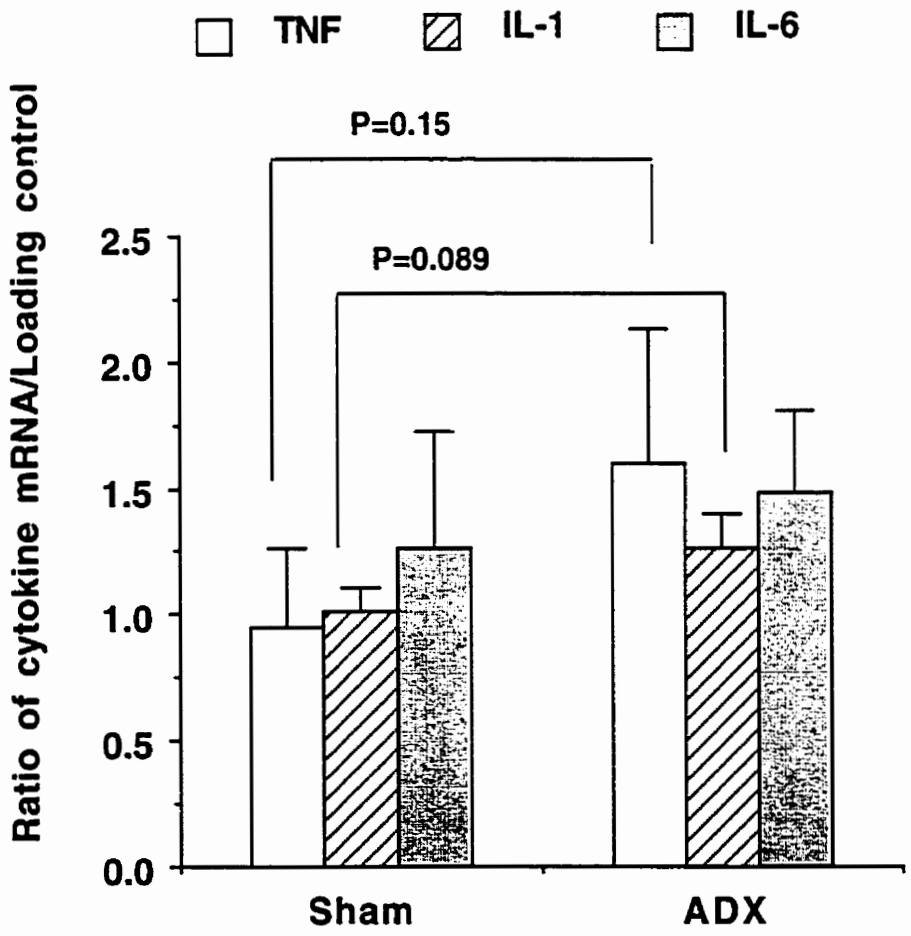


Figure 4-12: Combined effect of splenic nerve cut and adrenalectomy on splenic TNF mRNA one hour following 0.01μg of i.v. LPS. Rats with combinations of sham surgeries, ADX and splenic nerve cuts (NC) were injected with 0.01μg of i.v. LPS and killed at 1 hour post-injection. Splenic TNF mRNA levels [F(3,32)=1.14, p=0.36ns, n=26] were measured by Northern blotting and are expressed as a ratio relative to the loading control. The groups were compared by ANOVA and error bars represent +/-1 standard error of the mean.

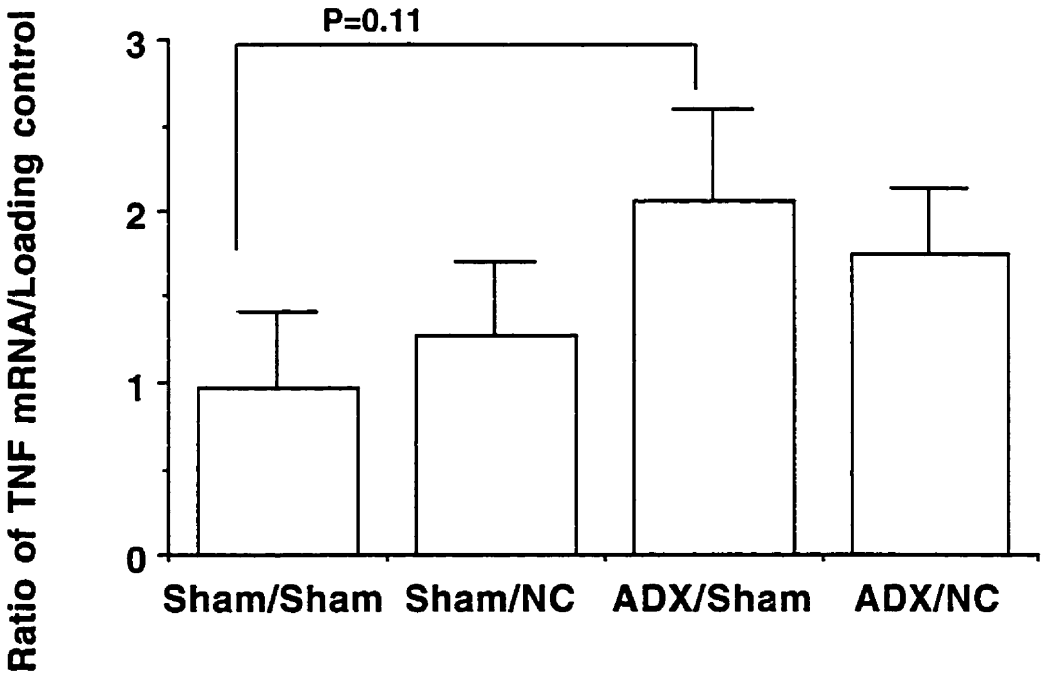


Figure 4-13: Combined effect of splenic nerve cut and adrenalectomy on splenic TNF mRNA one hour following 100μg of i.v. LPS. Rats with combinations of sham surgeries, ADX and splenic nerve cuts (NC) were injected with 100μg of i.v. LPS and killed at 1 hour post-injection. Splenic TNF mRNA levels [F(3,32)=0.947, p=0.43ns, n=36] were measured by Northern blotting and are expressed as a ratio relative to the loading control. The groups were compared by ANOVA and error bars represent +/-1 standard error of the mean.

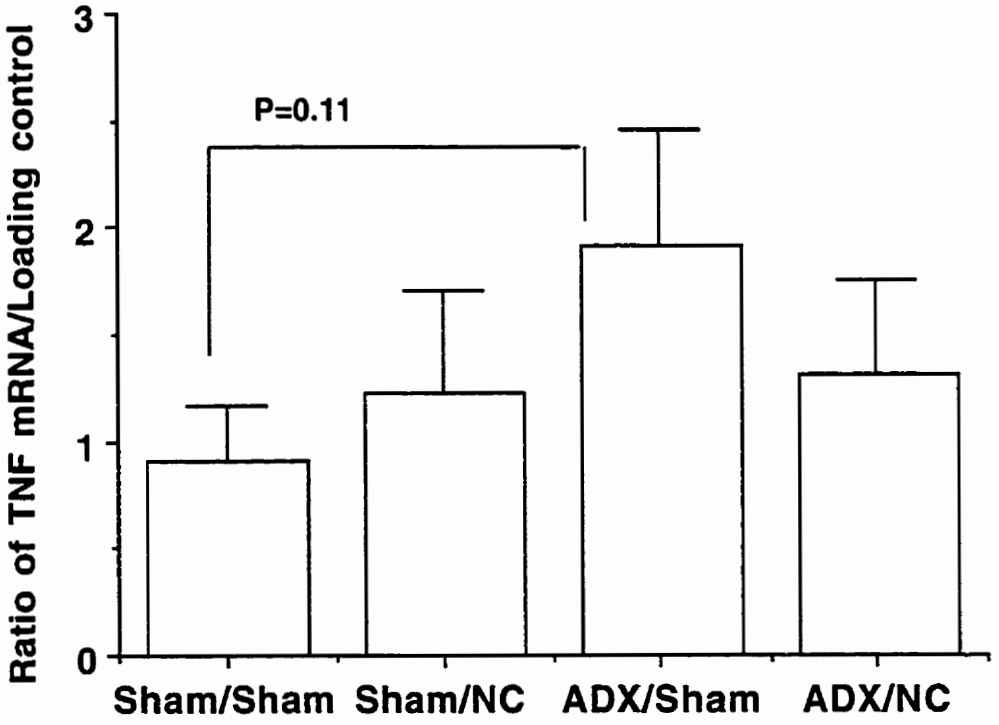


Figure 4-14: Combined effect of splenic nerve cut and adrenalectomy on splenic weight one hour following 100µg of i.v. LPS. Rats with combinations of sham surgeries, ADX and splenic nerve cuts (NC) were injected with 100µg of i.v. LPS and killed at 1 hour post-injection. Splenic weight [F(3,31)=4.85, p=0.007, n=35] was expressed as a ratio to body weight. The groups were compared by ANOVA and error bars represent +/-1 standard error of the mean.

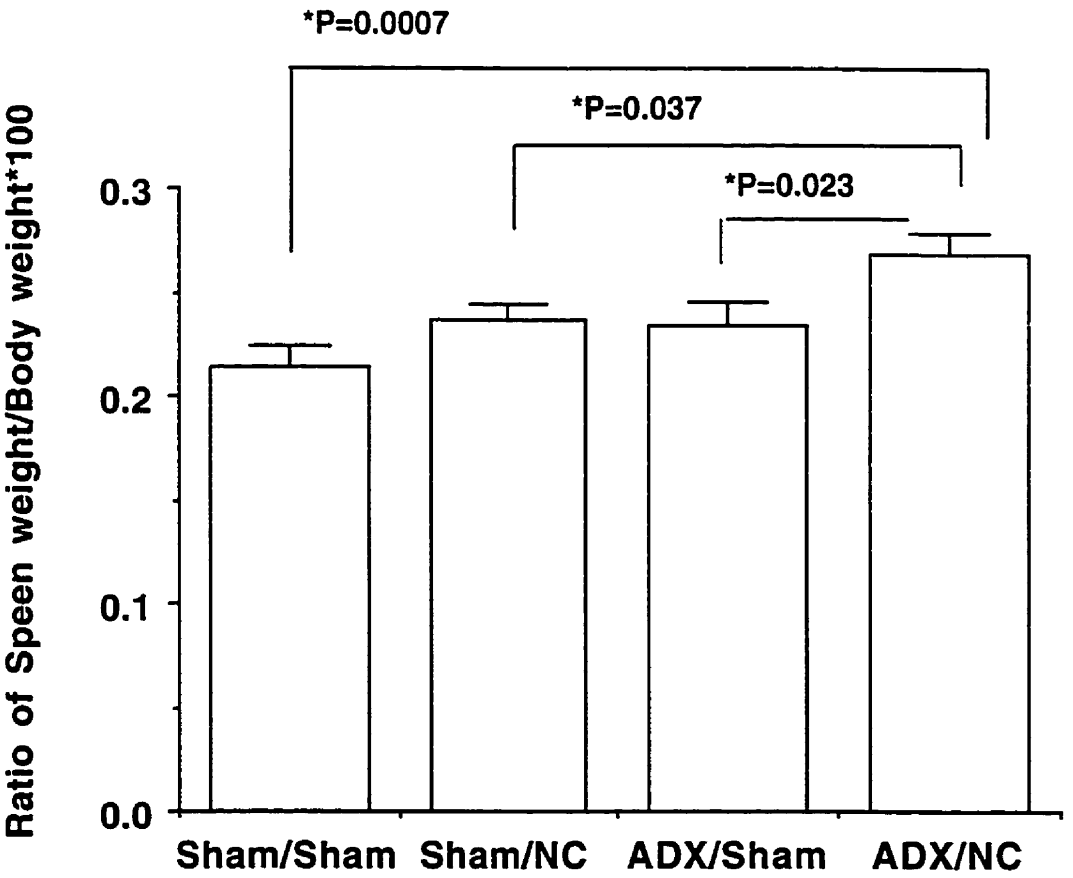
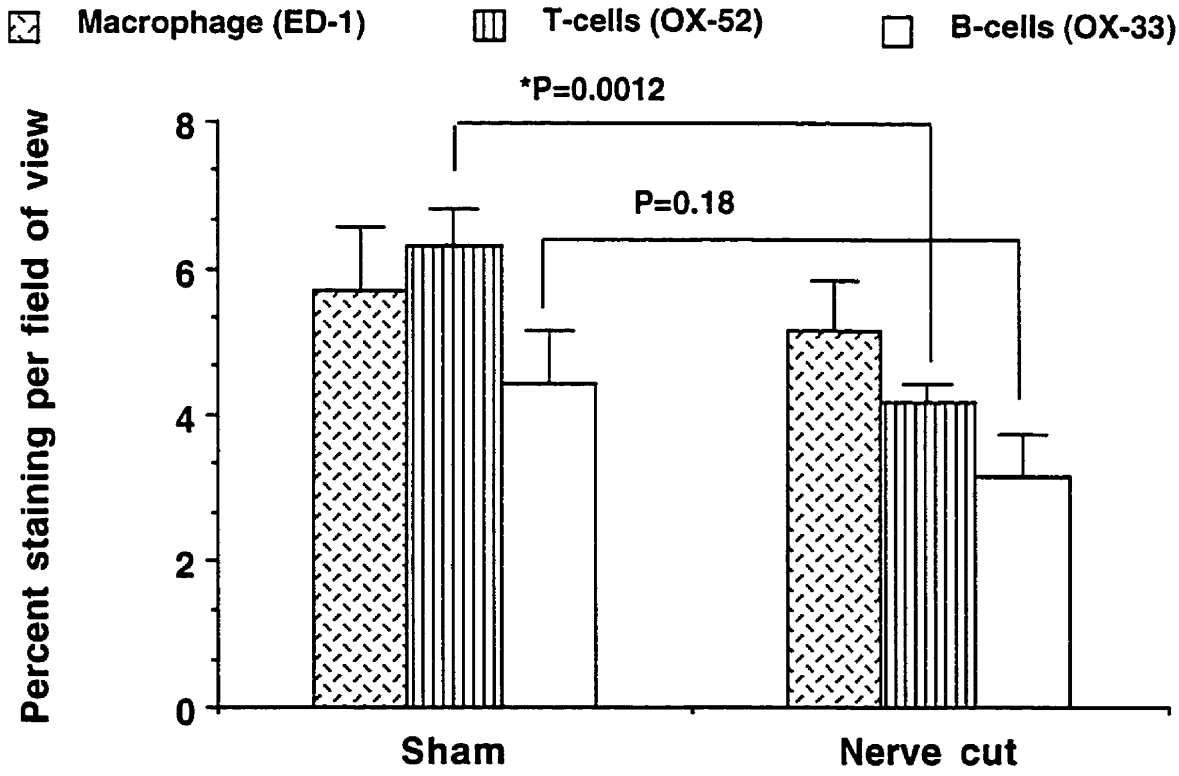


Figure 4-15: Effect of splenic nerve cut on the percentage of macrophage, T-cell and B-cell positive staining in the rat spleen 1 hr after 0.1μg of i.v. LPS. Control rats and rats with splenic nerve cuts were injected with 0.1μg of i.v. LPS and killed at 1 hr post-injection. Portions of the spleens were fixed and processed for immunohistochemistry with the rat macrophage cell marker, ED-1 [T(22)=0.487, p=0.63ns, n=24], the rat T-cell marker, OX-52 [T(22)=3.71, p=0.0012, n=24] and the rat B-cell marker, OX-33 [T(22)=1.38, p=0.18ns, n=24]. The percentage of positive staining per field of view was determined using NIH image in conjunction with a digital video microscope. The groups were compared by T-test and error bars represent +/-1 standard error of the mean.



4.8. Discussion

4.8.1. The effect of splenic nerve cut on splenic cytokine production in response to i.v. LPS.

Similar to in situ hybridization and immunohistochemistry [151,892], cytokine expression was not detectable by Northern blotting prior to LPS injection [891,892]. In contrast, other labs have shown with more sensitive techniques like RT-PCR that mRNA levels are detectable in saline treated animals [867]. In our experiments, the ELISA kits did detect low baseline expression for TNF, IL-1 and IL-6 protein. Unfortunately it is unknown if this basal expression represents normal endogenous levels of cytokine expression in non-pathogen free rats or is non-specific background.

The major objective for this set of experiments was to determine if the splenic nerve regulated in vivo cytokine production in response to i.v. LPS. Contrary to our hypothesis, we found that cutting the splenic nerve did not influence cytokine expression in response to i.v. LPS. This was unexpected since the majority of the in vitro literature and our in vivo model suggested a prominent role for NE in regulating splenic cytokine production.

Our model was initially based on the fact that Brown et al., demonstrated that the splenic nerve was inhibitory to splenic cytokines in vitro [89]. In agreement with Brown, Zhou et al., showed that macrophages from splenic nerve cut animals produced more cytokines in vitro than sham animals [890]. As reviewed in the rationale and in the literature review, the vast majority of in vitro reports also suggested that catecholamines

are inhibitory to cytokine production. In vivo, it is known that the splenic nerve, as compared to the renal nerve, can be selectively activated in response to LPS [76,645]. It is also known that immune stimuli can cause increased NE turnover in the spleen [219]. The activation of the nerve is dependent on the induction of central PGE2 [76], and when administered i.c.v., PGE2 suppresses splenic cytokine production [717]. Cutting the splenic nerve partially abrogated the immune suppression induced by i.c.v. PGE2 [717]. In other words LPS activates the splenic nerve which causes an increased release and/or turnover of splenic NE which is inhibitory to cytokine production. In spite of looking at both mRNA and protein levels to account for differences in post- transcriptional processing, no effect of the splenic nerve on cytokine production for any of the experiments was observed. The question thus remains as to why these results did not support our in vivo model and the majority of the in vitro studies.

One explanation as to why we did not observe a nerve cut effect could be due to the dose of i.v. LPS used in our studies. We first chose to investigate if cutting the splenic nerve would increase the peak of splenic cytokine production in response to LPS at 1 hr post-injection (the peak for TNF mRNA and protein). Related to this, we also wanted to determine if the time course in response to i.v. LPS would be altered in nerve cut animals as determined by cytokine levels at 2 hr post-injection. Based on the dose response data in the previous experiment it was postulated that larger doses of LPS incrementally activated different feedback systems which presumably served to keep cytokine levels in check. For example it was determined that splenic cytokine levels peaked in response to

1μg of i.v. LPS. The corticosterone response was also maximal in response to 1μg, while the epinephrine response was maximal after 10μg of LPS and splenic NE levels were reduced in response to 100μg of LPS. Therefore we wanted to test the peak cytokine response with a dose of LPS that also engaged the central systems controlling splenic nerve activity. This was important as it is possible that lower doses which do not affect homeostasis may not activate these central pathways. We also did not want to choose a dose of LPS too potent as our lab has shown [90,717] (which has been verified by Lacroix and Rivest [596]), that high doses of LPS may override any attempted blockade by central pathways on peripheral inflammation. Therefore we chose 10μg of LPS to induce the peak response because it was the lowest dose that induced maximal cytokine expression, consistently induced c-fos expression in the brain [105,860], increased plasma epinephrine (figure 3-17) and increased splenic nerve activity in 100% of the cases in anesthetized animals [76]. The results indicate that neither the peak levels of cytokines at 1 hr post-injection nor the levels measured at 2 hr were different in nerve cut animals in comparison to controls. Therefore it must be considered that either the 10μg dose was too potent and activated the immune response beyond what the central systems could downregulate, or that this dose was insufficient to engage the appropriate central pathways that control the splenic nerve.

Arguments that suggest the 10μg dose was too high a dose to observe the nerve cut effect include the fact that the cytokine expression is at its maximum and thus any negative signal transduced by the splenic nerve may be ineffective. If this is the case then

the drop in splenic NE levels observed in response to 100μg of LPS represents cardiovascular adaptations to LPS and the immune specific responses occur at lower doses of LPS. In the lowest dose measured, they reported that 4/6 animals responded to 0.4μg of i.v. LPS. However these studies were done in anesthetized animals, so arguably an even lower dose may activate the splenic nerve in conscious rats. Therefore if the nerve was activated in response to lower doses of LPS, and maximal doses could not be altered by nerve activation, then cutting the nerve should increase the cytokine response to a submaximal dose of LPS. However the results did not indicate any nerve cut effect in this paradigm either. Therefore it is likely that this dose of LPS (0.1μg), although capable of cytokine induction, did not activate the central counter-regulatory systems responsible for inhibiting peripheral cytokine production via the splenic nerve.

Another possibility is that the 10μg dose was also not potent enough to sufficiently activate the splenic nerve in our model. This is suggested by the fact although c-fos can be induced in the PVN, it may not be linked to the specific efferent pathways that control the splenic nerve. Also, since the 100μg dose of LPS caused a significant reduction in splenic NE levels relative to the 10μg dose (figure 3-19), this suggests that the splenic nerve is maximally activated in response to that dose of LPS. Therefore it is possible from a homeostatic perspective that the splenic nerve only serves to inhibit cytokine production in situations that overwhelm the other feedback pathways such as the HPAA and the adrenal medulla. However, cutting the splenic nerve had no effect on the response to 100μg of i.v. LPS in terms of cytokine production (figure 4-13).

Therefore our results indicate that cutting the splenic nerve does not regulate splenic cytokine production for the wide range of doses tested.

A second explanation as to why the there is an observed lack of regulation of splenic cytokines by the splenic nerve may have to do with a compensatory response. It is known that the HPAA and SNS are related in terms of central anatomical structures [94], central mediators [157,368] and can regulate each other in the periphery [365,366,379,380,382,383,385,386] and potentially through central feedback systems [366,369,370]. Therefore when one of these primary system are inhibited, the other systems may compensate. Evidence for this is suggested by Besedovsky et al., and [48] Brown et al., [89] who demonstrated more pronounced nerve cut effects in ADX rats. Similar to this, MacNeil et al., showed that acute ADX increased the sensitivity of the splenic nerve in response to LPS [378]. Also, ADX will increase peripheral NE turnover in response to stimuli [372] and some reports have suggested that the lack of corticosterone causes this increase in peripheral NE turnover [371]. Conversely, sympathectomy with 6OHDA will increase peripheral E [271], and increases peripheral and central HPAA activity [246,270-272]. It is known that ADX will cause changes in central mediators such as CRF and AVP [294,295,297,947,948] , however it is unknown if the c-fos and catecholamines induced centrally in response to 6OHDA represent compensation or are a non specific reaction to nerve damage. Therefore it is possible that the splenic nerve does regulate endogenous splenic cytokine production in response to LPS, but that the adrenal glands can compensate for the loss of neural input. Since

MacNeil's results were in acute ADX rats, the potential compensation mechanisms between systems must be immediate. This could also help explain the results of Elenkov et al., [195], who also investigated the effects of the SNS on cytokine production in vivo. They looked at TNF production in the plasma of mice given chlorisondamine and 30 minutes later challenged with 2 mg/kg of i.p. LPS. They found no effect of the ganglionic blocker on circulating levels of LPS-induced TNF. Interestingly, evidence to potentially refute the compensation theory was first presented by Brown [89] et al., (and verified in this experiment) in that plasma corticosterone and E levels were the same in both nerve cut and control rats. Therefore unless the increased levels of these mediators occurred before the time of sample collection, or there was a difference in free vs. bound corticosterone and/or E turnover, then compensation is not a viable explanation.

It is known that glucocorticoids are potent immunosuppressants and that corticosterone is induced in response to very low doses of LPS, sometimes even before cytokines can be induced [429]. Therefore it is possible that the splenic nerve does not normally regulate LPS-induced splenic cytokine production in an adrenal intact rat as corticosterone is sufficient for this purpose. However the in vitro studies suggest that the potential is there and may only be engaged in situations where the adrenal glands, and specifically the HPAA are not functioning to properly inhibit inflammation. Then any preferential activation of the splenic nerve in response to LPS in the intact animals may mediate cardiovascular signals that regulate blood flow and lymphocyte recirculation, which are known to be regulated in vivo by catecholamines [167,170-172].

4.8.2. The effect of the ADX on splenic cytokine production in response to i.v. LPS.

It is also possible that the effect of the splenic nerve on splenic cytokine production is either masked by the adrenal gland or is not engaged unless the adrenal gland is unable to maintain homeostasis on its own. Therefore in our model, the effect of the nerve on splenic immune function should be more pronounced in an ADX rat as observed by both Brown et al. [89], and Besedovsky et al. [48] in their experiments. However the optimal doses of LPS to use in ADX rats was first determined.

It is known that both ADX rats [344,345,679,944,949] and ADX mice [339,341-343,346,945,950] are more sensitive to the effects of LPS as compared to their sham counterparts. For example, Carli et al., demonstrated that ADX rats were 200X more sensitive to the lethal effect of LPS than sham operated rats [344]. This was confirmed in mice by Silverstein et al, [343]. In 1992, Ramachandra et al., [339] demonstrated that both surgical and chemical ADX mice were 500X more sensitive to the lethal effects of LPS than control rats. In agreement with previous reports [950] this also suggested that corticosteroids and not catecholamines were responsible for this effect. Ramachandra et al., also found that ADX mice produced 40-60X more TNF than control rats in response to the same dose of LPS. Other groups also found an increased cytokine production in ADX mice, but that the time course was similar, although slightly extended as compared to controls [341]. Increased IL-1 mRNA production was observed in ADX rats in response to i.p. LPS [944] as determined by Northern blotting, and macrophages

from ADX mice produced more IL-1 in vitro then their sham counterparts [951]. This is consistent with in vitro studies detailing the ability of glucocorticoids to inhibit cytokine production [328,329]. Therefore there was the possibility that the lethal effects of LPS in ADX mice were also due to increased cytokine production as well as lack of a pressor response and the dysregulation of energy metabolism [344]. Thus the dose response to LPS was repeated in ADX animals to verify the ADX effect on cytokine production and to determine the optimal dose to use in ADX/nerve cut studies.

In the ADX dose response we found that the 0.01μg dose of LPS-induced more prominent mRNA levels for TNF and IL-1 in ADX rats as compared to the dose response in sham operated controls. However, doses lower than 0.01μg did not induce cytokine levels higher than baseline. The 0.1μg dose was still submaximal in terms of inducing a TNF response, but induced the peak amount of IL-1 mRNA. This suggested that the ADX rats had an increased sensitivity to LPS as compared to the sham operated controls (up to 10X), but not near the magnitude suggested by Ramachandra et al. [339]. However later verification of the protein levels indicated that host response to these low doses of LPS-induced a similar pattern of TNF and IL-1 expression as compared to adrenal intact rats. It was found that the 0.1μg dose induced a submaximal response while the 0.01μg dose did not induce levels above baseline. The only differences were at the higher doses where 10μg of LPS-induced more cytokine protein than 1μg of LPS. This was in contrast to the protein data obtained in the intact rats and the mRNA data in ADX rats where the 1μg and 10μg doses induced equivalent responses. Therefore it is possible

that although mRNA levels have peaked, removal of the adrenal glands increased protein levels, most likely due to increased RNA stability. However this trend did not continue for doses above 10μg in that the 100μg and 1000μg doses did not increase cytokine expression above the 10μg dose. Therefore there is either a biological limit to the amount cytokines the spleen is able to produce or this is the point at which the nerve is actively inhibiting cytokine production.

The dose response of IL-6 mRNA in ADX rats was similar to intact animals in that peak levels were induced in response to the 10μg dose as compared to the 1μg dose (no induction was seen below 1μg). However the difference was that in the ADX dose response, the difference between the 1μg dose and the 10μg dose was statistically significant. It is unknown if this represents a differential sensitivity of ADX rats to LPS in terms of IL-6 production or is just biological variation as compared to the last experiment. Thus our results show that ADX rats are not many times more sensitive than control rats in terms of LPS-induced cytokine production. However the dose response experiments were performed on different sets of animals and thus direct comparisons were needed.

Three doses of i.v. LPS were then compared in sham and ADX rats: 0.01μg, 10μg and 100μg. In contrast to previous reports we found only a minor increase in cytokine production in ADX rats, as compared to sham operated controls. Interestingly, the trends were more prevalent with respect to TNF production than either IL-1 or IL-6. Thus the striking differences found by Ramachandra et al., in terms of TNF production in ADX

mice versus control mice were not repeated in our rat model. The reasons for the discrepancy between our results and the results reported by Ramachandra et al., are unknown. However possible explanations include the fact that both their end measure and animal model were different from ours. Because they assayed circulating levels of bioactive cytokines and not immunoreactive cytokines, the possibility exists that there is a difference between the two measures as TNF is susceptible to post-translational modification [548-550]. It also possible that since they measured circulating levels of cytokines, increased TNF turnover may be evident in the plasma but not in any individual organ. However we measured plasma TNF in response to 10μg and 100 μg of LPS in ADX rats and did not observe a difference as compared to controls (data not shown). Typical of many studies with glucocorticoids and cytokines, Grewe et al., found that dexamethasone decreased LPS-induced TNF mRNA by 50% in rat liver macrophage, yet totally abrogated the protein levels [327]. However the adminstration of synthetic glucocorticoids in vitro is different from the endogenous response as the microenvironment is lost. Also, dexamethasone binds GR more strongly than MR, where as corticosterone binds MR more strongly and GR is the predominant receptor type on immune cells.

In summary it is unknown if the nerve function has compensated for the missing adrenal glands or if cytokine production proceeds autonomously from central pathways as both the splenic nerve and the adrenal gland individually did not significantly impact on splenic cytokine production. Therefore if central systems function to modulate splenic

cytokine production in response to LPS, that modulation may only be observable upon removal of both the adrenal glands and the splenic nerve as this may be the only situation where homeostasis is in jeopardy.

4.8.3. The effect of the combination of splenic nerve cut and ADX on splenic cytokine production in response to i.v. LPS.

Although there were no observable effects of cutting the splenic nerve on splenic cytokine production in adrenal intact animals, it is still possible that the splenic nerve had a role in this capacity. This is because the adrenal glands may have masked any effect of the nerve or possibly compensated for its absence. Although trends were observed, ADX by itself also did not drastically alter splenic cytokine production in response to LPS. Therefore we employed the 2X2 experimental design (sham/sham, sham/nerve cut, ADX/sham and ADX/nerve cut) used by Brown et al., [89] and Besedovsky et al., [48] to determine if the splenic nerve regulated splenic cytokine production in ADX rats. The hypothesis was that the combination of ADX/nerve cut would eliminate the main regulatory systems that inhibit cytokine production, and, as a result cause elevated levels of splenic cytokine mRNA and protein in response to i.v. LPS as compared to the other three groups.

Similar to the rationale in the previous nerve cut experiments, we chose two doses of LPS that represented the two extremes of our dose response. The high dose, 100μg, was chosen because in intact animals the nerve was maximally activated in response to

this dose as determined by decreased NE levels. Therefore whatever the regulatory capability of the splenic nerve is, this capability is presumably maximum in response to 100μg of LPS. In intact and ADX rats cytokine production hits a ceiling in response to low doses of LPS as compared to the other physiological responses (changes in fever, blood pressure changes, body temperature etc.). Therefore if the adrenal gland, and the splenic nerve in the absence of the adrenal gland, function to maintain this ceiling, then presumably the higher the dose of LPS administered, the more obvious the nerve cut effect would become.

It is also possible that 100μg of LPS may overwhelm the immune system such that any manipulation would not alter cytokine production. If this was the case, this implies that any dose capable of inducing maximal cytokine production would be beyond the regulation of central inhibitory pathways. For this reason the 0.01μg dose of LPS was also chosen. The reasoning was if the nerve was activated in response to lower doses of LPS than indicated by splenic NE levels, then the submaximal response to 0.01μg of LPS could be potentiated in ADX/nerve cut animals as compared to controls. Both the 0.01 and 0.1μg doses induced submaximal responses but the 0.1μg dose induced maximal levels of IL-1 mRNA and thus was we selected the 0.01μg dose in these studies.

Similar to the other nerve cut experiments, there was no effect of splenic nerve cut on splenic cytokine production in response to either dose of LPS. This was in spite of removing the other potentially inhibitory mediators, the adrenal glands. Consistent with this, the effect of ADX on splenic cytokine production was not enhanced in nerve cut

animals suggesting that the compensatory mechanisms between the adrenal gland and the splenic nerve are not present for the conditions tested. Thus, for the doses tested, cytokine production was independent of central regulation save for the few modest changes observed in response to ADX. Therefore Besedovsky's feedback model involving the SNS and HPAA was not evident in response to LPS-induced cytokine production.

There are a few potential explanations as to why we were unable to observe a nerve cut effect on splenic cytokine production. Firstly, it is possible that the doses we used were too extreme. It is possible that the 100μg dose was too potent and caused maximal cytokine production irrespective of central activation. It was originally thought that since the 100μg dose caused splenic NE levels to drop, this was the point in which the splenic nerve was activated to restrain further cytokine production. However the lack of nerve cut effect in response to this dose of LPS suggests that the ceiling of cytokine production was reached independent of the splenic nerve. Therefore, if this dose was too potent, then the response to a lower dose of LPS (0.01μg), which did not cause maximal cytokine production, may be potentiated in ADX/nerve cut animals. The lack of an effect with this dose suggests that it was not potent enough to engage the central systems and the immune system dealt with it autonomously. Therefore it seems that the doses potent enough to engage the central systems have already activated the immune system beyond regulation and doses that induce submaximal responses do not activate the splenic nerve.

An alternate strategy would have been to utilize doses of LPS that induced discrepancies between cytokine mRNA and protein. For example, although IL-1 mRNA

levels in ADX rats peaked in response to 0.1μg of LPS, protein levels did not. Therefore a nerve cut effect in response to 0.1μg of i.v. LPS may have been observed for splenic IL-1 protein but not for splenic IL-1 mRNA. Likewise, in response to 1μg of LPS, mRNA for both TNF and IL-1 were at their peak levels while the protein levels were significantly less than those induced by 10μg of LPS. Therefore cutting the nerve may have increased cytokine protein but not mRNA in response to this dose as well. These studies would indicate if the splenic nerve, like glucocorticoids, could affect mRNA stability. Although these possibilities remain, there are a few points to be considered. First, although cytokines are susceptible to post-transcriptional regulation, there is no direct evidence that supports a for role NE or other neuropeptides in this capacity. As a matter of fact, Spengler's in vitro studies showed that NE affected TNF mRNA levels in a similar manner to the protein [187,193]. Also, although 10μg of LPS-induced more cytokine protein than 1μg of LPS, the magnitude of this difference was small (approximately 15-20%). It is unknown what the biological significance of this moderate a change would be.

Similarly, it is possible that NE regulates cytokine expression at the post-translation level as the majority of in vitro studies have measured secreted cytokines. If this were the case then measurements in any single organ may be uninformative with respect to potential increases in systemic spill-over into the plasma. However, since the spleen does not significantly contribute to the circulating pool of cytokines [923,924], measuring plasma levels in our experiments would not shed light on this possibility. Therefore systemic chemical sympathectomy would have to be done with either a

ganglionic blocker such as chlorisondamine or 6OHDA which destroys sympathetic post-ganglionic fibers. The former may be preferable as it could be administered more acutely to prevent long terms compensation and 6OHDA can cause undesirable secondary effects like an initial NE bolus and corticosterone release. Elenkov et al., performed this experiment with a ganglionic blocker (chlorisondamine) and did not observe a nerve blockade effect on plasma TNF production effect in adrenal intact mice [195]. However, they did not test the effects of chlorisondamine on LPS-induced TNF levels in ADX mice, a similar paradigm to Besedovsky's model. If we were to predict the result of this proposed experiment it is likely that the ablation of the SNS would not affect circulating cytokine production as in our model there was a close relationship between splenic mRNA, splenic protein and when measured, circulating protein. Surprisingly, this same group, Szelenyi et al., [198], recently found that LPS-induced plasma TNF levels could be increased in mice treated 24-48 hr prior to LPS with i.p. reserpine. Reserpine depletes catecholamines as opposed to ganglionic blockers which prevent communication between pre-ganglionic and post-ganglionic cells via blockade of the post-synaptic nicotinic receptors [134,141]. It is unknown why the results would differ between these studies as the function of these drugs should be similar in this paradigm in that they prevent catecholamine release in response to LPS. Interestingly, Besedovsky's group was able to abrogate LPS-induced increases in splenic blood flow with reserpine or splenic denervation but not hexamethonium (a ganglionic blocker) [952]. This suggests that LPS and cytokines may directly cause the release of NE from post-ganglionic nerve terminals.

However Szelenyi et al., gave no explanation of this phenomenon or of the apparent contradiction between their results and previously published reports from their lab. It is unclear why they observed an effect of the sympathetics on LPS-induced TNF in their model system and we did not. However it could be due to the pharmacological manipulation of their system with reserpine as it affects peripheral neurons, central neurons and the adrenal medulla, or the fact that their model system was mice and we used rats. We also found no noticeable difference in the mRNA levels for either TACE (which was undetectable) or ICE in response to 100μg of i.v. LPS among the four groups. However, since these molecules can themselves undergo post-translational regulation, measuring the levels of the mRNA does not provide definitive information on cytokine protein turnover.

Since it has been proven that compensation can occur between the efferent arms of the central regulatory system, another (albeit remote) possibility is that other systems may have compensated for the lack of inhibition from the adrenal glands and splenic nerve in response to splenic cytokine production. These mediators could be hormones from the pituitary, salivary glands or other endocrine glands. However there is no direct evidence to support this hypothesis.

The final possibility is that the nerve does not function to regulate cytokine production in response to LPS. Although there is a well documented role of catecholamines in regulating blood pressure and energy metabolism, our results contradict the many in vitro studies suggesting a role for NE in regulating LPS-induced cytokine

production. However other parameters of immune function such as cell movement [167,170,172] and phagocytosis may be mediated by NE [66,953]. For example, we found that cutting the splenic nerve increased splenic weight and significantly decreased the percentage of splenic T-cells per field of view. These observations seem to compliment each other as a fixed T-cell number in an increasing volume will result in a decreased T-cell percentage. Although the same pattern was evident for B-cells, it was of a significantly lesser magnitude; and, this relationship did not exist for macrophages. The reasons for these differential effects are unknown. However one possible explanation is that since NE can promote lymphocyte homing to the spleen [167], lack of NE may preclude the maintenance of the relative proportions of specific immune cell populations in the growing spleen. Alternatively it is possible that once certain cell types are in the spleen, it is more difficult for them to leave. Besedovsky's group (Rogausch et al., [172]) found in their splenic perfusion model that the lower the vascular resistance, the greater the movement of cells out of the spleen. However catecholamine agonists and antagonists had the ability to alter this relationship. For example although propanalol (a beta adrenergic antagonist) decreased vascular resistance, it did not cause an increased movement of cells out of the spleen as expected by its effect on the vasculature. Therefore the nerve may be mediating aspects of cell trafficking which are altered in response to LPS as indicated by the reduced latency of the increase in splenic nerve activity as compared to renal nerve activity [76]. Saline animals were not included in our experiments so it is unknown if the nerve cut effect on splenic weight we observed was

LPS independent. However it is unlikely that LPS could produce these changes in splenic morphology in only 1hr.

This putative role in regulating splenic weight and the proportions of the different cell types suggest the SNS may be more involved in regulating antigen surveillance as opposed to the acute inflammatory response. This is consistent with the notion that immune challenges which elicit a strong corticosterone response do not require additional regulation from the SNS. Therefore in antibody-antigen interactions, where physiological corticosterone is not a major factor [48,249-251], the nerve may be the primary mediator. This is suggested by Besedovsky's original report [48] which used sheep red blood cells as an immune measure and our lab has results that show that the splenic nerve can mediate IL-2-induced increases in the sheep red blood cell response. As well, Virginia Sanders and colleagues have published numerous critical studies on the effects of NE on antigen-antibody interactions [152,153,258-260,269,954,955]. Therefore it is possible that if the splenic nerve transduces an immune specific signal in response to LPS [76], it may not be meant for the innate response, but to prime the adaptive immune system for exposure to a potential pathogen. Thus it is possible that although LPS and live bacteria induce the same pattern of cytokine production, it is only during the chronicity of a bacterial infection that the role of the splenic nerve may be apparent. These studies have yet to be conducted.

We observed that all of the additional molecules we tested showed a similar pattern to the LPS-induced responses of TNF, IL-1 and IL-6 except for IFN-gamma and

IL-12 p40. Since these cytokines are involved in cell-mediated immunity it would be expected that they would follow a similar course to the others. For example, IL-12 p40 mRNA followed the trend of TNF in that it was increased by ADX. This is not surprising since IL-12 is a key mediator in the TH1 response which is inhibited by glucocorticoids. Along this line of reasoning it was predicted that cutting the splenic nerve cut would also increase IL-12 p40 mRNA levels as the environment would be more conducive to a TH1 response, however this was not observed. The expression pattern for IFN-gamma was exactly opposite to what we would have predicted in that the combination of nerve cut/ADX decreased mRNA expression relative to controls. It could be that this was a technical artifact as IFN-gamma levels were barely detectable at 1 hr post-LPS (their peak is at 2-4 hr post LPS). Further experiments would be required to validate these observations.

4.8.4. Perspectives

In summary we were unable to demonstrate in vivo that the splenic nerve regulates splenic cytokine production in response to LPS in either intact or ADX animals. We were also unable to demonstrate a significant effect of the adrenals on splenic cytokine production. Therefore it seems that once the inflammatory cascade is initiated, the brain cannot significantly impact on splenic cytokine production, at least with respect to the dose/time points tested. However, if the neural regulation of inflammation and natural immunity is merely a byproduct from the regulation of other systems such as

energy metabolism and cardiovascular function, why do macrophages have adrenergic and glucocorticoid receptors?

The answer may lie in the experimental paradigms that could demonstrate that the splenic nerve does regulate splenic immune function. For example our lab has shown that i.c.v. PGE2 reduced peripheral cytokine production in response to 0.1μg of i.v. LPS and that this effect was partially abrogated by splenic nerve cut [717]. This was similar to Brown's original paradigm of looking at cytokine production in macrophages from intact and splenic nerve cut animals that received i.c.v. IL-1 [89]. Others have shown similar effects with different measures of natural immunity. For example Lipton et al., found that i.c.v. MSH reduced skin inflammation and this effect was blocked by spinal cord transection [230]. Hori's lab found that central stimulation of certain brain areas either with lesions or substances administered i.c.v, caused increased splenic nerve activity and decreased NK cell function [160-162]; these effects were also abrogated by splenic denervation. Irwin et al., found a similar effect with i.c.v CRF and NK cell function [163]. Therefore a strong central stimulus that can effect immune function can potentially be abrogated by cutting the splenic nerve. These studies suggest that the potential for splenic nerve regulation of splenic cytokines exists, but can not be demonstrated in our paradigm. This is likely due to the fact that a dose of LPS required to activate the central systems, may also activate the immune system beyond normal physiological regulation. Thus once the LPS has activated the macrophages , any inhibitory signals are too late to affect cytokine production as the cascade is both rapid and transitory. By separating the central

stimulus from the peripheral stimulus (functionally and/or and temporally), maximal central activation can be tested in response to a peripheral challenge that will not overwhelm the system. Psychological stress is an ideal central stimulus because it will activate the same central systems as LPS, but not activate peripheral immune function. By imposing maximal central activation on a low dose of LPS it may be possible to determine if the splenic nerve can regulate splenic cytokine production.

5. Experiment 3

The role of the SNS and HPAA in regulating the effects of stress on LPS-induced splenic cytokine production.

5. Experiment 3: The role of the SNS and HPAA in regulating the effects of stress on LPS-induced splenic cytokine production.

5.1. Overview

The objectives of this experiment were to determine 1) if psychological stress (footshock) affects LPS-induced splenic cytokine production; and 2) if these affects are mediated by the splenic nerve, the adrenal glands or both of these structures. We modified a model used by Goujon et al., [867] where animals were injected with LPS prior to being exposed to stress.The exposure to stress and LPS injection were timed so that cytokines were measured at 1 hr post-injection (the peak for TNF mRNA, IL-1 mRNA and TNF protein) to remain consistent with our previous experiments. It is known that the HPAA and SNS are engaged more rapidly in response to stress than LPS, the mediators released from these systems are known to suppress immune function and that the dose of LPS used in our studies (0.1 μg) does not itself activate the SNS or HPAA. Therefore we predicted that stress will suppress LPS induced splenic cytokine production and that the splenic nerve and adrenal glands will mediate this effect. It was found that stress was immunosuppressive, but that neither the splenic nerve nor the adrenal gland were individually responsible for mediating the immunosuppressive effects of stress in our system. However removal of both the adrenal glands and the splenic nerve significantly abrogated the immunosuppressive effects of stress on splenic TNF and IL-1 mRNA and protein in response to i.v. LPS.

5.2. Objectives

1. To determine the effects of stress on LPS-induced splenic cytokine production.

2. To ascertain if the splenic nerve mediates the effects of stress on splenic cytokine production.

3. To assess the role of the adrenal glands in mediating the effects of stress on splenic cytokine production.

4. To examine the interaction between the adrenal glands and the splenic nerve in mediating the effects of stress on splenic cytokine production.

5.3. Hypotheses

It is predicted that stress will suppress LPS-induced splenic cytokine production and this will be partially mediated by the splenic nerve. Consistent with previous rationale, both the adrenal gland and the splenic nerve will have a role in mediating the effects of stress on splenic cytokine production in that the nerve cut effect will be more prominent in ADX rats.

5.4. Rationale

It is unknown why the previous set of experiments (experiment 2) failed to demonstrate that the splenic nerve had a role in regulating splenic cytokine production. However it is possible that once the inflammatory cascade is initiated, the SNS cannot significantly impact on LPS-induced splenic cytokine production. It is also possible that the doses required to engage the SNS in that study had activated the immune system beyond the influence of central regulation. Studies that effectively demonstrated a role for the splenic nerve on splenic immune function have all separated the immune stimulus from central activation. For example Pan et al., demonstrated that i.c.v. PGE2 inhibited peripheral cytokine production in response to 0.1 μg of i.v. LPS (this dose does not activate the central feedback systems) and this effect was partially abrogated by splenic nerve cut [717]. This was similar to Brown's paradigm which looked at cytokine production in macrophages from intact and splenic nerve cut animals that received i.c.v. IL-1 [89]. Similar effects were demonstrated by Macaluso et al.,[230], Hori et al.,[160-162] and Irwin et al., [163] in their respective paradigms.

For this set of experiments, we will employ psychological stress (sometimes referred to as just "stress") as our central stimulus and 0.1 μg of i.v. LPS as our peripheral stimulus. Psychological stress activates many of the same central and peripheral pathways as higher doses of LPS [32,90,757,758,807,815,831,956], without maximally activating the immune system. Thus this paradigm should effectively separate immune

activation from the central response as the 0.1 μg dose of LPS-induced moderate cytokine production in our system without concurrent increases in plasma corticosterone or splenic and plasma NE and E.

Additional rationale for objective 1: The first goal is to determine the effects of stress on LPS-induced splenic TNF, IL-1 and IL-6. In order to make a prediction as to whether stress will increase or decrease LPS-induced cytokine production, it is important to consider that the effects of stress on immune function may depend on the severity of the stress [862,957-959], the type of stress [884,959,960], the nature of the immune function that is being measured [884], the timing of the stress in relation to the immune challenge [808,837,883,957,958,961,962] and the strain and species of animals used [271,845,884,962,963]. Also, in vitro immune measures may not be accurate as Green-Johnson et al., demonstrated that cells removed from their microenvironment behave differently than they do in vivo [266,267].

We predict that stress will decrease LPS-induced levels of splenic TNF and IL-1, as catecholamines and glucocorticoids are known to inhibit LPS-induced cytokine production in vitro and in vivo, and these mediators are released in high amounts after stress. In fact the time course for stress-induced activation of the HPAA and SNS is reported to be more rapid than the LPS-induced response in that plasma catecholamines are increased almost immediately after the initiation of stress and can decrease to baseline by 1 hr [802,803,839,964]. Similarly, the plasma corticosterone level can be elevated as

rapidly as 2-5 minutes after the initiation of stress and drops to baseline by 1 hr, which is when LPS-induced corticosterone levels start to peak [15,790]. Therefore if the animals are exposed to stress in close proximity to LPS, catecholamines and corticosterone will be present in extremely high levels prior to cytokine induction. This is in contrast to the previous model where catecholamines and corticosterone are induced by high doses of LPS but may be too late to exert their effects at the initial stages of the inflammatory reaction.

Direct evidence to support stress-induced immunosuppression of LPS-induced cytokine production was reported by Goujon et al [867]. They found that 15 minutes of restraint stress (which is arguably less severe than our stress paradigm [835,840,841]) immediately following 10 μg of i.p. LPS, reduced cytokine levels in mice as measured by RT-PCR. Similarly, Beno and Kimura found that surgical stress also reduced LPS-induced plasma TNF levels [866]. However other reports suggest that stress can increase LPS-induced TNF and IL-1. For example, Kraal's group found that various types and intensities of stress increased LPS-induced levels of TNF and IL-1 in rat alveolar macrophages in culture [868,870]. Interestingly, they also found that these effects were abrogated by pre-treating the rats with chlorisondamine suggesting a role for the SNS in this effect [871]. Increases in LPS-induced cytokine levels by stress were also reported by Zhu et al., who looked at LPS-induced cytokine production in peritoneal macrophage from animals exposed to swimming stress [869]. The contrasting results demonstrated for this subject are indicative of the stress/immunity literature. Although many explanations

exist for this discrepancy, it is important to note that stress-induced increases in cytokines were only observed with in vitro assays.

Based on the literature, the prediction for the stress-induced alteration of IL-6 production is even less obvious. It would be expected that since IL-6 is produced in series with TNF and IL-1, and these cytokines positively influence the production of IL-6 [517,523,525-528], that the expression pattern of LPS-induced IL-6 in response to stress would be similar to TNF and IL-1. In support of this, glucocorticoids [326,328,332,333,336,337] and catecholamines [174,176] have been shown to decrease IL-6 production in response to LPS. In contrast, other reports suggest that although glucocorticoids inhibit IL-6 production, catecholamines can actually potentiate IL-6 production [965]. This latter effect has also been observed by others [173,200,965] and Straub et al., proposed that these divergent reports may be explained by the presence or absence of bacterial products. They found that if bacterial products are present then NE inhibits IL-6 secretion. If they are absent, then NE was found to potentiate IL-6 secretion [210]. In agreement with this latter observation, the majority of in vivo studies suggest that stress (in the absence of LPS) potentiates IL-6 production [204-207,873]. This phenomenon also occurs in models using non- psychological stress such as hemorrhage [966,967]. It is unknown how stress will affect IL-6 production in our paradigm as LPS is present. Therefore two-tailed tests were chosen for the initial experiments with respect to IL-6 production.

Goujon et al. [867], convincingly demonstrated a stress-induced suppression of

splenic cytokine production in vivo. Therefore we adapted their protocol by replacing the 15 minutes of restraint stress with 15 minutes of intermittent footshock. Goujon's 10 μg of i.p. LPS in the mouse was replaced with 0.1 μg of i.v. LPS in the rat. The qualitative differences are therefore that we used a milder immune challenge with a potentially more potent central stimulus [835,840,841] in order to better define the role of the SNS and HPAA in regulating splenic cytokine production.

Additional rationale for objectives 2-4: It is known that stress will activate the HPAA and SNS and cause the release of catecholamines and glucocorticoids [1]. It is also known that the splenic nerve is the only nerve to the spleen and it is entirely sympathetic [65,938]. In addition to this, splenic NE originates almost entirely (>95%) from the splenic nerve [144,937], splenic immune cells are in close proximity to splenic nerve fibers [151] and contain adrenergic receptors [66]. Combined with the fact that catecholamines can regulate immune function, and specifically cytokine production [180,187,188,192,968], it is likely that the effects of stress on splenic cytokine production are mediated at least in part by the splenic nerve.

In support of this Keller et al., found that stress was immunosuppressive to the in vitro mitogen response [57] and that this effect was present in ADX [56] and HYPOX [58] animals. Cunnick et al., also found that both corticosteroids and catecholamines were involved in stress-induced immune suppression of in vitro T-cell mitogenesis [859]. Although suggestive, these studies did not definitively determine if the

immunosuppressive effects of stress were mediated by the SNS. More direct studies from Irwin et al., showed that stress-induced immune suppression of NK function could be blocked by 6OHDA [163]. Sheridan's group used a model of restraint stress and exposure to influenza virus they also found that there were adrenal-dependent and independent mechanisms of immune suppression [863] as some of their stress-induced effects were blocked with 6OHDA [969]. Our lab was the first to show that surgically cutting the splenic nerve abrogated the immunosuppressive effects of stress on the response of splenocytes to mitogen and sheep red blood cells [860]. Based on these results it was predicted that the effects of stress on LPS-induced splenic cytokine production in vivo would be partially abrogated by cutting the splenic nerve.

It was also predicted that the nerve would have a more pronounced effect in ADX rats. This was because the SNS may compensate for the lack of an adrenal gland and have a more prominent role in mediating the stress-induced effects of splenic cytokine production as suggested by NE turnover studies in ADX rats [366,371,372]. In support of this, both Besedovsky [48] and Brown [89] saw increased effects of the splenic nerve cuts on splenic immunity in ADX rats in their respective paradigms.

5.5. Experimental Design

All studies utilized adult male Sprague-Dawley rats (225-250 g) that were obtained from Charles River, (Dorval Quebec). The rats were kept on a 12-12 light/dark

cycle and given food and water were given *ad libitum* (water for ADX rats contained 0.9% saline). Some animals underwent combinations of sham surgeries, ADX and splenic nerve cuts and were allowed to recover for 7-10 days. All animals were singly housed, handled for 2 days then taken through the experimental procedure (weighing, mock injection etc.) for 3 days before the actual experiment. On the day of the experiment, animals were weighed and then put under a heat lamp for 3 minutes to dilate the tail veins. They were then put in a restrainer and injected i.v. with 0.1μg of LPS (E.Coli serotype 055:B5 lot L-2637). Following this they were exposed to psychological stress for 15 minutes and killed 45 minutes following the completion of this procedure. Our model of psychological stress is 15 minutes of 1.6 mA intermittent footshock as described by Wan et al., [860]. Each shock lasted 5s and was preceded by a 15s warning tone. The average interval between shocks was 3.5 minutes with a range between 2-5 minutes. Homecage controls were left in their cage until LPS injection. The animals were killed by decapitation and blood and spleens were collected for various assays. Northern blots, ELISAs, immunhistochemistry, RIA for corticosterone and catecholamine determinations were performed as described in the materials and methods section. Experiments were analyzed by ANOVA, and one- and two-tailed T-tests as described in the materials and methods. The stress/ADX studies were analyzed by ANOVA in a 2X2 design looking at the effects of ADX, stress and the combination thereof on splenic cytokine production in response to 0.1 μg of i.v. LPS. In order to compare groups in different experiments, the individual values of the groups of interest were expressed as a difference from the mean of a group common to both

experiments. Although the principal was similar to Z scores were calculations, the units were not standardized in terms of standard deviations. Thus the data for experimental groups are expressed in the original units as increases or decreases from the mean of the group common to both experiments. All procedures were approved by the animal ethics committee at the University of Manitoba and the CCAC. The effectiveness of ADX surgeries were verified by measuring plasma corticosterone and epinephrine levels while splenic nerve cut surgeries were verified by measuring splenic NE.

5.6. Results

5.6.1. Objective 1 The effect of stress on LPS-induced splenic cytokine production when 0.1 μg of i.v LPS precedes the exposure to stress.

To verify that plasma corticosterone and catecholamine levels became elevated following exposure to stress, an abbreviated time course was performed in animals that received footshock alone. It was found that plasma corticosterone [$F(4,15)= 1.14$, $p=0.1571$ ns,], NE [$T(6)=2.394$, $p=0.027$] and E [$T(6)=1.599$, $p=0.08$ ns] were at their highest levels immediately after stress (time 0) and returned to baseline 1 hr later. Corticosterone levels (figure 5-1) were approximately 2X higher immediately after stress than in homecage control animals ($p=0.02$) or rats 1 hr after stress ($p=0.08$). These later two groups were not different from each other. A similar trend was demonstrated for plasma NE ($p=0.027$) and E ($p=0.08$) in that levels immediately after stress were higher

than homecage controls (figure 5-2). Levels of both NE and E returned to baseline at 1 hr post-stress (not shown).

In accordance with the above findings, animals that received stress immediately after LPS injection (figure 5-3), had lower levels of splenic TNF [T(18)=4.3, p=0.0002, one-tailed], IL-1 [T(18)=1.78, p=0.046, one-tailed] and IL-6 [T(11)=4.157, p=0.0016, two-tailed] mRNA than their non-stressed counterparts. Splenic TNF [T(13)=3.45, p=0.0021, one-tailed] and IL-6 protein [T(11)=3.564, p=0.0022, one-tailed] levels were also suppressed in response to stress. However, the splenic IL-1 concentration [T(13)=0.58, p=0.28 ns, one-tailed] was not different from that in control animals (figure 5-4). Immunostaining (figure 5-5) verified that stress reduced splenic TNF levels [T(18)=3.422, p=0.003, one-tailed], and determined that stress did not reduce the amount of ED-1 staining (macrophage cell marker) [T(18)=0.066, p=0.47 ns, one-tailed] in the spleen (figure 5-6). This suggests that stress affects the individual cells in the spleen and not the cell number. Interestingly, it was found that at the time of death that stressed animals in the LPS-injected group had a higher level of plasma corticosterone [T(18)=2.66, p=0.016] than LPS-injected non stressed rats (figure 5-7).

5.6.2. Objectives 2-4: The effect of splenic nerve cut and ADX on levels of splenic cytokine production in animals injected with 0.1 μg of i.v. LPS and subsequently exposed to stress.

To determine if the splenic nerve has a role in the immunosuppressive effects of stress on splenic cytokine production, sham operated and nerve cut animals were injected

with 0.1 μg of i.v. LPS and then exposed to 15 minutes of footshock. There were no significant differences between these groups for splenic TNF [T(18)=0.17, p=0.43 ns, one-tailed] and IL-1 mRNA [T(18)=0.30, p=0.76 ns, one-tailed] (figure 5-8). IL-6 mRNA levels were below our detection capabilities. There were also no differences between sham and nerve cut groups for splenic protein levels of TNF [T(13)=0.51, p=0.31 ns, one tailed], IL-1 [T(13)=1.17, p=0.13 ns, one tailed], and IL-6 [T(13)=0.121, p=0.45 ns, one-tailed] protein levels (figure 5-9).

Since we were unable to observe a nerve cut effect, the next goal was to determine if the adrenal glands were responsible for the stress-induced immune suppression. This experiment was designed as a 2X2 ANOVA looking at the effects of ADX and stress and the combination thereof on LPS-induced splenic cytokine levels (the groups were sham, ADX, sham/stress and ADX/stress).

Results for splenic TNF mRNA (figure 5-10) indicate a significant overall effect [$F(3,24)=74.87$, $p<0.0001$] and significant main effects for ADX [$F(1)=10.185$, $p=0.0039$] and stress [$F(1)=126.3$, $p<0.0001$] with no interactions. In terms of individual comparisons, the sham group had a trend for lower mRNA levels than the ADX group ($p=0.065$) and higher mRNA levels than sham/stress group ($p<0.0001$). The ADX/stress group had lower mRNA levels than the ADX group ($P<0.0001$) yet higher levels than the sham/stress group ($p<0.0001$) indicating that stress-induced immune suppression of splenic TNF mRNA was evident in ADX rats. Similar results were obtained for splenic TNF protein (figure 5-11) and plasma TNF protein (figure 5-12). There was a significant

overall effect for splenic TNF protein [F(3,24)=28.20, p<0.0001] and significant main effects for ADX [F(1)=21.24, p=0.0001] and stress [F(1)=61.67 p<0.0001] with no interaction. The differences between individual groups followed the same pattern as the mRNA where the sham group had lower splenic TNF levels than the ADX group (p=0.0013) and higher mRNA levels than sham/stress group (p<0.0001). The ADX/stress group had lower mRNA levels than the ADX group (P<0.0001) yet higher levels than the sham/stress group (p<0.0089) verifying that stress-induced immune suppression of splenic TNF mRNA was evident in ADX rats. Plasma TNF (figure 5-12) also showed a main overall effect [F(3,24)=9.21, p=0.0003] and significant main effects for ADX [F(1)=10.81 p=0.0031] and stress [F(1)=16.522 p=0.0004] with no interaction. The differences between individual groups followed the same pattern as splenic mRNA and protein where the sham group had lower plasma TNF levels than the ADX group (p=0.03) and higher mRNA levels than sham/stress group (p=0.01). Similarly, the ADX/stress group had lower mRNA levels than the ADX group (P=0.0058) yet higher levels than the sham/stress group (p=0.026).

This experiment also showed a significant overall effect for splenic IL-1 mRNA (figure 5-13) [F(3,24)=4.19, p=0.016], but there was only a significant main effect for stress [F(1)=12.06 p=0.002], not ADX [F(1)=0.03 p=0.55 ns], and there were no interactions. The differences between individual groups also showed a stress effect where the sham group had higher IL-1 mRNA levels than the sham/stress group (p=0.014) and the ADX group had higher levels that the ADX/stress group (p=0.03). Splenic IL-1

protein (figure 5-14) also showed a significant overall effect [F(3,24)=5.89, p=0.0036] and surprisingly significant main effects for both ADX [F(1)=4.72 p=0.04] and stress [F(1)=12.8 p=0.0015] with no interactions. The differences between individual groups only showed a stress effect where the sham group had higher splenic IL-1 protein levels than the sham/stress group (p=0.018) and the ADX group had higher levels that the ADX/sham group (p=0.018). There were no overall effects for plasma IL-1 [F(3,15)=1.849, p=0.19 ns] (figure 5-15); however, there was a significant main effect for ADX [F(1)=5.43, p=0.034] but not for stress [F(1)=0.005, p=0.94 ns] and no interactions. This was especially surprising since ADX rats had less plasma IL-1 than their sham operated counterparts.

The overall levels for splenic IL-6 mRNA were low, however (figure 5-16) there was an overall main effect [F(3,24)=11.495, p<0.0001] and a significant main effect for stress [F(1)=31.123, p<0.0001] but not for ADX [F(1)=0.654, p=0.42 ns] with no interactions. Comparisons between the individual groups also illustrated the stress effect in that the sham group had significantly higher levels of IL-6 mRNA than the sham/stress group (p=0.0071) and the ADX group had higher levels than the ADX/stress group (p<0.0001). Splenic IL-6 protein levels (figure 5-17) showed a similar pattern in that there was a significant overall effect [F(3,24)=13.798, p<0.0001] and a significant main effect for stress [F(1)=41.33, p<0.0001] but not for ADX [F(1)=0.087, p=0.77] and there were no interactions. Comparisons between individual groups illustrated a stress effect where the sham group had significantly higher levels of IL-6 mRNA than the

sham/stress group ($p<0.0001$) and the ADX group had higher levels than the ADX/stress group ($p=0.0002$). Unlike splenic mRNA and protein, there was no significant overall effect [$F(3,24)=0.201$, $p=0.89$ ns] or significant main effects of stress [$F(1)=0.560$, $p=0.46$ ns] or ADX [$F(1)=0.034$, $p=0.85$ ns] on plasma IL-6 levels (figure 5-18).

Since ADX rats were immunosuppressed in response to stress, it was then determined if cutting the splenic nerve would abrogate the immunosuppressive effects of stress on splenic cytokines in ADX rats. The ADX/nerve cut rats had significantly higher levels of TNF mRNA (figure 5-19) levels than the ADX/sham nerve cut group [$T(17)=3.74$, $p=0.0008$, one-tailed]. Splenic TNF protein (figure 5-20) showed a similar effect [$T(17)=4.26$, $p=0.0003$, one-tailed] as did splenic IL-1 mRNA [$T(17)=2.58$, $p=0.0098$, one-tailed] (figure 5-21) and protein [$T(17)=1.94$, $p=0.035$, one-tailed] (figure 5-22). IL-6 mRNA (figure 5-23) [$T(17)=0.148$, $p=0.44$ ns, one-tailed] and protein levels (figure 5-24) [$T(17)=0.305$, $p=0.38$ ns, one-tailed] were not different between the two groups. As an index of whether or not the splenic nerve cuts completely reversed the immunosuppressive of stress in ADX rats, ADX (non stress) and ADX/NC/stress groups were compared in terms of standard deviations away from the mean of the ADX/stress group in their respective experiments. The ADX and ADX/NC/stress groups were then compared by a two-tailed T-test. There was not a significant difference between the change in the ADX/NC/stress from the ADX/stress group in its respective experiment as compared to the change in the ADX (non stress) group from its respective ADX/stress group for splenic TNF mRNA [$T(14)=1.18$, $p=0.26$ns], IL-1 mRNA

[T(14)=0.22, p=0.83ns], TNF protein [T(14)=0.55, p=0.60ns] and IL-1 protein [T(14)=0.50, p=0.62ns] (figure 5-25).

5.7. Figures (* indicates that P≤0.05)

Figure 5-1: Time course for plasma corticosterone following 15 minutes of intermittent footshock. Rats were either left in their homecage or exposed to intermittent footshock for 15 minutes at 1.6 mA and killed at the indicated time points following the procedure (time 0 is immediately after footshock). Plasma corticosterone [F(4,15)=1.14, p=0.16ns, n=20] levels were measured by RIA and expressed as ng/ml. Error bars represent +/-1 standard error of the mean.

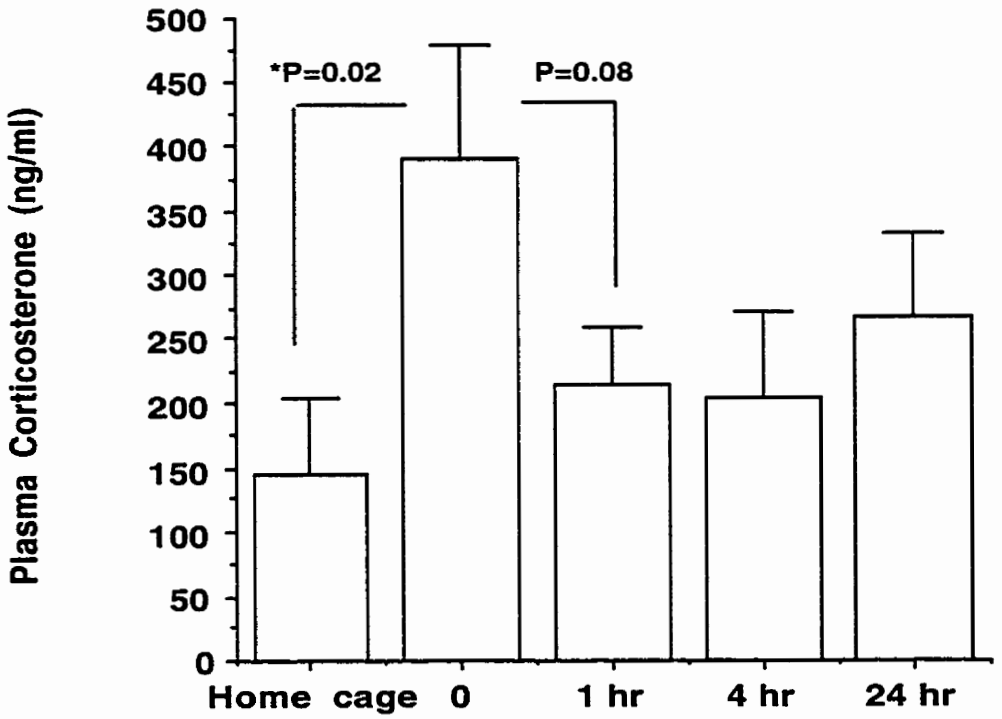


Figure 5-2: Effect of 15 minutes of intermittent footshock on plasma catecholamine levels. Rats were exposed to 1.6 mA of intermittent footshock for 15 minutes and killed immediately afterwards. Plasma catecholamine levels were measured by HPLC and compared to control rats with a one-tailed T-test. Both NE [T(6)=2.4, p=0.027, n=8] and E [T(6)=1.6, p=0.08, n=8] levels are expressed as pg/ml and error bars represent +/-1 standard error of the mean.

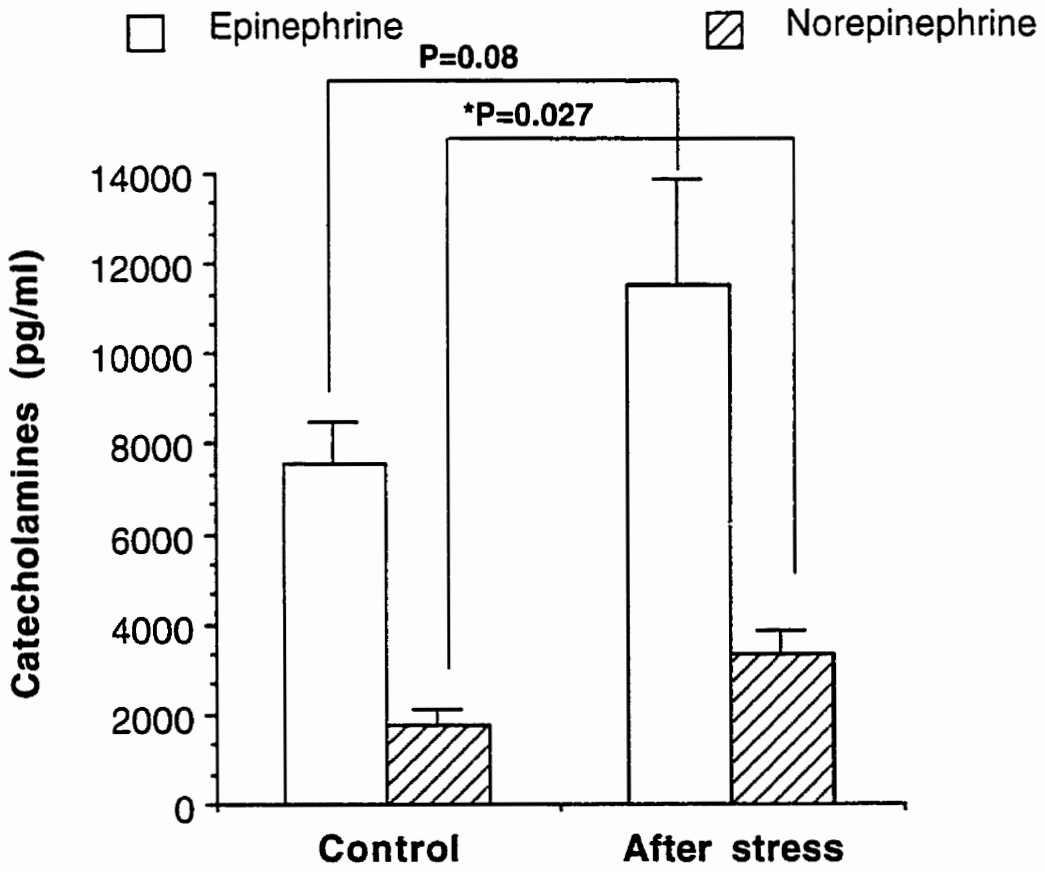


Figure 5-3: The effect of 15 minutes of intermittent footshock stress immediately following 0.1μg of i.v. LPS on splenic cytokine mRNA levels. Rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. Cytokine mRNA levels were analyzed by Northern blotting and expressed as a ratio relative to the loading control. TNF [T(18)=4.3, p=0.0002, n=20] and IL-1 [T(18)=1.78, p=0.046, n=20] mRNA levels were compared by one-tailed T-test and IL-6 [T(11)=4.15, p=0.0016, n=13] mRNA levels were compared by a two-tailed T-test. Error bars represent +/-1 standard error of the mean.

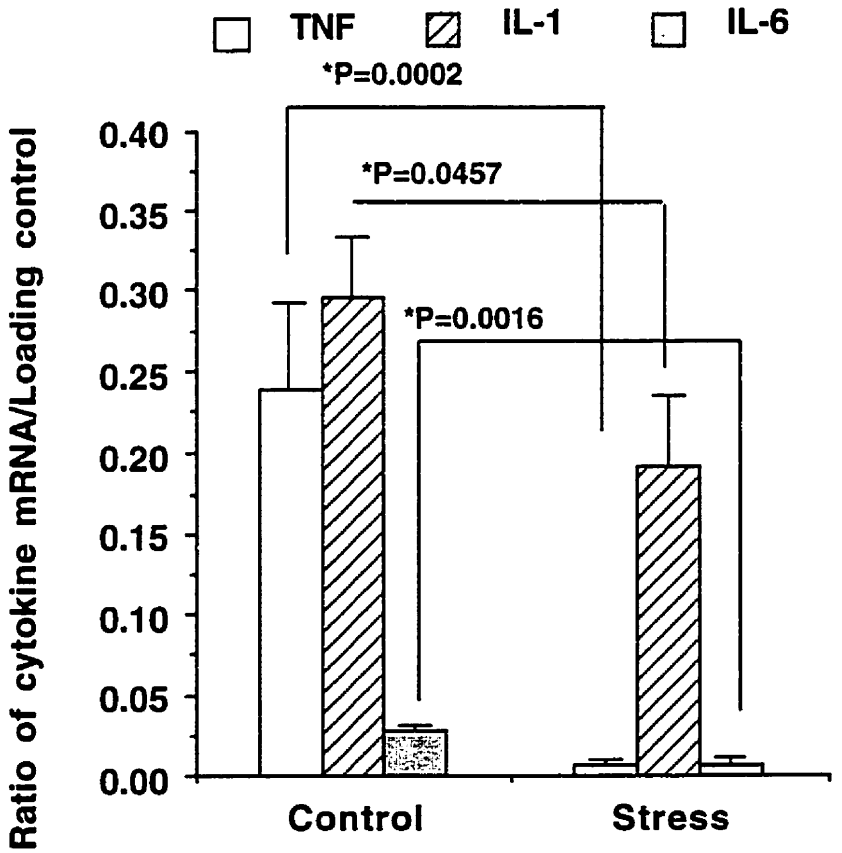


Figure 5-4: The effect of 15 minutes of intermittent footshock stress immediately following 0.1μg of i.v. LPS on splenic cytokine protein levels. Rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. Cytokine protein levels were measured by ELISA and expressed as pg/mg of splenic protein. TNF [T(13)=3.45, p=0.0021, n=15], IL-1 [T(13)=0.58, p=0.28ns, n=15] and IL-6 [T(11)=3.56, p=0.0022, n=13] protein levels were compared by one-tailed T-test and the error bars represent +/-1 standard error of the mean.

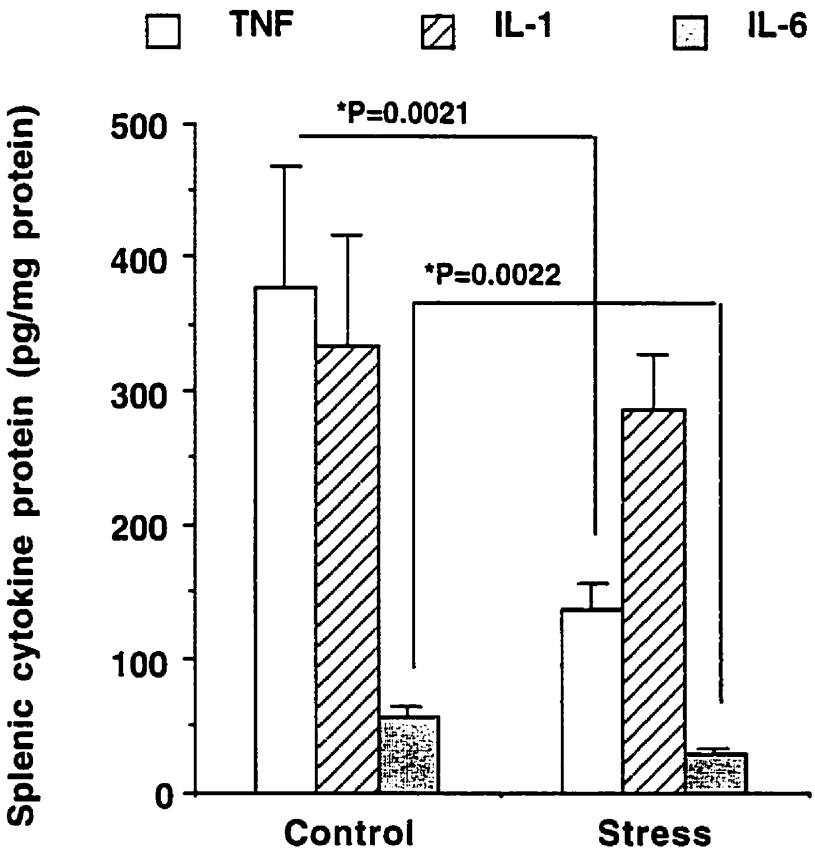


Figure 5-5: The effect of 15 minutes of intermittent footshock stress immediately following 0.1μg of i.v. LPS on TNF immunostaining. Rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. Splenic TNF levels [T(18)=3.422, p=0.003, n=20] were analyzed by immunohistochemistry and expressed as a percent of positive staining per spleen section. The groups were compared by one-tailed T-test and the error bars represent +/-1 standard error of the mean.

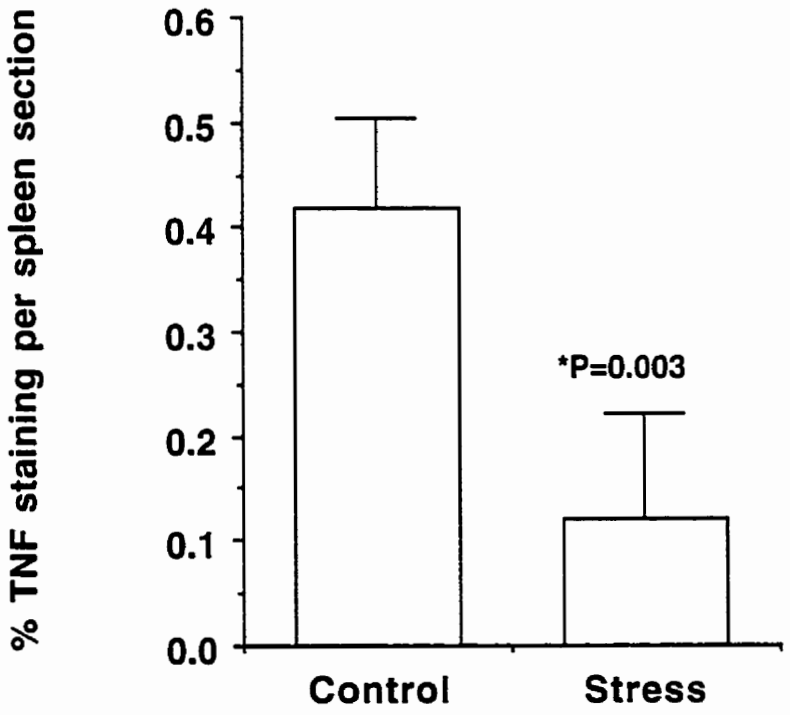


Figure 5-6: The effect of 15 minutes of intermittent footshock stress immediately following 0.1μg of i.v. LPS on ED-1 immunostaining. Rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. Splenic ED-1 levels [T(18)=0.066, p=0.47ns, n=20] were analyzed by immunohistochemistry and expressed as percent of positive staining per spleen section. The groups were compared by one-tailed T-test and the error bars represent +/-1 standard error of the mean.

Figure 5-7: The effect of 15 minutes of intermittent footshock stress immediately following 0.1μg of i.v. LPS on plasma corticosterone levels. Rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. Plasma corticosterone levels [T(18)=2.66, p=0.016, n=20] were analyzed by RIA and expressed as ng/ml. The groups were compared by two-tailed T-test and the error bars represent +/-1 standard error of the mean.

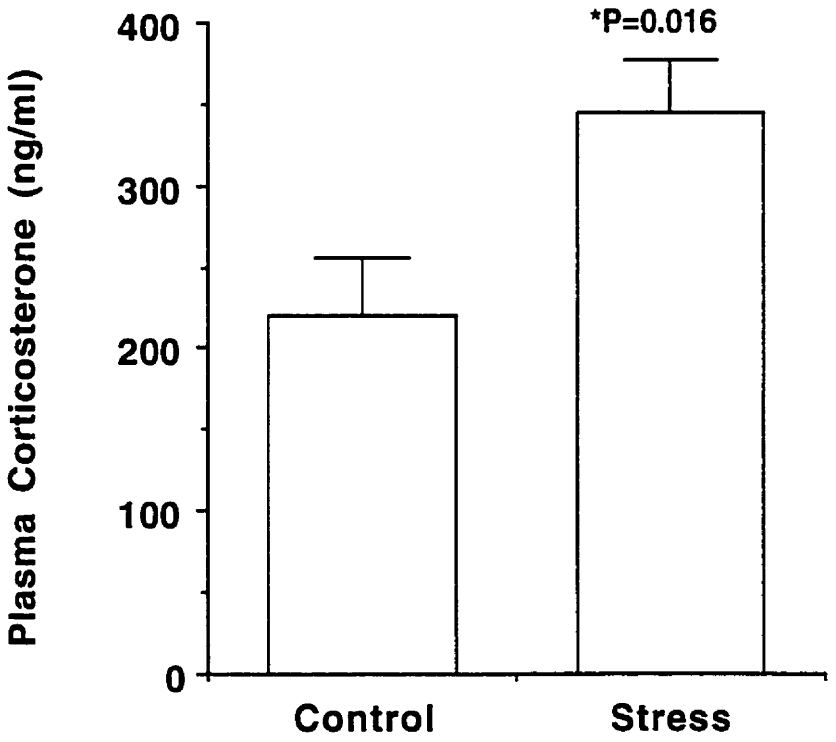


Figure 5-8: The effect of splenic nerve cut on splenic cytokine mRNA in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and splenic nerve cut rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. Cytokine mRNA levels were analyzed by Northern blotting and expressed as a ratio relative to the loading control. TNF [T(18)=0.17, p=043ns, n=20] and IL-1 [T(18)=0.3, p=0.76ns, n=20] mRNA levels were compared by two-tailed T-test and the error bars represent +/-1 standard error of the mean.

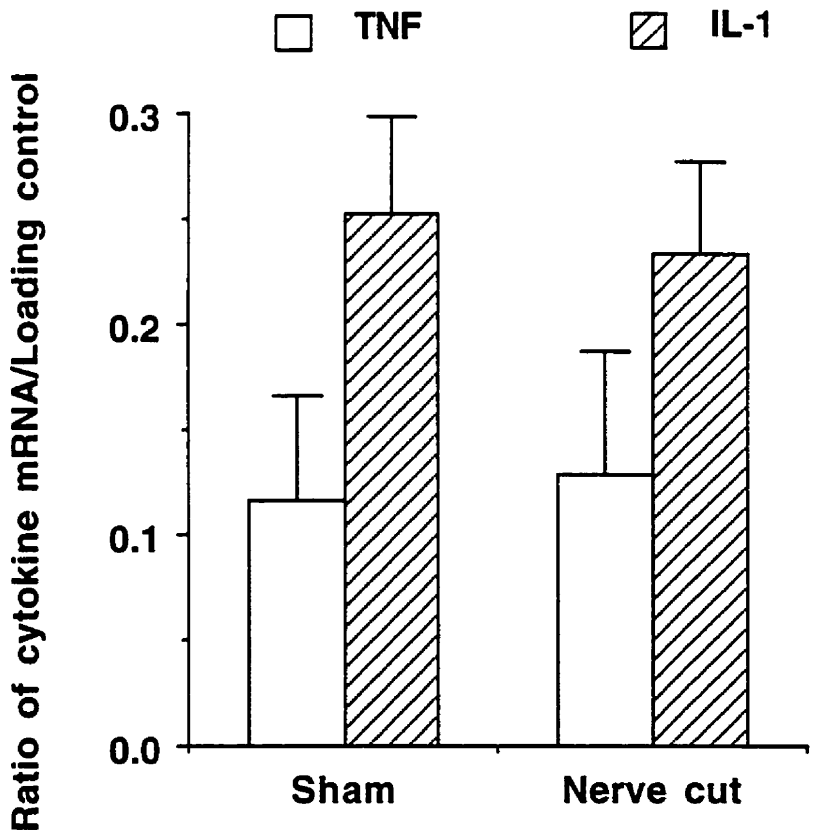


Figure 5-9: The effect of splenic nerve cut on splenic cytokine protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and splenic nerve cut rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. Cytokine protein levels were analyzed by ELISA and expressed as pg/mg of splenic protein. TNF [T(13)=0.51, p=0.31ns, n=15], IL-1 [T(13)=1.17, p=0.13, n=15] and IL-6 [T(13)=0.121, p=0.45ns, n=15] mRNA levels were compared by a two-tailed T-test and the error bars represent +/-1 standard error of the mean.

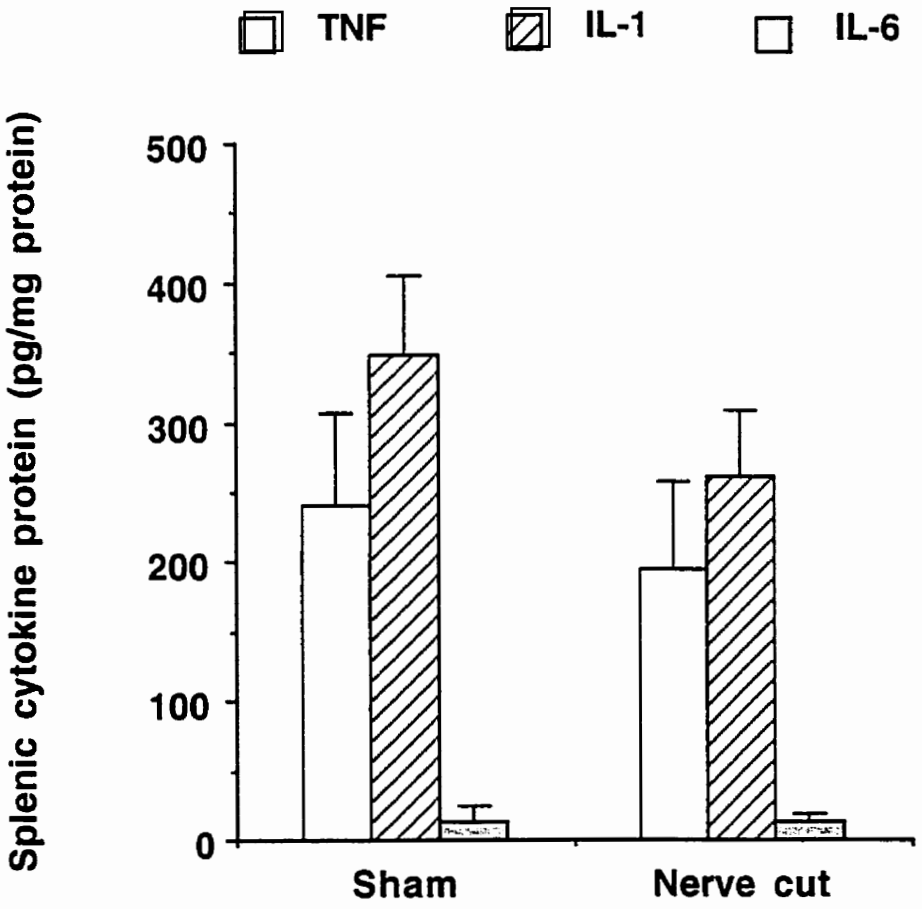
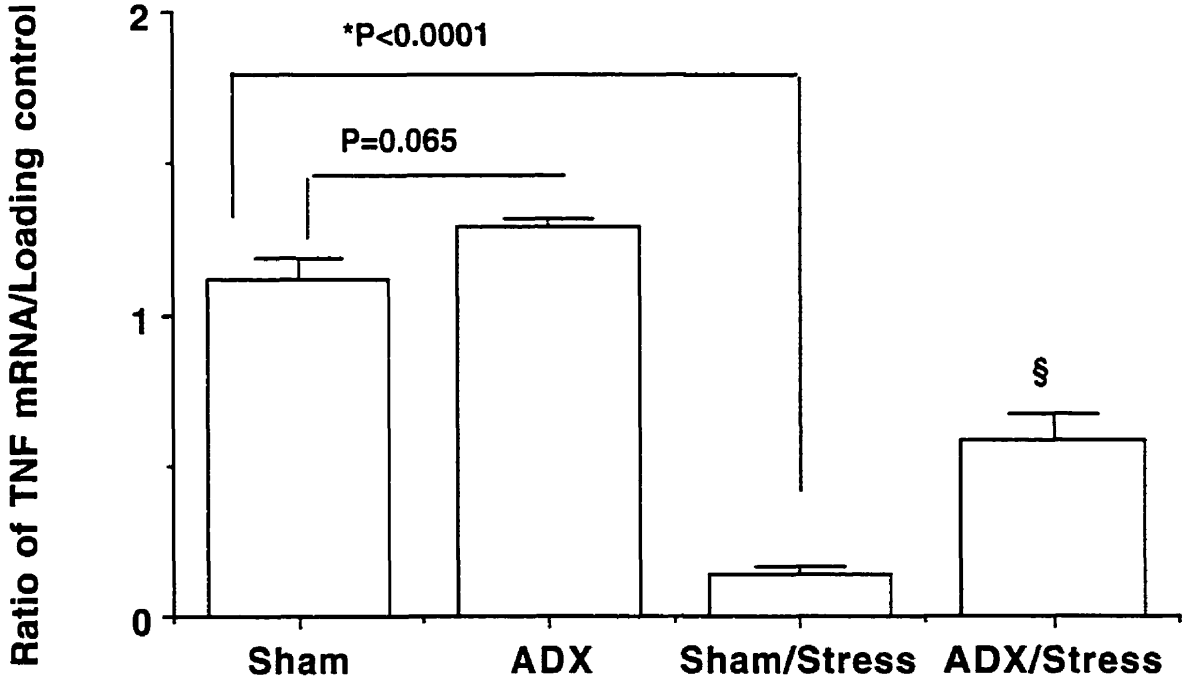


Figure 5-10: The effect of ADX on splenic TNF mRNA in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and ADX rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress (or left in their homecages for 15 minutes) and killed 45 minutes later. TNF mRNA levels [$F(3,24)=74.87$, $p<0.0001$, $n=28$] were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.



§ This group is significant (P<0.0001) from every other group.

Figure 5-11: The effect of ADX on splenic TNF protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and ADX rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress (or left in their homecages for 15 minutes) and killed 45 minutes later. TNF protein levels [$F(3,24)=28.20$, $p<0.0001$, $n=28$] were analyzed by ELISA and expressed as pg/mg of splenic protein. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

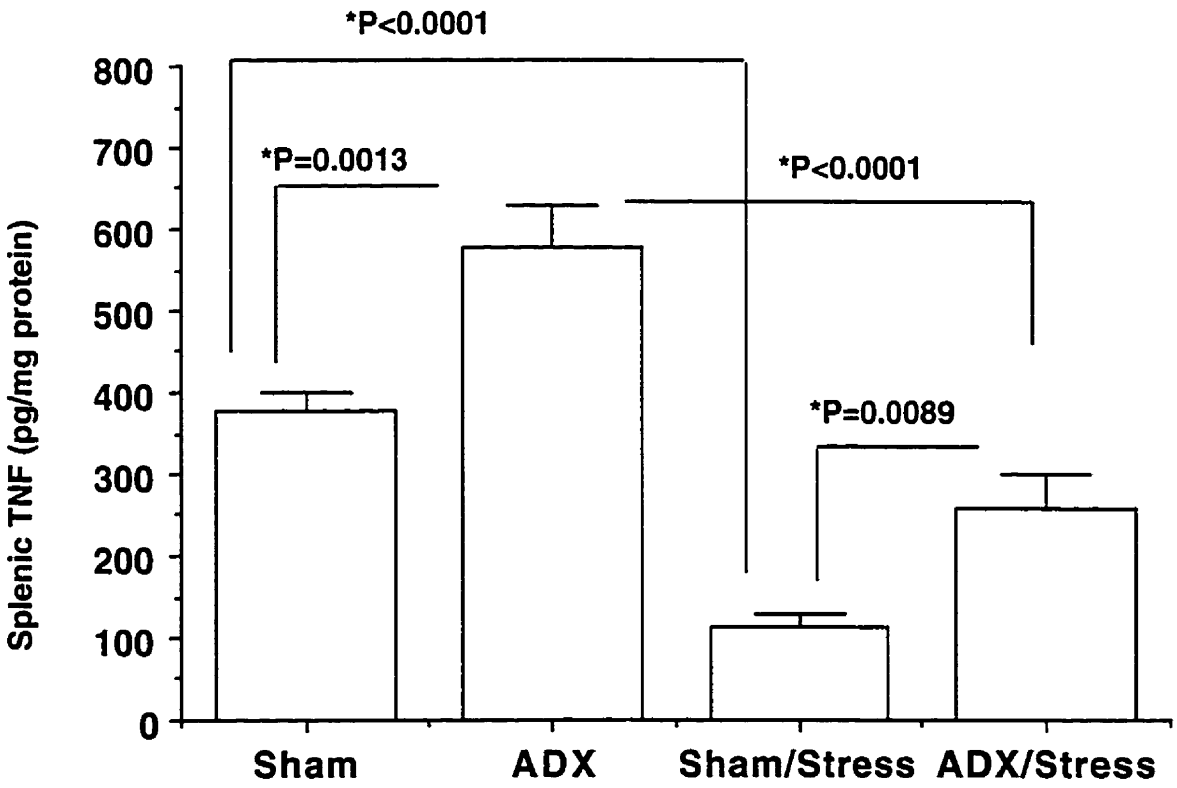


Figure 5-12: The effect of ADX on plasma TNF protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and ADX rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress (or left in their homecages for 15 minutes) and killed 45 minutes later. TNF plasma levels [F(3,24)=9.21, p=0.0003, n=28] were analyzed by ELISA and expressed as ng/ml. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

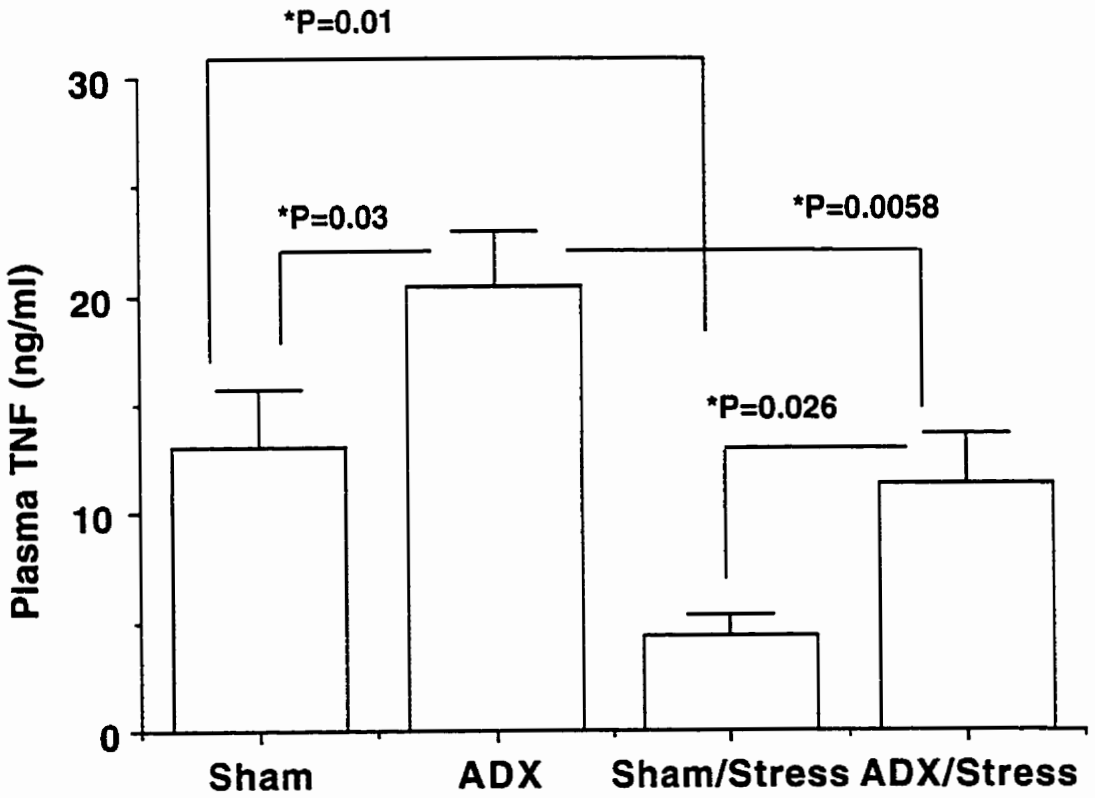


Figure 5-13: The effect of ADX on splenic IL-1 mRNA in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and ADX rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress (or left in their homecages for 15 minutes) and killed 45 minutes later. IL-1 mRNA levels [F(3,24)=4.19, p=0.016, n=28] were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

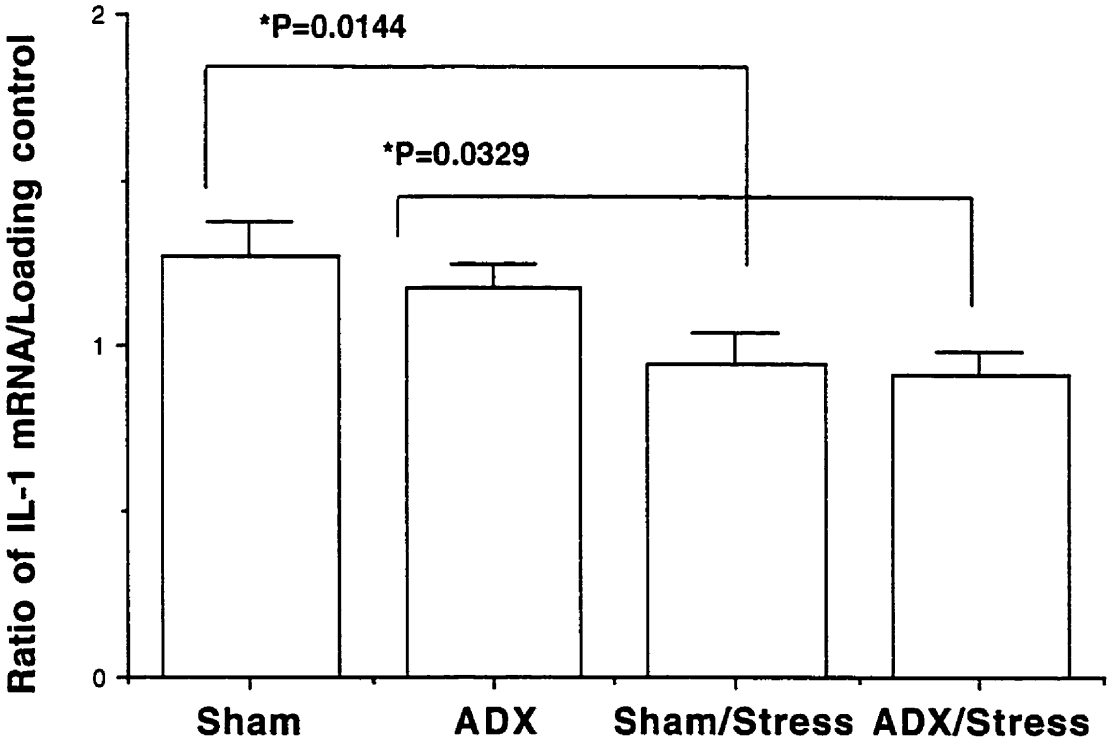


Figure 5-14: The effect of ADX on splenic IL-1 protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and ADX rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress (or left in their homecages for 15 minutes) and killed 45 minutes later. IL-1 protein levels [F(3,24)=5.89, p=0.0036, n=28] were analyzed by ELISA and expressed as pg/mg of splenic protein. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

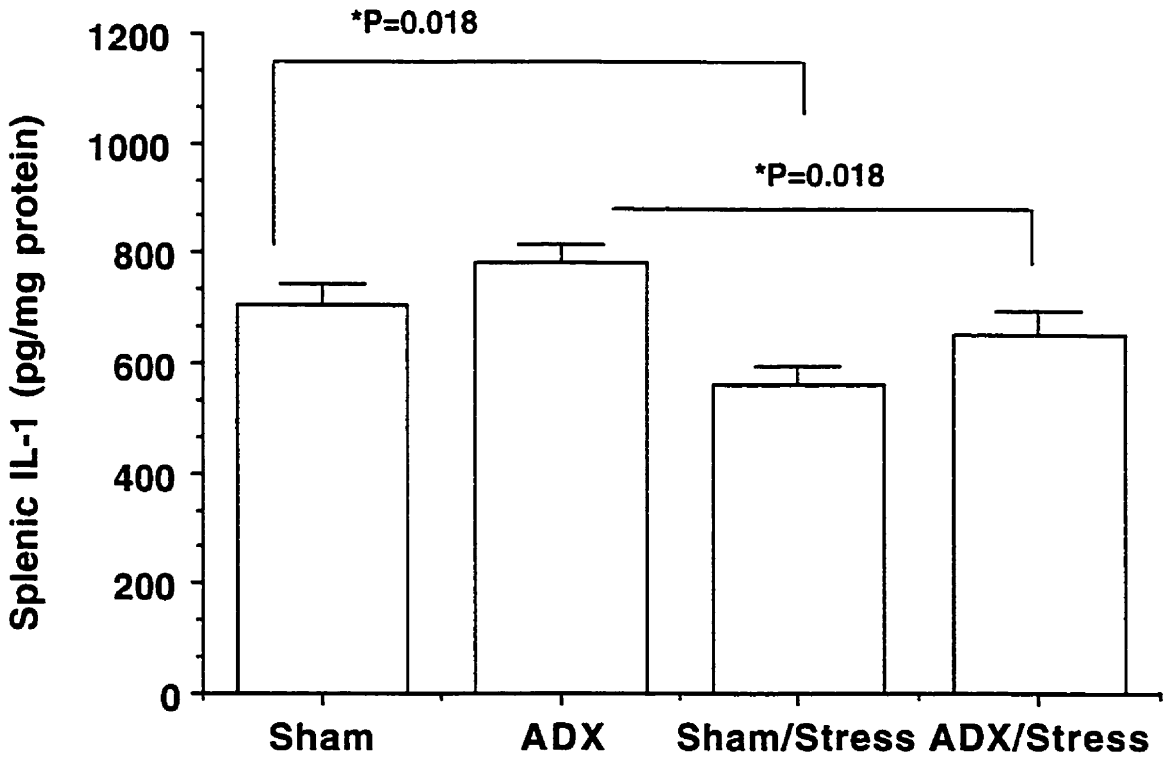


Figure 5-15: The effect of ADX on plasma IL-1 protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and ADX rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress (or left in their homecages for 15 minutes) and killed 45 minutes later. Plasma IL-1 levels [F(3,15)=1.85, p=0.19ns, n=19] were analyzed by ELISA and expressed as ng/ml. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

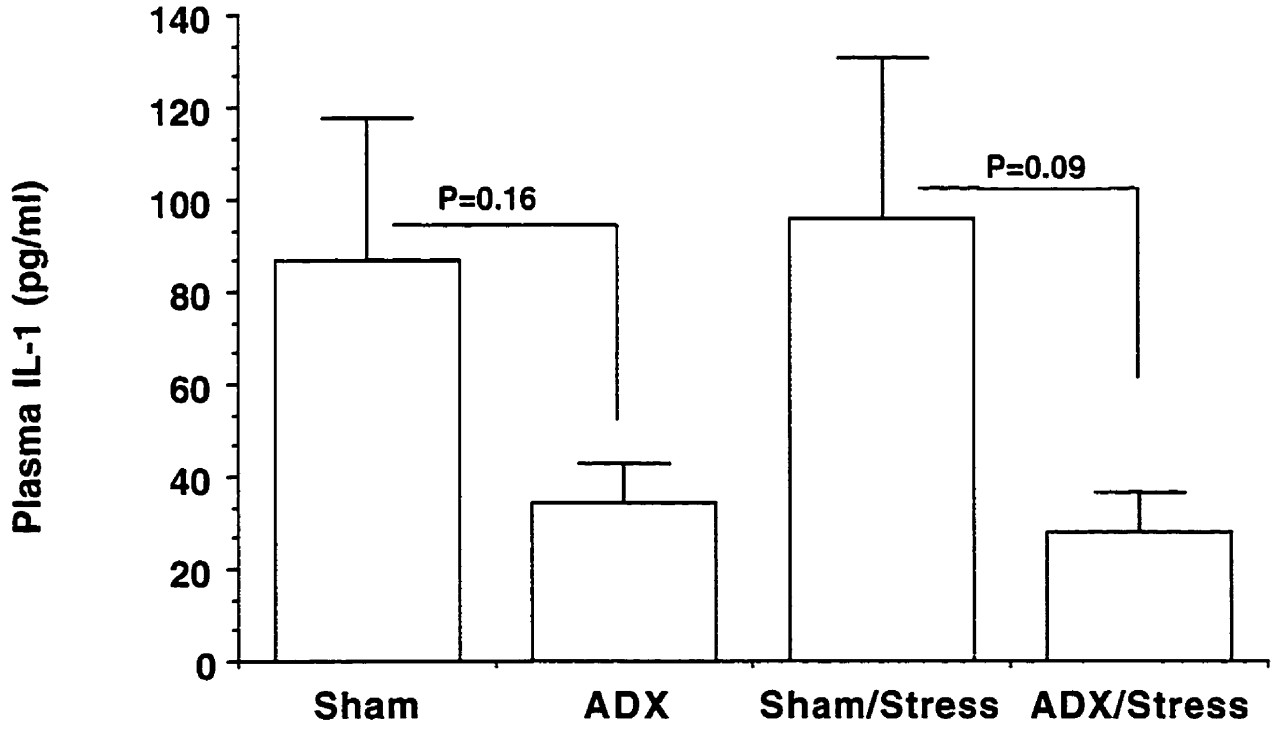


Figure 5-16: The effect of ADX on splenic IL-6 mRNA levels in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and ADX rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress (or left in their homecages for 15 minutes) and killed 45 minutes later. Splenic IL-6 mRNA levels [$F(3,24)=11.50$, $p<0.0001$, $n=28$] were analyzed by Northern blotting and expressed as ratio relative to the loading control. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

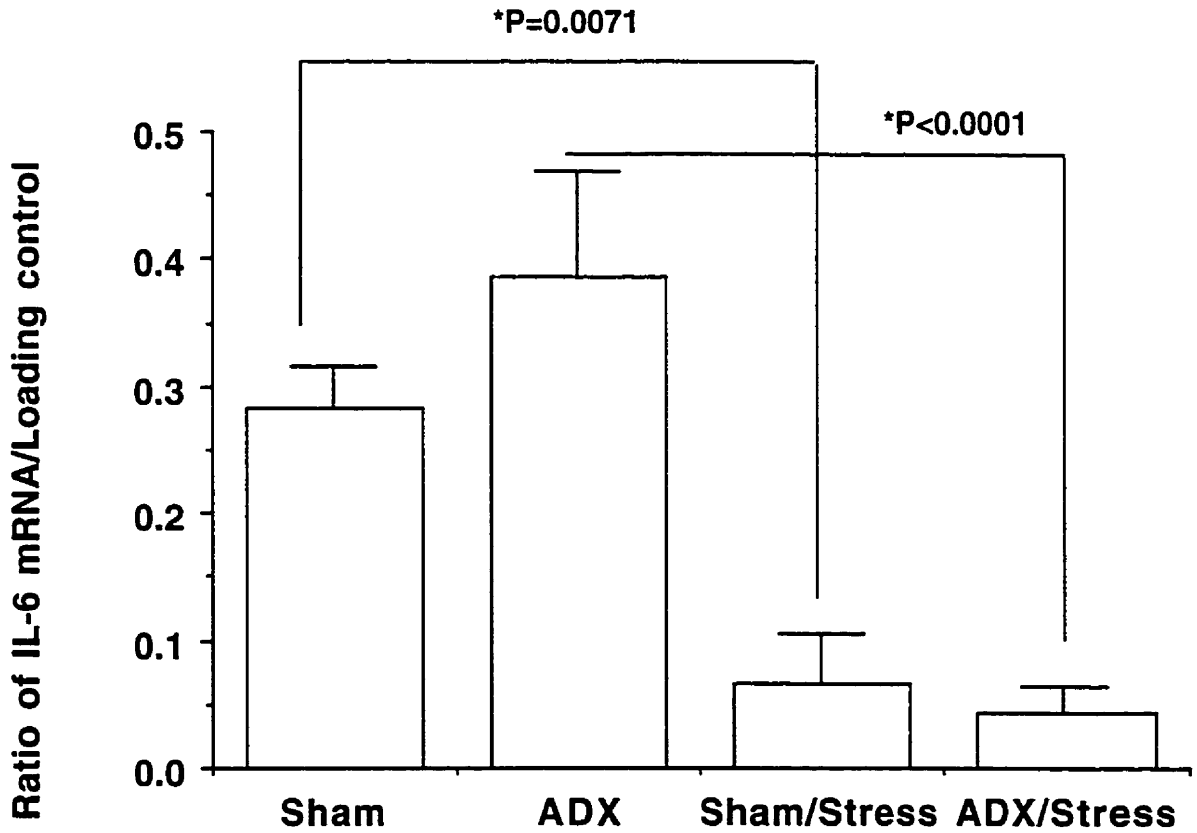


Figure 5-17: The effect of ADX on splenic IL-6 protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and ADX rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress (or left in their homecages for 15 minutes) and killed 45 minutes later. IL-6 protein levels [$F(3,24)=13.8$, $p<0.0001$, $n=28$] were analyzed by ELISA and expressed as pg/mg of splenic protein. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

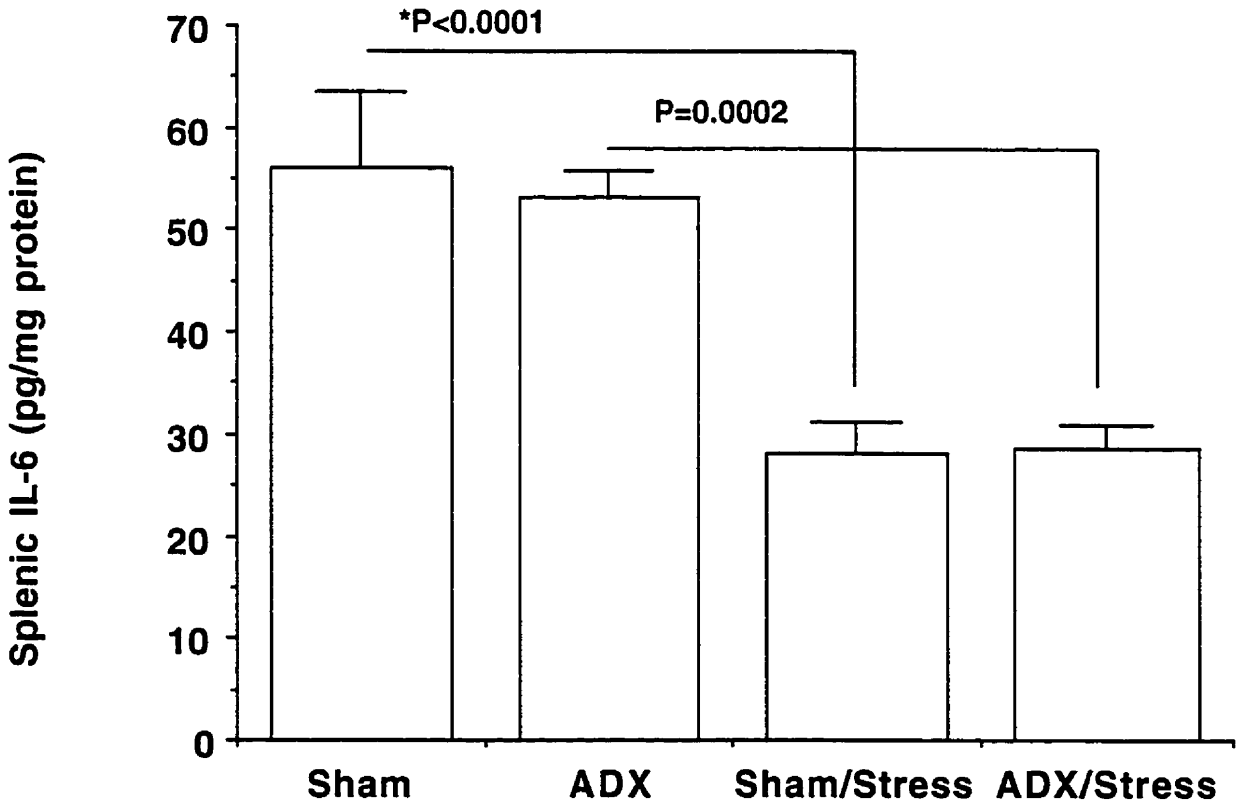


Figure 5-18: The effect of ADX on plasma IL-6 protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. Sham operated and ADX rats were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress (or left in their homecages for 15 minutes) and killed 45 minutes later. IL-6 plasma levels [F(3,24)=0.2, p=0.89ns, n=28] were analyzed by ELISA and expressed as pg/ml. The groups were compared by ANOVA and the error bars represent +/-1 standard error of the mean.

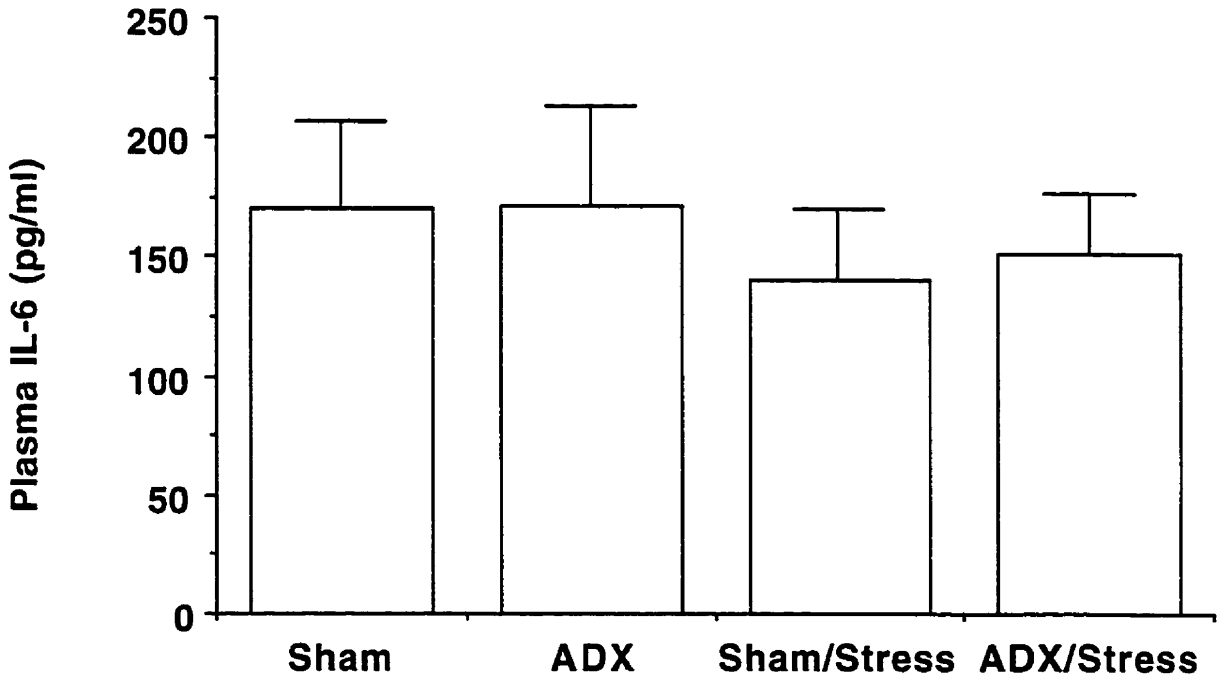


Figure 5-19: The effect of ADX and splenic nerve cut on splenic TNF mRNA in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. ADX rats with sham surgeries or splenic nerve cuts were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. TNF mRNA levels [T(17)=3.74, p=0.0008, n=19] were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The groups were compared by one-tailed T-test and the error bars represent +/-1 standard error of the mean.

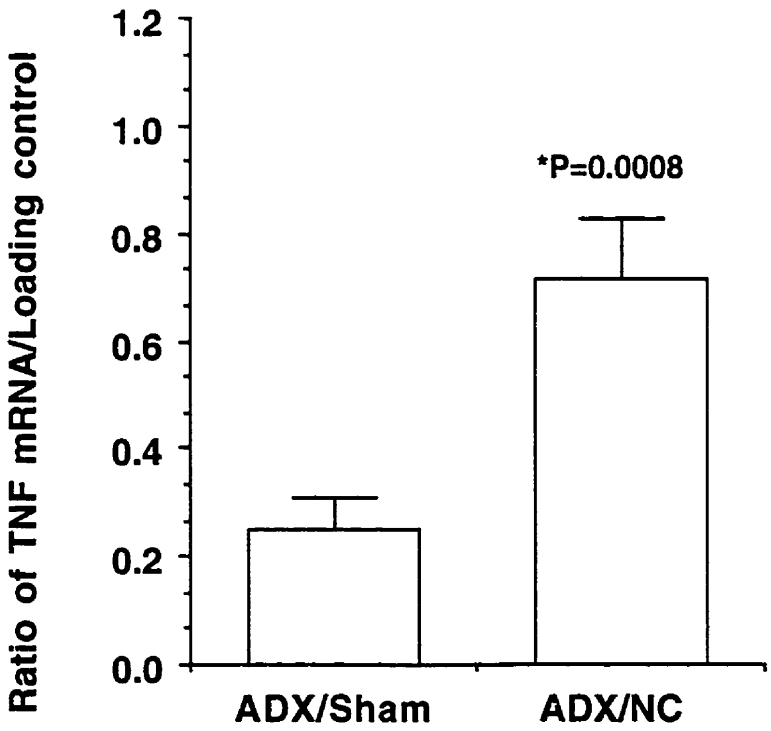


Figure 5-20: The effect of ADX and splenic nerve cut on splenic TNF protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. ADX rats with sham surgeries or splenic nerve cuts were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. TNF protein levels [T(17)=4.26, p=0.0003, n=19] were analyzed by ELISA and expressed as pg/mg of splenic protein. The groups were compared by one-tailed T-test and the error bars represent +/-1 standard error of the mean.

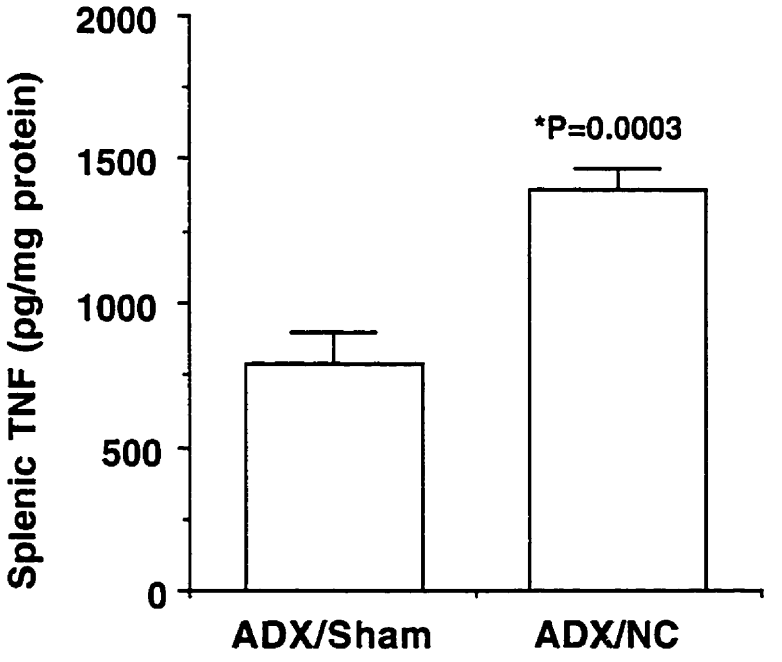


Figure 5-21: The effect of ADX and splenic nerve cut on splenic IL-1 mRNA in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. ADX rats with sham surgeries or splenic nerve cuts were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. IL-1 mRNA [T(17)=2.58, p=0.0098, n=19] levels were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The groups were compared by one-tailed T-test and the error bars represent +/-1 standard error of the mean.

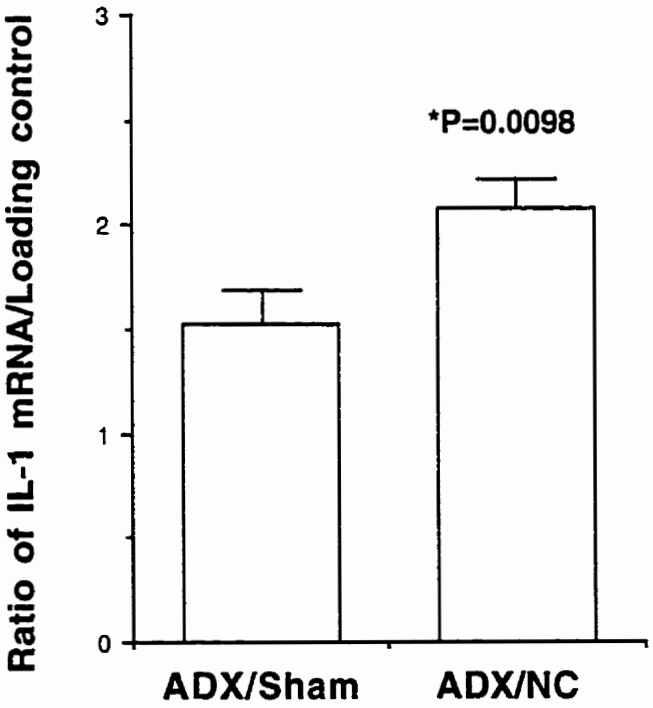


Figure 5-22: The effect of ADX and splenic nerve cut on splenic IL-1 protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. ADX rats with sham surgeries or splenic nerve cuts were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. IL-1 protein levels [T(17)=1.94, p=0.035, n=19] were analyzed by ELISA and expressed as pg/mg of splenic protein. The groups were compared by one-tailed T-test and the error bars represent +/-1 standard error of the mean.

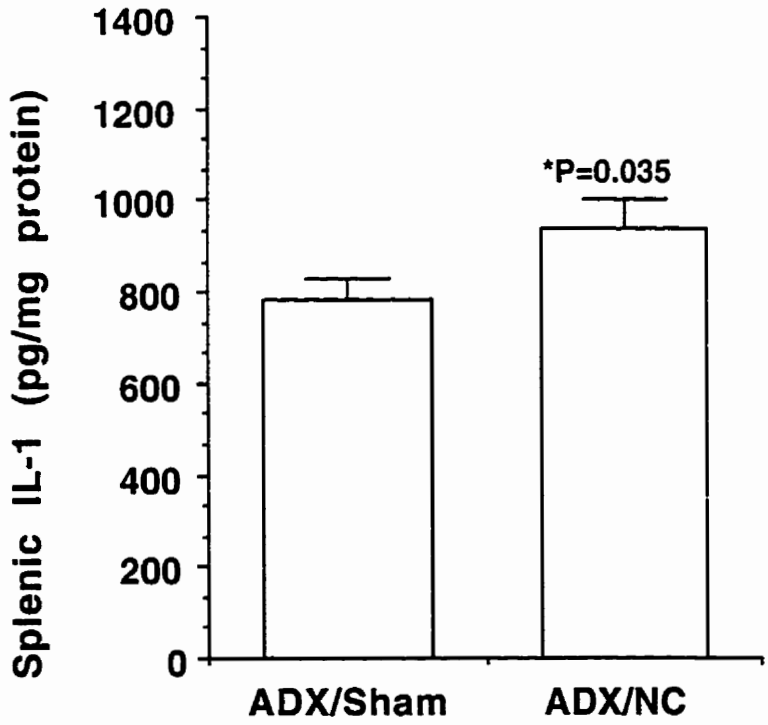


Figure 5-23: The effect of ADX and splenic nerve cut on splenic IL-6 mRNA in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. ADX rats with sham surgeries or splenic nerve cuts were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. IL-6 mRNA levels [T(17)=0.15, p=0.44ns, n=19] were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The groups were compared by one-tailed T-test and the error bars represent +/-1 standard error of the mean.

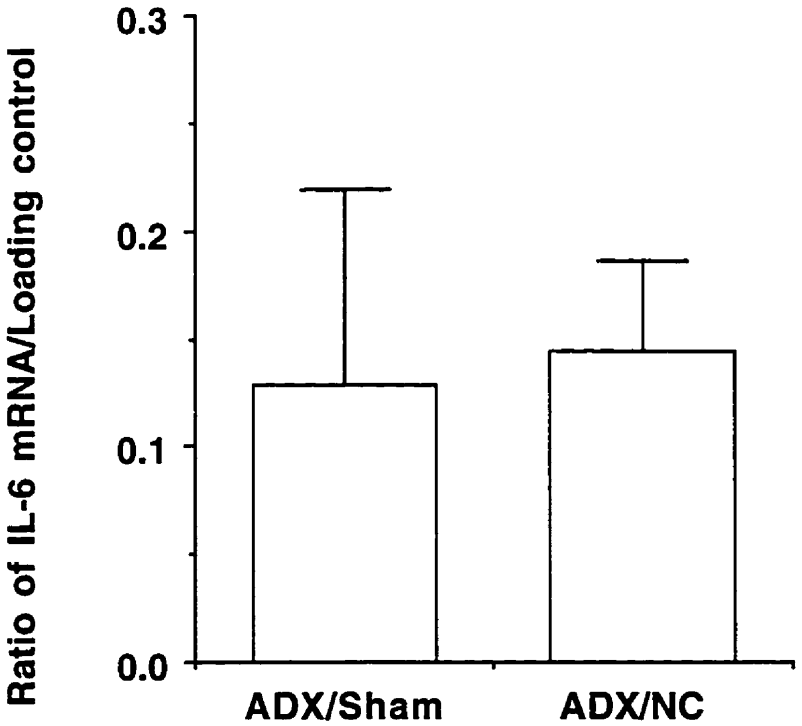


Figure 5-24: The effect of ADX and splenic nerve cut on splenic IL-6 protein in animals injected with 0.1μg of i.v. LPS and subsequently exposed to stress. ADX rats with sham surgeries or splenic nerve cuts were injected with 0.1μg of LPS, immediately exposed to 15 minutes of 1.6 mA intermittent footshock stress and killed 45 minutes after the completion of stress. IL-6 protein levels [T(17)=0.31, p=038ns, n=19] were analyzed by ELISA and expressed as pg/mg of splenic protein. The groups were compared by one-tailed T-test and the error bars represent +/-1 standard error of the mean.

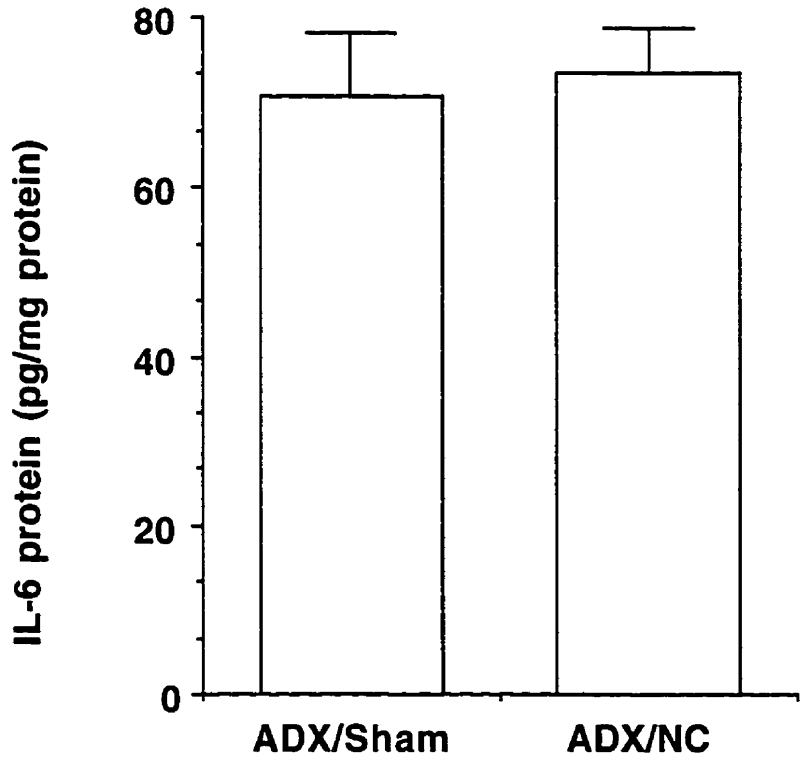
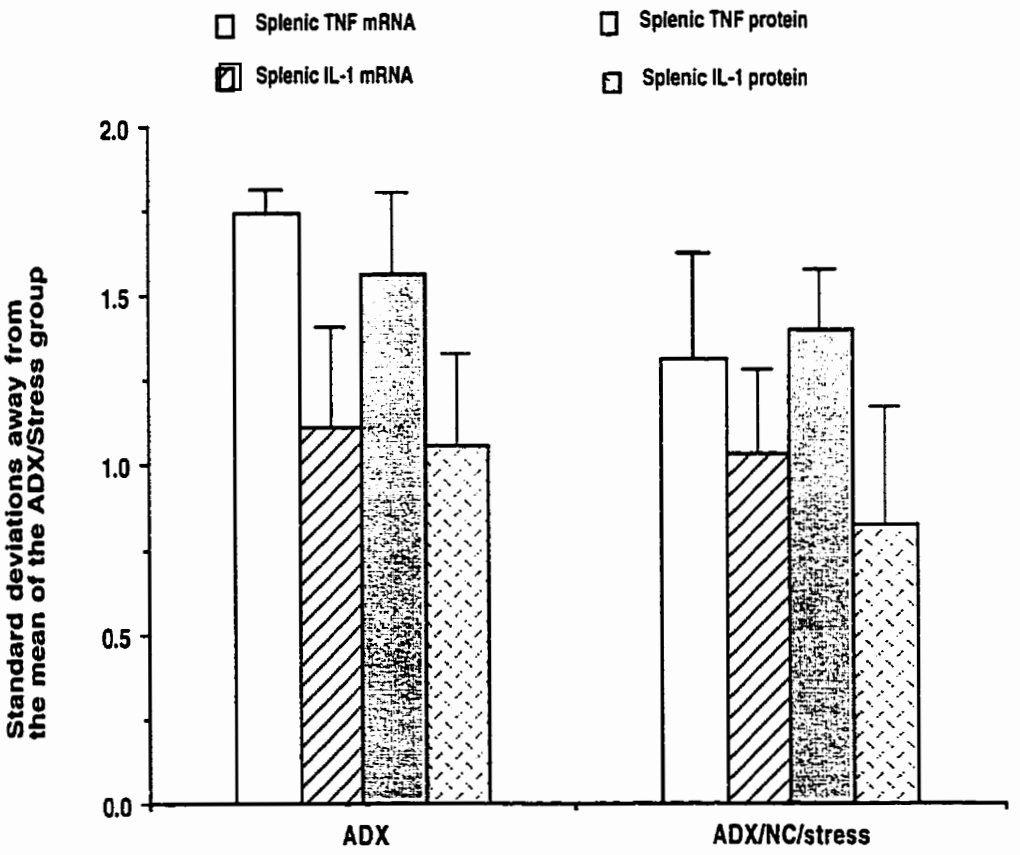


Figure 5-25: Comparison of splenic TNF and IL-1 mRNA and protein levels from the ADX/stress and ADX/nerve cut/stress experiments. The ratio of mRNA/Loading control and the level of protein for TNF and IL-1 for each animal from both the ADX (non stress) group and the ADX/nerve cut (NC)/stress group were normalized according to the ADX/stress group from their respective experiment. The data from individual animals were expressed as a difference from the corresponding ADX/stress group mean in terms of standard deviations (similar to a Z score). The groups were compared by a two-tailed T-test and the error bars represent +/-1 standard error of the mean [n=16].



5.8. Discussion

5.8.1. The effects of stress on LPS-induced splenic cytokine production when 0.1 μg of i.v LPS precedes the exposure to stress.

We found that LPS-induced levels of splenic TNF, IL-1 and IL-6 mRNA were decreased in animals that received footshock stress. Our results were consistent with other reports which suggested that the most likely reason for this suppression was the rapid elevation of both plasma corticosterone [15,790] and catecholamines [802,803,839,964] immediately following stress. Although LPS can also induce high levels of both catecholamines and corticosterone, the release of these molecules in response to LPS appears concurrently or subsequent to cytokine production. In contrast, these molecules were released prior to cytokine induction in our stress paradigm, and thus may be more effective at inhibiting splenic cytokine synthesis than they were in response to LPS alone.

In general, our results agreed with Goujon et al., in that stress reduced LPS-induced splenic cytokine production [867]. Although TNF and IL-1 mRNA were reduced, the magnitude for TNF reduction was more pronounced than IL-1. This is consistent with observations in experiment 2 of this thesis (with respect to the ADX effect) and studies by Pan et al., [717] (demonstrating the suppressive effect of i.c.v. PGE2 on splenic cytokines) where the experimental effect on IL-1 was reduced or non-existent as compared to the effect on TNF. These observations have also been made in

vitro [179,181] suggesting that although TNF, IL-1 and IL-6 are produced in series, regulatory influences affect these cytokines differentially. This is supported by the observations that splenic cytokine mRNA and protein for the same cytokine can be differentially regulated. A prominent example in the present study is that splenic IL-1 protein levels were not affected by stress as consistently as splenic IL-1 mRNA levels. Interestingly, this was also observed by Goujon et al., [867]. The reasons for this effect are unknown; however, it could be due to post-transcriptional processing of IL-1. Since we did not observe an increase in plasma levels of IL-1 in response to stress, it is unlikely that stress is differentially altering cytokine production in different organs as suggested by Takaki et al., for IL-6 [970]. It is known that cytokines like TNF and IL-1 have many points at which regulation can occur (post-transcriptional, post-translation etc. [551,583]). The biological significance of having different mRNA levels but not protein levels is unknown. However it is also possible that earlier in the time course, mRNA levels were the same and the protein levels measured at 1 hr are a reflection to an earlier time point. Thus in this paradigm where LPS precedes stress, it is possible that LPS activates the intracellular machinery prior to stress, and that when the stress activated signals reach the cell, cytokine production is rapidly turned off. This however does not explain similar results in the following sections where the paradigm is reversed and the stress-induced signals reach the cells prior to LPS.

It was interesting that despite the many reports to the contrary [204-207] , IL-6 was decreased in response to stress. The reasons for the difference between our results

and the other reports could be due to the presence of LPS as suggested by Straub et al., [210]. However further in vivo verification of this is warranted.

In order to explain how stress reduced splenic TNF and IL-1 mRNA and TNF and IL-6 protein it was important to determine if stress altered the splenic cell population or affected the cells' cytokine production mechanisms. It is known that stress [357] (via HPAA [853] and SNS [167-170,172]) and LPS [971] can cause changes in cell populations and migration rates. Although immunohistochemical studies suggested there was less staining for TNF in stressed rats as opposed to control rats, the ED-1 staining between stressed and non-stressed groups was comparable. Therefore the effects of stress are exerted at the cellular level and not due to a stress-induced cell redistribution at 1 hr post 0.1 μg of LPS. The image analysis program (NIH image) was set manually to recognize all staining above background. Therefore there were significantly fewer cells positive for TNF as no lightly stained cells were detected upon visual or densitometric inspection. Thus TNF staining as determined by immunohistochemistry and image analysis seemed to be an all or none phenomenon at the cellular level. It is unknown if the increased heart rate and blood pressure in stressed animals could be partly responsible for the stress-induced immune suppression as it could potentially increase the clearance rate of LPS. However it is important to remember that the stress-activated mediators such as glucocorticoids [327] and catecholamines [192] will reduce LPS-induced cytokine production in vitro. Not shown was that T-cell and B-cell populations were also unaffected by stress. However these results are preliminary in that pan-cell markers were

used and did not address the migration of any particular subsets of cells. As well, these data were not verified by flow cytometry.

5.8.2. The role of the adrenal gland and splenic nerve in regulating the stress-induced immunosuppression of splenic cytokine production.

In experiment 2 it was found that neither the adrenal gland nor splenic nerve had a major role in regulating LPS-induced splenic cytokine levels (although trends were observed with respect to ADX). However it was unknown whether the SNS and HPAA, alone or together, could potentially be responsible for stress-induced suppression of splenic cytokine production. The hypothesis that the splenic nerve is primarily responsible for this effect is suggested by the fact that both adrenal-independent [56,58,859] and 6OHDA-sensitive [863,969] mechanisms are known to mediate immune suppression. Most importantly, Wan et al., demonstrated that surgically cutting the splenic nerve abrogated the immunosuppressive effects of stress on the response of splenocytes to mitogens and sheep red blood cells [860].

Similar to the LPS studies, it was found that cutting the splenic nerve in an adrenal-intact animal did not abrogate the immunosuppressive effects of stress on LPS-induced cytokine production. This was consistent for both mRNA and protein for all cytokines tested. This was unexpected since with this paradigm, like the studies of Brown et al. [89], Pan et al., [717] and Wan et al. [860] (and unlike the paradigm in experiment 2), we activated the central systems without inducing maximal peripheral

cytokine production. Interestingly, the main difference between the experiments in this thesis and the earlier experiments by Brown et al. [89], and Wan et al. [860] was that their immune parameters were assayed in vitro. Although the studies by Pan et al.[717] were in vivo, they could only demonstrate a partial dis-inhibition of LPS-induced splenic cytokine production (in animals that received i.c.v. PGE2) upon cutting the splenic nerve. This was despite the robust effect of i.c.v. PGE2 on splenic cytokine production and the fact that PGE2 will increase splenic nerve activity [76,645]. The current studies are directly supported by other in vitro studies that suggest an inhibitory role for catecholamines on cytokine production [192]. Interestingly, preliminary results from our lab indicate that similar to in vitro pharmacological studies, cultured macrophages from splenic nerve cut animals produce more TNF and IL-1 than macrophagse from sham operated animals [890]. Therefore cutting the splenic nerve must have a very robust effect on the macrophage in order for the effect to be maintained in vitro. Even more impressive is the fact that the in vivo microenvironment seems to negate these seemingly robust alterations. Similar differences between in vivo and in vitro assays in neural immune studies were observed by Green-Johnson et al.[266,267], and Zhou et al., [204] in their respective paradigms. It is therefore still unclear as to when the in vitro potential of the SNS and HPAA to regulate LPS-induced cytokine production occurs in vivo.

Using similar rationale to experiment 2, it was postulated that the potential for splenic catecholamines to regulate cytokine production may not be evident in adrenal intact animals. This may be due to the fact that during stress there is such an

overwhelming amount of NE and E produced from the adrenal glands [143] that elimination of the splenic nerve alone may not be a major factor in the terms of the stress-induced regulation of splenic immunity. Similarly, it is also possible that since the SNS and HPAA are functionally interconnected [366,367], compensation may occur whereby a reduction of NE due to partial sympathectomy could cause an increase in catecholamine release from the adrenal glands. In support of this idea is the report that the adrenal gland can increase catecholamine production in response to sympathectomy [372]. However it is unknown if the adrenal glands would compensate if just one single organ (ie. the spleen) was denervated. Interestingly, the opposite may also be true as both Besedovsky [48] and Brown [89] saw increased effects of splenic nerve on splenic immunity in ADX rats in their respective paradigms. This is consistent with the observed increase in NE turnover in ADX rats upon exposure to various forms of stress [366,371,372] . Therefore in the absence of adrenals, the SNS may have a more prominent role in regulating the adaptive response to stress. Therefore the nerve cut effect should be more prominent in ADX animals. Prior to directly testing the effect of the nerve in ADX rats, we first tested the effect of ADX on stress levels of splenic cytokines to determine if the adrenal glands were either partially or totally responsible for all the immunosuppressive effects of stress in our system.

We used a 2X2 ANOVA design to investigate the effects of stress and ADX and the combination thereof on splenic cytokine production. Firstly, main effects for both stress and ADX were observed for TNF mRNA and protein production. The TNF

mRNA levels for the sham (non-stress) and ADX (non-stress) groups were comparable, while the ADX/stress group was significantly higher than the sham/stress group and significantly lower than ADX group. Thus it seemed that although stress-induced immune suppression was evident in ADX rats, the magnitude of suppression was significantly less than that observed for stressed sham rats. In terms of splenic TNF protein levels, the ADX effect was more prominent than it was for the mRNA and the magnitude of the stress-induced suppression of splenic cytokine levels was the same for sham and ADX rats. These effects were also evident for plasma TNF suggesting this phenomenon was consistent systemically. Thus, in agreement with Keller et al.[56,58], we found that immune function in ADX rats can still be suppressed by stress to the same magnitude as in adrenal-intact animals.

The results for IL-1 were similar to TNF, but less dramatic. For IL-1 mRNA there was a significant main effect of stress but not ADX, suggesting that ADX did not alter splenic IL-1 levels, but splenic IL-1 mRNA in ADX rats were still reduced in response to stress. However there was significant main effects for both stress and ADX with respect to splenic IL-1 protein. This is consistent with reports that glucocorticoids are able to regulate cytokines at a post-transcriptional level, via an increased stability of the mRNA that would result in more protein being produced [327,329,330]. It is unknown why the effects of stress on splenic IL-1 protein were observed in this experiment but not in the others. However the relatively small magnitude of this effect suggests that these changes may not have been detected in the previous experiments due to the fact that the

suppression of IL-1 protein in response to stress is not robust enough to be consistently demonstrated. Interestingly, plasma IL-1 was not affected by stress despite the fact that splenic IL-1 protein production was suppressed by stress. It is possible that the discrepancy Takaki et al. observed for IL-6 is true for IL-1 [970] in that other anatomical locations increased or maintained IL-1 production in response to stress despite the fact that splenic IL-1 was decreased. This would confirm studies by MacNeil et al. [76], Pettipher et al. [972] and Cunnick et al., [859] that demonstrate the functional specificity of neuroimmune regulation based on the anatomical location of the immune cells. It is also unknown why plasma IL-1 levels were detected in this experiment but not in any of the other experiments. However it is possible that since the levels of plasma IL-1 approached the lower detection limit of our assay (with many animals below this threshold), that minor variability in the experimental groups or with the ELISA kit could have precluded the detection of plasma IL-1 in the other experiments. Interestingly, it was also found that ADX rats in this experiment had reduced plasma IL-1 levels as compared to sham controls. This was surprising as both glucocorticoids and catecholamines have been shown to inhibit IL-1 production. The reasons for this ADX effect are unknown; however, this effect could be due to baseline levels of IL-1 being dependent on catecholamines. This is suggested by the fact that catecholamines can increase cytokines like IL-1 in response to certain stressors such as hemorrhage [906,973].

Although splenic IL-6 levels were low, it was clear that there was a stress effect for the mRNA, but no ADX effect in contrast to other reports [336]. Opposite to the

pattern displayed by TNF, the effects of stress were less prominent in the protein measures as opposed to the mRNA measures and non-existent in the plasma measures. The reasons for this are unknown but could be due to the fact IL-6 production from other parts of the body can be increased in spite of a decrease in splenic IL-6 as suggested by the results of Takaki et al [970]. This possibility is also supported by the fact that Straub et al., found that NE will increase IL-6 production if the cells are not concurrently exposed to bacterial products [210]. Therefore although NE transmission is systemic [1], the spleen is likely to be exposed to i.v. LPS faster and in larger quantities than most other parts of the body [913]. Therefore it is possible that the NE/LPS combination in the spleen does not occur in the same temporal pattern in all parts of the body allowing for other organs to increase IL-6 production despite decreased levels of splenic IL-6.

It is unclear why an ADX effect was present in this experiment as the effects of ADX on LPS-induced splenic cytokine production in previous experiments were only suggestive and not statistically significant. It is possible that the present dose of LPS (0.1 μg) induced an increased turnover rate in corticosterone and/or catecholamines in the adrenal gland that was not engaged in response to the 0.01μg dose of LPS from the last experiment. Thus, the trends observed for increased cytokines in ADX rats in response to the 0.01μg dose of LPS in the last experiment, may have been due to the absence of baseline levels of corticosterone and/or E, whereas the effects observed in this experiment were due to the absence of activated levels of corticosterone and/or E. With respect to the 10μg dose used in the last experiment, since this dose activated maximal levels of

cytokines, any effect of the adrenals may have been partially masked. Another explanation is that experimental variability was lower in the present experiment and the effects more obvious.

Since immune suppression occurs in ADX animals at a similar magnitude to adrenal intact animals, it is evident that another system outside of the HPAA is involved in mediating the immunosuppressive effects of stress on splenic cytokine production. It was unknown however if this other system was entirely responsible for the immunosuppressive effects of stress on cytokine production or just compensates for the loss of adrenal function in ADX animals. The former possibility would suggest that an endocrine factor such as opioids or prolactin is responsible for the observed stress-induced immune suppression as cutting the splenic nerve in adrenal intact animals had no effect. It is known that opioids are released in response to stress and are immune suppressive [419,882]. Therefore their removal may abrogate the immunosuppressive effects of stress. However this is inconsistent with Keller et al., [58] who found that HYPOX animals are still immune suppressed. Also, it has been shown that opioids are increased in response to LPS and do not affect TNF alpha production [974]. Therefore it is unlikely that these represent the primary mediators in our system. In contrast to opioids, prolactin is an immunocompetence factor [388,391] and can promote LPS-induced cytokine production [392,394]. Therefore prolactin could be the primary mediator if it was found that stress decreased prolactin levels. However many reports have observed an increase in prolactin levels in response to stress [843,932,975], thus this

explanation is also unlikely. Given this information it seemed all the more plausible that the immunosuppressive effects of stress on splenic cytokine production were engaging a dynamic multisystem process in which another system like the SNS could compensate for the removal of the adrenals, and potentially vice versa. If this was true, then cutting the splenic nerve in ADX rats would abrogate the immunosuppressive effects of stress on splenic cytokine production. Consistent with this, MacNeil et al., [378] demonstrated an increase in splenic nerve activity in response to LPS in animals with acute ADX.

Results revealed that cutting the splenic nerve in ADX rats abrogated the immunosuppressive effects of stress for TNF and IL-1 protein but not for IL-6 mRNA or protein. Thus the splenic nerve and SNS can compensate for the loss of adrenal function in ADX rats in terms of mediating the effects of stress on splenic cytokine production. The magnitude of the abrogation suggested the nerve was completely responsible for this effect. It is interesting to consider that if compensatory interactions exist between the HPAA and SNS, then other endocrine factors like opioids and prolactin may also be involved in this system. For example would HYPOX superimposed on ADX and splenic nerve cut increase cytokine production significantly above the ADX maximum, or have the opposite effect? The fact that IL-6 did not follow the pattern of the other two pro-inflammatory cytokines further suggests that the regulation of these cytokines is complex and is unique to each molecule. It is generally believed that LPS sequentially induces TNF which induces IL-1 which induces IL-6 production. Although this may be partially accurate, it is too simplistic considering the impact of stress on this regulatory system.

The actual mechanism of how immune suppression occurs in our system also merits further study. It is possible that catecholamines and glucocorticoids act on TNF and IL-1 mRNA and protein directly, as indicated by in vitro reports [192]. Another possibility is that the suppression results from an increase in anti-inflammatory molecules such as IKB and IL-10 [173,177,212,946]. As well, it may be possible that other mediators in the nerve such as substance P, VIP and NPY and not NE, are responsible for stress-induced immune suppression, as these factors can alter immune function as well [136-139,216-218] .

5.8.3. Perspectives

A main question generated by these experiments is that if the SNS and HPAA are generally in a dynamic equilibrium, is there is a physiological situation during which the SNS would function as the primary immunomodulatory system? For example, it is known that antigen-mediated immune responses can be regulated by NE [153,269,976] without large elevations in corticosterone [48,249]. As well, it has been found by Molina and Abumrad that the magnitude of the catecholamine increase was more prominent than the increase in glucocorticoids in response to hemorrhage. However, in terms of the LPS response, the reverse was true [353]. It is unknown if these effects were consistent throughout the response to the respective challenges as catecholamines and glucocorticoids were only measured at 90 minutes following the initiation of the stressor in that study. Importantly, they found that in response to hemorrhage, sympathectomy

increased hemorrhage-induced splenic cytokine levels [973]. Therefore, if immune suppression can be demonstrated without major changes in glucocorticoid levels, it is likely that the splenic nerve may be the main effector in that situation.

6. Experiment 4:

Characterization of the stress response when the exposure to stress precedes i.v. LPS.

6. Experiment 4: Characterization of the stress response when the exposure to stress precedes i.v. LPS.

6.1. Overview

The main objective of this experiment was to define a scenario where the nerve had the potential to mediate the effects of stress in adrenal intact animals. It is known that the expression of corticosterone in response to stress is highly transitory. By separating the exposure to stress and LPS such that glucocorticoid expression has returned to baseline hours prior to LPS injection, it was predicted that an observed suppression of LPS-induced splenic cytokine may be mediated by the splenic nerve. In order to accomplish this, the order of stress exposure and LPS injection was reversed where stress preceded the inflammatory challenge. The time intervals between stress and LPS that were tested included 0 hrs, 4 hrs, and 24 hrs. It was predicted that stress would be immunosuppressive at all the time points tested, with the effect dissipating as the interval between stress and LPS increased. It was found that TNF and IL-1 mRNA were only suppressed when LPS immediately followed stress. In contrast, IL-6 expression was potentiated when stress preceded LPS injection. These effects were observed in response to a low (0.1μg) and high (10μg) of LPS. This suggests a unique role for IL-6 (as compared to TNF and IL-1) in maintaining homeostasis in response to psychological and immunological stressors. Future studies should include determining the effect of the nerve in potentiating IL-6 production in this paradigm.

6.2. Objectives

1. To establish if the effects of stress on LPS-induced splenic cytokine production are the same when stress exposure precedes LPS administration.

2. To determine if the effects of stress on LPS-induced splenic cytokine production are evident in animals exposed to stress 4 or 24 hr prior to LPS injection.

3. To assess if the effects of stress on LPS-induced splenic cytokine production are evident in response to a dose of i.v. LPS that can induce maximal levels of splenic cytokines and plasma corticosterone.

6.3. Hypotheses

It is expected that immune suppression will be evident in animals irrespective of the dose of LPS and the sequence of LPS injection and stress exposure. Also, the effects of stress on splenic cytokine production will be less apparent the greater the time interval between the exposure to stress and LPS injection.

6.4. Rationale

To test the effects of stress on LPS-induced cytokine production, Goujon et al., first injected the mice with LPS then exposed them to stress [867]. However since cytokine production is rapid and transitory, this paradigm is only useful for testing the immediate effects of acute stress on cytokine production as cytokine levels will decrease after 1-2 hr post-injection. In order to test the temporal effectiveness of stress on LPS-induced splenic cytokine production, we reversed Goujon's paradigm so that the stress preceded the immune challenge. This allowed us to determine if stress 4 or 24 hr before LPS injection could impact on splenic cytokine production. We reasoned that the importance of testing these time intervals is that the further the immune challenge is separated from the initial corticosterone response, the more likely the nerve will play a major regulatory role on cytokine production.

Goujon et al., saw the effects of stress on LPS-induced cytokine production despite using a high dose of LPS (10 μg of i.p. LPS per mouse) [867]. Therefore we anticipated that the effects of stress on the cytokine production in response to 10μg of i.v. LPS in rats would be similar to the effects observed in response to 0.1 μg of LPS. This rationale was based on the fact that high levels of catecholamines and glucocorticoids are present prior to LPS administration and thus will be able to reduce the maximal levels of cytokines induced by 10μg of i.v. LPS.

6.5. Experimental Design

All studies utilized adult male Sprague-Dawley rats (225-250 g) that were obtained from Charles River, (Dorval Quebec). The rats were kept on a 12-12 light/dark cycle and given food and water were given *ad libitum*. All animals were singly housed, handled for 2 days then taken through the experimental procedure (weighing, mock injection etc.) for 3 days before the actual experiment. Animals were then exposed to 15 minutes of 1.6 mA intermittent footshock. Each shock lasted 5s and was preceded by a 15s warning tone. The average interval between shocks was 3.5 minutes with a range between 2-5 minutes. Apparatus controls are exposed to the shock apparatus and the intermittent warning tone for 15 minutes but not the electrical shock. Homecage controls were left in their cage until LPS injection (E.Coli serotype 055:B5 lot L-2637) and then treated like the other two groups. At various intervals following the stress exposure (0, 4, or 24 hr), animals were weighed and then put under a heat lamp for 3 minutes to dilate the tail veins. They were then put in a restrainer and injected i.v. with 0.1 or 10 μg of LPS and killed at 1 post-injection. The animals were killed by decapitation and blood and the spleens were collected for various assays. Northern blots, ELISAs, RIA for corticosterone and catecholamine determinations were performed as described in the materials and methods (section 2). Experiments were analyzed by ANOVA, and one- and two-tailed T-tests as described in the materials and methods. All procedures were approved by the animal ethics committee at the University of Manitoba and the CCAC.

6.6. Results

6.6.1. The effect of stress prior to 0.1 μg of i.v. LPS on LPS-induced splenic cytokine production.

The first two objectives were to determine if the effects of stress on splenic cytokine production are maintained if the sequence of stress exposure and LPS administration are reversed, and if so, how long this effect would persist. For cytokine mRNA (figure 6-1) the results of separate experiments are depicted together, with values expressed as a percentage of the cytokine values in homecage controls (HC). Although this presentation format suggests that ANOVA is the appropriate statistical test, T-tests were used as the only important comparisons were between the homecage controls and the experimental groups. Similar to our original paradigm, it was found that stress immediately before LPS suppressed splenic TNF [T(10)=2.69, p=0.011 one-tailed] and IL-1 [T(10)=1.85, p=0.047, one-tailed] mRNA levels while IL-6 mRNA levels were not detectable. No suppression was seen if the stress was given 4 and 24 hr before hand and thus splenic protein levels were not measured at these intervals. The splenic TNF protein (figure 6-2) levels at the 0 time interval was consistent with the mRNA levels in that a stress-induced suppression was observed [T(10)=3.89, p=0.0015 one-tailed]. However IL-1 protein levels were not different between groups [T(10)=0.617, p=0.27 ns one-tailed], similar to the effects observed in the previous experiment. Surprisingly, the level of IL-6 protein was increased in stressed animals as compared to controls [T(9)=4.85, p=0.001]. Also, plasma corticosterone (figure 6-3) was elevated at the time of

decapitation in stressed rats as compared to homecage controls [T(10)=2.627, p=0.013 one-tailed].

To verify that the effects of stress were due to the footshock and not due to the new environment of the shock apparatus, we measured plasma corticosterone (figure 6-4) and splenic mRNA levels (figure 6-5) in rats injected with 0.1 μg LPS at various intervals after exposure to the shock apparatus. There was not an overall effect of the apparatus exposure on plasma corticosterone [F(5,21)= 1.373, p=0.27 ns] however there were a few important observations. First of all animals, that were killed without being exposed to the injection procedure had the same levels of plasma corticosterone as homecage control (HC) rats killed one hour after 0.1 μg of LPS or saline injection. Also, it was found that animals exposed to the apparatus and immediately injected with LPS showed increased plasma corticosterone levels as compared to homecage controls (p=0.026). In terms of mRNA levels, no overall effects of exposure to the apparatus were observed for TNF [F(3,17)=2.148, p=0.14 ns] or IL-1 mRNA [F(3,17)=0.158, p=0.92 ns] and IL-6 was not detected. However the group that received LPS 24 hr after stress had higher levels of TNF mRNA that the group the received LPS immediately after stress (p=0.0412) This can be explained by the increased corticosterone levels immediately after stress and the experimental variability in the 24 hr group.

6.6.2. The effect of stress prior to 10 μg of i.v. LPS on LPS-induced splenic cytokine production.

The next question was to determine if the immunosuppressive effects of stress would be evident in response to a dose of LPS that maximally activated cytokines and both the HPAA and SNS (figure 6-6). Consistent with the results in response to 0.1 μg of LPS (figure 6-1), the only significant effects were observed in animals that received 10 μg of i.v. LPS immediately after stress (figure 6-6). It was found that TNF [T(10)=11.17, p<0.0001 one-tailed] and IL-1 [T(10)=2.83, p=0.009 one-tailed] levels were decreased as compared to controls while IL-6 levels were increased in stressed animals as compared to controls [T(10)=4.17, p=0.0019 one-tailed]. The effects of stress on splenic TNF and IL-6 protein levels (figure 6-7) were consistent with the effects on the mRNA levels in that TNF protein levels were decreased in stressed animals [T(10)=4.01, p=0.0012 one-tailed] while IL-6 levels were increased in stressed animals [T(9)=2.51, p=0.017]. Consistent with the other experiments, splenic IL-1 levels were not significantly between control and stressed animals [T(10)=0.315, p=0.38 ns one-tailed] despite the fact that the IL-1 mRNA level was decreased. In order to determine if splenic protein levels were consistent with systemic phenomenon, plasma TNF, IL-1 and IL-6 were measured. It was found that plasma TNF (figure 6-8) and IL-6 (figure 6-9) were consistent with splenic protein levels in that stress decreased plasma TNF [T(10)=3.29, p=0.0041 one-tailed] and increased plasma IL-6 levels [T(9)=2.617, p=0.014 one-tailed]. Plasma IL-1 was not detectable in this experiment. There was no difference in plasma corticosterone (figure 6-10) levels between stressed and control animals at the time of decapitation [T(10)=0.233, p=0.82 ns one-tailed]. In contrast to the 0.1 μg dose, there were no significant differences

between any of the apparatus control groups with respect to plasma corticosterone levels [F(3,17)= 0.914, p=0.46 ns] (figure 6-11), and splenic TNF [F(3,17)=1.03, p=0.40 ns], IL-1 [F(3,17)=0.525, p=0.67 ns] and IL-6 mRNA [F(3,17)=0.343, p=0.79 ns] (figure 6-12). However compared to homecage controls, splenic TNF mRNA levels were slightly decreased.

6.7. Figures (* indicates that P≤0.05)

Figure 6-1: The effect of 15 minute of intermittent footshock stress at various intervals prior to 0.1μg of i.v. LPS on LPS-induced splenic cytokine mRNA levels. Rats were exposed to 15 minutes of 1.6 mA of intermittent footshock stress, injected with 0.1μg of LPS at various intervals following this procedure and killed 1 hr following injection of LPS. Homecage controls (HC) were not exposed to the shock apparatus. The results from separate experiments are compiled and are presented together where each group is represented as a percentage of the HC group and only compared to this group by a one-tailed T-test. TNF and IL-1 mRNA levels were analyzed by Northern blotting and expressed as a ratio relative to the loading control. The error bars represent +/-1 standard error of the mean, n=6-9 per group.

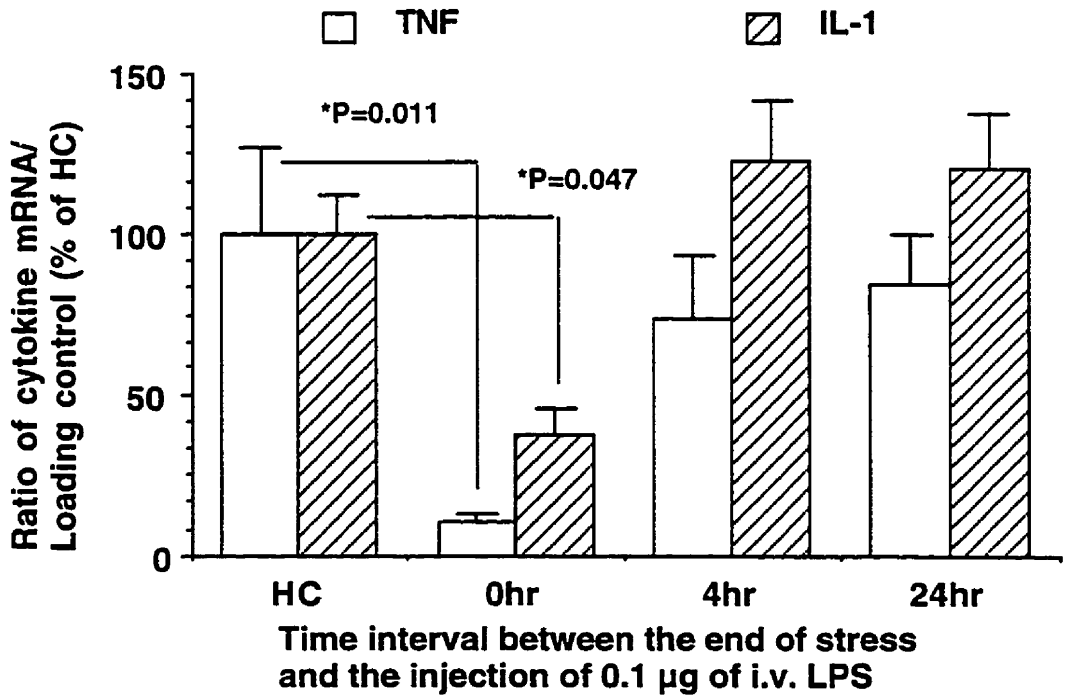


Figure 6-2: The effect of 15 minute of intermittent footshock stress immediately prior to 0.1μg of i.v. LPS on LPS-induced splenic cytokine protein levels. Rats were exposed to 15 minutes of 1.6 mA of intermittent footshock stress, immediately injected with 0.1μg of LPS and killed 1 hr following injection of LPS. Homecage controls (HC) were not exposed to the shock apparatus. Splenic TNF, IL-1 and IL-6 protein levels were measured by ELISA and expressed as pg/mg of splenic protein. TNF [T(10)=3.89, p=0.0015, n=12] and IL-1 levels [T(10)=0.62, p=0.27ns, n=12] were compared by one-tailed T-test while the IL-6 levels [T(9)=4.85, p=0.001, n=11] were compared with a two-tailed T-test. The error bars represent +/-1 standard error of the mean.

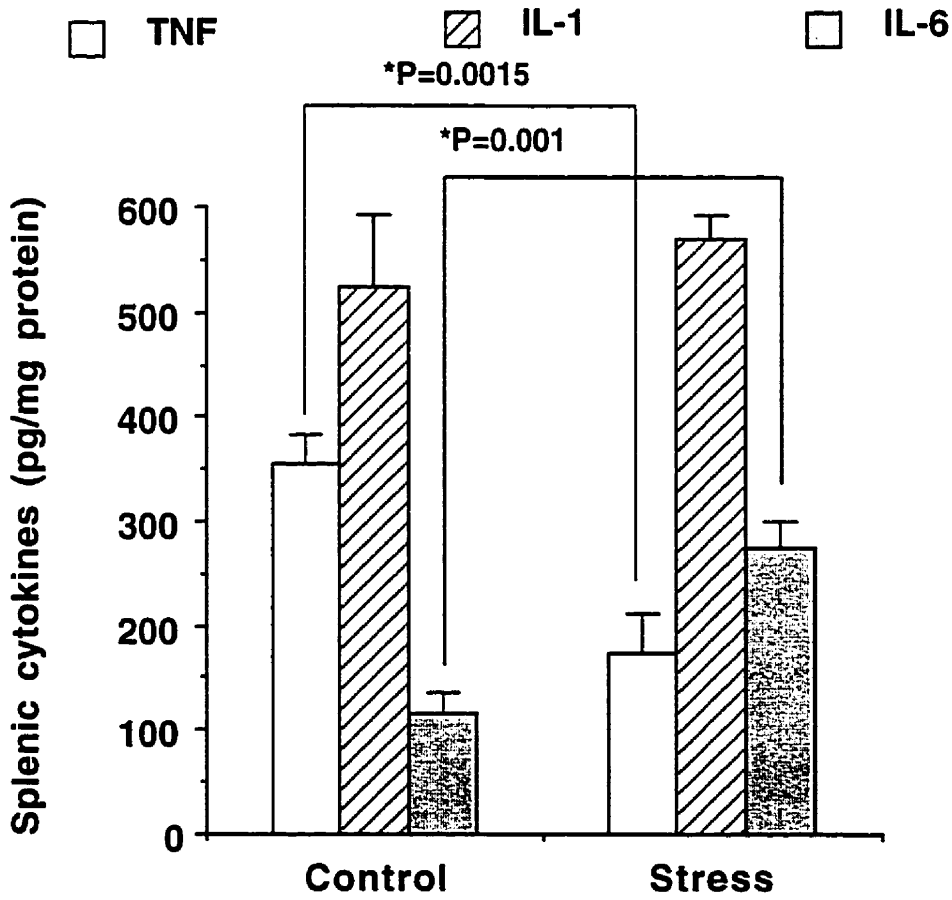


Figure 6-3: The effect of 15 minute of intermittent footshock stress immediately prior to 0.1μg of i.v. LPS on plasma corticosterone levels. Rats were exposed to 15 minutes of 1.6 mA of intermittent footshock stress, immediately injected with 0.1μg of LPS and killed 1 hr following injection of LPS. Homecage controls (control) were not exposed to the shock apparatus. Plasma corticosterone [T(10=2.63, p=0.0013, n=12] was measured by RIA and expressed as ng/ml. Error bars represent +/-1 standard error of the mean. Groups were compared with a one-tailed T-test.

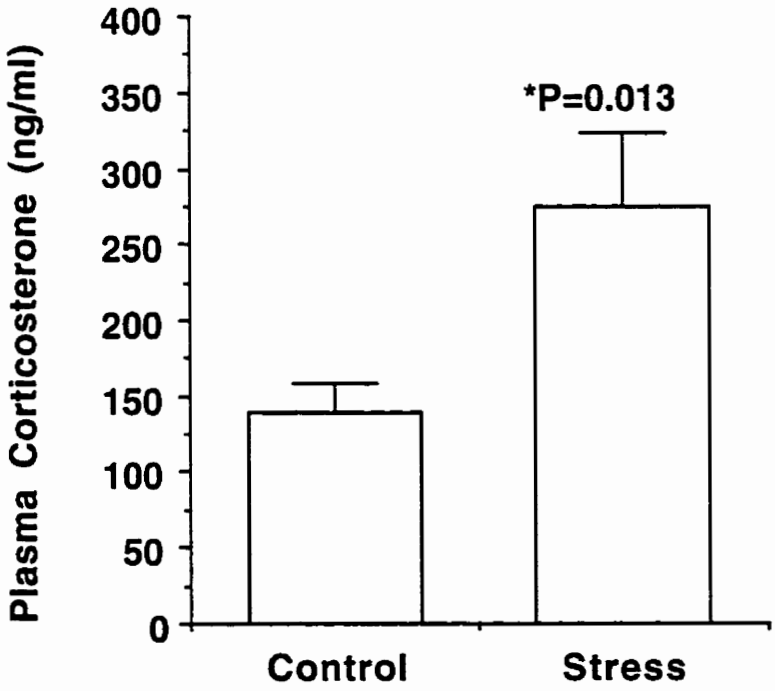


Figure 6-4: The effect of 15 minute apparatus control at various intervals prior to 0.1μg of i.v. LPS on plasma corticosterone levels. Rats were exposed to the shock apparatus for 15 minutes, injected with 0.1μg of LPS at various intervals following this procedure and killed 1 hr following injection of LPS. Homecage controls (HC) and saline injected rats were not exposed to the shock apparatus while other rats were taken directly from their cage and killed to control for the injection procedure (No inj.). Plasma corticosterone levels [F(5,21)=1.37,p=0.27ns, n=27] were measured by RIA and compared by ANOVA. Error bars represent +/-1 standard error of the mean.

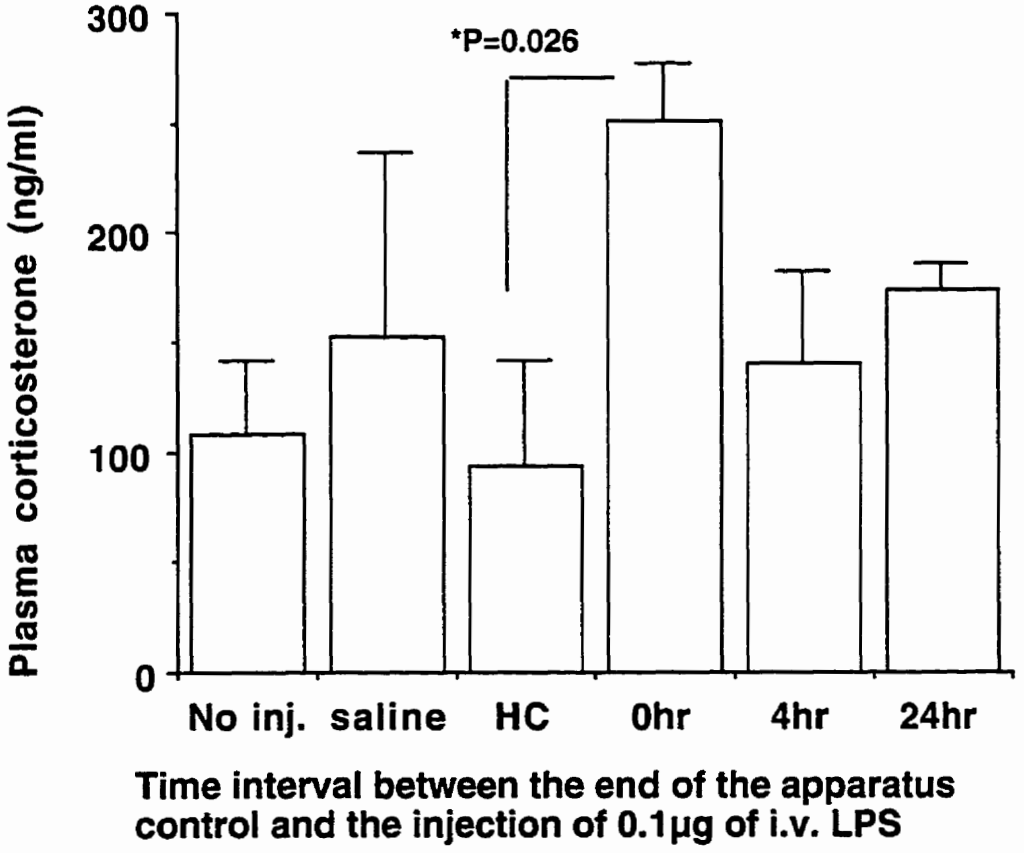


Figure 6-5: The effect of 15 minute apparatus control at various intervals prior to 0.1μg of i.v. LPS on LPS-induced splenic cytokine mRNA levels. Rats were exposed to the shock apparatus for 15 minutes, injected with 0.1μg of LPS at various intervals following this procedure and killed 1 hr following injection of LPS. Homecage controls (HC) were not exposed to the shock apparatus. Splenic TNF [F(3,17)=2.15, p=0.14ns, n=21] and IL-1 mRNA levels [F(3,17)=0.158, p=0.92ns, n=21] were analyzed by Northern blotting and expressed as a ratio relative to the loading control. Results were compared by ANOVA and error bars represent +/-1 standard error of the mean.

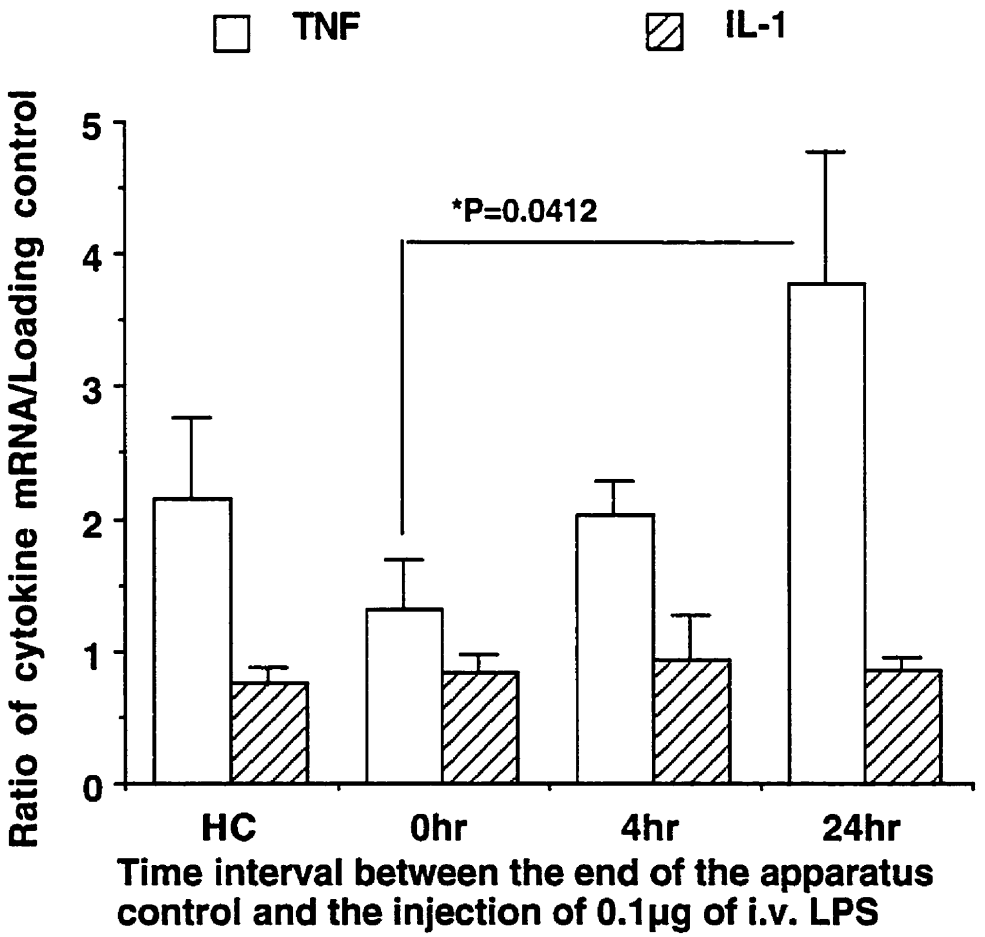


Figure 6-6: The effect of 15 minute of intermittent footshock stress at various intervals prior to 10μg of i.v. LPS on LPS-induced splenic cytokine mRNA levels. Rats were exposed to 15 minutes of 1.6 mA of intermittent footshock stress, injected with 10μg of LPS at various intervals following this procedure and killed 1 hr following injection of LPS. Homecage controls (HC) were not exposed to the shock apparatus. The results from separate experiments were compiled and are presented together where each group is represented as a percentage of the HC group and only compared to this group. Cytokine mRNA levels were analyzed by Northern blotting and expressed as a ratio relative to the loading control. Cytokine levels were compared by a one-tailed T-test and error bars represent +/-1 standard error of the mean, n=6-9 per group.

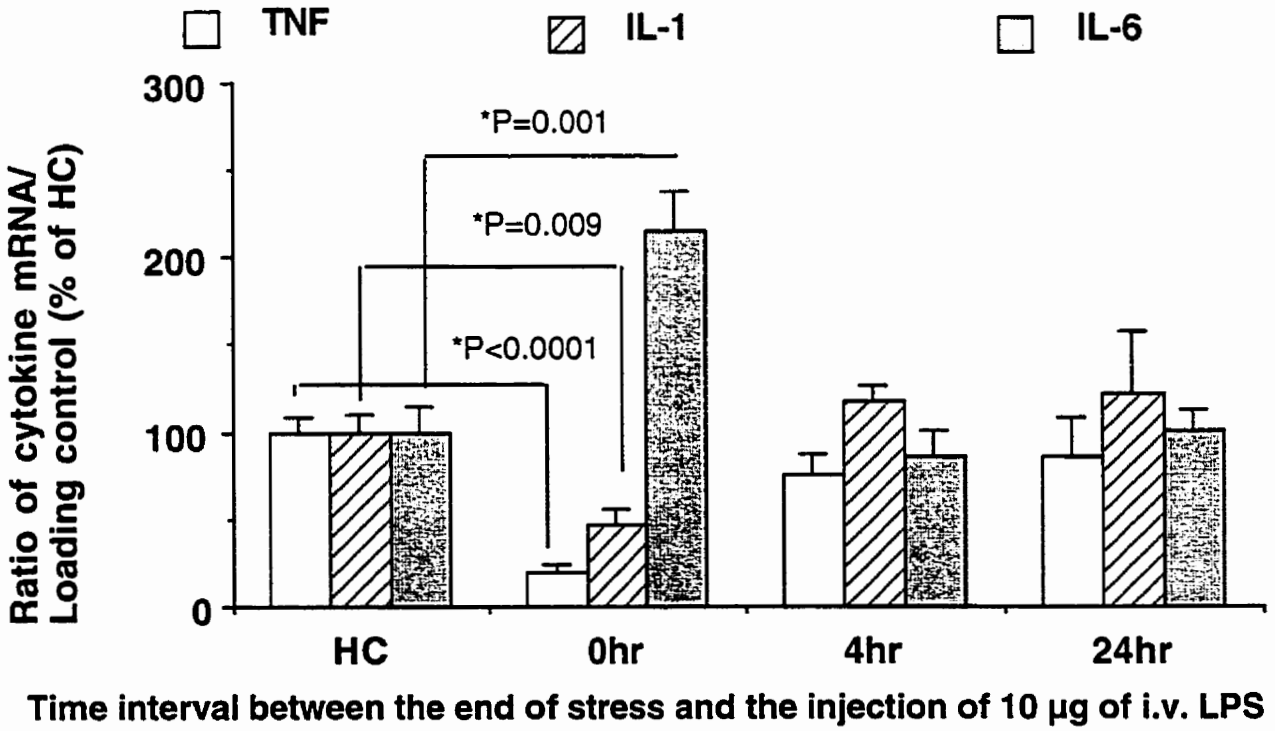


Figure 6-7 The effect of 15 minute of intermittent footshock stress immediately prior to 10μg of i.v. LPS on LPS-induced splenic cytokine protein levels. Rats were exposed to 15 minutes of 1.6 mA of intermittent footshock stress, immediately injected with 10μg of LPS and killed 1 hr following injection of LPS. Homecage controls (control) were not exposed to the shock apparatus. Splenic TNF [T(10)=4.01, p=0.0012, n=12], IL-1 [T(10)=0.315, p=0.38ns, n=12] and IL-6 [T(9)=2.51, p=0.017, n=11] protein levels were measured by ELISA and expressed as pg/mg of splenic protein. All protein levels were compared by one-tailed T-tests. The error bars represent +/-1 standard error of the mean.

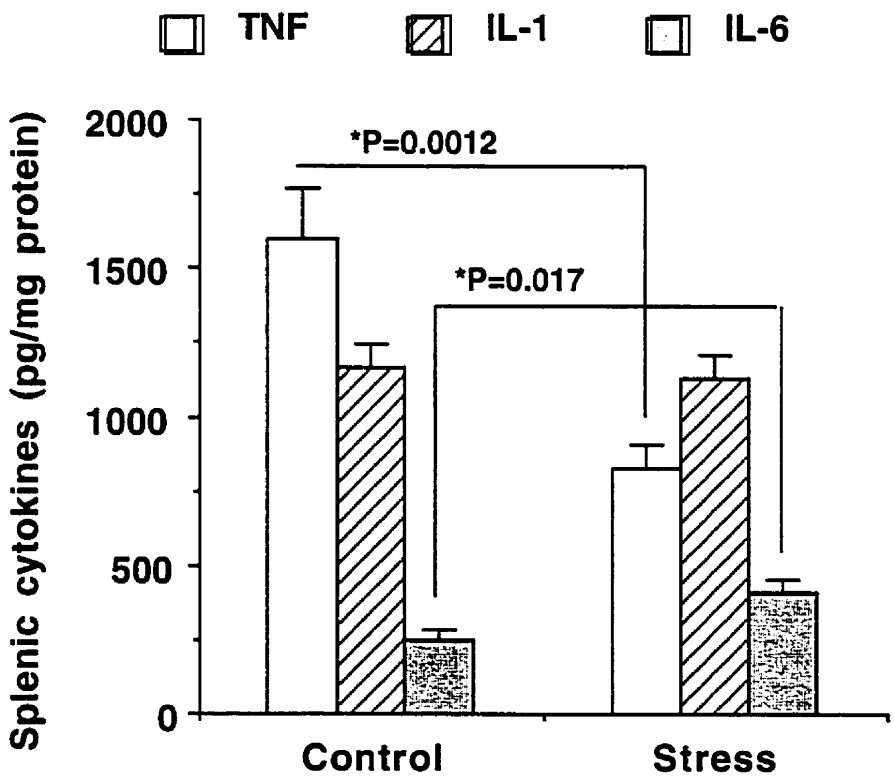


Figure 6-8: The effect of 15 minute of intermittent footshock stress immediately prior to 10μg of i.v. LPS on LPS-induced plasma TNF levels. Rats were exposed to 15 minutes of 1.6 mA intermittent footshock stress, immediately injected with 10μg of LPS and killed 1 hr following injection of LPS. Homecage controls (control) were not exposed to the shock apparatus. Plasma TNF [T(10)=3.29, p=0.0041, n=12] was measured by ELISA and expressed as ng/ml. Error bars represent +/-1 standard error of the mean. Groups were compared with a one-tailed T-test.

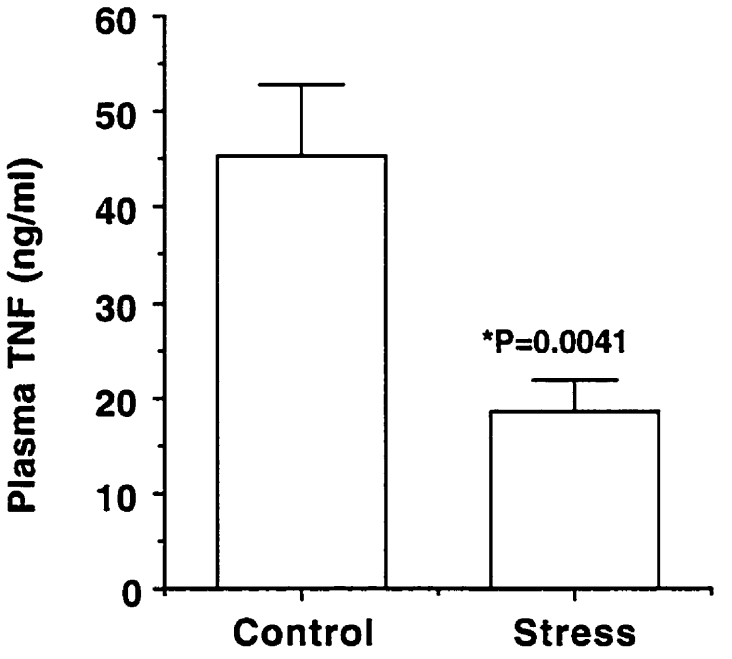


Figure 6-9: The effect of 15 minute of intermittent footshock stress immediately prior to 10μg of i.v. LPS on LPS-induced plasma IL-6 levels. Rats were exposed to 15 minutes of 1.6 mA intermittent footshock stress, immediately injected with 10μg of LPS and killed 1 hr following injection of LPS. Homecage controls (control) were not exposed to the shock apparatus. Plasma IL-6 [T(10)=2.617, p=0.014, n=12] was measured by ELISA and expressed as pg/ml. Error bars represent +/-1 standard error of the mean. The groups were compared with a one-tailed T-test.

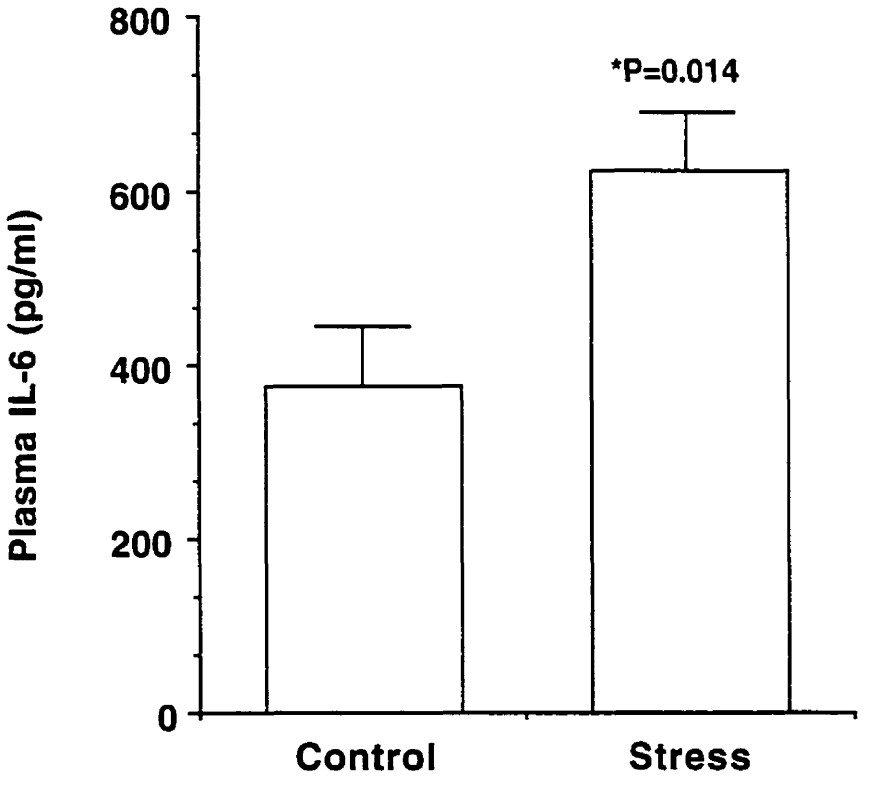


Figure 6-10: The effect of 15 minute of intermittent footshock stress immediately prior to 10μg of i.v. LPS on plasma corticosterone levels. Rats were exposed to 15 minutes of 1.6 mA of intermittent footshock stress, immediately injected with 10μg of LPS and killed 1 hr following injection of LPS. Homecage controls (control) were not exposed to the shock apparatus. Plasma corticosterone [T(10)=0.23, p=0.82, n=12] was measured by RIA and expressed as ng/ml. Error bars represent +/-1 standard error of the mean. Groups were compared with a one-tailed T-test.

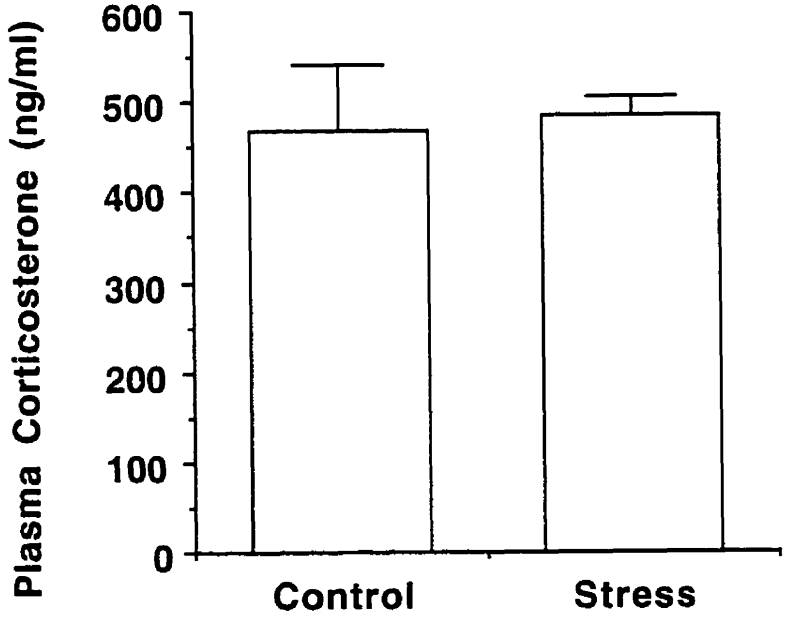


Figure 6-11: The effect of 15 minute apparatus control at various intervals prior to 10μg of i.v. LPS on plasma corticosterone levels. Rats were exposed to the shock apparatus for 15 minutes, injected with 10μg of LPS at various intervals following this procedure and killed 1 hr following injection of LPS. Homecage controls (HC) were not exposed to the shock apparatus. Plasma corticosterone [F(3,17)=0.914, p=0.46ns, n=21] was measured by RIA and expressed as ng/ml. Error bars represent +/-1 standard error of the mean.

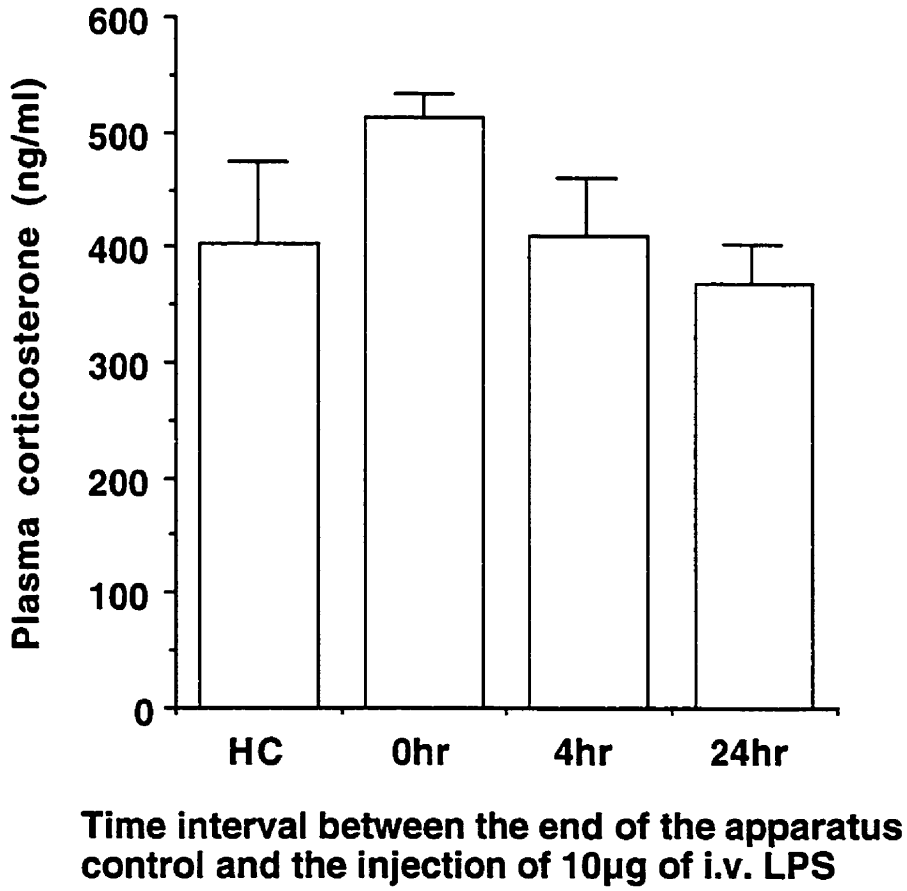
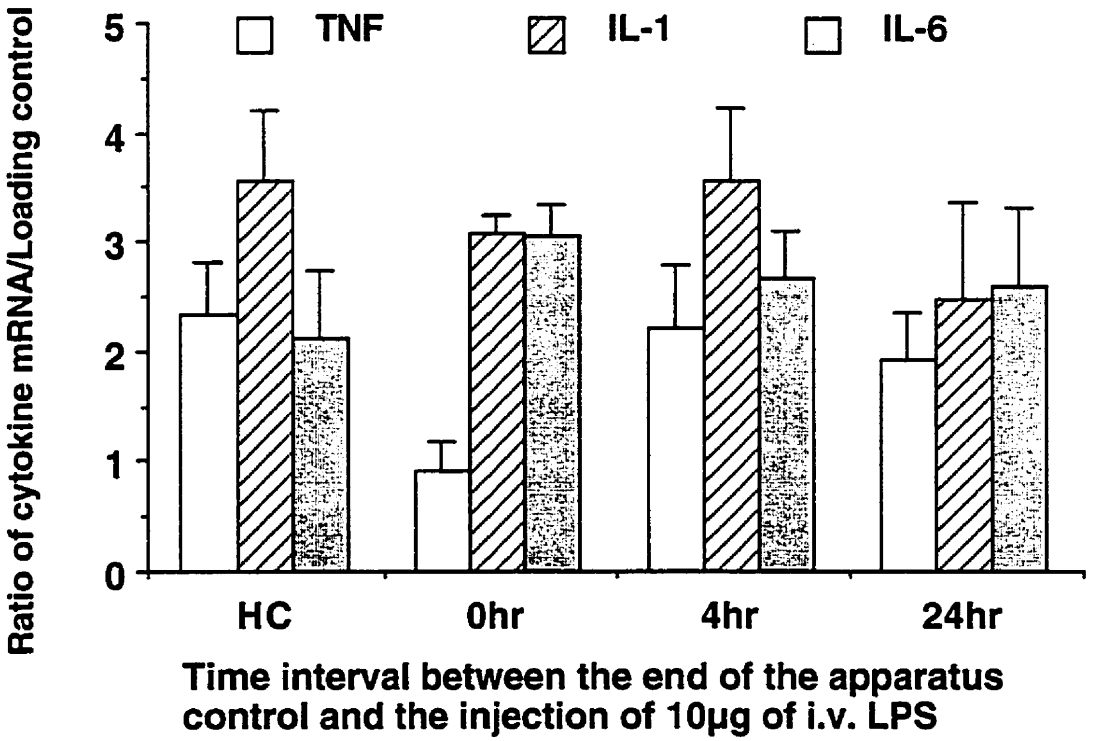


Figure 6-12: The effect of 15 minute apparatus control at various intervals prior to 10μg of i.v. LPS on LPS-induced splenic cytokine mRNA levels. Rats were exposed to the shock apparatus for 15 minutes, injected with 10μg of LPS at various intervals following this procedure and killed 1 hr following injection of LPS. Homecage controls (HC) were not exposed to the shock apparatus. Splenic TNF, IL-1 and IL-6 mRNA levels were analyzed by Northern blotting and expressed as a ratio relative to the loading control. Error bars represent +/-1 standard error of the mean.



6.8. Discussion

6.8.1. The effects of stress on LPS-induced plasma corticosterone levels when exposure to stress precedes 0.1 or 10 μg of i.v LPS.

Interestingly, it was found that two stressors that are subthreshold can interact to induce a corticosterone response. For example animals receiving footshock and 0.1 μg of i.v. LPS had higher levels of plasma corticosterone at 1 hr post LPS than animals just receiving LPS. This was in spite of the fact that the 0.1μg dose of LPS was unable to induce an increase in plasma corticosterone on its own at 1 hr post-injection, and that at 1 hr post-footshock, stress-induced corticosterone levels returned to baseline. This suggests that the magnitude of the HPAA response to stress represents an integrated response to diverse stressful stimuli. Therefore the hypothalamus and extra-hypothalamic regions can simultaneously process information from distinct stimuli and initiate a combined efferent response. This is plausible as Van Dijken et al., found animals exposed to 15 minutes of 1 mA footshocks had increased corticosterone responses to new stimuli despite the fact that baseline levels were not altered [19]. This was due to the increase in VP stores in the median eminence in response to the first stressor. Although the novelty-induced stress in this report was psychological in nature and not inflammatory like LPS, the central alterations were located in the median eminence, which is integral to all HPAA responses [19,827,977]. Increased corticosterone levels were not observed in animals exposed to stress and injected with 10 μg of LPS as compared to animals just injected with 10μg of

i.v. LPS. This was probably due to the fact that the 10μg dose of LPS induces a maximal level of corticosterone and the response can't be potentiated further (ceiling effect).

It is also possible that 0.1 μg dose of LPS by itself does induce a corticosterone response, that can be potentiated by footshock, but that our high baseline of corticosterone precluded the detection of this potentiation. As discussed in experiment 1, it was suggested by Beno and Kimura [866] that the high levels of corticosterone we found in our system at 1 hr post-injection may be due to our injection procedure, despite having habituated the animals to this protocol. We therefore compared handled LPS- and saline-treated animals at 1 hr post-injection with handled animals that were not exposed to the injection procedure. It was found that the levels of corticosterone were equal in all three groups. Therefore the high baseline levels of corticosterone observed in our experiments could have been a factor of the strain and supplier, as suggested by Turnbull and Rivier [930] or due to handling [842]. Although handling can reduce increases in corticosterone levels in response to routine procedures [837,838], it will still induce a mild stress response [842]. Therefore in future if we wanted to determine if the 0.1 μg dose of LPS did induce a corticosterone response, we would have to follow the protocol from Dr. C. Rivier's lab where animals: 1) could not be handled for 3 hr prior to LPS injection; 2) they would have to be injected by intravenous catheter; and 3) the guillotine would have to be directly beside the animal cages to significantly reduce handling induced increases in corticosterone levels immediately prior to decapitation. However this may present logistical problems as stress-related odours can increase corticosterone levels in

other animals in the same room [886]. Therefore the facilities needed to execute these experiments must prevent stress- or death- related odours and sounds from affecting the other animals.

We also found that our apparatus controls had higher levels of corticosterone than our homecage controls. This is consistent with reports by Keller et al., who also showed an increase in corticosterone in response to the apparatus control [56,57]. However in agreement with Keller at al., and Zalcman et al., [886], these mild increases did not significantly affect splenic immune function, even though small non-significant decreases were observed in our experiments with respect to TNF mRNA. Thus, although a component of the stress response was contributed by the novel environment, it did not affect our examination of main objective of this experiment which was to delineate the differences in splenic cytokine production between control and stressed rats.

6.8.2. The effects of stress on LPS-induced splenic and plasma cytokine levels when exposure to stress precedes 0.1 or 10 μg of i.v LPS.

It was clear from the last set of experiments (section 5) that the splenic nerve can inhibit cytokine production in ADX rats. Our next goal was to identify potential intervals in our paradigm where the immunosuppressive effects of stress were primarily transmitted by the splenic nerve in adrenal intact animals. It was evident from our previous experiments that to accomplish this goal we would have to select conditions where HPAA activity was at baseline. Molina and colleagues supported this strategy by

demonstrating that sympathectomy can alter cytokine production in adrenal intact animals if the stressor activates the SNS more vigorously than the HPAA [353,973]. To extend our previous studies, we decided to determine if stress-induced immune suppression was evident in animals that had a time delay between the exposure to stress and LPS injection. The concept was that if immune suppression was evident in animals that were stressed hours prior to LPS injection, then nerve-mediated effects would be more obvious as the adrenal response would have dissipated. This was based on the fact that the corticosterone and plasma catecholamine responses to stress are well documented and are at baseline at 1-2 hr post-stress [790,802,839,851,964,978,979]. However the time course of splenic nerve activity and splenic NE turnover in response to footshock stress is not known and could potentially still be active at these later intervals.

Since cytokine production was assayed one hour after LPS, separating the stress exposure and LPS injection could only be accomplished if we reversed our paradigm. We chose to expose the animals to stress immediately before LPS (time 0) as well as 4 and 24 hr before LPS. It was expected that the time 0 animals would be positive controls as glucocorticoids and catecholamines levels would be at their highest point immediately prior to injection (i.e. right after footshock) and thus be most capable of suppressing subsequent cytokine production induced by LPS. Another reason why this interval was included was based on studies by Zalcman et al., and Shanks and Anisman [808,980] that demonstrated that the timing of stress with respect to the immune challenge is critical for observing the experimental effects. Therefore if reversing the sequence of stress and LPS

injection changed the effects of stress on cytokine production, it should be most evident at time 0 as the SNS and HPAA are most active. We also tested whether immune suppression in response to 10μg of i.v. LPS would occur as maximal levels of splenic cytokines as well as maximal levels of plasma corticosterone were induced in response to that dose.

It was expected that immune suppression would be evident in the groups exposed to stress 4 and 24 hr prior to LPS, although not at the same magnitude as the animals exposed to stress immediately prior to LPS injection. However stress-induced effects on immune function were only evident in this latter group thus making it unlikely that a nerve cut effect would be present in adrenal intact animals in this paradigm. In the time 0 group, stress suppressed TNF and IL-1 mRNA in response to both 0.1 and 10 μg of LPS. This suggested that if splenic macrophages are exposed to glucocorticoids and/or catecholamine prior to cytokine production, that the central systems can regulate the LPS-induced inflammatory response. The fact that this effect was also seen in response to 10 μg of i.v LPS suggests that prior exposure of splenic macrophages to glucocorticoids and catecholamines is so robust a suppressive stimulus that even a dose of LPS that normally induces a maximal cytokine response, can be blunted. TNF protein and plasma protein mimicked splenic mRNA levels. However IL-1 protein did not follow the pattern of mRNA changes in splenic tissue; potential reasons for this were discussed in the previous experiment (section 5).

Perhaps the most interesting and surprising result of this set of experiments was

that IL-6 was increased, not decreased, in the animals exposed to stress immediately before LPS. Our results were consistent for responses to both 0.1 μg and 10 μg, and for both mRNA and protein, except that IL-6 mRNA was not detected in response to the 0.1 μg dose of LPS. These results were also confirmed by measuring plasma IL-6 levels in response to stress and 10 μg of LPS. Therefore the results by Takaki et al., [970] suggesting that organs respond differentially to stress in terms of IL-6 production are not applicable in this paradigm as increases were seen for all of splenic mRNA and protein and plasma protein levels of IL-6. These results support prior reports of stress-induced IL-6 production [204-207] and reports of catecholamines being able to increase IL-6 in vitro [200,965]. Also, this finding is consistent with the work by Straub et al., who found that the presence or absence of bacterial products will dictate whether NE decreases or potentiates IL-6 production [210]. This confirms that although the production of TNF, IL-1 and IL-6 are related, distinct regulatory pathways exist for each molecule in vivo depending on the microenvironment and physiological state of the animal. This is supported by the fact that although IL-6 functions to inhibit TNF production [981], IL-6 was only increased when the stress preceded the LPS injection whereas TNF was inhibited irrespective of the sequence of stress exposure and LPS injection. Therefore although TNF was reduced in irrespective of the order of LPS and stress, the mechanisms by which this happened must be different for each paradigm.

The fact that IL-6 expression is increased by simply reversing the order of stress and LPS injection was unexpected, despite Zalcman's findings [808,980]. Zalcman's

observations were made with a complex immune measure (the response to sheep red blood cells) that requires days to develop in vivo. Therefore it is plausible that stresses on different days during this response, would have different effects, as NE can either inhibit [48,252,253] or potentiate [152,153,258,259] the humoral immune response depending on the timing of NE exposure [269,955]. In contrast we used measures of innate immunity (cytokines) which are produced in a rapid and transitory fashion and although the timing of stress exposure and LPS injection was reversed, the difference was only 15 minutes. The question then, is how does do these diametrically opposed effects, based on the sequence of the two stimuli, occur in such a short span of time? It is likely that the stress-induced increase in catecholamines potentiated IL-6 production. It is possible that since LPS elicits more of an HPAA response than an SNS response (according to Molina and Abumrad) [353], and since glucocorticoids inhibit IL-6 [326,328,332] , exposing the animals to LPS prior to stress allows glucocorticoids to reach the immune cells prior to catecholamines. However if this was the whole story, then it would be expected that LPS by itself should reduce IL-6 production, when in fact the opposite was found. Therefore the effects observed by Straub et al., warrant further study as they seem fundamental to understanding the response to stress at a cellular level, as LPS itself, and not glucocorticoids, are probably responsible for rendering the cell unresponsive to NE [210]. The fact that IL-6 seems to be subject to additional complex regulation relative to the other cytokines suggests it is a key mediator in the response to a variety of stressors. Future studies should concentrate on the role of the splenic nerve in regulating the stress-

induced increase in IL-6 and whether the presence of an immune stimulus like LPS is required to achieve this effect.

7. General discussion and future directions

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Homeostasis is defined as the ability of an organism to ensure survival by maintaining a consistent internal environment [1,2]. Anything that disrupts this environment is referred to as stress [3]. The stress response includes a coordination of metabolic, physiological and behavioral alterations designed to nullify the change(s) imposed by the stressor and return the organism to its optimal homeostatic position. These responses are necessary for survival and are coordinated by the hypothalamus via multiple afferent and efferent systems. Although the response to stress in part depends on the nature of the stimulus (ie. immunological, physical, psychological), different stresses activate many of the same efferent systems and can elicit many of the same systemic effects. Therefore information regarding the regulation of the host's response to any single stressor may give insight into the overall stress response due to these commonalities. Delineation of these pathways are critical as dealing with stress is a major part of our existence and can impact on many aspects of health and disease [8,982].

Modern technology has given us the ability to create designer organisms and cell lines. However cells, tissues and organ systems do not exist autonomously, they interact with the rest of the body. Therefore in vitro studies or knockout studies may never fully expose the essence of the stress response due to its complexity and the overlapping functions of the many molecules involved in this essential biological process. Therefore the global objective of this thesis was to shed some light on the stress response using an

in vivo rat model. Specifically, we investigated the individual and combined roles of the HPAA and SNS in regulating splenic cytokine production in response to an inflammatory challenge and in response to a psychological stress superimposed on this inflammatory challenge. The inflammatory response was chosen as our experimental model due to its importance in mediating the early stages of tissue damage and repair, disease states and the response to foreign organisms [43,44,434].

Experiment 1: characterization of the in vivo response to i.v. LPS in the rat. The purpose of this experiment was to obtain the parametric data for our model system in response to i.v. LPS. LPS is a molecule from gram negative bacteria that is a potent activator of macrophages. Macrophage products like TNF, IL-1 and IL-6 mediate many of the effects of LPS on the host. The spleen was the target organ in our system because the innervation to the spleen is exclusively efferent and sympathetic and the nerve fibers are in close proximity to splenic macrophages [151].

We determined the time course and dose response for splenic TNF, IL-1 and IL-6 mRNA and protein in response to LPS. We also measured plasma corticosterone, plasma catecholamines and splenic catecholamine levels as indices of HPAA and SNS activation. The time course studies confirmed that TNF production peaked before IL-1 which peaked before IL-6. Critical parametric results were derived from our dose response study as these experiments were not comprehensively described in the prior literature. It was found that the dose response was relatively limited in that maximal cytokine production

was achieved in response to a relatively low dose of LPS (1-10μg) as compared to the dose required to elicit cardiovascular shock (roughly 100 times higher). The significance of this finding is that it suggests that although cytokines like TNF, IL-1 and IL-6 may initiate the host response to LPS and bacteria, they could not be the only mediators involved. There was a good relationship between cytokine mRNA and protein for the dose response and time course studies, suggesting that the effect of dose on post-transcriptional regulation of cytokine mRNA was minimal.

It was also found that only the 0.01 μg and the 0.1 μg doses of LPS-induced levels of cytokines that were above background and yet below maximal levels. Interestingly, these lower doses did not appear to activate either the SNS or HPAA, as changes in plasma corticosterone, plasma epinephrine and splenic NE were only detected in response to 1, 10 and 100 μg respectively. This was important because although the immune system, like the heart and enteric nervous system, can function autonomously, these systems are also subject to external regulation.

In terms of plasma corticosterone, plasma epinephrine and splenic NE levels, changes in the levels of these mediators also did not occur over a wide dose range in that they were either at baseline or maximal expression (however turnover rates were not measured). Also of note is that the peaks for each of these molecules occurred in response to successively higher doses of LPS. Therefore the response to increasing doses of LPS seemed to sequentially activate different (potentially inhibitory) regulatory systems as opposed to modulating the response of any single system. This reinforces the

observations of Cannon, Selye, and Kopin et al., of a coordinated response of the SNS and HPAA to stressors [1,2,10,11,248]. However the central signalling mechanisms which mediate these coordinated responses are not known despite the fact that the anatomical locations of the cells which project to the SNS and anterior pituitary have been identified [983]. Therefore it would be interesting to repeat the dose response study with the intention of measuring changes in the brain by dual labelling for c-fos and other markers of activation with peptides such as CRF, VP and OXY using confocal microscopy. It would be fundamental to determine the relationship between the dose of LPS and neurochemical and neuroanatomical measures of activation. Although previous studies have looked at the effect of LPS dose on c-fos expression in the hypothalamus and other areas of the brain [32,595,596], no study has identified specific mediators or groups of hypothalamic or extra-hypothalamic cells which could differentially activate the SNS as opposed to the HPAA, or the adrenal medulla as opposed to the splenic nerve.

There is no doubt that peripheral cytokines or central inflammatory stimuli can activate the brain. What is unknown is the role of central cytokines as signalling intermediates in the response to peripheral inflammation. The present experiments provided information on the subject by measuring cytokine levels in various brain areas at 1 hr post-injection of 1000μg of i.v. LPS. We proposed that since the majority of responses to LPS are initiated by 1 hr, then changes in cytokine levels in the brain at 1 hr post LPS may indicate a role for these molecules as central signalling intermediates. However, no differences were detected with respect to central cytokine levels between

any of the brain areas tested or in response to LPS. These results were consistent with Turnbull et al., who could inhibit the HPAA response to i.m. turpentine with the infusion of a central TNF antagonist despite not being able to detect changes in central TNF in response to peripheral turpentine [675]. This role of cytokines in central signalling pathways, especially pathways that are not immune specific, remains to be established [207,698,874]. One explanation for the effects of central cytokine antagonists on the response to peripheral inflammation could be that the molecules that are used to block central cytokines, are exerting effects irrespective of cytokine production as cytokine receptors are constitutively expressed in the brain. An interesting future study would be to block central TNF or IL-1 in response to LPS in mice where the cytokines or receptors for these molecules are knocked out. This would verify the specificity of these cytokine antagonists and hopefully help alleviate the controversy surrounding this issue. Turnbull et al., offered another explanation as to how the blockade of central cytokines can alter peripheral immune function. They found that centrally administered anti-TNF antibodies blocked the ACTH response to LPS through leakage into the periphery and neutralization of peripheral TNF [984]. However this could not be the only explanation because the infusion of a central TNF antagonist also blocked the ACTH response to turpentine, despite the fact that plasma TNF levels were below detectable limits [675]. It is also possible that the ability of central cytokines to influence peripheral immune function or the response to a peripheral immune stimulus can only occur with central inflammation. For example Woiciechowsky et al., found that brain injury can cause sympathetic storm

which increases inhibitory molecules like IL-10 in the periphery (probably via catecholamines) [926,927]. Therefore it is possible that the cannulation procedure, which is required to deliver substances to specific brain regions, is traumatic enough a stimulus for the induction of central cytokines and subsequent modification of the response to peripheral inflammation. Our own unpublished in situ hybridization studies support this notion as pro-inflammatory cytokines like IL-1 were produced along the cannula tract and increased at that location in response to i.c.v. LPS. As well, Woodroofe et al., demonstrated an increase in central cytokines following insertion of a microdialysis probe [687]. Therefore the central blockade of cytokines may be inhibiting the effects of central trauma on peripheral immune function and not the central signalling pathways that are exclusive to regulating to peripheral immune function. Proving this however is currently impossible because as mentioned, cannulation is the only procedure that allows the delivery of the various agonists and antagonists to specific brain areas.

Experiment 2: Effect of the SNS and HPAA on splenic cytokine in response to LPS. The main objective of the second experiment was to determine the individual and combined effect of splenic nerve cut and ADX on splenic cytokine production in response to various doses of LPS. This paradigm was originally used by Besedovsky et al., in the characterization of the central regulation of the antibody response to sheep red blood cells [48]. He found that both ADX and splenic nerve cut increased the response to sheep red blood cells and that the combination of the two procedures was additive. Our

main objective was to determine if this model was relevant to LPS-induced cytokine production. However we found that there was no effect of the splenic nerve on cytokine production, the effects ADX were minimal and there was no additive effect between these two procedures. These results suggest that although in vitro studies and studies utilizing pharmacological doses of agonists and antagonists can demonstrate the effects of the catecholamines and glucocorticoids on splenic cytokine production, the physiological in vivo situation is different. It is also possible that there was a potential nerve cut effect in response to high doses of LPS, but that it was masked due to the maximal activation of cytokine production. However if the SNS is an integral part of cytokine regulation in response to LPS, it would be expected that the nerve cut effect would be robust enough to be evident in one of the many scenarios we examined. It was not the case. This then raises the question that if the SNS and HPAA have the potential to regulate cytokine production and when is that regulation physiologically relevant or at least observable in vivo?

A clue may come from the studies of Molina and colleagues who found that sympathectomy can increase splenic cytokines in vivo if the stressor in question activates the SNS to a greater magnitude that the HPAA [353,973]. This may help explain our data as they found that the response to LPS activates the HPAA to a greater magnitude than the SNS. Interestingly, it has been found that repeated exposure to LPS reduces the corticosterone response [631,985,986] (however no measures of plasma catecholamines were made in those studies). Alternatively, McKechnie et al., demonstrated that in

response to a constant infusion of LPS for a 4 hr duration, NE levels did not drop over the course of the experiment despite the fact the E levels did [145]. Unfortunately, again plasma corticosterone levels were not measured in this study to support this contention. However clinical studies do support this possibility as 24% of septic shock patients suffer from adrenal insufficiency prior to glucocorticoid treatment [987]. Therefore in response to LPS, the nerve may function to inhibit cytokine production in more chronic scenarios. Future studies must determine if the splenic nerve could regulate splenic cytokine production in response to either a bacterial infection or constant infusion of LPS. Another clue as to when the control of cytokine production by the SNS is physiologically relevant are indicated the studies by Woiciechowsky et al. They found that brain injury can cause sympathetic storm which activates inhibitory molecules like IL-10 [926,927]. This would explain why Brown et al., using central IL-1 to mimic central inflammation and Pan et al., using central PGE2 could demonstrate a nerve cut effect on splenic cytokine production [89], while the experiments in this thesis, which used peripheral LPS, could not. Therefore one situation where the SNS may specifically function to inhibit cytokine production is when the brain itself is activated, such as in central inflammation or psychological stress, prior to the peripheral immune challenge.

It is clear from the studies by MacNeil et al., that the splenic nerve is preferentially activated in response to LPS and therefore is likely mediating some unique process in the spleen [76,645]. However since it does not appear to regulate cytokine production in response to LPS in non-stressed animals, then the questions remains as to

the nature of this unique signal. It is known that NE can alter cell movement irrespective of its effect on the cardiovascular system and that cutting the splenic nerve increased the weight of the spleen and altered the percentage of splenic T-cells per unit area. Since weight increased and the number of T-cells decreased per unit area in nerve cut animals, it is likely that the relative number of B cells and macrophages were increased in denervated spleens, while the total number of T-cells was constant in the enlarging spleen. However it is unknown if this effect was LPS-dependent [971] or represented a constitutive role of the nerve in cell trafficking.

There is no doubt that the innate and adaptive immune responses are in communication and can function autonomously from the CNS. However an interesting experiment would be to determine if the inflammatory response can influence the adaptive response via the SNS. This is based on the fact that LPS can selectively increase splenic nerve activity [76] despite the fact that the cutting the splenic nerve does not alter LPS-induced cytokine production. However since NE can affect the humeral response and cell trafficking, the increase in nerve activity may represent a signal to prime the adaptive immune system. Therefore it would be interesting to determine if the splenic nerve plays a role in the adaptive immune response to a foreign pathogen, and if this role is dependent on the potency of the initial inflammatory response (ie. if the splenic nerve was activated or not). Consistent with this, Zalcman et al., demonstrated that IL-2-induced potentiation of the response to SRBC was dependent on an intact splenic nerve [254].

With respect to LPS-induced splenic cytokine production, Besedovsky's feedback

model of neural immune regulation was not supported by our in vivo studies as neither the splenic nerve nor the adrenal glands significantly impacted on LPS-induced splenic cytokine production. Given the breadth of parameters in this study, it can safely be concluded that Besedovsky's model of inhibitory feedback can not be universally applied to all situations. It therefore remains to be determined if this feedback system is only relevant to adaptive immunity, measures of innate immunity not measured in our studies or are released to primarily correct perturbations of the metabolic and cardiovascular systems.

Experiment 3:the role of the SNS and HPAA in regulating the effects of stress on LPS-induced splenic cytokine production. Careful review of the available literature indicated that the nerve cut effect can be observed in paradigms where activation of the CNS was separated from a peripheral immune challenge. Therefore the objective of this experiment was to determine if the effects of stress on splenic cytokine production were transmitted by the splenic nerve. To accomplish this we utilized a paradigm where the animals were injected with a dose of LPS that did not induce a corticosterone response (0.1μg), immediately exposed to 15 minutes of footshock and killed 45 minutes later. The first study indicated that our results agreed with Goujon et al. [867] in that stress was immunosuppressive to cytokine mRNA and protein. This was most likely due to the appearance of glucocorticoids and catecholamines in the plasma prior to cytokine expression. It was also found that there were some discrepancies between concurrent

changes in levels of mRNA and protein, most notably with IL-1.

Cutting the splenic nerve in adrenal-intact animals did not abrogate the immunosuppressive effects of stress in our paradigm. Thus the objective of the next study was to determine if the stress-induced immune suppression of splenic cytokine production was entirely dependent on the adrenal glands. It was found that the magnitude of stress-induced suppression was comparable in ADX rats and adrenal-intact rats. Therefore neither the adrenal nor the splenic nerve alone mediates the immunosuppressive effects of stress on LPS-induced cytokine production. Although the pituitary gland was the likely source of immune suppression, Keller et al., had previously observed stress-induced immune suppression in HYPOX animals [58]. This information, combined with the fact that the splenic nerve activity was increased in ADX rats, lead us to believe that the SNS has a more predominant role than the HPAA in the stress response which occurs in the absence of the adrenal glands [378]. Therefore we looked at the effect of splenic nerve cut on stress-modified levels of splenic cytokine production in ADX rats.

Significantly, we found that cutting the splenic nerve abrogated the suppressive effects of stress on LPS-induced splenic cytokine production in ADX rats. Although it could not be unequivocally demonstrated that the nerve transmitted all the immunosuppressive effects of stress, the results clearly support this possibility. It is also interesting to consider how the SNS and adrenals are interacting in this situation. Given the fact that there was an ADX effect and a stress effect, the data suggests non redundant, and independent roles for catecholamines and glucocorticoids in this paradigm. For

example, removal of the adrenals increased baseline levels of some cytokines but did not impact on the magnitude of the stress effect. Also, splenic nerve cut alone did nothing. This suggests that either corticosterone and/or E are required for setting the ceiling of cytokine production in response to this dose of LPS, while catecholamines (or maybe just NE) are required for the stress effect. Thus it is possible that both the splenic nerve and the adrenals individually transmit immunosuppressive signals of sufficient magnitude to make the elimination of either one of these systems is insignificant. However it is also possible that there is an active compensation as described by MacNeil et al., who observed an increase in LPS-induced splenic nerve activity following acute ADX [378]. If such compensation does exist between the HPAA and SNS, is it due to actions at the post-ganglionic level, a lack of central glucocorticoid feedback or are other mechanisms involved? Would HYPOX superimposed on ADX and nerve cut increase cytokine production even more? Thus, similar to the dose response studies in experiment 1, it is important to investigate the events in the brain and /or periphery that are responsible for mediating this compensation as the organizational hierarchy appears to be fundamental to the stress response. Also, as described for experiment 2, it would be important to determine if the immune suppression of innate immunity applies to the adaptive immune response in a paradigm where both could be measured.

Although we demonstrated that the nerve could affect splenic cytokine production, it did so only after removal of the adrenals. Therefore in this paradigm the SNS may function as a biological emergency brake. Clinically, this backup system may be

critical in situations where adrenal function is lost, such as adrenal insufficiency brought on by chronic glucocorticoid excess, septic shock or other conditions such as tumors and Addison's disease [987-989].

Experiment 4: Characterization of the stress response when the exposure to stress precedes i.v. LPS. The final study thus set out to determine a scenario where the immunosuppressive effects of stress on splenic cytokine production could potentially be regulated by the splenic nerve in adrenal-intact animals. In an attempt to separate the immunosuppressive effects of stress from the HPAA-mediated response, we reversed the order of stress and LPS injection so that we could look at the more long-term effects of acute stress on splenic cytokine production while still measuring cytokine production at 1 hr post-LPS. We exposed the animals to stress 0, 4 and 24 hr prior to 0.1 μg of i.v. and measured splenic cytokines 1 hr post-injection. An additional series of studies were conducted to determine if the effects of stress 0, 4 and 24 hr prior to LPS injection were robust enough to suppress cytokines in response to 10μg of LPS, a dose that induces maximal splenic cytokine and plasma corticosterone levels. It was found that LPS-induced increases of splenic TNF and IL-1 expression were only suppressed when the animals were exposed to stress immediately prior to the LPS injection. No suppression was observed when the animals were exposed to stress 4 or 24 hr prior to LPS injections. The results were comparable in response to both 0.1μg and 10μg of i.v. LPS.

Although we were unable to a achieve our main goal of identifying an interval

during which the splenic nerve was potentially mediating the immunosuppressive effects of LPS on splenic TNF and IL-1 production separately from HPAA-mediated effects in adrenal-intact rats, this experiment generated some of the most interesting data in characterizing the stress response. In contrast to the original paradigm, it was found that if the sequence of stress exposure and LPS injection was reversed, so that the animal was exposed to stress prior to LPS, that splenic IL-6 levels were increased rather than suppressed. Plasma levels were also increased suggesting that this response was a systemic phenomenon. The ability of the stress response to drive such diverse reactions to what are seemingly very similar paradigms illustrates the dynamics of the regulatory system. These observations generate many additional questions regarding the interaction between stress and immunity. For example, do these results suggest that immune cells are committed to a certain path depending on whether they were first exposed to LPS or NE as suggested by Straub et al., [210]? Why are the sequence-dependent differential effects observed for IL-6 and not for TNF or IL-1? This suggests that IL-6 has an important function in the general response to stress, irrespective of the stressor, such as its role in regulating the acute phase response [45]. Importantly, these results lead to a series of experiments that test the nature of the stress response and the role of the SNS in the regulation of this system. For example it will be important to determine if the nerve is responsible for the potentiation of IL-6 in response to stress and if the role of the splenic nerve in potentiating IL-6 production is evident in the absence of LPS. Consistent with Besedovsky's model and prior studies in this thesis, the next set of experiments should

then determine the role of the adrenal gland in the stress-induced potentiation of IL-6 production and the interaction between the ADX and splenic nerve in this system. If either the nerve cut and/or ADX is found to regulate this response then future efforts should be concentrated on characterizing the central signals that mediate this effect. Otherwise the next experiments should determine the effects of habituation to chronic stress or conditioning on LPS-induced cytokine production and determine if the splenic nerve can mediate these effects in adrenal-intact animals.

Concluding remarks: Although it is clear that stress can regulate immune function, a larger question still remains as to whether this is by design or merely a side effect of the regulation of more critical systems such as blood pressure and metabolism. The fact that the stress response is not identical in response to different stressors suggests a potential for altering immune function by design. In agreement with this, Dhabhar and colleagues suggest that redistribution of lymphocytes to the periphery in response to stress is a critical part of the survival to a predator [317,358,359]. However the release and effects of glucocorticoid are rapid and highly transitory. Thus it is difficult to imagine that a system designed primarily to maintain homeostasis in response to short term stressors, would even consider the immune system a priority. Also, it is clear that the majority of the manuscripts (and almost all of the in vivo reports) that deal with the effects of stress on immunity, describe it as being a suppressive factor. This then questions the notion of immune regulation by stress as an adaptive response as their is no clear survival advantage

in having a suppressed immune system save for allergy and organ transplantation. It is therefore likely that the immune regulation occurs due to the chronic activation of a system that is meant to function in acute situations. Thus, the stress-immune relationship may represent a secondary consequence or by-stander effect as described by Cannon [2].

Relatively little has changed since Cannon proposed that secondary irrelevant effects of the stress response will materialize if an organism can not deal with stress [2]. This explanation is often invoked in that stress is often blamed for contributing, initiating, exacerbating or complicating many unrelated and diverse disease states (both physical and psychological), despite the fact that little is known regarding the mechanisms mediating these changes [8,982]. The detrimental effects of stress on health and immunity may be attributed to the fact that this regulatory system evolved to deal with situations rarely encountered in modern society. The fight or flight response served us well against predators, but in this day and age stresses are of a more chronic nature and often involve conceptual or perceived issues rather than physical threats. Therefore there is a physiological price to pay for chronically activating a system that is meant to function on an acute basis, and this price is paid at the expense of the organism's health.

What is known is that there is a primary link between the brain, immunity and the overall health of an individual. Thus, if stress is such a powerful phenomenon that it can exert its influence on diverse biological functions, then its impact on the health and economy of the world are incalculable. Therefore further funding and resources are required to better define the biochemical, anatomical and molecular processes which

control the response to stress and to devise strategies that may be better able to help the body defend itself against exogenous challenges.

The end

8. References

1. Dodd, J. and Role, L.W. The Autonomic Nervous System. In: Principals of Neural Science, edited by Kandel, E.R., Schwartz, J.H. and Jessel, T.M. Amsterdam: Elsevier, 1991, p. 761-775.

2. Kopin, I.J. Definitions of stress and sympathetic neuronal responses (1995). *Ann.N.Y.Acad.Sci.*771:19-30.

3. Herman, J.P. and Cullinan, W.E. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis (1997). *Trends.Neurosci.*20:78-84.

4. Cacioppo, J.T., Berntson, G.G., Malarkey, W.B., Kiecolt Glaser, J.K., Sheridan, J.F., Poehlmann, K.M., Burleson, M.H., Ernst, J.M., Hawkley, L.C. and Glaser, R. Autonomic, neuroendocrine, and immune responses to psychological stress: the reactivity hypothesis (1998). *Ann.N.Y.Acad.Sci.*840:664-673.

5. Cohen, S., Frank, E., Doyle, W.J., Skoner, D.P., Rabin, B.S. and Gwaltney, J.M.J. Types of stressors that increase susceptibility to the common cold in healthy adults [see comments] (1998). *Health Psychol.*17:214-223.

6. Olff, M. Stress, depression and immunity: the role of defense and coping styles [comment] (1999). *Psychiatry Res.*85:7-15.

7. De Gucht, V., Fischler, B. and Demanet, C. Immune dysfunction associated with chronic professional stress in nurses (1999). *Psychiatry Res.*85:105-111.

8. Stone, R. Stress:The invisible hand in eastern Europe's death rates (2000). *Science*288:1732-1733.

9. Cabot, J.B. Sympathetic Preganglionic Neurons: Cryoarchitecture, Ultrastructure and Biophysical Properties. In: *Central Regulation of Autonomic Functions*, edited by Loewy, A.D. and Spyer, K.M. New York: Oxford University Press, 1990, p. 44-67.

10. Selye, H. A Syndrome Produced by Diverse Nocous Agents (1936). *Nature*32:138.

11. Selye, H. Forty years of stress research: principal remaining problems and misconceptions (1976). *Can.Med.Assoc.J.*115:53-56.

12. Kvetnansky, R., Pacak, K., Sabban, E.L., Kopin, I.J. and Goldstein, D.S. Stressor specificity of peripheral catecholaminergic activation (1998). *Adv.Pharmacol.*42:556-560.

13. Ganong, W.F. Energy Balane, Metabolism and Nutrition. In: *Review of Medical Physiology*, Norwalk: Appleton & Lange, 1989, p. 233-265.

14. Kant, G.J., Bauman, R.A., Anderson, S.M. and Mougey, E.H. Effects of controllable vs. uncontrollable chronic stress on stress-responsive plasma hormones (1992). *Physiol.Behav.*51:1285-1288.

15. Muir, J.L. and Pfister, H.P. Time course of the corticosterone and prolactin response following predictable and unpredictable novelty stress in Rattus norvegicus (1987). *Physiol.Behav.*40:103-107.

16. Swenson, R.M. and Vogel, W.H. Plasma Catecholamine and corticosterone as well as brain catecholamine changes during coping in rats exposed to stressful footshock (1983). *Pharmacol.Biochem.Behav.*18:689-693.

17. Peters, M.L., Godaert, G.L., Ballieux, R.E., van Vliet, M., Willemsen, J.J., Sweep, F.C. and Heijnen, C.J. Cardiovascular and endocrine responses to experimental stress: effects of mental effort and controllability (1998). *Psychoneuroendocrinology.*23:1-17.

18. Menzaghi, F., Heinrichs, S.C., Pich, E.M., Weiss, F. and Koob, G.F. The role of limbic and hypothalamic corticotropin-releasing factor in behavioral responses to stress (1993). *Ann.N.Y.Acad.Sci.*697:142-154.

19. van Dijken, H.H., de Goeij, D.C., Sutanto, W., Mos, J., De Kloet, E.R. and Tilders, F.J. Short inescapable stress produces long-lasting changes in the brain-pituitary-adrenal axis of adult male rats (1993). *Neuroendocrinology*58:57-64.

20. Kant, G.J., Eggleston, T., Landman Roberts, L., Kenion, C.C., Driver, G.C. and Meyerhoff, J.L. Habituation to repeated stress is stressor specific (1985). *Pharmacol.Biochem.Behav.*22:631-634.

21. Licinio, J. and Wong, M.L. The role of inflammatory mediators in the biology of major depression: central nervous system cytokines modulate the biological substrate of depressive symptoms, regulate stress-responsive systems, and contribute to neurotoxicity and neuroprotection (1999). *Mol.Psychiatry*4:317-327.

22. Singh, L.K., Pang, X., Alexacos, N., Letourneau, R. and Theoharides, T.C. Acute immobilization stress triggers skin mast cell degranulation via corticotropin releasing hormone, neurotensin, and substance P: A link to neurogenic skin disorders (1999). *Brain Behav.Immun.*13:225-239.

23. Irwin, M. Immune correlates of depression (1999). *Adv.Exp.Med.Biol.*461:1-24.

24. Cohen, F., Kearney, K.A., Zegans, L.S., Kemeny, M.E., Neuhaus, J.M. and Stites, D.P. Differential immune system changes with acute and persistent stress for optimists vs pessimists (1999). *Brain Behav.Immun.*13:155-174.

25. Miller, G.E., Cohen, S., Rabin, B.S., Skoner, D.P. and Doyle, W.J. Personality and tonic cardiovascular, neuroendocrine, and immune parameters (1999). *Brain Behav.Immun.*13:109-123.

26. Solomon, G.F. Emotions, stress, the central nervous system, and immunity (1969). *Ann.N.Y.Acad.Sci.*164:335-343.

27. Solomon, G.F. Stress and antibody response in rats (1969). *Int.Arch.Allergy.Appl.Immunol.*35:97-104.

28. Feldman, S., Saphier, D. and Weidenfeld, J. Corticosterone implants in the paraventricular nucleus inhibit ACTH and corticosterone responses and the release of corticotropin-releasing factor following neural stimuli (1992). *Brain Res.*578:251-255.

29. Ovadia, H., Abramsky, O., Barak, V., Conforti, N., Saphier, D. and Weidenfeld, J.

Effect of interleukin-1 on adrenocortical activity in intact and hypothalamic deafferentated male rats (1989). *Exp.Brain Res.*76:246-249.

30. Deuchars, S.A., Spyer, K.M. and Gilbey, M.P. Stimulation within the rostral ventrolateral medulla can evoke monosynaptic GABAergic IPSPs in sympathetic preganglionic neurons in vitro (1997). *J.Neurophysiol.*77:229-235.

31. Nappi, R.E., Bonneau, M.J. and Rivest, S. Influence of the estrous cycle on c-fos and CRH gene transcription in the brain of endotoxin-challenged female rats (1997). *Neuroendocrinology*65:29-46.

32. Wan, W., Janz, L., Vriend, C.Y., Sorensen, C.M., Greenberg, A.H. and Nance, D.M. Differential induction of c-Fos immunoreactivity in hypothalamus and brain stem nuclei following central and peripheral administration of endotoxin (1993). *Brain Res.Bull.*32:581-587.

33. Dunn, A.J. and Swiergiel, A.H. The role of cytokines in infection-related behavior (1998). *Ann.N.Y.Acad.Sci.*840:577-585.

34. Dunn, A.J. Role of cytokines in infection-induced stress (1993). *Ann.N.Y.Acad.Sci.*697:189-202.

35. Horai, R., Asano, M., Sudo, K., Kanuka, H., Suzuki, M., Nishihara, M., Takahashi, M. and Iwakura, Y. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion (1998). *J.Exp.Med.*187:1463-1475.

36. Dunn, A.J., Powell, M.L., Meitin, C. and Small, P.A.,Jr. Virus infection as a stressor: influenza virus elevates plasma concentrations of corticosterone, and brain concentrations of MHPG and tryptophan (1989). *Physiol.Behav.*45:591-594.

37. Hermann, G., Tovar, C.A., Beck, F.M. and Sheridan, J.F. Kinetics of glucocorticoid response to restraint stress and/or experimental influenza viral infection in two inbred strains of mice (1994). *J.Neuroimmunol.*49:25-33.

38. Dunn, A.J. Stress-related activation of cerebral dopaminergic systems (1988). *Ann.N.Y.Acad.Sci.*537:188-205.

39. Chuluyan, H.E., Saphier, D., Rohn, W.M. and Dunn, A.J. Noradrenergic innervation of the hypothalamus participates in adrenocortical responses to interleukin-1 (1992). *Neuroendocrinology*56:106-111.

40. Feldman, S., Conforti, N. and Saphier, D. The preoptic area and bed nucleus of the stria terminalis are involved in the effects of the amygdala on adrenocortical secretion (1990). *Neuroscience.*37:775-779.

41. Li, H.Y., Ericsson, A. and Sawchenko, P.E. Distinct mechanisms underlie activation of hypothalamic neurosecretory neurons and their medullary catecholaminergic afferents in categorically different stress paradigms (1996). *Proc.Natl.Acad.Sci.U.S.A.*93:2359-2364.

42. Swiergiel, A.H., Smagin, G.N. and Dunn, A.J. Influenza virus infection of mice induces anorexia: comparison with endotoxin and interleukin-1 and the effects of indomethacin (1997). *Pharmacol.Biochem.Behav.*57:389-396.

43. Kumar, V., Cotran, R.S. and Robbins, S.L. Acute and Chronic Inflammation. In: *Basic Pathology*, edited by Kumar, V., Cotran, R.S. and Robbins, S.L. Philadelphia: W.B. Saunders, 1992, p. 61-46.

44. Abbas, A.K., Lichtman, A.H. and Pber, J.S. General Properties of Immune Responses. In: *Cellular and Molecular Immunology*, Philadelphia: W.B. Saunders, 1997, p. 4-14.

45. Abbas, A.K., Lichtman, A.H. and Pber, J.S. Cytokines. In: *Cellular and Molecular Immunology*, Philadelphia: W.B. Saunders, 1997, p. 250-277.

46. Abbas, A.K., Lichtman, A.H. and Pber, J.S. Immunity to Microbes. In: *Cellular and Molecular Immunology*, Philadelphia: W.B. Saunders, 1997, p. 342-361.

47. Solomon, G.F., Merigan, T.C. and Levine, S. Variation in adrenal cortical hormones within physiologic ranges, stress and interferon production in mice (1966). *Riv.Patol.Nerv.Ment.*87:74-79.

48. Besedovsky, H.O., del Rey, A., Sorkin, E., Da Prada, M. and Keller, H.H. Immunoregulation mediated by the sympathetic nervous system (1979). *Cell Immunol.*48:346-355.

49. Besedovsky, H., Sorkin, E., Felix, D. and Haas, H. Hypothalamic changes during the immune response (1977). *Eur.J.Immunol.*7:323-325.

50. Besedovsky, H., del Rey, A., Sorkin, E., Da Prada, M., Burri, R. and Honegger, C. The immune response evokes changes in brain noradrenergic neurons (1983). *Science*221:564-566.

51. Brooks, W.H., Cross, R.J., Roszman, T.L. and Markesbery, W.R. Neuroimmunomodulation: neural anatomical basis for impairment and facilitation (1982). *Ann.Neurol.*12:56-61.

52. Cross, R.J., Brooks, W.H., Roszman, T.L. and Markesbery, W.R. Hypothalamic-immune interactions. Effect of hypophysectomy on neuroimmunomodulation (1982). *J.Neurol.Sci.*53:557-566.

53. Cross, R.J., Markesbery, W.R., Brooks, W.H. and Roszman, T.L. Hypothalamic-immune interactions. I. The acute effect of anterior hypothalamic lesions on the immune response (1980). *Brain Res.*196:79-87.

54. Irwin, M. Stress-induced immune suppression. Role of the autonomic nervous system (1993). *Ann.N.Y.Acad.Sci.*697:203-218.

55. Cohen, N., Moynihan, J.A. and Ader, R. Pavlovian conditioning of the immune system (1994). *Int.Arch.Allergy.Immunol.*105:101-106.

56. Keller, S.E., Weiss, J.M., Schleifer, S.J., Miller, N.E. and Stein, M. Stress-induced

suppression of immunity in adrenalectomized rats (1983). *Science*221:1301-1304.

57. Keller, S.E., Weiss, J.M., Schleifer, S.J., Miller, N.E. and Stein, M. Suppression of immunity by stress: effect of a graded series of stressors on lymphocyte stimulation in the rat (1981). *Science*213:1397-1400.

58. Keller, S.E., Schleifer, S.J., Liotta, A.S., Bond, R.N., Farhoody, N. and Stein, M. Stress-induced alterations of immunity in hypophysectomized rats (1988). *Proc.Natl.Acad.Sci.U.S.A.*85:9297-9301.

59. Williams, J.M. and Felten, D.L. Sympathetic innervation of murine thymus and spleen: a comparative histofluorescence study (1981). *Anat.Rec.*199:531-542.

60. Williams, J.M., Peterson, R.G., Shea, P.A., Schmedtje, J.F., Bauer, D.C. and Felten, D.L. Sympathetic innervation of murine thymus and spleen: evidence for a functional link between the nervous and immune systems (1981). *Brain Res.Bull.*6:83-94.

61. Felten, D.L., Felten, S.Y., Bellinger, D.L., Carlson, S.L., Ackerman, K.D., Madden, K.S., Olschowki, J.A. and Livnat, S. Noradrenergic sympathetic neural interactions with the immune system: structure and function (1987). *Immunol.Rev.*100:225-260.

62. Romano, T.A., Felten, S.Y., Felten, D.L. and Olschowka, J.A. Neuropeptide-Y innervation of the rat spleen: another potential immunomodulatory neuropeptide (1991). *Brain Behav.Immun.*5:116-131.

63. Ackerman, K.D., Felten, S.Y., Dijkstra, C.D., Livnat, S. and Felten, D.L. Parallel development of noradrenergic innervation and cellular compartmentation in the rat spleen (1989). *Exp.Neurol.*103:239-255.

64. Felten, D.L., Ackerman, K.D., Wiegand, S.J. and Felten, S.Y. Noradrenergic sympathetic innervation of the spleen: I. Nerve fibers associate with lymphocytes and macrophages in specific compartments of the splenic white pulp (1987). *J.Neurosci.Res.*18:28-36, 118.

65. Nance, D.M. and Burns, J. Innervation of the spleen in the rat: evidence for absence of afferent innervation (1989). *Brain Behav.Immun.*3:281-290.

66. Abrass, C.K., O'Connor, S.W., Scarpace, P.J. and Abrass, I.B. Characterization of the beta-adrenergic receptor of the rat peritoneal macrophage (1985). *J.Immunol.*135:1338-1341.

67. Miles, K., Atweh, S., Otten, G., Arnason, B.G. and Chelmicka Schorr, E. Beta-adrenergic receptors on splenic lymphocytes from axotomized mice (1984). *Int.J.Immunopharmacol.*6:171-177.

68. Miles, K., Chelmicka Schorr, E., Atweh, S., Otten, G. and Arnason, B.G. Sympathetic ablation alters lymphocyte membrane properties (1985). *J.Immunol.*135:797s-801s.

69. Ganong, W.F. The Adrenal Medulla and Cortex. In: *Review of Medical Physiology*, Norwalk: Appleton & Lange, 1989, p. 301-325.

70. Levine, J.D., Clark, R., Devor, M., Helms, C., Moskowitz, M.A. and Basbaum, A.I. Intraneuronal substance P contributes to the severity of experimental arthritis (1984). *Science*226:547-549.

71. del Rey, A. and Besedovsky, H. Interleukin 1 affects glucose homeostasis (1987). *Am.J.Physiol.*253:R794-R798.

72. Terao, A., Kitamura, H., Asano, A., Kobayashi, M. and Saito, M. Roles of prostaglandins D2 and E2 in interleukin-1-induced activation of norepinephrine turnover in the brain and peripheral organs of rats (1995). *J.Neurochem.*65:2742-2747.

73. Kannan, H., Tanaka, Y., Kunitake, T., Ueta, Y., Hayashida, Y. and Yamashita, H. Activation of sympathetic outflow by recombinant human interleukin-1 beta in conscious rats (1996). *Am.J.Physiol.*270:R479-R485.

74. Niijima, A., Hori, T., Aou, S. and Oomura, Y. The effects of interleukin-1 beta on the activity of adrenal, splenic and renal sympathetic nerves in the rat (1991). *J.Auton.Nerv.Syst.*36:183-192.

75. Takahashi, H., Nishimura, M., Sakamoto, M., Ikegaki, I., Nakanishi, T. and Yoshimura, M. Effects of interleukin-1 beta on blood pressure, sympathetic nerve activity, and pituitary endocrine functions in anesthetized rats (1992). *Am.J.Hypertens.*5:224-229.

76. MacNeil, B.J., Jansen, A.H., Greenberg, A.H. and Nance, D.M. Activation and selectivity of splenic sympathetic nerve electrical activity response to bacterial endotoxin (1996). *Am.J.Physiol.*270:R264-R270.

77. Jones, S.B. and Romano, F.D. Plasma catecholamines in the conscious rat during endotoxicosis (1984). *Circ.Shock.*14:189-201.

78. Sapolsky, R., Rivier, C., Yamamoto, G., Plotsky, P. and Vale, W. Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor (1987). *Science*238:522-524.

79. Berkenbosch, F., van Oers, J., del Rey, A., Tilders, F. and Besedovsky, H. Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1 (1987). *Science*238:524-526.

80. Saphier, D., Mor, G., Ovadia, H., Maimon, A. and Abramsky, O. Absence of neural responses following suppression of the immune response by cyclophosphamide (1991). *Int.J.Neurosci.*56:277-282.

81. Saphier, D., Ovadia, H. and Abramsky, O. Neural responses to antigenic challenges and immunomodulatory factors (1990). *Yale J.Biol.Med.*63:109-119.

82. Kent, S., Kelley, K.W. and Dantzer, R. Effects of lipopolysaccharide on food-motivated behavior in the rat are not blocked by an interleukin-1 receptor antagonist (1992). *Neurosci.Lett.*145:83-86.

83. Kluger, M.J., Kozak, W., Leon, L.R., Soszynski, D. and Conn, C.A. Cytokines and fever (1995). *Neuroimmunomodulation.*2:216-223.

84. Saper, C.B. and Breder, C.D. The neurologic basis of fever [see comments] (1994). *N.Engl.J.Med.*330:1880-1886.

85. Kluger, M.J. Fever: role of pyrogens and cryogens (1991). *Physiol.Rev.*71:93-127.

86. Farrar, W.L., Kilian, P.L., Ruff, M.R., Hill, J.M. and Pert, C.B. Visualization and characterization of interleukin 1 receptors in brain (1987). *J.Immunol.*139:459-463.

87. Sundar, S.K., Cierpial, M.A., Kilts, C., Ritchie, J.C. and Weiss, J.M. Brain IL-1-induced immunosuppression occurs through activation of both pituitary-adrenal axis and sympathetic nervous system by corticotropin-releasing factor (1990). *J.Neurosci.*10:3701-3706.

88. Sundar, S.K., Becker, K.J., Cierpial, M.A., Carpenter, M.D., Rankin, L.A., Fleener, S.L., Ritchie, J.C., Simson, P.E. and Weiss, J.M. Intracerebroventricular infusion of interleukin 1 rapidly decreases peripheral cellular immune responses (1989). *Proc.Natl.Acad.Sci.U.S.A.*86:6398-6402.

89. Brown, R., Li, Z., Vriend, C.Y., Nirula, R., Janz, L., Falk, J., Nance, D.M., Dyck, D.G. and Greenberg, A.H. Suppression of splenic macrophage interleukin-1 secretion following intracerebroventricular injection of interleukin-1 beta: evidence for pituitary-adrenal and sympathetic control (1991). *Cell Immunol.*132:84-93.

90. Wan, W., Wetmore, L., Sorensen, C.M., Greenberg, A.H. and Nance, D.M. Neural and biochemical mediators of endotoxin and stress-induced c-fos expression in the rat brain (1994). *Brain Res.Bull.*34:7-14.

91. Levine, J.D., Dardick, S.J., Roizen, M.F., Helms, C. and Basbaum, A.I. Contribution of sensory afferents and sympathetic efferents to joint injury in experimental arthritis (1986). *J.Neurosci.*6:3423-3429.

92. Levine, J.D., Goetzl, E.J. and Basbaum, A.I. Contribution of the nervous system to the pathophysiology of rheumatoid arthritis and other polyarthritides (1987). *Rheum.Dis.Clin.North Am.*13:369-383.

93. Li, H.Y. and Sawchenko, P.E. Hypothalamic effector neurons and extended circuitries activated in "neurogenic" stress: a comparison of footshock effects exerted acutely, chronically, and in animals with controlled glucocorticoid levels (1998). *J.Comp.Neurol.*393:244-266.

94. Swanson, L.W. The Hypothalamus. In: *Chemical Neuroanatomy, Volume 5. Intergrated Systems of the CNS. Part I*, edited by Bjorkland, A., Hokfelt, T. and Swanson, L.W. Amsterdam: Elsevier, 1987, p. 1-124.

95. Sawchenko, P.E., Brown, E.R., Chan, R.K., Ericsson, A., Li, H.Y., Roland, B.L. and Kovacs, K.J. The paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stress (1996). *Prog.Brain Res.*107:201-222.

96. Gilman, . and Winans Newman, . Chemical Neuroanatomy. In: *Clinical Neuroanatomy and Neurophysiology*, Philadelphia: F.A. Davis Company, 1992, p. 250-261.

97. *Handbook of Chemical Neuroanatomy Volume 2: Classical Transmitters in the CNS Part 1*, 1984. Elsevier, Amsterdam.

98. *Handbook of Chemical Neuroanatomy Volume 3: Classical Transmitters and Transmitter Receptors in the CNS Part 2*, 1984. Elsevier, Amsterdam.

99. Hokfelt, T., Martensson, R., Bjorkland, A., Kleinau, S. and Goldstein, M. Distribution Maps of Tyrosine-hydroxylase-immunoreactive Neurons in the Rat Brain. In: *Handbook of Chemical Neuroanatomy Volume 2: Classical Transmitters in the CNS Part 1*, edited by Bjorkland, A. and Hokfelt, T. Amsterdam: Elsevier, 1984, p. 277-378.

100. Moore, R.Y. and Card, J.P. Noradrenaline-containing Neuron Systems. In: *Handbook of Chemical Neuroanatomy Volume 2: Classical Transmitters in the CNS Part 1*, edited by Bjorkland, A. and Hokfelt, T. Amsterdam: Elsevier, 1984, p. 123-156.

101. Sawchenko, P.E., Swanson, L.W., Grzanna, R., Howe, P.R., Bloom, S.R. and Polak, J.M. Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nucleus of the hypothalamus (1985). *J.Comp.Neurol.*241:138-153.

102. Cunningham, E.T.,Jr., Bohn, M.C. and Sawchenko, P.E. Organization of adrenergic inputs to the paraventricular and supraoptic nuclei of the hypothalamus in the rat (1990). *J.Comp.Neurol.*292:651-667.

103. Sawchenko, P.E., Arias, C. and Bittencourt, J.C. Inhibin beta, somatostatin, and enkephalin immunoreactivities coexist in caudal medullary neurons that project to the paraventricular nucleus of the hypothalamus (1990). *J.Comp.Neurol.*291:269-280.

104. Guyenet, P.G. Central noradrenergic neurons: the autonomic connection (1991). *Prog.Brain Res.*88:365-380.

105. Jackson, A.T.K. *Masters Thesis: Chemical Specificity of Endotoxin-induced C-fos Expressing Neurons in the Rat Hypothalamus*, 1999. Department of Physiology, University of Manitoba, Winnipeg, Canada.

106. Saphier, D. Electrophysiology and neuropharmacology of noradrenergic projections to rat PVN magnocellular neurons (1993). *Am.J.Physiol.*264:R891-R902.

107. Saphier, D. and Feldman, S. Catecholaminergic projections to tuberoinfundibular neurones of the paraventricular nucleus: III. Effects of adrenoceptor agonists and antagonists (1991). *Brain Res.Bull.*26:863-870.

108. Saphier, D. Catecholaminergic projections to tuberoinfundibular neurones of the paraventricular nucleus: I. Effects of stimulation of A1, A2, A6 and C2 cell groups (1989). *Brain Res.Bull.*23:389-395.

109. Ericsson, A., Arias, C. and Sawchenko, P.E. Evidence for an intramedullary prostaglandin-dependent mechanism in the activation of stress-related neuroendocrine circuitry by intravenous interleukin-1 (1998). *J.Neuro.*

110. Duan, Y.F., Kopin, I.J. and Goldstein, D.S. Stimulation of the paraventricular nucleus modulates firing of neurons in the nucleus of the solitary tract (1999). *Am.J.Physiol.*277:R403-R411.

111. Haas, H.S. and Schauenstein, K. Neuroimmunomodulation via limbic structures--the neuroanatomy of psychoimmunology (1997). *Prog.Neurobiol.*51:195-222.

112. Prewitt, C.M. and Herman, J.P. Anatomical interactions between the central amygdaloid nucleus and the hypothalamic paraventricular nucleus of the rat: a dual tract-tracing analysis (1998). *J.Chem.Neuroanat.*15:173-185.

113. Petrov, T., Krukoff, T.L. and Jhamandas, J.H. Convergent influence of the central nucleus of the amygdala and the paraventricular hypothalamic nucleus upon brainstem autonomic neurons as revealed by c-fos expression and anatomical tracing (1995). *J.Neurosci.Res.*42:835-845.

114. Veening, J.G., Swanson, L.W. and Sawchenko, P.E. The organization of projections from the central nucleus of the amygdala to brainstem sites involved in central autonomic regulation: a combined retrograde transport-immunohistochemical study (1984). *Brain Res.*303:337-357.

115. Mulders, W.H., Meek, J., Hafmans, T.G. and Cools, A.R. Plasticity in the stress-regulating circuit: decreased input from the bed nucleus of the stria terminalis to the hypothalamic paraventricular nucleus in Wistar rats following adrenalectomy (1997). *Eur.J.Neurosci.*9:2462-2471.

116. Cullinan, W.E., Herman, J.P. and Watson, S.J. Ventral subicular interaction with the hypothalamic paraventricular nucleus: evidence for a relay in the bed nucleus of the stria terminalis (1993). *J.Comp.Neurol.*332:1-20.

117. Sanchez, M.M., Aguado, F., Sanchez-Toscano, F. and Saphier, D. Adrenalectomy alters the response of neurons in the bed nucleus of the stria terminalis to electrical stimulation of the medial amygdala (1995). *Brain Res.Bull.*36:63-69.

118. Johnson, A.K. and Loewy, A.D. Circumventricular Organs and Their Role in Visceral Functions. In: *Central Regulation of Autonomic Functions*, edited by Loewy, A.D. and Spyer, K.M. New York: Oxford University Press, 1990, p. 247-267.

119. Cunningham, E.T.,Jr., Miselis, R.R. and Sawchenko, P.E. The relationship of efferent projections from the area postrema to vagal motor and brain stem catecholamine-containing cell groups: an axonal transport and immunohistochemical study in the rat (1994). *Neuroscience.*58:635-648.

120. Cunningham, E.T.,Jr. and Sawchenko, P.E. Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus (1988). *J.Comp.Neurol.*274:60-76.

121. Saphier, D. and Feldman, S. Effects of neural stimuli on paraventricular nucleus neurones (1985). *Brain Res.Bull.*14:401-407.

122. Saphier, D. and Feldman, S. Catecholaminergic projections to tuberoinfundibular

neurones of the paraventricular nucleus: III. Effects of adrenoceptor agonists and antagonists (1991). *Brain Res.Bull.*26:863-870.

123. Smith, J.E., Jansen, A.S., Gilbey, M.P. and Loewy, A.D. CNS cell groups projecting to sympathetic outflow of tail artery: neural circuits involved in heat loss in the rat (1998). *Brain Res.*786:153-164.

124. Strack, A.M., Sawyer, W.B., Platt, K.B. and Loewy, A.D. CNS cell groups regulating the sympathetic outflow to adrenal gland as revealed by transneuronal cell body labeling with pseudorabies virus (1989). *Brain Res.*491:274-296.

125. Strack, A.M., Sawyer, W.B., Hughes, J.H., Platt, K.B. and Loewy, A.D. A general pattern of CNS innervation of the sympathetic outflow demonstrated by transneuronal pseudorabies viral infections (1989). *Brain Res.*491:156-162.

126. Ding, Z.Q., Li, Y.W., Wesselingh, S.L. and Blessing, W.W. Transneuronal labelling of neurons in rabbit brain after injection of herpes simplex virus type 1 into the renal nerve (1993). *J.Auton.Nerv.Syst.*42:23-31.

127. Taylor, R.B. and Weaver, L.C. Spinal stimulation to locate preganglionic neurons controlling the kidney, spleen, or intestine (1992). *Am.J.Physiol.*263:H1026-H1033.

128. Pyner, S. and Coote, J.H. Evidence that sympathetic preganglionic neurones are arranged in target-specific columns in the thoracic spinal cord of the rat (1994). *J.Comp.Neurol.*342:15-22.

129. Appel, N.M. and Elde, R.P. The intermediolateral cell column of the thoracic spinal cord is comprised of target-specific subnuclei: evidence from retrograde transport studies and immunohistochemistry (1988). *J.Neurosci.*8:1767-1775.

130. Sawchenko, P.E. and Swanson, L.W. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat (1982). *J.Comp.Neurol.*205:260-272.

131. Swanson, L.W. and McKellar, S. The distribution of oxytocin- and neurophysin-stained fibers in the spinal cord of the rat and monkey (1979). *J.Comp.Neurol.*188:87-106.

132. Holets, V. and Elde, R. The differential distribution and relationship of serotoninergic and peptidergic fibers to sympathoadrenal neurons in the intermediolateral cell column of the rat: a combined retrograde axonal transport and immunofluorescence study (1982). *Neuroscience.*7:1155-1174.

133. MacNeil, B.J., Jansen, A.H., Greenberg, A.H. and Nance, D.M. Neuropeptide Involvement in Splenic and Renal Sympathetic Nerve Activity Induced by Central Injection of PGE2 (1997). *Psyconeuroimmunology Research Society Abstracts*P1.30.(Abstract)

134. Katzung, B.G. Introduction to Autonomic Pharmacology. In: *Basic and Clinical Pharmacology*, edited by Katzung, B.G. Norwalk: Appleton and Lange, 1995, p. 71-101.

135. Mueller, R.A., Thoenen, H. and Axelrod, J. Adrenal tyrosine hydroxylase:

compensatory increase in activity after chemical sympathectomy (1969). *Science*163:468-469.

136. Song, C., Earley, B. and Leonard, B.E. The effects of central administration of neuropeptide Y on behavior, neurotransmitter, and immune functions in the olfactory bulbectomized rat model of depression (1996). *Brain Behav.Immun.* 10:1-16.

137. Song, C. and Leonard, B.E. Comparison between the effects of sigma receptor ligand JO 1784 and neuropeptide Y on immune functions (1998). *Eur.J.Pharmacol.*345:79-87.

138. Friedman, E.M., Irwin, M.R. and Nonogaki, K. Neuropeptide Y inhibits in vivo specific antibody production in rats (1995). *Brain Behav.Immun.*9:182-189.

139. Nair, M.P., Schwartz, S.A., Wu, K. and Kronfol, Z. Effect of neuropeptide Y on natural killer activity of normal human lymphocytes (1993). *Brain Behav.Immun.*7:70-78.

140. vonHorsten, S., Ballof, J., Helfritz, F., Nave, H., Meyer, D., Schmidt, R.E., Stalp, M., Klemm, A., Tschernig, T. and Pabst, R. modulation of innate immune functions by intracerebrovenrticularly applied neuropeptide y: dose and time dependent effects (1998). *Life Sciences*63:909-922.

141. Hoffman, B.B Adrenoreceptor-Activating and Other Sympathomimetic Drugs. In: *Basic and Clinical Pharmacology*, edited by Katzung, B.G. Norwalk: Appleton and Lange, 1995, p. 115-131.

142. Micalizzi, E.R. and Pals, D.T. Evaluation of plasma norepinephrine as an index of sympathetic neuron function in the conscious, unrestrained rat (1979). *Life.Sci.*24:2071-2076.

143. Goldstein, D.S., McCarty, R., Polinsky, R.J. and Kopin, I.J. Relationship between plasma norepinephrine and sympathetic neural activity (1983). *Hypertension*5:552-559.

144. Shimizu, N., Kaizuka, Y., Hori, T. and Nakane, H. Immobilization increases norepinephrine release and reduces NK cytotoxicity in spleen of conscious rat (1996). *Am.J.Physiol.*271:R537-R544.

145. McKechnie, K., Dean, H.G., Furman, B.L. and Parratt, J.R. Plasma catecholamines during endotoxin infusion in conscious unrestrained rats: effects of adrenal demedullation and/or guanethidine treatment (1985). *Circ.Shock.*17:85-94.

146. Zhou, Z.Z. and Jones, S.B. Involvement of central vs. peripheral mechanisms in mediating sympathoadrenal activation in endotoxic rats (1993). *Am.J.Physiol.*265:R683-R688.

147. Qi, M., Zhou, Z.Z., Wurster, R.D. and Jones, S.B. Mechanisms involved in the rapid dissipation of plasma epinephrine response to bacterial endotoxin in conscious rats (1991). *Am.J.Physiol.*261:R1431-R1437.

148. Jones, S.B. and Romano, F.D. Dose- and time-dependent changes in plasma catecholamines in response to endotoxin in conscious rats (1989). *Circ.Shock.*28:59-68.

149. Morgan, A.E. and Lang, C.H. involvement of capsaicin-sensitive nerves in regulating the hormone and glucose metabolic response to endotoxin (1997). *American Journal Of Physiology Endocrinology And Metabolism*36:E328-E335.

150. Elenkov, I.J. and Vizi, E.S. Presynaptic modulation of release of noradrenaline from the sympathetic nerve terminals in the rat spleen (1991). *Neuropharmacology.*30:1319-1324.

151. Meltzer, J.C., Grimm, P.C., Greenberg, A.H. and Nance, D.M. Enhanced immunohistochemical detection of autonomic nerve fibers, cytokines and inducible nitric oxide synthase by light and fluorescent microscopy in rat spleen (1997). *J.Histochem.Cytochem.*45:599-610.

152. Sanders, V.M., Baker, R.A., Ramer-Quinn, D.S., Kasprowicz, D.J., Fuchs, B.A. and Street, N.E. Differential expression of the beta2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help (1997). *J.Immunol.*158:4200-4210.

153. Ramer-Quinn, D.S., Baker, R.A. and Sanders, V.M. Activated T helper 1 and T helper 2 cells differentially express the beta-2-adrenergic receptor: a mechanism for selective modulation of T helper 1 cell cytokine production (1997). *J.Immunol.*159:4857-4867.

154. Cook Mills, J.M., Cohen, R.L., Perlman, R.L. and Chambers, D.A. Inhibition of lymphocyte activation by catecholamines: evidence for a non-classical mechanism of catecholamine action (1995). *Immunology.*85:544-549.

155. Saito, M., Minokoshi, Y. and Shimazu, T. Accelerated norepinephrine turnover in peripheral tissues after ventromedial hypothalamic stimulation in rats (1989). *Brain Res.*481:298-303.

156. Okamoto, S., Ibaraki, K., Hayashi, S. and Saito, M. Ventromedial hypothalamus suppresses splenic lymphocyte activity through sympathetic innervation (1996). *Brain Res.*739:308-313.

157. Brown, M.R., Fisher, L.A., Spiess, J., Rivier, C., Rivier, J. and Vale, W. Corticotropin-releasing factor: actions on the sympathetic nervous system and metabolism (1982). *Endocrinology*111:928-931.

158. Rassnick, S., Sved, A.F. and Rabin, B.S. Locus coeruleus stimulation by corticotropin-releasing hormone suppresses in vitro cellular immune responses (1994). *J.Neurosci.*14:6033-6040.

159. Cross, R.J., Markesbery, W.R., Brooks, W.H. and Roszman, T.L. Hypothalamic-immune interactions: neuromodulation of natural killer activity by lesioning of the anterior hypothalamus (1984). *Immunology.*51:399-405.

160. Katafuchi, T., Ichijo, T., Take, S. and Hori, T. Hypothalamic modulation of splenic natural killer cell activity in rats (1993). *J.Physiol.Lond.*471:209-221.

161. Katafuchi, T., Take, S. and Hori, T. Roles of sympathetic nervous system in the suppression of cytotoxicity of splenic natural killer cells in the rat (1993). *J.Physiol.Lond.*465:343-357.

162. Take, S., Mori, T., Katafuchi, T. and Hori, T. Central interferon-alpha inhibits natural killer cytotoxicity through sympathetic innervation (1993). *Am.J.Physiol.*265:R453-R459.

163. Irwin, M., Hauger, R.L., Jones, L., Provencio, M. and Britton, K.T. Sympathetic nervous system mediates central corticotropin-releasing factor induced suppression of natural killer cytotoxicity (1990). *J.Pharmacol.Exp.Ther.*255:101-107.

164. Shakhar, G. and Ben Eliyahu, S. In vivo beta-adrenergic stimulation suppresses natural killer activity and compromises resistance to tumor metastasis in rats (1998). *J.Immunol.* 160:3251-3258.

165. Schedlowski, M., Hosch, W., Oberbeck, R., Benschop, R.J., Jacobs, R., Raab, H.R. and Schmidt, R.E. Catecholamines modulate human NK cell circulation and function via spleen-independent beta 2-adrenergic mechanisms (1996). *J.Immunol.* 156:93-99.

166. Hellstrand, K., Hermodsson, S. and Strannegard, O. Evidence for a beta-adrenoceptor-mediated regulation of human natural killer cells (1985). *J.Immunol.* 134:4095-4099.

167. Carlson, S.L., Fox, S. and Abell, K.M. Catecholamine modulation of lymphocyte homing to lymphoid tissues (1997). *Brain Behav.Immun.* 11:307-320.

168. Sudo, N., Yu, X.N., Sogawa, H. and Kubo, C. Restraint stress causes tissue-specific changes in the immune cell distribution (1997). *Neuroimmunomodulation.*4:113-119.

169. Altenburg, S.P., Martins, M.A., Silva, A.R., Cordeiro, R.S.B. and CastroFariaNeto, H.C. Ips-induced blood neutrophilia is inhibited by alpha(1)-adrenoceptor antagonists: a role for catecholamines (1997). *Journal Of Leukocyte Biology*61:689-694.

170. Carlson, S.L., Beiting, D.J., Kiani, C.A., Abell, K.M. and McGillis, J.P. Catecholamines decrease lymphocyte adhesion to cytokine-activated endothelial cells (1996). *Brain Behav.Immun.* 10:55-67.

171. Madden, K.S., Felten, S.Y., Felten, D.L., Hardy, C.A. and Livnat, S. Sympathetic nervous system modulation of the immune system. II. Induction of lymphocyte proliferation and migration in vivo by chemical sympathectomy (1994). *J.Neuroimmunol.*49:67-75.

172. Rogausch, H., del Rey, A., Oertel, J. and Besedovsky, H.O. Norepinephrine stimulates lymphoid cell mobilization from perfused rat spleen via beta-adrenergic receptors (1999). *Am.J.Physiol*276:R724-R730.

173. Szabo, C., Hasko, G., Zingarelli, B., Nemeth, Z.H., Salzman, A.L., Kvetan, V., Pastores, S.M. and Vizi, E.S. Isoproterenol regulates tumour necrosis factor, interleukin-10, interleukin-6 and nitric oxide production and protects against the development of vascular hyporeactivity in endotoxaemia (1997). *Immunology.*90:95-100.

174. van der Poll, T., Jansen, J., Endert, E., Sauerwein, H.P. and van Deventer, S.J. Noradrenaline inhibits lipopolysaccharide-induced tumor necrosis factor and interleukin 6 production in human whole blood (1994). *Infect.Immun.*62:2046-2050.

175. Siegmund, B., Eigler, A., Hartmann, G., Hacker, U. and Endres, S. adrenaline enhances lps-induced il-10 synthesis: evidence for protein kinase a-mediated pathway (1998). *International Journal Of Immunopharmacology*20:57-69.

176. Nakamura, A., Johns, E.J., Imaizumi, A., Abe, T. and Kohsaka, T. regulation of tumour necrosis factor and interleukin-6 gene transcription by beta(2)-adrenoceptor in the rat astrocytes (1998). *Journal Of Neuroimmunology*88:144-153.

177. Eigler, A., Siegmund, B., Emmerich, U., Baumann, K.H., Hartmann, G. and Endres, S. Anti-inflammatory activities of cAMP-elevating agents: enhancement of IL-10 synthesis and concurrent suppression of TNF production (1998). *J.Leukoc.Biol.*63:101-107.

178. Liao, J., Keiser, J.A., Scales, W.E., Kunkel, S.L. and Kluger, M.J. Role of epinephrine in TNF and IL-6 production from isolated perfused rat liver (1995). *Am.J.Physiol.*268:R896-R901.

179. Talmadge, J., Scott, R., Castelli, P., Newman Tarr, T. and Lee, J. Molecular pharmacology of the beta-adrenergic receptor on THP-1 cells (1993). *Int.J.Immunopharmacol.*15:219-228.

180. Severn, A., Rapson, N.T., Hunter, C.A. and Liew, F.Y. Regulation of tumor necrosis factor production by adrenaline and beta-adrenergic agonists (1992). *J.Immunol.*148:3441-3445.

181. Hetier, E., Ayala, J., Bousseau, A. and Prochiantz, A. Modulation of interleukin-1 and tumor necrosis factor expression by beta-adrenergic agonists in mouse ameboid microglial cells (1991). *Exp.Brain Res.*86:407-413.

182. Nakamura, A., Johns, E.J., Imaizumi, A., Yanagawa, Y. and Kohsaka, T. modulation of interleukin-6 by beta(2)-adrenoceptor in endotoxin-stimulated renal macrophage cells (1999). *Kidney International*56:839-849.

183. Seldon, P.M., Barnes, P.J., Meja, K. and Giembycz, M.A. Suppression of lipopolysaccharide-induced tumor necrosis factor-alpha generation from human peripheral blood monocytes by inhibitors of phosphodiesterase 4: interaction with stimulants of adenylyl cyclase (1995). *Mol.Pharmacol.*48:747-757.

184. Sekut, L., Champion, B.R., Page, K., Menius, J.A.,Jr. and Connolly, K.M. Anti-inflammatory activity of salmeterol: down-regulation of cytokine production (1995). *Clin.Exp.Immunol.*99:461-466.

185. Yoshimura, T., Kurita, C., Nagao, T., Usami, E., Nakao, T., Watanabe, S., Kobayashi, J., Yamazaki, F., Tanaka, H., Inagaki, N. and Nagai, H. Inhibition of tumor necrosis factor-alpha and interleukin-1-beta production by beta-adrenoceptor agonists from lipopolysaccharide-stimulated human peripheral blood mononuclear cells (1997). *Pharmacology.*54:144-152.

186. Guirao, X., Kumar, A., Katz, J., Smith, M., Lin, E., Keogh, C., Calvano, S.E. and Lowry, S.F. Catecholamines increase monocyte TNF receptors and inhibit TNF through beta 2-adrenoreceptor activation (1997). *Am.J.Physiol.*273:E1203-E1208.

187. Ignatowski, T.A., Gallant, S. and Spengler, R.N. Temporal regulation by adrenergic receptor stimulation of macrophage (M phi)-derived tumor necrosis factor (TNF) production post-LPS challenge (1996). *J.Neuroimmunol.*65:107-117.

188. Spengler, R.N., Chensue, S.W., Giacherio, D.A., Blenk, N. and Kunkel, S.L. Endogenous norepinephrine regulates tumor necrosis factor-alpha production from macrophages in vitro (1994). *J.Immunol.*152:3024-3031.

189. Pettipher, E.R., Eskra, J.D. and Labasi, J.M. The inhibitory effect of rolipram on TNF-alpha production in mouse blood ex vivo is dependent upon the release of corticosterone and adrenaline (1997). *Cytokine.*9:582-586.

190. Cheng, J.B., Watson, J.W., Pazoles, C.J., Eskra, J.D., Griffiths, R.J., Cohan, V.L., Turner, C.R., Showell, H.J. and Pettipher, E.R. The phosphodiesterase type 4 (PDE4) inhibitor CP-80,633 elevates plasma cyclic AMP levels and decreases tumor necrosis factor-alpha (TNFalpha) production in mice: effect of adrenalectomy (1997). *J.Pharmacol.Exp.Ther.*280:621-626.

191. van der Poll, T., Jansen, J., Endert, E., Sauerwein, H.P. and van Deventer, S.J. Noradrenaline inhibits lipopolysaccharide-induced tumor necrosis factor and interleukin 6 production in human whole blood (1994). *Infect.Immun.*62:2046-2050.

192. Hu, X.X., Goldmuntz, E.A. and Brosnan, C.F. The effect of norepinephrine on endotoxin-mediated macrophage activation (1991). *J.Neuroimmunol.*31:35-42.

193. Spengler, R.N., Allen, R.M., Remick, D.G., Strieter, R.M. and Kunkel, S.L. Stimulation of alpha-adrenergic receptor augments the production of macrophage-derived tumor necrosis factor (1990). *J.Immunol.*145:1430-1434.

194. Monastra, G. and Secchi, E.F. Beta-adrenergic receptors mediate in vivo the adrenaline inhibition of lipopolysaccharide-induced tumor necrosis factor release (1993). *Immunol.Lett.*38:127-130.

195. Elenkov, I.J., Hasko, G., Kovacs, K.J. and Vizi, E.S. Modulation of lipopolysaccharide-induced tumor necrosis factor-alpha production by selective alpha- and beta-adrenergic drugs in mice (1995). *J.Neuroimmunol.*61:123-131.

196. Hasko, G., Elenkov, I.J., Kvetan, V. and Vizi, E.S. Differential effect of selective block of alpha 2-adrenoreceptors on plasma levels of tumour necrosis factor-alpha, interleukin-6 and corticosterone induced by bacterial lipopolysaccharide in mice (1995). *J.Endocrinol.*144:457-462.

197. Hasko, G., Elenkov, I.J. and Vizi, E.S. Presynaptic receptors involved in the modulation of release of noradrenaline from the sympathetic nerve terminals of the rat thymus (1995). *Immunol.Lett.*47:133-137.

198. Szelenyi, J., Kiss, J.P. and Vizi, E.S. differential involvement of sympathetic nervous system and immune system in the modulation of tnf-alpha production by alpha(2)- and beta-adrenoceptors in mice (2000). *Journal Of Neuroimmunology*103:34-40.

199. Koff, W.C., Fann, A.V., Dunegan, M.A. and Lachman, L.B. Catecholamine-induced suppression of interleukin-1 production (1986). *Lymphokine.Res.*5:239-247.

200. van Gool, J., van Vugt, H., Helle, M. and Aarden, L.A. The relation among stress, adrenalin, interleukin 6 and acute phase proteins in the rat (1990). *Clin.Immunol.Immunopathol.*57:200-210.

201. Salas, M.A., Evans, S.W., Levell, M.J. and Whicher, J.T. Interleukin-6 and ACTH act synergistically to stimulate the release of corticosterone from adrenal gland cells (1990). *Clin.Exp.Immunol.*79:470-473.

202. Maimone, D., Cioni, C., Rosa, S., Macchia, G., Aloisi, F. and Annunziata, P. Norepinephrine and vasoactive intestinal peptide induce IL-6 secretion by astrocytes: synergism with IL-1 beta and TNF alpha (1993). *J.Neuroimmunol.*47:73-81.

203. Huang, Q.H., Takaki, A. and Arimura, A. Central noradrenergic system modulates plasma interleukin-6 production by peripheral interleukin-1 (1997). *Am.J.Physiol.*273:R731-R738.

204. Zhou, D., Kusnecov, A.W., Shurin, M.R., DePaoli, M. and Rabin, B.S. Exposure to physical and psychological stressors elevates plasma interleukin 6: relationship to the activation of hypothalamic-pituitary-adrenal axis (1993). *Endocrinology*133:2523-2530.

205. Kusnecov, A.W., Shurin, M.R., Armfield, A., Litz, J., Wood, P., Zhou, D. and Rabin, B.S. Suppression of lymphocyte mitogenesis in different rat strains exposed to footshock during early diurnal and nocturnal time periods (1995). *Psychoneuroendocrinology.*20:821-835.

206. Takaki, A., Huang, Q.H., Somogyvari Vigh, A. and Arimura, A. Immobilization stress may increase plasma interleukin-6 via central and peripheral catecholamines (1994). *Neuroimmunomodulation.*1:335-342.

207. Minami, M., Kuraishi, Y., Yamaguchi, T., Nakai, S., Hirai, Y. and Satoh, M. Immobilization stress induces interleukin-1 beta mRNA in the rat hypothalamus (1991). *Neurosci.Lett.*123:254-256.

208. Finck, B.N., Dantzer, R., Kelley, K.W., Woods, J.A. and Johnson, R.W. central lipopolysaccharide elevates plasma il-6 concentration by an alpha-adrenoreceptor-mediated mechanism (1997). *American Journal Of Physiology Regulatory Integrative And Comparative Physiology*41:R1880-R1887.

209. Kitamura, H., Okamoto, S., Shimamoto, Y., Morimatsu, M., Terao, A. and Saito, M. central il-1 differentially regulates peripheral il-6 and tnf synthesis (1998). *Cellular And Molecular Life Sciences*54:282-287.

210. Straub, R.H., Herrmann, M., Frauenholz, T., Berkmiller, G., Lang, B., Scholmerich, J. and Falk, W. Neuroimmune control of interleukin-6 secretion in the murine spleen. Differential beta-adrenergic effects of electrically released endogenous norepinephrine under various endotoxin conditions (1996). *J.Neuroimmunol.*71:37-43.

211. Hasko, G., Nemeth, Z.H., Szabo, C., Zsilla, G., Salzman, A.L. and Vizi, E.S.

isoproterenol inhibits il-10, tnf-alpha, and nitric oxide production in raw 264.7 macrophages (1998). *Brain Research Bulletin*45:183-187.

212. Siegmund, B., Eigler, A., Hartmann, G., Hacker, U. and Endres, S. Adrenaline enhances LPS-induced IL-10 synthesis: evidence for protein kinase A-mediated pathway (1998). *Int.J.Immunopharmacol.*20:57-69.

213. Hasko, G., Szabo, C., Nemeth, Z.H., Salzman, A.L. and Vizi, E.S. stimulation of beta-adrenoceptors inhibits endotoxin-induced il-12 production in normal and il-10 deficient mice (1998). *Journal Of Neuroimmunology*88:57-61.

214. Panina Bordignon, P., Mazzeo, D., Lucia, P.D., D'Ambrosio, D., Lang, R., Fabbri, L., Self, C. and Sinigaglia, F. Beta2-agonists prevent Th1 development by selective inhibition of interleukin 12 (1997). *J.Clin.Invest.*100:1513-1519.

215. Liu, Y., Li, Z. and Bakhiet, M. Upregulation of the chemokines Rantes, MCP-1, MIP-1a and MIP-2 in early infection with Trypanosoma brucei brucei and inhibition by sympathetic denervation of the spleen (1999). *Trop.Med.Int.Health*4:85-92.

216. Hernanz, A., Tato, E., De la Fuente, M., de Miguel, E. and Arnalich, F. Differential effects of gastrin-releasing peptide, neuropeptide Y, somatostatin and vasoactive intestinal peptide on interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha production by whole blood cells from healthy young and old subjects (1996). *J.Neuroimmunol.*71:25-30.

217. Chancellor Freeland, C., Zhu, G.F., Kage, R., Beller, D.I., Leeman, S.E. and Black, P.H. Substance P and stress-induced changes in macrophages (1995). *Ann.N.Y.Acad.Sci.*771:472-484.

218. Kincy Cain, T. and Bost, K.L. Substance P-induced IL-12 production by murine macrophages (1997). *J.Immunol.*158:2334-2339.

219. Vriend, C.Y., Zuo, L., Dyck, D.G., Nance, D.M. and Greenberg, A.H. Central administration of interleukin-1 beta increases norepinephrine turnover in the spleen (1993). *Brain Res.Bull.*31:39-42.

220. Terao, A., Oikawa, M. and Saito, M. Tissue-specific increase in norepinephrine turnover by central interleukin-1, but not by interleukin-6, in rats (1994). *Am.J.Physiol.*266:R400-R404.

221. Ichijo, T., Katafuchi, T. and Hori, T. Central interleukin-1 beta enhances splenic sympathetic nerve activity in rats (1994). *Brain Res.Bull.*34:547-553.

222. Sundar, S.K., Cierpial, M.A., Kamaraju, L.S., Long, S., Hsieh, S., Lorenz, C., Aaron, M., Ritchie, J.C. and Weiss, J.M. Human immunodeficiency virus glycoprotein (gp120) infused into rat brain induces interleukin 1 to elevate pituitary-adrenal activity and decrease peripheral cellular immune responses (1991). *Proc.Natl.Acad.Sci.U.S.A.*88:11246-11250.

223. Hodgson, D.M., Yirmiya, R., Chiappelli, F. and Taylor, A.N. Intracerebral interleukin-1beta impairs response to tumor invasion: involvement of adrenal catecholamines (1999). *Brain Res.*816:200-208.

224. Bell, R.C. and Lipton, J.M. Pulsatile release of antipyretic neuropeptide alpha-MSH from septum of rabbit during fever (1987). *Am.J.Physiol.*252:R1152-R1157.

225. Lipton, J.M., Macaluso, A., Hiltz, M.E. and Catania, A. Central administration of the peptide alpha-MSH inhibits inflammation in the skin (1991). *Peptides.* 12:795-798.

226. Ichiyama, T., Sakai, T., Catania, A., Barsh, G.S., Furukawa, S. and Lipton, J.M. Systemically administered alpha-melanocyte-stimulating peptides inhibit NF-kappaB activation in experimental brain inflammation (1999). *Brain Res.*836:31-37.

227. Galimberti, D., Baron, P., Meda, L., Prat, E., Scarpini, E., Delgado, R., Catania, A., Lipton, J.M. and Scarlato, G. Alpha-MSH peptides inhibit production of nitric oxide and tumor necrosis factor-alpha by microglial cells activated with beta-amyloid and interferon gamma (1999). *Biochem.Biophys.Res.Commun.*263:251-256.

228. Ichiyama, T., Sakai, T., Catania, A., Barsh, G.S., Furukawa, S. and Lipton, J.M. Inhibition of peripheral NF-kappaB activation by central action of alpha-melanocyte-stimulating hormone (1999). *J.Neuroimmunol.*99:211-217.

229. Sartin, J.L., Elsasser, T.H., Gunter, D.R. and McMahon, C.D. Endocrine modulation of physiological responses to catabolic disease (1998). *Domest.Anim.Endocrinol.* 15:423-429.

230. Macaluso, A., McCoy, D., Ceriani, G., Watanabe, T., Biltz, J., Catania, A. and Lipton, J.M. Antiinflammatory influences of alpha-MSH molecules: central neurogenic and peripheral actions (1994). *J.Neurosci.* 14:2377-2382.

231. Vriend, C.A.Y. *The Effects of Brain Infusion of Human Immunodeficiency Virus Glycoprotein (gp120) and Peripheral LPS on Splenic Macrophage IL-1 beta and TNF alpha and Splenic Sympathetic Nerve Activity*, 1999. Ph.D. Thesis: Department of Psychology, University of Manitoba, Winnipeg, Canada,

232. Vriend, C.A.Y., MacNeil, B.J., Meltzer, J.C., Sanders, V., Greenberg, A.H. and Nance, D.M. Central GP120 Alters the in vivo Production of Splenic Cytokines Following LPS (1997). *Psyconeuroimmunology Research Society Abstracts*(Abstract)

233. da Silva, B., Singer, W., Fong, I.W. and Ottaway, C.A. In vivo cytokine and neuroendocrine responses to endotoxin in human immunodeficiency virus-infected subjects (1999). *J.Infect.Dis.* 180:106-115.

234. Airaghi, L., Capra, R., Pravettoni, G., Maggiolo, F., Suter, F., Lipton, J.M. and Catania, A. Elevated concentrations of plasma alpha-melanocyte stimulating hormone are associated with reduced disease progression in HIV-infected patients (1999). *J.Lab.Clin.Med.* 133:309-315.

235. MacNeil, B.J., Jansen, A.H., Greenberg, A.H. and Nance, D.M. LPS induced Cytokine Responses in Chronic Spinal Cord Injured Rats (1998). *Society for Neuroscience Abstracts*737.5 in v.24 part 2:(Abstract)

236. Green, P.G., Luo, J., Heller, P. and Levine, J.D. Modulation of bradykinin-induced plasma extravasation in the rat knee joint by sympathetic co-transmitters (1993).

*Neuroscience.*52:451-458.

237. Lorton, D., Lubahn, C., Klein, N., Schaller, J. and Bellinger, D.L. Dual Role for Noradrenergic Innervation of Lymphoid Tissue and Arthritic Joints in Adjuvant-Induced Arthritis (1999). *BBI*13:315-334.

238. Levine, J.D., Coderre, T.J., Helms, C. and Basbaum, A.I. Beta 2-adrenergic mechanisms in experimental arthritis (1988). *Proc.Natl.Acad.Sci.U.S.A.*85:4553-4556.

239. Basbaum, A.I. and Levine, J.D. The contribution of the nervous system to inflammation and inflammatory disease (1991). *Can.J.Physiol.Pharmacol.*69:647-651.

240. Coderre, T.J., Basbaum, A.I., Dallman, M.F., Helms, C. and Levine, J.D. Epinephrine exacerbates arthritis by an action at presynaptic B2-adrenoceptors (1990). *Neuroscience.*34:521-523.

241. Tang, Y., Kohm, A.P., Sanders, V.M. and Jones, S.B. Soluble Protein Antigen Immunization Increases Efferent Sympathetic Activation in Immune Organs (1999). *Society for Neuroscience Abstracts*25 #381.4:942.(Abstract)

242. Saphier, D., Abramsky, O., Mor, G. and Ovadia, H. A neurophysiological correlate of an immune response (1987). *Ann.N.Y.Acad.Sci.*496:354-359.

243. Nance, D.M., Rayson, D. and Carr, R.I. The effects of lesions in the lateral septal and hippocampal areas on the humoral immune response of adult female rats (1987). *Brain Behav.Immun.* 1:292-305.

244. Wetmore, L., Green-Johnson, J., Gartner, J.G., Sanders, V. and Nance, D.M. The effects of kainic acid-induced lesions in the lateral septal area on cell-mediated immune function (1994). *Brain Behav.Immun.*8:341-354.

245. Yang, H., Wang, L., Huang, C.S. and Ju, G. Plasticity of GAP-43 innervation of the spleen during immune response in the mouse. Evidence for axonal sprouting and redistribution of the nerve fibers (1998). *Neuroimmunomodulation.*5:53-60.

246. Leo, N.A., Callahan, T.A. and Bonneau, R.H. Peripheral sympathetic denervation alters both the primary and memory cellular immune responses to herpes simplex virus infection (1998). *Neuroimmunomodulation.*5:22-35.

247. Kalinichenko, V.V., Mokyr, M.B., Graf, L.H.,Jr., Cohen, R.L. and Chambers, D.A. Norepinephrine-mediated inhibition of antitumor cytotoxic T lymphocyte generation involves a beta-adrenergic receptor mechanism and decreased TNF-alpha gene expression (1999). *J.Immunol.*163:2492-2499.

248. Webster, E.L., Elenkov, I.J. and Chrousos, G.P. The role of corticotropin-releasing hormone in neuroendocrine-immune interactions (1997). *Mol.Psychiatry*2:368-372.

249. Stenzel-Poore, M., Vale, W.W. and Rivier, C. Relationship between antigen-induced immune stimulation and activation of the hypothalamic-pituitary-adrenal axis in the rat (1993). *Endocrinology*132:1313-1318.

250. Delrue Perollet, C., Li, K.S. and Neveu, P.J. No change in plasma corticosterone and splenic norepinephrine during humoral and cellular immune responses to sheep erythrocytes in C3H mice (1995). *Neuroimmunomodulation.*2:36-43.

251. Shanks, N., Francis, D., Zalcman, S., Meaney, M.J. and Anisman, H. Alterations in central catecholamines associated with immune responding in adult and aged mice (1994). *Brain Res.*666:77-87.

252. Hall, N.R., McClure, J.E., Hu, S.K., Tare, N.S., Seals, C.M. and Goldstein, A.L. Effects of 6-hydroxydopamine upon primary and secondary thymus dependent immune responses (1982). *Immunopharmacology*5:39-48.

253. Kasahara, K., Tanaka, S. and Hamashima, Y. Suppressed immune response to T-cell dependent antigen in chemically sympathectomized mice (1977). *Res.Commun.Chem.Pathol.Pharmacol.*18:533-542.

254. Zalcman, S., Green-Johnson, J.M., Murray, L., Wan, W., Nance, D.M. and Greenberg, A.H. Interleukin-2-induced enhancement of an antigen-specific IgM plaque- forming cell response is mediated by the sympathetic nervous system (1994). *J.Pharmacol.Exp.Ther.*271:977-982.

255. Kouassi, E., Li, Y.S., Boukhris, W., Millet, I. and Revillard, J.P. Opposite effects of the catecholamines dopamine and norepinephrine on murine polyclonal B-cell activation (1988). *Immunopharmacology*16:125-137.

256. Li, Y.S., Kouassi, E. and Revillard, J.P. Differential regulation of mouse B-cell activation by beta-adrenoceptor stimulation depending on type of mitogens (1990). *Immunology.*69:367-372.

257. Alaniz, R.C., Thomas, S.A., Perez-Melgosa, M., Mueller, K., Farr, A.G., Palmiter, R.D. and Wilson, C.B. Dopamine beta-hydroxylase deficiency impairs cellular immunity (1999). *Proc.Natl.Acad.Sci.U.S.A.*96:2274-2278.

258. Sanders, V.M. and Munson, A.E. Beta adrenoceptor mediation of the enhancing effect of norepinephrine on the murine primary antibody response in vitro (1984). *J.Pharmacol.Exp.Ther.*230:183-192.

259. Sanders, V.M. and Munson, A.E. Kinetics of the enhancing effect produced by norepinephrine and terbutaline on the murine primary antibody response in vitro (1984). *J.Pharmacol.Exp.Ther.*231:527-531.

260. Kohm, A.P. and Sanders, V.M. Suppression of antigen-specific Th2 cell-dependent IgM and IgG1 production following norepinephrine depletion in vivo (1999). *J.Immunol.*162:5299-5308.

261. del Rey, A., Besedovsky, H.O., Sorkin, E., Da Prada, M. and Bondiolotti, G.P. Sympathetic immunoregulation: difference between high- and low- responder animals (1982). *Am.J.Physiol.*242:R30-R33.

262. Fuchs, B.A., Campbell, K.S. and Munson, A.E. Norepinephrine and serotonin content of the murine spleen: its relationship to lymphocyte beta-adrenergic receptor density and the

humoral immune response in vivo and in vitro (1988). *Cell Immunol.*117:339-351.

263. Roszman, T.L., Jackson, J.C., Cross, R.J., Titus, M.J., Markesbery, W.R. and Brooks, W.H. Neuroanatomic and neurotransmitter influences on immune function (1985). *J.Immunol.*135:769s-772s.

264. Frohman, E.M., Vayuvegula, B., Gupta, S. and van den Noort, S. Norepinephrine inhibits gamma-interferon-induced major histocompatibility class II (Ia) antigen expression on cultured astrocytes via beta-2-adrenergic signal transduction mechanisms (1988). *Proc.Natl.Acad.Sci.U.S.A.*85:1292-1296.

265. Frohman, E.M., Vayuvegula, B., van den Noort, S. and Gupta, S. Norepinephrine inhibits gamma-interferon-induced MHC class II (Ia) antigen expression on cultured brain astrocytes (1988). *J.Neuroimmunol.*17:89-101.

266. Green-Johnson, J.M., Zalcman, S., Vriend, C.Y., Dolina, S., Nance, D.M. and Greenberg, A.H. Role of norepinephrine in suppressed IgG production in epilepsy-prone mice (1996). *Life.Sci.*59:1121-1132.

267. Green-Johnson, J.M., Zalcman, S., Vriend, C.Y., Nance, D.M. and Greenberg, A.H. Suppressed T cell and macrophage function in the "reeler" (rl/rl) mutant, a murine strain with elevated cerebellar norepinephrine concentration (1995). *Brain Behav.Immun.*9:47-60.

268. Kruszewska, B., Felten, S.Y. and Moynihan, J.A. Alterations in cytokine and antibody production following chemical sympathectomy in two strains of mice (1995). *J.Immunol.*155:4613-4620.

269. Sanders, V.M. and Munson, A.E. Norepinephrine and the antibody response (1985). *Pharmacol.Rev.*37:229-248.

270. Callahan, T.A., Moynihan, J.A. and Piekut, D.T. Central nervous system activation following peripheral chemical sympathectomy: implications for neural-immune interactions (1998). *Brain Behav.Immun.*12:230-241.

271. Hermann, G., Beck, F.M., Tovar, C.A., Malarkey, W.B., Allen, C. and Sheridan, J.F. Stress-induced changes attributable to the sympathetic nervous system during experimental influenza viral infection in DBA/2 inbred mouse strain [published erratum appears in J Neuroimmunol 1994 Dec;55(2):221] (1994). *J.Neuroimmunol.*53:173-180.

272. Sudo, A. Decrease in adrenaline content of various organs of the rat after 6-hydroxydopamine (1985). *Eur.J.Pharmacol.*114:79-83.

273. Madden, K.S., Moynihan, J.A., Brenner, G.J., Felten, S.Y., Felten, D.L. and Livnat, S. Sympathetic nervous system modulation of the immune system. III. Alterations in T and B cell proliferation and differentiation in vitro following chemical sympathectomy (1994). *J.Neuroimmunol.*49:77-87.

274. Turnbull, A.V. and Rivier, C. Corticotropin-releasing factor, vasopressin, and prostaglandins mediate, and nitric oxide restrains, the hypothalamic-pituitary-adrenal response to acute local inflammation in the rat (1996). *Endocrinology*137:455-463.

275. Rivier, C. and Vale, W. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin (1983). *Nature*305:325-327.

276. Rivier, C. and Vale, W. Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion in vivo (1983). *Endocrinology*113:939-942.

277. Rivier, C., Rivier, J. and Vale, W. Inhibition of adrenocorticotropic hormone secretion in the rat by immunoneutralization of corticotropin-releasing factor (1982). *Science*218:377-379.

278. Sawchenko, P.E., Swanson, L.W. and Vale, W.W. Corticotropin-releasing factor: co-expression within distinct subsets of oxytocin-, vasopressin-, and neurotensin-immunoreactive neurons in the hypothalamus of the male rat (1984). *J.Neurosci.*4:1118-1129.

279. Makara, G.B. The relative importance of hypothalamic neurons containing corticotropin-releasing factor or vasopressin in the regulation of adrenocorticotropic hormone secretion (1992). *Ciba.Found.Symp.*168:43-51.

280. Schmidt, E.D., Janszen, A.W., Binnekade, R. and Tilders, F.J. Transient suppression of resting corticosterone levels induces sustained increase of AVP stores in hypothalamic CRH-neurons of rats (1997). *J.Neuroendocrinol.*9:69-77.

281. Turnbull, A.V. and Rivier, C. Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides (1997). *Proc.Soc.Exp.Biol.Med.*215:1-10.

282. Imaki, T., Nahan, J.L., Rivier, C., Sawchenko, P.E. and Vale, W. Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress (1991). *J.Neurosci.*11:585-599.

283. Makino, S., Takemura, T., Asaba, K., Nishiyama, M., Takao, T. and Hashimoto, K. differential regulation of type-1 and type-2 alpha corticotropin-releasing hormone receptor mrna in the hypothalamic paraventricular nucleus of the rat (1997). *Molecular Brain Research*47:170-176.

284. Reul, J.M., Labeur, M.S., Wiegers, G.J. and Linthorst, A.C. Altered neuroimmunoendocrine communication during a condition of chronically increased brain corticotropin-releasing hormone drive (1998). *Ann.N.Y.Acad.Sci.*840:444-455.

285. Boehme, S.A., Gaur, A., Crowe, P.D., Liu, X.J., Tamraz, S., Wong, T., Pahuja, A., Ling, N., Vale, W., De Souza, E.B. and Conlon, P.J. Immunosuppressive phenotype of corticotropin-releasing factor transgenic mice is reversed by adrenalectomy (1997). *Cell Immunol.*176:103-112.

286. Smith, G.W., Aubry, J.M., Dellu, F., Contarino, A., Bilezikjian, L.M., Gold, L.H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C.A., Sawchenko, P.E., Koob, G.F., Vale, W. and Lee, K.F. Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development (1998). *Neuron.*20:1093-1102.

287. Timpl, P., Spanagel, R., Sillaber, I., Kresse, A., Reul, J.M., Stalla, G.K., Blanquet, V., Steckler, T., Holsboer, F. and Wurst, W. Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1110ee comments] (1998). *Nat.Genet.*19:162-166.

288. Lovejoy, D.A., Aubry, J.M., Turnbull, A., Sutton, S., Potter, E., Yehling, J., Rivier, C. and Vale, W.W. Ectopic expression of the CRF-binding protein: minor impact on HPA axis regulation but induction of sexually dimorphic weight gain (1998). *J.Neuroendocrinol.*10:483-491.

289. Rivier, C., Rivier, J., Mormede, P. and Vale, W. Studies of the nature of the interaction between vasopressin and corticotropin-releasing factor on adrenocorticotropin release in the rat (1984). *Endocrinology*115:882-886.

290. Barberis, C. and Tribollet, E. Vasopressin and oxytocin receptors in the central nervous system (1996). *Crit.Rev.Neurobiol.*10:119-154.

291. Zingg, H.H. Vasopressin and oxytocin receptors (1996). *Baillieres.Clin.Endocrinol.Metab.*10:75-96.

292. Ma, X.M. and Lightman, S.L. the arginine vasopressin and corticotrophin-releasing hormone gene transcription responses to varied frequencies of repeated stress in rats (1998). *Journal Of Physiology London*510:605-614.

293. Dallman, M.F., Jones, M.T., Vernikos Danellis, J. and Ganong, W.F. Corticosteroid feedback control of ACTH secretion: rapid effects of bilateral adrenalectomy on plasma ACTH in the rat (1972). *Endocrinology*91:961-968.

294. Sawchenko, P.E. and Arias, C. Evidence for short-loop feedback effects of ACTH on CRF and vasopressin expression in parvocellular neurosecretory neurons (1995). *J.Neuroendocrinol.*7:721-731.

295. Sawchenko, P.E. Adrenalectomy-induced enhancement of CRF and vasopressin immunoreactivity in parvocellular neurosecretory neurons: anatomic, peptide, and steroid specificity (1987). *J.Neurosci.*7:1093-1106.

296. Plotsky, P.M. and Sawchenko, P.E. Hypophysial-portal plasma levels, median eminence content, and immunohistochemical staining of corticotropin-releasing factor, arginine vasopressin, and oxytocin after pharmacological adrenalectomy (1987). *Endocrinology*120:1361-1369.

297. Sawchenko, P.E. Evidence for a local site of action for glucocorticoids in inhibiting CRF and vasopressin expression in the paraventricular nucleus (1987). *Brain Res.*403:213-223.

298. Sawchenko, P.E., Swanson, L.W. and Vale, W.W. Co-expression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat (1984). *Proc.Natl.Acad.Sci.U.S.A.*81:1883-1887.

299. Conte Devolx, B., Guillaume, V., Boudouresque, F., Graziani, N., Magnan, E., Grino,

M., Emperaire, N., Nahoul, K., Cataldi, M. and Oliver, C. Effects of metyrapone infusion on corticotropin-releasing factor and arginine vasopressin secretion into the hypophysial portal blood of conscious, unrestrained rams (1992). *Acta Endocrinol.Copenh.* 127:435-440.

300. Imaki, T., Xiao Quan, W., Shibasaki, T., Yamada, K., Harada, S., Chikada, N., Naruse, M. and Demura, H. Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats (1995). *J.Clin.Invest.*96:231-238.

301. Dallman, M.F., Akana, S.F., Levin, N., Walker, C.D., Bradbury, M.J., Suemaru, S. and Scribner, K.S. Corticosteroids and the control of function in the hypothalamo-pituitary-adrenal (HPA) axis (1994). *Ann.N.Y.Acad.Sci.*746:22-31.

302. De Kloet, E.R., Oitzl, M.S. and Schobitz, B. Cytokines and the brain corticosteroid receptor balance: relevance to pathophysiology of neuroendocrine-immune communication (1994). *Psychoneuroendocrinology.*19:121-134.

303. Reul, J.M. and De Kloet, E.R. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation (1985). *Endocrinology*117:2505-2511.

304. Spencer, R.L., Kim, P.J., Kalman, B.A. and Cole, M.A. Evidence for mineralocorticoid receptor facilitation of glucocorticoid receptor-dependent regulation of hypothalamic-pituitary-adrenal axis activity (1998). *Endocrinology*139:2718-2726.

305. Bradbury, M.J., Akana, S.F. and Dallman, M.F. Roles of type I and II corticosteroid receptors in regulation of basal activity in the hypothalamo-pituitary-adrenal axis during the diurnal trough and the peak: evidence for a nonadditive effect of combined receptor occupation (1994). *Endocrinology*134:1286-1296.

306. Miller, A.H., Spencer, R.L., Stein, M. and McEwen, B.S. Adrenal steroid receptor binding in spleen and thymus after stress or dexamethasone (1990). *Am.J.Physiol.*259:E405-E412.

307. Spencer, R.L., Miller, A.H., Stein, M. and McEwen, B.S. Corticosterone regulation of type I and type II adrenal steroid receptors in brain, pituitary, and immune tissue (1991). *Brain Res.*549:236-246.

308. Herman, J.P., Patel, P.D., Akil, H. and Watson, S.J. Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat (1989). *Mol.Endocrinol.*3:1886-1894.

309. Herman, J.P. and Spencer, R. Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression in vivo (1998). *J.Neurosci.*18:7462-7473.

310. Sawchenko, P.E. and Bohn, M.C. Glucocorticoid receptor-immunoreactivity in C1, C2, and C3 adrenergic neurons that project to the hypothalamus or to the spinal cord in the rat (1989). *J.Comp.Neurol.*285:107-116.

311. Kovacs, K., Kiss, J.Z. and Makara, G.B. Glucocorticoid implants around the hypothalamic paraventricular nucleus prevent the increase of corticotropin-releasing factor and arginine vasopressin immunostaining induced by adrenalectomy (1986).

*Neuroendocrinology*44:229-234.

312. Herman, J.P., Cullinan, W.E., Young, E.A., Akil, H. and Watson, S.J. Selective forebrain fiber tract lesions implicate ventral hippocampal structures in tonic regulation of paraventricular nucleus corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA expression (1992). *Brain Res.*592:228-238.

313. Herman, J.P., Wiegand, S.J. and Watson, S.J. Regulation of basal corticotropin-releasing hormone and arginine vasopressin messenger ribonucleic acid expression in the paraventricular nucleus: effects of selective hypothalamic deafferentations (1990). *Endocrinology*127:2408-2417.

314. Jacobson, L. and Sapolsky, R. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis (1991). *Endocr.Rev.*12:118-134.

315. Terao, A., Oikawa, M. and Saito, M. Cytokine-induced change in hypothalamic norepinephrine turnover: involvement of corticotropin-releasing hormone and prostaglandins (1993). *Brain Res.*622:257-261.

316. Dhabhar, F.S., McEwen, B.S. and Spencer, R.L. Stress response, adrenal steroid receptor levels and corticosteroid-binding globulin levels--a comparison between Sprague-Dawley, Fischer 344 and Lewis rats (1993). *Brain Res.*616:89-98.

317. Dhabhar, F.S., Miller, A.H., McEwen, B.S. and Spencer, R.L. Differential activation of adrenal steroid receptors in neural and immune tissues of Sprague Dawley, Fischer 344, and Lewis rats (1995). *J.Neuroimmunol.*56:77-90.

318. Garrel, D.R. Corticosteroid-binding globulin during inflammation and burn injury: nutritional modulation and clinical implications (1996). *Horm.Res.*45:245-251.

319. Miller, A.H., Spencer, R.L., Pearce, B.D., Pisell, T.L., Tanapat, P., Leung, J.J., Dhabhar, F.S., McEwen, B.S. and Biron, C.A. 1996 Curt P. Richter Award. Effects of viral infection on corticosterone secretion and glucocorticoid receptor binding in immune tissues (1997). *Psychoneuroendocrinology.*22:455-474.

320. Scott, P.A., Cierpial, M.A., Kilts, C.D. and Weiss, J.M. Susceptibility and resistance of rats to stress-induced decreases in swim-test activity: a selective breeding study (1996). *Brain Res.*725:217-230.

321. Perretti, M., Duncan, G.S., Flower, R.J. and Peers, S.H. Serum corticosterone, interleukin-1 and tumour necrosis factor in rat experimental endotoxaemia: comparison between Lewis and Wistar strains (1993). *Br.J.Pharmacol.*110:868-874.

322. Wang, J. and Dunn, A.J. The role of interleukin-6 in the activation of the hypothalamo-pituitary-adrenocortical axis and brain indoleamines by endotoxin and interleukin-1 beta (1999). *Brain Res.*815:337-348.

323. Schotanus, K., Meloen, R.H., Puijk, W.C., Berkenbosch, F., Binnekade, R. and Tilders, F.J. Effects of monoclonal antibodies to specific epitopes of rat interleukin-1 beta (IL-1 beta) on IL-1 beta-induced ACTH, corticosterone and IL-6 responses in rats (1995). *J.Neuroendocrinol.*7:255-262.

324. Elenkov, I.J., Kovacs, K., Kiss, J., Bertok, L. and Vizi, E.S. Lipopolysaccharide is able to bypass corticotrophin-releasing factor in affecting plasma ACTH and corticosterone levels: evidence from rats with lesions of the paraventricular nucleus (1992). *J.Endocrinol.*133:231-236.

325. Chensue, S.W., Terebuh, P.D., Remick, D.G., Scales, W.E. and Kunkel, S.L. In vivo biologic and immunohistochemical analysis of interleukin-1 alpha, beta and tumor necrosis factor during experimental endotoxemia. Kinetics, Kupffer cell expression, and glucocorticoid effects (1991). *Am.J.Pathol.*138:395-402.

326. Derijk, R., Michelson, D., Karp, B., Petrides, J., Galliven, E., Deuster, P., Paciotti, G., Gold, P.W. and Sternberg, E.M. Exercise and circadian rhythm-induced variations in plasma cortisol differentially regulate interleukin-1 beta (IL-1 beta), IL-6, and tumor necrosis factor-alpha (TNF alpha) production in humans: high sensitivity of TNF alpha and resistance of IL-6 (1997). *J.Clin.Endocrinol.Metab.*82:2182-2191.

327. Grewe, M., Gausling, R., Gyufko, K., Hoffmann, R. and Decker, K. Regulation of the mRNA expression for tumor necrosis factor-alpha in rat liver macrophages (1994). *J.Hepatol.*20:811-818.

328. Doherty, G.M., Jensen, J.C., Buresh, C.M. and Norton, J.A. Hormonal regulation of inflammatory cell cytokine transcript and bioactivity production in response to endotoxin (1992). *Cytokine.*4:55-62.

329. Joyce, D.A., Steer, J.H. and Abraham, L.J. Glucocorticoid modulation of human monocyte/macrophage function: control of TNF-alpha secretion (1997). *Inflamm.Res.*46:447-451.

330. O'Leary, E.C., Marder, P. and Zuckerman, S.H. Glucocorticoid effects in an endotoxin-induced rat pulmonary inflammation model: differential effects on neutrophil influx, integrin expression, and inflammatory mediators (1996). *Am.J.Respir.Cell Mol.Biol.*15:97-106.

331. Papanicolaou, D.A., Tsigos, C., Oldfield, E.H. and Chrousos, G.P. Acute glucocorticoid deficiency is associated with plasma elevations of interleukin-6: does the latter participate in the symptomatology of the steroid withdrawal syndrome and adrenal insufficiency? (1996). *J.Clin.Endocrinol.Metab.*81:2303-2306.

332. Waage, A., Slupphaug, G. and Shalaby, R. Glucocorticoids inhibit the production of IL6 from monocytes, endothelial cells and fibroblasts (1990). *Eur.J.Immunol.*20:2439-2443.

333. Zanker, B., Walz, G., Wieder, K.J. and Strom, T.B. Evidence that glucocorticosteroids block expression of the human interleukin-6 gene by accessory cells (1990). *Transplantation.*49:183-185.

334. Zitnik, R.J., Whiting, N.L. and Elias, J.A. Glucocorticoid inhibition of interleukin-1-induced interleukin-6 production by human lung fibroblasts: evidence for transcriptional and post-transcriptional regulatory mechanisms (1994). *Am.J.Respir.Cell Mol.Biol.*10:643-650.

335. Breuninger, L.M., Dempsey, W.L., Uhl, J. and Murasko, D.M. Hydrocortisone regulation of interleukin-6 protein production by a purified population of human peripheral blood monocytes (1993). *Clin.Immunol.Immunopathol.*69:205-214.

336. Schobitz, B., Van Den Dobbelsteen, M., Holsboer, F., Sutanto, W. and De Kloet, E.R. Regulation of interleukin 6 gene expression in rat (1993). *Endocrinology*132:1569-1576.

337. Ray, A. and Sehgal, P.B. Cytokines and their receptors: molecular mechanism of interleukin-6 gene repression by glucocorticoids (1992). *J.Am.Soc.Nephrol.*2:S214-S221.

338. Sarlis, N.J., Stephanou, A., Knight, R.A., Lightman, S.L. and Chowdrey, H.S. Effects of glucocorticoids and chronic inflammatory stress upon anterior pituitary interleukin-6 mRNA expression in the rat (1993). *Br.J.Rheumatol.*32:653-657.

339. Ramachandra, R.N., Sehon, A.H. and Berczi, I. Neuro-hormonal host defence in endotoxin shock (1992). *Brain Behav.Immun.*6:157-169.

340. Pezeshki, G., Pohl, T. and Schobitz, B. Corticosterone controls interleukin-1 beta expression and sickness behavior in the rat (1996). *J.Neuroendocrinol.*8:129-135.

341. Parant, M., Le Contel, C., Parant, F. and Chedid, L. Influence of endogenous glucocorticoid on endotoxin-induced production of circulating TNF-alpha (1991). *Lymphokine.Cytokine.Res.*10:265-271.

342. Gonzalez, J.C., Johnson, D.C., Morrison, D.C., Freudenberg, M.A., Galanos, C. and Silverstein, R. Endogenous and exogenous glucocorticoids have different roles in modulating endotoxin lethality in D-galactosamine-sensitized mice (1993). *Infect.Immun.*61:970-974.

343. Silverstein, R., Hannah, P. and Johnson, D.C. Natural adrenocorticosteroids do not restore resistance to endotoxin in the adrenalectomized mouse (1993). *Circ.Shock.*41:162-165.

344. Carli, A., Auclair, M.C., Vernimmen, C. and Christeff, N. Potentiated cardiodepressant effect of serum by endotoxin in adrenalectomized rats (1986). *Circ.Shock.*19:39-45.

345. Johnson, R.W., Propes, M.J. and Shavit, Y. corticosterone modulates behavioral and metabolic effects of lipopolysaccharide (1996). *American Journal Of Physiology Regulatory Integrative And Comparative Physiology*39:R192-R198.

346. Goujon, E., Parnet, P., Cremona, S. and Dantzer, R. endogenous glucocorticoids down regulate central effects of interleukin-1 beta on body temperature and behaviour in mice (1995). *Brain Research*702:173-180.

347. Propes, M.J. and Johnson, R.W. role of corticosterone in the behavioral effects of central interleukin-1 beta (1997). *Physiology And Behavior*61:7-13.

348. Markel, E., Nyakas, C. and Endroczi, E. Footshock sensitivity of rats after adrenalectomy and glucocorticoid treatments (1984). *Acta Physiol.Hung.*64:123-127.

349. Goujon, E., Parnet, P., Aubert, A., Goodall, G. and Dantzer, R. Corticosterone regulates behavioral effects of lipopolysaccharide and interleukin-1 beta in mice (1995). *Am.J.Physiol.*269:R154-R159.

350. Stith, R.D. and McCallum, R.E. Down regulation of hepatic glucocorticoid receptors after endotoxin treatment (1983). *Infect.Immun.*40:613-621.

351. McCallum, R.E., Seale, T.W. and Stith, R.D. Influence of endotoxin treatment on dexamethasone induction of hepatic phosphoenolpyruvate carboxykinase (1983). *Infect.Immun.*39:213-219.

352. Sudo, A. Effects of adrenalectomy and chronic guanethidine treatment on tissue adrenaline concentrations in swimming-exposed rats (1987). *Jpn.J.Pharmacol.*45:197-201.

353. Molina, P.E. and Abumrad, N.N. differential effects of hemorrhage and lps on tissue tnf-alpha, il-1 and associate neuro-hormonal and opioid alterations (2000). *Life Sciences*66:399-409.

354. Karin, M., Yang Yen, H.F., Chambard, J.C., Deng, T. and Saatcioglu, F. Various modes of gene regulation by nuclear receptors for steroid and thyroid hormones (1993). *Eur.J.Clin.Pharmacol.*45 Suppl 1:S9-15.

355. Scheinman, R.I., Cogswell, P.C., Lofquist, A.K. and Baldwin, A.S.J. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids [see comments] (1995). *Science*270:283-286.

356. Fleshner, M., Brennan, F.X., Nguyen, K., Watkins, L.R. and Maier, S.F. RU-486 blocks differentially suppressive effect of stress on in vivo anti-KLH immunoglobulin response (1996). *Am.J.Physiol.*271:R1344-R1352.

357. Dhabhar, F.S. and McEwen, B.S. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking (1997). *Brain Behav.Immun.*11:286-306.

358. Dhabhar, F.S., Miller, A.H., McEwen, B.S. and Spencer, R.L. Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms (1995). *J.Immunol.*154:5511-5527.

359. Dhabhar, F.S., Miller, A.H., McEwen, B.S. and Spencer, R.L. Stress-induced changes in blood leukocyte distribution. Role of adrenal steroid hormones (1996). *J.Immunol.*157:1638-1644.

360. Kasting, N.W. Simultaneous and independent release of vasopressin and oxytocin in the rat (1988). *Can.J.Physiol.Pharmacol.*66:22-26.

361. Brouxhon, S.M., Prasad, A.V., Joseph, S.A., Felten, D.L. and Bellinger, D.L. Localization of corticotropin-releasing factor in primary and secondary lymphoid organs of the rat [In Process Citation] (1998). *Brain Behav.Immun.*12:107-122.

362. Hauger, R.L., Lorang, M., Irwin, M. and Aguilera, G. CRF receptor regulation and sensitization of ACTH responses to acute ether stress during chronic intermittent

immobilization stress (1990). *Brain Res.*532:34-40.

363. Mazzocchi, G., Rocco, S., Malendowicz, L.K., Rebuffat, P. and Nussdorfer, G.G. Bacterial lipopolysaccharide stimulates glucocorticoid secretion in hypophysectomized rats (1995). *Endocr.Res.*21:525-536.

364. Stachowiak, A., Macchi, C., Nussdorfer, G.G. and Malendowicz, L.K. Effects of oxytocin on the function and morphology of the rat adrenal cortex: in vitro and in vivo investigations (1995). *Res.Exp.Med.Berl.*195:265-274.

365. Axelrod, J. and Reisine, T.D. Stress hormones: their interaction and regulation (1984). *Science*224:452-459.

366. Kvetnansky, R., Pacak, K., Fukuhara, K., Viskupic, E., Hiremagalur, B., Nankova, B., Goldstein, D.S., Sabban, E.L. and Kopin, I.J. Sympathoadrenal system in stress. Interaction with the hypothalamic-pituitary-adrenocortical system (1995). *Ann.N.Y.Acad.Sci.*771:131-158.

367. Ehrhart Bornstein, M., Hinson, J.P., Bornstein, S.R., Scherbaum, W.A. and Vinson, G.P. Intraadrenal interactions in the regulation of adrenocortical steroidogenesis (1998). *Endocr.Rev.*19:101-143.

368. Brown, M.R. and Fisher, L.A. Corticotropin-releasing factor: effects on the autonomic nervous system and visceral systems (1985). *Fed.Proc.*44:243-248.

369. Golczynska, A., Lenders, J.W. and Goldstein, D.S. Glucocorticoid-induced sympathoinhibition in humans (1995). *Clin.Pharmacol.Ther.*58:90-98.

370. Brown, M.R. and Fisher, L.A. Glucocorticoid suppression of the sympathetic nervous system and adrenal medulla (1986). *Life.Sci.*39:1003-1012.

371. Kvetnansky, R., Fukuhara, K., Pacak, K., Cizza, G., Goldstein, D.S. and Kopin, I.J. Endogenous glucocorticoids restrain catecholamine synthesis and release at rest and during immobilization stress in rats (1993). *Endocrinology*133:1411-1419.

372. Takahashi, A., Ikarashi, Y., Ishimaru, H. and Maruyama, Y. Compensation between sympathetic nerves and adrenal medullary activity:effects of adrenodemedullation and chemical sympathectomy on catecholamine turnover (1993). *Life.Sci.*53:1567-1572.

373. Dailey, J.W. and Westfall, T.C. Effects of adrenalectomy and adrenal steroids on norepinephrine synthesis and monamine oxidase activity (1978). *Eur.J.Pharmacol.*48:383-391.

374. Haas, C.A., Frotscher, M. and Deller, T. Differential induction of c-Fos, c-Jun and Jun B in the rat central nervous system following unilateral entorhinal cortex lesion (1999). *Neuroscience.*90:41-51.

375. Sugimoto, T., Yoshida, A., Nishijima, K. and Ichikawa, H. c-Fos induction in the rat spinal dorsal horn partially deafferented by dorsal rhizotomy (1994). *Neurosci.Lett.*178:239-242.

376. Leah, J.D., Herdegen, T., Murashov, A., Dragunow, M. and Bravo, R. Expression of immediate early gene proteins following axotomy and inhibition of axonal transport in the rat central nervous system (1993). *Neuroscience.*57:53-66.

377. Viskupic, E., Kvetnansky, R., Sabban, E.L., Fukuhara, K., Weise, V.K., Kopin, I.J. and Schwartz, J.P. Increase in rat adrenal phenylethanolamine N-methyltransferase mRNA level caused by immobilization stress depends on intact pituitary-adrenocortical axis (1994). *J.Neurochem.*63:808-814.

378. MacNeil, B.J., Jansen, A.H., Greenberg, A.H. and Nance, D.M. The Effect of Acute Adrenalectomy on Sympathetic Activation Induced by Peripheral LPS or Central PGE2 (2000). *Am.J.Physiol*278:R1321-R1328.

379. Jasper, M.S. and Engeland, W.C. Splanchnicotomy increases adrenal sensitivity to ACTH in nonstressed rats (1997). *Am.J.Physiol.*273:E363-E368.

380. Dijkstra, I., Binnekade, R. and Tilders, F.J. Diurnal variation in resting levels of corticosterone is not mediated by variation in adrenal responsiveness to adrenocorticotropin but involves splanchnic nerve integrity (1996). *Endocrinology*137:540-547.

381. Holzwarth, M.A., Cunningham, L.A. and Kleitman, N. The role of adrenal nerves in the regulation of adrenocortical functions (1987). *Ann.N.Y.Acad.Sci.*512:449-464.

382. Hinson, J.P., Purbrick, A., Cameron, L.A. and Kapas, S. The role of neuropeptides in the regulation of adrenal zona fasciculata/reticularis function. Effects of vasoactive intestinal polypeptide, substance P, neuropeptide Y, Met- and Leu-enkephalin and neurotensin on corticosterone secretion in the intact perfused rat adrenal gland in situ (1994). *Neuropeptides.*26:391-397.

383. Laborie, C., Bernet, F., Dutriez Casteloot, I., Lesage, J. and Dupouy, J.P. Effect of cholinergic blockade on glucocorticoid regulation of NPY and catecholamines in the rat adrenal gland (1997). *Neuroendocrinology*66:98-105.

384. Gwosdow, A.R. Mechanisms of interleukin-1-induced hormone secretion from the rat adrenal gland (1995). *Endocr.Res.*21:25-37.

385. Walker, S.W., Lightly, E.R., Clyne, C., Williams, B.C. and Bird, I.M. Adrenergic and cholinergic regulation of cortisol secretion from the zona fasciculata/reticularis of bovine adrenal cortex (1991). *Endocr.Res.*17:237-265.

386. Walker, S.W., Lightly, E.R., Milner, S.W. and Williams, B.C. Catecholamine stimulation of cortisol secretion by 3-day primary cultures of purified zona fasciculata/reticularis cells isolated from bovine adrenal cortex (1988). *Mol.Cell Endocrinol.*57:139-147.

387. Nagy, E. and Berczi, I. Immunodeficiency in hypophysectomized rats (1978). *Acta Endocrinol.Copenh.*89:530-537.

388. Berczi, I. The role of the growth and lactogenic hormone family in immune function (1994). *Neuroimmunomodulation.*1:201-216.

389. Nagy, E., Berczi, I. and Sabbadini, E. Endocrine control of the immunosuppressive activity of the submandibular gland (1992). *Brain Behav.Immun.*6:418-428.

390. Rettori, V., Dees, W.L., Hiney, J.K., Lyson, K. and McCann, S.M. An interleukin-1-alpha-like neuronal system in the preoptic-hypothalamic region and its induction by bacterial lipopolysaccharide in concentrations which alter pituitary hormone release (1994). *Neuroimmunomodulation.*1:251-258.

391. Zellweger, R., Zhu, X.H., Wichmann, M.W., Ayala, A., DeMaso, C.M. and Chaudry, I.H. Prolactin administration following hemorrhagic shock improves macrophage cytokine release capacity and decreases mortality from subsequent sepsis (1996). *J.Immunol.*157:5748-5754.

392. DeVito, W.J. and Stone, S. Ethanol inhibits prolactin-induced activation of the JAK/STAT pathway in cultured astrocytes (1999). *J.Cell Biochem.*74:278-291.

393. Chikanza, I.C. Prolactin and neuroimmunomodulation: in vitro and in vivo observations (1999). *Ann.N.Y.Acad.Sci.*876:119-130.

394. Kumar, A., Singh, S.M. and Sodhi, A. Effect of prolactin on nitric oxide and interleukin-1 production of murine peritoneal macrophages: role of Ca2+ and protein kinase C (1997). *Int.J.Immunopharmacol.*19:129-133.

395. Zuckerman, S.H., Ahmari, S.E., Bryan Poole, N., Evans, G.F., Short, L. and Glasebrook, A.L. Estriol: a potent regulator of TNF and IL-6 expression in a murine model of endotoxemia (1996). *Inflammation.*20:581-597.

396. Ruh, M.F., Bi, Y., Cox, L., Berk, D., Howlett, A.C. and Bellone, C.J. Effect of environmental estrogens on IL-1beta promoter activity in a macrophage cell line (1998). *Endocrine.*9:207-211.

397. Ikejima, K., Enomoto, N., Iimuro, Y., Ikejima, A., Fang, D., Xu, J., Forman, D.T., Brenner, D.A. and Thurman, R.G. Estrogen increases sensitivity of hepatic Kupffer cells to endotoxin (1998). *Am.J.Physiol.*274:G669-G676.

398. Li, Z.G., Danis, V.A. and Brooks, P.M. Effect of gonadal steroids on the production of IL-1 and IL-6 by blood mononuclear cells in vitro (1993). *Clin.Exp.Rheumatol.*11:157-162.

399. Morishita, M., Miyagi, M. and Iwamoto, Y. Effects of sex hormones on production of interleukin-1 by human peripheral monocytes (1999). *J.Periodontol.*70:757-760.

400. Miller, L. and Hunt, J.S. Regulation of TNF-alpha production in activated mouse macrophages by progesterone (1998). *J.Immunol.*160:5098-5104.

401. Page, G.G. and Ben Eliyahu, S. The Effects of Sex and Estrous Phase on Surgery-induced Increases in Tumor cell Retention and its Attenutaion by Morphine (1996). *Psyconeuroimmunology Research Society Abstracts*(Abstract)

402. Shakhar, G., Rosenne, E., Haim, S. and Ben Eliyahu, S. Estrous Cycle Modulates the Effects of Metaproterenol on Breast Cancer Metastasis in F344 Rats (1996). *Psyconeuroimmunology Research Society Abstracts*(Abstract)

403. Wetmore, L. and Nance, D.M. Differential and sex-specific effects of kainic acid and domoic acid lesions in the lateral septal area of rats on immune function and body weight regulation (1991). *Exp.Neurol.*113:226-236.

404. Jessel, T.M. and Kelly, D.D. Pain and Analgesia. In: *Principals of Neural Science*, edited by Kandel, E.R., Schwartz, J.H. and Jessel, T.M. Amsterdam: Elsevier, 1991, p. 385-399.

405. Manfredi, B., Sacerdote, P., Gaspani, L., Poli, V. and Panerai, A.E. IL-6 knock-out mice show modified basal immune functions, but normal immune responses to stress (1998). *Brain Behav.Immun.*12:201-211.

406. Rivier, C. and Vale, W. Involvement of corticotropin-releasing factor and somatostatin in stress-induced inhibition of growth hormone secretion in the rat (1985). *Endocrinology*117:2478-2482.

407. Payan, D.G. and Katzung, B.G. Nonsteroidal Anti-inflammatory Drugs; Nonopiod Analgesics; Drugs used in Gout. In: *Opiod Analgesics and Antagonists*, edited by Katzung, B.G. Norwalk: Appleton and Lange, 1995, p. 536-559.

408. Ovadia, H., Nitsan, P. and Abramsky, O. Characterization of opiate binding sites on membranes of rat lymphocytes (1989). *J.Neuroimmunol.*21:93-102.

409. Shavit, Y., Lewis, J.W., Terman, G.W., Gale, R.P. and Liebeskind, J.C. Opioid peptides mediate the suppressive effect of stress on natural killer cell cytotoxicity (1984). *Science*223:188-190.

410. Lockwood, L.L., Silbert, L.H., Fleshner, M., Laudenslager, M.L., Watkins, L.R. and Maier, S.F. Morphine-induced decreases in in vivo antibody responses (1994). *Brain Behav.Immun.*8:24-36.

411. Bencsics, A., Elenkov, I.J. and Vizi, E.S. Effect of morphine on lipopolysaccharide-induced tumor necrosis factor-alpha production in vivo: involvement of the sympathetic nervous system (1997). *J.Neuroimmunol.*73:1-6.

412. Gomez Flores, R., Suo, J.L. and Weber, R.J. Suppression of splenic macrophage functions following acute morphine action in the rat mesencephalon periaqueductal gray (1999). *Brain Behav.Immun.*13:212-224.

413. Yamauchi, N., Shibasaki, T., Wakabayashi, I. and Demura, H. Brain beta-endorphin and other opioids are involved in restraint stress-induced stimulation of the hypothalamic-pituitary-adrenal axis, the sympathetic nervous system, and the adrenal medulla in the rat (1997). *Brain Res.*777:140-146.

414. Shavit, Y., Depaulis, A., Martin, F.C., Terman, G.W., Pechnick, R.N., Zane, C.J., Gale, R.P. and Liebeskind, J.C. Involvement of brain opiate receptors in the immune-suppressive effect of morphine (1986). *Proc.Natl.Acad.Sci.U.S.A.*83:7114-7117.

415. Flores, L.R., Dretchen, K.L. and Bayer, B.M. Potential role of the autonomic nervous system in the immunosuppressive effects of acute morphine administration (1996).

*Eur.J.Pharmacol.*318:437-446.

416. Fecho, K., Dykstra, L.A. and Lysle, D.T. Evidence for beta adrenergic receptor involvement in the immunomodulatory effects of morphine (1993). *J.Pharmacol.Exp.Ther.*265:1079-1087.

417. Appel, N.M., Kiritsy Roy, J.A. and Van Loon, G.R. Mu receptors at discrete hypothalamic and brainstem sites mediate opioid peptide-induced increases in central sympathetic outflow (1986). *Brain Res.*378:8-20.

418. Appel, N.M. and Van Loon, G.R. beta-Endorphin-induced stimulation of central sympathetic outflow: inhibitory modulation by central noradrenergic neurons (1986). *J.Pharmacol.Exp.Ther.*237:695-701.

419. Panerai, A.E., Sacerdote, P., Bianchi, M. and Manfredi, B. Intermittent but not continuous inescapable footshock stress and intracerebroventricular interleukin-1 similarly affect immune responses and immunocyte beta-endorphin concentrations in the rat (1997). *Int.J.Clin.Pharmacol.Res.*17:115-116.

420. Lipton, J.M., Catania, A. and Delgado, R. Peptide modulation of inflammatory processes within the brain (1998). *Neuroimmunomodulation.*5:178-183.

421. Ramaswamy, K., Mathison, R., Carter, L., Kirk, D., Green, F., Davison, J.S. and Befus, D. Marked antiinflammatory effects of decentralization of the superior cervical ganglia (1990). *J.Exp.Med.*172:1819-1830.

422. Mathison, R., Hogan, A., Helmer, D., Bauce, L., Woolner, J., Davison, J.S., Schultz, G. and Befus, D. Role for the submandibular gland in modulating pulmonary inflammation following induction of systemic anaphylaxis (1992). *Brain Behav.Immun.*6:117-129.

423. Aloe, L., Bracci Laudiero, L., Bonini, S. and Manni, L. The expanding role of nerve growth factor: from neurotrophic activity to immunologic diseases (1997). *Allergy.*52:883-894.

424. Aloe, L., Skaper, S.D., Leon, A. and Levi Montalcini, R. Nerve growth factor and autoimmune diseases (1994). *Autoimmunity.*19:141-150.

425. Romanovsky, A.A., Shido, O., Sakurada, S., Sugimoto, N. and Nagasaka, T. Endotoxin shock: thermoregulatory mechanisms (1996). *Am.J.Physiol.*270:R693-R703.

426. Lang, C.H., Bagby, G.J. and Spitzer, J.J. Glucose kinetics and body temperature after lethal and nonlethal doses of endotoxin (1985). *Am.J.Physiol.*248:R471-R478.

427. Christeff, N., Auclair, M.C., Benassayag, C., Carli, A. and Nunez, E.A. Endotoxin-induced changes in sex steroid hormone levels in male rats (1987). *J.Steroid.Biochem.*26:67-71.

428. Klosterhalfen, B., Hauptmann, S., Offner, F.A., Amo Takyi, B., Tons, C., Winkeltau, G., Affify, M., Kupper, W., Kirkpatrick, C.J. and Mittermayer, C. Induction of heat shock protein 70 by zinc-bis-(DL-hydrogenaspartate) reduces cytokine liberation, apoptosis, and mortality rate in a rat model of LD100 endotoxemia (1997). *Shock.*7:254-262.

429. Feuerstein, G., Hallenbeck, J.M., Vanatta, B., Rabinovici, R., Perera, P.Y. and Vogel, S.N. Effect of gram-negative endotoxin on levels of serum corticosterone, TNF alpha, circulating blood cells, and the survival of rats (1990). *Circ.Shock.*30:265-278.

430. Hewett, J.A. and Roth, R.A. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides (1993). *Pharmacol.Rev.*45:382-411.

431. Rietschel, E.T., Kirikae, T., Schade, F.U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A.J., Zahringer, U., Seydel, U., Di Padova, F. and et al , Bacterial endotoxin: molecular relationships of structure to activity and function (1994). *FASEB.J.*8:217-225.

432. Freudenberg, M.A. and Galanos, C. Bacterial lipopolysaccharides: structure, metabolism and mechanisms of action (1990). *Int.Rev.Immunol.*6:207-221.

433. Tobias, P.S., Tapping, R.I. and Gegner, J.A. Endotoxin interactions with lipopolysaccharide-responsive cells (1999). *Clin.Infect.Dis.*28:476-481.

434. Kumar, V., Cotran, R.S. and Robbins, S.L. Disorders of Vascular Flow and Shock. In: *Basic Pathology*, edited by Kumar, V., Cotran, R.S. and Robbins, S.L. Philadelphia: W.B. Saunders, 1992, p. 61-81.

435. Martich, G.D., Boujoukos, A.J. and Suffredini, A.F. Response of man to endotoxin (1993). *Immunobiology*187:403-416.

436. Rush, B.F.,Jr., Sori, A.J., Murphy, T.F., Smith, S., Flanagan, J.J.,Jr. and Machiedo, G.W. Endotoxemia and bacteremia during hemorrhagic shock. The link between trauma and sepsis? (1988). *Ann.Surg.*207:549-554.

437. Bhagat, K., Moss, R., Collier, J. and Vallance, P. Endothelial "stunning" following a brief exposure to endotoxin: a mechanism to link infection and infarction? (1996). *Cardiovasc.Res.*32:822-829.

438. Yoshikawa, D., Shiga, T., Saito, S., Morita, T., Imai, T. and Goto, F. Platelet-activating factor receptor antagonist attenuates endotoxin-induced vascular hyporeactivity in the pithed rat (1998). *Eur.J.Pharmacol.*342:241-245.

439. Paya, D. and Stoclet, J.C. Involvement of bradykinin and nitric oxide in the early hemodynamic effects of lipopolysaccharide in rats (1995). *Shock.*3:376-379.

440. Fish, R.E., Lang, C.H. and Spitzer, J.A. Regional blood flow during continuous low-dose endotoxin infusion (1986). *Circ.Shock*18:267-275.

441. Shirai, M., Shiga, J., Yoshimura, G., Fukuda, I., Kanegasaki, S. and Mori, W. The organ distribution of [3H]endotoxin following partial hepatectomy (1988). *Jpn.J.Exp.Med.*58:219-224.

442. Nakao, A., Taki, S., Yasui, M., Kimura, Y., Nonami, T., Harada, A. and Takagi, H. The fate of intravenously injected endotoxin in normal rats and in rats with liver failure (1994). *Hepatology*19:1251-1256.

443. Salomao, R., Rigato, O., Pignatari, A.C., Freudenberg, M.A. and Galanos, C. Bloodstream infections: epidemiology, pathophysiology and therapeutic perspectives (1999). *Infection.*27:1-11.

444. Rangel Frausto, M.S. The epidemiology of bacterial sepsis (1999). *Infect.Dis.Clin.North Am.*13:299-312, vii.

445. Carlet, J. Rapid diagnostic methods in the detection of sepsis (1999). *Infect.Dis.Clin.North Am.*13:483-94, xi.

446. Stelter, F., Witt, S., Furll, B., Jack, R.S., Hartung, T. and Schutt, C. Different efficacy of soluble CD14 treatment in high- and low-dose LPS models (1998). *Eur.J.Clin.Invest.*28:205-213.

447. Dellinger, R.P. Current therapy for sepsis (1999). *Infect.Dis.Clin.North Am.*13:495-509.

448. Smith, A.L. treatment of septic shock with immunotherapy (1998). *Pharmacotherapy*18:565-580.

449. Zeni, F., Freeman, B. and Natanson, C. Anti-inflammatory therapies to treat sepsis and septic shock: a reassessment [editorial; comment] (1997). *Crit.Care.Med.*25:1095-1100.

450. Opal, S.M. and Cross, A.S. Clinical trials for severe sepsis. Past failures, and future hopes (1999). *Infect.Dis.Clin.North Am.*13:285-97, vii.

451. Suffredini, A.F., Reda, D., Banks, S.M., Tropea, M., Agosti, J.M. and Miller, R. Effects of recombinant dimeric TNF receptor on human inflammatory responses following intravenous endotoxin administration (1995). *J.Immunol.*155:5038-5045.

452. Hawes, A.S., Rock, C.S., Keogh, C.V., Lowry, S.F. and Calvano, S.E. In vivo effects of the antiglucocorticoid RU 486 on glucocorticoid and cytokine responses to Escherichia coli endotoxin (1992). *Infect.Immun.*60:2641-2647.

453. Guglielmotti, A., Aquilini, L., Rosignoli, M.T., Landolfi, C., Soldo, L., Coletta, I. and Pinza, M. Benzydamine protection in a mouse model of endotoxemia (1997). *Inflamm.Res.*46:332-335.

454. Tateda, K., Matsumoto, T., Miyazaki, S. and Yamaguchi, K. Lipopolysaccharide-induced lethality and cytokine production in aged mice (1996). *Infect.Immun.*64:769-774.

455. Schreiber, W., Pollmacher, T., Fassbender, K., Gudewill, S., Vedder, H., Wiedemann, K., Galanos, C. and Holsboer, F. Endotoxin- and corticotropin-releasing hormone-induced release of ACTH and cortisol. A comparative study in men (1993). *Neuroendocrinology*58:123-128.

456. Vedder, H., Schreiber, W., Yassouridis, A., Gudewill, S., Galanos, C. and Pollmacher, T. Dose-dependence of bacterial lipopolysaccharide (LPS) effects on peak response and time course of the immune-endocrine host response in humans (1999). *Inflamm.Res.*48:67-74.

457. Smith, S.R., Calzetta, A., Bankowski, J., Kenworthy Bott, L. and Terminelli, C. Lipopolysaccharide-induced cytokine production and mortality in mice treated with Corynebacterium parvum (1993). *J.Leukoc.Biol.*54:23-29.

458. Tough, D.F., Sun, S. and Sprent, J. T cell stimulation in vivo by lipopolysaccharide (LPS) (1997). *J.Exp.Med.*185:2089-2094.

459. Matera, G., Cook, J.A., Hennigar, R.A., Tempel, G.E., Wise, W.C., Oglesby, T.D. and Halushka, P.V. Beneficial effects of a 5-lipoxygenase inhibitor in endotoxic shock in the rat (1988). *J.Pharmacol.Exp.Ther.*247:363-371.

460. Cudd, T.A. Thromboxane A2 acts on the brain to mediate hemodynamic, adrenocorticotropin, and cortisol responses (1998). *Am.J.Physiol.*274:R1353-R1360.

461. Ito, T., Asai, F., Oshima, T. and Kobayashi, S. Role of activated platelets in endotoxin-induced DIC in rats (1990). *Thromb.Res.*59:735-747.

462. Kruse Elliott, K.T., Albert, D.H., Summers, J.B., Carter, G.W., Zimmerman, J.J. and Grossman, J.E. Attenuation of endotoxin-induced pathophysiology by a new potent PAF receptor antagonist (1996). *Shock.*5:265-273.

463. Murakami, J., Ohtani, A. and Murata, S. Protective effect of T-686, an inhibitor of plasminogen activator inhibitor-1 production, against the lethal effect of lipopolysaccharide in mice (1997). *Jpn.J.Pharmacol.*75:291-294.

464. Fatehi Hassanabad, Z., Burns, H., Aughey, E.A., Paul, A., Plevin, R., Parratt, J.R. and Furman, B.L. Effects of L-canavanine, an inhibitor of inducible nitric oxide synthase, on endotoxin mediated shock in rats (1996). *Shock.*6:194-200.

465. Rosselet, A., Feihl, F., Markert, M., Gnaegi, A., Perret, C. and Liaudet, L. selective inos inhibition is superior to norepinephrine in the treatment of rat endotoxic shock (1998). *American Journal Of Respiratory And Critical Care Medicine*157:162-170.

466. Bucklin, S.E., Silverstein, R. and Morrison, D.C. An interleukin-6-induced acute-phase response does not confer protection against lipopolysaccharide lethality (1993). *Infect.Immun.*61:3184-3189.

467. Webb, D.J. and Gonias, S.L. a modified human alpha(2)-macroglobulin derivative that binds tumor necrosis factor-alpha and interleukin-1 beta with high affinity in vitro and reverses lipopolysaccharide toxicity in vivo in mice (1998). *Laboratory Investigation*78:939-948.

468. Brackett, D.J., Schaefer, C.F. and Wilson, M.F. The effects of H1 and H2 histamine receptor antagonists on the development of endotoxemia in the conscious, unrestrained rat (1985). *Circ.Shock.*16:141-153.

469. Rixen, D., Neugebauer, E., Lechleuthner, A., Buschauer, A., Nagelschmidt, M., Thoma, S. and Rink, A. Beneficial effect of H2-agonism and H1-antagonism in rat endotoxic shock (1994). *Shock.*2:47-52.

470. Weipert, J., Hoffmann, H., Siebeck, M. and Whalley, E.T. Attenuation of arterial blood pressure fall in endotoxin shock in the rat using the competitive bradykinin antagonist Lys-Lys-[Hyp2, Thi5,8, DPhe7]-Bk (B4148) (1988). *Br.J.Pharmacol.*94:282-284.

471. Opal, S.M. and Yu, R.L.J. Antiendotoxin strategies for the prevention and treatment of septic shock. New approaches and future directions (1998). *Drugs.*55:497-508.

472. Roeise, O., Garred, P., Mollnes, T.E. and Aasen, A.O. Studies on the dose dependence of endotoxin-induced in vitro activation of the complement system (1989). *Eur.Surg.Res.*21:34-41.

473. Turner, C.R., Esser, K.M., Wheeldon, E.B., Slivjak, M. and Smith, E.F. Cardiovascular and pulmonary effects of human recombinant tumor necrosis factor in the conscious rat (1989). *Circ.Shock.*28:369-384.

474. Beutler, B., Milsark, I.W. and Cerami, A.C. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin (1985). *Science*229:869-871.

475. Sanchez Cantu, L., Rode, H.N., Yun, T.J. and Christou, N.V. Tumor necrosis factor alone does not explain the lethal effect of lipopolysaccharide (1991). *Arch.Surg.*126:231-235.

476. Libert, C., Vink, A., Coulie, P., Brouckaert, P., Everaerdt, B., Van Snick, J. and Fiers, W. Limited involvement of interleukin-6 in the pathogenesis of lethal septic shock as revealed by the effect of monoclonal antibodies against interleukin-6 or its receptor in various murine models (1992). *Eur.J.Immunol.*22:2625-2630.

477. Ingalls, R.R., Monks, B.G., Savedra, R.,Jr., Christ, W.J., Delude, R.L., Medvedev, A.E., Espevik, T. and Golenbock, D.T. CD11/CD18 and CD14 share a common lipid A signaling pathway (1998). *J.Immunol.*161:5413-5420.

478. Le Roy, D., Di Padova, F., Tees, R., Lengacher, S., Landmann, R., Glauser, M.P., Calandra, T. and Heumann, D. Monoclonal antibodies to murine lipopolysaccharide (LPS)-binding protein (LBP) protect mice from lethal endotoxemia by blocking either the binding of LPS to LBP or the presentation of LPS/LBP complexes to CD14 (1999). *J.Immunol.*162:7454-7460.

479. Lamping, N., Dettmer, R., Schroder, N.W., Pfeil, D., Hallatschek, W., Burger, R. and Schumann, R.R. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria (1998). *J.Clin.Invest.*101:2065-2071.

480. Mukaida, N., Ishikawa, Y., Ikeda, N., Fujioka, N., Watanabe, S., Kuno, K. and Matsushima, K. Novel insight into molecular mechanism of endotoxin shock: biochemical analysis of LPS receptor signaling in a cell-free system targeting NF-kappaB and regulation of cytokine production/action through beta2 integrin in vivo (1996). *J.Leukoc.Biol.*59:145-151.

481. Lee, J.D., Kravchenko, V., Kirkland, T.N., Han, J., Mackman, N., Moriarty, A., Leturcq, D. and Ulevitch, R.J. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediateidentical cellular responses to endotoxin (1993).

*Proc.Natl.Acad.Sci.U.S.A.*90:9930-9934.

482. Qureshi, S.T., Lariviere, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P. and Malo, D. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4) [see comments] (1999). *J.Exp.Med.*189:615-625.

483. Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi Castagnoli, P., Layton, B. and Beutler, B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene (1998). *Science*282:2085-2088.

484. Ulevitch, R.J. Endotoxin opens the Tollgates to innate immunity [news] (1999). *Nat.Med.*5:144-145.

485. Gerard, C. Bacterial infection. For whom the bell tolls [news; comment] (1998). *Nature*395:217, 219.

486. Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J. and Gusovsky, F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction (1999). *J.Biol.Chem.*274:10689-10692.

487. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product (1999). *J.Immunol.*162:3749-3752.

488. Yang, R.B., Mark, M.R., Gurney, A.L. and Godowski, P.J. Signaling events induced by lipopolysaccharide-activated toll-like receptor 2 (1999). *J.Immunol.*163:639-643.

489. Yang, R.B., Mark, M.R., Gray, A., Huang, A., Xie, M.H., Zhang, M., Goddard, A., Wood, W.I., Gurney, A.L. and Godowski, P.J. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling [see comments] (1998). *Nature*395:284-288.

490. Kirschning, C.J., Wesche, H., Merrill Ayres, T. and Rothe, M. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide (1998). *J.Exp.Med.*188:2091-2097.

491. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S. differential roles of tlr2 and tlr4 in recognition of gram-negative and gram-positive bacterial cell wall components (1999). *Immunity*11:443-451.

492. Ishikawa, Y., Mukaida, N., Kuno, K., Rice, N., Okamoto, S. and Matsushima, K. Establishment of lipopolysaccharide-dependent nuclear factor kappa B activation in acell-free system (1995). *J.Biol.Chem.*270:4158-4164.

493. Weinstein, S.L., June, C.H. and DeFranco, A.L. Lipopolysaccharide-induced protein tyrosine phosphorylation in human macrophages is mediated by CD14 (1993). *J.Immunol.*151:3829-3838.

494. Weinstein, S.L., Sanghera, J.S., Lemke, K., DeFranco, A.L. and Pelech, S.L. Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated

protein kinases in macrophages (1992). *J.Biol.Chem.*267:14955-14962.

495. Viriyakosol, S. and Kirkland, T. Knowledge of cellular receptors for bacterial endotoxin--1995 (1995). *Clin.Infect.Dis.*21 Suppl 2:S190-S195.

496. Kraatz, J., Clair, L., Rodriguez, J.L. and West, M.A. Macrophage TNF secretion in endotoxin tolerance: role of SAPK, p38, and MAPK (1999). *J.Surg.Res.*83:158-164.

497. Nakano, M., Saito, S., Nakano, Y., Yamasu, H., Matsuura, M. and Shinomiya, H. Intracellular protein phosphorylation in murine peritoneal macrophages in response to bacterial lipopolysaccharide (LPS): effects of kinase-inhibitors and LPS-induced tolerance (1993). *Immunobiology*187:272-282.

498. Vogels, M.T., Mensink, E.J., Ye, K., Boerman, O.C., Verschueren, C.M., Dinarello, C.A. and van der Meer, J.W. Differential gene expression for IL-1 receptor antagonist, IL-1, and TNF receptors and IL-1 and TNF synthesis may explain IL-1-induced resistance to infection (1994). *J.Immunol.*153:5772-5780.

499. Spolarics, Z. and Spitzer, J.J. Acute endotoxin tolerance is accompanied by stimulated glucose use in macrophage rich tissues (1995). *Biochem.Biophys.Res.Commun.*211:340-346.

500. Freudenberg, M.A., Salomao, R., Sing, A., Mitov, I. and Galanos, C. Reconciling the concepts of endotoxin sensitization and tolerance (1998). *Prog.Clin.Biol.Res.*397:261-268.

501. Mekaouche, M., Siaud, P., Givalois, L., Barbanel, G., Malaval, F., Maurel, D., Assenmacher, I. and Ixart, G. Different responses of plasma ACTH and corticosterone and of plasma interleukin-1 beta to single and recurrent endotoxin challenges (1996). *J.Leukoc.Biol.*59:341-346.

502. Takemura, T., Makino, S., Takao, T., Asaba, K., Suemaru, S. and Hashimoto, K. hypothalamic-pituitary-adrenocortical responses to single vs. repeated endotoxin lipopolysaccharide administration in the rat (1997). *Brain Research*767:181-191.

503. Kraatz, J., Clair, L., Rodriguez, J.L. and West, M.A. In vitro macrophage endotoxin tolerance: defective in vitro macrophage map kinase signal transduction after LPS pretreatment is not present in macrophages from C3H/HeJ endotoxin resistant mice (1999). *Shock.*11:58-63.

504. Zhang, X. and Morrison, D.C. Lipopolysaccharide-induced selective priming effects on tumor necrosis factor alpha and nitric oxide production in mouse peritoneal macrophages (1993). *J.Exp.Med.*177:511-516.

505. Tolchard, S., Hare, A.S., Nutt, D.J. and Clarke, G. tnf alpha mimics the endocrine but not the thermoregulatory responses of bacterial lipopolysaccharide (lps): correlation with fos-expression in the brain (1996). *Neuropharmacology*35:243-248.

506. Sapolsky, R., Rivier, C., Yamamoto, G., Plotsky, P. and Vale, W. Interleukin-1 stimulates the secretion of hypothalamic corticotropin- releasing factor (1987). *Science*238:522-524.

507. Brown, A.R. and Fishman, M. Tumor necrosis factor-alpha analyzed within individual macrophages by combined immunocytochemistry and computer-aided image analysis (1990). *Cell Immunol.* 130:352-363.

508. Hofsli, E., Bakke, O., Nonstad, U. and Espevik, T. A flow cytometric and immunofluorescence microscopic study of tumor necrosis factor production and localization in human monocytes (1989). *Cell Immunol.* 122:405-415.

509. Chensue, S.W., Shmyr Forsch, C., Weng, A., Otterness, I.G. and Kunkel, S.L. Biologic and immunohistochemical analysis of macrophage interleukin- 1 alpha, - 1 beta, and tumor necrosis factor production during the peritoneal exudative response (1989). *J.Leukoc.Biol.* 46:529-537.

510. McCall, J.L., Yun, K., Funamoto, S. and Parry, B.R. In vivo immunohistochemical identification of tumor necrosis factor/cachectin in human lymphoid tissue (1989). *Am.J.Pathol.* 135:421-425.

511. Chensue, S.W., Remick, D.G., Shmyr Forsch, C., Beals, T.F. and Kunkel, S.L. Immunohistochemical demonstration of cytoplasmic and membrane-associated tumor necrosis factor in murine macrophages (1988). *Am.J.Pathol.* 133:564-572.

512. Singer, I.I., Scott, S., Hall, G.L., Limjuco, G., Chin, J. and Schmidt, J.A. Interleukin 1 beta is localized in the cytoplasmic ground substance but is largely absent from the Golgi apparatus and plasma membranes of stimulated human monocytes (1988). *J.Exp.Med.* 167:389-407.

513. Beesley, J.E., Bomford, R. and Schmidt, J.A. Ultrastructural localization of interleukin 1 in human peripheral blood monocytes; evidence for IL-1 beta in mitochondria (1990). *Histochem.J.* 22:234-244.

514. Agace, W., Hedges, S., Andersson, U., Andersson, J., Ceska, M. and Svanborg, C. Selective cytokine production by epithelial cells following exposure to Escherichia coli (1993). *Infect.Immun.* 61:602-609.

515. Malyak, M., Smith, M.F.,Jr., Abel, A.A. and Arend, W.P. Peripheral blood neutrophil production of interleukin-1 receptor antagonist and interleukin-1 beta (1994). *J.Clin.Immunol.* 14:20-30.

516. Goh, K., Furusawa, S., Kawa, Y., Negishi Okitsu, S. and Mizoguchi, M. Production of interleukin-1-alpha and -beta by human peripheral polymorphonuclear neutrophils (1989). *Int.Arch.Allergy.Appl.Immunol.* 88:297-303.

517. Schindler, R., Mancilla, J., Endres, S., Ghorbani, R., Clark, S.C. and Dinarello, C.A. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF (1990). *Blood.* 75:40-47.

518. de Bont, E.S., Niemarkt, A.E., Tamminga, R.Y., Kimpen, J.L., Kamps, W.A. and de Leij, L.H. Lipopolysaccharide-induced cytokine production in peripheral blood mononuclear cells: intracellular localization of tumor necrosis factor alpha and interleukin 1 beta detected with a three-color immunofluorescence technique (1996). *Histochem.Cell*

*Biol.*106:593-598.

519. Givalois, L., Dornand, J., Mekaouche, M., Solier, M.D., Bristow, A.F., Ixart, G., Siaud, P., Assenmacher, I. and Barbanel, G. Temporal cascade of plasma level surges in ACTH, corticosterone, and cytokines in endotoxin-challenged rats (1994). *Am.J.Physiol.*267:R164-R170.

520. de Kossodo, S., Critico, B. and Grau, G.E. Modulation of the transcripts for tumor necrosis factor-alpha and its receptors in vivo (1994). *Eur.J.Immunol.*24:769-772.

521. Remick, D.G., Strieter, R.M., Lynch, J.P., Nguyen, D., Eskandari, M. and Kunkel, S.L. In vivo dynamics of murine tumor necrosis factor-alpha gene expression. Kinetics of dexamethasone-induced suppression (1989). *Lab.Invest.*60:766-771.

522. Wollenberg, G.K., DeForge, L.E., Bolgos, G. and Remick, D.G. Differential expression of tumor necrosis factor and interleukin-6 by peritoneal macrophages in vivo and in culture (1993). *Am.J.Pathol.*143:1121-1130.

523. Shalaby, M.R., Waage, A., Aarden, L. and Espevik, T. Endotoxin, tumor necrosis factor-alpha and interleukin 1 induce interleukin 6 production in vivo (1989). *Clin.Immunol.Immunopathol.*53:488-498.

524. Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A., Beutler, B., Cerami, A., Figari, I.S., Palladino, M.A.,Jr. and O'Connor, J.V. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1 (1986). *J.Exp.Med.*163:1433-1450.

525. Gorospe, M., Kumar, S. and Baglioni, C. Tumor necrosis factor increases stability of interleukin-1 mRNA by activating protein kinase C (1993). *J.Biol.Chem.*268:6214-6220.

526. Beaty, C.D., Franklin, T.L., Uehara, Y. and Wilson, C.B. Lipopolysaccharide-induced cytokine production in human monocytes: role of tyrosine phosphorylation in transmembrane signal transduction (1994). *Eur.J.Immunol.*24:1278-1284.

527. Rivest, S. and Rivier, C. Stress and interleukin-1 beta-induced activation of c-fos, NGFI-B and CRF gene expression in the hypothalamic PVN: comparison between Sprague- Dawley, Fisher-344 and Lewis rats (1994). *J.Neuroendocrinol.*6:101-117.

528. Libert, C., Brouckaert, P., Shaw, A. and Fiers, W. Induction of interleukin 6 by human and murine recombinant interleukin 1 in mice (1990). *Eur.J.Immunol.*20:691-694.

529. Le Contel, C., Parant, F. and Parant, M. Indirect and selective down-regulation of serum tumor necrosis factor-alpha release by interleukin-1 beta (1992). *Immunobiology*186:199-213.

530. Di Santo, E., Alonzi, T., Poli, V., Fattori, E., Toniatti, C., Sironi, M., Ricciardi Castagnoli, P. and Ghezzi, P. Differential effects of IL-6 on systemic and central production of TNF: a study with IL-6-deficient mice (1997). *Cytokine.*9:300-306.

531. Kozak, W., Kluger, M.J., Soszynski, D., Conn, C.A., Rudolph, K., Leon, L.R. and Zheng, H. IL-6 and IL-1 beta in fever. Studies using cytokine-deficient (knockout) mice

(1998). *Ann.N.Y.Acad.Sci.*856:33-47.

532. van der Meer, M.J., Sweep, C.G., Rijnkels, C.E., Pesman, G.J., Tilders, F.J., Kloppenborg, P.W. and Hermus, A.R. Acute stimulation of the hypothalamic-pituitary-adrenal axis by IL-1 beta, TNF alpha and IL-6: a dose response study (1996). *J.Endocrinol.Invest.*19:175-182.

533. Perlstein, R.S., Whitnall, M.H., Abrams, J.S., Mougey, E.H. and Neta, R. Synergistic roles of interleukin-6, interleukin-1, and tumor necrosis factor in the adrenocorticotropin response to bacterial lipopolysaccharide in vivo (1993). *Endocrinology*132:946-952.

534. Vallieres, L. and Rivest, S. interleukin-6 is a needed proinflammatory cytokine in the prolonged neural activity and transcriptional activation of corticotropin-releasing factor during endotoxemia (1999). *Endocrinology*140:3890-3903.

535. Zalcman, S., Green-Johnson, J.M., Murray, L., Nance, D.M., Dyck, D., Anisman, H. and Greenberg, A.H. Cytokine-specific central monoamine alterations induced by interleukin- 1, -2 and -6 (1994). *Brain Res.*643:40-49.

536. Stefferl, A., Hopkins, S.J., Rothwell, N.J. and Luheshi, G.N. The role of TNF-alpha in fever: opposing actions of human and murine TNF-alpha and interactions with IL-beta in the rat (1996). *Br.J.Pharmacol.*118:1919-1924.

537. Utsumi, T., Akimaru, K., Kawabata, Z., Levitan, A., Tokunaga, T., Tang, P., Ide, A., Hung, M.C. and Klostergaard, J. Human pro-tumor necrosis factor: molecular determinants of membrane translocation, sorting, and maturation (1995). *Mol.Cell Biol.*15:6398-6405.

538. Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J. and Cerretti, D.P. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells (1997). *Nature*385:729-733.

539. Kim, K.U., Kwon, O.J. and Jue, D.M. Pro-tumour necrosis factor cleavage enzyme in macrophage membrane/particulate (1993). *Immunology.*80:134-139.

540. Tang, P., Hung, M. and Klostergaard, J. Human pro-tumor necrosis factor is a homotrimer (1996). *Biochemistry*35:8216-8225.

541. Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K. and et al , The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor (1995). *Cell*83:793-802.

542. Ledgerwood, E.C., Pober, J.S. and Bradley, J.R. Recent advances in the molecular basis of TNF signal transduction (1999). *Lab.Invest.*79:1041-1050.

543. Natoli, G., Costanzo, A., Guido, F., Moretti, F. and Levrero, M. Apoptotic, non-apoptotic, and anti-apoptotic pathways of tumor necrosis factor signalling (1998). *Biochem.Pharmacol.*56:915-920.

544. Bemelmans, M.H., Van Tits, L.J. and Buurman, W.A. Tumor necrosis factor: function, release and clearance (1996). *Crit.Rev.Immunol.*16:1-11.

545. Beutler, B., Greenwald, D., Hulmes, J.D., Chang, M., Pan, Y.C., Mathison, J., Ulevitch, R. and Cerami, A. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin (1985). *Nature*316:552-554.

546. Pasparakis, M., Alexopoulou, L., Episkopou, V. and Kollias, G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response [see comments] (1996). *J.Exp.Med.*184:1397-1411.

547. Zhan, Y.F. and Cheers, C. control of il-12 and ifn-gamma production in response to live or dead bacteria by tnf and other factors (1998). *Journal Of Immunology*161:1447-1453.

548. Ribeiro, S.P., Villar, J., Downey, G.P., Edelson, J.D. and Slutsky, A.S. Effects of the stress response in septic rats and LPS-stimulated alveolar macrophages: evidence for TNF-alpha posttranslational regulation (1996). *Am.J.Respir.Crit.Care.Med.*154:1843-1850.

549. Zuckerman, S.H., Evans, G.F., Snyder, Y.M. and Roeder, W.D. Endotoxin-macrophage interaction: post-translational regulation of tumor necrosis factor expression (1989). *J.Immunol.*143:1223-1227.

550. Snyder, Y.M., Guthrie, L., Evans, G.F. and Zuckerman, S.H. Transcriptional inhibition of endotoxin-induced monokine synthesis following heat shock in murine peritoneal macrophages (1992). *J.Leukoc.Biol.*51:181-187.

551. Han, J., Brown, T. and Beutler, B. Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level [published erratum appears in J Exp Med 1990 Mar 1;171(3):971-2] (1990). *J.Exp.Med.*171:465-475.

552. Beutler, B. and Kruys, V. Lipopolysaccharide signal transduction, regulation of tumor necrosis factor biosynthesis, and signaling by tumor necrosis factor itself (1995). *J.Cardiovasc.Pharmacol.*25 Suppl 2:S1-S8.

553. Udalova, I.A., Knight, J.C., Vidal, V., Nedospasov, S.A. and Kwiatkowski, D. complex nf-kappa b interactions at the distal tumor necrosis factor promoter region in human monocytes (1998). *Journal Of Biological Chemistry*273:21178-21186.

554. Goldfeld, A.E., Doyle, C. and Maniatis, T. Human tumor necrosis factor alpha gene regulation by virus and lipopolysaccharide (1990). *Proc.Natl.Acad.Sci.U.S.A.*87:9769-9773.

555. Kontoyiannis, D., Pasparakis, M., Pizarro, T.T., Cominelli, F. and Kollias, G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies (1999). *Immunity.*10:387-398.

556. Yang, Y., Chang, J.F., Parnes, J.R. and Fathman, C.G. t cell receptor (tcr) engagement

leads to activation-induced splicing of tumor necrosis factor (tnf) nuclear pre-mrna (1998). *Journal Of Experimental Medicine*188:247-254.

557. Udelsman, R., Blake, M.J., Stagg, C.A. and Holbrook, N.J. Endocrine control of stress-induced heat shock protein 70 expression in vivo (1994). *Surgery*115:611-616.

558. Kyriakis, J.M. Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family (1999). *Gene Expr.*7:217-231.

559. Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M. and Goeddel, D.V. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins (1995). *Cell*83:1243-1252.

560. Hsu, H., Xiong, J. and Goeddel, D.V. The TNF Receptor 1-Associated Protein TRADD Signals Cell Death and NFKB Activation (1995). *Cell*81:495-504.

561. Yeh, W.C., Hakem, R., Woo, M. and Mak, T.W. Gene targeting in the analysis of mammalian apoptosis and TNF receptor superfamily signaling (1999). *Immunol.Rev.*169:283-302.

562. Saklatvala, J., Davis, W. and Guesdon, F. Interleukin 1 (IL1) and tumour necrosis factor (TNF) signal transduction (1996). *Philos.Trans.R.Soc.Lond.B.Biol.Sci.*351:151-157.

563. Kolesnick, R. and Golde, D.W. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling (1994). *Cell*77:325-328.

564. Dinarello, C.A. Biologic basis for interleukin-1 in disease (1996). *Blood.*87:2095-2147.

565. Russell, D.A., Tucker, K.K., Chinookoswong, N., Thompson, R.C. and Kohno, T. Combined inhibition of interleukin-1 and tumor necrosis factor in rodent endotoxemia: improved survival and organ function (1995). *J.Infect.Dis.*171:1528-1538.

566. Schotanus, K., Tilders, F.J. and Berkenbosch, F. Human recombinant interleukin-1 receptor antagonist prevents adrenocorticotropin, but not interleukin-6 responses to bacterial endotoxin in rats (1993). *Endocrinology*133:2461-2468.

567. Beutler, B.A. and Cerami, A. Recombinant interleukin 1 suppresses lipoprotein lipase activity in 3T3-L1 cells (1985). *J.Immunol.*135:3969-3971.

568. Hirsch, E., Irikura, V.M., Paul, S.M. and Hirsh, D. Functions of interleukin 1 receptor antagonist in gene knockout and overproducing mice (1996). *Proc.Natl.Acad.Sci.U.S.A.*93:11008-11013.

569. Fantuzzi, G., Zheng, H., Faggioni, R., Benigni, F., Ghezzi, P., Sipe, J.D., Shaw, A.R. and Dinarello, C.A. Effect of endotoxin in IL-1 beta-deficient mice (1996). *J.Immunol.*157:291-296.

570. MacNeil, B.J., Greenberg, A.H. and Nance, D.M. Effect of Plasma Cytokine Levels on the Central Nervous System Responses to Endotoxin Injection in Mice (1999). *Psyconeuroimmunology Research Society Abstracts in*

*Neuroimmunomodulation*6:233.(Abstract)

571. Fantuzzi, G. and Dinarello, C.A. The inflammatory response in interleukin-1 beta-deficient mice: comparison with other cytokine-related knock-out mice (1996). *J.Leukoc.Biol.*59:489-493.

572. Labow, M., Shuster, D., Zetterstrom, M., Nunes, P., Terry, R., Cullinan, E.B., Bartfai, T., Solorzano, C., Moldawer, L.L., Chizzonite, R. and McIntyre, K.W. Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice (1997). *J.Immunol.*159:2452-2461.

573. Alexander, H.R., Doherty, G.M., Buresh, C.M., Venzon, D.J. and Norton, J.A. A recombinant human receptor antagonist to interleukin 1 improves survival after lethal endotoxemia in mice (1991). *J.Exp.Med.*173:1029-1032.

574. Luheshi, G., Miller, A.J., Brouwer, S., Dascombe, M.J., Rothwell, N.J. and Hopkins, S.J. Interleukin-1 receptor antagonist inhibits endotoxin fever and systemic interleukin-6 induction in the rat (1996). *Am.J.Physiol.*270:E91-E95.

575. Bluthe, R.M., Dantzer, R. and Kelley, K.W. Effects of interleukin-1 receptor antagonist on the behavioral effects of lipopolysaccharide in rat (1992). *Brain Res.*573:318-320.

576. Arend, W.P. Interleukin-1 receptor antagonist (1993). *Adv.Immunol.*54:167-227.

577. Dunn, A.J. The role of interleukin-1 and tumor necrosis factor alpha in the neurochemical and neuroendocrine responses to endotoxin (1992). *Brain Res.Bull.*29:807-812.

578. Opal, S.M., Fisher, C.J.,Jr., Dhainaut, J.F., Vincent, J.L., Brase, R., Lowry, S.F., Sadoff, J.C., Slotman, G.J., Levy, H., Balk, R.A., Shelly, M.P., Pribble, J.P., LaBrecque, J.F., Lookabaugh, J., Donovan, H., Dubin, H., Baughman, R., Norman, J., DeMaria, E., Matzel, K., Abraham, E. and Seneff, M. Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group [see comments] (1997). *Crit.Care.Med.*25:1115-1124.

579. Van Zee, K.J., Coyle, S.M., Calvano, S.E., Oldenburg, H.S., Stiles, D.M., Pribble, J., Catalano, M., Moldawer, L.L. and Lowry, S.F. Influence of IL-1 receptor blockade on the human response to endotoxemia (1995). *J.Immunol.*154:1499-1507.

580. Kanakaraj, P., Schafer, P.H., Cavender, D.E., Wu, Y., Ngo, K., Grealish, P.F., Wadsworth, S.A., Peterson, P.A., Siekierka, J.J., Harris, C.A. and Fung Leung, W.P. Interleukin (IL)-1 receptor-associated kinase (IRAK) requirement for optimal induction of multiple IL-1 signaling pathways and IL-6 production (1998). *J.Exp.Med.*187:2073-2079.

581. Bankers Fulbright, J.L., Kalli, K.R. and McKean, D.J. Interleukin-1 signal transduction (1996). *Life.Sci.*59:61-83.

582. Rossi, B. IL-1 transduction signals (1993). *Eur.Cytokine.Netw.*4:181-187.

583. Watkins, L.R., Hansen, M.K., Nguyen, K.T., Lee, J.E. and Maier, S.F. Dynamic regulation of the proinflammatory cytokine, interleukin-1beta: molecular biology for non-molecular biologists (1999). *Life.Sci.*65:449-481.

584. Dedrick, R.L. and Conlon, P.J. Prolonged expression of lipopolysaccharide (LPS)-induced inflammatory genes in whole blood requires continual exposure to LPS (1995). *Infect.Immun.*63:1362-1368.

585. Komaki, G., Gottschall, P.E., Somogyvari Vigh, A., Tatsuno, I., Yatohgo, T. and Arimura, A. Rapid increase in plasma IL-6 after hemorrhage, and posthemorrhage reduction of the IL-6 response to LPS, in conscious rats: interrelation with plasma corticosterone levels (1994). *Neuroimmunomodulation.*1:127-134.

586. Yoshimoto, T., Nakanishi, K., Hirose, S., Hiroishi, K., Okamura, H., Takemoto, Y., Kanamaru, A., Hada, T., Tamura, T., Kakishita, E. and et al , High serum IL-6 level reflects susceptible status of the host to endotoxin and IL-1/tumor necrosis factor (1992). *J.Immunol.*148:3596-3603.

587. Libert, C., Takahashi, N., Cauwels, A., Brouckaert, P., Bluethmann, H. and Fiers, W. Response of interleukin-6-deficient mice to tumor necrosis factor-induced metabolic changes and lethality (1994). *Eur.J.Immunol.*24:2237-2242.

588. Leon, L.R., White, A.A. and Kluger, M.J. role of il-6 and tnf in thermoregulation and survival during sepsis in mice (1998). *American Journal Of Physiology Regulatory Integrative And Comparative Physiology*44:R269-R277.

589. Fattori, E., Cappelletti, M., Costa, P., Sellitto, C., Cantoni, L., Carelli, M., Faggioni, R., Fantuzzi, G., Ghezzi, P. and Poli, V. Defective inflammatory response in interleukin 6-deficient mice (1994). *J.Exp.Med.*180:1243-1250.

590. Hoffman, G.E., Smith, M.S. and Verbalis, J.G. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems (1993). *Front.Neuroendocrinol.*14:173-213.

591. Angel, P. and Karin, M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation (1991). *Biochim.Biophys.Acta*1072:129-157.

592. Dragunow, M. and Faull, R. The use of c-fos as a metabolic marker in neuronal pathway tracing (1989). *J.Neurosci.Methods*29:261-265.

593. Sagar, S.M., Sharp, F.R. and Curran, T. Expression of c-fos protein in brain: metabolic mapping at the cellular level (1988). *Science*240:1328-1331.

594. Morgan, J.I., Cohen, D.R., Hempstead, J.L. and Curran, T. Mapping patterns of c-fos expression in the central nervous system after seizure (1987). *Science*237:192-197.

595. Hare, A.S., Clarke, G. and Tolchard, S. Bacterial lipopolysaccharide-induced changes in FOS protein expression in the rat brain: correlation with thermoregulatory changes and plasma corticosterone (1995). *J.Neuroendocrinol.*7:791-799.

596. Lacroix, S. and Rivest, S. Functional circuitry in the brain of immune-challenged rats:

partial involvement of prostaglandins (1997). *J.Comp.Neurol.*387:307-324.

597. Elmquist, J.K., Scammell, T.E., Jacobson, C.D. and Saper, C.B. Distribution of Fos-like immunoreactivity in the rat brain following intravenous lipopolysaccharide administration (1996). *J.Comp.Neurol.*371:85-103.

598. Sagar, S.M., Price, K.J., Kasting, N.W. and Sharp, F.R. Anatomic patterns of Fos immunostaining in rat brain following systemic endotoxin administration (1995). *Brain Res.Bull.*36:381-392.

599. Elmquist, J.K., Ackermann, M.R., Register, K.B., Rimler, R.B., Ross, L.R. and Jacobson, C.D. Induction of Fos-like immunoreactivity in the rat brain following Pasteurella multocida endotoxin administration (1993). *Endocrinology*133:3054-3057.

600. Goehler, L.E., Gaykema, R.P., Nguyen, K.T., Lee, J.E., Tilders, F.J., Maier, S.F. and Watkins, L.R. Interleukin-1beta in immune cells of the abdominal vagus nerve: a link between the immune and nervous systems? (1999). *J.Neurosci.*19:2799-2806.

601. Tkacs, N.C. and Strack, A.M. Systemic endotoxin induces Fos-like immunoreactivity in rat spinal sympathetic regions (1995). *J.Auton.Nerv.Syst.*51:1-7.

602. Herkenham, M., Lee, H.Y. and Baker, R.A. Temporal and spatial patterns of c-fos mRNA induced by intravenous interleukin-1: a cascade of non-neuronal cellular activation at the blood-brain barrier (1998). *J.Comp.Neurol.*400:175-196.

603. Lee, H.Y., Whiteside, M.B. and Herkenham, M. Area postrema removal abolishes stimulatory effects of intravenous interleukin-1beta on hypothalamic-pituitary-adrenal axis activity and c-fos mRNA in the hypothalamic paraventricular nucleus (1998). *Brain Res.Bull.*46:495-503.

604. Brady, L.S., Lynn, A.B., Herkenham, M. and Gottesfeld, Z. Systemic interleukin-1 induces early and late patterns of c-fos mRNA expression in brain (1994). *J.Neurosci.*14:4951-4964.

605. Ericsson, A., Kovacs, K.J. and Sawchenko, P.E. A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stress-related neuroendocrine neurons (1994). *J.Neurosci.*14:897-913.

606. Imaki, T., Shibasaki, T., Hotta, M. and Demura, H. Intracerebroventricular administration of corticotropin-releasing factor induces c-fos mRNA expression in brain regions related to stress responses: comparison with pattern of c-fos mRNA induction after stress (1993). *Brain Res.*616:114-125.

607. Miyata, S., Itoh, T., Lin, S.H., Ishiyama, M., Nakashima, T. and Kiyohara, T. Temporal changes of c-fos expression in oxytocinergic magnocellular neuroendocrine cells of the rat hypothalamus with restraint stress (1995). *Brain Res.Bull.*37:391-395.

608. Li, H.Y. and Sawchenko, P.E. Hypothalamic effector neurons and extended circuitries activated in "neurogenic" stress: a comparison of footshock effects exerted acutely, chronically, and in animals with controlled glucocorticoid levels (1998). *J.Comp.Neurol.*393:244-266.

609. Lacroix, S., Vallieres, L. and Rivest, S. C-fos mRNA pattern and corticotropin-releasing factor neuronal activity throughout the brain of rats injected centrally with a prostaglandin of E2 type (1996). *J.Neuroimmunol.*70:163-179.

610. Sabban, E.L., Hiremagalur, B., Nankova, B. and Kvetnansky, R. Molecular biology of stress-elicited induction of catecholamine biosynthetic enzymes (1995). *Ann.N.Y.Acad.Sci.*771:327-338.

611. Nankova, B., Kvetnansky, R., McMahon, A., Viskupic, E., Hiremagalur, B., Frankle, G., Fukuhara, K., Kopin, I.J. and Sabban, E.L. Induction of tyrosine hydroxylase gene expression by a nonneuronal nonpituitary-mediated mechanism in immobilization stress (1994). *Proc.Natl.Acad.Sci.U.S.A.*91:5937-5941.

612. Nankova, B., Devlin, D., Kvetnansky, R., Kopin, I.J. and Sabban, E.L. Repeated immobilization stress increases the binding of c-Fos-like proteins to a rat dopamine beta-hydroxylase promoter enhancer sequence (1993). *J.Neurochem.*61:776-779.

613. Hunt, S.P., Pini, A. and Evan, G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation (1987). *Nature*328:632-634.

614. Kozak, W., Conn, C.A. and Kluger, M.J. Lipopolysaccharide induces fever and depresses locomotor activity in unrestrained mice (1994). *Am.J.Physiol.*266:R125-R135.

615. Morimoto, A., Murakami, N., Nakamori, T., Sakata, Y. and Watanabe, T. Brain regions involved in the development of acute phase responses accompanying fever in rabbits (1989). *J.Physiol.Lond.*416:645-657.

616. Kjaer, A., Larsen, P.J., Knigge, U. and Warberg, J. Histaminergic activation of the hypothalamic-pituitary-adrenal axis (1994). *Endocrinology*135:1171-1177.

617. Nakamori, T., Morimoto, A., Yamaguchi, K., Watanabe, T., Long, N.C. and Murakami, N. Organum vasculosum laminae terminalis (OVLT) is a brain site to produce interleukin-1 beta during fever (1993). *Brain Res.*618:155-159.

618. Morimoto, A., Murakami, N., Nakamori, T. and Watanabe, T. Multiple control of fever production in the central nervous system of rabbits (1988). *J.Physiol.Lond.*397:269-280.

619. Morimoto, A., Murakami, N. and Watanabe, T. Effect of prostaglandin E2 on thermoresponsive neurones in the preoptic and ventromedial hypothalamic regions of rats (1988). *J.Physiol.Lond.*405:713-725.

620. Saigusa, T. Participation of interleukin-1 and tumor necrosis factor in the responses of the sympathetic nervous system during lipopolysaccharide-induced fever (1990). *Pflugers.Arch.*416:225-229.

621. Morrow, L.E., McClellan, J.L., Conn, C.A. and Kluger, M.J. Glucocorticoids alter fever and IL-6 responses to psychological stress and to lipopolysaccharide (1993). *Am.J.Physiol.*264:R1010-R1016.

622. Mouihate, A., Chen, X. and Pittman, Q.J. Interleukin-1beta fever in rats: gender

difference and estrous cycle influence (1998). *Am.J.Physiol.*275:R1450-R1454.

623. Nakamori, T., Morimoto, A. and Murakami, N. Effect of a central CRF antagonist on cardiovascular and thermoregulatory responses induced by stress or IL-1 beta (1993). *Am.J.Physiol.*265:R834-R839.

624. Smith, B.K., Conn, C.A. and Kluger, M.J. Experimental cachexia: effects of MCA sarcoma in the Fischer rat (1993). *Am.J.Physiol.*265:R376-R384.

625. Chuluyan, H.E., Saphier, D., Rohn, W.M. and Dunn, A.J. Noradrenergic innervation of the hypothalamus participates in adrenocortical responses to interleukin-1 (1992). *Neuroendocrinology*56:106-111.

626. Abraham, E. Effects of stress on cytokine production (1991). *Methods Achiev.Exp.Pathol.*14:45-62.

627. Kluger, M.J., Kozak, W., Leon, L.R., Soszynski, D. and Conn, C.A. Fever and antipyresis (1998). *Prog.Brain Res.*115:465-475.

628. Kluger, M.J., Kozak, W., Leon, L.R. and Conn, C.A. the use of knockout mice to understand the role of cytokines in fever (1998). *Clinical And Experimental Pharmacology And Physiology*25:141-144.

629. Zalcman, S., Murray, L., Dyck, D.G., Greenberg, A.H. and Nance, D.M. Interleukin-2 and -6 induce behavioral-activating effects in mice (1998). *Brain Res.*811:111-121.

630. Grota, L.J., Bienen, T. and Felten, D.L. Corticosterone responses of adult Lewis and Fischer rats (1997). *J.Neuroimmunol.*74:95-101.

631. Mekaouche, M., Siaud, P., Givalois, L., Barbanel, G., Malaval, F., Maurel, D., Assenmacher, I. and Ixart, G. different responses of plasma acth and corticosterone and of plasma interleukin-1 beta to single and recurrent endotoxin challenges (1996). *Journal Of Leukocyte Biology*59:341-346.

632. Maier, S.F., Watkins, L.R. and Nance, D.M. Multiple Routes of Action of Interleukin-1 on the Nervous System. In: *Psychoneuroimmunology (In press)*, edited by Ader, R., Cohen, N. and Felten, D.L. San Diego: Academic Press, 2000,

633. Dunn, A.J. Endotoxin-induced activation of cerebral catecholamine and serotonin metabolism: comparison with interleukin-1 (1992). *J.Pharmacol.Exp.Ther.*261:964-969.

634. Loewy, A.D. Central Autonomic Pathways. In: *Central Regulation of Autonomic Functions*, edited by Loewy, A.D. and Spyer, K.M. New York: Oxford University Press, 1990, p. 87-103.

635. Ericsson, A., Liu, C., Hart, R.P. and Sawchenko, P.E. Type 1 interleukin-1 receptor in the rat brain: distribution, regulation, and relationship to sites of IL-1-induced cellular activation (1995). *J.Comp.Neurol.*361:681-698.

636. Nadeau, S. and Rivest, S. effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and

p75) in the rat brain: a view from the blood-brain barrier (1999). *Neuroscience*93:1449-1464.

637. Lacroix, S., Feinstein, D. and Rivest, S. The bacterial endotoxin lipopolysaccharide has the ability to target the brain in upregulating its membrane CD14 receptor within specific cellular populations (1998). *Brain Pathol.*8:625-640.

638. Derijk, R., Van Rooijen, N., Tilders, F.J., Besedovsky, H.O., del Rey, A. and Berkenbosch, F. Selective depletion of macrophages prevents pituitary-adrenal activation in response to subpyrogenic, but not to pyrogenic, doses of bacterial endotoxin in rats (1991). *Endocrinology*129:330-338.

639. Lacroix, S. and Rivest, S. Effect of acute systemic inflammatory response and cytokines on the transcription of the genes encoding cyclooxygenase enzymes (COX-1 and COX-2) in the rat brain (1998). *J.Neurochem.*70:452-466.

640. Brochu, S., Olivier, M. and Rivest, S. neuronal activity and transcription of proinflammatory cytokines, i kappa b alpha, and inos in the mouse brain during acute endotoxemia and chronic infection with trypanosoma brucei brucei (1999). *Journal Of Neuroscience Research*57:801-816.

641. Nadeau, S. and Rivest, S. regulation of the gene encoding tumor necrosis factor alpha (tnf-alpha) in the rat brain and pituitary in response to different models of systemic immune challenge (1999). *Journal Of Neuropathology And Experimental Neurology*58:61-77.

642. Breder, C.D., Hazuka, C., Ghayur, T., Klug, C., Huginin, M., Yasuda, K., Teng, M. and Saper, C.B. Regional induction of tumor necrosis factor alpha expression in the mouse brain after systemic lipopolysaccharide administration (1994). *Proc.Natl.Acad.Sci.U.S.A.*91:11393-11397.

643. Lechan, R.M., Toni, R., Clark, B.D., Cannon, J.G., Shaw, A.R., Dinarello, C.A. and Reichlin, S. Immunoreactive interleukin-1 beta localization in the rat forebrain (1990). *Brain Res.*514:135-140.

644. Beutler, B.A., Milsark, I.W. and Cerami, A. Cachectin/tumor necrosis factor: production, distribution, and metabolic fate in vivo (1985). *J.Immunol.*135:3972-3977.

645. MacNeil, B.J., Jansen, A.H., Janz, L.J., Greenberg, A.H. and Nance, D.M. Peripheral endotoxin increases splenic sympathetic nerve activity via central prostaglandin synthesis (1997). *Am.J.Physiol.*273:R609-R614.

646. Scammell, T.E., Griffin, J.D., Elmquist, J.K. and Saper, C.B. Microinjection of a cyclooxygenase inhibitor into the anteroventral preoptic region attenuates LPS fever (1998). *Am.J.Physiol.*274:R783-R789.

647. Wong, M.L., Bongiorno, P.B., al Shekhlee, A., Esposito, A., Khatri, P. and Licinio, J. IL-1 beta, IL-1 receptor type I and iNOS gene expression in rat brain vasculature and perivascular areas (1996). *Neuroreport.*7:2445-2448.

648. Quan, N., Whiteside, M., Kim, L. and Herkenham, M. Induction of inhibitory factor kappaBalpha mRNA in the central nervous system after peripheral lipopolysaccharide

administration: an in situ hybridization histochemistry study in the rat (1997). *Proc.Natl.Acad.Sci.U.S.A.*94:10985-10990.

649. Laflamme, N. and Rivest, S. effects of systemic immunogenic insults and circulating proinflammatory cytokines on the transcription of the inhibitory factor kappa b alpha within specific cellular populations of the rat brain (1999). *Journal Of Neurochemistry*73:309-321.

650. Quan, N., Whiteside, M. and Herkenham, M. Time course and localization patterns of interleukin-1beta messenger RNA expression in brain and pituitary after peripheral administration of lipopolysaccharide (1998). *Neuroscience.*83:281-293.

651. Van Dam, A.M., Bauer, J., Tilders, F.J. and Berkenbosch, F. Endotoxin-induced appearance of immunoreactive interleukin-1 beta in ramified microglia in rat brain: a light and electron microscopic study (1995). *Neuroscience.*65:815-826.

652. Matsumura, K., Cao, C., Ozaki, M., Morii, H., Nakadate, K. and Watanabe, Y. brain endothelial cells express cyclooxygenase-2 during lipopolysaccharide-induced fever: light and electron microscopic immunocytochemical studies (1998). *Journal Of Neuroscience*18:6279-6289.

653. Kaufmann, W.E., Worley, P.F., Pegg, J., Bremer, M. and Isakson, P. COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex (1996). *Proc.Natl.Acad.Sci.U.S.A.*93:2317-2321.

654. Van Dam, A.M., Brouns, M., Man, A.H. and Berkenbosch, F. Immunocytochemical detection of prostaglandin E2 in microvasculature and in neurons of rat brain after administration of bacterial endotoxin (1993). *Brain Res.*613:331-336.

655. Ban, E., Milon, G., Prudhomme, N., Fillion, G. and Haour, F. Receptors for interleukin-1 (alpha and beta) in mouse brain: mapping and neuronal localization in hippocampus (1991). *Neuroscience.*43:21-30.

656. Gabellec, M.M., Griffais, R., Fillion, G. and Haour, F. Interleukin-1 receptors type I and type II in the mouse brain: kinetics of mRNA expressions after peripheral administration of bacterial lipopolysaccharide (1996). *J.Neuroimmunol.*66:65-70.

657. Cunningham, E.T.,Jr. and De Souza, E.B. Interleukin 1 receptors in the brain and endocrine tissues (1993). *Immunol.Today.*14:171-176.

658. Liu, L., Kita, T., Tanaka, N. and Kinoshita, Y. The expression of tumour necrosis factor in the hypothalamus after treatment with lipopolysaccharide (1996). *Int.J.Exp.Pathol.*77:37-44.

659. Lustig, S., Danenberg, H.D., Kafri, Y., Kobiler, D. and Ben Nathan, D. Viral neuroinvasion and encephalitis induced by lipopolysaccharide and its mediators (1992). *J.Exp.Med.*176:707-712.

660. Andersson, P.B., Perry, V.H. and Gordon, S. The acute inflammatory response to lipopolysaccharide in CNS parenchyma differs from that in other body tissues (1992). *Neuroscience.*48:169-186.

661. Montero Menei, C.N., Sindji, L., Garcion, E., Mege, M., Couez, D., Gamelin, E. and Darcy, F. Early events of the inflammatory reaction induced in rat brain by lipopolysaccharide intracerebral injection: relative contribution of peripheral monocytes and activated microglia (1996). *Brain Res.*724:55-66.

662. Montero Menei, C.N., Sindji, L., Pouplard Barthelaix, A., Jehan, F., Denechaud, L. and Darcy, F. Lipopolysaccharide intracerebral administration induces minimal inflammatory reaction in rat brain (1994). *Brain Res.*653:101-111.

663. Banks, W.A., Kastin, A.J. and Durham, D.A. Bidirectional transport of interleukin-1 alpha across the blood-brain barrier (1989). *Brain Res.Bull.*23:433-437.

664. Banks, W.A., Kastin, A.J. and Broadwell, R.D. Passage of cytokines across the blood-brain barrier (1995). *Neuroimmunomodulation.*2:241-248.

665. Katsuura, G., Arimura, A., Koves, K. and Gottschall, P.E. Involvement of organum vasculosum of lamina terminalis and preoptic area in interleukin 1 beta-induced ACTH release (1990). *Am.J.Physiol.*258:E163-E171.

666. Gaykema, R.P., Goehler, L.E., Tilders, F.J., Bol, J.G., McGorry, M., Fleshner, M., Maier, S.F. and Watkins, L.R. Bacterial endotoxin induces fos immunoreactivity in primary afferent neurons of the vagus nerve (1998). *Neuroimmunomodulation.*5:234-240.

667. Hansen, M.K., Taishi, P., Chen, Z. and Krueger, J.M. Vagotomy blocks the induction of interleukin-1beta (IL-1beta) mRNA in the brain of rats in response to systemic IL-1beta (1998). *J.Neurosci.*18:2247-2253.

668. Laye, S., Bluthe, R.M., Kent, S., Combe, C., Medina, C., Parnet, P., Kelley, K. and Dantzer, R. Subdiaphragmatic vagotomy blocks induction of IL-1 beta mRNA in mice brain in response to peripheral LPS (1995). *Am.J.Physiol.*268:R1327-R1331.

669. Goehler, L.E., Relton, J.K., Dripps, D., Kiechle, R., Tartaglia, N., Maier, S.F. and Watkins, L.R. Vagal paraganglia bind biotinylated interleukin-1 receptor antagonist: a possible mechanism for immune-to-brain communication (1997). *Brain Res.Bull.*43:357-364.

670. Ek, M., Kurosawa, M., Lundeberg, T. and Ericsson, A. Activation of vagal afferents after intravenous injection of interleukin-1beta: role of endogenous prostaglandins (1998). *J.Neurosci.*18:9471-9479.

671. Fleshner, M., Goehler, L.E., Schwartz, B.A., McGorry, M., Martin, D., Maier, S.F. and Watkins, L.R. thermogenic and corticosterone responses to intravenous cytokines (il-1 beta and tnf-alpha) are attenuated by subdiaphragmatic vagotomy (1998). *Journal Of Neuroimmunology*86:134-141.

672. Lenczowski, M.J.P., VanDam, A.M., Poole, S., Larrick, J.W. and Tilders, F.J.H. role of circulating endotoxin and interleukin-6 in the acth and corticosterone response to intraperitoneal lps (1997). *American Journal Of Physiology Regulatory Integrative And Comparative Physiology*42:R1870-R1877.

673. Kovacs, K.J. and Elenkov, I.J. Differential dependence of ACTH secretion induced by various cytokines on the integrity of the paraventricular nucleus (1995). *J.Neuroendocrinol.*7:15-23.

674. Dunn, A.J., Powell, M.L. and Gaskin, J.M. Virus-induced increases in plasma corticosterone (1987). *Science*238:1423-1425.

675. Turnbull, A.V., Pitossi, F.J., Lebrun, J.J., Lee, S., Meltzer, J.C., Nance, D.M., del Rey, A., Besedovsky, H.O. and Rivier, C. Inhibition of tumor necrosis factor-alpha action within the CNS markedly reduces the plasma adrenocorticotropin response to peripheral local inflammation in rats (1997). *J.Neurosci.*17:3262-3273.

676. Rivier, C. Effect of peripheral and central cytokines on the hypothalamic-pituitary-adrenal axis of the rat (1993). *Ann.N.Y.Acad.Sci.*697:97-105.

677. Turnbull, A.V., Lee, S. and Rivier, C. Mechanisms of hypothalamic-pituitary-adrenal axis stimulation by immune signals in the adult rat (1998). *Ann.N.Y.Acad.Sci.*840:434-443.

678. Kent, S., Bluthe, R.M., Dantzer, R., Hardwick, A.J., Kelley, K.W., Rothwell, N.J. and Vannice, J.L. Different receptor mechanisms mediate the pyrogenic and behavioral effects of interleukin 1 (1992). *Proc.Natl.Acad.Sci.U.S.A.*89:9117-9120.

679. Coelho, M.M., Souza, G.E. and Pela, I.R. Endotoxin-induced fever is modulated by endogenous glucocorticoids in rats (1992). *Am.J.Physiol.*263:R423-R427.

680. Kluger, M.J., Kozak, W., Leon, L.R. and Conn, C.A. The use of knockout mice to understand the role of cytokines in fever (1998). *Clin.Exp.Pharmacol.Physiol.*25:141-144.

681. Rivier, C., Vale, W. and Brown, M. In the rat, interleukin-1 alpha and -beta stimulate adrenocorticotropin and catecholamine release (1989). *Endocrinology*125:3096-3102.

682. MacNeil, B.J., Jansen, A.H., Greenberg, A.H. and Nance, D.M. Activation and selectivity of splenic sympathetic nerve electrical activity response to bacterial endotoxin (1996). *Am.J.Physiol.*270:R264-R270.

683. Hori, T., Oka, T., Hosoi, M. and Aou, S. Pain modulatory actions of cytokines and prostaglandin E2 in the brain (1998). *Ann.N.Y.Acad.Sci.*840:269-281.

684. Dunn, A.J. and Swiergiel, A.H. The role of cytokines in infection-related behavior (1998). *Ann.N.Y.Acad.Sci.*840:577-585.

685. Fabry, Z., Raine, C.S. and Hart, M.N. Nervous tissue as an immune compartment: the dialect of the immune response in the CNS (1994). *Immunol.Today.*15:218-224.

686. Probert, L., Akassoglou, K., Kassiotis, G., Pasparakis, M., Alexopoulou, L. and Kollias, G. TNF-alpha transgenic and knockout models of CNS inflammation and degeneration (1997). *J.Neuroimmunol.*72:137-141.

687. Woodroofe, M.N., Sarna, G.S., Wadhwa, M., Hayes, G.M., Loughlin, A.J., Tinker, A. and Cuzner, M.L. Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine

production (1991). *J.Neuroimmunol.*33:227-236.

688. Hillhouse, E.W. and Mosley, K. Peripheral endotoxin induces hypothalamic immunoreactive interleukin-1 beta in the rat (1993). *Br.J.Pharmacol.*109:289-290.

689. Ilyin, S.E., Gayle, D., Flynn, M.C. and Plata-Salaman, C.R. Interleukin-1beta system (ligand, receptor type I, receptor accessory protein and receptor antagonist), TNF-alpha, TGF-beta1 and neuropeptide Y mRNAs in specific brain regions during bacterial LPS-induced anorexia (1998). *Brain Res.Bull.*45:507-515.

690. Ban, E., Marquette, C., Sarrieau, A., Fitzpatrick, F., Fillion, G., Milon, G., Rostene, W. and Haour, F. Regulation of interleukin-1 receptor expression in mouse brain and pituitary by lipopolysaccharide and glucocorticoids (1993). *Neuroendocrinology*58:581-587.

691. Gabellec, M.M., Griffais, R., Fillion, G. and Haour, F. Expression of interleukin 1 alpha, interleukin 1 beta and interleukin 1 receptor antagonist mRNA in mouse brain: regulation by bacterial lipopolysaccharide (LPS) treatment (1995). *Brain Res.Mol.Brain Res.*31:122-130.

692. Breder, C.D., Dinarello, C.A. and Saper, C.B. Interleukin-1 immunoreactive innervation of the human hypothalamus (1988). *Science*240:321-324.

693. Breder, C.D., Tsujimoto, M., Terano, Y., Scott, D.W. and Saper, C.B. Distribution and characterization of tumor necrosis factor-alpha-like immunoreactivity in the murine central nervous system (1993). *J.Comp.Neurol.*337:543-567.

694. Hunt, J.S., Chen, H.L., Hu, X.L., Chen, T.Y. and Morrison, D.C. Tumor necrosis factor-alpha gene expression in the tissues of normal mice (1992). *Cytokine.*4:340-346.

695. Marquette, C., Van Dam, A.M., Ceccaldi, P.E., Weber, P., Haour, F. and Tsiang, H. Induction of immunoreactive interleukin-1 beta and tumor necrosis factor-alpha in the brains of rabies virus infected rats (1996). *J.Neuroimmunol.*68:45-51.

696. Bandtlow, C.E., Meyer, M., Lindholm, D., Spranger, M., Heumann, R. and Thoenen, H. Regional and cellular codistribution of interleukin 1 beta and nerve growth factor mRNA in the adult rat brain: possible relationship to the regulation of nerve growth factor synthesis (1990). *J.Cell Biol.*111:1701-1711.

697. Pitossi, F., delRey, A., Kabiersch, A. and Besedovsky, H. induction of cytokine transcripts in the central nervous system and pituitary following peripheral administration of endotoxin to mice (1997). *Journal Of Neuroscience Research*48:287-298.

698. Shintani, F., Nakaki, T., Kanba, S., Sato, K., Yagi, G., Shiozawa, M., Aiso, S., Kato, R. and Asai, M. Involvement of interleukin-1 in immobilization stress-induced increase in plasma adrenocorticotropic hormone and in release of hypothalamic monoamines in the rat (1995). *J.Neurosci.*15:1961-1970.

699. Nguyen, K.T., Deak, T., Owens, S.M., Kohno, T., Fleshner, M., Watkins, L.R. and Maier, S.F. Exposure to acute stress induces brain interleukin-1beta protein in the rat (1998). *J.Neurosci.*18:2239-2246.

700. Milligan, E.D., Nguyen, K.T., Deak, T., Hinde, J.L., Fleshner, M., Watkins, L.R. and Maier, S.F. The long term acute phase-like responses that follow acute stressor exposure are blocked by alpha-melanocyte stimulating hormone (1998). *Brain Res.*810:48-58.

701. Lang, C.H., Cooney, R. and Vary, T.C. central interleukin-1 partially mediates endotoxin-induced changes in glucose metabolism (1996). *American Journal Of Physiology Endocrinology And Metabolism*34:E309-E316.

702. Miller, A.J., Hopkins, S.J. and Luheshi, G.N. Sites of action of IL-1 in the development of fever and cytokine responses to tissue inflammation in the rat (1997). *Br.J.Pharmacol.*120:1274-1279.

703. Cartmell, T., Luheshi, G.N. and Rothwell, N.J. Brain sites of action of endogenous interleukin-1 in the febrile response to localized inflammation in the rat (1999). *J.Physiol.Lond.*518:585-594.

704. Luheshi, G.N., Stefferl, A., Turnbull, A.V., Dascombe, M.J., Brouwer, S., Hopkins, S.J. and Rothwell, N.J. Febrile response to tissue inflammation involves both peripheral and brain IL-1 and TNF-alpha in the rat (1997). *Am.J.Physiol.*272:R862-R868.

705. Sacoccio, C., Dornand, J. and Barbanel, G. Differential regulation of brain and plasma TNFalpha produced after endotoxin shock (1998). *Neuroreport.*9:309-313.

706. Ban, E., Haour, F. and Lenstra, R. Brain interleukin 1 gene expression induced by peripheral lipopolysaccharide administration (1992). *Cytokine.*4:48-54.

707. Sirko, S., Bishai, I. and Coceani, F. Prostaglandin formation in the hypothalamus in vivo: effect of pyrogens (1989). *Am.J.Physiol.*256:R616-R624.

708. Morimoto, A., Long, N.C., Nakamori, T. and Murakami, N. The effect of prostaglandin E2 on the body temperature of restrained rats (1991). *Physiol.Behav.*50:249-253.

709. Oka, T. and Hori, T. EP1-receptor mediation of prostaglandin E2-induced hyperthermia in rats (1994). *Am.J.Physiol.*267:R289-R294.

710. Scammell, T.E., Griffin, J.D., Elmquist, J.K. and Saper, C.B. Microinjection of a cyclooxygenase inhibitor into the anteroventral preoptic region attenuates LPS fever (1998). *Am.J.Physiol.*274:R783-R789.

711. Ando, T., Ichijo, T., Katafuchi, T. and Hori, T. Intracerebroventricular injection of prostaglandin E2 increases splenic sympathetic nerve activity in rats (1995). *Am.J.Physiol.*269:R662-R668.

712. Katafuchi, T., Ichijo, T. and Hori, T. Sequential relationship between actions of CRF and PGE2 in the brain on splenic sympathetic nerve activity in rats (1997). *J.Auton.Nerv.Syst.*67:200-206.

713. Feuerstein, G., Adelberg, S.A., Kopin, I.J. and Jacobowitz, D.M. Hypothalamic sites for cardiovascular and sympathetic modulation by prostaglandin E2 (1982). *Brain Res.*231:335-342.

714. Yokotani, K., Nishihara, M., Murakami, Y., Hasegawa, T., Okuma, Y. and Osumi, Y. Elevation of plasma noradrenaline levels in urethane-anaesthetized rats by activation of central prostanoid EP3 receptors (1995). *Br.J.Pharmacol.*115:672-676.

715. Lacroix, S. and Rivest, S. Functional circuitry in the brain of immune-challenged rats: partial involvement of prostaglandins (1997). *J.Comp.Neurol.*387:307-324.

716. Niimi, M., Sato, M., Wada, Y., Takahara, J. and Kawanishi, K. Effect of central and continuous intravenous injection of interleukin-1 beta on brain c-fos expression in the rat: involvement of prostaglandins (1996). *Neuroimmunomodulation.*3:87-92.

717. Pan, L., Pylypas, S., Sanders, V., Greenberg, A.H. and Nance, D.M. Central Injections of Prostaglandins Activate the Hypothalamus and Suppress Splenic Cytokine mRNA Production (1997). *Society for Neuroscience Abstracts*v23 #593.24:p1512.

718. Rivier, C. and Vale, W. Stimulatory effect of interleukin-1 on adrenocorticotropin secretion in the rat: is it modulated by prostaglandins? (1991). *Endocrinology*129:384-388.

719. Buller, K.M., Xu, Y. and Day, T.A. Indomethacin attenuates oxytocin and hypothalamic-pituitary-adrenal axis responses to systemic interleukin-1 beta (1998). *J.Neuroendocrinol.*10:519-528.

720. Rassnick, S., Zhou, D. and Rabin, B.S. Central administration of prostaglandin E2 suppresses in vitro cellular immune responses (1995). *Am.J.Physiol.*269:R92-R97.

721. Feuerstein, G., Adelberg, S.A., Kopin, I.J. and Jacobowitz, D.M. Cardiovascular and sympathetic responses to PGF2 alpha injection into hypothalamic nuclei (1981). *Brain Res.Bull.*6:203-207.

722. Rothwell, N.J. Central activation of thermogenesis by prostaglandins: dependence on CRF (1990). *Horm.Metab.Res.*22:616-618.

723. Terao, A., Matsumura, H. and Saito, M. Interleukin-1 induces slow-wave sleep at the prostaglandin D2-sensitive sleep-promoting zone in the rat brain (1998). *J.Neurosci.*18:6599-6607.

724. Cao, C., Matsumura, K. and Watanabe, Y. Induction of cyclooxygenase-2 in the brain by cytokines (1997). *Ann.N.Y.Acad.Sci.*813:307-309.

725. Mirtella, A., Tringali, G., Guerriero, G., Ghiara, P., Parente, L., Preziosi, P. and Navarra, P. Evidence that the interleukin-1 beta-induced prostaglandin E2 release from rat hypothalamus is mediated by type I and type II interleukin-1 receptors (1995). *J.Neuroimmunol.*61:171-177.

726. Weidenfeld, J., Crumeyrolle Arias, M. and Haour, F. Effect of bacterial endotoxin and interleukin-1 on prostaglandin biosynthesis by the hippocampus of mouse brain: role of interleukin-1 receptors and glucocorticoids (1995). *Neuroendocrinology*62:39-46.

727. Blatteis, C.M., Bealer, S.L., Hunter, W.S., Llanos, Q., Ahokas, R.A. and Mashburn, T.A.J. Suppression of fever after lesions of the anteroventral third ventricle in guinea pigs (1983). *Brain Res.Bull.*11:519-526.

728. Scammell, T.E., Elmquist, J.K., Griffin, J.D. and Saper, C.B. Ventromedial preoptic prostaglandin E2 activates fever-producing autonomic pathways (1996). *J.Neurosci.*16:6246-6254.

729. Morimoto, A., Murakami, N., Nakamori, T. and Watanabe, T. Evidence for separate mechanisms of induction of biphasic fever inside and outside the blood-brain barrier in rabbits (1987). *J.Physiol.Lond.*383:629-637.

730. Cao, C., Matsumura, K., Yamagata, K. and Watanabe, Y. Endothelial cells of the rat brain vasculature express cyclooxygenase-2 mRNA in response to systemic interleukin-1 beta: a possible site of prostaglandin synthesis responsible for fever (1996). *Brain Res.*733:263-272.

731. Elmquist, J.K., Breder, C.D., Sherin, J.E., Scammell, T.E., Hickey, W.F., Dewitt, D. and Saper, C.B. Intravenous lipopolysaccharide induces cyclooxygenase 2-like immunoreactivity in rat brain perivascular microglia and meningeal macrophages (1997). *J.Comp.Neurol.*381:119-129.

732. Breder, C.D. and Saper, C.B. Expression of inducible cyclooxygenase mRNA in the mouse brain after systemic administration of bacterial lipopolysaccharide (1996). *Brain Res.*713:64-69.

733. Cao, C., Matsumura, K., Ozaki, M. and Watanabe, Y. Lipopolysaccharide injected into the cerebral ventricle evokes fever through induction of cyclooxygenase-2 in brain endothelial cells (1999). *J.Neurosci.*19:716-725.

734. Matsumura, K., Cao, C., Ozaki, M., Morii, H., Nakadate, K. and Watanabe, Y. Electron microscopic evidence for induction of cyclooxygenase-2 in brain endothelial cells (1998). *Ann.N.Y.Acad.Sci.*856:278-280.

735. Cao, C., Matsumura, K., Yamagata, K. and Watanabe, Y. Cyclooxygenase-2 is induced in brain blood vessels during fever evoked by peripheral or central administration of tumor necrosis factor (1998). *Brain Res.Mol.Brain Res.*56:45-56.

736. Quan, N., Whiteside, M. and Herkenham, M. Cyclooxygenase 2 mRNA expression in rat brain after peripheral injection of lipopolysaccharide (1998). *Brain Res.*802:189-197.

737. Matsumura, K., Cao, C., Ozaki, M., Morii, H., Nakadate, K. and Watanabe, Y. Brain endothelial cells express cyclooxygenase-2 during lipopolysaccharide-induced fever: light and electron microscopic immunocytochemical studies (1998). *J.Neurosci.*18:6279-6289.

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

739. Laflamme, N. and Rivest, S. Effects of systemic immunogenic insults and circulating proinflammatory cytokines on the transcription of the inhibitory factor kappaB alpha within specific cellular populations of the rat brain (1999). *J.Neurochem.*73:309-321.

740. Inoue, H. and Tanabe, T. Transcriptional role of the nuclear factor kappa B site in the

induction by lipopolysaccharide and suppression by dexamethasone of cyclooxygenase-2 in U937 cells (1998). *Biochem.Biophys.Res.Commun.*244:143-148.

741. Crofford, L.J., Tan, B., McCarthy, C.J. and Hla, T. Involvement of nuclear factor kappa B in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes (1997). *Arthritis Rheum.*40:226-236.

742. Li, S., Wang, Y., Matsumura, K., Ballou, L.R., Morham, S.G. and Blatteis, C.M. The febrile response to lipopolysaccharide is blocked in cyclooxygenase-2(-/-), but not in cyclooxygenase-1(-/-) mice (1999). *Brain Res.*825:86-94.

743. Cao, C., Matsumura, K., Yamagata, K. and Watanabe, Y. Involvement of cyclooxygenase-2 in LPS-induced fever and regulation of its mRNA by LPS in the rat brain (1997). *Am.J.Physiol.*272:R1712-R1725.

744. Weidenfeld, J., Amir, I. and Shohami, E. Role of glucocorticoids in the regulation of brain prostaglandin biosynthesis under basal conditions and in response to endotoxin (1993). *Endocrinology*132:941-945.

745. Matsumura, K., Watanabe, Y., Onoe, H. and Hayaishi, O. High density of prostaglandin E2 binding sites in the anterior wall of the 3rd ventricle: a possible site of its hyperthermic action (1990). *Brain Res.*533:147-151.

746. Matsumura, K., Watanabe, Y., Imai Matsumura, K., Connolly, M., Koyama, Y. and Onoe, H. Mapping of prostaglandin E2 binding sites in rat brain using quantitative autoradiography (1992). *Brain Res.*581:292-298.

747. Fedyk, E.R. and Phipps, R.P. Prostaglandin E2 receptors of the EP2 and EP4 subtypes regulate activation and differentiation of mouse B lymphocytes to IgE-secreting cells (1996). *Proc.Natl.Acad.Sci.U.S.A.*93:10978-10983.

748. Sugimoto, Y., Shigemoto, R., Namba, T., Negishi, M., Mizuno, N., Narumiya, S. and Ichikawa, A. Distribution of the messenger RNA for the prostaglandin E receptor subtype EP3 in the mouse nervous system (1994). *Neuroscience.*62:919-928.

749. Zhang, J. and Rivest, S. distribution, regulation and colocalization of the genes encoding the ep2- and ep4-pge(2) receptors in the rat brain and neuronal responses to systemic inflammation (1999). *European Journal Of Neuroscience*11:2651-2668.

750. Bernardini, R., Chiarenza, A., Calogero, A.E., Gold, P.W. and Chrousos, G.P. Arachidonic acid metabolites modulate rat hypothalamic corticotropin-releasing hormone secretion in vitro (1989). *Neuroendocrinology*50:708-715.

751. Lundkvist, J., Chai, Z., Teheranian, R., Hasanvan, H., Bartfai, T., Jenck, F., Widmer, U. and Moreau, J.L. A non peptidic corticotropin releasing factor receptor antagonist attenuates fever and exhibits anxiolytic-like activity (1996). *Eur.J.Pharmacol.*309:195-200.

752. Liposits, Z., Phelix, C. and Paull, W.K. Adrenergic innervation of corticotropin releasing factor (CRF)-synthesizing neurons in the hypothalamic paraventricular nucleus of the rat. A combined light and electron microscopic immunocytochemical study (1986). *Histochemistry*84:201-205.

753. Liposits, Z., Phelix, C. and Paull, W.K. Electron microscopic analysis of tyrosine hydroxylase, dopamine-beta-hydroxylase and phenylethanolamine-N-methyltransferase immunoreactive innervation of the hypothalamic paraventricular nucleus in the rat (1986). *Histochemistry*84:105-120.

754. Sawchenko, P.E. and Swanson, L.W. The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat (1983). *J.Comp.Neurol.*218:121-144.

755. Linthorst, A.C.E., Flachskamm, C., Holsboer, F. and Reul, J.M.H.M. intraperitoneal administration of bacterial endotoxin enhances noradrenergic neurotransmission in the rat preoptic area: relationship with body temperature and hypothalamic-pituitary-adrenocortical axis activity (1995). *European Journal Of Neuroscience*7:2418-2430.

756. Pacak, K., Palkovits, M., Kvetnansky, R., Yadid, G., Kopin, I.J. and Goldstein, D.S. Effects of various stressors on in vivo norepinephrine release in the hypothalamic paraventricular nucleus and on the pituitary-adrenocortical axis (1995). *Ann.N.Y.Acad.Sci.*771:115-130.

757. Pacak, K., Palkovits, M., Kopin, I.J. and Goldstein, D.S. Stress-induced norepinephrine release in the hypothalamic paraventricular nucleus and pituitary-adrenocortical and sympathoadrenal activity: in vivo microdialysis studies (1995). *Front.Neuroendocrinol.*16:89-150.

758. Tanaka, M., Kohno, Y., Nakagawa, R., Ida, Y., Takeda, S., Nagasaki, N. and Noda, Y. Regional characteristics of stress-induced increases in brain noradrenaline release in rats (1983). *Pharmacol.Biochem.Behav.*19:543-547.

759. Plotsky, P.M. Facilitation of immunoreactive corticotropin-releasing factor secretion into the hypophysial-portal circulation after activation of catecholaminergic pathways or central norepinephrine injection (1987). *Endocrinology*121:924-930.

760. Itoi, K., Helmreich, D.L., Lopez Figueroa, M.O. and Watson, S.J. Differential regulation of corticotropin-releasing hormone and vasopressin gene transcription in the hypothalamus by norepinephrine (1999). *J.Neurosci.*19:5464-5472.

761. Orliaguet, G., Melik Parsadaniantz, S., Lenoir, V., Duval, P. and Kerdelhue, B. Norepinephrine but not epinephrine stimulates the release of corticotropin-releasing factor from in vitro superfused rat hypothalamus (1995). *J.Neurosci.Res.*42:236-241.

762. Guillaume, V., Conte Devolx, B., Szafarczyk, A., Malaval, F., Pares Herbute, N., Grino, M., Alonso, G., Assenmacher, I. and Oliver, C. The corticotropin-releasing factor release in rat hypophysial portal blood is mediated by brain catecholamines (1987). *Neuroendocrinology*46:143-146.

763. Sawchenko, P.E. Effects of catecholamine-depleting medullary knife cuts on corticotropin-releasing factor and vasopressin immunoreactivity in the hypothalamus of normal and steroid-manipulated rats (1988). *Neuroendocrinology*48:459-470.

764. Weidenfeld, J., Abramsky, O. and Ovadia, H. Evidence for the involvement of the central adrenergic system in interleukin 1-induced adrenocortical response (1989).

Neuropharmacology.28:1411-1414.

765. Szafarczyk, A., Alonso, G., Ixart, G., Malaval, F. and Assenmacher, I. Diurnal-stimulated and stress-induced ACTH release in rats is mediated by ventral noradrenergic bundle (1985). *Am.J.Physiol*.249:E219-E226.

766. Szafarczyk, A., Guillaume, V., Conte Devolx, B., Alonso, G., Malaval, F., Pares Herbute, N., Oliver, C. and Assenmacher, I. Central catecholaminergic system stimulates secretion of CRH at different sites (1988). *Am.J.Physiol*.255:E463-E468.

767. Gaillet, S., Malaval, F., Barbanel, G., Pelletier, G., Assenmacher, I. and Szafarczyk, A. Inhibitory interactions between alpha 2-adrenergic and opoid but not NPY mechanisms controlling the CRF-ACTH axis in the rat (1991). *Regul.Pept*.36:249-261.

768. Gaillet, S., Alonso, G., Le Borgne, R., Barbanel, G., Malaval, F., Assenmacher, I. and Szafarczyk, A. Effects of discrete lesions in the ventral noradrenergic ascending bundle on the corticotropic stress response depend on the site of the lesion and on the plasma levels of adrenal steroids (1993). *Neuroendocrinology*58:408-419.

769. Guo, A.L., Petraglia, F., Criscuolo, M., Ficarra, G., Salvestroni, C., Nappi, R.E., Trentini, G.P. and Genazzani, A.R. adrenergic and serotoninergic receptors mediate the immunological activation of corticosterone secretion in male rats (1996). *Gynecological Endocrinology*10:149-154.

770. Givalois, L., Siaud, P., Mekaouche, M., Ixart, G., Malaval, F., Assenmacher, I. and Barbanel, G. involvement of central histamine in the early phase of acth and corticosterone responses to endotoxin in rats (1996). *Neuroendocrinology*63:219-226.

771. Knigge, U., Kjaer, A., Jorgensen, H., Garbarg, M., Ross, C., Rouleau, A. and Warberg, J. Role of hypothalamic histaminergic neurons in mediation of ACTH and beta-endorphin responses to LPS endotoxin in vivo (1994). *Neuroendocrinology*60:243-251.

772. Lee, S., Barbanel, G. and Rivier, C. Systemic endotoxin increases steady-state gene expression of hypothalamic nitric oxide synthase: comparison with corticotropin- releasing factor and vasopressin gene transcripts (1995). *Brain Res*.705:136-148.

773. Harada, S., Imaki, T., Chikada, N., Naruse, M. and Demura, H. Distinct distribution and time-course changes in neuronal nitric oxide synthase and inducible NOS in the paraventricular nucleus following lipopolysaccharide injection (1999). *Brain Res*.821:322-332.

774. McCann, S.M., Karanth, S., Kimura, M., Yu, W.H. and Rettori, V. The role of nitric oxide (NO) in control of hypothalamic-pituitary function (1996). *Rev.Bras.Biol*.56 Su 1 Pt 1:105-112.

775. Lee, S., Kim, C.K. and Rivier, C. Nitric oxide stimulates ACTH secretion and the transcription of the genes encoding for NGFI-B, corticotropin-releasing factor, corticotropin-releasing factor receptor type 1, and vasopressin in the hypothalamus of the intact rat (1999). *J.Neurosci*.19:7640-7647.

776. Sawchenko, P.E., Swanson, L.W., Steinbusch, H.W. and Verhofstad, A.A. The

distribution and cells of origin of serotonergic inputs to the paraventricular and supraoptic nuclei of the rat (1983). *Brain Res.*277:355-360.

777. Givalois, L., Becq, H., Siaud, P., Ixart, G., Assenmacher, I. and Barbanel, G. Serotoninergic and suprachiasmatic nucleus involvement in the corticotropic response to systemic endotoxin challenge in rats (1999). *J.Neuroendocrinol.*11:629-636.

778. Laflamme, N., Feuvrier, E., Richard, D. and Rivest, S. Involvement of serotonergic pathways in mediating the neuronal activity and genetic transcription of neuroendocrine corticotropin-releasing factor in the brain of systemically endotoxin-challenged rats (1999). *Neuroscience.*88:223-240.

779. Conde, G.L., Renshaw, D., Lightman, S.L. and Harbuz, M.S. Serotonin depletion does not alter lipopolysaccharide-induced activation of the rat paraventricular nucleus (1998). *J.Endocrinol.*156:245-251.

780. Cohen, S., Doyle, W.J. and Skoner, D.P. Psychological stress, cytokine production, and severity of upper respiratory illness (1999). *Psychosom.Med.*61:175-180.

781. Song, C., Kenis, G., van Gastel, A., Bosmans, E., Lin, A., de Jong, R., Neels, H., Scharpe, S., Janca, A., Yasukawa, K. and Maes, M. Influence of psychological stress on immune-inflammatory variables in normal humans. Part II. Altered serum concentrations of natural anti-inflammatory agents and soluble membrane antigens of monocytes and T lymphocytes (1999). *Psychiatry Res.*85:293-303.

782. Diorio, D., Viau, V. and Meaney, M.J. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress (1993). *J.Neurosci.*13:3839-3847.

783. Pacak, K., Baffi, J.S., Kvetnansky, R., Goldstein, D.S. and Palkovits, M. Stressor-specific activation of catecholaminergic systems: implications for stress-related hypothalamic-pituitary-adrenocortical responses (1998). *Adv.Pharmacol.*42:561-564.

784. Pan, B., Castro Lopes, J.M. and Coimbra, A. Chemical sensory deafferentation abolishes hypothalamic pituitary activation induced by noxious stimulation or electroacupuncture but only decreases that caused by immobilization stress. A c-fos study (1997). *Neuroscience.*78:1059-1068.

785. Brown, M.R., Rivier, C. and Vale, W. Central nervous system regulation of adrenocorticotropin secretion: role of somatostatins (1984). *Endocrinology*114:1546-1549.

786. Rivest, S. and Rivier, C. Influence of the paraventricular nucleus of the hypothalamus in the alteration of neuroendocrine functions induced by intermittent footshock or interleukin (1991). *Endocrinology*129:2049-2057.

787. Pezzone, M.A., Dohanics, J. and Rabin, B.S. Effects of footshock stress upon spleen and peripheral blood lymphocyte mitogenic responses in rats with lesions of the paraventricular nuclei (1994). *J.Neuroimmunol.*53:39-46.

788. Chappell, P.B., Smith, M.A., Kilts, C.D., Bissette, G., Ritchie, J., Anderson, C. and Nemeroff, C.B. Alterations in corticotropin-releasing factor-like immunoreactivity in

discrete rat brain regions after acute and chronic stress (1986). *J.Neurosci.*6:2908-2914.

789. Hashimoto, K., Murakami, K., Takao, T., Makino, S., Sugawara, M. and Ota, Z. Effect of acute ether or restraint stress on plasma corticotropin-releasing hormone, vasopressin and oxytocin levels in the rat (1989). *Acta Med.Okayama.*43:161-167.

790. Bassett, J.R. and Cairncross, K.D. Time course for plasma 11-hydroxycorticosteroid elevation in rats during stress (1975). *Pharmacol.Biochem.Behav.*3:139-142.

791. Ma, X.M., Levy, A. and Lightman, S.L. Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: a study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA (1997). *Endocrinology*138:4351-4357.

792. Pacak, K., Palkovits, M., Kvetnansky, R., Matern, P., Hart, C., Kopin, I.J. and Goldstein, D.S. Catecholaminergic inhibition by hypercortisolemia in the paraventricular nucleus of conscious rats (1995). *Endocrinology*136:4814-4819.

793. Whitnall, M.H. Stress selectively activates the vasopressin-containing subset of corticotropin-releasing hormone neurons (1989). *Neuroendocrinology*50:702-707.

794. Diamant, M. and de Wied, D. Autonomic and behavioral effects of centrally administered corticotropin-releasing factor in rats (1991). *Endocrinology*129:446-454.

795. Rivier, C. and Vale, W. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin (1983). *Nature*305:325-327.

796. Scaccianoce, S., Muscolo, L.A., Cigliana, G., Navarra, D., Nicolai, R. and Angelucci, L. Evidence for a specific role of vasopressin in sustaining pituitary-adrenocortical stress response in the rat (1991). *Endocrinology*128:3138-3143.

797. Miaskowski, C., Ong, G.L., Lukic, D. and Haldar, J. Immobilization stress affects oxytocin and vasopressin levels in hypothalamic and extrahypothalamic sites (1988). *Brain Res.*458:137-141.

798. Windle, R.J., Shanks, N., Lightman, S.L. and Ingram, C.D. Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats (1997). *Endocrinology*138:2829-2834.

799. Kovacs, K.J. and Sawchenko, P.E. Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons (1996). *J.Neurosci.*16:262-273.

800. Herman, J.P. In situ hybridization analysis of vasopressin gene transcription in the paraventricular and supraoptic nuclei of the rat: regulation by stress and glucocorticoids (1995). *J.Comp.Neurol.*363:15-27.

801. Legradi, G., Holzer, D., Kapcala, L.P. and Lechan, R.M. Glucocorticoids inhibit stress-induced phosphorylation of CREB in corticotropin-releasing hormone neurons of the hypothalamic paraventricular nucleus (1997). *Neuroendocrinology*66:86-97.

802. McCarty, R. and Kopin, I.J. Sympatho-adrenal medullary activity and behavior during exposure to footshock stress: a comparison of seven rat strains (1978). *Physiol.Behav.*21:567-572.

803. Kvetnansky, R., Sun, C.L., Lake, C.R., Thoa, N., Torda, T. and Kopin, I.J. Effect of handling and forced immobilization on rat plasma levels of epinephrine, norepinephrine, and dopamine-beta-hydroxylase (1978). *Endocrinology*103:1868-1874.

804. Brown, M.R., Fisher, L.A., Rivier, J., Spiess, J., Rivier, C. and Vale, W. Corticotropin-releasing factor: effects on the sympathetic nervous system and oxygen consumption (1982). *Life.Sci.*30:207-210.

805. Irwin, M., Hauger, R.L., Jones, L., Provencio, M. and Britton, K.T. Sympathetic nervous system mediates central corticotropin-releasing factor induced suppression of natural killer cytotoxicity (1990). *J.Pharmacol.Exp.Ther.*255:101-107.

806. Irwin, M., Hauger, R.L., Brown, M. and Britton, K.T. CRF activates autonomic nervous system and reduces natural killer cytotoxicity (1988). *Am.J.Physiol.*255:R744-R747.

807. Dunn, A.J. Stress-related changes in cerebral catecholamine and indoleamine metabolism: lack of effect of adrenalectomy and corticosterone (1988). *J.Neurochem.*51:406-412.

808. Zalcman, S., Richter, M. and Anisman, H. Alterations of immune functioning following exposure to stressor- related cues (1989). *Brain Behav.Immun.*3:99-109.

809. Goldstein, L.E., Rasmusson, A.M., Bunney, B.S. and Roth, R.H. Role of the amygdala in the coordination of behavioral, neuroendocrine, and prefrontal cortical monoamine responses to psychological stress in the rat (1996). *J.Neurosci.*16:4787-4798.

810. Pacak, K., Palkovits, M., Kvetnansky, R., Fukuhara, K., Armando, I., Kopin, I.J. and Goldstein, D.S. Effects of single or repeated immobilization on release of norepinephrine and its metabolites in the central nucleus of the amygdala in conscious rats (1993). *Neuroendocrinology*57:626-633.

811. Pacak, K., McCarty, R., Palkovits, M., Kopin, I.J. and Goldstein, D.S. Effects of immobilization on in vivo release of norepinephrine in the bed nucleus of the stria terminalis in conscious rats (1995). *Brain Res.*688:242-246.

812. Shanks, N., Griffiths, J. and Anisman, H. Norepinephrine and serotonin alterations following chronic stressor exposure: mouse strain differences (1994). *Pharmacol.Biochem.Behav.*49:57-65.

813. Anisman, H. and Zacharko, R.M. Behavioral and neurochemical consequences associated with stressors (1986). *Ann.N.Y.Acad.Sci.*467:205-225.

814. Heinsbroek, R.P., van Haaren, F., Feenstra, M.G., Boon, P. and van de Poll, N.E. Controllable and uncontrollable footshock and monoaminergic activity in the frontal cortex of male and female rats (1991). *Brain Res.*551:247-255.

815. Dunn, A.J. Stress-related activation of cerebral dopaminergic systems (1988). *Ann.N.Y.Acad.Sci.*537:188-205.

816. Lachuer, J., Gaillet, S., Barbagli, B., Buda, M. and Tappaz, M. Differential early time course activation of the brainstem catecholaminergic groups in response to various stresses (1991). *Neuroendocrinology*53:589-596.

817. Knigge, U., Matzen, S. and Warberg, J. Histamine as a neuroendocrine regulator of the stress-induced release of peripheral catecholamines (1990). *Endocrinology*126:1430-1434.

818. Zalcman, S., Shanks, N. and Anisman, H. Time-dependent variations of central norepinephrine and dopamine following antigen administration (1991). *Brain Res.*557:69-76.

819. Shanks, N., Zalcman, S., Zacharko, R.M. and Anisman, H. Alterations of central norepinephrine, dopamine and serotonin in several strains of mice following acute stressor exposure (1991). *Pharmacol.Biochem.Behav.*38:69-75.

820. Cullinan, W.E., Herman, J.P., Battaglia, D.F., Akil, H. and Watson, S.J. Pattern and time course of immediate early gene expression in rat brain following acute stress (1995). *Neuroscience.*64:477-505.

821. Bohus, B., Koolhaas, J.M., Korte, S.M., Roozendaal, B. and Wiersma, A. Forebrain pathways and their behavioural interactions with neuroendocrine and cardiovascular function in the rat (1996). *Clin.Exp.Pharmacol.Physiol.*23:177-182.

822. Dunn, J.D. and Whitener, J. Plasma corticosterone responses to electrical stimulation of the amygdaloid complex: cytoarchitectural specificity (1986). *Neuroendocrinology*42:211-217.

823. Gray, T.S. Amygdaloid CRF pathways. Role in autonomic, neuroendocrine, and behavioral responses to stress (1993). *Ann.N.Y.Acad.Sci.*697:53-60.

824. Murakami, K., Akana, S.F. and Dallman, M.F. Dopamine-beta-hydroxylase activity is necessary for hypothalamo-pituitary-adrenal (HPA) responses to ether, and stress-induced facilitation of subsequent HPA responses to acute ether emerges as HPA responses are inhibited by increasing corticosterone (B) (1997). *J.Neuroendocrinol.*9:601-608.

825. Gaillet, S., Lachuer, J., Malaval, F., Assenmacher, I. and Szafarczyk, A. The involvement of noradrenergic ascending pathways in the stress-induced activation of ACTH and corticosterone secretions is dependent on the nature of stressors (1991). *Exp.Brain Res.*87:173-180.

826. Schmidt, E.D., Binnekade, R., Janszen, A.W. and Tilders, F.J. Short stressor induced long-lasting increases of vasopressin stores in hypothalamic corticotropin-releasing hormone (CRH) neurons in adult rats (1996). *J.Neuroendocrinol.*8:703-712.

827. Schmidt, E.D., Janszen, A.W., Wouterlood, F.G. and Tilders, F.J. Interleukin-1-induced long-lasting changes in hypothalamic corticotropin-releasing hormone (CRH)--neurons and hyperresponsiveness of the hypothalamus-pituitary-adrenal axis (1995). *J.Neurosci.*15:7417-7426.

828. Herman, J.P., Adams, D. and Prewitt, C. Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm (1995). *Neuroendocrinology*61:180-190.

829. Brzozowski, T., Konturek, S.J., Pytko Polonczyk, J. and Warzecha, Z. Gastric adaptation to stress: role of sensory nerves, salivary glands, and adrenal glands (1995). *Scand.J.Gastroenterol.*30:6-16.

830. Irwin, J., Ahluwalia, P. and Anisman, H. Sensitization of norepinephrine activity following acute and chronic footshock (1986). *Brain Res.*379:98-103.

831. Pacak, K., Armando, I., Fukuhara, K., Kvetnansky, R., Palkovits, M., Kopin, I.J. and Goldstein, D.S. Noradrenergic activation in the paraventricular nucleus during acute and chronic immobilization stress in rats: an in vivo microdialysis study (1992). *Brain Res.*589:91-96.

832. Melia, K.R., Ryabinin, A.E., Schroeder, R., Bloom, F.E. and Wilson, M.C. Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress (1994). *J.Neurosci.*14:5929-5938.

833. Konarska, M., Stewart, R.E. and McCarty, R. Predictability of chronic intermittent stress: effects on sympathetic-adrenal medullary responses of laboratory rats (1990). *Behav.Neural.Biol.*53:231-243.

834. Konarska, M., Stewart, R.E. and McCarty, R. Habituation of sympathetic-adrenal medullary responses following exposure to chronic intermittent stress (1989). *Physiol.Behav.*45:255-261.

835. Pitman, D.L., Ottenweller, J.E. and Natelson, B.H. Effect of stressor intensity on habituation and sensitization of glucocorticoid responses in rats (1990). *Behav.Neurosci.*104:28-36.

836. Dobrakovova, M., Kvetnansky, R., Oprsalova, Z. and Jezova, D. Specificity of the effect of repeated handling on sympathetic-adrenomedullary and pituitary-adrenocortical activity in rats (1993). *Psychoneuroendocrinology.*18:163-174.

837. Bhatnagar, S., Shanks, N. and Meaney, M.J. Plaque-forming cell responses and antibody titers following injection of sheep red blood cells in nonstressed, acute, and/or chronically stressed handled and nonhandled animals (1996). *Dev.Psychobiol.*29:171-181.

838. Viau, V., Sharma, S., Plotsky, P.M. and Meaney, M.J. Increased plasma ACTH responses to stress in nonhandled compared with handled rats require basal levels of corticosterone and are associated with increased levels of ACTH secretagogues in the median eminence (1993). *J.Neurosci.*13:1097-1105.

839. Keim, K.L. and Sigg, E.B. Physiological and biochemical concomitants of restraint stress in rats (1976). *Pharmacol.Biochem.Behav.*4:289-297.

840. Kohno, H. and Ohkubo, Y. Comparative glucoregulatory responses of mice to restraint and footshock stress stimuli (1998). *Biol.Pharm.Bull.*21:113-116.

841. Orr, T.E., Meyerhoff, J.L., Mougey, E.H. and Bunnell, B.N. Hyperresponsiveness of the rat neuroendocrine system due to repeated exposure to stress (1990). *Psychoneuroendocrinology.* 15:317-328.

842. Irwin, J., Ahluwalia, P., Zacharko, R.M. and Anisman, H. Central norepinephrine and plasma corticosterone following acute and chronic stressors: influence of social isolation and handling (1986). *Pharmacol.Biochem.Behav.*24:1151-1154.

843. Kant, G.J., Leu, J.R., Anderson, S.M. and Mougey, E.H. Effects of chronic stress on plasma corticosterone, ACTH and prolactin (1987). *Physiol.Behav.*40:775-779.

844. Ratner, A., Yelvington, D.B. and Rosenthal, M. Prolactin and corticosterone response to repeated footshock stress in male rats (1989). *Psychoneuroendocrinology.* 14:393-396.

845. Dhabhar, F.S., McEwen, B.S. and Spencer, R.L. Adaptation to prolonged or repeated stress--comparison between rat strains showing intrinsic differences in reactivity to acute stress (1997). *Neuroendocrinology*65:360-368.

846. Natelson, B.H., Ottenweller, J.E., Cook, J.A., Pitman, D., McCarty, R. and Tapp, W.N. Effect of stressor intensity on habituation of the adrenocortical stress response (1988). *Physiol.Behav.*43:41-46.

847. Magarinos, A.M. and McEwen, B.S. Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: comparison of stressors (1995). *Neuroscience.*69:83-88.

848. Armario, A., Lopez Calderon, A., Jolin, T. and Balasch, J. Response of anterior pituitary hormones to chronic stress. The specificity of adaptation (1986). *Neurosci.Biobehav.Rev.* 10:245-250.

849. Armario, A., Restrepo, C., Castellanos, J.M. and Balasch, J. Dissociation between adrenocorticotropin and corticosterone responses to restraint after previous chronic exposure to stress (1985). *Life.Sci.*36:2085-2092.

850. Tizabi, Y. and Aguilera, G. Desensitization of the hypothalamic-pituitary-adrenal axis following prolonged administration of corticotropin-releasing hormone or vasopressin (1992). *Neuroendocrinology*56:611-618.

851. Konarska, M., Stewart, R.E. and McCarty, R. Sensitization of sympathetic-adrenal medullary responses to a novel stressor in chronically stressed laboratory rats (1989). *Physiol.Behav.*46:129-135.

852. Culman, J., Kopin, I.J. and Saavedra, J.M. Regulation of corticotropin-releasing hormone and pituitary-adrenocortical response during acute and repeated stress in the rat (1991). *Endocr.Regul.*25:151-158.

853. Bhatnagar, S. and Meaney, M.J. Hypothalamic-pituitary-adrenal function in chronic intermittently cold-stressed neonatally handled and non handled rats (1995). *J.Neuroendocrinol.*7:97-108.

854. Hauger, R.L., Lorang, M., Irwin, M. and Aguilera, G. CRF receptor regulation and

sensitization of ACTH responses to acute ether stress during chronic intermittent immobilization stress (1990). *Brain Res.*532:34-40.

855. Hashiguchi, H., Ye, S.H., Morris, M. and Alexander, N. Single and repeated environmental stress: effect on plasma oxytocin, corticosterone, catecholamines, and behavior (1997). *Physiol.Behav.*61:731-736.

856. Lu, Z.W., Song, C., Ravindran, A.V., Merali, Z. and Anisman, H. Influence of a psychogenic and a neurogenic stressor on several indices of immune functioning in different strains of mice (1998). *Brain Behav.Immun.*12:7-22.

857. Lysle, D.T., Cunnick, J.E. and Rabin, B.S. Stressor-induced alteration of lymphocyte proliferation in mice: evidence for enhancement of mitogenic responsiveness (1990). *Brain Behav.Immun.*4:269-277.

858. Shurin, M.R., Zhou, D., Kusnecov, A., Rassnick, S. and Rabin, B.S. Effect of one or more footshocks on spleen and blood lymphocyte proliferation in rats (1994). *Brain Behav.Immun.*8:57-65.

859. Cunnick, J.E., Lysle, D.T., Kucinski, B.J. and Rabin, B.S. Evidence that shock-induced immune suppression is mediated by adrenal hormones and peripheral beta-adrenergic receptors (1990). *Pharmacol.Biochem.Behav.*36:645-651.

860. Wan, W., Vriend, C.Y., Wetmore, L., Gartner, J.G., Greenberg, A.H. and Nance, D.M. The effects of stress on splenic immune function are mediated by the splenic nerve (1993). *Brain Res.Bull.*30:101-105.

861. Toft, P., Dagnaes Hansen, F., Tonnesen, E. and Basse, P.M. The effect of surgical stress and endotoxin-induced sepsis on the NK-cell activity, distribution and pulmonary clearance of YAC-1 and melanoma cells (1999). *APMIS.*107:359-364.

862. Weiss, J.M., Sundar, S.K., Becker, K.J. and Cierpial, M.A. Behavioral and neural influences on cellular immune responses: effects of stress and interleukin-1 (1989). *J.Clin.Psychiatry*50 Suppl:43-53; discuss.

863. Bonneau, R.H., Sheridan, J.F., Feng, N.G. and Glaser, R. Stress-induced effects on cell-mediated innate and adaptive memory components of the murine immune response to herpes simplex virus infection (1991). *Brain Behav.Immun.*5:274-295.

864. Irwin, M., Vale, W. and Rivier, C. Central corticotropin-releasing factor mediates the suppressive effect of stress on natural killer cytotoxicity (1990). *Endocrinology*126:2837-2844.

865. Irwin, M., Hauger, R.L., Brown, M. and Britton, K.T. CRF activates autonomic nervous system and reduces natural killer cytotoxicity (1988). *Am.J.Physiol.*255:R744-R747.

866. Beno, D.W. and Kimura, R.E. Nonstressed rat model of acute endotoxemia that unmasks the endotoxin-induced TNF-alpha response (1999). *Am.J.Physiol.*276:H671-H678.

867. Goujon, E., Parnet, P., Laye, S., Combe, C., Kelley, K.W. and Dantzer, R. Stress downregulates lipopolysaccharide-induced expression of proinflammatory cytokines in the spleen, pituitary, and brain of mice (1995). *Brain Behav.Immun.*9:292-303.

868. Persoons, J.H., Schornagel, K., Breve, J., Berkenbosch, F. and Kraal, G. Acute stress affects cytokines and nitric oxide production by alveolar macrophages differently (1995). *Am.J.Respir.Crit.Care.Med.*152:619-624.

869. Zhu, G.F., Chancellor Freeland, C., Berman, A.S., Kage, R., Leeman, S.E., Beller, D.I. and Black, P.H. Endogenous substance P mediates cold water stress-induced increase in interleukin-6 secretion from peritoneal macrophages (1996). *J.Neurosci.*16:3745-3752.

870. Persoons, J.H., Moes, N.M., Broug Holub, E., Schornagel, K., Tilders, F.J. and Kraal, G. Acute and long-term effects of stressors on pulmonary immune functions (1997). *Am.J.Respir.Cell Mol.Biol.*17:203-208.

871. Broug Holub, E., Persoons, J.H., Schornagel, K., Mastbergen, S.C. and Kraal, G. Effects of stress on alveolar macrophages: a role for the sympathetic nervous system (1998). *Am.J.Respir.Cell Mol.Biol.*19:842-848.

872. Palleroni, A.V., Hajos, S., Wright, R.B. and Palleroni, N.J. Nitric oxide synthase induction in lines of macrophages from different anatomical sites (1998). *Cell Mol.Biol.Noisy.le.grand.*44:527-535.

873. Ando, T., Rivier, J., Yanaihara, H. and Arimura, A. Peripheral corticotropin-releasing factor mediates the elevation of plasma IL-6 by immobilization stress in rats (1998). *Am.J.Physiol.*275:R1461-R1467.

874. Shintani, F., Nakaki, T., Kanba, S., Kato, R. and Asai, M. Role of interleukin-1 in stress responses. A putative neurotransmitter (1995). *Mol.Neurobiol.*10:47-71.

875. Maier, S.F. and Watkins, L.R. Intracerebroventricular interleukin-1 receptor antagonist blocks the enhancement of fear conditioning and interference with escape produced by inescapable shock (1995). *Brain Res.*695:279-282.

876. Dobbs, C.M., Vasquez, M., Glaser, R. and Sheridan, J.F. Mechanisms of stress-induced modulation of viral pathogenesis and immunity (1993). *J.Neuroimmunol.*48:151-160.

877. Bonneau, R.H., Sheridan, J.F., Feng, N. and Glaser, R. Stress-induced modulation of the primary cellular immune response to herpes simplex virus infection is mediated by both adrenal-dependent and independent mechanisms (1993). *J.Neuroimmunol.*42:167-176.

878. Sheridan, J.F., Feng, N.G., Bonneau, R.H., Allen, C.M., Huneycutt, B.S. and Glaser, R. Restraint stress differentially affects anti-viral cellular and humoral immune responses in mice (1991). *J.Neuroimmunol.*31:245-255.

879. Hermann, G., Tovar, C.A., Beck, F.M., Allen, C. and Sheridan, J.F. Restraint stress differentially affects the pathogenesis of an experimental influenza viral infection in three inbred strains of mice (1993). *J.Neuroimmunol.*47:83-94.

880. Hermann, G., Beck, F.M. and Sheridan, J.F. Stress-induced glucocorticoid response modulates mononuclear cell trafficking during an experimental influenza viral infection (1995). *J.Neuroimmunol.*56:179-186.

881. Mizobe, K., Kishihara, K., Ezz Din El Naggar, R., Madkour, G.A., Kubo, C. and Nomoto, K. Restraint stress-induced elevation of endogenous glucocorticoid suppresses migration of granulocytes and macrophages to an inflammatory locus (1997). *J.Neuroimmunol.*73:81-89.

882. Jessop, J.J., West, G.L. and Sobotka, T.J. Immunomodulatory effects of footshock in the rat (1989). *J.Neuroimmunol.*25:241-249.

883. Zalcman, S. and Anisman, H. Acute and chronic stressor effects on the antibody response to sheep red blood cells (1993). *Pharmacol.Biochem.Behav.*46:445-452.

884. Lu, Z.W., Song, C., Ravindran, A.V., Merali, Z. and Anisman, H. Influence of a psychogenic and a neurogenic stressor on several indices of immune functioning in different strains of mice (1998). *Brain Behav.Immun.*12:7-22.

885. Zalcman, S., Minkiewicz-Janda, A., Richter, M. and Anisman, H. Critical periods associated with stressor effects on antibody titers and on the plaque-forming cell response to sheep red blood cells (1988). *Brain Behav.Immun.*2:254-266.

886. Zalcman, S., Kerr, L. and Anisman, H. Immunosuppression elicited by stressors and stressor-related odors (1991). *Brain Behav.Immun.*5:262-273.

887. Decker, D., Schondorf, M., Bidlingmaier, F., Hirner, A. and von Ruecker, A.A. Surgical stress induces a shift in the type-1/type-2 T-helper cell balance, suggesting down-regulation of cell-mediated and up-regulation of antibody-mediated immunity commensurate to the trauma (1996). *Surgery*119:316-325.

888. Kort, W.J., Weijma, I.M. and Westbroek, D.L. Effect of stress and dietary fatty acids on allograft survival in the rat (1979). *Eur.Surg.Res.*11:434-444.

889. Nance, D.M., Hopkins, D.A. and Bieger, D. Re-investigation of the innervation of the thymus gland in mice and rats (1987). *Brain Behav.Immun.*1:134-147.

890. Zhou, L. *Neuroendocrine and sympathetic control of splenic macrophage IL-1 secretion*, 1992. MsSc. Thesis: Department of Immunology, University of Manitoba, Winnipeg, Canada,

891. Meltzer, J.C., Sanders, V., Grimm, P.C., Chiasson, N., Hoeltke, H.J., Garrett, K.L., Greenberg, A.H. and Nance, D.M. Nonradioactive Northern blotting with biotinylated and digoxigenin-labeled RNA probes (1998). *Electrophoresis.*19:1351-1355.

892. Meltzer, J.C., Sanders, V., Grimm, P.C., Stern, E., Rivier, C., Lee, S., Rennie, S.L., Gietz, R.D., Hole, A.K., Watson, P.H., Greenberg, A.H. and Nance, D.M. Production of digoxigenin-labelled RNA probes and the detection of cytokine mRNA in rat spleen and brain by in situ hybridization (1998). *Brain Res.Brain Res.Protoc.*2:339-351.

893. Triest, S., Maiter, D. and Ketelslegers, J.M. Validation of northern blot analysis for

quantitating the expression of several genes in rat liver (1995). *Biotechniques.* 19:706-8, 710.

894. Barbu, V. and Dautry, F. Northern blot normalization with a 28S rRNA oligonucleotide probe (1989). *Nucleic Acids Res.* 17:7115.

895. Correa Rotter, R., Mariash, C.N. and Rosenberg, M.E. Loading and transfer control for Northern hybridization (1992). *Biotechniques.* 12:154-158.

896. Spanakis, E. Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls (1993). *Nucleic Acids Res.* 21:3809-3819.

897. Bursten, S.L., Stevenson, F., Torrano, F. and Lovett, D.H. Mesangial cell activation by bacterial endotoxin. Induction of rapid cytoskeletal reorganization and gene expression (1991). *Am.J.Pathol.* 139:371-382.

898. Bhatia, P., Taylor, W.R., Greenberg, A.H. and Wright, J.A. Comparison of glyceraldehyde-3-phosphate dehydrogenase and 28S-ribosomal RNA gene expression as RNA loading controls for northern blot analysis of cell lines of varying malignant potential (1994). *Anal.Biochem.* 216:223-226.

899. Finnegan, M.C., Goepel, J.R., Hancock, B.W. and Goyns, M.H. Investigation of the expression of housekeeping genes in non-Hodgkin's lymphoma (1993). *Leuk.Lymphoma.* 10:387-393.

900. Yamato, E., Ikegami, H., Takekawa, K., Fujisawa, T., Nakagawa, Y., Hamada, Y., Ueda, H. and Ogihara, T. Internal marker for quantitative analysis of mRNA by reverse transcriptase-polymerase chain reaction in pancreatic beta cells [letter] (1996). *Diabetologia.* 39:747-748.

901. Varesio, L., Clayton, M., Radzioch, D. and Bonvini, E. Selective inhibition of 28S ribosomal RNA in macrophages activated by interferon-gamma or -beta (1987). *J.Immunol.* 138:2332-2337.

902. Varesio, L. Imbalanced accumulation of ribosomal RNA in macrophages activated in vivo or in vitro to a cytolytic stage (1985). *J.Immunol.* 134:1262-1267.

903. de Leeuw, W.J., Slagboom, P.E. and Vijg, J. Quantitative comparison of mRNA levels in mammalian tissues: 28S ribosomal RNA level as an accurate internal control (1989). *Nucleic Acids Res.* 17:10137-10138.

904. Duhl, D.M., Gillespie, D.D. and Sulser, F. Ethidium bromide fluorescence of 28S ribosomal RNA can be used to normalize samples in northern or dot blots when analyzing small drug-induced changes in specific mRNA (1992). *J.Neurosci.Methods* 42:211-218.

905. Savonet, V., Maenhaut, C., Miot, F. and Pirson, I. Pitfalls in the use of several "housekeeping" genes as standards for quantitation of mRNA: the example of thyroid cells (1997). *Anal.Biochem.* 247:165-167.

906. Molina, P.E., Malek, S., Lang, C.H., Qian, L., Naukam, R. and Abumrad, N.N. Early

organ-specific hemorrhage-induced increases in tissue cytokine content: associated neurohormonal and opioid alterations (1997). *Neuroimmunomodulation.*4:28-36.

907. Hawes, A.S., Fischer, E., Marano, M.A., Van Zee, K.J., Rock, C.S., Lowry, S.F., Calvano, S.E. and Moldawer, L.L. Comparison of peripheral blood leukocyte kinetics after live Escherichia coli, endotoxin, or interleukin-1 alpha administration. Studies using a novel interleukin-1 receptor antagonist (1993). *Ann.Surg.*218:79-90.

908. Ge, Y., Ezzell, R.M., Tompkins, R.G. and Warren, H.S. Cellular distribution of endotoxin after injection of chemically purified lipopolysaccharide differs from that after injection of live bacteria (1994). *J.Infect.Dis.*169:95-104.

909. Kakizaki, Y., Watanobe, H., Kohsaka, A. and Suda, T. Temporal profiles of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in the plasma and hypothalamic paraventricular nucleus after intravenous or intraperitoneal administration of lipopolysaccharide in the rat: estimation by push-pull perfusion (1999). *Endocr.J.*46:487-496.

910. Brown, A.R. Immunological functions of splenic B-lymphocytes (1992). *Crit.Rev.Immunol.*11:395-417.

911. Van den Eertwegh, A.J., Boersma, W.J. and Claassen, E. Immunological functions and in vivo cell-cell interactions of T cells in the spleen (1992). *Crit.Rev.Immunol.*11:337-380.

912. Amlot, P.L. and Hayes, A.E. Impaired human antibody response to the thymus-independent antigen, DNP-Ficoll, after splenectomy. Implications for post-splenectomy infections (1985). *Lancet*1:1008-1011.

913. Lyons, A.B. and Parish, C.R. Are murine marginal-zone macrophages the splenic white pulp analog of high endothelial venules? (1995). *Eur.J.Immunol.*25:3165-3172.

914. Miyakawa, K., Matsuno, K., Ohmori, J. and Kotani, M. Localization in the rat spleen of carbon-laden macrophages introduced into the splenic artery: a subpopulation of macrophages entering the white pulp (1990). *Anat.Rec.*227:464-474.

915. Zuckerman, S.H., Evans, G.F. and Guthrie, L. Transcriptional and post-transcriptional mechanisms involved in the differential expression of LPS-induced IL-1 and TNF mRNA (1991). *Immunology.*73:460-465.

916. Novogrodsky, A., Vanichkin, A., Patya, M., Gazit, A., Osherov, N. and Levitzki, A. Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors (1994). *Science*264:1319-1322.

917. Zheng, H., Fletcher, D., Kozak, W., Jiang, M., Hofmann, K.J., Conn, C.A., Soszynski, D., Grabiec, C., Trumbauer, M.E., Shaw, A. and et al , Resistance to fever induction and impaired acute-phase response in interleukin-1 beta-deficient mice (1995). *Immunity.*3:9-19.

918. Stenzel Poore, M., Vale, W.W. and Rivier, C. Relationship between antigen-induced immune stimulation and activation of the hypothalamic-pituitary-adrenal axis in the rat (1993). *Endocrinology*132:1313-1318.

919. Givalois, L., Siaud, P., Mekaouche, M., Ixart, G., Malaval, F., Assenmacher, I. and Barbanel, G. Early hypothalamic activation of combined Fos and CRH41 immunoreactivity and of CRH41 release in push-pull cannulated rats after systemic endotoxin challenge (1995). *Mol.Chem.Neuropathol.*26:171-186.

920. Jones, S.B., Kotsonis, P. and Majewski, H. Endotoxin enhances norepinephrine release in the rat by peripheral mechanisms (1994). *Shock.*2:370-375.

921. Morrow, L.E., McClellan, J.L., Klir, J.J. and Kluger, M.J. The CNS site of glucocorticoid negative feedback during LPS- and psychological stress-induced fevers (1996). *Am.J.Physiol.*271:R732-R737.

922. Anisman, H. and Merali, Z. Chronic stressors and depression: distinguishing characteristics and individual profiles (1997). *Psychopharmacology.(Berl).*134:330-2; discussi.

923. Kohno, H., Yamamoto, M., Iimuro, Y., Fujii, H. and Matsumoto, Y. The role of splenic macrophages in plasma tumor necrosis factor levels in endotoxemia (1997). *Eur.Surg.Res.*29:176-186.

924. Kumins, N.H., Hunt, J., Gamelli, R.L. and Filkins, J.P. Partial hepatectomy reduces the endotoxin-induced peak circulating level of tumor necrosis factor in rats (1996). *Shock.*5:385-388.

925. Buttini, M. and Boddeke, H. Peripheral lipopolysaccharide stimulation induces interleukin-1 beta messenger RNA in rat brain microglial cells (1995). *Neuroscience.*65:523-530.

926. Woiciechowsky, C., Schoning, B., Lanksch, W.R., Volk, H.D. and Docke, W.D. Mechanisms of brain-mediated systemic anti-inflammatory syndrome causing immunodepression (1999). *J.Mol.Med.*77:769-780.

927. Woiciechowsky, C., Asadullah, K., Nestler, D., Eberhardt, B., Platzer, C., Schoning, B., Glockner, F., Lanksch, W.R., Volk, H.D. and Docke, W.D. Sympathetic activation triggers systemic interleukin-10 release in immunodepression induced by brain injury [see comments] (1998). *Nat.Med.*4:808-813.

928. Gartner, K., Buttner, D., Dohler, K., Friedel, R., Lindena, J. and Trautschold, I. Stress response of rats to handling and experimental procedures (1980). *Lab.Anim.*14:267-274.

929. Ebisui, O., Fukata, J., Murakami, N., Kobayashi, H., Segawa, H., Muro, S., Hanaoka, I., Naito, Y., Masui, Y., Ohmoto, Y. and et al , Effect of IL-1 receptor antagonist and antiserum to TNF-alpha on LPS-induced plasma ACTH and corticosterone rise in rats (1994). *Am.J.Physiol.*266:E986-E992.

930. Turnbull, A.V. and Rivier, C.L. Sprague-Dawley rats obtained from different vendors exhibit distinct adrenocorticotropin responses to inflammatory stimuli (1999). *Neuroendocrinology*70:186-195.

931. Menzies, R., Phelps, C., Wiranowska, M., Oliver, J., Chen, L., Horvath, E. and Hall, N.

the effect of interferon-alpha on the pituitary-adrenal axis (1996). *Journal Of Interferon And Cytokine Research*16:619-629.

932. Dobrakovova, M. and Jurcovicova, J. Corticosterone and prolactin responses to repeated handling and transfer of male rats (1984). *Exp.Clin.Endocrinol.*83:21-27.

933. Moga, M.M. and Saper, C.B. Neuropeptide-immunoreactive neurons projecting to the paraventricular hypothalamic nucleus in the rat (1994). *J.Comp.Neurol.*346:137-150.

934. Seggie, J.A. and Brown, G.M. Stress response patterns of plasma corticosterone, prolactin, and growth hormone in the rat, following handling or exposure to novel environment (1975). *Can.J.Physiol.Pharmacol.*53:629-637.

935. Paris, J.M., Lorens, S.A., Van de Kar, L.D., Urban, J.H., Richardson Morton, K.D. and Bethea, C.L. A comparison of acute stress paradigms: hormonal responses and hypothalamic serotonin (1987). *Physiol.Behav.*39:33-43.

936. Odio, M.R. and Maickel, R.P. Comparative biochemical responses of rats to different stressful stimuli (1985). *Physiol.Behav.*34:595-599.

937. Vriend, C.A.Y., Wan, W., Greenberg, A.H. and Nance, D.M. Cutting the splenic nerve differentially effects catecholamine and neuropeptide levels in the spleen (1993). *Society for Neuroscience Abstracts*(Abstract)

938. Trudrung, P., Furness, J.B., Pompolo, S. and Messenger, J.P. Locations and chemistries of sympathetic nerve cells that project to the gastrointestinal tract and spleen (1994). *Arch.Histol.Cytol.*57:139-150.

939. Felten, S.Y., Felten, D.L., Bellinger, D.L., Carlson, S.L., Ackerman, K.D., Madden, K.S., Olschowka, J.A. and Livnat, S. Noradrenergic sympathetic innervation of lymphoid organs (1988). *Prog.Allergy.*43:14-36.

940. Carlson, S.L., Felten, D.L., Livnat, S. and Felten, S.Y. Noradrenergic sympathetic innervation of the spleen: V. Acute drug- induced depletion of lymphocytes in the target fields of innervation results in redistribution of noradrenergic fibers but maintenance of compartmentation (1987). *J.Neurosci.Res.*18:64-9, 130-1.

941. Felten, S.Y. and Olschowka, J. Noradrenergic sympathetic innervation of the spleen: II. Tyrosine hydroxylase (TH)-positive nerve terminals form synapticlike contacts on lymphocytes in the splenic white pulp (1987). *J.Neurosci.Res.*18:37-48.

942. Bellinger, D.L., Felten, S.Y., Collier, T.J. and Felten, D.L. Noradrenergic sympathetic innervation of the spleen: IV. Morphometric analysis in adult and aged F344 rats (1987). *J.Neurosci.Res.*18:55-63, 126.

943. Ackerman, K.D., Felten, S.Y., Bellinger, D.L. and Felten, D.L. Noradrenergic sympathetic innervation of the spleen: III. Development of innervation in the rat spleen (1987). *J.Neurosci.Res.*18:49-54, 123.

944. Pezeshki, G., Pohl, T. and Schobitz, B. corticosterone controls interleukin-1 beta expression and sickness behavior in the rat (1996). *Journal Of*

*Neuroendocrinology*8:129-135.

945. Butler, L.D., Layman, N.K., Riedl, P.E., Cain, R.L., Shellhaas, J., Evans, G.F. and Zuckerman, S.H. Neuroendocrine regulation of in vivo cytokine production and effects: I. In vivo regulatory networks involving the neuroendocrine system, interleukin-1 and tumor necrosis factor-alpha (1989). *J.Neuroimmunol.*24:143-153.

946. van der Poll, T. and Lowry, S.F. Epinephrine inhibits endotoxin-induced IL-1 beta production: roles of tumor necrosis factor-alpha and IL-10 (1997). *Am.J.Physiol.*273:R1885-R1890.

947. Brown, E.R. and Sawchenko, P.E. Hypophysiotropic CRF neurons display a sustained immediate-early gene response to chronic stress but not to adrenalectomy (1997). *J.Neuroendocrinol.*9:307-316.

948. Sawchenko, P.E. and Swanson, L.W. Localization, colocalization, and plasticity of corticotropin-releasing factor immunoreactivity in rat brain (1985). *Fed.Proc.*44:221-227.

949. van der Meer, M.J., Sweep, C.G., Pesman, G.J., Tilders, F.J. and Hermus, A.R. Chronic stimulation of the hypothalamus-pituitary-adrenal axis in rats by interleukin 1beta: central and peripheral mechanisms (1996). *Cytokine.*8:910-919.

950. Bertini, R., Bianchi, M. and Ghezzi, P. Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor (1988). *J.Exp.Med.*167:1708-1712.

951. Perretti, M., Becherucci, C., Scapigliati, G. and Parente, L. The effect of adrenalectomy on interleukin-1 release in vitro and in vivo (1989). *Br.J.Pharmacol.*98:1137-1142.

952. Rogausch, H., delRey, A., Kabiersch, A., Reschke, W., Ortel, J. and Besedovsky, H. endotoxin impedes vasoconstriction in the spleen: role of endogenous interleukin-1 and sympathetic innervation (1997). *American Journal Of Physiology Regulatory Integrative And Comparative Physiology*41:R2048-R2054.

953. Lyte, M., Ernst, S., Driemeyer, J. and Baissa, B. Strain-specific enhancement of splenic T cell mitogenesis and macrophage phagocytosis following peripheral axotomy (1991). *J.Neuroimmunol.*31:1-8.

954. Sanders, V.M. and Munson, A.E. Role of alpha adrenoceptor activation in modulating the murine primary antibody response in vitro (1985). *J.Pharmacol.Exp.Ther.*232:395-400.

955. Sanders, V.M. and Powell Oliver, F.E. Beta 2-adrenoceptor stimulation increases the number of antigen-specific precursor B lymphocytes that differentiate into IgM-secreting cells without affecting burst size (1992). *J.Immunol.*148:1822-1828.

956. Pacak, K., Kvetnansky, R., Palkovits, M., Fukuhara, K., Yadid, G., Kopin, I.J. and Goldstein, D.S. Adrenalectomy augments in vivo release of norepinephrine in the paraventricular nucleus during immobilization stress (1993). *Endocrinology*133:1404-1410.

957. Komori, T., Fujiwara, R., Shizuya, K., Miyahara, S. and Nomura, J. The influence of physical restraint or fasting on plaque-forming cell response in mice (1996). *Psychiatry Clin.Neurosci.*50:295-298.

958. Zalcman, S., Minkiewicz Janda, A., Richter, M. and Anisman, H. Critical periods associated with stressor effects on antibody titers and on the plaque-forming cell response to sheep red blood cells (1988). *Brain Behav.Immun.*2:254-266.

959. Rinner, I., Schauenstein, K., Mangge, H., Porta, S. and Kvetnansky, R. Opposite effects of mild and severe stress on in vitro activation of rat peripheral blood lymphocytes (1992). *Brain Behav.Immun.*6:130-140.

960. Croiset, G., Heijnen, C.J., Veldhuis, H.D., de Wied, D. and Ballieux, R.E. Modulation of the immune response by emotional stress (1987). *Life.Sci.*40:775-782.

961. Shanks, N. and Anisman, H. Strain-specific effects of antidepressants on escape deficits induced by inescapable shock (1989). *Psychopharmacology.(Berl).*99:122-128.

962. Kerr, L., Drummond, L., Zaharia, M., Clelford, J. and Anisman, H. Stressor-induced alterations of the splenic plaque-forming cell response: strain differences and modification by propranolol (1996). *Pharmacol.Biochem.Behav.*53:235-241.

963. Shanks, N., Griffiths, J., Zalcman, S., Zacharko, R.M. and Anisman, H. Mouse strain differences in plasma corticosterone following uncontrollable footshock (1990). *Pharmacol.Biochem.Behav.*36:515-519.

964. Zukowska Grojec, Z., Konarska, M. and McCarty, R. Differential plasma catecholamine and neuropeptide Y responses to acute stress in rats (1988). *Life.Sci.*42:1615-1624.

965. Papanicolaou, D.A., Petrides, J.S., Tsigos, C., Bina, S., Kalogeras, K.T., Wilder, R., Gold, P.W., Deuster, P.A. and Chrousos, G.P. Exercise stimulates interleukin-6 secretion: inhibition by glucocorticoids and correlation with catecholamines (1996). *Am.J.Physiol.*271:E601-E605.

966. Molina, P.E. and Abumrad, N.N. Central sympathetic modulation of tissue cytokine response to hemorrhage (1999). *Neuroimmunomodulation.*6:193-200.

967. Komaki, G., Gottschall, P.E., Somogyvari Vigh, A., Tatsuno, I., Yatohgo, T. and Arimura, A. Rapid increase in plasma IL-6 after hemorrhage, and posthemorrhage reduction of the IL-6 response to LPS, in conscious rats: interrelation with plasma corticosterone levels (1994). *Neuroimmunomodulation.*1:127-134.

968. Ignatowski, T.A. and Spengler, R.N. Regulation of macrophage-derived tumor necrosis factor production by modification of adrenergic receptor sensitivity (1995). *J.Neuroimmunol.*61:61-70.

969. Feng, N., Pagniano, R., Tovar, C.A., Bonneau, R.H., Glaser, R. and Sheridan, J.F. The effect of restraint stress on the kinetics, magnitude, and isotype of the humoral immune response to influenza virus infection (1991). *Brain Behav.Immun.*5:370-382.

970. Takaki, A., Ito, K., Yagi, S., Kanemitsu, Y., Tsurogano, S., Shioda, S., Maeda, M. and Hori, T. Discrepency of tissue IL-6 expression between non-inflammatory stress and pyrogenic inflammation (1998). *Society for Neuroscience Abstracts*v24 # 738.11:1860.

971. Toft, P., Lillevang, S.T., Tonnesen, E., Svendsen, P. and Hohndorf, K. Redistribution of lymphocytes following E. coli sepsis (1993). *Scand.J.Immunol.*38:541-545.

972. Pettipher, E.R., Labasi, J.M., Salter, E.D., Stam, E.J., Cheng, J.B. and Griffiths, R.J. Regulation of tumour necrosis factor production by adrenal hormones in vivo: insights into the antiinflammatory activity of rolipram (1996). *Br.J.Pharmacol.*117:1530-1534.

973. Molina, P.E., Ajmal, M., Qian, L., Naukam, R., Volkow, M. and Abumrad, N.N. Differential regulation of hemorrhage-induced cytokine activation; central versus peripheral sympathetic nervous systems (1997). *Society for Neuroscience Abstracts*v23 #593.11:(Abstract)

974. Bundz, S., Molina, P.E., Lang, C.H. and Abumrad, N.N. Endogenous opiates do not modulate LPS-induced alterations in carbohydrate metabolism (1995). *Shock*4:397-402.

975. Kjaer, A., Knigge, U. and Warberg, J. Histamine- and stress-induced prolactin secretion: importance of vasopressin V1- and V2-receptors (1994). *Eur.J.Endocrinol.*131:391-397.

976. Sanders, V.M. The role of adrenoceptor-mediated signals in the modulation of lymphocyte function (1995). *Adv.Neuroimmunol.*5:283-298.

977. Schmidt, E.D., Binnekade, R., Janszen, A.W.J.W. and Tilders, F.J.H. short stressor induced long-lasting increases of vasopressin stores in hypothalamic corticotropin-releasing hormone (crh) neurons in adult rats (1996). *Journal Of Neuroendocrinology*8:703-712.

978. Fleshner, M., Goehler, L.E., Schwartz, B.A., McGorry, M., Martin, D., Maier, S.F. and Watkins, L.R. Thermogenic and corticosterone responses to intravenous cytokines (IL-1beta and TNF-alpha) are attenuated by subdiaphragmatic vagotomy (1998). *J.Neuroimmunol.*86:134-141.

979. Konarska, M., Stewart, R.E. and McCarty, R. Habituation and sensitization of plasma catecholamine responses to chronic intermittent stress: effects of stressor intensity (1990). *Physiol.Behav.*47:647-652.

980. Shanks, N. and Anisman, H. Stressor-provoked behavioral changes in six strains of mice (1988). *Behav.Neurosci.*102:894-905.

981. Nukina, H., Sudo, N., Komaki, G., Yu, X., Mine, K. and Kubo, C. The restraint stress-induced elevation in plasma interleukin-6 negatively regulates the plasma TNF-alpha level (1998). *Neuroimmunomodulation.*5:323-327.

982. Baum, A. and Posluszny, D.M. Health psychology: mapping biobehavioral contributions to health and illness (1999). *Annu.Rev.Psychol.*50:137-163.

983. Swanson, L.W. The Hypothalamus. In: *Handbook of Chemical Neuroanatomy*, edited by Bjorkland, A., Hokfelt, T. and Swanson, L.W. Elsevier, 1985, p. 1-124.

984. Turnbull, A.V. and Rivier, C.L. Intracerebroventricular passive immunization. I. The effect of intracerebroventricular administration of an antiserum to tumor necrosis

factor-alpha on the plasma adrenocorticotropin response to lipopolysaccharide in rats (1998). *Endocrinology*139:119-127.

985. Takemura, T., Makino, S., Takao, T., Asaba, K., Suemaru, S. and Hashimoto, K. Hypothalamic-pituitary-adrenocortical responses to single vs. repeated endotoxin lipopolysaccharide administration in the rat (1997). *Brain Res.*767:181-191.

986. Hadid, R., Spinedi, E., Daneva, T., Grau, G. and Gaillard, R.C. Repeated endotoxin treatment decreases immune and hypothalamo-pituitary-adrenal axis responses: effects of orchidectomy and testosterone therapy (1995). *Neuroendocrinology*62:348-355.

987. Soni, A., Pepper, G.M., Wyrwinski, P.M., Ramirez, N.E., Simon, R., Pina, T., Gruenspan, H. and Vaca, C.E. Adrenal insufficiency occurring during septic shock: incidence, outcome, and relationship to peripheral cytokine levels (1995). *Am.J.Med.*98:266-271.

988. Murakami, N., Fukata, J., Usui, T., Naito, Y., Tominaga, T., Nakai, Y., Masui, Y., Nakao, K. and Imura, H. Effects of repetitive administration of recombinant human interleukin-1 beta, an analog or corticotropin-releasing hormone combined with lysine vasopressin on rats with glucocorticoid-induced secondary adrenocortical insufficiency (1992). *J.Pharmacol.Exp.Ther.*260:1344-1348.

989. Krasner, A.S. Glucocorticoid-induced adrenal insufficiency [clinical conference] (1999). *JAMA*282:671-676.

9. Appendix-1:List of PCR primers

9. Appendix-1: List of PCR Primers

The primer sequences are written from 5' to 3'. The 5' primer is also known as the upper primer while the lower primer, T73', is designated as such since it has the binding sequence for T7 RNA polymerase. For IFN and 28S rRNA mouse and human primers were used respectively as the sequence homologies were almost identical to rat.

Rat TNF

5'	TACTGAACTTCGGGGTGATTGGTCC
T7 3'	TAATACGACTCACTATAGGGAGACAGCCTTGTCCCTTGAAGAGAACC

Rat RANTES

5'	TGCATCCCTCACCGTCATCC
T7 3'	TAATACGACTCACTATAGGGAGACTGGGTTGGCACACACTTGG

Rat GAPDH

5'	CAGTCTTCTGAGTGGCAGTGATGG
T7 3'	TAATACGACTCACTATAGGGAGAATGCTGGTGCTGAGTATGTCGTGG

Rat MIP-1 beta

5'	CTTCTCTCTCCTCCTGCTTGTG
T73'	TAATACGACTCACTATAGGGAGACAGATTTGCCTGCCTTTTTTGG

Rat MCP-1

5'	ACTCACCTGCTGCTACTCATTCAC
T73	TAATACGACTCACTATAGGGAGAGTTGTGGAAAAGAGAGTGGATGC

Rat IL-6

5' CTTCTTGGGACTGATGTTGTTGAC

T73' TAATACGACTCACTATAGGGAGACAGTATTGCTCTGAATGACTCTGG

Rat IL-18

5' GCGGAGCATAAATGACCAAGTTC

T73' TAATACGACTCACTATAGGGAGATGGCAAGCTAGAAAGTGTCCTTCA

Rat IL-12 p40

5' CATGTGGGAGCTGGAGAAAGATGTT

T73' TAATACGACTCACTATAGGGAGAGTGGAGCAGCAGATGTGAGTGG

Rat TACE

5' CCAAATGAGGACCAAGGAGGAAAG

T73' TAATACGACTCACTATAGGGAGACAGCAGGTGTCGTTGTTCAG

Rat ICE

5' CAGGATCTGGGCTATACTGTGAAA

T73' TAATACGACTCACTATAGGGAGATAATGATAACCTTGGGCTTGTCTT

Rat IL-1 beta

5' CTCTCCAGTCAGGCTTCCTTGTGC

T73' TAATACGACTCACTATAGGGAGACGAGTCACAGAGGACGGGCTC

Rat Actin

5' TGGGACGATATGGAGAAGATTTGG

T73' TAATACGACTCACTATAGGGAGATGACCGTCAGGCAGCTCATAGC

Mouse Interferon Gamma

5' GCTCTGAGACAATGAACGCTACAC

T73' TAATACGACTCACTATAGGGAGAATTCAATGACGCTTATGTTGTTGC

Human 28S rRNA

5' ACGCTCATCAGACCCCAGAAAAGG

T73' TAATACGACTCACTATAGGGAGACATGTTCAACTGCTGTTCAC

T7 Sequencing primer

AATACGACTCACTATAGG

10. Appendix-2:Previously published manuscripts

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The Journal of Histochemistry & Cytochemistry

TECHNICAL NOTE

Enhanced Immunohistochemical Detection of Autonomic Nerve Fibers, Cytokines and Inducible Nitric Oxide Synthase by Light and Fluorescent Microscopy in Rat Spleen

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SUMMARY We have developed enhanced immunohistochemical protocols for detecting autonomic nerve fibers and splenocyte-associated proteins in rat spleen. This includes norepinephrine-synthesizing enzymes (dopamine-βeta hydroxylase (DBH) and tyrosine hydroxylase (TH)), neuropeptide Y (NPY), tumor necrosis factor -α (TNF-α), interferon-γ (IFN-γ), c-fos protein, inducible nitric oxide synthase (iNOS), and the macrophage cell marker ED1. Animals were divided into sham-operated and splenic nerve-sectioned groups for detection of DBH, TH, and NPY. For immunodetection of TNF-α, iNOS, IFN-γ and c-fos, animals were injected iv with saline or 100 μg of lipopolysaccharide (LPS) and were sacrificed at various time intervals post injection. Rats were perfused with 4% paraformaldehyde, spleens removed and cryoprotected, and 50-μm floating sections were cut on a freezing microtome. Immunodetection was performed with various detection systems and substrate/chromogen solutions, and in some cases using pretreatment with proteinase K (PK) for antigen unmasking. PK pretreatment increased immunostaining for DBH, TH, NPY, IFN-γ, iNOS, and ED1, and the improvement was concentration-dependent. Using NPY immunostaining to index the signal-to-noise ratio for various substrates and detection systems, we found that an alkaline phosphatase detection system with NBT/BCIP as a substrate was the best procedure for light microscopy, whereas the CY3-labeled secondary antibody technique proved optimal for fluorescent microscopy. Surgical transection of the splenic nerve eliminated all nerve fiber staining for DBH, TH, and NPY. TNF-α, IFN-γ, c-fos, and iNOS proteins were observed in the spleen in a time-dependent manner after LPS stimulation. Fluorescent double labeling, visualized with fluorescent confocal scanning laser microscopy, revealed many NPY fibers distributed among the ED1-labeled macrophages. These results demonstrate that immunohistochemistry can be used to index the activational effects of an immune challenge on splenocytes in situ and verifies that splenic immune cells are innervated by the sympathetic nervous system. (J Histochem Cytochem 45:599–610, 1997)

KEY WORDS
dopamine β-hydroxylase
tyrosine hydroxylase
proteinase K
alkaline phosphatase
neuropeptide Y
TNF-α
IFN-γ
macrophage
c-fos
immunohistochemistry

NEUROANATOMIC and neuroendocrine studies have demonstrated a role for the nervous system in regulating immune function via the hypothalamic–pituitary–adrenal (HPA) axis and the autonomic nervous system. The sympathetic arm of the autonomic nervous system may play a major role in regulating immune function via direct sympathetic innervation of all immune organs (Trudrung et al. 1994; Nance and Burns 1989; Felten et al. 1987a,b). Typically the sympathetic nervous system exerts inhibitory control of immune function (Hu and Moller 1994; Dureus et al. 1993; Monastra and Secchi 1993; Wan et al. 1993b; Hu et al. 1991; Kouassi et al. 1988; Besedovsky et al. 1979). However, some immune responses have been shown to be potentiated by sympathetic activation (Zalcman et al. 1994).

The spleen is a model organ to study neural–immune interactions because of its well-described innervation (Trudrung et al. 1994; Nance and Burns

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Table 1 Dilutions of primary antibodies

Name	Dilution	Supplier	Reference
Anti-DBH	1:2000	Eugene Tech	Smith et al. 1991
Anti-TH	1:1500	Eugene Tech	Felten et al. 1987a
Anti-NPY	1:3000	Incstar	Pelletier et al. 1984
Anti-iNOS	1:500–1000	Transduction Labs	Western blot from company
Anti-iNOS	1:500–1000	Gift from Dr. H. Oshima	Bandelatova et al. 1993
Anti-TNF-α	1:5000	Genzyme	Diamond and Pesek 1991
Anti-IFN-γ	1:1000–2000	Biosource	Van der Meide et al. 1986
Anti-c-fos	1:10,000	Santa Cruz Biotechnology	Western blot from company
Anti-ED1	1:2000	Serotec	Damoiseaux et al. 1994

1989) and the ability to eliminate nerve fibers to the spleen by chemical or surgical sympathectomy (Zalcman et al. 1994; Vriend et al. 1993; Wan et al. 1993b; Romano et al. 1991; Nance and Burns 1989; Felten et al. 1987a,b; Besedovsky et al. 1979). Analysis of the effects of neural transmitters on splenic immune function indicate a functional role for norepinephrine (NE) and neuropeptide Y (NPY) (Hu and Moller 1994; Madden et al. 1994; Zalcman et al. 1994; Dureus et al. 1993; Fukushima et al. 1993; Monastra and Secchi 1993; Wan et al. 1993b; Hu et al. 1991; Spengler et al. 1990; Kouassi et al. 1988; Sanders and Munson 1985). Of particular interest are the effects of NE agonists and antagonists on the in vitro production of macrophage-associated cytokines, such as tumor necrosis factor-α (TNF-α) (Monastra and Secchi 1993; Spengler et al. 1990; Introna et al. 1986). Although these in vitro studies provide information on possible cellular interactions between sympathetic neural transmitters and the immune system, they may not accurately reflect the events that occur in vivo. Therefore, we have developed enhanced immunohistochemical protocols that enable us to examine the role of the autonomic nervous system in regulating splenic immune function by in situ localization of immune-related molecules and autonomic nerve fibers.

Materials and Methods

Chemicals

Lipopolysaccharide (LPS; *E. coli* serotype 055:B5), diaminobenzidine, glucose oxidase, nitroblue tetrazolium (NBT), levamisole, D-glucose, Fast Red, naphthol AS-MX phosphate, sodium nitroprusside, proteinase K (PK), glycine, BSA, and Triton X-100 were purchased from Sigma (St Louis, MO). Paraformaldehyde, sodium nitrite, and glycerol were purchased from BDH (Toronto, Ontario, Canada), 5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine salt (BCIP), ammonium chloride, *N,N*-dimethylformamide, sodium azide, methanol, and gelatin were purchased from Fischer (Fair Lawn, NJ). The Enzyme-Labeled-Fluorescence (ELF) kit was purchased from Molecular Probes (Eugene, OR). Normal goat serum was purchased from Cappel (Scarborough, Ontario, Canada) and RedPhos was purchased from Research Organics (Cleveland, OH).

Antibodies

Rabbit anti-c-fos was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-dopamine-β hydroxylase (DBH) and anti-tyrosine hydroxylase (TH) were purchased from Eugene Tech (Ridgefield Park, NJ). Rabbit anti-NPY was purchased from Incstar (Stillwater, MN). Rabbit anti-inducible nitric oxide synthase (iNOS) antibodies were a gift from Dr. H. Ohshima (International Agency for Research on Cancer; Lyon, France) or purchased from Transduction Labs (Lexington, KY). Rabbit anti-mouse TNF-α was purchased from Genzyme (Cambridge, MA). Rabbit anti-rat interferon-γ (IFN-γ) was purchased from Biosource (Camarillo, CA). Rabbit IgGs were purchased from Sigma, CY3-labeled goat anti-rabbit and FITC-labeled goat anti-mouse were purchased from Jackson Immunologicals (West Grove, PA), mouse monoclonal anti-ED1 was purchased from Serotec (Toronto, Ontario, Canada), mouse IgGs were purchased from Rockland Labs (Gilbertsville, PA), and unconjugated and alkaline phosphatase (AP)-conjugated goat anti-rabbit and rabbit peroxidase–anti-peroxidase (PAP) were purchased from Cappel (Scarborough, Ontario, Canada). Optimal dilutions of primary and secondary antibodies were determined empirically in preliminary experiments. Listed in Tables 1 and 2

Table 2 Dilutions of secondary antibodies

Name	Dilution
Goat anti-rabbit (GAR), unconjugated	1:150
GAR, alkaline phosphatase-conjugated	1:750–1:1000
GAR, CY3-conjugated	1:1000
Rabbit peroxidase–anti-peroxidase	1:300
Goat anti-mouse, FITC-conjugated	1:600

are the optimal dilutions of primary and secondary antibodies with the AP detection system using NBT/BCIP as a chromogen/substrate combination. References documenting the specificity of these antibodies also appear in Table 1.

Animals

For immunodetection of DBH, TH, and NPY fibers in the spleen, adult male (250–400 g) Sprague–Dawley rats (Charles River; Dorval, Quebec, Canada) were anesthetized (60 mg/kg sodium pentobarbital) and divided into two groups, sham surgeries and splenic nerve sections (described by Nance and Burns 1989) and allowed to recover for 7–10 days before tissue processing (six animals per group). For studies involving the immunodetection of TNF-α, c-fos, IFN-γ, and iNOS, animals were injected with saline or 100 μg of LPS via tail vein (at least three animals per time point) and were sacrificed at various intervals after injection. All procedures were approved by the animal ethics committee at the University of Manitoba and the CCAC.

Tissue Processing

Animals were sacrificed by an overdose of pentobarbital and then transcardially perfused with 100 ml of 1% sodium nitrite in phosphate buffer (PB), followed by 300 ml of 4% buffered paraformaldehyde (pH 7.3). Spleens were removed, postfixed for 2 hr, and cryoprotected in 30% sucrose. Serial 50-μm sections of spleen were cut on a freezing microtome and transferred to a 24-well culture plate containing 0.01 M PBS. Subsequent processing of the sections depended on the enzymatic or fluorescent detection system being tested.

PAP Detection

Sections were rinsed three times or were pretreated with 0–5 μg/ml of proteinase K (PK 0–5 μg/ml) in PK buffer (0.1 M Tris/50 mM EDTA, pH 8.3, at 25°C) for 30 min at 37C with agitation before the rinse steps (the first rinse after PK included 2 mg/ml glycine). Next, sections were either placed in primary antibody or were pretreated with 3% H_2O_2 and 0.1% azide (Li et al. 1987) at room temperature (RT) for 30 min to reduce endogenous peroxidase activity before being placed in primary antibody. Primary antibodies were diluted in 0.01 M PBS containing 2% bovine serum albumin (BSA), 1% normal goat serum (NGS), and 1% Triton X-100 (TTX). Trays were enclosed in humidified bags on a rocker table and incubated overnight at RT. The next day all sections were rinsed and placed in PBS/1% TTX/1% NGS for 90 min containing 1:150 dilution of unconjugated goat anti-rabbit antibody. Sections with and without previous pretreatment to reduce endogenous peroxidase activity were incubated for 30 min in 3% H_2O_2/ 5% methanol in PBS (Romano et al. 1991) or 1% sodium nitroprusside/ 0.074% HCl/100% methanol (Straus 1971) and rinsed. The sections were placed in 1:300 dilution of PAP in PBS/1% TTX/1% NGS for 90 min. Sections were rinsed and developed in 500 μl of 0.01 M PB/well containing 0.05% diaminobenzidine, 0.04% ammonium chloride, and 0.2% D-glucose. After addition of 50 μl of 0.006% glucose oxidase solution to each well, the sections were developed for 30–45 min and then rinsed in PBS, floated onto slides, dried overnight, and coverslipped in glycerol gel (50% glycerol/ 7.5% gelatin/0.1% azide in 0.1 M PB).

Alkaline Phosphatase Detection

Sections were rinsed and placed directly in primary antibody or first digested with PK as described above. After incubation in primary antibody, sections were rinsed and placed into a 1:750–1:1000 dilution of AP-conjugated goat anti-rabbit [whole antibody or $F(ab)_2$ fragments] for 2 hr, rinsed, and then developed with one of the following substrates: NBT/BCIP, NBT/Red-phos, Fast Red, or ELF (Larison et al. 1995). For NBT/BCIP and NBT/RedPhos detection, 0.4 mM NBT, 0.4 mM BCIP (or RedPhos), and 3 mM levamisole were added to 50 mM $MgCl_2$/100 mM Tris/100 mM NaCl, pH 9.3. Fast Red was developed by dissolving 20 mg of Fast Red salt in 20 ml of 100 mM Tris/3 mM levamisole (pH 8.2). To this solution, 4 mg ASMX-phosphate dissolved in 400 μl of dimethylformamide was added and stirred for 30 sec without filtering. ELF development was according to the manufacturer's instructions, except that the development solution was not filtered and sections were developed for 5–10 min. After development, sections were rinsed, floated onto slides, dried, and coverslipped as above.

CY3 Detection

Sections were rinsed and placed in primary antibody or pretreated with proteinase K as described above. After the primary incubation, sections were rinsed and incubated for 3–4 hr in a 1:1000 dilution of CY3-conjugated goat anti-rabbit antibody. Sections were rinsed, floated onto slides, air-dried, and coverslipped as above.

Fluorescent Double Labeling

Sections were incubated overnight with rabbit anti-NPY mixed with mouse anti-ED1 in 1% NGS, 2% BSA, and 1% TTX. Sections were then rinsed with PBS and incubated for 3–4 hr in goat anti-rabbit labeled with CY3 and goat anti-mouse labeled with FITC. Sections were rinsed in PBS and mounted as described above. Sections were then visualized with a Leitz epifluorescent microscope and with a Molecular Dy-

namics confocal scanning laser microscope equipped with an argon laser and dual detectors. The images were generated from unfiltered raw optical sections that were rendered as maximal intensity projections with ImageSpace software (Molecular Dynamics; Sunnyvale CA).

Controls

Control staining procedures included exclusion of primary and/or secondary antibodies and nonspecific rabbit or mouse immunoglobulins substituted for primary antibodies. The same controls were applied for double-labeling experiments, except that rabbit IgG and antibody to NPY were incubated with goat anti-mouse-conjugated FITC and mouse IgG and anti-ED1 were incubated with CY3-labeled goat anti-rabbit to check for crossreactivity.

Results

Immunohistochemistry for DBH with the PAP and AP Techniques

The focus of this study was to determine the location of nerve fiber- and splenocyte-associated proteins in rat spleen. Initially the PAP technique was used for immunolocalization of DBH and other molecules, but it was unsuitable because of the low signal-to-noise ratio. We observed that the spleen has two sources of background with the PAP technique: a diffuse pseudoperoxidase activity, probably originating from red blood cells and granulocytes, and clusters of very strongly positive cells located in the red pulp and marginal zones. Although the diffuse background staining did not significantly impair the analysis of results, the

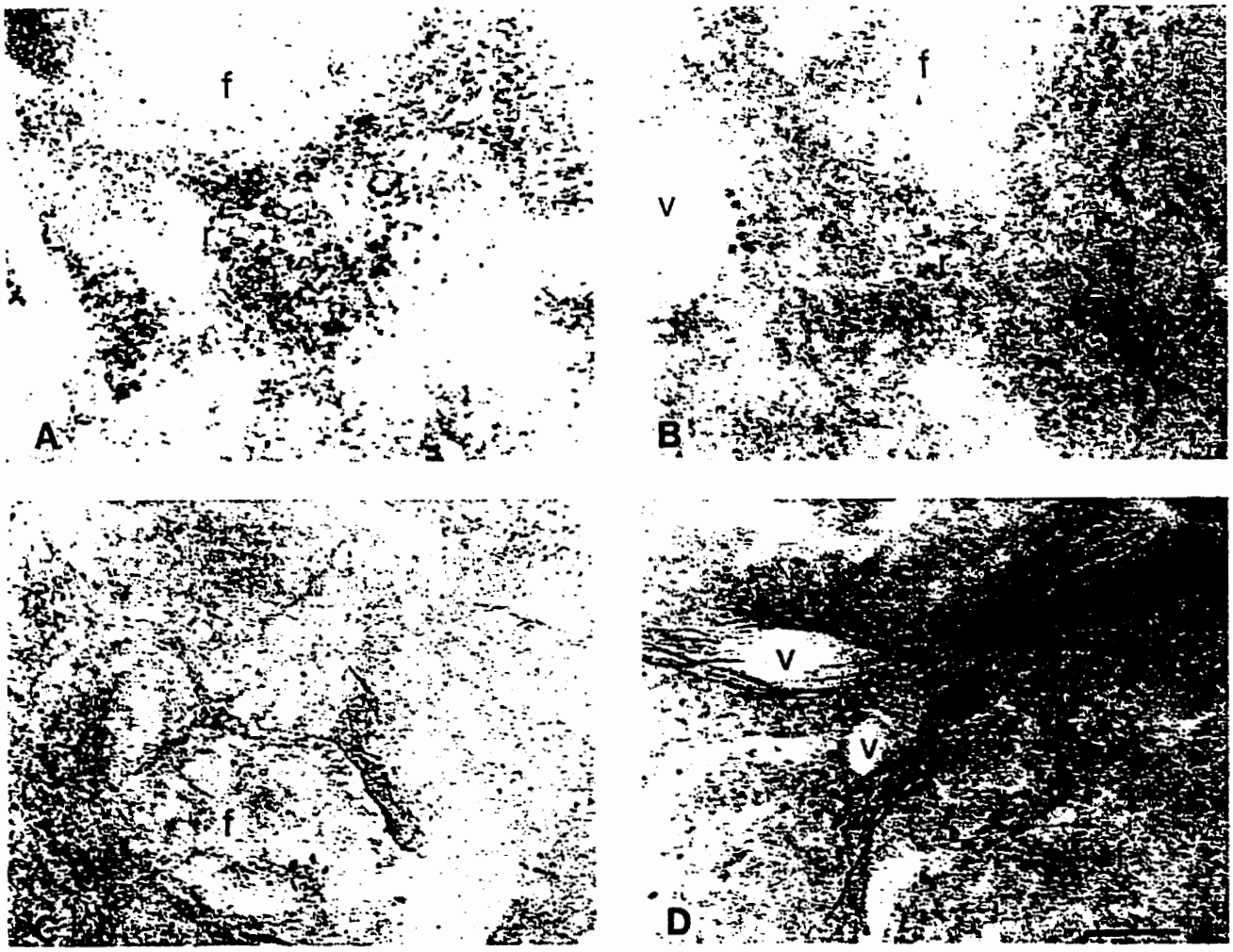

Figure 1 Photomicrographs showing endogenous peroxidase and endogenous alkaline phosphatase activity and the effect of proteinase K pretreatment on DBH immunostaining in the spleen with the PAP procedure. (A) Spleen section showing endogenous peroxidase activity. (B) Spleen showing endogenous alkaline phosphatase activity. (C) Spleen section showing DBH staining without PK. (D) Spleen showing DBH staining with PK treatment. v, blood vessel; f, follicle; r, red pulp. Bar = 100 μm.

strongly staining clusters of cells posed a major problem for analyzing cell-associated molecules. In an attempt to improve immunodetection with the PAP technique, we investigated various methods for reducing endogenous peroxidase activity (EPA), including peroxide (H_2O_2)/azide, H_2O_2/methanol, methanol/HCl/sodium nitroprusside, and a combination of H_2O_2/azide and H_2O_2/methanol. We found that all four methods reduced EPA to a similar extent, but the combination of H_2O_2/azide with H_2O_2/methanol consistently produced the best results in terms of signal-to-noise ratios (unpublished observations). The H_2O_2/methanol method was marginally better than H_2O_2/azide at reducing EPA, but H_2O_2/azide was better for preservation of tissue morphology. The third method, nitroprusside/methanol/HCl, was very effective at eliminating EPA, but it also decreased positive signal and strongly affected tissue morphology. Diluting the secondary antibody (goat anti-rabbit) or the PAP complex helped to reduce background staining in some spleen sections but often reduced positive staining as well. Some spleen sections continued to show clusters of cells with strong EPA, even after various bleaching methods, omission of antibodies, and PK treatment. Together, these observations indicated that tissue thickness, nonspecific binding of antibodies to Fc receptors and other antigens, and EPA were all likely contributors to the background staining with the PAP technique. In contrast to the PAP technique, the AP technique (Figure 1) demonstrated a high signal-to-noise ratio with minimal interference from endogenous AP activity. In side-by-side comparisons, the AP detection system was always qualitatively and esthetically superior to the PAP technique.

Antigen Unmasking with Proteinase K

The staining for DBH with both the PAP (Figure 1C) and the AP (Figure 2A) detection system was weak in undigested tissue sections. However, a dramatic and concentration-dependent improvement was observed for DBH staining with both detection systems if the

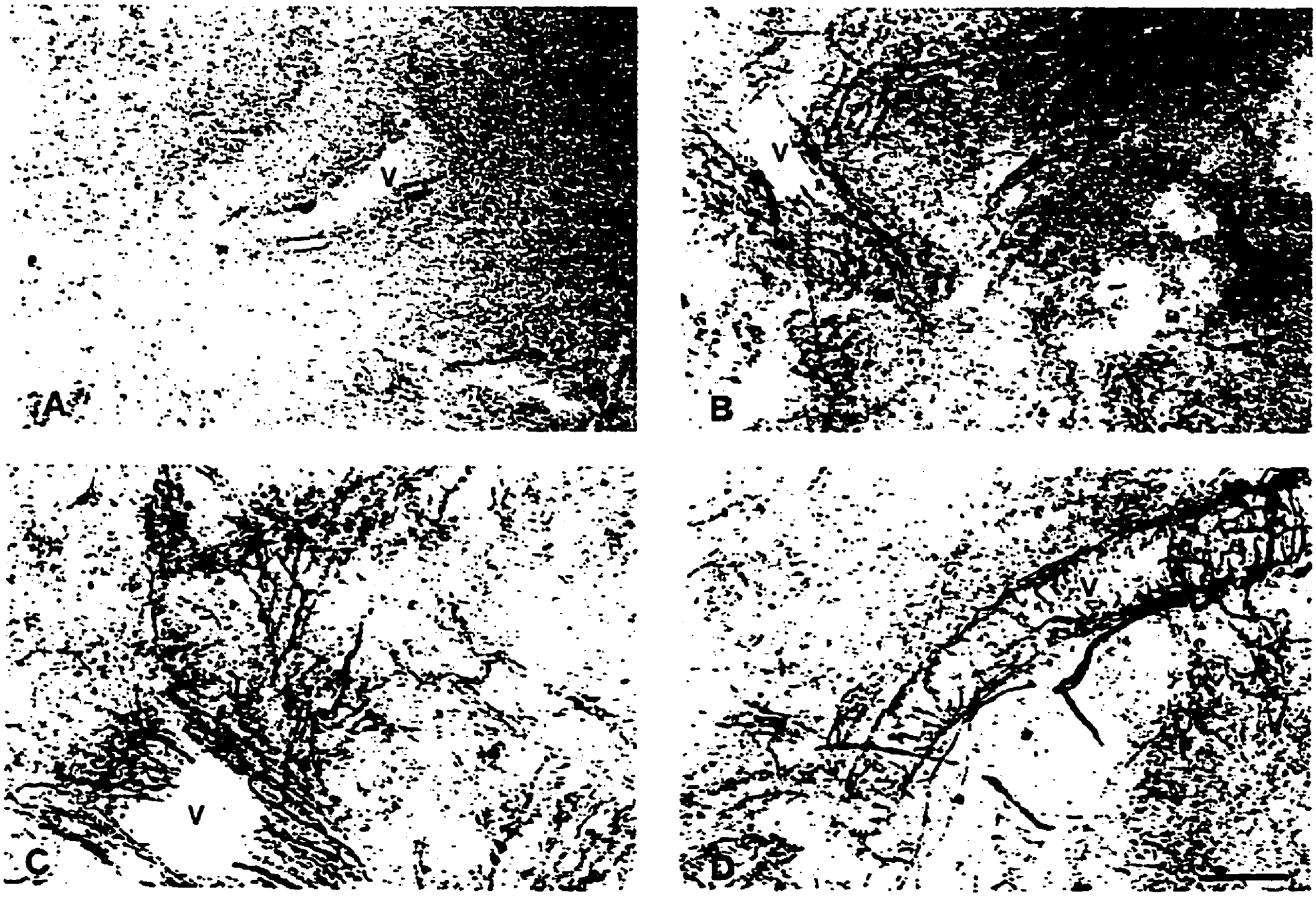

Figure 2 Photomicrographs showing the effect of different proteinase K (PK) concentrations on DBH immunodetection, as visualized by an alkaline phosphatase detection procedure with NBT/BCIP as substrate. (A) with 0.0 μg PK/ml; (B) with 0.31 μg PK/ml; (C) 0.625 μg PK/ml; (D) with 1.25 μg PK/ml. v, blood vessel. Bar = 100 μm.

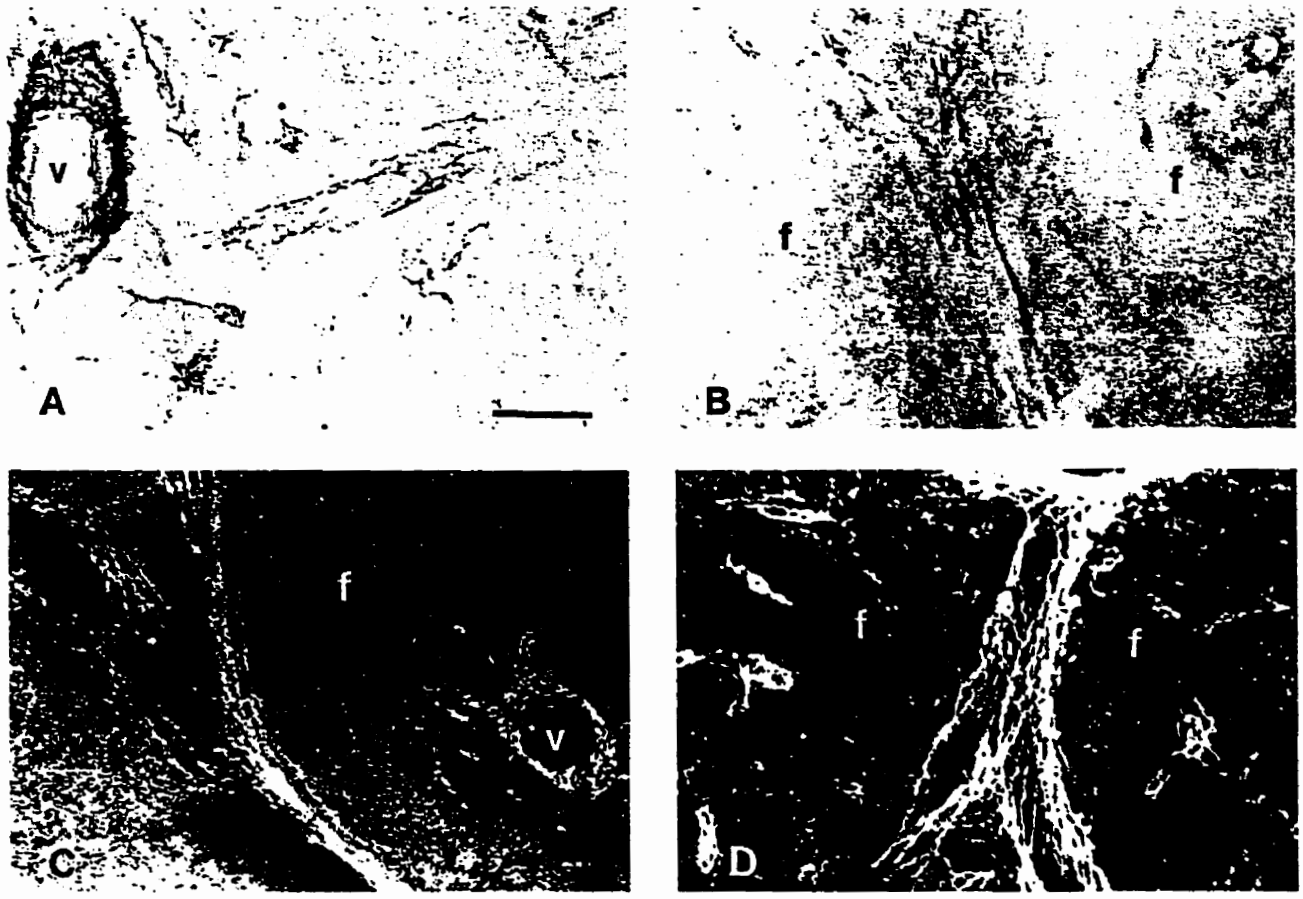

Figure 3 Photomicrographs showing immunostaining of PK-treated spleen sections for NPY-positive nerve fibers using different substrates and detection systems other than an alkaline phosphatase detection system with NBT/BCIP. (A) PAP with diaminobenzidine; (B) alkaline phosphatase with NBT/RedPhos; (C) alkaline phosphatase with ELF; (D) CY3-labeled secondary antibody. v, blood vessel; f, follicle; r, red pulp. Bar = 100 μm.

spleen sections were pretreated with PK (Figures 1D and 2B–2D). Similar observations were made with TH and NPY (not shown).

Comparison of Different Substrates for NPY Detection

To determine the optimal detection system and substrate, we compared NPY-positive immunodetection using AP substrates other than NBT/BCIP, as well as other detection systems (Figure 3). The substrates included NBT/RedPhos, Fast Red, and ELF, and the detection systems include the PAP technique and a fluorescent-conjugated antibody. Among the AP substrates, we found that NBT/BCIP was superior. Although the RedPhos worked well for NPY immunohistochemistry, it did not develop as quickly or stain as intensely as did NBT/BCIP. The Fast Red substrate could be visualized by light and fluorescence microscopy, but it produced a weak signal for both methods of visualization (not shown). Although detection of NPY with the ELF kit proved to be the better of the two fluorescent AP substrates, a CY3-labeled secondary antibody gave optimal results for NPY immunostaining. CY3 provided a high contrast with intense red nerve fibers against a dark background, and was highly resistant to photobleaching.

Effect of Splenic Nerve Sectioning on Immunostaining for Nerve Fibers

Using the AP system with NBT/BCIP, we demonstrated intense immunostaining for DBH, TH, and NPY in the spleens of sham-operated animals and a complete absence of staining for these molecules in nerve-sectioned animals (Figure 4).

Detection of TNF-α, IFN-γ, c-fos, and iNOS After LPS Injection

The results demonstrated that only a few TNF-α-positive cells and no c-fos or IFN-γ-positive cells were detectable in saline-treated rats. However, after LPS injections, many positive cells were observed for TNF-α,

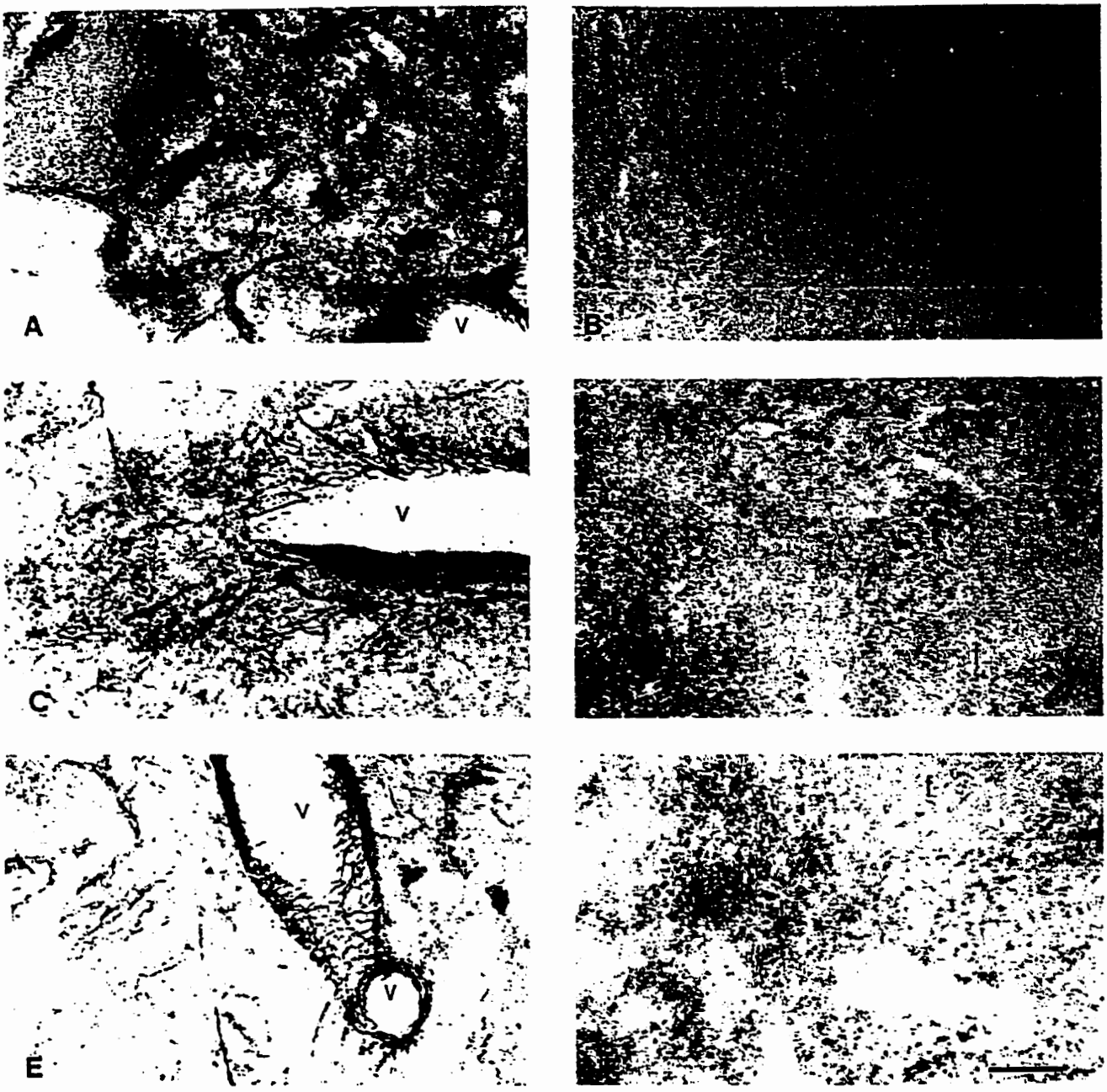

Figure 4 Photomicrographs showing DBH, TH, and NPY immunostaining in spleens of control and splenic nerve-sectioned animals. (A) DBH in sham-operated animal; (B) DBH after nerve sectioning; (C) TH in sham-operated animal; (D) TH after nerve sectioning; (E) NPY in sham-operated animal; (F) NPY following nerve sectioning. All spleen sections were pretreated with PK and developed with the alkaline phosphatase detection system using NBT/BCIP. v, blood vessel; f, follicle; r, red pulp. Bar = 100 μm.

IFN-γ and c-fos, distributed in a parafollicular pattern (Figure 5). We observed a major increase in TNF-α-positive cells from 30 min post LPS until 2 hr post LPS. C-fos-positive cells were first detected at 1 hr post LPS and maintained this intensity until 2 hr post LPS. At 4 hr post LPS there were no TNF-α-positive cells and only a few c-fos-positive cells. However, many IFN-γ-positive cells were present at 4 hr post LPS. A few iNOS-positive cells were present in saline-injected animals, but we observed a dramatic and time-dependent increase in iNOS staining in the spleen starting at 4 hr post LPS with maximal staining being observed at 6 hr post LPS (Figure 6). Antigen unmasking with PK improved immunostaining for iNOS, IFN-γ (not shown), and the macrophage cell marker ED1 (not shown) in the spleen.

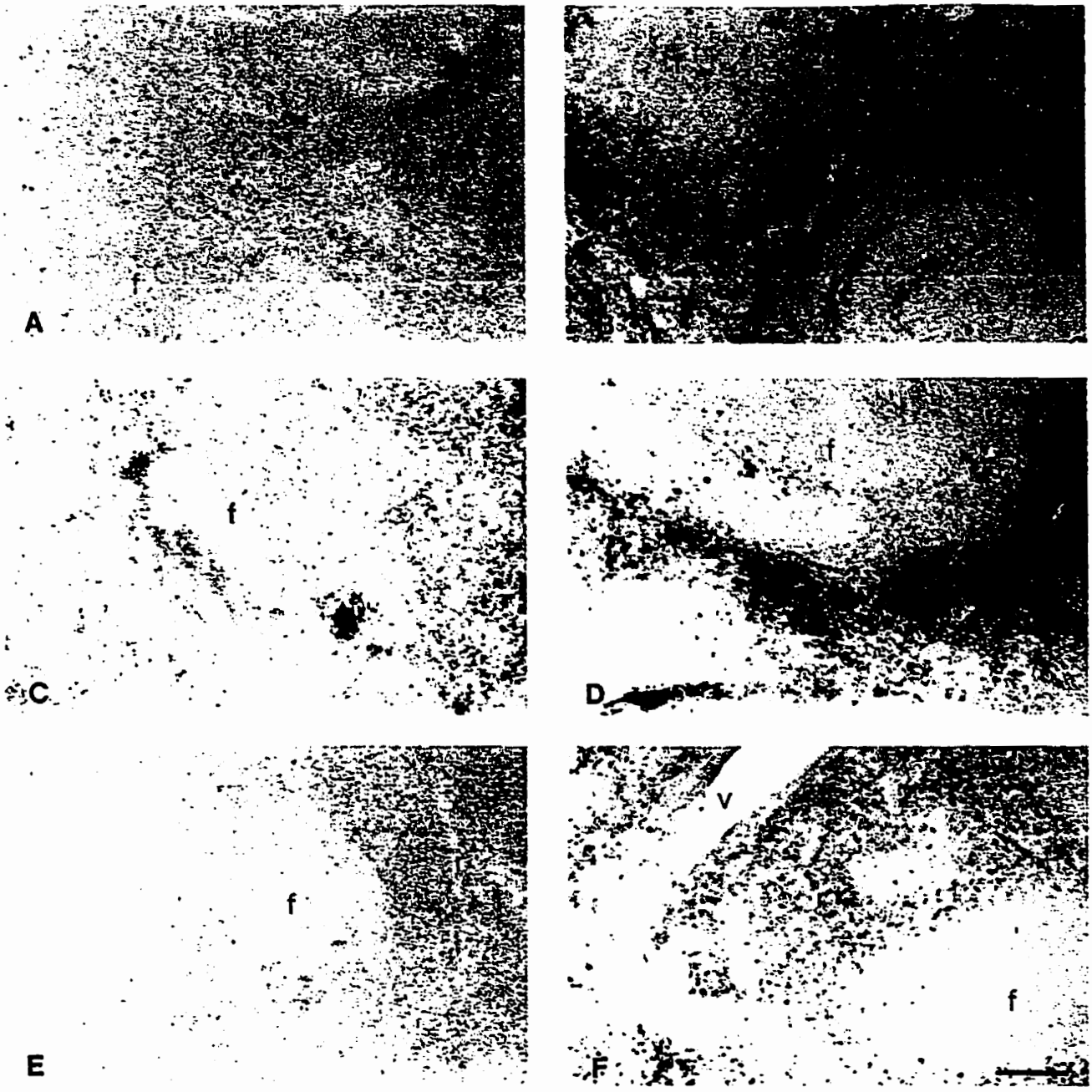

Figure 5 Photomicrographs showing TNF-α, IFN-γ and c-fos immunostaining in spleens of saline and LPS-treated animals. (A) TNF-α in a saline-treated animal; (B) TNF-α in spleen 90 min post LPS; (C) IFN-γ in a saline treated animal (section pretreated with PK); (D) IFN-γ in spleen 4 hr post LPS (section pretreated with PK); (E) c-fos in spleen of saline-treated animal; (F) c-fos in spleen 120 min post LPS injection. All spleen sections were developed with the alkaline phosphatase detection system using NBT/BCIP. v, blood vessel; f, follicle; r, red pulp. Bar = 100 μm.

Co-localization of NPY-positive Fibers with ED1-positive Cells

Using confocal microscopy, we demonstrated that macrophages (ED1-positive) are located in the same tissue compartment in the spleen as the sympathetic nerve fibers (NPY-positive) (Figure 7). This verifies previous anatomic and immunological studies that suggest a role for the sympathetic regulation of immune function.

Discussion

Although the PAP detection protocol provided excellent sensitivity for DBH (Figure 1D) and NPY (Figure 3A) immunodetection, the unpredictability of cellular background and the extra steps needed to remove this background suggested that it was not the optimal detection system for cell-associated antigens in the spleen. Both the AP detection system with NBT/BCIP

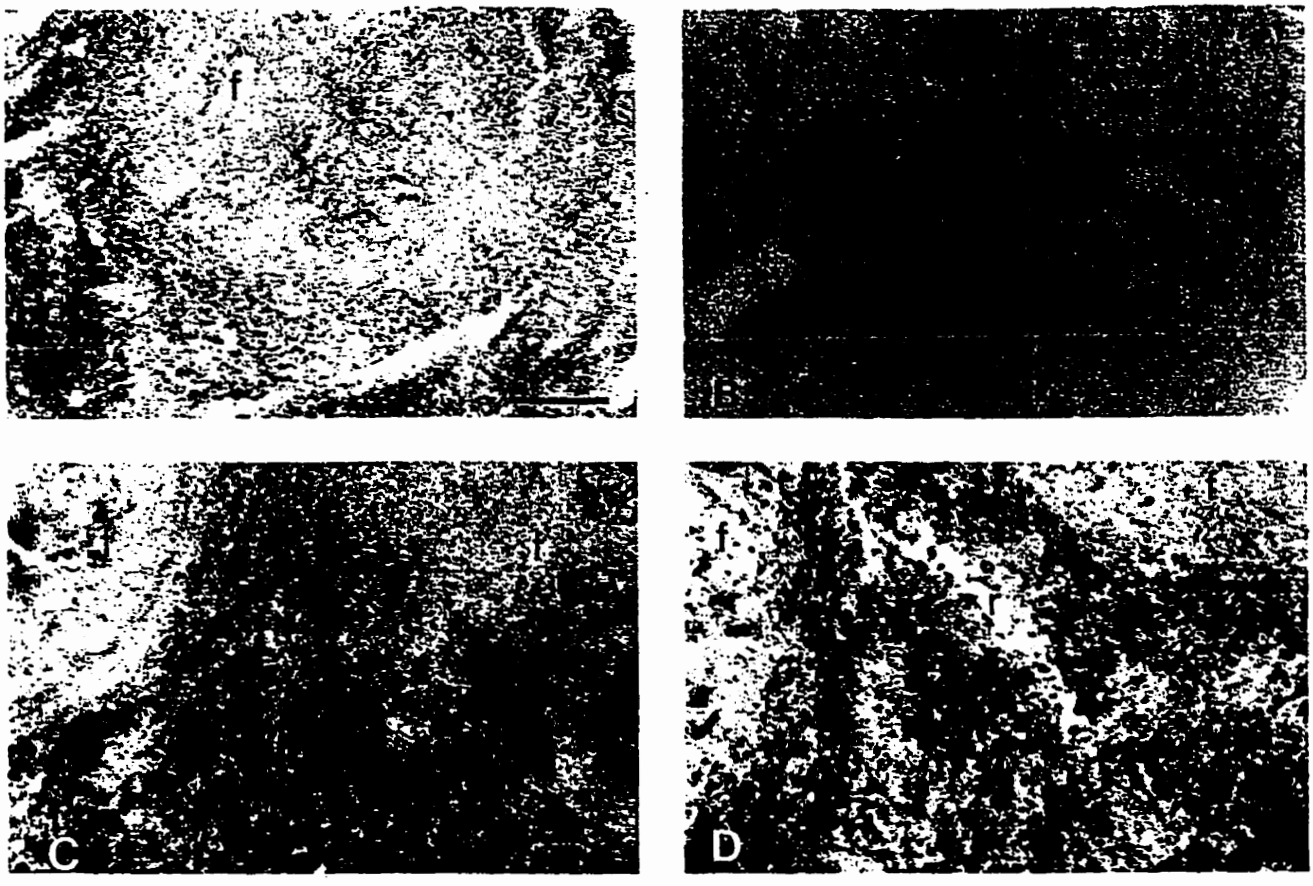

Figure 6 Photomicrographs showing iNOS immunostaining in spleens of saline- or LPS-treated rats with and without PK pretreatment. (A) Saline spleen with no PK; (B) saline spleen with PK; (C) 6-hr post-LPS spleen with no PK; (D) 6-hr post-LPS spleen with PK. All spleen sections were developed with the alkaline phosphatase detection system using NBT/BCIP. v, blood vessel; f, follicle; r, red pulp. Bar = 100 μm.

as a substrate and the CY3-conjugated antibody gave the best signal to noise ratio for detection of autonomic nerve fibers and immune-related proteins in the spleen. We also demonstrated that PK digestion dramatically improves nerve fiber staining for DBH, TH, and NPY and cellular staining for iNOS (Figure 6), IFN-γ, and ED1 (not shown). The optimal dose of PK to use for immunohistochemistry was variable among the different primary antibodies and even between groups of spleens perfused at different times, but the effective concentrations were 0–5 μg/ml, with the limiting step being the integrity of the tissue.

The use of proteolytic enzymes such as PK for antigen unmasking in immunohistochemistry has been described (Polak and Van Noorden 1982). However, the use of PK for improvement in nerve fiber immunodetection in the spleen is novel. Although it is unknown exactly why PK treatment enhances the immunostaining of some molecules, it is likely that several factors play important roles, including size and subcellular location of the molecule, type and amount of fixation, and the epitope recognized by the antibody.

Using optimized immunodetection techniques, we demonstrated that transection of the splenic nerve eliminated catecholamine-containing fibers in the spleen. This verifies previous data (Vriend et al. 1993) that showed a 98% depletion of NE levels in surgically denervated spleens, as measured by HPLC. Therefore, surgical sympathectomy is an effective method for removing the sympathetic control of the spleen and, relative to chemical sympathectomy, provides much greater anatomic specificity.

We also localized cytokines such as TNF-α (Diamond and Pesek 1991; Brown and Fishman 1990; Hofsli et al., 1989; Chensue et al. 1988) and IFN-γ (Heinzel et al. 1994; Heremans et al. 1994), transcription factors such as c-fos (Wan et al. 1993a,1994; Hamilton et al. 1989; Collart et al. 1987; Introna et al. 1986) and enzymes such as iNOS (Sato et al. 1995; Buttery et al. 1994; Cook et al. 1994; Bandaletova et al. 1993; Marletta 1993) in the spleen after injection of LPS (Hewett and Roth 1993). Only a few cells expressed the proteins for TNF-α, IFN-γ, c-fos and iNOS in rats injected with saline. However, after LPS

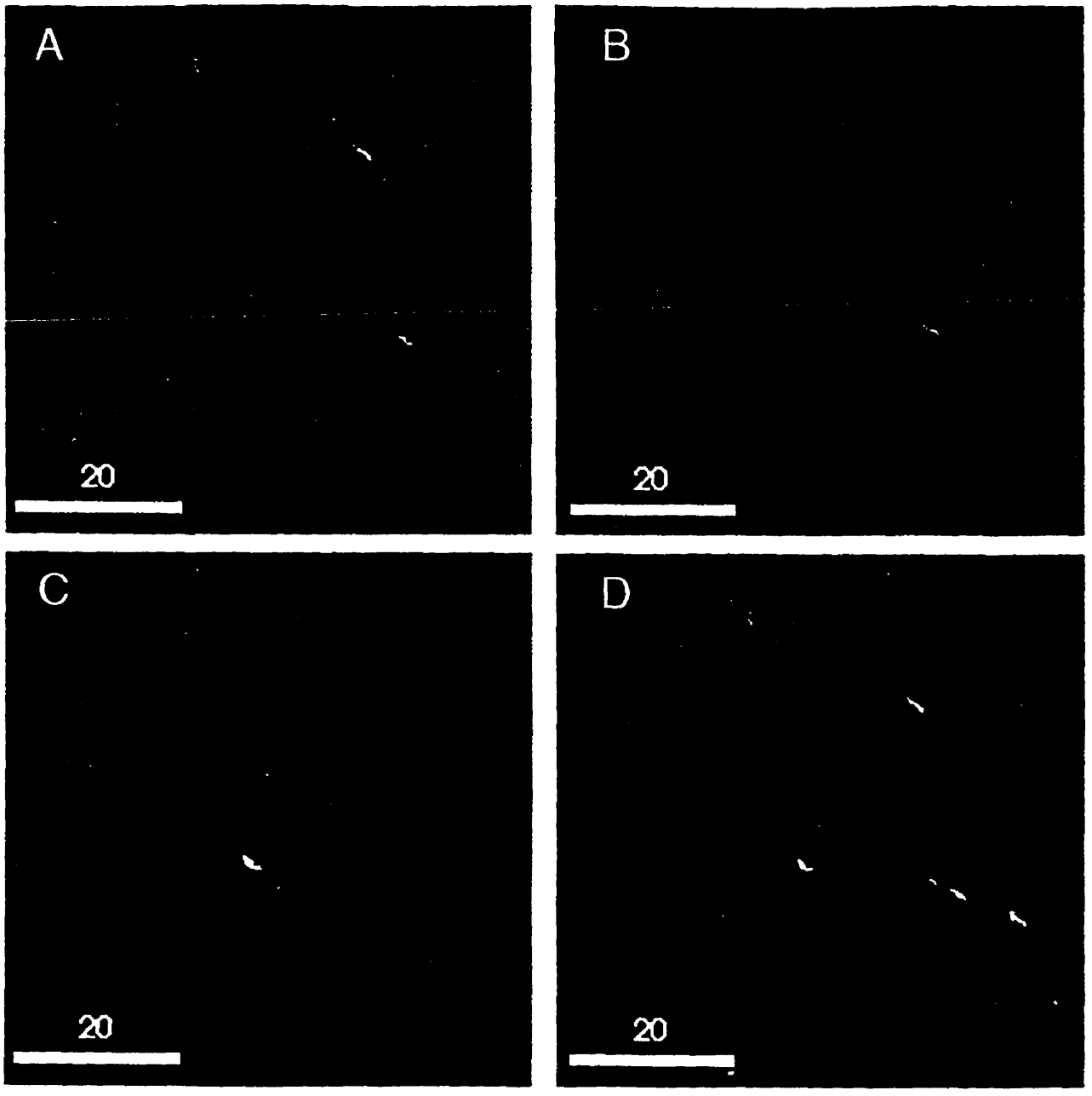

Figure 7 Digital prints of dual confocal microscope images of ED-1-positive macrophage (FITC) and NPY-positive nerve fibers (CY3) in rat spleen, visualized with a Molecular Dynamics confocal scanning laser microscope equipped with an argon laser and dual detectors. A, B, and C represent sequential renderings of five optical sections (1.47 μm/section) representing a total section thickness of 7.35 μm. D is a composite rendering of A–C and has a total section thickness of 22.05 μm. Resolution in the XY axis is 0.25 μm/pixel.

stimulation, many cells were immunopositive for these proteins in the marginal zone and red pulp, suggesting that they are probably macrophagic in origin and, in the case of IFN-γ, possibly NK- or T-cells (Heinzel et al. 1994; Heremans et al. 1994). We also demonstrated that ED1-positive cells can be co-localized with NPY-positive nerve fibers with immunofluorescence. Studies using confocal scanning laser microscopy demonstrated functional co-localization of sympathetic nerve fibers with immune effector cells in the spleen, confirming the anatomic co-localization reported by Felten et al. (1987a,b).

In conclusion, this article describes the ability to verify splenic nerve sections, to detect a variety of LPS-inducible proteins and to localize these proteins, to specific cell types by immunohistochemical tech-

niques. More importantly, this methodology may provide a valuable dependent measure of in situ immune function that can be utilized to assess the influence of the sympathetic nervous system on immune function.

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Literature Cited

Bandaletova T, Brouet I, Bartsch H, Sugimura T, Esumi H, Ohshima H (1993) Immunohistochemical localization of an inducible form of nitric oxide synthase in various organs of rats treated with Propionibacterium acnes and lipopolysaccharides. APMIS 101:330–336

Besedovsky HO, Del Rey A, Sorkin E, Da Prada M, Keller HH (1979) Immunoregulation mediated by the sympathetic nervous system. Cell Immunol 48:346–355

Brown AR, Fishman M (1990) Tumor necrosis factor alpha analyzed within individual macrophages by combined immunohistochemistry and computer-aided image analysis. Cell Immunol 130:352–363

Buttery LDK, Evans TJ, Springall DR, Carpenter A, Cohen J, Polak JM (1994) Immunochemical localization of inducible nitric oxide synthase in endotoxin-treated rats. Lab Invest 71:755–764

Chensue SW, Remick DG, Shmyr-Forsch C, Beals TF, Kunkel SL (1988) Immunohistochemical demonstration of cytoplasmic and membrane-associated tumor necrosis factor in murine macrophages. Am J Pathol 133:564–572

Collart MAD, Vassalli BJ, Vassalli P (1987) Modulations of functional activity in differentiated macrophages are accompanied by early and transient increase or decrease in c-fos gene transcription. J Immunol 139:949–955

Cook HT, Bune AJ, Jansen AS, Taylor GM, Loi RK, Cattell V (1994) Cellular localization of inducible nitric oxide synthase in experimental endotoxic shock in the rat. Clin Sci 87:179–186

Damoiseaux JGMC, Dopp EA, Calame W, Chao D, MacPherson GG, Dijkstra CD (1994) Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. Immunology 83:140–147

Diamond JR, Pesek I (1991) Glomerular tumor necrosis factor and interleukin 1 during acute aminonucleoside nephrosis. Lab Invest 64:21–28

Dureus P, Louis D, Grant AV, Bilfinger TV, Stefano GB (1993) Neuropeptide Y inhibits human and invertebrate immunocyte chemotaxis, chemokinesis, and spontaneous activation. Cell Mol Neurobiol 13:541–546

Felten DL, Ackerman KD, Wiegand SJ, Felten SY (1987a) Noradrenergic sympathetic innervation of the spleen: I. Nerve fibers associated with lymphocytes and macrophages in specific compartments of the splenic white pulp. J Neurosci Res 18:28–36

Felten DL, Felten SY, Bellinger DL, Carlson SL, Ackerman KD, Madden KS, Olschowka JA, Livnat S (1987b) Noradrenergic sympathetic neural interaction with the immune system: structure and function. Immunol Rev 100:221–260

Fukushima T, Sekizawa K, Jin Y, Yamaya M, Sasaki H, Takishima T (1993) Effects of beta-adrenergic receptor activation on alveolar macrophage cytoplasmic motility. Am J Physiol 265:L67–L72

Hamilton JA, Veis N, Bordun A, Vairo G, Gonda TJ, Phillips WA (1989) Activation and proliferation signals in murine macrophage: Relationships among c-fos and c-myc expression, phosphoinositide hydrolysis, superoxide formation, and DNA synthesis. J Cell Physiol 141:618–626

Heinzel FP, Rerko RM, Ling P, Hakimi J, Schoenhaut DS (1994) Interleukin-12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon. Infect Immun 62:4244–4249

Heremans H, Dillen C, van Damme J, Billiau A (1994) Essential role for natural killer cells in the lethal lipopolysaccharide-induced Shwartzman-like reaction in mice. Eur J Immunol 24:1155–1160

Hewett JA, Roth RA (1993) Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. Pharmacol Rev 45:381–411

Hofsli E, Bakke O, Nonstad U, Espevik T (1989) A flow cytometric and immunofluorescence microscopy study of tumor necrosis factor production and localization in human monocytes. Cell Immunol 122:405–415

Hu X, Goldmutz EA, Brosnan CF (1991) The effect of norepinephrine on endotoxin-mediated macrophage activation. J Neuroimmunol 31:35–42

Hu H, Moller G (1994) Lipopolysaccharide-stimulated events in B cell activation. Scand J Immunol 40: 221–227

Introna M, Hamilton TA, Kaufman RE, Adams DO, Bast Jr RC (1986) Treatment of murine peritoneal macrophages with bacterial lipopolysaccharide alters expression of c-fos and c-myc oncogenes. J Immunol 137:2711–2715

Kouassi E, Li YS, Boukhris W, Millet I, Revillard J (1988) Opposite effects of the catecholamines dopamine and norepinephrine on murine polyclonal B-cell activation. Immunopharmacology 16:125–137

Larison KD, Bremiller K, Wells KS, Clements I, Haugland RP (1995) Use of a new flurogenic phosphatase substrate in immunohistochemical applications. J Histochem Cytochem 43:77–83

Li C, Ziesmer SC, Lazcano-Villareal O (1987) Use of azide and hydrogen peroxide as an inhibitor for endogenous peroxidase in the immunoperoxidase method. J Histochem Cytochem 35:1457–1460

Madden KS, Moynihan JA, Brenner GJ, Felten SY, Felten DL, Livnat S (1994) Sympathetic nervous system modulation of the immune system. III. Alterations in T and B cell proliferation and differentiation in vitro following chemical sympathectomy. J Neuroimmunol 49:77–87

Marletta MA (1993) Nitric oxide synthase structure and mechanism. J Biol Chem 268:12231–12234

Monastra G, Secchi EF (1993) B-adrenergic receptors mediated in vivo the adrenaline inhibition of lipopolysaccharide-induced tumor necrosis factor release. Immunol Lett 38:127–130

Nance DM, Burns J (1989) Innervation of the spleen in the rat: evidence for absence of afferent innervation. Brain Behav Immun 3:281–290

Pelletier G, Guy J, Allen YS, Polak JM (1984) Electron microscope immunocytochemical localization of neuropeptide Y (NPY) in the rat brain. Neuropeptides 4:319–324

Polak JM, Van Noorden S (1982) Techniques in Immunocytochemistry. Vol 1. London, New York, Paris, San Diego, San Francisco, Toronto, Sao Paulo, Sydney, Tokyo, Academic Press

Romano TA, Felten SY, Felten DL, Olschowka JA (1991) Neuropeptide-Y innervation of the rat spleen: another potential immunomodulatory neuropeptide. Brain Behav Immun 5:116–131

Sanders VM, Munson AE (1985) Norepinephrine and the antibody response. Pharmacol Rev 37:229–248

Sato K, Miyakawa K, Takeya M, Hattori R, Yui Y, Sunamoto M, Ichimori Y, Ushio Y, Takahashi K (1995) Immunohistochemical expression of inducible nitric oxide synthase (iNOS) in reversible endotoxic shock studied by a novel monoclonal antibody against rat iNOS. J Leukocyte Biol 57:36–44

Smith PG, Hoffman TL, Reddy H (1991) Sprouting of aberrant neuropeptide Y-immunoreactive sympathetic nerves into neonatally denervated smooth muscle. Regul Peptides 35:103–113

Spengler RN, Allen RM, Remick DG, Strieter RM, Kunkel SL (1990) Stimulation of the alpha-adrenergic receptor augments the production of macrophage-derived tumor necrosis factor. J Immunol 145:1430–1434

Straus W (1971) Inhibition of peroxidase by methanol and by methanol-nitroferricyanide for use in immunoperoxidase procedures. J Histochem Cytochem 19:682–688

Trudrung P, Furness JB, Messenger JP (1994) Locations and chemistries of sympathetic nerve cells that project to the gastrointestinal tract and spleen. Arch Histol Cytol 57:139–150

Van der Meide PH, Dubbeld M, Vijverberg K, Kos T, Schellekens H (1986) The purification and characterization of rat gamma interferon by use of two monoclonal antibodies. J Gen Virol 67:1059–1071

Vriend CY, Wan W, Greenberg AH, Nance DM (1993) Cutting the splenic nerve differentially affects catecholamine and neuropeptide levels in the spleen. Soc Neurosci Abstr 388.5

Wan W, Janz L, Vriend CY, Sorenson CM, Greenberg AH, Nance DM (1993a) Differential induction of c-fos immunoreactivity in hypothalamus and brain stem nuclei following central and peripheral adminstration of endotoxin. Brain Res Bull 32:581–587

Wan W, Vriend CY, Wetmore L, Gartner JG, Greenberg AH, Nance DM (1993b) The effects of stress on splenic immune function are mediated by the splenic nerve. Brain Res Bull 30:101–105

Wan W, Wetmore L, Sorensen CM, Greenberg AH, Nance DM (1994) Neural and biochemical mediators of endotoxin and stress-induced c-fos expression in the rat brain. Brain Res Bull 34:7–14

Zalcman S, Green-Johnson JM, Murray L, Wan W, Nance DM, Greenberg AH (1994) Interleukin-2-induced enhancement of an antigen-specific IgM plaque-forming cell response is mediated by the sympathetic nervous system. J Pharmacol Exp Ther 271:977–982

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Nonradioactive Northern blotting with biotinylated and digoxigenin-labeled RNA probes

The application of nonradioactive RNA probes for Northern blotting offers the advantage of a rapid turn-around time for results without the loss of sensitivity for target mRNA detection. However, a problem that has impeded the widespread use of nonradioactive RNA probes for use in Northern blotting is the difficulty in stripping these probes from nylon membranes after hybridization. In this report we describe two protocols for stripping digoxigenin (Dig)-labeled RNA probes from nylon membranes. One protocol utilizes a phosphate-buffered formamide stripping solution to remove nonchemically modified (regular) RNA probes while the other method utilizes strippable probes that were produced with a chemically modified nucleotide (CTP) and removed by a specific stripping solution. This latter method was developed by Ambion Inc. and is called Strip-EZ™. We also describe a protocol for the detection of two separate rat mRNAs using both biotin and digoxigenin-labeled RNA probes that does not require stripping the membrane after hybridization. Finally, we describe the use of another new labeling technology, called Chem-Link™, that quickly and conveniently labels RNA for use in Northern blotting.

1 Introduction

The use of nonradioactive probes for Northern blotting offers many advantages over radioactive probes, including the rapid turn-around time for results, the increased safety relative to radioactive probes, the ability to store these probes for long periods of time, the ability to reuse these probes for multiple hybridizations [1] and the similar detection sensitivities of the two systems for nucleic acid hybridization [2, 3]. RNA probes have been used in Northern blotting because of their superior sensitivity when compared to cDNA probes [4, 5]. The major disadvantage in using hapten-labeled probes for blotting is that they are difficult to strip from nylon membranes [5–8]. Therefore, reprobing the membrane with a nonradioactive probe is difficult unless the mRNAs that are being detected are of sufficiently different sizes so as to prevent signal overlap, or each probe is labeled with a different hapten. Hoeltke *et al.* [9] described an elegant method where multiple mRNAs can be visualized at the same time with an alkaline phosphatase-based detection system. The only limitation of this system is that it utilizes chromogenic substrates that are much less sensitive than chemiluminescent substrates [10–13]. Therefore, in order to achieve the maximum sensitivity for the detection of different mRNAs on the same blot, chemiluminescent substrates must be used.

Probes were produced for tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and β-actin. TNF-α and IL-1β were chosen because of their well-documented inducibility by stimuli such as lipopolysaccharide (LPS) [14] and thus provide good positive and negative controls for our procedure, while β-actin was chosen because it is constitutively expressed and is often used as a loading control for Northern blots. This report describes the synthesis of nonchemically modified (regular) and Strip-EZ probes and describes the stripping protocols that can be used to remove these digoxigenin (Dig)-labeled RNA probes from nylon membranes. As well, we describe an alternative protocol using biotin as a second label for situations where the stripping protocols are not appropriate. Finally we describe an alternative method for labeling RNA called Chem-Link. Chem-Link is a *cis*-platinum compound which is chemically linked to either Dig or biotin and is able to coordinatively bind to the N7-position of guanosine and adenosine bases on nucleic acids (a coordinate bond is between a metal and its ligand [15]). Chem-Link offers advantages for researchers in that the labeling procedure is simple, quick, and does not require additional enzymes once the nucleic acid is synthesized.

2 Materials and methods

2.1 Northern blotting

Adult male (250–400 g) Sprague-Dawley rats were obtained from Charles River (Dorval, PQ), injected intravenously with saline or 0.1–100 μg of LPS (*E. coli* serotype 055:B5, Sigma, Mississauga, ON) and sacrificed 1–6 h after injection. Total RNA was isolated from fresh frozen rat spleens with Trizol™ reagent (Life Technologies, Burlington, ON) according to the manufacturer's instructions. The RNA samples were denatured (50% formamide/1 × MOPS/17.5% formaldehyde/10% glycerol and ethidium bromide) for 10 min at 65°C and run on a 1.2% agarose/17.9% formaldehyde gel for 2.5 h at 70 V. The RNA was transferred overnight to nylon membrane (Boehringer Mannheim, Laval, PQ) by capillary action

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DEPC, diethylpyrocarbonate; Dig, digoxigenin; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; SSC, sodium citrate/sodium chloride; TNF-α, tumor necrosis factor alpha

Keywords: Stripping / Chemiluminescence / Alkaline phosphatase / Nonradioactive blotting / Peroxidase

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with sodium citrate/sodium chloride (SSC) buffers. The membrane was baked for 2 h at 80°C to immobilize the RNA, after which the ethidium bromide-stained 28S and 18S ribosomal RNA bands were visualized using a UV transilluminator to verify the integrity of samples. The membranes were equilibrated with 2 × SSC and prehybridized for 1 h at 42°C (in 50% formamide, 5 × SSC, 0.1% sodium laurosarcosine, 0.2% SDS, and 2% Boehringer block). The membranes were then hybridized with biotin- or Dig-labeled RNA probes overnight at 60°C. After hybridization, membranes were washed twice in 2 × SSC/0.1% SDS for 15 min at room temperature and twice in 0.1 × SSC/0.1% SDS at 60°C for 20 min. Membranes were blocked for 1 h in 1% Boehringer block in maleic acid buffer, incubated with either sheep anti-Dig peroxidase-conjugated antibody (1/5000, Boehringer Mannheim), alkaline phosphatase-labeled streptavidin (1/5000, Amersham, Oakville, ON) or an alkaline phosphatase-labeled anti-biotin antibody (1/2000, New England Biolabs, Mississauga, ON) depending on the type of probe used for hybridization. Following three rinses in maleic acid buffer, the Dig-labeled bands were visualized with a chemiluminescent substrate for peroxidase (Boehringer Mannheim), while blots hybridized with biotinylated probes were visualized with the chemiluminescent alkaline phosphatase substrate, CDP star™ (1/100, Boehringer Mannheim) or with NBT/5-bromo-4-chloro-3-indolyl phosphate (BCIP), 0.4 mM NBT/0.4 mM BCIP in 50 mM $MgCl_2$/100 mM Tris in 100 mM NaCl, pH 9.3. Following chemiluminescent detection, the membranes were again prehybridized for 1 h and either hybridized with a second nonradioactive RNA probe or placed in prehybridization buffer overnight and reincubated with the same chemiluminescent substrate to assess the residual enzyme activity. Dig-labeled RNA probes from the first round of hybridizations were stripped from nylon membranes only when subsequent rounds of hybridizations included Dig-labeled probes. Stripping was accomplished by sealing the membrane in a plastic bag with 10 mM phosphate buffer (pH 7.5)/90% formamide and boiling for 10–15 min, by placing the membrane in a hybridization oven at 95°C with phosphate buffer (pH 7.5)/90% formamide for 15 min or by sealing in a plastic bag containing water with 0.1% sodium dodecyl sulfate (SDS) and autoclaving the bag for 15 min on the wet cycle (no drying time). Strip-EZ™ probes were removed from membranes according to the manufacturer's instructions (Ambion Inc., Austin, TX, USA). Briefly, after hybridization, the blots were rinsed in 1 × probe degradation buffer/0.1% SDS for 10 min at 68°C, followed by a 10 min wash at 68°C in blot reconstitution buffer/0.1% SDS and a 10 min wash at 68°C in 0.1% SDS. Chemiluminescent signals were exposed to film anywhere from 15 s to 30 min. Solutions were treated with 0.1% diethylpyrocarbonate (DEPC) as required.

2.2 Production of biotin- and Dig-labeled RNA probes by *in vitro* transcription

Templates for rat TNF-α, IL-1β and β-actin were produced as previously described [16, 17]. The biotin-labeled probe for β-actin and Dig-labeled RNA probes for TNF-α (sense and antisense), IL-1β and β-actin were synthesized from 4 μL of PCR product (containing a T7 RNA promoter sequence) using the *in vitro* transcription kit from Boehringer Mannheim. Dig-labeled Strip-EZ probes were synthesized according to instructions from Ambion, where the only significant modifications from Boehringer Mannheim's protocol were the inclusion of the modified Strip-EZ CTP to the reaction mix in limiting quantities (0.1 mM final concentration) and the concentration of the other nucleotides and T7 RNA polymerase was half of Boehringer Mannheim's recommended concentration. Probes were then dissolved in 20–100 μL of DEPC-treated water with RNase inhibitor (0.2 units/μL final concentration) and dissolved for 30 min at 37°C. The yields of Dig-labeled probes were determined by a combination of serial 1/10 and 1/4 dilutions that were spotted onto nylon membrane (Boehringer Mannheim) and detected with a 1/5000 dilution of alkaline phosphatase-labeled sheep anti-Dig antibody (Boehringer Mannheim) with NBT/BCIP as a substrate/chromogen combination. Concentrations of Dig-labeled probes were determined to range from 10–50 ng/μL by comparing serial dilutions of our Dig-labeled RNA probes with the Dig-labeled RNA standards provided in the Dig Nucleic Acid Detection Kit (Boehringer Mannheim). The yield of Strip-EZ probes were very low due to the limiting nucleotide concentrations and were subsequently dissolved in smaller amounts of DEPC-treated water. The biotin-labeled probe for β-actin was *in vitro* transcribed at the same time as its Dig-labeled counterpart and the yield was quantified by UV spectroscopy at 260 nm. Biotinylated RNA probes were also detected on the membrane with a 1/5000 dilution of alkaline phosphatase-labeled streptavidin (Amersham) and visualized with NBT/BCIP to confirm successful incorporation of the biotin-UTP. Solutions were treated with 0.1% DEPC as required.

2.3 Production of biotin- and Dig-labeled RNA probes using Chem-Link

TNF-α (sense and antisense) and β-actin RNA were *in vitro* transcribed with unlabeled nucleotides, purified according to the Dig RNA Labelling Kit (Boehringer Mannheim) and quantified by UV spectroscopy at 260 nm. Biotin and Dig Chem-Link™ (Boehringer Mannheim) were used to label TNF-α antisense, TNF-α sense, and β-actin antisense RNA. Four μL of Chem-Link was added to 4 μg of RNA in a volume of 20 μL. The mixture was incubated at 85°C for 30 min and the reaction was stopped with 5 μL of stop solution. The volume was adjusted to 40 μL with 14 μL of water and 1 μL of RNase inhibitor to a final concentration of 100 ng/μL of Dig-labeled RNA. The probes were then spotted onto nylon membrane alongside their *in vitro* transcribed counterparts and detected as described above.

3 Results

We observed that the antisense probes for TNF-α and IL-1β bound only to RNA from LPS-treated rat spleens, the β-actin probes bound to RNA from all animals and the sense probes did not hybridize in any lane (data not shown). We also found that stripping Dig-labeled RNA probes with a phosphate-buffered formamide solution or by the Strip-EZ method effectively eliminated most of the bound probe so that the residual signal did not inter-

fere with the visualization of subsequent probes (Fig. 1 and 2). Both of these procedures showed equivalent results through three rounds of hybridizations (Fig. 2). It was also clear from these experiments that the amount of target RNA plays a critical role in determining the efficiency of probe removal as the lanes with more bound probe also had a higher residual signal upon redetection (Figs. 1 and 2). This was observed for both stripping procedures.

Results also demonstrated that a blot initially hybridized with an antisense probe for TNF-α can be successfully reprobed with a biotinylated antisense probe for β-actin without the need for stripping (Fig. 3). The best signal-to-noise ratio achieved with chemiluminescent detection of the biotinylated β-actin probe was with the anti-biotin antibody. Both chemiluminescent substrates (for peroxidase and alkaline phosphatase) gave a very high background when used with streptavidin due to the nonspecific binding of the streptavidin to the membrane. This was verified when high background was obtained even in the absence of membrane-immobilized RNA and labeled probe (data not shown). However, the streptavidin worked well if NBT/BCIP was used for visualization. We also found that the enzymatic activities of both peroxidase and alkaline phosphatase were eliminated by overnight incubation at 60°C in hybridization solution (30 min exposure; data not shown).

The intensity of the color reaction generated by the serial dilutions of biotin- and Dig-labeled probes spotted on nylon membranes was used to determine the amount of probe from the Chem-Link preparations that would give an equivalent signal to the *in vitro* transcribed probes when both were used for hybridizations. The *in vitro* transcribed antisense Dig-labeled RNA probe for TNF-α was several times more sensitive than the Chem-Link counterpart even when the total amount of RNA was manyfold higher in the hybridization solution which contained the Chem-Link probe (Fig. 3A and B). The antisense β-actin probes labeled with Chem-Link biotin (Fig. 3D) and Chem-Link Dig (data not shown) showed a much closer detection sensitivity to their *in vitro* transcribed counterparts than did the Chem-Link, Dig-labeled antisense probe for TNF-α.

4 Discussion

In order to accurately quantitate and compare relative amounts of message in samples within a given experiment, it is necessary to reprobe the same membrane at least once with a loading control. In agreement with reports from other labs [5, 7, 8], our previous attempts to remove Dig-labeled RNA probes from nylon membranes proved to be ineffective (data not shown). It was necessary therefore to optimize a protocol which would effectively remove the Dig-labeled probe without damaging target RNA, and to have available an alternative protocol which utilized RNA probes labeled with a different hapten, such as biotin, in the event that the Dig-labeled RNA probe was not removable. The two stripping methods, 90% formamide/10 mM phosphate buffer and the Ambion Strip-EZ method, were both effective in removing Dig-labeled RNA probes from the previous hybridization, although it was always necessary to perform each stripping procedure twice. Although some residual signal was still evident in certain lanes after two rounds of stripping, it was detectable only after an exposure time which greatly exceeded the amount of time necessary to detect messages from the subsequent hybridization.

The removability of the probe is directly affected by both the amount of target RNA on the membrane and the concentration of probe used in the hybridization solution. Figure 1 shows that one round of formamide stripping successfully removes β-actin probe from lanes containing 5 μg or less of total RNA; however, both formamide and Strip-EZ methods had to be repeated in order to eliminate β-actin signal from lanes containing 10 μg of total RNA (Fig. 2). In both experiments β-actin probe concentration was kept constant. An unnecessarily high concentration of probe in the hybridization solution will result in a certain amount of nonspecific binding, as well as a specific signal so intense that even at the shortest exposure times prevents meaningful interpretation of the results Inconsistencies in probe removal were found to be eliminated when all stripping procedures were performed in a hybridization oven when compared to

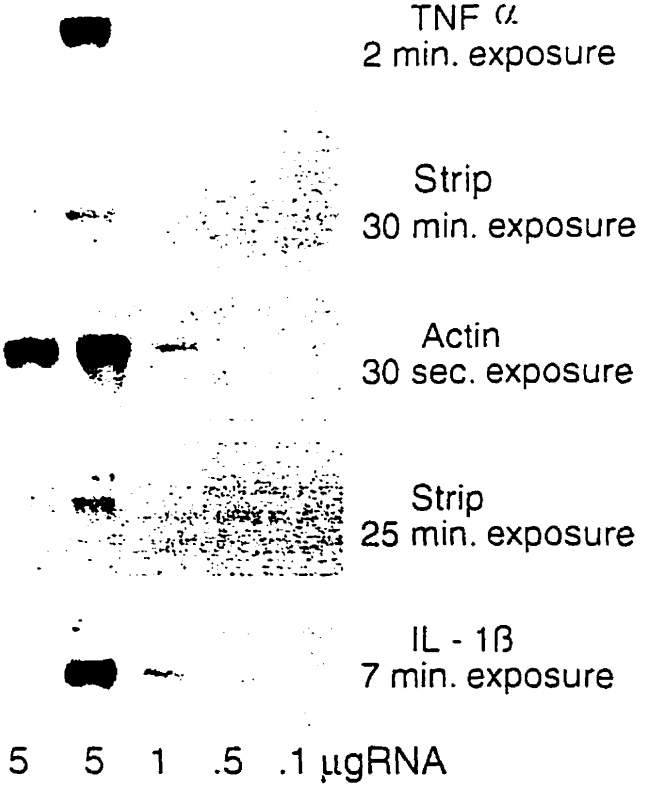

Figure 1. Northern blot sequentially probed with Dig-labeled RNA probes for TNF-α (10 ng/mL), β-actin (0.5 ng/mL), and IL-1β (10 ng/mL), demonstrating the effect of target RNA concentration on the amount of chemiluminescent signal before and after stripping. Dig-labeled probes were visualized with a chemiluminescent substrate for peroxidase and subjected to the stripping procedure utilizing phosphate-buffered formamide. The first lane of the blot has 5 μg of total RNA from a saline-treated rat while lanes 2–5 have total RNA from an LPS-treated rat in the following amounts, respectively: 5 μg, 1 μg, 0.5 μg and 0.1 μg.

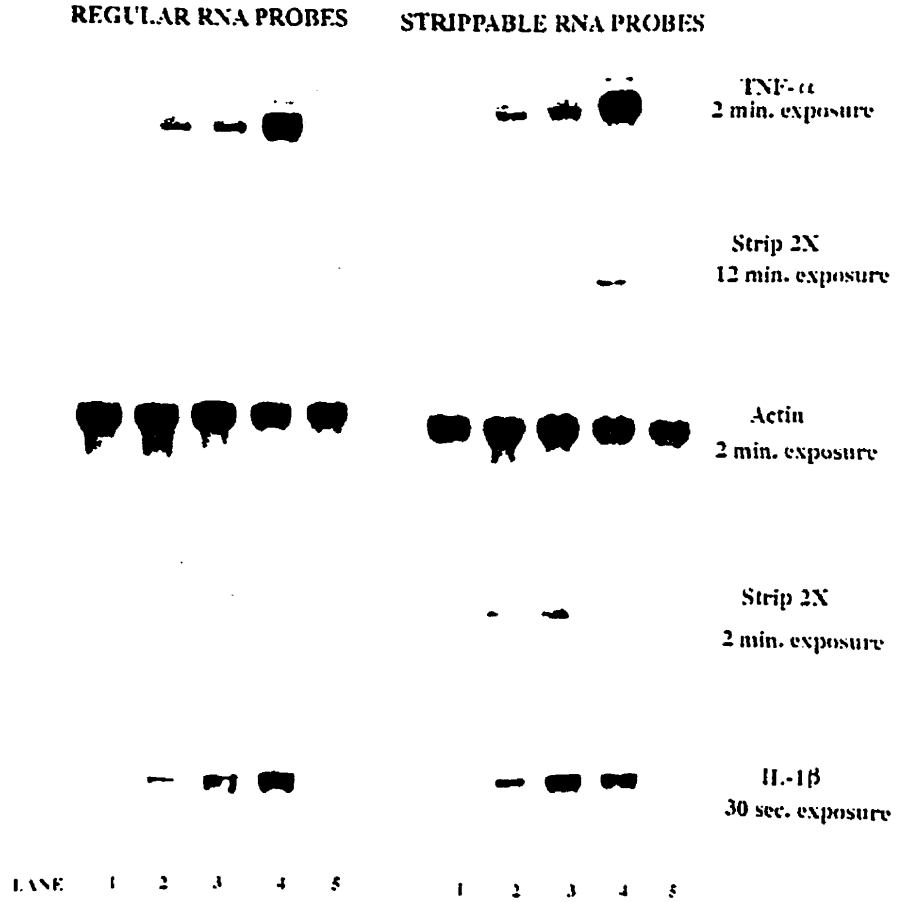

Figure 2. Northern blots sequentially probed with Dig-labeled RNA probes for TNF-α (10 ng/mL), β-actin (0.5 ng/mL), and IL-1β (10 ng/mL). The left side of the figure shows blots probed with non-modified (regular) probes while the right side shows blots probed with Strip-EZ probes. All Dig-labeled probes were visualized with a chemiluminescent substrate for peroxidase and subjected to the stripping procedure utilizing phosphate-buffered formamide or as recommended in the Strip-EZ kit. Each lane of the blot has 10 μg of total splenic RNA from various points post-LPS injection (100 μg i.v.). Lane (1) 6 h, (2) 4 h, (3) 2 h, (4) 1 h and (5) vehicle (saline) injection.

sealing the membranes in plastic bags with stripping solution and placing them in a boiling water bath. This was especially true with larger membranes. Stripping Dig-labeled RNA probes from nylon membranes by autoclaving them for 15 min in 0.1% SDS (suggested by Ambion technical services department in 1996) was also successful; however, we abandoned this approach due to inconvenience. We found that both stripping methods were equally effective when compared over three hybridizations (six rounds of stripping). The manufacturers of Strip-EZ suggest that their protocol is effective over nine hybridizations. It is unknown what effects the high temperature and concentration of formamide would have on target RNA following this many treatments; however, we were able to prove that, following six stripping treatments and three rounds of hybridizations, the membranes still showed prominent 28S and 18S bands (demonstrated by ethidium bromide staining; data not shown). The only clear advantage in using formamide as a stripping medium, apart from simplicity, would be if the RNA probes were being used for *in situ* hybridization as well as Northern blotting. This would allow the same probes to be used in both applications, and a separate set of "strippable" probes need not be generated specifically for Northerns.

In conclusion, it is safe to say that under optimized conditions of probe concentration and target abundance, both methods can be used effectively in RNA probe removal, thus expanding the use of individual membranes in message comparisons. It should be kept in mind, however, that those messages that are known to be abundant should be probed for last (unless the difference in molecular size is dramatic), and that the effectiveness of probe removal should always be tested for by detection, before any subsequent hybridizations are attempted. For the alternative protocol (the protocol utilizing both Dig- and biotin-labeled probes without the need for stripping) we knew that Dig-labeled probes offer a better signal-to-noise ratio than biotin-labeled robes [10, 11, 18]. Because of this, Dig-labeled probes with a chemiluminescent peroxidase substrate was used

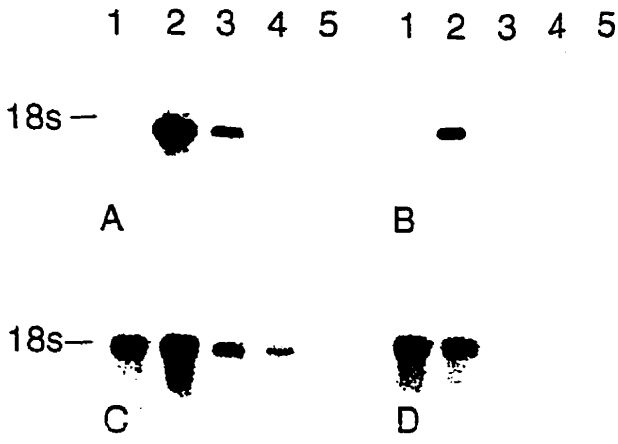

Figure 3. (A) Blot probed with an antisense TNF-α RNA probe made by *in vitro* transcription (25 ng/mL). (B) Blot probed with an antisense TNF-α RNA probe made with Chem-Link (100 ng/mL). (C) Same blot as shown in (A), reprobed with 50 ng/mL of biotinylated antisense β-actin RNA probe made by *in vitro* transcription. (D) Same blot as shown in (B) reprobed with 80 ng/mL of biotinylated antisense RNA for β-actin made with Chem-Link. Both blots are identical in that the first lane contains 5 μg of total RNA from a saline-treated rat while lanes (2)—(5) contain total RNA from an LPS-treated rat in the following amounts, respectively: 5 μg, 1 μg, 0.5 μg and 0.1 μg. All blots were exposed to film for 10 min. Blots (A), (B) were developed and exposed at the same time and blots (C), (D) were developed and exposed at the same time.

to detect our message of interest, while the loading control was detected with a biotin-labeled probe and the most sensitive chemiluminescent alkaline phosphatase substrate, CDP-star [11]. The detection/visualization protocol was designed to ensure there were no false positive due to residual chemiluminescence or enzymatic activity. We found that biotin can be used as a second label for Northern blotting in situations where stripping procedures are not optimal. However, the biotin-labeled probe should be used to detect the more abundant message because like other labs we found that the biotin-labeled probe did not give as good a signal-to-noise ratio as the Dig-labeled probe [10, 11, 18]. Likewise, if biotin is to be visualized with chemiluminescence, then it should be detected with an anti-biotin antibody because streptavidin may give too much background, depending on the protocol and type of membrane used. Chemiluminescent detection of nucleic acids is preferable to colorimetric detection because chemiluminescent substrates are more sensitive [10–13]. Also, the most sensitive chromogen, NBT/BCIP, is difficult to remove from nylon membranes [19], thereby complicating a potential third hybridization. Our results also demonstrate that overnight incubation in hybridization solution (with no probe) eliminated the chemiluminescent signal generated by both peroxidase and alkaline phosphatase after reexposure to their respective substrates. Both Dubitsky *et al.* [20] and Richterich and Church [21] demonstrated that antibodies can be successfully stripped of nylon membranes, reenforcing the view that the same chemiluminescent substrate can be used to detect all hapten-labeled probes if the proper precautions are taken.

Chem-Link is a *cis*-platinum compound that is chemically linked to either Dig or biotin and is able to coordinatively bind to the N7-position of guanosine and adenosine bases on nucleic acids. The sensitivity of detection for the Chem-Link probes was below that of the *in vitro* transcribed probes, and this may be attributed to many factors, including the sequence of the probe of interest (*i.e.* the amount of intrastrand guanosine or adenosine crosslinks that can be formed [22]) and the fact that proper controls for the Chem-Link reaction to measure incorporation efficiency have yet to be developed, thereby making direct comparisons difficult. However, with an abundant amount of target mRNA, as is the case with β-actin, the Chem-Link probes performed well. In conclusion, we found that it is possible to strip Dig-labeled RNA probes from nylon membranes and that biotin is an excellent second label to use in Northern blotting. These protocols help demonstrate that nonradioactive RNA probes are a viable alternative to radioactive cDNA probes for use in Northern blotting.

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5 References

[1] Hoeltke, H. J., Kessler, C., *Nucleic Acids Res.* 1990, *18*, 5843–5851.
[2] Engler-Blum, G., Meier, M., Frank, J., Muller, G. A., *Anal. Biochem.* 1993, *210*, 235–244.
[3] Sato, M., Murao, K., Mizobuchi, M., Takahara, J., *BioTechniques* 1993, *15*, 880–882.
[4] Rueger, B., Thalhammer, J., Obermaier, I., Gruenwald-Janho, S., *Biochemica* 1996, *3*, 35–38.
[5] Srivastava, R. A. K., Schonfeld, G., *BioTechniques* 1991, *11*, 584–587.
[6] Krueger, S. K., Williams, D. E., *Anal. Biochem.* 1995, *229*, 162–169.
[7] Moller, E. M., Schilling, A. G., Karlovsky, P., *BioTechniques* 1994, *17*, 682–684.
[8] Winkler, A., Fickel, J., Melzig, M. F., *BioTechniques* 1994, *16*, 61–62.
[9] Hoeltke, H. J., Ettl, I., Finken, M., West, S., Kunz, W., *Anal. Biochem.* 1992, *207*, 24–31.
[10] During, K., *J. Chromatogr.* 1993, *618*, 105–131.
[11] Hoeltke, H. J., Ankenbauer, W., Muhlegger, K., Rein, R., Sanger, G., Seibl, R., Walter, T., *Cell. Mol. Biol.* 1995, *41*, 883–905.
[12] Gurpreet, S., Sandhu, G. S., Eckloff, B. W., Kline, B. C., *BioTechniques* 1991, *11*, 14–16.
[13] Soumet, C., Ermel, G., Boutin, P., Boscher, E., Colin, P., *BioTechniques* 1995, *19*, 792–796.
[14] Hewett, J. A., Roth, R. A., *Pharmacol. Rev.* 1993, *45*, 381–411.
[15] Gillespie, R. J., Humphreys, D. A., Baird, N. C., Robinson, E. A., *Chemistry* Allyn and Bacon, Boston 1989, pp. 958, 983.
[16] Birk, P. E., Grimm, P. C., *J. Immunol. Methods* 1994, *167*, 83–89.
[17] Turnbull, A. V., Pitossi, F. J., Lebrun, J.-J., Lee, S., Meltzer, J. C., Nance, D. M., del Rey, A., Besodovsky, H. O., Rivier, C., *J. Neurosci.* 1997, *17*, 3262–3273.
[18] McQuaid, S., McMahon, J., Allan, G. M., *Biotech. Histochem.* 1995, *70*, 147–154.
[19] West, S., Schroder, J., Kunz, W., *Anal. Biochem.* 1990, *190*, 254–258.
[20] Dubitsky, A., Brown, J., Brandwein, H., *BioTechniques* 1992, *13*, 392–400.
[21] Richterich, P., Church, G. M., *Methods Enzymol.* 1993, *218*, 187–222.
[22] Fichtinger-Schepman, A. M. J., van der Veer, J. L., den Hartog, J. H. J., Lohman, P. H. M., Reedijk, J., *Biochemistry* 1985, *24*, 707–713.

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Brain Research Protocols 2 (1998) 339–351

BRAIN RESEARCH PROTOCOLS

Protocol

Production of digoxigenin-labelled RNA probes and the detection of cytokine mRNA in rat spleen and brain by in situ hybridization

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Abstract

Non-radioactive in situ hybridization is a sensitive method for determining the site of production for secretory molecules such as cytokines. We report here on the central and peripheral induction of proinflammatory cytokines by endotoxin, and outline procedures for the generation and application of rat-specific digoxigenin (Dig)-labelled RNA probes for the localization of mRNA by in situ hybridization. Rats were injected either intravenously (i.v.) or intracerebroventricularly (i.c.v.) with vehicle or lipopolysaccharide (LPS) and sacrificed at various time intervals post-injection. Rats were then perfused with 4% paraformaldehyde and the spleens and brains were removed and cryoprotected in 30% sucrose. Dig-labelled, rat-specific, antisense and sense RNA probes were generated by in vitro transcription from PCR-derived templates. Positive staining with all the antisense probes was cytoplasmic, whereas the sense probes showed no staining. Numerous tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) mRNA positive cells were observed in the marginal zone and in the red pulp of the spleen after iv LPS injections, whereas sections from saline-treated animals showed minimal cytokine mRNA expression. Cells positive for TNF-α and IL-1β mRNA were detectable in the brain after i.c.v. injections of LPS, but not after icv injection of vehicle. An antisense probe for c-*fos* was utilized in these studies as a positive control for our procedure due to its anatomically specific expression in the rat brain after LPS. In conclusion we have demonstrated that in situ hybridization with Dig-labelled RNA probes is an efficient, sensitive and reliable tool to localize cytokine mRNA production in rat tissue.

Themes: Endocrine and autonomic regulation

Topics: Neural-immune interactions

Keywords: In situ hybridization; Northern blotting; Tumor necrosis factor-alpha; Interleukin-1 beta; Floating section; Digoxigenin

1. Type of research

- Description of a protocol that is applicable to the generation of RNA probes in any system (where sequence information is known) to study the location of mRNA production and the mechanisms of cellular activation.

2. Purpose

- Determination of the role and localization of cytokines during neural inflammatory responses [8,13], fever [15] sickness behaviour [4,14,23] and the neural regulation of peripheral immune responses [6].
- Determination of how peripheral immune responses can influence cytokine production in the brain. Cytokines in the brain are reported to be induced by peripheral immune challenges [5,7,16,17,19]. What remains unclear is whether the presence of cytokines in the brain is due to infiltrating cells [11,23,24], activated brain cells

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[7,22,32], or if the cytokines cross the blood brain barrier via transport mechanisms [1] or damage [19,26].

2. Time required

- 1–11 days for preparation of animals
- 1–2 days for RNA isolation
- 1–3 days for RT-PCR (once primers are obtained)
- 3–10 days for sequencing/verification of the PCR product
- 2–3 days for in vitro transcription and quantification of RNA probe(s)
- 3–4 days for Northern blotting
- 4–6 days for in situ hybridization once tissue is collected

3. Materials

3.1. Animals

Adult male (200–400 g) Sprague–Dawley rats were obtained from Charles River, (Dorval, PQ) or Harlan Sprague–Dawley (Indianapolis, IN). Animals were fed rat chow and water ad lib and kept under standard light conditions (12 h light: 12 h darkness).

3.2. Special equipment

- Stereotaxic apparatus for surgery
- Homogenizer for RNA isolation
- PCR machine
- Primer design software (Oligo™, National Biosciences, Plymouth, MN)
- Microcentrifuge
- Variable temperature water bath
- Gel systems/boxes for DNA, RNA and sequencing gels
- Electroporater (BioRad, Mississauga, ON)
- Cryostat
- Freezing microtome
- Probe-on™ handles and slides for in situ hybridization (Fisher, Nepean, ON)

3.2.1. Special reagents for RNA isolation

- Trizol™ (Life Technologies, Burlington, ON)
- Diethylpyrocarbonate (DEPC-Sigma, Oakville, ON)

3.2.2. Special reagents for RT-PCR

- Superscript-2™ 1st strand synthesis kit (Life Technologies, Burlington, ON) or Expand™ Reverse Transcriptase (Boehringer Mannheim (BM), Laval, PQ)
- Taq Polymerase (Life Technologies, Burlington, ON)
- PCR primers were purchased from Clontech Labs (Palo Alto, CA) from amplimer kits (TNF-α and β-actin) or custom designed on Oligo™ Software (National Biosciences, Plymouth, MN) for Macintosh and obtained from Bio/Can (Mississauga, ON), see Table 1 for all primer information.
- DNA mass ladder (Life Technologies, Burlington, ON)
- Oligo (dt) primer-15-18 base pairs (Bio/Can, Mississauga, ON) if you use Boehringer Mannheim's kit.

3.2.3. Special reagents for in vitro transcription

- T7 RNA polymerase (BM, Laval, PQ)
- Digoxigenin (Dig)-UTP (BM, Laval, PQ)
- Sheep anti-Dig conjugated to alkaline phosphatase (BM, Laval, PQ)
- DEPC
- Dig nucleic acid production and detection kits (BM, Laval, PQ).

3.2.4. Special reagents for non-radioactive sequencing

- Low melting agarose (FMC, Rockland, ME)
- DNA silver staining kit (Promega, Madison, WI)
- Wizzard PCR-Purification columns (Promega, Madison, WI)

Table 1
Primer sequences, the Genbank accession number and the location of the PCR product on the sequence in question

Molecule	Primer sequences	Genebank reference	Product size (location)
TNF-α	5'-TACTGAACTTCGGGGTGATTGGTCC[a] 3'-CAGCCTTGTCCCTTGAAGAGAACC[a] T75'-TAATACGACTCACTATAGGGAGATACTGAACTTCGGGGTGATTGGTCC T73'-TAATACGACTCACTATAGGGAGACAGCCTTGTCCCTTGAAGAGAACC	X66539S40199	297 (243–537)
β-Actin[b]	5'-TTGTAACCAACTGGGACGATATGG[a] 3'-GATCTTGATCTTCATGGTGCTAGG[a] T73'-TAATACGACTCACTATAGGGAGATTCATGAGGTAGTCTGTCAGG	J00691	764 (1142–1905) 370 (1142–1509)
IL-1β	5'-CCTTGTGCAAGTGTCTGAAGCAG 3'-CTTCAAAGATGAAGGAAAAGAAGGTGC T75'-TAATACGACTCACTATAGGGAGACCTTGTGCAAGTGTCTGAAGCAG T73'-TAATACGACTCACTATAGGGAGACTTCAAAGATGAAGGAAAAGAAGGTGC	M98820	322 (166–485)
c-*fos*	5'-GCGGAGACAGATCAACTTGAAGACG 3'-GTTGCTAATGTTCTTGACCGGCTCC T73'-TAATACGACTCACTATAGGGAGAGTTGCTAATGTTCTTGACCGGCTCC	X06769	287 (633–917)

[a]These primers were obtained from Clontech's Amplimer kits.
[b]This mRNA sequence was deduced from genomic DNA sequence as specified in the Genebank accession number.

- Lone Ranger™ Gel solution (FMC, Rockland, ME)
- SigmaCote (Sigma, Oakville, ON)

3.2.5. Special reagents for radioactive sequencing
- S^{35} dATP (Mandel, Guelph, ON)
- Sequenase Version 2 kit (United States Biochemical Cleveland, OH).

3.2.6. Special reagents for blunt-end cloning
- SmaI (New England Biolabs, Mississauga, ON)
- Wizard PCR-Purification column (Promega, Madison WI)
- T4 polynucleotide kinase (Life Technologies, Burlington, ON)
- DNA polymerase I (Klenow fragment-Life Technologies Burlington, ON)
- T4 DNA ligase (BM, Laval, PQ)
- DH5-α cells (Life Technologies Burlington, ON)
- Specific restriction enzymes (see text)

3.2.7. Special reagents for Northern blotting
- Sheep anti-Dig conjugated to alkaline phosphatase or peroxidase (BM, Laval, PQ) and chemiluminescent substrates (BM, Laval, PQ).
- DEPC
- Nylon membrane (BM, Laval, PQ)

3.2.8. Special reagents for in situ hybridization
- Probe-on™ Slides and handles (Fisher, Nepean, ON)
- DEPC
- Proteinase K (BM, Laval PQ and Sigma, Oakville, ON)
- Levamisole (Sigma, Oakville ON)
- NBT (Sigma, Oakville, ON)
- BCIP (Fisher, Nepean, ON)
- Polyvinyl Alcohol (Sigma, Oakville, ON)
- Brij 35 (polyoxyethylene 23 lauryl ether, Sigma, Oakville, ON)
- RNase A (Sigma, Oakville, ON)

4. Detailed procedure

4.1. Animal preparation

Rats that received intracerebroventricular (i.c.v.) injections were prepared as follows.

(A) Anesthetized subcutaneously with a mixture (250 μl/100 g) consisting of 25% ketamine (25 mg/ml), 10% acepromazine (1 mg/ml), 25% xylazine (5 mg/ml) and 40% sterile water.

(B) Placed in the stereotaxic apparatus (David Kopf instruments, Tujunga, CA) with the incisor bar placed at 3.3 mm below the interaural line. The right lateral ventricle was reached using the coordinates from Paxinos and Watson [25]: A–P = 0.4 mm below Bregma, L = 1.4 mm, D–V = 3.8 mm below the dura as previously described [28].

(C) A 26-gauge stainless steel guide cannula was implanted close to the right lateral ventricle and secured with screws and dental cement (Plastic One, Roanoak, VA). An internal cannula (33-gauge, 1 mm projection beyond the tip of the guide cannula) was connected to the guide cannula. Animals were allowed to recover from surgery for 10 days.

(E) On the morning of the experiment, the rats were placed in individual buckets. The dummy cannula was removed and a catheter made of PE-50 tubing was attached to the internal guide cannula, which had been filled with 5 μl of either endotoxin-free water or 5 μl (100 ng/μl) of endotoxin (LPS; *E. coli* serotype 055:B5, injected over 2 min and dissolved in pyrogen free saline). Correct placement of the i.c.v. cannula was checked in each rat at the end of the assay by histological examination.

(F) Other rats were injected intravenously (i.v.) with endotoxin-free saline or 100 μg of lipopolysaccharide (dissolved in pyrogen-free saline). These animals were killed at various time points post-injection. All procedures were approved by the animal ethics committee at the University of Manitoba, the CCAC and the Salk Institute IACUC.

4.2. Isolation of RNA

RNA was isolated from rat spleen with Trizol™ reagent (Life Technologies Burlington, ON) according to the manufacturer's instructions. Briefly, the following procedures were carried out.

(A) Rat spleen was homogenized in Trizol™ reagent (1 ml of Trizol™/100 mg of tissue) and incubated at room temperature for 5 min.

(B) Two hundred microliters of chloroform was added for each ml of Trizol™; this mixture was then shaken vigorously and incubated at room temperature for 3 min.

(C) Samples were then centrifuged at 12,000 × *g* for 15 min at 4°.

(D) The aqueous phase was removed to a separate tube.

(E) Five hundred microliters of isopropanol/ml of Trizol™was then added to the aqueous phase to precipitate the RNA.

(F) Samples were mixed and then incubated at room temperature for 10 min.

(G) Samples were centrifuged at 12,000 × *g* for 15 min at 4°.

(H) The supernatants were removed and the pellets were washed in 75% Ethanol.

(I) The samples were centrifuged at 7500 × *g* for 5 min at 4°.

(J) The supernatant was aspirated with a pipet, and the residual liquid on the inside of tube was removed with a sterile cotton swab (carefully avoiding the pellet).

(K) The pellet was air dried for 5–10 min (not completely).

(L) The pellet was resuspended in Diethylpyrocarbonate (DEPC)-treated water, heated to 55°C for 10 min, and quantified by U.V. spectrophotometry at 260 and 280 nm.

4.3. First-strand cDNA synthesis and amplification

First-strand synthesis was performed with the Superscript-2™ pre-amplification system (Life Technologies Burlington, ON) or the Expand™ Reverse Transcriptase system (BM, Laval, PQ) according to the respective manufacturers' instructions. Both systems performed well, and below is the description of the protocol from Boehringer Mannheim. Briefly, the following procedures were carried out.

(A) One to three micrograms of total RNA was added to 100 pmol of Oligo (dt) primer and DEPC–water to a volume of 11 μl.

(B) The reaction was heated to 65°C for 10 min, briefly centrifuged and placed on ice. To this was added a final concentration of 1 mM nucleotides, 20 units of Rnase inhibitor, 129 × buffer and 50 units of Expand™RT enzyme. This reaction was incubated for 60 min at 42°C. These samples were either used right away for PCR or frozen at −70°C for later use.

(C) First-strand synthesis productt (0.2–2 μl) was added to a final concentration of 0.5 μm dNTP mix, 1.5 mM $MgCl_2$, *Taq* DNA polymerase (2 units), 1 × buffer and 20 pmol of each primer (see Table 1 for information) was added to a final volume of 50 μl.

(D) PCR cycling parameters were as follows: 80°C (5 min) then 35 cycles: 94°C (45 s), 60°C (45 s), 72°C (2 min) and 7-min extension at 72°C.

(E) PCR products were visualized by ethidium bromide on a 2% Agarose gel with a DNA mass ladder (Life Technologies Burlington, ON) to estimate yield.

4.4. Production of probe templates

(A) An aliquot of first-strand synthesis or first-strand amplification product (0.1–1 μl) was added to a PCR reaction tube containing sense or antisense primers with a T7 RNA polymerase promoter sequence as previously described [2] (see Table 1 for more information).

(B) Cycling parameters were identical to those mentioned above.

(C) PCR products were visualized by ethidium bromide on a 2% Agarose with a DNA mass ladder (Life Technologies Burlington, ON) to estimate yield.

4.5. Production of digoxigenin-labelled RNA probes

Dig-labelled RNA probes were produced and quantified using the Dig nucleic acid production and detection kits (BM, Laval, PQ). Briefly, the following procedures were carried out.

(A) PCR product (4–6 μl) was added to an in vitro transcription reaction containing final concentrations of 1 mM CTP, ATP, GTP, 0.65 M UTP, 0.35 M Dig-UTP, 2 units/μl of T7 RNA polymerase, 1 × buffer and 1 unit/μl RNase inhibitor. The reaction volume was brought up to 20 μl per sample and was incubated for 2 h at 37°C.

(B) One microliter (10 units/μl) of RNase-free DNase was added to each reaction tube. This reaction was incubated for 15 min at 37°C.

(C) To precipitate the probes, the following was added in order: 2 μl of 0.2M EDTA, 1 μl of molecular grade glycogen (BM, Laval, PQ), 2.5 μl of 4 M lithium chloride and 75 μl of cold ethanol. Samples were then placed at −70°C overnight.

(D) The probes were then centrifuged at 12,000 × *g* for 20 min at 4°C and rinsed in 70% ethanol.

(E) The probes were resuspended in 50–100 μl of DEPC-treated water (containing 0.2 units of Rnase inhibitor/μl water) and heated for 30 min at 37°C.

(F) Probe yield was determined by comparing serial dilutions of probe to Dig-labelled control RNA standards (BM, Laval, PQ). Samples were serially diluted (50% DEPC–water, 30% 20 × SSC, 20% formaldehyde) 1/10, spotted onto a nylon membrane (BM, Laval, PQ), and baked for 30 min at 120°C.

(G) Membranes were then blocked in 1% blocking reagent (BM, Laval, PQ) in maleic acid buffer for 30 min.

(H) Membranes were then incubated in sheep anti-Dig alkaline phosphatase-conjugated antibody (1/5000, BM, Laval, PQ) in blocking reagent/maleic acid buffer for 1 h at room temperature, rinsed 3 times in maleic acid buffer and then incubated in NBT/BCIP (0.46 mM NBT, 0.43 mM BCIP in 50 mM $MgCl_2$/100 mM Tris in 100 mM NaCl, pH 9.3) to visualize/quantitate the probe dilutions.

4.6. Verification of probe templates by dideoxy sequencing

Probe templates for c-*fos*, β-actin and TNF-α were sequenced by non-radioactive cycle sequencing, while the IL-1β cDNA fragment was blunt-end cloned into a plasmid and sequenced with S^{35} dATP. Non-radioactive cycle sequencing was performed according to the Silver Sequence DNA sequencing system (Promega, Madison, WI) and all reagents were from Promega unless otherwise specified. Radioactive sequencing was performed as described by manufacturer of the Sequenase Version 2 kit (United States Biochemical Cleveland, OH) and all reagents were from this kit unless otherwise specified.

4.6.1. Non-radioactive sequencing

(A) PCR products were eluted from a 3% low-melting agarose gel (FMC Rockland, ME), and purified by Wizard columns (Promega, Madison, WI). The yield was estimated by running an aliquot of the eluted product beside a known amount of DNA mass ladder (Life Technologies Burlington, ON).

(B) Eight to 20 ng of PCR product was added to 4.5 pmol of 3′ or 5′ primer, sequencing grade *Taq* polymerase (5 units) and 1 × buffer to a final volume of 16 μl. Four microliters of this mix was added to 4 tubes each containing 2 μl of one of the four dNTPs/ddNTPs termination mixes (included in kit).

(C) Samples were heated to 95°C for 2 min and put through 60 cycles at 95°C (30 s), 42°C (30 s), and 72°C (1 min). Three microliters of stop solution was added to each sample. Samples were run immediately or stored overnight at 4°C.

(D) The glass plates for the gel were prepared as follows: The plates were carefully cleaned with warm water and detergent, a few rinses of deionized water, wiped with an ethanol-saturated KimWipe™ and followed by an acetone-saturated KimWipe™. The short glass plate was treated with Bind Silane (from kit-for adherence of the gel to the glass plate) by diluting 1 μl of the Bind Silane in 1 ml of 95% ethanol/5% glacial acetic acid. This solution was used to saturate a KimWipe™, which was then used to apply the Bind Silane to the plate. The Bind Silane was allowed to sit on the plate for 5 min before it was gently wiped off with three separate ethanol-soaked KimWipes™. The long glass plate was coated with Sigma-Cote (Sigma, Oakville, ON) with a KimWipe™ and was allowed to sit for 10 min before being gently wiped with three separate ethanol-soaked KimWipes™.

(E) The gel (5% denaturing Long Ranger™ gel (FMC, Rockland, ME)) was prewarmed for 90 min at 95 W. The samples were heated for 2 min at 70°C and 3.5–4 μl of each sample was loaded per lane. The samples were run using a Bio-Rad power supply (Mississauga, ON) set to keep a constant temperature of 55°C (approximately 80–85 W).

(F) After the gel finished running, the plates were separated and the gel (on the short plate) was fixed in 10% glacial acetic acid for 50–60 min, and then rinsed three times in ultrapure water. All steps were carried out in large plastic trays.

(G) The gel was then stained for 50 min (2 g of silver nitrate and 3 ml of 37% formaldehyde in 2 l of ultrapure water).

(H) The gel was briefly set aside while the developer was prepared.

(I) The developing solution was prepared by adding 3 ml of 37% formaldehyde, and 500 μl of sodium thiosulfate (10 mg/ml) to the prechilled sodium carbonate solution (60 g of sodium carbonate in 2 l of ultrapure water-keep at 10°C in ice bath) right before use. Half of this solution was placed into the staining trays and used immediately for developing while the other half was kept on ice.

(J) The gel was dipped in ultrapure water and placed immediately in chilled developer. This dipping did not take longer than 10 s.

(K) The gel was agitated in developer until bands were seen. The developing solution was discarded and replaced with the 1 l that was kept on ice. Development was stopped by fixing for 2–3 min in 10% glacial acetic acid when faint bands were seen throughout the gel.

(L) The gel was rinsed twice in ultrapure water and air-dried at room temperature.

4.6.2. Blunt-end cloning and radioactive sequencing

IL-1β cDNA with a T7 RNA polymerase sequence at the 5′ end was blunt-end cloned into a SmaI (New England Biolabs, Mississauga, ON) site of a pUC 18 vector that had previously been treated with calf intestinal alkaline phosphatase (BM, Laval, PQ) to prevent self-ligation.

(A) Nine hundred nanograms of IL-1β cDNA was purified on a Wizard PCR purification column (Promega, Madison, WI) and concentrated by ethanol precipitation with 0.1 vol. of sodium acetate and 2.5 vol. of absolute ethanol.

(B) The pellet was then suspended in water, and 480 ng of sample was treated for 60 min with 5 μl (50 units) of T4 polynucleotide kinase (Life Technologies, Burlington, ON) in a final volume of 70 μl with 1 × ligation buffer (10 × buffer- 0.667 M Tris–Cl pH 7.5/0.05 M $MgCl_2$/ 0.05 M DTT and 10 mM dATP).

(C) The cDNA was purified by phenol/chloroform extraction, concentrated by ethanol precipitation and suspended in 20 μl of TE buffer.

(D) To blunt the 3′ end of the IL-1β cDNA, 16 μl (approximately 380 ng) of the kinased product was added to 2 μl of 10 × blunt ligation buffer (BLB) (0.5 M Tris–Cl pH 7.2/ 0.1 M $MgCl_2$) and 5 units of DNA polymerase fragment (Klenow, from Life Technologies Burlington, ON), the solution was gently mixed (to avoid dissociation of the Klenow) and incubated for 2 min at room temperature.

(E) To fill in the 5′ ends, 1 μl of 2 mM dNTPs were added to the above mixture for 30 min at room temperature.

(F) Three hundred nanograms of plasmid (0.5 μl), 0.5 μl of 50 mM dATP, 0.5 μl of 250 mM DTT and 5 Weiss units of T4 DNA ligase (Boehringer Mannheim, Laval, PQ) were added to this mixture and incubated overnight at 12–15°C.

(G) This product was precipitated, suspended in 20 μl of TE buffer, and a 2 μl aliquot was transformed into 50 μl of electroporation competent DH5-α cells (Life Technologies, Burlington, ON) using a Bio-Rad GenePulser™ with pulse controller set at 1.25 kV, 25 μFD, 400 Ω in a 0.1-cm electroporation cuvette (Bio-Rad Mississauga, ON).

(H) The transformed cells were grown overnight at 37°C on LB plates containing 20 μg/ml carbenicillin and 70 μl of 25 mg/ml 5 bromo-4 chloro-3-indolyl β-D-galactopyranoside (X-Gal) (American Biorganics, Niagara Falls, NY).

(I) Positive colonies (white) were placed in 2 ml of LB medium containing 50 μg/ml ampicillin and grown overnight at 37°C.

(J) Plasmids were isolated and purified as described by Birnboim and Doly [3] with slight modifications.

(K) Verification of successful cloning was made by linearizing the plasmid with a restriction enzyme site unique to the IL-1β insert (2 units) (Bse-R1, New England Biolabs, Mississauga, ON) and by cutting out the inserted IL-1β from the plasmid with *Bam*HI (10 units) (New England Biolabs, Mississauga, ON) and *Eco*RI (5 units) (Life Technologies, Burlington, ON).

(L) The plasmid miniprep (50 μl) was prepared for sequencing by treatment with RNase A (2 μl of 1 μg/μl stock solution-Sigma, Mississauga, ON) for 30 min at 37°C and precipitation with 33 μl of 2.5 M NaCl/20% polyethylene glycol 8000 (Sigma, Mississauga, ON) for 1 h on ice. The plasmid was resuspended in 20 μl of TE buffer and denatured in a final concentration of 0.2 M NaOH at room temperature for 5 min.

(M) The plasmid was precipitated with 8 μl of 5 M ammonium acetate and 100 μl of ethanol at −20°C for 1 h, and then resuspended in 7 μl of water. To this, 2 μl of buffer and 1 μl of primer (0.5 pmol of IL-1β 5′ primer) were added.

(N) The samples were heated to 65°C for 2 min, and the tubes allowed to gradually cool to room temperature over 30 min. The samples were then placed on ice.

(O) To this reaction we added (in order) 1 μl of 0.1 M DTT, 2 μl of diluted labelling mix, 0.5 μl of S^{35} dATP (Mandel, Guelph, ON) and 2 μl of diluted Sequenase enzyme. The samples were mixed thoroughly and incubated for 5 min at room temperature.

(P) A portion of this mix (3.5 μl) was then added to 4 pre-heated tubes each containing 2.5 μl of one of the four termination mixes.

(Q) Samples were incubated for 5 min at 37°C.

(R) Four microliters of stop solution to was then added to each of the samples.

(S) The samples were heated for 2 min at 80°C and loaded (2.5 μl per lane) onto a 6% denaturing acrylamide gel and run for 3.5 h at 2000 V. The gel was dried and exposed to film overnight.

4.7. Northern blotting

Briefly, the following procedure was performed according to Meltzer et al. [21]:

(A) Total RNA from spleen (10 μg per sample) was denatured (50% formamide/MOPS/17.5% formaldehyde/10% Glycerol and Ethidium bromide) for 10 min at 65°C.

(B) The samples were then loaded on a 1.2% agarose/formaldehyde gel and run for 3 h at 50 V.

(C) The samples were transferred to nylon membrane overnight (BM, Laval, PQ) by capillary action with SSC buffers.

(D) RNA was fixed on the membrane with a 2-h incubation at 80°C, and the integrity of the samples was verified by visualizing the ribosomal RNA subunits under UV light.

(E) The membranes were equilibrated in 2 × SSC and placed in pre-hybridized solution (50% formamide, 5 × SSC, 0.05 M sodium phosphate 0.1% SDS, Denhart's solution and dextran sulfate) at 42°C for 1 h.

(F) Membranes were then hybridized overnight at 60°C with Dig-labelled RNA probes (1–30 ng/ml depending on the probe).

(G) Membranes were washed two times in 2 × SSC/0.1% SDS for 15 min at room temperature and two times in 0.1 × SSC/0.1% SDS at 60°C for 25 min.

(H) Membranes were blocked for 1 h in 1% Boehringer block/ maleic acid buffer, incubated with sheep anti-Dig peroxidase-conjugated antibody (1/5000, BM, Laval, PQ) for 1 h at room temperature, rinsed 3 × in maleic acid buffer, and visualized using the Boehringer Mannheim chemiluminescence kit (Laval, PQ).

(I) The blots were exposed to film anywhere from 15 s to 5 min. All solutions were treated with 0.1% DEPC as required.

4.8. In situ hybridization for adhered spleen sections

Protocols were performed as previously described by Birk and Grimm [2], with modifications.

(A) Rats were overdosed with pentobarbitol and transcardially perfused with 100 ml of 1% sodium nitrite in phosphate buffer (PB) followed by 300 ml of 4% buffered paraformaldehyde (pH 7.3) in PB (for these steps, 0.1% DEPC was added immediately before use).

(B) The tissue was removed, post-fixed for 2 h and then cryoprotected in 30% sucrose (0.1% DEPC was added immediately before use). Once the spleens sank to the bottom of the glass bottle (1–2 days) they were embedded in Tissue Tek O.C.T. mounting compound (Immucor, Edmonton, AB) and stored at −70°C.

(C) Sections were cut (15 μm) on a freezing microtome, floated onto silanated Probe-On slides (Fisher), air-dried for 20 min at room temperature, and baked for 1 h at 60°C.

(D) Following this, sections were fixed for 20 min in cold 4% paraformaldehyde, rinsed 3 × in PBS, 1 × in 50% ethanol and stored dessicated at −70°C. For these steps, the solutions were made with 0.1% DEPC-treated water and molecular grade chemicals that were weighed using only baked utensils. DEPC (0.1%) was added again immediately before use.

(E) All buffers up to the RNase A step were made with DEPC-treated water and molecular grade chemicals that were weighed using only baked utensils. These buffers were then DEPC-treated and autoclaved. If the buffers contained Tris, DEPC was not added before autoclaving.

(F) Sections were gradually brought to room temperature and rinsed in PBS/0.1% Brij (Brij 35-polyoxyethylene 23 lauryl ether, Sigma, Oakville, ON).

(G) Sections were treated with 5 μg/ml of proteinase K (Boehringer Mannheim, Laval, PQ)/0.1% Brij in 0.1 M Tris/50 mM EDTA (pH 8.3 at 25°C) for 30 min at 37°C.

(H) The sections were refixed for 5 min in 4% paraformaldehyde/fresh DEPC.

(I) The sections were then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.1% Brij, pH 8.0, for 10 min, soaked in methanol for 5 min, and air-dried.

(J) Following this, the sections placed in prehybridization buffer (2 × SSC, 50% formamide, 1 mM Tris pH 7.5, 1 × Denhart's, 5% dextran and 1 mM EDTA) for 1 h at 37°C.

(K) Sections were then hybridized overnight at 50°C in prehybridization buffer with 400 pg to 1 ng of probe/μl of solution.

(L) Excess probe was removed with 2 × SSC/0.1% Brij (up to this point all solutions DEPC-treated and prepared with baked glassware).

(M) Sections were treated with 20 μg/ml RNase A (BM, Laval, PQ) in 2 × SSC/0.1% Brij, and rinsed consecutively in 2 ×, 1 × and 0.1 × SSC (5 washes each solution). All solutions contained 0.1% Brij.

(N) Sections were rinsed 5 times with 2 × SSC/Brij, followed by 5 rinses in sodium phosphate buffer/Brij.

(O) Sections were blocked for 1 h in 1% Boehringer block in maleic acid buffer/0.1% Brij and incubated with a 1/500 dilution of sheep anti-Dig alkaline phosphatase-conjugated antibody (BM, Laval, PQ) overnight at 4°C.

(P) Sections were rinsed 5 times in sodium phosphate buffer/0.1% Brij and then rinsed in 5 times in detection solution (50 mM $MgCl_2$/100 mM Tris in 100 mM NaCl, pH 9.3).

(Q) Sections were then developed in detection solution containing 0.4 mM NBT, 0.4 mM BCIP and 3 mM levamisole with 10% polyvinyl alcohol (PVA) (Sigma, St. Louis MO) for 1–5 h. The 10% PVA solution was made by dissolving 10% w/v PVA in detection solution at 90°C until the solution was clear. Once the solution was cooled down, the NBT and BCIP were added.

(R) Sections were then coverslipped in glycerol gel (50% glycerol/ 7.5% gelatin/ 0.1% azide in 0.1 M PB).

4.9. In situ hybridization for floating brain and spleen sections

The procedure was the same as outlined above for adherent section ISH, except that sections were cut at 50 μm on a freezing microtome and processed immediately. Sections were floated in 24-well culture plates throughout the procedure, treated with lower concentration of proteinase K (BM, Laval, PQ) (0.5–1 μg/ml) and the steps using methanol, ethanol, triethanolamine/acetic acid and refixing were omitted. The antibody step used 1% Triton-X 100 instead of 0.1% Brij, and the sections were developed in detection solution without polyvinyl alcohol. Sections were then floated onto coated slides and coverslipped with glycerol gel.

4.10. In situ hybridization for adhered brain sections

(A) Animals were deeply anesthetized intraperitoneally with 35% chloral hydrate and perfused via the ascending aorta with saline followed by cold 4% paraformaldehyde in 0.1 M borate buffer, pH 9.5.

(B) Brains were post-fixed for 3–4 h, and then transferred to 10% sucrose/4% paraformaldehyde/0.1 M borate buffer overnight at 4°C.

(C) Frozen sections were cut (30 μm) using a Histoslide microtome, collected in cryoprotectant (0.05 M sodium phosphate buffer, 30% ethylene glycol, 20% glycerol), and stored at −20°C until histochemical analysis.

(D) Prior to hybridization, tissue sections were mounted onto gelatin and poly-L-lysine coated slides, air-dried, and stored under a vacuum overnight.

(E) The slides were initially washed in PBS/0.1% Brij, pH 7.4, then treated with 5 μg/ml of proteinase K (EM Science, Gibbstown, NJ) in 0.1 M Tris pH 8.0, 50 mM EDTA/0.1% Brij for 20 min at 37°C.

(F) The tissue was refixed for 5 min in 4% paraformaldehyde freshly treated with 0.1% DEPC, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.1% Brij, pH 8.0, for 10 min, soaked in methanol for 5 min, and air-dried.

(G) The slides were treated in prehybridization mixture (50% formamide, 2 × SSC, 1 mM EDTA, 1 mM Tris pH

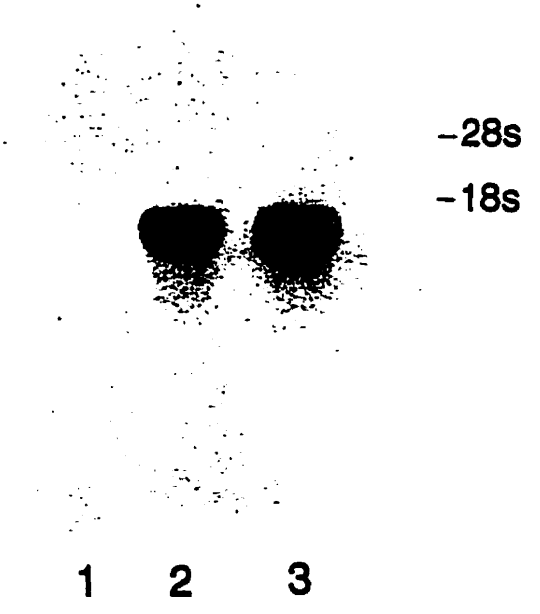
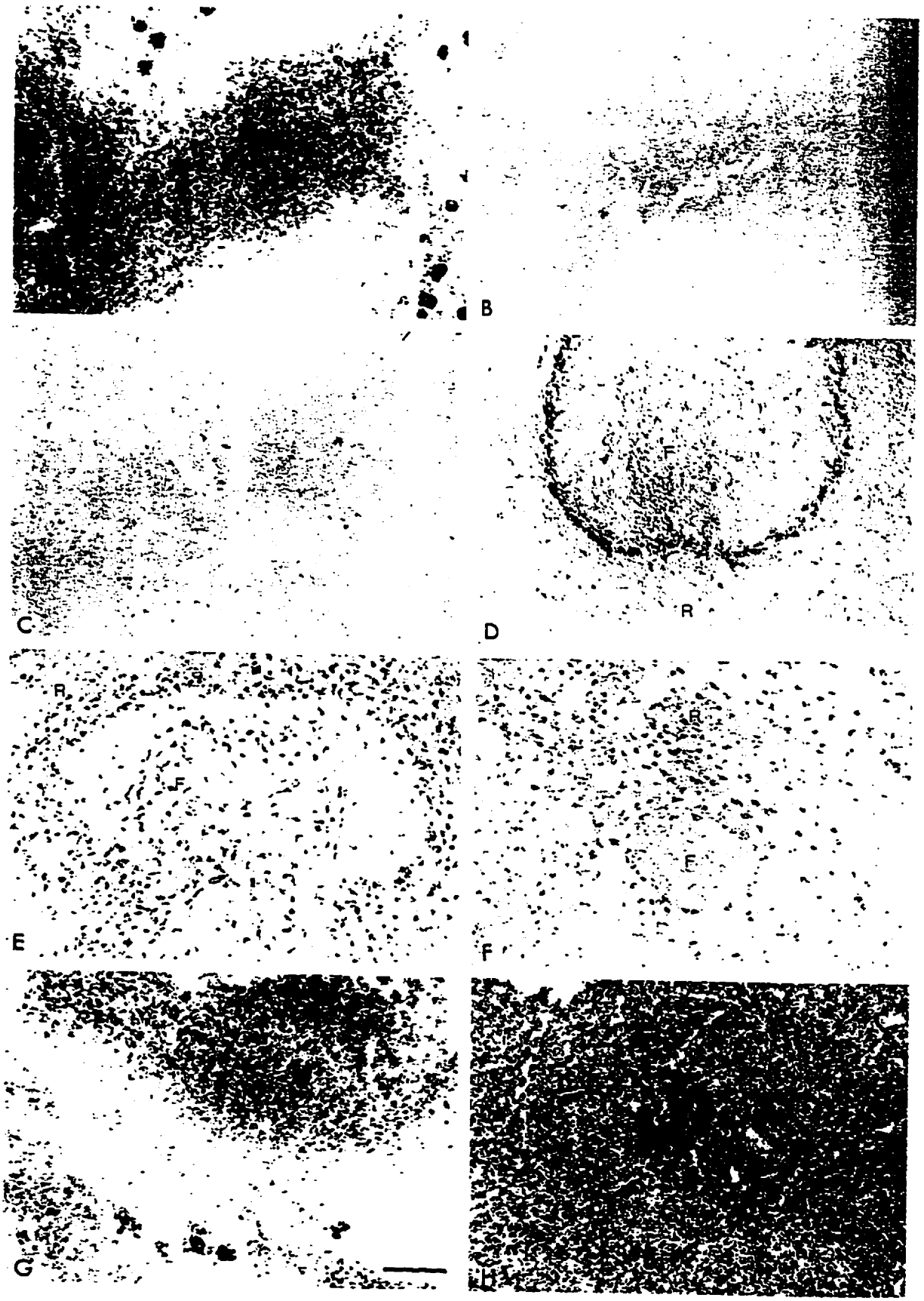


Fig. 1. Photograph illustrating a representative verification of RNA probes by Northern blotting. This blot was probed 10 ng/ml of the IL-1β RNA probe. Lane 1 contains 10 μg of total RNA from a saline-treated rat spleen, while lanes 2 and 3 contain 10 μg of total RNA from LPS-treated (1 h) rats. Abbreviations: 28S and 18S refer to the ribosomal RNA subunits which are approximately 4718 and 1874 bp in length, respectively.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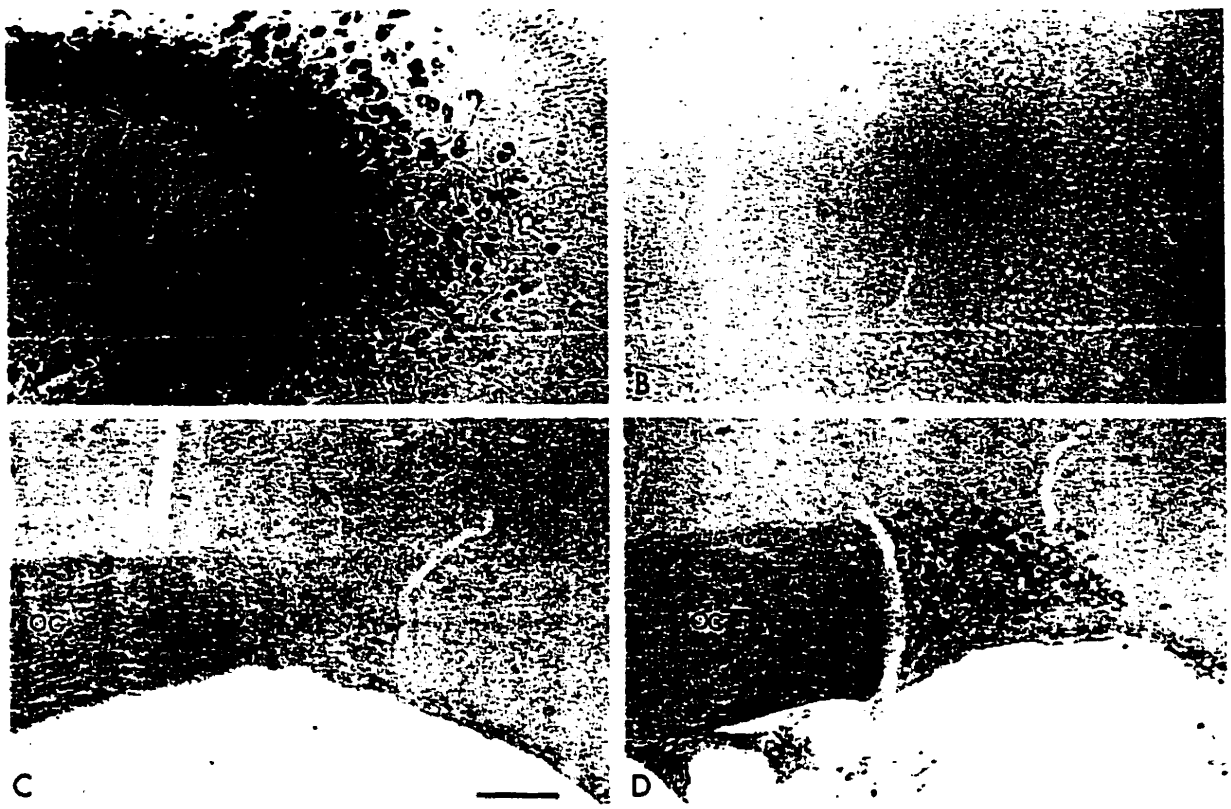

Fig. 3. A photomicrograph illustrating mRNA expression for β-actin and c-*fos* in the rat brain. (A) β-Actin mRNA expression in the CA3 region of the hippocampus of a saline-treated rat. (B) A section of brain from the same animal as (A) hybridized with a sense probe. (C) The supraoptic nucleus from the same animal as (A) hybridized with an antisense c-*fos* probe. (D) The supraoptic nucleus of an LPS-treated (1 h) rat hybridized with an antisense of c-*fos* probe. Abbreviations: optic chiasm (OC). Scale bar = 100 μm.

7.3, 1 × Denhardt's, 0.1 mg/ml yeast tRNA, 5% dextran sulfate) for 30 min at 37°C and then air-dried for about 5 min.

(H) Digoxigenin-labelled TNF-α or IL-β RNA probes (1 ng/μl final concentration) in prehybridization mixture were applied to each slide and then coverslipped. The slides were incubated at 95°C for 5 min, then allowed to hybridize overnight at 50°C.

(I) The coverslips were removed by gentle soaking in 2 × SSC/0.1% Brij and the sections were treated with 20 μg/ml RNase A (Sigma, St. Louis, MO) in 2 × SSC/Brij buffer for 30 min at 37°C.

(J) The slides were washed consecutively in 1 × SSC/0.1% Brij, 0.1 × SSC/0.1% Brij, and 2 × SSC/0.1% Brij, rinsed in a 10 mM NaPhosphate/0.1% Brij, and then blocked for 1 h in a 1% Boehringer block solution dissolved in 0.1 M maleic acid, 0.15 M NaCl/0.1% Brij.

(K) The slides were air-dried for about 5 min, then incubated overnight at 4°C with sheep anti-Dig alkaline phosphatase-conjugated antibody (1/250; BM, Indianapolis, IN) under coverslips.

(L) The coverslips were removed by gentle soaking in the 10 mM NaPhosphate/Brij buffer. Slides were air-dried for about 5 min and then developed in detection solution (10% Polyvinyl alcohol, 1 mM levamisole, 0.46 mM NBT, 0.43 mM BCIP in 50 mM $MgCl_2$/100 mM Tris in 100 mM NaCl, pH 9.3).

(M) The slides were coverslipped and incubated from 4 h to overnight at 30°C. The color reaction was stopped

Fig. 2. Photomicrographs demonstrating in situ hybridization for TNF-α, IL-1β and β-actin mRNA in rat spleen. (A) A floating section of spleen from a saline-treated rat hybridized with an antisense probe for β-actin. (B) A floating spleen section from an LPS-treated (1 h) rat hybridized with a sense probe for TNF-α. (C) A floating spleen section from a saline-treated rat hybridized with an antisense TNF-α probe. (D) A floating spleen section from an LPS-treated (1 h) rat hybridized with an antisense probe for IL-1β. (E) A floating spleen section from an LPS-treated (1 h) rat hybridized with an antisense probe for TNF-α. (F) An adhered section of spleen from an LPS-treated (1 h) rat hybridized with an antisense TNF-α probe. (G) A floating section of spleen from an LPS-treated (1 h) rat hybridized with a β-actin antisense probe. (H) Demonstrates an adhered spleen section from an LPS-treated (1 h) rat hybridized with a β-Actin antisense probe. Abbreviations: follicle (F), red pulp (R). Scale bar = 100 μm.

when the desired intensity was reached by soaking off the coverslips using a 10 mM Tris, pH 8.0/1 mM EDTA buffer.

(N) The sections were coverslipped in glycerol gel and examined microscopically. Placement of the i.c.v. cannulae was verified histologically, and only the rats with correct placement were included in the analysis of the results.

5. Results

5.1. Production of probes

Single band purity was obtained from the PCR reaction and purified by a combination of ethanol precipitations and gel purifications. Verification for the successful incorporation of the T7 polymerase promoter sequence into the PCR product was assessed by migration on an agarose gel as previously described [2], while verification of the cDNA templates were made by dideoxy sequencing. The specificities of all the Dig-labelled RNA probes were demonstrated by Northern blotting to total splenic RNA from saline and LPS-treated rat spleens as illustrated for the IL-1β probe (Fig. 1). Similar results were obtained with the TNF-α probe (data not shown). The sense RNA probes did not bind to the RNA from stimulated or control animals, while the β-actin probe bound in all lanes (data not shown).

5.2. Detection of TNF-α, IL-1β and β-actin mRNA in the spleen by ISH

Spleen sections from saline-treated rats showed very few TNF-α positive cells (Fig. 2C), and the TNF-α sense probe gave no positive staining (Fig. 2B). β-actin staining was strong in the spleens from both LPS (1 h post i.v. injection of LPS)- and saline-treated animals (Fig. 2A,G,H). IL-1β and TNF-α mRNA expression was detectable in LPS-treated animals mainly in the red pulp and marginal zones, with a few cells in the follicles (Fig. 2D,E,F). For TNF-α and β-actin mRNA detection, both floating and adhered sections of spleen were used. In general, the

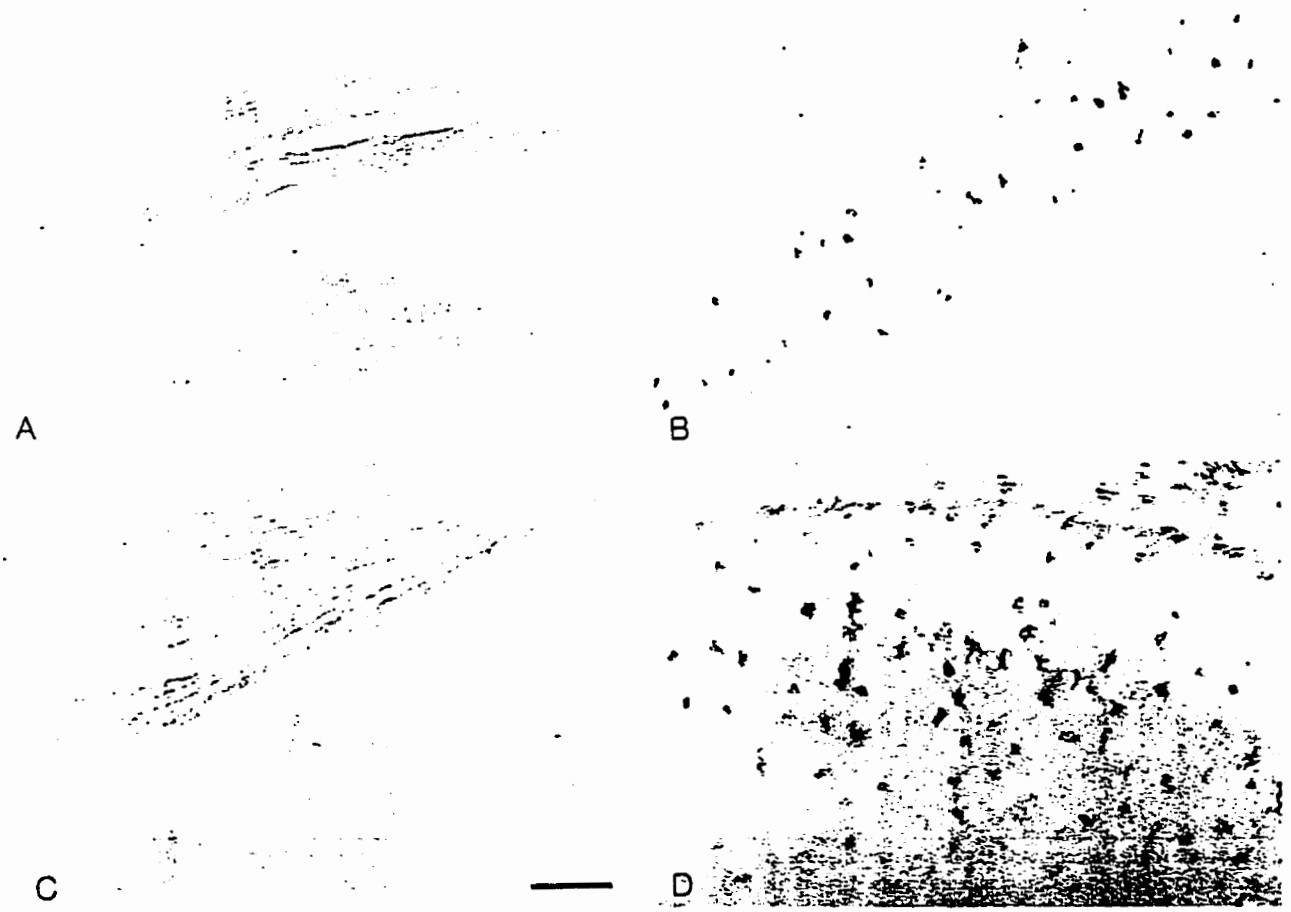

Fig. 4. Photomicrograph of TNF-α and IL-1β mRNA detection in the brains of rats injected i.c.v. with vehicle or LPS (500 ng). (A) Demonstrates the absence of cells positive for TNF-α mRNA in the hippocampus of a rat given i.c.v. vehicle (B) Demonstrates cells positive for TNF-α mRNA in the hippocampus of a rat 3 h following i.c.v. LPS. (C) Demonstrates the absence of cells positive for IL-1β mRNA in the hippocampus of a rat given i.c.v. vehicle. (D) Demonstrates cells positive for IL-1β mRNA in the hippocampus of a rat 3 h following i.c.v. LPS. Scale bar = 100 μm.

staining for TNF-α and β-actin mRNA in adhered sections was more intense after the same development time than the floating sections (Fig. 2F,H vs. Fig. 2E,G).

5.3. Detection of c-fos, β-actin, TNF-α and IL-β mRNA by ISH in the rat brain

Both LPS- and saline-treated rats showed strong β-actin staining, especially in the CA3 region of the hippocampus (Fig. 3A), while the sense probe showed no staining (Fig. 3B). We found that the level of β-actin mRNA was much more abundant than the other mRNAs examined in this study based on the much shorter chromogenic development required to generate a strong signal (i.e., 90 min vs. 5 h in spleen sections). A minimal number of cells positive for c-*fos* mRNA were observed in the paraventricular nucleus (data not shown) and the supraoptic nucleus (Fig. 3C) of the hypothalamus in saline-treated rats. In contrast, the paraventricular nucleus (data not shown) and the supraoptic nucleus (Fig. 3D) showed numerous c-*fos* positive cells following i.v. LPS injection. To demonstrate that cytokine mRNA can be induced in the brain, animals received an i.c.v. injection of vehicle or LPS (500 ng). Results demonstrate that in the hippocampus of saline-treated rats, there is no positive staining for either TNF-α or IL-β mRNA (Fig. 4A,C). In contrast, TNF-α and IL-1β mRNA was detectable by ISH in the hippocampus of rats 3 h after an i.c.v. injection of LPS (Fig. 4B,D).

6. Discussion

6.1. Troubleshooting

We have provided a detailed protocol for the production, verification and functional testing of TNF-α, IL-1β, β-actin and c-*fos* RNA probes using RT-PCR, PCR, dideoxy sequencing and Northern blotting. Although the production of RNA probes are labour-intensive relative to oligo probes, the benefits of these probes are their increased sensitivity relative to DNA probes [9,27,29], and the ability to eliminate non-specific binding by RNase treatment. Although there are many steps during probe production/application, where difficulties may arise, the following list represents the problems most commonly encountered in our experience.

(A) Isolating PCR fragments from gels was difficult with most commercial kits unless low melting agarose was used. However, we have had good success using the method of Girvitz et al. [12] for our subsequent band elutions. This provides a quick, reliable and inexpensive method for DNA isolation.

(B) Making RNA probes is a delicate procedure where the use of RNase free equipment and reagents are essential. Therefore, all the reagents must be molecular grade and nuclease free and all the glassware/metallic instruments must be baked for 8 h at 180°C or at the very least rinsed with chloroform or DEPC-treated water prior to use. Alternatively, sterile plasticware is assumed to be RNase-free and can be used in place of glass for some steps. It is also important that once the probes are in vitro transcribed, they are dissolved immediately in DEPC–water. We have found that the longer the drying time, the more difficult these probes are to dissolve. A 30-min incubation at 37–60°C may help dissolve probes that otherwise will not go into solution.

(C) We used both radioactive and non-radioactive sequencing methods with good success. The non-radioactive protocols may need more fine-tuning than the radioactive protocol in terms of the development/visualization steps; however, it is a viable alternative for labs that want to avoid the use radioactive nucleotides. Critical parameters include using the best quality water (double-distilled or Nanopue™), pre-chilling the developing reagent and only taking 5–10 s between removing the gel in the last rinse, and placing it in developing solution. Failure to adhere to these steps may result in high backgrounds or the absence of bands altogether. Also, we used 1/3 of the Bind Silane suggested in the manual, as we had problems with the plates sticking together. It was important to change gloves between applying the Bind Silane and Sigmacote, as a cross-contamination caused the gel to stick to both glass plates. We also observed that the sequencing of PCR products required fine tuning for the different template/primer combination; however, if the DNA fragment was first cloned into the plasmid, the conditions were uniform for all sequences. Sequencing the PCR products was more rapid than going through the cloning procedure, and is especially effective if the identity of the DNA fragment just needs to be verified.

(D) For Northern blotting, it is essential that all the equipment and solutions are RNase-free and that the pH of the buffers and the gel mix are correct. If not, this may cause sample degradation or poor transfer of the RNA to the membrane. Also, when testing new probes, we found that too much probe and/or too low a hybridization temperature results in too strong a signal, or a non-specific signal at the locations of the 28S and 18S ribosomal RNA subunits. Therefore, it is helpful to know the approximate size of your transcript of interest and to include known positive and negative samples to determine if you are getting specific hybridization.

(E) Penetration of the probe through the cellular matrix to hybridize to target mRNA is one of the most critical steps for in situ hybridization. The optimization of this step is dependent on both the amount of tissue fixation, and on the amount of tissue digestion; two factors that are in direct opposition. Although the optimal method of fixation may differ between experimental protocols and types of tissue, fixation should generally function to preserve tissue morphology and RNA retention without eliminating probe accessibility. Tissue digestion/degradation with proteinase

K or other substances (trypsin, HCl) should eliminate enough of the cellular/nuclear proteins, so the target sequence is open for hybridization, while leaving enough of the tissue matrix intact so that the RNA is not lost during the procedure. In our experience, we have noticed that both overdigestion and underdigestion can cause excessive background or a loss of signal depending on the circumstances. Therefore, it is very important to optimize the digestion/fixation parameters for each tissue before beginning this procedure on critically important samples. It is also important where possible to utilize conditions that provide known positive and negative controls to further verify the functional specificity of the probes in question. These controls can include hybridizing your probe to a section pre-digested with RNase, using a sense probe for the molecule of interest, unrelated sense probes, probes for housekeeping genes, unrelated antisense probes with known anatomical specificities, sections that are exposed to the detection procedure only, and tissues that are known to be positive and negative for your probe(s) of interest. This study utilized in situ hybridization for c-*fos* mRNA in the brain as a positive control because it is a well characterized gene that is inducible in distinct anatomical regions of the brain after LPS, and thus provides a more accurate positive control for the sensitivity of ISH protocols in the brain, because it is not as abundantly or ubiquitously expressed as β-actin.

6.2. Alternative protocols

We also determined if Dig-labelled RNA probes could be used in floating tissue sections. Floating section ISH has been described with radioactive [20] and non-radioactive probes [18,31]. The potential advantages of floating section ISH is that there is minimal background hybridization [20], detection of multiple mRNAs and proteins is possible [30,31], and that floating sections have superior tissue morphology (personal observations). We attribute the superior tissue morphology of the floating sections due to the floating sections being thicker, receiving less proteinase K digestion and the absence of the ethanol, and methanol steps that the adhered sections received. In agreement with LeGuellec et al. [18], we found that the chromogenic reaction for mRNA localization was less intense in the floating sections compared to the adhered sections. We attribute this to two things. First, the NBT/BCIP reaction with the adhered sections was intensified by the addition of polyvinyl alcohol [10], a step which was omitted in floating sections because it nonspecifically overdeveloped the floating sections (personal observations). Second, a higher concentration of proteinase K (5 μg/ml) was used for adhered sections (in comparison with 0.5 μg/ml for floating sections) which potentially provided more access for the RNA probes to hybridize to the mRNAs. Omitting proteinase K pre-treatment resulted in a significantly weaker positive signal, while using too much proteinase K on the floating sections caused the tissue to either disintegrate or fold on itself. We also observed that the antisense probe for β-actin demonstrated strong staining in the white pulp and identified either large cells, or compact clusters of cells that were more intensely stained than the rest of the section (Fig. 2A,G,H). A negative aspect associated with the floating section procedure was that more probe solution was required to keep the sections properly suspended during hybridization. However, these probes can be reused with satisfactory results (unpublished observations).

7. Quick procedure

- For probe production and positive controls harvest tissue that will contain message of interest. For in situ hybridization fix the tissue in paraformaldehyde.
- Isolate RNA from tissue of interest.
- Do RT-PCR to obtain template.
- Purify template and sequence.
- Demonstrate the specificity of the probe(s) by Northern blotting.
- Perform in situ hybridization with appropriate controls.

8. Essential references

Northern blotting [21]
In situ hybridization [2,9,10]
Floating section in situ hybridization [18,20,30,31]

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References

[1] W.A. Banks, A.J. Kastin, R.D. Broadwell, Passage of cytokines across the blood–brain barrier, Neuroimmunomodulation 2 (1995) 241–248.
[2] P.E. Birk, P.C. Grimm, Rapid nonradioactive in situ hybridization for interleukin-2 mRNA with RNA probes generated using the polymerase chain reaction, J. Immunol. Methods 167 (1994) 83–89.
[3] H.C. Birnboim, J. Doly, A rapid alkaline extraction procedure for screening recombinant plasmid DNA, Nucleic Acids Res. 7 (1979) 1513–1523.
[4] R.M. Bluthe, M. Pawlowski, S. Suarez, P. Parnet, Q. Pittman, K.W. Kelley, R. Dantzer, Synergy between tumor necrosis factor alpha and interleukin-1 in the induction of sickness behavior in mice, Psychoneuroendocrinology 19 (1994) 197–207.
[5] C.D. Breder, C. Hazuka, T. Ghayur, C. Klug, M. Huginin, K. Yasuda, M. Teng, C.B. Saper, Regional induction of tumor necrosis

factor alpha expression in the mouse brain after systemic lipopolysaccharide administration, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 11393–11397.
[6] R. Brown, Z. Li, C.Y. Vriend, R. Nirula, L. Janz, J. Falk, D.M. Nance, D.G. Dyck, A.H. Greenberg, Suppression of splenic macrophage interleukin-1 secretion following intracerebroventricular injection of interleukin-1 beta: evidence for pituitary–adrenal and sympathetic control, Cell. Immunol. 132 (1994) 84–93.
[7] M. Buttini, H. Boddeke, Peripheral lipopolysaccharide stimulation induces interleukin-1 beta messenger RNA in rat brain microglial cells, Neuroscience 65 (1995) 523–530.
[8] I.L. Campbell, M. Eddleston, P. Kemper, M.B.A. Oldstone, M.V. Hobbs, Activation of cerebral cytokine gene expression and its correlation with onset of reactive astrocyte and acute-phase response gene expression in scrapie, J. Virol. 68 (1994) 2383–2387.
[9] K.H. Cox, D.V. DeLeon, L.M. Angerer, R.C. Angerer, Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes, Dev. Biol. 101 (1984) 485–502.
[10] M. De Block, D. Debrouwer, RNA-RNA in situ hybridization using Digoxigenin-labelled probes: The use of high-molecular-weight polyvinyl alcohol in the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction, Anal. Biochem. 215 (1993) 86–89.
[11] Z. Fabry, C.S. Raine, M.N. Hart, Nervous tissue as an immune compartment: The dialect of the immune response in the CNS, Immunol. Today 15 (1994) 218–224.
[12] S.C. Girvitz, S. Bacchetti, A.J. Rainbow, F.L. Graham, A rapid and efficient procedure for the purification of DNA from agarose gels, Anal. Biochem. 106 (1980) 492–496.
[13] G.A. Higgins, J.A. Olschowka, Induction of interleukin-1B mRNA in the adult rat brain, Mol. Brain. Res. 9 (1991) 143–148.
[14] S. Kent, R.M. Bluthe, R. Dantzer, A.J. Hardwick, K.W. Kelley, N.J. Rothwell, J.L. Vannice, Different receptor mechanisms mediate the pyrogenic and behavioral effects of interleukin-1, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 9117–9120.
[15] M.J. Kluger, W. Koak, L.R. Leon, D. Soszynski, C.A. Conn, Cytokines and fever, Neuroimmunomodulation 2 (1995) 216–223.
[16] S. Laye, R.M. Bluthe, S. Kent, C. Combe, C. Medina, P. Parnet, K. Kelley, R. Dantzer, Subdiaphragmatic vagotomy blocks induction of IL-1 beta mRNA in mice brain in response to peripheral LPS, Am. J. Physiol. 268 (1995) R1327–R1331.
[17] S. Laye, P. Parnet, E. Goujon, R. Dantzer, Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice, Mol. Brain. Res. 27 (1994) 157–162.
[18] D. LeGuellec, A. Trembleau, C. Pechoux, F. Gossard, F. Morel, Ultrastructural non-radioactive in situ hybridization of GH mRNA in rat pituitary gland: Pre-embedding vs. ultra-thin frozen sections vs. post-embedding, J. Histochem. Cytochem. 40 (1992) 979–986.
[19] L. Liu, T. Kita, N. Tanaka, Y. Kinoshita, The expression of tumor necrosis factor in the hypothalamus after treatment with lipopolysaccharide, Int. J. Exp. Pathol. 77 (1996) 37–44.
[20] W. Lu, S.N. Haber, In situ hybridization histochemistry: a new method for processing material stored for several years, Brain Res. 578 (1992) 155–160.
[21] Meltzer, J.C., Sanders, V., Grimm, P.C., Chiasson, N., Hoeltke, H.J., Garrett, K.L., Greenberg, A.H., Nance, D.M., Non-radioactive Northern blotting with biotinylated and digoxigenin-labelled RNA probes, Electrophoresis (in press).
[22] C.N. Montero-Menei, L. Sindji, E. Garcion, M. Mege, D. Couez, E. Gamelin, F. Darcy, Early events of the inflammatory reaction induced in rat brain by lipopolysaccharide intracerebral injection: relative contribution of peripheral monocytes and activated microglia, Brain Res. 724 (1996) 55–66.
[23] T. Oka, K. Oka, M. Hosoi, S. Aou, T. Hori, The opposing effects of interleukin-1 beta microinjected into the preoptic hypothalamus and ventromedial hypothalamus on nociceptive behaviour in rats, Brain Res. 700 (1995) 271–278.
[24] T. Owens, T. Renno, V. Taupin, M. Krakowski, Inflammatory cytokines in the brain: does the CNS shape immune response?, Immunology 15 (1994) 566–571.
[25] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, 2nd edn., Academic Press, Orlando, 1986.
[26] V.J. Quagliarello, B. Wispelwey, W.J. Long Jr., W.M. Scheld, Recombinant human interleukin-1 induces meningitis and blood brain barrier injury in rats, J. Clin. Invest. 87 (1991) 1360–1366.
[27] B. Rueger, J. Thalhammer, I. Obermaier, S. Gruenwald-Janho, Experimental procedure for the detection of a rare human mRNA with the Dig system, Biochemica 3 (1996) 35–38.
[28] S. Rivest, G. Torres, C. Rivier, Differential effects of central and peripheral injection of interleukin-1 beta on brain c-*fos* expression and neuroendocrine functions, Brain Res. 587 (1992) 13–23.
[29] R.A.K. Srivastava, G. Schonfeld, Use of riboprobes for Northern blotting, Biotechniques 11 (1991) 584–587.
[30] A. Trembleau, F.E. Bloom, Enhanced sensitivity for light and electron microscopic in situ hybridization with multiple simultaneous non-radioactive oligodeoxynucleotide probes, J. Histochem. Cytochem. 43 (1995) 829–841.
[31] A. Trembleau, D. Roche, A. Calas, Combination of non-radioactive and radioactive in situ hybridization with immunohistochemistry: a new method allowing simultaneous detection of two mRNAs and one antigen in the same brain tissue section, J. Histochem. Cytochem. 41 (1993) 489–498.
[32] A.M. van Dam, M. Brouns, S. Louisse, F. Berkenbosch, Appearance of interleukin-1 in macrophage and in ramified microglia in the brain of endotoxin-treated rats: a pathway for the induction of non-specific symptoms of sickness?, Brain Res. 588 (1992) 291–296.