Chemical Specificity of Endotoxin–Induced *c*-fos Expressing Neurons in the Rat Hypothalamus

A Thesis presented to the University of Manitoba

In Partial Fufillment of the Requirements for the Degree

Master of Science

in

Physiology

by

Andora T. K. Jackson

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Acknowledgements and Dedication

First and foremost I would like to thank my advisor, Dr. Dwight Nance, for his guidance, support and exceptional patience while I have completed this thesis. His open and collegial manner made my experience as a graduate student a fulfilling one and I am honoured to have worked in his lab. I would have loved to stay for a Ph.D., but the rat allergy would probably kill me.

I also thank my committee, Drs. Arnold Greenberg and James Nagy, for their advice during my program and for their helpful critical comments in reviewing my thesis.

I thank all my friends in the lab, especially Susan, Brian, Lisa, Arno and Jon, who offered advice, acted as sounding boards, and lent helping hands.

I dedicate this thesis to my family. To my Mother, who always thought I'd be a brain surgeon—she just never expected it to be on *rat* brains. To my Father, who knows that it really takes hard work; his wise words from elementary school—"Study Harder"—have always guided me. To my sister and brother, Zoë and Cort, who never failed to asked if I was *ever* going to be finished (thanks for the encouragement). To my husband, James, who always knew I would finish (or was polite enough not to say otherwise).

Finally, I dedicate this thesis to my Grandparents, who gave me unwavering support and unconditional love. They had complete belief that I was the smartest grand-daughter ever (whether it was deserved or not). I wish they all could have seen me finish.

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ACTH	*adrenocorticotropin*
ACh	*acetylcholine*
ADH	*anti-diuretic hormone*
AVP	*arginine vasopressin*
cNOS	*constitutive form of NOS*
CNS	*central nervous system*
COX	*cyclooxygenase*
CRE	*Ca2+/cAMP response element*
CRF	*corticotropin-releasing factor*
DAB	*diaminobenzidine*
DBH	*dopamine-β-hydroxylase*
DNAB	*dorsal noradrenergic bundle*
EDRF	*endothelial-derived relaxing factor*
ELAM	*endothelial-leukocyte adhesion molecule*
FRA	*fos-related antigens*
GABA	*gamma-amino-butyric acid*
HPA	*hypothalamic-pituitary-adrenal*
ICE	*IL-1β converting enzyme*
ICAM	*intercellular adhesion molecule*
i.c.v.	*intracerebroventricular*
IFN-γ	*interferon-gamma*
IL-1	*interleukin-1*
IL-6	*interleukin-6*
IL-8	*interleukin-8*
IL-12	*interleukin-12*
iNOS	*inducible form of NOS*

i.p.	*intraperitoneal*
i.v.	*intravenous*
LHRH	*luteinizing hormone-releasing hormone*
LBP	*LPS binding protein*
LPS	*lipopolysaccharide*
MANOVA	*multivariate analysis of variance*
NADPH	*nicotinamide adenine dinucleotide phosphate*
NADPH-d	*nicotinamide adenine dinucleotide phosphate diaphorase*
NAME	*N nitro-L-arginine methylester*
NE	*norepinephrine*
NMDA	*N-methyl-D-aspartate*
NO	*nitric oxide*
NOS	*nitric oxide synthase*
OVLT	*organum vasculosum of the lamina terminalis*
OXY	*oxytocin*
PAP	*peroxidase anti-peroxidase*
PBS	*phosphate buffer saline*
PGE2	*prostaglandin E2*
PHA	*phytohemagglutinin*
POA	*preoptic area of the hypothalamus*
PVN	*paraventricular nucleus*
SNAP	*S-nitroso-N-acetylpenicillamine*
SON	*supraoptic nucleus*
SRE	*serum response element*
TNF	*tumour necrosis factor*
VCAM	*vascular cell adhesion molecule*
VNAB	*ventral noradrenergic bundle*

Abstract

It has been shown that intravenous (i.v.) injection of lipopolysaccharide (LPS), a powerful endotoxin derived from the outer coat of gram-negative bacteria, results in a dose- and time-dependent expression of the proto-oncogene c-fos in the brain. In particular, the region of the paraventricular nucleus (PVN) in the hypothalamus shows significant c-fos expression upon LPS treatment [134]. This thesis examined some of the characteristics of this c-fos induction.

Various staining procedures were used to determine the possible chemical specificity of the neurons activated during the endotoxin immune challenge. Many of the c-fos expressing cells stained positive for vasopressin or oxytocin, as well as, nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) reaction, indicating the presence of nitric oxide synthase. Double-staining procedures showed that colocalization also existed between NADPH-d and both OXY and AVP. OXY immunoreactivity, NADPH-d staining, and double labelled NADPH-d/OXY staining were all significantly increased in the rostral PVN after LPS treatment. This indicates a potential role for these substances in the central mediation of systemic immune challenges, and particularly a role for nitric oxide.

To examine more closely the role of nitric oxide in LPS-induced c-fos expression in the PVN, central injections of L-NAME, a nitric oxide synthase inhibitor, were used. L-NAME inhibited LPS-induced c-fos in the PVN and this inhibition was LPS dose-dependent. In addition, central administration of SNAP, a NO donor, resulted in a significant increase in plasma corticosterone levels.

To investigate further the possible mediators of the LPS-induced c-fos response, the effects of

central administration of prostaglandin E2 (PGE2), a potent mediator of the immune response to LPS, was examined. Centrally administered PGE2 induced c-fos in a pattern similar to that produced by i.v. LPS and this PGE2-induced c-fos also was inhibited by i.c.v. L-NAME. This suggests a role for nitric oxide in the neuronal signalling produced by LPS, and that PGE2 is a likely mediator in this pathway.

The role of ascending catecholamine tracts in the central expression of c-fos after i.v. LPS (immune stress) and footshock (psychological stress) were examined in order to determine the effects of these neural inputs in the central response to stress. Unilateral knife cuts of catecholamine tracts in the mesencephalic pontine region significantly attenuated LPS-induced c-fos expression in the PVN, but had no effect on footshock-induced c-fos expression. This inhibition of LPS-induced c-fos expression was even more evident after posteriorlateral hypothalamic knife cuts. While hypothalamic knife cuts did attenuate footshock-induced c-fos expression, this effect was limited to the caudal regions of the PVN. These results suggest a functional role for ascending catecholamine pathways in the central processing of the LPS immune challenge, but this pathway plays a limited role in processing psychological (footshock) stress.

Overall these results reveal that both cytokine and neural mediators are involved in the central c-fos response to peripheral immune challenge.

Introduction

When Besedovsky and his colleagues proposed their model for a bidirectional communications system between the immune system and the brain, their work began a major conceptual shift in neuroscience and immunology resulting in the emergent fields of neuroimmunology and psychoneuroimmunology. They proposed that response to immune challenges, and ultimately the maintenance of homeostatic wellbeing, is dependent on an effective network of communication between brain and body.

The idea that the mind can affect health is centuries old—ancient Chinese, Greek and Indian writings all held this belief. In modern science, the father of stress research, Han Selye, started to examine these interactions, but it is only in the past twenty years that research has begun to expose the complex interrelationship between the central nervous, behavioural, endocrine and immune systems. This thesis deals with basic components of an immense and complex system. Ultimately, a clearer understanding of the interactions between these systems will lead to improved understanding how psychological and physical health is maintained.

There are three main sections to this literature review. The first is a basic review of the role of the hypothalamus in the response to immune stimuli. The second explores the functions of the proto-oncogene c-fos, and its role in mapping functional pathways in the nervous system. The final section reviews the novel neurotransmitter nitric oxide.

I. The Immune Stress Response

Hypothalamic-Pituitary Axis

The hypothalamus, tucked away deep within the brain, consists of a heterogeneity of cells, processes, nuclei and areas. The hypothalamus is a region of the brain essential for the integration and homeostatic function of the body. It has been found to be involved in the control of somatic action, autonomic nervous system function, water balance and thirst, metabolism, energy balance and body temperature, appetite and the gastrointestinal system, sex and reproduction, hormonal levels, emotional expression, stress and adaption, and finally, immune function [review 66].

A stressor, including immune challenge, initiates the activation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in the hypothalamic release of corticotropin-releasing factor (CRF) into the median eminence. From there, CRF is transported by the portal blood supply to the anterior pituitary, where it activates the release of adrenocorticotropin (ACTH) which in turn regulates glucocorticoid secretion from the adrenal cortex. Glucocorticoids in turn feedback to the hypothalamus and/or pituitary and inhibit ACTH and CRF [129]. This relatively straight-forward, closed-loop feedback representation of the stress response belies the true complexity that surrounds the stress event. Although CRF is the main facilitator of ACTH, other factors have been discovered to modulate the stress response [129]. In inflammatory stress, such as that caused by endotoxin treatment, the cytokines TNF-α, IL-1, and IL-6 stimulate CRF and activate the HPA and sympathetic nervous system [151]. The type and duration of the stressor can also affect the hypothalamic response [46]. Researchers have appreciated the importance of the hypothalamus, and work within the field of neuroimmunology has blossomed as they

attempt to better understand how the hypothalamus acts as an integrator of endocrine and neural influences on the immune system.

Endocrine Control of the Immune System

Output from the hypothalamus is either neural or endocrine in nature [77, 138]. The importance of the hypothalamus during immune stress, and stress in general, has traditionally focused on the regulation of the pituitary [66]. Two regions in the hypothalamus that are very important in this control are the supraoptic (SON) and paraventricular (PVN) nuclei. These nuclei are morphologically separated into two major divisions; the parvocellular and magnocellular division [138]. The magnocellular system is comprised of a mixture of oxytocin and vasopressin-producing neuroendocrine cells [222]. This system has traditionally been known as the neuroendocrine pathway from the hypothalamus to the posterior pituitary. The magnocellular processes travel from the hypothalamus and descend down the neurohypophysial stalk into the pituitary and it is there that the release of oxytocin and vasopressin occurs into the blood [222]. The entire SON and part of the PVN is made up of magnocellular cells, however, these two nuclei only comprise around half of the magnocellular elements that project into the neurohypophysis—the other magnocellular cells are scattered throughout the hypothalamus [77].

The parvocellular division in the PVN consists of smaller cells, producing numerous peptides [65, 129, 221]. Parvocellular axons terminate at the median eminence and release peptides that travel, via the blood supply of the hypophyseal stalk, to the anterior pituitary [65, 138]. These peptides are the releasing and inhibiting factors of the anterior pituitary

hormones; among these peptides are CRF which is the releasing factor for ACTH [65]. The remaining parvocellular neurons have projections to numerous other brain areas, including brainstem nuclei and premotor autonomic nuclei in the spinal cord [143].

These two divisions of the PVN are not separate, divorced entities. A rich magnocellular dendritic tree has been shown to penetrate into the parvocellular division [77, 223]. Thus, events affecting one area, such as stress-activated releases of parvocellular CRF, do not go unnoticed, and possibly unaffected, by the other area.

Neural Control of the Immune System

Although the hypothalamo-neurohypophysial system is the best recognised route of magnocellular neurons, not all magnocellular elements project solely into the neurohypophysis [1, 22]. Some vasopressinergic efferents from the magnocellular PVN, and many neurons in the parvocellular region, project into the median eminence. Also, efferents from the PVN have been shown to extend to brainstem and spinal cord, some projections have been shown to reach as far as the sacral regions of the cord. The majority of these PVN efferents are oxytocinergic in nature [review in 77, 137].

Over a quarter of all oxytocin and vasopressin-staining cells in the PVN are found in the parvocellular division [143]. The median eminence is only one potential target for these neurons, a number of neurons in the parvocellular PVN project to autonomic neurons in the dorsal vagal complex in the brainstem and the intermediolateral cell column of the spinal cord, which in turn provides efferent fibres to visceral structures, such as immune organs, and may affect visceral function by modulating the parasympathetic and sympathetic systems

[143, 252]. Immune organs are well innervated by the noradrenergic sympathetic nervous system [266], and it has been noted that the release of norepinephrine (NE) from the sympathetic nervous system can suppress macrophage TNF production caused by LPS treatment [108]. Since the PVN has direct innervation to both the sympathetic and parasympathetic arms of the autonomic nervous system, this allows the hypothalamus to act as a central integrating centre for neuronal influences on the immune system, as well as its known endocrine functions [120].

Vasopressin, Oxytocin and associated neurotransmitters

The traditional roles of the nonapeptides, vasopressin and oxytocin, do not predict their effects in the immune system. Oxytocin causes the milk ejection reflex by the contraction of the myoepithelial in the mammary glands and is involved in the uterine contractions of parturition [37]. Vasopressin, also known as anti-diuretic hormone (ADH), causes the reabsorption of water from renal tubules [77]. However, evidence is mounting in favour of these substances as possible effectors in the stress response, including immune stresses. Both OXY and AVP have been implicated in behaviour [120, 37], and OXY has an important role in maternal and non-maternal affinity [37]. AVP, and to a lesser extent OXY, may function in the brain as an endogenous antipyretic during prostaglandin-accompanied fever [107, 220]. During certain stresses, increased levels of OXY in the portal blood supply has been observed [120, 37]. In response to osmotic stress, OXY neurons in the PVN and SON express c-fos [122, 123].

It has been shown that one neuron can express multiple biologically-active molecules [129, 131, 221]. Recent evidence suggest the colocalization of dynophin and enkephalin in

vasopressin- and oxytocin-producing cells [65]. Enkephalin plays an important role in the stress response, increasing CRF release from the hypothalamus in vitro [65]. OXY has also been found to potentiate the release of ACTH induced by CRF [140, 141]. This ACTH release may be induced by OXY binding the same receptors as CRF and having a common intracellular signal induction mechanism—probably induction of phospholipase C [253]. In the parvocellular region, CRF cells have been found to be colocalized with a multitude of peptides, including AVP [129, 131]. It is possible that AVP acts as a modulator of CRF within the parvocellular region, or perhaps at the level of the median eminence [129]. Other neurotransmitters known to be induced in the hypothalamus by stress include prolactin [34] and neurotensin [46]. It is important to note that there is species variability in the action of OXY and AVP on ACTH; contrary results have been reported between rodent and primate [35], and differences between sheep and rodent have been noted in the neurotransmitters released into the hypophyseal blood [79].

Oxytogeneric and vassopressinergic neurons in the PVN project to autonomic centres in the spinal cord and medulla [143]. Some of these projections innervate peripheral organs, including immune organs [266]. The function of the nonapepetides in peripheral immune function is only just being elucidated, although both oxytocin and vasopressin have been found in thymus extracts [269], and receptor sites for oxytocin in thymus and vasopressin in the spleen have been identified [268]. It appears that these nonapeptides might have a direct immunoregulatory effect on immune cells and functions [270, 271]. Vasopressin, and to a lesser extent oxytocin, act as immune regulators of T-cells and splenic function [271]; and oxytocin can exert control over splenic nerve activity [119].

Peripheral Effects of Lipopolysaccharide

Lipopolysaccharide (LPS), or endotoxin, has been recognised for over a century as a potent bacterial toxin derived from the outer coats of gram-negative bacteria [review 24]. Endotoxin is a potent stimulator of numerous immunological and physiological systems and treatment with systemic LPS can result in fever, cytokine upregulation, vascular changes, septic shock, and sometimes death. The LPS molecule occupies over 3/4 of the bacterial surface, and contains a Lipid A component that is the principle toxin and immunomodulating agent of LPS [206]. LPS, through its Lipid A component, interacts with host cells, including mononuclear cells, endothelial and smooth muscle cells, polymorphonuclear granulocytes, and thrombocytes [207].

Systemic LPS treatment results in a cascade of cytokines, including TNF, IL-1, IL-6, IL-8, and IL-12 [review 254]. There is a time variation in the release in LPS-induced cytokines—for example, TNF and IL-1 are induced early after LPS treatment and are considered immediate mediators of the cytokine immune response to LPS [254], whereas both IL-6 and IL-8 are delayed till 3 hours post-challenge [33, 255].

TNF, a substance that has the ability to induce necrosis of mouse tumours [208], is a potent mediator of the immune response to LPS, where its main targets are monocytes and macrophage [209]. Pretreatment with anti-TNF can attenuate LPS-induced septic shock [209]. Although TNF is a potent mediator in endotoxic actions, mediation of the LPS effects is due to a cascade of amplifying and associated agents, and interruption of that cascade by many factors, including IL-1 receptor antagonists [224], cyclooxygenase (COX) inhibitors [256], and anti-IL-12 [38]can disrupt the actions of LPS.

The actions of TNF are strongly synergized by interferon-gamma (IFN-γ) and IFN-γ can cause the upregulation of LPS-induced TNF-α in macrophages [224]. IFN-γ, traditionally regarded as a T cell-dependent cytokine, has been shown to be produced by naive natural killer (NK) cells after treatment with bacteria or LPS [225]. In fact, TNF-α and macrophages are two co-factors required for the generation of IFN-γ by NK cells [226]. Also, IL-12 can induce IFN-γ after high dose LPS treatment [38]. Although TNF alone can induce the expression of cell adhesion molecules, including ELAM, ICAM and VCAM [227], an interaction with IFN-γ is important in cellular recruitment as it enhances the role of TNF in endothelial adhesion molecule expression, and therefore expands and amplifies the inflammatory response [228].

Other substances are also released into the system upon LPS challenge. Systemic endotoxin, or intravenous TNF and IL-1 treatment, induce histidine decarboxylase, the enzyme that forms histamine [32] and LPS-induced septic shock results in a large increase in plasma histamine levels [109]. Macrophage-produced histamine can regulate IL-1 production by macrophage [105]. As well, endotoxemia levels of LPS substantially increases plasma catecholamine levels [109].

Plasma levels of CD14, a monocyte receptor, increase in response to LPS in a dose and time dependent manner [42]. LPS forms a 3-way complex with itself, CD14 and LBP (LPS binding protein). This complex is involved in LPS signalling, as well as in the clearance of LPS. [44].

Central Response to Lipopolysaccharide

Wan et. al. have shown that administration of LPS either via systemic or central routes results in a highly localised expression of c-fos within the brain [134, 135]. It has been shown that c-fos expression in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus is both dose and time-dependent following a peripheral injection of LPS. Similarly, the noradrenergic A2 cell group of the brainstem also contain c-fos–expressing cells following LPS injection [135]. It is possible that the factor(s) which mediate the increased c-fos expression upon LPS challenge are immune cell derived (for example, IL-1 or TNF-α) [135]. Thus, c-fos expression following LPS challenge may reflect an immune-CNS directed signalling pathway.

Following LPS challenge, immune cells will release multiple cytokines into the circulation, which may then signal the CNS directly through vascular paths (at blood-brain barrier free areas) or indirectly by inducing the central production of other mediators. Systemic injection of TNF acts on the hypothalamus to cause local release of prostaglandin E2 (PGE2) which induces fever [25], and central administration of IL-1β has been shown to induce a febrile response as well [34]. IL-1 binding sites are widely distributed in the rat brain. IL-1 immunoreactive fibres have been reported in the hippocampal CA3 region and PVN region of the hypothalamus [211]. IL-1 has been shown to stimulate hypothalamic CRF release, a basic response to immune stress [210].

Astrocytes and microglia release TNF, IL-1 and IL-6 in response to endotoxin [212], but it is unlikely that LPS induces this response directly via its LPS-LBP-CD14 complex since CD14 is barely detectable in the brain [42] and LPS is such a large molecule that its direct diffusion

through the blood-brain-barrier is unlikely.

Since the macromolecular cytokines and LPS are too large to pass directly through the blood-brain barrier, it is likely that these factors act at one of the circumventricular organs [120], quite possibly at the organum vasculosum of the lamina terminalis (OVLT), the subfornical organ or the area posterma [68]. TNF and IL-1 can effect the meninges [213], and increase the permeability of the blood-brain barrier [214]. The OVLT in particular has been shown to be a critical site for the febrile response [215], and is a central site where interleukins are produced in response to peripheral endotoxin [216].

Alternatively, activation of particular neural regions may underlie the LPS-induced central responses, including c-fos expression. The pattern of c-fos expression following LPS injection suggests that the afferent information arrives at the hypothalamus from the medulla oblongata via the solitario–paraventricular pathway [135, 139]. Other, higher-level inputs into the hypothalamus include neurons from the bed nucleus of the stria terminalis, which receives information from the neocortex and limbic system, including the hippocampus and amygdyla [77, 219]. Neurochemical changes following immune activation have been observed, indicating that alteration in neural activity in response to LPS is possible [217].

The ascending noradrenergic pathway from the brainstem nuclei are divided into dorsal and ventral bundles. The dorsal bundle starts in locus ceruleus (A6) innervates all cortical areas, hippocampus, cerebellum, amygdala, and anterior hypothalamus. The ventral bundle originates from A1, A2, A5 and A7 in pons and medulla oblongata and innervates the lower brainstem, hypothalamus, and limbic forebrain (including the amygdala, septum

and cingulum) [67, 137]. The A1 group of the venterolateral medulla innervates both magnocellular and parvocellular PVN and SON, and also connects to the locus ceruleus. There are catecholaminergic connections between the A1 and A2 groups [137]. The noradrenergic system of the brainstem has been shown to have an inhibitory effect on the antipyritic AVP cells in the PVN. Microinfusion of norepinephrine into the PVN enhances LPS fever, and chemical lesions of the noradrenergic PVN afferents with 6-hydroxydopamine reduces the response to LPS-induced fever [107].

Neural Disease and Immune Function

In humans with brain tumours, individuals with left side tumours have decreased in vitro responsiveness of peripheral blood lymphocytes to PHA (phytohemagglutinin). Right side tumours do not affect responsiveness. This indicates that anatomically discrete areas within the brain have the capacity to regulate immune function [3, 257]. Patients suffering from Alzhemier's disease show reduced CRF binding sites on blood immunocytes [153], and elevations in IL-1 may be a critical step in Alzheimer's disease plaque progression [28, 29]. AIDS patients have a decreased number of oxytocin immunoreactive neurons in the PVN, and it is possible that decreased function of the HPA decreases oxytocin in the brain [102].

Literature on human disease indicates links between dysfunction in the central nervous system and the immune system, and the neuroimmunological implication of these links are only recently being elucidated. Continued research into neuroimmunology has the potential to reveal some of the underlying mechanisms of diseases such as Alzheimer's, multiple sclerosis, AIDS, and stroke.

II. The Proto-oncogene Fos

C-fos is an immediate early-response proto-oncogene that codes for the nuclear protein fos as well as a series of fos-related antigens (FRA), which act either alone or in conjunction with other proto-oncogene proteins, such as jun, as transcriptional activators and may therefore be involved in gene regulation [5, 12, 186]. This protein has been found throughout the body and has been implicated and identified as a key component of a number of cellular functions.

The Function of C-fos: Cellular Growth

C-fos expression has been observed during cell growth, differentiation and development which may reflect activation of particular genomic elements characteristic of these different cellular stages [204, 205]. In cellular growth, c-fos as been identified as a trigger for a cascade of regulatory events where fos targets specific genes that result in eventual morphological change [2]. Unlike jun knock-out mice which die mid-gestation [206], knock-out mice for the c-fos gene are viable but show developmental and neurological defects [9], the reason for survival may be the other members of the fos family (FRA) are sufficient back-up. C-fos has also been associated with cellular death, although this involvement may not be direct but rather a side effect of the onset of death [49].

The Function of C-fos: Signal Transduction

C-fos has also been shown to have effects beyond that of growth and growth-related changes. Its induction occurs in response to a wide variety of extracellular stimuli, including both physiological and psychological stimuli. C-fos has been found to bind to either single or double stranded DNA [4, 5, 6], and this property seems to be intrinsic to the fos family of

proteins as a number of fos-related proteins also bind DNA [5, 93]. The effects of fos on transcription can either be inhibitory or activational in nature [9, 11, 12, 13, 101].

Perhaps the best known genetic target of fos is that of the AP-1 transcriptional control element (TGACTA), which is usually targeted by the fos-jun heterodimer [4, 12]. Although fos can bind the AP-1 site in a homodimeric form of fos-fos, it is the fos-jun heterodimer that shows the highest level of stability [61]. AP-1 has been shown to play an important role in the transcriptional control of a number of genes, particularly in long term responses to extracellular signal [4, 12]. Fos interacts at composite DNA elements containing weak AP-1 sites, including those for the glucocorticoid receptor [203], the T-cell transcription factor, NFAT [9], the glucocorticoid response element (cGRE) [101], vasopressin [50] and the proenkephalin gene [11].

C-fos has also been shown to be directly involved in the regulation of the c-fos promoter [13]. The self-repression of c-fos gene by its own product may play an important role in the regulation of the protein and its signal transduction activities. The complexity of the actions of c-fos are increased when one considers that c-fos may also regulate multiple transcriptional elements [13], developing a complex array of transcriptional inducers/repressors. The fact that c-fos is activated by so many types of stimuli, and has action on many potential transcriptional elements, often leads to the question of how this ubiquitous signal transducer is controlled. It has been hypothesised that there are multiple regulatory factors at work when c-fos is induced, including rate-limiting steps, hetero/homo-complexes, interaction between multiple regulatory sites, and the requirement for a complex mixture of both resident and induced transcription factors to

affect target genes [11, 61, 96, 101].

There are still many unknowns regarding the mechanisms of c-fos, but it is clear that its early appearance after cell stimulation, its nuclear location and its DNA binding ability imply a role in signal transduction, perhaps at the level of gene expression where it can function as a long-term transcriptional response to growth factors and other external stimuli. [4, 5]

The Induction of C-fos: Physiological Stimuli

A wide variety of physiological stimuli result in the induction of c-fos in the nervous system, including hormonal, vascular, and physical changes. Heat [10, 17, 59, 60], growth and differentiation factors [1, 16, 20, 73], seizure [21, 49], dehydration [22], immunological factors [34, 50, 113], caffeine [74], endotoxin [84], inflammation [86, 88], changes in ionic balance [92, 93], adrenalectomy [94], and hypoxia [58] have all been shown to induce c-fos in a variety of brain regions.

The Induction of C-fos: Psychological Stimuli

Stress, both physiological and psychological, induce c-fos in the nervous system. Psychological stressors that induce c-fos include immobilisation stress [52, 90], nociceptive stress [56] and footshock [258]. In fact immobilisation stress, intraperitpneal (i.p.) hypertonic saline (osmotic stress), and capsaicin nociceptive stress all induce c-fos in similar brain regions [92]. The neuronal c-fos response to stress varies depending on whether the stressor is acute or chronic [100, 258] . For chronic stressors, there is an attenuation in the activation of c-fos protein, perhaps due to a refractory period after initial induction [6]. It

is interesting to note that in early post-natal life, the central induction of c-fos by stress is attenuated in both mothers and pups and this change may be associated with lactation [95].

The Induction of C-fos: Mechanisms of Induction

The induction of c-fos is a complex process, which varies depending on the cell type and the stimulus inducing the c-fos expression [14, 15, 16, 19, 71, 83, 89]. There are two main mechanisms of c-fos induction in neuronal cells, based upon whether the activator is calcium dependent or calcium independent. Calcium independent induction occurs via a receptor-ligand interaction, whereas calcium dependent induction of c-fos involves the opening of calcium channels and a calmodulin or a calmodulin kinase mediated mechanism which leads to the transcription of c-fos [14]. C-fos induction in neurons does not require protein synthesis, but its inhibition and regulation might [19].

Glutamate in cerebellar cell cultures increases c-fos mRNA, possibly through the activation of a receptor-operated cationic channel, protein kinase C activation and increased calcium [69, 75]. In vivo, treatment with kainic acid or NMDA, two glutamate agonists, induces c-fos in the brain [70]. Additionally, calcium blockade studies have further defined the importance of calcium in c-fos expression [14, 218]. MK-801, a noncompetitive NMDA antagonist, blocked the c-fos expression in the brain that is usually induced by cortical damage [111]. In support of a calcium-dependent mechanism of c-fos induction in the LPS paradigm, it has been shown that MK-801 also blocks the expression of c-fos in the hypothalamus following i.p. or i.v. injection of LPS. [134].

There have been reports that activation of acetylcholine (ACh) receptors fails to induce c-fos

in cerebellar cell cultures [75], but other in vitro [274] and in vivo [272, 273] studies have shown that the muscarinic cholinergic system can induce c-fos mRNA expression. Activation of GABAa or GABAb receptors fail to induce c-fos mRNA [75], but GABA agonists have been shown to inhibit both photic-induced [275] and seizure-induced [276] c-fos expression, suggesting an regulatory role for this inhibitory transmitter. Dopamine agonists also increase in brain c-fos expression [53]. Norepinephrine (NE) released from A2-adrenergic neurons acts on beta adrenergic receptors to increase c-fos induction via a cAMP mechanism [91]. This activity can be enhanced by α2-adrenergic receptor antagonists which block the autoinhibitory effects of NE [74]. β-adrenergic receptor antagonist decrease, but does not abolish, c-fos induction. This indicates that multiple neurotransmitter receptor systems are involved in this regulatory process [91]. Although neurons express c-fos, it has been shown that direct electrical stimulation of dorsal horn nerves is not enough to sufficient to induce c-fos protein [59]. It is clear that c-fos expression after physiological stimuli can involve multiple factors, and it appears that the neurochemicals released during and as a result of synaptic transmission are necessary for c-fos induction.

Regulation of c-fos expression has been shown to involve the actions of multiple interdependent transcriptional elements, including serum response element (SRE), the fos AP-1 site and sis-inducible site, and Ca2+/cAMP response element (CRE) [89]. SRE and CRE are absolutely necessary for induction in mouse neurons [89]. Mutation of any of these elements resulted in a profound loss of stimulus-evoked gene expression of c-fos. It has been proposed that a rapid silencing of mRNA production following stimulus-induced activation may be one mechanism of c-fos control [62]. As well, there can be different mechanisms of c-fos-induction within a single cell type, which may account for some complex regulation on

the expression of c-fos [16].

C-fos: A marker of neuronal activation

The expression of c-fos can be used as an experimental marker of neuronal activation occurring in response to various physiological challenges [22, 23]. Basal background expression of c-fos is low in the central nervous system, thus c-fos has been used for anatomically specific metabolic mapping [22]. Different types of stimuli activate different parts of the brain, and c-fos staining can reflect these patterns of activation [23]. Compared to the mapping technique of 2-deoxyglucose autoradiography, c-fos immunostaining allows for cellular resolution [22]. However, c-fos also does not identify all of the same neurons as shown by the 2-deoxyglucose technique in some brain regions (eg substantia nigra)[23].

Sagar et al acknowledged that there is an uncertainty in the c-fos method for metabolic mapping studies due to the lack of knowledge concerning the range of stimuli that will produce alterations of c-fos synthesis in the CNS [22]. Nonetheless, c-fos has been shown to be expressed in groups of neurons distributed along activated neuronal pathways, such as neurons associated with the neuronal circuitry for nociceptive signalling [60, 63], the synaptic pathways for the motor-sensory cortex [22], siezure-induced pathways [6], and osmotic stress induced hypothalamo-neurohypophysial tract [22]. Wan et al [135] have suggested that the pattern of c-fos staining in the brain after peripheral immune challenge is indicative of activation of a neural-immune regulatory circuit.

III. Nitric Oxide

Physiology of Nitric Oxide: a novel neurotransmitter

Nitric oxide (NO) is not a typical neurotransmitter. The chemistry of NO is that of nitrogen monoxide, which involves an array of interrelated redox forms: nitrosonium cation (NO+), nitric oxide (NO) and nitroxyl anion (NO-). Under physiological conditions these forms can be interconverted but each has distinctive chemistry. NO is assumed to freely diffuse across aqueous media and cell membranes due to its charge neutrality. [118] This toxic, free radical gas with a half-life of about four seconds [187] is proving to be a pervasive messenger molecule [188]. Vascular smooth muscle relaxation [189, 190], inhibition of platelet aggregation [191], gastric secretory control [246], stimulation or enhancement of cytotoxicity [192] and the modulation of neurotransmission [193, 114, 121, 130, 132, 133] are a few of the biological actions in which NO is suspected to play an important part.

Physiology of Nitric Oxide: NO and EDRF

The physiological history of this molecule begins in the vascular system, with the discovery of a potent vasodilator released from the endothelial layer of blood vessels [189, 190]. Stimulation with a variety of neurotransmitters, including acetylcholine, bradykinin and histamine, resulted in the release of the elusive 'endothelial-derived relaxing factor' (EDRF)[187]. It was found that the vasodilatory effects of EDRF were the result of it targeting the heme bound to cytosolic guanylate cyclase, creating active cGMP. [126]. In 1987, Moncada and his colleagues confirmed that the biological activity of EDRF was actually attributable to nitric oxide [259].

The cerebellum is a region of high cGMP activity in the brain. Garthwaite et. al. [194]

found that a molecule similar to EDRF was released by the cerebellar cells upon glutamate stimulation of NMDA receptors. Increased stimulation of NMDA receptors resulted in a corresponding increase in cGMP levels [195, 194, 126]. The production of cGMP was antagonised by the addition of haemoglobin, a potent inhibitor of NO, to the cell culture media [194, 127]. This showed that NO was being released extracellularly from the stimulated cerebellar cells. The ability of the neuronal NO to effectively mimic the actions of EDRF linked both messenger substances as homologues.

Physiology of NOS: Constitutive and Inducible

Initial localisation of neurons producing NO was hampered by the short half-life of the molecule. However, efforts were made to determine the enzyme synthesising nitric oxide, i.e. nitric oxide synthase (NOS). NOS is responsible for the enzymatic deimidation of arginine to citrulline and the consequent production of NO [187]. Bredt and Snyder [117] isolated neuronal NOS and discovered it to be a 150 kD monomer that had an absolute requirement for calmodulin and calcium. The neuronal NOS was found to have recognition sites for NADPH, flavin adenine dinucleotide and flavin mononucleotide. The amino acid structure of NOS shows a close homology to cytochrome P-450 reductase [117].

Studies looking at the NOS produced by different cell types have shown that there are two major types of the enzyme—a constitutive form [117] and an inducible form [187, 196]. The constitutive form of NOS (cNOS) is present in the CNS neurons and in endothelial cells [117, 115]. This form is dependent on calcium calmodulin [117]. The inducible form of NOS (iNOS), found in macrophages, leukocytes and vascular smooth muscle, is not calcium dependent [196].

In the case of macrophages and neutrophils, NO is released in copious quantities during an endotoxin challenge, and it is believed that the released NO acts in a cytotoxic manner against immunogens [187]. It has also been shown that macrophages will produce NO in response to cytokines, such as TNF-α [187]. Initial research had attributed cNOS as the sole form of enzyme in neurons, however studies with cerebellar cultures have shown that neurons can express iNOS when treated with LPS and IFN-gamma [236]. Since iNOS is primarily a cytotoxic agent [187], iNOS in neurons could have a role in neurotoxcity [236].

Function of NO: Effects on Protein Kinases

Nitric oxide targets the heme bound to cytosolic guanylate cyclase [126]. It binds the iron atom that is complexed with protoporphyrin XI resulting in a NO-heme complex. The complexing of the heme portion of guanylate cyclase with NO results in a conformational change in the enzyme, creating active guanylate cyclase. The cGMP formed by the activated enzyme then acts on protein kinases, phosphodiesterases or ion channels which in turn result in cellular actions such as vasodilation [121]. This activity is seen in both constitutive and inducible NOS cells [48, 121, 126].

Function of NO: Effects on Proto-oncogenes

One of the earliest indications that nitric oxide might be involved in c-fos induction came from studies of noxious stimuli and spinal cord neurons. It was found that noxious stimulation resulted in c-fos expression in the spinal cord neurons that were in close opposition to NOS staining cells [54]. This lead to the hypothesis that nitric oxide induces guanylate cyclase in adjacent neurons which results in increased cGMP and subsequently c-fos-induction [54]. Later studies found that nitric oxide alone does not seem to have a direct

effect on transcription, but it can induce calcium and amplify calcium's effects on the transcription [47, 48]. It has also been shown that in some cases this NO-amplified calcium-induced c-fos can be the result of activation of protein kinase A-cAMP responsive element [47].

Function of NO: Effects as a neurotransmitter

Nitric oxide is not stored in synaptic vesicles and does not act upon membrane receptors [187]. Rather, its production is closely linked to synaptic activation and it must diffuse through the target cell membrane to reach its 'receptor', e.g. the heme in guanylate cyclase [126]. These properties, and also the fact that NO is a gas, do not promote NO as a potential neurotransmitter [188]. NOS immunoreactivity is localised only to neurons and to fibres which appear to make close connections with other neurons, and NO appears to act only on neurons in it immediate vicinity of release [48, 115]. It is becoming evident that NO may play an important part in neuronal plasticity [114], and may play a role in the later stages of the development of the nervous system [114]. In particular, recent evidence supports a possible neural role for NO in long-term potentiation (LTP) and long-term depression (LTD) of synaptic junctions [193, 130, 132, 133].

Function of NO: Neurodegeneration and Disease

In neurons, the level of NO released in normal situations, as a messenger molecule, is significantly lower than that released from cytotoxic macrophages [188, 187]. However, it has been demonstrated that excessive release of NO in the brain may be partially responsible for neurodegenerative diseases such as Huntington's Disease [188, 197] and glutamate neurotoxicity is mediated by NO [197]. Excessive stimulation of NMDA glutamate

receptors by increased levels of glutamate results in increased Ca^{2+} entry into the post-synaptic neuron [197]. This increase in intracellular calcium continuously activates NOS, resulting in pathological levels of NO. An interesting histochemical observation is the striking survival of NADPH-diaphorase neurons in brains of individuals with neurodegenerative diseases [188, 197]. This preferential survival may be due to the presence of anti-oxidants, such as superoxide dismutase in the NO-producing neuron [197]. NO reacts readily with superoxide free radicals producing toxic peroxynitrate anions [187]. Neurons to which NO diffuses may lack or be limited in their levels of anti-oxidants, and therefore not as well protected from peroxynitrate damage [197]. Also, NOS neurons may contain a factor which protects them from NO-mediated neurotoxicity. Macrophages may also produce some resistance factor to excess NO as they are not affected by their cytotoxic release of NO [187].

Nitric Oxide Synthase and the NADPH-diaphorase Stain

Purification of NOS allowed for immunohistochemical examinations of the brain. It was found that NOS immunostaining occurred in highly localised regions [115, 198], but perhaps most interesting was that the same areas in the brain that stained for NOS also stained for NADPH-diaphorase [199, 200, 201].

The NADPH-diaphorase reaction was described in the early 1960's by Thomas and Pearse. They showed that certain neurons in the brain were capable of NADPH-diaphorase dependent reduction of nitro blue tetrazolium salts into insoluble, visible formazans [202]. The result was a vivid, purple stain of discrete populations of neurons. However, it was not until NOS immunocytochemistry that a complete understanding of what was being stained by this technique was established [198].

Synder and his colleagues [199] determined through NADPH-diaphorase staining and NOS immunocytochemistry that there was a coincidence of NOS and NADPH-diaphorase in all positively stained regions of the brain and that areas of high-intensity NOS staining also expressed high-intensity NADPH-diaphorase staining. The diaphorase activity of NOS is thought to be due to the oxidative activity of NOS. Reduction of the nitro blue tetrazolium dye is thought to be caused by the reduction of a NADPH of a flavin associated with NOS which in turn reduces the dye to its formazan state.

In the hypothalamus, both the PVN and SON show NOS staining, and are therefore sites of NO production [115, 198]. A high concentration of neuronal staining in the magnocellular fibres that innervate the posterior pituitary has also been observed [115]. NADPH-diaphorase staining of the pituitary reveals intense staining in the posterior portion and weak to negligible staining in the anterior and intermediate lobes [202, 198].

Objectives and Hypothesis

The purpose of this thesis was to examine some of the characteristics of c-fos induction in the hypothalamic paraventricular nucleus after intravenous injection of lipopolysaccharide, a powerful endotoxin. Besedovsky et al [267] proposed a model for a bidirectional communications system between the immune system and the brain. It is possible that the pattern of c-fos staining in the brain after peripheral immune challenge is indicative of activation of a neural-immune regulatory circuitry [135], and that the mediators involved in c-fos induction have an important role in the central response to immune challenge.

Experiment 1 examined the chemical specificity of the c-fos expressing neurons activated during the endotoxin immune challenge using various staining procedures. Based on the results from the first experiment, it was clear that nitric oxide had an important role in the central response to peripheral LPS, so experiment 2 looked more closely at the role of nitric oxide in LPS-induced c-fos expression in the PVN.

Previous work in our lab [135] has shown that inhibition of central cyclooxygenase, the enzyme responsible for prostaglandin synthesis, attenuates LPS-induced c-fos expression in the PVN. To further investigate the effects of central prostaglandins, experiment 3 examined the effects of central prostaglandin E2 (PGE2), a potent mediator in the immune response to LPS, on c-fos expression in the PVN.

Finally, to determine the effects of neural, as opposed to cytokine, inputs on the central expression of c-fos after i.v. LPS treatment, experiment 4 investigated the role of ascending catecholamine tracts in the stress response.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (300-400 g; from Charles River Laboratories, St. Constant, Quebec) were used as subjects. They were housed in polystyrene cages and provided with food and water ad libitum. A 12h:12h light-dark cycle was employed in a temperature controlled housing facility.

Surgical and Injection Protocols

Anaesthesia

All animals undergoing surgical procedures were pretreated with atropine (0.03 ml s.c.) to counteract the respiratory distress resulting from pentabarbitol anaesthesia. Fifteen minutes post-atropine injection, subjects were anaesthetised with Somnotol (sodium pentabarbitol; 65 mg/kg, i.p.).

Cannulation

Animals were placed in a stereotaxic apparatus (David Kopf Instruments) and bregma was visualised. The skull was levelled horizontally relative to bregma and lambda. Using a small dental drill to allow access through the skull, a 26-guage stainless steel cannula was implanted into the left ventricle (coordinates from Bregma: AP: -0.8, Lat: 1.4, Dura: -4.0) and secured to the skull with skull screws and acrylic dental cement. The cannula was then sealed with a plug stylette to prevent clogging of the implanted guide stylette and also to prevent the entry of foreign material into the guide.

Post-surgery animals were housed individually and allowed to recover for 6-8 days before receiving intracerebroventricular (i.c.v.) infusions into the left lateral ventricle. The i.c.v. injections were preformed with a stainless-steel injector stylette that was placed within the implanted cannula guide. The animals were unrestrained within their cages during the i.c.v. injection (15 µl infused over four minutes).

Knife Cuts: Hypothalamus

Posterolateral hypothalamic knife cuts were performed with the skull in a level position (see above). A small section of the skull was removed to allow the entrance of the stainless steel knife. The blade (3.0mm in length) extended perpendicular to the shaft. The knife was descended along the midline with the blade pointed rostral (coordinates from Bregma: AP: -4.2, Lat: 0.0, Dura: -5.0) swung out to a 45° angle and then lowered to the base of the skull. Once the cut was complete, the knife was removed. The exit of the knife was accomplished by precisely reversing the entrance procedure, i.e. the exit and entry paths were identical. The wound was covered with Gel Foam and the skin sealed using stainless steel wound clips.

Knife Cuts: Brainstem

Knife cuts of the brainstem catecholamine tracts were performed with a Kopf retractable microsurgical knife. The knife was inserted via a small hole drilled in the skull (coordinates from Bregma: AP: -10.1, Lat: +2.0). It was slowly lowered to the base of skull and then raised 3.0mm. The knife was then extended towards the midline (2.0mm) and lowered back to the base of the skull. Then it was raised 4.0mm, the knife retracted and the assembly removed (technique courtesy of Drs. P.E. Sawchenko and A. Ericsson, personal

communications). The wound was sealed using wound clips and the animal allowed to recover. Further manipulation of knife-cut animals were performed no sooner than fourteen days post-surgery.

Intravenous Tail Vein Injections

Animals were briefly placed under a heat lamp (three minute exposure) to dilate the blood vessels in the tail. After heating, the subject was restrained and received an intravenous (i.v.) injection via the tail vein. To prevent undue stress to the animal, restraint was applied for no longer than one minute.

Tissue Processing

Perfusions

Animals were deeply anaesthetised with Somnotol (sodium pentabarbitol, 100 mg/kg i.p.). They were then perfused through the left ventricle with approximately 60 ml 1% sodium nitrite solution followed by 200-300 ml 4% paraformaldehyde solution in 0.1M phosphate buffer, pH 7.4. Brains were removed and post-fixed for two hours in 4% paraformaldehyde solution. Brains were then cryoprotected by placing them in a 30% sucrose solution and storing at 4° Celsius for at least 24 hours.

Immunocytochemistry

Coronal sections (40μm) of the brain were cut on a freezing microtome and collected into 0.01M phosphate buffer saline (PBS). Sections were selected for immunocytochemical or histochemical processing and washed three times in 0.01M PBS (10 minutes/rinse).

Sections were incubated overnight at room temperature with rabbit antibodies to either oxytocin (OXY), arginine vasopressin(AVP), c-fos or dopamine-ß-hydroxylase (DBH) in 24-well culture plates. The OXY and AVP antibodies were obtained from Incstar (Stillwater, MN), c-fos antibodies from Santa Cruz Laboratories (Santa Cruz, CA), and the DBH antibodies from Eugene Tech (Eugene OR). All antibodies were diluted with PBS solution containing 1% Triton-X, 1% normal goat serum, and 1% bovine serum albumin at the following ratios; OXY 1:3000, AVP 1:6000, c-fos 1:15,000, and DBH at 1:2000. Sections to be double-labelled with NADPH-diaphorase were treated with the histochemical stain first (see below) and then processed for immunocytochemistry with either OXY, AVP or c-fos antibodies.

Following incubation in the primary antibody for at least 16 hours (20 hours for the DBH antibody), sections were washed three times in PBS for ten minutes each wash and then placed in goat-anti-rabbit antibody (GAR; Cappel, diluted 1:150) for 90 minutes. Following this incubation, sections were again washed three times in PBS and placed for 90 minutes in rabbit peroxidase anti-peroxidase antibody (PAP; Cappel, diluted 1:300). After another series of three 10 minute rinses in PBS, the peroxidase reaction was visualised with a diaminobenzidine (DAB), d-glucose and glucose oxidase solution for 35-50 minutes, until development of a brown reaction product. The reaction was terminated by washing the sections in PBS twice. After washing,the sections were floated onto gelatin coated slides, air dried overnight, dehydrated in a series of alcohols, cleared in xylene and coverslipped.

Histochemistry

Brain sections were histochemically stained for NADPH-diaphorase using a staining

solution composed of 0.1M phosphate buffer (pH 8.0), 1 mM NADPH, 0.2 mM nitroblue tetrazolium, and 0.3% Triton-X. Also, 0.01 mM Dicumarol (3,3' methelene bis 4 hydroxy-coumarin) was added to prevent non-specific diaphorase staining. Sections were incubated in the NADPH-diaphorase stain for 25-30 minutes in an oven at 38-39° Celsius. The sections were then rinsed twice (30 minutes/rinse) in PBS before continuing with immunocytochemical processing.

Corticosterone Radioimmunoassay

Blood for corticosterone analysis was collected from the tail. Animals were restrained, the tip of the tail sliced with a scalpel, and the blood collected into polypropylene microcentrifuge tubes (~1.5 ml blood collected). The entire procedure was completed within 45 seconds and thus avoided the corticosterone peak induced by the tail cut and restraint. Animals were perfused immediately after the blood collection. Blood was mixed with 30 µl of 0.5 M EDTA to prevent hemoagglutination and stored on ice until centrifuged (1500 rmp for 15 minutes). Plasma was collected and stored at -70° Celsius until assayed for corticosterone.

Levels of plasma corticosterone were determined by radioimmunoassay as previously described [277]. Vials of supernatants and standards were thawed, and 10 µl was diluted into 0.5 ml of assay buffer (0.82% anhydrous sodium acetate, 0.01% sodium azide, and pH adjusted to 5.2 with 0.01% BSA in distilled water). Duplicate 0.1 ml volumes of unknown and standard dilutions were placed in 12 x 75 mm glass tubes. To this was added 0.1 ml of antiserum (diluted 1:4 with physiological saline containing 0.1% sodium azide then further diluted 1:100 with assay buffer). Then 0.1 ml of 3H-corticosterone (from New England

Nuclear; 250 μCi in 7.5 ml redistilled ETOH diluted with assay buffer to 25,000 cpm/100 μl) was added. All tubes were gently mixed and incubated at 4°C for 90 minutes. Then 1 ml of charcoal/dextran was added (0.025% dextran T-70 and 0.25% Norit A Charcoal in assay buffer). The tubes were well mixed and allowed to stand 10 minutes at 4 °C. The tubes were then centrifuged at 3000 rpm for 15 minutes in a 4 °C centrifuge. Supernatants were decanted into scintillation vials (0.8 ml) and 5 ml of the scintillation cocktail. Universol (from ICN Pharmaceuticals) was added. Vials were mixed vigorously for 1 minute, allowed to stand for at least 2 hours at room temperature and then counted in a Beckman (beta) scintillation counter for 10 minutes or 10^4 counts.

Experiment 1: Phenotypic specificity of endotoxin-induced c-fos expressing neurons in the PVN.

1.0 Introduction/Rationale

As reported by Wan et al [135], treatment with i.v. lipopolysaccharide (LPS), a powerful endotoxin derived from the outer coats of gram-negative bacteria, resulted in dose, time and site-dependent expression of the proto-oncogene c-fos in the hypothalamus, whereas control treatment with i.v. saline resulted in few and scattered c-fos expressing nuclei. Thus, expression of c-fos can be used as an experimental marker of neuronal activation occurring in response to physiological challenges [21, 22, 57, 58, 63], and these identified c-fos-expressing neurons presumably play a role in the neuroimmune response to systemic endotoxin.

The purpose of this study was to investigate the phenotypic specificity of those LPS-induced c-fos expressing neurons. Although discrete population of neurons in the hypothalamus have been described as c-fos-positive following an immune challenge, the chemical specificity of these neurons has only partially been identified [139, 148]. This study focused on two major hypothalamic neuropeptides: oxytocin (OXY) and vasopressin (AVP); as well as the novel neurotransmitter, nitric oxide.

Oxytocin and vasopressin containing neurons are found throughout the hypothalamus. They are the two major neuropeptides in magnocellular division of the PVN, and are also found in the parvocellular regions of the PVN. Traditional roles for oxytocin include milk ejection and uterine contraction [review 35], vasopressin is the endocrine hormone, anti-

diuretic hormone [review 77]. However, additional roles with regards to stress and immune regulation have been identified for both of these hypothalamic nonapeptides for the control of endocrine responses [102, 119, 129, 131, 140, 141] and the direct neural control of immune organs [143, 252, 268, 269, 270, 271].

Nitric oxide (NO), an atypical gaseous neurotransmitter, has been implicated in a number of neuronal roles, including synaptic plasticity and signal potentiation [130, 132, 133]. Its production in neurons requires the calmodulin-dependent enzyme, nitric oxide synthase (NOS). The NADPH-diaphorase reaction (as described in the *Introduction* section) was shown by Snyder at al [199] to be coincident with the presence of neuronal NOS. Thus, NADPH-diaphorase histochemistry was used in this study as a cellular marker of NO activity in the PVN.

1.1 Materials and Methods

1.1.1 Treatment

To determine the chemical specificity of c-fos expressing neurons in the hypothalamus after endotoxin challenge, subjects were divided into LPS-treated and saline-treated groups. Prior to injections, animals were placed under a heat lamp to dilate the tail vein. LPS-treated subjects were given 100 μg intravenous (i.v.) injection of lipopolysaccharide (Sigma, St. Louis, MO) in 0.2 ml of saline. Saline-treated subjects received an i.v. injection of 0.2 ml saline. Two hours following injection, the animals were deeply anaesthetised, and then perfused. Brains were removed for further processing (specifics on these procedures are located in *Materials and Methods* section).

1.1.2 Histochemical and Immunohistochemical Procedures

We determined that the sequence of labelling procedures was an important determinant of the quality of staining results. Thus, it was necessary to perform the NADPH-diaphorase staining first followed by other immunocytochemistry staining procedures. When the histochemical stain was processed after immunocytochemical staining the resulting sections showed reduced staining intensity and had a large amount of non-specific precipitate.

Coronal sections (40μm) of the brain were cut on a freezing microtome. Selected sections were first histochemically stained for NADPH-diaphorase (specifics on this procedures is located in *Materials and Methods* section).

Following the NADPH-diaphorase staining, sections from saline and LPS-treated animals were divided into three series and placed into 24-well culture plates. Each plate was

incubated with rabbit antibodies to either oxytocin, vasopressin or c-fos over night at room temperature. The dilution of the *c-fos* antibody varied between lots. For this experiment two separate lots were used, for Lot#K013 the dilution was 1:2000, and for Lot#E064 a dilution of 1:15,000 was used. The intensity of c-fos staining between the lots was similar when compared between animals treated with LPS-injection, as well as other known inducers of c-fos protein including footshock and exercise.

Following incubation in the primary antibody for at least 16 hours, sections were developed through the GAR-PAP-DAB procedure (specifics on this procedures is located in *Materials and Methods* section). The double-labelled sections were then mounted on slides.

It should be noted that attempts at triple-staining were made, with sections from an LPS-injected subject processed for NADPH-diaphorase, c-fos and oxytocin or vasopressin. The resulting sections had an excessive amount of background staining. Due to the overwhelming background 'noise', this technique was not further utilized.

1.2 Results and Analysis

As previously described [135], the treatment of 100 μg i.v. LPS resulted in site-specific expression of c-fos in the PVN, whereas control treatment with i.v. saline resulted in few and scattered c-fos expressing nuclei.

1.2.1 Analysis

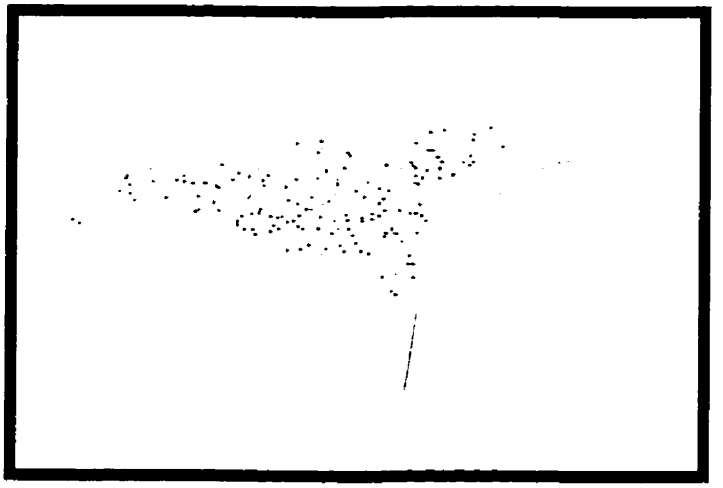
Three sections from the anterior hypothalamus were chosen to represent the caudal, medial and rostral regions of the PVN. The Rostral section consisted primarily the anterior parvocellular PVN. The Medial section was located 240μm caudally, consisting of a predominant magnocellular portion of the PVN as well as the medial parvocellular region. The the most Caudal section (480μm from the Rostral section) contained the posterior subnucleus of the PVN (Figure 1). In describing the nomenclature and anatomy of the hypothalamus, the primary source was the Handbook of Chemical Neuroanatomy [137]; but reference has also been made to Paxinos and Watson's atlas [136] and The Rat Nervous System [138].

The oxytocin (OXY) and vasopressin (AVP) immunoreactive cells contained a brown reaction product that was limited to the perikaryon cytoplasm and cellular processes. C-fos immunoreactive cells contained a brown reaction product that was specific to the nucleus. Double labelled cells were identified as those neurons with reaction product throughout the perikaryon and the nucleus (Figure 2).

The NADPH-diaphorase staining cells could be divided into two types—light staining and dark staining. As naming implies, the light staining NADPH-diaphorase cells were filled

A Rostral Section (0μm)

B Medial Section (+240μm)



C Caudal Section (+480μm)

Figure 1:

Representative Rostral, Medial and Caudal Sections through the Paraventricular Nucleus

Neurolucida drawings through the PVN of animals treated with intravenous LPS (100μg/rat) and sacrificed two-hours post-injection. Rostrocaudal sections of 30 μm were taken, and three sections representing the rostal, medial and caudal regions were selected for this study. Figure A shows the Rostral Section with staining in the anterior parvocellular region. Figure B shows the Medial Section with staining in the magnocellular region as well as the medial parvocellular region. Figure C shows the Caudal Section in the posterior PVN.

These representative sections have been processed to show OXY and c-fos immunoreactive neurons.

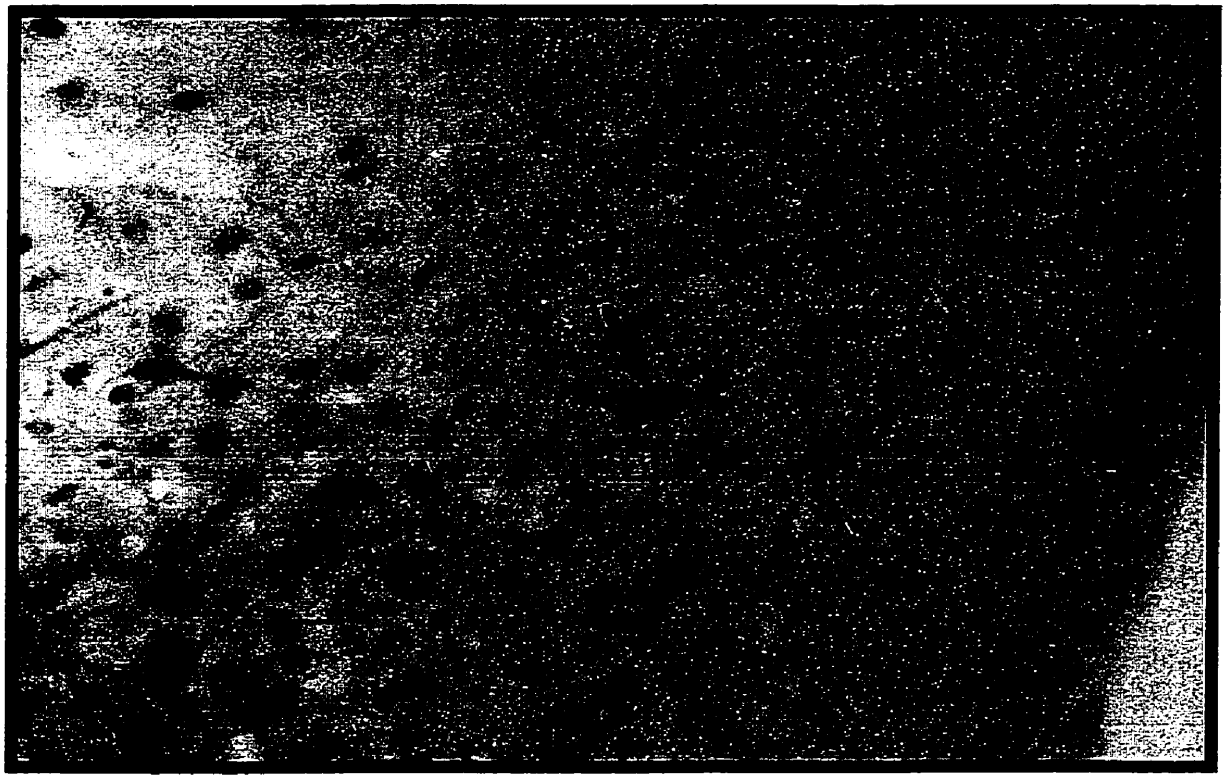


Figure 2: C-fos and Vasopressin Staining in the Paraventricular Nucleus

A high magnification photomicrograph demonstration c-fos and AVP staining in the PVN. A double-labeled neuron is indicated with the arrow.

with a light blue reaction product and the dark staining cells had a more intense blue stain. However, the two types were not clearly dichotomous as NADPH-diaphorase cells stained colours that fell within the blue spectrum between the most lightly and most darkly staining NADPH-diaphorase cells. For this study, all NADPH-diaphorase staining cells were considered, regardless of staining intensity. Double labelled NADPH-diaphorase/c-fos neurons were identified by their blue perikaryon and brown nuclei (Figure 3); double-labelled NADPH-diaphorase/nonapeptide cells contained both reaction products for the immunocytochemical and histochemical stain in the perikaryon so these double stained neurons appeared brown-blue in colour (Figure 4).

1.2.2 C-fos Immunoreactivity and Colocalization with OXY/AVP/NADPH-diaphorase

All stains in this study—OXY, AVP and NADPH-diaphorase—were colocalized with c-fos in the PVN. Colocalized cells (c-fos staining together with either OXY, AVP or NADPH-diaphorase) were most numerous in the medial section of the PVN. These double labelled cells were found scattered throughout the medial PVN, including both the magnocellular and medial parvocellular regions. Double-labelled cells were also located in the rostral region; however, more caudally in the posterior PVN, very few of the c-fos cells were stained with any of the nonapeptides or with NADPH-diaphorase.

On average the number of double labelled AVP/c-fos cells, OXY/c-fos cells, and NADPH-diaphorase/c-fos cells is similar. However, examination through the PVN levels (Figure 5) reveals that slightly more NADPH-diaphorase cells, relative to OXY or AVP cells, were double labelled with *c-fos* in the caudal region (posterior portion) of the PVN; in the rostral

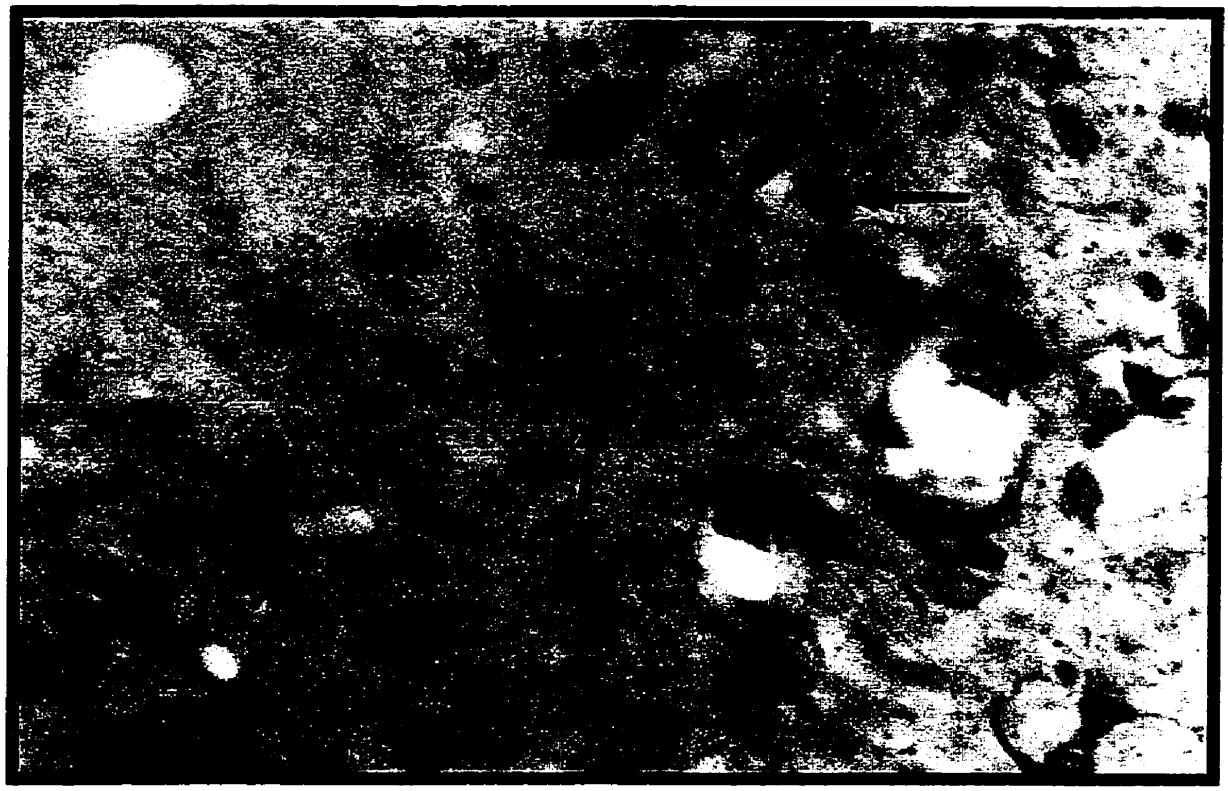


Figure 3: C-fos and NADPH-diaphorase Staining in the Paraventricular Nucleus

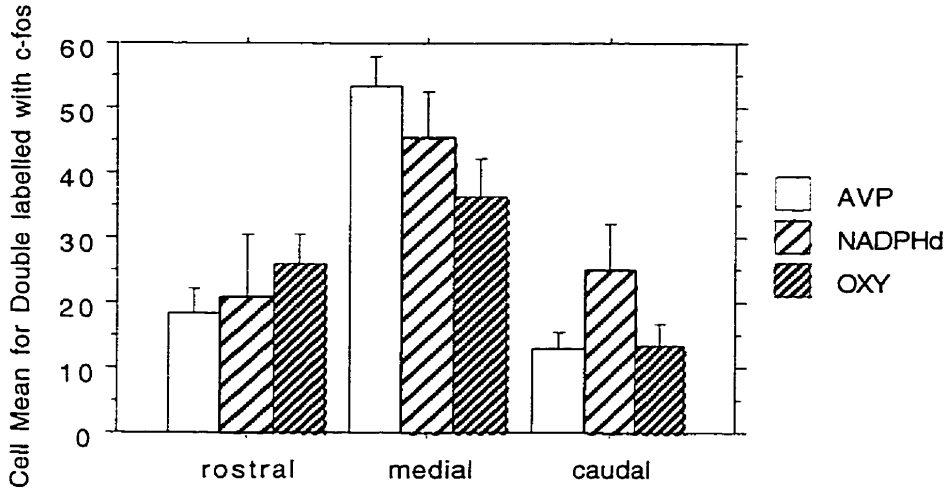
A high magnification photomicrograph of the PVN showing NADPH-diaphorase and c-fos staining. Light and dark NADPH-diaphorase cells colocalized with c-fos are indicated by arrows.

Figure 4: NADPH-diaphorase and oxytocin staining in the supraoptic nucleus

A high magnification photomicrograph showing NADPH-diaphorase and OXY staining. A double-labeled neuron is indicated with an arrow.

Figure 5: C-fos Immunoreactivity and Colocalization with OXY/AVP/NADPH-diaphorase

Histogram showing the ratio of NADPH-diaphorase positive neurons (n=5), OXY-immunoreactive neurons (n=6), and AVP-immunoreactive neurons (n=6) in the rostal, medial and caudal PVN that expressed fos after 100μg LPS i.v. Values are shown are mean +/- SE.



regions, there are more OXY/c-fos double labelled cells; and in the medial region, AVP/c-fos double labelled cells predominate.

As shown in the means table in Table 1, none of the substances investigated accounted for the majority of c-fos staining cells. The number of double stained cells accounted for less than a third of the fos staining population in all staining scenarios. This percentage dropped from the rostral through to the caudal regions of the PVN (for example, only about 13% the fos staining population in the caudal regions were double stained for AVP/c-fos compared to the 25.5% in the rostral region). However, when examining the number NADPH-diaphorase, AVP or OXY cells that were colocalized with c-fos, the overall percentage was higher. On average, 39.3% of the OXY, 32.8% of the NADPH-diaphorase and 32.6% of the AVP populations in the PVN of LPS-treated animals were doubled labelled with c-fos.

1.2.3 Effect of i.v. LPS on Expression of Vasopressin in the PVN

Systemic endotoxin treatment had no significant effect on the the number of AVP-expressing neurons in the PVN (Figure 6).

1.2.4 Effect of i.v. LPS on Expression of Oxytocin in the PVN

There was a significant increase ($p<.05$) in the number of oxytocin expressing cells in the rostral region of the PVN (anterior parvocellular portion) after treatment with i.v. LPS. The increase in the medial regions was not significant; and there was no difference in the caudal region of the PVN (Figure 7).

Table 1: Means table of percentage colocalization in the PVN after i.v. LPS treatment

Table 1a shows the percentage of c-fos population that is colocalized with either OXY, AVP or NADPH-diaphorase after i.v. LPS treatment. Table 1b shows the percentage of OXY, AVP and NADPH-diaphorase cells colocalized with c-fos after i.v. LPS treatment. (where d is the number of double labelled cells)

Table 1a:
% C-FOS population double labelled with AVP, OXY, or NADPH-d (mean ± S.E.)

	Rostral PVN	Medial PVN	Caudal PVN
%d/FOS with AVP (n=6)	25.5 ± 3.8	18.5 ± 1.7	12.7 ± 1.9
%d/FOS with NADPH-d (n=5)	29.8 ± 6.8	23.7 ± 5.8	22.8 ± 2.6
%d/FOS with OXY (n=6)	37 ± 6.2	14.1 ± 1.1	14.7 ± 3.2

Table 1b:
% population double labelled with c-fos (mean ± S.E.)

	Rostral PVN	Medial PVN	Caudal PVN
%d/AVP (n=6)	31.3 ± 4.2	39.4 ± 3.9	27.2 ± 4.3
%d/NADPH-d (n=5)	29.8 ± 8.0	38.9 ± 4.7	29.0 ± 7.2
%d/OXY (n=6)	36.6 ± 4.3	46.6 ± 7.9	34.8 ± 5.0

Figure 6: Effect of i.v. LPS on Expression of Vasopressin in the PVN

Effects of intravenous adminstration of 100μg LPS (circle, n=13) and 0.85% saline vehicle (square, n=4) on vasopressin immunoreactive staining in the rostral, medial and caudal PVN. Values are shown for the cell means +/- standard error. There is no significant difference between experimental and control treatment at any of the levels of the PVN.

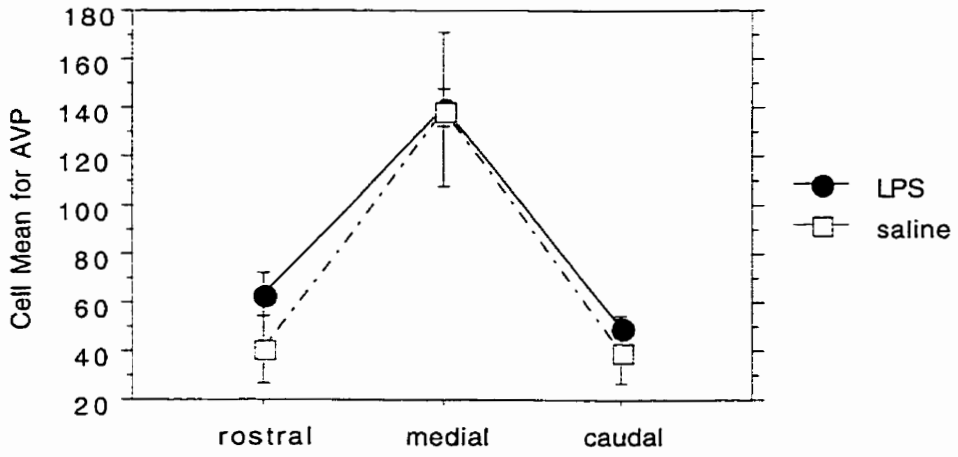
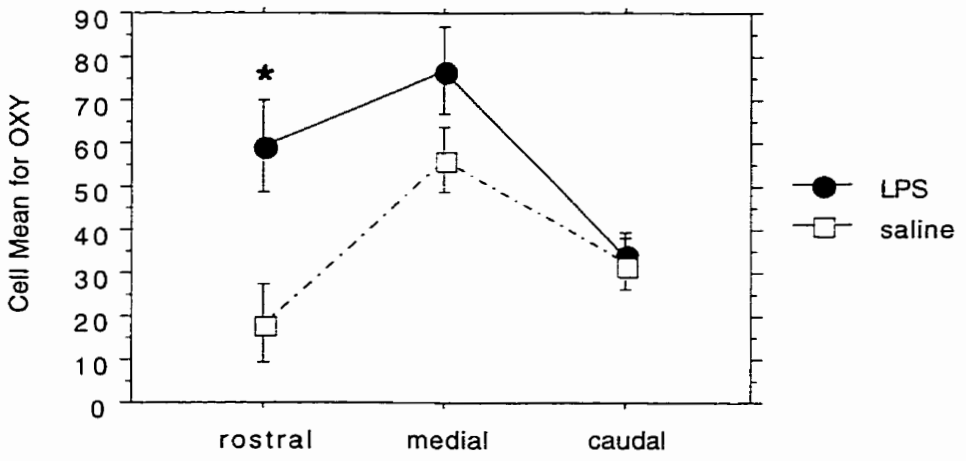


Figure 7: Effect of i.v. LPS on Expression of Oxytocin in the PVN

Effects of intravenous adminstration of 100µg LPS (circle, n=10) and 0.85% saline vehicle (square, n=5) on oxytocin immunoreactive staining in the rostral, medial and caudal PVN. Values are shown for the cell means +/- standard error. Asterisk (*) indicates a significant difference between LPS and saline treatment (ANOVA results; Post Hoc Scheffe's proceedure $P<0.05$, $F=6.359$).



1.2.5 Effect of i.v. LPS on Expression of NADPH-diaphorase in the PVN

There was also a significant increase ($p<.005$) in the number of NADPH-diaphorase expressing cells in the rostral PVN after endotoxin treatment. There is no significant difference between the LPS and control treatments in either of the medial or caudal regions of the PVN (Figure 8).

1.2.6 NADPH-diaphorase and Oxytocin/Vasopressin Colocalization

Double-labelled cells were visible in the PVN. The largest number of double labelled cells, for both OXY/NADPH-diaphorase and AVP/NADPH-diaphorase, were located in the rostral regions of the PVN, in the anterior parvocellular part. There is an obvious trend while progressing to the more caudal portions of the PVN for fewer cells to be double labelled.

The number of NADPH-diaphorase and AVP colocalized cells did not vary significantly between the LPS-challenged and saline-control animals (see Figure 9). In the rostral regions these cells accounted for approximately a third of the NADPH-diaphorase and a quarter of the AVP populations. In the caudal regions, only about 5% of the AVP and NADPH-diaphorase populations were double labelled (Figure 10, 11)

However, in the case of NADPH-diaphorase and OXY double labelling, there was a significant increase ($p<0.05$) in double labelled cells in the rostral portion of the PVN after LPS challenge (Figure 12). These double labelled cells accounted for about half of the NADPH-diaphorase population and close to 80% of the OXY stained cells (Figure 13, 14) in the rostral region. As with the AVP/NADPH-diaphorase staining, the number of double

labelled cells dropped while progressing caudally.

Figure 8: Effect of i.v. LPS on Expression of NADPH-d in the PVN

Effects of intravenous adminstration of 100μg LPS (circle, n=15) and 0.85% saline vehicle (square, n=11) on NADPH-diaphorase staining in the rostral, medial and caudal PVN. Values are shown for the cell means +/- standard error. Asterisk (*) indicates a significant difference between LPS and saline treatment (ANOVA results; Post Hoc Scheffe's proceedure $p<0.005$, $F=11.66$).

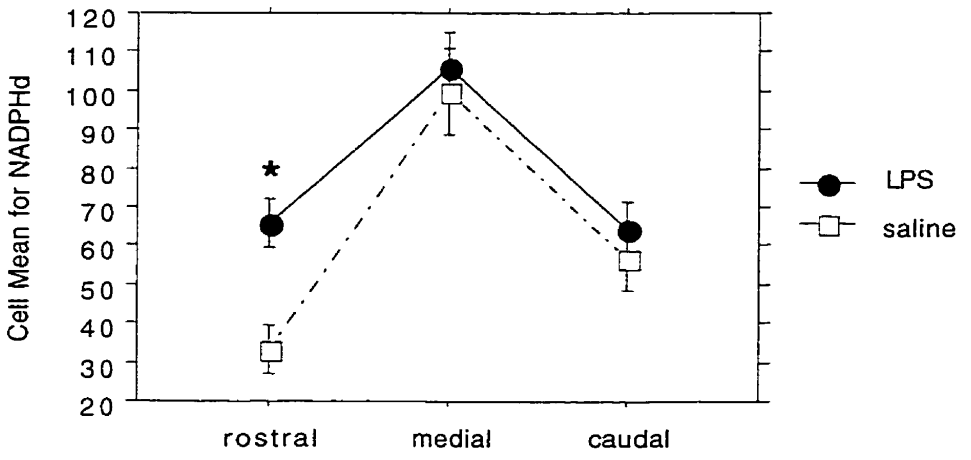


Figure 9: Effects of LPS treatment on colocalization of NADPH-d and AVP in the PVN

Effects of systemic lipopolysaccharide (LPS, 100µg i.v.) or vehicle control (0.85% saline i.v.) on the colocalization of NADPH-diaphorase and AVP staining in the rostral, medial and caudal PVN. Values are shown for the cell means +/- SE; n=7 (LPS, experimental) and n=4 (saline, control).

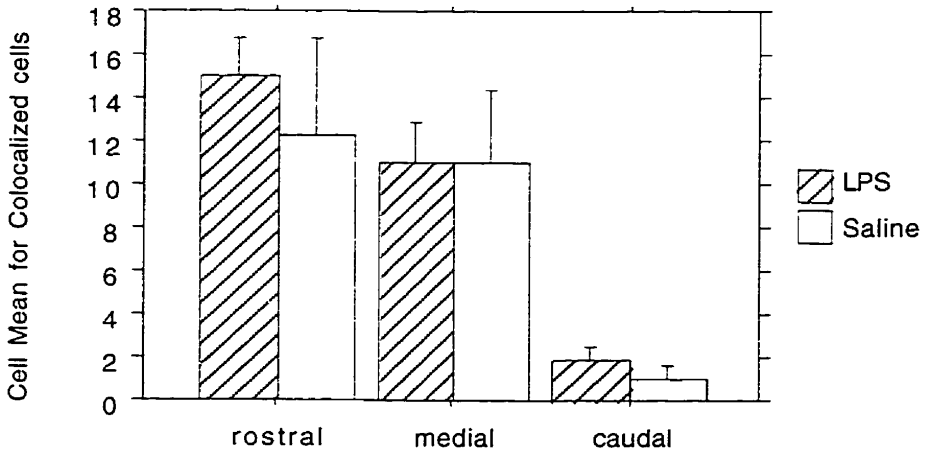


Figure 10:

Percentage of NADPH-d population colocalized with AVP in PVN after LPS challenge

Experimental animals treated with 100μg LPS (n=7); control animals with 0.85% saline (n=4). Values are shown for the percentage (+/- SE) of NADPH-diaphorase-positive neurons located in the rostral, medial and caudal PVN that exhibited AVP immunoreactivity.

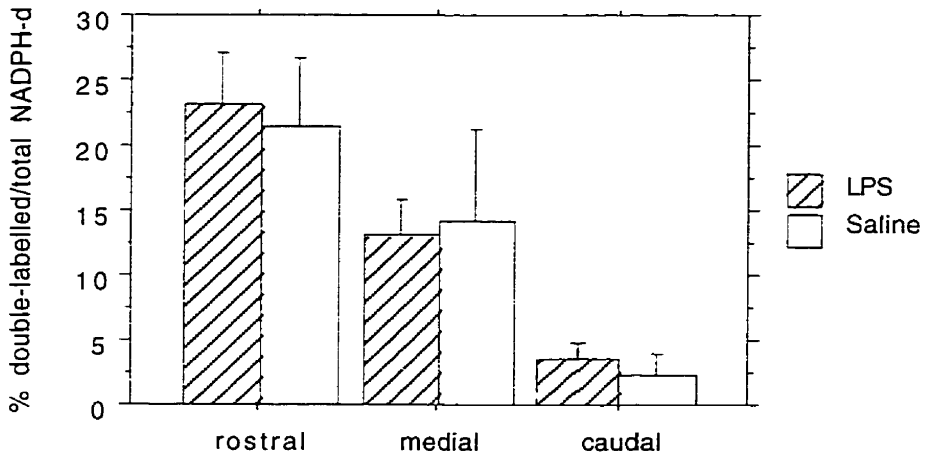


Figure 11:

Percentage of AVP population colocalized with NADPH-d in PVN after LPS challenge

Experimental animals treated with 100μg LPS (n=7); control animals with 0.85% saline (n=4). Values are shown for the percentage (+/- SE) of AVP immunoreactive neurons that contained NADPH-diaphorase located in the rostral, medial and caudal PVN.

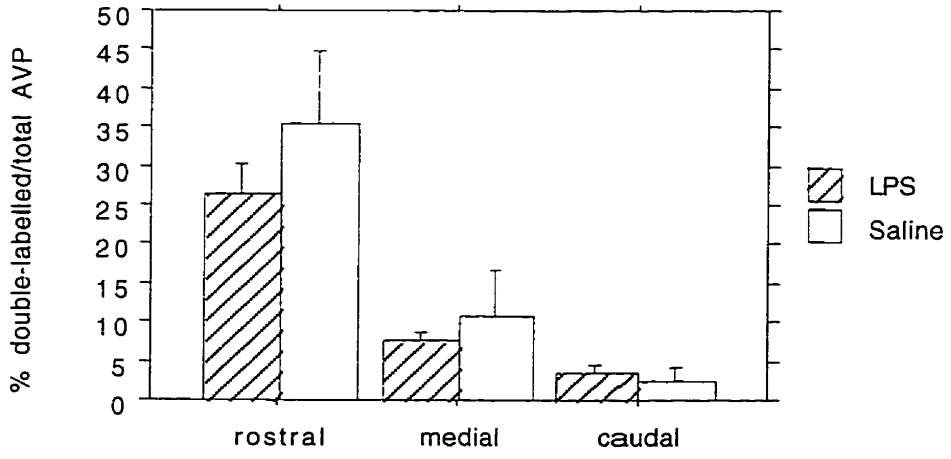


Figure 12: Effects of i.v. LPS on colocalization of NADPH-d and OXY in the PVN

Effects of systemic lipopolysaccharide (LPS, 100µg i.v.) or vehicle control (0.85% saline i.v.) on the colocalization of NADPH-diaphorase and OXY staining in the rostral, medial and caudal PVN. Values are shown for the cell means +/- SE; n=4 (LPS, experimental) and n=4 (saline, control). Asterisk (*) indicates a significant difference between LPS and saline treatment (ANOVA results; Post Hoc Scheffe's proceedure $P<0.05$, $F=6.549$).

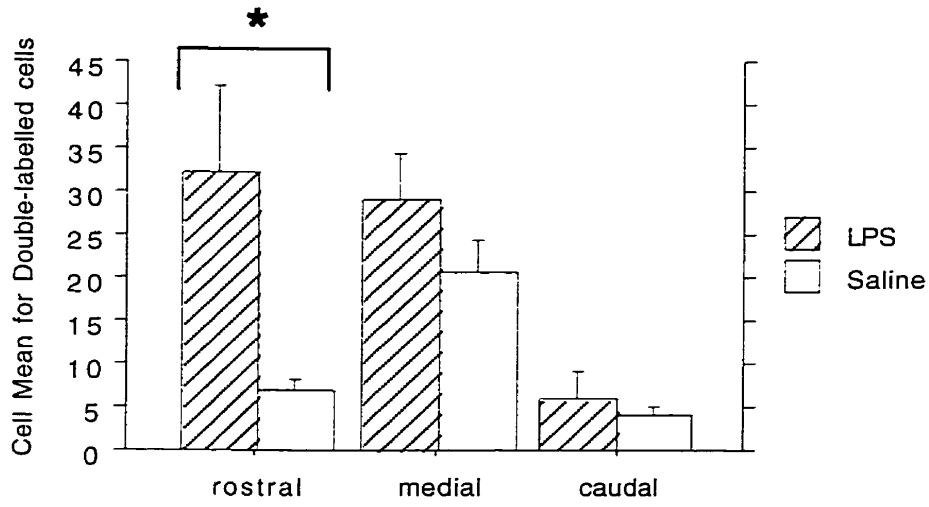


Figure 13:

Percentage of NADPH-d population colocalized with OXY in PVN after LPS challenge

Experimental animals treated with 100µg LPS (n=4); control animals with 0.85% saline (n=4). Values are shown for the percentage (+/- SE) of NADPH-diaphorase-positive neurons located in the rostral, medial and caudal PVN that exhibited oxytocin immunoreactivity.

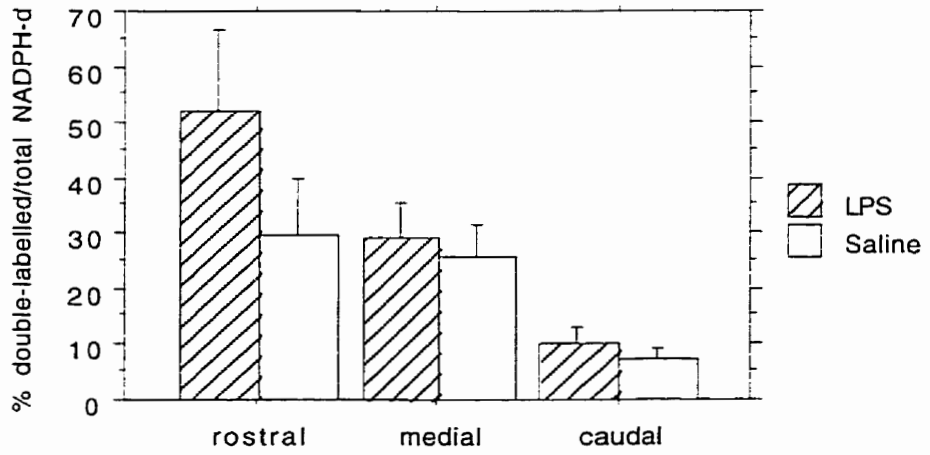
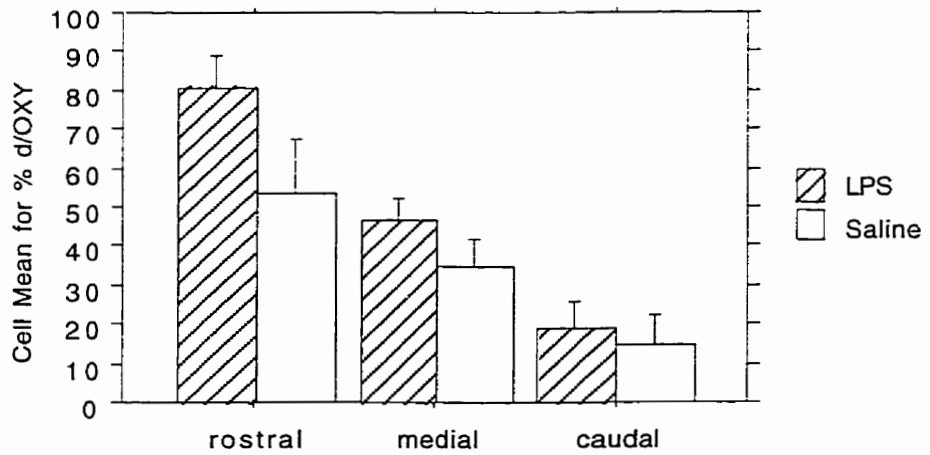


Figure 14:

Percentage of OXY population colocalized with NADPH-d in PVN after LPS challenge

Experimental animals treated with 100µg LPS (n=4); control animals with 0.85% saline (n=4). Values are shown for the percentage (+/- SE) of OXY immunoreactive neurons that contained NADPH-diaphorase located in the rostral, medial and caudal PVN.



1.3 Colocalization Study: Discussion/Summary

AVP, OXY and NADPH-d are colocalized with c-fos in the PVN after i.v. LPS treatment.

This study has identified the pattern of colocalization of c-fos with AVP, OXY or NADPH-diaphorase in the PVN after systemic endotoxin challenge. We report here that c-fos expressing neurons co-stained for OXY, AVP and NADPH-diaphorase in particular divisions of the PVN after intravenous LPS treatment. Hence, we suggest that these nonapeptides and the novel neurotransmitter nitric oxide, may be important in mediating elements of the central stress response to immune challenge. Importantly, none of the staining specificities accounted for the majority of c-fos staining cells, indicating that other neurochemically specific neurons also express c-fos following LPS challenge.

It has been demonstrated that different stressors can result in specific patterns of neuronal activation [56, 122, 123]. Select regions of the hypothalamus and brain are activated in a stressor specific manner and the type of neuropeptide or neurochemicals released and/or utilised may be unique to certain stressors [120, 129, 131]. Previously, both OXY and AVP neurons have been implicated in the central stress response [35, 37, 120]. Assuming that the colocalization of c-fos/OXY and c-fos/AVP is of functional importance in the immune stress response, it can be speculated that OXY and AVP may be important mediators that can modulate that response and, by implication, immune parameters.

Colocalization of NADPH-diaphorase staining cells and c-fos occurred throughout the PVN. The work of Hatakeyama at al. [149] has also noted this colocalization after very large (1000 µg) LPS i.p. administration. Increases in both iNOS mRNA after i.p. LPS [156, 164] and cNOS mRNA after i.v. LPS [145] have also been noted.

This study is one of the few looking at neuronal products in a quantitative fashion. A number of studies have examined changes in mRNA levels in PVN neurons after endotoxin treatment. Investigators have found increases in the following transcripts after an immune challenge: CRF mRNA, CRF-receptor mRNA, OXY mRNA, AVP mRNA, IL-1β mRNA, iNOS mRNA, cNOS mRNA, c-fos mRNA and NGF1-B mRNA [155, 156, 148, 164, 145, 142] Thus, multiple cell substances are activated during the immune stress: suggesting a stress processing mechanism which is extremely complex.

Although mRNA studies add to the understanding of the central response to a peripheral immune stimuli, the presence of transcripts does not always equate with increased protein production and release. In some cases, increases in neuronal c-fos mRNA have not resulted in increased c-fos protein production [99]. There are also issues of time course to consider: a nucleus that has an increase in mRNA must translate the transcript to have an increase in neuronal protein. Therefore examination of neuronal proteins give a view into the immediate state of the neuron, and not just their transcript changes that may result in future changes in the neuronal output.

Since this work began, there has been one publication specifically looking at colocalization of c-fos with other neuropeptides (proteins, not mRNA) in the PVN after i.v. LPS treatment. There are some consistencies as well as some contrasts between the two studies. Sagar et al [139] have also done double staining of fos and OXY and AVP after an intravenous endotoxin challenge. Their counts (n=2) in a representative PVN region found that 49% and 58% of oxytocin neurons were fos positive, while only 4% and 6% of the vasopressin neurons

were fos-positive. Our medial counts for OXY/c-fos double-labelling in the PVN are similar to that observed by Sagar; but our AVP/c-fos colocalization is significantly higher. The difference between results may be due to differences in our paradigms—in particular our differences in LPS dosage (our dosage was double that used by Sagar's group). The expression of c-fos is dependent on LPS dose; increases in dose being associated with increased and more widespread c-fos expression [134, 150]. For example, in studies with massive intraperitoneal LPS doses (250 μg/100 g), increases in AVP mRNA have been noted in the PVN that are not seen following lower dose treatments [155].

Staining for OXY and NADPH-diaphorase is significantly increased after i.v. endotoxin challenge, but not AVP.

Both OXY and NADPH-diaphorase staining in the rostral PVN were significantly increased after endotoxin challenge. These results support previous studies which showed that plasma oxytocin levels increase markedly after endotoxin challenge [261], and our observed increase in the number of OXY stained neurons after LPS challenge strongly implicates a role for OXY in the neuroimmune response to i.v. LPS. Since our preliminary report of LPS-induced increase in OXY protein expression [147], others have confirmed an increase in OXY mRNA in the PVN of sheep after i.v. LPS [142]. Some reports have not supported this conclusion; and pig reportedly show no increase in OXY after LPS treatment [260].

There has been speculation that OXY may have potentiating or modulating effects on CRF release and thus impact upon the hypothalamic-pituitary-adrenal axis which is a fundamental response system activated by stress [36, 53, 128, 129, 131, 140, 141]. OXY is a

known secretagogue of ACTH and has been shown to be colocalized with CRF in the magnocellular division of the anterior PVN [131, 313]. Alternatively, the effect of these peptides may be mediated at the level of the median eminence and thereby influence the level of CRF in the portal circulation and the subsequent activation of the pituitary [77, 129].

There are a number of potential roles for oxytocin in the neuroimmune response. Some oxytocinergic neurons in the PVN innervate the dorsal vagal complex [143] and OXY has been shown to have effects upon visceral target organs innervated by the vagus [144]. Oxytocinergic efferents of PVN origin have been found to innervate intermedial cell column of the spinal cord and the sympathetic pre-ganglionic neurons [77]. Research in our lab [119] has shown that the the effects of i.c.v. prostaglandin E2 (an immune mediator) on splenic nerve activity can be selectively suppressed by central pretreatment with an oxytocin receptor antagonist. It is possible that oxytocinergic central release from these hypothalamic efferents may modulate events in immune organs, affecting their response to an endotoxin challenge. As previously mentioned, OXY can also have a potentiating effect on CRF response [140, 141].

Neurons that project from the PVN to the spinal cord and dorsomedial medulla contain a variety of neurochemicals, including oxytocin, vasopressin, somatostatin, and enkephalin [137, 143]. Studies examining the presence of the nonapeptides in these descending projections have found that there are many more oxytocin-staining neurons than there are vasopressin ones. Most of these descending neurons are located in the the parvocellular regions of the PVN, although some are found scattered in the magnocellular regions [143].

Although our lack of significant change in AVP protein expression after LPS treatment is supported by reports that intravenous endotoxin produces no significant changes to the vasopressin mRNA levels in the PVN of rats [145] or sheep [142], there are a number of conflicting reports regarding changes in AVP levels after immune challenges. The lack of change in AVP expression does not rule out a role for AVP in the neuroimmune response. Treatment of hypothalamic explants with IL-1 have resulted in contrasting results, including increase in AVP release if pretreated with ACh [157] and other reports of no change in AVP release [159]. Rivest et al [155] have found that after a massive (250 μg/100 g) dose of i.p. endotoxin there is an increase in AVP mRNA in the parvocellular PVN.

AVP is a known CRF-secretagogue, capable of inducing ACTH release from the anterior pituitary [129]. These effects could be mediated at the neuronal level within the region of the PVN where AVP has been shown to be colocalized with CRF in parvocellular elements [129]. The ACTH response to endotoxin has been shown to have an AVP component [146], therefore it is possible that AVP has a role to play in the neuroimmune circuit that does not require increased AVP neuropeptide production or expression.

The increase of NADPH-diapohorase indicates a role for nitric oxide in the central response to peripheral endotoxin. There are many possible functions that nitric oxide may serve in immune challenged situations.

Synaptic plasticity such as long-term potentiation in the hippocampus [130, 132] and of long-term depression in the cerebellum [133] are thought to be in part mediated by NO. Importantly, the involvement of NO in these processes imply that NO is associated with

enhanced neuronal functioning. The role of NO as transmitter is controversial, but the possibility of a potentiator or enhancer of signalling has not been totally dismissed [114, 121, 130, 132, 133]. It is possible that within the hypothalamus, NO functions in a similar manner, to enhance neuronal function. The ability to modulate hypothalamic function in a precise and fast manner, as would be the case with NO, would be important for the hypothalamic regulatory system since the hypothalamus must maintain homeostasis under a variety of complex, but specific challenges.

NO is also known to be a potent activator of guanylyl cyclase, both intercellularly [126, 127] and intracellularly [124]. Guanylyl cyclase is the enzyme that converts GTP into cGMP, an important second messenger [121]. Possible targets for cGMP may be ion channels, cGMP-dependent protein kinases, and cGMP-stimulated or inhibited phosphodiesterases [121]. These targets may in turn be responsible for affecting ionic balance in the neuron (in particular the Ca2+ current) and create hyper- or hypopolarized environments. If NO acts in this manner in the hypothalamus it could then be involved in the modulation of neurotransmitter (including neuroendocrine) release. NO also has an amplification effect on the induction of PKA through a Ca2+ mechanism [47] It does not appear that NO mediates its effects directly on transcription, but it can amplify the effects of Ca2+ signals on transcription [47,48], including that of c-fos induction [47]. The possible effects of NO on c-fos induction may also be amplified through a cAMP-based mechanism [47, 89, 91].

Recent literature has suggested that NO may be an important vasodilator in the cerebral circulation [125]. Since this is the function of EDRF (NO) on the peripheral vascular system, it may be an appropriate one for intracerebral NO. Perhaps NO within the hypothalamic

cells may, upon release, affect the permeability of cerebral blood vessels, thereby altering the transport of immune factors.

Colocalization of NADPH-diaphorase and OXY in the rostral PVN occurs after i.v. LPS.

Studies of the rat hypothalamus have shown that in basal situations oxytocin-immunoreactive neurons in the PVN express NADPH-diaphorase activity, but very few vasopressin-immunoreactive neurons contain NADPH-diaphorase [279]. We have shown, and this has been confirmed by the work of Hatakeyama et al [149] that after LPS treatment this colocalization increases. The Hatakeyama study [149] only investigated the medial PVN, where they found increased colocalization of NADPH-diaphorase and OXY after peripheral LPS challenge; our results showed that colocalized cell means are increased in both the medial and the rostral PVN, but was statistically significant only for the rostral region.

It is possible that the colocalization of these two substances in the same neurons indicates a neuromodulatory relationship between NO and OXY. Oxytocin has been shown to stimulate the production of nitric oxide [282], and there is also evidence that nitric oxide can attenuate the release of oxytocin [281, 286, 284] and vasopressin [283]. Interestingly, there is also evidence that NO can activate PVN oxytocinergic neurons [286, 287]. Rettori et al [280] suggest that NO and oxytocin may interact in an ultrashort-loop negative feedback, with oxytocin stimulating NOS, resulting in an increased release of NO which in turn stimulates the release of other neurotransmitters. The released NO then acts back on oxytocinergic terminals to suppress the release of oxytocin. It is possible that NO and oxytocin function in a complex neuromodulatory role after a peripheral immune challenge resulting in both inhibitory and stimulatory effects.

The reason for the up regulation of these specific substances in the PVN after an immune challenge may be due to their intermediaries. For example, central PGE2, a known immune mediator which has been shown to induce the release of OXY [290, 285] in the brain, may be involved in this LPS-induced increase of OXY in the PVN. Increased OXY may in turn induce increased NO in the oxytocinergic neurons [280]. It is also possible that both OXY and NO are up regulated within the same neurons as a result of having the same inducer. Norepinephrine has a stimulatory effect on OXY [37, 285, 289] and NO [157, 180] release in the hypothalamus. Since the PVN receives innervation from medullary noradrenergic neurons [137, 288] and this pathway is activated during immune challenges [135, 251], it is possible that norepinephrine is an important mediator of the increase in colocalized NO and OXY in the PVN after peripheral endotoxin challenge .

Technical Consideration: Intravenous versus intraperitoneal administration.

The majority of studies of the central effects of peripheral immune challenges have either focused on LPS treatment of either an intravenous [139, 145, 142, 150, 162] or intraperitoneal [148, 149, 155, 156, 164] nature. Although these studies are useful in expanding our knowledge of the central effects of peripheral immune challenges, the results may be limited to the method of endotoxin delivery. Studies in our lab [134], and subsequent research by others [165], have shown that subdiaphramitic vagotomy can have a significant and dramatic attenuation on c-fos expression and immune measures after intraperitoneal LPS treatment. However, vagotomy has little to no effect on similar doses of intravenous endotoxin, which would seem to indicate separate activational pathway(s) exist between the periphery and central nervous system depending on the route of endotoxin exposure.

Maier et al [165] have attributed the variation between i.v. and i.p. LPS to the location of the vagotomy (i.e. subdiaphramic lesions). They proposed that i.v. LPS can activate c-fos in the brain due to vagal terminals in the lung that are left intact after a subdiaphramatic vagotomy. Since rats are unable to survive after a cervical vagotomy, they have been looking to confirm this hypothesis by examining the effects of direct injection of endotoxin into the lung in subdiaphramatic vagotomised animals. As yet they have not found c-fos expression in the brain after these treatments [personal communication S Maier to D Nance]. Recently, contradictory reports have been made as to the importance of the area postrema, the circumventricular component of the dorsal vagal complex, in mediating c-fos expression induced by intravenous IL-1. One study [314] has indicated that lesions of this area attenuate LPS-induced c-fos expression in the PVN, whereas another study [166] has reported no effect. Further investigation is required to determine the function of the vagus in the central response to i.v. endotoxin.

Experiment 2: Role of nitric oxide in the LPS-induced c-fos expression and in HPA activation.

2.0 Introduction/Rationale

The expression of c-fos can be used as an experimental marker of neuronal activation occurring in response to various physiological challenges. Endotoxin is a potent stimulator of numerous immunological and physiological systems. It has been shown that administration of lipopolysaccharide (LPS) either i.p., i.v. or i.c.v. results in a highly localised expression of c-fos within the hypothalamus, including the paraventricular nucleus (PVN) [135].

Nitric oxide (NO) is not a typical neurotransmitter. However, this toxic, free radical gas with a half-life of about four seconds is proving to be a pervasive messenger molecule. Vascular smooth muscle relaxation [189], inhibition of platelet aggregation [191], enhancement of cytotoxicity [192] and the modulation of neurotransmission [121] are just a few of the biological actions attributed to NO.

The enzyme responsible for the production of NO is nitric oxide synthase (NOS) [187]. In the hypothalamus, both the PVN and SON show NOS staining, and are therefore sites of NO production [199]. Previous studies in our lab have shown that levels of NADPH-diaphorase, a marker of NOS, were increased in the anterior parvocellular PVN after intravenous LPS treatment and that this histochemical marker was colocalized with and adjacent to c-fos expressing neurons. Others have verified these results showing increased iNOS mRNA [156] and increased cNOS mRNA [145] after systemic endotoxin challenge.

In order to examine in more detail the role of nitric oxide in the c-fos induction by immune stimuli, the effects of specific inhibition of central NOS activity by i.c.v. pretreatment with the N nitro-L-arginine methylester (L-NAME) on the central c-fos response to systemic endotoxin was examined. In addition, the effects of central administration of S-nitroso-N-acetylpenicillamine (SNAP), a nitric oxide donor, was also examined.

2.1 Materials and Methods

2.1.1 Treatment

Adult Sprague-Dawley male rats (300-380 g, Charles River) were used as subjects. Stainless steel cannulas were implanted into the left lateral ventricle. Cannulated animals were housed individually in a 12:12hr light/dark cycle with food and water ad libitum. Experiments were preformed no sooner than six days post-surgery.

To investigate the effects of central nitric oxide synthase inhibition on c-fos expression, experimental animals received 50 µg N nitro-L-arginine methylester (L-NAME) (Sigma) i.c.v. 15 minutes prior to an i.v. injection of either 10, 20 or 40 µg lipopolysaccharide (E.Coli, Sigma) via the tail vein. Control animals received 50 µg D-NAME, the inactive enantiomer of NAME, 15 minutes prior to an i.v. injection of either 10, 20 or 40 µg lipopolysaccharide. Two hours following injection, the animals were deeply anaesthetised, and then perfused. Brains were removed for further processing (specifics on these procedures are located in *Materials and Methods* section).

S-nitroso-amino-penicillamine (SNAP), a nitric oxide donor, was dissolved in vehicle of 6.5% absolute alcohol in 0.85% saline. Three injections of either SNAP (experimental; n= 11) or vehicle (control; n=8) were administered. Each injection was separated by 15 minutes. Fifteen minutes after the final injection, tail blood was collected and analysed for corticosterone content (specifics on RIA in *Materials and Methods* section).

2.1.2 Immunohistochemical Procedures

Coronal sections (40µm) of the brain were cut on a freezing microtome.Representative

sections through the hypothalamus were selected for immunohistochemical processing. Sections were incubated overnight at room temperature with rabbit antibodies to c-fos (1/15,000 Santa Cruz Laboratories). Sections were incubated for 18-20 hours in the antibody and then visualised via the PAP technique with DAB as a chromagen (details on immunocytochemical procedures in *Materials and Methods* section). Sections were then floated onto slides and coverslipped. Cell counts were made of comparable levels of the medial PVN where c-fos has been shown to be maximally induced in systemic LPS-treated animals [135].

2.2 Results and Analysis

2.2.1 Effects of the NO inhibitor L-NAME on LPS-induced c-fos expression.

Since our previous studies had used 100 μg LPS i.v., we initially preformed a series of NAME studies with this dose of endotoxin. We could not detect any variation between the c-fos expression of experimental (L-NAME) or control (D-NAME) animals (results not shown). Since our dose was originally chosen for maximal c-fos response, we decided to try a more moderate dose of 40 μg which, based on previous work in our lab [134], would elicit a significant and substantial response of c-fos in the PVN.

Pretreatment with 50 μg L-NAME i.c.v. prior to the 40 μg LPS i.v. resulted in a minor and insignificant decrease in c-fos expression in the PVN (mean difference = 45; $p > 0.1$). Since L-NAME appeared to be having some, albeit modest, effect on the c-fos expression, a still lower dose of LPS was examined. At 20 μg LPS i.v., pretreatment with L-NAME resulted in a significant decrease in endotoxin-induced c-fos. At a dose of 10 μg LPS i.v, L-NAME pretreatment continued to produce a significant decrease relative to controls (Figure 15, 16, 17).

Although 10, 20 and 40 μg of LPS induced increasing number of c-fos in the control D-NAME animals (mean c-fos at 10 μg=353 ± 35; mean c-fos at 20 μg=386 ± 35; mean c-fos at 10 μg=390 ± 40) these differences between the number of c-fos staining cells was not significant.

2.2.2 The effect of the NO donor, SNAP on c-fos and plasma corticosterone

Animals treated with SNAP i.c.v. had no change in c-fos expression in the PVN compared to controls. Intracerebroventricular treatment with other nitric oxide donors, including

Figure 15: Effects of central inhibition of nitric oxide synthase on LPS-induced c-fos expression in the PVN are LPS-dose dependent

Central NOS inhibition by i.c.v. L-NAME resulted in a significant attenuation of LPS-induced c-fos expression in the PVN after an LPS challenge of 20µg i.v. or less. Values are shown for the cell means +/- standard error. Asterisk (*) indicates a significant difference between L-NAME and D-NAME treatment (ANOVA results; Post Hoc Scheffe's proceedure; $p<0.05$).

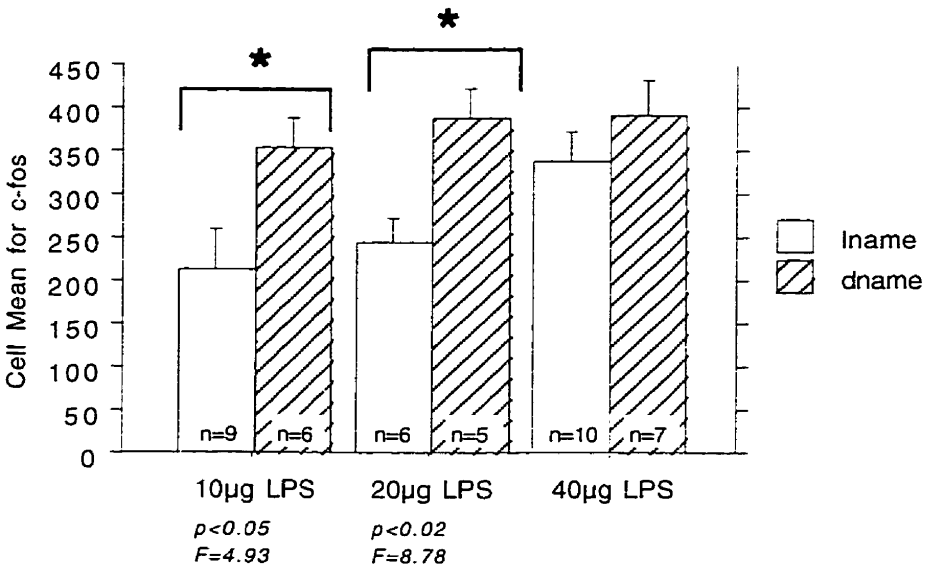


Figure 16: Central nitric oxide synthase inhibition can attenuate endotoxin-induced c-fos production at low doses of LPS

Photomicrographs showing the effects of L-NAME, a NOS inhibitor (Photo A, top) or D-NAME, and inactive enantimer (Photo B, bottom), on LPS-induced c-fos in the PVN after a 10μg i.v. dose of LPS. The c-fos production is significantly affected by central inhibition of nitric oxide (see Figure 15).

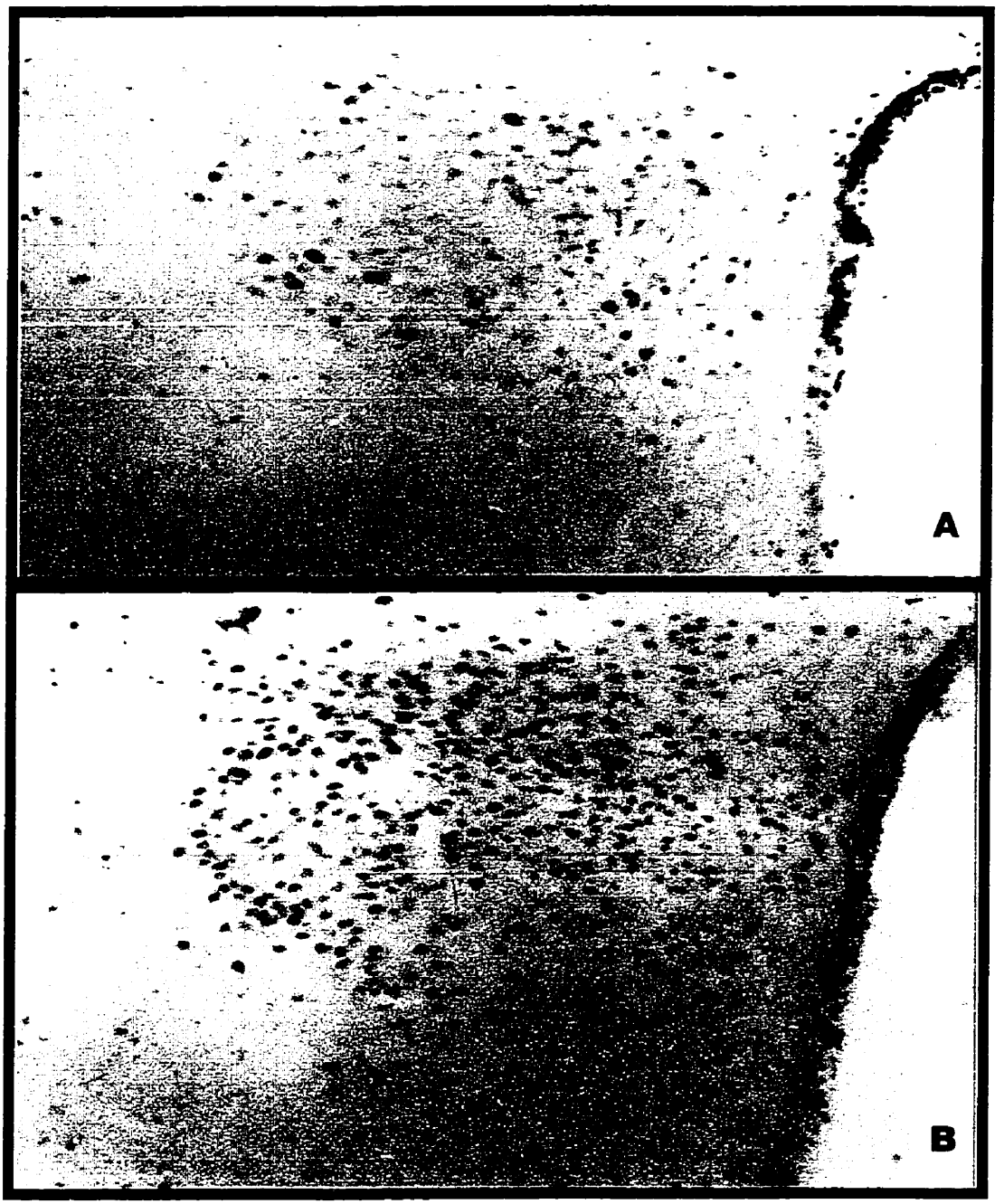
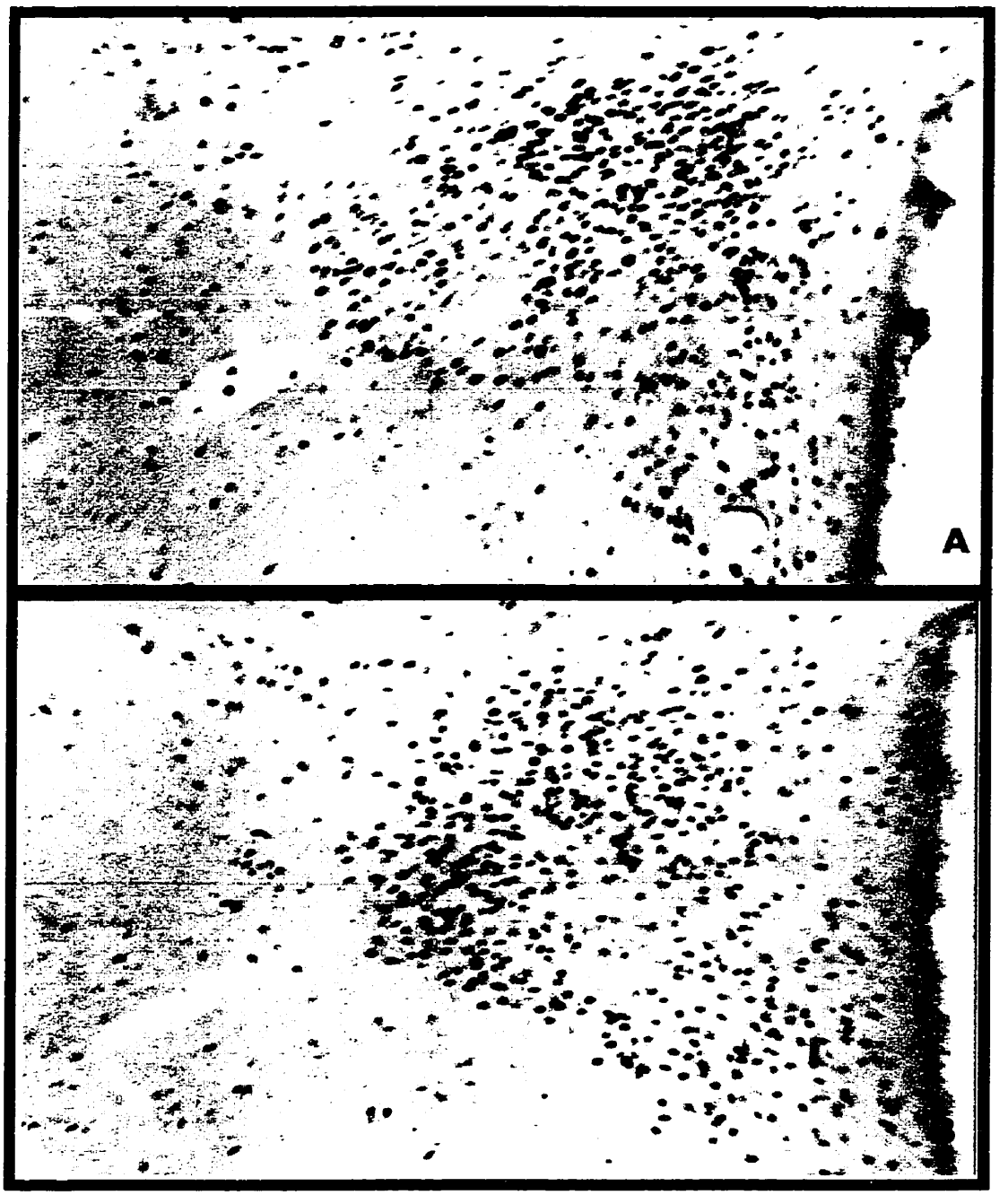


Figure 17: At high endotoxin doses, central nitric oxide synthase does not inhibit LPS-induced c-fos production in the PVN

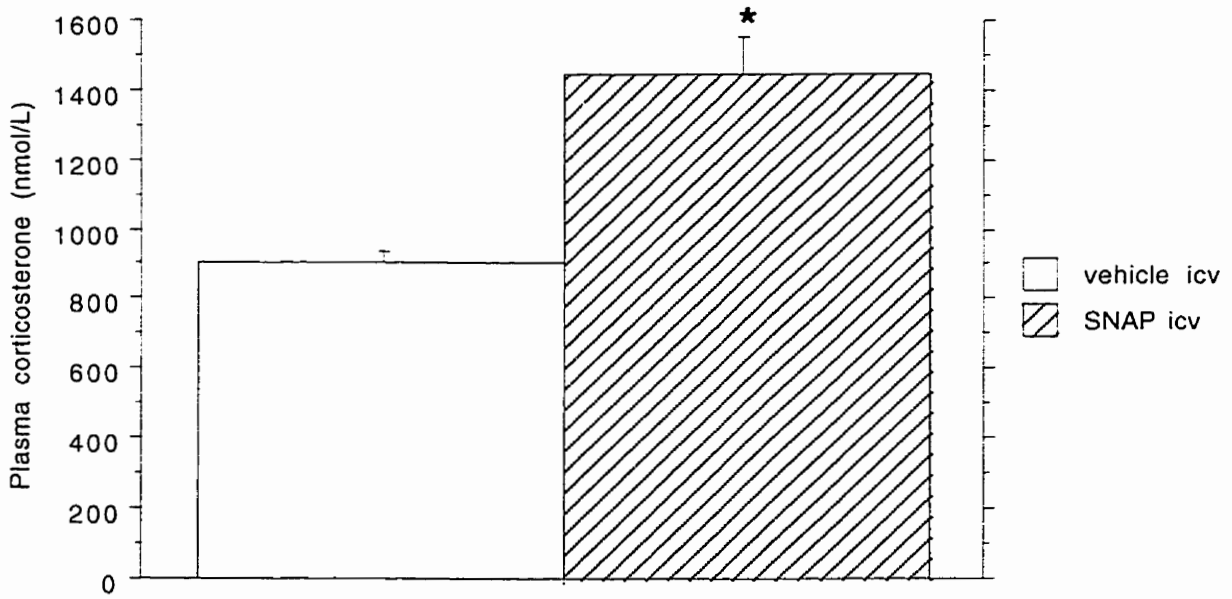
Photomicrographs showing the effects of L-NAME, a NOS inhibitor (Photo A, top) or D-NAME, and inactive enantimer (Photo B, bottom), on LPS-induced c-fos in the PVN after a 40μg i.v. dose of LPS. The c-fos production is not significantly affected by central inhibition of nitric oxide at this high dose.



sodium nitroprusside, also failed to elicit c-fos in the PVN (not shown). However, central treatment with SNAP i.c.v. resulted in a significant increase ($p<0.001$) in plasma corticosterone compared to i.c.v. vehicle treatment (Figure 18).

Figure 18: Effect of centrally administered SNAP, a nitric oxide donor, on plasma corticosterone levels

This graph shows the effects of central administration of S-nitroso-amino-penicillamine (SNAP), a nitric oxide donor, on plasma corticosterone levels. Experimental subjects received three injections of 30μg SNAP i.c.v. spaced 15 minutes apart (n=11), control animals received three injections of the vehicle (6.5% absolute alcohol in 0.85% saline) (n=8). Asterisk (*) indicates a significant potentiation in plasma corticosterone levels after SNAP treatment compared to vehicle (ANOVA results; Post Hoc Scheffe's proceedure $p<0.001$, $F=15.626$).



2.3 Nitric Oxide Study: Discussion/Summary

Central inhibition of nitric oxide synthase results in the attenuation of endotoxin-induced c-fos expression in the PVN.

We report here that central injection of L-NAME, a nitric oxide synthase inhibitor, inhibited LPS-induced c-fos in the paraventricular nucleus and this inhibition was LPS dose-dependent. Doses of 10, 20 or 40 μg LPS induced comparable levels of c-fos in the brain, but only the 10 and 20 μg doses were significantly inhibited by L-NAME. This suggests a functional role for central nitric oxide in low dose LPS-induced neuronal changes.

The lack of inhibition at higher doses may be due to extra-immunological effects induced by high doses of LPS. It is known that endotoxin has physiological effects as well as immunological ones, including effects on the gastrointestinal and cardiovascular systems [review 254], activation of which may also result in additional hypothalamic c-fos production [58, 100].

Assuming that the inhibition of LPS-induced c-fos expression by L-NAME is indicative of inhibition of the central response to peripheral immune challenge, then our results support an activational role for central NO in immune-CNS directed signalling. This is supported by other nitric oxide inhibitory studies: studies with NOS inhibitors have resulted in reduction in IL-1-induced CRF secretion in hypothalamic section studies [237] and hypothalamic cell culture studies [241]. However, these results seem to be in direct disagreement with the results of Costa et al [238] where NOS inhibitors had no effect on IL-1 induced CRF release, and indeed, NO donors were shown to inhibit CRF release.

Even further contradictory results come from in vivo studies by Rivier at al [183] where systemic administration of L-NAME alone results in a very small but significant increase in plasma ACTH, and that NOS inhibition potentiated the IL-1-induced ACTH response [184]. This result only appears contradictory if one assumes that our L-NAME, which attenuated endotoxin-induced c-fos expression in the PVN, is indicative of an inhibition of the HPA response to immune stimuli. Since the function of c-fos in the central immune response is unknown, we do not know if c-fos is involved in up-regulation or down-regulation of the HPA in response to immune stress. It is quite possible that c-fos induction occurs in both activational and inhibitory pathways. However, our results with central administration of SNAP would seem to indicate that central NO has a role to play in the up-regulation of the HPA.

It is best to be cautious when comparing the results of systemic and central treatment of L-NAME on brain activity. Iadecola et al [242] have shown that short-term systemically administered L-NAME in the rat results in a maximal inhibition of brain NOS activity by only 50%, and this partial inhibition develops over 1-2 hours. This slow time course may be due to the slow transport of L-NAME over the blood-brain-barrier [243]. Therefore the ability of systemic L-NAME treatment to modify central NO function is limited. It is also important to consider that systemic application of NOS inhibitors will affect the peripheral as well as central response to immune stimuli, and therefore central variations after systemic L-NAME may be the result of modifications to the peripheral immune response. It is likely that the differences in the results reported in this study and those reported by Rivier [183, 184] are due to our different modes administration of L-NAME.

Research seems to be pointing to a complex role for central nitric oxide where NO can either result in inhibitory or stimulatory action depending on the neuronal system activated. For example, glutamic acid induced (NMDA-mediated) [179] and norepinephrine induced [180] release of luteinizing hormone-releasing hormone (LHRH) from the HPA is stimulated by NO; NO also increases the potassium-induced release of GABA which inhibits LHRH release [176]. Another potentially dichotomous role for NO can be found in the release of histamine. NO acts to increase acetylcholine release which inhibits the release of histamine from the hypothalamus; but NO also induces increased glutamate release which stimulates histamine release [177]. The results of the current experiment indicate a role for nitric oxide in the induction of c-fos two hours after a peripheral immune challenge. However the function of that induction is not known, and it may be that some neurons are upregulated by this c-fos induction and others downregulated. However, it is possible that NO may have a different effects on c-fos induction under different stimuli, or perhaps under different time courses.

Sandi et al [237] suggest that seemingly contradictory effects of NO inhibitors on CRF release are attributable to experimental protocol. They suggest that pretreatment is necessary for NOS inhibitors to be effective, this is supported by studies in learning where the effects of NOS inhibitors are only detectable if the neurons have been pretreated for a minimum of 15 minutes prior to other experimental protocol [262].This is supported by their own work where simultaneous treatment of IL-1 and NOS inhibitors resulted in a lack of effect on CRF release, whereas pretreatment with the inhibitor showed an inhibitory effect [237]. As well, insufficient pretreatment has been implicated in conflicting studies that have examined the effects of NOS inhibitors on somatosensory -induced cerebral blood flow [242].

The experimental evidence seems to point to a modulatory role for NO, with many possible effects in the central response to immune challenge. At different points along the central cascade response to peripheral immune stimuli NO may act as an inhibitory or stimulatory mediator. The function of NO may be entirely dependent on the environment in which it is produced. Undoubtedly, as better understanding of when and where along this cascade nitric oxide is activated will lead to a clearer understanding of the overall central response to peripheral endotoxin.

Central treatment with SNAP, a NO donor, does not induce c-fos, but does increase plasma corticosterone levels.

The ability of NO donors to induce c-fos in neuronal cell cultures has been reported [47, 87, 244], however the mechanism by which the NO donors work seems to vary between sub-populations of neurons. In the case of embryonic striatial cell culture, SNAP treatment alone will induce c-fos mRNA [244], but in PC12 neuronal cultures SNAP alone has no effect, but will potentiate c-fos expression following calcium influx due to the calcium ionophore A23187 [47]. Our results would indicate that in the in vivo model, SNAP treatment alone is not sufficient to induce c-fos expression in the PVN.

The lack of c-fos induction by SNAP may also be attributable to the vehicle used. Attempts to dissolve SNAP in saline were unsuccessful and as a result, an alcoholic vehicle was used. Alcohol has been shown to attenuate stress-induced c-fos expression in the rat brain [181] and basal c-fos levels in the PVN [182]. This could account for the very "clean" immunocytochemistry results in both control and experimental animals.

The release of plasma corticosterone after SNAP treatment would indicate that this NO donor activated the HPA axis, although this was not reflected in c-fos activation of the PVN. Since c-fos expression is not an absolute requirement for neuronal activation, it is possible that the NO donor modified neurotransmitter release directly from the synapse, either affecting CRF release directly, or the action of a CRF agonist. Central administration of SNAP has been shown to elevate plasma vasopressin levels [235]. This activity may facilitate the increased corticotropin levels we observed, as vasopressin is a potential mediator of CRF release, and AVP and CRF are colocalized in the PVN [129]. As well, SNAP has been shown to induce a febrile response when injected into the OVLT, therefore NO may activate pyrogenic pathways [249] which in turn activate the HPA.

One theory on the role of NO in neurotransmitter release by the HPA has been put forward by McCann and his colleagues [180]. They suggest that NO in the PVN stimulates the activation of cyclooxygenase and subsequently prostaglandins which in turn mediate neurotransmitter release. It is possible that such a prostaglandin mechanism might be initiated by our SNAP treatment resulting in CRF release.

Experiment 3: Role of central prostaglandin E2 in PVN fos induction

3.0 Introduction/Rationale

Systemic treatment with lipopolysaccharide induces c-fos in specific brain regions, including the hypothalamic paraventricular nucleus (PVN) [134, 135]. LPS is known to induce a complex cascade of cytokines and immune mediators, including prostaglandins, which act on both the peripheral and central systems (see *Introduction*).

Since the 1970's prostaglandins have been recognised as inducers of fever in rats [229,230]. Recent studies indicate that prostaglandin activity in the preoptic region of the anterior hypothalamus is necessary for the production of endotoxin-induced fever [232]. Cyclooxygenase (COX), the heme-containing enzyme that produces prostaglandin E2, is induced throughout the brain vasculature and in the hypothalamus after systemic endotoxin treatment or systemic IL-1ß treatment [231]. Studies in our own lab have shown that inhibition of prostaglandin synthesis inhibits c-fos protein induction in the hypothalamus after LPS treatment [134]. Of all the prostaglandin types, PGE2 may specifically be responsible for mediating, in part, the CRF response to LPS [265]. Since PGE2 is a known mediator of LPS action, the purpose of this study was to examine more closely what role PGE2 might have in c-fos induction in the brain.

Some evidence suggests that nitric oxide may have a role in mediating prostaglandin's central effects after immune challenge. In glial cell cultures, viral-induced PGE is driven by NO [245], prostaglandin-induced fever is sensitive to NO inhibition [264], and prostaglandin-driven hypothalamic neurotransmitter release may also be driven by a NO

mechanism [176, 179]. Since our previous work (Experiments 1 and 2) indicated a role for nitric oxide in central response to endotoxin, and particularly in the induction of c-fos in the PVN, we examined the potential interaction between NO and PGE2 in c-fos expression in the PVN.

3.1 Methods

3.1.1 Treatment

Adult Sprague-Dawley male rats (300-380 g, Charles River) were used as subjects. Stainless steel cannulas were implanted into the left lateral ventricle. Cannulated animals were housed individually in a 12:12hr light/dark cycle with food and water ad libitum. Experiments were preformed no sooner than six days post-surgery.

To investigate the effects of central prostaglandin E2 (PGE2) treatment on c-fos expression, experimental animals received central administration of 0.5 µg, 2 µg, 4 µg or 7 µg of the long-acting PGE2 analogue, 15 (S)-15 methyl prostaglandin E2 (Caymen Chemicals), delivered in a saline vehicle. Control animals received 0.85% saline i.c.v.

To investigate the role of nitric oxide on this PGE2 response, animals were pretreated with 50 µg N nitro-L-arginine methylester (L-NAME) i.c.v. 15 prior to receiving an injection of 4 µg PGE2 i.c.v. The levels of fos induction after central treatment with 4 µg and 7 µg PGE2 analogue were comparable, so 4 µg dose was used for maximal fos induction. Control animals received the inactive enantiomer of NAME, D-NAME, 15 minutes prior to an i.c.v. injection of PGE2. Animals were perfused one hour after the PGE2 injection. Brains were removed for further processing (specifics on these procedures are located in *Materials and Methods* section).

3.1.2 Immunohistochemical Procedures

Coronal sections (40µm) of the brain were cut on a freezing microtome. Representative sections through the hypothalamus were selected for immunohistochemical processing.

Sections were incubated overnight at room temperature with rabbit antibodies to c-fos (1/15,000 Santa Cruz Laboratories). Sections were incubated for 18-20 hours in the antibody and then visualised via the PAP technique with DAB as a chromagen (details on immunocytochemical procedures in *Materials and Methods* section). Sections were then floated onto slides and coverslipped. Cell counts were made of comparable levels of the medial PVN where c-fos has been shown to be maximally induced in systemic LPS-treated animals [135].

3.2 Results and Analysis

3.2.1 Effects of the PGE2 on c-fos expression in the PVN.

Central administration of the PGE2 analogue resulted in a similar pattern of c-fos induction in the hypothalamus as seen with i.v. endotoxin treatment (Figure 19). The number of fos staining nuclei in the paraventricular nucleus (PVN) increased with increasing doses of PGE2. There was a significant increase ($p<0.05$) in fos expression in the PVN between the lowest dose of PGE2 administered (0.5 μg) and the highest dose (4 μg) (Figure 20).

3.2.2 Effects of NOS inhibition on PGE2-induced c-fos in the hypothalamus.

The PGE2-induced c-fos expression in the PVN was attenuated by central pretreatment with the nitric oxide synthase inhibitor L-NAME (Figure 21). All regions of the PVN examined—rostral, medial and caudal—all showed inhibition of fos induction with L-NAME administration. The decrease in the medial and caudal regions of the PVN was significant ($p<0.05$) (Figure 22)

Figure 19: Centrally administered PGE2 induces hypothalamic c-fos expression

Photomicrograph of c-fos staining in the paraventricular nucleus (A) and supraoptic nucleus (B) in the hypothalamus after the central administration of 4μg prostaglandin E2 analogue. This pattern of staining is similar to that observed in the hypothalamus after peripheral endotoxin treatment.

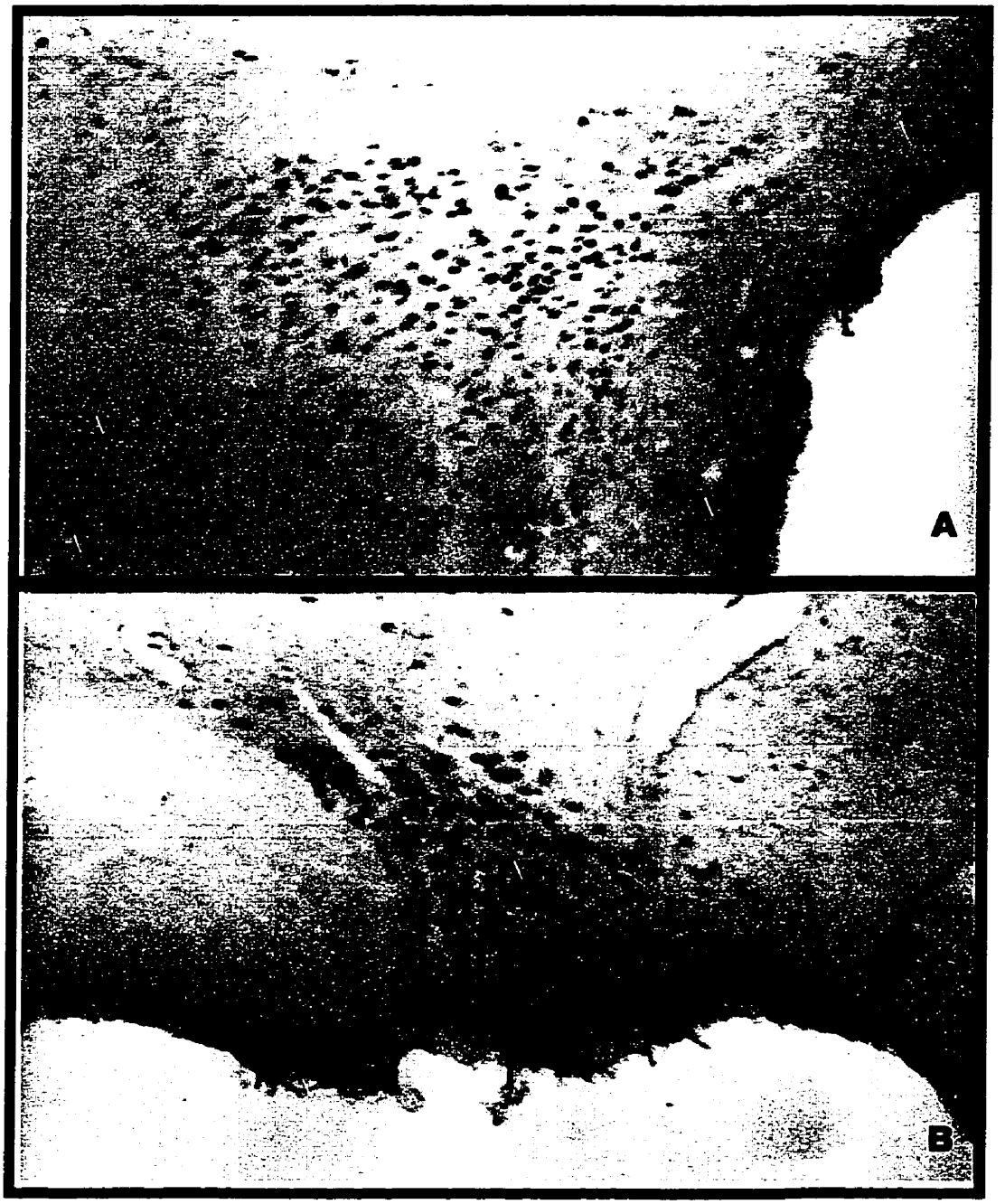


Figure 20:

Centrally administered PGE2 induces a dose-dependent expression of c-fos in the PVN

Effects of centrally-administered PGE2 on fos expression in the medial PVN. Fos-expression increased with increased dose of PGE2 administered. Values are shown for the cell means +/- standard error. Asterisk (*) indicates a significant difference between 0.5µg PGE2 (n=3) and 4µg PGE2 (n=3) treatment (ANOVA results; Post Hoc Scheffe's proceedure $p<0.05$, $F=5.45$).

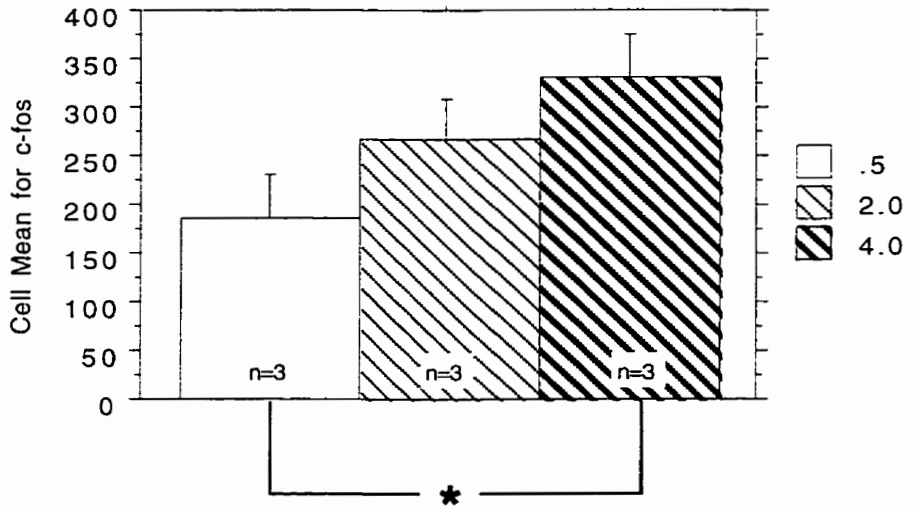


Figure 21: Central nitric oxide synthase inhibition can attenuate central PGE2-induced c-fos expression in the PVN

Photomicrographs showing the effects of L-NAME, a NOS inhibitor (Photo A, top) or D-NAME, and inactive enantimer (Photo B, bottom), on PGE2-induced c-fos in the PVN after a 4μg i.c.v. dose of PGE2 analogue. The c-fos production is significantly affected by central inhibition of nitric oxide (see Figure 22).

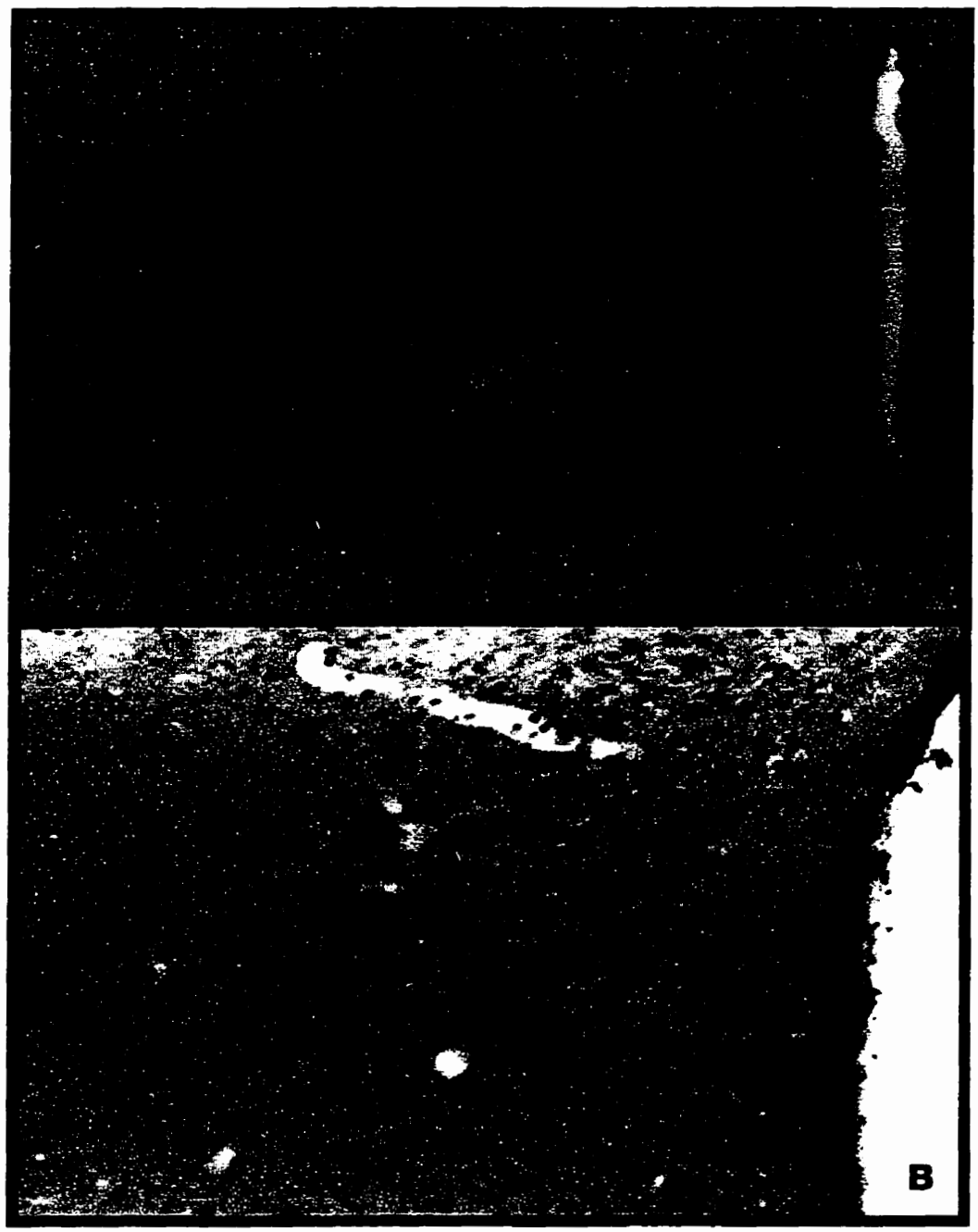
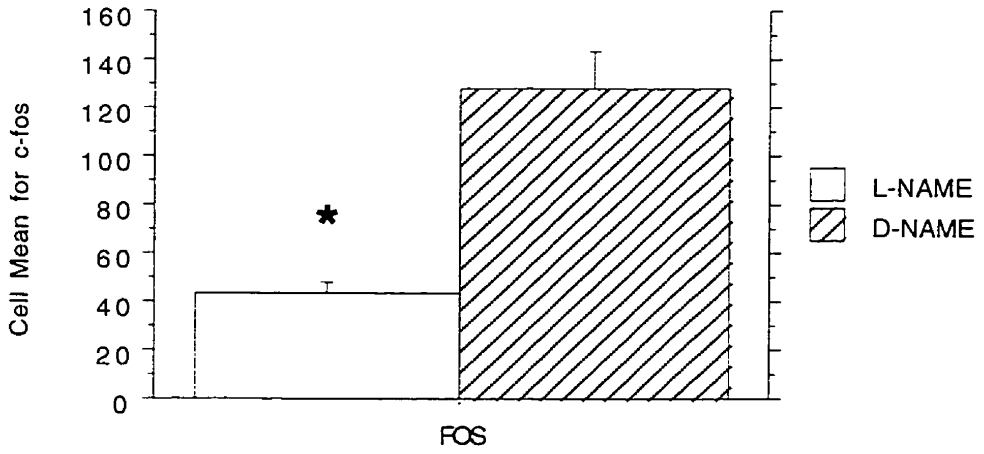


Figure 22: Effects of central inhibition of nitric oxide synthase on PGE2-induced c-fos expression in the PVN

Central NOS inhibition by i.c.v. L-NAME resulted in a significant attenuation of PGE2-induced c-fos expression in the PVN after an PGE2 challenge of 4µg i.c.v. Values are shown for the cell means +/- standard error. Asterisk (*) indicates a significant difference between L-NAME (n=5) and D-NAME (n=3) treatment (ANOVA results; Post Hoc Scheffe's proceedure $p<0.0001$, $F=35.248$).



3.3 Prostaglandin Study: Discussion/Summary

Central injections of PGE2 produce c-fos expression in the PVN.

The results of this study were originally reported at the 1996 Society for Neuroscience conference [185], and although at that time PGE2 was known to be an important immune mediator, only studies in piglets had shown that systemic i.v. treatment of PGE2 could induce c-fos [248], and little was known about the central mechanisms of PGE2. Since then, the effects of central PGE2 treatment on c-fos induction in the hypothalamus have been confirmed by others. Lacroix et al [186] found that central administration of PGE2 results in increased c-fos mRNA in the hypothalamus. In the parvocellular nucleus they noted that c-fos was expressed in both CRF and OXY immunoreactive neurons, and in the magnocellular region the transcript was colocalized primarily with OXY neurons. Our own results showing endotoxin-induced changes in oxytocin (Experiment 1) would indicate that this neurotransmitter is important in the neural-immune response. It has also been noted that microinjections of PGE2 into the medulla induce a c-fos staining in the PVN in a pattern similar to that obtained by systemic IL-1 injection. [166].

Site specific microinjection administration of PGE2 into the preoptic area of the hypothalamus (POA) has also been reported to induce fos in the PVN [247]. However this fos induction varies from that of systemic LPS, or i.c.v. PGE2 administration, in that only the parvocellular regions of the PVN had fos induction, and not both the magnocellular and parvocellular regions.

Although the c-fos-induction in our experiment was less pronounced compared to that achieved with endotoxin treatment, there was extensive induction in the PVN after i.c.v.

PGE2 treatment. The i.c.v. technique allows for a broad central distribution of the prostaglandin to potential brain targets. It is likely that PGE2 at the POA results in fever after LPS challenge [230, 232], and results in activation in the hypothalamus [247], but this is probably only one of many regions activated in the central response to immune challenge, many of which might use PGE2 as a mediator. In fact, even in the case of fever, the POA is probably only one of many PGE2 febrile-responsive sites [263]. The experimental evidence that inhibition of cyclooxygenase will inhibit LPS-induced c-fos in the brain [134] and that PGE2 induces fos in the hypothalamus in similar staining patterns to LPS-induced fos [186, this experiment], indicates that PGE2 plays a fundamental role in the induction of c-fos after a peripheral endotoxin challenge. It is likely that PGE2 mediates activation of hypothalamic neurons by its actions at the POA as well as other sites through out the brain.

PGE2-induced c-fos is mediated via a nitric oxide mechanism.

Just as we observed with LPS-induced c-fos, PGE2 -induced c-fos also was attenuated by central nitric oxide synthase inhibition. These results suggest a pathway for endotoxin-induced c-fos expression that involves the central production of both PGE2 and nitric oxide, and that the effects of PGE2 are mediated or potentiated via a nitric oxide mechanism.

Glucocorticoids, which are inhibitory for endotoxin-induced fever [234], have been shown to exert their antipyresis at the OVLT by inhibiting an NO pathway [233]. Since PGE2 is a pyrogen, it is possible that the action of glutocorticoids are an immunoregulatory response to an NO-activated PGE2 pathway.

Much of the literature looking at the links between NO and prostaglandins have revealed

that NOS inhibition results in inhibition of cyclooxygenase enzyme activity and prostaglandin production. Our results imply a role further downstream from that of PGE2 production, since our experimental protocol supersedes the necessity of COX activation.

It is interesting to note that c-fos is attenuated, but not completely eliminated by the L-NAME treatment, and that the attenuation observed for PGE2-induced c-fos is significantly greater than that seen with low-dose LPS induced fos. It is possible that although the NO mechanism of fos-induction is inhibited, other mechanisms of c-fos-induction are still in place. In the case of PGE2-induced c-fos, the inhibition of fos is about 70% of maximal. In the case of LPS (10 μg)-induced c-fos, the levels were reduced by only 40% of maximal.

There are a number of possible reasons why this variation exists between the PGE2 and LPS response. Central PGE2 alone probably provides only a limited number of routes for c-fos induction, and these pathways are sensitive to the NOS activity. It is likely that LPS induction of c-fos acts via a number of pathways, including a PGE2 mechanism.

The fact that inhibition of NO can attenuate PGE2-induced c-fos indicates that this pathway for c-fos activation requires NO. A likely sequence of events is that PGE2 induces NO which in turn induces c-fos.

Experiment 4: Role of central catecholamines in LPS-induced c-fos expression in the PVN.

4.0 Introduction/Rationale

Peripheral injection of lipopolysaccharide (LPS), a potent bacterial endotoxin, results in the central expression of the proto-oncogene c-fos. Areas of the brain that have increased c-fos expression include the paraventricular nucleus of the hypothalamus (PVN) and the catecholamine cell groups located in the dorsal and ventrolateral regions of the medulla [135]. The expression of c-fos can be used as an experimental marker of neuronal activation occurring in response to physiological challenges [22, 23]. Using this neuronal marker, it is possible to trace a neuroanatomical pathway, as indicated by fos staining after an LPS challenge, from the noradrenergic A1 and A2 cell groups of the brainstem up to the hypothalamus, and particularly for our studies, the paraventricular nucleus (PVN) [135, 139]. Thus, central c-fos expression following peripheral LPS stress may reflect an immune-CNS directed signalling pathway.

One means of immune activation of the central nervous system, and particularly the hypothalamus, involves the movement of blood borne cytokines that act via circumventricular organs, such as the OVLT and the subfornical organ [review 120]. However, the complete immune-CNS response does not act via this single pathway, and the actions of central catecholamines have been shown to play an important role in the central response to stress [266, 291]. It has been shown that catecholamines can influence the response of the hypothalamus not only to activation of the CRF system and the HPA, but also to fever [107] and cardiovascular [review 293] responses. Norepinephrine (NE) afferents have been shown

to innervate a number of hypothalamic cell types, including oxytocinergic, vasopressinergic and CRF-positive neurons [37, 137, 285, 289, 294]. Inhibitory as well as excitatory effects of NE on PVN neurons have been described [108, 137]. Activation of the HPA axis by stimulators of the immune system is associated with increased NE metabolism in the hypothalamus [104].

The major catecholamine nuclei are located in the pons and medulla and consist of adrenergic and noradrenergic neurons. These nuclei are divided into dorsal and ventral bundles. The dorsal noradrenergic bundle (DNAB) originates in the locus ceruleus (A6) and innervates the anterior hypothalamus, including regions of the PVN [67, 137]. However, this hypothalamic innervation is considered minor compared to that provided by the ventral noradrenergic bundle (VNAB). The ventral bundle originates in the A1, A2, A5 and A7 groups in the pons and medulla oblongata and provides the major noradrenergic innervation to the hypothalamus [137].

To investigate further the potential role of catecholamines in the the central expression of c-fos after peripheral immune stress (endotoxin treatment) and psychological stress (footshock treatment), unilateral brainstem or posteriorlateral hypothalamic knife cuts were used to eliminate ascending catecholamine input to the hypothalamus.

4.1 Materials and Methods

4.1.1 Treatment

Adult Sprague-Dawley male rats (300-380 g, Charles River) were used as subjects. One group of subjects underwent surgery to unilaterally transect the ascending catecholamine tracts in the brainstem, the other group recieved unilateral knife cuts of the posteriorlateral hypothalamus (specifics on these knife cut procedures are located in *Materials and Methods* section).

After the post-surgical recovery period of at least fourteen days, animals received 40 µg lipopolysaccharide (Sigma) via the tail vein. Animals were sacrificed two hours after the i.v.injection. Brains were removed for further processing (specifics on these procedures are located in *Materials and Methods* section).

4.1.2 Immunohistochemical Procedures

Coronal sections (40µm) of the brain were cut on a freezing microtome. Representative sections through the hypothalamus were selected for immunohistochemical processing. Sections were incubated overnight at room temperature with rabbit antibodies to c-fos (1/15,000 Santa Cruz Laboratories), or dopamine-β-hydroxylase (1/2000 Eugene Tech.). Sections were incubated for 18-20 hours in the antibody and then visualised via the PAP technique with DAB as a chromagen (details on immunocytochemical procedures in *Materials and Methods* section). Sections were then floated onto slides and coverslipped. Cell counts were made of eight representative sections through the PVN from the most rostral to most caudal parts of the nuclei.

4.2 Results and Analysis

4.2.1 Effects of mesencephalic-pontine cuts on LPS-induced and footshock-induced c-fos expression in the PVN.

Unilateral knife cuts of the ascending catecholamine tracts in the mesencephalic-pontine region of the brainstem partially eliminated the catecholamine afferents to the PVN, as indicated by attenuated dopamine-β-hydroxylase (DBH) staining in rostral, but not caudal, regions of the ipsilateral PVN. Regions with reduced DBH staining had a corresponding attenuation of endotoxin-induced c-fos (Figure 23). There were no significant changes between the cut and control sides of the c-fos expression in the PVN in footshock animals; however the LPS-induced c-fos expression was significantly attenuated in the anterior parvocellular portion of the rostral PVN and in the medial PVN which includes the medial parvocellular and posterior magnocellular regions, but not in the most rostral regions of the PVN and not in the most caudal region which includes the lateral parvocellular PVN (Figure 24). Multivariate analysis of variance (MANOVA) with repeated measures design was used to determine whether overall significant differences existed(Table 2).

4.2.2 Effects of the posteriorlateral hypothalamic cuts on LPS-induced and footshock-induced c-fos expression in the PVN.

Successful unilateral posteriorlateral hypothalamic knife cuts eliminated the majority of catecholamine afferents, as indicated by the reduction in DBH staining (Figure 25). An attenuation of endotoxin-induced c-fos was observed on the side of the PVN ipsilateral to the lesion (Figure 26). The intact control side showed normal DBH staining and c-fos staining. Footshock-induced c-fos was significantly inhibited on the lesioned side in caudal sections of the PVN (i.e. the lateral parvocellular and the caudal parts of the medial parvocellular

Figure 23: Reduction in dopamine-β-hydroxylase staining and LPS-induced c-fos expression in the anterior regions of the PVN after brainstem knife cuts of catecholamine afferents.

Photomicrographs showing reduced LPS-induced c-fos expression (Photo A, top) in the ipsilateral side (right) of the anterior parvocellular PVN after a brainstem cut which severed the catecholamine input to the PVN, as indicated by reduced dopamine-β-hydroxylase staining (Photo B, bottom). Possible reasons for the remianing DBH activity are discussed in Section 4.3.

Figure 24: Effects of mesencephalic-pontine cuts on LPS-induced and footshock-induced c-fos expression in the PVN.

Effects of knife cuts of the ascending catecholamine tracts in the mesencephalic-pontine region of the brainstem on 40μg i.v. LPS-induced c-fos in the PVN and on footshock-induced c-fos expression in the PVN. Asterisk (*) indicates a significant difference between cut and control sides. MANOVA analysis shown in Table 2.

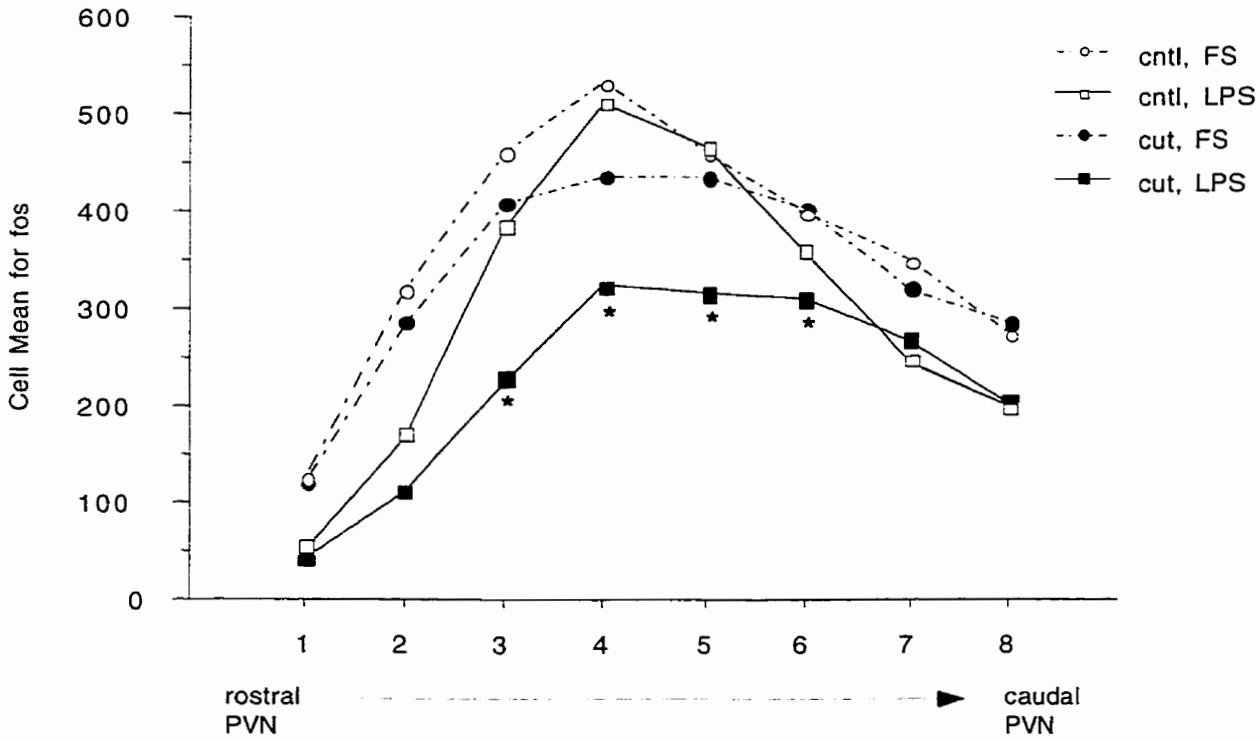


Table 2: Multivariate analysis of variance (MANOVA) with repeated measures design for effects of brainstem cuts

Three-way analysis of variance, with two within (lesioned versus intact sides (Effect 3); rostral-caudal level of the PVN (Effect 2)) and one between-group (endotoxin versus footshock (Effect 1)) variables, were performed using Statistica MANOVA analysis to determine whether overall significant differences existed.

STATISTICA GENERAL MANOVA	summary of all effects; design: 1-V1, 2-AP, 3-side					
Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	319096.9	7	248663.3	1.28325	0.2946007
2	7	257899.2	49	16589.9	15.54553	0.0000000
3	1	113238.7	7	3015.4	37.55405	0.0004777
12	7	6007.2	49	16589.9	0.36210	0.9197713
13	1	28810.4	7	3015.4	9.55456	0.0175412
23	7	13790.0	49	2337.2	5.90034	0.0000528
123	7	4346.7	49	2337.2	1.85983	0.0968658

Figure 25: Hypothalamic knife cuts result in the reduction of catecholamine input to the PVN

Dark-field photomicrograph of the PVN illustrate the impact of unilateral posteriorlateral hypothalamic knife cuts on dopamine-β-hydroxylase immunoreactivity. The cut side (left) has a reduction in DBH staining fibres, indicating a reduction in catecholaminergic input to the cut side of the PVN.

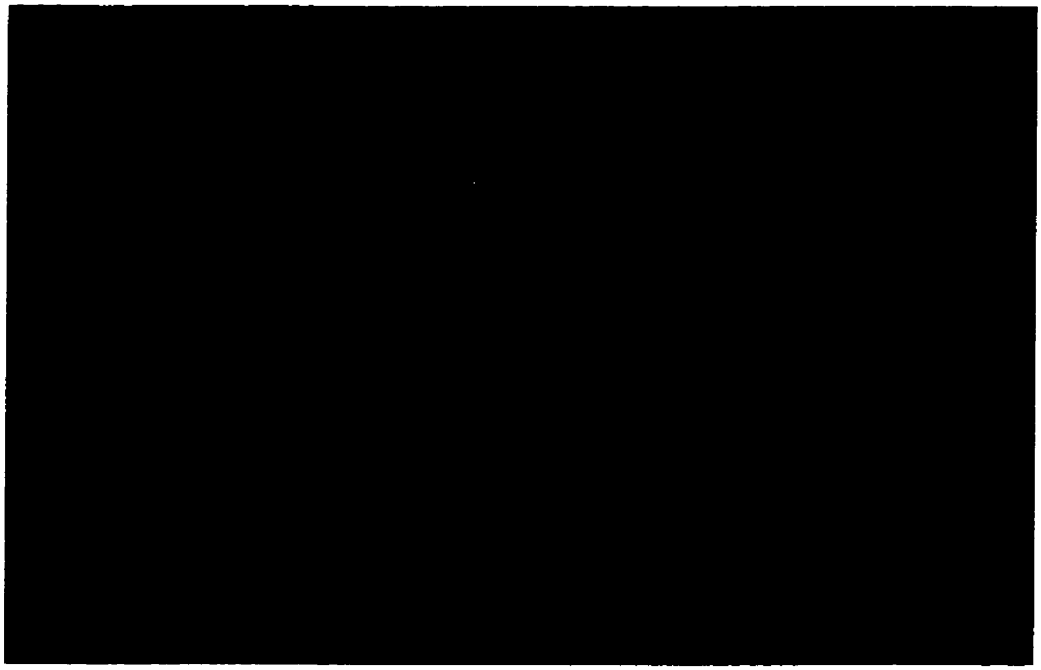
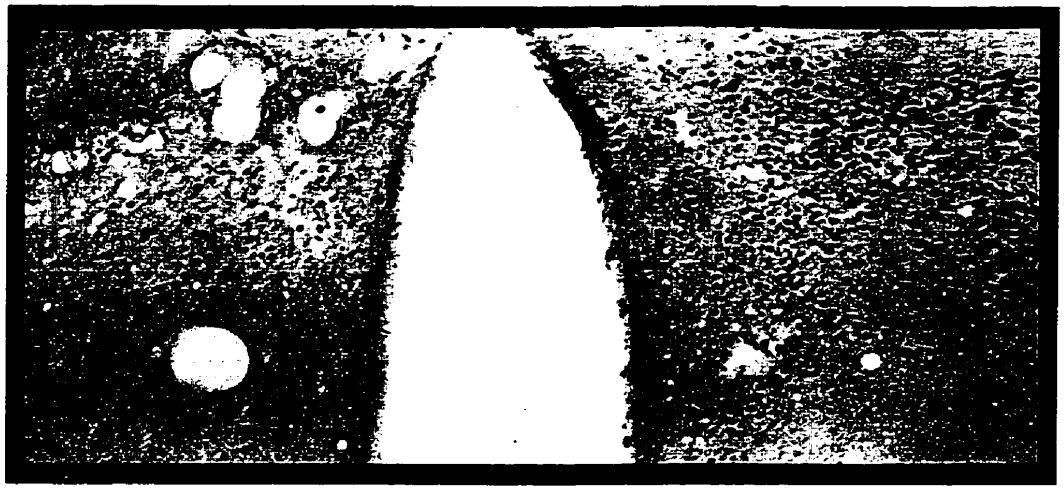


Figure 26: Reduction in endotoxin-induced c-fos expression on the ipsilateral side of the paraventricular nucleus after posteriorlateral knife cut.

Photomicrograph showing i.v. LPS-induced c-fos expression in the ipsilateral (left) and contralateral (right) sides of the paraventricular nucleus after a posteriorlateral knife cut which severed catecholamine input into the PVN.



regions of the PVN), but not in the rostral or medial sections of the PVN. Endotoxin-induced c-fos expression was significantly attenuated throughout the PVN on the cut side (Figure 27; MANOVA in Table 3).

Figure 27: Effects of the posteriorlateral hypothalamic cuts on LPS-induced and footshock-induced c-fos expression in the PVN.

Effects of knife cuts of the posteriorlateral hypothalamus on 40μg i.v. LPS-induced c-fos in the PVN and on footshock-induced c-fos expression in the PVN. Asterisk (*) indicates a significant difference between cut and control sides. MANOVA analysis shown in Table 3.

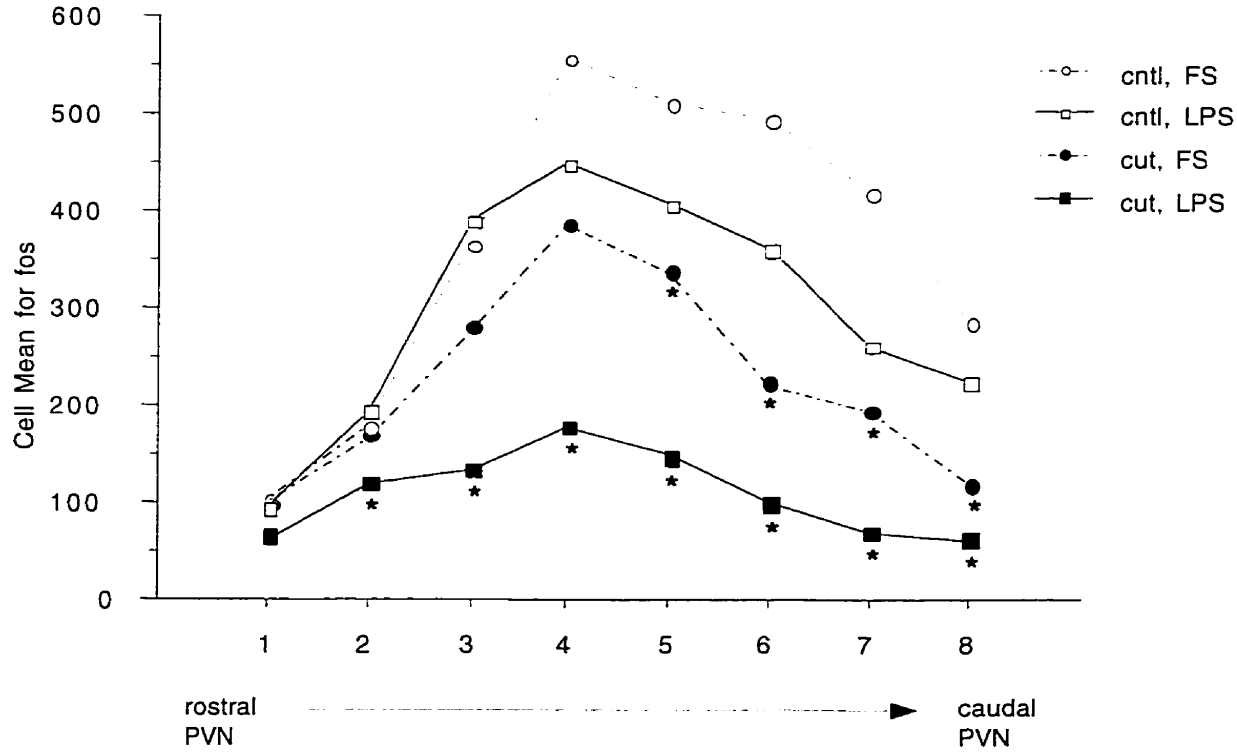


Table 3: Multivariate analysis of variance (MANOVA) with repeated measures design for effects of hypothalamic cuts.

Three-way analysis of variance, with two within (lesioned versus intact sides (Effect 3); rostral-caudal level of the PVN (Effect 2)) and one between-group (endotoxin versus footshock (Effect 1)) variables, were performed using Statistica MANOVA analysis to determine whether overall significant differences existed.

STATISTICA GENERAL MANOVA	summary of all effects; design: 1-V1, 2-AP, 3-side					
Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	297220.5	7	56659.39	5.24574	0.0557781
2	7	167904.9	49	5214.72	32.19829	0.0000000
3	1	848687.4	7	8597.78	98.71006	0.0000223
12	7	15190.4	49	5214.72	2.91299	0.0125194
13	1	18208.7	7	8597.78	2.11784	0.1889241
23	7	31823.4	49	2360.44	13.48202	0.0000000
123	7	5077.0	49	2360.44	2.15088	0.0553467

4.3 Knife Cut Study: Discussion/Summary

Brainstem cuts cause a significant attenuation of LPS-induced c-fos expression in the PVN.

The pattern of c-fos expression following LPS injection suggests that afferent information arrives at the hypothalamus from the medulla oblongata via the solitario–paraventricular pathway [135, 139, 292]. This hypothesis is supported by the results of this experiment which showed significant attenuation of endotoxin-induced c-fos expression in the rostral and medial PVN after lesions of the ascending noradrenergic pathways from brainstem nuclei. Our results are in agreement with the work of Sawchenko and his colleagues [251, 291] who noted an attenuation in IL-1-induced c-fos expression in the PVN after similar brainstem cuts. It is obvious that catecholamines have a role to play in the central response to peripheral immune stress, including c-fos expression [91], fever [107, 296], and HPA responses [review 299]. The fact that brainstem lesions of these ascending catecholamine tracts attenuate IL-1-induced [251,291] and LPS-induced c-fos expression in the PVN would seem to imply that these ascending pathways have significant involvement in mediating the central response to peripheral immune stress.

However, the effects of the cuts on LPS-induced c-fos in the PVN was not identical to that observed for IL-1-induced c-fos. Sawchenko noted that IL-1-induced c-fos expression in the ipsilateral PVN after unilateral knife cuts was attenuated to about 20% of the contralateral (control) side in the medial PVN. We too observed a decrease in contralateral (control) versus ipsilateral (cut) sides in fos induction after LPS challenge, however our reduction was not as pronounced as that of IL-1, although it was significant. Fos expression in the PVN on the side ipsilateral to the lesion was reduced to about 60% of that observed on the contralateral (control) side. Even in the subjects with greatest c-fos attenuation, the reduction

in fos expression on the cut side in the medial PVN was only about half of that observed on the control side.

There could be a number of reasons for these differences. Sawchenko and his colleagues [251] employed a much stricter evaluation of the effectiveness of their cuts, with the exclusion of any subject which did not have at least a 60% depletion of DBH-immunoreactivity in the ipsilateral PVN. Similarly, we used reduced DBH staining as the indicator for a successful lesion; however, we did not objectively determine the level of depletion. A subjective assessment was used to evaluate DBH depletion after knife cuts. Despite the obvious unilateral reduction in DBH staining, it is possible that our group of subjects contained animals with cuts that were not as effective as Sawchenko's group. However, there are reasons beyond technique that could account for this variation.

Much of the original work of neuroimmune activation has been focused on peripheral IL-1 challenge [159, 171]. Sawchenko's studies [251, 291] examined the effect of catecholaminergic afferents on i.v. Il-1-induced c-fos in the brain, unlike our study which focused on the effects of i.v. LPS-induced c-fos. It is possible that the variations in our results are due to using different immune stimuli. There are many reasons to assume some correlations between the effects of IL-1 and the effects expected from LPS. IL-1 is a major immune molecule with known immune functions including fever [174], increased corticosterone and ACTH levels [171], mediation of the inflammatory response [175], and is one of the primary cytokines induced after endotoxin challenge [254]. Like peripheral LPS treatment, systemic or central IL-1 treatment has been shown to induce c-fos mRNA in specific brain regions, including the PVN [173, 176]. Some studies have intimated that the

actions of systemic LPS are more dependent on the actions of central IL-1 compared to the actions of peripheral IL-1 production [172]. Despite the far-reaching immune actions of IL-1, it is only a single element in the endotoxin cascade that occurs after LPS treatment and therefore can only offer a limited view to the changes that an endotoxin challenge can induce.

There are some limitations to the coincidence that can be claimed between IL-1 actions and LPS actions. Research into the role of IL-1 has lead to results that question the importance of IL-1 in the central response to the endotoxin treatment. ICE knockout mice, which are deficient in the IL-1β converting enzyme (ICE), are unable to break the precursor IL-1 molecule into its active protein. The catalytic function of ICE is essential to the generation of mature IL-1β [169]. Our lab has found that these ICE mice respond in a similar fashion as controls after i.v. LPS treatment to a number of immune measures; including plasma corticosterone levels and activation of c-fos in the PVN [168]. This result is supported by other experiments using other knockout mice. Research using IL-1 receptor knockout (IL-R1 KO) mice have shown that although these genetic knockouts have a suppression of i.p. IL-1β induced fever compared to wild-type controls; i.p. LPS-induced fever has no such reduction suggesting a less critical role of IL-1 in the fever response and that other endogenous pyrogens released by endotoxin, such as TNF-α and IL-6, are sufficient to generate fever [170].

In the most caudal regions of the PVN, there were no differences between the endotoxin-induced fos expression on the ipsilateral (cut) and contralateral (intact) sides of the PVN. Since the reduction in fos expression in the medial regions seems to be due to the cuts of the

noradrenergic pathways from the brainstem, there may still be intact noradrenergic innervation to the rostral PVN.

The ascending NE pathway from the brainstem nuclei are divided into dorsal and ventral bundles. Our cuts transected the ventral bundle which originates from A1, A2, A5 and A7 in pons and medulla oblongata. The A2 region preferentially projects to the medial parvocellular PVN [278], which may account for the c-fos attenuation in this region when the ventral bundle is cut. While the VNAB is considered a major noradrenergic input to the hypothalamus, these cuts leave the dorsal bundle intact. The dorsal bundle originates in the locus ceruleus (A6) and innervates the hippocampus and, to a lesser extent, the hypothalamus [67, 137]. Also left intact are the noradrenergic inputs from parabrachial nucleus which have a direct route to PVN [294].

Interestingly, the brainstem cuts attenuate fos in the medial and parts of the rostral PVN, but not in the most rostral section, which contains the anterior magnocellular region. This region has been shown to consist primarily of oxytocin neurons [143], which according to our earlier experiment (Experiment 1) are the population that is increased in the rostral PVN after peripheral endotoxin challenge. It is possible that in the rostral PVN, the increases in oxytocin staining and its associated c-fos staining are not due to ascending norepinephrine inputs, but rather other central actions.

Work in our lab has shown an important role for central prostaglandins in the expression of c-fos in the hypothalamus (see Experiment 3), particularly after a peripheral immune challenge [134]. While basal production of prostaglandins in the hypothalamus seems to

requires intact NE innervation [295] and endotoxin-induced fever is mediated in part by central noradrenergic neurons [296], endotoxin-induced central prostaglandin synthesis can occur independently of catecholamines input [295]. Since prostaglandin activity in the preoptic region of the anterior hypothalamus is necessary for the production of endotoxin-induced fever [232], this would imply that there are redundant pathways for endotoxin fever induction, some requiring NE and other acting independent of NE. It is quite likely that c-fos is also induced via a number of different pathways. Our brainstem cuts attenuated those pathways that required medullary catecholamines, but left intact those that acted via other mechanisms, such as PGE2 which act at the anterior regions hypothalamus (such as the rostral PVN).

It is also possible that the hypothalamic c-fos expression that remains after the brainstem cuts is the result of LPS extra-immune actions. The cardiovascular system can be activated by high dose LPS [302], and these actions may be mediated by noradrenergic pathways outside the ventromedial medulla, such as the parabrachial nucleus [303].

Posteriorlateral hypothalamic cuts cause significant attenuation of endotoxin-induced c-fos expression throughout the PVN.

Lesions at the posterior hypothalamus produce transection of a number of major catecholaminergic pathways including the dorsal noradrenergic pathway from the locus ceruleus (A6) and the ventral noradrenergic bundle that originates in the pons and medulla oblongata (A1, A2, A5 and A7 groups). Inputs from the nigrostriatal dopaminergic pathway from the A9 region (substantia nigra) and the ascending mesolimbic dopaminergic pathway from the A10 region (ventral tegmentum area) are also severed by

these cuts [137, 304].

The more radical nature of these cuts ensured that the majority of the noradrenergic inputs from the caudal regions to the PVN, including the A1, A2 and A6 regions, were lesioned. This more complete removal of norepinephrine inputs may account for the increased attenuation, as well lesioning of dopaminergic inputs may also contribute to this attenuation. Catecholamines other than norepinephrine have been shown to have effect on the PVN after LPS treatment. Systemic endotoxin has been shown to increase turnover of dopamine in the PVN [297, 298, 301]. Dopamine also has been shown to induce c-fos in neurons [53, 300].

It is interesting to note that despite the significant attenuation of LPS-induced c-fos throughout the PVN, there is still no change in the most rostral region, which, as mentioned above, may be due to rostral inputs into this region of the PVN. It is also interesting to note that despite the profound attenuation, these knife cuts do not completely inhibit the c-fos response. Again, mediators other than catecholamine afferents may be sufficient to induce a partial response to the peripheral endotoxin challenge.

There is no significant effect on footshock-induced c-fos after brainstem lesions. After hypothalamic cuts there is a significant attenuation of footshock-induced c-fos expression in the caudal regions of the PVN.

Footshock-induced c-fos in the PVN was not significantly affected by transection of ascending catecholamine tracts from the brainstem. These results are in agreement with the work of Li et al [251] who also observed a similar lack of inhibition of the cuts on footshock-induced

c-fos protein. Although both psychological and physiological stressors have been shown to induce similar patterns of c-fos in the PVN [92, 134, 305], it is obvious that brainstem catecholamine afferents have a significant role in mediating c-fos induction in the PVN in response to physical (immune) stessors [our results, 251], but these ascending pathways do not have the same importance in mediating c-fos induction in response to psychological (footshock) stress.

The significant attenuation of footshock-induced c-fos expression in the caudal regions of the PVN after posteriorlateral hypothalamic cuts may be due to the more complete transection of the catecholamine ascending inputs. However, even these radical cuts could not produce significant attenuation in the rostral and medial portions of the PVN. This implies that there are additional mediators, other than the ascending catecholamines, that result in footshock-induced c-fos expression in the PVN. It has been shown that footshock stress induces c-fos in the amygdala [134, 306, 307, 311]. Since the amygdala known to be involved in the central response to psychological stressors [308, 309, 310, 312], it is possible that these limbic system inputs are responsible for c-fos activation in the rostral and medial portions of the PVN and these inputs can affect fos induction independent of ascending catecholamine innervation.

Summary

The purpose of this thesis was to examine some of the characteristics of c-fos induction in the hypothalamic paraventricular nucleus after intravenous injection of lipopolysaccharide, a powerful endotoxin. Each of the experiments within this thesis have revealed further information about LPS-induced c-fos induction in the PVN. This discussion will summarise the finding of these studies and offer a possible pathway for these mediators in the central response to peripheral immune challenge.

Experiment One: Phenotypic specificity of LPS-induced c-fos expressing neurons in the PVN

A portion of LPS-induced c-fos expressing neurons in the PVN co-stained with AVP, OXY and NADPH-diaphorase. Since c-fos expression in neurons during LPS injection is an indication that they are being activated in response to the immunological challenge, then the substances being produced in those neurons maybe possible targets for activational effects of endotoxin. Endotoxin treatment resulted in a significant increase in both NADPH-diaphorase (a marker for nitric oxide synthase) and OXY staining after systemic endotoxin treatment; this was not seen with the AVP staining. Colocalization also existed between NADPH-diaphorase and both AVP and OXY. There was a significant increase in double labelled NADPH-diaphorase/OXY cells in the rostral PVN after LPS treatment.

Our results confirm the work done by others [139, 149] , and expanded on those studies by examining rostral, medial and caudal regions of the PVN. The results imply a potential role of oxytocin and nitric oxide in the central response to a systemic immune challenge that is mediated from the anterior region of the PVN.

This study revealed the involvement of nitric oxide in LPS-induced c-fos expression in the hypothalamus. The next set of experiments investigated further the role of nitric oxide; in particular an examination of the effects of nitric oxide synthase blockers and donors, on the expression of c-fos.

Experiment Two: Effect of nitric oxide on LPS-induced c-fos and other immune measures

Our findings showed that c-fos expression in rats pretreated with central L-NAME, an inhibitor of nitric oxide synthase, and then injected with low dose (10 or 20 μg), but not high dose (40 or 100 μg), i.v. LPS had significantly decreased levels of c-fos in the PVN when compared to saline controls. We also found that central treatment with SNAP did not induce c-fos expression, but central injection of this NO donor did cause a significant increase in plasma corticosterone levels, indicating activation of the HPA axis.

This study revealed that endotoxin-induced c-fos expression involves the central production of nitric oxide. It also indicated that nitric oxide has a role in the release of CRF. The next set of experiments investigated further this nitric oxide pathway, specifically on the effects of prostaglandin E2, a known central immune mediator of systemic endotoxin treatment.

Experiment Three: Effect of PGE2 on c-fos induction in the PVN. Role for nitric oxide.

Central injection of PGE2 resulted in a similar c-fos pattern in the hypothalamus as has been seen after peripheral endotoxin challenge. This induction involved nitric oxide, as central pretreatment with L-NAME, an inhibitor of nitric oxide synthase, attenuated PGE2-induced c-fos expression in the PVN.

These results suggested a pathway for endotoxin-induced c-fos expression that involves the central production of both PGE2 and nitric oxide, and that the effects of PGE2 are mediated via a nitric oxide mechanism.

Experiment Four: Effect of ascending catecholamine inputs on LPS-induced c-fos in the PVN

The fact that brainstem lesions of ascending catecholamine tracts originating from the mesencephalic pontine region attenuated LPS-induced c-fos expression in the PVN, but did not affect footshock-induced fos, would seem to imply that these ascending pathways have significant involvement in mediating the central hypothalamic response to peripheral immune stress, but not psychological stress.

The lack of inhibition of fos induction and DBH staining in the posterior PVN after the brainstem cuts indicates that catecholamine afferents to the caudal regions of the PVN, such as those from the locus ceruleus, are spared by these cuts and provide sufficient input for c-fos expression. In support, hypothalamic knife cuts that eliminated most catecholamine inputs to the PVN, attenuated LPS-induced c-fos induction in all areas of the PVN, except the most caudal regions. The fact that there is never complete inhibition of the LPS-induced c-fos response in PVN indicates that mechanisms other than ascending catecholamine inputs are involved in mediating the fos response.

There is a complex interplay of mediators in the induction of c-fos after peripheral immune challenge, including biochemical mediators such as cytokines and neurotransmitters. Some immune activated c-fos induction in the PVN seems to be due, in part, to ascending catecholaminergic afferent information, but not completely and it is likely that mediators

from circumventricular organs such as PGE2 and nitric oxide also have significant roles. Figures 28 and 29 compile the results of this thesis and propose potential pathways of these different mediators on c-fos induction in the PVN.

As with many hypothalamic functions, there is probably a complex circuitry in place to regulate the central PVN response to LPS. It is possible that the c-fos induced in the nervous system after peripheral immune challenge indicates activation of both afferent signals into the central nervous system and efferent signals from the CNS to the periphery. We have managed in this study to examine some, but not all, of the mediators in this central circuit. Undoubtedly further research will continue to expand on the role of the PVN in the integration and response to peripheral immune challenge. The ability of the hypothalamus to mediate the myriad of inputs occurring after endotoxin challenge, and to provide appropriate responses, is a testament to the importance of this structure in the homeostatic well-being of an organism.

In summary, the observations of this thesis have revealed some of basic components of an immense and complex system, and provided a clearer understanding of the mediators involved in the central response to peripheral endotoxin challenge. However, it is obvious that further studies remain to be done to reveal the complex interrelationship between the central nervous, endocrine and immune systems that occurs in response to immune challenges. Ultimately, a clearer understanding of the interactions between these systems will lead to improved understanding how physical health is maintained.

Figure 28: Central mediators of peripheral endotoxin-induced c-fos expression in the PVN

This thesis identified a number of potential mediators of PVN c-fos induction after immune stimuli. These include a PGE2 mechanism which is mediated by nitric oxide, and catecholamine driven mechanisms. Interestingly, neither of these mechanisms can account for all the c-fos induction in the PVN. The role of nitric oxide in the central mediation of immune stimuli is of great interest as the function of this novel neurotransmitter is still being elucidated. The results of this thesis would imply that this molecule has an important role in mediating the central response to LPS. Its colocalization with oxytocin, which is significantly upregulated in the PVN after immune challenge, suggests other possible functions for this molecule. This thesis, while revealing some of the mechanisms involved in LPS-induced fos induction in the PVN, also leaves many more questions open for future study.

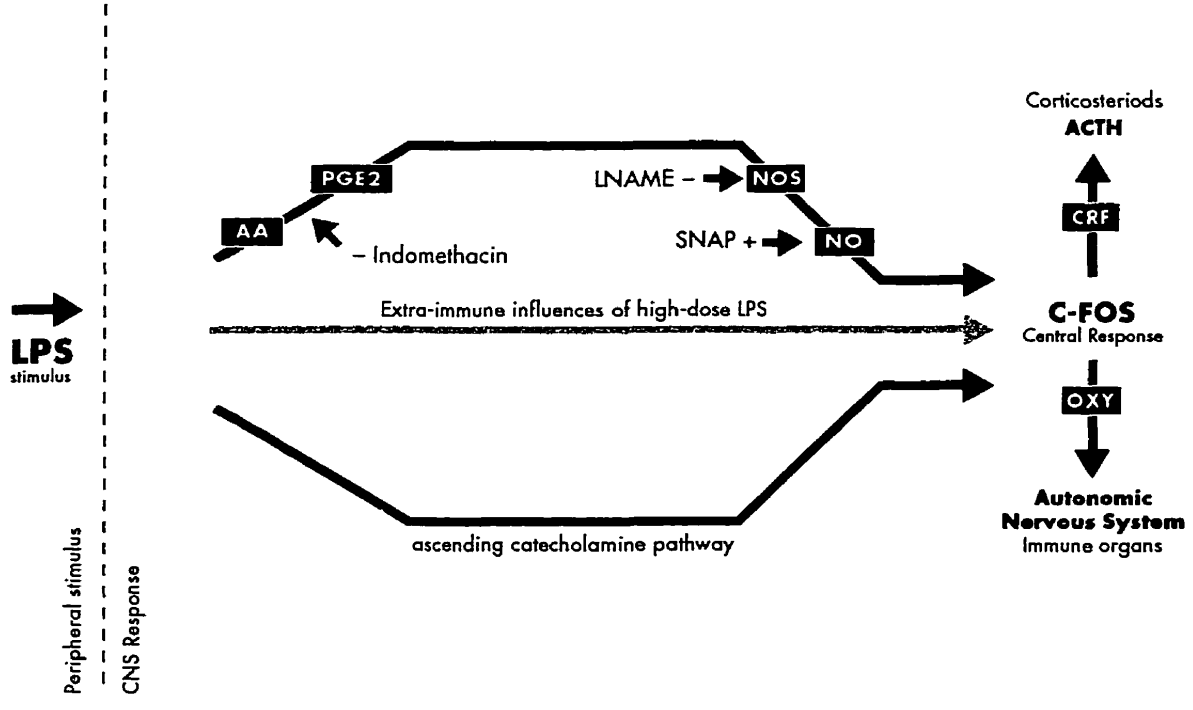
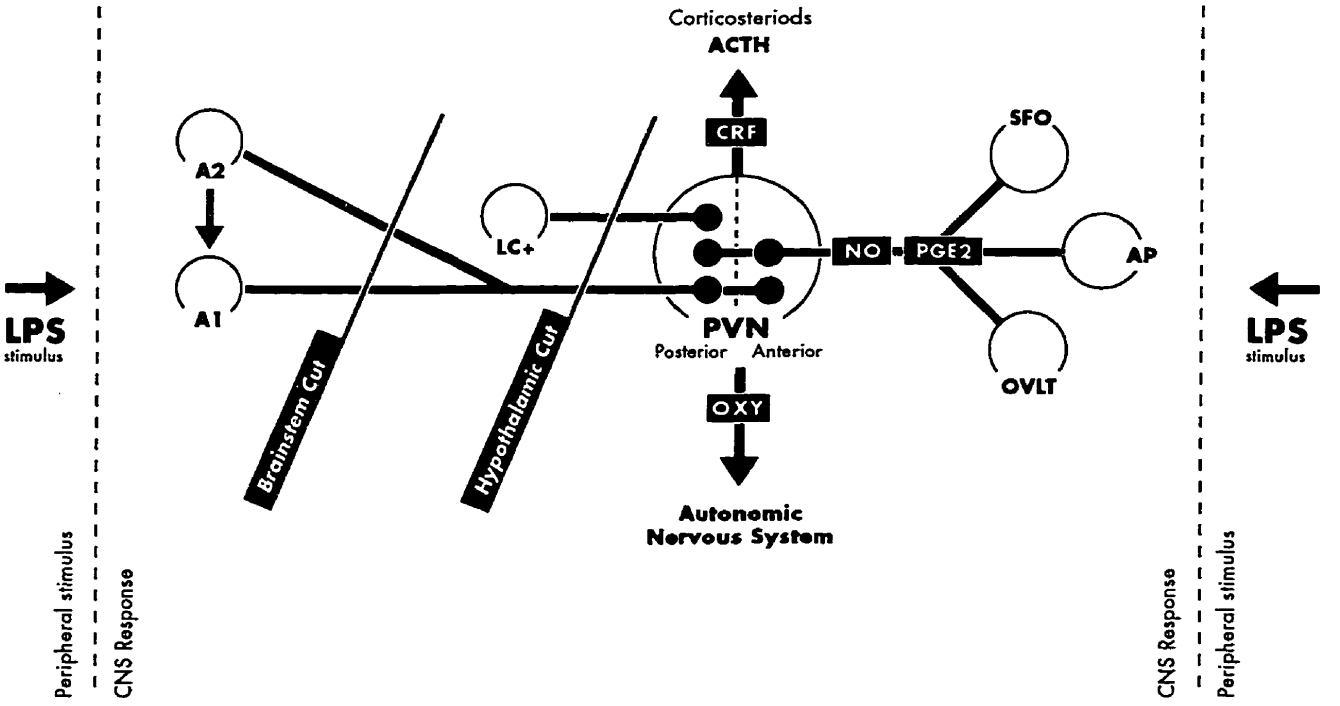


Figure 29: PVN integration of immune stimuli

This thesis identified both neural and cytokine mechanisms of c-fos induction in the PVN in response to peripheral immune stimuli. The PVN receives many inputs, including direct innervation from the noradrenergic ascending brainstem pathways, as well as major projections from the circumventricular organs where the blood-brain barrier is most permeable to immune cytokines. Activation of the PVN is a complex matter, with multiple inputs and also many potential responses. The results of this thesis and the work of others in our lab suggest that the activation of the HPA stress axis and CRF release is just one response to immune challenge, and it seems quite likely that other neurotransmitters and the activation of descending autonomic pathways have important, and perhaps primary, roles in the central response to peripheral immune challenges.



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