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NITRIC OXIDE REGULATES NEURONAL ACTIVATION AND IL-1β GENE EXPRESSION IN THE BRAIN IN RESPONSE TO IMMUNE STRESS

By

Wendy W. Yang

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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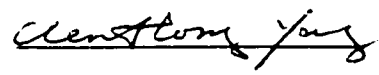

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: **Nitric Oxide Regulates Neuronal Activation and Central IL-1β Gene Expression in the Brain in Response to Immune Stress**, submitted by Wendy W. Yang in partial fulfillment of the requirements for the degree of Master of Science.

Teresa Krukoff

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Abstract

Immune stress induces changes in the hypothalamic-pituitary-adrenal axis activity and autonomic function. In the hypothalamus, the paraventricular nucleus (PVN) plays an integral role in this process. Cytokines in the PVN, particularly interleukin 1β (IL-1β), and nitric oxide (NO) are hypothesized to participate in the communication between the immune system and the brain. First, we tested the hypothesis (I) that putative NO-producing neurons are activated in the PVN in response to stress(es). We found that LPS activated putative NO-producing neurons in the PVN as well as in the brainstem. However, NO was likely not involved in LPS-induced signaling from the brainstem to the PVN. Finally, immune plus restraint stresses stimulated the NO system in the PVN to a greater extent than immune stress alone. Second, we examined the hypothesis (II) that NO regulates the PVN neuronal activity in response to endotoxin. We found that NO produced from eNOS inhibited the activation of neurons, putative NO-producing neurons and IL-1β gene expression in the PVN. The results support the hypothesis that NO plays an important role in regulating the PVN neuronal activity in response to immune stress.

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Special thanks to my parents, my husband and my daughter. This piece of work is dedicated to you.

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LIST OF ABBREVIATIONS

3v	third ventricle
7-Ni	7-nitroindazole
7-NiNa	7-nitroindazole sodium salt
aCSF	artificial cerebrospinal fluid
ap	anterior parvocellular division of the PVN
ACTH	adrenocorticotropin hormone
AG	aminoguanidine
AP	area postrema
AP-1	activator protein-1
BBB	blood-brain-barrier
BP	blood pressure
CNS	central nervous system
CRF	corticotropin releasing factor
CSF	cerebrospinal fluid
CVO	circumventricular organs
DAB	diaminobenzidine tetrahydrochloride
dp	dorsal parvocellular division of the PVN
eNOS	endothelial nitric oxide synthase
FLI	Fos-like immunoreactivity
HPA	hypothalamic-pituitary-adrenal
icv	intracerebroventricular

iNOS	inducible nitric oxide synthase
iv	intravenous
IL-1β	interleukin-1β
IL-6	interleukin-6
L-NAME	N^G-nitro-L-arginine methyl ester
L-NNA	N^G-nitro-L-arginine
lp	lateral parvocellular division of the PVN
LPS	lipopolysaccharide
MAP	mean arterial pressure
mp	medial parvocellular division of the PVN
NADPH	nicotinamide adenine dinucleotide phosphate
NADPH-d	nicotinamide adenine dinucleotide phosphate diaphorase
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NTS	nucleus of the tractus solitarius
ot	optic tract
OT	oxytocin
OVLT	organum vasculosum lamina terminalis
PBS	phosphate-buffered saline
PGE2	prostaglandin E2
pm	posterior magnocellular PVN
pv	periventricular PVN

PVN	paraventricular nucleus
SEM	standard error of mean
SSC	standard saline citrate
Tb	body temperature
TNF	tumor necrosis factor
VLM	ventrolateral medulla
VP	vasopressin

LIST OF SYMBOLS

⇩	LPS or saline injection
⬇	aCSF or NOS inhibitors injection
*†	significant difference
*	third ventricle

Chapter 1

INTRODUCTION

1.1 Overview

According to the classic point of view, most protein products of activated immune cells are restricted to areas outside the central nervous system. The brain was believed to be a privileged site that acted independently of the peripheral immune system. There is now growing recognition that the immune system does not act in isolation from the nervous system. Rather, information is transferred between the immune system and the nervous system to allow bidirectional regulation of immune and brain responses to infection. Many of the cytokine messengers produced by the immune system signal the brain that infection has occurred and many of the messengers produced by the brain have strong effects on the immune system.

1.2 Acute inflammation and cytokines

Detection of pathogens by macrophages of the immune system triggers these immune cells to synthesize and release a number of cytokine polypeptides, including interleukins and tumor necrosis factors. These cytokines attract other immune cells to the site of infection and participate in the destruction of the pathogen (Henderson et al., 1996).

The acute inflammation of an infection can be experimentally mimicked by peripheral injection of bacterial endotoxin lipopolysaccharide (LPS, endotoxic component of gram-negative bacterial wall) because LPS induces the secretion of several cytokines including interleukin-1β (IL-1β), tumor necrosis factor (TNF) and interleukin-6 (IL-6) from the immune system which occurs in a natural infection

(Chen et al., 1992). Circulating cytokines then cause significant increases in hypothalamic-pituitary-adrenal (HPA) activity.

So-called proinflammatory cytokines are made up of a large and diverse group of peptides whose sizes range from 8 to 26 kDa (Rothwell and Hopkins, 1995) and which, particularly IL-1β, have been implicated in the brain's response to disease (Hopkins and Rothwell, 1995). IL-1β, one of the chief mediators of the body's response to inflammation and immunological responses is produced throughout the body, including the brain (Dinarello, 1988). Therefore, IL-1β not only has a wide variety of effects in the periphery, but also has effects in the central nervous system.

1.3 Cytokines signalling pathways

How do peripherally produced cytokines gain access to the brain? This question is the source of some controversy. The large sizes of cytokines (e.g., IL-1β is MW~17,000) and their highly lipophobic characteristics suggest that it is unlikely for these molecules to diffuse passively across the blood-brain barrier (BBB) to reach the brain (Hopkins and Rothwell, 1995).

It has been proposed that cytokines can signal the brain via circumventricular organs (CVOs), cerebral vascular endothelium or activation of peripheral afferent nerves, such as the vagus nerve as described below.

1.3.1 *via CVOs*

The BBB excludes large molecular substances from the brain. This exclusiveness exists both due to the low rate of pinocytosis in endothelial cells and

to the existence of tight junctions joining adjacent endothelial cells of the capillary bed of the brain (Watkins et al., 1995a). There are a few regions in the brain, known as CVOs, where there is a lack of a BBB. Cytokines could passively cross into brain at these areas.

The lack of a BBB in CVOs within the brain has led to the hypothesis that cytokines could enter at these sites and activate other central regions with which these CVOs are neurally connected. In particular, the organum vasculosum lamina terminalis (OVLT) of the forebrain is thought to be penetrated by cytokines that bind to receptors on astrocytes surrounding the vascular network of the OVLT; binding of the receptors leads to the release of prostaglandin E2 (PGE2) from astrocytes (Katsurra et al., 1989). PGE2 is a small and lipophilic molecule that is then thought to cross into the brain and activate neurons in the neighbouring medial preoptic area that project to and affect the hypothalamus and other central areas (Katsurra et al., 1990; Komaki et al., 1992). Cytokines or PGE2 could activate OVLT neurons that themselves project to the medial preoptic area of the hypothalamus (Shibata and Blatteis, 1991). It has been confirmed that prostaglandins participate in the response of the brain to peripheral IL-1β (Katsurra et al., 1989; Katsurra et al., 1990; Terao et al., 1993; Bishai and Coceani, 1996) and that peripheral IL-1β injection increases the level of prostaglandins in the OVLT (Komaki et al., 1992). It has recently been suggested (Moltz, 1993) that prostaglandin production also occurs in vascular endothelial cells throughout the brain (Kluger, 1991; Banks et al., 1994), and not specifically at the OVLT.

Other CVOs, such as the area postrema (AP) in the brainstem, may act as "gates" through which cytokines reach the brain. Cytokine-activated cells in the AP stimulate neurons in the nucleus of the tractus solitarius (NTS) to which they are connected (Cunningham et al., 1994). The NTS, through its connections with autonomic centers in the forebrain (Calaresu et al., 1984), could then transmit the appropriate impulses to the paraventricular nucleus of the hypothalamus (PVN). The projection of cytokine-activated neurons in the NTS to the PVN has been verified with combined neuroanatomical techniques (Ericsson et al., 1994).

1.3.2 *Via cerebral vascular endothelium*

Cerebral vascular endothelial cells may be violated by circulating cytokines during inflammation, thereby allowing passage of mediators of illness into the brain (Furie and McHugh, 1989). On the other hand, there are IL-1 receptors in the endothelial cell. Binding of IL-1 to these receptors could result in alterations in endothelial cell metabolism and cause the release of neuroactive substances on the brain side of the BBB (Banks et al., 1991; Hashimoto et al., 1991; Banks et al., 1993). As mentioned previously, binding of cytokines to cerebral vascular endothelial cells can induce the production of PGE2, which can freely diffuse across the BBB and activate neurons in central sites.

1.3.3 *Via the vagus nerve*

An interesting possible mechanism for cytokine-brain communication is that a local increase in tissue cytokine levels activates afferent fibers in subdiaphragmatic branches of the vagus nerve and this information is then transmitted to the brainstem and then more rostrally in the brain (Banks et al., 1991). This hypothesis

was proposed on the basis of findings showing that subdiaphragmatic vagotomy blocks the fever, hypothalamic norepinephrine depletion, elevation in serum corticotropin, hyperalgesia, and behaviours produced by circulating cytokines or the endotoxin, LPS (Laye et al., 1995; Watkins et al., 1995b). Subdiaphragmatic vagotomy also blocks LPS- and cytokine-induced increases in expression of the immediate early gene, *c-fos* (used as a marker of activated neurons) in rat brain (Wan et al., 1994).

1.4 *c-fos* as a marker of activated neurons

The immediate early gene, *c-fos*, is a highly regulated gene whose transcription is elevated for a short time after the application of a stimulus (Curran and Morgan, 1995). In co-operation with similar proteins of the Jun family, *c-fos* acts as a trancriptional regulator by forming a protein complex that binds to the activator-protein-1 (AP-1) binding site of DNA (Curran and Teich, 1982; Lee et al., 1988; Sheng and Greenberg, 1990). Genes that contain the AP-1 binding site are activated by the Fos/Jun complex. Therefore, it is assumed that cells expressing *c-fos* can be expected to also express the so-called late-onset genes that encode differentiated neuronal products such as neurotransmitters (Sonnenberg et al., 1989; Armstrong and Montminy, 1993). Transcription of *c-fos* occurs within 5 min of application of a stimulus and accumulation of mRNA peaks at 30-45 min. The half-life of the Fos protein is about 2 hours (Müler et al., 1984).

The expression of *c-fos* has been widely used as a marker of activated neurons (Krukoff, 1998; Yang et al., 1999). Because *c-fos* is expressed at very low levels or not at all in unstimulated neurons (Krukoff, 1994; Herdegen et al., 1995;

Krukoff and Khalili, 1997), a high and rapid expression of *c-fos* in neurons can be attributed to the applied stimulus. Immunohistocheminstry is the most common use of recognizing the presence of Fos and Fos-related antigens within nuclei of neurons.

Although been widely used in many systems in response to stimulation, *c-fos* is not a universal marker of neuronal activity. The use of Fos expression has its limitations (Krukoff, 1998). First, careful controls are required to eliminate extraneous sources of background activity, because likely mild forms of handling of conscious animals could lead to Fos expression in the brain (Asanuma and Ogawa, 1994). Second, Fos study is limited to excitatory pathways, as neurons that are inhibited do not generally express Fos. Third, the technique may not be suitable for long-term experiments (when stimulus continues for days) if expression of *c-fos* is not required after the initial stimulus has been applied. Fourth, it is impossible to extinguish whether an identified pathway is uni- or multisynaptic using the expression of *c-fos*. Fifth, the presence of Fos provides no information about which subsequent pathway(s) are activated. Nevertheless, *c-fos* expression is a very popular technique to identify neurons as long as the limitations are kept in mind.

1.5 Responses of the PVN

Circulating cytokines entering the brain via the CVOs, the cerebral vascular endothelium or the vagus nerve lead to increase in *c-fos* mRNA in many brain regions: the NTS and the AP in the brainstem, and the PVN, central nucleus of the amygdala, bed nucleus of the stria terminalis, and medial preoptic area in the forebrain (Brady et al., 1994). The present study will focus on the investigation of

PVN neuronal activity.

The PVN consists of chemically and functionally distinct neuronal subpopulations (Swanson and Kuypers, 1980). Three subdivisions are magnocellular: the anterior, the medial and the posterior magnocellular parts (Swanson and Sawchenko, 1983). The anterior and medial magnocellular parts of the nucleus are composed almost exclusively of oxytocin (OT)-stained cells (Rhodes et al., 1981; Sawchenko and Swanson, 1982a). In the posterior magnocellular part of the PVN, OT-stained cells are concentrated anteroventromedially, and vasopressin (VP)-stained cells are concentrated posterodorsolaterally. VP- and OT-producing neurons in the magnocellular division of the PVN were shown to project to the posterior pituitary (Sawchenko and Swanson, 1982a). The parvocellular division of the PVN consists of five distinct parts: the periventicular, the anterior, the medial, the dorsal and the lateral parvocellular parts. On the basis of size, cells in the parvocellular division are, on the average, significantly smaller than those in the magnocellular division (Swanson and Sawchenko, 1983). Corticotropin releasing factor (CRF)-containing neurons are located in the medial parvocellular part of the PVN. Small populations of VP- and OT-producing neurons are also found in the parvocellular division of the PVN (Swanson and Sawchenko, 1983). Neurons in the medial region of the parvocellular division of the PVN mainly project to the median eminence and control anterior pituitary function (Swanson and Kuypers, 1980), and some of the medial parvocellular neurons in the PVN project to the autonomic areas of the brainstem and spinal cord (Sawchenko and Swanson, 1982a). Immunohistochemical studies

have shown that more than 30 putative transmitter substances, transmitter-related enzymes, and biologically active peptides are produced in cells or fibers within the PVN (Swanson and Sawchenko, 1983). The PVN has been implicated in the regulation of cardiovascular system (Sawchenko and Swanson, 1982b), feeding behavior (Sawchenko, 1982), thirst (Swanson and Mogenson, 1981) and other physiological responses. In summary, the anatomical and physiological evidence suggests that the PVN is involved in the integration of autonomic and endocrine responses.

PVN neuronal activity can be altered in response to immune stress and psychological stress. As indicated previously, circulating cytokines gain access to the brain via the CVOs or the vagus. The CVOs and the vagus nerve are neurally connected to specific autonomic centers, such as the NTS, ventrolateral medulla (VLM) in the brainstem, central nucleus of the amygdala, and medial preoptic area in the forebrain (Cunningham et al., 1994). These autonomic centers through their connections with the PVN in the forebrain, could then transmit the appropriate signals to alter PVN neuronal activity. It has been shown that circulating cytokines could induce central IL-1β gene expression or protein in the PVN (Dinarello, 1988; Lechan et al., 1990; Buttini and Boddeko, 1995; Hopkins and Rothwell, 1995). The centrally-produced IL-1β then stimulates other neurons in the PVN to release CRF (Berkenbosch et al., 1987; Sapolsky et al., 1987; Navarra et al., 1991; Kakucska et al., 1993) which stimulates release of adrenocorticotropin hormone (ACTH; Berkenbosch et al., 1987; Kakucska et al., 1993) from the anterior pituitary, and

glucocorticoids are then secreted by the adrenal glands (Vale et al., 1981). Thus, the HPA axis is activated in the communication between the immune system and the brain. Because the CRF-containing parvocellular neurons in the PVN also project to the brainstem or the spinal cord, the autonomic system may also be involved in the brain's response to immune stress.

Though the *in vivo* effects of circulating cytokines on VP or OT mRNA levels in the PVN have not been well studied, based on the distribution of LPS activated neurons in both the magnocellular regions of the PVN, the results imply that these neurons could produce VP or OT (Elmquist et al., 1996; Yang et al., 1999). As mentioned earlier, VP- and OT-producing neurons in the magnocellular region of the PVN project to the posterior pituitary, suggesting that the posterior pituitary may participate in the brain's response to immune stress as well. In addition, psychological stress, such as restraint or immobilization, leads to increased levels of CRF, VP or OT mRNA in the PVN (Bartanusz et al., 1993; Krukoff et al., 1999). These findings suggest that the PVN neuronal activity is altered in response to stresses.

1.6 Involvement of nitric oxide

It has been suggested that nitric oxide (NO) participates in the regulation of the PVN neuronal activity in response to stresses. The gas NO, first identified as the chemical responsible for endothelium-derived relaxing factor activity, is now well-established as a major endogenous vasorelaxant, and also acts as a nonconventional neuromessenger in the brain (Garthwaite, 1991; Moncada et al., 1991; Bredt and Snyder, 1992). The enzyme involved in the synthesis of NO, NO

synthase (NOS), exists in three major isoforms (Forstermann et al., 1991; Forstermann et al., 1995). Type I or neuronal NOS (nNOS) was first purified from rat and porcine cerebellum (Bredt and Snyder, 1990; Mayer et al., 1990; Schmidt et al., 1991). Immunohistochemistry using antibodies specific to nNOS suggests that the isoform is also expressed in certain areas of the spinal cord (Dun et al., 1992), in sympathetic ganglia and adrenal glands (Dun et al., 1993; Sheng et al., 1993), in epithelial cells of lung, uterus, stomach and kidney (Schmidt et al., 1992), and in human and rat skeletal muscle (Nakane et al., 1993; Kobzik et al., 1994). nNOS is constitutively expressed in the brain and nNOS activity is calcium-dependent (Forstermann et al., 1991; Forstermann et al., 1995). NOS catalyzes the synthesis of NO from L-arginine, soluble guanylyl cyclase, the target of NO, catalyses the production of the second messenger, cGMP, in target cells. cGMP can then affect ion channel or phosphodiesterase activity, or activate cGMP-dependent protein kinases to produce subsequent cellular events (Bredt and Snyder, 1989). The catalytic activity of NOS requires nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. NADPH, diaphorase and tetrazolium salts yield blue-colored precipitates, and this simple histological marker had been used to represent nNOS in the brain. It has been shown that nNOS and neuronal NADPH diaphorase (NADPH-d) are identical in the PVN when appropriate fixation has been used (Dawson et al., 1991; Hope et al., 1991). nNOS is found in neurons throughout the brain, many of which are known to participate in autonomic functions. In the PVN, both parvo- and magnocellular neurons contain nNOS (Arévalo et al., 1992; Vincent and Kimura, 1992; Vincent, 1994). nNOS coexists with CRF, VP or OT in

the PVN (Sánchez et al., 1994; Turnbull and Rivier, 1996).

Type II or inducible NOS (iNOS) can be induced in many cell types by cytokines, bacterial LPS and a variety of other agents. It was first isolated from mouse macrophages (Hevel et al., 1991; Stuehr et al., 1991). Immunohistochemical localization of iNOS in rats treated with LPS demonstrated the enzyme in macrophages, endothelial cells, hepatocytes, and glia (Bandaletova et al., 1993). iNOS mRNA is also specifically induced in the brain by intraperitoneal endotoxin (Wong et al., 1996; Jacobs et al., 1997; Satta et al., 1998). Under basal levels, iNOS is not expressed. iNOS is induced and its activity is calcium-independent. There is strong experimental evidence that iNOS is a key factor in the development of circulatory failure in endotoxic shock (Julou-Schaeffer et al., 1990; Thiemermann and Vane, 1990; Kilbourn and Griffith, 1992; Meyer et al., 1992; Suba et al., 1992; Wright et al., 1992; Szabó et al., 1993; Thiemermann, 1994).

Type III or endothelial NOS (eNOS) was first identified in endothelial cells. Immunohistochemical studies localized the enzyme to various types of endothelial cells in many tissues (Pollock et al., 1993; Myatt et al., 1993; Tracey et al., 1994). Surprisingly, eNOS immunoreactivity has also been described in neurons of the rat hippocampus and other brain regions (Dinerman et al., 1994). Like nNOS, eNOS is constitutively produced and calcium-dependent. The peripheral effects of eNOS on vasodilation have been well studied; on the other hand, studies of central effects of eNOS on neuronal activity have been few.

NO has been reported to have both stimulatory and inhibitory effects on hypothalamic neuronal activity. In support of NO's inhibitory effects, investigators

showed that NO donors inhibit IL-1β-stimulated CRF release in the rat hypothalamus *in vitro* (Costa et al., 1993), NO inhibits VP release by intravenous injection of the general NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; Goyer et al., 1994; Yamamoto et al., 1994), and NO inhibits the activity of vasopressinergic and oxytocinergic neurons in electrophysiological studies using slice preparations of the PVN (Bains and Ferguson. 1997). On the other hand, in support of stimulatory effects of NO, L-arginine has been shown to enhance interleukin 2-induced CRF release in the hypothalamus *in vitro* (Karanth et al., 1993) and peripherally administered blockers of NOS inhibit stress-induced activation of PVN neurons (Amir et al., 1997; Lee and Rivier, 1998).

These studies on effects of NO in hypothalamic neuronal activity are conflicting, and it was not indicated which specific isoform(s) of NOS was (were) involved in either the inhibitory or the stimulatory effects. One study showed that NO from eNOS suppressed the ACTH response to IL-1β, suggesting that eNOS might play an important role in regulating the HPA axis (Lee and Rivier, 1998). Therefore, the effect of NO in the brain is very complex and may depend on the type of stimulus.

A number of studies have shown that blockade of NO production leads to increased arterial pressure and renal sympathetic nerve activity (Sakuma et al., 1992; Harada et al., 1993), whereas administration of NO donor leads to hypotension (Lewis et al., 1991; Kagiyama et al., 1997). These results support the hypothesis that central NO acts to decrease sympathetic output to the periphery.

1.7 Hypothesis

The experiments described in this thesis were designed to test the following **hypotheses: (1) Putative NO-producing neurons are activated in the PVN in response to immune and psychological stress(es). (2) NO regulates PVN neuronal activity in response to immune stress.**

This thesis is presented in a paper format, and consists of two manuscripts. The first paper in chapter two addresses hypothesis 1, the second paper in chapter three addresses hypothesis 2.

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Chapter 2

Immune Stress Activates Putative Nitric Oxide-Producing Neurons in Rat Brain: Cumulative Effects with Restraint[‡]

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O. Oskin assisted with the processing of LPS + restraint animals. The data analysis was done by W. Yang.

2.1 ABSTRACT

Immune and restraint stresses induce changes in the hypothalamo-pituitary-adrenal axis activity and autonomic function. In the hypothalamus, the paraventricular nucleus (PVN) plays an integral role and nitric oxide (NO) is hypothesized to participate in this process. We used 1) intravenous injections of lipopolysaccharide (LPS, 125 μg/kg) to identify activated (Fos-positive) putative NO-producing neurons, 2) retrograde tracing to determine if autonomic medullary regions signal the PVN to mediate this activation, and 3) intravenous LPS injections plus restraint stress to determine if responses to restraint are altered by the presence of immune stress. At 2 hours after LPS injections, approximately 15% of putative NO-producing neurons were activated in the nucleus of the tractus solitarius (NTS) and ventrolateral medulla (VLM); about half of the putative NO neurons in the PVN were activated. In LPS + restraint rats, the percentage of activated putative NO neurons in the PVN was not significantly different from LPS-treated rats, but the numbers of putative NO neurons and activated NO neurons per section increased significantly. Retrogradely labeled neurons were found mostly in the middle NTS and VLM, and about 75% were activated. No neurons in the NTS or VLM were triple labeled. The results show that putative NO-producing neurons in the PVN, NTS, and VLM are activated by circulating LPS. However, the LPS-induced signaling to the PVN likely occurs through pathways other than the NO network of neurons in NTS or VLM. Finally, superimposition of restraint stress onto animals already exposed to immune

stress stimulates the NO system in the PVN to a greater extent than immune stress alone.

2.2 INTRODUCTION

Information transfer between the immune system and the brain allows bidirectional regulation of immune and brain responses to infection. Detection of pathogens by cells of the immune system triggers immune cells to synthesize and release a series of chemical messengers, including cytokines. Circulating cytokines including those whose release is stimulated by the endotoxin, lipopolysaccharide (LPS), act at several central sites to initiate the response of the central nervous system (CNS) to immune stress (Brady et al., 1994; Ericsson et al., 1994; Watkins et al., 1995). It is known that LPS administration activates autonomic neurons in the nucleus of the tractus solitarius (NTS), ventrolateral medulla (VLM) and the paraventricular nucleus of hypothalamus (PVN; Sagar et al., 1995; Wan et al., 1993). Furthermore, neurons in the NTS and VLM activated by cytokines project to the PVN (Ericsson et al., 1994). The hypothalamo-pituitary-adrenal (HPA) axis can be stimulated through these pathways or directly by centrally-produced cytokines via the PVN so that corticotropin releasing factor (CRF) is produced to stimulate the release of adrenocorticotropin hormone (ACTH) by the anterior pituitary gland (Kakucska et al., 1993; Berkenbosch et al., 1987). A subsequent step in this output includes the release of glucocorticoids by the adrenal glands (Vale et al., 1981).

Nitric oxide (NO) is a freely diffusible and short-lived gas that is produced by NO synthase (NOS; Bredt and Snyder, 1994; Marletta, 1989; Moncada et al., 1991) and is believed to regulate the output of sympathetic nervous activity (Krukoff, 1998). The neuronal isoform of NOS is produced in neurons throughout the brain, including autonomic centers such as the NTS, VLM, and PVN; in the PVN, both parvo- and magnocellular neurons produce NO (Arévalo et al., 1992; Hatakeyama et al., 1996; Sanchez et al., 1994; Siaud et al., 1994; Torres et al., 1993; Villar et al., 1994).

We have previously shown that restraint stress activates putative NO-producing neurons in the brain (Krukoff and Khalili, 1997). With regard to responses to immune system-brain interactions, blockade of NO synthesis leads to prolonged cytokine activation of the HPA axis (Rivier and Shen, 1994) and circulating LPS stimulates gene expression of neuronal NOS in the hypothalamus (Lee et al., 1995). Together, these results suggest that NO is an important neurotransmitter involved in mediating the brain's responses to both psychological and immune stress.

The purpose of the present study was to further investigate the role of NO in regulating the brain's responses to stress(es). First, we wished to determine whether LPS administration activates putative NO-producing neurons in autonomic centers of the brain. Second, we investigated the possibility that NO may act as a neurotransmitter in LPS-activated pathways from the NTS and VLM to the PVN. Third, by using the NO system, we explored the notion that the presence of one stressor (LPS) may lead to increased activation of the HPA axis

when another stressor (restraint) is superimposed upon the first. Fos immunohistochemistry was used to identify activated neurons, nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) staining was used to localize putative NO-producing neurons, and retrograde tracer labeling was used to localize neurons that project to the PVN.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Male Sprague-Dawley rats (250-300 g) were purchased from the Biological Animal Center, University of Alberta. They were housed two per cage under a 12:12 hour light-dark cycle at a temperature of 21°C. The rats were given free access to food and water. All protocols used in these experiments were approved by the University of Alberta Animal Welfare Committee.

2.3.2 Instrumentation and retrograde tracer injection

Rats were anesthetized with sodium pentobarbital (60 mg/kg; i.p.; Somnotol, M.T.C. Pharmaceuticals, Hamilton, Canada) and a silastic catheter (0.02 inch internal diameter x 0.037 inch external diameter) was inserted into the vena cava and sutured to the posterior abdominal wall. The free end of the catheter was passed under the skin and externalized between the scapulae with a 27-gauge stainless steel tubing. The line was closed with polyvinyl-pyroolidone and capped with silastic tubing. During the same surgical session, a subgroup of rats was placed in a stereotaxic frame. After exposing the skull and drilling a burr hole, a glass micropipette filled with 10-20 nl of rhodamine-labeled latex

microspheres (Lumafluor, Florida) was inserted in the PVN. An air-pressure system was used for ejecting the microspheres. After injection, the pipette was removed, the burr hole was sealed with bone wax, wounds were closed, and the animal was allowed to recover. Animals were handled daily after surgery so that they would become familiar with the manipulations of the experiment.

2.3.3 LPS administration

Five to 7 days after surgery, rats with tracer injections received intravenous injections of LPS (125 µg/kg; L-4005, Sigma Corp., St. Louis, MO) dissolved in saline (Elmquist et al., 1996) for a total of 0.3 ml/rat. Control rats were injected with the same amount of saline (iv).

2.3.4 LPS administration plus restraint stress

A group of rats without tracer received LPS injections as above. Sixty minutes later, the rats were restrained for one hour in hemicylinderical, well-ventilated, plexiglass tubes (Krukoff and Khalili, 1997).

2.3.5 Tissue preparation

Two hours after LPS injections or saline administration, and one hour following the restraint, rats were deeply anesthetized and perfused transcardially with 200 ml 0.9% saline followed by 500 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were isolated, postfixed in half-strength fixative and 10% sucrose for 1 hour, and stored in 20% sucrose overnight at 4°C. Coronal sections (50 µm) of the brain were cut in a cryostat. Sections of the brainstem and forebrain were collected in phosphate-buffered saline (PBS; pH 7.2) for further processing.

Sections were incubated for 30 minutes at 37°C in 0.1 M PBS (pH 7.2) containing 0.6% Triton X-100, 1 mg/ml β-NADPH, and 0.1 mg/ml nitroblue tetrazolium (Sigma). After a brief rinse in PBS, sections were incubated overnight with rabbit anti-c-*fos* antiserum (Oncogene, Uniondale, NY; 1.4 µg/ml) in 0.3% Triton X-100/PBS. The next day, sections were incubated with biotinylated anti-rabbit IgG (Vector; 1:200) for 1 hour and then with avidin-biotin complex (ABC Vecta Stain Kit; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. After a rinse in PBS, sections were exposed to 0.04% diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.005% hydrogen peroxide in PBS for 5-10 minutes. Sections were mounted onto glass microscope slides, air dried, coverslipped with Elvanol (Moviol, Calbiochem Corp. La Jolla, CA; dissolved in 40 ml PBS and 20 ml glycerol) and examined by using a Zeiss light/fluorescence microscope.

2.3.6 Analysis

Neurons with Fos-like immunoreactivity (FLI, stained in the nucleus) or positive for NADPH-d (stained in the cytosol) were counted with light microscopy; neurons with retrograde label with fluorescence microscopy. Labeled neurons were counted in every section of the NTS and VLM ipsilateral to the microsphere injections; in the PVN, they were counted on the sides contralateral to the injections. The PVN was divided into caudal, middle and rostral regions for purposes of analysis.

To avoid double-counting neurons in adjacent sections, data were expressed as numbers of labeled neurons *per section*. The following calculations were made:

LPS administration: (1) total numbers of neurons per section singly labeled for FLI, NADPH-d, or retrograde tracer, (2) total numbers of neurons per section double-labeled for FLI and NADPH-d, (3) total numbers of neurons per section double-labeled for FLI and retrograde tracer, (4) percentages of neurons with FLI as a proportion of neurons with either retrograde or NADPH-d, (5) percentages of neurons with retrograde tracer as a proportion of neurons with FLI, (6) percentages of neurons with NADPH-d as a proportion of neurons with FLI.

LPS + restraint: (1) total numbers of neurons per section in the PVN singly labeled for FLI or NADPH-d, (2) total numbers of neurons per section in the PVN double-labeled for FLI and NADPH-d, (3) percentages of neurons with FLI as a proportion of neurons with NADPH-d in the PVN, and (4) percentages of neurons with NADPH-d as a proportion of neurons with FLI in the PVN.

An analysis of variance test was used to determine whether values from control, LPS, and LPS + restraint rats were statistically different from each another. When levels of significance were indicated, the Student-Newman-Keuls test was used to indicate which pairs of data were significantly different from one another. $P < 0.05$ was taken to signify statistical significance.

2.4 RESULTS

Four injections sites in the PVN are illustrated in Figure 2-1.

2.4.1 Neurons with FLI

In control animals, few neurons showed FLI throughout the brain. LPS administration led to expression of FLI in neurons throughout the brain that were distributed similarly to that described previously by others (Elmquist et al., 1996); these results will not be repeated here. In the PVN, the largest numbers of neurons with FLI were found in the middle regions of both LPS and LPS + restraint rats. There were no significant differences between the numbers of neurons per section with FLI in the PVN between LPS and LPS + restraint rats, either when considered as a whole (Table 2-1) or as caudal to rostral regions (Table 2-2).

2.4.2 NADPH-d labeled neurons in the PVN

In control animals, 23 ± 5 NADPH-d positive neurons per section were found in the PVN (see Table 2-1). The number of NADPH-d containing neurons per section was increased with LPS injections (49 ± 7); a further increase in the number of NADPH-d labeled neurons was observed after LPS + restraint administration (90 ± 8). Numbers of NADPH-d labeled neurons were significantly higher in all regions of LPS + restraint rats compared to LPS rats with the largest numbers of these neurons found in the middle PVN (Table 2-2). Both parvo- and magnocellular regions of the PVN contributed to these increases (Figure 2-2).

2.4.3 FLI and NADPH-d labeled neurons

Very few double labeled neurons were found in the PVN of control rats (Table 2-1). LPS injections led to a significant increase in the number of double labeled neurons per section (25 ± 2) and a further significant increase was found

in LPS + restraint rats (43 ± 4). LPS + restraint rats showed significantly higher percentages of double labeled neurons as a percentage of neurons with FLI than LPS rats whereas there was no significant difference between the percentages of double labeled neurons as a percentage of NADPH-d neurons found in the LPS and LPS + restraint rats (Table 2-1).

Regionally, significantly larger numbers of double labeled neurons were found in the caudal and rostral PVN of LPS + restraint rats compared to LPS rats (Table 2-2). Double labeled neurons were found in both the parvo- and magnocellular regions of the PVN (Figures 2-2 and 2-3).

Small numbers of double-labeled neurons per section were found in the NTS (3 ± 0.7) and VLM (2 ± 0.4) following LPS injections (Table 2-3). The majority of these neurons was found in the region of the NTS just rostral to the area postrema and in the rostral VLM.

2.4.4 Retrogradely labeled neurons

Retrogradely labeled neurons in the NTS and VLM were found mostly at the level of the area postrema; 67% (± 8) and 79% (± 3) of the retrogradely labeled neurons in the NTS and VLM, respectively, also contained FLI in LPS rats (Table 2-3). On the other hand, no triple-labeled neurons were found in the NTS or VLM of LPS-treated rats.

2.5 DISCUSSION

The results of this study support the hypothesis that the neuronal NO system is important in the brain's responses to immune stress. We show that

putative NO-producing neurons in the PVN, NTS, and VLM are activated by circulating LPS, but that activated NADPH-d neurons in neither the NTS nor the VLM participate in direct signaling to the PVN during exposure to LPS. Finally, we have shown that the response of the NO system in the PVN to an immune stress plus restraint is greater than the response to the immune stress alone.

Most available evidence supports the view that NADPH-d staining in the brain represents NO synthase when fixation has been used (Dawson et al., 1991; Matsumoto et al., 1993; Norris et al., 1995; Rothe et al., 1998). An analysis of the effects of fixation on NADPH-d staining in rat striatum showed that, whereas staining density of neurons can be affected by fixation conditions, the numbers of neurons stained are not affected (Kuo et al., 1994). Lumme et al. (1997) compared numbers of neurons in the PVN and supraoptic nucleus stained for NADPH-d and NO synthase immunoreactivity, and found that the two values correlated very well even after axotomy or colchicine treatment. They concluded that NADPH-d staining represents NO synthase in these nuclei. Therefore, we believe that our NADPH-d staining is indicative of NO-producing neurons.

2.5.1 LPS activates NADPH-d positive neurons in the PVN.

Our results indicating that intravenous LPS activates neurons in central autonomic centers of the forebrain and brainstem are similar to those described previously for the same dose (Elmquist and Saper, 1996; Elmquist et al., 1996). We now show that significant proportions of NADPH-d neurons in the PVN are activated by circulating LPS.

Intravenous administration of LPS induces secretion of several cytokines from the immune system which, in turn, can signal the brain to begin the process that will rid the body of the toxic agent. Circulating cytokines such as the interleukins are known to activate neurons in the brain (Brady et al., 1994; Hanisch and Quirion, 1996; Callahan and Piekut, 1997) and our results suggest that some of these neurons produce nitric oxide. Furthermore, immune-brain interactions also contribute to the host defence response which includes changes in arterial pressure, body temperature, gastrointestinal activity, and other autonomic functions. These alterations may contribute to the activation of NADPH-d neurons in central autonomic centers. We have noted that LPS (100 µg/kg iv) elicits a rise in arterial pressure of approximately 20 mm Hg within the first 30 minutes after injection, a drop to normal levels within the next 30 minutes, and no significant change afterward (chapter 3). Larger doses of LPS (200 µg/kg iv), on the other hand, induce a drop in mean arterial pressure within 30 minutes following injections (Tkacs and Strack, 1995). LPS at the same dose (iv) as in the present study has been shown to lead to a drop in body temperature of 3°C after 90 minutes, a recovery of body temperature, and finally a rise in temperature after 4 hours (Elmquist et al., 1996). Therefore, it is likely that a combination of physiological responses contribute to the activation of NO-producing neurons in the brain.

NO is hypothesized to restrain the sympathetic nervous system (Krukoff, 1998) and may restrain the HPA axis during exposure to inflammatory or infectious agents. For example, NO donors inhibit interleukin 1β-stimulated CRF

release in the rat hypothalamus *in vitro* (Costa et al., 1993). In addition, release of ACTH in response to interleukin 1β is increased with blockade of NO synthase (Rivier and Shen, 1994; Kim and Rivier, 1998). On the other hand, L-arginine has been shown to enhance interleukin 2-induced CRF release in the hypothalamus *in vitro* (Karanth et al., 1993) and peripherally-administered blockers of NO synthase inhibit stress-induced activation of PVN neurons (Amir et al., 1997; Lee and Rivier, 1998). Therefore, NO's role in affecting the HPA axis is equivocal and may depend on the nature of the stress (Lee and Rivier, 1998). Nevertheless, because CRF synthesis occurs in the parvocellular neurons of the PVN (Swanson et al., 1986), the activated NADPH-d neurons in these regions may represent the NO-producing neurons that participate in the CRF response to LPS. NO has been shown to depolarize neurons in the parvocellular PVN (Bains and Ferguson, 1997b), thalamus (Pape and Mager, 1992), and dorsal motor nucleus of the vagus (Travagli and Gillis (1994). These responses are all dependent on activation of cGMP and may represent the mechanism through which NO can affect the HPA axis. Finally, the presence of activated NADPH-d neurons in the ventral medial parvocellular division of the PVN suggests that NO may participate in central autonomic pathways as these neurons project to the brainstem or spinal cord (Sawchenko and Swanson, 1982).

NADPH-d neurons activated by LPS or LPS + restraint were found in the magnocellular divisions of the PVN. The location of these neurons implies that they also produce either vasopressin or oxytocin. Indeed, colocalization of NADPH-d or NO synthase immunoreactivity with the neurohormones has been

demonstrated in magnocellular neurons (Sánchez et al., 1994; Hatakeyama et al., 1996). The role that NO may play in modulating the activity of magnocellular neurons has been addressed both *in vivo* and *in vitro*. Intracerebroventricular injections of the NO donor, SNAP, led to increases in vasopressin release (Ota et al., 1993). Nitroprusside application to hypothalamic slices also elicited release of vasopressin into the culture medium (Raber and Bloom, 1994). On the other hand, intravenous injections of N^G-nitro-L-arginine methyl ester (L-NAME) suggested that NO inhibits vasopressin release (Goyer et al., 1994). In agreement with the latter study, experiments with explant cultures of hypothalamus or neural lobe of the pituitary gland showed that NO inhibits release of vasopressin at both sites (Yasin et al., 1993; Lutz-Bucher and Koch, 1994). Finally, electrophysiological studies using slice preparations of the PVN (Bains and Ferguson, 1997a) or supraoptic nucleus (Liu et al., 1997) showed that NO inhibits the activity of vasopressinergic and oxytocinergic neurons, and that the inhibition in the PVN is at least partly mediated through the GABAergic neurotransmitter system (Bains and Ferguson, 1997a). Therefore, the majority of the above studies supports the concept that NO acts on PVN magnocellular neurons to inhibit the release of neurohormones.

2.5.2 Activated NADPH-d neurons in the NTS and VLM do not project to the PVN.

Our study confirms the finding that neurons in the NTS and VLM are activated following i.v. LPS injections and that a large proportion of these neurons projects to the PVN (Elmquist and Saper, 1996). We also show that

retrogradely labeled neurons in the NTS and VLM were found mainly at the level of the area postrema and that the majority of these neurons was activated. Additionally, we found that a few NADPH-d positive neurons in the NTS and VLM were activated. Activated NADPH-d positive neurons in the NTS and VLM did not, however, project to the PVN, suggesting that the communication of the LPS-induced signal to the PVN occurs through pathways other than the NO neuronal system of the NTS/VLM.

2.5.3 Restraint stress added to immune stress leads to increased stimulation of NADPH-d neurons in the PVN.

The effect of superimposing restraint stress onto rats already exposed to immune stress is intriguing. First, the numbers of neurons activated by LPS + restraint were not significantly different from the those activated by LPS alone. However, the numbers of neurons stained for NADPH-d were significantly increased in the PVNs of LPS + restraint rats compared to LPS rats. This type of finding is not without precedent. Increases in numbers of NADPH-d neurons have been observed in hypothalamic nuclei after axonal injury and colchicine treatment (Lumme et al., 1997), and in medial preoptic nucleus and ventromedial hypothalamus of female rats after estradiol treatment (Okamura et al., 1994a,b). NADPH-d staining in the supraoptic nucleus is also activity dependent (Pow, 1992). Numbers of neurons with NO synthase immunoreactivity in the PVN and supraoptic nucleus increased with chronic salt loading (Villar et al., 1994).

We have shown that the percentages of double labeled neurons as proportions of activated neurons were significantly elevated in LPS + restraint

rats compared to LPS rats, whereas the percentages of double labeled neurons as proportions of NADPH-d neurons were similar for LPS and LPS + restraint rats. This study also revealed that larger numbers of NADPH-d neurons in both the parvocellular and magnocellular divisions are activated by LPS + restraint compared to LPS alone. Together these results indicate, therefore, that significant numbers of neurons activated by LPS + restraint were recruited to express NADPH-d and they suggest that immune plus restraint stresses elicit increased stimulation of the NO system in the PVN compared to immune stress alone.

2.6 CONCLUSIONS

We have shown that LPS and LPS + restraint induce the activation of NADPH-d positive neurons in both the parvo- and magnocellular regions of the PVN. These results support the hypothesis that NO participates in the regulation of the HPA axis activity and autonomic functions. Furthermore, we have shown that superimposing restraint stress onto animals already exposed to immune stress leads to increased stimulation of the NO system in the PVN. These results have important implications for the physiological state of animals exposed to psychological stress when they are already responding to an immune stress.

Table 2-1: Total numbers of neurons per section single and double (Dbl) labeled for FLI and/or NADPH-d, percentages of neurons with FLI as a proportion of neurons with NADPH-d and percentages of neurons with NADPH-d as a proportion of neurons with FLI in the PVN of control, LPS and LPS + restraint (R) rats[1].

	Control	LPS	LPS + R
FLI	27 ± 0.6	139 ± 10*	111 ± 14*
NADPH-d	23 ± 5	49 ± 7* †	90 ± 8* †
Double	2 ± 0.2	25 ± 2* †	43 ± 4* †
%Dbl/FLI	6 ± 0.6	19 ± 2* †	39 ± 2* †
%Dbl/NADPH-d	8 ± 1	54 ± 6*	49 ± 4*

[1], n=3 for control, n=4 for LPS, n=4 for LPS + R.

*, significant difference compared to control.

†, significant difference between experimental groups.

Table 2-2: Numbers of neurons per section single and double (Dbl) labeled for FLI and/or NADPH-d, percentages of neurons with FLI as a proportion of neurons with NADPH-d and percentages of neurons with NADPH-d as a proportion of neurons with FLI in the caudal, middle and rostral PVN of control, LPS and LPS + restraint (R) rats[1].

	Caudal PVN			Middle PVN			Rostral PVN		
	Control	LPS	LPS+R	Control	LPS	LPS+R	Control	LPS	LPS+R
FLI	28 ± 3	122 ± 15*	87 ± 22*	40 ± 6	217 ± 11*	176 ± 39*	13 ± 4	77 ± 13*	73 ± 12*
NADPH-d	15 ± 2	46 ± 6* †	74 ± 9* †	37 ± 14	68 ± 8 †	120 ± 16* †	16 ± 5	34 ± 6* †	76 ± 10* †
Double	1 ± 0.4	14 ± 3* †	34 ± 3* †	3 ± 0.7	45 ± 5*	64 ± 12*	1 ± 0.4	17 ± 1* †	33 ± 4* †
%Dbl/FLI	4 ± 1	12 ± 1 †	43 ± 6* †	6 ± 0.7	21 ± 3* †	37 ± 2* †	6 ± 2	23 ± 3* †	47 ± 6* †
%Dbl/NADPH-d	7 ± 2	38 ± 9*	46 ± 3*	8 ± 3	70 ± 3* †	53 ± 5* †	8 ± 3	53 ± 1*	45 ± 6*

[1], n=3 for control, n=4 for LPS, n=4 for LPS + R.

*. Significant difference compared to control.

†. Significant difference between experimental groups.

Table 2-3. Total numbers of neurons per section labeled for FLI, NADPH-d or retrograde tracer, total numbers of neurons per section double labeled for FLI with NADPH-d or with retrograde tracer, percentages of neurons with FLI as a proportion of neurons with NADPH-d, percentages of neurons with NADPH-d as a proportion of neurons with FLI, percentages of neurons with FLI as a proportion of neurons with retrograde tracer, and percentages of neurons with retrograde tracer as a proportion of neurons with FLI in the NTS and VLM following LPS injections (n=4).

	NTS	VLM
FLI	80 ± 12	27 ± 3
NADPH-d	15 ± 3	17 ± 2
Tracer	8 ± 0.3	7 ± 0.8
FLI + NADPH-d	3 ± 0.7	2 ± 0.4
FLI + Tracer	5 ± 0.2	5 ± 0.8
% (FLI + NADPH-d) / FLI	4 ± 1	7 ± 0.6
% (FLI + NADPH-d) / NADPH-d	17 ± 5	13 ± 3
% (FLI + Tracer) / FLI	6 ± 0.6	22 ± 2
% (FLI + Tracer) / Tracer	67 ± 8	79 ± 3

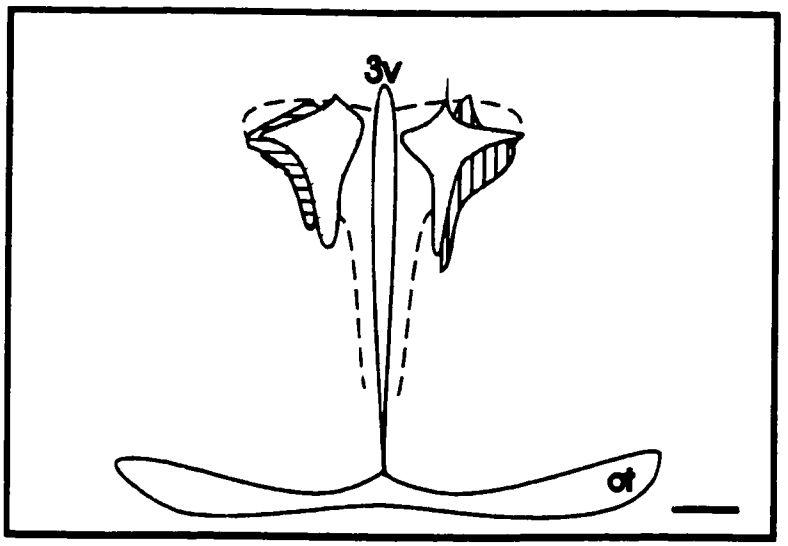

Figure 2-1: Schematic drawing illustrating unilateral injection sites of retrograde tracer in the paraventricular nucleus (PVN) of lipopolysaccharide-treated (LPS) rats. For convenience, two sites are shown on each side of the PVN and each site is indicated with a different type of shading. The dotted line indicates the location of the PVN. Abbreviations: 3v, third ventricle; ot, optic tract. Scale bar = 350 μm.

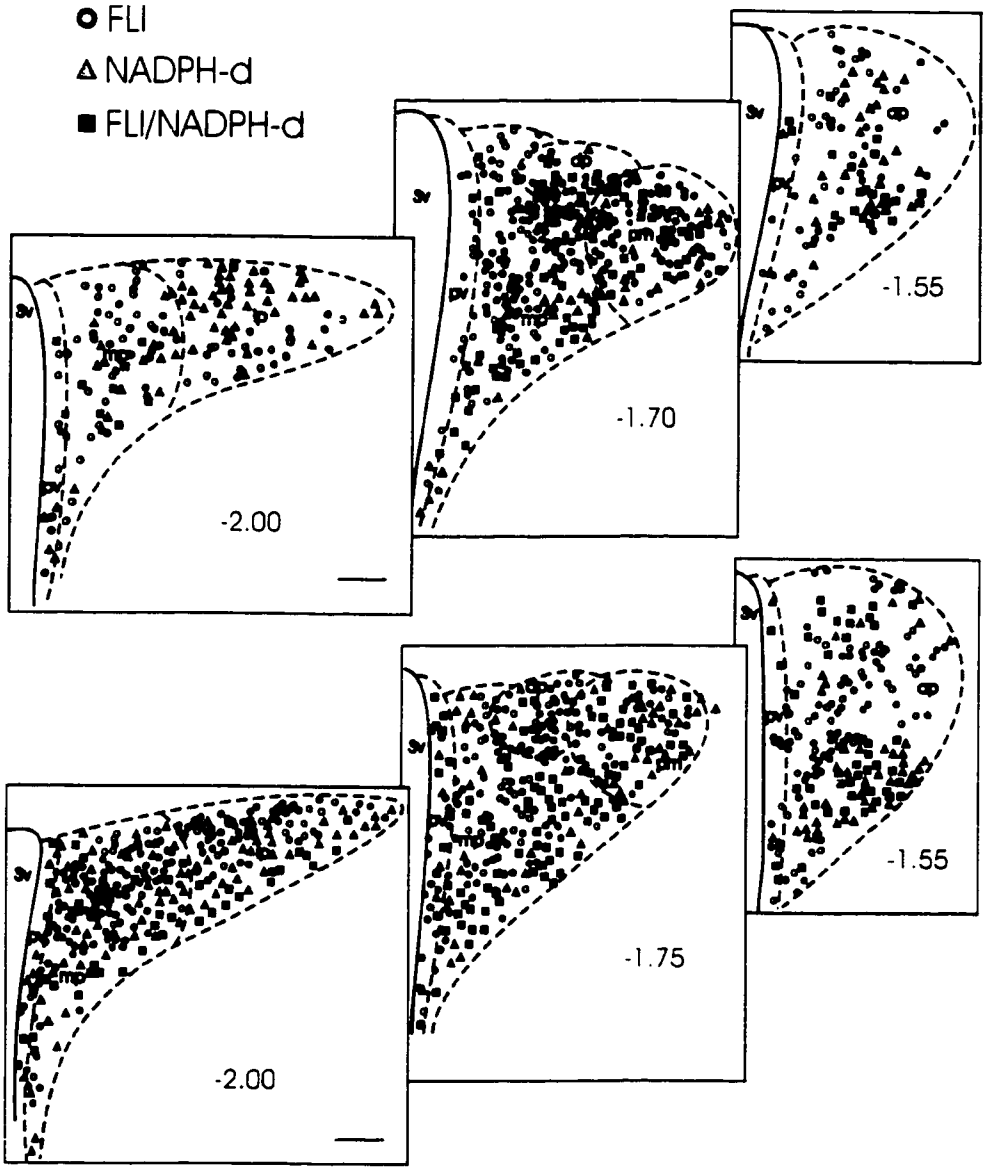

Figure 2-2: Composite drawings showing labeling at three levels of the PVN in LPS (top) and LPS + restraint (bottom) rats. Numbers indicate rostrocaudal coordinates relative to Bregma (Paxinos and Watson, 1986). Abbreviations: 3v, third ventricle; FLI, Fos-like immunoreactivity; NADPH-d, nicotinamide adenine dinucleotide phosphate-diaphorase; ap, dp, lp, and mp, anterior, dorsal, lateral, and medial parvocellular divisions of the PVN; pv, periventricular PVN; pm, posterior magnocellular PVN. Scale bars = 100 μm and refer to all panels.

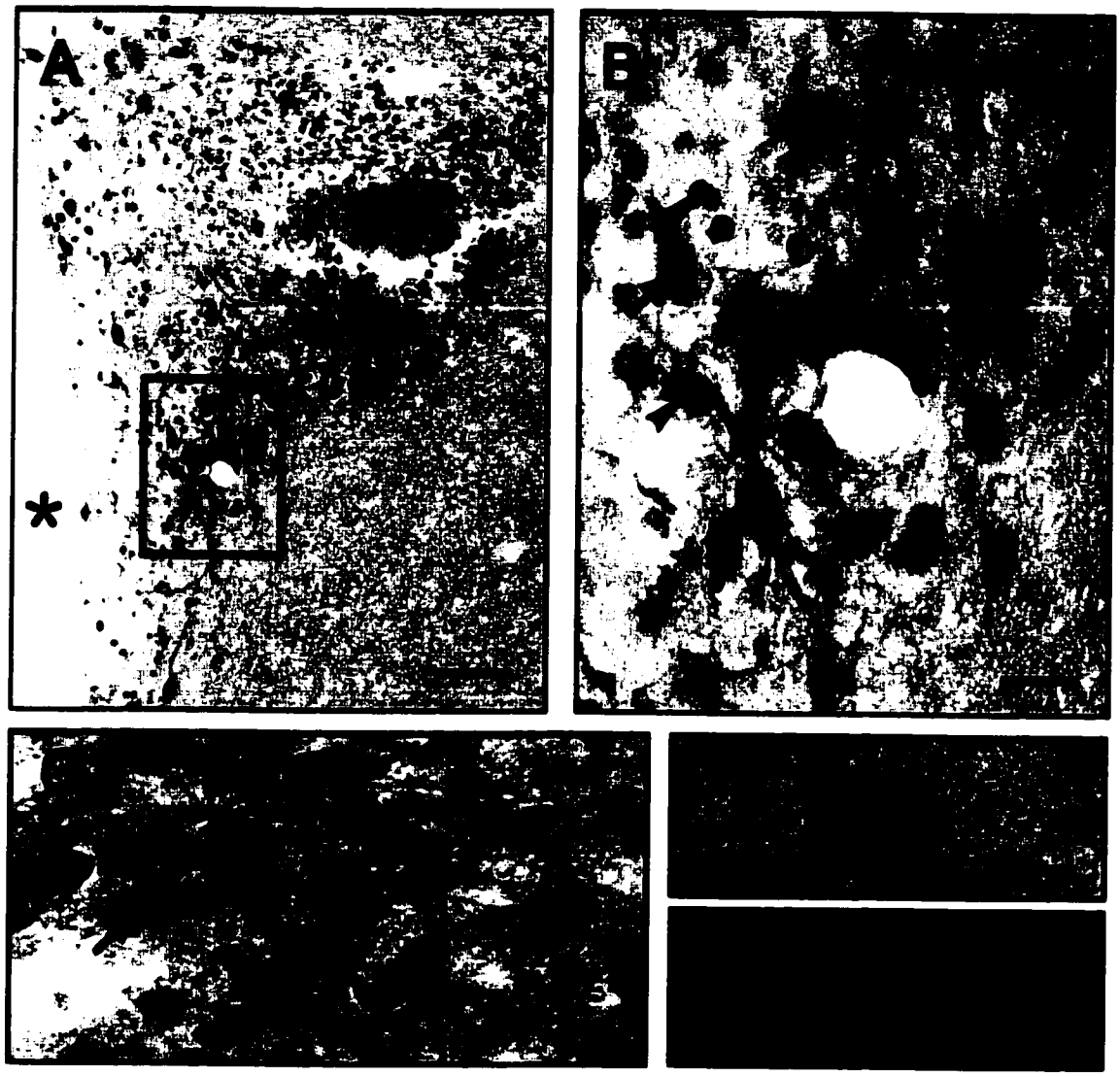

Figure 2-3: Fos-like immunoreactivity (FLI) and NADPH-d staining in the PVN (A,B) and NTS (C-E) of rats treated with LPS. The area within the rectangle in A is shown at higher magnification in B. Arrowheads in B and C represent single labeled neurons (FLI in nucleus or NADPH-d in cytosol); arrows represent double labeled neuron for FLI and NADPH-d (B and C) or FLI and retrograde tracer (D and E). Scale bars = 100 μm in A, 30 μm in B and C, and 12 μm in D and E. Asterisk, third ventricle.

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CHAPTER 3

NO REGULATES THE PVN NEURONAL ACTIVITY IN RESPONSE TO ENDOTOXIN[#]

[#] The data in this chapter has not yet been published. Used with permission from supervisor Dr. TL Krukoff.

3.1 ABSTRACT

Systemic administration of endotoxin induces changes in central neuronal activity, including gene expression of the cytokine, interleukin-1β (IL-1β). Nitric oxide (NO) has been also implicated in the modulation of neuroendocrine function. This study was performed to determine if NO regulates neuronal activation and IL-1β gene expression in the paraventicular nucleus of the hypothalamus (PVN) in response to intravenous endotoxin, lipopolysaccharide (LPS, 100 μg/kg). Intracerebroventricular injections of NO synthase (NOS) inhibitors, 7-nitroindazole sodium salt (7-NiNa, 10mM) for neuronal NOS (nNOS), N^G-nitro-L-arginine (L-NNA, 10mM) for nNOS and endothelial NOS (eNOS), and aminoguanidine (0.3 and 3mM) for inducible NOS, in artificial cerebrospinal fluid (aCSF) were made in conscious rats 30 minutes prior to LPS injections and again 2 hours after LPS. Control rats received aCSF + LPS. Four hours after LPS injections, brains were processed for Fos immunohistochemistry, NADPH-d histochemistry for nNOS neurons, and IL-1β *in situ* hybridization. Blood pressure (BP) and body temperature (Tb) were also recorded throughout the experiments. Rats receiving LPS + L-NNA showed consistent and significant increases in total numbers of activated neurons in the PVN. Increased numbers of activated NO-producing neurons and increased levels of IL-1β gene expression in the PVN were also observed in these rats compared to LPS controls. LPS + L-NNA or L-NNA rats showed significant differences on BP and Tb compared to controls, but L-NNA alone induced no neuronal activation and low levels of IL-1β gene expression in the PVN. Total numbers of NADPH-d neurons in

the PVN were unchanged. The results provide evidence that centrally-produced NO from eNOS inhibits the LPS-induced activation of neurons and IL-1β gene expression in the PVN, and they support the hypothesis that NO produced by eNOS restraints the central response to immune stress.

3.2 INTRODUCTION

Interleukin-1 (IL-1) is one of the primary groups of cytokines, which mediates the body's response to the peripheral immune challenge of endotoxin lipopolysaccharide (LPS) and is produced by a wide variety of cells throughout the body. In addition to its effects in the periphery, IL-1 can mediate various central nervous system responses including induction of fever, sleep, suppression of appetite, increased neuropeptide production, and altered neurotransmitter release (Berkenbosch et al., 1987; Dunn, 1988; Opp and Krueger, 1991; Rothwell and luheshi, 1994). Peripherally generated IL-1 is unlikely to be the source of centrally active IL-1 because only very small amounts of peripheral IL-1 have been found to cross the blood-brain barrier (BBB; Banks et al., 1995). Recent studies suggest that circulating cytokines influence the brain through circumventricular organs (CVOs), such as the area postrema (AP), and the subfornical organ, where there is a lack of BBB (Watkins et al., 1995). Cells of the AP stimulate neurons in the nucleus of tractus solitarius (NTS) to which they are connected (Cunningham et al., 1994). The NTS could then signal the paraventricular nucleus of the hypothalamus (PVN; Swanson and Sawchenko, 1983) to produce cytokines centrally (Dinarello, 1988;

Lechan et al., 1990; Buttini and Boddeko 1995; Hopkins and Rothwell, 1995). Centrally-produced IL-1, in particular IL-1β, activates the hypothalamo-pituitary-adrenal (HPA) axis by stimulating neurons in the PVN to release corticotropin releasing factor (CRF; Berkenbosch et al., 1987; Sapolsky et al., 1987; Navarra et al., 1991; Kakucska et al., 1993) which then acts on the anterior pituitary to stimulate release of adrenocorticotropin hormone (ACTH; Berkenbosch et al., 1987; Kakucska et al., 1993), and glucocorticoids are subsequently released by the adrenal glands (Vale et al., 1981). CRF-containing neurons in the PVN have also been shown to project to the brainstem and spinal cord to control autonomic function (Sawchenko and Swanson, 1982).

Nitric Oxide (NO) has been implicated in the modulation of neuroendocrine function. NO donors attenuate endotoxin-induced release of CRF *in vitro* (Costa et al., 1993), and nitric oxide synthase (NOS) inhibitors potentiate and prolong the activation of the HPA axis to endotoxin stimulus (Rivier and Shen, 1994; Rivier, 1995; Turnbull and Rivier, 1996). On the other hand, L-arginine (the substrate for NOS) has been shown to enhance interleukin 2-induced CRF release in the hypothalamus *in vitro* (Karanth et al., 1993) and peripherally-administered blockers of NOS inhibit stress-induced activation of PVN neurons (Amir et al., 1997; Lee and Rivier, 1998).

Investigators have attempted to identify which isoform of NOS is involved in regulating various brain responses to stresses. There are three isoforms of NOS (Forstermann et al., 1991; Forstermann et al., 1995), inducible NOS (iNOS), and

constitutively expressed neuronal NOS (nNOS) and endothelial NOS (eNOS). Earlier studies showed that intravenous injection of the non-selective NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) augmented IL-1β-induced ACTH release (Rivier and Shen, 1994; Rivier, 1995), but a recent report (Buxton et al., 1993) demonstrating that L-NAME, and other alkyl esters of arginine are muscarinic receptor antagonists, questions whether the effects of the first group observed were a results of the specific inhibition of NOS. Therefore, the Rivier group used a number of NOS inhibitors to investigate the ACTH response to IL-1β. The results showed that the effects of these inhibitors on the ACTH response to IL-1β were produced by inhibition of NO formation, and that NO involved in this effect was generated by endothelial isoform of NOS (Turnbull and Rivier, 1996).

Central IL-1β and NO both affect PVN neuronal activity in response to circulating LPS, and intracerebroventicular injection of IL-1β has been shown to stimulate nNOS gene expression in the PVN (Lee and Rivier, 1998), but no one has investigated the effects of NO on centrally-produced IL-1β or on neuronal activation. Thus, we proposed that NO acts as a mediator in regulating the PVN neuronal activity in response to LPS by altering centrally-produced IL-1β. The purpose of the present study was to determine if NO regulates PVN neuronal activity in response to LPS, if so, which isoform of NOS is responsible. First, we used three different NOS inhibitors, 7-Nitroindazole Sodium Salt (7-NiNa) for nNOS, N^G-Nitro-L-arginine (L-NNA) for eNOS and aminoguanidine (AG) for iNOS, to determine which, if any, alters neuronal activation in the PVN in response to LPS. Second, as our previous study (Yang et al.,

1999) showed that LPS-activated putative NO-producing neurons in the PVN, we wished to determine if any isoform of NOS affects LPS activation of putative NO-producing neurons in the PVN. Finally, we investigated the possibility that NO may regulate IL-1β gene expression in the PVN and the AP in response to LPS. Fos immunohistochemistry was used to identify activated neurons, nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) staining was used to localize putative NO-producing neurons, and *in situ* hybridization and image analysis were used to quantify IL-1β gene expression in the PVN and the AP.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Male Sprague-Dawley rats (250-300 g) were purchased from the Biological Animal Center, University of Alberta. They were housed two per cage under a 12:12 hour light-dark cycle at a temperature of 21°C. The rats were given free access to food and water. All protocols used in these experiments were approved by the University of Alberta Welfare Committee.

3.3.2 Instrumentation

Rats were anesthetized with sodium pentobarbital (60 mg/kg; i.p.; Somnotol, M.T.C. Pharmaceuticals, Hamilton, Canada).

3.3.2.1 *Venous catheterization*

As described in our previous studies (Krukoff et al., 1997; Yang et al., 1999), a 3-cm incision was made along the midline of the abdomen, and the vena cava was

exposed. A silastic catheter (0.02 inch internal diameter x 0.037 inch external diameter) was inserted into the vena cava and sutured to the posterior abdominal wall. The free end of the catheter was passed under the skin and externalized between the scapulae with a 23-gauge stainless steel tubing. The line was closed with polyvinyl-pyroolidone and capped with silastic tubing.

3.3.2.2 *Arterial catheterization*

The descending aorta was exposed, and a PE10 tubing (0.011 inch internal diameter x 0.024 inch external diameter) was inserted into the aorta and sutured to the posterior abdominal wall. The free end of the catheter was passed under the skin and externalized between the scapulae with a 27-gauge stainless steel tubing. The line was closed with polyvinyl-pyroolidone and capped with silastic tubing as described previously (Krukoff et al., 1997; Yang et al., 1999).

3.3.2.3 *Thermistor implantation*

A Copper/Constant thermistor (generously provided by Dr. L Wang, University of Alberta, Edmonton, AB) was implanted into the peritoneal cavity and sutured to the anterior abdominal wall. The free end of the thermistor was passed under the skin, externalized between the scapulae and positioned onto the skin.

3.3.2.4 *Intracerebroventricular (icv) cannulation*

The procedure we followed for icv cannulation was described in Herman et al. (1983). A sterile stainless 6 mm long C313G guide cannula (22 gauge, Plastic One Inc., Roanoke, VA) was fixed vertically in the arm of a stereotaxic frame, and a C313I internal cannula (28 gauge, Plastic One Inc., Roanoke, VA) was connected to a Accu-Rated Pump tubing (Fisher Scientic, Nepean, ON). Before the internal cannula was

inserted into the guide cannula, a bubble was made at the internal cannula end of the tubing using a Hamilton microsyringe so that outflow of cerebrospinal fluid (CSF) could be observed later. The internal cannula protruded 1mm beyond the tip of the guide cannula. Rats were then placed in the stereotaxic frame. The skull was exposed and an opening was drilled 1.0 mm posterior to the Bregma and 2.0 mm lateral to the midline. The guide cannula together with the internal cannula then were inserted 4.1 mm bellow the external surface of the skull into the lateral cerebral ventricle. To verify accurate placement, movement of the bubble and outflow of CSF were observed by withdrawing with the microsyringe. The internal cannula was removed, the guide cannula was positioned with three screws on the skull and dental acrylic cement, and was then closed with a C313DC dummy cannula (Plastic One Inc., Roanoke, VA) which protruded 0.5 mm from the guide tip.

Animal were allowed to recover and were handled daily after surgery so that they would become familiar with the manipulations of the experiment. Five to 7 days after the surgery, the experiments were performed.

3.3.3 Experimental Design

3.3.3.1 *Effects of NOS inhibitors on neuronal activation, NADPH-d, IL-1β gene expression, and arterial pressure in response to LPS*

Rats were instrumented with venous, arterial, and icv cannulae as described above. Five to 7 days after the surgery, the arterial line of each rat was connected for continuous recording of the arterial blood pressure. One hour was allowed for pressure to stabilize before the start of the experiment. Mean arterial pressure

(MAP, mmHg) was calculated for every 5 min interval. Rats received injections of artificial CSF (aCSF) or NOS inhibitors (dissolved in aCSF) over 60s intracerebroventricularly 30 min prior to LPS intravenous injections. LPS (100 μg/kg; Lee et al., 1995; L-4005, Sigma Corp., St. Louis, MI) dissolved in saline for a total of 0.3 ml/rat was injected intravenously. Two hours after LPS administration, rats received a second injection of aCSF or NOS inhibitors in aCSF. At 4 hours after LPS injections, rats were sacrificed (Figure 3-1). Rats were divided into five groups (n =4 for each group):

Group 1: LPS + aCSF (10 μl, composition: NaCl 138mM, KCl 5 mM, $CaCl_2$ 1.2 mM, NaH_2PO_4 1 mM, $NaHCO_3$ 10 mM: pH 7.5)

Group 2: LPS + 7-NiNa in aCSF (10 mM, 10 μl, Calbiochem)

Group 3: LPS + L-NNA in aCSF (10 mM, 10 μl, Calbiochem)

Group 4: LPS + AG in aCSF (0.3 mM, 10 μl, Sigma Corp., St. Louis, MI)

Group 5: LPS + AG in aCSF (3 mM, 10 μl)

At the end of the experiment, rats were deeply anesthetized and perfused transcardially with 200 ml 0.9% saline followed by 500 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were isolated, postfixed in half-strength fixative and 10% sucrose for 1 hour, and stored in 20% sucrose overnight at 4°C. Coronal sections (35 μm) of the brain were cut into 2 sets in a cryostat. Sections of the brainstem and forebrain were collected in phosphate-buffered saline (PBS; pH 7.2). Sections from the first set were processed for "Fos immunohistochemistry/NADPH-d histochemistry" and sections from the second set

were processed for "Fos immunohistochemistry/IL-1β *in situ* hybridization" as described below.

3.3.3.2 *Control experiments:*

To ensure that results obtained in the first experiment were not due to the effects of the NOS inhibitors themselves or changes in body temperature due to LPS, the following experiments were done:

3.3.3.2.1 *Effects of NOS inhibitors on neuronal activation, NADPH-d, and arterial pressure*

Rats were instrumented with venous, arterial, and icv cannulae as described above. Blood pressure was recorded continuously during the entire experiments through the arterial catheters as described previously. Rats received injections of aCSF or NOS inhibitors in aCSF over 60s intracerebroventricularly 30 min prior to saline intravenous injections. Two hours after saline administration, rats received another injections of aCSF or NOS inhibitors in aCSF. At 4 hours after saline injections, rats were sacrificed (Figure 3-1). Rats were divided into five groups (n = 3 for each group):

Group 1: saline + aCSF (10 µl)

Group 2: saline + 7-NiNa in aCSF (10 mM, 10 µl)

Group 3: saline + L-NNA in aCSF (10 mM, 10 µl)

Group 4: saline + AG in aCSF (0.3 mM, 10 µl)

Group 5: saline + AG in aCSF (3 mM, 10 µl)

At the end of the experiment, rats were deeply anesthetized and perfused

transcardially as described above. Coronal sections (35 µm) of the brain were cut in a cryostat. Sections of the brainstem and forebrain were collected in phosphate-buffered saline (PBS; pH 7.2) for "Fos immunohistochemistry/NADPH-d histochemistry" as described below.

3.3.3.2.2 *Effects of NOS inhibitors on body temperature in response to LPS*

Rats were instrumented with venous, icv cannulae, and thermistor as described above. The thermistor of each rat was connected to a digital thermometer (BAT-12, Bailey Instruments Inc., Saddlebrook NJ). Temperature recording began at least 30 minutes prior to injection of drug to assess baseline temperature and temperature was recorded every 10 minutes until the end of the experiment. Rats received injections of aCSF or NOS inhibitors in aCSF over 60s icv 30 min prior to LPS intravenous injections, and three more injections of aCSF or NOS inhibitors in aCSF were administered every 2 hours after LPS for 8 hours. At 8 hours, rats were sacrificed (Figure 3-1). Rats were divided into four groups (n = 4 for each group):

Group 1: LPS + aCSF (10 µl)

Group 2: LPS + 7-NiNa in aCSF (10 mM, 10 µl)

Group 3: LPS + L-NNA in aCSF (10 mM, 10 µl)

Group 4: LPS + AG in aCSF (3 mM, 10 µl)

At the end of the experiment, rats were deeply anesthetized and perfused transcardially as described previously. Coronal sections (35 µm) of the brain were cut in a cryostat. Sections were thaw-mounted onto Superfrost®/Plus slides (Fisher), dried and stored at –70°C for *in situ* hybridization as described below.

3.3.3.2.3 *Effects of NOS inhibitors on IL-1β gene expression*

Due to the data showed in the results, we tested the effect of icv injections of L-NNA alone on IL-1β gene expression. Rats were instrumented with icv cannulae and received two icv injections of L-NNA in aCSF (10 mM, 10 μl; n=6) over 60s two hours apart. For the positive control, rats received intravenous injections of LPS (100 μg/kg; n=6).

Four hours after L-NNA or LPS injections, rats were deeply anesthetized and perfused transcardially as described previously. Coronal sections (35 μm) of the brain were cut in a cryostat. Sections were thaw-mounted onto Superfrost®/Plus slides (Fisher), dried and stored at –70°C for *in situ* hybridization as described below.

3.3.4 Fos Immunohistochemistry/NADPH-d Histochemistry

Procedures for immunohistochemistry for Fos protein and histochemistry for NADPH-d are published previously (Krukoff and Khalili, 1997; Krukoff, 1998; Yang et al., 1999). Briefly, sections were incubated overnight with rabbit anti-c-*fos* antiserum (Oncogene, Uniondale, NY; 1.4 μg/ml) in 0.3% Triton X-100/PBS. According to the manufacturer, the antibody recognizes Fos and Fos-related antigens. Therefore, immunoreactivity will henceforth be called Fos-like immunoreactivity (FLI). The next day, sections were incubated with biotinylated anti-rabbit IgG (Vector; 1:200) for 1 hour and then with avidin-biotin complex (ABC Vecta Stain Kit; Vector laboratories, Burlingame, CA) for 1 hour at room temperature. Sections were exposed to 0.04% diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.005% hydrogen peroxide in PBS for 5-10 minutes.

After thorough washing, the sections were incubated for 30 minutes at 37°C in 0.1 M PBS (pH 7.2) containing 0.6% Triton X-100, 1 mg/ml β-NADPH, and 0.1 mg/ml nitroblue tetrazolium (Sigma Corp., St. Louis, MI). Sections were mounted onto glass microscope slides, air dried, coverslipped with Elvanol (Moviol, Calbiochem Corp. La Jolla, CA; dissolved in 40 ml PBS and 20 ml glycerol) and examined by using a Zeiss light microscope.

3.3.5 Fos Immunohistochemistry/IL-1β *in situ* Hybridization

Sections were stained for Fos as described above and were mounted onto Superfrost®/Plus slides, dried and stored at -70°C for processing at a later date. All the solutions and glassware used in this experiment were kept RNase free. An antisense ribonucleotide probe (Hurwitz et al., 1991) was transcribed from a rat IL-1β cDNA fragment containing 223-base pair with T7 RNA polymerase and [^{35}S] UTP. The plasmid was generously provided by Dr. Rohan (Children's Hospital, Boston, MA).

The protocol we followed for the *in situ* hybridization (ISH) was described in Krukoff et al. (1999). Briefly, frozen tissue sections were brought to room temperature, pretreated with 4% paraformaldehyde solution, 20 µg/ml proteinase K solution and 0.25% acetic anhydride in 0.1 M triethanolamin-HCl pH 7.4 solution, and dehydrated with ethanol. Radiolabeled probes were diluted in the riboprobe hybridization buffer and applied to brain sections (3-4 x 10^6 Million c.p.m/slide). After overnight incubation at 45°C in a humidified chamber, sections were washed in 4 x standard saline citrate (SSC) with 2-mercaptoethanol (1 µl/ml), in RNaseA (10 µg/ml in RNase buffer for 30 min at 37°C), and then in 2 x SSC, 0.1 x SSC (42°C for 40 min and 65°C for 45 min

respectively) solutions to reduce non-specific binding of the probe. The sections were then air dried for 3 h.

Slides were exposed at 4°C to X-ray film (X-OMAT AR, Kodak) for 48-72 hours and developed in an automatic film developer (X-OMAT, Kodak). To determine anatomical localization of hybridized probes at the cellular level, sections were dipped into Kodak NTB-2 emulsion (diluted 1:1 in water), exposed for 5-7 days and developed (D19, Kodak) for 5 min at 10-15°C.

3.3.6 Production of photomicrographs

Microscope images of sections were captured with a digitizing scanning camera (DC330, DAGE MTI Inc., Michigan IN) mounted directly on the microscope. The image editing software, CorelPhotopaint was used to process images. Only the sharpness, contrast and brightness were adjusted. All figures were printed on a dye sublimation printer (Kodak 8600).

3.3.7 Analysis

The PVN was divided into caudal, middle and rostral regions for purposes of analysis (Krukoff et al., 1999; Yang et al., 1999). The results were also expressed for the entire PVN. Neurons with Fos-like immunoreactivity (FLI, stained in the nucleus) or positive for NADPH-d (stained in the cytosol) were counted on one side of the PVN.

To avoid double-counting neurons in adjacent sections, data were expressed as numbers of labeled neurons *per section*. The following calculations were made: (1) numbers of neurons per section in the PVN singly labeled for FLI or NADPH-d, (2)

numbers of neurons per section in the PVN double-labeled for FLI and NADPH-d, (3) percentages of neurons with FLI as a proportion of neurons with NADPH-d in the PVN, and (4) percentages of neurons with NADPH-d as a proportion of neurons with FLI in the PVN.

Quantification of mRNA levels in the PVN and the AP was carried out under dark-field microscopy on nuclear emulsion-dipped slides using a digitizing scanning camera (DC330, DAGE MTI Inc., Michigan IN) coupled to a IBM compatible computer with Intel Pentium processor and Image software (Image-Pro Plus, Media Cybernetics, Silver Spring MD). Two sections from the middle region of the PVN (between –1.65 mm and –1.80 mm caudal to Bregma) and the middle subdivision of the NTS (between -14.0 mm and -13.5 mm caudal to Bregma) were analyzed in each animal. The frame used to define the area for quantifying the optical density was kept the same size and placed in the same location in the PVN and the AP respectively (Figure 3-11A,B,E). As shown in figure 8, the circle-shaped frame was placed to cover the majority of the PVN including both the parvo- and magnocellular regions. The rectangle-shaped frame was placed to cover approximately one third of the AP, the long axis of the rectangle was placed along the edge between the AP and the NTS, where the majority of the signal was found. Optical density was described as percentage of area covered by silver grains (Krukoff et at., 1999). Background signal was measured on nearby fiber tracts (internal capsule for the forebrain and pyramidal tract for the brainstem) and proportionally eliminated from the signal in the area of interest.

Data are expressed as means ± SEM. Repeated measures ANOVA was used

to analyze mean arterial pressure and body temperature data. Unpaired student's test was used in the analysis of Fos, NADPH-d and double labeling. $P < 0.05$ was taken to signify statistical significance.

3.4 RESULTS

3.4.1 Blood pressure

In LPS + aCSF rats, injections elicited an increase (10-20 mmHg) in arterial pressure at 25 min after LPS injection ($p < 0.05$) that lasted for approximately 10 min, a return to normal levels in the next 25 min, and no significant change afterward. LPS + NOS inhibitors in aCSF showed no significant differences from LPS + aCSF rats except for LPS + L-NNA in aCSF treated animals where a second increase in pressure (8 mmHg) occurred at 160 min for 5 min ($p < 0.05$; Figure 3-2).

Controls for investigating effects of NOS inhibitors themselves on arterial pressure showed no change in blood pressure throughout the experiment except that saline + L-NNA in aCSF rats showed significant increases (8-20 mmHg) 10 min after saline injection till the end of the experiment (Figure 3-3).

3.4.2 Body temperature (Tb)

In LPS + aCSF rats, 0.3-0.5°C drops in Tb occurred at 80 min after injection of LPS ($p < 0.05$) that lasted for 30 min. A recovery to baseline at 2-3 hours till the end of the experiment. LPS + NOS inhibitors in aCSF showed no significant differences compared to LPS + aCSF rats except for LPS + L-NNA in aCSF treated rats, which did not show the drops in Tb at 80-110 min but did produce an increase (0.3-0.5°C) at

300 min for 100 min after LPS injection ($p < 0.05$, Figure 3-4). Control rats (saline + aCSF) showed no change in Tb over 8 hours (Figure 3-5).

3.4.3 FLI labeled neurons in the PVN

In rats receiving LPS + aCSF, neurons with FLI were found in both the medial (parvocellular) and lateral (magnocellular) subdivisions of the PVN, and the largest numbers per section were found in the middle PVN in a caudal-rostral direction (Figure 3-6 and 3-7). This pattern of staining was similar in rats receiving LPS + NOS inhibitors in aCSF (Figure 3-6 and 3-8). Quantification revealed that there were no significantly differences in numbers of neurons per section with FLI in the caudal region of the PVN for any of the groups of LPS + NOS inhibitors in aCSF compared to LPS + aCSF rats. However, numbers of neurons per section with FLI were significant increased in LPS + L-NNA in aCSF in both the middle and rostral PVN, and in LPS + AG in aCSF (both doses) in the rostral PVN when compared to LPS + aCSF rats (Figure 3-8). When the data were combined for the entire PVN, only rats receiving LPS + L-NNA in aCSF had significantly higher numbers of neurons per section with FLI compared to rats with LPS + aCSF (Figure 3-8). Controls for investigating effects of NOS inhibitors themselves on neuronal activation (saline + NOS inhibitors in aCSF) showed few neurons per section (≤ 6) with FLI in the PVN (data not shown).

3.4.4 NADPH-d labeled neurons in the PVN

In rats receiving LPS + aCSF, neurons with NADPH-d were found in both the parvocellular and magnocellular subdivisions of the PVN (Figure 3-6 and 3-7). Rats treated with LPS + NOS inhibitors and controls (saline + NOS inhibitors in aCSF,

Figure 3-9) shared the similar pattern of staining (Figure 3-6). Quantification revealed that LPS + AG in aCSF (both doses) rats showed significant decreases in numbers of neurons per section with NADPH-d in the caudal PVN and significant increases in the rostral PVN compared to LPS + aCSF rats. However, in the middle PVN, there were no differences among groups (Figure 3-8). There were no significant differences in the total numbers of NADPH-d containing neurons per section in the PVN of LPS + NOS inhibitors in aCSF rats compared to LPS + aCSF rats (Figure 3-8). Controls for investigating effects of NOS inhibitors themselves on NADPH-d staining (saline + NOS inhibitors in aCSF) showed no significant differences among groups, either considered as a whole or as caudal to rostral regions (Figure 3-9).

3.4.5 FLI and NADPH-d labeled neurons in the PVN

In rats receiving LPS + aCSF, neurons with both FLI and NADPH-d were found in both the parvocellular and magnocellular subdivisions of the PVN, and the largest numbers per section were found in the middle PVN in a caudal-rostral direction (Figure 3-6 and 3-7). This pattern of staining was similar in rats receiving LPS + NOS inhibitors in aCSF (Figure 3-6 and 3-8). Quantification revealed that there were no significant differences in numbers of double-labeled neurons per section in the caudal regions of the PVN for any of the groups of LPS + NOS inhibitors in aCSF compared to LPS + aCSF rats. However, numbers of double-labeled neurons per section were significantly increased in LPS + L-NNA in aCSF in both the middle and the rostral PVN, and significantly decreased in LPS + 7-NiNa in aCSF and LPS + AG in aCSF (both doses) in the middle PVN when compared to LPS + aCSF rats. On the other hand, in the rostral PVN, numbers of double-labeled neurons per section were

significant increased in LPS + AG in aCSF (both doses) compared to LPS + aCSF rats. When the data were combined for the entire PVN, only rats receiving LPS + L-NNA in aCSF had significantly higher numbers of double-labeled neurons per section compared to rats with LPS + aCSF (Figure 3-8). Controls for investigating effects of NOS inhibitors themselves on neuronal activation and NADPH-d showed very few double-labeled neurons per section in the PVN (data not shown).

As a percentage of neurons with FLI, LPS + 7-NiNa in aCSF and LPS + AG in aCSF (both doses) showed lower percentages of double-labeled neurons compared to LPS + aCSF rats in the middle and the entire PVN (Figure 3-10). However, as a percentage of NADPH-d neurons, only in the middle regions of the PVN, LPS + L-NNA in aCSF showed an increase between the percentages of double-labeled neurons compared to LPS + aCSF rats (Figure 3-10).

3.4.6 IL-1β mRNA levels

Administration of LPS + aCSF led to gene expression of IL-1β in the PVN and the AP 4 hours after injection. The signal was equally distributed over, and confined to the PVN regions where the largest numbers of neurons with FLI were located (Figure 3-11A-D). LPS + NOS inhibitors in aCSF induced similar patterns of labeling. LPS + L-NNA in aCSF produced a significant increase in IL-1β gene expression in the PVN compared to LPS + aCSF (Figure 3-12). Rats receiving other NOS inhibitors showed no significant differences in IL-1β gene expression compared to LPS + aCSF (Figure 3-12). Switching between dark- and light-field microscopy showed that signal for IL-1β mRNA appeared to be localized to non-neuronal cells. The icv injections of L-NNA

alone showed low levels of IL-1β gene expression in the PVN (1.47 ± 0.03) compared to LPS controls (2.17 ± 0.04).

In the AP, IL-1β mRNA induced by LPS + aCSF were located at the edge between the AP and the NTS, and tended to aggregate in clusters (Figure 3-11E). This pattern of labeling was similar in rats receiving LPS + NOS inhibitors in aCSF. None of the groups of LPS + NOS inhibitors in aCSF showed significant differences in IL-1β gene expression compared to LPS + aCSF rats (Figure 3-12).

In rats treated with LPS + aCSF for 8 hours, IL-1β mRNA in the PVN was not observed above background signal. LPS + NOS inhibitors in aCSF showed no significant differences in IL-1β gene expression compared to LPS + aCSF (data not shown).

3.5 DISCUSSION

The results of this study support the hypothesis that NO plays an important role in regulating activity of cells within the PVN in response to the circulating endotoxin, lipopolysaccharide (LPS). We have confirmed that LPS activates neurons (including putative NO-producing neurons) as well as expression of the cytokine, IL-1β, in the PVN. We show that NO from endothelial NOS inhibits the activation of neurons including putative NO-producing neurons and IL-1β gene expression in the PVN in response to circulating LPS.

3.5.1 Technical consideration

3.5.1.1 *NOS inhibitors*

Of the three isoforms of NOS (Forstermann et al., 1991; Forstermann et al., 1995), neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, calcium-dependant, and are found in neurons of the central and peripheral nervous systems and in vascular endothelial cells, respectively. Inducible NOS (iNOS) is produced in many cell types, including hepatocytes, macrophages and glia, in response to endotoxin and is calcium-independent. Several NOS inhibitors show varying degrees of specificity for neuronal, endothelial and inducible NOS isoforms.

It has been shown that 7-Nitroindazole (7-Ni) selectively inhibits brain NOS (Moore et al., 1993a; Moore et al., 1993b). Administration of intraperitoneal 7-Ni (30 mg/kg) inhibits NOS activity in all brain regions studied with maximum inhibition (80–85%) observed 0.5 h following treatment. The inhibitory effect lasts 3–4 h without affecting blood pressure (Babbedge et al., 1993: Mackenzie et al., 1994). On the other hand, investigators found that the maximum inhibition and recovery from intraperitoneal 7-Ni were brain region- and dose-dependant (Kalisch et al., 1996), so that at doses of 7-Ni producing maximal NOS inhibition, the extent of NOS activity was more greatly inhibited in the cerebellum (16.6%) and substantia nigra (21.4%) than in the striatum (40.3%) and hippocampus (43.7%). In the above studies, 7-Ni was administered intraperitoneally because 7-Ni can be only dissolved in peanut oil. Therefore, its insolubility in physiological media has precluded its use in intracerebroventricular studies. On the other hand, the solubility of 7-Ni sodium salt (7-NiNa) in artificial CSF permits intracerebroventricular injection. So far, there are limited numbers of published reports on *in vivo* experiments investigating the central

effects of 7-NiNa. Only Silva et al. (1995) studied the dopamine release by continuously perfusing 7-NiNa (1mM) at a rate of 2 µl/min for 90 min into the striatum. In this study, we used 100 nmol for each injection, and repeated the injection every two hours.

L-NNA potently inhibits both eNOS and nNOS (Gross et al., 1990; Gross et al., 1991; Lambert et al., 1991; Lambert et al., 1992). L-NNA causes vasoconstriction, increases blood pressure, and reduces blood flow to organs when administered peripherally (Dinerman et al., 1993; Vane, 1994). Intravenous injection of L-NNA in conscious rats caused a sustained increase in MAP which reached a plateau response at approximately 10 min after injection, and the pressor effects lasted more than 2 hours (Wang et al., 1991). Intracerebroventricular administration of L-NNA (23 nmol and 14 nmol) do not elicit any significant change of baseline blood pressure (Mollace et al., 1992; Paczwa et al., 1997). Several groups have used intracerebroventricular injection of L-NNA, and the concentrations they used were variable (14 nmol-100 nmol). Most studies focused on peripheral effects, such as changes in mean arterial pressure. Only one group (Sandi et al., 1996) studied nervous system on locomotor activity by injecting 100 nmol L-NNA intracerebroventricularly. Therefore, we chose to inject 100 nmol every two hours in this study.

AG inhibits endotoxin-induced iNOS in many cell types, but has little or no effect on eNOS or nNOS *in vivo* (Griffiths et al., 1993; Hasan et al., 1993; Misko et al., 1993). Another iNOS inhibitor, S-Substituted isothiouras, which is found to be more selective for iNOS compared to AG (Garvey et al., 1994; Szabó et al., 1994; Southan

et al., 1995), has direct cytotoxic effects demonstrated on human colorectal adenocarcinoma cells (Garvey et al., 1994). In addition, intravenous administration S-ethyl isothiouras increases the blood pressure and decreases the heart rate in rabbits, while AG affects neither cardiovascular function (Seo et al., 1996). It has been shown that AG (iv) injected 20 min prior to LPS reversed the decrease in MAP induced by LPS, and the effect maintained for 3 hours (Wu et al., 1995). Only one study has investigated the central effects of AG *in vivo* (Brain Jr and Faraci, 1998), where tumor necrosis factor-α-induced dilation of cerebral arterioles was studied by flushing AG (0.3 mM) in 2 ml of aCSF every 30 min for a total of 4 hours with into cranial windows. In the present study, we chose to use two concentrations of AG (3 nmol and 30 nmol) for each injection, and the injection was repeated every two hours.

In the present study, NOS inhibitors were administered into the lateral ventricle of the brain so that peripheral effects are eliminated. We injected NOS inhibitors in aCSF 30 min prior to LPS injection and repeated the injections every two hours after LPS to ensure effectiveness of the inhibitors. L-NNA, which inhibits both nNOS and eNOS, affected the PVN neuronal activity in response to LPS, whereas 7-NiNa, which inhibits primarily nNOS, had no effects on the PVN neuronal response to LPS. These data provide strong evidence that the effect of L-NNA on altering the PVN neuronal activity in response to LPS is due to its inhibitory effect on eNOS, but not nNOS. Our control experiments show that the inhibitors had minimal, if any, effects on blood pressure, body temperature, or neuronal activation in the PVN. Therefore, we are confident that the effects of NOS inhibitors were not due to their indirect or toxic

effects.

3.5.1.2 *NADPH-d represents nNOS*

Most available evidence supports the view that NADPH-d staining in the brain represents neuronal NO synthase when fixation has been used (Dawson et al., 1991; Matsumoto et al., 1993; Norris et al., 1995; Rothe et al., 1998; Yang et al., 1999). Studies have shown that nNOS immunoreactive neurons correlate very well with NADPH-d stained neurons in the PVN even after axotomy or colchicine treatment (Lumme et al., 1997). Therefore, we believe that our NADPH-d staining is indicative of neuronal NO-producing neurons.

3.5.2 Systemic LPS activates neurons in the PVN

In the present study, LPS + aCSF administration showed similar neuronal activation in the PVN to that described previously (Elmquist et al., 1996; Yang et al., 1999). Neurons in both parvocellular and magnocellular regions of the PVN were activated by intravenous LPS. The PVN represents a center of homeostatic control mechanisms containing specific projections to autonomic and endocrine control sites involved in the acute phase reaction of infection (Swanson and Sawchenko, 1983; Saper, 1995). The neuroendocrine parvocellular division contains CRF neurons, small population of vasopressin (VP) and oxytocin (OT) neurons, and others, which project to the median eminence, the brainstem, the spinal cord and control the anterior pituitary and autonomic functions (Swanson and Sawchenko, 1983). The magnocellular neurons in the PVN release VP and OT and control the posterior pituitary function (Swanson and Sawchenko, 1983). Therefore, based on their locations, the LPS-activated neurons in both the parvo- and magnocellular

regions of the PVN which we described may also release CRF, VP or OT, and may be involved in regulating the HPA axis activity, autonomic and/or posterior pituitary functions.

Administration of LPS induces dose-dependent secretion of several cytokines from the immune system in a pattern mimicking natural infections (Chen et al., 1992). The relatively low dose of LPS (100 μg/kg) we administrated is thought to activate only the HPA axis without disrupting the BBB or causing toxic shock (Lee et al., 1995). Circulating cytokines gain access to the brain (Watkin et al., 1995) and stimulate central cytokines production in the PVN (Dinarello, 1988; Lechan et al., 1990; Buttini and Boddeko 1995; Hopkins and Rothwell, 1995). The HPA axis is then activated by centrally-produced cytokines via the secretion of CRF, ACTH and glucocorticoids. (Vale et al., 1981; Berkenbosch et al., 1987; Sapolsky et al., 1987; Navarra et al., 1991; Kakucska et al., 1993).

3.5.3 NO from eNOS inhibits neuronal activation in the PVN in response to LPS

We found that NO from eNOS inhibited neuronal activation in both the parvo- and magnocellular regions of the PVN after intravenous administration of LPS. In control experiments, we showed that eNOS inhibitor affected the blood pressure after LPS injection or alone. However, no neuronal activation was observed when rats were treated with eNOS inhibitor alone and blood pressure was elevated. The eNOS inhibitor also eliminated the drop in body temperature induced by LPS, and later produced a small increase at 340 min after LPS injection. As we processed brains for

Fos at 4 hours, however, the changes in body temperature at 340 min cannot account for the neuronal activation. Because the control experiments rule out the BP and Tb changes on affecting the neuronal activity, we conclude that the effect of eNOS on the neuronal activation is not due to its effect on BP or Tb.

3.5.4 NO from eNOS reduces numbers of activated NADPH-d neurons in the PVN in response to LPS

LPS activates NADPH-d neurons in both parvo- and magnocellular regions of the PVN (Yang et al., 1999). In the present study, LPS + aCSF showed a similar pattern of activated NADPH-d staining. Our results with NOS inhibitors now show that NO from eNOS inhibited activation of NADPH-d positive neurons in both the parvo- and magnocellular regions of the PVN after intravenous administration of LPS. In eNOS inhibitor treated animals, the increase in the percentage of double-labeled neurons as a proportion of NADPH-d neurons indicates that eNOS inhibitor caused more NADPH-d neurons to be activated. This intriguing finding suggests that NO from eNOS may inhibit nNOS activity in the PVN. This hypothesis remains to be tested. Because NADPH-d neurons in the PVN have also been shown to produce CRF, VP or OT (Torres et al., 1993; Miyagawa et al., 1994; Siaud et al., 1994; Hatakeyama et al., 1996), NO from eNOS may also affect CRF, VP or OT release.

Our results showing that NO inhibits neuronal activation in response to immune stress (LPS) can be compared with NO's actions in other transmitter processes. Investigators have reported that NO has both a stimulatory and an

inhibitory effect on CRF release. For example, NO inhibitor blocked IL-1β-induced CRF and ACTH release from rat hypothalamic cell culture *in vitro* (Brunetti et al., 1993; Sandi et al., 1995). On the other hand, release of ACTH in response to interleukin 1β was increased with blockade of NO synthase (Rivier and Shen, 1994). Similarly, NO has also been shown to both stimulate and inhibit the release of VP and OT. Intracerebroventricular injections of the NO donor, SNAP, led to increases in vasopressin release (Ota et al., 1993). Nitroprusside application to hypothalamic slices also elicited release of vasopressin into the culture medium (Raber and Bloom, 1994). These studies provide support for a stimulatory effect of NO. On the other hand, the following investigations provide evidence for a inhibitory effect of NO. Intravenous injections of N^G-nitro-L-arginine methyl ester (L-NAME) suggested that NO inhibits vasopressin release (Goyer et al., 1994; Yamamoto et al., 1994). In agreement with the latter study, experiments with explant cultures of hypothalamus or neural lobe of the pituitary gland showed that NO inhibits release of vasopressin at both sites (Yasin et al., 1993; Lutz-Bucher and Koch, 1994). Finally, electrophysiological studies using slice preparations of the PVN (Bains and Ferguson, 1997) or supraoptic nucleus (Liu et al., 1997) showed that NO inhibits the activity of vasopressinergic and oxytocinergic neurons, and that the inhibition in the PVN is at least partly mediated through the GABAergic neurotransmitter system (Bains and Ferguson, 1997).

The role of specific isoforms of NOS on the PVN neuronal activity has not been thoroughly studied. One group suggested that NO suppressed the ACTH

response to IL-1β, and that the NO involved in this effect is generated by the endothelial isoform of NOS (Turnbull and Rivier, 1996). We now show that eNOS may be the isoform involved in regulating neuronal activation in the PVN.

3.5.5 NO from eNOS inhibits IL-1β gene expression in the PVN in response to LPS

We confirm that LPS induces IL-1β gene expression in the PVN and the AP (Quan et al., 1997; Wong et al., 1997), and we now provide evidence to show that NO from eNOS may inhibit IL-1β gene expression in the PVN after intravenous LPS administration. Earlier studies demonstrated that the administration of 2.5-5.0 mg/kg LPS (intraperitoneal) caused a large increase of IL-1β mRNA in the PVN 6-8 hours after injection (Quan et al., 1997; Wong et al., 1997). In the present study, we intravenously administered a relatively low dose of LPS (100 μg/kg), and found a widespread distribution of IL-1β mRNA in the PVN 4 hours after injection. At 8 hours after LPS injection, however, we found that IL-1β gene expression was not above background levels in the PVN. Therefore, our results show that LPS-induced IL-1β gene expression is dose-, time- and route-dependant.

According to the FLI and IL-1β mRNA double labeling, we found that IL-1β gene expression was likely non-neuronal. These results are in general agreement with several previous studies, in which it was demonstrated that the cellular source of IL-1β in the rat brain after peripheral administration of endotoxin is mainly the microglial cell (Van Dam et al., 1992; Buttini and Boddeke, 1995; Van Dam et al., 1995).

We are the first to investigate the effects of NO on centrally-produced IL-1β. We show that eNOS inhibitor alone induced low levels of IL-1β mRNA in the PVN, and this can not explain the dramatic increase on IL-1β gene expression elicited by LPS plus eNOS inhibitor administration. Therefore, we conclude that NO from eNOS inhibits IL-1β gene expression in the PVN after intravenous LPS injections. Because the increased FLI and IL-1β signals are distributed in similar subdivisions of the PVN in eNOS inhibitor treated animals, these results suggest that centrally-produced IL-1β may itself participate in the neuronal activation in the PVN.

Based on the evidence that NO from eNOS may inhibit IL-1β gene expression in the PVN after LPS injections, that centrally-produced IL-1β may modulate PVN neuronal activity, and that the PVN is important in regulating the HPA axis, autonomic and the posterior pituitary responses to immune stress, we propose that NO from eNOS may play a significant role in regulating these functions during exposure to LPS.

In the AP, we show that IL-1β gene expression induced by intravenous LPS was not altered by NO from any of three isoforms of NOS. While the AP serves as a gate for circulating cytokines entering the brain (Watkins et al., 1995), Our results suggest that NO may be not involved in cytokine signaling pathways at the level of the AP in response to LPS.

3.6 CONCLUSION

We have shown that LPS (iv) plus eNOS inhibitors (icv) induce increased

neuronal activation, including NADPH-d neurons, and IL-1β gene expression in the PVN compared to LPS plus vehicle (aCSF). These results support the hypothesis that NO, produced from eNOS, may act as an important messenger in regulating the PVN neuronal activity and IL-1β gene expression in the PVN in response to circulating LPS.

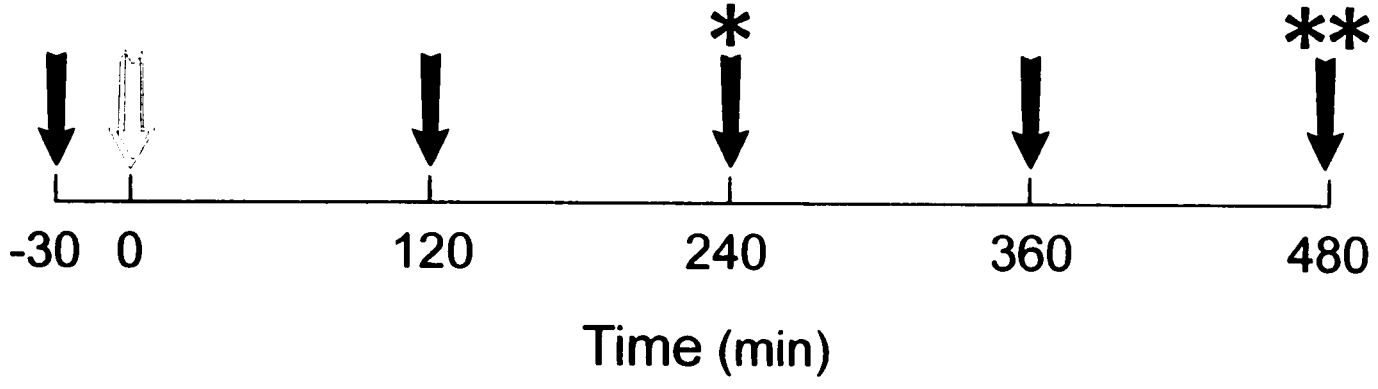

Figure 3-1: Drawing illustrating design of experiments performed. ⬇ , time at which aCSF, 7-NiNa, L-NNA or AG in aCSF was injected intracerebroventricularly. ⇩, time at which LPS was injected intravenously. *, time at which experimental and control rats used for investigating effects of NOS inhibitors on neuronal activation, NADPH-d and arterial pressure were killed. **, time at which control rats used for investigating effects of NOS inhibitors on body temperature in response to LPS were killed.

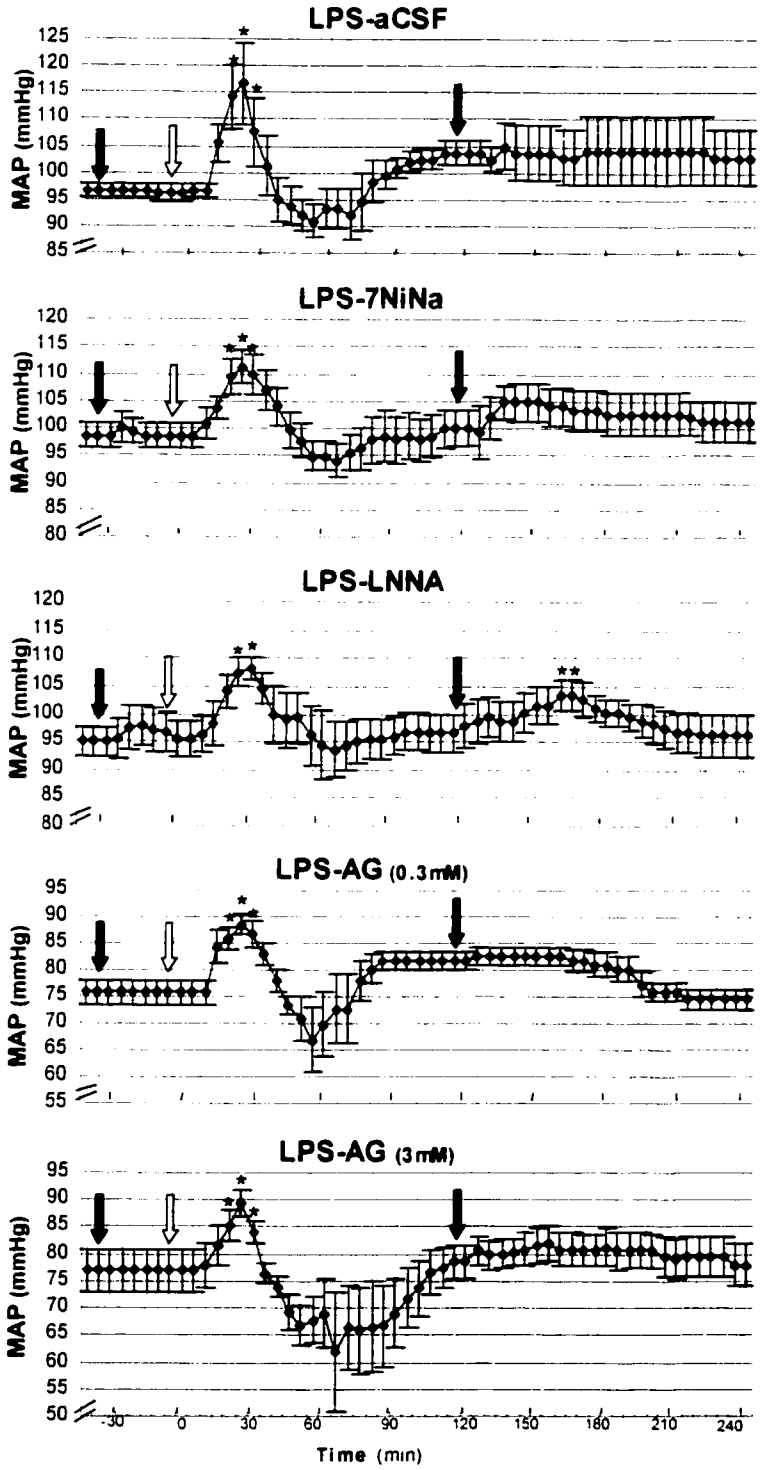

Figure 3-2: Changes in mean arterial pressure (MAP) after rats received aCSF or NOS inhibitors in aCSF (⬇), and LPS (↓, n = 4 for each group). Abbreviations: aCSF, artificial cerebrospinal fluid; 7-NiNa, 7-nitroindazole sodium salt; L-NNA, N^G-nitro-L-arginine; AG, aminoguanidine. *, significant difference ($p < 0.05$) compared to baseline.

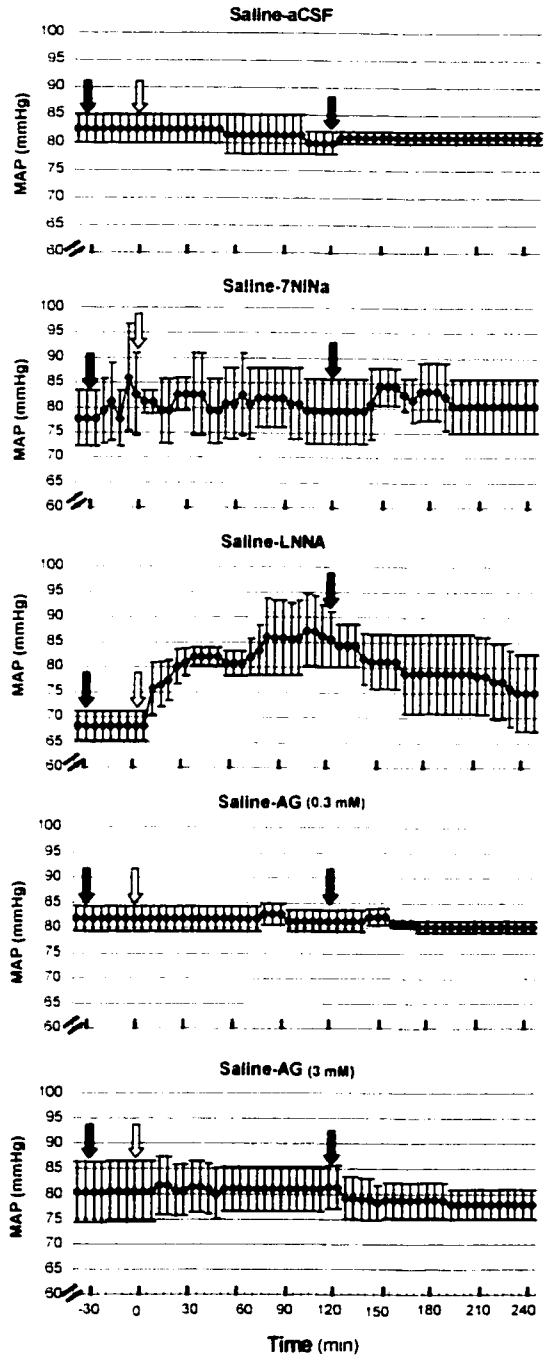

Figure 3-3: Graph demonstrating controls used for investigating effects of NOS inhibitors themselves in mean arterial pressure (MAP). Rats received intracerebroventricular injections of aCSF or NOS inhibitors in aCSF (⬇), and intravenous injections of vehicle (saline, ⬇, n = 3 for each group). Abbreviations: aCSF, artificial cerebrospinal fluid; 7-NiNa, 7-nitroindazole sodium salt; L-NNA, N^G-nitro-L-arginine; AG, aminoguanidine.

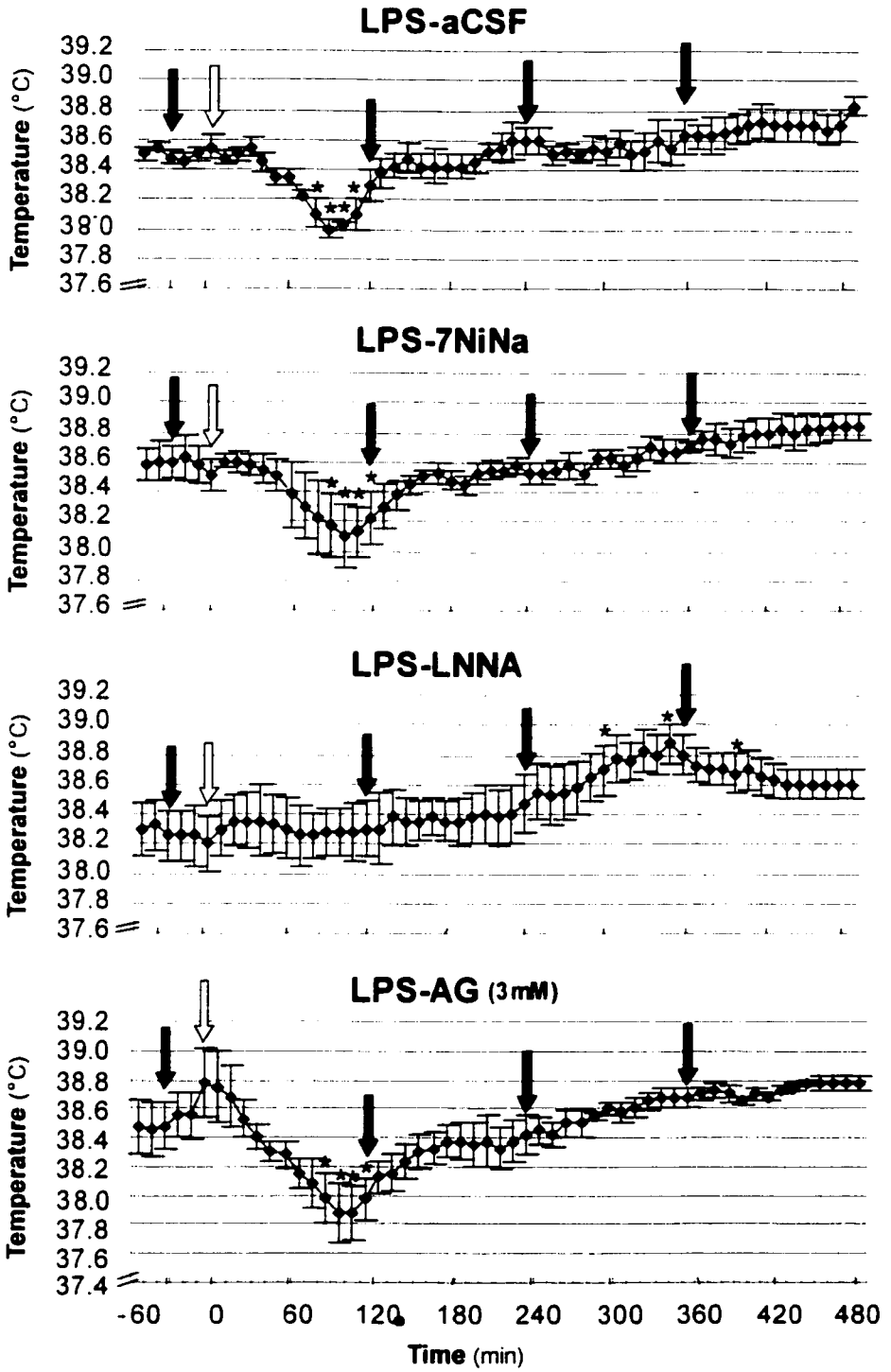

Figure 3-4: Changes in body temperature after rats received aCSF or NOS inhibitors in aCSF (⬇), and LPS (⬇, n = 4 for each group). Abbreviations: aCSF, artificial cerebrospinal fluid; 7-NiNa, 7-nitroindazole sodium salt; L-NNA, N^G-nitro-L-arginine; AG, aminoguanidine. *, significant difference ($p < 0.05$) compared to baseline.

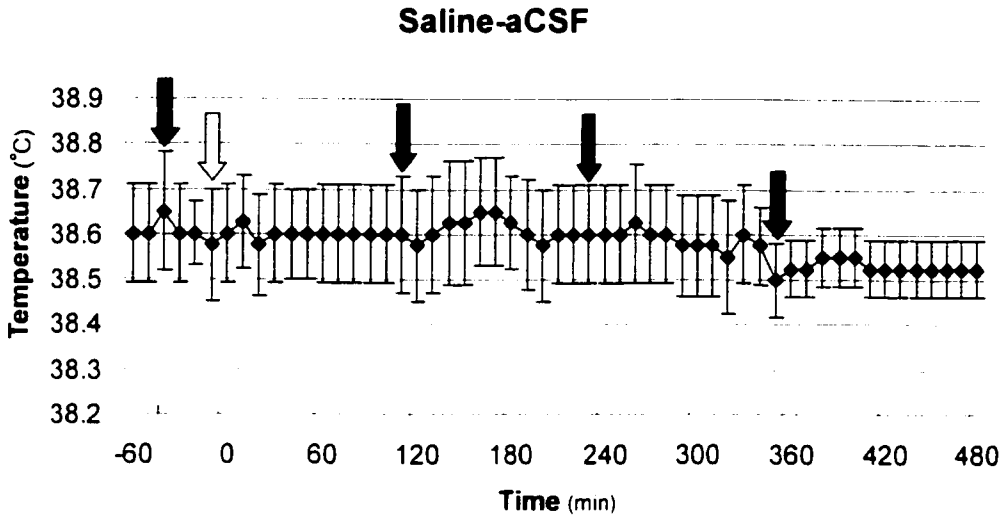

Figure 3-5: Graph demonstrating controls used for investigating changes in body temperature after rats received intracerebroventricular injections of vehicle (aCSF, ⬇), and intravenous injections of vehicle (saline, ⋯, n = 3 for each group). Abbreviations: aCSF, artificial cerebrospinal fluid.

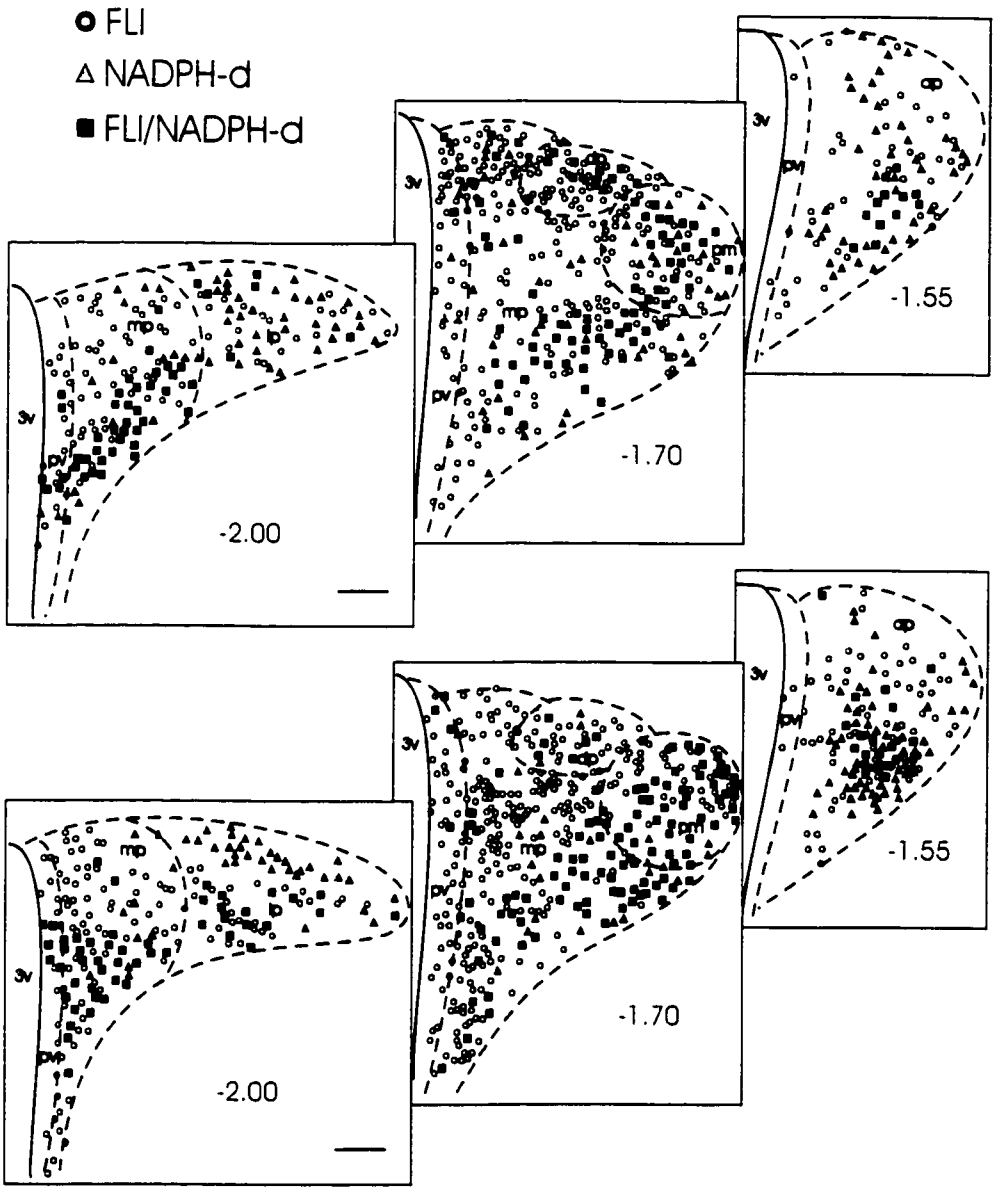

Figure 3-6: Composite drawing at three levels of the PVN in LPS + aCSF (top) and LPS + L-NNA in aCSF (bottom) rats. Numbers indicate rostrocaudal coordinates relative to Bregma (Paxinos and Watson, 1986). Abbreviations: 3v, third ventricle; FLI, Fos-like immunoreactivity; NADPH-d, nicotinamide adenine dinucleotide phosphate-diaphorase; ap, dp, lp, and mp, anterior, dorsal, lateral, and medial parvocellular divisions of the PVN; pv, periventricular PVN; pm, posterior magnocellular PVN. Scale bars = 100 μm and refer to all panels.

Figure 3-7: Fos-like immunoreactivity (FLI) and NADPH-d staining in the PVN of rats treated with LPS + aCSF. The area within the rectangle in A is shown at higher magnification in B. White arrowhead in B represents a FLI labeled neuron nuclei; black arrowhead represents NADPH-d labeled neuron cytosol; arrow represents double labeled neuron for FLI and NADPH-d. Scale bars = 100 μm in A, 30 μm in B. Asterisk, third ventricle. For color images of similar labeling, see our previous publication (Figure 3 in Yang et al., 1999).

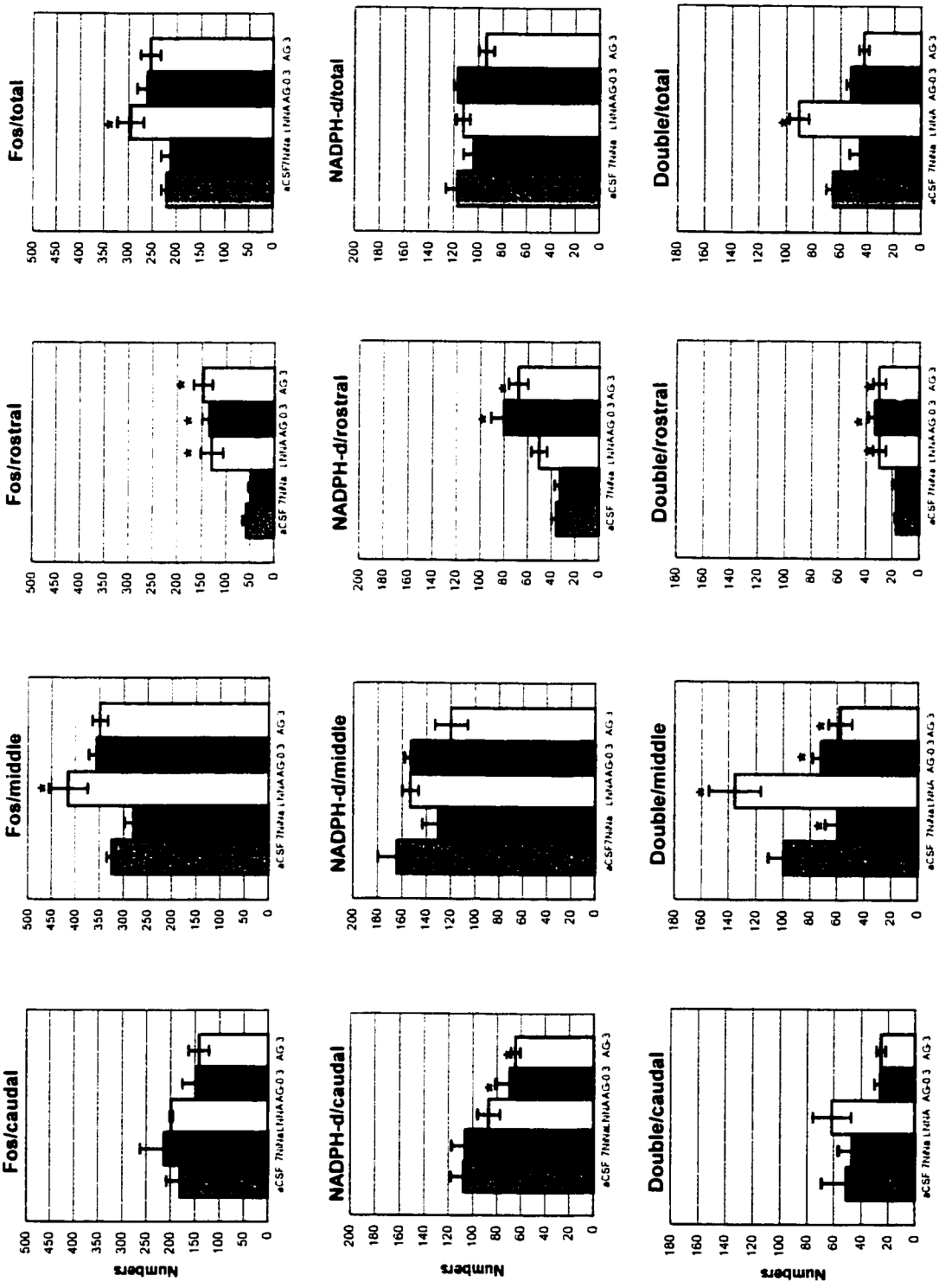
Fos/caudal
Fos/middle
Fos/rostral
Fos/total
NADPH-d/caudal
NADPH-d/middle
NADPH-d/rostral
NADPH-d/total
Double/caudal
Double/middle
Double/rostral
Double/total
Numbers
aCSF 7NAa LNAA AG-0 3 AG-3

Figure 3-8

Figure 3-8: Numbers of neurons per section single- and double-labeled for FLI and/or NADPH-d in the caudal, middle, rostral and for the entire (total) PVN of LPS + aCSF or NOS inhibitors in aCSF (n = 4 for each group). Abbreviations: aCSF, artificial cerebrospinal fluid; 7-NiNa, 7-nitroindazole sodium salt; L-NNA, N^G-nitro-L-arginine; AG-0.3, 0.3mM aminoguanidine; AG-3, 3mM aminoguanidine. *, significant difference ($p < 0.05$) compared to LPS + aCSF.

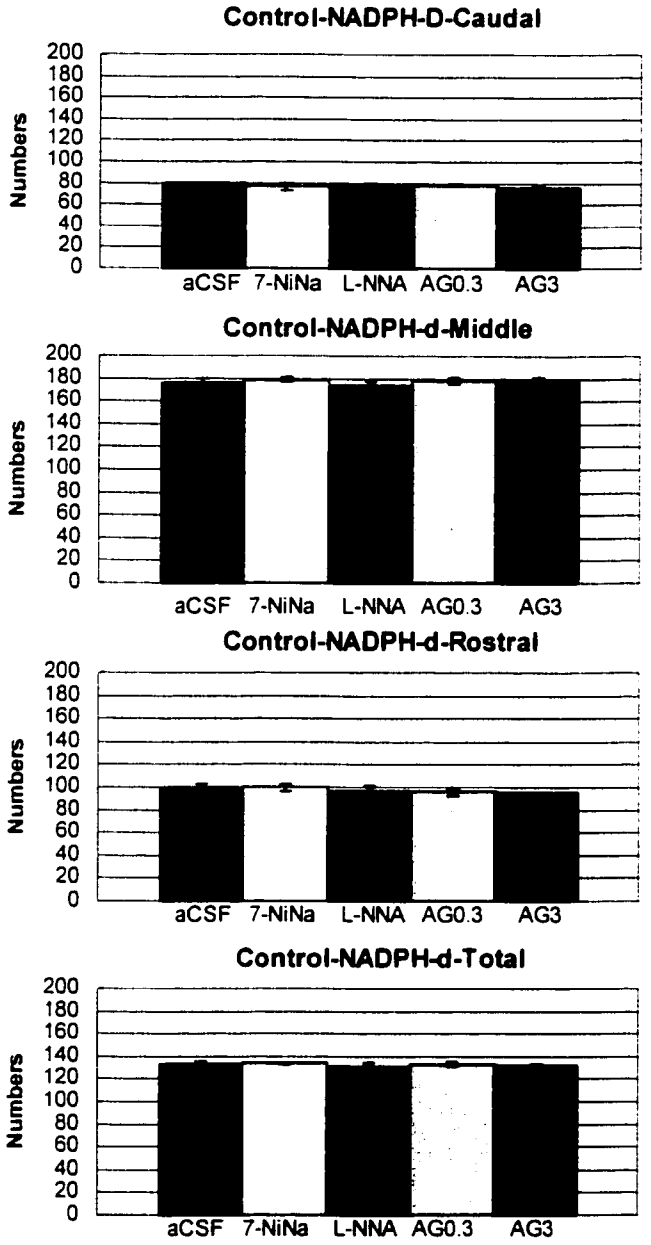

Figure 3-9: Graph demonstrating controls used for investigating effects of NOS inhibitors themselves on NADPH-d positive neurons. Rats received intracerebroventricular injections of aCSF or NOS inhibitors in aCSF, and intravenous injections of vehicle (saline, n = 3 for each group). Results are presented as numbers of neurons per section labeled for NADPH-d in the caudal, middle, rostral and for the entire (total) PVN. Abbreviations: aCSF, artificial cerebrospinal fluid; 7-NiNa, 7-nitroindazole sodium salt; L-NNA, N^G-nitro-L-arginine; AG-0.3, 0.3mM aminoguanidine; AG-3, 3mM aminoguanidine.

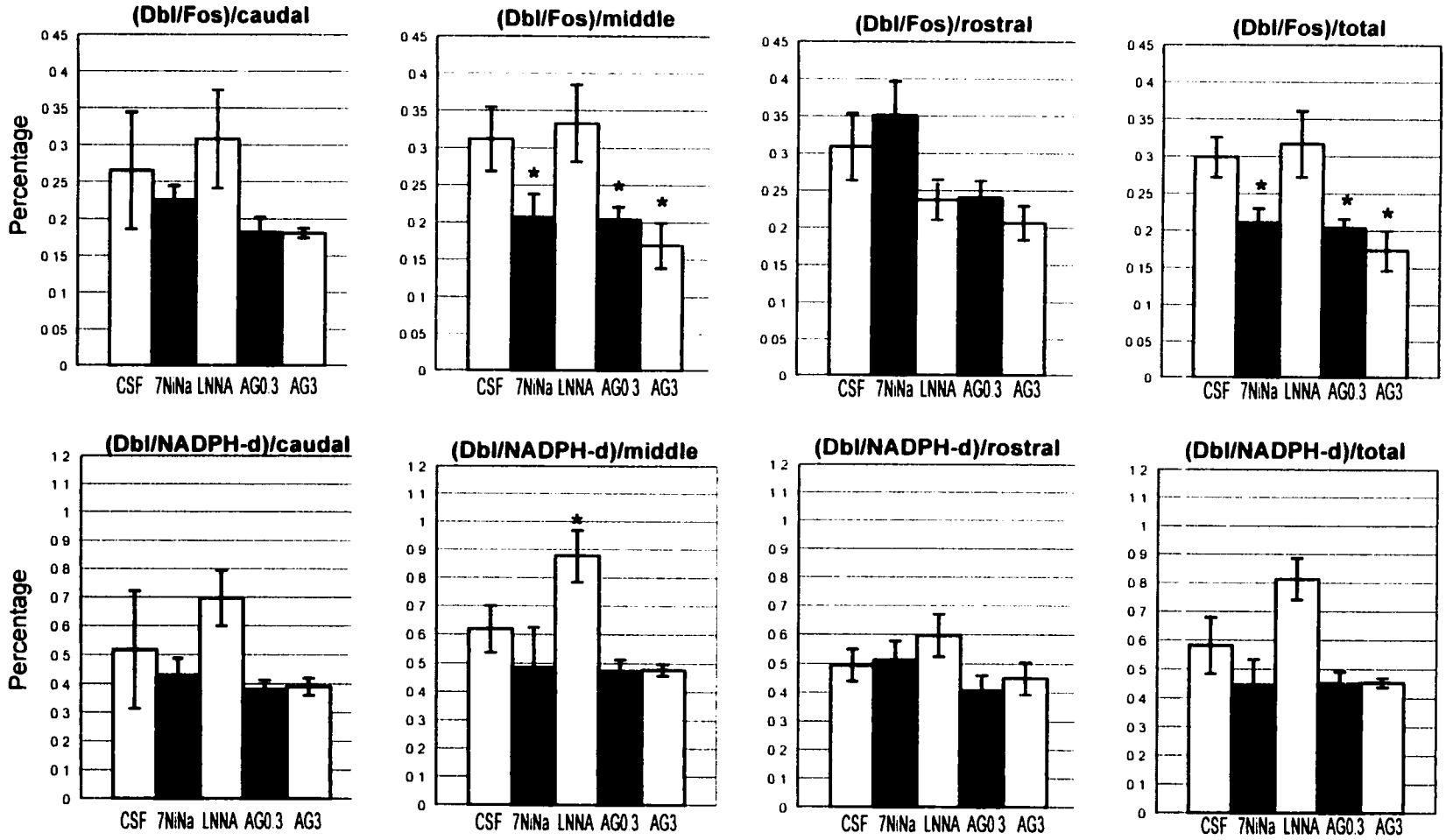

Figure 3-10: Percentages of neurons with FLI as a proportion of neurons with NADPH-d and percentages of neurons with NADPH-d as a proportion of neurons with FLI in the caudal, middle, rostral and for the entire (total) PVN of LPS + aCSF or NOS inhibitors in aCSF (n = 4 for each group). Abbreviations: aCSF, artificial cerebrospinal fluid; 7-NiNa, 7-nitroindazole sodium salt; L-NNA, N^G-nitro-L-arginine; AG-0.3, 0.3mM aminoguanidine; AG-3, 3mM aminoguanidine; Dbl, double. *, significant difference ($p < 0.05$) compared to LPS + aCSF.

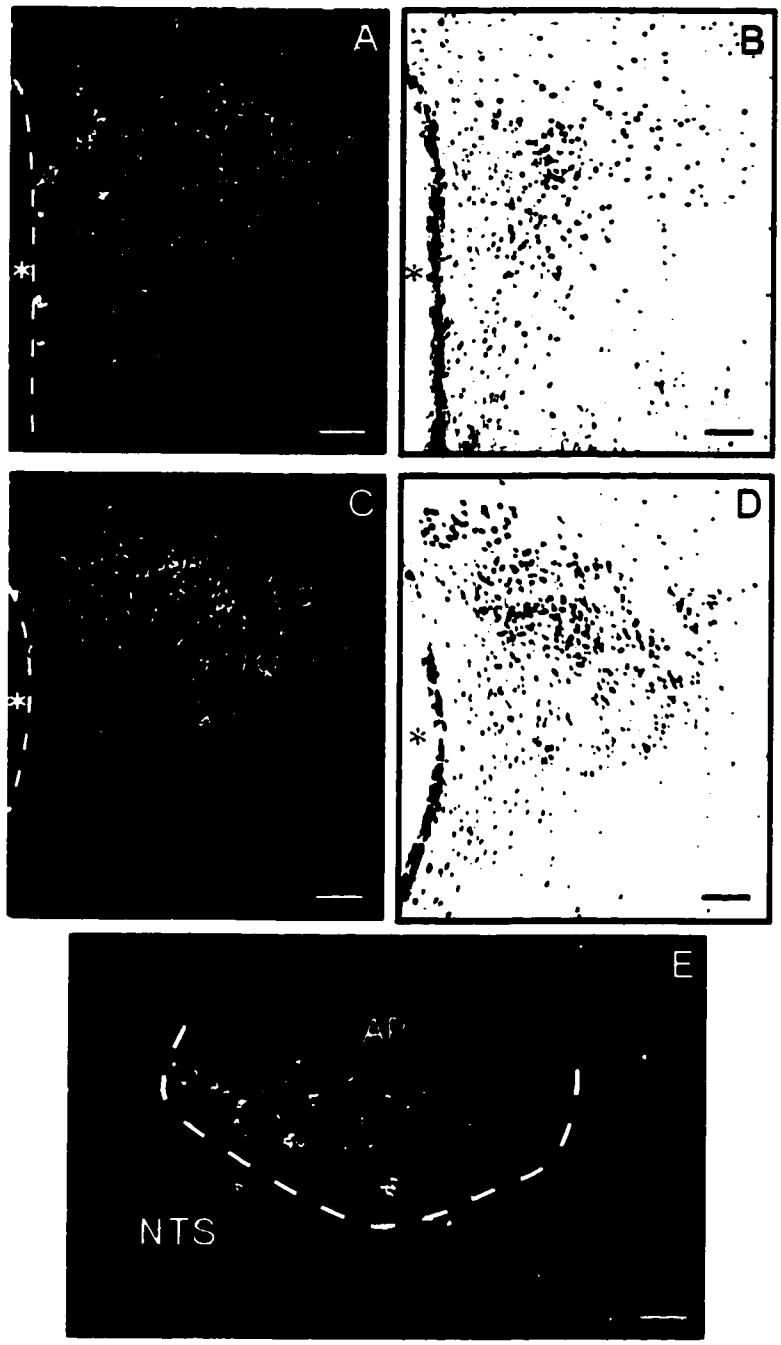

Figure 3-11: A-D, IL-1β mRNA and Fos-like immunoreactivity (FLI) in the PVN from LPS + aCSF (A,B) and LPS + L-NNA in aCSF (C,D) rats. A,C: dark-field photomicrographs showing IL-1β mRNA. Circles enclose the areas used to quantitate autoradiographic signal in the PVN. Dashed lines demarcate the third ventricle in A and C (*). B,D: FLI in same sections A and C, respectively. E, dark-field photomicrographs showing IL-1β mRNA in the area postrema (AP) from LPS + aCSF rat. Rectangle encloses the area used to quantitate signal in the AP. Dashed lines demarcate the AP in E. NTS, nucleus of the tractus solitarius. Scale bars = 100 μm in A-D and 50 μm in E.

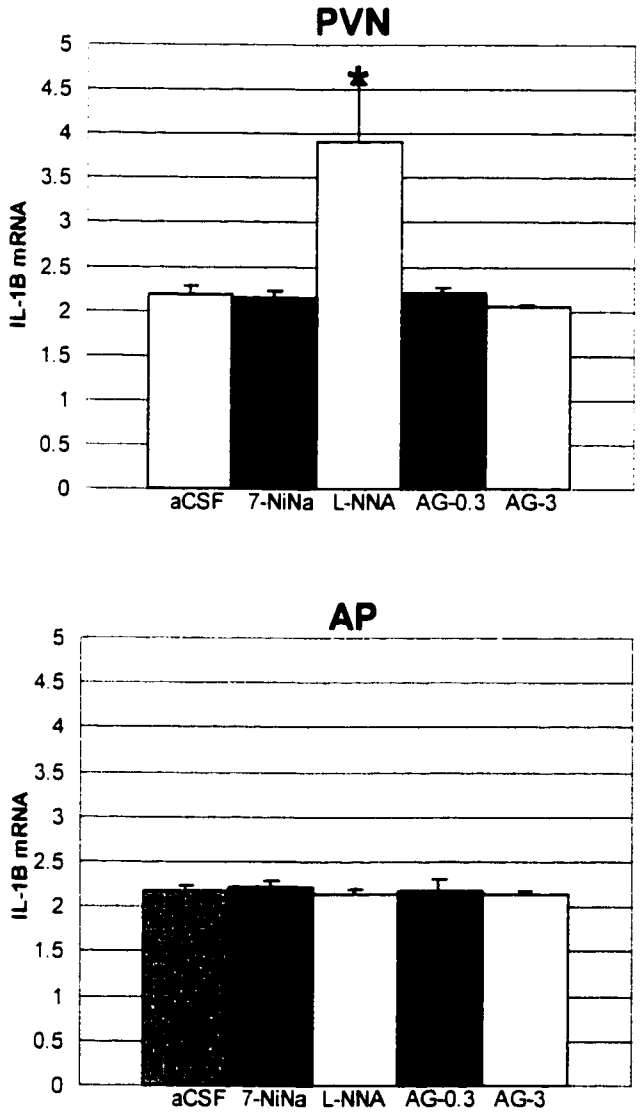

Figure 3-12: IL-1β mRNA labeling (no units) in the PVN and the AP of LPS + aCSF and LPS + NOS inhibitors in aCSF rats. Abbreviations: aCSF, artificial cerebrospinal fluid; 7-NiNa, 7-nitroindazole sodium salt; L-NNA, N^G-nitro-L-arginine; AG-0.3, 0.3mM aminoguanidine; AG-3, 3mM aminoguanidine; PVN, the paraventricular nucleus, AP, the area postrema. *, significant difference ($p < 0.05$) compared to LPS + aCSF.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 DISCUSSION and CONCLUSIONS

We have shown that (1) immune stress activated putative nitric oxide (NO)-producing neurons in the paraventicular nucleus (PVN), nucleus of the tractus solitarius (NTS) and ventroiateral medulla (VLM). (2) Activated putative NO-producing neurons in the NTS and VLM did not signal the PVN directly in response to immune stress. (3) Immune stress plus restraint stress led to increased stimulation of the NO system in the PVN compared to immune stress alone. (4) NO produced from endothelial nitric oxide synthase (eNOS) inhibited the activation of neurons, including putative NO-producing neurons in the PVN during exposure to immune stress. (5) NO generated from eNOS inhibited interleukin-1β (IL-1β) gene expression in the PVN in response to immune stress. These results support the hypothesis that NO participates in the regulation of PVN neuronal activity when animals are exposed to immune stress. The location of the activated neurons in the PVN and the known role for IL-1β in regulating corticotropin-releasing factor release suggest that NO acts as an important messenger in the modulation of the hypothalamic-pituitary-adrenal (HPA) axis activity, autonomic and/or the posterior pituitary functions in response to immune stress.

NO is known to be the principal endothelial vascular relaxing factor (Palmer et al., 1987; 1988), and is recognized as an intercellular messenger in regulating various other physiological functions, including macrophage cytotoxicity (Morris et al., 1994), neurotoxicity and plasticity in the brain (Dawson

et al., 1991; Schuman and Madison, 1994). Recent evidence also suggests that NO may act as a nonconventional neurotransmitter in the central nervous system (Garthwaite et al., 1988; Moncada et al., 1991; Bredt and Syder, 1992). NO has also been implicated in decreasing sympathetic output to the periphery (Lewis et al., 1991; Sakuma et al., 1992; Harada et al., 1993; Kagiyama et al., 1997; Krukoff and Khalili, 1997; Krukoff, 1998). The study of roles of NO in modulating PVN neuronal activity has led to controversial results, as NO has been reported to both stimulate (Brunetti et al., 1993; Ota et al., 1993; Raber and Bloom, 1994; Sandi et al., 1995) and inhibit (Yasin et al., 1993; Goyer et al., 1994; Lutz-Bucher and Koch, 1994; Rivier and Shen, 1994; Yamanoto et al., 1994; Bains and Ferguson, 1997) the secretion of corticotropin-releasing-factor (CRF), vasopressin (VP) and oxytocin (OT) by both *in vitro* and *in vivo*.

During exposure to immune stress, NOS and IL-1β gene expression are induced in the PVN (Dinarello, 1988; Lechan et al., 1990; Dinerman et al., 1994; Buttini and Boddeko, 1995; Hopkins and Rothwell, 1995; Lee et al., 1995; Wong et al., 1996; Quan et al., 1997; Wong et al., 1997; Jacobs et al., 1998; Lee and Rivier, 1998; Satta et al., 1998). Centrally-produced IL-1β initiates the activation of HPA axis to immune stress by stimulating the release of CRF in the PVN (Vale et al., 1981; Berkenbosch et al., 1987; Sapolsky et al., 1987; Navarra et al., 1991; Kakucska et al., 1993). These results suggest that NO and IL-1β are important regulators in the PVN's responses to immune stress.

In the first study presented in this thesis, the location of immune stress-activated putative NO-producing neurons in both the parvo- and magnocellular divisions of the PVN suggest that NO generated by neuronal isoform of NOS is involved in regulating the HPA axis activity, autonomic and/or the posterior pituitary functions. In the second study, we have shown that the role of NO may be to inhibit immune stress-activated neurons, putative NO-producing neurons and IL-1β gene expression in both the parvo- and magnocellular regions of the PVN, and that these effects are generated by the endothelial isoform of NOS.

In future experiments, we will investigate the interactions between isoforms of NOS. Our results show that NO from eNOS may inhibit neuronal NOS (nNOS) activity in the PVN in response to immune stress. We will determine if central inhibition of eNOS changes nNOS gene expression in the PVN during exposure to immune stress.

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