INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

University of Alberta

CENTRAL ENDOTHELIN A RECEPTORS MEDIATE THE DECREASE IN SYMPATHETIC ACTIVITY DURING RECOVERY FROM RESTRAINT STRESS

By

Avery Wah Chow Yip 

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

Department of Cell Biology

Edmonton, Alberta

Fall 2000

University of Alberta

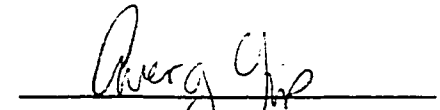
Library Release Form

Name of Author: Avery Wah Chow Yip

Title of Thesis: Central Endothelin A Receptors Mediate the Decrease in Sympathetic Activity During Recovery from Restraint Stress

Degree: Master of Science

Year Granted: 2000



905-10011 123rd Street
Edmonton, Alberta
T5N 1M9

June 28/00
Date

Abstract

While endothelin (ET) has been implicated in regulating the functions of many organ systems including the brain, the role of central ET receptors in autonomic regulation is not fully understood. We tested whether central ET_A receptors regulate sympathetic drive in response to restraint. Intracerebroventricular (i.c.v.) injections of the ET_A antagonist, BQ123, in rats subjected to restraint showed that, while the mean arterial pressure (MAP) increased equally in BQ123 and control rats in response to restraint, the MAP during the subsequent rest period was significantly greater in BQ123 rats. We also tested whether nitric oxide (NO) was involved in the decrease in sympathetic activity which occurred during recovery from restraint. Measurement of tissue nitrates/nitrites showed that NO content was decreased in the brainstems of BQ123-treated rats. I.c.v. injections of the constitutive NO synthase (cNOS) inhibitor, N^G-nitro-L-arginine, showed that inhibition of cNOS eliminated the decrease in MAP when rats were removed from restraint. These results support the hypothesis that central ET regulates sympathetic output and suggests that ET acts through ET_A receptors to decrease sympathetic activity through the central release of NO.

To Love is Being Alive
To Live is Being Loved

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Central Endothelin A Receptors Mediate the Decrease in Sympathetic Activity During Recovery From Restraint Stress** by Avery Wah Chow Yip in partial fulfilment of the requirements for the degree of Master of Science.

Teresa L. Krukoff

Robert B. Campenot

Jack H. Jhamandas

June 28, 2000

Date

For the friends

Rachel Carter, Marie Fitzgerald,
Tamara Uptigrove, and Christine Wiebe

and family

Mom, Dad, Li, and John

who keep me sane;
with much love and gratitude.

Acknowledgments

First and foremost, I would like to thank my supervisor, Teresa Krukoff, for giving me the opportunity to pursue graduate studies in her laboratory and to work on the projects described in this thesis. I have learned a great deal from her, my mentor, and my friend. I would also like to extend my gratitude to the members of my supervisory committee, Alan Bateson, Bob Campenot, and Jack Jhamandas for their guidance and many helpful suggestions throughout the course of my studies.

I would also like to thank the past and present members of our lab, Karen Atkinson-Leadbeater, Katie Davidson, Heather Edgell, Ewa Pedrycz, Jing Shan, Tev Stachniak, Yasmin Tharani, Yun Xia, and Wendy Yang for their friendship and smiling faces. Additional thanks goes out to Dave MacTavish and Kim Harris for their technical assistance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

3V	third ventricle
ACC	anterior cingulate cortex
ACTH	adrenocorticotropin hormone
Ang II	angiotensin II
AP	area postrema
ARC	arcuate nucleus
AVP	arginine vasopressin
BBB	blood-brain barrier
bMAP	baseline mean arterial pressure
BP	blood pressure
BQ 123	cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-)
BQ 788	N-*cis*-2,6-dimethyl-piperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine
cNOS	constitutive nitric oxide synthase
CNS	central nervous system
CO	cardiac output
CRF	corticotropin releasing factor
cRNA	complementary ribonucleic acid
CSF	cerebrospinal fluid
DAB	diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
dPm	dorsal parvocellular division of the PVN (medial portion)

dPv	dorsal parvocellular division of the PVN (ventral portion)
E	epinephrine
EAA	excitatory amino acid
ECE	endothelin-converting enzyme
eNOS	endothelial nitric oxide synthase
ET	endothelin
ET_A	endothelin receptor subtype A
ET_B	endothelin receptor subtype B
ET_C	endothelin receptor subtype C
FLI	Fos-like immunoreactivity
HPA	hypothalamic-pituitary-adrenal
HR	heart rate
i.c.	intracisternal
i.c.v.	intracerebroventricular
ILC	infralimbic cortex
IML	intermediolateral cell column of the spinal cord
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
i.v.	intravenous
IV^{th}	fourth ventrical
LC	locus coeruleus
L-NMA	N^{ω}-methyl-L-arginine
L-NNA	N^{G}-nitro-L-arginine

M	magnocellular division of the PVN
MAP	mean arterial pressure
mP	medial parvocellular division of the PVN
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NADPH-d	nicotinamide adenine dinucleotide phosphate diaphorase
NE	norepinephrine
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NO_x	nitrates and nitrites
NTS	nucleus of the tractus solitarius
PAG	periaqueductal gray
PBS	phosphate-buffered saline
PVN	paraventricular nucleus
RNase	ribonuclease
RNSA	renal nerve sympathetic activity
SEM	standard error of the mean
SNS	sympathetic nervous system
SON	supraoptic nucleus
SSC	standard saline citrate
STX	sarafotoxin
TH	tyrosine hydroxylase

VLM	ventrolateral medulla
VP	arginine vasopressin
WKY	Wistar-Kyoto

LIST OF SYMBOLS

$\forall$, time at which ET-1 was injected

$\Downarrow$, time at which BQ 123 or vehicle was injected

$\downarrow$, time at which L-NNA or vehicle was injected

$\otimes$, denotes significant difference $p < 0.005$

$*$, denotes significant difference $p < 0.05$

$\star$, denotes significant difference $p < 0.001$

∂, denotes significant difference $p < 0.01$

CHAPTER 1

INTRODUCTION

1.1. Overview

Although endothelin (ET)-1 (Yanagisawa et al., 1988) was first identified as a potent endothelium-derived vasoconstricting factor (Hickey et al., 1985; O'Brien et al., 1987), the physiological roles of ETs are now considered to be much more diverse. ETs have been implicated in regulating the functions of many organ systems, including the central and peripheral nervous systems (for reviews, Rubanyi & Polokoff ,1994; Kuwaki et al., 1997). While it is known that ET and its receptors are expressed in the central nervous system (CNS) (Jones et al., 1989; Koseki et al., 1989a,b; Lee et al., 1990; Giaid et al., 1991; Hori et al., 1992; Kurokawa et al., 1997), the role of ET in the brain is not yet fully understood.

1.2. The endothelin family

ETs are endogenous peptides consisting of 21 amino acids and characterised by four cysteine residues which form 2 intramolecular disulphide bonds (Fig. 1.1.) (Yanagisawa et al., 1988). The ET family includes three distinct isoforms: ET-1, ET-2, and ET-3 (Inoue et al., 1989), each potent constrictors of arterial smooth muscle *in vitro* and strong pressor agents *in vivo*. ETs are generated from a two-step process beginning with proteolytic cleavage of a 212 precursor polypeptide (preproET) to a relatively inactive 38 amino acid precursor, big-ET. Big-ET is then catalysed by endothelin-converting enzyme (ECE) to its mature 21 amino acid active form (Yanagisawa et al., 1988; Inoue et al., 1989). Although big-ET-1 is only 1% as potent as ET-1 in inducing

contractile activity in vascular strips, it is equipotent in raising blood pressure (BP) *in vivo* since big-ET is rapidly converted to ET by ECE (Sluck et al., 1999).

1.3. Endothelin receptors

ETs act on at least two distinct receptors, ET_A and ET_B (Masaki et al., 1994; Ohlstein et al., 1996). ET_A receptors preferentially bind ET-1 (ET-1 > ET-2 >> ET-3) (Arai et al., 1990), while ET_B receptors are non-selective and bind all three isoforms almost equally (ET-1 = ET-2 = ET-3) (Sakurai et al., 1990; Ogawa et al., 1991). The primary effects mediated by the ET_A receptor include vasoconstriction and vascular smooth muscle proliferation (Arai et al., 1990). On the other hand, the ET_B receptor mediates vasodilation through the release of nitric oxide (NO), and also mediates vasoconstriction in certain vascular beds as well as bronchoconstriction (Clozel et al., 1992; Hori et al., 1992; Hirata et al., 1993; Moreland et al., 1994; Sudjarwo et al., 1994; Mazzoni et al., 1999; Schmeck et al., 1999).

1.3.1. Receptor agonists and antagonists

ETs share a marked degree of structure and sequence homology to peptides isolated from the venom of the Israeli burrowing snake *Actrapsis engaddensis*, known as sarafotoxins (STXs) (Kloog and Sokolovsky, 1989). The STXs are also 21 amino acids long and contain the two characteristic intra-disulphide bonds (Fig. 1.1). Furthermore, STX S6b has been shown to be an ET_A selective agonist (Kumar et al., 1997) while STX S6c is an ET_B selective agonist (Williams, Jr. et al., 1991). Structural analysis of ETs and

STXs show that they have two moieties: a cyclic N terminus and a linear C terminus (Figure 1.1). Studies with analogues of ET-1 have shown that its N terminus is important for binding to, and activating ET_A receptors (Nakajima et al., 1989). On the other hand, the highly conserved C terminus has been shown to be important for the activation of ET_B receptors (Saeki et al., 1991). The finding that the linear domain alone can bind to and activate ET_B receptors is consistent with the notion that ET_B receptors are non-selective for the different ET isoforms.

These two receptor subtypes have been further characterised by the development of selective antagonists: BQ 123 selectively blocks ET_A receptors (Ihara et al., 1992, 1995; Vigne et al., 1993), and BQ 788 selectively blocks ET_B receptors (Ishikawa et al., 1994; Karaki et al., 1994).

1.3.2. ET receptor subtypes

With the development of new selective agonists and antagonists, it has become increasingly clear that all ET receptors cannot fit neatly into the simple classification of ET_A and ET_B. Studies have shown that some ET_A-mediated responses were insensitive to inhibition by BQ 123 (Sudjarwo et al., 1994). In addition, the BQ 123-sensitive ET_{A1} receptors were also more selective for ET-1 than ET-2. On the other hand, the BQ 123-insensitive ET_{A2} receptors were more selective for ET-2 than ET-1 (Gulati, 1991; Sudjarwo et al., 1994).

There is also pharmacological data indicating the existence of anatomically separate and distinct ET_B receptor subtypes; an ET_{B1} receptor on the endothelium that

invokes the release of NO to mediate vasodilation, and an ET_{B2} receptor on vascular smooth muscle that directly mediates vasoconstriction (Clozel et al., 1992; Hori et al., 1992; Hirata et al., 1993; Moreland et al., 1994; Sudjarwo et al., 1994; Schmeck et al., 1999;).

A unique ET receptor, designated ET_C, with slightly higher affinity for ET-3 than ET-1 has been cloned from *Xenopus laevis* dermal melanophores (Karne et al., 1993). There have also been reports of an additional ET_A-like receptor in Xenopus oocyte follicular membranes (Kumar et al., 1993), and heart (Kumar et al., 1994). These receptors showed selectivity for ET-1 vs. ET-3; however, BQ 123 was found to be ineffective in inhibiting ^{125}I-ET-1 binding to these receptors. It is not known whether these or related receptors exist in mammalian tissues.

1.4. Endothelin as a neuropeptide

1.4.1. Endothelin in the central nervous system

ET is widely distributed within the CNS. ET-1 mRNA as well as immunoreactivity, can be detected in the cerebral cortex, striatum, hippocampus, amygdaloid body, pituitary gland, supraoptic and paraventricular nuclei of hypothalamus, Purkinje cells of the cerebellum, raphe nuclei, dorsal motor nucleus of the vagus, and dorsal horn and intermediolateral (IML) cell column of the spinal cord (Lee et al., 1990; Yoshizawa et al., 1990; Giaid et al., 1991). The diverse distribution of ET-1 in the CNS suggests its influence on the control of many of functions. ET-1 is also present in glial cells and astrocytes, although its appearance seems to be limited to certain conditions

such as in culture, or during active gliosis, or under pathological states such as Alzheimer's disease (MacCumber et al., 1990; Ehrenreich et al., 1991; Jiang et al., 1993; Hama et al., 1997; Kuwaki et al., 1997).

More recently, Yamada and Kurokawa (1998) showed that, in the rat hypothalamus, magnocellular cell bodies in both supraoptic and paraventricular nuclei exhibited strong immunoreactivity for mature ET, whereas their nerve fibres did not. On the other hand, these neuronal cell bodies and nerve fibres showed positive immunostaining for big-ET-1. In fact, when axonal transport was blocked with colchicine, immunoreactivity for big-ET-1 in cell bodies increased, while immunoreactivity in nerve fibres decreased. These findings suggest that mature ETs are constitutively formed in cell bodies; however, big-ETs are transported along axons and processed either at nerve terminals or at the membrane of target cells (Yamada and Kurokawa, 1998).

While ET mRNA and immunoreactivity were also seen in some blood vessel endothelial cells of the brain (Giaid et al., 1991), it is unlikely that ET produced by endothelial cells can cross the blood-brain barrier (BBB) since systemically injected ^{125}I-ET-1 was found bound to only circumventricular structures of the median eminence, subfornical organ, and choroid plexus (Koseki et al., 1989a,b).

1.4.2. Endothelin receptors in the central nervous system

Both receptors are distributed widely, but not evenly, in the CNS. Autoradiographic studies using ^{125}I-ET-1 reveal that binding sites for ET are concentrated

in the basal ganglia, brainstem, hippocampus, choroid plexus, and cerebellum (Jones et al., 1989; Koseki et al., 1989b). In addition, ET-binding sites have been found in some areas of the brain which participate in the central control of autonomic functions. ET receptors have been found in the paraventricular nucleus (PVN), supraoptic nucleus (SON), nucleus of tractus solitarius (NTS), ventrolateral medulla (VLM), and pituitary (Koseki et al., 1989a,b; Shibata et al., 1997a,b). More recently, the development of a specific antibody to the rat ET_A receptor has demonstrated the topographic distribution of ET_A receptor containing neurons (Kurokawa et al., 1997). Immunoreactivity for ET_A receptors was detected not only in neuronal cell bodies, but also in neuronal fibres. Strong ET_A immunoreactivity was located in arcuate nucleus (ARC), periventricular and parvocellular portions of the PVN, NTS and its surrounding area, and locus coeruleus (LC). In addition, the magnocellular neurons of supraoptic and paraventricular nuclei showed weak immunoreactivity.

1.5. Effects of endothelin on brain and neural tissue

1.5.1. Cardiovascular effects of exogenous endothelin

Intravenous injections of ET in rats cause an initial fall in BP followed by a sustained increase in pressure (Kuwaki et al., 1994). The depressor effect is thought to be due to the release of NO from endothelial cells via ET_B receptors (Sakurai et al., 1990; Hirata et al., 1993). The pressor effect is mediated primarily through ET_A receptors on smooth muscle cells (Yanagisawa et al., 1988), although ET_B receptors have also been shown to exist on smooth muscle cells in some vascular beds and to contribute to the

pressor effect (Clozel et al., 1992; Moreland et al., 1994; Sudjarwo et al., 1994). In contrast to its peripheral effects, centrally administered ET-1 elicits an initial increase followed by a sustained decrease in BP in urethane-anaesthetised animals (Hashim and Tadepalli, 1992; Rebello et al., 1995; Gulati et al., 1995, 1996, 1997a,b; Rossi et al., 1997). The initial pressor effect is due to stimulation of the sympathetic nervous system (SNS) while the depressor effect is likely due to a decrease in sympathetic outflow. Both the pressor and depressor responses of centrally administered ET-1 were not observed in rats whose spinal cords were severed at the cervical level (Gulati et al., 1997b). The absence of the ET-1-induced cardiovascular responses in these animals suggest that both the pressor and depressor responses are centrally mediated. In addition, peripheral administration of adrenergic and/or ganglionic blockers prevented the circulatory changes (Yamamoto et al., 1992; Kumar et al., 1996; Nakamura et al., 1999) showing that the BP responses are mediated by the SNS. Furthermore, the changes in BP in response to intracerebroventricular (i.c.v.) ET-1 were shown to be mediated by ET_A receptors since pre-treatment with BQ 123 also eliminated these effects (Gulati et al., 1997b). Together, these findings suggest that centrally administered ET-1, via ET_A receptors, acts on vasoactive neurons to modulate sympathetic outflow. In support of these observations, central administration of STX 6b, an ET_A selective agonist, produced a comparable pressor and depressor response (Kumar et al., 1997). The pressor phase was due to an increase in sympathetic stimulation to the heart resulting in increased cardiac contractility, while the depressor effect was due, in part, to a reduction in cardiac

contractility. Again, the pressor and depressor responses were attenuated by BQ 123 (Kumar et al., 1997).

1.5.2. Other central effects of endothelins

ET-1 may be responsible for the release of hormones such as oxytocin (Yasin et al., 1994), arginine vasopressin (AVP) (Yamamoto et al., 1992; Yasin et al., 1994; Mosqueda-Garcia et al., 1995; Rossi et al., 1997), catecholamines (Makino et al., 1990), and adrenocorticotropin (ACTH) (Makino et al., 1990; Malendowicz et al., 1997), while inhibiting the *in vitro* release of prolactin (Samson et al., 1990). Interestingly, although ET-1 did not affect the basal release of corticotropin-releasing factor (CRF) *in vitro*, ET-3 inhibited CRF release from hypothalamic explants (Calogero et al., 1994). On the other hand, ET-1 increased ACTH release (Calogero et al., 1994) suggesting ET may participate in the autocrine and/or paracrine regulation of pituitary secretion (Schwartz and Cherny, 1992; Stojilkovic and Catt, 1992; Kanyicska and Freeman, 1993; Renner et al., 1996).

1.6. Sympathetic nervous system and the hypothalamic-pituitary-adrenal axis

The anterior cingulate cortex (ACC) represents one of the cortical components of the central autonomic network. The ACC participates in high-level regulation of the autonomic and endocrine function (Devinsky et al., 1995). Some of the connections of the infralimic cortex (ILC), one component of the ACC, are shown in Figure 1.2 (Hurley et al., 1991). The ILC projects to the PVN and other hypothalamic nuclei, amygdala and

bed nucleus of the stria terminalis, as well as autonomic nuclei of the brainstem and spinal cord including the periaqueductal gray (PAG) area, LC, NTS, VLM, and IML cell column (Hurley et al., 1991).

The catecholaminergic neurons of the LC and parvocellular corticotropin-releasing factor (CRF) neurons of the PVN are two of the central co-ordinators of the generalised stress response system, while the efferent sympathoadrenomedullary system and the hypothalamic-pituitary-adrenal (HPA) axis represent their peripheral limbs (Stratakis and Chrousos, 1995). The LC provides extensive norepinephrine (NE) innervation to all areas of the CNS involved in integration of sensory and motor responses to stressful external stimuli (Liozou, 1969; Korf et al., 1973; Swanson and Hartman, 1976; Grzanna and Molliver, 1980). Stress activates the SNS, while at the same time stimulates CRF neurons in the PVN, thereby activating the HPA axis (Calogero, 1995; Stratakis and Chrousos, 1995; Benarroch, 1997).

The sympathetic division of the autonomic nervous system provides a rapid mechanism that controls the acute response of the organism to a stressor. Centrally, the rostral VLM and NTS receive descending projections from the prefrontal and anterior cingulate cortices, amygdala, hypothalamus, and PAG and are critically involved in the cardiovascular response to stress (Spyer, 1989; Holstege, 1990; Spyer, 1994). This cardiovascular component includes sympathetic activation with increased heart rate (HR), increased cardiac output (CO) and increased BP. Peripherally, the SNS innervates many organs including the adrenal medulla (Barron and Van Loon, 1989; Benarroch, 1997).

Sympathetic stimulation of the adrenal medulla leads to the release of epinephrine (E) and NE into the circulation.

The PVN generates co-ordinated endocrine and autonomic responses to internal or external stressors (Swanson and Sawchenko, 1983). These include secretion of vasopressin, activation of adrenal cortical axis, and sympathoadrenal excitation (Fig. 1.2). Activation of the HPA axis begins with the release of CRF. The CRF neurons of the PVN project to the median eminence (Fig. 1.2). Upon release from neurons into the hypothalamic-hypophysial portal system, CRF is transported to the anterior pituitary where it stimulates the secretion of ACTH into the circulatory system. Circulating ACTH is the key regulator of glucocorticoid secretion by the adrenal cortex (Stratakis and Chrousos, 1995). Glucocorticoids are the final effectors of the HPA axis and participate in the adaptation of the organism to stress. The glucocorticoid, cortisol, acts in a negative feedback loop to inhibit CRF and ACTH release from the PVN and anterior pituitary, respectively. Additionally, control of this axis during stress also involves NE inputs from LC. Since there reciprocal neural connections between CRF and NE neurons, activation of one system enhances the activity the other (fig. 1.2) (Valentino et al., 1993; Kvetnanský et al., 1995; Pacák et al., 1995).

1.6.1. Involvement of nitric oxide

It has been suggested that NO regulates the output of sympathetic neuronal activity (Krukoff, 1998a, 1999). The synthesis of this non-conventional neuromessenger is catalysed by NO synthase (NOS) (Förstermann et al., 1991, 1995). NOS exists as three

isoforms: nNOS, eNOS, and iNOS . Both nNOS and eNOS are Ca^{+2}-dependent and constitutively expressed in brain and endothelium, respectively (Förstermann et al., 1991, 1995; Moncada, 1992). On the other hand, the Ca^{+2}-independent isoform, iNOS, is induced in many cell types by a variety of agents (Förstermann et al., 1991, 1995). Under normal conditions, iNOS is not expressed in cells (Moncada, 1992). The substrates in NO biosynthesis are L-arginine and O_2; and the final products are NO and citrulline (Moncada, 1992; Nathan, 1992). In addition to a number of co-factors, the catalytic activity of NOS requires nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor (Moncada, 1992).

A number of studies have provided evidence that NO is involved in regulating sympathetic output (Krukoff, 1998a, 1999a). Intravenous injections of NOS blockers led to increased BP (Wang et al., 1991; Sakuma et al., 1992) and increased renal nerve sympathetic activity (RNSA) (Sakuma et al., 1992). Intracisternal (i.c.) administration of N^{ω}-methyl-L-arginine (L-NMA), an inhibitor of NOS, also increases BP and RNSA (Togashi et al., 1992). Microinjection of NOS blockers into the NTS or rostral VLM increased BP and RNSA (Harada et al., 1993; Kagiyama et al., 1997), while the microinjection of a NO donor into the rostral VLM decreased BP (Kagiyama et al., 1997). These results support the notion that NO acts to decrease sympathetic output to the periphery.

The NADPH-diaphorase (NADPH-d) histochemical reaction has been widely used to localise putative NO-producing neurons in the brain (Krukoff, 1999). In fact, it has been shown that the population of neurons immunostained for nNOS and neurons

with NADPH-d staining are identical in the PVN when appropriate tissue fixation was used (Dawson et al., 1991). This histochemical technique is based on the presence of a diaphorase in neurons which catalyses the NADPH-dependent conversion of soluble tetrazolium salts to a blue precipitate (Hope et al., 1991; Scherer-Singler et al., 1983).

1.7. c-*fos* as a marker of activated neurons

Immediate-early genes are rapidly induced by extracellular stimuli and encode proteins that are required for subsequent cellular responses to occur (Curran and Morgan, 1995; Krukoff, 1998b). c-*fos* encodes a protein (Fos) that forms a complex with products from the related Jun family as a component of the transcription factor which binds to the activator protein 1 (AP-1) site on DNA (Curran and Franza, Jr., 1988; Lee et al., 1988; Curran and Morgan, 1995). Genes that contain the AP-1 binding site are activated by the Fos/Jun dimers, thereby allowing the expression of late-onset genes, including the ET genes (Yanagisawa et al., 1988; Inoue et al., 1989; Rubanyi and Polokoff, 1994; Kuwaki et al., 1997).

The expression of c-*fos* has been widely used as a marker of activated neurons (Krukoff et al., 1994, 1995, 1997; Krukoff, 1998b; Yang et al., 1999; Yang and Krukoff, 2000). Since the basal expression of c-*fos* is very low in unstimulated neurons (Krukoff et al., 1994; Herdegen et al., 1995; Krukoff and Khalili, 1997; Herdegen and Leah, 1998) and the transcription of c-*fos* occurs within minutes of application of stimulus (Müller et al., 1984; Krukoff, 1998b), a high and rapid expression of c-*fos* in neurons can be attributed to the applied stimulus. The most common technique for identifying activated

neurons is to visualise the presence of Fos within the nuclei of neurons using immunohistochemistry (Krukoff, 1998b).

1.8. Hypothesis

Based on the observations that central administration of ET-1 produces a sympathetic-induced pressor and depressor response via ET_A receptors, the experiments in this thesis were designed to test the hypothesis: **1) Central endothelin A receptors mediate the increase in sympathetic outflow during restraint stress and the decrease in sympathetic outflow during recovery from restraint.** Rats, receiving i.c.v. injections of the ET_A receptor antagonist, BQ 123, or vehicle, were subjected to a four hour restraint stress protocol. Mean arterial pressure (MAP) was used as a functional indicator of restraint-induced activation of SNS. Fos immunohistochemistry was used to identify activated neurons in the PVN, SON, ARC, LC, NTS, and VLM. NADPH-d histochemical staining was used to localise putative NO-producing neurons, and *in situ* hybridisation and image analysis were used to quantify TH mRNA (LC and NTS) (Chan and Sawchenko, 1995; Sabban et al., 1995; Nankova et al., 1999; Serova et al., 1999) and CRF mRNA (PVN) (Krukoff et al., 1999) gene expression. Since NO is believed to decrease sympathetic output to the periphery, experiments were also performed to test the hypothesis: **2) Central nitric oxide mediates the decrease in sympathetic outflow during recovery from restraint stress.** A nitrate/nitrite (NO_x) colourimetric assay kit was used to determine total NO content in hypothalamus and brainstem. In a separate set of experiments, N^G-nitro-L-arginine (L-NNA), an inhibitor of constitutive NOS (cNOS;

nNOS and eNOS) was used to block NO production in the brain. As with the first study, MAP was used as a functional indicator of restraint-induced activation of SNS. Fos immunohistochemistry was used to identify activated neurons and NADPH-d histochemical staining was used to localise putative NO-producing neurons.

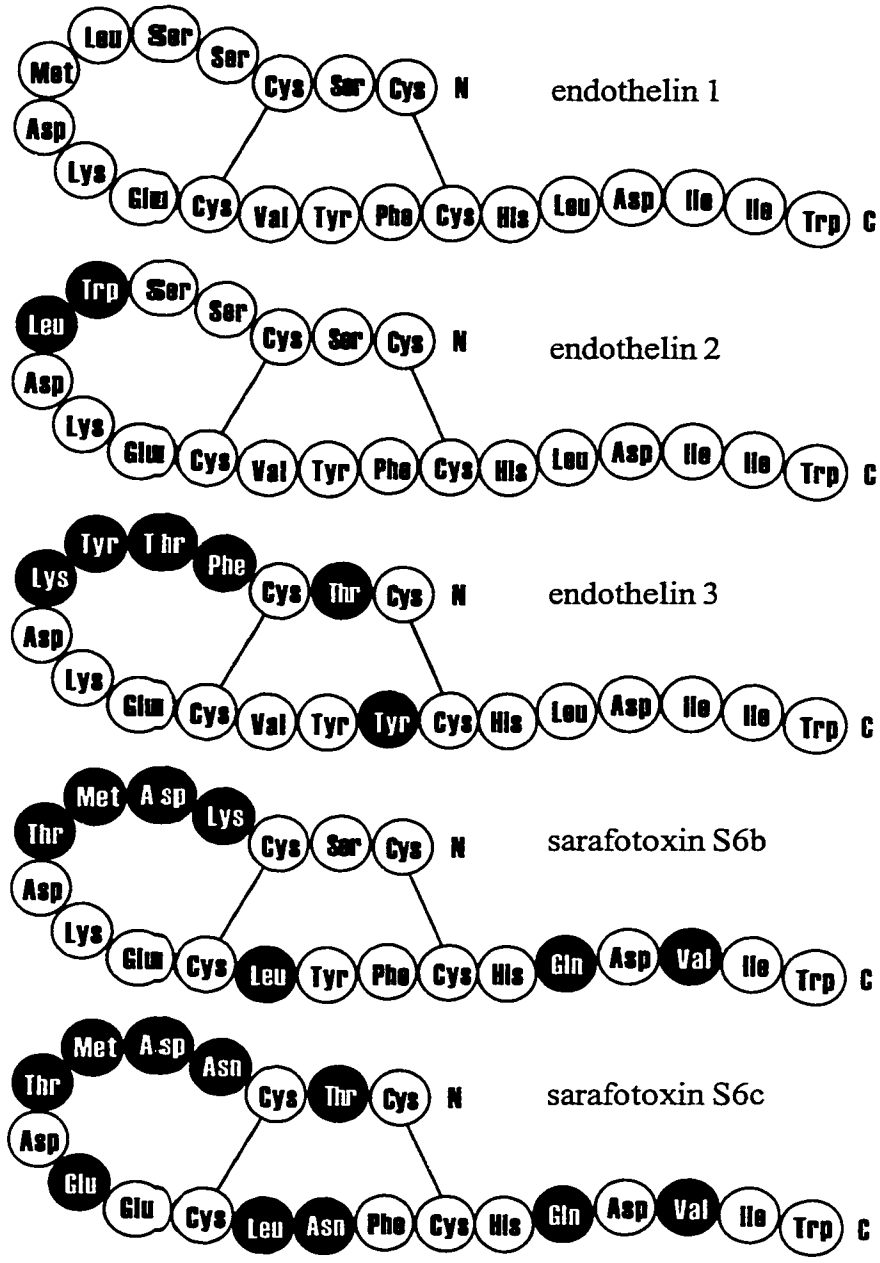


Figure 1.1. Structure of endothelins and sarafotoxins. **Filled circles** indicate residues that differ from the endothelin 1 sequence

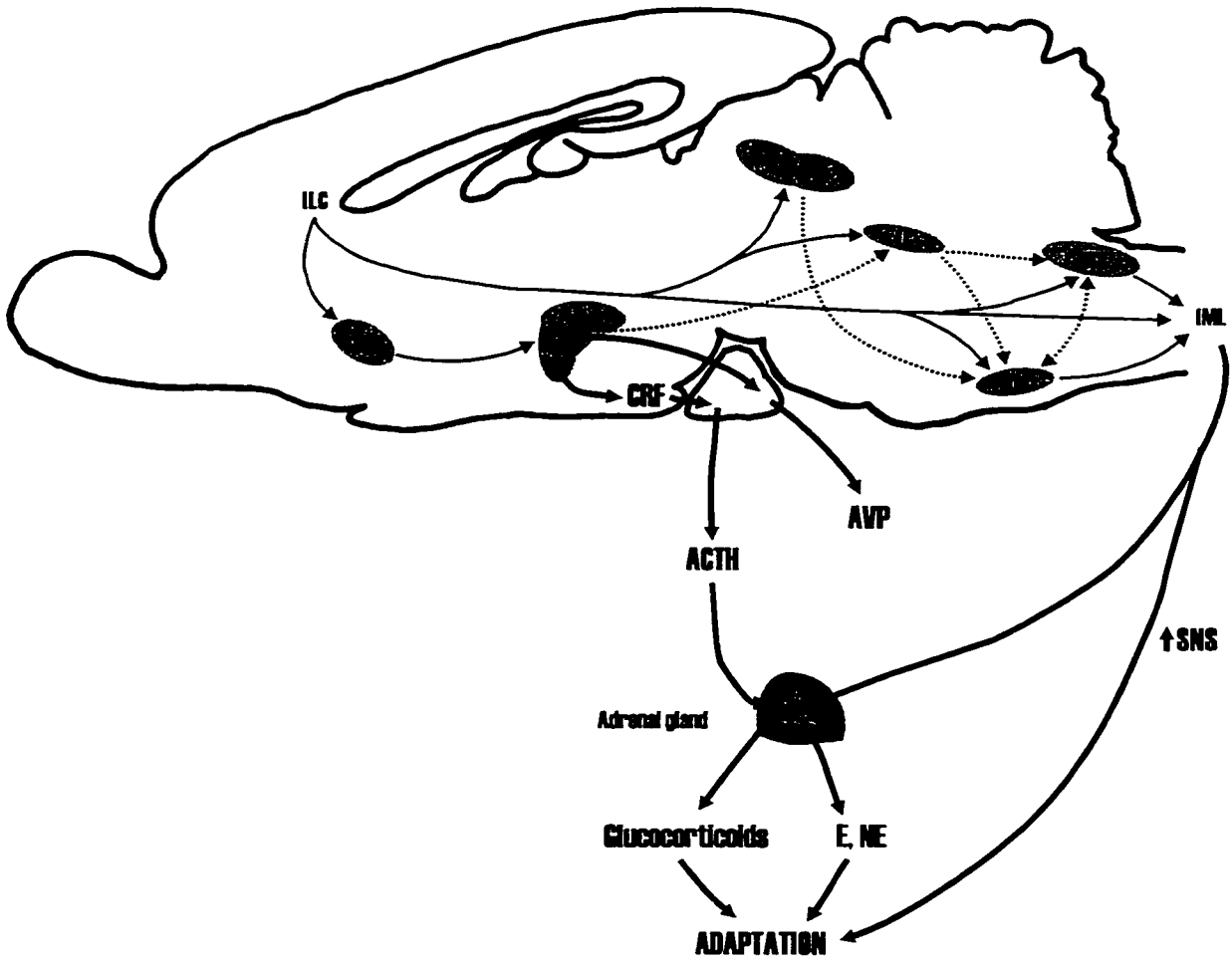


Figure 1.2. Hypothalamic-pituitary-adrenal axis and sympathetic nervous system activation in response to stress. Thin arrows represent neural connections. Thick arrows represent signalling pathway. Abbreviations: ACTH, adrenocorticotropin hormone; CRF, corticotropin-releasing factor; E, epinephrine; NE, norepinephrine; LC, locus coeruleus; NTS, nucleus of the tractus solitarius; PVN, paraventricular nucleus of the hypothalamus; SNS; sympathetic nervous system; VLM, ventrolateral medulla.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animals

Male Sprague-Dawley rats (200-300 g) were purchased from the Biological Sciences Animal Centre, University of Alberta. They were housed two per cage under a 12 hour light/dark schedule (lights on at 0700 h) at a temperature of 21°C. The rats were given food and water *ad libitum*. All protocols used in these experiments were approved by the University of Alberta Animal Welfare Committee.

2.2. Instrumentation

Rats were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.; Somnotol, M.T.C. Pharmaceuticals, Cambridge, ON) and received sterile penicillin G procaine suspension (30,000 U, i.m.; Ethacillin, Rogar/STB, London, ON), atropine sulphate (25 μg, s.c.; Ormond Veterinary Supply Ltd., Ancaster, ON), and buprenorphine hydrochloride (15 μg, i.m.; Buprenex® Injectable, Reckitt & Colman Pharmaceutical Inc., Richmond, VA).

2.2.1. *Intracerebroventricular cannulation*

Animals were implanted with a 22-gauge stainless steel guide cannula aimed at the lateral cerebral ventricle (C313G; Plastic One Inc., Roanoke, VA) (Yang and Krukoff, 2000). The skull was surgically exposed and an opening was drilled 1.0 mm posterior to Bregma and 2.0 mm lateral to the midline. The guide cannula was then inserted 4.1 mm below the external surface of the skull into the lateral ventricle. An internal cannula (C313I; 28 gauge; Plastic One) was connected to an Accu-Rated Pump

tube (Fisher Scientific, Nepean, ON) and a bubble was made at the cannula end of the tubing using a Hamilton microsyringe so that outflow of cerebrospinal fluid (CSF) could be observed by withdrawing with the microsyringe (Yang and Krukoff, 2000). The internal cannula was inserted into the guide cannula and accurate placement of cannula was confirmed by i) movement of the bubble and outflow of CSF; ii) observing a rise in blood pressure of at least 10 mmHg following injection of angiotensin II (5 pmol, i.c.v.; Bachem California, Torrance, CA) immediately after surgery; and iii) visual inspection of tissue sections for the tract left by the guide cannula. With the internal cannula removed, the guide cannula was secured to the skull with three screws and orthodontic resin (L.D. Chalk Company, Milford, Delaware), and was closed with a dummy cannula (C313DC; Plastic One).

2.2.2. Arterial and venous catheterisation

As described in previous studies (Krukoff et al., 1995; Krukoff et al., 1997; Yang et al., 1999), a midline abdominal incision was made and the descending aorta was exposed. Polyethylene tubing (PE10; 0.011 inch i.d. x 0.024 inch e.d.; Fisher Scientific) was inserted into the aorta and sutured to the posterior abdominal wall. The free end of the catheter was passed under the skin and externalised at the nape of the neck with 27-gauge stainless steel tubing (Small Parts Inc., Miami Lake, FL). The line was closed with polyvinylpyrolidone and capped with silastic tubing.

In the same surgical procedure, a silastic catheter (0.02 inch i.d. x 0.037 inch e.d.; Fred A. Dungey Inc., Scarborough, ON) was inserted into the inferior vena cava and

sutured to the posterior abdominal wall. The free end of the catheter was passed under the skin and externalised at the nape of the neck with 23-gauge stainless steel tubing (Small Parts). The line was capped with silastic tubing.

Animals were allowed to recover and were handled daily after surgery. Experiments were performed 5 to 7 days after surgery. On the day of the experiment, the arterial line of each rat was connected to a transducer for continuous recording of the arterial blood pressure. A baseline pressure was established for 30 minutes before the start of the experiment. Mean arterial pressure (MAP, mmHg) was calculated for every 5 minute interval.

2.3 Experimental Design

2.3.1 *Controls: Neuronal ET_A receptor blockade in urethane-anaesthetised rats by the selective antagonist, BQ 123*

The effectiveness of BQ 123 was determined as the inhibition of the rise in BP following i.c.v. administration of ET-1 (Rebello et al., 1995; Gulati et al., 1997b). Since i.c.v. ET-1 in conscious animals causes behavioural changes (Sirén and Feuerstein, 1989; Ouchi et al., 1989; Gross et al., 1993; Chew et al., 1994; Nagasaka et al., 1999) that impede measurement of blood pressure using our method, animals were anaesthetised with urethane (1g/kg, i.p.; Sigma Chemical, St. Louis, MO) (Mosqueda-Garcia et al., 1995). Rats received injections of BQ 123 (24 μg/kg, i.c.v.; American Peptide Company Inc., Sunnyvale, CA) (Clozel and Watanabe, 1993; Rebello et al., 1995) and/or ET-1 (200

ng/kg, i.c.v., (Gulati et al., 1997b; American Peptide) dissolved in 10 μl saline. Rats were divided into four groups (n=4 for each group):

Group 1: ET-1 given at time 0;

Group 2: BQ 123 given at time 0; ET-1 given after 15 minutes;

Group 3: BQ 123 given at time 0; ET-1 given after 120 minutes;

Group 4: BQ 123 given at time 0; ET-1 given after 240 minutes.

BP was recorded for the duration of the experiment and continued for 30 min following ET-1 administration. After each experiment, animals were given an overdose of sodium pentobarbital. From these experiments, it was determined (see results) that BQ 123 is effective for the 4 h restraint stress protocol (described below). Additionally, these studies confirmed that pre-treatment with i.c.v. BQ 123 for 15 minutes prevented the i.c.v. ET-1 pressor response (Rebello et al., 1995; Gulati et al., 1997a,b).

2.3.2. Controls: Effect of BQ 123 on MAP in conscious, non-stressed animals

To determine whether central BQ 123 itself alters BP in conscious animals, rats (n=4) received injections of BQ 123 (24 μg/kg, i.c.v.) dissolved in 10 μl saline. Control rats (n=4) were injected with the same amount of saline (i.c.v.). Both groups of animals were allowed to move freely in their cages for 4 hours and BP was recorded for the duration of the experiment. At the end of the experiment, rats were deeply anaesthetised and perfused transcardially as described below.

2.3.3. Effect of BQ 123 on MAP during restraint stress and recovery

Rats (n=8) received injections of BQ 123 (24 μg/kg, i.c.v.) dissolved in 10 μl saline. Control rats (n=8) were injected with the same amount of saline (i.c.v.). Fifteen minutes after BQ 123 or saline injections, rats were individually restrained in hemicylindrical, well-ventilated, plexiglass tubes (Krukoff and Khalili, 1997; Krukoff et al., 1999; Yang et al., 1999) according to a 4 h restraint stress protocol (alternating 1 h sessions of restraint/rest; Figure 2.) (Krukoff et al., 1999). This restraint protocol was found in previous experiments to yield results for c-*fos* expression which are similar to those for rats restrained for 1 h while allowing sufficient time for measurable changes in production of mRNA to occur (Krukoff et al., 1999). BP was recorded for the duration of the experiment. At the end of the experiment, rats were deeply anaesthetised and perfused transcardially as described below.

2.3.4. *Effect of BQ 123 and restraint stress on NO production in hypothalamus and brainstem*

Rats (n=4) received injections of BQ 123 (24 μg/kg, i.c.v.) dissolved in 10 μl saline. Control rats (n=4) were injected with the same amount of saline (i.c.v.). Fifteen minutes after BQ 123 or saline injections, rats were individually restrained as described above. At the end of the experiment, animals were given an overdose of sodium pentobarbital and decapitated. Hypothalamus and brainstem were dissected from each brain at 4°C, homogenised in 750 μl phosphate-buffered saline (PBS, pH 7.2) and centrifuged at 11,000 g for 20 minutes. The supernatant was centrifuged at 53,000 g for

15 minutes and the final supernatant was frozen for a NO_x colourimetric assay described below.

2.3.5. Effect of constitutive NOS inhibition on MAP during restraint stress and recovery

Rats were individually restrained as described above. Fifteen minutes prior to restraint, rats received i.c.v. injections of BQ 123 (24 μg/kg) dissolved in 10 μl saline or vehicle alone. At the same time, rats also received i.c.v. injections of L-NNA (88 μg/kg, Calbiochem, La Jolla, CA) dissolved in10 μl saline or vehicle alone. Immediately before the second hour of restraint, rats were given a second injection of L-NNA or vehicle to ensure continued inhibition of NO production (Yang and Krukoff, 2000)(Figure 2.). Rats were divided into four groups:

Group 1: Saline + Saline (n=4);

Group 2: Saline + L-NNA (n=5);

Group 3: BQ 123 + Saline (n=4);

Group 4: BQ 123 + L-NNA (n=5).

BP was recorded for the duration of the experiment. At the end of the experiment, rats were deeply anaesthetised and perfused transcardially as described below.

2.4. Tissue preparation

Rats were deeply anaesthetised and perfused transcardially with 200 ml ice-cold saline (0.9% NaCl) followed by 500 ml ice-cold 4% paraformaldehyde in PBS. The

brains were isolated, post-fixed in half-strength fixative and 15% sucrose for 1 hour, and stored in 20% sucrose overnight at 4°C. Coronal sections (35 μm) were cut in a cryostat. Sections of forebrain and brainstem either were thaw-mounted onto Superfrost®/Plus slides, air dried and stored at -70°C, or were collected in PBS for further processing.

2.5. Fos immunohistochemistry and NADPH-d histochemistry

Procedures for immunohistochemistry for Fos protein and histochemistry for NADPH-d have been published previously (Krukoff and Khalili, 1997; Krukoff, 1998b; Yang et al., 1999). Sections were kept at room temperature unless otherwise specified. Briefly, sections were incubated overnight with rabbit anti-c-*fos* antiserum (1:4000; Oncogene Research Products, Cambridge, MA) in 0.3% Triton X-100 (dissolved in PBS). According to the manufacturer, the antibody recognises Fos and Fos-related antigens. For this reason, immunoreactivity will be referred to as Fos-like immunoreactivity (FLI). The next day, sections were washed in PBS and incubated with a biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 hour. The sections were then washed in PBS and incubated with avidin-biotin complex (ABC Vecta Stain Kit, Vector Laboratories) for 1 hour. Sections were washed again in PBS and exposed to 0.04% diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.005% hydrogen peroxide in PBS for 15 to 20 minutes.

After another wash in PBS, the sections were incubated for 30 minutes at 42°C in PBS containing 0.6% Triton X-100, 1 mg/ml β-NADPH (Sigma), and 0.1 mg/ml nitroblue tetrazolium (Sigma). Sections were mounted onto glass microscope slides, air

dried, coverslipped with Elvanol (Mowiol, Calbiochem;10 g dissolved in 40 ml PBS and 20 ml glycerol) and examined by using a Zeiss light/fluorescence microscope.

2.6. *In situ* hybridisation

All solutions and glassware used in these procedures were RNase free. Hybridisation and histochemical localisation was carried out using ^{35}S-labelled cRNA probes. Protocols for riboprobe synthesis, hybridisation and autoradiographic localisation of mRNA signal have been described previously (Krukoff et al., 1999; Yang and Krukoff, 2000). Briefly, frozen tissue sections were brought to room temperature, pre-treated with 4% paraformaldehyde solution, 20 μg/ml proteinase K solution, 0.25% acetic anhydride in 0.1 M triethanolamine-HCl solution (pH 8), and dehydrated with ethanol. Radiolabelled probes were diluted in riboprobe hybridisation buffer and applied to brain sections (3 x 10^6 cpm/slide). After an overnight incubation at 46°C in a humidified chamber, sections were washed twice in 4x standard saline citrate (SSC) with 2-mercaptoethanol (1 μl/ml; Sigma) and twice again in 4x SSC. The sections were then incubated in RNase A (10 μg/ml in RNase buffer for 30 min at 37°C), and then in 2x SSC and 0.1x SSC (42°C for 40 min and 65°C for 45 min respectively) to reduce non-specific binding of the riboprobe. The sections were air dried for 3 hours.

After hybridisation, slides were exposed to X-ray film (X-OMAT AR, Kodak) for 48 to 72 hrs, dipped into NTB-2 Kodak photographic emulsion, exposed for 10 to 13 days, and developed for autoradiography. Tissues were dehydrated and coverslipped. Sections were examined with light- and darkfield microscopy.

2.6.1. *cRNA Probes*

cRNA probes for TH were generated from a 1.2kb *Eco* RI digest of rat TH cDNA containing the +14 to +1165 region of the coding sequence for the enzyme (Grima et al., 1985) that was subcloned into a pGEM-7Zf transcription vector (Promega Corp., Madison, WI). Antisense probes were transcribed with SP6 RNA polymerase (Promega) from plasmids linearised with *Apa* I; sense probes were transcribed with T7 RNA polymerase (Promega) from plasmids linearised with *Hind* III.

cRNA probes for CRF contained the +1283 to +2048 region of the peptide cloned into pGem-3Z (Promega) at the *Bam* H1 site. Antisense probes were generated with T7 RNA polymerase from plasmids linearised with *Hind* III; sense probes were generated with SP6 RNA polymerase from plasmids linearised with *Sac* I.

2.7. Nitrate/nitrite colourimetric assay

The method used to determine the amount of NO produced was the measurement of NO_x levels in tissue homogenates. The *ex vivo* measurement of NO_x has been shown to be a reliable method for the determination of constitutive NOS activity *in vivo* (Salter et al., 1996). Assays were done on homogenised tissue samples using a NO_x colourimetric assay kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Briefly, frozen supernatant samples were brought to room temperature, filtered using a 30 kDa molecular weight cut-off filter (Millipore, Bedford, MA), and 80 μl of elutant was incubated with 10 μl Enzyme Co-factor mixture and 10 μl Nitrate Reductase

mixture for 3 hours at room temperature. After incubation, 50 μl of each Griess Reagents R1 and R2 were added and the colour allowed to develop for 10 minutes at room temperature. The absorbance was read at 540 nm using an Elisa plate reader (MTX Lab System Inc., McLean, VA)(Yang and Krukoff, 2000).

2.8. Analysis

Neurons with FLI (stained in the nucleus) or positive for NADPH-d (stained in the cytoplasm) were counted in the PVN, NTS, VLM, and LC. Only neurons whose nuclei were visible (stained or unstained) were included in the counts. To avoid double-counting neurons in adjacent sections, data were expressed as numbers of labelled neurons *per section* (Krukoff, 1998b).

Quantification of mRNA levels in the PVN (CRF mRNA), NTS (TH mRNA), and the LC (TH mRNA) was carried out under light- and darkfield microscopy on nuclear emulsion-dipped slides using a digitising scanning camera (DC330, DAGE MTI Inc., Michigan, MI) coupled to a Pentium-based computer and imaging software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Two sections from each of the middle region of the PVN, the middle subdivision of the NTS, and the LC were analysed in each animal (Yang and Krukoff, 2000). The frame used to define the area for quantifying the silver grains was kept the same size for each region and placed in the same locations in the PVN, NTS, and the LC, respectively. Optical density was described as percentage of area covered by silver grains (Krukoff et al., 1999; Yang and Krukoff, 2000). Background signal was measured on nearby fibre tracts and subtracted from the signal in

the area of interest. Optical densities of two times that of background were considered to be positive hybridisation signals.

Data are expressed as means ± SEM. Arterial pressure tracings are expressed as percent of baseline MAP (% bMAP). In most experiments, data from experimental animals were compared to data from appropriate control animals with the unpaired Student's t test. In the L-NNA experiments, MAPs were compared by one-way ANOVA and the Newman-Keuls post hoc. $P < 0.05$ was taken to signify statistical significance. Graphical representation and statistical analysis were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA.

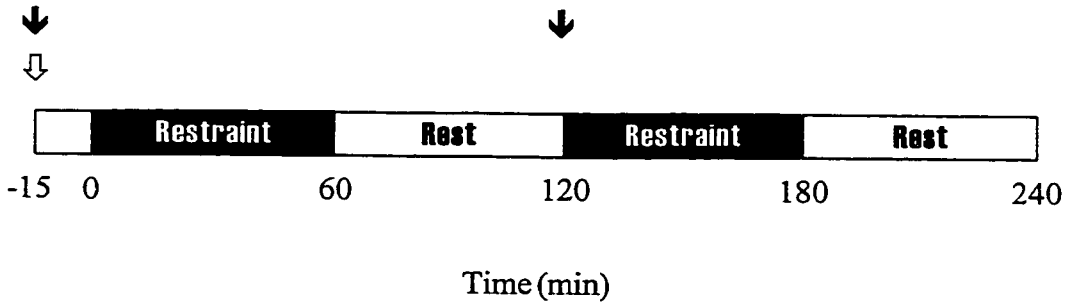


Figure 2. Drawing illustrating experimental protocol. ⇩, time at which BQ 123 or vehicle was injected intracerebroventricularly (i.c.v.). For L-NNA experiments, ↓, time at which L-NNA or vehicle was injected i.c.v.

CHAPTER 3

RESULTS

3.1. Controls: Neuronal ET_A receptor blockade in urethane-anaesthetised rats by the selective antagonist, BQ 123

In control experiments using urethane-anaethetised rats, injections of ET-1 produced an increase in arterial pressure (42-45 mmHg; 150% bMAP) that reached a plateau within 5 minutes after ET-1 injection (Fig. 3.1.a). While BQ 123 injection alone produced an increase in arterial pressure (17-25 mmHg; 120% bMAP; Figs. 3.1.b,c,d), pre-treatment with BQ 123 prevented the increase in arterial pressure in response to ET-1 at all time points investigated (Figs. 3.1.b,c,d).

3.2. Controls: Effect of BQ 123 in conscious, non-stressed animals

3.2.1. *Blood pressure*: The MAPs of conscious rats receiving BQ 123 were not significantly different from those receiving vehicle (Fig. 3.2.).

3.2.2. *Fos immunohistochemistry and NADPH-d histochemistry*: Except for a significant increase in the number of neurons per section with FLI in the LC of rats treated with BQ 123 (27.27 ± 5.6 vs. 9.19 ± 3.06 neurons; $p < 0.05$), quantification revealed that there were no significant differences in numbers of neurons per section with FLI, NADPH-d staining, or double-stained for FLI and NADPH-d in the PVN, SON, ARC, NTS, or VLM suggesting that BQ 123 itself does not affect neuronal activation.

3.2.3. *In situ hybridisation for TH mRNA and CRF mRNA*: Quantification revealed that there were no significant differences in the levels of TH mRNA or CRF mRNA expression in any of the areas investigated (Fig. 3.3.).

3.3. Effects of BQ 123 in conscious animals during restraint stress and recovery

3.3.1. Blood pressure: In conscious experimental rats, injection of BQ 123 produced a small but significant increase in arterial pressure (8-13 mmHg; 110% bMAP; $p < 0.05$; Fig. 3.4.) which subsided within 10 minutes. At the onset of restraint, arterial pressures in experimental and control animals rose approximately 40 mmHg (144% and 140% bMAP, respectively). During the first hour of restraint (0 to 60 min), there was a tendency for the arterial pressures in both groups of animals to decline slightly but not significantly. Although differences did not reach significance, the MAPs in rats receiving BQ 123 were consistently greater than in rats receiving vehicle.

The arterial pressures in both groups of animals decreased when the animals were removed from the restraint chamber (60 min). During the hour of rest (60 to 120 min), the arterial pressures in animals receiving vehicle continued to decrease and levelled off below baseline levels (92% bMAP). In animals receiving BQ 123, however, the arterial pressures decreased and then levelled off above baseline values (105% bMAP). The MAPs in experimental animals remained significantly higher than those in control animals (at least $p < 0.05$; see Fig. 3.4.). The average pressure difference between experimental and control animals during this period was 8 mmHg.

At the onset of the second period of restraint (120 min), the arterial pressures in both experimental and control animals rose approximately 35 mmHg (142% and 135% bMAP, respectively). During this second hour of restraint (120 to 180 min), the MAPs in animals receiving vehicle tended to decrease while the MAPs in animals receiving BQ 123 remained elevated. The MAPs in experimental rats were consistently greater than in control rats. Toward the end of the restraint period, the MAPs were significantly greater in experimental rats than in control rats, with an average pressure difference of 13 mmHg ($p < 0.05$; Fig. 3.4.).

The arterial pressures in both groups of animals decreased when the animals were removed from the restraint chamber (180 min) a second time. During this second hour of rest (180 to 240 min), the arterial pressures in animals receiving vehicle continued to decrease and levelled off below baseline levels (91% bMAP). In animals receiving BQ 123, the arterial pressures decreased slightly and levelled off above baseline values (109% bMAP). The arterial pressures in experimental animals remained significantly higher (by approximately 13 mmHg) than those in control animals (at least $p < 0.05$; Fig. 3.4.).

3.3.2. Fos immunohistochemistry and NADPH-d histochemistry: There were no significant differences in numbers of neurons per section with FLI, NADPH-d staining, or double-stained for FLI and NADPH-d for any of the areas investigated (Figs. 3.5., 3.6., and 3.7.).

3.3.3. *In situ hybridisation for TH mRNA and CRF mRNA*: Rats subjected to the restraint protocol following BQ 123 pre-treatment had a significant decrease in CRF mRNA in the parvocellular portion of the PVN ($p < 0.05$; Figs. 3.8. & 3.9.). There were no significant differences in the levels of TH mRNA expression in the NTS or LC.

3.4. Effect of BQ 123 and restraint stress on NO production in hypothalamus and brainstem of conscious animals

3.4.1. Nitrate/nitrite assay: Rats subjected to the restraint protocol following BQ 123 pre-treatment had a significant decrease in NO_x levels in the brainstem ($p < 0.05$; Fig. 3.10.). In the hypothalamus, however, there were no significant differences in NO_x levels.

3.5. Effects of constitutive NOS inhibition in conscious animals during restraint stress and recovery

3.5.1. Blood pressure: At the onset of restraint, arterial pressures in Saline + Saline, BQ 123 + Saline, Saline + L-NNA, and BQ 123 + L-NNA animals rose approximately 30 mmHg (135%, 135%, 133%, and 133% bMAP, respectively; Figs. 3.11. and 3.12.). During the first hour of restraint (0 to 60 min), there was a tendency for the arterial pressures in Saline + Saline, Saline + L-NNA and BQ 123 + L-NNA animals to decline, though these decreases were not significant. While not significant, during the last 35 minutes of restraint (25 to 60 min), the MAPs in BQ 123 + Saline rats were consistently greater than in the other groups (Figs. 3.11.b and 3.12.).

The arterial pressures in Saline + Saline and BQ 123 + Saline rats decreased when the animals were removed from the restraint chamber (60 min). During the hour of rest (60 to 120 min), the arterial pressures in Saline + Saline animals continued to decrease as described in Section 3.3.1 and levelled off below baseline levels (93% bMAP). In BQ 123 + Saline animals the arterial pressures decreased and levelled off above baseline values (106% bMAP). As described in Section 3.3.1, the arterial pressures in BQ 123 + Saline rats remained significantly elevated above baseline (by approximately 16 mmHg) as compared to MAPs in Saline + Saline rats ($p < 0.05$; see Figs. 3.11.b and 3.12.). The arterial pressures in the Saline + L-NNA and BQ 123 + L-NNA rats did not decrease significantly when the animals were removed from the restraint chamber (117% and 111% bMAP, respectively). Arterial pressures in BQ 123 + L-NNA rats were significantly greater (by approximately 9 mmHg) than MAPs in Saline + Saline rats (at least $p < 0.05$). The arterial pressures in Saline + L-NNA rats were significantly greater (by approximately 19 mmHg) than MAPs in Saline + Saline rats (at least $p < 0.05$). There were no significant differences in MAPs between BQ 123 + Saline and BQ 123 + L-NNA rats during the hour of rest (60 to 120 min). At 100 to 105 min, the MAPs in Saline + L-NNA rats were significantly greater than either BQ 123 + Saline or BQ 123 + L-NNA rats ($p < 0.05$; see Figs. 3.11. and 3.12.).

As described previously, the onset of the second hour of restraint (120 min) led to increased arterial pressures in Saline + Saline and BQ 123 + Saline animals of approximately 23 mmHg (123% and 124% bMAP, respectively) while the MAPs in BQ 123 + L-NNA and Saline + L-NNA animals rose approximately 19 mmHg (127% and

133% bMAP, respectively). During the hour of restraint (120 to 180 min), the MAPs in Saline + Saline and BQ 123 + L-NNA rats tended to decrease whereas the MAPs in BQ 123 + Saline and Saline + L-NNA rats remained elevated. While the differences did not reach significance, during the last 50 minutes of restraint (130 to 180 min), the MAPs in BQ 123 + Saline and Saline + L-NNA rats were consistently greater than in either Saline + Saline or BQ 123 + L-NNA groups (see Figs. 3.11. and 3.12.).

The arterial pressures in Saline + Saline and BQ 123 + Saline rats decreased when the animals were removed from the restraint chamber (180 min). During the hour of rest (180 to 240 min), the arterial pressures in Saline + Saline animals continued to decrease and levelled off below baseline levels (92% bMAP). In BQ 123 + Saline animals the arterial pressures decreased and then levelled off above baseline values (106% bMAP). The arterial pressures in the Saline + L-NNA and BQ 123 + L-NNA rats remained elevated. On the whole, there were few significant differences in MAP between Saline + L-NNA and BQ 123 + L-NNA rats. Generally, the arterial pressures in Saline + L-NNA rats were significantly greater (by approximately 27 mmHg) than MAPs in Saline + Saline rats (at least $p < 0.01$; see Figs. 3.11.a and 3.12.). The MAPs in Saline + L-NNA rats were significantly greater than MAPs in BQ 123 + Saline rats (at least $p < 0.05$; Figs. 3.11. and 3.12.). In general, the arterial pressures in BQ 123 + Saline and BQ 123 + L-NNA rats remained significantly elevated (by approximately 16 mmHg) above baseline as compared to MAPs in Saline + Saline rats (at least $p < 0.05$; see Figs. 3.11. and 3.12.). There were few significant differences in MAPs between BQ 123 + Saline and BQ 123 + L-NNA rats during the hour of rest (180 to 240 min).

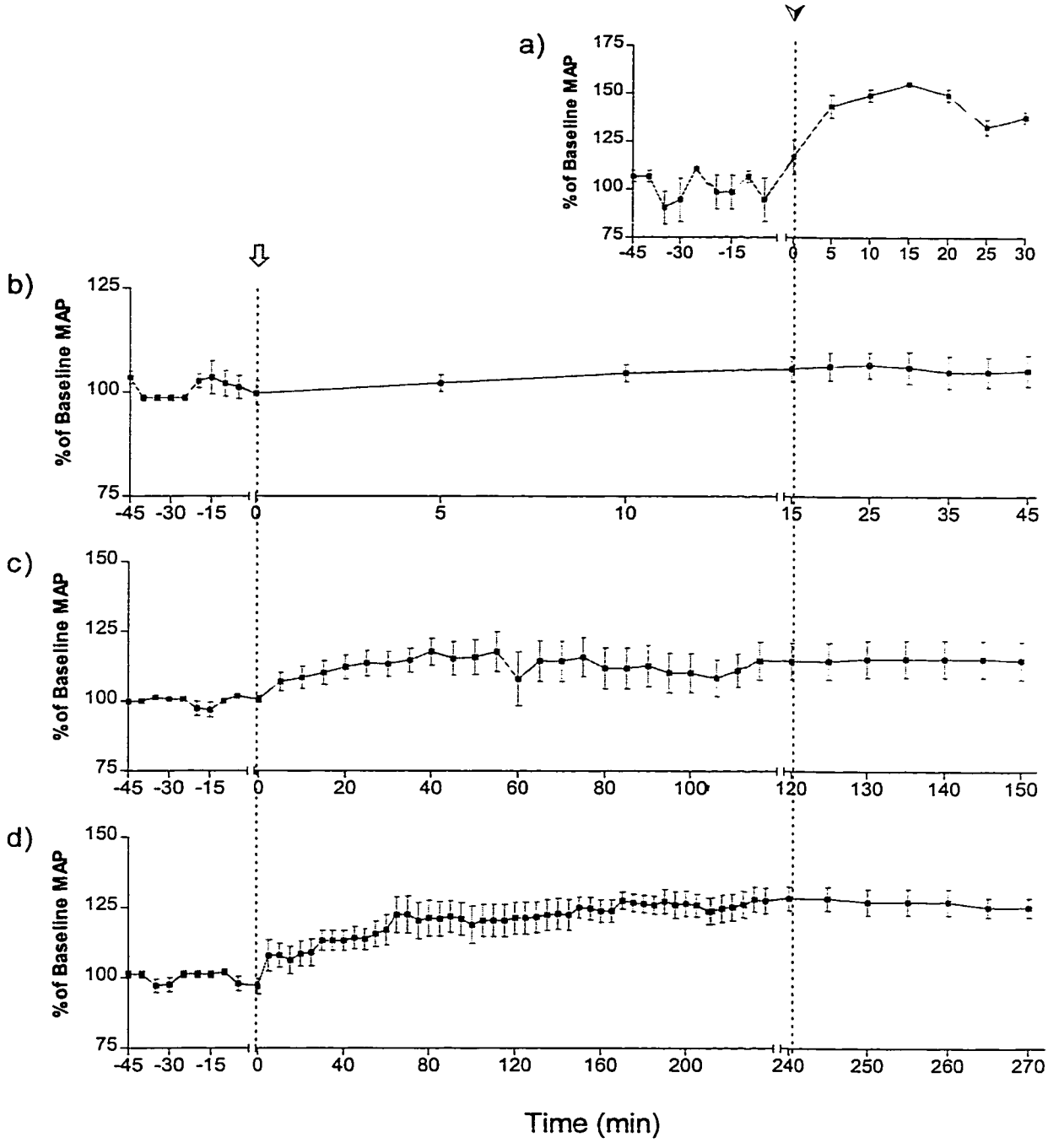


Figure 3.1. Changes in mean arterial pressure (MAP) after intracerebroventricular injections in urethane-anaesthetized rats. Data are expressed as mean ± SEM. ⩛, time at which ET-1 was injected. ⇩, time at which BQ 123 was injected. n=4 in each group.

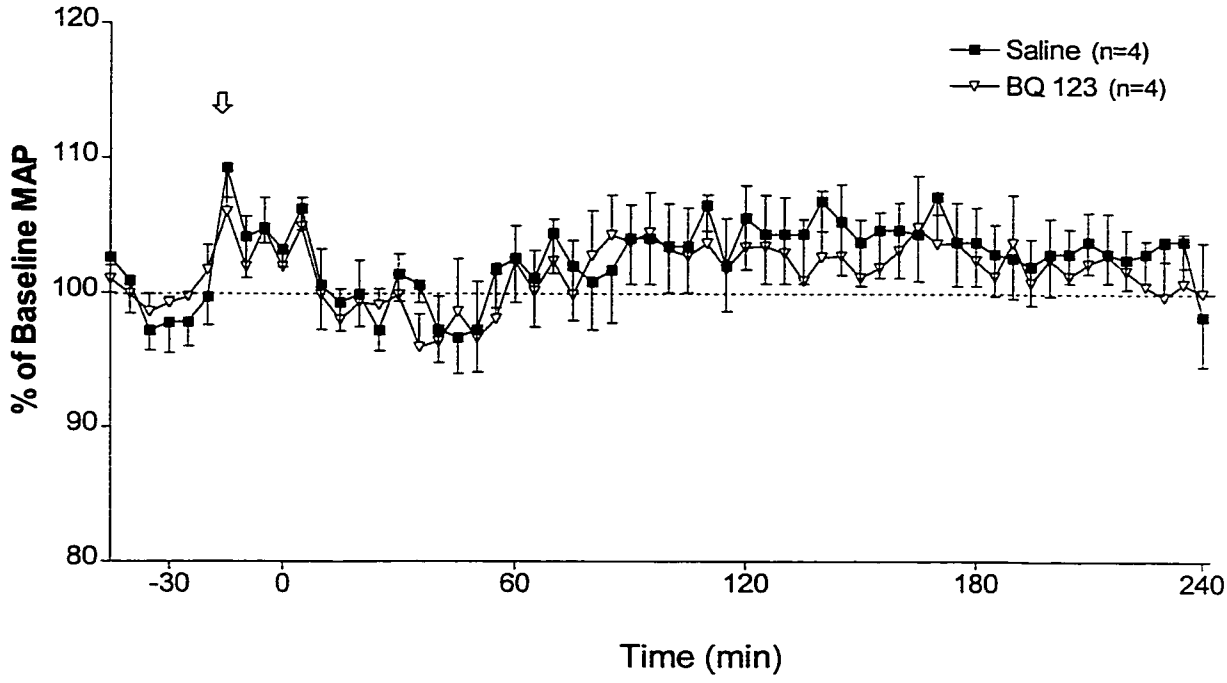


Figure 3.2. Changes in mean arterial pressure (MAP) after intracerebroventricular injections of ET_A antagonist, BQ 123 (24 μg/kg) or vehicle in non-stressed animals. Data are expressed as mean ± SEM. There were no significant differences between animals receiving BQ 123 or vehicle. ⇩, time at which injections were made.

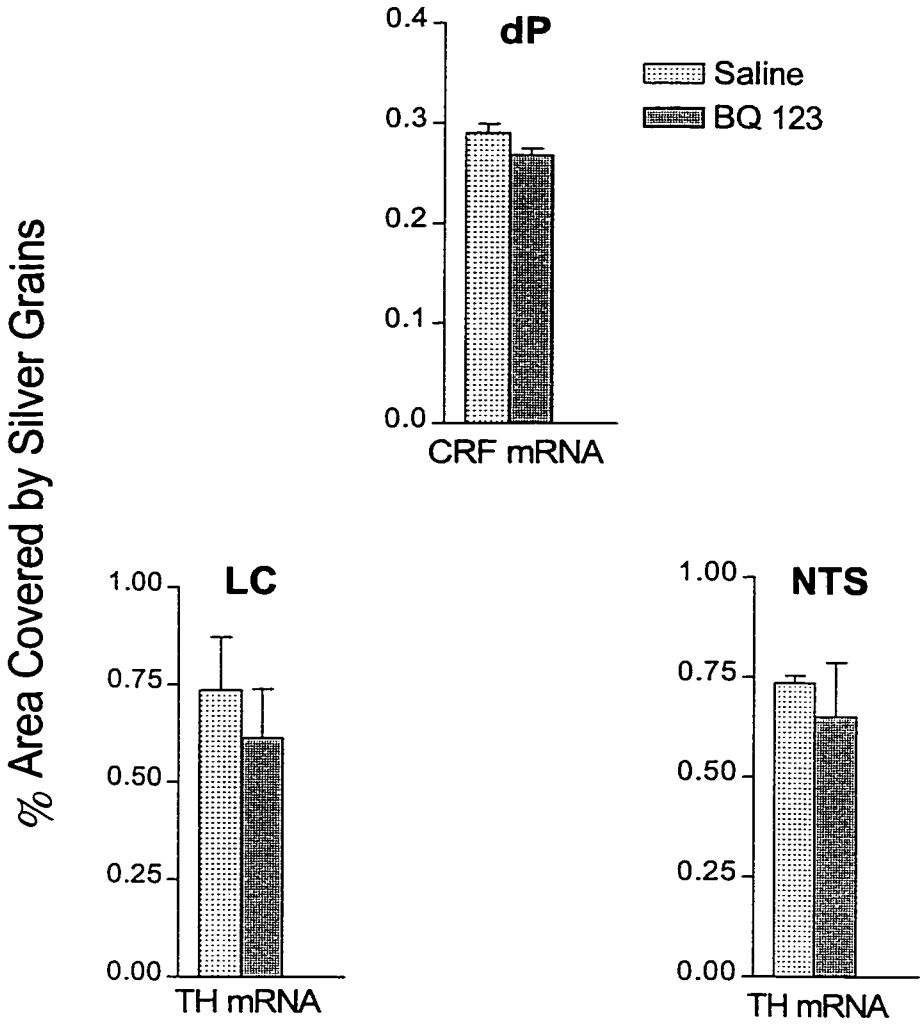


Figure 3.3. Expression of CRF mRNA in parvocellular PVN and TH mRNA in LC and NTS in non-stressed animals receiving intracerebroventricular injections of BQ 123 or vehicle. Data are expressed as mean ± SEM. There were no significant differences between animals receiving BQ 123 or vehicle. Abbreviations: CRF, corticotropin-releasing hormone; LC, locus coeruleus; NTS, nucleus of the tractus solitarius; PVN, paraventricular nucleus; TH, tyrosine hydroxylase; VLM, ventrolateral medulla

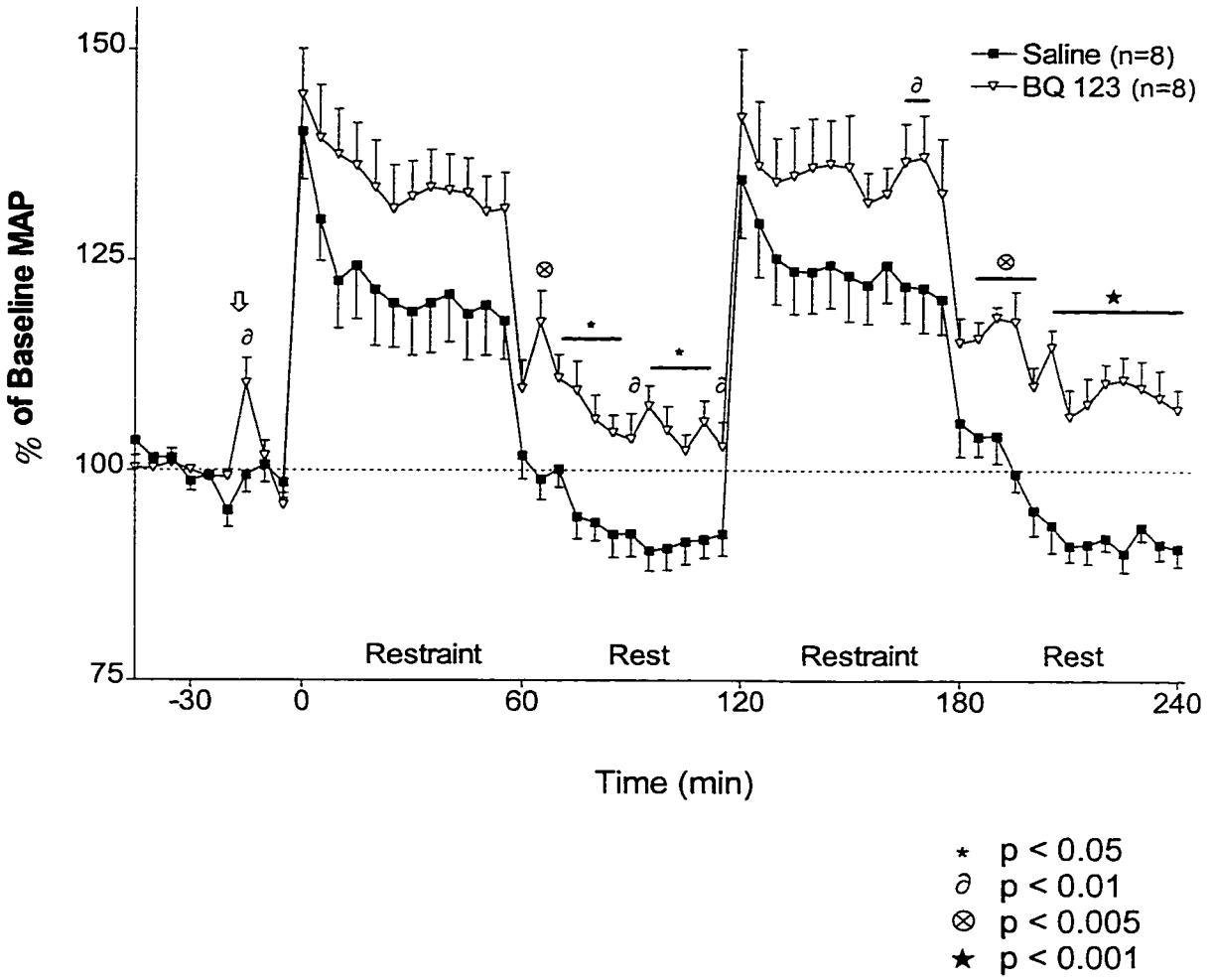


Figure 3.4. Changes in mean arterial pressure (MAP) during restraint stress and recovery in rats receiving ET_A antagonist, BQ 123, or vehicle. Data are expressed as mean ± SEM. ⇩, time at which BQ 123 (24 μg/kg) or vehicle was injected.

Figure 3.5. Numbers of neurons per section single- and double-labelled for FLI and/or NADPH-d in the rostral, middle, and caudal PVN in restraint stressed animals receiving BQ 123 or vehicle (n=4 for each group). Data are expressed as mean ± SEM. There were no significant differences between animals receiving BQ 123 or vehicle. Inset shows middle PVN divided into functional subdivisions. Abbreviations: PVN, paraventricular nucleus of the hypothalamus; rPVN, rostral PVN; mPVN, middle PVN; cPVN, caudal PVN; M, magnocellular division of mPVN; dPv, dorsal parvocellular division of PVN (ventral portion); dPm, dorsal parvocellular division of PVN (medial portion); mP; medial parvocellular division of PVN.

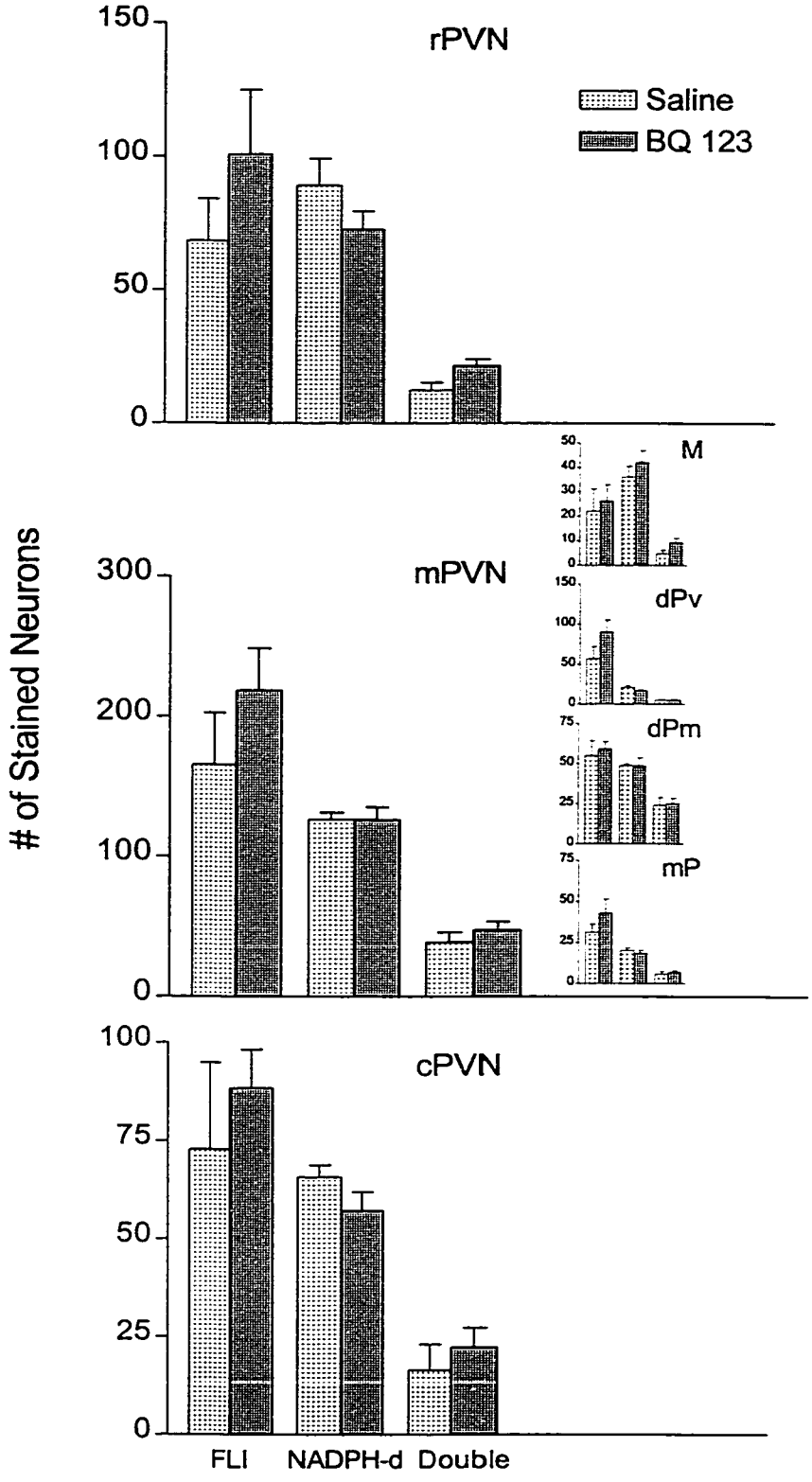

rPVN
Saline
BQ 123
150
100
50
0
M
50
40
30
20
10
0
mPVN
300
200
100
0
dPv
150
100
50
0
dPm
75
50
25
0
mP
75
50
25
0
cPVN
100
75
50
25
0
# of Stained Neurons
FLI
NADPH-d
Double

Figure 3.5.

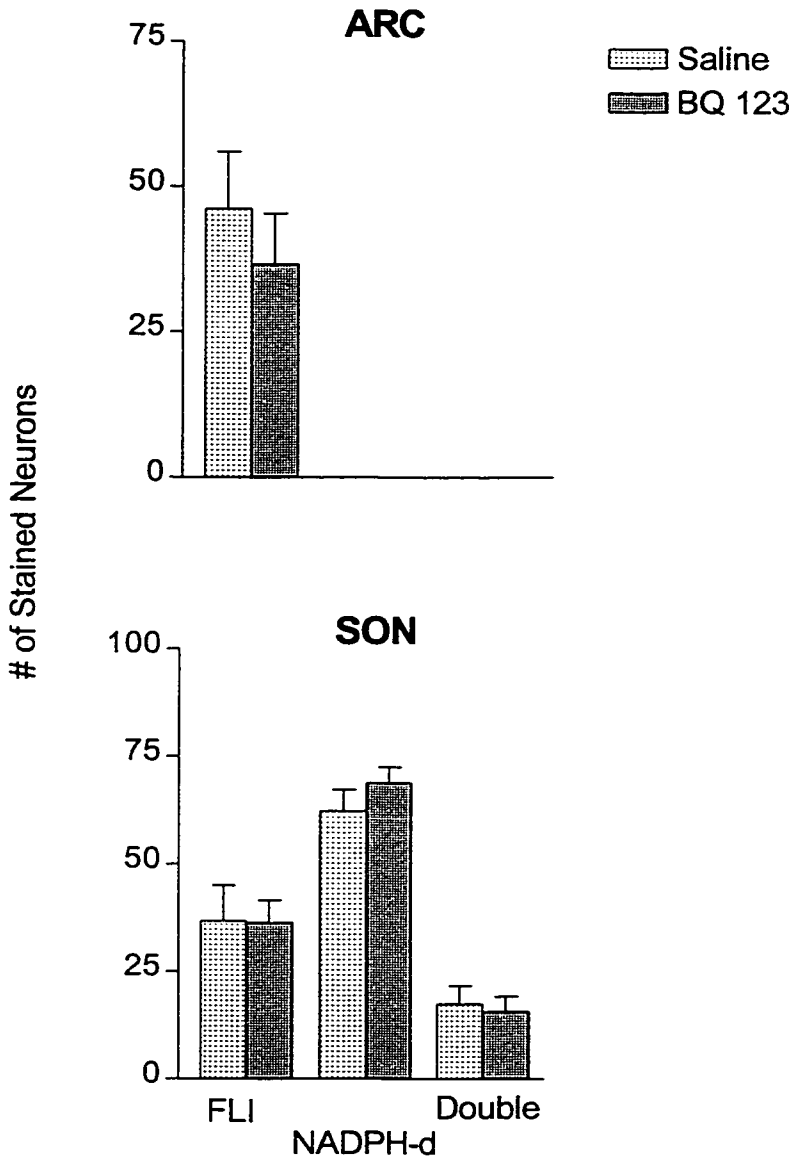


Figure 3.6. Numbers of neurons per section single- and double-labeled for FLI and/or NADPH-d in the arcuate and supraoptic nuclei of stressed animals receiving intracerebroventricular injections of BQ 123 or vehicle (n=4 for each group). Data are expressed as mean ± SEM. There were no significant differences between animals receiving BQ 123 or vehicle. Abbreviations: ARC, arcuate nucleus; FLI, Fos-like immunoreactivity; NADPH-d, nicotinamide adenine dinucleotide phosphate diaphorase; SON, supraoptic nucleus.

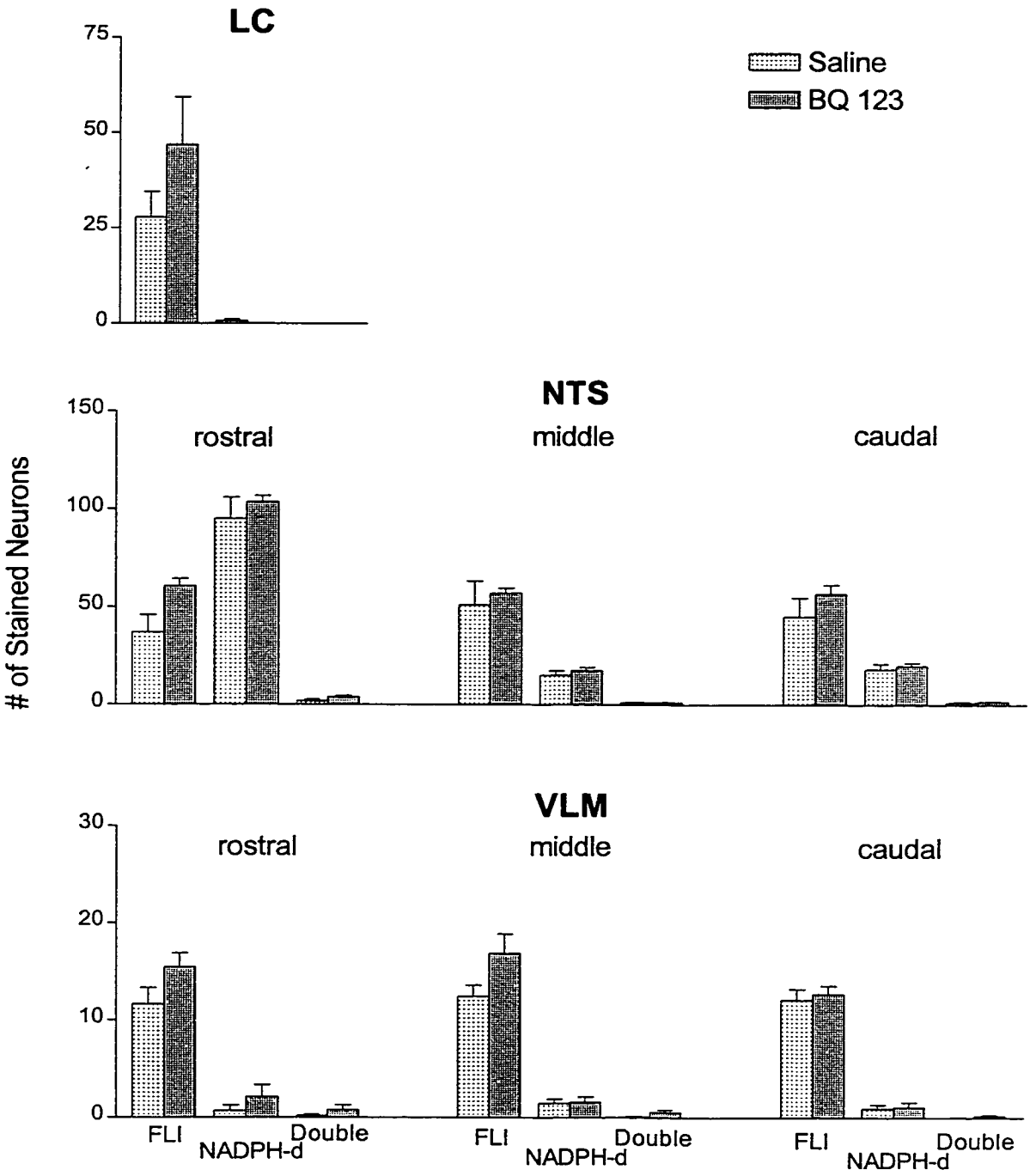


Figure 3.7. Numbers of neurons per section single- and double-labeled for FLI and/or NADPH-d in the LC, NTS, and VLM of stressed animals receiving intracerebroventricular injections of BQ 123 or vehicle (n=4 for each group). Data are expressed as mean ± SEM. There were no significant differences between animals receiving BQ 123 or vehicle. Abbreviations: FLI, Fos-like immunoreactivity; LC, locus coeruleus; NADPH-d, nicotinamide adenine dinucleotide phosphate diaphorase; NTS, nucleus of the tractus solitarius; VLM; ventrolateral medulla

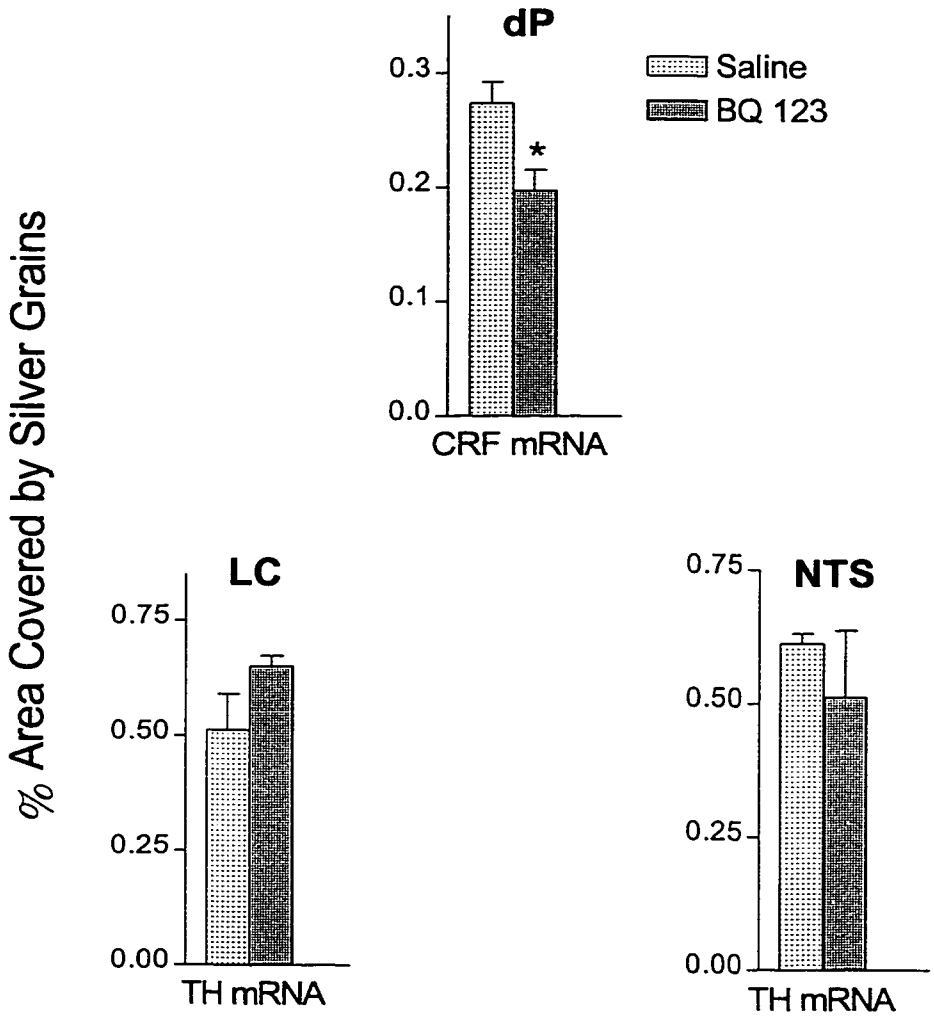


Figure 3.8. Expression of CRF mRNA in parvocellular PVN and TH mRNA in LC and NTS in stressed animals receiving intracerebroventricular injections of BQ 123 or vehicle. Data are expressed as mean ± SEM. *, significant difference $p < 0.05$. Abbreviations: CRF, corticotropin-releasing hormone; LC, locus coeruleus; NTS, nucleus of the tractus solitarius; PVN, paraventricular nucleus; TH, tyrosine hydroxylase; VLM, ventrolateral medulla

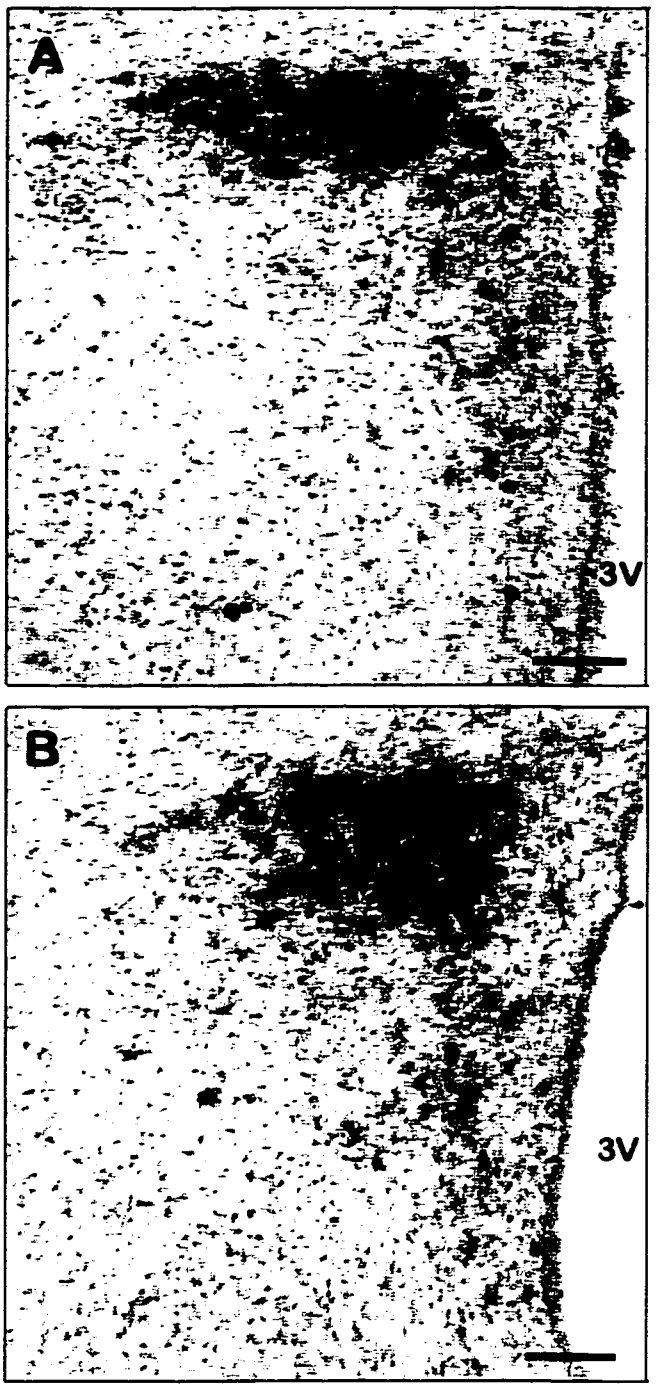


Figure 3.9. Expression of CRF mRNA in the paraventricular nucleus of restrained rats receiving: a) BQ 123 or b) vehicle. 3V, third ventricle. Bars represent 100 µm.

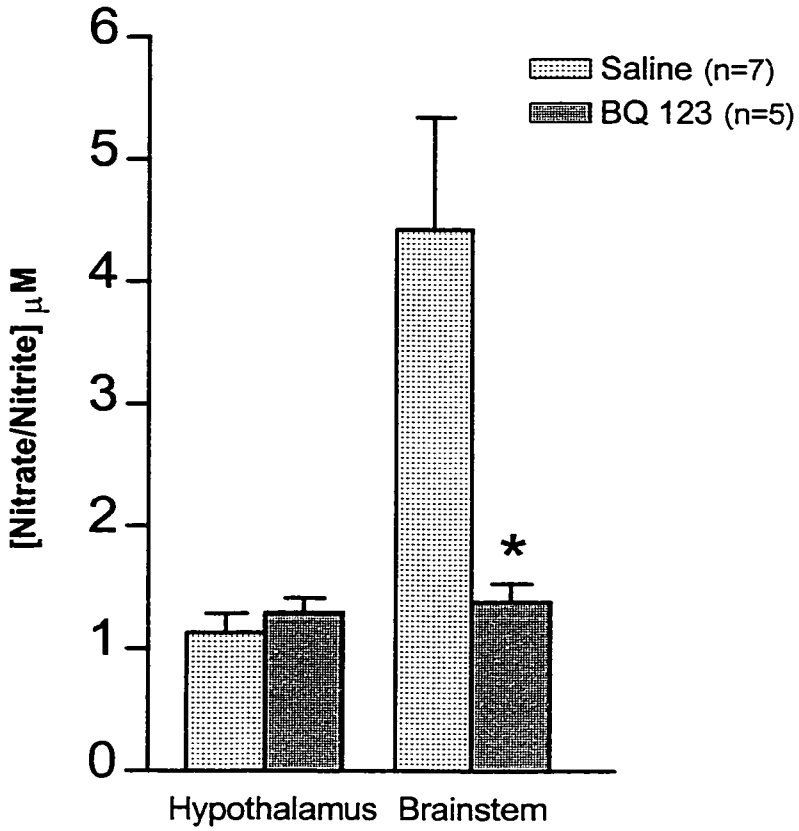


Figure 3.10. Nitrate/nitrite concentrations in hypothalamus and brainstem following restraint stress in rats receiving intracerebroventricular injections of BQ 123 or vehicle. Data are expressed as mean ± SEM. *, significant difference $p < 0.05$.

Figure 3.11. Changes in mean arterial pressure (MAP) during restraint stress and recovery in rats receiving a) constitutive NOS blocker, L-NNA, or vehicle; b) ET_A antagonist, BQ 123, and/or L-NNA. Data are expressed as mean ± SEM. ⇩, time at which BQ 123 (24 μg/kg) or vehicle was injected. ⬇, time at which L-NNA (88 μg/kg) or vehicle was injected. *, significant difference $p < 0.05$. δ, significant difference $p < 0.01$. ★, significant difference $p < 0.001$.

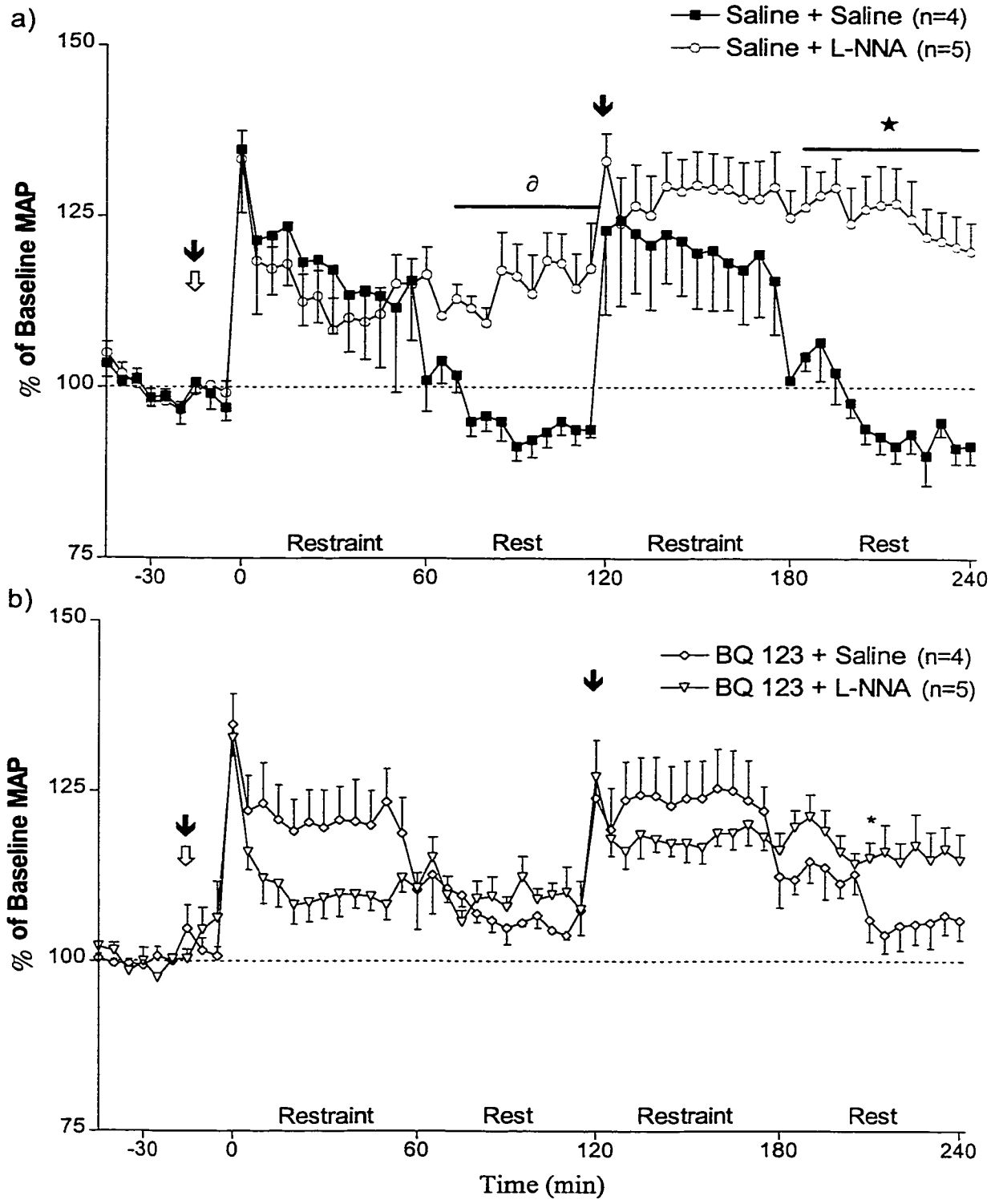

a)
Saline + Saline (n=4)
Saline + L-NNA (n=5)
% of Baseline MAP
150
125
100
75
∂
★
Restraint
Rest
Restraint
Rest
-30
0
60
120
180
240
b)
BQ 123 + Saline (n=4)
BQ 123 + L-NNA (n=5)
% of Baseline MAP
150
125
100
75
*
Restraint
Rest
Restraint
Rest
-30
0
60
120
180
240
Time (min)

Figure 3.11.

Figure 3.12. Changes in mean arterial pressure (MAP) during restraint stress and recovery in rats receiving ET_A antagonist, BQ 123, and/or constitutive NOS blocker, L-NNA. ⇩, time at which BQ 123 (24 μg/kg) or vehicle was injected. ⬇, time at which L-NNA (88 μg/kg) or vehicle was injected. Bars represent treatment group compared to Saline + Saline controls.

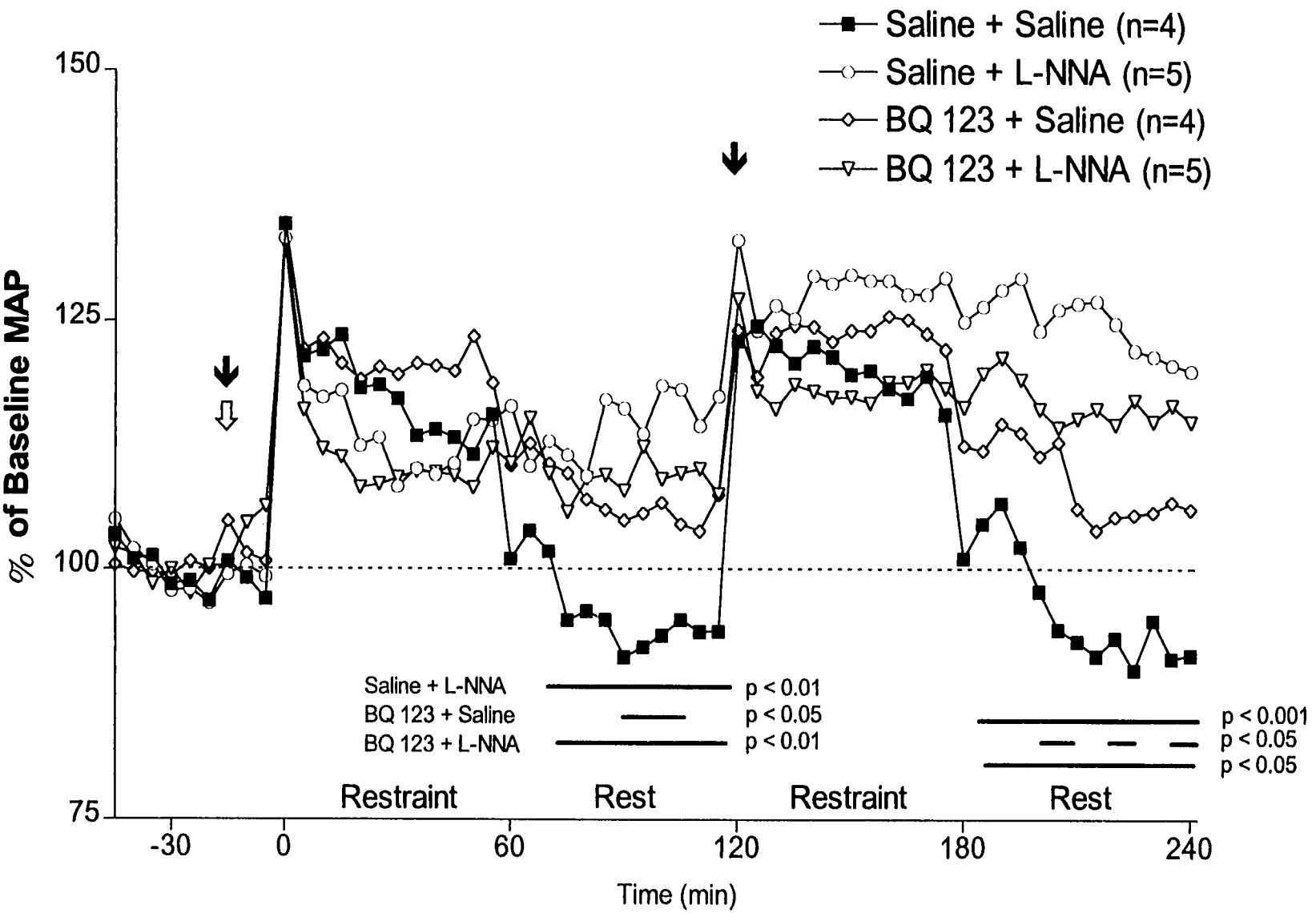

Saline + Saline (n=4)
Saline + L-NNA (n=5)
BQ 123 + Saline (n=4)
BQ 123 + L-NNA (n=5)
% of Baseline MAP
150
125
100
75
-30
0
60
120
180
240
Time (min)
Restraint
Rest
Restraint
Rest
Saline + L-NNA
BQ 123 + Saline
BQ 123 + L-NNA
p < 0.01
p < 0.05
p < 0.01
p < 0.001
p < 0.05
p < 0.05

Figure 3.12.

CHAPTER 4

DISCUSSION

We have shown that the MAPs of rats receiving vehicle increased in response to restraint and decreased slightly during the hour of restraint. Blockade of central ET_A receptors with BQ 123 did not affect the initial increase in MAP in response to restraint but the MAPs in rats receiving BQ 123 were consistently but not significantly greater than controls during the hour of restraint. Additionally, we have shown that the MAPs of control rats were depressed below baseline levels during the recovery from restraint. Pre-treatment with BQ 123 prior to restraint attenuated the decrease in MAP. The MAPs in BQ 123-treated rats remained significantly higher than controls during the rest periods. In addition, we have shown that there was a decrease in NO content in the brainstems of BQ 123-treated rats following restraint. Furthermore, blocking of constitutive NOS with L-NNA virtually eliminated the decrease in MAP when animals were removed from restraint. Together, these results support the hypothesis that central ET_A receptors mediate the decrease in sympathetic output during restraint and recovery from restraint. In addition, these results also suggest a role for NO in mediating this response.

4.1. Technical Considerations

4.1.1. Selectivity of BQ 123 antagonism

BQ 123 is potent and reversible inhibitor of ET_A receptors. BQ 123 has been shown to selectively inhibit [^{125}I]-ET-1 binding to ET_A receptors in vitro (Ihara et al., 1992; Wu-Wong et al., 1995). On the other hand, BQ 123 had no effect on [^{125}I]-ET-1 binding to ET_B receptors (Ihara et al., 1992). In addition, Ihara et al. (1995) showed that

[^{3}H]-BQ 123 binding was sensitive to ET-1 and ET-2 while relatively insensitive to ET-3 further suggesting BQ 123 is selective for ET_A receptors

In vivo studies have shown that pre-treatment with BQ 123 inhibited the peripheral ET-1-induced pressor responses in a dose-dependent fashion without affecting the ET-1-induced depressor responses (Ihara et al., 1991). Furthermore, central administration of BQ 123 in anaesthetised rats inhibited the cardiovascular effects of i.c.v. administration of the ET_A selective agonist STX 6b (Kumar et al., 1997). Together, these observations suggest that BQ 123 selectively inhibits the ET_A receptor.

4.1.2. Measurement of nitric oxide production

The *ex vivo* measurement of NO_x has been shown to be a reliable method for the determination of constitutive NOS activity *in vivo* (Salter et al., 1996). Since NO is readily converted to nitrates (Rhodes et al., 1995) and nitrites (Ignarro et al., 1993) in biological fluids, the measurement of nitrates and nitrites (NO_x) is an indicator of total NO production. While this method does not allow for the specific localisation of areas involved in NO production due to homogenisation of tissue, we have shown a decrease in the NO production in the brainstem of rats receiving BQ 123 and subjected to restraint. Since the brainstem contains many groups of NO-producing neurons, further studies using *in situ* hybridisation for NOS mRNA would be advantageous to identify those neurons which alter NOS activity in response to BQ 123 and restraint.

We have used the NADPH-d histochemical stain to identify putative NO-producing neurons in the brain. This method identifies neurons containing nNOS

(Dawson et al., 1991; Hope et al., 1991), but gives no indication of NO production. For instance, although there were no differences in the numbers of NADPH-d positive neurons in the brainstem of rats receiving BQ 123 or vehicle, there was a decrease in NO production in rats receiving BQ 123. On the other hand, there were no differences in the numbers of NADPH-d positive neurons in the PVN or in the levels of NO production in the hypothalamus. Since the PVN is one of the major integrators of sympathetic output, it is surprising that the levels of NO_x in the hypothalamus of rats receiving vehicle were less than in the brainstems of these rats. The amount of tissue analysed for each area may account for this difference. Although consistent between animals, the hypothalamus tissue blocks were approximately one third the size of the brainstem tissue blocks.

4.2. Controls

We have confirmed that central administration of ET-1 elicits a pressor response in urethane-anaesthetised rats. Several studies have reported a pressor effect of centrally administered ET-1 in conscious rats (Sirén and Feuerstein, 1989; Ouchi et al., 1989; Makino et al., 1990) and a biphasic pressor/depressor response in anaesthetised rats (Gulati et al., 1995, 1996, 1997a,b; Rebello et al., 1995). The absence of a depressor response in the present study following i.c.v. ET-1 using anaesthetised rats, however, may be due to the length of our observation period which was 30 min. Others have shown that the depressor response occurred at 30 min following ET-1 administration (Gulati et al., 1996, 1997a,b), at the time when we showed consistent but not significant decreases in MAP. Indeed, when observed for 25 min following i.c.v. ET-1 administration,

Nakamura *et al.* (1999) also did not detect a depressor effect. On the other hand, there are reports that ET-1 (i.c.v.) produced a pressor peak at 10 min and a sustained depressor response (Rebello et al., 1995).

The present study shows that central administration of BQ 123 alone increased MAP in urethane-anaesthetised animals. Others, on the other hand, have reported that BQ 123 does not alter basal BP levels in urethane-anaesthetised rats when administered i.c.v. (Gulati et al., 1997b; Nakamura et al., 1999), injected into the fourth ventricle (Tadepalli and Hashim, 1995), or microinjected into the raphe obscurus (D'Amico et al., 1999). Interestingly, BQ 123 microinjected into the NTS produced a brief increase followed by a decrease in BP and a return to baseline within 15 min (Mosqueda-Garcia et al., 1998). More importantly, however, we show that intracerebroventricular injections of BQ 123 in conscious animals did not alter MAP when compared to controls. This finding is in agreement with a study showing that i.c.v. BQ 123 did not alter the basal levels of sympathetic and cardiovascular activities in conscious Wistar-Kyoto (WKY) rats (Nakamura et al., 1999).

4.3. Fos immunohistochemistry, NADPH-d histochemistry and *in situ* hybridisation for CRF and TH mRNA in non-stressed rats

We observed a significant increase in the numbers of neurons with FLI in the LC of non-stressed rats treated with BQ 123. While the significance of this finding is unclear at the present time, it may suggest that ET_A receptors on LC neurons function to inhibit these neurons. Alternatively, this difference in the numbers of neurons with FLI may also

indicate that BQ 123 alters the signalling of upstream neurons resulting in the activation of neurons in the LC.

4.4. Fos immunohistochemistry, NADPH-d histochemistry and *in situ* hybridisation for CRF and TH mRNA in restraint-stressed rats

No significant differences in the numbers of neurons with FLI, NADPH-d, or double stained neurons were found between rats receiving BQ 123 or vehicle in any of the areas investigated in restraint-stressed animals. This finding is not surprising since c-*fos* is expressed rapidly in activated neurons (Müller et al., 1984; Krukoff, 1998b). In addition, an increase in MAP is known to increase c-*fos* expression (Li and Dampney, 1994; Krukoff et al., 1995). Since the MAPs in both BQ 123 and control rats increased equally at the onsets of restraint, the differences in MAPs seen during recovery from restraint may not be great enough to affect the number of activated neurons. As well, the protein product Fos, lasts for several hours (Müller et al., 1984; Herdegen and Leah, 1998; Krukoff, 1998b) which may obscure any differences in gene expression.

We observed a significant decrease in the expression of CRF mRNA in stressed rats treated with BQ 123 suggesting that ET has a stimulatory effect on CRF producing neurons in the PVN of restrained rats. While administration of ET-1 did not affect the basal release of CRF from hypothalamic explants (Yasin et al., 1994), ET-1 stimulated the basal ACTH secretion in rats (Malendowicz et al., 1997) which suggests that the *in vitro* effects of ET are different from their effects *in vivo*. Since administration of BQ 123 did not alter CRF mRNA levels in non-stressed animals, it is possible that ET only

augments CRF mRNA expression in parvocellular neurons of the PVN under heightened levels of sympathetic activation. Under these conditions, ET may act to enhance the level of HPA axis activation.

No significant differences in the levels of TH mRNA were found in either the LC or the NTS of restrained rats. As discussed previously, this finding is not surprising since the MAPs in BQ123 and control rats increased almost equally. It may be difficult to discern a difference in TH mRNA expression since transcription of mRNA may continue to be elevated even after the stimulus has been removed. For instance, immobilisation stress for 30 min or restraint stress for 1 h significantly increased the relative rate of TH transcription for several for hours (Smith et al., 1991; Nankova et al., 1999; Sands et al., 2000)

4.5. MAP during restraint stress and recovery

In response to restraint stress, sympathoadrenal system activation leads to increased heart rate and BP (Chen and Herbert, 1995; Irving et al., 1998). As demonstrated in the present study, the onset of restraint resulted in an increase in MAP in control rats. In another study, the MAPs in WKY rats also increased in response to restraint stress (McDougall et al., 2000). In both experiments, the MAPs tended to decrease slightly during both restraint sessions. This small reduction in MAP may indicate an unsuccessful attempt by the autonomic nervous system to return BP to baseline levels. However, the MAPs did not fully return to baseline during either hour of restraint suggesting continuous sympathetic activation during restraint.

To our knowledge, we are the first to measure the changes in MAP during the recovery from restraint stress. When animals were removed from restraint, MAPs quickly returned to baseline levels. Interestingly, as animals were allowed to rest, their MAPs decreased still further and levelled off below baseline levels during the hour of rest. This depressed MAP suggests that a compensatory decrease in sympathetic activity occurs during recovery from restraint.

4.6. Effect of BQ 123 during restraint stress and recovery

We have shown that the MAPs in both BQ 123-treated and control rats increased equally at the onset of restraint. As discussed in Section 1.4.4., it is currently believed that central ET increases sympathetic activity resulting in a pressor response which is followed by a decrease in sympathetic activity leading to the subsequent depressor response and that these responses are mediated by ET_A receptors. It is interesting then, that while BQ 123 abolishes the increase in MAP in response to i.c.v. ET-1, the blocking of central ET_A receptors does not affect the increase in MAP in response to restraint stress. This observation suggests that the increase in MAP, and presumably increase in sympathetic activation, at the onset of restraint stress is not mediated by ET_A receptors. An alternative explanation is that the inability of BQ 123 to affect the stress-induced pressor response may reflect the fact that a number of factors mediate sympathetic drive in response to restraint stress (McDougall et al., 2000).

Although the MAPs in BQ 123-treated and control rats increased equally, the physiological responses to increased sympathetic drive might be different in BQ 123-

treated rats. As discussed in Section 4.4., the MAPs in normal rats tend to decrease during restraint. Although not significantly different during the first restraint period, MAPs in BQ 123 rats remained higher than saline controls. Furthermore, by the end of the second period of restraint, the MAPs in BQ 123-treated rats were significantly higher than controls. These observations are in agreement with the notion that central ET mediates decreases in sympathetic activity (Gulati et al., 1997b). The blocking of ET_A receptors, thereby preventing the reduction in sympathetic drive would result in continual stimulation of the sympathetic nervous system. Constant sympathetic drive would then lead to the sustained elevation in MAPs during restraint seen in BQ 123 rats.

The decreased ability to reduce sympathetic activity in experimental rats was more apparent when the animals were removed from restraint and allowed to rest. In contrast to control rats where the resting MAPs decreased to below baseline, the MAPs in BQ 123-treated rats remained above baseline during this rest period. These results further suggest that ET_A receptors mediate the decrease in sympathetic drive during the recovery from restraint stress.

4.7. The depressor effect of central administration of ET-1

Several studies have examined the depressor response of centrally administered ET-1 in anaesthetised rats. Low doses of ET-1 applied to the fourth ventricle (IV^{th}) of ventilated rats (Hashim and Tadepalli, 1992,1994; Tadepalli and Hashim, 1995;), administered intracisternally (i.c.) (Mosqueda-Garcia et al., 1992), or administered intrathecally (Han et al., 1991) produced decreases in BP and HR; however, at higher

doses, the depressor response to ET-1 (IV^{th}) has also been shown to be preceded by a brief pressor response (Hashim and Tadepalli, 1992). Microinjection of ET-1 into the NTS (Hashim and Tadepalli, 1992; Mosqueda-Garcia et al., 1992) or area postrema (AP) (Mosqueda-Garcia et al., 1992) led to decreases in BP and HR. On the other hand, others have shown that ET-1 microinjected into NTS (Dai et al., 1997) or AP (Ferguson and Smith, 1990) produces pressor responses. These discrepancies could be explained by the total volume in which ET-1 was microinjected (Mosqueda-Garcia et al., 1992). In the studies showing decreases in BP following NTS or AP microinjection, a maximum volume of 60 nl was microinjected. In contrast, the studies showing increases in BP used much larger volumes. Talman and Reis (1981) have previously reported administration of vehicle in large volumes caused non-specific cardiovascular effects in small nuclei such as NTS and AP. Furthermore, others have demonstrated that the same dose of a microinjected substance has different effects on BP and HR if the volume of administration is different (Barraco et al., 1987).

Interestingly, the depressor effect of IV^{th} administration of ET-1 was attenuated when the rats were pre-treated with two excitatory amino acid (EAA) antagonists (Hashim and Tadepalli, 1994). This observation suggests that the depressor effect of central ET-1 may be the result of release of an endogenous EAA (Hashim and Tadepalli, 1994). In fact, the NTS is sensitive to both ET-1 and the EAA agonist, L-glutamate (Talman et al., 1980; Di Paola et al., 1991; Shihara et al., 1998). In addition, L-glutamate also produces depressor responses when injected into the NTS (Di Paola et al., 1991; Talman et al., 1980). Furthermore, the depressor response of L-glutamate has been shown

to be due to release of NO from the NTS (Di Paola et al., 1991). These observations suggest that the endothelin-induced decrease in sympathetic activity is mediated through the release of L-glutamate to increase NO production in the NTS.

4.8. Effect of BQ 123 and restraint stress on NO production in hypothalamus and brainstem

If our contention is correct, and the decrease in sympathetic drive seen in control animals during recovery from restraint is due to an ET_A receptor-mediated release of NO in the brainstem, then by blocking ET_A receptors, we would expect to see less NO in the brainstems of rats treated with BQ 123 following restraint. Indeed, we observed a significant decrease in the NO content in the brainstems of rats treated with BQ 123.

While we did not observe any significant differences in the numbers of activated putative NO-producing neurons in the NTS or VLM between experimental and control rats (see Section 4.3.), we cannot determine the level of NOS activity within those neurons using the NADPH-d histochemical marker. Blockade of ET_A receptors may alter NOS activity within neurons without affecting the number of activated NO-producing numbers. For instance, ET is known to mediate its cellular signalling through an increase in intracellular Ca^{+2} levels (Blomstrand et al., 1999). In addition, both constitutive forms of NOS are Ca^{+2}-dependent (Moncada, 1992; Förstermann et al., 1991,1995). Thus, by blocking ET signalling, the levels of intracellular Ca^{+2} may not be sufficient to sustain increased levels of NOS activity. Additionally, it is also possible that other areas of the brainstem may be involved in producing the different levels of NO seen in the present

study. Quantification for the numbers of neurons with FLI immunohistochemistry and NADPH-d histochemistry was limited to the NTS, VLM, and LC of the brainstem, whereas the tissue homogenate used in the NO_x assay included the entire brainstem.

In addition, the NO_x assay does not identify the source of NO. The decrease in NO in the brainstems of BQ 123-treated rats following restraint could also be due to decreased NO production from eNOS in endothelial cells. While much of the literature suggests that ET_B receptors are responsible for the release of NO from endothelial cells (Clozel et al., 1992; Hori et al., 1992; Hirata et al., 1993; Moreland et al., 1994; Sudjarwo et al., 1994; Mazzoni et al., 1999; Schmeck et al., 1999), one study has demonstrated that activation of ET_A receptors led to the release of NO from the bronchial epithelium (Naline et al., 1999). It is possible then, that brain endothelial cells mediate NO release via ET_A receptor activation and that the administration of BQ 123 decreases NO production from eNOS in these cells during recovery from restraint.

4.9. Effect of BQ 123 and L-NNA during restraint and recovery

To further investigate the role of NO in mediating the depressor response seen during recovery from restraint, we centrally blocked constitutive NOS with L-NNA. L-NNA has been shown to effectively inhibit NO production in the brain for four hours if 2 i.c.v. injections are made two hours apart (Yang and Krukoff, 2000).

The responses of Saline + Saline and BQ 123 + Saline rats were similar to those discussed in Sections 4.4. and 4.5., respectively. We now show that the MAPs in Saline + L-NNA and BQ 123 + L-NNA rats at the onset of, and during restraint, were similar to

their respective controls. L-NNA, however, eliminated the decrease in MAP seen in control animals when these animals were removed from restraint.

The results of the present study do not distinguish whether nNOS and/or eNOS is/are responsible for the production of NO which may mediate the decreased sympathetic activity seen during recovery from restraint. Since the MAPs in BQ 123 + Saline rats were intermediate between the MAPs of L-NNA treated and control rats, it is possible that both isoforms of NOS were involved in the decrease in sympathetic activity and that BQ 123 prevented NO production from only one of the isoforms. Indeed, blocking both nNOS and eNOS with L-NNA virtually eliminates the decrease in MAP when animals are removed from restraint. While we cannot conclusively determine which isoform of NOS is responsible for the ET-mediated production of NO, it is interesting to speculate a role for each isoform. As discussed previously, there is little or no evidence to suggest that endothelial cells contain ET_A receptors so the administration of BQ 123 would not affect NO production from eNOS in these cells. Therefore NO from eNOS could account for the initial decrease in MAP seen in BQ 123 + Saline rats. On the other hand, ET_A receptors have been shown to be present on neurons within many autonomic nuclei (Kurokawa et al., 1997; Yamada and Kurokawa, 1998). Thus, BQ 123 may block NO production from nNOS in these areas, preventing the decrease in sympathetic activity seen in control rats.

It is believed that NO regulates sympathetic output from the brain (Krukoff, 1998a; Krukoff, 1999). Psychological stress has been shown to activate NO-producing neurons in brain centres (Krukoff, 1999) including the PVN, raphe nuclei, NTS, and

VLM (Krukoff and Khalili, 1997). Although the present study did not reveal any significant differences in the number of activated NO-producing neurons in BQ 123 or control rats following restraint, the decrease in brainstem NO content and greater MAPs in seen in BQ 123 rats compared to control rats further suggest a role for NO in decreasing sympathetic activity during recovery from restraint.

Summary and Conclusions

To date, the literature indicates that centrally administered ET-1 acts on ET_A receptors in the brain to elicit both increases and decreases in sympathetic output. The results of the present study support the notion that ET_A receptors mediate a decrease in sympathetic activity and extend this idea by showing that endogenous ET decreases sympathetic activity during recovery from restraint through the ET_A receptor. We have shown that the MAPs of control rats fell below baseline levels during recovery from restraint. In contrast, the MAPs in BQ 123-treated rats remained significantly higher than in controls during the rest periods. In addition, we show that there was a decrease in NO content in the brainstems of BQ 123-treated rats following restraint. Furthermore, blocking of constitutive NOS with L-NNA eliminated the decrease in MAP when animals were removed from restraint and suggest that ET acts to decrease sympathetic activity through the central release of NO.

Figure 4 illustrates a proposed mechanism for endothelin-mediated decrease in sympathetic output. Based on our results, we propose that under heightened levels of sympathetic drive (restraint stress), endothelin in the brain acts on ET_A receptors in

autonomic centres to decrease sympathetic output possibly through the release of nitric oxide from the brainstem.

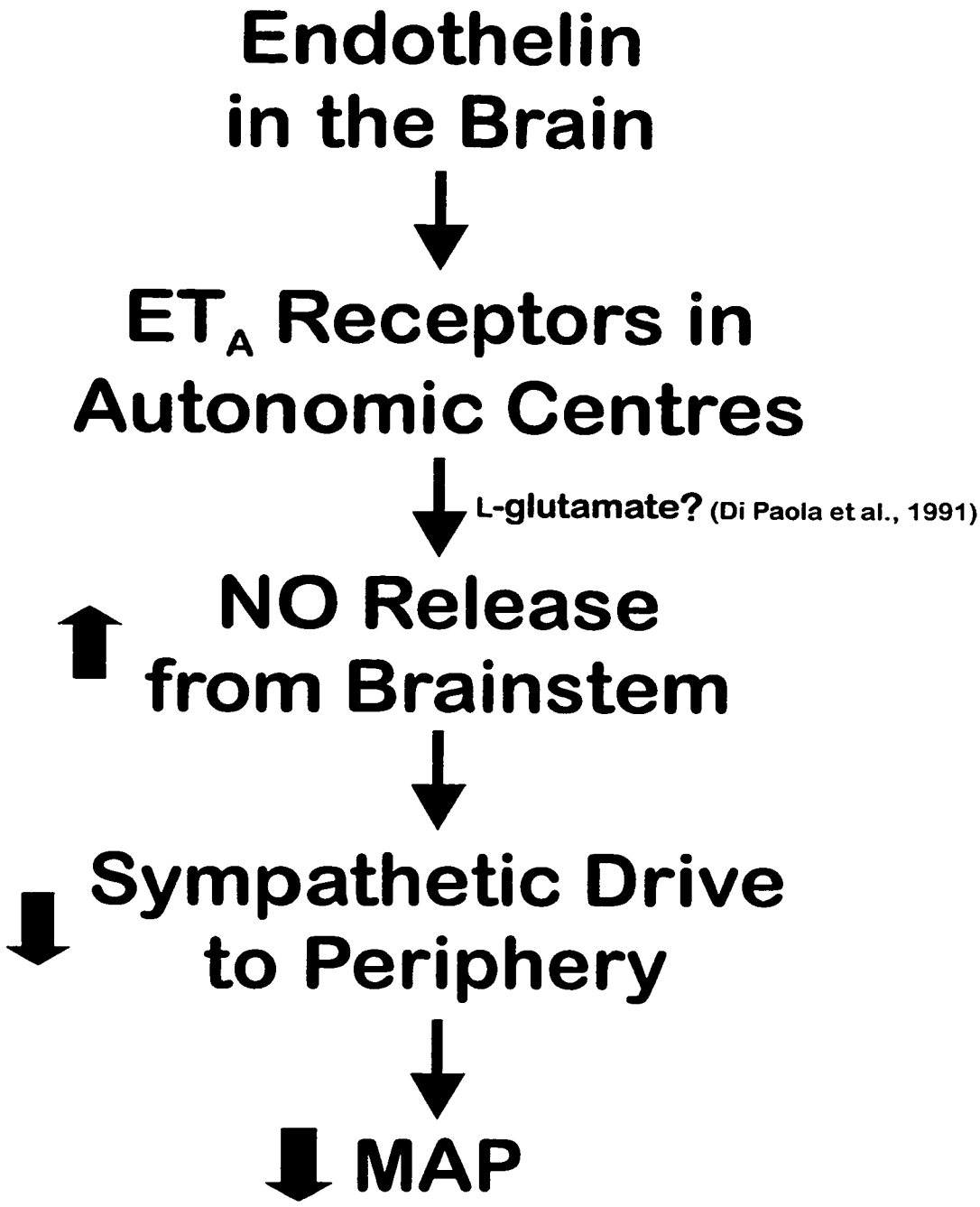


Figure 4. Schematic diagram representing hypothesised mechanism for endothelin-mediated decrease in sympathetic output to the periphery

References

Arai, H., Hori, S., Aramori, I., Ohkubo, H., and Nakanishi, S. 1990. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* **348(20):** 730-732.

Barraco, R., Polasek, P., Janusz, G., Campbell, W., and Schoener, E. 1987. Parameters affecting microinjections of adenosine into brainstem of rats. *Med Sci Res* **15:** 797-799.

Barron, B. and Van Loon, G. 1989. Role of sympathoadrenomedullary system in cardiovascular response to stress in rats. *J Auton Nerv Syst* **28:**179-188.

Benarroch, E. 1997. Central autonomic network and response to emotion and stress. In Central autonomic network: functional organization and clinical correlations. Futura Publishing Company Inc., New York. 373-404.

Blomstrand, F., Giaume, C., Hansson, E., and Rönnbäck, L. 1999. Distinct pharmacological properties of ET-1 and ET-3 on astroglial gap junctions and Ca^{+2} signaling. *Am J Physiol* **277(Cell Physiol. 46):** C616-C627.

Calogero, A. 1995. Neurotransmitter regulation of the hypothalamic corticotropin-releasing hormone neuron. *Ann NY Acad Sci* **771:** 31-40.

Calogero, A., Raiti, F., Burrello, N., D'Agata, R., and Mantero, F. 1994. Effects of endothelin-1 and endothelin-3 on rat hypothalamic corticotropin-releasing hormone and pituitary ACTH release *in vitro*. *J Endocrinol* **140:** 419-424.

Chan, R. and Sawchenko, P. 1995. Hemodynamic regulation of tyrosine hydroxylase messenger RNA in medullary catecholamine neurons: A c-*fos*-guided hybridization histochemical study. *Neurosci* **66(2):** 377-390.

Chen, X. and Herbert, J. 1995. Regional changes in c-*fos* expression in the basal forebrain and brainstem during adaptation to repeated stress: correlation with the cardiovascular, hypothermic and endocrine responses. *Neurosci* **64:**675-685.

Chew, B., Weaver, D., and Gross, P. 1994. Dose-related potent brain stimulation by the neuropeptide endothelin-1 after intraventricular administration in conscious rats. *Pharmacol Biochem Behav* **51(1):** 37-47.

Clozel, M., Gray, G., Breu, V., Löffler, B.-M., and Osterwalder, R. 1992. The endothelin ET_B receptor mediates both vasodilation and vasoconstriction *in vivo*. *Biochem Biophys Res Comm* **186(2):** 867-873.

Clozel, M. and Watanabe, H. 1993. BQ-123, a peptidic endothelin ET_A receptor antagonist, prevents the early cerebral vasospasm following subarachnoid hemorrhage after intracisternal but not intravenous injection. *Life Sci* **52:** 825-834.

Curran, T. and Franza, B., Jr. 1988. Fos and Jun: the AP-1 connection. *Cell* **55:** 395-397.

Curran, T. and Morgan, J. 1995. Fos: an immediate-early transcription factor in neurons. *J Neurobiol* **26:**403-412.

D'Amico, M., Di Filippo, C., Esposito, F., Rossi, F., and Masaki, T. 1999. Injection of endothelin-1 into the raphe obscurus of rats induces depressor responses predominantly through endothelin$_A$ receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* **359:** 471-476.

Dai, S.-M., Shan, Z.-Z., Miao, C.-Y., Yin, M., and Su, D.-F. 1997. Hemodynamic responses to endothelin-1 and endothelin antagonists microinjected into the nucleus tractus solitarius in rats. *J Cardio Pharmacol* **30:** 475-480.

Dawson, T., Bredt, D., Fotuhi, M., Hwang, P., and Snyer, S. 1991. Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc Natl Acad Sci USA* **88:** 7791-7801.

Devinsky, O., Morrell, M., and Vogt, B. 1995. Contributions of anterior cingulate cortex to behaviour. *Brain* **118:** 279-306.

Di Paola, D., Vidal, M., and Nisticò,G. 1991. L-Glutamate evokes the release of an endothelium-derived relaxing factor-like substance from the rat nucleus tractus solitarius. *J Cardio Pharmacol* **17:**S269-S272.

Ehrenreich, H., Anderson, R., Ogino, Y., Rieckmann, P., Costa, T., Wood, G., Coligan, J., Kehrl, J., and Fauci, A. 1991. Selective autoregulation of endothelins in primary astrocyte cultures: endothelin receptor-mediated potentiation of endothelin-1 secretion. *New Biologist* **3:**135-141.

Ferguson, A. and Smith, P. 1990. Cardiovascular responses induced by endothelin microinjection into area postrema. *Reg Peptides* **27:** 75-85.

Förstermann, U., Gath, I., Schwarz, P., Closs, E., and Kleinert, H. 1995. Isoforms of nitric oxide synthase. *Biochem Pharmacol* **50:**1321-1332.

Förstermann, U., Schmidt, H., Pollock, J., Sheng, H., Mitchell, J., Warner, T., Nakane, M., and Murad, F. 1991. Isoforms of nitric oxide synthase. *Biochem Pharmacol* **42:**1849-1857.

Giaid, A., Gibson, S., Herrero, M., Gentleman, S., Legon, S., Yanagisawa, M., Masaki, T., Ibrahim, N., Roberts, G., Rossi, M., and Polak, J. 1991. Topographical localisation of endothelin mRNA and peptide immunoreactivity in neurones of the human brain. *Histochem* **95:** 303-314.

Grima, B., Lamouroux, A., Blanot, F., Faucon-Biguet, N., and Mallet, J. 1985. Complete coding sequence of rat tyrosine hydroxylase mRNA. *Proc Natl Acad Sci USA* **82:** 617-621.

Gross, P., Beninger, R., Shaver, S., Wainman, D., Espinosa, F., and Weaver, D. 1993. Metabolic and neuroanatomical correlates of barrel-rolling and oculoclonic convulsions induced by intraventricular endothelin-1: a novel peptidergic signaling mechanism in visuovestibular and oculomotor regulation? *Expt Brain Res* **95:** 397-408.

Grzanna, R. and Molliver, M. 1980. The locus coeruleus in the rat: an immunohistochemical delineation. *Neurosci* **5(21):** 40.

Gulati, A. 1991. Characteristics of endothelin binding sites in the spinal cord of spontaneously hypertensive rats. *Eu J Pharmacol* **204:** 287-293.

Gulati, A., Kumar, A., Morrison, S., and Shahani, B. 1997a. Effect of centrally administered endothelin agonists on systemic and regional blood circulation in the rat: Role of sympathetic nervous system. *Neuropep* **31(4):** 301-309.

Gulati, A., Kumar, A., and Shahani, B. 1996. Cardiovascular effects of centrally administered endothelin-1 and relationship to changes in cerebral blood flow. *Life Sci* **58:** 437-445.

Gulati, A., Rebello, S., and Kumar, A. 1997b. Role of sympathetic nervous system in cardiovascular effects of centrally administered endothelin-1 in rats. *Am J Physiol* **273(Heart Circ. Physiol. 42):** H1177-H1186.

Gulati, A., Rebello, S., Roy, S., and Saxena, P. 1995. Cardiovascular effects of centrally administered endothelin-1 in rats. *J Cardio Pharmacol* **26(Suppl. 3):** S244-S246.

Hama, H., Kasuya, Y., Sakurai, T., Yamada, G., Suzuki, N., Masaki, T., and Goto, K. 1997. Role of endothelin-1 in astrocyte responses after acute brain damage. *J Neurosci Res* **47:**590-592.

Han, S.-P., Chen, H., Westfall, T., and Knuepfer, M. 1991. Characterization of the depressor effect of intrathecal endothelin in anaesthetized rats. *Am J Physiol* **260(Heart Circ. Physiol. 29):** H1685-H1691.

Harada, S., Tokunaga, S., Momhara, M., Masaki, H., Tagawa, T., Imaizumi, T., and Takeshita, A. 1993. Inhibition of nitric oxide formation in the nucleus tractus solitarius increases renal sympathetic nerve activity in rabbits. *Circ Res* **72:**511-516.

Hashim, M. and Tadepalli, A. 1992. Hemodynamic responses evoked by endothelin from central cardiovascular neural substrates. *Am J Physiol* **262(Heart Circ. Physiol. 31):** H1-H9.

Hashim, M. and Tadepalli, A. 1994. Hypotension to central endothelin-1: effect of glutamate receptor blockade. *Biochem Biophys Res Commun* **205(1):** 795-801.

Herdegen, T., Kovary, K., Buhl, A., Bravo, R., Zimmermann, M., and Gass, P. 1995. Basal expression of the inducible transcription factors c-*jun*, JunB, JunD, c-*fos*, FosB, and Krox-24 in the adult rat brain. *J Comp Neurol* **354:** 39-56.

Herdegen, T. and Leah, J. 1998. Inducible and constitutive transtription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res Rev* **28(3):** 370-490.

Hickey, K., Rubanyi, G., Paul, R., and Highsmith, R. 1985. Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *Am J Physiol* **248(Cell Physiology 17):** C550-C566.

Hirata, Y., Emori, T., Eguchi, S., Kanno, K., Imai, T., Ohta, K., and Marumo,F. 1993. Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *J Clin Invest* **91:**1367-1373.

Holstege, G. 1990. Subcortical limbic system projections to caudal brainstem and spinal cord. In The human nervous system. G.Paxinos, editor. Academic Press, San Diego. 261-286.

Hope, B., Michael, G., Knigge, K., and Vincent, S. 1991. Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc Natl Acad Sci USA* **88:** 2811-2814.

Hori, S., Komatsu, Y., Shigemoto, R., Mizuno, N., and Nakanishi, S. 1992. Distinct tissue distribution and cellular localization of the messenger ribonucleic acids encoding different subtypes of rat endothelin receptors. *Endocrinol* **130:** 1885-1895.

Hurley, K., Herbert, H., Moga, M., and Saper, C. 1991. Efferent projections of the infralimbic cortex of the rat. *J Comp Neurol* **308:** 249-276.

Ignarro, L.J., Fukuto, J.M., Griscavage, J.M., Rogers, N.E., and Byrns, R.E. 1993. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrite – comparison

with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci* **90:** 8103-8107.

Ihara, M., Ishikawa, K., Fukuroda, T., Saeki, T., Funabasi, K., Fukami, T., Suda, H., and Yano, M. 1992. *In vitro* biological profile of a highly potent novel endothelin (ET) antagonist BQ-123 selective for the ET_A receptor. *J Cardio Physiol* **20(Suppl. 12):** S11-S14.

Ihara, M., Yamanaka, R., Ohwaki, K., Ozaki, S., Fukami, T., Ishikawa, K., Towers, P., and Yano, M. 1995. [^{3}H]BQ-123, a highly specific and reversible radioligand for the endothelin ET_A receptor subtype. *Eu J Pharmacol* **274:** 1-6.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., and Masaki, T. 1989. The human endothelin family: Three structurally and pharmacologically distinct isopeptides by three separate genes. *Proc Natl Acad Sci USA* **86:** 2863-2867.

Irving, R., White, J., and Chan, R. 1998. The influence of restraint on blood pressure in the rat. *J Pharmacol Toxicol Meth* **38:**157-162.

Ishikawa, K., Ihara, M., Noguchi, K., Mase, T., Mino, N., Saeki, T., Fukuroda, T., Fukami, T., Ozaki, S., Nagase, T., Nishikibe, M., and Yano, M. 1994. Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proc Natl Acad Sci USA* **91:** 4892-4896.

Jiang, M., Höög, A., Ma, K.-C., Nie, X., Olsson, Y., and Zhang, W. 1993. Endothelin-1-like immunoreactivity is expressed in human reactive astrocytes. *Neuroreport* **4:**935-37.

Jones, C., Hiley, C., Pelton, J., and Mohr, M. 1989. Autoradiographic visualization of the binding sites for [^{125}I]endothelin in rat and human brain. *Neurosci Lett* **97:** 276-279.

Kadowaki, K., Kishimoto, J., Leng. G, and Emson, P.C. 1994. Up-regulation of nitric oxide synthase (NOS) gen expression together with NOS activity in the rat hypothalamo-hypophysial system after chronic salt loading: evidence of a neuromodulatory role of nitric oxide on arginine vasopressin and oxytocin secretion. *Endocrinol* **134:** 1011-1017.

Kagiyama, S., Tsuchihashi, Y., Abe, I., and Fujishima, M. 1997. Cardiovascular effects of nitric oxide in the rostral ventrolateral medulla of rats. *Brain Res* **757:**155-158.

Karaki, H., Sudjarwo, S., and Hori, M. 1994. Novel antagonist of endothelin ET_{B1} and ET_{B2} receptors, BQ-788: Effects on blood vessel and small intestine. *Biochem Biophys Res Comm* **205(1):** 168-173.

Karne, S., Jayawickreme, C., and Lerner, M. 1993. Cloning and characterization of an endothelin-3 specific receptor (ET_c receptor) from *Xenopus laevis* dermal melanophores. *J Biol Chem* **268:**19129-19133.

Kanyicska, B. and Freeman, M.E. 1993. Characterization of endothelin receptors in the anterior pituitary gland. *Am J Physiol* **256(Endocrinol. Metab.):** E601-E608.

Kloog, Y. and Sokolovsky, M. 1989. Similarities in mode and sites of action of sarafotoxins and endothelins. *TiPS* **10:**212-214.

Korf, J., Aghajanian, G., and Roth, R. 1973. Increased turnover of norepinephrine in the rat cerebral cortex during stress: role of the locus coeruleus. *Neuropharmacol* **12:**933-938.

Koseki, C., Imai, M., Hirata, Y., Yanagisawa, M., and Masaki, T. 1989a Autoradiographic distribution in rat tissues of binding sites for endothelin: A neuropeptide?. *Am J Physiol* **256(Reg. Int. Comp. Physiol. 25):** R858-R866.

Koseki, C., Imai, M., Hirata, Y., Yanagisawa, M., and Masaki, T. 1989b. Binding sites for endothelin-1 in rat tissues: An autoradiographic study. *J Cardio Physiol* **13(Suppl. 5):** S153-S154.

Krukoff, T. 1998b. c-*fos* expression as a marker of functional activity in the brain. In Neuromethods. A.Boulton, Baker,G., and Bateson,A., editors. Humana Press, Inc, 213-230.

Krukoff, T. 1998a. Central regulation of autonomic function: NO brakes? *Clin Exp Pharmacol Physiol* **25:** 474-478.

Krukoff, T. 1999. Central actions of nitric oxide in regulation of autonomic functions. *Brain Res Rev* **30:** 52-65.

Krukoff, T., Harris, K., Linetsky, E., and Jhamandas, J. 1994. Expression of c-*fos* protein in rat brain elicited by electrical and chemical stimulation of the hypothalamic paraventricular nucleus. *Neuroendocrinol* **59:** 590-602.

Krukoff, T. and Khalili, P. 1997. Stress-induced activation of nitric oxide-producing neurons in the rat brain. *J Comp Neurol* **377:** 509-519.

Krukoff, T., MacTavish, D., Harris, K., and Jhamandas, J. 1995. Changes in blood volume and pressure induce c-*fos* expression in brainstem neurons that project to the paraventricular nucleus of the hypothalamus. *Mole Brain Res* **34:** 99-108.

Krukoff, T., MacTavish, D., and Jhamandas, J. 1997. Activation by hypotension of neurons in the hypothalamic paraventricular nucleus that project to the brainstem. *J Comp Neurol* **385:** 285-296.

Krukoff, T., MacTavish, D., and Jhamandas, J. 1999. Hypertensive rats exhibit heightened expression of corticotropin-releasing factor in activated central neurons in response to restraint stress. *Mole Brain Res* **65:** 70-79.

Kumar, A., Morrison, S., and Gulati, A. 1997. Effect of ET_A receptor antagonists on cardiovascular responses induced by centrally administered sarafotoxin 6b: Role of sympathetic nervous system. *Peptides* **18(6):** 855-864.

Kumar, A., Shahani, B., and Gulati, A. 1996. Modification of systemic and regional circulatory effects of intracerebroventricular administration of endothelin-1 by propranolol in anesthetized rats. *Gen Pharmacol* **27(6):** 1025-1033.

Kumar, C., Mwangi, W., Nuthulaganti, P., Wu, H.-L., Pullen, M., Brun, K., Aiyar, H., Morris, R., Naughton, R., and Nambi, P. 1994. Cloning and characterization of a novel endothelin receptor from *Xenopus* heart. *J Biol Chem* **269:**13414.

Kumar, C., Nuthulaganti, P., Pullen, M., and Nambi, P. 1993. Novel endothelin receptors in the follicular membranes of *Xenopus laevis* oocytes mediate calcium responses by signal transduction through gap junctions. *Mol Pharmacol* **44:**153-157.

Kurokawa, K., Yamada, H., and Ochi, J. 1997. Topograpical distribution of neurons containing endothelin type A receptor in the rat brain. *J Comp Neurol* **389:** 349-360.

Kuwaki, T., Cao, W., and Kumada, M. 1994. Endothelin in the brain and its effect on central control by the circulation and other functions. *Jap J Physiol* **44:** 1-18.

Kuwaki, T., Kurihara, H., Cao, W., Kurihara, Y., Unekawa, M., Yazaki, Y., and Kumada, M. 1997. Physiological role of brain endothelin in the central autonomic control: from neuron to knockout mouse. *Prog Neurobiol* **51:** 545-579.

Kvetnanský, R., Pacák, K., Kukuhara, K., Viskupic, E., Hiremagalur, B., Nankova, B., Goldstein, D., and Kopin, I. 1995. Sympathoadrenal system in stress. *Ann NY Acad Sci* **771:**131-158.

Lee, M.-E., de la Monte, S. M., Ng, S.-C., Bloch, K. D., and Quertermous, T. 1990. Expression of the potent vasoconstrictor endothelin in the human central nervous system. *J Clin Invest* **86:** 141-147.

Lee, W., Lin, C., and Curran, T. 1988. Activation of the transforming potential of the human *fos* proto-oncogene requires message stabilization and results in increased amounts of partially modified *fos* protein. *Mol Cell Biol* **8:**5521-5527.

Li, Y.-W. and Dampney, R. 1994. Exrpession of Fos-like protein in brain following sustained hypertension and hypotension in conscious rabbits. *Neurosci* **61:**613-634.

Liozou, L. 1969. Projections of the nucleus locus coeruleus in the albino rat. *Brain Res* **15:** 563-566.

MacCumber, M., Ross, C., and Snyder, S. 1990. Endothelin in brain: receptors, mitogenesis, and biosynthesis in glial cells. *Proc Natl Acad Sci USA* **87:**2359-2363.

Makino, S., Hashimoto, K., Hirasawa, R., and Hattori, T. 1990. Central interaction between endothelin and brain natriuretic peptide on pressor and hormonal responses. *Brain Res* **534:** 117-121.

Malendowicz, L., Nussdorfer, G., Meneghelli, V., Nowak, M., Markowska, A., and Majchrzak, M. 1997. Effects of endothelin-1 on the rat pituitary-adrenocortical axis under basal and stressful conditions. *Endo Res* **23(4):** 349-364.

Masaki, T., Vane, J., and Vanhoutte, P. V. 1994. International union of pharmacology nomenclature of endothelin receptors. *Pharmacol Rev* **46(2):** 137-142.

Mazzoni, M., Breschi, M., Ceccarelli, F., Lazzeri, N., Guisti, L., Nieri, P., and Lucacchini, A. 1999. Suc-[Glu^{9},$Ala^{11,15}$]-endothelin-1 (8-21), IRL 1620, identifies two populations of ET_B receptors in guinea-pig bronchus. *Br J Pharmacol* **127:** 1406-1414.

McDougall, S., Paull, J., Widdop, R., and Lawrence, A. 2000. Restraint stress: differential cardiovascular responses in Wistar-Kyoto and spontaneously hypertensive rats. *Hypertens* **35:**126-129.

Moncada, S. The L-arginine:nitric oxide pathway. 1992. *Acta Physiol Scand* **145:** 201-227.

Moreland, S., McMullen, D., Abboa-Offei, B., and Seymour, A. 1994. Evidence for a differential location of vasoconstrictor endothelin receptors in the vasculature. *Br J Pharmacol* **112:** 704-708.

Mosqueda-Garcia, R., Appalsamy, M., Fernandez-Violante, R., and Hamakubo, T. 1998. Modulatory effects of endothelin on baroreflex activation in the nucleus of the solitary tract. *Eur J Pharmacol* **351:** 203-207.

Mosqueda-Garcia, R., Inagami, T., Appalsamy, M., Sugiura, M., and Robertson, R. 1992. Endothelin as a neuropeptide - Cardiovascular effects in the brainstem of normotensive rats. *Circ Res* **72:** 20-35.

Mosqueda-Garcia, R., Stainback, R., Fernandez-Violante, R., and Hamakubo, T. 1995. Cardiovascular effects of endothelin injected into brain nuclei regulating vasopressin release. *J Cardio Pharmacol* **26(Suppl. 3):** S159-S162.

Müller, R., Bravo, R., Burchkhardt, J., and Curran, T. 1984. Induction of c-*fos* gene and protein by growth factors preceeds activation of c-*myc*. *Nature* **312:** 716-720.

Nagasaka, J., Tsuji, M., Takeda, H., and Matsumiya, T. 1999. Role of endothelin receptor subtypes in the behavioral effects of the intracerebroventricular administration of endothelin in conscious rats. *Pharmacol Biochem Behavior* **64(1):** 171-176.

Nakajima, M., Kubo, S., Kumagaye, S., Nishio, H., Tsunemi, M., Inui, T., Kuroda, H., Chino,N., Watanabe,T., Kimura,T. *et al.* 1989. Structure-activity relationship of endothelin: importance of charged groups. *Biochem Biophys Res Comm* **163:**424-429.

Nakamura, K., Sasaki, S., Moriguchi, J., Morimoto, S., Miki, S., Kawa, T., Itoh, H., Nakata, T., Takeda, K., and Nakagawa, M. 1999. Central effects of endothelin and its antagonists on sympathetic and cardiovascular regulation in SHR-SP. *J Cardio Pharmacol* **33:** 876-882.

Naline, E., Bertrand, C., Biyah, K., Fujitani, Y., Okada, T., Bisson, A., and Advenier, C. 1999. Modulation of ET-1 induced contraction of human bronchi by airway epithelium-dependent nitric oxide release via ET_A receptor activation. *Br J Pharmacol* **126:** 529-535.

Nankova, B., Tank, A., and Sabban, E. 1999. Transient or sustained transcriptional activation of the genes encoding rat adrenomedullary catecholamine biosynthetic enzymes by different durations of immobilization stress. *Neurosci* **94(1):** 803-808.

Nathan, C. 1992. Nitric oxide is a secretory product of mammalian cells. *FASEB Journal* **6:**3051-3064.

O'Brien, R., Robbins, R., and McMurtry, I. 1987. Endothelial cells in culture produce a vasoconstrictor substance. *J Cell Physiol* **132:** 263-270.

Ogawa, Y., Nakao, K., Arai, H., Nakagawa, O., Hosoda, K., Suga, S.-I., Nakanishi, S., and Imura, H. 1991. Molecular cloning of a non-isopeptide-selective human endothelin receptor. *Biochem Biophys Res Comm* **178(1):** 248-255.

Ohlstein, E., Elliott, J., Feuerstein, G., and Ruffolo, R., Jr. 1996. Endothelin receptors: receptor classification, novel receptor antagonists, and potential therapeutic targets. *Med Res Rev* **16(4):** 365-390.

Ouchi, Y., Kim, S., Souza, A., Iijima, S., Hattori, A., Orimo, H., Yoshizumi, M., and Kurihara, H. 1989. Central effect of endothelin on blood pressure in conscious rats. *Am J Physiol* **256(Heart Circ. Physiol. 25):** H1747-H1751.

Pacák, K., Palkovits, M., Kvetnanský, R., Yadid, G., Kopin, I., and Goldstein, D. 1995. Effects of Various stressors on *in vivo* norepinephrine release in the hypothalamic

paraventricular nucleus and on the pituitary adrenocortical axis. *Ann NY Acad Sci* **771:** 115-130.

Pontieri, V., Venezuela, M.K., Scavone, C., and Michelini, L.C. 1998. Role of endogenous nitric oxide in the nucleus tractus solitarii on baroreflex control of heart rate in spontaneously hypertensive rats. *J Hypertens* **16(12 Pt. 2):** 1993-1999.

Rebello, S., Roy, S., Saxena, P., and Gulati, A. 1995. Systemic hemodynamic and regional circulatory effects of centrally administered endothelin-1 are mediated through ET_A receptors. *Brain Res* **676:** 141-150.

Renner, U., Pagotto, U., Arzt, E., and Stalla, G.K. 1996. Autocrine and paracrine roles of polypeptide growth factors, cytokines and vasogenic substances in normal and tumorous pituitary function and growth: a review. *Eur J Endocrinol* **135:** 515-532.

Rhodes, P.M., Leone, A.M., Francis, P.L., Struthers, A.D., and Moncada, S. 1995. The L-arginine-nitric oxide pathway is the major source of plasma nitrite in fasted humans. *Biochem Biophys Res Commun* **209:** 590-596.

Rossi, N., O'Leary, D., and Chen, H. 1997. Mechanisms of centrally administered ET-1-induced increases in systemic arterial pressure and AVP secretion. *Am J Physiol* **272(Endocrinol. Metab. 35):** E126-E132.

Rubanyi, G. and Polokoff, M. 1994. Endothelins: Molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* **46(3):** 325-414.

Sabban, E., Hiremagalur, B., Nankova, B., and Kvetnanský, R. 1995. Molecular biology of stress-elicited induction of catecholamine biosynthetic enzymes. *Ann NY Acad Sci* **771:**327-338.

Saeki, T., Ihara, M., Fukuroda, T., Yanagisawa, M., and Yano, M. 1991. [$Ala^{1,3,11,15}$]Endothelin analogs with ET_B agonistic activity. *Biochem Biophys Res Comm* **179:**286-292.

Sakuma, I., Togashi, H., Yoshioka, M., Saito, H., Yanagida, M., Tamura, M., Kobayashi, T., Yasuda, H., Gross, S., and Levi, R. 1992. N^G-methyl-L-arginine, an inhibitor of L-arginine-derived nitric oxide synthesis, stimulates renal sympathetic nerve activity *in vivo*. *Circ Res* **70:**607-611.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., and Masaki, T. 1990. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **348(20):** 732-735.

Salter, M., Duffy, C., Garthwaite, J., and Strijbos, P. 1996. *Ex vivo* measurement of brain tissue nitrate and nitrite accurately reflects nitric oxide synthase activity *in vivo*. *J Neurochem* **66:**1683-1690.

Samson, W. and Murphy, T. 1993. Antidipsogenic actions of endothelins are exerted via the endothelin-A receptor in the brain. *Am J Physiol* **265(Reg. Int. Comp. Physiol. 34):** R1212-R1215.

Samson, W., Skala, K., Huang, S., Gluntz, S., Alexander, B., and Gómez-Sánchez, C. 1991. Central nervous system action of endothelin-3 to inhibit water drinking in the rat. *Brain Res* **539:** 347-351.

Samson, W., Skala, K., Alexander, B., and Huang, F.-L. Pituitary site of action of endothelin: selective inhibition of prolactin release *in vitro*. 1990. *Biochem Biophys Res Commun* **169(2):** 737-743.

Sands,S., Strong, R., Corbitt, J., and Morilak, D. 2000. Effects of acute restraint stress on tyrosine hydroxylase mRNA expression in locus coeruleus of Wistar and Wistar-Kyoto rats. *Mole Brain Res* **75:**1-7.

Scherer-Singler, U., Vincent, S., Kimura, H., and McGeer, E. 1983. Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. *J Neurosci Meth* **9(3):** 229-234.

Schmeck, J., Gluth, H., Mihaljevic, N., Born, M., Wendel-Wellner, M., and Krafft, P. 1999. ET-1-induced pulmonary vasoconstriction shifts from ET_A- to ET_B-receptor mediated reaction after preconstriction. *J Appl Physiol* **87:**2284-2289.

Schwartz, J. and Cherny, R. 1992. Intercellular communication within the anterior pituitary influencing the secretion of hypophysial hormones. *Endo Rev* **13(3):** 453-475.

Serova, L., Nankova, B., Feng, Z., Hong, J., Hutt, M., and Sabban, E. 1999. Heightened transcription for enzymes involved in norepinephrine biosynthesis in the rat locus coeruleus by immobilization stress. *Biol Psych* **45:**853-862.

Shibata, S., Himeno, A., Shigematsu, K., Tsutsumi, K., Sakurai-Yamashita, Y., Yamashita, K. 1997a. Endothelin Receptors in Rat Pituitary Gland. *Cell Mol Neurobiol* **17:**89-100.

Shibata, S., Niwa, M., Himeno, A., Gana, N., Shigematsu, K., Matsumoto, M., Yamashita, K., Sumikawa, K., and Taniyama, K. 1997b. The endothelin ET_A receptor exists in the caudal solitary tract nucleus of the rat brain. *Cell Mol Neurobiol* **17:**151-156.

Shihara, M., Hirooka, Y., Hori, N., Matsuo, I., Tagawa, T., Suzuki, S., Akaike, N., and Takeshita, A. 1998. Endothelin-1 increases the neuronal activity and augments the responses to glutamate in the NTS. *Am J Physiol* **275(Reg. Int. Comp. Physiol. 44):** R658-R665.

Sirén, A. and Feuerstein, G. 1989. Hemodynamic effects of endothelin after systemic and central nervous system administration in the conscious rat. *Neuropep* **14:** 231-236.

Sluck, J., Lin, R., Katolik, L., Jeng, A., and Lehmann, J. 1999. Endothelin converting enzyme-1-, endothelin-1-, and endothelin-3-like immunoreactivity in the rat brain. *Neurosci* **91(4):** 1483-1497.

Smith, M., Bradly, L., Glowa, J., Gold, P.W., and Herkenham, M. 1991. Effect of stress and adrenalectomy on tyrosine hydroxylase mRNA levels in the locus ceruleus by *in situ* hybridization. *Brain Res* **544:**26-32.

Spyer, K. 1989. Neural mechanisms involved in cardiovascular control during affective behaviour. *Trend Neurosci* **12:** 506-513.

Spyer, K. 1994. Central nervous mechanisms contributing to cardiovascular control. *J Physiol* **474(1):** 1-19.

Stojilkovic, S.S., and Catt, K.J. 1992. Neuroendocrine actions of endothelins. *TiPS* **13(10):** 385-391.

Stratakis, C. and Chrousos, G. 1995. Neuroendocrinology and pathophysiology of the stress system. *Ann NY Acad Sci* **771:** 1-18.

Sudjarwo, S., Hori, M., Tanaka, T., Matsuda, Y., Okada, T., and Karaki, H. Subtypes of endothelin ET_A and ET_B receptors mediating venous smooth muscle contraction. 1994. *Biochem Biophys Res Comm* **200(1):** 327-633.

Swanson, L. and Hartman, B. 1976. The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent

connections in the rat utilizing dopamine-β-hydroxylase as a marker. *J Comp Neurol* **163:** 467-506.

Swanson, L. and Sawchenko, P. 1983. Hypothalamic integration: Organization of the paraventricular and supraoptic nuclei. *Ann Rev Neurosci* **6:** 269-324.

Tadepalli, A. and Hashim, M. 1995. Mechanisms of central endothelin-induced hypotension. *Naunyn-Schmiedeberg's Arch Pharmacol* **352:**108-112.

Talman, W., Perrone, M., and Reis, D. 1980. Evidence for L-glutamate as the neurotransmitter of baroreceptor afferent nerve fibers. *Science* **209:**813-814.

Talman, W. and Reis, D. 1981. Baroreflex actions of substance P microinjected into the nucleus tractus solitarii in rat: a consequence of local distortion. *Brain Res* **220:**402-407.

Togashi, H., Sakuma, I., Mitshiro, Y., Takeshi, K., Yasuda, H., Kitabatake, A., Saito, H., Gross, S., and Levi, R. 1992. A central nervous system action of nitric oxide in blood pressure regulation. *J Pharmacol Expt Ther* **262:**343-347.

Valentino, R., Foote, S., and Page, M. 1993. The locus coeruleus as a site for integrating corticotropin-releasing factor and noradrenergic mediation of stress responses. *Ann NY Acad Sci* **397:**173-188.

Vigne, P., Breittmayer, J., and Frelin, C. 1993. Competitive and non-competitive interactions of BQ-123 with endothelin ET_A receptors. *Eu J Pharmacol* **245:** 229-232.

Wang, Y.-X., Zhou, T., and Pang, C. 1991. Pressor effects of L and D enantiomers of N^G-nitro-arginine in conscious rats are antagonized by L- but not D-arginine. *Eur J Pharmacol* **200:** 77-81.

Williams, D., Jr., Jones, K., Pettibone, D., Lis, E., and Clineschmidt, B. 1991. Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. *Biochem Biophys Res Comm* **175:**556-561.

Wu-Wong, J.R., Chiou, W.J., Dixon, D.B., and Opgenorth, T.J. 1995. Dissociation characteristics of endothelin ET_A receptor agonists and antagonists. *J Cardio Pharmacol* **26(Suppl. 3):** S380-S384.

Yamada, H. and Kurokawa, K. 1998. Histochemical studies in endothelin and the endothelin-A receptor in the hypothalamus. *J Cardio Physiol* **31:**S215-S218.

Yamamoto, T., Kimura, T., Ota, K., Shoji, M., Inoue, M., Sato, K., Ohta, M., and Yoshinaga, K. Central effects of endothelin-1 on vasopressin release, blood pressure, and renal solute excretion. 1992. *Am J Physiol* **262(Endocrinol. Metab. 25):** E856-E862.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Misui, Y., Yazaki, Y., Goto, K., and Masaki, T. 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332:** 411-415.

Yang, W., Oskin, O., and Krukoff, T. 1999. Immune stress activates putative nitric oxide-producing neurons in the rat brain: Cumulative effects with restraint. *J Comp Neurol* **405:** 380-387.

Yang, W. and Krukoff, T. 2000. Nitric oxide regulates body temperature, neuronal activation and interleukin-1β gene expression in the hypothalamic paraventricular nucleus in response to immune stress. *Neuropharmacol* In press.

Yasin, S., Costa, A., Navarra, P., Pozzoli, G., Kostoglou-Athanassiou, I., Forsling, M., and Grossman, A. 1994. Endothelin-1 stimulates the *in vitro* release of neurohypophyseal hormones, but not corticotropin-releasing hormone via ET_A receptors. *Neuroendocrinol* **60:** 553-558.

Yoshizawa, T., Shinmi, O., Giaid, A., Yanagisawa, M., Gibson, S., Kimura, S., Uchiyama, Y., Polak, J., Masaki, T., and Kanazawa, I. 1990. Endothelin: a novel peptide in the posterior pituitary system. *Science* **247:**462-464.

Zhang, Z.G., Chopp, M., Gautam, S., Zaloga, C., Zhang, R.L., Schmidt, H.H.H.W., Pollock, J.S., and Förstermann, U. 1994. Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase containing neurons after focal cerebral ischemia in rat. *Brain Res* **654:** 85-95.