CHANGES IN CENTRAL AND PERIPHERAL REGULATION OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

ZURINA BINTI HASSAN

UNIVERSITI SAINS MALAYSIA

2012

CHANGES IN CENTRAL AND PERIPHERAL REGULATION OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

by

ZURINA BINTI HASSAN

Thesis submitted in fulfillment of the requirements

for the degree of

Doctor of Philosophy

April 2012

PERUBAHAN DALAM PENGAWALATURAN PUSAT DAN PERIFERI TIKUS DIABETIS TERARUH STREPTOZOTOSIN

oleh

ZURINA BINTI HASSAN

Tesis yang diserahkan untuk

memenuhi keperluan bagi

Doktor Falsafah

April 2012

ACKNOWLEDGEMENTS

First of all, I would like to thank Professor Munavvar Zubaid Abdul Sattar for allowing me to do this project. I am very grateful to him for all his excellent supervision, guidance and constant support towards the completion of my project and my thesis. Sincere thanks for the time he spent on reading and invaluable suggestions offered to improve the thesis.

I am also grateful to Associates Professor Dr Syed Azhar Syed Sulaiman (Dean of School of Pharmacy) for the facilities provided during the course of this study. My sincere thanks to Professor Ahmad Pauzi Md Yusof for his guidance and supervision in the project. I am indebted to Professor Mohd Zaini Asmawi and Dr Aidiahmad Dewa for their contribution.

I am also indebted to Nurul Hasnida and Farah Wahida for their excellent assistance and practical time they spent on teaching me the techniques in this project. I must single out three people in particular working together in physiological and pharmacology laboratory, Mr Roseli, Mr Farid and Mrs Yong. Many thanks and appreciation to the laboratory assistants and technicians who contributed to the success of this study.

Many thanks go to my family especially my children, Firdaus, aged 11 and Hafiz, aged 8 and Ellyana, aged 5 for their understanding, support and love all these years. To my husband, Khairil Faizi, the encouragement, motivation and helpful comments have been indispensable and made this time of writing pleasant and enjoyable, in spite of the pressures of the project.

Finally, many thanks to Ministry of Higher Education, Malaysia for financial assistance during my study.

TABLE OF CONTENTS

CHAPTER TWO: ROLE OF CENTRAL NERVOUS SYSTEM ON CARDIOVASCULAR REGULATION IN STZ-INDUCED DIABETIC RATS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABC	Avidin Biotin Complex
ADN	Aortic depressor nerve
ANOVA	Analysis of variance
ANS	Autonomic nervous system
ATP	Adenine triphosphate
AVP	Vasopressin
$CaCl_2$	Calcium chloride
$CaCl_2.2H_2O$	Calcium chloride dehydrate
cAMP	Cyclic adenine monophosphate
cGMP	Cyclic guanosine monophosphate
cm	centimetre
CNS	Central nervous system
CO	Cardiac output
CO_2	Carbon dioxide
CSN	Carotid sinus nerve
DAB	3, 3-diamino benzidine tetrahydrochloride
e.g.	Example
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelium nitric oxide synthase
et al.	et alii, others
g	gram
g/kg b.w.	gram per kilogram body weight
g/L	gram per litre
GABA	gamma (γ)-aminobutyric acid
GLU	Glutamate
HR	Heart rate
IML	Intermediolateral
iNOS	Inducible nitric oxide synthase

ir	immunoreactvity
K	Potassium
KCl	Potassium chloride
kg	kilogram
KH_2PO_4	Potassium hydrogen phosphate
KRB	Kreb Ringer Bicarbonate
L	litre
M	Molar
MAP	Mean arterial pressure
mg	milligram
mg/kg b.w.	milligram per kilogram body weight
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	minutes
mL	millilitre
mL/kg b.w.	millilitre per kilogram body weight
mm	millimetre
mM	millimolar
mmol/L	millimol per litre
n	Number of animals
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
nL	nanoliter
nmol	nanomol
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NTS	Nucleus tract solitarii
O_2	Oxygen
PBS	Phosphate buffer saline
PGI_2	Prostacyclin or 6-keto-PGF_1
PNS	Peripheral nervous system

PVN	Paraventricular nucleus
RVLM	Rostral ventrolateral medulla
s.e.m	Standard error of mean
SA	Sinoatrial
SNP	Sodium nitroprusside
STZ	Streptozotocin
SV	Stroke volume
µL	microlitre
µL/s	microlitre per second
µm	micrometre

LIST OF SYMBOLS

β	Beta
α	Alpha
^{0}C	Degree celcius
%	Percentage
~	approximately
<	less than
>	more than
±	plus minus
γ	Gamma

PERUBAHAN DALAM PENGAWALATURAN PUSAT DAN PERIFERI TIKUS DIABETIS TERARUH STREPTOZOTOSIN

ABSTRAK

Perubahan reflek kardiovaskular dalam diabetis menyumbang kepada penambahan fikiran yang tidak sihat dan kematian kardiovaskular. Perubahan ini mungkin berlaku pada aras saraf aferen, baroreflek pusat atau saraf eferen dalam lingkungan baroreflek. Dengan menggunakan tikus diabetis teraruh streptozotosin, kajian semasa direkabentuk untuk: (1) memeriksa secara sistematik perubahan diabetis teraruh pada komponen-komponen saraf pelbagai dengan menyiasat saraf aferen reflek baroreseptor, sumbangan baroreflek pusat dalam medula rostral ventrolateral (RVLM) dan nukleus paraventrikular (PVN) dan taburan saraf penggiat (c-Fos) dalam reaksi pengaktifan baroreseptor; (2) menilai reaksi vaskular tikus ternyahsaraf diabetis dalam ketidakhadiran baroreflek; dan (3) memeriksa potensi mekanisma bersel dalam gegelung aorta terasing *in vitro* yang mungkin menyumbang kepada disfungsi vaskular. Keputusan reflek baroreseptor dalam tikus diabetis teraruh STZ menunjukkan bradikardia baroreflek menurun dalam fenilefrina (PE)-teraruh meningkatkan tekanan arteri manakala takikardia reflek tidak berubah dalam natrium nitroprusida (SNP)-teraruh menurunkan tekanan arteri. Untuk menguji sumbangan baroreflek pusat, suntikan mikro dengan L-glutamat (1nmol dalam 100nL) menunjukkan perangsangan simpato. Manakala, suntikan mikro dengan GABA (50nM dalam 100nL) menunjukkan perencatan simpato dalam kawasan RVLM. Walaubagaimanapun, keputusan yang diperolehi tidak menunjukkan sebarang perbezaan antara kedua-dua kumpulan dan ini selaras dengan keputusan imunokimia c-Fos, yang menunjukkan bilangan saraf penggiat dalam NTS dan

RVLM adalah sama. Ini terbukti yang kerosakan tidak disebabkan oleh saraf sensori baroreseptor. Tambahan pula, perangsangan farmakologi saraf glutamat dengan asid DL-Homosistik (200mM dalam 100nL) dalam kawasan PVN menunjukkan kemelesetan ketara dalam kedua-dua MAP dan HR tikus diabetis seperti yang dilihat dalam metioda bikukulina (antagoni reseptor $GABA_A$, 1mM dalam 100nL), tetapi tidak kepada hidrobromida musimol (agoni reseptor $GABA_A$, 2mM dalam 100nL). Keputusan ini mungkin disebabkan oleh kelemahan kesan perencatan tonik saraf GABA ke atas perangsangan simpato saraf glutamat. HR tikus diabetis menunjukkan pengurangan ketara menandakan kemungkinan pengurangan dalam ganglia kardiak dan aktiviti saraf ganglia kardiak. Taburan saraf penggiat (c-Fos) dalam reaksi pengaktifan baroreseptor meningkat dalam saraf tunjang menandakan yang kedua-dua RVLM dan PVN mengarah saraf mereka ke saraf tunjang. Dalam model haiwan ternyahsaraf, perbezaan tekanan antara kedua-dua kumpulan hilang, manakala dalam haiwan tidak ternyahsaraf, tikus diabetis menunjukkan pengurangan ketara dalam MAP rehat. Penemuan ini mencadangkan yang pengurangan dalam MAP rehat dalam tikus diabetis sebelum ternyahsaraf disebabkan oleh perubahan dalam tona vasomotor simpatetic pusat kerana pengalihan aliran keluar simpatetik berkeputusan MAP yang sama dalam kedua-dua kumpulan. Penyumbangan fungsi NO lemah dan pengeluaran faktor pengecutan endotelium (EDCF) daripada endotelium meningkat dalam gegelung aorta terasing *in vitro* tikus diabetis. Disfungsi vaskular berlaku pada aras endotelium dan/atau otot licin vaskular. Umur dilihat sebagai faktor penyumbang yang kuat kepada bentuk pemecutan disfungsi vaskular.

CHANGES IN CENTRAL AND PERIPHERAL REGULATION OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

ABSTRACT

Alterations of cardiovascular reflexes in diabetes contribute to the increased cardiovascular morbidity and mortality. These alterations may be mediated at the level of afferent nerves, central baroreflex or efferent nerves within the baroreflex circuitry. Using the streptozotocin (STZ)-induced diabetic rats, the current study was designed to: (1) examine systematically diabetes-induced alteration of the multiple neural components by investigating the afferent nerves of the baroreceptor reflex, contribution of the central baroreflex in the rostral ventrolateral medulla (RVLM) and paraventricular nucleus (PVN) and distribution of activated neurons (c-Fos) in response to baroreceptor activation; (2) evaluate the vascular responsiveness of the diabetic pithed rats in the absence of baroreflex; and (3) examine the potential cellular mechanisms in isolated *in vitro* aortic rings that may contribute to vascular dysfunction. Results of baroreceptor reflex in STZ-induced diabetic rats showed baroreflex-mediated bradycardia was decreased in phenylephrine (PE)-induced increases in arterial pressure whereas reflex tachycardia was preserved in sodium nitroprusside (SNP)-induced decreases in arterial pressure. To test for the contribution of central baroreflex, microinjection of L-glutamate (1nmol in 100nL) showed sympathoexcitation. Meanwhile, microinjection with GABA (50nM in 100nL) showed sympathoinhibition in the RVLM regions. However, the results obtained did not show any difference between both groups and this was in line with the result of c-Fos immunohistochemistry, which demonstrated that the number of activated neurons in the

NTS and RVLM was similar. It is evident that the damage was not due to baroreceptor sensory nerves. In addition, the pharmacological stimulation of glutamatergic neurons with DL-Homocysteic acid (200mM in 100nL) in the PVN region showed a significant depression in both MAP and HR of diabetic rats as observed in to bicuculline methiodide ($GABA_A$ receptor antagonist, 1mM in 100nL), but not to muscimol hydrobromide ($GABA_A$ receptor agonist, 2mM in 100nL). These results are suggestive of the attenuation of GABAergic tonic inhibitory action on glutamatergic sympathoexcitation. The HR of diabetic rats showed significant reduction indicating the possible reduction in the cardiac ganglia and cardiac ganglionic neuronal activity. The distribution of activated neurons (c-Fos) in response to baroreceptor activation increased in the spinal cord indicating that both RVLM and PVN project their neurons to the spinal cord. In pithed animals model, the pressure difference observed between both groups was abolished, whereas in the non-pithed animals, the diabetic rats showed a significant reduction in the resting MAP. These findings suggested that the reduction in resting MAP in diabetic rats before pithing is due to the alteration in the intrinsic sympathetic vasomotor tone as removal of sympathetic outflow results in similar MAP in both groups. The functional contribution of NO was attenuated and the production of endothelium-derived contracting factor (EDCF) from the endothelium was enhanced in isolated *in vitro* aortic rings of diabetic rats. The vascular dysfunction occurred at the level of the endothelium and/or vascular smooth muscle. Age is seen to be a strong contributing factor to the accelerated form of vascular dysfunction.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Nervous system

The nervous system is an organ system containing a network of specialized cells called neurons. The neurons coordinate the actions and transmit signals between different parts of its body. The nervous system is made up of the central and peripheral nervous system.

1.1.1 Central nervous system

The central nervous system (CNS) comprises of the brain and spinal cord. The CNS is an integrative and control centre of the whole body. Anatomically, the regions of the brain are subdivided into the cerebrum, diencephalon, brainstem and cerebellum. The cerebrum is made up of cerebral hemispheres and diencephalon and both form the forebrain. Meanwhile the brainstem consists of the midbrain, pons and medulla oblongata. The brain also contains four interconnected cavities, the cerebral ventricles, which are filled with cerebral spinal fluid (Widmaier et al., 2006; Tortora and Derrickson, 2006).

The spinal cord lies within the bony vertebral column. In cross section, the central butterfly-shaped of gray matter is composed of interneurons, the cell bodies and dendrites of efferent neurons, the entering axons of afferent neurons and glial cells. The gray matter is surrounded by white matter, which consists of group of myelinated axons. Groups of afferent fibers enter the gray matter of the spinal cord from the peripheral nerves via the dorsal roots and their cell bodies in the dorsal root ganglia. On the other

hand, the axons of efferent neurons leave the spinal cord via the ventral roots. Both dorsal and ventral roots from the same level combine to form a spinal nerve on each side of the spinal cord (Widmaier et al., 2006; Tortora and Derrickson, 2006).

1.1.2 Peripheral nervous system

The peripheral nervous system (PNS) consists of sensory neurons, clusters of neurons called ganglia, and nerves connecting the rest of the body and to the CNS. These regions are all interconnected by means of complex neural pathways. The PNS has 43 pairs of nerves: 12 pairs of cranial nerves and 31 pairs of spinal nerves that connect with the spinal cord. The 31 pairs of spinal nerves are designated by the vertebral levels from which they exit: cervical (C1-C8), thoracic (T1-T12), lumbar (L1-L5), sacral (S1-S5) and coccygeal (CO1) (Widmaier et al., 2006; Tortora and Derrickson, 2006).

The PNS is divided into somatic and autonomic nervous system (ANS). Both the somatic and ANS consist of sensory nerve fibers (afferent) which propagate impulses from receptors to the CNS and motor nerve fibers (efferent) which conduct impulses from the CNS to effectors (Loewy and Spyer, 1990). The somatic motor neurons, which lead to muscle excitation, innervate the skin, joints, and skeletal muscles and they are made up of a single neuron between CNS and skeletal muscle. Meanwhile, the motor neurons of ANS (visceral motor) contain neurons that innervate the internal organs, blood vessels, and glands and they have two-neuron (connected by a synapse) between CNS an effector organ. It can either give excitation or inhibition. The first neuron has its cell body in the CNS. The synapse between the two neurons is outside the CNS in a cell cluster called the autonomic ganglion. The neurons between the CNS and the ganglia are

called preganglionic neurons whereas the neurons between the ganglia and the effector organ are known as postganglionic neurons (Loewy and Spyer, 1990; Guyenet 2006). The ANS itself consists of three parts: the sympathetic, parasympathetic and enteric nervous system. Both sympathetic and parasympathetic will be elucidated in this study. The enteric nervous system, a subsystem of the peripheral nervous system, functions independently in controlling the gastrointestinal system through its primary connection via the vagus nerve (Loewy and Spyer, 1990).

1.2 Cardiovascular physiology

The cardiovascular system consists of the pulmonary circulation and the systemic circulation. The pulmonary circulation starts from the right ventricle, moves to the lungs and then returns to the left atrium. The systemic circulation starts from the left ventricle, moves to all peripheral organs and tissues and then returns to the right atrium. In the systemic circulation, the large artery leaves the left side of the heart through the aorta and the large vein enters the right side of the heart through superior vena cava and inferior vena cava. The microcirculation of the arteries and veins are arterioles, capillaries and venules (Brownley et al., 2000).

The heart receives a rich supply of sympathetic and parasympathetic nerve fibers. The sympathetic postganglionic fibers innervate the entire heart and release norepinephrine while the parasympathetics terminate mainly on cells found in the atria and release primarily acetylcholine. The receptors for norepinephrine on cardiac muscle are mainly beta-adrenergic whereas the receptors for acetylcholine are the muscarinic type (Brodde et al., 2001; Olshansky et al., 2008).

Cardiac output (CO) is the volume of blood pumped by each ventricle, usually expressed in liters per minute. The cardiac output is determined by multiplying the heart rate (HR) and the stroke volume (SV). The heart rate is the number of beats per minute whereas the stroke volume is the blood volume ejected by each ventricle with each beat (Charkoudian et al., 2005). The beating of the heart is influenced by the nerves and hormones acting on the SA node. The sinoatrial (SA) node is a small group of conducting-system cells located in the right atrium near the entrance of the superior vena cava. The action potential spreads from the SA node throughout the atria and ventricles. Most of the parasympathetic and sympathetic postganglionic fibers end on the SA node. Parasympathetic (vagus) nerves cause a decrease in the heart rate, whereas activity in the sympathetic nerves causes an increase in the heart rate. Furthermore, epinephrine, the hormone liberated from the adrenal medulla, speeds the heart by acting on the same beta-adrenergic receptors in the SA node as norepinephrine released from neurons (Vaseghi and Shivkumar, 2008).

1.2.1 The vascular system

The aorta and other systemic arteries have thick walls of elastic tissue and serve as low-resistance conduits and as pressure reservoirs for maintaining blood flow to the tissues during ventricular relaxation. Arterial pressure is generally recorded as systolic/diastolic. The maximal aortic pressure following ejection is termed systolic pressure ($P_{systolic}$). As the left ventricle is relaxing and refilling, the pressure in the aorta falls. The lowest pressure in the aorta, which occurs just before the ventricle ejects blood into the aorta, is named the diastolic pressure ($P_{diastolic}$). The difference between systolic pressure and diastolic pressure is called the pulse pressure. The mean arterial pressure

(MAP) is the diastolic pressure plus one-third of the pulse pressure (Mohrman and Heller, 2010).

The sympathetic nervous system plays an essential role in the regulation of arterial blood pressure and keeps the blood vessels in a continual state of partial constriction called sympathetic tone (or vasomotor tone). The parasympathetic nervous system normally dominates the heart and activity levels of the digestive and urinary systems – these organs exhibit parasympathetic tone. The parasympathetic nervous system slows the heart but, the sympathetic division can override these effects during times of stress (Widmaier et al., 2006).

1.2.2 Factors affecting the arteriolar pressure

The sympathetic nerves innervate most arterioles via alpha-adrenergic receptors and cause vasoconstriction. In some situations, noncholinergic, nonadrenergic neurons which release nitric oxide or other noncholinergic vasodilators can also innervate the blood vessels. Some chemical inputs can act by stimulating the endothelial cells to release either vasodilators or vasoconstrictors which then act on the adjacent vascular smooth muscles to cause either relaxation or contraction. This will lead to vasodilation or vasoconstriction respectively (Toda and Okamura, 2003).

Endothelium, the largest organ in the body, senses mechanical stimuli such as pressure and shear stress, and hormonal stimuli such as vasoactive substances. It has been known to release agents that regulate vasomotor function, trigger inflammatory processes and affect homeostasis. Besides synthesizing and releasing many vasoactive compounds that modulate vascular diameter and hence tissue blood flow, it plays an important role in maintaining the vascular tone and luminal diameter of a blood vessel.

Hence, the vascular homeostasis depends on the net balance of vasoconstrictor and vasodilator forces as expounded by Dandona and co-workers (2003). It is widely accepted that three main endothelium-derived relaxing factors (EDRF) that act on vascular smooth muscle cells are nitric oxide (NO) (Furchgott & Zawadzki, 1980); prostacyclin (PGI_2) (Dusting et al., 1977) and endothelium-derived hyperpolarizing factors (EDHF)(Taylor & Weston, 1988; Komori and Vanhoutte, 1990; Luksha et al., 2009; Jin et al., 2011). Apart from EDRFs, endothelium is also a source of contracting factor namely endothelium-derived contracting factors (EDCF). Examples of the EDCF that are released by the endothelial cells in response to certain mechanical and chemical stimuli include endothelin-1 (ET-1), angiotensin II (Ang II), thomboxane A_2, norepinephrine, serotonin and reactive oxygen species (ROS) (Schiffrin, 2001; Verma & Anderson, 2002).

NO is synthesized from its precursor L-arginine by the enzyme NO synthase (NOS). The formation of NO requires calcium entry into the endothelium cells, oxygen, the cofactors NADPH, tetrahydrobiopterin (BH_4), calmodulin, heme (Snyder and Bredt, 1992; Knowles and Moncada, 1994; Toda et al., 2007). The NO diffuses from the endothelium to vascular smooth muscle to activate soluble guanylate cyclase which increases the levels of cGMP to resulting in relaxation of vascular smooth muscles (Mayhan, 2001). At the same time, cGMP inhibits the calcium entry into the cell resulting in reduction in the intracellular free Ca^{2+} concentration which prevents the Ca^{2+}-dependent activation of myosin light-chain kinase and muscle contraction (Toda and Okamura, 2003). NO also produces relaxation of blood vessels by the activation of potassium channels (Mayhan, 2001). Three distinct isoforms of nitric oxide synthase have been identified (eNOS – identified in vascular endothelium, nNOS – soluble

enzyme and expressed in the brain, peripheral nerves, including parasympathetic postganglionic nerves innervating blood vessels namely nitrergic nerve and iNOS – cytokine-inducible isoform) (Toda et al., 2007). NOS activity has been demonstrated in central and peripheral sites throughout the cardiac autonomic nervous system, including the receptors and effectors of the baroreflex pathway (Chowdhary and Townend, 1999). The endogenous and exogenous application of vasoactive agonists appears to bind to specific receptors on endothelial cells to stimulate the synthesis or release of nitric oxide or nitric oxide-containing compound that causes relaxation of vascular smooth muscles through activation of soluble guanylate cyclase (Vanhoutte and Mombouli, 1996; Toda et al., 2007).

Prostacyclin (PGI_2) is the major metabolite of arachidonic acid produced by cyclooxygenase in endothelial cells (Dusting et al., 1977). It is produced in the endothelial cells from prostaglandins H_2 (PGH_2) by the action of the enzyme prostacyclin synthase. It prevents formation of the platelet plug involved in blood clot formation. It is also an effective vasodilator. Its half-life is of seconds and broken down into 6-keto-PGF_1, which is a much weaker vasodilator. Prostacyclin-induced vasorelaxation involves the opening of one or more of types of potassium channels such as ATP-sensitive potassium channels (K-ATP), large conductance calcium-activated potassium channels (BK_{Ca}), inwardly rectifying potassium channels (K_{IR}) and/or voltage activated potassium channels (K_V) (Feletou and Vanhoutte, 2006).

Endothelium-derived hyperpolazing factor (EDHF) is synthesized and released from endothelium but its exact nature is still controversial. It hyperpolarizes vascular smooth muscle cells and causes these cells to relax and blood vessel to dilate. There is evidence to indicate that it links to endothelium-dependent relaxation by opening K^+

channels in vascular smooth muscle. K^+ hyperpolarizes the smooth muscle cells by activating inward rectifying potassium channels and/ or Na^+/K^+-ATPase, leading to relaxation in small arteries (Chen et al., 1988; Komori and Vanhoutte, 1990; Luksha et al., 2009). Furthermore, it has been reported that the contribution of EDHF to endothelium-dependent relaxations increases as the vessels size decreases (Tomioka et al., 1999; Luksha et al., 2009).

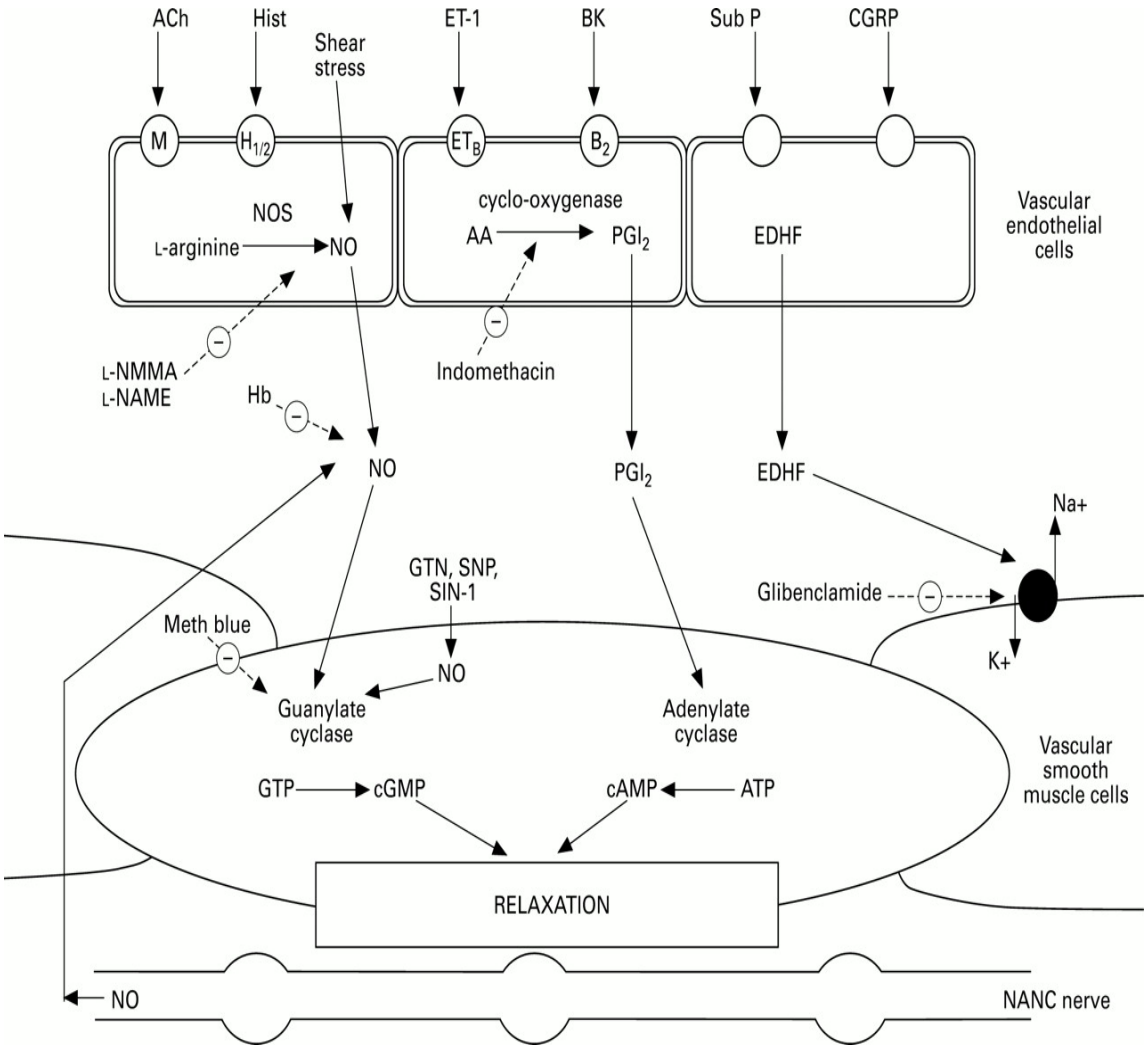

Figure 1.1 Mechanism of endothelium dependent relaxation showing the release of nitric oxide (NO), prostacyclin (PGI_2) and endothelium derived hyperpolarising factor (EDHF) from the endothelium. Agonists act on specific endothelial cell receptors and may stimulate the release of one or all mediators to induce smooth muscle relaxation. Abbreviations: Acetylcholine (ACh), histamine (Hist), endothelin-1 (ET-1), bradykinin (BK), substance P (Sub P), calcitonin gene related peptide (CGRP), nitric oxide synthase (NOS), arachidonic acid (AA), L-N^G monomethyl arginine (L-NMMA), L-N^Gnitroarginine methylester (L-NAME), haemoglobin (Hb), glyceryl trinitrate (GTN), sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1), methylene blue (Meth blue), cyclic guanosine/adenosine monophosphate (cGMP/cAMP), guanosine/adenosine triphosphate (GTP/ATP), non-adrenergic non-cholinergic (NANC), muscarinic receptor (M), histamine receptor ($H_{1/2}$), endothelin B receptor (ET_B), bradykinin receptor (B_2). (Buckley et. al., 1997).

1.3 Baroreceptor reflexes

Arterial baroreceptor reflexes play an important role in homeostasis adjustments to mean arterial pressure by preventing large fluctuations of arterial blood pressure (BP), by rapidly adjusting cardiac output and total peripheral resistance. They utilize mainly changes in the activity of the autonomic nerves (*i.e.* sympathetic and parasympathetic nervous system) supplying the heart and blood vessels, as well as changes in the secretion of the hormones (epinephrine, angiotensin II and vasopressin) through their effects on heart rate, venous return, contractility and peripheral resistance (Rovere and Raczak, 2006).

The heart and arterioles are controlled by sensory signals detecting changes in arterial pressure and pH (*i.e.* pCO_2 and pO_2) which modulate blood chemistry. Stretch-sensitive mechanoreceptors in the blood vessels serve as arterial baroreceptor that relay information about arterial blood pressure and blood volume through the glossopharyngeal nerve (from carotid sinus) and vagus nerve (from aortic arch) whose cell bodies lie within the nodose ganglion. Both afferent neurons travel from the carotid sinus and aortic arch through the IXth and Xth cranial nerves to the brainstem and provide excitatory input to secondary baroreceptor sensory neurons in the nucleus tractus solitarii (NTS) (Skrapari et al., 2006).

1.4 Central nervous mechanisms contributing to cardiovascular control

Cardiovascular homeostasis involves the regulation of sympathetic vasomotor tone to maintain blood plasma volume and arterial blood pressure (Guyenet, 2006). The PVN is primarily concerned with blood volume regulation (Lovick and Coote, 1988) and

the rostral ventrolateral medulla (RVLM) is the dominant brain region for tonic regulation of arterial blood pressure (Dampney, 1994; Sved, 2004).

The primary integrating center for the baroreceptor reflexes is called the medullary cardiovascular center which is located in the brainstem medulla oblongata. The central axons of the vagus and glossopharyngeal nerves terminate in the NTS in the posterior medulla. The fibers from NTS pass to the cardio-inhibitory and vasomotor centers of the medullary reticular formation, via a multisynaptic pathway involving an excitatory (glutamatergic neurons) projection from NTS to the caudal ventrolateral medulla (CVLM), an inhibitory (GABAergic neurons) projection from CVLM to the rostral ventrolateral medulla (RVLM) and an excitatory (glutamatergic) projection from RVLM to sympathetic preganglionic neurons via the intermediolateral cell column (IML) of the spinal cord (Dampney et al., 2003). These centers are also connected to the dorsal motor nucleus of the efferent vagal nerves, where parasympathetic outflow is regulated. Direct projections to the IML of the spinal cord originate predominantly from at least five areas of the brain: a) rostral ventrolateral medulla (RVLM), which plays a principal role in determining basal sympathetic nerve activity by balancing both inhibitory and excitatory inputs (Campos et al., 2008); b) rostral ventromedial medulla, c) caudal raphe nuclei, d) A5 cell group in the pons, and e) paraventricular nucleus of the hypothalamus (PVN)(Strack et al., 1989) and recently caudal pressor area (CPA)(Campos et al., 2008).

During cardiovascular reflexes, a rapid increase in arterial blood pressure results in increased afferent nerves to the vagal cardio-inhibitory center, which sends out impulses to the heart resulting in vagal slowing. At the same time, the sympathetic vasomotor center is inhibited, resulting in reduction of sympathetic vasoconstrictor tone.

On the other hand, decreasing arterial blood pressure is compensated by sympathetic excitation, with a consequent rise in cardiac output and vasoconstriction. Sympathetic stimulation also passes to the spleen and kidneys to increase blood volume. The spleen capsule contracts to release more blood into the circulation. At the same time, renin is released from the kidney to activate the renin-angiotensin-aldosterone system to promote water retention.

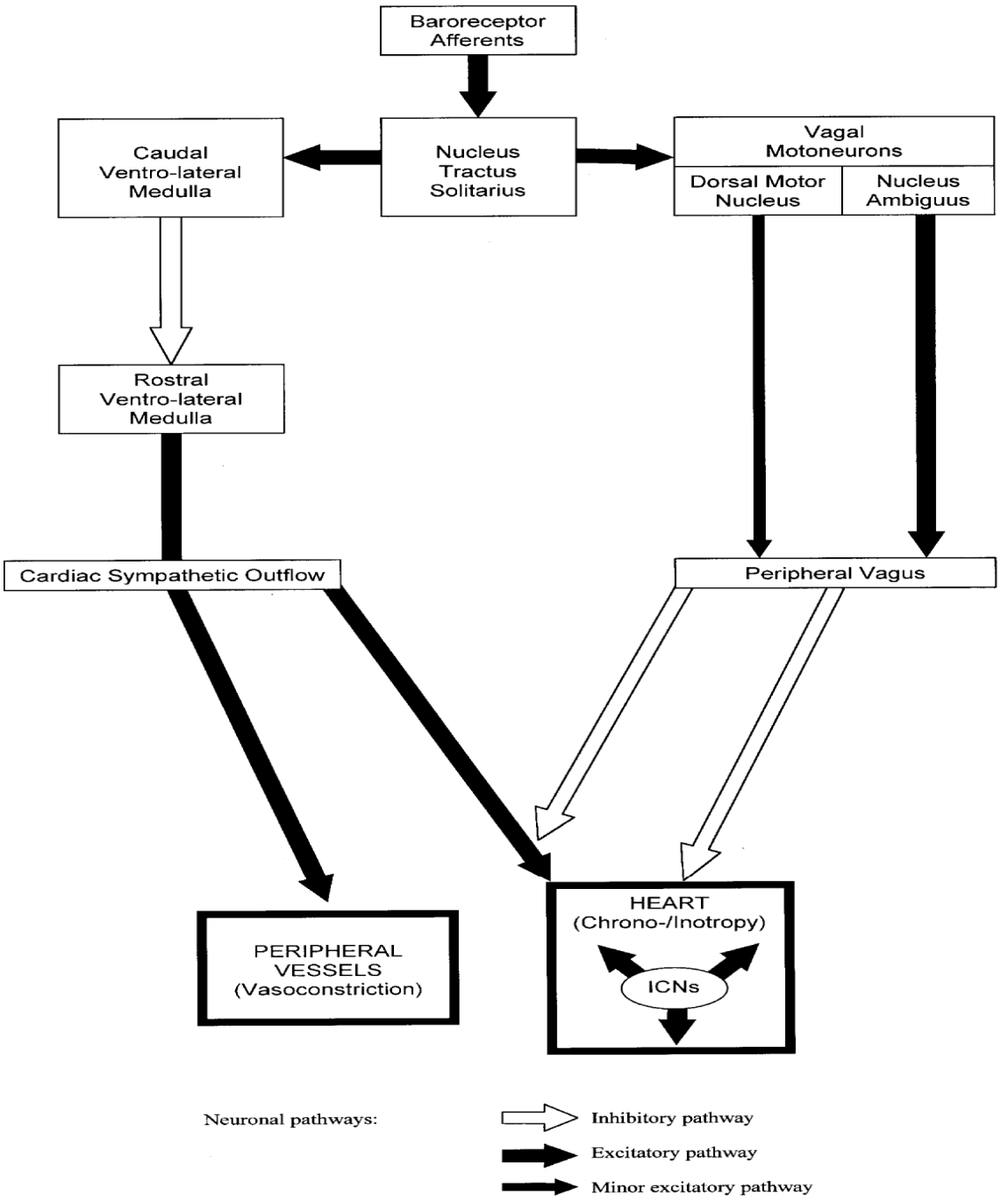

Figure 1.2 Cardiovascular autonomic control and baroreflex integration (Chowdhary and Townend, 1999). ICNs are intracardiac neurons.

1.4.1 Rostral ventrolateral medulla (RVLM)

RVLM is located 0-500 μm caudal to the facial motor (VII) nucleus, lateral to the inferior olivary nucleus and ventral to the compact formation of the nucleus ambiguous (Stocker et al., 2007; Kashihara et al., 2008). RVLM is involved in the modulation of arterial blood pressure through a variety of cardiovascular reflexes (Guyenet, 1990; Dampney, 1994; Reis et al., 1994; Lipski et al., 1996). It contains neurons that exert some control on the cardiovascular system in a manner that an electrical or chemical stimulation in this region can evoke large pressor responses (Leman et al., 2000). The pressor responses are mediated by activation of sympathetic preganglionic neurons (SPN) mainly located in the intermediolateral nucleus (IML) of the spinal cord between segments C8 to L3-L5 (Janig, 1985; Laskey and Polosa, 1988; Cabot, 1990). The properties of the sympathoexcitatory neurons in the RVLM are influenced by baroreceptor signals and have been extensively investigated (Dampney, 1994).

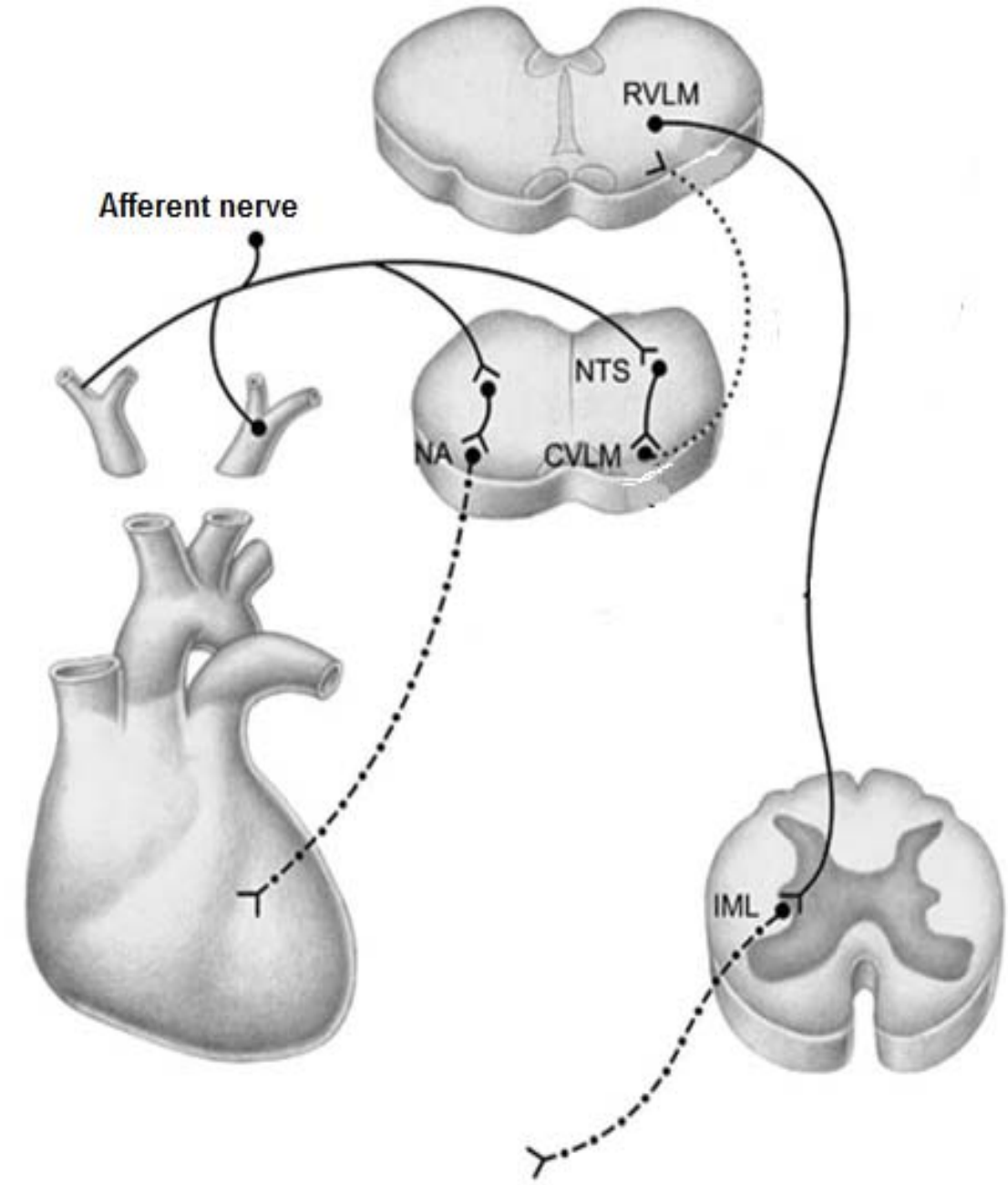

Figure 1.3 Schematic representation of pathways lead to the sympathetic vasomotor tone. RVLM = rostral ventrolateral medulla; CVLM = caudal ventrolateral medulla; NTS = nucleus of the tractus solitarius; IML = intermediolateral column; NA = nucleus ambiguous. Dotted line = GABAergic projection; continuous line = glutamatergic projection; dash-dot line = cholinergic projections (Modified from Campos et al., 2008).

1.4.2 The paraventricular nucleus (PVN) and cardiovascular control

Smith (1965) was first drawn to the existence of direct projection of fibers from the hypothalamus to the autonomic regions of the spinal cord by electrolytic lesions at sites in the hypothalamus, which on prior stimulation had elicited pressor and cardioacceleratory responses. Anterograde neuronal labeling studies revealed that there are groups of neurons with axons projecting directly to the spinal cord and terminating in the sympathetic lateral horn (Sawchenko & Swanson, 1982; Coote, 2004a).

The hypothalamic PVN is known to be involved in coordination of autonomic function such as regulation of food intake, responses to stress, modulating metabolic rate, thermoregulation and regulation of cardiovascular function (Schlenker, 2005). In addition, it influences neuroendocrine function and the secretion of vasopressin, thyrotropin-releasing hormone (TRH), orexin, corticotrophin-releasing factor (CRF) and oxytocin (Benarroch, 2005). It receives inputs directly or indirectly from rostral brain regions, including limbic system and amygdala and caudal brain stem regions such as NTS, pons, rostral ventrolateral medulla (RVLM) and locus coeruleus (Schlenker, 2005).

Anatomically, PVN is located on either side of the third ventricle in the hypothalamus. The PVN contains many different subgroups based on morphology and neuroanatomic projections. The subgroups are composed of magnocellular and parvocellular neurons. The magnocellular neurons project to the posterior pituitary and are responsible for secreting vasopressin (AVP) and oxytocin into the blood. The parvocellular PVN contains neurons that project to the intermediolateral cell column of the thoraco-lumbar spinal cord (IML) and neurons that innervate the pressor region of the RVLM (Badoer, 2001). The latter neurons are known to influence sympathetic nerve

activity. Accumulating evidence support that these neurons in the PVN can: (i) directly influence sympathetic nerve activity (via PVN-IML connections); (ii) indirectly influence sympathetic nerve activity (via PVN-RVLM connections); and (iii) both directly and indirectly influence sympathetic nerve activity (Dampney, 1994; Coote et al., 1998; Pyner and Coote, 2000; Badoer, 2001). The PVN is innervated by the A1 and A2 cell groups (Kannan et al., 1987) and receives afferent connections from peripheral baroreceptors.

The magnocellular neurons of the PVN play a key part in neuroendocrine control of fluid balance. The vagus nerve sends an impulse from atrial volume receptors located at the venous-atrial junctions to the brain. The first synapse is within the NTS of the medulla oblongata. The volume signals travel from the NTS to the hypothalamus to influence neurons in the PVN (Lovick and Coote, 1988; Pyner et al., 2002). Deering and Coote (2000) demonstrated that low amount of neurons excitant DL-Homocysteic acids preferentially evoke excitation of cardiac sympathetic activity and simultaneously evoke inhibition of renal sympathetic activity.

The most recent method in which excitatory amino acids are microinjected into the area of interest has overcome the disadvantage of electrical stimulation by selectively stimulating the cell bodies of interest (Goodchild et al., 1982). The changes in blood pressure and sympathetic nerve activity were reported to either decrease or increase when PVN was stimulated (Kannan et al., 1987, Malpas and Coote, 1994). Subsequently, Schlenker et al., (2001) reported that unilateral microinjection of bicuculline, a γ-aminobutyric acid $GABA_A$ receptor antagonist, into the PVN of conscious rats increased mean arterial pressure (MAP), breathing frequency, increased

the volume of a breath, heart rate and oxygen consumption for up to 10 minutes following injection.

1.4.3 Neurotransmitters in central cardiovascular regulation: Glutamate and GABA

Amino acid L-glutamate (GLU) is the major excitatory neurotransmitter in the mammalian central nervous system, whereas, γ-aminobutyric acid (GABA) appears to be the principal neurotransmitter mediating inhibitory synaptic currents. Both GLU and GABA play predominant roles in brainstem and spinal circuits that are essential for control of cardiovascular function by the CNS (Gordon and Sved, 2002).

Many experimental techniques have been used to explore the role of GLU and GABA in central pathways controlling cardiovascular function. Pharmacological studies involving direct microinjections of drugs into specific brainstem regions have been a continuous motivation among researchers since the last century. Microinjections of GLU and GABA were not only utilized to study neurotransmitters but also used to aid in the activation or inhibition of cells in a specific brain region.

The synaptic inputs to RVLM neurons are excitatory or inhibitory. These inputs are generally mediated via glutamate or GABA receptors. However, the RVLM presympathetic neurons also present receptors for other neurotransmitters or neuromodulators such as angiotensin II, enkephalin or ATP (Dampney 1994).

The PVN is a nucleus to which a number of excitatory and inhibitory neurotransmitters converge to influence its neuronal activity. The inhibition is dependent on GABA, which is mostly mediated by ionotropic $GABA_A$ receptors and NO. NO potentiates local GABAergic synaptic inputs onto the neurons in the PVN. Glutamate

and angiotensin II are excitatory neurotransmitters which modify the tonic inhibitory activity. Other neurotransmitters which have been shown to affect cardiovascular function are oxytocin, vasopressin and dopamine. They are found in neurons of the PVN and within the spinal cord (Pyner, 2009)

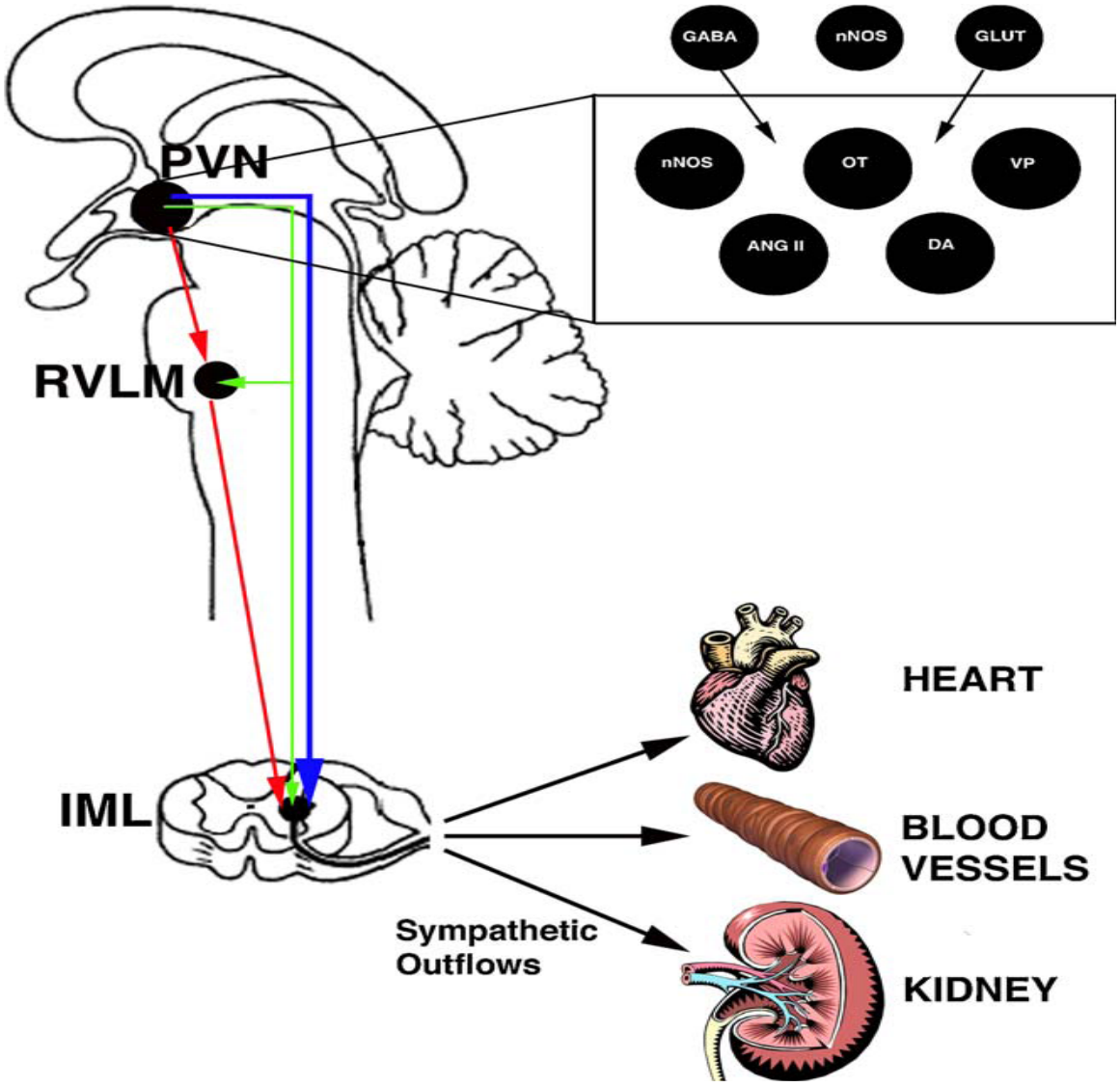

Figure 1.4 Schematic diagram illustrating the three main pathways by which the paraventricular nucleus of the hypothalamus (PVN) can influence sympathetic activity. A direct descending projection (blue) to the spinal intermediolateral cell column (IML); a projection to the vasomotor neurones (red) of the rostral ventrolateral medulla (RVLM) and a collateral projection to both RVLM and IML (green). In addition, the inset summarises the distribution of the neurotransmitters discussed: the parvocellular subdivisions of the PVN contain neurones capable of synthesising vasopressin (VP), oxytocin (OT), dopamine (DA), angiotensin II (ANG II) and nitric oxide (nNOS), although the majority of nitric oxide synthesising neurones are found in the magnocellular subdivision. Putative interneurones surrounding the PVN that contribute to the regulation of parvocellular neuronal activity contain GABA, glutamate (GLU) and nNOS (adapted from Pyner, 2009).

1.5 c-Fos immunohistochemistry

The early techniques that was used to metabolically identify regions of the brain that were involved in specific functions was the use of 2-deoxyglucose (2DG)(Kennedy et al., 1975). Following which, the histochemical localization of metabolic enzymes such as cytochrome oxidase and hexokinase has been used as a means to identify populations of neurons that respond to physiological stimuli (Krukoff, 1999). Although these techniques are inexpensive and technically straightforward there are limitations in their applications. The development of immediate-early genes described as c-Fos in neurons has provided a useful and popular marker of activated neurons. c-Fos encodes a protein (Fos) that participates with products of the related Jun family as a component of the protein complex that binds to the activator-protein-1(AP-1) binding site of DNA. Genes that contain AP-1 complex are activated by the Fos/Jun complex, thereby allowing the expression of the so-called late-onset genes that encode differentiated neuronal products such as neurotransmitters. Transcription of c-Fos occurs within minutes of application of a stimulus, amount of mRNA peaks at 30-45 minutes, and the half-life of the Fos protein is about 2 hours (Muller et al., 1984). In the central nervous system, basal levels of c-Fos immunoreactivity (ir) are low, but increase rapidly and transiently in response to stimulation (Krukoff, 1999). It has been suggested that c-Fos activation may be utilized as a high resolution metabolic marker for polysynaptic pathway tracing in the brain and spinal cord (Malakhova and Davenport, 2001). Fos immunohistochemistry stains the neuronal nucleus and detects neuronal changes (Dragunow and Faull, 1989).

1.6 Rational of the thesis

To summarize, sympathetic vasomotor outflow consists of efferent pathways that control vascular tone (vascular peripheral resistance) and cardiac output (contractility and heart rate), playing an essential role in maintaining the balance of blood pressure and bodily homeostasis. The integration of autonomic outflow to the cardiovascular system and the vasomotor tone is dependent on the activity of neurons located within the medulla which exhibit both excitatory and inhibitory inputs. The PVN and the RVLM appear to have a tonic effect on the control of sympathetic vasomotor tone of the five nuclei mentioned earlier (Dampney, 1994; Pan, 2004) and baroreceptor reflex pathway has been a tool in the research on brainstem-spinal control of cardiovascular regulation and identified to have pronounced effect on cardiovascular system.

Neurophysiology concerning the central and peripheral nervous system has been described to be an important issue in maintaining bodily homeostasis. However, the course of development of neurophysiology alterations such as CNS and PNS in STZ-induced diabetic rats is incompletely documented. With regard to the matter, the framework of the present study will be focusing on the role of CNS and PNS in cardiovascular regulation in STZ-induced diabetic rats. These include the baroreceptor reflex circuitry at different time frame, pharmacological stimulation of glutamatergic and GABAergic neurons within the RVLM and PVN regions and neuroanatomical changes during baroreceptor activation. Furthermore, an attempt was also made to study the vascular responsiveness in pithed animal model and vascular reactivity in *in vitro* aortic rings.

CHAPTER FOUR

GENERAL CONCLUSION

Diabetes mellitus can affect both the peripheral and the central nervous system. The nervous system plays an important role in regulating the cardiovascular functions. This study implicates both central and peripheral nervous system in cardiovascular regulation in diabetes. Some of the important findings in this study can be summarized below:

1. An impaired baroreflex-mediated bradycardia and preserved baroreflex-mediated tachycardia was observed in diabetic rats. The baroreflex sensitivity decreased in diabetic rats. The impaired baroreflex sensitivity may be mediated at the level of afferent nerves, central baroreflex or efferent nerves.
2. The pharmacological stimulation of excitatory neurons (glutamatergic neurons) with L-glutamate showed sympathoexcitation, whereas sympathoinhibition was seen when inhibitory neurons (GABAergic neurons) were activated with GABA at RVLM site. The results observed did not show any difference in these actions between non-diabetic and diabetic rats. However, the HR of diabetic rats showed significant reduction in both pharmacological stimulations.
3. Pharmacological stimulation of glutamatergic neurons with DL-Homocysteic acid showed significant reduction in both MAP and HR in diabetic rats. Similar results were obtained when stimulated with $GABA_A$

receptor antagonist, bicuculine methiodide into the PVN, *i.e.* significant reduction in sympathoexcitation, but not to $GABA_A$ receptor agonist, muscimol. These results may be due to the attenuation of GABAergic tonic inhibitory action on glutamatergic sympathoexcitation.

4. Results of c-Fos immunohistochemistry showed that the numbers of baroreceptor activated c-Fos-ir neurons in the NTS and RVLM were similar between non-diabetic and diabetic rats indicating that it is not likely to be due to the damage to the baroreceptor afferent fibers of the CSN and ADN.
5. In addition, the results of c-Fos immunohistochemistry also support the notion that RVLM and PVN project their neurons to the IML of the spinal cord directly and indirectly to influence sympathetic nerve activity since the c-Fos-ir neurons in the spinal cord are found to produce massive distribution during baroreceptor activation with PE.
6. It is reasonable to suggest that there is impairment in the baroreflex pathway involved in the cardiovascular regulation in STZ-induced diabetic rats.
7. In the second part of the study, a significant reduction in the resting MAP was observed in non-pithed diabetic rats, suggesting altered intrinsic sympathetic vasomotor tone as the removal of sympathetic outflow results in similar MAP in both groups. In addition, the functional contribution of NO is attenuated in pithed diabetic rats and an intact nervous system is not necessary for the synthesis and release of EDRF.

8. The depression in vascular responsiveness to PE and an impaired synthesis of NO were observed in pithed diabetic rats. Other EDRF such as prostacyclin and EDHF may also play a role in the regulation of vasomotor tone.
9. The potential cellular mechanisms that contribute to the vascular dysfunction in STZ-induced diabetic rats were also determined. It is suggested that the impairment occurred at the level of endothelium and/or vascular smooth muscle.
10. Time is also the primary contributing factor to the accelerated form of vascular dysfunction.
11. Finally, an understanding of mechanisms that leads to impaired reflex function and endothelial dysfunction will certainly lead to new and unique therapeutic strategies to reduce cardiovascular morbidity and mortality in diabetes mellitus.