STUDY ON ROLE OF EXCITATORY AMINO ACID INPUT ON ROSTRAL VENTROLATERAL MEDULLA NEURONS AND ASSESSMENT OF PERIPHERAL VASCULAR REACTIVITY IN OBESITY-INDUCED HYPERTENSION

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by

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KAJIAN KE ATAS PERANAN INPUT ASID AMINO PERANGSANG PADA NEURON ROSTRAL VENTROLATERAL MEDULA DAN PENILAIAN TINDAK BALAS VASKULAR PERIFERAL DALAM HIPERTENSI TERARUH OBESITI

oleh

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Tesis yang diserahkan untuk
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LIST OF ABBREVIATIONS

AGT	angiotensinogen
Amb	nucleus ambiguus
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
AT_1	angiotensin type 1 receptor
ATPase	adenosine triphosphatase
bpm	beats per minute
C	caudal from bregma
Ca^{2+}	calcium ion
CVLM	caudal ventrolateral medulla
EAA	excitatory amino acids
EPSP	excitatory postsynaptic potential
et al.	et alii, others
FFA	free fatty acid
G	gauge
g	gram
GABA	gamma-aminobutyric acid
GluRs	glutamate receptors
g/kg	gram per kilogram
HDL	high density lipid
HR	heart rate
IL	interleukin
iNOS	inducible nitric oxide synthase

IO	inferior olive
kDa	kilodalton
kg	kilogram
KYN	kynurenic acid
L	lateral from midline
LHA	lateral hypothalamus area
LF	low fat diet-treated rats
M	molar
MAP	mean arterial blood pressure
mg/kg	milligram per kilogram
Mg^{2+}	magnesium ion
min	minutes
mm	millimeter
mmHg	millimeter mercury
MSNA	muscle sympathetic nerve activity
n	number of animals
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanogram
nL	nanoliter
NMDA	N-methyl-D-aspartate
nM	nanomolar
NO	nitric oxide
NOS	nitric oxide synthase
Npr-C	natriuretic peptide receptor type C
NTS	nucleus tractus solitarius
OP	obesity-prone rats

OR	obesity-resistant rats
OSA	obstructive sleep apnea
O_2^-	superoxide ion
PAG	periaqueaductal gray
PGI_2	prostacyclin
PVN	paraventricular nucleus
py	pyramidal tract
RAS	renin angiotensin system
ROb	raphe obscurus
RVLM	rostral ventrolateral medulla
s	second
SEM	standard error of mean
SBP	systolic blood pressure
SHR	spontaneously hypertensive rats
SNS	sympathetic nervous system
SP5I	spinal trigeminal nucleus;
sp5	spinal trigeminal tract
TNF	tumor necrosis factor
TXA_2	thromboxane A_2
µg	microgram
µM	micromolar
µm	micrometer
μm^2	micrometer square
V	ventral from the skull surface
w/v	weight over volume

LIST OF SYMBOLS

α	alpha
β	beta
°C	degree Celsius
%	percentage
<	less than
>	more than

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ABSTRAK

Rostral ventrolateral medula (RVLM) memainkan peranan penting dalam pengawalan tonik dan berfasa tekanan darah. Kajian ini dijalankan bagi menentukan sama ada input asid amino perangsang (EAA) ke RVLM menyumbang kepada tekanan darah yang tinggi dan untuk menilai tindak balas vaskular periferal dalam hipertensi teraruh obesiti. Tikus jantan Sprague-Dawley diberi makan diet rendah-lemak atau diet sederhana tinggi-lemak (MHF) selama 16 minggu. Kemudian, tikus yang diberi makan diet MHF dibahagikan kepada tikus cenderung obes (OP) dan tikus rintang obes (OR) berdasarkan taburan berat badan. Tikus OR didefinisikan sebagai tikus dengan berat badan yang sama atau kurang dari tikus kawalan (LF) manakala tikus OP didefinisikan sebagai tikus dengan berat badan yang paling besar. Pada minggu ke-16, tikus digunakan untuk mengkaji peranan EAA ke atas neuron RVLM atau untuk mengkaji tindak balas vaskular periferal dalam tikus hipertensi teraruh obesiti. Dalam kajian yang pertama, L-glutamat (1 nmol) dan asid kainurenik (KYN) pada kepekatan 4 nM, 40 nM dan 4 µM disuntik ke dalam RVLM dan heksametonium (20 mg/kg) diberi secara intravena. Batang otak dikeluarkan untuk pengesahan histologi dan indeks adipositi dikira. Tikus OP menunjukkan perolehan berat badan yang lebih ketara, indeks adipositi yang lebih tinggi dan peningkatan tekanan darah. Tindakbalas presor terhadap L-glutamat adalah lebih besar dalam tikus OP berbanding tikus OR dan LF, dan keputusan ini mencadangkan peningkatan

tindak balas RVLM terhadap EAA dan peningkatan peranan input EAA kepada neuron vasomotor RVLM. Suntikan KYN pada 40 nM menurunkan tekanan darah arteri purata (MAP) dalam tikus OP sahaja manakala pada 4 nM, tiada sebarang perubahan yang signifikan pada MAP diperhatikan pada semua tikus. Suntikan KYN pada kepekatan 4 µM meningkatkan MAP dalam semua kumpulan tikus. Keputusan yang diperolehi mencadangkan terdapat ketidakseimbangan input EAA kepada RVLM dalam tikus yang obes, di mana, keseimbangan disesarkan ke arah perangsang yang menjurus kepada peningkatan tekanan darah. Yang kedua, pertambahan perencatan kepada laluan glutamat perangsang oleh KYN berkemungkinan mendedahkan laluan perangsang yang lain yang memerlukan penyelidikan lanjutan. Heksametonium menghasilkan penurunan MAP yang lebih besar dalam tikus OP berbanding tikus OR dan LF dan keputusan ini mencadangkan peningkatan tonik simpatetik vasomotor dalam tikus OP. Untuk kajian tindak balas vaskular periferal, pelbagai dos intravena noradrenalina (0.15-5 µg/kg), fenilefrina (1-32 µg/kg) dan angiotensin II (3.75-120 ng/kg) menghasilkan tindak balas presor bergantungan dos yang lebih besar pada MAP dalam tikus OP yang ternyahsaraf berbanding tikus OR dan LF yang ternyahsaraf. Keputusan ini menggambarkan peningkatan dalam tindak balas vaskular periferal terhadap agen-agen vasoaktif yang mungkin telah menyumbang sebahagiannya kepada tekanan darah yang tinggi dalam tikus hipertensi teraruh obesiti.

STUDY ON ROLE OF EXCITATORY AMINO ACID INPUT ON ROSTRAL VENTROLATERAL MEDULLA NEURONS AND ASSESSMENT OF PERIPHERAL VASCULAR REACTIVITY IN OBESITY-INDUCED HYPERTENSION

ABSTRACT

Rostral ventrolateral medulla (RVLM) plays a pivotal role in the tonic and phasic regulation of blood pressure. The present study was carried out to determine whether the excitatory amino acid (EAA) input to the RVLM contributed to elevated blood pressure and to assess the peripheral vascular reactivity in obesity-induced hypertension. Male Sprague-Dawley rats were fed with low-fat diet or moderately high-fat (MHF) diet for 16 weeks. Then, rats on MHF diet were segregated into obesity-prone (OP) and obesity-resistant (OR) rats based on the body weight distribution. OR rats were defined as rats with body weight similar or less than control rats (LF rats) while OP rats were defined as rats with greatest body weight. At week 16, rats were subjected to study the role of EAA on RVLM neurons or to study the peripheral vascular reactivity in obesity-induced hypertension. In the former study, L-glutamate (1 nmol) and kynurenic acid (KYN) at concentrations of 4 nM, 40 nM and 4 μM were microinjected into RVLM and hexamethonium (20 mg/kg) was administered intravenously. Brain stem was removed for histological verification and adiposity index was calculated. OP rats exhibited significantly larger weight gain, higher adiposity index and also elevated blood pressure. Pressor response to L-glutamate was greater in OP rats than in OR and LF rats, suggesting an increased responsiveness of the RVLM towards EAA and increased role of EAA input to RVLM vasomotor neurons. KYN injection at 40 nM reduced mean arterial

blood pressure (MAP) in OP rats only while at 4 nM no significant change in MAP was observed in any of the groups. KYN injection at 4 µM increased MAP in all groups. Results obtained suggest an imbalance of EAA input to the RVLM of obese rats, whereby, the balance has shifted towards the excitation leading to elevation of blood pressure. Secondly, increased inhibition of glutamate excitatory pathway by KYN may unmask other excitatory pathway that awaits further investigation. Hexamethonium produced a greater decrease in MAP in OP rats than OR and LF rats thus suggesting the presence of elevated sympathetic vasomotor tone in OP rats. For peripheral vascular reactivity study, different doses of intravenous noradrenaline (0.15-5 µg/kg), phenylephrine (1-32 µg/kg) and angiotensin II (3.75-120 ng/kg) produced greater dose-dependent pressor responses in MAP of pithed OP rats than in pithed OR and LF rats, reflecting an enhancement in peripheral vascular responsiveness to vasoactive agents that might have partly contributed to the elevated blood pressure in obesity-induced hypertensive rats.

CHAPTER ONE

INTRODUCTION

1.1 Obesity

Obesity is a major health problem which is associated with poor quality of life and decreased life expectancy especially in the developed countries (Doll *et al*., 2002; Davy and Orr, 2009), and worryingly in developing countries, the prevalence of obesity is increasing (Antic *et al*., 2003). In human, obesity is defined as having body mass index (BMI = body weight (kg) / height $(m)^2$) greater than 30 (Barton *et al*., 2003; Caballero, 2003; Stapleton *et al*., 2008).

1.2 Characteristics of obesity

Obesity develops when a person's energy intake exceeds the expenditure, and body fat accumulates to an extent that can negatively affect health (Bruce-Keller *et al*., 2008; Gooren, 2008). This happens when there is lack of physical activity in lifestyle but excess calories intake which plays a dominant role in the development of obesity (Sowers, 2003; Eikelis and Esler, 2005).

There is a body of evidence that shows obesity as a result of inherited gene that confers susceptibility, and also the environment that is becoming more obesogenic (Diaz, 2002; Sowers, 2003; Mercer and Archer, 2008). Cigarettes smoking, nutritional factors, such as dietary fat and psychological stress also contribute to the development of the obesity (Barton *et al*., 2003).

Adipose tissue particularly the visceral adipose tissues express predominantly β_3-adrenergic receptors which play a pivotal role in the thermogenesis. Studies in human have shown that β_3-adrenergic receptor polymorphism affects the regulation of lipolysis and energy expenditure which eventually can act as a precursor to the pathology of obesity (Yasuda *et al*., 2006).

In obesity-prone individuals, the thresholds for sensing hormonal and metabolic signals are genetically greater. These signals are important in inhibiting weight gain through their action on the network of metabolic sensing neurons that regulate the energy homeostasis. Thus, the raised threshold will diminish the effects of inhibitory signals which convey the message to the brain in the situation of excess of energy stores (Levin, 2005).

Obesity has been associated with a number of diseases including hypertension, type II diabetes mellitus, atherosclerosis, focal-segmented glomerulosclerosis, albuminuria, dyslipidemia, insulin resistance and proinflammatory states (Diaz, 2002; Barton *et al*., 2003; Sowers, 2003; Grundy, 2004). Obstructive sleep apnea also presents in obese hypertensive patients, and obesity is also a predisposing factor for the psychological problem such as anger, anxiety and depression (Naderali, 2008).

1.3 Obesity-related hypertension

Obesity is intimately linked with hypertension in a way that an increase in blood pressure is closely related to the magnitude of weight gain (Landsberg, 1986; 1989; Rocchini *et al*., 1989; Hall *et al*., 1993). However, the exact mechanism linking both obesity and hypertension remains poorly understood. Previous studies have reported that approximately 75% of cases of hypertension can be attributed to obesity. For an increase of 10 kg of body weight, there is a parallel increase of 3.0 mmHg systolic and 2.3 mmHg of diastolic blood pressure. This minor increase in blood pressure can portend a 12% increase in coronary heart disease risk and a 24% increase in stroke risk (Mark *et al*., 1999; Aneja *et al*., 2004; Rahmouni *et al*., 2005a; Francischetti and Genelhu, 2007; Mittendorfer and Peterson, 2008).

1.3.1 Characteristics of obesity-related hypertension

Some alterations in hemodynamic parameters have been observed in obesity-related hypertension. Cardiac output and blood flow are significantly increased with weight gain (Hall *et al*., 1993; 1999). Expanded blood volume and elevated ventricular filling pressure lead to the increase in stroke volume whereas; diminished cardiac vagal tone leads to an increase in resting heart rate. Increases in these two parameters thereby, contributing to the higher resting cardiac output. Blood flow tends to increase in obesity-induced hypertension in order to perfuse the excess adipose tissue mass as well as other regions, and the summation raises the cardiac output (Hall *et al*., 2000; Davy and Hall, 2004).

Obese hypertensive human has been shown to exhibit activated renin-angiotensin system (RAS) and the sympathetic nervous system, elevated circulating leptin, and reduced growth hormone concentration (Montani *et al*., 2002). In addition, the levels of triglycerides and dyslipidemia are elevated that indicate low levels of HDL-cholesterol. Glomerulosclerosis in the kidney may occur as a result of hyperlipidemia which eventually leads to alteration in kidney function (Hall, 2003). Increased oxidative stress is also observed in obesity-related hypertension that predisposes to atherosclerosis (Dobrian *et al*., 2000).

1.3.2 Sympathetic nervous system in obesity

Sympathetic nervous system (SNS) plays a crucial role in the regulation of metabolic and cardiovascular homeostasis. Several lines of evidence have shown that there is an elevated sympathetic nervous activity in obesity (Esler, 2000; Montani *et al*., 2002; El Atat *et al*., 2003). This long-term overactivity of SNS causes a rise in blood pressure via peripheral vasoconstriction and increased in renal tubular sodium reabsorption (Kassab *et al*., 1995; Rahmouni *et al*., 2005a). It is further supported by the observation that there is an increased muscle sympathetic nerve activity (MSNA) in obese normotensive subjects and lean hypertensive subjects. This study also demonstrates a greater increase in the MSNA of obese hypertensive subjects (Grassi *et al*., 2000; Alvarez *et al*., 2002). Elevated level of SNS can be stimulated by increasing the caloric intake whereas fasting can reduce the sympathetic nervous activity (Antic *et al*., 2003; Davy and Hall, 2004; Rahmouni *et al*., 2005a).

1.3.3 Aetiology of obesity-related hypertension

There are several potential mechanisms that contribute to the SNS activation in obese human. One of these is through RAS which seems to be activated in obesity (Boustany *et al*., 2004). In normotensive humans, infusion of angiotensin II increases the MSNA while inhibition of angiotensin converting enzyme decreases the activity (Davy and Orr, 2009). Treatment with angiotensin-converting enzyme inhibitor in obese dogs and obese hypertensive humans attenuates sodium retention and also decreases blood pressure thus supporting the significant role of angiotensin II in stimulating the renal sodium retention which then contributes to the elevation of blood pressure in obesity (Francischetti and Genelhu, 2007). Furthermore, it is well known that adipose tissues can release angiotensinogen (AGT) which is then converted to the active form of angiotensin II (Dandona *et al*., 2005; Rahmouni *et al*., 2005a; Mittendorfer and Peterson, 2008). Frederich and his colleagues had demonstrated that food intake plays a pivotal role in the expression of AGT in rat's adipose tissue. They reported that level of AGT expression was significantly reduced when the rat was fasting while refeeding caused a marked increase in AGT expression of adipose tissue. This phenomenon is accompanied with parallel changes in blood pressure whereby, fasting leads to a fall and refeeding causes a rise in blood pressure, respectively. Thus, this finding suggests that changes in AGT expression in adipose tissue might contribute to the changes in systemic blood pressure associated with fasting and refeeding as the level of plasma AGT and hepatic AGT expression had not changed. It has been proposed that the presence of tumor necrosis factor (TNF) α might be the possible stimulator for the AGT gene expression, as there was an overexpression of this TNF-α mRNA and protein in adipose tissue of obese

subjects and animals. In addition, stimulation of AGT expression by TNF-α has been suggested in rat liver (Frederich *et al*., 1992; Engeli *et al*., 2000).

It has also been reported that there is an upregulation of renin activity and aldosterone in obesity which lead to an increase in plasma volume and contraction of vascular smooth muscle, and in the end may result in increased blood pressure. Furthermore, previous study has also proven that weight loss is accompanied with a reduction in plasma renin activity and aldosterone which lead to a fall in blood pressure. Obesity may also be associated with an imbalance in the sympathovagal system. This was shown by heart rate variability studies and renal noradrenaline spillover studies. Obesity may increase the blood pressure as a result of increased sympathetic tone which leads to α_1-adrenoceptor stimulation (El Atat *et al*., 2003; Mittendorfer and Peterson, 2008).

Leptin, a 164-amino acid protein derived from adipocytes, promotes weight loss by reducing the food intake and increasing energy expenditure through an action on the arcuate nucleus of the hypothalamus. Plasma leptin level is elevated in obesity due to resistance to its anorectic and weight-reducing effects (Haynes, 2005). However, elevated level of leptin in obesity does preserve the activation of the renal sympathetic nerve activity and exerts its effects through activation of the ventromedial and dorsomedial hypothalamus. This condition is referred as selective leptin resistance (Aizawa-Abe *et al*., 2000; Zhang and Reisin, 2000; Rahmouni *et al*., 2005b; Francischetti and Genelhu, 2007). Administration of chronic leptin infusion in rats had significantly increased arterial blood pressure and heart rate as a result of increased sympathetic activity. This was confirmed by blockade of the adrenergic pathway which abolishes the effect (Monassier *et al*., 2006; Tentolouris *et al*., 2006).

Obese subjects are always accompanied with elevated insulin levels. Insulin acts on regions of the brain to promote reduction of food intake and increase of energy expenditure via sympathetically mediated thermogenesis. However, there is often a resistance to the actions of insulin in peripheral tissues. It has been reported that high levels of insulin in obese human and dogs do not have any effect on the salt excretion, sympathetic activity and blood pressure. Moreover, aspirin therapy to improve insulin resistance does not prevent the development of hypertension in high-fat diet treated dogs, thus indicating that insulin resistance is not responsible for obesity-associated hypertension. Additionally, SNS activity and blood pressure are not elevated in patients with insulinoma, eventhough there is a presence of high fasting insulin plasma levels compared to lean subjects (Francischetti and Genelhu, 2007). In dogs fed with high fat diet, clonidine pretreatment, a centrally active α_2 – agonist which inhibits central nervous sympathetic activation prevents the development of insulin resistance and hypertension (Rocchini *et al*., 1999). Further study by Rocchini *et al.* (2004) showed that peripheral α_1– and β-blockade did not prevent the obesity-induced insulin resistance, indicating that hypertension and insulin resistance in obesity are not directly related. In contrast, high levels of insulin in rats do elevate blood pressure that may be mediated via complex interaction between insulin, RAS and thromboxane (TXA_2) activity (El Atat *et al*., 2003; Rahmouni *et al*., 2005a).

Obstructive sleep apnea (OSA) is another potential mechanism that can contribute to the activation of SNS in obesity-induced hypertension. OSA which is common in obesity is believed to evolve from episodic nocturnal sympathetic stimulation into ongoing, daytime sympathetic nervous activation. This is supported

by study on lean subjects with OSA which shows that there is some elevation of sympathetic tone. In addition, study on obese subjects with OSA shows a greater elevation of sympathetic tone (Esler *et al*., 2006).

Obesity also shows a characteristic of low-grade systemic inflammatory state as the adipocytes itself can release pro-inflammatory cytokines, interleukin-6 (IL-6) and TNF-α. Emerging data has shown the link between increased inflammation with a rise in blood pressure (Grundy, 2004; Mittendorfer and Peterson, 2008).

Elevated free fatty acids (FFA) in obesity may cause a rise in blood pressure through two major mechanisms, namely endothelial dysfunction and sympathetic activation. High levels of FFA may inhibit the nitric oxide synthase activity thus blunts the vasodilatory response. FFA also has the capability to stimulate generation of oxygen radical from vascular endothelial and smooth muscle cells via activation of NADPH oxidase, thereby adding to the decreased nitric oxide bioavailability (Montani *et al*., 2002). It also can interact with the SNS by enhancing the vascular response to α-adrenergic agonists (Antic *et al*., 2003). Engeli and Sharma (2001) have also proposed that adipocyte-derived fatty acid can boost the release of a hepatic stimulator of aldosterone synthesis.

Obesity has also been associated with several alterations in renal structure and functions. Adipose tissue which surrounds the kidney may increase the intrarenal pressure leading to impairment of pressure natriuresis, hence, contributing to the development of hypertension in obesity. Alterations in the histology of the renal medulla, such as increased interstitial cells and extracellular matrix mainly by glycosamino-glycan and hyaluran component, cause a rise in renal interstitial fluid pressure and tissue oedema. Consequently, the vasa recta blood flow is reduced

while tubular reabsorption is increased due to compression of the thin Henle's loops (Sharma, 2004; Francischetti and Genelhu, 2007).

Natriuretic peptides act on its specific receptors to exert its effects on the plasma volume, renal sodium handling and blood pressure. These peptides can dampen the baroreceptor function and lower the activation threshold of vagal efferents which results in suppression of reflex tachycardia and vasoconstriction due to decreased extracellular volume. In obese subjects, there is an overexpression of the natriuretic peptide receptor type C (Npr-C) in adipose tissue which can negatively affect the systemic activity and actions of these peptides. As a result, these will lead to sodium retention and development of hypertension. Studies have shown that in obese subjects, urinary sodium excretion has been delayed whilst response of plasma ANP to saline load is blunted (Zhang and Reisin, 2000; El Atat *et al*., 2003; Aneja *et al*., 2004).

Adiponectin, a 30 kDa protein which is also secreted by white fat, is known to enhance insulin sensitivity and also can stimulate oxidation of fatty acid in muscle. An *in vitro* study has shown that adiponectin can relax the aorta and mesenteric arteries. Adiponectin also can exert its effects on blood vessels whereby, its plasma levels have been correlated to vasodilator responses in human. Obesity has been associated with low plasma level of adiponectin while weight loss is associated with high plasma level of adiponectin (Sharma, 2004; Morris, 2008).

Sympathetic activity can affect the baroreflex sensitivity. It has been shown that baroreflex sensitivity is reduced in obese man as compared to lean where men with higher visceral fat had lower baroreflex sensitivity than those with lower visceral fat (Grassi *et al*., 2000; Tentolouris *et al*., 2006).

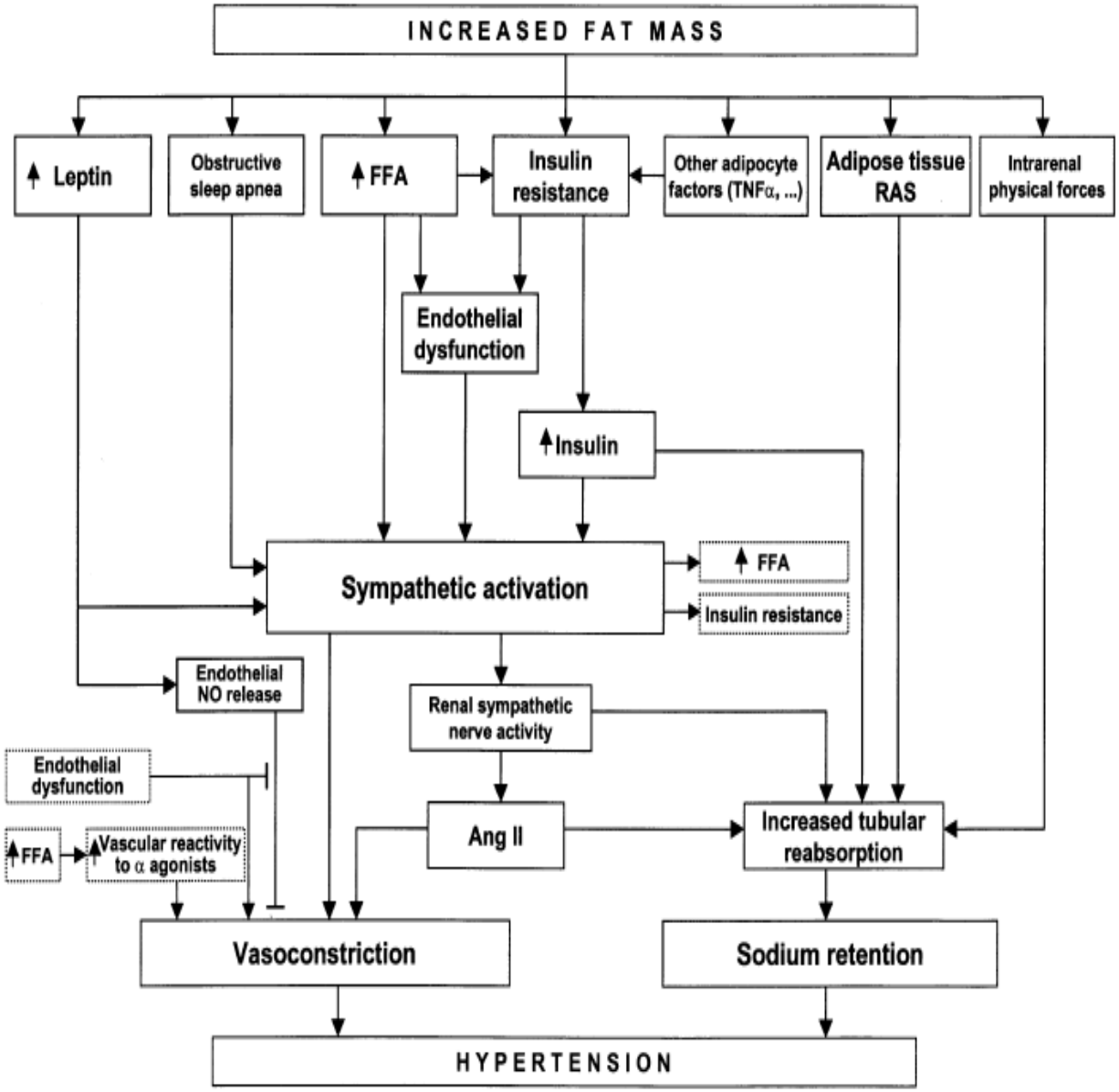

Figure 1.1: General scheme of possible mechanisms involved in obesity-induced hypertension. A number of factors induced or potentiated by obesity lead to sympathetic activation, vasoconstriction and sodium retention, all promoting hypertension. FFA, free fatty acids; TNF-α, tumor necrosis factor; RAS, renin-angiotensin system; NO, nitric oxide; Ang II, angiotensin II. Adapted from Montani *et al*. (2002).

1.4 Rat's model of obesity-induced hypertension

Animal models have been widely used as a tool to study the susceptibility to obesity in human. It includes genetically obese animals (Zucker rats), surgically induced obese rats (ventromedial hypothalamus-lesioned rat), spontaneously obese animals (rhesus monkeys) and diet-induced obese rat and mouse. Among these models, the dietary model developed by Levin in 1983 is the most relevant with regard to human obesity as it allows one to examine resistance and susceptibility to obesity. It also represents the genetic and environmental factors that may contribute to the development of obesity (Lauterio *et al*., 1994; 1998; Rahmouni *et al*., 2005b; Reuter, 2007) and also allows one to dissociate between the factors related to diet and obesity per se (Dobrian *et al*., 2000). However, that particular study by Levin (1983) did not measure any cardiovascular variables.

In this model, rats on moderately high-fat diet (32% kcal as fat) for a certain period of time will be segregated into different groups based on the body weight distribution. One third of the rats whose body weight gain is greater, is considered as obesity-prone rats while one third lower is considered as obesity-resistant rats and the remainder is excluded from the study (Lauterio *et al*., 1998). A number of studies demonstrated the presence of elevated level of blood pressure, cholesterol and insulin, activation of RAS and increase in renal oxidative stress in obesity-prone rats (Carroll *et al*., 2006). Other than that, obesity-prone rats also exhibit characteristics that mimic the obese hypertensive patients, such as increased plasma noradrenaline response to intravenous glucose, increased plasma leptin concentration and decreased growth hormone secretion and synthesis (Dobrian *et al*., 2000).

1.5 Central baroreceptors pathway

Baroreceptor reflex is important in maintaining the perfusion pressure in the face of disturbances of circulatory homeostasis through a number of neuronal and humoral regulatory adjustments. Alteration in the pressure load at the specialized pressure sensors which are located on the walls of aortic arc and carotid sinuses initiates these adjustments (Stauss, 2002).

Baroreceptor afferent fibers that run from glossopharyngeal and vagal nerves provide tonic, excitatory input to neurons of the nucleus of the solitary tract (NTS) which is subjacent to the area postrema in the dorsomedial medulla during the resting blood pressure levels. Then, second-order NTS neurons that convey the baroreceptor signals project to caudal and intermediate part of the ventrolateral medulla (VLM) and excite the neurons. Neurons from the caudal part of the VLM (CVLM) project to rostral part of the VLM (RVLM) and inhibit the sympathoexcitatory neurons. It has been demonstrated that blockade of the inhibitory pathway to RVLM eliminate the baroreflex (Dampney *et al*., 2001; Alzamora *et al*., 2006; Kawabe *et al*., 2008).

The neurons from the RVLM further project to sympathetic preganglionic neurons in the intermediolateral column of the spinal cord which is important in controlling the sympathetic vasomotor outflow (Bergamaschi *et al*., 1995; Gordon and Sved, 2002; Morrison, 2003; Iigaya *et al*., 2007). Baroreceptor sympathetic efferents regulate the heart, kidneys and also noradrenaline release (Guyenet, 2006). Meanwhile, alteration in the cardiac parasympathetic nerve activity which is evoked by baroreceptor is mediated by a direct excitatory projection from second order neurons in the NTS to the preganglionic parasympathetic neurons in the nucleus ambiguus (Sved *et al*., 2001).

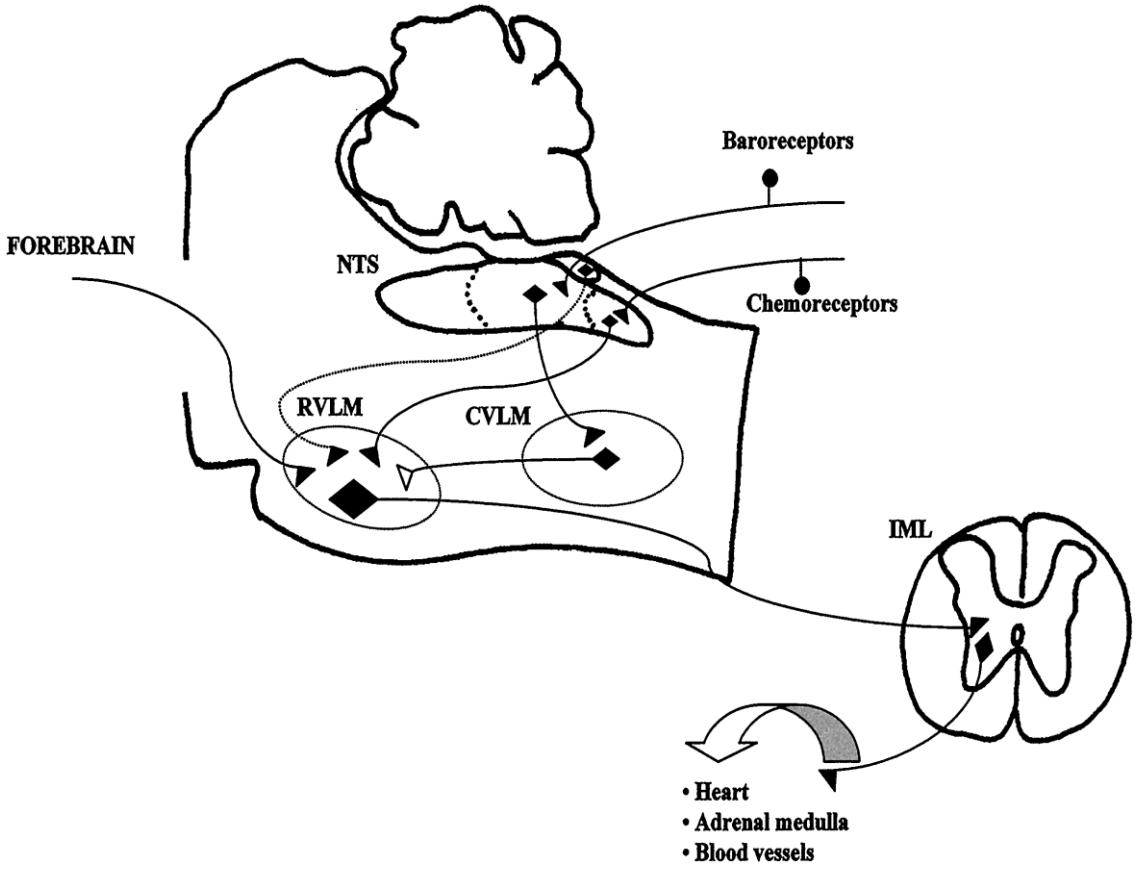

Figure 1.2: Schematic sagittal view of the medulla oblongata depicting neural pathways involved in neurogenic hypertension. Premotor neurons of the RVLM provide excitatory drive to preganglionic neurons in the intermediolateral cell column (IML), which provide sympathetic output to target organs. RVLM neurons receive excitatory (▲) inputs from (1) forebrain structures, (2) commissural NTS, and (3) area postrema, and inhibitory (Δ) inputs from the CVLM. The intermediate and commissural NTS represent the primary site of projection of afferent fibers arising from baro- and chemoreceptors. Increased excitatory inputs and/or decrease of inhibitory inputs result in enhancement of RVLM activity, thus increasing sympathetic output, a common feature of different forms of hypertension. Adapted from Colombari *et al*. (2001).

1.5.1 Central baroreceptor pathway in obesity-induced hypertension

Baroreflex play a pivotal role in the acute and chronic regulation of body fluid volumes and arterial pressure but in chronic hypertension, baroreflex function is often impaired. There is an intriguing finding that shows the baroreflexes rapidly adapt and reset towards the existing level of the arterial pressure, but the magnitude and the time course of resetting remain unclear. Recently, a number of studies have suggested that baroreflexes resetting is incomplete in hypertension (Lohmeier *et al*., 2002; 2003; 2007).

Study using Fos-Li immunoreactivity in model of obesity-induced hypertension has shown that there is a chronic activation of the neurons subserving the baroreflexes. In this study, increased Fos staining in NTS and CVLM were observed thus indicating important roles of these regions in mediating baroreflex inhibition of sympathoexcitatory cells in RVLM. This finding is supported by the observation in animals with intact baroreflexes which shows similar response during acute elevations in arterial pressure, and also study in animals without baroreflexes which shows no increase in Fos staining in NTS and CVLM. Therefore, these findings indicate that activation of baroreflex is sustained during obesity hypertension and thus baroreflex-mediated sympathoinhibition is a long-term compensatory response in this model of hypertension. Eventhough activation of central baroreflex pathway is sustained in obesity hypertension, it has been shown that Fos-Li expression is significantly increased in the RVLM of obese dogs (Lohmeier *et al*., 2003), thereby supports other studies that showed increased sympathetic activity to the kidney and other vascular beds in obesity hypertension (Esler, 2000; Hall *et al*., 1999). Additionally, the presence of increased number of

Fos-Li cells in obese dogs may reflect the dominant action of excitatory inputs over chronic inhibitory effects of the baroreflex to the RVLM (Lohmeier *et al*., 2003).

Another study in obesity-induced hypertensive rats with intact baroreflex showed that reflex bradycardia was reduced during pressor responses to phenylephrine but no difference was observed in reflex tachycardia during depressor responses to sodium nitroprusside in both obese hypertensive rats and control. Similar inhibition of reflex tachycardia was seen in both groups after either cholinergic or β-adrenergic blockade by methylatropine or propranolol. Meanwhile, reflex bradycardia was reduced more in obese-hypertensive rats than in control rats but equally reduced after β-adrenergic and cholinergic blockade respectively. Hence, it is postulated that selective impairment of reflex bradycardia might be due to deficient of parasympathetic activity since blockade of β-adrenergic would leave only residual reflex responses by parasympathetic activity (Bunag *et al*., 1990).

1.5.2 Rostral ventrolateral medulla

Rostral ventrolateral medulla (RVLM) plays a pivotal role in the tonic and phasic regulation of cardiovascular function (Lipski *et al*., 1996; Araujo *et al*., 1999; Campos and McAllen, 1999; Ito *et al*., 2001; 2002; Mayorov and Head, 2002; Granata and Cohen, 2004; Oshima *et al*., 2006; Menezes and Fontes, 2007). RVLM bulbospinal neurons that project directly to sympathetic preganglionic neurons in the spinal cord, thus called ‘presympathetic’ neurons are a major source of basal tonic sympathetic vasomotor activity (Yang and Coote, 1998; Tagawa and Dampney, 1999; Potts *et al*., 2000; Hu *et al*., 2002; Horiuchi and Dampney, 2002; Dampney *et*

al., 2003; Oshima *et al.*, 2006;). These tonic excitations of sympathetic vasomotor outflow are important in maintaining normal arterial blood pressure (Brooks *et al*., 2004; Horiuchi *et al*., 2004). Therefore, any neuronal inhibition of RVLM will markedly result in decrease in arterial pressure. These RVLM neurons receive both excitatory and inhibitory synaptic inputs resulted from stimulation of peripheral receptors and also higher center in the brain (Horiuchi *et al*., 2004).

Numerous studies have shown that RVLM neurons are tonically active but the tonic excitatory drive to the neurons remains unclear (Horiuchi *et al*., 2004; Stocker *et al*., 2007). However, it is believed that the tonic excitatory input arise from one or combination of afferents to the RVLM. Studies on neuroanatomical has documented some brain regions which project to the vasomotor area of the RVLM. In rats, some regions have been identified including CVLM, NTS, area postrema, raphe nuclei, A5 neurons, hypothalamic paraventricular neurons (PVN), central amygdaloid nucleus, periaqueductal gray (PAG), parabrachial nucleus and lateral hypothalamic area (LHA). Inhibition of these brain regions individually did not lead to marked decrease in arterial pressure indicating that there might be two or more regions that drive tonic excitatory input to the RVLM. Thus, marked decrease in arterial pressure would only be observed after inhibition of correct combination of these regions (Sved *et al*., 2001; Pilowsky and Goodchild, 2002; Dampney *et al*., 2003).

1.5.2.1 Neurotransmitters and neuromodulators in RVLM

RVLM neurons consist of heterogenous cell population whereby, different genes, different complement of receptors, transmitters, enzymes and other proteins are found in different subpopulations of bulbospinal RVLM neurons. These includes glutamate, catecholamines, preproenkephalin/enkephalin, substance P, neuropeptide Y, cocaine- and amphetamine-regulated transcript, preprogalanin, calbindin, $GABA_A$ and $GABA_B$ receptors, P2X receptors, $alpha_{2A}$-adrenergic receptors, $angiotensin_{1A}$ receptors, and mu opioid receptors. Among these, the key for neurochemical within RVLM are those involved glutamate and GABA transmission (Pilowsky and Goodchild, 2002).

Glutamate is an excitatory amino acid (EAA) which acts as a principle neurotransmitter in the central nervous system (Goren *et al*., 2000; Gonzalez and Robinson, 2004; Maragakis and Rothstein, 2004; Shigeri *et al*., 2004; Bridges and Esslinger, 2005). Glutamate is released into the synaptic cleft and bind to glutamate receptors resulted in propagation of action potential. Apart from glutamate receptors, glutamate transporters also play a role in modulation of the synaptic activity by removing the glutamate from the synaptic cleft. Studies have proven the importance of glutamate in normal central nervous system synaptic function. However, excess accumulation of glutamate in the synaptic cleft can lead to neurotoxicity. Defects in the function of glutamate transporters lead to a rise in extracellular glutamate which then results in the development of neurologic disease. Glutamate transporters dysfunction might be a result of alteration in transcription or splicing, increased turnover of the transporters, altered trafficking of glutamate transporter, abnormal

protein phosphorylation and reduced transport capacity (Maragakis and Rothstein, 2004; Boeck *et al*., 2005).

Glutamate can act on two main classes of receptors which are ionotropic (ion-channel linked) and metabotropic receptors (G-protein linked) (Tsuchihashi *et al*., 1994). The latter receptors couple to the intracellular second messenger cascades via G-proteins (Tsuchihashi *et al*., 2000; Herlenius and Lagercrantz, 2004; Vandenberghe and Bredt, 2004; Galik *et al*., 2008). Ionotropic glutamate receptors can be subdivided into two types which are N-methyl-D-aspartate (NMDA) and non-NMDA receptors based on their sensitivity to agonists and differing molecular structures. Non-NMDA receptors can be further classified into kainate and AMPA receptors. Each class of glutamate receptors (GluRs) has their own subunits whereby, NMDA receptors consist of NR1and NR2A-NR2D. Meanwhile, GluR1-GluR4 represents the AMPA-sensitive family and GluR5-GluR7, KA1 and KA2 belong to kainate family (Smart, 1997; Pamidimukkala *et al*., 2002).

Gamma-aminobutyric acid (GABA) is a dominant inhibitory neurotransmitter in the central nervous system of the mature animal. GABA can acts on two types of receptors namely, $GABA_A$ and $GABA_B$ receptor. $GABA_A$ receptor is a transmembrane protein wherein, other substances like barbiturate and benzodiazepines can bind to specific sites and thus modulating the opening properties of the chloride channel. These receptors have different pharmacological and electrophysiological properties depending on their subunit composition. $GABA_B$ receptor (G protein-coupled) which is present in lower level in the central nervous system, performs its function late in the development of central nervous system (Herlenius and Lagercrantz, 2004).

Nerve cells contain high concentration of chloride during the early development of the central nervous system. Therefore, opening of the chloride channel by GABA leads to depolarization (i.e., excitation) to occur. By contrast, the decrease in chloride concentration during maturation led to an opposite effect of GABA whereby, chloride ions are pumped out and cell hyperpolarization occurs. Hence, GABA switches its role from excitatory to inhibitory neurotransmitter in the mature animal (Herlenius and Lagercrantz, 2004).

Kynurenic acid (KYN), an endogenous tryptophan metabolite is present in the mammalian central nervous system. Increase level of KYN in the brain is related with sedation, analgesia and reduction of post-ischaemic or post-inflammatory damage. KYN has the capability to reduce the neurotoxic effects of glutamate by acting on the glutamate ionotropic receptors (Stone and Addae, 2002; Prescott *et al*., 2006; Moroni *et al*., 2007).

Nitric oxide (NO) is present with varying amounts in most of the brain regions. NO is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS) (Raevskii, 1997). NO plays a vital role in many functions of the nervous system including memory, learning, vision and cardiovascular regulation. NO can act centrally, decreasing the sympathetic nerve activity and blood pressure (Dominiczak and Bohr, 1995; Zhang and Patel., 1998; Patel *et al*., 2001). Recent study reported that high levels of NO can reduce glutamate presynaptic release via inhibition of presynaptic N-type Ca^{2+} -channel activity thereby, depressing the RVLM neurons (Tai *et al*., 2005).

1.5.2.2 Tonic glutamatergic input to RVLM presympathetic neurons

Numerous studies have demonstrated that RVLM presympathetic neurons receive excitatory glutamatergic synaptic input which originate from both medullary and supramedullary regions (Dampney *et al*., 2003). There are some possible sources of this glutamatergic synaptic input such as input from pontine reticular formation, lateral tegmental field and also from the PVN of the hypothalamus. Blockade of ionotropic glutamate receptors in the RVLM of cat and rabbit caused a profound reduction in blood pressure indicating that glutamatergic synaptic input is the main source of tonic excitatory drive to RVLM (Barman *et al*., 2000; Hourichi and Dampney, 2002; Sved *et al*., 2002).

1.5.2.3 Tonic GABAergic input to RVLM presympathetic neurons

Studies have reported that there is a presence of tonic GABAergic input to the RVLM neurons. This GABAergic input to RVLM is mediated partly via neurons of the caudal aspect of the VLM (CVLM) (Sved and Gordon, 1994). Furthermore, tonic GABAergic input to the RVLM presympathetic neurons are also partly engaged in baroreceptor-independent regulatory mechanism. Inhibitory action of GABA on RVLM neurons is mediated preferentially through activation of $GABA_A$ receptor. Blockade of this $GABA_A$ receptor in the sympathoexcitatory regions of the RVLM eliminates the action of inhibitory vasomotor neurons from the caudal part thus results in large increase in sympathetic nerve activity and blood pressure (Moreira *et al*., 2005; Menezes and Fontes, 2007).

1.5.2.4 Intrinsic pacemaker of RVLM

Apart from synaptic input which is driven by neurons located elsewhere in the brain, it has been speculated that tonic activity of RVLM neurons can also be generated by intrinsic pacemaker activity which is capable of generating action potentials (autoactivity) (Lewis and Coote, 1993; Dampney *et al*., 2003; Coote, 2006). This theory arises due to failure of blockade of glutamate receptors to cause significant reduction of tonic discharge of RVLM neurons. In addition, removal of synaptic input by blockade of excitatory or inhibitory EAA receptors or during perfusion of low-Ca^{2+} high-Mg^{2+} solution did not affect the regular and irregular discharge of RVLM neurons in brain slice. This theory is further supported by the fact that action potential in RVLM neurons was not initiated by EPSPs, the regular discharge was reset by an experimentally given stimulus and the discharge was eliminated by membrane hyperpolarization with no residual EPSPs. Besides, other membrane properties which were exhibited by the cells could also contribute to the intrinsic oscillating depolarization (Lewis and Coote, 1993; Coote, 2006).

Nevertheless, such intrinsic activity of 'pacemaker' neurons was only recorded *in vitro* in isolated medulla and spinal cord but not *in vivo*. Intracellular recordings of identified RVLM presympathetic neurons *in vivo* showed irregular low-frequency action potentials which triggered by EPSPs (Lipski *et al*., 1996; Coote, 2006) and in accordance with the pattern of discharge from synaptic drive observed in sympathetic preganglionic neurons (Coote, 2006). This theory also failed to explain the basic rhythmic oscillations of activity in RVLM presympathetic neurons which are related with similar activity in postganglionic sympathetic nerves.

It also cannot explain the non-uniformity of sympathetic activity in different vasomotor nerves.

Therefore, synaptic drive is the most probably source of tonic activity in RVLM neurons in unchallenged adult animal. However, some considerable condition should be taken into account. For instance, specific slow synaptic input due to its removal could induce activation of selective inward currents which has been shown to trigger beating activity in RVLM neurons *in vitro* as a response to challenging environmental stimuli (Coote, 2006).

1.5.2.5 RVLM in obesity-induced hypertension

Sved and his colleagues had proposed that sympathetic vasomotor outflow from RVLM neurons is maintained by balance of excitatory and inhibitory input (Sved *et al*., 2001). In normotensive rats, administration of KYN, an EAA antagonist into RVLM did not produce any effect on the arterial blood pressure (Ito *et al*., 2002). This indicated that under resting conditions, direct excitatory and indirect inhibitory influences of EAA are in perfect balance whereby, activation of the CVLM inhibitory mechanisms counterbalances the excitatory drive to the RVLM neurons. Blockade of the EAA receptors in the RVLM simultaneously removed both of the influence of tonic excitation and inhibition which led to no net change in sympathetic vasomotor activity and arterial blood pressure (Sved *et al*., 2002; Horiuchi *et al*., 2004). However, blockade of EAA receptors in RVLM in combination with CVLM inhibition using muscimol (GABA agonist) did cause a profound fall in arterial blood pressure indicating that RVLM does receive important

tonic excitatory drive. In contrast, injection of muscimol into CVLM alone results in increase of both sympathetic activity and arterial blood pressure due to release of excitatory mechanisms to the RVLM (Moreira *et al*., 2005).

However, in models of experimental hypertension, the excitatory and inhibitory balance is altered (Ito *et al*., 2002). In Dahl-sensitive model, the balance of excitatory amino acid input was disrupted, whereby; the balance is shifted towards excitation in Dahl-sensitive rats as compared with Dahl-resistant rats. A significant drop in mean arterial pressure was recorded after microinjection of KYN into the RVLM (Ito *et al*., 2001). In addition, disruption in the balance of excitatory and inhibitory input was also observed in spontaneously hypertensive rat (SHR) (Ito *et al*., 2000).

In addition, recent study has proven that the RVLM neurons do contribute to obesity-induced hypertension in rats. However, the finding only indicates that the tonic activity of RVLM neurons is necessary for the elevated blood pressure in obesity-hypertension rats but it does not demonstrate that the tonic activity of RVLM neurons is elevated (Stocker *et al*., 2007).

Thus, this present study aims to investigate the neural pathways that underlie the activation of RVLM neurons by determining whether EAA input particularly L-glutamate within the RVLM neurons do contribute to elevated blood pressure in obesity. For this purpose, we investigate further if the microinjections of EAA-receptor antagonist into RVLM vicinity result in a reduction of arterial pressure in obesity-induced hypertensive rats.

1.6 Study on peripheral vascular reactivity

Previous study has demonstrated that elevated blood pressure in diet-induced obesity rats is associated with an enhanced vascular contractility (Boustany-Kari *et al*., 2007). However, such finding was observed in *in vitro* preparation of the mesenteric arteries of the rats, thus not presenting the whole animal system. Numerous studies has been carried out using pithed rats (Fozard and Leach, 1968; Barret, 1971; Dong *et al*., 1995; Gavin and Docherty, 1996; Zhou and Vargas, 1996; Dendorfer *et al*., 1999; Elayan *et al*., 2002; 2008; Cobos-Puc *et al*., 2007) as pithing eliminates the central reflex responses to the pressor effects upon peripheral stimulation. Therefore, availability of this pithing technique enables us to assess *in vivo* peripheral vascular reactivity in the rat's model of obesity-induced hypertension.

1.6.1 Endothelial dysfunction in obesity-induced hypertension

Obesity is closely related with increased risk for hypertension whereby, inflammation, vascular remodeling and changes in vascular reactivity are the leading role in the pathogenesis of this disease (Mundy *et al*., 2007). Abnormalities of arterial function and structures have been observed in obese human and in certain animal models of obesity including the changes in release of vasoactive substances from the endothelial cells and also elasticity of the arterial wall. Naderali and his colleagues have reported that endothelial dysfunction can occur as a result of acute consumption of a palatable diet long before development of any significant obesity. Short term withdrawal of this palatable diet incompletely corrected the endothelial dysfunction. However, endothelial dysfunction can be corrected completely by

chronic removal of palatable diet which indicates that diet per se plays an important role in the development of vascular abnormalities in animal models of obesity (Naderali *et al*., 2004).

Endothelial dysfunction in obesity is due to alteration in the synthesis/release of prostanoids and nitric oxide. These substances play a vital role in the regulation of the vascular tone (Mascher *et al*., 2006). Prostacyclin (PGI_2) is mainly generated in endothelial cells from arachidonic acid. PGI_2 can cause vasodilation through activation of protein kinase A which reduce the myosin light chain kinase activity and also can inhibit platelet aggregation (Khazaei *et al*., 2008).

Experimental studies in animal model of obesity has shown the presence of oxidative stress which can lead to the endothelial dysfunction. Oxidative stress can block the nitric oxide synthase and also inactivate the action of nitric oxide, thus impairing the NO-mediated vasorelaxation. It can also cause an increase in generation of endothelin-1 and increase effects of the superoxide anion and hydrogen peroxide on vascular smooth muscle cells (Wolk *et al*., 2003).

Insulin acts as a vasodilator that can affect the peripheral resistance in normal vasculature. Insulin acts by inhibiting the voltage-gated Ca^{2+} influx and stimulating the glucose transport and glucose phosphorylation, thus leading to activation of Ca^{2+} ATPase transcription and also increase in cellular Ca^{2+} efflux. As a result, there is decrease in vascular resistance due to net decrease of intracellular Ca^{2+}. However, the presence of insulin resistance in obesity blunt these effects, thereby increases the vascular resistance (Zhang and Reisin, 2000).