T.R.

ESKISEHIR OSMANGAZI UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF MEDICAL PHARMACOLOGY

INVESTIGATION THE EFFECTS OF HYDROGEN SULFIDE TREATMENT ON GLIA-MEDIATED NEUROINFLAMMATION IN HYPERTENSION

DOCTORAL THESIS

BASAK DONERTAS

ADVISORS

Prof. BASAR SIRMAGUL

Assist. Prof. JASENKA ZUBCEVIC

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Funded by The Scientific and Technological Research Council of Turkey (TUBITAK), 2214-A International Research Fellowship Programme for PhD Students

ACCEPTANCE AND APPROVAL FORM

This study entitled "Investigation the effects of hydrogen sulfide treatment on glia-mediated neuroinflammatıon in hypertension" prepared by Basak DONERTAS has been "**ACCEPTED**" as a Doctorate Thesis in accordance with the related article of the Graduate Education and Training Regulation of Eskisehir Osmangazi University.

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ÖZET

Hipertansiyonda glia aracılı nöroinflamasyonda hidrojen sülfür tedavisinin etkilerinin araştırılması

Otonom nöral yolakların aktivasyonu hipertansiyon patogenezinde önemli rol oynamaktadır. Hipotalamik paraventriküler nükleus (PVN) kan basıncı ve semptatik aktivitenin kontrolünde önemli bir kardiyoregülatör merkezdir. Hidrojen sülfür (H_2S) nöromodülatör, antiinflamatuvar, antioksidan ve antihipertansif özelliklere sahip endojen bir moleküldür. Bu araştırmada, kronik intraserebroventriküler (icv) sodyum hidrosülfit (NaHS) uygulamasının, sıçanlarda, anjiyotensin (Ang) II ile indüklenen hipertansiyon ve sempatik aktivite artışı üzerindeki etkilerinin araştırılması amaçlanmıştır. Erişkin, erkek, Sprague Dawley sıçanların abdominal aortuna kan basıncı ölçümü için radyotelemetri transmitterleri yerleştirilmiştir. Bazal kan basıncı ölçümlerinin ardından sıçanlar rastgele 6 gruba ayrılmıştır (i) Kontrol; (ii) Hipertansiyon (HTN); (iii) icv 30 nmol/s NaHS; (iv) icv 30 nmol/s+HTN; (v) icv 60 nmol/s NaHS; (vi) icv 60 nmol/s NaHS+HTN. HTN oluşturmak için sıçanlara subkütan (sc) olarak yerleştirilen mini ozmotik pompalar aracılığıyla 4 hafta boyunca kronik Ang II (200 ng/kg/dk) uygulanmıştır. Icv NaHS ya da icv PBS, sc Ang II ya da salin infüzyonuyla eş zamanlı olarak uygulanmıştır. Deney sonunda sıçanların beyin, kalp dokuları ve kan ve serebnospinal sıvıları (SS) analiz için toplanmıştır. HTN grubuyla karşılaştırıldığında, 4. haftadaki ortalama arteriyal kan basıncının icv NaHS ile tedavi edilen HTN grubundaki sıçanlarda anlamlı düzeyde daha düşük olduğu saptandı ($P<0.001$). Kan basıncı değerleri ile uyumlu olarak kontrol grubuna göre HTN grubunda sol ventrikül kalınlığının anlamlı derecede yüksek olduğu ($p<0.0001$), 60 nmol/s NaHS+HTN grubunda anlamlı derecede düşük olduğu saptandı ($p<0.0001$). 30 nmol/s NaHS+HTN grubunda bu bakımdan anlamlı bir farklılık görülmedi. Icv 60 nmol/h NaHS tedavisinin Ang II ile indüklenen sempatik aktivite artışını hafiflettiği saptandı. Plazma ve SS'deki H_2S düzeylerinde gruplar arasında anlamlı farklılık saptanmadı. PVN'deki $Iba1^+$ mikroglia hücrelerinin

sayısının HTN grubunda kontrol ve 30 NaHS/s+HTN grubuna göre anlamlı düzeyde düşük olduğu saptandı ($p<0.05$). Elde edilen sonuçlara göre santral H_2S uygulaması, Ang II ile indüklenen kan basıncı ve sempatik aktivite artışını azaltmakta ve bu etkide PVN'deki nöroinflamasyonu hafifletici etkisinin olabileceği düşünülmektedir.

Anahtar kelimeler: Hidrojen sülfür, hipertansiyon, mikroglia, nöroinflamasyon, PVN

SUMMARY

Effects of hydrogen sulfide treatment on glia-mediated neuroinflammation in hypertension

Activation of autonomic neural pathways plays a significant role in pathogenesis of hypertension (HTN) and the paraventricular nucleus (PVN) is a major cardioregulatory brain region in regulating blood pressure (BP) and sympathetic nerve activity (SNA). Hydrogen sulfide (H_2S) is an important gaseous signaling molecule with neuromodulatory, anti-inflammatory, anti-oxidant and anti-hypertensive effects. The aim of this study was to explore whether chronic intracerebroventricular (icv) infusion of NaHS, an H_2S donor, would alleviate BP increase and sympathetic overactivity in Angiotensin (Ang II)-induced hypertensive rats via attenuation of neuroinflammation. Adult, male, Sprague Dawley rats were implanted with radiotelemetry transmitters in the descending aorta. Following baseline BP measurements, rats were divided into 6 groups: (i) Control; (ii) HTN; (iii) 30 nmol/h NaHS; (iv) 30 nmol/h NaHS-treated HTN (v) 60 nmol/h NaHS; (vi) 60 nmol/h NaHS-treated HTN rats. HTN was induced by chronic infusion of Ang II (200 ng/kg/min) for 4 weeks using subcutaneously placed mini-osmotic pumps. Icv NaHS or phosphate buffered saline (PBS) was administered simultaneously with subcutaneuos Ang II or saline infusion. At endpoint, brain and heart tissues and plasma and cerebrospinal fluid (CSF) were collected for further analysis. At week 4, mean arterial pressure (MAP) was significantly reduced icv NaHS treated HTN groups compared to HTN group ($P<0.001$). In line with MAP data, left ventricular (LV) wall thickness was increased in Ang II-induced HTN group compared to control ($p<0.0001$), which was significantly decreased by chronic 60 nmol/h ($p<0.0001$) but not in the 30 nmol/h NaHS treated group. Icv 60 nmol/h NaHS but not 30 nmol/h NaHS, was able to normalize the Ang II–perturbed sympathetic overactivity. No significant difference was found in H_2S levels in plasma and CSF among groups. Quantification of $Iba1^+$ microglial cells in the PVN of Ang II-infused rats showed that Ang II infusion significantly increased the number of microglial cells compared

to control ($p<0.05$), while our preliminary data show that icv 30 nmol/h NaHS treatment normalized the numbers of microglia in the PVN of Ang II-infused rats. These findings suggest that central H_2S attenuates sympathetic activity and hypertensive response, which are partly due to attenuation of neuroinflammation within the PVN in Ang II-induced HTN.

Key words: Hydrogen sulfide, hypertension, microglia, neuroiflammation, PVN

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LIST of ABBREVIATIONS

ACE: Angiotensin converting enzyme

ACEI: Angiotensin converting enzyme inhibitors

Ang: Angiotensin

ANS: Autonomic nervous system

ARB: Angiotensin receptor blockers

ATP: Adenosine triphosphate

AT1R: Ang II type 1 receptor

AT2R: Ang II type 2 receptor

BBB: Blood brain barrier

BP: Blood pressure

CAT: Cysteine aminotransferase

cATP: Cyclic adenosine triphospate

CO: Cardiac output

CO: Carbon monoxide

COX: Cyclooxygenase

CBS: Cystathionine ß-synthase

CCB: Calcium channel blockers

cGMP: Cyclic guanosine monophospate

CSE: Cystathionine γ-lyase

CSF: Cerebrospinal fluid

CNS: Central nervous system

CVD: Cardiovascular diseases

CVO: Circumventricular organs

LIST of ABBREVIATIONS *(Continued)*

DBP: Diastolic blood pressure

FBS: Fetal bovine serum

HF: High frequency

HR: Heart rate

HRV: Heart rate variability

HTN: Hypertension

H_2S: Hydrogen sulfide

Iba: Ionized calcium-binding adapter molecule 1

Icv: Intracerebroventricular

IHC: Immunohistochemistry

IL: Interleukin

IML: Intermediolateral column

Ip: Intraperitoneal

IS: Immune system

MAP: Mean arterial pressure

PI: Pulse interval

LF: Low frequency

LV: Left ventricul

NA: Norepinephrine

NADPH: Nicotinamide adenine dinucleotide phosphate

NaHS: Sodium hydrosulfide

NO: Nitric oxide

NF-κB: Nuclear factor kappa B

LIST of ABBREVIATIONS *(Continued)*

NMDA: N-Methyl-D-aspartate

NTS: Nucleus of the solitary tract

PVN: Paraventricular nucleus

RAS: Renin-angiotensin system

ROS: Reactive oxygen species

RVLM: Rostral ventral lateral medulla

SD: Standard deviation

SEM: Standard error of mean

Sc: Subcutaneous

SD: Sprague Dawley

SHR: Spontaneously hypertensive rats

SBP: Systolic blood pressure

SFO: Subfornical organ

SNS: Sympathetic nervous system

STS: Sodium thiosulfate

TNF: Tumor necrosis factor

TP: Total power

VLF: Very low frequency

3-MST: 3-Mercaptopyruvate sulfurtransferase

1- INTRODUCTION and OBJECTIVES

Hypertension (HTN) is an important risk factor for cardiovascular, cerebrovascular events, chronic kidney disease and mortality (Franklin & Wong, 2013; Kjeldsen, 2018). Approximately 1 billion people worldwide has HTN (Kearney et al., 2005). Despite significant advancement in the treatment, up to twenty percent of hypertensive patients is resistant or refractory to available anti-hypertensive drugs (Pimenta & Calhoun, 2012). This in part may be due to neurogenic mechanisms which are driven by chronic hyperactivity of the sympathetic nervous system (SNS), (T. Yang, Richards, Pepine, & Raizada, 2018).

Dysfunction in the central nervous system (CNS) has been suggested as a key contributor to the development of HTN (Haspula & Clark, 2018). Renin-angiotensin system (RAS), oxidative stress and glial-mediated neuroinflammation in cardioregulator brain regions are implicated as key factors in augmenting sympathetic activity in HTN (Haspula & Clark, 2018). Hence, it becomes essential to investigate the role of glia in angiotensin (Ang)-II mediated sympathoexcitation in HTN and to attenuate disruptive effects of neuroinflammation on cardioregulatory centers in the brain thereby reduce blood pressure (BP).

Conventional anti-hypertensive medications were inadequate to pass the blood brain barrier (BBB) and some of them could also stimulate central sympathetic outflow (Fisher & Fadel, 2010). Alternatively, adrenergic neuron blockers and ganglion blockers have been used, but despite their BP reducing effect, their use has been restricted due to side-effects (DeQuattro & Li, 2002). Alfa $(\alpha)_2$ or imidazoline receptor agonists (α-metildopa and clonidine) have also been used as central sympatholytic agents, but their tendency to produce orthostatic intolerance complicates their therapeutic application (Amery, Bossaert, Fagard, & Verstraete, 1972). Besides pharmacological treatments, renal nerve ablation, carotid baroreflex stimulation and deep brain stimulation are being developed to target SNS in treatment-resistant HTN, and early results are positive (Fisher & Paton, 2012).

However, these are invasive procedures that are still in the experimental stages. Novel therapies are needed to better address the problem of resistant HTN.

Hydrogen sulfide (H_2S) is a gaseous signaling molecule with anti-hypertensive (van Goor, van den Born, Hillebrands, & Joles, 2016) and anti-inflammatory properties (Gemici & Wallace, 2015). Increasing evidence suggests that impairment in H_2S homeostasis plays a role in the development of HTN (van Goor et al., 2016), (Meng, Ma, Xie, Ferro, & Ji, 2015). Consistently, H_2S donors are suggested being useful for the treatment of HTN. In Ang II-induced hypertensive animal models, intraperitoneal (ip) injection of H_2S donors, sodium hydrosulfide (NaHS) and sodium thiosulfate (STS), were found to reduce BP (Al-Magableh, Kemp-Harper, & Hart, 2015; Snijder et al., 2015; Snijder et al., 2014). In diabetic spontaneously hypertensive rats (SHR), ip injection of NaHS was found to lower BP (Ahmad et al., 2012), while central NaHS injection in the hypothalamus of freely moving rats was found to reduce mean arterial pressure (MAP), (Dawe, Han, Bian, & Moore, 2008).

Recently, H_2S and its donors have also been shown to attenuate glia-mediated neuroinflammation. It has been shown that NaHS or STS treatment decreased the release of pro-inflammatory cytokines by in vitro glial cells activated by lipopolysaccharide/interferon-γ or interferon-γ (M. Lee, McGeer, & McGeer, 2016). Moreover, pre-treatment with ip injection of NaHS has been shown to reduce pro-inflammatory cytokines as well as the extensive astrogliosis, microgliosis in hippocampus (Xuan et al., 2012). Our objective is to examine whether NaHS can help to alleviate Ang II-induced HTN by attenuating neuroinflammation in hypotalamic paraventricular nucleus (PVN), a cardioregulatory region implicated in elevation of SNS. Chronic Ang II infusion is an established animal model of HTN and is characterized by elevated sympathetic drive, and activation of microglia in the PVN (Braga et al., 2011; King & Fink, 2006; P. Shi, Diez-Freire, et al., 2010), and as such is ideal for our current hypothesis and experimental protocol.

2- GENERAL INFORMATION

2.1- Definition of Blood Pressure

BP is referred as the pressure on the walls of vessels. The stretch of the walls of the arteries increases in systole and decreases after diastole (Magder, 2018). BP is measured in millimeters of mercury and usually expressed as the systolic blood pressure (SBP) over the diastolic blood pressure (DBP), (Brzezinski, 1990).

2.2- Regulation of Blood Pressure

BP depends on the balance between cardiac output (CO) and the peripheral vascular resistance (PVR), (Rhian M. Touyz, 2014). BP regulation is a complex process including the interactions of baroreceptors, RAS, adrenergic system, vasoactive factors such as nitric oxide (NO), endothelin-1 and reactive oxygen species (ROS), (Rhian M. Touyz, 2014). Many organs are included in the control of BP, (Figure 2.1).

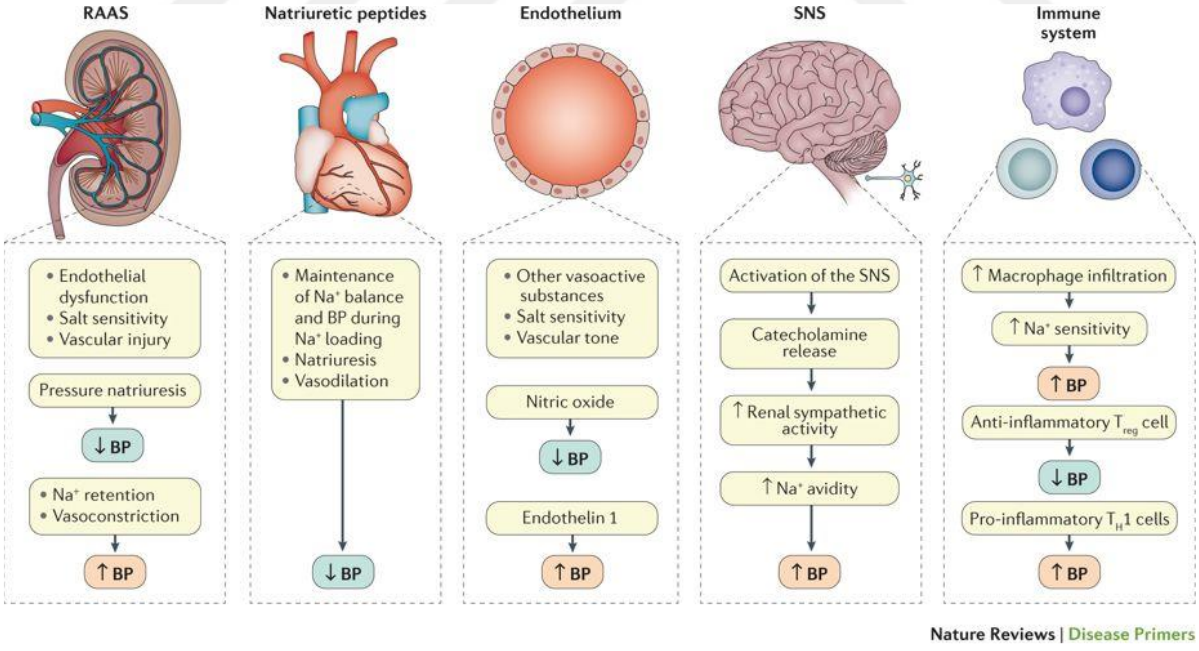

Figure 2-1 Physiological mechanisms involved in the regulation of blood pressure (Oparil et al., 2018).

2.3- Definition of Hypertension

HTN is a chronic condition in which BP in the arteries is elevated (Oparil et al., 2018). High BP can result from an increase in CO, an increase in total PVR, or a combination of both (Mayet & Hughes, 2003). The underlying cause of HTN is unknown in the majority of patients (ninety–ninety five percent) which is called primary or essential HTN (Oparil et al., 2018). If high BP has an identifiable cause, it is termed secondary HTN (Oparil et al., 2018).

2.3.1- Classification of blood pressure

BP is categorized as normal, elevated, or stage 1 or 2 HTN by American Heart Association (Whelton et al., 2018). HTN is defined as a BP of 130/80 mmHg or greater (Table 2.1).

Table 2-1 Blood pressure categories (Whelton et al., 2018).

Blood Pressure Categories	**Systolic Blood Pressure (mmHg)**		**Diastolic Blood Pressure (mmHg)**
Normal	<120	and	<80
Elevated	120-129	and	<80
Hypertension			
Stage 1	130–139	or	80–89
Stage 2	≥140	or	≥90

European Society of Hypertension define HTN as a SBP ≥140 mmHg or DBP ≥90 mmHg (Table 2.2), (Williams et al., 2018).

Table 2-2 Categories of blood pressure in adults (Williams et al., 2018).

Blood Pressure Categories	**Systolic Blood Pressure (mmHg)**		**Diastolic Blood Pressure (mmHg)**
Optimal	<120	and	<80
Normal	120-129	and/or	80-84
High normal	130–139	and/or	85-89
Hypertension			
Grade 1	140–159	and/or	90–99
Grade 2	160–179	and/or	100-109
Grade 2	160–179	and/or	≥90

2.3.1- Epidemiology of hypertension

HTN is a risk factor for mortality and disability. Elevated BP effects approximately 1 billion people worldwide (Kearney et al., 2005) and continues to be the biggest single contributor to the global burden of disease and of mortality (S. S. Lim et al., 2012).

HTN is also one of the strongest risk factor for cardiovascular diseases (CVD), (Franklin & Wong, 2013; Kjeldsen, 2018). HTN accounts for an estimated fifty four percent of all strokes and fourty seven percent of all ischemic heart disease events globally (Lawes, Vander Hoorn, Rodgers, & International Society of, 2008). Despite significant advancement in the treatment, up to twenty percent of hypertensive patients is resistant or refractory to available anti-hypertensive drugs (Pimenta & Calhoun, 2012). Successful prevention and treatment of HTN are crucial to reduce the burden of the disease and increase life expectancy.

2.3.2- Pathophysiology of hypertension

The underlying cause of HTN is unknown in the ninety–ninety five percent of hypertensive patients which is called primary or essential HTN (Oparil et al., 2018). Primary HTN is a multi-factorial disease and genetic and environmental factors

play an important role in the pathogenesis of the disease (Carretero & Oparil, 2000). Some pathophysiological mechanisms including RAS overactivity (Navar, 2010), autonomic nervous system (ANS) dysfunction (Mancia & Grassi, 2014), and peripheral and central inflammation (Harrison et al., 2011) have been related with the genesis of HTN.

Renin-angiotensin system and hypertension

RAS affect the control of BP and plays a crucial role in the pathophysiology of HTN. Renin, synthesized from prorenin in the juxtaglomerular cells of the kidney, is secreted in response to various stimuli such as renal baroreceptor mechanism, SNS, prostanoids and NO, Ang II-mediated feedback, calcium modulators (Kurtz, 2012). Renin converts angiotensinogen to Ang-I. Angiotensin converting enzyme (ACE), then, hydrolyzes Ang-I to potent vasoconstrictor Ang II (Figure 2.2). Ang II mediates vasoconstriction as well as aldosterone release from the adrenal gland, resulting in sodium retention and increased BP (Oparil et al., 2018). Ang II is also associated with endothelial dysfunction and has profibrotic and pro-inflammatory effects, mediated in large part by increased oxidative stress, resulting in renal, cardiac and vascular injury (Oparil et al., 2018), (Figure 2.2) Ang II exerts its effects by binding to Ang II type 1 receptor (AT1R) and type 2 (AT2R) receptor (Goodfriend, Elliott, & Catt, 1996). Most of the physiological actions of Ang II including vasoconstriction, release of aldosterone, inhibition of renin release and reabsorption of renal tubular sodium, as well as many undesirable effects including fibrosis and inflammation are mediated by the AT1R (Atlas, 2007). Abnormal activation of AT1R leads to a number of pathophysiologies including HTN (Karnik et al., 2015). AT2R are predominant in various brain areas such as the locus coeruleus and the amygdaloid nucleus however its expression declines after birth (K. D. Singh & Karnik, 2016). Although ACE is the primary enzyme of the RAS cascade, ACE-2 that converts Ang II to Ang-(1-7) has gained a great interest in the pathophysiology of HTN over the last decade (Varagic, Ahmad, Nagata, & Ferrario, 2014).

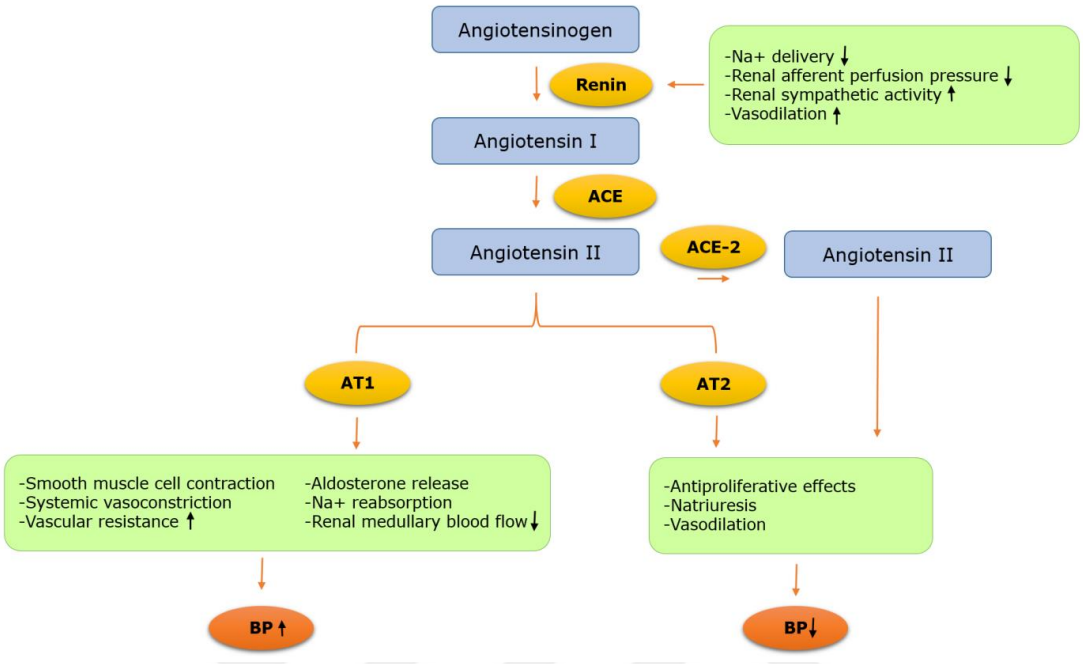

Figure 2-2 Renin–angiotensin–aldosterone system in the regulation of blood pressure (ACE: Angiotensin converting enzyme; AT: Angiotensin receptor; BP: Blood pressure. Adapted from (Oparil et al., 2018).

The RAS components have also been shown to exist locally in several tissues including the heart, lung, adrenal gland, kidney, blood vessels and brain (Fountain & Lappin, 2019). The brain RAS is involved in the modulation of cardiovascular and fluid–electrolyte homeostasis (Campos, Bader, & Baltatu, 2011). Over-activity of the brain RAS has been implicated in neurogenic HTN (Grobe et al., 2010).

Sympathetic nervous system and hypertension

SNS mediates the short- and long-term regulation of BP through its efferent innervations on heart, vasculature, kidney, adrenal medulla, and gut (Charkoudian & Rabbitts, 2009; T. Yang et al., 2018). Sympathetic nerves control PVR and CO by increasing myocardial contractility, HR and causing vasoconstriction (Charkoudian & Rabbitts, 2009).

SNS overactivation in the pathogenesis of HTN is well-established (Mancia & Grassi, 2014). SNS stimulation of heart, vasculature and kidneys increases BP by augmenting CO, PVR and fluid retention (Esler, 2011). Evidence suggests that there is an increased SNS activity in both humans and animal models of HTN and it's not only a consequence of HTN, but can be a crucial triggering mechanism (Esler, 2011; Takahashi, 2012). However, to date, the mechanism that potentiates the increase in SNS activity has not been fully elucidated (Wyss, 1993). Several mechanisms have been proposed to explain the sympathetic overdrive seen in individuals with HTN such as baroreflex dysfunction, inflammation, and RAS overactivity, among other (Figure 2.3), (Mancia & Grassi, 2014).

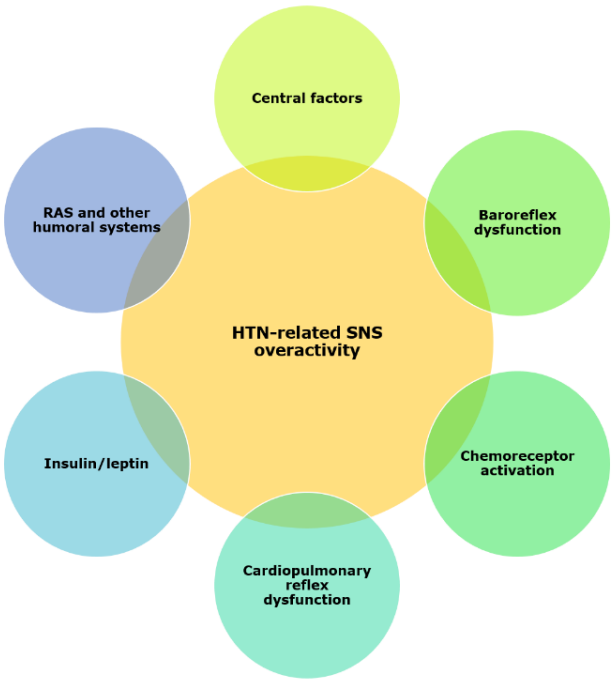

Figure 2-3 Potential mechanisms for sympathetic activation in hypertension (SNS: Sympathetic nervous system; RAS: Renin-angiotensin system. Adapted from (Mancia & Grassi, 2014).

Inflammation and hypertension

Mounting evidence implicates a key role for peripheral and neuroinflammation with sympathetic overactivity in the pathophysiology of HTN in both humans and animal models (Bautista, Vera, Arenas, & Gamarra, 2005; Harrison, 2014; M. V. Singh, Chapleau, Harwani, & Abboud, 2014; Zubcevic, Santisteban, et al., 2014). SNS also acts as an integrative interface between the brain and the IS (Paton & Waki, 2009; Winklewski, Radkowski, & Demkow, 2014). Lymphoid organs are innervated by autonomic, mostly sympathetic efferent nerves and it modulates the function of immune cells (Nance & Sanders, 2007). However, inflammation is not only a consequence but may also contribute to development and/or establishment of HTN. For instance, mice lacking T and B cells have blunted HTN and do not develop abnormalities of vascular function during Ang II infusion or desoxycorticosterone acetate (DOCA)–salt and transfer of T cells, but not B cells, restored the hypertensive response, indicating that T cells play an important role in generation of HTN (Guzik et al., 2007). Dahl salt-sensitive rat lacking T and B cells, demonstrated a significant blunting of salt-sensitive HTN and renal disease (Mattson et al., 2013). Recent studies have also supported the role of T cells in the development of HTN (Trott et al., 2014).

Inflammation of the cardioregulatory brain regions has been implicated in the pathogenesis of HTN (Paton & Waki, 2009; Winklewski, Radkowski, Wszedybyl-Winklewska, & Demkow, 2015). However, whether neuroinflammation is a cause or consequence of HTN is not yet known. Pro-inflammatory cytokines produced in the periphery can pass through the BBB and cause inflammation and elevate sympathetic outflow (Winklewski et al., 2015), while pro-inflammatory cytokines produced by glia and neurons in cardioregulatory regions can also cause peripheral inflammation, which may further contribute to the development of HTN (de Kloet et al., 2013; Paton & Waki, 2009).

2.4 Neuroinflammation and Sympathetic Overactivity in Angiotensin II induced Hypertension

Uncontrolled, treatment resistant HTN has been suggested to be partly due to neurogenic mechanisms which are driven by chronic hyperactivity of the SNS (T. Yang et al., 2018). The hypothesis of SNS in the initiation, maintenance and progression of HTN has been well-studied. Results from experimental and human studies implicate the 'neurogenic hypothesis of HTN', emphasizing pathophysiological relevance of the sympathetic and parasympathetic neural abnormalities (i.e. a sympathetic activation with a parasympathetic inhibition) in patients with essential HTN (Grassi, Seravalle, & Quarti-Trevano, 2010). However, the specific neural mechanisms underlying the development and progression of neurogenic HTN are incompletely understood.

Emerging studies indicate that elevated sympathetic activity in HTN may be associated with neuroinflammation in cardioregulatory brain regions (Santisteban et al., 2015; Z. Shi, Jiang, Wang, Xu, & Guo, 2014). However, the cellular mechanism of neuroinflammation and the cause and effect relation between HTN and neuroinflammation is unknown. It has been suggested that pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1ß, and ROS within nuclei that regulate cardiovascular homeostasis promote sympathetic drive and increase BP (Lob, Schultz, Marvar, Davisson, & Harrison, 2013; Marvar, Lob, Vinh, Zarreen, & Harrison, 2011; P. Shi, Raizada, & Sumners, 2010), a mechanism proposed to involve microglial activation (X. Z. Shen et al., 2015; P. Shi, Diez-Freire, et al., 2010).

2.4.1- Autonomic regions involved in regulation of sympathetic nervous system activity and blood pressure

SNS efferent transmission from brain to the peripheral tissues is controlled by several forebrain and hindbrain nuclei. Sympathetic preganglionic neurons in the intermediolateral (IML) column, a region of gray matter of the spinal cord, receive excitatory drive from many CNS areas including the hypothalamus, medulla

oblongata, pons, and amygdala (Dampney, 1994). Hypothalamic PVN, subfornical organ (SFO), the rostral ventral lateral medulla (RVLM), and the nucleus of the solitary tract (NTS) are the most important cardioregulatory regions (Kasparov & Teschemacher, 2008; Szczepanska-Sadowska, Cudnoch-Jedrzejewska, Ufnal, & Zera, 2010), (Figure 2.4). PVN, a forebrain nucleus that integrates and responds to a variety of neural and humoral signals regulating sympathetic drive and extracellular fluid volume (Coote, 2005). PVN is sympatho-excitatory and it might be activated by increases in the level of circulating Ang II, chronic stress or anxiety, or peripheral receptors (Zucker, Wang, Brandle, Schultz, & Patel, 1995). RVLM, a point of convergence for signals from forebrain and hindbrain centers that determines the intensity of sympathetic activity (Felder, Yu, Zhang, & Wei, 2009). SFO is concerned primarily with thirst and sodium appetite and PVN receives direct input from higher forebrain areas, such as the SFO (Felder et al., 2009). PVN and RVLM contain neurons with long descending IML of the spinal cord, ultimately affecting sympathetic drive to the heart and the vascular tree and, importantly, renal handling of sodium and water and renin release (Felder et al., 2009). NTS is the primary site of cardiorespiratory reflex integration and functions as a comparator between its renal nervous system and axons that innervate the preganglionic sympathetic neurons in the cardiovascular receptor afferents and projects to nuclei that regulate the circulatory variables (Zanutto, Valentinuzzi, & Segura, 2010). PVN is one of the major direct projections to the NTS (Dampney, 1994).

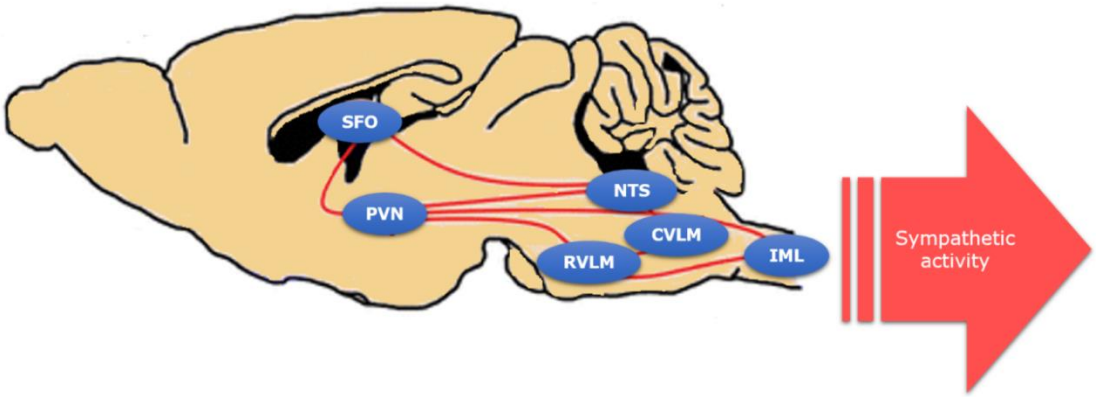

Figure 2-4 Schematic diagram of the brain regions involved in the sympathetic regulation of cardiovascular functions (CVLM: caudal ventral lateral medulla; IML intermediolateral nucleus; NTS: nucleus tractus solitarii; PVN: paraventricular nucleus of the hypothalamus; RVLM: rostral ventral lateral medulla; SFO: subfornical organ. Adapted from: (Hurr & Young, 2016).

2.4.2- Inflammation of autonomic regions in hypertension: Participation of microglia

Neuroinflammation

Neuroinflammation is defined as the inflammatory response of CNS which is mediated by the cytokines, chemokines, ROS, and secondary messengers released by resident CNS glia, endothelial cells, and peripherally derived immune cells (DiSabato, Quan, & Godbout, 2016). Neuroinflammation has been implicated in the pathogenesis of various neurodegenerative (Singhal, Jaehne, Corrigan, Toben, & Baune, 2014), psychiatric (Brites & Fernandes, 2015; Meyer, 2013), cardiovascular diseases (Sharma et al., 2018; Winklewski et al., 2015). Inflammation of autonomic brain regions has been implicated in the sympathetic overactivity in HTN (Paton & Waki, 2009; Winklewski et al., 2015) in which microglia activation plays an important role (Dheen, Kaur, & Ling, 2007; Santisteban et al., 2015).

Microglia activation and neuroinflammation

The CNS consists of two major cell types, neurons and glial cells (astrocytes, oligodendrocytes and microglia), (Dheen et al., 2007). Microglia are the resident immune cells of the brain involved in the maintenance of the neural environment (Kraft & Harry, 2011). Microglia are distributed throughout the brain but appear to have varied roles in specific regions (Gosselin et al., 2014; Grabert et al., 2016). Microglia are active players of pathological states in the brain (Bechade, Cantaut-Belarif, & Bessis, 2013). Microglia are activated upon various noxious stimulus or inflammatory states (Dheen et al., 2007). Activated microglia release pro-inflammatory cytokines (Takeuchi et al., 2006) and also undergo morphology changes characterized by large cell soma and short processes (Kreutzberg, 1996), (Figure 2.5).

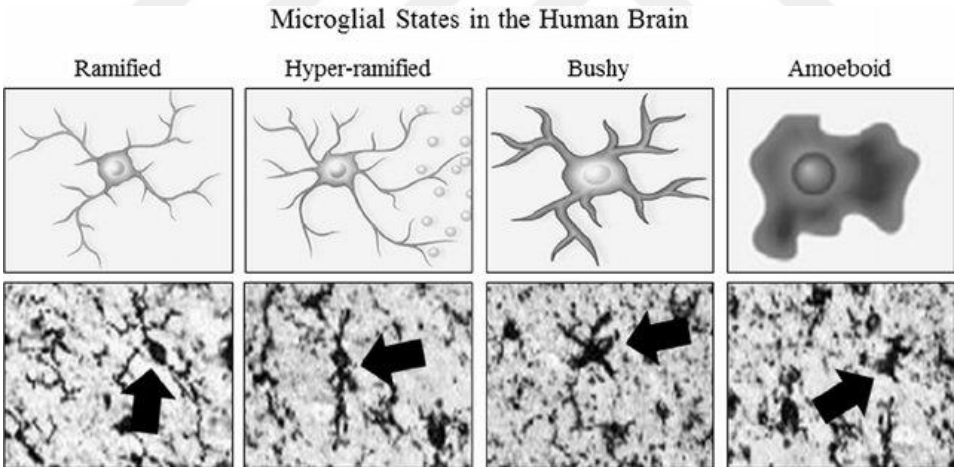

Figure 2-5 Activated morphology of microglia (Crews & Vetreno, 2016).

Effects of microglia on neuronal function

Microglia release soluble factors, including cytokines and prostaglandins, which influence and modulate neuronal function during physiological and pathological conditions (Delpech et al., 2015; Stellwagen & Malenka, 2006; Yirmiya & Goshen, 2011). Moreover, microglia-derived cytokines can indirectly affect

neurons through gliotransmission mediated by astrocytes (Santello & Volterra, 2012). For instance, TNF-α released by activated microglia potentiates glutamate release from astrocyte, which can modulate synaptic plasticity and even lead to neurotoxicity (Bezzi et al., 2001). In addition, adenosine triphosphate (ATP) released by microglia is shown to induce glutamate release by astrocytes thereby acutely exciting proximal neurons (Pascual, Ben Achour, Rostaing, Triller, & Bessis, 2012).

Microglia can also support adaptive synaptic plasticity through the release of neurotrophic factors, such as brain-derived neurotrophic factors (Parkhurst et al., 2013). Microglia actively phagocytose synapses during neurodevelopment (Stevens et al., 2007).

2.4.3- Angiotensin II, microglia activation, sympathetic overactivity

Sympathetic overactivity has been reported in Ang II mediated HTN suggesting the involvement of central mechanisms (Kumagai et al., 2012; LaGrange, Toney, & Bishop, 2003; Osborn, Fink, Sved, Toney, & Raizada, 2007). Ang II acts in the CNS to modulate neurohumoral pathways involved in sympathoexcitation and BP regulation. Ang II exerts its actions by binding to neuronal AT1R in the circumventricular organs (CVO), including the SFO and organum vasculosum lamina terminalis, and subsequently activating hypothalamic and brain stem sites such as PVN and RVLM, contributing to sympathoexcitation and hypertensive response (Simpson, 1981). It's hypothesized that Ang II increases the production and/or release of pro-inflammatory cytokines from glia. Subsequently, the released pro-inflammatory cytokines increase ROS production. Furthermore, Ang II, via stimulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, increases ROS formation in both neurons and microglia, via a cytokine-independent mechanism. In turn, ROS, can act to increase neuronal discharge, thereby contributing to increase in the sympathetic outflow and BP (P. Shi, Raizada, et al., 2010; Zubcevic, Waki, Raizada, & Paton, 2011), (Figure 2.6).

Increased levels of plasma Ang II are known to induce vascular inflammation (Rodriguez-Iturbe et al., 2001; P. Shi, Raizada, et al., 2010). This has

also been reported to occur in the brain vasculature because of systemic infusion of Ang II resulting from increase in leukocyte adhesion in the brain vasculature and disruption of BBB (M. Zhang, Mao, Ramirez, Tuma, & Chabrashvili, 2010). Circulating Ang II can penetrate the brain either via brain regions lacking the BBB (M. Zhang et al., 2010) or via the disrupted BBB during HTN (Biancardi, Son, Ahmadi, Filosa, & Stern, 2014). The brain RAS also produces Ang peptides locally in PVN, SFO, RVLM, area postrema, and NTS (Davisson, 2003; Gironacci, Cerniello, Longo Carbajosa, Goldstein, & Cerrato, 2014).

The effects of RAS in the CNS are not only a consequence of the activity of circulating RAS, acting through the CVO but also local and independent RAS has activity in the brain (Phillips & de Oliveira, 2008). Astrocytes are the major source of brain angiotensinogen (Stornetta, Hawelu-Johnson, Guyenet, & Lynch, 1988), with only a small contribution from neurons (Kumar, Rassoli, & Raizada, 1988; Thomas, Greenland, Shinkel, & Sernia, 1992). Over-activity of the brain RAS has also been implicated in HTN (Grobe et al., 2010). Within the CNS, Ang II promotes a hypertensive state by enhancing sympathetic neural outflow, altering the release of hormones involved in BP regulation, as well as modulating inflammatory processes. Emerging evidence also suggests that brain Ang II may alter bone marrow derived hematopoietic stem and progenitor cells and thus exacerbate hypertensive vascular pathologies (Jun et al., 2012; Zubcevic, Jun, et al., 2014; Zubcevic, Santisteban, et al., 2014), (Figure 2.6).

It has been demonstrated that crosstalk between brain prostaglandin E2, its receptor EP1 and ROS signaling in the development of Ang II-induced HTN (Figure 2.6). In response to systemic Ang II infusion, HTN and SFO-ROS production were abolished in mice with null mutations in the EP1 receptor and cyclooxygenase (COX)-1 (Cao et al., 2012). Genetic reconstitution of the EP1 receptor selectively in the SFO restored these responses, demonstrating that COX-1 derived prostaglandin E2 ROS generation in the forebrain SFO is required for Ang II dependent HTN (Cao et al., 2012).

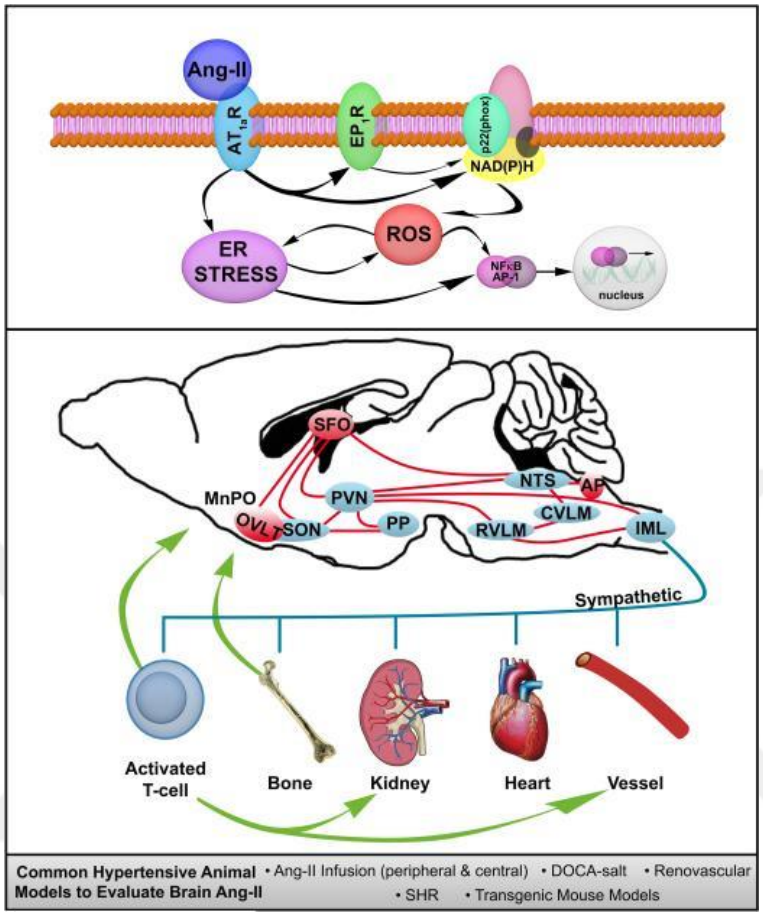

Figure 2-6 Schematic illustration of the signaling pathways, neural networks and sympathetic nervous system influenced physiological outputs involved in the development of hypertension due to peripherally- or locally-generated Angiotensin-II (Ang-II) action in the brain (Young & Davisson, 2015) demonstrated crosstalk between brain prostaglandin E2, its receptor EP1 and ROS signaling in the development of Ang-II-induced hypertension.

2.4.4- Effets of angiotensin II within paraventricular nucleus

PVN, located against the third ventricle within the hypothalamus, is a critical autonomic control center of the hypothalamus (Powers-Martin, Phillips, Biancardi, & Stern, 2008; Swanson & Sawchenko, 1980). More specifically, it has been implicated in central cardiovascular and volume control, including BP regulation (Ramchandra, Hood, Frithiof, McKinley, & May, 2013). PVN is an important integrative site within the brain composed of magnocellular and parvocellular

neurons which is known to influence sympathetic nerve activity (Ramchandra et al., 2013). PVN neurons can influence sympathetic nerve activity directly or indirectly (Badoer, 2001). It has been shown that neurons in the PVN with projections to the IML or RVLM may be activated by decreases in blood volume (Badoer, 2001). PVN also containes integrative neurons consisting of glutamate and GABA containing cells (Ferguson, Latchford, & Samson, 2008).

Ang II was first suggested to be a neurotransmitter utilized by SFO neurons projecting to the PVN (Lind, Swanson, & Ganten, 1985). Studies have shown that Ang II administration into PVN caused vasopressin release (Bains, Potyok, & Ferguson, 1992; Shoji, Share, & Crofton, 1989) and augmented the cardiac sympathetic afferent reflex mediated by AT1R (Zhu, Patel, Zucker, & Wang, 2002) which has been blocked by AT1R antagonist losartan (Z. Li, Bains, & Ferguson, 1993; Z. Li & Ferguson, 1993). Ang II has been shown to depolarize magnocellular neurons and also increase the frequency of excitatory postsynaptic potentials in these neurons which was dependent on an increase in glutamatergic input (Latchford & Ferguson, 2004). Studies also have suggested that glutamate interneurons have an important role in mediating the excitatory effects of Ang II (Latchford & Ferguson, 2004). Ang II has been shown to excite spinally projecting PVN neurons by attenuation of GABAergic synaptic inputs through activation of presynaptic AT1R (D. P. Li, Chen, & Pan, 2003).

It has been reported that AT1R activation by Ang II within PVN is a major contributor to chronic sympathoexcitation (Paton & Raizada, 2010). Chronic inhibition of ACE in PVN has been shown to attenuate sympathoexcitation and ROS production and modulate expression of cytokine in RVLM in renovascular HTN (P. Shi et al., 2014). It has been demonstrated that direct injection of inflammatory cytokine IL-1ß into PVN increased BP whereas increasing the expression of the anti-inflammatory IL-10 specifically within the PVN reduced BP in Ang II induced HTN in rats (P. Shi, Diez-Freire, et al., 2010).

2.5 Treatment of Hypertension

Systematic reviews and meta-analyses involving hypertensive patients have shown that lowering BP reduces the risk of major cardiovascular, cerebrovascular events, comorbidities and mortality (Ettehad et al., 2016; Thomopoulos, Parati, & Zanchetti, 2014). Management of high BP requires both non-pharmacological and pharmacological treatments.

2.5.1- Non-pharmacological therapy

Non-pharmacological treatments include lifestyle modifications, diet, probiotics, reducing salt intake, moderate alcohol consumption, regular physical activity, weight loss, avoiding stress, and minimizing alcohol consumption (Mahmood et al., 2019).

2.5.2- Pharmacological therapy

The current recommendations for treatment of HTN are mainly based on the use, alone or in combination, of anti-hypertensive medications which include: angiotensin receptor blockers (ARB) and angiotensin converting enzyme inhibitors (ACEI), calcium channel blockers (CCB), beta (ß)-blockers and diuretics. All of these medications have proved to be adequate to decrease BP enough to be maintained below the threshold levels for the aged population (James et al., 2014). There is however a >30% of patients whose elevated BP cannot be controlled (Yoon et al., 2015) with the current recommendations and require novel interventions or additional drugs to their treatment regimen with variable rate of success (Persell, 2011). Therefore, the question of finding new treatment strategies is still a major concern in pharmacological research.

Initial treatment of HTN starts with the first-line anti-hypertensive medications either as monotherapy or combination therapy (Williams et al., 2018). However, many patients with HTN require combination therapy since monotherapy does not achieve BP goals (Lithell et al., 2003).

Renin–angiotensin system blockers (angiotensin-converting enzyme inhibitors and angiotensin receptor blockers)

ACEI and ARB are among the most frequently used anti-hypertensive drugs which have similar effectiveness (Reboldi et al., 2008). ACEI block RAS system. Inhibition of ACE causes decreased production of Ang II thereby it enhances natriuresis, lowers BP, and prevents remodeling of smooth muscle and cardiac myocytes. It is also hypothesized that ACEI interfere with the degradation of vasodilator peptide bradykinin (Herman & Bashir, 2019).

ARB displace Ang II from AT1R and lowers BP by antagonizing Ang II induced vasoconstriction, aldosterone release, catecholamine release etc. (Barreras & Gurk-Turner, 2003).

Calcium channel blockers

CCB are widely used in the treatment of HTN and have similar effectiveness as other major drug classes on BP (Thomopoulos, Parati, & Zanchetti, 2015). They dilate arteries by inhibiting calcium influx through voltage-dependent L-type calcium channels. When, inward calcium flux is inhibited, vascular smooth muscle cells relax, resulting in vasodilation. In cardiac muscle, contractility is reduced and the sinus pacemaker and atrioventricular conduction velocities are slowed (Elliott & Ram, 2011). CCB increases renal blood flow, dilates afferent arterioles, and increases glomerular filtration pressure thereby causes natriuresis (Elliott & Ram, 2011).

Beta blockers

It has been proposed that anti-hypertensive effects of ß-blockers result from reduction of CO, effects on the CNS, inhibition of the RAS, plasma volume reduction, vasomotor tone reduction, PVR reduction, improvements in vascular compliance, resetting of the baroreceptor, reductions in norepinephrine (NA) release secondary to drug effects on prejunctional ß receptors, attenuation of the pressor

response to exercise and stress-induced catecholamines (Gorre & Vandekerckhove, 2010).

Diuretics

Diuretics are the second most commonly prescribed class of anti-hypertensive medication because of their efficacy, low cost, low side effects profile, their synergistic effect when combined with other anti-hypertensive agents (Roush & Sica, 2016). Diuretics are divided into 4 major groups including carbonic anhydrase inhibitors; loop diuretics; thiazides and related sulphonamide compounds; and potassium sparing agents according to their primary site of action within the renal tubule (Shah, Anjum, & Littler, 2004). Diuretics administered alone or in combination with other agents form the basis of therapy for the majority of hypertensive patients.

2.5.3- Conventional anti-hypertensive medications for sympathetic overactivity in hypertension

ACEI, ß-blockers or ARB have been reported to generate minimal alterations in muscle sympathetic nerve activity whereas diuretics and dihydropyridine CCB could stimulate central sympathetic outflow (Fisher & Fadel, 2010). These findings highlight the inadequacies of conventional anti-hypertensive medications to control excessive central sympathetic drive in HTN. Alternatively, adrenergic neuron blockers (i.e. reserpine and guanethidine) and ganglion blockers (i.e. hexamethonium) have been used, but despite their BP reducing effect, their use has been restricted due to side-effects (DeQuattro & Li, 2002). α_2 or imidazoline receptor agonists (α-metildopa and clonidine) have also been used as central sympatholytic agents, but their tendency to produce orthostatic intolerance complicates their therapeutic application (Amery et al., 1972). Besides pharmacological treatments, renal nerve ablation, carotid baroreflex stimulation and deep brain stimulation are being developed to target SNS in resistant HTN, and early results are positive (Fisher & Paton, 2012). However, these are invasive procedures that are still in the

experimental stages. Novel therapies are needed in order to better address the problem of treatment-resistant HTN.

2.6 Hydrogen Sulfide

H_2S, traditionally known as a highly toxic gas with the smell of rotten eggs, is an endogenously produced signaling molecule in bacteria, plants, and animals including mammals (Bouillaud & Blachier, 2011; Olson, 2012). The physiological importance of H_2S was recognized in 1996 when reported it acts as a novel neuromodulator (Abe & Kimura, 1996). Studies in animals and humans have shown H_2S to be involved in diverse physiological and pathophysiological processes, such as regulation of BP, inflammation, neurodegenerative diseases and metabolic disorders, including obesity and diabetes (Szabo, 2007).

2.6.1- Biosynthesis and catabolism

H_2S can be generated from L-homocysteine and L-cysteine via the methionine transsulfuration pathway or from dietary cysteine (Liu et al., 2012; Olson & Straub, 2016), (Figure 2.7). H_2S is produced by enzymatic or non-enzymatic pathways in the body, however the nonenzymatic pathway only accounts for a small portion of H_2S production (Wang, 2012). Cysteine and methionine are metaolized to H_2S by endogenous enzymes, cystathionine ß-synthase (CBS), cystathionine γ-lyase (CSE), and cysteine aminotransferase (CAT) in conjunction with mercaptopyruvate sulfurtransferase (3-MST), (Liu et al., 2012; S. Singh & Banerjee, 2011). Expression of these enzymes differs in tissues. CSE is the predominant H_2S producing enzyme in cardiovascular system while CBS is in nervous system and 3-MST is found in mitochondria and cytoplasm (Liu et al., 2012).

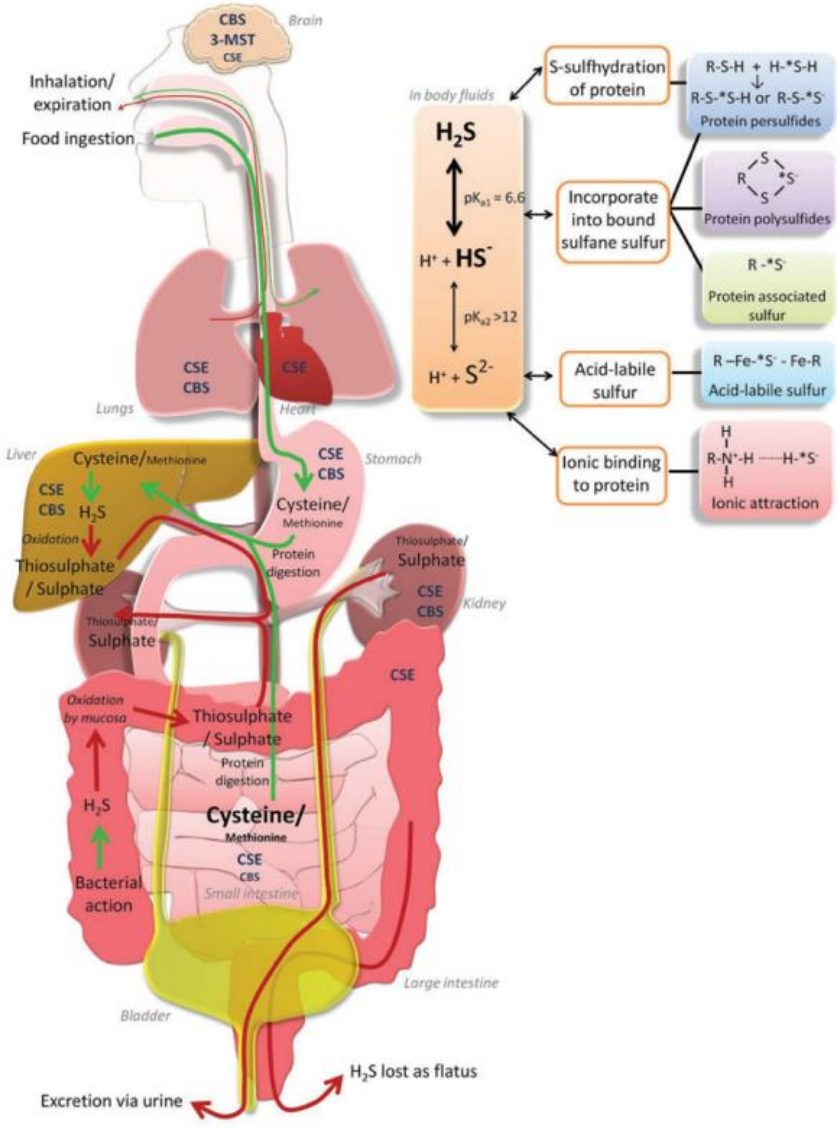

Figure 2-7 Biosynthesis of hydrogen sulfide (Liu et al., 2012).

The activity of CBS is regulated presumably at the transcriptional level by glucocorticoids and cyclic AMP. The activity of CBS can be directly inhibited by NO and carbon monoxide (CO), (Puranik et al., 2006). Regulation of CSE is less understood, but there is evidence that myeloid zinc finger 1 and specificity protein 1 play roles in its basal transcriptional activity, and the enzyme can be upregulated by bacterial endotoxin (Ishii et al., 2004; Miles & Kraus, 2004).

The enterobacterial flora is another source of H_2S. The intestinal epithelium expresses specialized enzyme systems that efficiently degrade sulphide to thiosulphate and sulphate - presumably to protect itself against high local concentrations of sulphide, and to prevent an excessive entry of H_2S into the systemic circulation (Fiorucci, Distrutti, Cirino, & Wallace, 2006; Furne, Springfield, Koenig, DeMaster, & Levitt, 2001). There are also several inorganic sources of H_2S in the body, including a non-enzymatic reduction of elemental sulphur using reducing equivalents obtained from the oxidation of glucose, as described in erythrocytes (Searcy & Lee, 1998).

Once produced in mammalian cells, free H_2S can immediately exert its biological effects or can be absorbed and stored in acid-labile sulfur or bound sulfane sulfur forms and released later in response to a physiological signal (Searcy & Lee, 1998). Acid-labile sulfur pool releases sulfur atoms under acidic conditions from the iron-sulfur complexes of mitochondrial enzymes (Searcy & Lee, 1998). Bound sulfane sulfur pool is localized to the cytoplasm and releases H_2S in alkaline enviroment under reducing conditions (Wang, 2012). There are several catabolic pathways including expiration and excretion, oxidation, methylation, scavenging for H_2S (Wang, 2012).

2.6.2- Physical and chemical properties

H_2S is a colorless gas with a rotten-eggs odor. Since, H_2S is highly lipophilic, it can freely cross the membranes without any membrane transporter (Mathai et al., 2009). In body fluids, free sulfide exists as H_2S, HS^-, and S^{2-}. Free sulfide is dissolved H_2S gas, which is a weak acid and in solution exists in the equilibrium $H_2S \leftrightarrow HS^- \leftrightarrow S^{2-}$. Amount of H_2S and HS^- within the cell is nearly equal, and approximately a 20%H_2S/80% HS^- ratio in extracellular fluid and plasma at 37°C and pH 7.4 (Wang, 2012). Since all these three forms are found in aqueous solutions, it is not possible to determine which one of them is biologically active. Thus, the terminology of "the H_2S concentration" usually refers to the sum of H_2S, HS^-, and S^{2-} (Liu et al., 2012). When produced in the body, H_2S is rapidly oxidized to sulfate or

can be stored in proteins. Sulfides are also bound to proteins in blood and tissues (Levitt, Abdel-Rehim, & Furne, 2011).

2.6.3- Role of hydrogen sulfide in hypertension

After the discovery of H_2S in the body as a physiological mediator and its endogenous production in vascular tissues (Hosoki, Matsuki, & Kimura, 1997; W. Zhao, Zhang, Lu, & Wang, 2001), it brought the question whether H_2S might have any physiological functions in the cardiovascular system. H_2S has been shown to be an important vasoactive factor relaxing rat aortic tissue (Abe & Kimura, 1996; W. Zhao et al., 2001). Following these, the pathophysiological role of H_2S in cardiovascular diseases, its potential role in HTN has come into question. It has been shown that expression and activity of H_2S producing enzyme CSE and plasma levels of H_2S have diminished in SHR (Yan, Du, & Tang, 2004), L-NAME-induced hypertensive (Zhong, Chen, Cheng, Tang, & Du, 2003) and 2-kidney-1-clip hypertensive rats (Xiao et al., 2016). CSE deficient and CBS heterozygous mice also displayed HTN (Sen et al., 2010; G. Yang et al., 2008). Chronic administration CSE and CBS enzyme inhibitors to normotensive rats resulted in a decrease in urinary excretion rate of sulfate which is considered as an indicator for endogenous H_2S production. The changes in this rate were also found to be associated with increases in MAP in the combination of enzyme inhibitors (Roy, Khan, Islam, Prieto, & Majid, 2012).

In patients with HTN, plasma H_2S concentrations were found to be low (Sun, Xi, Yang, Ma, & Tang, 2007). Cysteine and the variants of the CSE and CBS genes, respectively, have been shown to influence the hypertensive phenotype (Lucock et al., 2013). It has been found that H_2S dependent contribution to vasodilation was functionally absent in hypertensive adults, likely due to a reduction in the endogenous production of H_2S within the vasculature (Greaney et al., 2017). All of these findings suggest a possible role for H_2S in its pathogenesis of HTN.

Anti-hypertensive effect of H_2S in different hypertensive models has been identified by many studies. In SHR, chronic ip administration of NaHS (56 μmol/kg/day) for 5 weeks or 3 months (10, 30, and 90 μmol/kg/day) and a slow-releasing H_2S compound GYY4137 (133 μmol/kg/day) for 2 weeks attenuated the elevation of BP (L. Li et al., 2008; Y. X. Shi et al., 2007; Yan et al., 2004), lessened vascular remodeling and collagen accumulation (Y. X. Shi et al., 2007; Yan et al., 2004) and also reduced the myocardial ROS production (Y. X. Shi et al., 2007). In L-NAME-induced hypertensive rats, chronic ip administration of NaHS inhibited the development of HTN and cardiac hypertrophy (Zhong et al., 2003). In Ang II induced HNT, chronic ip treatment with H_2S donors NaHS and STS attenuated development of HTN (Snijder et al., 2015; Snijder et al., 2014) proteinuria, renal damage, renal function loss and prevented the development of cardiac hypertrophy associated with Ang II infusion (Snijder et al., 2014). It has also been shown that NaHS could directly inhibit the specific binding and could decrease the binding affinity of the AT1R (X. Zhao et al., 2008), However, the mechanism by which H_2S regulates Ang II-induced HTN is not clear. The effect of H_2S was also studied on animals with 2-kidney-1-clip model of renovascular HTN. It has been found that chronic ip NaHS (5.6 mg/kg/day) treatment over 4 weeks attenuated the development of HTN, the accumulation of the renin level and suppressed the upregulated renin mRNA level in these rats (Lu, Liu, et al., 2010). In contrast, these effects have not been observed in 1-kidney-1-clip rats, suggesting that the anti-hypertensive effect of H_2S may be greater in HTN associated with higher plasma renin activity (Lu, Liu, et al., 2010).

Mechanisms for anti-hypertensive effects of hydrogen sulfide

Several mechanisms have been proposed to contribute to the effects of H_2S on vessel tone (Figure 2.8). H_2S has been shown to induce vascular smooth muscle relaxation through the activation of K_{ATP} channels leading to membrane hyperpolarization (W. Zhao et al., 2001). H_2S has been suggested to dilate blood vessels in synergy with NO (Hosoki et al., 1997). H_2S has been shown to react with S-nitrosothiol species to release NO (Rodriguez, Maloney, Rassaf, Bryan, & Feelisch,

2003) and NO has also been shown to enhance H_2S production from vascular tissues (W. Zhao et al., 2001).

Recently, it has also been shown that H_2S dilates vessels by increasing intracellular cyclic guanosine monophosphate (cGMP) levels by inhibiting phosphodiesterase activity (Bucci et al., 2010). H_2S has also been reported to attenuate vascular inflammation (Pan, Liu, Gong, Wu, & Zhu, 2011), reduce ROS production (Al-Magableh et al., 2015), inhibit the synthesis and release of renin (Lu, Liu, et al., 2010) and ACE activity (Laggner et al., 2007).

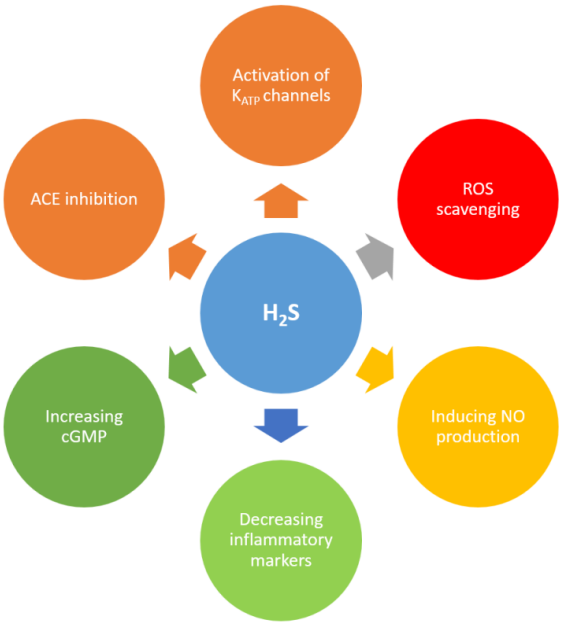

Figure 2-8 Possible mechanisms that may underlie hydrogen sulfide (H_2S)-induced blood pressure lowering effects. (ACE: Angiotensin converting enzyme, cGMP: cyclic guanosine monophosphate, NO: Nitric oxide, ROS: Reactive oxygen species, Adapted from: (Al Disi, Anwar, & Eid, 2015).

2.6.4- Role of hydrogen sulfide in neuroinflammation

H_2S is neuromodulator and neuroprotective in the nervous system (X. Zhang & Bian, 2014). It can freely cross cell membrane and regulates various intracellular signaling processes (X. Zhang & Bian, 2014) H_2S decreases intracellular pH in

primary cultured rat microglia and astrocytes, possibly via enhancing the activity of $Cl^-/HCO3^-$ exchanger and inhibiting that of Na^+/H^+ exchanger (Lu, Choo, et al., 2010). H_2S increases intracellular Ca^{2+} in various cell types such as neurons (Yong, Choo, Tan, Low, & Bian, 2010), astrocytes (Nagai, Tsugane, Oka, & Kimura, 2004) and microglial cells which is likely mediated by both extracellular Ca^{2+} influx and Ca^{2+} release from intracellular store (S. W. Lee et al., 2006). H_2S facilitates hippocampal long-term potentiation by enhancing N-methyl-d-aspartate (NMDA) receptor-mediated responses, which is possibly mediated by the cyclic adenosine monophosphate (cAMP)/protein kinase pathway and sulfhydration of NMDA (Abe & Kimura, 1996; Kimura, 2013). A wide range of H_2S's beneficial effects are mediated by its anti-inflammatary, anti-oxidant, anti-endoplasmic reticulum stress, and anti-apoptosis effects (X. Zhang & Bian, 2014).

Recently, H_2S and its donors have been shown to attenuate glia-mediated neuroinflammation in neurodegenerative diseases (M. Lee, McGeer, Kodela, Kashfi, & McGeer, 2013; M. Lee et al., 2016; Xuan et al., 2012). In an in vitro glial-mediated neuroinflammatory model, NaHS and STS were found to reduce the release of pro-inflammatory cytokines such as TNF-α and IL-6 (M. Lee et al., 2016), both markers of microglial activation. In cognitive impairment condition induced by hepatic ischemia and reperfusion, NaHS was found to attenuate neuroinflammation by reducing pro-inflammatory cytokine levels in the hippocampus, and lowering the expression of ionized calcium-binding adaptor molecule 1 (Iba1), (Tu, Li, Wang, Li, & Chu, 2016), another marker of microglial activation. In a 6-hydroxydopamine-induced rat model of Parkinson's Disease, NaHS was found to inhibit 6-hydroxydopamine-evoked NADPH oxidase activation and microglial activation in the substantia nigra, and reduce accumulation of pro-inflammatory factors in the striatum via nuclear factor kappa B (NF-κB) pathway (Hu et al., 2010).

2.6.5- Hydrogen sulfide and paraventricular nucleus

Recent evidence suggests that PVN may act as a site of action for H_2S. CBS activity and H_2S level in PVN have been found to be decreased in chronic heart failure rats (Gan et al., 2012). The bilateral infusion of GYY4137 into PVN for 6 weeks decreased MAP, attenuated plasma NA levels and H_2S levels and CBS expressions in PVN in high salt-induced hypertensive rats by the downregulation of NADPH oxidase and ROS and lower expressional levels of IL-1β and increased expression of IL-10 in PVN (Liang et al., 2017).

2.6.6- Hypothesis

The above-mentioned anti-inflammatory, anti-oxidant, and blood pressure-lowering roles of H_2S raise the possibility that NaHS, an H_2S donor, may have therapeutic potential in HTN with reactive oxidative and neuroinflammatory components (Figure 2.9).

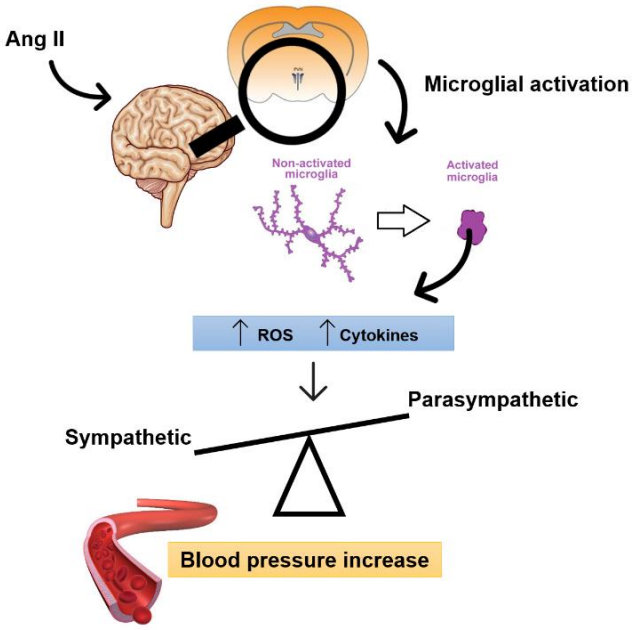

Figure 2-9 Neuroinflammation, microglia activation and sympathetic overactivity in Angiotensin II-induced hypertension.

2.6.7- Aim of the study

Here, we focused on the effects of NaHS, an H_2S donor, on microglial activity modulation in the PVN using Ang II induced rat model of HTN, largely based on the following rationale: firstly, enhanced sympathetic tone can be controlled by PVN; secondly, Ang II is associated with neuroinflammation in the PVN in HTN; and lastly, recent studies have shown that H_2S attenuates glial-mediated neuroinflammation.

3- MATERIALS and METHODS

3.1- Materials

3.1.1- Chemical substances

Table 3-1 Chemical substances used in the study.

Angiotensin II acetate salt	BACHEM
Artificial cerebrospinal fluid	Tocris Bioscience
Bacitracin zinc neomycin sulfate polymyxin B sulfate	Equate
Buprenorphine hydrochloride	Buprenex injection 0.3 mg/mL
Chlorhexidine gluconate	Bimeda Inc.
Enrofloxacin	Baytril 100 100 mg/mL
Fetal bovine serum (FBS)	CORNING
Hydrochloric acid (HCL)	Fisher
Hydrogen peroxide	Fisher CAS 7732-18-5
Iron trichloride ($FeCl_3$)	Alfa Aeser
Isoflurane	USP Patterson veterinary for use in horses and dogs
Jet Denture Repair Powder	Lang Dental
N-dimethyl-p-phenylenediamine oxalate	Fisher
Ortho-Jet Liquid	Lang Dental
Oxygen	
Phosphate buffered saline (PBS), 1X without calcium and magnesium	CORNING cellgro
Physiological saline	
Puralube Vet Ointment, petrolatum ophthalmic ointment	Dechra
Sodium hydrosulfide hydrate (NaHS)	Sigma
Tissue adhesive	3M Vetbond
Trichloroacetic acid	Fisher
TritonX-100	Fisher
Zinc acetate, anhydrous, 99.9+%	Alfa Aesar

3.1.2- Devices

Table 3-2 Devices used in the study.

AM radio	
Autoclave	
Blood Collection Tube	BD Vacutainer K2 EDTA 13mm x 100 mm 6 mL Pink BD Hemogard Closure Plastic Tube
pH meter	Fisher
Centrifuge	Eppendorf Centrifuge 5810 R
Cotton swabs	
Drill	
Forceps	
Gauze squares	Fisher
Heating pad	
Isoflurane anesthesia machine	Parkland Scientific
Light	
Magnet	
Micropipettes	Gilson
Micropipettes' tips	Thermo Fisher Scientific
Microplate reader	BioTek SYNERGYMX
Microscope slides	Fisher Superfrost plus
Microtome	Sakura Accu-Cut SRM
Micrtome	MICROM HM 505 E
Microtome blades	Accu-edge
Needles	
Plastic centrifuge tubes	Fisher
Radio-telemetry system	Data Science International (DSI)
Surgical microscope	
Surgical scissors	
Scale	OHAUS Adventurer
Shaving machine	
Sterile gloves	Kimberly-Clark
Stereotaxic frame	David Kopf Instruments
Straight hemostats	
Syringes	
Vortex	Fisher
Water bath	Precision Scientific
Water bath	Boekel Scientific
Wound clip applier and wound clips	Alzet

3.2- Methods

3.2.1- Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee and complied with the standards stated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight-week-old, male Sprague Dawley (SD) rats (n=4-7 per group) were purchased from Charles River Laboratories (United States). All animals were housed in a temperature-controlled room (22°C to 23°C) of University of Florida Animal Care Service Facility with a 12:12-hour light-dark cycle (lights on at 6 AM) with food and water available *ad libitum*. Rats were kept in facility at least for three days for acclimation before experiments.

3.2.2- Study design

After 3-5 days of acclimatization, telemetry transducers were implanted into the abdominal aorta of rats as previously described (Huetteman & Bogie, 2009). Following this, animals were randomly divided into groups and were allowed to recover for 1 week. Baseline MAP, SBP, DBP, and HR were recorded over fourty eight hours before Ang II (200 ng/kg/min) was delivered chronically using subcutaneous (sc) osmotic pumps (ALZET) to induce high BP. Ang II or saline was continuously infused using mini-osmotic pumps placed subcutaneously for 4 weeks (Figure 3.1). On the day of the placement of osmotic mini-pumps, all rats also received intracerebroventricular (icv) cannulae delivering either NaHS (30 or 60 nmol/h) or phosphate buffered saline (PBS). Rats were allowed to recover for 1 week prior to any measurements. Telemetric measurements were taken once a week for 4 weeks. At endpoint, rats were sacrificed and blood and whole brains were collected for further analysis (Figure 3.1).

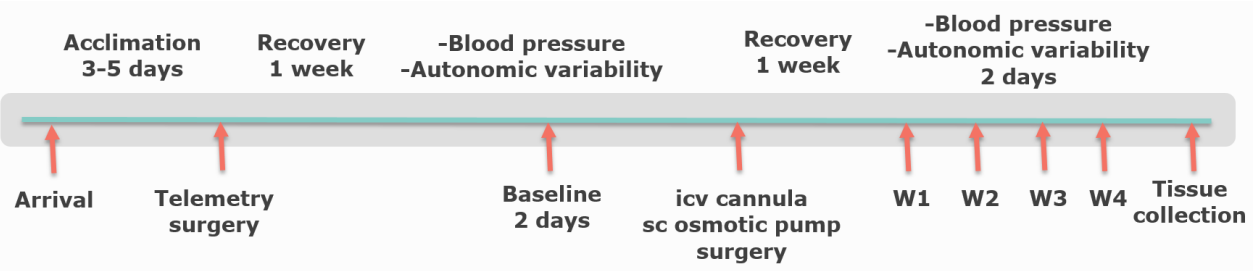

Figure 3-1 Study design (W: Week).

3.2.3- Radiotelemetry, blood pressure measurements and spectral analysis

Abdominal aorta cannulation with intraperitoneal device placement

Aseptic conditions are assured by using autoclaved instruments and sterilized materials and disinfecting the work bench. Radio-telemetry transmitters were implanted into abdominal aorta of rats as previously described (Huetteman & Bogie, 2009).

Briefly, rats were anesthetized with a mixture of O_2 (1 L/min) and 3% to 4% isoflurane. Anesthesia was maintained using an O_2/isoflurane (2%) mixture delivered through a specialized nose cone for the duration of the surgery. Body temperature was maintained at 37±1°C using a heating pad. The abdomen was shaven and the skin cleaned with chlorhexidine followed by ethanol. A 4-5-cm midline incision was made from the lower thorax along to the abdomen and the skin was gently dissected from the abdominal wall. A second incision was made into the peritoneal cavity using blunt scissors. The intestines were gently placed using moistened cotton applicators and retracted using moistened gauze sponges to allow good visualization of the descending aorta located along the dorsal body wall (Figure 3.2). Surrounding fat and connective tissue were carefully dissected from the aorta using cotton applicators. The connective tissue between the aorta and the vena cava was gently separated with the closed tips of the forceps. A 4-0 silk suture tie was grasped with forceps tips and threaded between the aorta and the vena cava to temporarily occlude blood flow at the time of vessel cannulation. An additional occlusion suture between the vena cava and the aorta just cranial to the iliac

bifurcation was placed to simplify sealing of the catheter by improving the hemostasis of the vessel (Figure 3.2).

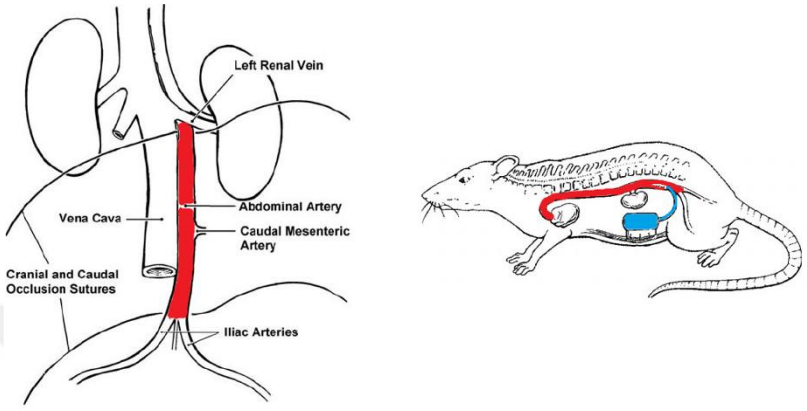

Figure 3-2 Depiction of a rat instrumented with a blood pressure telemetry device. The catheter is sealed into the abdominal aorta and the transmitter is anchored to the abdominal wall closure (Huetteman & Bogie, 2009).

Tip of a 23-gauge needle was bended downward to a 90° angle such that the open part of the bevel is on the outside of the bend. Blood flow in the aorta was restricted by applying firm traction to the occlusion sutures. Implantable telemetry transmitters for rats (DSI, St. Paul, MN, USA, model PA-C40) were used in the surgery. The catheter of the transmitter was held in forceps and bent-tipped syringe needle was used to incise the vessel wall and introduce the catheter into the artery. Then, needle was withdrawn and the catheter was advanced until the distal tip of the catheter gently contacted the restriction at the anterior occlusion suture. The puncture site was dried with cotton applicators to ensure good bonding of the tissue adhesive (3M™ Vetbond™ Tissue Adhesive). A tiny drop of tissue adhesive was applied to the catheter and allowed to flow completely around the puncture site. After adhesive's set, the cranial occlusion suture was slowly released and the catheterization site was observed for blood leakage. A small rectangle of fiber material approximately 5×7 mm was prepared to anchor the catheter until connective tissue forms. The fiber patch was placed over the catheterization site and

additional adhesive was applied to anchor the catheter to the surrounding tissues. All gauze sponges and retraction were removed without disturbing the catheter. The abdominal cavity was irrigated with warm saline. Tissue hydration was maintained with sterile saline throughout the entire procedure. The intestines were gently restored to their original position and the device was placed on top of the intestines, parallel to the long axis of the body with the catheter attachment directed caudally. The abdominal wall incision was closed with non-absorbable suture. The device was anchored by incorporating the longitudinal suture ridge on the device into the abdominal wall closure. The skin incision was closed with wound clips and topical antibiotic was applied (Bacitracin). Rats received a single dose of buprenorphine (0.1 mL/g body weight; Buprenex, Pfizer, NY) subcutaneously during surgery and were left to recover for 1 week before baseline telemetric measurements were taken.

Blood pressure measurement and heart rate variability analysis using telemetry system

BP data of rats were monitored and collected using telemetry system (DSI, St. Paul, MN, USA), (Figure 3.3). Telemetry system allows monitoring physiologic functions in awake and freely moving laboratory animals while minimizing stress-associated artifacts (Braga & Prabhakar, 2009).

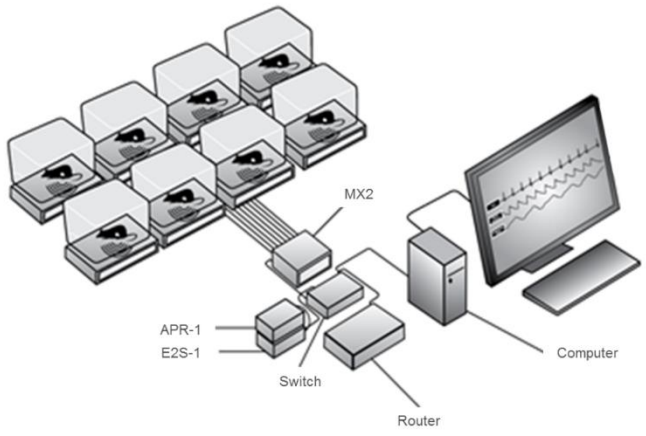

Figure 3-3 Radio-telemetry system (https://www.datasci.com/images/default-source/default-album/8-animal-physiotel-hd.jpg?sfvrsn=64eee365_0).

BP measurements were conducted 1 week after telemetry surgery as baseline measurements (week 0) and once a week for 4 weeks following the recovery after implantation of mini-osmotic pumps. On these days, the measurements were taken on the same day and time starting from 10.00 am to 09.10 am continuously for 5 minutes at 1000 Hz every hour over 48-hour period throughout the 12-hour light/dark cycle. MAP, SBP, DBP, and heart rate (HR) were recorded using the Ponemah software v.6.11 (DSI, St. Paul, MN, USA).

Heart rate variability (HRV) analaysis was also performed as described by manufacturer in order to derive ANS variables. HRV analysis enables to assess cardiac health and the sympathetic and parasympathetic function of the ANS (Rajendra Acharya, Paul Joseph, Kannathal, Lim, & Suri, 2006). Sympathetic stimulation causes an increase in HR and parasympathetic activity decreases HR, providing a regulatory balance in physiological autonomic function (Rajendra Acharya et al., 2006).

Analysis methods for HRV data exist in the time-domain and frequency-domain. In this study, HRV data was analyzed via frequency domain analysis. Frequency domain techniques were performed on the inter-beat-interval signal, a plot of the R-R intervals (ms) versus time. There are typically three main frequency components of HRV; Very Low Frequency (VLF), Low Frequency (LF), High Frequency (HF). Cut-off frequency ranges for VLF at 0.05–0.25 Hz (VLF, indicative of humoral effects on sympathetic drive), LF at 0.25–1 Hz (LF, indicative of overall vasomotor drive), and HF at 1–3 Hz (HF, indicative of cardiac parasympathetic activity) bands were used as defined for rats (Zubcevic et al., 2009). The said parameters were automatically derived from the BP waveform signal using Ponemah software v.6.11. The values for both BP and variability analysis were averaged for every 1 h of recording.

After the 4-week measurement, rats were euthanized. Blood, brains and hearts were collected for further analysis, as detailed below.

3.2.4- Angiotensin II induced hypertension

HTN was established by chronic infusion of Ang II (200 ng/kg/min) using mini-osmotic pumps (0.25-0.28 µl per hour, 28 days, ALZET model 2004). Mini-osmotic pumps were implanted subcutaneously 1 week after the surgery of telemetry transmitters and following the 48-hour baseline recordings. Control animals received saline in osmotic pumps.

Preparation of mini-osmotic pumps

Mechanism of implantable mini-osmotic pump

Mini-osmotic pumps deliver drugs at continuous or controlled rates. It is consisted of three concentric layers named rate controlling, semi-permeable membrane; osmotic membrane and impermeable drug reservoir (Figure 3.4).

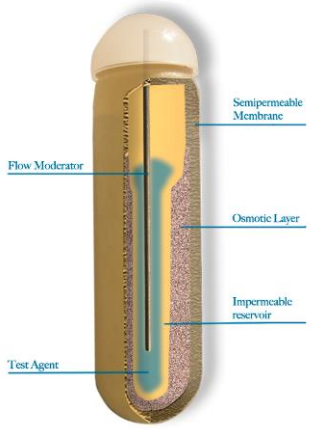

Figure 3-4 Structure of a mini-osmotic pump.

Pump works by osmotic pressure difference between osmotic layer, and the tissue environment in which the pump is implanted. The high osmolality of the osmotic layer causes water to flux into the pump through semipermeable membrane, the outer surface of the pump. As the water enters the osmotic layer, it compresses the flexible reservoir, displacing the drug from the pump at a controlled rate

(Theeuwes & Yum, 1976). The rate of delivery is controlled by the water permeability of the semipermeable membrane. In our experiment, mini-osmotic pumps delivering drugs at a rate of 0.25-0.28 µl per hour (ALZET model 2004, 28 days) were used.

Calculation of angiotensin II amount

Treatment dose of Ang II (200 ng/kg/min) used in the study was determined based on previous studies (Iulita et al., 2018; Santisteban et al., 2015). Ang II was prepared the day before icv surgery in sterile saline. Total volume of saline and total amount of Ang II needed were calculated using the formulas below:

I) Target dose (µg/h) = [Target dose (ng/kg/min) * Average weight of rats (kg)]/ 1000*60

II) Concentration (µg/µL) = Target dose (µg/h)/ Pump rate (µL/h)

III) Total volume saline (µL) = [Pump volume (µL) + Extra pump volume (50 µL)] * Number of pumps (n)

IV) Total mass Ang II (µg) = Concentration (µg/µL) * Total volume saline (µL)

The calculated amount of Ang II was weighed out in a sterile plastic tube since Ang II solution has a high affinity for glass. Then, the calculated volume of sterile saline was added into the plastic tube with Ang II and mixed thoroughly until the solution became clear.

Osmotic pump filling

Gloves were worn while handling pumps since natural oils from hands may damage the exterior of the pump casings. One cc syringe and 25 G needle were used to fill the pumps. Air drawn into syringe along with the Ang II solution was minimized. All bubbles were removed carefully. Syringe was inserted gently into the pump and the pump was filled slowly. Filling was stopped as soon as fluid rose out of the pump. Needle was removed carefully from the hole and it was replaced with

the lid of the pump. Pumps for control animals were filled with saline for sc or PBS for icv placement. Filled pumps were placed into 50 mL conical centrifuge tubes (Falcon, Fisher) filled with 30 mL saline and incubated in the water bath (35 ^{o}C) overnight.

Implantation of mini-osmotic pumps with angiotensin II or saline

Implantation of Ang II or saline pumps was performed on the same day as icv cannulations. Details of the latter surgery are explained under icv cannula implantation below.

3.2.5- Delivery of NaHS via intracerebroventricular infusion

One week after the telemetry surgery, all rats were assigned to subgroups (Table 3.3) to receive either chronic icv NaHS (30 or 60 nmol/h) or PBS infusion via mini-osmotic pump (Brain infusion kit 1 3-5 mm, ALZET), in addition to either chronic Ang II or saline sc pumps. Pumps lasted for 4 weeks from the day of the drug preparation.

Table 3-3 Experimental groups in the study.

	Groups (n= 4-7)	**Treatment**
I	Control rats	icv PBS+ sc saline
II	Hypertensive rats	icv PBS+ sc Ang II
III	icv 30 nmol NaHS-treated rats	icv 30 nmol/h NaHS+ sc saline
IV	icv 60 nmol NaHS-treated rats	icv 60 nmol/h NaHS+ sc saline
V	icv 30 nmol NaHS-treated hypertensive rats	icv 30 nmol/h NaHS+ sc Ang II
VI	icv 60 nmol NaHS-treated hypertensive rats	icv 60 nmol/h NaHS+ sc Ang II

Calculation of NaHS amount

Treatment doses of NaHS were based on a previous study showing effects of acute doses of NaHS (Sikora, Drapala, & Ufnal, 2014). NaHS was prepared the day before the icv surgery using PBS. Concentrations and volumes needed were calculated using the formulas below:

I) Concentration (ng/µL) = Target dose (ng/h)/ Pump rate (µL/h)

II) Total volume PBS (µL) = [Pump volume (µL) + Extra pump volume (20 µL)] * Number of pumps (n)

III) Total mass NaHS (ng) = Concentration (ng/µL) * Total volume PBS (µL)

The calculated amount of NaHS was weighed out in a glass tube. Then, the calculated volume of PBS was added into the tube with NaHS and mixed thoroughly until the solution became clear. pH of the resulting solution was adjusted to 7.5 by addition of small volume of 1 N HCL.

Osmotic pump filling

Pumps were filled with NaHS or PBS as described above. Filled pumps were placed into 50 mL conical centrifuge tubes (Falcon, Fisher) filled with 30 mL saline and incubated in water bath (35 ^{o}C) overnight to activate mini-osmotic pumps.

Intracerebroventricular cannulation and implantation of mini-osmotic pumps

On the day of implantation, all surgical procedures were carried out on a surgical work bench equipped with a surgical microscope. Icv cannulas were implanted in rats as previously described (DeVos & Miller, 2013). Briefly, rats were anesthetized with a mixture of O_2 (1 L/min) and 4% isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) as shown in Figure 3.5. Anesthesia was maintained using an O_2/ isoflurane (2%) mixture delivered through a specialized nose cone for the duration of the surgery. Body temperature was maintained using heating pad.

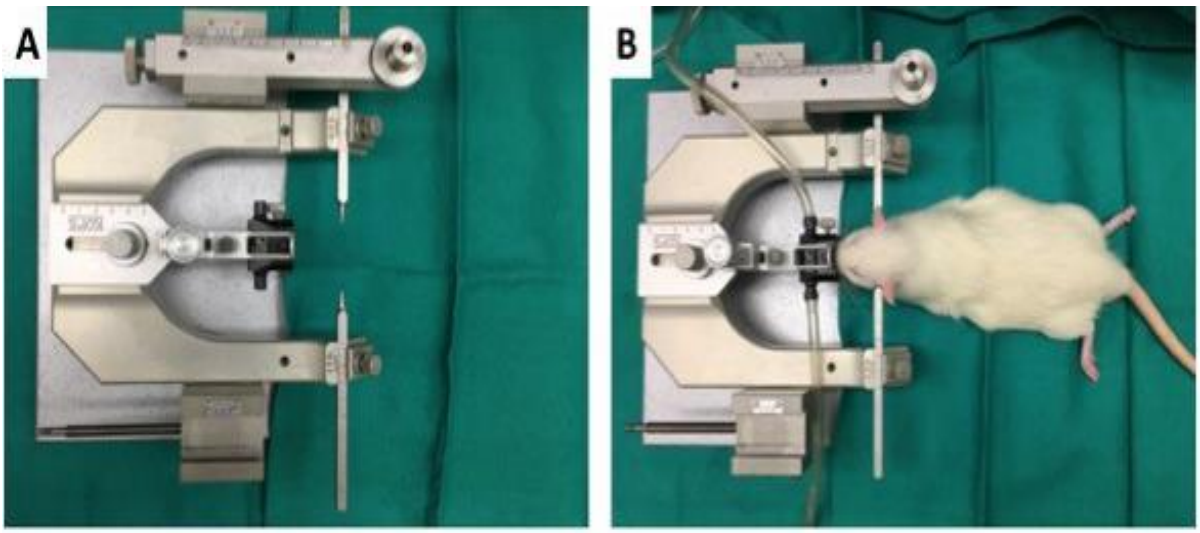

Figure 3-5 Mounting of rats in a stereotaxic surgical device. **A.** Stereotaxic head frame for rodents. **B.** Rat mounted in a stereotaxic frame with secured ear bars (https://bio-protocol.org/e2861).

Then, head was shaved and scrubbed with chlorhexidine and ethanol. A midline incision was made using a small pair of scissors in the skin starting at the back of the eyes and extending posteriorly for about 2 cm. Periosteum was also incised. Periosteum was rolled to the edges of incision by cotton swabs dipped in hydrogen peroxide to enhance visualization of the bregma. Retractors were attached to the periosteum to keep skin and membrane away from the surgical area. The tip of the dental drill, clipped onto the electrode carrier of the stereotaxic instrument, was positioned over bregma and the coordinates on the Digital display were set to zero. Then, the tip of the drill was moved to the calculated coordinates. Stereotaxic coordinates for the lateral cerebroventricle were as follows: 1.3 AP (anterior-posterior) (bregma), 1.50 ML (medial-lateral), and 4.50 DV (dorsal-ventral) (from skull surface), according to the Paxinos and Watson Rat Brain Atlas. A hole for infusion cannula and two holes for brain screws to fix cannula were drilled carefully in the skull bone.

Using curved forceps and small screw driver, two stainless steel screws were placed into the two smaller holes. Then, infusion cannula (Brain infusion kit 1 3-5 mm, ALZET) was placed onto the carrier of the stereotaxic instrument. A 4-week mini-osmotic pump was also connected to this infusion cannula via the catheter tube to deliver drug into the brain. Cannula was moved to the calculated location and slowly lowered into the pre-drilled hole. Skin starting at the neck and extending posteriorly about 4 cm was carefully retracted from the muscle in order to place the mini-osmotic pump subcutaneously. Using the cannula and pumps, either NaHS (30 or 60 nmol/h; SigmaAldrich, St. Louis, MO) or PBS (CORNING cellgro) was infused for 4 weeks into the left cerebroventricle at a flow rate of 0.25-0.28 μL/h. A 4-week mini-osmotic pump with Ang II or saline (0.25-0.28 μL/h) was also implanted in all rats. After the placement of pumps, a small amount of dental cement was applied around the cannula and screws to fix the cannula in place. The wound was closed with wound clips and topical antibiotic (Bacitracin) was applied. Rats received a single dose of buprenorphine (0.1 mL/g body weight; Buprenex, Pfizer, NY) subcutaneously during surgery and were monitored daily throughout the experimental period. At the end of experiment rats were sacrified and brain, heart, plasma and cerebrospinal fluid (CSF) were collected for further analysis detailed below.

3.2.6- Measurement of hydrogen sulfide in plasma and cerebrospinal fluid

H_2S concentration in plasma and CSF was assayed spectrophotometrically as described previously (X. Shen et al., 2011). Briefly, plasma and CSF were collected from rats followed by centrifugation. 75 μL plasma or CSF mixed with 250 μL 1% (w/v) zinc acetate (Alfa Aeser) and 425 μL distilled water in a tube. Then, 20 mM N-dimethyl-p-phenylenediamine oxalate (Fisher) in 7.2 μM HCL (133 μL) and 30 mM $FeCI_3$ (Alfa Aeser) in 1.2 μM HCL (133 μL) were added to the test tube and incubated 10 minutes at room temperature. Protein in the plasma was removed by adding 250 μL of 50% trichloroacetic acid (Fisher) to the reaction mixture and pelleted by centrifugation at 12000 g for 15 min. 300 μL of samples were put into

each well and absorbance of the solution was read with a spectrophotometer (BioTek SynergyMx) at 670 nm in a 96-well plate (Fisher). All samples were assayed in duplicate and blank substracted absorbance values were averaged. The zinc acetate assay measures free H_2S plus related species including HS^- and S^{2-} (L. Li et al., 2008). Accordingly, results for plasma or CSF H_2S reported herein indicate the sum total of these species.

3.2.7- Cardiac hypertrophy

Tissue samples were fixed in 2% paraformaldehyde for 48 hours followed by 70% ethanol until infiltration process. Fixed tissues were dehydrated, embedded in paraffin, sectioned at 4 µm thick, and stained with hematoxylin-eosin (H&E). Slides were imaged on a Keyence Flourescenct microscope under equal conditions for all slides, using the bright field setting on the scope. Slides were scanned and stitched using a 4x objective and the analyzed using a 20x objective. Left ventricular (LV) thickness was measured using ImageJ.

3.2.8- Immunohistochemistry

Rats were euthanized using 4% isoflurane in 95/5 O_2/CO_2. Whole brain tissue was collected in 2% paraformaldehyde overnight, and then transferred to 30% sucrose until they dropped to bottom of 50 mL conical tube, confirming cryoprotection. Then, whole brain was placed in OCT Compound (Tissue-Tek), frozen and stored at -80°C until sectioning. Transverse sections (40 µm) were cut on a freezing microtome to obtain a range of slices corresponding to the PVN regions as per Paxinos and Watson Rat Brain Atlas coordinates. PVN sections were mounted (40 µm thick) on superfrost plus slides (Fisher). Slides have been stored at -20°C until immunohistochemistry (IHC) staining. Slides have allowed to reach room temperature before starting IHC and stained following protocol. First, slides were blocked for one hour in 4% normal goat serum (Jackson ImmunoResearch Labs)-superblock solution (ThermoScientific). IHC was performed with rabbit anti-Iba1 primary antibody (1:300 dilution; Jerry Shaw; Encore) in 1% normal goat serum/PBS solution incubated 16 hours at 4°C, followed by a secondary antibody

(Anti-rabbit AF-647) incubation (1:100 dilution in 1% normal serum in 1XPBS; Invitrogen; A-11008 or A-11012) for 3 hours at room temperature. Slides were mounted wiFth VECTASHIELD mounting medium containing nuclear stain DAPI (Vectorlabs). The micrographs were taken using taken on a Keyence Fluorescence Microscope, all under the same conditions for all slides. Slides were scanned and stitched using a 4x objective and the analyzed using a 20x objective. Total number of microglia cells in PVN was quantified in 1000 µm x 1000 µm bin by counting microglial marker Iba1 positive cells using Image J.

3.3- Data and Statistical Analysis

Descriptive statistics are expressed as mean±standard error of the mean (SEM) and median (Q1-Q3) and categorical data are expressed as frequency and percentage. Shapiro wilk test is used to test the normality of data. Kruskal–Wallis H test is used for the analysis of the continious data with a-non normal distribution. Repeated measures analysis of variance test is used for the analysis of the BP data by weeks. $P<0.05$ was considered statistically significant. SPSS 21.0 was used for statistical analysis.

4. RESULTS

4.1- Blood Pressure Results

4.1.1- Mean arterial pressure, systolic blood pressure, diastolic blood pressure and heart rate by weeks

In total, 9 rats have been implanted Ang II pumps subcutaneously to induce HTN. However, 4 of 9 rats did not have elevated BP at the end of the 4-week infusion period (Figure 4.1 Week 0 MAP in HTN developed Ang II: 97.715±1.655 and in non-HTN developed Ang II: 98.124±2.382; Week 4 MAP in HTN developed Ang II: 154.543±8.188 and in non-HTN developed Ang II: 104.215±3.842).

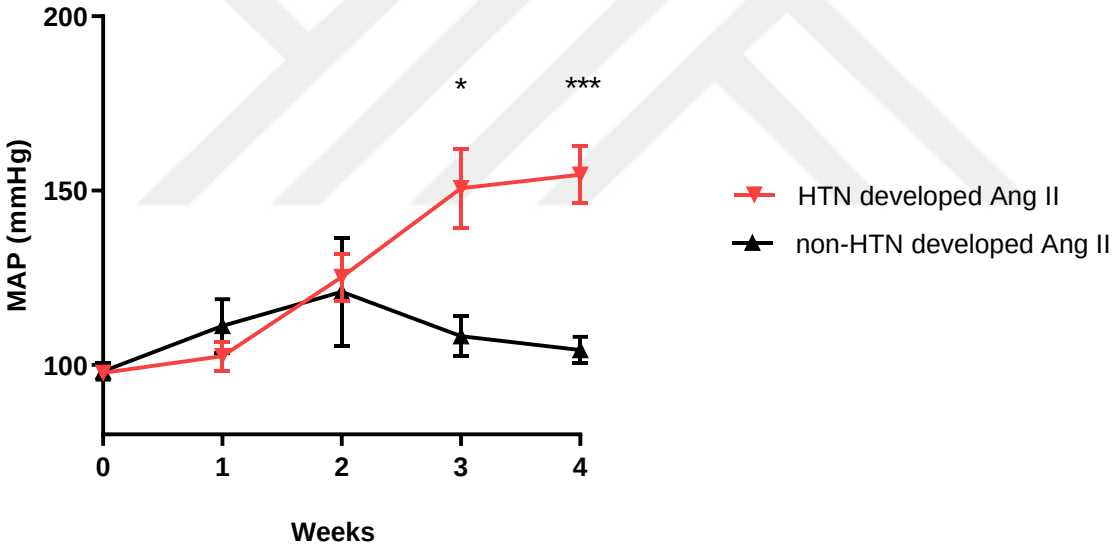

Figure 4-1 Mean arterial pressure (MAP) in animals receiving subcutaneous infusion of Ang II (n=9) for 4 weeks (HTN: Hypertension. Ang II: Angiotensin II). *p<0.05; ***p<0.001 HTN developed Ang II infused rats (n=5) vs non-HTN developed Ang II infused rats (n=4). Data is presented as the mean ± SEM.

Systemic infusion of Ang II increased blood pressure as measured by telemetry

As shown in Figure 4.2 baseline MAP (Figure 4.2A and Table 4.1), SBP (Figure 4.2B and Table 4.2), DBP (Figure 4.2C and Table 4.3) and, HR (Figure 4.2D and Table 4.4) were similar in rats before beginning the infusion protocol. In response to Ang II infusion (n=5), MAP, SBP, DBP started to significantly increase at week two (P<0.001) and HR at week three (P<0.001) and remained elevated throughout 4-week infusion period compared to saline-infused control rats (n=4).

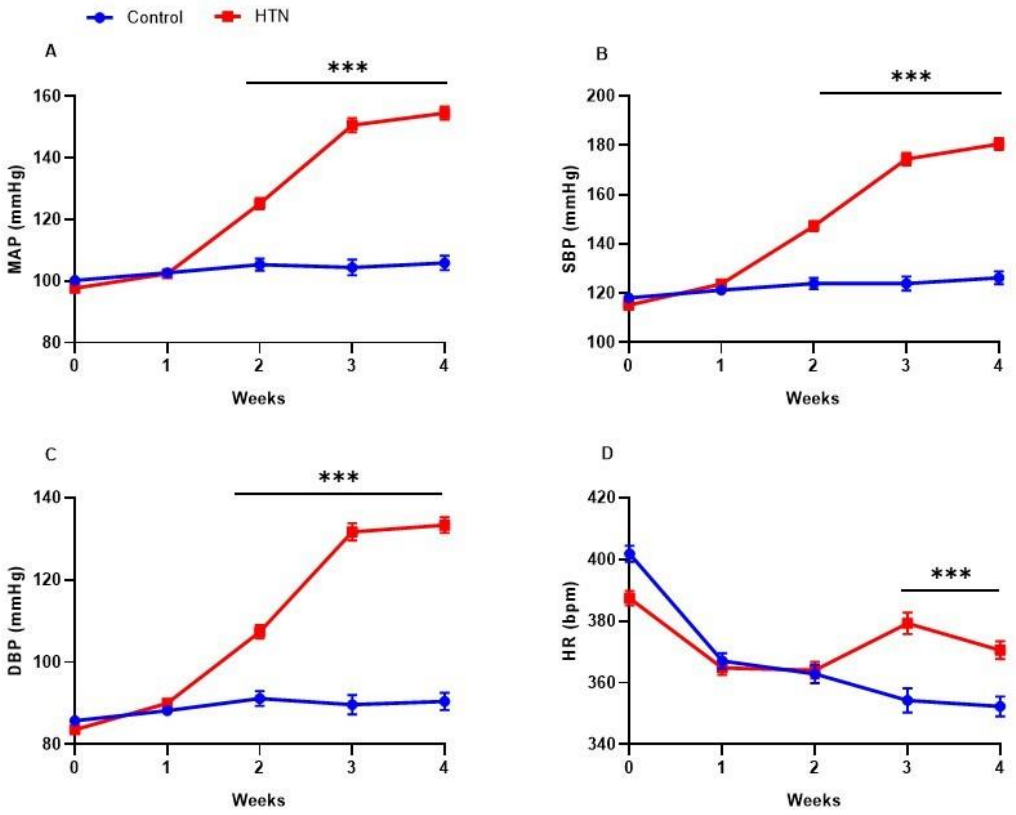

Figure 4-2 Effects of systemic Ang II infusion on blood pressure**.** A) Mean arterial pressure (MAP), B) Systolic blood pressure (SBP), D) Diastolic blood pressure (DBP), E) Heart rate (HR). ***p<0.001 intracerebroventricular (icv) PBS+ sc Ang II (n=5) vs icv PBS+ sc saline (n=4). Data is presented as the mean ± SEM.

Central administration of NaHS attenuated Ang II-dependent increase in blood pressure

Icv 30 nmol/h or 60 nmol/h NaHS infusions for 4 weeks alone had no statistically significant effect on MAP, SBP, DP and HR (Figure 4.3A and Table 4.1, Figure 3B and Table 4.2, Figure 3C and Table 4.3, Figure 3D and Table 4.4, respectively). Concomitant infusions of icv 30 nmol/h or 60 nmol/h NaHS along with Ang II infusion significantly attenuated BP increase starting at week 3 (Figure 4.3A $P<0.001$: MAP in HTN: 150.623±2.273; 30 nmol/h: 136.705±1.921; 60 nmol/h: 129.040±1.921) till week 4 (Figure 4.3A $P<0.001$: MAP in HTN: 154.543±2.103; 30 nmol/h: 137.157±1.778; 60 nmol/h: 127.803±1.778) compared to HTN group. Decrease in MAP was significantly more pronounced in 60 nmol/h NaHS treated HTN group compared to 30 nmol/h NaHS treated one at week 3 (Figure 4.3A $P<0.05$: MAP difference= -7.664) and week 4 (Figure 4.3A $P<0.001$: MAP difference= -9.353).

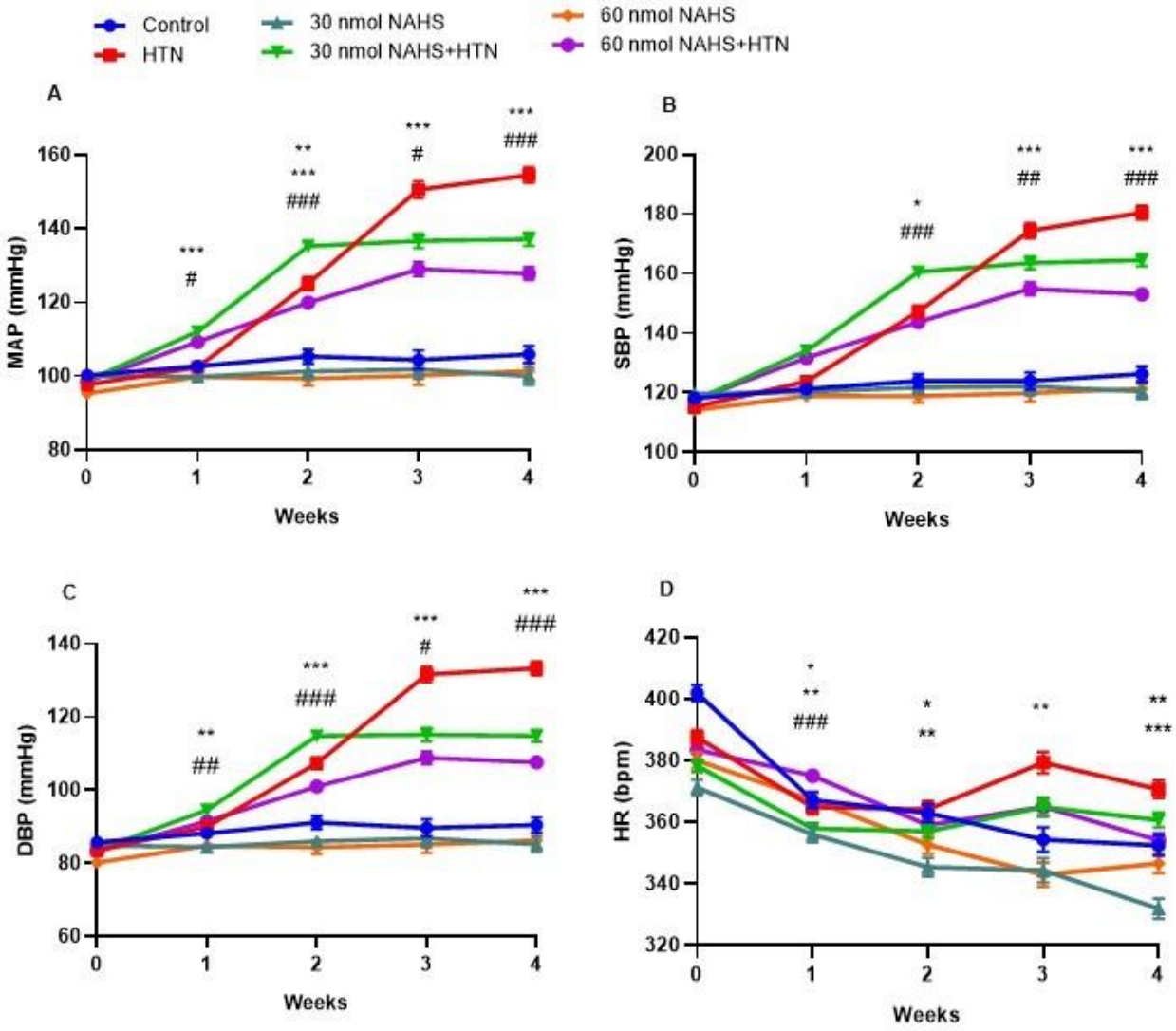

Figure 4-3 Effects of intracerebroventricular (icv) 30 nmol/h NaHS or 60 nmol/h NaHS treatment on blood pressure. A) Mean arterial pressure (MAP), B) Systolic blood pressure (SBP), D) Diastolic blood pressure (DBP), E) Heart rate (HR). *p<0.05; **p<0.01, p<0.005; ***p<0.001: icv 30 nmol/h NaHS+ sc Ang II (n=7) and icv 60 nmol/h NaHS+ sc Ang II (n=7) vs. icv PBS+ sc Ang II (n=5). #p<0.05; ##p<0.01, p<0.0 05; ###p<0.001: icv 30 nmol/h NaHS+ sc Ang II vs. icv 60 nmol/h NaHS+ sc Ang II. Data is presented as the mean ± SEM.

Table 4-1 Comparisions of mean arterial pressure (MAP) by weeks and groups (HTN: Hypertension. Ang II: Angiotensin II. Data is presented as the mean ± SEM).

MAP (mmHg) / **Groups** / **Weeks (W)**	**Control (1) (n=4)**	**HTN (2) (n=5)**	**30 nmol NAHS (3) (n=4)**	**30 nmol NAHS +HTN (4) (n=7)**	**60 nmol NAHS (5) (n=4)**	**60 nmol NAHS +HTN (6) (n=7)**	**Multiple comparisons p values**
W0 (a)	100.253 ±0.593	97.715 ±0.531	100.033 ±0.593	98.781 ±0.448	95.302 ±0.593	98.589 ±0.448	**p<0.001:** 1-5; 3-5; 4-6; 5-1,3,4,6; **p<0.005:** 1-2; 2-1,3,5; 3-2; 5-2 **p<0.05:** 1-4; 1-6; 4-1; 4-1; 6-1,5
W1 (b)	102.702 ±1.190	102.442 ±1.065	99.866 ±1.190	112.092 ±0.900	99.811 ±1.190	109.297 ±0.900	**p<0.001:** 1-4,6; 2-4,6: 3-4,6; 4-1,2,3,5; 5-4,6; 6-1,2,3,5 **p<0.05:**4-6; 6-4
W2 (c)	105.394 ±1.978	125.168 ±1.770	101.295 ±1.978	135.315 ±1.496	99.391 ±1.978	119.964 ±1.496	**p<0.001:** 1-2,4,6; 2-1,3.4,5; 3-2,4,6; 4-1,2,3,5,6; 5-2,4,6; 6-1,3,4,5 **p<0.05:** 1-5; 2-6; 5-1; 6-2
W3 (d)	104.438 ±2.541	150.623 ±2.273	101.805 ±2.541	136.705 ±1.921	100.142 ±2.541	129.040 ±1.921	**p<0.001:** 1-2,4,6; 2-1,3,4,5,6; 3-2,4,6; 4-1,2,3,5; 5-2,4,6; 6-1,2,3,5 **p<0.05:** 4-6; 6-4
W4 (e)	105.914 ±2.351	154.543 ±2.103	100.060 ±2.351	137.157 ±1.778	101.385 ±2.351	127.803 ±1.778	**p<0.001:**1-2,4,6; 2-1,3,4,5,6; 3-2,4,6; 4-1,2,3,5,6; 5-2,4,6; 6-1,2,3,4,5
Multiple comparisons p values	**p<0.05:** a-b,c,e; b-a; c-a; e-a	**p<0.001:** a-b,c,d,e; b-a,c,d,e; c-a,b,d,e; d-a,b,c; e,a,b,c **p<0.05** d-e; e-d		**p<0.001:** a-b,c,d,e; b-a,c,d,e; c-a,b; d-a,b; e-a,b	**p<0.001:** a-b: b-a **p<0.05:** a-c,e; c-a; e-a	**p<0.001:** a-b,c,d,e; b-a,c,d,e; c-a,b,d,e; d-a,b,c; e-a,b,c	**p<0.001**

Table 4-2 Comparisions of systolic blood pressure (SBP) by weeks and groups (HTN: Hypertension. Ang II: Angiotensin II. Data is presented as the mean ± SEM).

SBP (mmHg) / Groups / Weeks (W)	Control (1) (n=4)	HTN (2) (n=5)	30 nmol NAHS (3) (n=4)	30 nmol NAHS +HTN (4) (n=7)	60 nmol NAHS (5) (n=4)	60 nmol NAHS +HTN (6) (n=7)	Multiple comparisons p values
W0 (a)	118.148 ±0.758	115.243 ±0.678	119.674 ±0.758	117.499 ±0.573	114.097 ±0.758	117.583 ±0.573	**p<0.001:** 1-5; 2-3; 3-2,5; 4-5; 5,1,3,4,6; 6-5 **p<0.01:** 1-2;2-1,4,6; 4-2; 6-2 **p<0.05:** 3-4,6; 4-3; 6-3
W1 (b)	121.271 ±1.457	123.830 ±1.303	120.322 ±1.457	134.020 ±1.101	118.999 ±1.457	131.669 ±1.101	**p<0.001:** 1-4,6; 3-4,6; 4-1,2,3,5; 5-4,6; 6-1,2,3,5 **p<0.01:** 2-5
W2 (c)	123.948 ±2.228	147.287 ±1.993	121.760 ±2.228	160.589 ±1.684	118.914 ±2.228	143.819 ±1.684	**p<0.001:** 1-2,4,6; 2-1,3,4,5; 3-2,4,6; 4-1,2,3,5,6; 5-2,4,6; 6-1,3,4,5
W3 (d)	123.919 ±2.844	174.482 ±2.544	122.087 ±2.844	163.643 ±2.150	119.904 ±2.844	154.982 ±2.150	**p<0.001:** 1-2,4,6; 2-1,3,4,5,6; 3-2,4,6; 4-1,2,3,5; 5-2,4,6; 6-1,2,3, **p<0.01:** 4-6; 6-4
W4 (e)	126.268 ±2.647	180.533 ±2.367	120.499 ±2.647	164.569 ±2.001	121.368 ±2.647	153.128 ±2.001	**p<0.001:** 1-2,4,6; 2-1,3,4,5,6; 3-2,4,6; 4-1,2,3,6; 5-2,4,6; 6-1,2,3,4,5
Multiple comparisons p values	**p<0.01:** a-c,e; c-a; e-a **p<0.05:** a-b,d;b-a; d-a	**p<0.001:** a-b,c,d,e; b-a,c,d,e; c-a,b,d,e; d-a,b,c,e; e-a,b,c,d		**p<0.001:** a-b,c,d,e; b-a,c,d,e; c-a,b **p<0.05:** c-e	**p<0.001:** a-b; b-a **p<0.01:** a,e; e-a **p<0.05** a,c,d; c-a; d-a	**p<0.001:** a-b,c,d,e; b-a,c,d,e; c-a,b,d,e; d-a,b,c; e-a,b,c	**p<0.001**

Table 4-3 Comparisions of diastolic blood pressure (DBP) by weeks and groups (HTN: Hypertension. Ang II: Angiotensin II. Data is presented as the mean ± SEM).

DBP (mmHg) / Groups / Weeks (W)	Control (1) (n=4)	HTN (2) (n=5)	30 nmol NAHS (3) (n=4)	30 nmol NAHS +HTN (4) (n=7)	60 nmol NAHS (5) (n=4)	60 nmol NAHS +HTN (6) (n=7)	Multiple comparisons p values
W0 (a)	85.789 ±0.511	83.587 ±0.457	84.903 ±0.511	83.658 ±0.387	80.237 ±0.511	83.372 ±0.387	**p<0.005:** 1-2,4; 2-1; 4-1 **p<0.001:** 1-5,6; 2-5; 3-5; 4-5; 5-1,2,3,4,6; 6-1,5 **p<0.05:** 3-6; 6-3
W1 (b)	88.208 ±1.054	90.018 ±0.943	84.487 ±1.054	94.559 ±0.797	84.815 ±1.054	91.478 ±0.797	**p<0.001:**1-4; 2-3,4,5; 3-2,4,6; 4-1,2,3,5; 5-2,4,6; 6-3,5 **p<0.05:**1-3,5,6; 3-1; 5-1; 6-1 **p<0.01:** 4-6; 6-4
W2 (c)	91.194 ±1.815	107.379 ±1.624	86.0619 ±0.87	114.801 ±1.372	84.459 ±1.815	101.006 ±1.372	**p<0.001:** 1-2,3,4,6; 2-1,3,5; 3-1,2,5,6; 4-1,5,6; 5-2,3,4,6; 6-1,3,4,5 **p<0.005:** 2-4,6; 4-2; 6-2 **p<0.01:**1-5; 5-1 **p<0.05:** 3-4; 4-3
W3 (d)	89.682 ±2.368	131.660 ±2.118	86.814 ±2.368	115.148 ±1.790	85.169 ±2.368	108.865 ±1.790	**p<0.001:** 1-2,4,6; 2-1,3,4,5,6; 3-2,4,6; 4-1,2,3,5; 5-2,4,6; 6-1,2,3,5 **p<0.05:** 4-6; 6-4
W4 (e)	90.481 ±2.112	133.311 ±1.889	85.261 ±2.112	114.832 ±1.597	86.218 ±2.112	107.642 ±1.597	**p<0.001:**1-2,4,6; 2-1,3,4,5,6; 3-2,4,6; 4-1,2,3,5; 5-2,4,6; 6-1,2,3,5 **p<0.005:** 4-6; 6-4
Multiple comparisons p values	**p<0.005:** a-c; c-a **p<0.05:** a-b,e; b-a,c c-b; e-a	**p<0.001:** a-b,c,d,e; b-a,c,d,e; c-a,b,d,e; d-a,b,c; e-a,b,c		**p<0.001:** a-b,c,d,e; b-a,c,d,e; c-a,b; d-a,b; e-a,b;	**p<0.001:** a-b; b-a **p<0.01:** a-e; e-a **p<0.05:** a-c,d; c-a; d-a	**p<0.001:** a-b,c,d,e; b-a,c,d,e; c-a,b,d,e; d-a,b,c; e-a,b,c	**p<0.001**

Table 4-4 Comparisions of heart rate (HR) by weeks and groups (HTN: Hypertension. Ang II: Angiotensin II. Data is presented as the mean ± SEM).

HR (bpm) / Groups / Weeks (W)	Control (1) (n=4)	HTN (2) (n=5)	30 nmol NAHS (3) (n=4)	30 nmol NAHS +HTN (4) (n=7)	60 nmol NAHS (5) (n=4)	60 nmol NAHS +HTN (6) (n=7)	Multiple comparisons p values
W0 (a)	401.858 ±2.622	387.446 ±2.345	371.193 ±2.622	378.203 ±1.982	379.994 ±2.622	383.459 ±1.982	**p<0.001:** 1-2,3,4,5,6; 2-1,3; 3-1,2,6; 4-1; 5-1; 6-1,3 **p<0.005:** 2-4; 4-2 **p<0.05:** 2-5; 3-4,5; 4-3; 5-2,3
W1 (b)	367.119 ±2.579	364.861 ±2.307	355.972 ±2.579	357.905 ±1.950	367.141 ±2.579	375.036 ±1.950	**p<0.001:** 3-6; 4-6; 6-3,4 **p<0.005:** 1-3; 2-6; 3-1,5; 4-5; 5-3,4; 6-2 **p<0.01:** 4-1 **p<0.05:**1-4,6; 2-3,4; 3-2; 4-2; 5-6; 6-1,5
W2 (c)	362.896 ±3.003	364.125 ±2.686	345.378 ±3.003	356.932 ±2.270	352.657 ±3.003	358.981 ±2.270	**p<0.001:**1-3; 2-3; 3-1,2,6; 6-3 **p<0.005:** 3-4; 4-3 **p<0.01:** 2-5; 5-2 **p<0.05:** 1-5; 2-4; 4-2; 5-1
W3 (d)	354.314 ±3.932	379.327 ±3.517	344.265 ±3.932	364.887 ±2.973	342.875 ±3.932	364.992 ±2.973	**p<0.001:** 1-2; 2-1,3,5; 3-2,4,6; 4-3,5; 5-2,4,6; 6-3,5 **p<0.005:** 2-4,6; 4-2; 6-2 **p<0.05:** 1-4,5,6; 4-1; 5-1; 6-1
W4 (e)	352.358 ±3.228	370.592 ±2.887	331.857 ±3.228	360.596 ±2.440	346.505 ±3.228	354.017 ±2.440	**p<0.001:** 1-2,3; 2-1,3,5,6; 3-1,2,4,6; 4-3; 5-2; 6-2,3 **p<0.005:** 3-5; 4-5; 5-3,4 **p<0.01:** 2-4; 4-2 **p<0.05:** 1-4; 4-1
Multiple comparisons p values	**p<0.001:** a-b,c,d,e; b-a,e; c-a; d-a; e-a,b **p<0.005:** b-d; d-b **p<0.01:** c-e; e-c **p<0.05:** c-d; d-c	**p<0.001:** a-b,c,e; b-a,d c-a,d;d-b,c;e-a **p<0.01:** d-e; e-d **p<0.05:** a-d; d-a	**p<0.001:** a-b,c,d,e b-a,e; c-a; d-a,e-a,b **p<0.005:** b-c;c-b,e d-e;e-c,d **p<0.01:** b-d; d-b	**p<0.001:** a-b,c,d,e; b-a; c-a; d-a; e-a **p<0.05:** b-d; c-d; d-b,c	**p<0.001:** a-b,c,d,e b-a,c,d,e c-a,b d-a,b e-a,b **p<0.05:** c-d; d-c	**p<0.001:** a-c,d,e b-c,e c-a,b d-a,e e-a,b,e **p<0.005:** a-b; b-a,d d-b	**p<0.001**

4.1.2- Very low frequency, low frequency, high frequency, total power by weeks

Systemic Ang II infusion caused autonomic imbalance

Four-week Ang II infusion significantly increased LF (sympathetic vasomotor tone) starting at week 1 till week 3, yet the latter failed to reach significance and total power (TP) starting at week 3 and week 4 compared to control group (Figure 4.4, Table 4.6 and Table 4.8, respectively). Ang II significanlty increased the cardiac parasympathetic drive measured by HF (pulse interval (PI)) starting at week 3 and week 4 compared to control group (Figure 4.4, Table 4.7), which is probably due to compensation due to increase in LF. Nevertheless – the compensation was not sufficient since LF/HF which is an indicator of vasovagal balance was decreased in Ang II (Figure 4.4, Table 4.9).

Central administration of NaHS along with Ang II infusion attenuated autonomic imbalance in hypertension

Icv 60 nmol NaHS but not 30 nmol NaHS, was able to normalize the Ang II–perturbed LF, HF and TP (Figure 4.5, Table 4.6, Table 4.7 and Table 4.8, respectively).

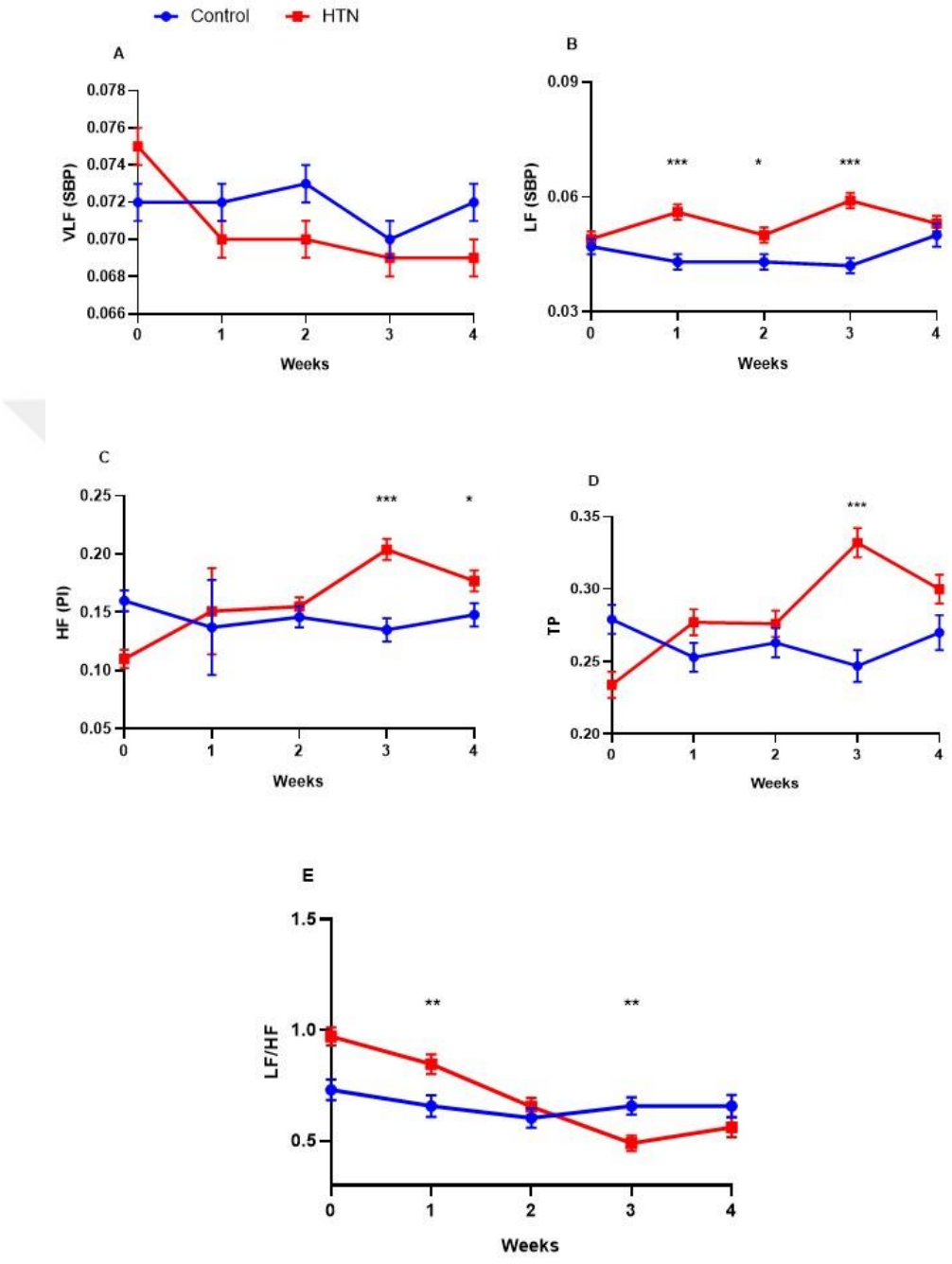

Figure 4-4 Effects of systemic Ang II infusion on autonomic variables. A) Very low frequency (VLF), B) Low frequency (LF), C) High frequency (HF), D) Total power (TP), E) Low Frequency/High Frequency (LF/HF). *p<0.05; **p<0.01; p<0.005; ***p<0.001: intracerebroventricular (icv) PBS+ sc Ang II (n=5) vs icv PBS+ sc saline (n=4). Data is presented as the mean ± SEM.

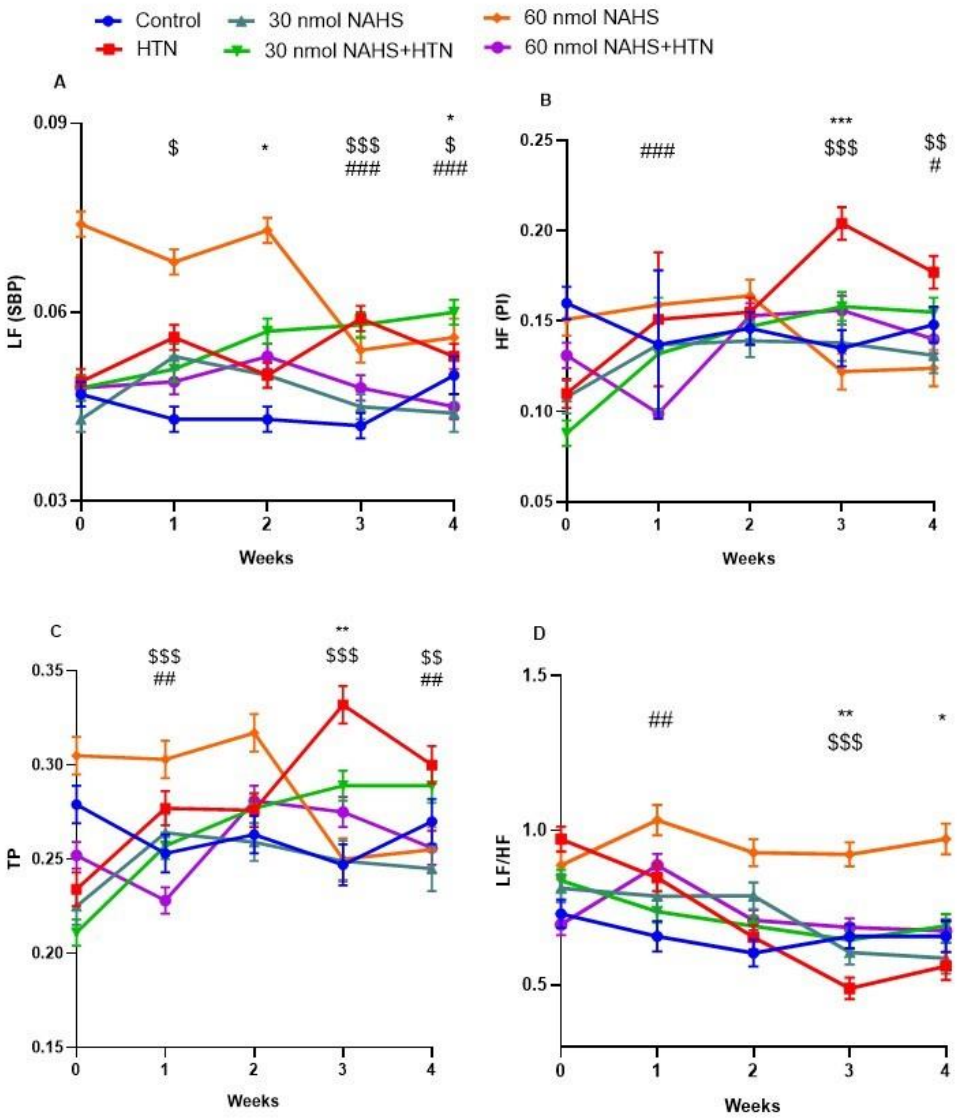

Figure 4-5 Effects of intracerebroventricular (icv) 30 nmol/h NaHS or 60 nmol/h NaHS treatment on autonomic variables. A) Low frequency (LF), B) High frequency (HF), C) Total power (TP), D) Low Frequency/High Frequency (LF/HF). *p<0.05; **p<0.01; p<0.0 05; ***p<0.001: icv 30 nmol/h NaHS+ sc Ang II (n=7) and $p<0.05; $$p<0.01; p<0.0 05; $$$p<0.001: icv 60 nmol/h NaHS+ sc Ang II (n=7) vs. icv PBS+ sc Ang II (n=5). #p<0.05; ##p<0.01, p<0.005; ###p<0.001: icv 30 nmol/h NaHS+ sc Ang II vs. icv 60 nmol/h NaHS+ sc Ang II. Data is presented as the mean ± SEM.

Table 4-5 Comparisions of very low frequency (VLF) by weeks and groups (HTN: Hypertension. Ang II: Angiotensin II. SBP (mmHg): Systolic blood pressure. Data is presented as the mean ± SEM).

VLF (SBP) Groups / Weeks (W)	Control (1) (n=4)	HTN (2) (n=5)	30 nmol NAHS (3) (n=4)	30 nmol NAHS +HTN (4) (n=7)	60 nmol NAHS (5) (n=4)	60 nmol NAHS +HTN (6) (n=7)	Multiple comparisons p values
W0 (a)	0.072 ±0.001	0.0752 ±0.001	0.0742 ±0.001	0.0742 ±0.001	0.0802 ±0.001	0.0722 ±0.001	**p<0.001:** 1-5; 2-5; 3-6; 4-5; 5-1,2,3,4,5; 6-5 **p<0.05:** 1-2; 2-1,6; 6-2
W1 (b)	0.0722 ±0.001	0.0702 ±0.001	0.0732 ±0.001	0.0742 ±0.001	0.0762 ±0.001	0.0752 ±0.001	**p<0.001:** 2-5; 5-2 **p<0.005:** 2-6; 6-2 **p<0.01:** 2-4; 4-2 **p<0.05:** 1-5; 2-3; 3-2; 5-1
W2 (c)	0.0732 ±0.001	0.0702 ±0.001	0.0702 ±0.001	0.0732 ±0.001	0.0792 ±0.001	0.0742 ±0.001	**p<0.001:** 2-5; 3-5; 4-5; 5,2,3,4 **p<0.005:** 1-5; 5-1,6; 6-5 **p<0.01:** 3-6; 6-3; 4-6 **p<0.05:** 1-3; 2-4; 2-6; 3-1; 3-4; 4-2,3; 6-2
W3 (d)	0.0702 ±0.001	0.0692 ±0.001	0.0652 ±0.001	0.0732 ±0.001	0.0742 ±0.001	0.0702 ±0.001	**p<0.001:**3-4,5; 4-3 **p<0.005:** 2-4,5; 3-6; 4-2; 6-3 **p<0.01:**1-3; 3-1 **p<0.05:** 1-4,5; 2-3; 3-2; 4-1,6; 6-4,5
W4 (e)	0.0722 ±0.001	0.0692 ±0.001	0.0712 ±0.001	0.0732 ±0.001	0.0752 ±0.001	0.0702 ±0.001	**p<0.005:** 2-5; 5-2; 5-6; 6-5 **p<0.05:** 2-4; 3-5; 4-2,6; 5-3; 6-4
Multiple comparisons p values	**p<0.01:** c-d; d-c	**p<0.001:** a-b,c,d,e; b-a; c-a; d-a; e-a	**p<0.001:** a-d; b-d; c-d; d-a,b,c,e; e-d **p<0.01:** b-c; c-b **p<0.05:** a-c; c-a		**p<0.001:** a-d; a-e; c-d; d-a; d-c **p<0.005:** a-b; b-a; e-a **p<0.05:** b-c; c-b; c-e; e-c	**p<0.001:** b-d,e; c-d; d-b,c; e-b **p<0.005:** c-e; e-c **p<0.05:** a,b,d; b-a; d-a	**p<0.001**

Table 4-6 Comparisions of low frequency (LF) by weeks and groups (HTN: Hypertension. Ang II: Angiotensin II. SBP (mmHg): Systolic blood pressure. Data is presented as the mean ± SEM).

LF (SBP) Groups / Weeks (W)	Control (1) (n=4)	HTN (2) (n=5)	30 nmol NAHS (3) (n=4)	30 nmol NAHS +HTN (4) (n=7)	60 nmol NAHS (5) (n=4)	60 nmol NAHS +HTN (6) (n=7)	Multiple comparisons p values
W0 (a)	0.047 ±0.002	0.049 ±0.002	0.043 ±0.002	0.048 ±0.002	0.074 ±0.002	0.048 ±0.002	**p<0.001:** 1-5; 2-5; 3-5; 4-5; 5-1,2,3,4,6; 6-5 **p<0.05:** 2-3; 3-2
W1 (b)	0.043 ±0.002	0.056 ±0.002	0.053 ±0.002	0.051 ±0.002	0.068 ±0.002	0.049 ±0.002	**p<0.001:** 1-2,5; 2-1,5; 3-5; 4-5; 5-1,2,3,4,6; 6-5 **p<0.005:** 1-3; 3-1 **p<0.05:** 1-4; 2-6; 4-1; 6-2
W2 (c)	0.043 ±0.002	0.050 ±0.002	0.050 ±0.002	0.057 ±0.002	0.073 ±0.002	0.053 ±0.002	**p<0.001:**1-4,5; 2-5; 3-5; 4-1,5; 5-1,2,3,4,6; 6-5 **p<0.005:**1-6; 6-1 **p<0.05:** 1-2,3; 2-1,4; 3-1,4; 4-2,3
W3 (d)	0.042 ±0.002	0.059 ±0.002	0.045 ±0.002	0.058 ±0.002	0.054 ±0.002	0.048 ±0.002	**p<0.001:** 1-2,4,5; 2-1,3,6; 3-2,4; 4-1,3,6; 5-1; 6-2,4 **p<0.05:** 1-6; 3-5; 5-3; 6-1
W4 (e)	0.050 ±0.003	0.053 ±0.002	0.044 ±0.003	0.060 ±0.002	0.056 ±0.003	0.045 ±0.002	**p<0.001:** 3-4; 4-5,6; 6-5; 6-4 **p<0.005:** 1-4; 3-5; 4-1; 5-3,6 **p<0.01:** 2-3; 3-2 **p<0.05:** 2-4,6; 4-2; 6-2
Multiple comparisons p values	**p<0.05:** c-e; d-e; e-c,d	**p<0.001:** a-d; d-a **p<0.005:** c-d; d-c **p<0.01:** a-b; b-a **p<0.05:** b-c; c-b; d-e; e-d	**p<0.001:** a-b; b-a **p<0.005:** a-c; b-e; c-a; e-b **p<0.05:** b-d; c-e; d-b; e-c	**p<0.001:** a-c,d,e; b-e; c-a; d-a; e-a,b **p<0.005:** b-c,d; c-b; d-b	**p<0.001:** a-d,e; b-d,e; c-d,e; d-a,b,c; e-a,b,c **p<0.05:** a-b; b-a	**p<0.005:** c-e; e-c **p<0.01:** a-c; c-a **p<0.05:** b-c; c-b,d; d-c	**p<0.001**

Table 4-7 Comparisions of high frequency (HF) by weeks and groups (HTN: Hypertension. Ang II: Angiotensin II. PI: Pulse interval (ms). Data is presented as the mean ± SEM).

HF (PI) Groups / Weeks (W)	Control (1) (n=4)	HTN (2) (n=5)	30 nmol NAHS (3) (n=4)	30 nmol NAHS +HTN (4) (n=7)	60 nmol NAHS (5) (n=4)	60 nmol NAHS +HTN (6) (n=7)	Multiple comparisons p values
W0 (a)	0.160 ±0.009	0.110 ±0.008	0.108 ±0.009	0.088 ±0.007	0.151 ±0.009	0.131 ±0.007	**p<0.001:** 1-2,3,4; 2-1,5; 3-1,5; 4-1,5,6; 5-2,3,4; 6-4 **p<0.05:** 1-6; 2-4,6; 3-6; 4-2; 6-1,2,3
W1 (b)	0.137 ±0.041	0.151 ±0.037	0.137 ±0.041	0.132 ±0.031	0.159 ±0.009	0.100 ±0.007	**p<0.001:** 2-6; 5-6; 6-2,5 **p<0.005:** 1-6; 3-6; 4-6; 6-1,3,4 **p<0.05:** 4-5; 5-4
W2 (c)	0.146 ±0.009	0.155 ±0.008	0.139 ±0.009	0.147 ±0.007	0.164 ±0.009	0.153 ±0.007	
W3 (d)	0.135 ±0.010	0.204 ±0.009	0.138 ±0.010	0.158 ±0.008	0.122 ±0.010	0.156 ±0.008	**p<0.001:** 1-2; 2-1,3,4,5,6; 3-2; 4-2; 5-2; 6-2 **p<0.005:** 4-5; 5-4 **p<0.01:** 5-4; 6-5
W4 (e)	0.148 ±0.010	0.177 ±0.009	0.131 ±0.010	0.155 ±0.008	0.124 ±0.010	0.140 ±0.008	**p<0.001:** 2-5; 5-2 **p<0.005:** 2-3,6; 3-2; 6-2 **p<0.05:** 1-2; 2-1; 4-5; 5-4
Multiple comparisons p values	**p<0.05:** a-b,d; b-a; d-a	**p<0.001:** a-b,c,d,e; b-a,d; c-a,d; d-a,b,c; e-a **p<0.01:** b-e; d-e; e-b,d **p<0.05:** c-e; e-c	**p<0.005:** a-c,d; c-a; d-a **p<0.01:** a-b; b-a **p<0.05:** a-e; e-a	**p<0.001:** a-b,c,d,e; b-a; c-a; d-a; e-a **p<0.005:** b-d; d-b **p<0.01:** b-e; e-b **p<0.05:** b-c; c-b	**p<0.001:** c-d,e; d-c; e-c **p<0.005:** b-d,e; d-b; e-b **p<0.01:** a-d; d-a **p<0.05:** a-e; e-a	**p<0.001:** a-b; b-a,c,d,e; c-b; d-b; e-b **p<0.005:** a-c,d; c-a; d-a **p<0.05:** d-e; e-d	**p<0.001**

Table 4-8 Comparisions of total power (TP) by weeks and groups (HTN: Hypertension. Ang II: Angiotensin II. SBP (mmHg): Systolic blood pressure. Data is presented as the mean ± SEM).

TP (SBP) Groups / Weeks (W)	Control (1) (n=4)	HTN (2) (n=5)	30 nmol NAHS (3) (n=4)	30 nmol NAHS +HTN (4) (n=7)	60 nmol NAHS (5) (n=4)	60 nmol NAHS +HTN (6) (n=7)	Multiple comparisons p values
W0 (a)	0.279 ±0.010	0.234 ±0.009	0.225 ±0.010	0.211 ±0.007	0.305 ±0.010	0.252 ±0.007	**p<0.001:** 1-3,4; 2-5; 3-1,5; 4-1,5,6; 5-2,3,4,6; 6-4,5 **p<0.005:**1-2; 2-1 **p<0.05:**1-6; 2-4; 3-6; 4-2; 6-1,3
W1 (b)	0.253 ±0.010	0.277 ±0.009	0.264 ±0.010	0.257 ±0.007	0.303 ±0.010	0.228 ±0.007	**p<0.001:** 1-5; 2-6; 4-5; 5-1,4,6; 6-2,5 **p<0.01:** 3-5,6; 4-6; 5-3; 6-3,4 **p<0.05:** 1-6; 6-1
W2 (c)	0.263 ±0.010	0.276 ±0.009	0.259 ±0.010	0.277 ±0.008	0.317 ±0.010	0.281 ±0.008	**p<0.001:** 1-5; 3-5; 5-1,3 **p<0.005:** 2-5; 4-5; 5-2,4 **p<0.01:** 5-6; 6-5
W3 (d)	0.247 ±0.011	0.332 ±0.010	0.249 ±0.011	0.289 ±0.008	0.250 ±0.011	0.275 ±0.008	**p<0.001:** 1-2; 2-1,3,5,6; 3-2; 5-2; 6-2 **p<0.005:** 1-4; 2-4; 3-4; 4-1,2,3 **p<0.01:** 4-5; 5-4 **p<0.05:** 1-6; 6-1
W4 (e)	0.270 ±0.012	0.300 ±0.010	0.245 ±0.012	0.289 ±0.009	0.255 ±0.012	0.256 ±0.009	**p<0.001:** 2-3; 3-2 **p<0.005:** 2-5,6; 3-4; 4-3; 5-2; 6-2 **p<0.01:** 4-6; 6-4 **p<0.05:** 4-5; 5-4
Multiple comparisons p values	**p<0.01:** a-d; d-a **p<0.05:** a-b; b-a	**p<0.001:** a-b,c,d,e; b-a,d;c-a,d; d-a,b,c; e-a **p<0.005:** d-e; e-d **p<0.05:** b-e; c-e; e-b,c	**p<0.005:** a-b,c; b-a; c-a **p<0.05:** a-d; d-a	**p<0.001:** a-b,c,d,e b-a; c-a; d-a; e-a **p<0.005:** b-d,e; d-b; e-b **p<0.05:** b-c; c-b	**p<0.001:** a-d,e; b-d,e; c-d,e; d-a,b,c; e-a,b,c	**p<0.001:** a-c; b-c,d; c-a,b; d-b **p<0.01:** a-b,d; b-a,e d-a; e-b **p<0.05:** c-e; d-e; e-c,d	**p<0.001**

Table 4-9 Comparisions of low frequency/high frequency (LF/HF) by weeks and groups (HTN: Hypertension. Ang II: Angiotensin II. Data is presented as the mean ± SEM).

LF/HF Groups / Weeks (W)	Control (1) (n=4)	HTN (2) (n=5)	30 nmol NAHS (3) (n=4)	30 nmol NAHS +HTN (4) (n=7)	60 nmol NAHS (5) (n=4)	60 nmol NAHS +HTN (6) (n=7)	Multiple comparisons p values
W0 (a)	0.730 ±0.046	0.971 ±0.041	0.813 ±0.046	0.838 ±0.035	0.887 ±0.046	0.696 ±0.035	**p<0.001:** 1-2; 2-1,6; 6-2 **p<0.005:** 4-6; 5-6; 6-4,5 **p<0.05:** 1-5; 2-3,4; 3-2,6; 4-2; 5-1; 6-3
W1 (b)	0.657 ±0.049	0.847 ±0.044	0.787 ±0.049	0.737 ±0.037	1.033 ±0.049	0.887 ±0.037	**p<0.001:** 1-5,6; 3-5; 4-5; 5-1,3,4; 6-1 **p<0.005:** 1-2; 2-1; 4-6; 6-4 **p<0.01:** 2-5; 5-6,2; 6-5
W2 (c)	0.603 ±0.044	0.654 ±0.039	0.788 ±0.044	0.690 ±0.033	0.928 ±0.044	0.709 ±0.033	**p<0.001:** 1-5; 2-5; 4-5; 5-1,2,4,6; 6-5 **p<0.005:** 1-3; 3-1 **p<0.05:** 2-3; 3-2,5; 5-3
W3 (d)	0.657 ±0.039	0.489 ±0.035	0.605 ±0.039	0.645 ±0.029	0.922 ±0.039	0.686 ±0.029	**p<0.001:** 1-5; 2-5,6; 3-5; 4-5; 5-1,2,3,4,6; 6-2,5 **p<0.005:** 1-2; 2-1,4; 4-2 **p<0.05:** 2-3; 3-2
W4 (e)	0.657 ±0.050	0.561 ±0.045	0.586 ±0.050	0.691 ±0.038	0.972 ±0.050	0.675 ±0.038	**p<0.001:** 1-5; 3-5; 4-5; 5-1,2,3,4,6-5 **p<0.05:** 2-4; 4-2
Multiple comparisons p values	**p<0.05:** a-c; c-a	**p<0.001:** a-c,d,e; b-c,d,e; c-a,b,d; d-a,b,c e-ab **p<0.005:** a-b; b-a	**p<0.001:** a-d,e; b-d,e; c-e,d; d-a,b,c; e-a,b,c	**p<0.001:** a-c,d,e; c-a; d-a; e-a **p<0.01:** a-b; b-a **p<0.05:** b-d; d-b	**p<0.005:** a-b; b-a **p<0.05** b-c,d; c-b; d-b	**p<0.001:** a-b; b-a,c,d,e: c-b; d-b; e-b	**p<0.001**

4.1.3- Hydrogen sulfide levels in plasma and cerebrospinal fluid

There was no significant difference in H_2S levels in plasma and CSF among groups (Table 4.10 and Figure 4.6 and 4.7, respectively).

Table 4-10 Hydrogen sulfide levels in plasma and cerebnospinal fluid (HTN: Hypertension. Ang II: Angiotensin II. CSF: Cerebrospinal fluid).

Groups		n	Absorbance (Arbitary unit)	
			Mean±SD	Median (Q1-Q3)
Plasma	**Control**	4	0.021±0.002	0.020 (0.019-0.023)
	HTN	4	0.020±0.001	0.020 (0.019-0.021)
	30 nmol/h NaHS	4	0.023±0.004	0.022 (0.021-0.028)
	30 nmol/h NaHS+ HTN	5	0.023±0.004	0.022 (0.021-0.026)
	60 nmol/h NaHS	4	0.022±0.002	0.022 (0.021-0.024)
	60 nmol/h NaHS+ HTN	6	0.020±0.001	0.020 (0.019-0.021)
CSF	**Control**	4	0.038±0.015	0.038 (0.024-0.053)
	HTN	4	0.040±0.005	0.042 (0.035-0.044)
	30 nmol/h NaHS	3	0.044±0.002	0.044 (0.043-0.044)
	30 nmol/h NaHS+ HTN	5	0.043±0.001	0.042 (0.042-0.044)
	60 nmol/h NaHS	4	0.042±0.005	0.043 (0.037-0.045)
	60 nmol/h NaHS+ HTN	6	0.030±0.009	0.030 (0.022-0.039)

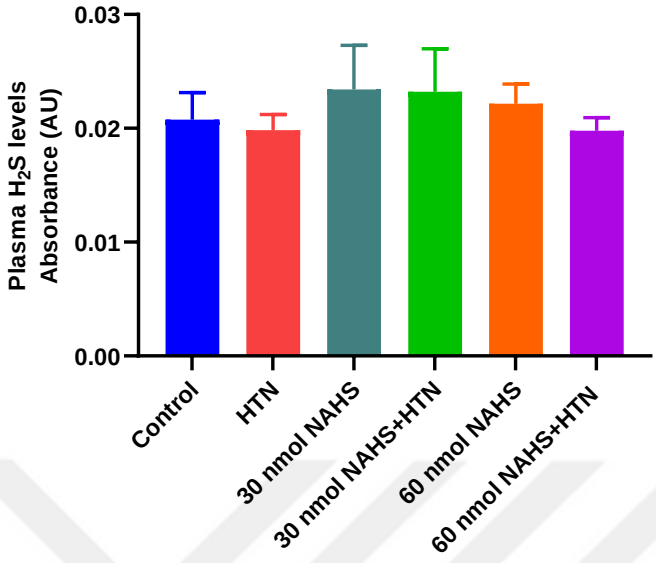

Figure 4-6 Hydrogen sulfide levels in plasma (HTN: Hypertension. Ang II: Angiotensin II. AU: Arbitary unit. Control (n=4), icv PBS+ sc Ang II (n=4), icv 30 nmol/h NaHS (n=4), icv 30 nmol/h NaHS+ sc Ang II (n=5), icv 60 nmol/h NaHS (n=4), icv 60 nmol/h NaHS+ sc Ang II (n=6). Data is presented as the mean ± SD).

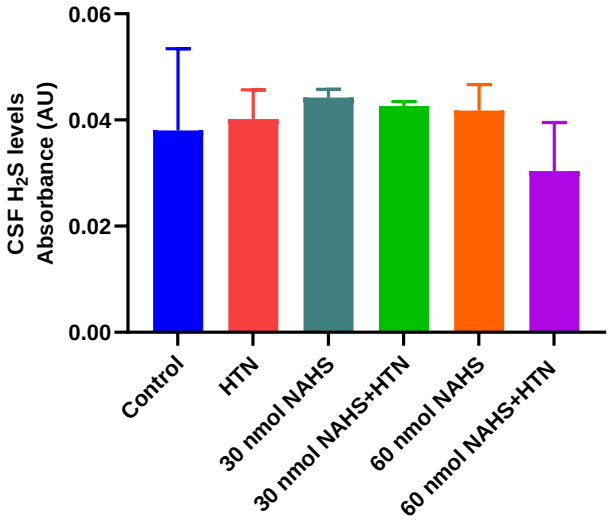

Figure 4-7 Hydrogen sulfide levels in cerebrospinal fluid (HTN: Hypertension. Ang II: Angiotensin II. AU: Arbitary unit. Control (n=4), icv PBS+ sc Ang II (n=4), icv 30 nmol/h NaHS (n=3), icv 30 nmol/h NaHS+ sc Ang II (n=5), icv 60 nmol/h NaHS (n=4), icv 60 nmol/h NaHS+ sc Ang II (n=6). Data is presented as the mean ± SD).

Cardiac hypertrophy

Ang II infusion caused cardiac hypertrophy with increased LV wall thickness compared to control (Figure 4.8 P<0.0001: HTN: 1474±57.53, Control: 575.6±97.13 and Figure 4.9) and 60 nmol/h NaHS treatment also attenuated the development cardiac hypertrophy in hypertesive rats (Figure 4.8 P<0.0001: 60 nmol/h NaHS+ HTN: 824.7±36.12, HTN: 1474±57.53 Figure 4.9) whereas no significant change was detectable in 30 nmol/h NaHS treated HTN group (Figure 4.8, 1394±67.16).

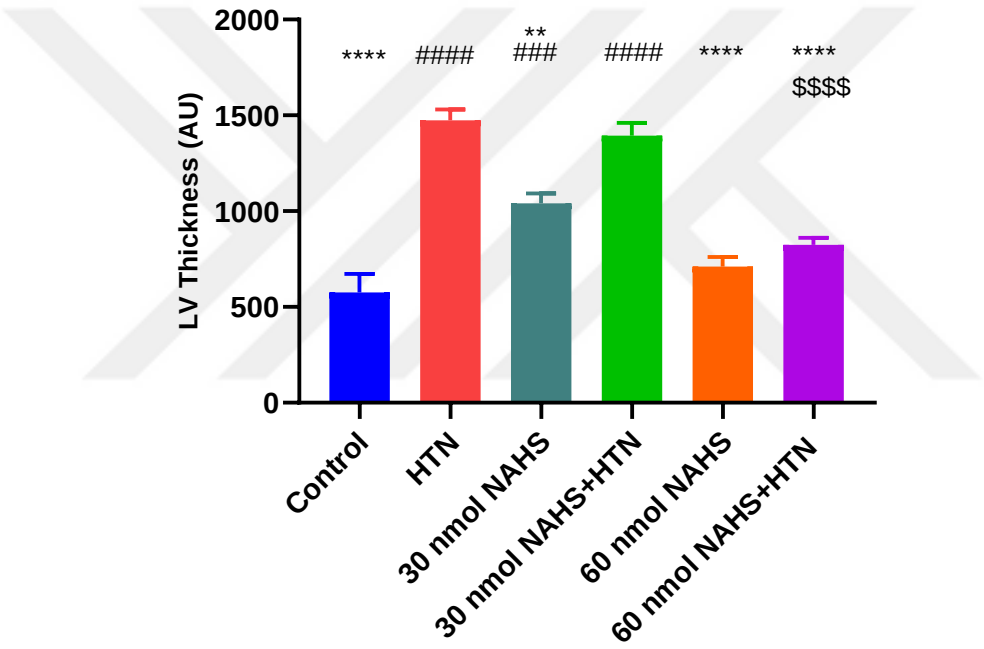

Figure 4-8 Left ventricular hypertrophy. Left ventricular thickness. **p<0.01, ****p<0.0001 vs. icv PBS+ sc Ang II (n=3). ###p<0.001, ####p<0.0001 vs. icv PBS+ sc saline (n=3). $$$$p<0.0001 vs. icv 30 nmol/h NaHS+ sc Ang II (n=5). icv 60 nmol/h NaHS (n=4), icv 60 nmol/h NaHS+ sc Ang II (n=7), (HTN: Hypertension. Ang II: Angiotensin II, LV: Left ventricul. Data is presented as the mean ± SEM).

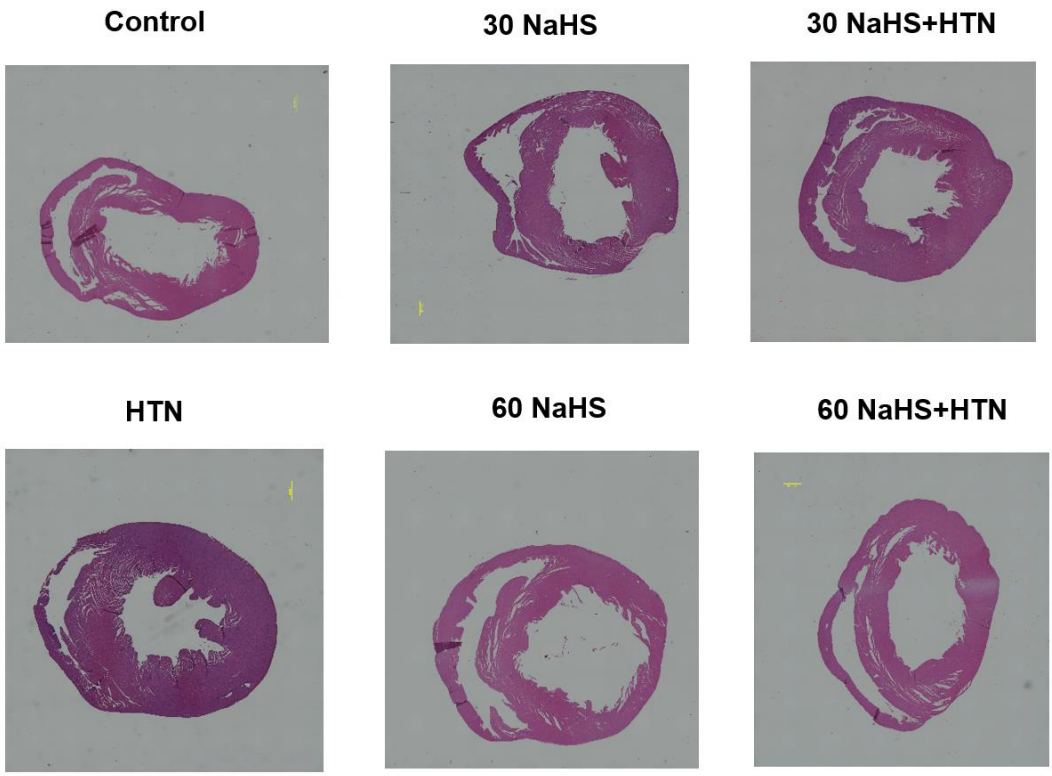

Figure 4-9 Representative images of hemotoxylin and eosin stained ventricular sections of experimental groups (HTN: Hypertension, Ang II: Angiotensin II. (magnification: 4x). Scale bar= 1000 and 50 μm).

Microglia activation

We compared the levels of activated microglia in different experimental groups to determine if the change in MAP was associated with changes in microglial activation in the PVN. Quantification of Iba1^{+} microglial cells in the PVN of Ang II showed a significant increase in total number of microglia chronic Ang II infusion group (Figure 4.10).

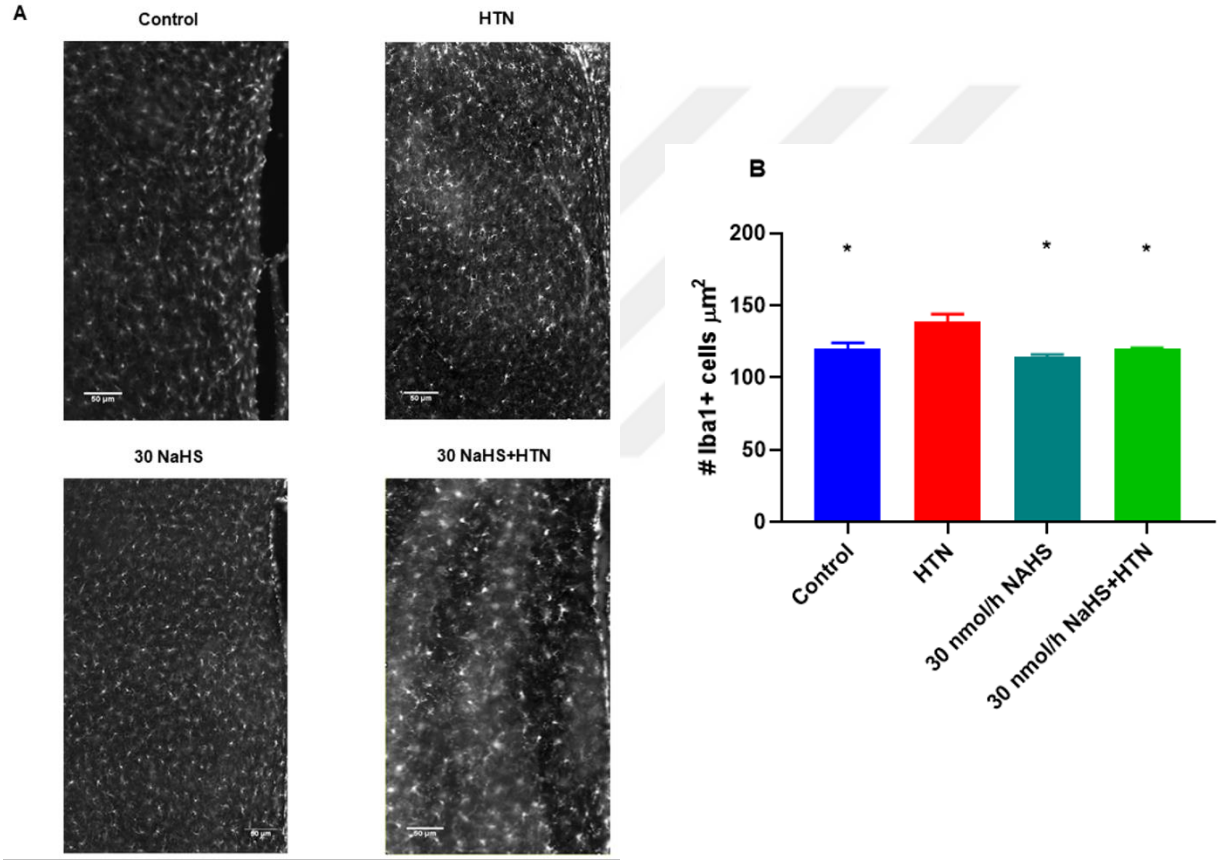

Figure 4-10 Effect of chronic Ang II infusion and NaHS treatment on microglial activation in the PVN. A) Representative images of PVN microglial cells (magnification: 20x). Scale bar=50 µm). B) Total number of Iba1^{+} cells in 1000x1000 bin in PVN. *p < 0.05 vs. HTN (n=2). Control (n= 2), 30 nmol/h NaHS (n= 2), 30 nmol/h NaHS+HTN (n= 3), (HTN: Hypertension. Data is presented as the mean ± SEM).

5. DISCUSSION

Ang II is associated with neuroinflammation in the PVN in HTN, a key cardioregulatory brain region that regulates the sympathetic tone (Paton & Raizada, 2010). Recent studies show that H_2S can attenuate glial-mediated neuroinflammation (M. Lee et al., 2013; M. Lee et al., 2016; Xuan et al., 2012), and that PVN may act as a site of action for H_2S (Liang et al., 2017), thus raising the possibility that H_2S donor may have a therapeutic potential in HTN.

Rationale of using angiotensin II induced-hypertension to investigate the central effects of NaHS on neuroinflammation in hypertension

Chronic Ang II infusion is an established animal model of HTN which is partly mediated by activation of the SNS (Yu & Dickinson, 1971) and microglia (Santisteban et al., 2015) thus, this model was used in our current experimental protocol.

Previous studies have established that HTN is associated with increases in pro-inflammatory cells (Jun et al., 2012; Zubcevic, Jun, et al., 2014) which can pass through the BBB to cause inflammation and elevate sympathetic outflow (Winklewski et al., 2015). Thus, we decided to investigate the circulating levels of CD3, CD4 (representative of T cells) and CD90 (angiogenic progenitor cells) in H_2S-infused groups by flow cytometry (Santisteban et al., 2015). We found no significant difference in levels of circulating CD3, CD4 and CD90 between our groups (%mononuclear cells (MNCs): CD3 in 30 nmol/h NaHS: 44.96±1.47; 30 nmol/h NaHS-treated HTN: 48.77±3.02; HTN: 42.89±3.23. CD4 in 30 nmol/h NaHS: 27.98±0.79; 30 nmol/h NaHS-treated HTN: 32.41±1.86; HTN: 26.70±0.87. CD90 in 30 nmol/h NaHS: 9.62±0.81; 30 nmol/h NaHS-treated HTN: 7.95±0.81; HTN: 11.44±0.49). This confirmed that 4 weeks of Ang II infusion did not induce systemic inflammation. This finding was also supported by our previous finding that more than 4 weeks of Ang II infusion is required to cause systemic inflammation in SD

rats (Jun et al., 2012). Thus, we did not further investigate systemic inflammation in our model.

Failure of angiotensin II to increase blood pressure in a proportion of SD rats

In the current study, normotensive male SD rats (n=9) were subjected to a 4-week Ang II infusion (200 ng/kg/min), (Q. Li, Dale, Hasser, & Blaine, 1996; Santisteban et al., 2015; P. Shi, Diez-Freire, et al., 2010) to induce high BP and neuroinflammation. Four-week Ang II infusion resulted in a significant increase in MAP in 5 out of 9 rats ($P<0.001$, Figure 4.2A and Table 4.1), as expected. However, in the 4 remaining rats, Ang II infusion failed to induce BP increase (Figure 4.1). This was not due to failure of our Ang II delivery method, as the mini-osmotic pumps were examined at the end of the experiment and it was confirmed that Ang II was delivered appropriately. Furthermore, same batch and lot number Ang II was used in all rats. Thus, the failure of some SD rats may have arose from natural variability of SD rats to respond to Ang II.

The mechanisms of HTN caused by chronic Ang II infusion are not completely understood; however, direct action on vascular smooth muscle to increase PVR (Brooks & Osborn, 1995; Wong et al., 1991), actions within the kidney to promote sodium resorption (Hall, 1986), and effects on neural pathways (Takahashi, Yoshika, Komiyama, & Nishimura, 2011) have been proposed in HTN. The vast majority of Ang II actions occur via the AT1R, including vasoconstriction, cellular proliferation, and activation of the SNS (R. M. Touyz & Schiffrin, 2000). Renal and liver AT1R gene expression has been shown to be maintained in Ang II-induced HTN (Harrison-Bernard, El-Dahr, O'Leary, & Navar, 1999). Moreover, suppressed plasma renin activity (Gonzalez-Villalobos et al., 2008) and increased intrarenal Ang II content caused by Ang II infusion has been shown to play a significant role in increase in BP during Ang II-induced HTN (Gonzalez-Villalobos et al., 2009). The failure to downregulate AT1R and renin levels in addition to low intrarenal Ang II

may be the reason why we did not see an increase in BP in some of our rats. However, renin measurement in patients with essential HTN shows that the plasma renin activity is normal in 60% of patients (Mulatero, Verhovez, Morello, & Veglio, 2007) which may not reflect the Ang II induced BP increase whereas Ang II kidney contents were found to be high in Ang II infused rats (Von Thun, Vari, el-Dahr, & Navar, 1994; Zou et al., 1996). We did not measure intrarenal Ang II levels in our study, which would confirm the effects of Ang II in Ang II-infused rats with NaHS treatment. Thus, we consider this a limitation of our study. However, we show a dose-response effect of our H_2S treatment which gives us confidence in our results ($P<0.001$, Figure 4.3A). Moreover, H_2S treatments did not completely normalize Ang II HTN, which, if it had happened, would have raised doubts about the effect of Ang II in the H_2S-treated rats (Figure 4.3A and Table 4.1). However, we should measure intrarenal Ang II levels of animals in future studies in order to better confirm the success of Ang II and treatment agent.

Attenution of blood pressure increase and autonomic dysfunction in angiotensin II induced-hypertension by central NaHS treatment

Dysfunction of vascular H_2S synthase/H_2S pathways are related to the pathogenesis of HTN (Yan et al., 2004; Zhong et al., 2003) and H_2S donors and precursors reportedly decrease BP in animal models of HTN (Ahmad et al., 2012; Ahmad et al., 2014; Y. X. Shi et al., 2007). In Ang II-induced HTN, chronic ip treatment with H_2S donors NaHS and STS attenuated development of HTN (Snijder et al., 2015; Snijder et al., 2014). However, the central effect of H_2S in HTN remains uknown.

Considering that elevated sympathetic activity in HTN may be associated with neuroinflammation in cardioregulatory brain regions (Santisteban et al., 2015; Z. Shi et al., 2014), and that H_2S and its donors have been shown to attenuate glia-mediated neuroinflammation in neurodegenerative diseases (M. Lee et al., 2013; M. Lee et al., 2016; Xuan et al., 2012), we investigated whether central administration

of NaHS, an H_2S donor, would alleviate Ang II HTN and attenuate neuroinflammation.

Our data indicated that chronic central administration of NaHS (30 and 60 nmol/h) attenuated the Ang II-induced increase in MAP in a dose-dependent manner ($P<0.001$, Figure 4.3A and Table 4.1). Evaluation of sympathetic/parasympathetic balance provides an insight into autonomic function in CVD (Waki et al., 2006). Sympathetic overactivity has been reported in Ang II-mediated HTN suggesting the involvement of central mechanisms (Kumagai et al., 2012; LaGrange et al., 2003; Osborn et al., 2007). Therefore, we investigated the effects of central NaHS treatment on autonomic dysfunction in rodent Ang II HTN (Carthy, 2014). For this reason, HRV, the fluctuation in the time intervals between adjacent heartbeats (McCraty & Shaffer, 2015), has been determined automatically by telemetry software in order to derive VLF band (representative of myogenic activity), LF band (sympathetic/baroreflex modulation), HF band (reflective of cardiac parasympathetic tone), TP band (reflects total variance in heart rate pattern over length of recording), and LF:HF (an index of vasovagal balance), (Waki et al., 2006). Although it didn't reach significant level, HTN rats showed a trend in decrease in VLF compared to control group during the 4 week-infusion period (Figure 4.4A and Table 4.5), which suggested impaired myogenic vascular function in response to BP increase (Stauss, Petitto, Rotella, Wong, & Sheriff, 2008). Moreover, at week 4 of Ang II infusion, VLF was significantly increased in 30 nmol/h- ($P<0.05$, 0.073±0.00) and 60 nmol/h NaHS-treated HTN (0.073±0.00) compared to the HTN group (0.069±0.00), (Table 4.5). Ang II infusion also significantly increased HF, reflective of the cardiac parasympathetic drive, starting at week 3 and week 4 compared to control group (Figure 4.4, Table 4.7), probably due to compensation to increase in LF. Nevertheless – the compensation was not sufficient to maintain homeostasis, since LF/HF, an indicator of vasovagal balance, was decreased in Ang II (Figure 4.4, Table 4.9). Importantly, the 60 nmol but not 30 nmol NaHS treatment normalized the Ang II–perturbed LF, HF and TP

(Figure 4.4, Table 4.6, Table 4.7 and Table 4.8, respectively). Previous studies have found that chronic infusion of GYY4137, an H_2S donor, into PVN decreased MAP and plasma NA levels in high salt-induced hypertensive rats (Liang et al., 2017). Thus, it can be concluded that H_2S may act to reduce Ang II-induced SNS over activation.

LV hypertrophy is a secondary manifestation of HTN resulting from adaptiation of heart muscle in order to accomodate the increased cardiac work by increasing muscle mass through the compensatory hypertrophic response (J. Li et al., 2019). In line with our MAP data, we observed increased LV wall thickness in Ang II-induced HTN group compared to control (Figure 4.8), which was significantly decreased by chronic 60 nmol/h but not the 30 nmol/h NaHS, treatment (Figure 4.8).

Gender-associated differences in BP have been observed in animals as well as in humans (Reckelhoff, 2001). In animal studies, androgens have been shown to downregulate vasodilatory AT2R expression levels in aorta (Mishra, Hankins, & Kumar, 2016) and plasma ACE activity was found to be higher in male mice (Y. K. Lim et al., 2002). Our study examined only male rats and potential sex differences in the response to NaHS and Ang II cannot be discarded. Thus, we consider this another limitation of our study that should be addressed in future experiments.

Endogenous plasma levels of H_2S are also decreased in hypertensive patients and the SHR (Du, Yan, & Tang, 2003; Sun et al., 2007; Yan et al., 2004). Thus, we investigated if Ang II-induced HTN will be related to low levels of H_2S. However, we observed that Ang II-induced HTN was not accompanied by decreased plasma H_2S (Table 4.10 and Figure 4.6 and 4.7, respectively). This may be related to both the dose (200 ng/kg/min) and duration of Ang II treatment used in the current study. Indeed, low doses (50–200 ng/kg/min) of Ang II infusion reportedly produce minimal to no impairment of endothelial function (Gomolak & Didion, 2014); thus, this may not affect endogenous H_2S. In the current study we used methylene blue method to detect H_2S levels in plasma as previously described (Zheng et al., 2012). This may

have interfered with our detection of H_2S. H_2S values based on colorimetric assays or ion selective electrode assays depend on harsh chemical treatment (strong acid or base, respectively). Due to this, and contrary to recent reports, H_2S gas at concentrations of <100 nM was essentially undetectable with this method (Whitfield, Kreimier, Verdial, Skovgaard, & Olson, 2008). In future studies, analytical techniques including gas/ion chromatography, HPLC, polarographic electrodes etc. may be preferred to measure H_2S levels since they seem more sensitive than colorimetric methods (Kolluru, Shen, Bir, & Kevil, 2013).

H_2S is produced in the CNS by CBS and 3-MST, and both astrocytes and microglia reportedly generate H_2S in the brain (M. Lee, Schwab, Yu, McGeer, & McGeer, 2009). Although it is generally accepted that the action of CSE on the cardiovascular system is responsible for the effect of H_2S on cardiovascular function, the activity of CBS may also play an important role in modulating vascular tone via its effects on ANS (Kulkarni et al., 2009). Both CBS protein levels and H_2S production are reportedly decreased in the RVLM of SHR (Duan et al., 2015). Moreover, CBS and 3-MST were down-regulated in rats with subarachnoid hemorrhage, while NaHS delivery increased H_2S in the brain and enhanced the activity of these enzymes (Cui et al., 2016). In our study, we investigated if decrease in MAP would correlate to the H_2S levels in CSF. We observed no difference in H_2S between our experimental groups (Table 4.10 and Figure 4.7) As above, this may be related the low detection ability of our current assay. Moreover, the dose and duration of Ang II used in the current study may have to increase in order to diminsh the activity of CBS. Lastly, it has been shown that the reduction in vascular H_2S production required downregulation of both CSE and CBS simultaneously, suggesting that the deficiency in the activity of one enzyme could be compensated by the activity of the other enzyme in maintaining the endogenous production level of H_2S (Roy et al., 2012). All these possibilities will be addressed in future studies.

Relation between attenuation of glia-mediated neuroinflammation in paraventricular nucleus and sympathetic overactivity induced by angiotension II

Ang II acts in the CNS to modulate neurohumoral pathways involved in sympathoexcitation and BP regulation. It has been reported that AT1R activation by Ang II within PVN is a major contributor to chronic sympathoexcitation (Paton & Raizada, 2010). Hence, we investigated whether attenuation of BP increase and autonomic dysfunction induced by Ang II is mediated via attenuation of glia-mediated neuroinflammation in the PVN. Microglial cells have been reported to exhibit the expression of the Iba1, a microglia/macrophage-specific calcium-binding protein (Sasaki, Ohsawa, Kanazawa, Kohsaka, & Imai, 2001) involved in the membrane ruffling processes of macrophages/microglia, thus considered one of the most important molecules in the motile properties of these cells (Kanazawa, Ohsawa, Sasaki, Kohsaka, & Imai, 2002). Quantification of $Iba1^+$ microglial cells in the PVN of Ang II-infused rats showed that Ang II infusion significantly increased the number of microglial cells compared to control, while our preliminary data show that 30 nmol/h ICV NaHS treatment normalized the numbers of microglia in the PVN of Ang II-infused rats (Figure 4.10). This shows that, in HTN group, microglial activation in the PVN leads microglial proliferation, which is measurable as an increase in the density of $Iba1^+$ cells.

Increased levels of plasma Ang II are known to induce vascular inflammation (Rodriguez-Iturbe et al., 2001; P. Shi, Raizada, et al., 2010) which in turn can activate microglia (Hoogland, Houbolt, van Westerloo, van Gool, & van de Beek, 2015). Our flow data showed that 4 weeks of Ang II infusion was not enough to induce systemic inflammation since more than 4 weeks of Ang II infusion was required (Jun et al., 2012). Thus, Ang II-induced inflammation in the PVN may be resulted from the brain infusion of systemic admistered Ang II via disrupted BBB (M. Zhang et al., 2010) or beacuse of the locally produced Ang peptides in PVN, SFO, RVLM, area postrema, and NTS (Davisson, 2003; Gironacci et al., 2014).

It's hypothesized that Ang II increases the production and/or release of pro-inflammatory cytokines from glia. Subsequently, the released pro-inflammatory cytokines increase ROS production. Furthermore, Ang II, via stimulation of NADPH oxidase, increases ROS formation in both neurons and microglia, via a cytokine-independent mechanism. In turn, ROS, can act to increase neuronal discharge, thereby contributing to increase in the sympathetic outflow and BP (P. Shi, Raizada, et al., 2010; Zubcevic et al., 2011). In our study, we did not investigate the underlying mechanisms of H_2S in attenuation of neuroinflammation. Others have shown that bilateral infusion of an H_2S donor, GYY4137, into PVN for 6 weeks decreased MAP, attenuated plasma NA levels and H_2S levels, and CBS expressions in PVN in high salt-induced hypertensive rats by downregulation of NADPH oxidase and ROS production and reduction in IL-1ß, but increase in expression of IL-10 in the PVN (Liang et al., 2017). Although not investigated, H_2S may also attenuate neuroinflammation via its anti-oxidant and anti-inflammatory effects in the current study and this will be investigated in future experiments using *in vitro* methods.

6. CONCLUSION and FUTURE STUDIES

Our data show that central administration of an H_2S donor, NaHS, attenuates BP increase and aoutonomic dysfunction in Ang II-induced HTN. This is associated with attenuation of Ang II-induced neuroinflammation in the PVN. Thus, H_2S has a potential for being a therapeutic agent for neurogenic HTN. However, the underlying mechanisms of central H_2S effects in HTN need to be adressed in future studies. Morever, gut dysbiosis has been related with the pathogenesis of neuroinflammatory diseases (Wohleb & Godbout, 2013) including HTN (T. Yang et al., 2015) and H_2S is produced by both gut bacteria and the host gut (Liu et al., 2012). However, what is currently not known is the extent of contribution of the gut-derived H_2S is to the host overall H_2S levels and if bacteria could affect the production of H_2S by the host. We will address this in our future studies.

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