MECHANISMS BY WHICH RENAL DENERVATION CHRONICALLY LOWERS BLOOD PRESSURE IN THE SPONTANEOUSLY HYPERTENSIVE RAT

By

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ABSTRACT

MECHANISMS BY WHICH RENAL DENERVATION CHRONICALLY LOWERS BLOOD PRESSURE IN THE SPONTANEOUSLY HYPERTENSIVE RAT

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Clinical management of hypertension (HTN) presents a persistent and problematic issue for primary care physicians. Under half of patients receiving anti-hypertensive drug therapy reach therapeutic goals meaning most treated patients still carry considerable risk for developing further cardiovascular complications. Sympathetic activation is known to be an important factor in the pathophysiology of many forms of hypertension, and has been a rationale for using the adrenergic system as a therapeutic target for lowering blood pressure (BP). Sympathetic activity to the kidney in particular has been of great interest as renal sympathetic nerves activate many physiological pathways that can impact blood pressure: renin release, sodium reabsorption, and direct renovascular vasoconstriction. Sensory nerves supplying the kidneys have also been demonstrated to modulate BP perhaps through alterations in central sympathetic outflow. Recently, catheter-based renal nerve ablation (CBRNA) was developed as a non-pharmacological treatment modality for managing difficult-to-treat HTN. Clinical studies have shown that in patients not reaching goal blood pressure during pharmacological interventions, ablation of the renal nerves significantly and chronically lowers blood pressure. Responses to CBRNA in humans have been quite variable in magnitude, which has prompted investigators to ask important questions such as 1) For which hypertensive patients should this treatment option be indicated? 2) What patient

characteristics predict a good response to the new therapy? and 3) How do concomitant antihypertensive medication regimens affect the fall in BP seen after CBRNA? These questions all derive from the fact that we do not yet understand the mechanisms that mediate the BP response to CBRNA. Technical and ethical considerations limit our ability to investigate questions of mechanism in human subjects, thus highlighting the importance of studying this problem in a pre-clinical setting.

The studies performed in the following dissertation use an animal model of human hypertension in an attempt to identify the mechanisms responsible for the long-term reduction in blood pressure after CBRNA. The central hypotheses of this dissertation are that 1) renal denervation in the spontaneously hypertensive rat (SHR) will decrease BP to a degree similar to the effect of CBRNA in human patients with drug-resistant hypertension, and 2) that the BP response to renal denervation in the SHR is due to suppression of both renal sympathetic nerve activity (RSNA) and non-renal sympathetic nerve activity) (NRSNA). The ultimate goals of this work were to 1) understand the relevant physiological and pharmacological mechanisms that influence the blood pressure lowering effect of renal denervation, and 2) provide pre-clinical evidence to help guide more effective selection of patients likely to response to CBRNA with a clinically significant fall in blood pressure.

ACKNOWLEDGEMENTS

Under this quote I have entered the lab almost daily for the past 5 years, "*I hate ingratitude more in a man than lying, vainness, babbling, drunkenness, or any taint of vice whose corruption inhabits our frail blood (-Viola, Twelfth Night).*" To honor this truth, I offer the following:

The entirety of this work would not be possible without the generous guidance and support of Dr. Gregory Fink. I am deeply grateful for the opportunity to work and train under his mentorship. His lessons of diplomacy, scholarship, and excellence were taught by example. While I am still a poor measure of the embodiment of these lessons, he should know they have not gone unheeded. Greg, you have my sincerest respect.

Additional appreciation must be extended to my thesis committee members: Dr. James Galligan, Dr. Ann Dorrance, and Dr. Donna Wang. I thank you for the expertise you have brought to this project, your commitment to my education, and the invaluable guidance passed along to me as a young scientist. Working with each of you has been a pleasure.

Furthermore, I'd like to acknowledge the skillful and irreplaceable technical talents of Hannah Garver and Robert Burnett. Without Hannah's assistance, I would still be struggling to get these experiments off the ground. She is a masterful surgeon and a good friend. Robert and his HPLC machine, whether he knows it or not, are the backbone of much our lab's work. We have processed many samples together, and I am thankful for his help. I must also thank him for watching me struggle to improve my golf game.

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KEY TO ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ACE	angiotensin converting enzyme
ACE-I	angiotensin converting enzyme inhibitor
ADH	anti-diuretic hormone
AngI	angiotensin I
AngII	angiotensin II
AR	adrenergic receptors
AT1	angiotensin II receptor type I
ATP	adenosine triphosphate
AV3V	anteroventral third ventricle
BP	blood pressure
bpm	beats per minute
cAMP	cyclic adenosine monophosphate
cap	capsaicin
CBRNA	catheter-based renal nerve ablation
CGRP	calcitonin-gene related peptide

CKD	chronic kidney disease
CNS	central nervous system
CO	cardiac output
CVD	cardiovascular disease
DAG	diacyl-glycerol
ESRF	end stage renal failure
Hct	hematocrit
HPLC	high pressure liquid chromatography
hr	hour
HR	heart rate
HTN	hHypertension
Hz	heHertz
IP3	inositol triphosphate
JGGC	juxtaglomerular complex
JNC	Joint National Committee
LK	left kidney
LRP	left renal pelvis

LS	lLow Salt
LSNA	lumbar sympathetic nerve activity
mmHg	millimeters of mercury
MSNA	muscle sympathetic nerve activity
NaCl	sSodium chloride
NE	norepinephrine
NHE3	sodium hydrogen exchanger 3
NPY	neuropeptide Y
NRSNA	non-renal sympathetic nerve activity
NTS	nucleus tractus solitarius
PRA	plasma renin activity
PVN	paraventricular nucleus
RAAS	renin-angiotensin aldosterone system
RDX	surgical renal denervation
RK	right kidney
RRP	right renal pelvis
RSNA	renal sympathetic nerve activity

RSR	renin secretion rate
RVLM	rostral ventrolateral medulla
RVR	renal vascular resistance
SFO	subfornical organ
SHR	spontaneously hypertensive rat
SHRSP	stroke prone SHR
SNA	sympathetic nervous system activity
SO	sham operated
sodium	Na+
SP	substance P
TPR	total peripheral resistance
VIP	vVasoactive intestinal peptide
wk	week
yr	year

CHAPTER 1: INTRODUCTION

Overview

Catheter-based renal nerve ablation (CBRNA) was recently developed as a non-pharmacological treatment for drug-resistant essential hypertension (HTN) [1-3]. Hypertension remains a prominent clinical concern as it is the most common condition encountered in primary care, and uncontrolled hypertension is a major risk factor for developing significant cardiovascular disease [4]. Drug-resistant hypertension is defined as blood pressure (BP) that remains above goal levels suggested in various treatment guidelines despite combined use of 3 different anti-hypertensive medications including a diuretic [5]. The proportion of HTN cases actually controlled with current therapies is quite low: only about half of all cases are successfully managed to goal BP [6]. This suggests additional therapies are necessary to lower the BP of patients that are not responding to current treatment modalities. It is known that activation of the renal sympathetic nerves occurs in the pathogenesis of both experimental and human hypertension [7]. Increased renal nerve activity impacts many physiological pathways that contribute to BP regulation, and in HTN inappropriately elevated nerve activity is thought to contribute to the pathophysiological elevation of BP in some but not all patients. Therefore, investigators developed CBRNA as a method to physically interrupt elevated renal sympathetic nerve activity with the goal of providing a novel clinical approach to treating drug-resistant HTN. And in fact disruption of the renal nerves located in the adventitia of the renal arteries with CBRNA recently has been shown to promote a significant reduction in office and ambulatory BP in some patients with drug-resistant HTN that persists at least several years beyond the initial procedure [8, 9].

However, the precise mechanisms explaining the prolonged reduction in BP remain unknown. In addition to interruption of sympathetically-regulated renal functions, some initial studies suggested that decreased non-renal sympathetic activity could account for the antihypertensive response to CBRNA [2, 3]. Few detailed investigations have been undertaken to identify the relative importance of the various mechanisms that account for the chronic reduction in BP. Without this information, clinicians have little ability to predict which patients will respond to CBRNA with a fall in BP or how concomitant medical therapies might affect the response. This dissertation describes experimental studies designed to identify the mechanisms by which renal denervation lowers BP, and how those mechanisms are influenced by concomitant anti-hypertensive drug therapy. The first chapter aims to summarize information currently known about renal innervation, renal nerve physiology, role of the renal nerves in the pathophysiology of hypertension, and clinical trials evaluating renal nerve ablation in patients. Finally, I provide a brief discussion of the current clinical questions in need of experimental exploration.

Overview of renal nerve anatomy and physiology

General renal anatomy

In humans, the kidneys are bilateral, retroperitoneal organs located below the diaphragm at the level of spinal segments T12-L3 along the posterior abdominal wall. The normal kidney has a concave shape and is highly perfused, giving the organ a deep red hue. The blood supply to each kidney comes from an ipsilateral renal artery branching from the abdominal aorta. The kidneys are drained through separate renal

veins that communicate blood to the inferior vena cava. Along with the renal pelvis, which carries urine to the ureters, the blood vessels enter the kidney at the renal hilum. Arterial blood supply to the kidney is provided by end-artery circulation, meaning the divisions arising from these main arteries do not anastomose with other arterial blood supplies. After penetrating the renal hilum, the renal artery gives rise to anterior and posterior segmental arteries. Each segmental artery supplies a distinct division of the kidney through interlobar, arcuate, and interlobular arteries. The interlobular arteries gives rise to the afferent and efferent glomerular arterioles. Coursing along and embedded in the main renal artery and its divisions are both the efferent and afferent nerves that supply the kidney (Figure 1). More specific aspects of these nerve types are discussed below. In addition to the neural and vascular components, the kidneys contain filtration machinery consisting of the glomeruli and tubular structures (i.e. the nephron) that enable the formation of urine. These tubules carry filtered fluid deep into the kidney to be collected in ducts and transmitted to the bladder via the ureter [10].

Anatomy of renal innervation: Efferent and afferent renal nerves

The kidneys are densely innervated. The nerve fibers are separated into either efferent “motor” nerves or afferent sensory nerve fibers. The anatomical and functional significance of each neural axis has been comprehensively reviewed previously [7, 11, 12]. The following two sub-chapters will draw upon these reviews to provide a brief overview of the detailed neuroanatomy and physiological function of the renal nerves.

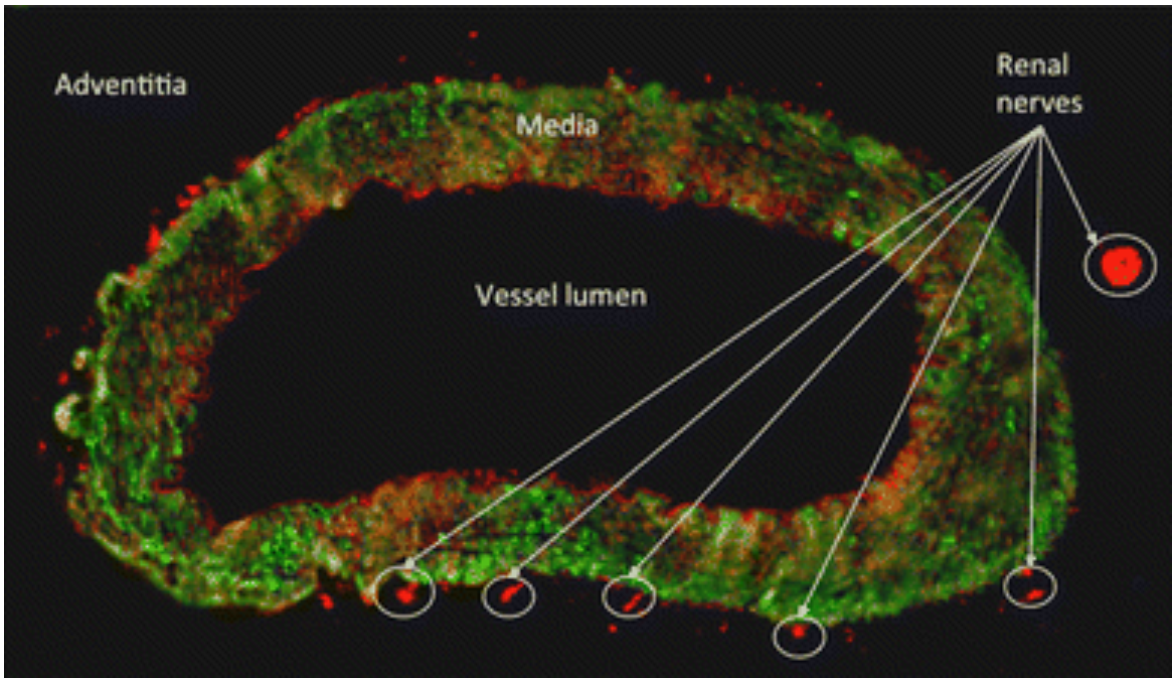


Figure 1. **Cross-section of the renal artery from the Sprague-Dawley rat.** This histological preparation demonstrates the relationship between renal sympathetic nerves and arterial anatomy (red, tyrosine hydroxylase; green, α-smooth muscle actin; blue, DAPI). Reproduced with permission from Sobotka et al. [13].

Renal efferent nerve fibers are primarily sympathetic nerves with pre-ganglionic cell bodies located in the intermediolateral cell column from T9-L1. The schematic representation of the major contributors to renal sympathetic innervation in humans are outlined in figure 2. Post-ganglionic fibers have origins in the celiac plexus, with the celiac ganglion and aorticorenal ganglion containing a majority of the post-ganglionic cell bodies that supply the kidneys. Renal efferent sympathetic fibers supply all segments of the renal vasculature with the highest density of innervation located at the glomerular arterioles. In addition to supplying the renal vasculature, the efferent sympathetic fibers also synapse at the juxtaglomerular apparatus and all parts of the nephron.

Renal afferent nerve fibers originate from cell bodies located in the dorsal root ganglion at T6-L4. The majority of renal afferent nerve fibers supply the renal pelvis [14]. Afferent fibers enter into the ipsilateral spinal cord, and most synapse on interneurons located in laminae I, III-V of the dorsal horn. A small proportion of renal afferent fibers cross the midline of the spinal cord to innervate the contralateral kidney [15]. Sensory information relayed through interneurons in the spinal cord is conducted superiorly by second-order neurons organized into ascending tracts that project to higher centers in the central nervous system. Approximately 8% of the renal afferent fibers directly project to the medulla without synapsing on interneurons [16]. Sensory information is transmitted to several nuclei within the brainstem and hypothalamus, namely the nucleus tractus

solitarius (NTS), rostral ventrolateral medulla (RVLM), subfornical organ (SFO), and paraventricular nucleus (PVN). Labeling studies utilizing the pseudorabies virus have also shown renal afferent nerve projections to the nodose ganglion [15].

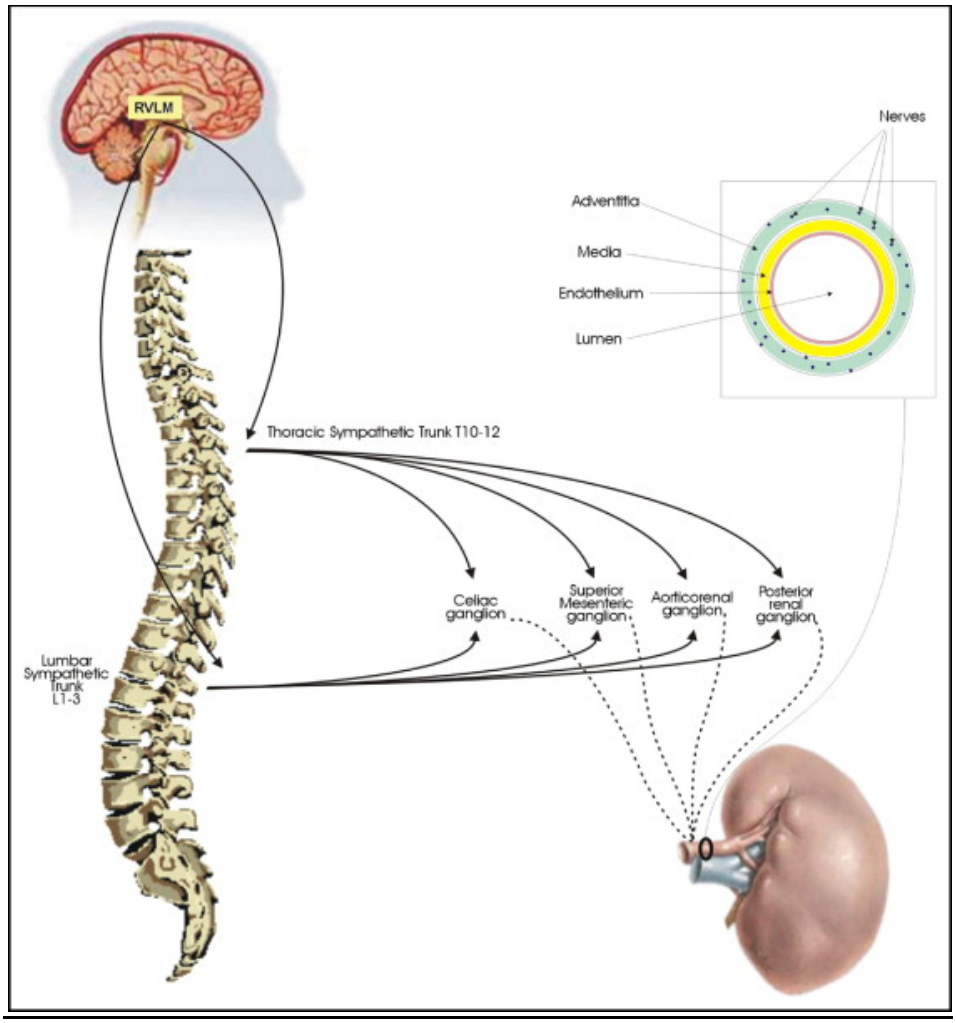


Figure 2: <u>Schematic representation of the sympathetic innervation of the kidneys</u>. Hypothalamic neurons communicate through connections to pre-ganglionic nerves in the spinal cord. Pre-ganglionic projections synapse at several pre-aortic ganglia where post-ganglionic fibers arise and innervate the kidney [17]. Reproduced with permission.

Renal nerve physiology: Efferent and afferent nerves

Renal efferent nerves are primarily noradrenergic, i.e. release norepinephrine (NE) as their primary neurotransmitter. Neuropeptide Y (NPY) and adenosine triphosphate (ATP) are also co-released with NE. While there is some evidence for dopaminergic and cholinergic signaling machinery within the kidneys, there is little support for separate renal nerves that exclusively release dopamine or acetylcholine [7].

The molecular signal transduction of NE released from the renal efferent nerves is mediated by α- and β-adrenergic receptors (AR), which are both G-protein-coupled receptors. Renal α-ARs are classified as either α1- or α2-AR. The receptors can be further identified as α1A-AR, α1B-AR or α2A-, α2B-, or α2C-AR subtypes. The α1-ARs are coupled to Gq/11 proteins that increase phospholipase C activity when activated. Through generation of second messengers, inositol triphosphate (IP3) and diacyl-glycerol (DAG), α1-AR activation promotes release of intracellular calcium and protein kinase C activation. The α2-ARs are coupled to Gi/o proteins. Activation of the α2-AR inhibits adenylyl cyclase and reduces Ca2+ influx through certain Ca2+-permeable ion channels. The β-AR subtypes expressed within the kidney are the β1-AR and β2-AR. All β-AR are coupled to Gs proteins, which stimulate cyclic adenosine monophosphate (cAMP) production through adenylyl cyclase activation.

The physiological effects of adrenergic receptor activation within the kidney are diverse (**Figure 3)**. Noradrenergic signaling through α1A-AR promotes vasoconstriction of afferent and efferent arterioles. The α1B-AR, located primarily on the tubules, facilitates

the reabsorption of sodium and water by stimulating sodium hydrogen exchanger 3 (NHE3) and Na+/K+ ATPase activity. Activation of α2-AR mediates suppression of NE release from presynaptic nerve terminals and may alter expression of sodium transporters within the lumen of the renal tubules. At the juxtaglomerular apparatus, β1-AR functions to promote renin release, leading to the activation of the renin-angiotensin system.

Under normal physiological conditions the renal sympathetic nerves are tonically active meaning there is a continual basal release of NE. Under basal conditions the renal sympathetic nerve activity fluctuates between 0.5-2.0Hz. At this level of activity, the sympathetic nerves influence renin release, sodium reabsorption, and renal vascular resistance[11, 18]. Renal sympathetic nerve activty and renal blood flow are inversely related, and both paramters fluctuate diurnally[18]. During resting conditions, RBF is higher as RSNA falls; However, RBF is reduced as RSNA increases, which can occur with even normal ambulation [18]. This suggests the renal nerves are directly involved in regulating renovascular resistance. Renal sympathetic nerve activity becomes elevated above normal levels in conditions where blood volume drops and input to cardiopulmonary baroreceptors is reduced. For example, a reduction in blood volume due to severe hemorrhage or body sodium depletion (e.g. low sodium diet, diuretic administration, dehydration) will decrease cardiopulmonary baroreceptor activity and increase renal sympathetic nerve firing. Conversely, states of volume expansion activate cardiopulmonary baroreceptors and reduce renal sympathetic nerve activity. As discussed in later sections, renal efferent activity can become elevated with aging, increased body mass, and under pathological conditions such as HTN. The

mechanisms responsible for the elevation in renal efferent activity are not fully understood [11, 19].

Renal afferents nerves are sensory nerves, which exist as either myelinated or unmyelinated fibers. They are peptidergic, containing one or a combination of the following peptide neurotransmitters: substance P (SP), vasoactive intestinal peptide (VIP), or calcitonin-gene related peptide (CGRP).

The renal afferent nerves have been demonstrated to respond to either mechanical distention or chemical stimuli [11]. Experimental manipulation of either renal perfusion pressure or renal pelvic pressure has been shown to increase renal afferent nerve activity through stimulation of mechanoreceptor fibers embedded in the walls of the renal arteries, veins, or renal pelvis. The afferent nerve fibers also respond to chemical stimuli through chemoreceptors, termed R1 and R2 chemoreceptors. The R1 chemoreceptor is silent during resting conditions and only activated during periods of complete renal ischemia. In contrast, the R2 chemoreceptor is active under resting conditions and will respond to various experimental chemical stimuli. The R2 chemoreceptor has been documented to respond to both non-diuretic urine (i.e., elevated ionic concentration in the urine) and renal ischemia.

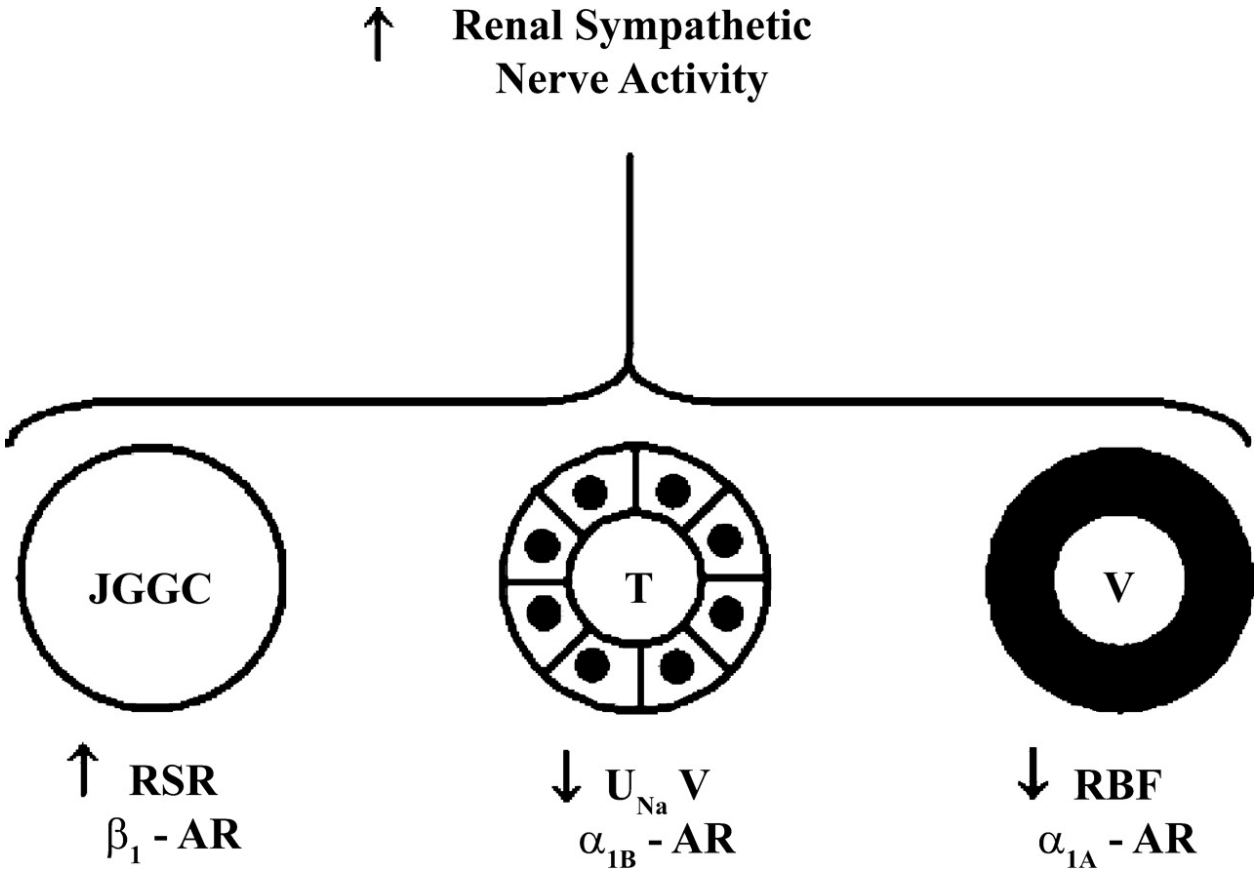


FIGURE 3: <u>Effects of increased renal sympathetic activity on renal function</u>. Elevated renal sympathetic nerve activity can increase renin release, decrease sodium excretion, and decrease renal blood flow [20]. Reproduced with permission

Afferent renal nerve activity increases during intra-renal infusion of bradykinin or adenosine. However, it is unclear whether the R2 chemoreceptor is mediating this rise in renal afferent nerve activity or if additional receptors are involved [12].

The physiological contributions of the renal afferent nerves are poorly characterized compared to the renal efferent nerves. Conventionally, the renal afferent nerves are described as serving a sympatho-inhibitory function through the reno-renal reflex. Experimental evidence demonstrates that excitation of renal afferent nerves produces a reduction in contralateral renal efferent nerve activity [7]. This reflex is thought to be important in maintaining sodium and water excretion during the challenge of a unilateral ureteral obstruction or in the setting of increased sympathetic drive to the kidney. Therefore, it is thought that changes in the responsiveness of the inhibitory reno-renal reflex may reduce its inhibitory control over renal efferent nerve activity, leading to excess sodium reabsorption, thus promoting salt-sensitive elevations in BP.

Conversely, reports also suggest the renal afferent nerves may have an excitatory function that increases BP. In the 5/6 nephrectomy model of chronic kidney disease, severing renal afferent nerve input into the CNS with dorsal rhizotomy resulted in decreased BP and reduced markers of glomerulosclerosis [21]. In a separate study, injection of 10% phenol into the kidney produced a sustained increase in BP and secretion of norepinephrine from the posterior hypothalamus. Denervation of the injured kidney reversed these findings [22]. These studies reveal the renal afferent nerves have additional physiological contributions beyond the inhibitory reno-renal reflex. Ultimately, the sum of the evidence suggests renal afferent nerves are important contributors to the

regulation of BP, and therefore, could play an important role in the development and maintenance of pathological elevations in BP (i.e., HTN).

BP regulation and the clinical importance of HTN

BP regulation

Arterial BP is determined by cardiac output (CO) and total peripheral resistance (TPR). As outlined in Figure 4**,** many other physiological variables affect these main factors and thus arterial BP**.** Traditionally, controllers of BP have been classified as acute (seconds to minutes), intermediate (minutes to hours), and long-term (hours to days) regulatory mechanisms [23].

Acute BP regulation is dominated by the arterial baroreceptor reflex, which is a neurally mediated feedback mechanism that corrects for beat-to-beat fluctuations in BP. Increased BP is detected by pressure-sensitive stretch receptors (baroreceptors) located in the carotid sinus and cardiopulmonary structures. These signals are transmitted to the central nervous system (CNS) by afferent nerves and integrated in the CNS. Efferent sympathetic output to the heart and certain vascular beds is suppressed, which lowers BP. In the absence of baroreceptor activation, efferent sympathetic support for BP is relatively uninhibited [23] and BP increases. Although the baroreceptor reflex defends against acute changes in BP, it is well established that the reflex desensitizes and resets to a higher-pressure threshold of activation when challenged by a sustained increase in BP [24]. Traditionally this has limited the acceptance of baroreflex mechanisms as long-term controllers of BP [25]. Current computational modeling of the long-term regulation of BP disputes this view and

predicts that baroreceptor function may also be important in defending against chronic perturbations in BP. The authors point out that the adaptation and resetting of the baroreflex arc to a higher BP occurs as a consequence of vascular stiffness i.e. more pressure is required to distend the stiffer arterial walls and thus, the baroreflex arc is not active until the higher pressures are reached. The authors conclude that dysregulation of BP control occurs, in part, as a consequence of baroreflex setting, not independent of it [26]. The importance of the baroreflex arc in chronic BP control is also highlighted by studies that use baroreflex stimulation to chronically reduce BP [27].

The renin-angiotensin-aldosterone system (RAAS) is an important mediator of intermediate BP control. Activation of RAAS begins with renin release from the juxtaglomerular apparatus when stimulated by reduced renal afferent arteriole BP, increased renal sympathetic nerve activity (RSNA), or decreased sodium chloride (NaCl) content at the macula densa [28]. Renin, a proteolytic enzyme, cleaves circulating angiotensinogen to angiotensin I (Ang I). Angiotensin converting enzyme (ACE), primarily located in the lungs, converts AngI into angiotensin II (AngII). The AngII peptide plays a key role in intermediate BP regulation, as its actions involve stimulation of direct reabsorption of sodium (Na+) from the renal tubules, stimulates thirst and release of anti-diuretic hormone (ADH), vasoconstriction in resistance vessels, secretion of aldosterone (ALDO), and activation of the sympathetic nervous system [23, 28]. ALDO is a salt-conserving hormone synthesized and released from the zona glomerulosa in the adrenal cortex. Within the kidney, ALDO supports the maintenance of BP by increasing reabsorption of Na+ from the collecting tubules.

Long-term control of BP is traditionally thought to be governed exclusively by the renal pressure natriuresis mechanism. It regulates plasma volume, cardiac output and BP by controlling Na+ and water balance [29]. To maintain blood volume, and thus BP, at a set level, the kidneys regulate urine formation by filtering salt and water from the blood. The pressure natriuresis mechanism is proposed to regulate BP as follows: Increases in BP above a set point are corrected for by directly increasing the excretion of sodium and water from the kidney in the form of urine, thus reducing blood volume and BP back to the “desired” levels. A reduction in BP below the set point reduces urine formation, increases plasma volume, and restores BP to the desired level [23, 25, 29]. Much importance has been placed on the pressure-natriuresis mechanism; specifically, chronic alterations in BP are assumed by many to be impossible without a change in the pressure-natriuresis mechanism.

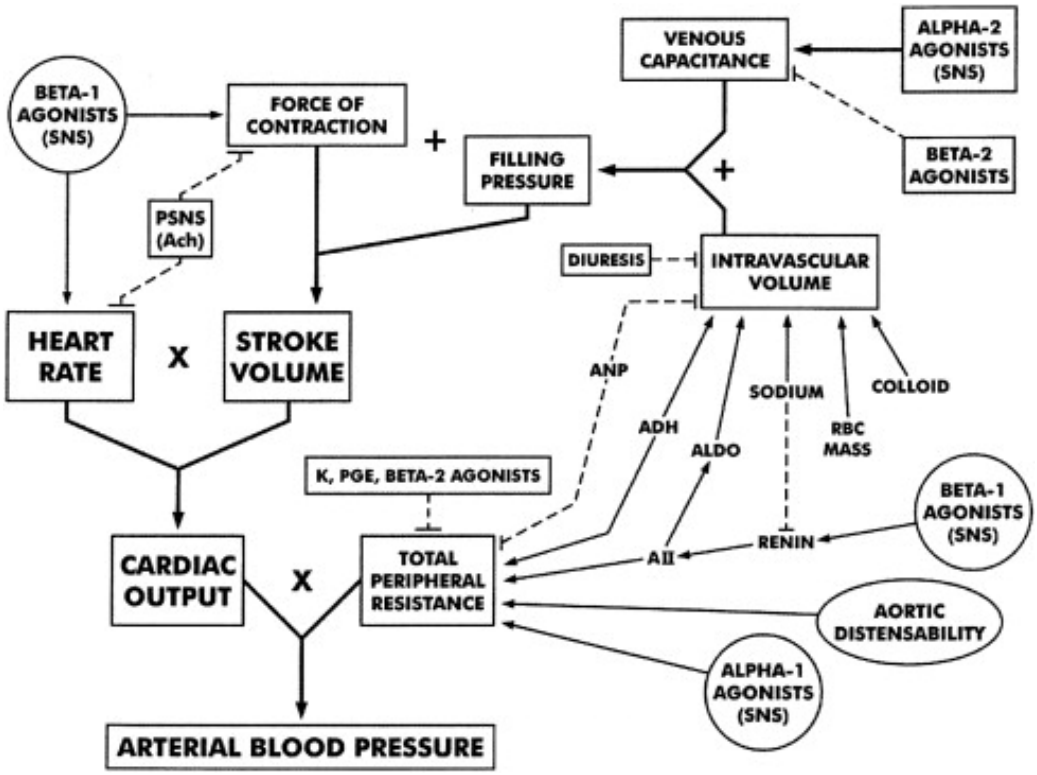


Figure 4: <u>Schematic representation of BP control systems</u> [23]. Arterial pressure is a product of cardiac output and total peripheral resistance. However, many physiological parameters and signaling mechanisms influence these two factors and thus help to regulate arterial pressure. Reproduced with permission.

Hypertension epidemiology and clinical significance

Hypertension is a chronic, pathological elevation of arterial BP that is thought to occur as a result of failure in one or more BP regulation mechanisms. According to the eighth report of the Joint National Committee (JNC), hypertension is the most common condition encountered in primary care [4]. The previous JNC report defined HTN as a systolic pressure greater than 140mmHg or a diastolic pressure above 90mmHg [30]. Untreated HTN is a serious risk factor for developing cardiovascular disease (CVD), which may manifest as stroke, myocardial infarction, kidney disease, and congestive heart failure [24]. Hypertension and related sequlae are an enormous national health problem with a growing prevalence. As of 2012, 76.4 million Americans over the age of 20 met the criteria for HTN, meaning 1 in 3 American adults have the condition. Current projections suggest an additional 27 million Americans will develop HTN by 2030. Recent estimates also place the total annual cost of treating HTN to be as high as $50.6 billion. Furthermore, only 47.8% of patients receiving treatment for HTN actually achieve BP control, i.e., a BP below the 140/90 threshold [6]. Taken together, these data highlight the current and forecasted clinical burden posed by untreated HTN and underscores the need for increased efforts in better treating HTN. Moreover, given the low rate of hypertension control, it is clear that gaining additional understanding of the pathophysiological mechanisms involved in hypertension may be necessary to better manage human HTN.

Hypertension etiology

Hypertension is characterized clinically as either primary or secondary hypertension [24]. Secondary hypertension is a pathological elevation in BP caused by an identifiable cause and represents the minority of high BP cases. As an example, patients with an actively secreting adrenal pheochromocytoma develop hypertension due to excess norepinephrine and epinephrine released from the tumor. Resection of the underlying neoplasm abolishes the excessive adrenergic stimulus, and the hypertension resolves. In contrast, the etiology of primary hypertension is generally idiopathic and multifactorial. The discussion of hypertension in this dissertation will focus on primary hypertension.

Essential hypertension is the most prevalent form of hypertension, representing approximately 90% of the hypertensive cases observed in patients [31]. Essential hypertension is hereditable, but seldom attributable to a single-gene mutation; it is generally presumed to be related to dysfunction of multiple mechanisms involved in BP regulation.

The dominant viewpoint regarding the etiology of HTN, i.e., the Guyton hypothesis, emphasizes the importance of sodium and water retention due to dysfunction in the renal pressure-natriuresis mechanism as the principal initiating mechanism for the development and maintenance of hypertension [24, 25, 29]. This theory of BP control and dysregulation is derived from experimental animal studies and computer simulations. Guyton and colleagues posit that the kidneys themselves have “overriding dominance” in long-term BP control, although they acknowledge the influence of other

systems on renal sodium and water retention [29]. This dominance is due to the supposed “infinite gain” (in a control system sense) of the renal pressure-natriuresis mechanism, which is proposed to slowly but fully correct for any transient sodium/water non-equilibrium states by increases or decreases in BP (as described earlier in this dissertation). Guyton specifically states, “once the net intake of water and salt have been set, and once the functional capabilities of the kidneys in relation to arterial pressure have been set, there is only one single BP level that will cause equilibrium between input and output of water and salt [29].” One could conclude, therefore, that BP will always be determined by the relationship between input/output of sodium/water and the functional capacity of the kidney. Under this hypothesis, HTN is assumed to occur because the dominant pressure-natriuresis mechanism is either “reset” by intrinsic changes in the kidneys themselves, or due to input from other physiological BP control mechanisms, such as excessive renin-angiotensin system activation, sympathetic overactivity, or changes in circulating levels of various vasoactive or sodium-retaining substances (figure 5).

While the Guyton hypothesis of BP regulation and hypertension development has been broadly accepted, there are some notable controversies. Investigators have long struggled to find any biological mechanism(s) that could encode a renal BP “set-point” [25]. Additionally, in contrast to what might be expected, changes in blood volume are seldom observed to precede or accompany changes in BP in either hypertensive patients or experimental models of hypertension [32, 33]. Others have reported that in animals fed high salt diets, natriuresis occurred without a change in BP, suggesting the two variables are not as inseparably linked as Guyton proposed [34].

A new mathematical model has challenged the importance of the pressure-natriuresis mechanism in the Guyton-Coleman model and has suggested that long-term BP control could be determined by sympathetic nervous system activity (SNA) [35]. As pointed out by these authors, increases in RSNA, non-renal sympathetic nerve activity (NRSNA), or both variables can facilitate increases in BP in the absence of an intrinsic renal abnormality in pressure-natriuresis. These observations support the notion that the pressure-natriuresis mechanism may not be as dominant in BP control as originally posited by Dr. Guyton and that dysregulation of the sympathetic nervous system may be primarily responsible for the pathologically elevated BP observed in hypertensive patients (the “neurogenic hypothesis”). The sympathetic nervous system exerts a tonic influence over most key cardiovascular parameters affecting BP such as vascular tone, heart rate and cardiac contractility, in addition to affecting the pressure-natriuresis mechanism.

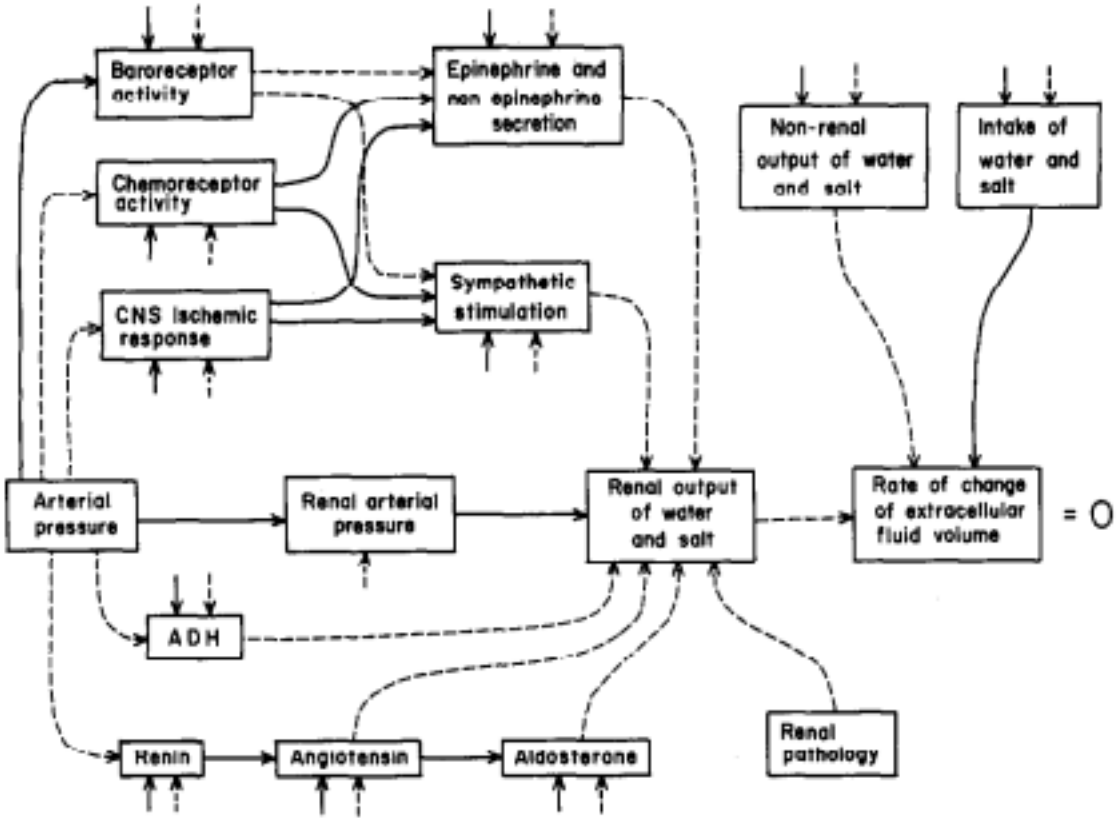


Figure 5: <u>Representation of the Guyton hypothesis of the long-term regulators of BP</u> [29]. The fundamental principle in this hypothesis is that renal sodium and water excretion must match intake, or BP will rise. Many components can influence the output parameter; however, the Guytonian hypothesis suggests that the renal output mechanism is the dominant mechanism involved in BP regulation and is therefore central in the pathogenesis of hypertension. Reproduced with permission.

Evidence for SNS as a cause for HTN

Evidence of a SNS component in experimental HTN

The evidence that supports SNA as a critical component in the pathogenesis of certain forms of experimental hypertension has been thoroughly reviewed elsewhere [36-40]. In brief, the evidence is best summarized as follows.

In experimental HTN, animals are not born hypertensive, but high BP either develop over time (e.g. genetic models of HTN) or HTN is induced by an experimental intervention (e.g. renal artery clipping, angiotensin infusion, DOCA-salt treatment, etc). Investigators have shown that destruction of the SNS prior to the development of HTN with 6-hydroxydopamine is sufficient to prevent many forms of experimental HTN. This is especially true in DOCA-salt HTN [36]. Chemical and surgical approaches have been used to create lesions within the CNS to evaluate how SNA supports HTN. Destruction of SNS sites within the CNS can cause, prevent the development of, or reverse HTN depending on the location of the lesion [36, 38, 39]. These findings confirm that a variety of SNS structures play critical roles in HTN. Direct nerve recording studies have shown that sympathetic nerve activity is increased in certain animals models of HTN [37]. Plasma NE, an indirect measurement of sympathetic nerve activity, has also been documented to be increased in experimental HTN [40]. Treatment with sympatholytic drugs, such as clonidine, results in a significant lowering BP in a variety of animal models of HTN. The BP effect of clonidine is also accompanied by reductions in plasma NE and urinary NE excretion supporting the notion that the SNS is contributing to the elevated BP in these models [40]. Augmentation of alpha-adrenergic receptor density

has also been documented [40]. In summary, the importance of the SNS in HTN has been broadly investigated across many animal models of HTN. Dysfunction in the SNS, from the anatomical level to the molecular signaling machinery, has been shown to have a potential role in the inappropriate elevation of BP. This evidence clearly demonstrates that SNA contributes to the pathophysiology of HTN. It is also important to note that these studies reinforce the notion that animal models are important investigative tools that can be used for understanding how SNS mechanisms and sympatholytic therapies influence the natural course of HTN.

Evidence of a neurogenic component in human HTN

At the beginning to mid-twentieth century, physicians began treating severe cases of hypertension by surgically removing components of the sympathetic nervous system [41-45]. Using evidence from early studies in experimental animals as a guide, the clinical rationale at the time was that removing the neural vasoconstrictor influence on arteries would reduce systemic vascular resistance in patients with malignant hypertension. These physicians hoped that this technique would lower BP in these patients and protect cerebral and retinal arteries that are vulnerable to high pressure [46]. Thoraco-lumbar sympathectomy plus splanchnicectomy was a widely utilized procedure in which surgeons excised selected thoracic and lumbar sympathetic chain ganglia and the splanchnic nerves. Smithwick and Thompson, the two surgeons that pioneered this procedure, reported that in a total of 1266 surgically treated hypertensive patients and 467 medically treated patients the 5-year survival rate was much higher in the surgical cohort. Patients that responded to the procedure with a reduction in BP had a 99% survival rate, whereas those that did not respond with a BP decrease after

surgery had a 75% survival rate. Medically treated patients did much poorer, with a survival rate of only 38% [42, 43]. Smithwick's data also showed that 45% of patients that survived surgery had a significant reduction in BP post-operation, and the reduction in pressure was documented to at least 10 years. These findings suggest that the sympathetic nervous system may be a key element in the pathophysiology of certain forms of HTN in humans. Unfortunately, surgical treatment of hypertension caused dramatic adverse effects, such as orthostatic hypotension, erectile dysfunction, and syncope. As a result, the surgical targeting of large portions of the sympathetic nervous system was largely abandoned with the advent of more efficacious pharmacological agents that produced fewer untoward events.

Evidence that supports SNA as a cause of HTN has also been gained from hypertensive subjects treated with sympatholytic drugs developed after surgical sympathectomy was halted. Infusion of the ganglionic blocker trimethaphan into hypertensive subjects promoted a significantly greater reduction in SBP compared to normotensive controls. Most importantly, ganglionic blockade in the hypertensive subjects returned SBP to normotensive levels. This finding indicates that SNA was a major contributor to the elevated BP in these subjects [47]. Evaluation of the BP lowering effects of older sympatholytic drugs, such as clonidine and reserpine, further supports the notion that SNA is a key component to HTN. Early JNC recommendations for HTN treatment suggested that reserpine, an agent that depletes vesicular catecholamines stores, should be added to the anti-hypertensive drug regimen of patients not adequately controlled by a diuretic alone. The recommendation was based on the observation that addition of reserpine to the diuretic treatment lowered BP an

average of 20-23mmHg [48]. The anti-hypertensive effect of clonidine, a centrally acting sympatholytic, has been correlated with a reduction in plasma NE [48]. These observations with sympatholytic agents in human subjects demonstrate that the SNS is an important contributor in the pathophysiology of HTN. Unfortunately, use of these agents has been largely abandoned due to unwanted side-effects.

In addition to these observations, additional investigative techniques reveal that sympathetic activity is often inappropriately elevated in patients with HTN compared to normotensive subjects. Plasma norepinephrine content, a crude marker of sympathetic activation, has been demonstrated to be elevated in hypertensive patients compared to age-matched controls [49]. However, plasma norepinephrine has not always been found to correlate with HTN [49, 50]. This may be due to the inherent insensitivity of the analytical technique, owing largely to its inability to account for catecholamine reuptake and metabolism [51-53]. To overcome this limitation, investigators have also used another indirect assessment of SNA – whole-body NE spillover – which is a dilution method requiring a radiolabeled NE tracer. From this technique, one can calculate NE clearance, allowing a more accurate and less ambiguous characterization of sympathetic activity. Using this approach, researchers have shown sympathetic activity is significantly elevated in patients with HTN compared to normotensive controls [50, 51, 54, 55]. Additionally, more direct approaches to measuring SNA have been developed; the most important has been microneurography. This technique utilizes a recording electrode placed directly against postganglionic sympathetic nerve fibers in the peroneal nerve. This allows recording of multi- and single-unit firing bursts from sympathetic nerves to skeletal muscle in conscious patients. Muscle sympathetic nerve activity

(MSNA) in some hypertensive patients is double to triple the activity observed in subjects with normal BP [51].

It is important to note that the SNS can contribute to the elevation without an increase in nerve firing. Increased adrenergic activity can arise from modifications of molecular machinery at several locations in the adrenergic signaling pathway. These potential alterations are well described in an older, but thorough review by Abboud [36]. First, it possible that increased sympathetic signaling could occur in the presence of defective alpha-2 adrenergic receptors on the pre-junctional sympathetic nerve. This alteration would allow augmented NE release even at basal firing patterns due to loss of negative feedback. In fact, this pattern of impaired function of the alpha-2 adrenergic receptor has been documented in the DOCA-salt rat [56]. Increased alpha-1 adrenergic receptor expression on the vasculature has also been proposed as a mechanism by which adrenergic activity could increase SNS activity independent of increases in sympathetic nerve firing [36]. It is worth pointing out that withdrawal of vagal control of BP can also create in increase in sympathetic signaling without a rise in nerve firing. As is the case with prejunctional alpha-2 adrenergic receptors, muscarinic receptors are also expressed at prejunctional sympathetic fibers and function in the same manner. Therefore, autonomic imbalance between vagal and sympathetic signaling pathways could lead to excessive sympathetic tone without any elevation in sympathetic nerve firing [36]. Regardless of the precise mechanisms that precipitate sympathetic overactivity, it is clear that the SNS is a major component in the development and maintenance of HTN. Although this section demonstrates the importance of sympathetic activation in the pathogenesis of HTN, it should be noted that the pattern of sympathetic

activation observed in human hypertensives is not global. Instead, as will be discussed in the preceding sections, sympathetic activation in HTN can involve only discrete regional locations without full activation of the entire SNS[57, 58].

Variables associated with sympathetic activation and hypertension

Sympathetic activation is not universal for all subjects with hypertension, but some estimates suggest as many as 50% of all human HTN cases are neurogenic, meaning SNA is a contributing factor to the BP dysregulation [19, 35, 36]. Key biological factors associated with increased sympathetic activity are age, sex, and body mass. It is well known that BP increases with age [59]. Investigators have also shown that SNA also rises with age. Work by Seals and colleagues demonstrated SNA increases as human subjects' age. In healthy, aged adults, whole-body NE spillover and MSNA were significantly elevated compared to healthy, young adults [57, 58]. Seals' group further demonstrated greater sympathetic support for BP in older individuals as treatment with the ganglionic blocker, trimethephan, produced a significantly greater fall in BP in older subjects compared to the young adults. It should be pointed out, however, that in contrast to what is seen with hypertensive patients, during normal aging, SNA increases to skeletal muscle and hepatomesentric targets but not to the kidneys [58].

Sex also effects sympathetic activation and BP, but this relationship is highly dependent upon age. Studies have shown that hypertension is more likely to occur in young adult males compared to young adult females; However, older, post-menopausal women have a higher prevalence of CVD and HTN than age-matched men [60]. Similar

observations have been made regarding SNA. Hogarth et al reports MSNA is significantly higher in young, adult male subjects compared to aged-matched females [61]. However, while SNA increases with age in both sexes, MSNA is actually higher in older, post-menopausal women compared to age-matched men [62].

It is also known that obesity is associated with BP. Population studies show that BP increases with body mass index [63]. Data also show that body mass is also associated with increased SNA. Analysis of regional sympathetic activity with NE spillover in normotensive, obese subjects has revealed increased SNA to the kidneys and skeletal muscle, but not to the skin or heptaomesenteric circulation compared to normotensive controls. Additionally cardiac sympathetic activity is reduced in these normotensive, obese subjects. However, in hypertensive obese patients, cardiac activity becomes unchanged or increased and SNA to the kidney and skeletal muscle remains elevated [64]. One should note that these patterns of sympathetic activation are clearly different from what is described for healthy, aging individuals. To further support obesity as a predictor of sympathetic activation, treatment of hypertension with adrenergic receptor antagonists has been shown to be more effective in obese hypertensives patients compared to lean hypertensive patients [65].

Evidence of RSNA as a component of HTN

Sympathetic activation does not always occur uniformly to all sites of sympathetic innervation. As mentioned previously, in ageing human subjects, sympathetic activity increases only to skeletal muscle and the gut [58]. In normotensive, obese individuals, the skeletal muscle and kidneys receive increased sympathetic drive while the gut is

spared [64]. These observations underscore the idea that the sympathetic nervous system can be activated in a regionally specific manner. The precise mechanisms governing regional increases in SNA are unknown. A key observation that has emerged in the evaluating of regional alterations in sympathetic activity is that increased RSNA is a common pathophysiological phenomenon associated with HTN.

Evidence of a RSNA component in experimental HTN

Intra-renal infusion of low doses of NE in uninephrectomized, conscious dogs resulted in a significant elevation in BP. Blood pressure in these dogs returned to normal upon cessation of the NE infusion. In contrast, infusion of NE systemically had no sustained influence on BP [66, 67]. This study suggests that increased adrenergic activity to the kidney could be an important mediator in some forms of pathologically elevated BP. The authors further documented that this elevated adrenergic activity to the kidney was sufficient to shift the pressure-natriuresis mechanism [68]. The best demonstration of the importance of the sympathetic nervous system in HTN comes from studies where components of the sympathetic nervous system are surgically targeted in hypertensive animal models. Surgical denervation of the kidneys (RDX) has been used to demonstrate the involvement of RSNA in the initiation and development of various forms of experimental HTN (Table 1) [20]. The surgical denervation method involves surgically stripping away the neural tissue surround the renal vasculature and eliminating any remaining tissue with phenol. A major advantage with this procedure is that surgical denervation almost completely denervates the kidney, as markers of sympathetic nerves are reduced to 4% of normal within 24hrs after surgery [69]. The procedure must be performed carefully as to not denervate surrounding tissues.

Analysis of markers of sympathetic innervation, such as tissue NE content, in surrounding non-renal tissues, is used to confirm specificity of denervation. This allows any observed effect to be directly attributed to loss of renal nerves alone. Generally, it has been concluded from these studies that RDX prevents or blunts the development of most forms of experimental HTN [11]. The altered course of the development of HTN in these models usually has been attributed to denervation natriuresis and diuresis. As a parallel to what was discussed previously regarding intra-renal infusion of NE in dogs, elimination of renal nerve activity in other animal models shifts the pressure natriuresis curve in the opposite direction, i.e. a larger natriuresis for any given pressure [11, 68]. Thus, neurogenic activation within the kidneys during HTN development may increase BP by altering the pressure natriuresis mechanism. Most work examining the effect of RDX on the development and maintenance of experimental HTN has been performed in the SHR. This work will be discussed in subsequent sections of this chapter.

Evidence of a RSNA component in human HTN

As noted earlier, it is estimated that as many as 50% of all cases of primary hypertension have a neurogenic cause [70]. In these hypertensive patients, sympathetic activity to the heart, kidneys, and skeletal muscle is increased. Sympathetic nerve activity to the kidneys and heart alone can account for up to 50% of all SNA [71]. In normotensive patients, the heart contributes only 3% of total SNA, and the kidneys contribute 17% [52]. Renal sympathetic activity (RSNA), measured by renal NE spillover, is increased 2-3 times in patients with essential hypertension as compared to normotensive subjects [70]. As discussed earlier, increased RSNA can elevate BP through sodium and water reabsorption by direct action at the proximal tubule and also

indirectly by activating the RAAS. Additionally, excess RSNA can increase TPR by augmenting renal vascular resistance (RVR) by constricting renal arteries and arterioles. In fact, renal blood flow (RBF) is reduced in some subjects with HTN [72]. Furthermore, α-AR antagonists have no effect on renal blood flow in normotensive patients, but increase RBF in patients with HTN[72]. Combined with the findings of normal renovascular responsiveness to intrarenal NE infusion in HTN, these data support the hypothesis that there is greater sympathetic nerve activity to the renal vasculature in human HTN [72].

In summary, the experimental and clinical evidence demonstrate that sympathetic activity to the kidneys is an important contributor to the natural course of some forms of HTN. Given the previous discussion indicating the need for better treatment options for HTN, these reports would indicate that the renal nerves are a logical therapeutic target.

Medical management of hypertension

Pharmacological treatment strategies

In 2013 the JNC issued updated guidelines for the treatment of HTN. For patients under the age of 60 years and those with diabetes, the committee still recommends initiating treatment when BP rise above 140/90mmHg, and sets a therapeutic goal of lowering BP below 140/90mmHg. In non-diabetic patients older than 60 years, the threshold for initiating treatment is 160/90mmHg with a therapeutic goal of lowering BP below 150/90mmHg. However, the committee concedes that BP can be further lowered in older patients as long at treatment is well-tolerated [4].

The current approaches used in the clinical management of HTN can be divided into two separate categories: lifestyle modification and pharmacological therapies. Lifestyle modifications include recommendations to lose weight (BMI<25kg/m^2), adapt to a low sodium diet, and increase physical activity [73]. While these interventions are known to lower BP in some individuals, they are often unsuccessful due to patient non-compliance.

Pharmacological management of HTN is often achieved using one or more of several classes of anti-hypertensive medications. The drug classes commonly used in treating HTN include diuretics, beta-blockers, alpha antagonists, central sympatholytics, ACE inhibitors, angiotensin II receptor antagonists, calcium channel blockers, and direct vasodilators. Current guidelines suggest that the initial anti-hypertensive agent should be chosen based on the patient's ethnicity. In non-black patients, it is recommended that the first agent be a thiazide-type diuretic, calcium channel blocker, ACE inhibitor, or angiotensin receptor blocker. In black patients, evidence suggests that starting with a thiazide-type diuretic or a calcium channel blocker is more appropriate [4]. The guidelines also recommend follow-up within 1 month after initiating a medication. If the initial medication failed to lower BP sufficiently, it is recommended that the dose be increased or an additional medication should be added to the regimen. Should BP fail to be controlled with 2 medications, addition of a third is advised. After medications are added, if BP is still uncontrolled, the patient is considered to have resistant hypertension. At this point, it is recommended that the patient be sent to a hypertension specialist for additional anti-hypertensive drug therapy [4].

Models	
Spontaneously hypertensive rat (SHR)	DOCA-NaCl (rat)
Borderline hypertensive rat	DOCA (pig)
Stroke-prone SHR	Grollman renal wrap (rat)
New Zealand SHR	Low sodium, 1 kidney hypertension (rat)
Goldblatt 1 kidney, 1 clip (rat)	Angiotensin II hypertension (rat)
Goldblatt 2 kidney, 1 clip (rat)	Obesity hypertension (dog)
Aortic coarctation (dog)	NaCl (baroreflex-impaired rabbit)
Aortic nerve transection (rat)	

TABLE 1: Models of experimental hypertension in which renal denervation prevents or delays the development of HTN. Adapted and reproduced with permission [20].

Due to the considerable cardiovascular damage that occurs with uncontrolled HTN, it is important to continue to investigate the pathophysiology of HTN and seek new treatment modalities that will lower BP in drug resistant HTN. This goal was the motivation of the investigators that developed the device-based approach CBRNA.

Catheter-based renal nerve ablation

The previously discussed thoraco-lumbar sympathectomy was effective in lowering BP by eliminating sympathetic innervation to multiple targets, including the kidneys. The extensive nature of that approach, however, caused many untoward effects. In principle, a more restricted denervation could produce fewer adverse effects and still lower BP as long as disruption of neural connections critical to BP regulation was achieved. CBRNA was designed to lower BP by destroying the renal nerve fibers that travel adjacent to and through the renal arteries without damaging the neural connections to other organs. To this end, Sobotka and colleagues developed a specialized endovascular catheter, the Symplicity catheter (Ardian Inc, Palo Alto, CA, US), which allows highly localized radiofrequency energy to be applied across the renal artery resulting in damage to nerve fibers within and near the arterial wall. Figure 6 shows CT-angiographs of a patient receiving CBRNA on the right kidney [74].

The first reported patient to receive CBRNA was a 59-year old male with long-standing drug-resistant HTN. In fact, this patient was on 7 different anti-hypertensive medications. The patient's BP prior to the intervention was 161/107mmHg. Following the procedure, BP fell to 141/90 and 127/81 at 30 days and 12 months, respectively [3]. The procedure was associated with a reduction in NE spillover from both kidneys,

halving of plasma renin activity, an increase in renal blood flow, and a reduction in MSNA [3]. It is logical to expect that interruption of the renal sympathetic nerves would reduce renal NE spillover. In fact, the investigators reported a 75% reduction in right kidney NE spillover and a 48% reduction in left kidney NE spillover 30 days after CBRNA in this patient[3]. These findings suggested that the BP lowering effect of CBRNA required only partial denervation of the kidneys. Additionally, it was expected that removing neural influences on renin release would reduce the circulating levels of renin, and thus plasma renin activity. One would also anticipate a decrease in renovascular resistance as the vasoconstrictor influence provided by renal sympathetic innervation would be diminished. In fact, Mahfoud and colleagues in later studies also showed a modest but significant reduction in the renal resistive index in patients undergoing CBRNA [75]. Interestingly, although sodium and water balance are believed to play a role in the BP lowering effect of CBRNA [76], no studies have directly evaluated the possible natriuretic and diuretic effects of CBRNA.

The reduction in MSNA observed in the first published report regarding CBRNA in humans [3] was unexpected and suggests that the BP lowering effect of CBRNA could be in part through interruption of non-renal sympathetic nerve activity (NRSNA). This observation provides a rationale for considering the renal afferent nerves, and related CNS pathways, as potential mediators of the fall in BP provided by CBRNA. Certain studies in rats, the findings of which are discussed below, have shown that the renal afferent nerves are capable of modulating SNA and elevating BP. Whether CBRNA lowers BP by eliminating sympathoexcitatory sensory signals originating from the kidneys of human subjects remains to be proven.

The Symplicity HTN trials, which were multi-center, international clinical studies, also revealed reductions in office-BP in patients receiving CBRN [1, 2]. In Symplicity HTN 1, office BP was gradually, but persistently reduced at 1, 3, 6, 9, and 12 months. At the 12-month endpoint, BP was reduced 27/17mmHg. Control subjects showed an increase in BP over the same period [2]. Ambulatory BP was also measured in a subset of 12 patients: average 24-hour systolic pressure was reduced 11mmHg. Symplicity HTN 2 reported significant reductions in office BP (Figure 7). At the 6-month follow-up period, the primary end point for the study, office BP was significantly reduced 32/12 mmHg in CBRNA-treated patients with no reduction reported in those patients receiving standard medical therapy. Ambulatory pressure recorded in 20 CBRNA-treated patients was found to be reduced 11/7 mmHg. In 25 control subjects, ambulatory BP did not change. One should note that the activity of neurohumoral factors regulating BP was not carefully examined in these patients.

Patients in the Symplicity HTN 2 control group that were crossed over to the CBRNA treatment showed a 23/8mmHg reduction in BP 6 months after treatment. As a reminder, BP was not reduced in these patients while they were continued on standard medical treatment [9].

Interestingly, long-term monitoring of patients receiving CBRNA demonstrated a prolonged BP lowering effect. Follow-up in 153 CBRNA patients by Symplicity HTN 1 investigators revealed a continued reduction in BP up to at least 24 months after the procedure. In 18 patients that were 2 years removed from the CBRNA intervention, BP was still reduced 32/14mmHg [8].

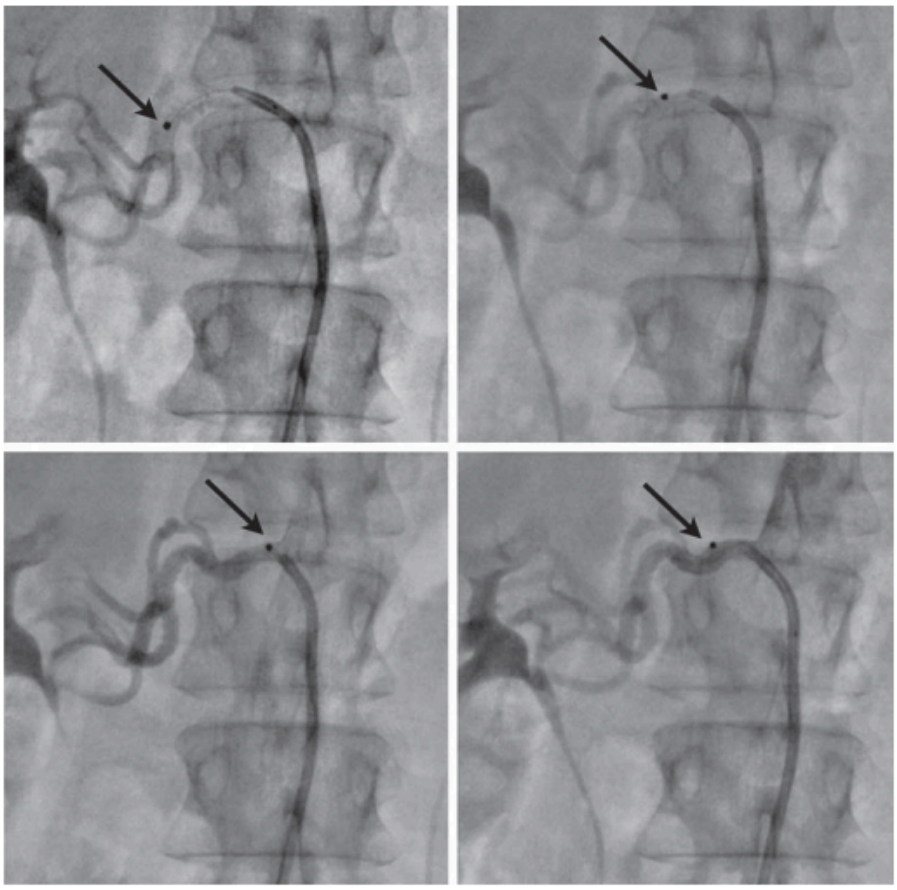


FIGURE 6. **Radiofrequency ablation of the right renal artery by Symplicity catheter at four different locations**. The arrow marks the tip of the ablation catheter. Reproduced with permission from Goliasch et al. [74]. Original figure legend translated and adapted using Google Translate.

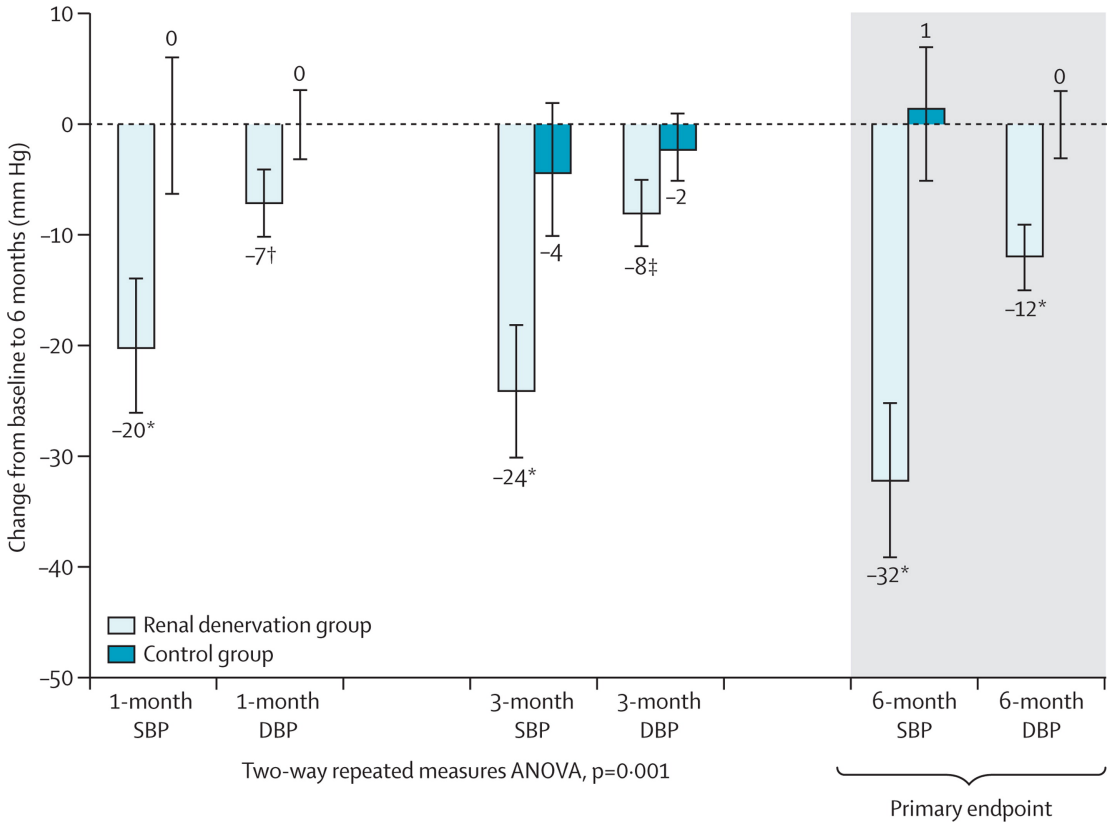


Figure 7. **Effect of CBRNA on office-BP.** BP changes at 1, 3, and 6 months after catheter-based renal nerve ablation as reported from Symplicity HTN 2 [1]. Reproduced with permission from Esler et al. [1].

Collectively, these data support the idea that CBRNA is an effective treatment for chronically lowering BP in patients with difficult-to-treat HTN. Moreover, as contrasted with the non-selective, thoraco-lumbar sympathectomy, the procedure was documented to be quite safe, and without difficult convalescence, prolonged orthostatic hypotension, or other autonomic problems.

Current challenges associated with catheter-based renal nerve ablation

Although the clinical effectiveness and safety of CBRNA has been demonstrated in several human trials, important questions remain. Firstly, some studies of CBRNA linked the reduction in BP with a decrease in whole-body sympathetic activity. As mentioned previously, renal NE spillover showed a 75% reduction in the right kidney and a 48% reduction in the left kidney; however, a 42% reduction in whole-body NE spillover also was found [3]. A recent report has shown that CBRNA in 35 patients with drug-resistant HTN lowered MSNA 6±12 bursts/min 12 months after the procedure[77]. The reduction in whole-body NE spillover and MSNA has been proposed to be due to central inhibition of SNA, i.e. that CBRNA suppresses sympathoexcitatory signals transmitted to the CNS by renal afferent nerves. This conclusion was derived from the following reasons: 1) The renal contribution to whole-body NE spillover is only about 17%, and whole-body NE spillover dropped more than 17% despite incomplete renal denervation. Therefore, a reduction in NRSNA must also be occurring to explain this finding. 2) MSNA, evaluated by microneurography, was also reduced. This finding further supports the hypothesis that a reduction in NRSNA could explain the BP response to CBRNA. In contrast to these results, Brinkmann et al. did not find MSNA to be reduced in patients undergoing CBRNA [78]. In fact, they comment, “central

sympathetic inhibition may be the exception rather than the rule after renal nerve ablation in unselected patients with difficult-to-control hypertension [78].” Therefore, one important question is: *Does CBRNA lower BP by affecting the RSNA or are effects on NRSNA necessary as well*?

A second important issue is that patients undergoing CBRNA are on multiple anti-hypertensive medications. For example, Symplicity HTN 1 patients averaged 4.7 anti-hypertensive medications, patients enrolled in Symplicity HTN 2 averaged 5.2 [1, 2], and in Brinkmann et al. the average number of BP-lowering drugs per patient was 7 [78]. Given the discrepancy of the findings between these investigators and the lack of a standard medication regimen for the patients, it has been proposed that drug therapy could influence the BP response to CBRNA [79]. Interestingly, in a follow-up analysis on the durability of the BP effect of CBRNA in 153 patients, multivariate analysis suggested that patients treated with the centrally acting sympatholytic drug clonidine were more likely to show a BP reduction following CBRNA [8]. This is somewhat counterintuitive as clonidine would be expected to decrease both RSNA and NRSNA. Overall, however, little research has been performed to investigate how drug therapies might impact the response to CBRNA [79].

A third important question for clinicians has been “How can we identify patients that are most likely to respond with a durable BP reduction after CBRNA?” Retrospective analysis of the first patients to receive CBRNA (153 patients) revealed that only higher baseline systolic BP, and use of a central sympatholytic agent at baseline, predicted a fall in BP with CBRNA [8]. Multivariate analysis of the baseline characteristics of 43 patients receiving CBRNA also showed that higher systolic BP (>150mmHg) predicted a

fall in BP with CBRNA [80]. Additional investigation has shown that higher basal blood levels of sFLT-1, ICAM-1, and VCAM-1, markers associated with endothelial dysfunction and HTN, were predictive of a BP reduction with CBRNA [81]. The biomarkers listed above are not routinely measured in the management of HTN [24], and a systolic BP >150mmHg could occur in many forms of HTN. This means these characteristics are too non-specific to guide clinicians in a clear direction regarding which patients should receive CBRNA. Therefore, more pre-clinical effort is necessary to understand the precise patient populations where CBRNA could have the greatest therapeutic effect.

Finally, and of particularly great importance, the very recent Symplicity HTN 3 study, the first double-blinded, placebo-controlled trial of CBRNA to treat resistant hypertension, failed to meet its primary efficacy endpoint for BP lowering[82]. One could conclude that CBRNA is a failed approach that should be abandoned, however many have noted flaws in the trial and encouraged additional investigation {Joyner, 2014 #2684;Messerli, 2014 #2685}. Particularly critical questions are: 1) How important is the thoroughness of denervation to the BP-lowering action of CBRNA? and 2) How can we identify the patients who are most likely to respond to CBRNA with a significant and persistent fall in BP?

Clearly, much more investigation is necessary to enable physicians and scientists to better understand the limits of CBRNA and regional sympathetic modulation. As pointed out by Jordan et al., “without data from proper clinical trials, the real benefit of this highly promising intervention may never be appreciated. Conceivably, the lack of data could

even lead to underutilization of renal nerve ablation in other areas such as mild-moderate hypertension, sleep apnea, pre-diabetes, or congestive heart failure [83]."

Unfortunately, addressing these questions in a clinical setting is often difficult or even impossible. It is for this reason that identifying an animal model that responds to renal denervation with reductions in BP similar to those seen in clinical trials could provide a great advantage in determining how CBRNA lowers BP in humans and the conditions under which a good response would be most likely to be achieved.

Renal denervation in the SHR

The SHR is a genetic model of HTN; however, as is the case in essential HTN, the pathogenesis of the HTN is not attributable to a single gene mutation. In contrast to other models of HTN, the SHR develops high BP without any specific intervention i.e. HTN develops spontaneously as the animals age.. Moreover the pattern of the development of HTN, the magnitude of the HTN and the resulting end-organ damage parallels the human condition [84]. For this reason many investigators have preferred the SHR as an experimental animal model for human essential HTN. As mentioned previously, renal denervation has been performed in many models of hypertension including the SHR. As with most studies in other animal models, these experiments examined the ability of RDX to prevent HTN in the SHR. This experimental paradigm has limited relevance to the clinical setting, where treatment of hypertension typically begins only many years after high blood pressure is detected. Therefore, a more clinically relevant experimental approach is to determine if renal denervation lowers BP in established hypertension. Many studies investigating reversal of established HTN in

the SHR via RDX have been negative, but the overall data are inconsistent. The discussion below provides a rationale for continuing to explore RDX in the SHR despite numerous previous investigations on the topic.

RDX in the SHR: A rational for further exploration

Several investigations in the SHR show RDX delays the development of HTN i.e. RDX performed in pre-hypertensive rats delays the expression of high BP [85-88]. At least two studies show that RDX performed in 7 week old SHR blunted the development of HTN [85, 88]. However, when HTN becomes established as SHR age, some investigators find that RDX has little effect on BP [88]. Winternitz and colleagues also found an increase in fractional excretion of sodium in pre-hypertensive rats subjected to RDX but did not find a similar result in rats with established HTN[88]. The authors' main conclusions were that the preventive effect of RDX on HTN was likely related to the natriuretic effect of denervation, and that the renal nerves are involved in the development but not the maintenance of HTN in the SHR. This has been the dominant narrative in the field, and as a consequence most studies in other models use renal RDX as a method for exploring mechanisms involved in the development rather than the maintenance of HTN. On the other hand several reports indicate that RDX may be effective at lowering BP in SHR even after HTN is established. Gattone et al [89] report a significant reduction in BP (-24mmHg in MAP) after RDX in adult SHR with established HTN. In 2013, Paton's group published similar results showing that RDX significantly reduced BP in adult SHR with established HTN [90]. Walsh's group published findings showing RDX significantly lowered BP for at least five days in 6 month old SHR [91, 92], however, no longer-term evaluation of the BP response was

performed. Importantly, the BP lowering effect of RDX was not associated with sodium loss. This could be interpreted to mean that RDX is lowering BP in established HTN by a mechanism not associated with natriuresis, which was the mechanism described to support the blunted rise in BP in younger SHR. Collectively these studies argue that the renal nerves could be involved in the maintenance of hypertension in the SHR. Since relatively few studies used RDX as a means of reversing HTN in the SHR after it had developed, our understanding of the mechanisms involved are limited. Nevertheless, these observations provide a rationale for additional exploration of RDX in the SHR. The work of this dissertation aims to: 1) confirm in SHR that RDX can reverse established HTN, 2) assess the durability of the fall in BP, and 3) characterize the mechanisms responsible for the BP reduction.

Additional clinically relevant questions to be addressed using RDX in the SHR

A major point of debate regarding how CBRNA lowers BP in humans is whether the effect is due to interruption of the renal afferent and renal efferent nerves. Similar efforts have been undertaken in the SHR although the main focus was on mechanisms involved in the prevention of HTN. Work from Janssen et al employed the use of dorsal rhizotomy to address the role of renal afferent nerves in the development and maintenance of HTN. These experiments showed that interruption of renal sensory nerves through dorsal rhizotomy does not alter the development of HTN. Unexpectedly, dorsal rhizotomy in 13 week old SHR with established HTN modestly, but significantly, lowered BP. When the authors compared the magnitude of BP reduction from other total renal denervation studies, the observed fall was much greater with total renal denervation. The authors concluded that renal afferent nerves mediate only a small part

of the overall BP response to renal denervation [93]. Unlike the previous studies to which those authors compared their results, where both kidneys were intact, the rats in their studies underwent a unilateral nephrectomy 4-5 weeks before the dorsal rhizotomy. This anatomical difference makes the author's comparisons harder to interpret. It is unclear what effect this uninephrectomy might have on the renal afferent nerves of the contralateral kidney. Therefore further work is needed to assess how interruption of renal afferent nerves in intact SHR might influence established HTN. It is plausible to hypothesize that interruption of renal afferent nerves could lower BP as it is known that the renal afferent nerves in rats project to both the NTS and the nodose ganglion [15]. The NTS is a key cardiovascular control center in the CNS, and a major nuclei involved in the baroreflex[19]. Modification of input into the NTS could influence SNA at non-renal targets. It has been further demonstrated that sensory information carried by the renal afferent nerves is sufficient to elevate BP [22]. Additionally, damage to renal afferent nerves during RDX leads to alterations in non-renal cardiovascular function[90, 91]. Walsh showed that the BP reduction in 6 month old SHR was associated with decreased vascular resistance to both renal and splanchnic vascular beds within hours after surgery[91]. The effect on vascular resistances persisted up to 5 days after RDX. Hindlimb vascular resistance also fell considerably, however the reduction was not statistically significant [91]. Further temporal evaluation of vascular resistances in these animals was not performed. Paton and colleagues showed that in addition to lowering BP, RDX significantly reduces lumbar sympathetic nerve activity and improves baroreflex sensitivity in 12 week old SHR [90]. These findings also suggest that the BP lowering effect of RDX could be attributed to a reduction in

sympathetic activity to non-renal targets, thus underscoring the possible importance of severing renal afferent nerves. One aim of my dissertation is to explore the relative roles of afferent and efferent renal nerve mechanisms in the BP response to RDX using the SHR.

There is also substantial concern about the impact of kidney reinnervation on BP after CBRNA. Currently there are no studies in humans addressing this issue. Previous work in the SHR suggests reinnervation of the kidneys after RDX. Winternitz et al reported that the prolonged, RDX-mediated natriuretic effect presumed to delay the onset of HTN in the SHR disappeared at the same time that the kidneys reinnervated [88]. In a particularly important study, Kline et al showed that the kidneys are functionally reinnervated 2 weeks post-RDX even though kidney NE content (a common measure of extent of denervation) is still depressed [94]. Unfortunately, these investigators did not also measure BP. Norman and Dzielak showed that repeated RDX at three week intervals is sufficient to prevent the full expression of HTN even as the SHR enters adulthood [87]. This suggests that regrowth of the renal nerves could mitigate any BP reductions gained from the initial RDX procedure. This has not been explored in SHR with established HTN and therefore requires further investigation.

Significance of telemetric BP recording in the SHR

A significant limitation to interpreting previous work on renal denervation in SHR is that the majority of studies were performed prior to the implementation of continuous 24hr/day telemetric BP recording. Most relied on the use of tail-cuff BP, which is much less sensitive than telemetry and involves an element of stress as the animal must be

restrained. In human studies, RDX lowers both office BP and ambulatory BP. In rodents there is no true equivalent of office BP, but telemetric measurements are similar to ambulatory measurements in patients. As mentioned previously, the average reduction in ambulatory systolic BP in Symplicity HTN 1 and HTN 2 patients was modest, i.e. ~11 mmHg. It is unlikely that tail-cuff BP measurements would be able to detect such a small change as statistically significant in small groups of animals. With the use of telemetric recording, it is possible to statistically detect such a difference in BP with 5-7 rats. Therefore, should RDX lower BP in the SHR with established HTN by the same magnitude observed in the clinical trials, we will be able to reliably capture this response with telemetric BP recording.

Central hypothesis, scope of the project, and overall significance

Although CBRNA has shown promise in small clinical trials and is being performed in hypertension clinics throughout the world, relatively little information is available on how this procedure lowers BP in humans. As a consequence, important factors for procedure utilization and outcome optimization, such as patient selection and integration with existing drug therapies, remain undefined. *The work presented in this dissertation was conducted to provide knowledge on how CBRNA reduces BP in human patients, and the optimal conditions under which it would do so, by using RDX in SHR with well-established HTN as an animal model.* While keeping the limitations of experimental animal research in mind, this approach may provide guidance for additional clinical studies aimed at improving and refining the therapeutic potential of CBRNA in HTN management. The studies presented here have three goals. First, I test the appropriateness of the SHR as an animal model to study the antihypertensive effect

of renal denervation in established HTN. Second, I attempt to identify physiological mechanisms responsible for the fall in BP after renal denervation in SHR. Third, I investigate in SHR how co-treatment with clinically relevant anti-hypertensive drugs alters the BP response to renal denervation.

The central hypotheses of this dissertation are: 1) that RDX in the SHR will decrease BP to a degree similar to the effect of CBRNA in human patients with resistant hypertension, and 2) that the BP response to renal denervation in the SHR is due to suppression of both renal sympathetic nerve activity (RSNA) and non-renal sympathetic nerve activity)(NRSNA)(Figure 8).

These central hypotheses were evaluated by testing the following sub-hypotheses:

Sub-hypothesis 1: SHR validation (Chapter 3)

RDX in SHR will cause a persistent decrease in BP similar in magnitude to that observed after CBRNA in human patients with resistant hypertension.

Sub-hypothesis 2: Clonidine study (Chapter 4)

If the BP effect of RDX in the SHR is mediated by interruption of RSNA and/or NRSNA, then prior suppression of SNA with the centrally acting sympatholytic drug clonidine should prevent the expected chronic drop in BP associated with RDX.

Sub-hypothesis 3: Losartan study (Chapter 5)

If the BP effect of RDX in the SHR is mediated by interruption of sympathetically mediated activation of the RAAS, then prior suppression of the RAAS with the

angiotensin II receptor blocker (ARB), losartan, should prevent the fall in BP associated with RDX.

Sub-hypothesis 4: Low sodium study (Chapter 6)

If the BP response to RDX is mediated by a diuretic/natriuretic effect due to loss of RSNA, then the magnitude of the BP response will be inversely proportional to the animal's steady-state sodium intake (as is observed with the antihypertensive response to diuretic drugs).

Sub-hypothesis 5: Adrenergic antagonists study (Chapter 7)

If the BP effect of RDX is mediated by reduced activation of adrenergic receptors either in the kidney or elsewhere, then the α1-AR antagonist, prazosin, and/or the β1-AR antagonist, atenolol, will prevent the fall in BP associated with RDX.

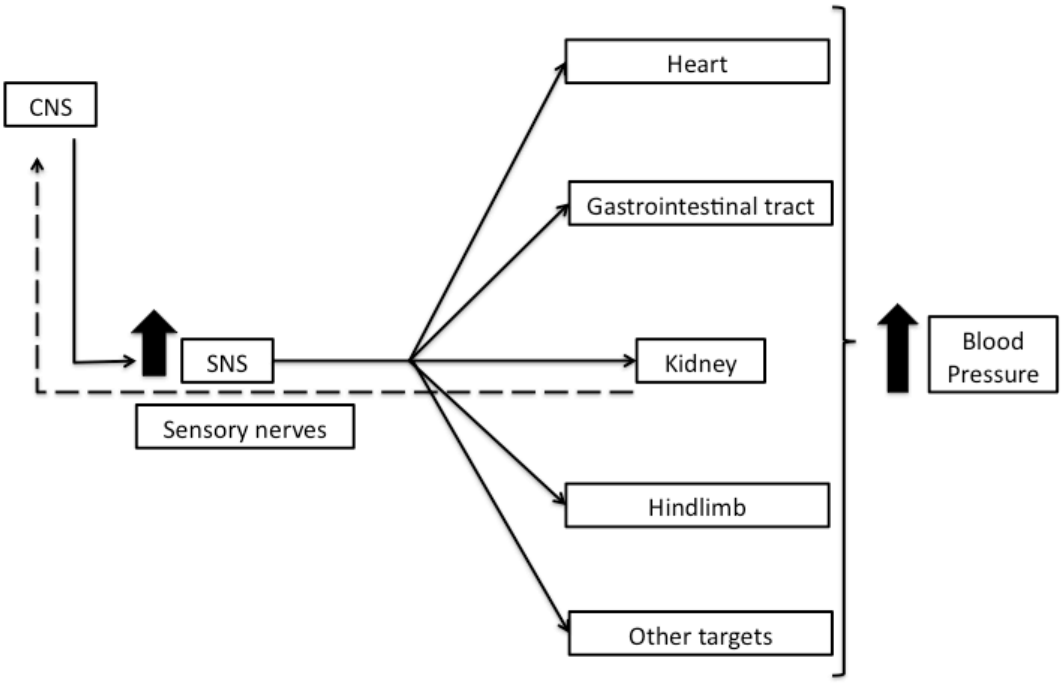
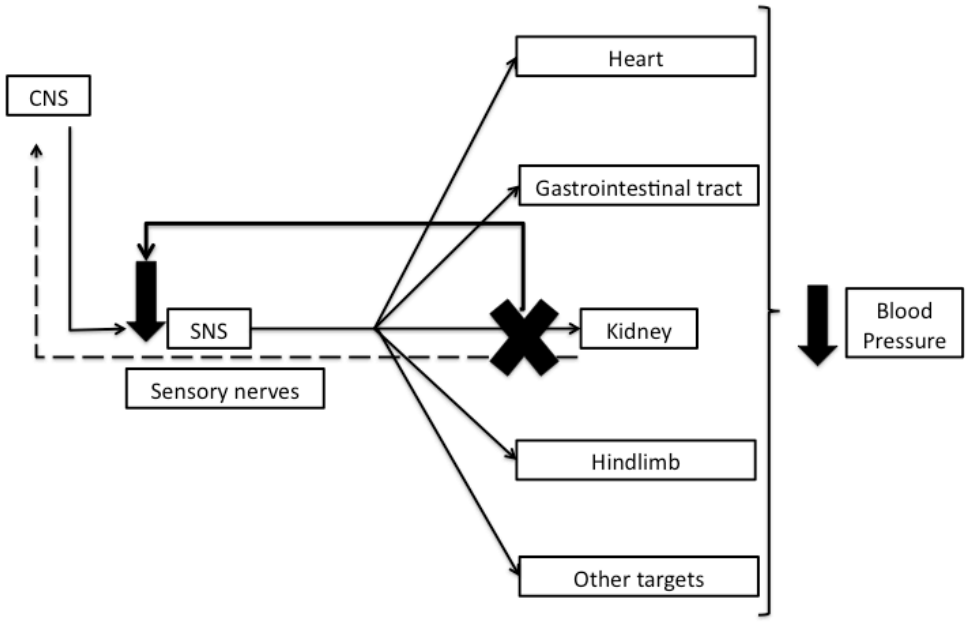


FIGURE 8. Schematic of central hypothesis. TOP: Elevated BP in the aged SHR is supported by increased sympathetic drive. BOTTOM: Renal denervation lowers BP in the aged SHR by suppressing a mechanism related to sympathetic nervous system activity.

Figure 8 (cont'd)



CHAPTER 2. METHODS

General surgical procedures

All animal procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University. Prior to all surgical procedures, analgesia and antibiotic considerations were addressed with carprofen (5 mg/kg; SC), enrofloxacin (2.5 mg/kg; IM), and piperacillin (120mg/kg; SC). Surgical anesthesia was induced with 4% isoflurane in 100% oxygen and maintained throughout the procedures at 2-3%. Post-operative recovery occurred under a heat lamp until animals were conscious and stable. Health and welfare of the rats were continuously monitored throughout the

Rats

General considerations

Rats were singly housed with ad libitum access to food and distilled water. Normal laboratory chow consisted of Harlan Teklad Diet 8640. Select studies utilized different sodium intakes -- either high sodium intake consisting of 0.4% NaCl (Harlan Teklad diet 8640), supplemented with 1% NaCl drinking water, or low sodium intake consisting of 0.1% NaCl (Harlan Teklad diet 7034) low sodium diet. All animals were purchased from Charles River Laboratories, Portage, IN. Euthanasia was performed by exsanguination in deeply anesthetized rats (5% isoflurane) concomitantly injected with a lethal dose of sodium pentobarbital (100mg/kg).

Spontaneously hypertensive rats

Male, spontaneously hypertensive rats (SHR) were purchased at various ages ranging from 13 weeks to retired breeder status. The vendor estimated the retired breeder rats to be 24-52 weeks of age (median age: 36 weeks).

Sprague-Dawley rats

Male Sprague-Dawley rats (275g; 8 wks of age; Charles River) were generously donated by Dr. Peter Cobbett. Animals were aged an additional 8 weeks after procurement before studies were undertaken.

Telemetry and hemodynamic recording

Radiotelemeters (TA11PA-C40, Data Science International) were used to record BP in conscious rats. The catheter of the telemeter was advanced from the femoral artery to the abdominal aorta in anesthetized rats (Image 1). The telemeter body was housed subcutaneously (Image 2). Rats were allowed 5-7 days of recovery from telemeter implantation before hemodynamic variables (SBP, DBP, MAP, HR, PP) were recorded. Measurements were collected for ten seconds every 10 minutes (24 hr/day) throughout the experimental period. Recorded variables were stored on a computer and analyzed using Dataquest ART 4.1 software.

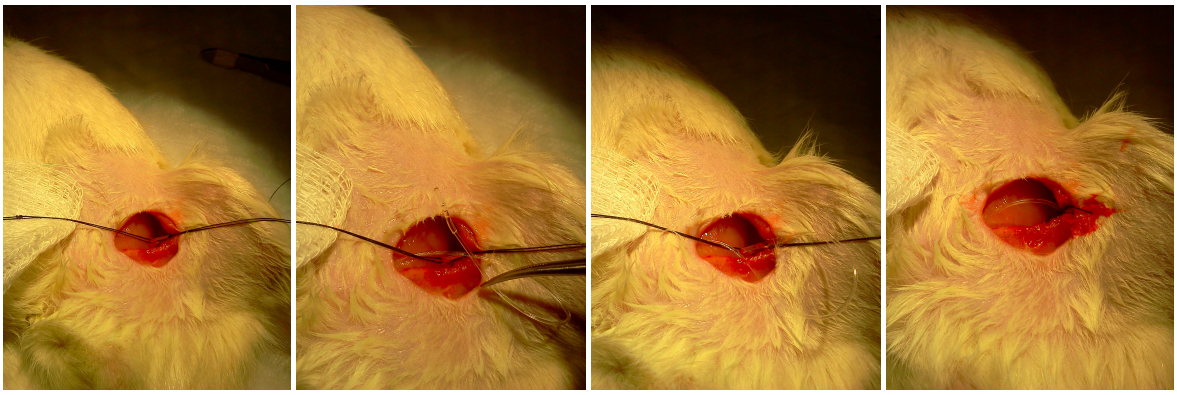


Figure 9. **Placement of BP telemeter catheter in abdominal aorta by way of femoral artery**. From left to right: Femoral artery is ligated and accessed by incision. Catheter is carefully guided into position and secured with surgical suture. Images by JT Phelps.

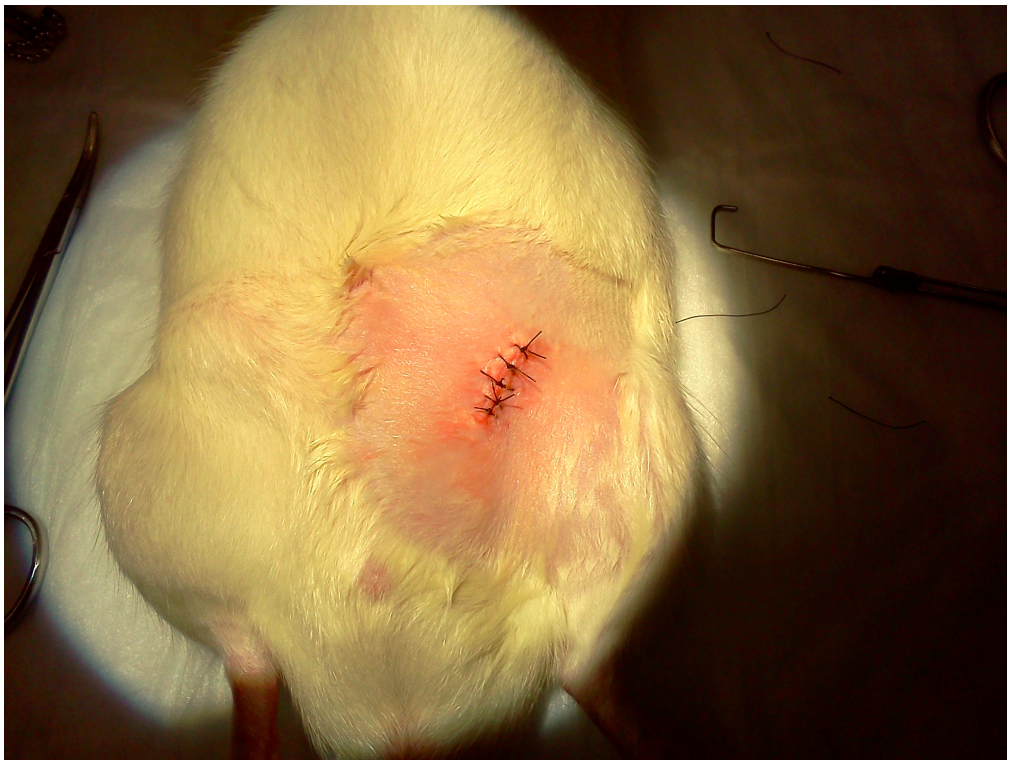


Figure 10. **A representative image of the surgical incision closure and subcutaneous securing of telemetry transmitter**. Telemeter body is the bulbous structure left of femoral incision. Image by JT Phelps.

Bilateral renal denervation

The renal vasculature was accessed through a midline incision in anesthetized rats. Abdominal contents were gently displaced and kept hydrated using 0.9% saline. The renal arteries were blunt dissected away from the renal vein, and nerve fibers within the connective tissue were mechanically stripped away. A phenol solution (20% phenol in 100% ethanol) was applied to the renal vasculature to destroy any remaining nerve fibers. The abdominal contents were returned, and the incision was closed with 5-0 silk sutures. Sham operation (SO) consisted of exposing the renal vessels without further blunt dissection or application of phenol.

Bilateral renal de-afferentation

Renal vasculature was accessed as described in section 2.4. Instead of separating the renal artery from vein, the connective tissue surrounding the vascular bundle was gently disrupted to create a small tunnel. A piece of gauze soaked in a 33mM capsaicin solution (90% saline, 5% ethanol, 5% Tween 80) was advanced through the tunnel and packed around the blood vessels for 5-7 minutes. Gauze was extracted and the incision closed with 5-0 silk suture.

Unilateral renal de-efferentation

The left renal artery and vein were approached through a midline abdominal incision in anesthetized rats. A similar tunnel was made around the renal vasculature as described in section 2.5, and a piece of gauze soaked in 6-hydroxydopamine (0.5mg/mL) was wrapped around the vessels for 10 minutes. The gauze was removed and the incision closed with 5-0 silk.

Drug administration

Hexamethonium

Hexamethonium, a non-depolarizing, nicotinic receptor antagonist, was administered at a dose of 30mg/kg through intraperitoneal injection. This dose has been routinely used by my lab to evaluate neurogenic support of BP [95-97].

Clonidine

Clonidine, an α2-AR antagonist, was dissolved in 0.9% saline and delivered to rats subcutaneously using an ALZET osmotic mini-pump (model 2006). The dose of clonidine administered was 125 μg/kg/day. It has been previously observed that BP and HR are chronically reduced in SHR using this dose and delivery method without development of tolerance to clonidine treatment [98].

Prazosin

Prazosin, an α1-AR antagonist, was dissolved in the rats' drinking water using a Branson 2510 sonicator to achieve a concentration of 85.7mg/L. The dose administered to the rats was 3mg/kg/day. This dose was used previously shown to chronically suppress α_1-AR signaling [99].

Atenolol

Atenolol, a selective β1-AR antagonist, was dissolved in the rats' drinking water at a concentration of 1mg/mL. This dose was previously shown to chronically suppress β_1-AR activity [100].

Losartan

Losartan, an angiotensin II receptor antagonist, was dissolved in the rats' drinking water and administered at a dose of 10mg/kg/day. This dose has been shown to be well-tolerated during chronic administration, effective in lowering BP, and sufficient to block the AT_1R in the SHR [101].

Chlorthalidone

Chlorthalidone, a thiazide-like diuretic (antagonist of the NaCl transporter in the distal convoluted tubule), was dissolved in the rats' drinking water at a concentration of 100mg/L. This concentration was selected based on previously unpublished studies in the laboratory of Greg Fink.

Furosemide

Furosemide, a loop diuretic (antagonist of the Na-K-2Cl transporter in the thick ascending limb of the nephron), was dissolved in 2% ethanolamine in distilled water (pH=8) and delivered subcutaneously using ALZET osmotic mini-pumps (model 2ML1). The dose administered to the rats was 0.5mg/hr or 12mg/day. DiBona and colleagues have shown this dose and delivery method are well tolerated chronically and effective at increasing sodium excretion [102].

Amlodipine

Amlodipine, an L-type calcium channel antagonist, was dissolved in the drinking water at a concentration of 60mg/L. The dose administered to the rats was 10mg/kg/day. This

dose has been used previously to block calcium channels and lower BP in rat models of HTN [103-105].

Blood collection and processing

Blood collection

Rats were briefly anesthetized with isoflurane, and 0.3-0.5mL of blood was drawn from the tail vein into a heparinized syringe using a 25G needle. Blood samples were collected into pre-chilled, heparinized microcentrifuge tubes for further processing.

Hematocrit measurements

Heparinized, micro-hematocrit capillary tubes were used to collect a sample from the blood collected as described in section 2.7.1. The tubes were sealed on one end and centrifuged at 10,000rpm for 10 minutes. The hematocrit measurements were taken using a reader and instructions provided by the manufacturer (Hermle Z 300).

Plasma collection

Samples were centrifuged at 3000rpm for 10 minutes to separate the plasma from the blood cells. Plasma was carefully aspirated from the sample and stored at -80°C until analyzed.

Evaluation of sympathetic activity

Neurogenic pressor activity

The magnitude of the sympathetic nervous system support of BP was determined by calculating the maximum fall in BP within the first 30 minutes after hexamethonium injection (30mg/kg, IP).

Plasma norepinephrine (NE) concentration

Plasma NE content was determined from duplicate plasma samples by alumina extraction and reverse-phase high performance liquid chromatography (HPLC) separation with coulometric detection.

NE and other catecholamines were extracted from a 100L plasma sample using 6.7-7.3mg of activated acid-washed alumina in a microcentrifuge tube. The internal standard DHBA (15μL) and 0.4mL 2M Tris/0.5M EDTA (pH = 8.1) were added to the tube. After 20 minutes on a vortex shaker, samples were centrifuged for 3 minutes and the supernatant discarded. Samples were washed with 0.4mL 18 MOhm water and shaken for 3 minutes. After centrifugation for 3 minutes, the supernatant was discarded. Catecholamines were then eluted from the alumina pellet with 100μL 0.2mM acetic acid. Samples were shaken (3 minutes) and centrifuged (3 minutes) with the supernatant collected for direct injection into the HPLC machine (40μL injection). All HPLC assays wer expertly peformed by Robert Burnett.

Verification of renal denervation

Tissue collection

At the conclusion of each study, the kidneys and spleen were collected from the euthanized rats. The renal pelvis was blunt dissected from the medial aspect of the kidneys for analysis of afferent denervation. The lateral aspect of the kidneys was saved to evaluate efferent sympathetic denervation. The spleen was collected to serve as a negative control for the efferent sympathetic denervation procedure. The tissues were flash frozen in liquid nitrogen and stored at -80°C until analyzed.

Afferent renal denervation

The efficacy of afferent sympathetic denervation was determined by measuring calcitonin-gene related peptide in the renal pelvis using an ELISA-based method, which was performed by John Osborn's laboratory at the University of Minnesota.

Efferent renal denervation

The efficacy of efferent sympathetic denervation was determined by measuring norepinephrine content in the tissue samples by reverse-phase high performance liquid chromatography analysis with electrochemical detection. Robert Burnett performed these analyses.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 software. All studies utilized a mixed-model repeated measures design. Between group differences were analyzed using a mixed-model two-way ANOVA, and a post-hoc analysis at each time

point was performed when appropriate using a Bonferroni's multiple comparison test. When only two groups were compared at a single time point, a Student's t-test was used. Correlation analyses were performed using a Pearson's correlation test. Regression analyses utilized the Deming (Model II) linear regression. A p-value < 0.05 was considered significant. Hemodynamics reported in 24hr averages. Steady-state hemodynamics values were determined by averaging the values reported during last three days of a treatment interval once parameters had stabilized. All results are reported at mean ± SEM. The p-value reporting scheme used in Graph Pad prism is also used in all figures (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$).

CHAPTER 3 – Renal denervation lowers BP in older adult spontaneously hypertensive rats similar to observations in humans

CBRNA is emerging as a treatment option for difficult-to-treat hypertension in the absence of a detailed mechanistic understanding of how this treatment modality actually lowers BP. Although current clinical investigation is ongoing, the primary endpoints for these studies are clinical effectiveness with little exploration into mechanism [106]. Due to ethical constraints, pursuing some mechanisms in human studies may not be possible. Fortunately surgical removal of the renal nerves in animal models of hypertension is feasible and well documented [11].

While RDX has been performed in many experimental models of hypertension, the results leave current investigators three major challenges. 1) Most of these studies involved denervation during the developmental phase of HTN, whereas very little research has been conducted on the reversal of high BP after HTN has been established. The latter is more analogous to the human situation in which CBRNA is being used. 2) Several of the experimental models of HTN that are affected by RDX are not, in fact, models of essential HTN, but better reflect secondary HTN pathologies [107, 108]. 3) Even within models that more closely resemble human essential hypertension, studies across labs have yielded different effects of RDX on BP, making identification of physiological mechanisms difficult. The purpose of this study was to re-examine RDX in the SHR to determine if it could serve as a suitable model for exploring how CBRNA lowers BP in humans.

The SHR was chosen for study because this strain of rat is often considered a model of human essential HTN in that all rats develop high BP over the course of their lifespans without addition of external stimuli such as salt or a pressor hormone i.e. the HTN is hereditable and “primary” [108]. Furthermore, this rat strain develops many of same end-organ complications associated with human HTN [107, 108] i.e. cardiac hypertrophy, congestive heart failure and renal dysfunction are commonly seen in this model.

With regard to the effectiveness of RDX in lowering BP in the SHR, published results are inconsistent. As previously reviewed, RDX performed in SHR 5-8wks of age is reported to delay, but not prevent the development of hypertension (HTN) [88]. In SHR aged 12-18wks, most investigators report RDX has no persistent effect on BP or renal function [85, 86, 88, 109]. However, one group claims that RDX at this age produces a prolonged reduction in BP [89]. Additionally, there are other reports demonstrating an anti-hypertensive effect of RDX in 6-month-old SHR [91, 92]. These findings suggest SHR may respond to RDX only when they are older, or after a prolonged period of elevated BP. Therefore, I concluded that further evaluation of the adult SHR was necessary to determine if advancing age would predict a larger BP lowering effect of RDX.

In the studies described in this Chapter, RDX was performed in the SHR at 13wks and 36wks of age. I had three main objectives: 1) to compare the effect of RDX on BP in SHR at different ages; 2) to evaluate the magnitude and durability of the BP-lowering effect of RDX using continuous telemetric recording of BP; and 3) to determine if the

BP-lowering and other effects of RDX in SHR resembled those observed in humans subjected to CBRNA.

METHODS

Animals

At total of 38 male SHR were used in this study, which consisted of two separate experiments: 1) assessing the chronic effect of RDX on BP in younger vs. older adult SHR and 2) determining how effectively RDX denervated the kidneys without affecting sympathetic innervation to other abdominal organs. The BP effect of RDX was compared in 13wk SHR (N=15; Sham-operated (SO): n=7; RDX: n=8) and 36wk SHR (N=16; SO: n=8; RDX: n=8). An additional 7 SHR were used to evaluate the effect of RDX on tissue norepinephrine content (SO: n=3; RDX: n=4). In rats where BP was recorded, telemeters were implanted 1wk prior to beginning recording.

Experimental protocol

Initial baseline BP was recorded for 5-7 days prior to RDX. Following RDX, BP was measured in both age groups for at least 2wks. BP in the 36wk SHR was followed for 11wks after RDX. Blood was collected from the tail vein at baseline and 1wk after RDX. Kidneys and spleen were collected at the end of the BP studies for analysis of tissue catecholamine content, an index of sympathetic innervation density. Kidney, spleen, duodenum, and liver samples were collected at 2wks post-RDX from an additional 7 SHR to analyze the effectiveness and specificity of RDX in denervating the kidney. This 2wk time point was chosen as others have reported RDX maximally depletes renal nerve markers within this time frame [69].

RESULTS

The hemodynamic response to RDX in 36wk SHR is shown in Figure 11. During the final day of the baseline period, before surgical intervention, average 24hr MAP (pre-SO: 156.6±3.0; pre-RDX: 157.3±2.0mmHg) and HR (pre-SO: 291.0±4.0; pre-RDX: 294.0±3.0bpm) were indistinguishable between groups. Statistical analysis of 24hr MAP recorded during the baseline period and the 2wk time period following RDX revealed a significant interaction between RDX and day of study ($p<0.05$), as well as a significant main effect of RDX ($p<0.05$). BP was maximally reduced in RDX-treated animals compared to sham-operation at 48 hours following surgery (SO: 158.4±3.1; RDX: 136.5±4.6mmHg). MAP in RDX-treated SHR gradually increased over the following 3 days, but even after MAP stabilization, the RDX group maintained a significantly lower BP compared to sham-operation ($p<0.05$). Statistical analysis of HR over the same interval also revealed a significant interaction term ($p<0.05$) but not a significant main effect for RDX treatment. The increase in HR (SO: 281±4; RDX: 308±10bpm) peaked in the RDX group 48hrs after treatment when BP was at a nadir. No difference in HR between groups was seen from 5 days after surgery onward.

RDX had a lesser effect on hemodynamics in the 13wk SHR (Figure 12). Immediately prior to RDX, average 24hr BPs (SO: 143.9±3.4; RDX: 139.3±4.1mmHg) were similar between groups. However, at baseline, HR was significantly lower in rats that were to receive RDX (SO: 322±3; RDX: 307±4bpm, $p<0.05$). Within 48hrs of RDX, MAP fell significantly in RDX-treated SHR (SO: 147.6±2.6; RDX: 132.3±2.4mmHg). However, the reduction in MAP did not persist, recovering to values similar to the control period by 3 days after RDX.

Comparison of the change in steady-state MAP from baseline to 2wks post-RDX between the two age groups is shown in Figure 13. RDX in 13wk SHR did not produce a reduction in steady-state 24hr MAP after intervention when compared to sham-operation ($p>0.05$). However, RDX in 36wk SHR significantly reduced steady-state MAP compared with control rats (SO: +2.5±0.8 vs. RDX: -5.2±2.0mmHg; $p<0.05$).

Telemetric monitoring of BP occurred for an additional 11wks after RDX in the 36wk SHR. The MAP observed during the final 5 days of the study is shown in Figure 14. At this time, MAP was still significantly reduced compared to sham-operation ($p<0.05$). Evaluation of the reduction in steady-state MAP from baseline to the end of the study demonstrates that MAP was significantly reduced in RDX-treated rats compared to sham-operated rats (SO: -1.6±1.8 vs. RDX: -9.4±1.4mmHg; $p<0.05$).

To test the effectiveness and specificity of RDX, NE was measured in the left kidney, right kidney, duodenum, and spleen 2wks after RDX in a separate group of 36wk SHR (Figure 13). RDX significantly lowered NE content in both the left (SO: 165.5±10.2 vs. RDX: 3.8±2.2 ng/g; $p<0.05$) and right kidneys (SO: 156.9±33.2 vs. RDX: 33.1±29.9ng/g; $p<0.05$). No other tissues showed a significant difference in NE content between groups ($p>0.05$).

At the end of the BP study in the 36wk SHR, tissue NE content was analyzed in the left kidney (LK), right kidney (RK), and spleen (Figure 16). Eleven weeks after RDX, NE content was significantly reduced in both the LK (SO: 127.9±5.9 vs. RDX: 53.5±12.3ng/g; $p<0.05$) and RK (SO: 131.1±5.1 vs. RDX: 78.3±11.2ng/g; $p<0.05$). Splenic NE content was not different between the groups ($p>0.05$).

For comparative purposes, the change in average 24hr BP 30 days after CBRNA in humans and at various time periods after RDX in adult SHR are shown in Table 2 and Figure 17. Since clinical studies focus on changes in SBP and DBP in the human patients and not MAP, the same parameters are given in Table 2, although SEM was not reported in the clinical studies. In the SYMPLICITY HTN trials, the reduction in SBP was 11mmHg. In a recent study examining the change in 24hr ambulatory BP in 22 patients receiving CBRNA, the drop in SBP was 9mmHg [110]. In the 36wk SHR, SBP dropped 7.0±2.6mmHg within two weeks following surgery and was 10.2±1.8mmHg below baseline 11wks after intervention. Similarly, DBP fell 3.7±1.5mmHg at 2wks after RDX and was reduced 7.0±1.3mmHg 11wks after RDX. The 13wk SHR showed a 2.4±1.1mmHg increase in SBP within 2wks post-operation, and DBP changed 0.1±0.7mmHg. Diastolic pressure was reduced 7mmHg in Symplicity HTN 2 and 6mmHg in Volz et al. We report a reduction in the 36wk SHR of 4mmHg within 2wks of RDX and a 7mmHg reduction 11wks later. There was no effect on diastolic pressure in the 13wk SHR.

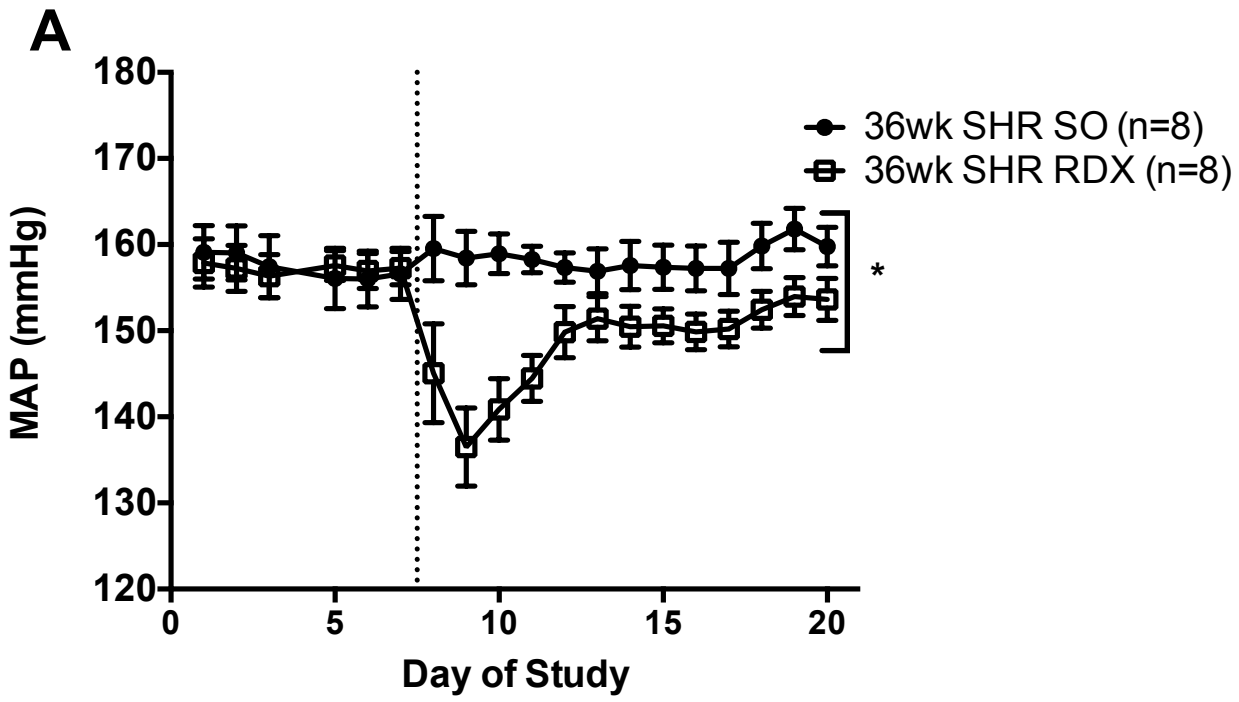
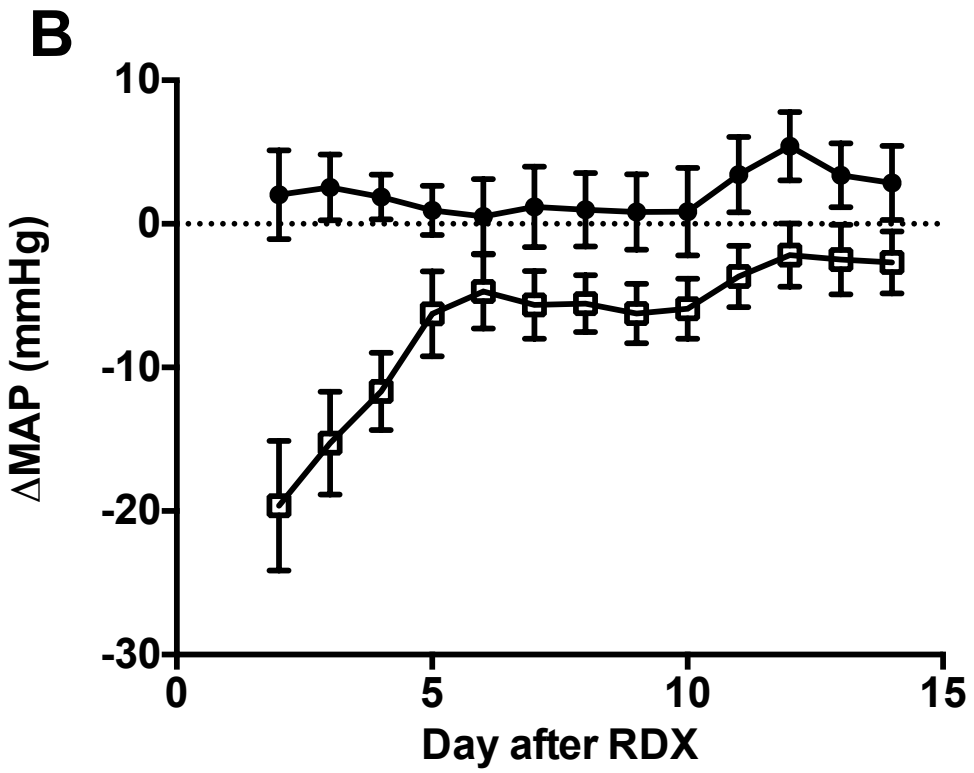


FIGURE 11. <u>The effect of RDX on hemodynamics in 36wk SHR.</u> The effect of RDX on (A.) mean arterial pressure, (B.) change in mean arterial pressure from baseline, and (C.) heart rate. BP stabilized significantly lower in RDX-treated 36wk SHR. As MAP stabilized, HR was similar between groups. Vertical dashed line indicates day RDX was performed (* $p<0.05$).

Figure 9 (cont'd)



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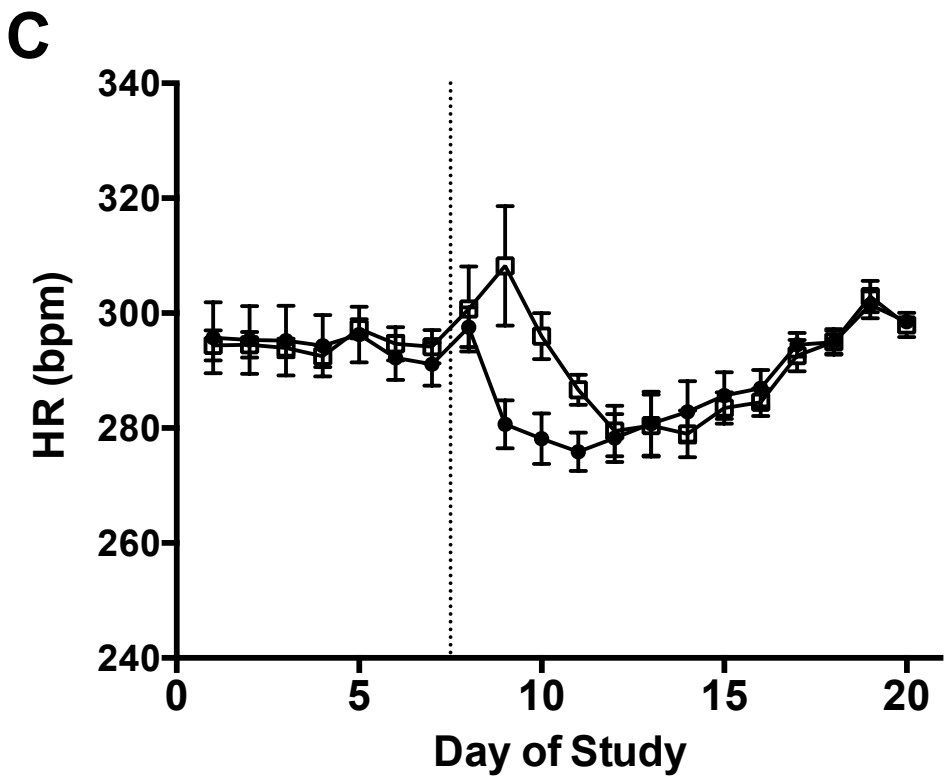


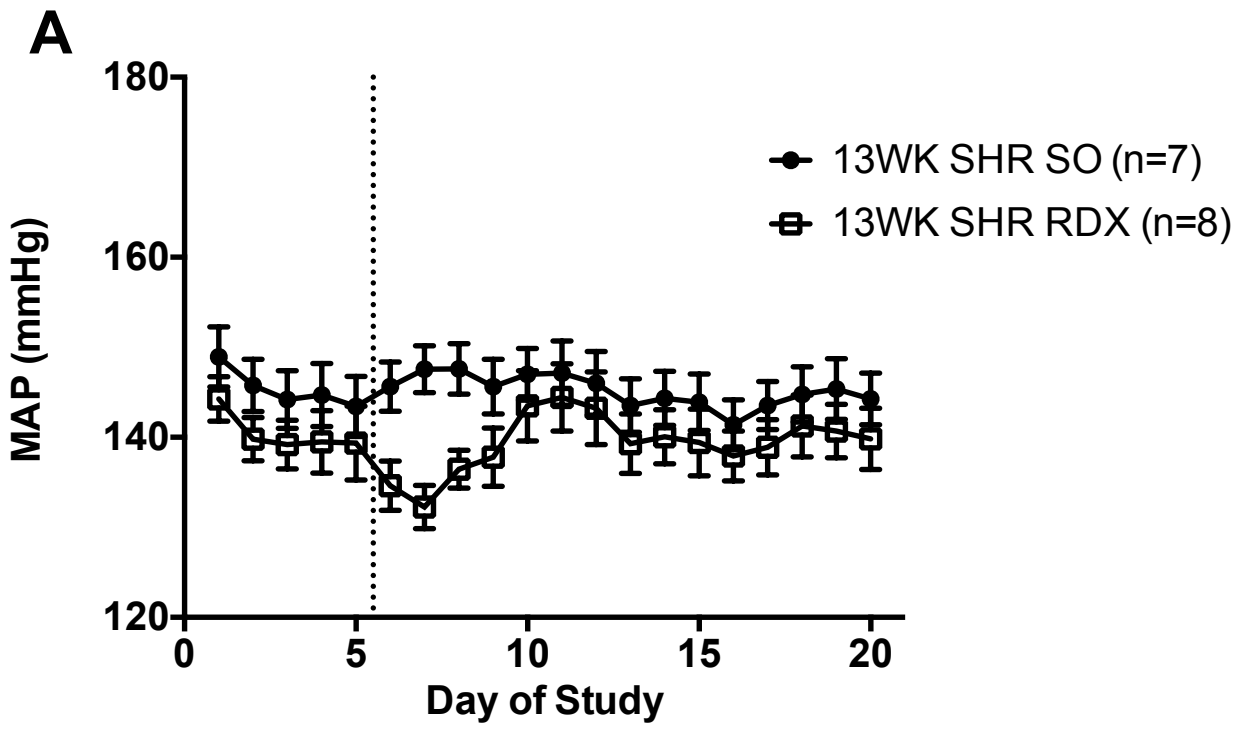
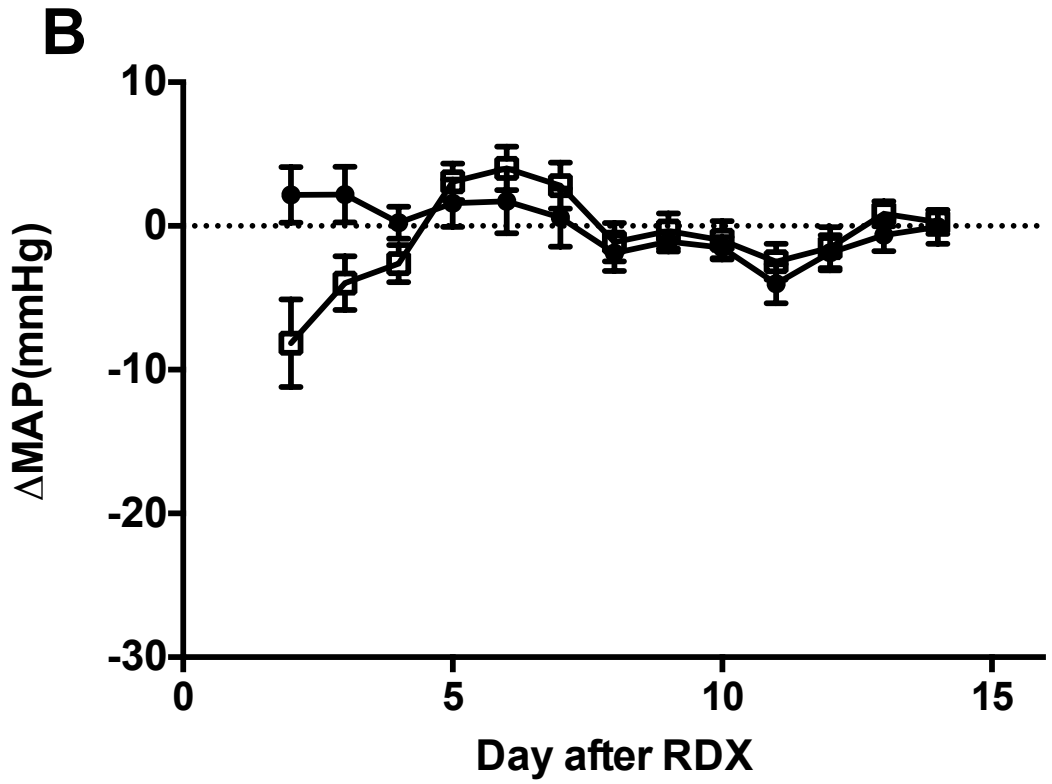


FIGURE 12. <u>The effect of RDX on hemodynamics in the 13wk SHR.</u> The effect of RDX on (A.) mean arterial pressure, (B.) change in mean arterial pressure from baseline, and (C.) heart rate. After BP stabilized, RDX did not chronically lower BP in the 13wk SHR. HR was significantly different between groups prior to surgical intervention. After MAP stabilized, HR was also not altered by RDX. Vertical dashed line indicates day RDX was performed (* $p<0.05$).

Figure 12 (cont'd)



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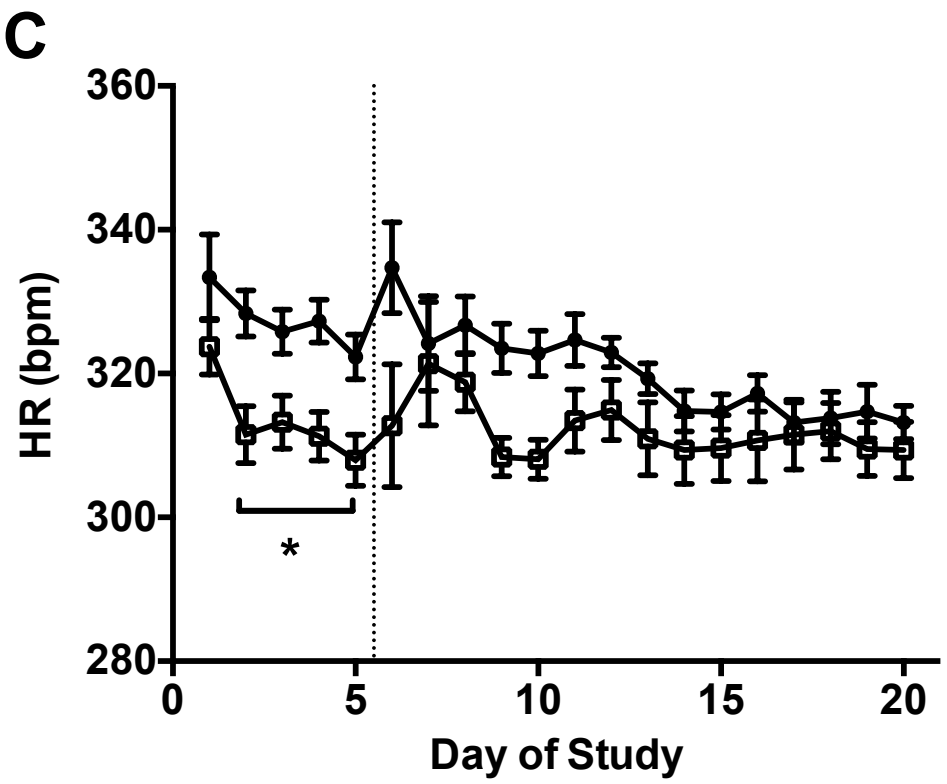


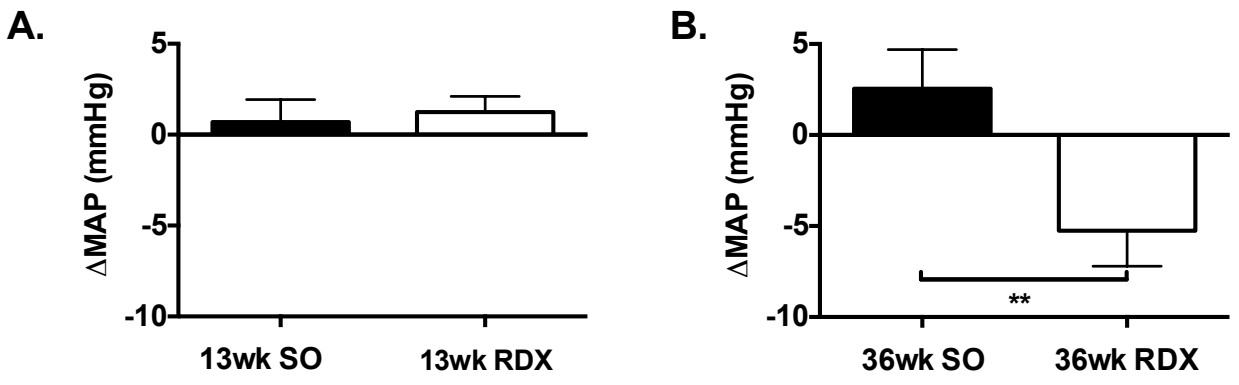


FIGURE 13. <u>Comparison of the change in steady-state MAP response 2wks after RDX.</u> The change in steady-state MAP is shown for (A.) 13wk and (B.) 36wk SHR. There was no reduction in MAP in 13wk SHR. However, MAP was significantly reduced by RDX in 36wk SHR compared to sham-operated rats (**: $p < 0.01$).

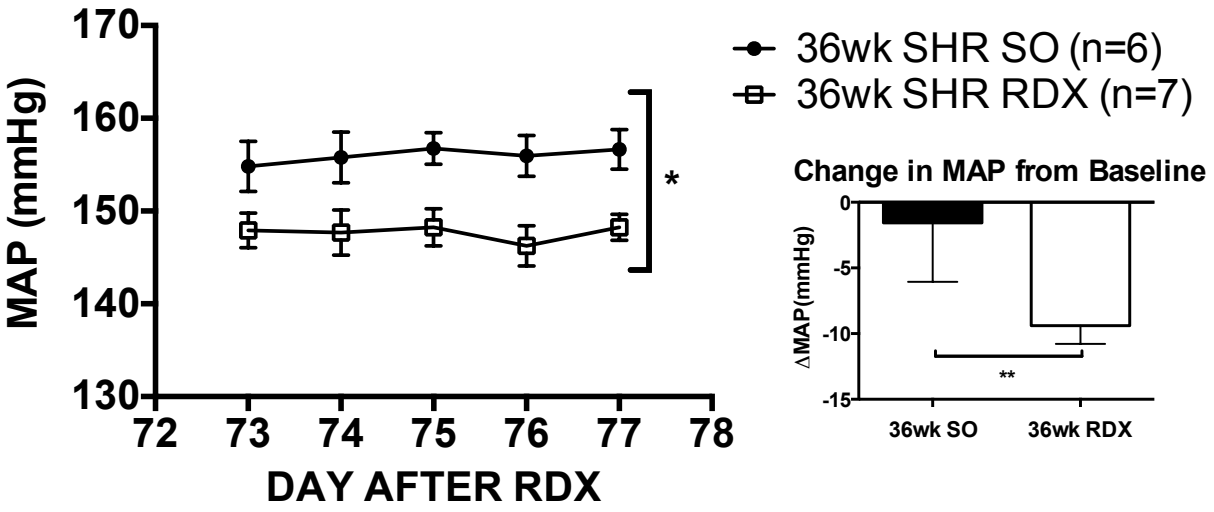


FIGURE 14. **<u>The durability of BP response to RDX in 36wk SHR</u>**. MAP remained reduced in RDX-treated SHR 11wks after intervention. *Inset*: The change in steady-state MAP from baseline to the end of the study shows RDX has a significantly greater fall in BP compared to sham operation (*p<0.05; **p<0.01).

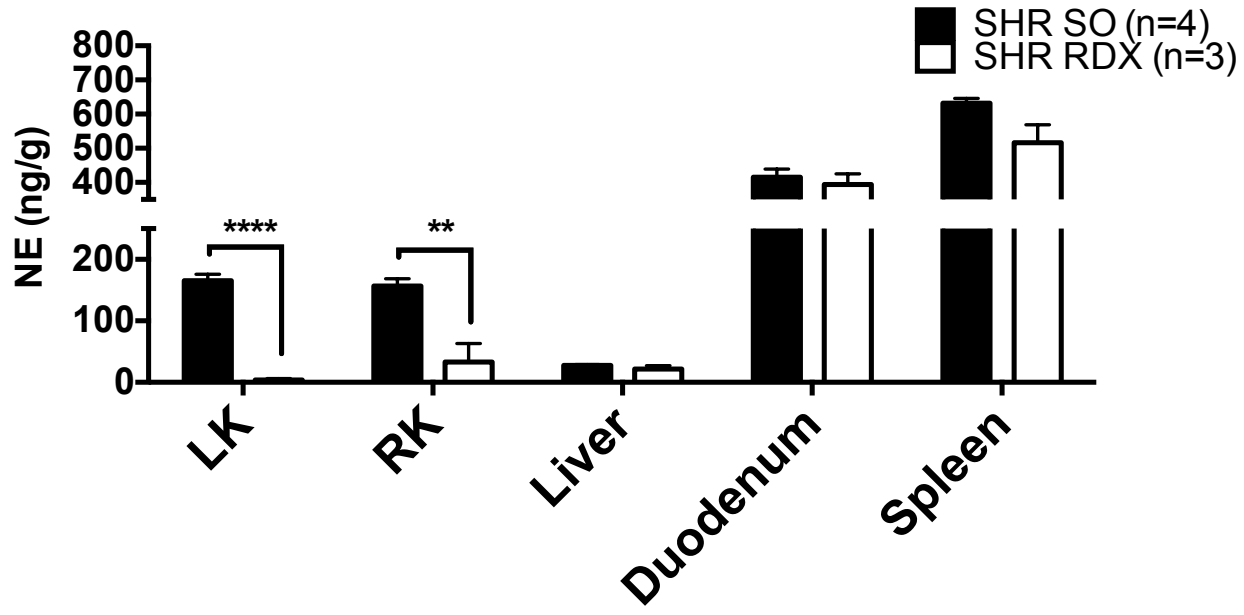


FIGURE 15. <u>Tissue NE content measured 2wks after RDX in a separate group of 36wk SHR rats.</u> Left kidney (LK) and right kidney (RK) NE content were significantly reduced 2wks after RDX. NE content in adjacent tissues was not affected, indicating the specificity of RDX (**p<0.01; ****p<0.0001).

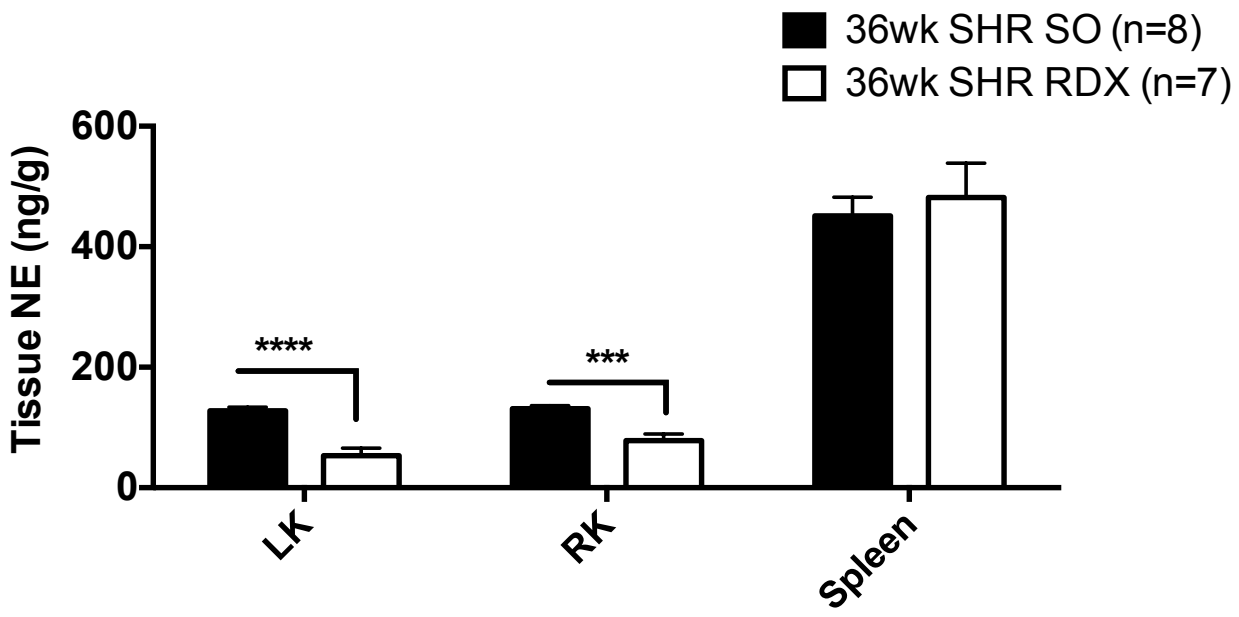


FIGURE 16. <u>Tissue NE content in 36wk SHR measured 11wks after RDX</u>. Left kidney (LK) and right kidney (RK) NE content were significantly reduced compared to sham-operation (***p<0.001; ****p<0.0001). Splenic NE content was not significantly altered, again emphasizing the specificity of denervation.

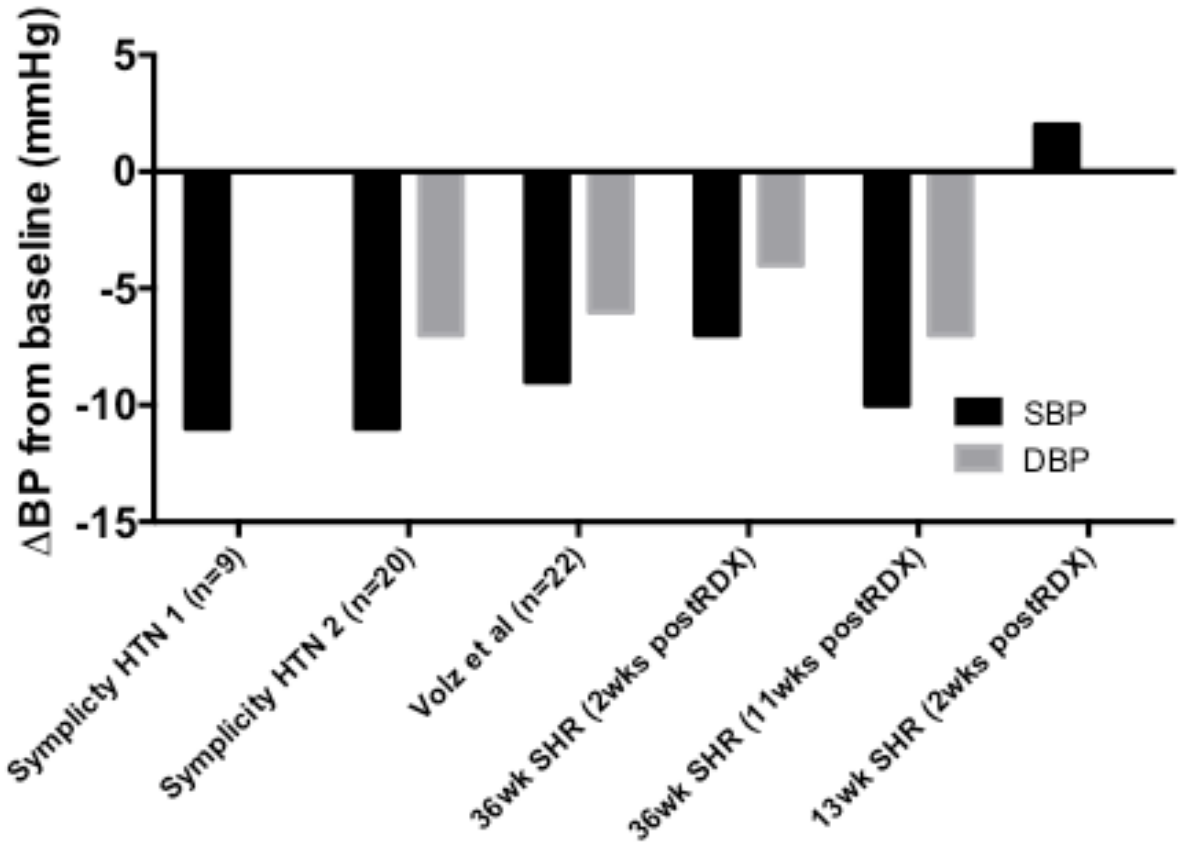


FIGURE 17. **Comparison of the change in ambulatory BP in patients treated with CBRNA and adult SHR treated with RDX**. Reductions in ambulatory BP in the 36wk SHR are of similar magnitude of those values reported in humans

Study	ΔSBP (mmHg)	ΔDBP (mmHg)
Symplicity HTN 1 (n=9)	-11	n/a
Symplicity HTN 2 (n=20)	-11	-7
Volz et al. (n=22)	-9	-6
36wk SHR (2wks after RDX)	-7.0±2.6	-3.7±1.5
36wk SHR (11wks after RDX)	-10.2±1.8	-7.0±1.3
13wk SHR (2wks after RDX)	+2.4±1.1	0.1±0.7

TABLE 2. Comparison of the change in ambulatory BP in patients treated with CBRNA and adult SHR treated with RDX. Reductions in ambulatory BP in the 36wk SHR are of similar magnitude of those values reported in humans. Standard error of the mean was not reported in the clinical studies.

DISCUSSION

This study demonstrates that effective bilateral removal of the nerves supplying the kidneys will chronically lower BP in older (36wk) male SHR. These observations also support previous work showing that interruption of the renal nerve supply does not consistently reduce BP in younger adult SHR, i.e., the 13wk SHR. Furthermore, to my knowledge, this is the first study to show that the BP reduction caused by RDX in the SHR persists for at least eleven weeks after surgery. Most importantly, I suggest that the 36wk SHR should be considered as a candidate model in future mechanistic studies that examine how CBRNA lowers BP in humans, in part because the magnitude of BP response to renal denervation in the older SHR closely mirrors the responses observed in human patients (TABLE 2).

In my hands, RDX in the 13wk SHR did not cause a persistent decrease in BP. These results are similar to the findings of Winternitz et al. who found that RDX in 18wk old SHR produced a small, non-significant reduction of SBP that returned to pre-surgical levels within 10 days [88]. Although my SHR were somewhat younger, an initial significant reduction in BP after RDX was lost within 3 days. In agreement with Winternitz et al., we conclude RDX does not consistently produce a chronic reduction in BP in young adult SHR with established hypertension.

Gattone et al. [89] observed a significant reduction in BP occurring not until 11-14 days after RDX in young adult SHR (12wks of age). One potential explanation for the discrepancy with my data may lie in the different methods used to record BP. Gattone et al. utilized the tail-cuff method, whereas I employed telemetry. I would have anticipated

detecting their reported 24mmHg reduction in SBP with telemetric measurements. My data from 13wk SHR also do not align with a recent report from Hart et al. wherein bilateral renal denervation in 12wk old SHR produced a significant reduction in BP for at least 10 days [90]. In a preliminary study, I found that RDX in 12-13wk SHR significantly reduced BP compared to sham-operation up to 30 days post-operation. However, I was unsuccessful in repeating these findings in later experiments. Collectively these results suggest that the contribution of renal SNA to HTN in the young adult SHR is variable. Perhaps, this variability could be dependent on the commercial vendor that supplied the rats. In Gattone et al, rats were purchased from Harlan. Hart et al, which performed the experiments in Bristol, UK, does not report the supplier of the rats. I purchased my rats from Charles River. Drift within the separated rat colonies may be affecting the response to RDX. This hypothesis, however, has not been tested.

To my knowledge, there is only one group that has reported a BP-lowering effect of RDX in older adult SHR (24wks) [91, 92]. Lee et al. reported an average reduction of 25mmHg in renal-denervated SHR at least 5 days after the procedure [92]. From the same group using the 24wk SHR, Krueger et al. also reported a significant 20-27% reduction in MAP after RDX, although the exact magnitude was not specified. In that study the reduction in BP also lasted at least 5 days. In support of those data, I showed a significant reduction in BP after RDX in adult SHR whose ages range from 24-52wks (median: 36wks). In my study, I showed a 22mmHg reduction in MAP 48hrs after the procedure, whereas the anti-hypertensive effect 5 days after RDX was approximately 6 mmHg. The difference in outcomes could be attributed to the methodology used to record BP. My animals were continuously recorded throughout the study, while the

duration of the BP recordings by the other groups is unclear. Krueger and Lee do not report long-term recording of BP, which may have revealed a more modest effect of RDX. Regardless of the difference in the magnitude of the fall in BP following RDX, it is interesting to note that in all studies involving older adult SHR, RDX produced a significant reduction in MAP compared to sham-operated controls.

The overall goal of my studies was to evaluate the SHR as a potential model for understanding how CBNRA lowers BP in humans. Prior to my study, few investigators have sought to identify an animal model that responds to renal denervation in a manner similar to that seen in human patients [111]. Using SYMPLICITY HTN-2 as an example, there are several additional similarities between patients and the 36wk SHR. First, baseline BPs in both the SHR and human patients are quite elevated. Average baseline BP in humans that were chosen for renal denervation was recorded as 178/97mmHg [1]. Prior to RDX in the 36wk SHR, steady-state BP was approximately 186/129mmHg. Although BP was somewhat higher in SHR, particularly in terms of DBP, the high BP seen in human and rat carries significant risk for cardiovascular complications. It should be noted that while DBP is lower in the SYMPLICITY trials compared to SHR presented here, there is at least one report of renal denervation being successfully performed on a patient with similar DBP [74]. Therefore it is reasonable to conclude that the basal BP in the SHR is representative of BP one might expect in drug-resistant HTN. Second, it is interesting to note that in both the SHR and human, renal denervation caused a reduction in BP in older subjects. The average patient receiving renal nerve ablation in SYMPLICITY HTN-2 was 58 years old, or approximately 79% of their average life expectancy as calculated from recent WHO reports [112]. The lifespan of the untreated

SHR ranges between 10 and 21 months [107]. Thus, at 9 months of age the SHR is also well into its average life expectancy (42-90%). Moreover, 71% of the patients in SYMPLICITY HTN 2 had received anti-hypertensive therapy more than 5 years prior to intervention with renal nerve ablation, suggesting these patients had long-term exposure to poorly controlled high BP. SHR reach the full expression of HTN at 10wks of age [84]. Therefore by 36wks, SHR will have been exposed to high BP for a significant fraction of their lifespan. While it is not immediately clear what role aging *per se* versus chronic exposure to uncontrolled hypertension may play in the response to renal nerve ablation, the 36wk SHR appears to be a candidate model for exploring both variables. This could be investigated by controlling the HTN in SHR from an early age with anti-hypertensive therapies and then performing RDX once the rats reach 36wks. It would be expected that RDX would lower BP in these treated SHR if the response was truly attributable to an aging phenomenon. If RDX does not lower BP in these SHR, one might conclude that the RDX effect is linked to chronic exposure to high BP.

Finally, as noted earlier (Figure 15 and Table 2), the magnitude of BP reduction following RDX in the 36wk SHR is similar to what is seen in human patients after CBRNA. SYMPLICITY-HTN 1 reports office BP measured 6 months after the procedure fell an average of 22/11 mmHg [2]. However, 24hr ambulatory SBP in office responders only fell an average of 11mmHg, and changes in DBP were not reported. Similar results were recorded in SYMPLICITY HTN-2: office BP was reduced 32/12mmHg 6 months after intervention, while 24hr ambulatory pressure measured in a subset of 20 patients fell an average of 11/7mmHg [1]. I did not observe a chronic change in HR with RDX in the aged SHR. Sympliciity HTN 2 also did not report a significant change in HR with

CBRNA[1]. Acutely, however, I did observe a significant increase in HR following RDX. I interpreted this response to be mediated by a reduction in baroreflex stimluation as MAP was reduced almost 20mmHg within 24hrs after RDX. Interestingly, both CBRNA and RDX have been shown to improve baroreflex sensitivity in humans and rats, respectively [90]. Although I did not directly measure baroreflex sensitivity, it is possible that RDX could also alter baroreflex function in this model too. If baroreflex sensitivity were improved, one might expect a modest suppression of HR at lower BP. With this in mind, I would have expect HR to have been reduced at some time point after RDX even as MAP was reduced. While HR did fall in the RDX SHR compared to pre-surgical levels, the same response occurred in SO rats. It is currently unclear what role baroreflex modulation might have in the BP response to RDX in this model. I can only conclude that HR is acutely influenced by RDX in this aged SHR. Interestingly, in the 36wk SHR I studied, RDX lowered BP -7/-4mmHg within 2wks of RDX, and -10/-7mmHg by the end of the experiment. Although few investigations have examined the chronic effect of CBRNA on ambulatory BP, one investigation has reported that 24hr ambulatory BP also gradually falls in human patients [113]. I interpret this shared response between rat and human as further support for using this model to study CBRNA.

An issue of major importance in for the therapeutic use of CBRNA is the durability of the BP response. Factors that could influence the duration of response to the procedure include reinnervation of the kidneys after denervation, prior anti-hypertensive drug treatment, or the degree of initial renal denervation. In most clinical trials the fall in BP persists at least 6 months. Follow-up of SYMPLICITY HTN-1 patients shows the effect

on office BP was sustained at least 24 months; however, investigators did not measure ambulatory pressure at this time [114]. The reduction in BP after RDX in the older SHR that I studied persisted at least 11wks. This was surprising in light of reports that the kidney should have functionally reinnervated within 14-24 days [94]. My measurements of reinnervation after 11wks based on kidney NE content suggest that kidneys of the SHR are partially reinnervated, but not to sham-operated levels; however, I do not have any assessment of functional reinnervation. When considering the expectation of functional sympathetic reinnervation of the kidneys in this model and the duration of the BP effect reported in my studies, these findings suggest that mechanisms not associated with efferent renal sympathetic nerves could be involved in the BP lowering response to RDX.

There are important limitations to this study. The renal denervation technique I used almost completely removes both afferent and efferent nerves supplying the kidneys. Clinical data, however, suggests CBRNA causes a less complete denervation: for example, renal sympathetic activity as measured by regional NE spillover only falls by about 50% 1 month after CBRNA [2]. Therefore, the model I used does not replicate that aspect of the clinical procedure. As search of the literature shows that the impact of partial versus full bilateral renal denervation on BP has not been explored extensively in animal models. Also, in this study I did not measure the degree of renal afferent denervation. It should be noted that Mulder et al report that the RDX procedure eliminates SP, a marker of renal afferent innervation, in addition to markers of renal efferent innervation [69]. Finding a method to document the extent of renal denervation

in a clinically practical way would be a major advance in the application of CBRNA to human patients.

It is also important to note that unlike human hypertensive patients receiving CBRNA, the aged SHR used in my study were not drug treated and it is unknown whether they would be resistant to drug therapy of their hypertension. It is unlikely however considering that the SHR is known to respond well to several anti-hypertensive therapies such as RAAS inhibitors, calcium channel blockers, and direct vasodilators [107]. Unfortunately, there are no established rat models of drug-resistant hypertension.

The BP response to RDX in my animals was not evaluated beyond 11 weeks. Further evaluation of the BP effect of RDX is warranted primarily due to the fact that the office-BP response in humans has been documented up to 3yrs [115]. Technical limitations such as telemeter battery life could make very long-term follow up studies impractical. In clinical studies, investigators document changes in both office BP and ambulatory BP at discrete pre-determined time points. In the rat, there is no method for measuring office BP. However, using telemetric recording I can continuously and chronically measure BP before and after RDX. This approach is superior to the human studies, where ambulatory measurements are generally taken on a single day either 30 or 180 days after CBRNA [1, 106]. Currently, the clinical record does not reveal whether BP is affected immediately following the procedure or how the fall in BP might progress over the next thirty days. This pattern of BP reduction could provide an indication of which mechanisms are at play. Therefore, documenting the BP continuously over the hours days, weeks, and months using telemetry in the aged SHR may provide much greater

insight into the mechanisms that support the reduction in BP associated with CBRNA than what is available from clinical studies.

The most critical limitation to this study is that rats are not humans. While BP in the aged SHR certainly responds to RDX in a way similar to what is observed clinically, there are many important differences between free-living human patients and laboratory rats. For example, the rats' environment is tightly controlled while the human experience is highly diverse. Therefore, extrapolating these conclusions to the human condition should be done cautiously.

In conclusion, here I provide evidence that the older adult SHR is a credible model for further exploring the mechanisms responsible for the long-term reduction in BP following bilateral renal denervation in human patients with drug-resistant hypertension. This information could provide insight into factors affecting the magnitude and duration of BP response to CBRNA, such as drug treatment, patient age, ethnicity, sex, salt intake, basal hemodynamics and renal function, and even existing co-morbidities. In addition, this experimental model could help reveal other beneficial or untoward physiological effects that might accompany renal denervation in the clinical setting.

CHAPTER 4 – Chronic administration of the centrally acting sympatholytic drug clonidine prevents the expected fall in BP associated with bilateral renal denervation in the spontaneously hypertensive rat

The exact mechanism(s) responsible for the reduction in BP after renal denervation is(are) not known for humans or animals, but for ethical reasons it is possible to do far more detailed investigations of those mechanisms in experimental animals. In the previous chapter, I proposed that RDX in the aged adult SHR is a credible (though not perfect) animal model for understanding how catheter-based renal nerve ablation CBRNA lowers BP in humans. Therefore, in the studies described in this Chapter, I used the aged SHR as a model to investigate the hypothesis that the mechanism of the BP-lowering effect of RDX is suppression of sympathetic activity.

As I reviewed earlier, CBRNA damages both renal sensory afferent nerve fibers and efferent sympathetic nerve fibers in humans. Since renal afferent nerve traffic modulates non-renal SNA, CBRNA could decrease BP by modulating physiological effects of mainly renal SNA, mainly NRSNA or of both [7, 11]. Specifically how might that occur?

Elevation of renal SNA could theoretically cause hypertension by increasing renin secretion, the tubular reabsorption of sodium, and/or renal vasoconstriction. In patients undergoing CBRNA, sympathetic activity within the kidney has been documented to fall after the procedure [2]. This suggests that intra-renal sympatholysis could lower BP by inhibiting renin secretion, causing natriuresis, decreasing renal vascular resistance, or some combination of all three effects.

Renal afferent nerves project to cardioregulatory nuclei in the CNS and mediate sympathoexcitatory responses. Loss of this sympathoexcitatory drive after CRBNA could reduce NRSNA and BP [21, 116]. The potential importance of reductions in NRSNA due to loss of renal afferent nerve activity after CBRNA is strongly supported by the numerous non-renal outcomes associated with CBRNA. These outcomes, such as reduced cardiac hypertrophy, decreased MSNA, and improvement in insulin resistance, are known to be influenced by NRSNA [3, 117, 118],

Although it seems obvious that impaired sympathetic effects on the cardiovascular system would at least partially explain the BP response to renal denervation, there has been surprisingly little detailed investigation of that idea. Therefore here I set out to evaluate whether sympatholysis is indeed the dominant mechanism involved; and to determine which renal nerve axis is mainly responsible for the reduction in BP.

In study 1, I tested the general hypothesis that the BP-lowering effect of renal denervation requires a reduction in SNA by performing RDX during pharmacological suppression of sympathetic activity with the centrally acting sympatholytic drug, clonidine [98]. I predicted that clonidine-induced SNA suppression would prevent the BP response to RDX, but the effect would return once clonidine was discontinued. I also measured plasma NE as an index of overall sympathetic activity (RSNA and NRSNA) to further explore the relationship between BP and sympathetic activity after RDX.

In study 2, I tested the hypothesis that the sympatholytic mechanism explored in study 1 required renal afferent nerve signaling. To this end I used a newly developed technique for selectively damaging the renal afferent nerves while sparing the renal sympathetic

efferents: perivascular application of the neurotoxin capsaicin[119]. I predicted that selective renal deafferentation would lead to a similar reduction in BP (and SNA) as observed after RDX, thus proving that afferent renal nerve signaling plays a critical part in the antihypertensive response to renal denervation.

METHODS

Animals

This study used a total of 23 male SHR (median age 36wks; range 24-52wks). Rats were divided into 2 studies: 1) evaluation of RDX during clonidine treatment (N=14); and 2) evaluation of the contribution of renal afferent nerves to the BP-lowering effect of RDX (N=9). Rats were fitted with telemetry transmitters at least 1wk prior to recording baseline hemodynamics.

Experimental protocol

Study 1

Baseline BP was recorded for 4 days. Rats were then allocated to the sham-operation (SO; n=7) or RDX (n=7) group in a way that ensured equivalent MAPs between groups. Rats then received a subcutaneous mini-osmotic pump containing clonidine (125μg/kg/day), and BP was continuously monitored for 8 days. Justification for this dose is described in Chapter 2. Rats then underwent the RDX or sham procedure. After 9 days of post-operative BP monitoring, clonidine pumps were removed. BP was recorded over the next 12 days after clonidine treatment was stopped. Blood was drawn from the tail vein at the end of each treatment interval to evaluate plasma NE content.

At the end of the study, tissue NE was analyzed in the LK, RK, and spleen to confirm the effectiveness and selectivity of RDX.

Study 2

Rats were allocated to either sham-operation (SO; n=4) or renal deafferentation (ARDX; n=5) groups. Baseline BP was monitored for 4 days. ARDX or sham operations were performed (as described in Chapter 2) at the end of the baseline period. Post-operative hemodynamic variables were continuously monitored for the next 13 days. At the end of the study, sympathetic innervation density was assessed by measuring tissue NE in the LK, RK, and spleen. Verification of deafferentation of the kidneys was assessed by measuring CGRP in the left renal pelvis (LRP) and right renal pelvis (RRP) [69].

RESULTS

The effect of RDX on hemodynamics during chronic clonidine administration is shown in Figure 18. Rats administered clonidine had not yet been subjected to RDX or SO. They are referred to as “pre-SO” or “pre-RDX.” Immediately prior to implantation of the clonidine pumps, baseline average 24hr MAP (pre-SO: 161.3±2.3; pre-RDX: 160.8±2.1 mmHg; $p>0.05$) and HR (pre-SO: 302.0±3.0; pre-RDX: 308.0±6.0bpm; $p>0.05$) were not different between groups. Steady-state MAP dropped in both groups from baseline after implantation of the clonidine pumps (Figure 19), and the response between groups was not different (pre-SO: -15.34±0.7 vs. pre-RDX: -15.34±1.1mmHg; $p>0.05$). Plasma NE concentration also dropped significantly in both groups following clonidine administration (Baseline: pre-SO: 255.8±26, pre-RDX: 240.1±23.0 pg/mL; Clonidine: pre-SO: 182.9±15, pre-RDX: 163.7±30.3pg/mL; $p<0.05$).

RDX was performed 1wk after initiating clonidine treatment. Rats receiving sham operation are herein known as “SO” whereas RDX-treated rats are known as “RDX.” Statistical analysis of MAP after surgical intervention revealed a significant interaction term ($p<0.05$) and a significant main effect of RDX ($p<0.05$). In clonidine-treated rats RDX significantly lowered MAP within 48hrs compared to rats given sham operation (SO: ΔMAP: 0.4±1.8; RDX: ΔMAP: -11.0±1.3mmHg; $p<0.05$); however, this reduction in pressure was not sustained beyond 3 days after RDX. The change in steady-state MAP from the clonidine treatment period to the stabilization of MAP 1wk after RDX demonstrates no significant difference between SO and RDX responses (FIGURE 17. SO: -1.2±1.0 vs. RDX: -2.9±2mmHg; $p>0.05$). The HR response was identical between groups.

Figure 20 shows hemodynamic responses after clonidine treatment was discontinued on day 25 of the study. Within 48hrs, MAP peaked, but the change in MAP in RDX-treated rats was significantly less compared to sham-operated rats (SO: ΔMAP: 25.3±2.3; RDX: ΔMAP 18.0±2.3mmHg, $p<0.05$). In 7 days BP stabilized, and MAP was significantly lower over the next 6 day period in RDX-treated rats compared to SO ($p<0.05$). On the final day during this treatment interval, 12 days after cessation of clonidine treatment, MAP was 156.5±1.2mmHg in sham-operated rats and 148.4±2.1mmHg in RDX-treated rats. HR also increased after clonidine withdrawal; however, the response was the same in the two groups. Comparison of plasma NE concentrations during the clonidine treatment and RDX period (Table 3) to the time period after clonidine cessation reveals a significantly higher plasma NE content in both groups after the central sympatholytic was removed (Clonidine, +RDX: SO: 141.1±16.8,

RDX: 113.2±7.9pg/mL; RDX, no clonidine: SO: 199.8±13.5, RDX: 213.6±14.1pg/mL; $p<0.05$).

Figure 21 demonstrates the reduction in steady-state MAP associated with RDX as measured from baseline to the end of the study (after rats had recovered from clonidine treatment). SHR that received RDX showed a significantly greater reduction in steady-state MAP compared to sham-operated rats (SO: -3.8±1.0 vs. RDX: -12.8±1.8mmHg; $p<0.05$). Effects of clonidine alone, or RDX alone, on MAP within the RDX group reveals a similar magnitude of response to both interventions (clonidine: -15.3±1.2 vs. RDX: −12.8±1.8mmHg; $p>0.05$).

The contribution of capsaicin-sensitive renal afferent nerves to the hemodynamic effect of RDX in the 36wk SHR is shown in Figure 22. ARDX did not lower MAP within 2wks following the intervention compared to SO ($p>0.05$). Analysis of HR revealed a significant interaction term ($p<0.05$) but no main effect of RDX. HR was transiently elevated for 24hrs after ARDX compared to SO (SO: 318.0±4.0; RDX: 343.0±11.0bpm), but this effect on HR did not persist.

At the end of both studies, tissue neurotransmitter levels were measured. In study 1, tissue NE was measured. Figure 23 shows the tissue NE content as measured 7wks after RDX. NE content in the left (SO: 150.4±4.0 vs. RDX: 16.7±5.5 ng/g; $p<0.05$) and right kidney (SO: 128.5±3.3 vs. RDX: 42.8±9.0ng/g; $p<0.05$) was significantly reduced by RDX. Splenic NE was not altered ($p>0.05$).

In the study 2, tissue NE and CGRP were analyzed (Figure 24). Treatment with CAP significantly lowered CGRP content in both the right (SO: 215.3±38.8 vs. RDX: 7.7±1.3

pg/mg; $p<0.05$) and left (SO: 128.2±23.2 vs. RDX: 4.9±1.3pg/mg; $p<0.05$) renal pelvis. Tissue NE content was not altered in the kidneys or the spleen ($p>0.05$).

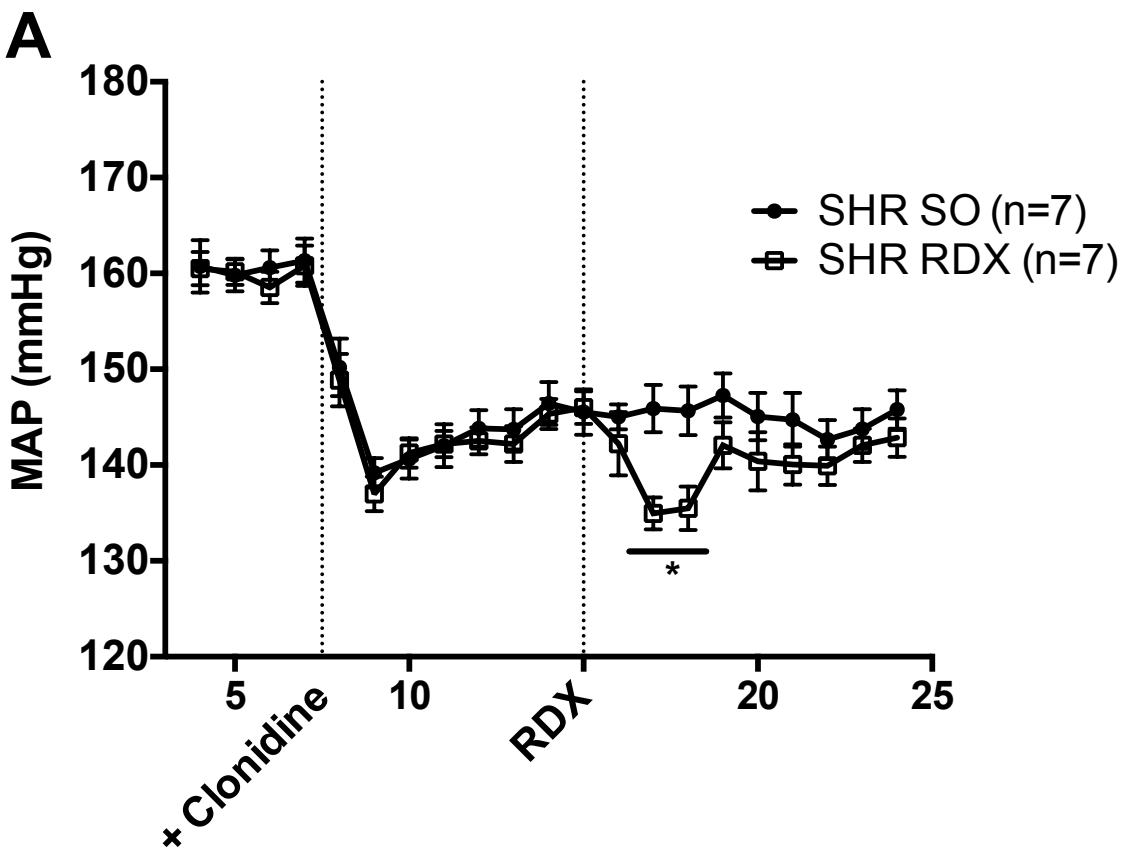
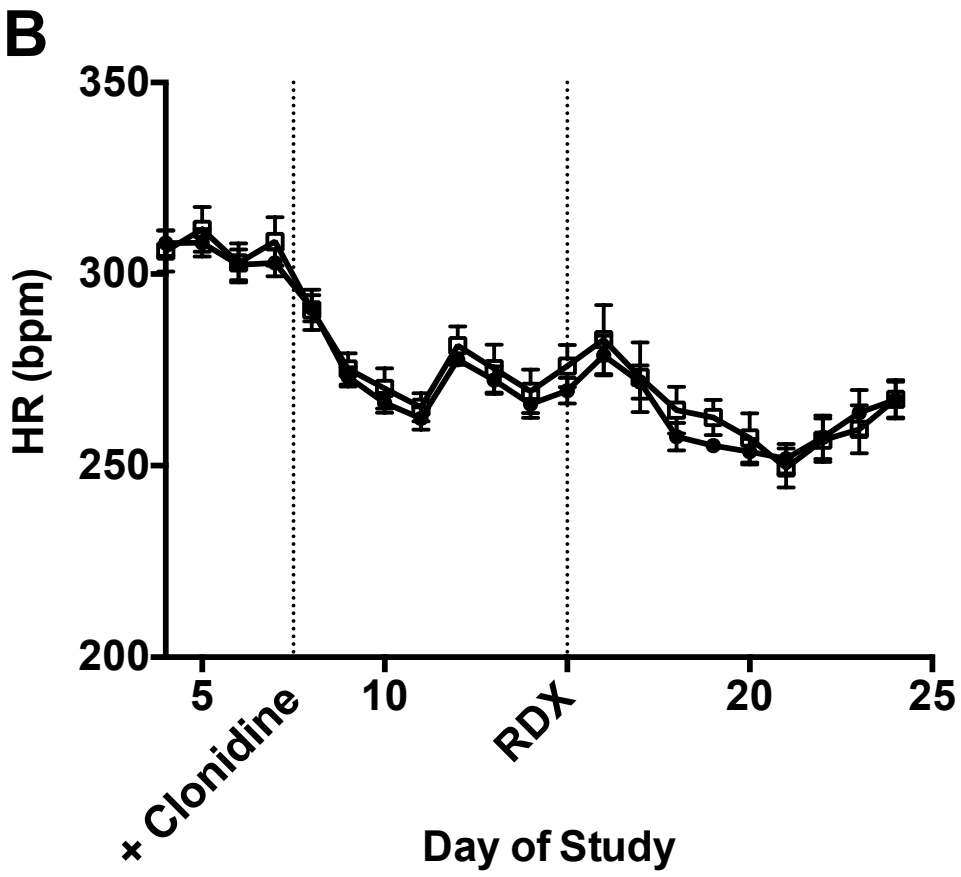


FIGURE 18. <u>The effect of RDX on hemodynamics during clonidine administration.</u>

The effect of RDX on average daily (A.) MAP and (B.) HR during chronic administration of clonidine. RDX during the clonidine treatment significantly lowered MAP for two days only (*$p<0.05$), however MAP did not remain significantly lower in RDX SHR compared to SO SHR. HR was suppressed by clonidine therapy, but the response was similar between groups.

Figure 18 (cont'd)



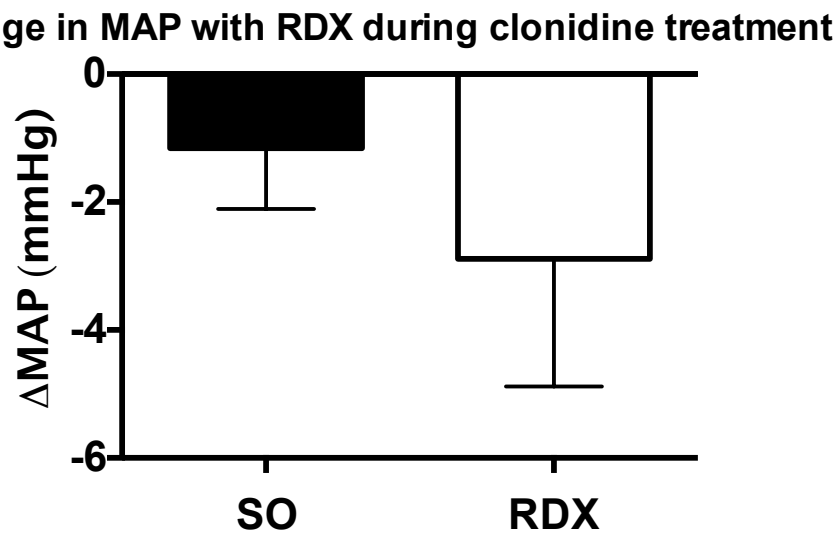


FIGURE 19. <u>The change in steady-state MAP following RDX during clonidine treatment.</u> There was no significant difference in BP response between the groups. RDX did not lower steady-state MAP during clonidine administration ($p>0.05$).

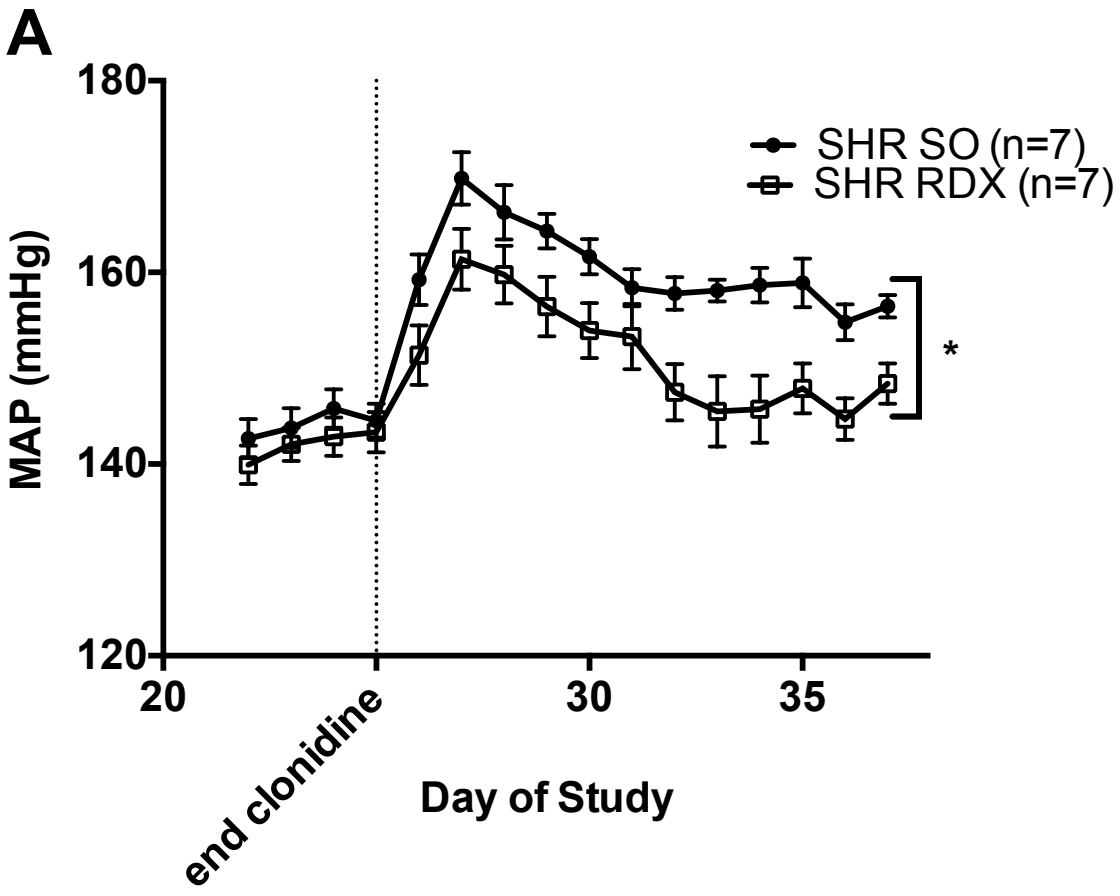
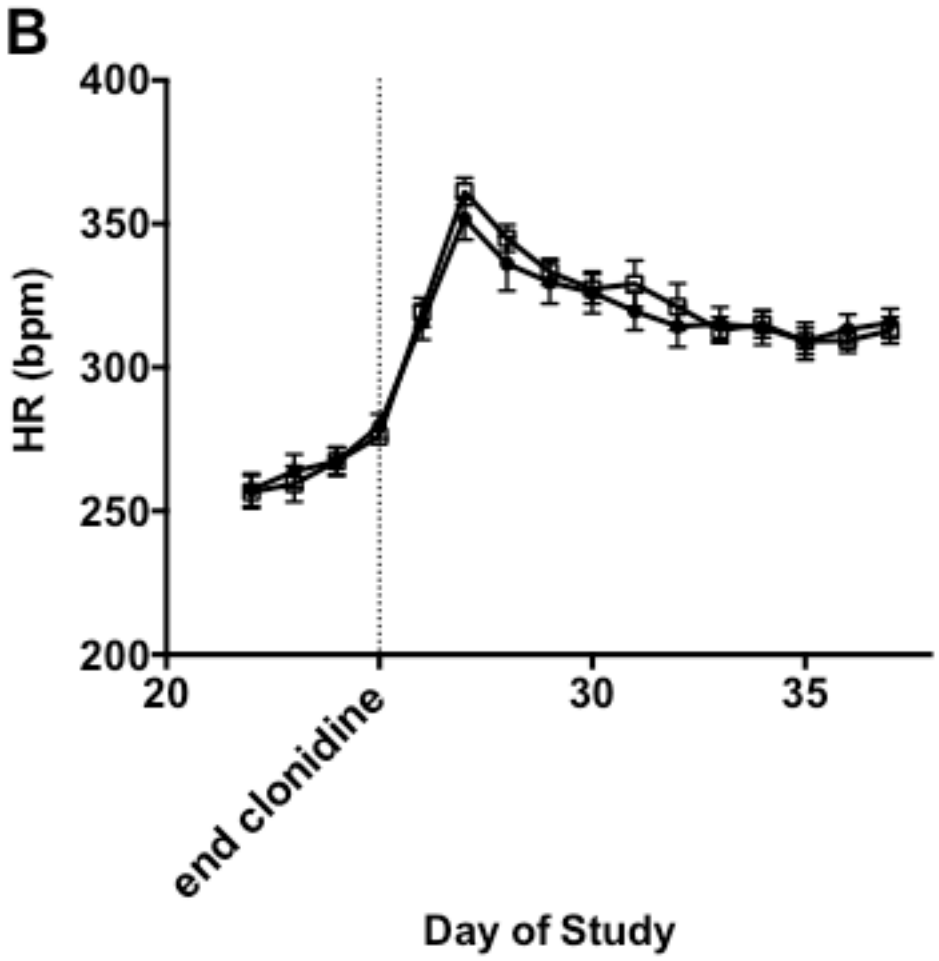


FIGURE 20. **Hemodynamic response after discontinuation of clonidine treatment in RDX and sham-operated SHR.** (A.) MAP increased in both groups after cessation of clonidine; however, RDX treatment significantly lowered MAP compared to sham. (B.) HR increased identically in both groups. (*$p<0.05$)

Figure 20 (cont'd)



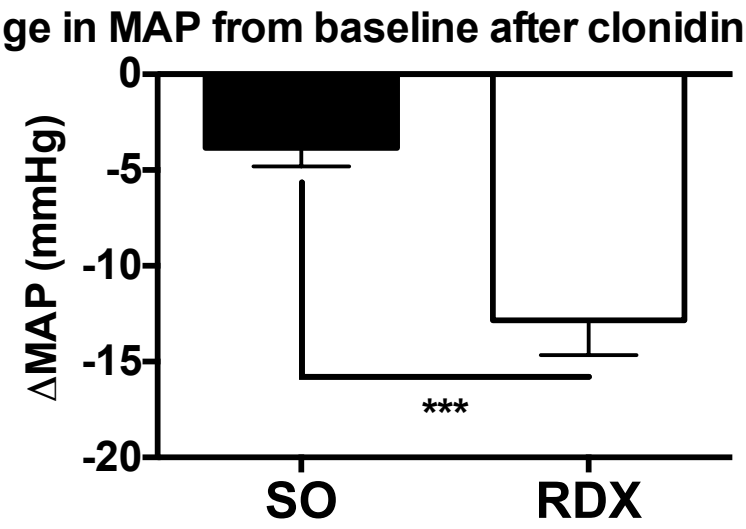


FIGURE 21. <u>The change in average steady-state 24hr MAP from baseline to the RDX treatment period after removal of clonidine</u>. SHR treated with RDX had a significantly greater reduction in MAP once clonidine was discontinued than those treated with sham-operation. ($p<0.001$)

	SO	RDX
	plasma NE concentration (pg/mL)	
Baseline (pre-surgery)	255.8±26.0	240±23.0
Clonidine (pre-surgery)	182.9±15.0****	163±30.0****
Clonidine (+), RDX (+)	141.1±16.8^{++++}	113.2±7.9^{++++}
Clonidine (-), RDX (+)	199.8±13.5****	213.6±14.1****

TABLE 3. Comparison of plasma NE concentrations in RDX or sham-operated rats before and after clonidine withdrawal. Clonidine significantly reduced plasma NE levels independent of surgical intervention. Discontinuation of clonidine administration significantly increased plasma NE concentration to near baseline levels independent of surgical intervention. There was no difference in plasma NE between the groups. ($^{++++}p<0.0001$ vs baseline; $^{****}p<0.0001$ vs clonidine(+), RDX(+)).

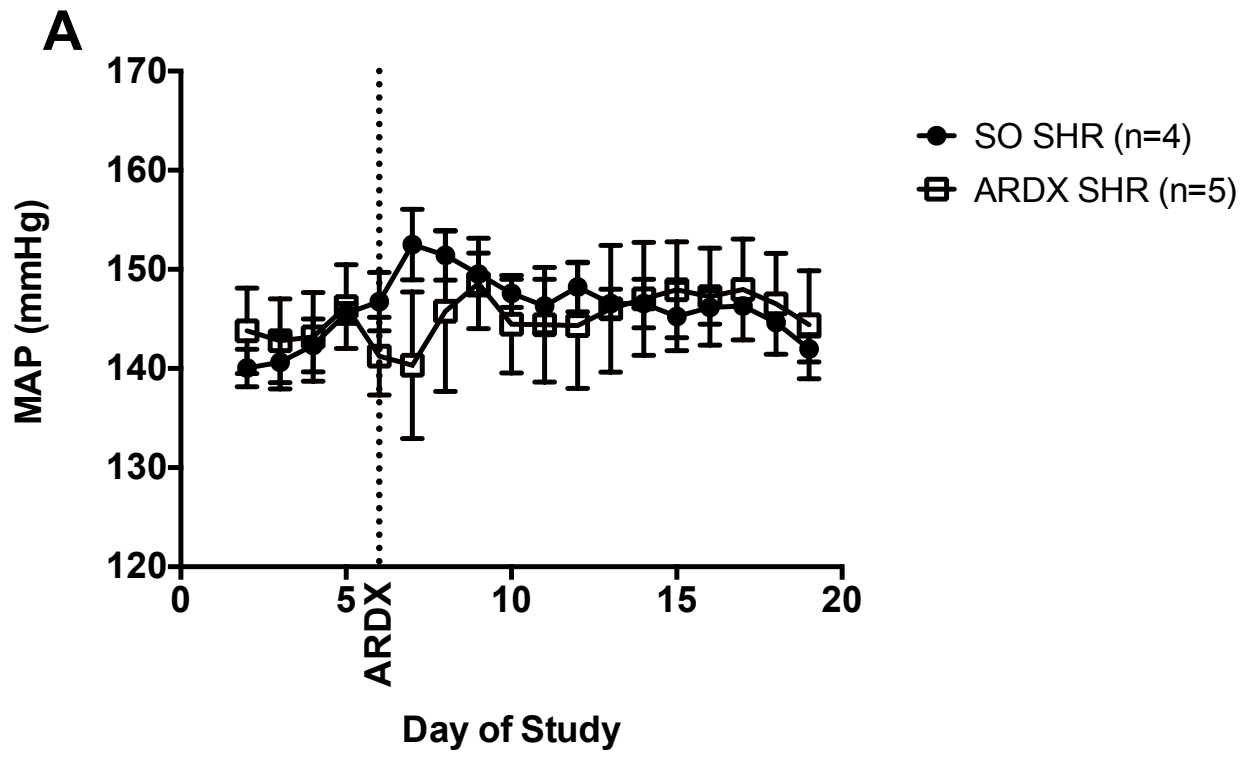
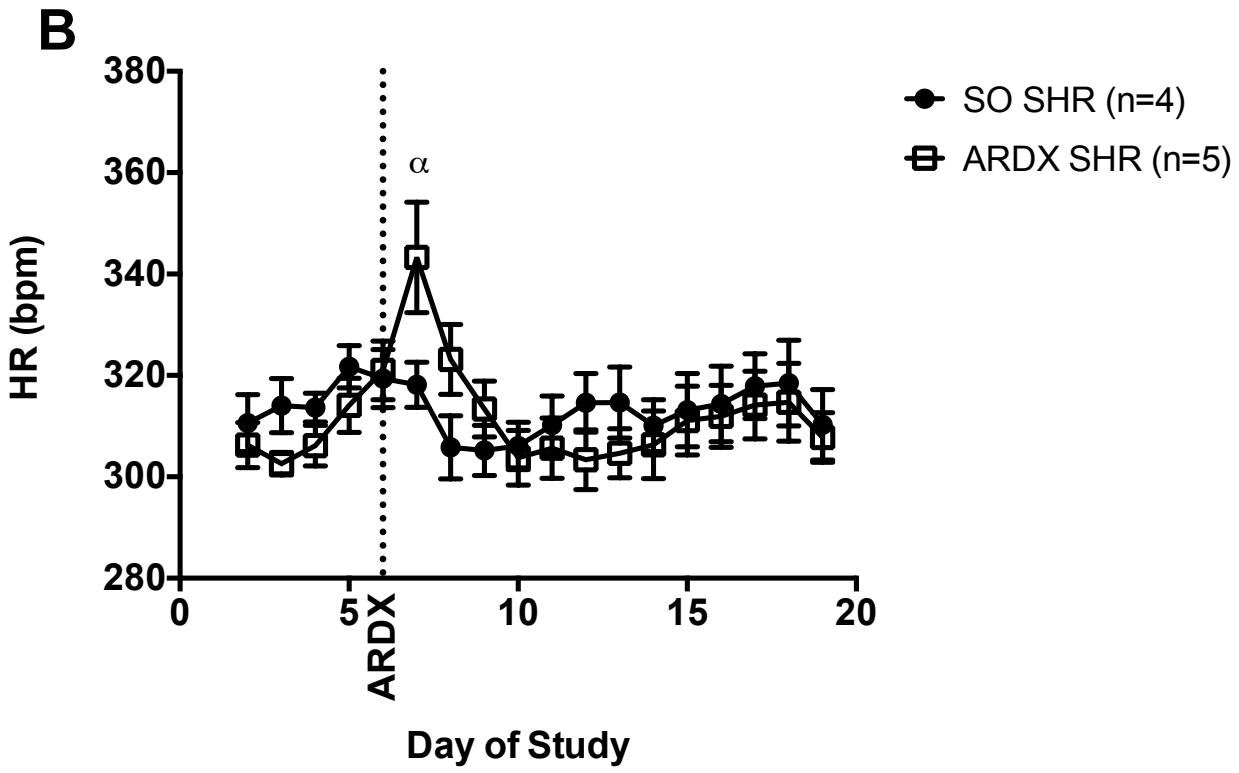


FIGURE 22. **The contribution of capsaicin-sensitive renal afferent nerves to the hemodynamic effect of RDX**. (A.) MAP was not reduced by capsaicin treatment. (B.) HR was elevated for 24hrs after capsaicin treatment, but returned to sham levels. (α, $p<0.001$ for interaction term)

Figure 22 (cont'd)



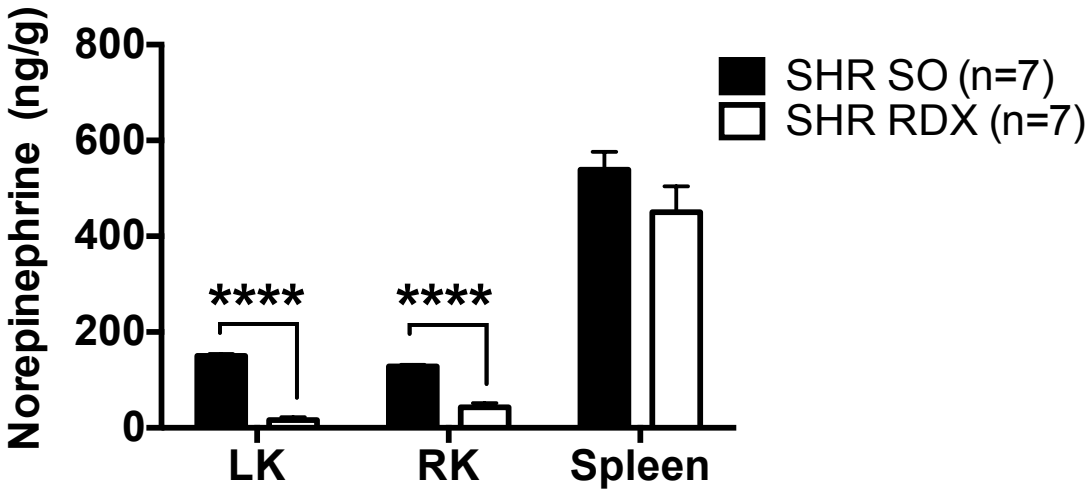


FIGURE 23. <u>Tissue NE analyzed 7 weeks after RDX. In</u> study 1, Left kidney (LK) and right kidney (RK) NE content were significantly reduced, while splenic NE was not altered. (****p<0.0001)

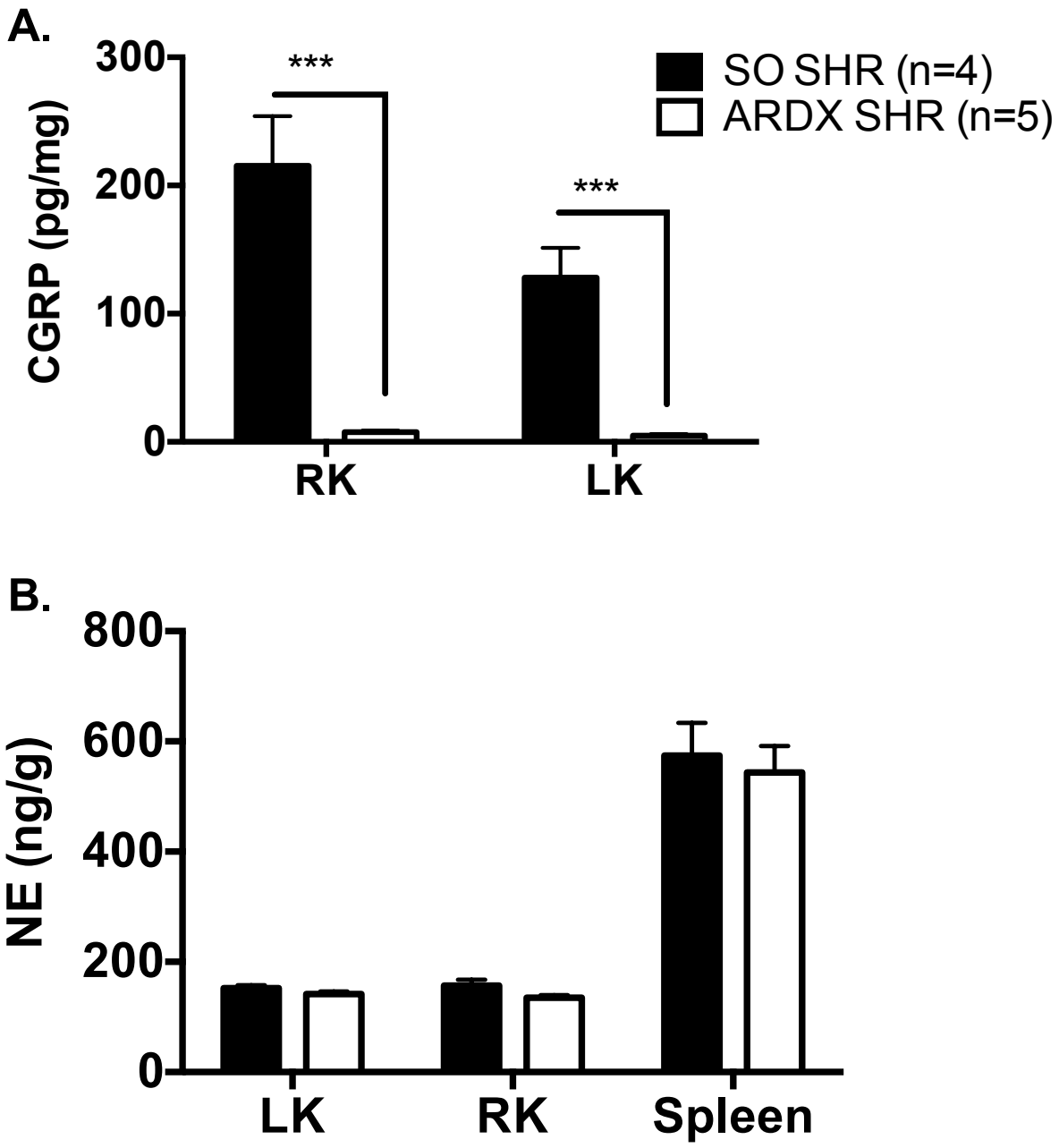


FIGURE 24. <u>Tissue neurotransmitter content after ARDX. (</u>A.) Renal CGRP content was significantly reduced by ARDX procedure; however, (B.) Tissue NE was not altered. (LK: left kidney; RK: right kidney; ***p<0.001)

DISCUSSION

The studies described in this chapter tested the hypothesis that the BP-lowering effect of RDX would be attenuated in a setting of low SNA induced by the centrally acting sympatholytic drug clonidine, or when possible afferent renal nerve mediated sympathoexcitatory effects were prevented. From the findings I conclude that: 1) the BP-lowering effect of RDX is not effective when SNA is low, as prior sympatholysis with clonidine eliminated the effect; and 2) that the BP-lowering effect of RDX is not due to loss of afferent signaling, since elimination of capsaicin-sensitive renal afferents while sparing sympathetic efferent nerves did not reduce BP.

RDX during clonidine-mediated suppression of sympathetic activity did not elicit the expected, sustained reduction in MAP. One might argue that this was due to incomplete RDX in this group of rats. However, after clonidine withdrawal, MAP in rats that previously received RDX was, as expected, significantly lower than MAP in sham-operated controls. This provides strong evidence that the RDX was complete; later measurements of kidney NE content support that conclusion. Therefore, I interpret this result to mean that the BP-lowering effect of RDX was attenuated due to the low SNA at the time of the denervation. Although clonidine's sympatholytic effects are well-accepted [120, 121], I attempted to confirm its efficacy in my study by measuring plasma NE as an index of "whole-body" SNA. Plasma NE fell significantly in SHR treated with clonidine and was elevated after termination of treatment. Heart rate, another rough index of SNA, also declined significantly during clonidine treatment. These finding suggest that I successfully reduced whole-body SNA with clonidine treatment. Therefore, it is

reasonable to conclude that clonidine prevented the expected BP response to RDX by reducing SNA. However, this does not tell us whether the critical SNA involved was renal, non-renal or a combination of both.

Interestingly, the magnitude of the fall in BP observed with RDX treatment was nearly identical to the reduction in BP found with clonidine alone. This similarity, and the failure of RDX to lower BP further during clonidine treatment, suggests that the BP reduction after RDX may be dependent *entirely* on eliminating sympathetic nerve activity. The tachycardia that was observed after RDX in untreated SHR (Chapter 3) did not occur in the presence of clonidine treatment even though MAP did fall immediately after RDX. This could be due to suppression of central sympathetic outflow, which decreases cardiac sympathetic nerve activity[122]. I did, however, observe a significant increase in HR within 24hrs after ARDX compared to SO rats. This suggests to me that the RDX procedure influences HR in this model through capsaicin-sensitive renal nerves. Since BP was not significantly reduced by ARDX, it is uncertain whether this tachycardia is related to a baroreflex mechanism. One could speculate that ARDX is acutely interfering with baroreflex control of BP, however, further evaluation of this hypothesis is necessary. Assuming baroreflex mechanisms are being modified by ARDX, these data suggest that the BP lowering effect of RDX is not solely mediated by alterations in the baroreflex.

One advantage of animal studies on renal denervation is the opportunity to measure hemodynamics variables continuously before and after the procedure. This information

can provide insight into the mechanisms responsible for BP changes, since as I reviewed earlier, the impact of these mechanisms are usually first observed within distinct time frames (e.g. neural reflexes in seconds to minutes, pressure-natriuresis over hours to days, and vascular remodeling over days to weeks). In this study RDX during clonidine treatment lowered MAP during the first three days following surgery. It is not clear what mechanisms explain this acute event. However, given the transient nature of the reduction in MAP, these mechanisms are less likely to play an important role in the chronic reduction in BP observed after RDX. No one has reported on the very short-term BP responses to CBRNA in human patients, so the clinical relevance of the findings also is unclear.

My findings here lend support to the suggestion by other investigators that the BP response to a short trial of clonidine could be used to predict which patients will have an anti-hypertensive response to renal nerve ablation [123]. Patients that respond to clonidine with a fall in BP should also respond to CBRNA. Although my studies were not designed to directly evaluate the positive predictive value of a clonidine regimen as outlined by Katholi et al., the present observations do suggest that clonidine and renal denervation are lowering BP by interrupting similar pathophysiological mechanisms. However, surprisingly, a previous retrospective analysis indicated that patients being treated with a central sympatholytic drug were actually *more likely* to show a favorable BP response to CBRNA [8]. This could indicate that mechanisms other than sympatholysis are important to the BP-lowering effect of CBRNA. Alternatively, since the subjects were all drug-resistant (i.e. did not show an adequate decrease in BP on drug therapy), it may show that patients with high SNA *despite* central sympatholytic

therapy are good subjects to receive CBRNA. Further clinical and experimental investigations are necessary to better understand how the BP response to a central sympatholytic could be used to predict the BP lowering effects of renal denervation.

To my knowledge, this is the first study to measure the hemodynamic response to selective renal deafferentation in the adult SHR. My data suggest that signaling through capsaicin-sensitive renal afferent nerves does not provide continuing support for the hypertension in older SHR. Although MAP was slightly reduced after ARDX, there was no persistent influence on BP such as I observed with total RDX in this model. One potential explanation for this finding is that capsaicin treatment failed to cause loss of CGRP-dependent renal afferent function. This is unlikely given the almost complete loss of CGRP I observed in the kidneys from capsaicin-treated rats. But functional assessment would provide a more reliable indicator of the status of renal afferent nerve signaling.

Another explanation for the negative outcome could be that the capsaicin-sensitive afferent nerves mainly serve a sympatho-inhibitory function, and that their activity is already suppressed in the SHR [124]. Interruption of a suppressed negative regulator of BP would be expected to mildly increase rather than reduce BP. Since I did not observe any increase in MAP following ARDX, it's possible that capsaicin treatment eliminated both sympathoexcitatory and sympathoinhibitory renal afferent effects with a resulting neutral effect on BP. Paton's group reports that in addition to BP, lumbar sympathetic nerve activity (LSNA) is reduced by RDX, suggesting that renal afferent nerves may be

capable of modulating non-renal sympathetic activity and BP [85]. One way to test this idea would be to examine the BP response to capsaicin treatment in normotensive rats. I would expect a mild increase in BP or SNA after loss of the renal afferent innervation if this subset of renal nerves was mainly serving a sympatho-inhibitory role.

Dorsal rhizotomy (a less selective approach to renal deafferentation) from T8-L1 significantly lowered MAP by 5% within 7-10 days after surgery in 16-18wk male SHR [93]. Given the non-selective nature of dorsal rhizotomy, it is possible other non-renal afferent nerve fibers could be responsible for the fall in BP.

Overall, the data presented in this study do not support the hypothesis that the BP reduction after RDX is linked to interruption of capsaicin-sensitive renal afferent nerves. While this approach virtually abolished CGRP-positive nerve fibers, it is known that a rat kidney contains sensory nerves that do not synthesize CGRP [14]. However, it is not known at this time whether these fibers are capsaicin-sensitive. Therefore, it is possible that a population of capsaicin-insensitive nerves could be mediating the modulation of BP and sympathetic activity in the SHR. A more sophisticated means of organ-specific deafferentation is needed to understand the relationship between renal afferent nerves, SNA and BP.

In summary, the BP effect of RDX in the aged adult SHR is related to elimination of sympathetic activity but it is not immediately clear whether this is due to reduction in RNSA alone, NRSNA or both. On the assumption that plasma NE in the intact rat reflects both RNSA and NRSNA, but after RDX reflects only NRSNA, a few tentative conclusions can be drawn. Since plasma NE values were equivalent between sham-

operated and RDX rats both at baseline (pre-RDX) and after RDX at the end of the study, this could mean that RSNA is very low in aged SHR. This would argue against the BP effect of RDX being due primarily to eliminating RSNA. Alternatively, NRSNA may have actually increased in RDX-treated rats, perhaps as a compensation for loss of RSNA. If so this would suggest that the BP lowering effect of RDX is attributable mainly to loss of RSNA. It should be noted, however, while that plasma NE is often used to approximate SNA, it is a crude marker and must be interpreted cautiously [52]. Only one group has directly measured NRSNA after renal denervation in SHR and they found lumbar SNA to be decreased [85]. On the whole then, existing data support the conclusion that RDX lowers BP by reducing SNA, but do not allow a secure conclusion about which components of the SNS are most critical. My data therefore support the hypothesis that evaluation of sympathetic activity prior to CBRNA could be used as a predictor for a positive antihypertensive response to CBRNA. Above all, evaluation of additional mechanisms associated with efferent renal nerve activity is needed to better explain the long-term BP lowering effect of renal denervation.

CHAPTER 5 – The BP lowering effect of renal denervation is not prevented by interruption of the renin-angiotensin system using the angiotensin II receptor blocker, losartan.

In the previous chapter, I showed that the BP lowering effect of RDX in the aged SHR was prevented by pre-treating the rats with clonidine. This demonstrated that the BP response to RDX is attributable to interruption of RSNA, NRSNA, or both. Given the virtual certainly of complete loss of RSNA with RDX, and the lack of clear evidence in my studies for a role of NRSNA in the BP response to RDX, it seemed reasonable to proceed on the assumption that the BP effect is simply due to loss of an intra-renal sympathetic signaling process.

As discussed in Chapter 1, RSNA is known to activate RAAS by promoting renin release through a β1-adrenergic receptor mechanism within the juxtaglomerular apparatus [11]. In fact, the initial case report describing the long-term response to CBRNA documented a reduction in RSNA and a halving of plasma renin activity (PRA) [3]. Previous investigation into RDX in SHR has shown RDX influences PRA and kidney renin content (KRA), although this finding is not always consistent [88, 91, 109]. Thus, these data suggest that one potential mechanism to explain the reduction in BP in the SHR may be a suppression of sympathetically mediated activation of the renin-angiotensin system RAAS. In this study I investigated that possibility in the aged SHR. I hypothesized that if the BP effect of RDX is attributable to interruption of RAAS activity, then prior pharmacological suppression of most RAAS biological activity with the angiotensin II AT_1 receptor blocker (ARB) losartan would prevent a reduction in BP after RDX.

METHODS

Animals and experimental protocols

Rats were fitted with a telemetry transmitter 1wk prior to recording baseline hemodynamics. SHR were allocated to either the sham-operation (SO; n=5) or RDX (n=8) groups so that basal MAP was equivalent between groups. Water consumption was measured during the baseline BP recording period to calculate losartan concentration necessary to deliver losartan in the drinking water at a dose of 10mg/kg/day [101, 125, 126]. Losartan treatment was initiated on day 9 of the study. Nine days after beginning losartan treatment, rats underwent RDX or SO. Losartan treatment continued for 11 days after RDX and then was discontinued. BP was continuously recorded to the end of the study. Blood was drawn from the tail vein at end of the baseline, losartan, and losartan+RDX periods. At the study's termination, the left kidney (LK), right kidney (RK), and spleen were harvested to analyze tissue NE content.

RESULTS

The response to RDX during losartan treatment is shown in Figure 25. Analysis of the baseline 24hr MAP and HR, when rats had not yet received surgical intervention, revealed no statistical difference between groups ($p>0.05$). Immediately prior to initiating losartan treatment, MAP (pre-SO: 159.5±6.0; pre-RDX: 158.4±4.1mmHg) and HR (pre-SO: 296.0±4.0; pre-RDX: 290.0±5.0bpm) were almost identical. Similarly, analysis of MAP and HR during the period in which losartan was administered, but rats had not yet received surgery, demonstrated no difference between groups ($p>0.05$). Evaluation of the change in steady-state 24hr MAP from baseline to the losartan treatment period

revealed both groups responded to the ARB similarly (pre-SO: −14.6±1.6 vs. pre-RDX: −19.9±1.9mmHg; $p>0.05$). Twenty-four hours prior to performing RDX, there was no difference in MAP (pre-SO: 144.5±4.4; pre-RDX: 140.1±3.7mmHg) or HR (pre-SO: 307.0±7.0; pre-RDX: 295.0±5.0bpm) between RDX and SO rats. RDX during losartan administration produced a significant reduction in MAP compared to sham-operation that lasted 11 days ($p<0.05$ for main effect of RDX); however, HR was not significantly altered ($p>0.05$). Evaluation of the change in steady-state MAP following RDX during losartan treatment (Figure 26) shows RDX significantly lowered steady-state MAP compared to sham-operation (SO: −2.5±0.7 vs. RDX: −12.1±1.4mmHg; $p<0.05$).

The BP response in RDX or SO rats after losartan withdrawal is shown in Figure 27. After withdrawal of the ARB, BP rose in both groups with MAP remaining lower in RDX−treated SHR; however, MAP in the RDX group was not statistically different from the SO rats ($p>0.05$). Telemetry from one rat in the RDX group (SHR RDX 4) was not working during four days of this 13-day interval and was excluded from this analysis. Comparison of the change in steady-state MAP in surgically treated rats during losartan treatment showed RDX-treated SHR maintained a significantly reduced MAP compared to SO rats (Figure 28. SO: −3.9±0.6 vs. RDX: −14.1±2.0mmHg; $p<0.05$). Hemodynamic values were continually measured for up to 8wks after RDX in this study. Figure 29 shows the effect of RDX on MAP after losartan treatment was discontinued for at least 2wks.

MAP was significantly reduced over the last 13 days of the experiment in RDX compared to SO rats ($p<0.05$). The change in steady-state MAP from baseline at the

end of the study was significantly greater in RDX rats (Inset Figure 29. SO: 0.8±1.7 vs. RDX: -21.8±5.6mmHg; $p<0.05$).

Plasma NE concentration measured at baseline, losartan alone, and the losartan + RDX treatment intervals is listed in Table 4. Statistical analysis reveals a significant interaction term ($p=0.008$) with a post-hoc analysis showing a significantly elevated plasma NE in the pre- RDX group at baseline ($p<0.05$). There were no other differences between groups.

Terminal tissue NE content is shown in Figure 30. RDX significantly lowered NE from SO levels in the LK (SO: 117.9±7.8 vs. RDX: 7.7±2.0ng/g; $p<0.05$), RK (SO: 110.8±10.7 vs. RDX: 30.0±7.5ng/g; $p<0.05$), and the spleen (SO: 432.1±29.1 vs. RDX: 300.9±41.3ng/g; $p<0.05$).

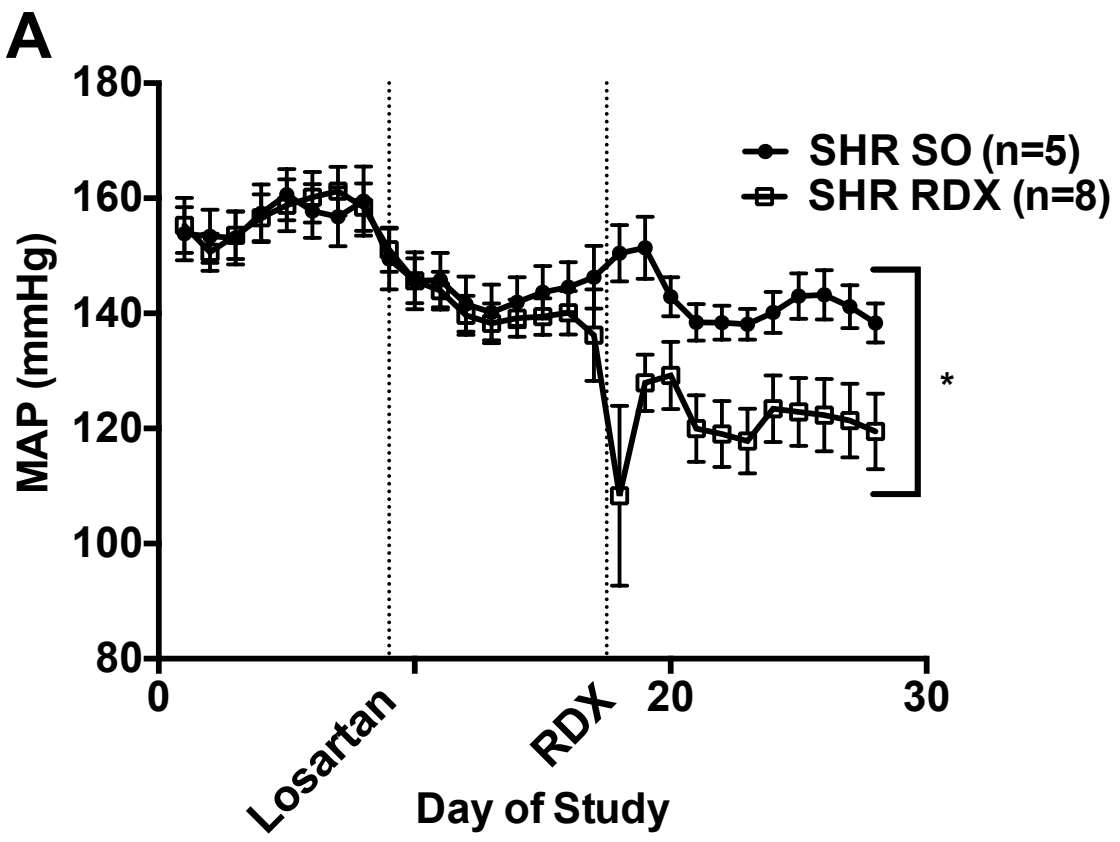
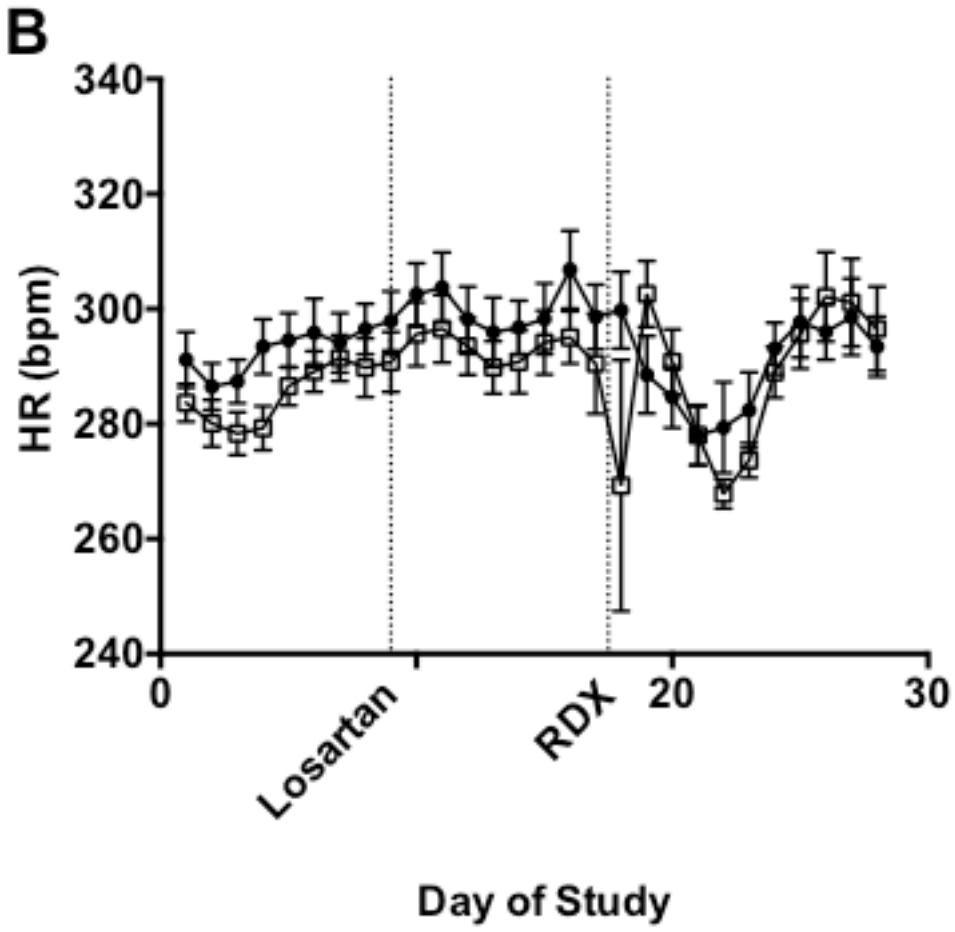


FIGURE 25. <u>The hemodynamic response to RDX during losartan treatment in the aged adult SHR</u>. A: MAP was reduced and stabilized significantly lower in RDX SHR compared to SO SHR during losartan treatment. B: HR response was not different between groups. (*$p<0.05$)

Figure 25 (cont'd)



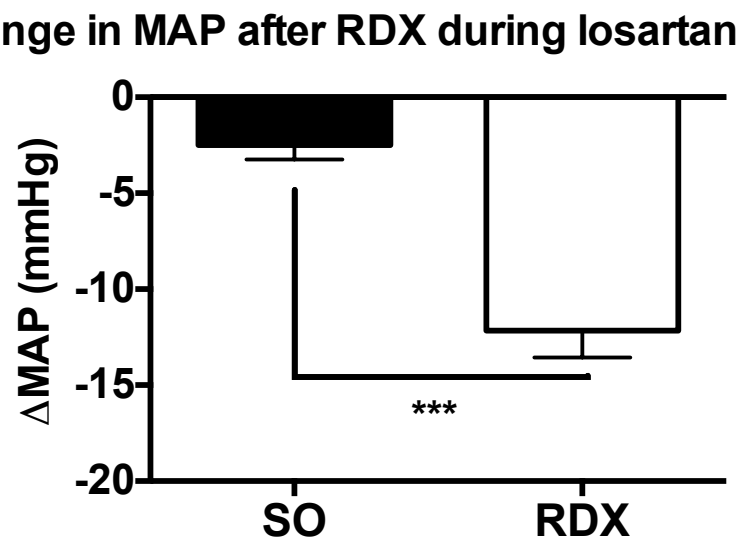


FIGURE 26. <u>The change in steady-state MAP following RDX during the losartan treatment period.</u> MAP was significantly reduced by RDX compared to sham-operation. (***p<0.001)

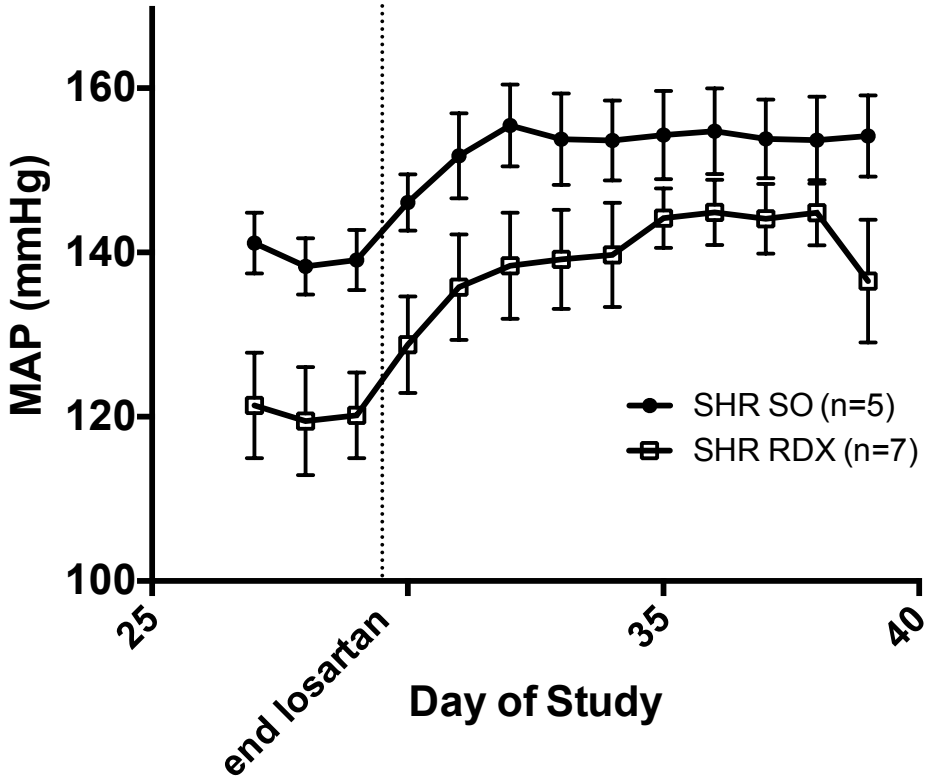


FIGURE 27. <u>The BP response following discontinuation of losartan in RDX or sham-operated SHR</u>. BP remained lower in RDX animals, but this reduction was not statistically significant over the time interval. (p=0.06)

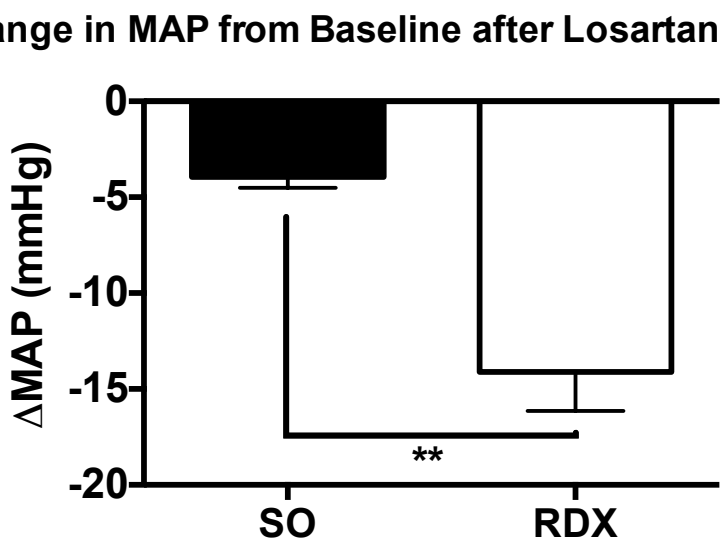


FIGURE 28. <u>Change in steady-state MAP after losartan withdrawal in RDX or sham-operated SHR</u>. MAP was significantly reduced from baseline in RDX-treated SHR compared to sham. (**p<0.001)

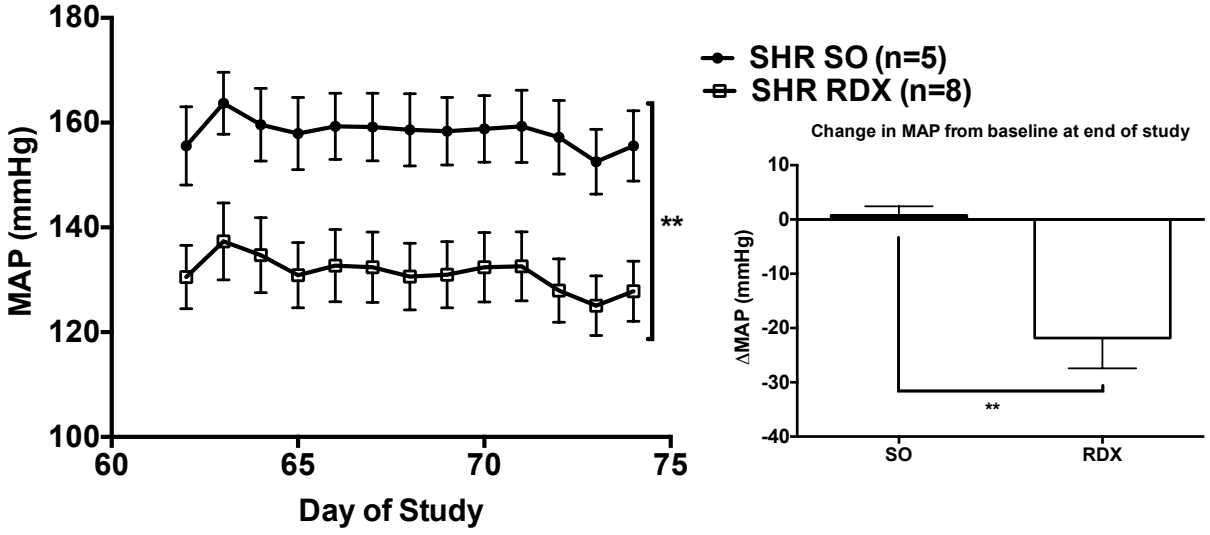


FIGURE 29. MAP in RDX- or sham-operated SHR 8wks after intervention. RDX-treated rats maintained significantly lower MAP compared to sham controls. Inset: Change in steady-state MAP from baseline to the end of the study. RDX group responded with a much larger fall in MAP compared to sham (p**<0.01;**p<0.01).

Treatment Interval	SHR SO	SHR RDX
Baseline	194.1±13.4	291.5±20.2 $^{\alpha,*}$
Losartan	273.3±20.5	253.6±21.1
Losartan, + RDX	234.0±12.2	233.4±17.1

TABLE 4. **Plasma norepinephrine levels in losartan-treated SHR.** Plasma NE concentration (pg/mL) was significantly elevated in the RDX group at baseline. However, there was no difference between groups with any other treatment. ($^{\alpha}$ $p<0.01$ for interaction term, *$p<0.05$ vs. sham Bonferroni post-hoc multiple comparisons)

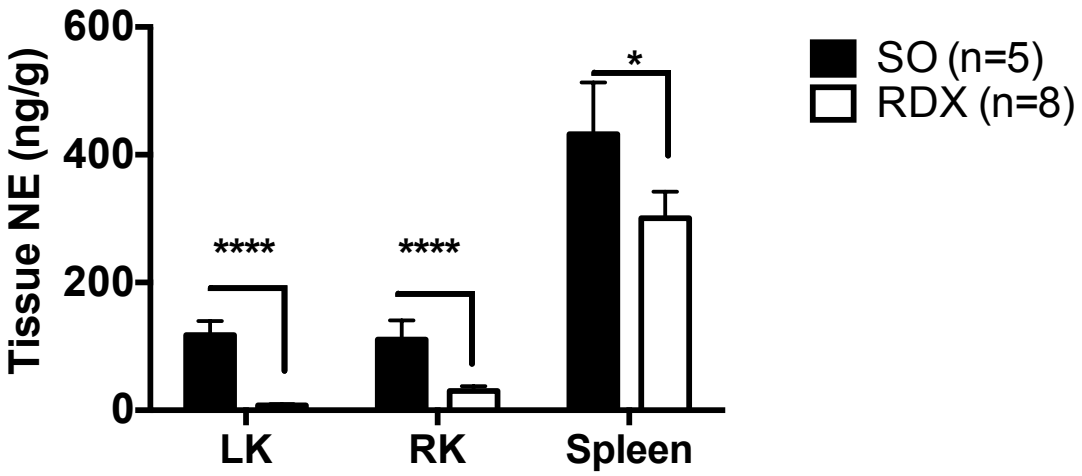


FIGURE 30. **<u>Tissue NE content in aged SHR 56 days after RDX or sham operation</u>**.

RDX significantly lowered tissue NE in all tissues. (LK: left kidney; RK: right kidney: ****$p<0.0001$; *$p<0.05$ compared to sham)

DISCUSSION

This study examined whether the BP response to bilateral renal denervation in the aged adult SHR model requires interruption of sympathetically derived RAAS support of BP. My prior data supported the hypothesis that interruption of SNA is the major mechanism for the fall in BP after RDX in the aged SHR and that the BP effect may be more attributable to loss of RSNA than NRSNA, although this conclusion is not definitive. It is well known that sympathoexcitation and RAAS activation are closely linked [20, 127]. In the SHR, it has been shown that renal sympathetic nerve support of BP and RAAS activity increase with age in the SHR [91, 128]. Therefore, it is logical to propose that the BP response to RDX I observed in aged SHR could be attributed to a reduction in sympathetically mediated RAAS support of BP. As previously discussed, activation of RAAS through elevated RSNA occurs by increasing renin release, and ultimately leads to the generation of AngII. Although other molecular components of the signaling cascade are involved in the RAAS pathway, it is AngII that serves as the principal mediator of RAAS activity [24]. AngII signaling through the AT_1 receptor in particular serves most of the cardiovascular homeostatic functions associated with RAAS activity, e.g. vasoconstriction, aldosterone synthesis, catecholamine release from the adrenal medulla, release of anti-diuretic hormone, dipsogenesis and NE release from sympathetic neurons [24]. Therefore, blocking RAAS activity at the AT_1 receptor could be thought of as pharmacological inhibition of RAAS. I hypothesized in this study that if the BP lowering effect of RDX was linked to suppression of sympathetically-mediated renin release and RAAS activation, then prior pharmacological inhibition of AngII activity at the AT_1 receptor should prevent a further fall in BP with RDX. The main finding from

this study was that pharmacologic blockade of AngII with an ARB did not interfere with the fall in BP associated with RDX in the aged adult SHR. Therefore, I conclude from these findings that the BP lowering effect is not linked to reduced AT_1 receptor activation due to loss of sympathetic regulation of renin release.

Losartan treatment in similarly aged SHR to mine was previously employed by Mihailovic-Stanojevic et al. [129]. In 9- and 18-month-old rats, administration of losartan (10mg/kg/day) for 4wks reduced SBP by about 20mmHg compared to untreated controls**.** These investigators documented a reduction in RVR and an increase in RBF and aortic blood flow. Losartan treatment also produced smaller heart weights, indicating a reduction in cardiac hypertrophy, a known complication of excess AngII signaling. These findings suggest that losartan (10mg/kg/day) is sufficient to suppress RAAS-signaling in the aged adult SHR. In my rats, which were treated with the same dose of losartan, BP fell a similar magnitude prior to surgical intervention **(Figure 25:** pre-SO: −16.6±1.4, pre-RDX: −22.1±2.0mmHg). This observation supports the idea that RAAS was antagonized at this dose.

In my study, RDX still lowered MAP compared to the sham-operated SHR after 8 days of losartan administration (10mg/kg/day). BP was significantly lower in RDX-treated rats for eleven days after surgical intervention with steady-state MAP falling 12.1±1.4mmHg in RDX-treated rats and 2.5±0.7 SO rats. At the end of the study, 8wks after surgical intervention and 45 days after ARB withdrawal, steady-state MAP was still significantly lower in RDX rats (SO: 0.8±1.7 vs. RDX: -21.8±5.6mmHg). These observations suggest that interruption of RAAS at the AT1R is not a critical mechanism driving the reduction in BP associated with RDX. RDX could lower BP by affecting other signaling

components in the RAAS upstream from the AT1R (e.g. renin). This logic would suggest that renin is elevating BP independent of other components of the RAAS. Clinical trials have shown that this scenario is unlikely as direct renin inhibition in patients taking an ARB or ACE-I does not result in better BP control [130]. This hypothesis could be tested however by treating aged SHRs with either a direct renin inhibitor, ARB or a combination of the two prior to RDX. One would expect that if RDX is lowering BP through a RAAS-independent mechanism then BP would still be reduced by RDX in these treated rats. Overall, however, it is likely that suppression of other intra-renal mechanisms linked to SNA explain the reduction in BP after RDX. These signaling mechanisms could include altered sodium/water handling leading to prolonged natriuresis/diuresis or reduced renal vascular resistance.

Recently, a study in Kuming dogs demonstrated that CBRNA caused a significant reduction in plasma renin, AngII, and aldosterone, in addition to lowering BP [131]. These data are consistent with clinical studies by Schlaich et al. and others that report CBRNA reduces renin activity and aldosterone [132, 133]. However, the modest reductions in RAAS activity observed might not be sufficient to affect BP. These studies did not assess the consequence of blocking RAAS signaling prior to renal denervation to show that the fall in BP associated with CBRNA was indeed mediated by RAAS suppression. Additionally, it should be noted that plasma renin activity fluctuates, especially in the presence of anti-hypertensive therapies [122]. Central sympatholytics and beta-blockers cause PRA to decrease, however PRA can increase with the administration of ACE-I, ARBs, diuretics, or direct vasodilators. As mentioned previously, the main function of renin activity seems to be generation of AngII, which is

the critical effector molecule of the RAAS. Again, one would expect that if RDX was lowering BP by diminishing RAAS activity that prior blockade of the AT1 receptor should prevent any RDX effect on BP. The data presented here demonstrate that, at least in the aged SHR, RAAS inhibition at the level of the AT1 receptor does not interfere with the ability of RDX to lower BP.

In this study rats allocated to the RDX group had a significantly higher baseline plasma NE compared to the sham-operated controls. This finding could be interpreted to mean that this group had a greater basal sympathetic activity. Since all other measured parameters between these two groups were identical, this observation could have been a random event. Plasma NE was reduced in pre-RDX-treated rats after losartan treatment suggesting sympathetic activity was decreased by the ARB. However, in the sham group, plasma NE increased compared to basal measurements. The cause of this differential response to losartan is not clear. From these plasma NE data, I conclude that I have no evidence to suggest the losartan treatment significantly affects SNA.

Surprisingly, in this study, RDX lowered tissue NE content in both the kidneys and the spleen. This could be interpreted as a non-specific denervation procedure, a result that is inconsistent with all other RDX procedures we have performed. As in all my other studies, surgical stripping of the tissue around the renal artery was undertaken carefully. No other tissues were mechanically disturbed. Application of phenol was also handled judiciously as to limit the spread of the solution to prevent denervation of nearby tissues. Given the close proximity of the spleen and celiac ganglia, the origin of the post-ganglionic fibers that innervate the spleen, it is possible that these structures were damaged during surgery. However, work from my lab and collaborating labs have

shown that damage to these structures would virtually abolish tissue NE [111, 134]. While one cannot argue against the statistical difference between RDX and sham-operated splenic NE content, given my considerable experience with the technique, this outcome may be explained as an event of chance.

In summary, RDX in the aged SHR lowers BP through a sympatholytic mechanism not linked to suppression of RAAS signaling via the AT1 receptor. It is likely therefore that interruption of another sympathetic signaling pathway is mediating the fall in BP, but the specific mechanism remains to be identified. Although my earlier results suggest that the effect was likely attributable to elimination of RSNA, the current data are consistent with the hypothesis that NRSNA could be involved in the BP response. With regard to CBRNA in difficult-to-treat hypertensive patients, these data suggest that it is unlikely that prior treatment with an ARB or any other RAAS inhibitor would mitigate the expected reduction in BP. However, more investigation into the interactions between pharmacotherapies and CBRNA is needed to confirm this observation in humans.

CHAPTER 6 – Changes in body sodium do not alter the BP response to RDX in the aged SHR.

Previously conducted studies described in Chapter 4 show the BP response to RDX in the aged SHR is linked to interruption of a sympathetic mechanism. Additional experiments have shown that RDX is not lowering BP by mitigating activities of the RAAS mediated through the AT_1 receptor. As previously discussed, it is likely that interruption of another intra-renal sympathetic signaling mechanism better explains the reduction in BP associated with RDX.

Renal sympathetic nerve activity directly participates in body sodium homeostasis [11]. As discussed in Chapter 1, increased RSNA causes sodium and water reabsorption from the nephron. This could lead to blood volume expansion and an increase in BP via the mechanisms originally proposed by Guyton and colleagues. Additionally, as mentioned previously, the BP effect of RDX in younger SHR is associated with a prolonged natriuretic, diuretic response [88]. Therefore, it could be argued that RDX is lowering BP in the aged SHR by altering renal handling of sodium and water; more simply, RDX could be acting as a diuretic.

In this study, I examined the possibility that the BP lowering response to RDX could be attributed to a natriuretic/diuretic mechanism. The BP-lowering response to RDX was evaluated during low (LS), normal (NS), and high (HS) salt conditions. I hypothesized that if RDX is lowering BP due to a diuretic/natriuretic mechanism then the BP response would be inversely related to the body sodium state. To be more specific, as is classically observed with diuretic therapy [122], I expected that if RDX was lowering BP

through a diuretic action, then the BP lowering effect of RDX would be greatest during sodium depletion, while salt loading would mitigate the BP response to RDX.

METHODS

Experimental protocols

This study used a total of 15 male SHR (36wks). Rats were fitted with telemetry transmitters 1wk prior to recording of hemodynamic parameters. Rats were then allocated into groups (Sham-operated (SO): n=7 and RDX: n=8) so that basal MAP was equivalent between groups. After baseline hemodynamic values were recorded, rats were placed on a 0.1% NaCl diet (Low salt: LS). BP was continuously recorded for 7 days before RDX or SO was performed. SHR remained on a LS diet for 14 days after RDX. Additional sodium depletion was performed using the diuretics chlorthalidone (CHLOR: 100mg/dL in drinking water) or furosemide (FURO: 12mg/day [102]). Justification for these doses is described in Chapter 2. CHLOR treatment was administered for 4 days before being withdrawn and BP was allowed to stabilize. FURO was administered for 7 days before being withdrawn and BP was allowed to stabilize. Rats were then placed on the standard 0.4% NaCl diet, and hemodynamics recorded for 14 days. Animals then entered the high salt (HS) condition that consisted of the 0.4% NaCl chow plus 1.0% NaCl drinking water for 16 days. Distilled water was returned at the end of the HS period, and recordings continued for 20 days. Blood was collected from the tail vein at the end of each treatment period to measure plasma NE content. At the conclusion of the study, SHR were euthanized and renal and splenic tissues were collected to confirm the effectiveness of denervation.

RESULTS

The BP effect of RDX during LS conditions is shown in Figure 31. Baseline recording of average 24hr MAP did not reveal a significant difference between groups ($p>0.05$). Immediately prior to introduction of the LS diet, which was prior to surgical intervention, MAP (pre-SO: 159.6±1.4; pre-RDX: 161.2±1.6 mmHg) and HR (pre-SO: 303.0±2.0; pre-RDX: 308.0±2.0bpm) were similar between groups. Introduction of a LS diet occurred on day 10 of the study, and the change in dietary sodium load did not lower MAP. The change in steady-state MAP after initiating the LS diet was not different between groups (pre-SO: -3.3±1.3 vs. pre-RDX:-0.5±1.7mmHg; $p>0.05$). RDX occurred 7 days after dietary intervention. Analysis of MAP over 14 days after RDX revealed a significant interaction between groups ($p <0.05$) and a significant RDX effect ($p<0.05$). Steady-state MAP was significantly reduced from the LS treatment period in RDX-treated SHR compared to SO (Figure 32: SO: −1.5±1.1 vs. RDX: −9.9±2.5mmHg; $p<0.05$).

Hemodynamic responses to diuretic treatment during the LS diet period in RDX and sham-operated SHR are shown in Figure 33. Analysis of MAP during the 4 day CHLOR treatment revealed a significant RDX effect ($p<0.05$). MAP was still significantly reduced in RDX treated SHR compared to SO rats. The HR response did not differ between groups. After CHLOR had been withdrawn, subsequent FURO eliminated the BP lowering effect of RDX. MAP 24hrs prior to FURO withdrawal was not different between groups (SO: 131.4±2.4; RDX: 126.1±4.7mmHg; $p>0.05$). HRs were significantly elevated in RDX animals compared to SO rats during FURO treatment ($p<0.05$ for main effect of RDX). At peak response, HR was 316.0±2.0 bpm in sham-operated SHR and 327.0±4.0 bpm in RDX SHR. The change in steady-state MAP during CHLOR treatment

was significantly greater in RDX SHR compared to SO (SO: -3.2±0.3 vs RDX: -7.4±1.0). The change in steady-state MAP was similar in the two groups of rats during FURO administration (SO: −29.1±2.9 vs. RDX −34.0±4.6mmHg; $p>0.05$).

SHR were returned to 0.4%NaCl diet on day 59 of the study. Over the next 14 days, neither MAP nor HR were significantly lower in RDX animal compared to shams ($p>0.05$). The change in steady-state MAP from the initial baseline period was larger in the RDX rats but the difference was not statistically significant (SO: −7.2±3.8 vs. RDX: −12.9±1.9mmHg; $p>0.05$).

Rats were transitioned to HS conditions on day 75. Hemodynamic responses during and after the HS period are shown in Figure 34. Statistical analysis of MAP during the HS condition revealed a significant effect of RDX ($p<0.05$) as MAP was lower in RDX treated animals. Similar analysis of HR demonstrated a significant interaction between groups as HR rose over time and then fell again as it stabilized ($p<0.05$). The change in steady-state MAP from the pre-surgical baseline revealed MAP remained −6.9±1.9mmHg below baseline in RDX treated SHR, while MAP rose 3.6±2.2mmHg in SO rats ($p<0.05$). On day 92, SHR were returned to distilled drinking water, while remaining on normal salt diet; in other words, to the conditions that held at beginning of the protocol. Analysis of the MAP during this period revealed a significant effect of RDX, as MAP remained lower compared to SO rats ($p<0.05$). HR was not different between groups during this period. At the end of the study, 94 days after RDX, steady-state MAP was significantly reduced from the pre-surgical baseline in RDX rats compared to SO rats (SO: 1.4±3.2 vs. RDX: −10.8±2.0mmHg; $p=0.005$).

The difference in the change in steady-state MAP (ΔΔsteady-state MAP) between SO and RDX rats was calculated to be the BP lowering effect of RDX at each treatment interval of this study (TABLE 5). Analysis shows there is no significant difference in the BP response during any treatment ($p>0.05$).

Plasma NE content for baseline, LS, RDX during LS, and HS conditions are reported in TABLE 6. Statistical analysis did not reveal any significant differences in plasma NE during any treatment.

Tissue norepinephrine content analyzed from renal and splenic tissue at the conclusion of the study is shown in Figure 35. Left (Sham: 126.9±8.0 vs. RDX: 35.9±5.5ng/g; $p<0.0001$) and right (Sham: 127.2±11.2 vs. RDX: 71.7±12.5ng/g; $p=0.005$) kidney NE content was significantly reduced, while splenic NE was not different between groups (p.0.05). Analysis was performed 94 days after RDX or sham-operation.

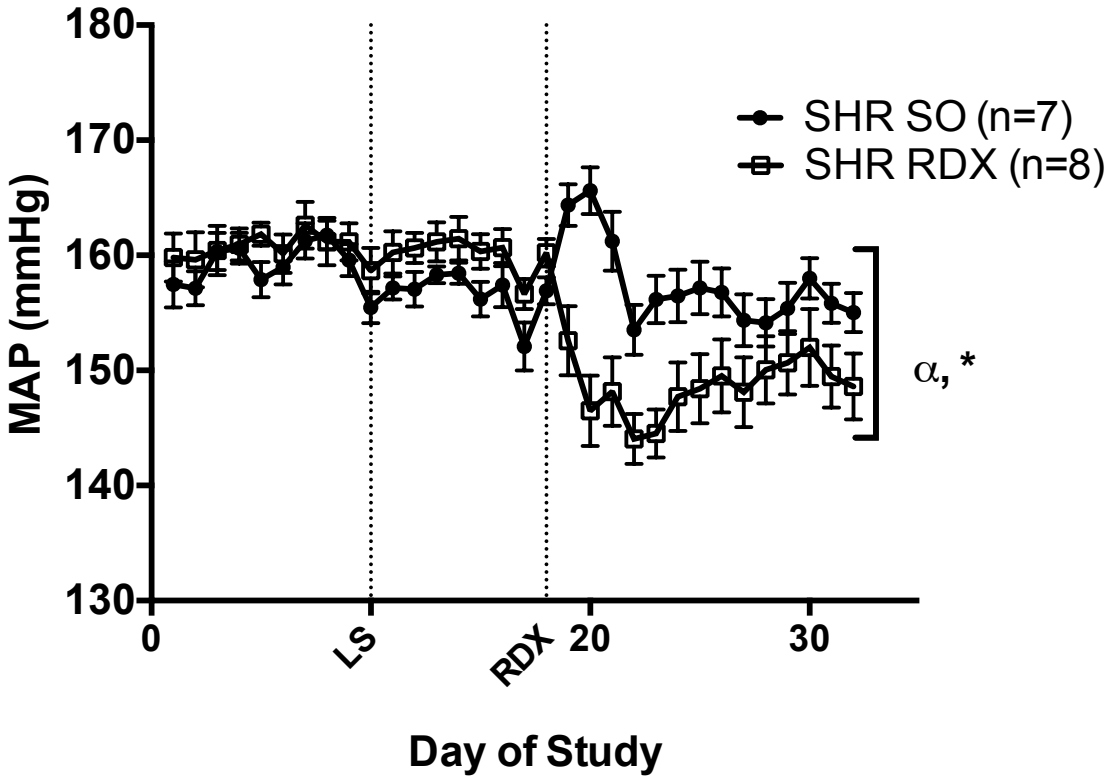
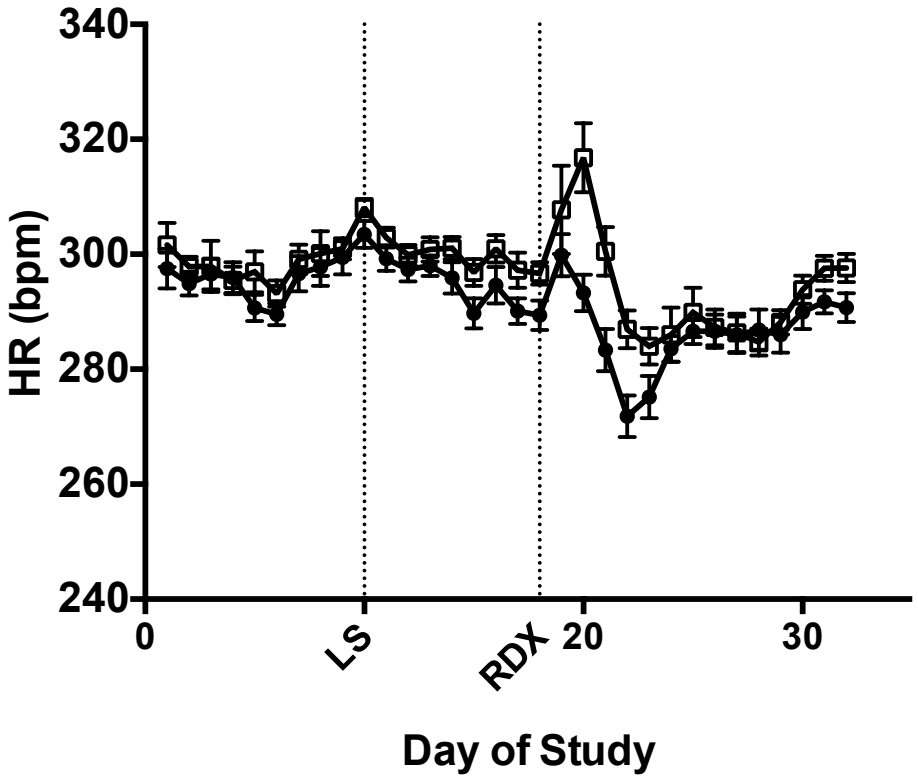


FIGURE 31. <u>The hemodynamic effect of RDX during LS diet</u>. TOP: MAP stabilized significantly lower in RDX-treated SHR compared to SO SHR after 1wk of 0.1% NaCl diet. BOTTOM: HR was not significantly increased after RDX once MAP stabilized. ($^{\alpha}$ $p<0.05$ for interaction term; *$p<0.05$ for RDX term)

Figure 31 (cont'd)



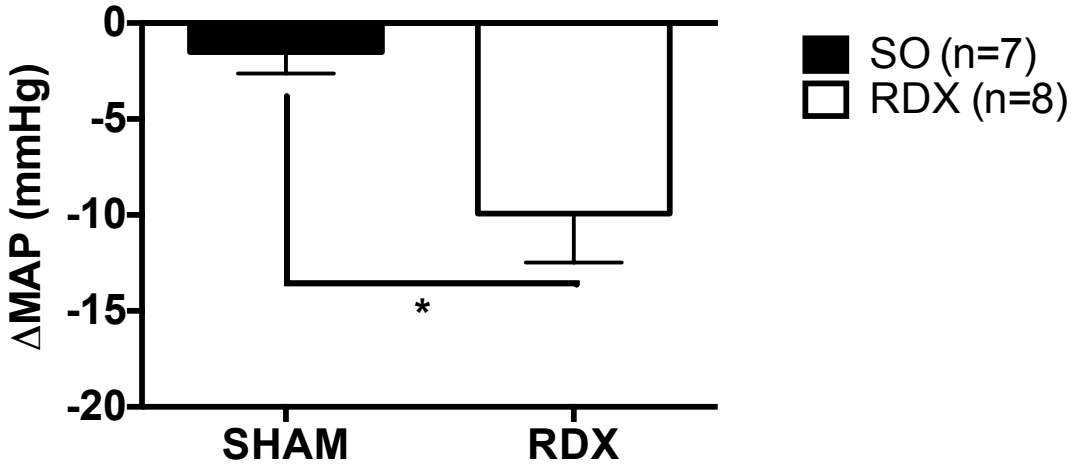


FIGURE 32. <u>The change in steady-state MAP after RDX in LS-treated SHR</u>. SHR receiving RDX had significantly reduced MAP during LS treatment compared to sham-operated rats. (*p<0.05)

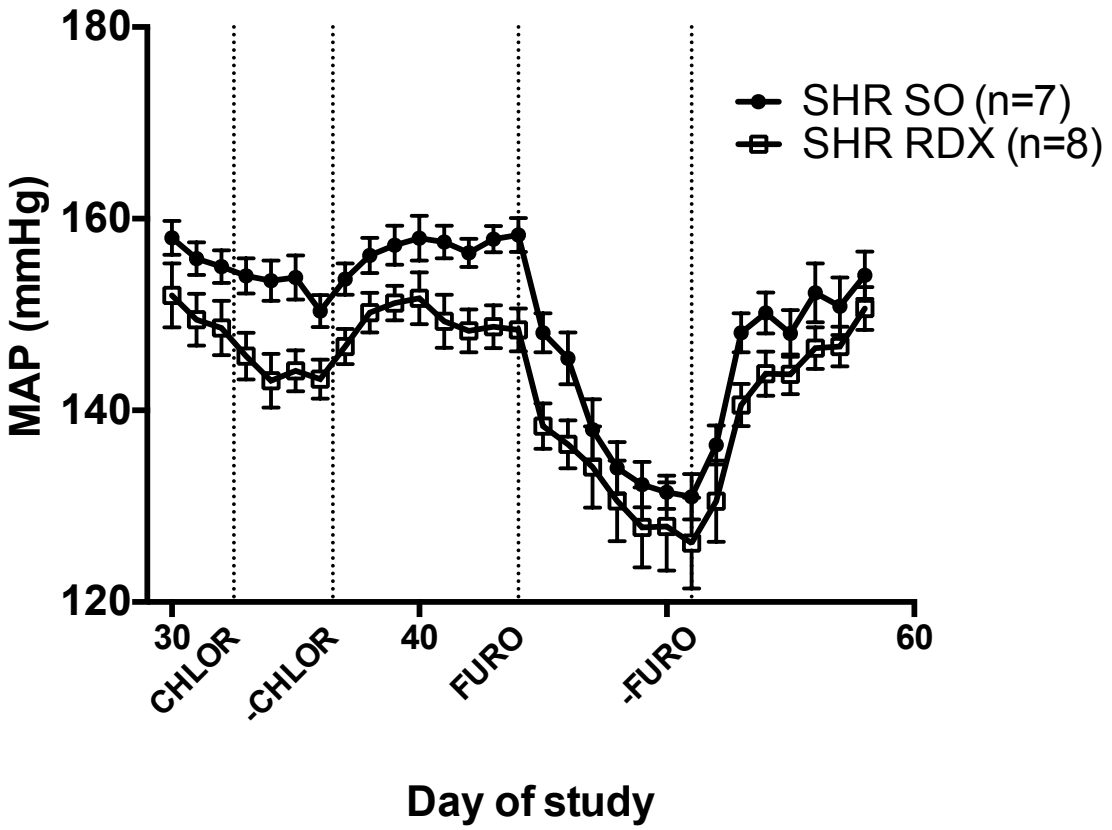
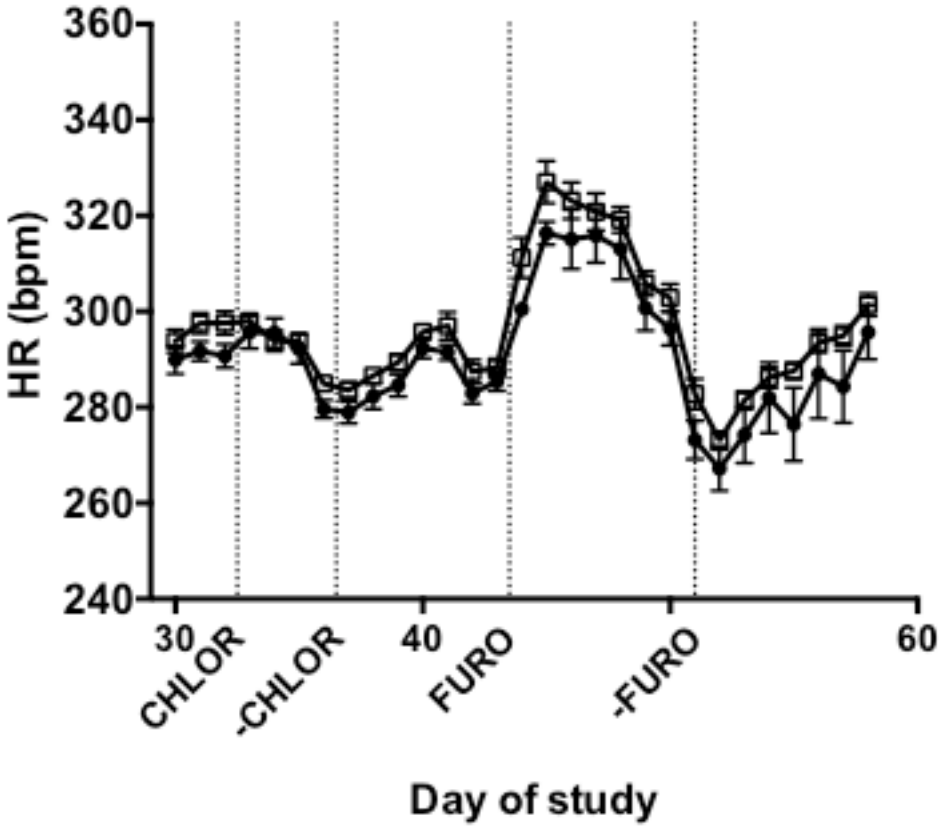


FIGURE 33. **The hemodynamic response to diuretics in RDX- and Sham-operated SHR on LS diet**. TOP: MAP was significantly reduced in SHR RDX during CHLOR treatment but not during FURO treatment. BOTTOM: HR was not different between groups. Statistics described in text.

Figure 33 (cont'd)



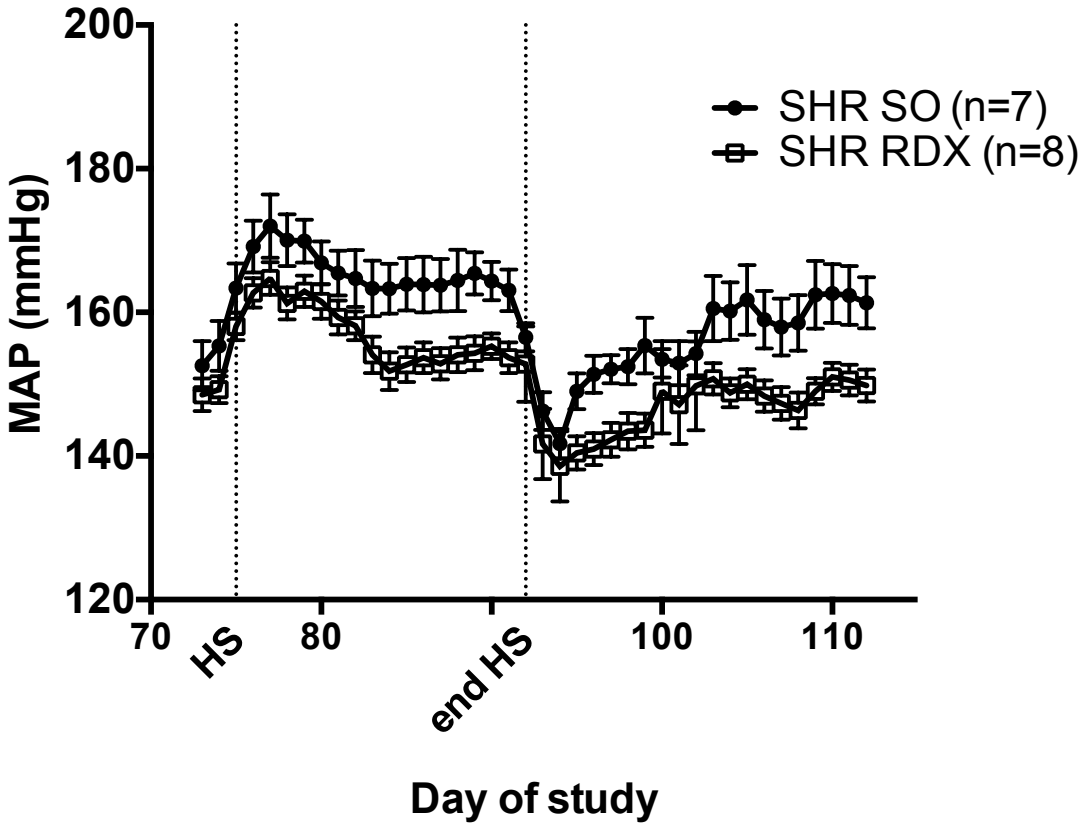
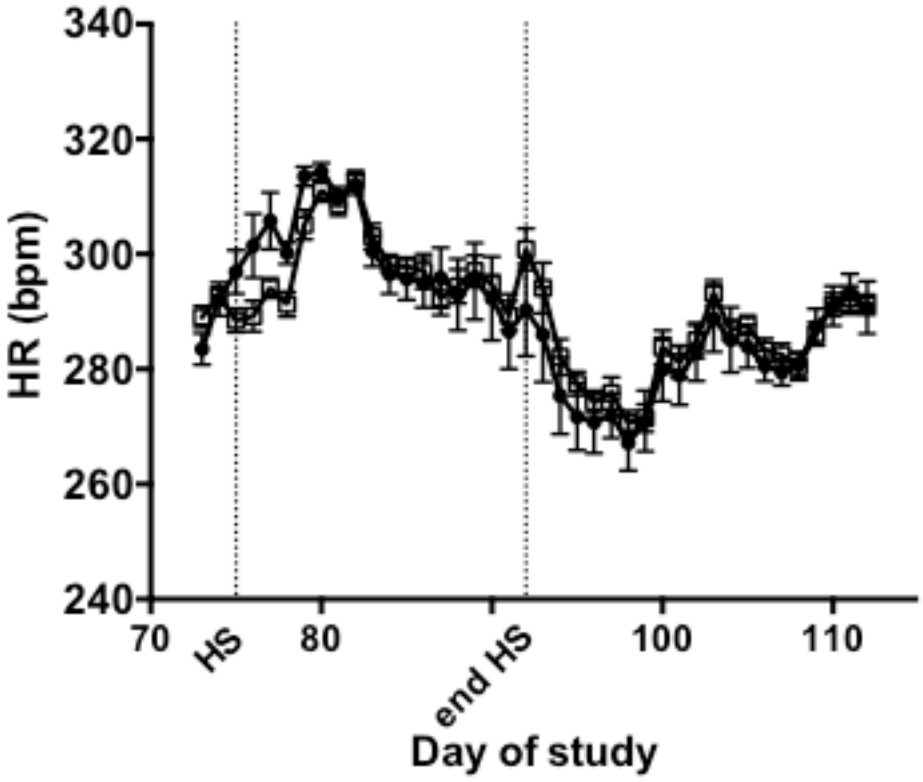


FIGURE 34. **Hemodynamic response to high salt conditions in RDX- and sham-denervated SHR**. TOP: MAP was significantly reduced in HS conditions compared to sham-operated rats and remained lower after HS was removed. BOTTOM: HR response did not differ between groups. Statistical analysis described in text.

Figure 34 (cont'd)



	ΔΔSteady-state MAP between groups (mmHg)
LS	-8.4±2.9
LS, CHLOR	-4.1±1.1
LS, FURO	-4.9±5.6
NS	-5.7±4.1
HS	-10.5±2.9
NS (end of study)	-12.3±3.7

TABLE 5. The BP lowering effect of RDX in various sodium conditions. The BP response attributable to RDX was not significantly different at any time throughout the study ($p>0.05$). (LS: low sodium; CHLOR: chlorthalidone; FURO: furosemide; NS: normal sodium; HS: high sodium)

Treatment Interval	SO	RDX
Baseline	250.7±27.1	262.4±10.8
Low Sodium	264.7±32.9	234.7±16.2
RDX during Low Sodium	250.0±37.0	242.4±24.8
High Sodium	251.0±14.1	226.3±26.6

TABLE 6. **Plasma NE content during baseline, LS, RDX during LS, and HS conditions.** There was no difference in plasma NE between groups at any time during the study.

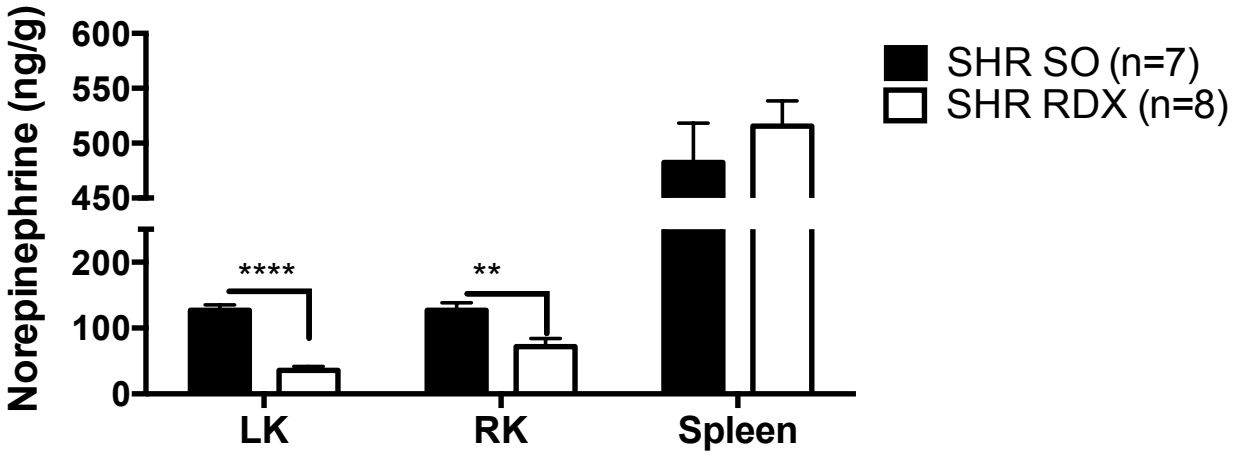


FIGURE 35. **Tissue NE content analyzed 94 days after RDX**. Left and right kidney NE content was significantly lowered by RDX compared to sham, while splenic NE content was not influenced by RDX. (****p<0.001; **p<0.01)

DISCUSSION

In this study I examined the hypothesis that the BP response to RDX is due to a natriuretic/diuretic effect caused by loss of RSNA-mediated renal sodium and water reabsorption. One direct approach to testing this hypothesis would have been to carefully measure sodium and water balance, and perhaps blood volume, in rats before and after RDX (and sham-operation). Such measurements are extremely difficult, however, and potentially could be confounded by simultaneous changes in MAP and sodium excretion due to the operation of the pressure-natriuresis mechanism. Instead I chose to use the following indirect approach to the hypothesis. Some anti-hypertensive drugs, i.e. the diuretics, are *known* to lower BP by inducing natriuresis and diuresis [122]. Furthermore, the efficacy of these drugs in lowering BP is inversely related to the sodium and water intake of the subject receiving the drugs for therapy; specifically, the drugs are much less effective at lowering BP in sodium-replete states and more effective when dietary sodium intake is restricted [122]. Therefore, I based the experiments here on the assumption that if RDX were lowering BP by a diuretic/natriuretic mechanism due to loss of RSNA then the BP response to RDX would be largest when the rats were sodium depleted and would be diminished when the rats were salt loaded. My main finding was that the BP response to RDX did not appear to be closely related to body sodium status. In fact, the BP response to RDX was numerically larger when the rats were salt loaded than when they were salt depleted (Table 5). I conclude from this finding that RDX is not lowering BP primarily through a natriuretic/diuretic mechanism. Supporting this interpretation are data from these and my earlier experiments showing that the fall in BP after RDX is very rapid in onset. One

would expect that if RDX were lowering BP through an effect on salt and water regulation, then the BP response to RDX would occur more gradually, i.e. take several days to achieve the maximum effect, as discussed in Chapter 1.

Although it is largely true that the BP response to RDX was similar under all sodium conditions (TABLE 5), during administration of furosemide average daily MAP was no longer significantly different between sham-operated and RDX rats. Since furosemide should have produced the greatest degree of sodium depletion of any of the study conditions, according to my hypothesis the difference in BP between sham-operated and RDX rats should have been the largest during furosemide treatment. This finding could mean that RDX lowers BP not via sodium loss but instead through the same physiological mechanisms affected by furosemide. Furosemide prevents sodium reabsorption in the thick ascending limb of the nephron by blocking the NKCC2 transporter protein[122]. Since with furosemide treatment BP fell in sham-operated rats to levels equivalent to those seen in RDX rats, RDX could be lowering BP by altering expression of NKCC2. Indeed, other investigators have reported that RDX reduces renal NKCC2 expression in the thick ascending limb [135, 136]. Therefore, it is plausible that RDX could lower BP in the aged SHR by acting as a diuretic/natriuretic agent through altered expression of NKCC2. I find this unlikely, however, given the results during HS intake. As mentioned previously, in the presence of high sodium intake any diuretic/natriuretic effect of RDX would be greatly reduced in size, as would the BP lowering effect of RDX. These data show that even in a high salt condition, RDX lowered MAP compared to SO rats. Therefore, it is unlikely that RDX is primarily lowering MAP by a diuretic mechanism involving altering NKCC2 expression. Another

explanation for the loss of the BP effect of RDX following furosemide treatment could be a technical oversight. In this phase of the study, BP was never allowed to reach a steady-state level before drug discontinuation. The Alzet mini-osmotic pump employed in the delivery of the furosemide dose operated for a maximum of 7 days. Since treatment with chlorthalidone treatment yielded little change in BP after 4 days of the diuretic, we anticipated seeing a stabilization of MAP within the 7-day window of furosemide administration. This stabilization did not occur. It is clear from the later observations in the HS condition that BP may not stabilize within 7 days. A more chronic examination of the BP-lowering effect of RDX during furosemide treatment is necessary to rule out the likelihood that these initial observations were due to technical error.

While these data do not support a natriuretic/diuretic mechanism as the primary mediator of the BP lowering effect of RDX, a complicating factor in interpreting the data is that sodium intake has uncertain effects on RSNA. In general, RSNA is expected to have a greater impact on renal sodium reabsorption during sodium depletion. For example, long-term feeding of a LS (0.06%) diet in the normotensive Wistar rat increased RSNA, whereas RSNA was lower in rats on a HS diet (3.12%) [137]. Recent studies in rats show that that inhibition of RSNA during HS intake is critical to maintenance of sodium balance and BP [138]. And renal denervation was reported to impair the ability of rats to achieve sodium balance when salt restricted [139]. Other investigators, however, failed to find an effect of renal denervation on sodium balance [140-144]. Furthermore, in conscious rabbits, large changes in sodium intake did not affect directly recorded RSNA [145]. Therefore, the influence of sodium intake on RSNA is not clear, but obviously could have affected the outcome of my studies. For example,

it would be logical to expect that if an elevation in RSNA accompanies a decrease in sodium intake, then during salt depletion RDX rats would exhibit an exaggerated fall in BP compared to sham-operated rats. Similarly if RSNA falls with an increase in sodium intake, then RDX rats should show a reduced fall in BP compared to SO rats. As mentioned earlier, I did not observe a consistently exaggerated fall in steady-state BP in RDX rats during salt depletion. The fall in BP in RDX rats on chlorthalidone was modest but still significantly greater than what was observed in similarly treated SO SHR (−3.2±0.3 vs RDX: −7.4±1.0). As previously noted, treatment with furosemide to produce maximal sodium depletion produced a similar fall in BP between groups and actually reduced the difference in BP between RDX and sham-operated rats. The cause for this differential response to sodium depletion is not known, but it is clear that sodium depletion was not associated with a larger BP response to RDX. Most importantly, I did not observe a reduced BP response to RDX compared to SO SHR when rats were salt loaded. These findings argue against the idea that RSNA is inversely related to sodium intake.

I did not directly measure RSNA in my experiments. However, I did measure plasma NE as an indicator of overall SNA, and it seems reasonable to assume that RSNA and NRSNA usually will change in parallel. There is a large literature on how varying salt intake affects plasma NE. Many studies show an inverse correlation between salt intake and plasma NE [146-149], while others report little or no relationship [150-152]. In my experiments, in which sodium intakes varied over 10-fold (0.1% to at least 1%), I observed no statistically significant differences in plasma NE in either SO or RDX rats. In RDX rats plasma NE presumably derives almost entirely from NRSNA. This is

consistent with other evidence cited earlier that neither RSNA nor NRSNA are consistently changed by even large differences in salt intake.

In Chapter 4, I showed that the steady-state BP effect of RDX is prevented by treatment with a central sympatholytic drug that significantly reduced plasma NE. Based on that finding I had anticipated that I could cause sympathoexcitation (including RSNA) using sodium depletion and thereby exaggerate the BP response to RDX; but this did not occur. Different results might be obtained in situations in which RSNA is clearly increased, such as stress [153], heart failure[154] or prolonged hypotension[155].

In this study, similar to the results reported in Chapter 3, the BP response to RDX persisted after 94 days after RDX. In chapter 3, RDX treated SHR maintained a significantly lower BP compared to sham-operated SHR for 11wks after surgery. In this study, RDX treated rats had a significantly lower BP compared to sham-operated SHR over 13wks after surgery. This observation shows the BP lowering effect persists much longer than previously recorded. Tissue norepinephrine was significantly reduced in RDX treated SHR, suggesting that kidneys were still denervated. However the functional status of the renal innervation in my rats is uncertain, since as discussed in Chapter 3, the kidney has been reported to be functionally reinnervated within 30 days of total denervation despite reduced tissue NE content [94]. If the kidneys of the rats in this study are functionally reinnervated as described by Kline's group, these findings suggest that non-renal mechanisms are likely driving the long-term fall in BP. The lack of a connection between the BP effect of RDX and the intra-renal sympathetic mechanisms evaluated in Chapters 5 and 6 (AT1 receptor antagonism, salt load) further support this hypothesis that non-renal mechanisms could be involved.

In this study, HR fell with sham-operation as MAP increased. This bradycardic event likely explained by an increase in activity of the baroreflex. In previous studies, MAP and HR were stable after sham-operation. I do not have any data to explain this unexpected response. As shown in previous chapters, HR was significantly elevated within 24hrs after RDX compared to SO rats. This response could have occurred as a consequence of decreased baroreflex activity as BP fell in RDX treated rats. However, as observed previously, deafferentation elicited a similar rise in HR without a significant change in BP. At this time, I think this repeat finding adds further support to the hypothesis that RDX influences cardiac function although the mechanism is not addressed by my data. Further exploration could be undertaken to examine the precise mechanisms that support the change in HR.

In summary, these data demonstrate that the BP lowering effect of RDX in the aged SHR is similar in the presence of sodium excess or depletion. This finding suggests that natriuresis and diuresis are not the primary drivers of the BP-lowering effect of RDX, perhaps in part because SNA is the aged SHR is not modulated by body salt status. The findings therefore indicate that some other mechanism associated with RSNA is mainly responsible for the BP response to RDX. More investigation into other renal sympathetic nerve targets such as α-AR and β-AR is necessary to better understand how RDX might be lowering BP in the aged SHR.

CHAPTER 7 – The BP lowering effect of RDX in the aged SHR is lost during adrenergic receptor blockade.

The BP lowering effect of RDX in the aged SHR has been linked in my dissertation to interruption of a sympathetically-mediated process, however it is not yet known which sympathetic signaling mechanism(s) may be most responsible for the fall in BP. Processes connected to intra-renal sympathetic activity such as RAAS signaling through the AT_1 receptor and anti-natriuresis/anti-diuresis do not appear to be involved. Therefore, it is likely that interruption of an alternative sympathetic signaling mechanism better explains the fall in BP after RDX. This signaling mechanism could be of renal or non-renal origin, or even a combination of both.

As discussed in the introduction to this dissertation, adrenergic receptors mediate signal transduction initiated by norepinephrine (NE), the primary sympathetic neurotransmitter of the renal nerves. The alpha(α)$_1$-adrenergic receptor (α_1-AR) has been shown to activate both renal vasoconstriction and sodium reabsorption from the tubules [11]. The beta(β)$_1$-AR is expressed on the juxtaglomerular cells and facilitates neutrally mediated renin release [11]. In this study, I hypothesized that if elimination of activity at the adrenergic receptors, α_1-AR or β_1-AR, were driving the BP response to RDX, then prior antagonism of either these receptors would prevent the BP response to RDX. Given my previous findings that RAAS inhibition at the AT_1 receptor prior to RDX does not influence the RDX effect on BP, I did not expect suppression of renin release to be linked to the BP response of RDX. Thus, I did not anticipate that β_1-AR antagonism would interfere with a BP response to RDX. Therefore, I expected that if suppression of

adrenergic receptor activity was mediating the BP response to RDX, then blockade of the α_1-AR would prevent the BP response to RDX in the aged SHR.

METHODS

This study used a total of 13 male SHR (SO: n=6; RDX; n=7). Two identical experiments were conducted separately, and the data were combined. In the first experiment, 7 SHR were used (SO: n=3; RDX: n=4). The second experiment used 6 SHR (SO: n=3; RDX: n=3). In both studies, SHR were instrumented with telemetry transmitters 1wk prior to recording of hemodynamics, and rats were allocated into treatment groups so as to create equivalent basal MAP between groups. Baseline hemodynamic variables were recorded for 4 days. The α_1 adrenergic receptor antagonist prazosin (3mg/kg/day [99]) was administered in drinking water beginning on day 5. The BP effects of prazosin treatment were recorded over the next 9 days. At that time, on day 14, RDX or sham operation was performed on SHR receiving prazosin treatment. After 10 days, the β_1 adrenergic receptor antagonist atenolol (1mg/mL [100]) was added to the drinking water that also contained prazosin. Justification for use of these drugs is in Chapter 2. Ten days later, all medications were withdrawn, and BP was allowed to stabilize. SHR were euthanized at the end of study, and tissues (left kidney, right kidney, spleen) were harvested to determine the effectiveness of renal denervation.

RESULTS

The hemodynamic response to RDX during prazosin treatment is shown in Figure 36. Analysis of average daily MAP and HR during the baseline period shows both

parameters were similar between groups ($p>0.05$). Twenty-four hours prior to initiating prazosin treatment, when SHR had not yet undergone RDX surgery, MAP was 160.5±3.2 in the SO group and 159.3±5.0 in the RDX group. Analysis of the BP response during the prazosin treatment interval reveals a similar response to drug between groups ($p>0.05$). Prazosin lowered MAP on the first day of treatment; however, MAP gradually returned back to baseline levels. Twenty-four hours prior to surgical intervention, MAP was 161.7±4.3 in the rats allocated to receive SO and 156.7±3.1 in rats allocated to receive RDX. RDX or SO was performed on day 14 of the study while rats were still receiving prazosin therapy. Statistical analysis of the MAP response during this period demonstrated a significant interaction term ($p<0.05$), although the main effect of RDX was not significant ($p>0.05$). MAP in RDX rats stabilized at a lower level than in SO rats for the next 9 days. The change in steady state MAP after RDX is shown in Figure 37. MAP was significantly reduced from the prazosin treatment period by RDX compared to SO (SO: 1.54±1.3 vs. RDX: −4.0±1.6; $p<0.05$).

Analysis of HR during the baseline and the prazosin treatment interval showed no significant difference between groups ($p>0.05$). HR became maximally elevated during prazosin treatment on day 7 of the study (pre-SO: 335.0±5.0bpm; pre-RDX: 335.0±4.0bpm) but gradually decreased. On day 13, 24hrs prior to RDX, HR was 300.0±3.0 bpm in SO and 300.0±6.0 bpm in RDX groups. Statistical analysis of HR after RDX also shows a significant interaction term between factors ($p<0.05$). Immediately after RDX, 24hr HR increased in RDX-treated SHR (318.0±4.0 bpm) but not in SO (306.0±2.0 bpm). HR then fell in RDX-treated SHR and remained reduced for 1wk. HR returned to SO levels within 10 days.

The hemodynamic response to the addition of atenolol to prazosin treated SHR after RDX or SO is shown in Figure 38. As previously discussed, prior to initiating beta-blocker therapy, MAP was significantly lower in RDX-treated SHR on prazosin compared to similarly treated SO SHR. MAP was maximally reduced by atenolol treatment within 4 days of administration. The maximal response to atenolol was similar between groups (SO: −27.8±0.6 vs RDX: −23.9±2.7mmHg; $p>0.05$). Analysis of MAP during atenolol treatment shows BP was not significantly different between groups: the change in steady-state MAP after atenolol administration was not different between groups (SO: -20.8±2.0 vs RDX: -19.9±1.8mmHg; $p>0.05$). HR was also reduced by beta-blocker therapy, but there was no difference between groups ($p>0.05$).

All drug treatments were discontinued after day 33 of the study, and hemodynamics were recorded for the next 11 days until the study was terminated (Figure 39). Although MAP was lower in RDX-treated animals, statistical analysis did not reveal a significant reduction between groups. Analysis of the change in steady-state MAP from baseline (Figure 40) also demonstrated a reduction in MAP compared to SO, but this was not statistically significant (SO: 3.0±4.0 vs. RDX: −7.7±3.5mmHg; $p>0.05$). HR was unchanged between groups after discontinuation of drugs ($p>0.05$).

Tissue NE contents measured from the LK, RK, and spleen at the end of the study are shown in Figure 41. SHR treated with RDX had a lower NE content in the LK (SO: 156±9.6 vs. RDX: 10.8±4.1 ng/g; $p<0.05$) and RK (SO: 147.1±8.5 vs. RDX: 62.1±13.2 ng/g; $p<0.05$), but not the spleen ($p>0.05$).

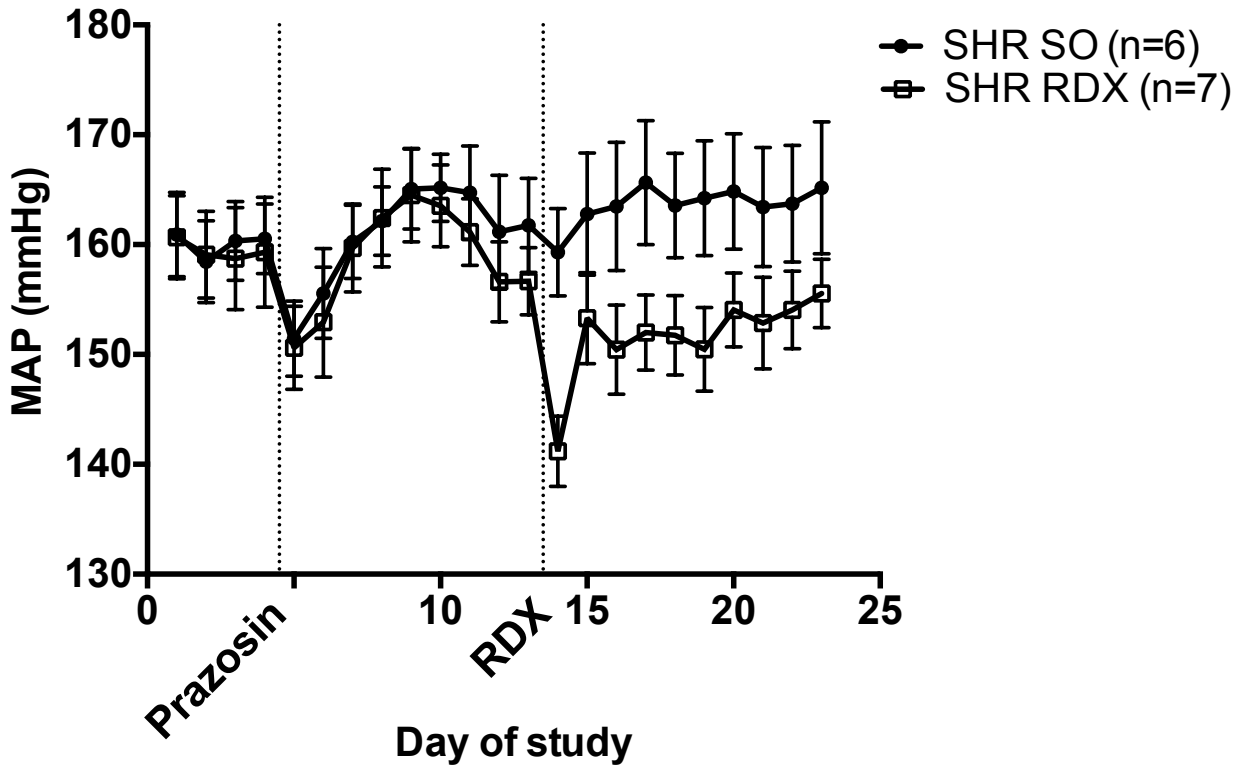
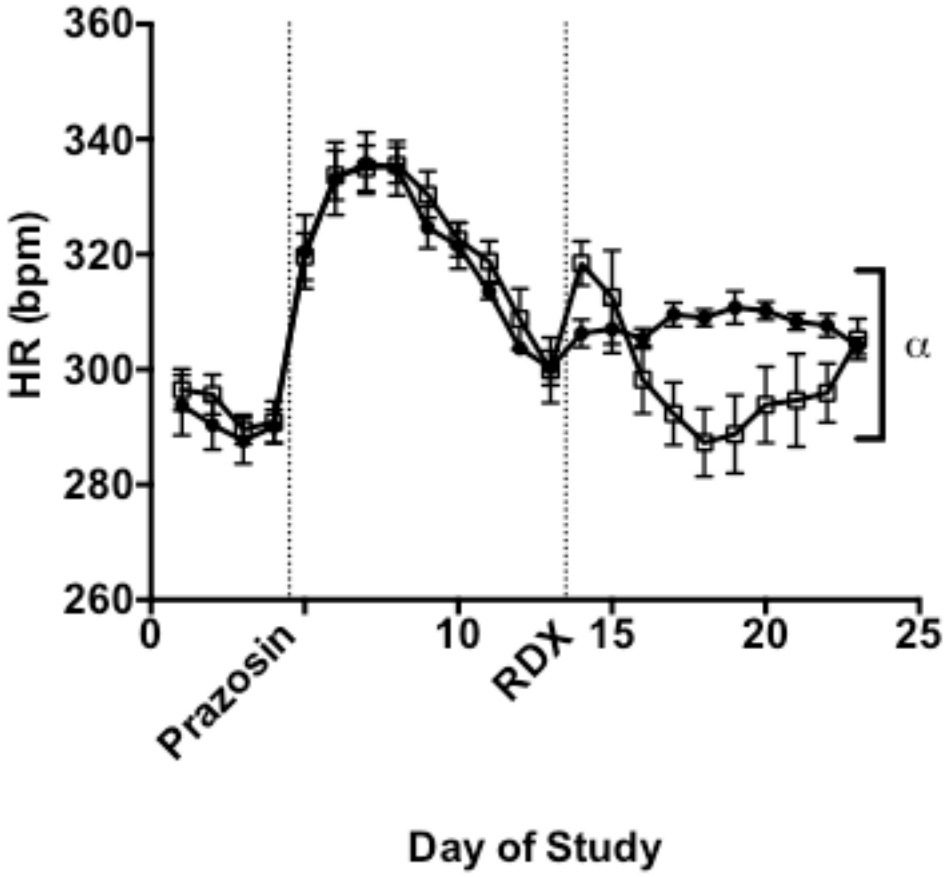


FIGURE 36. **<u>Hemodynamic response to RDX during prazosin treatment</u>**. TOP: MAP was reduced in RDX-treated SHR compared to sham operation once BP stabilized, although this reduction was not statistically significant (p=0.09). BOTTOM: HR was significantly reduced by RDX when BP stabilized; however, this effect did not persist beyond 10 days after RDX ($^{\alpha}p<0.05$ for interaction).

Figure 36 (cont'd)



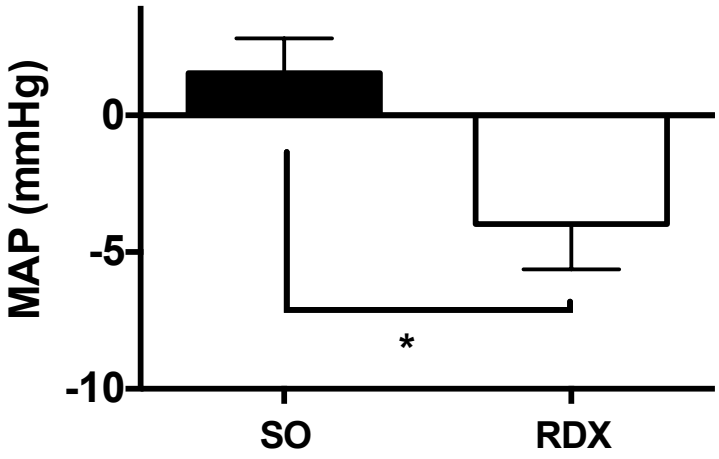


FIGURE 37. <u>The change in steady-state MAP after RDX during prazosin treatment</u>.

MAP was significantly reduced after RDX in prazosin-treated SHR. (*p<0.05)

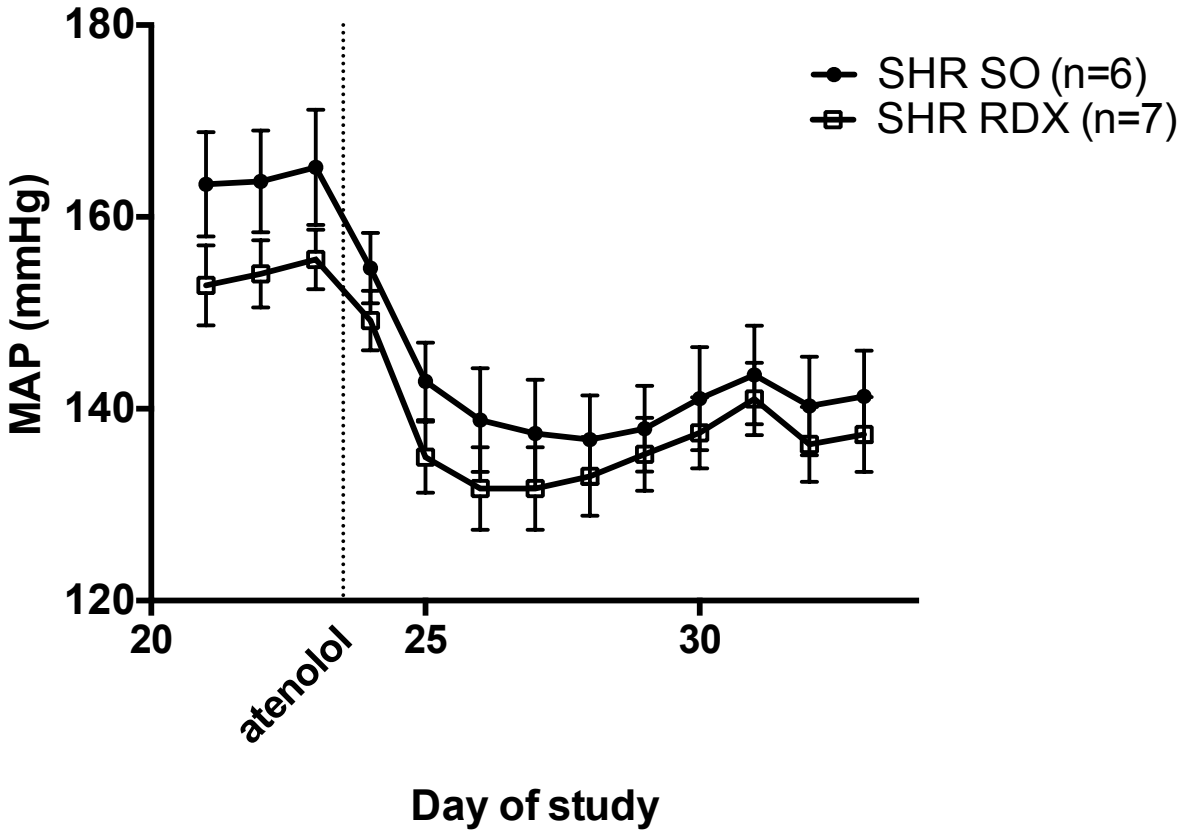
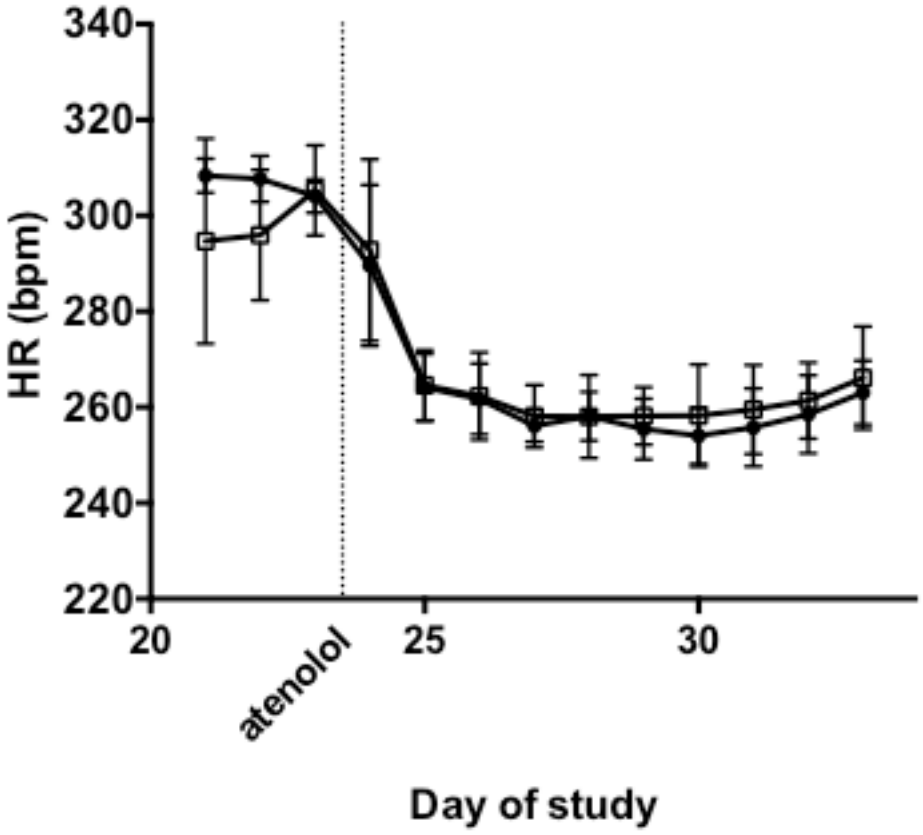


FIGURE 38. <u>The hemodynamic response to atenolol in prazosin-treated SHR that received RDX or sham surgery</u>. TOP: MAP fell similarly between RDX and sham-operated animals. BOTTOM: The fall in HR was nearly identical between groups.

Figure 38 (cont'd)



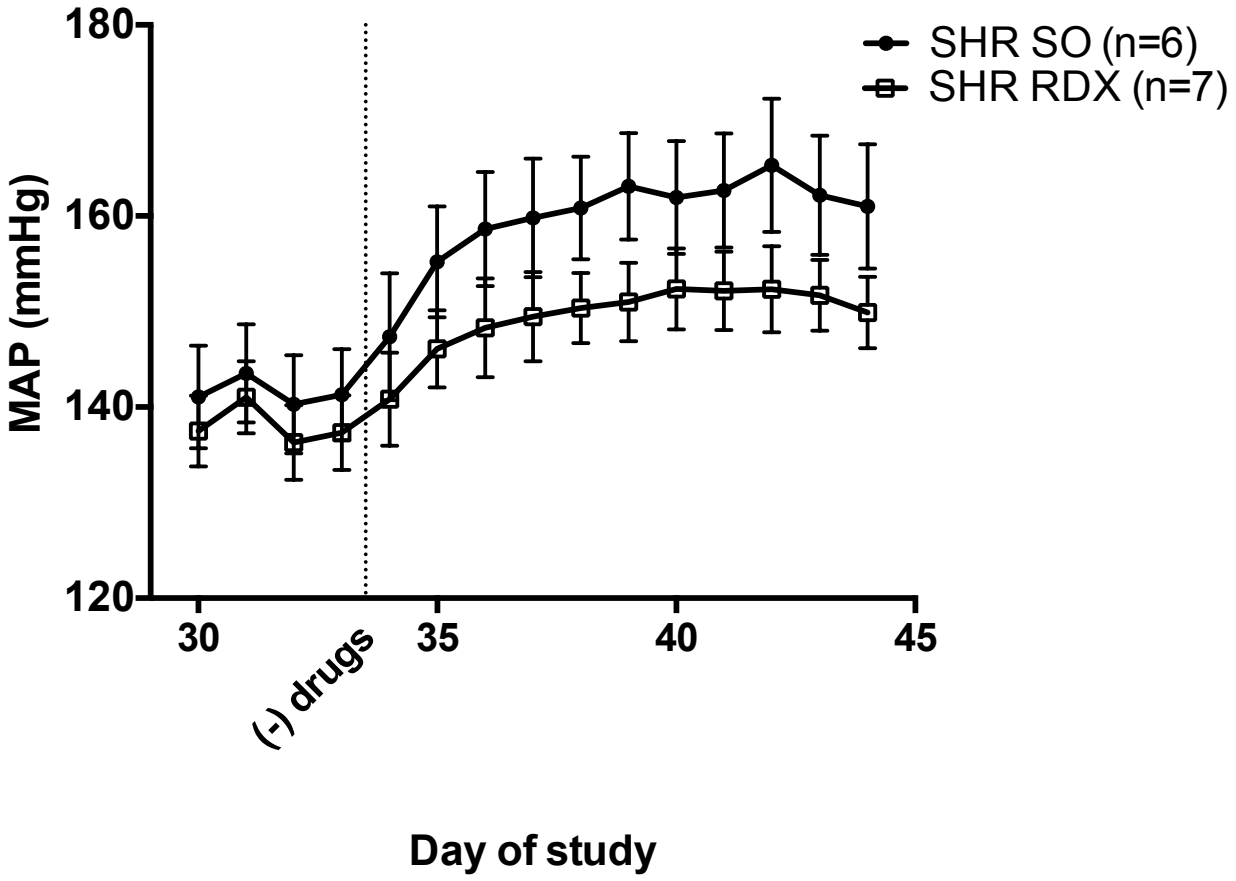
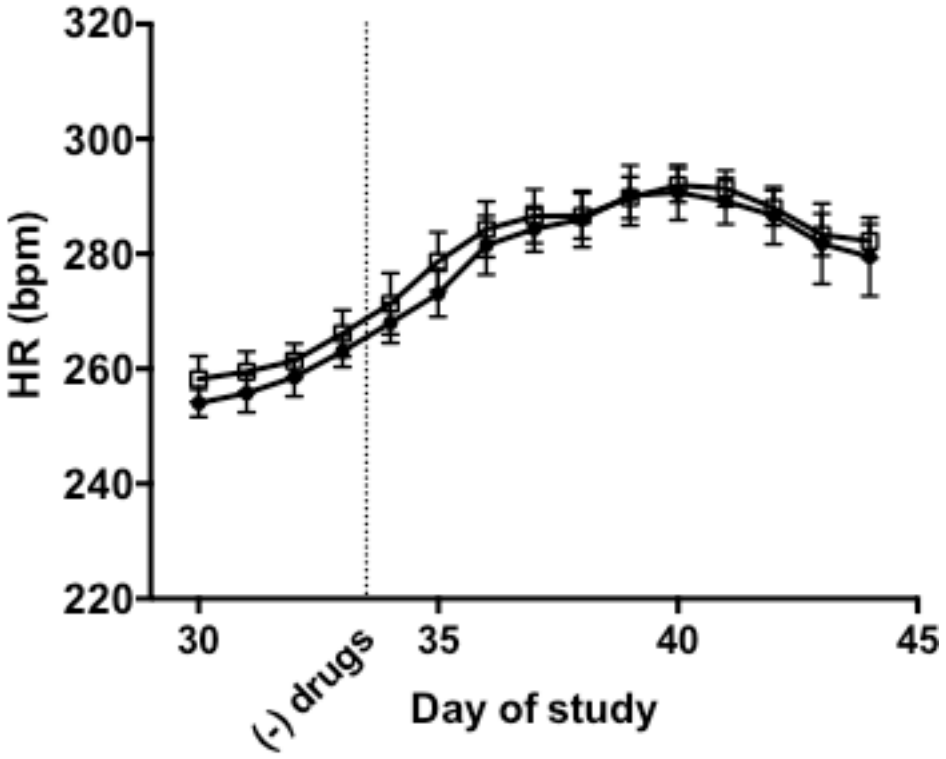


FIGURE 39. **Hemodynamic response after cessation of adrenergic antagonist therapy in SHR treated with RDX or sham procedure**. TOP: MAP is lower in RDX treated SHR; however, this was not statistically significant. BOTTOM: HR was not different between groups.

Figure 39 (cont'd)



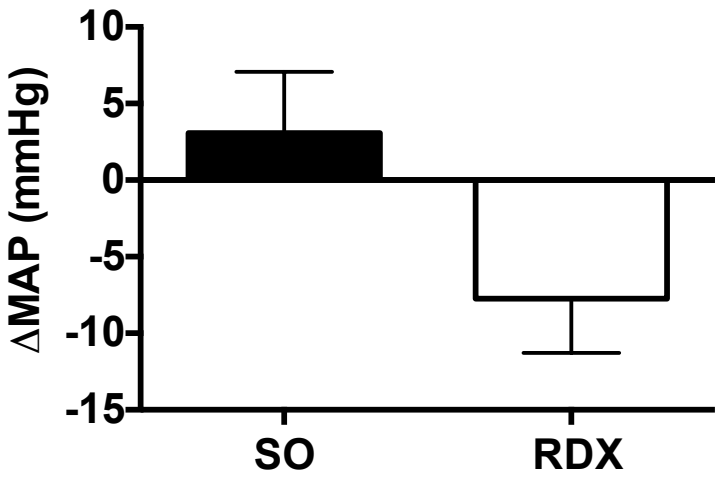


FIGURE 40. <u>The change in steady-state MAP from baseline at the end of the study.</u> RDX-treated SHR exhibited greater reduction in MAP from baseline compared to sham-operation; however, this was not statistically significant. (p=0.06)

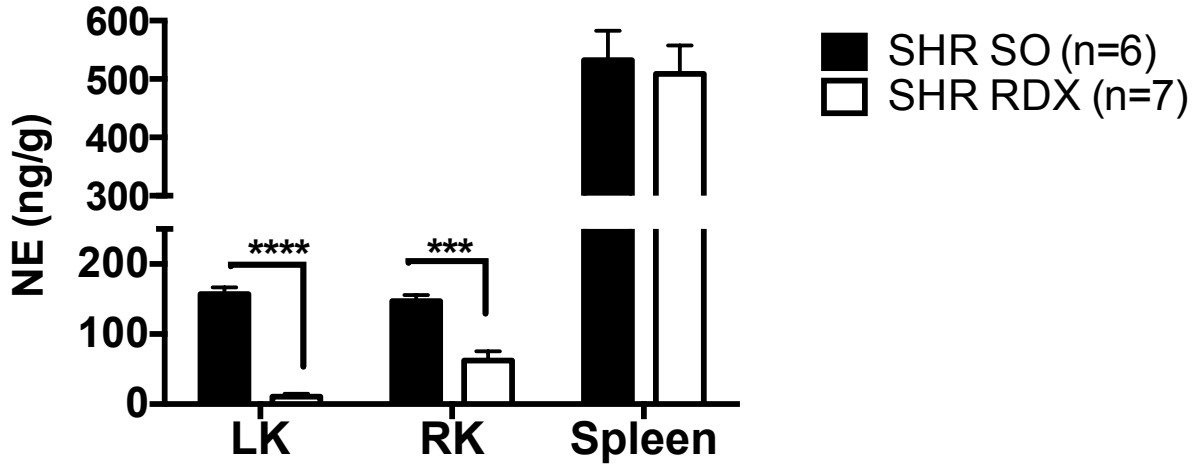


FIGURE 41. <u>Tissue NE content 30 days after RDX</u>. NE was significantly reduced in both the left kidney (LK) and right kidney (RK) by RDX, whereas splenic NE content was unchanged. (***p<0.001; ****p<0.0001)

DISCUSSION

The main finding from this study is that RDX lowers BP during α_1-AR antagonism but not during combined α_1-AR and β_1-AR antagonism. Unlike the observations from previous studies of RDX in the aged SHR, this study also shows that HR is significantly depressed after RDX in aged SHR being treated with a α_1-AR antagonist. These findings suggest that RDX is primarily lowering BP by suppressing a mechanism attributable to a β_1-AR signaling pathway, and that the BP response may also involve decreased cardiac function

In a previous study in pithed rats, a dose of prazosin (1mg/kg iv) prevented any change in BP following electrical stimulation of the SNS through the spinal cord [156]. In my studies, water intake in the SHR ranged from 30-50mL/day and mean SHR weight was approximately 400 g. With the bioavailability of oral prazosin at 56%, the prazosin concentration given in this study (3mg/ml) would be expected to suppress any α1-AR activity in these SHR rats [157]. However, I did not directly evaluate the degree of α1-AR caused by prazosin in my study.

Initiation of prazosin treatment is known to be accompanied by a reflex tachycardia [127]. Antagonism of the α1-AR reduces sympathetically mediated vasoconstriction, causing a fall in TPR and BP and thus a diminution of baroreceptor input to the CNS. The resulting baroreflex mediated elevation in HR boosts CO, and thus BP is partially restored to pretreatment levels. In my SHR I observed a similar response to prazosin.

MAP fell within 24hrs after prazosin administration but later steadily rose to baseline values, presumably due in part to elevated HR and CO (although I did not measure this directly).

Bilateral RDX in the prazosin-treated SHR lowered MAP immediately after the procedure and it remained lower than in SO rats as BP stabilized over the next ten days, although this was not statistically significant. The absolute difference in MAP between SO and RDX rats (−5.5 mmHg) was smaller than in most of my previous experiments. I expected that if RDX lowers BP primarily by interfering with a signaling process mediated through α_1-AR (either intrarenal or systemic), then there would be no chronic separation in MAP between SO and RDX-treated SHR. Although not statistically significant, MAP remained lower in RDX-treated SHR compared to SO rats. The lack of statistical significance could be explained by the high variability observed in the SO rats which has not been observed in my previous studies. This variability could be attributed to the fact that two independent studies were combined into one dataset. It is worth noting that in both independent studies, RDX during prazosin treatment produced an almost identically reduction in chronic MAP compared to SO SHR. I interpreted this repeatable finding to mean RDX lowers BP independent of the α_1-AR. Therefore my results do not support the hypothesis that RDX is primarily lowering BP through interruption of an α_1-AR mediated mechanism. This is surprising considering my earlier observations: in the presence of the sympatholytic drug clonidine I observed a loss of the BP response to RDX, as well as a reduction in plasma NE, a marker of sympathetic activity. These findings were interpreted to mean that the BP response to RDX is due to

a reduction in sympathetic activity. Given that RDX is certain to drastically reduce sympathetic activity to the kidneys, I thought it logical to conclude that the BP response was simply due to interruption of renal sympathetic activity. However, results from investigation of two renal processes linked to intra-renal adrenergic signaling, i.e. RAAS activity and sodium reabsorption, argued against that idea. Since renal sympathetic function governs the triad of RAAS activation, sodium reabsorption, and renal vasoconstriction, I therefore anticipated that blockade of α_1-AR mediated vasoconstriction within the kidney would prevent the BP response to RDX. This hypothesis was supported by work from DiBona's group that show that α_1-AR activity is the principal mediator of vascular activity within the kidney of the SHR [158]. This expected outcome did not occur. As noted earlier though, the reduction in BP after RDX in prazosin treated rats was not as robust as previously observed. In the aged SHR study from Chapter 3, RDX lowered MAP -5.2±2.0mmHg in the first two-week period. The reduction in MAP from baseline following RDX in prazosin-treated SHR was only −3.9±1.6mmHg during the same time period. While the difference between responses was quite small, RDX in the other studies produced a consistent reduction in MAP on the order of 10mmHg. Even in the untreated aged SHR study from Chapter 3, the reduction in MAP from baseline was −9.4±1.4mmHg at the end of the study. At the end of this study, steady-state MAP in RDX rats was only reduced 7.7±3.5mmHg. This may mean that the long-term BP response to RDX is partially due to loss of an α_1-AR mediated signaling process. My studies do not reveal whether the α_1-AR signaling process is intrarenal or systemic, and I did not directly measure the degree to which

renovascular resistance or systemic vascular resistance changed after RDX in either untreated or prazosin-treated rats.

Compensation from RAAS could also explain the blunted BP response to RDX during α_1-AR antagonism. Previous work has shown that in SHR treated with α_1-AR antagonists, BP falls more in response to an AT_1R antagonist than in normal rats, suggesting that the maintenance of HTN after α1-AR antagonism is driven in part by elevated RAAS activity [159]. As shown in Chapter 5, RDX effects on BP were not dependent upon RAAS activity at the AT_1R. Therefore, the pressor effects of the AT_1R would not be expected to be abated by RDX, and therefore they could defend against BP reductions during α_1-AR antagonism. This idea could be tested in future experiments.

There are several pieces of evidence that support the notion that α_1-AR would not be the dominant mechanism involved in a long-term BP reduction following RDX. Kline and Mercer documented development of super-sensitivity of the renal vasculature to NE within 2wks following RDX [94]. Later investigation documented a significant increase in renal α_1-AR in rats undergoing unilateral renal denervation without a change in the expression of other adrenergic receptors [160]. One would expect that in the presence of super-sensitivity and increasing α_1-AR expression after RDX, any reduction in BP attributable to this pathway would be lost over time, especially as reinnervation occurred. Instead, I documented in several studies that BP remains reduced in RDX-treated animals over several weeks after surgical intervention, and that prazosin

treatment only modestly reduced the treatment response to RDX. Therefore, it is unlikely that interruption of α_1-AR signaling in the kidney is mediating the long-term fall in BP. Measurement of renovascular resistance after RDX in untreated and prazosin-treated animals would help resolve this question. The acute fall in BP, observed 24-48hrs after RDX, also seems to be unaffected by α_1-AR blockade. The initial fall in MAP after RDX observed in untreated SHR (Chapter 3) was -19.6±4.5mmHg. In prazosin treated SHR, RDX initially lowered MAP -15.5±2.4. This observation would suggest that the acute fall in BP following is only slightly mediated by interruption of loss of the α_1-AR. Examination of the initial fall in BP other studies shows that it was blunted by both clonidine and low-sodium conditions where BP only fell -7.3±1.1mmHg and -6.7±3.9mmHg, respectively. This suggests that the initial fall in BP could be explained in part to loss of sympathetically- mediated sodium reabsorption. Curiously, as reviewed previously, the RSNA is thought to influence sodium reabsorption from the nephron by acting on the $\alpha_{1\beta}$-AR[11]. Prazosin is known to have similar potency at $\alpha_{1\alpha}$- and $\alpha_{1\beta}$-AR. Therefore, one would have expected prazosin to interfere with the initial fall in BP if it were mediated by sodium reabsorption mechanism connected to the $\alpha_{1\beta}$-AR. At this time, my data do not provide an explanation for initial large reduction in BP following RDX.

Addition of the selective β_1-AR antagonist, atenolol, during prazosin treatment in SHR further suppressed the BP-lowering effect of RDX. BP fell to a similar level in the SO and RDX rats, eliminating the statistical difference in BP between groups. After discontinuation of all medications, BP rose again but was lower in RDX-treated SHR. This suggested that the difference in BP between the RDX and sham SHR was linked to

a β_1-AR mechanism. Although we do not have a complete study showing the effect of β_1-AR antagonism alone, I do have preliminary results that demonstrate atenolol alone was sufficient to eliminate the BP response of RDX. In the second group of SHR entering this experimental protocol, atenolol alone was administered 12 days after discontinuation of all medications, and again the difference in BP between groups was lost. Discontinuation of the atenolol therapy restored the BP effect of RDX. While a larger study is necessary to confirm this finding, I am encouraged by the reproducibility and reversibility of this finding. I interpret these data to mean that the BP response to RDX is heavily influenced by interruption of a β_1-AR-mediated process.

Unexpectedly, I observed a significant depression in HR in prazosin-treated SHR during the first week after RDX. Heart rate increased after the initial fall in BP within the first 24hrs following RDX and returned to pre-operative levels within 48hrs. This observation was consistent with previous findings and suggests that the baroreflex is compensating for the sudden drop in BP observed after RDX. At 72hrs after RDX, HR began to fall and remained lower for several days. This may reflect a decrease in cardiac sympathetic drive, an increase in vagal activity or both. This could be explained by a decrease in sympathetic outflow from the brain which has been supported in both SHR and human studies [90, 113]. Other investigators have also demonstrated alterations in cardiac autonomic tone with RDX. Zucker's group reported that in a model of congestive heart failure, unilateral RDX reduced sympathetic tone to the heart and restored baroreflex sensitivity [161]. In an anesthetized porcine model of obstructive sleep apnea and atrial fibrillation, bilateral RDX was able to alter atrial fibrillation inducibility and atrial

effective refractory period during application of the negative tracheal pressure stimulus. The investigators noted that these anti-arrhythmic effects of RDX were attributable to the ability of the procedure to modulate autonomic balance at the heart [162]. In human patients receiving CBRNA, clinicians have documented reductions in markers of cardiac hypertrophy with improvement in diastolic function, even in patients without changes in BP; these effects have been suggested to occur due to reduced neurohumoral signaling to the heart [117]. In my study, I did not observe a difference in the fall in HR between groups with atenolol. If RDX was reducing adrenergic drive through cardiac β_1-ARs, then atenolol should have produced a lesser fall in BP in RDX-treated SHR in contrast with SO rats. However, in my study, initiation of selective beta blockade occurred after HR had normalized between groups. Earlier administration of a β_1-AR may be necessary to better capture this expected difference.

Interestingly, β_1-AR antagonism eliminated the difference in BP between SO and RDX rats after the relative bradycardia had resolved. This observation may point to the importance of a non-cardiac β_1-AR signaling process mediating the RDX effect. Given my previous finding that losartan therapy did not suppress the BP-lowering effect of RDX, it is unlikely that this response is related to a reduction in β_1-AR-mediated renin release. Therefore, other explanations are necessary. Although atenolol is less lipophilic compared to other beta-blockers, it has been documented to penetrate into the CNS [163]. Furthermore, atenolol was shown to suppress NE spillover in the SHR, suggesting the drug causes a global reduction in sympathetic activity [164]. One group has reported that atenolol suppresses efferent renal sympathetic activity [165]. DiBona

and colleagues also reported that intracerebroventricular administration of non-selective beta-blockers (propranolol and timolol) reduces the increased renal sympathetic nerve activity induced by stress [166]. These findings suggest that beta-adrenergic signaling in the brain can directly modulate RSNA function; however, it is not known what other specific sympathetic nerves could be altered by central beta-adrenergic receptor activity. One could interpret these data to mean that because atenolol eliminates the BP response to RDX in the SHR and acts to suppress RSNA, RDX is affecting BP through mechanisms driven by RSNA. This conclusion could prove true, but it seems premature. If RDX is lowering BP through a RSNA-dependent mechanism then AT_1R or α_1-AR antagonism should have prevented the BP response. My data do not support this thinking. Therefore, at this time my data are insufficient to conclude that NRSNA is not involved in the BP response to RDX. If the renal nerves are capable of influencing NRSNA, it would likely involve transmission of information along the renal sensory afferent nerves. Recall from Chapter 4 that renal deafferentation did not produce a BP response in my model. However, as discussed earlier, the extent to which capsaicin treatment can completely eliminate all functional afferent nerves from the kidney is not currently known. If NRSNA support of BP is reduced by RDX, it is likely that renal sensory nerves unaffected by capsaicin treatment are mediating this process. Further work is necessary to confirm whether capsaicin-insensitive renal sensory nerves regulate SNA via a pathway involving β1-AR in the brain.

It should be mentioned as a caveat to this study that renal sympathetic nerves release other neurotransmitters beyond NE. As mentioned in Chapter 1, NPY and ATP are co-

released with NE, but the actions of these substances are largely ignored in this study. Both NPY and ATP may serve roles to potentiate or inhibit NE release, but the primary mediator of the renal sympathetic nerve support of BP is NE [11]. Therefore, it was logical to focus exclusively on the interruption of noradrenergic signaling pathways before exploring the involvement of alternative neurotransmitters.

In summary, this study confirms my clonidine data by suggesting that the BP-lowering effect of renal denervation is indeed connected to loss of one or more sympathetic mechanisms. Data shown in this chapter point especially to the importance of a β_1-AR-signaling pathway that is not exclusively related to cardiac function. This sympatholytic effect may be centrally mediated, a finding which underscores the importance of afferent renal nerve signaling to the brain.

CHAPTER 8 – CONCLUDING DISCUSSION

The experiments described in this dissertation aimed to use the aged spontaneously hypertensive rat to understand the mechanisms underlying the fall in BP associated with renal denervation. In this final chapter, I will summarize my main findings, discuss my overall interpretation for my observations, detail possible implications for CBRNA in the clinic, and provide some ideas for future research.

Overall discussion and future directions

RDX in the aged SHR as a model for CBRNA in humans

In Chapter 3, I showed that bilateral renal denervation significantly lowered ambulatory BP in the aged SHR. The BP reduction was modest, stable over long periods of time, and the fall in BP was of similar magnitude to what is reported in humans. I also showed that steady state HR is not affected by renal denervation which was also reported in humans. My conclusion from this initial study was that RDX in the aged SHR should be considered as a credible model for understanding how CBRNA influences BP in the human.

The aged SHR was not the first model I tested in the search for an experimental animal with established hypertension that would respond to RDX with a sustained fall in BP. Although the data were not shown in this dissertation, I attempted RDX in both the DOCA-salt model and the stroke-prone SHR (SHRSP). Both of these models are listed in TABLE 1 as having been shown to respond to RDX with a delay in or blunting of the development of HTN. The result in the DOCA-salt rat is not too surprising as recent telemetric recording of BP in DOCA-salt rats receiving renal denervation or a sham-

operation showed that the renal nerves are more important in the pathogenesis of the HTN in this model than in the maintenance of the high BP [167, 168]. Curiously, a recent paper indicated that RDX in the 9-wk-old salt-loaded SHRSP was sufficient to lower BP and provide significant survival benefits, possibly due to attenuated oxidative stress in the brain [169]. In my hands, RDX in older SHRSP (12-14wks) did not significantly lower BP. Intervening at an earlier age in the SHRSP may have yielded similar results to what was published in Nakagawa et al., but I would question the clinical relevance of using this model because in human patients anti-hypertensive treatments are not administered during HTN development. Considering the BP responses I observed in much older SHR, it would be interesting to examine whether RDX in much older SHRSP might produce similar results on BP and oxidative stress in the brain. Additionally, one could also evaluate oxidative stress in the brains of aged SHR treated with RDX. If this is a shared mechanism between models, it could provide an explanation as to how RDX might be influencing β_1-AR signaling in the brain, which is a mechanism I propose could mediate the fall in BP in the aged SHR.

The usefulness of the aged SHR as a model for exploring RDX became more apparent with each additional study I performed. First, in each study presented herein, RDX was shown to lower BP compared to the sham controls. The consistent reproducibility of the BP-lowering effect following RDX makes for a compelling argument that this response is indeed real. Second, beyond the consistent nature of the BP response, I think the comparable magnitude and duration of the BP changes in aged SHR compared to humans is noteworthy. As pointed out in TABLE 2, the magnitude of change in

ambulatory BP following RDX is well within the range of what is generally seen in patients. This suggests to me that the renal nerve contribution to blood pressure regulation in aged SHR is very similar to hypertensive humans and therefore similar pathological processes may be responsible. Interestingly, the reduction in BP after RDX was present to the end of every study I performed in the SHR. Regardless of what additional pharmacological interventions were performed in these rats, RDX SHR always showed lower BP at the end of my studies compared to sham-operated rats. While the results in the prazosin study may be an exception to this statement, as MAP was not significantly reduced from sham-operated levels, average MAP was lower in RDX rats compared to SO rats at the end of that study. Also like in humans with resistant hypertension that are treated using CBRNA, RDX in the SHR did produced a gradually increasing fall in BP over time [113].Human patients also show a reduction in BP following CBRNA that lasts at least 5 years [8]. In my studies I did not show that the BP lowering effect of RDX in SHR persists for years, but it was documented to exist at least 13wks. Considering that the lifespan of an SHR rats is only approximately 12-20 months, compared to the human lifespan of 73yrs, this 13wk reduction in BP should be considered lengthy in duration and quite significant [107, 112]. What is most interesting about this long-term reduction in BP in the aged SHR is that it persists beyond the time point when the kidneys are expected to reinnervate [69, 94]. One would expect that if sympathetic reinnervation occurs in humans and rats subjected to renal denervation, and the BP-lowering effect is mainly due to loss of renal sympathetic activity, then the effect of renal denervation should be abolished once the nerves regrow and regain function. Since we do not see this effect in either species, it could mean that the

restored renal nerves are either non-functional or functionally different from the pre-surgical nerves. Although I did not address this hypothesis in my studies, I think additional efforts should be directed at understanding how RDX may be altering nerve function in regenerated renal nerves.

As mentioned in chapter 1, early reports of CBRNA in humans included measures of renal NE spillover as an index of effectiveness of CBRNA in producing denervation of the kidneys [3, 170]. Thus far NE spillover has been the only clinical marker of "successful denervation" of the kidneys. Esler's group showed that renal NE spillover was reduced an average of 47% when measured 30 days after CBRNA [170]. By contrast, the open surgical procedure I used reduced renal NE content by as much as 98% within two weeks after surgery. Eleven weeks after RDX, the reduction in renal NE content in the aged SHR is more modest, ranging between a 40% to 60% reduction. Thus one concern is that the surgical technique for RDX may not adequately represent what occurs in patients with CBRNA. In my opinion there are three arguments that mitigate this limitation of my experimental model. The first is practicality: There is no device available to subject rats to CBRNA. Second, there are currently no data available that CBRNA in humans is incapable of depleting tissue NE content (hence the degree of renal sympathetic innervation) to an extent similar to that in rats. The earliest time point renal NE spillover has been measured is 30 days after CBNRA. No human studies have looked at the effect of CBRNA on these parameters at earlier time points. It is possible that CBRNA reduces renal sympathetic innervation to a greater extent than what has been described to date. For example, my results from Chapter 3 clearly show that renal NE content is reduced more at 2wk than at 11wks after RDX demonstrating tissue NE is

restored over time. Kopp and colleagues have recently reported similar results [69]. In fact, reinnervation after renal denervation has been well studied and known to occur for some time [94]. Therefore, one could hypothesize that similar events are occurring within the 30 days that pass after CBRNA in patients, i.e. NE content (as reflected by renal NE spillover) is much lower immediately after CBRNA. A more complete examination of NE spillover in humans would verify the validity of this claim, but this is unlikely to be done because of ethical constraints.

Third, the technique I used allows the question of the relationship between the degree of denervation and the BP response to be addressed more quantitatively. From all of my studies, I have gathered renal NE content, final MAP, and change in MAP from baseline from each animal that underwent the RDX or sham procedure. From these data, I can begin to ask the questions, “Does terminal renal NE content predict the BP response to RDX?” and (if so), “How well does this predictive model align my data with what is reported in human patients?” A correlation analysis shows a significant positive correlation between the change in MAP from baseline and total renal NE content (Figure 42; $r = 0.50$; $p=0.001$). A Deming linear regression analysis also shows a statistically significant relationship between renal NE content and change in BP after RDX ($p=0.00$; $y=5.452x+157.8$). Similar significant relationships held for the change in systolic BP ($y=5.430x+166.4$) and diastolic BP ($y=5.495x+151.7$). Kidneys from SO rats had an average NE content of 133.4±2.8ng/g.

This analysis reveals several important points. First, the equations presented above (MAP, SBP, or DBP equations) allow me to predict that in the event of no change in BP following RDX, the kidneys would have renal NE content similar to what is observed in

sham kidneys (i.e. no denervation). Second, my analysis shows that in the event of maximal renal NE depletion (i.e., the perfect renal denervation), MAP would be expected to fall a maximum of 29 mmHg (x-intercept). One might expect the greatest fall in MAP to occur with the first few days after RDX when renal NE is maximally depleted. In none of my studies do I report a fall in BP greater than 29mmHg. The greatest reduction in MAP after RDX was 28mmHg (losartan study), and subsequently, these animals also had the lowest tissue NE at study termination. These data emphasize the strong relationship between the degree of renal sympathetic efferent denervation and the magnitude of the BP effect [94, 160][94, 160][94, 160][94, 160][94, 160][89, 154][89, 154]. One of the largest challenges currently facing interventionalists utilizing the CBRNA technology is the determination of the degree of renal denervation immediately following RF energy application. If the BP response is indeed correlated with the degree of denervation it will be important to accurately predict the efficacy of nerve ablation intra-operatively. In my opinion, more effort should be directed at identifying biochemical or functional markers of nerve damage to optimize the BP response to renal denervation.

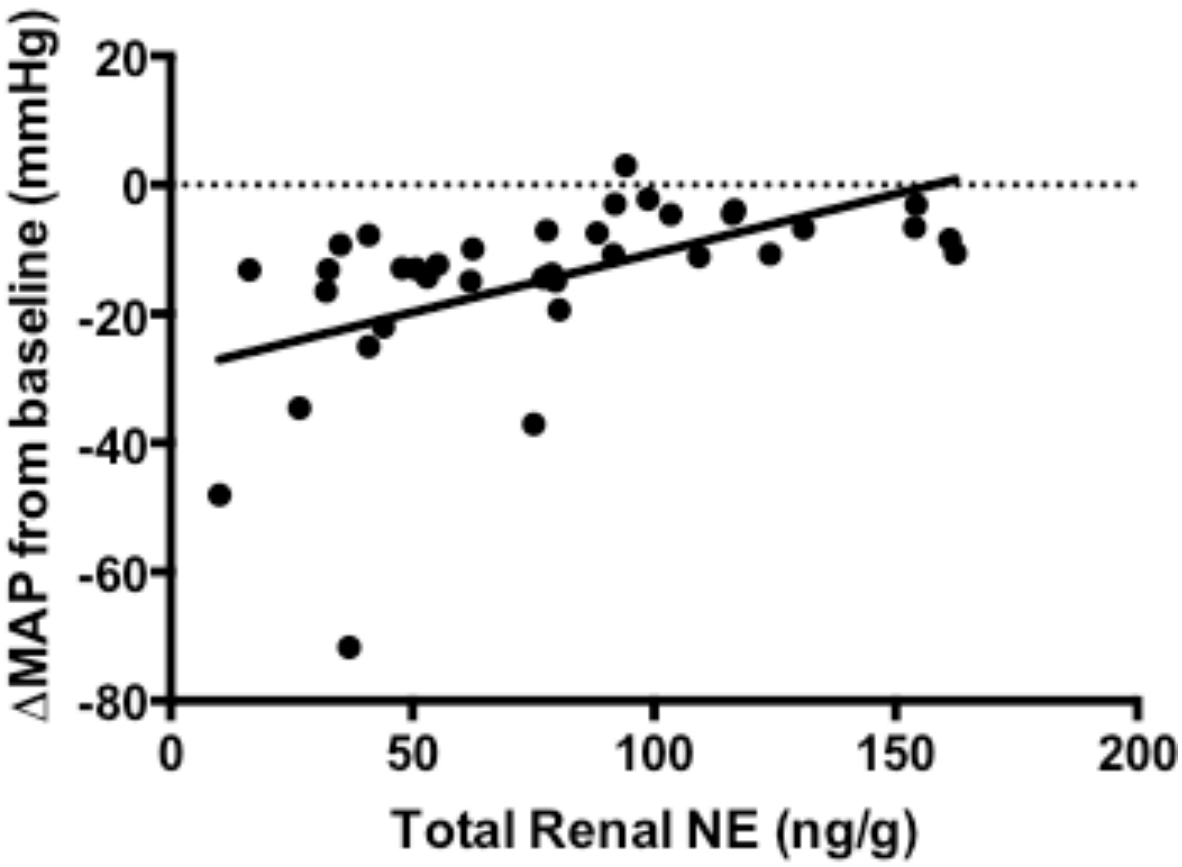


FIGURE 42. **Scatterplot of total renal NE content plotted against change in MAP from baseline from every RDX SHR presented in this dissertation**. Correlation analysis shows a significant positive relationship between the two variables (p=0.001). Deming linear regression also reports a significant relationship and describes the line of best fit as y=0.1834x – 28.9.

Finally, it is important to note how my data relate to Esler's work in humans. The Symplicity HTN 1 trial reported that in 10 patients the average reduction in renal NE spillover was 47% of pre-intervention measurements [2]. Using the regression analysis from my data, a 47% reduction in renal NE content would be expect to elicit a 14.6mmHg decrease in ambulatory SBP. The reported reduction in ambulatory SBP in the trial was 11mmHg [2]. While I do not have enough data to statistically compare these two observations, it is interesting to note the similarity between the expected and observed responses. Overall, I conclude that my model is behaving very similarly to what has been described in patients with resistant hypertension, and that the aged SHR is a credible model for studying how CBRNA lowers BP in humans.

Mechanisms by which RDX lowers BP in the aged SHR

The main focus of this dissertation work was to identify the mechanisms that explain the RDX-mediated fall in BP. In Chapter 3, using a very straightforward approach, I demonstrated that the BP response to RDX is primarily caused by a sympatholytic event. In the presence of clonidine, the anticipated reduction in BP after RDX did not occur. Upon cessation of clonidine treatment, BP was lower in RDX treated SHR compared to SO rats. I conclude that the mechanism that promotes the sustained fall in BP after RDX is also affected by clonidine. As discussed previously, clonidine is an α2-AR agonist, but it also binds to imidazoline receptors. The exact cellular mechanism by which clonidine lowers BP is not fully understood. [122]. Nevertheless, clonidine exerts its hypotensive effect mainly by decreasing sympathetic outflow from the CNS and by suppressing catecholamine release from adrenergic nerve terminals [122]. Specifically, clonidine has been documented to decrease nerve activity in pre-ganglionic splanchnic

fibers and in post-ganglionic cardiac and renal nerve fibers [120, 122]. In support of the hypothesis that clonidine causes sympatho-inhibition I documented a reduction in plasma NE and HR from baseline in SO and pre-RDX SHR treated with clonidine administration. I also showed an elevation of both parameters upon clonidine withdrawal.

It is noteworthy that BP was lowered an almost identical amount in aged SHR by clonidine induced sympatholysis and by RDX. Furthermore, cessation of clonidine therapy led to very little change in BP in RDX rats. Therefore it is tempting to speculate that clonidine would not be effective in lowering BP in RDX rats. I do not have data to support this claim, however, and I think it unlikely to be true. Clonidine significantly suppressed plasma NE and HR while RDX in untreated SHR did not alter either parameter. I interpret this to mean that RDX is not suppressing SNA to the same extent as clonidine, and clonidine should still elicit a hypotensive effect in previously renal denervated SHR. In the human trials, there is very little detailed data regarding changes to medications in patients that received CBRNA as adjustments in medications are typically avoided by trial design. In a follow-up study to Symplicity HTN 2, investigators report that 46% of patients receiving CBRNA had a medication change with 18.6% requiring an additional medication [9]. We currently have a very limited understanding about how adding a central sympatholytic, or other sympatholytic drugs, to renal denervated patients might alter BP. This information will likely be valuable, as some patients receiving CBRNA will need changes to their medication regimen to sustain a BP reduction. However, as discussed previously, a retrospective analysis indicated that patients being treated with a central sympatholytic drug were actually *more likely* to

show a favorable BP response to CBRNA [8]. This could indicate that mechanisms other than sympatholysis are important to the BP-lowering effect of CBRNA although my data in this dissertation do not support this conclusion. Alternatively, since the subjects were all drug-resistant (i.e. did not show an adequate decrease in BP on drug therapy), it may show that patients with high SNA *despite* central sympatholytic therapy are good subjects to receive CBRNA. Further clinical and experimental investigations are necessary to understand the relationship between central sympatholytic drug treatment and BP response to CBRNA.

My findings with clonidine also support the notion that higher basal sympathetic activity at baseline might predict a larger response to CBRNA. Esler and colleagues recently published a study demonstrating a reduction in MSNA and BP in 35 patients undergoing CBRNA [77]. Analysis of the basal MSNA and the BP response to CBRNA showed a non-statistically significant trend toward a larger BP reduction in those patients with higher basal MSNA [77]. Analysis of basal plasma NE (as an indicator of global SNA) and the final BP response to RDX in my aged SHR rats also showed a similar trend ($p=0.07$). Although not statistically significant, I think these observations are clues that basal sympathetic activity could be an important variable for predicting the response to CBRNA. Further work is necessary to confirm this hypothesis.

Importantly, it is not clear whether the reduction in sympathetic activity caused by RDX (or clonidine) occurs only in the kidneys or whether non-renal SNA also is reduced due to loss of sympathoexcitatory drive from renal afferent nerves as hypothesized by other

authors [21, 116, 171-174]. Although the sources of sympathoexcitatory afferent activity from the kidney have not been rigorously identified, elimination of CGRP containing afferent nerve fibers with capsaicin treatment did not chronically alter MAP in my animals. I concluded that these afferent nerve fibers are not involved in the BP response to RDX.

As discussed earlier, deafferentation via dorsal rhizotomy was shown to elicit a BP reduction in adult SHR (18wks) compared to sham-operated controls [93]. Since I could not replicate this finding using the more selective renal capsaicin approach, the discrepancy could be explained by the non-selective nature of dorsal rhizotomy. Total sensory denervation of kidney with dorsal rhizotomy would damage capsaicin-sensitive and insensitive nerve fibers, both of which may be sympathoexcitatory [93, 174]. Alternatively sensory denervation of other organs by dorsal rhizotomy could also explain the fall in BP. More rigorous understanding of the role capsaicin-insensitive renal sensory nerves play in BP regulation is necessary. There is currently no method available to selectively denervate those nerve fibers. In addition to requiring better understanding the renal afferent nerves, we have little evidence to suggest which region of the brain may be mediating their reported effect on BP. Pseudorabies virus labeling studies show renal sensory nerves feed to the nucleus tractus solitarius (NTS) and the nodose ganglion of the brain [15]. We currently have no data to show whether denervation methods differentially effects nerve pathways projecting to either nuclei. Attention to biochemical or electrophysiological changes in these areas of the brain after RDX may prove to be valuable for determining how interruption of renal sensory information is lowering BP through central targets.

Finally, it is conceivable that it may be necessary to denervate both neural axes simultaneously to achieve a full BP response to RDX. Kopp and colleagues demonstrated that some renal afferent nerves are important in mediating the reno-renal reflex, which serves as a sympathoinihibitory feedback mechanism on renal efferent sympathetic activity [7]. Her group reports that the reno-renal reflex in the SHR is impaired [124], and her work also shows that renal deafferentation through dorsal rhizotomy may promote hypertension primarily through loss of regulation of the efferent renal nerve axis [175]. Wang's group at Michigan State has also shown that global loss of capsaicin-sensitive sensory nerves contributes to elevations in BP [176, 177]. Given the reported dysregulation of efferent renal nerve activity that can occur in the absence of certain renal sensory nerves, removing both efferent and afferent nerves may be critical in lowering BP. Therefore, development of a method to selectively remove renal efferent nerves would aid in our understanding of the interplay between renal sensory nerves and sympathetic support of BP. In an unpublished experiment, I sought to use topical application of 6-hydroxydopamine (6-OHDA) on the renal vasculature to selectively destroy nerve fibers that expressed the catecholamine reuptake transporter, i.e., renal efferent nerves [178]. I was unable to deplete tissue NE content with this approach. However, further optimization of this approach or use of similar techniques could provide valuable insights into the question of whether or not elimination of both renal nerve axes is required to lower BP chronically.

As noted earlier although the clonidine study revealed the importance of sympatholysis in the BP response to RDX, I could not determine from that study whether reductions in

RSNA, NRSNA or both were required. Therefore, I undertook additional studies to evaluate whether the BP response to RDX was associated with interruption of two known RNSA-dependent physiological BP control mechanisms: RAAS activation and renal tubular sodium reabsorption.

The losartan study presented in this dissertation demonstrated that blockade of the RAAS pathway at the AT_1R with the ARB losartan had no effect on the BP response to RDX. I was able to significantly lower BP with RDX even after BP had already been decreased with the losartan (10mg/kg) treatment. Furthermore, discontinuation of the ARB caused BP to rise without influencing the BP response to RDX. This suggested to me that RDX and losartan are lowering BP by two separate mechanisms, and I concluded that the sympatholytic event associated with RDX in the SHR is not inhibition of the RAAS (presumably via renin release). From a clinical perspective these findings demonstrate that pharmacological agents that influence RAAS activity, particularly at the AT_1R, are not likely to interfere with the BP response to CBRNA. Therefore, it may be advantageous to pair CBRNA and RAAS inhibitors to optimize BP control in certain patients. As discussed previously, there are a few caveats to my losartan study to consider. I repeat them here only to add insight into additional experiments that could performed to validate my conclusions. First, the tissue NE results showed a very atypical reduction in splenic NE. This indicates that the BP response to RDX could have been due to non-specific denervation. As explained earlier, this conclusion may be erroneous given the selective nature of every other RDX study I performed. I attribute this finding to random chance. Repeating this study would provide additional evidence as to whether the BP response to RDX requires only renal denervation or not. Second,

the RAAS pathway has many signaling mediators upstream from AngII-AT1R interaction. It would be interesting to observe whether direct renin inhibitors or angiotensin converting enzyme inhibitors (ACE-I) have different effects than an ARB on the BP response to RDX. Such a study would indicate that these upstream signaling mechanisms are more important in the BP drop compared to the AT1R.

Very large alterations in sodium intake in the aged SHR had no effect on the steady-state difference in BP between SO and RDX-treated rats. I concluded from this observation that the long-term reduction in BP observed with RDX is likely not connected to a diuresis/natriuresis mechanism, which is classically blunted in sodium-replete conditions [122]. This suggests that the BP response to CBRNA should not be influenced by a patient's sodium intake, i.e. a patient does not need to be on a low-sodium diet (or be further sodium depleted with diuretic therapy) to maximize their BP response to CBRNA. This is important as patient compliance with low-sodium diets can be problematic without the use of extensive motivation tools and elaborate training mechanisms [179]. Although it is currently recommended that patients with HTN avoid excess sodium intake, it is encouraging to note that the response to CBRNA will likely not be influenced by patient diet. It is also important to note that the current definition of drug-resistant HTN includes that patients be on 3 or more medications including a diuretic [5]. While I do not present any data to evaluate the BP response to RDX during combination anti-hypertensive treatment, my data do suggest that addition of a diuretic to a drug regimen would not impede the BP lowering effect.

My final set of experiments examined the BP response to RDX under conditions of adrenergic blockade, since adrenergic receptors mediate the renovascular, renin and sodium transport actions of RSNA. Furthermore, the cardiovascular effects of NRSNA also are largely produced by adrenergic receptor activation. Although this overlap complicates the interpretation of experiments employing systemic administration of adrenergic blockers, I obtained some interesting and informative results.

First, antagonism of the α_1-AR with prazosin did not prevent RDX from lowering BP compared to SO rats. MAP fell immediately after RDX and stabilized at a significantly lower level than in the SO group. It should be noted, however, that the steady-state MAP response to α_1-AR antagonism was numerically less than what I typically found in untreated 36wk SHR. This may indicate some role for loss of α_1-AR signaling activity in the BP-lowering effect of RDX. Nonetheless, because α_1-AR signaling occurs in most tissues of the body, it is still not possible for me to identify which sympathetic nerve targets are most influenced by RDX. Electrophysiological recording of select regional sympathetic nerves in SO and RDX-treated SHR would allow better discrimination of where RDX is exerting its influence over BP. Paton's group has already shown that RDX reduces lumbar sympathetic nerve activity in 12wk old SHR [90]. My lab is currently working on telemetric recording of regional sympathetic activity beyond the lumbar nerve in conscious, freely moving rats. Additional experiments involving combined regional sympathectomy and renal denervation could be useful in resolving

possible involvement of non-renal targets as well. Theoretically, use of drug delivery methods to promote tissue specific α_1-AR antagonism could also be employed to evaluate where RDX is exerting its effect. For example, by implanting a catheter into the renal or supra-renal artery, an α_1-AR antagonist could be delivered directly into the kidney prior to RDX. Additionally, other experiments could use this delivery system to administer an α_1-AR agonist after RDX to attempt to reverse the BP lowering effect. This may only be a theoretical approach as catheter placement could itself cause inadvertent renal denervation. One could consider approaching the question by developing SHR strains with tissue specific α1-AR deletions, but this process is likely to be time intensive and cost prohibitive. A mouse model would be much more conducive to this sort of molecular genetic approach.

During α_1-AR antagonism I observed a sub-chronic reduction in HR in RDX treated SHR that was not observed in my other studies. Addition of the beta-blocker atenolol after HR had returned to sham levels attenuated the difference in MAP between RDX and sham SHR. BP was allowed to stabilize during combined atenolol and prazosin treatment and the BP lowering effect of RDX was no longer evident. I concluded from these findings that RDX likely lowers BP in the SHR at least in part through a β_1-AR-mediated mechanism. I proposed that these mechanisms are likely originating in the brain and are not related to suppression of neurally mediated renin-release. The losartan data support this claim. However, it is not entirely clear what central β_1-AR receptor processes are involved or the brain region involved. β_1-AR antagonists are not traditionally thought to lower BP due to actions in the brain, especially since beta-blockers with lower lipophilicity show anti-hypertensive effects [122]. It is clear from several studies,

however, that relatively hydrophilic beta-blockers, such as atenolol, can penetrate into the brain and bind β_1-AR [163, 180]. This demonstrates that the original notion about certain beta-blockers not being able to enter the CNS is likely incorrect. Furthermore, it has been shown that atenolol equally lowers BP in rats treated with either a sham-operation or stellate ganglionectomy to sympathetically denervate the heart [100]. Since, as expected, HR was not altered by atenolol treatment in rats with stellate ganglionectomy, the BP lowering effect of the drug was due to interruption of β_1-AR signaling at sites other than at the heart. It has been suggested that use of a β_1-AR antagonist lowers BP by decreasing SNA to the muscle as MSNA fell in patients receiving the drug [181]. This paper suggests that β_1-AR activity could be mediating central sympathetic outflow to different vascular beds. However, a recent publication reports that MSNA and calf vascular resistance were not changed in 14 patients treated for 8wks with atenolol [182]. The authors of both studies also discussed the possibility that modification of central sympathetic activity during chronic β_1-AR antagonism could involve adaptation of a baroreflex loop. Indeed, others have shown atenolol increases baroreflex sensitivity in addition to lowering BP [183]. If RDX is lowering BP by interrupting a central β_1-AR mechanism connected to the baroreflex, then SHR subjected to RDX should show improvements in baroreflex function. In fact, Paton's group has demonstrated RDX in SHR does improve baroreflex sensitivity [90]. They have also shown similar findings in humans. Although it is unclear what precise β_1-AR pathway might be mediating the BP response to RDX, these data indirectly support my conclusion that RDX lowers BP by acting on a β_1-AR mediated pathway and that it may involve changes in baroreflex function. Although my data do not specificially address

baroreflex function after RDX, I do show a consistent elevation in HR after RDX compared to SO rats. This even occurs with renal deafferentation where there is no significant BP change. This certainly suggests that the baroreflex is modified by RDX, but development of more carefully designed experiments is necessary to confirm this hypothesis. Additionally, it is well known that while beta-blockers are generally safe, side-effects associated with these drugs include drowsiness, sleep disorder, depression, and hallucinations. This indicates that, again, these drugs can influence brain function. I conclude that RDX is likely reducing BP by affecting a central β_1-AR signaling pathway. This may be mediated by interruption of capsaicin-insensitive renal afferent nerves.

I do not have complete data from a separate study where RDX is performed during atenolol treatment. As previously mentioned though I do have preliminary data showing that atenolol alone is sufficient to eliminate the BP response to RDX. These preliminary results support my claim that RDX is lowering BP through interruption of β_1-AR mechanisms. An appropriately powered study examining the BP lowering effect of RDX in atenolol treated SHR would provide definitive insight into whether these preliminary results are accurate. My lab is currently undertaking this experiment.

I should note that the relevant β_1-AR mechanisms could be both distinct and time specific. My data show early reductions in MAP may be supported by a decrease in HR, which could be explained by a decrease in sympathetic drive to the heart, while at later time points when HR is restored, other non-cardiac β_1-AR receptor signaling processes

may be involved. An additional experiment could be undertaken to understand the mechanism behind the subchronic reduction in HR observed in prazosin-treated SHR receiving RDX. We do not currently know if this response fosters the BP reduction following RDX or if it is an independent result. RDX performed during combined α_1- and β_1-AR blockade would answer this question.

An obvious criticism conclusion would be that elimination of the sympathoexcitatory central β_1-AR should lead to decreased activity at vasoconstrictor α_1-ARs. Therefore, one should expect that prior inhibition of α1-AR activity should prevent a fall in BP after RDX. I do not observe this expected outcome. A logical explanation could be that central β_1-ARs act through an alternative vasoconstrictor mechanism, i.e. other than through the α_1-AR. Data from Lohmeier's group, which uses electrical stimulation of the baroreflex to lower BP in dogs, shows that the post-junctional α_2-AR can be involved in maintaining BP. Electrical baroreflex stimulation activates afferent nerve pathways of the carotid baroreceptors to suppress central sympathetic outflow. Lohmeier and colleagues showed that the BP lowering effect of electrical baroreflex activation is less effective during α_2-AR antagonism [184]. This finding suggests that postjunctional α_2-AR can mediate a significant proportion of the sympathetic support of BP. Other investigations have shown that the α_2-AR is an important mediator of the actions of the SNS on venomotor tone [185] Although not explored in my research, it is possible that RDX is influencing BP by decreasing α_2-AR mediated venomotor tone by interrupting a central β_1-AR mediated pathway. While I did not block the post-junctional α_2-AR or venomotor tone in my studies, future efforts in these areas could help to explain the BP response to RDX. There is evidence for an alternative vasoconstrictor mechanism in

the SHR that involves central β_1-ARs and peripheral β_2-ARs [186]. This mechanism is currently under-explored, but could help explain my findings. Future evaluation of this mechanism using central and peripheral infusions of selective and non-selective beta-blockers would be valuable in revealing how this pathway is involved in the BP response to RDX.

The obvious conclusion from the adrenergic antagonist study would be that CBRNA is unlikely to lower BP in patients already taking β1-AR antagonists. In Symplicity HTN 2, for example, 83% of CBRNA patients were on a beta-blocker, and yet 84% of CBRNA patients responded with a reduction in office BP of at least 10mmHg [1]. One could interpret this to mean that there is no relationship between beta-blocker use and BP response to CBRNA. However, such a conclusion may be incorrect for several reasons. First, although the patients in the SYMPLICITY studies are prescribed beta-blockers, there may be wide variation in beta-blocker effectiveness from patient to patient. Based on my observations in the SHR one would expect a blunting of the BP response to CBRNA in patients that respond with a large BP fall to beta-blockade. Further investigation into the individual exposure levels of patients receiving CBRNA could provide evidence of such a drug effect. This assumes, however, that these patients are not resistant to the beta-blocker treatment. It is important to remember that patients in the CBRNA trials are resistant to combination anti-hypertensive therapy. This could explain the lack of a relationship between beta-blocker use and BP response. Second, patient adherence to drug therapy is not tightly controlled in the clinical trials. In Symplicity HTN 2, for example, patients were required to keep a medication compliance journal two weeks prior to CBRNA, but little was documented beyond this period [1].

Without documentation of plasma drug levels, there is no definitive proof that these patients were receiving the prescribed drug therapy. In an effort to assess non-compliance in patients with difficult-to-treat HTN, serum drug levels were analyzed in 84 patients prescribed a 3 drug regimen for uncontrolled HTN. In this cohort, 65% of patients failed to take their medication as directed [187]. This study highlights the challenge of patient non-compliance even in the setting of clinical trials. It could be speculated that patients entering CBRNA clinical trials face similar compliance issues. Therefore, one might expect that if patients were more compliant with the anti-hypertensive therapies involving beta-blockers, the BP response to renal denervation would be much lower. More evidence needs to be collected to support this claim. Serum drug screens would be an excellent addition to future CBRNA studies.

In conclusion, my findings demonstrate that RDX in the aged SHR is indeed a credible model for understanding how CBRNA lowers BP in humans. Experiments within this model suggest that the BP lowering effect of RDX in the aged SHR is mediated by a sympatho-inhibitory mechanism associated with the β_1-AR (Figure 43). This β_1-AR mechanism does not appear to be related to suppression of renin release suggesting that the mechanism is likely extra-renal. This indicates that renal afferent nerves and suppressed NRSNA are likely more important in the BP lowering effect of RDX than reduced RSNA. Although my studies did not investigate capsaicin-insensitive renal afferent nerves, nor locate the exact brain regions and neurocircuitry involved with these fibers, I conclude that the BP lowering effect of RDX is mediated by a mechanism in the brain. Future investigation should seek to understand how RDX modifies sympathetic outflow from the brain.

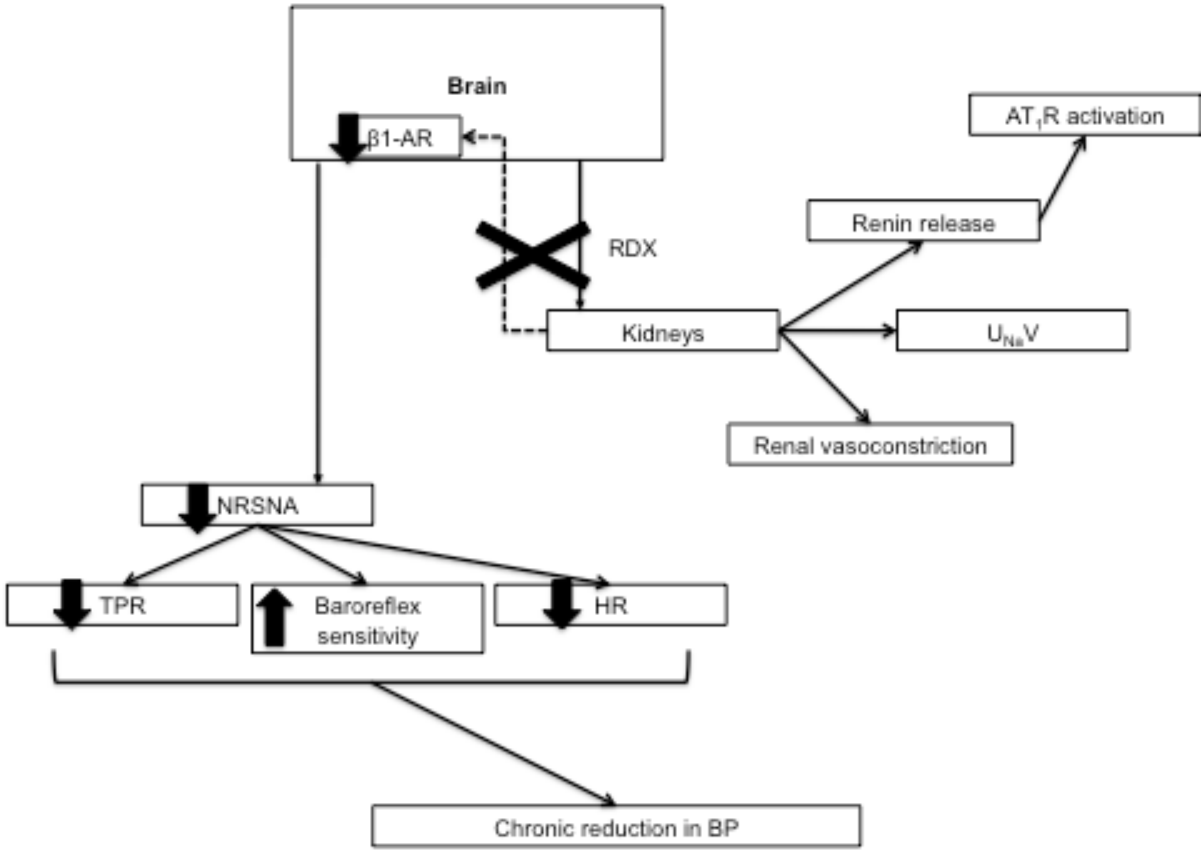


Figure 43. Overall schematic for how RDX lowers BP in aged SHR. RDX chronically reduces BP by suppressing β_1-AR activity in the brain leading to a reduction in NRSNA. Mechanisms known to be associated with RSNA do not appear to be involved. Possible mechanisms connected to NRSNA that could explain the BP reduction include reduced total peripheral resistance, improved baroreflex sensitivity, and reduced heart rate.

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