Role of the Renal Nerves in Hypertension

A DISSERTATION SUBMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

Jason David Foss

IN PARTIAL FULFILLMENT OF THE REQUIERMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: John W. Osborn, PhD

November, 2014

Acknowledgements

I would like to thank my advisor, Dr. John Osborn who has been a phenomenal mentor throughout my time in his lab and without whom none of this work would have happened. I would also like to thank the members of my thesis committee Dr. Scott O'Grady, Dr. Stephen Katz and especially Dr. William Engeland who helped us develop our novel method for selective ablation of afferent renal nerves. I would also like to thank Dr. Gregory Fink who has been a collaborator and coauthor on all of my papers. His input has been invaluable.

Much of this work would not have been possible without instruction and assistance from the current and past members of the Osborn lab. In particular I would like to thank Dusty Van Helden, Pilar Ariza Guzman, Marcus Kuroki, Britta Veitenheimer-Rupp, Ruijun Han, and Xinying Wang. I also thank Jeff Larson, Eric Homan and Madeline Gauthier for their help with some of the less glamourous parts of this work.

Dedication

I dedicate this thesis to my amazing parents, Jim and Carol Foss. Their unending love, support and encouragement continue to push me toward bigger and better things.

Abstract

High blood pressure (hypertension; HTN) is the leading risk factor for death, yet the precise causes are unclear. The nervous system is known to play a role in some forms of HTN and research has pointed to the kidneys as a likely neural target in HTN. This possibility has been strengthened by recent clinical trials showing that ablation of the renal nerves (renal denervation; RDNX) has a significant antihypertensive effect in drug resistant patients. However, failure of the most recent sham-controlled trial has raised many questions regarding this treatment. Chief among them is whether the antihypertensive effect of RDNX is due to changes in kidney function secondary to ablation of sympathetic (efferent) renal nerves, or due to a reduction in non-renal sympathetic nerve activity secondary to ablation of sensory (afferent) renal nerves. In order to address this question, I first identified an animal model of HTN in which RDNX had an antihypertensive effect. Importantly, I showed that RDNX had roughly the same effect on blood pressure in hypertensive Dahl salt-sensitive (S) rats as has been reported in clinical trials. I then developed and validated a novel method for selective ablation of afferent renal nerves (renal-CAP treatment). Using this method, I showed that afferent renal nerves are not necessary to maintain cardiovascular or sodium/water homeostasis in normotensive rats subjected to dietary sodium loading. We also showed that renal-CAP treatment and complete RDNX caused the same attenuation of deoxycorticosterone-salt HTN, suggesting that the antihypertensive effect of RDNX in this model is due to ablation of afferent renal nerves. Lastly, we showed that RDNX has the same antihypertensive effect in Dahl S rats with mild HTN (after three weeks of high salt feeding) and in those with severe HTN (after nine weeks of high salt feeding). These results suggest that the antihypertensive effect of RDNX in the Dahl S rat is not dependent on duration of high salt feeding or pretreatment blood pressure and that the antihypertensive effect of RDNX in this model is not due to ablation of afferent renal nerves.

Table of contents

List of tables

Chapter three

Chapter Four

List of figures

Chapter One

Chapter Two

Chapter three

Chapter Four

Contributions

Aside from Jason Foss and John Osborn, the following individuals performed significant portions of the work described in this thesis:

- Robert Burnett, who works in the laboratory of Dr. Gregory Fink, performed the HLPC assays to assess NE content (data in chapters two, three and four).
- Xinying Wang performed the ELISA to assess renal pelvic CGRP content (data in chapters three and four).
- Lori Garcia, who works in the laboratory of Dr. Stefan Tunev performed the tissue preparation, immunohistochemical staining and imaging for TH and CGRP (data in chapter three).
- Dr. Richard Wainford performed the assessment of afferent renal nerve functionality using bradykinin (data in chapter three).
- Dusty Van Helden performed the DOCA-salt protocol (data in chapter three). In addition, he contributed greatly to the daily sodium and water balance measurements (data in chapters two and three).

Chapter One

Introduction

1.1 Background

High blood pressure (hypertension, HTN) is a major contributor to death and disability, worldwide. In fact, due to the causal links between HTN and many major health problems including heart failure and stroke, HTN is currently the number one risk factor for death and the number three risk factor for disease burden worldwide (108). Moreover, the prevalence of HTN is staggering. An estimated one billion people currently have HTN and this number is expected to rise to 1.5 billion by the year 2025 (90). Despite these worrying figures, the causes of HTN are still largely unknown and treatment remains poor with pharmacological therapy failing to reduce blood pressure to an acceptable range in an estimated 10-15% of hypertensive patients (1).

While it is difficult to pin down the precise etiology of HTN in most patients, decades of experiments performed in people as well as various animal models have revealed a number of possible contributors. Perhaps the most well studied contributor to HTN is the kidney. In fact, renal excretory function has been proposed to be the "rate limiting step" in HTN in that arterial pressure (AP) cannot increase chronically unless renal excretory function is impaired. The reasoning behind this hypothesis is that AP is correlated with urinary sodium excretion and urine flow rate (pressure-

natriuresis/diuresis) such that increases in AP lead to excretion of salt and water, a decrease in blood volume and return of AP to normal levels. Therefore, it has been suggested that in order for AP to be increased chronically, pressure-natriuresis/diuresis must be impaired (58). While this obligatory impaired pressure-natriuresis/diuresis hypothesis is intriguing, mounting evidence suggest that other factors can and do contribute to HTN in the absence of changes in renal sodium and water handling.

The sympathetic nervous system (SNS) is one such factor that has been implicated in the pathogenesis of HTN. Global, renal, cardiac and skeletal muscle sympathetic activity have been shown to be elevated in human hypertensives (142) and pharmacological as well as surgical blockade of the SNS have been shown to reduce AP in various animal models of HTN as well as in hypertensive patients (38, 134). Poor tolerance to global sympathetic blockade and invasive surgical procedures led to the abandonment of these therapies for treatment of HTN for many years. Recently however, technological advances have reignited interest in targeting the SNS for treatment of HTN.

It has been known for over a century that the nervous system exhibits great influence over renal function. Some of the earliest experiments were performed by Claude Bernard in the 1850s in which he denervated the kidneys and noted ureteral distension. Later studies showed that stimulation of the renal nerves had an antidiuretic effect. Decades of research since then have elucidated a great deal more about the nature of neural influence on renal function. Additional research has revealed the kidney as a sensory organ that sends information back to the central nervous system (CNS) via afferent sensory nerves.

Sympathetic innervation of the kidneys is known to have pro-hypertensive effects such as enhancing sodium reabsorption, increasing renal vascular resistance and stimulating renin release. Because of these sympathetic actions, the role of the renal nerves in HTN has been investigated for decades. Specifically, ablation of the renal nerves (renal denervation, RDNX) has been performed in nearly every major hypertensive animal model with varying effects on AP. While it does not always produce

an antihypertensive effect, RDNX has been shown to lower AP both in normotensive rats and in many animal models of HTN (38, 76).

Numerous studies demonstrating the possible therapeutic effect of RDNX coupled with recent technological advances have led to the development of a catheter based RDNX for the treatment in humans. During this minimally invasive procedure, a device is introduced into the renal arteries through a catheter in the femoral artery and, using radiofrequency ablation, the device destroys the nerves running to and from the kidneys. Initial clinical data showed a significant and sustained antihypertensive effect of RDNX in severely hypertensive, drug resistant patients (42, 101). Although these results were exciting, they sparked a number of important questions regarding the antihypertensive mechanisms underlying the therapeutic effect of this new treatment.

The mechanistic questions surrounding RDNX have become even more important in the past year wherein data from the first major multicenter sham-controlled double blinded clinical trial of RDNX were released (13). In this study, not only did RDNX have a much smaller antihypertensive effect than in previous clinical trials, but patients who underwent RDNX did not have a statistically significant decrease in AP when compared to sham operated individuals. While these results have sparked a great deal of debate over why this trial failed after the plethora of positive data previously reported, they also highlight two important points. First, these data suggest that RDNX may not be an effective treatment for HTN in all people. Second, these data highlight the importance of further basic science research, which must be done in order to better understand how RDNX lowers AP when it does in order to optimize treatment efficacy.

Perhaps the most discussed mechanistic question surrounding this treatment is whether the antihypertensive effect of RDNX is due to ablation of afferent or efferent renal nerves. When this treatment was initially developed, the rationale was that because the renal sympathetic nerves (efferent renal nerves) influence sodium reabsorption, renal vascular tone and plasma renin concentration, removal of the neural influence on these pro-hypertensive mechanisms would cause a reduction in AP. Indeed there is some evidence to suggest that this may explain the antihypertensive effect of RDNX.

Specifically, it has been shown that a decrease in renal resistive index accompanies a fall in AP in patients following RDNX (112). While this is compelling, the lack of additionally evidence makes it less than conclusive. Additionally, there is no evidence that the antihypertensive effect of RDNX is associated with a decrease in plasma renin concentration or an unloading of salt and water.

An alternate explanation for the antihypertensive effect of RDNX is that ablation of sympathoexcitatory renal sensory nerves (afferent renal nerves) causes a reduction in sympathetically mediated peripheral vasoconstriction. While this has not been shown directly, there are a number of observations that suggest sympathetic outflow is indeed reduced by RDNX. Specifically, RDNX has been associated with reductions in muscle sympathetic nerve activity, plasma glucose concentration, cardiac arrhythmias and episodes of sleep apnea (67, 149, 171). It should be noted that one study found no change in muscle sympathetic nerve activity following RDNX; however, there was also no change in AP leaving open the possibility that the blood pressure lowering effect of RDNX is due to reductions in sympathetic nerve activity (18).

To begin to address the differential role of afferent and efferent renal nerves in HTN, I will first review what is currently known about these nerves. I will discuss the anatomy, functions and mechanisms of acute and chronic activation of both efferent and afferent renal nerves and then review what is known about the differential role of afferent and efferent renal nerves in various forms of HTN before presenting my own work.

1.2 Efferent renal nerves

Gross anatomy of the renal nerves

The gross anatomy of the renal nerves is depicted in Figure 1. The renal nerves course along the renal arteries and veins in a sort of meshwork that penetrates the walls of the vessels into the tunica media (8). While the majority of the renal nerve fibers run from the aorta to the hilum of the kidney, there are some renal nerve bundles that course toward and join the renal nerve plexus at various points between the aorta and kidney. As is true with many nerves throughout the body, the renal nerves contain multiple different fiber types. Specifically, the renal nerves contain both sympathetic (efferent) fibers which course from sympathetic ganglia to the kidneys, and sensory (afferent) fibers which course from the kidneys to the spinal cord. The specific anatomy and mechanisms of neurotransmission of both afferent and efferent renal nerves are discussed below.

Anatomy of efferent renal nerves

As with many sympathetic nerves, the cell bodies of the renal sympathetic preganglionic neurons reside in the intermediolateral (IML) cell column of the spinal cord. These neurons then send axons out of the spinal cord via the ventral horns which synapse onto sympathetic postganglionic fibers in the sympathetic ganglia. The IML, in turn receives input from various brain regions. With regard to the renal sympathetic nerves specifically, the primary neural input into the IML arises from the rostral ventrolateral medulla (RVLM). This important cardiovascular regulatory region of the medulla receives input from a number of other brain nuclei. By in large, the RVLM receives excitatory input from the paraventricular nucleus of the hypothalamus (PVN) and inhibitory input from the nucleus of the solitary tract (NTS) and the caudal ventrolateral medulla (CVLM). It should be noted that the PVN also has direct input into the IML bypassing the RVLM. The PVN in turn receives excitatory input from three important regions of the forebrain, the median preoptic nucleus (MnPO) and two of the circumventricular organs (CVOs), the subfornical organ (SFO) and the organum

vasculosum of the lamina terminalis (OVLT). This network of brain regions is illustrated in Figure 2. The importance of these nuclei in the regulation of efferent renal sympathetic nerve activity (ERSNA) will be discussed in the following sections of this chapter.

The sympathetic preganglionic neurons that give rise to the renal nerves exit the spinal cord and synapse in the paravertebral ganglia (T6-L4) and the aorticorenal, celiac and the superior mesenteric prevertebral ganglia with significant interspecies variability (38, 148). The sympathetic postganglionic fibers exit their respective ganglia, course along the renal artery and renal vein, and enter the kidney via the hilum. Within the kidney, sympathetic fibers innervate multiple structures. The most densely innervated of these structures include the afferent and efferent glomerular arterioles, the juxtaglomerular cells and renal tubular epithelial cells (82).

Sympathetic innervation of the renal resistance vessels has been well characterized. Specifically, efferent renal nerves have been shown to innervate the vascular smooth muscle cells of both the afferent and efferent arterioles as well as the interlobar, interlobular and arcuate arteries. Efferent renal nerves also directly innervate the juxtaglomerular (JG) cells that account for the majority of renin production in the body. Lastly, the entire renal tubular system is innervated by sympathetic fibers although the most densely innervated segment is the proximal tubule.

Functions of the efferent renal nerves

As with all sympathetic nerves, the primary neurotransmitter of the renal sympathetic postganglionic nerves is norepinephrine (NE). In addition, there is evidence that these neurons also release a number of cotransmitters such as dopamine, nitric oxide, neuropeptide Y, vasoactive intestinal peptide and adenosine. While it is likely that these neurotransmitters play some role in the sympathetic control of renal function, the importance of such cotransmitters in the kidney is largely unknown. Therefore, this section will focus on the role of NE and its receptors on renal function.

As previously mentioned, the three structures that are primarily innervated by efferent renal nerves are the renal resistance vessels, JG cells and renal tubular epithelial

cells. As a result, the three primary physiological functions influenced by ERSNA are renal vascular resistance, renin release and renal sodium reabsorption. Changes in all of these parameters follow stimulation of the target cells by NE; however, the adrenergic receptors (ARs) and intracellular signaling pathways involved are quite different.

The JG cells express the β_1AR on their plasma membrane. NE released from sympathetic nerve terminals binds to the β_1AR leading to activation of adenylyl cyclase resulting in increased levels of intracellular cyclic adenosine monophosphate (cAMP). The cAMP signaling pathway in turn promotes increased production and release of renin from the JG cells (59, 81). The renal resistance vessels are stimulated by efferent renal nerves primarily via the α_{1A}AR. Stimulation of the α_{1A}AR results in phospholipase C signaling and increased intracellular Ca^{2+} leading to contraction of the smooth muscle cells and thus vasoconstriction (82). Sympathetic stimulation of the tubular epithelial cells occurs primarily through α1BAR which, through mitogen activated protein kinase (MAPK) signaling, results in increased apical expression of the sodium/hydrogen exchanger (NHE3). Additionally, through Ca^{2+}/calcineurin signaling, α_{1B}AR activation results in increased activity of the sodium potassium ATPase (Na^+/K^+ATPase) on the basolateral membrane. Increased expression of NHE3 and activity of Na^+/K^+ATPase facilitate the reabsorption of sodium from the tubular lumen into the blood (82).

It is well accepted that renin release, renal vascular resistance and sodium reabsorption are influenced by ERSNA; however, there is some debate as to the importance of neural control of these parameters under normal physiological conditions. It has been shown that electrical stimulation of the renal nerves caused frequency-dependent activation of these functions (38). Increased release of renin occurred at a relatively low frequency of renal nerve stimulation. At slightly higher frequency of stimulation, sodium reabsorption was increased. It was not until a relatively high frequency of stimulation that renal blood flow (RBF) was decreased. It has been suggested that because the frequency of stimulation required to evoke reductions in RBF were so high in this experiment, ERSNA is likely not a factor in every day control of RBF. This assertion was recently challenged by Yoshimoto et al., who continuously

recorded renal nerve activity and RBF in conscious, freely moving rats (179). The results of this study showed that changes in RBF that occurred during normal changes in behavioral state were accompanied by changes in RSNA. Malpas et al., performed a similar study in rabbits and showed that while the presence or absence of renal nerves does not alter 24 hour averaged RBF, RSNA does influence episodic changes in RBF (11). Taken together, the results of these two studies suggest that RSNA can indeed influence RBF under normal physiological conditions.

It is also important to note that while ERSNA has been shown to influence renin release, sodium reabsorption and renal vascular resistance, there are a number of redundant control mechanisms that influence all three of these parameters. In fact, when the renal nerves are removed by RDNX most studies have found that these parameters remain, on average, within normal physiological ranges (11, 77, 78). It is therefore likely that chronic changes in these parameters would be observed following RDNX only in situations of inappropriately high ERSNA.

Acute regulation of efferent renal nerve activity

A significant amount of work has gone into elucidating the mechanisms by which ERSNA is controlled. By in large, these have been acute experiments performed in anesthetized animals. While the results of such experiments may not relate to the mechanisms underlying chronic regulation of ERSNA, these important experiments have revealed a number of factors that can influence ERSNA acutely.

As discussed earlier, there are many regions of the brain that are involved in the regulation of sympathetic outflow. With regard to the renal nerves, it is thought that the RVLM is primary controller of ERSNA (57). The RVLM receives excitatory input from the PVN and inhibitory input from the NTS. In turn, the PVN and NTS receive various inputs from sensory pathways creating numerous possible mechanisms for the regulation of ERSNA.

Baro-, chemo- and cardiopulmonary reflexes: There are many neural and humoral factors that can influence the firing of the central sympathomodulatory pathways, the best

studies of which is the baroreceptor reflex (baroreflex). Stretch receptors in the aortic arch and carotid sinuses, called baroreceptors, become activated as increased AP causes stretching of the aorta and carotid arteries (64). When the baroreceptors are activated, they send signals back to the brain via afferent nerves exciting neurons in the NTS. In turn, the NTS sends excitatory input into the CVLM which increases inhibitory input to the RVLM and ultimately reduces sympathetic outflow to various targets of cardiovascular importance including the kidney (34). Therefore, increases in AP, sensed by the aortic and carotid baroreceptors, result in a suppression of ERSNA (14). While this has been demonstrated repeatedly in acute experiments, the role of baroreflex control of ERSNA chronically has been shown to be minimal. In fact removal of baroreceptor input by sinoaortic denervation (SAD) has been shown to cause elevations in ERSNA and AP; however, these parameters return to normal levels within days of surgery suggesting that the baroreflex plays a role in short term rather than long term control of ERSNA (73). In addition to baroreflex control of ERSNA, the low pressure cardiopulmonary receptors and peripheral arterial chemoreceptors have been shown to regulate the efferent renal nerves through their input into the same regulatory regions of the brain (70, 169).

Visceral reflexes: Many acute studies have demonstrated the existence of reflex arcs that originate in various organs and reflexively regulate ERSNA. Of these visceral reflexes, the most well studied is the so called reno-renal reflex in which afferent renal nerve activity (ARNA) has been shown to inhibit ERSNA (52). In addition, osmosensitive afferents in the hepatoportal circulation have been shown to influence ERSNA (129). There is even evidence for a spleno-renal reflex (61). Taken together, these studies and many more demonstrate the vast neural networks that are involved in the regulation of ERSNA; however, it should be noted that these are acute studies and the role of these various neural inputs in the chronic regulation of ERSNA is not known.

Plasma and CSF osmolality: Another factor that can contribute to the regulation of ERSNA is the ionic composition of the blood and cerebrospinal fluid (CSF). Most studies have shown that infusion of hypertonic saline into the CSF (intracerebroventricular; i.c.v.) causes a decrease in ERSNA, although some studies have

shown increases in ERSNA with i.c.v. hypertonic saline, suggesting that there are regions of the central sympathoregulatory circuit that are sodium-sensitive (71, 177). Additionally, intravenous infusion of hypertonic saline has been shown to alter ERSNA (10). Taken together, these studies suggest that there are osmosensitive regions in the brain that alter SNA to the kidneys. Moreover, the fact that increases in both plasma osmolality and CSF osmolality increase ERSNA suggests that the osmosensitive region(s) of the brain likely lie outside of the blood brain barrier (BBB). In fact, both the SFO and OVLT, which lack a BBB have been shown to be osmosensitive making them the likely sensory regions that respond to changes in plasma or CSF osmolality (6, 17). In addition, to plasma or CSF osmolality, the SFO and OVLT are thought to respond to various circulating factors including angiotensin II (AngII), aldosterone and leptin. The effect of these hormones on ERSNA will be discussed in the next section as they have been shown to influence ERSNA both acutely and chronically.

Stress: It should also be noted that various types of stressors, such as air jet stress and cold water stress, have been shown to activate the renal nerves (37, 84). While many studies have shown acute responses of ERSNA to these stressors, the role that stress plays in chronic elevations in SNA and AP is largely unknown at this point, although much work is currently being done to investigate this possible link.

Chronic regulation of ERSNA

Acute experiments have uncovered many factors that can regulate ERSNA, but when considering the role of renal nerves in chronic HTN, it is more important to determine what factors contribute to the long term regulation of renal sympathetic outflow. While this is a much more difficult task, a number of important studies have expanded our understanding of the long term regulators of ERSNA. Using direct or indirect indices of ERNSA, a number of hypertensive stimuli have also been shown to cause long term changes in renal nerve activity. In this section I will review what is known about the roles of AngII, aldosterone and leptin in chronic regulation of ERSNA.

Angiotensin II: The functional end product of the renin-angiotensin system, AngII, is known to have many prohypertensive properties as it causes vasoconstriction, sodium retention and increased production of the salt retaining hormone, aldosterone. Additionally, AngII receptors have been identified in the CVOs of the brain and electrolytic lesioning of both the SFO and OVLT have been shown to attenuate HTN produced by chronic infusion of exogenous AngII (65, 163). Interestingly, renal nerve activity has been shown to inhibited or enhanced in a species-dependent manner during chronic AngII infusion. Using direct nerve recording techniques in chronically instrumented rats, our lab has shown that AngII causes a reduction in ERSNA (178). Similar results have been shown in rabbits receiving chronic AngII infusion (12). Renal NE overflow, an index of ERSNA, was also shown to be decreased with chronic AngII infusion in dogs (24). Denervation studies have shown mixed results as to the ability of RDNX to reduce AP in the AngII induced HTN (20, 66, 91). While these data, when considered together, constitute a rather unclear story, it is undeniable that AngII can influence the activity of the renal nerves chronically, although the direction and magnitude of this regulation may be model-dependent.

Aldosterone and leptin: In addition to AngII, aldosterone and leptin have been shown to act in the CNS and are both associated with neurogenic HTN (31, 51). Moreover, denervation studies have repeatedly shown that RDNX attenuates HTN associated with these hormones, suggesting that aldosterone and leptin can act though the renal nerves to increase AP (60, 77). Receptors for aldosterone and leptin receptors have been identified in various regions of the brain including the hypothalamus and it is likely that these sympathoexcitatory substances act through many of the same central pathways as AngII (60, 106).

Much of what is known about the chronic regulation of ERSNA comes from studies in which naturally occurring stimuli are given in excess exogenously. While these studies are useful in establishing proof of concept, they do not provide solid evidence for the mechanisms involved in the naturally occurring over activity of renal nerves. A significant amount of work is needed to address this point.

1.3 Afferent renal nerves

Anatomy of the afferent renal nerves

The cell bodies of the sensory (afferent) neurons of kidneys have been found in the dorsal root ganglia (DRG) at spinal levels from T8 to L4 (Fig. 1) with interspecies variability (2, 104, 139, 170). The peripheral axons of these neurons course out of the DRG, join the renal nerve bundles near the renal artery and vein, and enter the kidney via the hilum. Inside the kidney primary afferent renal nerves innervate, almost exclusively, the wall of the renal pelvis, although a small number of renal afferent fibers have been shown to innervate the interlobar and arcuate arteries and, to a lesser extent, the interlobular arteries and afferent arterioles (45, 116).

The central axons of the DRG cells course through the dorsal roots and into the dorsal horn where they synapse onto second order neurons. Sensory information from the kidneys then ascends via the spinoreticular and spinothalamic tracts (3-5). It should be noted that a very small portion of afferent renal nerves have been shown to project monosynaptically to the medulla (94, 174). Fos labeling in the brain following afferent renal nerve stimulation has revealed increased activity in the OVLT, SFO, MnPO, PVN, NTS, supraoptic nucleus (SON) and many other nuclei (147). It is through input into this vast network of autonomic regulatory regions of the brain that ARNA is thought to influence various homeostatic processes.

Primary Afferent fibers are often classified into various fiber types as defined by fiber diameter, conduction velocity and myelination state. The renal primary afferents have been shown to be mostly (75-80%) slow conducting unmyelinated C-fibers with a much smaller population (~20%) of faster conducting thinly myelinated A-δ fibers (92, 93, 128). Additionally, a very small population (~5%) of rapidly conducting (12-32 m/s) myelinated afferent renal nerve fibers has been reported.

Functions of the afferent renal nerves

Renal Pain: The kidneys, like many visceral organs, relay many different sensory modalities to the central nervous system and therefore activation of afferent renal nerves results in many different functional outcomes. Perhaps the most intuitive of these is the sensation of pain resulting from renal injury. Indeed damage to the kidneys resulting from insults such as kidney disease and kidney stones causes a great deal of pain to the patient. This sensation of pain results from afferent renal pathways converging with somatic nociceptive pathways from the flank and abdomen within the spinothalamic tract of spinal cord (3). Noxious stimuli in the kidney therefore are interpreted by the brain as flank or abdominal pain. This type of referred pain is common with visceral organs and exists to alert the brain of internal problems.

Modulation of cardiovascular function: In addition to the sensation of renal pain, activation of afferent renal nerves has been shown to have a number of various autonomic consequences. Many studies have shown that activation of afferent renal nerves results in robust alterations in cardiovascular function. Specifically, most studies have found that increases in ARNA are associated with increases in mean arterial pressure (MAP) and heart rate (HR) as well as increases in regional and total peripheral vascular resistance (TPR), suggesting the presence of sympathoexcitatory afferent renal nerves (7, 44, 150). Interestingly, these same types of studies, when performed in rabbits, typically result in the opposite effects (109). That is stimulation of afferent renal nerves in the rabbit generally results in a decrease in MAP, HR and TPR, suggesting the presence of sympathoinhibitory afferent renal nerves in the rabbit.

Reno-renal reflex: While stimulation of afferent renal nerves typically causes increases in SNA to various cardiovascular targets, it has been consistently shown that it also results in a suppression of SNA to the kidneys (29, 180). This so called "renorenal reflex" has been extensively studied by a number of labs throughout the past three decades. In short, when activated by a number of different stimuli, afferent renal nerves act to inhibit ERSNA and promote natriuresis and diuresis. While this reflex has been shown extensively in acute anesthetized preparations and, to a lesser extent, in acute

experiments in conscious animals, its role in the long term regulation of ERSNA and salt and water homeostasis is more controversial. It has been proposed that this inhibitory renorenal reflex is important in maintaining sodium and water homeostasis in situations of dietary sodium loading (82), although my own work (presented in chapter three) directly contradicts this theory.

Release of vasopressin and oxytocin: As mentioned earlier, renal afferent signaling results in the excitation of neurons in a number of different regions of the brain. Among these is the SON, a part of the brain that is critical for the release of the hormones vasopressin and oxytocin (35). Moreover, it has been shown that stimulation of afferent renal nerves results in increased plasma vasopressin levels (25, 144). Although less well studied, increased ARNA has also been shown to increase levels of oxytocin in the blood (144). Since vasopressin acts on the kidney to promote water retention, it makes sense that signals from the kidney can stimulate its release. Moreover, misregulation of this kidney-SON axis may explain some forms of HTN that have been linked to both increased ARNA and vasopressin release. This will be discussed in relation to the deoxycorticosterone acetate (DOCA)-salt model of HTN in chapter three.

Acute regulation of afferent renal nerve activity

Acute experiments have shown that afferent renal nerves respond to many different stimuli. In general this has led to the classification of afferent renal nerves into three functional categories: mechanosensitive, chemosensitive or nociceptive.

Mechanosensitive: Because the wall of the renal pelvis to densely innervated by afferent renal nerves(45), it is not surprising that changes within the pelvis can stimulate these nerves. One such intrapelvic stimulus is mechanical stretch. It has been shown that increases in renal pelvic pressure result in increases in ARNA. Forceful pressurization of the renal pelvis, occlusion of the ureter, and increases in urine flow rate following acute volume loading or administration of diuretics have all been shown to induce increases in ARNA (5, 53, 99). Other mechanical stimuli have been shown to increase activity of afferent renal nerves as well. Among these are increased renal arterial, venous or

interstitial pressure (124). While these responses have been clearly shown, it is unclear whether these stimuli act on the same population of afferent fibers that are stimulated by increased pelvic pressure, or if a separate population of fibers is responsible for the response.

Chemosensitive: In addition to the presence of mechanosensitive afferent renal nerves, it has been repeatedly demonstrated that afferent renal nerves respond to chemical stimuli within the kidney. Among the stimuli most studied are adenosine, bradykinin, substance P and prostaglandin E2. Infusion of these compounds into the renal pelvis or renal artery has been shown to elicit increases in ARNA (87, 96, 111, 146). Some studies have also shown that increases in pelvic sodium concentration by intrapelvic infusion of hypertonic saline can lead to activation of afferent renal nerves (182). Lastly, renal ischemia caused by acute reductions in RBF is known to stimulate ARNA (110).

Nociceptors: As mentioned earlier, some of the afferent neural pathways from the kidneys have been shown to ascend the spinal cord through the spinothalamic tract (3). Moreover, neurons in this pathway are responsive to both renal and cutaneous noxious stimuli. These observations demonstrate the basis for referred renal pain that is experienced in renal disease and in the presence of kidney stones.

Although afferent renal nerves can be classified into three functional categories, it should be noted that there is some evidence of mechanistic overlap. Single afferent renal neurons likely respond to multiple types of stimuli. For example, both mechanical stretch and intrapelvic substance P administration have been shown to activate a single afferent renal nerve unit (111).

Chronic regulation of afferent renal nerve activity

While acute stimuli of ARNA have been fairly well studied, it is less clear what may regulate the activity of these nerves chronically. There is, however, a significant body of evidence suggesting that these nerves may be perpetually overactive in the setting of chronic kidney disease (CKD). This hypothesis is derived from a number of pieces of evidence. First, as discussed earlier, acute stimulation of afferent renal nerves

has been shown to increase SNA and AP in many studies. Second, many patients with advanced CDK or end-stage renal disease (ESRD) have elevated levels of muscle SNA (15, 127). Lastly, it has been reported that when these hypertensive ESRD patients receive bilateral, but not unilateral kidney transplants, muscle SNA and AP are reduced (63).

A possible role of renal pathology in chronic overactivity of afferent renal nerves is supported by studies in which unilateral denervation attenuates HTN induced by some type of insult to the denervated kidney. In this regard, Katholi e. al., showed that HTN induced by renal artery stenosis is partially reversed by denervation of the ischemic kidney (88, 89). Moreover, they showed that this chronic hyperactivity of the afferent renal nerves is dependent on adenosine signaling in the kidney (86). Similar studies by Campese et al., showed that renal damage induced by injection of phenol into the kidney results in HTN that is prevented by ipsilateral but not contralateral removal renal afferent input (21). Taken together, these studies suggest that chronic renal pathology can cause afferent renal nerve dependent HTN.

Data from these studies in people and animals provide convincing evidence that afferent renal nerves are a link between kidney disease and HTN; however, the mechanisms by which CKD may lead to chronic activation of afferent renal nerves remain unclear. One possibility that has been proposed is that inflammatory signals in the kidneys, induced by renal damage, stimulate afferent renal nerves. Indeed significant infiltration of immune cells into the kidneys has been shown repeatedly in various models of HTN (62, 141). Moreover, it has been shown that some cytokines, which are released from inflammatory immune cells, can activate sensory fibers (27). Taken together, these observations raise the possibility that the renal damage associated with CKD leads to infiltration of inflammatory immune cells into the kidney which release cytokines that then act on sympathoexcitatory afferent renal nerves to drive HTN.

1.4 Differential role of afferent and efferent renal nerves in hypertension

The antihypertensive effect of RDNX has been extensively documented in various animal models of HTN and, although somewhat controversial, in human hypertensives as well (13, 38). While the results of such studies highlight the promise of RDNX as an effective treatment modality, there are currently many questions surrounding the mechanisms underlying the antihypertensive effect of RDNX. At the forefront of these questions is whether the therapeutic effects of RDNX are due to ablation of afferent or efferent renal nerves. This distinction is important with regard to optimizing the efficacy of RDNX or developing more specific ablation techniques in order to minimize possible side effects. While this treatment was originally developed with the intent of disrupting overactive ERSNA which could contribute to HTN by increasing renal vascular resistance, renin release or tubular sodium and water reabsorption; clinical evidence suggests that ablation of afferent renal nerves has observable functional consequences. Namely, RDNX has been shown to decrease: plasma glucose, muscle sympathetic nerve activity, incidence of sleep apnea and cardiac arrhythmias, suggesting that RDNX causes alterations in sympathetic outflow to non-renal targets (67, 149, 171). These observations indicate the further possibility that RDNX reduces AP by reducing sympathetic outflow to targets of cardiovascular importance such as the splanchnic vascular bed, skeletal muscle vascular bed or kidneys. In this section, I will review the preclinical studies that have used various denervation techniques to explore the differential role of afferent and efferent renal nerves in the antihypertensive effect of RDNX.

Dorsal rhizotomy

In 1985, Brody et al., showed that surgical sectioning of the dorsal roots (dorsal rhizotomy) at spinal levels T10-L1 in order to produce a selective ablation of afferent renal nerves (105). Indeed this technique permanently eliminates the majority of renal

afferent input into the spinal cord; however, because a large number of non-renal afferent pathways enter the spinal cord at these levels as well, this technique is not selective for renal afferent neurons (26, 103, 120). Moreover, this technique fails to ablate the small portion of afferent renal nerve fibers that enter the spinal cord above T9 and below L1. Despite these limitations, studies using DRX have provided significant insight into the possible physiological roles of afferent renal nerves.

Removal of renal afferent input by DRX in normotensive rats has been shown to result in salt-sensitivity of blood pressure (97). That is, DRX rats become hypertensive when fed a high salt diet as compared to sham operated rats. Moreover, these rats have been shown to retain sodium and water compared to sham rats, indicating elevated ERSNA, i.e. diminished renorenal reflex. These results have been interpreted to mean that high dietary consumption of sodium causes an increase in ARNA which then suppresses ERSNA leading to natriuresis and diuresis to maintain sodium balance. DRX rats therefore become hypertensive when fed a high sodium diet because they are unable to suppress ERSNA leading to inappropriately high sodium and water retention and, as a result, increased blood volume. This finding, however, is complicated by the non-selective nature of RDX and is directly challenged by my own results presented in chapter three.

Systemic administration of capsaicin

Systemic administration of the transient receptor potential vanilloid 1 (TRPV1) receptor agonist, capsaicin to neonatal rata pups has been used for decades to ablate sensory fibers throughout the body. While this whole body deafferentation has largely been used to study sensory pathways involved in pain, Wang et al., have used this technique extensively to study the role of capsaicin sensitive afferent fibers in cardiovascular regulation. As is true with RDX, these studies have demonstrated that high sodium intake causes marked HTN in rats subjected to systemic capsaicin as neonatal pups as compared to vehicle treated rats (164). Again, these results have been interpreted to suggest that ablation of sympathoinhibitory afferent renal nerves leads to

inappropriately elevated ERSNA during high salt intake. This assertion will also be challenged in chapter three.

Investigations into the role of afferent renal nerves in animal models of HTN

In the 1980s and ‘90s, there were a number of experiments that attempted to assess the role of afferent renal nerves in HTN using complete RDNX or DRX. These studies, while imperfect in the use of nonselective denervation techniques, established a possible role of afferent renal nerves in various forms of HTN.

Among the most studied models of HTN are the Goldblatt models of renovascular HTN. In these models, HTN develops secondary to renal artery stenosis which is created by placing a clip around the renal artery reducing RBF. Goldblatt HTN is typically induced by clipping one (2 kidney, 1 clip; 2K1C) or both (2 kidney, 2 clip; 2K2C) kidneys, or by removing one kidney and clipping the remaining kidney (1 kidney, 1 clip; 1K1C). Interestingly, denervation of the clipped kidney partially reverses 2K1C HTN, whereas denervation of the unclipped kidney has no effect on 2K1C HTN (88). Likewise, it has been shown that RDNX partially attenuates 1K1C HTN (89, 130). Because the stimulus for HTN in these models clearly comes from the clipped kidney, these results were taken to suggest that activation of afferent renal nerves was responsible, in part, for the HTN induced by renal artery stenosis. Later studies supported this conclusion more directly by showing that DRX also attenuates 2K2C and 1K1C HTN (167, 173).

In addition to the Goldblatt models, DRX has been shown to attenuate a number of other models of HTN. Campese et al., have extensively characterized the phenol renal injury model of HTN in which phenol is injected directly into the cortex of one kidney. The resulting renal injury leads to increased SNA and HTN, both of which are prevented by DRX, suggesting that renal injury leads to afferent renal nerve-dependent increases in SNA and AP (21, 22). Additionally, cyclosporine A-induced HTN in attenuated by DRX suggesting that the HTN associated with post-renal transplant cyclosporine treatment is caused by activation of ARNs (181).

It is important to note that DRX has failed to attenuate a number of other models of HTN. Most importantly, DRX did not attenuate the development of HTN in the spontaneously hypertensive rat (SHR) (175). This is a critical finding since this genetically spontaneous form of HTN is considered to be one of the more relevant models of human HTN. Additionally, HTN induced by infusion of NE into the renal artery or by wrapping and compressing one kidney with suture (renal wrap model) is not attenuated by DRX (80, 133). Taken together, these studies suggest that ARNs likely do not play a role in all forms of human HTN.

1.5 Thesis overview

While it has been shown that, at least in some people, RDNX can lower AP, it remains unclear whether this effect is due to ablation of afferent or efferent renal nerves. The importance of this question has been highlighted by the failure of a recent clinical trial of RDNX. In order to address this question, I have 1) established a model of HTN in which RDNX lowers AP, 2) developed a technique to selectively ablate afferent renal nerves, and 3) used this technique to test the central hypothesis that the antihypertensive effect of RDNX is due to ablation of afferent rather than efferent renal nerves (Figure 3).

The first of these points is addressed in chapter two. This chapter contains data from experiments that establish a genetic model of salt-sensitive HTN, the Dahl salt-sensitive (S) rat, as a model in which RDNX reduced AP. This project also established that, in addition to the renal nerves, the splanchnic sympathetic nerves are important in the maintenance of HTN in this model. Moreover, the antihypertensive effect of both RDNX and celiac ganglionectomy (CGX) to remove splanchnic sympathetic innervation, were unrelated to salt and water unloading. The results of this study showed the potential of targeting at multiple sympathetic end organs for the treatment of HTN.

Chapter three focuses on the development and validation of a novel method for selective ablation of afferent renal nerves, termed renal-CAP treatment. We showed that 1) renal-CAP treatment causes loss of afferent, but not efferent neural markers from

kidneys as assessed by immunohistochemistry and tissue assays, 2) renal-CAP treatment causes loss of afferent renal nerve functionality as assess by physiological responses to pharmacological activation of afferent renal nerves, 3) afferent renal nerves do not play a significant role in cardiovascular or sodium/water homeostasis during chronic dietary sodium loading and 4) the antihypertensive effect of RDNX in the DOCA-salt model is due to ablation of afferent renal nerves.

Having established the Dahl S rat as model HTN in which RDNX lowers AP, chapter four discusses data from a study in which we tested the hypothesis that the antihypertensive effect of RDNX in the Dahl S rat is due to ablation of afferent renal nerves. In this study we showed that the antihypertensive effect of RDNX in the Dahl S rat is identical whether it is done after 3 weeks of high salt (when MAP is ~140 mmHg) or whether it is done after 9 weeks of high salt (when MAP is ~170 mmHg). Moreover, we showed that, while RDNX lowered MAP ~10 mmHg compared to SHAM, renal-CAP treatment had absolutely no effect on AP. Taken together, these results suggest that the antihypertensive effect of RDNX in the Dahl S rat is not dependent on duration of high salt feeding or pretreatment blood pressure and that the antihypertensive effect of RDNX in this model is not due to ablation of afferent renal nerves.

In the final chapter, I will summarize all of these findings and lay out the significance of this work. This chapter will highlight the importance of the work presented and discuss how our findings not only inform the basic science aspect of HTN, but how they may impact the future of HTN treatment.

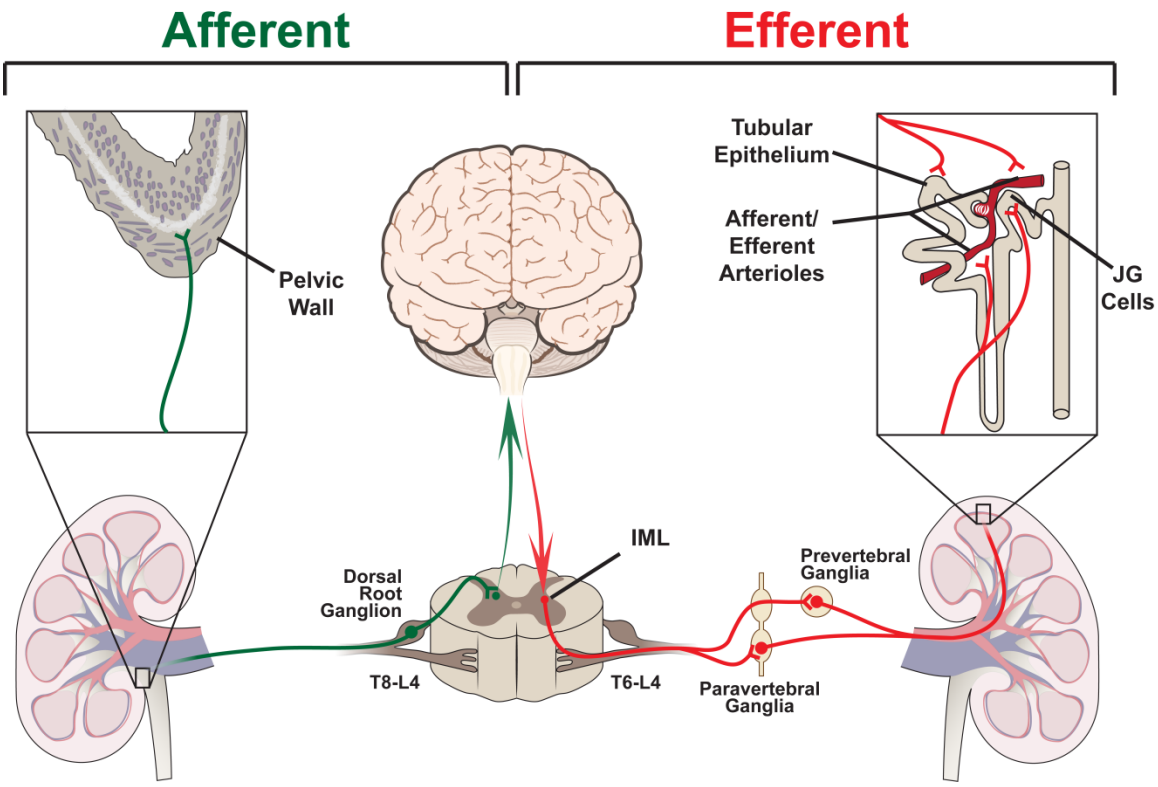

Figure 1. Gross anatomy of the renal nerves. Renal afferent pathways are depicted on the left. The cell bodies of renal primary afferent neurons are located in the dorsal root ganglia (DRG) at spinal levels T8-L4. These neurons have peripheral axons, which innvervate primarily the renal pelvic wall, and central axons, which synapse on secondary afferent neurons in the dorsal horn of the spinal cord. Renal efferent pathways are depicted on the right. Presympathetic neurons in the brain synapse onto sympathetic preganglionic neurons in the intermediolater cell column (IML) of the spinal cord. These neurons in turn have axons that synapse onto sympathetic post ganglionic neurons in the paravertebral ganglia (T6-L4) and the celiac, superior mesenteric and aorticorenal prevertebral ganglia. The axons of these postganlionic neurons course along the renal artery and vein, into the kidney and innervate primarily the glomerular arterioles, renal tubules and juxtaglomerular (JG) cells.

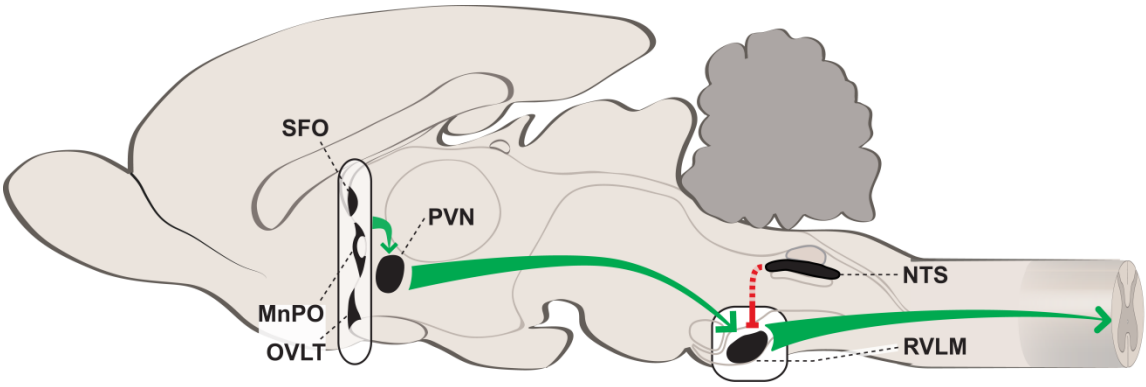

Figure 2. Sympathoregulatory brain regions involved in control of efferent sympathetic renal nerve activity. SFO = subfornical organ, MnPO = median preoptic nucleus, OVLT = organum vasculosum of the lamina terminalis, PVN = paraventricular nucleus, NTS = nucleus of the solitary tract, RVLM = rostral ventrolateral medulla.

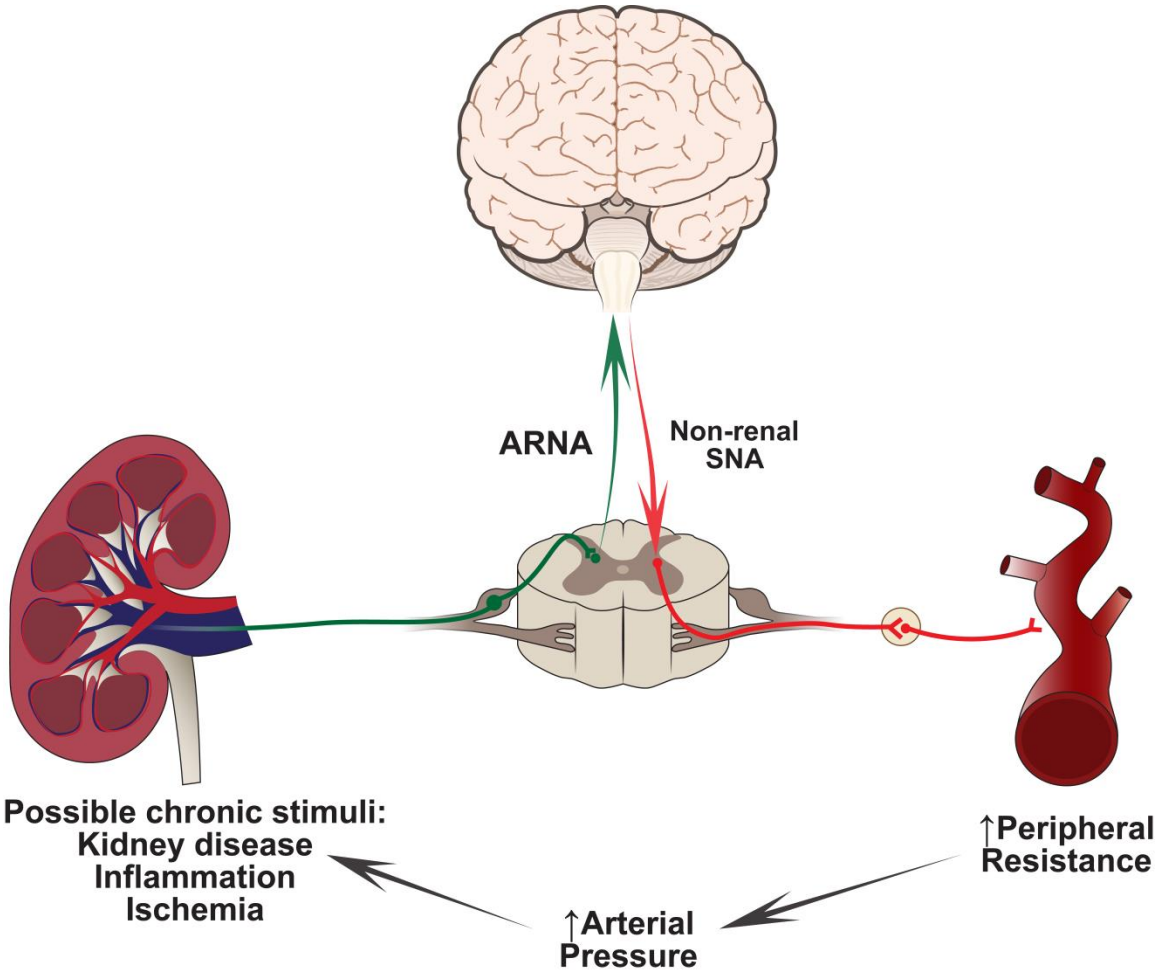

Figure 3. Hypothetical model. Chronic stimuli such as kidney disease, renal inflammation and renal ischemia cause increases in afferent renal nerve activity (ARNA). In turn, this drives sympathetic outflow to non-renal targets causing an increase in peripheral resistance and thus arterial pressure. Chronic increases in arterial pressure lead to feed back to cause additional insult to the kidney further driving ARNA.

Chapter Two

Reversal of Genetic Salt-Sensitive Hypertension by Targeted Sympathetic Ablation

Jason Foss1, Gregory D. Fink2 and John W. Osborn1

1University of Minnesota
Department of Integrative Biology and Physiology
Minneapolis, MN

2Michigan State University
Department of Pharmacology and Toxicology
East Lansing, MI

Published in Hypertension. 2013;61:806-811

2.1 Summary

The sympathetic nervous system plays an important role in some forms of human hypertension as well as the Dahl salt-sensitive rat model of hypertension; however, the sympathetic targets involved remain unclear. To address this, we examined the role of the renal and splanchnic sympathetic nerves in Dahl hypertension by performing either sham surgery (n = 10) or targeted sympathetic ablation of the renal nerves (renal denervation, n = 11), the splanchnic nerves (celiac ganglionectomy, n = 11) or both renal and splanchnic nerves (n = 11) in hypertensive Dahl rats. Mean arterial pressure increased from ~120 mmHg while on a 0.1% sodium chloride diet, to ~140 mmHg after being fed a 4.0% sodium chloride diet for three weeks. At that point rats underwent sham or targeted sympathetic ablation. Four weeks after treatment, mean arterial pressure was lower in renal denervated (150.4 ± 10.4) and celiac ganglionectomized (147.0 ± 6.1) rats compared to sham rats (165.0 ± 3.7), and even lower in rats that underwent both ablations (128.4 ± 6.6). There were no differences in heart rate or fluid balance between sham and renal denervated rats; however, rats that underwent either celiac ganglionectomy or both ablations exhibited marked tachycardia as well as sodium and water retention following treatment. These data suggest that targeted sympathetic ablation is an effective treatment for established hypertension in the Dahl rat and that the kidneys and the splanchnic vascular bed are both independently important targets of the sympathetic nervous system in this model.

2.2 Introduction

Hypertension is the leading risk factor for death worldwide (108), yet the underlying causes are poorly understood as evidenced by the staggering number of uncontrolled cases of hypertension (33, 40, 131, 136). Recent studies have shown that catheter-based renal nerve ablation results in a sustained reduction of arterial pressure in drug resistant hypertensives, suggesting that targeted sympathetic ablation may be an effective treatment for hypertension that avoids the side effects of globally acting sympatholytic drugs (100, 152, 153).

While the kidneys appear to be sympathetic targets in some forms of hypertension, other vascular beds, such as the splanchnic circulation, are also likely important as evidenced by the ability of splanchnic nerve stimulation to increase arterial pressure (102) and surgical splanchnic sympathectomy to attenuate drug resistant hypertension in humans (56, 145). Additionally, we have shown that the splanchnic, but not renal nerves contribute to the pathophysiology of angiotensin II-salt (AngII-salt) hypertension (91). Together, these findings suggest that renal nerve ablation may be effective in some, but not all, forms of hypertension and that targeted sympathetic ablation of non-renal vascular beds may be an effective strategy for the treatment of hypertension in some individuals.

With that in mind, the present study compared the effects of renal and splanchnic nerve ablation on arterial pressure and body fluid balance in a well-accepted and widely studied genetic model of neurogenic hypertension - the Dahl salt-sensitive (Dahl S) rat. Similar to a significant fraction of humans with essential hypertension, the Dahl S rat becomes hypertensive when fed a high salt diet, has increased sympathetic activity (19, 50, 54, 55, 117, 155) and has increased renal and splanchnic vascular resistance (16). Although renal nerve ablation has been consistently reported to have no effect on the development of Dahl S hypertension (75, 126, 132, 176), the ability of renal nerve ablation to reverse hypertension in this model not been reported. This is an important distinction with respect to the development of new therapies, which for the foreseeable

future will focus on the treatment rather than prevention of hypertension. To our knowledge, the effect of splanchnic nerve ablation on either the developmental or maintenance phase of Dahl S hypertension has not been reported.

The present study was designed to address the following questions. First, once hypertension is initiated does renal nerve ablation decrease arterial pressure in the Dahl S rat? If so, is this response due to increased renal excretion of sodium and water? Second, does ablation of sympathetic nerves innervating the splanchnic vascular bed decrease arterial pressure in the Dahl S rat and, if so, how does that response compare to renal nerve ablation? Third, is combined regional sympathectomy more effective in lowering arterial pressure than denervation of a single target?

2.3 Methods

Animals and General Procedures

Male Dahl S rats were purchased from Charles River Laboratories (Wilmington, MA) and housed in pairs in a temperature and light controlled room until the beginning of the study, at which time they were 64-70 days old and 250-340g. The rats were allowed access to standard rat chow and distilled water ad libitum during this pre-experimental period. All procedures were approved by the University of Minnesota Animal Care and Use Committee and were conducted in accordance with the institutional and National Institutes of Health guidelines. For all surgeries, rats were anesthetized with 2.0% isoflurane. Atropine sulfate (0.4 mg/kg, IP) and gentamicin sulfate (10 mg/kg, IM) were administered prior to surgery. For three days following surgery, buprenorphine (0.015 mg, SQ) was given twice per day and the drinking water was supplemented with amoxicillin (1 mg/ml).

Experimental Protocol

The timeline for the experimental protocol is shown in Figure 1. Rats were placed on a low salt diet (0.1% NaCl; Research Diets, New Brunswick, NJ) and instrumented

with radio telemeters (model TA11PA-C40, DSI, Intl. St. Paul, MN) for monitoring of mean arterial pressure (MAP) and heart rate (HR) as previously described (162). After a 7-day recovery period, rats were individually housed in metabolic cages (Techniplast 3701M001, Buguggiate, Italy) and allowed to acclimate for four days. Three days of baseline data were then collected (see below for details) and rats were placed on a high salt diet (4.0% NaCl; Research Diets) for the remainder of the protocol. After 21 days of high salt intake, rats were anesthetized with isoflurane and, via a midline approach, subjected to a sham (SHAM; n = 10), renal denervation (RDNX; n = 11), celiac ganglionectomy (CGX; n = 11) or combined renal denervation and celiac ganglionectomy (RDNX-CGX; n = 11) procedure. SHAM, RDNX and CGX procedures were performed as previously described (76, 107) and the combined RDNX-CGX was achieved by performing the RDNX and CGX in single procedure. Rats were returned to their cages and monitored for an additional 4 weeks. Upon completion of the study, rats were anesthetized with isoflurane and the duodenum, liver, spleen and both kidneys of each rat were harvested, weighed, immediately frozen with liquid nitrogen and stored at −80 °C until they were assayed for tissue norepinephrine content as previously described (107).

Daily Measurements

The transmitter signal was monitored by a receiver (Data Sciences, model RPC-1) mounted on the side of the metabolic cage and connected to a Data Exchange Matrix (Data Sciences, Int). The arterial pressure signal was sampled at 500 samples/second for 10 seconds every 4 minutes using commercially available software (Data Sciences, Int.). HR was determined from the arterial pressure profile using the same software. 24 hour (24h) averages of MAP and HR were determined and plotted for each day of the study.

24h food intake, water intake and urine output were measured gravimetrically. 24h sodium intake was calculated by multiplying food intake (grams) and sodium content of the diet (0.1% NaCl = 0.01711 mmol Na+/g food; 4.0% NaCl = 0.6844 mmol Na+/g food). 24h sodium excretion was calculated by multiplying 24h urine output (ml) and

urinary sodium concentration (mmol Na+/ml), which was measured using an ion specific electrode (NOVA-5+ electrolyte analyzer, Nova Biomedical, Waltham, MA). 24h sodium and water balances were calculated as 24h intake minus 24h excretion. Cumulative sodium and water balances were determined from sequential summation of daily balances over the duration of the protocol.

Statistical Analysis

Data were analyzed by 2-way analysis of variance for repeated measures followed by the Holm-Sidak method for all post-hoc comparisons (SigmaPlot version 10.0). A p value less than 0.05 was considered to be statistically significant.

2.4 Results

Cardiovascular Responses to Targeted Sympathetic Ablation

As shown in Figure 2, MAP increased in all groups from ~120 mmHg on the final day of 0.1% NaCl diet (SHAM = 120.2 ± 2.2, RDNX =119.5 ± 2.0, CGX = 119.8 ± 1.3, RDNX-CGX = 118.3 ± 1.8 mmHg) to ~140 mmHg after three weeks of 4.0% NaCl (SHAM = 140.1 ± 3.1, RDNX = 140.9 ± 5.0, GCX = 139.4 ± 2.4, RDNX-CGX = 138.5 ± 3.4 mmHg). Following SHAM surgery, MAP fell transiently, most likely due to a transient decrease in food (and therefore sodium) intake (please see http://hyper.ahajournals.org Figures S1), but returned to the pre-surgery trajectory within 7 days, reaching 165.0 ± 3.7 mmHg on the final day of the protocol. In contrast, MAP also fell in the RNDX group but did not rebound to the same trajectory and, by the end of the protocol, MAP was ~15 mmHg lower (150.4 ± 10.4) than SHAM rats. The magnitude and time course of the MAP response to CGX was similar to that of RDNX rats with MAP reaching a final level of 147.0 ± 6.1 mmHg. Finally, RDNX-CGX resulted in a much greater decrease in MAP than RDNX or CGX alone with a final MAP of 128.4 ± 6.6, ~35 mmHg lower than SHAM rats and ~20 mmHg lower than RDNX and CGX rats on the final day of the protocol. Moreover, the maximum pressure response was

greater in RDNX-CGX than all other groups (please see http://hyper.ahajournals.org Figures S2).

The effects of SHAM and targeted sympathetic ablation on HR are also shown in Figure 2. HR on the final day of baseline was similar in all groups (SHAM = 422.5 ± 2.7, RDNX =416.9 ± 4.9, CGX = 425.1 ± 4.1, RDNX-CGX = 420.6 ± 4.8 bpm) and decreased to a similar level after three weeks of high salt intake (SHAM = 380.0 ± 3.1, RDNX = 385.2 ± 3.0, CGX = 383.6 ± 2.8, 384.3 ± 2.9 bpm). Following treatment surgery, HR transiently increased and then gradually fell in SHAM and RDNX rats such that HR was not statistically different between these groups throughout the protocol. In contrast both CGX and RDNX-CGX rats exhibited a marked biphasic increase in HR following treatment. During the first peak, HR increased to 425.87 ± 10.5 in CGX rats and 450.22 ± 6.2 in RNDX-CGX rats and began to fall but then increased to a second peak before falling to levels slightly higher than SHAM and RDNX rats by the end of the protocol.

Sodium and Water Balance Responses to Targeted Sympathetic Ablation

Daily sodium and water intake, excretion and balance measurements were similar between all groups during the baseline period, increased transiently when the diet was increased to 4.0% NaCl, and returned to baseline levels until the time of SHAM or targeted sympathetic ablation (please see http://hyper.ahajournals.org Figures S1 and S3). There were no differences in these parameters between SHAM and RDNX rats at any time during the 4 weeks following surgery with the exception of the day after surgery when urine output was higher in RDNX than SHAM rats. Sodium and water intake, excretion and balance decreased transiently after SHAM and RDNX but returned to pre-procedure levels within 10 days.

Cumulative sodium and water balances over the duration of the protocol were calculated from the daily balance measurements in all 4 groups (Figure 3). There were no differences for cumulative sodium or water balance between RDNX and SHAM rats over the entire protocol. On the other hand, CGX rats retained sodium and water following

nerve ablation such that cumulative balances were significantly higher by the end of the protocol as compared to SHAM rats. This response was significantly attenuated by combining CGX with RDNX.

Tissue Norepinephrine Content

Tissues were collected at the end of the study to determine the extent of denervation 4 weeks post-procedure. Results of the norepinephrine assay clearly show that RDNX selectively denervated the kidneys, CGX selectively denervated the gut and RDNX-CGX denervated both (Figure 4).

2.5 Discussion

The sympathetic nervous system plays an important role in some forms of human hypertension; however, the degree to which sympathetic activity to the kidneys, relative to non-renal vascular beds, contributes to the pathogenesis and maintenance of essential hypertension remains unclear(57, 83, 115) . The recent success of catheter based renal nerve ablation to treat drug resistant hypertension supports the concept that the kidneys are a key target of the sympathetic nervous system in hypertension, but it is still not clear whether other vascular beds are also important.

The present study was designed to address this question by measuring the cardiovascular and fluid balance responses to targeted ablation of sympathetic nerves to the kidneys and the splanchnic vascular bed, separately and in combination, in Dahl S rats. The main findings of this study were: 1) RDNX decreased arterial pressure independent of changes in sodium and water balance; 2) CGX decreased arterial pressure to a similar magnitude as RDNX despite compensatory increases in HR and sodium and water balance; 3) combined RDNX and CGX induced the greatest fall in arterial pressure suggesting that the responses to RDNX and CGX are mediated by separate mechanisms. Overall, these results suggest that targeted sympathetic ablation is an effective treatment

for established hypertension in the Dahl S rat and that both the renal and splanchnic vascular beds are important sympathetic targets in this model.

Renal denervation partially reverses salt-induced hypertension in Dahl S rats

RDNX has no effect on the developmental phase of Dahl S hypertension (75, 132, 176). However, the ability of RDNX to reverse this model of hypertension has not been reported. Compared to SHAM rats, arterial pressure was ~15 mmHg lower in RDNX rats throughout the 4 week post-procedure period. The mechanism(s) by which RDNX attenuate(s) any form of hypertension is a subject of great debate. One explanation is that RDNX increases renal sodium and water excretion and causes a subsequent contraction of blood volume (36) by suppressing sympathetically mediated renin secretion and/or sodium reabsorption. However, we found no differences in daily or cumulative sodium and water balance between SHAM and RDNX rats. This is consistent with our previous reports that RDNX decreases arterial pressure in normotensive Sprague Dawley rats independent of sodium balance or renin release (76, 78). It is important to note that RDNX did not affect the salt sensitivity of arterial pressure in Sprague Dawley rats (76) or Dahl S rats.

Another possibility is that RDNX results in renal vasodilation. Although this hypothesis remains to be tested in the Dahl S rat, it was recently reported that renal nerve ablation in patients with drug resistant hypertension decreases renal vascular resistance with no change in glomerular filtration rate (112).

A third possibility is that RDNX attenuates hypertension by ablation of centrally projecting afferent renal nerves reducing sympathetic activity to non-renal vascular beds. As Dahl S rats become increasingly hypertensive, renal injury worsens (151, 156, 157), and evidence suggests that kidney disease can drive afferent renal nerve-dependent sympathetically mediated hypertension (30, 63). The progressive nature of this proposed kidney disease dependent sympathoexcitation may explain the difference in effectiveness of RDNX to reverse, rather than prevent Dahl S hypertension. However, it is important to

note that the arterial pressure response to RDNX may not require augmented afferent signaling since it occurs in normotensive Sprague-Dawley rats (76, 77).

Celiac Ganglionectomy Partially Reverses Hypertension in the Dahl S Rat: Possible Role of Blood Volume Redistribution

A novel finding of this study is that CGX decreased arterial pressure in Dahl S rats to a similar magnitude as RDNX. To our knowledge this the first study, in any experimental model, demonstrating the ability of CGX to reverse hypertension. Although the mechanisms by which CGX reversed Dahl S hypertension were not explored, it is likely that, similar to our studies in the AngII-salt model (91), CGX decreased splanchnic vascular resistance and increased total vascular conductance, both of which would promote a redistribution of blood from the arterial to the highly compliant venous compartment. This hypothesis is in line with our recently published mathematical model of salt-sensitive hypertension in which the distribution of blood volume between a high compliant (i.e. splanchnic) and low compliant (i.e. kidney) vascular bed is determined by neural input to each vascular bed (9). Additional experiments will be needed to test this hypothesis.

Sympathetic nerve activity was not measured in this study and therefore it remains to be tested whether the responses to CGX were due to decreased sympathetic nerve activity per se. Alternative explanations include the possibility that ablation of sensory nerves in the gut may affect arterial pressure in non-sympathetically mediated ways (i.e. changes in immune system function or circulating levels of vasopressin). However, we have reported in other models that CGX decreases non-hepatic splanchnic norepinephrine spillover (85), and increases total vascular capacitance (91). These findings combined with measurements of tissue NE in the present study are most consistent with idea that CGX reduces sympathetic input to splanchnic vasculature resulting in decreased arterial pressure. This hypothesis remains to be tested by studies of the splanchnic hemodynamic responses to CGX in the Dahl S rat.

The magnitude and time course of the arterial pressure response to CGX was nearly identical to RDNX suggesting these procedures may act via a common pathway. However, the responses of the other variables suggest that RDNX and CGX reduced arterial pressure by separate mechanisms. Specifically, CGX resulted in marked tachycardia and sodium and water retention compared to SHAM and RDNX rats. These responses are consistent with activation of compensatory mechanisms to maintain arterial pressure following loss of sympathetic input to the splanchnic vascular bed which may reduce effective blood volume and therefore cardiac output (via increased venous capacitance and reduced atrial filling) and total peripheral resistance (46). These results are consistent with compensatory increases in both cardiac and renal sympathetic activity following CGX. The greater tachycardic response to RDNX-CGX compared to CGX suggests a baroreflex mediated increase in cardiac sympathetic activity since the antihypertensive effect of RDNX-CGX was much greater than that of CGX. The blunted sodium and water retention following RDNX-CGX compared to CGX is consistent with the activation of renal sympathetic nerves following CGX. In addition, reduced atrial filling would be expected to inhibit the release of atrial natriuretic peptide (ANP) promoting sodium retention. The extent to which these mechanisms buffer the arterial pressure response to CGX in Dahl S rats remains to be determined.

Another possibility is that the compensatory HR and fluid balance responses to CGX were secondary to volume depletion due to decreased sodium and water absorption from the small intestine, since some rats exhibited diarrhea following CGX similar to previous studies using CGX (49, 118). While we cannot discount this entirely, we do not feel it was a major contributor to the responses to CGX for several reasons. First, in past studies we have shown that CGX increases total vascular capacitance but has no effect on absolute blood volume (91). Second, although some rats exhibited transient diarrhea (1-2 weeks), sodium retention persisted during the 2-3 weeks following the cessation of diarrhea. Finally, there was no correlation between final body weight (an index of nutrient absorption) and final arterial pressure in any group (please see http://hyper.ahajournals.org Figure S4). We conclude that the compensatory responses of

HR and fluid balance to CGX are secondary to a decrease in effective blood volume rather than a reduction in absolute volume. This hypothesis will be tested in future studies in which we measure the effect of CGX on mean circulatory filling pressure in Dahl S rats.

Additive Effects of Renal and Splanchnic Denervation in the Treatment of Hypertension

Further evidence that RDNX and CGX act via separate pathways is the fact that the combination of these treatments resulted in a greater response than either treatment alone. In addition, based on analysis of tissue norepinephrine content, RDNX had no effect on the duodenum, liver or spleen and CGX had no effect on the kidneys. Taken together we conclude that the renal nerves and splanchnic nerves contribute to the maintenance of Dahl S hypertension independently of one another and therefore RDNX and CGX reduce arterial pressure via two distinct mechanisms.

It is worth noting that the compensatory increases in HR and sodium and water retention seen in CGX rats were also observed in RDNX-CGX rats. The increase in HR during the first peak tended to be greater in RDNX-CGX rats than in CGX rats, which is consistent with a baroreflex mediated response as discussed above. Similarly, sodium retention in RDNX-CGX rats may have resulted from the greater fall in renal perfusion pressure, via the pressure-natriuresis relationship or suppression of plasma ANP as discussed above.

2.6 Perspectives

Renal nerve ablation has been shown to decrease arterial pressure in some human hypertensives; however, the lack of a method to quantitate the extent of denervation in humans makes it impossible to establish the efficacy of this treatment since failure to respond may be due to incomplete denervation or the fact that renal nerves do not contribute to all forms of human essential hypertension. The Dahl S rat is an excellent

animal model for preclinical studies to address this issue. The response of arterial pressure to renal denervation in this model suggests that this approach can partially reverse hypertension but sympathetic nerves to other target organs are also important. Specifically, our study suggests that targeted splanchnic nerve ablation may have an additional therapeutic effect and should be pursued as a possible stand-alone or adjunct treatment for hypertension in the future. Further studies are needed to establish the mechanisms underlying the antihypertensive effects of these interventions.

Acknowledgments

The authors would like to thank Dusty Van Helden and Marcos Kuroki for their help with metabolic data collection and analysis.

Sources of funding

This research was supported by NIH grant R01 HL076312

Conflict(s) of interest/disclosure(s) statement

J.W.O. is a paid consultant of Medtronic CardioVascular, Inc. Santa Rosa, CA

Novelty and significance

What Is New?

- RDNX and CGX both decrease arterial pressure in hypertensive Dahl S rats.
- CGX and RDNX-CGX, but not RDNX cause increases in heart rate as well as sodium and water retention.
- RDNX-CGX has an additive antihypertensive effect.
- The responses to RDNX and CGX are mediated by different mechanisms.

What Is Relevant?

- RDNX decreased arterial pressure to a magnitude similar to that reported in humans.
- CGX decreased arterial pressure as has been reported in human hypertensives.

Summary

The results of this study suggest that both the renal and splanchnic nerves contribute to the maintenance of Dahl S hypertension and that targeted ablation of the splanchnic sympathetic nerves should be considered as treatment option for hypertensive patients.

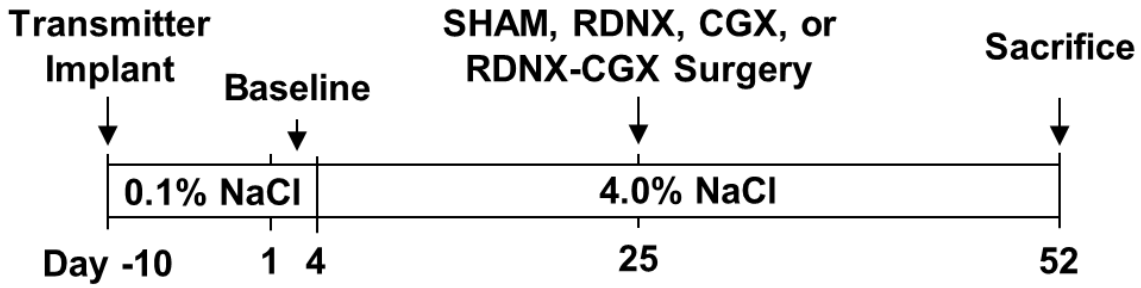

Figure 1. Protocol timeline. CGX = celiac ganglionectomy, RDNX = renal denervation, RDNX-CGX = combined celiac ganglionectomy and renal denervation, and SHAM = sham surgery.

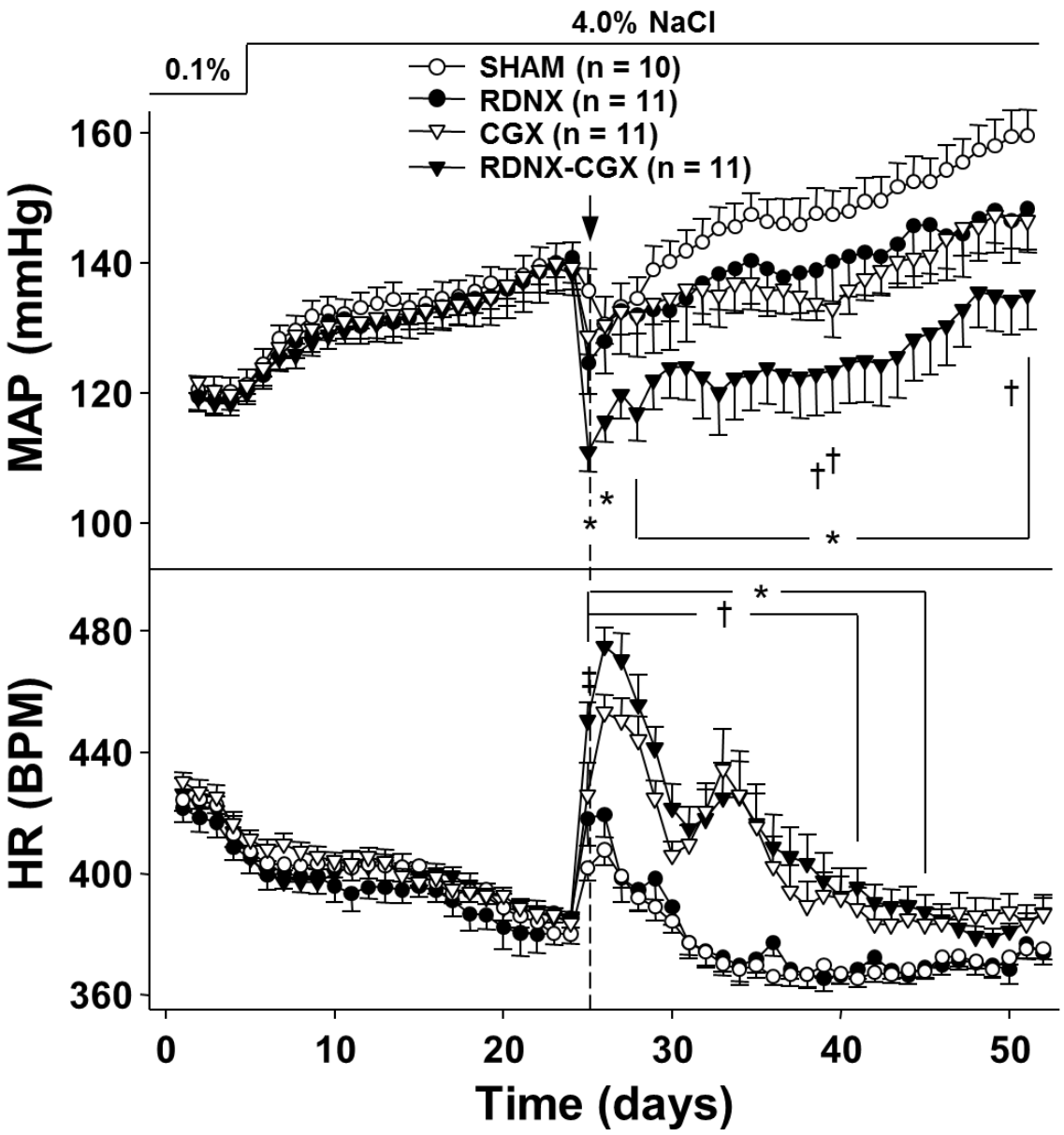

Figure 2. Effect of SHAM, RDNX, CGX and RDNX-CGX on MAP and HR. * = $p < 0.05$ for RDNX-CGX vs. SHAM. † = $p < 0.05$ for CGX vs. SHAM. ‡ = $p < 0.05$ for RDNX vs. SHAM.

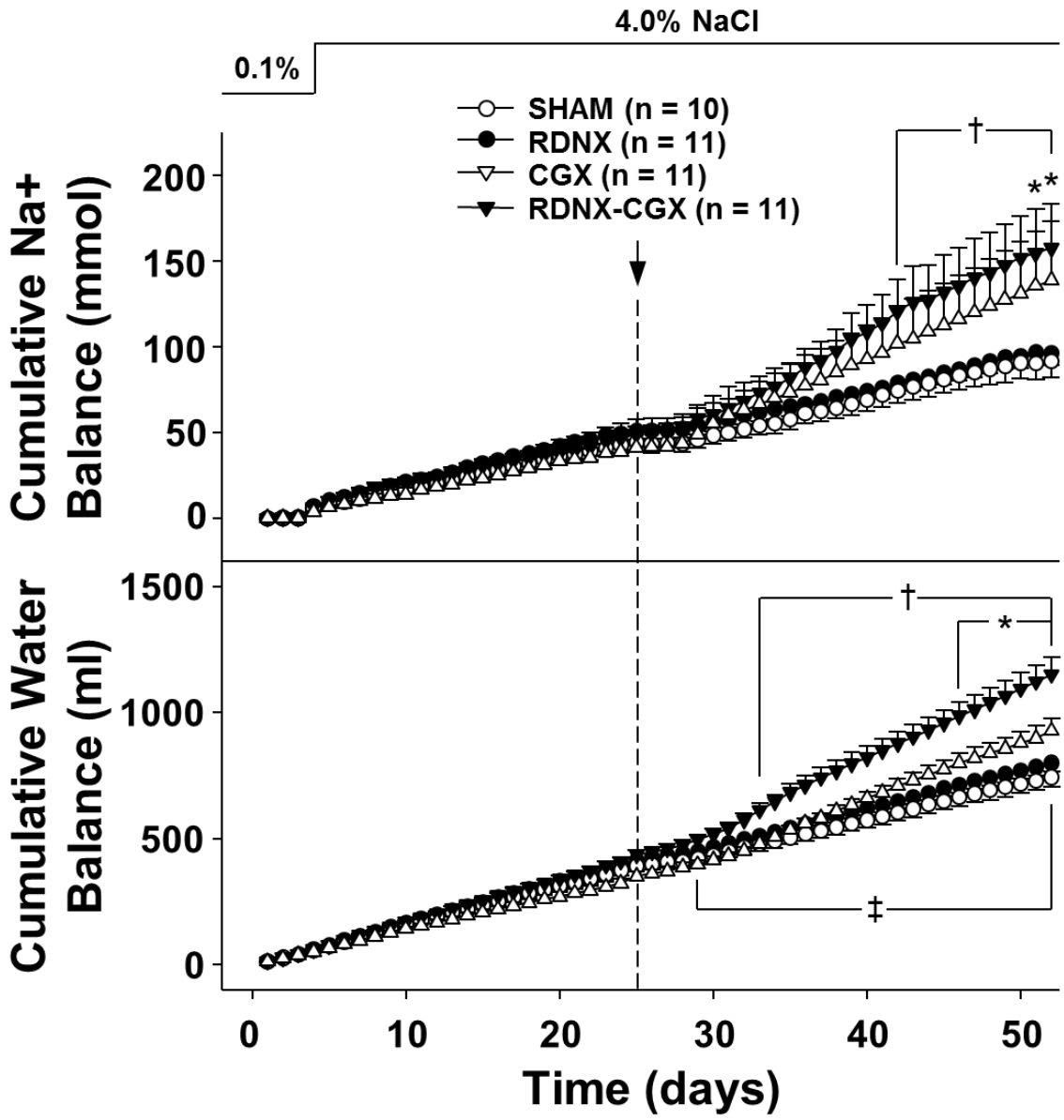

Figure 3. Effect of SHAM, RDNX, CGX and RDNX-CGX on cumulative sodium and water balance. * = $p < 0.05$ for RDNX-CGX vs. SHAM. † = $p < 0.05$ for CGX vs. SHAM. ‡ = $p < 0.05$ for CGX vs. RDNX-CGX.

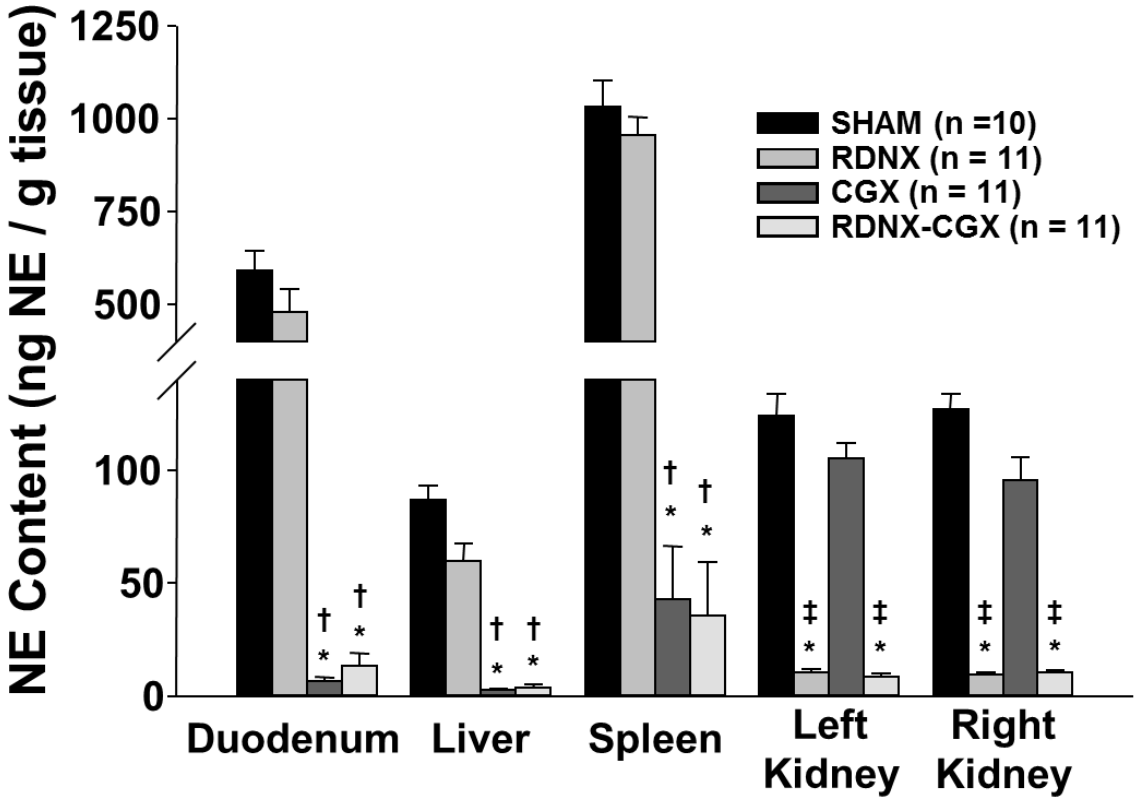

Figure 4. Effect of SHAM, RDNX, CGX and RDNX-CGX on norepinephrine (NE) content in the duodenum, liver, spleen, left kidney and right kidney. * = $p < 0.05$ vs. SHAM. † = $p < 0.05$ vs. RDNX. ‡ = $p < 0.05$ vs. CGX.

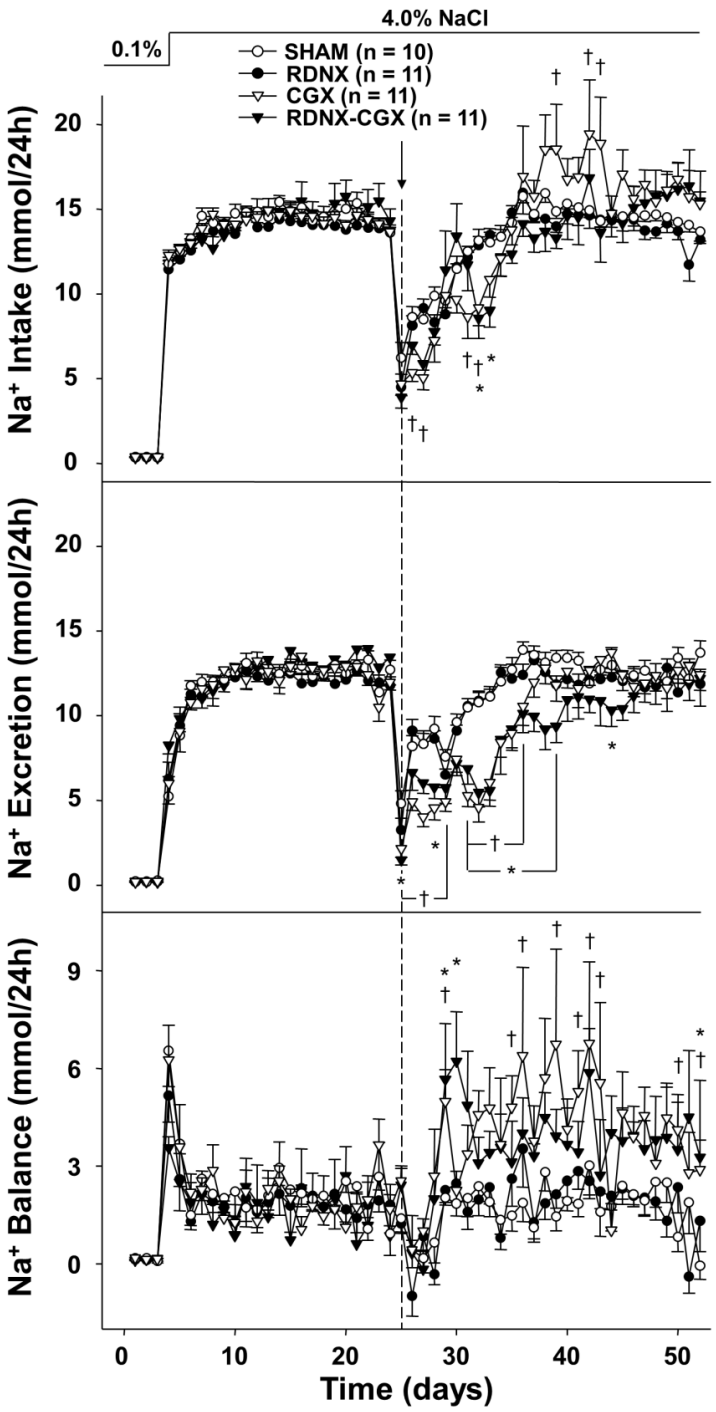

Figure S1. Effect of SHAM, RDNX, CGX and RDNX-CGX on sodium intake, excretion and balance. * = $p < 0.05$ for RDNX-CGX vs. SHAM. † = $p < 0.05$ for CGX vs. SHAM. ‡ = $p < 0.05$ for RDNX vs. SHAM.

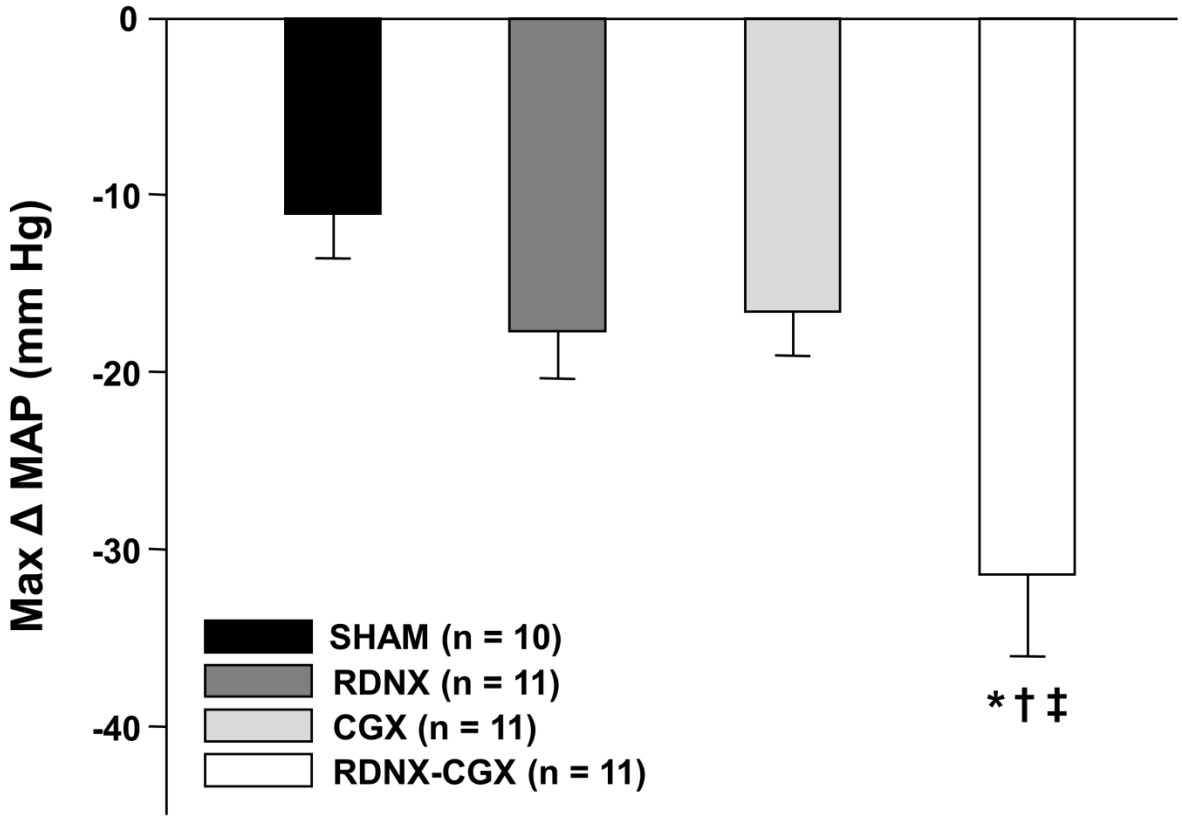

Figure S2. Maximum arterial pressure response to SHAM, RDNX, CGX and RDNX-CGX (Max ΔMAP; the lowest MAP following surgery minus MAP on the day before surgery). * = $p < 0.05$ vs. SHAM. † = $p < 0.05$ vs. RDNX. ‡ = $p < 0.05$ vs. CGX.

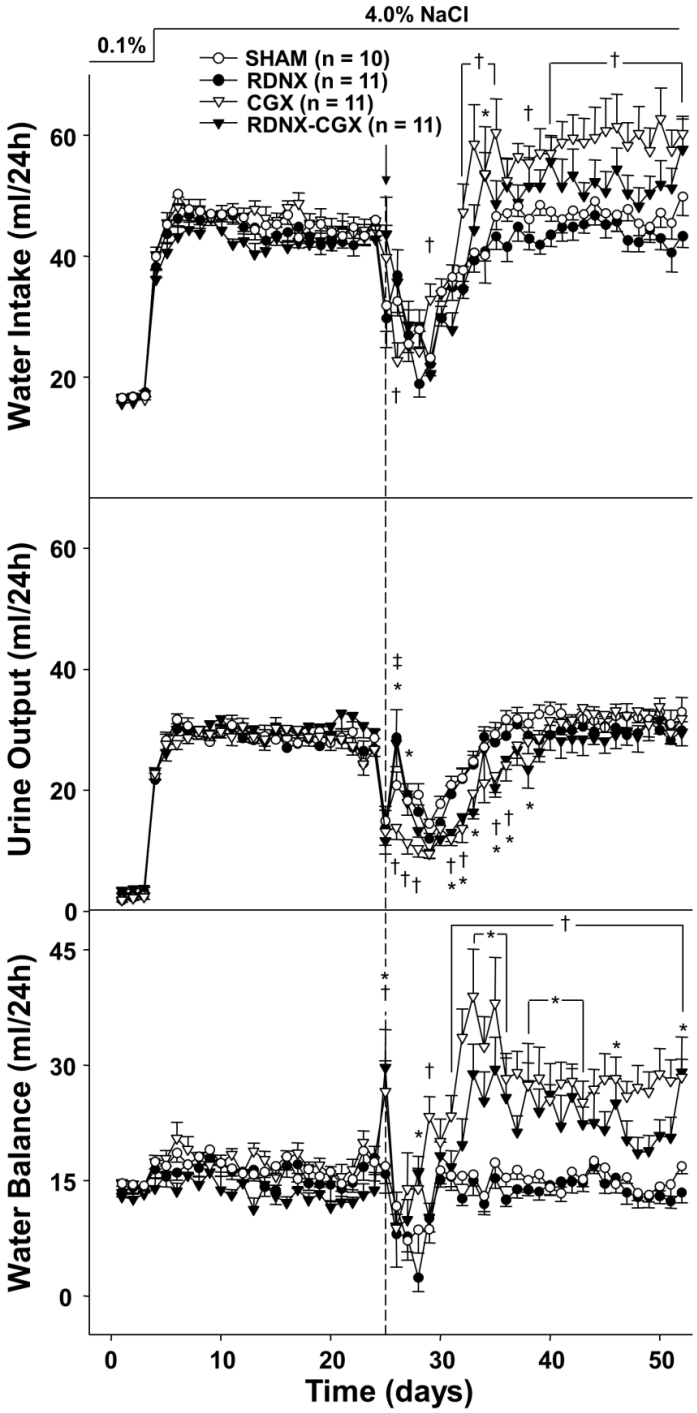

Figure S3. Effect of SHAM, RDNX, CGX and RDNX-CGX on water intake, urine output and water balance. * = $p < 0.05$ for RDNX-CGX vs. SHAM. † = $p < 0.05$ for CGX vs. SHAM. ‡ = $p < 0.05$ for RDNX vs. SHAM.

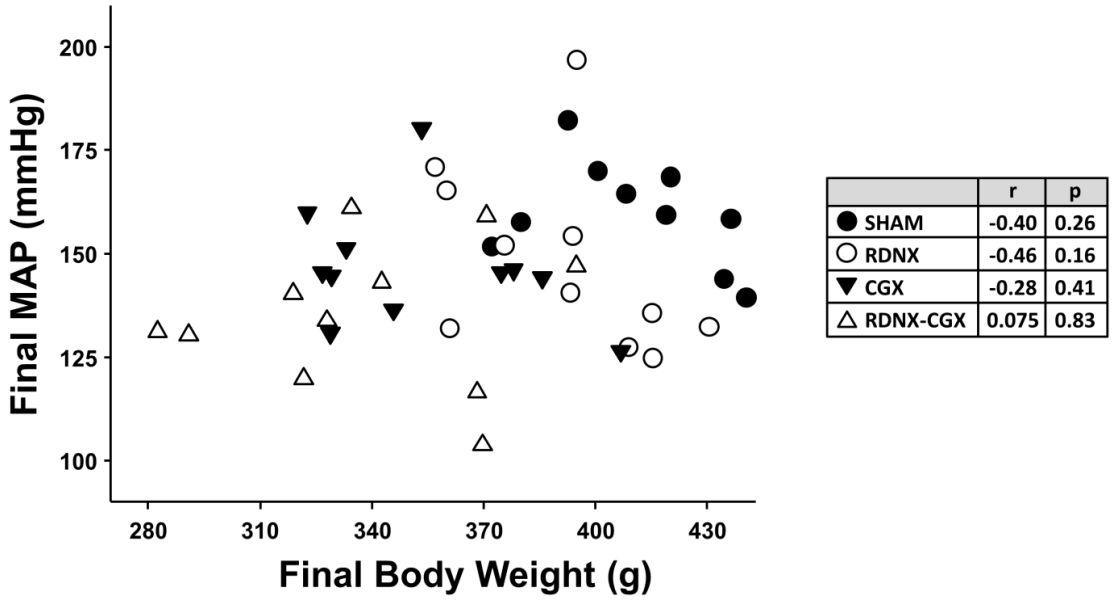

Figure S4. Final MAP vs. final body weight. No correlation exists between final MAP and final body weight in any group.

Chapter three

A Novel Method of Selective Ablation of Afferent Renal Nerves by Periaxonal Application of Capsaicin

Jason D. Foss1, Richard D. Wainford2, William C. Engeland3, Gregory D. Fink4 and John W. Osborn1

University of Minnesota

Departments of 1Integrative Biology and Physiology, and 3Neuroscience

Minneapolis, MN

2 Boston University School of Medicine

Department of Pharmacology & Experimental Therapeutics and the Whitaker Cardiovascular Institute

Boston, MA

4Michigan State University

Department of Pharmacology and Toxicology

East Lansing, MI

Currently in review for publication in the American Journal of Physiology - Regulatory, Integrative and Comparative Physiology

3.1 Summary

Renal denervation has been shown to lower arterial pressure in some hypertensive patients, yet it remains unclear whether this is due to ablation of afferent or efferent renal nerves. To investigate the role of afferent renal nerves in arterial pressure regulation, previous studies have used methods that disrupt both renal and non-renal afferent signaling. The present study was conducted to develop and validate a technique for selective ablation of afferent renal nerves that does not disrupt other afferent pathways. To do this, we adapted a technique for sensory denervation of the adrenal gland by topical application of capsaicin and tested the hypothesis that exposure of the renal nerves to capsaicin (renal-CAP treatment) causes ablation of afferent but not efferent renal nerves. Renal-CAP treatment had no effect on renal content of the efferent nerve markers tyrosine hydroxylase and norepinephrine; however, the afferent nerve marker, calcitonin gene-related peptide was largely depleted from the kidney 10 days after treatment, but returned to roughly half of control levels by seven weeks post-treatment. Moreover, renal-CAP treatment abolished the cardiovascular responses to acute pharmacological stimulation of afferent renal nerves. Renal-CAP treated rats showed normal weight gain as well as cardiovascular and fluid balance regulation during dietary sodium loading. To some extent, renal-CAP treatment did blunt the bradycardic response, and increase the dipsogenic response to increased salt intake. Lastly, renal-CAP treatment significantly attenuated the development of deoxycorticosterone acetate-salt hypertension. These results demonstrate that renal-CAP treatment effectively causes selective ablation of afferent renal nerves in rats.

3.2 Introduction

Numerous clinical trials have highlighted catheter based renal nerve ablation (renal denervation; RDNX) as a possibly viable antihypertensive treatment in patients with drug-resistant hypertension (43, 101, 114), although other studies have failed to show a significant antihypertensive effect of the treatment (13, 18). Despite the controversy surrounding these clinical data, RDNX has been consistently shown to reduce blood pressure in numerous animal models of hypertension (38) suggesting that when sufficient denervation is achieved, RDNX can have an antihypertensive effect in certain forms of hypertension. The failure of RDNX to reduce blood pressure in some cases highlights the importance of further understanding the mechanisms underlying the antihypertensive effect of RDNX. Specifically, since renal nerves contain both motor (efferent) and sensory (afferent) fibers, it is not known whether the antihypertensive response to RDNX is due to ablation of efferent nerves, afferent nerves, or both. Since elevated efferent renal nerve activity increases renal sodium reabsorption, renal vascular resistance and renin release, one hypothesis is that RDNX lowers arterial pressure secondary to a reduction in one or more of these parameters (36). Alternatively, elevated afferent renal nerve activity has been shown to increase sympathetic nerve activity to renal and non-renal vascular beds (135, 146) suggesting that RDNX may decrease arterial pressure by reducing sympathetic tone, not only to the kidney, but to other targets as well. Recent studies in humans with drug resistant hypertension support both of these hypotheses. Specifically, RDNX has been shown to decrease renal resistive index, likely resulting from a loss of efferent neural control of renal vascular tone (112). It has also been reported that RDNX decreases skeletal muscle sympathetic activity (67, 143), plasma glucose (113), incidence of cardiac arrhythmias (137, 158), and the frequency of episodes of sleep apnea (171). These responses likely result from ablation of afferent renal nerves. However, it should be noted that at least one study has shown no reduction in skeletal muscle sympathetic activity following RDNX (18).

Surgical RDNX has been used for decades in animal models to study the role of renal nerves in the regulation of renal and cardiovascular function (38). However, as is true with catheter based renal nerve ablation in humans, this method is non-selective since it disrupts both afferent and efferent neural pathways. To study the functions of afferent renal nerves specifically, a procedure termed dorsal rhizotomy has been used (97, 98). This surgical technique interrupts afferent neural pathways from the kidney by sectioning the dorsal roots of the spinal cord at levels T9-L1. While this procedure removes a large portion of the renal afferent input into the spinal cord, it is not specific to renal afferents since it also disrupts all visceral, somatic and cutaneous afferent inputs at these spinal levels. This is particularly important since osmosensitive and sodium-sensitive hepatoportal afferents, which are known to regulate efferent renal nerve activity, at least acutely (74, 121, 122), are also sectioned by this procedure. Moreover, we have previously shown that denervation of these afferents chronically increases arterial pressure in normal rats although we did not establish the role of renal nerves in this response (23). Another method used to disrupt afferent renal nerve signaling is systemic administration of the transient receptor potential (TRP) V1 receptor agonist, capsaicin, to neonatal rat pups (72, 164, 165, 168). It is well documented that capsaicin ablates small, unmyelinated C-fibers (47, 69) and this method destroys afferent neurons in the kidney; however, it is not specific for renal afferents since systemic delivery of capsaicin has been shown to cause degeneration of TRPV1+ sensory fibers throughout the body. Nonetheless, it is interesting to note that both dorsal rhizotomy and systemic capsaicin treatment have been reported to cause salt-sensitive hypertension in the rat (97, 164) and these findings have been interpreted as evidence that disruption of renal afferent signaling may be the cause of this salt-sensitive hypertension (97). However, since both methods are not selective for renal afferents, the possibility remains that the observed salt-sensitive hypertension resulted from the ablation of other sensory afferents, such as hepato-portal osmoreceptors(122, 123).

There is clearly a strong need for a technique to selectively ablate afferent renal nerves experimentally. Such a technique would be a valuable tool for studying the role of

renal afferent signaling in the regulation of renal and cardiovascular function, and could also pave the way for development of more refined renal nerve ablation therapies in humans. The present study was conducted to address this need. The approach we used was adapted from our previous findings that topical application of capsaicin to the adrenal gland causes selective ablation of adrenal afferent nerves in the rat(159-161). Because the majority of renal afferent fibers are unmyelinated (or thinly myelinated) and capsaicin sensitive (93, 166), and TRPV1 receptors are localized along the axons of sensory fibers as well as nerve terminals (154), we hypothesized that periaxonal application of capsaicin to the renal nerves (renal-CAP treatment) would cause selective ablation of afferent renal nerves.

We tested this hypothesis by quantifying the content of neuronal markers in the kidney at various time points following renal-CAP treatment using the afferent nerve marker, calcitonin gene related peptide (CGRP), and the efferent nerve markers tyrosine hydroxylase (TH) and norepinephrine (NE). We also assessed the ability of renal-CAP treatment to abolish the afferent renal nerve-dependent cardiovascular responses to intrarenal infusions of bradykinin in conscious rats. Additionally, since previous studies using non-selective methods to ablate afferent renal nerves have suggested that impaired renal afferent signaling may be a determinant of salt-sensitive hypertension, we assessed the effect renal-CAP treatment on the regulation of arterial pressure and sodium/water balance in rats fed varying levels of dietary sodium. Finally, we tested whether the development of deoxycorticosterone acetate (DOCA)-salt hypertension, which has been proposed to be driven by afferent renal nerves (77), is attenuated by renal-CAP treatment.

3.3 Materials and methods

Animals and General Procedures

With the exception of the experiments using intrarenal and intravenous bradykinin infusion, all experiments were performed at the University of Minnesota. For these studies, male Sprague Dawley rats were purchased from Charles River Laboratories

(Wilmington, MA) and housed in pairs in a temperature and light controlled room until the beginning of the study. Rats were allowed access to standard rat chow and distilled water ad libitum during this pre-experimental period. For all surgical procedures performed in these experiments, rats were anesthetized with 2.0% isoflurane and atropine sulfate (0.2 mg/kg, intraperitoneal) and ketoprofen (5 mg/kg, subcutaneous) and gentamicin sulfate (2.5 mg/kg, intramuscular]) were administered prior to surgery. For three days following surgery, ketoprofen (2.5 mg/kg, subcutaneous) was given once per day and the drinking water was supplemented with amoxicillin (1 mg/ml). All procedures were approved by the University of Minnesota Animal Care and Use Committee and were conducted in accordance with the institutional and National Institutes of Health guidelines.

The experiments to assess cardiovascular responses to intrarenal and intravenous infusions of bradykinin were conducted at Boston University. For these studies, male Sprague Dawley rats were purchased from Harlan Laboratories, Inc. (Indianapolis, IN) and housed individually in a temperature and light controlled room. Following completion of surgical procedures, rats received a standard rat chow and water ad libitum. All procedures were approved by the Boston University School of Medicine Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical Procedures

Renal-CAP Treatment: Rats were anesthetized, a midline abdominal incision was made and the visceral organs were externalized and reflected to expose the left kidney. A hole was made in the peritoneal membrane to expose the renal artery and vein. The fat surrounding the renal artery and vein was then gently dissected away from the vessels to expose the renal nerves. A small piece of gauze soaked in a capsaicin solution (33 mM in 5% ethanol, 5% tween 80 and 90% normal saline) was wrapped around the renal artery and vein for 15 minutes. A small piece of parafilm was placed under the renal artery and vein prior to the placement of capsaicin-soaked gauze to prevent any non-renal capsaicin

exposure. Following 15 minutes of capsaicin exposure, the gauze and parafilm were removed, the area was dried, and the procedure repeated on the contralateral side if called for in the experimental protocol. At the end of the procedure, the viscera were replaced and the abdominal muscles and skin were closed separately with 3-0 silk suture. The sham control treatment was performed by externalizing the viscera, visualizing the renal artery and vein, replacing the organs and then closing the wound as described above.

Complete Renal Denervation (RDNX): Denervation of both afferent and efferent renal nerves was performed as previously described (77). Briefly, rats were anesthetized with 2% isoflurane and the left renal nerves were then exposed through a midline abdominal incision. Using a dissecting microscope, the renal vein and artery were dissected out of the surrounding fascia and stripped of all visible renal nerve bundles. Following dissection, the renal artery was painted with 10% phenol in ethanol to ensure the destruction of any remaining renal nerve fibers. This procedure was then repeated on the contralateral side and the incisions closed.

Instrumentation for Acute Assessment of Afferent Renal Nerve Functionality: On the day of acute bradykinin experiments, rats were anesthetized with sodium methohexital (20mg/kg, intraperitoneal; supplemented with 10 mg/kg, intravenous as required). To allow for intrarenal infusion of bradykinin, the left kidney was exposed through a small flank incision. A catheter (polyethylene; PE 10) was then placed in a branch of the left renal artery and exteriorized through the flank incision and the incision was closed. Collection of urine was achieved through a catheter (polyethylene; PE 240) inserted into the urinary bladder via a midline abdominal incision. The catheter was exteriorized through this incision and the incision was closed. An additional incision was made in the left inguinal area. The left femoral vein was catheterized (polyethylene; PE 50) for intravenous infusion of saline or drugs and the left femoral artery was catheterized (polyethylene; PE 50) for measurement of mean arterial pressure (MAP) and heart rate (HR). Both catheters were exteriorized at the incision site and the incision was closed.

Validation of renal-CAP treatment by IHC

To test the efficacy of renal-CAP treatment to cause selective ablation of afferent renal nerves, and to determine whether reinnervation occurs over time, male Sprague Dawley rats (58-65 days old, 220-330g) were subjected to either bilateral renal-CAP or SHAM treatment and sacrificed after 10 days or 4 weeks. Kidneys were then collected for immunohistochemical labeling of the efferent nerve-specific marker TH and the afferent nerve-specific marker CGRP. Additionally, IHC data for a 7-week post-treatment time point were obtained from rats that underwent the salt-sensitivity protocol described below.

At the endpoint, rats were anesthetized with 2% isoflurane, euthanized by exsanguination and kidneys were immediately removed, rinsed in normal saline and placed in 10% neutral buffered formalin until the time of sectioning. Kidneys were embedded in paraffin and cut longitudinally at a thickness of 5 µm. Sections were deparaffinized and subjected to heat induced epitope retrieval (HIER) in a Decloaking Chamber (Biocare Medical; Model DC2008US) using Antigen Decloaker solution (Biocare Medical; CB910M) at 95°C. Sections were incubated in Protein Block Serum-Free (Dako; X0909) then in a polyclonal CGRP antibody (Enzo; BML-CA1134-0100) diluted to 1:500 in DaVinci Green Universal Diluent (Biocare Medical; PD900). Endogenous peroxidases were blocked with Peroxidazed 1 (Biocare Medical; PX968M) and sections were incubated in an anti-rabbit:HRP polymer (Invitrogen; 87-9263). Betazoid DAB Chromogen (Biocare Medical; BDB2004MM) was then applied to each slide and sections were incubated with Protein Block Serum-Free. Sections were then incubated in a rabbit polyclonal TH antibody (Abcam; ab112) diluted to 1:1000 followed by Mach 3 Probe (Biocare Medical) and Mach 3 Rabbit AP-Polymer (Biocare Medical; M3R533L). Sections were then incubated in Warp Red Chromogen (Biocare Medical; WR806S) and counterstained with Mayer's Hematoxylin (Electron Microscopy Sciences; 26043-06). Each slide was dipped in xylene and a coverslip was applied with Surgipath Micromount permanent mounting media (Leica; 3801731).

To quantify the labeling of CGRP and TH, IHC slides were digitally photographed and the images were analyzed using the Positive Pixel Count v9 algorithm in ImageScope software v11 (Aperio Technologies, Vista, CA). Because CGRP labeling was confined to the renal pelvis, CGRP content was quantified as the area of CGRP+ labeling (μm2) per length of pelvic wall analyzed (mm). Because TH labeling was most robust in the large nerve bundles found in the hilum of the kidney, TH was quantified as the percent area of a major nerve bundle that was labeled positive for TH. Quantification was done by individuals blinded to the experimental group.

Validation of renal-CAP treatment by ELISA and HPLC

We also used an enzyme-linked immunosorbent assay (ELISA) for CGRP and high-performance liquid chromatography (HPLC) for NE to quantify the extent of selective afferent renal nerve ablation and the time course of reinnervation. Male Sprague Dawley rats (59-74 days old, 230-375g) were subjected to unilateral renal-CAP treatment of the left kidney, with the right kidney serving as a sham-operated control. Rats were sacrificed 10 days, 4 weeks or 7 weeks after treatment and kidneys were collected and assayed for content of CGRP and NE.

Kidneys were removed immediately after death; the renal artery, renal vein, ureter and capsule were removed; and the kidneys were placed in a cold normal saline bath for further dissection. Renal parenchymal samples were taken from the poles and lateral portion of the kidney and flash frozen in liquid nitrogen. The renal pelvis was then carefully dissected from the remaining portion of kidney and flash frozen in liquid nitrogen. All frozen samples were stored at -80°C until being assayed.

The parenchymal samples were assayed for NE using HPLC as previously described (107). The isolated renal pelvic samples were assayed for CGRP content using a commercially available ELISA kit (Cayman Chemicals; Ann Arbor, MI; Item Number 589001). Tissues were homogenized in 1M acetic acid and CGRP was extracted using C18 Sep-columns (Peninsula Laboratories; San Carlos, CA; Item Number Y-1000). To

eliminate any interassay variance, all pelvic samples were run on single 96 well ELISA plate.

Functional validation of renal-CAP treatment

A test of afferent renal nerve functionality was conducted in male Sprague Dawley rats (275-300 g) 7-10 days after being subjected to either SHAM or bilateral renal-CAP treatment (n = 6 per treatment group). On the day of the acute experiment, rats were instrumented as described above. Rats were then placed in a plexiglass holder and an intravenous infusion of sterile isotonic saline (20µl/min) was maintained for a 2 hour surgical recovery period prior to experimentation to enable the animal to regain full consciousness and cardiovascular and renal excretory function to stabilize.

MAP and HR were continuously recorded via the surgically implanted femoral artery cannula using computer-driven BIOPAC data acquisition software (MP150 and AcqKnowledge 3.8.2, CA) connected to an external pressure transducer (P23XL; Viggo Spectramed Inc., CA). MAP and HR were measured during a 20 minute control period. Bradykinin was then infused, in a random order, at concentrations of 5, 10, 20 and 40 µg/kg/min either intravenously or intrarenally for a period of 5 min per dose per administration route. MAP and HR were recorded as the average value of the last 2 minutes of each infusion period.

Effect of renal-CAP treatment on cardiovascular and fluid balance responses to increased salt intake

In order to determine whether renal-CAP treatment adversely affects cardiovascular and fluid homeostasis under conditions of chronic sodium loading, rats were subjected to the following protocol. Male Sprague Dawley rats (71-72 days old, 330-390 g) underwent bilateral renal-CAP (n = 8) or SHAM treatment (n = 8) and were implanted with radiotelemeters (model TA11PA-C40, Data Sciences, Int., St. Paul, MN) to measure MAP and HR as previously described (162). The rats were fed a 0.1% NaCl diet (Research Diets, New Brunswick, NJ) and allowed to recover for 10 days. After a 4

day baseline period, the diet was increased to 4% NaCl (Research Diets) for 3 weeks then 8% NaCl (Research Diets) for 2 weeks. The animals were housed in metabolic cages (Techniplast 3701M001, Buguggiate, Italy) for daily measurement of sodium intake, sodium excretion, water intake and urine output. At the end of the protocol, rats were sacrificed and kidneys were collected for immunohistochemical analysis of TH and CGRP.

The radiotelemeter signal was monitored by a receiver (model RPC-1, Data Sciences, Int.) mounted on the side of the metabolic cage and connected to a Data Exchange Matrix (Data Sciences, Int.). The arterial pressure signal was sampled at 500 samples/second for 10 seconds every 4 minutes using Dataquest A.R.T.™ Platinum Acquisition software (version 4.30, Data Sciences, Int.). HR was determined from the arterial pressure profile using the same software. 24-hour (24h) averages of MAP and HR were determined and plotted for each day of the study.

24h food intake, water intake and urine output were measured gravimetrically. 24h sodium intake was calculated by multiplying food intake (grams) and sodium content of the diet (0.1% NaCl = 0.01711 mmol Na+/g food; 4.0% NaCl = 0.6844 mmol Na+/g food; 8.0% NaCl = 1.3688 mmol Na+/g food). 24h sodium excretion was calculated by multiplying 24h urine output (ml) and urinary sodium concentration (mmol Na+/ml), which was measured using an ion specific electrode (NOVA-5+ electrolyte analyzer, Nova Biomedical, Waltham, MA). 24h sodium and water balances were calculated as 24h intake minus 24h excretion.

Effect of renal-CAP treatment on the development of DOCA-salt hypertension

We have previously reported that RDNX attenuates the development of hypertension in the DOCA-salt model by 50% and hypothesized this was due to ablation of renal afferent nerves (77). Therefore, to test whether the antihypertensive effect of RDNX in the DOCA-salt model is due to ablation of afferent renal nerves, rats underwent either SHAM, RDNX or renal-CAP treatment and were subjected to DOCA-salt treatment as previously described (77). Briefly, male Sprague-Dawley rats (285-400g)

underwent left unilateral nephrectomy and were allowed two weeks for compensatory renal hypertrophy. Rats were then subjected to SHAM, RDNX or renal-CAP and implanted with a radiotelemeter (Data Sciences, Int.) for measurement of MAP and HR. After ten days of recovery and three days of baseline, silicone pellets containing a total of 100 mg DOCA were implanted subcutaneously between the scapulae. 3 weeks after DOCA implant, rats were sacrificed and kidneys were harvested for tissue assays of CGRP and NE content as described earlier.

DOCA implants were made by mixing 100 mg DOCA into 2 ml of silicone (Sylgard 184 silicone elastomer base; Dow Corning, Midland, MI). Once the DOCA was homogenously mixed into the silicone, silicone elastomer curing agent (0.2 ml) was added. The DOCA implants were allowed to cure at room temperature for 24 hours and were then stored at 4°C until implantation. Each DOCA implant was cut into 2 to 3 mm cubes that were then implanted subcutaneously between the scapulae.

Statistical Analysis

The magnitude of the changes in cardiovascular and renal excretory parameters following intrarenal or intravenous infusion of bradykinin as well as differences in daily measurements generated from the salt-sensitivity and DOCA-salt protocols were analyzed by 2-way analysis of variance for repeated measures followed by the Bonferroni method for post-hoc comparisons (SigmaPlot version 12.3, Systat Software Inc., San Jose, CA). All body weight, IHC, ELISA, HPLC and baseline MAP and HR data were analyzed by one-way analysis of variance followed by the Bonferroni method for post-hoc comparisons (SigmaPlot version 12.3). For the time course of reinnervation, a linear regression (SigmaPlot version 12.3) was performed. For all statistical analyses, a p value less than 0.05 was considered to be statistically significant.

3.4 Results

Validation of renal-CAP treatment by immunohistochemistry

Figure 1 shows representative immunohistochemistry images of a SHAM (top) and a renal-CAP (bottom) treated kidney 10 days post-treatment. Robust labeling of TH is apparent in both kidneys while the significant amount of CGRP labeling seen in the SHAM kidney is absent in the renal-CAP kidney. Because careful examination of all sections revealed that CGRP labeling was observed in the renal pelvis exclusively, CGRP was quantified as area of CGRP+ labeling per length of pelvic wall. TH labeling was found throughout the kidney but was most robust in the large nerve bundles near the hilum of the kidney, so TH was quantified as percent area of a nerve bundle with TH+ labeling. Labeling of CGRP and TH was quantified in SHAM and renal-CAP kidneys 10 days, 4 weeks and 7 weeks post treatment (Figure 2A and 2B). TH labeling was not different between SHAM and renal-CAP kidneys at any time point. In contrast, CGRP labeling was nearly abolished 10 days and 4 weeks post renal-CAP treatment. By 7 weeks post-treatment, CGRP labeling returned to approximately 50% of that observed in SHAM treated kidneys.

Validation of renal-CAP treatment by ELISA and HPLC

While IHC is useful for visualizing neurons of interest, more accurate quantification of neuromarkers can be obtained by assaying tissues for content of neurotransmitters. Therefore, we measured pelvic CGRP content by ELISA and renal NE content by HPLC at 10 days, 4 weeks and 7 weeks after unilateral renal-CAP treatment, using the non-treated kidney as a SHAM control. As shown in Figure 2C, renal NE content was not different between SHAM and renal-CAP kidneys at any time point. Conversely, renal pelvic CGRP was undetectable 10 days after renal-CAP treatment. Pelvic CGRP began to return to detectable levels at 4 weeks and, by 7 weeks, had returned to roughly 50% of control levels (Figure 2D). Figure 3 shows CGRP content over time in renal-CAP treated kidneys plotted as % of control (the ratio of CGRP

content of the left renal pelvis to right renal pelvis X 100) and suggests that the time course of afferent reinnervation is roughly linear, i.e., once initiated, afferent reinnervation occurs at a fairly constant rate.

Effect of renal-CAP treatment on cardiovascular responses to activation of afferent renal nerves in conscious rats

Infusion of bradykinin into the renal artery has been shown to activate afferent renal nerves (4, 172) and increase MAP and HR (146). Therefore, to determine whether renal-CAP treatment causes functional ablation of afferent renal nerves, experiments were conducted in conscious rats in which the physiological responses to direct pharmacological activation of afferent renal nerves by bradykinin were assessed. There were no significant differences between groups in baseline MAP (SHAM = 124 ± 3 mmHg, renal-CAP = 121 ± 2 mmHg) or HR (SHAM = 414 ± 18 BPM, renal-CAP = 427 ± 16 BPM). More importantly, as shown in Figure 4, bradykinin infused into the renal artery evoked a dose dependent increase in MAP and HR in SHAM, but not renal-CAP rats. The same doses of bradykinin administered intravenously had no effect on MAP or HR in either SHAM or renal-CAP rats. Sodium excretion was unaffected by bradykinin infusion given by either route (data not shown).

Effect of renal-CAP treatment on cardiovascular and fluid balance regulation during chronic salt loading

An additional experiment was conducted to determine if renal-CAP treatment disrupts cardiovascular and fluid balance regulation during chronic increases in dietary salt intake. Such dysregulation could occur as a result of non-specific effects of renal-CAP treatment (i.e., ablation of efferent renal nerves) or secondary to disruption of normal dietary sodium-induced renal afferent signaling. Figure 5 shows MAP and HR in SHAM and renal-CAP treated rats fed increasing levels of dietary salt. Renal-CAP treatment had no effect on the basal level of MAP when rats were being fed a low (0.1%) salt diet. Similarly, the response of MAP to a 40-fold (4.0% NaCl) and 80-fold (8.0%

NaCl) increase in salt intake was not different between renal-CAP and SHAM treated rats. Conversely, the progressive bradycardia observed in SHAM rats in response to increasing dietary sodium was significantly attenuated in renal-CAP rats.

Figure 6 shows sodium intake, excretion and balance throughout the protocol. There were no differences in sodium intake between SHAM and renal-CAP treated rats at any level of dietary salt; 0.1%, (~ 0.4 mmol/day), 4.0% (~ 17 mmol/day) and 8.0% (~ 31 mmol/day). Furthermore, the renal sodium excretory responses to these large increases in salt intake were not different between renal-CAP and SHAM treated rats throughout the protocol. As a result, there were no differences in sodium balance between groups over the 40-day protocol. Likewise, water intake and urine output were largely similar between groups throughout the protocol with the exception that renal-CAP rats tended to drink more water and excrete more urine than SHAM rats initially when diets were changed from 0.1% to 4.0% and from 4.0% to 8.0% (Figure 7). Water balance was similar between groups on all days of the protocol.

Table 1 shows body weight at the time of treatment (bilateral renal-CAP or SHAM) and sacrifice 10 days, 4 weeks or 7 weeks post-treatment as well as the total weight gain from time of treatment to the time of sacrifice. Consistent with our findings that renal-CAP treatment does not adversely affect food intake or maintenance of body fluid balance during chronic dietary salt loading, these data demonstrate that renal-CAP treatment does not affect the rate of body weight gain over the same period of time.

Effect of renal-CAP treatment on the development of DOCA-salt hypertension

An additional experiment was conducted to test whether renal-CAP treatment attenuates a model of hypertension that is thought to be mediated by afferent renal nerves, the DOCA-salt model. As shown in Figure 8A, baseline MAP trended lower in RDNX rats as compared to SHAM and renal-CAP rats, and baseline HR was not different between groups. After 3 weeks of DOCA treatment, MAP in the SHAM group had increased ~ 30 mmHg above baseline (Figure 8B). In confirmation with data we have previously reported (77), this increase was attenuated by ~50% in RDNX rats.

Importantly, renal-CAP treatment had a nearly identical antihypertensive effect to RDNX. Neither RDNX nor renal-CAP had an effect on the change in HR observed with DOCA implantation (Figure 8B). As shown in Figure 9, both RDNX and renal-CAP treatment caused significant reductions in the renal pelvic content of the afferent nerve marker, CGRP, whereas RDNX and not renal-CAP treatment drastically reduced renal content of the efferent nerve marker, NE in DOCA-salt rats.

3.5 Discussion

The role of renal nerves in arterial pressure regulation and hypertension has been investigated in various animal models for years (38, 48, 77). Furthermore, recent technological advances have raised interest in targeting the renal nerves for the treatment of human hypertension (43, 101, 114) as well as other diseases such as diabetes (113) and sleep apnea (171). However, the promising clinical trials using RDNX have sparked many questions that need to be answered in order to optimize the effectiveness of renal nerve ablation. Chief among these is whether the antihypertensive effect of RDNX is due to ablation of afferent or efferent renal nerves. Clarification of this point is critical to our understanding of the pathophysiological mechanisms involved and to the development of more targeted nerve ablation approaches.

In the present study we investigated the ability of periaxonal application of the TRPV1 receptor agonist, capsaicin, on renal nerves to selectively ablate afferent renal nerves while leaving efferent nerves intact. While capsaicin is known to activate sensory neurons acutely, it is also neurotoxic following prolonged exposure (69, 154). Systemic administration of capsaicin to neonatal rat pups results in whole body sensory nerve denervation (79), and local periaxonal application of capsaicin has been shown to selectively ablate sensory fibers of specific organs, such as the adrenal gland(159-161). Moreover, since the TRPV1 receptor is expressed along the axons of sensory fibers, but is not expressed in sympathetic efferent neurons (154), we hypothesized that periaxonal

application of capsaicin could be used to selectively ablate sensory fibers in the kidney while leaving efferent fibers intact.

Renal-CAP treatment causes temporary selective loss of afferent nerve markers from the kidney as assessed by IHC and tissue assays

Confirmation of sympathetic denervations has typically been achieved by assessing the content of NE in the denervated tissue and measurement of renal NE content by HPLC is the most commonly used and widely accepted method to confirm RDNX as successful in animals. We have performed numerous studies investigating the effect of RDNX on the pathogenesis of experimental hypertension and, using HPLC, typically report renal NE content to be reduced by 90-95% in renal denervated rats compared to control animals (48, 76-78, 91). While useful, it is important to note that this method does not assess ablation of afferent renal nerves, which is typically done by measurement of the neurotransmitters CGRP, or substance P (SP).

In the present study, we quantified the content of neurochemical markers of efferent (TH and NE) and afferent (CGRP) nerves in SHAM or renal-CAP treated kidneys. We found that renal-CAP treatment virtually eliminated renal pelvic CGRP content without affecting renal NE or TH content. Pelvic CGRP was undetectable 10 days post-treatment by ELISA, but did recover over time. This recovery occurred linearly over time with levels ~20% and ~50% of control at 4 and 7 weeks post-treatment respectively.

The time course of afferent reinnervation following renal-CAP treatment observed in the present study is strikingly similar to a recent investigation of the reinnervation pattern following complete surgical RDNX in rats (125). In this study, Mulder and coworkers used IHC to label markers of efferent nerves (TH and neuropeptide Y; NPY) and afferent nerves (CGRP and SP) in renal denervated and sham operated kidneys at various time points after treatment. Labeling for the efferent makers TH and NPY in the renal cortex was nearly eliminated 4-5 days after RDNX, but returned to ~50% by 4 weeks. A very similar pattern was observed for the afferent markers CGRP

and SP in the pelvic wall. In the present study, IHC revealed that renal-CAP treatment reduced pelvic CGRP labeling to ~10 and 20% of control at 10 days and 4 weeks post treatment, respectively. In addition, using an ELISA for CGRP, we found that renal-CAP reduced renal pelvic content to undetectable levels at 10 days post-treatment and 20% of control at 4 weeks. In our study both methods (IHC and ELISA) demonstrated that pelvic CGRP returns to ~50% of control levels by 7 weeks post treatment. Although this time point was not measured in the study by Mulder and colleagues, they did report that NPY, TH, CGRP and SP returned to 100% of control by 12 weeks post treatment. In the present study, linear regression analysis of pelvic CGRP versus time post treatment suggests that pelvic GGRP would return to 100% of control 14 weeks post treatment. Based on the result of two independent measurements of the presence of afferent renal nerves, IHC for CGRP and ELISA for CGRP content, we conclude that renal-CAP treatment results in ablation of afferent renal nerves as effectively as, if not more effectively than, surgical RDNX. Moreover, in contrast to surgical RDNX, this technique does not cause ablation of efferent renal nerves as assessed by renal NE and TH content.

Renal-CAP treatment causes functional ablation of afferent renal nerves

Because depletion of neural markers does not necessarily correspond with a loss of neuronal function, we performed experiments in conscious rats to determine whether renal-CAP treatment causes functional ablation of afferent renal nerves. In these experiments, we measured the cardiovascular responses to intrarenal infusions of bradykinin, which has been shown to stimulate afferent renal nerves (4, 172) and increase MAP and HR (68, 146). To control for non-renal effects of bradykinin, intravenous infusions were given at the same doses.

Bradykinin infused into the renal artery of SHAM rats caused a dose dependent increase in MAP and HR and these responses were absent in renal-CAP rats. Since intravenous infusions at the same doses caused no changes in MAP or HR, we conclude that the responses to intrarenal infusion were indeed due to intrarenal actions of bradykinin. More importantly, abolition of these responses by renal-CAP treatment

confirms previous reports that these responses are mediated by activation of afferent renal nerves and that renal-CAP results in complete functional ablation of afferent renal nerves. These results suggest that, in addition to causing loss of the neurotransmitter CGRP, renal-CAP treatment results in a complete loss of afferent neuronal function.

Renal-CAP treatment does not affect the regulation of arterial pressure, fluid balance or weight gain during chronic dietary salt loading

In addition to examining the effectiveness of renal-CAP treatment in selectively ablating afferent renal nerves, we sought to determine whether renal-CAP treatment causes a disruption in the regulation of fluid balance and arterial pressure during chronic increases in dietary salt intake. This could potentially occur as a result of non-specific effects of renal-CAP treatment (i.e. ablation of renal efferent nerves) or secondary to disruption of normal renal afferent signaling as suggested by earlier studies using non-selective methods of afferent renal denervation such as dorsal rhizotomy (97) and systemic administration of capsaicin (164, 165, 168). In the present study, renal-CAP treatment did not affect the regulation of arterial pressure or sodium and water balance in response to increasing dietary salt intake 40 and 80-fold from baseline over a period of 5 weeks. Additionally, renal-CAP treated rats gained weight to a similar extent as SHAM rats. We conclude that, while renal-CAP is an effective treatment for targeted ablation of CCRP+ afferent renal nerves, this treatment does not interfere with regulation of arterial pressure, fluid balance or body weight gain in normotensive Sprague Dawley rats subjected to dietary sodium loading.

Previous studies have shown that non-selective renal "deafferentation" causes salt-sensitive hypertension (97, 164, 165, 168). This was not observed in the present study using a more selective method for ablation of afferent renal nerves. In addition, we have previously reported that complete surgical RDNX (ablation of both afferents and efferents) also has no effect on the salt-sensitivity of arterial pressure or regulation of sodium and water balance in normotensive Sprague Dawley rats (76, 78). There are at least two explanations for the discrepancy between the results of the present study and

those of previous studies. The first is that the non-specific methods used previously also target non-renal visceral afferent pathways that are salt-sensitive such as hepatoportal osmoreceptors(122, 123). These sodium sensors have been shown to be important in the inhibition renal sympathetic nerve activity following hypertonic saline infusion into the portal vein and cause suppression of efferent renal nerve activity following ingestion of meals high in sodium content (74, 121, 129). Therefore it is possible that any method that disrupts afferent signaling from these receptors may result in sodium retention and hypertension. The second possible explanation for the differences between studies is the method of arterial pressure measurement. In the current study, arterial pressure was measured continuously by radio telemetry in unrestrained rats. In contrast, previous studies employed methods that are known to induce acute stress responses such as indirect tail cuff measurements in restrained rats (164, 165, 168), acute direct recordings in anesthetized rats (164, 165), or acute direct recordings in conscious rats days after catheter implantation (97). Thus, it is possible that in the earlier studies, afferent renal nerve ablation led to exaggerated arterial pressure responses to acute stress in rats consuming a high salt diet. Both of these possibilities remain to be investigated. It is important to note that, whereas renal-CAP treatment had no effect on the basal level of arterial pressure in rats consuming a normal salt diet, we have consistently found that complete surgical RDNX decreases arterial pressure ~10 mmHg (76, 78). This suggests that the chronic hypotensive response to complete RDNX in non-hypertensive Sprague Dawley rats is due to loss of efferent renal nerves exclusively.

Renal-CAP treatment amplifies the dipsogenic response and attenuates the bradycardic response to increased dietary sodium intake

Another interesting finding in this study was that renal-CAP treated rats drank more water than control rats during the initial increases in salt intake. This occurred when dietary salt was increased from 0.1% to 4.0% NaCl and from 4.0% to 8.0% NaCl. Although we did not measure plasma vasopressin (AVP) in this study, it has been suggested that afferent renal nerves influence AVP release (25, 28, 35, 144). One

explanation for our findings is that renal-CAP rats had an impaired AVP response to acute increases in plasma osmolality and compensated by increasing water intake. Additionally, it is possible that afferent renal nerves play a direct regulatory role in the dipsogenic response to increases in dietary sodium load. While this is an interesting finding, the mechanism underlying this observation remains to be investigated.

Finally, while we saw no differences in MAP between sham and renal-CAP rats, we did observe an effect of renal-CAP on chronic regulation of HR during increased dietary salt intake. Specifically, the bradycardic response to dietary sodium loading that was observed in SHAM rats was significantly attenuated by renal-CAP treatment. This suggests a role of afferent renal nerve signaling in the regulation of HR under conditions of high salt intake. This hypothesis remains to be tested by further studies.

Afferent renal nerves mediate the development of DOCA-salt Hypertension

Having shown that afferent renal nerves play a minimal role in the regulation of arterial pressure in normotensive rats, we performed one additional experiment to explore the role of these nerves in hypertension. We have previously reported that RDNX attenuates the development of DOCA-salt hypertension by 50% and hypothesized this was mediated by, at least in part, ablation of afferent renal nerves (77). This hypothesis was based on the difference in magnitudes of the bradycardic response to DOCA-salt treatment in SHAM and RDNX rats. Although HR decreased in both groups, it was significantly higher in SHAM rats despite the fact that arterial pressure was also higher in these rats. We hypothesized that afferent renal nerves modulate baroreflex control of HR in DOCA-salt rats and that RDNX blocks this modulation secondary to ablation of renal afferent nerves (77). Moreover, we hypothesized that the antihypertensive effect of RDNX was due to ablation of afferent renal nerves.

To test this hypothesis, we subjected DOCA-salt rats to SHAM, RDNX or renal-CAP treatment. The results of present experiment show clearly that selective ablation of afferent renal nerves by renal-CAP treatment attenuates the development of DOCA-salt hypertension over the same time course and by the same magnitude as RDNX, suggesting

that the antihypertensive effect of RDNX in the DOCA-salt model is due to ablation of afferent rather than efferent renal nerves.

Neither renal-CAP treatment nor RDNX had a significant effect on the HR response to DOCA implantation over the 21 day protocol which is consistent with our previous report in which we observed no significant difference in HR between SHAM and RDNX rats until after 4 weeks of DOCA (77). This observation is also consistent with the salt-sensitivity study in which renal-CAP treatment had no effect on HR compared to SHAM until after 3 weeks of high salt. While the results of our previous DOCA-salt study and the current salt-sensitivity study suggest a role of afferent renal nerves in HR regulation, this role seems to be minor and only apparent during long-term protocols. Further studies will be needed to fully elucidate the role of afferent renal nerves in HR regulation.

The results of this study are important because they raise the possibility that the antihypertensive effect of RDNX in some humans may be due to ablation of afferent rather than efferent renal nerves. Further studies will be needed in order to determine the mechanisms underlying this antihypertensive effect, but some possibilities include decreased sympathetic outflow to renal and/or non-renal targets or decreased plasma AVP levels. Understanding the mechanisms underlying this antihypertensive effect may be critical to our understanding of the therapeutic effects of RDNX and to the development of more refined treatments.

3.6 Limitations

The primary limitation to the present study is that renal-CAP treatment is effective in temporarily ablating CGRP containing afferent renal neurons, it is not clear to what extent this procedure is effective in total “deafferentation” of the kidney as it does not target sensory neurons in the kidney that lack the TRPV1 receptor. Although the conventional view is that the vast majority of renal afferents are indeed TRPV1+, this is open to further investigation. Additionally, it is possible that the antihypertensive effect

of renal-CAP treatment is due to a non-specific action of capsaicin; however, this is unlikely since the capsaicin solution is well contained to the treatment area during the application period and the effects of RDNX and renal-CAP on arterial pressure were identical.

3.7 Perspectives and Significance

While RDNX has been shown to lower arterial pressure in some humans with resistant hypertension, it is not known whether the antihypertensive effect is due to ablation of afferent or efferent renal nerves. To this point, it is possible that there are some patients for whom RDNX will work by disrupting renal efferent signaling and some for whom it will work by reducing renal afferent signaling. Therefore, it is crucially important to address 1) whether afferent renal nerves are involved in hypertension, 2) if they are involved, whether we can target afferent renal nerves specifically in order to optimize the efficacy and reduce the possible side effects of RDNX and 3) whether we can identify patients who would benefit from afferent- or efferent-specific renal nerve ablation. As a first step toward addressing these points, we have developed a method for selective ablation of afferent renal nerves in the rat. This procedure, termed renal-CAP, effectively ablates afferent renal nerves, leaving efferent renal nerves intact. This experimental tool may be valuable in elucidating the role of afferent renal nerves in animal models of hypertension and other cardiovascular diseases and may establish a rationale for developing a means for permanent ablation of afferent renal nerves in humans. Moreover, the results of our DOCA-salt experiment suggest that afferent renal nerves may be involved in some forms of hypertension, making afferent renal nerves both an important subject for future basic science research as well as a possible target for human hypertension.

Acknowledgments

The authors would like to thank Dr. Stefan Tunev and Lori Garcia of Medtronic CardioVascular, Inc. for their work in performing IHC and their help in analyzing the resulting images. We thank Ruijun Han and Xinying Wang for performing the ELISA analysis of pelvic CGRP as well as Robert Burnett for performing the tissue NE measurements. We would also like to thank Dusty Van Helden, Jeff Larson and Marcos Kuroki for their help with the collection of metabolic data.

Grants

This research was supported by National Institutes of Health grants R01HL076312 (JWO), RO1HL107330 and K02HL112718 (RDW) and by National Science Foundation grant IOS1025119 (WCE).

Disclosures

J.W.O. is a paid consultant of Medtronic CardioVascular, Inc. Santa Rosa, CA.

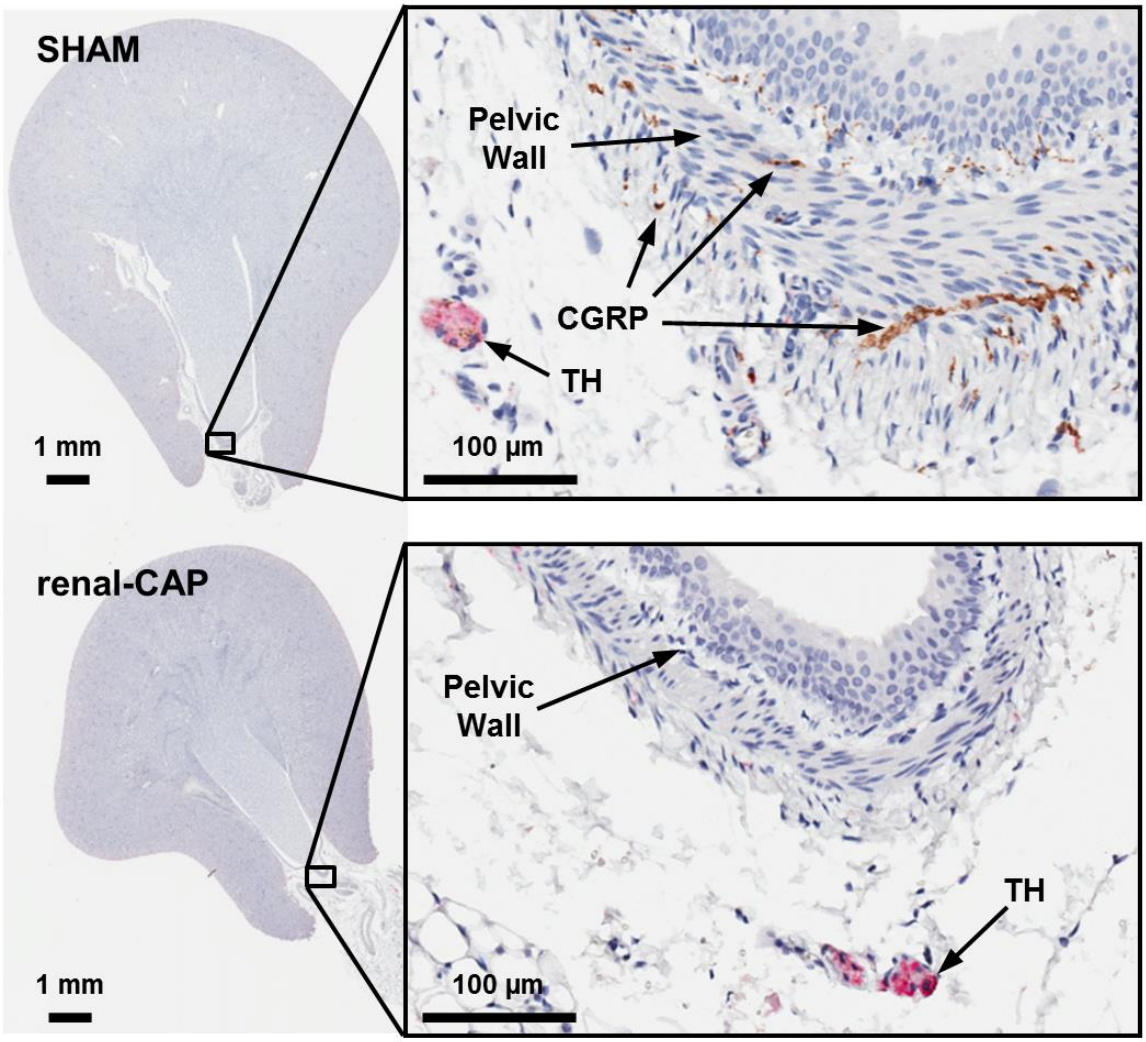

Figure 1. Representative IHC images stained for CGRP and TH from a SHAM and a renal-CAP treated kidney 10 days after treatment. The image on left shows the entire slice of the kidney. The boxed in portion of the left image is magnified in the image on the right. Brown = CGRP, pink = TH.

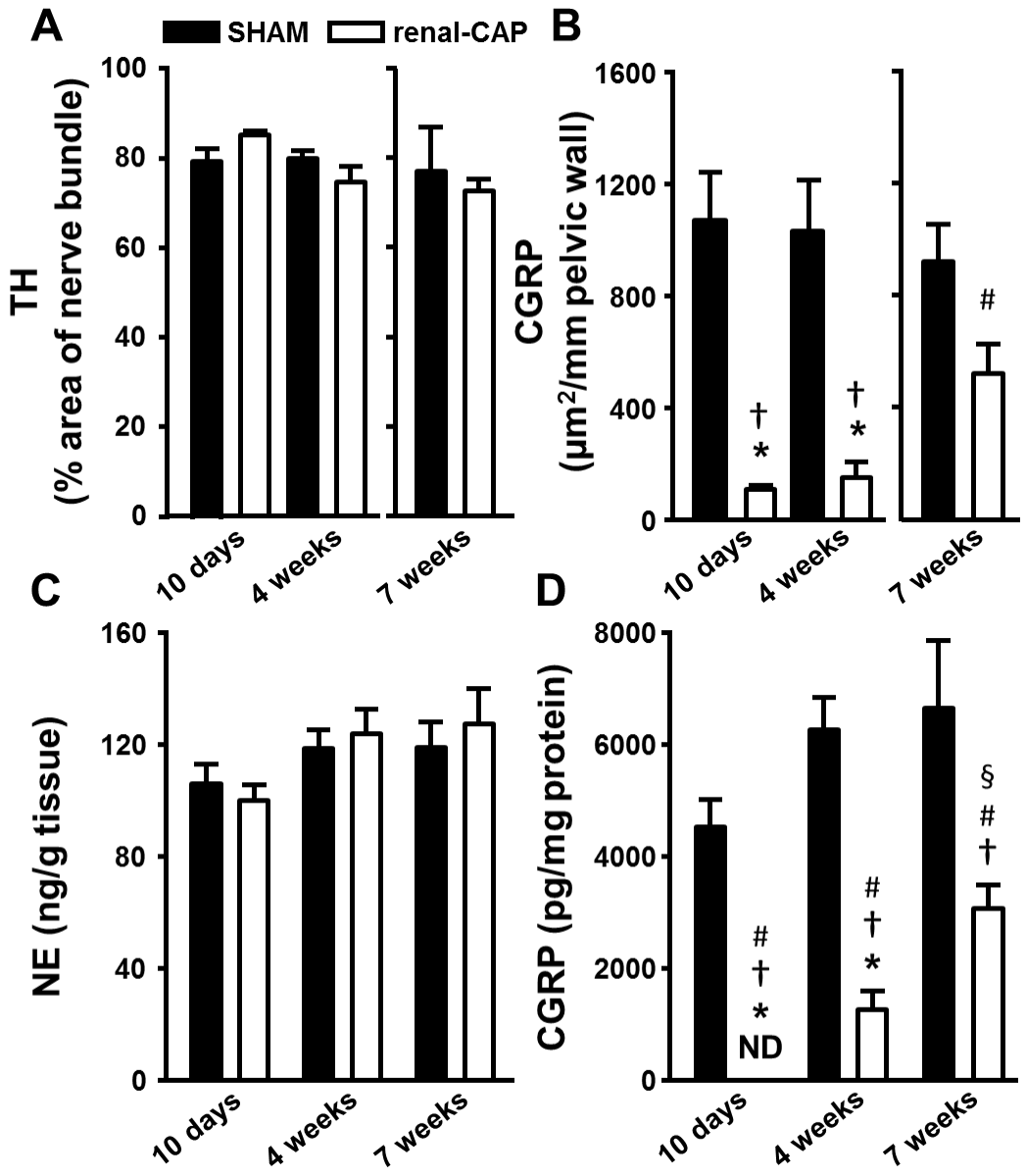

Figure 2. Tissue content of neurochemical markers 10 days, 4 weeks and 7 weeks following SHAM or renal-CAP treatment. A.) IHC labeling of TH. B.) IHC labeling of CGRP. C.) NE content of the renal parenchyma by HPLC. D.) CGRP content of the pelvic wall by ELISA. ND = not detectable. * = $p < 0.05$ compared to the 10 day SHAM group, † = $p < 0.05$ compared to the 4 week SHAM group, # = $p < 0.05$ compared to the 7 week SHAM group. § = $p < 0.05$ compared to the 10 day renal-CAP group.

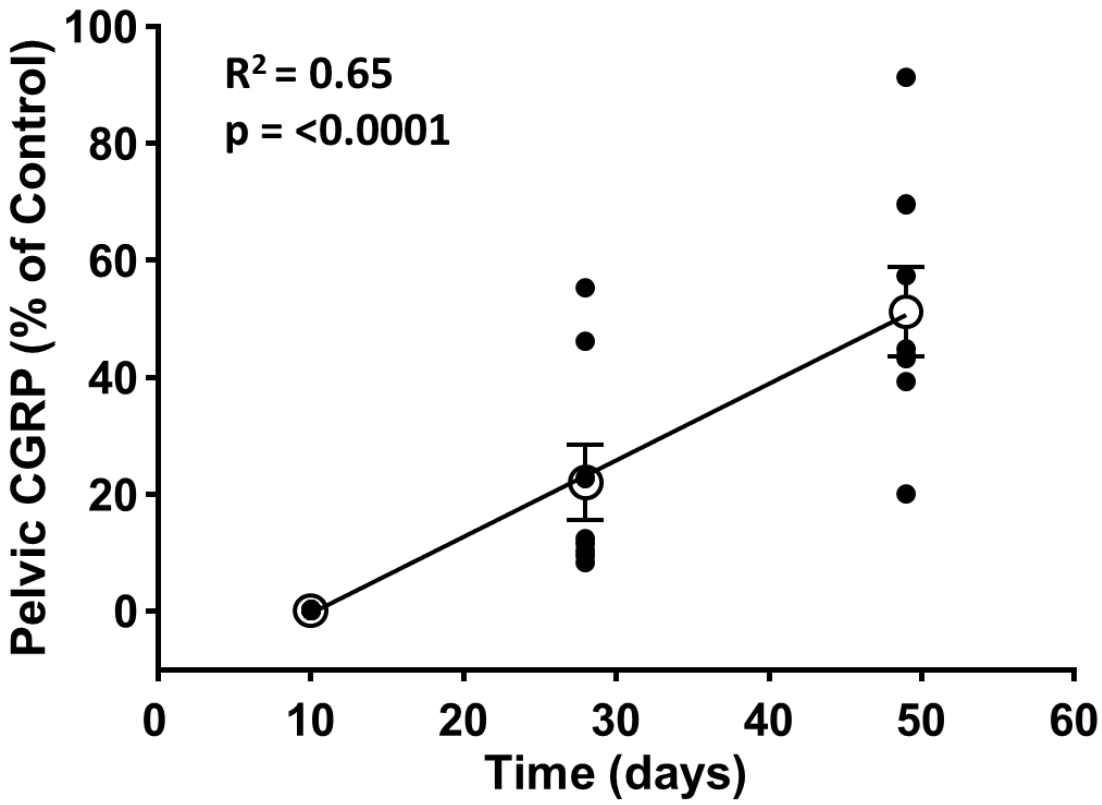

Figure 3. Time course of afferent reinnervation following renal-CAP treatment. Renal pelvic CGRP content of renal-CAP treated (left) kidneys is expressed as % of within animal control (right) kidneys. A linear regression was performed (R2 = 0.65, $p < 0.0001$) and group averages ± SE are overlaid.

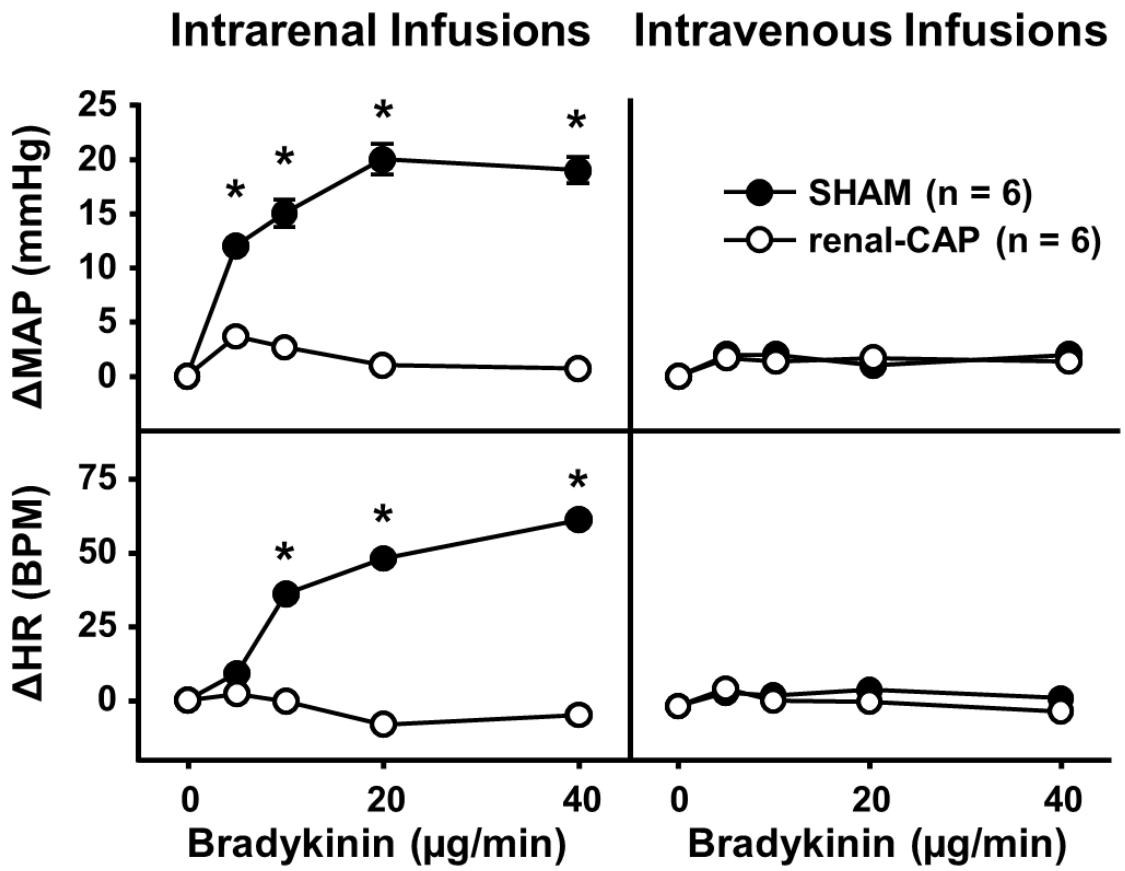

Figure 4. Physiological responses to pharmacological stimulation of afferent renal nerves in SHAM and renal-CAP treated rats. Changes in MAP (ΔMAP, top) and HR (ΔHR, bottom) following intrarenal (left) and intravenous (right) infusions of bradykinin. * = $p < 0.05$ for SHAM vs. renal-CAP.

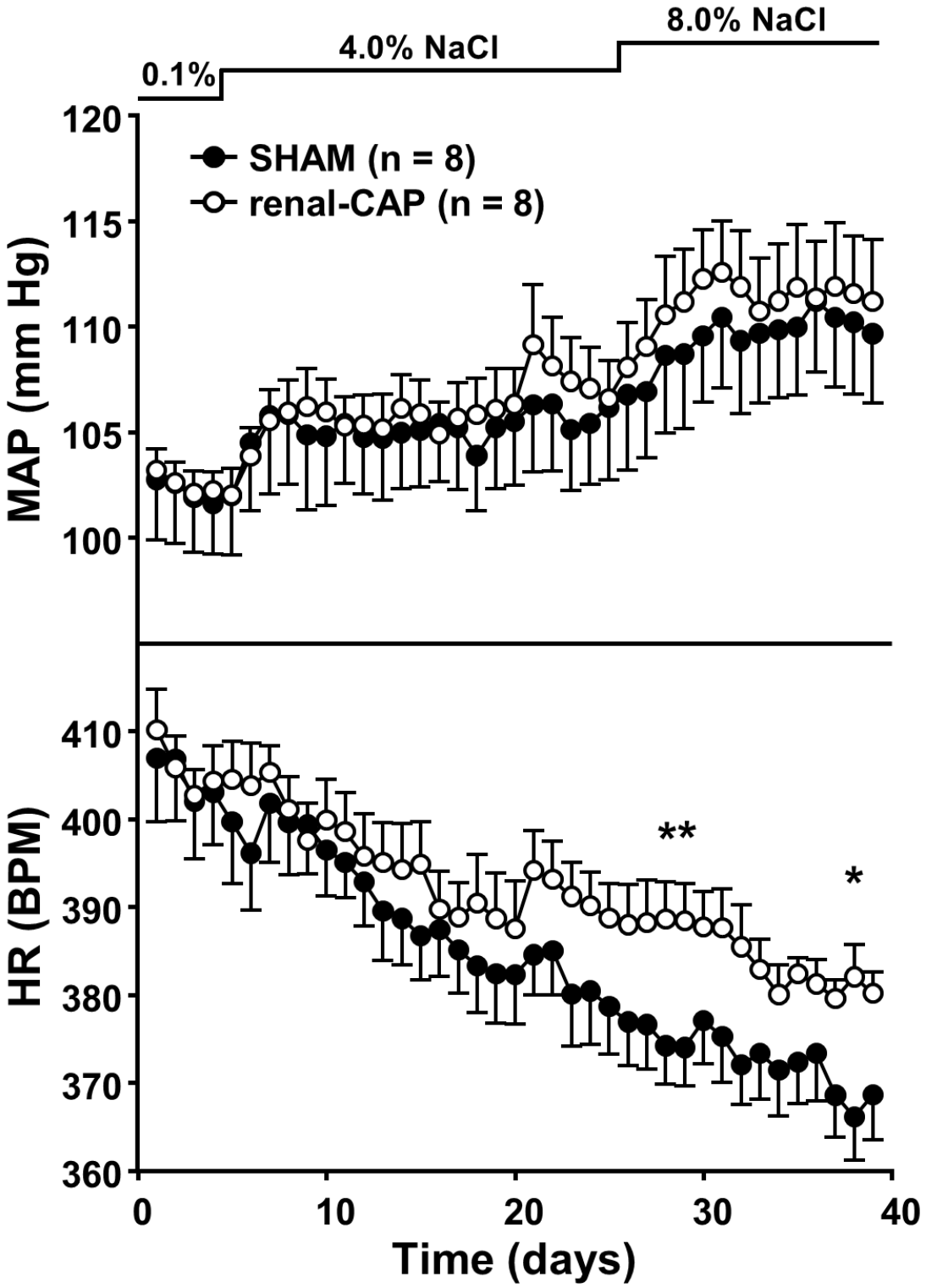

Figure 5. MAP and HR in SHAM and renal-CAP treated rats subjected to the salt-sensitivity protocol. * = $p < 0.05$ for SHAM vs. renal-CAP.

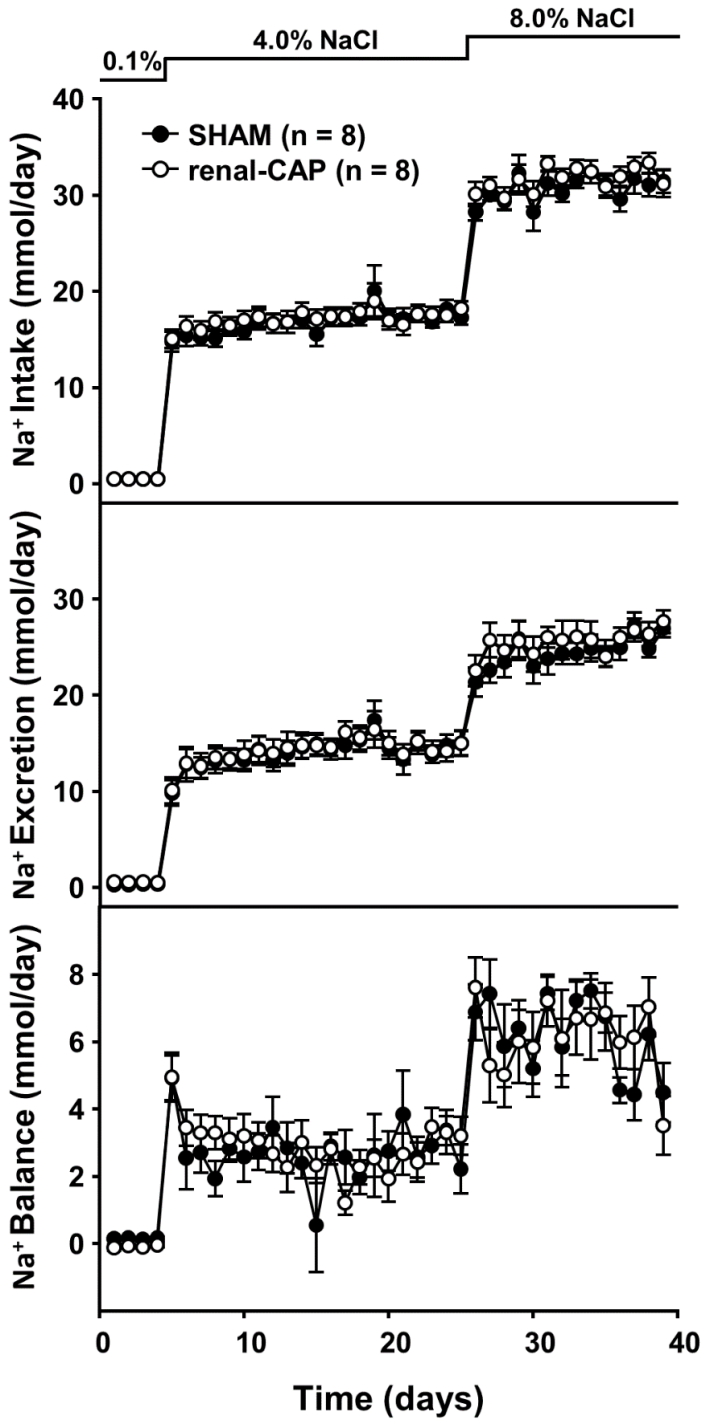

Figure 6. Sodium intake, excretion and balance in SHAM and renal-CAP treated rats subjected to the salt-sensitivity protocol. There were no significant differences between groups.

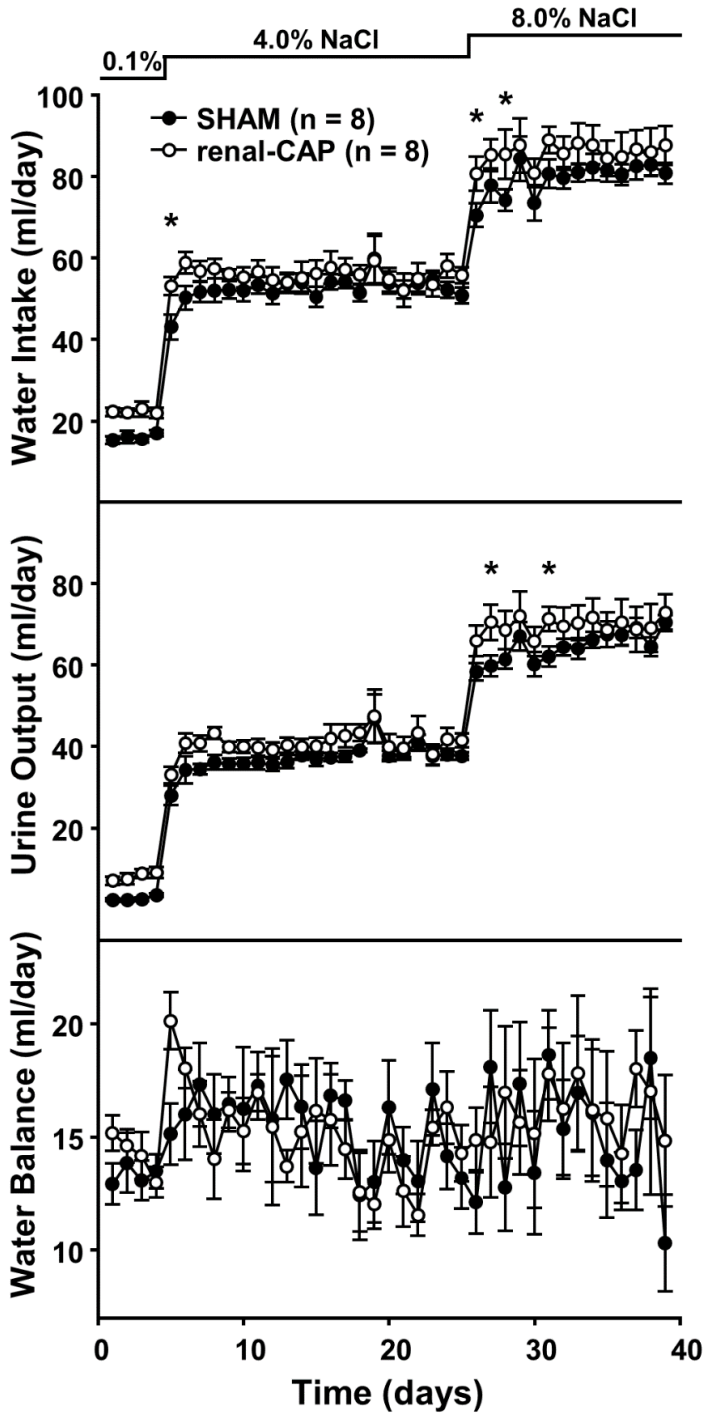

Figure 7. Water intake, urine output and water balance in SHAM and renal-CAP treated rats subjected to the salt-sensitivity protocol. * = $p < 0.05$ for renal-CAP vs. SHAM.

	10 day		4 week		7 week	
	SHAM	renal-CAP	SHAM	renal-CAP	SHAM	renal-CAP
BW at Tx (g)	242.0 ± 4.2	250.2 ± 4.3	307.7 ± 5.9	309.2 ± 5.2	357.5 ± 7.5	355.7 ± 4.1
Final BW (g)	294.0 ± 3.4	280.8 ± 5.5	462.8 ± 14.5	451.3 ± 16.0	512.9 ± 25.8	513.2 ± 17.5
ΔBW (g)	52.0 ± 6.7	30.6 ± 8.8	155.1 ± 10.6	142.0 ± 13.0	155.5 ± 21.7	157.5 ± 16.3

Table 1. Body weight at the time of treatment (BW at Tx), body weight at sacrifice (Final BW) and weight gain from treatment to sacrifice (ΔBW) in SHAM and renal-CAP rats that underwent a 10 day, 4 week or 7 week protocol. No significant differences were found between SHAM and renal-CAP rats that underwent the same protocol.

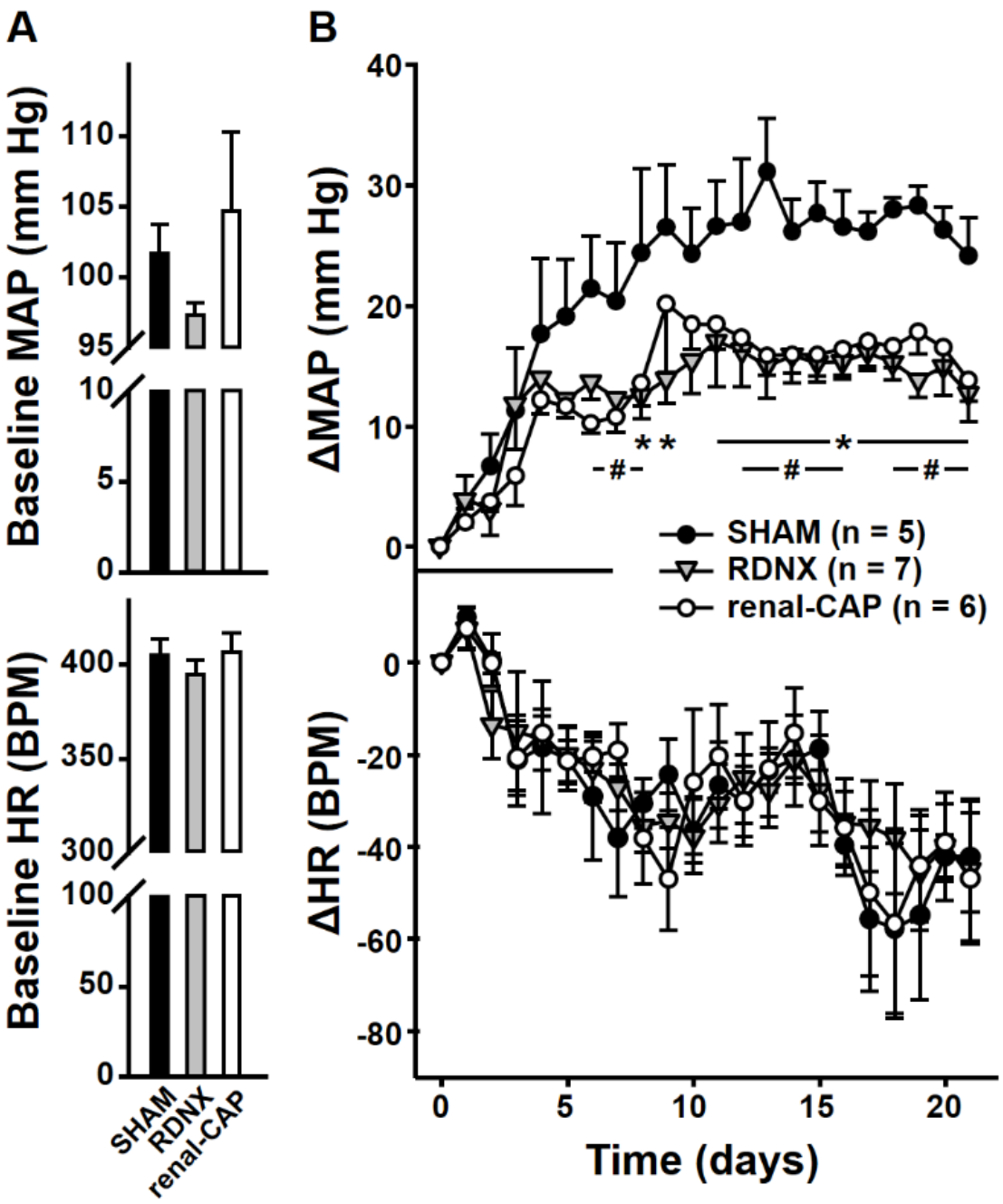

Figure 8. A.) Average baseline MAP and HR and B.) change in MAP from baseline (ΔMAP) and change in HR from baseline (ΔHR) in SHAM, RDNX and renal-CAP rats upon DOCA implantation. * = $p < 0.05$ for RDNX vs. SHAM. # = 0.05 for renal-CAP vs. SHAM.

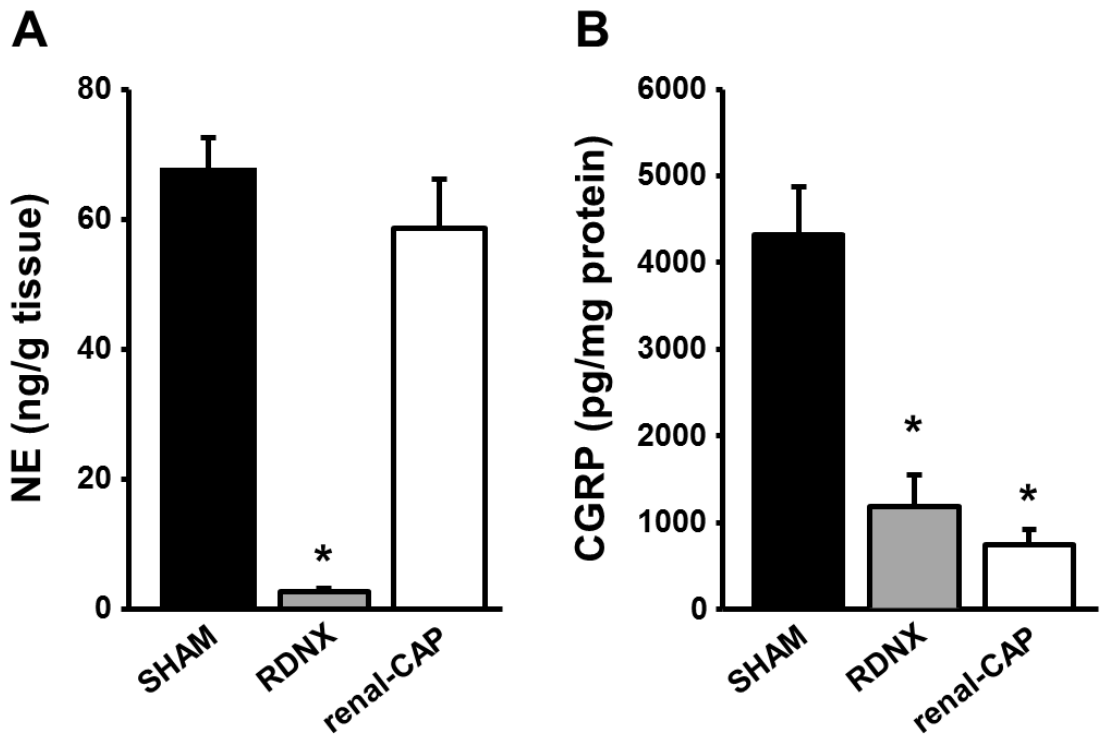

Figure 9. Tissue content of neurochemical markers in the kidneys of rats that underwent the DOCA-salt protocol A.) NE content in the renal parenchyma. B.) CGRP content in the renal pelvic wall. * = $p < 0.05$ compared to SHAM.

Chapter Four

Differential role of afferent and efferent renal nerves in the maintenance of early and late phase Dahl S hypertension

Jason Foss[1], Gregory D. Fink[2] and John W. Osborn[1]

[1]University of Minnesota
Department of Integrative Biology and Physiology
Minneapolis, MN

[2]Michigan State University
Department of Pharmacology and Toxicology
East Lansing, MI

To be submitted for publication in Hypertension

4.1 Summary

Recent clinical data suggest that ablation of renal nerves (renal denervation; RDNX) may be a plausible treatment for human hypertension. These studies have also raised the question whether the antihypertensive effect of RDNX is due to ablation of afferent or efferent renal nerves. Chronic insults to the kidneys have been linked to afferent renal nerve-dependent hypertension, and we have previously shown that RDNX lowers arterial pressure in hypertensive Dahl salt-sensitive (S) rats, which exhibit renal pathology. Using this model, we tested the hypothesis that the antihypertensive effect of RDNX is due to ablation of afferent renal nerves by comparing the effect of complete RDNX to selective ablation of afferent renal nerves (renal-CAP) during two phases of hypertension in the Dahl S rat. In the "early phase" rats underwent treatment after 3 weeks of high NaCl feeding when mean arterial pressure (MAP) was ~140 mmHg and kidney disease is known to be mild. In the "late phase" rats underwent treatment after 9 weeks of high NaCl feeding when MAP was ~170 mmHg and kidney disease is known to be severe. RDNX reduced MAP ~10 mmHg compared to SHAM in both the early and late phase. Renal-CAP had no antihypertensive effect compared to SHAM. These results suggest that, in the Dahl S rat, the antihypertensive effect of RDNX is not dependent on pretreatment arterial pressure nor is it due to ablation of afferent renal nerves, even in the context of prolonged high NaCl feeding and more severe hypertension and renal pathology.

4.2 Introduction

Despite the staggering worldwide prevalence of hypertension and its significant association with death and disability, little progress has been made in the treatment of this disease (108, 131). Recent technological advancements have pointed to catheter based ablation of renal nerves (renal denervation; RDNX) as a promising treatment for drug resistant hypertension in humans. The efficacy and safety of this device-based approach were demonstrated by two small clinical trials, Symplicity HTN-1 and Symplicity HTN-2. However, a larger sham-controlled trial, Symplicity HTN-3, failed to demonstrate a significant therapeutic effect (13). Although the reason for the failure of Symplicity HTN-3 is currently unknown, the results are certainly surprising given the large body of earlier clinical and preclinical data demonstrating the efficacy of RDNX in the prevention and treatment of hypertension (38, 42, 101). It is clear that further studies designed to address the mechanisms underlying the antihypertensive effect of RDNX are needed to further refine and optimize this treatment strategy.

Since the first clinical studies of RDNX were published, it has been debated whether RDNX lowers arterial pressure (AP) secondary to ablation of afferent or efferent renal nerves (100). Reductions in efferent renal nerve activity could reduce AP by decreasing renin release, tubular sodium reabsorption and/or renal vascular resistance – the three primary renal functions influenced by efferent renal nerve activity (38). Indeed data from one clinical study suggest that RDNX may reduce renal vascular resistance as indicated by a reduction in renal resistive index (112). However, there are no clinical studies regarding the effect of RDNX on plasma renin concentrations or sodium balance.

Another possibility is that the antihypertensive response to RDNX is due to ablation of sympathoexcitatory afferent renal nerves resulting in decreased non-renal sympathetic activity. This hypothesis is supported by clinical studies demonstrating reductions in muscle sympathetic nerve activity, plasma glucose, cardiac arrhythmias and events of sleep apnea following RDNX (67, 149, 171). While these studies have revealed

significant physiological consequences of afferent renal nerve ablation, it is still unclear whether the antihypertensive effect of RDNX is due to ablation of afferent renal nerves.

The notion that hypertension can result from elevated afferent renal nerve activity is based on a number of studies. First, stimulation of afferent renal nerves has been shown to increase AP acutely (135, 150). Second, insults to the kidneys, such as damage induced by cortical injection of phenol or chronic ischemia caused by renal artery stenosis, have been shown to increase AP chronically in an afferent renal nerve-dependent manner (22, 167, 173). Lastly, end-stage renal disease patients are known to have elevated AP and sympathetic nerve activity both of which are reduced following bilateral, but not unilateral renal transplantation, suggesting that signals arising from the diseased kidney drive sympathetic outflow and HTN in renal failure patients (63). Importantly, patients enrolled in the RDNX trials, on average, had mild kidney disease as indicated by reduced glomerular filtration rate (43, 152). Therefore, it is possible that the antihypertensive effect of RDNX in people is secondary to a reduction in kidney disease-induced afferent renal nerve activity.

We have recently reported that RDNX partially reverses hypertension in the Dahl salt-sensitive (Dahl S) rat (48). This genetic model of salt-sensitive hypertension is associated with activation of the sympathetic nervous system (138) and marked kidney disease that worsens with both age and the progression of hypertension (32). Interestingly, the antihypertensive effect of RDNX that we reported is similar to that reported following RDNX in patients with ambulatory monitoring of AP (114). However, similar to catheter based RDNX in humans, it is unknown whether the antihypertensive response to RDNX in Dahl S rats is due to ablation of afferent or efferent renal nerves.

To investigate the role of afferent renal nerves in hypertension, we recently developed and validated a novel method for selective ablation of afferent renal nerves in the rat (chapter three). This selective ablation, termed renal-CAP treatment, is achieved by periaxonal application of capsaicin, a transient receptor potential vanilloid (TRPV) 1 receptor agonist, to the renal nerves. Since the majority of afferent fibers in the kidney express TRPV1 receptors (166), but efferent renal nerves do not, and because prolonged

activation of TRPV1 leads to neurodegeneration (160, 161), renal-CAP causes selective degeneration of afferent renal nerves while leaving efferent nerves intact. In this report we demonstrated that renal-CAP treatment and RDNX both blunted the development of deoxycorticosterone acetate (DOCA)-salt hypertension to a similar extent. This finding suggests that the antihypertensive effect of RDNX in the DOCA-salt model is due to ablation of afferent renal nerves and provides a "proof of concept" for the utility of this novel method to study the role of afferent renal nerves in other preclinical models of hypertension.

In the present study, we used this novel method for selective ablation of afferent renal nerves to investigate the mechanisms mediating the antihypertensive effect of RDNX in Dahl S rat. We tested the hypothesis that the response to RDNX is due to ablation of afferent renal nerves specifically by comparing the antihypertensive effect of complete RDNX to that of renal-CAP treatment. Moreover, since kidney disease is a possible culprit of afferent renal nerve-dependent hypertension, and Dahl S rats exhibit renal pathology that worsens with duration of high NaCl feeding and hypertension, we tested this hypothesis in both the early phase (after 3 weeks of high NaCl feeding when HTN and renal pathology are mild) and the late phase (after 9 weeks of high NaCl feeding when HTN and renal pathology are more severe) of hypertension.

4.3 Methods

Animals and General Procedures

Male Dahl S rats were purchased from Charles River Laboratories (Wilmington, MA) and housed in pairs in a temperature and light controlled room until the beginning of the study. Rats were allowed access to standard rat chow and distilled water *ad libitum* during this pre-experimental period. All procedures were approved by the University of Minnesota Animal Care and Use Committee and were conducted in accordance with the institutional and National Institutes of Health guidelines. For all surgeries, rats were anesthetized with 2.0% isoflurane. Atropine sulfate (0.4 mg/kg,

intraperitoneal) and gentamicin sulfate (10 mg/kg, intramuscular) were administered prior to surgery. For three days following surgery, ketoprofen (2.5 mg/kg, subcutaneous) was given once per day and the drinking water was supplemented with amoxicillin (1 mg/ml).

Experimental Protocol

Rats were subjected to one of the protocols outlined in Figure 1 and described below.

Early phase protocol: 67-74 day old rats were placed on a low salt diet (0.1% NaCl; Research Diets, New Brunswick, NJ) and instrumented with radio telemeters (model TA11PA-C40, DSI, Intl. St. Paul, MN) for monitoring of mean arterial pressure (MAP) and heart rate (HR) as previously described (162). After a 7-day recovery period, rats were individually housed in metabolic cages (Techniplast 3701M001, Buguggiate, Italy) and allowed to acclimate for four days. Three days of baseline data were then collected (see below for details) and rats were placed on a high salt diet (4.0% NaCl; Research Diets) for the remainder of the protocol. After 21 days of high salt intake, rats were anesthetized with isoflurane and, via a midline approach, subjected to sham (SHAM; n = 10), renal denervation (RDNX; n = 10) or selective ablation of afferent renal nerves (renal-CAP; n = 10) as previously described (162) (chapter three). Rats were returned to their cages and monitored for an additional 4 weeks.

Late phase protocol: 62-64 day old rats were placed on a high salt diet (4.0% NaCl; Research Diets) and, after 49-50 days, were instrumented with radio telemeters (DSI, Intl.) for monitoring of MAP and HR. After a 6-7-day recovery period, rats were individually housed in metabolic cages (Techniplast) and allowed to acclimate for four days. Three days of baseline data were then collected. After 9 weeks total of high salt diet, rats were anesthetized with isoflurane and, via a midline approach, subjected to a sham (SHAM; n = 8), renal denervation (RDNX; n = 9) or selective ablation of afferent renal nerves (renal-CAP; n = 9). Rats were returned to their cages and monitored for an additional 2 weeks.

At the end of the study, rats were anesthetized, exsanguinated and kidneys were removed immediately after death. The renal artery, renal vein, ureter and capsule were removed; and the kidneys were placed in a cold normal saline bath for further dissection. Renal parenchymal samples were taken from the poles and lateral portion of the kidney and flash frozen in liquid nitrogen. The renal pelvis was then carefully dissected from the remaining portion of kidney and flash frozen in liquid nitrogen. All frozen samples were stored at -80°C until being assayed.

The parenchymal samples were assayed for norepinephrine (NE), a marker of efferent renal nerves, using HPLC as previously described (107). The isolated renal pelvic samples were assayed for calcitonin gene-related peptide (CGRP), a marker for afferent renal nerves, using a commercially available ELISA kit (Cayman Chemicals; Ann Arbor, MI; Item Number 589001). Tissues were homogenized in 1M acetic acid and CGRP was extracted using C18 Sep-columns (Peninsula Laboratories; San Carlos, CA; Item Number Y-1000). To eliminate any interassay variance, all pelvic samples were run on single 96 well ELISA plate.

Daily Measurements

The transmitter signal was monitored by a receiver (Data Sciences, model RPC-1) mounted on the side of the metabolic cage and connected to a Data Exchange Matrix (Data Sciences, Int). The AP signal was sampled at 500 samples/second for 10 seconds every 4 minutes using commercially available software (Data Sciences, Int.). HR was determined from the AP profile using the same software. 24-hour (24h) averages of MAP and HR were determined and plotted for each day of the study.

24h food intake, water intake and urine output were measured gravimetrically. 24h sodium intake was calculated by multiplying food intake (grams) and sodium content of the diet (0.1% NaCl = 0.01711 mmol Na^+/g food; 4.0% NaCl = 0.6844 mmol Na^+/g food). 24h sodium excretion was calculated by multiplying 24h urine output (ml) and urinary sodium concentration (mmol Na^+/ml), which was measured using an ion specific electrode (NOVA-5+ electrolyte analyzer, Nova Biomedical, Waltham, MA). 24h sodium

and water balances were calculated as 24h intake minus 24h excretion. Cumulative sodium and water balance were then calculated by sequential summation of daily balances beginning on the day of treatment surgery.

Statistical Analysis

MAP, HR and cumulative Na^+/H_2O balance data were analyzed by 2-way analysis of variance (ANOVA) for repeated measures followed by the Bonferroni method for post-hoc comparisons GraphPad Prism v6 (GraphPad Software, San Diego, CA). Baseline MAP and HR as well as CGRP and NE data were analyzed by one-way ANOVA. A p value less than 0.05 was considered to be statistically significant.

4.4 Results

Early Phase Protocol

As shown in Table 1, baseline MAP before treatment (after 3 weeks of high NaCl feeding) was similar in all three groups (SHAM = 138.6 ± 3.1 mmHg, RDNX = 139.9 ± 3.1 mmHg, renal-CAP = 138.1 ± 1.6 mmHg). MAP dropped transiently in all three groups following treatment and then continued to rise throughout the remainder of the protocol (Figure 2) and on the final day, MAP was similar in SHAM (161.7 ± 4.5 mmHg) and renal-CAP (160.1 ± 4.0 mmHg) rats. However, MAP in RDNX rats was ~10 mmHg lower than SHAM and renal-CAP throughout the protocol and MAP on the final day was 150.5 ± 3.2 mmHg.

HR was similar in SHAM (396.4 ± 3.4 BPM), RDNX (401.4 ± 4.2 BPM) and renal-CAP (396.0 ± 4.2 BPM) rats on the day before treatment (Table 1). In all three groups, HR transiently increased following treatment and then fell slightly below baseline (Figure 2). There were no differences in HR between SHAM and renal-CAP rats throughout the protocol. Two days after treatment, HR was significantly higher in RDNX compared to SHAM and renal-CAP rats. HR was then similar in all groups until about

two weeks after treatment when HR in the RDNX group trended lower and was statistically significantly lower than SHAM on days 22 and 28.

As shown in Figure 2, there were no differences in cumulative sodium or water balance between groups at any time following treatment in the early phase protocol.

Late Phase Protocol

In rats fed a high NaCl diet for 9 weeks (late phase), pretreatment MAP was nearly 30 mmHg higher than rats fed a high NaCl diet for 3 weeks (Table 1). However, MAP was similar in all three late phase groups prior to treatment. MAP dropped transiently in all three groups after treatment and then continued to rise throughout the remainder of the protocol (Figure 3). The initial drop was much greater in the late phase protocol than in the early phase protocol; however, MAP was similar in SHAM (178.3 ± 6.1 mmHg) and renal-CAP (182.0 ± 5.4 mmHg) rats by the end of the protocol. As in the early phase protocol, MAP in RDNX rats was ~10 mm Hg lower than SHAM and renal-CAP rats throughout the late phase protocol and MAP on the final day was 172.7 ± 6.4 mmHg.

As shown in Table 1, the pretreatment HR in the late phase protocol was similar between groups and similar to the pretreatment HR in the early phase protocol. On the day of treatment HR was significantly higher in renal-CAP rats compared to SHAM and significantly higher in RDNX rats compared to both renal-CAP and SHAM. HR was then similar between groups throughout the protocol with the exception of day 9 when HR was significantly lower in RNDX rats compared to SHAM.

As with the early phase protocol, there were no differences in cumulative water balance between groups at any time following treatment in the late phase protocol (Figure 3). However, cumulative sodium balance trended slightly lower in RDNX and renal-CAP rats compared to SHAM, but these differences were only significant on the final day of the late phase protocol.

Renal NE and CGRP content

To confirm the denervations, we measured renal tissue content of the afferent nerve marker, CGRP and the efferent nerve marker, NE. In both the early and late phase protocols, NE and CGRP were significantly reduced in the kidneys of RDNX rats as compared to SHAM rats (Figure 4). In both protocols, renal-CAP treatment resulted in a reduction in pelvic CGRP content that was even greater than RDNX. Compared to SHAM, renal NE content was not reduced in renal-CAP rats in the late phase protocol, but was somewhat reduced in renal-CAP rats in the early phase protocol.

4.5 Discussion

Although both preclinical and clinical studies suggest that RDNX has great potential for treating drug resistant hypertension, it is unclear whether the antihypertensive effect of RDNX is due to ablation of afferent or efferent renal nerves. However, mounting clinical evidence suggests that the therapeutic effects of RDNX may indeed be due to ablation of afferent renal nerves (67, 149, 171). To address this possibility, we recently developed a method to selectively ablate afferent renal nerves and have shown that this method attenuates the development of DOCA-salt hypertension to a similar extent as RDNX (chapter three). These results suggest that attenuation of DOCA-salt hypertension by RDNX is due to ablation of afferent rather than efferent renal nerves.

We have also previously shown that RDNX partially reverses the early phase of Dahl S hypertension (48). In the present study, we investigated whether the antihypertensive effect of RDNX in the Dahl S rat is due to ablation of afferent renal nerves by comparing the effects of RDNX and renal-CAP treatment. Moreover, because kidney disease has been linked to afferent renal nerve-dependent hypertension (63) and because Dahl S rats exhibit progressive renal pathology (32), we tested this hypothesis in both the early phase and late phase of Dahl S hypertension.

Renal denervation, but not selective ablation of afferent renal nerves, partially reverses Dahl S hypertension

To test whether the antihypertensive effect of RDNX in the Dahl S rat is due to ablation of afferent renal nerves, we compared the effect of RDNX and renal-CAP treatment on MAP in both the early and phase of Dahl S hypertension. In both protocols, RDNX lowered MAP ~10 mmHg compared to SHAM rats. Importantly, selective ablation of afferent renal nerves by renal-CAP treatment had no effect on MAP as compared to SHAM treatment in either protocol. These data show that, in contrast to our hypothesis, and our recent report in the DOCA-salt model, the antihypertensive effect of RDNX in the Dahl S rat is not due to ablation of afferent renal nerves. This discrepancy is important because it suggests that the mechanisms underlying the antihypertensive effect of RDNX vary from model to model and will likely vary from patient to patient.

It is also interesting to note that, while we have not performed RDNX in Dahl S rats prior to high NaCl feeding, we have shown that RDNX lowers MAP ~10 mmHg in normotensive Sprague-Dawley rats whereas renal-CAP has no effect on basal MAP (76, 78). Moreover, RDNX in human subjects has been shown to reduce 24h averaged ambulatory MAP ~10 mmHg (114). Taken together, these results suggest that, in the absence of stimuli that cause inappropriately high afferent or efferent renal nerve activity, basal efferent renal nerve activity may contribute ~10 mmHg to MAP.

The antihypertensive effect of RDNX is similar in both the early and late phase of Dahl S hypertension

Because kidney disease has been linked to afferent renal nerve-dependent hypertension and Dahl S rats exhibit progressive renal pathology (32, 63), we subjected Dahl S rats to RDNX and renal-CAP treatment in both the early phase (after 3 weeks of high NaCl feeding), when MAP was ~140 mmHg and renal pathology is known to be moderate; and in the late phase (after 9 weeks of high NaCl feeding), when MAP was ~170 mmHg and renal pathology is known to be more severe (32). Interestingly, the antihypertensive effect of RDNX was nearly identical in both the early and late phase of

Dahl S hypertension, and renal-CAP had no effect in either phase. These results have two important implications. First, the degree to which RDNX reverses HTN in the Dahl S rat appears not to be dependent on the duration of high salt feeding or pretreatment AP. This suggests that, in contrast to previous reports, baseline AP may be a poor predictor of the antihypertensive effect of RDNX (152). Second, these data suggest that, even in the presence of renal pathology, afferent renal nerves may not drive hypertension.

This study suggests that kidney disease is not necessarily associated with afferent renal nerve-dependent hypertension. However, we cannot exclude the possibility that kidney disease may drive hypertension via afferent renal nerves in other models. We have recently shown that renal-CAP treatment is effective in attenuating the development of DOCA-salt hypertension, a model in which rats are uninephrectomized and severe renal pathology results in the remaining kidney (119). Therefore, while renal pathology did not lead to afferent renal nerve-dependent hypertension in either phase of Dahl S hypertension, it may play a role in the DOCA-salt model. Further studies will be needed to determine the precise circumstances in which kidney disease can causes elevations in afferent renal nerve activity and AP.

The antihypertensive effect of RDNX is not due to sodium and water unloading

While total assessment of the efferent renal nerve specific responses to RDNX (i.e. plasma renin concentration and renal vascular resistance) was outside of the scope of this study, we did measure whole body cumulative sodium and water balance. These measurements revealed that the antihypertensive effect of RDNX did not correspond with a reduction in sodium or water balance. These results demonstrate that a reduction in AP can occur in the absence of enhanced natriuresis and diuresis. This finding is consistent with our earlier report on the effect of RDNX in Dahl S rats (48). These data, combined with our findings that afferent renal nerves do not play role in this model, suggest that the antihypertensive effect of RDNX in the Dahl S rat is likely due to either reduced activity of the renin angiotensin system or a reduction in renal vascular resistance. Further investigation will be needed to test these possibilities.

HR is largely unaffected by RDNX and renal-CAP

Our previous work has suggested that renal nerves may play a minor role in the long-term regulation of heart rate. Specifically, renal-CAP treatment resulted in a blunting of the bradycardic response to dietary sodium loading in normotensive Sprague-Dawley rats. Alternatively, the bradycardic response to DOCA-salt hypertension was actually enhanced in RDNX rats compared to SHAM rats despite the antihypertensive effect of RDNX (77). Interestingly, there was a slightly enhanced bradycardia in the early phase of Dahl S hypertension in this study. While the effect was minor and only significant on a few days, these results are consistent with some minor role of the renal nerves in heart rate regulation. This recurring phenomenon merits further investigation in order to uncover the mechanisms involved.

4.6 Perspectives

While RDNX appears to be a promising treatment option for drug resistant hypertension, the mechanisms underlying this therapeutic effect remain unclear. In order to further optimize this treatment, it is important to determine whether the antihypertensive effect is due to ablation of afferent or efferent renal nerves. To this end, we have shown that the antihypertensive effect of RDNX in the Dahl S rat is not due to ablation of afferent renal nerves. This is in contrast to our previous study in the DOCA-salt model in which renal-CAP treatment blunted the development of hypertension (chapter three). These results are critically important because they suggest that the mechanisms underlying the antihypertensive effect of RDNX will likely vary from patient to patient. Therefore, it may be optimal to develop selective ablation techniques, which could be used to target either subset of renal nerves in order to optimize efficacy and minimize side effects. Furthermore, it may be possible to develop clinical tests to identify patients that would benefit from such selective ablations. Further animal studies will be critical in developing such tests.

Acknowledgments

The authors would like to thank Dusty Van Helden, Jeff Larson, Eric Homan, Madeline Gauthier and Marcos Kuroki for their help with metabolic data collection and analysis. We would also like to thank Robert Burnett for his work on the NE assay as well as Ruijun Han and Xinying Yang for their work on the CGRP ELISA.

Sources of funding

This research was supported by NIH grant R01

Conflict(s) of interest/disclosure(s) statement

J.W.O. is a paid consultant of Medtronic CardioVascular, Inc. Santa Rosa, CA

Novelty and significance

What Is New?

- The antihypertensive effect of RDNX in the Dahl S rat is unrelated to the duration of high NaCl feeding, pretreatment AP or degree of kidney disease
- The antihypertensive effect of RDNX in the Dahl S rat appears not to be due to ablation of afferent renal nerves.

What Is Relevant?

- The recent failure of one clinical trial has highlighted the importance of understanding how RDNX lowers arterial pressure when it does.
- The degree to which RDNX lowers MAP in the Dahl S rat is similar to what has been reported in human patients
- The mechanisms underlying the antihypertensive effect of RDNX appear to vary from model to model and will likely vary from patient to patient

Summary

The results of this study suggest that the antihypertensive effect of RDNX in the Dahl S rat is not dependent on pretreatment AP or degree of kidney disease nor is it due to ablation of afferent renal nerve. If the same is true in some people, selective ablation of efferent renal nerves may be preferred.

EARLY PHASE	SHAM	RDNX	renal-CAP
Pretreatment MAP (mm Hg)	138.56 ± 3.05	139.88 ± 3.13	138.11 ± 1.57
Pretreatment HR (BPM)	396.40 ± 3.44	401.35 ± 4.20	396.04 ± 4.22

LATE PHASE	SHAM	RDNX	renal-CAP
Pretreatment MAP (mm Hg)	165.90 ± 4.26	168.83 ± 5.99	169.39 ± 5.29
Pretreatment HR (BPM)	385.86 ± 3.23	392.24 ± 5.33	395.90 ± 5.69

Table 1. Average mean arterial pressure (MAP) and heart rate (HR) on the day before treatment surgery in SHAM, RDNX and renal-CAP rats in the early phase (top) and late phase (bottom) protocols. There were no statistical differences in MAP or HR between groups in either protocol.

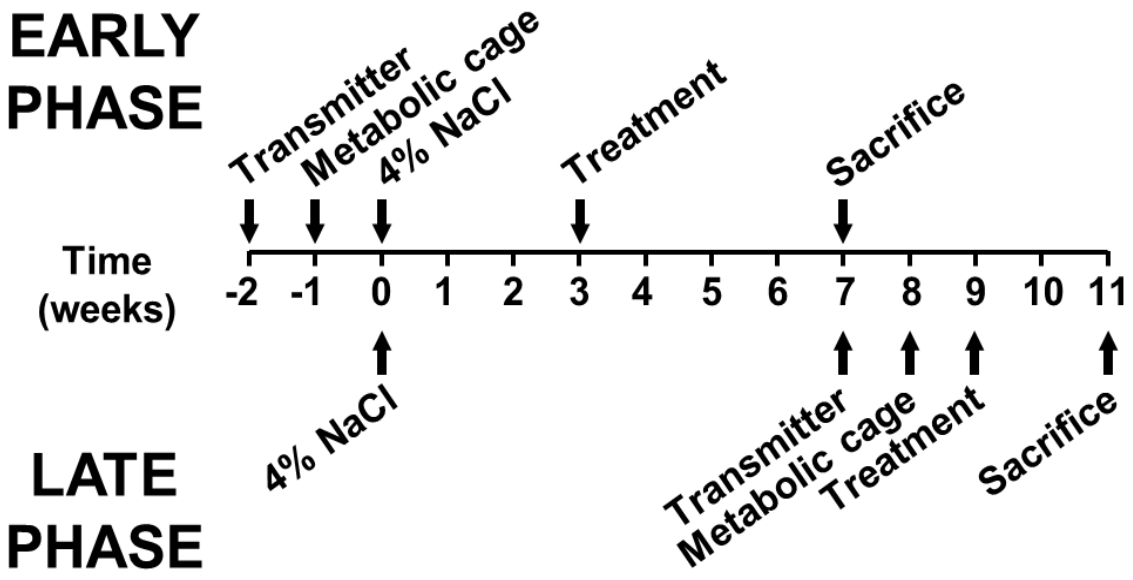

Figure1. Detailed description of the early phase and late phase protocols.

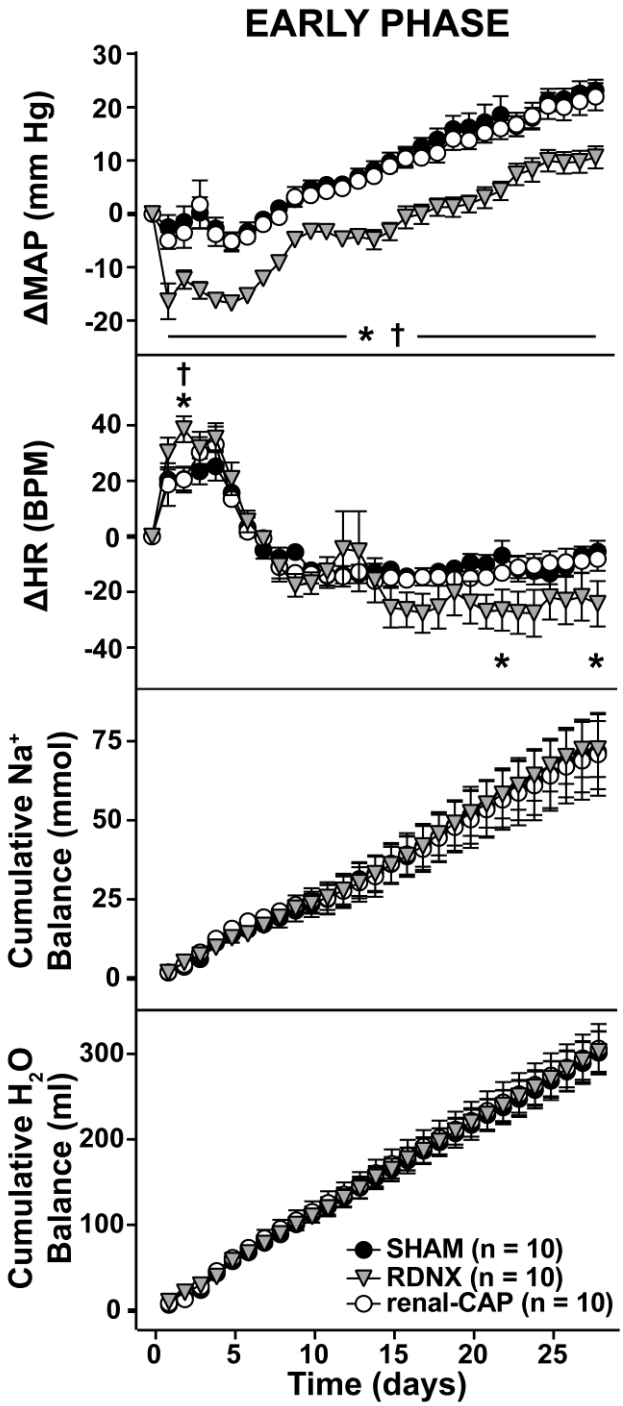

Figure 2. Change in mean arterial pressure (ΔMAP), change in heart rate (ΔHR), cumulative sodium (Na+) balance, and cumulative water (H2O) balance following SHAM, RDNX or renal-CAP treatment in the early phase protocol. * = p <0.05 for RDNX vs. SHAM. † = = p <0.05 for RDNX vs. renal-CAP.

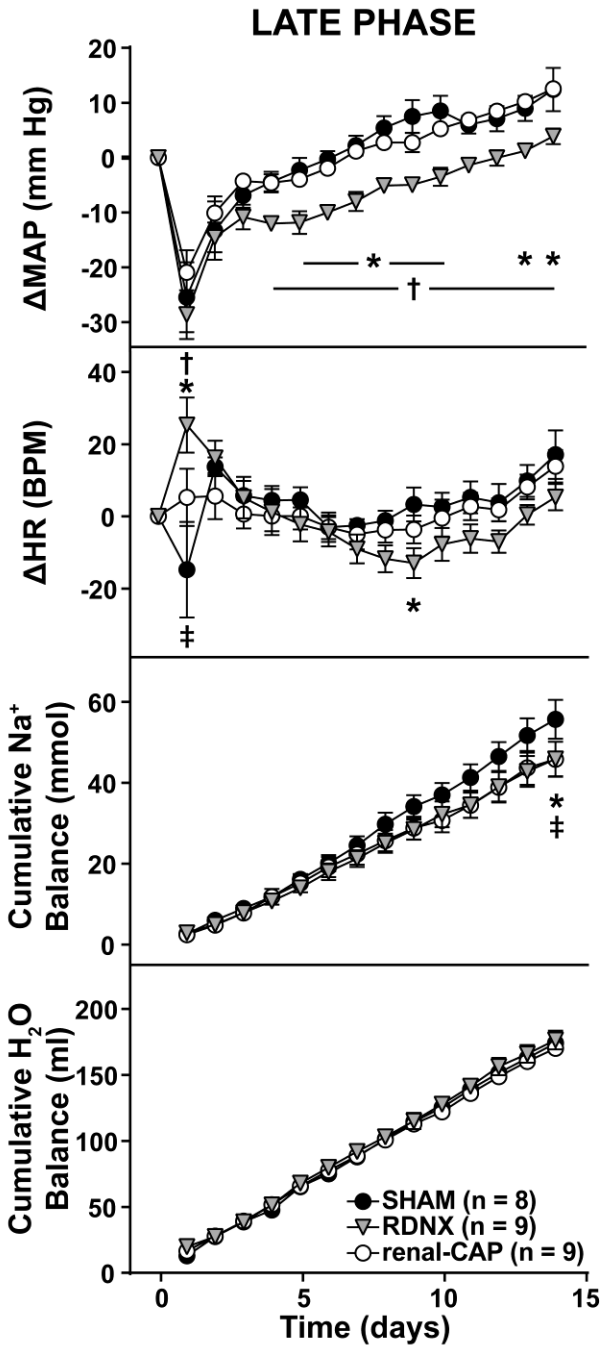

Figure 3. Change in mean arterial pressure (ΔMAP), change in heart rate (ΔHR), cumulative sodium (Na+) balance, and cumulative water (H2O) balance following SHAM, RDNX or renal-CAP treatment in the late phase protocol. * = $p < 0.05$ for RDNX vs. SHAM. † = = $p < 0.05$ for RDNX vs. renal-CAP, ‡ = $p < 0.05$ for renal-CAP vs. SHAM.

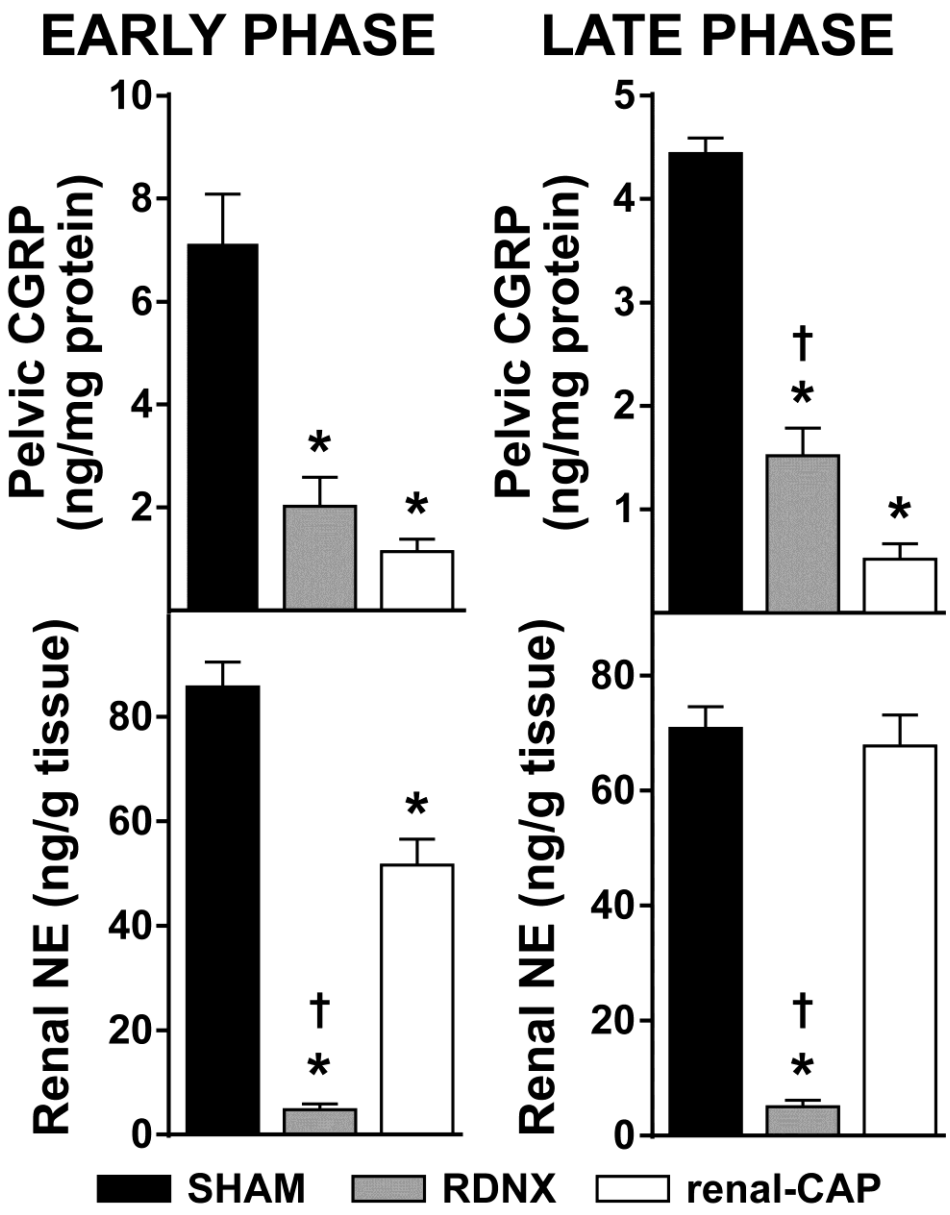

Figure 4. Content of the afferent nerve marker calcitonin gene-related peptide (CGRP; top) and the efferent nerve marker norepinephrine (NE; bottom) in the kidneys of SHAM, RDNX and renal-CAP rats at the end of the early phase (left) and late phase (right) protocols. * = $p < 0.05$ for RDNX vs. SHAM. † = = $p < 0.05$ for RDNX vs. renal-CAP.

Chapter Five

Conclusions

Clinical data suggest that RDNX may be an effective antihypertensive treatment in drug resistant human patients, but the mechanisms underlying this therapeutic effect remain unclear. Because RDNX works by destroying all types of nerves in the kidney, it is of great interest whether the decrease AP seen with this treatment is secondary to ablation of afferent or efferent renal nerves. The focus of my work has been centered on the hypothesis that the antihypertensive effect of RDNX is due to ablation of afferent rather than efferent renal nerves. In order to test this hypothesis, I first identified an animal model of HTN in which RDNX lowers AP. We found that RDNX had a significant antihypertensive effect in a commonly used genetic model of salt-sensitive HTN, the Dahl S rat. In this model, RDNX lowered MAP ~10-15 mmHg, a similar antihypertensive effect to the one observed in 24 hr. averaged ambulatory measurements in people. I then developed and validated a novel method for selective ablation of afferent renal nerves. We showed that 15 minute exposure of the renal nerves to capsaicin (renal-CAP treatment) causes both anatomical and functional ablation of afferent, but not efferent renal nerves. Using this technique, I then tested whether the antihypertensive effect of RDNX is due to ablation of afferent renal nerves in mildly hypertensive Dahl S rats fed a high salt diet for three weeks (early phase) and in severely hypertensive Dahl S

rats fed a high salt diet for nine weeks (late phase). The results of this final experiment suggested that the antihypertensive effect of RDNX in Dahl S is not due to ablation of afferent renal nerves. Moreover, the therapeutic effect of RDNX was equal in both phases of Dahl S HTN, suggesting that the antihypertensive effect of RDNX is not dependent on the duration of high salt feeding or pretreatment MAP. In this final chapter I will discuss the significance of these finding as well the importance of some of the ancillary findings of these experiments.

Renal-CAP treatment: a novel method for selective ablation of afferent renal nerves

Perhaps the most significant contribution of my work to the field of HTN research is the development and validation of a novel method for selective ablation of afferent renal nerves. While other methods have been used to indirectly investigate the physiological roles of afferent renal nerves, our renal-CAP method is the first technique developed to selectively and specifically ablate afferent renal nerves. That is, it causes ablation of afferent renal nerves without affecting efferent renal nerves or any non-renal nerves. Because this technique allows for specific investigation into the role of afferent renal nerves, and because the debate over the role of afferent vs efferent renal nerves in HTN is a hot one, this technique has great potential to influence future basic science research, which may in turn inform future clinical research.

Additive effect of multiple targeted sympathetic denervations

Another important finding was that independent denervations have the potential to have an additive antihypertensive effect. The observation that, in the Dahl S rat, RDNX and CGX each have an antihypertensive effect was interesting, but even more interesting was that these two procedures had an additive effect when done in conjunction. This important result suggests that human hypertensives may benefit from a multipronged approach. That is, certain patients who undergo an ablation of one sympathetic target may benefit from an additional ablation procedure. While current technology does not allow for any simple means of denervating non-renal targets, future technological

advances may make it feasible to target multiple sympathetic pathways with added benefit.

RDNX reduces AP in the absence of unloading of sodium and water

One prominent hypothesis regarding the mechanisms underlying the antihypertensive effect of RDNX is centered on the idea that removal of efferent renal nerves causes natriuresis and diuresis and thereby decreases AP. While this hypothetical model seems feasible, my data directly challenge this idea. Multiple experiments done in the Dahl S rat showed significant reductions in AP with RDNX independent of changes in sodium and water balance compared to sham operated controls. These data add to the growing body of evidence suggesting that chronic reductions in AP can occur in lieu of changes in renal excretory function.

Challenging previous studies: ablation of afferent renal nerves does not result in salt-sensitive HTN

Also important was the observation that afferent renal nerves are not required to maintain cardiovascular or salt and water homeostasis in the rat. Our data showing that rats are able to regulate sodium/water balance as well as AP and, to a large extent, HR challenge the hypothesis that afferent renal nerves are crucial in maintaining cardiovascular and salt/water homeostasis in the context of high dietary sodium intake. While further studies are needed to explain why afferent denervation that is not specific to the kidney (i.e. dorsal rhizotomy or systemic capsaicin) results in salt-sensitive HTN, it is clear from my data that selective ablation of afferent renal nerves does not lead to salt-sensitive HTN. This point becomes increasingly important when we begin to consider selective ablation procedures. Specifically, it may be preferable to perform selective ablation of afferent renal nerves in some patients. It is therefore important to know that this type of selective ablation would not result in salt-sensitivity, which would be a major side effect.

How this work informs the future of clinical HTN treatment

When data from the initial human RDNX trials were published, it appeared as though RDNX may be a universally effective treatment for drug resistant HTN. However, at least two recent trials have failed to show a significant antihypertensive effect of RDNX (13, 18). The reason for the discrepancy in these results remains unclear at present time, but there are a number of hypotheses ranging from different device design to user error on the interventionalists' part. While we may never know the exact cause for the failure of these trials, our animal studies shed some light on this controversy.

In the course of my studies, I have performed RDNX in various animal models of HTN. Moreover, RDNX has been used in an attempt to either prevent or reverse nearly every animal model of HTN in the past (38). In this thesis I have described the effect of RDNX on the reversal of Dahl S HTN and the prevention of DOCA-salt HTN. While RDNX did have an antihypertensive effect in both of these models, our lab has previously shown that RDNX has no effect on the development of a commonly used model of HTN, the AngII-salt model in which AngII is given subcutaneously and the rats are fed a high sodium diet (91). In addition, I performed a study (not included in this thesis) showing that RDNX does not prevent the development of the i.v. AngII-salt model in which AngII is given intravenously and rats are fed a high sodium diet.

The question then arises, which model is the right model for human HTN? The answer to this question is rather unsatisfying – we don't know. The reason that we cannot know for certain which model of HTN mimics human HTN best is that, just as all models of HTN are different, not all human hypertensives are the same. Therefore, the cautionary lesson to be learned from all of this is that RDNX likely will not be an effective antihypertensive treatment for all patients. The plethora of animal data and the growing body of clinical data supporting this assertion suggest that it would be logical for the device industry to take a step back and focus on a couple of key points. First, as RDNX likely will not work for all hypertensive patients, there is a great need to develop a reliable means to determine which patients will benefit from RDNX. Second, the

mechanisms underlying the antihypertensive effect of RDNX need to be identified in order to optimize the treatment.

Within the field, there has been a great deal of debate over whether RDNX lowers AP, when it does, by removing sympathetic control of the kidneys, or by removing sympathoexcitatory input from afferent renal nerves. While it has been impossible to answer this question in human patients, there is some clinical data supporting both hypotheses. Specifically, RDNX has been shown to reduce renal resistive index (presumably due to ablation of efferent renal nerves) as well as non-renal SNA, plasma glucose, incidence of sleep apnic events and severity of cardiac arrhythmias (all presumably due to ablation of afferent renal nerves) (67, 112, 149, 171).

While clinicians have not been able to resolve this debate in people, the animal studies described in this thesis shed some light on a possible answer. Using two different animal models of HTN, I have shown that the antihypertensive effect of RDNX can be due to ablation of either afferent or efferent renal nerves. Specifically, while RDNX attenuated HTN both in the Dahl S rat and the DOCA-salt model, selective ablation of afferent renal nerves with renal-CAP treatment attenuated only DOCA-salt HTN. As stated earlier, we do not know which model better represents human HTN since there are many forms of human HTN. Therefore, these results suggest that when RDNX lowers AP in people, it may be due to ablation of afferent or efferent renal nerves depending on the type of HTN the patient has. This is an important point because we may be able to develop selective ablation techniques to target either afferent or efferent renal nerves, specifically.

If it is indeed true that there are patients who could benefit from selective ablation of either afferent or efferent renal nerves, there may be a number of benefits to developing selective ablation techniques. First, because there is an issue achieving complete denervation with current technology (41), selective ablation techniques may result in more complete ablation of either afferent or efferent renal nerves. Indeed, my data showed consistently that renal-CAP treatment reduces pelvic CGRP content to greater extent than complete RDNX. Furthermore, since nearly all afferent renal nerves

are thought to innervate the pelvic wall, intrapelvic administration of a selective neurotoxin may result in a more complete ablation of afferent renal nerves, although this anatomical advantage does not exist for selective ablation of efferent renal nerves.

The second possible benefit of selective ablation is a minimization of side effects. It should be noted that there have been very few side effects reported following RDNX. However, there is a concern that if a renal denervated patient is in an extreme situation such as hemorrhage or extreme water deprivation, the absence of sympathetic control of the kidney would be detrimental. Therefore, if a patient's HTN is driven by ARNA, selective ablation of afferent renal nerves would be preferred as it would preserve the efferent renal nerves for these types of extreme circumstances. Likewise, preservation of the sensory fibers of the kidneys may be preferred if they are not contributing to HTN. For example, if a patient has a kidney stone, we know that the kidney sends important pain signals to the brain. In the absence of afferent renal nerves, kidney stones would likely go undetected and untreated causing greater damage. In this situation, it would be preferable to selectively ablate efferent renal nerves. Of course, the key to this treatment strategy would be to identify patients who would benefit from selective ablation of either afferent or efferent renal nerves.

In order to better identify those patients who would benefit from RDNX or selective ablation, there is a need to develop clinical tests that would help determine patient eligibility. To date, the only factors that have been identified as a predictors of the efficacy of RDNX to low AP are pretreatment MAP and use of sympatholytic drugs (152). That is, RDNX has been shown to be a more effective antihypertensive treatment in patients who are more hypertensive and those who respond well to sympatholytic drugs. While this may be of some help, identification of biomarkers that could help predict the efficacy of RDNX to lower AP would be preferred. Moreover, biomarkers may be able to help doctors identify those patients who would benefit from selective ablation of afferent or efferent renal nerves.

While little effort has been made to identify such predictive biomarkers, it is not inconceivable. For example, patients with elevated afferent renal nerve activity will likely

have elevated levels of afferent neurotransmitters in their urine since these fibers reside predominantly in the renal pelvis which is the final collection point for urine in the kidney. An index of ERSNA may be more difficult to identify as urinary NE and dopamine content have been shown to be poor indicators of ERNSA (95). While difficult, the development of such clinical indicators of ARNA and ERSNA merits further research.

Unanswered questions

If RDNX lowers AP in the Dahl S rat secondary to ablation of efferent renal nerves, what specific efferent renal nerve function is responsible for the antihypertensive effect? While, we do not have a definitive answer to this question, we can eliminate the possibility that the drop in AP is secondary to increased natriuresis and diuresis as RDNX does not alter sodium or water balance in the Dahl S rat. The other two primary candidates then are changes in plasma renin concentration or renal vascular resistance.

Renin, the rate limiting enzyme in the renin-angiotensin system, is well known to play a role in some forms of HTN and therefore a reduction in circulating renin levels following RDNX could explain the antihypertensive effect of the treatment. However, this is unlikely to explain the therapeutic effect of RDNX in the Dahl S rat since this model has been shown to be a low renin model (138). That is, because hypertensive Dahl S rats have very low levels of plasma renin, RDNX likely does not effectively lower renin levels further. While it is unlikely that RDNX lowers renin levels in Dahl S rats, this still merits investigation to explicitly exclude the possibility.

A much more likely explanation is that RDNX lowers AP in the Dahl S rat secondary to reductions in renal vascular resistance. Because the kidneys receive roughly one fifth of the total cardiac output (140), and because efferent renal nerve activity has been shown to exhibit powerful control over renal blood flow (179), it is logical to posit that RDNX causes a reduction in renal vascular resistance and thereby total peripheral resistance and systemic AP. This hypothesis is strengthened by the clinical observation in one study that the decrease in AP following catheter based RDNX is associated with a

reduction in renal resistive index (112). Again, this possible explanation remains to be tested.

Why is it that the antihypertensive effect of RDNX is due to ablation of afferent renal nerves in the DOCA-salt model, but due to ablation of efferent renal nerves in the Dahl S rat? While it is important and interesting that the antihypertensive effect of RDNX appears to be due to ablation of afferent renal nerves in the DOCA-salt model, but due to ablation of efferent renal nerves in the Dahl S rat, this discrepancy raises the question of why. Rather, what is it that causes afferent renal nerves to drive HTN in one model and what causes the efferent renal nerves to drive HTN in another. The reason this question is so important is that, as discussed earlier, there are likely some patients who could benefit from selective ablation of either afferent or efferent renal nerves and therefore identifying factors that drive each subset of nerves will be crucial in determining which treatment would benefit which patients.

One possibility is that kidney disease can drive HTN via activation of afferent renal nerves. The results shown in chapter four demonstrate that, in the Dahl S rat, which is known to exhibit marked kidney disease, the antihypertensive effect of RDNX in not due to ablation of afferent renal nerves. However, this does not exclude the possibility that renal damage can lead to afferent renal nerve-dependent HTN. This may in fact be the case in the DOCA-salt model. It is important to remember that in this model, one kidney is removed and, after significant compensatory renal hypertrophy occurs, a salt retaining hormone is supplemented and rats are given saline rather than water to drink. In other words, renal excretory capacity is impaired and rats take in excessive amounts of salt. It is perhaps not surprising that the remaining kidney incurs significant damage as a result. It is therefore possible that the afferent renal nerve-dependent HTN observed in the DOCA-salt model is a result of excessive renal inflammation that occurs as a result of such severe renal damage.

Moreover, while kidney disease clearly does not cause afferent renal nerve-dependent HTN in the Dahl S rat, it is possible that if the kidneys incurred greater damage, that the afferent renal nerves would become overactive, further driving HTN in

this model. Although the results of the Dahl S studies suggest that kidney disease does not necessarily drive afferent renal nerves and HTN, the results of the DOCA-salt study along with a small body of clinical data warrant further research into the possible link between kidney disease and afferent renal nerve-dependent HTN.

Are there afferent renal nerve fibers that are unaffected by renal-CAP treatment? The likely answer to this question is yes. Because the vast majority of afferent fibers in the kidney have been shown to be CGRP+, unmyelinated or thinly myelinated, and capsaicin sensitive; we have assumed that renal-CAP treatment affects nearly all of the afferent renal nerves (39, 92, 128, 166). However, it is likely that some afferent renal nerves are TRPV1- and therefore unaffected by capsaicin treatment. Because we used CGRP as our marker for afferent renal nerves, we are unable to assess the presence of CGRP- fibers in the kidneys. While we were unable to devise a method to quantify the entire afferent renal nerve population, it may be possible. Use of a pan-neuronal marker may be useful in this regard. It may be possible, using IHC, to label all neurons in the kidney and double label for TH in order to exclude efferent fibers. The remaining fibers that are positive only for the pan-neuronal marker would be assumed to be the entire population of afferent renal nerves. This type of experiment merits further consideration.

Final Thoughts

The original goal of my work was to determine the differential role of afferent and efferent renal nerves in HTN. In order to achieve this goal, I conducted numerous experiments, the results of which have a number of important implications. First, my work has supplied the field with a new method that will be helpful in answering important questions regarding the physiology of afferent renal nerves. Second, my work informs the clinical treatment of HTN in that it suggests that 1) it is likely that not every patient will benefit from RDNX, 2) selective ablation procedures may be both feasible and preferred to complete RDNX and 3) additional ablation procedures performed either alone or in conjunction with RDNX should be pursued as possible treatment modalities for clinical HTN.

Bibliography

1. **Acelajado MC, Pisoni R, Dudenbostel T, Dell'Italia LJ, Cartmill F, Zhang B, Cofield SS, Oparil S, and Calhoun DA**. Refractory hypertension: definition, prevalence, and patient characteristics. *Journal of clinical hypertension* 14: 7-12, 2012.

2. **Ammons WS**. Bowditch Lecture. Renal afferent inputs to ascending spinal pathways. *The American journal of physiology* 262: R165-176, 1992.

3. **Ammons WS**. Electrophysiological characteristics of primate spinothalamic neurons with renal and somatic inputs. *Journal of neurophysiology* 61: 1121-1130, 1989.

4. **Ammons WS**. Spinoreticular cell responses to intrarenal injections of bradykinin. *The American journal of physiology* 255: R994-1001, 1988.

5. **Ammons WS**. Spinoreticular cell responses to renal venous and ureteral occlusion. *The American journal of physiology* 254: R268-276, 1988.

6. **Anderson JW, Washburn DL, and Ferguson AV**. Intrinsic osmosensitivity of subfornical organ neurons. *Neuroscience* 100: 539-547, 2000.

7. **Ashton N, Clarke CG, Eddy DE, and Swift FV**. Mechanisms involved in the activation of ischemically sensitive, afferent renal nerve mediated reflex increases in hind-limb vascular resistance in the anesthetized rabbit. *Canadian journal of physiology and pharmacology* 72: 637-643, 1994.

8. **Atherton DS, Deep NL, and Mendelsohn FO**. Micro-anatomy of the renal sympathetic nervous system: a human postmortem histologic study. *Clinical anatomy* 25: 628-633, 2012.

9. **Averina VA, Othmer HG, Fink GD, and Osborn JW**. A new conceptual paradigm for the haemodynamics of salt-sensitive hypertension: a mathematical modelling approach. *The Journal of physiology* 590: 5975-5992, 2012.

10. **Badoer E, Ng CW, and De Matteo R**. Glutamatergic input in the PVN is important in renal nerve response to elevations in osmolality. *American journal of physiology Renal physiology* 285: F640-650, 2003.

11. **Barrett CJ, Navakatikyan MA, and Malpas SC**. Long-term control of renal blood flow: what is the role of the renal nerves? *American journal of physiology Regulatory, integrative and comparative physiology* 280: R1534-1545, 2001.

12. **Barrett CJ, Ramchandra R, Guild SJ, Lala A, Budgett DM, and Malpas SC**. What sets the long-term level of renal sympathetic nerve activity: a role for angiotensin II and baroreflexes? *Circulation research* 92: 1330-1336, 2003.

13. **Bhatt DL, Kandzari DE, O'Neill WW, D'Agostino R, Flack JM, Katzen BT, Leon MB, Liu M, Mauri L, Negoita M, Cohen SA, Oparil S, Rocha-Singh K, Townsend RR, Bakris GL, and Investigators SH-**. A controlled trial of renal denervation for resistant hypertension. *The New England journal of medicine* 370: 1393-1401, 2014.

14. **Bishop VS, Hasser EM, and Nair UC**. Baroreflex control of renal nerve activity in conscious animals. *Circulation research* 61: I76-81, 1987.

15. **Blankestijn PJ**. Sympathetic hyperactivity in chronic kidney disease. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 19: 1354-1357, 2004.

16. **Boegehold MA, Huffman LJ, and Hedge GA**. Peripheral vascular resistance and regional blood flows in hypertensive Dahl rats. *The American journal of physiology* 261: R934-938, 1991.

17. **Bourque CW, Ciura S, Trudel E, Stachniak TJ, and Sharif-Naeini R**. Neurophysiological characterization of mammalian osmosensitive neurones. *Experimental physiology* 92: 499-505, 2007.

18. **Brinkmann J, Heusser K, Schmidt BM, Menne J, Klein G, Bauersachs J, Haller H, Sweep FC, Diedrich A, Jordan J, and Tank J**. Catheter-based renal nerve ablation and centrally generated sympathetic activity in difficult-to-control hypertensive patients: prospective case series. *Hypertension* 60: 1485-1490, 2012.

19. **Bugenhagen SM, Cowley AW, Jr., and Beard DA**. Identifying physiological origins of baroreflex dysfunction in salt-sensitive hypertension in the Dahl SS rat. *Physiological genomics* 42: 23-41, 2010.

20. **Burke SL, Evans RG, Moretti JL, and Head GA**. Levels of renal and extrarenal sympathetic drive in angiotensin II-induced hypertension. *Hypertension* 51: 878-883, 2008.

21. **Campese VM, and Kogosov E**. Renal afferent denervation prevents hypertension in rats with chronic renal failure. *Hypertension* 25: 878-882, 1995.

22. **Campese VM, Kogosov E, and Koss M**. Renal Afferent Denervation Prevents the Progression of Renal-Disease in the Renal Ablation Model of Chronic-Renal-Failure in the Rat. *American Journal of Kidney Diseases* 26: 861-865, 1995.

23. **Carlson SH, Osborn JW, and Wyss JM**. Hepatic denervation chronically elevates arterial pressure in Wistar-Kyoto rats. *Hypertension* 32: 46-51, 1998.

24. **Carroll RG, Lohmeier TE, and Brown AJ**. Chronic angiotensin II infusion decreases renal norepinephrine overflow in conscious dogs. *Hypertension* 6: 675-681, 1984.

25. **Caverson MM, and Ciriello J**. Effect of stimulation of afferent renal nerves on plasma levels of vasopressin. *The American journal of physiology* 252: R801-807, 1987.

26. **Cervero F, and Tattersall JE**. Somatic and visceral sensory integration in the thoracic spinal cord. *Progress in brain research* 67: 189-205, 1986.

27. **Chiu IM, von Hehn CA, and Woolf CJ**. Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. *Nature neuroscience* 15: 1063-1067, 2012.

28. **Ciriello J**. Afferent renal inputs to paraventricular nucleus vasopressin and oxytocin neurosecretory neurons. *The American journal of physiology* 275: R1745-1754, 1998.

29. **Colindres RE, Spielman WS, Moss NG, Harrington WW, and Gottschalk CW**. Functional evidence for renorenal reflexes in the rat. *The American journal of physiology* 239: F265-270, 1980.

30. **Converse RL, Jr., Jacobsen TN, Toto RD, Jost CM, Cosentino F, Fouad-Tarazi F, and Victor RG**. Sympathetic overactivity in patients with chronic renal failure. *The New England journal of medicine* 327: 1912-1918, 1992.

31. **Correia ML, Morgan DA, Sivitz WI, Mark AL, and Haynes WG**. Leptin acts in the central nervous system to produce dose-dependent changes in arterial pressure. *Hypertension* 37: 936-942, 2001.

32. **Cowley AW, Jr., Ryan RP, Kurth T, Skelton MM, Schock-Kusch D, and Gretz N**. Progression of glomerular filtration rate reduction determined in conscious Dahl salt-sensitive hypertensive rats. *Hypertension* 62: 85-90, 2013.

33. **Cushman WC, Ford CE, Cutler JA, Margolis KL, Davis BR, Grimm RH, Black HR, Hamilton BP, Holland J, Nwachuku C, Papademetriou V, Probstfield J, Wright JT, Jr., Alderman MH, Weiss RJ, Piller L, Bettencourt J, Walsh SM, and Group ACR**. Success and predictors of blood pressure control in diverse North American settings: the antihypertensive and lipid-lowering treatment to prevent heart attack trial (ALLHAT). *Journal of clinical hypertension* 4: 393-404, 2002.

34. **Dampney RA**. Functional organization of central pathways regulating the cardiovascular system. *Physiological reviews* 74: 323-364, 1994.

35. **Day TA, and Ciriello J**. Afferent renal nerve stimulation excites supraoptic vasopressin neurons. *The American journal of physiology* 249: R368-371, 1985.

36. **DiBona GF, and Esler M**. Translational medicine: the antihypertensive effect of renal denervation. *Am J Physiol-Reg I* 298: R245-R253, 2010.

37. **DiBona GF, and Jones SY**. Analysis of renal sympathetic nerve responses to stress. *Hypertension* 25: 531-538, 1995.

38. **DiBona GF, and Kopp UC**. Neural control of renal function. *Physiological reviews* 77: 75-197, 1997.

39. **Ditting T, Tiegs G, Rodionova K, Reeh PW, Neuhuber W, Freisinger W, and Veelken R**. Do distinct populations of dorsal root ganglion neurons account for the sensory peptidergic innervation of the kidney? *American journal of physiology Renal physiology* 297: F1427-1434, 2009.

40. **Egan BM, Zhao Y, Axon RN, Brzezinski WA, and Ferdinand KC**. Uncontrolled and apparent treatment resistant hypertension in the United States, 1988 to 2008. *Circulation* 124: 1046-1058, 2011.

41. **Esler M**. Illusions of truths in the Symplicity HTN-3 trial: generic design strengths but neuroscience failings. *Journal of the American Society of Hypertension : JASH* 8: 593-598, 2014.

42. **Esler MD, Bohm M, Sievert H, Rump CL, Schmieder RE, Krum H, Mahfoud F, and Schlaich MP**. Catheter-based renal denervation for treatment of patients with treatment-resistant hypertension: 36 month results from the SYMPLICITY HTN-2 randomized clinical trial. *Eur Heart J* 35: 1752-1759, 2014.

43. **Esler MD, Krum H, Schlaich M, Schmieder RE, Bohm M, Sobotka PA, and Symplicity HTNI**. Renal sympathetic denervation for treatment of drug-resistant hypertension: one-year results from the Symplicity HTN-2 randomized, controlled trial. *Circulation* 126: 2976-2982, 2012.

44. **Faber JE, and Brody MJ**. Afferent renal nerve-dependent hypertension following acute renal artery stenosis in the conscious rat. *Circulation research* 57: 676-688, 1985.

45. **Ferguson M, and Bell C**. Ultrastructural localization and characterization of sensory nerves in the rat kidney. *The Journal of comparative neurology* 274: 9-16, 1988.

46. **Fink GDaJWO**. The splanchnic circulation. In: *Primer on the autonomic nervous system*, edited by Robertson D. Amsterdam: Elsevier Academic Press, 2012, p. 211-214.

47. **Fitzgerald M**. Capsaicin and sensory neurones--a review. *Pain* 15: 109-130, 1983.

48. **Foss JD, Fink GD, and Osborn JW**. Reversal of genetic salt-sensitive hypertension by targeted sympathetic ablation. *Hypertension* 61: 806-811, 2013.

49. **Freedman MA, Hallenbeck GA, and Code CF**. The effect of vagotomy and of methantheline bromide on the diarrhea produced by celiac and superior mesenteric ganglionectomy. *Surgical forum* 481-486, 1953.

50. **Friedman R, Tassinari LM, Heine M, and Iwai J**. Differential development of salt-induced and renal hypertension in Dahl hypertension-sensitive rats after neonatal sympathectomy. *Clinical and experimental hypertension* 1: 779-799, 1979.

51. **Fujita M, and Fujita T**. The role of CNS in salt-sensitive hypertension. *Current hypertension reports* 15: 390-394, 2013.

52. **Genovesi S, Pieruzzi F, Centonza L, Wijnmaalen P, Zanchetti A, and Stella A**. Electrophysiological evidence of ipsilateral reno-renal reflexes in the cat. *Journal of the autonomic nervous system* 65: 45-48, 1997.

53. **Genovesi S, Pieruzzi F, Wijnmaalen P, Centonza L, Golin R, Zanchetti A, and Stella A**. Renal afferents signaling diuretic activity in the cat. *Circulation research* 73: 906-913, 1993.

54. **Gordon FJ, Matsuguchi H, and Mark AL**. Abnormal baroreflex control of heart rate in prehypertensive and hypertensive Dahl genetically salt-sensitive rats. *Hypertension* 3: I135-141, 1981.

55. **Goto A, Ikeda T, Tobian L, Iwai J, and Johnson MA**. Brain lesions in the paraventricular nuclei and catecholaminergic neurons minimize salt hypertension in Dahl salt-sensitive rats. *Clinical science* 61 Suppl 7: 53s-55s, 1981.

56. **Grimson KS, Orgain ES, Anderson B, and D'Angelo GJ**. Total thoracic and partial to total lumbar sympathectomy, splanchnicectomy and celiac ganglionectomy for hypertension. *Annals of surgery* 138: 532-547, 1953.

57. **Guyenet PG**. The sympathetic control of blood pressure. *Nature reviews Neuroscience* 7: 335-346, 2006.

58. **Guyton AC**. Dominant role of the kidneys and accessory role of whole-body autoregulation in the pathogenesis of hypertension. *American journal of hypertension* 2: 575-585, 1989.

59. **Hackenthal E, Paul M, Ganten D, and Taugner R**. Morphology, physiology, and molecular biology of renin secretion. *Physiological reviews* 70: 1067-1116, 1990.

60. **Hall JE, da Silva AA, do Carmo JM, Dubinion J, Hamza S, Munusamy S, Smith G, and Stec DE**. Obesity-induced hypertension: role of sympathetic nervous system, leptin, and melanocortins. *The Journal of biological chemistry* 285: 17271-17276, 2010.

61. **Hamza SM, and Kaufman S**. Splenorenal reflex modulates renal blood flow in the rat. *The Journal of physiology* 558: 277-282, 2004.

62. **Harrison DG, Guzik TJ, Lob HE, Madhur MS, Marvar PJ, Thabet SR, Vinh A, and Weyand CM**. Inflammation, immunity, and hypertension. *Hypertension* 57: 132-140, 2011.

63. **Hausberg M, Kosch M, Harmelink P, Barenbrock M, Hohage H, Kisters K, Dietl KH, and Rahn KH**. Sympathetic nerve activity in end-stage renal disease. *Circulation* 106: 1974-1979, 2002.

64. **Heesch CM**. Reflexes that control cardiovascular function. *The American journal of physiology* 277: S234-243, 1999.

65. **Hendel MD, and Collister JP**. Contribution of the subfornical organ to angiotensin II-induced hypertension. *American journal of physiology Heart and circulatory physiology* 288: H680-685, 2005.

66. **Hendel MD, and Collister JP**. Renal denervation attenuates long-term hypertensive effects of Angiotensin ii in the rat. *Clinical and experimental pharmacology & physiology* 33: 1225-1230, 2006.

67. **Hering D, Lambert EA, Marusic P, Walton AS, Krum H, Lambert GW, Esler MD, and Schlaich MP**. Substantial reduction in single sympathetic nerve firing after renal denervation in patients with resistant hypertension. *Hypertension* 61: 457-464, 2013.

68. **Hoagland KM, Maddox DA, and Martin DS**. Intrarenal infusion of bradykinin elicits a pressor response in conscious rats via a B2-receptor mechanism. *Canadian journal of physiology and pharmacology* 77: 563-570, 1999.

69. **Holzer P**. Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacological reviews* 43: 143-201, 1991.

70. **Honig A**. Peripheral arterial chemoreceptors and reflex control of sodium and water homeostasis. *The American journal of physiology* 257: R1282-1302, 1989.

71. **Huang BS, and Leenen FH**. Sympathoexcitatory and pressor responses to increased brain sodium and ouabain are mediated via brain ANG II. *The American journal of physiology* 270: H275-280, 1996.

72. **Huang Y, and Wang DH**. Role of renin-angiotensin-aldosterone system in salt-sensitive hypertension induced by sensory denervation. *American journal of physiology Heart and circulatory physiology* 281: H2143-2149, 2001.

73. **Irigoyen MC, Moreira ED, Ida F, Pires M, Cestari IA, and Krieger EM**. Changes of renal sympathetic activity in acute and chronic conscious sinoaortic denervated rats. *Hypertension* 26: 1111-1116, 1995.

74. **Ishiki K, Morita H, and Hosomi H**. Reflex control of renal nerve activity originating from the osmoreceptors in the hepato-portal region. *Journal of the autonomic nervous system* 36: 139-148, 1991.

75. **Iwata T, Muneta S, Kitami Y, Okura T, Ii Y, Murakami E, and Hiwada K**. Effect of renal denervation on the development of hypertension in Dahl-Iwai salt-sensitive rats. *Nihon Jinzo Gakkai shi* 33: 867-871, 1991.

76. **Jacob F, Ariza P, and Osborn JW**. Renal denervation chronically lowers arterial pressure independent of dietary sodium intake in normal rats. *Am J Physiol-Heart C* 284: H2302-H2310, 2003.

77. **Jacob F, Clark LA, Guzman PA, and Osborn JW**. Role of renal nerves in development of hypertension in DOCA-salt model in rats: a telemetric approach. *American journal of physiology Heart and circulatory physiology* 289: H1519-1529, 2005.

78. **Jacob F, LaBine BG, Ariza P, Katz SA, and Osborn JW**. Renal denervation causes chronic hypotension in rats: Role of beta(1)-adrenoceptor activity. *Clin Exp Pharmacol P* 32: 255-262, 2005.

79. **Jancso G, Kiraly E, and Jancso-Gabor A**. Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. *Nature* 270: 741-743, 1977.

80. **Janssen BJ, van Essen H, Vervoort-Peters LH, Thijssen HH, Derkx FH, Struyker-Boudier HA, and Smits JF**. Effects of complete renal denervation and selective afferent renal denervation on the hypertension induced by intrarenal norepinephrine infusion in conscious rats. *Journal of hypertension* 7: 447-455, 1989.

81. **Johns EJ**. An investigation into the type of beta-adrenoceptor mediating sympathetically activated renin release in the cat. *British journal of pharmacology* 73: 749-754, 1981.

82. **Johns EJ, Kopp UC, and Dibona GF**. Neural control of renal function. *Comprehensive Physiology* 1: 731-767, 2011.

83. **Joyner MJ, Charkoudian N, and Wallin BG**. A sympathetic view of the sympathetic nervous system and human blood pressure regulation. *Experimental physiology* 93: 715-724, 2008.

84. **Kanbar R, Orea V, Barres C, and Julien C**. Baroreflex control of renal sympathetic nerve activity during air-jet stress in rats. *American journal of physiology Regulatory, integrative and comparative physiology* 292: R362-367, 2007.

85. **Kandlikar SS, and Fink GD**. Splanchnic sympathetic nerves in the development of mild DOCA-salt hypertension. *Am J Physiol-Heart C* 301: H1965-H1973, 2011.

86. **Katholi RE, McCann WP, and Woods WT**. Intrarenal adenosine produces hypertension via renal nerves in the one-kidney, one clip rat. *Hypertension* 7: I88-93, 1985.

87. **Katholi RE, Whitlow PL, Hageman GR, and Woods WT**. Intrarenal adenosine produces hypertension by activating the sympathetic nervous system via the renal nerves in the dog. *Journal of hypertension* 2: 349-359, 1984.

88. **Katholi RE, Whitlow PL, Winternitz SR, and Oparil S**. Importance of the renal nerves in established two-kidney, one clip Goldblatt hypertension. *Hypertension* 4: 166-174, 1982.

89. **Katholi RE, Winternitz SR, and Oparil S**. Role of the renal nerves in the pathogenesis of one-kidney renal hypertension in the rat. *Hypertension* 3: 404-409, 1981.

90. **Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, and He J**. Global burden of hypertension: analysis of worldwide data. *Lancet* 365: 217-223, 2005.

91. **King AJ, Osborn JW, and Fink GD**. Splanchnic circulation is a critical neural target in angiotensin II salt hypertension in rats. *Hypertension* 50: 547-556, 2007.

92. **Knuepfer MM, Akeyson EW, and Schramm LP**. Spinal projections of renal afferent nerves in the rat. *Brain research* 446: 17-25, 1988.

93. **Knuepfer MM, and Schramm LP**. The conduction velocities and spinal projections of single renal afferent fibers in the rat. *Brain research* 435: 167-173, 1987.

94. **Knuepfer MM, and Schramm LP**. Properties of renobulbar afferent fibers in rats. *The American journal of physiology* 248: R113-119, 1985.

95. **Kopp U, Bradley T, and Hjemdahl P**. Renal venous outflow and urinary excretion of norepinephrine, epinephrine, and dopamine during graded renal nerve stimulation. *The American journal of physiology* 244: E52-60, 1983.

96. **Kopp UC, Cicha MZ, Nakamura K, Nusing RM, Smith LA, and Hokfelt T**. Activation of EP4 receptors contributes to prostaglandin E2-mediated stimulation of renal sensory nerves. *American journal of physiology Renal physiology* 287: F1269-1282, 2004.

97. **Kopp UC, Cicha MZ, and Smith LA**. Dietary sodium loading increases arterial pressure in afferent renal-denervated rats. *Hypertension* 42: 968-973, 2003.

98. **Kopp UC, Jones SY, and DiBona GF**. Afferent renal denervation impairs baroreflex control of efferent renal sympathetic nerve activity. *American journal of physiology Regulatory, integrative and comparative physiology* 295: R1882-1890, 2008.

99. **Kopp UC, Smith LA, and Pence AL**. Na(+)-K(+)-ATPase inhibition sensitizes renal mechanoreceptors activated by increases in renal pelvic pressure. *The American journal of physiology* 267: R1109-1117, 1994.

100. **Krum H, Schlaich M, Whitbourn R, Sobotka PA, Sadowski J, Bartus K, Kapelak B, Walton A, Sievert H, Thambar S, Abraham WT, and Esler M**. Catheter-based renal sympathetic denervation for resistant hypertension: a multicentre safety and proof-of-principle cohort study. *Lancet* 373: 1275-1281, 2009.

101. **Krum H, Schlaich MP, Bohm M, Mahfoud F, Rocha-Singh K, Katholi R, and Esler MD**. Percutaneous renal denervation in patients with treatment-resistant hypertension: final 3-year report of the Symplicity HTN-1 study. *Lancet* 2013.

102. **Kubicek WG, Kottke FJ, Laker DJ, and Visscher MB**. Renal function during arterial hypertension produced by chronic splanchnic nerve stimulation in the dog. *The American journal of physiology* 174: 397-400, 1953.

103. **Kuo DC, and de Groat WC**. Primary afferent projections of the major splanchnic nerve to the spinal cord and gracile nucleus of the cat. *The Journal of comparative neurology* 231: 421-434, 1985.

104. **Kuo DC, Nadelhaft I, Hisamitsu T, and de Groat WC**. Segmental distribution and central projections of renal afferent fibers in the cat studied by transganglionic transport of horseradish peroxidase. *The Journal of comparative neurology* 216: 162-174, 1983.

105. **Lappe RW, Webb RL, and Brody MJ**. Selective destruction of renal afferent versus efferent nerves in rats. *The American journal of physiology* 249: R634-637, 1985.

106. **Leenen FH**. The central role of the brain aldosterone-"ouabain" pathway in salt-sensitive hypertension. *Biochimica et biophysica acta* 1802: 1132-1139, 2010.

107. **Li M, Galligan J, Wang D, and Fink G**. The effects of celiac ganglionectomy on sympathetic innervation to the splanchnic organs in the rat. *Autonomic neuroscience : basic & clinical* 154: 66-73, 2010.

108. **Lopez AD, Mathers CD, Ezzati M, Jamison DT, and Murray CJ**. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* 367: 1747-1757, 2006.

109. **Ma G, and Ho SY**. [Hemodynamic effects of renal interoreceptor and afferent nerve stimulation in rabbit]. *Sheng li xue bao : [Acta physiologica Sinica]* 42: 262-268, 1990.

110. **Ma G, and Ho SY**. [Observation on the afferent nerve activity induced by stimulation of renal receptors in the rabbits]. *Sheng li xue bao : [Acta physiologica Sinica]* 42: 269-276, 1990.

111. **Ma MC, Huang HS, Wu MS, Chien CT, and Chen CF**. Impaired renal sensory responses after renal ischemia in the rat. *Journal of the American Society of Nephrology : JASN* 13: 1872-1883, 2002.

112. **Mahfoud F, Cremers B, Janker J, Link B, Vonend O, Ukena C, Linz D, Schmieder R, Rump LC, Kindermann I, Sobotka PA, Krum H, Scheller B, Schlaich M, Laufs U, and Boehm M**. Renal Hemodynamics and Renal Function After Catheter-Based Renal Sympathetic Denervation in Patients With Resistant Hypertension. *Hypertension* 60: 419-424, 2012.

113. **Mahfoud F, Schlaich M, Kindermann I, Ukena C, Cremers B, Brandt MC, Hoppe UC, Vonend O, Rump LC, Sobotka PA, Krum H, Esler M, and Bohm M**. Effect of renal sympathetic denervation on glucose metabolism in patients with resistant hypertension: a pilot study. *Circulation* 123: 1940-1946, 2011.

114. **Mahfoud F, Ukena C, Schmieder RE, Cremers B, Rump LC, Vonend O, Weil J, Schmidt M, Hoppe UC, Zeller T, Bauer A, Ott C, Blessing E, Sobotka PA, Krum H, Schlaich M, Esler M, and Bohm M**. Ambulatory blood pressure changes after renal sympathetic denervation in patients with resistant hypertension. *Circulation* 128: 132-140, 2013.

115. **Malpas SC**. Sympathetic nervous system overactivity and its role in the development of cardiovascular disease. *Physiological reviews* 90: 513-557, 2010.

116. **Marfurt CF, and Echtenkamp SF**. Sensory innervation of the rat kidney and ureter as revealed by the anterograde transport of wheat germ agglutinin-horseradish peroxidase (WGA-HRP) from dorsal root ganglia. *The Journal of comparative neurology* 311: 389-404, 1991.

117. **Mark AL**. Sympathetic neural contribution to salt-induced hypertension in Dahl rats. *Hypertension* 17: I86-90, 1991.

118. **Marlett JA, and Code CF**. Effects of celiac and superior mesenteric ganglionectomy on interdigestive myoelectric complex in dogs. *The American journal of physiology* 237: E432-443, 1979.

119. **Matsumura Y, Kuro T, Kobayashi Y, Konishi F, Takaoka M, Wessale JL, Opgenorth TJ, Gariepy CE, and Yanagisawa M**. Exaggerated vascular and renal pathology in endothelin-B receptor-deficient rats with deoxycorticosterone acetate-salt hypertension. *Circulation* 102: 2765-2773, 2000.

120. **Mohamed AA, Parker TL, and Coupland RE**. The innervation of the adrenal gland. II. The source of spinal afferent nerve fibres to the guinea-pig adrenal gland. *Journal of anatomy* 160: 51-58, 1988.

121. **Morita H, Ishiki K, and Hosomi H**. Effects of hepatic NaCl receptor stimulation on renal nerve activity in conscious rabbits. *Neuroscience letters* 123: 1-3, 1991.

122. **Morita H, Matsuda T, Furuya F, Khanchowdhury MR, and Hosomi H**. Hepatorenal reflex plays an important role in natriuresis after high-NaCl food intake in conscious dogs. *Circulation research* 72: 552-559, 1993.

123. **Morita H, Yamashita Y, Nishida Y, Tokuda M, Hatase O, and Hosomi H**. Fos induction in rat brain neurons after stimulation of the hepatoportal Na-sensitive mechanism. *The American journal of physiology* 272: R913-923, 1997.

124. **Moss NG**. Electrophysiology of afferent renal nerves. *Federation proceedings* 44: 2828-2833, 1985.

125. **Mulder J, Hokfelt T, Knuepfer MM, and Kopp UC**. Renal sensory and sympathetic nerves reinnervate the kidney in a similar time-dependent fashion after renal denervation in rats. *American journal of physiology Regulatory, integrative and comparative physiology* 304: R675-682, 2013.

126. **Nagasu H, Satoh M, Kuwabara A, Yorimitsu D, Sakuta T, Tomita N, and Kashihara N**. Renal denervation reduces glomerular injury by suppressing NAD(P)H oxidase activity in Dahl salt-sensitive rats. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 25: 2889-2898, 2010.

127. **Neumann J, Ligtenberg G, Klein, II, Koomans HA, and Blankestijn PJ**. Sympathetic hyperactivity in chronic kidney disease: pathogenesis, clinical relevance, and treatment. *Kidney international* 65: 1568-1576, 2004.

128. **Niijima A**. Observation on the localization of mechanoreceptors in the kidney and afferent nerve fibres in the renal nerves in the rabbit. *The Journal of physiology* 245: 81-90, 1975.

129. **Nishida Y, Sugimoto I, Morita H, Murakami H, Hosomi H, and Bishop VS**. Suppression of renal sympathetic nerve activity during portal vein infusion of hypertonic saline. *The American journal of physiology* 274: R97-103, 1998.

130. **Norman RA, Jr., Murphy WR, Dzielak DJ, Khraibi AA, and Carroll RG**. Role of the renal nerves in one-kidney, one clip hypertension in rats. *Hypertension* 6: 622-626, 1984.

131. **Ong KL, Cheung BM, Man YB, Lau CP, and Lam KS**. Prevalence, awareness, treatment, and control of hypertension among United States adults 1999-2004. *Hypertension* 49: 69-75, 2007.

132. **Osborn JL, Roman RJ, and Ewens JD**. Renal nerves and the development of Dahl salt-sensitive hypertension. *Hypertension* 11: 523-528, 1988.

133. **Pan JY, Bishop VS, Ball NA, and Haywood JR**. Inability of dorsal spinal rhizotomy to prevent renal wrap hypertension in rats. *Hypertension* 7: 722-728, 1985.

134. **Parati G, and Esler M**. The human sympathetic nervous system: its relevance in hypertension and heart failure. *Eur Heart J* 33: 1058-1066, 2012.

135. **Patel KP, and Knuepfer MM**. Effect of afferent renal nerve stimulation on blood pressure, heart rate and noradrenergic activity in conscious rats. *Journal of the autonomic nervous system* 17: 121-130, 1986.

136. **Persell SD**. Prevalence of resistant hypertension in the United States, 2003-2008. *Hypertension* 57: 1076-1080, 2011.

137. **Pokushalov E, Romanov A, Corbucci G, Artyomenko S, Baranova V, Turov A, Shirokova N, Karaskov A, Mittal S, and Steinberg JS**. A randomized comparison of pulmonary vein isolation with versus without concomitant renal artery denervation in patients with refractory symptomatic atrial fibrillation and resistant hypertension. *J Am Coll Cardiol* 60: 1163-1170, 2012.

138. **Rapp JP**. Dahl salt-susceptible and salt-resistant rats. A review. *Hypertension* 4: 753-763, 1982.

139. **Rosas-Arellano MP, Solano-Flores LP, and Ciriello J**. c-Fos induction in spinal cord neurons after renal arterial or venous occlusion. *The American journal of physiology* 276: R120-127, 1999.

140. **Sapirstein LA, Sapirstein EH, and Bredemeyer A**. Effect of hemorrhage on the cardiac output and its distribution in the rat. *Circulation research* 8: 135-148, 1960.

141. **Schiffrin EL**. Inflammation, immunity and development of essential hypertension. *Journal of hypertension* 32: 228-229, 2014.

142. **Schlaich MP, Lambert E, Kaye DM, Krozowski Z, Campbell DJ, Lambert G, Hastings J, Aggarwal A, and Esler MD**. Sympathetic augmentation in hypertension: role of nerve firing, norepinephrine reuptake, and Angiotensin neuromodulation. *Hypertension* 43: 169-175, 2004.

143. **Schlaich MP, Sobotka PA, Krum H, Lambert E, and Esler MD**. Renal sympathetic-nerve ablation for uncontrolled hypertension. *The New England journal of medicine* 361: 932-934, 2009.

144. **Simon JK, Kasting NW, and Ciriello J**. Afferent renal nerve effects on plasma vasopressin and oxytocin in conscious rats. *The American journal of physiology* 256: R1240-1244, 1989.

145. **Smithwick RH, and Thompson JE**. Splanchnicectomy for essential hypertension; results in 1,266 cases. *Journal of the American Medical Association* 152: 1501-1504, 1953.

146. **Smits JF, and Brody MJ**. Activation of afferent renal nerves by intrarenal bradykinin in conscious rats. *The American journal of physiology* 247: R1003-1008, 1984.

147. **Solano-Flores LP, Rosas-Arellano MP, and Ciriello J**. Fos induction in central structures after afferent renal nerve stimulation. *Brain research* 753: 102-119, 1997.

148. **Sripairojthikoon W, and Wyss JM**. Cells of origin of the sympathetic renal innervation in rat. *The American journal of physiology* 252: F957-963, 1987.

149. **Steinberg JS, Pokushalov E, and Mittal S**. Renal denervation for arrhythmias: hope or hype? *Current cardiology reports* 15: 392, 2013.

150. **Stella A, Weaver L, Golin R, Genovesi S, and Zanchetti A**. Cardiovascular effects of afferent renal nerve stimulation. *Clinical and experimental hypertension Part A, Theory and practice* 9 Suppl 1: 97-111, 1987.

151. **Sterzel RB, Luft FC, Gao Y, Schnermann J, Briggs JP, Ganten D, Waldherr R, Schnabel E, and Kriz W**. Renal disease and the development of hypertension in salt-sensitive Dahl rats. *Kidney international* 33: 1119-1129, 1988.

152. **Symplicity HTNI**. Catheter-based renal sympathetic denervation for resistant hypertension: durability of blood pressure reduction out to 24 months. *Hypertension* 57: 911-917, 2011.

153. **Symplicity HTNI, Esler MD, Krum H, Sobotka PA, Schlaich MP, Schmieder RE, and Bohm M**. Renal sympathetic denervation in patients with treatment-resistant hypertension (The Symplicity HTN-2 Trial): a randomised controlled trial. *Lancet* 376: 1903-1909, 2010.

154. **Szallasi A, and Blumberg PM**. Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacological reviews* 51: 159-212, 1999.

155. **Takeshita A, Mark AL, and Brody MJ**. Prevention of salt-induced hypertension in the Dahl strain by 6-hydroxydopamine. *The American journal of physiology* 236: H48-52, 1979.

156. **Tian N, Moore RS, Phillips WE, Lin L, Braddy S, Pryor JS, Stockstill RL, Hughson MD, and Manning RD, Jr.** NADPH oxidase contributes to renal damage and dysfunction in Dahl salt-sensitive hypertension. *American journal of physiology Regulatory, integrative and comparative physiology* 295: R1858-1865, 2008.

157. **Tian N, Thrasher KD, Gundy PD, Hughson MD, and Manning RD, Jr.** Antioxidant treatment prevents renal damage and dysfunction and reduces arterial pressure in salt-sensitive hypertension. *Hypertension* 45: 934-939, 2005.

158. **Ukena C, Bauer A, Mahfoud F, Schreieck J, Neuberger HR, Eick C, Sobotka PA, Gawaz M, and Bohm M**. Renal sympathetic denervation for treatment of electrical storm: first-in-man experience. *Clinical research in cardiology : official journal of the German Cardiac Society* 101: 63-67, 2012.

159. **Ulrich-Lai YM, Arnhold MM, and Engeland WC**. Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH. *American journal of physiology Regulatory, integrative and comparative physiology* 290: R1128-1135, 2006.

160. **Ulrich-Lai YM, Fraticelli AI, and Engeland WC**. Capsaicin-sensitive nerve fibers: a potential extra-ACTH mechanism participating in adrenal regeneration in rats. *Microscopy research and technique* 61: 252-258, 2003.

161. **Ulrich-Lai YM, Marek DJ, and Engeland WC**. Capsaicin-sensitive adrenal sensory fibers participate in compensatory adrenal growth in rats. *American journal of physiology Regulatory, integrative and comparative physiology* 283: R877-884, 2002.

162. **Veitenheimer B, and Osborn JW**. Role of spinal V1a receptors in regulation of arterial pressure during acute and chronic osmotic stress. *American journal of physiology Regulatory, integrative and comparative physiology* 300: R460-469, 2011.

163. **Vieira AA, Nahey DB, and Collister JP**. Role of the organum vasculosum of the lamina terminalis for the chronic cardiovascular effects produced by endogenous and exogenous ANG II in conscious rats. *American journal of physiology Regulatory, integrative and comparative physiology* 299: R1564-1571, 2010.

164. **Wang DH, Li J, and Qiu J**. Salt-sensitive hypertension induced by sensory denervation: introduction of a new model. *Hypertension* 32: 649-653, 1998.

165. **Wang DH, Wu W, and Lookingland KJ**. Degeneration of capsaicin-sensitive sensory nerves leads to increased salt sensitivity through enhancement of sympathoexcitatory response. *Hypertension* 37: 440-443, 2001.

166. **Wang H, Wang DH, and Galligan JJ**. P2Y2 receptors mediate ATP-induced resensitization of TRPV1 expressed by kidney projecting sensory neurons. *American journal of physiology Regulatory, integrative and comparative physiology* 298: R1634-1641, 2010.

167. **Wang Q, Fan XP, Chen Z, Zhao QH, Chen SQ, and Wan ZH**. [Role of afferent renal nerves in 2K2C Goldblatt hypertension]. *Sheng li xue bao : [Acta physiologica Sinica]* 47: 366-372, 1995.

168. **Wang Y, Chen AF, and Wang DH**. ET(A) receptor blockade prevents renal dysfunction in salt-sensitive hypertension induced by sensory denervation. *American journal of physiology Heart and circulatory physiology* 289: H2005-2011, 2005.

169. **Weaver LC**. Cardiopulmonary sympathetic afferent influences on renal nerve activity. *The American journal of physiology* 233: H592-599, 1977.

170. **Weiss ML, and Chowdhury SI**. The renal afferent pathways in the rat: a pseudorabies virus study. *Brain research* 812: 227-241, 1998.

171. **Witkowski A, Prejbisz A, Florczak E, Kadziela J, Sliwinski P, Bielen P, Michalowska I, Kabat M, Warchol E, Januszewicz M, Narkiewicz K, Somers VK, Sobotka PA, and Januszewicz A**. Effects of renal sympathetic denervation on blood pressure, sleep apnea course, and glycemic control in patients with resistant hypertension and sleep apnea. *Hypertension* 58: 559-565, 2011.

172. **Wu YM, and He RR**. Biphasic activation of renal afferent by intrarenal artery injection of bradykinin in anesthetized rabbits. *Sheng li xue bao : [Acta physiologica Sinica]* 51: 651-659, 1999.

173. **Wyss JM, Aboukarsh N, and Oparil S**. Sensory denervation of the kidney attenuates renovascular hypertension in the rat. *The American journal of physiology* 250: H82-86, 1986.

174. **Wyss JM, and Donovan MK**. A direct projection from the kidney to the brainstem. *Brain research* 298: 130-134, 1984.

175. **Wyss JM, Oparil S, and Sripairojthikoon W**. Neuronal control of the kidney: contribution to hypertension. *Canadian journal of physiology and pharmacology* 70: 759-770, 1992.

176. **Wyss JM, Sripairojthikoon W, and Oparil S**. Failure of renal denervation to attenuate hypertension in Dahl NaCl-sensitive rats. *Canadian journal of physiology and pharmacology* 65: 2428-2432, 1987.

177. **Yasuda Y, Honda K, Negoro H, Higuchi T, Goto Y, and Fukuda S**. The contribution of the median preoptic nucleus to renal sympathetic nerve activity increased by intracerebroventricular injection of hypertonic saline in the rat. *Brain research* 867: 107-114, 2000.

178. **Yoshimoto M, Miki K, Fink GD, King A, and Osborn JW**. Chronic angiotensin II infusion causes differential responses in regional sympathetic nerve activity in rats. *Hypertension* 55: 644-651, 2010.

179. **Yoshimoto M, Sakagami T, Nagura S, and Miki K**. Relationship between renal sympathetic nerve activity and renal blood flow during natural behavior in rats. *American journal of physiology Regulatory, integrative and comparative physiology* 286: R881-887, 2004.

180. **Zanchetti A, Stella A, Golin R, and Genovesi S**. Neural control of the kidney--are there reno-renal reflexes? *Clinical and experimental hypertension Part A, Theory and practice* 6: 275-286, 1984.

181. **Zhang W, and Victor RG**. Calcineurin inhibitors cause renal afferent activation in rats: a novel mechanism of cyclosporine-induced hypertension. *American journal of hypertension* 13: 999-1004, 2000.

182. **Zhu Y, Xie C, and Wang DH**. TRPV1-mediated diuresis and natriuresis induced by hypertonic saline perfusion of the renal pelvis. *American journal of nephrology* 27: 530-537, 2007.