Roles and mechanisms of the kidney sodium-chloride cotransporter (NCC) in salt-sensitive hypertension

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Boston University
ROLES AND MECHANISMS OF THE KIDNEY SODIUM-CHLORIDE COTRANSPORTER (NCC) IN SALT-SENSITIVE HYPERTENSION

by

AKSHAY NILESH DESAI

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ACKNOWLEDGMENTS

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ABSTRACT

Hypertension is both a domestic and international health issue – diagnosed in 1 in 3 U.S. adults and classified by the World Health Organization as the number one risk factor for mortality worldwide. It has been established that salt plays a role in the development of hypertension, and that a salt-sensitive phenotype indicates heightened sensitivity to salt consumption. Here, we studied the roles of the afferent renal nerves, which travel from the kidney to the central nervous system, and the sodium-chloride cotransporter in fluid and electrolyte homeostasis and blood pressure regulation.

Our laboratory utilized a novel technique of afferent renal nerve ablation on Sprague-Dawley rats to examine the effects of afferent renal nerve mechanoreceptors and chemoreceptors in response to acute sympathoinhibitory challenges. Additionally, salt-sensitive and salt-resistant rats were randomly subjected to chronic normal salt (0.6% NaCl) or high salt (8% NaCl) diets, and examined for levels of norepinephrine and substance-P release. A different group of salt-resistant and salt-sensitive rats were subcutaneously infused with terazosin, a selective α-1 adrenoreceptor antagonist, or propranolol, a selective β-adrenoreceptor antagonist, and then randomly subjected to normal salt (0.6% NaCl) or high salt (4% NaCl) diets for 21 days. We subsequently examined these rats, and analyzed the effects of high salt intake on blood pressure,
sodium-chloride cotransporter activity, and expression of the sodium-chloride cotransporter and its relevant kinases.

In response to an acute mechanoreceptor-specific stimulus, Sprague-Dawley rats that underwent afferent renal nerve ablation were unable to modulate blood pressure or natriuresis after regaining consciousness. Chronic high salt (8% NaCl) consumption in salt-sensitive rats resulted in increased levels of plasma norepinephrine, renal norepinephrine, and norepinephrine-evoked Substance-P release. In addition, salt-sensitive rats subjected to a 21-day high salt (4% NaCl) diet exhibited increased blood pressure, elevated sodium-chloride cotransporter activity, and upregulated levels of the sodium-chloride cotransporter and the kinases that regulate it. However, these observed increases in blood pressure, protein activity, and protein expression were abolished in salt-sensitive rats experiencing α-1 adrenoreceptor antagonism due to terazosin administration.

In conclusion, our findings indicate that mechanoreceptor-driven afferent renal nerve activation is needed to maintain fluid and electrolyte homeostasis and regulate blood pressure in response to acute sympathoinhibitory challenges and chronic high salt intake. In addition, our data demonstrates that the sodium-chloride cotransporter is aberrantly upregulated in salt-sensitive rats through a norepinephrine-α1-adrenoreceptor gated pathway, and this upregulation results in excessive salt reabsorption. Thus, our experiments have generated new data that reveals selective α1-adrenoreceptor antagonism and renal denervation as potential treatment options for hypertensive individuals.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ARN</td>
<td>Afferent Renal Nerve</td>
</tr>
<tr>
<td>BN</td>
<td>Brown Norway</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DCT</td>
<td>Distal Convoluted Tubule</td>
</tr>
<tr>
<td>DSR</td>
<td>Dahl Salt-Resistant</td>
</tr>
<tr>
<td>DSS</td>
<td>Dahl Salt-Sensitive</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial Sodium Channel</td>
</tr>
<tr>
<td>HCTZ</td>
<td>Hydrochlorothiazide</td>
</tr>
<tr>
<td>HS</td>
<td>High Salt</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>im</td>
<td>intramuscularly</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean Arterial Pressure</td>
</tr>
<tr>
<td>MTZ</td>
<td>Metolazone</td>
</tr>
<tr>
<td>NCC</td>
<td>Sodium-Chloride Cotransporter</td>
</tr>
<tr>
<td>NCCs</td>
<td>Sodium-Chloride Cotransporters</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NS</td>
<td>Normal Salt</td>
</tr>
<tr>
<td>OxSR1</td>
<td>Oxidative Stress Response Kinase 1</td>
</tr>
<tr>
<td>pNCC</td>
<td>Phosphorylated Sodium-Chloride Cotransporter</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular Nucleus (in Hypothalamus)</td>
</tr>
</tbody>
</table>
RAAS ......................................................... Renin-Angiotensin-Aldosterone-System
Renal-CAP ................................................. Afferent Renal Nerve Ablation by Exposure to Capsaicin
RPP .................................................................... Renal Pelvic Pressure
RDNX .................................................................. Renal Denervation
RSNA .............................................................. Renal Sympathetic Nervous Activity
RVLM .............................................................. Rostral Ventral Lateral Medulla (in Brainstem)
sc ........................................................................ subcutaneous
SD ...................................................................... Sprague-Dawley
SHR ...................................................................... Spontaneously Hypertensive
SNSA .............................................................. Sympathetic Nervous System Activity
SPAK .............................................................. STE20/SPS1-related Proline/Alanine-rich Kinase
SS-13-BN .......................................................... Salt-Sensitive Brown Norway
UNaV .............................................................. Urinary Sodium Excretion/Natriuresis
ΔUNaV .............................................................. Peak Natriuretic Response
V ........................................................................ Urine Output
WHO ..................................................................... World Health Organization
WKY ...................................................................... Wistar-Kyoto
WNK ...................................................................... With-No-Lysine Kinase (1 or 4)
INTRODUCTION

History and Overview of Hypertension

According to the World Health Organization (WHO), hypertension is the #1 risk factor for mortality worldwide\textsuperscript{1,2}. It is common among Americans, affecting one in three adults in the United States\textsuperscript{1,2}. Hypertension is also the #1 risk factor for disability-adjusted life years, and is expected to affect 41\% of all Americans by 2030\textsuperscript{2–10}.

Furthermore, hypertension has been implicated as a major risk factor for many cardiovascular and renal diseases\textsuperscript{11}. Stages of hypertension are classified by blood pressure. Table 1 indicates these classifications of blood pressure, as was established during The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure\textsuperscript{12}.

Table 1: JNC 7 Blood Pressure Classification. (Taken from a the JNC 7\textsuperscript{12})

<table>
<thead>
<tr>
<th>Blood Pressure</th>
<th>Systolic Blood Pressure</th>
<th>Diastolic Blood Pressure</th>
</tr>
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<tbody>
<tr>
<td>Classification</td>
<td>mmHg</td>
<td>mmHg</td>
</tr>
<tr>
<td>Normal</td>
<td>&lt;120</td>
<td>and &lt;80</td>
</tr>
<tr>
<td>Prehypertension</td>
<td>120-139</td>
<td>or 80-89</td>
</tr>
<tr>
<td>Hypertension Stage 1</td>
<td>140-159</td>
<td>or 90-99</td>
</tr>
<tr>
<td>Hypertension Stage 2</td>
<td>$\geq$ 160</td>
<td>or $\geq$ 100</td>
</tr>
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Though the classification of hypertension is relatively straightforward, its pathogenesis is more elaborate. Hypertension involves the incorporation of several important regulatory systems, including the central nervous system (CNS). The CNS has been identified by multiple researchers as having a significant role in the development of hypertension. In fact, it has been proposed that 50% of clinical hypertension cases are designated as neurogenic essential hypertension.

One key component of the CNS is the hypothalamus, which serves as a mediator and implementer of signal transduction in response to different stimuli. Therefore, changes in the functioning capabilities of the hypothalamus can lead to changes in sympathetic nervous system activity which consequently affect other body functions. For example, parvocellular neurons in the paraventricular nucleus (PVN) influence sympathetic nervous activity (SNA) to mediate neural components of cardiovascular reflexes. Figure 1, taken from a 2013 publication in Hypertension, shows the sympathetic nervous system and the brain sites that interact with it.

Figure 1 – Sympathetic Nervous System Interactions with Specific Brain Sites. (From Hirooka et al. 18.)
History and Implications of Salt-Sensitive Hypertension

Hypertension’s role as a risk factor in numerous cardiovascular and renal diseases makes it important to understand. One factor relevant to hypertension is salt, which is consumed in increasing quantities in the modern American diet. The kidney, which consists of many protein transporters that regulate sodium reabsorption, plays a significant role in sodium excretion. In fact, a hallmark of urinary tract obstruction is the failure of the kidney’s ability to regulate urinary excretion of water and sodium. A long-term reduction in sodium intake has been clearly established to lower blood pressure and thus reduce the potential for cardiovascular risk. Furthermore, several studies have shown that a reduction in salt intake can reduce blood pressure in both hypertensive and normotensive patients.

There is great variability, however, in how different individuals respond to different quantities of salt intake. A small increase in sodium intake may lead to drastic increases in blood pressure for certain individuals, while others may experience little change despite consuming a significantly greater amount of sodium in their diet.

A formal definition of salt-sensitivity was given in 1996 by Dr. Myron Weinberger, who concluded that individuals are salt-sensitive if they exhibit a 10% increase in mean arterial pressure in response to a high salt (HS) vs low salt challenge. Approximately 50% of all hypertensive individuals are salt-sensitive, while 26% of normotensive individuals are considered salt-sensitive. Specific genetic polymorphisms have been identified that can predispose an individual to becoming salt-sensitivity. Specifically, genes that increase or decrease expression of proteins involved...
in renal sodium transport are of interest. Gitelman’s Syndrome, for example, results from mutations in the sodium-chloride cotransporter (NCC) located at the distal convoluted tubule of the nephron (DCT)\textsuperscript{34–37}. Individuals afflicted with this syndrome experience hypokalemia, hypomagnesemia, and metabolic alkalosis\textsuperscript{34–37}.

Figure 2, taken from a recent publication in *Adv Exp Med Biol*, illustrates findings from these many studies that demonstrate the effect of salt reduction on blood pressure in both hypertensive (A) and normotensive (B) patients.

**Figure 2 – Effect of Salt Reduction on Blood Pressure in Hypertensive (A) and Normotensive Patients (B). (Taken from Rust et al.\textsuperscript{11})**

A

<table>
<thead>
<tr>
<th>Reference</th>
<th>Extent of salt reduction</th>
<th>Reduction of blood pressure [mm Hg]</th>
</tr>
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<tr>
<td>Midgley et al., 1996</td>
<td>-5.9 g NaCl/d</td>
<td>-3.8</td>
</tr>
<tr>
<td>Cotter et al., 1997</td>
<td>-5.8 g NaCl/d</td>
<td>-4.8</td>
</tr>
<tr>
<td>Graudal et al., 1998</td>
<td>-6.9 g NaCl/d</td>
<td>-3.9</td>
</tr>
<tr>
<td>He &amp; MacGregor, 2002</td>
<td>-4.6 g NaCl/d</td>
<td>-1.9</td>
</tr>
<tr>
<td>Aburto et al., 2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>He et al., 2013</td>
<td>no average value indicated</td>
<td>-2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-2.8</td>
</tr>
</tbody>
</table>

\( \square \) RR systolic \( \square \) RR diastolic

B

<table>
<thead>
<tr>
<th>Reference</th>
<th>Extent of salt reduction</th>
<th>Reduction of blood pressure [mm Hg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midgley et al., 1996</td>
<td>-7.4 g NaCl/d</td>
<td>-1.6</td>
</tr>
<tr>
<td>Cotter et al., 1997</td>
<td>-4.4 g NaCl/d</td>
<td>-1.9</td>
</tr>
<tr>
<td>Graudal et al., 1998</td>
<td>-9.3 g NaCl/d</td>
<td>-1.2</td>
</tr>
<tr>
<td>He &amp; MacGregor, 2002</td>
<td>-4.3 g NaCl/d</td>
<td>-1.0</td>
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<td>no average value indicated</td>
<td>-1.4</td>
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<tr>
<td>He et al., 2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1.0</td>
</tr>
</tbody>
</table>

\( \square \) RR systolic \( \square \) RR diastolic
Significance of the Afferent Renal Nerves in Salt Sensitive Hypertension

The hypothalamus is not only integral for controlling blood pressure, but also for regulating fluid and electrolyte homeostasis. It is known that renal nerves conduct sympathetic nervous activity (RSNA), and play a crucial role in the regulation of the renin-angiotensin-aldosterone system (RAAS)\textsuperscript{18}. Once considered to be of little consequence by prominent physiologist Homer Smith, renal nerves have since been demonstrated to cause changes in many physiological parameters including arterial pressure, glomerular filtration rate, and renal blood flow\textsuperscript{38}. These changes affect urinary sodium excretion (UNaV), also known as natriuresis, and demonstrate the impact that RSNA has on electrolyte homeostasis\textsuperscript{38}. The importance of the renal nerves is further underscored by the fact that renal denervation leads to decreases in renal tubular sodium reabsorption and RAAS inhibition, resulting in overall decreases in blood pressure\textsuperscript{18}.

The afferent renal nerve (ARN) is also important in regulating RSNA. Originating in the kidney and projecting to nuclei such as the RVLM and PVN in the brainstem and hypothalamus\textsuperscript{39–41} respectively, the ARN functions as a negative feedback circuit\textsuperscript{42}. The ARN conducts an inhibitory reno-renal reflex, which is activated by efferent (from brain to kidney) RSNA and/or chemical or mechanical stimuli\textsuperscript{42–44}. This inhibitory reno-renal reflex suppresses both sodium reabsorption and efferent RSNA\textsuperscript{42,44–49}. The ARN inhibitory reno-renal reflex requires the release of several important molecular compounds including prostaglandin E2, calcitonin g-related peptide, and substance P\textsuperscript{48–51}. This inhibitory reno-renal reflex is conducted by renal $\alpha$-2 adrenoreceptors and both endothelin A and endothelin B (ETAR and ETBR) receptors\textsuperscript{48–51}.
Animal models demonstrate the importance of the ARN. Spontaneously hypertensive ( SHR) rats model neurogenic hypertension, and have been shown to exhibit decreased $\alpha$-2-dependent adrenoreceptor activity$^{48-51}$. Contrastingly, salt-resistant Sprague-Dawley (SD) rats exhibit increased ETBR dependent ARN activity during HS diets$^{48-51}$. In fact, removal of all afferent renal inputs leads to salt-sensitive hypertension in normal SD rats$^{52}$. Removal of ARN inputs via selective ARN ablation, however, remain of interest and are a focal point of current renal research.

**Significance of the Sodium-Chloride (NCC) Cotransporter**

Renal sodium reabsorption is carried out by protein transporters, one of which is the sodium-chloride cotransporter or NCC. The NCC belongs to a family of solute carriers in which electroneutrality is established through the simultaneous transport of both a cation and an anion in a 1:1 stoichiometric relationship$^{53}$. The NCC is integral to the fine-tuning of sodium reabsorption that occurs after tubuloglomerular feedback, and it is responsible for the reabsorption of 5-10% of filtered sodium and potassium$^{54,55}$. The rate at which the NCC transports sodium and chloride is generated and maintained by the sodium-potassium ATPase located at the basolateral membrane of renal tubular epithelial cells$^{56}$.

The NCC is sensitive to thiazides, a group of molecules that prevent sodium reabsorption by inhibiting sodium-chloride cotransporters (NCCs)$^{53,56}$. Thiazides, such as Metolazone (MTZ) and Hydrochlorothiazide (HCTZ), prevent chloride reabsorption by NCCs located in the DCT$^{56-58}$. There is significant evidence regarding the effectiveness of thiazides and thiazide diuretics in treating hypertension$^{59-63}$, and they are widely
considered the first line of therapy for hypertension treatment\textsuperscript{64,65}. By inhibiting chloride reabsorption in the DCT, thiazide diuretics hamper the ability of NCCs to transport sodium and chloride ions simultaneously. Therefore, administration of thiazides causes attenuated sodium reabsorption. The NCC’s mechanism of action is illustrated by Figure 3, taken from Dr. Gerardo Gamba’s 2009 publication in \textit{Am J Renal Physiol}\textsuperscript{56}.

\textbf{Figure 3 – Salt Reabsorption in the Early Part of the Distal Tubule of Nephron. DCT 1 – Distal Convoluted Tubule 1, DCT 2 – Distal Convoluted Tubule 2, NCC – Sodium-Chloride Cotransporter, ENaC – Epithelial Sodium Channel, CNT – Connecting Tubule. (From Gamba et al.\textsuperscript{56})}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\end{figure}

It had been determined in the late 1980s that sodium and chloride transport occurred to a greater extent in the early distal tubule rather than the later distal tubule\textsuperscript{66}. This increased transport is due to an increased density of solute transporters located in the early portion of the DCT\textsuperscript{56}. This first portion of the DCT, designated as DCT 1,
exclusively populated by NCC transporters, while the second portion, termed DCT 2, consists of both NCC and epithelial sodium channels (ENaC)\(^6\). Interestingly, the NCC directly regulates luminal potassium secretion and magnesium secretion, but inversely regulates calcium reabsorption\(^5\). Figures 4 and 5, taken from publications by Dr. Erika Moreno, illustrate the primary and secondary structure of the NCC cotransporter\(^5,6\).

Figure 4 - NCC Secondary Structure and Molecular Interactions. Na – Sodium ion, Cl – Chloride ion, MTZ – Metolazone (Thiazide diuretic). (From Moreno et al.\(^5\))

![NCC Secondary Structure and Molecular Interactions](image1)

Figure 5 – NCC Secondary Structure and Primary Structures in Human, Rat, Mouse, and Flounder NCC. (From Moreno et al.\(^6\))

![NCC Secondary Structure and Primary Structures](image2)
The NCC and Salt-Sensitive Hypertension

The relationship between salt-sensitive hypertension, the NCC, and excess RSNA has been of great interest. In 2011, a group of researchers analyzed the effects of salt intake on a protein known as With-No-Lysine kinase 4 (WNK4)\textsuperscript{68}. WNK proteins have been identified as having significant roles in salt reabsorption\textsuperscript{69}. These researchers, led by Dr. ShengYu Mu, determined that salt loading results in \( \beta \)-adrenoreceptor mediated downregulation of WNK4, NCC activation in the DCT, and consequently increased salt retention and blood pressure\textsuperscript{68}. A study conducted in 2014 delved deeper into NCC activation, analyzing the effect of norepinephrine (NE) on NCC activation and phosphorylation\textsuperscript{70}. NE is an endogenous adrenergic compound that can increase blood pressure\textsuperscript{71}. It has been established that NE mediates some of its effects by acting on adrenoreceptors located on renal tubular epithelial cells throughout the nephron\textsuperscript{38,70,72}.

Using mice as a model, researchers analyzed the effects of a chronic infusion of NE. Their results suggested that NE may bind to both \( \alpha \)-1 and \( \beta \) adrenoreceptors in the aforementioned DCT 1 region, and that this binding affects WNK4 through activation of the protein kinase OxSR1 (Oxidative Stress Response Kinase 1)\textsuperscript{70}. OxSR1 phosphorylates NCC cotransporters, which stimulates them and increases their abundance at the apical membrane of tubular cells\textsuperscript{70}. A phosphorylated NCC (pNCC) is active, and reabsorbs sodium and chloride\textsuperscript{70}. Thus, substantial phosphorylation of NCC transporters causes increased sodium retention and, therefore, increased blood pressure\textsuperscript{70}.
Figure 6, taken from Terker et al.’s 2014 publication in *Hypertension*, illustrates the hypertensive effects of chronic NE infusion, which include increases in both NCC and pNCC levels in DCT tubular cells.70

**Figure 6 – Effects of Chronic Norepinephrine Infusion on Blood Pressure, NCC Abundance, and pNCC Abundance.** A – Control vs NE chronic infusion effect on BP, B – NCC and actin levels in response to chronic NE infusion, C – pNCC-T53, WNK4, and actin levels in response to chronic NE infusion. (Reproduced from Terker et al.)

Through ARN ablation by exposure to capsaicin (Renal-CAP), our laboratory aims to demonstrate the ARN’s significant role in maintaining fluid and electrolyte homeostasis. Given the ARN’s role as a modulator of efferent RSNA, deficiencies in its ability to regulate sympathetic outflow can lead to increased sodium reabsorption, via the NCC, and increased risk of hypertension. Findings should indicate that the ARN’s mechanosensitive reno-renal reflex influences inhibition of excess RSNA. Further analyses will elucidate the mechanisms of the NCC, and detail more information concerning the signal transduction pathways it needs to function properly. Our data will
enrich pre-existing cardiovascular-renal research and highlight alternative therapeutic targets in sympathetically driven hypertension.
SPECIFIC AIMS

1. To establish that mechanoreceptor-dependent afferent renal nerve activation facilitates fluid and electrolyte homeostasis and blood pressure regulation.

2. To establish that NE regulates NCC activity, via an \( \alpha-1 \) adrenoreceptor gated WNK-OxSR1 signal transduction pathway, to mediate sodium homeostasis and long-term blood pressure regulation.
METHODS

Animals

Sprague–Dawley (SD), Dahl Salt-Sensitive (DSS), Dahl Salt-Resistant (DSR), Wistar-Kyoto (WKY), SHR (Envigo, Indianapolis, IN, USA), and Salt-Sensitive Brown Norway rats (SS-13-BN) (Charles River Laboratories, Wilmington, MA, USA) weighing 275–300 g were individually housed in a temperature (range: 20–26 °C) and humidity-controlled (range: 30–70%) environment under a 12-hour light/dark cycle and were randomly assigned to experimental treatment groups. Rats were allowed tap water and standard rodent diet (Test Diet, St. Louis, MO, USA) ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee in accordance with the guidelines of Boston University School of Medicine and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Generation of Salt-Sensitive Brown Norway (SS-13-BN) Rats

The SS-13-BN rat line was derived at the Medical College of Wisconsin by using inbred normotensive Brown Norway (BN) rats and DSS rats. Residual heterozygosity and genetic contamination were eliminated by using a set of 182 microsatellite markers (Research Genetics) for genotyping that provided even coverage of the 21 chromosomes (10-cM intervals). The progenitor rats used for the present study were homozygous for all regions tested, and each of these parental strains underwent a periodic total genome scan to ensure allelic homogeneity.

The generation of a panel of reciprocal SS-13-BN rats with the use of DSS and BN rats was initiated by using a single F1 male, originating from a male DSS rat and a female
BN rat, backcrossed to 2 or 3 DSS and BN females. For subsequent backcrosses, each selected male breeder was backcrossed to 3 to 6 parental females. DNA was extracted from tail tips of males and genotyped with markers spaced every 10 cM for the introgressed chromosome. Rats not heterozygous for the entire chromosome were rejected. Remaining male progeny were genotyped by using a subset of genetic markers that characterized the mixed chromosomes from the previous generation to select the best next breeders.

This process was continued until there were no longer mixed chromosomes in the (recipient) genetic background. The rats carrying full-length heterozygous target chromosomes were crossed to fix the donor chromosome, and a total genome scan was performed to verify that the line was isogenic. The SS^{13}-BN line was then maintained by brother-sister matings.

**Surgical Procedures**

**ARN Ablation by Exposure to Capsaicin (Renal-CAP)**

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 dissected away from the vessels to expose the renal nerves. A 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 para-film was placed under the renal artery and vein before placement of the capsaicin-soaked gauze to prevent any nonrenal capsaicin exposure. After 15 minutes
of capsaicin exposure, the gauze and para-film were removed. The area was immediately dried, and the procedure was repeated on the contralateral side if necessary. 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 was performed by externalizing the viscera, visualizing the renal artery and vein, replacing the organs, and then closing the wound.

**Acute Femoral Artery, Vein, and Bladder Cannulation**

On day of study, all animals were anesthetized with sodium methohexital [20 mg/kg intraperitoneally (ip)] and supplemented with 10 mg/kg intravenously as required. Once anesthetized, rats were instrumented with catheters in the left femoral artery, left femoral vein, and bladder for the measurement of arterial blood pressure, intravenous (IV) administration of saline and/or drugs, and renal function, respectively. Rats were subsequently placed in a Plexiglas holder, and an IV infusion of isotonic saline (20 μl/min) was maintained for a 2-hour recovery period before experimentation to allow the animal to regain full consciousness and to stabilize cardiovascular, renal, and excretory functions.

Mean arterial pressure (MAP) and heart rate (HR) were recorded through the implanted femoral artery cannula using BIOPAC data acquisition software (MP150 and AcqKnowledge 3.8.2; BIOPAC Systems, Goleta, CA) connected to an external pressure transducer (P23XL; Viggo Spectramed, Oxnard, CA).
Subcutaneous Osmotic Minipump Implantation

Animals were anesthetized (sodium methohexital, 20 mg/kg ip) and surgically instrumented with an osmotic minipump (Alzet, osmotic pump model 2ML2, Palo Alto, CA) that was placed subcutaneously in the subscapular region. After subcutaneous (sc) surgical placement, all animals were returned to their home cage following administration of penicillin (300,000 units/ml, 0.3 ml intramuscularly [im]).

Experimental Treatment Groups

Naïve Animals

Naïve animals were randomly assigned to receive a NS (0.6% NaCl) or HS (4% or 8% NaCl) diet for a 21-day experimental period. WKY and SHR rats were kept on NS diet.

Isotonic Saline Vehicle Infusion

Animals underwent implantation of an osmotic minipump delivering a sc infusion of isotonic saline (flow rate 5 μl/h) prior to random assignment to either a NS or HS diet for a 21-day experimental period.

Norepinephrine Infusion

Animals underwent implantation of an osmotic minipump delivering a sc infusion of NE (Sigma, St. Louis, MO; cat. no. A7256) dissolved in isotonic saline (NE; 600 ng/min, flow rate 5 μl/h^24) prior to random assignment to either a NS or HS diet for a 21-day experimental period.

Terazosin Infusion

Animals underwent implantation of an osmotic minipump delivering a sc infusion of the α-1 adrenoreceptor antagonist terazosin (10mg/kg/day) dissolved in isotonic saline
prior to random assignment to either a NS or HS diet for a 21-day experimental period. Note in Figure 17, terazosin was administered from days 22-42 of the 42-day experimental period.

Propranolol Infusion

Animals underwent implantation of an osmotic minipump delivering a sc infusion of the β-adrenoreceptor antagonist propranolol (10mg/kg/day) dissolved in isotonic saline prior to random assignment to either a NS or HS diet for a 21-day experimental period.

Acute Experimental Protocols

The following cardiovascular, intravenous, and renal sodium transporter activity were performed consecutively in a single experiment in each animal following 21 days of NS or HS intake.

Cardiovascular Function

Following a 2-hour surgical recovery period, baseline MAP was recorded continuously over a 30-minute period in conscious rats via the surgically implanted femoral artery cannula.

Intravenous (iv) 5% Body Weight Volume Expansion

For a 30-minute period, rats underwent a 5% body weight (BW) volume expansion (VE) calculated [.05 x BW] IV isotonic saline administration. A 90-minute recovery period followed the VE administration. CV and renal parameters were monitored throughout.
2-Hour Intravenous (iv) 1M NaCl Infusion

Following a 60-minute control period of IV saline, 1M NaCl was administered for 2 hours (flow rate: 20 μl/min). CV and renal parameters were continuously monitored, and a 2-hour recovery period followed.

Renal Sodium Transporter Activity

After cardiovascular function protocol concluded, renal sodium transporter activity was assessed. All animals received an IV infusion of isotonic saline (flow rate 20 μl/min) for 1-hour, followed by an IV bolus (2 mg/kg) of amiloride, which inhibits ENaC75-77, preceding a 1-hour IV infusion of amiloride (2 mg/kg, flow rate 20 l/min), and an IV bolus (2 mg/kg) of HCTZ, administered to inhibit NCC activity53,56, preceding a 1 hour IV infusion of amiloride (2 mg/kg, flow rate 20 μl/min78) in combination with HCTZ (2 mg/kg, flow rate 20 μl/min78).

During the 3-hour protocol, HR and MAP were recorded and urine was collected in 10-minute intervals to assess peak natriuresis to IV amiloride or HCTZ (n = 6/group). The peak natriuretic response ($\Delta$UNaV; μeq/min) was determined by subtracting the baseline UNaV value from the maximum natriuretic value observed during each hour of drug administration. Baseline UNaV values were determined by averaging the UNaV values from the last two 10-minute time points during the previous hours of the study [hour 1: IV saline (40–50 min, 50–60 min) or hour 2: IV amiloride (100–110 min, 110–120 min)]. The maximum natriuretic response to amiloride occurred during the 10–20 min time point after drug infusion. The maximum natriuretic response to amilorideHCTZ occurred during the 20–30 min time point following drug administration.
**Analytical Techniques**

Urine volume was determined gravimetrically, assuming 1 g = 1 ml. Urinary and plasma sodium concentrations were measured using a flame photometer (model 943; Instrumentation Laboratories, Bedford, MA;).

**Plasma Norepinephrine (NE) Measurement**

Naïve SD rats were placed on a NS or HS diet for the 21-day experimental protocol. After the protocol was completed, animals were decapitated while conscious and whole blood was collected. Plasma NE content was determined via ELISA (Immuno-Biological Labs America, Minneapolis, MN; cat. no. IB89552).

**CNS Tissue Collection**

At baseline and following completion of the IV VE or IV 2 hour 1M NaCl protocol (including recovery), rats were anaesthetized with sodium methohexital (10 mg kg iv) and immediately perfused transcardially with 0.2–0.3 L of 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS (4 °C, 0.3–0.5 L). The brains were removed and placed in a vial containing 4% PFA in PBS overnight, and then switched to a 30% w/v sucrose solution for 2 days. The PVN was sectioned into three separate sets of serial 40-μm coronal sections that were collected into a cryoprotectant, consisting of 30% sucrose + 30% ethylene glycol + 1% polyvinylpyrrolidone in 0.1 M PBS. These three sets were stored at 20 °C until they were processed for immunohistochemistry.
**Fos\(^+\) Immunoreactivity**

Free-floating sections from each brain were processed for Fos immunohistochemistry using a rabbit polyclonal anti-Fos antibody (Calbiochem, San Diego, CA, USA) as previously described\(^79\). Sections were brought to room temperature and rinsed twice for 30 min in 0.1 M PBS to remove cryoprotectant. Sections were incubated in 0.3% hydrogen peroxide in distilled water for 30 min at room temperature and then rinsed for 30 min in 0.1 M PBS. Sections were then incubated for 2 hours at room temperature in PBS diluent (3% normal horse serum in 0.1 M PBS containing 0.25% Triton-100; Sigma-Aldrich). The rabbit polyclonal anti-Fos antibody was diluted to 1 : 30 000 in PBS diluent, and the sections were incubated in the primary antibody for 48 h at 4 °C.

After two 30-minute rinses in 0.1 M PBS, sections were incubated in a biotinylated horse anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted to 1 : 200 in PBS diluent for 2 h at room temperature. The tissue was reacted with an avidin–peroxidase conjugate (ABC-Vectastain kit; Vector Laboratories) and PBS containing 0.04% 3,3-diaminobenzidine hydrochloride and 0.04% nickel ammonium sulphate (Sigma-Aldrich). Sections were mounted on gel-coated slides, processed through a series of dehydrating alcohols followed by xylenes, and placed under a coverslip with Permount mounting medium.

Tissue sections were analyzed using an Olympus microscope (BX41) equipped for epifluorescence and an Olympus DP70 digital camera with DP MANAGER software (v 2.2.1) (Olympus, Center Valley, PA, USA). The PVN was identified using a
stereotaxic atlas\textsuperscript{80} and sampled at three rostral–caudal levels to assess for neuronal activation\textsuperscript{81}. Care was taken to ensure that the sections used were from the same rostral–caudal plane in each brain. Analysis was performed on two sets of tissue for each animal. The number of Fos-positive cells was visually quantified by participants blind to the experimental conditions using National Institutes of Health IMAGEJ software (NIH, Bethesda, MD, USA), and the counts for each PVN subnucleus was averaged for each animal.

**Kidney Cortical Preparation**

Kidneys were harvested from animals following completion of acute experimental protocols and stored at –80°C. Kidney cortex tissue was homogenized on ice using a hand-held pestle in a homogenizing buffer (10 mM triethanolamine, 250 mM sucrose, 100 mM NaN3, 10 mM PMSF, and 1 mM leupeptin). The resulting homogenate was centrifuged at 4,000 g for 10 min at 4°C. The supernatant was collected and centrifuged at 17,000 g for 60 min at 4°C. Following centrifugation, the membrane pellet was re-suspended in 400 μl of homogenizing buffer, and protein content was quantified via the BCA assay. Membrane preparations were stored at –80°C prior to use in immunoblotting studies.

**Determination of Renal Sodium-Chloride Cotransporter Levels**

Protein extracted from membrane preparations of kidney cortex tissue were loaded at a concentration of 20 μg of protein per lane. Membranes were blocked in 5% milk for 1 hour and incubated overnight at 4°C with anti-NCC (1:2,000; Millipore, Billerica, MA; cat. no. AB3553) or anti--actin (1:5,000; Sigma, St. Louis, MO; cat. no.
A5316) in 0.1% PBS-Tween. Afterwards, membranes were exposed to either a secondary horseradish peroxidase donkey anti-rabbit IgG (1:5,000; Promega, Madison, WI; cat. no. V7951) or antimouse IgG peroxidase antibody (1:10,000; Sigma; cat. no. A9044) in 0.1% PBS-Tween for 1 hour at room temperature. Bound antibodies were visualized using chemiluminescence (GE signal enhancer; GE, Buckinghamshire, UK). Densitometric analysis was performed using Quantity One software (Bio-Rad, Hercules, CA), and band densities were normalized to β-actin.

**Statistical Plan/Power Calculations**

Results are expressed as mean ± SEM. The effects of our experimental variable (e.g., VE, ARN ablation) on CV, renal, PVN and/or metabolic parameters at different time points are evaluated by comparing the average of these values with the respective group control value via a one-way repeated measures ANOVA with Dunnett’s post-hoc test. Differences between treatment groups were assessed by two-way repeated measures ANOVA with treatment being one fixed effect and time the other, with the interaction included. The time (days, etc.) is the repeated factor with Bonferroni’s post-hoc test. Data was verified for normal distribution and equality of variances. Statistical significance is defined as p<0.05.
RESULTS

Effects of ARN Ablation and Physiological Activation of ARN Mechanoreceptors and Chemoreceptors on Cardiovascular and Renal Parameters

Figure 7 illustrates the activation of ARN mechanoreceptors and chemoreceptors in response to acute IV 5% BW VE and 1M NaCl infusions respectively. The effects of Renal-CAP on mean arterial pressure (MAP), urine output (V), and UNaV are shown in Figures 7A and 7B. Our results show that MAP increased in Renal-CAP rats during the 90-minute recovery period after IV 5% BW VE (Figure 7A). In addition, natriuresis was notably reduced during IV VE in Renal-CAP rats compared to sham rats (Figure 7A).

Figure 7 – Effect of ARN ablation by exposure to capsaicin (Renal-CAP) in male SD rats on changes in mean arterial pressure (MAP), urine output (V) and natriuresis (UNaV) evoked by an IV 5% bodyweight (BW) volume expansion (VE) (A), and a 2 hour IV 1M NaCl infusion (B). Effect of IV 5% BW VE (C) and 1M NaCl (D) infusion on renal pelvic pressure (RPP) in anesthetized male SD rats. *p<0.05 vs resp. group control/baseline, τ p<0.05 vs resp. sham Renal-CAP group value.
While IV 5% BW VE resulted in statistically significant differences in the MAP, V, and UNaV of Renal-CAP and sham rats (Figure 7A), IV infusion of 1M NaCl caused no notable differences between these parameters in Renal-CAP and sham rats (Figure 7B). In Figure 7A, we noted that Renal-CAP mitigated natriuresis and caused increased MAP throughout an IV 5% BW VE. Contrastingly, a 2 hour IV infusion of 1M NaCl caused no statistically significant differences between the MAP and V of Renal-CAP and sham rats (Figure 7B). Although Renal-CAP and sham rats exhibited changes in UNaV that were significant compared to the respective group control, as marked by *, they do not experience changes in UNaV that are statistically significant between one another, as indicated by τ (Figs. 7A and 7B).

We also examined the effects of an IV 5% BW VE (Figure 7C) and an IV 1M NaCl infusion (Figure 7D) on renal pelvic pressure (RPP) in anesthesized male SD rats. Our results show that these anesthesized male SD rats experience statistically significant increases in RPP throughout the 30-minute IV VE administration period and during the first 30 minutes of recovery (Figure 7C). However during both the control and 2-hour IV 1M NaCl administration periods, no increases in RPP were observed (Figure 7D).

**PVN Parvoceullar Neuronal Activation in Response to Acute ARN Mechanoreceptor and Chemoreceptor Activation.**

In addition to assessing certain cardiovascular and renal parameters in response to IV 5% BW VE and IV 1 M NaCl infusion (Figure 7) we also analyzed PVN neuronal activation in response to these acute sympathoinhibitory challenges, as illustrated on the following page by Figure 8.
Figure 8 – A & B (Top) Representative level 2 images of Renal-CAP (A) and sham (B) 5% VE PVN Fos+ nuclei. (C) Mean Fos+ parvocellular PVN nuclei averaged across all rostral-caudal levels at baseline & 120-minutes post 5% VE or 1M NaCl challenge. 3V – Third Ventricle. Note: *p<0.05 vs resp. group control/baseline, τ p<0.05 vs resp. sham Renal-CAP group value.
Level 2 representative images of both Renal-CAP and sham rats showed little to no activation of PVN parvocellular neurons at baseline prior to IV 5% BW VE (Figs. 8A and 8B). 120-minutes post 5% BW VE, however, we observed activation of these neurons in both Renal-CAP and sham rats (Figs. 8A & 8B). Critically, PVN parvocellular neuron activation was substantially greater in sham rats (Figure 8A) than in Renal-CAP rats (Figure 8B). This suggests that the ARN is responsible for inducing the activation of PVN parvocellular neurons in response to an acute mechanoreceptor sympathoinhibitory challenge, such as an IV 5% BW VE.

In Figure 8C, mean Fos⁺ parvocellular PVN nuclei averaged across all rostral-caudal levels at baseline & 120-minutes post IV 5% VE or 1M NaCl are shown. At baseline, very few Fos⁺ parvocellular PVN nuclei were observed across all PVN sub-regions (Figure 8C). This changed 120-minutes post 5% VE and 1M NaCl, as significantly greater parvocellular nuclei were quantified in both Renal-CAP and sham rats. Figure 8C reaffirms the level 2 images of Figures 8A and 8B, again showing that Renal-CAP rats exhibited significantly less Fos⁺ parvocellular PVN nuclei than sham rats across all PVN sub-regions (Figure 8C). This again implies that an intact ARN is necessary to evoke PVN parvocellular neuronal activation in response to an acute mechanoreceptor stimulus.

**ARN Responses as a Function of High Salt Intake**

Our results indicate that HS intake (HS; 8% NaCl) evokes both global and renal sympathoinhibition in male SD, DSR, and SS-13-BN rats, as demonstrated by Figure 9.
After 21 days on the HS diet previously described, both plasma and renal NE levels were reduced in SD, DSR, and SS13-BN rats (Figs. 9A and 9B). Notably, DSS rats on the same 21-day HS diet experienced statistically significant increases in plasma and renal NE (Figs. 9A and 9B). In addition, renal pelvic assay substance-P release in response to NE was greater in SD and DSR rats on HS diets than those on normal salt (NS) diets (Figure 9C). Although DSS rats on HS diets did experience a slight increase in NE-evoked substance-P release, this release was attenuated compared to SD and DSR rats on the same diet (Figure 9C). Interestingly, substance-P release in response to NaCl was unchanged across SD, DSR, and DSS rats regardless of salt diet (Figure 9D).
SD rats on HS diets demonstrated increased UNaV in response to RPP that was statistically significant with respect to both baseline values and NS group values (Figure 10A). However, SD rats on NS diets did not demonstrate any statistically significant increase in UNaV until a 10mmHg increase in RPP occurred (Figure 10A). In contrast, DSS rats demonstrated impaired natriuresis in response to increases in RPP (Figure 10A). Also noteworthy, increases in renal pelvic NaCl after HS intake were unable to generate enhanced UNaV in both SD and DSS rats (Figure 10B). These results suggest that increased RPP, a stimulus of ARN mechanoreceptors, occurs after HS intake in SD rats but not after HS intake in DSS rats.

**The Relationship Between Salt Consumption, Sympathetic Nervous System Activity, and Regulation of the NCC**

The effects of HS consumption (HS; 8% NaCl) for 21 days were assessed in groups of male rats that were salt-resistant, salt-sensitive, and in SHR rats (Figs. 11 and 12). The group of salt-resistant rats was composed of sc saline infused SD rats and DSR rats. In these rats, MAP was unaffected by HS intake as illustrated in Figure 11A. In
concordance with their salt-resistant phenotype, both sc saline infused SD rats and DSR rats downregulated both NCC activity, defined as ΔUNaV to iv HCTZ (Figure 11B), and NCC expression in response to HS intake (Figs. 11B and 12A).

Figure 11 – Effect of 21 day high-salt intake (HS, 8% NaCl) on: A – MAP and B – NCC activity (defined as peak natriuresis to HCTZ infusion) in male sc saline or NE (600 ng/min) infused SD rats, DSR and DSS rats, Renal-CAP SD rats, and naïve WKY and SHR rats. WKY and SHR rats were maintained on normal salt diets. Note: *p<0.05 vs resp. normal salt (NS;0.6% NaCl) value, τp<0.05 vs resp. DSR value, Φp<0.05 vs SD saline HS value.
Assessed salt-sensitive rats included DSS rats, sc NE (600 ng/min) infused SD rats, and Renal-CAP SD rats (Figs. 11-12). In response to HS intake, all three rats exhibited statistically significant increases in MAP in comparison to their respective NS and salt-resistant counterparts (Figure 11A). Unlike their salt-resistant counterparts, these salt-sensitive rats were also unable to downregulate either NCC activity or expression following HS intake (Figs 11B and 12A).

Figure 12 – Effect of 21-day high salt intake (HS, 8% NaCl) on: A – renal NCC protein expression and B – representative immunoblots of NCC protein levels in male sc saline or NE infused SD, DSR, and DSS rats. Note: *p<0.05 vs resp. normal salt (NS;0.6% NaCl) value, τp<0.05 vs resp. DSR value, Φp<0.05 vs SD saline HS value
By comparing naïve WKY and SHR rats maintained on NS diets (NS; 0.6% NaCl), we could study MAP and NCC activity in essential neurogenic hypertension (Figure 11). We observed that both MAP (Figure 11A) and NCC activity (Figure 11B) were enhanced in SHR rats compared to WKY rats, which indicated that the SHR rats demonstrated sympathoexcitation. Taken together, Figures 11 and 12 indicate that in both salt-sensitive and essential neurogenic hypertension, the NCC is not downregulated as it should be. Representative immunoblots of NCC levels in all salt-resistant and salt-sensitive rats analyzed are depicted in Figure 12B. These immunoblots bolster the data presented Figures 11 and 12A, as NCC protein levels were successfully downregulated in salt-resistant rats on HS diets but not in salt-sensitive rats on HS diets (Figure 12B).

**α-1 Adrenoreceptors, β-Adrenoreceptors, and Their Roles in NCC Regulation**

Figures 11 and 12 illustrated how MAP, NCC activity, and NCC expression changed in groups of salt-resistant and salt-sensitive rats consuming NS and HS (8% NaCl) diets. In the following pages, Figures 13-16 show how MAP and protein levels of important kinases involved in NCC regulation are impacted by the administration of terazosin and propranolol – α-1 adrenoreceptor and β-adrenoreceptor antagonists respectively. Three separate groups of rats were assessed: sc saline infused SD rats on NS or HS diets, sc NE infused SD rats on NS or HS diets, and naïve DSS rats on HS diets (Figs. 13-16).

SD rats subcutaneously infused with saline exhibited no differences in MAP on HS (4% NaCl) diets versus NS diets (Figure 13A). In conjunction with either sc terazosin (10mg/kg/day) or propranolol (10mg/kg/day) administration, sc saline infused SD rats on
HS diets still demonstrated comparable MAP to those on NS diets (Figure 13A). In addition, consumption of a HS diet did not affect sodium-evoked downregulation of NCC activity in this group of rats. Saline infused SD rats and SD rats infused with a combination of saline and either terazosin or propranolol exhibited unaltered baseline NCC activity on NS diets and successfully downregulated NCC activity on HS diets (Figure 13B). Thus, administration of these adrenoreceptor antagonists did not alter baseline MAP, NCC activity, or sodium-evoked suppression of NCC activity in sc saline infused SD rats (Figure 13). In addition, these rats responded to HS intake by suppressing the expression of both the NCC and the important kinases that regulate it (Figs. 14-16).

Figure 13 – Effect of 21 day 4% NaCl intake (High Salt) in sc saline or NE infused SD rats and male DSS rats receiving sc terazosin (Teraz; 10mg/kg/day) or propranolol (Pro; 10mg/kg/day) on MAP (A) and NCC activity (B) (peak natriuresis to HCTZ). *p<0.05 vs resp. normal salt (NS) group, τp<0.05 vs resp. HS group value.
In contrast, sc NE infused SD rats exhibited increased MAP as a function of HS intake (Figure 13A). Notably, administration of either terazosin or propranolol eliminated these increases in MAP and abolished the salt-sensitive of blood pressure in sc NE infused SD rats on HS diets (Figure 13A).
In these SD rats, NE infusion eliminated the ability of salt-intake to downregulate NCC activity, NCC expression, and the expression of the following proteins involved in NCC activation or regulation: pNCC, OxSR1, pOxSR1, and STE20/SPS1-related proline/alanine-rich kinase (SPAK) (Figs. 13-16). NE binds to renal adrenoreceptors and causes activation of WNK, which phosphorylates both SPAK and OxSR1. These kinases then phosphorylate NCC, upregulating it and making it active.

Figure 15 – Effect of 21 day 4% NaCl intake (High Salt) in sc saline or NE infused SD rats and naïve male DSS rats receiving sc terazosin (Teraz; 10mg/kg/day) or propranolol (Pro; 10mg/kg/day) on OxSR1 expression (A) and pOxSR1 expression (B). *p<0.05 vs resp. normal salt (NS) group, τp<0.05 vs resp. HS group value.
Critically, selective $\alpha$-1 adrenoreceptor via terazosin administration restored not only downregulation of NCC activity in response to salt-intake, but also downregulation of NCC, pNCC, OxSR1, pOxSR1, and SPAK expression (Figs. 13-16). Propranolol administration eliminated NE-evoked increases in MAP (Figure 13A). Unlike terazosin, propranolol was unable to reestablish sodium-evoked suppression of NCC activity or expression (Figs. 13B and 14A). It was similarly unsuccessful in restoring sodium-evoked suppression of pNCC, OxSR1, and pOxSR1 (Figs. 14B-15).

Figure 16 – Effect of 21 day 4% NaCl intake (High Salt) in sc saline or NE infused SD rats and naïve male DSS rats receiving sc terazosin (Teraz; 10mg/kg/day) or propranolol (Pro; 10mg/kg/day) on STE20/SPS1-related proline/alanine-rich kinase (SPAK) expression (A). Sample Immunoblots (B). *p<0.05 vs resp. normal salt (NS) group, $\tau$p<0.05 vs resp. HS group value.
In naïve DSS rats on HS diets sc terazosin administration blunted the observed increases in MAP (Figure 13A), indicating terazosin’s ability to attenuate the salt-sensitivity of blood pressure in naïve DSS rats. In contrast, propranolol was unable to attenuate the salt-sensitivity of blood pressure in DSS rats on HS diets, given that these rats still experienced notable increases in MAP despite propranolol administration (Figure 13A).

Just as propranolol was unable to restore sodium-evoked suppression of NCC activity or expression in NE infused SD rats, DSS rats receiving the β-adrenoreceptor antagonist did not regain sodium-evoked suppression of either NCC parameter (Figs. 13B and 14A). Phosphorylated NCC, OxSR1, and pOxSR1 remained upregulated in DSS rats receiving sc propranolol (Figs. 14B-15). Interestingly, SPAK expression was downregulated after propranolol administration in both sc NE infused SD rats and naïve male DSS rats (Figure 16A). Sample immunoblots (Figure 16B) illustrate terazosin’s ability and propranolol’s inability to restore sodium-evoked suppression of NCC, pNCC, and other kinase levels.

**Antagonism of the Renal α-1 Adrenoreceptor in Established Salt-Sensitive Hypertension and Established Essential Neurogenic Hypertension**

We analyzed sc NE infused SD rats and SHR rats to assess terazosin’s effects on two forms of established hypertension: salt-sensitive hypertension (sc NE infused SD) and essential neurogenic hypertension (SHR) (Figure 17). During a 42-day experimental period, SD rats on NS and HS diets (HS; 4% NaCl) received sc NE for the first 21 days. These rats consequently developed salt-sensitive hypertension. To test terazosin’s ability on established salt-sensitive hypertension, some of these sc NE infused SD rats received
sc terazosin during days 22-42 of the experimental period (Figure 17). We examined terazosin’s effects on established essential neurogenic hypertension by comparing Naïve NS (NS; 0.6% NaCl) fed SHR rats to those receiving the α-1 antagonist subcutaneously (Figure 17). Critically, terazosin administration lowered blood pressure and reduced NCC activity in established essential neurogenic hypertension. In addition, it reestablished sodium-evoked suppression of NCC activity and abolished salt-sensitivity of blood pressure in established NE-evoked salt-sensitive hypertension (Figure 17).

Figure 17 – Effect of sc terazosin (10mg/kg/day) on: MAP (A) and peak natriuresis (ΔUNaV) to iv HCTZ (B) in groups of naïve normal salt (NS; 0.6% NaCl) fed male SHR and SD rats receiving either NS or high salt (HS; 4% NaCl) during 42-day sc saline or NE (600 ng/min) infusion or 42-day NE infusion in combination with 21-day sc terazosin for days 22-42. *p<0.05 vs resp. group NS (0.6% NaCl) value, τp<0.05 vs HS NE.

[Image of bar charts showing MAP and peak natriuresis data]
DISCUSSION

Through a novel experimental paradigm and refined analyses of the cardiovascular and renal systems, our laboratory has produced important evidence regarding the respective roles of the ARN and the NCC in the regulation of fluid and electrolyte homeostasis. Acutely, we have demonstrated a role of the ARN’s mechanoreceptors in mediating a crucial sympathoinhibitory reno-renal renal reflex that maintains blood pressure and increases UNaV in response to an IV 5% BW VE: an acute sympathoinhibitory challenge that is mechanoreceptor-specific. Chronically, our experiments have yielded data that suggests that ARN mechanoreceptors are activated by long-term HS intake in salt-resistant rats, but not salt-sensitive rats. Furthermore, our results have indicated that this same mechanoreceptor-driven ARN sympathoinhibitory reno-renal reflex indirectly influences NCC activity and expression. Collectively, our experiments have provided innovative mechanistic information on both the ARN and NCC, and have provided insight into new a new therapeutic target, the DCT α-1 adrenoreceptors, that can be used to treat sympathetically-driven hypertension in humans.

Acute Role of the ARN

Mechanoreceptors in the ARN are activated by RPP increases of 2.5 to 3 mmHg increments, and are important in conducting the ARN sympathoinhibitory reno-renal reflex that exerts a tonic inhibition on efferent RSNA. Through administration of an acute IV 5% BW VE, we isolated renal mechanoreceptors and activated them by increasing total body fluid and sodium content, which increased RPP. In contrast, we used a 1M NaCl infusion to isolate renal chemoreceptors. This
infusion activated the chemoreceptors by increasing total body and urinary sodium, neither of which affected RPP\textsuperscript{19,88,89}. Both of these infusion protocols have been established as acute sympathoinhibitory challenges\textsuperscript{82–86} that are uniquely able to activate ARN mechanoreceptors or chemoreceptors.

Our data suggests that ARN mechanosensitive receptors have a significant role in mediating an acute ARN sympathoinhibitory reno-renal reflex that regulates both natriuresis and MAP. While previous studies, notably those conducted by Dr. Ulla Kopp, examined the effects and activation mechanisms of renal mechanosensory nerves\textsuperscript{44,85,90,91}, our experiment analyzed how ARN mechanoreceptor-dependent functions were affected by a novel ARN ablation technique – Renal-CAP\textsuperscript{73}.

By comparing the responses of conscious Renal-CAP and sham surgery rats during and after acute IV 5\% BW VE\textsuperscript{74,82,84,92,93} (ARN mechanoreceptor stimulus) and iv 1M NaCl infusion\textsuperscript{85,86} (ARN chemoreceptor stimulus), we used a new experimental paradigm to assess how changes in MAP, V, and UNaV were acutely affected in conscious, ARN denervated rats. This is the first time that a physiological role of the ARN sympathoinhibitory reno-renal reflex has been demonstrated in a conscious animal.

Furthermore, our quantification of PVN parvocellular neuronal activation at baseline and 120-minutes post-VE revealed new visual evidence that shows that IV 5\% BW VE activates PVN inhibitory neurons that modulate CNS sympathetic outflow, and thus regulate both blood pressure and UNaV. These findings illustrate the acute role of ARN mechanoreceptors in maintaining fluid and electrolyte homeostasis through the
sympathoinhibitory reno-renal reflex, and highlight its protective abilities against excess efferent SNSA.

**Chronic Role of the ARN**

The ARN mechanoreceptor-driven sympathoinhibitory reno-renal reflex is not only significant during acute sympathoinhibitory challenges, but also in response to chronic HS intake. Our results indicated that salt-resistant rats depend on the mechanosensitive ARN sympathoinhibitory reno-renal reflex to regulate blood pressure during chronic HS consumption. This relates to Dr. Kopp’s previous findings concerning the ability of ARNs in normotensive rats to inhibit excessive efferent SNSA\textsuperscript{43,44,91}. In contrast, salt-sensitive rats suffer from an impairment in the ARN sympathoinhibitory reno-renal reflex which prevents them from controlling blood pressure in response to chronic HS intake.

These findings suggest that ARN mechanoreceptors are activated by HS intake in salt-resistant rats, potentially due to increased RPP resulting from increased urine output. Given that the urine flows from renal pelvis of the kidney to reach the bladder, where urine is stored\textsuperscript{94,95}, it is logical that increased urine output would require increased urine volume flowing through the renal pelvis and, therefore, lead to increased RPP.

The enhanced mechanosensitive propagation of the ARN sympathoinhibitory reno-reflex in response to HS intake is shown to be vital for salt-resistant rats to regulate blood pressure and UNaV. Clinically, patient screening for this protective ARN reno-renal reflex can prevent its unnecessary removal and, by doing so, ensure a preexisting mechanism of regulating efferent SNSA is kept intact.
The NCC and Blood Pressure

The NCC plays a critical role in fine-tuning the sodium reabsorption that occurs after the tubuloglomerular feedback that occurs in the proximal convoluted tubule. The NCC is regulated by a complex and multifaceted kinase network, which consists of the proteins WNK, SPAK, and OxSR1. Our data provides evidence that the NCC is indirectly affected by the mechanoreceptor-driven ARN sympathoinhibitory reno-renal reflex through a NE-α1-adrenoreceptor gated signal transduction pathway. It has been established that the mechanosensitive ARN sympathoinhibitory reno-renal reflex suppresses excess efferent RNSA.

Our data suggests that the efferent renal nerve, which projects from the CNS back to the kidney, releases NE that binds to α1 adrenoreceptors in the DCT. This binding results in a kinase cascade in which a now activated WNK1 phosphorylates the intermediary kinases SPAK and OxSR1, the latter of which phosphorylates stationary NCCs and increases their activity. Since the mechanosensitive ARN sympathoinhibitory reno-renal reflex attenuates central sympathetic outflow in response to chronic HS consumption, it prevents excess efferent RSNA and, thus, prevents excessive renal NE release and the aberrant NCC-mediated sodium reabsorption that would consequently occur. On the following page, Figure 18 illustrates the modulation of efferent RNSA by the ARN sympathoinhibitory reno-renal reflex in response to HS intake at the level of organ systems and at DCT cells.
Our experiments have demonstrated the ability of a selective α1-adrenoreceptor antagonist (terazosin) to mitigate the salt-sensitivity of blood pressure in salt-sensitive rats and shown that terazosin can restore sodium-evoked suppression of NCC activity and sodium-evoked suppression of NCC, pNCC, OxSR1, and pOxSR1. Critically, we demonstrated the ability of terazosin administration to lower blood pressure and reduce NCC activity in rats with established salt-sensitive hypertension and with established
essential neurogenic hypertension. This provides important evidence that \( \alpha_1 \)-adrenoreceptor antagonism is a viable therapeutic approach to precisely treat salt-sensitive and essential neurogenic hypertension. The need to identify novel therapeutic targets to treat hypertension is underscored by the fact the development of antihypertensive drugs has not been very productive\(^{106} \). Given the incidence of individuals with salt-sensitive or essential neurogenic hypertension\(^{17,30,107–113} \), our findings hold significance in revealing that \( \alpha_1 \)-adrenoreceptor antagonism as a precise therapeutic mechanism that can potentially be used to treat these hypertensive individuals.

**Overall Significance, Limitations, and Future Considerations**

Our results have demonstrated that the mechanoreceptor-based ARN sympathoinhibitory reno-reflex plays a role in PVN neuronal activation and maintenance of blood pressure in salt-resistant rats, and that failure of this pathway occurs in rats with salt-sensitive and essential neurogenic hypertension. These findings show that increased propagation of the mechanosensitive ARN sympathoinhibitory reno-reflex occurs in response to chronic HS intake, and that this reno-reflex is necessary to regulated blood pressure and natriuresis. In a clinical context, patient screenings for this protective ARN reno-renal reflex can prevent it from being removed and consequently ensure patients keep a preexisting mechanism of regulating efferent SNSA in place.

One limitation our laboratory faced is the lack of pharmacological antagonists for either mechanoreceptors or chemoreceptors. Thus, our experiments necessitated the use of physiological manipulations, IV 5\% BW VE and IV 1M NaCl infusion, to activate and assess mechanoreceptors and chemoreceptors respectively. Should mechanoreceptor and
chemoreceptor pharmacological antagonists be developed and become available, additional studies will be conducted to ensure our results are valid. It should also be noted that our quantification of active PVN inhibitory interneurons was limited through Fos+ immunohistochemistry. This is primarily because Fos indicates activation and does not stain based on the phenotypes of active cells. Therefore, we are unable to definitively say that the activated neurons we quantified are inhibitory or excitatory because there Fos+ only indicates activation, not cell phenotype.

We have also indicated the ARN’s important role in facilitating sodium-evoked maintenance of chronic NCC activity by regulating sympathetic tone, and that this regulation occurs via a NE-α1-adrenoreceptor-WNK1-OxSR1 pathway. In addition, our data presents compelling evidence that NCC regulation is hampered in salt-sensitive rats, which are unable to modulate efferent RSNA due to an impaired mechanosensitive ARN sympathoinhibitory reno-renal reflex.

Another limitation faced involved our ability to isolate physiological NCC activity. To selectively examine physiological NCC activity, we administered prior ENaC blockade (achieved through amiloride administration) and continued ENaC blockade during NCC inhibition (achieve through amiloride HCTZ administration). In addition, we were limited in that we signified NCC phosphorylation as an indicator of NCC activity. We understand that the weakness of using this experimental protocol and assessment technique is that they do not directly assess NCC mediated sodium transport. However, these techniques have been widely used and are the only experimental approaches available to evaluate NCC activity in vivo.
We have provided important evidence on potential treatments for salt-sensitive and essential neurogenic hypertension. Our data provides insight into α-1 adrenoreceptor antagonism as a new therapeutic mechanism that can increase activity of the mechanosensitive ARN sympathoinhibitory reno-renal reflex and natriuresis in individuals with salt-sensitive or essential neurogenic hypertension. Since anti-hypertensive drug development has been unproductive\(^{106}\), our data is significant in revealing a new therapeutic mechanism that can treat these hypertensive individuals in a more precise and effective manner.

In addition, our results have demonstrated the potential therapeutic benefits of renal denervation in humans. Considering the role that excessive efferent RSNA has in NCC upregulation and hypertension development, ablation of renal sympathetic nerves is a worthwhile treatment avenue to consider. Hypertensive individuals who suffer from impaired or nonexistent mechanosensitive ARN sympathoinhibitory reno-renal reflex activity could greatly benefit from ablation of renal sympathetic nerves. In these hypertensive individuals, impairment of the mechanosensitive ARN sympathoinhibitory reno-renal reflex would be measured as UNaV in response to clinically manipulated increases in RPP.
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Circ Res  
Circulation Research

Clin Exp Pharmacol Physiol  
Clinical and Experimental Pharmacology and Physiology

Clin Sci Lond Engl  
Clinical Science

Cochrane Database Syst Rev.  
The Cochrane Database of Systematic Reviews

Compr Physiol  
Comprehensive Physiology

Curr Hypertens Rep  
Current Hypertension Reports

Curr Hypertens Rev  
Current Hypertension Reviews

Curr Opin Cell Biol  
Current Opinion in Cell Biology

Curr Opin Nephrol Hypertens  
Current Opinion in Nephrology and Hypertension

Curr Opin Pediatr  
Current Opinion in Pediatrics

Exp Physiol  
Experimental Physiology

Eur Heart J  
European Heart Journal

FASEB J  
Federation of American Societies for Experimental Biology Journal

Hypertens Dallas Tex  
Hypertension

Indian Heart J  
Indian Heart Journal

JAMA  
The Journal of the American Medical Association

J Am Coll Nutr  
The Journal of the American College of Nutrition

J Am Soc Nephrol JASN  
Journal of the American Society of Nephrology
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