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Contribution of the sympathetic nervous system to the pathogenesis of salt-sensitive hypertension

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CONTRIBUTION OF THE SYMPATHETIC NERVOUS SYSTEM TO THE PATHOGENESIS OF SALT-SENSITIVE HYPERTENSION

by

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For Mom, Dad, Mark, Jacob, Mae, Tess, and Rudy.
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ABSTRACT

Dysregulation of the sodium-chloride cotransporter (NCC) is believed to significantly impact blood pressure. Recent studies have implicated overactivity of the sympathetic nervous system as a mechanism driving renal NCC dysregulation to evoke the development of salt-sensitive hypertension. It is proposed that the sympathetic nervous system accomplishes this by norepinephrine (NE)-mediated over-activation of the beta2-adrenergic receptors located in the distal tubules of the kidney. Beta2-adrenoreceptor activation is hypothesized to stimulate the protein kinases SPAK and OxSR1 to phosphorylate and thus activate NCC. This beta2-receptor-SPAK/OxSR1-NCC pathway was elucidated in studies that challenged salt-resistant mice with high-salt diets, bilateral adrenalectomies, and NE infusion. To expand the scope of these studies, we investigated the effects of elevated circulating NE on blood pressure, NCC activity, and expression of NCC proteins, SPAK, and OxSR1 in a different salt-resistant animal species (the Sprague-Dawley rat).

In this study we implanted male Sprague-Dawley rats with osmotic minipumps delivering a subcutaneous infusion of either saline, NE, a 50:50 solution of DMSO/isotonic saline, a combination of NE and the NCC antagonist hydrochlorothiazide, or a combination of NE and the beta-adrenoreceptor antagonist propranolol. Following implantation of the pumps the rats were randomly assigned to
either a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl) for two weeks. After fourteen days all animals underwent acute femoral artery, vein, and bladder cannulation in order to monitor heart rate and blood pressure, administer drugs intravenously, and track renal function, respectively. Following surgical recovery, blood pressure and heart rate were measured continuously, and urine was collected in ten-minute intervals in order to assess peak natriuretic responses to amiloride and hydrochlorothiazide. Following this protocol the rats received an intravenous bolus of hexamethonium (30 mg/kg), and their peak drops in blood pressure were recorded. Afterwards both kidneys were harvested and frozen at -80 °C for measurement of NCC proteins, SPAK, and OxSR1 expression.

This study demonstrates that increased circulating NE induces salt-sensitive hypertension in the naturally salt-resistant Sprague-Dawley rat. Chronic infusion of NE raised the blood pressure of the rats, and a high-salt diet exacerbated this effect. Furthermore, NE prevented salt-evoked suppression of NCC activity and NCC, SPAK, and OxSR1 protein expression. Co-infusion of hydrochlorothiazide with NE attenuated NE-mediated hypertension and caused no variance in the blood pressures between the standard salt and high-salt groups. This indicates that chronically antagonizing NCC eliminated the salt-sensitive component of NE-mediated hypertension. Beta-receptor antagonism combined with NE infusion completely eliminated the hypertensive influence of NE and downregulated the expression of NCC proteins, SPAK, and OxSR1. However, NCC activity still remained at a level comparable with that observed in the NE-infused rats, demonstrating dissociation between protein expression and function.
These data, the first report in a rat model of an interaction of NE and a high salt intake that impairs NCC function, demonstrate that increased levels of NE in combination with a high dietary salt intake result in NCC dysregulation and the development of NE-mediated salt-sensitive hypertension. To an extent the data also support the proposition that NE activates the beta-adrenergic receptors to influence the activity of NCC and the expression of NCC proteins, SPAK, and OxSR1. Beta-antagonism combined with NE infusion attenuated the effects of NE on blood pressure and the expression of NCC proteins, SPAK, and OxSR1. However, the NE-mediated elevation of NCC activity still remained high. We propose that the beta-receptors are not the only adrenergic receptors that can influence NCC activity. The presence of alpha-adrenergic receptors in the distal tubules suggests that they may be able to keep NCC activity elevated through a pathway independent of the beta-receptors, SPAK, and OxSR1.
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<td>Angiotensin II</td>
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<td>BPM</td>
<td>Beats Per Minute</td>
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<td>CD</td>
<td>Collecting Duct</td>
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<td>CVD</td>
<td>Cardiovascular Disease</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DT</td>
<td>Distal Tubule</td>
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<tr>
<td>ECF</td>
<td>Extracellular Fluid</td>
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<tr>
<td>ENaC</td>
<td>Epithelial Sodium Channel</td>
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<tr>
<td>FHht</td>
<td>Familial Hyperkalemic Hypertension</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>HCTZ</td>
<td>Hydrochlorothiazide</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<td>KS-WNK1</td>
<td>Kidney-Specific WNK1 Isoform</td>
</tr>
<tr>
<td>L-WNK1</td>
<td>Long WNK1 Isoform</td>
</tr>
<tr>
<td>MABP</td>
<td>Mean Arterial Blood Pressure</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean Arterial Pressure</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid Receptor</td>
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<tr>
<td>NCC</td>
<td>Sodium-Chloride Cotransporter</td>
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<td>NHE3</td>
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<td>NKCC2</td>
<td>Sodium-Potassium-Two Chloride Cotransporter</td>
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NE ........................................................................................................ Norepinephrine
ODU ........................................................................................................ Optical Density Unit
OxSR1 ..................................................................................................... Serine/Threonine-Protein Kinase
PBS-T ...................................................................................................... Phosphate-Buffered Saline/Tween-20
PHAIIT ................................................................. Pseudohypoaldosteronism Type II
PMSF ...................................................................................................... Phenylmethanesulfonylfluoride
pNCC ................................................................. Phosphorylated NCC
PT .............................................................................................................. Proximal Tubule
RAAS ................................................................. Renin-Angiotensin-Aldosterone System
Rac1 ................................................ Ras-Related C3 Botulinum Toxin Substrate 1
ROMK ................................................................. Renal Outer Medullary Potassium Channel
s.c. ............................................................................................................. Subcutaneous
SD ............................................................................................................. Sprague-Dawley
SEM ......................................................................................................... Standard Error of the Mean
SGK1 ................................................................. Serum/Glucocorticoid-Regulated Kinase 1
SNS ............................................................................................................. Sympathetic Nervous System
SPAK ................................................ STE20/SPS1-Related Proline/Alanine-Rich Kinase
SR .............................................................................................................. Salt-Resistant
SS ............................................................................................................. Salt-Sensitive
TAL .............................................................................................. Thick Ascending Limb
UNaV .............................................................................................. Urinary Excretion of Sodium
WNK .............................................................................................. With-No-Lysine
INTRODUCTION

Cardiovascular disease (CVD) is a global pandemic. While CVD was once a relatively minor disease, it has matured into the killer responsible for 30% of global deaths each year.\(^1\) Although its prevalence is high and its manifestation assumes the forms of numerous diseases, many of those who suffer from CVD share a commonality: high blood pressure.\(^2\) By increasing the chances of stroke, congestive heart disease, and heart failure, hypertension is a major risk factor of CVD. As of the year 2000, one billion of the world’s population had hypertension, with the number expected to rise to 1.56 billion by 2025.\(^1\) In Western countries alone, 18% of CVD mortalities are a result of hypertension.\(^3\) In the United States, one-third of the adult population has hypertension, and half of those people will die from it. A fifth of them do not even know they have high blood pressure because of its asymptomatic nature. Even though antihypertensive therapy can decrease the risk of stroke by 30%, congestive heart failure by 40%-50%, coronary heart disease by 10%-20%, and overall mortality by 10%,\(^2\) only half of those undergoing treatment succeed in lowering their blood pressure below the recommended threshold of 140/90 mm Hg.\(^4\) The limitations of current treatment, coupled with the high economic burden of hypertension\(^3\), have created a drive to find cheaper, more efficacious treatments.
Salt Sensitivity

Hypertension exists in various forms such as secondary, resistant, and pseudo-resistant. While the pathogeneses of pseudo-resistant and secondary hypertension are known and treatable, the causes of resistant hypertension remain elusive. Resistant (also known as idiopathic or essential) hypertension is a blood pressure exceeding 140/90 mm Hg that refuses to drop despite being treated with three or more drugs (one of which is a diuretic) at maximum doses. Obesity, increasing age, and kidney disease are suspected of contributing to resistant hypertension, but the exact mechanisms remain unclear. Peaking the curiosity of researchers is that one of the most common traits in those with resistant hypertension is excessive salt consumption. This led to studies of how salt intake affects blood pressure. When experimenting on single individuals, researchers found a positive correlation between salt intake and blood pressure, but when they tested this correlation in large populations, it diminished. To account for this discrepancy, the theory of salt sensitivity was developed. For some individuals a high salt intake raises their blood pressure, but for others the excess salt only exerts marginal effects on pressure. The former group was called salt-sensitive (SS), and the latter group salt-resistant (SR).

The Pressure-Natriuresis Curve

To understand salt sensitivity, one must first understand sodium balance in an SR individual. Suppose an SR person eats a salty meal. The ingested sodium is absorbed into the blood stream. There it draws water from the cells into the extracellular fluid (ECF) space. This expansion of the ECF raises the cardiac output, blood pressure, and
subsequently the perfusion pressure on the kidney. The kidney responds twofold: it filters more sodium and water and reabsorbs less. Sodium and water excretion rises, and blood pressure returns to normal. What is present here then is a positive correlation between blood pressure and natriuresis. Graphing this relationship yields the pressure-natriuresis curve, and it helps us understand a potential contributor to salt-sensitive hypertension.

Figure 1. Relationship between salt intake, blood pressure, and natriuresis. A: The equilibrium between pressure, sodium intake, and natriuresis in a salt-resistant individual. B: Increased salt intake in a salt-resistant individual. The slope steepens and shifts left. Natriuresis increases with little change in blood pressure. C: The equilibrium between pressure, sodium intake, and natriuresis in a salt-sensitive individual. The slope decreases and shifts right. More pressure is needed to excrete excessive sodium. MABP, mean arterial blood pressure. Figure taken from Mullins and Mullins.

Guyton, an early proponent of the pressure-natriuresis curve, hypothesized that chronic hypertension was not solely caused by high vascular resistance which was a
popular idea during his time. He believed factors that altered the position and slope of the pressure-natriuresis curve were culpable as well. These factors would be changes in salt intake and alterations in kidney function. In an SS person the pressure-natriuresis curve has shifted to the right, and the slope has decreased. This means that it takes a greater increase in blood pressure to excrete the same amount of sodium that an SR person excretes at a lower blood pressure. The physiological difference between SR and SS individuals is not in the extent of their natriuresis but in the efficiency with which they achieve it.\textsuperscript{7,10}

**Kidney Sodium Transporters**

The decreased efficiency in sodium excretion present in SS individuals is attributed in part to overactivity of the sodium transporters in the kidney.\textsuperscript{13} These transporters and ion channels line the apical membranes of the tubular cells of the nephron in a non-uniform manner, thus granting each part of the nephron a unique reabsorption capacity. The proximal tubule (PT) is the site of bulk transport, reabsorbing 60\%-70\% of the filtered sodium through the Na/H exchanger NHE3. Another 20\%-30\% is reabsorbed in the thick ascending limb (TAL) by the Na/K/2Cl cotransporter NKCC2. The final 5\%-7\% is reabsorbed in the distal tubule (DT) mostly by the Na/Cl cotransporter NCC but also by the epithelial sodium channel ENaC. The collecting duct (CD) performs last minute sodium adjustments primarily through ENaC.\textsuperscript{14}
The Sodium-Chloride Cotransporter (NCC)

Despite only reabsorbing a small fraction of the filtered sodium, overactivity of NCC may contribute to salt-sensitive hypertension. The NCC is a 12-transmembrane-spanning homodimer which cotransports sodium and chloride in an electroneutral 1:1 ratio. The influence of the NCC on blood pressure has been elucidated through observations of diseases that cause its over- and underexpression. Gordon’s syndrome (also known as pseudohypoaldosteronism type II (PHAII) or familial hyperkalemic hypertension (FHht)) is a genetic disease in which a mutation in the NCC regulatory pathway causes NCC overexpression. NCC activity rises, and elevated blood pressure, hypercalciurea, and hyperkalemic acidosis ensue. Conversely, mutations which decrease NCC activity cause hypotension, hypokalemia, and low calcium and magnesium levels in a disorder known as Gitelman’s syndrome. The response of NCC to thiazides is also noteworthy. Thiazides are some of the oldest and most efficacious diuretics used to treat hypertension, and they do so by blocking NCC. However, they are only effective in hypertensive patients (such as those with Gordon’s syndrome). Their effect on normotensive patients is negligible at best. These findings suggest that NCC possesses a substantial influence on blood pressure.

While thiazides have their benefits, they also induce multiple side effects such as hypercalcinemia, hypokalemia, and insulin resistance. If a drug could target NCC with greater precision, it may achieve a greater antihypertensive effect while forgoing the side effects. The fact that Gordon’s and Gitelman’s syndromes are caused by activating/inactivating mutations in the regulatory pathway has sparked further research.
into how NCC makes its way to the apical membrane and what molecules regulate this process. It was found that NCC is transcribed from the gene SLC12A3, processed in the endoplasmic reticulum and Golgi apparatus, and shipped to the plasma membrane. There it is activated by phosphorylation. This presents two areas for regulation: the amount of NCC expressed on the apical membrane (Type 1) and the level of its phosphorylation (Type 2). These processes are regulated by a series of kinases that constitutes the WNK-SPAK/OxSR1-NCC kinase chain.

**WNKs**

The most upstream kinases of this pathway are the With-No-Lysine (WNK) kinases. They exist all over the body and serve various functions. In the kidney they regulate NCC, ENaC, NKCC2, and ROMK (renal outer medullary potassium channel). Their activation is brought about through self-phosphorylation. How exactly they know when to self-phosphorylate is unclear, but low intracellular chloride, hyperosmotic conditions, and decreased cell volume seem to promote it. Once the WNKs are phosphorylated, their N-terminal regions RFXV/I bind to the unique conserved C-terminals in the downstream kinases SPAK (STE20/SPS1-related proline/alanine-rich kinase) and OxSR1 (serine/threonine-protein kinase) and phosphorylate key threonine residues (Thr233 in SPAK, Thr185 in OxSR1). This encourages SPAK and OxSR1 to bind to the peptide motif RFXV/I in the N-terminal region of NCC, phosphorylate Thr53, Ser71, and T58, and thus activate NCC. Degradation of WNKs is mediated by the ubiquitin E3 ligase. Two components form this heterodimeric complex: kelch-like protein
3 and cullin. Kelch-like protein 3 recognizes the substrates, and cullin facilitates their ubiquitylation. Losing either one of these proteins confers a gain of function on WNKs and results in NCC and NKCC2 hyperactivity.\textsuperscript{20}

The existence of multiple WNK isoforms accounts for their differing functions. The most significant and most puzzling is WNK4. In vitro studies of \textit{Xenopus laevis} oocytes showed WNK4 inhibiting NCC by either directing it for lysosomal degradation or interfering with its processing in the Golgi apparatus.\textsuperscript{14,19,22,23} It was also found in the salt-resistant Sprague Dawley (SD) rats that a low-salt diet decreased WNK4 expression and increased expression of NCC mRNA, while a high-salt diet exerted the opposite effects.\textsuperscript{22,24} Therefore, it was perplexing when later in vivo studies painted WNK4 as a stimulator of NCC. Overactive WNK4 in vivo increased SPAK and OxSR1 phosphorylation and caused a Gordon’s syndrome-like phenotype.\textsuperscript{25} Meanwhile, total loss of WNK4 decreased both NCC activity and blood pressure.\textsuperscript{17} To settle these conflicting functions, it has been hypothesized that the cellular environment modulates the function of WNK4.\textsuperscript{19} Angiotensin II (AII)-mediated increases in intracellular calcium seem to switch WNK4 from inhibitory to stimulatory actions. Other WNK isoforms may also have a similar influence.\textsuperscript{16}

One of these isoforms is WNK1. A common form of WNK1 is a mutant in which not all of its exons have been spliced out. This long WNK1 (L-WNK1) powerfully activates SPAK by inhibiting WNK4 (which again suggests an inhibitory role for WNK4).\textsuperscript{19,26} L-WNK1 also forms homodimers and heterodimers with itself, WNK4, and WNK3 by interacting with the conserved HQ motifs located on the C-terminal sides. This
dimerization modulates their activities, but to what effect remains unclear. Another form of WNK1 is the kidney-specific WNK1 (KS-WNK1) which inhibits L-WNK1, thus inhibiting NCC.\textsuperscript{26} WNK3 is another important isoform that stimulates NCC. How it does this is not known, but the ratio of WNK3 to WNK4 appears to indicate whether NCC is being inhibited (a low ratio) or being stimulated (a high ratio).\textsuperscript{19}

\textbf{Figure 2. Regulation of NCC.} WNKs can regulate NCC by influencing its expression on the apical membrane (Type 1 regulation) and/or its level of phosphorylation (Type 2 regulation). WNK1 stimulates NCC by either inhibiting WNK4 or stimulating SPAK. WNK4 may inhibit NCC by preventing its expression on the apical membrane or stimulate NCC by phosphorylating SPAK. Figure taken from Glover et al.\textsuperscript{14}
**SPAK and OxsR1**

WNKs can be potentially useful targets for drug therapy, but their pleiotropic functions and influence over multiple transporters may result in undesirable side effects. However, SPAK and OxsR1 may make excellent targets. Lying downstream of the WNKs, SPAK and OxsR1 are protein kinases that possess similar N-terminal catalytic domains and C-terminal regulatory domains\(^ {25}\), and both can phosphorylate and activate NCC. Though SPAK and OxsR1 share similar structures and functions, differences do exist. SPAK occurs primarily in the DT to activate NCC. OxsR1 is there too, but it also works in the TAL to activate NKCC2.\(^ {13,18}\) An in vivo study by Chu et al. demonstrated the power SPAK and OxsR1 wield in sodium transporter regulation. Mice with overactive WNK4 showed Gordon’s syndrome symptoms when OxsR1 was either present or knocked out. However, when overactive WNK4 was coupled with a SPAK knockout model, a normal phenotype was observed. Losing both OxsR1 and SPAK resulted in hypotension despite the overactive WNK4. This suggests that SPAK is the primary phosphorylator of NCC. OxsR1 can help compensate for the loss of SPAK, but on its own it fails to achieve the same levels of phosphorylation.\(^ {16,25}\)

**Table 1. Summary of in vivo kinase study by Chu et al.**\(^ {25}\) Overactive WNK4 increases NCC activity with or without OxsR1. Losing SPAK prevents WNK4 stimulation of NCC. Loss of both SPAK and OxsR1 results in hypotension.

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<tr>
<th><strong>Mouse Model</strong></th>
<th><strong>Phenotype</strong></th>
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<tr>
<td>Overactive WNK4</td>
<td>PHAII (hypervolemia, hypertension, hyperkalemia, increased renin)</td>
</tr>
<tr>
<td>Overactive WNK4 + OxsR1 Knockout</td>
<td>PHAII</td>
</tr>
<tr>
<td>Overactive WNK4 + SPAK Knockout</td>
<td>Normal phenotype</td>
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<tr>
<td>Overactive WNK4 + OxsR1 Knockout + SPAK Knockout</td>
<td>Hypotension, hypokalemia</td>
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The RAAS System

An understanding of the regulation of NCC allows us to comprehend how extrarenal stimuli modulate NCC expression and contribute to SS hypertension. The sympathetic nervous system (SNS) is one such stimulus. It has been shown that increased activity of the efferent renal sympathetic nerves can work indirectly to increase sodium reabsorption. One way it does this is by vasoconstricting the afferent renal arterioles, which decreases the amount of sodium filtered. The SNS also stimulates the renin-angiotensin-aldosterone system (RAAS). The RAAS works to retain sodium and water in the face of hypovolemia and/or hypotension. Sensing decreased tubular flow, the juxtaglomerular cells of the kidney release renin. Renin is then converted into angiotensin I and later into angiotensin II (AII) which acts on multiple levels to retain sodium. AII further vasoconstricts the renal arterioles and, as mentioned previously, is suspected of switching WNK4 from an inhibitor to a stimulator of NCC. Thus AII-mediated sodium retention relies on high salt intake and WNK4. If either of these two is lacking then the hypertensive effects of AII are attenuated.

AII increases sodium retention a third way by stimulating aldosterone release. Similar to AII, aldosterone increases sodium reabsorption in response to decreasing ECF sodium levels. It accomplishes this by binding to mineralocorticoid receptors (MR) in the distal nephron. The activated receptors increase the transcription of the kinase SGK1 (serum/glucocorticoid-regulated kinase 1), which goes on to phosphorylate two serine residues near the carboxyl terminus of WNK4. This phosphorylation either activates
WNK4 or frees NCC from WNK4 suppression. Which one of these actions is the case is unclear.\textsuperscript{26}

Because aldosterone powerfully stimulates NCC, it would be natural to assume that SS individuals would have higher aldosterone levels than SR ones. However, this is not so. Salt loading has been found to decrease aldosterone levels in both SS and SR persons.\textsuperscript{23} This does not mean that SGK1 cannot contribute to salt-sensitive hypertension. Rac1, a member of the Rho family of GTPases, can activate MRs independently of aldosterone.\textsuperscript{8} In SR individuals, salt intake decreases both aldosterone and Rac1 levels; thus MR activation is minimal. SS people also have lowered aldosterone in response to salt, but their Rac1 levels rise, leading to increased MR activation, SGK1 transcription, and NCC activity. This highlights the value that an MR blocker can bring to hypertension therapy.\textsuperscript{8,22}

**The Sympathetic Nervous System**

If chronically elevated aldosterone and AII are not a direct source of salt-sensitive hypertension, the question remains as to what is responsible. New studies indicate that in addition to *indirectly* increasing sodium reabsorption the SNS can *directly* increase sodium reabsorption by stimulating NCC in a pathway independent of AII, aldosterone, and renal hemodynamics.\textsuperscript{29-31} This was first suspected when it was found that SS individuals had higher plasma concentrations of norepinephrine (NE) than SR individuals.\textsuperscript{32} When later studies tested the effects of salt loading, both groups exhibited no differences in their sodium balance and had similar levels of AII and aldosterone. The
only difference was in their plasma NE levels which decreased in the SR group but increased in the SS group.\textsuperscript{33} The discovery of both alpha- and beta-receptors on kidney tubular cells reinforced the idea that the sympathetic nervous system can directly influence tubular function.\textsuperscript{34–36} Early renal denervation trials found that cutting the kidney off from efferent sympathetic influence produced a considerable and sustained drop in blood pressure in patients with resistant hypertension.\textsuperscript{27,37,38} This implies that aberrant SNS activity can contribute to the pathogenesis of salt-sensitive hypertension.

The dispersion of adrenergic receptors on the tubular cells of the kidney suggests that each part of the nephron has a unique response to sympathetic stimulation. Alpha1-adrenergic receptors have been found to increase sodium reabsorption in the PT,\textsuperscript{38} beta1-adrenergic receptors have been found to stimulate the release of renin,\textsuperscript{39} and now studies have implicated the beta2-adrenergic receptors in the DT as accountable for increasing the activity of NCC.\textsuperscript{7,15,40} An early micropuncture study showed that the DT is responsive to beta agonists and antagonists. Isoproterenol, an agonist, increased sodium reabsorption in the DT, while propranolol, an antagonist, decreased sodium reabsorption.\textsuperscript{41} A few decades later a study by Mu et al.\textsuperscript{42} proposed an intracellular pathway. They observed that beta2-adrenergic receptor activation stimulated protein kinase A to phosphorylate histone deacetylase 8. Once phosphorylated, histone deacetylase 8 was unable to act on histones 3 and 4. Because they were still acetylated, histones 3 and 4 were able to bind to the promoter region of the WNK4 gene. As this was happening, glucocorticoid receptors (GR) in the DT were activated. These activated GRs, along with negative glucocorticoid response elements, also bound to the promoter region. WNK4 transcription dropped as a
result, and NCC was freed from inhibition. For this mechanism to increase blood pressure, GR activation and excessive salt intake had to be present. Losing either one of these conditions nullified the effect of beta2-receptor activation. On the other hand, MR activation and AII generation were not involved in this pathway.

**Figure 3. Actions of the renal sympathetic nervous system in response to salt.** Salt-sensitive individuals exhibit increased renal SNS activity in response to salt. This activates the beta2-adrenergic receptors in the DT, leading to inhibition of WNK4 transcription, increased NCC activity, and ultimately hypertension. Figure taken from Fujita.22

The pathway proposed by Mu et al. further implicates the sympathetic nervous system in the development of salt-sensitive hypertension and nominates the beta2-adrenergic receptors as targets for therapeutic treatment.42 However, further experiments could expand the scope of this study. Mu et al. supported their hypothesis of the beta2-
receptor-SPAK/OxSR1-NCC pathway using data collected from salt-resistant mice. The limitation of mice is that it is difficult to induce salt-sensitivity in them. To do so, Mu et al. had to perform bilateral adrenalectomy and challenge them with a high-salt diet and infusions of NE, propranolol, or isoproterenol.\textsuperscript{42} While this does not nullify their results, their proposed pathway would be further validated if it were demonstrated in an additional salt-resistant animal species.

It should also be noted that not all researchers are in unanimous agreement on the specifics of this pathway. In replicating these experiments, Uchida et al. found no change in WNK4 mRNA, NCC, and pNCC (phosphorylated NCC) levels upon activation of the beta2-receptors.\textsuperscript{43} Ellison et al. were also unable to detect any changes in WNK4 mRNA levels in response to beta2-receptor activation.\textsuperscript{15} These results further emphasize the importance of testing this pathway in more than one animal species.

**Aims of the Present Study**

The question our study proposes to answer is whether the sympathetic nervous system can generate salt-sensitive hypertension. If so, then we will test the hypothesis that it is mediated through dysregulation of the sodium-chloride cotransporter NCC. If this is true as well, then we will assess the role of beta-adrenergic receptors in this pathway. While answering these questions, we will perform our experiments in the Sprague-Dawley rat. Like mice, they are salt-resistant. However, by using them we will be expanding the scope of previous studies and establishing that the beta-receptor pathway proposed by Mu et al.\textsuperscript{42} is applicable in a salt-resistant animal species apart from
the mouse. In doing so we hope to demonstrate in the Sprague-Dawley rat that the sympathetic nervous system can contribute to the pathogenesis of salt-sensitive hypertension by dysregulating the activity of NCC through a beta-adrenergic pathway.
METHODS

Animals

We used male Sprague-Dawley (SD) rats (270-300 g bodyweight; Harlan Laboratories, Inc., Indianapolis, IN, USA) for these experiments. Each rat was kept in its own cage where the environment was controlled in both temperature (range 68-79 °F) and humidity (range 30%-70%) and operated under a twelve-hour light/dark cycle. The SD rats underwent implantation of a subcutaneous osmotic minipump and were assigned to either a standard rodent diet (0.4% NaCl [0.174 mEq Na/kg]) or a high-salt diet (8% NaCl [1.378 mEq Na/kg]) for fourteen days. Both groups had ad libitum access to water. The Boston University School of Medicine Institutional Animal Care and Use Committee approved all of our protocols, and our procedures were conducted in compliance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

Surgical Procedures

1. Subcutaneous Osmotic Minipump Implantation: All SD rats (except the naïve group) underwent subcutaneous osmotic minipump implantation. Animals were given 20 mg/kg of sodium methohexital intra-peritoneally (i.p.) to anesthetize them and were then implanted subcutaneously with an osmotic minipump (model 2ML2, Alzet, Palo Alto, CA, USA) in the subscapular region. After receiving the pumps, the animals were returned to their home cages.
2. **Acute Femoral Vein, Artery, and Bladder Cannulation:** After fourteen days of living on either a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl), all animals underwent acute femoral vein, artery, and bladder cannulation. Again, 20 mg/kg of sodium methohexital was given i.p. to anesthetize the animals. If necessary, an additional 10 mg/kg i.p. was administered. Cannulas were then inserted into the left femoral artery to measure blood pressure and heart rate, left femoral vein to administer saline and/or drugs intravenously (i.v.), and bladder to monitor renal function. Following surgery the rats recovered in Plexiglas holders and received an i.v. infusion of saline for two hours. This enabled the rats to regain full consciousness and allowed their cardiovascular and renal functions to stabilize. Their blood pressures and heart rates were constantly recorded by means of the femoral artery cannula using computer-driven BIOPAC data acquisition software (BIOPAC, Goleta, CA, USA) connected to an external pressure transducer (model P23XL; Viggo Spectramed Inc., Oxnard, CA, USA).

**Chronic Experimental Treatment Groups**

1. **Naïve Animals:** Naïve SD rats were randomly assigned to receive either a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl) for fourteen days (N=6/group).

2. **Isotonic Saline Infusion:** SD rats were implanted with osmotic minipumps delivering a subcutaneous (s.c.) infusion of isotonic saline (flow rate 5 µL/h)
before being randomly assigned to either a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl) for fourteen days (N = 5/group).

3. **Norepinephrine Infusion**: SD rats were implanted with osmotic minipumps delivering an s.c. infusion of norepinephrine dissolved in isotonic saline (NE; 600 ng/min; flow rate 5 µL/h) before being randomly assigned to either a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl) for fourteen days (N = 6/group).

4. **DMSO/Saline Vehicle Infusion**: SD rats were implanted with osmotic minipumps delivering an s.c. infusion of DMSO/isotonic saline (50:50 solution; flow rate 5 µL/h) before being randomly assigned to either a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl) for fourteen days (N = 6/group).

5. **NE + Hydrochlorothiazide (HCTZ) Infusion**: SD rats were implanted with osmotic minipumps delivering an s.c. infusion of norepinephrine (NE 600 ng/min; flow rate 5 µL/h) in combination with hydrochlorothiazide (HCTZ 4 mg/kg/day; flow rate 5 µL/h) dissolved in DMSO/isotonic saline (50:50 solution) before being randomly assigned to either a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl) for fourteen days (N = 6/group).

6. **NE + Propranolol Infusion**: SD rats were implanted with osmotic minipumps delivering an s.c. infusion of norepinephrine (NE; 600 ng/min; flow rate 5 µL/h) in combination with the beta-adrenergic receptor antagonist propranolol (propranolol 10 mg/kg/day; flow rate 5 µL/h) dissolved in DMSO/isotonic
saline (50:50 solution) before being randomly assigned to either a standard diet (0.4% NaCl) or high-salt diet (8% NaCl) for fourteen days (N = 6/group).

**Acute Experimental Protocols**

After living on a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl) for fourteen days, the SD rats were subjected to the following protocols. The protocols were performed consecutively in a single experiment.

1. **Blood Pressure Measurement**: After a two-hour recovery period, the baseline mean arterial pressure (MAP; measured in mm Hg) of each rat was recorded continuously over a thirty-minute period using the femoral artery cannula. Baseline MAP was calculated as the overall average of the MAP during the thirty-minute period.

2. **Kidney Sodium Transporter Activity**: After the MAP measurement, all rats received a one-hour control i.v. infusion of isotonic saline (flow rate 20 µL/min) followed by a one-hour i.v. infusion of amiloride (2 mg/kg; 20 µL/min) and finished with a one-hour i.v. infusion of amiloride (2 mg/kg; 20 µL/min) combined with hydrochlorothiazide (2 mg/kg; 20 µL/min)\(^{23}\). Heart rate and MAP were continuously monitored during all three of the infusion periods, and the urine was collected in ten-minute intervals in order to assess peak natriuresis to i.v. amiloride or hydrochlorothiazide.

3. **Assessment of Beta-Receptor Blockade**: Upon completion of the kidney sodium transporter activity protocol, the NE + propranolol-infused rats were
given an i.v. bolus (7 µg) of the beta-agonist isoproterenol. The peak changes in blood pressure (peak ΔMAP) and heart rate (Δ heart rate) were measured in order to verify that propranolol successfully blocked the beta-receptors. Both peak ΔMAP and Δ heart rate were defined as the difference between the values of MAP/heart rate immediately before the rats began recovering from isoproterenol administration and the values of MAP/heart rate immediately before isoproterenol was first administered. Once the rats recovered, they underwent the autonomic function protocol in a manner similar to the other animal groups.

4. **Autonomic Function:** Upon completion of the kidney sodium transporter activity protocol on all rats (and the assessment of beta-receptor blockade protocol on NE + propranolol-infused rats), an i.v. bolus of hexamethonium (30 mg/kg) was administered, and the peak response in mean arterial blood pressure (peak ΔMAP) was recorded. Peak ΔMAP was defined as the difference between the MAP immediately before the rats began recovering from hexamethonium administration and the MAP before hexamethonium was administered. Afterwards both kidneys were harvested from the rats and frozen at -80 °C for measurement of NCC proteins, SPAK, and OxSR1 in the outer cortex of the kidney.
Analytical Techniques

Urine volume was measured gravimetrically with the assumption that 1 g = 1 ml. Flame photometry (model 943; Instrumentation Laboratory, Bedford, MA, USA) was used to measure the sodium concentration (in mM) in the urine. Natiruressis (UNaV) was calculated using the following equation: UNaV = (rate of urine excretion (µL/min) x sodium concentration (mmol/L))/1000. Peak natriuresis (peak ΔUNaV) was calculated by taking the average natriuresis of the last two ten-minute intervals of an infusion and subtracting it from the natriuresis of the first ten-minute interval after a drug was administered.

Kidney Cortex Membrane Preparation

The harvested kidneys were collected and stored at -80 °C upon completion of the acute experiment. The kidney cortex tissue (~200 mg) was homogenized on ice in a buffer (10 mM triethanolamine, 250 mM sucrose, 100 mM sodium azide (NaN₃), 10 mM phenylmethanesulfonylflouride (PMSF), and 1 mM leupeptin) using a handheld pestle. The tissue homogenate was centrifuged at 4,000 x g for ten minutes at 4 °C, and the resulting supernatant was collected and further centrifuged at 17,000 x g for an hour at 4°C. Afterwards the membrane pellet was resuspended in 300 µL of the homogenization buffer, and a bicinchoninic acid (BCA) assay was used to quantify the protein content. All membrane preparations were stored at -80 °C.
Determination of Renal Sodium Chloride Cotransporter, SPAK, and OxSR1 Levels

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 an hour and probed overnight at 4 °C with either anti-NCC (1:2000), anti-SPAK (1:2000), or anti-OxSR1 (1:2000) antibodies in 0.5% phosphate-buffered saline with Tween 20 (PBS-T). Afterwards the membranes were exposed to a secondary horseradish peroxidase donkey anti-rabbit IgG (H+L) (1:5000) in 0.5% PBS-T for an hour at room temperature. Chemiluminescence was used to visualize the bound antibodies. Densitometric analysis was performed using Quantity One software (Bio-Rad, Waltham, MA, USA), and band densities were normalized to β-actin (1:5000).

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences among groups were assessed by a two-way analysis of variance (ANOVA), followed by a Tukey post-hoc test to compare variations between groups. Statistical analysis was carried out using the software program GraphPad Prism (v6; GraphPad Software, La Jolla, CA, USA). Statistical significance was defined as p < 0.05.
RESULTS

Effect of Salt and Norepinephrine Infusion on Mean Arterial Pressure

Because the sympathetic nervous system is suspected of playing a role in the pathogenesis of salt sensitivity, we first compared the mean arterial pressure (MAP) values of norepinephrine (NE)-infused Sprague-Dawley (SD) rats with those of naïve and saline (vehicle)-infused SD rats (Figure 4). Diet was also taken into consideration, with animals in each group subsisting on either a standard rodent diet (0.4% NaCl) or a high-salt diet (8% NaCl).

Figure 4. Mean arterial pressures of naïve, saline (vehicle), and NE-infused rats. Increased salt intake fails to increase the blood pressures of naïve and saline-infused rats. NE infusion elevates MAP. A high-salt diet exacerbates this effect. (*p < 0.05 vs. respective naïve group; †p < 0.05 vs. respective saline (vehicle) group; ‡p < 0.05 vs. NE + normal salt; mean ± SEM; N = 5-6.)

Because SD rats are naturally salt-resistant, a high-salt diet failed to significantly increase the MAP in naïve and saline-infused rats. Rats infused with NE experienced
elevated blood pressure while living on a standard diet. A high-salt diet intensified the hypertensive effect of NE.

**Effect of Salt and Norepinephrine on Response to Hexamethonium**

To determine how much of the elevated blood pressure was due to vascular vasoconstriction we administered an i.v. bolus of the ganglionic blocker hexamethonium and measured the peak changes in MAP (peak ΔMAP) across the six groups (Figure 5).

![Graph showing the effect of hexamethonium on MAP (peak ΔMAP) across different groups](image)

**Figure 5. Hexamethonium-induced depressions of MAP in naïve, saline, and NE-infused rats.** NE-infused rats experience the same response to hexamethonium regardless of their diet. (*p < 0.05 vs. respective naïve group; ′p < 0.05 vs. respective saline group; mean ± SEM; N = 5-6.)

NE-infused rats on both a standard and high-salt diet displayed similar hexamethonium-evoked drops in MAP. Their responses exceeded those of the naïve and saline-infused rats on both standard and high-salt diets.
Effect of Salt and Norepinephrine on Sodium Transporter Activity

Because recent studies suspect that the sympathetic nervous system exerts some of its hypertensive effects by upregulating sodium transporter activity in the kidneys,\textsuperscript{15,42} we investigated the activities of the epithelial sodium channel (ENaC) and the sodium-chloride cotransporter (NCC). In order to assess sodium transporter activity, we administered infusions of specific sodium transporter blockers and measured their peak natriuretic responses (peak ΔUNaV).

To determine ENaC activity, we gave the rats the ENaC antagonist amiloride (Figure 6).

![Figure 6. Natriuretic response to amiloride in naïve, saline, and NE-infused rats. NE-infused rats have a response to amiloride similar to the response of naïve rats. (*p < 0.05 vs. respective normal salt group; mean ± SEM; N = 5-6.)](image)

In native SD rats a high-salt diet has been found to downregulate ENaC; thus amiloride only elicits a mild natriuresis in animals on a high-salt diet. However, as shown in Figure 6, naïve and NE-infused rats had a large response to amiloride when on a
standard diet (indicating high ENaC activity) and a blunted response when on a high-salt diet (indicating low ENaC activity).

NCC activity was measured by i.v. infusion of the NCC antagonist hydrochlorothiazide (HCTZ) (Figure 7).

![Figure 7. Natriuretic response to HCTZ in naïve, saline, and NE-infused rats. NE-infused rats experience no salt-induced suppression of NCC activity. (*p < 0.05 vs. respective normal salt group, †p < 0.05 vs. respective saline group; mean ± SEM; N = 5-6.)](image)

NCC activity was downregulated in naïve and saline-infused rats living on a high-salt diet. However, in the presence of NE, a high-salt diet failed to downregulate NCC activity.

**Effect of Norepinephrine on NCC, SPAK, and OxSR1 Levels**

After determining the degree of NCC activity, we quantified the expression of NCC proteins in the kidney. To do this, we harvested the kidney cortexes of the saline-infused and NE-infused rats and measured their levels of NCC expression (Figure 8).
Figure 8. Expression of NCC proteins in the kidney cortex of saline and NE-infused rats. A high-salt diet results in no downregulation of NCC proteins in NE-infused rats. (ODU, optical density units; *p < 0.05 vs. saline + normal salt group; mean ± SEM; N = 5-6.)

A high-salt diet decreased the expression of NCC proteins in the saline-infused rats. However, infusion of NE prevented excessive salt from downregulating NCC protein expression. As a follow-up we measured the levels of SPAK and OxSR1, two known phosphorylators of NCC (Figures 9 and 10).
Figure 9. Expression of SPAK in the kidney cortex of saline and NE-infused rats. SPAK drops in response to excess salt in saline-infused rats. A high-salt diet fails to downregulate SPAK in NE-infused rats. (ODU, optical density units; *p < 0.05 vs. respective normal salt group; †p < 0.05 vs. respective saline group; mean ± SEM; N = 5-6.)
In saline-infused rats a high-salt diet significantly downregulated SPAK but not OxsR1. NE-infused rats experienced no salt-induced suppression of SPAK and OxsR1 and exhibited elevated levels of OxsR1.

**Effect of Norepinephrine and Hydrochlorothiazide Co-Infusion on Mean Arterial Pressure**

NCC is believed to command a significant influence on blood pressure as a result of the hypertensive effects observed when it is overactive (Gordon’s syndrome) and the
hypotensive effects when it is impaired (Gitelman’s syndrome)\textsuperscript{14,42}. To test this influence we gave SD rats a co-infusion of NE combined with the NCC antagonist hydrochlorothiazide (HCTZ), assigned them to either a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl) for two weeks and compared their blood pressures (MAP values) with those of 50:50 DMSO/saline-infused (control) and NE-infused rats (Figure 11).

![Figure 11. Mean arterial pressures of DMSO, NE, and NE + HCTZ-infused rats. Co-infusion of HCTZ with NE eliminates the salt-sensitive component of NE-mediated hypertension. (*p < 0.05 vs. respective normal salt group, †p < 0.05 vs. respective DMSO/saline group, ‡p < 0.05 vs. NE + high salt group; mean ± SEM; N = 6.)](image)

Infusion of norepinephrine significantly increased blood pressure, and excess salt exacerbated this effect. The addition of HCTZ produced a heightened blood pressure similar to that of the NE-infused rats living on a standard diet. Diet did not cause a noteworthy variation of blood pressure within the NE + HCTZ group.
Effect of Norepinephrine and Hydrochlorothiazide Co-Infusion on the Response to Hexamethonium

To determine the extent of the contribution of vascular vasoconstriction to the blood pressures of DMSO, NE, and NE + HCTZ-infused rats, we gave them an i.v. bolus of hexamethonium and measured their peak depressions in MAP (peak ΔMAP) (Figure 12).

![Graph showing peak ΔMAP for different conditions](image)

**Figure 12. Hexamethonium-induced depressions of MAP in DMSO, NE, and NE + HCTZ-infused rats.** NE + HCTZ-infused rats on a standard diet have a response to hexamethonium similar to the control DMSO-infused rats. A high-salt diet in NE+HCTZ animals leads to a heightened response resembling the NE-infused rats. (*p < 0.05 vs. respective DMSO vehicle group; mean ± SEM; N = 6.)

NE + HCTZ co-infusion caused the rats on a standard diet to have a response to hexamethonium similar to the responses seen in the DMSO-infused rats. By contrast, a high-salt diet led the NE + HCTZ-infused rats to respond in a manner comparable with the NE-infused rats.
Effect of Norepinephrine and Hydrochlorothiazide Co-Infusion on Sodium Transporter Activity

To ascertain the effects of chronic NE + HCTZ co-infusion on ENaC and NCC activity, we gave the rats infusions of amiloride and HCTZ and measured their peak natriuretic responses (peak ΔUNaV) (Figures 13 and 14).

Figure 13. Natriuretic response to amiloride in DMSO, NE, and NE + HCTZ-infused rats. NE and NE + HCTZ-infused rats experience diet-evoked suppression of ENaC activity. (*p < 0.05 vs. respective normal salt group; mean ± SEM; N = 6.)

Figure 14. Natriuretic response to HCTZ in DMSO, NE, and NE + HCTZ-infused rats. Rats co-infused with NE and HCTZ have a significantly diminished natriuretic response to HCTZ compared with DMSO and NE-infused rats. (*p < 0.05 vs. respective normal salt group, τp < 0.05 vs. respective DMSO/saline group, φp < 0.05 vs. respective NE group; mean ± SEM; N = 6.)
NE and NE + HCTZ-infused rats exhibited similar natriuretic responses to amiloride while on a standard diet and experienced an attenuated natriuresis when placed on a high-salt diet. These groups, however, differed in their responses to HCTZ. Rats infused solely with NE had a high natriuresis regardless of diet, whereas the co-infusion of HCTZ with NE led to severely blunted natriuretic responses regardless of diet.

**Effect of Norepinephrine and Propranolol Co-Infusion on Mean Arterial Pressure**

Recent studies claim that activation of the beta2-adrenergic receptors in the distal tubules upregulates NCC, SPAK, and OxSR1. To test this claim we implanted SD rats with osmotic minipumps delivering a simultaneous infusion of the beta-blocker propranolol with NE. After a two-week period of subsisting on either a standard diet (0.4% NaCl) or a high-salt diet (8% NaCl), the NE + propranolol-infused rats exhibited a blockage of their beta-receptors as a result of the chronic infusion of propranolol (see isoproterenol testing in the Appendix). At this time we compared the blood pressures (MAP values) of these rats with the blood pressures of their saline and NE-infused counterparts (Figure 15).
Figure 15. Mean arterial pressures of saline, NE, and NE + propranolol-infused rats. Propranolol eliminates NE-mediated hypertension. (*p < 0.05 vs. respective saline group; †p < 0.05 vs. respective normal salt group; Φp < 0.05 vs. respective NE group; mean ± SEM; N = 6.)

The addition of propranolol abolished the hypertensive effects of norepinephrine. Moreover, diet caused no significant difference in the blood pressures within the NE + propranolol-infused rats.

Effect of Norepinephrine and Propranolol Co-Infusion on Response to Hexamethonium

To quantify the amount of vascular vasoconstriction resulting from co-infusion of NE and propranolol, we administered hexamethonium to the rats and measured their peak drop in MAP (peak ΔMAP) (Figure 16).
Figure 16. Hexamethonium-induced depressions of MAP in saline, NE, and NE + propranolol-infused rats. Co-infusion of NE and propranolol results in a smaller response to hexamethonium than observed for the saline and NE-infused rats. (*p < 0.05 vs. respective saline group, †p < 0.05 vs. respective NE group; mean ± SEM; N = 6.)

Because propranolol blocks all beta-receptors, including those located on the vasculature, NE + propranolol-infused rats exhibited the smallest drop in MAP compared with saline and NE-infused rats.

**Effect of Norepinephrine and Propranolol Co-Infusion on Sodium Transport Activity**

To determine the level of sodium transporter activity in the NE + propranolol-infused rats, we again used amiloride and HCTZ to gauge ENaC and NCC activity, respectively. Their amiloride-evoked natriuresis (peak ∆UNaV) followed the same trend as seen in NE-infused rats; namely, a standard diet produced a high response, and a high-salt diet produced an attenuated response (Figure 17).
Figure 17. Natriuretic response to amiloride in saline, NE, and NE + propranolol-infused rats. NE + propranolol-infused rats have a response to amiloride similar to those found in NE-infused rats. (\(*p < 0.05\) vs. respective normal salt group; mean ± SEM; \(N = 6\).)

Propranolol infusion hardly affected the response of the rats to HCTZ. The NE + propranolol-infused rats on a standard diet had a natriuresis similar to those seen in the saline groups. A high-salt diet failed to downregulate their natriuretic response, which resembled that belonging to the NE-infused rats (Figure 18).

Figure 18. Natriuretic response to HCTZ in saline, NE, and NE + propranolol-infused rats. NE + propranolol-infused rats exhibit a natriuretic response to HCTZ that refuses to be downregulated by excess salt. (\(*p < 0.05\) vs. respective saline group, \(^\tau\) \(p < 0.05\) vs. respective normal salt group; mean ± SEM; \(N = 6\).)
Effect of Norepinephrine and Propranolol Co-Infusion on NCC, SPAK, and OxSR1 Levels

After measuring their natriuresis, we harvested the kidney cortexes of the NE + propranolol-infused rats, quantified their expression of NCC proteins, and compared them with those of the saline and NE-infused rats (Figure 19).

![Expression of NCC proteins in the kidney cortex of saline, NE, and NE + propranolol-infused rats.](image)

Previously we found that infusion of NE alone prevented the salt-evoked suppression of NCC protein expression. However, the addition of propranolol resulted in
a significant decrease in NCC protein expression in response to a high-salt diet. To explore this further we measured the levels of SPAK and OxSR1 (Figures 20 and 21).

![Graph showing expression levels of SPAK and β-actin in different groups of rats](image)

**Figure 20. Expression of SPAK in the kidney cortex of saline, NE, and NE + propranolol-infused rats.** Rats co-infused with NE and propranolol exhibit attenuated levels of SPAK expression compared with NE-infused rats. (ODU, optical density units; *p < 0.05 vs. respective normal salt group; †p < 0.05 vs. respective saline group; ‡p < 0.05 vs. respective NE group; mean ± SEM; N = 6.)
Figure 21. Expression of OxSR1 in the kidney cortex of saline, NE, and NE + propranolol-infused rats. Rats infused with NE and propranolol have a lower expression of OxSR1 compared with NE-infused rats. (ODU, optical density units; p < 0.05 vs. respective NE group; mean ± SEM; N = 6.)

Blocking the beta-receptors led the NE + propranolol-infused rats to have significantly lower levels of SPAK and OxSR1 than observed in the NE-infused rats, regardless of their diet.
DISCUSSION

Key Findings in Norepinephrine-Infused Rats

Our results further support the contribution of the sympathetic nervous system (SNS) in the development of salt-sensitive hypertension. Chronic infusion of norepinephrine (NE) generated hypertension in the classically salt-resistant Sprague-Dawley (SD) rat, and a high-salt diet magnified this effect. However, this rise in blood pressure cannot be attributed to renal mechanisms alone. Because the osmotic-mini pumps implanted in the rats were delivering NE on a systemic level, the observed effects were not the same as they would have been if NE was solely being produced locally at the kidneys through the renal efferent sympathetic nerve terminals.\textsuperscript{31,39} The ganglionic blocker hexamethonium allowed us to determine how much of the hypertension was due to vasoconstriction of the vasculature. We found that although the NE-infused rats on a high-salt diet had a greater MAP than their standard diet counterparts, the responses of both groups to hexamethonium were nearly identical. This indicated that the greater MAP in the high-salt group was due to more than just changes in vascular tone.

We then used amiloride and hydrochlorothiazide (HCTZ) to gauge the activity of the sodium transporters ENaC and NCC, respectively. Both naïve and NE-infused rats demonstrated decreased natriuretic responses to amiloride when placed on a high-salt diet, implying that increased sympathetic nervous system activity did not interfere with diet-evoked suppression of ENaC. However, when we gave the rats HCTZ, only the naïve and saline-infused rats experienced a high-salt mediated attenuation in their
natriuretic responses. Rats infused with NE exhibited a natriuretic response to HCTZ that could not be suppressed by a high-salt diet. Also for the NE-infused rats, we found that a high-salt diet failed to downregulate the expression of NCC and its phosphorylators SPAK and OxSR1. Therefore, it appears that chronic norepinephrine infusion, in mimicking overactive SNS activity, impedes the downregulation of both NCC function and expression during periods of excessive salt consumption.

The Sympathetic Nervous System Dysregulates NCC When Contributing to Salt-Sensitive Hypertension

To confirm that NCC is the primary transporter which the SNS dysregulates when contributing to salt-sensitive hypertension, we chronically antagonized NCC by co-infusing SD rats with NE and HCTZ. As a result of this treatment, rats on a standard diet developed similar blood pressures to those of the NE-infused rats subsisting on a standard diet, but rats on a high-salt diet failed to exhibit the significantly increased pressures shown by their NE-infused counterparts. These trends suggested that antagonizing NCC eliminated the salt-sensitive component of NE-mediated hypertension. Rats co-infused with NE and HCTZ also displayed amiloride-evoked natriureses similar to those of the NE-infused rats. Thus blocking NCC did not prevent the salt-induced suppression of ENaC. Based on our animal model, we can surmise that excessive SNS activity dysregulates NCC to drive the development of salt-sensitive hypertension.
Propranolol Partially Counters the Effects of Norepinephrine Infusion

Our data in part support the propositions of Mu and Ellison that the SNS activates the beta-adrenergic receptors to dysregulate NCC function and expression. When SD rats were co-infused with NE and the beta-antagonist propranolol, no significant rise in blood pressure occurred. Curiously, propranolol did not correct the dysregulation of NCC activity. Despite their beta-receptors being blocked, NE + propranolol-infused rats reacted with a heightened natriuresis to HCTZ similar to the response in the NE-infused rats. However, upon quantifying the expression of NCC, SPAK, and OxSR1 in the kidney, we found that NE and propranolol co-infusion substantially lowered the expression of all proteins compared with NE infusion alone. Since our measurement of NCC proteins did not differentiate between phosphorylated and non-phosphorylated states, it is possible that in the NE + propranolol-infused rats, all of the observed NCC proteins were phosphorylated, allowing function to remain high despite lowered protein count. The implications of this are two-fold: (1) beta-adrenergic receptor activation can prevent the salt-induced downregulation of NCC activity and expression of its proteins, SPAK, and OxSR1, and (2) NE may not solely rely on the beta-receptors to keep NCC activity high.

Possible Influence of the Alpha-Adrenergic Receptors

The study by Mu and colleagues did not investigate the role of the alpha-adrenergic receptors in the regulation of NCC. Previous studies have already shown that the alpha-receptors can facilitate sodium retention either by increasing renin release or by
directly stimulating sodium reabsorption in the proximal tubules.\textsuperscript{34,39} Our chief concern lies in their possible actions in the distal tubules (DTs). The distribution of alpha- and beta-adrenergic receptors in the DT cells varies. Some cells only have beta2-receptors; others have a mix of alpha-, beta1-, and beta2-receptors.\textsuperscript{39} When we co-infused rats with NE and propranolol, expression of NCC proteins, SPAK, and OxSR1 dropped, yet NCC activity remained at a level comparable with that of the NE-infused rats. We hypothesize that the alpha-receptors may be responsible for this action by operating through a WNK4-dependent process, which is independent of the beta-receptors and SPAK/OxSR1 (Figure 22).

![Figure 22. Possible alternative adrenergic influence on NCC. Beta-adrenergic receptors may not be the only conduit through which norepinephrine affects NCC. The alpha-receptors may be able to prevent the downregulation of NCC activity through a WNK4-dependent process.](image)

It should be mentioned that Ellison and coworkers did investigate the actions of the alpha-receptors on NCC activity. In this study mice were given either an alpha- or beta-agonist and had their levels of phosphorylated NCC (pNCC) measured thirty
minutes later. The beta-agonist group displayed a significant increase in pNCC, while the alpha-agonist group did not. When the mice were given both alpha- and beta-agonists simultaneously, they exhibited the greatest elevation of pNCC, which suggests a synergistic interaction between the two receptors. What concerns us in this experiment is that the alpha-receptors were only tested under acute conditions, and whether these results are applicable to a chronic model is unclear.

**Limitations of Study and Future Directions**

Although our study generated useful data, the work lacks a firm grasp on the role of the alpha-adrenergic receptors during the regulation of NCC under conditions of chronic NE elevation. Future experiments could address this issue by chronically co-infusing the rats with NE and the alpha-antagonist prazosin. In addition, because propranolol is a non-specific beta-antagonist, this study did not differentiate the effects of the beta1-receptors from those of the beta2-receptors. Chronically infusing the rats with NE and the beta1-antagonist atenolol could remedy this situation in future work.

Our study examined how elevated NE coupled with a high-salt diet induced salt-sensitive hypertension in the salt-resistant Sprague-Dawley rat. We also found that activation of a beta-receptor-SPAK/OxSR1-NCC pathway led to the inability of excess salt to downregulate the expression of NCC proteins, SPAK, and OxSR1. However, our study, along with the work of Mu and Ellison, has only looked at the beta-receptor pathway in salt-resistant animal species. It would be interesting to determine if this pathway exists in a naturally salt-sensitive species such as the Dahl Salt-Sensitive rat. If
variances in the pathway do exist between the two animal models, this would emphasize the importance of the pathway as a therapeutic target in the treatment of salt-sensitive hypertension.

**Conclusion**

In conclusion, our study further demonstrates that elevated sympathetic nervous system (SNS) activity can give rise to salt-sensitive hypertension. Whereas the experiments led by Mu and Ellison induced salt sensitivity in the salt-resistant mouse model,\textsuperscript{15,42} we were able to do the same in the naturally salt-resistant Sprague-Dawley rat without the aid of bilateral adrenalectomy. In an attempt to mimic the actions of an overactive sympathetic nervous system, we chronically infused the rats with norepinephrine and found that it generated salt-sensitive hypertension to a certain extent by preventing the diet-evoked suppression of both NCC activity and the expression of NCC proteins, SPAK, and O\textsuperscript{x}SR\textsubscript{1}. To see if NE brought about these changes through a beta-adrenergic receptor pathway, we co-infused the rats with NE and the beta-antagonist propranolol. This condition abolished the hypertensive influence of NE and attenuated the expression of NCC proteins, SPAK, and O\textsuperscript{x}SR\textsubscript{1}. However, NCC activity remained elevated. Thus we can say that our data partially support the claims of Mu and Ellison. It does appear that the SNS can dysregulate NCC by activating the beta-receptors. The inability of beta-antagonism to lower NCC activity suggests that NE does not rely solely on the beta-receptors to affect NCC. The presence of alpha-adrenergic receptors in the
distal tubules implies that NE may activate them to affect NCC function and expression, and future studies will investigate this mechanism.
APPENDIX

The inability of isoproterenol to significantly increase both blood pressure and heart rate for NE + propranolol-infused rats indicated that chronic infusion of propranolol had succeeded in blocking the beta-adrenergic receptors (Figures A1 and A2).

Figure A1. Isoproterenol-evoked changes in the mean arterial pressure of NE + propranolol-infused rats. Isoproterenol fails to drastically increase the mean arterial pressure in rats co-infused with NE and propranolol. (MAP, mean arterial pressure; mean ± SEM; N = 6.)
Figure A2. Isoproterenol-evoked changes in the heart rate of NE + propranolol-infused rats. Isoproterenol fails to significantly increase heart rate in NE + propranolol-infused rats. (BPM, beats per minute; mean ± SEM; N = 6.)
LIST OF JOURNAL ABBREVIATIONS

Am J Clin Nutr................................................. The American Journal of Clinical Nutrition
Am J Med.......................................................... The American Journal of Medicine
Am J Physiol ...................................................... The American Journal of Physiology
Am J Physiol Renal Physiol............... American Journal of Physiology. Renal Physiology
Ann Med .......................................................... Annals of Medicine
Cardiovasc Ther ................................................ Cardiovascular Therapeutics
Cell Mol Life Sci ........................................... Cellular and Molecular Life Sciences: CMLS
Clin Exp Pharmacol Physiol .....Clinical and Experimental Pharmacology and Physiology
Curr Hypertens Rep ....................................... Current Hypertension Reports
Dtsch Arztebl Int.............................................. Deutsches Ärzteblatt International
Exp Cell Res ....................................................... Experimental Cell Research
J Am Coll Cardio ............................................. Journal of the American College of Cardiology
J Am Soc Nephrol............................. Journal of the American Society of Nephrology
J Cardiovasc Pharmacol.......................... Journal of Cardiovascular Pharmacology
J Clin Invest ..................................................... The Journal of Clinical Investigation
J Pharmacol Sci............................................. Journal of Pharmacological Sciences
Kidney Int ........................................................... Kidney International
Nat Med ............................................................. Nature Medicine
Naunyn Schmiedebers Arch Pharmacol .......... Naunyn-Schmiedeberg's Archives of Pharmacology
Pflugers Arch ...................................................... European Journal of Physiology
Physiol Rev .......................................................... Physiological Reviews

Proc Natl Acad Sci USA ............... Proceedings of the National Academy of Sciences of the United States of America

Sci Signal ............................................................... Science Signaling
REFERENCES


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CURRICULUM VITAE

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SUMMARY:
• Currently enrolled in Boston University School of Medicine’s Masters of Medical Science Program.
• Graduated with a B.A. in Biology and Pre-Medicine.
• Significant exposure to cadaver dissection, surgery, and the ER.
• Strong background in the performance arts.

EDUCATION:

Master’s of Science in Medical Science, August 2013 to Present
Boston University School of Medicine, Boston, Massachusetts
Current GPA: 3.49

Courses taken included:

Human Physiology       Biochemistry
Pharmacology           Cellular Organization of Tissues
Biostatistics          Biomedical Information

Bachelor of Arts in Biology and Pre-Medicine, May 2012
Augustana College, Rock Island, Illinois
Graduated with a cumulative GPA of 3.19

Courses taken included:

Cadaver Dissection       Human Physiology
Neuroanatomy             Human Anatomy
Genetics                 Elementary Greek
Parasitology             Early Modern/Renaissance Poetry
EXPERIENCE:

Masters Research Program, September 2014 to Present
Whitaker Cardiovascular Institute, Boston, Massachusetts

• Spent 30-40 hours/week assisting post-doctorate students in researching salt-sensitive hypertension.
• Helped gather and analyze data.

Emergency Room Volunteer, October 2012 to June 2013
Northwest Community Hospital, Arlington Heights, Illinois

• Volunteered 4-8 hours per week in Northwest Community Hospital’s emergency room.
• Transported patients to and from CT, MRI, X-Ray, and Ultrasound departments.
• Ensured the comfort of patients.

Trinity Hospital Internship, November 2011 to February 2012
Trinity Medical Center, Bettendorf, Iowa

• Shadowed Dr. Nathan Fierce, a general surgeon, eight hours per week.
• Accompanied Dr. Fierce as he conducted patient rounds.
• Attended seminars on breast cancer research and new methods of teaching upcoming surgeons.
• Watched several surgical procedures from a scrubbed in perspective.

Shadowing at Asperius Hospital, August 2010
Asperius Hospital, Wausau, Wisconsin

• Shadowed Dr. William Mason, an anesthesiologist on a daily basis.
• Gained exposure to general, cardiovascular, and orthopedic surgical procedures.
ACTIVITIES:

Warden, Phi Mu Alpha Sinfonia, April 2010 to May 2012

• Served on Phi Mu Alpha’s executive board as Warden for two years.
• Maintained the decorum of weekly fraternity meetings.
• Oversaw the production of the fraternity’s bi-annual initiation ceremony. This required casting and coaching the actors, scheduling rehearsal times, and creating props and costumes.

Member, Phi Mu Alpha Sinfonia, November 2008 to May 2012

• Member of Phi Mu Alpha, a national music fraternity, for four years.
• Participated in volunteer projects, fundraisers, and campus competitions.

Theatre, September 2009 to February 2012

• Acted in several of Augustana’s theatre productions, ranging from main-stage productions to comedy shows to one-act plays.
• Cast, directed, and presented a one-act play.

Augustana Chamber Strings, November 2011 to May 2012

• Performed in Augustana’s classical guitar ensemble.

EMPLOYMENT HISTORY:

Server, iPic Theatres, December 2012 to August 2013
South Barrington, Illinois

• Worked as a waiter at a hybrid movie theatre/restaurant. Responsibilities included the management of up to eight tables simultaneously, money handling, and general cleaning tasks.
Server, Cubby Bear North, June 2012 to December 2012  
Lincolnshire, Illinois

• Worked as a waiter at a sports bar. Took care of customers’ orders, handled money, and cleaned.

Dining Services, Augustana College, Spring 2010, Fall 2010, Fall 2011  
Rock Island, Illinois

• Cooked and served food in Augustana’s cafeteria.

Lifeguard, Aquaguard Management, Summer 2010, Summer 2011  
Vernon Hills, Illinois

• Ensured the safety of pool patrons. Certified in aquatic rescue and CPR. Maintained the pool’s chemical and pH levels.

Maintenance Worker, Christian Liberty Academy, Summer 2008  
Arlington Heights, Illinois

• Maintained the upkeep of the school’s grounds, cleaned the lockers, floors, carpets, classrooms, etc… Helped construct the school’s new playground.

INTERESTS:

• Guitar
• Weight lifting
• Nutrition
• Reading
• Theatre/Film