Pathophysiology of age-dependent hypertension in male Sprague-Dawley rats
PATHOPHYSIOLOGY OF AGE-DEPENDENT HYPERTENSION IN
MALE SPRAGUE-DAWLEY RATS

by

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PATHOPHYSIOLOGY OF AGE-DEPENDENT HYPERTENSION IN
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JERRY DU HAI
ABSTRACT

Background: Hypertension is a major health concern with a myriad of possible causes. Sodium is a major component of blood pressure regulation implicated in the maintenance of fluid volume. The blood pressure response to changes in salt intake varies considerably among individuals; salt-sensitive individuals exhibit increases in blood pressure parallel to elevated sodium intake, whereas salt-resistant individuals maintain constant blood pressure regardless of variations in sodium intake. Salt-Sensitive Hypertension (SSH) develops due to an impairment of normal mechanisms that react to an elevated sodium load. Aging is another major risk factor for the development of hypertension. The effects of aging have a profound impact on the cardiovascular, renal, and nervous systems, which work together to regulate blood pressure. The development of SSH and impact of aging on blood pressure have been well-established, but the neurophysiological mechanisms implicated in SSH and aging, have only been recently explored.

Objective: To provide mechanistic insight regarding the integrated roles of the renal and nervous systems in age-dependent hypertension in male Sprague-Dawley (SD) rats.

Methods: Male SD rats of various ages were randomly assigned to various experimental protocols. 3-month-old male SD rats were randomly assigned to
receive Renal-Capsaicin surgery (Renal-CAP) to ablate the afferent renal nerve (ARN) or sham surgery, followed by an acute 5% body weight i.v.-isotonic volume expansion protocol (5%-VE) in which natriuretic response and cardiovascular functions (HR/MAP) were continuously monitored and analyzed for the duration of the experimental period. 3/8/16-month-old male SD rats without Renal-CAP surgery were similarly exposed to the 5%-VE protocol. C-fos immunohistochemistry (c-Fos IHC) was performed on brain slides prepared from rats assigned to the 5%-VE protocol to assess PVN parvocellular neuronal activation, as a marker for ARN activity. 3/8/16-month-old male SD rats on normal-salt and high-salt (NS/HS; 0.6%/4% sodium chloride respectively) diets were assigned to an amiloride and hydrochlorothiazide protocol (AM-HCTZ) to evaluate NCC activity, and exposed to i.p. hexamethonium to account for the sympathetic contribution to blood pressure. Renal/plasma NE content was assessed via ELISA to further account for sympathetic tone. Immunoblotting was performed on 3-month-old control saline-infused, s.c.-norepinephrine (NE), and s.c.-norepinephrine+terazosin/propranolol male SD rats to assess NCC, phosphorylated NCC (pNCCT58), SPAK, WNK1, OxSR1, and phosphorylated OxSR1 protein levels, to evaluate the roles of the α1/β-adrenoceptors in the NCC-implicated ARN-mediated sympathoinhibitory pathway.

**Results:** In response to the 5%-VE protocol, the natriuretic response was attenuated in Renal-Cap rats. Renal-CAP rats also experienced a significant increase in MAP in response to 5%-VE, while sham Renal-CAP rats did not. Both
Renal-CAP and sham Renal-CAP rats experienced a significant increase in the mean number of PVN Fos-positive cells (Fos+) post-expansion, although the increase in Fos+ cells in Renal-CAP rats was smaller in magnitude in comparison to their sham Renal-CAP counterpart. An increase in MAP and decrease in urinary volume/sodium excretion was observed in male SD rats of increasing age in response to 5%-VE. An increase in the mean number of Fos+ cells was observed post-expansion in all age groups, while 3-month-old rats experienced a larger increase in Fos+ cell count relative to 8-month-old rats.

An increase in MAP was observed in rats of increasing age on NS-intake. An increase in MAP was also observed in rats of the same age with an increase in dietary salt intake from a NS to HS-intake. An increase in MAP drop due to hexamethonium was observed in rats on HS-intake and of older age. An increase in renal/plasma NE content was observed in rats of increasing age on NS-intake, and increases in salt intake in the same age group led to a significant increase in NE content.

An increase in NCC activity was observed in s.c.-NE/Renal-CAP 3-month-old rats in response to HS-intake, and in rats of increasing age on NS-intake. In response to HS-intake, a decrease in NCC activity occurred in rats treated with terazosin but not propranolol. Furthermore, a decrease in NCC/pNCC/WNK1/pOxSR1 expression was observed in rats treated with terazosin in response to HS-intake, whereas protein levels in rats treated with propranolol varied independently of dietary salt intake.
**Conclusion:** This present study has provided abundant evidence regarding the integrated roles of renal sodium handling and the ARN-mediated sympathetic tone in the pathophysiology of age-dependent hypertension in male SD rats. Through the demonstration of a hypertensive mechanism, involving an impairment of fluid and electrolyte homeostasis that implicates the renal, cardiovascular, and nervous systems, this study provides a crucial stepping stone for the development of mechanistic treatments for age-dependent hypertension in elderly individuals with elevated sympathetic tone.
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LIST OF ABBREVIATIONS

AM-HCTZ .................. Amiloride Hydrochlorothiazide (Experimental Protocol)
ARN ................................................................. Afferent Renal Nerve
c-Fos IHC .......................................................... cfos-immunohistochemistry
DP ................................................................. dorsal parvocellular
ENaC ................................................................. Epithelial Sodium Channel
Fos+ ................................................................. Fos-positive parvocellular nucleus
HS .......................................................... High Salt Diet (4% NaCl)
HTN ................................................................. hypertension
i.p ................................................................. intraperitoneal
i.v ................................................................. intravenous
LP ................................................................. lateral parvocellular
MP ................................................................. medial parvocellular
NCC ................................................................. Sodium Chloride Cotransporter
ND ................................................................. not determined
NE ................................................................. Norepinephrine
NS ................................................................. Normal Salt Diet (0.4% NaCl)
Pro ................................................................. propranolol
PVN ................................................................. Paraventricular Nucleus
RAAS ................................................................. Renin-Angiotensin-Aldosterone System
Renal-CAP ........................................................ Renal Capsaicin (ARN Ablation) Protocol
s.c ................................................................. subcutaneous
SD ................................................................. Sprague-Dawley
SHR ............................................................... Spontaneously Hypertensive Rat
SSH .............................................................. Salt-Sensitive Hypertension
Teraz ............................................................... Terazosin
VLP ................................................................. ventrolateral parvo cellular
WKY ............................................................... Wistar Kyoto Rat
3V ................................................................. third ventricle
5%-VE ......................................................... Acute Volume Expansion (Experimental Protocol)
INTRODUCTION

The Growing Impact of Hypertension

Hypertension is defined as having a systolic and diastolic blood pressure greater than or equal to 140/90 mmHg respectively (Yoon et al. 2015). It is a significant and growing health concern in both the developing and developed worlds, such that it has been identified as the leading risk factor for mortality (Kearney et al. 2005). An estimated one billion adults worldwide are affected by hypertension, and this number is expected to reach 1.5 billion by 2025 (Imprialos et al. 2016). According to the Framingham heart study, both men and women over the age of 55 have a greater than 90% lifetime risk of developing hypertension (Vasan et al. 2002). A standard classification of hypertension is shown in Table 1 below.

Table 1. Classification of hypertension for adult human population. Table taken and adapted from Herz (Dorner et al. 2013).

<table>
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<th>Category</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
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<td>Optimal</td>
<td>&lt;120</td>
<td>(And) &lt;80</td>
</tr>
<tr>
<td>Normal</td>
<td>120–129</td>
<td>(And/or) 80–84</td>
</tr>
<tr>
<td>Above Normal</td>
<td>130–139</td>
<td>(And/or) 85–89</td>
</tr>
<tr>
<td>Stage 1 Hypertension (low)</td>
<td>140–159</td>
<td>(And/or) 90–99</td>
</tr>
<tr>
<td>Stage 2 Hypertension (intermediate)</td>
<td>160–179</td>
<td>(And/or) 100–109</td>
</tr>
<tr>
<td>Stage 3 Hypertension (high)</td>
<td>≥180</td>
<td>(And/or) ≥110</td>
</tr>
<tr>
<td>Isolated Systolic Hypertension</td>
<td>≥140</td>
<td>(And) &lt;90</td>
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Hypertension is also a major risk factor for cardiovascular and kidney disease, stroke, premature death, and disability (Frame, Carmichael, and Wainford 2016), such that it is responsible for up to 45% of deaths due to heart disease and 51% of deaths due to stroke (World Health Organization 2013). In fact, mortality rates due to heart disease and stroke double with every 20 mmHg increase in systolic blood pressure (SBP) and every 10 mmHg increase in diastolic blood pressure (DBP) (Yoon et al. 2015).

Hypertension is a medical condition with a myriad of possible causes (e.g. dysfunctions in renal mechanisms, sympathetic nervous system, vasculature, hormonal levels, renin-angiotensin-aldosterone system (RAAS), and/or the endothelin system (Elijovich et al. 2016)), and is due to the existence of many moving physiological parts that work in tandem to maintain the normotensive state. Cases of hypertension are often categorized as either primary or secondary hypertension. In secondary hypertension, there usually exists a distinct cause that is responsible for the hypertensive condition. However, the majority of hypertensive cases are of the primary nature, in which there exists no clear cause (Nadar and Lip 2009).

**Impact of Salt on Hypertension**

Salt, namely sodium chloride, is an essential component to one’s daily diet. Sodium is a major component of blood pressure regulation, as it is implicated in the maintenance of fluid volume. Sodium content is balanced through the
involvement of various cardiovascular and renal mechanisms that use ion channels, carriers and pumps to move the cation from one location to another. In the kidney, the sodium chloride cotransporter (NCC), and the epithelial sodium channel (ENaC), located in the renal distal tubules, are crucial in maintaining proper sodium balance (Kong et al. 2016). In the normal physiological state, the NCC/ENaC transporters allow the reabsorption of sodium from the distal tubule. The sodium balance determines the volume of the extracellular fluid, a critical determinant of blood pressure. Dysfunction of one or more mechanisms in blood pressure regulation, such as sodium balance or sympathetic tone, often leads to a higher risk of abnormal blood pressure.

The prevalence of hypertension is escalating in large part due to an increase in dietary salt intake throughout the world; the current estimated dietary salt intake is 9-12 grams per day (g/day) in most countries, significantly higher than the 5 g/day recommended by the World Health Organization (Rust & Ekmekcioglu, 2016). The effect of dietary salt intake on blood pressure has also been well-documented; in the famous INTERSALT study, a 100 mmol increase in urinary sodium excretion was shown to correlate with a 3-6 mmHg increase in systolic blood pressure (Kjeldsen et al. 2017). Furthermore, in an analysis of the impact of salt consumption on cardiovascular disease, a reduction of 2.30 grams of sodium per day was associated with a mean arterial pressure (MAP) reduction of 3.82 mmHg (Mozaffarian et al. 2014). In addition to its direct effects on blood pressure regulation, dietary salt intake is also implicated in other functions of the
cardiovascular system, such as endothelial dysfunction, and ventricular hypertrophy (Baldo, Rodrigues, and Mill 2015); this in turn may further exacerbate the impairment of blood pressure regulation due to salt alone. Therefore, hypertension due to dietary salt intake is a definitive global health issue.

**Pathophysiology of Salt-Sensitive Hypertension**

While numerous studies have identified a correlation between salt intake and blood pressure, the blood pressure response to changes in salt intake varies considerably among individuals. As shown in figure 1 (Myron H. Weinberger 1996), increases in dietary salt intake lead to rises in blood pressure in all normotensive individuals, but the extent to which blood pressure rises varies significantly. Thus, individuals are often divided into salt-sensitive and salt-insensitive groups; salt-sensitive individuals exhibit increases in blood pressure parallel to elevated sodium intake, whereas salt-insensitive individuals maintain relatively constant blood pressure levels, regardless of variations in sodium intake (Rust and Ekmekcioglu 2016).
Salt-Sensitive Hypertension (SSH) is estimated to exist in up to 50% of hypertensive individuals (Felder et al. 2013). SSH develops due to an impairment of normal mechanisms that react to an elevated sodium load in the cardiovascular system; an inability to maintain sodium balance results in elevated blood pressure via fluid retention involving the RAAS, the actions of the antidiuretic vasopressin, and various other mechanisms, to maintain fluid volume (Myron H. Weinberger 1996). The specific impairment that leads to SSH is highly variable, and can involve a combination of a lack of activation of...
natriuretic pathways and overactive anti-natriuretic pathways, which result in defective physiological mechanisms.

In particular, the sympathetic nervous system’s interactions with the kidney are essential in maintaining proper fluid and electrolyte homeostasis. It acts via the neurotransmitter norepinephrine (NE) to increase salt and water retention, and thus blood pressure. Multiple studies have implicated NE as a contributor to the activation of the sodium chloride cotransporter (NCC) in SSH (Terker et al. 2014), and more recently, NE has been shown to prevent a dietary sodium-evoked suppression of the NCC, independent of the RAAS (Walsh et al. 2016).

In the kidneys, sodium that reaches the renal distal convoluted tubule is reabsorbed through the operation of the NCC and ENaC; thus, an impairment in the proper functioning of either transporter may also lead to the development of SSH (Pavlov and Staruschenko 2016). Regulation of the NCC in particular involves the activation of adrenergic receptors triggered by NE. This in turn leads to the activation of a signaling cascade involving the WNK kinases, SPAK kinase, and the OxSR1 serine/threonine-protein kinase (Terker et al. 2014); in tandem, WNK, SPAK, and OxSR1 are part of a complex kinase network that regulates NCC activity and expression via phosphorylation of NCC to pNCC (phosphorylated NCC). While previous studies have suggested that β-adrenoceptors stimulate the NCC, and that OxSR1 provides an essential role in modulating NCC activity, the precise mechanisms surrounding the regulation of the NCC remain unknown.
(Terker et al. 2014). A proposed schematic representation of NCC regulation is provided in figure 2 below.

**Figure 2. Proposed schematic representation of NCC regulation.** In this model of NCC regulation, the WNK kinases are activated (green arrows; red arrows = inhibition) by aldosterone, angiotensin-II (Ang II), decreased salt (NaCl) intake, and increased potassium (K+) intake. The WNK kinases use the intermediary SPAK/OxSR1 (OSR1) kinases to mediate phosphorylation, and thus activation, of the NCC cotransporter. Figure adapted and taken from *Kidney Int.* (Johns 2013).

While the development of SSH and the body’s mechanisms against it have been well-established, the neurophysiological mechanisms implicated in mediating bodily responses to SSH have only been recently explored. Specifically, the renal sympathetic nerves (RSN), and in particular, the afferent renal nerve (ARN), has been recently implicated in the long-term regulation of blood pressure and pathophysiology of SSH (Johns 2013). Afferent renal nerves carry information from renal chemoreceptors, which respond to sensory mechano- and
chemoreceptors in the renal pelvis, leading to a reno-renal reflex that evokes sympathoinhibition (Frame, Carmichael, and Wainford 2016). Renal sympathetic outflow has been shown to be regulated via the hypothalamic paraventricular nucleus (PVN) in fluid volume expansion (Ramchandra et al. 2013), and the ARN in particular has also been shown to evoke neural responses in the PVN (Xu et al. 2015). Moreover, central brain Gα-pathways have been identified as being essential in facilitating fluid and sodium homeostasis, and thus normotension, in response to acute physiological stresses and/or chronic changes in dietary salt intake (Carmichael and Wainford 2015). Dietary high salt (HS) intake has been shown to trigger upregulation of Gαq-protein concentrations in the paraventricular nucleus (PVN) in Dahl Salt-Sensitive rats, suggesting that their salt-sensitive phenotypes may be caused in part by reduced Gαq-protein concentrations, which have a critical counter-regulatory role in preventing vasopressin hypersecretion in salt-resistant phenotypes (Wainford and Kapusta 2010).

**Impact of Aging on Hypertension**

Aging has been a well-documented risk factor for the development of various forms of hypertension (Rust and Ekmekcioglu 2016). The effects of aging are multifaceted and complex, and have a profound impact on the proper functioning of the cardiovascular, renal, and nervous systems. These various organ systems work in tandem to regulate blood pressure, and abnormalities due
to aging can affect one or more mechanisms implicated in blood pressure regulation, leading to an increase in risk factors associated with hypertension.

The impact of aging on SSH in particular has been well-documented in the aforementioned INTERSALT Study. Salt sensitivity was shown to be positively correlated with age, as shown in figure 3, and more so in already hypertensive individuals than normotensive one. Moreover, salt-sensitive individuals were observed to have an elevated blood pressure significantly higher after 10 years when compared with salt-resistant individuals (M. H. Weinberger and Fineberg 1991). Thus, aging is a major risk factor in the diagnosis and treatment of SSH, one that involves various physiological mechanisms.

**Figure 3. Change in salt sensitivity as a function of age in normotensive and hypertensive subjects.** Salt sensitivity, which increased with age, was measured as the change in MAP resulting from sodium depletion following volume expansion. There was a significant difference between the salt sensitivity of normotensive (white bars) and hypertensive (dashed bars) subjects in the 31-40, 41-50, and 51-60 age groups. Figure taken from *Hypertension*, American Heart Association Journal (M. H. Weinberger and Fineberg 1991).
In the cardiovascular system, aging has been associated with arterial stiffness, wall thickening due to endothelial dysfunction, and cardiac dysfunction, which may all contribute to an inability to properly regulate blood pressure. These developments are caused primarily by the loss of smooth muscle cells, calcification of the tunica intima and tunica media layers of blood vessels, and increased collagen deposition in vessel walls (Dao et al. 2005). As a result, the ability of the body to regulate blood pressure deteriorates.

In the kidneys, aging has been associated with declines in renal function with various causes. On average, kidney mass has been observed to decrease by 10% for each decade of aging past the fifth decade; furthermore, creatinine clearance has been shown to decline 30-40% in individuals over the age of 80 (Duarte, Santos-Araújo, and Leite-Moreira 2011). These observations are often caused by disruptions to glomerular filtration (nonfunctional glomeruli, glomerulosclerosis), and various fluid/electrolyte disturbances may be caused by a decline in activity of both the glomeruli and of the NCC/ENaC transporters as well as impairments in renin release by the juxtaglomerular cells (Epstein 1996). These factors also contribute to abnormal blood pressure regulation, and may potentially lead to SSH.

While the impact of aging on hypertension has been well-established, there remain multiple challenges to the treatment of hypertension in the elderly. While multiple studies have suggested benefits for morbidity and mortality with a blood pressure reduction to $\leq 150/90$ mmHg, there is uncertainty concerning the
benefits of further reduction, as well as concerning blood pressure guidelines for individuals aged 80 or older (Imprialos et al. 2016). As is the case with various other disease states, there are also risks associated with polypharmacy and comorbidities, which increase with age; this is of particular concern in the treatment of hypertension due to the need for treatments involving multiple drug classes to obtain aggressive blood pressure goals (Imprialos et al. 2016). Lastly, the treatment of hypertension in the elderly also commonly involves essential lifestyle modifications, such as the cessation of smoking, alcohol, and increases in dieting and exercise. These lifestyle modifications may prove more difficult in elderly populations, due again to the presence of comorbidities and other potential health-related issues associated with aging (Imprialos et al. 2016).

**Goals of Present Study**

While aging has been definitively associated with the pathogenesis of hypertension (Dorner et al. 2013), there remains a lack of knowledge surrounding the integrated renal and neural mechanisms involved in regulating sodium balance in age-dependent hypertension. Therefore, the present study aims to further explore the effect of aging on the regulation of blood pressure, and fluid and electrolyte homeostasis in male Sprague-Dawley (SD) rats, to gain novel mechanistic insight into the pathophysiology of age-dependent hypertension involving the ARN. An analysis was performed regarding the ability of male SD rats of various ages to manage acute physiological and pharmacological
challenges and on the chronic management of fluid and electrolyte balance, which may prove useful in deriving new therapeutic and mechanistic targets for the treatment of SSH in humans.

The ability to excrete water and sodium through urine was evaluated among rats of various ages in parallel to young, middle-aged, and elderly human ages. Physiologically, acute stresses were presented in the form of volume expansion. The activity of the NCC cotransporter was evaluated pharmacologically. Norepinephrine (NE) content was also evaluated, and c-Fos immunohistochemistry (c-Fos IHC) was performed on various brain slides from male SD rats to estimate activation of PVN parvocellular neurons implicated in the natriuretic response. In addition, NCC expression and regulation was evaluated by immunoblotting to determine the effect of sympathetically-mediated SSH on levels and expression of the NCC and its regulatory kinases (WNK1, OxSR1, SPAK).
SPECIFIC AIMS

1. To evaluate the role of the ARN in the sympathoinhibitory and natriuretic responses to acute i.v. volume expansion in young (3-month-old) male SD rats.

2. To evaluate the effect of age on the sympathoinhibitory reno-renal reflex-mediated natriuretic response to acute i.v. volume expansion.

3. To evaluate the effect of aging and salt intake (lifelong 0.4% sodium chloride normal salt [NS] vs. 21-day 4% sodium chloride high salt [HS] intake) on blood pressure, sympathetic tone, and NCC activity.

4. To evaluate the effect of salt intake (lifelong NS vs. 21-day HS-intake) on NCC regulation in normal young male SD rats, s.c.-NE infused, and renal-CAP (33 mM capsaicin solution wrapped around renal artery and vein) male SD rats.
METHODS

Studies were conducted using male Sprague-Dawley (SD) rats supplied from Harlan Laboratories (Indianapolis, IN, USA). Rats were individually housed in a controlled 20-26°C and 30-70% humidity environment with 12 hour light/dark cycles. Rats were randomly assigned to experimental treatment groups and fed a normal-salt (NS) diet containing 0.6% sodium chloride (Teklad Global Diet, Harlan Laboratories, Indianapolis, IN; Teklad Global 18% protein rodent diet no. 2918, 18% protein, 5% crude fat, 5% fiber, 102 mEq Na+/kg) with tap water readily available, or a high-salt (HS) diet containing 4% sodium chloride (Teklad Global Diet, Harlan Laboratories, Indianapolis, IN; Teklad Global 19% protein rodent diet no. 3095, 19% protein, 5% crude fat, 3% fiber, ~696 mEq Na+/kg). All experimental protocols were approved by the Institutional Animal Care and Use Committee in accordance with the guidelines of the Boston University School of Medicine and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were classified by their age group (3-month-old = young human equivalent, 8-month-old = adult human equivalent, 16-month-old = old human equivalent), for their designated acute experimental protocols (i.v.-isotonic volume expansion or amiloride/hydrochlorothiazide infusion), and for all relevant pre-experimental surgical procedures performed (Renal-CAP surgery, subcutaneous osmotic minipumps).
**Afferent Renal Nerve (ARN) Ablation**

3-month-old male SD rats were randomly assigned to receive Renal-Capsaicin surgery (Renal-CAP; 33 mM Capsaicin in 5% ethanol, 5% Tween 80 and 90% normal saline) surgery. In the Renal-CAP surgery, a flank incision was made, and the left renal artery and vein were exposed through a small hole in the peritoneal membrane. The fat surrounding the renal artery and vein was then dissected away, and parafilm was placed under the vessels. A small piece of gauze soaked in a capsaicin solution was wrapped around the renal artery and vein for 15 min. The gauze and parafilm were removed, the area was dried, and the procedure was repeated on the contralateral side. 3-month-old male SD rats were also randomly assigned to receive sham Renal-CAP surgery, in which the left and right renal nerves were visualized through a midline abdominal incision without physical disruption of the area.

**Subcutaneous Osmotic Minipump Implantation**

Rats were supplied with a subcutaneous osmotic minipump containing a control saline, NE, NE/terazosin, or NE/propranolol at 10 mg/kg/day prior to their dietary assignments. Rats designated for osmotic minipump implantation were anesthetized with sodium methohexital (20mg/kg i.p.) and surgically instrumented with an osmotic minipump (Alzet, osmotic pump model 2ML2, Palo Alto, CA, USA) placed subcutaneously (s.c.) in the subscapular region.
Following implantation, all rats were returned to their home cage following administration of penicillin (300,000 units/ml, 0.3ml).

**Acute Surgical Procedure**

On the day of the study, rats were anesthetized with sodium methohexital (20 mg/kg i.p. supplemented with 10 mg/kg i.v. as required), and surgical implantation of cannulae for the left femoral vein, artery, and bladder, were subsequently performed to allow for intravenous infusions, measurement of heart rate (HR) and mean arterial pressure (MAP), and collection of plasma and urine samples for collection of data regarding renal function.

Following surgical cannulation, rats were placed in a Plexiglas holder and i.v.-infused isotonic saline (20 µL/min) was maintained for a 2 hour surgical recovery period prior to the start of each experimental protocol; this allowed the rat to regain full consciousness, and to stabilize its cardiovascular and renal functions.

During the experimental period, HR/MAP were continuously recorded using a BIOPAC data acquisition software (MP150 and ACKNOWLEDGE 3.8.2, BIOPAC Systems, Inc, Goleta, CA, USA) connected to an external pressure transducer (P23XL; Viggo Spectramed, San Francisco, CA, USA), and urine was collected in 10-minute intervals throughout the duration of the experiment. Following the recovery period, rats were treated with an acute physiological or pharmacological challenge in the form of 5% body weight i.v.-isotonic volume
expansion (5% VE) or i.v.-amiloride and hydrochlorothiazide infusion (AM-HCTZ).

**Acute Volume Expansion Protocol**

For the 5%-VE experimental protocol, HR/MAP were first recorded continuously over a 20-minute control period (20 µL/min i.v.-isotonic saline). This was followed by a 30-minute expansion period in which isotonic saline was infused intravenously equivalent to 5% of the rat’s body weight. This was followed by a 90-minute recovery period in which i.v.-isotonic saline was again infused at a baseline rate of 20 µL/min.

**NCC Activity Protocol**

For the NCC Activity experimental protocol, HR/MAP were first recorded continuously over a 60-minute control period (20 µL/min i.v.-isotonic saline). This was followed by an i.v. infused bolus of amiloride (2 mg/kg) used to block the ENaC, and a 60-minute period of continuous i.v. amiloride infusion (2 mg/kg at 20 µL/min). This was followed by an i.v.-infused bolus of a combined amiloride and hydrochlorothiazide solution (2 mg/kg) and a 60-minute period of continuous i.v.-amiloride/hydrochlorothiazide infusion (2 mg/kg at 20 µL/min).
**Sympathetic Nervous System Protocol**

Following completion of the AM-HCTZ experimental protocol, the peak change in MAP (ΔMAP) in response to hexamethonium (30 mg/kg i.p.) was assessed. Baseline MAP was determined as the average MAP recorded over a 10 minute control period prior to i.p. hexamethonium injection. Post-injection, MAP was monitored for an additional 30 minute period. The peak depressor response was assessed over the post-injection time period, and occurred within 1 minute of the injection.

**Post-Acute Experimental Protocol Data Analysis**

All measurements performed on acute studies were compiled and calculated via Microsoft Excel. Analysis of sodium and potassium urine and plasma content was conducted using a flame photometer (model 943; Instrumentation Laboratories, Bedford, MA). Analysis of urine and plasma osmolality was conducted using a vapor pressure osmometer (Vapro 5600, Wescor, Logan, UT). Urine volume was determined gravimetrically assuming 1g = 1ml. HR/MAP were monitored and recorded continuously throughout the experimental protocol. Renal and plasma NE levels were determined via ELISA (Immuno-Biological Labs America, Minneapolis, MN; cat. no. IB89552).

In the AM-HCTZ protocol, the peak natriuretic response (ΔUNaV; μeq/min) was determined by subtracting the baseline UNaV value from the maximum natriuretic value observed during each hour of drug infusion (i.v.-
amiloride or i.v.-amiloride and hydrochlorothiazide). Baseline UNaV values were determined by averaging the UNaV values from the last two 10-min time points during the previous hours of the study; for the amiloride period, baseline hours were taken from the 40–50 min/50–60 min isotonic saline time slots, and for the amiloride-hydrochlorothiazide period, from the 100–110 min/110–120 min IV-amiloride time slots.

**Post-Acute Experimental Protocol Studies**

Following acute protocol completion, rats were either decapitated while conscious, allowing for the collection of both kidneys, which were immediately frozen at −80°C, or rats were deeply anesthetized with sodium methohexital (10 mg/kg i.v.) and immediately perfused transcardially with 0.2–0.3L of 0.1 m phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 m PBS (4°C, 0.3–0.5L).

**C-Fos Immunohistochemistry (c-Fos IHC) Studies**

Rat 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 (30% sucrose + 30% ethylene glycol + 1% polyvinyl-pyrrolidone in 0.1M PBS) and stored at −20°C until they were processed for immunohistochemistry.
Free-floating sections from each brain were processed for c-Fos IHC using a rabbit polyclonal anti-Fos antibody (Calbiochem, San Diego, CA, USA). Sections were briefly brought to room temperature and rinsed twice for 30 min in 0.1M PBS to remove cryoprotectant. Sections were incubated in 0.3% hydrogen peroxide in distilled water for 30 minutes at room temperature and then rinsed for 30 minutes in 0.1M PBS. Sections were then incubated for 2 hours at room temperature in PBS diluent (3% normal horse serum in 0.1M 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 2 days at 4°C. After two 30 minutes rinses in 0.1M 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 hours 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 Permoun 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 (Paxinos & Watson, 1998, *The Rat Brain in*
Stereotaxic Coordinates, San Diego, CA) and sampled at three rostral–caudal levels (Stocker, Cunningham, and Toney 2004). 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 Institute of Health image software (NIH, Bethesda, MD, USA), and the counts for each PVN subnucleus was averaged for each animal.

After designated acute experimental protocols, rats were sacrificed and kidneys were immediately extracted, harvested, and stored at -80°C. Approximately 200 mg of kidney cortical tissue was homogenized on ice using hand-held pestles and 1.5 ml microcentrifuge tubes containing a homogenizing buffer consisting of 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 minutes at 4°C. The supernatant was collected and centrifuged at 17,000 g for 60 minutes at 4°C. Following centrifugation, the membrane pellet was resuspended in 400 µl of homogenizing buffer, and protein content was quantified via BCA assay. Membrane preparations were stored at −80°C prior to use in western blotting studies.

**Immunoblotting Studies**

For the immunoblotting, protein extracted from membrane preparations of kidney cortex tissue were loaded at a concentration of 40 µg of protein per
lane. Membranes were blocked in 5% milk for 1 hour and probed overnight at 4°C with anti-NCC (1:2,000; Millipore, Billerica, MA; cat. no. AB3553), anti-wnk1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. SC20470), anti-SPAK (1:1000; Abcam; cat. no. AB79045), anti-OxSR1 (1:1000; Abcam; cat. no. AB74003), anti-pNCCT58 (1:1000; (Lee et al. 2013)), or anti-pOxSR1 (1:1000; Abcam; cat. No. AB138655) primary antibody in 0.1% PBS-Tween. On the next day, membranes were exposed to a secondary horseradish peroxidase (HRP) donkey anti-rabbit IgG (1:5,000; Promega, Madison, WI; cat. no. V7951) in 0.1% PBS-Tween for 1 hour at room temperature. Blots were subsequently exposed to ECL reagent for 5 minutes, then bound antibodies were visualized using chemiluminescence (GE signal enhancer; GE, Buckinghamshire, UK).

Densitometric analysis was performed using Quantity One software (Bio-Rad, Hercules, CA). All immunoreactive bands were normalized to β-Actin (1:1000, A2228, Sigma) and semi-quantified via the Quantity One software.
RESULTS

**ARN Ablation and Acute Volume Expansion**

Natriuresis in response to a 5% body weight i.v.-isotonic volume expansion (5%-VE) was significantly attenuated (p<0.05) in selective afferent renal nerve (ARN; via Renal-CAP protocol) ablated 3-month-old male SD rats in comparison to their sham counterpart (figure 4). Additionally, Renal-CAP rats experienced a significant increase (p<0.05) in MAP in response to 5%-VE relative to control baseline values, while sham Renal-CAP rats did not.

*Figure 4. Effect of afferent renal nerve (ARN) ablation via the Renal-CAP protocol on changes in MAP, urine output (V; µL/min), and natriuresis (UNaV; µEq/min) in response to a 5% body weight i.v.-isotonic volume expansion. *: p<0.05 vs. respective group/control baseline values; †: p<0.05 vs. respective sham Renal-CAP counterpart.*
Both Renal-CAP and sham Renal-CAP rats experienced a significant increase (p<0.05) in the mean number (per section) of Fos-positive parvocellular nuclei (Fos+) in all PVN subregions in response to volume expansion, relative to their respective group control/baseline counterparts (figures 5 and 6). However, the increase in Fos+ cell count post-expansion in Renal-CAP rats was smaller in magnitude in comparison to their sham Renal-CAP counterpart, such that 5%-VE exposed Renal-CAP rats exhibited a significant decrease (p<0.05) in Fos+ cells in all PVN subregions relative to its 5%-VE sham Renal-CAP counterpart.

Figure 5. Mean Fos-positive (Fos+) PVN nuclei averaged across all rostral-caudal levels at baseline & 120 minutes post 5%-VE. *: p<0.05 vs. respective group/control baseline values; τ: p<0.05 vs. respective sham Renal-CAP counterpart. DP = dorsal parvocellular, MP = medial parvocellular, VLP = ventrolateral parvocellular, LP = lateral parvocellular
Figure 6. Representative images of sham and Renal-CAP pre- and post-5%-VE PVN Fos+ nuclei. 3V = third ventricle.
**Age-Dependent Acute Volume Expansion**

An increase in MAP was observed in male Sprague-Dawley (SD) rats of increasing age (figure 7). A significant increase (p<0.05) was observed between the MAP of 8-month-old rats in comparison to its 3-month-old counterpart (p<0.05) and between the MAP of 16-month-old rats in comparison to their 8-month-old counterpart. However, there was no observed change in MAP in male SD rats of any age in response to 5%-VE.

**Figure 7. Effect of age on MAP in response to a 5% body weight i.v.-isotonic volume expansion.** τ: p<0.05 vs. respective 3-month-old counterpart; #: p<0.05 vs. respective 8-month-old counterpart.

An increase in urinary volume excretion was observed in male SD rats of all ages in response to a 5%-VE (figure 8). However, a significant decrease (p<0.05) was observed between the percent of urine load excreted of both 8/16-month-old rats when compared to their respective 3-month-old counterparts and
between the percent of urine load excreted of 16-month-old rats in comparison to their 8-month-old counterpart, during the recovery period.

**Figure 8.** Effect of age on urinary volume excretion (measured as percent of load excreted) in response to a 5% body weight i.v.-isotonic volume expansion. τ: p<0.05 vs. respective 3-month-old counterpart; #: p<0.05 vs. respective 8-month-old counterpart.

An increase in urinary sodium excretion was observed in male SD rats of all ages in response to a 5%-VE (figure 9). However, a significant decrease (p<0.05) was observed between the percent of sodium load excreted of both 8/16-month-old rats when compared to their respective 3-month-old counterparts and between the percent of sodium load excreted of 16-month-old rats in comparison to their 8-month-old counterpart, during the recovery period.
Figure 9. Effect of age on urinary sodium excretion (measured as percent of sodium load excreted) in response to a 5% body weight i.v.-isotonic volume expansion. τ: p<0.05 vs. respective 3-month-old counterpart; #: p<0.05 vs. respective 8-month-old counterpart.

**Age-Dependent PVN Activation**

At baseline prior to volume expansion, no difference was observed in the mean number of Fos-positive parvocellular nuclei (Fos+) at all rostral-caudal levels between 3 and 8 month-old groups (figures 10 and 11). Following volume expansion, the Fos+ cell count was significantly higher (p<0.05) in 3-month-old male SD rats relative to their 8/16-month-old counterparts. This difference was most pronounced in the medial parvocellular (MP) level of the PVN.
Figure 10. Mean number (per section) of baseline and 120-minute post-expansion Fos-positive parvocellular nuclei averaged across all rostral-caudal levels. *: p<0.05 vs. respective baseline value; τ: p<0.05 vs. respective 3-month-old counterpart at same time point; #: p<0.05 vs. respective 8-month-old counterpart at same time point. DP = dorsal parvocellular, MP = medial parvocellular, VLP = ventrolateral parvocellular, LP = lateral parvocellular. ND = not determined.
Figure 11. Representative images of PVN fos-positive nuclei in male Sprague-Dawley rats aged 3, 8, and 16 months at baseline and 120 minutes post-volume expansion. 3V = third ventricle.
Aging and Salt Intake

An increase in MAP was observed in male SD rats of increasing age (figure 12). Furthermore, an increase in MAP was observed in male SD rats of the same age with an increase in dietary salt intake from a NS to HS-intake. A significant increase (p<0.05) was observed between the MAP of both NS/HS-intake 8-month-old rats when compared to their respective NS/HS-intake 3-month-old counterparts. A significant increase (p<0.05) was also observed between the MAP of 16-month-old NS-intake rats when compared to its 8-month-old NS-intake counterpart.

Figure 12. Effect of lifelong normal salt (NS; 0.6% NaCl) vs. 21-day high salt (HS; 4% NaCl) intake on MAP in 3/8/16-month-old male SD rats. τ: p<0.05 vs. respective 3-month-old counterpart; #: p<0.05 vs. respective 8-month-old counterpart. ND = not determined.
An increase in the MAP drop (ΔMAP) due to hexamethonium (i.p. 30 mg/kg) was observed in male SD rats of older age on NS-intake (figure 13) relative to its 3-month-old counterpart. HS-intake in 3-month-old rats led to a significant decrease in ΔMAP (p<0.05) relative to its NS-intake counterpart. However, HS-intake in the older 8-month-old rats did not lead to a decrease in ΔMAP in comparison to its NS-intake counterpart.

Figure 13. Peak ΔMAP to i.p. hexamethonium in 3/8/16-month-old male Sprague-Dawley rats on NS/HS diets. *: p<0.05 vs. respective 3-month-old NS control value; τ: p<0.05 vs. respective 3-month-old counterpart. ND = not determined.
An increase in renal NE content was observed in male SD rats of increasing age on NS-intake (figure 14). HS-intake in 3-month-old rats led to a significant decrease (p<0.05) in renal NE content relative to its 3-month-old NS-intake counterpart. However, HS-intake in the older 8-month-old rats did not alter renal NE content significantly, in comparison to its NS-intake counterpart.

Figure 14. Renal norepinephrine content in 3/8/16-month-old male Sprague-Dawley rats on NS/HS diets. Renal NE content was determined via ELISA. *: p<0.05 vs. respective 3-month-old NS control value; τ: p<0.05 vs. respective 3-month-old counterpart; #: p<0.05 vs. respective 8-month-old counterpart. ND = not determined.
An increase in plasma NE content was observed in male SD rats of increasing age on NS-intake (figure 15). HS-intake in 3-month-old rats led to a significant decrease (p<0.05) in plasma NE content relative to its 3-month-old NS-intake counterpart. However, HS-intake in the older 8-month-old rats did not alter plasma NE content significantly, in comparison to its NS-intake counterpart.

Figure 15. Plasma norepinephrine content in 3/8/16-month-old male Sprague-Dawley rats on NS/HS diets. Plasma NE content was determined via ELISA. *: p<0.05 vs. respective 3-month-old NS control value; τ: p<0.05 vs. respective 3-month-old counterpart. ND = not determined.
**Salt Sensitivity of Blood Pressure**

No change in MAP was observed in control s.c.-saline male SD rats in response to HS-intake. However, a significant increase (p<0.05) in MAP was observed in s.c.-NE and Renal-CAP 3-month-old male SD rats in response to HS-intake, relative to their NS-intake counterparts (figure 16). A significant increase (p<0.05) in MAP was also observed in NS-intake 8-month-old rats relative to its 3-month-old counterpart, and in NS-intake 16-month-old rats relative to its 8-month-old counterpart.

**Figure 16.** Effect of lifelong normal salt (0.6% NaCl) intake vs. high salt (4% NaCl for 21 days) intake on MAP in male SD rats of various ages and aging models. *: p<0.05 vs. respective normal salt value; τ: p<0.05 vs. respective 3-month-old counterpart; #: p<0.05 vs. respective 8-month-old counterpart. ND = not determined.
HS-intake in 3-month-old s.c.-saline male SD rats led to a significant decrease (p<0.05) in NCC activity (figure 17), whereas HS-intake in salt-sensitive s.c.-NE and Renal-CAP rats did not. A significant increase (p<0.05) in NCC activity was also observed in NS-intake 8-month-old and 16-month-old rats in comparison to its 3-month-old counterpart. However there was no significant decrease in NCC activity of 8-month-old rats with HS-intake compared with same age NS-intake.

Figure 17. Effect of lifelong normal salt (0.6% NaCl) intake vs. high salt (4% NaCl for 21 days) intake on NCC activity (measured as peak natriuresis to i.v. HCTZ infusion) in male SD rats of various ages and aging models. *: p<0.05 vs. respective normal salt value; τ: p<0.05 vs. respective 3-month-old counterpart; #: p<0.05 vs. respective 8-month-old counterpart. ND = not determined, HTN = hypertension.
**α/β-Adrenoceptor Antagonism**

In control s.c.-saline 3-month-old male SD rats, neither treatment with terazosin nor propranolol led to changes in MAP. In s.c.-NE-infused rats, treatment with terazosin led to a significant decrease (p<0.05) in MAP in response to HS-intake relative to its non-terazosin-treated counterpart, whereas treatment with propranolol resulted in a lower MAP independent of diet. In 16-month-old rats, treatment with terazosin led to a significant decrease (p<0.05) in MAP in response to HS-intake, relative to its naïve counterpart.

**Figure 18.** Effect of lifelong normal salt (0.6% NaCl) intake vs. high salt (4% NaCl for 21 days) intake on MAP in male SD rats of various ages/models receiving s.c. terazosin (10 mg/kg/day) or propranolol (9.9 mg/kg/day). *: p<0.05 vs. respective control normal salt counterpart; φ: p<0.05 vs. respective s.c. saline counterpart; τ: p<0.05 vs. respective high-salt counterpart; #: p<0.05 vs. respective 3-month-old naïve counterpart. NE = norepinephrine, Pro = propranolol, Teraz = terazosin.
In control s.c.-saline 3-month-old male SD rats, a significant decrease (p<0.05) in NCC activity was observed in response to HS-intake, both with and without treatment with terazosin or propranolol. In s.c NE-infused rats, treatment with terazosin also resulted in a significant decrease (p<0.05) in NCC activity relative to its NS-intake counterpart, whereas treatment with propranolol did not. In 16-month-old rats, treatment with terazosin also resulted in a significant decrease (p<0.05) in NCC activity relative to its naïve counterpart.

**Figure 19.** Effect of lifelong normal salt (0.6% NaCl) intake vs. high salt (4% NaCl for 21 days) intake on NCC activity (measured as peak natriuresis to i.v. HCTZ) in male SD rats of various ages/models receiving s.c. terazosin (10 mg/kg/day) or propranolol (9.9 mg/kg/day). *: p<0.05 vs. respective control normal salt counterpart; †: p<0.05 vs. respective high-salt counterpart; #: p<0.05 vs. respective 3-month-old naïve counterpart. NE = norepinephrine, Pro = propranolol, SHR = spontaneously hypertensive rat, Teraz = terazosin.
**Immunoblotting**

**Figure 20.** Sample immunoblots in saline and NE-infused 3-month-old male SD rats. Band densities were normalized to β-actin.

In control saline male SD rats, HS-intake resulted in a significant decrease (p<0.05) in NCC and pNCC expression relative to its NS-intake counterpart (figures 20, 21, and 22). In s.c. NE-infused rats, no difference in NCC and pNCC expression was observed in response to HS-intake. However, HS-intake NCC and pNCC expression was significantly higher (p<0.05) relative to its control saline counterpart. Treatment with terazosin resulted in a significant decrease (p<0.05) in NCC and pNCC expression relative to its NS-intake counterpart. However, treatment with propranolol did not lead to changes in NCC/pNCC expression in response to HS-intake.
Figure 21. Renal NCC protein levels in saline and NE-infused 3-month-old male SD rats. *: p<0.05 vs. respective control normal salt counterpart; τ: p<0.05 vs. respective high-salt counterpart; φ: p<0.05 vs. respective s.c. saline counterpart. Pro = propranolol, Teraz = terazosin.

Figure 22. Renal phosphorylated NCC (pNCCT58) protein levels in saline and NE-infused 3-month-old male SD rats. *: p<0.05 vs. respective control normal salt counterpart; τ: p<0.05 vs. respective high-salt counterpart; φ: p<0.05 vs. respective s.c. saline counterpart. Pro = propranolol, Teraz = terazosin.
In control saline male SD rats, HS-intake resulted in a significant decrease (p<0.05) in SPAK expression relative to its NS-intake counterpart. (figures 20 and 23). In s.c. NE-infused rats, a slight increase in SPAK expression was observed in response to HS-intake. HS-intake SPAK expression was also significantly higher (p<0.05) relative to its control saline counterpart. Treatment with either terazosin or propranolol resulted in a significant decrease in NCC/pNCC expression relative to its NS-intake counterpart.

Figure 23. Renal SPAK protein levels in saline and NE-infused 3-month-old male SD rats. *: p<0.05 vs. respective control normal salt counterpart; τ: p<0.05 vs. respective high-salt counterpart; φ: p<0.05 vs. respective s.c. saline counterpart. Pro = propranolol, Teraz = terazosin.

In control saline male SD rats, HS-intake resulted in a significant decrease (p<0.05) in WNK1 expression relative to its NS-intake counterpart. (figures 20 and 24). In s.c. NE-infused rats, no difference in WNK1 expression was observed in response to HS-intake. However, HS-intake WNK1 expression was
significantly higher (p<0.05) relative to its control saline counterpart. Treatment with terazosin resulted in a significant decrease in WNK1 expression in response to HS-intake relative to its control saline NS-intake counterpart. However, treatment with propranolol did not lead to changes in WNK1 expression in response to HS-intake.

Figure 24. Renal WNK1 protein levels in saline and NE-infused 3-month-old male SD rats. *: p<0.05 vs. respective control normal salt counterpart; τ: p<0.05 vs. respective high-salt counterpart; ϕ: p<0.05 vs. respective s.c. saline counterpart. Pro = propranolol, Teraz = terazosin.

In control saline male SD rats, HS-intake resulted in a significant decrease (p<0.05) in OxSR1 expression relative to its NS-intake counterpart. (figures 20 and 25). In s.c. NE-infused rats, a significant increase (p<0.05) in OxSR1 expression was observed relative to its control saline counterpart, independent of diet. Treatment with terazosin resulted in a significant decrease in OxSR1
expression independent of diet. However, treatment with propranolol did not lead to changes in OxSR1 expression.

**Figure 25. Renal OxSR1 protein levels in saline and NE-infused 3-month-old male SD rats.** *: p<0.05 vs. respective control normal salt counterpart; τ: p<0.05 vs. respective high-salt counterpart; φ: p<0.05 vs. respective s.c. saline counterpart. Pro = propranolol, Teraz = terazosin.

In control saline male SD rats, HS-intake resulted in a significant decrease (p<0.05) in pOxSR1 expression relative to its NS-intake counterpart. (figures 20 and 26). In s.c. NE-infused rats, an increase in pOxSR1 expression was observed in response to HS-intake. HS-intake pOxSR1 expression was also significantly higher (p<0.05) relative to its control saline counterpart. Treatment with terazosin resulted in a significant decrease in pOxSR1 expression in response to HS-intake. However, treatment with propranolol did not lead to changes in pOxSR1 expression in response to HS-intake.
Figure 26. Renal pOxSR1 protein levels in saline and NE-infused 3-month-old male SD rats. *: p<0.05 vs. respective control normal salt counterpart; τ: p<0.05 vs. respective high-salt counterpart; φ: p<0.05 vs. respective s.c. saline counterpart. Pro = propranolol, Teraz = terazosin.
DISCUSSION

**ARN Ablation and Acute Volume Expansion**

ARN ablation (via the Renal-CAP protocol) in young 3-month-old male Sprague-Dawley (SD) rats resulted in an attenuation of natriuresis and sympathoinhibitory PVN parvocellular neuron activation, as well as increased MAP during i.v.-isotonic volume expansion. This observation is in agreement with previous studies implicating the ARN in elevated sympathetic tone (Patel and Knuepfer 1986). The data also suggests that the mechanosensitive ARN may contribute to the regulation of natriuresis and MAP via a PVN-implicated sympathoinhibitory reno-renal reflex. This suggestion is also in agreement with previous studies that implicate renal denervation of both the efferent and afferent nerves, as opposed to selective ARN ablation, in the reduction of blood pressure (Foss, Fink, and Osborn 2016).

**Age-Dependent Acute Volume Expansion**

In the acute isotonic volume expansion studies involving male SD rats of varying ages, the data suggest that the hypertensive state in adult (8-month) and aged (16-month) male SD rats may be due to an impaired ability to excrete fluid and sodium through the urine. The attenuated natriuretic response in adult and aged male SD rats with age-dependent hypertension may be due to an inhibition of the aforementioned sympathoinhibitory reno-renal reflex that acts to mediate
natriuresis. This is also supported by the observation of attenuated PVN neuronal activation identified by the mean number of Fos+ parvocellular nuclei, which suggests a decrease in sympathoinhibitory signal propagation via the ARN in response to acute volume expansion in older male SD rats. Collectively, the data significantly suggests that age-dependent hypertension in male SD rats progressively results in an attenuated sympathoinhibitory reno-renal reflex-mediated natriuresis, as well as a decreased in PVN neuronal activation, in response to acute 5%-VE. The data is in agreement with various prior studies that historically relate sympathetic nervous system dysfunction with the pathogenesis of hypertension (Mancia and Grassi 2014).

**Aging and Salt Intake**

The observation of increased MAP in older male SD rats on lifetime NS-intake in conjunction with an increased sympathetic tone suggests that their age-dependent hypertension may be due to a lack of sympathoinhibition. Hexamethonium is a known ganglionic blocker that as expected, led to a depressor response in all rat groups (Walsh et al. 2016). As a ganglionic blocker, i.p. hexamethonium served as an indicator of the contribution of sympathetic tone to the overall MAP. Thus, the elevated depressor response due to i.p. hexamethonium in older male SD rats further suggests that their age-dependent hypertension may be due to an increase in sympathetic tone. This is further suggested by the lack of sympathoinhibition (elevated renal/plasma NE, elevated
ΔMAP to i.p. hexamethonium) observed in HS-intake adult (8-month-old) male SD rats.

As previously mentioned, NE has been shown to contribute towards NCC regulation (Terker et al. 2014), and more specifically, to act towards preventing a dietary sodium-evoked suppression of the NCC (Walsh et al. 2016). Thus, the observation of a lack of sympathoinhibition may be due to changes in NCC expression and activity, which was evaluated in subsequent studies.

**Salt Sensitivity of Blood Pressure**

In the salt-sensitivity studies, the data suggest that young 3-month-old male SD rats maintain normotension via downregulation of NCC activity and expression in response to elevated sodium intake; this is suggested by the parallel decrease in both NCC and phosphorylated NCC levels in s.c.-saline male SD rats. In the NE-infused rats, used as a model of age-related increases in sympathetic tone, there is a failure to downregulate NCC activity in response to HS-intake, suggesting that NCC activity serves a critical role in the pathophysiology of SSH in young male SD rats. In the Renal-CAP male SD rat, another model of sympathoexcitation caused by attenuation of sympathoinhibitory influence from the ARN, there is also a failure to reduce NCC activity in response to HS-intake, suggesting a critical connection between ARN-mediated sympathetic tone and NCC activity. These aging and sympathoexcitation models coincide with the observation of increased NCC activity in the adult/aged 8/16-month-old male SD
rats, further suggesting an age-related dysfunction involving ARN-mediated sympathetic tone and NCC activity. In addition, the observation of higher MAP and HCTZ-induced natriuresis in older male SD rats on a NS diet suggests an increase in basal NCC activity in age-dependent hypertension. This further suggests the involvement of sympathetic tone in NCC activity and regulation, and in the overall pathogenesis of age-dependent hypertension in the older male SD rats.

**α₁/β-Adrenoceptor Antagonism**

α₁ and β-adrenoceptor antagonism (via terazosin and propranolol respectively) did not affect NCC activity or regulation in control saline-infused young 3-month-old male SD rats. However, α₁-antagonism significantly decreased MAP and NCC activity in aged 16-month-old male SD rats with established hypertension. Furthermore, α₁-antagonism in young NE-infused male SD rats resulted in the abolishment of salt-sensitivity of MAP (as indicated by similar MAP in both s.c.-NE/terazosin NS/HS groups) as well as the restoration of HS-intake-dependent NCC downregulation. Collectively, this suggests that the α₁-adrenoceptor is implicated in the regulation of the sympathetically-mediated NCC activity, which is involved in the development of age-dependent hypertension. This challenges the suggestion in recent studies that suggests sympathetically-mediated NCC activity acts via the β-adrenoceptor (Terker et al. 2014).
The immunoblotting studies suggest that in young NE-infused male SD rats, the restoration of HS-intake induced NCC downregulation via α₁-antagonism, accompanied by the downregulation of WNK₁ expression, operates via a mechanism that hinders OxSR₁ upregulation and phosphorylation. While β-antagonism also abolished NE-induced hypertension in young male SD rats, it did so independently of salt-intake, suggesting that the β-adrenoceptor may not be directly involved in the aforementioned age-dependent hypertension seen in aged male SD rats. This is further suggested by the lack of suppression of NCC, WNK₁, or OxSR₁ activity due to HS-intake in s.c.-NE/propranolol-infused male SD rats. Collectively, the data suggest that the lack of NCC activation implicated in age-dependent hypertension may be due to abnormalities in the α₁-adrenoreceptor pathway, which involves WNK₁, SPAK, and OxSR₁ expression. The data contributes novel insight into the suggestion in previous studies that the WNK, SPAK, and OxSR₁ kinases are all involved in NCC regulatory responses to changes in salt intake (Vallon 2008).

**Limitations and Alternative Strategies**

While this present study has soundly suggested a role of the ARN in mediating the sympathoinhibitory and natriuretic responses to an acute challenge in fluid and electrolyte balance in the form of 5%-VE, as well as in preventing the development of SSH in young male SD rats, it may be limited by the lack of available mechano- or chemoreceptor pharmacological antagonists to
support the premise that such receptors are activated and thus affecting the ARN. Therefore, an additional alternative strategy is to incorporate the use of such receptor antagonists, when available. While c-Fos IHC is considered an established method of assessing neuronal activation (Sundquist and Nisenbaum 2005), it is still not without certain limitations, such as the lack of a phenotype for labelled cells. Thus, future alternative strategies may also use additional methods to account for c-Fos IHC limitations in data analysis, through consultation and collaboration with experts in c-Fos IHC analysis.

This present study has also suggested that the sympathetically-mediated age-dependent hypertension in male SD rats acts via α₁-adrenoceptor-gated NCC activity. However, while the isolation of the NCC cotransporter via ENaC blockade prior to and during NCC antagonism has been widely accepted and reproduced, as is the assessment of NCC phosphorylation (Pedersen et al. 2010), such techniques provide markers of NCC activity, without directly assessing NCC-mediated sodium transport. Thus, future considerations and experiments should make use of alternative and more explicit methods of measuring sodium transport via the NCC, once available.

**Significance and Future Considerations**

This present study has provided abundant evidence regarding the integrated roles of renal sodium handling and ARN-mediated sympathetic tone in the pathophysiology of age-dependent hypertension in male SD rats. As such,
it helps to significantly generate mechanistic insight into the development of age-dependent hypertension. Additionally, this study is clinically relevant to the global epidemic of hypertension, as it is not only models the growing risk of developing hypertension with age, but also models the global trend towards excess dietary salt intake, which as previously mentioned, drastically increases the risk of developing SSH (M. H. Weinberger and Fineberg 1991).

Future studies surrounding the mechanisms of age-dependent hypertension should look to further define mechanistic targets in the treatment of the condition, as such targets may prove useful in the use of CRISPR/cas9 technology for genetic manipulation. Future studies may also target alternative animal models; however, for such experimental approaches to come into fruition, established models of hypertension, as well as surgical techniques such as the renal pelvic cannulation performed in this present study, need to be developed first in animals beyond rats alone. In the clinical setting, blood pressure and NE levels are already reliably measured. However, further translating the findings and suggestions of this present study into the clinical setting requires more definitive considerations surrounding the nature of salt intake in patients and novel markers of NCC activity beyond phosphorylation alone.

Currently, the vast majority of anti-hypertensive treatments available have proven to be less effective than expected, especially for the elderly population (Imprialos et al. 2016). This is due, among many other possible reasons, primarily to risks of increased adverse effects as well as to comorbidities and
other complications that arise from aging, and in the case of SSH, a lack of routine diagnosis of salt-sensitivity in the elderly human population (Myron H. Weinberger 1996). Thus, making significant progress in the treatment of age-dependent hypertension requires a paradigm shift from a non-specific reduction of blood pressure to a mechanistic and personalized approach that targets the pathophysiological cause of the hypertension itself. Overall, through the demonstration of a hypertensive mechanism, involving an impairment of fluid and electrolyte homeostasis that implicates the renal, cardiovascular, and nervous systems, this study provides a crucial stepping stone for the development of the mechanistic treatment of hypertension in elderly individuals with elevated sympathetic tone.
REFERENCES


Terker, Andrew S., Chao-Ling Yang, James A. McCormick, Nicholas P. Meermeier, Shaunessy L. Rogers, Solveig Grossmann, Katja Trompf, Eric

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Yoon, Sung Sug, Qiuping Gu, Tatiana Nwankwo, Jacqueline D. Wright, Yuling Hong, and Vicki Burt. 2015. “Trends in Blood Pressure Among Adults