Supplemental Data and Methods

Failure to Vasodilate in Response to Salt Loading Blunts Renal Blood Flow and Causes Salt-Sensitive Hypertension

Short Title: Impaired Vasodilation in Salt Induced Hypertension

Jing Wu[1,2*], Larry N. Agbor[2], Shi Fang[1,2], Masashi Mukohda[2], Anand R. Nair[2], Pablo Nakagawa[1,2], Avika Sharma[3], Donald A. Morgan[2], Justin L. Grobe[1,2], Kamal Rahmouni[2,4,5], Robert M. Weiss[5], James A. McCormick[3], Curt D. Sigmund[1,2*]

[1]Department of Physiology, Cardiovascular Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226;
[2]Department of Neuroscience and Pharmacology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242;
[3]Division of Nephrology and Hypertension, Department of Medicine, Oregon Health and Science University, Portland, Oregon 97239;
[4]Veteran Affairs Health Care System, Iowa City, Iowa 52242;
[5]Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242;

*Co-corresponding Authors:

Curt D. Sigmund, Ph.D.
Department of Physiology
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, WI 53226-0509
Phone: 414-955-8277
E-mail: csigmund@mcw.edu

Jing Wu, Ph.D.
Department of Physiology
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, WI 53226-0509
Phone: 414-955-8277
E-mail: jingwu@mcw.edu

Parameters	Time	NT Chow Diet			NT HSD			S-P467L Chow Diet			S-P467L HSD		
		Mean	**S.E.**	**n**	**Mean**	**S.E.**	**n**	**Mean**	**S.E.**	**n**	**Mean**	**S.E.**	**n**
Body Weight (g)	Baseline	25.6	1.20	10	27.05	0.68	10	26.79	1.30	14	26.67	0.87	15
	Week 3	26.08	0.19	10	26.88	0.51	10	26.42	0.92	14	26.69	0.62	15
Daily Food Intake (g)	Baseline	3.40	0.17	5	3.36	0.18	8	3.43	0.18	6	3.16	0.14	9
	Week 3	3.86	0.04	4	2.72*	0.22	8	3.58	0.19	6	3.28	0.21	9
Daily Feces (g)	Baseline	0.95	0.06	5	0.99	0.09	8	0.92	0.20	6	0.85	0.07	9
	Week 3	1.30	0.06	4	0.56*	0.06	8	1.00	0.13	6	0.78	0.11	9
Daily Water Intake (mL)	Baseline	2.82	0.15	5	2.93	0.08	8	2.25	0.51	6	2.60	0.15	6
	Week 3	2.64	0.12	4	4.88*	0.48	8	2.59	0.28	5	5.47*	0.39	8
Daily Urine (mL)	Baseline	0.66	0.20	5	0.48	0.10	7	0.37	0.16	5	0.33	0.06	7
	Week 3	0.71	0.15	4	2.07*	0.42	7	0.50	0.14	6	1.89*	0.21	8

Table S1. Metabolic Cage Study Before and During the 4-Week Study.
Metabolic cage studies were performed for two consecutive days at baseline and on Day 21 of the 4-week study. Body weight was recorded right before mice were placed in metabolic cages. Mice were acclimated to the cages in the first 24 hours and data in the second 24 hours were analyzed. Data are presented as mean ± SEM and were analyzed by 2-way ANOVA Repeated Measurements. * $p<0.05$, HSD compared to Chow Diet, Tukey's multiple comparisons test, two-way ANOVA.

Parameters	Time	NT Chow Diet			NT HSD			S-P467L Chow Diet			S-P467L HSD		
		Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n
LV Thickness (mm)	Baseline	1.08	0.06	10	0.98	0.09	4	1.03	0.05	10	0.95	0.05	9
	Week 3	1.10	0.06	10	1.02	0.09	4	1.06	0.05	10	1.02	0.06	8
LV Mass (mg)	Baseline	88.39	4.26	10	79.14	4.70	4	79.14	6.11	10	74.90	3.49	9
	Week 3*	89.88	5.14	10	85.69	1.45	4	78.22	3.94	10	87.35#	5.04	8
EDV (µL)	Baseline	32.47	1.81	10	31.81	1.88	4	29.60	2.52	10	29.83	2.16	9
	Week 3*	36.77	2.20	10	35.77	2.74	4	35.16	1.67	10	32.64	1.53	8
ESV (µL)	Baseline	5.25	0.51	10	4.61	0.45	4	4.72	0.51	10	4.57	0.45	9
	Week 3	5.45	0.65	10	5.24	0.44	4	4.98	0.68	10	4.66	0.42	8
Stroke Volume (µL)	Baseline	27.22	1.54	10	27.20	1.55	4	24.89	2.13	10	25.24	1.90	9
	Week 3*	31.31	1.73	10	30.54	2.47	4	30.18	1.09	10	27.98	1.29	8
CO (mL/min)	Baseline	18.08	1.00	10	18.71	0.69	4	16.97	1.26	10	17.48	1.36	9
	Week 3*	22.42	1.17	10	21.49	1.86	4	21.34	0.86	10	20.36	1.12	8
EF (%)	Baseline	83	1.2	10	86	1.0	4	84	1.0	10	85	1.1	9
	Week 3	86	1.1	10	85	1.0	4	86	1.4	10	86	1.0	8
HW/BW (mg/g)	Week 4	3.96	0.08	15	3.97	0.12	7	4.11	0.08	14	4.26†	0.05	13

Table S2. Cardiac Dimensions and Function at Baseline and During the 4-Week Study.
Cardiac ultrasound was performed in mice under light sedation at baseline and on Day 21 of the 4-week study. LV, left ventricular; EDV, end-diastolic volume; ESV, end-systolic volume; CO, cardiac output; EF, ejection fraction. Data are presented as mean ± SEM. Cardiac ultrasound data were analyzed using two-way ANOVA with repeated measurements (RM). * $p<0.05$, effect of time in two-way ANOVA RM (meaning values for all groups together in week 3 were different from all groups together at baseline). # $p<0.05$ vs Baseline, Sidak's multiple comparisons test. HW/BW indicates heart weight/body weight measured at sacrifice at the end of the 4-week study (on Day 28). HW/BW data were analyzed by Two-way ANOVA; † $p<0.05$ vs NT Chow Diet.

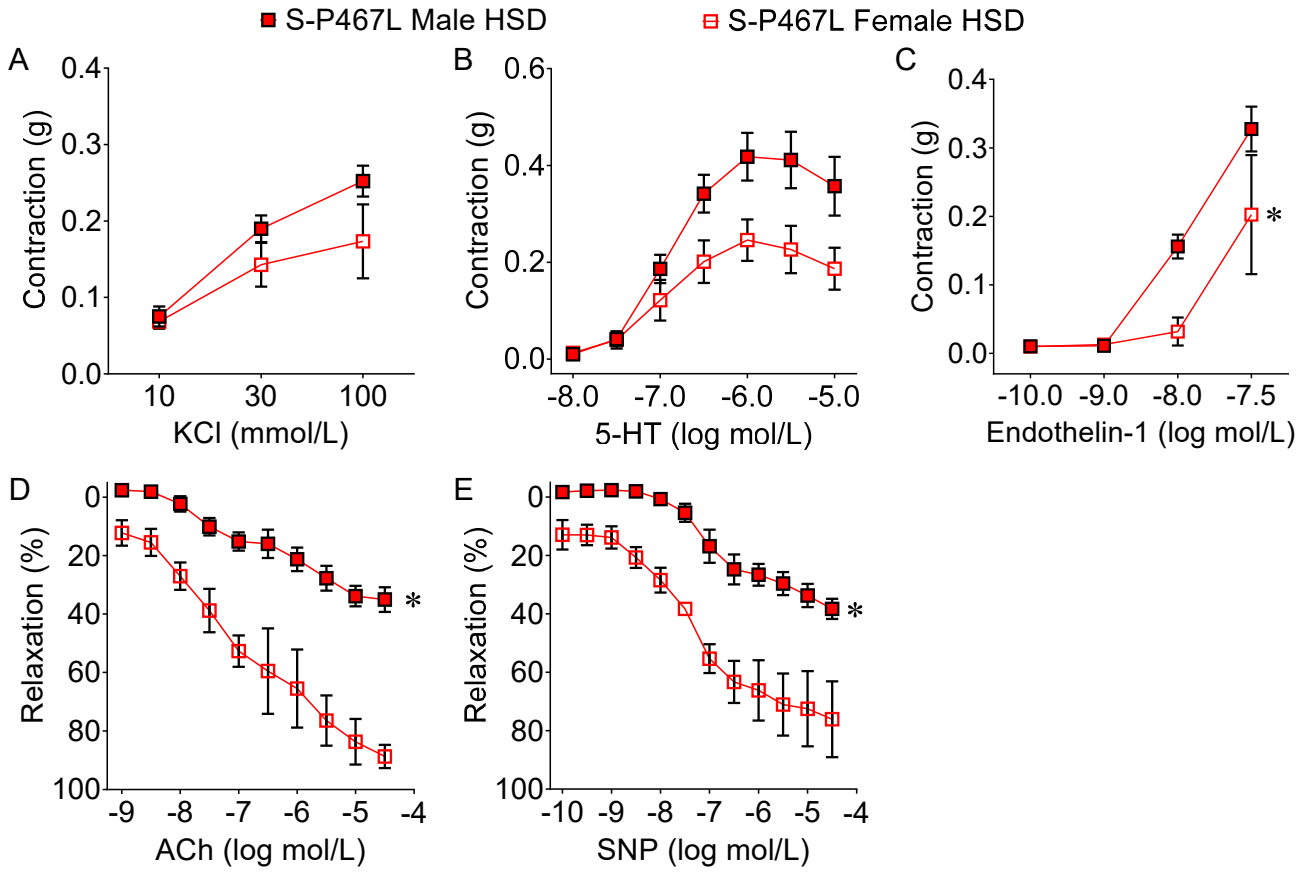

Figure S1. Gender Differences in Vascular Function.
In an initial trial, male and female S-P467L transgenic mice were fed HSD for 8 weeks and isometric tension was measured with wire myography in carotid artery. To show differences between male (n=6-8) and females (n=3), data for the male mice were replotted from Figure S4 and Figure 3A-B. A-C) Cumulative concentration-response curves for KCl (A), 5-HT (B), and Endothelin-1 (C). D-E) Cumulative concentration-response curves for acetylcholine (ACh) and sodium nitroprusside (SNP). Data are plotted as mean ± SEM and were analyzed with two-way ANOVA RM. * $p<0.05$, male vs female.

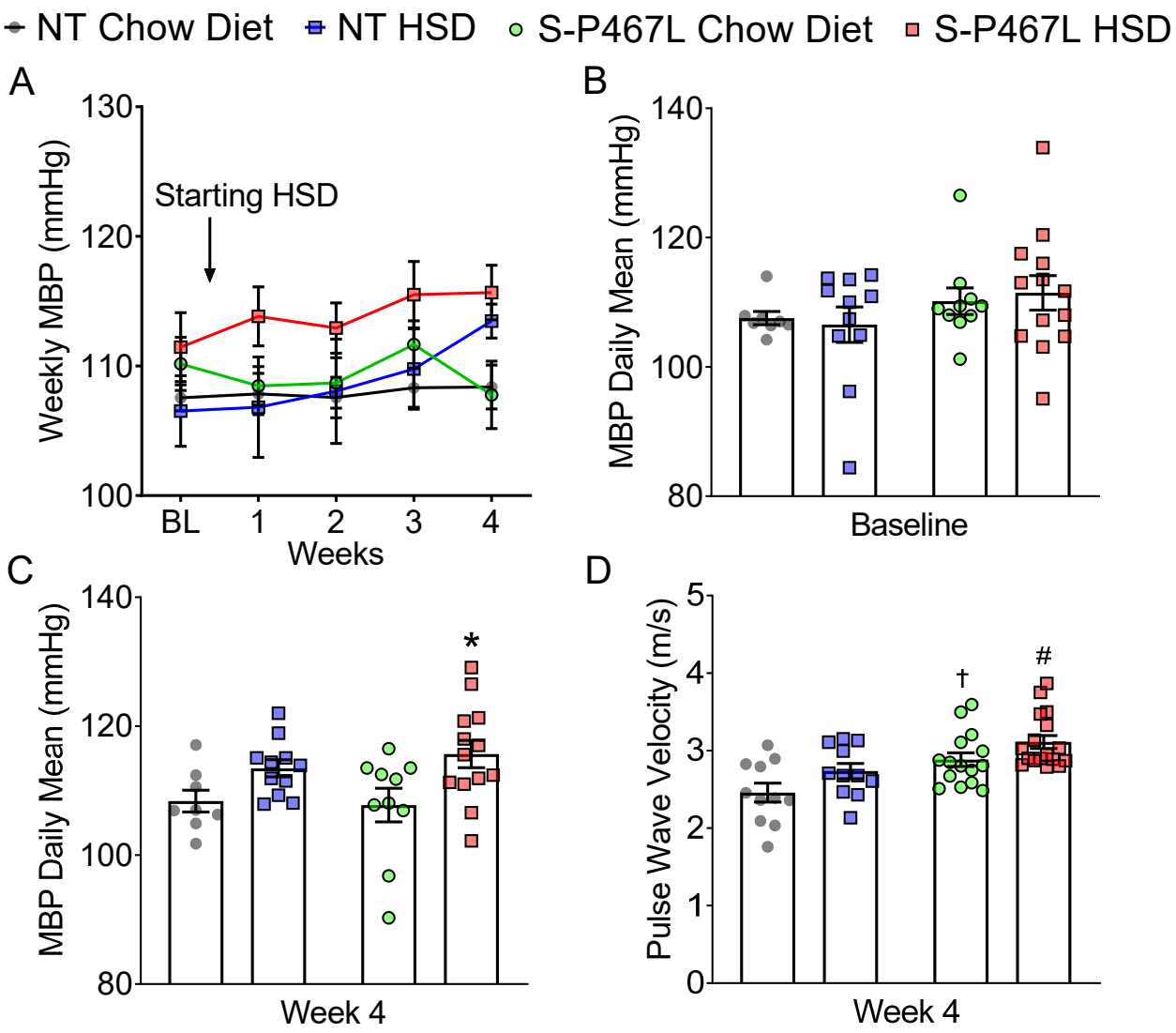

Figure S2. Mean Blood Pressure and Arterial Stiffness.
Blood pressure was measured by radiotelemetry at baseline and weekly during the 4-week study (n=8-13). A) Weekly mean values of mean blood pressure (MBP) were displayed throughout the study. B-C) MBP at baseline and at the end of week 4 was shown as daily means. D) Pulse wave velocity was measured in week 4 with vascular ultrasound in a separate cohort of mice without implanted radiotelemeters and calculated using the transit time method as described in methods. Data are presented as mean ± SEM. Weekly MBP was analyzed by 2-way ANOVA with RM and mean data were analyzed by 2-way ANOVA with Tukey's multiple comparison tests. * $p<0.01$ vs S-P467L Chow Diet. † $p<0.05$ vs NT Chow Diet. # $p<0.05$ vs NT HSD.

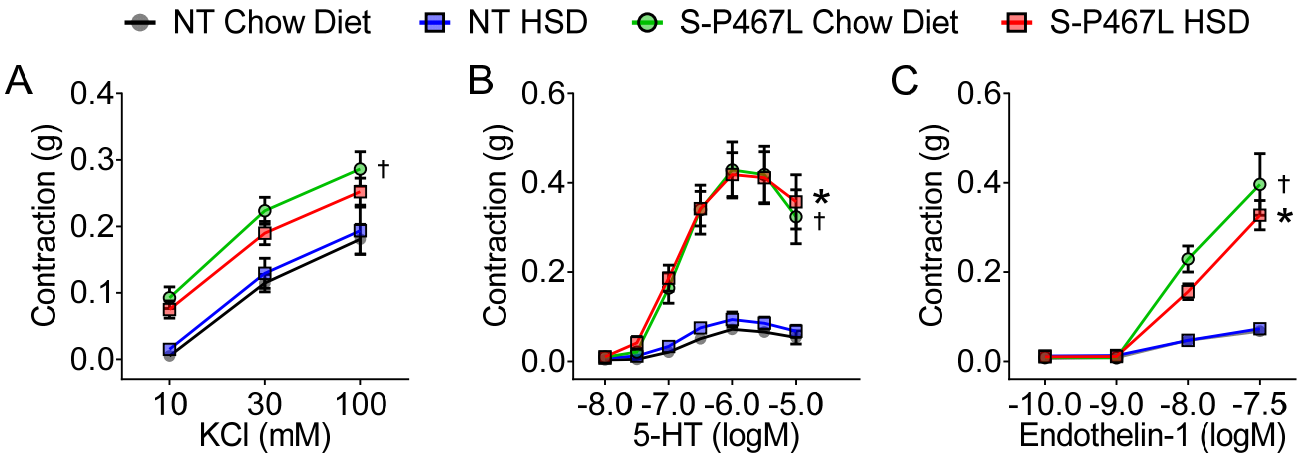

Figure S3. Vascular Contraction.
A-C) Cumulative concentration-response curves for KCl (A, n=4-6), 5-HT (B, n=5-7), and Endothelin-1 (C, n=5-8) were determined in the carotid artery using wire myography at the end of the study. Data are presented as mean ± SEM. Two-way ANOVA RM was performed for statistical analysis. * $p<0.05$, S-P467L HSD vs NT HSD. † $p<0.05$, S-P467L Chow Diet vs NT Chow Diet.

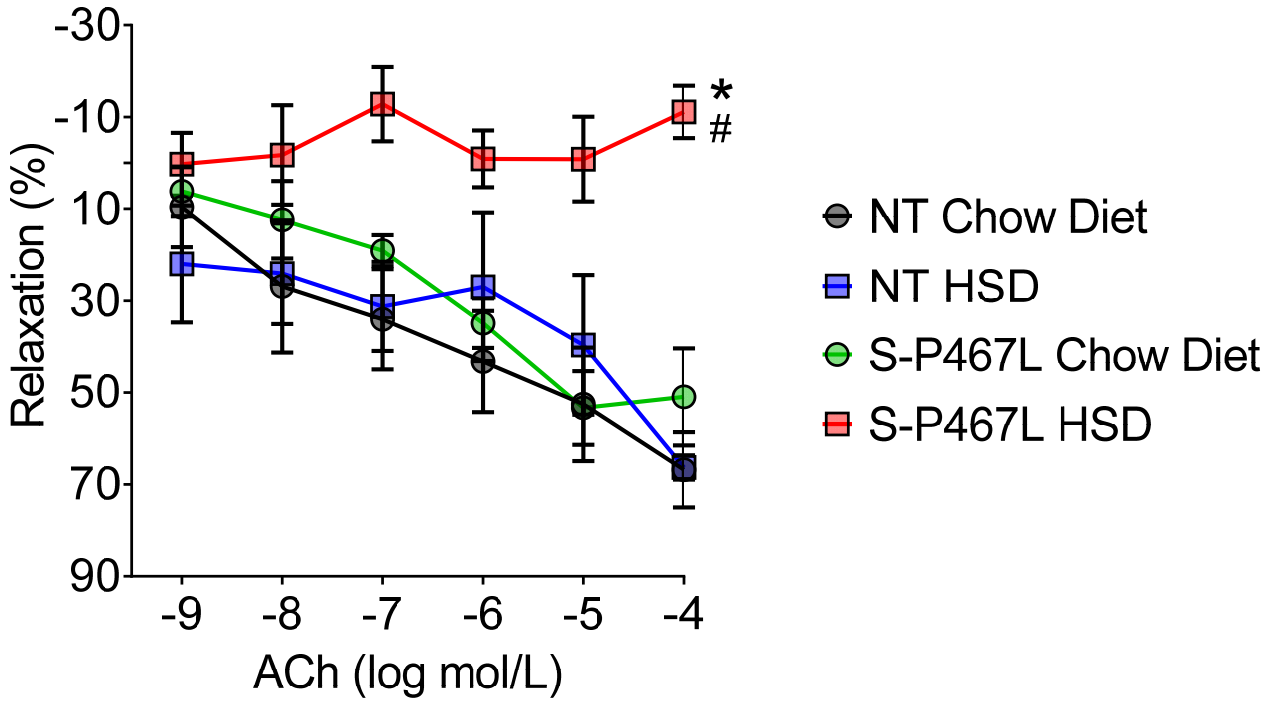

Figure S4. Acetylcholine Response in Basilar Artery.
Acetylcholine (ACh)-induced vasodilation was determined by pressure myography in basilar artery at the end of the study (NT Chow n=4, NT HSD n=3, S-P467L Chow n=5, S-P467L HSD n=5). Data are plotted as mean ± SEM. Two-way ANOVA RM was performed for statistical analysis. * $p<0.05$, S-P467L HSD vs NT HSD. # $p<0.05$, S-P467L HSD vs S-P467L Chow Diet.

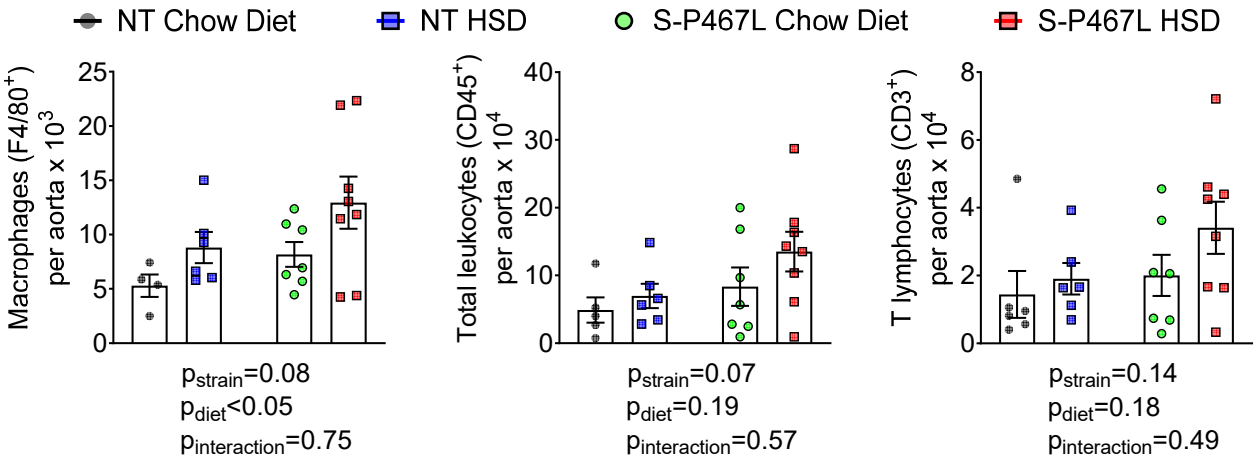

Figure S5 Aortic Inflammation.
Flow cytometry was performed in aortas immediately after sacrifice at the end of the study. Anti-mouse F4/80, anti-mouse CD45 and anti-mouse CD3 antibodies were used as markers of macrophages, leukocytes, and T lymphocytes in the aortic tissue (n=4-8). Data are presented as mean ± SEM. Two-way ANOVA RM was performed for statistical analysis and p values were reported below each panel.

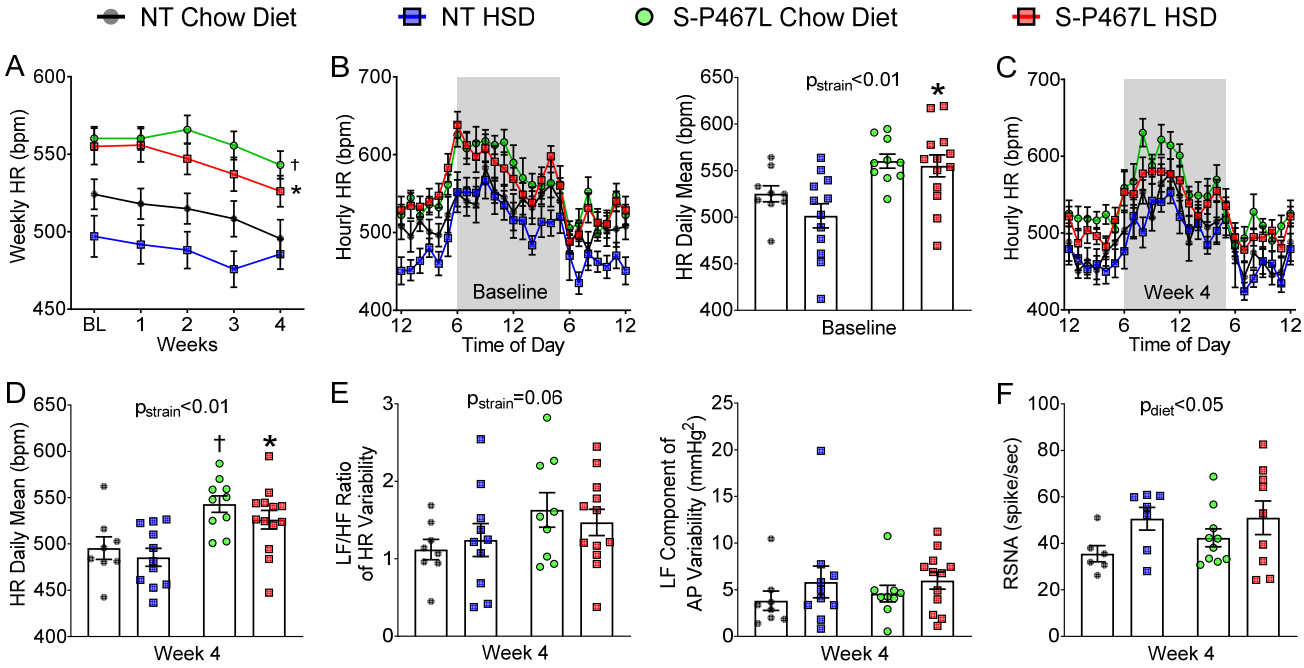

Figure S6. Heart Rate and Sympathetic Nerve Activity.

A) Time course of weekly means of heart rate (HR) measured by radiotelemetry at baseline (BL) and during the 4-week study. B-D) HR at baseline and at the end of Week 4 is shown as hourly and daily means. n=8-13. E) HR variability and arterial pressure (AP) variability were derived by power spectral analysis using continuous HR recordings from a subset of mice in the BP study. n=8-12. F) Renal sympathetic nerve activity (RSNA) was recorded in separate animals without implanted radiotelemeters (n=6-10). Data are plotted as mean ± SEM. Two-way ANOVA RM was employed to analyze weekly HR. Two-way ANOVA was performed for HR daily means, HR variability, AP variability and RSNA. Tukey's multiple comparison procedures were performed for pairwise comparisons. * $p<0.05$, S-P467L HSD vs NT HSD; † $p<0.05$, S-P467L Chow Diet vs NT Chow Diet. The effects of strain in two-way ANOVA are also indicated.

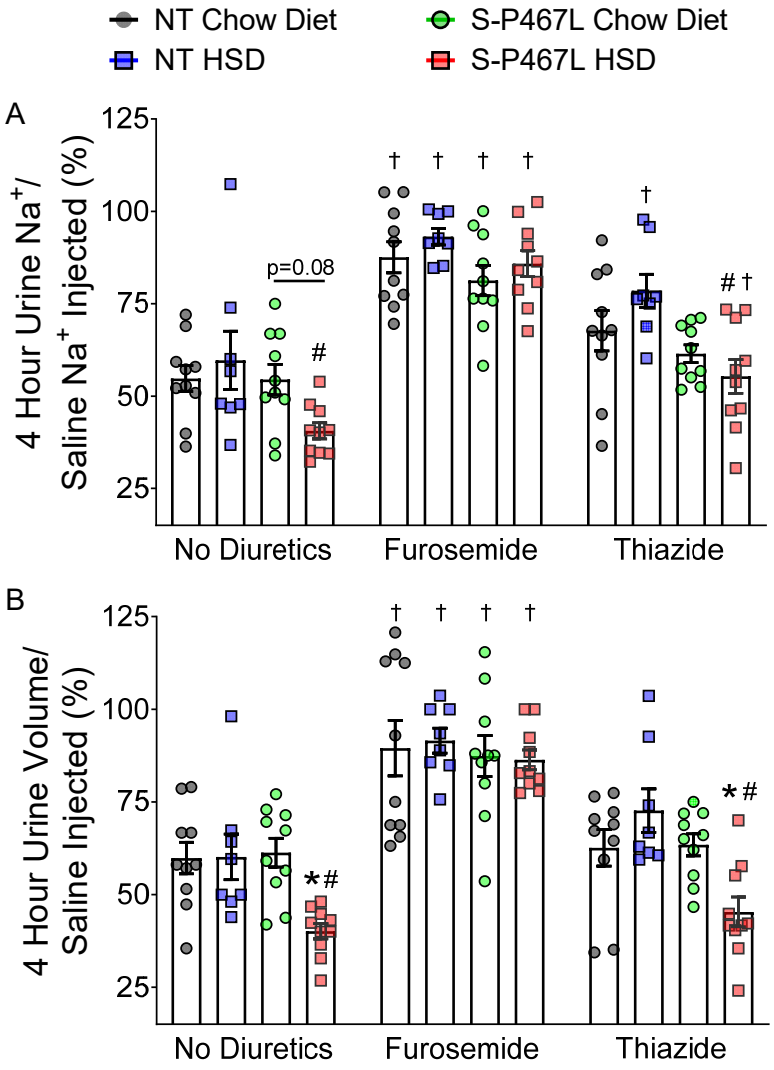

Figure S7 Acute Natriuresis/Diuresis Test.
In separate cohort of animals, a single i.p. injection of normal saline equal to 10% of body weight was administered on day 21 without diuretics, repeated on day 24 with furosemide (20 mg/kg), and repeated again on day 27 with hydrochlorothiazide (40 mg/kg), respectively. A-B) Sodium excretion and urine volume in the subsequent 4 hours following each injection was monitored and plotted as percentages of saline sodium and volume injected (n=8-10). Data are plotted as mean ± SEM. * $p<0.05$ HSD vs chow diet. # $p<0.05$, S-P467L HSD vs NT HSD. † $p<0.05$, diuretic vs no diuretic.

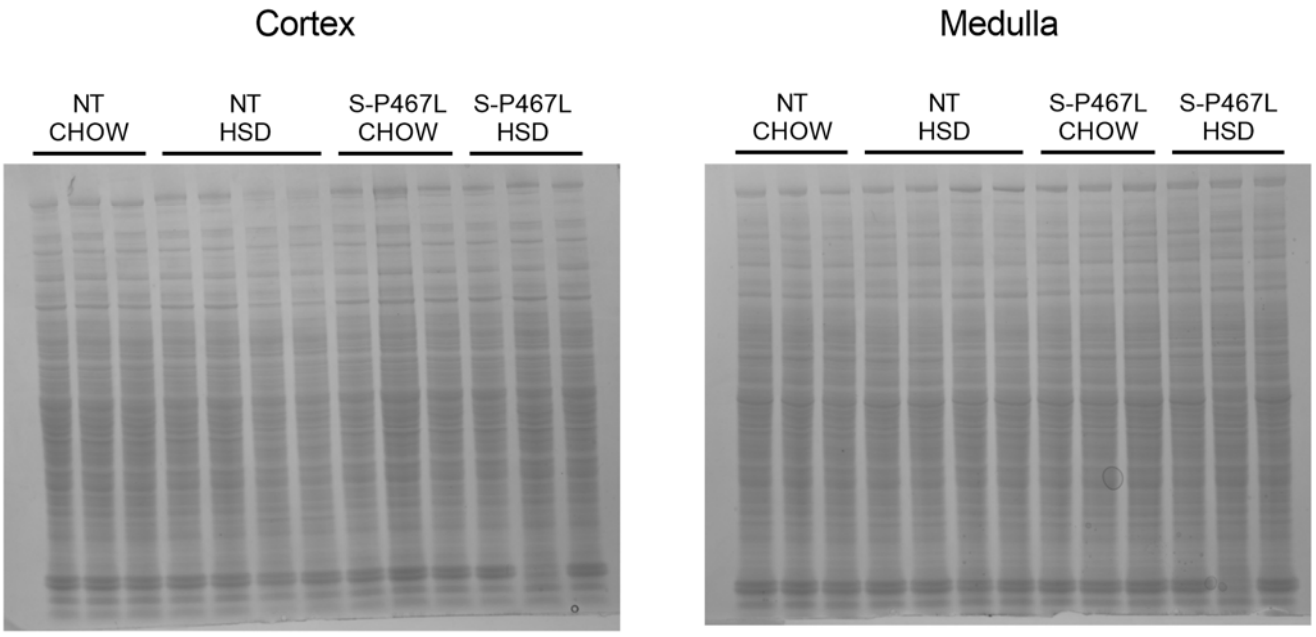

Figure S8 Coomassie Brilliant Blue Staining for Total Protein.
Kidneys were harvested at the end of 4 weeks, cortex and medulla were manually separated and homogenized. Protein concentration was determined using the BioRad DC assay and 20 µg Protein was loaded on a 4%-12% Bis-tris acetate gel. Densitometric quantification of total protein was performed after Coomassie brilliant blue staining. Western blotting for NCC and NKCC2 in Figure 6 was performed by loading 20 µg adjusted protein on additional gels following the protocol described in Methods.

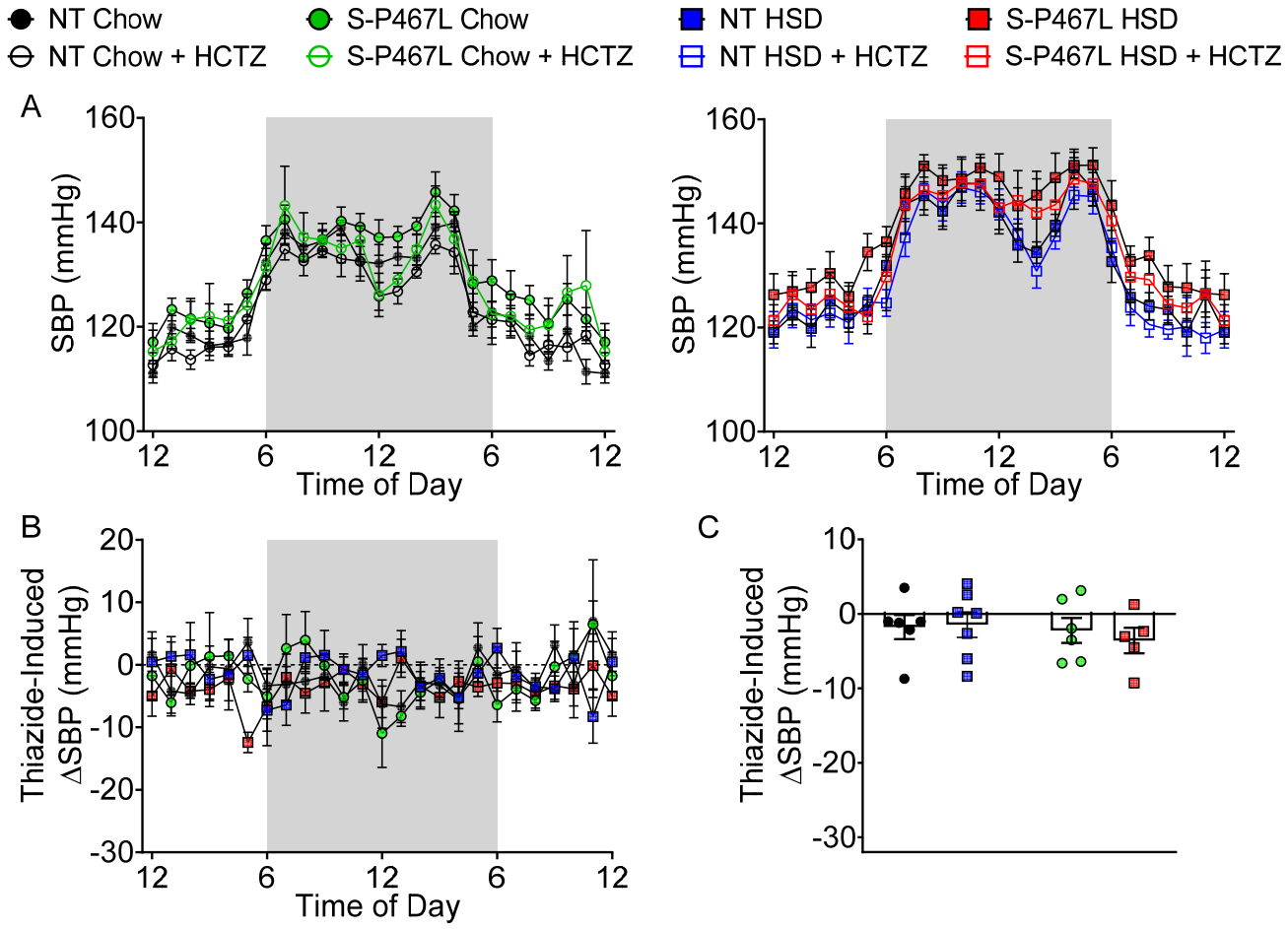

Figure S9 Blood Pressure Response to Hydrochlorothiazide.
A) Separate cohorts of mice received hydrochlorothiazide (300 mg/L) in drinking water for 5 days on while on chow diet (black and green). Then the same animals consumed HSD for 3 weeks and were treated with hydrochlorothiazide for 5 days in week 4 (blue and red). B-C) Thiazide-induced changes in SBP were calculated as differences before and after the thiazide treatment and consolidated into hourly and daily means. Data are plotted as mean ± SEM. Hourly ΔSBP was analyzed by two-way ANOVA RM. Daily means of ΔSBP were analyzed with two-way ANOVA with Tukey's multiple comparison procedures. n=5-7. HCTZ, hydrochlorothiazide.

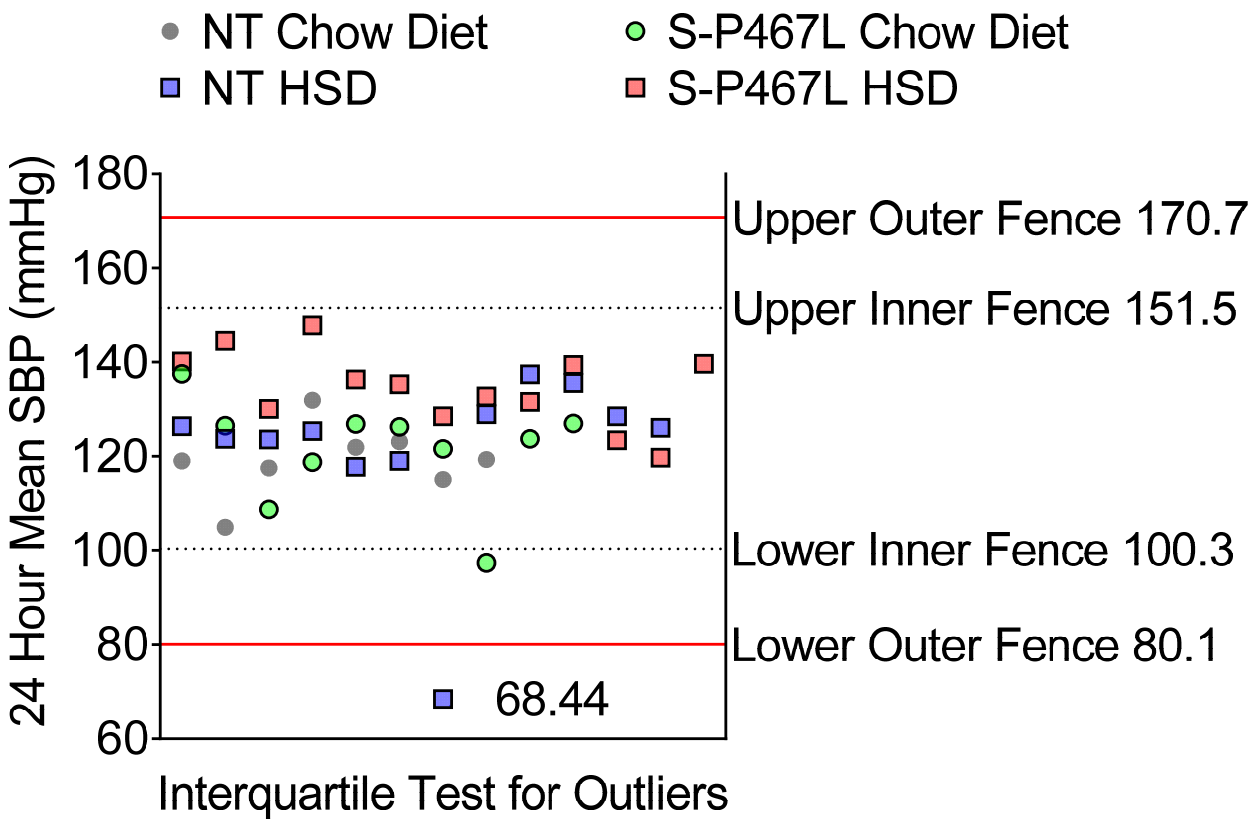

Figure S10. The Interquartile Test for Outliers in Systolic Blood Pressure.
Systolic blood pressure at the end of the 4-week study (same dataset as Figure 2A plus the outlier) were arranged in ascending order to determine the median, the first quartile (Q1, i.e. 25 percentile) and the third quartile (Q3, i.e. 75 percentile). The interquartile range (IQR) was calculated by subtracting the first quartile from the third quartile, i.e. IQR = Q3 - Q1. The Lower Inner Fence and the Lower Outer Fence were calculated as Q1 - 1.5 x IQR and Q1 - 3 x IQR, respectively. The Upper Inner Fence and the Upper Outer Fence were calculated as Q3 + 1.5 x IQR and Q3 + 3 x IQR, respectively. The inner fences are indicated with dashed lines and the outer fences are indicated with solid lines. One data point in the NT HSD group (68.44 mmHg) fell below the Lower Outer Fence. This data point was also detected as an outlier in the ROUT test and Grubb's test. This mouse, which had an occluded radiotelemetry catheter identified in the post-mortem inspection, was thus excluded from the datasets in Figure 1, Figure S2, Figure S3 and Figure 4.

Supplementary Methods

Mice: Most mice in this study (performed at the University of Iowa) were maintained on a laboratory rodent chow diet containing 0.3% sodium (the NIH-31 Modified Open Formula Mouse/Rat Sterilizable Diet, Teklad Catalog # 7013). Some mice in this study (performed at the Medical College of Wisconsin) were maintained on a chow diet containing 0.1% sodium (Teklad Global Soy Protein-Free Extruded Rodent Diet, Catalog # 2920X). Mice were switched to the 4% high salt diet (TD03095).

Mice are housed within their rooms in either static or ventilated micro-isolation cages. All manipulations involving the animals, or cages, are performed in laminar flow hoods or biosafety cabinets and gloved hands are sprayed with a chlorine dioxide product in between cages. A sentinel program monitors for the presence of specific viral, bacterial and parasitic infections. For mouse colonies, MCW tests/screens for the following mouse agents via use of dirty bedding sentinels (serological) and/or environmental testing (PCR of rack exhaust dust): Clostridium piliforme, Mycoplasma pulmonis, CAR bacillus, Ectromelia, EDIM, LCMV, MAV1, MAV2, MCMV, MHV, MNV, MPV, MVM, Polyoma, PVM, REO3, Sendai, TMEV, Encephalitozoon cuniculi, Corynebacterium bovis, Aspiculuris tetraptera and Syphacia obvelata (for pinworms), and Myocoptes, Radfordia, and Myobia (for fur mites). Murine Norovirus (MNV) and Helicobacter are considered endemic at MCW as they are in most other academic institutions.

Measurement of blood pressure and heart rate: Blood pressure and heart rate were measured with radiotelemetry at a sampling rate of 2000 Hz. Mice at approximately 12 weeks of age were anesthetized with ketamine/xylazine and a radiotelemetry BP catheter (TA11PA-C10, DSI) was implanted into the left common carotid artery. The mice were given a 10-day recovery period before baseline blood pressure was recorded for 10 minutes every hour for 2-6 consecutive days. During the 4-week study, blood pressure and heart rate was recorded for 24-48 hours at the end of each week. Data from each animal were averaged hourly, and corresponding times across each recording period were averaged for each animal to create a single consolidated 24-hour tracing. This hourly tracing was further averaged to generate daily means and weekly means of blood pressure and heart rate.

A total of 53 mice were implanted with radiotelemetry transmitters. Eleven mice (~20%) did not produce complete datasets and were therefore excluded from the blood pressure analysis (Figures 1-2 and Figure S2) and the heart rate analysis (Figure S6). These exclusions occurred because of radiotelemeter battery failure (n=8) and catheter occlusion (n=2). One mouse in the NT HSD group with unusually low blood pressure values in week 4 was excluded because it was detected as an outlier by three different tests: the interquartile range outlier test (Figure S10), the ROUT test and the Grubb's test. The exclusion was further justified by an occluded radiotelemetry catheter identified post-mortem.

In studies presented in Figure 7, a subset of animals were kept beyond week 4 for the assessment of the blood pressure-lowering effects of diuretics in week 5 and week 6. A single dose of furosemide was injected via i.p. at 20 mg/kg in week 5. Due to the short half-life of the drug in rodents (<2 hours), furosemide-induced decreases in BP were calculated by comparing the average of SBP 24 hours prior to and 24 hours following the injection.[1] Seven days after the furosemide study, the same animals received hydrochlorothiazide (300 mg/L) in drinking water for 5 days as previously described by others.[2] The differences of SBP before and after the

thiazide treatment were calculated.

To rule out the possibility of interference between the two diuretics, we recruited an additional set of mice to test the effect of hydrochlorothiazide alone. Mice received hydrochlorothiazide twice, first time while on chow diet (Figure S9A left panel) and second time after the establishment of salt-induced hypertension in week 4 (Figure S9A right panel), allowing in a before-after self-control. The differences of SBP before and after the thiazide treatment was calculated in Figure S9B-C.

Power spectral analysis: Continuous radiotelemetry recording at the end of week 4 was used in power spectral analysis for heart rate variability and blood pressure variability as previously described.[3, 4] Beat-by-beat heart rate and blood pressure time series were derived from the blood pressure waveforms and converted to an equidistant sampling rate using cubic spline interpolation. Those equidistant heart rate time series were subjected to a fast Fourier transform to calculate spectral power in the very low frequency (VLF, 0.02-0.20 Hz), low frequency (LF, 0.2-0.6 Hz, reflecting mainly sympathetic cardiac modulation) and high frequency (HF, 1.0-5.0 Hz, reflecting para-sympathetic cardiac modulation) bands. Relative LF and HF were calculated as the relative value of each power component in proportion to the total power minus the VLF component. The LF/HF ratio of heart rate variability was used an indication of sympathetic nerve activity to the heart; while the LF component of the arterial pressure was used an indication of sympathetic nerve activity in the vascular tone regulation.[4, 5]

Renal sympathetic nerve activity: In week 4 of the protocol, NT controls and S-P467L mice were anesthetized with ketamine/xylazine and catheterized in the left common carotid artery for measurement of arterial pressure and heart rate. The right jugular vein was catheterized for maintenance of anesthesia with intravenous α-chloralose. Body temperature was maintained at 37.5°C using a temperature controlled surgical table and lamp. The left kidney was exposed and a renal nerve was carefully dissected free and placed on a bipolar 36-gauge platinum-iridium electrode. When an optimal recording of renal sympathetic nerve activity was obtained, the electrode was covered with silicone gel (World Precision Instruments). Electrodes were attached to a high-impedance probe (HIP-511, Grass Instruments), and the nerve signal was amplified to 10^5 times with a Grass P5 AC preamplifier, filtered at a 100 and 1000 Hz cutoff, and routed to a speaker system and to an oscilloscope (model 54501A; Hewlett-Packard) for both auditory and visual confirmation of the nerve signal. The amplified, filtered nerve signal was also directed to a MacLab analog-digital converter (model 8S ADInstruments) to a cursor modular that would count the action potentials (spikes/sec) that exceeded the background noise threshold and to a resetting voltage integrator (RVI, model B600C, University of Iowa Bioengineering) that sums the total voltage output (background noise plus nerve activity) to a unit of 1 V*sec/min before resetting to zero. At the end of the study, the background noise was determined after death and were subtracted from the total sympathetic nerve activity. Data were recorded and analyzed using a PowerLab unit and associated Chart software (ADInstruments). All surgical preparations were performed by the same individual (DAM). Non-stimulated recordings of sympathetic nerve activity were kept consistent using the same recording conditions throughout the study.

Pulse wave velocity measurements: Pulse wave velocity (PWV) was measured using the transit time method with Doppler ultrasound (Mouse Doppler TM, Indus Instruments, Texas) as

previously described.[6] Mice were anesthetized with isoflurane and placed at a supine position on a heated platform (38°C) with ECG electrodes taped to their paws for continuous recordings. Pressure waveforms using a 20 MHz probe were imaged at the descending aorta and abdominal aorta 1 mm above the exit to iliac arteries. Arrival and transit times were calculated and averaged over 10 cardiac cycles, and the distance between the descending and abdominal aorta was measured using a ruler. PWV (m/s) was calculated by dividing the distance by the transit time.

Cardiac output and peripheral vascular resistance: Echocardiogram was performed at baseline and on Day 21 of the study protocol as previously described.[7] In brief, mice were lightly sedated using midazolam (0.15 mg subcutaneous). Parasternal long- and short-axis views were obtained using a high-frequency echocardiography system (Vevo 2100, VisualSonics) to assess left ventricular thickness, left ventricular mass, end-systolic volume, end-diastolic volume, stroke volume and ejection fraction. Cardiac output is multiplication of stroke volume and HR determined simultaneously at the time of echocardiogram. Total peripheral vascular resistance was computed by dividing mean blood pressure with cardiac output.

Renal blood flow and resistance index: Mice were anesthetized by isoflurane inhalation on Day 21 and vascular ultrasound was performed on a high-frequency echocardiography system (Vevo 2100, VisualSonics) to determine renal artery diameter and renal blood flow to the right kidney. Studies were performed on the right kidney because the right renal artery was longer than the left and was anatomically easier to access by ultrasound. The right renal artery luminal diameter (D, mm) and blood flow velocity V (mm/s) was measured at the midpoint between the abdominal aorta and the entry to the right kidney. The peak systole blood flow velocity ($V_{systole}$, mm/s) and the end-diastolic blood flow velocity ($V_{diastole}$, mm/s) were employed to determine renal artery resistance index (RI) using the following equation.[8]

$$RI = \frac{Vsystole - Vdiastole}{Vsystole}$$

The renal artery luminal cross-sectional area (A, mm^2) was determined by the luminal diameter (D, mm) using the equation below.

$$\mathrm{A} = \frac{1}{4}\pi\, D^2$$

The mean renal blood flow (RBF) is a function of luminal cross-sectional area, mean blood flow velocity (V_{mean}, mm/s) and time (seconds) as indicated by the following equation (Because 1 Liter is equal to 10^3 cm^3 or 10^6 mm^3, 1 mm^3 is 1 µL).

$$RBF\ (\mu L/min) = A \times Vmean \times 60$$

RBF was normalized to body weight (µL/min/g) to eliminate the effect of age in this manuscript.

Analysis of urinary sodium, nitrate/nitrite, and albumin: At baseline or on Day 21, mice were individually placed into metabolic cages (MMC100, Hatteras Instruments) for 48 hours. Mice were acclimated to the cages in the first 24 hours. Food intake and water intake in the second 24 hour were recorded while feces and urine were collected and measured. Sodium ingestion was calculated from food intake while urinary sodium excretion was analyzed by a flame photometer (Cole-Parmer, IL) for the computation of net sodium balances as described.[9] Urine samples were diluted 1:20 for the measurement of total nitrate and nitrite levels using a commercially available kit (Enzo Life Sciences, Catalog # ADI-917-020). Briefly, nitrate was reduced to nitrite by nitrate reductase and the total nitrite was determined in a colorimetric

detection that converts nitrite to a colored azo dye product of the Griess Reaction that absorbs visible light at 540 nm. In separate mice, urinary albumin was quantified in week 4 with a fluorescent assay that utilized Albumin Blue 580 dye (Molecular Probes) and a fluorescent plate reader (FL600, Bio-Tek) as previously described.[10]

Acute diuretic/natriuretic challenge: Diuretic/natriuretic challenge studies were carried out to assess as described.[9, 11] In brief, mice were placed in metabolic cages on day 21 immediately after being injected i.p. with saline equal to 10% of their body weight and urine output in the next 4 hours were collected. Three days later, the experiment was repeated in the same animals with furosemide (20 mg/kg) dissolved in the saline load. Another three days after this, the experiment was performed in the same animals again with hydrochlorothiazide (40 mg/kg) dissolved in the saline load. Urinary sodium in this manuscript was analyzed by a flame photometer (Cole-Parmer, IL) by the same technician.

ELISA assay for plasma renin: At the end of the study protocol, mice were euthanized by pentobarbital overdose (150 mg/kg given i.p. in 0.1 mL volume) and blood was collected through cardiac puncture. Blood was collected into a heparinized microcentrifuge tube, gently mixed and immediately centrifuged at 5000 rcf for 3 minutes at room temperature. Plasma was carefully separated and immediately frozen in liquid nitrogen and then transferred to -80 ^{o}C until the renin ELISA assay was performed. Concentration of renin in the plasma was measured using a commercially available ELISA kit (RayBiotech) per manufacturer's instructions as previously described.[12]

Vascular function: Wire myography studies in the carotid artery was performed as previously described.[6, 12] Briefly, the left and right common carotid arteries were dissected free of adventitial fat and each cut into two segments. Likewise, the first-order renal artery branches (segmental arteries) were also dissected from both kidneys. Carotid and renal arterial rings were then equilibrated for 45 min under a resting tension of 0.25 g, and concentration-dependent response to acetylcholine (ACh, 1 nmol/L-30 μmol/L) and sodium nitroprusside (SNP, 0.1 nmol/L-30 μmol/L) were performed after an initial submaximal precontraction (40%-60%) with a thromboxane A2 receptor agonist U46619 (60 nmol/L). Vasoconstriction was recorded in response to KCl (10-100 mmol/L), 5-hydroxytryptamine (5-HT, i.e. serotonin, 10 nmol/L-10 μmol/L), and endothelin-1 (0.1 nmol/L-0.3 μmol/L). ACh, SNP, KCl, and endothelin-1 (E7764) were obtained from Sigma Aldrich. U46619 (CAS 56985-40-1) was obtained from Cayman Chemical.

Basilar arteries were isolated and cannulated onto glass micropipettes filled with oxygenated Krebs buffer (in mmol/L: 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 11 Glucose) in an organ chamber as we described previously.[6, 12] Arteries were transferred to a pressurized myograph system (Model 110p, Danish Myograph Technologies) and equilibrated for 30 min at 60 mmHg luminal pressure under no-flow conditions. The viability of vessels was first examined by exposure to 100 mmol/L KCl. The vessels were initially precontraction to 30% of maximal contractile capacity with U46619 for the assessment of vasodilation responses to ACh and SNP. The level of precontraction was similar in NT and S-P467L mice with chow diet or HSD.

Flow Cytometry: Flow cytometry was performed in aortic tissues as previously described.[11, 13] Briefly, immediately after mice were sacrificed, 20 mL ice-cold PBS was perfused into the left

ventricle to remove blood from the circulation. Aortas were harvested and minced before being incubated in a digestion buffer (phenol-free RPMI 1640 containing 1 mg/mL collagenase A, 1 mg/mL collagenase B and 0.1 mg/mL DNase I, Roche) at 37°C for 20 minutes. The suspension was passed through a cell strainer with a pore diameter of 70 µm to collect single cells. This single cell suspension was subsequently treated with a red blood lysis buffer (Invitrogen) to remove red blood cells. Fc receptors were blocked with anti-mouse CD16/CD32 (BD Biosciences, clone 2.4G2) for 20 min at 4°C prior to the staining of surface markers. The antibodies used were: Alexa Fluor 488 anti-mouse CD45, APC-Cy7 anti-mouse CD3, and APC anti-mouse F4/80. Each aortic cell sample was incubated in 100 µl of FACS buffer containing 1.5 µl of each antibody for 35 minutes. The cells were then washed twice with FACS buffer and immediately analyzed on an LSR II flow cytometer with DIVA software (BD Biosciences). Dead cells were eliminated from analysis using Hoechst 33528 (Sigma-Aldrich). For each experiment, we performed flow minus one (FMO) controls for each fluorophore to establish gates. Data analysis was performed using FlowJo 10.2 software (Tree Star, Inc.).

Western Blot: Kidneys were harvested and cortex and medulla were manually dissected. Separated cortex and medulla were flash frozen in liquid nitrogen and stored and -80°C until processing. Cortex or medulla was homogenized using a Potter homogenizer in 1 ml cold homogenization buffer containing lysis buffer containing 300 mM sucrose, 50 mM Tris-HCl (pH 7.4;), 1 mM EDTA, 1 mM EGTA, 1 mM NaVO4, 50 mM NaF, 1 mM ditiothreitol (MilliporeSigma), 1 mM phenylmethane sulfonyl fluoride, 1 µg/mL aprotinin, and 4 µg/mL leupeptin. Homogenate was centrifuged at 6000 g for 15 min at 4°C and supernatant was transferred to a new tube and stored at −80°C. Protein concentration was determined using the Bio-Rad DC assay and adjusted for loading by densitometric quantitation of total protein after running on a 4%–12% Bis-tris acetate gel (Invitrogen) and Coomassie staining.[14, 15] For blotting, 20 µg adjusted protein were separated on a 4%–12% Bis-tris acetate gel (Invitrogen) and transferred to a polyvinylidene fluoride membrane by using the Trans-Blot Turbo Transfer System (Bio-Rad). The membrane was blocked with 5% nonfat milk in PBS-Tween, followed by incubation with the primary antibody overnight at 4°C. Cortex was blotted with anti-total NCC antibody (1:6000),[16] while medulla was blotted with anti-total NKCC2 antibody (1:1000, Stressmarq SPC-401). Membranes were washed, incubated with HRP-goat anti-rabbit IgG (1:2,500, Invitrogen, 65-6120) for 1 h at room temperature, washed again, and finally incubated with Western Lightning ECL (Perkin Elmer). ECL signal was detected with a Syngene Pxi4 imager. Densitometry was performed with ImageJ (http://rsbweb.nih.gov/ij/).

References

1. Zaumseil J and Braunlich H. [Age dependent differences in the pharmacokinetics of furosemide in rats]. *Arch Int Pharmacodyn Ther*. 1976;219:167-76.
2. Ueda K, Nishimoto M, Hirohama D, Ayuzawa N, Kawarazaki W, Watanabe A, Shimosawa T, Loffing J, Zhang MZ, Marumo T and Fujita T. Renal Dysfunction Induced by Kidney-Specific Gene Deletion of Hsd11b2 as a Primary Cause of Salt-Dependent Hypertension. *Hypertension*. 2017;70:111-118.
3. Borges GR, Morgan DA, Ketsawatsomkron P, Mickle AD, Thompson AP, Cassell MD, Mohapatra DP, Rahmouni K and Sigmund CD. Interference with peroxisome proliferator-activated receptor-gamma in vascular smooth muscle causes baroreflex impairment and autonomic dysfunction. *Hypertension*. 2014;64:590-6.
4. Shinohara K, Liu X, Morgan DA, Davis DR, Sequeira-Lopez ML, Cassell MD, Grobe JL, Rahmouni K and Sigmund CD. Selective Deletion of the Brain-Specific Isoform of Renin Causes Neurogenic Hypertension. *Hypertension*. 2016;68:1385-1392.
5. Stauss HM. Identification of blood pressure control mechanisms by power spectral analysis. *Clin Exp Pharmacol Physiol*. 2007;34:362-8.
6. Mukohda M, Fang S, Wu J, Agbor LN, Nair AR, Ibeawuchi SC, Hu C, Liu X, Lu KT, Guo DF, Davis DR, Keen HL, Quelle FW and Sigmund CD. RhoBTB1 protects against hypertension and arterial stiffness by restraining phosphodiesterase 5 activity. *J Clin Invest*. 2019;130.
7. Weiss RM, Chu Y, Brooks RM, Lund DD, Cheng J, Zimmerman KA, Kafa MK, Sistla P, Doshi H, Shao JQ, El Accaoui RN, Otto CM and Heistad DD. Discovery of an Experimental Model of Unicuspid Aortic Valve. *J Am Heart Assoc*. 2018;7.
8. Bude RO and Rubin JM. Relationship between the resistive index and vascular compliance and resistance. *Radiology*. 1999;211:411-7.
9. Zhang J, Rudemiller NP, Patel MB, Karlovich NS, Wu M, McDonough AA, Griffiths R, Sparks MA, Jeffs AD and Crowley SD. Interleukin-1 Receptor Activation Potentiates Salt Reabsorption in Angiotensin II-Induced Hypertension via the NKCC2 Co-transporter in the Nephron. *Cell Metab*. 2016;23:360-8.
10. Abais-Battad JM, Alsheikh AJ, Pan X, Fehrenbach DJ, Dasinger JH, Lund H, Roberts ML, Kriegel AJ, Cowley AW, Jr., Kidambi S, Kotchen TA, Liu P, Liang M and Mattson DL. Dietary Effects on Dahl Salt-Sensitive Hypertension, Renal Damage, and the T Lymphocyte Transcriptome. *Hypertension*. 2019;74:854-863.
11. Wu J, Saleh MA, Kirabo A, Itani HA, Montaniel KR, Xiao L, Chen W, Mernaugh RL, Cai H, Bernstein KE, Goronzy JJ, Weyand CM, Curci JA, Barbaro NR, Moreno H, Davies SS, Roberts LJ, 2nd, Madhur MS and Harrison DG. Immune activation caused by vascular oxidation promotes fibrosis and hypertension. *J Clin Invest*. 2016;126:50-67.
12. Nair AR, Agbor LN, Mukohda M, Liu X, Hu C, Wu J and Sigmund CD. Interference With Endothelial PPAR (Peroxisome Proliferator-Activated Receptor)-gamma Causes Accelerated Cerebral Vascular Dysfunction in Response to Endogenous Renin-Angiotensin System Activation. *Hypertension*. 2018;72:1227-1235.
13. Mukohda M, Stump M, Ketsawatsomkron P, Hu C, Quelle FW and Sigmund CD. Endothelial PPAR-gamma provides vascular protection from IL-1beta-induced oxidative stress. *Am J Physiol Heart Circ Physiol*. 2016;310:H39-48.
14. Ferdaus MZ, Miller LN, Agbor LN, Saritas T, Singer JD, Sigmund CD and McCormick JA. Mutant Cullin 3 causes familial hyperkalemic hypertension via dominant effects. *JCI Insight*. 2017;2.

15. McDonough AA, Veiras LC, Minas JN and Ralph DL. Considerations when quantitating protein abundance by immunoblot. *Am J Physiol Cell Physiol*. 2015;308:C426-33.
16. McCormick JA, Mutig K, Nelson JH, Saritas T, Hoorn EJ, Yang CL, Rogers S, Curry J, Delpire E, Bachmann S and Ellison DH. A SPAK isoform switch modulates renal salt transport and blood pressure. *Cell Metab*. 2011;14:352-64.