SYMPATHETIC STIMULATION OF THIAZIDE-SENSITIVE SODIUM-CHLORIDE COTRANSPORT IN THE GENERATION OF SALT-SENSITIVE HYPERTENSION
Online Supplement

Andrew S. Terker[#], Chao-Ling Yang[#,*], James A. McCormick[#], Nicholas P. Meermeier[#,*], Shaunessy L. Rogers[#], Solveig Grossmann[$], Katja Trompf[$], Eric Delpire[§], Johannes Loffing[$], and David H. Ellison[#,*]

[#] Division of Nephrology & Hypertension, Department of Medicine, Oregon Health & Science University, Portland, OR, 97239,
[*] VA Medical Center, Portland, OR, USA,
[$] Institute of Anatomy, University of Zurich, Zurich, Switzerland, and
[§] Department of Anesthesiology, Vanderbilt University Medical School, Nashville, TN.

David H. Ellison, M.D.
Professor of Medicine and Physiology & Pharmacology
Division of Nephrology & Hypertension, CH12R
Oregon Health & Science University
3181 SW Sam Jackson Park Road
Portland, OR 97239
Phone 503 494-4465
Fax: 503 494-5330
Email: ellisond@ohsu.edu

Detailed Methods

Animals

All animal studies were approved by Oregon Health and Science University's Institutional Animal Care and Usage Committee (Protocol IS918). All mice were male littermates (12–16 weeks, 25–30 g) and had a C57Bl/6 background. All strains are backcrossed to the appropriate wild-type mice every 10 generations to maintain genetic backgrounds. SPAK$^{-/-}$ mice,[21,22] and Angiotensin II receptor type 1a (AT_{1a})$^{-/-}$ mice[23] have all been reported previously. Doxycycline-inducible kidney-specific (KS) OxSR1$^{-/-}$ mice were generated by crossing mice that were homozygous for floxed OxSR1 with *lox*P sites flanking the 113 bp exon 2 with mice that express both the reverse tetracycline transactivator (rtTA) under the control of the renal epithelial cell-specific Pax8 promoter[24] and the LC1 transgene.[25] Wild-type controls for the KS OxSR1$^{-/-}$ mice were those littermates that had two floxed OxSR1 alleles, but lacked either the LC1 transgene, the Pax8 transgene, or both. Prior to use in reported studies, KS OxSR1$^{-/-}$ mice were treated with doxycyline (2 mg/ml in 5% sucrose in drinking water) for 15 days to induce Cre-mediated recombination. Wild-type controls also received doxycycline.

PCR Genotyping

Standard PCR on tail snips was used for animal genotyping. For $AT_{1a}$$^{-/-}$ mice, the following primers were used to distinguish knockout from wild-type animals: 5'-TGAGAACACCAATATCACTG-3', 5'-TTCGTAGACAGGCTTGAG-3', and 5'-CCTTCTATCGCCTTCTTGACG-3'. To distinguish mice carrying at least one modified SPAK allele from wild-type mice, a previously reported approach was used.[22] Western blots on protein extracts from cultured tail cells were then used to further distinguish heterozygous SPAK mice from homozygous SPAK null mice. For KS OxSR1$^{-/-}$ mice, the following primers were used to identify animals that were homozygous for floxed exon 2 within the OxSR1 gene and also carried at least one copy each of the Cre and Pax8 rtTA transgenes: OxSR1FloxF: 5'-AGCTCAGGCTCCCTCCACGGAG, OxSR1FloxR: 5'-AAGACACATTGATACTCTGTTTCTCGAAGG; CreF: 5'-TTTCCCGCAGAACCTGAAGATG, CreR: 5'-TCACCGGCATCAACGTTTTCTT; Pax8F: 5'-CCATGTCTAGACTGGACAAGA, Pax8R: 5'-CAGAAAGTCTTGCCATGACT.

Animal studies

Systolic blood pressures (SBPs) were measured with tail-cuffs using volumetric pressure recording (CODA-6; Kent Scientific). This method has been validated for SBP measurements in mice, showing correlation with radiotelemetry.[26,27] The mice were first acclimated to the experimental procedure for five consecutive days; baseline SBP was then measured for one week on normal salt (0.49% NaCl). At the start of the second week, NE (2.5 mg/kg/d, Sigma) or vehicle was administered via osmotic minipump (Alzet) for two weeks while SBP measurements were continued. During the third week, all mice were switched to a high salt diet (8% NaCl, Harlan Teklad) and

SBP measurements were continued. For short-term adrenergic stimulation studies, norepinephrine (1250 ug/kg), phenylephrine (1250 ug/kg), isoproterenol (1250 ug/kg), or vehicle was injected intraperitoneally as indicated.

Immunoblotting

Mouse kidneys were harvested and snap-frozen in liquid nitrogen. Kidneys were then homogenized on ice in chilled homogenization buffer containing protease and phosphatase inhibitors. Protein (20–80 μg) was separated on 3–8% (wt/vol) Tris acetate or 4-12% (wt/vol) Bis-Tris gel (Invitrogen). All primary antibodies have been characterized and were specific to the following proteins: NCC, pNCC at threonine-53, and WNK4 (developed in laboratory of DHE), pNCC at threonine-58 and pNCC at serine-71 (developed in laboratory of JL), SPAK, OxSR1,[28] β-actin (Abcam). Densitometry was performed using ImageJ (http://rsbweb.nih.gov/ij/). All bands were normalized to actin. Representative images are shown. Densitometry is in the online supplement.

Immunofluorescence

Mice were anesthetized with isoflurane and kidneys perfusion-fixed by retrograde abdominal aortic perfusion of 3% paraformaldehyde in PBS (pH 7.4). After overnight cryoprotection with 800 mOsm sucrose and freezing, 5 μm sections were cut, washed in 1× PBS, incubated in 1× PBS with 0.5%Triton X for 30 min, washed in PBS, and blocked in 5% milk in PBS for 30 min. Primary antibody in 5% milk in 1× PBS was added for 1 hr, followed by a wash in PBS. Sections were incubated for 45 min with secondary antibody in block, then washed. Images were acquired on a high resolution wide field Core DV system (Applied Precision™). The system was an Olympus IX71 inverted microscope with a proprietary XYZ stage enclosed in a controlled environment chamber; differential interference contrast (DIC) transmitted light and a solid state module for fluorescence. The camera used was a Nikon Coolsnap ES2 HQ. Representative images are shown.

DCT-Specific PCR

DCT cDNA was obtained from mice expressing the enhanced green fluorescent protein (EGFP) under the control of the parvalbumin promoter. GFP+ tubule segments were isolated via a Complex Object Parametric Analyzer and Sorter (COPAS) and RNA and cDNA were prepared all as described previously.[29] The following primers were used were for the designated RT-PCR reactions. β_1-adrenergic receptor forward: 5'-ATCGTTCTGCTCATCGTGGTGGGTAACG, β_1-adrenergic receptor reverse: 5'-CGTCAGCAAACTCTGGTAGCGAAAGGG; β_2-adrenergic receptor forward: 5'-ATGGGGCCACACGGGAACGAC, β_2-adrenergic receptor reverse: 5'-GGCGTAGGCCTGGTTCGTGAAGAAGTC; NCC forward: 5'-TCACCATCAGCACACTGATGGGAGG, NCC reverse: 5'-ATCACTCCCCAGATGTTGAGCATGCAAC; AQP2 forward: 5'-

GAGATTACCCCTGTAGAAATCCGCGGGG, AQP2 reverse: 5'-GGGTCCGATCCAGAAGACCCAGTGATC; GAPDH forward: 5'-ATGGTGAAGGTCGGTGTGAACGGATTTG, GAPDH reverse: 5'-GCCTTCTCCATGGTGGTGAAGACACCAG.

Ex vivo stimulation of COPAS-sorted DCT with Isoproterenol

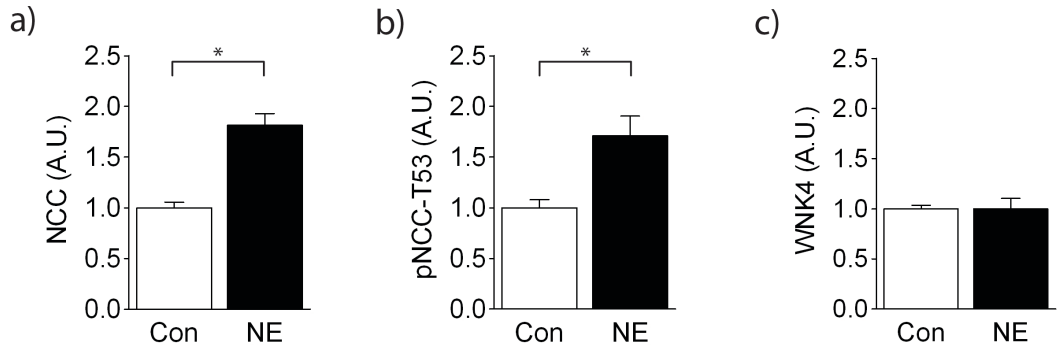
DCTs were sorted with COPAS from one mouse expressing the enhanced green fluorescent protein (EGFP) under the control of the parvalbumin promoter as described previously[29]. Per condition, 1000 DCTs were sorted into 35 mm Petri dishes (on ice) in an alternating manner (control, isoproterenol, control, isoproterenol, etc.) to avoid time-dependent sorting effects. Once 1000 DCTs per condition were sorted, Petri dishes were placed in an incubator for 5 min until 37°C were reached. Cells were then stimulated with either vehicle or 100 nM isoproterenol (DL-isoproterenol hydrochloride, Sigma) at 37°C for 10 min. The supernatant was collected and the remaining cells washed off the petri dish with ice cold KREBS and collected as well. Cells were spun at 800xg, 4°C for 5 min and the pellet re-disolved in 30 ul 1x Laemmli. 500 tubules were loaded on a 10 % SDS PAGE to detect either NCC or pNCC.

Statistical Analyses

Unpaired t-tests were used to compare groups. $p<0.05$ was considered significant. When necessary, corrections for multiple comparisons were used as indicated. Two-way ANOVA with repeated measures was used to compare blood pressure across time in vehicle- and norepinephrine-treated mice.

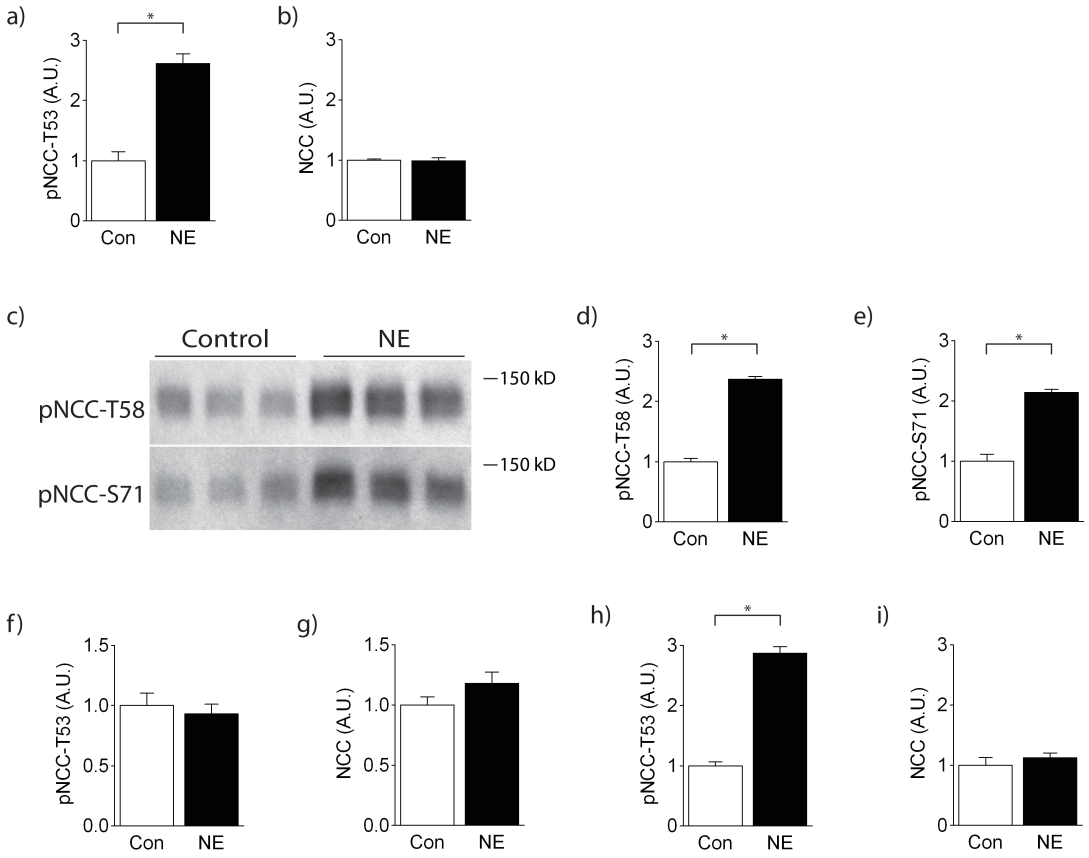
Supplemental Figure Legends

S1



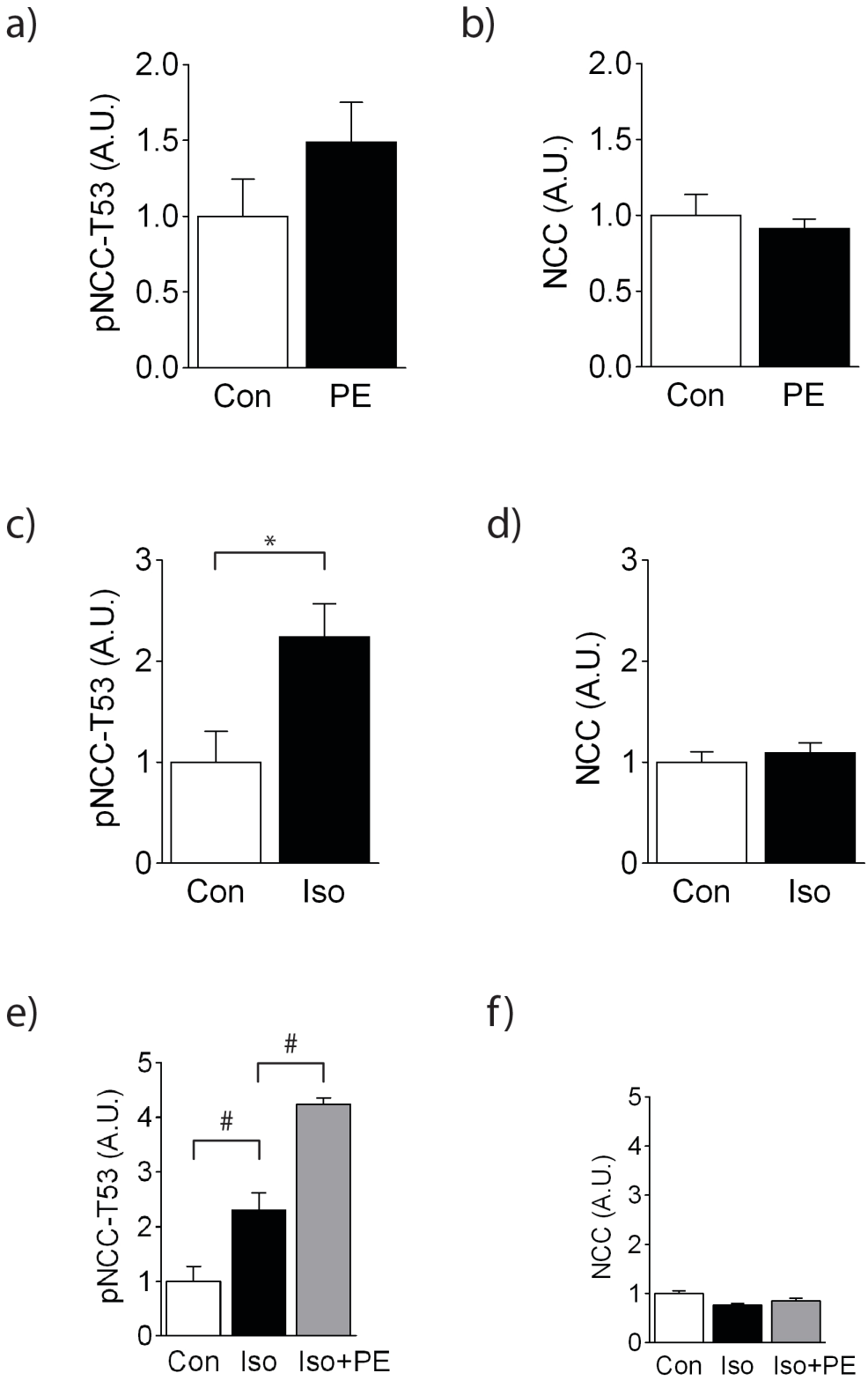
S1: Western blot quantification for chronic NE treatment. Panel A: Chronic NE treatment increased NCC abundance. **Panel B:** Chronic NE treatment increased pNCC-T53 abundance. **Panel C:** WNK4 abundance was unchanged by chronic NE treatment. Graphs depict mean ± s.e.m. * $p<0.05$ by unpaired t-test.

S2



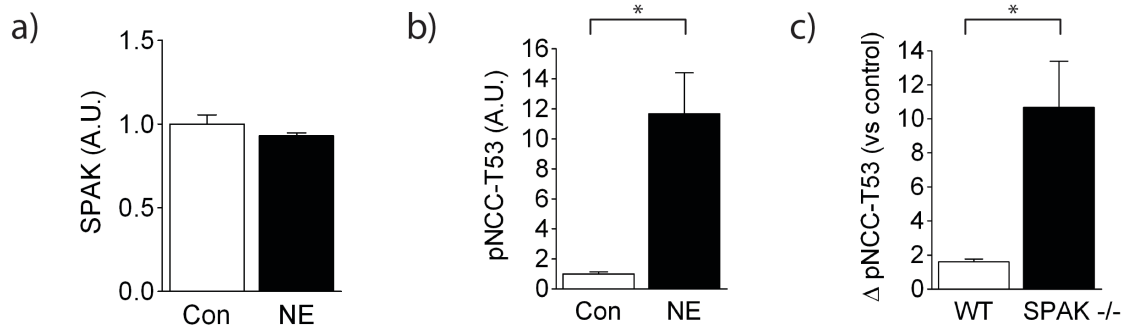
S2: Western blot quantification for acute NE treatment. Panel A: Treatment with NE for 30 minutes increased pNCC-T53 abundance. **Panel B:** Treatment with NE for 30 minutes did not change NCC abundance. **Panel C:** Treatment with NE for 30 minutes also increased pNCC-T58 and pNCC-S71 abundance. **Panel D:** Immunoblot quantification for pNCC-T58 abundance. **Panel E:** Immunoblot quantification for pNCC-S71 abundance. **Panel F:** pNCC abundance in $AT_{1a}^{-/-}$ mice is not statistically different from wild-type controls. **Panel G:** NCC abundance in $AT_{1a}^{-/-}$ mice is not statistically different from wild-type controls. **Panel H:** Treatment with NE for 30 minutes increased pNCC-T53 abundance in $AT_{1a}^{-/-}$ mice. **Panel I:** Treatment with NE for 30 minutes did not change NCC abundance in $AT_{1a}^{-/-}$ mice. Graphs depict mean ± s.e.m. * $p<0.05$ by unpaired t-test.

S3


a)
pNCC-T53 (A.U.)
2.0
1.5
1.0
0.5
0.0
Con
PE
b)
NCC (A.U.)
2.0
1.5
1.0
0.5
0.0
Con
PE
c)
pNCC-T53 (A.U.)
*
3
2
1
0
Con
Iso
d)
NCC (A.U.)
3
2
1
0
Con
Iso
e)
pNCC-T53 (A.U.)
#
#
5
4
3
2
1
0
Con
Iso
Iso+PE
f)
NCC (A.U.)
5
4
3
2
1
0
Con
Iso
Iso+PE

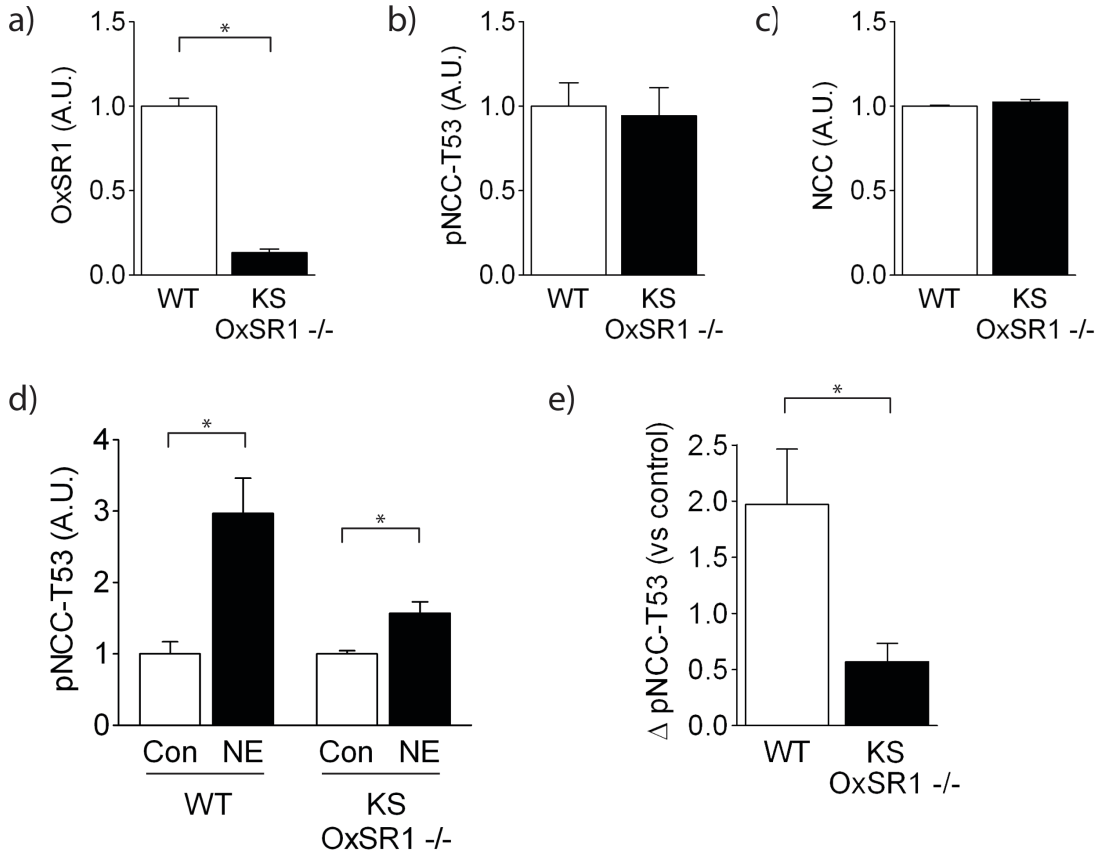
S3: Western blot quantification for acute PE and Iso treatment. Panel A: Treatment with the α-receptor agonist, phenylephrine (PE), for 30 minutes did not significantly increase pNCC-T53 abundace. **Panel B:** Treatment with PE for 30 minutes did not change NCC abundance. **Panel C:** Treatment with the β-receptors agonist, isoproterenol (Iso), for 30 minutes significantly increased pNCC-T53 abundance. **Panel D:** Treatment with Iso for 30 minutes did not change NCC abundance. **Panel E:** Treatment with both agonists together increased pNCC-T53 abundance greater than either agonist alone. **Panel F:** Treatment with both agonists together did not alter NCC abundance. Graphs depict mean ± s.e.m. * $p<0.05$ by unpaired t-test. # $p<0.016$ by unpaired t-test.

S4



S4: Western blot quantification for acute NE treatment in SPAK$^{-/-}$ mice. **Panel A:** Treatment with NE for 30 minutes did not change SPAK abundance in wild-type mice. **Panel B:** Treatment with NE for 30 minutes increased pNCC-T53 abundance in SPAK$^{-/-}$ mice. **Panel C:** Treatment with NE for 30 minutes increased pNCC-T53 abundance in SPAK$^{-/-}$ mice greater than in wild-type controls. Graphs depict mean ± s.e.m. * $p<0.05$ by unpaired t-test.

S5



S5: Western blot quantification for acute NE treatment in KS OxSR1$^{-/-}$ mice. Panel A: KS OxSR1$^{-/-}$ mice had significantly decreased abundance of OxSR1 in their kidneys compared with wild-type controls. **Panel B:** The abundance of pNCC-T53 was unaltered in KS OxSR1$^{-/-}$ mice at baseline. **Panel C:** The abundance of NCC protein was unaltered in KS OxSR1$^{-/-}$ mice at baseline. **Panel D:** Treatment with NE for 30 minutes increased pNCC-T53 abundance in KS OxSR1$^{-/-}$ mice. **Panel E:** Treatment with NE for 30 minutes increased pNCC-T53 abundance in KS OxSR1$^{-/-}$ mice less than in wild-type controls. Graphs depict mean ± s.e.m. * $p<0.05$ by unpaired t-test.

Supplemental Movie 1: NE increased DCT OxSR1 apical localization in SPAK$^{-/-}$ mice. Treatment with NE for 30 minutes increased the apical localization of OxSR1 in the DCT of SPAK$^{-/-}$ mice.