Overexpression of the Sodium Chloride Cotransporter Is Not Sufficient to Cause Familial Hyperkalemic Hypertension

James A. McCormick, Joshua H. Nelson, Chao-Ling Yang, Joshua N. Curry, David H. Ellison

Abstract—The sodium chloride cotransporter (NCC) is the primary target of thiazides diuretics, drugs used commonly for long-term hypertension therapy. Thiazides also completely reverse the signs of familial hyperkalemic hypertension (FHHt), suggesting that the primary defect in FHHt is increased NCC activity. To test whether increased NCC abundance alone is sufficient to generate the FHHt phenotype, we generated NCC transgenic mice; surprisingly, these mice did not display an FHHt-like phenotype. Systolic blood pressures of NCC transgenic mice did not differ from those of wild-type mice, even after dietary salt loading. NCC transgenic mice also did not display hyperkalemia or hypercalciuria, even when challenged with dietary electrolyte manipulation. Administration of fludrocortisone to NCC transgenic mice, to stimulate NCC, resulted in an increase in systolic blood pressure equivalent to that of wild-type mice (approximately 20 mm Hg). Although total NCC abundance was increased in the transgenic animals, phosphorylated (activated) NCC was not, suggesting that the defect in FHHt involves either activation of ion transport pathways other than NCC, or else direct activation of NCC, in addition to an increase in NCC abundance. (Hypertension. 2011;58:00-00.) ● Online Data Supplement

Key Words: hypertension ● hyperkalemia ● sodium chloride symporters ● thiazides ● mice ● transgenic

Familial hyperkalemic hypertension (FHHt) is characterized by hyperkalemia, hypertension, and metabolic acidosis, with normal glomerular filtration rate. Plasma renin levels are low, but plasma aldosterone is often in the normal range but inappropriately low with respect to the observed high level of plasma potassium, a strong stimulus of aldosterone secretion. Importantly, administration of thiazide diuretics, which inhibit the distal convoluted tubule (DCT)–specific sodium chloride cotransporter (NCC), is uniquely effective at ameliorating these abnormalities. FHHt is caused by mutations in 2 members of the with-no-lysine (K) (WNK) kinases, so named because of the unusual positioning of the lysine involved in coordinating ATP. Deletion of part of the first intron of WNK1 increases its expression in leukocytes and was proposed to be a gain-of-function mutation. Mice heterozygous for WNK1 display lower blood pressure than wild-type controls, supporting the hypothesis that WNK1 acts to increase blood pressure. Missense mutations within the WNK4 gene also lead to FHHt. WNK4 strongly inhibits NCC activity in Xenopus oocytes, whereas mutant WNK4 stimulates it; WNK1 increases NCC activity both through suppression of WNK4 and by activating STE20- and-SPS1-related proline/alanine-rich kinase (SPAK). Subsequent studies have shown that WNK1 itself is inhibited by a kidney-specific isoform lacking the kinase domain (KSWNK1). Dysregulation of NCC activity has therefore been proposed to be the primary defect underlying FHHt. In vitro studies, however, have revealed that the WNK kinases regulate a wide variety of ion channels and transporters besides NCC (reviewed in), resulting in controversy regarding the central role of NCC in the etiology of FHHt. Two mouse models that closely resemble FHHt have been reported. In the first, transgenic mice expressing 2 copies of WNK4 with an FHHt-causing mutation, in addition to the 2 endogenous wild-type alleles, were generated. These mice displayed an FHHt-like phenotype, including elevated blood pressure, hyperkalemia, hypercalciuria, and hyperplasia of the distal convoluted tubule, the nephron segment to which NCC is restricted. Interestingly, mice expressing an additional copy of wild-type WNK4 displayed an opposite phenotype. Another model was generated in which an FHHt-causing WNK4 mutation was knocked in, and similarly, an FHHt phenotype was observed. In both cases, the FHHt phenotype was completely reversed by administration of thiazides. Therefore, overexpression of NCC, achieved by other means, should be sufficient to cause an FHHt-like phenotype. The current experiments were designed to test this hypothesis.

Methods

Generation of NCC Transgenic Mice
All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Oregon Health & Science University Institutional Animal Care and Use Committee.

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Figure 1. Generation of sodium chloride cotransporter (NCC) transgenic mice. A, BAC clone RP-24-263E2 was modified by recombinating in Escherichia coli to remove Herpud1 and the region of Nup43 extending to its 3' untranslated region. The resulting NCC transgene contains 12.5 kb of sequence upstream of the NCC transcription start and the 3' untranslated region of NCC (vector sequence is not shown). B, Semiquantitative polymerase chain reaction on genomic DNA extracted from the tails of potential founders identified lines 727 and 743 as containing >12 and 5 copies of the NCC transgene; amplification of the β-globin gene confirmed equal template input. M indicates DNA markers; WT, wild-type.

Animals and approved by the Institutional Animal Care and Use Committee of the Oregon Health & Science University (protocol number A858). To generate mice overexpressing NCC, a bacterial artificial chromosome (BAC) clone containing the entire mouse NCC gene was obtained from CHORI. The closed circular BAC was purified using the Qiagen Large Construct kit, microinjected into (C57BL/6 X SJL)F2 mouse eggs, and surgically transferred to recipients. Founders were crossed with C57BL/6 wild-type mice, and offspring of interbreeding of the resulting N2 generation were used in subsequent experiments. The numbers of animals used for each procedure are given in the Results.

Western Blotting and Immunofluorescence

Animals were killed by CO2 asphyxiation, and kidneys were harvested. Homogenized samples were separated on a 4% to 12% NuPage Bis-Tris Gel (Novex, Invitrogen Corp) and transferred to polyvinylidene difluoride paper. Following antibody incubation, detection was performed using the Western Lightning kit (PerkinElmer) according to the manufacturer’s protocol. For immunofluorescence on kidney sections, mice were anesthetized with ketamine/xylazine/acepromazine (50/50/0.5 mg/kg) and perfusion fixed with 4% paraformaldehyde via the abdominal aorta. Kidneys were frozen, and 7-μm sections were prepared on a cryostat. Standard procedures were used for immunostaining, using 5% fat-free milk in PBS as block. Primary antibodies against NCC and NCC phosphorylated at threonine 53 were developed in our laboratory (see Supplemental Figure I, available online at http://hyper.ahajournals.org, for validation of antiphospho-p-53-NCC antibody); all other antibodies were purchased.

Blood Pressure Measurements

Blood pressure was measured in male mice aged 3 to 4 months by tail cuff, using a Coda 6 tail-cuff apparatus (Kent Scientific). This method is recommended for high-throughput studies in mice, including initial characterization of genetically modifications; it has been extensively validated and, over the physiological range, gives values similar to radiotelemetry.15

Plasma and Urine Chemistry

Blood was collected by cardiac puncture and analyzed immediately with an iSTAT blood chemistry analyzer (Abbott Laboratories). For dietary K+ manipulation, blood was collected from the lateral saphenous vein into heparinized tubes and centrifuged at 2000g for 5 minutes at room temperature. Plasma [Mg2+] was determined using a colorimetric assay (Pointe Scientific). Plasma [Na+] and [K+] were determined using a model 2655-10 flame photometer (Cole-Parmer). Urinary calcium and creatinine were measured by colorimetric assays (Pointe Scientific) on spot urine samples. Plasma aldosterone was measured by ELISA, according to the manufacturer’s protocol (IBL America); the plasma renin concentration assay is described in the supplemental material.

Dietary Manipulations

All diets used were obtained from Harlan Teklad: TD07309 (0.8% K+, control diet for K+ studies), TD07278 (5% K+), TD96208 (0.49% NaCl, control diet for NaCl studies), and TD92012 (8% NaCl). Mice were placed on each diet for 10 days.

Results

Generation of NCC Transgenic Mice

To generate mice overexpressing NCC, a BAC clone containing the entire mouse NCC gene, modified by recombinating to remove other genes, was used (Figure 1A). Twelve founders were identified, and semiquantitative polymerase chain reaction using primers targeting the NCC gene confirmed that the mice carried additional copies of the NCC gene (Figure 1B). A single transgene integration site was confirmed by fluorescence in situ hybridization analysis (Supplemental Figure II). Two lines were selected for further analysis, line 727, which carries >20 copies of the transgene, and line 743, which carries 5 copies (Figure 1B). Line 727 was not characterized extensively because of 80% perinatal mortality, which was most likely due to integration site of the transgene (see below and Supplemental Figure II), and hereafter “NCC transgensics” will refer to line 743, which has 5 copies of the transgene integrated. A single transgene integration site in line 743 was confirmed by fluorescence in situ hybridization analysis (Supplemental Figure II).

Western blotting revealed a 1.7-fold increase in total NCC expression in the kidneys of NCC transgenic mice (Figure 2A and 2B), similar to the increase observed in KS-WNK knockout mice.14 Expression levels of epithelial sodium channel (EnaC) subunits, the sodium-hydrogen antiporter 3 (NHE3), total and phospho-NKCC2, and the NCC regulatory protein SPAK did not differ significantly between wild-type and transgenic mice (Supplemental Figure III). Immunofluorescence on kidney sections, using an anti-NCC antibody, confirmed that there was no ectopic expression of total NCC in the kidney (Figure 3C).

Phosphorylation and Cellular Localization of NCC Is Not Altered in NCC Transgenic Mice

WNK1 activates NCC indirectly, through phosphorylation and activation of SPAK kinase,15 which then phosphorylates NCC, a prerequisite for full transport activity.16,17 Western
blotting using an antibody that recognizes p-T53 NCC revealed that the abundance of phosphorylated NCC did not differ between wild-type and NCC transgenics (Figure 3A and 3B), indicating that the ratio of phosphorylated to total NCC (Figure 2A and 2B) was reduced by 41% in the NCC transgenic animals. In addition to phosphorylation, for NCC to transport NaCl it must be localized to the apical membrane. Immunofluorescence, however, indicated that total and phospho-NCC cellular localization do not differ significantly between wild-type and transgenic mice (Figure 3C), but it could not clarify whether NCC expression in the luminal membrane was significantly different between genotypes.

**Overexpression of NCC Does Not Alter Basal Blood Chemistry Values or Body Weight**

There were no significant differences in plasma chemistry values between wild-type and transgenics, including plasma K\(^+\), which is typically elevated in FHHt (Table). Plasma renin concentration and aldosterone levels were determined and did not differ significantly, nor did body weights (Table). Significantly, line 727, which carries 20 copies of the transgene, did not display hyperkalemia or hypocalciuria (data not shown).

### Table. Plasma Chemistry and Body Weights of Wild-Type and Sodium Chloride Cotransporter (NCC) Transgenic Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type, ±SD (n)</th>
<th>NCC Transgenic, ±SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g), males</td>
<td>31.6±3.6 (10)</td>
<td>28.9±2.4 (6)</td>
</tr>
<tr>
<td>Body weight (g), females</td>
<td>23.6±1.9 (9)</td>
<td>23.2±2.6 (17)</td>
</tr>
<tr>
<td>Na(^+) (mmol/L)</td>
<td>147.5±1.86 (11)</td>
<td>147.9±3.08 (11)</td>
</tr>
<tr>
<td>K(^+) (mmol/L)</td>
<td>3.85±0.29 (11)</td>
<td>3.85±0.31 (11)</td>
</tr>
<tr>
<td>Mg(^2+) (mmol/L)</td>
<td>2.15±0.29 (11)</td>
<td>2.20±0.31 (11)</td>
</tr>
<tr>
<td>Cl(^-) (mmol/L)</td>
<td>107.3±0.9 (11)</td>
<td>107.8±1.7 (11)</td>
</tr>
<tr>
<td>iCa(^2+) (mmol/L)</td>
<td>1.22±0.03 (11)</td>
<td>1.25±0.05 (11)</td>
</tr>
<tr>
<td>TCO(_2) (mmol/L)</td>
<td>24.0±2.8 (11)</td>
<td>23.2±2.0 (11)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>241±37 (11)</td>
<td>226±45 (11)</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>21.8±2.7 (11)</td>
<td>22.6±2.9 (11)</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.24±0.07 (11)</td>
<td>0.23±0.05 (11)</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.42±0.01 (11)</td>
<td>0.42±0.03 (11)</td>
</tr>
<tr>
<td>Plasma renin concentration (ng/mL per h)</td>
<td>79±3.6 (10)</td>
<td>108±3.9 (9)</td>
</tr>
<tr>
<td>Aldosterone (nmol/L)</td>
<td>0.79±0.12 (18)</td>
<td>0.90±0.29 (18)</td>
</tr>
</tbody>
</table>

For plasma values, 5 males and 6 females were used, except for renin activity (5 males and 5 females for wild-type; 5 males and 4 females for transgenic) and aldosterone (9 of each gender per group).

iCa\(^2+\) indicates ionized calcium; TCO\(_2\), total CO\(_2\); BUN, blood urea nitrogen.

Figure 2. Sodium chloride cotransporter (NCC) transgenic mice display increased total NCC protein expression. **A**, Western blot analysis of whole kidney extracts from wild-type (WT) and NCC transgenic (NCC-TG) mice was performed using antibodies against NCC and β-actin. **B**, Densitometric quantitation was performed normalizing to β-actin, and expression of total NCC in NCC-TG (filled bars) relative to WT (open bars) mice was calculated. WT, n=13; NCC-TG, n=16. Values are shown ±SEM; *P=0.005.

Figure 3. Sodium chloride cotransporter (NCC) phosphorylation and cellular distribution are not altered in NCC-transgenic (NCC-TG) mice. **A**, Western blot analysis of whole kidney extracts from wild-type (WT) and NCC-TG mice was performed using antibodies against phospho (p)-NCC (T53) and β-actin. **B**, Densitometric quantitation was performed normalizing to β-actin, and expression of p-NCC in NCC-TG (filled bars) relative to WT (open bars) mice was calculated. n=12 for each group. Values are shown ±SEM. **C**, Immunofluorescence showed that in both WT and NCC-TG mice, total and p-NCC displayed expression at the apical membrane of the distal convoluted tubule.
activity of NCC, so to ensure full activation of NCC, tisone has previously been shown to increase expression and tension (Figure 4). The synthetic mineralocorticoid fludrocortisone does not lead to salt-sensitive hypertension in wild-type or NCC transgenic mice, indicating that overexpression of NCC does not lead to salt-sensitive hypertension (Figure 4). The absence of functional differences likely reflected the fact that the abundance of total and phospho-NCC did not differ between wild-type and transgenic animals (Supplemental Figure IV). These data indicate that activation of NCC by fludrocortisone does not elicit a hypertensive phenotype in mice overexpressing NCC at the message level because NCC abundance at the protein level and NCC activity are not enhanced. Immunofluorescence showed that the degree of phospho-NCC expression at the apical membrane was similar in both groups (data not shown), suggesting that activation of NCC regulatory pathways, such as WNK/SPAK, may be required to fully stimulate the overexpressed NCC.

Mice Overexpressing NCC Are Not Hypertensive
Systolic blood pressure values on a standard diet containing 0.49% NaCl did not differ between genotypes (Figure 4) on the standard diet, nor did heart rates (wild-type 896 ± 4 versus NCC transgenic 918 ± 7, n = 10). The mice were placed on a high-NaCl (8% NaCl) diet for 10 days, which can elicit a hypertensive phenotype in mice normotensive on standard salt diets. Salt loading did not lead to an increase in blood pressure in wild-type or NCC transgenic mice, indicating that overexpression of NCC does not lead to salt-sensitive hypertension (Figure 4). The synthetic mineralocorticoid fludrocortisone has previously been shown to increase expression and activity of NCC, so to ensure full activation of NCC, wild-type and transgenic mice on the high NaCl diet were provided with fludrocortisone in their drinking water. Systolic blood pressure increased in both groups, but to a similar degree (19 mm Hg in wild-type and 22 mm Hg in transgenics) (Figure 4). The presence of functional differences likely reflected the fact that the abundance of total and phospho-NCC did not differ between wild-type and transgenic animals (Supplemental Figure IV). These data indicate that activation of NCC by fludrocortisone does not elicit a hypertensive phenotype in mice overexpressing NCC at the message level because NCC abundance at the protein level and NCC activity are not enhanced. Immunofluorescence showed that the degree of phospho-NCC expression at the apical membrane was similar in both groups (data not shown), suggesting that activation of NCC regulatory pathways, such as WNK/SPAK, may be required to fully stimulate the overexpressed NCC.

NCC Transgenic Mice Display Normal Responses to Dietary Electrolyte Modification
A distinctive characteristic of FHHt is the presence of hyperkalemia. On a control diet containing 0.8% potassium, NCC mice were normokalemic; increasing the dietary potassium level to 5% did not elicit hyperkalemia (Figure 5A). Similarly, NCC transgenic mice did not display a change in plasma sodium after 10 days on the high-potassium diet (Figure 5B). Western blotting revealed that expression of total NCC was not altered by potassium loading in either genotype, but phospho-NCC trended to lower levels in NCC transgenic mice (Supplemental Table I). In the distal convoluted tubule, sodium reabsorption is correlated inversely to calcium absorption. In FHHt, patients with mutations in WNK4 are hypercalciuric, whereas patients with mutations in WNK1 are normocalciuric. This suggests that mutant WNK4 may cause hypercalciuria by an interaction of WNK4 with a calcium channel or transporter. In contrast, inactivating mutations of NCC result in Gitelman syndrome, characterized by hypochloremic metabolic alkalosis, hypokalemia, and hypocalciuria. Analysis of spot urine samples revealed no significant difference in urinary calcium levels between NCC transgenic and wild-type mice (Figure 5C). Furthermore, manipulation of the diet to provide 8%

Figure 4. Sodium chloride cotransporter (NCC) transgenic mice are normotensive. Systolic blood pressure was measured in wild-type (WT) (open bars) and NCC mice transgenic (TG) (filled bars) mice after 2 weeks on standard (0.49% NaCl) and high-salt (8% NaCl) diets and 2 weeks of high-salt diet with fludrocortisone in drinking water (17 mg/L). There were no significant differences in systolic blood pressure on standard or high-salt diet. Fludrocortisone treatment led to a similar, significant increase in systolic blood pressure in both wild-type and NCC transgenic mice.

Figure 5. Normal electrolyte homeostasis in sodium chloride cotransporter (NCC) transgenic mice. Plasma K⁺ (A) and plasma Na⁺ (B) did not differ between wild-type (WT) (open bars) and NCC transgenic (TG) (filled bars) mice on standard (0.8% K⁺) or high-potassium (5% K⁺) diets. Values are means±SEM, n = 19 to 22. C, Urinary calcium:creatinine, measured from spot urine collections, did not differ between wild-type (open bars) and NCC transgenic mice (filled bars) on standard (0.49% NaCl) or high-NaCl (8%) diets. Values are means±SEM, n = 18 to 22.
NaCl for 7 days did not induce hypercalciuria in either genotype (Figure 5C). Salt loading caused a trend toward lower expression of α-ENaC but significantly increased expression of γ-ENaC in both wild-type and NCC transgenic mice (Supplemental Table I); surprisingly, β-ENaC expression tended to increase in wild-type mice but decrease in NCC transgenic mice.

Responses of aldosterone secretion to both potassium and sodium loading did not differ between wild-type and NCC transgenic mice, significantly increasing in response to high K⁺ and significantly decreasing in response to high NaCl (Supplemental Figure V). Similarly, no differences in plasma renin activities were observed between wild-type and NCC transgenic mice following dietary manipulation (Supplemental Figure V).

Discussion

The current results add to an increasing body of evidence suggesting that increased NCC abundance alone is insufficient to cause hyperkalemia and hypertension. Initial studies in mice, coupled with clinical observations in humans, suggested that the predominant mechanism by which mutations in WNK kinases lead to FHHt is by increasing NCC abundance or trafficking to the apical membrane. As noted above, KS-WNK1 is believed to be a dominant-negative regulator of WNK1, thereby suppressing the effects of WNK1 to stimulate NCC. In contrast to the mild phenotypes observed in these studies, other mouse models, in which FHHt mutants of WNK4 were overexpressed or knocked in, developed clear hyperkalemic hypertension in association with increased NCC expression; the abundance of NCC increased by 2-fold in 1 model. One possible reason for the observed differences between the models results from differential effects on other transporters. Expression of ENaC is downregulated in KS-WNK1 knockout mice, whereas it is upregulated in mice overexpressing an FHHt mutant of WNK4. Taken together with our observations in NCC transgenic mice, these data suggest that WNKs must alter the activity of several transport proteins to induce the FHHt phenotype.

Another possibility is that WNKs generate hyperkalemic hypertension by activating NCC via SPAK, in addition to increasing its abundance. In NCC transgenic mice, although total NCC expression was increased, there was no difference in the phospho-NCC level between wild-type and transgenic mice (Figure 3). We reasoned that by increasing NCC abundance and phosphorylation, fludrocortisone might uncover a difference between wild-type and transgenic mice, but fludrocortisone increased blood pressure similarly in both groups. Notably, the phenotype of KS-WNK1 knockout mice, in which both total and phospho-NCC levels are elevated, is intermediate between the phenotype observed in transgenic NCC mice and the FHHt-mutant WNK4 mice, with a mild increase in diastolic pressure under basal conditions. Thus, it seems unlikely that an increase in phospho-NCC alone would generate the complete FHHt phenotype.

There are several possible explanations for the ability of thiazides to completely correct FHHt that are not directly related to their inhibition of NCC. First, it is well established that thiazide diuretics inhibit activity of carbonic anhydrase in the proximal tubule. More recently, it has been shown that NCC knockout mice treated with thiazides still display a significant increase in urinary sodium output, showing that thiazides act on other sodium transport mechanisms. Finally, thiazide treatment leads to changes in kidney structure, including apoptosis and dedifferentiation in the DCT, where NCC is the predominant sodium entry pathway. These changes may result from reduced intracellular sodium concentration or increased intracellular calcium levels. Atrophy of the DCT is also observed in NCC knockout mice and in transgenic mice overexpressing wild-type WNK4, in which
NCC activity is presumably lower. Therefore, it is possible that some of the effects of thiazides in patients with FHHt are secondary to structural changes in the distal nephron.

Surprisingly, salt loading did not increase urinary calcium excretion in wild-type mice. In humans, urinary calcium excretion was ≈5 times as high in salt-loaded rats compared with controls drinking deionized water, irrespective of dietary calcium content. In their mouse model of FHHt, Lalioti et al saw little change in urinary calcium excretion by wild-type mice on an 8% NaCl diet, the same level of NaCl provided in our studies. These data suggest that renal calcium handling in mice may respond differently to salt loading. Another explanation is that in the mouse studies, animals were provided with 8% NaCl for only 10 days, whereas the rat studies involved 8 weeks of salt loading.30

Perspectives

These unanticipated data suggest that NCC overexpression alone is insufficient to induce hyperkalemic hypertension accompanied by hypercalciuria and that dysregulation of NCC activity or of other transporters/channels plays a significant role in the etiology of FHHt. In vitro, the WNK kinases have been shown to regulate the activities of a broad range of sodium and potassium transport mechanisms in the kidney (reviewed in). Therefore, it is likely that the WNK kinases play a broad role in ion homeostasis in normal physiology. Alternatively, the mouse may not precisely model human physiology and pathophysiology with regard to NCC function, because NCC knockout mice do not precisely mimic Gitelman syndrome caused by inactivating mutations in the human NCC gene.33,34 Although the thiazides are extremely effective in Gitelman syndrome, their pleiotropic actions make them a broad-spectrum medication, and their use is somewhat complicated by their pleiotropic actions.32

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Disclosures

None.

References


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Supplemental information
Expanded Methods

**Generation of NCC transgenic mice**

All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University (protocol number A858). To generate mice over-expressing NCC, a BAC clone containing the entire mouse NCC gene was obtained from CHORI. In addition to the NCC gene, this BAC (RP24-263E2) contained one entire gene downstream (*Herpud1*), and a 70 kilobase fragment extending to the coding region of the upstream *Nup43* gene. To remove the *Herpud1* gene, recombineering was performed using the Red/ET kit (Open Biosystems) according to the manufacturer’s protocol. The primers used to add homology arms to the counter-selection marker were:

5’ATGACAAAGTATTAGTCATTGATTCCAGTCTTTGATTTGTATACAAAATTGGGCCTTGTTAGGATGGCGGG3’; 5’CAACTGATACAGAAAAAGCCTTTTGACAGATCCAACGCTATCTTTTGTCGAAGAAGCTCAGTAAGAAA3’.

The primer used to remove the counter-selection marker was:

5’ATGACAAAGTATTAGTCATTGATTCCAGTCTTTGATTTGTATACAAAATTGCACGAAAAATAGGCCGTTGGATCTGATCAGTTG3’.

Removal of the *Herpud1* gene was confirmed by restriction analysis and DNA sequencing. The closed circular BAC was purified using the Qiagen Large Construct kit and microinjected into (C57BL/6 X SJL)F2 mouse eggs and surgically transferred to recipients by the University of Michigan Transgenic Core. Founders were crossed with C57BL/6 wild type mice and offspring of interbreeding of the resulting N2 generation were used in subsequent experiments.

**Identification of transgenic founders**

Crude genomic DNA extracts were prepared from tail snips by heating at 95°C for 45 minutes in NaOH, followed by neutralization with Tris-HCl. 4µl of extract was used directly in PCR reactions. For routine genotyping PCR, primers targeting the BAC vector pTARBAC2.0 were used: forward 5’TTCGCCAGATCCCGCCTTTCT3’; reverse 5’ACGTGCGATCAACGGTCTCA3’. Mouse NCC primers: forward 5’AGGGTCAAGGGCACGGGTTGGC3’; reverse 5’GGTAAAGGGAGCGGGTCCAGGAGCAGG3’. Mouse beta-globin primers: forward 5’CCAATCTGCTCAGACAGGATAGAGGAGG3’; reverse 5’CCTTGAAGGCTGTCAAAGTGGATCCAGGCAATCG3’.

**FISH analysis**

FISH analysis was performed on primary fibroblasts cultured from tail snips. The uncut BAC transgene was be used as template to generate a probe using the DIG Nick-translation Kit (Roche), according to the manufacturer’s protocol. The labeled probe was combined with Cot-1 DNA (Invitrogen) and hybridized overnight at 37°C to the fixed cells in a solution containing 50% formamide, 10% dextran sulfate, 0.1% SDS and 2× SSC (0.03 M trisodium citrate, pH 7.0, and 0.3 M NaCl). The slides were washed twice in 0.5X SSC/0.1% SDS at 45°C for 5 min, covered with PBS/0.5% BSA for 5 mins then incubated with 1:200 dilution of Anti-dig-fluorescein Fab fragments (Roche) room temperature for 1 hour. After washing twice with PBS, slides were mounted and examine by fluorescent microscopy.
**Blood pressure measurements**

Blood pressure was measured in male mice aged 3-4 months by tail-cuff, using a Coda 6 tail-cuff apparatus (Kent Scientific). Mice were trained for 5 days prior to recording of blood pressure. After training, each day, ten acclimation cycles, followed by fifteen measurement cycles, were performed on alternating channels while maintaining body temperature between 34 °C and 36 °C. The multiple blood pressure data for each animal from 3 days of measurement were averaged.

**Plasma renin activity**

Plasma renin activity (PRA) was measured as the amount of angiotensin I generated after incubation with excess angiotensinogen (MP Biomedicals). Two microliters of plasma were incubated with excess porcine angiotensinogen (4µM) in a 10µl reaction containing sodium acetate (50mM, pH6.5), AEBSF (2.5mM), 8-hydroxyquinoline (1mM) and EDTA (5mM) for 15 minutes at 37°C. The assay was linear for at least 30 minutes. The reaction was stopped by boiling for 5 minutes, diluted 1:50, and angiotensin I was measured by ELISA (Phoenix Pharmaceuticals).

**Statistical analyses**

Data were analyzed using SPSS (Version 17.0, Chicago, IL, USA). All values are expressed as mean ± standard error of the mean. Comparisons between two groups were performed using the Student’s t-test (paired or unpaired, as appropriate); multi-group comparisons were performed using 1-way analysis of variance (ANOVA) followed by a post-hoc test. In animals, blood pressure data were analyzed using 2-way ANOVA to assess whether the changes from average baseline blood pressure to final day blood pressure in the two groups were different.

**Thiazide response test**

Mice maintained on 0.49% NaCl control diet had their bladders emptied via manual massage. Mice were then placed in metabolic cages, and urine was then collected under water-saturated light mineral oil for 3 hours. Hydrochlorothiazide (dissolved in 1.7% ethanolamine) was injected intraperitoneally at 25mg/kg and a further 3 hour urine collection performed. Urinary sodium and creatinine levels were then assayed.

**References**

Table S1

Effects of potassium loading on total and p-NCC expression in wild type and NCC transgenic mice

Wild type

<table>
<thead>
<tr>
<th>Channel/transporter</th>
<th>Control diet</th>
<th>High K+ diet</th>
<th>P value</th>
<th>Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NCC</td>
<td>100 ± 33</td>
<td>86 ± 12</td>
<td>0.66</td>
<td>No change</td>
</tr>
<tr>
<td>p-NCC</td>
<td>100 ± 33</td>
<td>38 ± 11</td>
<td>0.09</td>
<td>Down</td>
</tr>
</tbody>
</table>

NCC transgenic

<table>
<thead>
<tr>
<th>Channel/transporter</th>
<th>Control diet</th>
<th>High K+ diet</th>
<th>P value</th>
<th>Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NCC</td>
<td>100 ± 44</td>
<td>117 ± 50</td>
<td>0.74</td>
<td>No change</td>
</tr>
<tr>
<td>p-NCC</td>
<td>100 ± 44</td>
<td>107 ± 48</td>
<td>0.72</td>
<td>No change</td>
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</table>

Effects of sodium chloride loading on transporter and channel expression

Wild Type

<table>
<thead>
<tr>
<th>Channel/transporter</th>
<th>Control diet</th>
<th>High NaCl diet</th>
<th>P value</th>
<th>Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha ENaC</td>
<td>100 ± 20</td>
<td>60 ± 10</td>
<td>0.20</td>
<td>Down</td>
</tr>
<tr>
<td>Beta ENaC</td>
<td>100 ± 20</td>
<td>210 ± 52</td>
<td>0.07</td>
<td>Up</td>
</tr>
<tr>
<td>Gamma ENaC</td>
<td>100 ± 5</td>
<td>160 ± 19</td>
<td>0.01*</td>
<td>Up</td>
</tr>
<tr>
<td>NHE3</td>
<td>100 ± 20</td>
<td>113 ± 18</td>
<td>0.69</td>
<td>No change</td>
</tr>
<tr>
<td>Total NCC</td>
<td>100 ± 6</td>
<td>88 ± 16</td>
<td>0.29</td>
<td>No change</td>
</tr>
<tr>
<td>pNCC</td>
<td>100 ± 24</td>
<td>130 ± 52</td>
<td>0.23</td>
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</tr>
<tr>
<td>Total NKCC2</td>
<td>100 ± 7</td>
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<td>0.03</td>
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NCC Transgenic

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<th>P value</th>
<th>Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha ENaC</td>
<td>100 ± 20</td>
<td>55 ± 10</td>
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<td>Down</td>
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<tr>
<td>Beta ENaC</td>
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<td>49 ± 52</td>
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<tr>
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<td>254 ± 19</td>
<td>0.003*</td>
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</tr>
<tr>
<td>NHE3</td>
<td>100 ± 20</td>
<td>136 ± 18</td>
<td>0.19</td>
<td>No change</td>
</tr>
<tr>
<td>Total NCC</td>
<td>100 ± 3</td>
<td>141 ± 16</td>
<td>0.09</td>
<td>Up</td>
</tr>
<tr>
<td>pNCC</td>
<td>100 ± 24</td>
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<tr>
<td>Total NKCC2</td>
<td>100 ± 8</td>
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</table>
Figure S1: Validation of anti-pT53-NCC antibody

(A) 25 μg of protein extract from whole kidney from rat (R) and mouse (M) were resolved on a 4-12% NuPage Bis-Tris gel and western blotting performed using two antibodies generated against p-T53-NCC (antibodies R39 and R40). Specificity of the antibodies for p-T53-NCC was confirmed by treating rat protein extracts with 1 unit/μg calf intestinal phosphatase for 1 hour at 60°C (R+CIP).

(B) Specificity against NCC was confirmed by blotting kidney extracts from wild type (WT) and NCC knockout (KO) mice.
Figure S2: Initial characterization of NCC transgenic lines

(A) Fish analysis on primary tail fibroblasts from line 743 confirmed a single integration site for the transgene. The arrowhead marked with * indicates the transgene, while the other two arrowheads indicate the two endogenous NCC alleles. (B) Survival curves for wild type and NCC transgenic mice, n=11. (C) FISH analysis of metaphase spreads from line 727 primary tail fibroblasts indicate that the transgene (probe is the yellow band) has integrated in to the distal end of band B of chromosome 2 (red paint). There are 5 genes in this region, of which 2 have been disrupted in mice by gene targeting. Lrp1b<sup>−/−</sup> disruption results in no phenotype, while disruption of Zeb2 results in defects in neuronal development<sup>2</sup>. 
Figure S3: Western blotting reveals no differences in other sodium regulatory proteins between wild type and NCC transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>Alpha ENaC</th>
<th>Beta ENaC</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>TG</td>
</tr>
<tr>
<td></td>
<td>100 ± 7</td>
<td>105 ± 3</td>
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<tr>
<td>Actin</td>
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<td>p=0.45</td>
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<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
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<td>TG</td>
</tr>
<tr>
<td></td>
<td>100 ± 9</td>
<td>82 ± 8</td>
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<tr>
<td>Actin</td>
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<table>
<thead>
<tr>
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<th>Total NKCC2</th>
<th>p-NKCC2</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>TG</td>
</tr>
<tr>
<td></td>
<td>100 ± 9</td>
<td>127 ± 9</td>
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<tr>
<td>Actin</td>
<td></td>
<td>p=0.10</td>
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<table>
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<tbody>
<tr>
<td></td>
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<td>TG</td>
</tr>
<tr>
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<td>90 ± 6</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>p=0.22</td>
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Western blotting was performed on protein extracts from whole kidney using the indicated antibodies. Densitometric analysis was performed, with values normalized to actin. Average expression in wild types (WT) was set to 100%, and relative expression in NCC transgenics (TG) determined. Values expressed are +/- S.E.M., n = 4. p values for comparisons between WT and TG are shown.
Figure S4: Total and p-NCC do not significantly differ between wild type and NCC transgenic mice on a high (8%) NaCl diet following treatment with fludrocortisone

Western blotting was performed on whole kidney extracts from wild type (WT) and NCC transgenic (TG) mice following 10 days on a high NaCl diet with concurrent administration of fludrocortisone, suggesting that NCC was maximally activated in both groups. Average expression in wild types was set to 100%, and relative expression in NCC transgenics determined. Values expressed are +/- S.E.M., n = 4. p values for comparisons between WT and TG are shown.
Figure S5: NCC transgenic mice display reduced response to thiazides, but no differences in aldosterone and plasma renin activity compared to wild type mice.

(A) NCC transgenic mice display a blunted response to thiazide diuretic administration compared with wild type mice. Values shown are means +/- S.E.M., n = 7, *, p<0.05.

(B) NCC transgenic mice display the expected changes in plasma aldosterone to dietary electrolyte manipulation. Values shown are means +/- S.E.M., n = 5, *, p<0.05.

(C) No differences in plasma renin activity were observed between wild type and NCC transgenic mice following dietary electrolyte manipulations. Values shown are means +/- S.E.M., n = 5.