Over-expression of the sodium chloride cotransporter (NCC) is not sufficient to cause familial hyperkalemic hypertension

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'ATGACCAAAGTATTAGTCATTGATTCAGTCTTTGATTTGTATACAAATTGGGCCTGGT GATGATGGCGGG3';5'CAACTGATACAGAAAAAGCCTTTGACAGATCCAACGCCTATCTT TTCGTGTCAGAAGAACTCGTCAAGAA3'.
The primer used to remove the counter-selection marker was:
5'ATGACCAAAGTATTAGTCATTGATTCAGTCTTTGATTTGTATACAAATTGCACGAAAA GATAGGCGTTGGATCTGTCAAAGGCTTTTTCTGTATCAGTTG3'.
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: forward5'TTCCCAGATCCGCGCTTCT3'; reverse5'ACGTGCCGATCAACGTCTCA3'. Mouse NCC primers: forward 5'AGGGTCAAGGGCACGGTTGGC3; reverse 5'GGTAAAGGGAGCGGGTCCGAGG. Mouse beta-globin primers: forward 5' CCAATCTGCTCACACAGGATAGAGAGGGCAGG 3'; reverse 5' CCTTGAGGCTGTCCAAGTGATTCAGGCCATCG 3'.

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 37ºC 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. Ccomparisons 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

1. Marschang P, Brich J, Weeber EJ, Sweatt JD, Shelton JM, Richardson JA, Hammer RE, Herz J. Normal development and fertility of knockout mice lacking the tumor suppressor gene lrp1b suggest functional compensation by lrp1. *Mol Cell Biol*. 2004;24:3782-3793.
2. Van de Putte T, Maruhashi M, Francis A, Nelles L, Kondoh H, Huylebroeck D, Higashi Y. Mice lacking zfhx1b, the gene that codes for smad-interacting protein-1, reveal a role for multiple neural crest cell defects in the etiology of hirschsprung disease-mental retardation syndrome. *Am J Hum Genet*. 2003;72:465-470.

Table S1

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

Wild type

Channel/transporter	Control diet	High K+ diet	P value	Trend
Total NCC	100 ± 33	86 ± 12	0.66	No change
p-NCC	100 ± 33	38 ± 11	0.09	Down

NCC transgenic

Channel/transporter	Control diet	High K+ diet	P value	Trend
Total NCC	100 ± 44	117 ± 50	0.74	No change
p-NCC	100 ± 44	107 ± 48	0.72	No change

Effects of sodium chloride loading on transporter and channel expression

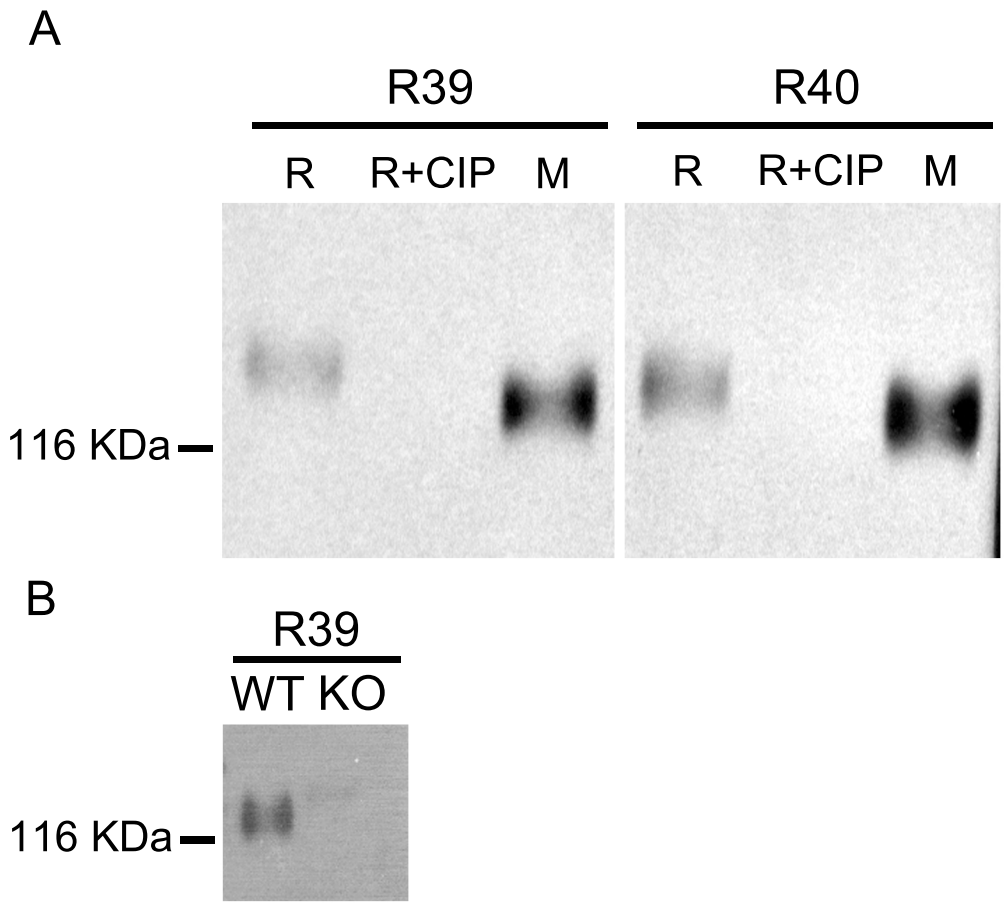
Wild Type

Channel/transporter	Control diet	High NaCl diet	P value	Trend
Alpha ENaC	100 ± 20	60 ± 10	0.20	Down
Beta ENaC	100 ± 20	210 ± 52	0.07	Up
Gamma ENac	100 ± 5	160 ± 19	0.01*	Up
NHE3	100 ± 20	113 ± 18	0.69	No change
Total NCC	100 ± 6	88 ± 16	0.29	No change
pNCC	100 ± 24	130 ± 52	0.23	Up
Total NKCC2	100 ± 7	124 ± 2	0.03	Up

NCC Transgenic

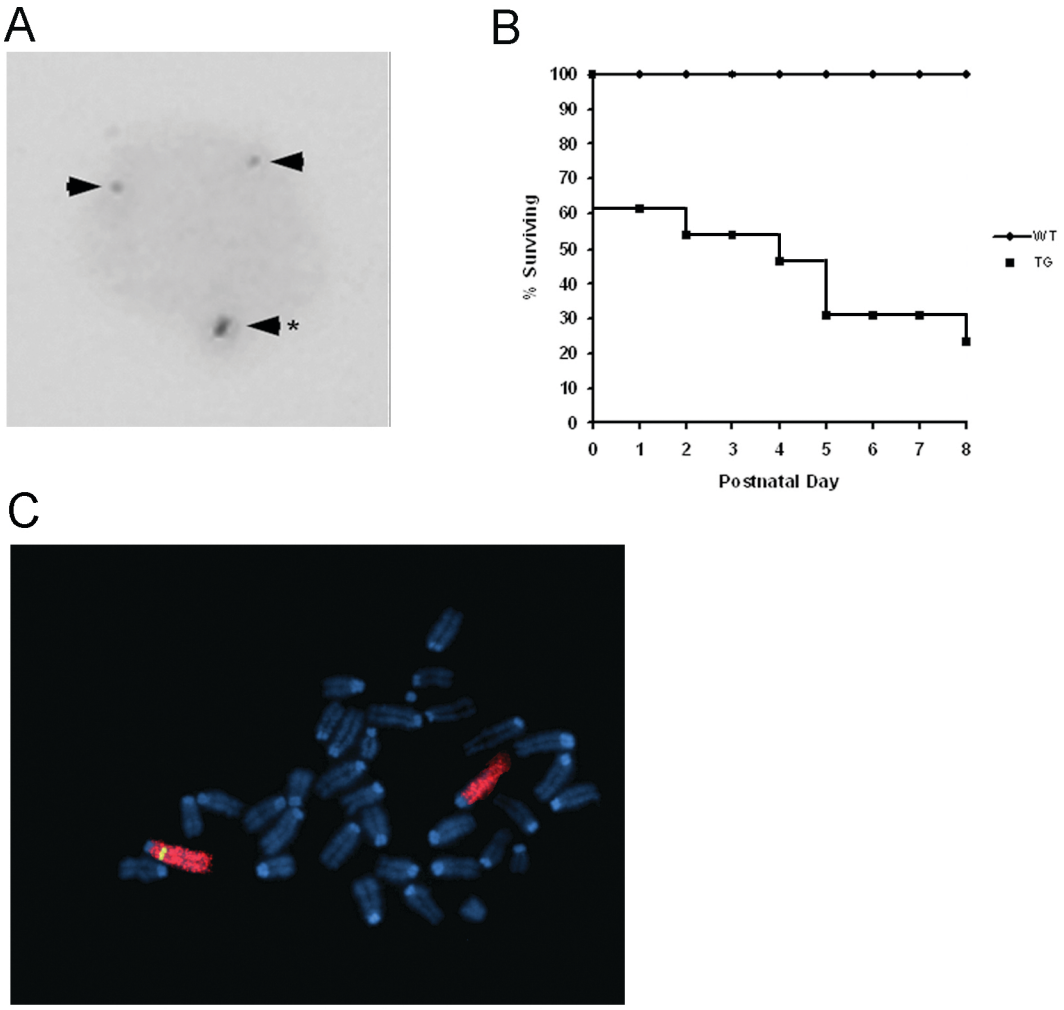
Channel/transporter	Control diet	High NaCl diet	P value	Trend
Alpha ENaC	100 ± 20	55 ± 10	0.07	Down
Beta ENaC	100 ± 44	49 ± 52	0.26	Down
Gamma ENac	100 ± 5	254 ± 19	0.003*	Up
NHE3	100 ± 20	136 ± 18	0.19	No change
Total NCC	100 ± 3	141 ± 16	0.09	Up
pNCC	100 ± 24	143 ± 52	0.40	Up
Total NKCC2	100 ± 8	127 ± 5	0.04	Up

Figure S1: Validation of anti-pT53-NCC antibody



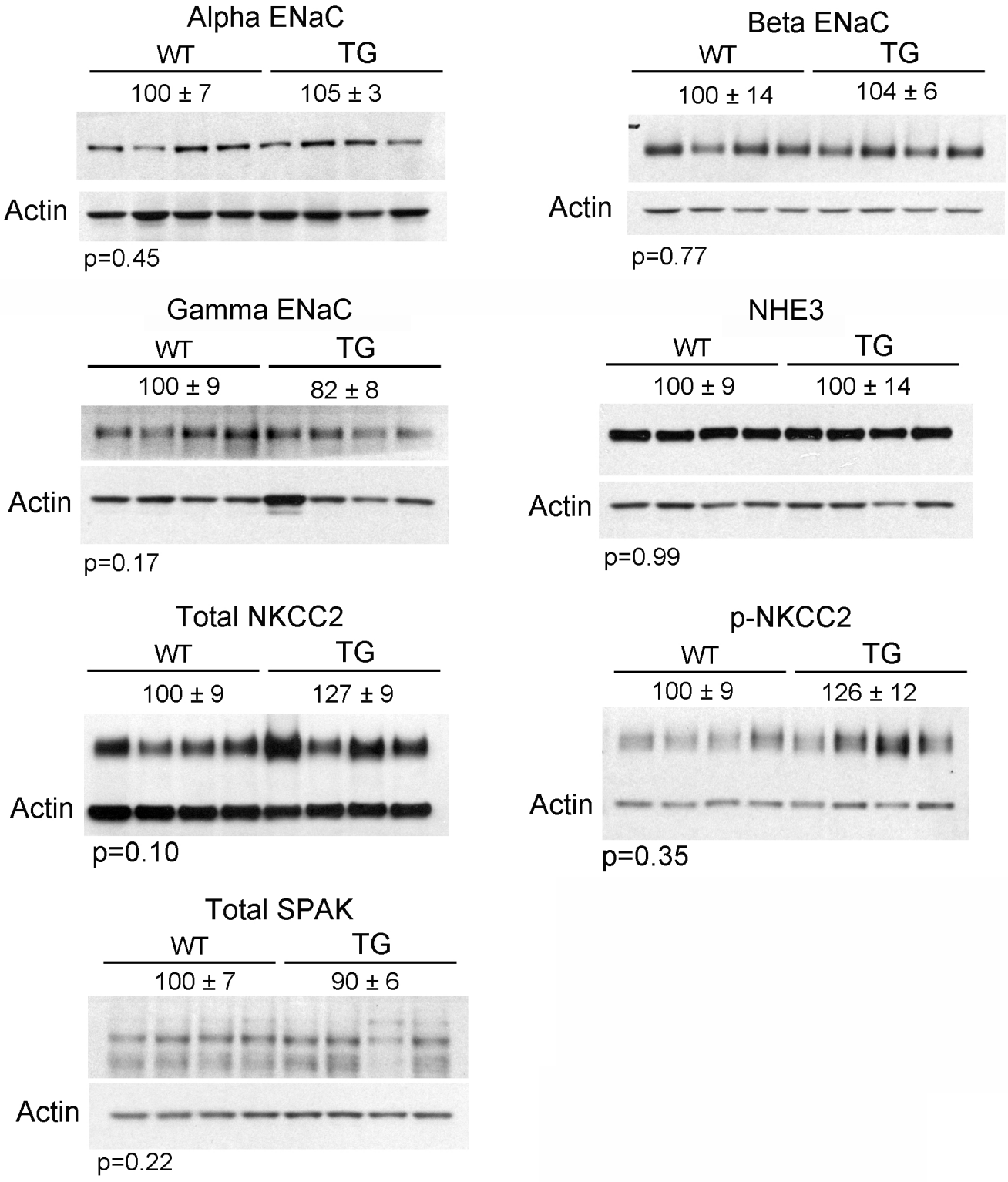
(A) 25 µg of protein extract from whole kidney from rat (R) and mouse (M) were resolved on a 4-12% NuPage Bis-Tris gel and western blotting performed using two antibodies generated against p-T53-NCC (antibodies R39 and R40). Specificity of the antibodies for p-T53-NCC was confirmed by treating rat protein 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



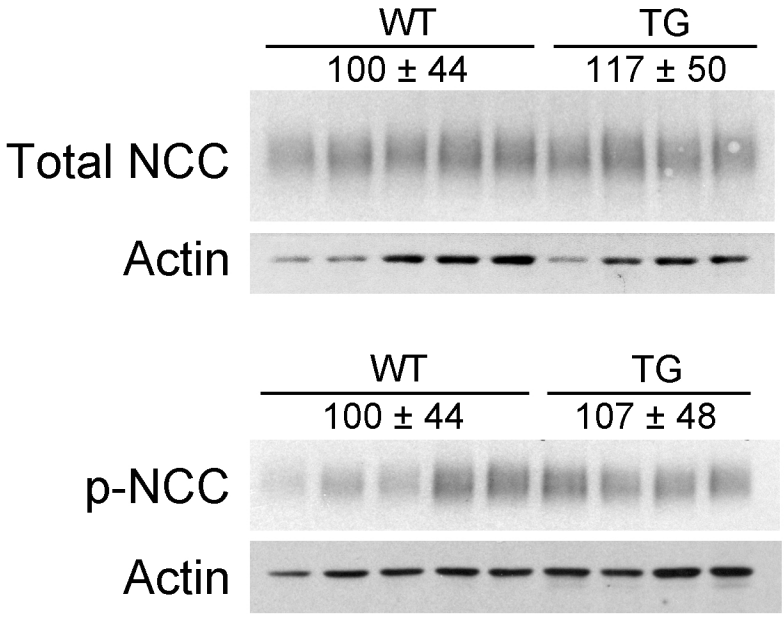
(A) Fish analysis on primary tail fibroblasts from line 743 confirmed a single integration site for the transgene. The arrowhead marked with * indicates the transgene, while the other two arrowheads indicate the two endogenous NCC alleles. (B) Survival curves for wild type and NCC transgenic mice, n=11. (C) FISH analysis of metaphase spreads from line 727 primary tail fibroblasts indicate that the transgene (probe is the yellow band) has intgrated in toe 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 [1] disruption results in no phenotype, while disruption of Zeb2 results in defects in neuronal development [2].

Figure S3: Western blotting reveals no differences in other sodium regulatory proteins between wild type and NCC transgenic mice



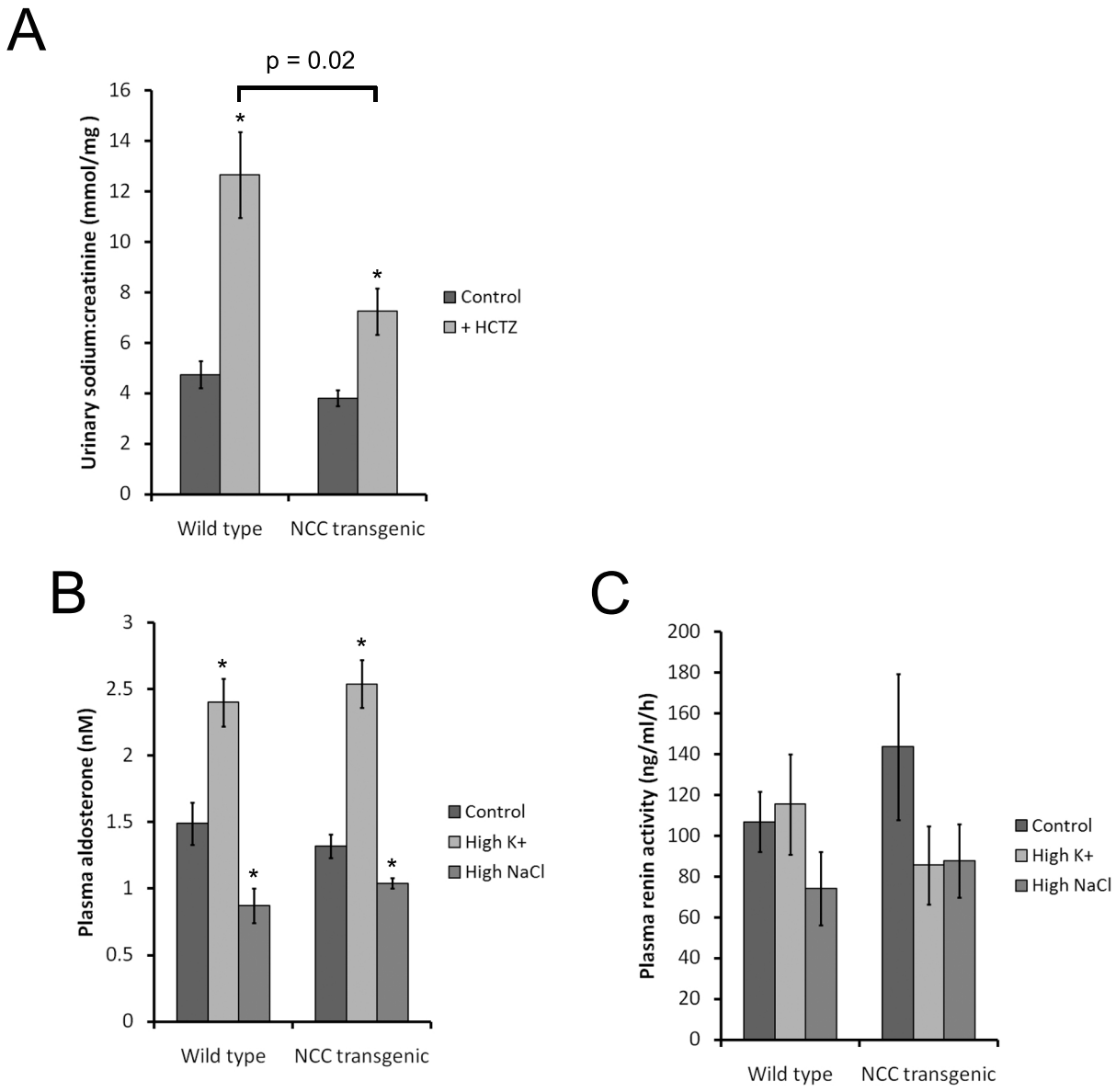
Western blotting was performed on protein extracts from whole kidney using the indicated antibodies. Densitometric analysis was performed, with values normalized to actin. Average expression in wild types (WT) was set to 100%, and relative expression in NCC transgenics (TG) determined. Values expressed 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.