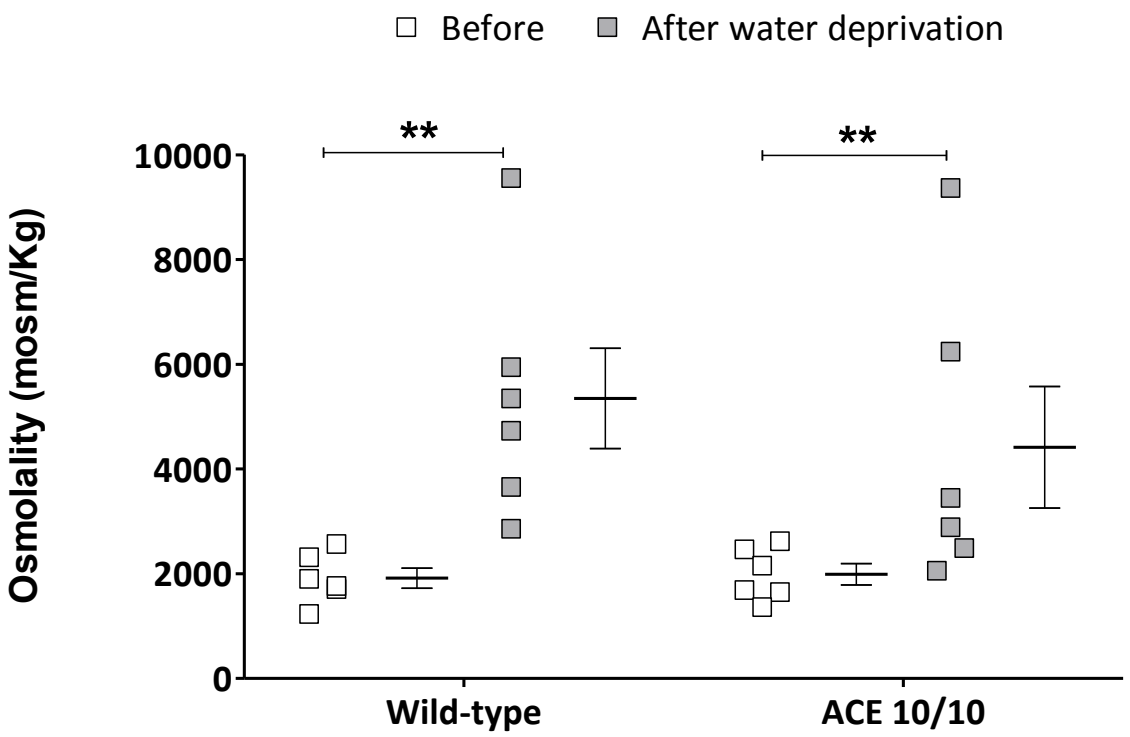
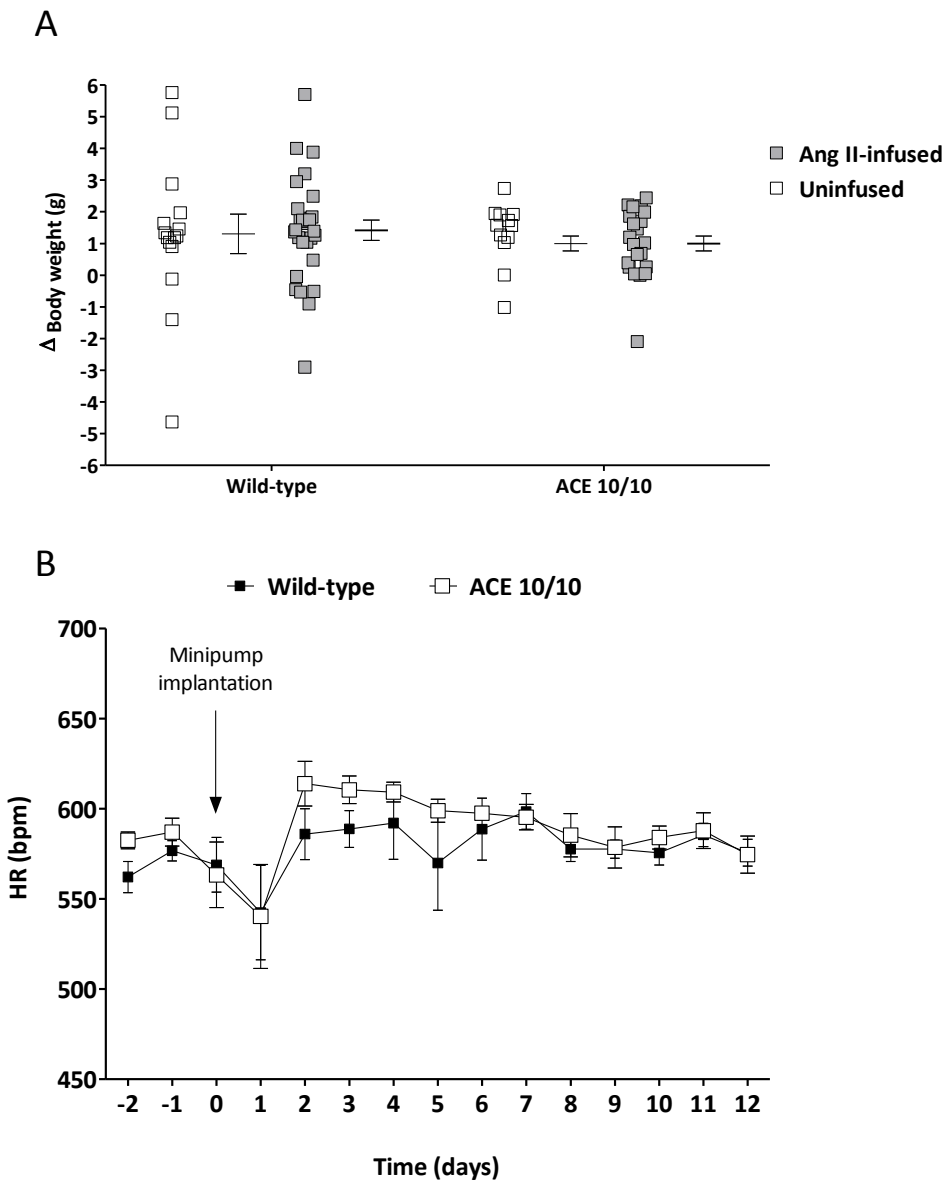
SUPPLEMENTAL DATA



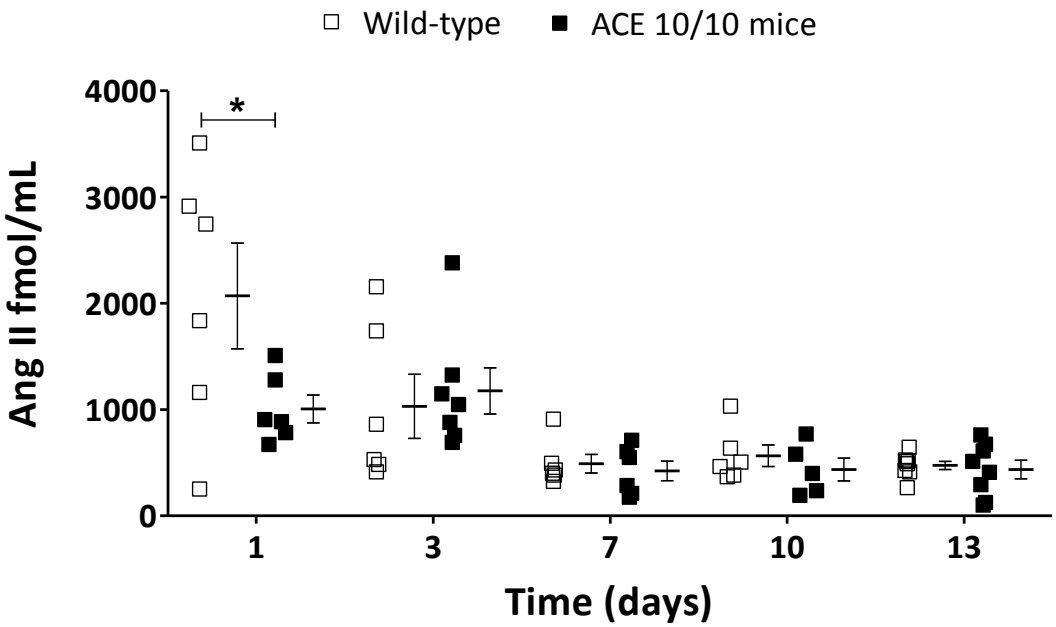
Supplemental Figure 1. Urine concentration capacity is preserved in the ACE 10/10 mice. Urine osmolality was measured in wild-type and ACE 10/10 mice before and after overnight water deprivation by vapor pressure osmometry.
n=6 per group. Values are individual records and means ± SEM. **=$p<0.01$.



Supplemental Figure 2. Body weight and heart rate changes of wild-type and ACE 10/10 mice in response to Ang II infusion.

(A) Body weight differential (Δ) was calculated subtracting the initial measure from the weight 2 weeks after sham-operation of or Ang II infusion. *n* = 6-22 per group.

(B) Heart rate (HR) was recorded in conscious unrestrained conditions by telemetry. *n* = 7-8 per group. Ang II = Angiotensin II (400 ng/kg/min via minipump), uninfused = sham-operated mice. Bpm = Beats per minute. Values are individual records and means ± SEM.

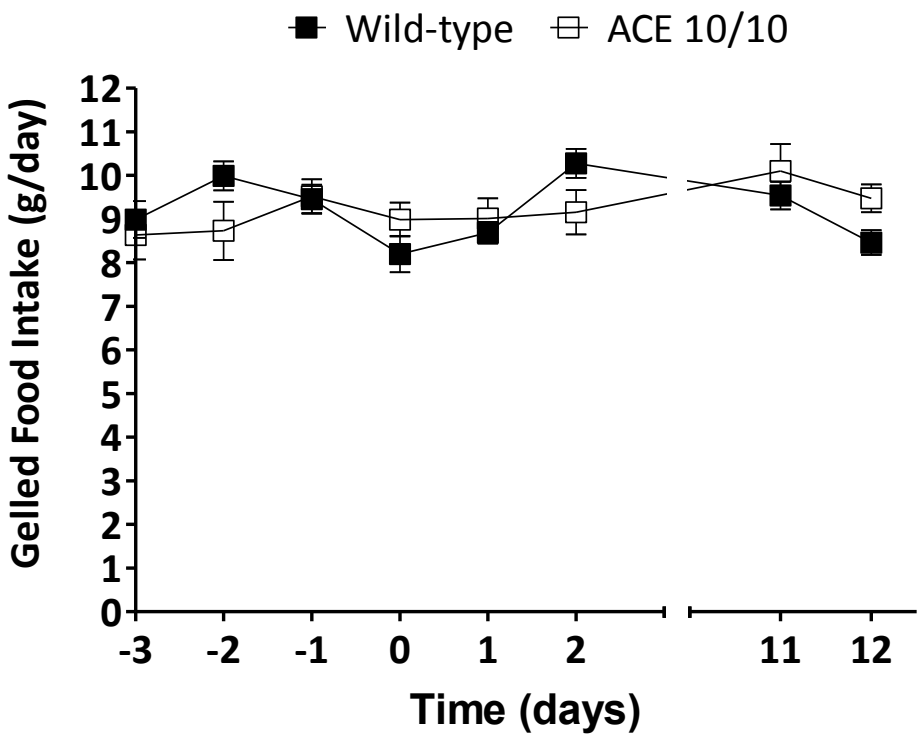


Supplemental Figure 3. Plasma Ang II levels in wild-type and ACE 10/10 mice during chronic Ang II infusion.

Trunk blood was collected from wild-type and ACE 10/10 mice during Ang II infusion (400 ng/kg/min) by conscious decapitation at each time point. Plasma Ang II concentration was measured by radioimmunoanalysis.

n = 5-7 per group. * = $p<0.05$. Values are individual records and means ± SEM.

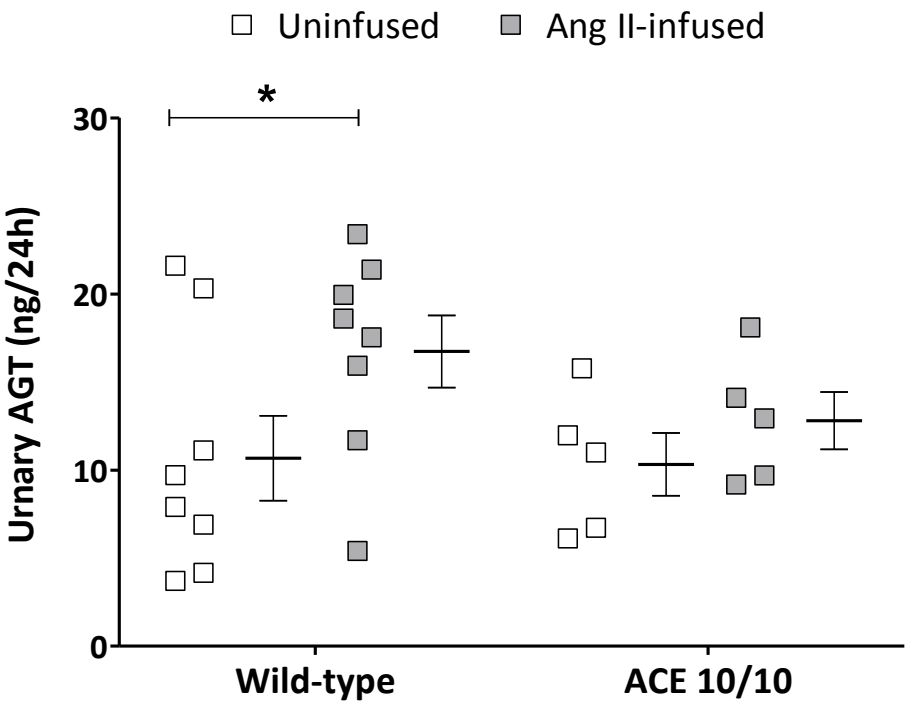
There were no overall statistical differences between the two groups (two-way ANOVA), the only difference was detected at day 1 (post-test analysis).



Supplemental Figure 4. Food and water consumption during chronic Ang II infusion. Wild-type and ACE 10/10 mice were housed individually in metabolic cages with free access to a gelled diet containing all nutrients and water. Minipumps delivering Ang II (400 ng/kg/min via minipump) were implanted at day 0.

n = 6-12 per group. Values are means ± SEM.

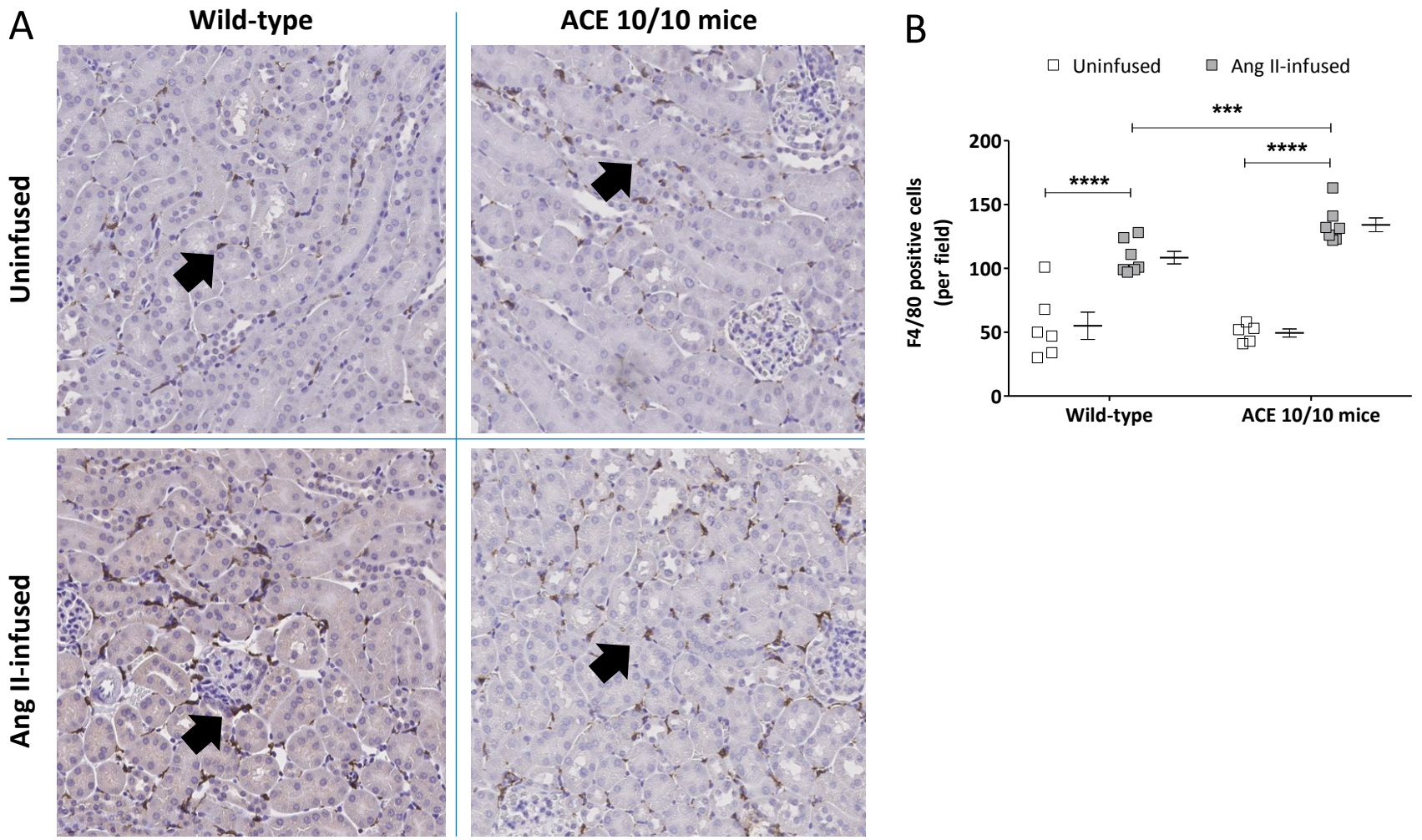
There were no statistical differences between the two groups (two-way ANOVA).



Supplemental Figure 5. Urinary angiotensinogen excretion in wild-type and ACE 10/10 mice during chronic Ang II infusion.

Angiotensinogen (AGT) concentration was measured by ELISA and corrected for urine volume to calculate daily excretion (16). Values are individual records and means ± SEM.

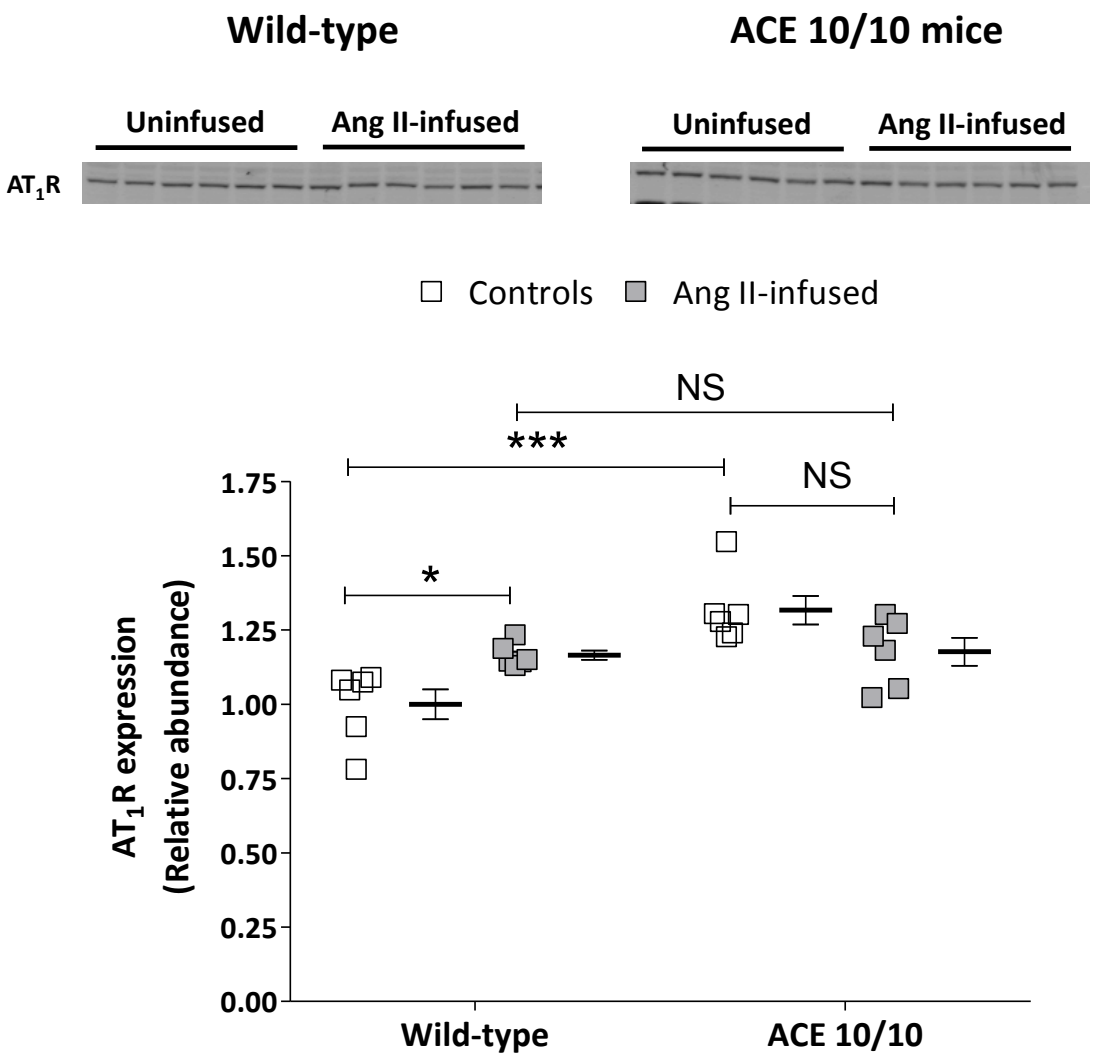
n = 5-8 per group. Ang II = Angiotensin II (400 ng/kg/min via minipump), uninfused = sham-operated mice, * = $p<0.05$.



Supplemental Figure 6. Renal macrophage infiltration in wild-type mice and ACE 10/10 mice during chronic Ang II infusion.

A. Macrophages were identified using an antibody against the specific surface marker F4/80. They are shown as brown cells in the pictures (black arrows). Images are representative kidneys from every group.

B. Quantification of F4/80 positive cells. Values are individual records and means ± SEM.
n = 7-8 per group. Ang II = Angiotensin II (400 ng/kg/min via minipump), uninfused = sham-operated mice, *** = $p<0.001$, **** = $p<0.0001$.

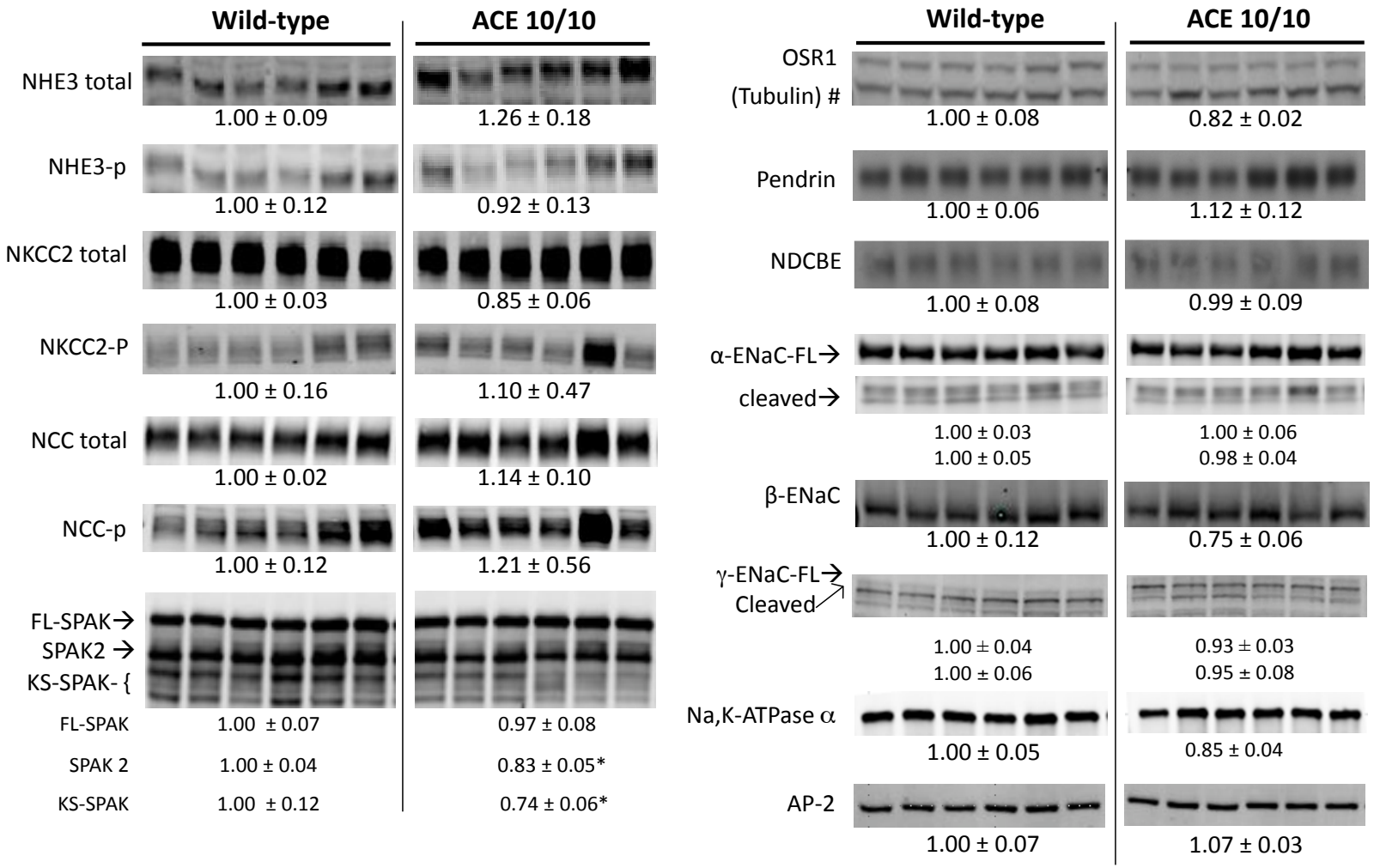


Supplemental Figure 7. Changes in renal angiotensin II type 1 receptor (AT_1R) protein expression in wild-type mice and ACE 10/10 mice during Ang II infusion.

AT_1R expression was analyzed in total tissue homogenates from wild-type and ACE 10/10 mice after 2 weeks of sham-operation (uninfused group) or Ang II infusion (400 ng/kg/min via minipump).

(A) Immunoblots of whole kidney homogenates were run at a constant amount of protein/lane (20 µg).

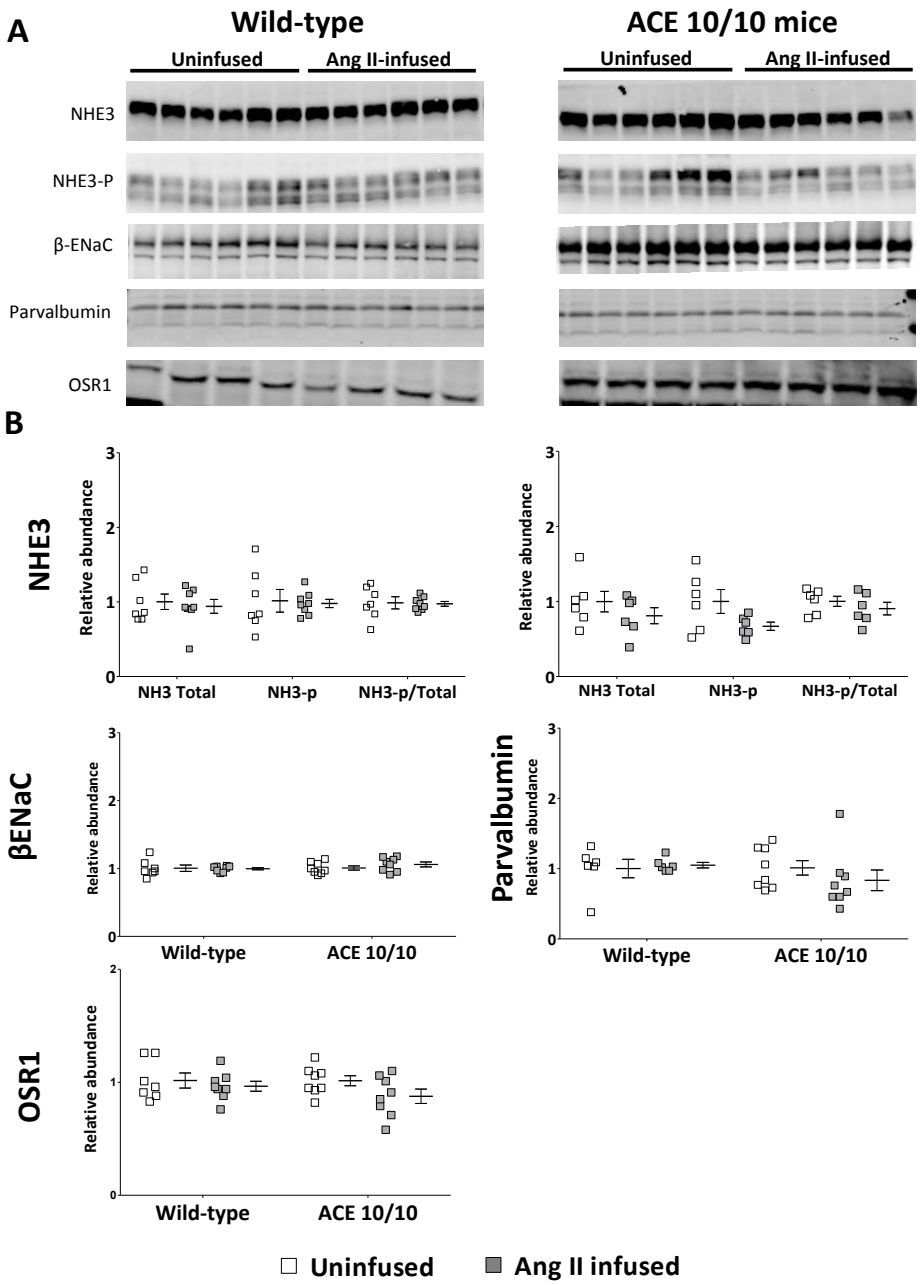
(B) Relative abundance of AT_1 receptor is displayed as individual records and means ± SEM. n = 6 per group. * = p<0.05, *** = p<0.001.



Supplemental Figure 8. Baseline renal sodium transporter profile of wild-type and ACE 10/10 mice.

Transporters expression was analyzed in total kidney homogenates from uninfused wild-type and mutant mice. Immunoblots of whole kidney homogenates were run at a constant amount of protein/lane (Table S1). The clathrin adapter AP-2 was chosen to be analyzed on the same blots as a loading control because it is not expected to be regulated by Ang II and it is linear in the same protein loading range as the transporters. Vertical lines represent separation of noncontiguous lanes of samples run in the same blot.

n = 6-8 per group * = $p<0.05$ vs. WT. Values are means ± SEM.

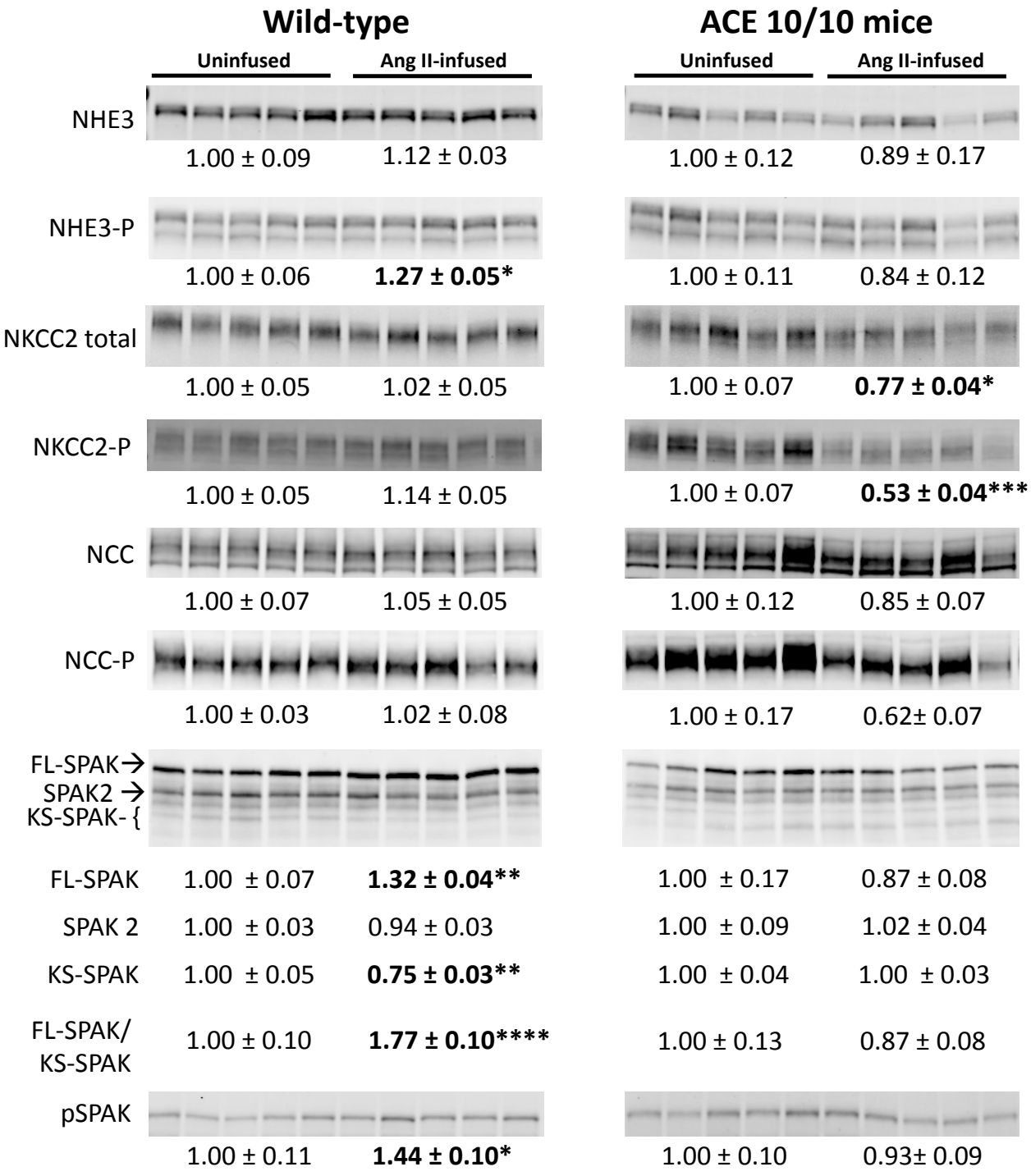


Supplemental Figure 9. Absence of changes in the sodium/hydrogen exchanger 3 (NHE3), the β subunit of ENaC, parvalbumin and OSR1.

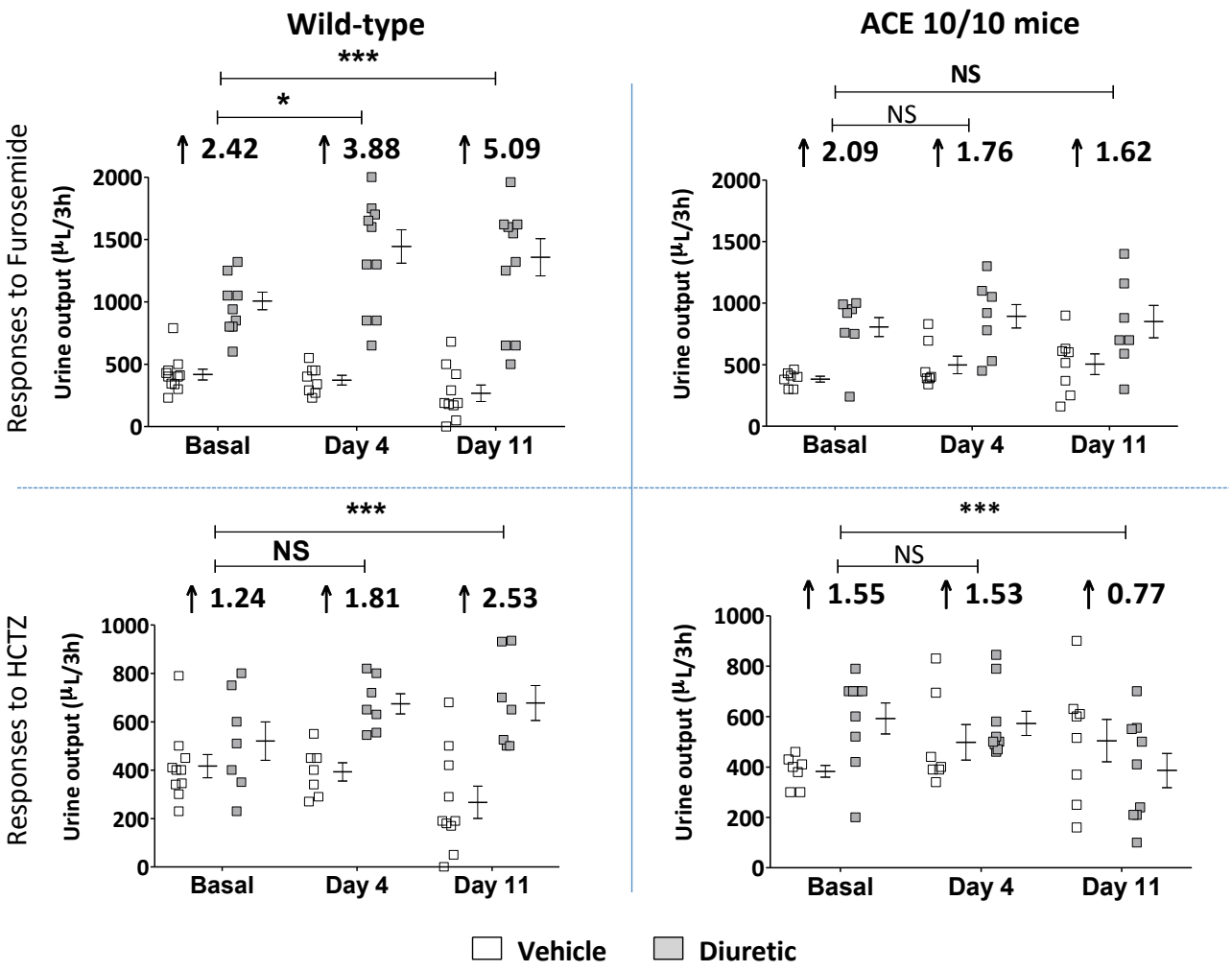
Transporters expression was analyzed in total tissue homogenates from wild-type and ACE 10/10 mice after 2 weeks of sham-operation (uninfused group) or Ang II infusion (400 ng/kg/min via minipump).

(A) Immunoblots were run at a constant amount of protein/lane (Table S1) for every transporter. Parvalbumin, expressed only in the DCT 1 was analyzed to estimate whether there was any change in the length of the DCT during Ang II infusion and there was not.

(B) Relative abundance is displayed as individual records and means ± SEM.
n = 6-8 per group.



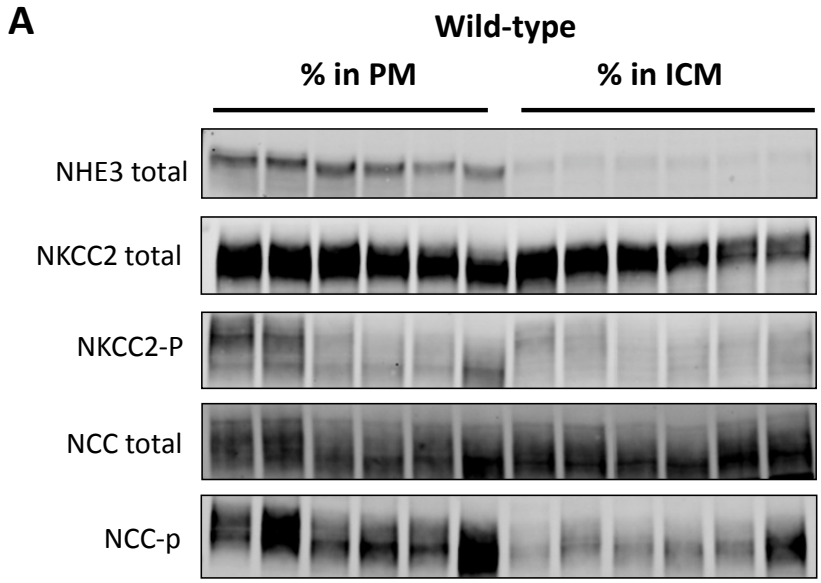
Supplemental Figure 10. Sodium transporter profile after 4 days of angiotensin II infusion.
Transporters expression was analyzed in total tissue homogenates from wild-type and ACE 10/10 mice after 4 days of sham-operation (uninfused group) or Ang II infusion (400 ng/kg/min via minipump).Immunoblots were run at a constant amount of protein/lane (Table S1). Relative abundance is displayed as individual records and means ± SEM.
n = 5 per group. * = $p<0.05$, ** = $p<0.01$, **** = $p<0.0001$



Supplemental Figure 11. The absence of kidney ACE prevents the in vivo activation of $Na^+/K^+/2Cl^-$ co-transporter (NKCC2) and the NaCl co-transporter (NCC) induced by chronic Ang II infusion as measured by diuretic responses to specific blockers

Transport activity in wild-type mice and ACE 10/10 mice was assessed by measuring the diuretic responses after a single IP bolus of furosemide (an NKCC2 blocker, 25 mg/kg) or hydrochlorothiazide (HCTZ, a NCC blocker, 25 mg/kg). Testing was performed before and after Ang II infusion (400 ng/kg/min). Numbers on top of the figures represent fold change over response in time-matched mice injected with vehicle.

n= 7-10. *** = $p<0.01$, **** = $p<0.0001$. Values are individual records and means ± SEM.



B

	% in PM	% in ICM
NHE3 total	96.7 ± 0.4	3.3 ± 0.4
NKCC2 total	85.8 ± 1.6	14.2± 1.6
NKCC2-P	88.5 ± 3.0	11.5 ± 3.0
NCC total	81.6 ± 1.2	18.4 ± 1.2
NCC-p	91.0 ± 2.2	9.0 ± 2.1

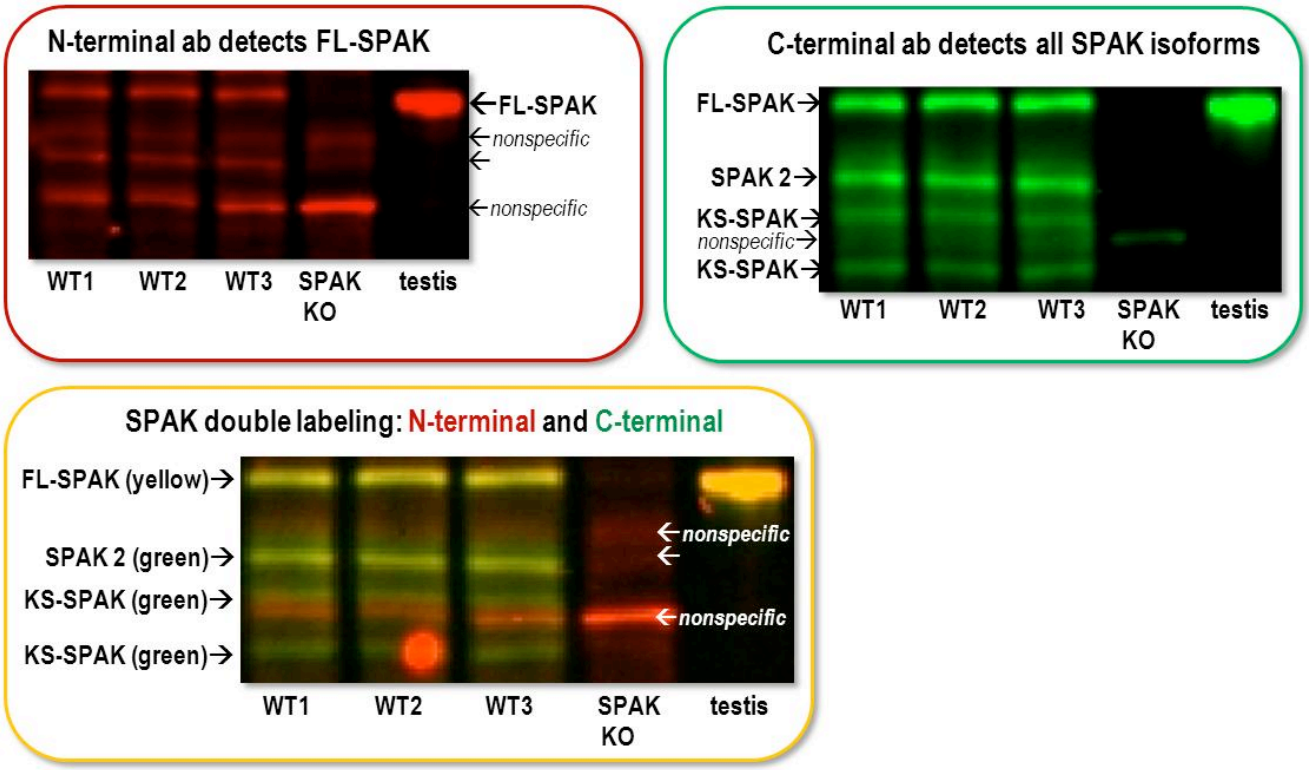
Supplemental Figure 12. Subcellular distribution of NKCC and NCC after Ang II infusion in kidneys from wild-type mice.

The fraction of NKCC2 and NCC in plasma membranes (PM) versus intracellular membranes (ICM) from Ang II-infused wild-type mice (n=6) was assessed after a differential fractionation protocol that enriches for PM vs. ICM (54). Using this method, and average of 2.2 ± 0.1 of PM and 0.48 ± 0.1 mg ICM were recovered per kidney.

(A) Immunoblots were run at a constant amount of protein/lane (14 μg) of PM and ICM.

(B) The percentage of transporter in PM vs. ICM was calculated from the signal intensity (A) corrected for the recovery of PM and ICM membrane protein in each sample, and expressed as percentage of transporter abundance in total kidney homogenates. Values are mean ± SEM.

Using this method, more than 95% of NHE3 was localized to PM, confirming immunohistochemistry findings (28) and validating this approach and 90% of NKCC-P and NCC-P were localized to the enriched PM, with only 10% localized to enriched ICM. In contrast, higher fractions of unphosphorylated NKCC and NCC were localized to enriched ICM. From this analysis we conclude that changes in NKCC-P and NCC-P represent changes in apical PM transporter pools. Similar percentages were obtained in Ang II-infused ACE 10/10 mice.



Supplemental Figure 13. Identification of SPAK isoforms.
SPAK isoforms were identified using a combination of custom-made antibodies. The antibody against the N-terminus (top left in red) does not identify the truncated forms of SPAK (SPAK 2 and KS-SPAK). The antibody against the C-terminus of SPAK (top right in green) identifies all three forms. In yellow (bottom) is an overlay of the two images in which only the band for the full length (FL)-SPAK overlaps. WT1, WT2 and WT3 = Kidney homogenates from wild-type mice, SPAK-KO = kidney homogenate from a SPAK KO mouse (Provided by Delpire Lab).

Antibody Target	Apparent Mobility (kDa)	Protein µg/lane (2 amts for linearity)	Primary antibody supplier	Ab host	dilution	Incubation time	secondary antibody supplier	host and target	dilution2	Incubation time
IMMUNOBLOTS:										
ACE	~190	20	Santa Cruz Bio	Goat	1:500	O/N	LiCor	DAG 800	1:10000	1 hr
Angiotensinogen	~50	20	Sernia (Queensland)	Sheep	1:1000	O/N	LiCor	DAG 800	1:10000	1 hr
AP2	~100	20,10	Sigma	Mu	1:3000	2hr	LiCor	GAM 800	1:5000	1hr
AT_1R	~50	20,10	Santa Cruz Bio	Rb	1:500	O/N	LiCor	GAR 800	1:15000	1 hr
ENaC -α	~90 & ~25	50,25	Loffing (Zurich)	Rb	1:1000	O/N	Invitrogen	GAR 680	1:5000	1hr
ENaC -β	~90	40,20	Palmer (Cornell)	Rb	1:1000	O/N	Invitrogen	GAR 680	1:5000	1hr
ENaC -g	~80-60	50,25	Palmer (Cornell)	Rb	1:1000	O/N	Invitrogen	GAR 680	1:500	1hr
NaPi2	~83	50, 25	McDonough (USC)	Rb	1:1000	O/N	Invitrogen	GAR 680	1:5000	1hr
NCC	~150	40,20	Ellison (OHSU)	Rb	1:1000	O/N	Invitrogen	GAR 680	1:5000	1hr
NCC pS71	~150	40,20	Bachmann (Charite)	Rb	1:1500	2 hr	Invitrogen	GAR 680	1:5000	1hr
NDCBE	~140	15	Eladari (INSERM)	Rb	1:500	O/N	Biorad	HRP-GAR	1:10,000	2 hr
NHE3	~83	50, 25	Millipore	Rb	1:3000	O/N	Invitrogen	GAR 680	1:5000	1hr
NHE3pS552	~83	50, 25	Millipore	Mu	1:1000	2hr	LiCor	GAM 800	1:5000	1hr
NKA α1	~90	1,0.5	Kashgarian (Yale)	Mu	1:200	O/N	Invitrogen	GAM 680	1:5000	1hr
NKCC2	~150	5, 2.5	C. Lytle (UCR)	Mu	1:1000	O/N	Invitrogen	GAM 680	1:5000	1hr
NKCC2pT96T101	~150	5, 2.5	Forbush (Yale)	Rb	1:2000	2hr	LiCor	GAR 800	1:5000	1hr
Pendrin	~120	15	Aronson (Yale)	Rb	1:10,000	O/N	Biorad	HRP-GAR	1:10,000	2 hr
Parvalbumin	~20	40,20	Santa Cruz Bio	Goat	1:1000	O/N	Invitrogen	DAG 680	1:5000	1hr
Pendrin	~120	15	Aronson (Yale)	Rb	1:10,000	O/N	Biorad	HRP-GAR	1:10,000	2 hr
OSR1	~60	40,20	DSTT, Dundee, UK	Sheep	1:1000	O/N	Invitrogen	DAS 680	1:5000	1hr
SPAK-C term	~70-50	20,10	Delpire (Vanderbilt)	Rb	1:3000	O/N	Invitrogen	GAR 680	1:5000	1hr
SPAK-N term	~70	20,10	Delpire (Vanderbilt)	Rb	1:1000	O/N	Invitrogen	GAR 680	1:5000	1hr
SPAKpS373, OSR1pS325	~70	40,20	DSTT, Dundee, UK	Sheep	1:1000	2 hr	Invitrogen	DAS 680	1:5000	1hr
IMMUNOFLUORESCENCE:										
NCC-P			Bachmann (Charite)	Rb	1:2000	1.5	Molec. Probes	GAR 568	1:200	1hr
NKCC2-P			Forbush (Yale)	Rb	1:1600	1.5	Molec. Probes	GAR 568	1:200	1hr
IMMUNOHISTOCHEMISTRY:										
F4/80	~160		AbD Serotec	Rat	1:100	45 min	Biocare	Rat on mouse HRP-polymer	1X	15min

Table S1. Immunoblot and Immunofluorescence antibody details.

Mu = mouse, Rb = rabbit, O/N = overnight