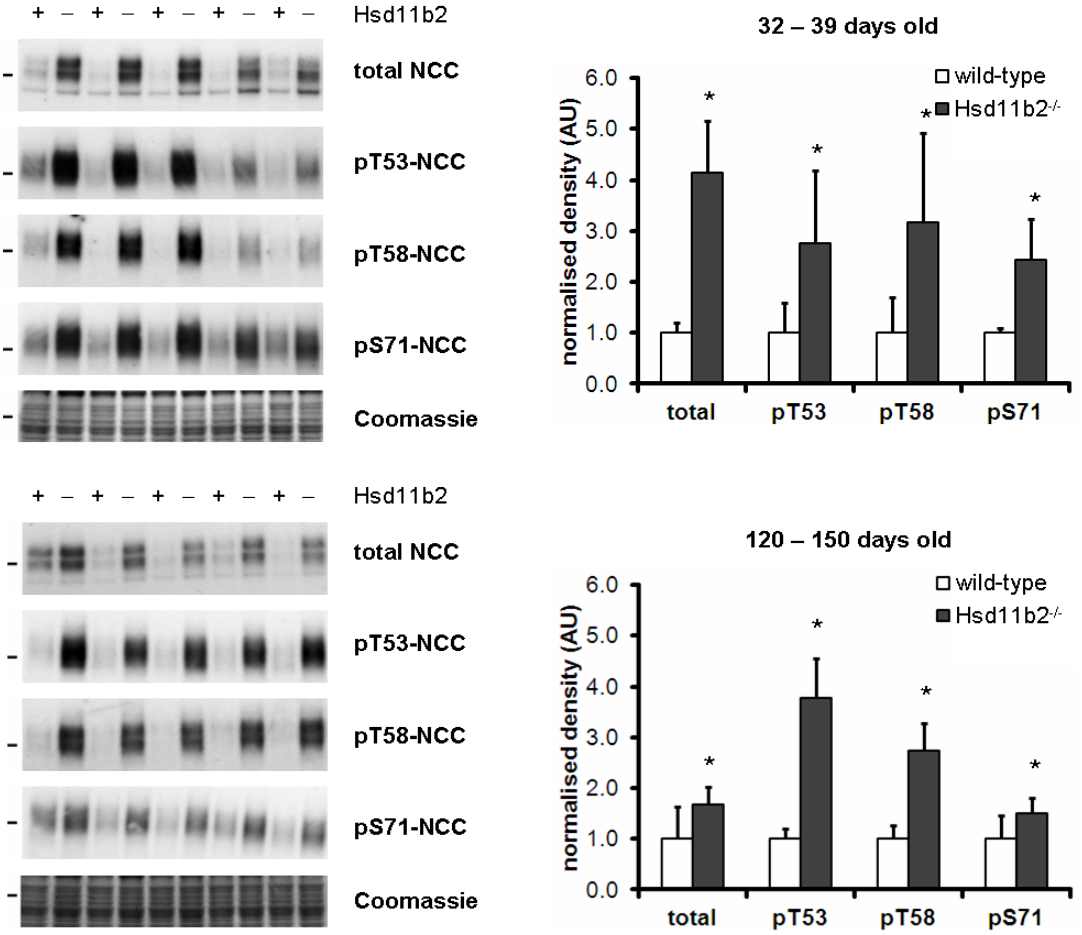
SUPPLEMENTAL FIGURES & TABLES

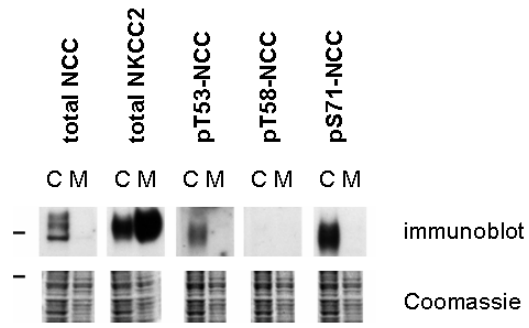
JASN-2013-06-0634

Title:

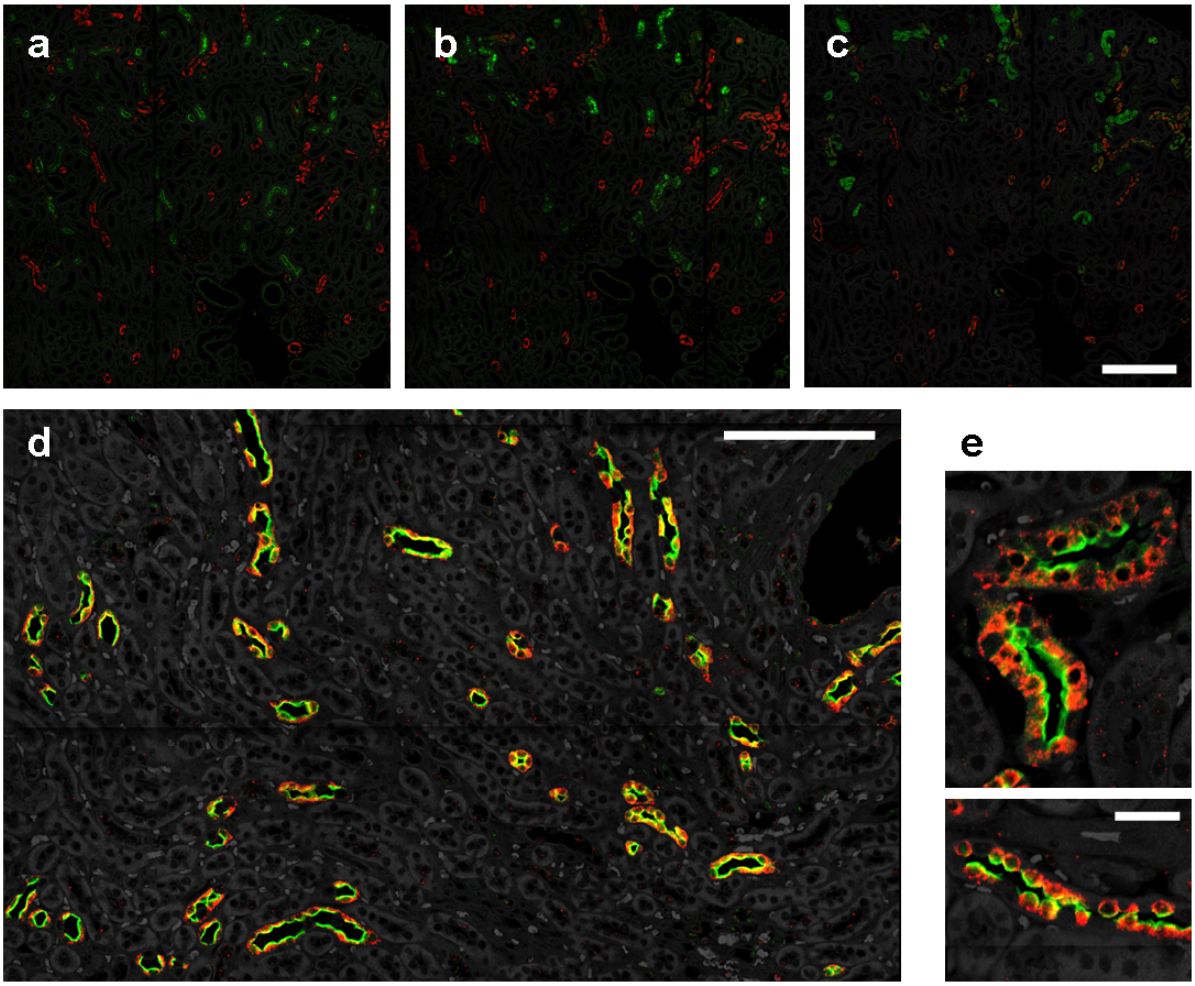
Hypertrophy in the Distal Convoluted Tubule of an 11β-Hydroxysteroid Dehydrogenase Type 2 Knockout Model



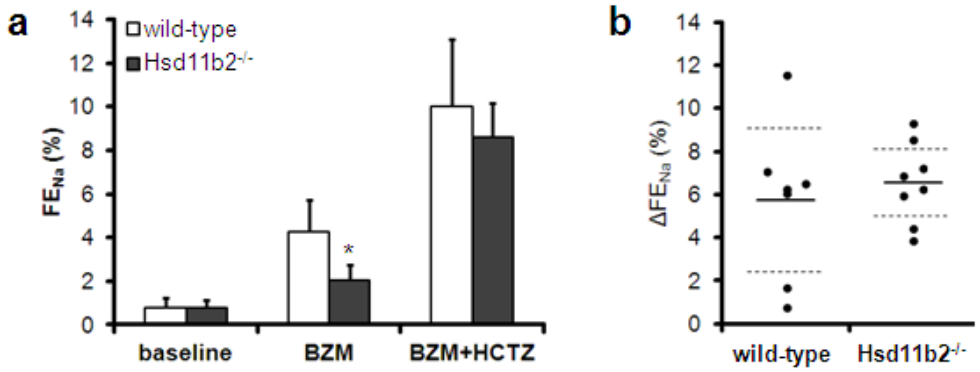
Supplemental Figure S1. Total and phosphorylated forms of NCC in wild-type and Hsd11b2$^{-/-}$ kidneys at c. 30 and 120 – 150 days of age. Immunoblots of total cellular protein (fraction 'S0') from whole kidney homogenates from wild-type (Hsd11b2 '+') or knockout ('–') mice culled at 32 – 39 or 120 – 150 days of age. 12 μg loaded per lane. A representative Coomassie stain (in each case taken from the gel used for the total NCC immunoblot) is shown for each age group. Size markers, 117 kDa for each blot and 55 kDa for each gel. Band densities are in arbitrary units relative to the wild-type group; mean and 95 % confidence interval, n = 5. * $p < 0.05$, genotypes compared by unpaired t-test.



Supplemental Figure S2. Immunoblotting with antibodies to pNCC on preparations of renal cortex and medulla. 8 μg of total cellular protein (fraction S0) prepared from a homogenate of cortex (C) or medulla (M) of wild-type kidney were loaded per lane. Total NCC could be detected in cortex but not medulla, whereas NKCC2 was detected in both. The antibodies to the phosphorylated forms of NCC (pT53-, pT58- and pS71-) recognised antigen in kidney cortex but not medulla, confirming the specificity of these antibodies for NCC. The results for anti-pT58 are subject to the caveat that only a very faint signal was recognised in kidney cortex, perhaps reflecting the fact that this site is not heavily phosphorylated under control conditions. Size markers, 117 kDa (blots) and 55 kDa (Coomassie stains).



Supplemental Figure S3. Expression of 11β-HSD2 in the renal tubule of male C57BL/6J mice at 55 – 65 days of age. (a) – (c) Serial sections showing the degree of co-localisation between 11β-HSD2 (red) and, in green, NCC (a), parvalbumin (b) and calbindin-D28k (c). Parvalbumin and 11β-HSD2 did not co-localise. In serial sections, parvalbumin and NCC co-localised almost completely. This conflicts with the observation of a 'late DCT' or 'DCT2' in rats and female mice, in which parvalbumin expression is absent[1] and may reflect the fact that this subsegment is poorly developed in male mice[2]. Calbindin D28k exhibited a partial overlap with 11β-HSD2; all tubules expressing 11β-HSD2 also expressed calbindin D28k, but some tubules (located in the cortex and outside the medullary rays) expressed calbindin D28k but not 11β-HSD2. **(d)** 11β-HSD2 (red) and AQP2 (green) were expressed in a common set of renal tubules, although the subcellular site of expression differed, with AQP2 expressed at the apical cell membrane and 11β-HSD2 expressed in the perinuclear region. **(e)** High-power view of tubules labelled with 11β-HSD2 (red) and APQ2 (green). Scale bars, 200 μm (a – c), 100 μm (d), 25 μm (e).



Supplemental Figure S4. Natriuretic response to thiazides in anaesthetised mice at 120 – 150 days of age. (a) FE_{Na} in anaesthetised mice in a renal clearance study (wild-type n = 7, Hsd11b2$^{-/-}$ n = 8) determined at baseline, following benzamil (BZM) and following BZM plus hydrochlorothiazide (HCTZ, 2 mg per kg IV). * $p < 0.05$ for comparison between genotypes by 2-way repeated measures ANOVA and *post-hoc* Bonferroni's test. **(b)** Data presented as the thiazide-induced increment in FE_{Na} for each mouse. The mean and 95 % confidence interval for each group and depicted by the solid and dashed lines respectively. There was no significant difference between genotypes ($p > 0.05$ by unpaired t-test).

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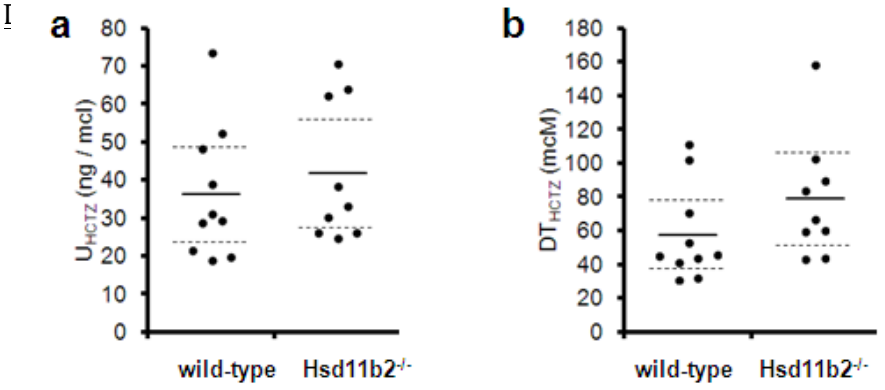
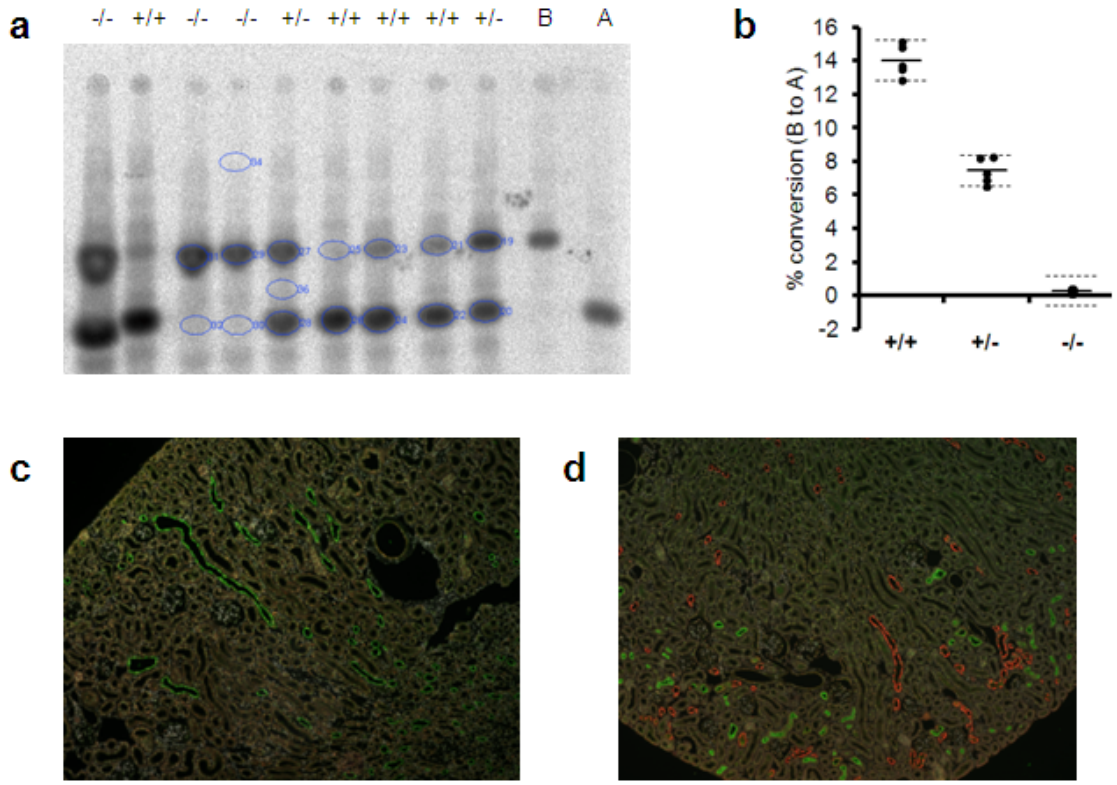


Supplemental Figure S5. Urinary HCTZ excretion in wild-type and Hsd11b2$^{-/-}$ mice. (a) Urinary [HCTZ] following an intravenous bolus of HCTZ (2 mg per kg body weight) in the renal clearance study presented in Figure 8. There was no significant difference between genotypes ($p > 0.05$ by unpaired t-test). **(b)** The same data were used to derive the estimated concentration of HCTZ in the distal renal tubule: $DT_{HCTZ} = U_{HCTZ} . (P_{Osm} / U_{Osm})$. There was no significant difference between genotypes.



Supplemental Figure S6. 11β-HSD2 expression and activity are absent in Hsd11b2 null kidneys. **(a) & (b)** 11β-HSD2 activity in kidney homogenates was assayed by using thin-layer chromatography to detect conversion of [^{3}H]corticosterone (B) to [^{3}H]dehydrocorticosterone (A). **(a)** Representative TLC blot. Steroid standards were loaded in the two lanes at the right-hand side. **(b)** % conversion of B to A in kidney homogenates during a 60 minute equilibration; kidneys were from wild-type ('+/+'), Hsd11b2 haploinsufficient ('+/-') or Hsd11b2 null ('-/-') mice. The conversion in Hsd11b2 null kidneys was no different from zero. **(c) & (d)** 11β-HSD2 protein was not detectable by immunofluorescence in Hsd11b2 null kidneys. Representative fluorescent micrographs from Hsd11b2 null and wild-type kidneys are presented, using identical imaging parameters. **(c)** Dual-label immunofluorescence for 11β-HSD2 protein (red) and AQP2 (green) in Hsd11b2 null kidney. **(d)** Dual-label immunofluorscence for 11β-HSD2 (red) and NCC (green) in wild-type kidney.

	wild-type (n = 5)		Hsd11b2$^{-/-}$ (n = 5)	
	number of glomerular cross-sections per mm^2			
total	17.3	(14.9 - 19.7)	12.6	(9.8 - 15.5) **
	number of tubular cross-sections per mm^2			
all tubules	426	(378 - 475)	344	(256 - 432)
NKCC2 +	102.0	(89.0 - 115.0)	65.0	(50.0 - 80.0) **
NCC +	49.7	(42 - 57.4)	47.0	(42.3 - 51.7)
AQP2 +	60.1	(52.2 - 68.1)	34.0	(26.0 - 42.0) **
	% of total tubular cross-sections			
NCC +	11.7	(9.3 - 14.2)	13.5	(10.5 - 16.6)
	tubular cell nuclei per mm^2			
NKCC2 +	543	(459 - 626)	465	(366 - 565)
NCC +	405	(335 - 475)	627	(545 - 709) **
AQP2 +	532	(438 - 626)	344	(287 - 401) **
	tubular cell nuclei per tubule cross-section			
NKCC2 +	5.32	(4.80 - 5.85)	7.23	(6.03 - 8.43) **
NCC +	8.48	(7.90 - 9.06)	13.37	(11.77 - 14.96) **
AQP2 +	8.84	(7.80 - 9.87)	10.25	(8.51 - 11.99)

Supplemental Table S1. Quantitative analysis of distal tubular structure in Hsd11b2$^{-/-}$ mice. Wild-type and Hsd11b2$^{-/-}$ mice were culled at 55 – 65 days of age. 'NKCC2 +', 'NCC +' and 'AQP2 +' refer to those cortical tubules expressing NKCC2, NCC and AQP2. Data for 'all tubules' were obtained by counting all tubular cross-sections in those sections stained for NCC and therefore represent all cortical nephron segments (and not just those of the distal renal tubule). Data are presented as mean (95 % confidence interval). ** $p < 0.01$ (comparison between genotypes by unpaired t-test).

	wild-type (n = 5)		Hsd11b2$^{-/-}$ (n = 5)	
	number of glomerular cross-sections included in analysis per subject			
glomeruli	68.8	(59.3 - 78.3)	50.2	(38.9 - 61.5)
	number of tubular cross-sections included in analysis per subject			
all tubules	443	(393 - 493)	357	(266 - 449)
NKCC2 +	150	(131 - 169)	95.0	(73.0 - 118.0)
NCC +	125	(106 - 144)	118	(106 - 130)
AQP2 +	88.4	(76.7 - 100.1)	50.0	(38.3 - 61.7)
	number of tubular cell nuclei included in analysis per subject			
NKCC2 +	798	(675 - 920)	684	(538 - 831)
NCC +	952	(603 - 1301)	1574	(1368 - 1780)
AQP2 +	781	(643 - 920)	505	(422 - 589)

Supplemental Table S2. Number of histological features counted per experimental subject as part of the quantitative analysis presented in Figure 2. Values are mean (95 % confidence interval). 'NKCC2 +', 'NCC +' and 'AQP2 +' refer to data derived from tubular cross-sections expressing NKCC2, NCC or AQP2.

cohort	haematocrit	plasma Osm / mOsm	plasma [Na^+] / mM	plasma [K^+] / mM	GFR / μl per min per 100 g
			cohorts aged 55 – 65 days		
wild-type (n = 9)	43.0 (42.1 – 43.9)	297.4 (291.8 – 303.0)	139.8 (137.2 – 142.3)	5.9 (5.7 – 6.1)	703.2 (520.1 – 886.2)
Hsd11b2$^{-/-}$ (n = 8)	46.8 * (45.5 – 48.1)	308.9 * (299.6 – 318.2)	146.6 * (144.0 – 149.2)	2.7 * (2.3 – 3.2)	700.3 (516.3 – 884.2)
			cohorts aged 120 – 150 days		
wild-type (n = 7)	42.2 (41.3 – 43.2)	297.4 (285.3 – 309.6)	141.9 (139.8 – 143.9)	5.2 (5.0 – 5.3)	546.4 (400.8 – 692.1)
Hsd11b2$^{-/-}$ (n = 8)	43.6 (42.5 – 44.7)	316.5 * (309.9 – 323.1)	149.9 * (148.8 – 151.0)	2.6 * (2.3 – 2.8)	796.9 (578.5 – 1015.2)
furosemide-treated (n = 6)	44.2 (41.3 – 47.0)	319.0 * (309.7 – 328.3)	149.7 * (148.4 – 151.0)	4.6 (4.0 – 5.3)	616.2 (467.9 – 764.5)

Supplemental Table S3. Characteristics of the wild-type, Hsd11b2$^{-/-}$ and furosemide-treated cohorts used for renal clearance experiments. Haematocrit and GFR were determined during the baseline sampling period; osmolality, [Na^+] and [K^+] were determined on a terminal blood sample. Data are mean and 95 % confidence interval. * $p < 0.05$ for comparison with wild-type control group of the same age (by unpaired t-test at 55 – 65 days or by 1-way ANOVA and *post-hoc* Dunnett's test at 120 – 150 days of age).

gene	accession number	forward primer	reverse primer	UPL
18S rRNA	NR_003278.1	Ctcaacacgggaaacctcac	cgctccaccaactaagaacg	77
TBP	NM_013684	Gggagaatcatggaccagaa	gatgggaattccaggagtca	97
cyclophilin A	NM_008907.1	Acgccactgtcgcttttc	gcaaacagctcgaaggagac	46
11βHSD2	NM_008289.2	Cactcgaggggacgtattgt	gcaggggtatggcatgtct	26
SGK1	NM_001161845	Gattgccagcaacacctatg	ttgatttgttgagagggacttg	91
NHE3	NM_001081060	tccatgagctgaatttgaagg	tacttggggagcgaatgaag	5
NKCC2 (total)	NM_183354 (isoform A)	Tgctggtgccaacatctct	atggtccctctggggatg	85
NCC	NM_019415	Cctccatcaccaactcacct	ccgcccacttgctgtagta	12
ENaC alpha	NM_011324.2	ccaagggtgtagagttctgtga	agaaggcagcctgcagttta	45
NDCBE	NM_021530.2	Agaggggaagatgctgagt	tcctccacatcctcttcaaac	83
parvalbumin	NM_013645	ttctggacaaagacaaaagtgg	tgaggagaagcccttcagaat	88
calbindin D28k	NM_009788	aaggcttttgagttatatgatcagg	ttcttctcacacagatctttcagc	42
NCX1	NM_011406	ccatgctagagatcatccgatt	gtcagtggctgcttgtcatc	6
TRPV5	NM_001007572	Gagagggacgagctctgga	acaggaaacgaggcattttc	67
WNK4	NM_175638	Tccgatttgatctggatgg	gggcaggatgaactcattgta	26
WNK1-total	AY309076	Ccagcaagcagttctggag	tgactgtgttattggagtttgttct	68
WNK1-L	AY309076	cttttgccaagagtgtgataggt	caacggattcatcatatttctcc	92
WNK1-KS	AY311934 / AY309076	Tgctgctgttctcaaaagga	acttcaggaattgctactttgtca	20
SPAK-total	ENSMUST00000102715	tttaaaaacgttgacatttaagttgg	cccgatcagcttcacttcat	71
SPAK-L	ENSMUST00000102715	gtacgagctccaggaggttatc	tcttgcctgggtttgcat	27
SPAK-KS	JN368425	ttaccgtcattcctaactttactgc	gaatgcgcttactccaaaatct	18
OSR-1	NM_133985	Tgccttcaaaaggatccaga	tggaaaaatttgtgcctcaac	84
PP4	ENSMUST00000032936.6	Tggactcgccagtcacagta	gggacatcgccacctactc	17

Supplemental Table S4. Primers and probes used for Q-PCR. 'UPL' refers to the reference number of the probe in the Roche Universal ProbeLibrary.

sp.	reference	Method					expression of 11β-HSD2 (H) & MR (M)					
		ISH	IHC	Cre	activity	SN-PCR		TALH	DCT-1	DCT-2	CNT	CCD
RABBIT	Velazquez *et al.* (1998) [3]				★	★	**H**	?	+++ *	+++ *	?	++
							M	?	?	?	?	?
	Bonvalet *et al.* (1990) [4]				★		**H**	++	?	?	?	++
							M	?	?	?	?	?
	Farman *et al.* (1991) [5]		★				**H**	?	?	?	?	?
							M	++	++ *	++ *	++	++
RAT (Sprague-Dawley)	Bostanjoglo *et al.* (1998) [6]	★	★				**H**	–	–	++	++++	+++
							M	+	+	++	++	++
	Schmitt *et al.* (1999) [7]	★	★				**H**	–	–	++	++	++
	1 – 8 days old						**M**	?	?	?	?	?
	Smith *et al.* (1997) [8]		★				**H**	+	++ *	++ *	++ *	++
	embryonic – adult						**M**	?	?	?	?	?
HUMAN	Kyossev *et al.* (1996) [9]		★				**H**	+	?	?	+++	+++
							M	?	?	?	?	?
	Hirasawa *et al.* (1997) [10]		★				**H**	++	++ *	++ *	?	++
							M	++	++ *	++ *	?	++
MOUSE	Campean *et al.* (2001) [2]		★				**H**	–	–	±	+++	+++
	NMRI mice						**M**	?	?	?	?	?
	Cole (1995) [11]	★					**H**	–	++ *	++ *	++ *	++
	C57BL6 mice						**M**	?	?	?	?	?
	Naray-Fejes-Toth & Fejes-Toth (2007) [12]		★	★			**H**	–	–	–	++	++
	HSD2 / iCre × ROSA26 mice						**M**	?	?	?	?	?

Supplemental Table S5. 11β-HSD2 expression in the distal renal tubule: a literature review. All studies were conducted in adult animals unless stated otherwise. Methods used to detect 11β-HSD2 expression were in situ hybridisation (ISH), immunohistochemistry (IHC), a Cre-Lox genetic reporter system (Cre), enzyme activity studies using radiolabelled tracers in microdissected nephron segments (activity) or single-nephron RT-PCR in microdissected nephron segments. Key: '–' = reported as absent; '+' to '++++' = reported as present; '?' = not reported; '*' denotes study in which no attempt was made to identify DCT1, DCT2 or CNT.

COMPLETE METHODS

Animals

All experiments were conducted in accordance with UK Home Office regulations and the Animals (Scientific Procedures) Act 1986. Mice were housed in groups ($n \leq 5$) and were given free access to water and a standard mouse chow containing 0.25 % Na, 0.38 % Cl and 0.67 % K. C57BL/6JOlaHsd wild-type mice were supplied by Harlan UK. Hsd11b2$^{-/-}$ mice were derived from a colony carrying a null mutation in the Hsd11b2 gene on the C57BL/6JOlaHsd background[13]. We confirmed that 11β-HSD2 protein and enzyme activity were absent from Hsd11b2$^{-/-}$ kidneys (Supplemental Figure S7). Experimental animals were the male offspring of parents that were both homozygous for the null mutation; wild-type controls were age and sex-matched. Separate cohorts of wild-type and Hsd11b2$^{-/-}$ mice were used to prepare tissue for histological analysis, protein extraction, RNA extraction and for the analysis of Na^+ transport function.

Immunofluorescence

Administration of BrdU

Wild-type and Hsd11b2$^{-/-}$ mice were implanted with an osmotic pump (Alzet® model 1007D) containing 50 mg / ml BrdU in 50 % DMSO, which had been primed in 0.9 % NaCl at 37 ° C overnight. Mice received inhaled isofluorane anaesthesia and subcutaneous analgesia (buprenorphrine 50 μg per kg body weight as Vetergesic®) before the pump was implanted into a subcutaneous pocket at the back of the neck. Pumps remained *in situ* for 7 days before the animals were culled by perfusion fixation. The dose of BrdU delivered to the wild-type group was 25.6 ± 1.0 mg / kg / day (mean ± SD); the knockout group received 28.3 ± 3.3 mg / kg / day.

Perfusion fixation

Kidneys were fixed *in situ* using a perfusion fixation protocol adapted from that described by Loffing and Kaissling[1,14,15]. Mice were anaesthetised with Sagatal (sodium pentobarbital, 50 mg / kg IP), and the infra-renal aorta was cannulated to permit retrograde perfusion with a vent in the vena cava. 10 ml heparinised saline (20 units / ml in PBS) were infused followed immediately by 50 ml fixative (fresh 4 % PFA in PBS, pH 7.4), delivered at a rate of 15 ml / min by a Gilson Minipuls 3 peristaltic pump. The right kidney was

removed, the poles cut off and discarded, and the central portion immersed in 4 % PFA at 4 ° C for 24 hours before being embedded in paraffin wax. 5 micron sections were cut for indirect immunofluorescence.

Immunofluorescence

Sections were de-waxed and hydrated through graded alcohols and then heated in the presence of Novocastra Bond Epitope Retrieval Solution 1, pH 6.0 (ER1, Leica). Indirect immunofluorescent detection of target antigens was performed using a Leica BOND-MAX ™ robot. Primary antibodies and their dilutions were: sheep anti-11β-HSD2 (Millipore, AB1296) at 1:6000; rabbit anti-NCC (Millipore, AB3553) at 1:2000; sheep anti-NKCC2 (Division of Signal Transduction Therapy, Dundee University) at 1:4000; goat anti-AQP2 (Santa-Cruz, sc-9882) at 1:4000; rabbit anti-parvalbumin (Swant, PV25) at 1:60000; rabbit anti-calbindin D-28k (Swant, CB38) at 1:100000; sheep anti-BrdU (Fitzgerald, 20-BS17) at 1:250. Secondary antibodies and their dilutions were: goat anti-rabbit IgG-HRP (Abcam, AB6112) at 1:500; rabbit anti-sheep IgG-HRP (Nordic immunology) at 1:500; rabbit anti-goat IgG-HRP (Millipore, AP106P) at 1:500. The binding sites of HRP-conjugated secondary antibodies were detected using a tyramide-labelled fluorophore (either Cy3 or Cy5). For double-stainings (in which two different fluorophores were used to stain two different targets on the same sample) the sample was heated for 10 mins in the presence of ER1 solution after the first detection, in order to strip off the first primary and secondary antibodies. All sections were counter-stained with DAPI (4',6-diamidino-2-phenylindole).

Image acquisition and analysis

Images were obtained using a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope. A blue diode 405 nm laser and HeNe 546 and 633 nm lasers were used to excite DAPI, Cy3 and Cy5 respectively, with appropriate detection. To facilitate the quantitative analysis of morphological parameters, a set of fluorescent micrographs was obtained from cohorts of wild-type and Hsd11b2$^{-/-}$ kidneys using the following standard method. A region of the cortex was selected at random whilst viewing through the DAPI channel (thus blinded to the fluorescent signals providing information regarding the localisation of specific nephron segments and proliferating cells). A merged image was then obtained through all three channels (Cy3, Cy5 and DAPI) and stored for quantitative analysis. A different region of cortex was then selected using the DAPI filter-set and the process repeated. In this way, a

series of five images representing a random discontinuous sample of kidney cortex (total area approximately 1.5 mm^2) were obtained from each kidney section. In each case these were drawn from regions distributed roughly evenly throughout the cortex. Images were analysed using ImageJ 1.46r (http://imagej.nih.gov/ij) with the Cell Counter plugin. Each kidney specimen was assigned a random 4-digit code at the time of tissue fixation. Images were therefore acquired and analysed blinded to the identity of the sample. Furthermore they were analysed in a random order (so that the five images obtained from each kidney section were not necessarily examined in sequential order). The total number of histological features counted to generate the quantitative analysis of distal tubular structure are presented in Supplemental Table S2.

Semi-quantitative immunoblotting

Protein sample preparation

Kidneys were homogenised in a buffer containing phosphatase, kinase and protease inhibitors (250 mM sucrose,10 mM triethanolamine, 2 mM EDTA, 50 mM NaF, 25 mM Na β-glycerophosphate, 5 mM Na pyrophosphate, 1 mM Na orthovanadate, 1 % Protease Inhibitor Cocktail Set III, Calbiochem ®, pH 7.6). Supernatants from two 15 minute centrifugations at 4000 *g* were pooled to form a total cellular protein fraction ('S0'), free from gross cellular and nuclear debris. This fraction was subject to a further centrifugation at 16000 *g* for 32 mins to pellet a fraction ('P1') enriched in plasma membranes; the supernatant from this spin was centrifuged at 200000 *g* for 60 mins to pellet a fraction ('P2') enriched in subapical membrane vesicles[16].

SDS-PAGE and immunoblotting

Samples were heated to 70 ° C for 15 mins in sample buffer (NuPAGE LDS sample buffer, Invitrogen) containing 50 mM DTT, separated by SDS-PAGE on NuPAGE Novex ™ 3 – 8 % Tris-Acetate gels (Invitrogen) and then transferred to an Amersham Hybond ™-P PDVF membrane (GE Healthcare). Membranes were incubated with blocking buffer (5 % w/v non-fat dry milk powder / 0.2 % v/v Tween-20 in PBS) on a rolling shaker at room temperature for 1 hr and then incubated with primary antibody overnight at 4 ° C. Primary antibodies and their dilutions were: rabbit anti-NCC (Millipore, AB3553) at 1:1000; sheep anti-pT53-NCC, anti-pT58-NCC and anti-pS71-NCC (Division of Signal Transduction Therapy, Dundee University), each at 1:500 (= 0.2 – 1.2 μg / ml); sheep anti-NKCC2 (DSTT, Dundee) at

1:10000. For Western blots designed to recognise specific phosphorylated forms of NCC, the corresponding non-phosphorylated peptide was included in the solution of primary antibody at a final concentration of 10 μg / ml. After three five-minute washes with wash buffer (0.2 % v/v Tween-20 in PBS) the membrane was incubated with an HRP-conjugated secondary antibody for 1 – 2 hrs at room temperature and then washed again (three ten-minute washes). Secondary antibodies and their dilutions were: goat anti-rabbit IgG-HRP (Santa-Cruz, sc-2030) at 1:2000; donkey anti-sheep IgG-HRP (Sigma, A3415) at 1:20000. Peroxidase activity was revealed using SuperSignal® West Pico Chemiluminescent Substrate to expose photographic film.

Densitometry

Films were photographed using a digital SLR camera (Nikon D40) with the exposure set to maximise dynamic range without significant pixel saturation. Images were analysed as 8-bit .tiffs in ImageJ (version 1.43u). A rectangular region of interest (ROI) was drawn to encompass the entire vertical range of each lane and the area under this curve was taken as a measure of the band density. No background subtraction step was applied. The bottom portion of each gel was excised prior to transfer to the PVDF membrane and stained with Coomassie blue. The density of each lane on the film was divided by the density of the corresponding Coomassie lane in order to correct for variation in the total amount of protein loaded for each sample. The final data were normalised so that for each assay, the wild-type group had a mean value of 1.0.

Q-PCR

RNA extraction and cDNA preparation

RNA was extracted from frozen mouse kidneys using the QIAGEN RNeasy® Mini Kit, using a protocol that included an on-column treatment with DNaseI. The integrity of the RNA preparations was verified by agarose gel electrophoresis and staining with ethidium bromide. cDNA was transcribed from 500 ng of total RNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit.

Quantitative real-time PCR (Q-PCR)

Q-PCR assays were designed to permit the detection of specific product by the real-time measurement of fluorescence using the Roche Universal ProbeLibrary. The primers and probes used for each assay are presented in Supplemental Table S4. Reactions were

assembled in 10 μl volumes, each containing 2 μl of sample cDNA (diluted 1:40), forward and reverse primers each at a final concentration of 200 nM and the relevant probe at a final concentration of 100 nM. All plates included a 7-point standard curve, in which aliquots from all of the cDNA samples were pooled and diluted 1:10 to form a '1 × standard' which was then subjected to serial 1:2 dilutions, and three negative controls (water, no-template and no-reverse transcriptase). All samples, standards and controls were performed in triplicate. Reactions were run on a Roche Lightcycler 480 using the following cycling conditions: 95 ° C for 5 mins, then 95 ° C for 10 secs, 60 ° C for 30 secs repeated for 60 cycles, then 40 ° C for 30 sec. The threshold cycle (Cp) for each well was identified by finding the maximum value in a plot of the second derivative of fluorescence vs. time. Triplicate results were analysed and replicates excluded if the Cp standard deviation was > 0.4. The results of any given assay were only accepted if the standard curve was satisfactory (efficiency 1.7 – 2.1 and error < 0.05) and all negative controls (water, no template and reverse transcriptase-negative) were negative. For each assay, Cp values were converted to mean template abundance (in arbitrary units), using the 7-point standard curve. 18S rRNA, TBP and cyclophilin A were selected as endogenous control genes, as these transcripts did not differ in abundance between Hsd11b2$^{-/-}$ and wild-type kidneys. All other assays were expressed relative to the endogenous control genes – i.e. for each sample, the abundance of a 'test' transcript was divided by the mean abundance of the three endogenous control transcripts in that sample. The final data were normalised so that for each assay, the wild-type group had a mean value of 1.0.

11β-HSD2 enzyme activity

11β-HSD2 enzyme activity in kidney homogenates was assessed using thin-layer chromatography to measure conversion of [^{3}H]corticosterone to [^{3}H]dehydrocorticosterone as previously described[17].

Tubule segment micro-dissection

Micro-dissection and preparation of RNA and cDNA

Wild-type male C57BL/6 mice were subjected to terminal anaesthesia. The left kidney was perfused *in situ* though a catheter placed in the abdominal aorta with 1 ml ice-cold heparinised 0.9 % NaCl followed by 2 ml HBSS (in mM: NaCl 140, KCl 5, $MgSO_4$ 0.8, $MgCl_2$ 1, Na_2HPO_4 0.33, NaH_2PO_4 0.44, $CaCl_2$ 0.5, glucose 5, HEPES 10, NaOH 5, pH 7.4) then 2 ml

collagenase solution containing 250 μg / ml collagenase type IA (Sigma) in HBSS. The kidney was decapsulated and cut into thin cortico-medullary wedges, which were incubated in collagenase solution for 30 mins at 37 °C. These were then transferred to microdissection medium (0.1 % BSA, 10 mM ribonucleoside vanadyl complex in HBSS) and placed on ice during 1 – 2 hrs of microdissection. PCTs, DCTs and CCDs were dissected from the cortex.

c. 0.5 mm lengths of tubule were transferred into 400 μl denaturing solution (4M guanidine thiocyanate, 25 mM Na citrate pH 7, 0.5 % sarcosyl, 100 mM β-mercaptoethanol). RNA was prepared by phenol-chloroform extraction (adding 0.1 vols 2M Na acetate pH 4.0, 1 vol phenol, 0.2 vols chloroform and 0.004 vols isoamylalcohol). RNA was precipitated from the aqueous phase by the addition of 450 μl isopropanol in the presence of 20 μg glycogen. After three ethanol washes, the pellet was re-suspended in 10 μl 2 mM DTT containing 0.5 mcl RNaseOUT™. All 10 μl were used as the template for cDNA synthesis using the Applied Biosystems High Capacity cDNA Reverse Transcriptase kit.

PCR

Template cDNA was amplified by PCR in 20 μl reactions containing 0.1 mM dNTPs, 0.5 μM each of forward and reverse primers, 1.5 mM $MgCl_2$, 0.025 U μl^{-1} Taq DNA polymerase (ThermoScientific) and template cDNA from c. 25 μm of tubule. Positive control template was cDNA prepared from 1 ng of total kidney RNA. Negative control templates were reverse-transcriptase negative cDNA preparation from total kidney RNA and water. PCR conditions were: 95 °C for 3 mins, then 33 cycles of 94 °C for 20 secs, 59 °C for 30 secs, 72 °C for 45 secs then 72 °C for 7 mins. Intron-spanning primers were designed to amplify gene products from Hsd11b2 (5′-ctgctggctgctctcgactg & 5′-ccagaacacggctgatgtcctc), Slc12a3 (5′-ctaccaatggcaaggtcaag & 5′-taggagatggtggtccagaa) and Scnn1g (5′-ccaaagccagcaaataaacaaa & 5′-gcggcgggcaataatagaga).

Thiazide-sensitive tubular Na^+ reabsorption

Metabolic cages (acute HCTZ)

Mice were housed individually in metabolic cages that enabled the independent collection of urine and faeces and were left to acclimatise for four days before any experimental data were obtained. Each mouse was given an intraperitoneal injection of vehicle (c. 0.2 ml 2 % DMSO), immediately followed by a 6-hour urine collection. 24 hours

later mice received an injection of HCTZ (20 mg per kg body weight), followed by a further 6-hour urine collection. Urine collections were timed to fall during the animal's active phase (i.e. the dark phase of a 24 hr dark-light cycle).

Metabolic cages (chronic HCTZ)

Mice were housed individually in metabolic cages and acclimatised as above. Each mouse was given an intraperitoneal injection of vehicle (c. 0.2 ml 2 % DMSO) daily for 3 days during the 'baseline' period and then an injection of HCTZ (20 mg per kg body weight) daily for 7 days during the test period. Urine samples were collected over 24 hours.

Renal clearance

Renal clearance experiments were performed as previously described [13]. Mice were anaesthetised with Inactin® (thiobutabarbital sodium salt hydrate, Sigma) and catheters were inserted into the trachea, jugular vein, carotid artery and bladder. A bolus dose (0.1 ml per 10 g body weight) of physiological saline solution was given via the jugular catheter as soon as intravenous access was established, followed by a continuous infusion of 0.2 ml per 10 g per hour. This infusate comprised 100 mM NaCl, 5 mM KCl, 15 mM $NaHCO_3$, 0.25 % FITC-inulin, pH 7.4. FITC-inulin had been dialysed in a 2000 Da cut-off dialysis cassette (Slide-A-Lyzer®, Thermo) against at least 1600 ml 0.9 % NaCl, changed thrice over 24 hrs. Mean arterial blood pressure was recorded from the carotid catheter in real time. A 60 minute equilibration period was followed by three 40-minute collection periods, during which urine was collected under water-saturated mineral oil. After the first 'control' collection, a bolus dose of benzamil (BZM, 2 mg per kg body weight IV) was administered, followed by a continuous infusion (1 mg per kg per hr for the remainder of the experiment). A 20-minute period of re-equilibration was followed by a second 40-minute urine collection. Mice then received a bolus dose of hydrochlorothiazide (HCTZ, 2 mg per kg body weight), followed by another 20 minute re-equilibration and then a final 40-minute urine collection. This BZM dosing regime was found to produce a stable natriuresis during the length of the study; HCTZ was administered at the smallest dose that was found to elicit maximal natriuresis in wild-type mice (data not shown; to be published elsewhere). ~80 μl blood samples were obtained from the arterial line at the end of the first equilibration period and after each urine collection; these were used to determine plasma [inulin]. At the end of the

protocol, a 1 ml sample of blood was obtained for the measurement of plasma [Na^+], [K^+] and osmolality.

A subset of mice was used in a renal clearance study to determine the acute natriuretic effect of bendroflumethiazide (BFZ, 12 mg per kg body weight IV). A 40-minute baseline collection period was followed by the BFZ bolus, followed by 20 minutes of re-equilibration and then a second 40-minute collection period.

Analysis of urine and plasma

[FITC-inulin] in plasma and urine was determined by measuring fluorescence intensity at pH 7.4, relative to a 9-point standard curve. [Na^+] in plasma and urine was determined by ion-sensitive electrode using a calibrated Roche 9180 Electrolyte Analyzer. Osmolality was determined by freezing-point depression on a Vogel Osmometer OM 801. [HCTZ] in urine was determined by HPLC triple quadrupole mass spectrometry, using a method that has been submitted for publication elsewhere.

Chronic furosemide treatment

Mice were implanted with a subcutaneous osmotic pump (Alzet® model 2001) containing furosemide (80 mg ml^{-1} in 50 % DMSO, pH 8.0), delivering a dose of c. 90 mg kg^{-1} day^{-1}. The pumps remained *in situ* for 7 days, during which time the mice had access to both tap water and salt solution (0.8 % NaCl, 0.1 % KCl) in order to prevent excessive volume and electrolyte losses. The mice were then either culled to yield tissue for immunoblotting or used for a renal clearance study.

Statistical analysis

Data are presented as the mean and 95 % confidence interval for the mean. Experimental groups were compared by unpaired t-test or by ANOVA with *post-hoc* Bonferroni's or Dunnett's tests as appropriate. For multiple comparisons by t-test, the Dunn-Sidák method was used to apply a correction to maintain the family-wise error rate (π) at 0.05, where the individual error rate, $\alpha = 1 - (1 - \pi)^{1/n}$, for n independent tests.

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