ONLINE SUPPLEMENT

Failure to down-regulate the epithelial sodium channel causes salt-sensitivity in *Hsd11b2* heterozygote mice

Eilidh Craigie, Louise C Evans, John J Mullins, Matthew A Bailey

British Heart Foundation Centre for Cardiovascular Science, The University of Edinburgh, UK

Supplementary Methods

Experiments were carried out in *Hsd11b2* wild-type (*Hsd11b2*$^{+/+}$) and *Hsd11b2*$^{+/-}$ littermates generated from *Hsd11b2*$^{+/-}$ x *Hsd11b2*$^{+/-}$ crosses. Mice were genotyped by Southern hybridization, as described[1] and were used between 100-200 days of age.

Renal Clearance Experiments: Renal function was measured in age-matched cohorts of *Hsd11b2*$^{+/-}$ or *Hsd11b2*$^{+/+}$ mice, maintained on either a low (LS; 0.03 %), standard (SS; 0.25 %) or high sodium (HS; 2.5 %) diet (Special Diet Services, Essex, UK) for 18-21 days. Mice were then anesthetised (Thiobutabarbital sodium (Inactin), 100 mg/kg; IP) and cannulae placed in the jugular vein (for IV infusion) and carotid artery (for measurement of BP and blood sampling). A tracheotomy was performed to maintain a clear airway and a catheter placed in the bladder. Urine was collected directly into pre-weighed tubes under mineral oil. Mice were infused throughout (0.2 ml/h/10 g bodyweight IV) with a saline solution (120 mM NaCl, 15 mM NaHCO3, 5 mM KCl) containing 0.5 % FITC-labeled inulin and 2% p-aminohippurate acid (PAH). After a 40-minute equilibration period, a 40-minute control urine collection was made. A bolus dose of amiloride (2 mg/kg; IV) was injected 10 minutes before the second 40-minute collection was started. Arterial blood samples of ~50 μl were taken at the beginning and end of each urine collection period. Mean arterial BP was measured continuously (PowerLab, AD Instruments, UK) via a carotid cannula. At the end of the experiment, a ~500 μl sample of arterial blood was taken for measurement of plasma sodium, potassium, aldosterone and corticosterone concentrations.

Urine flow rate was calculated by weight, assuming a density equal to that of water. FITC-inulin and PAH in urine and plasma were used to calculate glomerular filtration rate (GFR) and effective renal plasma flow (eRPF), respectively, by standard clearance equations. The urinary and plasma concentrations of sodium and potassium were measured by ion selective electrode (Electrolyte analyzer 9180, Roche, UK) and multiplied by urine flow to calculate excretion rates. Fractional excretion of electrolytes is the urinary excretion expressed as a percentage of filtered load. The effect of amiloride on sodium and potassium excretion was taken as the difference between excretion rates in the first and second urine collections, as described [2].

Chronic Inhibitor Administration: These experiments were performed on mice maintained on a HS diet and receiving one of three co-treatments: spironolactone, RU486 or benzamil.

Spironolactone and RU486 were mixed into a Silastic matrix (a gift from Dow-Corning, USA) and formed into pellets, which were cured overnight at 37ºC. Two pellets, each containing ~30mg of the drug were implanted subcutaneously under isofluorane anesthesia, five days before feeding HS diet. In vitro studies confirmed that drug release from the matrix was at a constant rate over the experimental period.

The concentration of drug in terminal plasma was measured by mass-spectrometry. The active metabolite of spironolactone, canrenone, was ~75 nmol/l; that of RU486 was ~100nmol/l. These concentrations were previously shown to exert a hypotensive effect against concentrations of corticosterone similar to that reported here [3].

Benzamil was administered via an osmotic minipump (model 2004, Alzet, Charles River, UK) at a dose rate of 0.7 µg/g body weight/day. Minipumps were implanted subcutaneously under isoflurane gas anesthesia and IV buprenorphine analgesic and surgical wounds were closed with auto-wound clips. The mice were allowed five days to recover from the effects of surgery before experimental measurements commenced.

Sodium Balance in Conscious Mice: Mice were housed continuously in metabolic cages (Techniplast, Italy). During the equilibration and control periods, mice were fed SS. After three consecutive days of stable sodium balance (control period), mice underwent surgery for minipump implantation to allow for the chronic administration of benzamil (described above) or vehicle. Sodium excretion was again measured over three consecutive days before the diet was changed to HS. Sodium balance was measured over a 3-day period as before. Water and food intake was monitored daily, as was urine and fecal output and mouse body weight. Urinary sodium concentration was measured by flame photometry (BWB-1, BWB technologies, UK) and used to calculate urinary sodium excretion over a 24h period. Fecal sodium concentration was also measured by flame photometry, following extraction into nitric acid. Daily sodium balance was calculated as dietary sodium intake – (fecal sodium excretion + urinary sodium excretion). The data are expressed a cumulative balance over a 3-day period.

Steroid Measurements: Plasma aldosterone concentrations were measured in 100µl of plasma, collected under terminal anesthesia, by radioimmunoassay (Coat-A-Count, DPC, CA, USA). Plasma corticosterone concentrations were measured as follows: briefly, plasma samples were diluted 1 in 10 in borate buffer (0.133 M boric acid, 67.5 mM NaOH, 0.5 % BSA, 1 % methanol, 0.1 % ethylene glycol, pH7.4 with HCl). Diluted plasmas were then incubated at 80°C for 30 minutes. Aliquots of the diluted plasma were mixed in duplicate in a 96 well plate (BD Falcon, 96 well flexible plate) with 1:4000 dilution of sheep antibody to corticosterone (Micropharm Ltd, UK), 1.5 M ^{3}H-labelled corticosterone, and anti sheep scintillation proximity assay reagent (1:4 dilution; GE Healthcare). This mixture was incubated overnight at room temperature, and sample wells were counted in a liquid scintillation counter (1450 Microbeta Plus model, Wallac).

qPCR analysis of mRNA abundance: RNA was isolated from homogenized kidney using an RNeasy mini kit (Qiagen, UK) and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). Quantitative PCR was performed using a Universal Probe Library kit (Roche, UK) and primers designed using ProbeFinder version 2.45 for Mouse, (Roche Diagnostics, UK). The following target genes were assessed: *nr3c1, nr3c2, scnn1a, scnn1b and sgk1*. Quantification was performed using the second derivative maximum method and target gene expression was normalized to mean concentration values. Values were normalized to the abundance Ppia, which was not different between groups and had a similar CT (~25). 18S rRNA was also found not to significantly differ across groups but has a CT of ~8 and was therefore not used to normalize target gene expression. Primer sequences are detailed in Table S1.

References

1. Bailey MA, Craigie E, Livingstone DE, Kotelevtsev YV, Al-Dujaili EA, Kenyon CJ, Mullins JJ. Hsd11b2 haploinsufficiency in mice causes salt sensitivity of blood pressure. *Hypertension.* 2011;57:515-520.

2. Ashek A, Menzies RI, Mullins LJ, Bellamy CO, Harmar AJ, Kenyon CJ, Flatman PW, Mullins JJ, Bailey MA. Activation of Thiazide-Sensitive Co-Transport by Angiotensin II in the cyp1a1-Ren2 Hypertensive Rat. *PLoS One.* 2012;7:e36311.

3. Bailey MA, Mullins JJ, Kenyon CJ. Mineralocorticoid and glucocorticoid receptors stimulate epithelial sodium channel activity in a mouse model of Cushing syndrome. *Hypertension.* 2009;54:890-896.

Table S1: Target genes for qPCR, with primer sequences and the Roche UPL Probe number

Gene name	Genbank ID	Left Primer	Right Primer	Probe Number
Ppia (Peptidylprolyl isomerase A)	NM_008907.1	acgccactgtcgcttttc	gcaaacagctcgaaggagac	46
Nr3c2 (Mineralocorticoid receptor)	NM_001083906.1	caaaagagccgtggaagg	tttctccgaatcttatcaataatgc	11
Nr3c1 (Glucocorticoid receptor)	NM_008173.3	caaagattgcaggtatcctatgaa	cttggctcttcagaccttcc	91
Scnn1a (Epithelial sodium channel subunit alpha)	NM_011324.2	ccaagggtgtagagttctgtga	agaaggcagcctgcagttta	45
Scnn1b (Epithelial sodium channel subunit beta)	NM_011325.1	ttcaactggggcatgacag	ccgatgtccaggatcaactt	29
Sgk1 (Serum/glucocorticoid regulated kinase 1)	NM_001161845.2	gattgccagcaacacctatg	ttgatttgttgagagggacttg	91

Supplementary Results

Figure S1: Quantitative PCR in kidney extracts. A) *Scnn1a*, which encodes for the αENaC subunit; C) *Scnn1b*, which encodes for the βENaC subunit and C) *Sgk1*, which encodes for serum and glucocorticoid-inducible kinase 1. *Hsd11b2*$^{+/+}$ mice (open bars, n=6) and *Hsd11b2*$^{+/-}$ mice (grey bars, n=7) were maintained on a high sodium (2.5%) diet for 3 weeks. Data are mean ± SEM of gene expression normalized to that of *Ppia*, **=P<0.01 by Student's unpaired t-test.

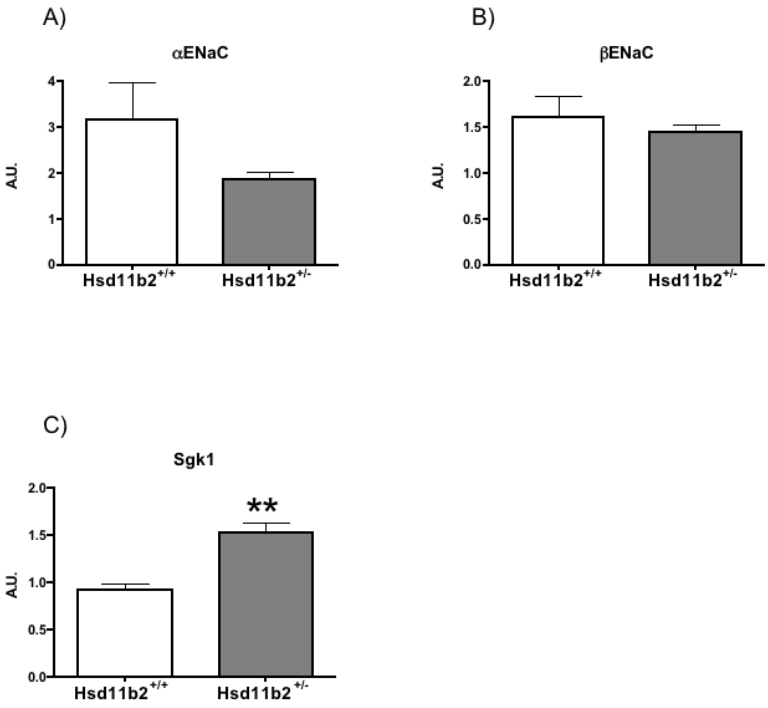

Figure S2. Plasma corticosterone concentration in conscious, unrestrained mice. *Hsd11b2*$^{+/+}$ mice (open bars, n=6) and *Hsd11b2*$^{+/-}$ mice (grey bars, n=6) were initially fed a control sodium (0.25%) diet and measurements were taken at 7am and 7pm. Mice were then fed a high sodium (2.5%) diet for three weeks, before measurements were again made. Data are mean ± SEM. Statistical analysis was by 2-way ANOVA with repeated measures, indicating a significant effect of genotype (P<0.001), diet (P<0.01) and interaction (P<0.01). Bonferroni post-hoc test P values are as shown.

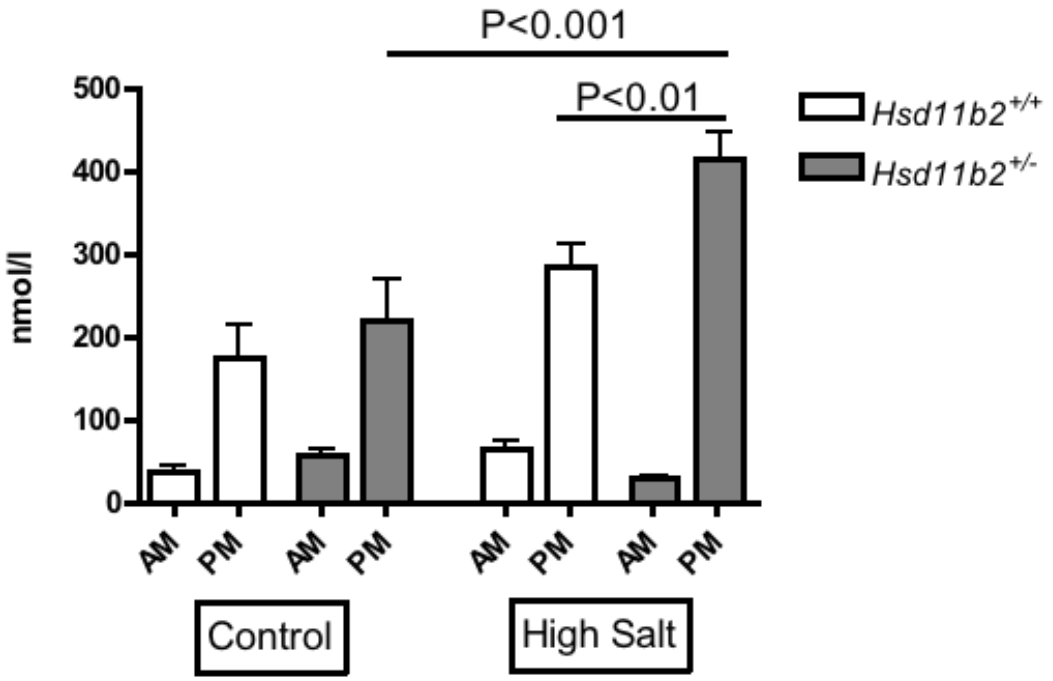

Figure S3: Quantitative PCR in kidney extracts. A) *Nr3c2*, encoding for the mineralocorticoid receptor and B) *Nr3C1*, encoding for the glucocorticoid receptor *Hsd11b2*$^{+/+}$ mice (open bars, n=6) and *Hsd11b2*$^{+/-}$ mice (grey bars, n=7), were maintained on a high sodium (2.5%) diet for 3 weeks. Data are mean ± SEM of gene expression normalized to that of *Ppia*. **=P<0.01 by Student's unpaired t-test.