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Severe salt-losing syndrome and hyperkalemia in adult kidney-specific αENaC knockouts

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Abstract

Systemic pseudohypoaldosteronism type-1 (PHA-1) is a severe salt-losing syndrome caused by loss-of-function mutations of ENaC characterized by neonatal life-threatening hypovolemia and hyperkalemia. Plasma aldosterone reaches very high plasma levels under hypovolemic and hyperkalemic challenge, that could respectively lead to either increased or decreased sodium reabsorption via the Na^+/Cl^- co-transporter NCC. We generated adult inducible nephron-specific αENaC knockout mice ($Scnn1a^{Pax8/LC1}$) that exhibit hyperkalemia and body weight loss when kept under regular salt diet mimicking PHA-1. Despite high plasma aldosterone levels, NCC protein expression and phosphorylation are downregulated, indicating that NCC is aldosterone-insensitive under hyperkalemia. However, although plasma aldosterone level remains significantly increased, NCC expression is restored to control levels following high sodium and reduced potassium diet (rescue diet), and body weight, plasma and urinary electrolyte concentrations and excretion are mostly normalized. Finally, shift to regular diet after rescue diet reinstates severe PHA-1 syndrome accompanied by significantly reduced NCC phosphorylation. In conclusion, lack of ENaC-mediated sodium transport along the nephron cannot be compensated by other sodium channels and/or transporters, but solely by high sodium and reduced potassium diet. We conclude that hyperkalemia becomes the determining factor to downregulate NCC activity regardless of sodium loss in the ENaC-mediated salt-losing PHA-1 phenotype.

Introduction

In case of decrease in volume of blood plasma, the mineralocorticoid hormone aldosterone mediates at least in part Na^+ retention by activating the Renin-Angiotensin-Aldosterone System (RAAS) and thus salt transport in the distal nephron. In this condition K^+ secretion remains unchanged. Aldosterone is also released if plasma K^+ is increased, allowing K^+ secretion in the distal nephron without affecting Na^+ reabsorption. This mechanism is commonly referred as the "aldosterone paradox" but how aldosterone exerts these apparently opposite effects is not yet completely understood [1]. Na^+ reabsorption in the distal nephron occurs through two different means: the electroneutral thiazide-sensitive Na^+/Cl^- co-transporter (NCC) expressed mainly in the DCT1 with lower expression in the DCT2, and the amiloride-sensitive epithelial Na^+ channel (ENaC) expressed in the aldosterone sensitive distal nephron (ASDN) namely the DCT2, CNT and CD. Na^+ and K^+ are the most important cations for the transmembrane potential across the plasma membrane, and electrogenic Na^+ reabsorption through ENaC increases the driving force for K^+ transport and thus K^+ excretion. ENaC consists of three different subunits α, β and γ organized in a heteromultimeric complex. The role of ENaC in humans was demonstrated by mutations in the channel causing Mendelian forms of hypertension and hypotension, namely Liddle´s syndrome and pseudohypoaldosteronism type 1 (PHA-1), respectively [2, 3]. PHA-1 is a salt-losing syndrome accompanied by hyperkalemia and metabolic acidosis. Systemic PHA-1 is an autosomal recessive form characterized by a severe neonatal salt-losing syndrome accompanied by (often lethal) hyperkalemia and metabolic acidosis. The majority of the pathogenic mutations map to αENaC predicting near-complete truncations of the protein.

To define the physiological role of ENaC *in vivo,* a mouse model with constitutive inactivation of the α subunit of ENaC has been generated which leads to death soon after birth. αENaC knockout mice display lung fluid clearance failure, hyperkalemia and sodium loss [4]. The constitutive lack of β and γENaC subunits in mice leads to a milder pulmonary phenotype, but the kidney phenotype characterized by hyperkalemia and metabolic acidosis is predominant, and accompanied by elevated plasma aldosterone levels. The β and γENaC knockouts also die within 48h hours after birth [5, 6]. α, β and γ ENaC knockouts thus present with renal phenotypes similar to that of humans with PHA type 1, but did not allow analysing the consequence of ENaC deletion specifically in the kidney and/or during adulthood [4-6]. To further dissect the role of ENaC along the nephron, we previously reported that mice with αENaC inactivated in the CD are able to maintain sodium and potassium balance [7]. This suggested that the late DCT and/or the CNT are rather involved. More recently, we studied the phenotype of renal CNT/CD-specific αENaC knockout mice. Only under low salt diet these mice develop a mild PHA-1 with higher urinary sodium excretion accompanied by a higher urinary volume and a lower osmolarity. Under sodium-deficient diet, a significant lower body weight, a higher urinary sodium excretion and hyperkalemia were observed [8]. These data thus demonstrate that αENaC deletion in the CNT is sufficient to induce clinical symptoms of PHA-1 suggesting that the CNT plays a critical role in achieving sodium and potassium balance. The model, however, does not recapitulate the severe often lethal phenotype observed in newborns suffering from PHA-1 with deletion or truncation mutations in the αENaC gene locus [9, 10].

The aim of the present work was to develop an inducible renal tubule-specific αENaC knockout in adulthood to determine whether ENaC-deficiency along the nephron mimics

the severe PHA-1 phenotype. Our data clearly demonstrate that (i) αENaC expression is indispensable in adult kidney for sodium and potassium regulation, and the mice develop a severe pseudohypoaldosteronism that mimics the human PHA-1, (ii) the knockout mice can be rescued with high sodium and reduced potassium diet allowing restoration of normal sodium and potassium excretion, (iii) a down-regulation of NCC expression and phosphorylation occurs when knockout animals are subjected to a normal salt diet. The increased sodium delivery to the CNT/CCD may be an attempt to prevent death caused by hyperkalaemia.

Results

Generation of inducible nephron-specific Scnn1a$^{Pax;LC1}$ knockout mice

To induce the deletion of the αENaC (*Scnn1a*) gene locus in adulthood, we treated 1-month-old Scnn1a$^{Pax8/LC1}$ triple transgenic animals (carrying the Scnn1a$^{lox/lox}$, Pax8-rtTA and LC1 transgenes, respectively) and their control littermates (Scnn1a$^{lox/lox}$;Pax8-rtTA and Scnn1a$^{lox/lox}$;LC1, namely Scnn1a^{Pax8} and Scnn1a^{LC1}) with doxycycline. We assessed the presence of the deleted *Scnn1a* allele (Δ) by PCR on genomic DNA extracted from kidney, lung, and liver, and identified the *Scnn1a* Δ allele in kidney and in liver of Scnn1a$^{Pax8/LC1}$ mice but not in lung or in Scnn1a^{Pax8}, Scnn1a^{LC1} and Scnn1a$^{lox/lox}$ controls (**Supplementary Fig.1A**). Analysis of *Scnn1a* mRNA transcript expression in kidney by real-time PCR demonstrated a significant reduction to 20% of controls in the Scnn1a$^{Pax8/LC1}$ knockout mice, whereas the expression of βENaC (Scnn1b) and γENaC (Scnn1g) was not affected (**Fig.4A**). Immunofluorescence on kidneys from animals under standard salt diet revealed efficient recombination of the *Scnn1a* gene locus. In overviews on the renal cortex, control mice showed numerous αENaC-positive renal tubules, while Scnn1a$^{Pax8/LC1}$ knockout mice revealed only a few remaining αENaC-positive renal tubules. In contrast, γENaC was similarly detectable in control and Scnn1a$^{Pax8/LC1}$ knockout mice (**Supplementary Fig.1B**). High magnifications revealed that αENaC was efficiently deleted in all distal tubule cells that express the Cre protein. Only a few single cells in the ASDN did not express Cre and continued to express αENaC (**Supplementary Fig.1C**). Although the recombination of the *Scnn1a* allele (Δ) occurs also in liver (**Supplementary Fig.1A**), αENaC mRNA and protein expression levels in this organ did

not differ between control and Scnn1a$^{Pax8/LC1}$ knockout mice (**Supplementary Fig.2A and B**).

αENaC expression is crucial to maintain sodium and potassium homeostasis in adulthood

One-month-old Scnn1a$^{Pax8/LC1}$ knockout and control animals were fed with a regular salt diet and placed in metabolic cages for four consecutive days to determine their urinary and plasma Na$^+$ and K$^+$ concentrations (**Supplementary Fig. 3A**). Following doxycycline treatment control animals kept gaining weight in their growing phase, whereas Scnn1a$^{Pax8/LC1}$ knockout mice rapidly lost body weight and manifested severe sickness (**Fig. 1A and Supplementary Fig. 4**). All knockout animals analysed lost more than 10% of their initial body weight and were thus sacrificed. Moreover, Scnn1a$^{Pax8/LC1}$ knockout mice presented with signs of hyponatremia, and developed a severe hyperkalemia (**Fig. 1B**). Creatinine levels did not vary in urine, but were significantly increased in plasma of Scnn1a$^{Pax8/LC1}$ knockout mice (**Supplementary Fig. 5A and B**). Creatinine clearance was not significantly different among the two groups, despite an almost 50% reduction in knockouts (**Supplementary Fig. 5C**). During this period, we observed no difference in water intake or urine output compared to water intake with the exception of a decrease in food intake and in amount of feces at the fourth day following doxycycline induction in knockout mice (**Supplementary Fig. 6A-D**). This was accompanied by significantly increased cumulative 24h-urinary sodium and a decreased 24h-urinary potassium excretion (**Fig. 1C and D**). Finally, a significant increase in plasma aldosterone levels (controls: 3±1 nM, n=8 versus knockouts: 32±5 nM, n=7; *P*≤0.001) was detected in the

kidney-specific knockout mice mimicking a severe pseudohypoaldosteronism type 1 phenotype.

High Na$^+$ and reduced K$^+$ diet restores body weight gain and electrolyte balance in Scnn1a$^{Pax8/LC1}$ knockout mice

The animals were induced by doxycycline during three days under a standard salt diet. At the third day of the doxycycline treatment, the diet was changed to high Na$^+$ and reduced K$^+$ during two months to compensate for Na$^+$ loss and reduced K$^+$ excretion, and animals were analysed during the last three days of high Na$^+$ and reduced K$^+$ treatment (**Supplementary Fig. 3B**). Nephron-specific Scnn1a$^{Pax8/LC1}$ knockout mice displayed body weight gain as control animals, and presented with normal natremia and kalemia, although plasma K$^+$ of knockouts remained significantly higher than that of controls (**Fig. 2A and B and Supplementary Fig. 4**). The cumulative urinary sodium and potassium balance in Scnn1a$^{Pax8/LC1}$ knockout mice was re-established in comparison with the controls (**Fig. 2C and D**), and food and water intake, feces amount, urine volume compared to water intake became indistinguishable between the two groups (**Supplementary Fig. 7A-D**). These data indicate that the salt-losing phenotype of Scnn1a$^{Pax8/LC1}$ knockout mice can be restored by compensating sodium and potassium intake, and nearly 80% of the initial Scnn1a$^{Pax8/LC1}$ knockout mice survived (28 out of 36). Interestingly, plasma aldosterone levels were 45-fold higher compared to controls (0.2 ± 0.03 nM, n=14 in controls and 10 ± 2 nM, n=13 in knockouts, *P*<0.001).

The return to a standard diet reinstates a severe type 1 PHA characterized by metabolic acidosis

When we returned to the standard diet following two months of high Na^+ and reduced K^+ treatment (**Supplementary Fig. 3C**), Scnn1a$^{Pax8/LC1}$ knockout mice rapidly lost body weight (**Fig. 3A and Supplementary Fig. 4**), and presented with significantly lower plasma sodium, but normalized cumulative sodium excretion (**Fig. 3B and C**) most likely because of reduced food intake and thus reduced Na^+ input (**Supplementary Fig. 8A and D**) and hyperkalemia accompanied by reduced cumulative potassium excretion (**Fig. 3B and D**). With exception of water intake and urine volume to water intake ratio (**Supplementary Fig. 8B and C**)**,** Scnn1a$^{Pax8/LC1}$ knockout animals significantly reduced food intake with consequences on feces output (**Supplementary Fig. 8A and D**)**.** Aldosterone levels stayed significantly increased in knockouts (6 ± 2 nM, n=8 in controls and 53 ± 8 nM, n=6 in knockouts, $P<0.01$). While plasma Ca^{2+} and Cl^- levels were comparable between the two groups, blood pH was significantly reduced in knockout mice (**Table 1**). To get insights into acidosis, we analysed pCO_2, cBase(Ecf) and $cHCO_3^-$ in blood. While pCO_2 did not change, we found a significant decrease in the levels of cBase(Ecf) and $cHCO_3^-$ in Scnn1a$^{Pax8/LC1}$ knockout mice, revealing the inability of these animals to excrete acid via the kidney (**Table 1**).

Downregulation of NCC phosphorylation despite severe salt-losing syndrome

To analyse whether the expression of β and γENaC subunits changes when αENaC is absent, we analysed β and γ mRNA and protein levels under standard diet, under rescue diet (high sodium and reduced potassium) and back to standard diet after rescue diet (**Supplementary Fig. 3A-C**). β and γENaC mRNA expression was not modified in all three diets (**Fig. 4A-C**). At the protein level, we observed that γENaC expression did not change, but βENaC levels were increased under standard salt diets (**Fig. 4D-F**)

suggesting an attempt to compensate for αENaC absence. The sodium-chloride co-transporter, also known as thiazide-sensitive sodium-chloride co-transporter NCC reabsorbs sodium and chloride ions from the tubular fluids in distal convoluted tubules of the nephron, and plays a major role in Na$^+$ balance [11]. To verify whether absence of αENaC in adult kidney might have any effect on NCC regulation, we analysed NCC mRNA transcript and protein expression following induction of αENaC-deficiency under the three different diet phases. Total mRNA and protein NCC levels were decreased under standard salt diet before the rescue phase, and surprisingly did not vary when back to standard diet after the high Na$^+$ and reduced K$^+$ treatment (**Fig. 4A-C**, and **Fig. 5A-C**). Despite the establishment of a severe PHA-1 phenotype under the standard diets, Scnn1a$^{Pax8/LC1}$ knockout mice presented with a significantly decreased NCC T53 and T58 phosphorylation (**Fig. 5A-C**). In contrast, high Na$^+$ and reduced K$^+$ restored the phosphorylated state of NCC to control levels (**Fig. 5B**). Altogether these data indicate that the absence of αENaC in the nephron leads to decreased NCC activity, and that a diet rich in Na$^+$ and reduced in K$^+$ is sufficient to restore electrolyte balance and NCC phosphorylation.

Discussion

Deletion of αENaC/Scnn1a along the nephron leads to a severe PHA-1 phenotype

Scnn1a$^{Pax8/LC1}$ knockout mice develop a severe pseudohypoaldosteronism syndrome with rapid weight loss, disturbance of plasma Na$^+$/K$^+$ concentrations, significantly increased urinary Na$^+$ loss and decreased K$^+$ excretion presenting all clinical features of the human PHA-1. The adult phenotype mimics as well that of the newborns with constitutive deletion of the αENaC (Scnn1a), βENaC (Scnn1b) and γENaC (Scnn1g) subunits [4-6] (**Fig. 1**) and confirms the critical role of ENaC function within the ASDN. Although we could not asses it directly because the animals are too small and sick to be measured, the Scnn1a$^{Pax8/LC1}$ knockouts are most likely in a severe hypovolemic state that, together with reduced food intake, could explain body weight loss. The phenotype observed in Scnn1a$^{Pax8/LC1}$ knockout mice is more severe than the one with CNT/CD-specific ENaC inactivation [8] and closely reproduces the pharmacological inactivation of ENaC by acute administration of amiloride in one-week salt-depleted rats [12]. Pax8 expression has been described in liver, and we observed partial DNA recombination at the Scnn1a gene locus also in this organ (**Supplementary Fig. 1A**). However, αENaC mRNA and protein levels in liver did not change between control and knockout animals (**Supplementary Fig. 2A and B**) and no relevant ENaC function has been described so far in liver that is linked to sodium and potassium homeostasis. Whole heterozygous mutant αENaC knockout mice maintain blood pressure and sodium balance even upon different sodium diets [13]. Moreover, no DNA recombination at the αENaC (*Scnn1a*) gene locus was observed in lung (**Supplementary Fig. 1A**) where ENaC function in alveolar fluid clearance is well known

[14]. The results presented in this article clearly demonstrate that ENaC deficiency along the nephron cannot be compensated by other sodium-absorbing channels.

Rescue of sodium and potassium homeostasis in *Scnn1a^Pax8/LC1* mice by high sodium and reduced potassium diet

The constitutive mineralocorticoid receptor knockout mice that show impaired ENaC activity resembling inborn PHA-1 when untreated, can be rescued by NaCl addition to the diet, however retaining sodium-losing defects [15]. We thus asked whether Scnn1a^Pax8/LC1 knockout mice could compensate sodium loss and hyperkalemia with a high Na^+ and reduced K^+ diet. Indeed, a diet rich in Na^+ and reduced in K^+ is sufficient to almost completely restore body weight and plasma and urinary electrolytes in Scnn1a^Pax8/LC1 knockout mice (**Fig. 2, Supplementary Fig. 4 and 7**) and it would be interesting to explore whether high salt alone (with normal potassium) can correct the hyperkalaemia. The localization of the mineralocorticoid receptor in the ASDN, and in other renal cell types [16] and/or a largely aldosterone-independent ENaC function in the DCT2/CNT [17] may contribute to the complexity of corticosteroid effects on ASDN function. A crosstalk between the angiotensin II membrane receptor and the mineralocorticoid receptor signalling pathways is well established, as demonstrated by Shibata et al. and Terker et al. [18, 19]. Conditional inactivation of the mineralocorticoid receptor in the CD and late CNT is only compensated under standard diet, but no longer when sodium supply is limited [20]. The phenotype is thus comparable to that of late CNT/CD-specific ENaC knockout where the same AQP2-Cre transgenic line was used [7]. Again, the relatively mild phenotype in the CNT/CD ENaC knockout mice can be explained by a compensation of renal ENaC activity via the RAAS system in more proximal ENaC-containing nephron segments, like

the "early" CNT and "late" DCT. This may point to a crucial mineralocorticoid receptor function in more proximal nephron segments, like the DCT1, CTAL, OMTAL independent from regular ENaC activity. Interestingly, the return to the standard diet after two months of high Na^+ and reduced K^+ treatment reinstates a PHA type 1 phenotype. Despite sodium loss, sodium may be delivered to more distal nephron segments to favour potassium excretion (**Fig. 3 and Supplementary Fig. 4 and 8**). We have recently demonstrated that activation of the RAAS system in the kidney can compensate for the absence of αENaC in colon in a mouse model of αENaC deletion in intestinal superficial cells [21]. Thus the intestine of the Scnn1a$^{Pax8/LC1}$ knockout mice may play a compensatory role with the attempt to prevent Na^+ loss and K^+ retention. When αENaC is deleted along the nephron, β and γENaC subunits cannot form fully functional channels [22]. By contrast, when β or γENaC is deleted, the remaining αγ or αβ channels may induce sufficient activity to maintain sodium balance. Indeed Knepper and coworkers have shown *in vivo* that the protein abundance of the αENaC subunit was regulated by salt diet and aldosterone, while the γENaC was cleaved in response to aldosterone and dietary Na^+ [23, 24]. In this study we observed no difference in the cleaved γENaC subunit in both wild-type and knockout animals under the different diets.

Aldosterone-independent regulation of NCC in Scnn1a$^{Pax8/LC1}$ mice

Described as an "aldosterone paradox", aldosterone can trigger differential regulation of Na^+ and K^+ transport between DCT1 and the ASDN [1]. Following induction of ENaC deletion along the nephron, NCC is significantly less phosphorylated. This finding is unexpected, as the Scnn1a$^{Pax8/LC1}$ mice suffer from hyponatremia and hyperkalemia (**Fig. 1**) accompanied by high plasma aldosterone levels. The hyperkalemia may trigger the

apparent contradictory aldosterone-induced NCC down-regulation. Indeed, a high-K^+ diet decreases NCC [25, 26]. The K^+-loading-induced NCC down-regulation may occur rapidly in response to both an oral potassium intake and an intravenous potassium infusion [27, 28] is aldosterone-independent [29] and was shown to overrule hypovolemic NCC stimulation [30]. The NCC downregulation may thus improve renal K^+ excretion.

This novel animal model points to the DCT2/CNT as crucial aldosterone-sensitive nephron segment. However, we do not exclude that the CD may still play an important role under challenging conditions, even if ENaC-deletion per se in this segment does not seem to be a prerequisite for sodium and potassium balance [7]. Indeed, two recent studies unveil an ENaC regulation largely independent from aldosterone [17] and likely dependent on vasopressin [31] suggesting that sodium, but also potassium handling might be regulated in a cell-type and nephron segment-specific manner. In conclusion, Scnn1a$^{Pax8/LC1}$ knockout mice fully reproduce the PHA-1 phenotype, and hyperkalemia remains the predominant and life-threatening feature to be avoided even at the expense of increased sodium loss.

Concise methods

Generation of inducible nephron-specific αENaC-deficient mice.

To inactivate the *Scnn1a* gene in all proximal and distal tubules, and the entire collecting duct system of the kidney, we took advantage of Tet-On and Cre-loxP systems. Nephron-specific αENaC-deficient mice (Scnn1a$^{lox/lox}$;Pax8-rtTA$^{tg/0}$;TRE-LC1$^{tg/0}$) and littermate controls (Scnn1a$^{lox/lox}$;Pax8-rtTA$^{tg/0}$, Scnn1a$^{lox/lox}$;TRE-LC1$^{tg/0}$, and Scnn1a$^{lox/lox}$) were obtained by interbreeding Scnn1a$^{lox/lox}$;Pax8-rtTA$^{tg/0}$ with Scnn1a$^{lox/lox}$;TRE-LC1$^{tg/0}$ mice. Genotyping of the mice was performed by PCR analysis of ear biopsies [32] at the age of weaning using the following primers: Pax8-rtTA ST1: 5'-CCATGTCTAGACTGGACAAGA-3', Pax8-rtTA ST2: 5'-CTCCAGGCCACATATGATTAG-3'; LC-1 Cre3: 5'-TCGCTGCATTACCGGTCGATGC-3'; LC-1 Cre4: 5'-CCATGAGTGAACGAACCTGGTCG-3'. Animals were housed in a temperature- and humidity-controlled room with an automatic 12-hour light/dark cycle and had free access to food and tap water. Experimental procedures and animal maintenance followed federal guidelines and were approved by local authorities, and adhered to the NIH Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/olaw/references/phsol.htm).

Induction of nephron-specific αENaC-deficient mice.

We generated inducible renal tubule-specific αENaC knockout mice using the αENaC floxed allele (*Scnn1a$^{lox/lox}$* [32]), the Pax8-rtTA$^{tg/0}$ transgenic mice expressing the reverse tetracycline transactivator under control of the *Pax8* promoter that is driving the expression in all proximal and distal tubular cells along the nephron [33], and the TRE-LC1$^{tg/0}$ transgenic mice where the expression of the Cre recombinase and luciferase is

under the control of the tetracycline response element (TRE) [34]. In the presence of doxycycline the reverse tetracycline transactivator binds and activates the tetracycline-responsive element, thereby triggering Cre recombinase expression. To induce the deletion of the *Scnn1a* gene, 4-week-old Scnn1a$^{lox/lox}$;Pax8-rtTA;LC1 mice and control littermates were treated with 2 mg/mL doxycycline and 2% sucrose in the drinking water, following 2 days of 2% sucrose in the drinking water. The doxycycline hydrochloride (Sigma, Deisenhofen, Germany) was protected from light, and prepared fresh every two days. The recombination of the floxed *Scnn1a* alleles in kidney, lung and liver was investigated by DNA-based PCR analysis (using the primers described above) and at the mRNA level using TaqMan PCR (Applied Biosystems 7500).

Quantitative RT-PCR

Organs were homogenized using Tissue Lyzer (QIAGEN) and RNA was extracted with the guanidinium thiocyanate-phenol-chloroform extraction method (QIAzol lysis reagent, QIAGEN). 1.5 µg of RNA were reverse transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). Quantitative RT-PCR was performed by TaqMan PCR using Applied Biosystems 7500. Each measurement was taken in duplicate. Quantification of fluorescence was normalized to β-actin. Primer sequences were published previously [35].

Salt diets and metabolic cages

The diets were given as solid food (pellet, standard cages) or as powder (metabolic cages). After 3 days of doxycycline treatment under standard salt diet (0.17% sodium and 0.97% potassium given as powder, ssniff Spezialdiäten GmbH) mice were fed for 3 days

with a diet rich in sodium and low in potassium (3.5% sodium, potassium < 0.1% given as powder, ssniff Spezialdiäten GmbH), that was supplemented with 0.2% potassium in drinking water during the following 2 months. At the end of this period, mice were fed again with a normal salt diet for 3 days (0.17% sodium and 0.97% potassium given as powder, ssniff Spezialdiäten GmbH). For the metabolic cage studies, experimental mice and controls from the same litter were placed in individual metabolic cages (Tecniplast, Buguggiate, Italy) and fed with the different salt diets. The high concentration of plasma aldosterone in control animals on the standard salt diet may reflect the choice of a control diet relatively lower in Na^+ and higher in K^+ content than other standard rodent chows. During the experiments the animals had free access to food and water. Body weight, food and water intake, urine excretion and quantity of feces were monitored once-daily at the same time. At the end of the experiments, blood was collected, mice were sacrificed (by decapitation), and kidney, lung and liver were collected for molecular analyses.

Urine and Serum/Plasma Analysis

Urine and serum/plasma osmolarity as well as sodium, potassium, and bicarbonate concentrations were analysed by using a flame photometer (Cole-Parmer). Plasma aldosterone levels were measured according to standard procedures using a RIA (Coat-A-Count RIA kit; Siemens Medical Solutions Diagnostics, Ballerup, Denmark). Mouse samples with values >1200 pg/ml were further diluted using a serum pool with a low aldosterone concentration (<50 pg/ml). The urinary and plasmatic creatinine concentrations were measured by enzyme-linked immunosorbent assay (ELISA) at the Zurich Integrative Rodent Physiology (ZIRP) platform.

Blood gas analyzes

The mice were anesthetized with 3% isoflurane and 97% atmospheric air mixture. The thorax was opened and the heart was exposed. 150-300 µl of blood was withdrawn from the right atrium with a heparin coated syringe (Pico50, Radiometer, Denmark). The blood was immediately measured by a blood gas analyzer (ABL800 Flex, Radiometer, Denmark).

Western blot analyzes

Frozen tissues were homogenized and protein extracted as described [36]. Anti-α, β, γENaC, NCC and pT53-NCC and pT58-NCC antibodies were obtained and used as described [27]. Phospho-antibodies were diluted 1/1000. Anti-β-actin antibody (Sigma-Aldrich) was used as loading control.

Immunofluorescence on kidney sections

Kidneys were fixed by vascular perfusion and processed for immunohistochemistry as previously described [37]. Serial cryosections (5 µm) were incubated overnight at 4°C with polyclonal rabbit antisera against either Cre (dilution 1:10'000) [38], α-ENaC (dilution 1:1'000) [27] , or γ-ENaC (dilution 1:20'000) [39]. The primary antibodies were revealed with a Cy3-conjugated donkey anti-rabbit IgG (Jackson Immuno Research Labs, West Grove, PA) diluted 1:1'000. Moreover, some sections were incubated also with FITC-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories) diluted 1:100. Images were acquired with a Leica DFC 350 FX charge-coupled device camera (Leica, Heerbrugg, Switzerland) and processed by Leica Application Suite software before

importing into Adobe Photoshop CS3 and Powerpoint for image arrangement and labeling. The primary antibodies were omitted in control experiments.

Statistical analysis

Results are presented as mean ± SEM. Data between control and $Scnn1a^{lox/lox}$;$Pax8$-$rTta$;$LC1$ mice were analysed by unpaired t test. P values <0.05 were considered statistically significant.

Abstract: R. Perrier, B.C. Rossier, E. Hummler. Nephron-specific gene targeting of a ENaC in adulthood leads to a severe PHA type 1 phenotype. J. Am. Soc. Nephrol., 20 (2009), p. 382A.

Statement of competing financial interests

The authors declare that no conflict of interest exists.

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Figure legends

Figure 1 Inducible Scnn1a knockout mice develop a PHA type 1 phenotype under regular diet.

(**A**) Body weight changes (Δ body weight) in percentage of initial body weight monitored during four consecutive days following doxycycline administration at day 0. A total of 19 control (straight line) and 20 Scnn1a knockout mice (Scnn1a$^{Pax8/LC1}$, dashed line) were analyzed. (**B**) Plasma Na$^+$ and K$^+$ measurements (mmol) in control (n=14) and knockout mice (n=11). (**C**) 24h- cumulative urinary sodium and (**D**) potassium excretion (mmol) of control (n=11) and knockout mice (n=10). Results are presented as mean ± SEM and data were analysed by unpaired t test. P values <0.05 were considered statistically significant.

Figure 2 High Na$^+$ and reduced K$^+$ diet normalizes body weight loss and plasma and urinary electrolyte concentration.

(**A**) Body weight changes (Δ body weight) in percentage of initial body weight in control (n=11) and knockout mice (n=11) at the end of two months of high Na$^+$ and reduced K$^+$ diet (rescue diet). (**B**) Plasma Na$^+$ and K$^+$ concentrations in control (n=6) and knockout mice (n=4). (**C**) 24h-urinary cumulative sodium and (**D**) potassium excretion (mmol) of control (n=11) and knockout mice (n=11). -3, -2, -1 and 0 correspond to the last days of the rescue diet. Results are presented as mean ± SEM and data were analysed by unpaired t test. P values <0.05 were considered statistically significant.

Figure 3 The switch to standard diet reinstates a severe PHA type 1 phenotype.

(**A**) Body weight changes (Δ body weight) in percentage of initial body weight in control

(n=8) and Scnn1a$^{Pax8/LC1}$ knockout mice (n=8) during three days of standard diet after

two months of high Na$^+$ and reduced K$^+$ diet. (**B**) Plasma Na$^+$ and K$^+$ concentrations in

control (n=15) and knockout mice (n=13). (**C**) 24h-urinary sodium excretion of control

and knockout mice during three days of standard diet (+1, +2, +3) after two months of

high Na$^+$ and reduced K$^+$ diet. (**D**) 24h-urinary potassium excretion of control and

knockout mice. n= 8 mice per genotype. Results are presented as mean ± SEM and

data were analysed by unpaired t test. P values <0.05 were considered statistically

significant.

Figure 4 βENaC protein expression increases in absence of αENaC.

α, β, γENaC and NCC mRNA transcript expression in the kidney determined by

quantitative real-time PCR and normalized to β-actin in control (white) and knockout

(black column) mice (**A**) under standard diet, (**B**) following rescue (high sodium and

reduced potassium) diet and (**C**) three days following the return to the standard diet, n≥4

per genotype. (**D**) Representative Western blot analyses for α, β and γENaC on the

whole kidney of control and Scnn1a$^{Pax8/LC1}$ knockout mice under standard diet (n=4 per

genotype), (**E**) following a high Na$^+$/reduced K$^+$ (rescue) diet (controls: n=7; knockouts

n=7), and (**F**) three days following the switch to the standard diet (controls: n=13;

knockouts n=13). Protein expression was normalized to the amount of β-actin and

reported relative to control values. Results are presented as mean ± SEM and data were

analysed by unpaired t test. P values <0.05 were considered statistically significant.

Figure 5 NCC phosphorylation is normalized following rescue diet.

(**A**) Representative Western blot analyses for total NCC and phosphorylated pT53- and pT58-NCC on the whole kidney of control (n=14) and Scnn1a$^{Pax8/LC1}$ knockout mice (n=14) under standard diet, (**B**) following a high Na$^+$/reduced K$^+$ (rescue) diet (controls: n=12; knockouts n=11), and (**C**) three days following the switch to the standard diet (controls: n=6; knockouts n=8). Protein expression was normalized to the amount of β-actin and reported relative to control values. Results are presented as mean ± SEM and data were analysed by unpaired *t* test. *P* values <0.05 were considered statistically significant.

Table 1

Blood parameters of mice after the return to the standard salt diet following two months of rescue diet.

	Control (n=7)	Experimental group (n=6)
Na$^+$ (mmol.l^{-1})	145±0,4	137±1,3 ***
K$^+$ (mmol.l^{-1})	5±0,2	8±0,5 ***
Ca^{2+} (mmol.l^{-1})	1,3±0,01	1,2±0,01
Cl$^-$ (mmol.l^{-1})	113±0,9	111±1,2
pH	7±0,02	7±0,02 *
pCO$_2$ (mmHg)	41±1,9	40±2,5
cBase (Ecf)	-4±0,8	-8±0,9 ***
cHCO$_3^-$ (aP,st)	19±0,6	16±0,5 ***

Data are average ± SEM. * P <0.05; *** P <0.001. cBase (Ecf): standard base excess.

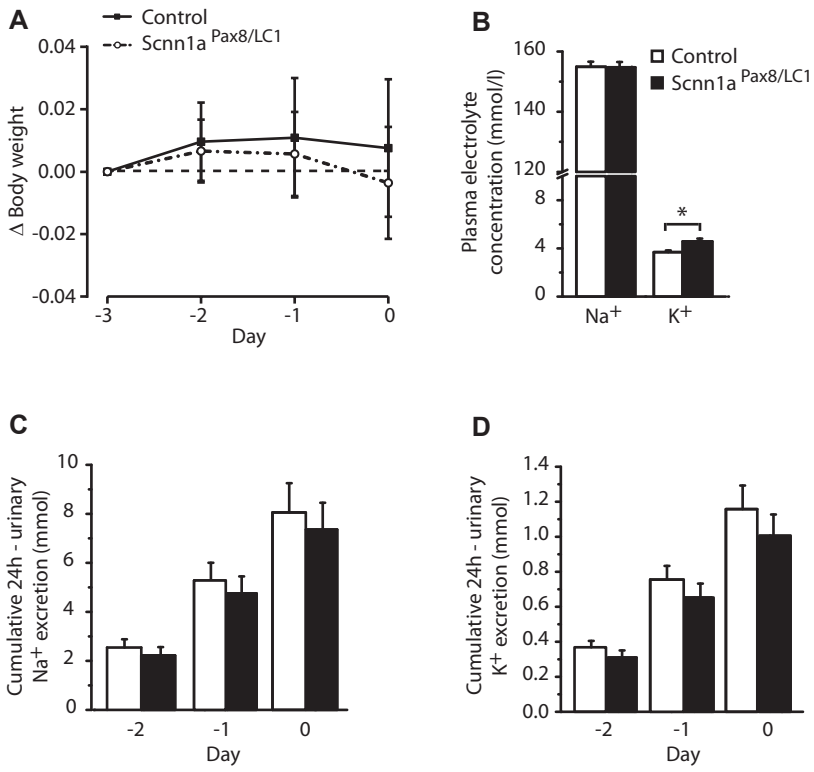cHCO$_3^-$ (aP,st): standard bicarbonate.
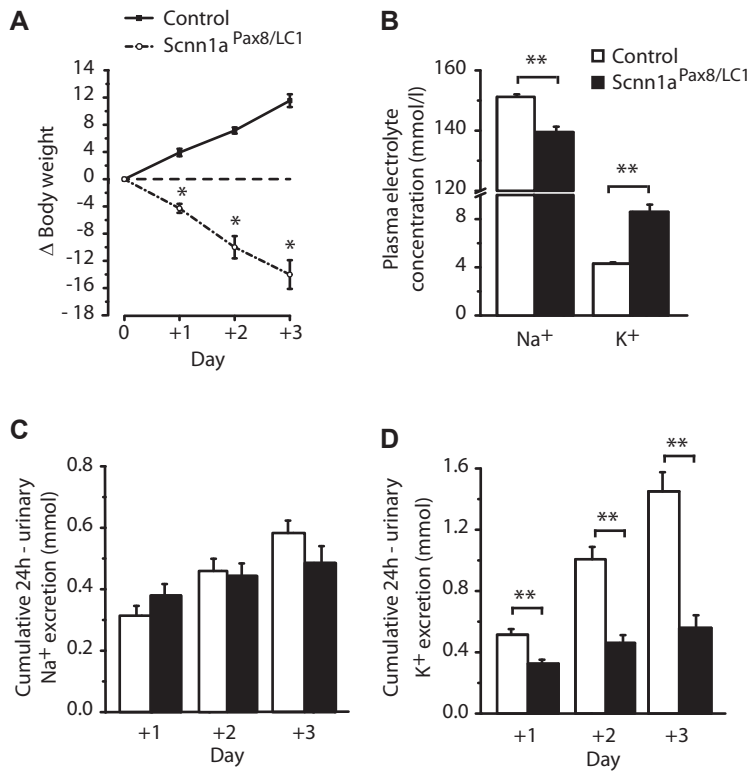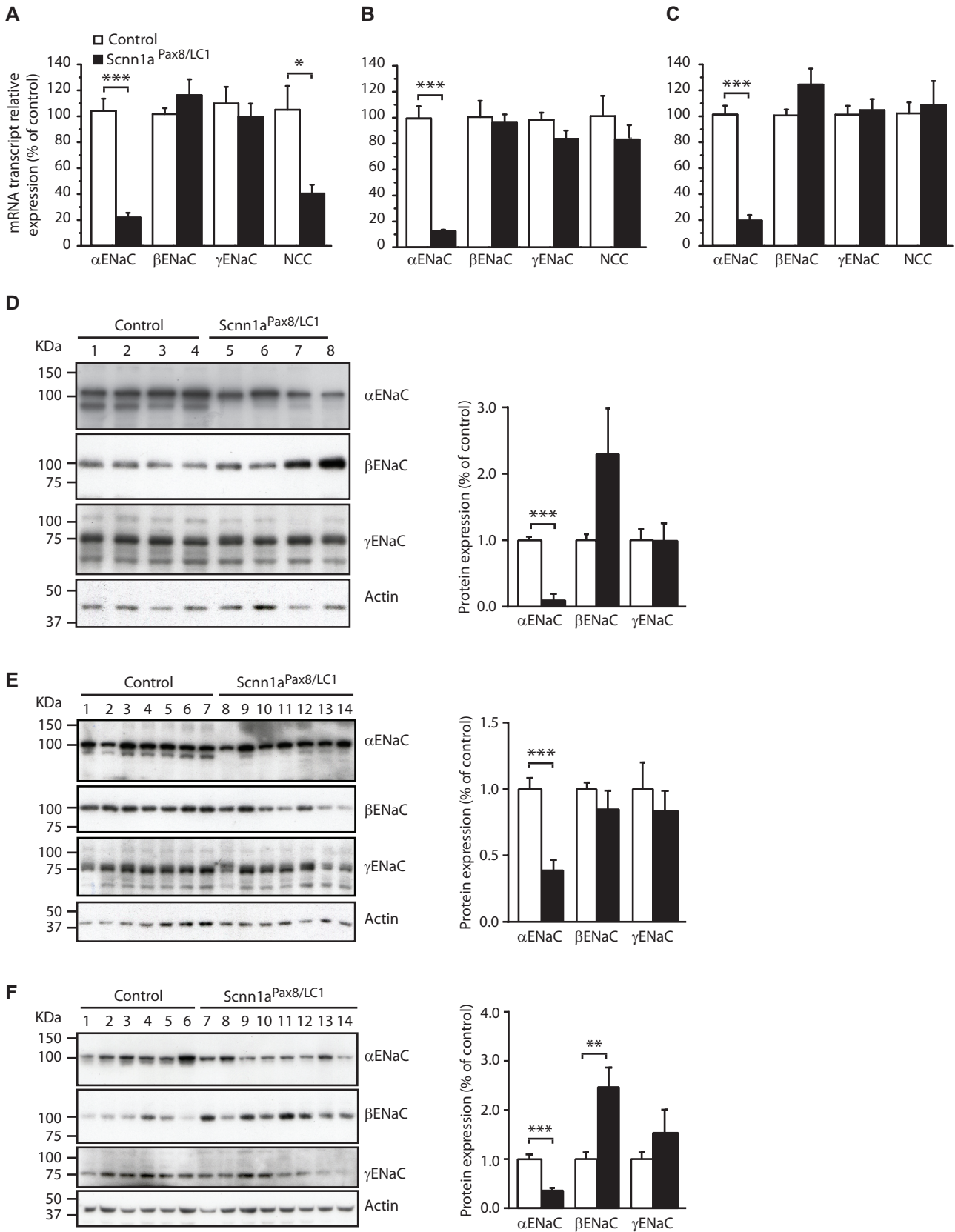
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5