Water and Electrolyte Homeostasis in a Mouse Model with Reduced ENaC Gamma Subunit Expression

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Running Title: Mouse with Reduced ENaC Gamma Subunit Expression

Keywords: ENaC, Potassium Excretion, Salt Sensitivity, Extracellular Fluid Volume, Quantitative magnetic resonance

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Abstract

The epithelial Na^+ channel (ENaC) promotes the absorption of Na^+ in the aldosterone-sensitive distal nephron, colon, and respiratory epithelia. Deletion of genes encoding ENaC's subunits results in early post-natal mortality. We present initial characterization of a mouse with dramatically suppressed expression of the γ subunit. We use this hypomorphic (γ^{mt}) allele to explore the importance of ENaC's γ subunit in homeostasis of electrolytes and body fluid volume. At baseline, γ subunit expression in $\gamma^{mt/mt}$ mice is markedly suppressed in kidney and lung, while electrolytes resemble those of littermate controls. Challenge with a high K^+ diet does not cause significant differences in blood K^+, but provokes higher aldosterone in $\gamma^{mt/mt}$ mice than controls. Quantitative magnetic resonance (QMR) measurement of body composition reveals similar baseline body water, lean tissue mass, and fat tissue mass in $\gamma^{mt/mt}$ mice and controls. Surprisingly, euvolemia is sustained without significant changes in aldosterone or atrial natriuretic peptide. $\gamma^{mt/mt}$ mice exhibit a more rapid decline in body water and lean tissue mass in response to a low Na^+ diet than controls. Replacement of drinking water with 2% saline induces dramatic increases in body fat in both genotypes, and a selective transient increase in body water and lean tissue mass in $\gamma^{mt/mt}$ mice. While ENaC in renal tubules and colon work to prevent extracellular fluid volume depletion, our observations suggest that ENaC in non-epithelial tissues may have a role in preventing extracellular fluid volume overload.

Introduction

The epithelial Na^+ channel (ENaC) has a key role in regulation of extracellular fluid volume and blood pressure, and renal K^+ secretion. ENaC loss of function mutations in humans cause pseudohypoaldosteronism type 1 (PHA1), associated with urinary Na^+ wasting, polyuria, hypotension, hyperkalemia and elevated aldosterone, reflecting extracellular fluid volume contraction (37). Gain-of-function mutations cause Liddle syndrome, characterized by hypertension, suppressed aldosterone, and hypokalemia secondary to enhanced renal tubular Na^+ absorption and K^+ secretion (37). The importance of ENaC in regulating Na^+ handling and extracellular fluid volume suggest that ENaC activity may play a role in salt-sensitive hypertension (16, 25, 28).

The essential nature of the genes encoding ENaC's subunits, α, β, or γ, has impaired investigation of the functional contributions of these subunits. A hypomorphic β subunit allele has proven useful in assessing the physiologic importance of this subunit, though complicated by inclusion of a Liddle syndrome mutation in the hypomorphic β subunit allele (29). Mice expressing this allele demonstrate more than twenty-fold reduction in β subunit expression but survive to adulthood, exhibiting a PHA1-like phenotype. Subsequent studies capitalized on this useful genetic tool to demonstrate the importance of the β subunit in modulation of respiratory fluid clearance, arterial myogenic vasoconstriction, renal vascular blood flow, and baroreception (9, 11, 14, 31).

Here, we present initial characterization of a gene-targeted mouse strain expressing a hypomorphic γ subunit allele, resulting in dramatically reduced expression in both kidney and lung but supporting viability in the homozygous state ($\gamma^{mt/mt}$). This hypomorphic allele allows exploration of the importance of ENaC's γ subunit in homeostasis of electrolytes and body fluid volume.

Methods

Generation and care of γ subunit hypomorphic mice: Gene-targeted mice were produced using homologous recombination in embryonic stem (ES) cells. *Scnn1g* exon 2 and 7 to 8 kB of flanking DNA were cloned from a Chromosome 7 bacterial artificial chromosome (BACPAC Genomics). The cDNA for the only known transcript of *Scnn1g* was inserted, in frame, distal to the start codon in *Scnn1g* exon 2 (Figure 1). An accompanying neomycin cassette decreased expression of the targeted locus. (27) Embryonic stem cells were electroporated with this construct. Correct insertion of the transgene was confirmed in G418-resistant stem cells by Southern blot. Chimeric mice carrying the targeted allele were produced at Charles River Inc. by microinjection of blastocysts with correctly targeted ES cell clones. Male progeny with germline incorporation of the transgene were selected as founders of independent mouse lines. Progeny were back-crossed for four to five generations into the 129S2/SvPasCrl (129sv, Charles River) or C57B/6 (Jackson Labs) background. Heterozygous ($\gamma^{+/mt}$) mice from the two separate lines were cross-bred to generate experimental mice and littermate controls. Genotyping was performed in a single reaction with the following three primers: pER31 (intron 1 common forward primer: ACC TTA CTT GGC TCC TCT GTC CCT TC), pER32 (transgenic exon 2-3 junction reverse primer: GGA GGT CAC TCA CAG CAC TGT ACT TGT AG), and pER35 (wild-type intron 2 reverse primer: GGA GGC AGA TGC TAA CCT CAT TTC AGG). Amplification products were 633 bp (γ^{+}) and 400 bp (γ^{mt}). All control mice were littermates of $\gamma^{mt/mt}$ mice.

Regular diet included 0.94% K^+ and 0.23% Na^+ (Prolab Isopro RMH 3000, LabDiet). High K^+ diet contained 5.2% K^+ (as KCl) and 0.3% Na^+ (Teklad TD.09075, Envigo). Low Na^+ diet (TD.90228) contained 0.01-0.02% Na^+ and 0.8% K^+. 2% saline was unbuffered 2 g/dL NaCl in dH_20 (w/v). All animal work was approved by University of Pittsburgh Institutional Animal Care and Use Committee.

Measurement of electrolytes and hormones: Whole blood was collected upon mouse sacrifice via aspiration from a cardiac ventricle and measurements were performed immediately using an iSTAT

handheld device (Abbott Point of Care, Inc.). ELISAs for aldosterone (Enzo Life Sciences, catalog number ADI-900-173) or ANP (Sigma-Aldrich catalog number RAB00385) were performed on plasma diluted 1:25 to 1:200 or 1:4, respectively.

Immunoblotting: Tissues were stored at -80 degrees C° until use. Approximately 50 mg of tissue was homogenized using a Dounce homogenizer in 0.15 mL (for kidney) or 0.2 mL (for lung) HEPES-buffered saline plus Phosphatase Inhibitor Cocktail Set II and Protease Inhibitor Cocktail Set III (Calbiochem). Protein was measured using a Pierce™ BCA protein assay kit (Thermo Fisher) and mixed 1:1 with 2x Laemmli buffer (Bio-Rad) plus 5% β-mercaptoethanol. Sixty μg of protein were loaded per well in a 4-15% acrylamide TGX gel (Bio-Rad) and run in 1x Tris-Glycine SDS buffer. Proteins were transferred to nitrocellulose, and blocked in 10% milk in TBST. Primary antibodies included anti-γ subunit (StressMarq; catalogue number SPC-405; 0.5 μg/mL), anti-β subunit (StressMarq; catalogue number SMC-241; 1 μg/mL), and anti-GAPDH (ProteinTech, 0.1 μg/mL). After incubation with appropriate HRP-linked secondary antibodies, blots were digitally analyzed a ChemiDoc Imaging System (Bio-Rad).

Body composition analysis: In vivo mouse body composition was measured by quantitative magnetic resonance using a 100H Body Composition Analyzer (EchoMRI™)(40, 43). QMR was recently shown to be effective at detecting progressive differences in body water in response to changes in dietary Na^+ and water or mineralocorticoid treatment (24). Measurements were performed in late afternoon. Tag-free, un-anesthetized, ~10 week-old mice were weighed, immediately placed in a restraint tube (EchoMRI), and inserted into the analyzer. The tube was carefully examined to ensure absence of urine or other fluid from previously measured mice. Choice of restraint tube influenced measurements, therefore the same tube was used for all measurements. Each measurement required less than 3 minutes. After measurement, each mouse was returned immediately to its cage. After all mice were measured once, the all mice were individually weighed and measured again. Data shown represent mean of two

118 measurements. Mouse weights and body water declined by 0.2 to 0.6 g between measurements (~1
119 hour apart), so mice were measured in the same order each session.

120 *Statistics:* Outliers were removed using the ROUT method (ROUT coefficient of 1%). Pair-wise
121 comparisons were performed by Student's *t*-test ($\alpha = 0.05$). For multiple, independent comparisons, *p*
122 was adjusted using Sidak's method (38). For non-independent, repeated measurements (body
123 composition over time), false discovery rates were controlled using Benjamini, Krieger, and Yekutieli's
124 two-stage step-up method (Q = 0.05), and *p* values adjusted accordingly (3). Analyses were performed
125 using Prism 8.4.2 (GraphPad). Reported errors represent standard deviation.

126 **RESULTS**

127 Immunoblots of whole kidneys and lungs from transgenic mice ($\gamma^{mt/mt}$) and littermate controls ($\gamma^{+/+}$)
128 revealed reduced γ subunit expression (Figure 2). In kidney, γ subunit (normalized to $\gamma^{+/+}$) was reduced
129 from 100 ± 10 % (N = 5) to 8 ± 4 % (N = 6; $p < 0.0001$). In lung, it was reduced from 100 ± 43 % (N = 6) to
130 16 ± 4% (N = 4; $p < 0.01$). β subunit expression was not significantly different: 100 ± 60 % (N = 5) in
131 control kidneys versus 62 ± 21 % (N = 6, p = NS) in $\gamma^{mt/mt}$ kidneys, and 100 ± 49 % (N = 6) in control lungs
132 versus 144% ± 132 % (N = 5) in $\gamma^{mt/mt}$ lungs (p = NS).

133 Because deletions of ENaC subunits exhibit early post-natal mortality, we examined litter size and
134 genotype ratios in $\gamma^{+/mt}$ crosses. (2) Litter size at weaning was 4.4 ± 1.8 for 129sv pups (25 litters) and 5.2
135 ± 1.9 for C57BL/6 pups (5 litters). These resemble previously reported wild-type litter sizes of 4.6 for
136 129sv mice and 5.5 for C57BL/6 mice (12). Analysis of litters from 23 heterozygote crosses in the 129sv
137 background revealed that live $\gamma^{mt/mt}$ pups were present at near-Mendelian ratios (29 out of 103
138 compared to 28/103 and 46/103 for $\gamma^{+/+}$ and $\gamma^{+/+}$ pups, respectively). Weights of $\gamma^{mt/mt}$ mice resembled

age- and sex-matched controls. These data indicate no significant pre- or peri-natal mortality or abnormal growth of $\gamma^{mt/mt}$ mice.

Pharmacologic or genetic inhibition of ENaC causes hyperkalemia (2, 32, 44). On a regular diet, blood K^+, Na^+, Cl^-, total CO_2 (tCO_2), urea nitrogen (BUN), and hemoglobin (Hb) from $\gamma^{mt/mt}$ mice resembled controls (Table 1 and Figure 4). Measurements in 129sv mice resembled C57B6/J mice. Blood K^+ also did not differ between genotypes on a high K^+ diet (Table 2 and Figure 5). The point-estimate for blood K^+ in $\gamma^{mt/mt}$ males exceeded that of $\gamma^{+/+}$ males, but did not reach significance (adjusted p = 0.09). Blood Na^+ was lower in $\gamma^{mt/mt}$ females than in $\gamma^{+/+}$ females (adjusted p = 0.04).

Increased blood K^+ stimulates aldosterone secretion, as does decreased blood volume. Either of these could increase aldosterone in the plasma of $\gamma^{mt/mt}$ mice. Elevated aldosterone enhances urinary K^+ excretion and would attenuate any increase in blood K^+ in $\gamma^{mt/mt}$ mice. On a regular diet, plasma aldosterone from $\gamma^{mt/mt}$ mice were similar to those of $\gamma^{+/+}$ mice ($\gamma^{+/+}$: 580 ± 720 pg/mL, N = 11; $\gamma^{mt/mt}$: 880 ± 610, N = 9, p = NS) Atrial natriuretic peptide (ANP) levels were also similar between groups (Table 1). Mean plasma aldosterone levels increased in both $\gamma^{mt/mt}$ and $\gamma^{+/+}$ mice when mice were placed on a high K^+ diet for 10 days, but they were higher in $\gamma^{mt/mt}$ mice than in controls (Figure 6 and Table 2; $\gamma^{+/+}$ 3100 ± 2500 pg/mL, N = 19; $\gamma^{mt/mt}$ 4900 ± 2500, N = 23, $p < 0.05$). This difference was not evident when males and females were analyzed separately.

We hypothesized that $\gamma^{mt/mt}$ mice would exhibit similar total body water and lean tissue mass when compared against $\gamma^{+/+}$ littermates on a regular diet, given that plasma aldosterone and ANP levels were similar. Quantitative magnetic resonance revealed no significant differences in percent body water, lean tissue mass, or fat tissue mass (Figure 7). For $\gamma^{+/+}$ mice, body water was 67.7 ± 2.7 % (N = 10; 1 female and 9 males), compared to 67.4 ± 2.6 % for $\gamma^{mt/mt}$ mice (N = 7; 2 female and 5 males; p = NS). Lean tissue

mass was 79.9 ± 2.5 % vs. 79.4 ± 3.0 % (p = NS), and fat composition was 12.0 ± 3.1 % vs. 12.8 ± 3.1 % (p = NS).

$\gamma^{mt/mt}$ mice did not have evidence of volume depletion at baseline. To evaluate whether compensatory mechanisms could be overcome by physiologic stress, animals were given a Na^+-depleted (0.01-0.02% Na^+) diet (Figure 7). Over six days, body weights decreased significantly overall ($p < 0.0001$). However, genotype-dependent differences in body weight were not observed. Similarly, normalized body water percentage decreased over this period ($P < 0.0001$). The $\gamma^{mt/mt}$ mice exhibited lower normalized body water at day 2 than controls (99.1 ± 2.1% for $\gamma^{+/+}$ mice, N = 10; 96.3 ± 2.7 % for $\gamma^{mt/mt}$ mice, N = 7; adjusted p = 0.03; Figure 7). Subsequently, differences between genotypes lost significance. Normalized lean tissue mass also decreased overall (p = 0.002). At day 2, $\gamma^{mt/mt}$ mice exhibited lower normalized lean mass than controls (100.3 ± 2.1% for $\gamma^{+/+}$ mice, N = 10; 97.1 ± 1.9 % for $\gamma^{mt/mt}$ mice, N = 7; adjusted p < 0.01). Body fat did not change in either group.

The ability of ENaC to promote renal and gastrointestinal Na^+ retention has led to the hypothesis that the channel may contribute to salt-sensitive hypertension (16, 34, 35). We therefore asked whether $\gamma^{mt/mt}$ mice were protected from fluid volume overload during an obligatory increase in Na^+ intake associated with replacement of electrolyte-free drinking water with 2% saline. Mice prefer saline to electrolyte-free water and consume more than 6 mL of 2% saline per day (19). The saline increased body weights significantly by 9 days, with no difference between groups (106 ± 6.8 %, N = 10 $\gamma^{+/+}$ mice and 107 ± 4.2 %, N = 7 $\gamma^{mt/mt}$ mice, on day 9, p = NS; Figure 7). Body water increased in $\gamma^{mt/mt}$ mice relative to controls at day two (to 101.7 ± 2.1% vs. 98.9 ± 2.6%, respectively; adjusted p = 0.03). $\gamma^{mt/mt}$ mice also exhibited higher lean tissue mass on day two (101.9 ± 1.7% vs. 99.1 ± 1.7%, respectively; p = 0.04). Body water and lean tissue masses did not differ significantly on later days. Body fat increased dramatically, but similarly, in $\gamma^{+/+}$ and $\gamma^{mt/mt}$ mice over 9 days (to 140.7 ± 30.7 % and 149.7 ± 28.3 %, respectively).

Discussion

These results demonstrate a grossly healthy mouse model with reduced expression of ENaC's γ subunit. Homozygous mice have no evidence of volume-depletion or impaired K^+ handling at baseline, but exhibit aldosterone on a high K^+ diet suggesting impaired K^+ secretion. They experience enhanced sensitivity of body fluid volume to changes in dietary Na^+, with more rapid loss of fluid and lean tissue on a low Na^+ diet. Surprisingly, a transient larger increase in fluid and lean tissue mass was observed when water was replaced with 2% saline to drink. A dramatic increase in body fat content occurs in both $\gamma^{mt/mt}$ and $\gamma^{+/+}$ mice given 2% saline. Finally, these studies illustrate the utility of quantitative magnetic resonance in assessing fluid volume in dietary and genetic mouse models.

$\gamma^{mt/mt}$ mice should prove valuable for further investigations into the physiologic role of ENaC's γ subunit, an essential protein. Floxed models allow targeted deletion in specific tissues, but depend upon available Cre-expressing mouse lines (21, 36). A β subunit hypomorph with a β subunit Liddle syndrome variant has previously been reported (29, 39). The contribution of different subunits to the physiology of various cell-types likely differs, and this mouse will facilitate investigation of the physiologic role of the γ subunit.

The $\gamma^{mt/mt}$ mice exhibit significantly higher aldosterone on a high K^+ diet, suggesting impaired K^+ secretion. In the aldosterone-sensitive distal nephron, ENaC-mediated Na^+ reabsorption is tightly linked to K^+ excretion. Pharmacologic or genetic impairment of ENaC activity reduce K^+ secretion and promote hyperkalemia (4, 6). It is therefore surprising that $\gamma^{mt/mt}$ mice exhibit blood K^+ levels similar to controls. On a high K^+ diet, the renal tubule increases K^+ secretion through pathways that are both ENaC-dependent and ENaC-independent (13). This adaptation includes up-regulation of ROMK channels in principal cells, and BK channels in intercalated cells (7, 33). The $\gamma^{mt/mt}$ mice may compensate for impaired ENaC-dependent K^+ secretion by upregulating alternative K^+ secretory pathways.

The $\gamma^{mt/mt}$ animals exhibit no evidence of body fluid depletion at baseline, as indicated by: i) aldosterone and ANP levels that are indistinguishable from controls; ii) hemoglobin levels that reveal no signs of hemoconcentration; iii) BUN levels that are not elevated; and iv) body water and lean tissue mass that do not differ from controls. These findings stand in contrast to the induced γ subunit knock-out in the kidney of adult mice, which causes weight loss, hemoconcentration, and elevated aldosterone (5). Residual ENaC activity likely contributes, but additional compensatory mechanisms are likely. A downregulation of ENaC in non-epithelial sites (see below) might also contribute.

Dietary Na^+ restriction produces a more rapid decline in body water and lean tissue in $\gamma^{mt/mt}$ mice than in controls. More surprising is the response of $\gamma^{mt/mt}$ mice given saline as drinking fluid. We predicted that impaired enteric and renal Na^+ absorption would attenuate increases in body water associated with increased Na^+ consumption. Instead, saline causes a significant, transient increase in total body fluid in $\gamma^{mt/mt}$ animals but not controls. If confirmed, these findings may point to a role for ENaC's γ subunit not only in promoting Na^+ retention in the context of dietary Na^+ depletion, but also in preventing volume-overload in the context of increased Na^+ consumption.

Such a role may depend upon the function of ENaC in tissues beyond the kidney tubule. One group observed that global β subunit suppression increases blood pressure, as measured with intravascular telemetry catheters (9). Another group found no change in blood pressure, although they measured blood pressures in anesthetized mice using physically coupled catheters (29). Increased blood pressure and our finding of increased fluid volume sensitivity to dietary saline might reflect ENaC activity in extra-epithelial tissues where the channel is expressed, such as arterial baroreceptors, vascular endothelium and myocytes, dendritic cells, or central nervous system osmosensors (8, 10, 23, 41). Providing mice with saline to drink increases Na^+ consumption, but also deprives them of electrolyte-free water. Further

studies will be required to confirm these findings and to determine whether they are the result of fluid volume overload or relative water deprivation and increased serum tonicity.

A recent report demonstrated increased lipolysis in the context of replacement of drinking water with 2% saline (20, 30). The authors observed increased fat catabolism when dietary calorie in-take was fixed. However, the authors also noted that mice given 2% saline to drink and *ad libitum* food experience weight gain (20). As our mice were also provided food *ad libitum*, our observations are consistent with the previously reported increase in body weight, and show that the increase in weight with 2% saline specifically reflects an increase in fat mass. This illustrates an important caveat relating to the conclusion that increased salt intake stimulates lipolysis. Fat break-down may only occur when increased calorie consumption is prevented.

Finally, this study illustrates the usefulness of quantitative magnetic resonance (QMR) in evaluating body fluid content in genetic mouse models. Though percent changes in total body water were small, they likely under-represent change in extracellular water, which contributes only 1/3 of total body water (15). QMR has been used extensively for evaluation of body-fat content, but a recent study showed changes in body water due water deprivation or aldosterone administration (24). The appeal of this method stems from the ability to perform repeated measures in live, unanesthetized animals. Measurements detect changes not detectable by weight alone.

The findings in this manuscript demonstrate a novel genetic tool for exploring the importance of ENaC's γ subunit *in vivo*. These findings, if confirmed, would suggest a surprising role for ENaC in salt-sensitivity, protecting against changes in body volume due to acute dietary increases in Na^+ consumption.

Acknowledgments

This work was supported by grants from the National institutes of Health, including K08 DK110332, R01 HL147818, T32 DK061296 and P30 DK079307.

Figure Legends

Figure 1. Generation of an *Scnn1g* Hypomorphic Allele. A schematic of the wild-type and transgenic *Scnn1g* locus (encoding the ENaC γ subunit) is shown in **A**. The genomic region corresponding to the targeting construct is represented as a hatched box. Open, boxes represent exons. Gray bars (with *) represent radiolabeled probes used for Southern blot. These are external to the targeting construct. Neo: neomycin-resistance cassette. γ ENaC-V5: cDNA for ENaC's γ subunit, with C-terminal V5 tag, driven in frame by the start codon of the native gene. Restriction sites used for Southern blot are marked. **B.** Homologous recombination of the 5' arm was analyzed by Southern blot of SacI-digested ES cell genomic DNA with the 5' probe (not shown). Shown is a Southern blot with the 3' probe of NdeI-digested genomic DNA of three representative clones from the 5' analysis, confirming homologous recombination of the 3' arm, which manifests as a shift in the size of the probed fragment from 8.0 to 12.6 kb**.** *+*: clones with correct homologous recombination of the 3' arm. M: KB markers.

Figure 2. $\gamma^{mt/mt}$ mice express significantly less γ subunit in kidney and lung. Immunoblots of kidney and lung tissue lysates were probed with antibodies against the β or γ subunit of ENaC, then stripped and probed with antibodies against GAPDH. Arrowheads on the left of each blot show molecular weight marker positions. Carrots on the right of each blot indicate approximate region taken for signal quantification. Dot plots show protein normalized to mean signal from $\gamma^{+/+}$ mice. Squares represent males, circles represent females. Open or shaded symbols represent data from 129sv or C57BL/6 mice, respectively. γ subunit protein levels in $\gamma^{mt/mt}$ mouse kidneys were reduced from 100 ± 10 % (mean ± SD, N = 5 mice) to 8 ± 4 % (N = 6). ***: $p < 0.0001$ (two-tailed Student's t-test). In lung, the γ subunit was

reduced from 100 ± 43 % (N = 6) to 16 ± 4% (N = 4) **: $p < 0.01$ (two-tailed Student's t-test). The kidney β subunit point estimate decreased from 100 ± 60 % (N = 6) to 62 ± 9% (N = 6), but the difference was not significant. Lung β subunit protein signal also did not differ significantly between groups: 100 ± 49 % (N = 6) for $\gamma^{+/+}$ vs. 144 ± 132 % for $\gamma^{mt/mt}$ (N = 5).

Figure 3. Pups born to $\gamma^{+/mt}$ parents exhibit genotype frequencies consistent with Mendelian inheritance and gain weight normally. Left and right plots demonstrate that $\gamma^{mt/mt}$ mice exhibit weights similar to age- and sex-matched $\gamma^{+/+}$ littermate controls. 8 to 10 week-old mice: $\gamma^{+/+}$ males weighed 26.0 ± 0.9 g, N = 5; $\gamma^{mt/mt}$ males weighed 27.1 ± 0.9 g, N = 4, p = NS. $\gamma^{+/+}$ females weighed 21.1 ± 0.4 g, N = 7; $\gamma^{mt/mt}$ females weighed 21.0 ± 0.8 g, N = 3, p = NS. 22 to 24 week-old mice: $\gamma^{+/+}$ males weighed 34.4 ± 6.1 g, N = 3; $\gamma^{mt/mt}$ males weighed 33.3 ± 3.3 g, N = 3, p = NS; $\gamma^{+/+}$ females weighed 26.7 ± 2.3 g, N = 3; $\gamma^{mt/mt}$ females weighed 25.35 ± 2.2 g, N = 4, p = NS. (Comparisons examined using two-tailed Student's t-test.) Open or shaded symbols represent data from 129sv or C57BL/6 mice, respectively.

Figure 4. Blood analytes are similar in $\gamma^{mt/mt}$ and $\gamma^{+/+}$ mice. Blood parameters from mice on a regular diet are shown. Electrolytes, urea nitrogen, hemoglobin, and creatinine were measured in whole blood. Open symbols represent mice in the 129sv background; shaded symbols represent the C57BL/6 background. There were no significant differences between $\gamma^{+/+}$ mice and $\gamma^{mt/mt}$ mice, as determined by two-tailed Student's t-test with adjustment of *p* values for multiple comparisons (two sexes) using Sidak's method. Please see Table 1 for means and standard deviations.

Figure 5. On a high K^+ diet, blood electrolyte, BUN and Hb concentrations in $\gamma^{mt/mt}$ mice largely resemble those of $\gamma^{+/+}$ mice. 129sv mice were provided with 5.2% K^+ (as KCl) diet for 10 days before blood collection. Electrolytes, urea nitrogen, hemoglobin, and creatinine were measured in whole blood. Blood Na^+ was higher in female $\gamma^{+/+}$ mice than in $\gamma^{mt/mt}$ mice (146 ± 2, N = 9 vs. 144 ± 2, N = 7; adjusted p

296 = < 0.05). There were no other significant differences between $\gamma^{+/+}$ mice and $\gamma^{mt/mt}$ mice. Groups were
297 compared using two-tailed Student's t-test with adjustment of *p* values for multiple comparisons in two
298 sexes using Sidak's method. See Table 2 for means and standard deviations.

299 **Figure 6. Plasma aldosterone levels in $\gamma^{mt/mt}$ mice are similar to controls at baseline, but are higher**
300 **than $\gamma^{+/+}$ littermate controls on a high K^+ diet.** 129sv mice were maintained on a regular (0.94% K^+) diet,
301 or transitioned to a 5.2% K^+ (as KCl) diet for 10 days. Squares represent males, circles represent females.
302 On a regular diet, there was no differences in plasma aldosterone between $\gamma^{+/+}$ mice (590 ± 720 pg/mL,
303 N = 11) and $\gamma^{mt/mt}$ mice (880 ± 610 pg/mL, N = 9). On the high K^+ diet, aldosterone increased in both
304 groups (to 3100 ± 2500 pg/mL, N = 19 in $\gamma^{+/+}$ mice, adjusted $p < 0.05$ compared to $\gamma^{+/+}$ mice on a regular
305 diet; and 4900 ± 2500 pg/mL, N = 23 in $\gamma^{mt/mt}$ mice, adjusted $p < 0.001$ compared to regular diet).
306 Aldosterone on the high K^+ diet was higher in $\gamma^{mt/mt}$ mice (* p =0.01, as determined by Student's t-test,
307 adjusted for multiple comparisons using Sidak's multiple comparisons test.) Please see Table 2 for
308 analysis by sex.

309 **Figure 7. Body composition analysis reveals no significant difference in body water, lean tissue mass,**
310 **or fat mass on a regular diet, but shows differences of body water and lean tissue mass to in response**
311 **to a low or high salt diet.** Body composition in 129sv mice was measured using quantitative magnetic
312 resonance. Circles represent female mice, squares represent males. Starting weights were similar
313 between groups. Prior to dietary manipulation, as a percentage of total body weight, there were no
314 differences in body composition. These animals were placed on a Na^+-depleted (0.01-0.02% Na^+) diet,
315 and subsequent body composition variables were normalized to baseline for each animal. Triangles and
316 diamonds represent $\gamma^{mt/mt}$ and $\gamma^{+/+}$ mice, respectively. Weight declined but did not differ between
317 genotypes. At day 2, normalized body water was lower in $\gamma^{mt/mt}$ mice than in $\gamma^{+/+}$ mice ($p = 0.03$), and
318 normalized lean tissue mass was lower in $\gamma^{mt/mt}$ mice ($p < 0.01$). Normalized fat content did not differ

between $\gamma^{+/+}$ and $\gamma^{mt/mt}$ mice. Composition did not differ on subsequent days. After a 10 day recovery on regular diet, effect of 2% saline on body composition was evaluated. 2% saline increased weights overall, but genotypes did not differ. On day 2, normalized body water and lean tissue increased in $\gamma^{mt/mt}$ mice compared to controls ($p = 0.03$ and $p = 0.04$, respectively). Thereafter, composition did not differ between genotypes. Body fat increased similarly in $\gamma^{+/+}$ and $\gamma^{mt/mt}$ mice. Baseline differences between genotypes were assessed by two-tailed t-test. Changes in body composition following dietary changes were analyzed using two-way ANOVA (†: < 0.05, †: < 0.01, †††: $p < 0.0001$ for time as a source of variation). Differences in genotypes over time were assessed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (*: $p < 0.05$. **: $p < 0.01$).

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Table 2. Blood measurements on a high K^+ diet.

	$\gamma^{+/+}$	$\gamma^{mt/mt}$	Male $\gamma^{+/+}$	Male $\gamma^{mt/mt}$	Female $\gamma^{+/+}$	Female $\gamma^{mt/mt}$
Na^+ (mmol/L)	147 ± 2 (19)	147 ± 3 (24)	147 ± 2 (10)	148 ± 2 (17)	146 ± 2 (9)	144 ± 2 (7)*
K^+ (mmol/L)	5.2 ± 0.6 (19)	5.4 ± 0.7 (24)	5.0 ± 0.7 (10)	5.5 ± 0.7 (17)	5.5 ± 0.5 (9)	5.2 ± 0.7 (7)
Cl^- (mmol/L)	119 ± 3 (19)	119 ± 3 (23)	118 ± 3 (10)	119 ± 3 (16)	119 ± 4 (9)	119 ± 4 (7)
tCO_2 (mmol/L)	20 ± 2 (19)	20 ± 2 (24)	21 ± 2 (10)	21 ± 1 (17)	20 ± 2 (9)	18 ± 2 (7)
Urea nitrogen (mg/dL)	26 ± 4 (19)	28 ± 4 (24)	28 ± 4 (10)	28 ± 4 (17)	25 ± 3 (9)	29 ± 6 (7)
Hemoglobin (mg/dL)	13.3 ± 0.9 (19)	13.5 ± 0.9 (24)	13.4 ± 0.7 (10)	13.6 ± 0.8 (17)	13.2 ± 1.1 (9)	13.4 ± 1 (7)
Creatinine (mg/dL)	< 0.2 md/dL	< 0.2 md/dL	< 0.2 md/dL	< 0.2 md/dL	< 0.2 md/dL	< 0.2 md/dL
Aldosterone (pg/mL)	3100 ± 2500 (19)	4900 ± 2500 (23)*	3100 ± 2800 (10)	4600 ± 2500 (17)	3000 ± 2200 (9)	5800 ± 2700 (6)

Animals were given high K^+ diet (5.2% K^+, as KCl) for 10 days. Electrolytes, urea nitrogen, hemoglobin, and creatinine were assayed in whole blood. Creatinine values were all below the detection threshold. Aldosterone was measured in plasma. Differences were assessed using a two-tailed Student's t-test, with adjustment of *p* values for multiple comparisons (two sexes) using Sidak's method. *: $p < 0.05$ for difference between $\gamma^{+/+}$ and $\gamma^{-/-}$.

Table 1. Blood measurements on a regular diet

	$\gamma^{+/+}$	$\gamma^{mt/mt}$	Male $\gamma^{+/+}$	Male $\gamma^{mt/mt}$	Female $\gamma^{+/+}$	Female $\gamma^{mt/mt}$
Na^+ (mmol/L)	145 ± 2 (21)	144 ± 2 (16)	145 ± 1 (13)	145 ± 2 (10)	144 ± 2 (8)	143 ± 1 (6)
K^+ (mmol/L)	4.9 ± 0.7 (20)	4.9 ± 0.6 (16)	5.0 ± 0.7 (12)	4.9 ± 0.4 (10)	4.7 ± 0.7 (8)	4.8 ± 0.8 (6)
Cl^- (mmol/L)	113 ± 2 (14)	113 ± 3 (16)	113 ± 2 (9)	113 ± 2 (10)	114 ± 2 (5)	112 ± 3 (6)
tCO_2 (mmol/L)	24.8 ± 3.3 (20)	24.5 ± 2.3 (16)	26.5 ± 3.1 (12)	25.2 ± 1.6 (10)	22.3 ± 1.7 (8)	23.3 ± 2.9 (6)
Urea Nitrogen (mg/dL)	27 ± 5 (14)	28 ± 5 (16)	28 ± 5 (9)	28 ± 5 (10)	25 ± 5.5 (5)	27 ± 6 (6)
Hemoglobin (mg/dL)	13.1 ± 0.9 (20)	13.6 ± 0.8 (16)	13.3 ± 0.5 (12)	13.5 ± 0.8 (10)	12.9 ± 1.3 (8)	13.8 ± 1.0 (6)
Creatinine (mg/dL)	< 0.2 md/dL	< 0.2 md/dL	< 0.2 md/dL	< 0.2 md/dL	< 0.2 md/dL	< 0.2 md/dL
Aldosterone (pg/mL)	590 ± 720 (11)	880 ± 610 (9)	420 ± 290 (8)	880 ± 580 (5)	1040 ± 1380 (3)	890 ± 750 (4)
ANP (pg/mL)	221 ± 114 (11)	191 ± 101 (10)	259 ± 112 (8)	227 ± 104 (6)	121 ± 11 (3)	136 ± 77 (4)

Blood parameters from mice on a regular diet are shown: mean ± standard deviation (N). Electrolytes, urea nitrogen, hemoglobin, and creatinine were assayed in whole blood. All creatinine values were below the detection threshold. Aldosterone and atrial natriuretic peptide (ANP) measurements were measured in plasma. There were no significant differences between $\gamma^{+/+}$ mice and $\gamma^{mt/mt}$ mice, either in male mice or female mice, or when mice from both sexes were pooled, as determined by two-tailed Student's t-test with adjustment of *p* values for multiple comparisons (two sexes) using Sidak's method.

Figure 1.

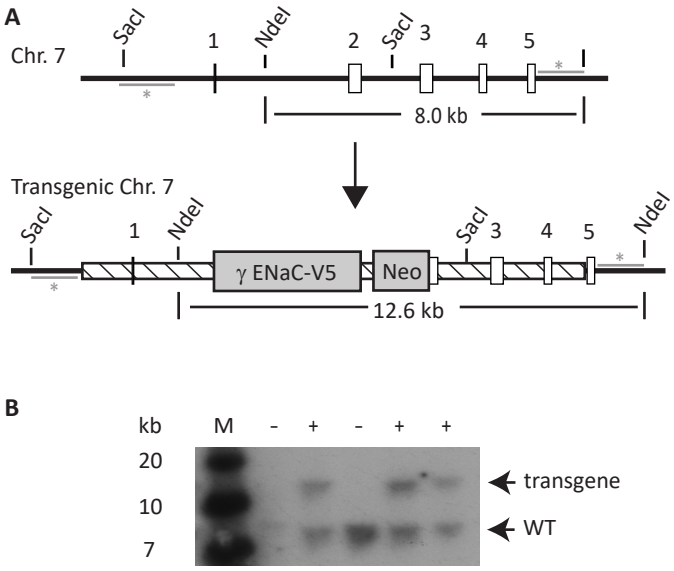


Figure 2.

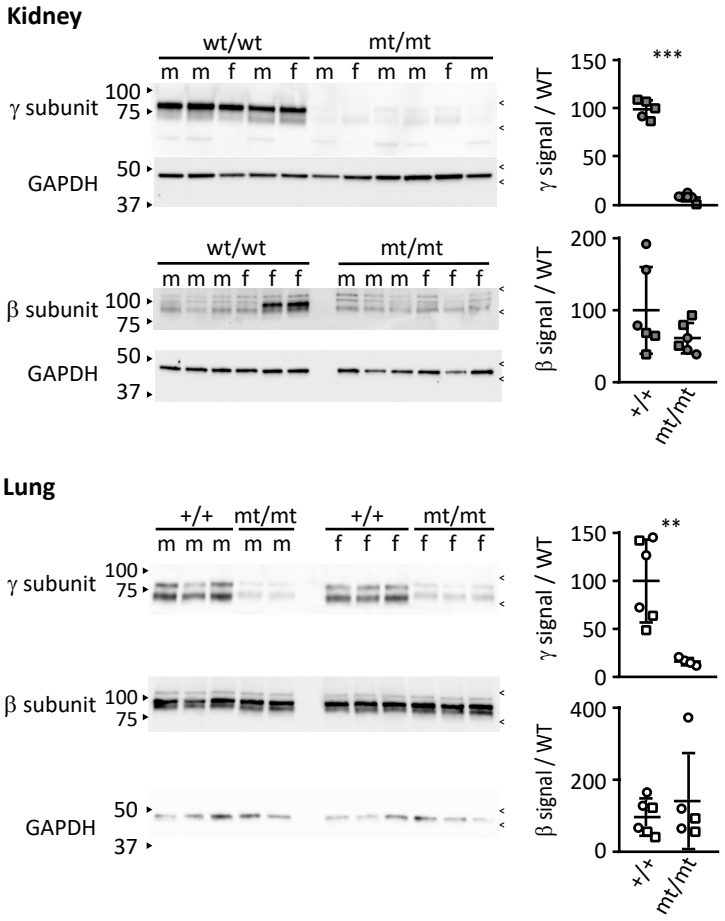
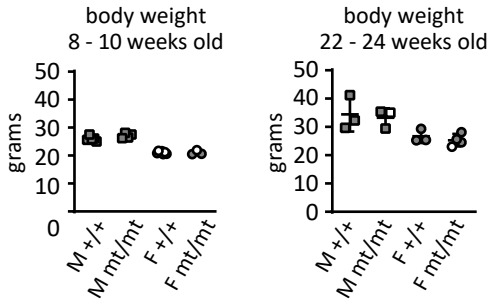


Figure 3.



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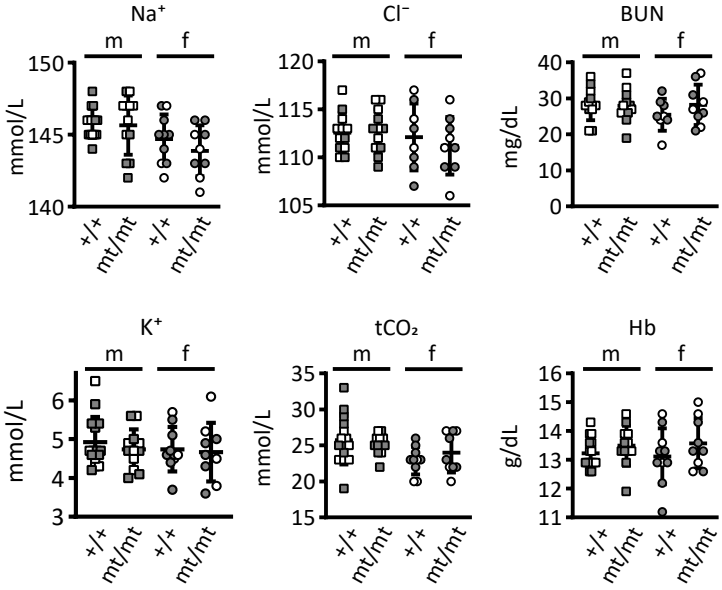

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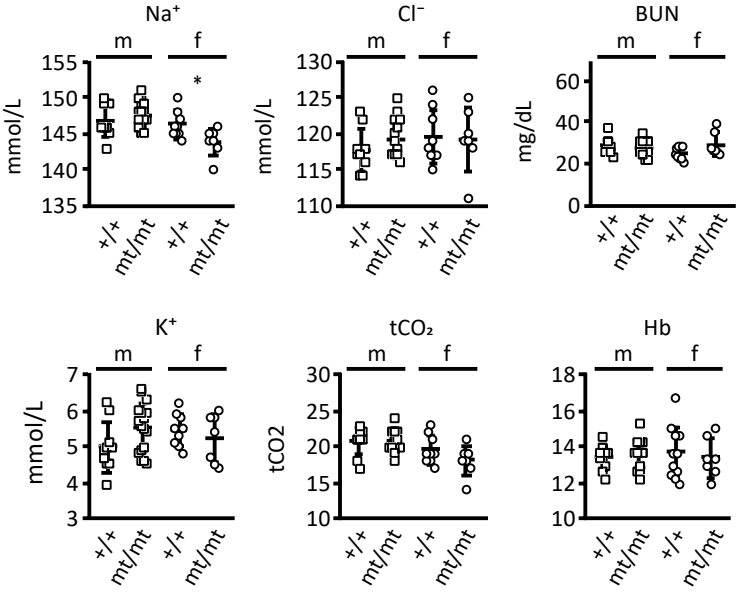

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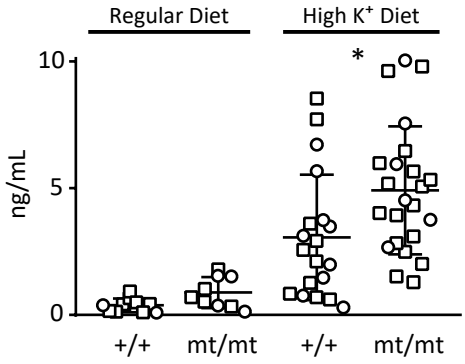

Figure 7.