ONLINE ONLY DATA SUPPLEMENT

Sexual dimorphic pattern of renal transporters and electrolyte homeostasis

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PART I: RATS

Expanded Methods

Immunofluorescence. As previously described,[1] rats were anesthetized with Inactin (110 mg/kg, i.p.) plus intramuscular ketamine (100 µl) and maintained at 37°C. Polyethylene catheters (PE-50) were inserted into the carotid artery to monitor blood pressure and into the jugular vein for infusion of 4% BSA in 0.9% saline at 50 µl/min to maintain euvolemia. The left kidney was exposed via an abdominal incision and blood pressure confirmed to remain within autoregulatory range (80–110 mmHg mean arterial pressure). At the completion of all surgical preparation and 15 min equilibration, the capsule of the left kidney was gently removed and kidney placed in small Plexiglas cup and bathed *in situ* with ice cold PLP fixative (2% paraformaldehyde, 75 mM lysine, and 10 mM Na-periodate, pH 7.4) for 5 min to avoid changes in perfusion pressure. Kidney was removed, bisected on midsagittal plane, and post fixed in PLP fixative for 2-4 hr. The fixed tissue was rinsed with PBS, cryoprotected overnight in 30% sucrose-PBS, embedded in Tissue-Tek OCT Compound, and frozen at -80°C. Male and female cryosections (5 µm) were cut and transferred to charged glass slides (male and female samples side-by-side), air dried, rehydrated in PBS for 10 min, washed 10 min in 50 mM NH_4Cl in PBS and subjected to antigen retrieval with 1% SDS in PBS for 5 min. After two 5 min washes in PBS, sections were blocked with 1% BSA in PBS to reduce background. Sections were double-labeled with polyclonal antiserum to NHE3 and monoclonal antibody to villin (both 1:100) in 1% BSA in PBS for 2 hr at room temperature. Using the same protocol, NaPi2 was detected using polyclonal antiserum to NaPi2 (1:50) and monoclonal antibody to villin (1:100). After three 5 min washes in

PBS, the sections were incubated with a mixture of Alexa 488-conjugated goat anti-rabbit and Alexa 568-conjugated goat anti-mouse secondary antibodies diluted 1:500 in 1% BSA in PBS for 1 hr, washed three times with PBS, mounted in ProLong Antifade, and dried overnight at room temperature. Samples were viewed with a ZEISS LSM 510 confocal system with differential interference contrast overlay and microscopy. Results shown in Figure 1 are representative of three sets of male and female rats examined. For analysis of villin in Figure 2B, fluorescence excitation and detector settings were the same for imaging sections from male versus female samples on the same slide.

Stationary in vivo microperfusion. As previously described,[2] male and female Sprague Dawley rats were anesthetized by intramuscular administration of Tiletamine/Zolazepam (50 mg/kg) and Xylazine (5 mg/kg). After tracheostomy, the left jugular vein was cannulated for infusion of saline with 3% mannitol (0.05 ml/min). The kidney was isolated using a lumbar approach, immobilized *in situ* using Ringer-agar in a Lucite cup under a microscope and adequately illuminated. The tubule was perfused using a double-barreled micropipette: one side was filled with Sudan black-colored castor oil, the other with the luminal perfusion solution colored with 0.05% FD & C green. The intratubular pH was measured as the voltage difference between the two asymmetric sides of the H^+ ion-sensitive microelectrode: the larger barrel contained a H^+-sensitive ion-exchange resin (Fluka Chemika, Buchs, Switzerland), and the smaller barrel contained 1 mM KCl colored by FD & C green (reference barrel). Intratubular pH changes were recorded continuously in the same tubule with a microcomputer equipped with an analog-to-digital conversion board (Lynx, São Paulo, Brazil) for data acquisition and processing. Net bicarbonate reabsorption ($JHCO_3^-$) was measured by injecting a

droplet of the luminal perfusion solution between the oil columns and following the intratubular pH changes toward the steady-state level [$(pH)_s$]. From the intratubular pH values measured along this curve and from systemic PCO_2, the intratubular concentrations of HCO_3^- were calculated at one-second intervals using the Henderson-Hasselbach equation. The rate of tubular acidification was expressed as the half-time ($t_{1/2}$) of the reduction of the injected HCO_3^- levels to their stationary level. Net HCO_3^- reabsorption ($JHCO_3^-$) per centimeter^2 was calculated from the following equation:

$$JHCO_3^- = k\,[(HCO_3^-)_i - (HCO_3^-)_s]\,r/2$$

where k is the rate constant of the reduction of luminal bicarbonate [$k = \ln 2/(t_{1/2})$], r is the tubule radius, and $(HCO_3^-)_i$ and $(HCO_3^-)_s$ are the concentrations of HCO_3^- at the injected level and at the stationary level, respectively.

Assessment of stage in estrus cycle. The cells lining the vagina of the female rat and mouse respond to the levels of circulating hormones and can provide detailed information on the estrus cycle. The classic stages of the rat estrus cycle can be designated as estrus, metestrus, diestrus and proestrus.[3] Vaginal smears were obtained by lavage as described previously.[4] Briefly, after terminal anesthesia, cells were flushed from the vaginal lining by introducing 0.25-0.3 ml of sterile saline (NaCl 0.9% w/v) into the vagina using a disposable plastic pipette and placing two drops of the resulting cell suspension onto a slide. Unstained samples were evaluated immediately under an inverted microscope with a 10X objective (Nikon TMS, Japan). Rodent estrus cycles are very sensitive to changes in the light/dark cycle, so animals were kept in a 12:12 light/dark system and vaginal smears were taken between 10 AM-12 PM in all cases.

Homogenate preparation. As described,[5] renal cortex and medulla were quickly dissected on ice, cortices were homogenized in 5 ml and medullas in 3 ml of isolation buffer [5% sorbitol, 0.5 mM disodium EDTA, and 5 mM histidine-imidazole buffer, pH = 7.5, with the addition of 0.2 mM phenylmethylsulfonyl fluoride, 9 μg/ml aprotinin, and 5 μl/ml of a phosphatase inhibitor cocktail (Sigma P2850)]. Each sample was homogenized for 5 min at a low-speed setting with an Ultra-Turrax T25 (IKA-Labortechnik) and then centrifuged at 2,000 g for 10 min. Supernatants were retained and the cortex (not medulla) pellet was rehomogenized in another 5 ml of isolation buffer, recentrifuged, and pooled with the first supernatant. The 2,000 g supernatant (So) samples were stored as single use aliquots, quick frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined using the BCA assay (Pierce Thermo Scientific, Rockford, IL).

Analytical Methods. Titratable acidity (TA) defined as the base equivalents (meq) necessary to bring the pH of the urine to 7.4, was determined using 0.1N NaOH (Sigma) and a standard pH meter. Urinary phosphate (Pi) concentration was determined with the Sigma-Aldrich Phosphate Colorimetric Kit (MAK030-1KT). Both TA and Pi were assessed in urine collected during overnight fasted rats that had free access to water. Urinary excretion rate was calculated by correcting for urine volume collected over 16 hrs.

Table S1. Rat immunoblot protocol and antibody details. ~kDa refers to apparent molecular weight determined by SDS-PAGE molecular weight markers. μg protein per lane: to ensure linearity of the detection system, 1 and 1/2 amounts of each sample were assessed. Only one amount is shown in figures, although both amounts are used to calculate the changes in abundance, as described in Methods and illustrated in Figure S1B. Ab = antibody, Mu = mouse, Rb = rabbit, Sh = sheep, Go = Goat, O/N = overnight, GAR = goat anti-rabbit, GAM = goat anti-mouse, DAS = donkey anti-sheep, SAM = sheep anti-mouse, DAR = donkey anti-rabbit, NA = Not assayed.

Antibody Target	~kDa	μg /lane cortex	μg/lane medulla	Primary Ab supplier	Ab host	Dilution	Time	Secondary Ab supplier	Host and target	Dilution	Time
ACE1	190	60, 30	NA	Bernstein (Cedars Sinai)	Rb	1:1000	O/N	Invitrogen	GAR 680	1:5000	1 hr
ACE2	92	30, 15	NA	Abcam	Rb	1:2000	O/N	Invitrogen	GAR 680	1:5000	1 hr
AQP-1	35 24	10, 5	NA	Maunsbach (Denmark)	Rb	1:1000	1 hr	Invitrogen	GAR 680	1:5000	1 hr
AQP-2	37 23	20, 10	13, 6.5	Santa Cruz (sc 9882)	Go	1:500	2 hr	Invitrogen	DAG 680	1:5000	1 hr
Claudin-2	23	30, 15	NA	ThermoFisher (#32-5600)	Mu	1:2000	O/N	GE Healthcare Life Sciences	HRP-SAM	1:5000	1 hr
Claudin-7	23	30, 15	NA	ThermoFisher (#34-9100)	Rb	1:1000	O/N	GE Healthcare Life Sciences	HRP-DAR	1:5000	1 hr
Claudin-10	21	30, 15	NA	ThermoFisher (#38-8400)	Rb	1:250	O/N	GE Healthcare Life Sciences	HRP-DAR	1:5000	1 hr
DPP IV	100	15, 7.5	NA	Farquhar (UCSD)	Rb	1:2000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
ENaC-α	100 30	80, 40	20, 10	Loffing (Zurich)	Rb	1:5000	O/N	Invitrogen	GAR 680	1:5000	1 hr
ENaC-β	100	60, 30	20, 10	Loffing (Zurich)	Rb	1:1500	O/N	Invitrogen	GAR 680	1:5000	1 hr
ENaC-γ	80 60	60, 30	20, 10	Palmer (Cornell)	Rb	1:1000	O/N	Invitrogen	GAR 680	1:5000	1 hr
HO-1	32	80, 40	40, 20	Abcam	Rb	1:2000	O/N	Invitrogen	GAR 680	1:5000	1 hr
Megalin	500	10, 5	NA	Farquhar (UCSD)	Rb	1:5000	O/N	Invitrogen	GAR 680	1:5000	1 hr
Myosin IIA	250	1.0, 0.5	NA	Biomed Tech (BT-561)	Rb	1:2000	2 hr	Invitrogen	GAR 680	1:5000	1 hr

Table S1. Continued

Antibody Target	~kDa	µg /lane cortex	µg/lane medulla	Primary Ab supplier	Ab host	Dilution	Time	Secondary Ab supplier	Host and target	Dilution	Time
Myosin VI	145	1.0, 0.5	NA	Proteus (25-6791)	Rb	1:2000	O/N	Invitrogen	GAR 680	1:5000	1 hr
NaPi2	85	60, 30	NA	Biber (Zurich)	Rb	1:3000	O/N	Invitrogen	GAR 680	1:5000	1 hr
NBCe1	130	10, 5	NA	Kurtz (UCLA)	Rb	1:5000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
NCC	150	60, 30	NA	McDonough	Rb	1:5000	O/N	Invitrogen	GAR 680	1:5000	1 hr
NCCpT53	150	60, 30	NA	Loffing (Zurich)	Rb	1:5000	O/N	Invitrogen	GAR 680	1:5000	1 hr
NCCpS71	150	20, 10	NA	Loffing (Zurich)	Rb	1:5000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
NHE3	~83	15, 7.5	8, 4	McDonough	Rb	1:2000	O/N	Invitrogen	GAR 680	1:5000	1 hr
NHE3-pS552	83	5, 2.5	8, 4	Santa Cruz (53962)	Mu	1:1000	2 hr	LI-COR	GAM 800	1:5000	1 hr
NHERF-1	50	10, 5	NA	Weinman (U. Maryland)	Rb	1:2000	3 hr	LI-COR	GAR 800	1:5000	1 hr
NKCC2	160	15, 7.5	8, 4	C. Lytle (UCR)	Mu	1:6000	O/N	LI-COR	GAM 800	1:5000	1 hr
NKCC2-pT96T101	160	15, 7.5	8, 4	Forbush (Yale)	Rb	1:2000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
Na,K-ATPase α1	100	1.0, 0.5	1.0, 0.5	Kashgarian (Yale)	Mu	1:200	2 hr	Invitrogen	GAM 680	1:5000	1 hr
Na,K-ATPase β1	50	NA	2, 1	McDonough	Rb	1:500	O/N	Invitrogen	GAR 680	1:5000	1 hr
Podocin	35-40	60, 30	NA	GeneTex	Rb	1:1000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
ROMK	50 40	60, 30	NA	Alomone (2009 batch)	Rb	1:1000	O/N	Invitrogen	GAR 680	1:5000	1 hr
SPAK	60-70	20, 10	NA	Delpire (Vanderbilt)	Rb	1:2000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
SPAK-pS373	70	80, 40	NA	DSTT, Dundee	Sh	1:4000	2 hr	Invitrogen	DAS 680	1:5000	1 hr
Villin	100	30, 15	NA	Immunotech	Mu	1 :2000	1 hr	LI-COR	GAM 800	1:5000	1 hr

A.

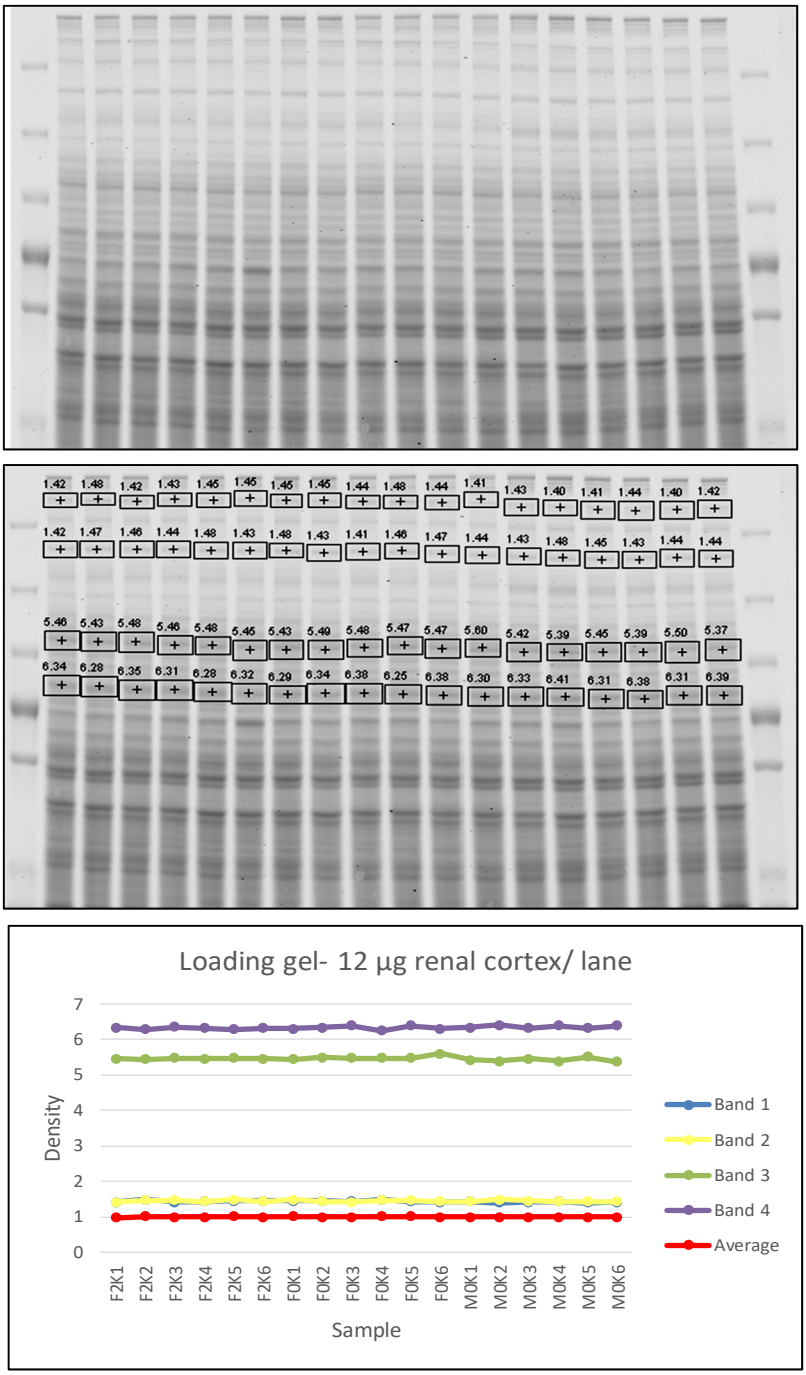

Figure S1. A) Direct protein staining of the gel was implemented to assess equivalent protein loading per lane. *Top:* Image of a Coomassie stained SDS-PAGE gel (not blot) of renal cortex homogenate samples loaded at 12 µg/lane. *Middle image* highlights 4 unidentified protein bands arbitrarily chosen for quantitation by the LI-COR Odyssey system (1 to 4 top to bottom). *Bottom:* summary display of the density of each of the 4 arbitrary bands (color coded) in each sample as well as the average of the 4 bands (red). The sample to sample variation was less than 5%, viewed as acceptable.

B.

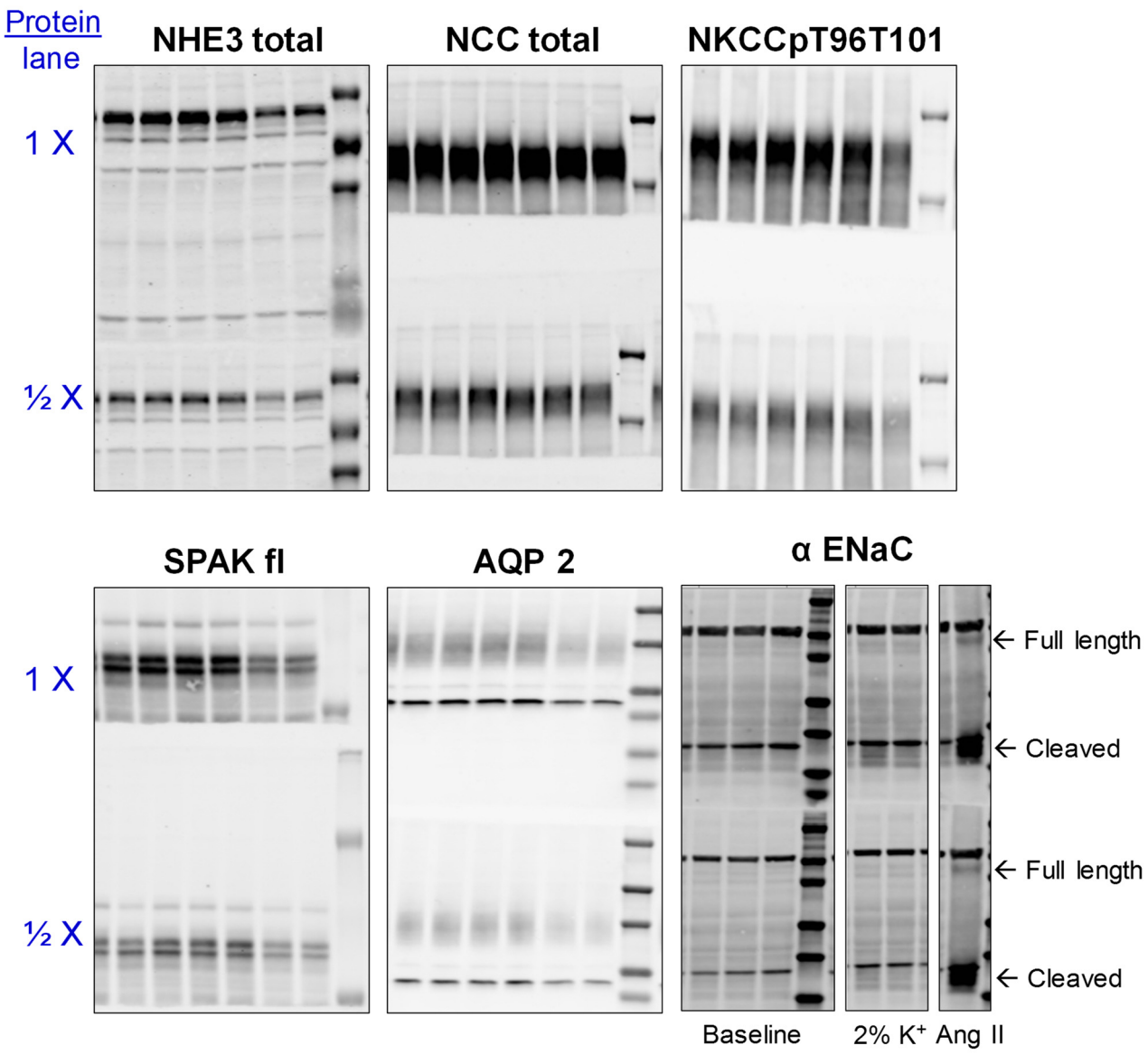

Figure S1. B) Linearity of the detection system in rat samples. Illustration of the layout of the quantitative immunodetection system: 2 gels of samples loaded at 1X on one gel and ½X on another gel are placed on one piece of blot matrix for transfer and subsequent antibody incubations and quantitation. Shown are the molecular weight lanes and adjacent baseline samples; ENaC subunit panels include AngII and high K^+ diet treated samples (which both increase ENaC abundance and activity) on the same blots because the baseline samples have low baseline abundance. The bands illustrate that loading ½ reduced the signal proportionately. See Table S1 for mobility, amounts loaded per lane and antibodies used.

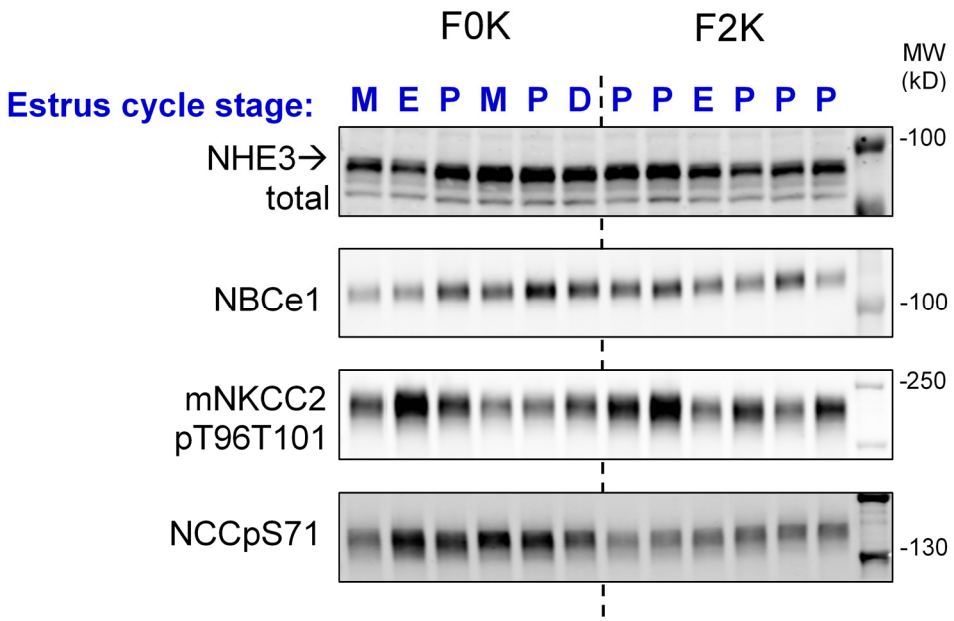

Figure S2. Stages of estrus cycle and renal expression of proteins in female rats. During terminal anesthesia, vaginal smears were obtained by lavage and stage of estrus cycle evaluated by microscopy. The full estrus cycle in rats, occurs over 4 or 5 days and can be divided into four stages: P= proestrus, E= estrus, M= metestrus, D= diestrus. Vaginal smears were taken between 10 AM -12 PM in all cases.
Abundance of renal cortical NHE3 total, NBCe1, NCCpS71 and medullary NKCC2pT96T101 support the conclusion that there is no correlation between the stage of the estrus cycle and these proteins abundance or activity.

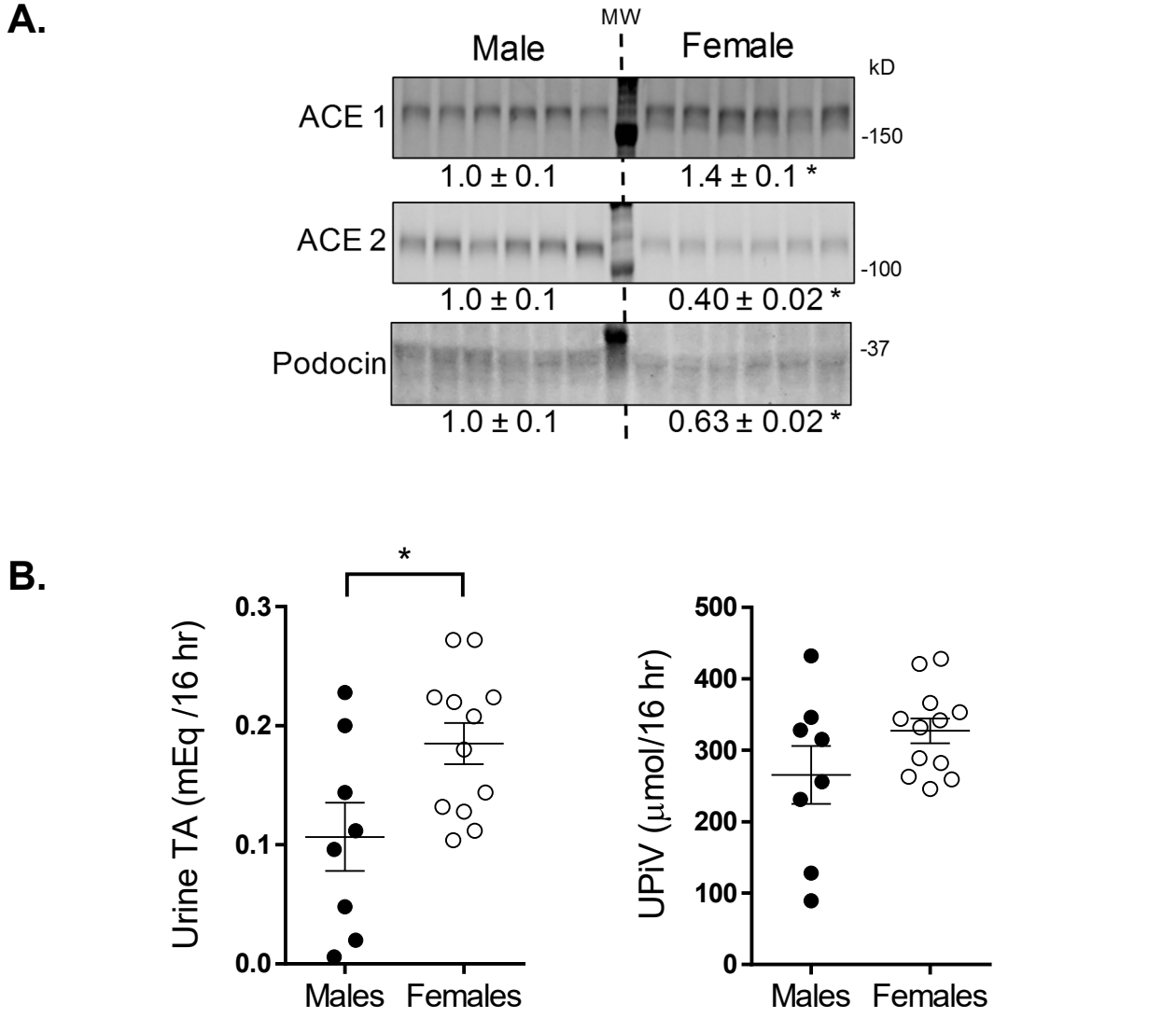

Figure S3. A. Abundance of renal Angiotensin-converting enzyme (ACE), Angiotensin-converting enzyme 2 (ACE2) and podocin in female vs. male rats. Immunoblots of renal cortical homogenates. Both 1 and ½ amounts of protein were loaded (only one amount is displayed, see antibody table S1 for details). Density values, normalized to Male group = 1.0, displayed as means ± SEM (n=6). ★=P<0.05 vs. Male.

Figure S3 B. Excretion of urinary titratable acidity and phosphate in female vs. males was determined in urine collected overnight in fasted rats with free access to water. Urine TA = urinary titratable acidity excreted over 16 hr collection, UPiV = urinary phosphate excretion over the 16 hr collection. Values represent individual measurements and mean ± SEM (n=8-12). *P<0.05 vs. Males

PART II: MICE

Methods

Animals and metabolic protocols

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Keck School of Medicine of the University of Southern California and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Experiments were performed on male and female C57BL/6 mice (16 wk old) purchased from Jackson Laboratories (Bar Harbor, ME). Mice were weighed and placed in metabolic cages for acclimation at 11:00 AM with free access to food and water. At 3:00 PM the following day, food was removed. Fasting overnight urine was collected from 3:00 PM to 7:00 AM (16 hr). From 7:00 AM to 10:00 AM mice had free access to either 0% K diet or 2% K diet, during which time urine was collected. Following the meal, mice were anesthetized as described above and a blood sample was collected from the cheek (to avoid hemolysis) for plasma $[K^+]$ and $[Na^+]$ assessment. Urine was collected from the bladder and pooled with 3 hr urine from metabolic cage. Kidneys were rapidly removed and dissected into cortex and medulla for immediate homogenization as described for the rat homogenization.

Body fluid measurements

Urine volumes, urinary and plasma $[Na^+]$, $[K^+]$ and $[Li^+]$, and osmolality were measured as described for rats. During terminal anesthesia, vaginal smears were obtained by lavage introducing 0.1-0.2 ml of sterile saline and stage of estrus cycle evaluated by microscopy.

Homogenate preparation

As previously described for rats, renal cortex and medulla were quickly dissected on ice, both homogenized in 1 ml of isolation buffer plus inhibitors for 5 min at a low-speed setting and then centrifuged at 2,000 g for 10 min. Supernatants were retained and the cortex (not medulla) pellet was rehomogenized in another 1 ml of isolation buffer, re-centrifuged, and pooled with the first supernatant. Samples were quick frozen as single use aliquots in liquid N_2 and stored at -80°C. Protein concentrations were determined as described above.

Saline challenge

A saline challenge was performed in a set of unfasted male and female C57BL/6 mice (16 wk old, n=4-5). Mice were acclimated to the metabolic cages for 24 hr with free access to water and control gelled diet to assure hydration. At 11:00 AM mice were acutely anesthetized with isoflurane, injected i.p. with a volume 0.9% w/v NaCl (warmed to 37°C) equivalent to 10% of their body weight and returned immediately to metabolic cages without food (they quickly woke up). Urine was collected hourly for 5 hr. The same protocol was applied to Cld-2 KO male and female mice with the exception that urine was pooled throughout the 5 hr collection. Urine volumes and Na^+ concentration were measured as described for rats. Excretion results were expressed as the percentage of the Na^+ and volume load injected.

Table S2. Mouse immunoblot protocol and antibody details. ~kDa refers to apparent molecular weight determined by SDS-PAGE molecular weight markers. μg protein per lane: to ensure linearity of the detection system, 1 and 1/2 amounts of each sample were assessed. Only one amount is shown in figures, although both amounts are used to calculate the changes in abundance, as described in Methods and illustrated in Figure S1B. Ab = antibody, Mu = mouse, Rb = rabbit, Sh = sheep, Go = Goat, O/N = overnight, GAR = goat anti-rabbit, GAM = goat anti-mouse, DAS = donkey anti-sheep, SAM = sheep anti-mouse, DAR = donkey anti-rabbit, NA = Not assayed.

Antibody Target	~kDa	μg /lane cortex	μg/lane medulla	Primary Ab supplier	Ab host	Dilution	Time	Secondary Ab supplier	Host and target	Dilution	Time
AQP-1	35 24	10, 5	NA	Maunsbach (Denmark)	Rb	1:1000	1 hr	Invitrogen	GAR 680	1:5000	1 hr
AQP-2	37 23	20, 10	10, 5	Santa Cruz (sc 9882)	Go	1:500	2 hr	Invitrogen	DAG 680	1:5000	1 hr
Claudin-2	23	22.5, 11.3	NA	ThermoFisher (#32-5600)	Mu	1:2000	O/N	GE Healthcare Life Sciences	HRP-SAM	1:5000	1 hr
Claudin-7	23	22.5, 11.3	NA	ThermoFisher #34-9100)	Rb	1:1000	O/N	GE Healthcare Life Sciences	HRP-DAR	1:5000	1 hr
ENaC-α	100 30	40, 20	15, 7.5	Loffing (Zurich)	Rb	1:5000	O/N	Invitrogen	GAR 680	1:5000	1 hr
ENaC-β	100	40, 20	15, 7.5	Loffing (Zurich)	Rb	1:1500	O/N	Invitrogen	GAR 680	1:5000	1 hr
ENaC-γ	80 60	50, 25	15, 7.5	Loffing (Zurich	Rb	1:1000	O/N	Invitrogen	GAR 680	1:5000	1 hr
Megalin	500	10, 5	NA	Farquhar	Rb	1:5000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
Myosin IIA	250	5, 2.5	NA	Biomedical Tech (BT-561)	Rb	1:2000	O/N	Invitrogen	GAR 680	1:5000	1 hr
Myosin VI	145	5, 2.5	NA	Proteus (25-6791)	Rb	1:2000	O/N	Invitrogen	GAR 680	1:5000	1 hr
NaPi2	85	40, 20	NA	McDonough	Rb	1:1000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
NBCe1	130	5, 2.5	NA	Kurtz (UCLA)	Rb	1:5000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
NCC	150	40, 20	NA	McDonough	Rb	1:5000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
NCCpT53	150	40, 20	NA	Loffing (Zurich)	Rb	1:5000	O/N	Invitrogen	GAR 680	1:5000	1 hr
NCCpS71	150	40, 20	NA	Loffing (Zurich)	Rb	1:5000	2 hr	Invitrogen	GAR 680	1:5000	1 hr

Table S2. continued

Antibody Target	~kDa	µg /lane cortex	µg/lane medulla	Primary Ab supplier	Ab host	Dilution	Time	Secondary Ab supplier	Host and target	Dilution	Time
NHE3	83	40, 20	10, 5	McDonough	Rb	1:2000	O/N	Invitrogen	GAR 680	1:5000	1 hr
NHE3-pS552	83	5, 2.5	10, 5	Santa Cruz (53962)	Mu	1:1000	2 hr	LI-COR	GAM 800	1:5000	1 hr
NHERF-1	50	20, 10	NA	Weinman (U.Maryland)	Rb	1:2000	O/N	Invitrogen	GAR 680	1:5000	1 hr
NKCC2	160	20, 10	10, 5	C. Lytle (UCR)	Mu	1:6000	O/N	LI-COR	GAM 800	1:5000	1 hr
NKCC2-pT96T101	160	20, 10	10, 5	Forbush (Yale)	Rb	1:2000	2 hr	Invitrogen	GAR 680	1:5000	1 hr
Na,K-ATPase α1	100	5, 2.5	2, 1	Kashgarian (Yale)	Mu	1:200	2 hr	Invitrogen	GAM 680	1:5000	1 hr
SPAK	60-70	20, 10	NA	Delpire (Vanderbilt)	Rb	1:2000	O/N	Invitrogen	GAR 680	1:5000	1 hr
SPAK-pS373	70	40, 20	NA	DSTT, Dundee	Sh	1:4000	2 hr	Invitrogen	DAS 680	1:5000	1 hr
Villin	100	20, 10	NA	Immunotech	Mu	1 :2000	1 hr	LI-COR	GAM 800	1:5000	1 hr

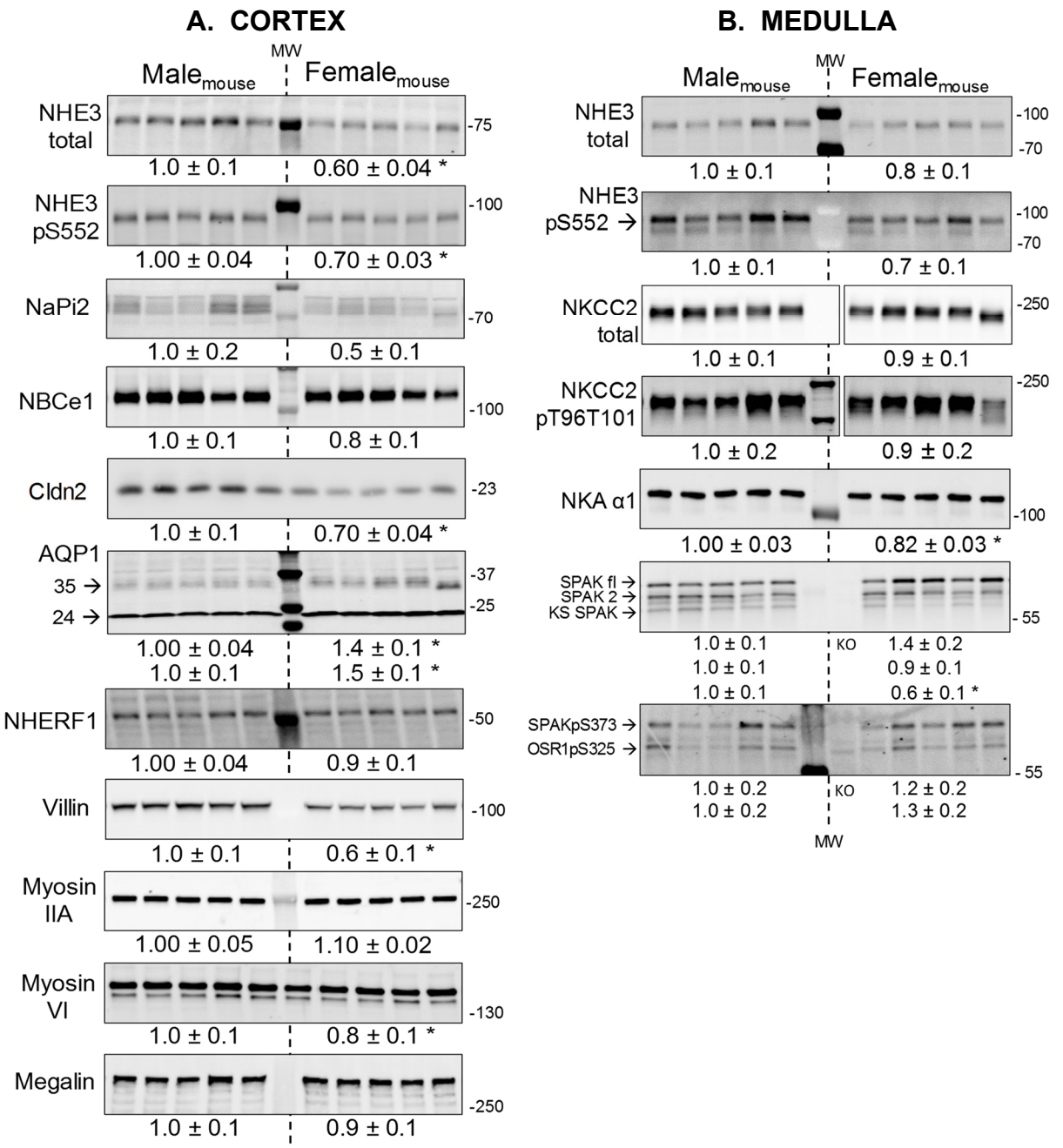

Figure S4. **Sexual dimorphic expression of renal proximal nephron proteins in female vs. male C57BL/6 mice.** Immunoblots of renal homogenates. Both 1 and ½ amounts of protein were loaded (only one amount is displayed, see antibody table S2 for details). Density values, normalized to $Male_{mouse}$ group=1.0, displayed as means ± SEM (n=5). ✱=P<0.05 vs. $Male_{mouse}$. **A)** Renal cortex samples, **B)** Renal medulla samples. *Abbreviations:* NHE3: Na^+/H^+ exchanger isoform 3; NHE3pS552: NHE3 phosphorylated at Ser 552; NaPi2: Na^+/Pi cotransporter isoform 2; NBCe1: $Na^+/HCO3^-$cotransporter 1; Cldn2: claudin-2; AQP1-35, -24: aquaporin 1- 35 and -24 kD forms; NHERF 1: NHE regulatory factor 1; NKCC2: Na^+-K^+-$2Cl^-$ cotransporter; NKCC2pT96T101: NKCC2 phosphorylated at Thr 96 and 101; SPAK: STE20/SPS1-related proline alanine-rich kinase; fl=full length; KO=$SPAK^{-/-}$; NKA: Na^+,K^+-ATPase α1 subunit

C

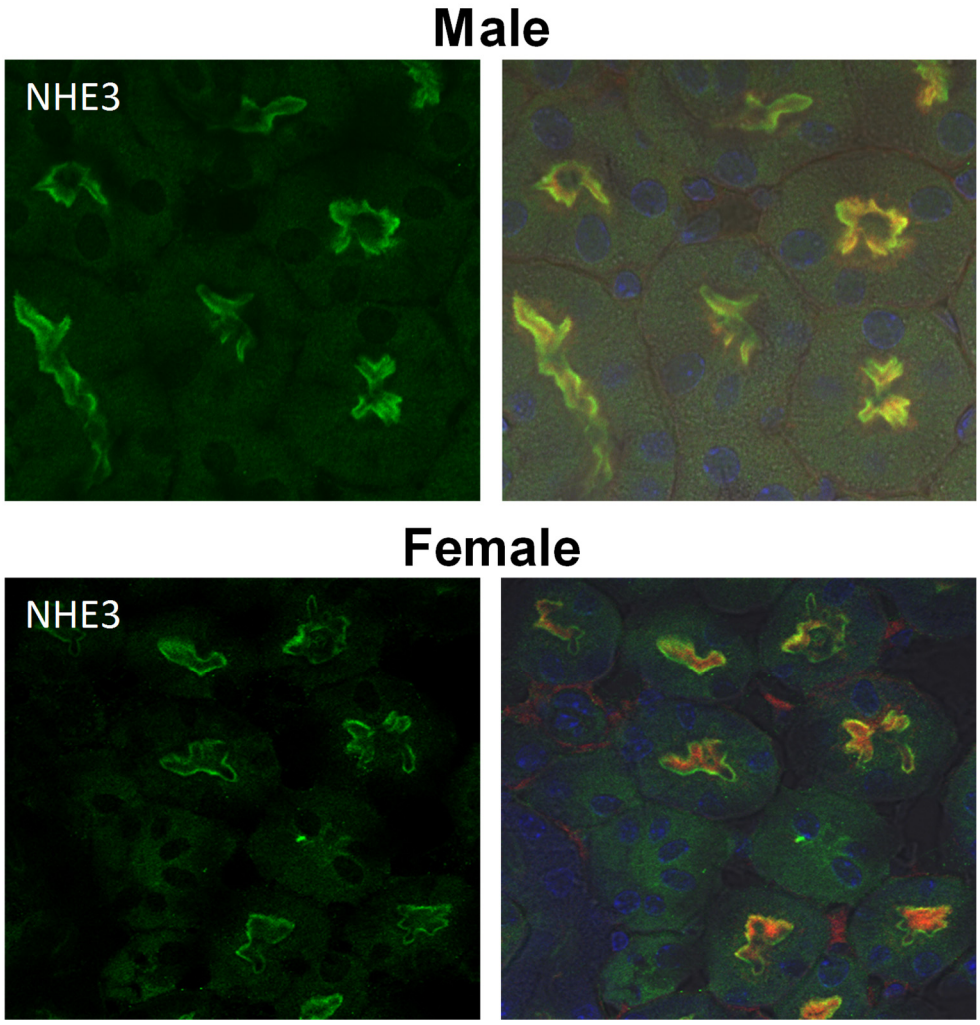

Figure S4 continued. **C)** NHE3 preferentially distributed to the base of the microvilli in females. Indirect immunofluorescence microscopy of the Na^+/H^+ exchanger isoform 3 (NHE3) distribution and abundance in kidney samples from untreated male and female C57BL/6 mice processed side by side on the same slide; two different sets of mouse kidneys were analyzed with similar results. NHE3 detected with polyclonal anti-NHE3 (1:100) detected with the secondary antibody AlexaFluor 488 (green) and microvilli identified using anti-villin (1:100) detected with the secondary antibody AlexaFluor 568 (red). In males, the microvilli are primarily yellow, indicating co-localization of NHE3 with villin in the body of the villi, while in the females, relatively more NHE3 is located at the base of the microvilli, revealing more red (villin) stained microvilli.

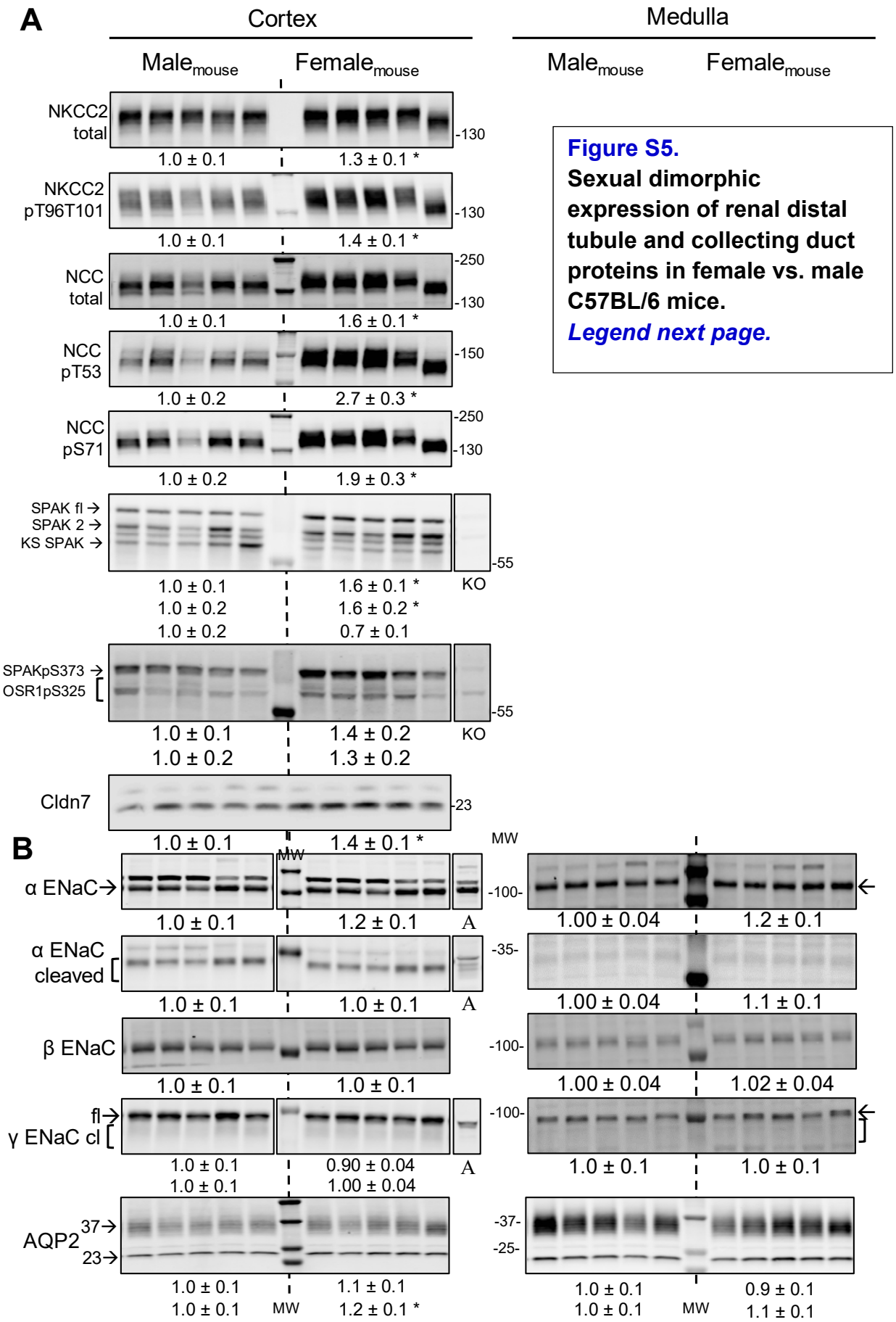

Figure S5.
Sexual dimorphic expression of renal distal tubule and collecting duct proteins in female vs. male C57BL/6 mice.
Legend next page.

Figure S5. **Sexual dimorphic expression of renal distal tubule and collecting duct proteins in female vs. male C57BL/6 mice.** Immunoblots of renal homogenates. Both 1 and ½ amounts of protein were loaded (only one amount is displayed, see antibody table S2 for details). Density values, normalized to $Male_{mouse}$ group= 1.0, displayed as means ± SEM (n=5). ✱=*P*<0.05 vs. $Male_{mouse}$. **A)** Renal cortex samples probed for thick ascending limb and distal tubule proteins. **B)** Renal cortex and medulla samples probed for collecting duct channels; A indicates samples from AngII infused rats, which express high levels of activated ENaC subunits. *Abbreviations:* NKCC2: Na^+-K^+-$2Cl^-$ cotransporter; NKCC2pT96T101: NKCC2 phosphorylated at Thr 96 and 101; NCC: Na^+-Cl^- cotransporter; NCCpT53 and NCCpS71: NCCp at Thr 53 and Ser 71, respectively; SPAK: STE20/SPS1-related proline alanine-rich kinase; fl=full length, KS SPAK: kidney specific SPAK; KO=$SPAK^{-/-}$; Cldn7: claudin-7; ENaC: epithelial Na^+ channel; AQP2-23 -37: aquaporin 2-23 and -37 kD forms; ROMK: renal outer medullary K^+ channel partial glycosylated (pg) and fully glycosylated (fg) forms.

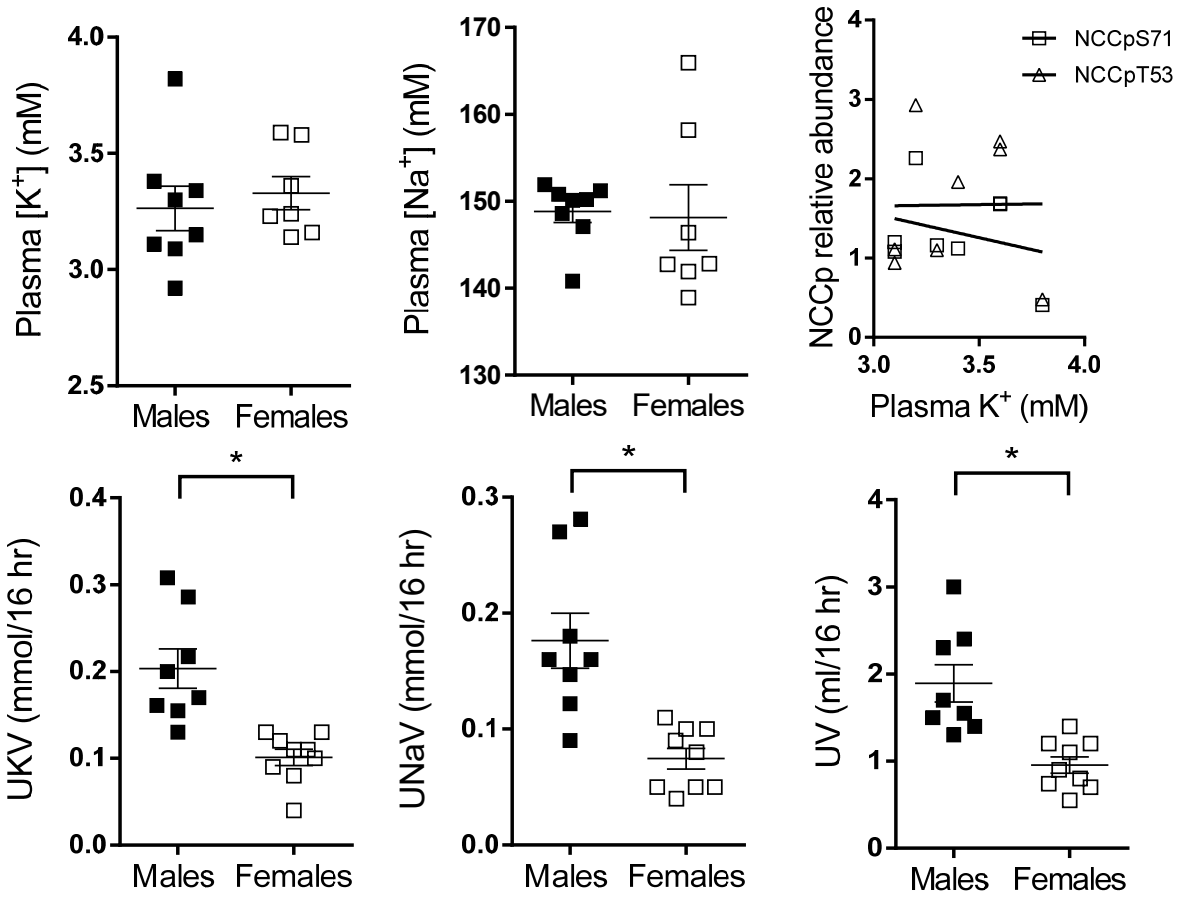

Figure S6. Electrolytes in plasma and urine measured after overnight fast plus 3 hr 0% K^+ meal. Male and female C57BL/6 mice were fasted overnight then fed a meal containing 0% K^+ for 3 hr. Overnight and during feeding, urine was collected in metabolic cages. At 3 hr, plasma was collected. UKV=urinary K^+ excretion, UNaV=urinary Na^+ excretion. Values represent individual measurements and mean ± SEM (n=4-9). **P*<0.05 vs. Males. **Upper right panel** summarizes NCCpT53 and NCCpS71 abundance from Figure S4 plotted against plasma [K^+], combining both males and females and indicates no inverse relationship between plasma [K^+] and NCCp at baseline in mice (r<0.08).

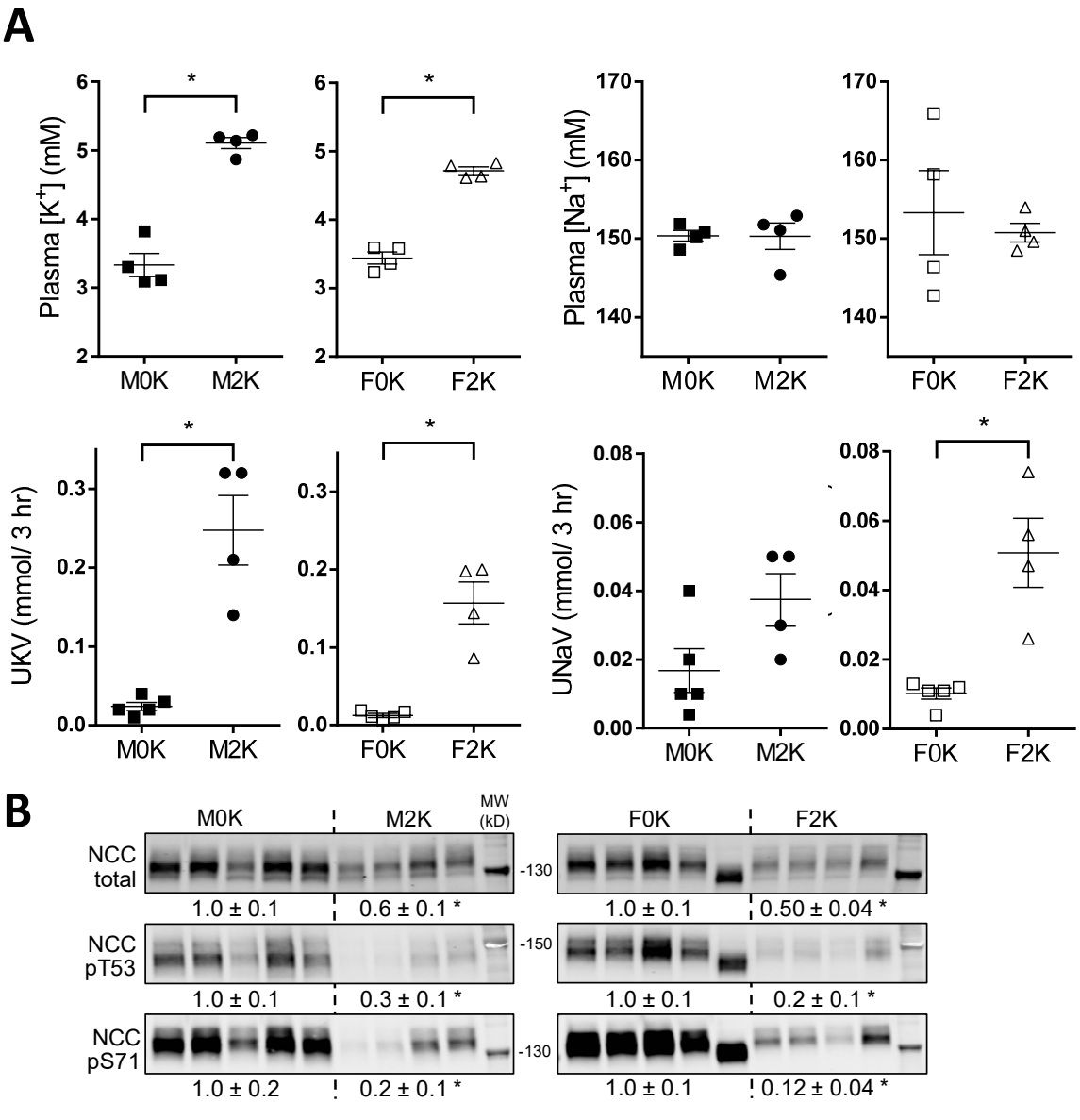

Figure S7. Effects of a 3 hr 2% vs. 0% K^+ meal in male vs. female mice. After overnight fast, mice were fed either 2%K or 0%K 3 hr meal, during which urine was collected. Blood and kidneys were collected at 3 hr. **A)** Plasma [K^+], UKV (urinary K^+ excretion) and UNaV (urinary Na^+ excretion) increase after K^+ rich meal. **B)** NCC and NCCp abundance decrease in response to K^+ rich meal. Density values, normalized to 0K=1.0, displayed as means ± SEM (n=4-5). ★=*P*<0.05 vs. 0K group. **C)** Food intake during 3 hr meal.

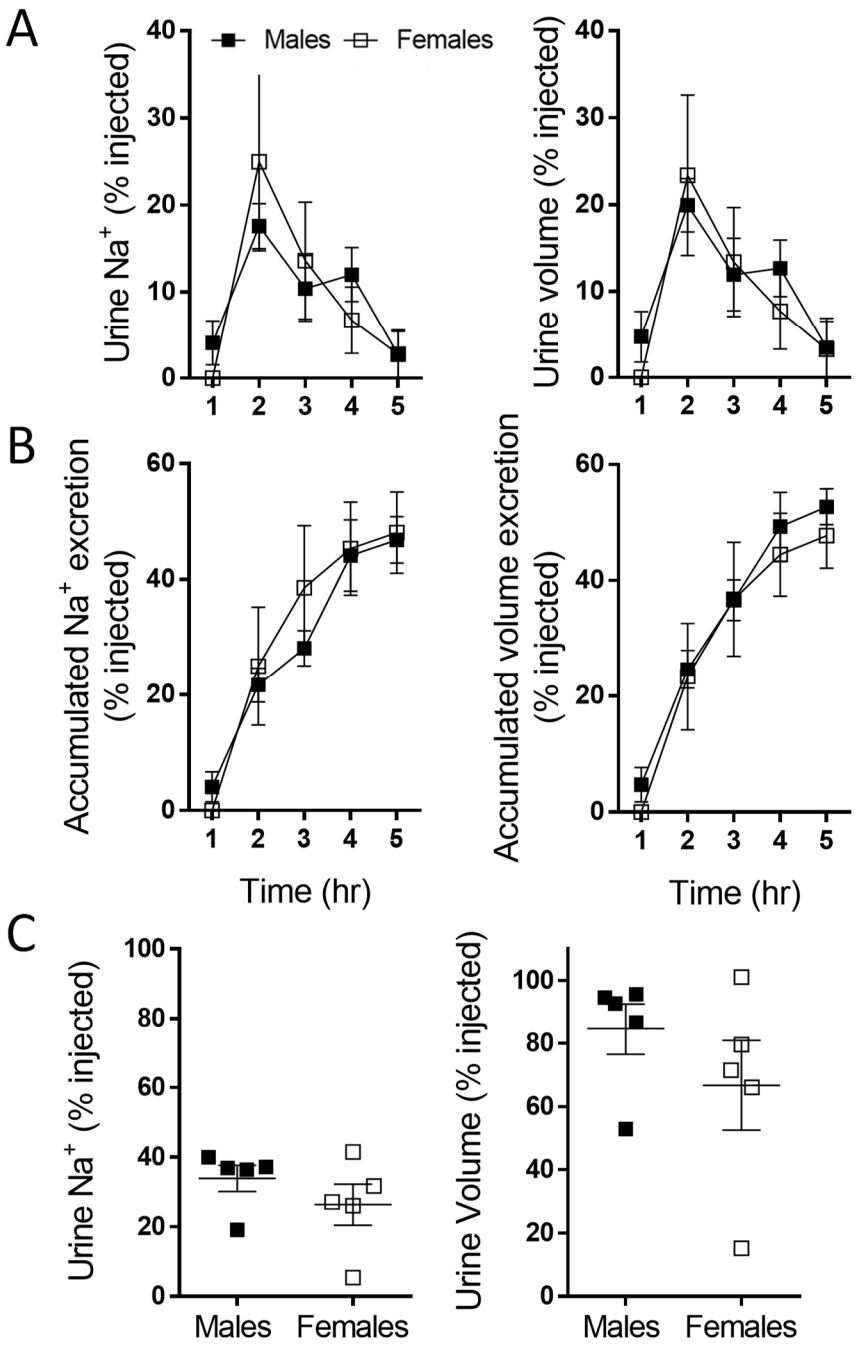

Figure S8. Diuretic and natriuretic responses to a saline load are indistinguishable in male vs. female wild type or Cld-2$^{-/-}$ mice.

Wild type C57BL/6 mice were challenged with an i.p. bolus of warmed saline equivalent to 10% of their body weight and placed in metabolic cages for urine collection.

A) Fraction of injected Na^+ and volume excreted over 5-hour collection period.

B) Accumulated excretion of Na^+ and volume.

C) Same protocol applied to Cld-2$^{-/-}$ mice. Results expressed as fraction of injected Na^+ and volume excreted at the end of the 5-hour collection period.

Data are expressed as mean ± SEM, n=4-5 per group.

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