# SUPPLEMENTAL MATERIAL

## Vasopressin increases Urinary Acidification via V1a Receptor of Collecting Duct Intercalated Cells

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**SUPPLEMENTAL MOVIE LEGEND**

Subcellular distribution of V1aR in perfusion-fixed mouse kidney; immunofluorescence staining. (A, B) 3D reconstruction of structured illumination microscopy (3D-SIM) images show V1aR (green) in an A-IC (A) with signal distribution along the basolateral membrane and a B-IC (B) with subapical accumulation of V1aR immunoreactivity.

**SUPPLEMENTAL MOVIE**

Please refer to the video file: Giesecke et al. V1aR_SuppMov.avi

## SUPPLEMENTAL FIGURE LEGENDS

**Supplemental Figure 1.** Overview of V1aR distribution in mouse kidney. (A) Triple labeling for V1aR, aquaporin 2 (AQP2) and pendrin reveals basolateral V1aR distribution in macula densa cells and AQP2- and pendrin-negative type A intercalated cells, whereas pendrin-positive type B intercalated cells show intracellular to subapical V1aR distribution; kidney zones are specified (Cortex, OSOM – outer stripe of outer medulla, ISOM – inner stripe of outer medulla, IM – inner medulla).

**Supplemental Figure 2.** Distribution of V1aR in macula densa cells of rodent and human kidneys. (A, B) Immunoperoxidase staining of perfusion-fixed mouse kidney shows basolateral V1aR signal in macula densa cells (between black bars in A). Signal is absent when primary antibody was omitted for a negative control (B; neg. ctrl.); bright field microscopy, differential interference contrast optics. (C, D) Immunofluorescence labeling of V1aR shows strong basolateral signal in macula densa cells of wild type mouse kidney (C; WT), but no significant labeling of V1aR-knockout macula densa cells (D; V1aR-KO); immunofluorescence combined with bright field microscopy. (E, F) Immunofluorescence staining of perfusion-fixed rat (E) and immersion-fixed human kidneys (F) show no V1aR immunoreactivity in macula densa cells (between bars; Gl - glomerulus). Nuclear counterstaining with DAPI (blue).

**Supplemental Figure 3.** V1aR distribution in renal vasculature of mouse kidney. (A-C) Representative confocal images showing an artery in a mouse kidney section double-labeled for V1aR (green signal) and PECAM1 (CD31; red signal). (D) Representative confocal image of rat kidney inner medulla showing double labeling for V1aR (green signal) and aquaporin 2 (AQP2; magenta signal) with V1aR-positive vasa recta (arrows) and AQP2-positive collecting ducts. Nuclear counterstaining with DAPI (blue).

**Supplemental Figure 4.** Examples of microdissected nephron segments. (A) Overview of unsorted glomeruli and renal tubules. (B-F) Isolated glomeruli (Glom.), proximal tubules (PT),

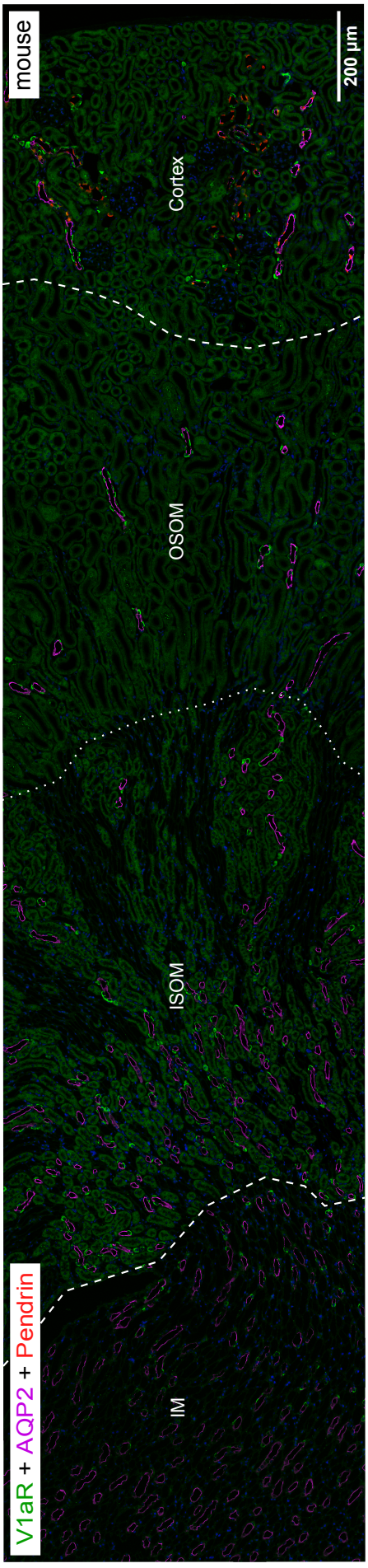
thick ascending limbs (TAL), distal convoluted tubules (DCT) and connecting tubules with collecting ducts (CNT/CD), sorted according to morphological criteria.

**Supplemental Figure 5.** V1aR distribution in kidneys of Brattleboro rats and effects of the V1aR agonist. (A, B) Representative confocal microscopic images of kidneys from AVP-deficient Brattleboro rats, labeled for V1aR (green signal), pendrin (red signal) and AQP2 (magenta signal). Images show basolateral V1aR signal in AQP2- and pendrin-negative A-ICs (A; open arrows) vs. diffused cytoplasmic V1aR signal in AQP2-negative, pendrin-expressing B-ICs (B; closed arrow). (C, D) Higher magnifications of inserts (A and B) demonstrating basolateral V1aR distribution in A-ICs (C) vs. perinuclear/apical V1aR distribution in B-ICs (D); note the absence of V1aR signal in the luminal membrane of B-ICs (A, C). Nuclei are counterstained with DAPI (blue signal). (E) Effects of V1aR agonist (A0-4-7, 2 $\mu$g/kg body weight for 2h; n=7) vs. vehicle (n=5) on plasma pH and $HCO_3^-$ levels in Brattleboro rats. Data are means ± SEM; $^{**}P<0.01$ vs. vehicle.

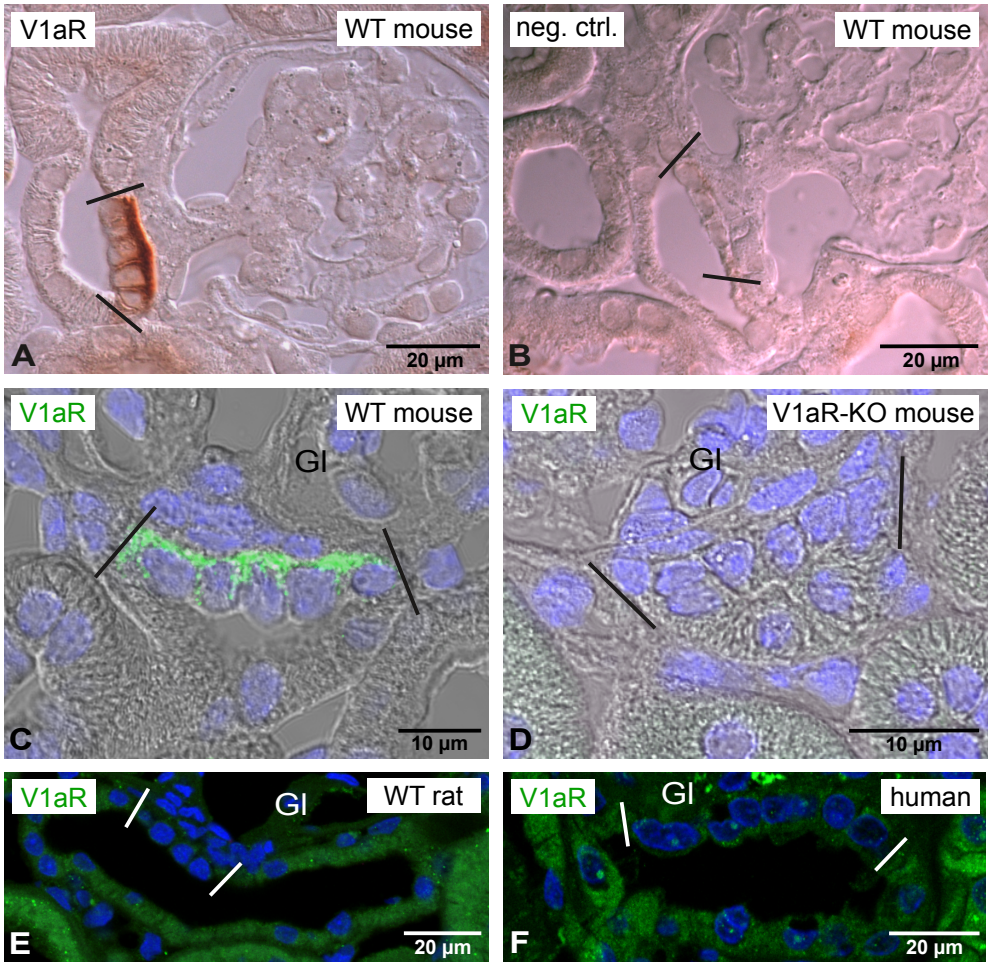
**Supplemental Figure 6.** Verification of metabolic acidosis in mice fed with a $NH_4Cl$ enriched diet. (A-H) Plasma pH, $K^+$, $Cl^-$ and $HCO_3^-$ levels in control mice receiving regular diet (ctrl) vs. mice fed with $NH_4Cl$ enriched ($NH_4Cl$) diet for physiological experiments (A-D) or morphological evaluation (E-H). Decreased plasma pH and $HCO_3^-$ levels along with increased $Cl^-$ levels confirmed induction of metabolic acidosis.

**Supplemental Figure 7.** Effects of metabolic acidosis on cellular V1aR distribution. (A, B) Representative confocal microscopic images of kidneys from mice fed with regular diet (Control) vs. acid-loaded mice ($NH_4Cl$, 3d) show similar V1aR distribution patterns.

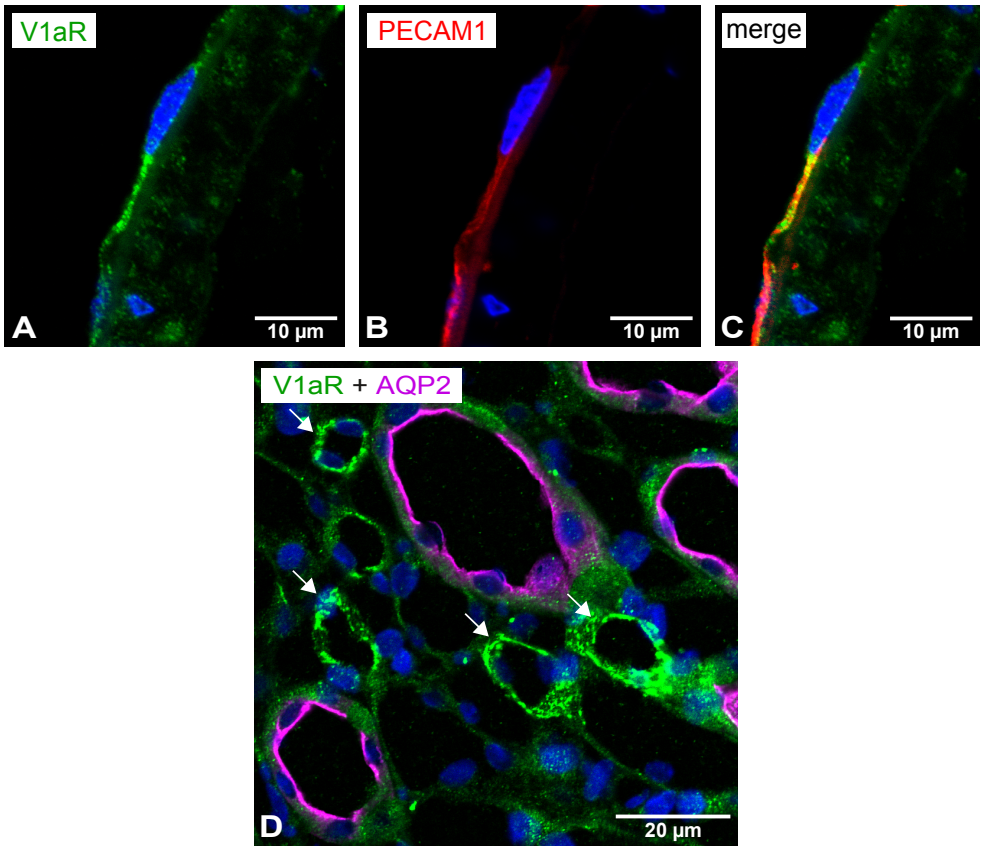
# Supplemental Figure 1



# Supplemental Figure 2



# Supplemental Figure 3



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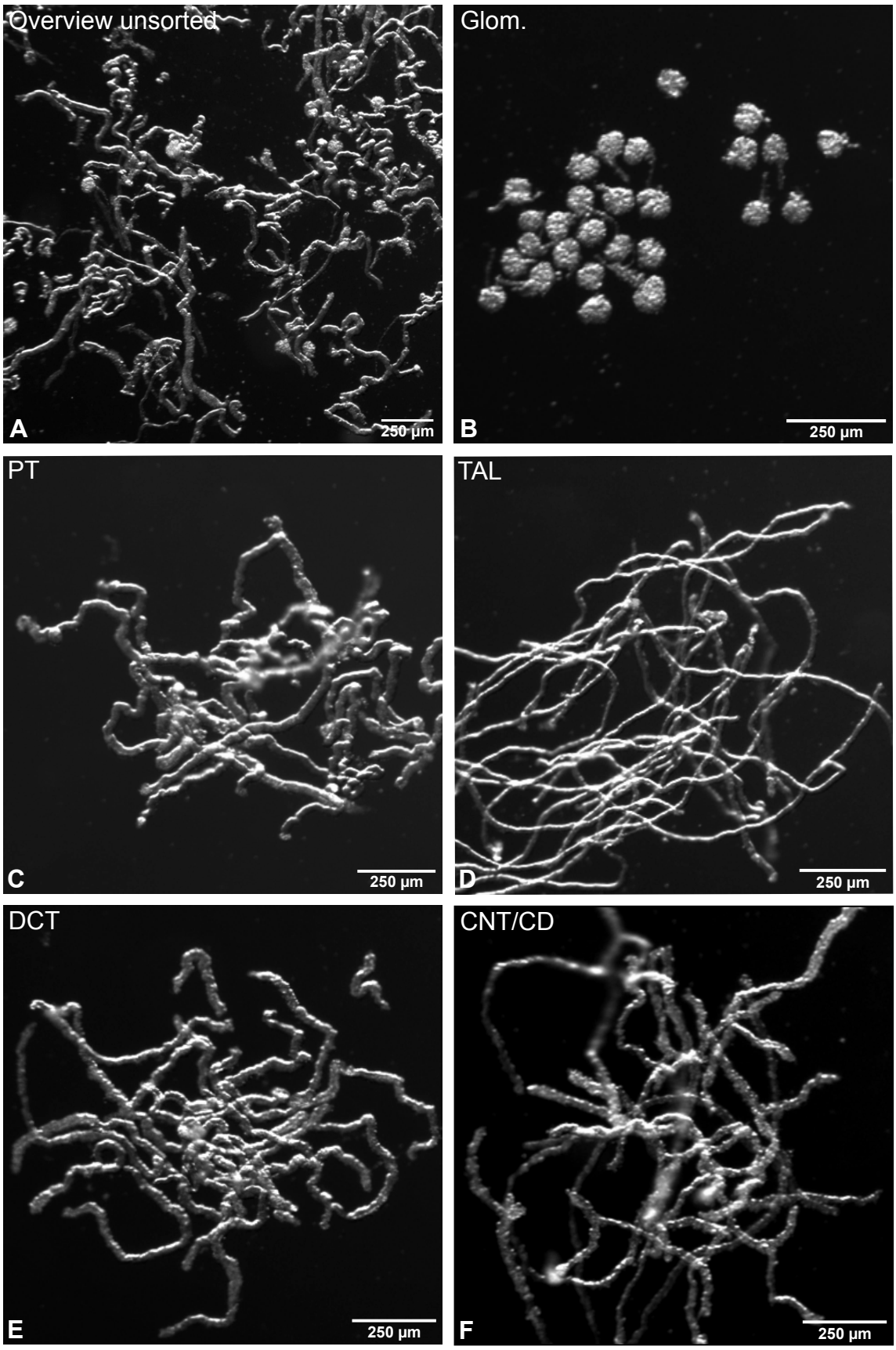

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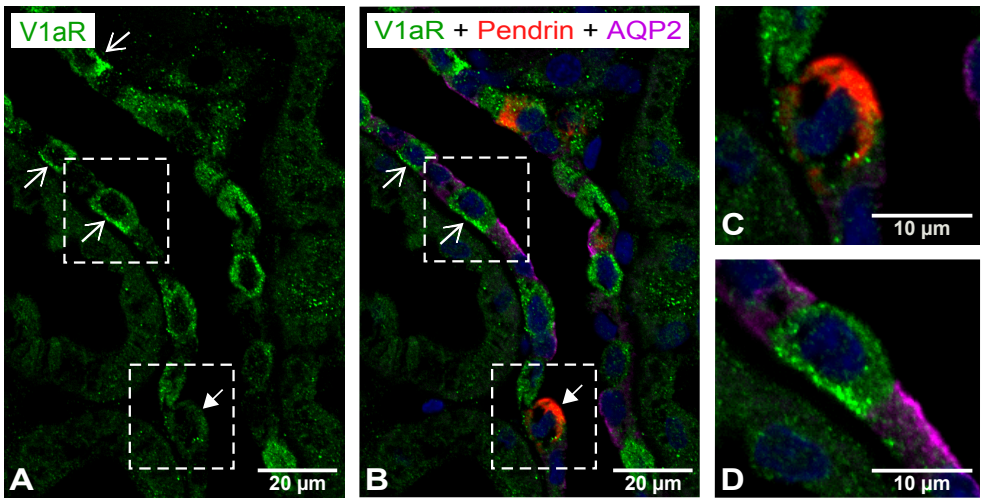
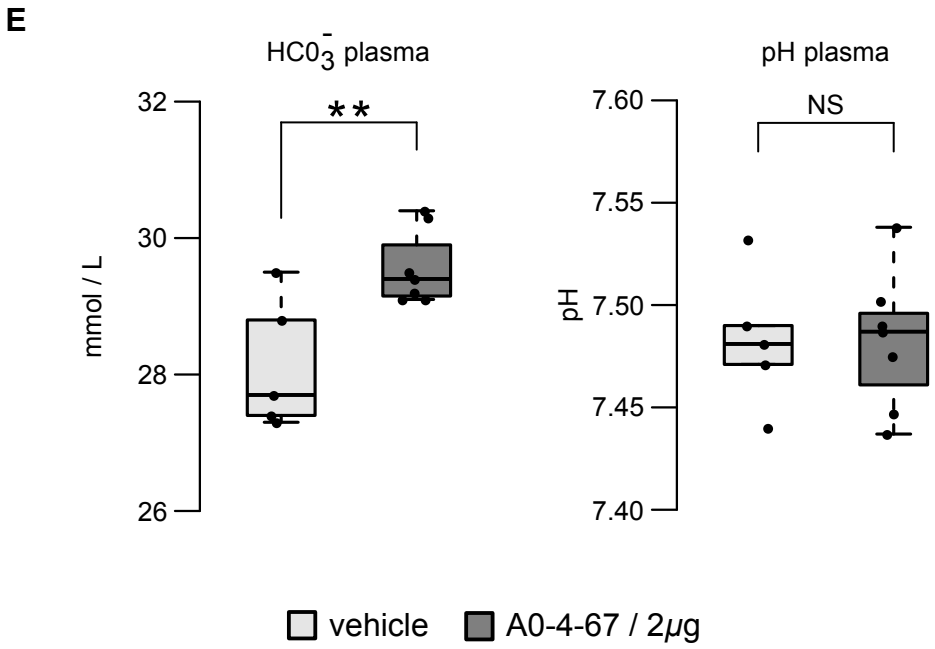
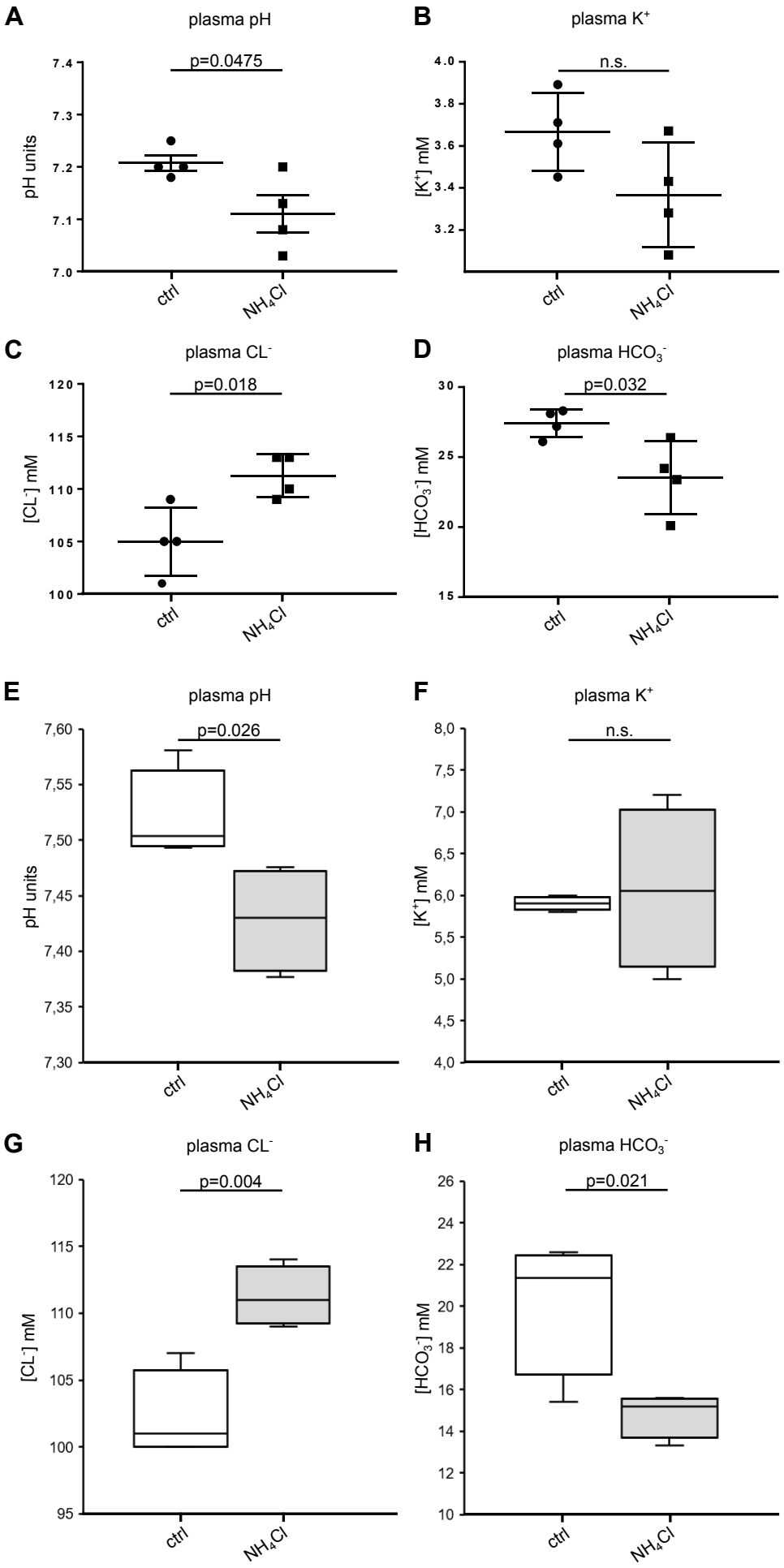
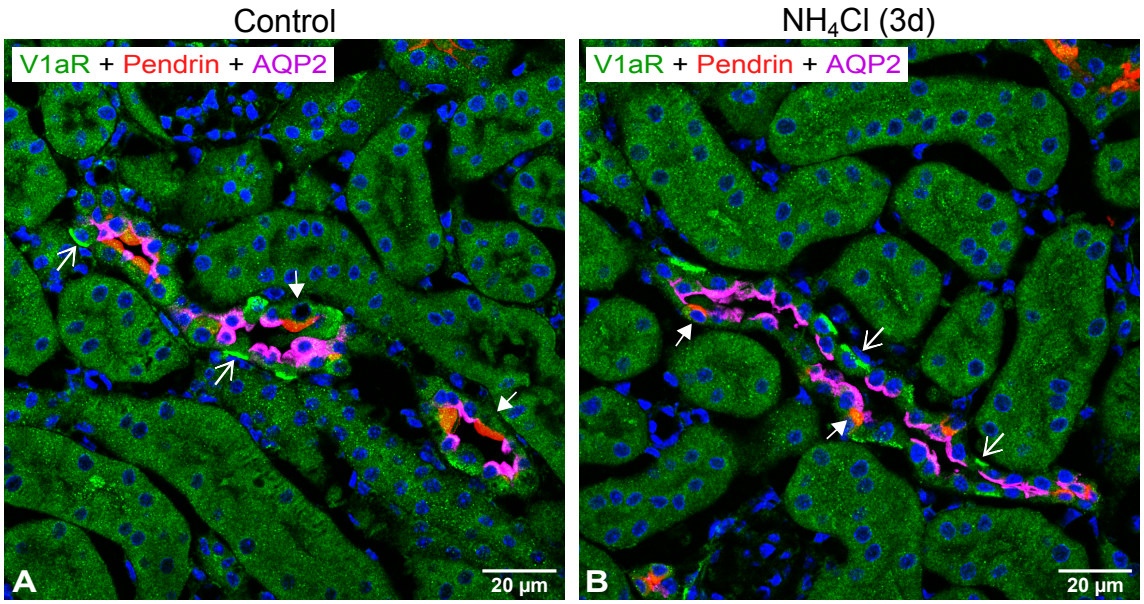


# Supplemental Figure 6



# Supplemental Figure 7



**Supplemental Table 1.** Proportion of cell types in control vs. acidotic mice

| Category | Cortex % | | | Medulla % | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Outer Medulla | | | | Inner Medulla | |
| | | | | Outer Stripe | | Inner Stripe | | | |
| | PCs | A-ICs | B-ICs | PCs | A-ICs | PCs | A-ICs | PCs | A-ICs |
| Control | 61.93 | 13.35 | 24.72 | 79.27 | 20.73 | 82.96 | 17.04 | 92.53 | 7.47 |
| $NH_4Cl$ (3d) | 63.80 | 13.58 | 22.62 | 76.74 | 23.26 | 85.25 | 14.75 | 92.99 | 7.01 |
| Delta | 1.87 | 0.23 | -2.10 | -2.53 | 2.53 | 2.29 | -2.29 | 0.46 | -0.46 |
| P-Value | 0.30 | 0.84 | 0.26 | 0.19 | 0.19 | 0.17 | 0.17 | 0.72 | 0.72 |

**Supplemental Table 2.** Measurements of titratable acid minus bicarbonate (TA), ammonium ($NH_4^+$) and net acid excretion (NAE) in the same sample of human urine (500 $\mu$l, pH 7.04)

| Number of measurement | 0.1 N NaOH ($\mu$l) for titration of TA* | 0.1 N NaOH ($\mu$l) for titration of $NH_4^+$* | TA (mmol/l) | $NH_4^+$ (mmol/l) | NAE (mmol/l) |
|---|---|---|---|---|---|
| 1 | 522.5 | 316.0 | -12.4 | 18.7 | 6.3 |
| 2 | 529.0 | 306.0 | -11.2 | 16.8 | 5.6 |
| 3 | 525.0 | 305.0 | -11.9 | 16.6 | 4.7 |
| 4 | 527.0 | 314.0 | -11.5 | 18.3 | 6.8 |
| 5 | 522.0 | 313.0 | -12.5 | 18.1 | 5.6 |
| Mean | | | -11.9 | 17.7 | 5.8 |
| SD | | | ± 0.51 | ± 0.85 | ± 0.71 |
| SEM | | | ± 0.19 | ± 0.32 | ± 0.27 |

*Endpoint of titration: pH 7.40 at $PCO_2$=0 mmHg and 37°C; 0.1 N NaOH = 0.096 M NaOH

**Supplemental Table 3.** Measurements of titratable acid minus bicarbonate (TA), ammonium ($NH_4^+$) and net acid excretion (NAE) in the same sample of brattleboro rat urine (500 $\mu$l, pH 7.43)

| Number of measurement | 0.1 N NaOH ($\mu$l) for titration of TA* | 0.1 N NaOH ($\mu$l) for titration of $NH_4^+$* | TA (mmol/l) | $NH_4^+$ (mmol/l) | NAE (mmol/l) |
|---|---|---|---|---|---|
| 1 | 581.0 | 232.0 | 1.2 | 1.9 | 3.1 |
| 2 | 578.5 | 235.5 | 0.7 | 2.6 | 3.3 |
| 3 | 575.0 | 230.0 | 0.0 | 1.5 | 1.5 |
| 4 | 568.0 | 236.5 | -1.3 | 3.0 | 1.6 |
| 5 | 566.5 | 234.0 | -1.6 | 2.5 | 0.9 |
| Mean | | | -0.2 | 2.3 | 2.1 |
| SD | | | ± 1.09 | ± 0.51 | ± 0.93 |
| SEM | | | ± 0.41 | ± 0.19 | ± 0.35 |

*Endpoint of titration: pH 7.40 at $PCO_2$=0 mmHg and 37°C; 0.1 N NaOH = 0.096 M NaOH

**Supplemental Table 4.** Measurement of blank (distilled water)

| 0.1 N NaOH ($\mu$l) for titration of TA* | 0.1 N NaOH ($\mu$l) for titration of $NH_4^+$* |
|---|---|
| 575.0 | 222.0 |

*Endpoint of titration: pH 7.40 at $PCO_2$=0 mmHg and 37°C; 0.1 N NaOH = 0.096 M NaOH

## COMPLETE METHODS

### Approval of Animal Experiments

All animal experiments were performed in accordance with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes. The Berlin Animals Ethics Committee at the Landesamt für Gesundheit und Soziales approved the studies on Brattleboro rats (*permission G0220/12 and G0148/18*), the Danish Animal Welfare Regulation Authority gave the permission for the studies with C57/BI6J mice (*permission 2016-15-02101-01129*) and the experiments on isolated collecting duct segments were performed in accordance with the Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume of Schleswig-Holstein (*permission V312-72241.121-2*). All animals were housed under a controlled temperature, air humidity and a 12/12 hour day/night light cycle. They had free access to table water and food ad libitum.

### Generation and characterization of anti-V1aR antibody

To produce the anti-V1aR antibody we chose the peptide sequence NH2-CKDSPKSSKSIRFIPVST-CONH2 due to its negligible homology with the vasopressin V2 and V1b receptors and high conservation between rodent and human species. Eighteen rabbit pre-immune serum samples were analyzed for potential background and cross-reactivity signals in mouse kidney using immunofluorescence to choose three animals for immunization. Peptide synthesis, immunization of animals and affinity purification of anti-V1aR antibodies from the three rabbits were performed by Pineda Antibody-Service (*Berlin, Germany*). The antibody producing the strongest signal in mouse kidney was used for localization studies. The specificity of this anti-V1aR antibody was verified using kidneys from V1aR-deficient mice as negative controls and transient transfection of human GFP-tagged V1aR in HEK293 cells as positive controls. For further validation of the antibody immunoprecipitation and western blot analysis were performed. PCR was used to insert the FLAG epitope, DYKDDDDK, between the initial methionine residue and the second amino acid of the mouse V1b receptor. Construction of the expression plasmid for mouse V1a receptor was described previously by Kashiwazaki[34]. Empty vector and mammalian

expression plasmids for V1a or FLAG-V1b receptor were transiently transfected into HEK cells in 100-mm dishes using Fugene HD reagent (*Promega*). Twenty four hours after transfection, the cells were washed with PBS and were then lysed in lysis buffer (50mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.5% Nonidet P-40, and a proteinase inhibitor cocktail, *Roche*). For immunoprecipitation, cellular lysate and 1 µg of antibody was incubated at 4 ºC for 1 h and precipitated with protein G Sepharose (*GE Healthcare Life Sciences*). For western blot analysis, anti-V1a antibody and FLAG antibodies were used at dilutions of 1:1000 and 1:1500, respectively. The signals from peroxidase-conjugated secondary antibody were detected using enhanced chemiluminescence (*GE Healthcare Life Sciences*). For this part of the study we used the following antibodies: Anti-FLAG tag M2 mouse monoclonal antibody *(#F3165, Sigma Aldrich, Japan*), horseradish peroxidase-conjugated antibody against mouse immunoglobulin G *(#W4021, Promega, 1:20000*), secondary horseradish peroxidase-conjugated antibodies for rabbit *(#458, Medical & Biological Laboratories Co. LTD, Japan, 1:5000*).

**Cell culture experiments**

To verify our anti-V1aR antibody, we cultured HEK293 cells in Minimum Essential Medium Eagle *(#M4526, SIGMA ALDRICH CHEMIE GmbH, Steinheim, Germany*) containing 5% fetal calf serum (*#10270, GibcoTM, Life technologies™, Carlsbad, CA, US*) and 1% GlutaMAX™-I 100X *(#35050-038, Gibco™, Life technologies™, Carlsbad, CA, US)* at 37°C, 95% humidity and 5% $CO_2$ (*Incubator CB150, Binder GmbH, Tuttlingen, Germany*). Cells were seeded on coverslips (*Ø 12mm, #P231.1, Carl Roth GmbH+Co.KG, Karlsruhe, Germany*) placed in 24 well tissue culture plates *(#353047, Falcon®, Corning Incorporated, Corning, NY, USA*). We transfected the cells either with GFP-tagged V1aR plasmid (*#67846, Addgene, Cambridge, MA 02139, USA*) or with the control GFP plasmid (pEFGP-N1) using jetPEI® DNA transfection reagent *(#101-40N, Polyplus-transfection-Bioparc, Illkirch, France*), for 48 hours at 37°C. Coverslips with cells were fixed with 3% paraformaldehyde *(#30525-89-4, Merck KGaA, Darmstadt, Germany*) in TBS (pH 7.57) for 10 minutes. After washing with TBS, cells were permeabilized using 0.1% Triton X-100

(*#9036-19-5, Merck KGaA, Darmstadt, Germany*) in TBS for 10 minutes, blocked with 5% BSA (*#11930.04, SERVA Electrophoresis GmbH, Heidelberg, Germany)* in TBS for 30 minutes. Slides were then incubated with the anti-V1aR antibody in 5% BSA in TBS for 30 minutes at room temperature followed by overnight incubation at 4°C followed by an anti-rabbit Cy3-coupled IgG (*711-165-152, DIANOVA GmbH, Hamburg, Germany*) for two hours at room temperature. This was followed by the incubation with mouse-anti-GFP antibody *(ab291-50, abcam, Cambridge, UK*) and detection with anti-mouse Alexa Fluor® 488 coupled IgG antibody (*DIANOVA GmbH, Hamburg, Germany*) with the same protocol. Each incubation step was separated by three 5-minute washing steps. Samples were evaluated under an LSM 5 Exciter confocal microscope (*Carl Zeiss Microscopy GmbH, Jena, Germany*).

Primary inner medullary collecting duct (IMCD) cells were harvested from the kidneys of adult (12-14 weeks) male Wistar rats and cultured as described by the group of E. Klussmann at the Max Delbrück Center for Molecular Medicine in Berlin.[28] Briefly, 0.4 *µ*m pore size, 24 mm polyester Transwell® plates *(#3450, Corning Inc., Kennebunk, ME, US*) were coated with Collagen Type IV *(#356233, BD Biosciences, Le Pont de Claix, France*). Six rats per experiment were anesthetized by isoflurane inhalation *(#B506, Forene®, AbbVie Deutschland, Germany*) and sacrificed by decapitation. The kidneys were removed, and the kidney inner medulla was surgically separated and immediately dissected in ice-cold DPBS *(#14190144, Gibco™, Life technologies™, Carlsbad, CA, US)* and digested in freshly prepared enzyme solution containing 1 mg/ml hyaluronidase *(#37326-33-3, Merck KGaA, Darmstadt, Germany*), 2.2 mg/ml collagenase (*C2-22, Biochrom GmbH, Berlin, Germany*), gentamicin *(#15710064, Thermo Fisher Scientific, Karlsruhe, Germany*) and nystatin *(#N4014, Merck KGaA, Darmstadt, Germany*) in DPBS *(#14190144, Gibco™, Life technologies™, Carlsbad, CA, US*) for 2 hours at 37°C at 200-300 rpm in a shaking water bath (*Typ 1083, GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany*). The homogenate was then filtered through a Cell Strainer with a pore size of 70 *µ*m (*#4117401, BD Falcon™, BD Bioscience, Bedford, MA, USA*) and centrifuged at 300 g (*Laborfuge 400e,*

*Heraeus Holding GmbH, Hanau, Germany*) at 16°C for 5 minutes to obtain the IMCD cells in the pellet. Cells were then resuspended in freshly prepared DMEM culture containing 4.5 g/L glucose (*#P04-03550, PAN Biotech, Aidenbach, Germany*), adjusted with urea (*#U5378-100G, SIGMA-ALDRICH CHEMIE GmbH, Steinheim, Germany*) and sodium chloride to 600 mosmol (*#1064040500, Merck KGaA, Darmstadt, Germany*) and supplemented with 1% GlutaMAX™-I 100X (*#35050-038, Gibco™, Life technologies™, Carlsbad, CA, US*), 1% non-essential amino acids (*#SH30238.01, GE Healthcare Lifescience, Chalfont St Giles, Great Britain*), 1% Ultroser (*#15950-017, Cytogen GmbH, Wetzlar, Germany*), 500 *µ*M Dibutyryl-cAMP (*#D009, BIOLOG Life Science Institute, Bremen, Germany*), 20 U/ml nystatin (*#N6261, Sigma-Aldrich, St. Louis, MO, USA*) and 0.25 *µ*g/ml gentamicin (*#G1264, Sigma-Aldrich, St. Luis, MO, USA*).

Cells were grown on permeable filter support *(#3450, Transwell® Permeable Supports, Corning Incorporated, Kennebunk, USA*) to full confluence and treated with A0-4-67 (1.3 *µ*M) or vehicle (0.9 % saline solution) from the basolateral side for 4 hours. After that, cells were fixed in 4% paraformaldehyde (*#30525-89-4, Merck KGaA, Darmstadt, Germany*) for 10 minutes, washed in TBS, incubated with a V-ATPase B1/B2 antibody *(#sc-20943, Santa Cruz Biotechnology, Dallas, TX, USA*) and the primary antibody was detected using an IgG Cy3-coupled antibody (*711-165-152, DIANOVA GmbH, Hamburg, Germany*). Nuclei were counterstained with DAPI (*D9542-5MG, SIGMA-ALDRICH CHEMIE GmbH, Steinheim, Germany*). V-ATPase B1/B2 signals were detected by a confocal microscope (*Zeiss LSM 5 Exciter*), processed with a ZEN 2008 software (*Carl Zeiss Microscopy GmbH, Jena, Germany*) and quantified using Fiji v2.0 (*National Institutes of Health, Bethesda, MD, USA*).

**Immunofluorescence and immunohistochemistry**

Paraffin-embedded kidneys were cut into 4 *µ*m sections using a microtome (*Leica RM2125RT, Leica Microsystems, Wetzlar, Germany*) and were dewaxed and hydrated with xylene *(#8080, J.T.Baker, Avantor Performance Materials B.V., Deventer, Netherlands*) and a series of descending grades of ethanol *(#K928.4, Carl Roth GmbH+Co.KG, Karlsruhe, Germany*). Sections were then boiled in citrate buffer (pH 6.0) for 6 minutes for antigen

retrieval. Coverslips with fixed cultured cells were permeabilized for 30 minutes in 0.5% 0.5% Triton® X-100 *(#9036-19-5, Merck KGaA, Darmstadt, Germany*). After washing in TBS, kidney sections or cultured cells were incubated in a wet chamber with 5% skim milk *(#232100, BD Difco™ Skim MILK, Becton, Dickinson and Company, Sparks, MD, USA*) in TBS to block unspecific protein interactions. Primary antibodies to V1aR (*own antibody*), AQP2 (*sc-9882, Santa Cruz Biotechnology, Dallas,TX, USA*), pendrin (*Gift from CA Wagner, Zürich, Schweiz*) and V-ATPase (*sc-20943, Santa Cruz Biotechnology, Dallas, TX, USA*) were applied for 1 hour at room temperature followed by overnight incubation at 4°C. For double-labelling, the primary antibodies were applied consecutively, separated by 3 washing steps in TBS each lasting 5 minutes. Signals were generated using fluorescent Cy2-, Cy3- or Cy5 conjugated (*Dianova, Hamburg, Germany*) or HRP-conjugated secondary antibodies *(#P0399, Dako, Glostrup, Denmark*). Immunofluorescent stains were evaluated under an LSM 5 Exciter confocal microscope (*Carl Zeiss Microscopy GmbH, Jena, Germany*) equipped with 40× and 63× EC Plan-NEOFLUAR oil immersion objectives (N.A. 1.3/1.4). The confocal microscope was equipped with 405 nm/50 mW diode laser, 458 nm/476 nm/488 nm/514 nm/50 mW argon laser, 543 nm/2 mW helium neon laser and 633 nm/12 mW helium neon laser lines. Filters for excitation/emission were set to 405/BP 420-480 for DAPI, 488/BP 505-550 for Cy2, 543/BP 560–615 for Cy3 and 633/LP 650 for Cy5 (BP = bandpass, LP = longpass). Bright-field images were taken with a Leica DMRB upright microscope (*Leica Microsystems GmbH, Wetzlar, Germany*) fitted with a 100× PL FLUOTAR oil immersion objective and an Axio Cam MR3 camera (*Carl Zeiss Microscopy GmbH, Jena, Germany*). Images were processed with either a ZEN 2008 or Axio Vision SE 64 4.8.3 software (*Carl Zeiss Microscopy GmbH, Jena, Germany)*. Brightness and contrast were adjusted in Fiji v2.0 (*National Institutes of Health, Bethesda, MD, USA*).

3D SIM images were acquired using 568 nm and 647 nm laser lines, standard filter sets and 125 nm z-sectioning using the Delta Vision OMX V4 Blaze™ (*GE Healthcare, Chalfont St Giles, Buckinghamshire, Great Britain*) system. 100 nm fluorescent beads *(#T7284, Tetraspeck, Thermo Fischer Scientific, Karlsruhe, Germany*) were used for registration of the

detection channels, achieving less than 40 nm registration error for all channels. Images and movies were exported with the SoftWoRx software (*version 6.5.2, GE Healthcare, Chalfont St Giles, Buckinghamshire, Great Britain*) and Fiji v2.0 (*National Institutes of Health, Bethesda, MD, USA*).

**Effects of the V1aR agonist (A0-4-67) in mice**

Male adult (10-12 weeks) C57/Bl6J mice (*Janvier Labs, Le Genest-Saint-Isle, France*) were randomly assigned to the experimental and control group. Anaesthesia was induced by an intraperitoneal bolus injection of a ketamine (10 mg/ml) / xylazine (1 mg/ml) mix at a dose of 0.1 ml/10 g body weight and subsequently maintained by intravenous infusion of the same mixture at a third of the induction dose per hour via one of the animal's tail veins. Throughout the whole experiment, each lasting 90 minutes, urine volume was measured every 5 minutes by calibrated glass capillaries and urine samples were collected every 5 minutes. After assessing the depth of anaesthesia by testing the withdrawal reflexes on the lower limbs of the mouse, the urinary bladder was catheterized via a small incision in the abdomen to allow continuous urine collection. A micro pH electrode (*Ø 200 µm; Unisense A/S, Aarhus, Denmark*) was placed in the outflow of the catheter to measure the urine pH continuously; data were obtained every second (*pH/mV-Meter, Unisense A/S, Aarhus, Denmark*). After establishing the baseline pH values for 30 minutes, the mice received the V1aR agonist A0-4-67 (2 µg/kg body weight i.p.) or the vehicle (0.9% saline) by intraperitoneal bolus injection. Measurements of pH and urine collection were continued for another 60 minutes. Since the acute effect of the V1aR agonist on urine pH can only be observed provided that the baseline of urine pH is alkaline, we excluded one experiment in the urine pH analysis that started with a much lower urine pH baseline compared to all other experiments.

**Effects of the V1aR agonist (A0-4-67) in Brattleboro Rats**

Effects of V1aR activation were studied in male Brattleboro rats (n=16), 13 to 15 weeks old, weighing 300-380g. Animals had free access to distilled water and normal rat chow. Rats were randomly divided into two groups (each n=8). Starting with the first subgroup (n=8), animals were coincidentally divided into an experimental (n=4) and a control group (n=4) and

were placed in 8 metabolic cages. After three days of recovery the groups were changed. The experimental group (n=4) received the vehicle and served as the control group, whereas the control group (n=4) was treated with the V1aR agonist and served as the experimental group. The same procedure was conducted with the second subgroup (n=8). Each approach involving 4 rats in the control and 4 rats in the experimental group was considered as an independent experiment. Each independent experiment started at the respective day at 12:00 AM with 120 minutes of adaptation time. All urine samples were collected under mineral oil *(#M5904, Sigma-Aldrich, St. Luis, MO, USA*). After collecting baseline urine samples at the end of the adaptation time, rats were treated on the respective day always between 2:00 and 2:30 PM via intraperitoneal bolus injection with vehicle (0.9% saline) or A0-4-67 (200 ng/kg body weight i.p.). Urine samples were collected hourly at 3:30 PM (t1), 4:30 PM (t2), 5:30 PM (t3) and 6:30 PM (t4) and were directly measured after collection for acid base parameters using an ABL800 FLEX analyzer (*Radiometer GmbH, Krefeld, Germany*). The rats were then placed in normal cages for three days and the experiment was repeated two times using higher A0-4-67 doses (2 µg/kg body weight and 10 µg/kg body weight i.p.) with a 3-day interval for recovery in normal cages between the experiments. After application of these three doses and a further recovery period in normal cages for three days, the treatment groups were exchanged so that the vehicle-treated rats now received the three A0-4-67 doses, whereas the V1aR-agonist group received the vehicle according to the protocol above. This experimental design resulted in consecutive treatment of all 16 Brattleboro rats with vehicle and the three different A0-4-67 doses. After pilot analysis of the urine data, we have chosen the A0-4-67 dose of 2 µg/kg body weight and the treatment time of 2 hours for evaluation of effects on plasma acid-base parameters. After 2 hours, animals were anesthetized with isoflurane (*#B506, Forene®, AbbVie Deutschland GmbH & Co.KG*), and scarificed by decapitation. Blood samples were taken from the cervical arteries directly into 1.3 ml micro tubes containing EDAT or Heparin *(#41.1504.005 and #41.1503.005, SARSTEDT AG & Co., Nürnbrecht, Germany*) and centrifuged for 10 minutes at 4°C at 1000 RPM (*MIKRO 200R, Andreas Hettich GmbH & Co.KG*). Supernatant was pipetted to fresh

1.5 ml SafeSeal tubes *(#72706, SARSTEDT AG & Co., Nürnbrecht, Germany*) and stored at 4°C. Plasma samples were measured on the same day with the ABL 800 Flex analyzer (*Radiometer GmbH, Krefeld, Germany*).

**Urinary net acid excretion in Brattleboro rats**

Determination of urinary net acid excretion (NAE) in Brattleboro rats after V1aR stimulation with the V1aR Agonist A0-4-67 (10 *µ*g/kg body weight i.p.) was carried out by manual titration with 0.1 N NaOH as described by Chan, J. (1972)[30] to the endpoint of pH 7.40 at $P_{CO2}$=0 mmHg, at a temperature of 37°C. We evaluated the experimental setting and the protocol for a reduced urine volume of 500 *µ*l per sample by measuring the same urine probe from a healthy human male individual and from a male brattleboro rat multiple times (Supplemental Table 2-4). The sodium hydroxide was dissolved in distilled water and the 0.1 M NaOH solution was standardized using benzoic acid to 0.1 N NaOH. The titration was carried out using Eppendorf pipettes (*Eppendorf Research® plus 0.1 – 2.5 µl, Eppendorf Research® plus 0.5 – 10 µl, Eppendorf Research® plus 10 – 100 µl, Eppendorf Multipette® Xstream*). The pH was measured adjusted to temperature by an electronic pH-Meter (*pH-Meter CG 812, SCHOTT Instruments GmbH, Mainz, Germany*) with a mirco pH-electrode *(#285105151, N 6000 A, SI Analytics GmbH, Mainz, Germany*). All urine samples of the Brattleboro rats were stored at -25°C. 500 µl of urine per animal were mixed with 500 µl 0.1 M HCL, boiled for 4 minutes and placed on ice for 5 minutes and then in a water bath of 37°C to allow for temperature adjustment. For assessment of titratable acid (TA) the titration was started with 0.1 N NaOH using the titration mode of the Eppendorf Multipette® Xstream. Because HCl converts bicarbonate $HCO_3^-$ to $CO_2$, which was removed by boiling, TA represents titratable acid minus $HCO_3^-$. Adding of 500 µl of 8% formaldehyde solution exposed the protons bound to ammonia and caused the pH to drop. The sample was titrated back to the endpoint of pH 7.40. The blank was treated identically. Ammonia, titratable acid and net acid excretion were calculated in units of mmol/l as described by Chan; briefly:

$$(d\ V_{NaOH}\ ml / 0.5\ ml) \times 0.1\ N\ NaOH\ mol/l \times 1000\ mmol/Eq,$$

Net acid excretion = [titratable acid (minus bicarbonate)] + ammonia.

The results were adjusted to the urine volume per hour and the body weight of the rats.

**Isolated perfused Collecting ducts**

All chemicals for the following experimental part were obtained by Merck Chemicals GmbH, Darmstadt, Germany or Carl Roth GmbH + Co.KG, Karlsruhe, Germany if not stated otherwise. Adult C57 Bl6/N mice were sacrificed by decapitation after isoflurane anesthesia. Kidneys were removed immediately, cut in thin coronary sections and placed in ice-cold (4°C) dissection solution that was adjusted to a pH of 7.4. The dissection solution contained: 145 mmol/l NaCl, 0.4 mmol/l $KH_2PO_4$, 1.6 mmol/l $K_2HPO_4$, 1 mmol/l $MgSO_4$, 10 mmol/l sodium acetate, 1 mmol/l alpha ketogloterate, 1.3 mmol/l calcium gluconate, 5 mmol/l glycine, 48 mg/l trypsin inhibitor, 25 mg/l DNase I, 0.05 % albumin. Collecting ducts were dissected from the transition zone between cortex and outer medulla and processed for measurements of intracellular calcium concentrations $[Ca^{2+}]_i$ or luminal pH. We used fura-2 (*#F1221, Thermo Fischer Scientific, Karlsruhe, Germany*) as an indicator for calcium concentration measurement and BCECF (*#B1151, Thermo Fischer Scientific, Karlsruhe, Germany*) as a marker for the pH. Four to six collecting ducts were analyzed in each experimental setting. The collecting ducts were perfused with a double barreled perfusion system of concentric pipettes (Greger R, Hampel W: A modified system for in vitro perfusion of isolated renal tubules. Pflugers Arch 389(2): 175-6, 1981).

The luminal perfusion solution contained: 145 mmol/l NaCl, 3.6 mmol/l potassium gluconate, 5 mmol/l glucose, 1 mmol/l $MgCl_2$, 1.3 mmol/l calciumgluconat and was adjusted to a pH of 7.4 and a temperature of 37°C. All measurements were performed in a pre-gassed (95% $O_2$ 5% $CO_2$) bath solution with a bath exchange rate of 5-6 ml/minute.

The bath chamber solution consisted of: 120 mmol/l NaCl, 0.4 mmol/l $KH_2PO_4$, 1.6 mmol/l $K_2HPO_4$, 1 mmol/l , 5 mmol/l glucose, 1 mmol/l $Mg_2Cl$, 1.5 mmol/l $CaCl_2$ and was adjusted with $NaHCO_3$ to a pH of 7.4 and maintained at a temperature of 37°C.

For $[Ca^{2+}]_i$ measurements, collecting ducts were incubated for 50 to 60 minutes at room temperature in the dissection solution with 10 $\mu$mol/l Fura-2-AM. Tubules were transferred to

the bath chamber and fluorescence intensities at 340 nm and 380 nm were monitored using an Axiovert 55m inverted microscope (*Carl Zeiss Microscopy GmbH, Jena, Germany*) with a Visichrome High Speed Polychromator System (*Visitron Systems GmbH, Puchheim, Germany*) and the MetaFluor® Fluorescence Ratio Imaging Software (*version 7.6.1.0, Molecular Devices LLC, San Jose, CA, USA*). The 340/380 nm excitation ratio was calculated and used as an indicator for the intracellular $Ca^{2+}$ concentration. After obtaining baseline values, the collecting ducts were treated with the V1aR agonist A0-4-67 (50 nM or 100 nM) from the basolateral side for 3 minutes followed by a washout period of 7-8 minutes and the consecutive application of AVP (50 nM) for 3 minutes. The goal was to compare the effect caused by the V1aR-agonist with the AVP response. In each experiment, one perfused collecting duct was analyzed by measurement of 5 regions in the respective segment. The peak height of the effects of the V1aR agonist A0-4-67 and of the AVP effects are given as the delta of the 340/380 nm excitation ratio in comparison to the pre-value.

For the assessment of the luminal pH, collecting ducts were perfused with 100 *µ*mol/l BCECF. Collecting ducts were closed at the open, unperfused side with a holding pipette and luminal fluorescence intensities at 486 nm and 440 nm were monitored to calculate the ratio 486/440 as a measure of luminal pH. After an equilibration period of 5-10 minutes, the V1aR agonist was applied at a concentration of 100 nM for approximately 4 minutes followed by a wash-out period of 10 minutes and the consecutive application of AVP at a concentration of 50 nM for over 4 minutes. A luminal region near the perfusion side was analyzed for each tubule and the ratio of 486/440 nm normalized to the mean of 30 seconds shortly before the application of the V1aR agonist. The effects of the V1aR agonist and of AVP are given as the delta relative ratio of 486/440 nm in comparison to the mean of pre- and post-value from 6 independent experiments. In addition, 4 experiments were performed with only the application of 50 nM AVP without preceding V1aR application and analyzed accordingly.

**Statistical analyses**

Data are shown as mean and standard error of the mean (SEM). For experimental series n reflects the number of mice, rats or collecting ducts. We assumed normal distribution of our

results. Comparisons between experimental conditions were performed with unpaired two-tailed Student’s t-test (Figure 5 and 7). Since the acute effect of V1aR agonist on urine pH can apparently only be observed under the premise that baseline urine pH is alkaline, we excluded one experiment in the urine pH analysis, starting with a much lower urine pH baseline compared to all other experiments (Figure 5, A). The effects of V1aR agonist and AVP in isolated collecting ducts were tested by paired Student´s t-test vs. pre-control (Fura-2 measurements) and vs. mean of pre- and post-control (luminal BCECF), respectively. Effects between different V1aR-AG concentrations and AVP were compared by a Kruskal-Wallis-test followed by Dunn's multiple comparisons test (Figure 8). A p value of $<0.05$ was chosen to indicate statistical significance.