Aus dem Institut für Funktionelle Anatomie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Die Aktivierung des Vasopressin Type 1a Rezeptors in der Niere induziert die Harnansäuerung und moduliert den Säure-Basen Haushalt

-

Vasopressin type 1a receptor promotes the urinary acidification and modulates the acid-base balance

zur Erlangung des akademischen Grades
Medical Doctor - Doctor of Philosophy (MD/PhD)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Torsten Giesecke

aus Meißen

Datum der Promotion: 04.06.2021

Table of Contents

Abbreviations

3D-SIM ..... 3D-structured illumination microscopy
ADH ..... antidiuretic hormone
A-ICs ..... type A intercalated cells
AQP2 ..... aquaporin-2
AVP ..... [arg^{8}]vasopressin
BCA ..... bicinchoninic acid
BCECF ..... 2',7'-Bis-(2-Carboxyethyl)-5-(and -6)-Carboxyfluorescein
B-ICs ..... type B intercalated cells
BP ..... bandpass
BSA ..... bovine serum albumin
BW ..... body weight
cAMP ..... cyclic adenosine monophosphate
CDs ..... collecting ducts
CNT ..... connecting tubule
DAPI ..... 4′,6-Diamidin-2-phenylindol
DCT ..... distal convoluted tubule
DMEM ..... dulbecco's modified eagle medium
DPBS ..... dulbecco’s phosphate-buffered saline
dRTA ..... distal renal tubular acidosis
EDAT ..... ethylenediaminetetraacetic acid
GAPDH ..... anti-Glyceraldehyde 3-phosphate dehydrogenase
GFP ..... green fluorescent protein
GPCRs ..... G-protein-coupled receptors
HEK293 ..... human embryonic kidney cells
HRP ..... horseradish peroxidase
ICs ..... intercalated cells
IgG ..... immunoglobulin G
IMCD ..... inner medullary collecting duct
IP ..... intraperitoneal
kDa ..... kilodalton
LP ..... longpass
mRNA ..... messenger ribonucleic acid
MW ..... molecular weight
NA ..... numerical aperture
NAE ..... net acid excretion
PBS ..... phosphate-buffered saline

PCR ..... polymerase chain reaction
PCs ..... principal cells
pH ..... power of hydrogen
PLC ..... phospolipase C
PT ..... proximal tubule
RAAS ..... renin-angiotensin-aldosterone system
RNA ..... ribonucleic acid
RT-PCR ..... real-time polymerase chain reaction
SDS ..... sodium dodecyl sulfate
SDS-PAGE ..... sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM ..... standard error of the mean
SIM ..... structured illumination microscopy
TA ..... titratable acid
TAL ..... thick ascending limb
TBS ..... tris-buffered saline
Tris ..... tris(hydroxymethyl)aminomethane
V1aR ..... vasopressin type 1a receptor
V1bR ..... vasopressin type 1b receptor
V2R ..... vasopressin type 2 receptor
vasopressin-deficient ..... VP-deficient
V-ATPase ..... vacuolar H^+-ATPase
VP ..... vasopressin
vs. ..... versus

Abstract

Vasopressin (VP) enables antidiuresis via activation of the vasopressin type 2 receptor (V2R) in the kidneys. The vasopressin type 1a receptor (V1aR) is also expressed in renal tissue, but its function receives little attention. V1aR signaling has been linked to the acid-base handling in collecting duct intercalated cells (ICs), although the underlying mechanisms remain elusive.

In this study, we tested the hypothesis that V1aR activation in type A intercalated cells (A-ICs) of the collecting duct induces urinary H^+ secretion, thereby promoting urinary acidification and net acid excretion (NAE).

To study renal V1aR distribution, we generated an anti-V1aR antibody and verified its specificity using V1aR knockout tissue and V1aR-transfected cultured cells. Localization studies in mice, rats, and human tissue were performed using immunofluorescence and confocal microscopy, combined with 3D-structured illumination microscopy (3D-SIM). For functional studies *in vivo*, the V1aR-specific agonist, AO-4-67, was administered in VP-deficient Brattleboro rats and wild-type (C57BL/6J) mice (0.2, 2, or 10 μg/kg BW; 1 to 4h). The V1aR antagonist, CL-14-102, was used to evaluate V1aR effects in acidotic mice fed with NH_4Cl in chow for three days. Urine was collected in metabolic cages or via a ureteral catheter, and plasma samples were obtained at the end of experiments. The *in vivo* studies were complemented by *ex vivo* experiments in isolated, microperfused mouse collecting ducts, and cultured inner medullary collecting duct cells.

Localization of V1aR in mouse, rat, and human kidneys produced a basolateral signal in A-ICs and a perinuclear to subapical signal in type B intercalated cells of connecting tubules and collecting ducts throughout these species. Basolateral V1aR signal was further detected in macula densa cells of mouse but not of rat or human kidneys. The V1aR agonist significantly decreased the urinary pH in Brattleboro rats (pH 7.38 to 6.71 after 1-hour, $P<0.01$) and C57BL/6J mice (pH 7.18 to 6.78 after 20 minutes, $P<0.05$) and tripled the urinary net acid excretion in Brattleboro rats. In contrast, the administration of the V1aR-antagonist in acidotic mice induced no changes in urinary pH. Basolateral treatment of isolated perfused medullary collecting ducts with the V1aR agonist or VP increased intracellular Ca^{2+} levels in ICs and decreased luminal pH (pH -5% after 3 minutes, $P<0.05$) suggesting V1aR-dependent Ca^{2+} release and stimulation of proton secreting proteins. Basolateral treatment of inner medullary collecting duct cells with the V1aR agonist induced luminal translocation of vacuolar H^+-ATPase (V-ATPase) in A-ICs (apical signal intensity +93%, $P<0.001$).

In summary, our results show that V1aR activation contributes to urinary acidification via H^+ secretion by A-ICs. Pharmacological targeting of V1aR may have clinical implications for disorders of renal acid-base handling, such as distal renal tubular acidosis.

Zusammenfassung

Die antidiuretischen Effekte des Hormons Vasopressins (VP) werden durch Aktivierung des Vasopressin Typ 2 Rezeptors (V2R) in der Niere vermittelt. Der Vasopressin Typ 1a Rezeptor (V1aR) wird ebenfalls in der Niere exprimiert und hier in Verbindung mit der Aufrechterhaltung der Säure-Base Homöostase gebracht. Diese Funktion wurde jedoch bisher nur unzureichend charakterisiert. In der vorliegenden Arbeit haben wir daher die Hypothese untersucht, dass die Aktivierung des V1aR in den Typ A Schaltzellen des Sammelrohres der Niere die Säuresekretion in den Urin steigert.

Für die Charakterisierung der renalen V1aR Verteilung wurde ein V1aR-Antikörper hergestellt und seine Spezifität validiert. Die Lokalisationsstudien in Gewebe von Mäusen, Ratten und humanen Proben wurden mit immunhistochemischen Verfahren durchgeführt und mit hochauflösenden mikroskopischen Verfahren ausgewertet. Im Rahmen der funktionellen Untersuchungen *in vivo*, wurde ein V1aR Agonist (A0-4-67) bei Vasopressin defizienten Brattleboro Ratten und normalen Mäusen verwendet (0.2, 2, or 10 µg/kg BW; 1 to 4h). Darüber hinaus wurde der V1aR Antagonist (CL-14-102) Mäusen im Rahmen einer experimentellen metabolischen Azidose verabreicht. Urin- und Plasmaproben wurden ausgewertet. Isolierte, mikroperfundierte Sammelrohre der Niere wurden hinsichtlich der luminalen Veränderungen des pH Wertes und kultivierte Zellen des inneren Marks der Niere bezüglich der Regulation der protonensezernierenden V-ATPase untersucht.

In Geweben von Maus, Ratte und Mensch zeigte der V1aR ein basolaterales Signal in den Typ A und ein perinukleäres bis subapikales Signal in den Typ B Schaltzellen des Verbindungstubulus und des Sammelrohres aller drei Spezies. Die Applikation des V1aR Agonisten führte zur einer signifikanten Reduktion des pH-Wertes im Urin, sowohl in den Brattleboro Ratten (10 *µ*g/kg Körpergewicht; pH 7.38 auf 6.71 nach 1 Stunde, $P<0.01$), als auch in Mäusen (2 *µ*g/kg Körpergewicht; pH 7.18 auf 6.78 nach 20 Minuten, $P<0.05$). Dabei war die Netto-Säure-Sekretion im Urin der Brattleboro Ratten dreifach erhöht. Die basolaterale Stimulation von isolierten Sammelrohren des Nierenmarks mit dem V1aR Agonisten oder mit VP führte zu einem Anstieg des intrazellulären Calciums in den Schaltzellen sowie zu einem reduzierten pH-Wert im Lumen der Sammelrohre (pH -5% nach 3 Minuten, $P<0.05$). Diese Ergebnisse sprechen für eine über den V1aR vermittelte Calciumfreisetzung und Stimulation der Protonensekretion. In der Zellkultur von Sammelrohrzellen führte eine basolaterale Stimulation zur luminalen Translokation der V-ATPase in den Typ A Schaltzellen (apikale Signalintensität +93%, $P<0.001$).

Zusammengefasst zeigen die Ergebnisse, dass die Aktivierung des V1aR zur Ansäuerung des Urins durch Stimulation der Protonensekretion in den Typ A Schaltzellen der Niere beiträgt. Zukünftig könnten daher V1aR Agonisten oder Antagonisten als Behandlungsoption bei Störungen des Säure-Basen Haushaltes in Betracht gezogen werden.

1 Introduction

Vasopressin (VP) is a small neurohypophyseal hormone (MW=1080), containing nine amino acids in a ring structure. It is synthesized by magnocellular neurons of the supraoptic- and paraventricular hypothalamic nuclei. VP is derived from a prepro-hormone consisting of a signal peptide, vasopressin, neurophysin II and a C-terminal peptide called copeptin. The hormone precursor is proteolytically cleaved in neurosecretory granules to VP, neurophysin II and copeptin. After transport to the neurosecretory endings of the magnocellular neurons that project to the posterior pituitary gland, the cleavage products are released in equimolar amounts to the bloodstream.[1] The critical stimulus for the VP secretion is an increase in plasma osmolality. Other triggers include decreased arterial pressure, reduced cardiac filling, stress, and cold temperatures.[2]

Three receptor-subtypes can mediate the effects of VP: the vasopressin type 1a receptor (V1aR), the vasopressin type 1b receptor (V1bR), and the vasopressin type 2 receptor (V2R). They are G-protein-coupled receptors (GPCRs), using either calcium (V1aR and V1bR) or cyclic adenosine monophosphate (cAMP) as a second messenger (V2R).[3]

Activation of these receptors leads to diverse central and peripheral effects. Stimulation of V1aR and V1bR in the central nervous system may affect several functions such as social behavior, aggression, anxiety, learning, memory, or thermoregulation.[4] The peripheral effects include the constriction of vessels via the V1aR.[5] These vasoconstrictive effects were first discovered in the nineteenth century. Extracts of the posterior pituitary caused elevation of blood pressure *in vivo* and vasoconstriction of isolated blood vessels *in vitro*.[6] These observations gave the hormone its present name: vasopressin.[7] However, more important is its role in the water-electrolyte balance, mediated via the V2R in the kidney. The kidneys help to maintain the water and electrolyte balance through the regulation of water and electrolyte excretion.[8]

The functional unit of the kidney, the nephron, consists of the glomerulus and several types of tubules, including the proximal tubule (PT), the loop of Henle, the distal convoluted tubule (DCT), and the connecting tubule (CNT). The ensuing collecting ducts (CDs) also fulfill several vital tasks for the water-electrolyte balance. The glomerulus filters the blood into the tubular system, which is lined by a single layer of epithelial cells and surrounded by capillaries. Through their close anatomical relationship, the epithelial cells can reabsorb solutes back from the tubular lumen to the blood and secrete toxic substances into the lumen. The epithelial cells of the PT are responsible for the reabsorption of approximately two-thirds of filtered sodium chloride, water, and significant amounts of other solutes. The coordinated work of the distal nephron and CDs enables the urinary concentration, thereby adjusting the excretion of water and electrolytes to the needs of the body.[9]

The V2R is expressed in the distal nephron, comprising the thick ascending limb (TAL), DCT, and CNT, as well as the cortical and medullary CDs. V2R activation enables the urinary concentration via stimulation of relevant salt transport or water transport proteins. Effects of V2R activation include insertion of the aquaporin-2 (AQP2) water channels into the luminal membrane of principal cells (PCs) and stimulation of Na^+-(K^+)-Cl^- transporters in TAL and DCT, leading to enhanced salt and water reabsorption.

The crucial role of the VP-V2R signaling for the water and electrolyte homeostasis can be illustrated by disease-causing mutations either in the VP genes or V2R genes. Mutations in the VP gene, which impair the VP-production or secretion, lead to the clinical picture of central diabetes insipidus with pronounced polyuria and polydipsia. Similarly, non-responsiveness of V2R to VP due to loss-of-function mutations causes nephrogenic diabetes insipidus with similar symptoms.[10]

The V1aR is also expressed in the kidney, but its functional relevance has been studied to a lesser extent compared to the V2R receptor. Several studies addressed the distribution of V1aR, but the results remain controversial. The lack of specific antibodies robustly recognizing this receptor type was a critical barrier to the understanding of its renal distribution and physiological roles. Recent characterization of genetically engineered V1aR knockout mice suggested that the renal V1aR promotes renin release, thereby stimulating the renin-angiotensin-aldosterone system (RAAS), which affects the blood pressure.[11,12]

Further studies revealed that V1aR-deficient mice also exhibit an impaired acid-base balance. They show a phenotype that is consistent with the distal renal tubular acidosis (dRTA) caused by hyporeninemic hypoaldosteronism. The impaired renin release and low RAAS activity lead to low blood pressure, metabolic acidosis, and hyperkalemia in V1aR knockout mice.[13]

Recent single-cell ribonucleic acid (RNA) sequencing, as well as some older localization studies, suggested that the V1aR is expressed in the intercalated cells (ICs) of the CNT and CD.[14–16] Moreover, metabolic acidosis increased the V1aR messenger ribonucleic acid (mRNA) expression and protein abundance in CDs of rats, suggesting that V1aR is involved in adaptive changes during acidosis.[17,18]

The collecting duct is composed of two different cell types, principal cells (PCs) and intercalated cells (ICs). PCs are responsible for water reabsorption in response to V2R stimulation. The ICs can be morphologically and functionally divided into the proton-secreting type A intercalated cells (A-ICs) and bicarbonate-secreting type B intercalated cells (B-ICs). A third population, the so-called non-A, non-B ICs may represent a transition state between the other two types. In a simplified view, A-ICs secrete protons via different proton pumps expressed in their apical membrane, and B-ICs are responsible for the secretion of hydroxide ion equivalents via a

chloride-bicarbonate exchanger expressed in their apical membrane. Both subtypes are essential in maintaining the acid-base balance by responding to changes of the acid-base status, helping to dispose of acid generated by dietary intake, which cannot be eliminated by the lungs.[19]

Several lines of evidence so far have suggested that V1aR is expressed in CD intercalated cells, where the receptor participates in renal acid-base handling, although the underlying mechanisms remain to be characterized in detail. We hypothesized that the VP-V1aR axis promotes the urinary proton secretion by A-ICs, thereby increasing the net acid excretion (NAE) and acidifying the urine.[13]

2 Aims of this work

The aims of this work include the characterization of the V1aR distribution in rodent and human kidneys and elucidation of the functional relevance of the V1aR signaling in renal acid-base handling. To this end, we followed the hypothesis that the V1aR is expressed in A-ICs, and that its activation stimulates the proton secretion in this cell type. The study was designed to enable a high-quality morphological analysis of renal V1aR distribution using a new antibody generated for this purpose. The physiological *in vivo* experiments recruit relevant models such as VP-deficient Brattleboro rats and a metabolic acidosis model in mice receiving a V1aR agonist or antagonist. The *in vivo* data were supported by *ex vivo* experiments in isolated mouse CDs and cultured primary rat CD cells to demonstrate local effects of V1aR activation in the absence of concomitant systemic changes.

3 Methods

3.1 Approval of animal experiments

The animals used for experiments in this study were treated in accordance with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes. We got the permission of the Berlin Animals Ethics Committee at the Landesamt für Gesundheit und Soziales for our studies on Brattleboro rats (permission G0220/12 and G0148/18), the group of Prof. Leipziger received the permission for the studies with C57BL/6J mice (permission 2016-15-02101-01129) from the Danish Animal Welfare Regulation Authority and the group of Prof. Bleich performed the experiments on isolated collecting duct segments after approval from the Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume of Schleswig-Holstein (permission V312-72241.121-2). All animals were housed under controlled temperature, air humidity, and under a 12/12 hour day/night light cycle with free access to table water and food ad libitum.

3.2 Generation and characterization of the anti-V1aR antibody

For the generation of the anti-V1aR antibody, we collaborated with the company Pineda Antikörper-Service in Berlin, represented by Dr. Julio Pineda de Castro. For immunization of the animals, we chose the peptide sequence NH2-CKDSPKSSKSIRFIPVST-CONH2 due to its little homology with the vasopressin type 2 (V2R) and vasopressin type 1b receptors (V1bR) and high conservation between rodent and human species. After analyzing of eighteen rabbit pre-immune serum samples for potential background and cross-reactivity signals using immunofluorescence, we 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). We tested the sera of all three rabbits on day 60, 90, and at day 150 for specific V1aR signals by immunofluorescence microscopy. After an immunization time of 156 days, the sera of all three animals were used for affinity purification of three different anti-V1aR antibodies. After evaluating all three antibodies, we selected the antibody that produced the strongest signal in mouse kidney for our 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 green fluorescent protein-tagged (GFP-tagged) V1aR in human embryonic kidney cells (HEK293) as positive controls. For further validation of the antibody immunoprecipitation and western blot analysis were performed. Polymerase chain reaction (PCR) was used to insert the FLAG epitope, DYKDDDDK, between the initial methionine residue and the second amino acid of the mouse V1bR. Construction of the expression plasmid for mouse V1aR was described previously by Kashiwazaki A.[20] Empty vector and mammalian expression plasmids for V1aR, or FLAG-V1bR were transiently transfected into HEK293 cells in 100-mm dishes using Fugene HD reagent

(Promega). Twenty-four hours after transfection, the cells were washed with phosphate-buffered saline (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 4ºC for 1 h and precipitated with protein G Sepharose (GE Healthcare Life Sciences). For western blot analysis, anti-V1aR 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 (HRP) conjugated antibody against mouse immunoglobulin G (#W4021, Promega, 1:20000), secondary HRP-conjugated antibodies for rabbit (#458, Medical & Biological Laboratories Co. LTD, Japan, 1:5000).

3.3 Cell culture experiments

3.3.1 Overexpression of the V1aR in HEK293 cells

We further verified our anti-V1aR antibody by overexpression experiments with V1aR- and control plasmids in cell culture. For this purpose 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. Cells on coverslips were then were fixed with 3% paraformaldehyde (#30525-89-4, Merck KGaA, Darmstadt, Germany) in tris-buffered saline (pH 7.57) for 10 minutes. After fixation, we washed the cells shortly with tris-buffered saline (TBS). After washing, cells were permeabilized using 0.1% Triton X-100 (#9036-19-5, Merck KGaA, Darmstadt, Germany) in TBS for 10 minutes, blocked with 5% bovine serum albumin (BSA) (#11930.04, SERVA Electrophoresis GmbH, Heidelberg, Germany) in TBS for 30 minutes. The coverslips with the attached cells 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 treatment with an anti-rabbit Cy3-coupled IgG (711-165-152, DIANOVA GmbH, Hamburg, Germany) for two hours at room temperature. Next, followed 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) using 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).

3.3.2 Primary cell culture of inner medullary collecting duct cells

We used the protocol published by the group of E. Klussmann at the Max Delbrück Center for Molecular Medicine in Berlin to culture inner medullary collecting duct (IMCD) cells.[21] 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 adult (12-14 weeks) male Wistar rats per experiment were anesthetized by isoflurane inhalation (#B506, Forene®, AbbVie Deutschland, Germany) and sacrificed by decapitation. We removed the kidneys and separated the inner medulla from the rest of the kidney with a sharp, curved scissor and place the material immediately in ice-cold dulbecco's phosphate-buffered saline (DPBS) (#14190144, Gibco™, Life technologies™, Carlsbad, CA, US). The tissue was then digested in a 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 re-suspended in freshly prepared dulbecco's modified eagle m*edium* (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. Luis, MO, USA) and 0.25 µg/ml gentamicin (#G1264, Sigma-Aldrich, St. Luis, MO, USA). We seeded the cells on permeable filter support (#3450, Transwell® Permeable Supports, Corning Incorporated, Kennebunk, USA). After growing to full confluence, we treated the cells with the V1aR agonist A0-4-67 ([Phe^2,Orn^8]VT([Phe^2]OVT); 1.3 *µm*) or with 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 4′,6-Diamidin-2-phenylindol (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).

3.4 Immunofluorescence and immunohistochemistry

For our localization studies, we used paraffin-embedded kidneys of mice and rats. The animals were anesthetized by an intraperitoneal (IP) injection of 0.07 mg/g body weight pentobarbital sodium (#6088986, Narcoren®, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany). After laparotomy and preparation of the big abdominal vessels, we perfused the kidneys through the infrarenal aorta for 15 seconds with PBS followed perfusion-fixation with 3% paraformaldehyde (#1.04005, Merck KGaA, Darmstadt, Germany) in PBS over 5 minutes. Kidneys were then removed, dissected, placed in tissue cassettes (#10794582, Thermo ScientificTM ShandonTM Gewebekassetten, Fisher Scientific GmbH, Schwerte, Germany) and stored in a solution of PBS/sucrose (330 mOsm/kg H20, pH 7.4). Paraffin embedding was performed shortly after the preparation of the kidneys by the pathology department of the Charité – Universitätsmedizin Berlin. Paraffin blocks were then cut in 4 μm sections using a microtome (Leica RM2125RT, Leica Microsystems, Wetzlar, Germany), placed on object slides (Super Frost Plus®, R. Langenbrinck GmbH Labor- und Medizintechnik, Emmendingen, Germany) using a water bath (water bath 1052, GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany) and dried overnight at a temperature of 37 degrees (Drying cabinet, GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany). For immunofluorescence staining, the slices were dewaxed with xylene (#8080, J.T.Baker, Avantor Performance Materials BV, Deventer, Netherlands) and rehydrated with 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-labeling, the primary antibodies were applied consecutively, separated by three 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 (NA 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. 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).

3.5 Immunoblotting

Kidney, livers, and brains of mice (C57BL/6J) were cut in little pieces using a razor blade, placed in 2 ml microtubes (#72.691, SARSTEDT AG & Co., Nürnbrecht, Germany) and stored in liquid nitrogen. After freezing, probes were crushed in a mortar, filled with liquid nitrogen using a pestle. The tissue powder of each sample was collected with a cold scalpel, transferred in a fresh precooled 1.5 ml microtube (#72706, SARSTEDT AG & Co., Nürnbrecht, Germany), and temporarily stored in liquid nitrogen. Depending on the amount of tissue, 500 µl to 750 µl of homogenization buffer containing 250 mM sucrose, 10 mM triethanolamine, protease inhibitors (complete, #11697498001, Roche Diagnostics GmbH, Mannheim, Germany), and phosphatase inhibitors (PhosSTOP *EASYpack*, #04906845001, Roche Diagnostics GmbH, Mannheim, Germany) were added and samples thawed on ice. The disintegration of the homogenized probes was further achieved by sonication (Sonoplus GM70, BANDELIN electronic GmbH & Co. KG, Berlin, Germany). Cell debris and nuclei were removed by centrifugation at 1000 x g for 10 min at 4°C (MIKRO200R, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). The

supernatant was secured in a fresh 1.5 ml microtube (#72706, SARSTEDT AG & Co., Nürnbrecht, Germany) tube and stored on ice; the pellet was discharged. For a colorimetric detection and quantitative analysis of the total protein amount in the supernatant, a bicinchoninic acid protein assay reagent kit (#23235, Micro BCA Protein Assay Kit, Fisher Scientific GmbH, Schwerte, Germany) was used. The resulting water-soluble complex has an absorbance at 562 nm, linear with the protein concentration. Samples were incubated for 2 hours at 37°C and absorbance was measured (Microplate reader ASYS Expert 96, BioChrom Ltd., Cambridge, United Kingdom). Protein concentration was then calculated from a standard curve using albumin (#11930, Albumin Bovine Fraction V, ph 7.0, SERVA Electrophoresis GmbH, Heidelberg, Germany). The Samples were dissolved in a buffer (2 % SDS, 10 % glycerol, 5 % ß-mercaptoethanol, 1 % bromphenol blue, 95 mM Tris, pH 6,8) and incubated for 15 minutes at 65°C. Protein separation was carried out by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an electrophoresis chamber(Bio-Rad Laboratories GmbH, München, Germany) filled with buffer (SDS 0,1 %, glycin 192 mM, Tris 25 mM; pH 8,3; 8–10% acrylamide gel). Proteins were transferred to a nitrocellulose membrane (#741280, Porablot NCP, Macherey-Nagel GmbH & Co. KG, Düren, Germany) in a tank-plotting-system (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The membranes were incubated with 0.1% Ponceau red staining (#P3504, Merck KGaA, Darmstadt, Germany) to verify the successful transfer and equity of protein loading. Blocking of unspecific binding was performed with 5% skim milk (#232100, Difco™ Skim Milk, BD, Becton, Dickinson and Company, Franklin Lakes USA) in PBS. Membranes were sealed in plastic film and incubated with the respective primary antibodies overnight at 4°C. Polyclonal rabbit anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) was used to normalize all data for expression of the housekeeping gene GAPDH. Proteins were finally visualized by chemiluminescence, and signals were detected by as ECL- and Fluorescence Imager (Chemostar, Intas Science Imaging Instruments GmbH) followed by evaluation with Fiji Image J Software (National Institutes of Health, Bethesda, MD, USA).

3.6 Effects of V1aR agonist and antagonist in mice

We randomly assigned male adult (10-12 weeks) C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) to the experimental and control group and induced anesthesia 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. After induction, anesthesia was 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. The depth of anesthesia was assessed during the experiment by testing the withdrawal reflexes on the lower limbs of the mice. Each experiment lasted 90 minutes, and urine volume was measured every 5 minutes by calibrated glass capillaries, whereas urine samples were

collected every 5 minutes. For continuous pH measurement and urine collection, the urinary bladder was catheterized via a small incision in the abdomen. 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. The 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, [Phe^2,Orn^8]VT([Phe^2]OVT)[22] (2 µg/kg body weight) or the vehicle (0.9% saline) by intraperitoneal bolus injection. Measurements of pH and urine collection were continued for another 60 minutes. Experiments were repeated using the V1aR antagonist, CL-14-102, d(CH_2)$_5$[Tyr(Me)^2,Dab^5]AVP.[23] Metabolic acidosis was induced by providing NH_4CL enriched chow for three days as prescribed previously.

3.7 Effects of V1aR stimulation in vasopressin-deficient rats

For further evaluation of V1aR stimulation *in vivo*, we studied the effects of the V1aR agonist AO-4-67, [Phe^2,Orn^8]VT([Phe^2]OVT),[22] in VP-deficient Brattleboro rats that were placed in metabolic cages during the experiments. We used a total number of 16 male Brattleboro rats, 13 to 15 weeks old, each weighing 300-380g that had free access to distilled water and standard rat chow. Rats were randomly divided into two groups (each n=8). Starting with the first subgroup (n=8), animals were coincidentally divided into experimental (n=4) and control group (n=4) and placed in 8 metabolic cages. After three days of recovery in regular cages, 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 control and 4 rats in the experimental group was considered as an independent experiment. Each independent experiment started on 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 at 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 IP). 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 regular 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 IP) with a 3 days interval for recovery in regular cages between the experiments. After the application of these three doses and a further recovery period in regular 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 vehicle according to the protocol above. This

experimental design resulted in consecutive treatment of all 16 Brattleboro rats with a 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 sacrificed by decapitation. Blood samples were taken from the cervical arteries directly into 1.3 ml microtubes containing ethylenediaminetetraacetic acid (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). The 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).

3.8 Measurement of urinary net acid excretion

The determination of urinary net acid excretion (NAE) in Brattleboro rats after V1aR stimulation with the V1aR agonist A0-4-67 (10 µg/kg body weight IP) was carried out by manual titration with 0.1 N NaOH as described by Chan JC to the endpoint of pH 7.40 at P_{CO2}=0 mmHg, at a temperature of 37°C.[24] We evaluated the experimental setting and the protocol for a reduced urine volume of 500 µl per sample by measuring the same urine probe from a healthy human male individual and from a male Brattleboro rat multiple times. 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 micro 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 to cool down and then in a water bath of 37°C to allow temperature adjustment. For the 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 JC; briefly:

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

$$\text{Net acid excretion} = \text{titratable acid} - \text{bicarbonate} + \text{ammonia}.$$

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

3.9 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 C57BL/6J 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-ketoglutarate, 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 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein (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 as described by Greger R and Hampel W.[25] 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: 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 μmol/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 ([arg^8]vasopressin, 50 nM) for 3 minutes. The goal was to compare the effect caused by the V1aR agonist, AO-4-67, [Phe^2,Orn^8]VT([Phe^2]OVT),[22] 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 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 μmol/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 washout 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.

3.10 Data analysis

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. 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. The effects between different V1aR-AG concentrations and AVP were compared by a Kruskal-Wallis- test followed by Dunn's multiple comparisons test. A p-value of <0.05 was chosen to indicate statistical significance.

4 Results

VP is crucial for maintaining water homeostasis in the body by activating renal salt and water conservation mechanisms mainly by the activation of the V2R in the kidney.[8] The V1aR in the kidney has been studied to a lesser extent compared to the V2R. Several studies have addressed its renal distribution. However, the results are inconclusive. Particularly little information is available about the intracellular localization of the receptor. [15,16,26]

To provide more valid localization data, we generated a new polyclonal anti-V1aR antibody. We immunized six rabbits using a synthetic peptide with a high homology to rat and human V1aR and low similarities to V1bR and V2R. The resulting affinity-purified anti-V1aR antibodies were applied for localization studies.

4.1 V1aR distribution in rodent and human kidneys

Firstly, we performed immunoperoxidase stainings of mouse kidneys using the new anti-V1aR antibody and evaluated them by light microscopy. A basolateral V1aR signal was present in scattered cells of the connecting tubule (CNT) and collecting duct (CD), which were identified as intercalated cells (ICs) according to morphologic criteria such as bulging out into the luminal space.[9] Other ICs demonstrated a perinuclear to apical V1aR immunoreactivity.

To assign these distinct patterns of V1aR immunoreactivity to the intercalated cell types, we performed multi-labeling of principal cell- and intercalated cell-marker proteins in mouse, rat, and human kidney sections. Aquaporin-2 (AQP2) was applied as a marker for principal cells (PCs), and pendrin served as a marker for type B intercalated cells (B-ICs). A basolateral V1aR signal was present in AQP2- and pendrin-negative type A intercalated cells (A-ICs), whereas perinuclear and apical signal patterns, were observed in pendrin-positive B-ICs across the studied species.

In contrast, PCs showed no significant V1aR immunoreactivity. This finding is significant since previous data suggested the presence of the V1aR in both intercalated cells (ICs), and principal cells(PCs).[16] A minor proportion of ICs showed intermediate diffuse V1aR signal patterns likely reflecting the transition state of non-A, non-B ICs.[19] Apart from in the CDs, substantial basolateral V1aR signal was identified in macula densa cells of the mouse kidney. However, these could not be reproduced in the rat or human species. Other nephron segments showed no significant V1aR immunoreactivity in either species.

To study the distinct intracellular V1aR distribution patterns in more detail, we took advantage of the structured illumination microscopy (SIM) combined with 3D-reconstruction. This high-resolution technique showed V1aR-positive, vesicle-like structures, which were associated with the basolateral membrane in A-ICs and enriched in the subapical and perinuclear cell compartments of B-ICs.

Furthermore, we used microdissected mouse nephron segments to investigate the expression of the V1aR along the nephron on mRNA level by real-time polymerase chain reaction (RT-PCR). We found strong signals in the connecting tubule and collecting duct segments, as well as in glomeruli with attached macula densa cells. In contrast to earlier studies, we could not confirm its presence in the thick ascending limb (TAL).[13,18,27] Nevertheless, these results are consistent with the findings of our antibody study at protein level.

Since the V1aR localization data were obtained with a new antibody, we paid particular attention to the verification of its specificity using several positive and negative controls. Labeling of kidney sections from V1aR-deficient mice with the anti-V1aR antibody produced no significant signal, suggesting that immunoreactive patterns obtained in rodent or human kidneys reflect the specific binding of the antibody to V1aR. Immunoblotting using this antibody produced strong immunoreactive bands of predicted molecular size (45 kDa) in extracts from mouse liver, and weaker signals in lysates from mouse brain or kidney tissues.[15,28] Transfection of cultured HEK293 cells with either V1aR or V1bR followed by immunoblotting using the anti-V1aR antibody produced significant V1aR immunoreactivity in V1aR- but not in V1bR-transfected cells.

Therefore, the specificity of our anti-V1aR antibody was confirmed by several independent control experiments, which validated our localization data.

4.2 Effects of V1aR-deficiency on the distribution of Intercalated cells

Because of the reported morphological plasticity of ICs, we decided to evaluate the effects of V1aR signaling on the proportions of A-ICs vs. B-ICs using V1aR-deficient mice.[29,30] Quantification of PCs, A-ICs, and B-ICs in kidney sections, triple-labeled for AQP2, pendrin, and V-ATPase, showed no significant differences between wild-type and V1aR-deficient mice, suggesting that V1aR-signaling is not essential for proportional distribution of IC-types under normal conditions.

4.3 V1aR mediated effects *in vivo*

To characterize functional effects mediated by V1aR activation, we performed *in vivo* studies using the V1aR agonist, AO-4-67, [Phe^2, Orn^8]VT([Phe^2]OVT),[22] and the V1aR antagonist, CL-14-102, d$(CH_2)_5$[Tyr(Me)^2,Dab^5]AVP,[23] kindly provided by Prof. Manning (College of Medicine, Toledo, OH, USA).

4.3.1 Effects of V1aR stimulation in mice

We treated urinary bladder-catheterized mice (C57BL/6J) with the V1aR agonist or vehicle. Compared to the vehicle, the treatment of mice with the V1aR agonist significantly decreased the urinary pH from 7.18 to 6.78, suggesting urinary acidification.

4.3.2 Effects of V1aR stimulation in vasopressin-deficient rats

To further characterize the effects of V1aR activation, we treated vasopressin-deficient Brattleboro rats with the V1aR agonist and evaluated their acid-base balance using metabolic cages. The animals received three different doses of the V1aR agonist as a single intraperitoneal injection (200 ng/kg, 2 µg/kg, or 10 µg/kg body weight). We collected the urine hourly under mineral oil to avoid contamination of the probes with carbon dioxide from the air. The highest dose significantly reduced the urinary pH [from 7.40 to 6.71, 6.71, 6.56, and 6.53 after 1, 2, 3, and 4 hours; $P<0.01$], diminished urinary bicarbonate excretion (-82%, -92%, and -85% after 2, 3 and 4 hours; $P<0.001$) and produced a trend for increased ammonia (NH_4^+). Calculation of the net acid excretion (NAE) revealed significant increases in animals receiving the V1aR agonist. The NAE was measured by manual titration of the urine probes according to the protocol published by Chan JC with some adaptations.[24] We calculated the NAE with the formula: NAE = titratable acid - bicarbonate + ammonia.

4.3.3 V1aR mediated effects during metabolic acidosis

Since V1aR-induced urinary proton secretion may be involved in renal adaptations to acidotic states, we studied the role of V1aR during acid load. To this end, we have taken advantage of an established mouse-model of metabolic acidosis resulting from NH_4Cl administration with chow for three days.[31] Neither intracellular V1aR distribution in A-ICs or B-ICs, nor proportional distribution of different IC-types was altered in acidotic mice. Moreover, the application of the V1aR antagonist in acidotic, bladder-catheterized mice produced no significant changes in urinary pH compared to the vehicle group suggesting that V1aR signaling is not involved in the compensation of metabolic acidosis, at least in this setting.

4.4 V1aR mediated effects *ex vivo*

4.4.1 Effects of V1aR stimulation in primary inner medullary collecting duct cells

Two transporters are mainly responsible for proton secretion in A-ICs, both localized in the apical membrane. The V-ATPase is a uniporter that only secretes protons. In contrast, the H^+/K^+-ATPase transports protons into the lumen in exchange for potassium.[19] We tested V1aR-mediated effects on V-ATPase in inner medullary collecting duct (IMCD) cells isolated from rat kidney papilla.[21] Labeling of cultured IMCD cells for V1aR and V-ATPase revealed the presence of both products in A-ICs, which were scattered between AQP2-positive PCs. We treated these cells grown on a permeable support, to full confluence from the basolateral side with the V1aR agonist, AO-4-67 (1 μm), or vehicle for four hours and evaluated the V-ATPase signal intensity and distribution in A-ICs using confocal *z*-stacks. Treatment with the V1aR agonist substantially enhanced the apical V-ATPase signal intensity in A-ICs, suggesting the V1aR-mediated activation of V-ATPase. Assuming the same effect in our *in vivo* experiments, stimulation of V-ATPase may partially explain for the observed urinary acidification.

4.4.2 Effects of V1aR stimulation in isolated collecting duct segments

In an additional set of experiments, we assessed V1aR-mediated effects in isolated perfused outer medullary CDs from mouse kidney. Binding of VP to the V1aR has been reported to induce intracellular calcium release via activation of the phospholipase C (PLC) pathway.[4] Therefore, we evaluated the intracellular Ca^{2+} levels $[Ca^{2+}]_i$, as well as the luminal pH in CDs using Ca^{2+}- or pH-sensitive fluorescent dyes. Application of the V1aR agonist or AVP to the basolateral side of the collecting ducts significantly increased $[Ca^{2+}]_i$ in ICs to a comparable extent. Parallel measurement of the luminal pH revealed decreased levels after the application of the V1a agonist or AVP, suggesting that V1aR activation promotes luminal proton secretion.

5 Discussion

The hormone vasopressin (VP), also known as the antidiuretic hormone (ADH), is critical to the urinary concentration and volume homeostasis.[8] While the water reabsorption is chiefly governed by the V2R-mediated response to VP, activation of V1aR appears to exert modulating effects on the renal electrolyte handling, particularly, on the acid-base homeostasis.[13,17] The role of renal V1aR remained elusive, but the recent characterization of V1aR knockout mice shed some light on its functions in the kidney.[11] The phenotyping of V1aR-deficient mice revealed impaired acid-base balance.[17] These results may have significant clinical impact since dysregulation of the extracellular pH, and impaired acid-base status have been associated with higher morbidity and mortality in humans. Chronic metabolic acidosis contributes to osteopenia, osteoporosis, and peripheral insulin resistance.[32,33] Insufficient urinary acidification may facilitate kidney stone formation, leading to recurrent stones in the urogenital tract. A low urinary pH prevents crystal formation, which is seen as the starting point of stone development.[34] Despite these clinical conditions, associated with impaired acid-base homeostasis, the role of VP and V1aR herein has received only little attention even after the generation of V1aR knockout mice. This study extends the morphological and functional information related to the role of V1aR in the renal acid-base handling.

Previous studies demonstrated V1aR expression in the connecting tubules (CNTs) and collecting ducts (CDs), but its cell type-specific and intracellular distribution remained debatable.[15,16] Early functional studies with isolated CDs of rabbit kidneys suggested apical V1aR distribution in intercalated (ICs) or principal cells (PCs). In these experiments, the luminal application of [arg^8]vasopressin (AVP) exerted effects on the transepithelial resistance and intracellular calcium levels, indicating the presence of an apical vasopressin receptor.[35] The hypothesis of apical V1aR distribution is promising since VP is filtered into the urine and reaches concentrations that are 50-100 times higher than those in plasma. The high luminal hormone levels would then rapidly activate any apical vasopressin receptor.[36] However, previous studies provided no convincing evidence for the apical V1aR localization. In contrast, other studies demonstrated basolateral effects of V1aR stimulation in rodents but failed to define the responsive cell type.[37]

To clarify this issue, we addressed the segment- and cell type-specific aspects of V1aR distribution in mouse, rat, and human kidneys using a new anti-V1aR antibody. The clear basolateral, membrane-bound V1aR signal in type A intercalated cells (A-ICs) suggests that this cell type responds to the plasma vasopressin rather than to the filtered hormone. Although type B intercalated cells (B-ICs) showed a perinuclear to subapical V1aR signal, high-resolution imaging revealed no association of the V1aR signal with the apical membrane in this cell type. Nevertheless, minor luminal surface expression of the receptor below the detection limit of our

antibody may enable a particular responsiveness of ICs to the luminal VP, especially when considering its high urinary concentrations. In this context, the intracellular accumulation of the receptor in B-ICs may reflect a rapid AVP-binding to the luminal V1aR followed by the internalization of the receptor.[38] Following this reasoning, one would expect a clear membrane-bound signal in VP-deficient Brattleboro rats. However, these animals lacked the apical V1aR signal as well. Based on our localization data, we suggest that B-ICs do not directly respond to vasopressin.

This coincides with previous studies, showing that neither V1aR deletion in mice nor V1aR knockdown of the receptor in cultured ICs altered the expression of pendrin, which is the major transporter of B-ICs.[17] In contrast, pendrin expression was suppressed in V2R-deficient mice, whereas the treatment of VP-deficient Brattleboro rats with the V2R agonist desmopressin increased pendrin expression. Since V2R receptor is not expressed in ICs[39], we conclude that these effects may result from paracrine signaling events between ICs and the neighboring V2R-expressing PCs.[29,40]

Notably, apart from the clear basolateral V1aR signal in A-ICs or sub-apical signals in B-ICs, we observed a small population of ICs showing discrete V1aR signal patterns, which do not match those of A-ICs or B-ICs. We believe that these cells represent a transitional state and belong to the so-called non-A, non-B intercalated cells. In general, adaptation to the stress of metabolic acidosis can be accomplished either by changes in the number of intercalated cells or by a shift from type B to type A intercalated cells.[19] The transition from a type B to a type A intercalated cell depends on the presence of particular proteins, such as the extracellular matrix protein hensin or galectin 3. Mice with a defect in the protein hensin develop metabolic acidosis due to the heavily reduced number of A-ICs in the cortex or the kidney.[30] Our morphological analysis of V1aR-deficient mice did not reveal any changes in proportions of A-ICs vs. B-ICs, suggesting that V1aR-signaling is not critical to the proper differentiation of intercalated cells.

Our functional experiments supported our localization data and showed direct effects of VP in A-ICs. We used a specific V1aR agonist or antagonist in rodents to investigate changes in parameters of acid-base status. In VP-deficient Brattleboro rats, stimulation with the V1aR agonist leads to decreased urinary pH and increases the net acid excretion (NAE). Given the absence of endogenous VP we expected a particularly strong response to the V1aR agonist, but only a relatively high dose was efficient. One possible explanation for this finding could be the altered urinary buffering capacities in Brattleboro rats due to extreme diuresis and volume depletion.

Similar to Brattleboro rats, the application of a high V1aR-agonist dose rapidly decreased the urinary pH in bladder catheterized mice. This effect may be related to V1aR-induced activation of A-ICs.

To characterize local effects of V1aR in the absence of concomitant systemic stimuli, we performed *ex vivo* experiments in primary cell culture of inner medullary collecting duct (IMCD) cells and in isolated perfused collecting ducts (CDs). Basolateral application of the V1aR agonist or VP induced several effects supporting the *in vivo* data. These effects include intracellular calcium release and decrease of luminal pH in isolated perfused CDs, as well as apical V-ATPase immunoreactivity in cultured IMCD cells. The latter likely reflect V-ATPase trafficking[41], although increased protein abundance due to stimulated translation may contribute as well. Other studies reported angiotensin II and aldosterone as potent stimuli for the V-ATPase.[41] Interestingly, aldosterone seems to require the presence of the V1aR to mediate the proton secretion in ICs. Knockdown of the V1aR *in vitro* abrogated the effects of aldosterone on V-ATPase stressing the role of V1aR signaling in the regulation of this proton pump.[17] Further work is necessary to elucidate the effects of V1aR activation on the H^+/K^+-ATPase, which is another proton secreting transporter in the apical membrane of A-ICs. Due to the lack of suitable technical tools, we were not able to address this issue.

Since our results implicated V1aR in urinary proton secretion, we assumed that this mechanism might contribute to renal adaptations to systemic shifts of the acid-base homeostasis, such as metabolic acidosis. In this context, previous work reported elevated V1aR mRNA expression in ICs after acid load.[13] We therefore evaluated the V1aR distribution in mice with metabolic acidosis due to an NH_4CL enriched diet.[31] In contrast to previous data, our results showed no effects of metabolic acidosis on V1aR protein abundance or distribution, suggesting the absence of compensatory alterations at this level. Application of the V1aR antagonist did not affect urinary pH in this model as well, pointing to a minor role of V1aR signaling in adaptation to metabolic acidosis.

In conclusion, this study significantly extends morphological and physiological information on the V1aR-mediated signaling in the kidney and provides translational perspectives for modulation of the renal acid-base handling using selective V1aR agonists and antagonists.

6 References

1. Treschan TA, Peters J. The vasopressin system: physiology and clinical strategies. *Anesthesiology*. 2006;105(3):599-612; quiz 639-640. doi:10.1097/00000542-200609000-00026

2. Bankir L, Bichet DG, Morgenthaler NG. Vasopressin: physiology, assessment and osmosensation. *J Intern Med*. 2017;282(4):284-297. doi:10.1111/joim.12645

3. Birnbaumer M. Vasopressin receptors. *Trends Endocrinol Metab TEM*. 2000;11(10):406-410. doi:10.1016/s1043-2760(00)00304-0

4. Koshimizu T, Nakamura K, Egashira N, Hiroyama M, Nonoguchi H, Tanoue A. Vasopressin V1a and V1b receptors: from molecules to physiological systems. *Physiol Rev*. 2012;92(4):1813-1864. doi:10.1152/physrev.00035.2011

5. Share L. Role of vasopressin in cardiovascular regulation. *Physiol Rev*. 1988;68(4):1248-1284. doi:10.1152/physrev.1988.68.4.1248

6. Oliver G, Schäfer EA. On the Physiological Action of Extracts of Pituitary Body and certain other Glandular Organs: Preliminary Communication. *J Physiol*. 1895;18(3):277-279. doi:10.1113/jphysiol.1895.sp000565

7. Bankir L, Bouby N, Ritz E. Vasopressin: a novel target for the prevention and retardation of kidney disease? *Nat Rev Nephrol*. 2013;9(4):223-239. doi:10.1038/nrneph.2013.22

8. Knepper MA, Kwon TH, Nielsen S. Molecular physiology of water balance. *N Engl J Med*. 2015;372(14):1349-1358. doi:10.1056/NEJMra1404726

9. Alpern R, Caplan M, Moe OW, eds. *Seldin and Giebisch's The Kidney: Physiology and Pathophysiology*. 5 edition. Academic Press; 2013.

10. Christ-Crain M, Bichet DG, Fenske WK, Goldman MB, Rittig S, Verbalis JG, Verkman AS. Diabetes insipidus. *Nat Rev Dis Primer*. 2019;5(1):54. doi:10.1038/s41572-019-0103-2

11. Koshimizu T, Nasa Y, Tanoue A, Oikawa R, Kawahara Y, Kiyono Y, Adachi T, Tanaka T, Kuwaki T, Mori T, Takeo S, Okamura H, Tsujimoto G. V1a vasopressin receptors maintain normal blood pressure by regulating circulating blood volume and baroreflex sensitivity. *Proc Natl Acad Sci U S A*. 2006;103(20):7807-7812. doi:10.1073/pnas.0600875103

12. Aoyagi T, Izumi Y, Hiroyama M, Matsuzaki T, Yasuoka Y, Sanbe A, Miyazaki H, Fujiwara Y, Nakayama Y, Kohda Y, Yamauchi J, Inoue T, Kawahara K, Saito H, Tomita K, Nonoguchi H, Tanoue A. Vasopressin regulates the renin-angiotensin-aldosterone system via V1a receptors in macula densa cells. *Am J Physiol Renal Physiol*. 2008;295(1):F100-107. doi:10.1152/ajprenal.00088.2008

13. Yasuoka Y, Kobayashi M, Sato Y, Zhou M, Abe H, Okamoto H, Nonoguchi H, Tanoue A, Kawahara K. The intercalated cells of the mouse kidney OMCD(is) are the target of the vasopressin V1a receptor axis for urinary acidification. *Clin Exp Nephrol*. 2013;17(6):783-792. doi:10.1007/s10157-013-0783-y

14. Chen L, Lee JW, Chou CL, Nair AV, Battistone MA, Păunescu TG, Merkulova M, Breton S, Verlander JW, Wall SM, Brown D, Burg MB, Knepper MA. Transcriptomes of major renal collecting duct cell types in mouse identified by single-cell RNA-seq. *Proc Natl Acad Sci U S A*. 2017;114(46):E9989-E9998. doi:10.1073/pnas.1710964114

15. Gonzalez CB, Figueroa CD, Reyes CE, Caorsi CE, Troncoso S, Menzel D. Immunolocalization of V1 vasopressin receptors in the rat kidney using anti-receptor antibodies. *Kidney Int*. 1997;52(5):1206-1215. doi:10.1038/ki.1997.445

16. Carmosino M, Brooks HL, Cai Q, Davis LS, Opalenik S, Hao C, Breyer MD. Axial heterogeneity of vasopressin-receptor subtypes along the human and mouse collecting duct. *Am J Physiol Renal Physiol*. 2007;292(1):F351-360. doi:10.1152/ajprenal.00049.2006

17. Izumi Y, Hori K, Nakayama Y, Kimura M, Hasuike Y, Nanami M, Kohda Y, Otaki Y, Kuragano T, Obinata M, Kawahara K, Tanoue A, Tomita K, Nakanishi T, Nonoguchi H. Aldosterone requires vasopressin V1a receptors on intercalated cells to mediate acid-base homeostasis. *J Am Soc Nephrol JASN*. 2011;22(4):673-680. doi:10.1681/ASN.2010050468

18. Tashima Y, Kohda Y, Nonoguchi H, Ikebe M, Machida K, Star RA, Tomita K. Intranephron localization and regulation of the V1a vasopressin receptor during chronic metabolic acidosis and dehydration in rats. *Pflugers Arch*. 2001;442(5):652-661. doi:10.1007/s004240100590

19. Roy A, Al-bataineh MM, Pastor-Soler NM. Collecting duct intercalated cell function and regulation. *Clin J Am Soc Nephrol CJASN*. 2015;10(2):305-324. doi:10.2215/CJN.08880914

20. Kashiwazaki A, Fujiwara Y, Tsuchiya H, Sakai N, Shibata K, Koshimizu T. Subcellular localization and internalization of the vasopressin V1B receptor. *Eur J Pharmacol*. 2015;765:291-299. doi:10.1016/j.ejphar.2015.08.043

21. Faust D, Geelhaar A, Eisermann B, Eichhorst J, Wiesner B, Rosenthal W, Klussmann E. Culturing Primary Rat Inner Medullary Collecting Duct Cells. *JoVE J Vis Exp*. 2013;(76):e50366-e50366. doi:10.3791/50366

22. Manning M, Sawyer WH. Design, synthesis and some uses of receptor-specific agonists and antagonists of vasopressin and oxytocin. *J Recept Res*. 1993;13(1-4):195-214. doi:10.3109/10799899309073655

23. Chan WY, Wo NC, Cheng LL, Manning M. Isosteric substitution of Asn5 in antagonists of oxytocin and vasopressin leads to highly selective and potent oxytocin and V1a receptor antagonists: new approaches for the design of potential tocolytics for preterm labor. *J Pharmacol Exp Ther*. 1996;277(2):999-1003.

24. Chan JC. The rapid determination of urinary titratable acid and ammonium and evaluation of freezing as a method of preservation. *Clin Biochem*. 1972;5(2):94-98. doi:10.1016/s0009-9120(72)80014-6

25. Greger R, Hampel W. A modified system for in vitro perfusion of isolated renal tubules. *Pflugers Arch*. 1981;389(2):175-176. doi:10.1007/bf00582110

26. Ostrowski NL, Young WS, Knepper MA, Lolait SJ. Expression of vasopressin V1a and V2 receptor messenger ribonucleic acid in the liver and kidney of embryonic, developing, and adult rats. *Endocrinology*. 1993;133(4):1849-1859. doi:10.1210/endo.133.4.8404628

27. Terada Y, Tomita K, Nonoguchi H, Yang T, Marumo F. Different localization and regulation of two types of vasopressin receptor messenger RNA in microdissected rat nephron segments using reverse transcription polymerase chain reaction. *J Clin Invest*. 1993;92(5):2339-2345.

28. Morel A, O'Carroll AM, Brownstein MJ, Lolait SJ. Molecular cloning and expression of a rat V1a arginine vasopressin receptor. *Nature*. 1992;356(6369):523-526. doi:10.1038/356523a0

29. Schwartz GJ, Gao X, Tsuruoka S, Purkerson JM, Peng H, D'Agati V, Picard N, Eladari D, Al-Awqati Q. SDF1 induction by acidosis from principal cells regulates intercalated cell subtype distribution. *J Clin Invest*. 2015;125(12):4365-4374. doi:10.1172/JCI80225

30. Gao X, Eladari D, Leviel F, Tew BY, Miró-Julià C, Cheema FH, Miller L, Nelson R, Paunescu TG, McKee M, Brown D, Al-Awqati Q. Deletion of hensin/DMBT1 blocks conversion of - to -intercalated cells and induces distal renal tubular acidosis. *Proc Natl Acad Sci*. 2010;107(50):21872-21877. doi:10.1073/pnas.1010364107

31. Nowik M, Kampik NB, Mihailova M, Eladari D, Wagner CA. Induction of Metabolic Acidosis with Ammonium Chloride (NH_4Cl) in Mice and Rats – Species Differences and Technical Considerations. *Cell Physiol Biochem*. 2010;26(6):1059-1072. doi:10.1159/000323984

32. DeFronzo RA, Beckles AD. Glucose intolerance following chronic metabolic acidosis in man. *Am J Physiol-Endocrinol Metab*. 1979;236(4):E328. doi:10.1152/ajpendo.1979.236.4.E328

33. Jehle S, Zanetti A, Muser J, Hulter HN, Krapf R. Partial Neutralization of the Acidogenic Western Diet with Potassium Citrate Increases Bone Mass in Postmenopausal Women with Osteopenia. *J Am Soc Nephrol*. 2006;17(11):3213-3222. doi:10.1681/ASN.2006030233

34. Tessitore N, Ortalda V, Fabris A, D'Angelo A, Rugiu C, Oldrizzi L, Lupo A, Valvo E, Gammaro L, Loschiavo C, Panzetta G, Panebianco R, Bedogna V, Maschio G. Renal acidification defects in patients with recurrent calcium nephrolithiasis. *Nephron*. 1985;41(4):325-332. doi:10.1159/000183609

35. Ando Y, Tabei K, Asano Y. Luminal vasopressin modulates transport in the rabbit cortical collecting duct. *J Clin Invest*. 1991;88(3):952-959. doi:10.1172/JCI115398

36. Bankir L. Antidiuretic action of vasopressin: quantitative aspects and interaction between V1a and V2 receptor-mediated effects. *Cardiovasc Res*. 2001;51(3):372-390.

37. Naruse M, Yoshitomi K, Hanaoka K, Imai M, Kurokawa K. Electrophysiological study of luminal and basolateral vasopressin in rabbit cortical collecting duct. *Am J Physiol-Ren Physiol*. 1995;268(1):F20-F29. doi:10.1152/ajprenal.1995.268.1.F20

38. Terrillon S, Barberis C, Bouvier M. Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with β-arrestin and their trafficking patterns. *Proc Natl Acad Sci U S A*. 2004;101(6):1548-1553. doi:10.1073/pnas.0305322101

39. Mutig K, Borowski T, Boldt C, Borschewski A, Paliege A, Popova E, Bader M, Bachmann S. Demonstration of the functional impact of vasopressin signaling in the thick ascending limb by a targeted transgenic rat approach. *Am J Physiol Renal Physiol*. 2016;311(2):F411-423. doi:10.1152/ajprenal.00126.2016

40. Pearce D, Soundararajan R, Trimpert C, Kashlan OB, Deen PMT, Kohan DE. Collecting duct principal cell transport processes and their regulation. *Clin J Am Soc Nephrol CJASN*. 2015;10(1):135-146. doi:10.2215/CJN.05760513

41. Wagner CA, Devuyst O, Bourgeois S, Mohebbi N. Regulated acid–base transport in the collecting duct. *Pflüg Arch - Eur J Physiol*. 2009;458(1):137-156. doi:10.1007/s00424-009-0657-z

7 Statutory declaration (Eidesstattliche Versicherung)

„Ich, Torsten Giesecke, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Die Aktivierung des Vasopressin Type 1a Rezeptors in der Niere induziert die Harnansäuerung und moduliert den Säure-Basen Haushalt (Vasopressin type 1a receptor promotes the urinary acidification and modulates the acid-base-balance) selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Erstbetreuer angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

8 Declaration of contribution

The experiments in the study:

Giesecke T, Himmerkus N, Leipziger J, Bleich M, Koshimizu TA, Fähling M, Smorodchenko A, Shpak J, Knappe C, Isermann J, Ayasse N, Kawahara K, Schmoranzer J, Gimber N, Paliege A, Bachmann S, Mutig K: Vasopressin increases Urinary Acidification via V1a Receptor of Collecting Duct Intercalated Cells. J Am Soc Nephrol. 2019 Jun; 30(6): 946-961.

were carried out on a collaborative basis by different research groups under the lead of the group of PD Dr. Kerim Mutig at the Charité - Universitätsmedizin Berlin, Institute of Functional Anatomy (former Institute of Vegetative Anatomy).

The following parts and experiments were performed by myself: Localization studies for the V1aR using immunohistochemistry techniques and confocal microscopy in mice, rat and human tissue (Figure 1, A-F; Figure 2, A-G; Supplemental Figure 1-3), study of the subcellular distribution of the receptor with structured illumination microscopy (Figure 1, G and H), analysis of V1aR expression by RT-PCR in microdissected mouse nephron segments (Figure 1, I), validation of the anti-V1aR antibody in V1aR deficient tissue of mice (Figure 2, A-D), western blot analysis of V1aR expression in different organs of mice (Figure 3, E), overexpression of V1aR in HEK293 cells (Figure 3, H-K), analysis of collecting duct cell types in wild-type versus vasopressin V1a-receptor-deficient kidneys (Table 1), in vivo experiments in Brattleboro rats housed in metabolic cages and stimulated with the V1aR agonist A0-4-67 (Figure 4, C-F and Supplemental Figure 5), establishing of the method for titration of the net acid exertion in urine of Brattleboro rats and measurement of all probes of the experiment (Figure 4, E-F and Supplemental Table 2-4), immunocytochemistry and stimulation experiments in primary cell culture of rat inner medullary collecting duct cells (Figure 5 and Figure 6), analysis of collecting duct cell types under acid load in mice (Supplemental Table 1 and Supplemental Figure 6-7), statistical analysis of the data, creation of the manuscript, preparation of the figure tables, submission and resubmission of the manuscript, coordination of the submission process.

The following people contributed in part to the work named above in the following manner: Dr. Nina Himmerkus and Prof. Markus Bleich provided the microdissected nephron segments for further RT-PCR analysis (Figure 1, I); Dr. Jan Schmoranzer and Dr. Niclas Gimber helped Torsten Giesecke with the structured illumination microscopy (equal contribution of Torsten Giesecke and Dr. Jan Schmoranzer/Dr. Niklas Gimber); Carolin Knappe assisted during the experiments with the Brattleboro rats; Julia Sphak helped as technical assistant in culturing HEK293 cells and primary cell culture of rat inner medullary collecting duct cells; Prof. Sebastian Bachmann, Dr. Kerim Mutig and Torsten Giesecke wrote the manuscript (equal contribution); Dr. Alexander Paliege contributed human kidney samples and helped Torsten

Giesecke with the immunohistochemistry of this special type of tissue (equal contribution); Alina Smorodchenko helped with technical issues regarding immunohisto- and immunocytochemistry (minor contribution); PD Dr. Kerim Mutig was principal investigator of the project.

Dr. Nina Himmerkus, Prof. Dr. Marcus Bleich, and Julian Isermann performed the experiments on isolated perfused collecting ducts of male C57BL/6J mice in the Institute of Physiology, at the Christian-Albrechts-Universität zu Kiel (Figure 7, A-D) and provided the images of microdissected nephron segments (Supplemental Figure 4).

Prof. Dr. Jens Leipziger and Niklas Ayasse from the Department of Biomedicine at the Aarhus University were responsible for *in vivo* experiments with C57BL/6J mice using the V1aR agonist A0-4-67 and the V1aR antagonist CL-14-102 (Figure 4, A and B).

Prof. Katsumasa Kawahara and Prof. Taka-Aki Koshimizu contributed perfusion fixated tissue from the V1aR knockout mice (Figure 3, A-D).

Prof. Taka-Aki Koshimizu furthermore supported the validation of the anti-V1aR antibody by immunoprecipitation experiments and western blot analysis (Figure 3, F and G).

Date Signature

9 Publication

Giesecke T, Himmerkus N, Leipziger J, Bleich M, Koshimizu TA, Fähling M, Smorodchenko A, Shpak J, Knappe C, Isermann J, Ayasse N, Kawahara K, Schmoranzer J, Gimber N, Paliege A, Bachmann S, Mutig K: Vasopressin increases Urinary Acidification via V1a Receptor of Collecting Duct Intercalated Cells. J Am Soc Nephrol. 2019 Jun; 30(6): 946-961.[Impact Factor 2017: 8.655]

https://doi.org/10.1681/ASN.2018080816

9.1 Journal Summary List (ISi Web of Knowledge[SM])

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"UROLOGY and NEPHROLOGY"** Selected Category Scheme: WoS

Gesamtanzahl: 76 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	EUROPEAN UROLOGY	30,723	17.581	0.070590
2	Nature Reviews Nephrology	4,668	14.101	0.017100
3	JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY	37,796	8.655	0.056820
4	KIDNEY INTERNATIONAL	42,587	8.429	0.049960
5	Nature Reviews Urology	2,966	8.089	0.008660
6	AMERICAN JOURNAL OF KIDNEY DISEASES	23,490	7.129	0.034740
7	Clinical Journal of the American Society of Nephrology	15,858	5.835	0.035740
8	JOURNAL OF UROLOGY	50,944	5.381	0.051660
9	BJU INTERNATIONAL	20,875	4.688	0.030530
10	NEPHROLOGY DIALYSIS TRANSPLANTATION	25,654	4.600	0.038260
11	PROSTATE CANCER AND PROSTATIC DISEASES	2,022	4.099	0.004890
12	UROLOGIC ONCOLOGY-SEMINARS AND ORIGINAL INVESTIGATIONS	4,787	3.397	0.013310
13	CURRENT OPINION IN NEPHROLOGY AND HYPERTENSION	3,324	3.370	0.006500
14	Kidney International Supplements	1,810	3.357	0.008600
15	SEMINARS IN NEPHROLOGY	2,705	3.350	0.004690
16	PROSTATE	7,414	3.347	0.009420
17	Journal of Sexual Medicine	9,343	3.339	0.015850
18	NEUROUROLOGY AND URODYNAMICS	5,737	3.263	0.007220
19	ASIAN JOURNAL OF ANDROLOGY	3,502	3.259	0.006300
20	AMERICAN JOURNAL OF PHYSIOLOGY-RENAL PHYSIOLOGY	15,665	3.164	0.020520

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
20	AMERICAN JOURNAL OF PHYSIOLOGY-RENAL PHYSIOLOGY	15,665	3.164	0.020520
21	KIDNEY & BLOOD PRESSURE RESEARCH	1,524	3.000	0.002640
22	WORLD JOURNAL OF UROLOGY	4,944	2.981	0.010580
23	AMERICAN JOURNAL OF NEPHROLOGY	4,472	2.884	0.008650
24	JOURNAL OF NEPHROLOGY	2,647	2.724	0.004300
25	JOURNAL OF RENAL NUTRITION	1,799	2.651	0.003210
26	ADVANCES IN CHRONIC KIDNEY DISEASE	1,611	2.633	0.004030
27	PEDIATRIC NEPHROLOGY	8,625	2.627	0.011860
28	EUROPEAN UROLOGY SUPPLEMENTS	625	2.585	0.000850
29	Clinical Genitourinary Cancer	1,628	2.539	0.005000
30	Aging Male	718	2.500	0.000890
31	BMC Nephrology	3,373	2.395	0.011410
32	UROLOGY	21,383	2.300	0.023960
33	CardioRenal Medicine	406	2.221	0.001300
34	NEPHRON	3,051	2.203	0.002390
35	NEPHROLOGY	3,115	2.178	0.006050
36	UROLOGIC CLINICS OF NORTH AMERICA	1,861	2.149	0.001730
37	INTERNATIONAL UROGYNECOLOGY JOURNAL	6,083	2.078	0.009650
38	JOURNAL OF ENDOUROLOGY	6,286	2.038	0.008470
38	Urolithiasis	693	2.038	0.002010
40	Clinical and Experimental Nephrology	2,107	2.016	0.004440
41	PERITONEAL DIALYSIS INTERNATIONAL	3,530	2.009	0.003320
42	Current Urology Reports	1,203	1.983	0.002990
43	World Journal of Mens Health	294	1.981	0.000930
44	INTERNATIONAL JOURNAL OF UROLOGY	3,957	1.941	0.006530
45	Journal of Pediatric Urology	2,551	1.935	0.006680

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
46	BLOOD PURIFICATION	1,864	1.919	0.002970
47	CURRENT OPINION IN UROLOGY	1,642	1.830	0.003250
48	SEMINARS IN DIALYSIS	2,539	1.818	0.003820
49	BMC Urology	1,187	1.792	0.003350
50	International Neurourology Journal	436	1.726	0.001050
51	INTERNATIONAL UROLOGY AND NEPHROLOGY	3,612	1.692	0.007080
52	Scandinavian Journal of Urology	518	1.684	0.001860
53	INTERNATIONAL JOURNAL OF IMPOTENCE RESEARCH	1,946	1.517	0.001230
54	UROLOGIA INTERNATIONALIS	2,671	1.508	0.003320
55	Sexual Medicine	172	1.457	0.000660
56	Minerva Urologica E Nefrologica	352	1.449	0.000470
57	RENAL FAILURE	3,001	1.440	0.005110
58	THERAPEUTIC APHERESIS AND DIALYSIS	1,478	1.416	0.002130
59	CLINICAL NEPHROLOGY	3,230	1.352	0.003120
59	Canadian Journal of Urology	959	1.352	0.001840
61	Actas Urologicas Espanolas	889	1.260	0.001090
62	Hemodialysis International	1,392	1.237	0.002600
63	Iranian Journal of Kidney Diseases	644	1.192	0.001160
64	CUAJ-Canadian Urological Association Journal	1,650	1.179	0.004520
65	NEFROLOGIA	1,006	1.167	0.001520
66	LUTS-Lower Urinary Tract Symptoms	182	1.038	0.000610
67	International Braz J Urol	1,285	0.976	0.002030
68	Contributions to Nephrology	1,362	0.931	0.001410
69	Urology Journal	706	0.880	0.001130
70	PROGRES EN UROLOGIE	993	0.819	0.001010

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
71	Nephrology Nursing Journal	484	0.744	0.000440
72	Nephrologie & Therapeutique	277	0.479	0.000390
73	ARCHIVOS ESPANOLES DE UROLOGIA	509	0.465	0.000480
74	UROLOGE	622	0.437	0.000520
75	AKTUELLE UROLOGIE	93	0.235	0.000080
76	Revista de Nefrologia Dialisis y Trasplante	5	0.019	0.000000

9.2 Giesecke T, Himmerkus N, Leipziger J, Bleich M, Koshimizu TA, Fähling M, Smorodchenko A, Shpak J, Knappe C, Isermann J, Ayasse N, Kawahara K, Schmoranzer J, Gimber N, Paliege A, Bachmann S, Mutig K: Vasopressin increases Urinary Acidification via V1a Receptor of Collecting Duct Intercalated Cells. J Am Soc Nephrol. 2019 Jun; 30(6): 946-961. [Impact Factor 2017: 8.655]

https://doi.org/10.1681/ASN.2018080816

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

Blank page instead of the original article due to copyright restrictions

10 Curriculum vitae

„Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.“

11 Publication list

Boldt C, Röschel T, Himmerkus N, Plain A, Bleich M, Labes R, Blum M, Krause H, Magheli A, **Giesecke T**, Mutig K, Rothe M, Weldon SM, Dragun D, Schunck WH, Bachmann S, Paliege A: Vasopressin lowers renal epoxyeicosatrienoic acid levels by activating soluble epoxide hydrolase. Am J Physiol Renal Physiol. 2016 Dec 1; 311(6):F1198-F1210. [Impact Factor 2017: 3.164]

Giesecke T, Kawahara K, Koshimizu T, Gimber N, Schmoranzer J, Smorodchenko A, Bachmann S, Mutig K, Distribution of vasopressin receptors within the renin-angiotensin-aldosterone system. Acta Physiologica. 2017 March; vol. 219 Supplement 711: B01-6. [Impact Factor 2017: 5.930]

Giesecke T, Giesecke Y, Kawahara K, Koshimizu T, Gimber N, Schmoranzer J, Smorodchenko A, Bachmann S, Mutig K: Segmental and Cellular V1a Receptor Distribution in the Mammalian Kidney. The FASEB Journal. April 2017; vol.31 no.1: Supplement 857.6. [Impact Factor 2018: 5.391]

Zhang, L, Hernández V, Swinny J, Verma A, **Giesecke T**, Emery A, Mutig K, Garcia Segura LM, Eiden L: A GABAergic cell type in the lateral habenula links hypothalamic homeostatic and midbrain motivation circuits with sex steroid signaling. Transl Psychiatry. 2018 Feb 26; 8(1):50. [Impact Factor 2017: 4.691]

Campos-Lira E, Kelly L, Seifi M, Jackson T, **Giesecke T**, Mutig K, Koshimizu TA, Hernandez VS, Zhang L, Swinny JD: Dynamic Modulation of Mouse Locus Coeruleus Neurons by Vasopressin 1a and 1b Receptors. Front Neurosci. 2018 Dec 10; 12:919. [Impact Factor 2017: 3.877]

Giesecke T, Himmerkus N, Leipziger J, Bleich M, Koshimizu TA, Fähling M, Smorodchenko A, Shpak J, Knappe C, Isermann J, Ayasse N, Kawahara K, Schmoranzer J, Gimber N, Paliege A, Bachmann S, Mutig K: Vasopressin increases Urinary Acidification via V1a Receptor of Collecting Duct Intercalated Cells. J Am Soc Nephrol. 2019 Jun; 30(6): 946-961. [Impact Factor 2017: 8.655]

12 Acknowledgments

This work would not have been possible without the continuous support of many peoples throughout the long time between my enrollment in medical school and PhD thesis defense.

Firstly, I would like to express my deep gratitude to my whole family, especially to my wife, my daughter, and my parents. Without them, this thesis would not have been possible.

Furthermore, I would like to thank PD Dr. Kerim Mutig, Professor Sebastian Bachmann, and Professor Kai Schmidt-Ott for their excellent supervision and continued support throughout my PhD, particularly PD Dr. Kerim Mutig for the transfer of the research topic.

My thanks also go to all members of the Bachmann/Mutig lab. Thank you all for the great time I had during these last few years. I am very grateful.