Kidney collecting duct cells make vasopressin in response to NaCl induced hypertonicity

Juan Pablo Arroyo [1,2*], Andrew S. Terker [1,2], Yvonne Zuchowski [1], Jason A. Watts [3], Fabian Bock [1,2], Cameron Meyer[1], Wentian Luo [1], Meghan Kapp [4,5], Edward R. Gould [1], Elizabeth Hammock [6], Matthew H. Wilson [1,7], Roy Zent [1,2,7], Mingzhi Zhang [1,2], Gautam Bhave [1,2], and Raymond C. Harris [1,2,7*]

Affiliations:

[1] Division of Nephrology and Hypertension, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee

[2] Vanderbilt Center for Kidney Disease, Vanderbilt University Medical Center, Nashville, Tennessee.

[3] Epigenetics and Stem Cell Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina

[4] Division of Renal Pathology, Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee

[5] Department of Pathology, Case Western Reserve University, University Hospitals, Cleveland, Ohio.

[6] Department of Psychology, Florida State University, Tallahassee, Florida

[7] Department of Veterans Affairs, Tennessee Valley Healthcare System, Nashville, Tennessee.

*Address correspondence to:

Juan Pablo Arroyo
Division of Nephrology and Hypertension
Vanderbilt University Medical Center
S-3223 Medical Center North
1161 21st Ave South
Nashville, TN 37232-2372

Or

Raymond C Harris
Division of Nephrology and Hypertension
Vanderbilt University Medical Center
C-3121 MCN
1161 21st Ave South

Nashville, TN 37232

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Abstract

Vasopressin has traditionally been thought to be produced by the neurohypophyseal system and then released into the circulation where it regulates water homeostasis. The syndrome of inappropriate secretion of anti-diuretic hormone (vasopressin) made us question if vasopressin could be produced outside of the brain and whether the kidney could be a source of vasopressin. We found that mouse and human kidneys expressed vasopressin mRNA. Using an antibody that detects the pre-pro-vasopressin, we found that immunoreactive pre-pro-vasopressin protein is found in mouse and human kidneys. Moreover, we found that murine collecting duct cells make biologically active vasopressin which increases in response to NaCl mediated hypertonicity, and that water restriction increases the abundance of kidney-derived vasopressin mRNA and protein expression in mouse kidneys. Thus, we provide evidence of biologically active production of kidney-derived vasopressin in kidney tubular epithelial cells.

Introduction

Vasopressin is a nine amino-acid peptide hormone that plays a key role in water and blood pressure homeostasis (1). Vasopressin is the end-product of a highly processed 164 amino acid pre-pro-peptide. Processing of the vasopressin pre-pro-peptide results in three distinct peptides with a 1:1:1 ratio: vasopressin, neurophysin II, and copeptin. Vasopressin is the biologically active hormone, neurophysin II is a carrier protein for vasopressin, and copeptin is the c-terminal glycosylated end-product. Physiologic vasopressin production is currently thought to be limited to the brain under physiologic conditions. The main physiologic stimuli for vasopressin production in the hypothalamus are increased extra-cellular fluid tonicity and hypotension (1). Vasopressin binds to three distinct G-coupled protein receptors, V1a, V1b, and V2. The V2 receptor (V2R) is mainly expressed in the kidney along the distal nephron and is a critical regulator of abundance and trafficking of the water channel, Aquaporin-2 (AQP2) (2-4). Vasopressin signaling through V2R leads to AQP2 phosphorylation and translocation to the apical membrane of connecting and collecting duct cells (5), which leads to increased apical water permeability with consequent water retention and increased urine concentration.

Unregulated vasopressin production can lead to excessive water retention and decreased serum sodium (Na^+) levels. This common clinical scenario, known as Syndrome of Inappropriate Antidiuretic Hormone secretion (SIADH) occurs in several diseases processes including, malignancy, pulmonary disorders, central nervous system disorders, non-hypothalamic tumors, and certain medications (6). Moreover, there is a large body of work that shows that vasopressin is involved in a broad range of physiologic and pathophysiologic states that go beyond water and blood pressure homeostasis (7-20). This raises the question of whether biologically active vasopressin is also produced outside of the brain under physiologic conditions. Vasopressin production have been the topic of multiple studies elucidating the work of vasopressin…

To answer this question, we investigated whether kidney epithelial cells produce vasopressin and if this production is regulated by changes in extracellular fluid tonicity.

We found evidence of vasopressin gene expression and protein production in mouse and human kidney epithelial cells. We demonstrated that this vasopressin activated V2R in vitro, and its production was increased when cells were placed in hypertonic NaCl solution. Finally, we provide evidence that whole kidney vasopressin mRNA and protein expression increased in water deprived mice. Thus, we conclude that kidney epithelial cells produce vasopressin that can be increased by NaCl mediated hypertonicity under physiological conditions.

Methods

Cells – *Wild-type* C57BL/6J inner medullary collecting duct cells (IMCDs) were isolated and transfected with SV40 as described previously (21). Cells were cultured in DMEM high glucose, pyruvate containing medium supplemented with 10% FBS and 100 I.U./mL penicillin an 100 (µg/mL) streptomycin. Cells from passages 2-7 were plated at a density of $5x10^5$ cells per well (12-well dish) prior to experiments.

Hypertonic stimulation –Confluent IMCDs were serum starved for 24 hrs, after which new FBS-free DMEM and 100 mmol NaCl were added in the presence or absence of decreasing doses of V2R inhibitor, OPC 31260. Cells were kept in the NaCl +/- OPC supplemented medium for 24 hrs after which cells were harvested in Trizol (Invitrogen) for RNA extraction or RIPA (Thermo Scientific) for total protein extraction. To compare hypertonic stimuli, serum starved confluent IMCDs were stimulated with 100 mmol NaCl, 200 mmol glucose, or 200 mmol mannitol for 24 hrs. Cells were then harvested in Trizol (Invitrogen) for RNA extraction.

Conditioned medium - Confluent IMCDs were serum starved for 24 hrs, after which new FBS-free DMEM +/- 100 mmol NaCl was added. After 24 hrs the medium was collected from both NaCl treated and control groups and the NaCl conditioned medium was diluted to achieve a calculated osmolality similar to that of the control medium (~320 mOsm) (Figure 4A). The control and NaCl conditioned media were then added to confluent serum starved HEK-hV2R-CRE-Luc cells or IMCDs or (see below) for 3 hrs. The 3 hr time point was chosen since prior work by Hasler et al. showed that 3 hrs of hypertonic stimulation decreased AQP2 expression (22). IMCDs were then harvested in RIPA buffer (Thermo Scientific) or fixed on a slide with 4% PFA for immunolabeling and HEK-hV2R-CRE-Luc cells underwent the luciferase reporter assay as described below.

V2R luciferase reporter cell assay - *piggyBac* transposon vectors (23) were designed and ordered from Vectorbuilder (Chicago, IL) to express the human V2R along with

puromycin resistance (PB-Puro-CMV-hAVPR2) or cyclic AMP (cAMP) response element driving luciferase (pPB-CREminiP-Luc) in separate vectors. HEK293 cells were transfected in 6 well plates at 60% confluence using lipofectamine LTX (Invitrogen) with 1µg of each transposon vector along with 0.5µg of pCMV-m7PB hyperactive transposase (24), according to the manufacturer's instructions. One day after transfection, cells were split to 100mm dishes and selected with 3µg/ml of puromycin for two weeks. For luciferase assays, stably transfected cells (HEK-hV2R-CRE-Luc) were treated with different reagents as indicated for 3-18 hours in 24 well plates. Two µl of 30mg/ml of XenoLight D-Luciferin (PerkinElmer) was added to wells after treatment and incubated for 5 minutes. Luciferase expression was quantitated by capturing photons/sec using a PerkinElmer IVIS imaging system.

HEK-293 transient transfection - HEK-293 cells were cultured in 6 well plate to 60% confluence and transfected using lipofectamine LTX (Invitrogen) using 2.5 ug of pCMV6-XL5 or pCMV6-XL5-*Avp* (Origene) following the manufacturer's instructions. Cells were collected in RIPA buffer (Thermo Scientific) 24 hrs after transfection.

siRNA transfection – IMCDs were plated at a density 7.5 x10^5 cells per well and upon reaching 30% confluency they were transfected with Silencer Select (ThermoFisher) Avp siRNA (AssayID: s232140) or ctrl siRNA (AssayID: 4390843) according to the manufacturer's instructions. Cells were lysed in RIPA buffer (Thermo Scientific) upon reaching 90% confluency 24-36 hrs later and vasopressin protein amount was evaluated with immunoblotting (see below).

Mice. All animal experiments were performed in accordance with the guidelines and with the approval of the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center. Age-matched male wild type C57BL/6J (8-12 weeks old) were used for the experiments. *Water restriction* - Twenty-four hours prior to euthanasia, water was removed from cages of the water restricted group. *Water loading* – Twenty-four hours prior to euthanasia, food in the water loaded group was changed to a gelled diet (25). Briefly, 65 grams of crushed 4.5% fat mouse chow (5L0D - LabDiets)

was mixed with 7 g of gelatin and dissolved in 120 mL of water. Gel was solidified in plastic cups and then served as the sole source of food for 24 hrs, 9:00 AM to 9:00 AM. Ad-lib access to water was maintained throughout.

Reverse Transcription and Real Time qPCR – RNA from cells and kidneys was isolated with Trizol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized from equal amounts of total RNA from each sample using SuperScript IV First-strand Synthesis Sytem kit (Invitrogen). *Reverse Transcription* - PCR was performed using Q5 High-Fidelity DNA Polymerase (New England BioLabs), forward primer (ATGCTCGCCAGGATGCTCAACACTACG) reverse primer (TCAGTAGACCCGGGGCTTGGCAGAATCCACGGACTC). *Quantitative RT-qPCR* - was carried out using TaqMan real-time PCR (7900HT, Applied Biosystems). All gene probes and master mix were purchased from Applied Biosystems. The probes used in the experiments were: mouse *Avp* (Mm00437761_g1), mouse *Aqp2* (Mm00437575_m1), mouse Akr1b3 (Mm01135578_g1), mouse Slc14a2 (Mm01261839_m1), mouse RPS18 (Mm02601777).

Immunoblotting – Protein was extracted from cells and whole kidneys using RIPA buffer (Thermo Scientific) with protease and phosphatase inhibitors (Roche), total protein was then quantified with BCA Assay (Pierce), and equal amounts of protein were loaded in MiniProteanTGX polyacrylamide precast gels (Bio-Rad) and transferred to nitrocellulose using Transblot Turbo (Bio-Rad). Nitrocellulose membranes were stained for total protein using Ponceau-S (Thermo Fisher) and blocked with 5% non-fat dry milk for 1 hour room temperature. Primary antibodies used were: anti-pre-pro-vasopressin (PhosphoSolutions) 1:1000 overnight, anti-aquaporin E2 sc-515770 (Santa Cruz) 1:100 overnight, anti-aquaporin phosphor serine269 (26) (Phosphosolutions - p112-269), 1:1000 overnight. Secondary antibodies were HRP coupled anti-mouse (AB_10015289) and anti-rabbit (AB_2337938) from Jackson Immuno-Research. Band density quantification was performed with ImageJ.

Immunofluorescence – *Cells* – IMCDs were plated on Corning 6-well 0.4 μM pore transwell inserts or Nunc-Lab Tech II Chamber Slides (Thermo) and grown to confluency. Cells were then stimulated with isotonic FBS-free control or isotonic FBS-free NaCl conditioned medium per above. After 3 hrs of stimulation, cells were washed in ice-cold PBS, fixed with 4% PFA for 30 min at RT, blocked with 5% BSA in PBS 0.2% Tween for 1 hr RT and probed with anti-aquaporin E2 FITC conjugated sc-515770 (Santa Cruz) 1:100 overnight or anti-pre-pro-vasopressin (PhosphoSolutions) 1:1000 1hr room temperature and ActinRed555 (Life technologies) per manufacturers protocol. Cells were imaged on Nikon TiE fully motorized inverted fluorescent microscope. *Kidneys* - Following euthanasia, kidneys were removed and incubated at room temperature overnight in 3.7% formaldehyde, 10 mM sodium m-periodate, 40 mM phosphate buffer, and 1% acetic acid. The fixed kidney was dehydrated through a graded series of ethanol, embedded in paraffin, sectioned (5 μm), and mounted on glass slides. Immunostaining was carried out as described previously (27). Primary antibodies used: anti-pre-pro-vasopressin (PhosphoSolutions) 1:1000 1hr room temperature, anti-aquaporin E2 FITC conjugated sc-515770 (Santa Cruz) 1:100 overnight, or Dolichos Biflorus Agglutinin (DBA) – Rhodamine labeled (Vector) and Lotus Tetragonolobus Lectin (LTL) – Fluorescein labeled, or ActinRed 555 ReadyProbes (Thermo). Secondary antibody used: goat-anti-Rabbit AF647 coupled (Invitrogen - A27040).

Human tissue samples – All human tissue samples were obtained in accordance with and following the Vanderbilt University Medical Center IRB. Human tissue samples were obtained as unidentified frozen human tissue procured through the Collaborative Human Tissue Network (CHTN) and the formalin fixed paraffin embedded de-identified blocks procured through the biorepository of the Vanderbilt University Medical Center Department of Pathology.

Statistical analyses. All values are expressed as mean ± SEM. Between group comparisons were made using one-way ANOVA with post-hoc Tukey test or Mann-

Whitney test as indicated in figure legend $p<0.05$ was used for significance threshold. Analysis was performed using Prism 9 software.

Results

Vasopressin mRNA is present in human and mouse kidneys

We initially interrogated the Genotype-Tissue Expression Project (GTEx) database to identify potential sites of extra-hypothalamic vasopressin production in humans (28). Vasopressin mRNA expression was highest in the brain, followed by testis, kidney cortex, and kidney medulla (Supplemental Figure 1A). We confirmed the database-defined expression of AVP mRNA in mouse brain and kidney tissue by performing RT-PCR on various tissues (Supplemental Figure 1B). We also found that vasopressin mRNA was present in principal cells of mice utilizing the mouse kidney single cell RNA sequencing data set available through the Kidney Interactive Transcriptomics website (http://humphreyslab.com/SingleCell) (Supplemental Figure 1C). Together, these data suggest that the vasopressin gene is expressed in the kidney, and that principal cells are likely expressing *Avp* mRNA at baseline.

Hypertonicity regulates *Avp* mRNA expression in collecting duct cells *in-vitro*

As the scRNAseq data suggested there was vasopressin expression in collecting duct cells *in-vivo*, we performed quantitative RT-PCR to explore whether *Avp* mRNA was present in mouse collecting duct cells *in-vitro*. Vasopressin mRNA was detectable at baseline and increased when the cells were exposed to 100 mmol of NaCl (Figure 1A). To determine whether increased vasopressin expression was due to increased concentration of NaCl *per se* or was a response to the increased hypertonicity of the medium, we added either 100 mmol of NaCl or 200 mmol of mannitol, or glucose to the DMEM medium. The increased *Avp* transcription was only seen in the presence of the excessive NaCl, suggesting the effect was specific to the NaCl stimulation. (Figure 1B). In contrast, expression of aldose reductase (*Akr1b3*), which is sensitive to increased extracellular tonicity, increased significantly in response to both NaCl and mannitol (Figure 1C) (29). *As Aqp2* expression increases with activation of the V2R and hypertonicity (22) we measured the expression of this gene in response to NaCl,

glucose or mannitol and found that only 100 mmol of NaCl increased its expression. (Figure 1 D). This increase was likely due to autocrine or paracrine vasopressin production by the IMCD cells as Aqp2 expression was significantly inhibited by addition of the non-peptide V2R antagonist OPC-31260 (30, 31) (Figure 1E). By contrast *Akr1b3* expression induced by NaCl was not affected by the V2R antagonist (Figure 1F), suggesting that there was similar hypertonic stress between groups, and that the effect of the drug was specific. Taken together, these results indicate that cultured IMCD cells produce vasopressin mRNA, which is regulated by NaCl induced hypertonicity. In addition, they suggest that IMCD cells produce locally active vasopressin that can regulate *Aqp2* expression *in-vitro*.

Development of an antibody that detects locally produced pre-pro-vasopressin

We next set out to confirm that kidney collecting ducts cells produced biologically active vasopressin. To do this we needed to develop novel tools as vasopressin protein is difficult to quantify. Currently vasopressin is quantified by an ELISA assay for copeptin, which is used as a surrogate for vasopressin levels (32). Moreover, the epitopes for the commercially available antibodies target end-products of pre-pro-vasopressin processing making it impossible to distinguish site of production from peripheral tissue uptake. To confirm that kidney collecting duct cells produced biologically active vasopressin, we developed a custom antibody in which the epitope spans the known cleavage sites for the vasopressin precursor peptide (Figure 2A), which would allow us to measure locally produced vasopressin. We characterized the antibody by performing a Western blot on brain from a wild-type mouse in the presence and absence of the blocking peptide. The antibody detected an intense band at the expected weight for pre-pro-vasopressin (~20kDa) in the brain and the signal was abolished with the blocking peptide (Supplementary Figure 2A). We then confirmed the antibody did not detect the nine amino acid variants of vasopressin or oxytocin (Supplemental Figure 2B). Moreover, immunostaining of mouse brain sections showed an intense signal in the supraoptic nucleus, containing vasopressin-producing cell bodies, and the internal layer

of the median eminence, containing axons of vasopressin-producing neurons that project to the posterior pituitary (Supplemental Figure 2C) (33). We further characterized the antibody by performing a Western blot on brain, kidney and plasma. The antibody detected an intense band at the expected weight for pre-pro-vasopressin (~20kDa) in the brain and the kidney (Figure 2B), but not in the plasma. To confirm the presence of vasopressin in the kidneys *in vivo*, we obtained *wild-type* mouse whole kidney lysates and incubated them with anti-vasopressin primary antibody with and without incubation with the blocking peptide. We detected a band of the expected weight and the signal was eliminated by the blocking peptide (Figure 2C). To verify that IMCD cells produced vasopressin, we performed immunoblots on cell lysates. We saw the expected single 20kDa band that decreased when the IMCDS cells were transfected with *Avp* siRNA (Figure 2D). Thus, we had produced a highly specific antibody that could identify un-cleaved pre-pro vasopressin.

IMCDS cells produce biologically active vasopressin

To assess if vasopressin protein increased in the same conditions as vasopressin mRNA, we stimulated IMCDs with NaCl for 24 hrs and determined that indeed vasopressin precursor protein expression increased (Figure 3A). Immunofluorescence staining of IMCDs stimulated with NaCl also showed increased staining for vasopressin (Figure 3G) vs controls (Figure 3D) and the signal was once again abolished with the blocking peptide (Figure 3J).

To confirm that the vasopressin produced by the IMCDS cells was biologically active we developed a novel cell line; stably transfected HEK293 cells with the human V2 receptor (hV2R) and a cAMP response element driving luciferase expression (HEK-hV2R-CRE-Luc) that can be activated by V2 receptor activation. We then developed a bioassay in which we collected medium from control or NaCl stimulated IMCDs, illustrated in (Figure 4A). Serum starved HEK-hV2R-CRE-Luc cells were then subjected to the following: 1) no medium change, 2) fresh FBS-free DMEM, 3) fresh FBS-free DMEM + 100 mmol NaCl, 4) IMCD control medium, 5) IMCD NaCl medium, and 6) IMCD NaCl medium +

OPC31260 (Figure 4B). We found that medium from IMCDs stimulated luciferase production. NaCl medium luciferase activity was higher than IMCD control medium, and this effect was abolished with the addition of OPC-31260 (Figure 4C). Together, these data suggest that there is biologically active vasopressin in IMCD conditioned medium at baseline which increases after NaCl treatment, and the NaCl dependent increase in luciferase activity was due to V2R activation as it was prevented with V2R antagonist OPC-31260.

We further confirmed the presence of biologically active vasopressin in IMCD conditioned medium by adding it to serum starved IMCDs after which we assessed the expression of AQP2 (Figure 4 D-N) in response to control or NaCl conditioned IMCD medium (Figure 4A). As expected, the addition of conditioned medium of IMCDS cells exposed to 100mMol of NaCl resulted in increased expression and phosphorylation of AQP2 in serum starved IMCDS cells and this increase was blocked by the addition of OPC-31260 as measured by immunoblotting and immunofluorescence. Together, these data suggest that IMCD cells make biologically active vasopressin when stimulated with NaCl, and local vasopressin can regulate AQP2 total protein abundance and phosphorylation.

Vasopressin mRNA and protein increased with water restriction *in vivo*

To confirm regulated expression of vasopressin by the kidney *in-vivo*, we water restricted, and water loaded *wild type* mice for 24 hrs, assessed whole kidney mRNA and protein for vasopressin expression and confirmed localization via immunofluorescence. Both vasopressin mRNA and protein were higher in water restricted vs water loaded mice (Figure 5 A-C). Consistent with our *in vitro* data, vasopressin protein was found in connecting and collecting ducts (Figure 5 D-K). Moreover, vasopressin signal was higher in water restricted vs water loaded mice (Figure 5 F, J).

Vasopressin protein is made by human kidneys

Whole kidney mRNA data (Supplemental Figure 1A) suggested vasopressin expression in human kidneys. Therefore, we obtained samples of frozen, and formalin fixed paraffin embedded human kidney tissue to test for vasopressin protein expression. We found that human kidneys can express vasopressin protein (Figure 6 A), and vasopressin is found in connecting and collecting ducts (Figure 6 B-F). Together, these data indicate that vasopressin is found in human CD cells *in vivo*.

Discussion

Vasopressin is thought to be made primarily in the brain, and that this is the sole source of vasopressin stimulating vasopressin V2 receptors of the kidney. In the present study we challenge the current dogma. We developed a novel antibody that recognizes the pre-pro-vasopressin peptide to study local production of vasopressin in the kidney. We used this antibody to confirm that vasopressin was made in mouse collecting ducts cells *in vitro* and *in vivo*. We then showed that kidney-derived vasopressin production was responsive to water restriction, which increased mRNA and protein expression in mice. Finally, we also determined that vasopressin protein was found in healthy human kidney collecting ducts. Thus, we provide novel evidence of physiologic biologically active extra-hypothalamic production and regulation of kidney-derived vasopressin from kidney epithelial cells.

There are multiple prior reports of vasopressin production outside of the brain (34-41), and the publicly available scRNA-sequencing data, we confirmed vasopressin mRNA in mouse and human kidneys (Supplementary Figure 1) and protein in collecting duct cells *in-vitro.* We also found that *Avp* is expressed at baseline in vitro and in vivo and that NaCl increases *Avp* mRNA (Figure 1). These data are consistent with the regulation of hypothalamic *Avp* by NaCl mediated hypertonicity (42).

Interestingly, NaCl is known to increase *Aqp2* independent of exogenous vasopressin, but the contribution of endogenous vasopressin had never been studied (5, 22). We found that V2R was activated *in-vitro* by endogenous vasopressin in response to hypertonic stress. Moreover, the addition of a V2R antagonist prevented hypertonicity induced increase in *Aqp2* expression in a dose dependent manner (Figure 1 E). Thus, these data suggest that vasopressin is produced by collecting duct cells *in vitro* and plays a role regulating *Aqp2* expression.

Vasopressin is difficult to quantify (43, 44). Commercially available methods to measure vasopressin including ELISAs and antibodies all detect terminally processed

peptides, preventing discrimination between local production versus peripheral uptake of hypothalamic vasopressin or related peptides. To circumvent this, we developed an antibody that specifically detects the pre-pro-vasopressin peptide, as the epitope spans the cleavage regions (Figure 2A). Our antibody detected a band at the expected weight (~20 kDa) in mouse brain and kidney (Figure 2B, Supplementary Figure 2A), it stained regions of the brain known to produce vasopressin (Supplementary Figure 2C) as well as murine IMCDs (Figure 3). With this antibody, we were able to determine that immunoreactive vasopressin can be found in both mouse and human whole kidney lysates (Figure 2C,D Figure 6A,B) and localizes to the distal nephron. To our knowledge this is the only available method that can detect the vasopressin pre-pro-peptide. This is critical, as peripheral uptake of circulating vasopressin or copeptin could account for non-specific staining with other methodologies.

We designed a bioassay to assess if the locally produced vasopressin was biologically active (Figure 4A) and found that biologically active vasopressin was present in medium from IMCDs (Figure 4B-N). Our bioassay relied on V2R activation by a ligand found in IMCD supernatant to assess the biologic activity of vasopressin. In our model, it possible that luciferase and/or V2R being activated independent of vasopressin. However, the decrease in luciferase signal seen with the addition of OPC-31260 (Figure 4B lanes 5-6) suggests that there is a V2R specific ligand in IMCD medium. Our data does not preclude the possibility that local vasopressin could be signaling through other pathways including intracellular binding to the V2R (45). Although cancer cell lines have been reported to make vasopressin (46), and there are scattered reports of vasopressin mRNA expression in tissues outside of the hypothalamus (34-41), the production of biologically active vasopressin by non-malignant cells has not been reported.

Hypertonicity is a known regulator of hypothalamic vasopressin production, and we found that both *Avp* mRNA (Figure 5A) and vasopressin protein (Figure 5B,C) were higher in kidneys from water restricted vs water loaded animals. These *in vivo* observations corroborated our *in vitro* data and suggest that local vasopressin could be playing a role in urine concentration and dilution *in vivo*. From these results we can

conclude that kidney-derived vasopressin is produced by collecting duct cells *in vivo* and production is regulated by hypertonic stress.

The physiologic pathways through which hypothalamic vasopressin is involved in regulation of water and blood pressure homeostasis have been known for over 50 years (1). However, there is a large body of literature on the non-water non-blood pressure effects of vasopressin for which physiologic feedback loops have not been clearly defined (7-20). Non-osmotic vasopressin production has been ascribed to hypothalamic stimulation via other mechanisms e.g., nausea and pain (6, 47), but to our knowledge there is no prior reported data on vasopressin production outside of the brain under physiologic conditions. Interestingly, the vasopressin peptide evolved prior to the development of the vertebrate neurohypophyseal system (48). This finding could explain why cells outside of the neurohypophyseal system express vasopressin. As such, non-malignancy associated extra-hypothalamic production of vasopressin has also been reported previously in the heart, ovaries, testis, and adrenal glands (34-41). However, these studies did not establish whether this extra-hypothalamic vasopressin was biologically active.

Our study extends the current model in which vasopressin is solely produced in the brain under physiologic conditions. Many questions remain regarding the *in-vivo* relevance of our observations. Given that kidney-derived vasopressin is stimulated by hypertonicity and patients with diabetes insipidus (DI) have low medullary tonicity, we would expect patients with central or nephrogenic DI to have low to absent levels of kidney-derived vasopressin. This might explain why kidney-derived vasopressin is unable to overcome the concentrating defect in patients with nephrogenic diabetes insipidus. Interestingly, V2R stimulation has differing effects depending on whether vasopressin is apical or basolateral, and reports of intracellular activation of the V2R open the door to the possibility that local vasopressin production may regulate V2R from within the cell (45, 49). Moreover, there are data that suggest that kidney-derived vasopressin mRNA is upregulated in humans with CKD (Supplemental Figure 3A) and

in mice after kidney injury (Supplemental Figure 3B), implying that the local kidney-vasopressin system could be involved in pathways beyond water homeostasis.

In conclusion, our data show that there is regulated expression of the vasopressin gene in the kidney in both mice and humans and kidney epithelial cells make biologically active vasopressin in response to NaCl mediated hypertonicity. It is well known that vasopressin contributes to progression of non-diabetic, diabetic, and polycystic kidney disease (18, 50-52). Our identification of a local vasopressin system in the kidney could provide insight as to how the vasopressin system contributes to kidney function in health and disease.

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Author Contributions

JPA, GB, MW, and RCH, conceived the study and designed experiments. JPA, YZ, CM, FB, MK, WL performed experiments. JPA, AST, FB, JAW, ERG MK, RZ, EH, MHW, GB, MZ, MW, and RCH analyzed data. JPA, AST, EH, RZ, GB, and RCH wrote and edited the manuscript.

Conflict of Interest

The authors have declared that no conflict of interest exists

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Figure 1 - NaCl mediated hypertonicity increased vasopressin (*Avp*) mRNA in kidney epithelial cells. Incubation with NaCl (500 mOsm) for 24 hrs increased vasopressin mRNA in mouse inner medullary collecting duct cells ($p<0.001$ vs control) **(A)**. NaCl ($p<0.01$) but not mannitol or glucose increase *Avp,* mRNA **(B)**. Aldose reductase (*Akr1b3*), a marker of hypertonic stress, increased with NaCl ($p<0.0001$ vs control) and mannitol ($p<0.001$ vs control) but not glucose **(C)**. Aquaporin 2 (*Aqp2*) mRNA increased after treatment with NaCl but not glucose or mannitol **(D)**. Vasopressin receptor 2 antagonist OPC-31260 (20 nm) blunted the NaCl mediated increase in *Aqp2* (**E**) despite similar hypertonic stress (**F**). Data are presented as mean ± SE and analyzed with 1-way ANOVA with Tukey's post hoc analysis, with a minimum of 4 independent replicates per group ** $p<0.01$, *** $p<0.001$, and ****$p<0.0001$ vs control.

Figure 2 - Pre-pro-vasopressin was found in mouse brain and kidney. Our novel custom antibody targets the cleavage site of the vasopressin precursor peptide (**A**). The antibody detected a specific band at 20 kDa in mouse brain and kidney samples, but not in plasma (**B**). We detected a specific band in WT mouse kidneys (n=5), and the signal was inhibited if the primary antibody was incubated with the blocking peptide (**C**). Vasopressin was found in IMCD cells and expression was decreased with the use of siRNA that targets *Avp* (**D**). Data in D is presented as mean ± SE and analyzed with an unpaired Mann-Whitney test n=4 independent replicates ** $p=0.002$

Figure 3 – NaCl increased pre-pro-vasopressin protein in IMCD cells. Treatment of inner medullary collecting duct cells (IMCD) with NaCl for 24 hrs increased the abundance of vasopressin by immunoblot (**A,B**) and immunofluorescence (**C-K**) i. Pre-incubation of the primary antibody with the blocking peptide abolished the signal (**I-K**). Data in B are presented as mean ± SE and analyzed with an unpaired Mann-Whitney test, n=9 independent replicates * $p=0.024$

Figure 4 – **IMCD cell medium contains a V2R stimulating ligand**. Bioassay experimental design to obtained conditioned medium from treated and untreated inner

medullary collecting duct cells (IMCD) (**A**). HEK-V2R-Luc cells stimulated with control and NaCl treated IMCD medium had increased luciferase activity (**B,C** lanes 4,5). The NaCl mediated increase was blocked with the V2R antagonist OPC-31260 (**B,C** lane 6). Addition of isotonic NaCl conditioned medium increased both phosphorylation of serine 269 and total abundance of AQP2, this effect is prevented with OPC-31260 (**D, E**). In IMCDs (**F-N**), NaCl treated medium increased the staining of aquaporin 2 (AQP2) (green), relative to isotonic control medium (**G** vs **J**). The increase was prevented with OPC-31260 (**M**). Data in C and E are presented as mean ± SE of total flus (photons per second) analyzed with 1-way ANOVA with Tukey's post hoc analysis, with a minimum of 4 independent replicates per group # $p<0.0001$ vs control, **** $p<0.0001$ vs isotonic control, ** $p=0.0018$ vs Isotonic NaCl, and * $p<0.05$ vs Isotonic Control.

Figure 5 – Kidney-derived vasopressin increases after water restriction. *Avp* mRNA increased in water restricted mice relative to water loaded controls (**A**). Kidney-derived pre-ppro-vasopressin protein increased after water restriction relative to water loading (**B**). In mouse kidney (**D-O**)Kidney-derived pre-pro-vasopressin signal (white) in collecting ducts (DBA – red) increased in water restricted animals (**J vs F**) and the signal was abolished with primary antibody pre-incubation with blocking peptide (**N vs J**) . Data in A and C are presented as mean ± SE and analyzed with an unpaired Mann-Whitney test, * $p=0.041$, ** $p = 0.013$, water restricted n=5, water loaded n=5.

Figure 6 – Human kidneys make vasopressin. Pre-pro-vasopressin was found in healthy human kidney lysates (A). Pre-pro-vasopressin (white) was found in the collecting ducts (DBA – red), but not in the proximal tubules (LTL – green) (**B – E**).

Supplementary Figure 1 – Mouse and human kidneys have vasopressin mRNA

Vasopressin gene mRNA is present in humans and mice and localizes to medullary collecting duct cells, **a)** GTEx database – bulk tissue gene expression of *Avp* in human tissue, **b)** mouse RT-PCR which shows vasopressin mRNA in brain and kidney tissue (expected size 494 base pairs), **c)** mouse kidney single cell RNAseq from the Kidney Interactive Transcriptomics (http://humphreyslab.com/SingleCell/) that shows expression of the vasopressin gene in medullary collecting duct principal cells (PC2) at baseline. (Used with permission). (PTS1, proximal tubule segment 1; PTS2, proximal tubule segment 2; PTS3, proximal tubule segment 3; NewPT1/PT2, proliferating proximal tubule; DTL-ATL, thin descending and ascending limb of the loop of Henle; MTAL, medullary thick ascending of the loop of Henle; CTAL1-2, cortical thick ascending limb of the loop of Henle; MD, macula Densa; DCT, distal convoluted tubule; PC1, cortical principle cell; PC2, medullary collecting duct principal cells; ICA/ICB, alpha and beta intercalated cells; Uro, urothelium; Pod, podocytes; PEC, parietal epithelial cell; EC1/2 endothelial cells; Fib, fibroblast; Per, pericytes; MØ , macrophage; Tcell, T-lymphocytes)

Supplementary Figure 2 – Characterization of our pre-pro-vasopressin antibody

Mouse whole brain lysates were run and incubated with anti-pre-pro-vasopressin alone or pre-incubated with the blocking peptide. Primary antibody alone recognized a single band at the expected weight (20kDa), and the signal was abolished when the primary antibody was pre-incubated with the blocking peptide (**A**). To corroborate that our antibody only detected the pre-pro-vasopressin we blotted 1 ug of recombinant vasopressin or oxytocin on nitrocellulose and then probed with anti-pre-pro-vasopressin primary. No signal was detected, confirming that our antibody only detects the un-processed vasopressin (**B**). We then confirmed that our antibody detected vasopressin in the expected anatomic regions in a WT mouse brain (**C**).

Supplementary Figure 3

Whole kidney mRNA from non-CKD and CKD kidney biopsies shows a 1.8 fold increased expression of *Avp* mRNA in patients with CKD (**A**). Mouse kidney single cell RNAseq from the Kidney Interactive Transcriptomics (http://humphreyslab.com/SingleCell/) that shows expression of the vasopressin gene is induced in multiple nephron segments after ischemia reperfusion injury (**B**). (PTS1, proximal tubule segment 1; PTS2, proximal tubule segment 2; PTS3, proximal tubule segment 3; NewPT1/PT2, proliferating proximal tubule; DTL-ATL, thin descending and ascending limb of the loop of Henle; MTAL, medullary thick ascending of the loop of Henle; CTAL1-2, cortical thick ascending limb of the loop of Henle; MD, macula Densa; DCT, distal convoluted tubule; PC1, cortical principle cell; PC2, medullary collecting duct principal cells; ICA/ICB, alpha and beta intercalated cells; Uro, urothelium; Pod, podocytes; PEC, parietal epithelial cell; EC1/2 endothelial cells; Fib, fibroblast; Per, pericytes; MØ , macrophage; Tcell, T-lymphocytes)

Figure 1

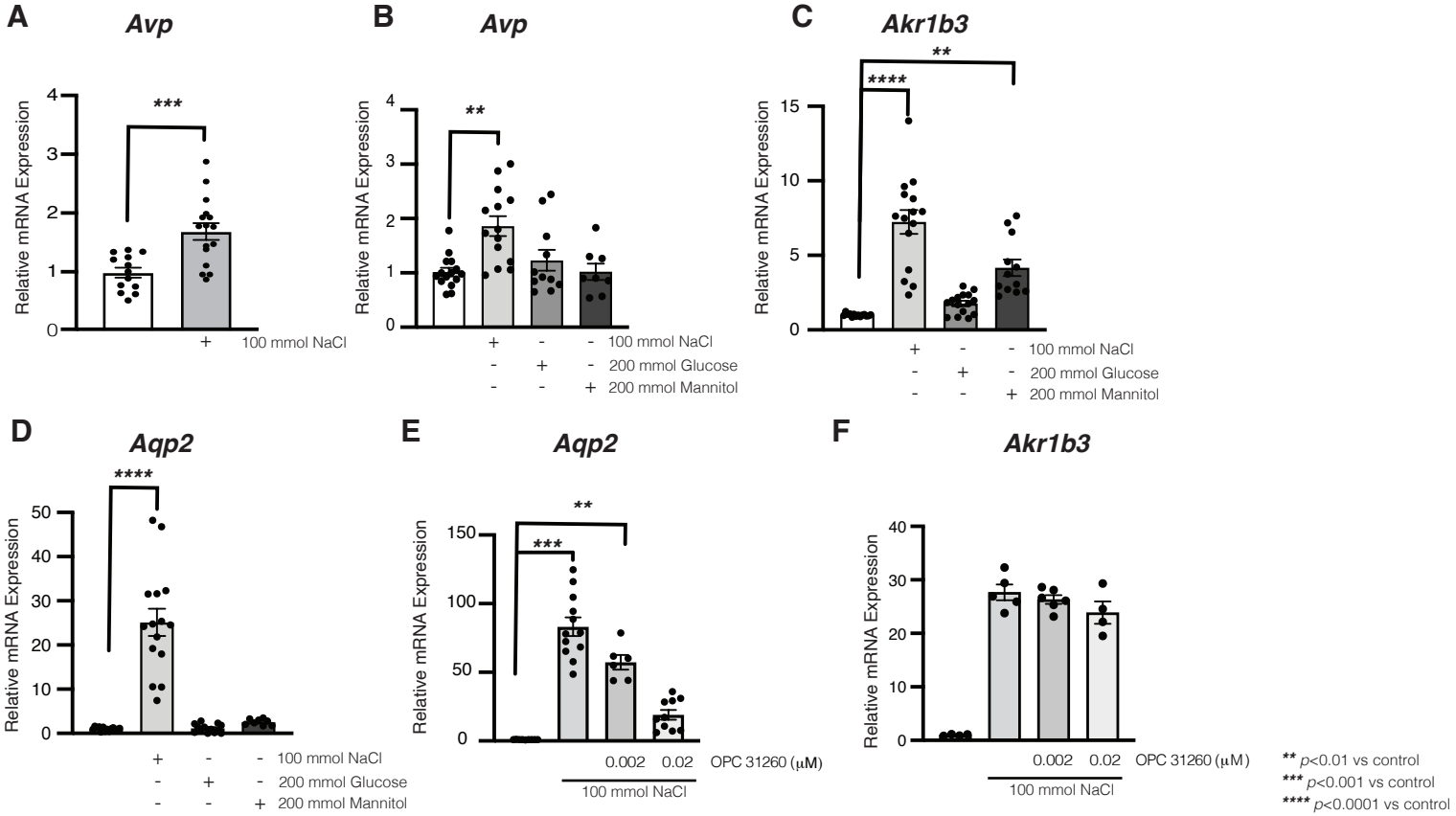

A
Avp
Relative mRNA Expression
***
+ 100 mmol NaCl
B
Avp
Relative mRNA Expression
**
+ - - 100 mmol NaCl
- + - 200 mmol Glucose
- - + 200 mmol Mannitol
C
Akr1b3
Relative mRNA Expression
**
****
+ - - 100 mmol NaCl
- + - 200 mmol Glucose
- - + 200 mmol Mannitol
D
Aqp2
Relative mRNA Expression
****
+ - - 100 mmol NaCl
- + - 200 mmol Glucose
- - + 200 mmol Mannitol
E
Aqp2
Relative mRNA Expression
**
***
0.002 0.02 OPC 31260 (μM)
100 mmol NaCl
F
Akr1b3
Relative mRNA Expression
0.002 0.02 OPC 31260 (μM)
100 mmol NaCl
** p<0.01 vs control
*** p<0.001 vs control
**** p<0.0001 vs control

Figure 2

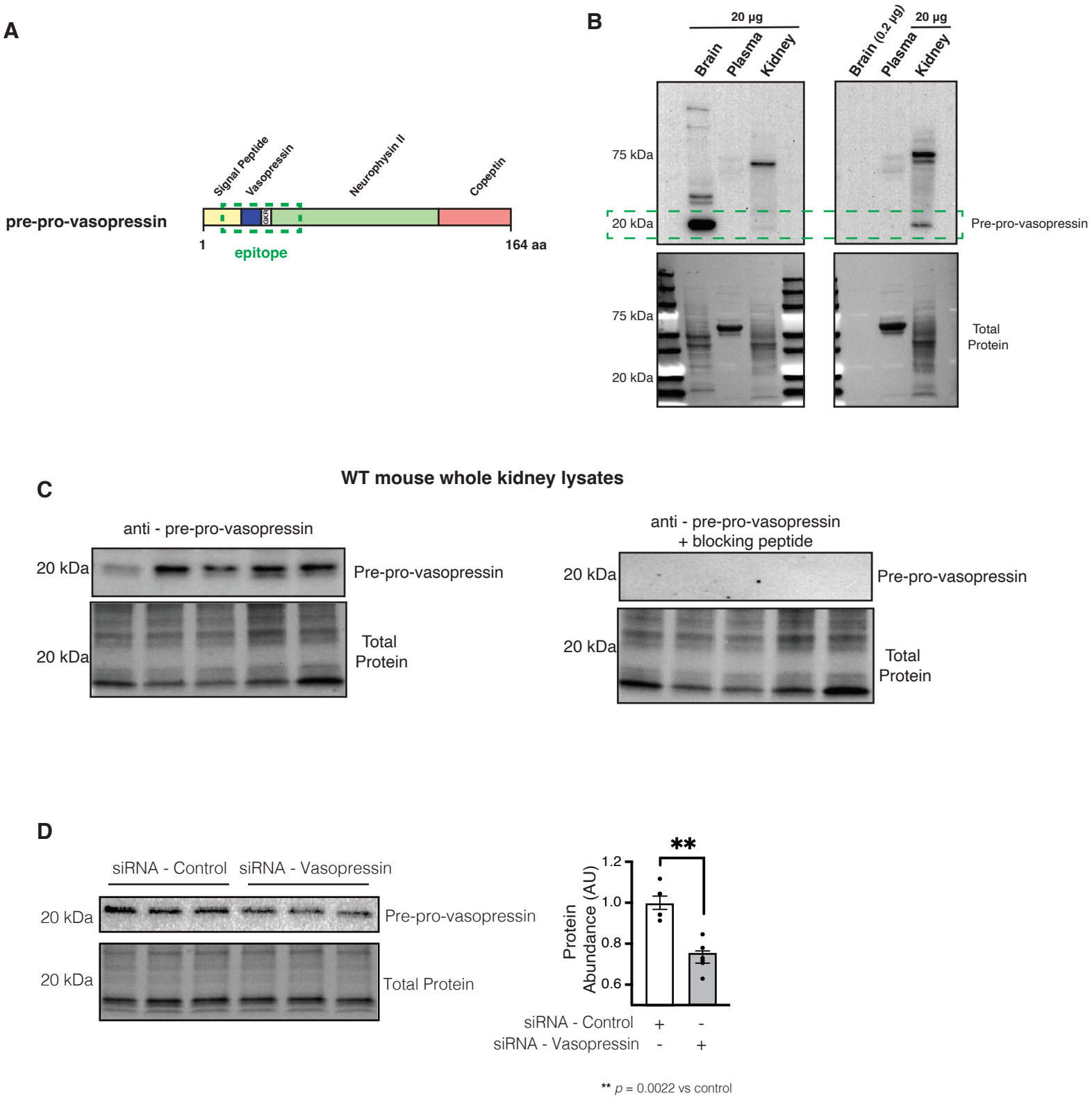

A
pre-pro-vasopressin
Signal Peptide
Vasopressin
Neurophysin II
Copeptin
CKR
1
164 aa
epitope
B
20 µg
Brain
Plasma
Kidney
Brain (0.2 µg)
20 µg
Plasma
Kidney
75 kDa
20 kDa
Pre-pro-vasopressin
75 kDa
20 kDa
Total Protein
C
WT mouse whole kidney lysates
anti - pre-pro-vasopressin
20 kDa
Pre-pro-vasopressin
20 kDa
Total Protein
anti - pre-pro-vasopressin
+ blocking peptide
20 kDa
Pre-pro-vasopressin
20 kDa
Total Protein
D
siRNA - Control
siRNA - Vasopressin
20 kDa
Pre-pro-vasopressin
20 kDa
Total Protein
**
Protein Abundance (AU)
1.2
1.0
0.8
0.6
siRNA - Control + -
siRNA - Vasopressin - +
** p = 0.0022 vs control

Figure 3

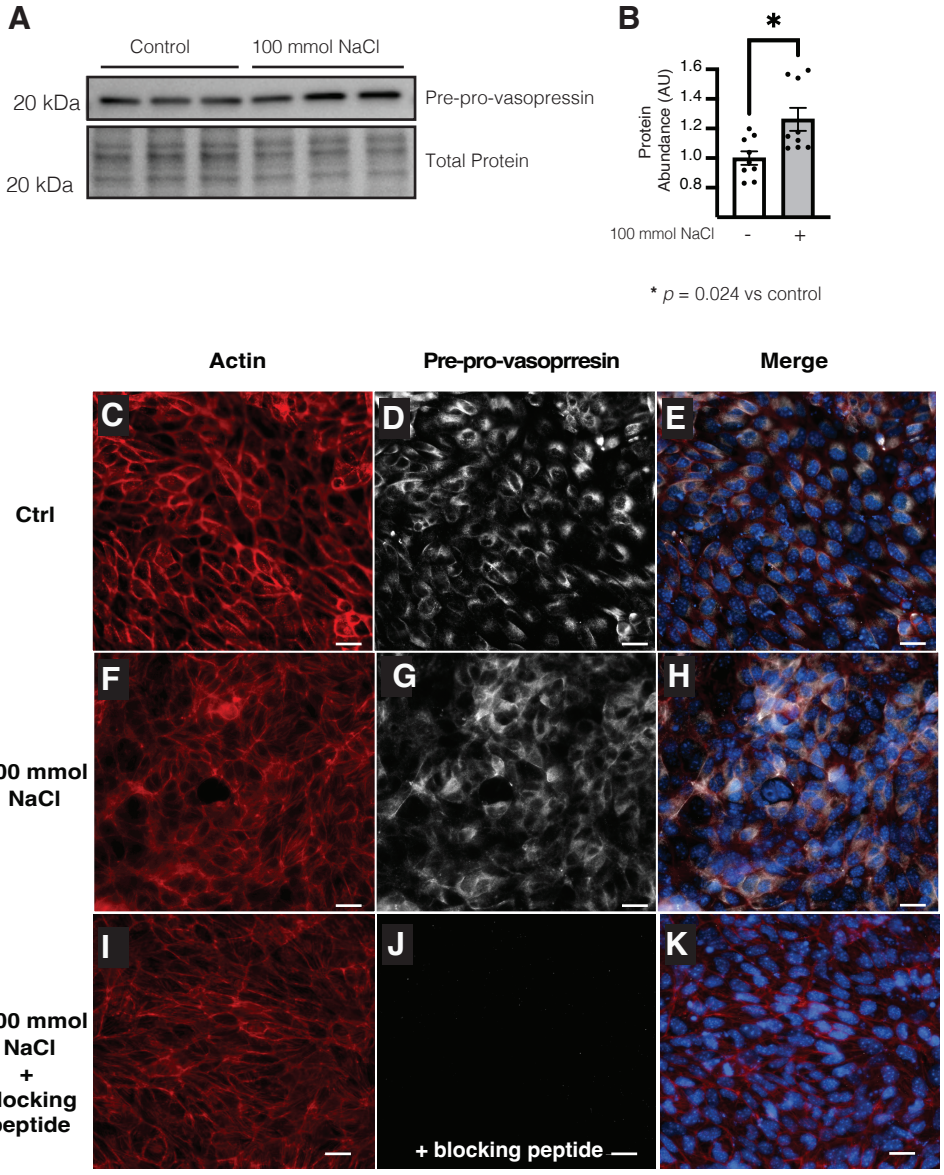

A
Control
100 mmol NaCl
20 kDa
Pre-pro-vasopressin
20 kDa
Total Protein
B
*
Protein Abundance (AU)
1.6
1.4
1.2
1.0
0.8
100 mmol NaCl
-
+
* p = 0.024 vs control
Actin
Pre-pro-vasoprresin
Merge
Ctrl
100 mmol NaCl
100 mmol NaCl + blocking peptide
C
D
E
F
G
H
I
J
K
+ blocking peptide

Figure 4

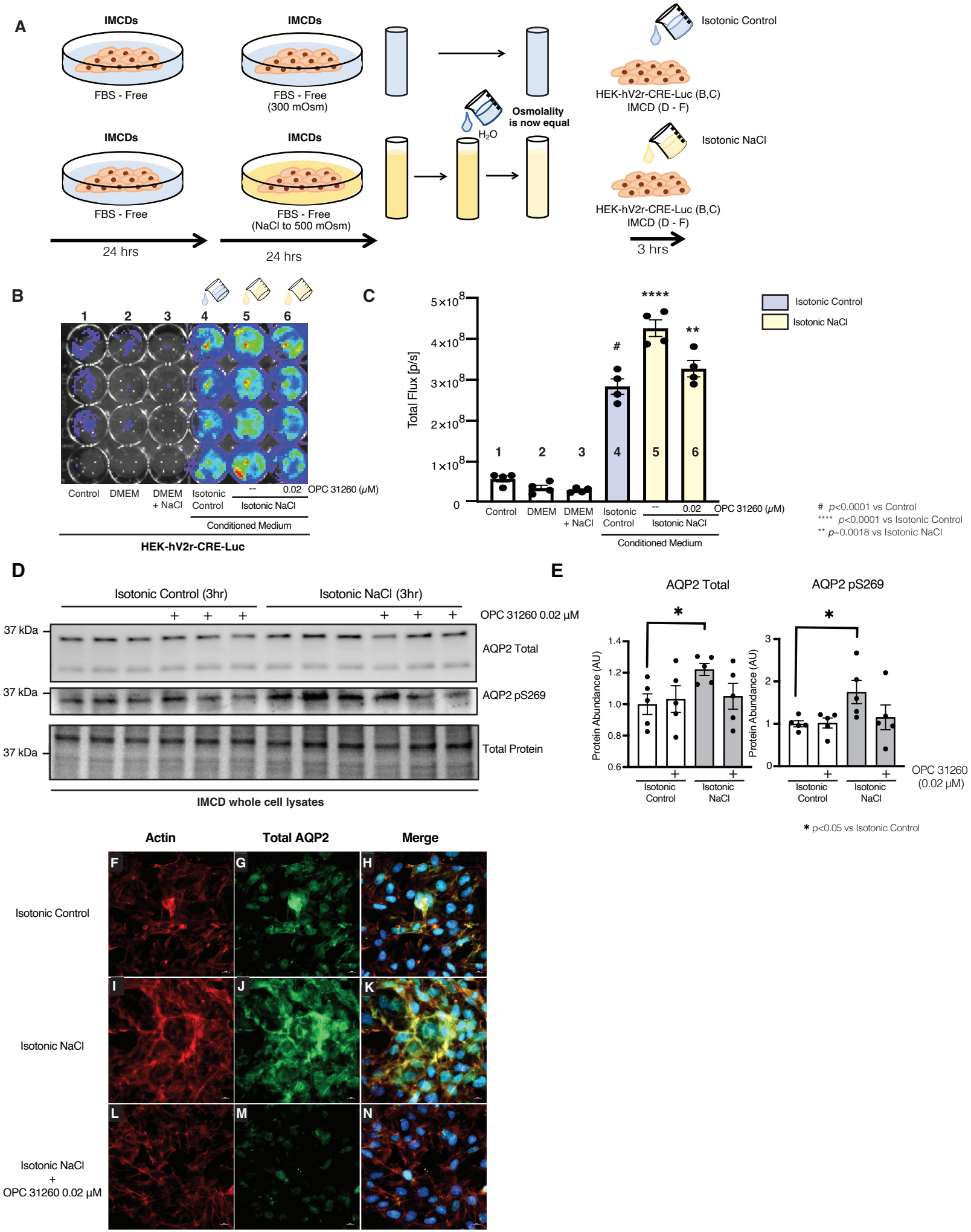
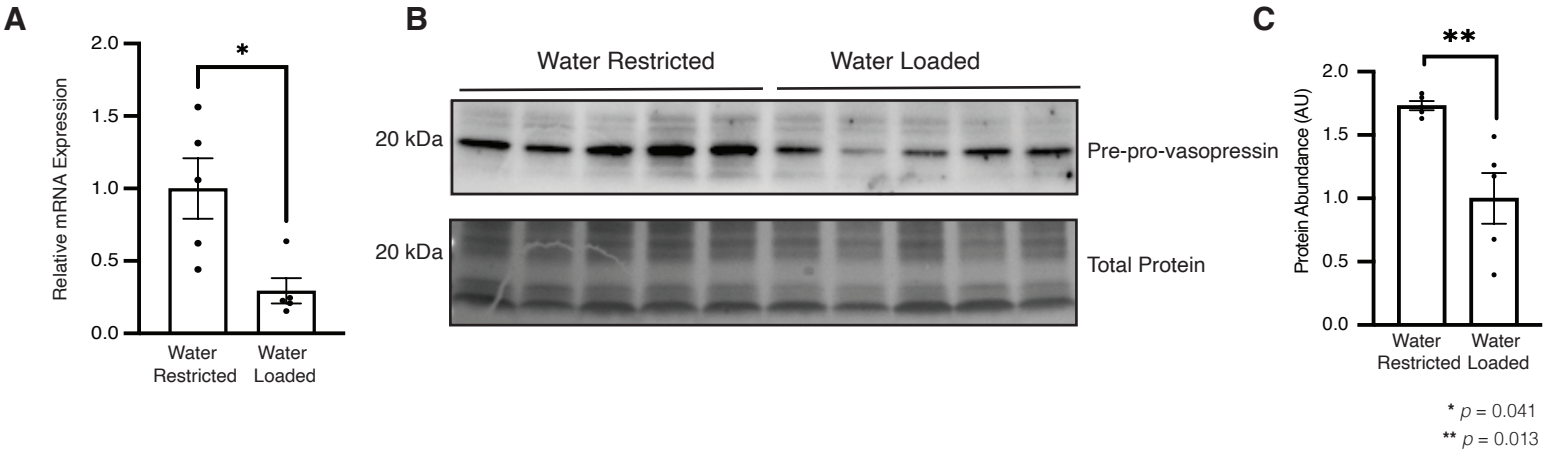

A
IMCDs
FBS - Free
IMCDs
FBS - Free
(300 mOsm)
IMCDs
FBS - Free
IMCDs
FBS - Free
(NaCl to 500 mOsm)
24 hrs
24 hrs
H2O
Osmolality
is now equal
Isotonic Control
HEK-hV2r-CRE-Luc (B,C)
IMCD (D - F)
Isotonic NaCl
HEK-hV2r-CRE-Luc (B,C)
IMCD (D - F)
3 hrs
B
1 2 3 4 5 6
Control
DMEM
DMEM + NaCl
Isotonic Control
-- 0.02 OPC 31260 (μM)
Isotonic NaCl
Conditioned Medium
HEK-hV2r-CRE-Luc
C
Total Flux [p/s]
5×10^8
4×10^8
3×10^8
2×10^8
1×10^8
0
****
**
#
Isotonic Control
Isotonic NaCl
Control
DMEM
DMEM + NaCl
Isotonic Control
-- 0.02 OPC 31260 (μM)
Isotonic NaCl
Conditioned Medium
# p<0.0001 vs Control
**** p<0.0001 vs Isotonic Control
** p=0.0018 vs Isotonic NaCl
D
Isotonic Control (3hr)
Isotonic NaCl (3hr)
OPC 31260 0.02 μM
37 kDa
AQP2 Total
37 kDa
AQP2 pS269
37 kDa
Total Protein
IMCD whole cell lysates
E
AQP2 Total
AQP2 pS269
Protein Abundance (AU)
Isotonic Control
Isotonic NaCl
OPC 31260 (0.02 μM)
* p<0.05 vs Isotonic Control
Actin
Total AQP2
Merge
Isotonic Control
Isotonic NaCl
Isotonic NaCl + OPC 31260 0.02 μM
F G H I J K L M N

Figure 5


A
Relative mRNA Expression
2.0
1.5
1.0
0.5
0.0
*
Water Restricted
Water Loaded
B
Water Restricted
Water Loaded
20 kDa
Pre-pro-vasopressin
20 kDa
Total Protein
C
Protein Abundance (AU)
2.0
1.5
1.0
0.5
0.0
**
Water Restricted
Water Loaded
* p = 0.041
** p = 0.013

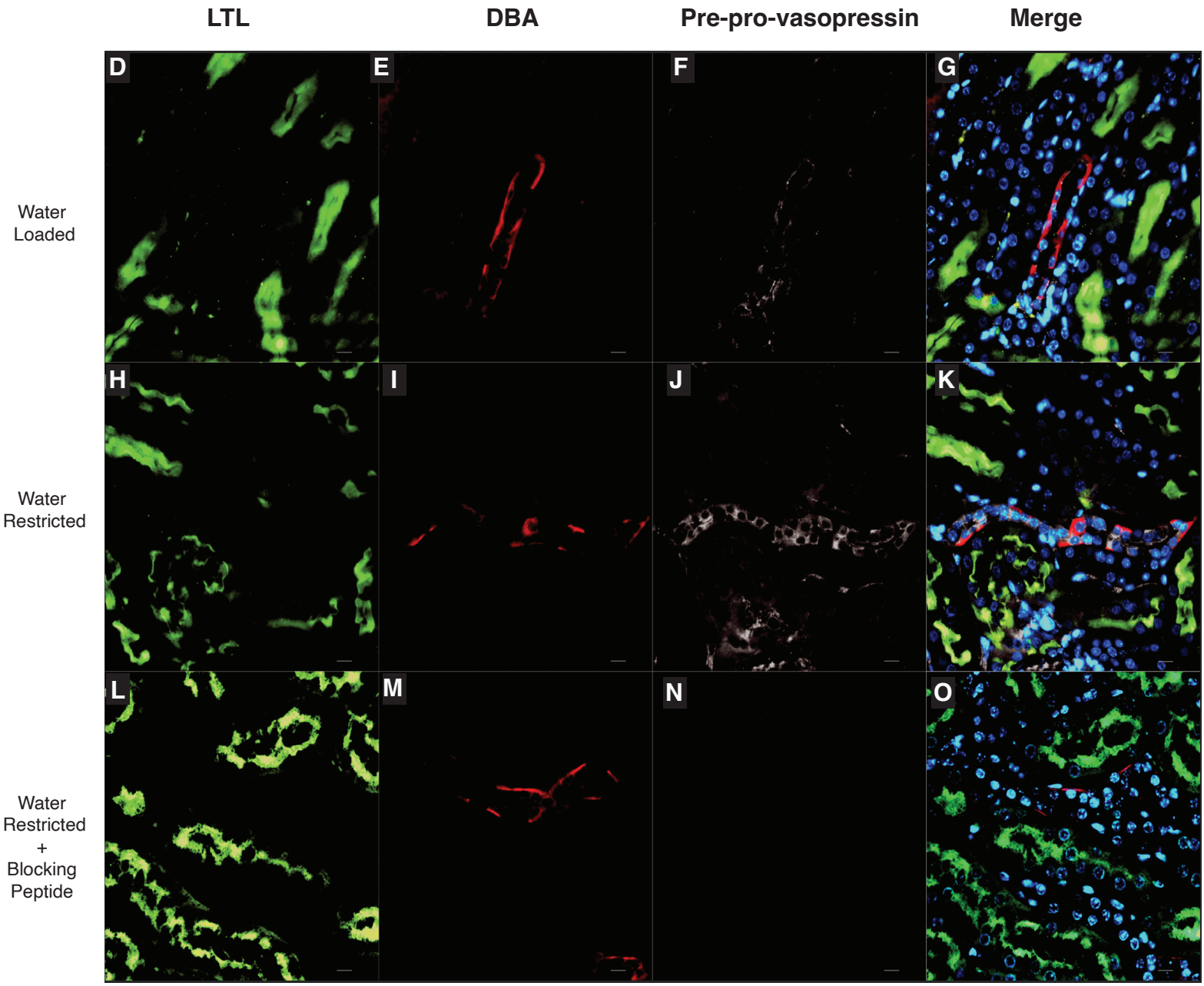
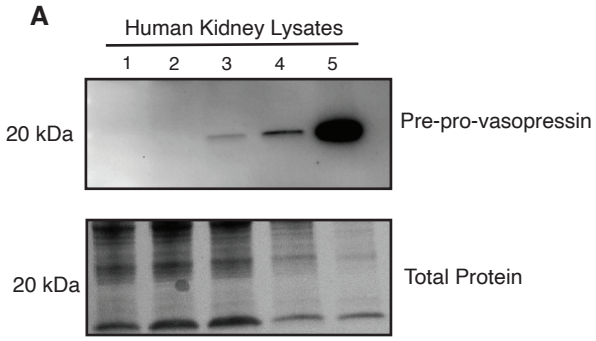
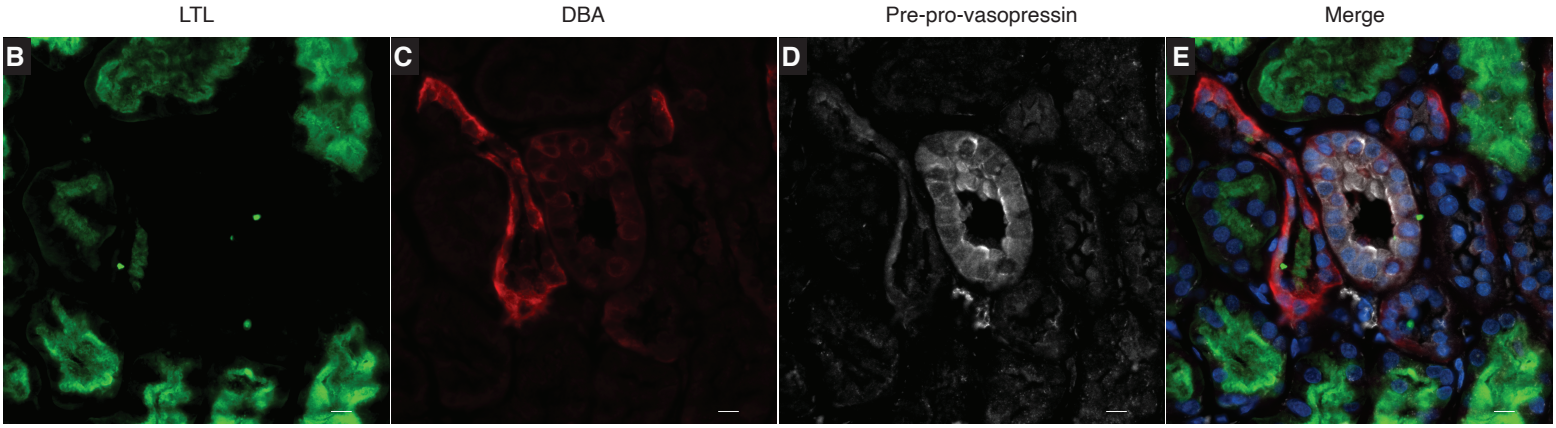

LTL
DBA
Pre-pro-vasopressin
Merge
Water Loaded
Water Restricted
Water Restricted + Blocking Peptide
D
E
F
G
H
I
J
K
L
M
N
O

Figure 6


A
Human Kidney Lysates
1
2
3
4
5
20 kDa
Pre-pro-vasopressin
20 kDa
Total Protein


LTL
DBA
Pre-pro-vasopressin
Merge
B
C
D
E