Supplemental material

Title

"Role of Angiotensin-II type 1a receptor (AT1aR) of renal tubules in regulating inwardly-rectifying potassium channels 4.2 (Kir4.2), Kir4.1 and epithelial Na^+ channel (ENaC)."

Author Name

[1&4]Xin-Peng Duan, [2&4] Yu Xiao, [3]Xiao-Tong Su, [4]Jun-Ya Zheng, [3]Susan Gurley,[3]Jacqueline Emathinger, [3]Chao-Ling Yang, [3]James McCormick, [3]David H. Ellison, [4]Dao-Hong Lin and [4]Wen-Hui Wang

Affiliation

[1] Department of Physiology, Xuzhou Medical University, Xuzhou, China,

[2]Department of Physiology, Qiqihar Medical College, Heilongjiang, China;

[3]Department of Medicine, Oregon Health & Science University, Portland, Oregon,

[4]Department of Pharmacology, New York Medical College, Valhalla, NY.

Corresponding author

Wen-Hui Wang, MD

Department of Pharmacology

New York Medical College

Valhalla, NY 10595

914 594 4139 (phone)

914 347 4956 (Fax)

Wenhui_wang@nymc.edu

Methods

Metabolic Cage and Measurement of systolic blood pressure

The male/female (m/f) mice (one mouse per cage) were placed into a metabolic cage (MMC100, Hatteras Instruments, Cary, NC) for three-day adaptation before 24-hr urine collection. We recorded the food and water intakes and collected urine after 24 hr. Urine volume was measured and Na^+ and K^+ contents of the urine were determined by flame photometers (Cole-Parmer). Systolic blood pressure (SBP) of the mice was measured by tail-cuff methods (Model CODA, Kent Scientific, Torrington, CT). Male and female *Agtr1a*$^{flox/flox}$ and Ks-AT1aR KO mice (12-14 weeks old) were trained on the equipment for a minimum of five days during which data was not recorded. Baseline SBP was then determined for three consecutive days.

Preparation of the renal tubules

Mice were sacrificed by CO_2 inhalation plus cervical dislocation. The abdomen of the mice was quickly opened to expose the left kidney which was then perfused with 2 ml L-15 medium (Life Technology) containing type 2 collagenase (250 unit/ml) (Worthington Bio.). After the perfusion, the left kidney was removed for harvesting the renal cortex, which was further cut into small pieces and incubated in collagenase-containing L-15 media for 30-50 min at 37ºC. The tissue was then washed three times with fresh L-15 medium and transferred to an ice-cold chamber for dissection. The isolated PT, DCT and CCD tubules were placed on a small cover glass coated with poly-lysine and the cover glass was placed on a chamber mounted on an inverted microscope.

Measurement of ROMK and ENaC

We used the last 100 μm DCT before the start of the CNT, which has a smaller tubule diameter than DCT2. However, it was possible that some measurements were performed in the initial CNT because it is not always obvious to identify the beginning of the CNT. Thus, we have referred that the study was performed in the DCT2/CNT. The CCD was identified after the first branch of connecting tubule. After forming a high resistance seal, the membrane capacitance was monitored until the whole-cell patch configuration was formed. For measuring TPNQ (400 nM)-sensitive K^+ currents (ROMK), the tip of the pipette was filled with a pipette solution containing (in mM) 140 KCl, 2 MgATP, 1 EGTA, 0.5 mM MgATP and 10 HEPES (pH 7.4). The pipette was then back-filled with the pipette solution containing amphotericin B (20 μg/0.1 ml). The bath solution contains (in mM) 140 KCl, 2 $MgCl_2$, 1.8 $CaCl_2$ and 10 HEPES (pH=7.4). For the measurement of amiloride-sensitive Na^+ currents (ENaC), the pipette solution contained (in mM) 125 K-gluconate, 15 KCl, 2 MgATP, 1 EGTA, and 10 HEPES (pH 7.4), whereas the bath solution contained 130 Na-gluconate, 10 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 5 HEPES (pH 7.4). ENaC currents were determined by adding amiloride (10 μM) in the bath solution. The currents were low-pass filtered at 1 KHz, digitized by an Axon interface with 4 KHz sampling rate (Digidata 1440A). Data were analyzed using the pClamp software system 9.0 (Axon).

Immunoblotting

Whole kidney protein extract was obtained from frozen kidney homogenized in a buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1mM EGTA, 1 mM DTT supplemented with phosphatase and protease inhibitor cocktails (Sigma). Protein (40-60 μg) was separated on 4-12% (wt/vol) Tris-Glycine gel (Novex™, TheromoFisher Scientific) and transferred to nitrocellulose membrane. The membranes were incubated 1 hour with LI-COR blocking buffer (PBS) and then incubated overnight at 4ºC with primary antibodies including anti-pNHE_3, anti-NHE_3, anti-NCC, anti-pNCC at threonine-53, anti-Kir4.1, anti-Kir4.2, anti-Kir5.1, anti-αENaC, anti-βENaC and anti-γENaC. All antibodies used in the experiments are validated and the information is included in table s2. An Odyssey infrared imaging system (LI-COR) was used to capture the images at a wave-length of 680 or 800 nM.

Measurement of AT1A mRNA levels by qRT-PCR.
We followed the protocol published previously to measure relative levels of *Agtr1a* mRNA using renal cortex tissue in both *Agtr1a*$^{flox/flox}$ and Ks-AT1aR KO mice [1]. The mRNA was isolated and reverse transcription was performed using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). SYBR Green-based quantitative PCR was carried out using the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Controls tubes lacking cDNA and containing RNA were included in each run. After PCR cycling was ended, we performed melting curve analyses. Representative PCR products were run on agarose gel and visualized by SYBR® safe staining (Invitrogen). For calculating *Agtr1a* mRNA expression, the amount of *Agtr1a* gene relative to endogenous control *Gapdh* was determined by the ΔCT method. The following primer sequences were used: AT1a Forward-5'-ACTCACAGCAACCCTCCAAG-3' , AT1a Reverse-5'-ATCACCACCAAGCTGTTTCC-3' (Amplicon size: 236bp), GAPDH Forward-5'-TCACCACCATGGAGAAGGC-3' ,GAPDH Reverse-5'-GCTAAGCAGTTGGTGGTGCA-3' (Amplicon size: 168bp).

Kidney perfusion fixation and Immunofluorescence
Mice were anesthetized with a ketamine-xylazine-acepromazine cocktail (50:5:0.5 mg/kg). The right kidney was tied off, removed, and flash frozen in liquid nitrogen for isolation of protein. The left kidney was perfusion fixed with retrograde abdominal aortic perfusion of 3% paraformaldehyde in PBS (pH 7.4). After perfusion, the left kidney was removed, dissected, and cryopreserved in 800 mOsm sucrose in PBS overnight before being embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA). Slides were prepared by cutting 5-µm sections and were stored at -80°C until use. Immunofluorescent staining was prepared as follows. Slides were incubated with 0.5% Triton X-100 in PBS for 30 min. Sections were then blocked with 5% milk in PBS for 30 min followed by incubation with primary antibody, diluted in blocking buffer, for 1 h at room temperature or overnight at 4°C. Sections were washed with PBS three times and incubated with fluorescent dye-conjugated secondary antibody, diluted in blocking buffer, for 1 h at room temperature. Sections were washed with PBS three times and stained with DAPI before being mounted with ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific, Carlsbad, CA). Images were captured using a KEYENCE BZ-X800 microscope (Itasca, IL). Image processing was completed using BZ-X800 analyzer software (KEYENCE CORPORATION OF AMERICA, Itasca, IL). A list of antibodies used for immunofluorescence is provided in table s2.

Measurement of plasma Angiotensin-II levels
Plasma angiotensin II peptide levels were determined with an enzyme immunoassay (ELISA Kit S-1133, BMA Biomedicals Switzerland). For this, plasma was mixed with ice-cold methanol at a ratio of 1:2. After centrifugation at 4,000 rpm for 30 minutes at 4°C, the collected supernatant was dried by centrifugal evaporation. Dried pellets were rehydrated with the buffer provided in the kit. AngII was measured following manufacturer's instruction as previously described (PMID: 30185469) and expressed as picogram per mL of plasma.

RNAScope Method
Agtr1a mRNA expression was examined with the BaseScope™ Detection Reagent Kit v2 – RED (Cat. No. 323900, ACD Bio, CA) by following the manufacturer's instructions for FFPE slides. Hybridization signals were detected using Fast-Red staining and counterstained with 50% Gil's Hematoxylin I solution. Slices were mounted with VectaMount (ACD Bio) and viewed with an inverted Microscope. Positive controls were routinely enclosed. The RNAscope probes used are targeted exon 3 (Cat. # 1273921-C1 BaseScope Probe - BA-Mm-Agtr1a-3zz-st-C1, ACD Bio).

Supplemental reference

1. Sparks MA, Stegbauer J, Chen D, Gomez JA, Griffiths RC, Azad HA, Herrera M, Gurley SB, Coffman TM. Vascular type 1a angiotensin ii receptors control bp by regulating renal blood flow and urinary sodium excretion. *Journal of the American Society of nephrology*. 2015;26:2953
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Table s1 Primers for genotyping and RNAscope

Gene	**Primer sequence**	**size**
Agtr1a flox	Forward 5’ GCTTTCTCTGTTATGCAGTCT-3’ Reverse 5’ ATCAGCACATCCAGGAATG-3’	1600bp flox 1400bp WT
Pax8rtTA	Forward 5’ CCATGTCTAGACTGGACAAGA-3’ Reverse 5’ CAGAAAGTCTTGCCATGACT-3’	220bp
LC1-CRE	Forward 5’ TTTCCCGCAGAACCTGAAGATG-3’ Reverse 5’ TCACCGGCATCAACGTTTTCTT-3’	190 bp

Table s2 Information of Antibodies used in experiments

Antibody	Species	Dilution for WB	Source
pT53-NCC*	Rabbit	1:3000	PhosphoSolutions [2]
NCC*	Rabbit	1:2000	Millipore[2]
Kir4.2 (APC-058)	Rabbit	1: 500	Alomone lab [3]
Kir5.1	Rabbit	1:200	Alomone [4]
Kir4.1	Rabbit	1:200	Alomone lab [2]
GAPDH	Rabbit	1:1000	Cell Signaling [5]
ENaC-α	Rabbit	1:1000	StressMarq[5]
ENaC-β	Rabbit	1:1000	StressMarq[5]
ENaC-γ	Rabbit	1:1000	StressMarq[5]
pNHE3	mouse	1.1000	Novus Biologicals[6]
NHE3	mouse	1:1000	Santa Cruz[6]

Table s3 Density of *Agtr1a* mRNA

	Glomerulus (arbitrary number)	100 μm² renal tubules (arbitrary number)
***Agtr1a*$^{flox/flox}$ (m)**	31±5	27±5
Ks-AT1aR KO (m)	29±6	4±1*

Asterisk indicates the significant difference between KO and corresponding control mice. Density of the probe is calculated by Fiji ImageJ software. The values are arbitrary and it is presented as 100 □M square of kidney section after subtracting the back ground value.

Table s4 Blood pressure (BP) and heart rate(HR) in the WT and Ks-AT1aR-KO mice

	***Agtr1a*$^{flox/flox}$ (m)**	Ks-AT1aR KO(m)	***Agtr1a*$^{flox/flox}$ (f)**	Ks-AT1aR KO (f)
SBP (mmHg)	119±3 (n=8)	102±3 * (n=8)	115±2(n=8)	101±3 *
DBP (mmHg	95±3(n=8)	82±2 * (n=8)	95±3(n=8)	83±2*
HR	451±18(n=8)	435±18 (n=8)	501±28 (n=8)	471±17 (n=8)

Asterisk indicates the significant difference between KO and corresponding control mice.

Supplemental Figures

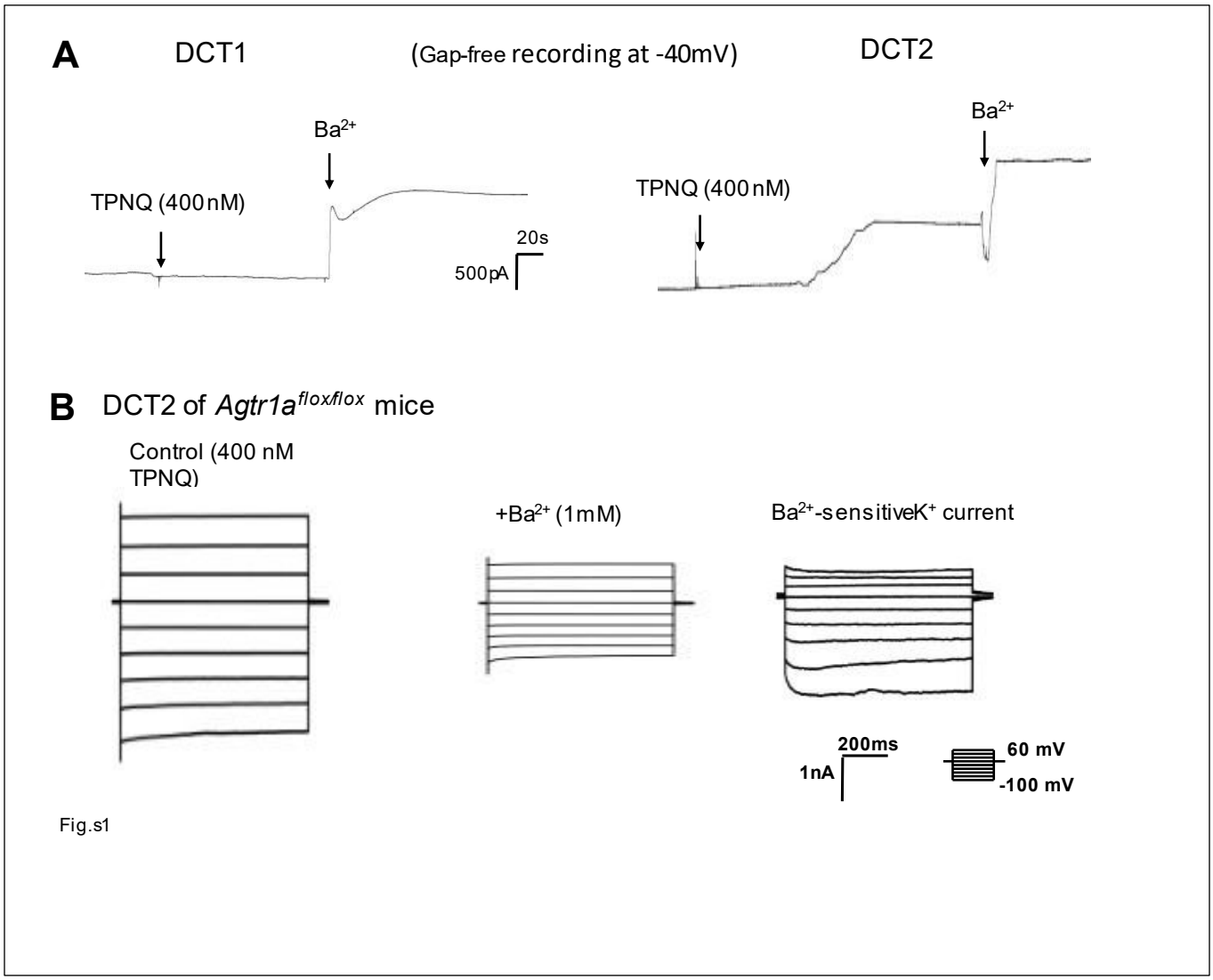

Fig.s1 **Measurement of whole-cell K^+ currents in DCT1 and DCT2** (A) a whole-cell recording showing TPNQ-sensitive currents (ROMK) and TPNQ-insensitive/ Ba^{2+}-sensitive Kir4.1/Kir5.1-mediated K^+ currents using gap-free protocol in DCT1 or DCT2 (at -40 mV). (B) A whole-cell recording showing Ba^{2+}-sensitive Kir4.1/Kir5.1-mediated K^+ currents measured with step protocol from -100 to 60 mV in the DCT2 in the presence of 400 nM TPNQ.

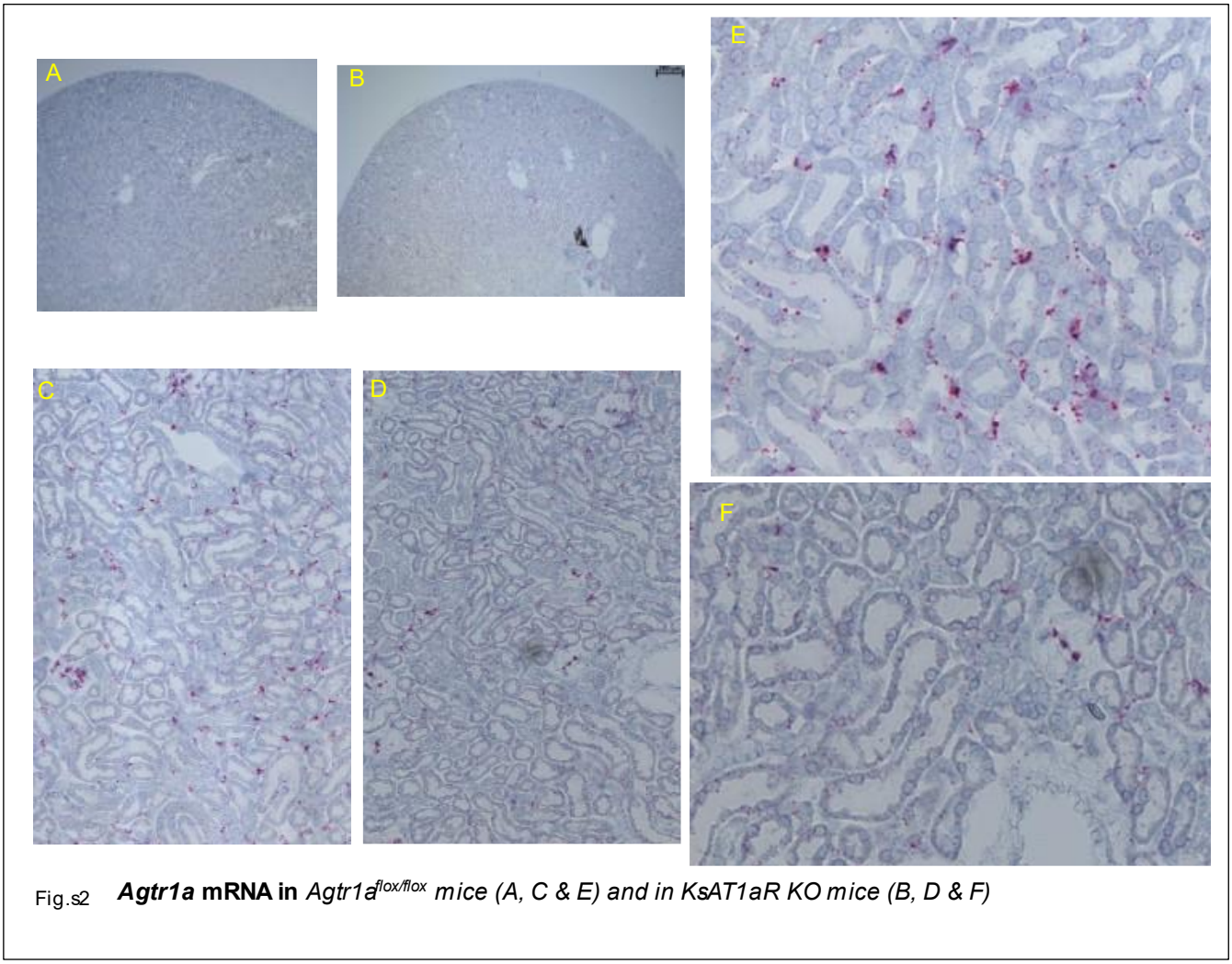

Fig.s2 ***Agtr1a* mRNA in *Agtr1a*$^{flox/flox}$ mice (A, C & E) and in KsAT1aR KO mice (B, D & F)**

Fig.s2a ***Agtr1a* mRNA expression in *Agtr1a*$^{flox/flox}$ mice and Ks-AT1aR KO mice**. Hybridization signals were detected using Fast-Red staining and counterstained with 50% Gil's Hematoxylin I solution. (A, C, E) Images with low, intermediate and large magnifications show the *Agtr1a* mRNA expression (red dots) in *Agtr1a*$^{flox/flox}$ mice. (B, D, F) Images with low, intermediate and large magnifications show the *Agtr1a* mRNA expression in Ks-AT1aR KO mice. *Agtr1a* mRNA expression is detected in the glomerulus but not in the tubule structure in KO mice. RNAscope probes used are targeted exon 3 (Cat. # 1273921-C1 BaseScope Probe - BA-Mm-Agtr1a-3zz-st-C1, ACD Bio). (A)

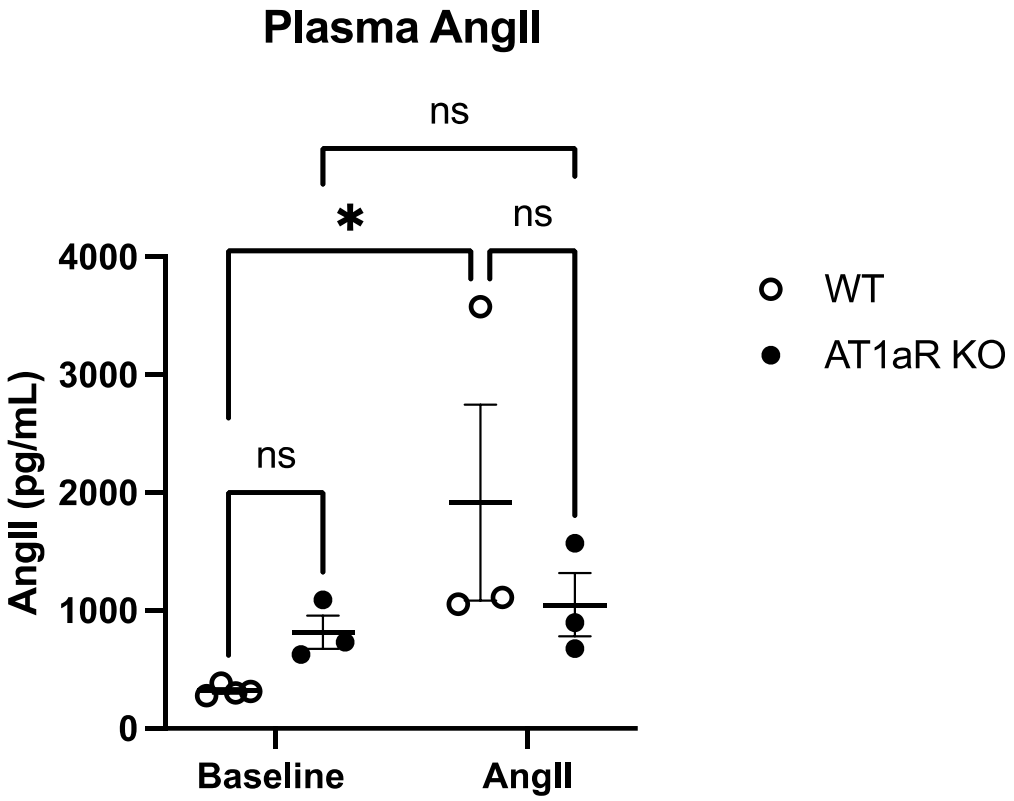

Fig.s2b **Plasma angiotensin-II level in WT and Ks-AT1aR-KO mice.** The experiments were performed in male WT (*Agtr1a*$^{flox/flox}$) and Ks-AT1aR-KO mice under control conditions (Baseline) and during angiotensin-II perfusion (AngII). Asterisk indicates a significant difference.

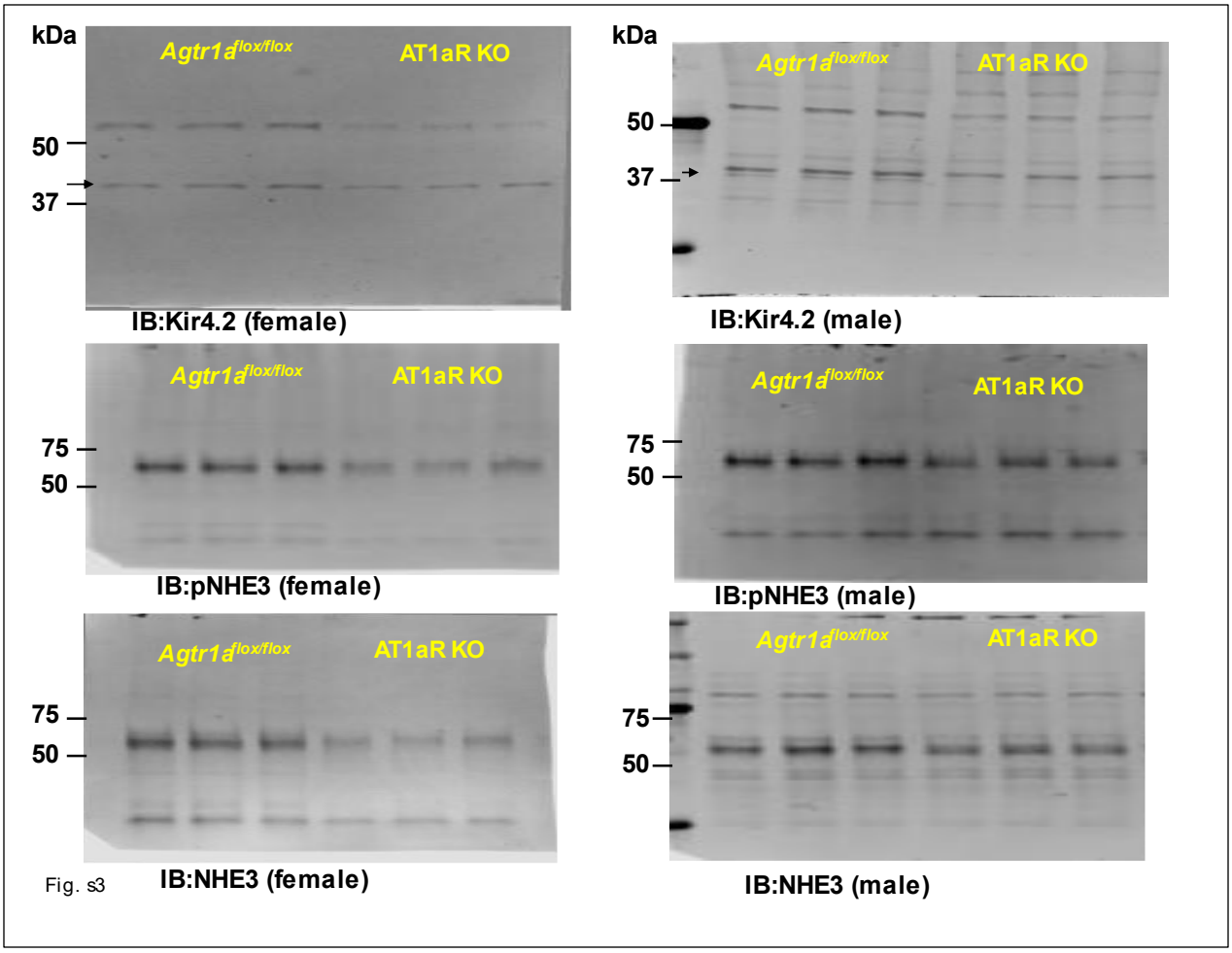

Fig.s3 Deletion of AT1aR decreases Kir4.2 expression. A set of western blot shows the expression of Kir4.2, phosphor (P)-NHE_3 (S^{552}) and total NHE_3 in female and male Ks-AT1aR KO mice and *Agtr1a*$^{flox/flox}$ mice. Kir4.2 monomer is indicated by an arrow.

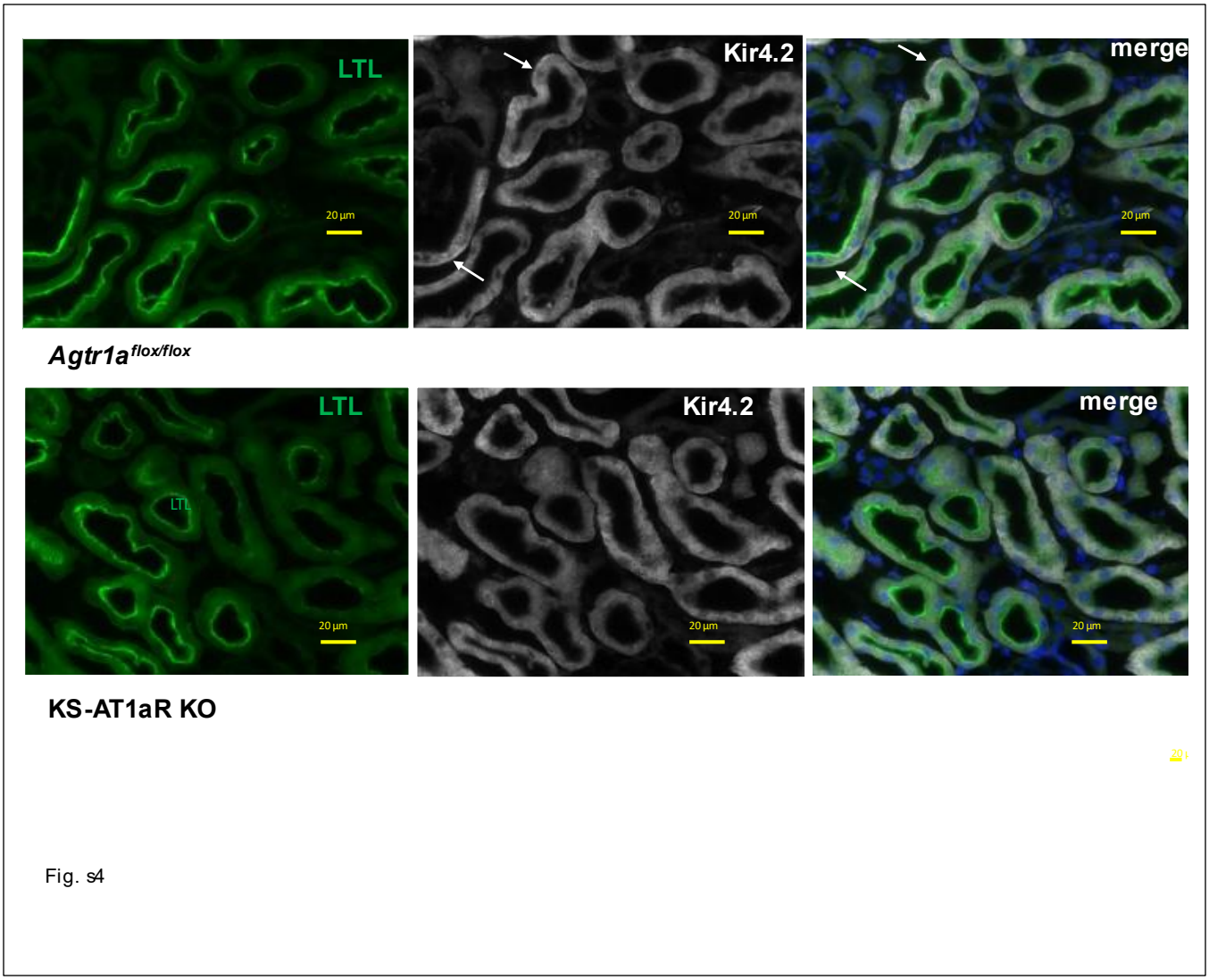

Fig.s4 Kir4.2 expression in proximal tubule of *Agtr1a*$^{flox/flox}$ (control) mice and KS-AT1aR KO mice. Immunofluorescence images of Kir4.2 (grey) and lotus tetragonolobus lectin (LTL), a proximal tubule marker (green) in control and KS-AT1aR KO mice. Nuclei are stained using DAPI in blue. White arrow indicates the Kir4.2 staining in the basolateral membrane of proximal tubule of the control mice.

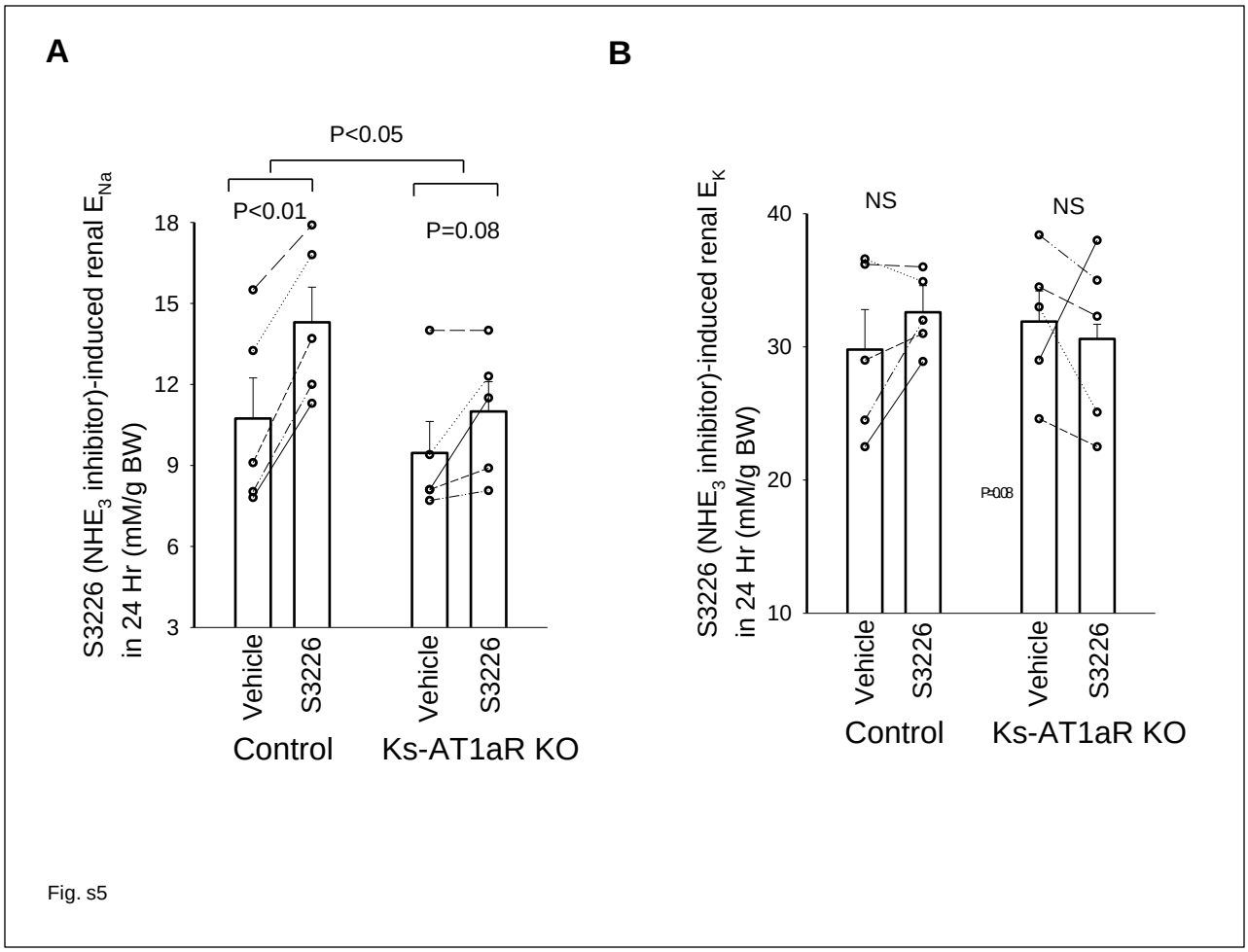

Fig.s5 Deletion of AT1aR attenuates S3226 (NHE_3 inhibitor)-induced renal natriuresis (A) A bar graph with scatter plots shows the effect of $3226 on renal natriuresis (ENa) in male control (*Agtr1a*$^{flox/flox}$) and Ks-AT1aR KO mice. (B) A bar graph with scatter plots shows the effect of $3226 on renal potassium excretion (E_K) in the control (*Agtr1a*$^{flox/flox}$) and Ks-AT1aR KO mice. S3226 was resolved in saline (1 mg S3226 in 1 ml) and the mice were fed S3226 (3mg per Kg body weight) with gavage. After S3226 application, the mice were kept in a metabolic cage for 24 hours to collect urine.

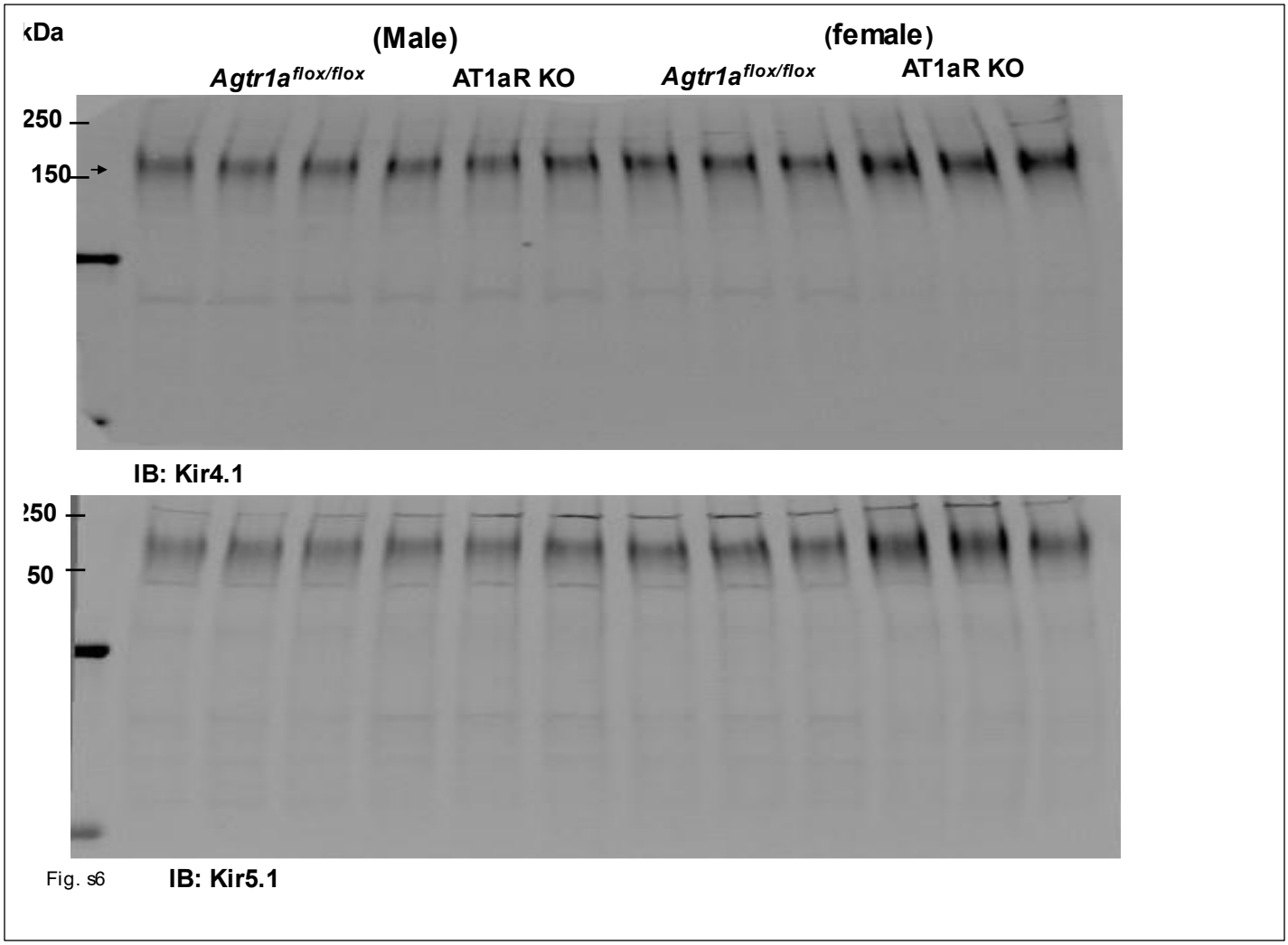

Fig. s6

Fig.s6 Deletion of AT1aR did not decrease the expression of Kir4.1 and Kir5.1 heterotetramer. A set of western blot shows the expression of Kir4.1 and Kir5.1 in male and female Ks-AT1aR KO mice and *Agtr1a*$^{flox/flox}$ mice. Kir4.1 band is indicated by an arrow.

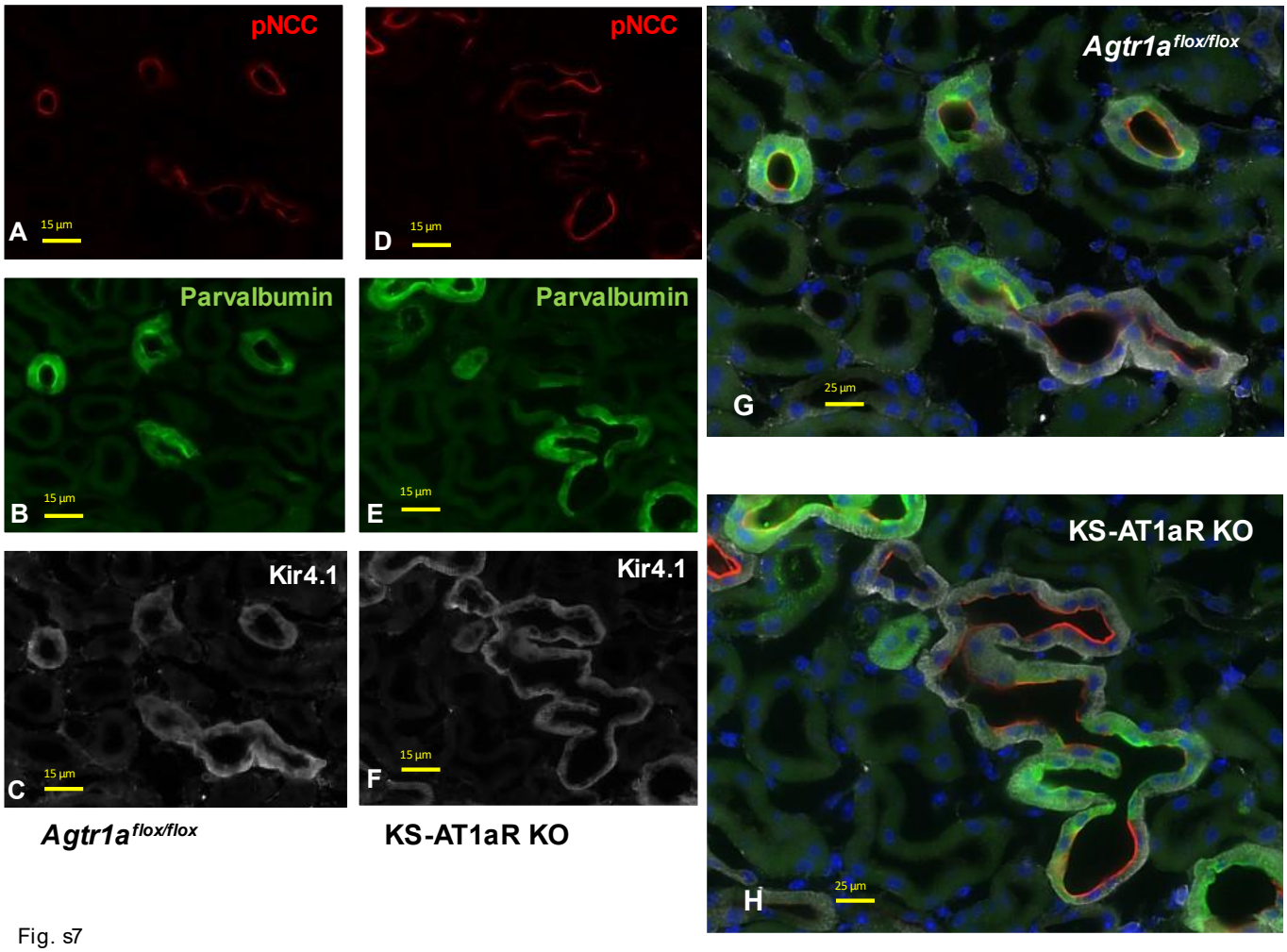

Fig. s7

Fig.s7 Kir4.1 expression in DCT of *Agtr1a*$^{flox/flox}$ (control) mice and KS-AT1aR KO mice.
Immunofluorescence images of pNCC (red), parvalbumin (green) and Kir4.1 (grey). (A-C) Kir4.1 staining in *Agtr1a*$^{flox/flox}$ mice. ((D-F) Kir4.1 staining in Ks-AT1aR KO mice. A merged view of pNCC, parvalbumin and Kir4.1 in *Agtr1a*$^{flox/flox}$ mice (G) and in Ks-AT1aR KO mice (H), respectively.

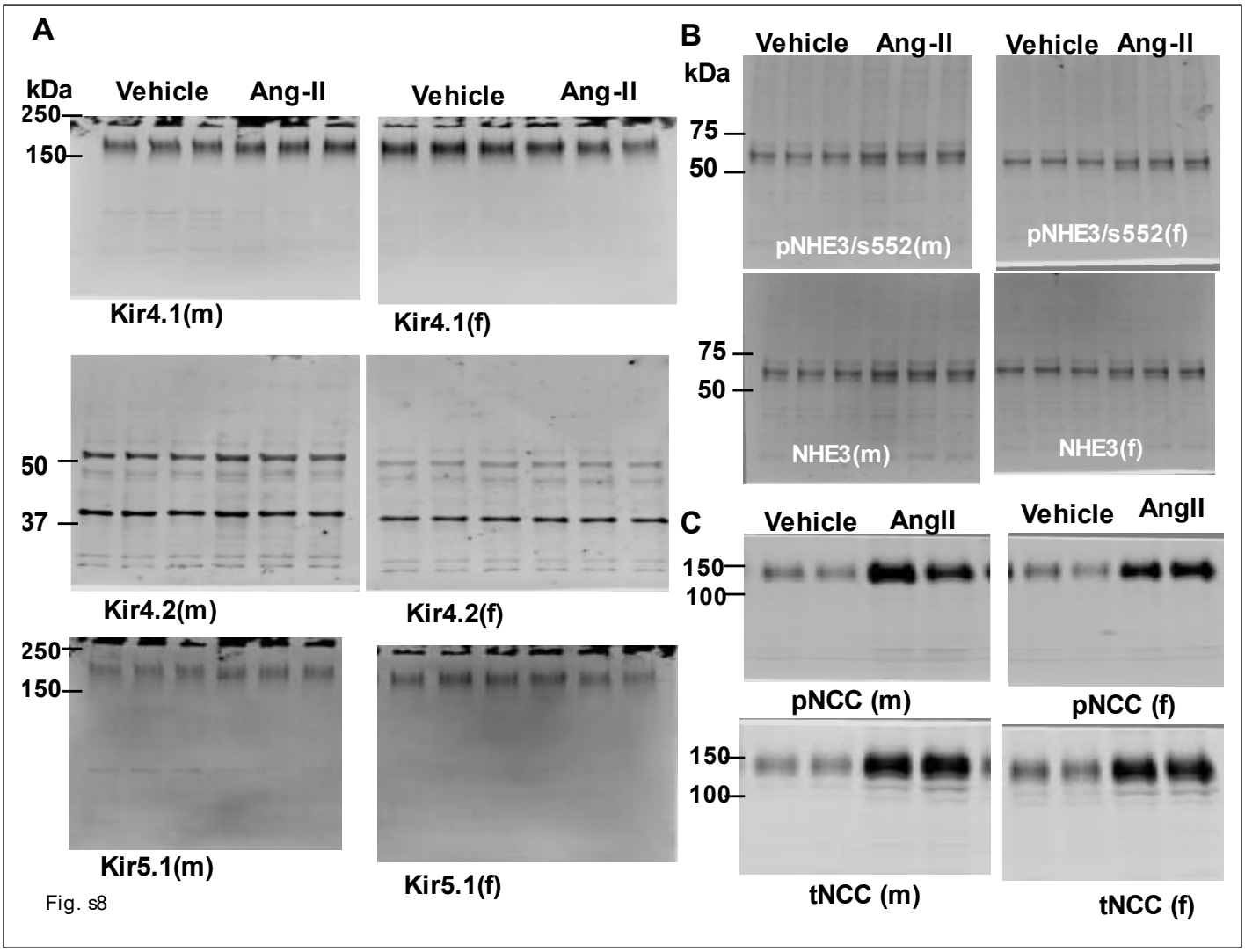

Fig. s8 Effect of angiotensin II perfusion (7 days) on the expression of Kir4.1, Kir4.2, Kir5.1, NHE_3 and NCC. (A) A set of western blot shows the expression of Kir4.2, Kir4.1, Kir5.1 in male (m) and female (f) mice. (B) A western blot shows the expression of $pNHE_3$ (S^{552}) and total NHE_3 in m/f mice. (C) A western blot shows the expression of pNCC and tNCC in m/f mice. Male and female control mice were treated with angiotensin-II perfusion for 7 days (at 200 ng/min /Kg) through an installed osmotic pump.

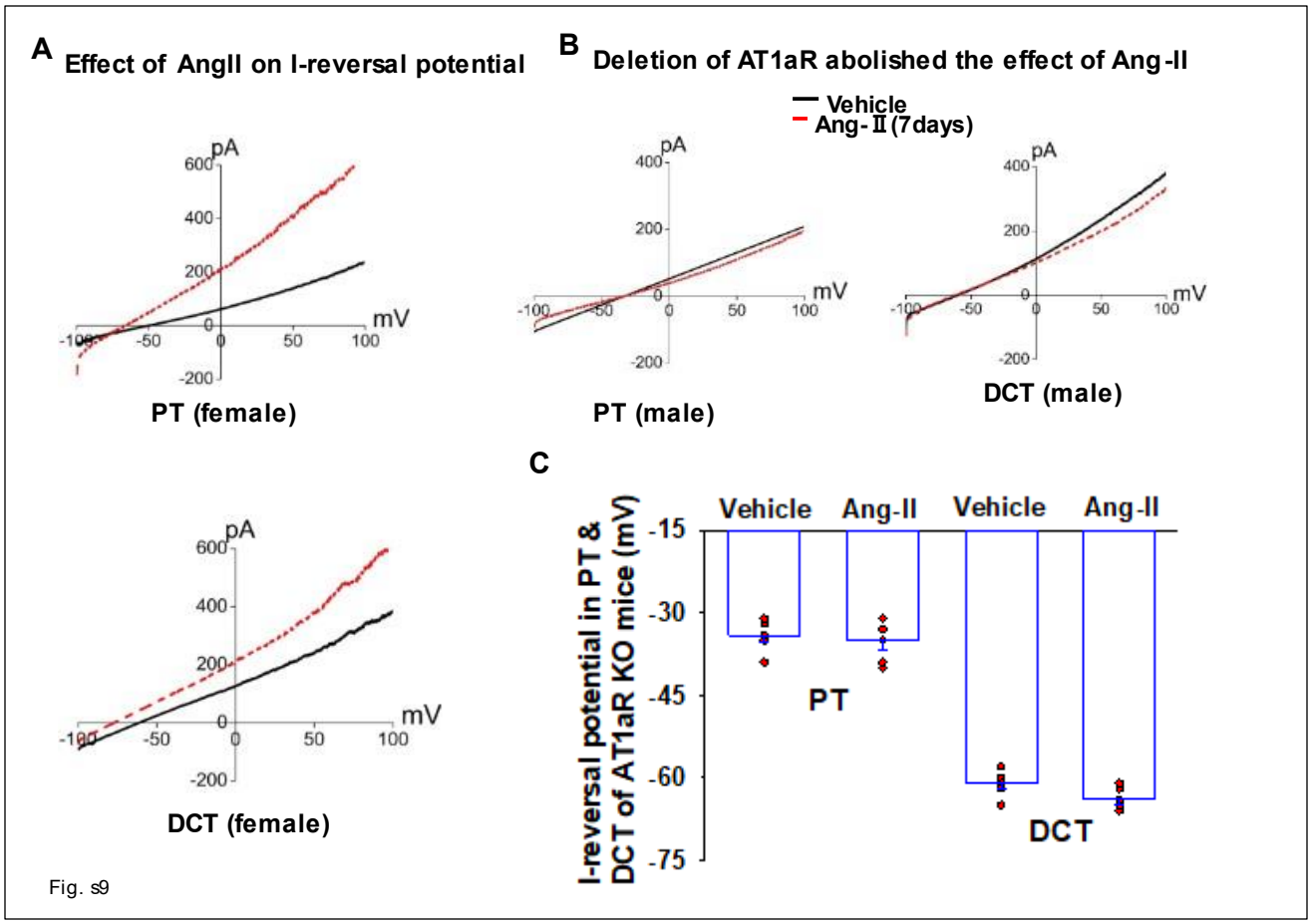

Fig.s9 Deletion of AT1aR abolished angiotensin-II effect on the membrane potential of PT and DCT. (A) A set of I-reversal potential traces measured with whole-cell voltage clamp from -100 to 100 mV in the PT and DCT1 of female control mice treated with angiotensin-II for 7 days (red) and vehicle (black), respectively. (B) A set of I-reversal potential traces measured with whole-cell voltage clamp from -100 to 100 mV in the PT and DCT1 of male Ks-AT1aR KO mice treated with vehicle and angiotensin-II for 7 days, respectively. (C) A bar graph with a scatter plot summarizes the results of experiments in which I-reversal potentials were measured in the PT and DCT1 of male Ks-AT1aR KO mice treated with vehicle and angiotensin-II.

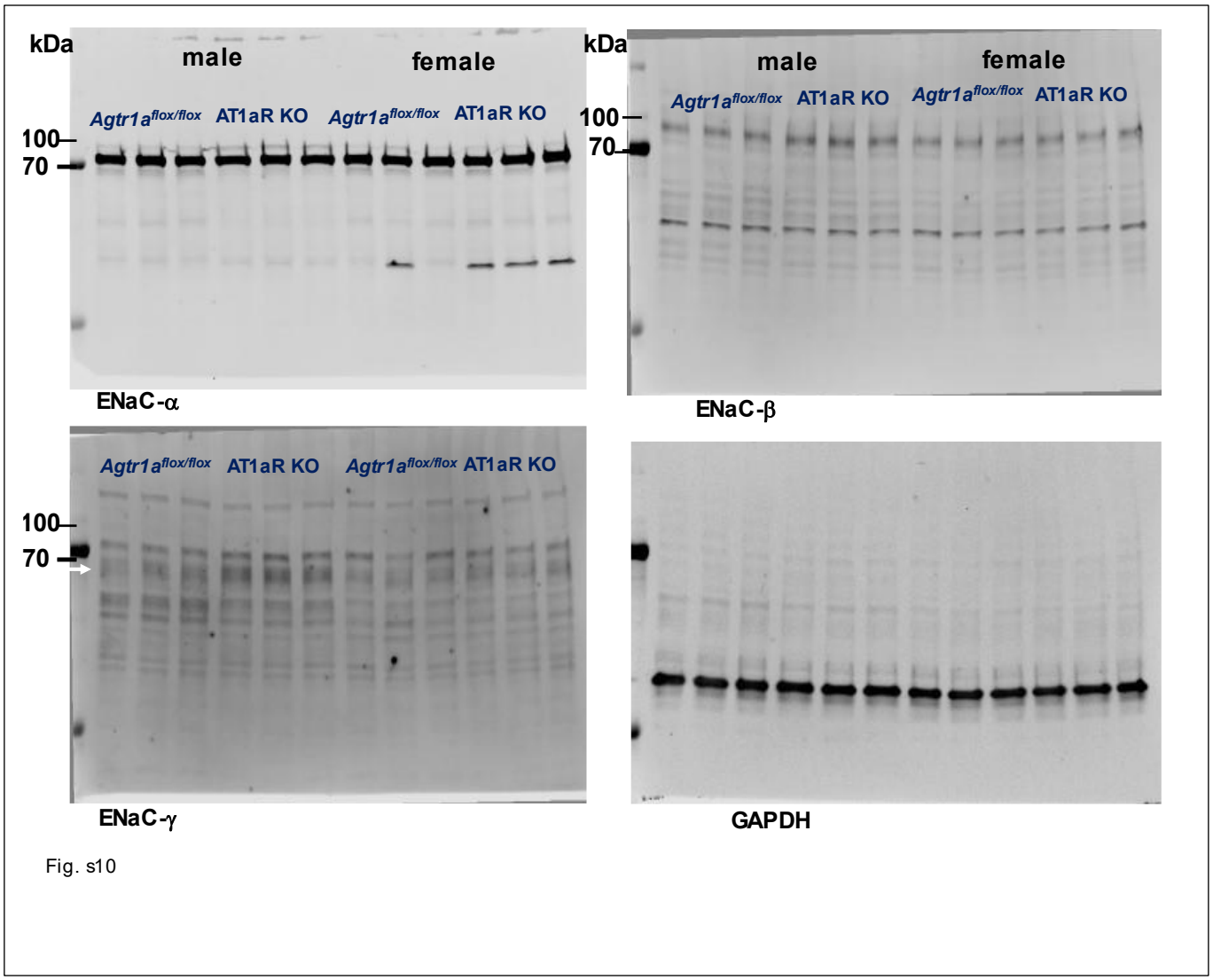

Fig. s10 Deletion of AT1aR slightly stimulates the expression of cleaved γENaC . A set of western blot shows the expression of αENaC, βENaC, γENaC, cleaved γENaC and GAPDH in male and female Ks-AT1aR KO mice and *Agtr1a*$^{flox/flox}$ mice. The cleaved γENaC is indicated by an arrow.

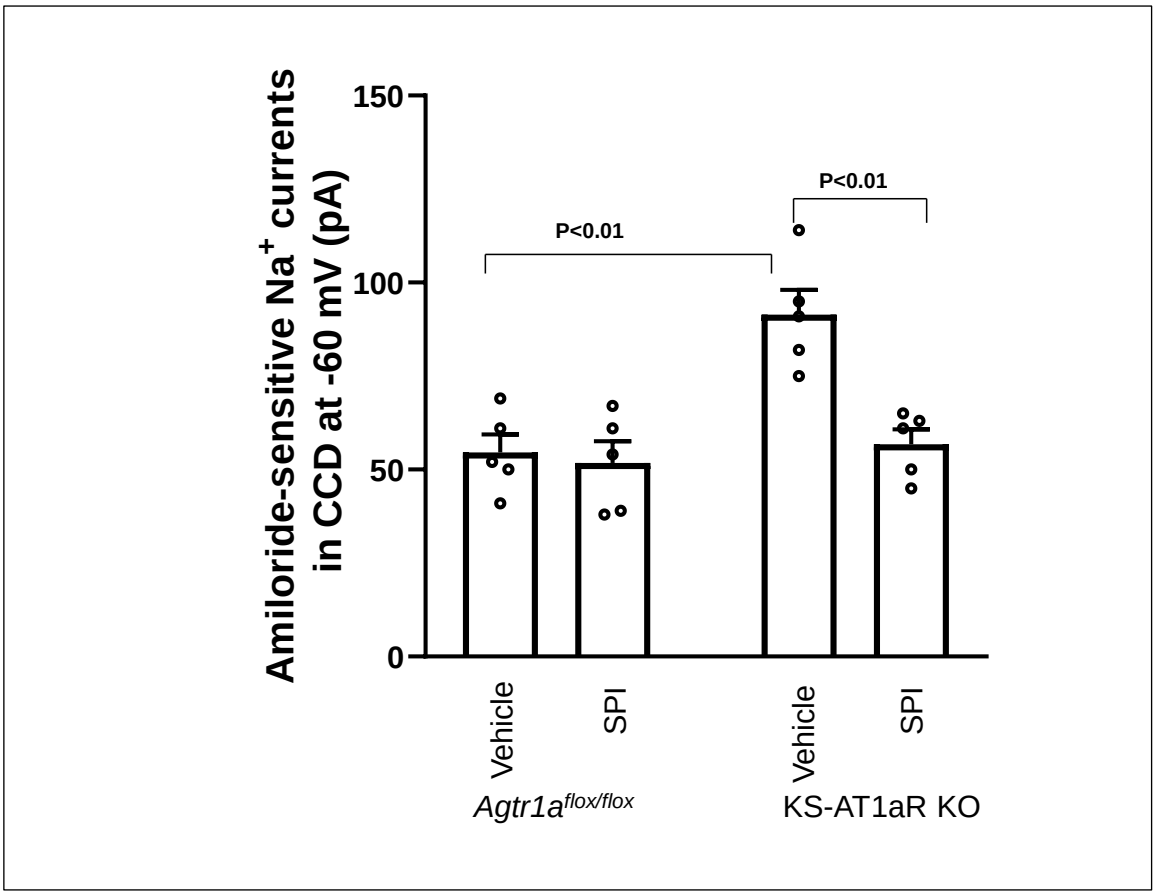

Fig.s11 Spironolactone (SPI) decreased amiloride-sensitive Na^+ currents of the CCD in Ks-AT1aR KO mice. A bar graph with scatter plots summarizes the results of experiments in which the whole-cell amiloride-sensitive Na^+ currents were measured in the CCD of male control (*Agtr1a*$^{flox/flox}$) and Ks-AT1aR KO mice treated with vehicle or SPI for 7 days. SPI was dissolved in ethanol and further diluted at a1:200 ratio into drinking water. By measuring the volume of water intake, the daily dose of spironolactone for each mouse was 40 mg/kg.