Online Data Supplement

Norepinephrine-induced stimulation of Kir4.1/Ki5.1 is required for the activation of Na-Cl transporter in distal convoluted tubule.

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Running Title: Norepinephrine and NCC

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Material and Methods

Preparation of the DCT

Mice were sacrificed by CO_2 inhalation plus cervical dislocation. After mice were sacrificed, the abdomen was opened to expose the left kidney, we perfused the left kidney with 2 ml L-15 medium (Life Technology) containing Type 2 collagenase (250 unit/ml). The collagenase-perfused kidney was then removed for further dissection. The renal cortex was separated and cut into small pieces for additional incubation in collagenase-containing L-15 media for 30-50 min at 37^{o}C. The tissue was then washed three times with fresh L-15 medium and transferred to an ice-cold chamber for dissection. The isolated DCT 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. For continuous perfusion of norepinephrine (2.5mg/d/Kg) or vehicles for 7 days, the mice were implanted subcutaneously with an osmotic mini-pump (Model. 1007D, Alzet). The animal use protocol was approved by independent animal usage committee in both NYMC and Harbin Medical University.

Generating KS-Kir4.1 knockout (KO) mice

Mice expressing Pax8-rtTA and tet-on LC-1 transgene were crossed with *Kcnj10*-floxed mice to generate inducible kidney-specific *Kcnj10* knockout mice (KS-*Kcn10*$^{-/-}$). *Kcnj10* deletion was carried out in 8-week-old male and/or female mice homozygous for floxed *Kcnj10* gene and heterozygous for Pax8-rtTA/LC-1 transgene by providing doxycycline (5mg/ml, 5% sucrose) in the drinking water for 2 weeks. This was followed by at least 2 additional weeks without doxycycline treatment, before performing experiments. Littermate mice of the same age and genetic background drinking 5% sucrose were used as controls (*Kcnj10*$^{+/+}$).

Genotyping

Tail DNA was PCR amplified with the following primers: *kcnj10* forward 5'-TGATGTATCTCGATTGCTGC-3' and reverse 5'-CCCTACTCAATGCTCTTAAC-3' yielding a 550 bp product from the floxed Kcnj10 gene and a 420 bp product from the wild-type allele; Pax8rtTA forward 5'CCATGTCTAGACTGGACAAGA-3' and Pax8rtTA reverse 5'-CAGAAAGTCTTGCCATGACT-3' which yields a 220 bp product; and LC1-CRE forward 5'-TTTCCCGCAGAACCTGAAGATG-3' and reverse 5'-TCACCGGCATCAACGTTTTCTT-3' which yields a 190 bp product.

Single channel recording

Single channel patch-clamp experiments were performed in the basolateral membrane of both DCT1 and DCT2. Single K^+ channel currents were recorded with an Axon200B amplifier (Axon), low-pass filtered at 1 KHz, and digitized by an Axon interface (Digidata 1332) with sampling rate of 4 KHz. The bath solution contains (in mmol/l) 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 1.8 $MgCl_2$, and 10 HEPES (pH 7.40) and the pipette solution contains 140 KCl, 1 EGTA, 2 $MgCl_2$, and 10 HEPES (pH=7.4 titrated with KOH). We determine the channel activity by calculating NP_o (a product of channel number and open probability) which was calculated from data samples of 60 seconds duration in the steady state. NP_o was determined using the following equation:

$$NP_o = \Sigma(t_1 + 2t_2 + ...it_i)$$

where t_i is the fractional open time spent at each of the observed current levels. The channel conductance was determined by measuring the current amplitudes over several voltages. Data were low-pass filtered at 1 KHz, digitized by an Axon interface and analyzed using the pCLAMP Software System 10.3 (Axon).

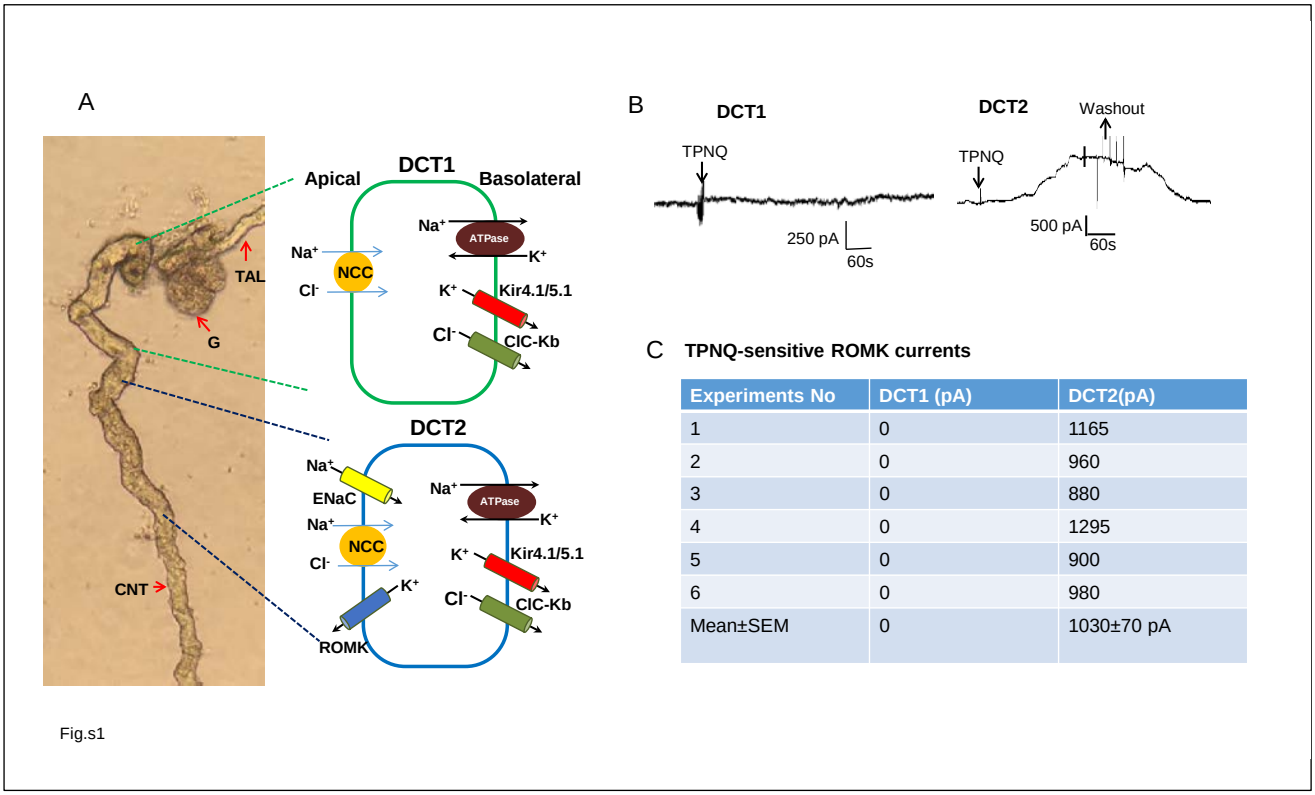
Whole cell recording

For the measurement of Ba^{2+}-sensitive whole-cell K^+ currents and I_K reversal potential, whole-cell patch-clamp recording were performed in the early portion of the DCT (DCT1). The tip of the pipette was filled with pipette solution containing (in mmol/l) 140 KCl, 2 $MgCl_2$, 1 EGTA, and 5 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 for the measurement of I_K reversal potential is the same as that used to perform the single channel recordings. For the measurement of whole-cell Ba^{2+}–sensitive K^+ currents, the bath solution contains (in mmol/L) 140 KCl, 2 $MgCl_2$, 1.8 $CaCl_2$ and 10 HEPES (pH=7.4). An Axon 200A patch-clamp amplifier was used for the whole-cell patch-clamp recording and data were analyzed using the pCLAMP Software System 10.3 (Axon). After forming a high resistance seal (>2 GΩ), the membrane capacitance was monitored until the whole-cell patch configuration was formed. The currents were low-pass filtered at 1 KHz, digitized by an Axon interface with 4 KHz sampling rate (Digidata 1440A).

Procedures for renal clearance

Animal were anesthetized by 2-4% isoflurane through inhaling mask. The mice were placed on a heated small blanket to maintain body temperature at 37^{o}C. The trachea was canulated to clear any mucus that may be produced during the experiment. A carotid artery was catheterized with PE10 tubing for blood sample collections, jugular vein was also cannulated for iv infusion. The bladder was exposed and catheterized via a suprapubic incision with a 10 cm piece of PE-10 tubing for urine collections. After completion of surgery, isotonic saline was given intravenously for 4 hr (0.3 ml/1 hr 0.9% saline) to replace surgical fluid losses and to maintain hemodynamics. To study the effect of norepinephrine on HCTZ-induced urinary Na^+ and K^+ excretion, one time perfusion of HCTZ (25mg/Kg BW) was performed in both control (vehicle) and NE-treated mice. Urine collections started one hr after infusion of 0.3 ml saline and total 6 collections (every 30 minutes) were performed (2 for controls and 4 for experiments). After renal clearance experiment, the mice were sacrificed by IV somnasol. A dual-channel flame photometer with internal lithium standard (Cole-Parmer Instrument, Vernon Hills, IL) was used for measuring urinary or plasma Na^+ and K^+.

Results



C **TPNQ-sensitive ROMK currents**

Experiments No	DCT1 (pA)	DCT2(pA)
1	0	1165
2	0	960
3	0	880
4	0	1295
5	0	900
6	0	980
Mean±SEM	0	1030±70 pA

Fig.s1

Fig. s1 ROMK is expressed only in the late DCT (DCT2). (A) A DCT tubule image showing the DCT1 and DCT2. A corresponding cell model for DCT1 (green) and DCT2 (blue) demonstrates that ROMK and ENaC are only expressed in the apical membrane of the DCT2. (B) A set of whole-cell recordings showing that application of 400 nM TPNQ decreased K^+ (ROMK) currents in the DCT2 but not in DCT1. (C) A table summarizes the results of 6 experiments in which the whole-cell recording was used to detect TPNQ (400nM)-sensitive K^+ (ROMK) currents in the split open DCT1 or DCT2.

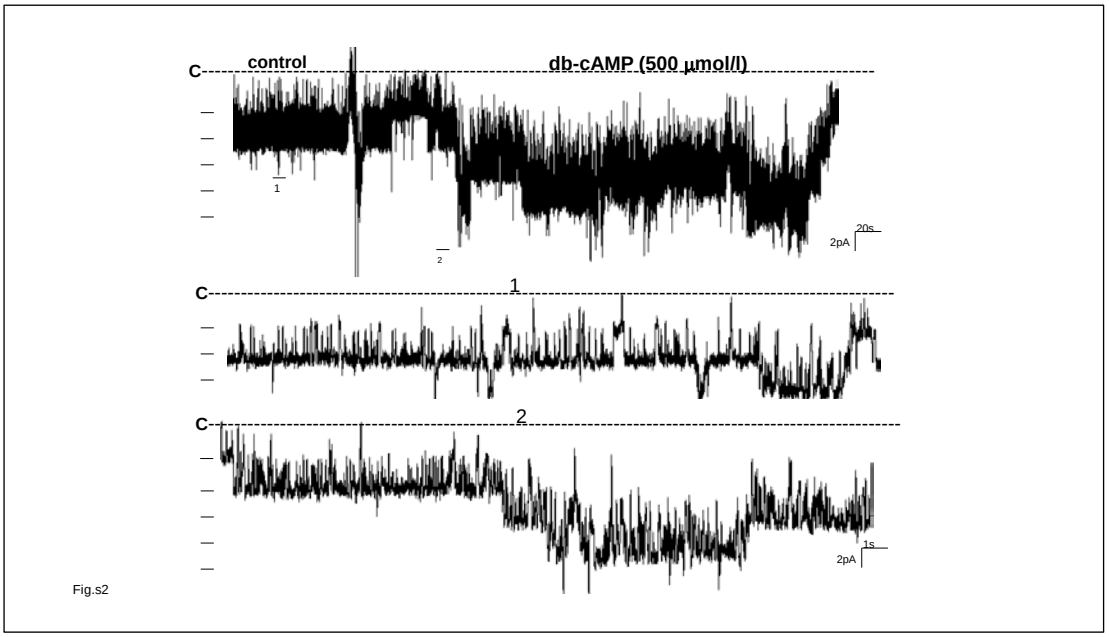


Fig.s2

Fig. s2 Membrane-permeable cAMP analogue stimulates the basolateral K channel in the DCT. A single channel recording shows the effect of db-cAMP on the basolateral 40 pS K^+ channel in the DCT. The top trace illustrates the time course of the experiments. Two parts of the recording indicated by numbers are extended to demonstrate the fast time resolution. The experiments were performed in cell-attached patches. The pipette holding potential was 0 mV and the channel closed level is indicated by a dotted line.

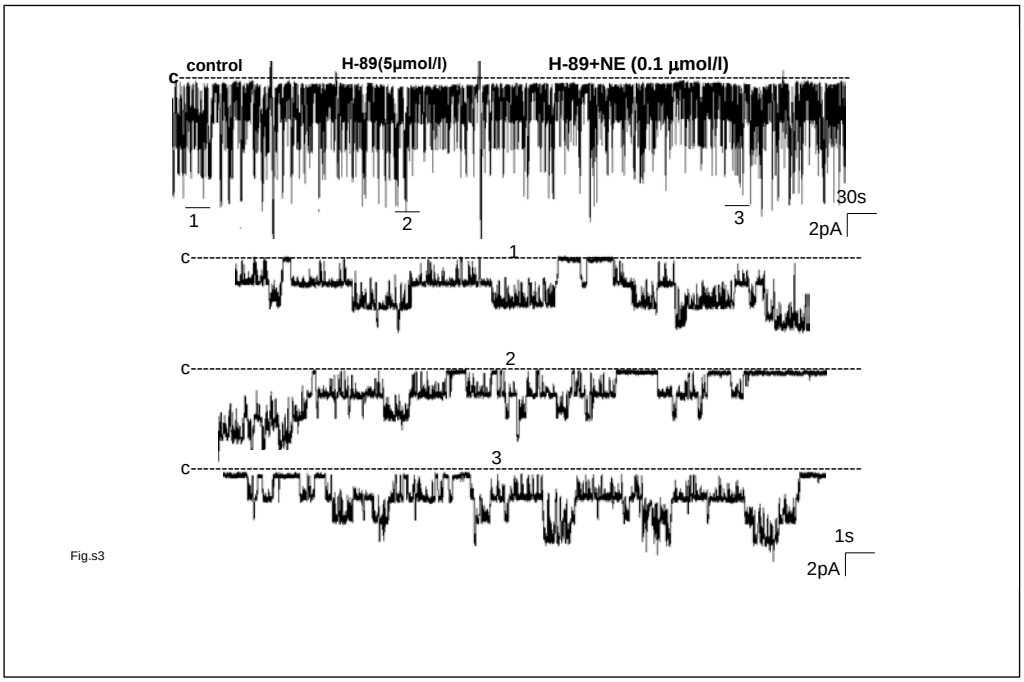


Fig.s3 Inhibition of PKA abolished the effect of NE on the K^+ channel. A single channel recording shows the effect of NE on the basolateral 40 pS K^+ channel in the DCT in the presence of H89. The top trace illustrates the time course of the experiments. Three parts of the recording indicated by numbers are extended to demonstrate the fast time resolution. The experiments were performed in cell-attached patches and the pipette holding potential was 0 mV. The channel closed level is indicated by a dotted line.

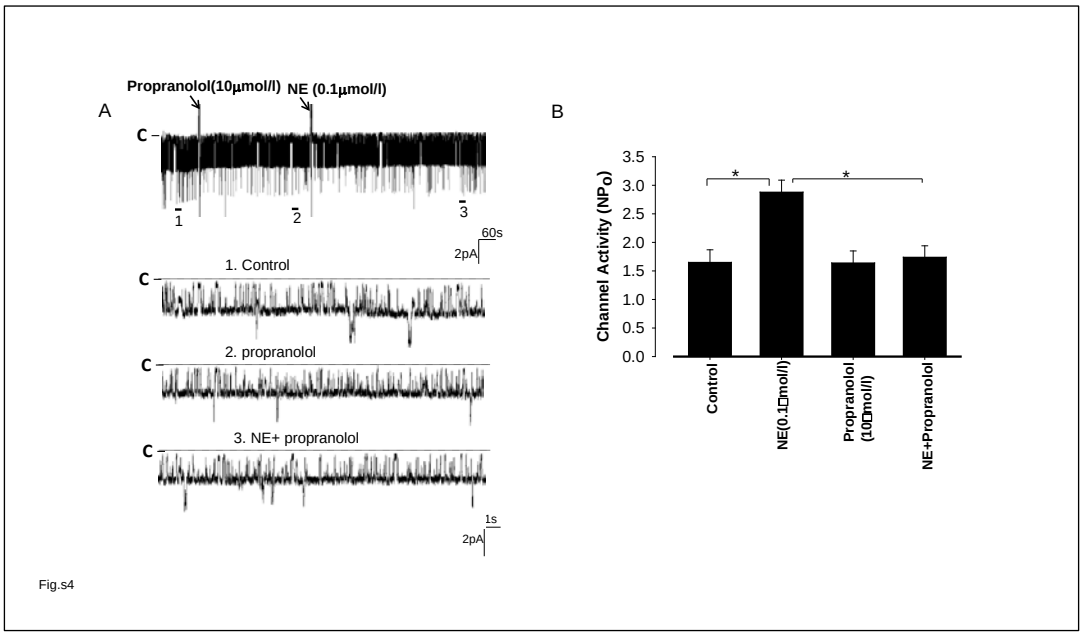


Fig.s4

Fig.s4 β-adrenergic receptor mediates the effect of NE on the K^+ channel. (A) A single channel recording shows the effect of 0.1 μmol/l NE on the basolateral 40 pS K^+ channel in the DCT. The top trace illustrates the time course of the experiments. Three parts of the recording indicated by numbers are extended to demonstrate the fast time resolution. The experiments were performed in cell-attached patches with (in mmol/l) and the pipette holding potential was 0 mV. The channel closed level is indicated by a dotted line. (B) A bar graph summarizes the results of experiments in which effect of NE (0.1 μmol/l) on the K^+ channel activity was examined in the presence or in the absence of propranolol (10 μmol/l). "*" indicates the significant difference ($p<0.05$).

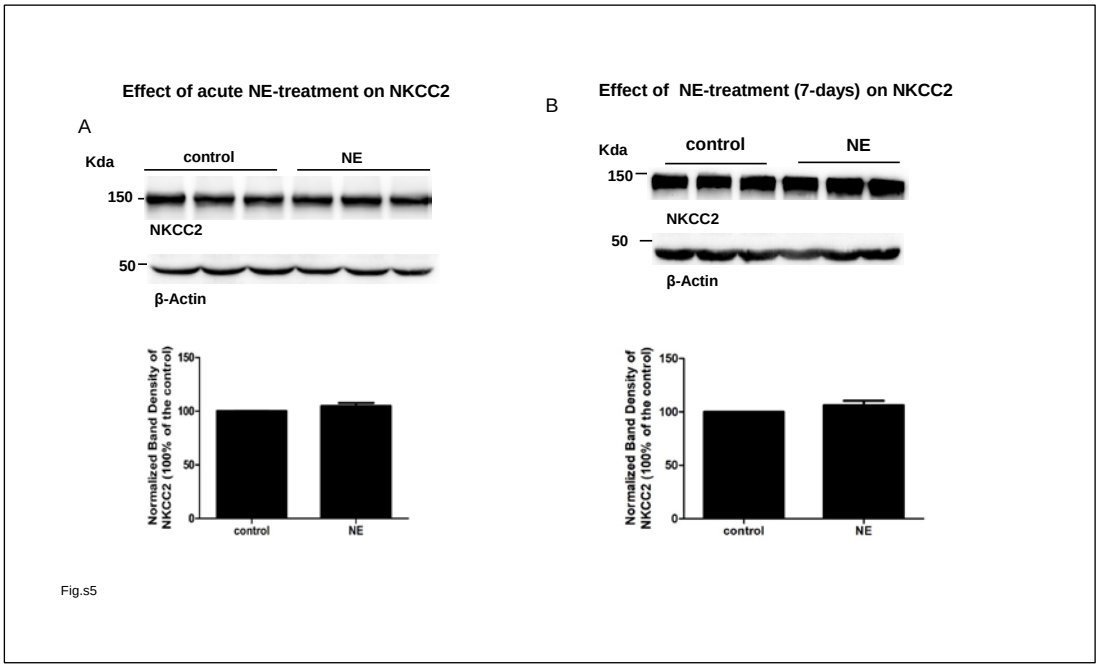


Fig.s5

Fig.s5 NE treatment does not increase the expression of NKCC2. Western blot showing the expression of NKCC2 in the mice treated acutely with NE (peritoneal injection of NE 30 min before experiments) (A) or in the mice received with NE-infused (7 days) (B). The normalized band density

is shown in the lower panel of corresponding Western blot. NE was continuously infused for 7 days (2.5 mg/kg/day) through a subcutaneously installed osmotic mini-pump.

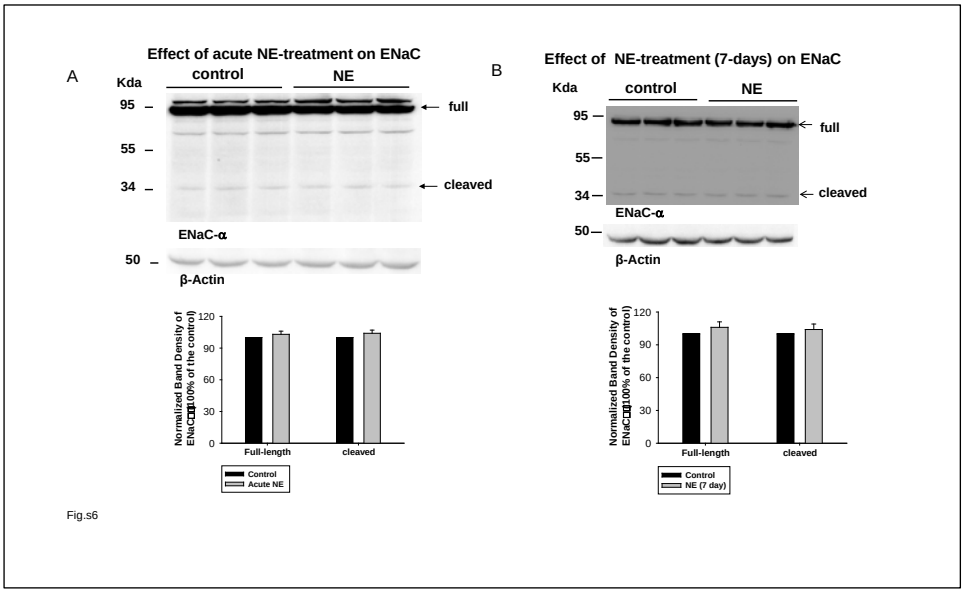


Fig.s6 NE treatment does not increase the expression of ENaC-α. Western blot showing the expression of ENaC in the mice treated acutely with NE (peritoneal injection of NE 30 min before experiments) (A) or in the mice received with NE-infused (7 days) (B). The normalized band density is shown in the lower panel of the corresponding Western blot. NE was continuously infused for 7 days (2.5 mg/kg/day) through a subcutaneously installed osmotic mini-pump.

Effect of NE infusion (7 days) on plasma Na^+ and K^+.

	WT	WT+NE-infusion	Ks-*Kcnj10*$^{-/-}$	Ks-*Kcnj10*$^{-/-}$ +NE-infusion
P_{Na^+} (mmol/l)	144.5±2.2	144.4±1.2	143.4±0.9	143.4±0.4
P_{K^+} (mmol/l)	3.82±0.16	3.74±0.2	2.53±0.10*	2.52±0.04*

Fig.s7

Fig.s7 The plasma Na^+ and K^+ concentrations in WT and Ks-*Kcnj10*$^{-/-}$ mice treated or untreated with NE infusion (7 days) through osmatic pumps. "*" indicates the significant difference in comparison to WT control. The experimental numbers (mice) are four.