Supporting Information

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SI Methods

Materials. All chemicals and materials were from Sigma and/or Fisher Scientific unless noted otherwise. All reagents were HPLC-grade or better.

Isolated, Split-Open ASDN Preparation. Mice were killed by CO_2 administration followed by cervical dislocation, and kidneys were removed immediately. Kidneys then were cut into thin slices (<1 mm), and the slices were placed into ice-cold physiologic saline solution buffered with Hepes (pH 7.4). The ASDN was identified as merging of the connecting tubule into the collecting duct and was mechanically isolated from cortical sections of kidney slices by microdissection using watchmaker forceps under a stereomicroscope. Isolated ASDN was allowed to settle onto 5 × 5-mm cover glass coated with poly-L-lysine. Cover glass containing ASDN was placed in a perfusion chamber mounted on an inverted Nikon Eclipse TE2000 microscope and superfused with room temperature Hepes-buffered (pH 7.4) saline solution. ASDN were split open with two sharpened micropipettes controlled with different micromanipulators to gain access to the apical membrane of principal cells. Isolated, split-open ASDN were used for patch-clamp analysis within 2 h after euthanizing.

Patch-Clamp Electrophysiology. Gap-free single-channel current data from gigaohm seals (recording pipette resistance 7–8 megaohms) were acquired (and subsequently analyzed) with an Axopatch 200B (Axon Instruments) or EPC-9 (HEKA Instruments Inc.) patch-clamp amplifier interfaced via a Digidata 1322A (Axon Instruments) to a PC running the pClamp 9.2 suite of software (Axon Instruments). Currents were low-pass filtered at 100 Hz with an eight-pole Bessel filter (Warner Instruments). Unitary current (i) was determined from all-point amplitude histograms fitted with single- or multi-Gaussian curves by using the standard 50% threshold criterion to differentiate between events. Events were inspected visually before acceptance. Channel activity, defined as NP_o, was calculated by using the standard equation:

$$NP_0 = (t_1 + 2t_2 + \ldots + nt_n),$$

where N and P_o are the number of ENaC in a patch and the mean open probability of these channels, respectively, and t_n is the fractional open time spent at each of the observed current levels. P_o was calculated by dividing NP_o by the number of active channels within a patch as defined by all-point amplitude histograms.

Immunohistochemistry/Immunofluorescence. Washed kidneys cleared of blood were rapidly immersed in HistoPrep frozen tissue embedding medium (Fisher Scientific) and snap-frozen on dry ice. Sagittal and (cortical) horizontal slices (5 μm) were cryosectioned by using a Leica CM1850 UV cryostat (Leica Microsystems). Slices were mounted on Superfrost Plus glass slides (Fisher Scientific) and fixed in 10% paraformaldehyde (pH 7.4). For immunofluorescence, nonspecific binding was blocked with PBS (pH 7.4) containing 1% BSA for 30 min at room temperature. Slices were probed overnight at 4 °C with a mixture of goat-anti-AQP2 (1:100 dilution; Santa Cruz) and rabbit-subunit-specific-anti-ENaC (1:1,000) antibodies in PBS containing 1% BSA. Slices were then washed and incubated at room temperature for 1 h with secondary antibodies: Cy5 anti-rabbit (1:500; Invitrogen) and FITC anti-goat (1:500; Invitrogen). Following another wash, slices were stained with 300 nM DAPI (Calbiochem). Slices were mounted with Immu-mount (Thermo Scientific) and then imaged with epifluorescence.

Analysis of Hormones and Electrolytes. Blood was collected on heparin by cardiac puncture immediately after euthanization. Samples were then centrifuged at 1,500 × g for 15 min at 4 °C. Urinary and plasma [Na^+] and [K^+] were quantified by using a PFP7 flame photometer (Techne). Plasma osmolality was determined with a vapor pressure osmometer (Wescor Inc.).

For determination of plasma [AVP], AVP was extracted with acetone and petroleum ether following a standard protocol (1). Plasma corticosterone concentration was quantified with HPLC by using a Waters PC600 pump-controlled module. For HPLC, data were collected and analyzed with Empower Pro software (Waters Corp.). The mobile phase consisted of 1.0 L of 50% MeOH and 50% 20 mM potassium dihydrogen phosphate (pH 6.7). Column flow rate was 1.5 mL/min. Elution of corticosterone peaked at 214 nm after 22.5 ± 0.02 min. The concentration of corticosterone was determined with regression analysis by using an external standard. The linear calibration range was from 0 to 1 μg/mL with a limit of detection at 9.4 ng/mL and a limit of quantification of 31.3 ng/mL.

1. Roos KP, Strait KA, Raphael KL, Blount MA, Kohan DE (2012) Collecting duct-specific knockout of adenylyl cyclase type VI causes a urinary concentration defect in mice. *Am J Physiol Renal Physiol* 302:F78–F84.

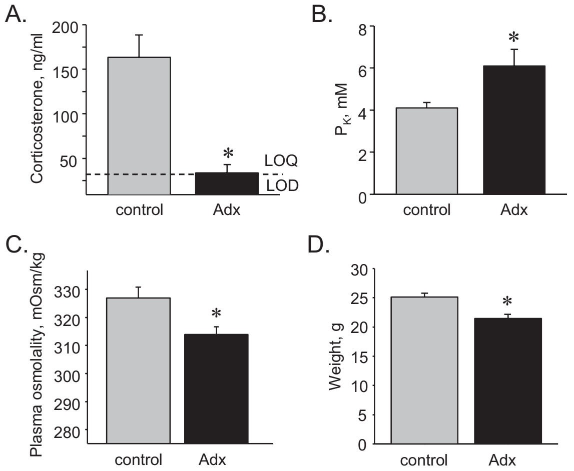

Fig. S1. Adrenal gland function is compromised in Adx mice. (*A*) Summary graph of plasma corticosterone concentration in control (gray) vs. Adx (black) mice ($n \geq 5$). The limit of quantification and the limit of detection were 31.3 and 9.4 ng/mL, respectively. *Significantly less than control. (*B*) Summary graph of plasma [K^+] in control (gray; $n = 15$) vs. Adx (black; $n = 5$) mice. *Significantly greater compared with control. (*C*) Summary graph of plasma osmolality in control (gray) vs. Adx (black) mice ($n \geq 7$) maintained with tap water. *Significantly less than control. (*D*) Summary graph of weight of control (gray) vs. Adx (black) mice maintained with tap water ($n = 10$). *Significantly less than control.

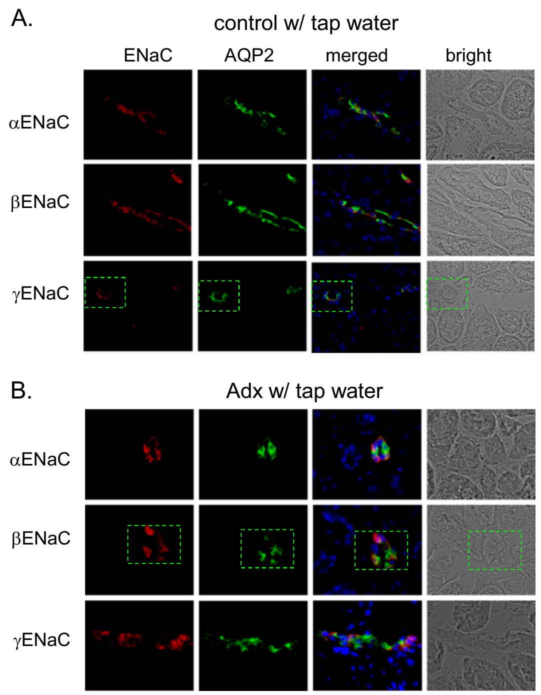

Fig. S2. All three ENaC subunits are expressed in the ASDN of Adx mice. Representative ($n \geq 3$) fluorescence micrographs of ASDN from control (*A*) and Adx (*B*) mice maintained with tap water probed with anti-ENaC (*Left*, red; α, *Top*; β, *Middle*; γ, *Bottom*) and anti-AQP2 (second from left; green) antibodies, and corresponding merged (third from left) and bright field images (*Right*). Nuclear staining (blue) with DAPI is included in merged images. Green-dashed boxes indicate the regions enlarged and shown in Fig. 2.

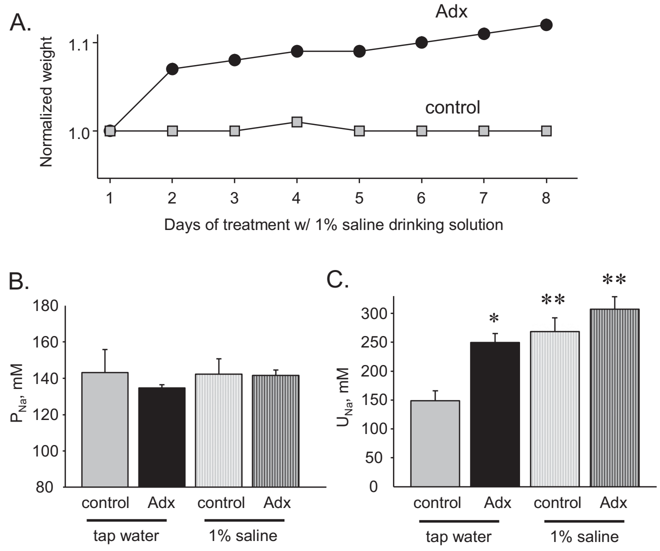

Fig. S3. Maintenance with 1% saline eases the volume loss and hyponatremia of Adx mice. (*A*) Summary graph of mean normalized (to starting day) weight gain for control (gray squares) and Adx (black circles) mice maintained with 1% saline ($n = 5$). By day eight there was no longer a significant difference in the mean weights of control and Adx mice. (*B* and *C*) Summary graphs of the mean plasma (*B*; $n \geq 5$) and urinary (*C*; $n \geq 7$) [Na^+] for control (gray) and Adx (black) mice maintained with tap water (filled bars) and 1% saline (striped bars) drinking solution. P_{Na} trended lower in the Adx group compared with control maintained with tap water and trended higher ($P < 0.07$) in the Adx group maintained with 1% saline compared with tap water. *Significantly greater compared with control on the same drinking regimen. **Significantly greater compared with the same group on tap water.

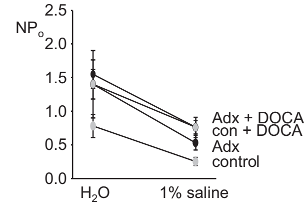

Fig. S4. Feedback regulation of ENaC is compromised in Adx mice. Summary graph of the activity of ENaC in control (gray) and Adx (black) mice drinking tap water and 1% saline solution in the absence and presence of DOCA. Data are from Table 1.

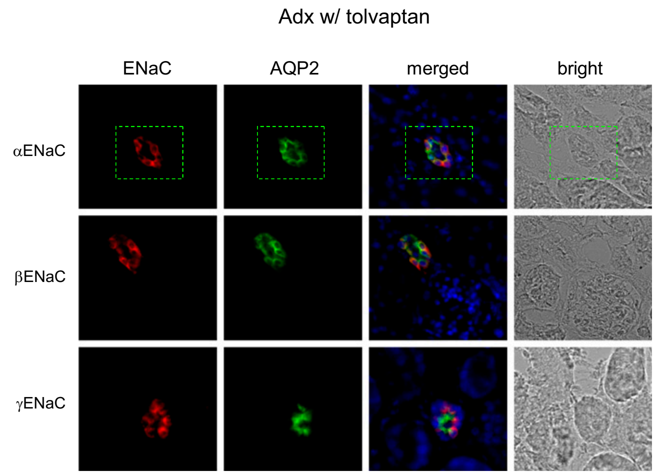

Fig. S5. AVP stimulates ENaC through a posttranslational mechanism. Representative ($n = 7$) fluorescence micrographs of ASDN from Adx mice treated with tolvaptan probed with anti-ENaC (*Left*, red; α, *Top*; β, *Middle*; γ, *Bottom*) and anti-AQP2 (second from left; green) antibodies, and corresponding merged (third from left) and bright field images (*Right*). Nuclear staining (blue) with DAPI is included in merged images. Green-dashed boxes indicate the regions enlarged and shown in Fig. 7.