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ORGAN PHYSIOLOGY

TRPV4 is associated with central rather than nephrogenic osmoregulation

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Abstract TRPV4 is a polymodal cation channel expressed in osmosensitive neurons of the hypothalamus and in the mammalian nephron. The segmental distribution and role(s) of TRPV4 in osmoregulation remain debated. We investigated the renal distribution pattern of TRPV4 and the functional consequences of its disruption in mouse models. Using qPCR on microdissected segments, immunohistochemistry, and a *LacZ* reporter mouse, we found that TRPV4 is abundantly expressed in the proximal tubule, the late distal convoluted tubule, and throughout the connecting tubule and collecting duct, including principal and intercalated cells. TRPV4 was undetectable in the glomeruli and thick ascending limb and weakly abundant in the early distal convoluted tubule. Metabolic studies in $Trpv4^{+/+}$ and $Trpv4^{-/-}$ littermates revealed that the lack of TRPV4 did not influence activity, food and water intake, renal function, and urinary concentration at baseline. The mice showed a similar response to furosemide, water loading and deprivation, acid loading, and dietary NaCl restriction. However, $Trpv4^{-/-}$ mice showed a significantly lower vasopressin synthesis and release after water deprivation, with a loss of the positive correlation between plasma osmolality and plasma vasopressin levels, and a delayed water intake upon acute administration of hypertonic saline. Specific activation of TRPV4 in primary cultures of proximal tubule cells increased albumin uptake, whereas no effect of TRPV4 deletion could be observed at baseline. These data reveal that, despite its abundant expression in tubular segments, TRPV4 does not play a major

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role in the kidney or is efficiently compensated when deleted. Instead, TRPV4 is critical for the release of vasopressin, the sensation of thirst, and the central osmoregulation.

Keywords Osmoregulation · TRPV4 · Tubular functions · Vasopressin · Thirst

Introduction

Osmoregulation is essential for every organism. In mammals, the homeostatic regulation of body water content involves a concerted action of the brain and the kidney. While the kidney is mainly an effector handling water, the control center is in the hypothalamus, where are located the most influential set of osmosensitive neurons [7, 10]. At the cellular level, ion channels are implicated in numerous osmotic and mechanical stimulus detection [15, 26]. Studies based on mouse models [21, 22, 24] and human genetics [39] suggested that TRPV4 could be involved in systemic osmoregulation. However, although some evidence supported a physiological role of TRPV4 in the hypothalamic osmosensitive nuclei [11, 21, 24], others showed opposite results [12, 17]. Furthermore, the effect of TRPV4 disruption on NaCl and water handling by the nephron has not yet been studied.

TRPV4 is a cation channel that belongs to the transient receptor potential (TRP) vanilloid subfamily [36]. TRPV4 responds to a large variety of stimuli, including osmotic cell swelling, mechanical stress, temperature, endogenous arachidonic acid metabolites, and phorbol esters [20, 27, 42, 43]. In view of its expression pattern in the brain, which includes neurons of the subfornical organ, the median preoptic area, and the organum vasculosum of the lamina terminalis [7, 11, 20], TRPV4 is likely to play a role in osmoregulation. Indeed, these neurons are endogenously osmosensitive and project to magnocellular neurons in the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus [8, 9]. Magnocellular neurons possess intrinsic osmosensitivity [28, 29] and synthesize arginine vasopressin (AVP); they also express TRPV4 [11]. In response to increased plasma osmolality, AVP is released from the posterior pituitary gland into the blood and binds to the AVP receptor type 2 (AVPR2) in the principal cells lining the collecting ducts of the kidney. This interaction leads to a signaling cascade causing phosphorylation and apical insertion of the water channel aquaporin-2 (AQP2), which facilitates osmotically driven water reabsorption [30]. The role of TRPV4 in central osmosensors has been studied in two distinct *Trpv4* knockout mouse models [21, 24]. Under water deprivation, Mizuno et al. reported an *exaggerated* AVP secretion in $Trpv4^{-/-}$ mice, while Liedtke et al. reported *blunted* AVP secretion and thirst mechanism. Recently, using several hypertonic challenges, Kinsman et al. questioned TRPV4 implication in thirst sensation [17].

TRPV4 is abundantly expressed in the kidney [2, 18, 20, 40]. However, its localization along the nephron is controversial, possibly reflecting the lack of specificity of antibodies raised against TRP channels. A first study stated that TRPV4 was restricted to tubular segments constitutively impermeable to water: thick ascending thin limb (TAL), distal convoluted tubule (DCT), and collecting ducts (CDs) [40]. Along these segments, TRPV4 was found in the basolateral side of the cells. Noteworthy is that TRPV4 was absent from nephron segments exhibiting constitutive water permeability, i.e., the proximal tubules (PTs) and descending thin limbs. A subsequent study showed that TRPV4 was restricted to connecting tubules (CN) and CDs [2]. In view of these discrepant results, the physiological role of TRPV4 in the kidney remains unclear.

In order to clarify the role of TRPV4 in the kidney and in osmoregulation in general, we characterized the expression of TRPV4 in the mouse kidney by using quantitative polymerase chain reaction (qPCR) on isolated nephron segments, immunohistochemistry, and a *Trpv4* reporter mouse model. We then evaluated the functional consequences of TRPV4 disruption in terms of renal function, general behavior, and specific parameters related to the osmoregulation.

Materials and methods

Animals and sampling

Experiments were conducted on 12-week-old, male $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice, generated by targeted deletion of exon 12 of the *Trpv4* gene [21]. The mice were backcrossed on a C57BL/6 background before starting the study. They were housed in a light- and temperature-controlled room with ad libitum access to tap water and standard diet (AO3, SAFE, France). Basal activity was evaluated in individual metabolic cages (Physiocage, Panlab-Bioseb, Vitrolles, France) during 48 h after 24 h of habituation. Overnight urine collections were obtained in individual metabolic cages, after appropriate training. Blood was collected by venous puncture or decapitation (for vasopressin measurement), and plasma was stored at −20 °C. Tissue samples were obtained after anesthesia with Sevoflurane (Abbott, Ottignies, Belgium) and were immediately processed for fixation and RNA/protein extraction. All protocols complied with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the local Ethics Committee.

Investigation of renal function

Mice were placed in metabolic cages to assess diuresis and renal handling of ions at baseline and during the following protocols. The urinary concentrating ability was tested after 24-h water deprivation. The capacity to excrete a water load was tested after intraperitoneal (IP) injection of sterile water (100 μL/g) [1]. Parameters were also obtained after administration of a single dose of furosemide (10 mg/kg, IP) or NaCl solution (0.5 M, 0.8 mL/20 g, IP). The effect of Na^+-depleted diet was investigated in *Trpv4*$^{-/-}$ mice receiving deionized water ad libitum and a standard diet (1 % NaCl) followed by a 0.01 % NaCl diet for 17 days.

Analytic procedures

Sodium, potassium, and chloride values were determined using a Kodak Ektachem DT60 II Analyzer (Johnson & Johnson, New Brunswick, NJ, USA). Other electrolytes, creatinine, and urea were measured on a Synchron CX5 analyzer (Beckman Coulter, Fullerton, CA, USA). Osmolality was measured on a Fiske osmometer (Needham Heights, MA, USA). Plasma AVP levels were measured using EIA (Peninsula Laboratories, San Carlos, CA).

Microdissection of individual nephron segments and primary culture

Male *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice were decapitated and the kidneys were removed and decapsulated. The dissection was done as described previously [31]. Mouse proximal tubule cell (mPTC) culture and fluorescein isothiocyanate (FITC)-labeled albumin uptake assays were conducted as described previously [38].

Calcium measurement

mPTCs were incubated for 30 min at room temperature with 5 μM Fura2-AM (Calbiochem, Camarillo, CA, USA), then washed for 1 h in Krebs-HEPES buffer and mounted in the same medium on a Zeiss Axiovert 200 M inverted microscope (Zeiss Belgium, Zaventem, Belgium). Intracellular calcium ($[Ca^{2+}]_i$) in individual cells was measured using alternative excitation of Fura-2/AM (0.5 Hz) at 340 and 380 nm using a Lambda DG-4 Ultra High Speed Wavelength Switcher (Sutter Instrument, Novato, CA, USA). Images were acquired with a Zeiss Axiocam camera coupled to a 510-nm emission filter and analyzed with Axiovision software.

Antibodies

The antibodies used for Western blotting and immunostaining are listed in Supplementary Material 1: Table 1.

Western blotting

Extraction and immunoblotting of kidney samples were performed as described previously [16]. Normalization was obtained after membrane stripping and reprobing.

Immunostaining

Mice were anesthetized with ketamine/xylazine (IP) and perfused through the left ventricle with phosphate-buffered saline (PBS) followed by paraformaldehyde–lysine–periodate fixative. Five-micrometer-thick cryosections (TRPV4) or sections from paraffin-embedded tissue blocks (AQP2, pAQP2, and NOS1) were incubated overnight at 4 °C with primary antibodies. Binding sites were revealed by Cy3-conjugated goat

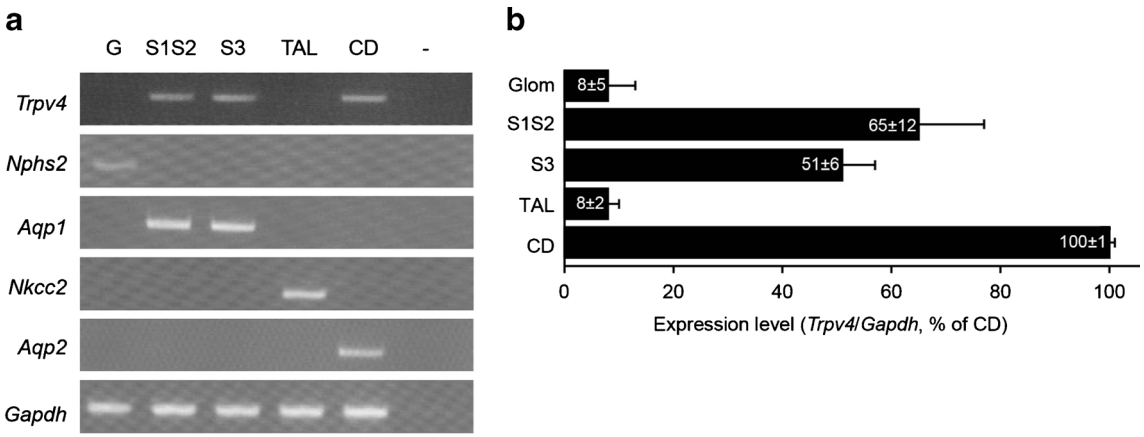

Fig. 1 Differential expression of *Trpv4* in microdissected nephron segments from mouse kidney. **a** RT-PCR of *Trpv4* mRNA with segment-specific markers. *Nphs2* (Podocin) is expressed in the glomeruli, *Aqp1* in PT, *Nkcc2* in TAL, and *Aqp2* in CD. RT-PCR products were size-fractionated on 1.5 % agarose gel. **b** Semiquantitative RT-PCR on microdissected segments demonstrates that *Trpv4* is expressed in the pars convoluta (*S1S2*) and the pars recta (*S3*) of the PT and in the CD ($n = 5$ fractions)

anti-rabbit IgG antibodies. DAPI was used to counterstain nuclei. Sections were studied by epifluorescence on a Zeiss microscope.

Real-time PCR

RNA extraction, retrotranscription, and qPCR were performed as described previously [16]. For detailed conditions and primers, see Supplementary Material 1: "Materials and Methods" and Table 2.

Reporting of *Trpv4* expression

A mouse strain was generated by homologous recombination inserting a *lacZ* reporter gene downstream the endogenous *Trpv4* promoter. Kidneys from 3-month-old heterozygous mice ($Trpv4^{Wt/lacZ}$) were isolated, cryoprotected, and frozen-sectioned (10 μM). For β-galactosidase activity detection, sections were fixed in 2 % glutaraldehyde, washed, and stained overnight at 37 °C in PBS supplemented with 20 mM $K_3Fe(CN)$, 20 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/mL Xgal. They were then washed and counterstained with Nuclear Fast Red. No staining was detected on sections from wild-type mice treated in the same conditions. For parallel immunostaining, frozen sections were fixed in methanol, then blocked and incubated with segment-specific antibodies. Binding sites were revealed using the AEC kit (Vector Laboratories).

Statistical analyses

Values are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were assessed by ANOVA or two-tailed Student's *t* test (GraphPad, San Diego, CA), as appropriate. *P* values <0.05 were considered significant.

Results

Expression and distribution of TRPV4 in mouse kidney

We first investigated the segmental distribution of *Trpv4* mRNA in mouse kidney using microdissected fractions characterized for segment-specific markers including podocin (*Nphs2*) for glomeruli, AQP1 (*Aqp1*) for PT, Na^+–K^+–$2Cl^-$ co-transporter (*Nkcc2*) for TAL, and AQP2 (*Aqp2*) for CD (Fig. 1a). The expression of *Trpv4* mRNA was detected in the convoluted (S1–S2) and straight (S3) parts of the PT, as well as in the CD, but neither in the glomerulus nor in TAL segments (Fig. 1b).

The distribution pattern of TRPV4 was established by using well-characterized antibodies that evidenced a single, specific 90-kDa band for TRPV4 by immunoblotting as well as a distinct tubular staining in wild-type $Trpv4^{+/+}$ mouse kidney (Supplementary Material 1: Fig. 1). Immunofluorescence showed that TRPV4 was widely expressed in the mouse kidney (Fig. 2). Based on co-distribution with segment-specific markers, TRPV4 was evidenced in the apical and basolateral membrane of PT cells (NaPi-2a; Fig. 2a). In the distal nephron, a slight basolateral staining for TRPV4 was detected in the first part of DCT, positive for the Na^+–Cl^- co-transporter (NCC), becoming more prominent in DCT2 (Fig. 2b) and continuing throughout the CN and the CD (AQP2; Fig. 2d). Type A intercalated cells in the CD were clearly TRPV4-positive (H-ATPase; Fig. 2f). However, intercalated cells in CN were weakly stained (Fig. 2e). Importantly, TRPV4 immunoreactivity was absent from the TAL (NKCC2; Fig. 2c).

These results were validated using a *LacZ* reporter mouse, allowing to assess the segment-specific expression of *Trpv4*

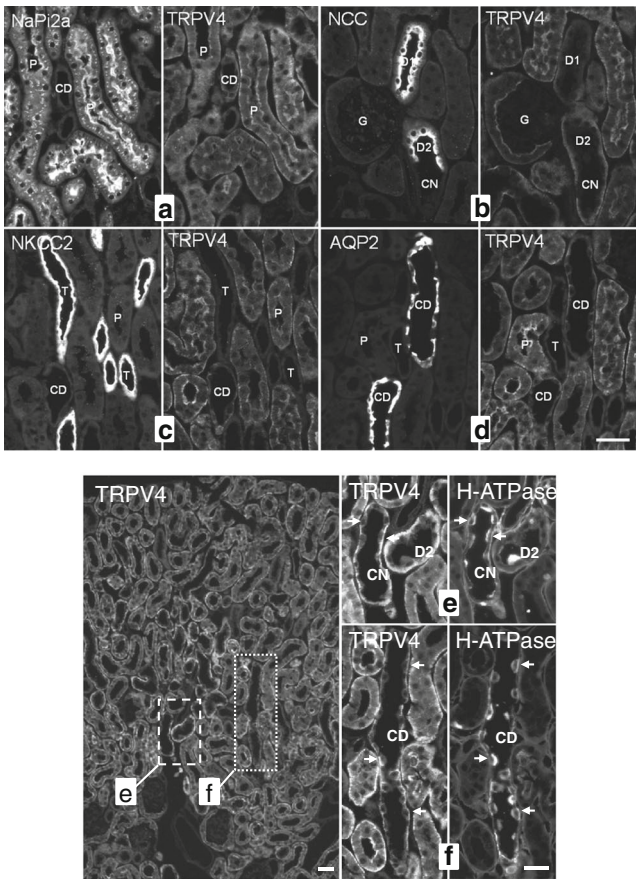

Fig. 2 Parallel staining of TRPV4 with different nephron segment-specific markers. **a** Immunofluorescence staining of NaPi2a and TRPV4 showing coincident staining in proximal tubules (*P*). **b** Immunostaining of NCC and TRPV4 depicting co-expression in DCT1 (*D1*) and DCT2 (*D2*). TRPV4 immunoreactivity was absent in the glomerulus (*G*). **c** Double staining of NKCC2 and TRPV4 showing the absence of immunoreactivity in the TAL. **d** Collecting ducts (*CD*) show basolateral expression of TRPV4 and luminal AQP2 staining. **e**, **f** Details of TRPV4 staining in the distal nephron. H-ATPase-positive intercalated cells in CN are weakly stained (*e*). However, intercalated cells in CD are clearly TRPV4-positive (*f*). *Scale bar*, 50 μm

by co-staining of β-galactosidase activity (Fig. 3a–e). Confirming TRPV4 immunolocalization, the β-galactosidase activity detection reported *Trpv4* expression in the PT (AQP1), the DCT (NCC), and the CD (AQP2), but not in the glomerulus and the TAL (UMOD).

Functional consequences of TRPV4 disruption

We next investigated whether the disruption of TRPV4 influences renal function in mouse. $Trpv4^{-/-}$ mice had normal appearance, body weight, water intake, and food intake and showed no obvious behavioral abnormalities (testing in Physiocage) as compared to $Trpv4^{+/+}$ littermates (Table 1). Similarly, there were no significant alterations in the baseline biological parameters in plasma and urine (Table 1). Notably, $Trpv4^{-/-}$ mice did not exhibit urinary loss of phosphate, glucose, calcium, or low-molecular-weight (CC16) protein, pleading against a defect in PT function at baseline.

In order to test the physiological role of TRPV4 in the distal nephron, we administered a single dose of furosemide (10 mg/kg, IP) to the mice (Fig. 4). As expected, furosemide induced a strong diuretic response after 2 h, with decreased urine osmolality and increased urinary excretion of Na^+, K^+, and Ca^{2+} (Fig. 4a–e). The response was similar in $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice, suggesting functional integrity of the TAL and the CD.

We then evaluated whether the effect of sodium deprivation was affected by the deletion of TRPV4 (Fig. 4f, g). *Trpv4* mice were fed a Na^+-replete diet (1 % NaCl) at baseline, followed by a Na^+-depleted diet (0.01 % NaCl) for 17 days. The dietary change resulted in very similar variations in the urinary excretion of Na^+ and K^+ in $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice both in terms of time course and absolute levels during the sodium depletion test.

Because TRPV4 has also been detected in intercalated cells, we investigated whether the lack of TRPV4 could disrupt the ability to excrete ammonium during an oral acid load (Fig. 4h, i). The response to both short-term (2 days) and long-term (6 days) NH_4Cl acid load showed that both the decrease in urine pH (Fig. 4h) and the increase in urinary ammonium excretion (Fig. 4i) were similar in wild-type and $Trpv4^{-/-}$ mice. These data indicate that TRPV4 is not essential for the mineralocorticoid response or for the urinary acidification during metabolic acidosis.

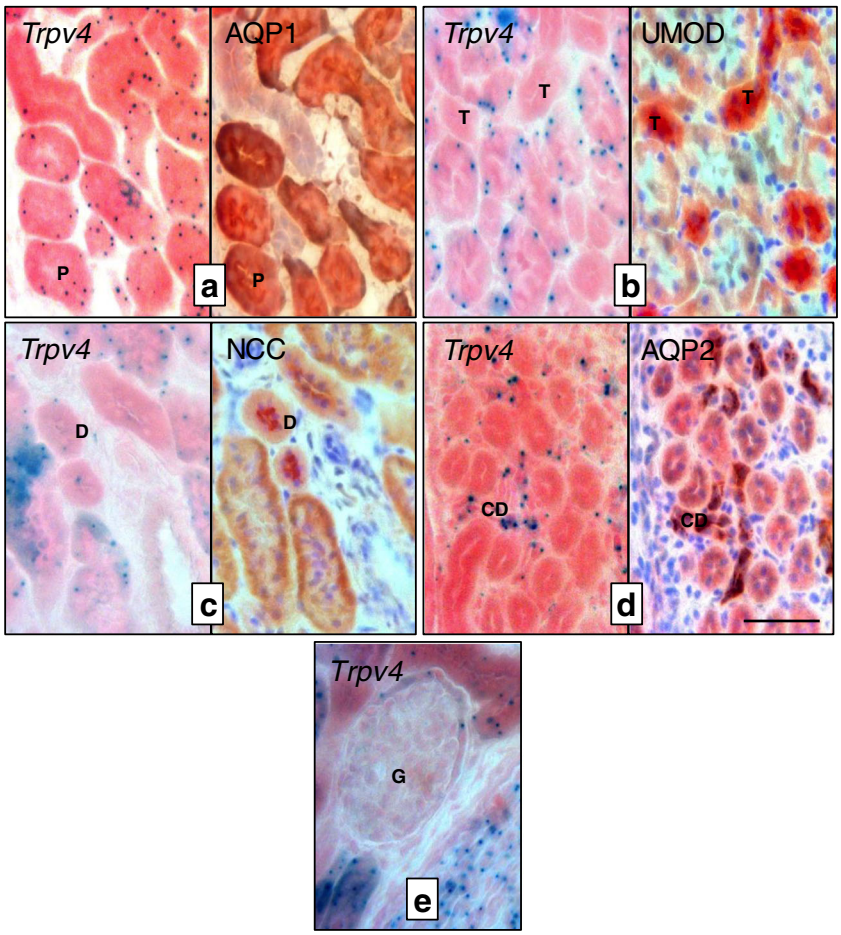

Fig. 3 Segmental distribution of TRPV4 in the reporter mouse kidney. Serial sections of $Trpv4^{Wt/lacZ}$ kidney (*Trpv4* expression is reported by beta-galactosidase staining) co-stained with segment-specific markers. **a** Staining of *Trpv4* and AQP1 showing co-expression in proximal tubules (*P*). **b** Staining of *Trpv4* and UMOD showing the absence of expression in the TAL (*T*). **c** Staining of *Trpv4* and NCC showing co-expression in the DCT (*D*). **d** Staining of *Trpv4* and AQP2 showing co-expression in the CD. **e** *Trpv4* is not expressed in the glomerulus (*G*). *Scale bar*, 50 μm

Table 1 Physical activity and biological parameters in *Trpv4* mice at baseline

Parameter	*Trpv4*$^{+/+}$	*Trpv4*$^{-/-}$	*P*
Body weight (g)	21.81 ± 0.63	21.15 ± 0.49	0.42
Water intake (mL/g body weight)	0.37 ± 0.01	0.39 ± 0.03	0.53
Food intake (g/g body weight)	0.35 ± 0.04	0.38 ± 0.03	0.59
Activity (movements/48 h)	24177 ± 4988	27028 ± 3591	0.65
Rearing (standings/48 h)	6073 ± 741	6412 ± 831	0.77
Plasma			
Osmolality (mOsm/kg H_2O)	321 ± 1.95	322 ± 2.26	0.68
Creatinine (mg/dL)	0.11 ± 0.01	0.13 ± 0.01	0.08
Urea (mg/dL)	22 ± 0.90	22 ± 1.56	0.78
Na^+ (mM)	148 ± 0.65	150 ± 0.90	0.14
K^+ (mM)	5.08 ± 0.15	4.84 ± 0.15	0.27
Cl^- (mM)	121 ± 1.52	120 ± 1.41	0.80
AVP (ng/mL)	2.72 ± 0.42	3.57 ± 0.50	0.22
Urine			
Diuresis (μL min^{-1} g^{-1} body weight)	0.036 ± 0.004	0.033 ± 0.004	0.57
Osmolality (mOsm/kg H_2O)	2327 ± 133	2316 ± 88	0.94
pH	6.89 ± 0.13	6.59 ± 0.12	0.07
Na^+/creatinine (nmol/ng creatinine)	0.29 ± 0.02	0.31 ± 0.03	0.49
K^+/creatinine (nmol/ng creatinine)	0.60 ± 0.02	0.58 ± 0.03	0.67
Cl^-/creatinine (nmol/ng creatinine)	0.37 ± 0.01	0.40 ± 0.03	0.34
Ca^{2+}/creatinine (ng/ng creatinine)	0.11 ± 0.01	0.09 ± 0.01	0.31
Mg^{2+}/creatinine (ng/ng creatinine)	1.20 ± 0.11	0.98 ± 0.08	0.12
Glucose (mg/dL)	82.9 ± 2.60	86.6 ± 3.64	0.43
Phosphate (mg/dL)	124 ± 24.1	153 ± 21.8	0.38
CC16 (μg/L)	9.44 ± 3.71	12.90 ± 3.98	0.54

Data are mean ± SEM; $n = 10$ mice per group

CC16 Clara cell 16-kDa protein

Effect of TRPV4 disruption on central osmoregulation

Previous studies have demonstrated that TRPV4 is located within the forebrain lamina terminalis, a structure that is essential for both thirst and vasopressin secretion. To evaluate the central response to hyperosmotic stimuli, we challenged *Trpv4* mice with an IP injection of a hyperosmotic solution (0.5 M NaCl, 0.04 mL/g body weight). Mice were single-housed, allowing the precise measurement of cumulative water intake (Physiocage testing). Under these conditions, water intake was significantly delayed in *Trpv4*$^{-/-}$ mice (*Trpv4*$^{-/-}$ $T_{1/2} = 1056 \pm 245$ s versus *Trpv4*$^{+/+}$ $T_{1/2} = 672 \pm 83$ s; Fig. 5a, b), suggesting a central defect in osmoregulation.

In a second set of experiments, *Trpv4* mice were exposed to a 24-h water deprivation (Fig. 6). *Trpv4*$^{-/-}$ mice and their control littermates had the same urine output and osmolality at baseline, with water deprivation resulting in a similar drop in urine volume (Fig. 6a) and a similar increase in urine osmolality (U_{Osm}) in both groups (Fig. 6b). Of interest is that *Trpv4*$^{-/-}$ mice exhibited significantly higher levels of plasma osmolality (P_{Osm}) after water deprivation (Fig. 6c) as well as a lower increase in AVP mRNA expression (Fig. 6d) and plasmatic AVP (Fig. 6e) as compared to control littermates. Furthermore, the positive correlation between P_{Osm} and plasma AVP levels, as observed in *Trpv4*$^{+/+}$ mice, was abolished in *Trpv4*$^{-/-}$ mice (Fig. 6f).

Effect of TRPV4 disruption on peripheral osmoregulation

The potential role of TRPV4 on the renal response to water deprivation was then investigated. Immunoblotting (whole kidney membrane fraction) showed similar membrane AQP2 and pS269 AQ2 levels in both genotypes at baseline and after water deprivation (Fig. 7a, b). The levels of p256 and p261 AQP2 were also found to be similar between *Trpv4*$^{-/-}$ mice and controls (Supplementary Material 1: Fig. 2). Immunostaining showed a similar increase in AQP2 signal intensity, membrane localization, and phosphorylation in the CDs of *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice (Fig. 7c, d). Of note is that expression studies on whole kidney (real time (RT)-PCR) showed that *Aqp2* mRNA expression levels, which were

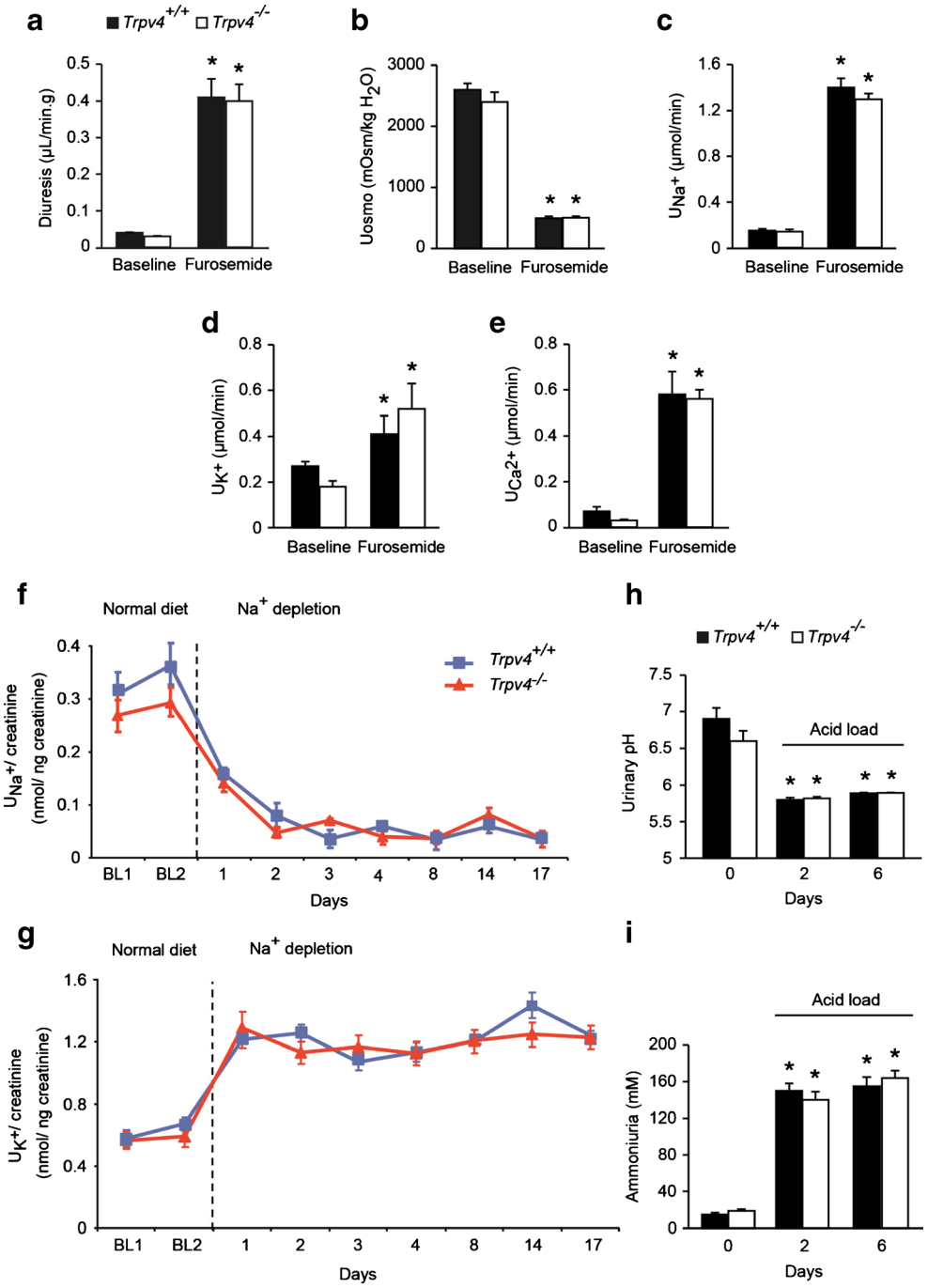

Fig. 4 Renal function testing in *Trpv4* mice. **a**–**e** Effects of furosemide on $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice. Furosemide injection induced an increase in diuresis (**a**) and a significant decrease in urine osmolality (**b**). Natriuresis (**c**), kaliuresis (**d**), and calciuresis (**e**) were increased as well. No difference was observed between $Trpv4^{+/+}$ and $Trpv4^{-/-}$ ($n = 5$ pairs). **f**, **g** Urinary Na^+ and K^+ excretion obtained with normal and Na^+-depleted diets. At baseline (*BL1* and *BL2*), mice were fed a diet containing 1 % NaCl. This baseline was followed by a Na^+-depleted diet (0.01 % NaCl) for 17 days (*1–17*). The amount of Na^+ (**f**) and K^+ (**g**) excreted in the urine of $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice was determined. Results are expressed as nanomoles of Na^+ and K^+ excreted per nanogram of creatinine. No significant difference in Na^+ and K^+ excretion was observed between $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice at baseline and during sodium depletion ($n = 5$ pairs). **h**, **i** Response to acid loading. Five pairs of mice were exposed to NH_4Cl load for 0, 2, or 6 days. In $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice, the acid load reduced the urinary pH (**h**) and induced an increase in urinary excretion of ammonium (**i**). The two groups showed a similar response to this challenge. $^*P < 0.05$ vs. baseline

similar between both genotypes at baseline, showed a significantly lower rise after water deprivation in $Trpv4^{-/-}$ mice. Expressions of *Aqp3*, *AvpR1*, and *AvpR2* were similarly regulated in both genotypes (Fig. 8a–d). Investigations of water handling were completed by injecting the V2R selective agonist dDAVP (1 ng/g; Fig. 9a). This treatment resulted in a similar increase in U_{Osm} (after 5 h) in both genotypes. In a reverse experiment, acute water loading (100 µL/g, IP) demonstrated that $Trpv4^{-/-}$ and $Trpv4^{+/+}$ mice have a similar capacity to excrete water (Fig. 9b).

These results, and the lack of reactivity of plasma AVP under water deprivation, suggest the activation of a vasopressin-independent pathway to increase membrane AQP2 in $Trpv4^{-/-}$ mice [4, 6, 25]. In order to test whether the nitric oxide (NO) pathway could be involved, we compared the expression of nitric oxide synthases (NOS) in water-deprived $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice. There was a significant upregulation of NOS1 in $Trpv4^{-/-}$ kidneys compared to controls, corresponding to enhanced NOS1 level in their inner medulla, with the highest signal in the vasculature

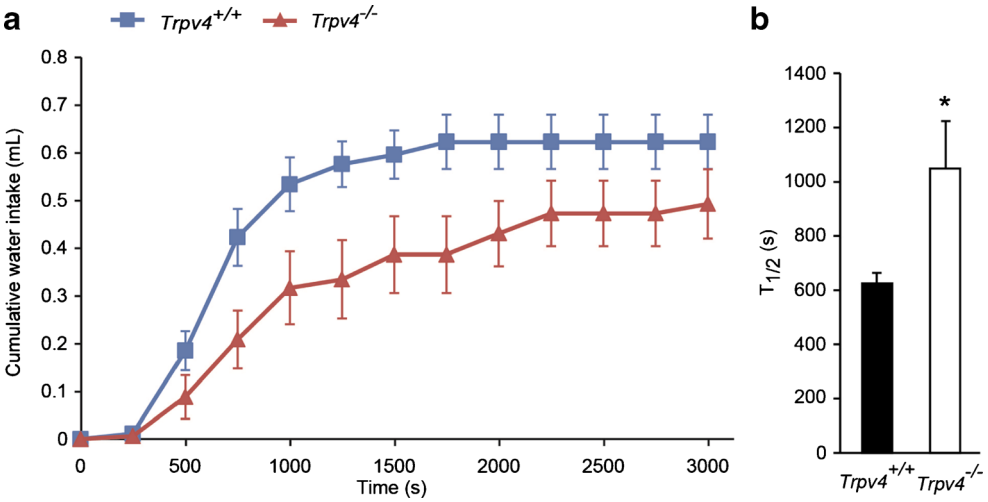

Fig. 5 Hypertonic saline stimulation and water intake in *Trpv4* mice. After adaptation in Physiocage, mice were injected 0.5 M NaCl (IP) and monitored for subsequent water intake. As compared to wild-type littermates, $Trpv4^{-/-}$ mice show a decreased cumulative water intake (**a**) (area under the curve: $Trpv4^{-/-} = 953 \pm 159$ mL vs. $Trpv4^{+/+} = 1435 \pm 143$ mL; $P = 0.04$) and a significantly delayed water intake (**b**) ($T_{1/2}$: KO = 1056 ± 245 s vs. WT = 672 ± 83 s; $P = 0.03$, $n = 10$ pairs). $^{*}P < 0.05$ vs. control

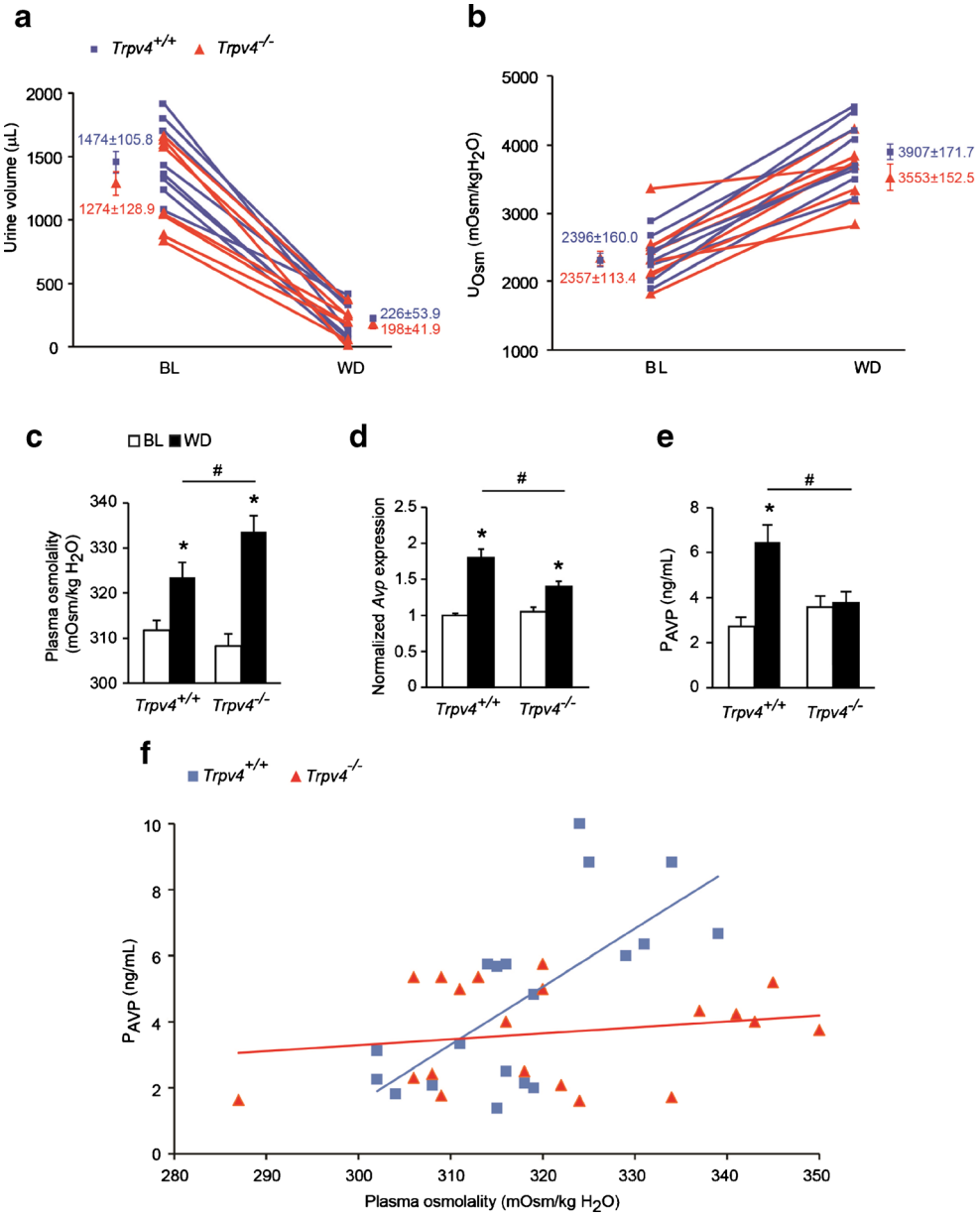

Fig. 6 Effects of water deprivation on water balance in *Trpv4* mice. Urine output (**a**) and urine osmolality (**b**) obtained at baseline (*BL*) and after 24 h of water deprivation (*WD*) show the same variations in both strains. In comparison with $Trpv4^{+/+}$ littermates, $Trpv4^{-/-}$ mice exhibited a significantly higher rise in plasma osmolality (**c**), but a significantly lower upregulation of the levels of *Avp* mRNA in the brain (**d**) and of the plasma levels of AVP (P_{AVP}) (**e**) after water deprivation. **f** Relationship between plasma osmolality (P_{Osm}) and vasopressin (P_{AVP}). After 24 h of water deprivation, in wild-type mice, the increase in P_{Osm} was reflected by an important increase in P_{AVP} ($R^2 = 0.47$). This relationship was lost in $Trpv4^{-/-}$ mice ($R^2 = 0.04$; $n = 10$ pairs, except for (**f**) $n = 20$ pairs). $^{*}P < 0.05$ vs. baseline; $^{\#}P < 0.05$ vs. controls

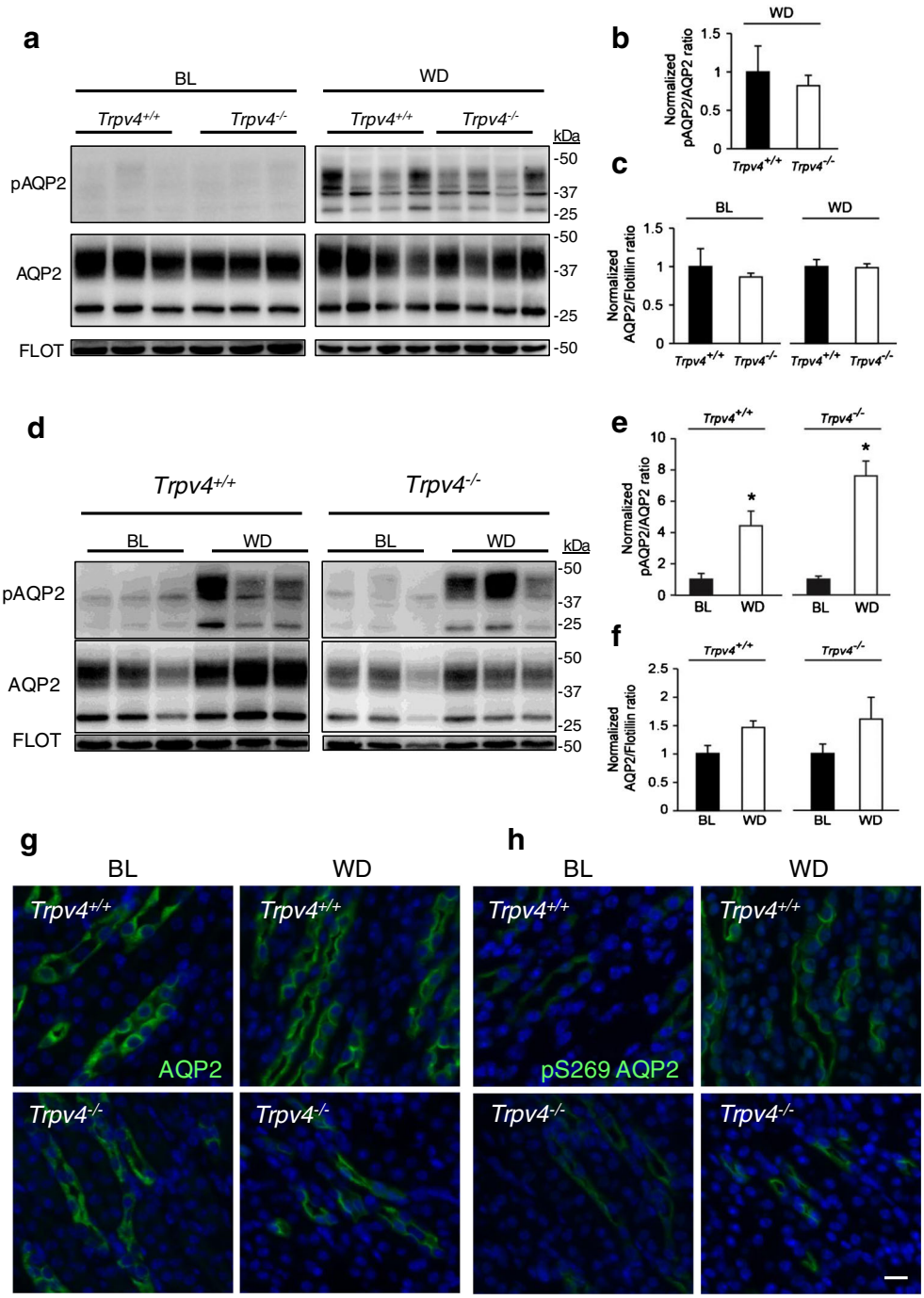

Fig. 7 Effects of water deprivation on AQP2 protein in *Trpv4* kidneys. **a** Immunoblotting of pS269 (pAQP2) and total AQP2 comparing $Trpv4^{+/+}$ and $Trpv4^{-/-}$ kidneys at baseline (*BL*) and after water deprivation (*WD*). **b**, **c** Quantification of the immunobloting signals. Flotillin (*FLOT*) is used as a membrane fraction loading control. No difference was found at baseline or after water deprivation. **d** Immunoblotting of pS269 and total AQP2 analyzing the response to water deprivation within each genotype. **e**, **f** Quantification of the immunobloting signals. Like controls, $Trpv4^{-/-}$ kidneys showed a significant rise in AQP2 phosphorylation after water deprivation. $*P < 0.05$ vs baseline. **g**, **h** Immunostaining of AQP2 and pS269 AQP2 in CD under strictly controlled conditions showed a similar increase in signal and membrane localization in $Trpv4^{+/+}$ and $Trpv4^{-/-}$ CD after water deprivation. *Scale bar*, 50 mm

(Fig. 10a, b). Other isoforms were not upregulated (data not shown).

Effect of TRPV4 activation on proximal tubule cells

As TRPV4 was found to be highly expressed in the PT cells, which are characterized by a very efficient apical endocytosis, we further analyzed its role on the endocytic uptake of albumin in primary cultures of mPTCs [38]. We first verified that we could activate TRPV4 in mPTCs with the specific agonist GSK1016790A: stimulation of $Trpv4^{+/+}$ mPTC with 100 nM GSK1016790A induced a large increase in $[Ca^{2+}]_i$, which was not observed in $Trpv4^{-/-}$ mPTC (Fig. 11a). Then, we compared endocytosis of FITC-labeled albumin [38] between $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mPTCs (Fig. 11b). In standard conditions, the endocytosis of FITC-labeled albumin was similar between the two genotypes. However, activation of TRPV4 with GSK1016790A enhanced albumin endocytosis in $Trpv4^{+/+}$ mPTC. This effect was not observed in $Trpv4^{-/-}$ mPTC.

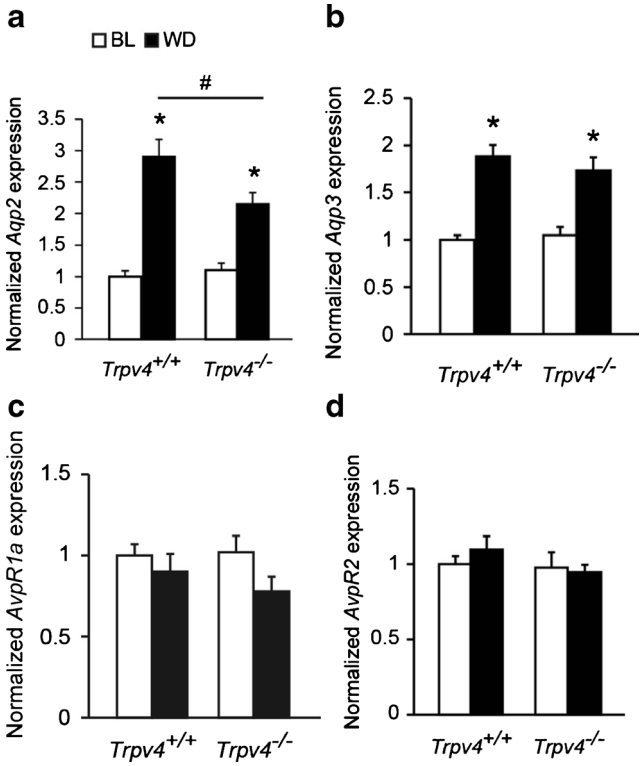

Fig. 8 Effects of water deprivation on the expression of genes involved in osmoregulation in *Trpv4* kidneys. **a** Expression studies on whole kidney (RT-qPCR) revealed similar levels in the mRNA expression of *Aqp2* between both genotypes at baseline, but a significantly lower rise after water deprivation in $Trpv4^{-/-}$ mice. The levels of expression of *Aqp3* (**b**), *Avpr1a* (**c**), and *Avpr2* (**d**) mRNAs were similar between both genotypes at baseline and after water deprivation. $^{*}P < 0.05$ vs. baseline; $^{\#}P < 0.05$ vs. controls

Discussion

In this study, we detailed the expression profile of TRPV4 in mouse kidney and analyzed the consequences of the genetic deletion of TRPV4 on the central and nephrogenic components of osmoregulation. In contrast to previous studies, we demonstrate that TRPV4 is absent from the TAL, but abundantly expressed in proximal and distal tubular segments. Despite the widespread expression pattern of TRPV4 in the kidney, its deletion has no detectable effect on a large range of renal function parameters. Conversely, the absence of TRPV4 is associated with abnormal thirst regulation and vasopressin release, demonstrating a key role in central osmoregulation.

Knockout (KO) mice for TRPV4 express a relatively subtle phenotype [17, 21, 24]. Indeed, Mizuno et al. reported no perturbation under normal conditions, but an exaggerated secretion of AVP in response to hyperosmolarity. Using another line, Liedtke et al. reported that $Trpv4^{-/-}$ mice drank less and were slightly hyperosmolar. AVP secretion in response to hyperosmotic stimulation was diminished, with lower fluid intake and increased latency of drinking. In a third study, Kinsman et al. found no difference in water intake following hyperosmotic challenge between this latter model and controls. Besides these KO models, other studies linked TRPV4 to AVP secretion in SON [11], modulated thirst with a TRPV4 agonist [41], or showed its role in osmosensitivity of the PVN [13, 14]. TRPV4 has also been described as a peripheral osmoreceptor in nerve endings surrounding hepatic blood vessels [19]. However, the fact that human patients with denervated liver (liver-transplanted patients) have an elevated plasma osmolality in combination with elevated levels of the C-terminal pro-AVP suggests an appropriate central response in the absence of this peripheral osmoreceptor.

Our studies, based on the $Trpv4^{-/-}$ mouse model developed by Liedtke et al. which was backcrossed on an inbred C57BL/6 background, reveal a significantly impaired ability of *Trpv4* KO mice to increase AVP expression and secretion upon water deprivation. Furthermore, $Trpv4^{-/-}$ mice had a significantly

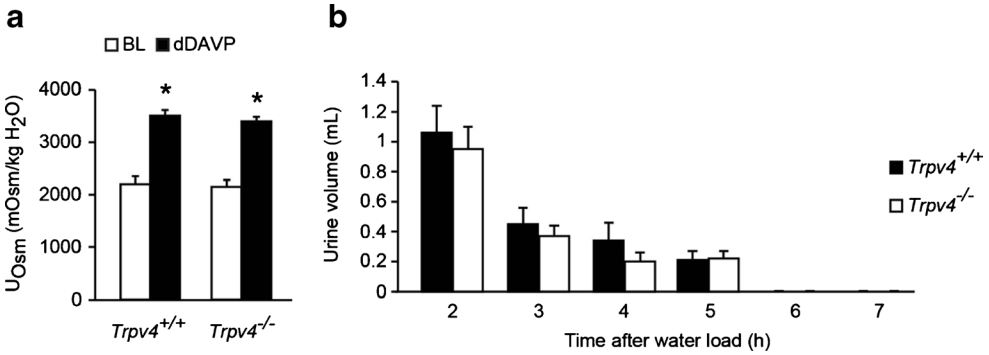

Fig. 9 Investigations on water handling by the *Trpv4* kidney. **a** Effect of dDAVP treatment on urine osmolarity in $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice. Urine osmolality (U_{Osm}) was measured at baseline and 5 h after injection of 1 ng/g dDAVP (Minirin®, IP). The two groups of mice showed similar U_{Osm} values at baseline (2238 ± 175 vs. 2245 ± 144 mOsm/kg H_2O). dDAVP injection induced an increase of U_{Osm}; however, no significant difference was observed between $Trpv4^{-/-}$ mice and wild-type littermates (3371 ± 78 vs. 3534 ± 110 mOsm/kg H_2O; $n = 6$ pairs of mice). $^{*}P < 0.05$ vs. baseline. **b** Response to water loading in $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice. Ten pairs of mice were administered an IP injection of water (100 μL/g) and urine volume (in milliliters) was measured every hour. Each group showed the same ability to excrete water after injection

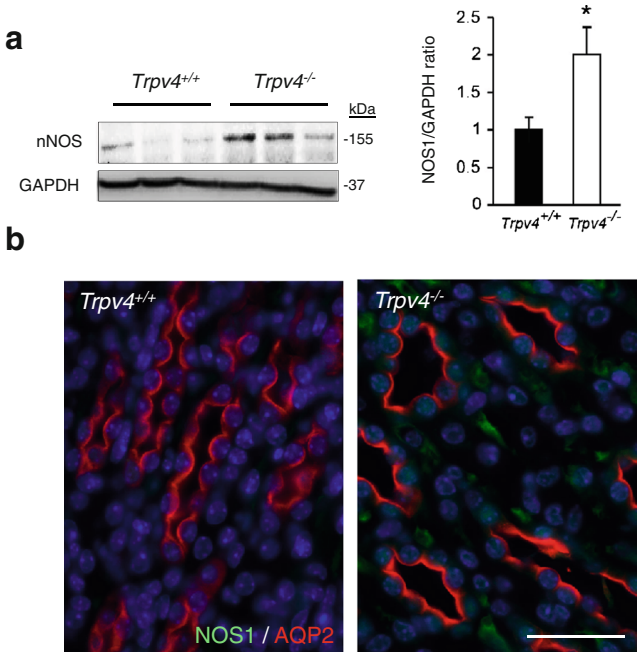

Fig. 10 Effects of water deprivation on NOS1 expression in *Trpv4* kidneys. **a** Immunoblotting of NOS1 conducted on whole kidney extracts. Comparison of NOS1 levels in $Trpv4^{+/+}$ and $Trpv4^{-/-}$ kidneys after water deprivation revealed a significantly higher level in $Trpv4^{-/-}$ mice. GAPDH is used as a loading control. $^{*}P<0.05$ vs. controls. **b** Immunostaining on kidneys from water-deprived mice showed a higher NOS1 level in $Trpv4^{-/-}$ inner medulla, particularly in the vessels, in comparison with the controls. AQP2 is used to stain principal cells of the CD. *Scale bar*, 50 μm

higher plasma osmolality and a reduced *Aqp2* mRNA upregulation after 24-h water deprivation. The latter findings are probably related to defective AVP secretion, as also evidenced by the loss of the typical positive correlation between plasma osmolality and plasma AVP levels. This correlation was also lost in $Trpv1^{-/-}$ mice (Fig. 6f from this article can be paralleled to Fig. 6 from [35]), indicating that TRPV1 and TRPV4 are both needed in the mechanism of AVP secretion. These findings indicate that the loss of TRPV4 alters the function of central osmoreceptors implicated in the regulation of AVP secretion. The central defect is also reflected by an alteration of the mechanism of thirst, as evidenced by the significant delay in the water intake in $Trpv4^{-/-}$ mice after hypertonic saline injection.

The distribution and physiological role of TRPV4 in the kidney remain debated [23, 32]. Our studies, based on RT-qPCR, immunostaining, and gene expression reporting in mouse kidney, show consistently that TRPV4 is abundantly expressed in the apical and basolateral areas of PT cells and essentially at the basolateral membrane of the late DCT, the CN, and the CD, including principal and intercalated cells. In contrast, TRPV4 is absent from the glomerulus and the TAL. This pattern of distribution differs from that published in two previous reports [2, 40]. Immunostaining for TRPV4 on rat and mouse kidneys performed by Tian et al. suggests that it is expressed in the basolateral membrane of the TAL and the downstream nephron segments, but was not validated on a KO model. On the other hand, Berrout et al. showed expression (predominantly apical) from the CN through the papillary CD. While the antibody properties and the immunostaining conditions could explain the absence of positive PT staining in the latter study, our pattern is strongly supported by non-immunological evidences that are RNA expression and *lacZ* reporting studies.

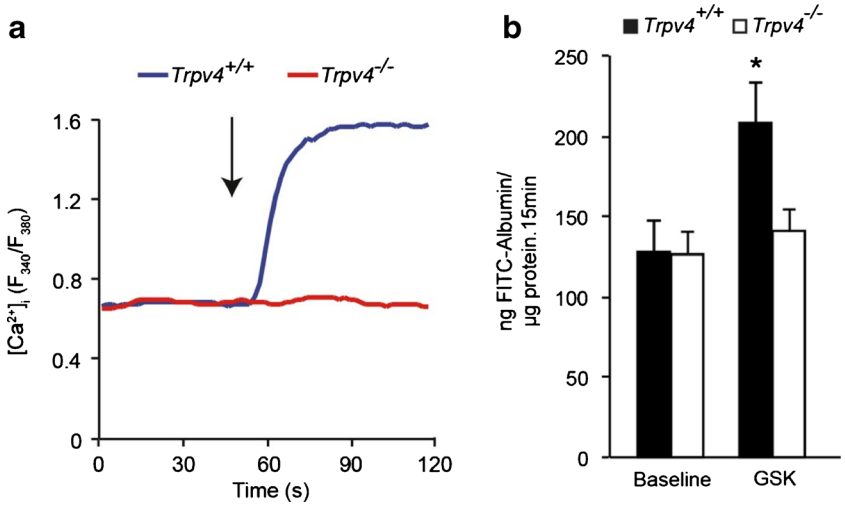

Fig. 11 Role of TRPV4 on albumin endocytosis in mPTC. **a** Expression of a functional TRPV4 channel in the mPTC was verified by monitoring $[Ca^{2+}]_i$ in the presence of the TRPV4 agonist GSK1016790A (100 nM, *arrow*). Specificity of the agonist was verified on $Trpv4^{-/-}$ mPTC. The graph represents the average response of ten cells for each condition and is representative of five different experiments. **b** $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mPTCs were exposed to 0.5 mg/mL FITC-labeled albumin for 15 min and the uptake was quantified. No significant difference was found at baseline, but in the presence of 100 nM GSK1016790A (*GSK*), albumin uptake was enhanced in $Trpv4^{+/+}$ mPTC but not in $Trpv4^{-/-}$ mPTC ($n=9$ filters from three different mice for each condition). $^{*}P<0.05$ vs. $Trpv4^{+/+}$ baseline

Several lines of evidence were used to assess the potential role(s) of TRPV4 in these nephron segments. Studies performed at baseline failed to show any difference in behavior and plasma and urine parameters between *Trpv4*$^{-/-}$ and *Trpv4*$^{+/+}$ mice. An extensive set of functional tests was also used, failing to show significant differences in the renal response between *Trpv4*$^{-/-}$ and *Trpv4*$^{+/+}$ mice: furosemide treatment, NaCl depletion, acid loading, water deprivation, dDAVP treatment, and water loading. These negative results have to be interpreted in view of in vitro studies that have demonstrated a physiological role of TRPV4 as a flow sensor on mouse renal epithelial cell line [44] and isolated CD [2, 3, 37]. Although there was no biological sign of PT dysfunction in *Trpv4*$^{-/-}$ mice at baseline, we show that TRPV4 activation can enhance endocytosis in mPTC. Further investigations will be needed to evaluate the potential role of TRPV4 in the mecanosensors regulating the PT functions [33, 34]. The fact that the kidney is a site of high TRPV4 expression compared to other organs [18, 20], with TRPV4 being one of the most expressed TRP channels [18], may suggest that redundant mechanisms compensate for the lack of TRPV4 in *Trpv4*$^{-/-}$ kidneys. Despite the defect in AVP secretion, the renal response to 24-h water deprivation was found to be functional in *Trpv4*$^{-/-}$ mice as the urine concentrating ability and AQP2 trafficking and phosphorylation were not significantly different between *Trpv4*$^{-/-}$ mice and controls. Interestingly, we found an upregulation of NOS1 in the inner medulla of water-deprived *Trpv4*$^{-/-}$ mice, where NO signaling could be an alternative pathway to increase membrane AQP2 in the absence of AVPR2 stimulation [4, 5].

In conclusion, despite abundant TRPV4 expression in the proximal and distal nephron segments, the *Trpv4*$^{-/-}$ mouse does not highlight a major renal dysfunction at baseline. Instead, TRPV4 is critical for the release of vasopressin, the thirst sensation and the central regulation of osmolality.

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Compliance with ethical standards

Conflict of interest The authors declare that that they have no conflicts of interest.

Research involving animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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