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Beta-3 Adrenergic Receptor (β_3-AR) In The Kidney: A Possible New Player In Sympathetic Regulation Of Renal Function

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ABSTRACT

To date, the study of the sympathetic regulation of renal function has been restricted to the important contribution of β_1- and β_2-adrenergic receptors. This study aims to investigate the expression and the possible physiological role of β_3-adrenergic receptor (β_3-AR) in mouse kidney.

Here we found that β_3-AR is expressed in most of the nephron segments expressing the type-2 vasopressin receptor (AVPR2), including the thick ascending limb and the cortical and outer medullary collecting duct. *Ex vivo* experiments in mouse kidney tubules showed that β_3-AR stimulation with the selective agonist BRL37344 increased intracellular cAMP levels and promoted two key processes in the urine concentrating mechanism: accumulation of the water channel aquaporin 2 at the apical plasma membrane in the collecting duct and activation of the Na-K-2Cl symporter in the thick ascending limb. Both effects are prevented by the β_3-AR antagonist L748,337 or by the protein kinase A inhibitor H89. Interestingly, we show here that in mice genetic inactivation of β_3-AR is associated with significantly increased urine excretion of water, Na, K and Cl, while stimulation of β_3-AR significantly reduced urine excretion of water and the same electrolytes. Moreover,

BRL37344 promotes a potent antidiuretic effect in AVPR2-null mice with X-linked nephrogenic diabetes insipidus.

Taken together, these findings are of potential physiological importance as they uncover the antidiuretic effect of β_3-AR stimulation in the kidney and suggest that β_3-AR agonism might be useful to bypass AVPR2-inactivating mutations.

INTRODUCTION

In the kidney the antidiuretic hormone arginine vasopressin (AVP) is a critical regulator of water and electrolytes homeostasis. AVP is released from the pituitary gland into the bloodstream and binds to the type-2 vasopressin receptor (AVPR2) [1], a G protein-coupled receptors localized in the thick ascending limb of Henle (TAL), distal convolute tubule (DCT) and collecting duct (CD) acting mainly through the cAMP-PKA pathway.

In particular, in the TAL, AVP stimulates NaCl reabsorption across the Na-K-Cl cotransporter NKCC2, increasing its phosphorylation at a regulatory threonine in the amino terminus [2], thus generating and maintaining the cortico-medullary osmotic gradient providing the driving force for water reabsorption in the kidney tubules.

Concomitantly, in the CD, AVP stimulates the exocytosis of the water channel aquaporin 2 (AQP2) [3] from intracellular storage vesicles toward the apical membrane of the principal cells, dramatically increasing water reabsorption at this site (for a review see [4]).

Inactivating mutations of AVPR2 gene cause X-linked nephrogenic diabetes insipidus (XNDI) characterized by constant diuresis and risk of severe dehydration [5]. In recent years, many studies showed that hormones different from AVP also exhibit anti-diuretic effect [6-10]. Therefore, uncovering novel hormone systems able to regulate diuresis independently from the AVP-AVPR2 axis may impact on the management of diseases such as XNDI.

The β-adrenergic system plays a fundamental role in the control of several renal functions. In particular activation of type 1 and 2 β-adrenoreceptors ($\beta_{1\text{-}2}$-AR) [11] regulates renal blood flow, glomerular filtration rate, sodium and water reabsorption, acid-base balance and secretion of renin from the juxtaglomerular granular cells (for a review see [12]).

Among β-ARs, the β_3-AR is the last identified member of this receptor family. At first it was shown to regulate lipolysis and thermogenesis in adipose tissue [13], while subsequently it has been described to play important roles in the pathophysiology of the cardiovascular system [14] and the urinary tract [15]. However, its expression and possible physiological role in the kidney remains to be fully clarified. There is indication in mice that β_3-AR mRNA is expressed by renal arteries where β_3-AR might contribute to enhance vasoreactivity to angiotensin II

[16]. In addition, in rat kidney, a cDNA microarray screening showed that β_3-AR is expressed in the kidney medulla [17]. Moreover, in humans, β_3-AR polymorphisms seem to be associated with the diuretic effect of thiazide diuretics [16, 18] suggesting a role for β_3-AR in regulating water renal reabsorption. In this respect, demonstrating this novel role of β_3-AR in renal physiology is particularly intriguing in light of potential therapeutic applications of β_3-AR-acting drugs as novel strategies to counteract diseases characterized by altered diuresis. In fact, β_3-AR is relatively resistant to agonist-induced desensitization as it lacks the phosphorylation sites involved in short-term receptor desensitization [19] a feature that would ensure prolonged pharmacological stimulation of β_3-AR *in vivo*. In addition, due to the reduced number of tissues expressing β_3-AR, compared to the other β-AR isoforms, β_3-AR agonists are supposed to show low systemic off-target effect [14].

Here we show, for the first time, evidences suggesting a novel physiological role of kidney-expressed β_3-AR in triggering antidiuresis, likely supporting the possible use of β_3-AR agonists in the treatment of pathologies characterized by altered diuresis.

RESULTS

β3-AR expression in the mouse kidney

Reverse transcriptase-PCR (RT-PCR) revealed that β_3-AR mRNA was clearly detectable in the RNA samples from mouse kidney, brown adipose and bladder (Fig. 1A). In particular, the intron-spanning primers amplified two bands, of 234 bp and 337 bp, representing β_{3a}-AR and β_{3b}-AR transcripts, respectively [20]. Sequencing confirmed the specificity of the obtained bands (data not shown).

Immunoblotting analysis revealed that mouse kidney cortex and total medulla expressed a band of 44 kDa, for the core protein and one at 68 kDa for the glycosylated form in all samples (Fig. 1B). Both bands were also revealed in β_3-AR-expressing control tissues.

Immunolocalization of β3-ARs in the mouse kidney

The specific localization of the β_3-AR along the mouse nephron was evaluated by double antibody staining using a series of renal cell type-specific antibodies (Fig. 2). β_3-AR was expressed at the apical and basolateral membrane of the epithelial cells of the thin ascending limb (tAL), identified by the presence of the kidney-specific chloride channel (ClC-K1) [21, 22]. β_3-AR was also localized at the basolateral membrane of the epithelial cells of: i) the TAL, expressing the apical NKCC2 cotransporter [23]; ii) the DCT, expressing the apical the thiazide-sensitive NaCl symporter (NCC) [24]; iii) the cortical and the outer medullary CD (CCD, OMCD, the latter not shown), expressing AQP2 at the apical membrane [25]. The staining for β_3-AR completely disappeared when the anti β_3-AR antibody used for immunofluorescence was pre-adsorbed on its immunizing peptide (see Fig. 1 of supplemental material).

We also demonstrated that β_3-AR was neither expressed in the proximal convolute tubule (PT) nor in the thin descending limb of Henle's loop (TDL), the inner medullary CD (IMCD) and the *Vasa Recta* (Supplemental Fig. 2). Overall, the present data show that β_3-AR is localized in those nephron tracts also expressing AVPR2.

Effect of β3-AR activation on cAMP production, AQP2 trafficking and NKCC2 phosphorylation: *ex vivo* experiments

Our finding that β3-AR is expressed in the AVPR2-positive kidney segments, prompted us to investigate whether β3-AR activation may mimic the effect of AVP on cAMP production, AQP2 intracellular trafficking and NKCC2 activation. Using an *ex vivo* model consisting of freshly-isolated mouse kidney tubule suspensions, we measured changes in intracellular cAMP concentrations in response to either the specific β3-AR agonist BRL37344 (BRL; 1, 10, 100μM) or the AVP analogue 1-deamino-8-D-arginine-vasopressin (dDAVP, 10^{-7}M), used as positive control for cAMP production (Fig. 3A). Results are reported as % of the cAMP concentration measured in resting tubules. Treatment with BRL37344 led to concentration-dependent increases in intracellular cAMP levels, with the maximal effect observed at 10μM (+173%, P<0.0001).

Accordingly, we used 10μM BRL37344 for all the following experiments performed in freshly-isolated live mouse kidney slices, left untreated (resting) or incubated either with dDAVP or with BRL37344 (Fig. 3B). Confocal microscopy showed that both BRL37344 and dDAVP promoted AQP2 accumulation at the luminal plasma membrane of CCD cells (Fig. 3B, white arrows), compared with the cytoplasmic localization of AQP2 observed in control slices (Fig. 3B, white arrowheads). In line with the absence of β3-AR in the IMCD, BRL37344 failed to induce AQP2 apical accumulation in this portion of the CD (not shown). Importantly, the effect of BRL37344 was prevented by preincubation either with the β3-AR-selective antagonist L748,337 [26] or with the PKA inhibitor H89 [27].

Next, we evaluated the level of NKCC2 phosphorylation (p-NKCC2) in the same experimental conditions, using an antibody against the regulatory phospho-threonine residues in the N-terminus of NKCC2 [28]. Western blotting (Fig. 3C, D) showed that p-NKCC2 increased by about five-fold after BRL37344 treatment compared to resting conditions, an effect comparable to that obtained by dDAVP. Pre-treatment with either L748,337 or H89 significantly prevented this effect of BRL37344. Of note, incubation of kidney slices either with L748,337 or with H89 alone did not change AQP2 subcellular localization or NKCC2 phosphorylation, compared to resting slices (not shown).

To confirm that these effects of BRL37344 were ascribable to β_3-AR stimulation, we repeated these experiments on live kidney slices from β_3-AR-null mice (β_3-AR$^{-/-}$) [29]. Importantly, in the absence of β_3-AR functional expression, BRL37344 promoted neither AQP2 apical accumulation (Fig. 4A) nor NKCC2 phosphorylation (Fig. 4B, C). In addition, in β_3-AR$^{-/-}$ mice, 10μM BRL37344 was unable to promote intracellular cAMP elevation in isolated kidney tubules (not shown).

Effect of β_3-AR knockout on water and electrolytes handling in the mouse kidney.

The effect of β_3-AR agonism on AQP2 and NKCC2, the major players involved in antidiuresis, prompted us to investigate whether β_3-AR inactivation may affect water and electrolytes handling in the kidney *in vivo*. To this purpose, we evaluated these parameters in β_3-AR$^{-/-}$ mice [29], lacking β_3-AR functional expression, and $\beta_{1\text{-}2}$-AR$^{-/-}$ knockout mice [30], in which β_3-AR is the only expressed β-AR. Age-matched wt animals of each strain were used as controls (β_3-AR$^{+/+}$ and $\beta_{1\text{-}2}$-AR$^{+/+}$). Strikingly, in β_3-AR$^{-/-}$ diuresis was higher (by 77%), urine osmolality was lower (by 30%) and water intake was increased (by 40%) compared with β_3-AR$^{+/+}$ (Fig. 5A). On the contrary, urine parameters and water intake were comparable between $\beta_{1\text{-}2}$-AR$^{+/+}$ and $\beta_{1\text{-}2}$-AR$^{-/-}$ mice (Fig.5B). No significant differences in food intake were observed between mouse strains (not shown).

In line with these results, immunofluorescence analysis showed that, compared to control β_3-AR$^{+/+}$ mice, β_3-AR$^{-/-}$ mice have reduced AQP2 plasma membrane expression and increased subapical localization (Fig. 5C).

Analysis of urine electrolytes, reported in Table 1, showed that β_3-AR$^{-/-}$ mice display significantly higher urine excretion of Na^+, K^+ and Cl^- compared with their age-matched β_3-AR$^{+/+}$. Instead, the plasma concentration of the same electrolytes and the GFR were comparable between β_3-AR$^{-/-}$ and β_3-AR$^{+/+}$. These results suggest a reduced activity of the NKCC2 transporter in β_3-AR$^{-/-}$ mice.

Immunofluorescence analysis showed that in β_3-AR$^{-/-}$ mice the antibody against pNKCC2 detected a lower amount of activated NKCC2 in the outer medulla, compared to β_3-AR$^{+/+}$ animals (Fig. 5D).

To further support this evidence we analyzed the effects of bumetanide injection on natriuresis in both β_3-AR$^{-/-}$ and β_3-AR$^{+/+}$mice (Supplemental Fig.3C, D). Natriuresis was higher in β_3-AR$^{+/+}$ than in β_3-AR$^{-/-}$ (in %,

355.4±21.55 versus 287±27.5; p<0.0001), confirming that β3-AR$^{-/-}$ mice have less basal NKCC2 cotransporter activity to inhibit.

Of note, the maximal urine concentrating ability of β3-AR$^{-/-}$ mice under water deprivation test was comparable to that of β3-AR$^{+/+}$ mice (Supplemental Fig. 3A, B).

Effect of β3-AR stimulation on diuresis.

Next, to uncover the possible antidiuretic effect of pharmacological stimulation of β3-AR in mice, we examined whether BRL37344 could *per se* induce antidiuresis. β3-AR$^{+/+}$ and β3- received a single i.p. injection of BRL37344 (0,6 mg/kg), or PBS alone (vehicle). Urine samples were collected for 4 h after injections, the first time point at which all BRL37344-treated animals began to urinate. Diuresis, urine osmolality and urine electrolytes excretion were analyzed and reported in Fig. 6. Notwithstanding the different diuresis of β3-AR$^{-/-}$ and β3-AR$^{+/+}$ mice, we expressed our results as % of values measured in vehicle-treated animals of each genotype. Strikingly, BRL37344 strongly reduced the diuresis in β3-AR$^{+/+}$ but not in β3-AR$^{-/-}$ mice (Fig. 6A). Concomitantly, BRL37344 significantly increased urine osmolality only in β3-AR$^{+/+}$ mice (Fig. 6B). Interestingly, in β3-AR$^{+/+}$ mice, urine excretion of Na^+, K^+, Cl^-, normalized to the volume of diuresis, were significantly reduced by BRL37344 (Fig. 6C, D, E). Of note, the GFR of β3-AR$^{+/+}$ animals, measured at 1, 2, 3 and 4h after BRL37344 treatment, was not affected (Fig. 6F).

Effect of β3-AR stimulation on diuresis of mice lacking AVPR2

Next, we investigated whether the potent antidiuretic effect of BRL37344 observed in β3-AR$^{+/+}$ mice, could bypass the inactivation of the AVP signaling in mice lacking AVPR2 [12, 31]. Mice received a single i.p. injection of BRL37344 (0,6 mg/kg) or PBS alone (vehicle). Urine samples were collected every hour for 3 hours and diuresis (Fig. 7A) and urine osmolality (Fig. 7B) reported. Strikingly, 1 h after the injection, the urine output of all BRL37344-treated mice was reduced to zero compared to vehicle-treated mice (Fig. 7A, 1h). Therefore, we could not measure urine osmolality at this time point (Fig. 7B, 1h). Two hours after injection, the diuresis of BRL37344-treated mice was still dramatically reduced compared to vehicle-treated animals (Fig. 7A, 2h) and

urine osmolality increased (Fig. 7B, 2h). Three hours *post* injection the effect BRL37344 on diuresis still persisted (Fig. 7A, 3h), while that on urine osmolality partially reversed (Fig. 7B, 3h).

DISCUSSION

The possible expression and physiological role of β_3-AR in the kidney has not been deeply investigated so far, although the well-known contribution of β_1- and β_2-ARs to the sympathetic regulation of renal function.

The present results show β_3-AR expression in the same AVPR2-expressing tubules. Since it is known that, similarly to AVPR2, β_3-AR activates the cAMP pathway [32], we hypothesized that pharmacological stimulation of β_3-AR might regulate the trafficking/activity of AQP2 and NKCC2 that are known to be involved in the AVP-elicited antidiuretic response in the kidney [2, 3].To confirm this hypothesis, we first demonstrated that BRL37344 significantly increases cAMP production and promotes both AQP2 apical accumulation and NKCC2 phosphorylation/activation indicating that, similarly to AVP, β_3-AR agonists may effectively increase kidney reabsorption of water and solutes.

The pharmacological profile of BRL37344 is quite complex. For instance, there is indication that BRL37344 may have an intrinsic activity at β_1- or β_2-ARs [33] thus indicating that BRL37344 may exert its effects *via* β_1- or β_2-ARs. As shown by the present results, BRL37344 effects on AQP2 and NKCC2 are prevented by the β_3-AR antagonist L748,337 and are not observed in β_3-AR-null mice thus supporting the notion that BRL37344 acts selectively at β_3-AR at the dose used in the present study, and excluding that it can exert an off-target effect.

As shown by the present results, β_3-AR$^{-/-}$ are characterized by mild polyuria, lower urine osmolality and increased urinary excretion of Na^+, K^+ and Cl^- but not Ca^{++}. Increased water excretion in the absence of β_3-AR is in line with our observation of reduced plasma membrane expression of AQP2 in the CCD of β_3-AR$^{-/-}$. In addition, increased Na^+, K^+ and Cl^- excretion in β_3-AR$^{-/-}$ is in line with decreased NKCC2 activity as also supported by the findings that β_3-AR$^{-/-}$ show less activated NKCC2 at the plasma membrane and the natriuretic response to bumetanide is less pronounced in β_3-AR$^{-/-}$ than in β_3-AR$^{+/+}$.

The fact that food consumption in β_3-AR$^{-/-}$ is comparable to that of control β_3-AR$^{+/+}$ (Table 1) seems to exclude that solute diuresis can explain the polyuria of β_3-AR$^{-/-}$. Neither defect of AVP release (central polydipsia) can explain the polyuria of β_3-AR$^{-/-}$ since these mice show normal urine concentrating abilities under water deprivation challenge.

As also shown by the present results, β_3-AR$^{-/-}$ have normal plasma levels of Na^+, K^+, Cl^- and Ca^{++} indicating that their polyuric phenotype is neither induced by hypercalciuria/hypercalcemia nor by hypokalemia differently from that previously observed in rat models of potassium deprivation or in collecting duct cells cultured in high Ca^{++} [34-36]. In addition, the polyuria in β_3-AR$^{-/-}$ is not a consequence of increased GFR, which does not differ between β_3-AR$^{-/-}$ and β_3-AR$^{+/+}$. On the other hand, $\beta_{1\text{-}2}$-AR$^{-/-}$ do not show a renal phenotype, in terms of alterations of diuresis and urine osmolality, suggesting that the lack of β_1- and β_2-AR does not affect urine parameters and indicating that these parameters are mainly regulated by β_3-AR. However, the question whether β_3-AR is more important than β_1- and β_2-AR during baseline renal function cannot be solved by the present results as we cannot correctly compare the water balance ability of β_3-AR$^{-/-}$ to that of $\beta_{1\text{-}2}$-AR$^{-/-}$. Indeed, the two strains result from different genetic background and it is known from early studies in the field of renal physiology that renal parameters significantly differ in mice of different strains [37].

In line with the stimulatory effect of β_3-AR activation on AQP2 subcellular localization and NKCC2 phosphorylation, BRL37344 exerts a potent antidiuretic effect in β_3-AR$^{+/+}$ but not in β_3-AR$^{-/-}$ thus confirming the *ex vivo* data on its specific action at β_3-AR. The additional finding that in β_3-AR$^{+/+}$ BRL37344 reduces urinary excretion of Na^+, K^+ and Cl^-, but not Ca^{++} and induces a 70% reduction of the diuresis, while urine osmolality is increased by about 40%, may be explained by assuming that β_3-AR stimulation promotes not only water but also salt reabsorption in the kidney tubule. In line with this possibility, the strong reduction of diuresis observed in BRL37344-treated mice is independent on the reduction of GFR.

It is known that β_3-ARs are expressed in the hypothalamus, where their activation increases the release of serotonin [38]. Thus, the possibility exists that the antidiuretic effect of BRL37344 may be due to an extra renal action of the agonist, for instance to a hypothalamic regulation of AVP release. The present results in the vital conditional model of AVPR2-null mice [31] seem to exclude this possibility. In these mice, AQP2 is dramatically down regulated and does not traffic to the apical plasma membrane in response to AVP. As a consequence, AVPR2-null mice develop X-linked NDI (XNDI) whose main symptoms are polyuria, polydipsia and hypernatremia [39-41]. As shown here, a single i.p. injection of BRL37344 greatly reduces the diuresis and increases urine osmolality supporting the notion that, *in vivo,* β_3-AR agonism elicits an AVP-independent

antidiuretic effect. In addition, results in live kidney slices demonstrating that BRL37344 induces cAMP production, AQP2 accumulation at the apical plasma membrane of CDs and NKCC2 phosphorylation /activation in the TAL provide an additional, although indirect, evidence that BRL37344 triggers its effect independently of central β_3-AR activation.

The present results cannot exclude that the effects of BRL37344 on urine output may be related on systemic effects of the drug as, for example, on arterial pressure. However, it has been established in rats that BRL37344 reduces arterial pressure by about 14% [42] therefore it is unlikely that such an effect may be responsible for the observed 70% reduction in diuresis. In conclusion, our experimental data indicate that: i) in mouse kidney β_3-ARs are expressed in most of the AVP-sensitive nephron segments, ii) β_3-AR stimulation promotes AQP2 plasma membrane accumulation and NKCC2 activation, thus increasing water and salt reabsorption in the kidney tubule; iii) this effect is likely mediated by an increase of intracellular cAMP concentration; iv) β_3-AR agonism induces antidiuresis in mice lacking AVPR2.

Taken together, the present data suggest an unexplored role of sympathetic stimulation *via* β_3-AR in promoting antidiuresis under physiological conditions. A number of evidence indicates that there is a synaptic contact between renal sympathetic nerve fiber varicosities and renal tubular epithelial cell basolateral membranes [12, 43]. In this respect, the present data support the hypothesis that sympathetic stimulation of β_3-AR, upregulating NKCC2 and AQP2 activity at the apical plasma membrane, can enhance solutes and water reabsorption in the nephron, thus eliciting an antidiuretic effect.

In this study we restricted our investigation to the regulatory role of β_3-AR on AQP2 and NKCC2, but the possible effect of β_3-AR stimulation on other Na/Cl transporters or additional AQPs, participating to the countercurrent multiplier system, is worth further investigation.

The observation that β_3-AR$^{-/-}$ are polyuric but show normal urine concentrating ability during water deprivation suggest that, under physiological conditions, β_3-AR activation by sympathetic nerves does not seem to provide an additional mechanism corroborating the kidney antidiuretic response to AVP. Although much work remains to be done in order to understand the role of β_3-AR on water and salt reabsorption during sympathetic activation, the present results are potentially relevant for the development of novel pharmacological

approaches for the treatment of those diseases caused by AVPR2 altered signaling, including XNDI, polycystic kidney diseases and the syndrome of inappropriate secretion of antidiuretic hormone. For instance, in XNDI patients, β_3-AR agonists may bypass the lack of AVPR2 function, rescuing NKCC2 and AQP2 activities and improving the unpaired urine concentration mechanism. It has to be underlined that patients with autosomal dominant or recessive forms of NDI [40], due to mutations of the AQP2 gene, would not benefit from this potential treatment.

Further studies are needed to verify this proof-of-concept, but the ameliorative effect of BRL37344 on renal concentrating abilities of AVPR2-null mice, strongly encourages studies toward this direction. In particular, we suggest that agonists of the human β_3-AR, like Mirabegron [44], already used to treat overactive bladder, may either improve the impaired concentrating ability of the kidney or increase the beneficial effects of the current NDI therapy.

MATERIALS AND METHODS

Antibodies and reagents

Rabbit and goat polyclonal antibodies against β3-AR (cat.# sc-50436 and cat.# sc-1473) were from Santa Cruz Biotechnology (www.scbt.com) and were previously validated for Western blotting and immunofluorescence analysis, respectively [45, 46]. Rabbit polyclonal antibodies against AQP1 (cat.# sc-20810), and CD-31 (cat.# sc-1506), BRL37344 (cat.# sc-200154), L748,337 (cat.# sc-204044) were also from Santa Cruz Biotechnology. H-89 dihydrochloride hydrate (cat.# B1427), and [deamino-Cys[1], D-Arg[8]]-Vasopressin acetate salt hydrate (dDAVP, cat.# V-1005) were from Sigma (www.sigmaaldrich.com). Rabbit anti-CLC-K antibody (cat.# ACL-004) was from Alomone Labs (www.alomone.com). Rabbit anti-NKCC2 (cat.# AB3562P) antibodies were from Millipore (www.merckmillipore.com). Rabbit anti-NCC antibody (cat.# SPC-402D) was from StressMarq Biosciences Inc. (www.stressmarq.com). Rabbit affinity-purified polyclonal antibody against human AQP2 was previously described [47]. The antibody against the phosphorylated threonines 96 and 101 of mouse NKCC2 (p-NKCC2) [28] was kindly provided by Prof. Biff Forbush, Yale University, New Haven, CT (USA).

β3-AR pharmacology

BRL37344 is a well-known β3-AR agonist [48], which has been previously used in mice [49-51]. BRL37344 displays a rank order of potency at the human β-ARs that is β3-AR>β2-AR>β1-AR, with an approximately 20-fold and 100-fold higher selectivity for β3-AR versus β2-AR and β1-AR, respectively [52]. BRL37344 has been found to be effective at 10 μM in the human isolated internal anal sphincter model [53], in human retinal endothelial cells [54] and in mouse retinal explants [55].

According to the literature, the pharmacological profile of the β3-AR antagonist L748,337 is very complex. For instance, it has been reported as one of the very few antagonists with high selectivity for β3-AR [56]. Several reports indicate that L748,337 affinity (and perhaps selectivity) at β3-AR is lower with rodent than with the

human receptors [57, 58]. Nonetheless, L748,337 remains the most suitable β3-ARantagonist currently available [26].

RNA isolation and RT-PCR

Total RNA was extracted from adult mouse brown adipose tissue, bladder and kidney by the TRIzol® reagent and then was reverse-transcribed into cDNA using Super-Script® VILO™ cDNA Synthesis Kit.

The intron-spanning primers (forward: 5'-TCTAGTTCCCAGCGGAGTTTTCATCG -3', reverse: 5'-CGCGCACCTTCATAGCCATCAAACC -3') were previously reported [20].

As a positive control, a fragment of mouse β-actin complementary DNA was amplified using specific primers.

PCR amplifications were performed using Taq DNA Polymerase recombinant (www.lifetechnologies.com) according to the following program: (94 °C, 3 min) x1 cycle; (94 °C, 45 sec; 55 °C, 30 sec; 72 °C, 1 min) x40 cycles. Amplified products were analyzed by 3% agarose gel followed by ethidium bromide staining. Sequencing were performed by BMR-genomics (www.bmr-genomics.it, Padua, Italy), using the method of Sanger.

Cell and Tissue fractionation and immunoblotting

Brown adipose, bladder and kidney cortex/total medulla were isolated from male C57BL/6J mice and homogenized in RIPA buffer [59]. Where reported, kidney slices were lysed in antiphosphatase buffer [2].

15 µg of each lysate were separated by standard SDS-PAGE and analyzed by Western blotting.

After blocking with 3% bovine serum albumin, blots were incubated with anti- β3-AR antibody (#SC-50436, 1:200) and anti p-NKCC2 antibodies (1:1000). Membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibody.

Reactive proteins were revealed by enhanced chemiluminescence, detected with Chemidoc XRS detection system imaged with Image Lab Software (www.bio-rad.com) and quantified with ImageJ software (http://imagej.nih.gov/ij/).

Immunofluorescence

Mouse kidneys were fixed overnight with 4% paraformaldehyde in PBS at 4°C, dehydrated in graded ethanol, and embedded in paraffin wax. Serial sections, 5 µm thick, were deparaffinised, rehydrated and subjected to immunofluorescence analysis. Antigen retrieval was performed by boiling sections in citrate buffer (10mM sodium citrate, pH6). Nonspecific binding sites were blocked with 1% BSA in PBS for 30 min and sections were then incubated with the primary antibodies (dilutions: β_3-AR (#SC1473) 1:100, AQP2 1:1000; AQP1 1:200, CLC-K 1:300, NKCC2 1:500, CD31 1:100, NCC 1:200, p-NKCC2 1:500) in the same solution.

After washes in PBS, sections were incubated with AlexaFluor-conjugated secondary antibodies (www.lifetechnologies.com). Confocal images were obtained with a confocal microscope Leica TSC-SP2.

Preparation of kidney tubule suspensions and cAMP assay

Kidneys from FVB/C57/129/DBA mice (10- week-old males) were minced and enzymatically digested as previously reported [10]. For each individual experiment, tubule suspensions from the kidneys of three mice were pooled. Aliquots of tubule suspensions were preincubated with the phosphodiesterase inhibitor IBMX for 10 min at 37 °C. Subsequently, BRL37344 (1, 10 and 100µM) or dDAVP (100 nM) were added, and reactions were carried out for 45 min at 37 °C. Total intracellular cAMP was determined *via* ELISA as previously reported [10].

Kidney tissue slices: preparation and treatment

C57BL/6J male mice were anesthetized with an i.p. injection of tribromoethanol (250 mg/kg;) and euthanized by cervical dislocation. Kidneys were rapidly excised and thin transversal slices (250 µm) were cut using a McILWAIN Tissue Chopper (www.tedpella.com).

The kidney slices were incubated at 37 °C for 15 min in DMEM-F12 medium pre-equilibrated in a 5% CO_2 incubator. Slices were stimulated for 40 min at 37 °C with dDAVP (10^{-7} M) or BRL37344 (10^{-5} M), the latter alone or after 30 min of preincubation with either L748,337 (10^{-7} M) or the PKA inhibitor H89 (10^{-5} M). Slices

were either processed for immunoblotting analysis or fixed in 4% PFA in PBS, cryopreserved in 30% sucrose in PBS. Ultra-thin cryosections (5 µm) collected and subjected to immunofluorescence analysis for AQP2 and β_3-AR as described above.

Animal studies

All animal experiments were approved by the Institutional Committee on Research Animal Care, in accordance with the Italian Institute of Health Guide for the Care and Use of Laboratory Animals. Mice were maintained on a 12 h light/12 h dark cycle, with free access to water and food.

β_3-AR $^{-/-}$ and β_3-AR $^{+/+}$ mice [60] were purchased from Jackson Laboratory (www.jax.org). $\beta_{1\text{-}2}$-AR $^{-/-}$ and $\beta_{1\text{-}2}$-AR $^{+/+}$ mice [30] were generated as previously reported [61, 62]. Mice (n=8 for each group) were kept in metabolic cages (www.tecniplast.it) for 5 days to measure 24h diuresis, osmolality and water intake. They received a single i.p. injection of BRL37344 (0,6 mg/kg) or PBS alone.

Urinary and plasma electrolytes were measured with the ion selective electrode (ISE) method.

GFR of conscious mice was measured by fluorescein isothiocyanate-labeled sinistrin clearance as previously reported [63].

The generation of the conditional AVPR2 knockout mice ($V2R^{fl/fl}$ and $V2R^{fl/y}$ Esr1-Cre mice) and deletion of the AVPR2 gene has been previously described [31].

$V2R^{fl/y}$ Esr1-Cre mice (n=5 for each group) received a single i.p. injection of BRL37344 (0,6 mg/kg) or PBS alone. Mice were housed into metabolic cages and urine output and osmolality were monitored every hour for 3 hours. Urine osmolality was measured using a vapor pressure osmometer (www.wescor.com).

Statistical analysis

For statistical analysis, GraphPad Prism software (version 5.00, GraphPad Software, San Diego, California, USA) was used. For each experiments the statistical analysis performed is indicated in the figure legends.

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Figure Legends

Figure 1. Expression of β_3-ARs mRNA and protein in mouse kidney.
A) Total RNA from mouse kidney was probed for the presence of mRNA coding β_3-AR. Brown adipose tissue and bladder were used as positive controls. Two amplicons corresponding to β_{3b}-AR (337bp) and β_{3a}-AR (234bp) were visualized in all samples. Control RT-PCR was performed using primers amplifying mouse β-actin.
B) Total protein extracts from mouse kidney cortex and total medulla were analyzed by Western blotting using anti β_3-AR antibodies. Two bands, corresponding to the core and the glycosylated protein were detected in the kidney fractions at the same molecular size of those revealed in brown adipose and urinary bladder.
Experiments were repeated three times with comparable results.

Figure 2. Immunolocalization of β_3-AR in mouse kidney
Paraffin-embedded kidney sections (C57BL6/J, wt) were immunostained with anti β_3-AR antibodies (green) and co-stained with antibodies against specific markers of different segments of the kidney tubule: CLC-K channel for the thin ascending limb (tAL), NKCC2 for the thick ascending limb (TAL), NCC for the distal convolute tubule (DCT) and AQP2 for the cortical collecting duct (CCD) (all in red). Overlay of the each double staining experiment indicated significant expression of β_3-AR in the tAL, TAL, DCT and CCD. Drawings of the nephron on the right column indicated in light green the β_3-AR-positive segments.
Same results were obtained in 5 different animals. (Bar=20μm)

Figure 3. *ex vivo* β_3-AR activation in kidney tubule: intracellular cAMP measurements, AQP2 subcellular localization and NKCC2 phosphorylation.
A) dDAVP and BRL37344-induced cAMP production in mouse kidney tubule suspensions. Freshly isolated tubule suspensions from wt mice (12-week-old males; n= 3 per individual experiment) were pooled and equally distributed into 24-well plates. Samples were treated with dDAVP (10^{-7}M) or with the indicated concentrations of BRL34377 for 60 min at 37°C. Total cAMP generated in each well was normalized to the protein content. Three independent experiments were carried out. Data are expressed as % of the cAMP content measured in resting cells ± SEM. Significant differences between means were tested by One-way analysis of variance ANOVA with Newman-Keuls's post-test. Significance was accepted for p values < 0.05.
***P<0.0001, **P<0.001, compared to resting tubules. ##P<0.001, compared to BRL1μM.
B) Freshly-isolated kidney slices (250 μm) where rapidly cut after sacrifice, maintained in CO_2-equilibrated culture medium at 37°C, left untreated (resting) or incubated with desmopressin (dDAVP, 10^{-7} M) or with BRL37344 (BRL, 10μM). BRL37344 was also incubated after preincubation either with the β_3-AR-selective antagonists (L748,337, 10^{-7} M) or with the PKA inhibitor (H89, 10^{-5} M). Slices were treated as described above, fixed and ultra-thin sections were stained for AQP2 and β_3-AR, and subjected to confocal microscopy. BRL37344 was as effective as dDAVP in promoting AQP2 expression at the apical plasma membrane of cortical and outer medullary collecting duct cells (white arrows), compared with the intracellular localization of AQP2 observed in untreated samples (resting) or samples incubated with BRL37344 after preincubation wit L748,337 or H89 (white arrowheads). (Bar=15μm)
C) Kidney slices were treated as above, then lysed and total protein extracts subjected to Western blotting analysis using the anti pNKCC2 and the anti total NKCC2 antibodies. **D)** Densitometric analysis showed a five-folds increase of pNKCC2 (normalized to total NKCC2) in samples treated with BRL37344 or dDAVP and the effect of BRL37344 was significantly prevented by L748,337 and H89. Data are provided as mean±SEM and expressed as % of the resting condition. Significant differences between means were tested by One-way analysis of variance ANOVA with Newman-Keuls's post-test. ***p<0.001
Comparable results were obtained in three different mice.

Figure 4. BRL37344 failed to induce AQP2 apical expression and NKCC2 phosphorylation in the kidney of β3-AR-null mice.

A) Freshly-isolated kidney slices (250 µm) where were obtained from $\beta_3^{-/-}$AR mice, maintained in CO_2-equilibrated culture medium at 37°C, left untreated (resting) or incubated with desmopressin (dDAVP, 10^{-7} M) or with BRL37344 (BRL, 10µM). Slices were fixed and ultra-thin sections (5 µm) were stained for AQP2 and subjected to confocal laser-scanning microscopy. In $\beta_3^{-/-}$AR mice BRL37344 was unable to promote AQP2 expression at the apical plasma membrane of cortical and outer medullary collecting duct cells. dDAVP was used as internal control to promote AQP2 apical expression. (Bar=10µm) **B)** Slices were also lysed and protein extracts subjected to Western blotting analysis with anti pNKCC2 and total NKCC2 antibodies. **C)** Densitometric analysis of pNKCC2, normalized to total NKCC2, showed that in $\beta_3^{-/-}$AR mice BRL37344 was unable to increase NKCC2 phosphorylation compared to dDAVP. Data are provided as mean±SEM and expressed as % of the resting condition. Significant differences between means were tested by One-way analysis of variance ANOVA with Newman-Keuls's post-test. ***p<0.0001

Comparable results were obtained in three different mice.

Figure 5. Mice lacking functional expression of β3-AR showed mild polyuria and reduced urine osmolality.

A) β3-AR-null mice (β_3-$AR^{-/-}$) and their age-matched controls (β_3-$AR^{+/+}$) (N=8 for each group), were individually housed in metabolic cages for 5 days and 24h urine output, urine osmolality and water intake measured daily. The analysis reports the mean values ± SEM relative to 24h urine collection. In β_3-$AR^{-/-}$ mice urine output was nearly 77% higher, urine osmolality 30% lower and water intake 41% higher compared with control β_3-$AR^{+/+}$ mice. Statistical analysis was performed by unpaired *t*-test. *p<0.05.

B) The same experimental protocol was applied to $\beta_{1\text{-}2}$-AR-null mice ($\beta_{1\text{-}2}$-$AR^{-/-}$) and their age-matched controls ($\beta_{1\text{-}2}$-$AR^{+/+}$) (N=8 for each group). No statistically significant difference was observed on urine parameters and water intake between the two experimental groups.

C) Immunofluorence analysis showed that β_3-$AR^{-/-}$ mice have reduced plasma membrane expression and higher subapical localization of AQP2, compared to control β_3-$AR^{+/+}$ mice. (Bar=10µm)

D) Immunofluorescence analysis with the anti pNKCC2 showed also that β_3-$AR^{-/-}$mice have reduced levels of activated NKCC2 (pNKCC2). (Bar=30µm)

Comparable results were obtained in three different mice.

Figure 6. Effect of β3-AR stimulation on urine concentrating ability in β_3-$AR^{+/+}$ mice.

β3-AR-null mice (β_3-$AR^{-/-}$) and their age-matched controls (β_3-$AR^{+/+}$) (n=10 for each genotype), were individually acclimatized in metabolic cages for 48h then 5 for each group received a single i.p. injection of BRL37344 (0,6 mg/kg) while 5 control animals received PBS alone (vehicle). Urine samples were collected for 4h after injection. **A)** urine output and **B)** urine osmolality measured in β_3-$AR^{+/+}$ and β_3-$AR^{-/-}$ expressed as percent of control values measured in vehicle-injected animals ±SEM. Urine output of β_3-$AR^{+/+}$ animals was reduced of about 70% and urine osmolality increased of about 40% following BRL37344 injection. No significant effect was seen in β_3-$AR^{-/-}$ mice. Significant differences between means were tested by One-way analysis of variance ANOVA with Newman-Keuls's post-test. *P<0.05, ***P<0.0001

C,D,E) Urine excretion of Na^+, K^+, Cl^-, normalized for the urine volume, measured in β_3-$AR^{+/+}$ mice. Data are reported as percent of the values measured in vehicle-injected animals ± SEM. Significant differences between means were tested by Mann-Whitney test. *P<0.05.

F) GFR of β_3-$AR^{+/+}$ mice conscious mice was measured at 1, 2, 3 and 4h after injection of BRL37344 or vehicle alone. No significant difference was seen, at each time point, between BRL37344- and vehicle-injected mice. Significant differences between means were tested by two-way ANOVA with Bonferroni post-test.

Figure 7. β3-AR stimulation promotes antidiuresis in mice lacking functional expression of the AVPR2
Ten *V2R*$^{fl/y}$*Esr1-Cre* mice were acclimatized into mice metabolic cages for 48h then 5 received a single i.p. injection of BRL37344 (0,6 mg/kg) while 5 control animals received PBS alone (vehicle). Urine samples were collected every hour for 3 hours from both groups and **(A)** urine output and **(B)** urine osmolality at each time point reported.
One hour after the injection, the urine output of treated mice (BRL37344) was reduced to zero compared to vehicle-injected animals (vehicle) (Diuresis, 1h). Two hours after injection, urine output of treated-mice was still dramatically reduced compared to control animals (Diuresis, 2h). Urine osmolality increased in BRL37344-injected animals (Urine osmolality, 2h). At 3 hours post injection, the effect BRL on the urine output was still persistent (Urine output, 3h), while that on urine osmolality partially reversed (Urine osmolality, 3h). The analysis reports the mean values ± SEM.
Significant differences between measurements were tested by two-way ANOVA with Bonferroni post-test for diuresis and by one-way ANOVA with Bonferroni post-test. *p<0.05; ***p<0.001

Table 1

	Electrolytes	β_3-AR$^{+/+}$	β_3-AR$^{-/-}$	
Plasma				
	Na^+ (mEq/L)	139.0 ± 5.57	141.3 ± 0.67	*NS*
	K^+ (mEq/L)	6.73 ± 0.29	6.43 ± 0.19	*NS*
	Cl^- (mEq/L)	108.0 ± 3.22	106.3 ± 2.67	*NS*
	Ca^{2+} (mEq/L)	3.16 ± 0.53	3.34 ± 0.44	*NS*
Urine				
	Na^+ (mEq/24h)	019 ± 0.02	0.27 ± 0.01	**
	K^+ (mEq/24h)	0.18 ± 0.02	0.23 ± 0.01	*
	Cl^- (mEq/24h)	0.45 ± 0.04	0.59 ± 0.03	**
	Ca^{2+} (mEq/24h)	0.005 ± 0.0006	0.005 ± 0.0005	*NS*
	GFR (µl/min)	235.5 ± 20.76	253.8 ± 30.94	*NS*
	Food Intake (g)	5.07 ± 0.08	5.14 ± 0.08	*NS*

Plasma electrolyte concentrations, renal 24-h electrolyte excretion and GFRs and food intake in β_3-AR$^{+/+}$and β_3-AR$^{-/-}$ mice. Values are means ± SEM of measurements in N=8 mice/genotype. Statistical analysis was performed using Unpaired t-test.

*p<0,05, **p< 0,01

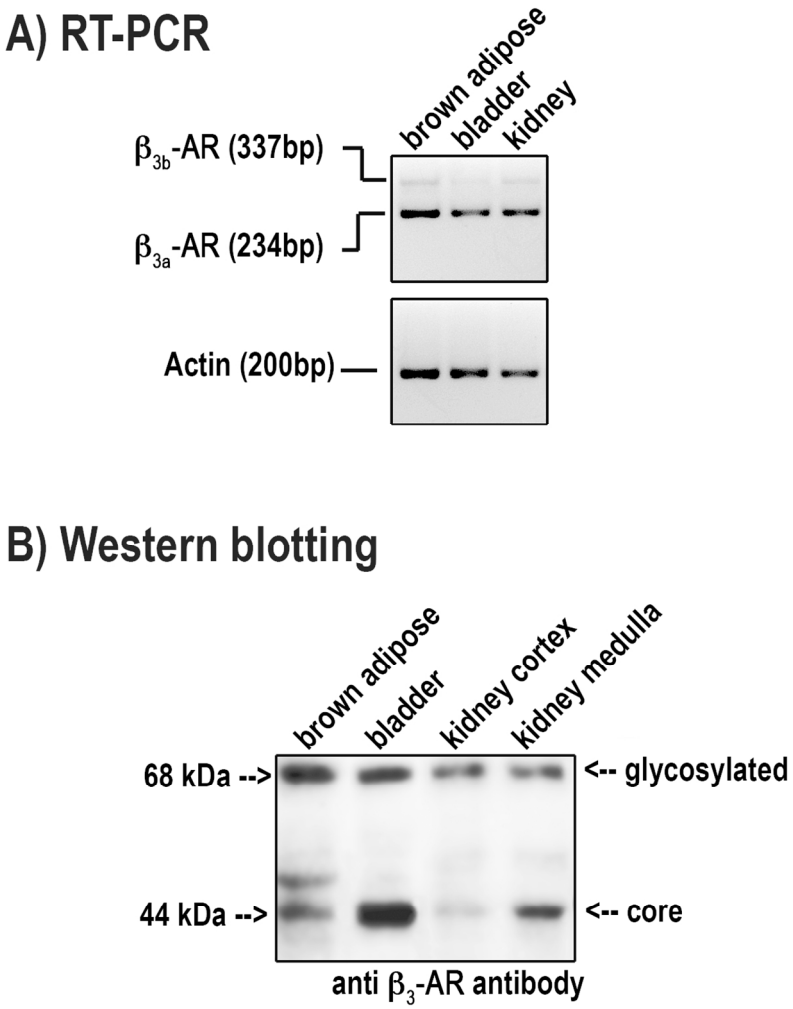

Fig. 1

Figure 1. Expression of β3-ARs mRNA and protein in mouse kidney.
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B) Total protein extracts from mouse kidney cortex and total medulla were analyzed by Western blotting using anti β3-AR antibodies. Two bands, corresponding to the core and the glycosylated protein were detected in the kidney fractions at the same molecular size of those revealed in brown adipose and urinary bladder.
Experiments were repeated three times with comparable results.

108x153mm (300 x 300 DPI)

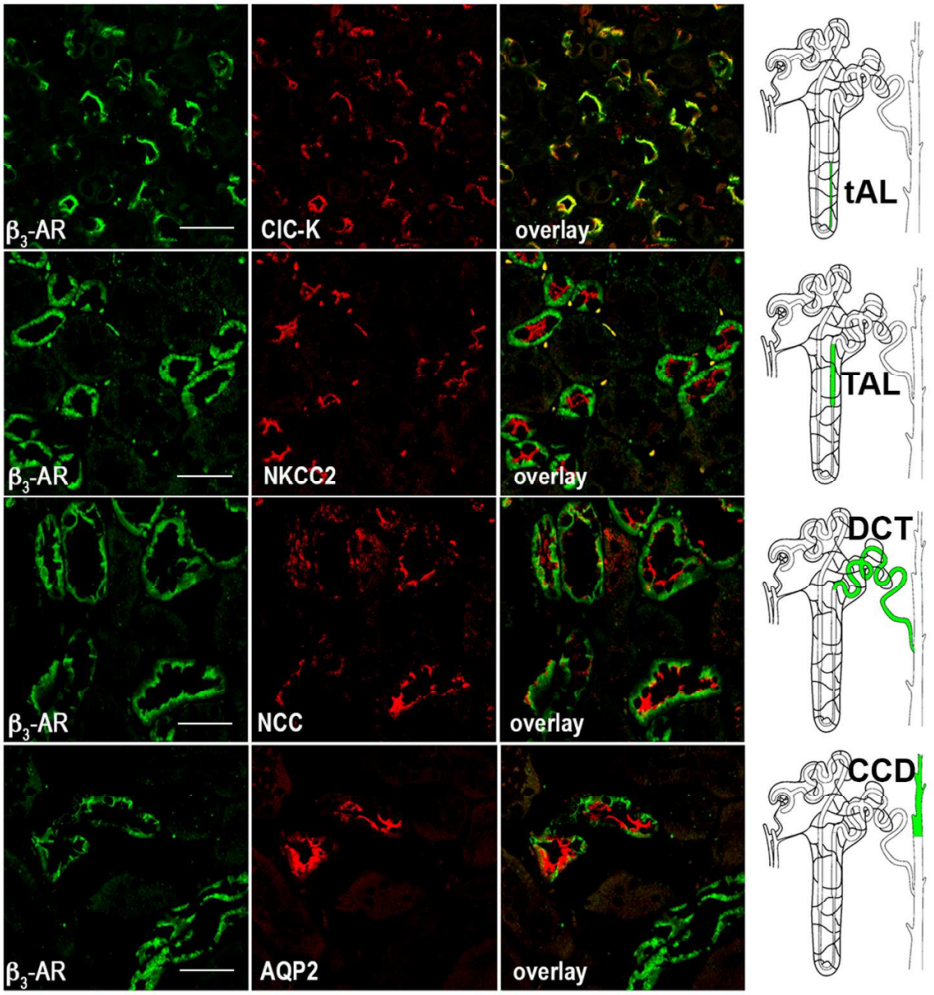

Fig.2

Figure 2. Immunolocalization of β3-AR in mouse kidney
Paraffin-embedded kidney sections (C57BL6/J, wt) were immunostained with anti β3-AR antibodies (green) and co-stained with antibodies against specific markers of different segments of the kidney tubule: CLC-K channel for the thin ascending limb (tAL), NKCC2 for the thick ascending limb (TAL), NCC for the distal convolute tubule (DCT) and AQP2 for the cortical collecting duct (CCD) (all in red). Overlay of the each double staining experiment indicated significant expression of β3-AR in the tAL, TAL, DCT and CCD. Drawings of the nephron on the right column indicated in light green the β3-AR-positive segments. Same results were obtained in 5 different animals. (Bar=20μm)

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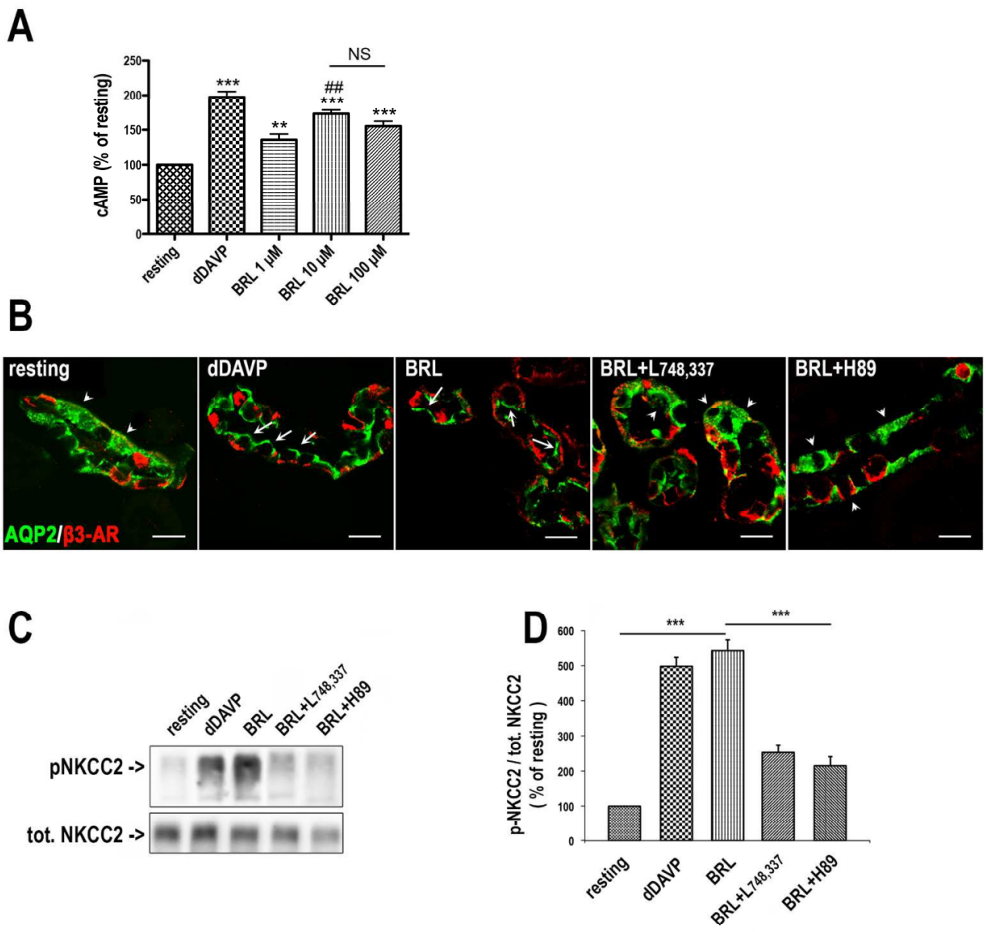

Fig. 3

Figure 3. ex vivo β3-AR activation in kidney tubule: intracellular cAMP measurements, AQP2 subcellular localization and NKCC2 phosphorylation.

A) dDAVP and BRL37344-induced cAMP production in mouse kidney tubule suspensions. Freshly isolated tubule suspensions from wt mice (12-week-old males; n= 3 per individual experiment) were pooled and equally distributed into 24-well plates. Samples were treated with dDAVP (10-7M) or with the indicated concentrations of BRL34377 for 60 min at 37°C. Total cAMP generated in each well was normalized to the protein content. Three independent experiments were carried out. Data are expressed as % of the cAMP content measured in resting cells ± SEM. Significant differences between means were tested by One-way analysis of variance ANOVA with Newman-Keuls's post-test. Significance was accepted for p values < 0.05. ***P<0.0001, **P<0.001, compared to resting tubules. ##P<0.001, compared to BRL1µM.

B) Freshly-isolated kidney slices (250 µm) where rapidly cut after sacrifice, maintained in CO2-equilibrated culture medium at 37°C, left untreated (resting) or incubated with desmopressin (dDAVP, 10-7 M) or with BRL37344 (BRL, 10µM). BRL37344 was also incubated after preincubation either with the β3-AR-selective antagonists (L748,337, 10-7 M) or with the PKA inhibitor (H89, 10-5 M). Slices were treated as described above, fixed and ultra-thin sections were stained for AQP2 and β3-AR, and subjected to confocal microscopy. BRL37344 was as effective as dDAVP in promoting AQP2 expression at the apical plasma membrane of cortical and outer medullary collecting duct cells (white arrows), compared with the intracellular localization of AQP2 observed in untreated samples (resting) or samples incubated with BRL37344 after preincubation wit L748,337 or H89 (white arrowheads). (Bar=15µm)

C) Kidney slices were treated as above, then lysed and total protein extracts subjected to Western blotting analysis using the anti pNKCC2 and the anti total NKCC2 antibodies. D) Densitometric analysis showed a five-folds increase of pNKCC2 (normalized to total NKCC2) in samples treated with BRL37344 or dDAVP and the effect of BRL37344 was significantly prevented by L748,337 and H89. Data are provided as mean±SEM and expressed as % of the resting condition. Significant differences between means were tested by One-way analysis of variance ANOVA with Newman-Keuls's post-test. ***p<0.001
Comparable results were obtained in three different mice.

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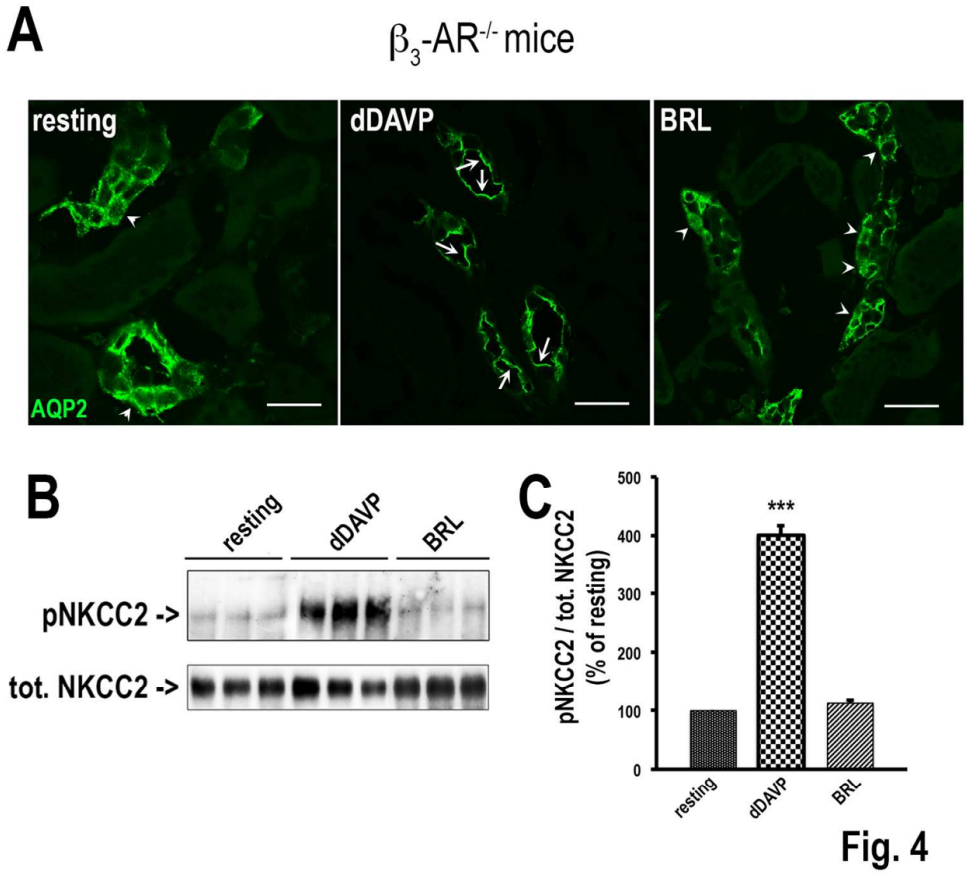

Figure 4. BRL37344 failed to induce AQP2 apical expression and NKCC2 phosphorylation in the kidney of β3-AR-null mice.

A) Freshly-isolated kidney slices (250 µm) where were obtained from β3-/-AR mice, maintained in CO2-equilibrated culture medium at 37°C, left untreated (resting) or incubated with desmopressin (dDAVP, 10-7 M) or with BRL37344 (BRL, 10µM). Slices were fixed and ultra-thin sections (5 µm) were stained for AQP2 and subjected to confocal laser-scanning microscopy. In β3-/-AR mice BRL37344 was unable to promote AQP2 expression at the apical plasma membrane of cortical and outer medullary collecting duct cells. dDAVP was used as internal control to promote AQP2 apical expression. (Bar=10µm) B) Slices were also lysed and protein extracts subjected to Western blotting analysis with anti pNKCC2 and total NKCC2 antibodies. C) Densitometric analysis of pNKCC2, normalized to total NKCC2, showed that in β3-/-AR mice BRL37344 was unable to increase NKCC2 phosphorylation compared to dDAVP. Data are provided as mean±SEM and expressed as % of the resting condition. Significant differences between means were tested by One-way analysis of variance ANOVA with Newman-Keuls's post-test. ***p<0.0001

Comparable results were obtained in three different mice.

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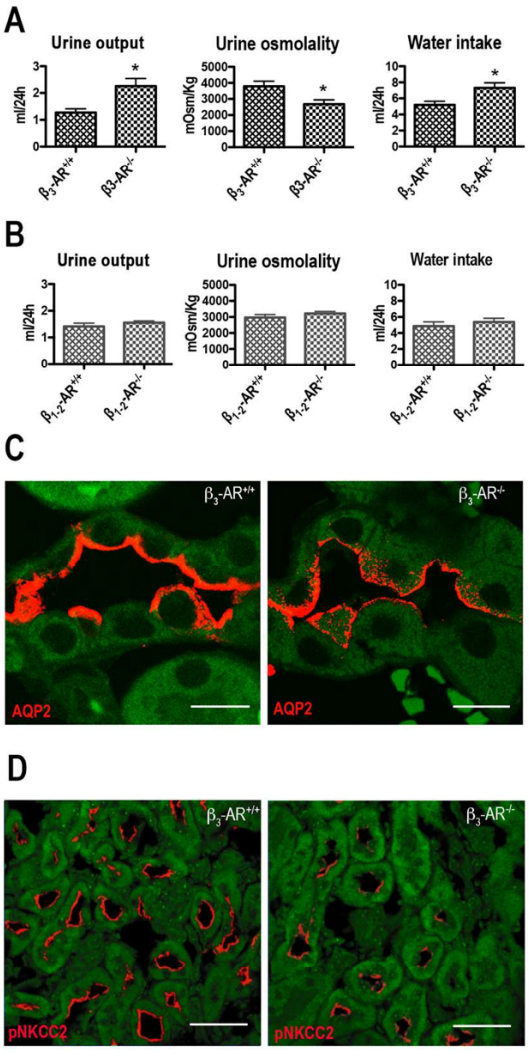

Fig. 5

Figure 5. Mice lacking functional expression of β3-AR showed mild polyuria and reduced urine osmolality. A) β3-AR-null mice (β3-AR-/-) and their age-matched controls (β3-AR+/+) (N=8 for each group), were individually housed in metabolic cages for 5 days and 24h urine output, urine osmolality and water intake measured daily. The analysis reports the mean values ± SEM relative to 24h urine collection. In β3-AR-/- mice urine output was nearly 77% higher, urine osmolality 30% lower and water intake 41% higher compared with control β3-AR+/+ mice. Statistical analysis was performed by unpaired t-test. *$p<0.05$. B) The same experimental protocol was applied to β1-2-AR-null mice (β1-2-AR-/-) and their age-matched controls (β1-2-AR+/+) (N=8 for each group). No statistically significant difference was observed on urine parameters and water intake between the two experimental groups. C) Immunofluorence analysis showed that β3-AR-/- mice have reduced plasma membrane expression and higher subapical localization of AQP2, compared to control β3-AR+/+ mice. (Bar=10μm) D) Immunofluorescence analysis with the anti pNKCC2 showed also that β3-AR-/-mice have reduced levels of activated NKCC2 (pNKCC2). (Bar=30μm)

Comparable results were obtained in three different mice.

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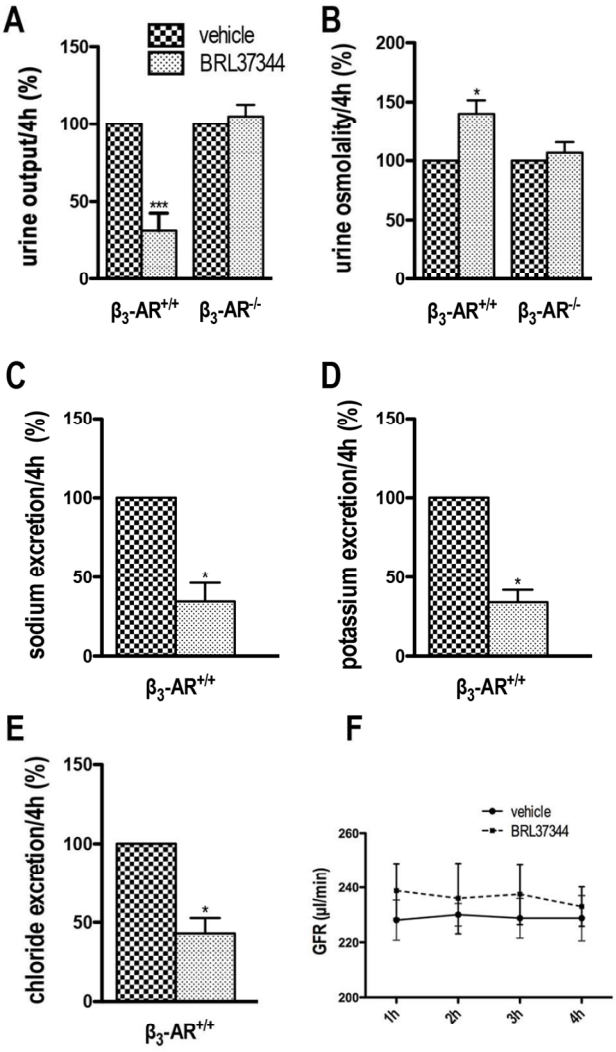

Fig. 6

Figure 6. Effect of β3-AR stimulation on urine concentrating ability in β3-AR+/+ mice.
β3-AR-null mice (β3-AR-/-) and their age-matched controls (β3-AR+/+) (n=10 for each genotype), were individually acclimatized in metabolic cages for 48h then 5 for each group received a single i.p. injection of BRL37344 (0,6 mg/kg) while 5 control animals received PBS alone (vehicle). Urine samples were collected for 4h after injection. A) urine output and B) urine osmolality measured in β3-AR+/+ and β3-AR-/- expressed as percent of control values measured in vehicle-injected animals ±SEM. Urine output of β3-AR+/+ animals was reduced of about 70% and urine osmolality increased of about 40% following BRL37344 injection. No significant effect was seen in β3-AR-/- mice. Significant differences between means were tested by One-way analysis of variance ANOVA with Newman-Keuls's post-test. *P<0.05, ***P<0.0001

C,D,E) Urine excretion of Na+, K+, Cl-, normalized for the urine volume, measured in β3-AR+/+ mice. Data are reported as percent of the values measured in vehicle-injected animals ± SEM. Significant differences between means were tested by Mann-Whitney test. *P<0.05.

F) GFR of β3-AR+/+ mice conscious mice was measured at 1, 2, 3 and 4h after injection of BRL37344 or vehicle alone. No significant difference was seen, at each time point, between BRL37344- and vehicle-injected mice. Significant differences between means were tested by two-way ANOVA with Bonferroni post-test.

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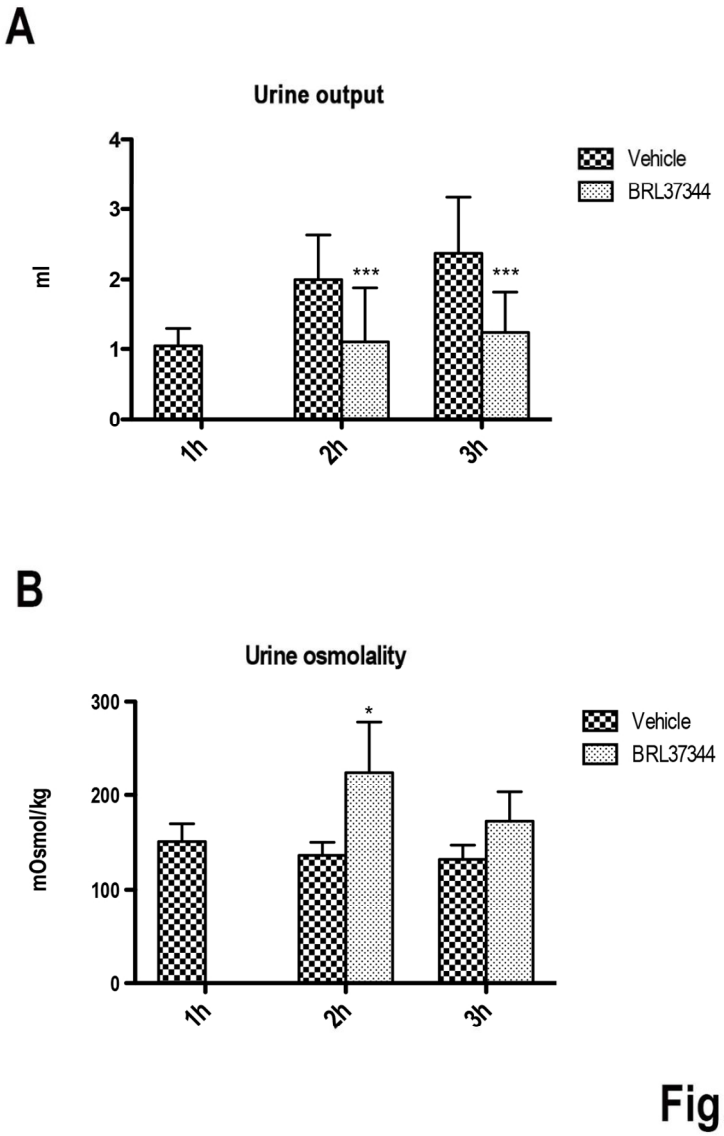
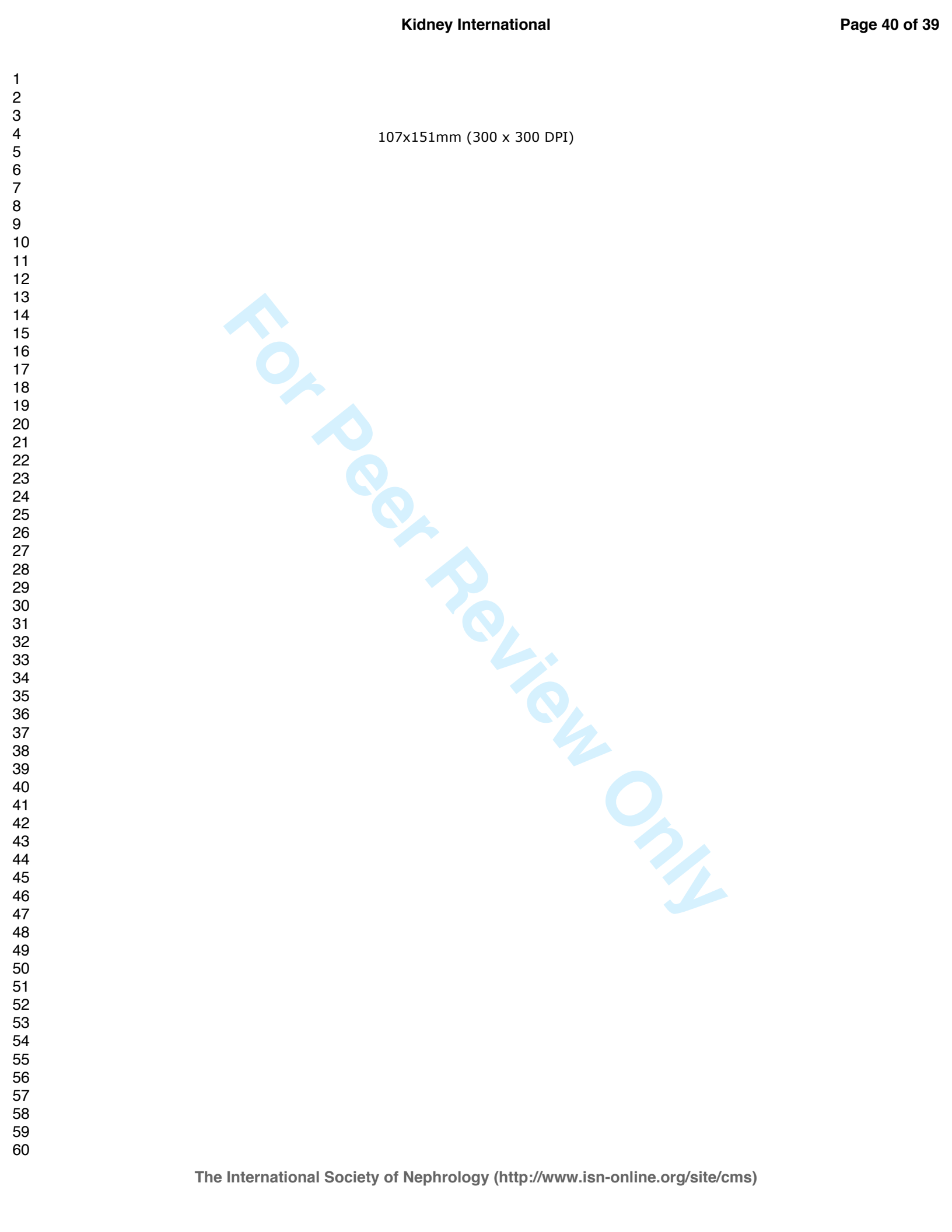


Fig. 7

Figure 7. β3-AR stimulation promotes antidiuresis in mice lacking functional expression of the AVPR2
TenV2Rfl/yEsr1-Cre mice were acclimatized into mice metabolic cages for 48h then 5 received a single i.p. injection of BRL37344 (0,6 mg/kg) while 5 control animals received PBS alone (vehicle). Urine samples were collected every hour for 3 hours from both groups and (A) urine output and (B) urine osmolality at each time point reported.
One hour after the injection, the urine output of treated mice (BRL37344) was reduced to zero compared to vehicle-injected animals (vehicle) (Diuresis, 1h). Two hours after injection, urine output of treated-mice was still dramatically reduced compared to control animals (Diuresis, 2h). Urine osmolality increased in BRL37344-injected animals (Urine osmolality, 2h). At 3 hours post injection, the effect BRL on the urine output was still persistent (Urine output, 3h), while that on urine osmolality partially reversed (Urine osmolality, 3h). The analysis reports the mean values ± SEM.
Significant differences between measurements were tested by two-way ANOVA with Bonferroni post-test for diuresis and by one-way ANOVA with Bonferroni post-test. *p<0.05; ***p<0.001



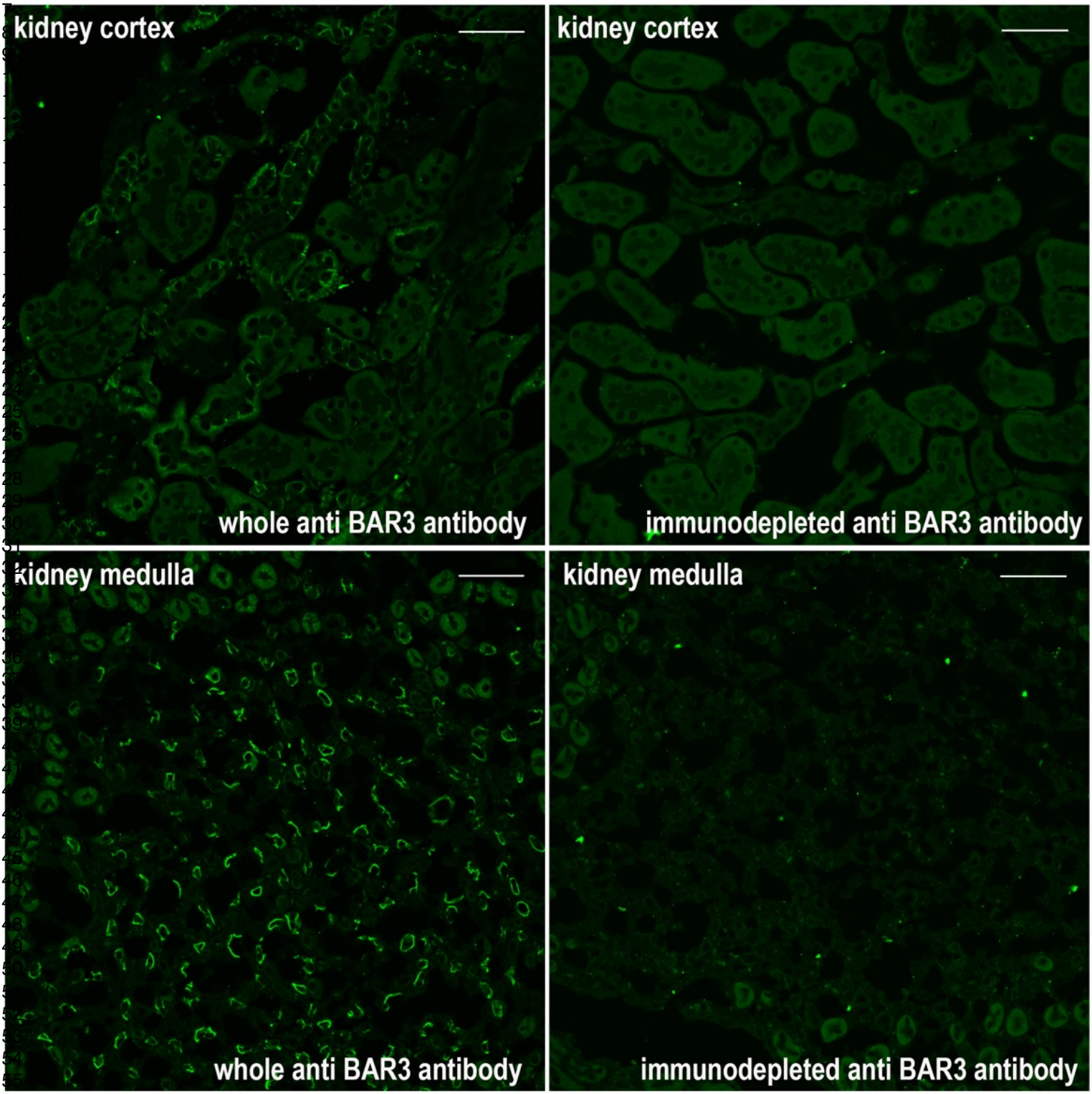


Fig.1 Supplemental

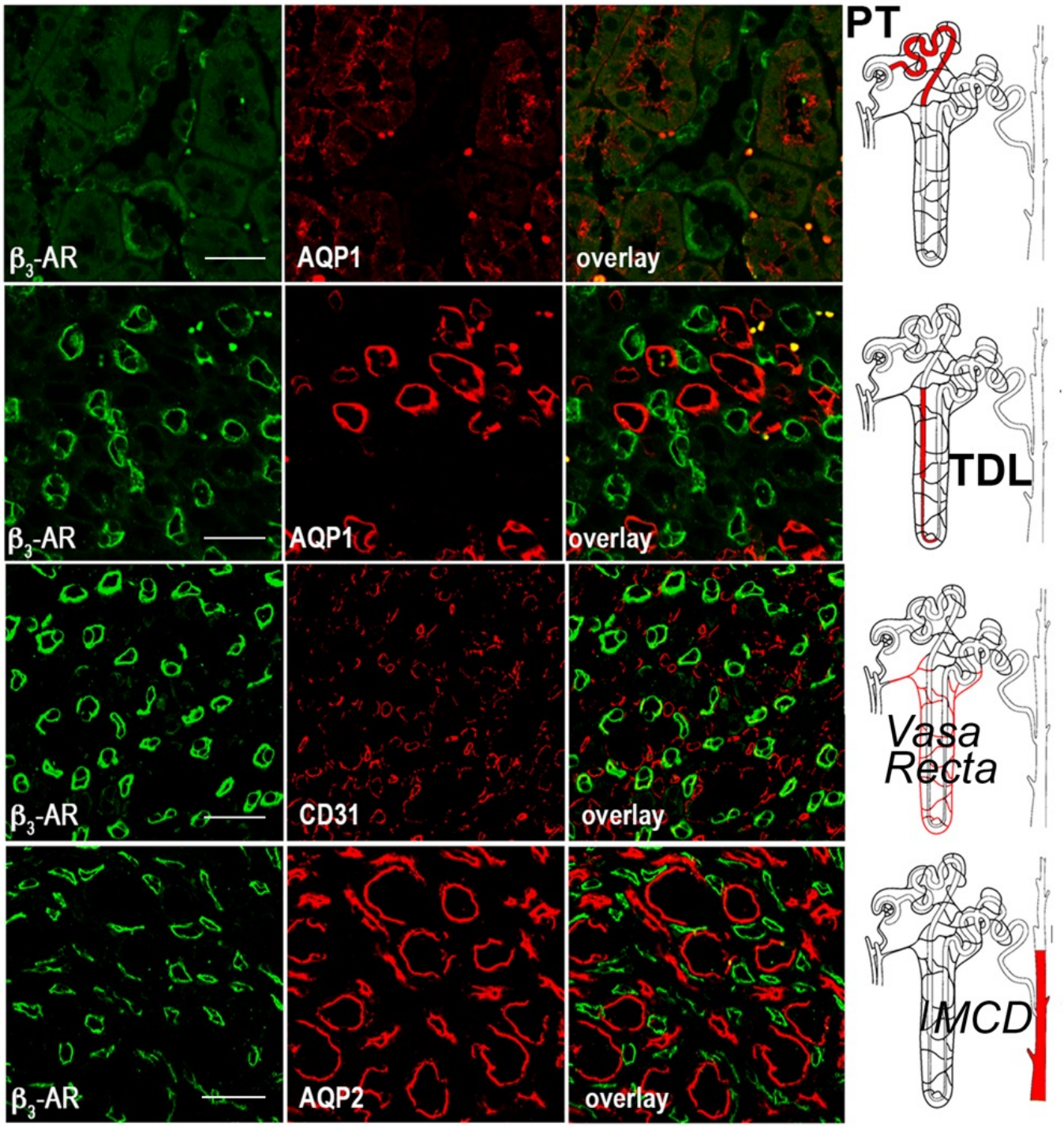


Fig. 2 supplemental

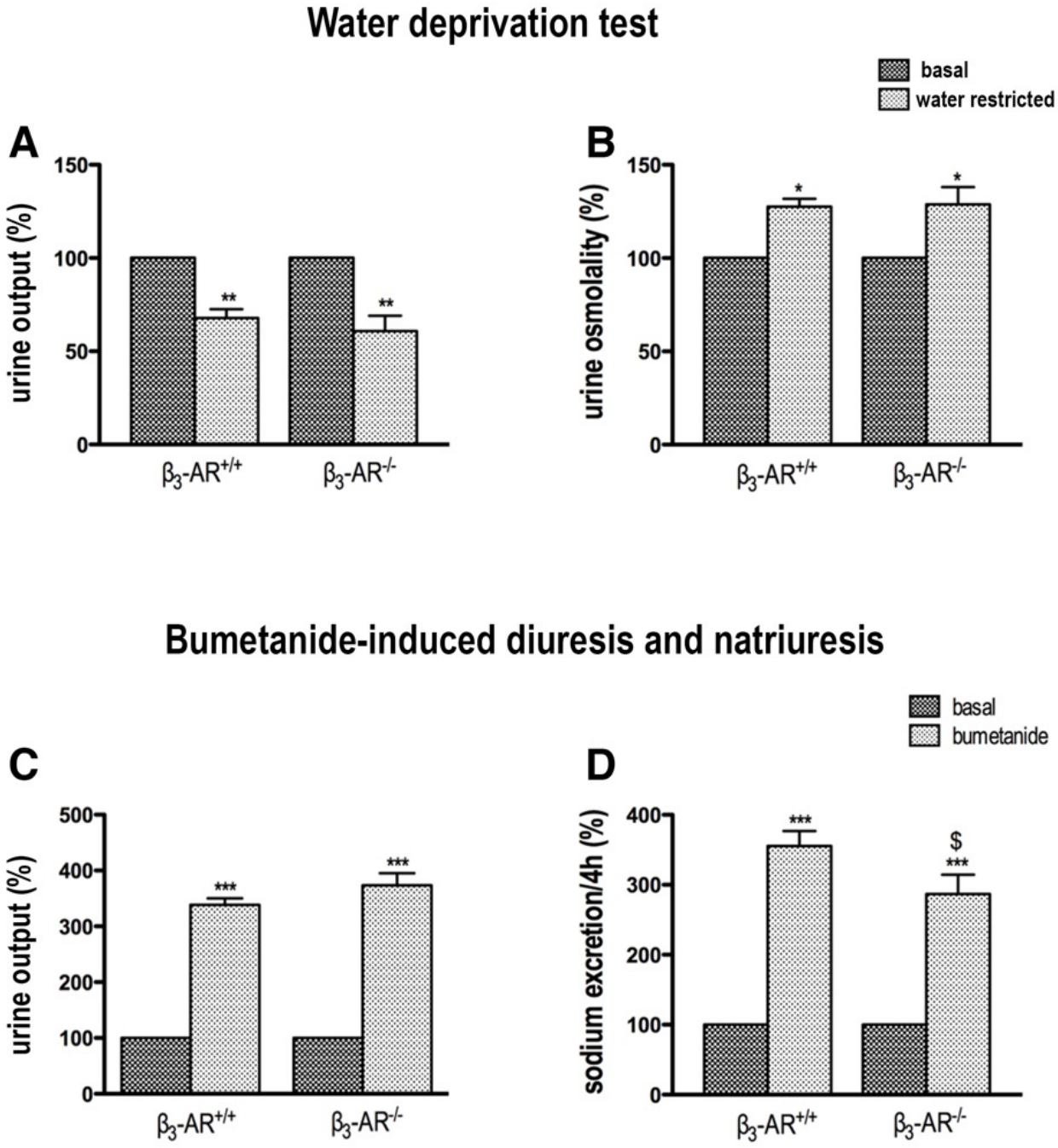


Fig.3 Supplemental

Legends to Supplemental Figures

Fig. 1. Pre-adsorption of anti β_3-AR antibody on its immunizing peptide completely abolished the immunostaining of β_3-AR in mouse kidney sections.
Goat antiβ_3-AR (#SC1473) was preadsorbed on its immunizing peptide (#SC1473p) and used to immunostained paraffin-embedded mouse kidney sections. Compared to whole antibody, immunodepleted antibody failed to detect β_3-AR-positive tubule in both kidney cortex and medulla. (Bar=50µm)

Fig.2. Immunolocalization of β_3-AR in mouse kidney.
Paraffin-embedded kidney sections (C57BL6/J, wt) were immunostained with anti β_3-AR antibodies (green) and co-stained with antibodies against specific markers of different segments of the kidney tubule or vasculature: AQP1 for the proximal tubule (PT) and the thin descending limb (TDL), AQP2 for the inner medullary collecting duct (IMCD) and CD31 for the endothelium of *Vasa Recta* (all in red). Overlay of the each double staining experiment indicated that β_3-AR was neither expressed in the PT nor in the TDL nor in the IMCD nor in the *Vasa Recta*. Drawings of the nephron on the right column indicated in red the β_3-AR-negative tubule or vascular portions. β_3-AR was expressed at the basolateral plasma membrane in all the tubules where it is expressed. Same results were obtained in at least 5 animals. (Bar=20µm)

Fig. 3 Water deprivation test and bumetanide-induced diuresis and natriuresis.
β_3-AR-null mice (β_3-AR$^{-/-}$) and their age-matched controls (β_3-AR$^{+/+}$) (N=8 for each group), were individually housed in metabolic cages for 24h, then 4 animals per group were subjected to water deprivation for 24 hours, while 4 animals had free access to water (basal). The 24h urine output **(A)** and urine osmolality **(B)** of control animals were set as 100%. Urine output of water-deprived animals was reduced of 32% in β_3-AR$^{+/+}$ mice and of 40% in β_3-AR$^{-/-}$ mice. Urine osmolality of water-deprived animals was increased of 27% in β_3-AR$^{+/+}$ mice and of 28% in β_3-AR$^{-/-}$ mice. Data are provided as mean±SEM. Significant differences between means were tested by one-way analysis of variance ANOVA with Newman-Keuls's post-test. **p<0.001, *P<0.05 No significant interstrain differences were observed.
Urine output **(C)** and natriuresis **(D)** after the i.p. injection of vehicle or 40 mg/Kg bumetanide for 4h in β_3-AR$^{+/+}$ and β_3-AR$^{-/-}$ mice. The 4h urine output and urine Na^+ excretion of control animals was set as 100%. (n=5). Data are provided as mean±SEM. Significant differences between means were tested by one-way analysis of variance ANOVA with Newman-Keuls's post-test. ***P <0.0001 for intrastrain differences between vehicle and bumetanide treatments; §P<0.01 for interstrain differences in the effects of bumetanide. The bumetanide-induced natriuresis was significantly attenuated in β_3-AR$^{-/-}$ mice compared with β_3-AR$^{+/+}$.