Orphan Transporter SLC6A18 Is Renal Neutral Amino Acid Transporter B0AT3

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Abstract

The orphan transporter Slc6a18 (XT2) is highly expressed at the luminal membrane of kidney proximal tubules and displays approximately 50% identity with Slc6a19 (B(0)AT1), which is the main neutral amino acid transporter in both kidney and small intestine. As yet, the amino acid transport function of XT2 has only been experimentally supported by the urinary glycine loss observed in xt2 null mice. We report here that in Xenopus laevis oocytes, co-expressed ACE2 (angiotensin-converting enzyme 2) associates with XT2 and reveals its function as a Na(+)- and Cl(-)-dependent neutral amino acid transporter. In contrast to its association with ACE2 observed in Xenopus laevis oocytes, our experiments with ace2 and collectrin null mice demonstrate that in vivo it is Collectrin, a smaller homologue of ACE2, that is required for functional expression of XT2 in kidney. To assess the function of XT2 in vivo, we reanalyzed its knock-out mouse model after more than 10 generations of backcrossing into C57BL/6 background. In addition to the previously published glycminuria, we observed a urinary loss of several other amino acids, in particular beta-branched and small neutral ones. Using telemetry, we confirmed the previously described link of XT2 absence with hypertension but only in physically restrained animals. Taken together, our data indicate that the formerly orphan transporter XT2 functions as a sodium and chloride-dependent neutral amino acid transporter that we propose to rename B(0)AT3.
Orphan transporter SLC6A18 is renal neutral amino acid transporter B0AT3

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The orphan transporter Slc6a18 (XT2) is highly expressed at the luminal membrane of kidney proximal tubules and displays ~50% identity with Slc6a19 (B0AT1) which is the main neutral amino acid transporter in both kidney and small intestine. As yet, the amino acid transport function of XT2 had only been experimentally supported by the urinary glycine loss observed in xt2 null mice. We report here that in Xenopus laevis oocytes, co-expressed angiotensin-converting enzyme 2 (ACE2) associates with XT2 and reveals its function as a Na+- and Cl--dependant neutral amino acid transporter. In contrast to its association with ACE2 observed in Xenopus laevis oocytes, our experiments with ace2 and collectrin null mice demonstrate that in vivo it is Collectrin, a smaller homologue of ACE2, that is required for functional expression of XT2 in kidney. To assess the function of XT2 in vivo, we re-analysed its knock-out mouse model after more than 10 generations of backcrossing into C57BL/6 background. In addition to the previously published glycinuria, we observed a urinary loss of several other amino acids, in particular beta-branched and small neutral ones. Using telemetry, we confirmed the previously described link of XT2 absence with hypertension, but only in physically restrained animals. Taken together, our data indicate that the formerly orphan transporter XT2 functions as a sodium and chloride dependent neutral amino acid transporter that we propose to rename B0AT3.

The SLC6 family is composed of a variety of transporters that transfer small organic solutes such as neurotransmitters and amino acids across the plasma membrane. These substrates are co-transported with sodium and in many cases also with chloride. The substrates transported by most of the different family members have been identified and include neurotransmitters ((nor)epinephrine, dopamine, serotonin, GABA), osmolytes (taurine, creatinine) and amino acids. Despite this extensive characterisation, no data directly demonstrating the function of XT2 (Slc6a18) in an expression system has been reported. In particular, the expression of XT2 in Xenopus laevis oocytes and cultured mammalian cells failed to result in uptake of a variety of tested substrates (1,2).

However, XT2 exhibits approximately 50% identity to B0AT1 (Slc6a19), the Na⁺ cotransporter of neutral amino acids the defect of which causes Hartnup disorder. The XT2 gene is arranged in
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tandem with that of B₀AT1 (Slc6a19) on chromosome 5 in human and chromosome 13 in mouse, and thus presumably arose by gene duplication. A cloning study identified, besides the A12 isoform that resembles most the other Slc6 family members, five shorter transcripts the physiological significance of which is not established (1). In a localization study performed using mouse tissues, we have shown that XT2 is mainly expressed in the kidney, where it localises to the brush border membrane of the late proximal tubule (S2, S3), in a complementary fashion to B₀AT1 that localises to the early proximal tubule (S1) (3).

A xt2 null mouse model was generated and analyzed previously (2). The high amounts of glycine found in the urine of these mice supported the hypothesis that the orphan gene product XT2 functions as an amino acid transporter. Measurements of uptake in brush border membrane vesicles additionally demonstrated its function as a high affinity transport system of glycine. Surprisingly, the xt2 null mice displayed a systolic blood pressure that was 15 - 20 mmHg higher than that of their wild-type littermates, a difference that was abolished upon glycine supplementation in drinking water. Such an impact of XT2 on blood pressure was however not confirmed in human, where a SNP within the SLC6A18 gene present in 46.7% of a general Japanese population corresponds to a nonsense mutation (Y319X) presumably leading to a loss of function, and is not associated with hypertension (4).

We have recently demonstrated that in the proximal kidney tubule, the expression of Slc6 B₀ cluster amino acid transporters requires their association with Collectrin (Tmem27, Coll), a membrane protein homologous to the membrane anchor domain of the renin angiotensin system enzyme ACE2 (5). Furthermore, we have shown that the enzyme ACE2, highly expressed in kidney and intestine, plays the role of associated protein in small intestine for luminal Slc6 amino acid transporters (6).

The first aim of the present study was therefore to retest the function of XT2 in the X. laevis expression system using co-expression of both the kidney and the small intestine associated proteins Coll and ACE2, and second, to retest the impact of the absence of XT2 in the xt2 null mouse model after backcrossing it more than 10 times into C57BL/6 background. It appears, based on telemetric measurements, that XT2 is involved in blood pressure control only under stress conditions in the C57BL/6 background. We show in this study that the product of Slc6a18 called XT2 is a Na⁺- and Cl⁻-dependent neutral amino acid transporter and displays, compared to B₀AT1, a lower Kₐ₅ and a different substrate selectivity. Because of its broad transport selectivity for neutral amino acids we suggest to rename it B₀AT3.

**Experimental Procedures**

cRNA preparation of mouse XT2, Collectrin and ACE2 - The constructs of the A12 isoform of mouse XT2-KSM, Collectrin-pcDNA3.1Hygro and ACE2-pcDNA3 were linearized and used as template for RNA synthesis (mMESSAGE mMACHINE, Ambion, Austin, Texas, USA).

Transport studies in X. laevis oocytes - Expression studies and uptake measurements in X. laevis oocytes were performed as described previously (7). Ten minutes uptakes were performed with buffer containing 0.1 mM of the corresponding L-AA (2 uCi ¹⁴C-L-AA / ml or 20 uCi ³H-L-AA / ml). All experiments were done in buffer containing NaCl (100 mM) or NMDG-Cl (100 mM) for ion dependency experiments. Chloride was substituted by corresponding gluconate (Glc) salts. Kinetics experiments were performed with increasing concentrations of L-Ile (0.01, 0.03, 0.1, 0.3, and 1 mM) or L-Gly (0.01, 0.1, 0.3, 1, and 3 mM), in the presence of NaCl. Data is expressed in pmol / h / oocyte and values obtained for non-injected oocytes are subtracted. Multiple comparisons within groups were performed by repeated-measures one way ANOVA, followed by Tukey post test.

**Animals** - The xt2, ace2 and collectrin wild-type and knock-out mice were housed in standard conditions and fed a standard diet. Generation of the knock-out mice was described elsewhere (2,5,8). All procedures for mice handling were according to the Swiss Animal Welfare laws and approved by the Kantonales Veterinäramt Zürich.

**Metabolic Cages** - Animals were adapted to metabolic cages (Tecniplast, Buguggiate, Italy) for 3 days before data collection, where they had free access to standard mouse diet (18.5% crude fat, 20% protein, 61.5% carbohydrate) and water.
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protein, Kliba-Nafag, Kaiseraugst, Switzerland) and drinking water. After a first day of data collection in standard conditions, the diet was either switched to low protein (<0.5% crude protein, Kliba-Nafag) for two days or water access was removed for 24 h. Daily food/water intake, urine/feces output and body weights were measured. Urinary pH was measured using a pH microelectrode (691 pH-meter, Metrohm). Urinary creatinine was measured by the Jaffe method (9). Urinary electrolytes (Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻, SO₄²⁻) were measured by ion chromatography (Metrohm ion chromatograph, Herisau, Switzerland).

**Amino acid analysis** - Mouse blood was collected by decapitation and 1 μl of heparin-Na⁺ 25000 I.E./5 ml (B. Braun, Melsungen, Germany) was added. Plasma was collected after centrifugation at 5900 g and 4°C. Ice-cold methanol deproteinization of the plasma was performed as described elsewhere (10). Hundred microliters of deproteinised sample was dried and resuspended in 45 μl of borate buffer. Samples were then derivatized using AccQ Tag (Waters, Milford, USA) and analyzed on an Acquity UPLC (Waters) according to the manufacturers instructions by the Functional Genomics Center Zurich (FGCZ) (11).

One microlitre mouse urine collected over 24 h was diluted up to 100 μl by 50 mM HCl containing the internal standards norvaline and sarcosine in a concentration of 50 pmol/μl. The solutions were centrifuged, and transferred into new hydrolysing tube. One microlitre was injected for pre-column derivatization with ortho-phthaldialdehyde (OPA) and analyzed on an Amino Quant amino acid analyzer (Agilent Technologies GmbH, Böblingen, Germany) at the Functional Genomics Center Zurich (FGCZ).

**RNA and real time qRT-PCR** - RNA was extracted from frozen kidneys and real time qRT-PCR was performed as previously described (3). Three samples per mouse and genotype were run and the abundance of the target mRNAs was calculated relative to Hypoxanthine Guanine Phosphoribosyltransferase (HPRT) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference. Relative expression ratios were calculated as R = 2^(Ct(reference) – Ct(test)); Ct: cycle number at the threshold, test: tested mRNAs. Primers and probes were chosen as described elsewhere for B₀AT1 (3), XT3 (3), SIT1 (3), XT2 (3), ACE2 (12), Coll (5), GAPDH (3) and HPRT (13).

**Protein Preparation and Western blot Analysis** - Oocyte homogenization and protein preparation was done as described previously (14). Surface labelling of oocytes expressing XT2 alone or co-expressed with ACE2 or Collectrin using MTSEA-Biotin (Sigma-Aldrich, Buchs Switzerland) and streptavidin precipitation were performed as previously described (15). For immunoprecipitation, the lysate equivalent to 8 oocytes was coupled to 25 μl of anti-XT2 serum (3) in EBC solution (20 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP40) for 8 h at 4°C on a rotator. The immunocomplexes were coupled to Immobilized Protein A/G beads (Pierce, Rockford, USA) O/N at 4°C on a rotator. The beads were washed 6 times with NET-N solution (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% NP40, 1 mM EDTA), and the immunoprecipitate was eluted, denatured and reduced by heating at 95°C for 5 min in Laemmli Buffer. The equivalent of 2.4 oocytes was analysed by Western blotting. Brush Border Membrane Vesicles (BBMV) were prepared from whole mouse kidney using the Mg²⁺ precipitation technique as described elsewhere (15). Immunoblotting of the equivalent of 1 oocyte or 20 μg of BBMVs was performed as described elsewhere (3). Primary antibodies were diluted (1:2000) for rabbit Affinity Purified (AP) anti-B₀AT1 (3) / anti-XT2 (3) / anti-XT3 (3), (1:2000) for guinea-pig serum anti-Coll (antigen peptide: NH2-VQSAIRKNRNRINSAC-CONH2 as in (16), Pineda, Berlin, Germany), (1:1000) for AP goat anti-mACE2 (R&D Systems, Minneapolis, USA), and (1:10000) for mouse anti-mb-actin (Sigma, St Louis, USA). Secondary antibodies were diluted (1:5000) for ECL™ Anti-rabbit IgG and Anti-mouse IgG Alkaline Phosphatase Conjugate (Promega). Antibody binding was detected with Immobilon Western Chemiluminescent HRP or AP substrate (Millipore, Billerica, USA). Chemiluminescence was detected with a DIANA III camera (Raytest, Dietikon, Switzerland). For quantification relative
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Organ fixation - Mice were anesthetized with ketamine and xylazine (90 mg/kg body weight, Narketan 10, Vétoquinol, Lure, France) and Xylazine (10 mg/kg body weight, Xylazin, Streuli, Uznach, Switzerland) intraperitoneally and perfused through the left cardiac ventricle with phosphate-buffered saline (PBS, 0.9% NaCl in 10 mM phosphate buffer, pH 7.4) followed by a buffered paraformaldehyde solution (4%, pH 7), as previously described (17). Kidneys were then harvested, incubated overnight in paraformaldehyde solution, washed several times with PBS and stored in PBS-0.02% sodium azide at 4°C. Tissues were then mounted with Kryostat OCT (Medite, Nunningen, Switzerland), frozen in liquid propane and stored at -80°C.

Immunofluorescence - Immunofluorescence was performed as previously described (3). Primary antibodies were diluted (1:200) for rabbit Affinity Purified (AP) anti-mXT2 (3), (1:100) for AP goat anti-mACE2 (R&D), and (1:1000) for rabbit serum anti-mCollectin (antigen peptide: NH2-CDPLDMKGGHINDGFLT-CONH2 as in (16), Pineda). Secondary antibodies were diluted (1:500) for Alexa Fluor 594 donkey anti-rabbit IgG and Alexa Fluor 488 donkey anti-goat IgG (Molecular Probes, Invitrogen, Carlsbad, USA). For actin staining, Texas Red - X phalloidin (Molecular Probes) was diluted (1:500). Digital images were viewed using a Nikon Eclipse TE300 epifluorescence microscope (Nikon Instruments Inc., Melville, USA) equipped with a DS-5M Standard CCD camera (Nikon) and acquired with NIS-Elements (Nikon).

Telemetry measurement of blood pressure - Eight weeks old mice were anaesthetised (see Organ Fixation) and implanted with PA-C10 transmitter (Data Sciences International (DSI), St. Paul, USA) through the carotid artery as previously described and following manufacturer information to place the pressure sensitive tip in the aortic arch (18,19). For analgesia purposes, 100 μl of bupivacain hydrochloride 0.25% (Bucain, DeltaSelect GmbH Pfullingen, Germany) was injected directly in the wound at the end of the procedure and carprofenum (5mg/kg, Rimadyl, Pfizer, New York, USA) was injected subcutaneously every 12 h for 2 days. Twenty-four hours continuous measurements were recorded after 2 weeks of recovery with Dataquest ART version 3.1 and RespiRate (DSI). Systolic, diastolic and mean blood pressure, heart frequency, activity and respiratory rate were analysed separately for the light vs. dark phases and activity=0 vs. activity>0. To mimic tail-cuff manometry stress, mice were restrained during 20 min in a perforated 50 ml Falcon tube, when the data was continuously measured.

RESULTS

Since XT2 has been shown to be associated in kidney proximal tubule with Coll (5), we tested the possibility that XT2 would be expressed functionally in X. laevis oocytes in the presence of this associated protein. XT2 was therefore co-expressed in X. laevis oocytes with Coll and/or its homologue ACE2 and the uptake of L-isoleucine was tested. Unexpectedly, uptake of L-isoleucine was observed only when XT2 was co-expressed with ACE2, whereas no transport was observed when XT2 was expressed alone or together with Coll (Fig. 1A). Furthermore, co-expression of ACE2 and Coll with XT2 did not further increase the transport rate compared to XT2 + ACE2.

The L-isoleucine transport induction upon ACE2 co-expression is accompanied by a slight increase of XT2 surface expression of 1.64 ± 0.39-fold when compared to XT2 alone (Fig. 1B). Furthermore, co-immunoprecipitation of ACE2 from oocyte membranes using XT2 antibody shows that ACE2 interacts with XT2 (Fig. 1C). These results suggest that ACE2 acts as a chaperone for XT2 slightly increasing its surface expression and more strongly its function in X. laevis oocytes. This co-expression renders the measurement of XT2 transport function possible in an expression system for the first time.

XT2 co-expression with ACE2 allowed the characterization of its transport selectivity, showing the transport of a broad range of neutral amino acids (A, M, V, I > G, S, L) (Fig. 2A). Concentration-dependence experiments with L-Ile and Gly revealed a K0.5 of XT2 for these substrates (0.21 mM and 0.53 mM, respectively) that is lower than that of B0AT1 (Figs. 2B-C) (20). In contrast to B0AT1, but similarly to other members of the Slc6 family such as the GABA transporter GAT1 (Slc6a1), the transport of L-Ile by XT2 is not only Na+- but also Cl--dependant as well as pH-
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The Na⁺ dependence of Gly transport is sigmoidal with a Hill coefficient that is higher than one suggesting that more than one sodium molecule is involved (data not shown). The precise ion transport stoichiometry of XT2 could not be investigated by two-electrode voltage clamp due to the low substrate transport rates. To assess the situation of the different partners of XT2 in vivo, we first checked the localisation of XT2, Coll and ACE2 in mouse kidney. As previously shown, XT2 localises to the apical membrane in distal segments of the proximal tubule (S2,S3) and is not present in the initial S1 segment that begins at the glomerulus (3) (Fig. 4A). In contrast, ACE2 and Coll are both localised along the entire proximal tubule, but with opposed gradients increasing towards the distal and proximal tubular ends, respectively (Fig. 4A, Supplemental Table 2). This shows that the main site of ACE2 expression coincides with the localisation of XT2, suggesting that XT2 may interact with ACE2 in the proximal tubule, additionally to its known interaction with Coll (5). To test the role of these two potential associated proteins on XT2 expression in vivo, we used ace2 and coll null mice. At the mRNA level in kidney, XT2 expression was unchanged in coll null mice, as shown previously (5), and in ace2 null mice (Fig. 4B). However, Western blot analysis of kidney brush border membrane proteins showed that XT2 was absent in the coll⁺ mice, in contrast to ace2⁻²⁻ and wild-type mice (Figs. 4C-D). Immunofluorescence performed on kidney sections confirmed that XT2 is lacking in coll⁻⁺ mice proximal tubule, whereas it is normally expressed in ace2⁻⁻ as in wild-type control mice (Figs. 4E-F). Amino acid fractional excretions of ace2⁻⁻ and ace2⁻⁻⁻ mice were compared to assess the impact of ACE2 on XT2 function (Table 1). The results indicate that amino acid handling was not significantly different in the kidney of ace2 null mice compared to wild-type littersmates. In contrast, aminoaciduria was very high in coll null mice, as reported previously (5). Note however that in these mice several other amino acid transporters are affected that contribute to the aminoaciduria. These results confirm that the partner of XT2 in mouse kidney is indeed Coll and not ACE2, despite the fact that the expression gradient of Coll along the proximal tubule is opposed to that of both XT2 and ACE2 and that Coll unlike ACE2 does not allow XT2 function in X. laevis oocytes.

In the original study, xt2 null mice displayed, compared to wild-type littersmates, a surprising elevation of blood pressure as measured by tail
cuff manometry. This difference was then abolished when drinking water was supplemented with glycine (0.1 g/ml) (2). The blood pressure of xt2 null mice was now retested using mice backcrossed more than 10 times into C57BL/6 background and using telemetry (Datasciences). The measurements showed no differences in blood pressure between the xt2−/− and xt2+/+ mice during the light phase when mice were not active (Fig. 5A). Similar results were obtained during the light and dark phases, whether the mice were active or not (data not shown). To test whether the previously measured increase in blood pressure was provoked by the stress of the tail cuff manometry method, telemetry measurements were repeated while physically restraining the mice. Submitting the animals to this stress provoked a higher mean blood pressure in xt2−/− mice than in wild-type littermates (130.8 ± 0.89 mmHg versus 125.3 ± 1.40 mmHg), and also a higher respiratory rate (Fig. 5B). This suggests an increased stress-induced reaction possibly involving a dysregulation of the sympathetic nervous system in xt2−/− mice.

DISCUSSION

The first aim of this study was to directly characterise the function of the orphan transporter XT2 in the X. laevis oocyte expression system using its in vivo associated protein Coll. However, not Coll but only its homologue ACE2 supported the functional expression of XT2 in X. laevis oocytes such that its transport function of neutral amino acids expected from its homology with B0AT1 could be measured. This raised the suggestion that ACE2 might interact with XT2 in mouse kidney in addition to Coll. However, our experiments with the ace2 and coll null mice confirmed that the in vivo partner of XT2 is Coll and not ACE2. Indeed XT2 expression and localisation is normal in the proximal kidney tubule of ace2 null mice and absent in coll null mice. Furthermore, the absence of ACE2 does not affect the reabsorption of amino acids, whereas that of Coll leads to a strong aminoaciduria that is due to the lack of expression of XT2 and of other proximal tubule amino acid transporters (5). These observations suggest that during evolution, Coll took over the role of ACE2 in amino acid transport due to the increasing involvement of the latter as a key player in the renin-angiotensin system. It is however not clear why the restriction of functional XT2 association with ACE2 or Coll is just opposite in X. laevis oocytes as compared to the in vivo situation in kidney. Surprisingly, it appears that the difference observed in X. laevis oocytes is species specific. Indeed, as suggested to us by S. Bröer (personal communication), co-expression of the human Coll ortholog with mouse XT2 generates an uptake similar to that with mouse ACE2 (data not shown). Further experiments will be required to identify potential additional cell-specific players that account for these differences.

The functional expression of XT2 together with ACE2 in oocytes allowed the long sought characterisation of the function of this orphan transporter. Interestingly, its broad transport selectivity for neutral amino acids is clearly different from that of B0AT1. Particularly, whereas beta-branched amino acids are very good substrates for XT2, as it is the case for B0AT1, aromatic amino acids are apparently less efficiently transported. Importantly, L-Ala, L-Met, Gly, L-Ser and L-Cys are also efficiently transported. In contrast to B0AT1 that displays a Na⁺-dependant electrogenic neutral amino acid transport, XT2 is shown here to display a transport mode that is not only Na⁺-, but also Cl⁻-dependant with a K0.5 for its preferred substrates that is lower than that of B0AT1. In that sense, the axial arrangement of XT2 that follows B0AT1 along the proximal tubule is similar to the sequential arrangement of low and high affinity glucose and peptide transporters SGLT2 and 1 and Pept1 and 2, respectively.

To correlate the measured in vitro function of XT2 with its in vivo function we re-analysed the xt2 null mouse model. As the knock-out mice didn’t display compensatory changes of other amino acid transporters of the SLC6 cluster, or of associated proteins, we hypothesise that the urinary phenotype of xt2 null mice is essentially due to the absence of XT2. In accordance to the in vitro results we measured a broad aminoaciduria. Strikingly, there was not only a substantial loss of glycine (60-fold higher than in xt2+/+) but also of L-glutamine (20-fold) and of all other neutral amino acids (Fig. 3). XT2 thus appears to be required for reabsorbing the leftover tubular amino acids that have not been reabsorbed in the early
proximal kidney tubule segments by B0AT1 or the amino acids that would eventually backleak into the late proximal tubule segments through the paracellular route (22). Additionally, XT2 might also be required for the reabsorption of neutral amino acids that the luminal heterodimeric cystine and cationic amino acids transporter b0,+AT-rBAT releases in exchange for its uptake substrates in the tubular lumen. Thereby XT2 would interfere with L-arginine metabolism and nitric oxide (NO) synthesis (23). This latter hypothesis could be an explanation for the slightly decreased L-arginine plasma level and the tendency for elevated blood pressure observed in xt2 null mice.

Interestingly, XT2 had originally been identified in rat kidney as a renal osmotic stress-induced transcript (ROSIT) (24). However, the experiments that we performed in mice could not verify such a regulation (E. Romeo and F. Verrey, unpublished results). This is either due to a species difference or to the less drastic conditions that we used which might not have been sufficient to trigger this regulation.

In their original description, xt2 null mice displayed a significantly elevated blood pressure (2). This was however not observed in the present study after backcrossing these mice 10 times in a C57BL/6 background and using a telemetry system for the blood pressure measurements. Previous reports have already evidenced discrepancies between tail cuff manometry and telemetric blood pressure measurements and also highlighted the importance of the genetic background. In particular, the original xt2 null mouse had Sv129 genetic background, a strain that contains two renin genes and the renal blood flow of which was suggested to be more sensitive toward NO than that of C57BL/6 mice (25). We could however observe in the present study a stress-sensitive hypertension and a stress-induced increase in respiratory rate in xt2 null mice, suggesting a possible impact of the lack of this transporter on regulatory functions of the sympathetic nervous system. Interestingly, plasma L-arginine of xt2-/- mice was slightly lower than in the xt2+/+ mice and L-arginine is the precursor of nitric oxide that, when released by endothelial cells, leads to vasodilatation. Slightly lower circulating and renal levels of L-arginine might therefore induce susceptibility to an increase in blood pressure (26).

The role of XT2 in human seems to be limited since SLC6A18 has not been found associated with iminoglycinuria (R. Kleta, personal communication). Furthermore, a frequent SNP that leads to a stop codon in SLC6A18 has not been linked to increased blood pressure (4). However, it is not excluded that the individual variability in stress-induced blood pressure increase could be linked to the function of this renal neutral amino acid transporter.

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FOOTNOTES

ACE2: Angiotensin-converting enzyme 2
SLC6A18 is neutral amino acid transporter B₀AT3

Coll: Collectrin (Tmem27)
BBMV: Brush border membrane vesicles
HPRT: Hypoxanthine Guanine Phosphoribosyltransferase
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

FIGURE LEGENDS

**Fig. 1.** XT2 and ACE2 functional interaction *in vitro*. A. Co-expression of ACE2 but not of Coll supports the function of XT2 in *X. laevis* oocytes. *X. laevis* oocytes were injected with 10 ng of each cRNA and incubated for 6 days. Na⁺-dependent uptake of L-Ile 0.1 mM (10 min) was assayed. The data represent 16-24 oocytes from 2-4 independent experiments. Bars indicate mean value ± SEM. ***P<0.001. B. Surface-biotinylated proteins of *X. laevis* oocytes injected with XT2 alone, co-expressed with Coll or with ACE2 were analysed by Western blot using anti-XT2 antibody. The data correspond to the means ± SEM (n = 3). C. Total membranes of *X. laevis* oocytes were used for immunoprecipitation with an anti-XT2 antibody and immunocomplexes were analysed by Western blot with anti-ACE2 antibody.

**Fig. 2.** Transport selectivity and kinetics of XT2 co-expressed with ACE2. A. Substrate selectivity of XT2 co-expressed with ACE2 in *X. laevis* oocytes. Na⁺-Cl⁻-dependent uptake selectivity of L-AA (0.1 mM) was assayed for 10 min. Amino acids are abbreviated with the single letter code. B, C. Concentration dependence of the XT2-ACE2 mediated transport in oocytes. Curves corresponding to Michaelis-Menten kinetics were fitted to Na⁺-Cl⁻-dependent uptake of L-Ile (B) and Gly (C). Data represent means of 18-39 oocytes ± SEM from 3-5 independent experiments. D. Transport induced by XT2 co-expressed with ACE2 is Na⁺- and Cl⁻-dependent. Data are means of 13-16 oocytes ± SEM from two independent experiments. E. Transport of L-Ile was not influenced by pH. The transport of L-Ile was assayed in the presence of Na⁺ and Cl⁻ at different pH’s. Means from 8 oocytes ± SEM. Open bars show transport rate of XT2-, black bars of XT2+ACE2- and grey bars of ACE2-expressing oocytes (D, E).

**Fig. 3.** The *xt2* null mice have increased levels of neutral amino acids in urine, and a slight but not significant decrease of plasma glycine (G) and L-arginine (R). Plasma (A) and urine (B) of *xt2*+/− and *xt2*−/− mice was deproteinized and analysed by HPLC. Bars indicate mean values ± SEM, n=7 (A) and n=6 (B) mice per genotype. *P<0.05, **P<0.01, ***P<0.001. ND: not determined.

**Fig. 4.** Lack of XT2 in *coll*−/− but not in *ace2*−/− mouse kidney. A. Immunofluorescence of wild-type kidney co-labelled with XT2 and ACE2, and on a consecutive section co-labelled with Coll and Actin. G: glomerulus. B. Real time RT-PCR of XT2 and Coll on *ace2*+/− and *ace2*−/− total kidney RNA. Bars indicate mean value ± SEM, n=3 mice per genotype. C, D. Kidney brush border membrane vesicles (BBMV) (20 µg) from *coll*+/− and *coll*−/− mice (C) and *ace2*+/− and *ace2*−/− mice (D) were analysed by Western blot using antibodies against XT2, Coll (with a specific signal at 42 kDa, see supplemental data), ACE2, and β-actin (βa). Each lane corresponds to material prepared from one mouse. Immunofluorescence of *coll+* kidney co-labelled with XT2 and ACE2 (E), and *ace2+* kidney labelled with XT2 and on a consecutive section with Coll (F). Immunofluorescence experiments were performed at least three times with similar results for each antibody. Representative images are shown in the figures. As shown also by Western blot in C, D, XT2 expression is lacking in kidney of *coll* null mice, whereas it is expressed in *ace2* null mice.

**Fig. 5.** The *xt2* null mice display a stress-induced increase of blood pressure and respiratory rate. The *xt2*+/− and *xt2*−/− mice were implanted with blood pressure telemetry sensors (DSI). Mean pressure, pulse pressure, heart rate (BPM: beats per minute) and respiratory rate (RCPM: Respiratory Cycle Per Minute) of *xt2*+/− and *xt2*−/− mice during the light period, when the mice were not active (A) or during a 20 min
SLC6A18 is neutral amino acid transporter B0AT3

physical restrain stress (B). Bars indicate mean values ± SEM, n=7 mice per genotype. *P<0.05, **
P<0.01.

Table 1. Fractional urinary amino acid excretion
Fractional excretion was calculated using an estimate of the glomerular filtration rate of 150 μl/min in
females and 240 μl/min in males (21), the measured amino acid concentrations in plasma and urine and
the volume of urine excreted over 24 h. 2ND: not determined. 3Males n=3, females n=3. 4Males n=5.
5Males n=3

|       | 3xt2+/+ | 3xt2-/- | fold change | 4ace2+/y | 4ace2-/- | fold change | 5coll+/y | 5coll-/- | fold change |
|-------|---------|---------|-------------|----------|----------|-------------|----------|----------|-------------|
| G     | 0.53    | 51.37   | 96.25       | 0.73     | 0.51     | 0.69        | 1.95     | 82.13    | 42.19       |
| A     | 0.59    | 4.49    | 7.60        | 0.43     | 0.69     | 1.61        | 0.60     | 56.42    | 94.47       |
| V     | 0.33    | 3.50    | 10.75       | 0.13     | 0.07     | 0.51        | 0.21     | 26.36    | 125.00      |
| L     | 1.23    | 11.71   | 9.50        | 0.45     | 0.25     | 0.56        | 1.55     | 54.56    | 35.22       |
| I     | 2.30    | 8.97    | 3.90        | 0.39     | 0.15     | 0.37        | 0.41     | 36.45    | 88.31       |
| M     | 5.00    | 33.41   | 6.68        | 1.61     | 1.19     | 0.74        | 6.20     | 58.52    | 9.43        |
| S     | 0.47    | 6.66    | 14.07       | 2.48     | 1.77     | 0.72        | 1.99     | 77.69    | 39.00       |
| T     | 1.04    | 7.33    | 7.06        | 0.57     | 0.35     | 0.61        | 1.55     | 114.24   | 73.78       |
| C     | ND      | ND      | ND          | ND       | ND       | ND          | ND       | ND       | ND          |
| P     | 0.33    | 0.59    | 1.79        | 1.71     | 1.13     | 0.66        | 0.46     | 65.93    | 144.59      |
| N     | 2.91    | 6.76    | 2.33        | ND       | ND       | ND          | ND       | ND       | ND          |
| Q     | 0.11    | 2.94    | 26.72       | 0.32     | 0.32     | 1.00        | 0.36     | 63.69    | 177.61      |
| F     | 0.39    | 5.87    | 15.21       | 0.87     | 0.67     | 0.77        | 0.52     | 21.25    | 40.83       |
| Y     | 0.34    | 2.14    | 6.38        | 0.61     | 0.49     | 0.80        | 1.70     | 16.45    | 9.70        |
| W     | 1.97    | 4.24    | 2.16        | 0.30     | 0.39     | 1.30        | 10.99    | 22.78    | 2.07        |
| K     | 1.58    | 2.98    | 1.88        | 0.10     | 0.09     | 0.88        | 2.46     | 4.50     | 1.83        |
| R     | ND      | ND      | ND          | ND       | ND       | ND          | ND       | ND       | ND          |
| H     | 1.74    | 9.66    | 5.56        | 1.25     | 1.11     | 0.89        | ND       | ND       | ND          |
| D     | 2.62    | 4.12    | 1.57        | ND       | ND       | ND          | ND       | ND       | ND          |
| E     | 11.46   | 16.03   | 1.40        | 4.05     | 3.61     | 0.89        | 2.17     | 6.52     | 3.01        |
Figure 1

A

![Graph showing L-Ile production rate per hour/oocyte with conditions: + + + + XT2 ACE2 Coll with ns and *** ns annotations.](image)

B

![Western Blot showing XT2 protein expression with oocytes conditions: + + + + XT2 ACE2 Coll.](image)

C

![Western Blot showing ACE2 protein expression with conditions: + + XT2 ACE2.](image)
Figure 2

A

B

C

D

E

\[
V_{\text{max}} = 477 \pm 44 \text{ pmol/hour} \\
K_{0.5} = 0.21 \pm 0.06 \text{ mM}
\]

\[
V_{\text{max}} = 226 \pm 26 \text{ pmol/hour} \\
K_{0.5} = 0.53 \pm 0.17 \text{ mM}
\]

\[
\text{ns}
\]
Figure 3

A

B
Figure 4

A

ace2<sup>+/y</sup> and coll<sup>+/y</sup>

**XT2**

**Coll**

ACE2

Actin

B

XT2

Coll

C

coll<sup>+/y</sup> and coll<sup>-/y</sup>

**XT2**

βα

ACE2

βα

D

dace2<sup>+/y</sup> and coll<sup>-/y</sup>

**XT2**

βα

Coll

βα

E

coll<sup>-/y</sup>

**XT2**

ACE2

F

dace2<sup>-/y</sup>

**XT2**

ACE2

Coll
Figure 5

A

B

xt2+/+ xt2-/- xt2+/+ xt2-/- xt2+/+ xt2-/- xt2+/+ xt2-/-
Orphan transporter SLC6A18 is renal neutral amino acid transporter B\textsuperscript{0}AT3

**Supplemental Data**

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**Supplemental Table 1.** Summary of metabolic cage and urine data from male and female mice.
Male and female mice were fed a normal protein diet (A. 18.5 %, 2 d), a low protein diet (B. <0.5 %, 2 d) or were deprived of water (C. 24 h) A. Males n=9, females n=8, total n=17. B. Males n=4, females n=5, total n=9. C. Males n=5, females n=3, total n=8. Data indicate means ± SEM. Urinary excretion was normalized to creatinine (crea). BW: body weight. ND: not determined.

|                  | xt2\textsuperscript{+/+} | xt2\textsuperscript{-/-} | xt2\textsuperscript{+/+} | xt2\textsuperscript{-/-} | xt2\textsuperscript{+/+} | xt2\textsuperscript{-/-} |
|------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Males body weight (g) | 25.28±0.39               | 25.62±0.51               | 22.98±0.50               | 23.73±0.33               | 23.36±0.19               | 23.08±0.75               |
| Females body weight (g) | 21.65±0.43               | 21.65±0.39               | 20.28±0.09               | 20.44±0.35               | 19.17±0.82               | 18.93±0.65               |
| Males and females: |                          |                          |                          |                          |                          |                          |
| Food (g/g BW)     | 0.155±0.0075             | 0.158±0.008              | 0.142±0.005              | 0.147±0.003              | 0.110±0.010              | 0.113±0.005              |
| Water (ml/g BW)   | 0.24±0.02                | 0.22±0.01                | 0.45±0.05                | 0.39±0.05                | 0                      | 0                       |
| Feces (g/g BW)    | 0.056±0.004              | 0.053±0.005              | 0.018±0.004              | 0.018±0.002              | 0.032±0.003              | 0.0310±0.002             |
| Urine (ml/g BW)   | 0.031±0.005              | 0.039±0.005              | 0.206±0.026              | 0.162±0.023              | 0.012±0.003              | 0.018±0.003              |
| Creatinine (mM)   | 5.44±0.42                | 5.18±0.34                | 1.40±0.17                | 1.39±0.17                | 6.55±0.61               | 5.49±0.21               |
| Osmolality (mOsm/kg) | 3266±209                | 3079±14                  | 457.3±44.2               | 509.8±47.0               | 4680±356                | 4540±377                |
| pH               | 6.23±0.07                | 6.28±0.12                | 6.22±0.14                | 6.27±0.06                | ND                     | ND                      |
| Na\textsuperscript{+}/crea | 33.08±4.08              | 38.44±4.71               | 81.93±5.20               | 122.9±21.4               | 44.11±7.38              | 41.91±8.60              |
| K\textsuperscript{+}/crea | 112.9±13.0               | 122.2±14.8               | 58.02±5.19               | 78.99±11.29              | 127.8±2.285             | 111.1±11.66             |
| Ca\textsuperscript{2+}/crea | 0.97±0.24               | 1.08±0.22                | 3.15±1.193               | 2.26±0.25                | 0.36±0.07               | 0.28±0.07               |
| Mg\textsuperscript{2+}/crea | 10.25 ± 1.11            | 11.44 ± 1.56             | 8.10 ± 0.73              | 10.19 ± 1.56             | 8.76 ± 0.50             | 9.52 ± 0.73             |
| Cl\textsuperscript{−}/crea | 58.78 ± 1.90            | 60.15 ± 2.55             | 83.54 ± 2.72             | 100.4 ± 12.12            | 135.9 ± 21.12           | 154.5 ± 26.59           |
| PO\textsubscript{4}\textsuperscript{3−}/crea | 9.03 ± 0.64             | 10.05 ± 1.23             | 28.62 ± 1.61             | 30.56 ± 1.58             | 31.50 ± 5.24            | 27.18 ± 4.34            |
| SO\textsubscript{4}\textsuperscript{2−}/crea | 14.34 ± 0.87            | 14.13 ± 0.74             | 4.82 ± 0.48              | 4.83 ± 0.38              | 24.82 ± 2.71            | 28.62 ± 5.06            |
**Supplemental Table 2.** Immunofluorescence quantification in early (S1) and late (S3) segments of the proximal tubule show opposing axial gradients of Coll and ACE2. XT2 and B⁰AT1 were quantified as controls. Brush border staining was quantified (intensity/area) in early proximal tubule (after the narrowing of Bowman’s capsule) and late proximal tubule (long straight segments towards the medulla) with AnalySIS 5 (SoftImaging System, Munster, Germany). A 100% staining was set in the segments with the strongest signal for each antibody. The analysis was performed for 2-4 animals with 2-3 early and late proximal tubule segments per section for each antibody. Picture acquisition conditions were kept constant between the regions of a same kidney section.

| Signal intensity (%) | Proximal tubule | early | late |
|----------------------|-----------------|-------|------|
| Coll                 | 100.00          | 51.19 |
| ACE2                | 56.23           | 100.00|
| XT2                  | 0.00            | 100.00|
| B⁰AT1               | 100.00          | 0.00  |

**Supplemental Fig. 1.** Absence of compensatory regulation of other renal SLC6 amino acid transporters or associated proteins in xt2 null mice. A. Real time RT-PCR of B⁰AT1, XT3 (Slc6a20a) and SIT1 (Slc6a20b) on ace2⁺/⁺ and ace2⁻/⁻ total kidney RNA. Bars indicate mean value ± SEM, n=2 mice per genotype. B. Western blot analysis on kidney BBMV (20 μg) from xt2⁺/⁺ and xt2⁻/⁻ mice using antibodies against B⁰AT1, XT3, ACE2, Coll, and β-actin (βa).

**Supplemental Fig. 2.** Specificity of the guinea-pig serum anti-Coll was tested on coll⁺/⁺ and coll⁻/⁻ BBMVs with and without deglycosylation (PNGase F). A specific signal is detected around 40 kDa. Hydrolysis of Asn-oligosaccharides was carried out using endo-beta-N-acetylglucosaminidase F enzyme (PNGase F) (New England Biolabs, USA).
Supplemental Figure 2

| coll | +/y | +/y |
|------|-----|-----|
| PNGase F | -   | +   | -   | +   |

Coll

- 170
- 130
- 95
- 72
- 55
- 43
- 34
- 26