The heterodimeric amino acid transporter 4F2hc/y$^+$LAT2 mediates arginine efflux in exchange with glutamine

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The cationic amino acid arginine, due to its positive charge, is usually accumulated in the cytosol. Nevertheless, arginine has to be released by a number of cell types, e.g. kidney cells, which supply other organs with this amino acid, or the endothelial cells of the blood–brain barrier which release arginine into the brain. Arginine release in mammalian cells can be mediated by two different transporters, y$^+$LAT1 and y$^+$LAT2. For insertion into the plasma membrane, these transporters have to be associated with the type-II membrane glycoprotein 4F2hc [Torrents, Estevez, Pineda, Fernandez, Lloberas, Shi, Zorzano and Palacin (1998) J. Biol. Chem. **273**, 32437–32445]. The present study elucidates the function and distribution of y$^+$LAT2. In contrast to y$^+$LAT1, which is expressed mainly in kidney epithelial cells, lung and leucocytes, y$^+$LAT2 has a wider tissue distribution, including brain, heart, testis, kidney, small intestine and parotis. When co-expressed with 4F2hc in *Xenopus laevis* oocytes, y$^+$LAT2 mediated uptake of arginine, leucine and glutamine. Arginine uptake was inhibited strongly by lysine, glutamate, leucine, glutamine, methionine and histidine. Mutual inhibition was observed when leucine or glutamine was used as substrate. Inhibition of arginine uptake by neutral amino acids depended on the presence of Na^+, which is a hallmark of y$^+$LAT-type transporters. Although arginine transport was inhibited strongly by glutamate, this anionic amino acid was only weakly transported by 4F2hc/y$^+$LAT2. Amino acid transport via 4F2hc/y$^+$LAT2 followed an antiport mechanism similar to the other members of this new family. Only preloaded arginine could be released in exchange for extracellular amino acids, whereas marginal release of glutamine or leucine was observed under identical conditions. These results indicated that arginine has the highest affinity for the intracellular binding site and that arginine release may be the main physiological function of this transporter.

Key words: antiporter, arginine transport, astrocyte, brain metabolism, glutamine transport.

INTRODUCTION

Recently, a new family of heterodimeric amino acid transporters has been identified (for review see [1]). The heterodimers are constituted of the type-II membrane glycoprotein 4F2hc (CD98) [2,3], which is covalently bound, via a disulphide bridge, to a polytopic membrane protein (also called light chain [4,5]). The transport pore of the heterodimer is most likely constituted of the light chain [6], whereas the 4F2hc protein is necessary for the translocation of the complex into the plasma membrane [4,5,7]. Western-blotting and immunofluorescence studies showed that 4F2hc is located in the plasma membrane in the absence of a light chain, whereas the light chain, when expressed alone, does not reach the surface of the oocyte [4,5]. The expanding family of 4F2hc-linked light chains now include the system L-associated isoforms LAT1 and LAT2, the system y$^+$L-associated isoforms y$^+$LAT1 and y$^+$LAT2 and the glutamate/cystine exchanger xCT [2,8–12].

The transporters of this family are likely to be involved in interorgan and intercellular transfer of amino acids [1,13,14]. In epithelial tissues trafficking of the 4F2hc subunit ensures a basolateral location, where the transporters allow the release of neutral or cationic amino acids into the blood. Since all 4F2hc-associated transporters identified so far display antiport mechanisms upon expression in *Xenopus laevis* oocytes, net transport of amino acids across blood–tissue barriers remains elusive on a molecular level. However, there are inconsistent reports about the ability of the isoform LAT2, which is strongly expressed at blood–tissue barriers [11], to mediate net amino acid transport [10,11,15].

There is increasing evidence for active metabolic traffic between different cell types in the brain. This includes the possible transfer of lactate [16], of branched-chain amino acids and keto acids [14,17] and the transfer of glutamine [18,19] between astrocytes and neurons. The transfer of amino acids between brain cells is thought to contribute to nitrogen balance in the brain without giving rise to the release of neurotoxic ammonia. It is well established that in neurons, synaptosomes and also in astrocytes, transport of leucine and glutamine is partially Na^+-dependent and can be inhibited by arginine [20–23]. These characteristics are typical for y$^+$L-like amino acid transport systems [24]. Expression of the system y$^+$L isoform y$^+$LAT1 is largely confined to the kidney [8,9]. We therefore wondered whether the isoform y$^+$LAT2 might be involved in the transfer of amino acids in the brain, a tissue with high levels of 4F2hc expression [25]. The y$^+$LAT2 cDNA was first deposited as an unknown gene from the myeloblast cell line KG-1 [26] and was identified recently as an amino acid transporter [8,9].

The present study was performed to elucidate a possible involvement of y$^+$LAT2 in the transfer of neutral and cationic amino acids between brain cells. The results indicate that y$^+$LAT2 is a glutamine(leucine)/arginine exchanger which could play a role in the Na^+-dependent uptake of leucine and glutamine into neurons as well as in the supply of arginine for certain brain cells.

Abbreviations used: cRNA, complementary RNA; RT, reverse transcriptase.

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MATERIALS AND METHODS

Materials

L-[U-^{14}C]Isoleucine (12.6 GBq/mmol), L-[U-^{14}C]leucine (11.4 GBq/mmol), L-[4,5-^{3}H]leucine (11.4 GBq/mmol), L-[U-^{14}C]glutamine (8.8 GBq/mmol), L-[U-^{14}C]glutamate (11.4 GBq/mmol) and L-[U-^{14}C]arginine (10.3 GBq/mmol) were purchased from Amersham Buchler, Braunschweig, Germany. The RNA cap structure analogue 7 mG(5′)ppp(5′)G was purchased from New England Biolabs, Schwalbach, Germany. Restriction enzymes, nucleotides and RNA polymerases were from Life Technologies, Eggenstein, Germany. Collagenase A (EC 3.4.24.3; 0.3 units/mg from *Clostridium histolyticum*) was from Roche (Mannheim, Germany); lots were tested for their suitability for oocyte preparation. All other chemicals were of analytical grade and were supplied by E. Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Roche.

Expression in *X. laevis* oocytes

For expression studies the rat 4F2hc subcloned into plasmid pGEM-He-Juel was used [2]. The human y^{+}LAT2 cDNA (originally designated KIAA0245 cDNA; EMBL/GenBank accession no. D87432) was kindly provided by Francois Verrey (University of Zürich, Zürich, Switzerland). For *in vitro* transcription the 4F2hc plasmid DNA was linearized with *Not*I and transcribed *in vitro* with T7 RNA polymerase in the presence of a cap analogue (mMessage mMachine Kit, Ambion, Austin, TX, U.S.A.). The y^{+}LAT2-containing plasmid was linearized with *Pst*I and transcribed *in vitro* with SP6 RNA polymerase in the presence of a cap analogue. The protocol supplied with the polymerase was followed, with the exception that all nucleotides and the cap analogue were used at 2-fold concentrations (1 mM) to increase the yield of complementary RNA (cRNA). Template plasmids were removed by digestion with RNase-free DNase I. The cRNA was purified by phenol/chloroform extraction followed by precipitation with 0.5 vols of 7.5 M ammonium acetate and 2.5 vols of ethanol to remove unincorporated nucleotides. After determination of the amount of cRNA by measuring absorption at 260 nm, the integrity of the transcript was verified by denaturing agarose-gel electrophoresis.

X. laevis females were purchased from the South African *Xenopus* facility (Knysna, South Africa). Oocytes (stages V and VI) were isolated as described in [27] and allowed to recover overnight. They were microinjected with 20 nl of a mixture of 4F2hc and y^{+}LAT2 cRNA in water at a concentration of 1 μg/μl, by using a microinjection device (WPI Instruments, Berlin, Germany). Controls remained uninjected.

Flux measurements

Uptake experiments were performed as described before [27], with the exception that ND96 (96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes; titrated with NaOH to pH 7.4) was used as incubation buffer. In Na^{+}-free incubation buffer NaCl was replaced by *N*-methyl-D-glucamine chloride. The concentration of ^{14}C-labelled compounds was taken into account for the adjustment of final substrate concentrations. For efflux, oocytes were preloaded for 10 min with [^{14}C]glutamine, [^{14}C]arginine or [^{14}C]leucine (0.1 mM). Subsequently, oocytes were washed four times with 4 ml of ice-cold ND96 buffer. To initiate efflux, the washing buffer was aspirated and replaced by ND96 buffer at room temperature which was supplemented with 1 mM of the indicated amino acid. Antiport activity was demonstrated by dual-label counting. For this, oocytes were preloaded with 100 μM [^{14}C]arginine for 2 h to allow equilibration of the specific activity. Extracellular radioactivity was removed by washing oocytes three times with 4 ml of ND96 at room temperature. Subsequently, 1 ml of 100 μM [^{3}H]leucine of similar specific activity was added to the oocytes. At the indicated times samples of seven oocytes were washed three times with ice-cold incubation buffer to remove extracellular radioactivity. The oocyte's [^{14}C]arginine and [^{3}H]leucine contents were determined by scintillation counting using a calibrated quench-corrected dual-label programme.

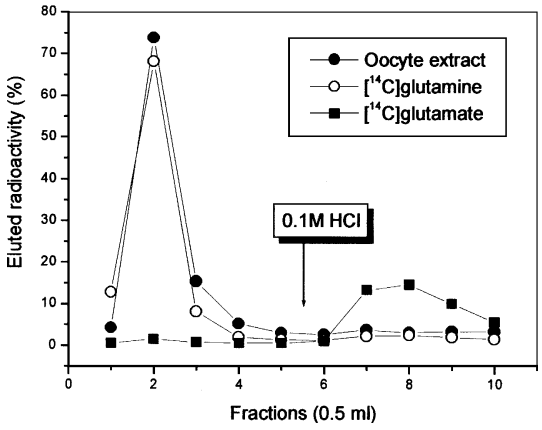

Figure 1 Metabolism of glutamine in 4F2hc/y^{+}LAT2-expressing *X. laevis* oocytes

Oocytes were preloaded with [^{14}C]glutamine for 60 min. Subsequently, oocytes were homogenized and 100 μl of the extract (●) was applied to a Dowex 1 × 8-50 anion-exchange column. Equivalent samples of 100 μM [^{14}C]glutamine (○) and [^{14}C]glutamate (■) were applied to the column for comparison. Bound radioactivity was eluted by flushing with 0.1 M HCl.

Metabolism of [^{14}C]glutamine and [^{14}C]leucine in *X. laevis* oocytes

For efflux experiments oocytes were preloaded with labelled glutamine. To test whether glutamine could have been metabolized during the preloading time, advantage was taken of the fact that primary glutamine metabolites are all anionic compounds (glutamate, α-ketoglutarate, citric acid-cycle intermediates). Ten oocytes were preloaded with 100 μM [^{14}C]glutamine for 60 min. Oocytes were washed three times with ice-cold incubation buffer and subsequently homogenized by trituration in 500 μl of water. The homogenate was centrifuged and the clear supernatant was removed carefully, avoiding contamination with floating lipids.

To separate glutamine from its metabolites, a bed of 2 ml of Dowex 1 × 8-50 anion exchanger was prepared in a Pasteur pipette. The anion exchanger was regenerated by washing the column with 5 vols of 1 M HCl. Subsequently, the column was washed intensively with water to remove the acid. Three samples were applied to the column: (i) a 100-μl aliquot of 100 μM L-[U-^{14}C]glutamine (170 Bq), (ii) a 100-μl aliquot of 100 μM L-[U-^{14}C]glutamate (170 Bq) and (iii) 100 μl of oocyte extract (40 Bq). Labelled glutamate was removed completely, whereas 92 % of the glutamine standard passed through the column unhindered (Figure 1). Bound glutamate could be eluted from the column by washing with 0.1 M HCl. The complete radioactivity of the oocyte extract (100 %) passed through the column as the glutamine standard; no radioactivity could be released by washing with 0.1 M HCl (Figure 1), indicating that glutamine is not significantly metabolized in oocytes.

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Metabolism of branched-chain amino acids was investigated in one of our earlier studies [27] and was found to be negligible.

Cell culture

Astroglia-rich primary cultures were prepared from newborn-rat brains as described in [28]. Briefly, total brains were passed successively through two nylon nets of 211 μm and then of 135 μm mesh width. The suspended cells of a complete litter (10–15 animals) were collected by centrifugation and resuspended in 20 ml of Dulbecco's modified Eagle's medium/10 % fetal calf serum. After determination of the cell number, cells were diluted in Dulbecco's modified Eagle's medium/10 % fetal calf serum to a final density of 200000 cells/ml. Aliquots were dispensed in cell-culture dishes of appropriate size and incubated in a cell incubator at 37 °C in 90 % air/10 % CO_2 for 14–21 days. Medium was renewed every week. For all experiments 14–21-day-old confluent cultures were used. At least 90 % of these cells expressed glial acidic fibrillary protein. The cultures contained limited numbers of oligodendroglial, ependymal and microglial cells, but were devoid of neurons (results not shown).

Neuron-rich primary cultures were derived from embryonal rat brains (E16) as described in [29]. Briefly, embryos were removed from the uteri of pregnant rats after 16 days of gestation. Total brains were passed successively through two nylon nets of 135 μm and then of 20 μm mesh width: 1×10^6 cells were seeded on to poly-D-lysine-coated 35-mm dishes. After 3 days in culture, cells were treated for 24 h with cytosine arabinoside at a final concentration of 0.5 μM to kill dividing cells. Subsequently, cells were incubated in glia-conditioned neuron culture medium [29]. The cultures were used at a culture age of 5–7 days. These cultures contained some astroglial cells but no oligodendroglial or ependymal cells, as determined by cell-specific markers [29].

Reverse transcriptase (RT)-PCR

RT reaction

Total RNA was isolated from different mouse tissues, embryonal rat brain, adult rat brain, astroglia-rich rat primary cultures and neuron-rich rat primary cultures by the 'acid-guanidinium-thiocyanate-phenol-chloroform extraction' method of [30]. For reverse transcription, 0.5 μg of oligo$(dT)_{15}$ was added to 1 μg of total RNA in a total volume of 12 μl. The mixture was incubated for 10 min at 65 °C and then chilled on ice. Dithiothreitol, dNTPs, 5 × RT buffer and 30 units of RNasin (Promega, Madison, WI, U.S.A.) were added and the whole mixture (20 μl) was incubated further at 37 °C for 2 min. For cDNA synthesis, 200 units of Superscript II-RT (Life Technologies) were added followed by incubation at 37 °C for 1 h. The cDNA was purified by using a PCR-product purification kit (Roche).

PCR

A standard PCR protocol with 100 pmol of each primer and a 10-μl aliquot of the purified cDNA was used for amplification of the fragment during 30 cycles (95 °C, 30 s; 50 °C, 45 s; 72 °C, 1 min) in a Thermocycler using *Taq* polymerase (Qiagen, Hilden, Germany). After amplification, the samples were extracted once with chloroform and 20 μl-aliquots were analysed by electrophoresis on a 1 % agarose gel.

For detection of the mouse y+LAT2 mRNA the sense primer 5′-TCAGGCCATCATCGCCATCAC-3′ and the antisense primer 5′-CAGCACTGTGTAAAAGGCCAC-3′ were used to amplify a specific fragment of 463 bp. The partial mouse y+LAT2 sequence was derived from two overlapping mouse expressed sequence tags (EMBL/GenBank accession numbers D76889 and AV047005), which encode homologues of the human y+LAT2 sequence. To verify that the mouse or rat y+LAT2 cDNA was amplified, representative bands were isolated from agarose gels, cloned into plasmid pCR-Blunt (Invitrogen, Groningen, The Netherlands) and analysed by dye-terminator sequencing (IFKZ, Sequencing Facility, University of Tübingen, Tübingen, Germany).

Electrophysiology

Two-electrode voltage-clamp experiments were performed 15–48 h after injection of the cRNA at room temperature using a Geneclamp 500 amplifier and a MacLab D/A converter. Experiments were normally performed at a holding potential of −50 mV if not otherwise stated. Data were filtered with 100 Hz. ND96 was used as the external control solution. The flow rate of the solution was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s.

Calculations

All data are given as means ± S.E.M., with n indicating the number of experiments. All measurements were performed with equal numbers of cRNA-injected and non-injected oocytes. In experiments with labelled compounds, all values represent net uptake rates calculated as: (mean uptake rate of seven cRNA-injected oocytes) − (mean uptake rate of seven non-injected oocytes) = (mean net uptake rate) using Gauss' law of error propagation for the calculation of the final S.E.M. values. Each experiment presented was performed at least twice with similar results.

RESULTS

To investigate the function of y+LAT2 as an amino acid transporter we co-expressed its cRNA (10 ng per oocyte) with

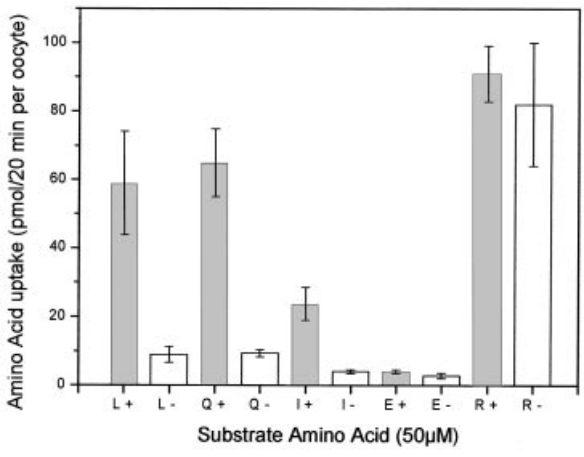

Figure 2 Amino acid uptake by 4F2hc/y+LAT2-expressing *X. laevis* oocytes

X. laevis oocytes were injected with 10 ng of each subunit. After a 2-day expression, uptake of [^{14}C]leucine (L), [^{14}C]isoleucine (I), [^{14}C]glutamine (Q), [^{14}C]arginine (R) and [^{14}C]glutamate (E) was determined at a substrate concentration of 50 μM in the presence (shaded bars) and absence (white bars) of Na^+.

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Table 1 Kinetic parameters of amino acid uptake by 4F2hc/y$^+$LAT2-expressing *X. laevis* oocytes

X. laevis oocytes were injected with 10 ng of each subunit. After a 2-day expression, uptake of [^{14}C]leucine, [^{14}C]glutamine, [^{14}C]arginine and [^{14}C]glutamate was determined at different substrate concentrations in the presence or absence of Na^+ over an incubation period of 10 min.

Amino acid	Na^+	K_m value (μM)	V_{max} value (pmol/10 min)
Glutamine	+	295 ± 42	200 ± 11
Leucine	+	236 ± 41	177 ± 9
Arginine	+	120 ± 35	149 ± 10
Arginine	−	138 ± 23	192 ± 8

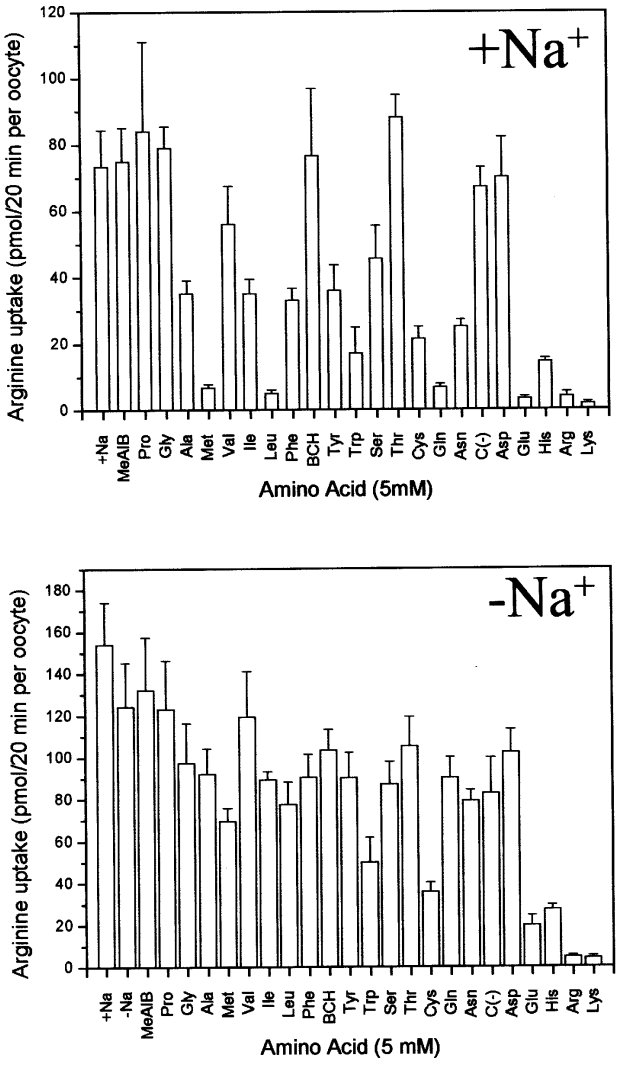

Figure 3 Inhibition of arginine uptake in 4F2hc/y$^+$LAT2-expressing *X. laevis* oocytes

X. laevis oocytes were injected with 10 ng of each subunit. After a 2-day expression, uptake of [^{14}C]arginine (50 μM) was determined in the presence of a variety of amino acids [all 5 mM except cystine, C(−), 300 μM)] in Na^+-containing ND96 buffer (top panel) and after 3 days of expression in ND96 buffer in which NaCl was replaced by *N*-methyl-D-glucamine chloride (bottom panel). The differences in uptake activities of the control samples between panels resulted from the extended expression period of the oocytes used for the experiment in the bottom panel. BCH, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid; MeAIB, *N*-methylamino-isobutyric acid.

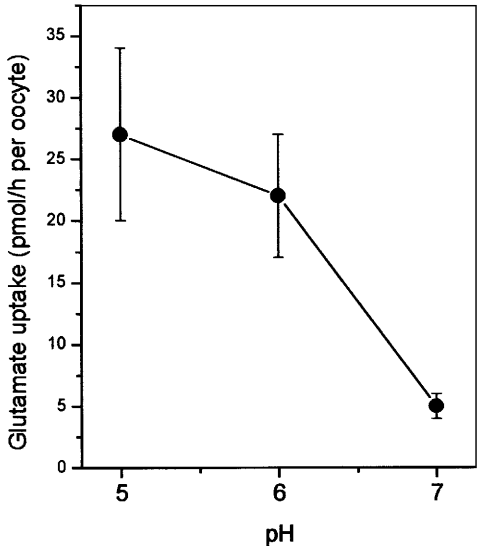

Figure 4 Dependence of glutamate uptake on the extracellular pH

X. laevis oocytes were injected with 10 ng of each subunit. After a 2-day expression, uptake of [^{14}C]glutamate was determined at a substrate concentration of 100 μM at different pH values.

the 4F2hc cRNA (10 ng per oocyte) in *X. laevis* oocytes. To avoid an association of oocyte endogenous transporters with the expressed 4F2hc subunit, all measurements were performed after a 2-day expression period. At this time, expression of 4F2hc alone resulted in marginally increased transport activity (results not shown). In contrast, after a 2-day expression of 4F2hc plus y$^+$LAT2 we observed strong uptake of arginine, leucine and glutamine and moderately increased uptake of isoleucine (Figure 2). In agreement with the known properties of system y$^+$L [24], uptake of neutral amino acids was found to be Na^+-dependent, whereas arginine uptake was unaffected by replacement of Na^+ by *N*-methyl-D-glucamine. In contrast to oocytes expressing 4F2hc alone, even isoleucine uptake depended almost entirely on the presence of Na^+. This confirmed that the 4F2hc subunit did not interact significantly with oocyte endogenous subunits, which mediate Na^+-independent uptake of isoleucine [2].

To characterize the amino acid transport mediated by the 4F2hc/y$^+$LAT2 heterodimer, kinetic constants were determined for arginine, glutamine and leucine (Table 1). For uptake, similar values were found for all three substrates in the presence of Na^+. The V_{max} values ranged between 149 and 200 pmol/10 min per oocyte. The K_m value for arginine (120 μM) was lower than the corresponding values for neutral amino acids, which amounted to 236 and 295 μM for leucine and glutamine, respectively. The K_m value in the absence of Na^+ could only be determined for arginine (138 μM), whereas the transport activity of the neutral amino acids was too low for quantification.

Competition assays were performed in the presence and absence of Na^+ to evaluate the substrate specificity of the transporter. When added at a 100-fold excess in the presence of Na^+, arginine uptake (50 μM) was strongly inhibited by lysine, glutamate, leucine, glutamine, methionine and arginine itself. Other neutral amino acids still showed significant inhibition, although to a lower extent (Figure 3, top panel). In the absence of Na^+, strong inhibition of arginine uptake was restricted to arginine, lysine, histidine and glutamate (Figure 3, bottom panel).

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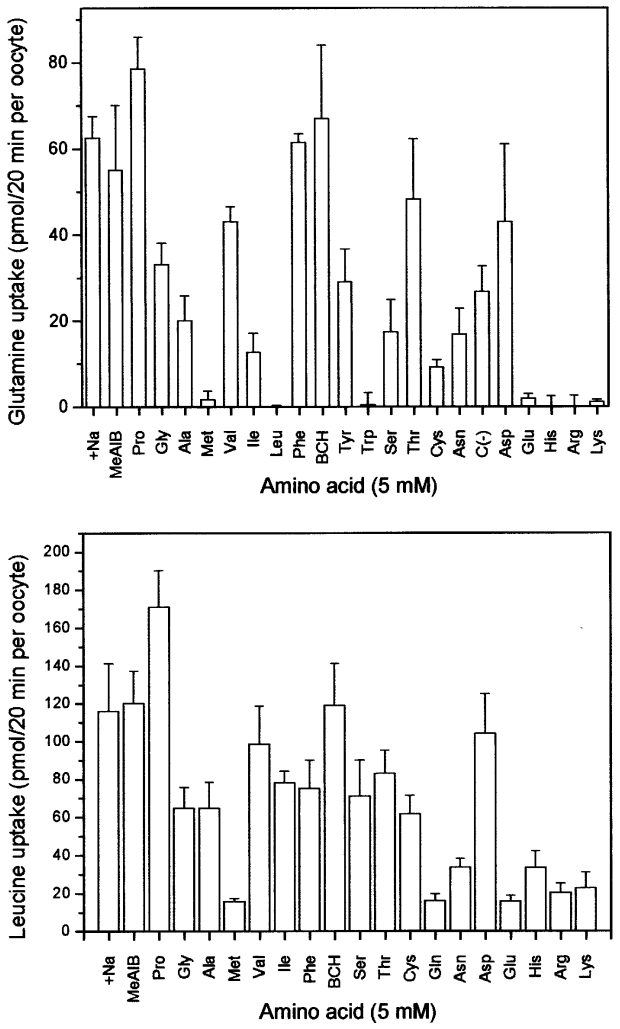

Figure 5 Inhibition of glutamine and leucine uptake in 4F2hc/y+LAT2-expressing *X. laevis* oocytes

X. laevis oocytes were injected with 10 ng of each subunit. After a 2-day expression, uptake of [^{14}C]glutamine (50 μM, upper panel) or [^{14}C]leucine (50 μM, lower panel) was determined in the presence of a variety of amino acids [all 5 mM except cystine, C(—), 300 μM] in Na^+-containing ND96 buffer. BCH, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid; MeAIB, *N*-methylaminoisobutyric acid.

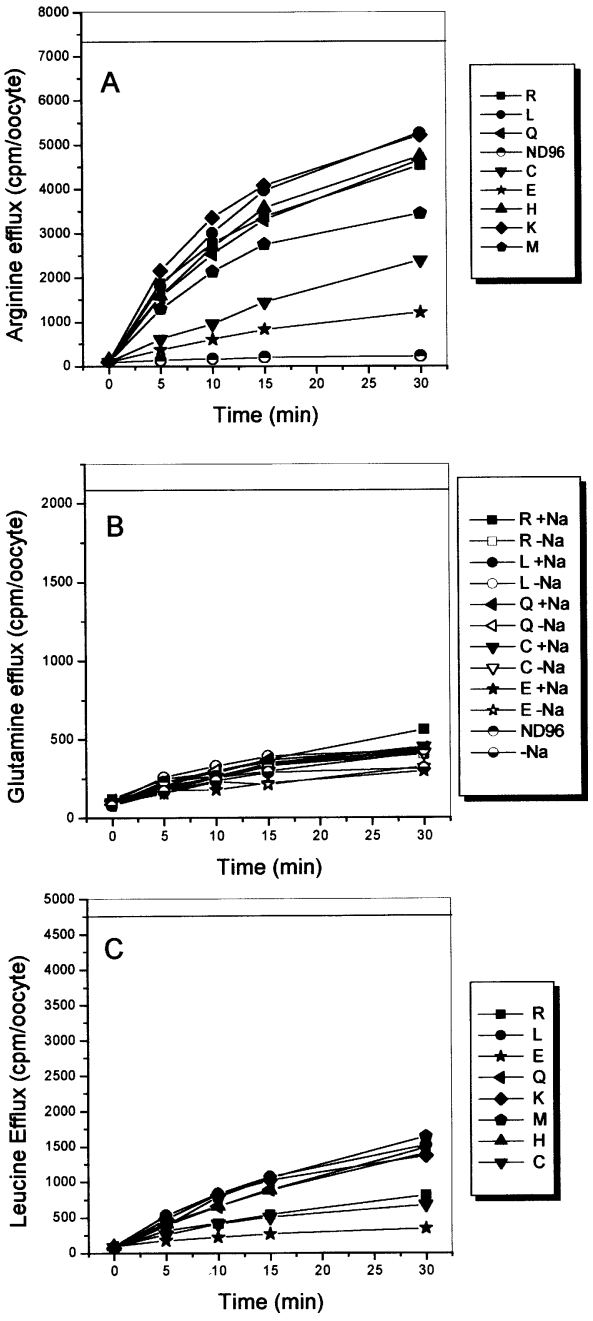

Figure 6 Efflux of preloaded amino acids from 4F2hc/y+LAT2-expressing *X. laevis* oocytes

X. laevis oocytes were injected with 10 ng of each subunit. After a 2-day expression, groups of seven oocytes were preloaded with (**A**) [^{14}C]arginine (50 μM), (**B**) [^{14}C]glutamine (50 μM) or (**C**) [^{14}C]leucine (50 μM) for 10 min. Subsequently, oocytes were washed three times with ice-cold ND96 buffer and then suspended in 1 ml of the same buffer at ambient temperature containing the indicated amino acid (1 mM; R, arginine; L, leucine; Q, glutamine; C, cysteine; E, glutamate; H, histidine; K, lysine; M, methionine). In the case of glutamine-preloaded oocytes, efflux was investigated in both ND96 buffer (+Na) and sodium-free buffer (—Na). Samples were removed from the supernatant at the indicated time points. The released radioactivity was integrated over time and corrected for the number of oocytes in the assay. The intracellular radioactivity accumulated during the preloading period is indicated by a horizontal line at the top of each panel, to allow a comparison of the efflux activity.

Most neutral amino acids exerted only weak inhibition of arginine uptake. Inhibition of arginine uptake by cysteine, tryptophan and glutamate, in contrast, depended only weakly on the presence of Na^+. In view of the strong inhibition of arginine uptake by glutamate we investigated whether 4F2hc/y+LAT2 also mediated the uptake of glutamate (Figure 2). Despite the strong inhibition of arginine uptake by glutamate, only a small but significant glutamate uptake activity was detected in 4F2hc/y+LAT2-expressing oocytes. In agreement with the comparable inhibition in the presence and absence of Na^+, the uptake activity was similarly low under both conditions. As 4F2hc/y+LAT2 mainly accepts neutral and basic amino acids as substrates, we investigated whether an increase of the zwitterionic form of glutamate would augment its uptake. In agreement with this notion, we found a strong increase in glutamate uptake with decreasing pH (Figure 4). Arginine uptake was, however, resistant to inhibition by aspartate, which is in agreement with the observation that amino acids with short side chains, with the exception of cysteine, are generally weak inhibitors of amino acid transport via 4F2hc/y+LAT2.

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As indicated above, inhibition of glutamine uptake could only be investigated in the presence of Na^+ (Figure 5, upper panel). Under these conditions, glutamine uptake (50 μM) was inhibited strongly by a 100-fold excess of arginine, lysine, histidine, leucine, tryptophan, methionine, glutamate and cysteine. Other neutral amino acids exerted only partial but significant inhibition. A similar inhibition profile was determined for leucine uptake in the presence of Na^+ (Figure 5, lower panel). Again the most significant inhibition was observed by an excess of arginine, lysine, histidine, glutamine, methionine, glutamate and cysteine.

As exemplified by glutamate, good inhibitors of amino acid transport via 4F2hc/y^+LAT2 were not necessarily good substrates of the transporter. To investigate the substrate specificity, oocytes were preloaded with labelled arginine, glutamine and leucine, and subsequently, trans-stimulation of the efflux of the preloaded amino acid was investigated. Under these conditions it is expected that only amino acids which are translocated by the transporter will stimulate efflux of the preloaded amino acid, whereas pure inhibitors of the transporter should also inhibit efflux of the preloaded amino acid.

Similar to the other members of this family [4,5,8–12], y^+LAT2 was found to obey an antiport mechanism (Figure 6). Efflux of preloaded amino acids was almost undetectable in the absence of extracellular amino acids. Addition of saturating concentrations (1 mM) of substrate amino acids to the supernatant, in contrast, resulted in a significant efflux of the preloaded amino acid. Although the uptake activities for arginine, glutamine and leucine were similar in 4F2hc/y^+LAT2-expressing oocytes (see Table 1), the efflux activity strongly discriminated between the three amino acids.

The antiport mechanism was most evident when arginine efflux was investigated (Figure 6A). In the absence of extracellular amino acids no significant release of labelled arginine could be detected. However, addition of substrate amino acids (1 mM) to the supernatant resulted in a rapid release of arginine (Figure 6A) from the oocytes. The efficacy of efflux induction decreased in the order lysine = leucine = histidine = glutamine = arginine > methionine $\gg$ cysteine > glutamate. This order coincided with the inhibitory action of these amino acids and confirmed that glutamate is a weakly but significantly transported substrate. Similarly, cysteine, the inhibitory action of which did not depend on the presence of Na^+, was also a weak substrate of the transporter.

Much lower efflux activity was observed with preloaded leucine (Figure 6C) and almost no significant efflux activity was observed when glutamine was preloaded into the oocytes (Figure 6B). The most significant leucine efflux was observed with extracellular methionine, leucine, glutamine, histidine and lysine. Arginine, in contrast, induced only a low efflux of preloaded leucine. Efflux of glutamine generally was very low and did not depend on the presence or absence of amino acids or the presence of Na^+.

The low efficacy of glutamine efflux suggested that preloading of oocytes with glutamine should decrease the subsequent uptake of amino acids via y^+LAT2. In agreement with this notion, preloading of oocytes for 60 min with 0.1 and 1 mM unlabelled glutamine indeed resulted in a decrease in [^{14}C]glutamine (100 μM) uptake by 29 % (64 $\pm$ 15 pmol/10 min per oocyte) and 37 % (57 $\pm$ 11 pmol/10 min per oocyte), respectively, relative to the control value of 90 $\pm$ 22 pmol/10 min per oocyte. To determine the stoichiometry of the antiport mediated by y^+LAT2, oocytes were preloaded with [^{14}C]arginine (100 μM) for 120 min to equilibrate the intracellular pool to about the same specific activity as the extracellular arginine pool. Subsequently, extracellular arginine was removed and replaced by [^{3}H]leucine (100 μM). Both compounds were used at similar specific activities.

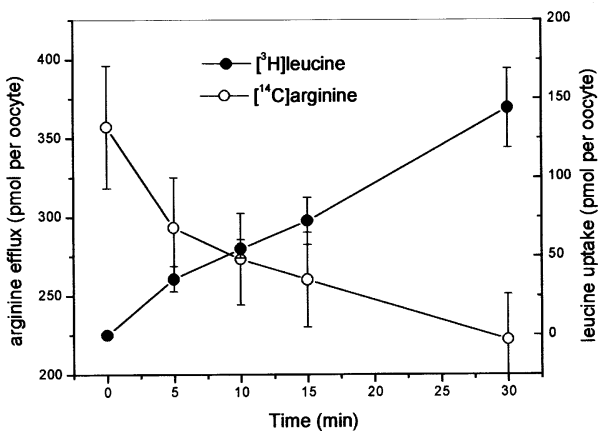

Figure 7 Uptake of leucine is accompanied by an efflux of arginine

Oocytes were preloaded with 100 μM [^{14}C]arginine (○) for 2 h and subsequently incubated in 100 μM [^{3}H]leucine (●). Fluxes of both isotopes were determined by liquid scintillation counting using a dual-label programme.

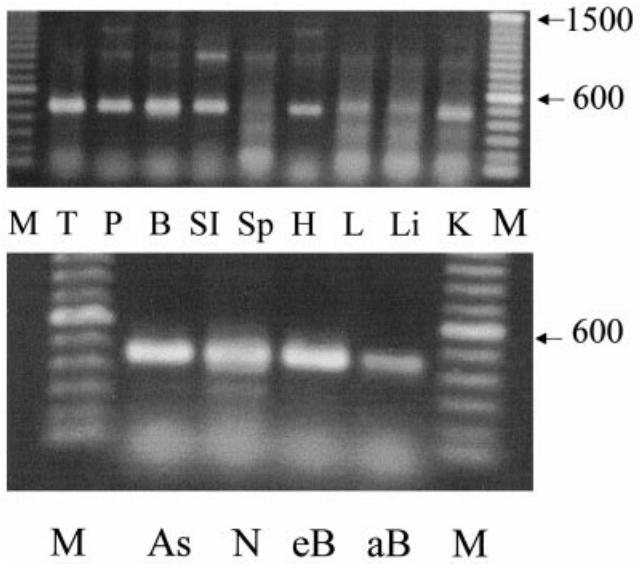

Figure 8 Expression pattern of y^+LAT2 in different mouse tissues and different cultured rat brain cells

Total RNA was isolated from different mouse tissues (upper panel) and from cultured rat astrocytes, neurons, embryonic and adult brain (lower panel). RNA (1 μg) was reverse transcribed into cDNA and an aliquot was used for RT-PCR using y^+LAT2-specific primers. The y^+LAT2-specific fragment (463 bp) was amplified for 30 cycles. The following abbreviations are used: M, marker; T, testis; P, parotis; B, brain; SI, small intestine; Sp, spleen; H, heart; L, lung; Li, liver; K, kidney; As, cultured rat astrocytes; N, cultured rat neurons; eB, embryonic brain; aB, adult brain.

The fluxes of both amino acids were monitored simultaneously by dual-label counting (Figure 7). An efflux of approx. 150 pmol of arginine during 30 min was accompanied by an uptake of about 150 pmol of leucine.

Two types of current were associated with the expression of the 4F2hc/y^+LAT2 heterodimer in *Xenopus* oocytes. First, a basic substrate-independent conductance, which is similar to the non-specific cation conductance of 4F2hc/LAT1 expressing

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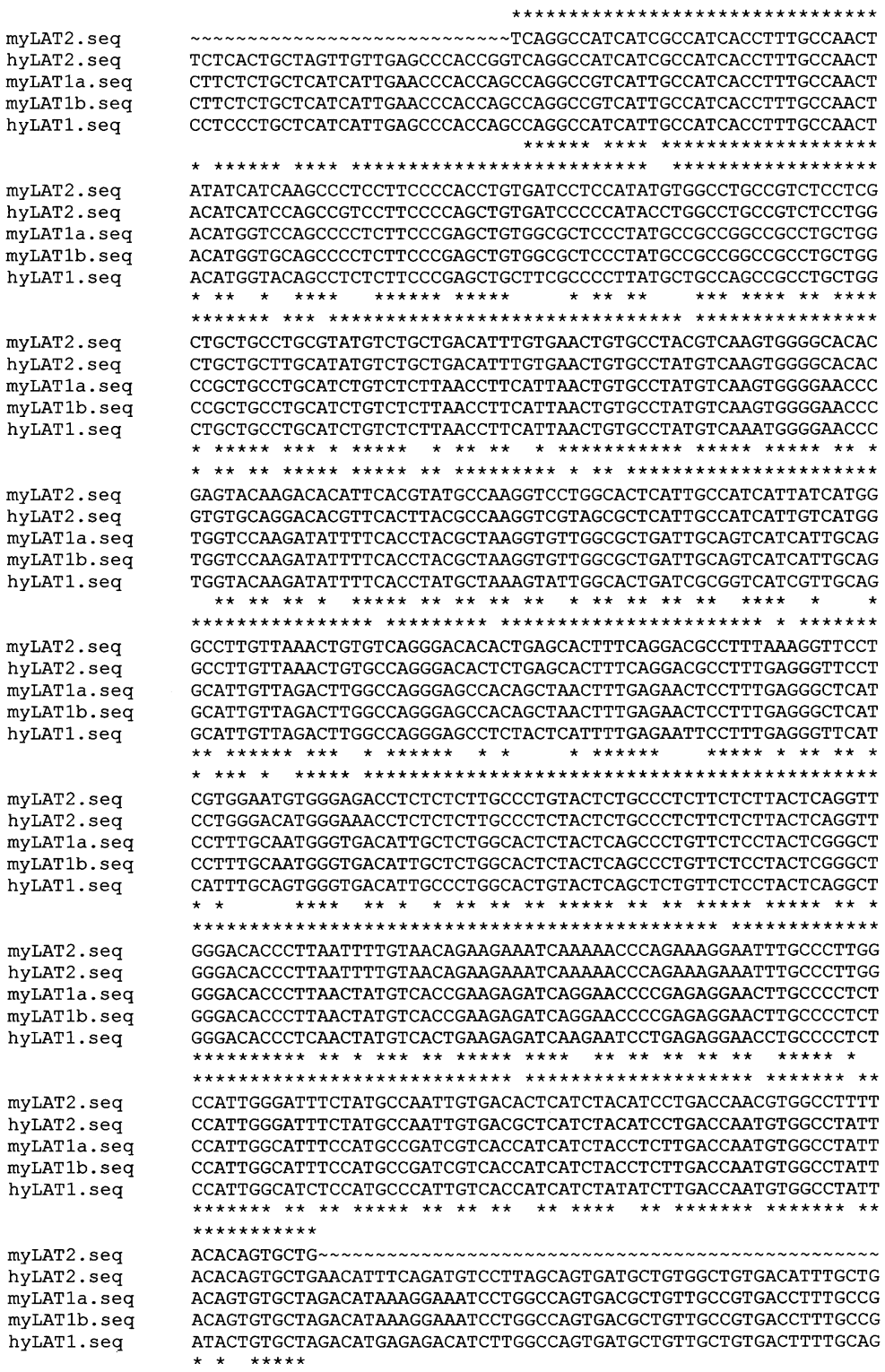

Figure 9 Sequence comparison of different y+LAT isoforms

The sequence of the amplified 463-bp fragment from mouse testis was determined and aligned with other known y+LAT-isoform cDNAs. Identical nucleotides between the mouse cDNA fragment and the human y+LAT2 are marked by asterisks above the alignment, identities in all sequences are marked below the alignment. m, mouse; h, human.

oocytes [31], and secondly, small substrate-induced outward directed currents, which were induced by arginine as well as by glutamate (results not shown).

To gain insight into the physiological functions of the 4F2hc/y+LAT2 transporter we investigated the tissue expression of this transporter (Figure 8). By RT-PCR we could detect the mRNA

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for y+LAT2 in mouse testis, parotis, brain, small intestine, heart and kidney. The strongest signal was detected in brain. No or very weak signals were obtained in RNA samples from spleen, lung and liver. When analysed on a cellular level, both cultured neurons and cultured astrocytes were found to express y+LAT2 mRNA. Embryonic brain contained higher levels of y+LAT2 mRNA than adult brain. The amplified mouse y+LAT2 cDNA fragment showed a higher sequence similarity to the human y+LAT2 cDNA (92% identity), than to mouse and human y+LAT1 cDNAs (74 and 73% identities), thereby confirming its assignment as mouse y+LAT2 cDNA (Figure 9).

DISCUSSION

In agreement with previous studies [9] we found that transport of neutral amino acids by y+LAT2 was Na^+-dependent, whereas arginine transport was independent of the ionic composition of the medium. These are typical features of amino acid transport via system y+L [24]. The dual-label and efflux experiments demonstrated that the y+LAT2 transporter mediates an exchange of neutral and cationic amino acids. Although complete equilibration might not have been achieved after equilibration for 2 h, it is likely that the 4F2hc/y+LAT2 heterodimer operates with a stoichiometry of 1:1. This antiport mechanism is expected to be electroneutral because the positive charge of the cationic amino acid is compensated for by the Na^+ ion accompanying the neutral amino acid. Nevertheless, we observed substrate-induced outward currents with glutamate as well as arginine. As the transport rate of glutamate is much slower than the rate of arginine uptake, and both substrates elicited currents of a similar size and in the same direction, it is unlikely that these currents reflect transporter currents. We have recently identified a non-specific cation-conductance in 4F2hc/LAT1-expressing oocytes [31], which we also detected in oocytes expressing 4F2hc/y+LAT2. The substrate-induced outward currents might therefore reflect inhibition of the cation conductance, which at the resting potential of the oocyte mediates inward currents.

The requirement of Na^+ for the inhibitory action of neutral amino acids varied with the individual amino acid. In particular, cysteine, glutamate and tryptophan exerted inhibition on arginine transport in the absence and presence of Na^+. A comparison of the Na^+-dependence of isoleucine transport with the Na^+ requirement for inhibition of arginine transport by isoleucine suggests that Na^+ is essential for translocation but not for inhibition. This suggests that neutral amino acids are able to bind to the transporter in the absence of Na^+, albeit with lower affinity. This ternary complex does not allow translocation and therefore is inhibitory. Na^+ also might be replaced at lower pH by H^+. The increase in glutamate uptake with decreasing pH could be interpreted in this way. In the case of glutamate we find it tempting to suggest that the zwitterionic form of glutamate is recognized by the transporter.

The antiport mechanism of the transporter allowed us to determine the substrate specificity without the need for labelled substrates. Only substrates that are translocated by the transporter will elicit efflux in this assay. In contrast, non-transported inhibitors will even inhibit efflux of preloaded substrates. Investigation of arginine, glutamine and leucine efflux revealed two properties of the transporter. First, the y+LAT2 amino acid transporter, in contrast to y+LAT1 [9], has a more narrow substrate specificity, which includes only arginine, lysine, leucine, glutamine, histidine and methionine. Secondly, only preloaded arginine is efficiently released by addition of amino acids to the extracellular medium. Since oocytes have significant intracellular concentrations of all amino acids [32], it can be concluded that the intracellular K_m value clearly favours binding of arginine and much less the binding of neutral amino acids. This was corroborated by the observed decrease in the glutamine-uptake rate subsequent to preloading of oocytes with glutamine. Similarly, the apparent affinity of the transporter for extracellular arginine is higher than for extracellular leucine and glutamine, but only by a factor of two. Given the fact that most extracellular fluids in organisms contain much more glutamine than any other amino acid, the physiological transport mode of y+LAT2 will probably be an arginine/glutamine exchange. Observations on amino acid transport and metabolism in cultured brain cells are in agreement with this conclusion. Nagaraja and Brookes [22] showed that about 50% of glutamine uptake into cultured astrocytes could be inhibited by an excess of arginine and also that in cultured neurons about 30% of the Na^+-dependent glutamine uptake is sensitive to inhibition by this amino acid [23]. At the blood–brain barrier, arginine has to be released on the abluminal side of the endothelial cells. As brain produces a surplus of glutamine, an exchange of arginine against glutamine at the blood–brain barrier could mediate the supply of the brain with arginine. Although we did not investigate expression of y+LAT2 in brain microvessels, the occurrence of a transporter in astrocytes frequently coincides with expression at the blood–brain barrier. Release of arginine might also play a role in tissues where arginino-succinate synthetase and nitric oxide synthase are located in different cell types (e.g. [33]). Cells which only express nitric oxide synthase need extracellular arginine for NO production. This arginine could be supplied by neighbouring cells releasing it in exchange for glutamine.

The kidney, by conversion of plasma citrulline, supplies the body with arginine [34]. Although y+LAT2 is expressed in the kidney, the massive expression of y+LAT1 in this tissue [8,9] suggests that this isoform is responsible for the release of arginine in the kidney. Other tissues and cell types with lower transport capacity are likely to use y+LAT2 for release of arginine.

Analysis of the properties of the 4F2hc/y+LAT2 heterodimer also revealed an evolutionary relationship between the glutamate/cystine exchanger 4F2hc/xCT [12] and the other family members. Similar to the xCT transporter, y+LAT2 discriminates strongly between aspartate and glutamate. Aspartate did not act as a competitive inhibitor of glutamine, arginine or leucine transport. Furthermore, glutamate is able to release preloaded arginine from the oocytes, albeit with low efficacy.

The heterodimeric amino acid-transporter isoform y+LAT2 is a widely distributed glutamine/arginine exchanger. The mechanism allows the intercellular and interorgan transfer of arginine, a mechanism that could play a physiological role in the transfer of glutamine and arginine in the brain and other tissues.

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