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ATA2 Is Predominantly Expressed as System A at the Blood-Brain Barrier and Acts as Brain-to-Blood Efflux Transport for L-Proline

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ABSTRACT

Although system A is present at the blood-brain barrier (BBB), the physiological roles of system A have not been clarified. The efflux transport of the substrates of system A, such as L-proline (L-Pro), glycine (Gly), and α-methylaminoisobutyric acid (MeAIB), across the BBB was investigated using the in vivo Brain Efflux Index method. Over a period of 40 min, L-[^{3}H]Pro and [^{3}H]Gly underwent efflux from the brain, whereas [^{3}H]MeAIB did not. The efflux of L-[^{3}H]Pro was inhibited by the presence of unlabeled L-Pro and MeAIB, suggesting that carrier-mediated efflux transport of L-Pro across the BBB is involved in system A. L-[^{3}H]Pro uptake by TR-BBB cells, used as an in vitro BBB model, was Na^+-dependent with high-affinity (K_{m1} = 425 μM) and low-affinity (K_{m2} = 10.8 mM) saturable processes. The manner of inhibition of L-[^{3}H]Pro uptake for amino acids was consistent with system A. Although GlnT, ATA2, and ATA3 mRNA were all expressed in TR-BBB cells, ATA2 mRNA was predominant. Under hypertonic conditions, ATA2 mRNA in TR-BBB cells was induced by up to 373%, and it activated [^{3}H]MeAIB uptake. In light of these observations, our results indicate that L-Pro and Gly are transported from the brain across the BBB, whereas MeAIB is retained in the brain. System A is involved in efflux transport for L-Pro at the BBB. The predominantly expressed ATA2 mRNA at the BBB may play a role in maintaining the concentration of small neutral amino acids and cerebral osmotic pressure in the brain under pathological conditions.

The blood-brain barrier (BBB), which is formed by complex tight junctions of the brain capillary endothelial cells, segregates the circulating blood from interstitial fluid in the brain (Cornford, 1985). The BBB is well known to regulate not only the supply of nutrients and drugs to the brain from the circulating blood (Cornford, 1985; Pardridge et al., 1990), but also the efflux transport of compounds, such as P-glycoprotein, which transports anticancer and other drugs (Tsuji et al., 1992; Schinkel et al., 1994). Another transporter at the BBB is system A, a transporter of small neutral amino acids, which accepts L-alanine, L-proline, glycine, and α-methylaminoisobutyric acid (MeAIB) as substrates. It has been suggested that system A is present in the abluminal (brain) side of the BBB because MeAIB, which is a specific nonmetabolizable substrate for system A (Norman and Mann, 1986), is taken up in an Na^+-dependent manner from the brain side using isolated rat brain capillaries (Betz and Goldstein, 1978) and isolated abluminal membrane vesicles from bovine brain endothelial cells (Sanchez del Pino et al., 1995). Several investigations using brain-uptake methods have established that system A substrates undergo limited influx transport across the BBB (Oldendorf, 1971; Sershen and Lajtha, 1979). These investigations suggest that system A plays a role in the efflux transport of small neutral amino acids at the BBB to maintain their concentration in the brain (Betz and Goldstein, 1978). However, it does not fully explain this hypothesis, because the neutral amino acids are believed to be supplied from the circulating blood (Cornford, 1985). Re-

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ABBREVIATIONS: BBB, blood-brain barrier; BEI, Brain Efflux Index; L-[^{3}H]Pro, L-[2,3-^{3}H]proline; [^{3}H]Gly, [2-^{3}H]glycine; [^{14}C]inulin, [^{14}C]carboxyl-inulin; [3-*o*-^{3}H]MG, 3-*o*-[methyl-^{3}H]methyl-D-glucose; [^{3}H]MeAIB, *N*-[methyl-^{3}H]methylaminoisobutyric acid; Par2, parietal cortex area 2; ECF, extracellular fluid; HPLC, high-performance liquid chromatography; K_{eff}, apparent brain efflux rate constant; V_{brain}, brain distribution volume; TR-BBB, conditionally immortalized rat brain capillary endothelial cell line; TR-BBB13, conditionally immortalized rat brain capillary endothelial cell line 13; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s).

cently, three Na^+-dependent small neutral amino acid transporters have been identified as system A isoforms, namely, GlnT/ATA1/SAT1 (Varoqui et al., 2000), ATA2/SAT2/SA1 (Reimer et al., 2000; Sugawara et al., 2000a; Yao et al., 2000), and ATA3 (Sugawara et al., 2000b). Moreover, Alfieri et al. (2001) recently suggested that ATA2 expression and its amino acid transport activity in porcine endothelial cells is under osmotic regulation. To understand the regulation and physiological and/or pathophysiological functions of system A at the BBB, it is important to identify isoforms of system A and clarify the transport functions of system A at the BBB under disease conditions. The osmo-regulation in the brain may play a role in detoxification in the brain to protect it against adverse events such as brain edema, stroke hyponatremia, head injures, ischemia, and hydrocephalus (Phillis et al., 1999; De Petris et al., 2001).

The development of the Brain Efflux Index (BEI) method (Kakee et al., 1996) allowed us to investigate the direct in vivo efflux transport at the BBB of several compounds, such as excitatory neurotransmitters [e.g. L-glutamic acid and L-aspartic acid (Hosoya et al., 1999)], neuroactive steroids [e.g., estrone-3-sulfate and dehydroepiandrosterone sulfate (Asaba et al., 2000; Hosoya et al., 2000a)], and a suppressive neurotransmitter [e.g., γ-aminobutyric acid (Kakee et al., 2001)].

The purpose of the present study was to investigate the function and molecular characteristics of system A at the BBB. The in vivo efflux transport of L-Pro, Gly, and MeAIB was characterized using the BEI method. The transport characteristics for L-Pro and mRNA regulation of system A under normal and hypertonic conditions were investigated using a conditionally immortalized rat brain capillary endothelial cell line (TR-BBB) as an in vitro model of the BBB (Hosoya et al., 2000b; Terasaki and Hosoya, 2001).

Experimental Procedures

Animals. Male Wistar rats weighing 250 to 300 g were purchased from Charles River (Yokohama, Japan). This study was approved by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Reagents. L-[2,3-^{3}H]Proline (L-[^{3}H]Pro, 45.0 Ci/mmol), [2-^{3}H]glycine ([^{3}H]Gly, 41.1 Ci/mmol), and 3-*o*-[methyl-^{3}H]methyl-D-glucose ([3-*o*-^{3}H]MG, 72.4 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA); [^{14}C]carboxyl-inulin ([^{14}C]inulin, 2.10 mCi/g) was from ICN Pharmaceuticals (Costa Mesa, CA); *N*-[methyl-^{3}H]methylaminoisobutyric acid ([^{3}H]MeAIB, 85.0 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO); L-Pro was from Nacalai Tesque (Kyoto, Japan); and MeAIB was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and were available commercially.

BEI Study. The in vivo brain efflux experiments were performed using the intracerebral microinjection technique reported previously (Kakee et al., 1996). A Wistar rat was anesthetized by intramuscular injection of ketamine-xylazine (1.22 mg/kg xylazine and 125 mg/kg ketamine) and placed in a stereotaxic frame (SR-6; Narishige Co., Tokyo, Japan), which determines the coordinates of the rat brain coinciding with the parietal cortex area 2 (Par2). The applied solution (0.50 μl) containing 0.2 μCi of each ^{3}H-labeled substrate and 10 nCi of [^{14}C]inulin as a reference compound in extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM HEPES), pH 7.4, was then administered to the brain. The radioactivity remaining in the brain was measured in a liquid scintillation counter equipped with an appropriate crossover correction for ^{3}H and ^{14}C (LS-6500; Beckman Coulter, Inc., Fullerton, CA). The BEI value, the percentage of substrate remaining in the ipsilateral cerebrum (100 − BEI), and the apparent brain efflux rate constant (K_{eff}) were determined according to methods used in a previous report (Kakee et al., 1996).

HPLC Analysis. Metabolism of L-Pro was evaluated by measuring ^{3}H-labeled compound in brain and jugular venous plasma after intracerebral microinjection of L-[^{3}H]Pro (25 μCi) using an HPLC system equipped with an analytical column (Capcell Pak SCX UG80, 4.6 mm i.d. × 250 mm; Shiseido, Tokyo, Japan). The mobile phase, 10 mM KH_2PO_4, pH 3.0, was pumped through the column at a rate of 1.0 ml/min at 40°C. The radioactivity in each fraction (0.50 ml) was determined by a liquid scintillation counter.

Brain Slice Uptake Study. The brain distribution volume (V_{brain}) of L-Pro was determined by the in vitro brain slice uptake method as described previously (Kakee et al., 1996) to estimate the apparent efflux clearance across the BBB using the equation

$$CL_{BBB,efflux} = K_{eff} \times V_{brain} \quad (1)$$

Cell Culture. TR-BBB13 cells (passage number 18–23) were grown routinely in collagen type-1 coated tissue-culture flasks (BD Biosciences, Bedford, MA) at 33°C under 5% CO_2/air, as described previously (Hosoya et al., 2000b). The osmolality of the normal culture medium (Dulbecco's modified Eagle's medium supplemented with 100 U/ml benzylpenicillin, 100 μg/ml streptomycin sulfate, 10% fetal bovine serum, and 15 μg/ml endothelial cell growth factor) was approximately 290 mOsm/kg. Hypertonic culture medium (450 mOsm/kg) was prepared by adding 160 mM sucrose to normal culture medium.

Uptake Study by Cultured TR-BBB13 Cells. TR-BBB13 cells were seeded on 24-well plates (BD Biosciences) at a density of 1.0×10^5 cells/well and were cultured for 48 h. The uptake of L-[^{3}H]Pro or [^{3}H]MeAIB (1.25 μCi) by TR-BBB13 cells was measured in the presence of [^{14}C]inulin (0.25 μCi) as a correction for water adhesion at 37°C as described previously (Hosoya et al., 2000b). Na^+-free ECF buffer was prepared by equimolar replacement of NaCl and $NaHCO_3$ with choline chloride and choline bicarbonate, respectively. The radioactivity was measured by using a liquid scintillation counter. The protein content of cultured cells was measured using a DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

RNA Extraction and RT-PCR Study. Total RNA was isolated from the rat brain capillary-rich fraction and TR-BBB13 cells using Trizol reagent (Invitrogen, Carlsbad, CA). Isolation of the rat brain capillary-rich fraction was performed as described previously (Hosoya et al., 2000b). Total (1 μg) RNA from each sample was transcribed into first-strand cDNA with a ReverTra Ace kit (Toyobo, Osaka, Japan). The primers used for amplification had the following sequence: GlnT (sense, 5′-TGA TCT TCG GAG CCA CCT CTC-3′; antisense, 5′-TTA CCA TCA CCA CCA ACA CTC G-3′), ATA2 (sense, 5′-ACA TAA GGC ATA CGG TCT GGC T-3′; antisense, 5′-CAG CCC ATT CGT ATC TTC AAT GTT-3′), or ATA3 (sense, 5′-GGC ATG AAC TTC ATG GTG GAC TA-3′; antisense, 5′-AGC TGT ATC AAA TGT GTA GAC TTT-3′). The amplification conditions were as follows: denaturation for 30 s at 95°C, annealing for 1 min at 60°C, and synthesis for 1 min at 72°C, for 40 cycles, followed by further incubation for 10 min at 72°C. The polymerase chain reaction products were separated on 2.5% agarose gel containing ethidium bromide (0.6 μg/ml) and were visualized with an imager (EPIPRO 7000; Aisin, Aichi, Japan). The RT-PCR product was subcloned into pGEM-T Easy Vector (Promega, Madison, WI) for sequence analysis. DNA sequencing was performed by use of the dideoxynucleotide chain-termination method using an automated DNA sequencer (model 4200; LI-COR, Lincoln, NE).

Real-Time Quantitative RT-PCR Study. Real-time quantitative RT-PCR analysis was performed using an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA) with

2× SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. To quantitate the amount of specific mRNA in the samples, a standard curve was generated for each run using the plasmid (pGEM-T Easy Vector System I, Promega) containing the gene of interest (dilution ranging from 10^{-7} ng/μl to 1 ng/μl). This enabled standardization of the initial RNA content of TR-BBB13 cells relative to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Polymerase chain reaction was performed using GlnT, ATA2, ATA3, or GAPDH-specific primers through 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min after preincubation at 95°C for 10 min. The specific primers for ATA3 are listed above. GlnT, ATA2, and GAPDH primers are the following: GlnT: sense, 5′-TGA TCT TCG GAG CCA CCT CTC-3′; antisense, 5′-TTA CCA TCA CCA CCA ACA CTC G-3′; ATA2: sense, 5′-CCT GTG GAA GTG GCT TTG ATG-3′; antisense, 5′-AGT TCC CAC GAT CGC AGA GTA-3′; and GAPDH: sense, 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′; antisense, 5′-TCC TTG GAG GCC ATG TAG GCC AT-3′.

Data Analysis. The kinetic parameters of L-Pro uptake by TR-BBB13 cells were estimated by fitting the uptake rate (V) versus L-Pro concentration data to eq. 2 using the nonlinear least-squares regression analysis program, MULTI (Yamaoka et al., 1981).

$$V = \frac{V_{max1} \times [S]}{(K_{m1} + [S])} + \frac{V_{max2} \times [S]}{(K_{m2} + [S])} \quad (2)$$

where [S] is the L-Pro concentration, V_{max1} is the maximum uptake rate for the high-affinity process, K_{m1} is the corresponding Michaelis-Menten constant, V_{max2} is the maximum uptake rate for the low-affinity process, and K_{m2} is the corresponding Michaelis-Menten constant. An unpaired, two-tailed Student's t test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined by analysis of variance followed by the modified Fisher's least-squares difference method.

Results

Efflux Transport of L-Pro, Gly, and MeAIB from Rat Brain across the BBB. The in vivo brain-to-blood efflux transport of L-Pro, Gly, and MeAIB, substrates of system A, across the BBB was determined by the BEI method. Each test compound was microinjected into the Par2 region of the rat brain with [^{14}C]inulin as an impermeable marker. L-[^{3}H]Pro and [^{3}H]Gly were eliminated in a time-dependent manner with a K_{eff} of $9.25 \times 10^{-3} \pm 1.38 \times 10^{-3}$ min^{-1} and $8.61 \times 10^{-3} \pm 1.85 \times 10^{-3}$ min^{-1} (mean ± S.D.), respectively (Fig. 1, B and C). In contrast, [^{3}H]MeAIB did not undergo any significant elimination over a 40-min period (Fig. 1A), like the [^{14}C]inulin (data not shown). Figure 2 shows typical HPLC chromatograms of L-[^{3}H]Pro in the ipsilateral cerebrum and jugular venous plasma after intracerebral microinjection. The amount of L-[^{3}H]Pro in the cerebrum and plasma was 90.7 and 41.2% of the total radioactivity of the sample, respectively. Although there was approximately 10 and 59% of unknown L-[^{3}H]Pro metabolite in the cerebrum and plasma, respectively, at least a part of the L-Pro in the brain interstitial fluid is transported in intact form from the brain to the circulating blood across the BBB.

Measurement of Efflux Clearance of L-Pro across the BBB. The distribution volume of L-Pro in the brain, V_{brain}, was determined using the in vitro brain slice uptake method to estimate the efflux clearance of L-Pro across the BBB, $CL_{BBB,\ efflux}$. The V_{brain} value was evaluated as the value of the steady-state brain slice-to-medium ratio of L-[^{3}H]Pro. Figure 3 shows the time course of L-[^{3}H]Pro uptake by brain slices. No significant difference in the slice-to-medium ratio between the 80- and 120-min incubation was observed, giving a V_{brain} of 5.05 ± 0.19 ml/g of brain. Incorporating K_{eff} ($9.25 \times 10^{-3} \pm 1.38 \times 10^{-3}$ min^{-1}; Fig. 1B) and V_{brain} (5.05 ± 0.19 ml/g of brain) into eq. 1, the $CL_{BBB,\ efflux}$ of L-Pro across the BBB was found to be $4.67 \times 10^{-2} \pm 0.88 \times 10^{-2}$ ml/min/g of brain.

Effect of L-Pro and MeAIB on Efflux Transport of L-[^{3}H]Pro or [3-*o*-^{3}H]MG across the BBB. To characterize the L-Pro efflux transport process at the BBB in vivo, the effects of L-Pro and MeAIB, specific substrates of system A on L-[^{3}H]Pro efflux transport from rat brain were investigated (Table 1). L-Pro and MeAIB were chosen as indicators of carrier-mediated transport and system A for L-[^{3}H]Pro efflux transport at the BBB, respectively. L-[^{3}H]Pro remaining in the ipsilateral cerebrum at 40 min was increased to 163 and 167% by preadministering 50 μl of 100 mM L-Pro and 100 mM MeAIB to the Par2 region, respectively, the estimated concentration in the brain being 100 mM (Kakee et al., 1996). In contrast, preadministration of L-Pro or MeAIB did not affect [3-*o*-^{3}H]MG efflux transport from the brain (Table 1), supporting the hypothesis that this preadministration does not cause any nonspecific damage to the BBB because [3-*o*-^{3}H]MG, a substrate of GLUT1, was used as a transcellular transport marker at the BBB (Kakee et al., 1996). These results indicate that L-[^{3}H]Pro efflux transport is inhibited by

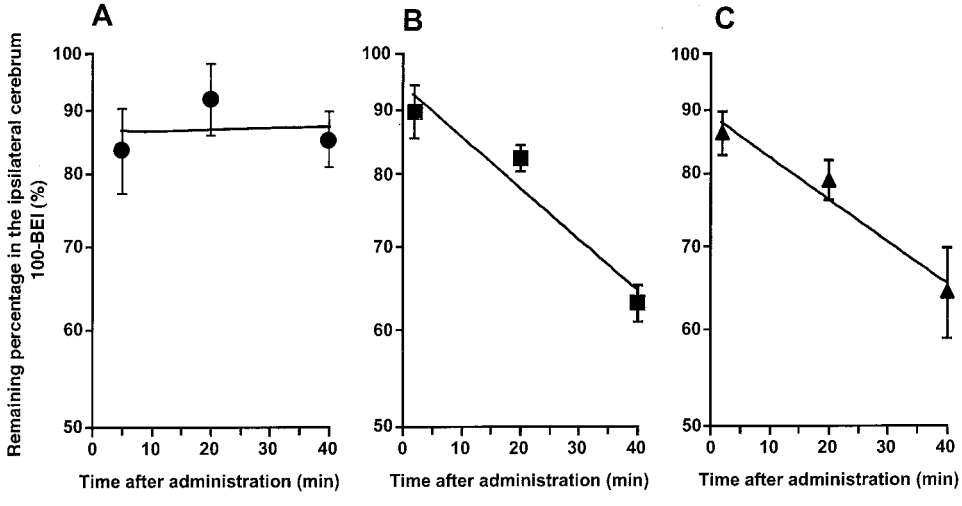

Fig. 1. Time-courses of [^{3}H]MeAIB (A), L-[^{3}H]Pro (B), and [^{3}H]Gly (C) remaining in the ipsilateral cerebrum after intracerebral microinjection into the Par2 region of the rat brain. Each ^{3}H-labeled test compound (0.2 μCi) and [^{14}C]inulin (10 nCi) dissolved in 0.50 μl of ECF buffer was injected into the Par2 region of rat brain. Solid lines were fitted using the nonlinear least-squares regression analysis program. Each point represents the mean ± S.E.M. (n = 4–5).

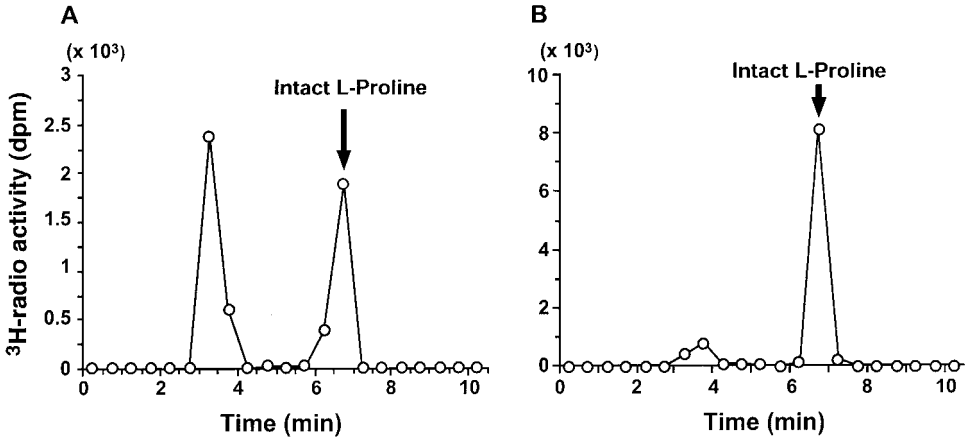

Fig. 2. Typical HPLC chromatograms of L-[^{3}H]Pro in jugular venous plasma (A) and ipsilateral cerebrum (B). An aliquot of L-[^{3}H]Pro (25 μCi) was injected into the Par2 region of rat brain. Venous blood was collected from the ipsilateral jugular vein 5 min after microinjection. Ipsilateral brain was removed at 10 min. HPLC analysis was performed at a flow rate of 1.0 ml/min. Each point represents the radioactivity measured in the respective fraction (0.50 ml each).

unlabeled L-Pro and MeAIB and suggest that system A may be involved in L-Pro transport across the BBB.

Time Course and Concentration-Dependence of L-Pro Uptake by TR-BBB13 Cells. Although system A is most probably involved in L-Pro efflux transport across the BBB, kinetic parameters, such as the Michaelis-Menten constant (K_m) of L-Pro for system A, remain unknown. To determine the kinetic parameters of L-Pro for system A and characterize L-Pro transport at the BBB, L-[^{3}H]Pro uptake was performed using TR-BBB13 cells as an in vitro BBB model. The L-[^{3}H]Pro uptake by TR-BBB13 cells exhibited a time-dependent increase and was linear for at least 10 min with an initial uptake rate of 5.46 μl/min/mg of protein (Fig. 4).

Figure 5 shows concentration-dependent L-[^{3}H]Pro uptake by TR-BBB13 cells. The Eadie-Scatchard plot (Fig. 5, inset) gave two straight lines, indicating that two saturable processes were involved in L-Pro uptake by TR-BBB13 cells. Kinetic analysis of the uptake data using eq. 2, by nonlinear least-squares regression analysis, yielded a K_{m1} of 425 ± 151 μM, a V_{max1} of 1.93 ± 0.48 nmol/min/mg of protein, a K_{m2} of 10.8 ± 1.6 mM, and a V_{max2} of 13.4 ± 0.3 nmol/min/mg of protein (mean ± S.D.).

Effect of Na^+-Free Conditions and Several Amino Acids on L-[^{3}H]Pro Uptake by TR-BBB13 Cells. The effect of Na^+-free conditions and several amino acids on the L-[^{3}H]Pro uptake by TR-BBB13 cells at 5 min is summarized in Table 2. Under Na^+-free conditions, L-[^{3}H]Pro uptake was reduced by 93.9%. It was significantly inhibited by system A substrates, such as methylated amino acids (MeAIB, by 70.7%; *N*-methyl-L-alanine, by 78.4%) and small neutral amino acids (L-Pro, by 87.0%; Gly, by 70.2%; L-Ala, by 93.5%). In contrast, basic and acidic amino acids, such as L-glutamic acid and L-lysine, produced no marked inhibition.

Real-Time Quantitative RT-PCR Analysis of mRNA of System A Isoforms in TR-BBB13 Cells. The manner of inhibition of L-[^{3}H]Pro uptake by TR-BBB13 cells supports the hypothesis that system A is involved in the uptake process. Therefore, the mRNA expression of system A isoforms in TR-BBB13 cells and rat brain capillary-rich fraction as an in vivo BBB was examined. The bands for GlnT, ATA2, and ATA3 in the rat brain capillary-rich fraction (lane 1) and in TR-BBB13 cells (lane 2) were visualized at 159 bp, 154 bp, and 390 bp, respectively (Fig. 6A). The control lacking the reverse-transcriptase enzyme was assayed in parallel to monitor any possible genomic contamination (right-hand side of each lane). Sequence analysis indicated that the RT-PCR product from the TR-BBB13 cells was 98, 99, and 99% identical to rat GlnT, ATA2, and ATA3, respectively, at the nucleotide level (Sugawara et al., 2000a,b; Varoqui et al., 2000). Then the mRNA expression levels of GlnT, ATA2, and ATA3 were determined in TR-BBB13 cells by real-time quantitative RT-PCR analysis. The quantity of expressed mRNA, compensated with GAPDH, for GlnT, ATA2, and ATA3 was $3.21 \pm 0.24 \times 10^{-4}$, $2.99 \pm 0.40 \times 10^{-2}$ and $1.4 \pm 0.16 \times 10^{-5}$, respectively (Fig. 6B). Accordingly, the expression of ATA2 mRNA was 93- and 2140-fold greater than GlnT and ATA3 mRNA in TR-BBB13 cells, respectively.

Effect of Hypertonic Conditions on [^{3}H]MeAIB Uptake by TR-BBB13 Cells. To clarify the osmotic regulation of system A at the BBB, the hypertonic effect on system A transport activity was determined using TR-BBB13 cells exposed to hypertonic culture medium (450 mOsm/kg) for 17 h (Fig. 7). In this study, [^{3}H]MeAIB was used as a specific substrate for system A to avoid the effects of metabolism and other amino transporters. The hypertonic condition of 450 mOsm/kg was chosen for treatment of TR-BBB13 cells because a preliminary study suggested that this led to the

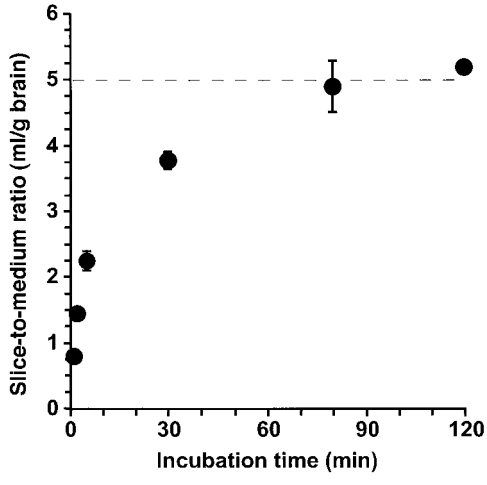

Fig. 3. Time course of the apparent uptake of L-[^{3}H]Pro by brain slices. Rat brain slices were incubated with 0.05 μCi/ml L-[^{3}H]Pro and 0.01 μCi/ml [^{14}C]inulin as a marker of water adhesion at 37°C. The broken line shows the V_{brain} value of 5.05 ± 0.19 ml/g brain using steady-state values between 80 and 120 min. Each point represents the mean ± S.E.M. (n = 3).

TABLE 1

Effect of L-proline and MeAIB on the efflux of L-[^{3}H]proline and [3-*o*-^{3}H]MG from rat brains

L-[^{3}H]Proline or [3-*o*-^{3}H]MG dissolved with [^{14}C]inulin in 0.5 μl of ECF buffer was injected into the Par2 region. The brain concentration of L-[^{3}H]proline and [3-*o*-^{3}H]MG was estimated from the injectant concentration divided by the dilution factor (i.e., 30.3), which was reported previously (Kakee et al., 1996). Data were determined at 40 and 20 min after intracerebral microinjection for L-[^{3}H]proline and [3-*o*-^{3}H]MG, respectively. Each value represents the mean ± S.E.M. (percentage of control) (n = 5–8). Inhibitor solution (50 μl) was administered to the Par2 region just before injection of L-[^{3}H]proline or [3-*o*-^{3}H]MG.

Inhibitors	Percentage Remaining in the Brain	
	l-[^{3}H]Proline (293 nM)	[3-*o*-^{3}H]MG (182 nM)
Control	75.6 ± 8.1 (100)	20.9 ± 3.2 (100)
100 mM L-PROLINE	123 ± 12 (163)*	23.4 ± 3.9 (112)
100 mM MeAIB	126 ± 13 (167)*	17.0 ± 3.9 (81.3)

* $p < 0.05$, significantly different from control.

greatest [^{3}H]MeAIB uptake compared with 390 and 540 mOsm/kg. Under hypertonic conditions, [^{3}H]MeAIB uptake was increased in a time-dependent manner up to 8 h, and then it reached steady state. The [^{3}H]MeAIB uptake activity in TR-BBB13 cells exposed to hypertonic culture medium for 8 h was 8.4-fold greater than that under normal culture conditions.

Hypertonic Regulation of GlnT, ATA2, and ATA3 mRNA Expression in TR-BBB13 Cells. The hypertonic effect on the mRNA levels of GlnT, ATA2, and ATA3 is shown in Fig. 8. The GlnT and ATA3 mRNA levels in TR-BBB13 cells were increased in a time-dependent manner with the exposure time. They were significantly (1.7- and 47-fold, respectively) greater than that under normal culture conditions (control) over 24 h. On the other hand, the ATA2 mRNA level increased up to 4 h and then gradually fell. It was 3.7- and 1.4-fold greater than that under normal culture conditions for 4 h and 24 h, respectively.

Discussion

In this study, we obtained in vivo evidence to prove that system A substrates, such as L-Pro and Gly, in brain interstitial fluid are transported across the BBB (Fig. 1, B and C). Moreover, significant L-[^{3}H]Pro was found in the jugular venous plasma, indicating that L-Pro undergoes efflux transport in intact form from the brain to the circulating blood across the BBB (Fig. 2). This is consistent with previous reports that L-Pro and Gly were limited to transport from the blood to the brain using brain-uptake methods (Preston et al., 1995; Benrabh and Lefauconnier, 1996). This is supported by the finding that the apparent L-Pro $CL_{BBB,\ efflux}$ value of 46.7 μl/(min·g of brain), determined by a combination of the BEI method (Fig. 1B) and brain slice uptake studies (Fig. 3), was 14-fold greater than that of the apparent influx clearance value [3.3 μl/(min·g of brain)] determined by the in situ perfusion method (Benrabh and Lefauconnier, 1996). The V_{brain} of L-Pro at 5.05 ml/g brain, determined in the steady-state brain slice uptake study, exhibited concentrative uptake (Fig. 3) caused by the brain-specific, high-affinity Na^+-dependent L-Pro transporter PROT (Renick et al., 1999). The inhibition of L-Pro and MeAIB at 100 mM for L-[^{3}H]Pro efflux transport across the BBB suggests that system A is the transporter responsible for the carrier-mediated efflux transport of small amino acids at the BBB (Table 1). However, another system A substrate, MeAIB, did not cross the BBB (Fig. 1A). This suggests that system A is present in the abluminal side and some other amino acid transporter may carry L-Pro and Gly, but not MeAIB, in the luminal side of the BBB (Fig. 9). From a physiological point of view, the efflux

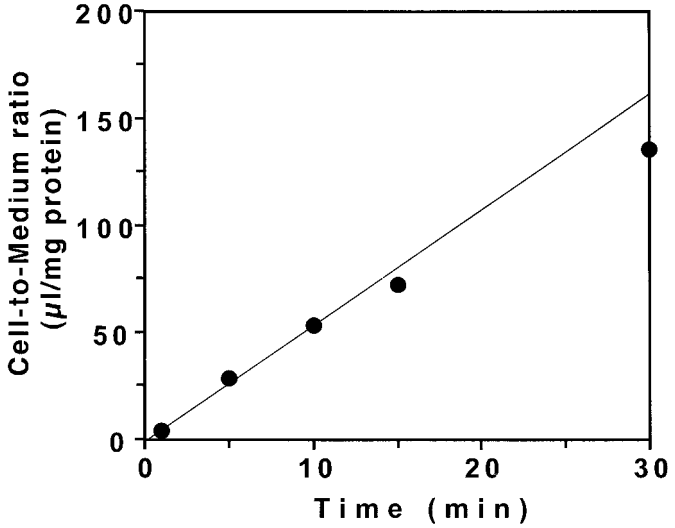

Fig. 4. Time course of L-[^{3}H]Pro uptake by TR-BBB13 cells. L-[^{3}H]Pro uptake (0.1 μM) was performed at 37°C. The error bar is smaller than the size of the symbol. Each point represents the mean ± S.E.M. (n = 4).

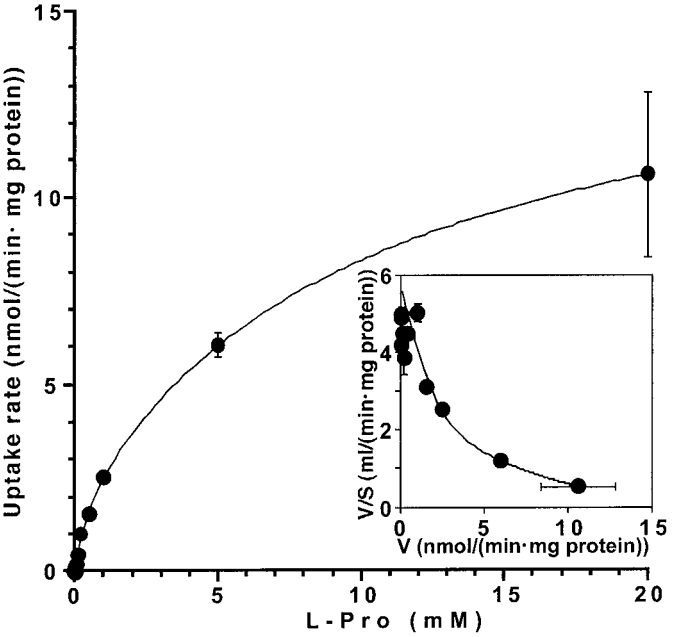

Fig. 5. Concentration-dependence of L-[^{3}H]Pro uptake by TR-BBB13 cells. L-[^{3}H]Pro uptake (0.1 μM) was performed at 37°C in the presence or absence of unlabeled L-Pro. Each point represents the mean ± S.E.M. (n = 4). The inset graph shows the Eadie-Scatchard plot. Solid lines were fitted using the nonlinear least-squares regression analysis program. The following parameters were obtained according to equation [2]: K_{m1} = 425 ± 151 μM, V_{max1} = 1.93 ± 0.48 nmol/min/mg of protein, K_{m2} = 10.8 ± 1.6 mM, and V_{max2} = 13.4 ± 0.3 nmol/min/mg of protein (mean ± S.D.).

TABLE 2

Effect of Na^+-free conditions and several amino acids on L-[^{3}H]proline uptake by TR-BBB13 cells

L-[^{3}H]Proline uptake by TR-BBB13 cells was measured in the presence or absence of 10 mM inhibitor at 37°C for 5 min. Under Na^+-free conditions, L-[^{3}H]proline uptake was performed in the presence of choline instead of Na^+. Each value represents the mean ± S.E.M. (n = 3–4).

Conditions	Percentage of Control
Control	100 ± 4
Na^+-free	6.1 ± 0.2*
MeAIB	29.3 ± 1.3*
N-methyl-L-alanine	21.6 ± 1.3*
L-Proline	13.0 ± 0.7*
Glycine	29.8 ± 2.0*
L-Alanine	6.5 ± 0.8*
L-Glutamate	105 ± 2*
L-Lysine	92.9 ± 10.7

* $p < 0.001$, significantly different from control.

transport systems for L-Pro and Gly at the BBB play a key role in maintaining the concentration of L-Pro and Gly in the brain interstitial fluid. These efflux transporters, as well as PROT and GLYT, which are transporters of Gly, expressed in neuronal cells regulate the concentration of L-Pro and Gly in the brain. These transporters help to maintain very low concentrations in the fluid surrounding the brain parenchymal cells because both amino acids exhibit neuroactivity (Shank and Campbell, 1984; Barmack et al., 1999). Moreover, L-Pro and Gly are neurotoxic, and an L-Pro metabolism disorder, hyperprolinemia type II, is associated with seizures and mental retardation (Nadler et al., 1988; Cohen and Nadler, 1997). Other findings support the hypothesis that amino acids are released into interstitial spaces of the cerebral cortex during ischemia (Phillis et al., 1999). Therefore, our findings regarding the efflux transport of L-Pro and Gly may help to elucidate the mechanism of efflux transport at the BBB and the role of the detoxifying system operating in the brain.

High- and low-affinity transport processes were found in TR-BBB13 cells used as an in vitro BBB model. The corresponding K_m values were 425 μM and 10.8 mM, respectively (Fig. 5). L-[^{3}H]Pro uptake by TR-BBB13 cells was completely inhibited under Na^+-free conditions (Table 2). Therefore, even the low-affinity transport process seems to be Na^+-dependent because it would not be saturable in the presence of unlabeled L-Pro under Na^+-free conditions. L-[^{3}H]Pro uptake by TR-BBB13 cells was significantly inhibited by system A substrates, such as MeAIB, *N*-methyl-L-alanine, Gly, and L-Ala, by more than 70% (Table 2). The high-affinity process for L-Pro uptake by TR-BBB13 cells is most likely involved in system A because the K_m value of L-Pro for system A ranges from 0.11 to 1 mM at the BBB (Hwang et al., 1983; Smith and Stoll, 1998). Although nothing is presently known about the low-affinity process, other isoforms of system A (Fig. 6) and other Na^+-dependent neutral amino acid transporters, such as system ASC and system B, seem to be present at the BBB (Tayarani et al., 1987).

Although TR-BBB13 cells and the rat brain capillary-rich fraction used in vivo at the BBB expressed all three isoforms of system A—GlnT, ATA2, and ATA3—(Fig. 6A), real-time quantitative RT-PCR analysis supported the hypothesis that ATA2 is predominantly expressed in TR-BBB13 cells (Fig. 6B). Therefore, ATA2 seems to be the responsible transporter for system A at the BBB. This finding is consistent with a major isoform of the virtually ubiquitous amino acid transport system A, as reported elsewhere (Sugawara et al., 2000a). The up-regulation of MeAIB uptake by TR-BBB13 cells in response to hypertonic stress occurred simultaneously with the induction of ATA2 mRNA (Figs. 7 and 8B). Although GlnT and ATA3 mRNAs were also induced 1.7- and 47-fold, respectively, under hypertonic culture conditions for 24 h, this may only make a minor contribution to the solute uptake activity. This is because the ATA2 mRNA level was 93-, 344-, and 74-fold greater than that of GlnT mRNA under normal, 4-h, and 24-h hypertonic culture conditions, respectively, and 2140-, 4350-, and 61-fold greater than that of ATA3 mRNA under normal, 4-h, and 24-h hypertonic culture conditions, respectively. Moreover, the up-regulation ATA2

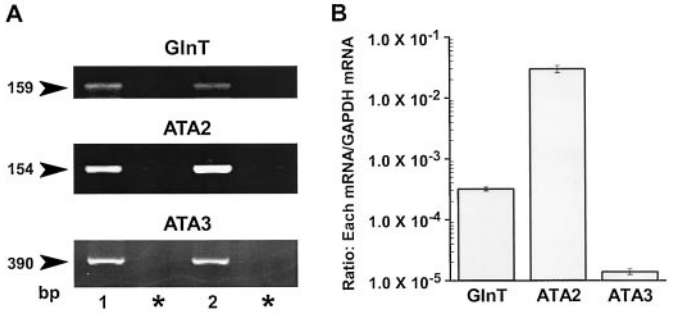

Fig. 6. RT-PCR analysis of GlnT, ATA2, and ATA3 in rat brain capillary-rich fraction and TR-BBB13 cells (A) and the amount of GlnT, ATA2, and ATA3 in TR-BBB13 cells (B). A, lane 1, rat brain capillary-rich fraction; lane 2, TR-BBB13 cells. The size of the expected RT-PCR product of GlnT, ATA2, and ATA3 was 159, 154, and 390 bp, respectively. B, the amount of GlnT, ATA2, and ATA3 in TR-BBB13 cells was determined by real-time quantitative RT-PCR analysis. Each column represents the mean the mean ± S.E.M. (n = 4–8). The GlnT, ATA2, and ATA3 mRNA content relative to the amount of GAPDH (GlnT/GAPDH, ATA2/GAPDH, and ATA3/GAPDH) was 3.21 ± 0.24 × 10^{-4}, 2.99 ± 0.40 × 10^{-2} and 1.4 ± 0.16 × 10^{-5}, respectively. Accordingly, the ratio of ATA2 was 93- and 2140-fold greater than that of GlnT and ATA3, respectively.

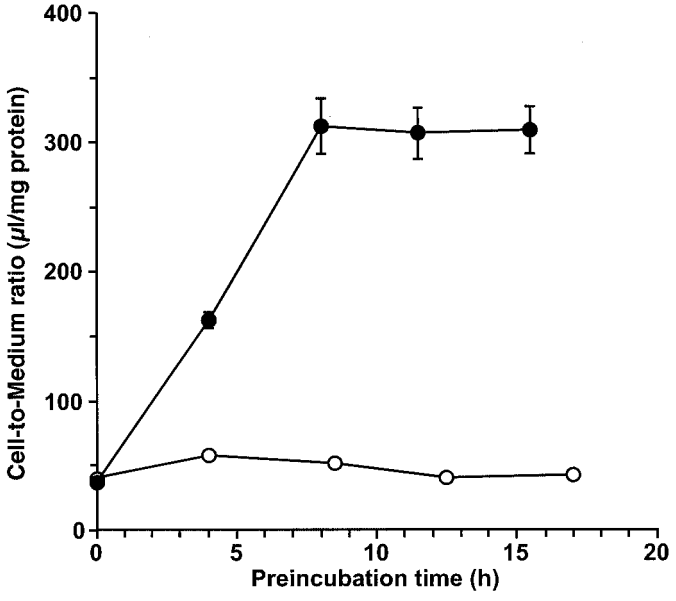

Fig. 7. Time courses of the hypertonic activation of [^{3}H]MeAIB uptake by TR-BBB13 cells. [^{3}H]MeAIB uptake (73 nM) was determined after incubation in normal (290 mOsm/kg, ○) and hypertonic (450 mOsm/kg, ●) culture medium. The error bar for normal culture medium incubation is smaller than the size of the symbol. Each point represents the mean ± S.E.M. (n = 3–4).

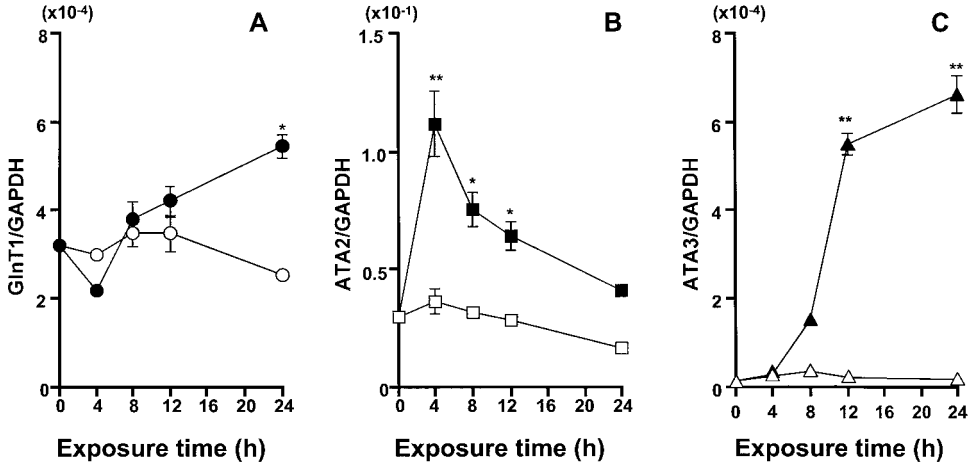

Fig. 8. Time-dependence of the hypertonic effect on the expression of GlnT (A), ATA2 (B), and ATA3 (C) mRNA in TR-BBB13 cells. TR-BBB13 cells were incubated in normal (290 mOsm/kg, open symbols) and hypertonic (450 mOsm/kg, filled symbols) culture medium over 24 h. Each point represents the mean ± S.E.M. (n = 4–8). ⋆, $p < 0.01$; ⋆⋆, $p < 0.001$ are significantly different from normal culture medium incubation (control).

mRNA is faster than that of GlnT and ATA3 mRNA (Fig. 8). The ATA2 mRNA level was increased for 4 h by hypertonic stress and then decreased thereafter (Fig. 8B). A similar finding was reported for cultured porcine endothelial cells during a 16-h exposure to hypertonic culture medium (Alfieri et al., 2001). The lack of agreement between the time course of MeAIB uptake and the ATA2 mRNA level needs some explanation. Conceivably, there may have been a time difference between transcription and the function of ATA2. MeAIB did not undergo efflux transport across the BBB (Fig. 1A). However, MeAIB uptake by TR-BBB13 cells occurred (Fig. 7). These results are in agreement with a previous report (Betz and Goldstein, 1978) and support the hypothesis that MeAIB can be taken up and interacts with L-Pro on the abluminal side but may not undergo efflux on the luminal (blood) side (Fig. 9). The uptake study using TR-BBB13 cells may represent the abluminal side of the BBB because of the lack of polarity. However, further studies are needed to clarify whether MeAIB undergoes efflux from TR-BBB13 cells to elucidate the efflux from the luminal side. Taken together, the up-regulation of solute transport and the ATA2 mRNA level in response to hypertonic stress in TR-BBB13 cells seems to be responsible for the osmo-regulation of system A at the BBB.

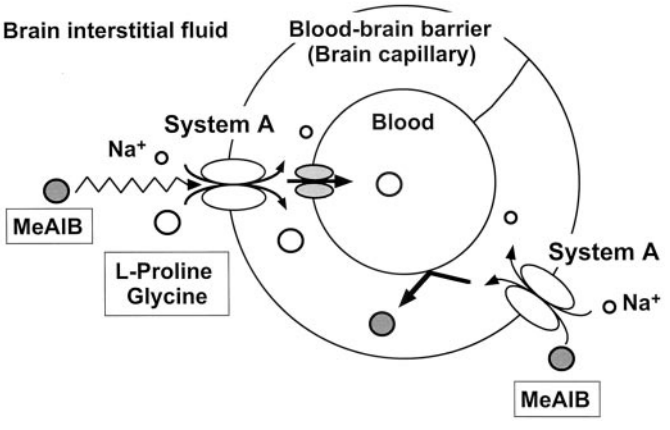

Fig. 9. The efflux transporter, system A, at the blood-brain barrier. System A is present in the abluminal side and carries L-Pro, Gly, and MeAIB. The other amino acid transporter may be present on the luminal side and carry L-Pro and Gly, but not MeAIB. MeAIB also inhibits system A–mediated L-Pro on the abluminal side. L-Pro and Gly undergo efflux transport across the BBB, but MeAIB does not.

The possible physiological and pathophysiological roles for the induction of system A at the BBB involve detoxification in the brain to maintain a constant environment for the neurons as well as a barrier function. Alterations in the osmolality and water distribution in the brain and cerebrospinal fluid compartments are a common occurrence in many neuropathological conditions such as brain edema, stroke hyponatremia, head injures, and hydrocephalus (De Petris et al., 2001). Therefore, the osmoregulatory transporters at the BBB play a pivotal role in maintaining osmolality in the brain. Although in the case of small neutral amino acids it is not clear whether they act as cellular osmolytes, the Na^+-coupled transporters at the BBB may be involved in the regulation of Na^+ efflux from the brain across the BBB. Together with the Na^+-dependent betaine/GABA transporter, GAT2/BGT-1 (Takanaga et al., 2001), and the Na^+-dependent taurine transporter (Komura et al., 1996) present at the BBB, these Na^+-coupled organic solute cotransporters also seem to be involved in the regulation of osmolality in the brain and in the maintenance of fluid balance across the BBB. GAT2/BGT-1 and TauT are also induced under hypertonic conditions (Bitoun and Tappaz, 2000). The cell volume regulation of brain capillary endothelial cells is also important for maintaining a barrier like the BBB because hypertonic conditions lead to the opening of the BBB (Tomiwa et al., 1982).

In conclusion, L-Pro and Gly, substrates of system A, were eliminated from rat brain, whereas MeAIB was retained (Fig. 9). This is the first direct evidence to prove the efflux mechanism of these neuroactive amino acids, and it also allows a better understanding of the function of the BBB with regard to the regulation of the concentration of amino acids in the brain. Up-regulation of the ATA2 mRNA level and transport activity seems to regulate the osmolality in the brain and the cell volume in brain capillary endothelial cells to maintain a stable environment in the brain and permit the BBB to function under pathological conditions.

Acknowledgments

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References

Alfieri RR, Petronini PG, Bonelli MA, Caccamo AE, Cavazzoni A, Borghetti AF, and Wheeler KP (2001) Osmotic regulation of ATA2 mRNA expression and amino acid transport system A activity. *Biochem Biophys Res Commun* **283:**174–178.

Asaba H, Hosoya K, Takanaga H, Ohtsuki S, Tamura E, Takizawa T, and Terasaki T (2000) Blood-brain barrier is involved in the efflux transport of a neuroactive steroid, dehydroepiandrosterone sulfate, via organic anion transporting polypeptide 2. *J Neurochem* **75:**1907–1916.
Barmack NH, Guo H, Kim HJ, Qian H, and Qian Z (1999) Neuronally modulated transcription of a glycine transporter in rat dorsal cochlear nucleus and nucleus of the medial trapezoid body. *J Comp Neurol* **415:**175–188.
Benrabh H and Lefauconnier JM (1996) Blood-endothelial cell and blood-brain transport of L-proline, alpha-aminoisobutyric acid, and L-alanine. *Neurochem Res* **21:**1227–1235.
Betz AL and Goldstein GW (1978) Polarity of the blood-brain barrier: neutral amino acid transport into isolated brain capillaries. *Science (Wash DC)* **202:**225–227.
Bitoun M and Tappaz M (2000) Gene expression of the transporters and biosynthetic enzymes of the osmolytes in astrocyte primary cultures exposed to hyperosmotic conditions. *Glia* **32:**165–176.
Cohen SM and Nadler JV (1997) Proline-induced potentiation of glutamate transmission. *Brain Res* **761:**271–282.
Cornford EM (1985) The blood-brain barrier, a dynamic regulatory interface. *Mol Physiol* **7:**219–259.
De Petris L, Luchetti A, and Emma F (2001) Cell volume regulation and transport mechanisms across the blood-brain barrier: implications for the management of hypernatraemic states. *Eur J Pediatr* **160:**71–77.
Hosoya K, Asaba H, and Terasaki T (2000a) Brain-to-blood efflux transport of estrone-3-sulfate at the blood-brain barrier in rats. *Life Sci* **67:**2699–2711.
Hosoya K, Sugawara M, Asaba H, and Terasaki T (1999) Blood-brain barrier produces significant efflux of L-aspartic acid but not D-aspartic acid: in vivo evidence using the brain efflux index method. *J Neurochem* **73:**1206–1211.
Hosoya K, Takashima T, Tetsuka K, Nagura T, Tomi M, Ohtsuki S, Takanaga H, Ueda M, Yanai N, Obinata M, et al. (2000b) mRNA expression and transport characterization of conditionally immortalized rat brain capillary endothelial cell lines: a new in vitro BBB model for drug targeting. *J Drug Target* **8:**357–370.
Hwang SM, Miller M, and Segal S (1983) Uptake of L-[^{14}C]proline by isolated rat brain capillaries. *J Neurochem* **40:**317–323.
Kakee A, Takanaga H, Terasaki T, Naito M, Tsuruo T, and Sugiyama Y (2001) Efflux of a suppressive neurotransmitter, GABA, across the blood-brain barrier. *J Neurochem* **78:**1–10.
Kakee A, Terasaki T, and Sugiyama Y (1996) Brain efflux index as a novel method of analyzing efflux transport at the blood-brain barrier. *J Pharmacol Exp Ther* **277:**1550–1559.
Komura J, Tamai I, Senmaru M, Terasaki T, Sai Y, and Tsuji A (1996) Sodium and chloride ion-dependent transport of beta-alanine across the blood-brain barrier. *J Neurochem* **67:**330–335.
Nadler JV, Wang A, and Hakim A (1988) Toxicity of L-proline toward rat hippocampal neurons. *Brain Res* **456:**168–172.
Norman PS and Mann GE (1986) Transport characteristics of system A in the rat exocrine pancreatic epithelium analyzed using the specific non-metabolized amino acid analogue alpha-methylaminoisobutyric acid. *Biochim Biophys Acta* **861:**389–394.
Oldendorf WH (1971) Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. *Am J Physiol* **221:**1629–1639.
Pardridge WM, Triguero D, Yang J, and Cancilla PA (1990) Comparison of in vitro and in vivo models of drug transcytosis through the blood-brain barrier. *J Pharmacol Exp Ther* **253:**884–891.
Phillis JW, Song D, and O'Regan MH (1999) Effects of hyperosmolarity and ion substitutions on amino acid efflux from the ischemic rat cerebral cortex. *Brain Res* **828:**1–11.
Preston JE, al-Sarraf H, and Segal MB (1995) Permeability of the developing blood-brain barrier to ^{14}C-mannitol using the rat in situ brain perfusion technique. *Brain Res Dev Brain Res* **87:**69–76.
Reimer RJ, Chaudhry FA, Gray AT, and Edwards RH (2000) Amino acid transport system A resembles system N in sequence but differs in mechanism. *Proc Natl Acad Sci USA* **97:**7715–7720.
Renick SE, Kleven DT, Chan J, Stenius K, Milner TA, Pickel VM, and Fremeau RT Jr (1999) The mammalian brain high-affinity L-proline transporter is enriched preferentially in synaptic vesicles in a subpopulation of excitatory nerve terminals in rat forebrain. *J Neurosci* **19:**21–33.
Sanchez del Pino MM, Peterson DR, and Hawkins RA (1995) Neutral amino acid transport characterization of isolated luminal and abluminal membranes of the blood-brain barrier. *J Biol Chem* **270:**14913–14918.
Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CA, van der Valk MA, Robanus-Maandag EC, te Riele HP, et al. (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77:**491–502.
Sershen H and Lajtha A (1979) Inhibition pattern by analogs indicates the presence of ten or more transport systems for amino acids in brain cells. *J Neurochem* **32:**719–726.
Shank RP and Campbell GL (1984) Amino acid uptake, content, and metabolism by neuronal and glial enriched cellular fractions from mouse cerebellum. *J Neurosci* **4:**58–69.
Smith QR and Stoll J (1998) Blood-brain barrier amino acid transport, in *Introduction to the Blood-Brain Barrier* (Pardridge WM ed) pp 188–197, Cambridge University Press, Cambridge.
Sugawara M, Nakanishi T, Fei YJ, Huang W, Ganapathy ME, Leibach FH, and Ganapathy V (2000a) Cloning of an amino acid transporter with functional characteristics and tissue expression pattern identical to that of system A. *J Biol Chem* **275:**16473–16477.
Sugawara M, Nakanishi T, Fei Y, Martindale RG, Ganapathy ME, Leibach FH, and Ganapathy V (2000b) Structure and function of ATA3, a new subtype of amino acid transport system A, primarily expressed in the liver and skeletal muscle. *Biochim Biophys Acta* **1509:**7–13.
Takanaga H, Ohtsuki S, Hosoya K, and Terasaki T (2001) GAT2/BGT-1 as a system responsible for the transport of γ-aminobutyric acid at the mouse blood-brain barrier. *J Cereb Blood Flow Metab* **21:**1232–1239.
Tayarani I, Lefauconnier JM, Roux F, and Bourre JM (1987) Evidence for an alanine, serine, and cysteine system of transport in isolated brain capillaries. *J Cereb Blood Flow Metab* **7:**585–591.
Terasaki T and Hosoya K (2001) Conditionally immortalized cell lines as a new in vitro model for the study of barrier functions. *Biol Pharm Bull* **24:**111–118.
Tomiwa K, Hazama F, and Mikawa H (1982) Reversible osmotic opening of the blood-brain barrier. Prevention of tissue damage with filtration of the perfusate. *Acta Pathol Jpn* **32:**427–435.
Tsuji A, Terasaki T, Takabatake Y, Tenda Y, Tamai I, Yamashima T, Moritani S, Tsuruo T, and Yamashita J (1992) P-glycoprotein as the drug efflux pump in primary cultured bovine brain capillary endothelial cells. *Life Sci* **51:**1427–1437.
Varoqui H, Zhu H, Yao D, Ming H, and Erickson JD (2000) Cloning and functional identification of a neuronal glutamine transporter. *J Biol Chem* **275:**4049–4054.
Yamaoka K, Tanigawara Y, Nakagawa T, and Uno T (1981) A pharmacokinetic analysis program (MULTI) for microcomputer. *J Pharmacobiodyn* **4:**879–885.
Yao D, Mackenzie B, Ming H, Varoqui H, Zhu H, Hediger MA, and Erickson JD (2000) A novel system A isoform mediating Na^+/neutral amino acid cotransport. *J Biol Chem* **275:**22790–22797.

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