City University of New York (CUNY)

CUNY Academic Works

Publications and Research

LaGuardia Community College

2006

Cationic amino acid transport across the blood-brain barrier is mediated exclusively by system y⁺

Robyn L. O'Kane

CUNY La Guardia Community College

Juan R. Viña

Universitat de Valencia

Ian Simpson

Penn State University

Rosa Zaragozá

Univseritat de Valencia

Ashwini Mokashi

The Chicago Medical School

See next page for additional authors

[How does access to this work benefit you? Let us know!](#)

More information about this work at: https://academicworks.cuny.edu/lg_pubs/53

Discover additional works at: <https://academicworks.cuny.edu>

This work is made publicly available by the City University of New York (CUNY).

Contact: AcademicWorks@cuny.edu

Authors

Robyn L. O'Kane, Juan R. Viña, Ian Simpson, Rosa Zaragozá, Ashwini Mokashi, and Richard A. Hawkins

Cationic amino acid transport across the blood-brain barrier is mediated exclusively by system y^+

Robyn L. O'Kane,¹ Juan R. Viña,^{2,4} Ian Simpson,³
Rosa Zaragoza,² Ashwini Mokashi,⁴ and Richard A. Hawkins⁴

¹Natural and Applied Science Department, LaGuardia Community College/City University of New York, Long Island City, New York; ²Departamento de Bioquímica & Biología Molecular, Facultad de Medicina, Universitat de Valencia, Valencia, Spain; ³Department of Neural and Behavioral Sciences, Milton S. Hershey Medical Center, Penn State University College of Medicine, Hershey, Pennsylvania; and ⁴Department of Physiology & Biophysics, The Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois

Submitted 5 January 2006; accepted in final form 24 March 2006

O'Kane, Robyn L., Juan R. Viña, Ian Simpson, Rosa Zaragoza, Ashwini Mokashi, and Richard A. Hawkins. Cationic amino acid transport across the blood-brain barrier is mediated exclusively by system y^+ . *Am J Physiol Endocrinol Metab* 291: E412–E419, 2006. First published March 28, 2006; doi:10.1152/ajpendo.00007.2006.—Cationic amino acid (CAA) transport is brought about by two families of proteins that are found in various tissues: Cat (CAA transporter), referred to as system y^+ , and Bat [broad-scope amino acid (AA) transporter], which comprises systems $b^{0,+}$, $B^{0,+}$, and y^+L . CAA traverse the blood-brain barrier (BBB), but experiments done in vivo have only been able to examine the BBB from the luminal (blood-facing) side. In the present study, plasma membranes isolated from bovine brain microvessels were used to identify and characterize the CAA transporter(s) on both sides of the BBB. From these studies, it was concluded that system y^+ was the only transporter present, with a prevalence of activity on the abluminal membrane. System y^+ was voltage dependent and had a K_m of $470 \pm 106 \mu M$ (SE) for lysine, a K_i of $34 \mu M$ for arginine, and a K_i of $290 \mu M$ for ornithine. In the presence of Na^+ , system y^+ was inhibited by several essential neutral AAs. The K_i values were 3–10 times the plasma concentrations, suggesting that system y^+ was not as important a point of access for these AAs as system L1. Several small nonessential AAs (serine, glutamine, alanine, and glycine) inhibited system y^+ with K_i values similar to their plasma concentrations, suggesting that system y^+ may account for the permeability of the BBB to these AAs. System y^+ may be important in the provision of arginine for NO synthesis. Real-time PCR and Western blotting techniques established the presence of the three known nitric oxide synthases in cerebral endothelial cells: NOS-1 (neuronal), NOS-2 (inducible), and NOS-3 (endothelial). These results confirm that system y^+ is the only CAA transporter in the BBB and suggest that NO can be produced in brain endothelial cells.

amino acid active transport; brain capillaries; endothelial cells; essential amino acids; nonessential amino acids; polarity

THE BLOOD-BRAIN BARRIER (BBB) surrounds almost the entire central nervous system and separates the brain from the circulation. Cerebral capillary endothelial cells, which have a larger number of tight junctions than what is found in other continuous capillaries, form the BBB. These tight junctions restrain paracellular movement and divide the membranes of the endothelial cells into two distinct sides, luminal (blood side) and abluminal (brain side), thereby creating polarity (50, 51).

Different populations of both lipids and intrinsic proteins (e.g., transporters) exist on the luminal and abluminal sides (3, 4, 46). Therefore, hydrophilic nutrients, such as amino acids (AAs), must pass two sheaths of membrane, the combined characteristics of which determine which molecules traverse the barrier and how quickly.

Studies of AA transport across the BBB in vivo and in vitro showed the BBB to be polarized with respect to AAs. The facilitative transport system L1 that transports large neutral amino acids (LNAA) exists on both the luminal and abluminal membranes (38, 39). The system X_{AG}^- that transports glutamate exists only on the luminal membrane, as does the system N that transports glutamine (26). However, no Na^+ -dependent systems have been found on the luminal membrane (1, 9, 32, 40, 41, 44). On the other hand, five transport systems have been found to date that are located on the abluminal membrane. These transporters include systems A, ASC, and N, which collectively transport neutral amino acids (NAA) (31), and Na^+ -LNAA, which transports LNAA (primarily essential). In addition there are at least three members of system EAAT (excitatory AA transporter) that transport acidic (or anionic) amino acids (AAA). These Na^+ -dependent systems may couple the Na^+ gradient that exists between the extracellular fluid (ECF) and endothelial cells to actively remove AAs from the brain's ECF. The organization of the Na^+ -dependent transporters on the abluminal membrane and facilitative transporters on the luminal membrane of the BBB explains why the concentrations of all AAs (except glutamine) are much lower in the ECF of brain than in the plasma; ECF concentrations are ~10% of the plasma values (42).

The situation for cationic amino acids (CAA; lysine, arginine, and ornithine) is less well known. The concentration of CAA in ECF is 10–30% that of plasma. A facilitative transporter of CAA exists, at least on the luminal side (32), and experiments in vivo indicate system y^+ as the primary mechanism for CAA transport (43). Although it has been proposed that system y^+ exists on both sides, this has not been measured directly. The other potential transporters of CAA include system y^+L , a broad-scope AA transporter that was first identified in human erythrocytes (16, 17, 34); system $B^{0,+}$, a Na^+ -dependent carrier that carries CAA (as well as NAA, although with less affinity) (53); and system $b^{0,+}$, a facilitative

Address for reprint requests and other correspondence: R. A. Hawkins, Dept. of Physiology & Biophysics, The Chicago Medical School, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, IL 60064–3095 (e-mail: RAH@post.harvard.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

transporter (53–55). Whether any other system besides γ^+ is present on the BBB has not been evaluated.

The lower concentration of CAA in ECF could be explained by brain metabolism or by the presence of a Na^+ -dependent transport system on the abluminal membranes. Therefore, the first objective was to study CAA transport using isolated luminal and abluminal plasma membrane vesicles from bovine BBB to identify and characterize the transporter(s) responsible.

One of the CAA, arginine, is the precursor of nitric oxide (NO). NO is a diffusible gas, originally called endothelium-derived relaxing factor, that regulates numerous physiological actions, including smooth muscle contraction, blood flow, and pressure (35). NO also acts as a signal to regulate gene expression, apoptosis, cell cycle, and differentiation (20). The biosynthesis of this physiological messenger requires L-arginine and O_2 for the NO synthase (NOS)-catalyzed reaction. Three isoforms of NOS have been identified: neuronal (NOS-1), inducible (NOS-2), and endothelial (NOS-3) (61). Endothelial cells do not have the ability to synthesize arginine de novo (60). Therefore, endothelial cells must rely on an external source of arginine; it seems that the availability of arginine is the determining factor in NO production by endothelial cells (60, 61).

Which forms of NOS exist in the BBB has not been studied. Thus the second objective of this study was to examine the presence of the enzymes required for NO production within the endothelial cells of brain.

MATERIALS AND METHODS

Materials. L-[^{14}C]lysine (290 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO), AAs, collagenase type IA, methyl-amino isobutyric acid (MeAIB), and 2-aminobicyclo(2.2.1)heptane-2-carboxylic acid (BCH) from Sigma (St. Louis, MO), and the Bio-Rad protein assay from Bio-Rad Laboratories (Hercules, CA).

Animals. Fresh bovine brains were bought from Aurora Meat Packing (North Aurora, IL). The cows were killed for food under US Department of Agriculture supervision, and the meat was sold for human consumption.

Preparation of membrane vesicles. Membrane vesicles from brain endothelial cells were prepared as previously described (38). Briefly, isolated microvessels from bovine cerebral cortices were obtained as described by Pardridge et al. (36). With the exception of one experiment wherein NOSs were studied, the microvessels were digested with collagenase type IA to remove the basement membrane, pericytes, and glial fragments. The refined microvessels were homogenized to release endothelial cell membranes containing both luminal and abluminal domains. In one experiment where the relative activity of CAA transport was to be determined, the membranes were further separated into luminal and abluminal membranes as previously described (38). The luminal vesicles were 90% pure, whereas the abluminal membranes were 80% pure. The assessment of purity was as described by Sánchez del Pino et al. (37, 39) using γ -glutamyl transpeptidase as a luminal membrane marker and MeAIB transport (system A) activity as an abluminal marker. The vesicles were stored at -70°C until they were used.

RNA extraction and PCR analysis. Total RNA was isolated from bovine microvessels (both treated with collagenase type IA and untreated) using the TRIzol reagent (Invitrogen Life Technologies, Frederick, MD). Reverse transcription (RT) and polymerase chain reaction (PCR) were performed in one step using the TTh DNA polymerase kit (Roche Diagnostics). The mRNA expression was studied by real-time PCR (iCycler iQ real-time PCR detection system), and specific oligonucleotides for NOS-1, 5'-TGTTGGTGGAT-

GCTTGTGT-3' and 5'-AGTGGTTTCCTGGTGAGGTG-3'; NOS-2, 5'-CTCCAGAATCCCTGAGCAAG-3' and 5'-TTTTGGGGTTCATGATGGAT-3'; NOS-3, 5'-CCAGCTCAAGACTGGAGACC-3' and 5'-TCAATGTCATGCAGCCTCTC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, 5'-GGGTCATCATCTCTGCACCT-3' and 5'-GGTCATAAGTCCCTCCACGA-3'; were used. The mRNA detection was carried out by measuring the binding of the fluorescent dye SYBR Green I to double-stranded DNA. The threshold cycle (C_T) was determined, and then the relative gene expression was expressed as follows: fold change = $2^{-\Delta(\Delta C_T)}$, where $\Delta C_T = C_T$ (target) – C_T (housekeeping) and $\Delta(\Delta C_T) = \Delta C_T$ (treated) – ΔC_T (control).

For semiquantitative PCR, the resulting PCR products were separated by electrophoresis in a 1.2% agarose gel in Tris-borate-EDTA (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0) and stained with ethidium bromide. The expected sizes for the PCR products were 176 (GAPDH), 221 (NOS-1), 171 (NOS-2), and 156 bp (NOS-3).

Protein extraction and immunoblotting. The “digested” and “undigested” cow capillaries (0.1 g) were homogenized (Ultra-Turrax; IKA-Werke, Staufen, Germany) in 1 ml of extraction buffer (0.2 mM HEPES, pH 7.9, 20% glycerol, 250 mM KCl, 2 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 5 mM NaF, 0.5 mM Na_3VO_4 , and 0.1% Triton X-100) in the presence of protease inhibitors (5 $\mu\text{l/ml}$ of protease inhibitor cocktail from Sigma). The resulting homogenate was centrifuged at 20,000 g for 20 min at 4°C , and supernatants were normalized for protein concentration using the bicinchoninic acid protein assay reagent (Pierce Chemical, Rockford, IL).

Equal amounts of protein (20 μg) were then boiled in sample buffer for 5 min and separated by SDS-PAGE. After electrophoresis, the proteins were transferred to 0.45- μm pore-size nitrocellulose. The procedure was the same for all antibodies. The membranes were incubated in blocking solution (5% wt/vol nonfat dry milk with 0.05% vol/vol Tween-20) for 1 h at room temperature. This was followed by three washes with Tween-Tris-buffered saline (TTBS; 25 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% vol/vol Tween-20). Next, blots were incubated with primary antibodies to NOS-1, NOS-2, and NOS-3. Rabbit polyclonal antibodies were from Affinity Bioreagents, and mouse monoclonal α -tubulin was from Santa Cruz Biotech (Santa Cruz, CA) in TTBS for 1 h at room temperature. Blots were washed again with TTBS and incubated with the secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotech) for 1 h at room temperature. Finally, blots were washed with TTBS, and antibody was detected with luminol reagent (Supersignal West Pico; Pierce Chemical).

Measurement of transport rates. Experiments were performed by a rapid filtration method (39). Membrane vesicles were thawed, centrifuged at 37,500 g for 25 min at 4°C , and suspended in storage buffer (290 mM mannitol, 10 mM HEPES, pH 7.4). Vesicles were allowed to equilibrate overnight at 4°C . The final concentration of protein was between 2.5 and 5 μg protein/ μl . The vesicle suspensions were divided into 5- μl aliquots that were preincubated at 37°C for 1 min before initiation of transport measurements. Reaction medium containing ^3H - or ^{14}C -labeled substrate, with or without competing substrate, was added to initiate the reaction. The concentration of extravesicular NaCl, KCl, or choline Cl at the start of the measurements was 100 mM, and the internal concentration was zero unless otherwise stated (osmolarity was adjusted to 300 by including 90 mM mannitol, 10 mM HEPES, pH 7.4). Reaction times were 10, except for time course studies. Reactions were stopped by the addition of 1 ml of an ice-cold stopping solution (145 mM NaCl and 10 mM HEPES, pH 7.4) and the immediate filtering on a 0.45- μm Gelman Metricel filter (Ann Arbor, MI) under vacuum. The filtered membranes were immediately washed four times with 1-ml aliquots of stopping solution, after which the filters were counted by liquid scintillation spectroscopy. Unless otherwise indicated, all rates were corrected for nonspecific transport, binding, or trapping, as measured in the presence of a saturating dose of unlabeled substrate.

Determination of kinetic characteristics. Apparent K_m and V_{max} values for lysine were determined by measuring the initial rates of transport at various concentrations. From these data, a nonlinear regression analysis was performed using Sigma Plot (SPSS, Chicago, IL), and apparent K_m and V_{max} were calculated. The initial rates of clearance were obtained by dividing the rate of transport of tracer by the substrate concentrations or by dividing the V_{max} by the K_m .

Inhibition studies: K_i calculations. In some experiments, AAs or inhibitors were included in the reaction medium. The velocity with and without the putative substrates was measured, and the percentage of inhibition was determined.

Transmembrane potential effect on transport. To create different initial transmembrane potentials, valinomycin (12.5 $\mu\text{g}/\text{mg}$ protein), a K^+ -specific ionophore, was used to increase the permeability of vesicles to K^+ . Different ratios of internal and external concentrations of K^+ were used to establish potential differences ranging from -80.7 to 10.7 mV. The vesicles were prepared overnight with equilibrating solutions containing 25 or 100 mM KCl, 10 mM HEPES, and mannitol to bring the final osmolarity to 300 mOsm.

The reaction was initiated by the addition of an appropriate volume and composition of extravascular solution to create the desired initial transmembrane potential as calculated by the Nernst equation.

To measure voltage sensitivity, the equation for a sigmoid curve was used to fit the data, using Sigma Plot and Microsoft Excel:

$$y = \frac{a}{1 + \exp\left(-\frac{x - x_0}{k}\right)}$$

where a is the maximal initial rate, x_0 is the midpoint, and k is the steepness of the curve at the midpoint. Once k was determined, the effective valence (z) associated with transport was calculated (22):

$$k = \frac{RT}{zF}$$

At 37°C , RT/F is 26.7 mV (R is the gas constant, $8.3 \text{ J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$, T is the temperature in Kelvin, and F is Faraday's constant, $9.7 \times 10^4 \text{ C/mol}$).

Protein determination. Protein concentrations were determined using the Bio-Rad Protein Microassay, with bovine serum albumin as the standard, on the basis of the method of Bradford (6).

Statistical analyses. Curves were fitted by Sigma Plot and/or Microsoft Excel, and data were analyzed with StatView (SAS, Cary, NC) using ANOVA and Fisher's least significant difference test. Values were considered significant at $P < 0.05$.

RESULTS

Lysine transport is not Na^+ dependent. To determine whether Na^+ -dependent CAA transport occurs in BBB membranes, the uptake of lysine was measured in the presence of an inwardly directed Na^+ or choline $^+$ gradient using a mixed-membrane preparation (both luminal and abluminal membranes). To date, all Na^+ -dependent carriers have been found only on the abluminal membrane (19). However, by using a mixed-membrane preparation it was possible to exclude Na^+ dependency from both membranes in one experiment. The presence of a Na^+ gradient did not enhance transport; rather, it inhibited the rate of uptake (Fig. 1). Because no Na^+ -dependent transport could be detected, mixed membranes were used for the remaining experiments, except where otherwise indicated.

Lysine transport is not mediated by system $b^{0,+}$. Although the most common transporter of CAA is system y^+ (27), other facilitative systems of CAA have been described. One such

Fig. 1. Lysine uptake in the presence and absence of a Na^+ gradient. The uptake of [^{14}C]lysine (30 μM) was measured over time in the presence of an inwardly directed Na^+ or choline gradient (100 mM external, internal nil). Mixed membranes (both luminal and abluminal) were used to determine whether any evidence for Na^+ -dependent transport could be detected. Each point represents the mean of 3 individual determinations $\pm$ SE expressed as $\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$.

system is $b^{0,+}$. It is Na^+ independent and was originally described in blastocysts and later shown to be important in human kidney (17, 27, 34). To explore whether $b^{0,+}$ is active in the BBB, we measured the uptake of lysine in the presence of harmaline, a cation that is a particularly strong inhibitor of system $b^{0,+}$ (54). We detected no significant inhibition of lysine uptake in the presence of harmaline and, therefore, no evidence for system $b^{0,+}$ activity (Fig. 2).

Lysine transport is inhibited by homoarginine. System y^+ activity is identifiable by the use of homoarginine, a CAA (58). Therefore, we tested the effect of homoarginine on lysine uptake. Lysine transport was inhibited almost completely in the presence of either an inwardly directed Na^+ gradient or a choline $^+$ gradient (Fig. 3). These results support that lysine transport is mediated by system y^+ .

Kinetics and voltage sensitivity of lysine uptake. The kinetic characteristics of lysine transport were measured in the presence and absence of a -18.4 -mV initial transmembrane potential (Fig. 4). The apparent K_m values at 0 and -18 mV were 470 ± 106 and $920 \pm 310 \mu\text{M}$ SE (not different statistically, $P > 0.05$), respectively; the corresponding V_{max} values were 1.3 ± 0.1 and 2.2 ± 0.3 (SE) $\text{nmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$. These K_m values are slightly higher than those reported in some cell types (10, 12, 15), yet they were within the range for isoforms of the y^+ transporter reported by others (13). The corresponding clearance values were 1.9 and $2.4 \mu\text{l}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, respectively. Although it appeared that the negative transmembrane potential (-18 mV) increased the rate of transport, the results were not definitive; the V_{max} values were statistically significant ($P < 0.05$), but the K_m values were not. Therefore, the influence of transmembrane potential was further analyzed by measuring the initial rate of lysine transport over a range of transmembrane potentials (Fig. 5). The data (expressed as $\text{pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$) had a maximal rate of $520 \pm 60 \text{ pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, the midpoint of the curve was 28 ± 9 mV, and the slope of the curve at the midpoint was -27 ± 6 mV. The data indicated that lysine transport was voltage sensitive and were consistent with one positive charge translocated per lysine molecule.

Distribution of system y^+ on the BBB. The distribution of functional carriers between the luminal and abluminal mem-

Fig. 2. Lysine uptake is not inhibited by harmaline. Uptake of [14 C]lysine (70 μ M) was measured in the presence and absence of 2 mM harmaline (a potent inhibitor of the facilitative system $b^{0,+}$) with and without an inwardly directed Na^+ or K^+ gradient (100 mM external, internal nil). (In this instance, K^+ was used instead of choline as the control.) Each point represents the mean of 5 individual determinations $\pm$ SE expressed as $\text{pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$. There were no statistically significant differences.

brane domains was calculated using an “ f value” analysis, as described by Sánchez del Pino et al. (37). An f value of 1 means that carrier activity is limited to the luminal membrane, whereas an f value of 0 indicates an abluminal position. Intermediate numbers describe the relative distribution of active carriers between both membranes. Membranes were separated into five fractions, as described by Sánchez del Pino et al. (38). The f value associated using data from the purist fractions 1 (90% pure) and 3 (70% pure) was 0.17 ± 0.04 SE. Therefore, the location of CAA transport activity was predominantly abluminal, with some activity on the luminal membrane.

Potential substrates of system y^+ transport. System y^+ has been reported to transport some NAA in the presence of Na^+ (10, 11). Lysine transport was measured in the presence of alanine and serine (20 mM each), and a range of NaCl concentrations from 0 to 100 mM to determine the optimal concentration of Na^+ (Fig. 6). In the absence of NaCl, alanine and serine did not inhibit lysine uptake significantly. However,

Fig. 3. Lysine uptake is inhibited by homoarginine (HA). HA has been identified as a potent inhibitor of the y^+ system (58). Therefore, we tested the effect of HA on lysine transport. Uptake of [14 C]lysine (90 μ M) was measured in the presence and absence of HA (20 mM) in the presence of an inwardly directed choline (Ch) gradient (100 mM external, internal nil). Each point represents the mean of 5 individual determinations $\pm$ SE expressed as $\text{pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$. There were no statistically significant differences found between any pairs of data.

Fig. 4. Substrate-velocity curves of lysine at 2 different transmembrane potentials. [14 C]lysine uptake over a range of concentrations (40 μ M to 3 mM) and at 2 different transmembrane potentials (0 and -18.4 mV). Transmembrane potentials were calculated by the Nernst equation (MATERIALS AND METHODS). Each point represents the mean of 5 individual determinations $\pm$ SE expressed as $\text{pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$. *Statistical significance ($P < 0.05$) compared with their respective control values.

when NaCl was present, lysine uptake was inhibited by both amino acids in a dose-dependent manner up to 100 mM, the maximum concentration of NaCl tested.

The uptake of lysine was measured in the presence of various AAs (2 mM) to determine the potential substrates of system y^+ (Table 1). Lysine uptake was inhibited mostly by the two CAA arginine and ornithine (98 and 80% respectively); thus the affinity of system y^+ is arginine > lysine > ornithine.

Additionally, several NAA manifested significant inhibition. However, the K_i values for these essential NAA were 3–10 times greater than the reported plasma concentrations (42, 43). Therefore, whereas system y^+ may supplement the activity, the primary carrier of essential NAA is a Na^+ -independent system L1 (44). Interestingly, the K_i values for several nonessential NAA, i.e., serine, glutamine, alanine, and glycine are in the same range as their plasma concentrations (42).

System y^+ may supply arginine for NO synthesis in microvessels. NO is synthesized from O_2 and arginine, and in addition to the activity of NOS, the availability of arginine is a

Fig. 5. Uptake of lysine is sensitive to a transmembrane potential. Uptake of [14 C]lysine (30 μ M) was measured over a range of transmembrane potentials (-80.7 to 10.7 mV). Transmembrane potentials were calculated by the Nernst equation (MATERIALS AND METHODS). Each point represents 1–3 individual determinations $\pm$ SE expressed as $\text{pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$. Maximal initial rate was 520 ± 60 (SE) $\text{pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$, the midpoint of the curve was 28 ± 9 mV, and the slope of the curve at the midpoint was -27 ± 6 mV. Effective valence associated with lysine transport was 1.

Fig. 6. Determination of the Na⁺ concentration necessary to demonstrate inhibition of system y⁺ by small neutral amino acids. Uptake of [¹⁴C]lysine (30 μ M) was measured over a range of Na⁺ concentrations (0–100 mM) in the presence of alanine and serine (20 mM each), 2 neutral amino acids that were reported to inhibit system y⁺ (10, 11). Velocity values were normalized to the controls (uptake of lysine at each concentration of Na with no inhibitors) and normalized to a value of 100 $\pm$ (SE). *Statistical significance ($P < 0.05$) compared with their respective control values.

determining factor (14, 27). RT-PCR was used to probe two preparations for mRNA that was specific to the three known NOS (neuronal, endothelial, and inducible) in microvessels that were not treated with collagenase IA and microvessels that were. (Collagenase was used to remove the basement membrane, adhering astrocyte membranes, and pericytes.) The results indicated that all three forms were expressed at the same time in the BBB (Fig. 7). Quantitative PCR showed similar results (data not shown).

Table 1. Amino acid spectrum of system y⁺ in the presence of Na⁺

Inhibitor	Normalized Velocity, %	Inhibition, %	K _i , μ M
None (NaCl) (9)	100 $\pm$ 5		
Arginine (4)	2 $\pm$ 4	98	34
Ornithine (2)	20 $\pm$ 5	80	290
Cysteine (4)	44 $\pm$ 10	56	530
Serine (5)	45 $\pm$ 5	55	540
Phenylalanine (4)	52 $\pm$ 4	48	590
Glutamine (5)	56 $\pm$ 9	44	620
Histidine (5)	58 $\pm$ 12	42	630
Alanine (5)	59 $\pm$ 8	41	640
Threonine (4)	63 $\pm$ 8	37	670
Valine (4)	64 $\pm$ 3	36	680
Glycine (5)	64 $\pm$ 5	36	680
Methionine (4)	66 $\pm$ 10	34	690
Leucine (5)	71 $\pm$ 7		
Isoleucine (4)	71 $\pm$ 8		
Tyrosine (4)	74 $\pm$ 7		
Proline (5)	77 $\pm$ 14		
Tryptophan (5)	102 $\pm$ 28		
Asparagine (5)	109 $\pm$ 27		

Velocity values were normalized to the control velocity and are means $\pm$ SE, with the number of determinations in parentheses. Membrane vesicles were incubated with 73 μ M [¹⁴C]lysine ($K_m = 470 \mu$ M) in the presence of 100 mM NaCl. Inhibition of [¹⁴C]lysine transport by several basic and neutral amino acids (2 mM) was determined. Background was measured in the presence of 20 mM homoarginine. Percent inhibition and K_i values were calculated only for those amino acids that were statistically significant ($P \leq 0.05$). Statistical analyses were performed using ANOVA and Fisher's least significant difference test for multiple comparisons. See MATERIALS AND METHODS for the formula to calculate the K_i values.

Fig. 7. mRNA exists in endothelial cells for neuronal nitric oxide synthase (NOS-1), inducible NOS (NOS-2), and endothelial NOS (NOS-3). Electrophoretic analysis of RT-PCR was performed on a 1.2% agarose gel stained with ethidium bromide. Total RNA was amplified by RT-PCR using specific primers (MATERIALS AND METHODS). A: RNA from undigested microvessels. B: RNA from microvessels that were treated with collagenase IA to remove the basement membranes, pericytes, and any adhering astrocyte processes.

Membrane proteins from each preparation of microvessels, collagenase-treated and untreated (20 μ g protein/lane), were electrophoresed and immunoblotted with specific antibodies (Fig. 8). The results corroborated the mRNA data; all three forms of NOS were expressed with the greatest intensity in the endothelial cell layer (collagenase digested preparation).

DISCUSSION

As mentioned, plasma membrane CAA transport is carried out by two families of proteins: Cat, commonly referred to as the system y⁺; and Bat, which comprises systems b^{0,+}, B^{0,+}, and y⁺L (13). These transporters and some of their characteristics are shown in Table 2. In a series of studies employing enriched luminal and abluminal vesicles, we have shown that several Na⁺-dependent AA transport systems exist on the abluminal membrane (29–31). No Na⁺-dependent systems have been found on the luminal membrane. There are one or more Na⁺-dependent systems capable of transporting all nat-

Fig. 8. Western blot analysis of NOS-1, NOS-2, and NOS-3 expression. Membrane proteins (20 μ g/lane) were electrophoresed and immunoblotted with specific antibodies (MATERIALS AND METHODS). Lane A was from collagenase-treated microvessels. Lane B was from untreated microvessels.

Table 2. *Cationic amino acid transport systems*

System	Na ⁺ Dependence	Primary Substrates	Inhibitors	Voltage Sensitive
y ⁺	No	Lys, Arg, Orn NAA (weak)	homoarginine	Yes
b ^{0,+}	No	Lys, Arg, Leu	harmaline	No
y ⁺ L	No	Lys, Arg, Orn	N-methylmaleimide	
	Yes	Leu, Met, Gln other NAA		
B ^{0,+}	Yes	Lys, Arg, Ala NAA	BCH	

NAA, neutral amino acids; BCH, 2-aminobicyclo(2.2.1)heptane-2-carboxylic acid. Primary references: y⁺ (10); b^{0,+} (55); y⁺L (18); B^{0,+} (52).

urally occurring NAA and AAA from the ECF to the endothelial cells (29–31).

Transporter system B^{0,+} is the only Na⁺-dependent carrier that carries CAA (as well as NAA, although with less affinity) and is inhibited by BCH (53). We found no evidence for system B^{0,+} in the BBB (29). First, the uptake of lysine was not stimulated by a Na⁺ gradient; rather, uptake in the presence of a Na⁺ gradient was depressed compared with lysine uptake in the presence of choline⁺ (Fig. 1). Second, it was previously shown that the rate of lysine transport was not inhibited by the presence of concentrations of BCH up to 10 mM (29). The CAA are unique because all other naturally occurring AAA and NAA examined to date have Na⁺-dependent transporters on the abluminal membrane that are capable coupling the Na⁺ gradient existing between the ECF and BBB endothelial cells to transport AAs out of the ECF (19); facilitative transport seems to be the only mechanism on the BBB to allow the movement of CAA. There are three known facilitative transport systems (Na⁺ independent), b^{0,+}, y⁺L, and y⁺, that transport CAA. To obtain a more complete understanding of CAA transport across the BBB, we determined which of these transporters are participating in the movement of CAA across the BBB. 1) System b^{0,+} is a Na⁺-independent AA transporter discovered in mouse blastocysts (53, 55). System b^{0,+} accepts CAA as well as bulky NAA that do not branch at the β -carbon. In humans, a defect in expression of b^{0,+} leads to cystinuria (17, 27, 34). The cation harmaline is a particularly strong inhibitor of system b^{0,+} (54). We detected no statistically significant inhibition of lysine uptake (Fig. 2) and, therefore, no support for the hypothesis that b^{0,+} is active in cerebral endothelial cells. 2) System y⁺L is a broad-scope AA transporter that was first identified in human erythrocytes (16, 18, 34). System y⁺L has two distinctive properties; it can bind and translocate CAA as well as NAA, and its specificity varies depending on the ionic composition of the medium. In a Na⁺ medium it transports a variety of LNAA with a particular affinity for leucine, isoleucine (27). The data do not support the hypothesis that y⁺L is active in the BBB. First, lysine transport in the BBB is voltage sensitive (Fig. 5), whereas system y⁺L is not (27). Second, leucine, for which y⁺L has a strong affinity, did not inhibit lysine transport significantly (Table 1). Third, system y⁺L has a strong affinity for lysine in the low μ M range, whereas the affinity in membrane vesicles was 470 μ M. These observations, together with the demonstration that homoarginine eliminates lysine transport almost completely (Fig. 3), leads to the conclusion that system y⁺L is not active,

or at best could be only a minor component. 3) System y⁺ is a CAA transport system that carries lysine, arginine and ornithine (10). System y⁺ is voltage sensitive, inhibited by homoarginine and by NAA in the presence of alkali metal ions Na⁺ > Li⁺ > K⁺ (17, 27, 48, 58). In Fig. 3, it is shown that lysine uptake was inhibited almost completely by homoarginine. Therefore, the majority of observable lysine transport could be ascribed to system y⁺. Our kinetic data support those of Stoll et al. (45), who reported that the presence of carrier y⁺ is 40 times higher in cerebral microvessels than whole brain.

Our data show that lysine transport in BBB membranes is sensitive to the transmembrane potential, which is another feature of system y⁺ (23). The data were consistent with one positive charge translocated per lysine molecule. Because Na⁺ does not stimulate transport and lysine has a positive charge at pH 7, it may be inferred that lysine is drawn into endothelial cells by the negative transmembrane potential that normally exists (2). The combined electrical and chemical driving forces will determine the direction of CAA flux. Assuming cerebral spinal fluid concentrations of CAA are similar to those of the ECF (about 10–30% those in plasma) (42), the net movement of CAA would occur from plasma to brain.

Although Stoll et al. (45) measured lysine flux from plasma to brain, y⁺ could only be inferred to exist on both luminal and abluminal membranes (44). However, with their experimental technique, no direct measurements of abluminal transport activity could be made to support this assumption. Our data show that, although lysine transport activity was present on both membranes, the abluminal membranes had about four times the activity.

Several nonessential NAA (e.g., serine, glutamine, alanine, and glycine) inhibited y⁺ transport, presumptive evidence that small NAA may be transported by system y⁺. Interestingly, the K_i values were in the same range as the reported plasma concentrations (42). In a seminal article on BBB permeability, Oldendorf (32) reported that essential AA uptake was greater than nonessential AA uptake but did not rule out transport of nonessential AA. Review of Oldendorf's data showed some transport of serine, alanine, proline, and glycine did occur. It seems likely that these small, nonessential AA enter the brain at a slow rate on the y⁺ system. Although these small AAs are not necessary, nor are neurotransmitters (e.g., glycine), Na⁺-dependent mechanisms exist on the abluminal membrane that are capable of returning small NAA to the plasma (29, 31).

Arginine availability is essential for the biosynthesis of the vasodilator NO, formally known as endothelium-derived relaxing factor because of its ability to relax smooth muscle and cause vasodilation (35, 56). The synthesis of NO is mediated by the enzyme NOS that converts arginine and molecular oxygen to citrulline and NO. Three distinct isoforms of the enzyme have been identified: Ca²⁺/calmodulin-dependent NOS-1, NOS-2, and NOS-3. Aside from the distribution of NOS enzymes, it appears that the astrocyte is the major storage site for arginine (59), and a neuron-astrocyte shuttle has been proposed to provide the neuron with arginine (57). Thus the detection of greater activity of system y⁺ on the abluminal membrane and the proximity of the astrocytic end feet may enable rapid equilibration with the ECF and thereby provide arginine to the astrocytes and neurons. In any event, our RT-PCR and Western blotting studies revealed that all three NOS enzymes are present in endothelial cells and suggests that

the endothelial cell has a significant capacity to produce NO. However, the function of the NO generated is not clear, as the endothelial cells of the BBB are not ensheathed with smooth muscle (5), and thus it is unlikely that NO is mediating changes in blood flow.

NO may play a role in the maintenance of the BBB (25, 28, 47), communication with astrocytes (8, 24, 49), the control of ion and nutrient transport (7, 27), and the control of platelet aggregation (33, 56). The last may explain an observation of Hernandez et al. (21). They fed monkeys an atherogenic diet and observed extensive atherosclerosis throughout the body, with the exception of cerebral vessels. Thus cerebral vessels appear to be protected, or perhaps the progression of atherosclerotic cerebrovascular disease lags behind that of the rest of the body.

In conclusion, y^+ seems to be the primary system for transporting CAA into the brain. No evidence could be found for other transporters. In contrast to AAA and NAA, for which Na^+ -dependent carriers are present on the abluminal membrane, there were no detectable Na^+ -dependent transport systems found for CAA.

GRANTS

This work was supported by National Institutes of Health Grants NS-041405 (I. Simpson); Ministerio de Ciencia y Tecnología y Plan Nacional (BFU2004-01965); Conselleria de Educación, Cultura y Deporte (GRUPOS04/01), and Instituto de Salud Carlos III (RCMN, RC03/08), Madrid, Spain (J. R. Viña); the Carmen y Severo Ochoa postdoctoral fellowship (supported by the Ayuntamiento de Valencia, Valencia, Spain; R. Zaragosa); and the International Glutamate Technical Committee (R. A. Hawkins).

REFERENCES

- Battistin L, Grynbaum A, and Lajtha A. The uptake of various amino acids by the mouse brain in vivo. *Brain Res* 29: 85–99, 1971.
- Betz AL. Transport of ions across the blood-brain barrier. *Fed Proc* 45: 2050–2054, 1986.
- Betz AL, Firth JA, and Goldstein GW. Polarity of the blood-brain barrier: distribution of enzymes between the luminal and antiluminal membranes of brain capillary endothelial cells. *Brain Res* 192: 17–28, 1980.
- Betz AL and Goldstein GW. Polarity of the blood-brain barrier: neutral amino acid transport into isolated brain capillaries. *Science* 202: 225–226, 1978.
- Bradbury M. *The Concept of a Blood-Brain Barrier*. New York: John Wiley & Sons, 1979.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Anal Biochem* 72: 248–254, 1976.
- Chen Y, McCarron RM, Golech S, Bembry J, Ford B, Lenz FA, Azzam N, and Spatz M. ET-1- and NO-mediated signal transduction pathway in human brain capillary endothelial cells. *Am J Physiol Cell Physiol* 284: C243–C249, 2003.
- Chen Y and Swanson RA. Astrocytes and brain injury. *J Cereb Blood Flow Metab* 23: 137–149, 2003.
- Christensen HN. Developments in amino acid transport, illustrated for the blood-brain barrier. *Biochem Pharmacol* 28: 1989–1992, 1979.
- Christensen HN and Antonioli JA. Cationic amino acid transport in the rabbit reticulocyte. Na^+ -dependent inhibition of Na^+ -independent transport. *J Biol Chem* 244: 1497–1504, 1969.
- Christensen HN and Handlogten ME. Reactions of neutral amino acids plus Na^+ with a cationic amino acid transport system. *FEBS Lett* 3: 14–17, 1969.
- Closs EI, Albritton LM, Kim JW, and Cunningham JM. Identification of a low affinity, high capacity transporter of cationic amino acids in mouse liver. *J Biol Chem* 268: 7538–7544, 1993.
- Closs EI, Simon A, Vekony N, and Rotmann A. Plasma membrane transporters for arginine. *J Nutr* 134: 2752S–2759S, discussion 2765S–2767S, 2004.
- Cylwik D, Mogielnicki A, and Buczek W. L-arginine and cardiovascular system. *Pharmacol Rep* 57: 14–22, 2005.
- Deves R, Angelo S, and Chavez P. N-ethylmaleimide discriminates between two lysine transport systems in human erythrocytes. *J Physiol* 468: 753–766, 1993.
- Deves R, Angelo S, and Rojas AM. System y^+ : the broad scope and cation modulated amino acid transporter. *Exp Physiol* 83: 211–220, 1998.
- Deves R and Boyd CA. Transporters for cationic amino acids in animal cells: discovery, structure, and function. *Physiol Rev* 78: 487–545, 1998.
- Deves R, Chavez P, and Boyd CA. Identification of a new transport system (y^+) in human erythrocytes that recognizes lysine and leucine with high affinity. *J Physiol* 454: 491–501, 1992.
- Hawkins RA, O’Kane RL, Simpson IA, and Vina JR. Structure of the blood-brain barrier and its role in the transport of amino acids. *J Nutr* 136: 218S–226S, 2006.
- Hemish J, Nakaya N, Mittal V, and Enikolopov G. Nitric oxide activates diverse signaling pathways to regulate gene expression. *J Biol Chem* 278: 42321–42329, 2003.
- Hernandez M, Brennan R, White W, and Brierly J. Cerebral vascular reactivity in the arteriosclerotic monkey (Abstract). *3rd International Joint Meeting on Stroke and Cerebral Circulation*, New Orleans, LA, 1978.
- Hille B. *Ionic Channels of Excitable Membranes*. Sunderland, MA: Sinauer Associates, 1992.
- Kavanaugh MP. Voltage dependence of facilitated arginine flux mediated by the system y^+ basic amino acid transporter. *Biochemistry* 32: 5781–5785, 1993.
- Krizanac-Bengez L, Kapural M, Parkinson F, Cucullo L, Hossain M, Mayberg MR, and Janigro D. Effects of transient loss of shear stress on blood-brain barrier endothelium: role of nitric oxide and IL-6. *Brain Res* 977: 239–246, 2003.
- Krizbai IA and Deli MA. Signalling pathways regulating the tight junction permeability in the blood-brain barrier. *Cell Mol Biol (Noisy-le-grand)* 49: 23–31, 2003.
- Lee WJ, Hawkins RA, Viña JR, and Peterson DR. Glutamine transport by the blood-brain barrier: a possible mechanism for nitrogen removal. *Am J Physiol Cell Physiol* 274: C1101–C1107, 1998.
- Mann GE, Yudilevich DL, and Sobrevia L. Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol Rev* 83: 183–252, 2003.
- Mayhan WG. Regulation of blood-brain barrier permeability. *Microcirculation* 8: 89–104, 2001.
- O’Kane RL and Hawkins RA. Na^+ -dependent transport of large neutral amino acids occurs at the abluminal membrane of the blood-brain barrier. *Am J Physiol Endocrinol Metab* 285: E1167–E1173, 2003.
- O’Kane RL, Martinez-Lopez I, DeJoseph MR, Viña JR, and Hawkins RA. Na^+ -dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) of the blood-brain barrier. A mechanism for glutamate removal. *J Biol Chem* 274: 31891–31895, 1999.
- O’Kane RL, Vina JR, Simpson I, and Hawkins RA. Na^+ -dependent neutral amino acid transporters (A, ASC, and N) of the blood-brain barrier: mechanisms for neutral amino acid removal. *Am J Physiol Endocrinol Metab* 287: E622–E629, 2004.
- Oldendorf WH. Uptake of radiolabeled essential amino acids by brain following arterial injection. *Proc Soc Exp Biol Med* 136: 385–386, 1971.
- Olin JW, Jang J, Jaff MR, Beckman JA, and Rooke T. The top 12 advances in vascular medicine. *J Endovasc Ther* 11, Suppl 2: II21–II31, 2004.
- Palacin M, Estevez R, Bertran J, and Zorzano A. Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* 78: 969–1054, 1998.
- Palmer RM, Ferrige AG, and Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524–526, 1987.
- Pardridge WM, Eisenberg J, and Yamada T. Rapid sequestration and degradation of somatostatin analogues by isolated brain microvessels. *J Neurochem* 44: 1178–1184, 1985.
- Sánchez del Pino MM, Hawkins RA, and Peterson DR. Biochemical discrimination between luminal and abluminal enzyme and transport activities of the blood-brain barrier. *J Biol Chem* 270: 14907–14912, 1995.
- Sánchez del Pino MM, Hawkins RA, and Peterson DR. Neutral amino acid transport by the blood-brain barrier: membrane vesicle studies. *J Biol Chem* 267: 25951–25957, 1992.

39. **Sánchez del Pino MM, Peterson DR, and Hawkins RA.** Neutral amino acid transport characterization of isolated luminal and abluminal membranes of the blood-brain barrier. *J Biol Chem* 270: 14913–14918, 1995.
40. **Schain RJ and Watanabe KS.** Distinct patterns of entry of two non-metabolizable amino acids into brain and other organs of infant guinea pigs. *J Neurochem* 19: 2279–2288, 1972.
41. **Sershen H and Lajtha A.** Capillary transport of amino acids in the developing brain. *Exp Neurol* 53: 465–474, 1976.
42. **Siegal GJ, Agranoff AB, Albers RW, Fisher SK, and Uhler MD.** *Basic Neurochemistry*. Philadelphia, PA: Lippincott-Raven, 1998.
43. **Smith QR.** The blood-brain barrier and the regulation of amino acid uptake and availability to brain. In: *Fuel Homeostasis and the Nervous System*, edited by Vranic M. New York: Plenum, 1991, p. 55–71.
44. **Smith QR and Stoll J.** Blood-brain barrier amino acid transport. In: *Introduction to the Blood-Brain Barrier: Methodology, Biology, and Pathology*, edited by Pardridge WM. Cambridge, UK: Cambridge Univ. Press, 1998, p. 188–197.
45. **Stoll J, Wadhvani KC, and Smith QR.** Identification of the cationic amino acid transporter (System y⁺) of the rat blood-brain barrier. *J Neurochem* 60: 1956–1959, 1993.
46. **Tewes BJ and Galla HJ.** Lipid polarity in brain capillary endothelial cells. *Endothelium* 8: 207–220, 2001.
47. **Thiel VE and Audus KL.** Nitric oxide and blood-brain barrier integrity. *Antioxid Redox Signal* 3: 273–278, 2001.
48. **Thomas EL, Shao TC, and Christensen HN.** Structural selectivity in interaction of neutral amino acids and alkali metal ions with a cationic amino acid transport system. *J Biol Chem* 246: 1677–1681, 1971.
49. **Utepbergenov DI, Mertsch K, Sporberr A, Tenz K, Paul M, Haseloff RF, and Blasig IE.** Nitric oxide protects blood-brain barrier in vitro from hypoxia/reoxygenation-mediated injury. *FEBS Lett* 424: 197–201, 1998.
50. **van Meer G, Gumbiner B, and Simons K.** The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next. *Nature* 322: 639–641, 1986.
51. **van Meer G and Simons K.** The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO J* 5: 1455–1464, 1986.
52. **Van Winkle L, Campione A, and Farrington B.** Development of system B^{0,+} and a broad-scope Na-dependent transporter of zwitterionic amino acids in preimplantation mouse conceptuses. *Biochim Biophys Acta* 1025: 225–233, 1990.
53. **Van Winkle L, Christensen H, and Campione A.** Na⁺-dependent transport of basic, zwitterionic, and bicyclic amino acids by a broad-scope system in mouse blastocysts. *J Biol Chem* 260: 12118–12123, 1985.
54. **Van Winkle LJ, Campione AL, and Gorman JM.** Inhibition of transport system b^{0,+} in blastocysts by inorganic and organic cations yields insight into the structure of its amino acid receptor site. *Biochim Biophys Acta* 1025: 215–224, 1990.
55. **Van Winkle LJ, Campione AL, and Gorman JM.** Na⁺-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distinguish between these substrates. *J Biol Chem* 263: 3150–3163, 1988.
56. **Vane JR and Botting RM.** The role of chemical mediators released by the endothelium in the control of the cardiovascular system. *Int J Tissue React* 14: 55–64, 1992.
57. **Vega-Agapito V, Almeida A, Hatzoglou M, and Bolanos JP.** Peroxynitrite stimulates L-arginine transport system y(+) in glial cells. A potential mechanism for replenishing neuronal L-arginine. *J Biol Chem* 277: 29753–29759, 2002.
58. **White MF.** The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochim Biophys Acta* 822: 355–374, 1985.
59. **Wiesinger H.** Arginine metabolism and the synthesis of nitric oxide in the nervous system. *Prog Neurobiol* 64: 365–391, 2001.
60. **Wu G and Meininger CJ.** Regulation of L-arginine synthesis from L-citrulline by L-glutamine in endothelial cells. *Am J Physiol Heart Circ Physiol* 265: H1965–H1971, 1993.
61. **Wu G and Meininger CJ.** Regulation of nitric oxide synthesis by dietary factors. *Annu Rev Nutr* 22: 61–86, 2002.