Glutamine transport by the blood-brain barrier: a possible mechanism for nitrogen removal

WHA-JOON LEE,1 RICHARD A. HAWKINS,2 JUAN R. VIÑA, and DARRYL R. PETERSON1

1Department of Physiology and Biophysics, Finch University of Health Science/The Chicago Medical School, North Chicago, Illinois 60064-3095; and 2Departamento de Bioquímica y Biología Molecular, Facultades de Medicina y Farmacia, Universitat de Valencia, Valencia 46010, Spain

Lee, Wha-joon, Richard A. Hawkins, Juan R. Viña, and Darryl R. Peterson. Glutamine transport by the blood-brain barrier: a possible mechanism for nitrogen removal. Am. J. Physiol. 274 (Cell Physiol. 43): C1101–C1107, 1998.—Glutamine and glutamate transport activities were measured in isolated luminal and abluminal plasma membrane vesicles derived from bovine brain endothelial cells. Facilitative systems for glutamine and glutamate were almost exclusively located in luminal-enriched membranes. The facilitative glutamine carrier was neither sensitive to 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid inhibition nor did it participate in accelerated amino acid exchange; it therefore appeared to be distinct from the neutral amino acid transport system L1. Two Na-dependent glutamine transporters were found in abluminal-enriched membranes: systems A and N. System N accounted for ~80% of Na-dependent glutamine transport at 100 µM. Abluminal-enriched membranes showed Na-dependent glutamate transport activity. The presence of 1) Na-dependent carriers capable of pumping glutamine and glutamate from brain into endothelial cells, 2) glutaminase within endothelial cells to hydrolyze glutamine to glutamate and ammonia, and 3) facilitative carriers for glutamine and glutamate at the luminal membrane may provide a mechanism for removing nitrogen and nitrogen-rich amino acids from brain.

GluTAMINE, NORMAllY THE most abundant amino acid in plasma, has several important metabolic roles, including as an energy source for intestinal, endothelial, and lymphocytic cells (22), a regulator of nitric oxide synthesis by endothelial cells (4), and a nontoxic transport vehicle to carry ammonia from peripheral tissues to the liver where ammonia is metabolized to urea (35).

In brain, ammonia is incorporated into glutamine in astrocytes where glutamine synthetase is most concentrated (24). This leads to an accumulation of glutamine in brain (6 µmol/g) and its extracellular fluid (0.5 µmol/ml) (10).

Glutamine moves from the plasma across the blood-brain barrier (BBB) by a facilitated process, albeit slowly, compared with other neutral amino acids (34). It has been assumed that glutamine is transported across the BBB on the neutral amino acid transporter designated L1, which has a high affinity for most large neutral amino acids (34). However, a definitive demonstration of system L1 involvement in glutamine transport at the BBB has not been made.

Na-dependent amino acid transport systems A, N, and ASC are involved in glutamine transport in various cell types (2, 21). Of these transporters, system N shows the greatest specificity for amino acids with a nitrogen group in the side chain (i.e., glutamine, asparagine, and histidine) (21). Whereas system A transport activity is present in the abluminal membrane of the bovine BBB (30), system ASC is absent (30, 32). The presence of system N transport in the BBB has not been studied.

Glutamate is an amino acid that functions as an excitatory neurotransmitter and normally does not enter the brain (12). In addition, reports indicate that it may be actively extruded from brain (12, 15, 28). A high-affinity concentrative transport system for glutamate has been described, presumably on the abluminal membrane (15), and a low-affinity system seems to exist on the luminal membrane (3, 25).

The purpose of the current experiments is to examine glutamine and glutamate transport at the luminal (blood-facing) and abluminal (brain-facing) borders of the BBB. For these studies, a technique was employed in which vesicles suitable for the study of transport were isolated from the luminal and abluminal membranes of cerebral capillary endothelial cells (30, 31). The results suggest that the endothelial cells of the BBB are involved in brain nitrogen balance by providing a mechanism for the removal of nitrogen in the form of glutamine, glutamate, and ammonia.

MATERIALS AND METHODS

Materials. L-[U-14C]glutamine (264.6 mCi/mmol), L-[3H]glutamate (17.8 Ci/mmol), N-methylamino-[L-14C]isobutyric acid (56 mCi/mmol), L-[3H]phenylalanine (46 Ci/mmol), and [U-14C]sucrose (475 mCi/mmol) were bought from DuPont NEN. Collagenase type IV, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH), and N-(methylamino)isobutyric acid (MeAIB) were bought from Sigma. The Bio-Rad protein assay was purchased from Bio-Rad Laboratories (Hercules, CA).

Isolation and characterization of membrane vesicles. Membrane vesicles from bovine brain endothelial cells were prepared as previously described (31). Briefly, cerebral capillaries were isolated (30) and treated with collagenase to remove remnants of the basement membrane, pericytes, and adherent membrane fragments of astrocytic end-feet. The capillaries were then homogenized, and the released membranes were segregated on a discontinuous Ficoll gradient (31). Luminal-enriched and abluminal-enriched membrane vesicles were identified in the gradient using γ-glutamyl transpeptidase and MeAIB (system A transport activity) as the respective markers (31). Enriched membrane fractions were collected at the 0%/5% (luminal-enriched) and 10%/15% (abluminal-enriched) interfaces on fractionation and frozen at −80°C in storage buffer (290 mM mannitol and 10 mM HEPES-Tris, pH 7.4). The integrity of the vesicles was determined by measuring the uptake of radiolabeled sucrose for which no transport system exists (32).
Uptake measurements. Transport of radiolabeled [14C]glutamine and [3H]glutamate into membrane vesicles was measured by rapid filtration (32). All experiments were conducted at 37°C. After thawing, the vesicles were resuspended in storage buffer to a protein concentration of 2.5–5.0 µg/µl. Uptake experiments were initiated by adding 10 µl of a reaction mixture (storage buffer, substrate, and either 100 mM NaCl or 100 mM KCl) to 10 µl of the membrane suspension and incubating in a water bath. Thus the concentration of NaCl or KCl at the start of the experiment was always 50 mM. The reaction was stopped by adding 1 ml of ice-cold stopping solution (145 mM NaCl and 10 mM HEPES-Tris, pH 7.4) and rapidly filtering on a 0.45-µm Gelman Metricel filter 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. To control for binding or trapping of substrate, the apparent uptake at time zero was subtracted from all experimental values.

The time course of [14C]glutamine (100 µM) or [3H]glutamate (2 µM) uptake was measured in the presence of NaCl or KCl. The contribution of system A to the Na-dependent transport of glutamine was determined by adding 2 mM MeAIB, a specific inhibitor of system A (7, 19). The residual activity was taken to be attributable to another Na-dependent glutamine transporter (e.g., system N). All time-course data were normalized to the 60-min equilibrium value.

Glutamine uptake experiments were conducted in the presence of a KCl gradient with and without 1 mM BCH, a defining substrate of the L transport system (8), to determine the contribution of facilitative system L1 to glutamine flux. To further examine the possibility of system L1 transport, luminal vesicles were preloaded with glutamine (2–20 mM), and the initial rate of [3H]phenylalanine transport (10 µM) was used to determine the exact contribution of each membrane domain to the activity being measured (31). The integrity of the membrane vesicles was determined by measuring the uptake of 10 µM [14C]sucrose as an index of leakage. The initial rates of uptake, expressed as the permeability-surface area product (PSA), were found to be 0.23 ± 0.02 (SE) and 0.18 ± 0.01 µl·min⁻¹·mg protein⁻¹ in luminal- and abluminal-enriched vesicles, respectively. These values were consistent with those obtained previously (32) and represented only a small proportion (3–10%) of the maximum PSA of the various transport systems studied (Table 1).

### Table 1. Na gradient, kinetic constants, and PSA for luminal- and abluminal-enriched vesicles

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Na Gradient, mM</th>
<th>Vₘₐₓ, nmol·mg⁻¹·min⁻¹</th>
<th>Kᵥₒ, mM</th>
<th>Kᵥₐ, µl·min⁻¹·mg⁻¹</th>
<th>PSA, µl·min⁻¹·mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal-enriched</td>
<td>0</td>
<td>4.0 ± 0.71</td>
<td>2.3 ± 0.18</td>
<td>0.49 ± 0.01</td>
<td>2.2</td>
</tr>
<tr>
<td>Abluminal-enriched</td>
<td>0</td>
<td>1.0 ± 0.16*</td>
<td>2.4 ± 1.4</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>Abluminal-enriched</td>
<td>50</td>
<td>11.0 ± 0.3*</td>
<td>1.4 ± 0.2</td>
<td>0.64 ± 0.01*</td>
<td>8.5</td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal-enriched</td>
<td>0</td>
<td>6.4 ± 0.90</td>
<td>2.4 ± 1.3</td>
<td>0.03 ± 0.01</td>
<td>2.7</td>
</tr>
<tr>
<td>Abluminal-enriched</td>
<td>0</td>
<td>0.1 ± 0.02*</td>
<td>0.8 ± 1.2</td>
<td>0.01 ± 0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>Abluminal-enriched</td>
<td>50</td>
<td>22.5 ± 2.9*</td>
<td>6.8 ± 3.1</td>
<td>0</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Values for kinetic constants are means ± SE (asymptotic). Kinetic constants calculated from the experiments shown in Fig. 3 by nonlinear regression analysis. Permeability-surface area product (PSA), an index of permeability at low concentrations, was calculated as Vₘₐₓ/Kᵥₒ + Kᵥₐ (where Vₘₐₓ is maximal velocity, Kᵥₒ is the Michaelis-Menten constant, and Kᵥₐ is the dissociation constant). *P < 0.05.
branes, the Na gradient, but not the K gradient, caused 
$[^{14}\text{C}]$glutamine to achieve an intravesicular concen-
tration that transiently exceeded the equilibrium value
(overshoot) (Fig. 1A). The overshoot was observed in
the presence of 2 mM MeAIB (data not shown), indicat-
ing that a secondary active transport system, other
than system A, was involved in Na-dependent gluta-
mine transport into abluminal-enriched membrane
vesicles.

Analysis of the luminal-enriched membranes showed
no overshoot in the presence of either an inwardly
directed Na or K gradient (Fig. 1B). The apparent
stimulation of glutamine uptake in the presence of an
Na gradient, compared with a K gradient, was attrib-
uted to contamination by abluminal vesicles. This was
substantiated by a calculated f value of 0, indicating
that all Na-dependent glutamine transport activity
was attributable to the abluminal membrane.

The initial rate of glutamine uptake in the presence
of Na was found to be inhibited by 80% in the presence
of 3 mM histidine but only by ~20% with 2 mM MeAIB
(Fig. 2). The data suggested that an N-type system was
responsible for the majority of abluminal Na-depen-
dent glutamine transport (21), with a smaller fraction
entering by the A system. Analysis of the velocity vs.
substrate relationship measured in the presence of 2
mM MeAIB (Fig. 3A) revealed a $K_m$ of 1.4 mM and a
$V_{max}$ of 11 nmol·min$^{-1}$·mg protein$^{-1}$ in addition to an
apparently “nonsaturable” component. The nature of
the latter component could not be discerned from the
data.

Facilitative transport of glutamine. Michaelis-Menten
plots demonstrated facilitative transport of glutamine
across the luminal-enriched membranes (Fig. 3A, Ta-
ble 1). Again, as was seen with the Na-dependent
abluminal system, there was a saturable and a nonsatu-
rable component. In the absence of an Na gradient,
the abluminal-enriched membranes appeared to be only
slightly, if at all, permeable to glutamine. This was
substantiated by an f value of 0.9 from which it was
concluded that virtually all the facilitative transporters
were in the luminal membranes.

System L1 transport activity is inhibited by saturat-
ing concentrations of BCH and participates in an
accelerated exchange mechanism with other amino
acids (2, 7, 8, 21). In the current study, the initial rate
of glutamine uptake in luminal-enriched vesicles was not
inhibited by the addition of 1 mM BCH to the Na-free
(KCl) reaction medium (data not shown). Preloading
luminal-enriched vesicles with 2–20 mM glutamine did
not augment phenylalanine influx. The initial rate of

![Fig. 1](1) Time course of glutamine uptake in abluminal (A) and
luminal (B) membranes. Uptake of 100 µM $[^{14}\text{C}]$glutamine was
measured in the presence of 50 mM external NaCl or KCl at 37°C
over a 60-min period. In the presence of an initial Na gradient (●),
abluminal membrane vesicles (A) concentrated glutamine above the
equilibrium value (overshoot). No overshoot was seen in the absence
of an Na gradient (○). Luminal membrane vesicles (B) showed
acceleration of transport but no overshoot in the presence of an Na
gradient. All values were normalized to the equilibrium value.

![Fig. 2](2) Characterization of glutamine uptake into abluminal vesicles.
Net uptake of 100 µM $[^{14}\text{C}]$glutamine was measured over a 15-s
period at 37°C in the presence of 50 mM external NaCl or KCl.
Na-dependent glutamine uptake into abluminal membranes was
inhibited by the addition of 2 mM N-(methylamino)isobutyric acid
(MeAIB) or 3 mM histidine (His) to the reaction medium. Data are
means ± SE of 3 separate experiments. *P < 0.05.
phenylalanine uptake was 942 ± 36 (SE) in control (unfilled) vesicles and 954 ± 77 (SE) in vesicles pre-loaded with glutamine. It therefore appeared that facilitated glutamine transport across the luminal membrane of the BBB occurred by a system that was distinct from system L1.

Glutamate transport. A Michaelis-Menten analysis of glutamate uptake demonstrated considerable facilitative transport activity in luminal-enriched membranes that could be described as a single saturable component (Fig. 3B, Table 1). Although the identity of this system was not further investigated, a recent report indicated that glutamate transport from blood to brain was not inhibited by \( L \)-cystine and therefore unlikely to be \( system \ n \). This carrier showed no inhibition by BCH or evidence of amino acid transstimulation and was designated as “system n.”

Hepatic encephalopathy is associated with hyperammonemia and an accumulation of glutamine in brain (14). Also, there is a markedly increased permeability of the BBB to neutral amino acids, leading to high concentrations of aromatic amino acids in brain. James and co-workers (17) proposed a “unified theory of portal-systemic encephalopathy” to explain the BBB permeability changes. They suggested that brain glutamine facilitates the transport of neutral amino acids at the BBB by an exchange mechanism, thereby enhancing amino acid entry into brain. In support, Cangiano et al. (5) found that bovine brain microvessels preloaded with glutamine stimulated the uptake of other neutral amino acids. Although they felt that the L system was in-
volved, the effect occurred only in the presence of Na and was blocked by MeAIB, indicating the participation of the Na-dependent A system. The “unified theory” was criticized by Mans et al. (14, 23) who pointed out that glutamine is poorly transported by the neutral amino acid transporter and would, therefore, be kinetically unsuitable for this exchange mechanism and the transport of different neutral amino acids increases to different degrees under conditions of portal encephalopathy. In the present experiments, the facilitated transport of glutamine could not be attributed to system L1 but rather to a distinct transporter, possibly system n (27). There was no evidence for glutamine enhancement of phenylalanine transport, a representative substrate of system L1, suggesting that glutamine does not directly stimulate neutral amino acid flux from plasma to brain.

Na-dependent systems N, A, and ASC are reported to be responsible for glutamine transport in various cell types (2, 4, 21). System ASC is not present at the bovine BBB, but system A is in the abluminal membrane (30, 32) and could have accounted for ~20% of abluminal Na-dependent glutamine transport activity. Another Na-dependent system was shown to mediate the majority of glutamine transport across the abluminal membrane. This transporter was inhibited by histidine (3 mM) and was refractory to inhibition by MeAIB, an amino acid analog specifically transported by system A (8, 20). The substrate affinity for glutamine obtained in this study ($K_m = 1.4$ mM) was similar to values reported for the N system in rat liver (16, 20) and in human skeletal muscle (1). Thus the characteristics of the histidine-sensitive, Na-dependent system most closely resemble those of the hepatocyte amino acid system N transporter (20). These two potent Na-dependent transporters (systems A and N) are in a position to pump glutamine from the extracellular space into the endothelial cell from where it can cross the luminal membrane by facilitated diffusion.

Many cells, including endothelial cells, are known to consume glutamine (35). In endothelial cells, the capacity to metabolize glutamine is reflected by high activity of the initiating enzyme, glutaminase (22). Indeed, substantial glutaminase activity was confirmed to be present in bovine endothelial cells used in this study.

It has now become clear that the reuptake of glutamate by neurons (18, 38) and astrocytes (29, 36) terminates excitatory signals and prevents the accumulation of excitotoxic levels of glutamate in the brain. Glutamate levels in cerebrospinal fluid are normally in millimolar concentrations (8, 20). The substrate affinity for glutamine obtained in this study ($K_m = 6.8$ mM) is lower than that reported for other Na-dependent glutamate transporters (18, 29, 36). Yet, the PSA values for glutamate transport by the luminal and abluminal membrane domains are nearly equal, suggesting similar functional capabilities (Table 1). Thus, in addition to astrocytes and neurons, the endothelial cells of cerebral capillaries may also play a role in the maintenance of low glutamate concentrations in the extracellular fluid of brain.

Taken together, these observations indicate that the two membranes of the BBB are organized to remove glutamine and glutamate from the brain. The Na-dependent carriers are capable of pumping both glutamine and glutamate from the brain extracellular fluid into endothelial cells where glutaminase hydrolyzes glutamine to glutamate and ammonia. The luminal facilitative carriers for both glutamate and glutamine can then transport these nitrogen-rich molecules into the plasma, effectively removing them from the brain (Fig. 4).

These observations may also provide an explanation for the long-standing puzzle regarding brain ammonia metabolism. Various measurements have shown that 20–50% of the ammonia circulating through brain passes the BBB (10). The ammonia that enters brain is almost completely incorporated into the amide group of glutamate by astrocytes. It is puzzling, however, that it has been impossible to consistently measure arteriovenous differences of ammonia (9, 10). If there were no mechanism for the removal of glutamine, it would accumulate in brain, thereby raising the osmolarity considerably. From this study, it seems likely that glutamine pumped into endothelial cells is at least

![Fig. 4. Glutamine metabolism in endothelial cells of the blood-brain barrier. Powerful Na-dependent transport systems located on the abluminal membrane are in a position to actively transport glutamine (Gln) and glutamate (Glu) from brain extracellular fluid into the endothelial cells. Facilitative transport systems exist on the luminal membrane for both glutamine and glutamate, but there is little if any transport activity for either of these metabolites on the abluminal membrane. Within the endothelial cell, glutamine may be hydrolyzed to glutamate and ammonia by glutaminase (Glnase). This arrangement of carriers provides a mechanism for removing nitrogen-rich molecules from the extracellular fluid and directing them toward the plasma. A, Na-dependent system A; N, Na-dependent system N; G, Na-dependent glutamate transporter.](http://advcell.physiology.org.org/)
partially metabolized to ammonia and glutamate, which may then diffuse across the luminal membrane. In this way, the ammonia released from brain to blood would be in a compartment (endothelial cells) that is on the blood side of the astrocytic barrier.

The results of the current studies also explain why the entry of glutamine and glutamate to the central nervous system is greatly restricted, even though carrier activities for both amino acids have been described (13, 25, 34). Glutamine and glutamate can traverse luminal membranes on facilitative systems. However, movement into brain across the abluminal membranes would be slow because of the steep Na gradient that exists between brain extracellular fluid and the cell interior.

In conclusion, the BBB is arranged in such a manner as to not only inhibit the entry of glutamine and glutamate into brain but to expel these nitrogen-rich amino acids and possibly ammonia. Therefore, the BBB may participate in the regulation of brain nitrogen metabolism and protect against the development of neurotoxicity by preventing the accumulation of these nitrogen-rich molecules in the brain.

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Address for reprint requests: R. A. Hawkins, Dept. of Physiology and Biophysics, Finch Univ. of Health Sciences/The Chicago Medical School, 3333 Green Bay Road, North Chicago, IL 60064.

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REFERENCES


