Na⁺-dependent Glutamate Transporters (EAAT1, EAAT2, and EAAT3) of the Blood-Brain Barrier

A MECHANISM FOR GLUTAMATE REMOVAL*

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Na⁺-dependent transporters for glutamate exist on astrocytes (EAAT1 and EAAT2) and neurons (EAAT3). These transporters presumably assist in keeping the glutamate concentration low in the extracellular fluid of the brain. Recently, Na⁺-dependent glutamate transport was described on the abluminal membrane of the blood-brain barrier. To determine whether the above-mentioned transporters participate in glutamate transport of the blood-brain barrier, total RNA was extracted from bovine cerebral capillaries, cDNA for EAAT1, EAAT2, and EAAT3 was observed, indicating that mRNA was present. Western blot analysis demonstrated all three transporters were expressed on abluminal membranes, but none was detectable on luminal membranes of the blood-brain barrier. Measurement of transport kinetics demonstrated voltage dependence, K⁺-dependence, and an apparent Kₐ of 14 mM (aggregate of the three transporters) at a transmembrane potential of −61 mV. Inhibition of glutamate transport was observed using inhibitors specific for EAAT2 (kainic acid and dihydrokainic acid) and EAAT3 (cysteine). The relative activity of the three transporters was found to be approximately 1:3:6 for EAAT1, EAAT2, and EAAT3, respectively. These transporters may assist in maintaining low glutamate concentrations in the extracellular fluid.

Glutamate is an amino acid that functions as an excitatory neurotransmitter. When its concentration in the extracellular fluid becomes elevated, glutamate can be toxic to neurons (1) and has been associated with serious brain damage (2). Therefore, the concentration of glutamate in the extracellular fluid is kept low (1–3 μmol/liter) (3, 4), even though there is 12 μmol/g of whole brain (5). A possible mechanism to maintain the low concentration in the extracellular fluid has been postulated with the finding of active glutamate transport into neurons (6, 7) and astrocytes (8). The role of the blood-brain barrier in this control mechanism has not yet been delineated.

A major function of the blood-brain barrier is to regulate the movement of nutrients and other molecules into and out of the brain (9). The tight junctions connecting endothelial cells of the capillaries make most substances impermeant (9, 10). Therefore, only lipid-soluble substances or substances with a transport mechanism can traverse the barrier.

Mechanisms may exist on the blood-brain barrier to control the influx of glutamate and possibly aid in its efflux due to its excitotoxic properties at high concentrations. Evidence of such a mechanism was first described in the mid-1970s when Oldendorf and Szabo (11) showed glutamate transport by the blood-brain barrier in vivo. Later, Drewes et al. (12) demonstrated a net efflux of glutamate from isolated perfused dog brains. This efflux was against a concentration gradient, suggesting that an energy-dependent transport system mediated the efflux (13). In 1985, Hutchison et al. (14) performed glutamate transport studies on isolated microvessels. These studies showed a high affinity Na⁺-dependent glutamate transporter on the abluminal membrane of the blood-brain barrier. In addition to active transport on the abluminal membrane, facilitative transport of glutamate has been described across the luminal membrane (15, 16), possibly to allow glutamate to move into endothelial cells or out to blood. The accumulation of this data led to two questions. What are the characteristics of the carrier(s) responsible for the high affinity glutamate transport across the blood-brain barrier, and is there more than one transporter present and active?

To date, two Na⁺-dependent glutamate transporters have been isolated and cloned from rat brain, GLAST (17) and GLT-1 (18), and one transporter has been cloned from rabbit brain, EAAC1 (7). Homologues of each of these transporters have recently been found in human brain (19): EAAT1 (GLAST), EAAT2 (GLT-1), and EAAT3 (EAAC1). In addition, EAAT4 (20) and EAAT5 (21) have been isolated and cloned from human cerebellum and retina, respectively. We focused our studies of blood-brain barrier glutamate transporters on EAAT1, EAAT2, and EAAT3 due to their ubiquitous location in the brain.

The purpose of the current experiments was to determine the Na⁺-dependent glutamate transporters on the blood-brain barrier and their kinetic characteristics. The results indicate that the blood-brain barrier contains at least three Na⁺-dependent glutamate carriers with different activities that, combined, show a high affinity for glutamate transport. Therefore, the
blood-brain barrier could be the site of an important control mechanism for maintaining nontoxic levels of glutamate.

**EXPERIMENTAL PROCEDURES**

**Materials**—L-[2,3,4-3H]Glutamic acid (60 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Glutamic acid, cysteine, valinomycin, kainic acid, dihydrokainic acid, and collagenase Type IA were bought from Sigma. The Bio-Rad protein assay was purchased from Bio-Rad.

**Isolation and Characterization of Membrane Vesicles**—Membrane vesicles from brain endothelial cells were prepared as described previously (22). Briefly, isolated microvesicles from bovine cerebral cortices were digested with collagenase Type IA (180 units collagenase/ml) and homogenized, and the released membranes were separated into five fractions at the interfaces of a discontinuous Ficoll gradient (15, 2, 5, 10, and 20%) (22). These membrane fractions are referred to as F1, 2, 3, and 4, respectively, with the remaining pellet as F5. The amount of abluminal and luminal membrane in each fraction was determined by the activities of two markers: system A transport of N-(methylamino)isobutyric acid for abluminal membrane and γ-glutamyl transpeptidase for luminal membrane. The percentage of luminal membrane in each fraction was: F1, 80%; F2, 57%; F3, 31%; F4, 26%; F5, 29%.

**Isolation and Analysis of mRNA Expression by RT-PCR**—Brain microvesicles were isolated (22), digested with collagenase, suspended in freezing buffer (280 mM sucrose, 2 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.4), and stored at −80 °C. A single-step method of mRNA isolation was used on the microvesicles (23). The RT-PCR reactions were conducted in one step with Titan™ One Tube RT-PCR, following the instructions of the manufacturer (Roche Molecular Biochemicals). EAAT1 expression levels were determined using the following primers (5′ to 3′): CCTGCGATGTATACGTAGT and ATGTTGAGTATGACGCT; primers for EAAT2, GAGAAAAACCCATTCTTTTT and CGGATGGGAGGACGAAATC; primers for EAAT3, GGGACAGATTCTGCTGCACGAACTGCG. Reverse transcription and amplification conditions were 50 °C for 30 min and 94 °C for 2 min. For EAAT2, EAAT3, MAP-2a, glutamine synthetase, and 26S ribosomal protein RNA, the procedure was 10 cycles of denaturation (94 °C for 30 s), annealing (51 °C for 30 s), and extension (68 °C for 1 min) and 15 cycles at 94 °C for 30 s, 51 °C for 30 s, and 68 °C for 1 min plus 1 s per cycle with a final extension of 68 °C for 7 min. For EAAT1 mRNA, the procedure was 10 cycles of denaturation (94 °C for 30 s), annealing (54 °C for 30 s), and extension (68 °C for 1 min) and 20 cycles at 94 °C for 30 s, 54 °C for 30 s, and 68 °C for 1 min plus 1 s per cycle with a final extension of 68 °C for 7 min. The resulting PCR products were separated on a 7% SDS-polyacrylamide gel electrophoresis (24). After electrophoresis, the gels were stained with ethidium bromide. Total RNA from microvessels—mRNA in isolated brain microvesicles. Electrophoretic analysis of RT-PCR (20% of total reaction volume) was performed on a 1.2% agarose gel stained with ethidium bromide. Total RNA from microvessels was amplified by RT-PCR using specific primers (see “Experimental Procedures”). Lane 1, molecular weight markers; lane 2, 26 S ribosomal protein RNA (253 bp); lane 3, EAAT1 (242 bp); lane 4, EAAT2 (150 bp); lane 5, EAAT3 (370 bp).

**DNA Sequencing**—The identity of the PCR products was determined by sequencing of the cDNAs in an ABI 373 automatic sequencer using the Dye Deoxy™ terminator cycle sequencing kit (Perkin-Elmer).

**Electrophoresis and Immunoblotting**—Membrane proteins were separated on a 7% SDS-polyacrylamide gel electrophoresis (24). After electrophoresis, the proteins were blotted onto nitrocellulose membranes (0.45 μm, Schleicher & Schuell). For detection of EAAT1, EAAT2, and EAAT3 proteins, blots were incubated in blocking solution (5% (w/v) nonfat dry milk with 0.05% (v/v) Tween 20) for 1 h at room temperature with shaking. After three washes with a Tris buffer (TTBS: 25 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% (v/v) Tween 20), blots were incubated with primary antibodies in TTBS for 1 h at room temperature with gentle agitation. Blots were washed again with TTBS and incubated with secondary antibody conjugated with alkaline phosphatase for 1 h. After 1 h, blots were washed and developed. Polyclonal (rabbit) anti-EAAT2 was purchased from Affinity Bioreagents, Inc. (Golden, CO). Anti-EAAT1 antibody was generously supplied by Dr. Wilhelm Novak, University of Florida College of Medicine, Gainesville, FL.

**Statistical Analyses**—All data were analyzed with an unpaired Student’s t test, based on the method of Bradford (26).

**RESULTS**

**Glutamate Transporter Expression in Isolated Microvesicles**—cDNA (from RT-PCR) was separated by electrophoresis. Specific probes identified the Na+ dependent glutamate transporters EAAT1, EAAT2, and EAAT3 (Fig. 1). The results indicated the three transporters were expressed by brain microvesicles. cDNA sequencing of the bands demonstrated greater than 95% homology in each compared with other species (EAAT1 (17), EAAT2 (18), EAAT3 (6, 7)).

**Localization of the Na+-dependent Glutamate Transporters**—To determine the localization of EAAT1, EAAT2, and EAAT3, membrane proteins (2 μg/lane) from the five fractions were electrophoresed and immunoblotted with specific antibodies (Fig. 2). Fractions 1 and 2 were enriched in luminal membrane (80% and 57%, respectively), whereas fractions 3, 4, and 5 were enriched in abluminal membrane (69, 74, and 71%, respectively). Protein for the three transporters was found only in fractions 3, 4, and 5, indicating an abluminal location.
Microvessel Preparation—The presence of astrocytes or neurons could contaminate the microvessel preparation, thereby skewing the results. To test this, markers were used to assess the purity of the preparation (glutamine synthetase for astrocytes (27) and microtubule-associated protein (MAP-2a) for neurons (28)). mRNA for glutamine synthetase and MAP-2a were detectable in the brain extract showing glutamine synthetase (astrocyte marker); lane 2, no detectable glutamine synthetase in microvessels; lane 3, whole brain extract showing MAP-2a (neuron marker); lane 4, no detectable MAP-2a in microvessels; lane 5, molecular weight standards.

Na\(^+\) Dependence of Glutamate Transport—Preliminary experiments measured glutamate uptake in the presence and absence of Na\(^+\) (choline was used as an inert ion). Glutamate transport in the absence of Na\(^+\) was 50–70% less than in the presence of Na\(^+\) (data not included). Once Na\(^+\) dependence was established, all further experiments were done in the presence of Na\(^+\) using a saturating concentration of glutamate to determine the base line.

Transmembrane Potential Comparison—Glutamate transport has been shown to be voltage-dependent (29). Therefore, to assess the voltage sensitivity of blood-brain barrier glutamate transport, uptake was determined at three transmembrane potentials was measured. The initial rate was measured as the change in glutamate uptake between 0 and 15 s. Valinomycin, a potassium-specific ionophore, was used to make the vesicles permeable to K\(^+\), and different concentration gradients of KCl created the desired potentials (18.4, 0, −18.4, −42.6, −80.7, −100.3 mV), according to the Nernst equation (30), as follows.

\[
E_K = 61 \log \frac{[K_{out}]}{[K_{in}]} \quad \text{(at 37 °C)} \quad \text{(Eq. 1)}
\]

The initial rate increased as transmembrane potential became more negative (Fig. 4). The following equation was used to fit the curve.

\[
y = \frac{a}{1 + \exp \left(\frac{x-x_m}{k}\right)} \quad \text{(Eq. 2)}
\]

where \(a\) is the maximal initial rate (1.6 ± 0.3), \(x_m\) is the midpoint (−10.8 ± 15), and \(k\) is the steepness of the curve (−33.3 ± 16). Once \(k\) was determined, the net charge (\(z\)) associated with glutamate transport was calculated (30) as follows.

\[
k = \frac{RT}{zF} \quad \text{(Eq. 3)}
\]

At 37 °C, \(RT/F\) (\(R\) is the gas constant, 8.315 J-K\(^{-1}\)-mol\(^{-1}\), \(T\) is the temperature in Kelvin, and \(F\) is Faraday’s constant, 9.648 × 10\(^4\) C-mol\(^{-1}\)) is 26.73 mV. Solving for \(z\), a net charge of 0.8 is obtained, indicating a net movement of one positive charge.

Potassium Sensitivity—Barbour et al. (31) showed a dependence of glutamate transport on intracellular potassium. To determine if the blood-brain barrier glutamate transporter was dependent on K\(^+\), Na\(^+\)-dependent glutamate uptake was measured with and without intravesicular K\(^+\). Vesicles with K\(^+\) showed a 56.5% greater uptake than vesicles without K\(^+\). This increased transport was compared with uptake with a −61-mV transmembrane potential (obtained by valinomycin and intravesicular KCl). Glutamate uptake with a −61-mV transmembrane potential was 20% greater than intravesicular K\(^+\) alone (data not shown).

\(K_m\) Determination—A range of glutamate concentrations (0.2 \(\mu\)M to 1 \(\mu\)M) was used to determine the kinetics of glutamate uptake. The results, plotted in Eadie-Hofstee fashion (Fig. 5), showed a \(K_m\) of 14 ± 4 \(\mu\)M (asymptotic standard error) and a \(V_{max}\) of 151 ± 20 pmol-mg\(^{-1}\)-min\(^{-1}\).

Activity of Transporters—To determine the activity of the three Na\(^+\)-dependent glutamate transporters, inhibitors (1 \(\mu\)M for EAAT2 (kainic acid and dihydrokainic acid (19)) and EAAT3 (cysteine (32)) were used (Fig. 6). Arriza et al. (19) reported \(K_m\) values for kainic acid (17 \(\mu\)M) and dihydrokainic acid (9 \(\mu\)M) (19). Zerangue and Kavanaugh (32) reported that 1 \(\mu\)M cysteine “completely” inhibited glutamate transport by EAAT3. Using 1 \(\mu\)M each of inhibitors and assuming the concentration of substrate was negligible, near complete (>98%)
The activities of EAAT1, EAAT2, and EAAT3 was approximately 1:3:6, and specific inhibition by each blocking agent, the ratio of inhibition of 37 ± 6% (S.E.), kainic acid inhibited 24 ± 6%, and dihydrokainic acid (DHK) inhibited 37 ± 8% of glutamate transport; both inhibit EAAT2. * statistically significant compared with control (p < 0.05). V̇, pmol·mg⁻¹·min⁻¹; n = 5.

Inhibition of glutamate transport was expected according to the following formula

\[ \% \text{ Inhibition} = \frac{I}{K_m \cdot \left(1 + \frac{[S]}{K_m} + [I]\right)} \quad (\text{Eq. 4}) \]

where S is substrate and I is inhibitor. Cysteine inhibited 58 ± 5% (S.E.), kainic acid 24 ± 6%, and dihydrokainic acid inhibited 37 ± 8% of glutamate uptake. Assuming complete and specific inhibition by each blocking agent, the ratio of activities of EAAT1, EAAT2, and EAAT3 was approximately 1:3:6.

**DISCUSSION**

The purpose of these experiments was to determine whether Na⁺-dependent glutamate carriers exist on the blood-brain barrier. Our results established that mRNA for three glutamate transporters, EAAT1, EAAT2, and EAAT3, was present in brain endothelial cells and that each transporter protein existed on the abluminal membrane of the blood-brain barrier. Examination of transporter characteristics revealed voltage dependence and K⁺ sensitivity of glutamate transport in abluminal vesicles, a high affinity for glutamate transport (combined activity), and an approximate ratio of activity of 1:3:6 for EAAT1, EAAT2, and EAAT3, respectively.

Previously, we had identified low affinity glutamate transport across the abluminal membrane of the blood-brain barrier (Kₐ of 6.8 mM (16)). Other laboratories had reported high affinity glutamate transport with an apparent Kₐ in the µM range (14, 19). Additionally, voltage dependence of glutamate uptake had been shown by Brew and Attwell (29). Therefore, we reexamined glutamate transport and affinity in the presence of varying transmembrane potentials and observed an increase in initial rate as the transmembrane potential was made more negative, as well as an apparent Kₐ in the µM range. Reports of K⁺ dependence of glutamate transport (31) prompted us to question whether our observations were of voltage dependence or of K⁺ sensitivity. Our results corroborated a voltage dependence as well as a K⁺ sensitivity of blood-brain barrier glutamate transport.

The number of Na⁺ ions transported with glutamate is still the source of controversy. Some suggest at least two Na⁺ molecules translocated with a glutamate molecule (29, 33, 34), whereas others report three Na⁺ molecules/glutamate (35–37). Our calculations of a net charge of 0.8 support at least two Na⁺ ions per glutamate.

Once high affinity glutamate transport was observed, the next question was which of the three transporters was active. Reported Kₐ values for EAAT1, EAAT2, and EAAT3 are very similar (19), and it would be difficult to determine the activity of the transporters on the basis of kinetics alone. Because of this, we relied on the known pharmacology of the transporters by using inhibitors for EAAT2 (dihydrokainic acid and kainic acid) and EAAT3 (cysteine). Relative activity obtained was approximately 1:3:6 for EAAT1, EAAT2, and EAAT3. A possible explanation for the different percent inhibition of dihydrokainic acid versus kainic acid is the finding of partial inhibition of glutamate transport through a cortical neuronal transporter by dihydrokainic acid (38).

With the current observations of three active transporters for glutamate on the abluminal membrane combined with work previously published (16), a general scheme of glutamate-glutamine interactions including the roles of astrocytes and neurons can be deduced (Fig. 7). Extracellular glutamate is transported into astrocytes, neurons, and endothelial cells of the blood-brain barrier by at least three Na⁺-dependent transporters. Once in astrocytes, glutamate is converted to glutamine and released into the extracellular space. Glutamate transported into neurons can be stored for future release during synaptic transmission.

Glutamate transported into endothelial cells combined with catabolism of glutamine to glutamate by glutaminase (16) (Fig. 7) transiently increases intracellular glutamate concentration. When the concentration of glutamate in endothelial cells becomes greater than the concentration in plasma (0.1 mM (39)), glutamate is facilitatively transported across the luminal mem-

**Fig. 5. Kinetics of glutamate transport.** Na⁺-dependent glutamate uptake was measured over a range of concentrations (0.2 µM to 1 mM) at a transmembrane potential of -61 mV. The results were plotted in Eadie-Hofstee fashion using data obtained from the Michaelis-Menten plot (see inset). An apparent Kₐ of 14 ± 4 µM (asymptotic standard error) and Vₘₐₓ of 151 ± 20 pmol·mg⁻¹·min⁻¹ were obtained. V̇, pmol·mg⁻¹·min⁻¹. For each point, n = 4–5.

**Fig. 6. Relative activity of EAAT1, EAAT2, and EAAT3.** Inhibition of [³H]glutamate by 1 mM cysteine, kainic acid, and dihydrokainic acid was compared with glutamate uptake without inhibitors (Control). Vesicles were preset at -61 mV. Cysteine (inhibitor of EAAT3) inhibited 58 ± 5% (S.E.) of glutamate transport. Kainic acid (KA) inhibited 24 ± 6%, and dihydrokainic acid (DHK) inhibited 37 ± 8% of glutamate transport; both inhibit EAAT2. * statistically significant compared with control (p < 0.05). V̇, pmol·mg⁻¹·min⁻¹; n = 5.

**Fig. 7. Diagrammatic representation of glutamate uptake into neurons, astrocytes, and endothelial cells of the blood-brain barrier.** Glutamine transport and synthesis are shown for completeness.
brane into blood (15, 16). Also, the flux of glutamate from blood into endothelial cells is possible via facilitative carriers (Fig. 7), but movement of glutamate from the cells to brain would be difficult due to the steep Na⁺ gradient that exists between extracellular fluid and endothelial cells (39). The absence of facilitative carriers on the abluminal membrane (16) further prevents movement of glutamate into the brain from the cells. Therefore, the blood-brain barrier not only restricts net glutamate entry into the central nervous system but also expels it, providing a possible explanation for why glutamate is not observed entering the brain in vivo (40).

Astrocytes, containing two Na⁺-dependent glutamate transporters, are in close apposition with neurons (shown diagrammatically in Fig. 7), providing a milieu for termination of glutamate transmission (8). The role of the blood-brain barrier is not as obvious unless certain pathological states are considered, such as transient ischemia or hypoxia. In these conditions, nerve cells and astrocytes depolarize, causing glutamate transporters to run “backward” (41, 42), increasing the glutamate concentration in extracellular fluid. In addition, as cellular metabolism slows, intracellular acidosis occurs. This acidosis exacerbates the reversal of the astrocyte transporters (43), further increasing the glutamate concentration in extracellular fluid to near toxic levels. The transporters present on the blood-brain barrier would then be in a position to remove glutamate from the extracellular fluid (Fig. 7), maintaining nontoxic levels. The transporters present on the blood-brain barrier would then be in a position to remove glutamate from the extracellular fluid (Fig. 7), maintaining nontoxic levels. Other members of the glutamate transporter family may also exist. Recently, homologues of EAAT1, EAAT2, and EAAT5 have been cloned (sEAAT1, sEAAT2A, sEAAT2B, sEAAT5A, and sEAAT5B (44)). In the case of the blood-brain barrier, we only examined EAAT1, EAAT2, and EAAT3, and whether other transporters are present has to be determined. However, the three transporters accounted for the glutamate transport activity detected by our technique.

In conclusion, at least three Na⁺-dependent glutamate transporters exist on the abluminal membrane of the blood-brain barrier with two possible functions: to restrict glutamate entry to the brain and to remove glutamate from the extracellular fluid in conjunction with facilitative transporters on the luminal membrane. This net transport out of brain provides a possible protective mechanism against glutamate neurotoxicity.

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